

MYCOLOGY

VOLUME 22

Handbook of Industrial Mycology

edited by
Zhiqiang An

Handbook of Industrial Mycology

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Foreword

The economic importance of fungi is almost impossible to exaggerate. Not only are they integral to the great ecological cycles of the natural world, and not only do their mycorrhizal associations provide the foundation for agriculture and forestry, they are also essential to biotechnology. In fact, modern biotechnology has its roots in fungal fermentations. Yeasts have been used since antiquity in brewing and baking, while molds have been used to improve the flavor of cheese and in the production of Asian condiments such as soy sauce and miso. During the twentieth century, industrial fermentations were developed for production of organic acids, diastatic enzymes, plant growth hormones, natural and semisynthetic antibiotics, antitumor agents, and immunomodulators. The commercial development of penicillin, the first and most important of the clinically useful antibiotics, laid the groundwork for much of what has come after, especially the technology for growing filamentous fungi in submerged culture. With new discoveries, it has become apparent that the potential applications of fungal metabolism are greater than we once suspected.

The original intent of the Marcel Dekker mycology series was to establish “a niche for publication of work dealing with all aspects of mycology.” A volume on *Industrial Mycology* is long overdue. Biotechnologists have made great progress in discovering how to manipulate yeasts and molds at the biochemical and molecular level. They have exploited two major physiological characteristics of fungi: their ability to degrade simple and complex polymers and their ability to biosynthesize a huge variety of complex secondary metabolites. Fungi can be engineered to produce many heterologous proteins and they remain an important source of novel drugs, including anticancer drugs and immune function regulators. Several species (e.g., *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Neurospora crassa*) are superb models.

This new volume covers both theoretical and practical aspects of industrial mycology with emphasis on the dramatic advances in the application of bioinformatics, molecular biology, “reverse genetics,” combinatorial chemistry, metabolic engineering, genomics, proteomics, and cognate fields to the manipulation of fungi. Collectively, these techniques have altered what is possible in the discovery laboratory.

Divided into eight sections and 26 chapters, *Industrial Mycology* is a comprehensive guide to the new mycology of the 21st century. It describes how fungi contribute directly

to the contemporary pharmaceutical industry, and how they can be manipulated in order to contribute more in the future. Dr. Zhiqiang An, editor, has done an excellent job of bridging the traditional gulf between academic and industrial studies of fungi. In addition, he has brought together a stellar group of international experts to write the chapters. As a result of his efforts, *Industrial Mycology* presents up-to-date information on more than two dozen separate topics, thereby providing an invaluable reference for anyone involved in applied mycology. This book will guide researchers so that they can apply new scientific approaches for using fungi to solve many real world problems. It is intended to inspire as well as to inform. The pharmacological potential of fungi is immense and awaits further exploitation.

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Preface

Fungi are nutrition-absorptive eukaryotic organisms found in every ecological niche. Because of their simplistic and rapid life cycles, yet complex genetics, metabolism, and morphology, fungi have been studied in the laboratory as model systems (such as *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Neurospora crassa*) in every aspect of the biological sciences. The economic importance of fungi is equally impressive, with uses in the food and beverage industries, agriculture, the pharmaceutical and agrochemical industries, and medicine. The significance of fungi as both laboratory model organisms and industrial processes is reflected by the large number of specialized disciplines dedicated to the study of mycology, such as plant pathology, medical mycology, food mycology, environmental mycology, and industrial mycology. This book provides an overview of recent developments in industrial mycology, with emphasis on discovery of bioactive metabolites, biocatalysts, and the underlying genetics of fungi.

Fungi are of interest to the pharmaceutical and agrochemical industries because of the diverse bioactive metabolites produced by these organisms. Since the treatment of bacterial infections using partially purified penicillin two-thirds of a century ago, bioactive fungal metabolites have strongly influenced the development of the modern pharmaceutical and agrochemical industries. Mevinolin, cyclosporin A, β -lactam antibiotics, pneumocandins, ergotamine, strobilurins, and mycophenolic acid are examples of revolutionary pharmaceuticals and agrochemicals that have a fungal origin. Chapter 3 provides a comprehensive review of the bioactive fungal metabolites discovered to date. The isolation of sordarins is one example to illustrate the complexity of the bioactive metabolite discovery process (Chapter 11). In spite of the success of bioactive fungal metabolites as pharmaceuticals and agrochemicals, fungi remain an essentially untapped source of medicines because only a small fraction of the vast fungal kingdom has been explored for bioactive metabolite production. The potential for discovering new bioactive metabolites from fungi is unlimited.

The industrial discovery of bioactive fungal metabolites is a complex, integrated, but somewhat empirical process. Major steps in this process include: 1) collection and identification of natural material (Chapter 6); 2) chemical extraction (Chapter 8); in vitro assay for biological activities (Chapter 10); 4) isolation and structural elucidation of the active components from the extracts (Chapter 8); 5) characterization of the in vivo biologi-

cal efficacy and toxicity profiles; and 6) strain and fermentation improvement for higher production titer and reduction of undesirable side products (Chapters 19 and 20). Although one of most critical steps in fungal natural products drug discovery, *in vivo* biological efficacy and toxicity profiling of fungal metabolites is not addressed in this book because it is mostly a mammalian clinical issue, and not a mycological one, which is the focus of this book.

Industrial fungal bioactive metabolite discovery has been largely an empirical process and this empirical approach has been and will continue to be effective. However, recent advances in the genetics of microbial secondary metabolite biosynthesis, genomics, and metabolic engineering will play an ever-increasing role in facilitating fungal bioactive metabolites discovery. This book attempts to provide readers with comprehensive discussions on the genetics of fungal secondary metabolism (Chapters 12B18). Another major advance in fungal bioactive metabolite discovery lies in detection and prediction methods, such as chemometric and genetic methods (Chapter 9). Genetic engineering will also help to expand the recovery of bioactive metabolites and enzymes from unculturable and difficult-to-grow fungi by expressing secondary metabolite-encoding and enzyme-encoding genes and gene clusters in heterologous hosts (Chapter 7).

To serve as a stand-alone reference book, overviews of industrial mycology (Chapter 1), the fungal kingdom (Chapter 2), fungal cell cycle control (Chapter 4), and fungal genetic transformation (Chapter 5) are included. Several chapters addressing other industrial mycology topics are also included: industrial fungal bioconversions (Chapter 21), metabolomics (Chapter 22), engineering of fungal metabolic biosynthetic pathways (Chapter 23), heterologous protein and enzyme expression in fungi (Chapter 24), mycotoxin (Chapter 25), and fungi as biocontrol agents (Chapter 26).

Finally, I would like to express my gratitude to Dr. Joan Bennett for the opportunity to contribute to the Mycology series, and I am indebted to the expert authors who agreed to contribute to this endeavor. I also want to thank Ms. Anita Lekhwani and Joseph Cacciottoli of Marcel Dekker, Inc., for their dedicated assistance throughout the project.

Zhiqiang An

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1

Industrial Mycology: Past, Present, and Future

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1. INTRODUCTION

1.1. Background

The versatility of fungal biosynthesis is enormous. The use of yeasts dates back to ancient days. Before 7000 B.C., beer was produced in Sumeria. Wine was made in Assyria in 3500 B.C. and ancient Rome had over 250 bakeries which were making leavened bread by 100 B.C. Milk has been made into Kefyr and Koumiss using *Kluyveromyces* in Asia for many centuries. Today, fungi are producers of five leading fermentation products in terms of tons per year worldwide. These are beer (60 million), wine (30 million), citric acid (900 thousand), single cell protein and fodder yeast (800 thousand), and baker's yeast (600 thousand). Fungi are also used in many other industrial processes, such as the production of enzymes, vitamins, polysaccharides, polyhydric alcohols, pigments, lipids, and glycolipids. Some of these products are produced commercially while others are potentially valuable in biotechnology. Fungal secondary metabolites are extremely important to our health and nutrition and have tremendous economic impact. The antibiotic market, which includes fungal products (Fig. 1), amounts to almost 30 billion dollars and includes about 160 antibiotics. Other important pharmaceutical secondary metabolites produced by fungi are hypocholesterolemic agents and immunosuppressants, some having markets of over 1 billion dollars per year. In addition to the multiple reaction sequences of fermentations,

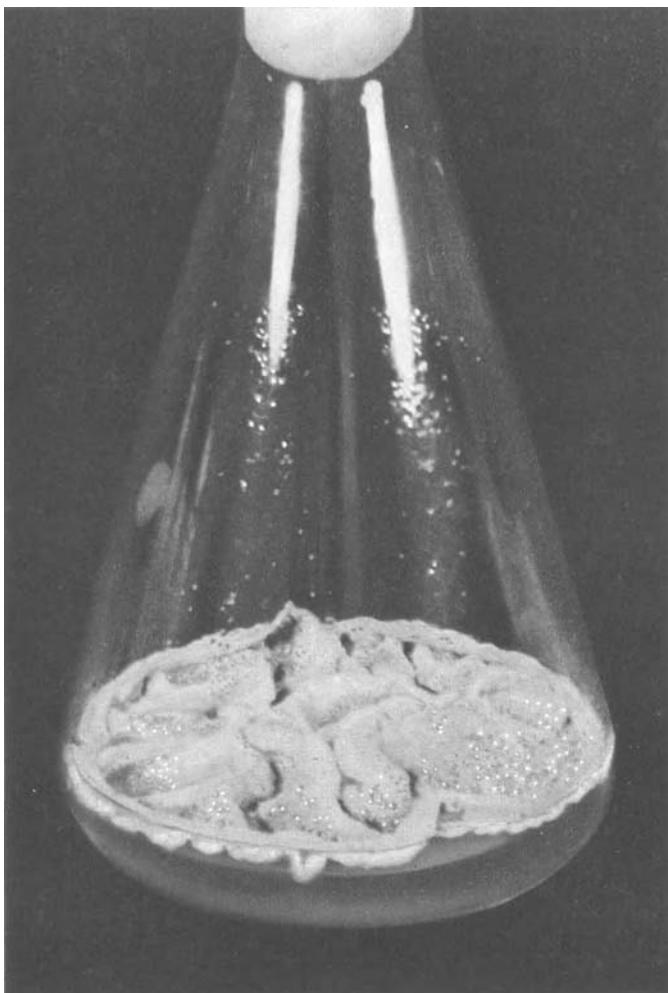


Figure 1 The mold *Penicillium notatum* growing in a flask of culture medium for the production of penicillin in the early days of the antibiotic age. (From Ref. 166.)

fungi are extremely useful in carrying out biotransformation processes. These are becoming essential to the fine chemical industry in the production of single-isomer intermediates. The molecular biotechnology industry has made a major impact in the business world, biopharmaceuticals (recombinant protein drugs, vaccines, and monoclonal antibodies) having a market of 15 billion dollars. Recombinant DNA technology, which includes yeasts and other fungi as hosts, has also produced a revolution in agriculture and has markedly increased markets for microbial enzymes. Molecular manipulations have been added to mutational techniques as means of increasing titers and yields of microbial processes and in discovery of new drugs. Today, fungal biology is a major participant in global industry. The best is yet to come as fungi move into the environmental and energy sectors.

1.2. Overview

Microorganisms are important to us for many reasons, but the principal one is that they produce things of value to us. These may be very large materials such as proteins, nucleic acids, carbohydrate polymers, or even cells, or they can be smaller molecules, which we usually separate into metabolites essential for vegetative growth and those inessential—that is, primary and secondary metabolites, respectively. The power of the microbial culture in the competitive world of commercial synthesis can be appreciated by the fact that even simple molecules such as lactic acid are made by fermentation rather than by chemical synthesis. Most natural products are made by fermentation technology because they are complex and contain many centers of asymmetry. For the foreseeable future, they probably will not be made commercially by chemical synthesis.

Although microbes are extremely good in presenting us with an amazing array of valuable products, they usually produce them only in amounts that they need for their own benefit; thus, they tend not to overproduce their metabolites. Regulatory mechanisms have evolved in microorganisms that enable a strain to avoid excessive production of its metabolites. Thus, they can compete efficiently with other forms of life and survive in nature. On the other hand, the fermentation microbiologist desires a “wasteful” strain that will overproduce and excrete a particular compound that can be isolated and marketed. During the screening stage, the microbiologist searches for organisms with weak regulatory mechanisms. Once a desired strain is found, a development program is begun to improve titers by modification of culture conditions, mutagenesis, and recombinant DNA technology. The microbiologist is actually modifying the regulatory controls remaining in the original culture so that its “inefficiency” can be further increased and the microorganism will excrete tremendous amounts of these valuable products into the medium. The main reason for the use of microorganisms to produce compounds that can otherwise be isolated from plants and animals or synthesized by chemists is the ease of increasing production by environmental and genetic manipulation. Thousand-fold increases have been recorded for small metabolites.

The importance of the fermentation industry resides in five important characteristics of microorganisms: (1) a high ratio of surface area to volume, which facilitates the rapid uptake of nutrients required to support high rates of metabolism and biosynthesis; (2) a tremendous variety of reactions that microorganisms are capable of carrying out; (3) a facility to adapt to a large array of different environments, allowing a culture to be transplanted from nature to the laboratory flask or the factory fermentor, where it is capable of growing on inexpensive carbon and nitrogen sources and producing valuable compounds; (4) the ease of genetic manipulation, both *in vivo* and *in vitro*, to increase productivity, to modify structures and activities, and to make entirely new products; and (5) an ability to make specific enantiomers, usually the active ones, in cases in which normal chemical synthesis yields a mixture of active and inactive enantiomers.

2. PRODUCTION OF FUNGAL PRIMARY METABOLITES

2.1. Alcohols

Ethyl alcohol is a primary metabolite that can be produced by fermentation of a sugar, or a polysaccharide that can be depolymerized to a fermentable sugar. Yeasts are preferred for these fermentations, but the species used depends on the substrate employed. *Saccharomyces cerevisiae* is employed for the fermentation of hexoses, whereas *Kluyveromyces*

fragilis or *Candida* sp. may be utilized if lactose or pentoses, respectively, are the substrates. Under optimum conditions, approximately 10% to 12% ethanol by volume is obtained within 5 days. Such a high concentration slows growth and the fermentation ceases. With special yeasts, the fermentation can be continued to alcohol concentrations of 20% by volume. However, these concentrations are attained only after months or years of fermentation. Ethanol is mainly manufactured by fermentation at a level of 4 million tons per year. Because of the elimination of lead from gasoline in the United States, ethanol is being substituted as a blend to raise gasoline's octane rating. Production of ethanol from starchy crops (mainly corn) reached 1.6 billion gallons in the United States in 2000 [1], whereas in Brazil ethanol produced from cane sugar amounted to 12.5 billion L/yr being used either as a 22% blend or as a pure fuel [2].

With regard to beverage ethanol, some 60 million tons of beer and 30 million tons of wine are produced each year. The fungi used for alcoholic products are wine yeasts (including special flocculent strains for the production of champagne and film-forming strains for the production of flor sherry), sake yeast, top- and bottom-fermenting brewing strains (varying in the degree of flocculation occurring during fermentation), and distiller's strains for alcohol production from cereal starch. Strain improvement by genetic manipulation and protoplast fusion has contributed superior strains for the above processes. About 2 million tons of yeast are produced for the distilling, brewing, and baking industries each year.

Production of glycerol is usually done by chemical synthesis from petroleum feedstocks, but good fermentation processes are available [3]. Osmotolerant yeast strains (*Candida glycerinogenes*) can produce up to 137 g glycerol of per liter with a yield of 65% based on consumed glucose and a productivity of 32 g/L/day [4]. In China, 10,000 tons per year are made by fermentation, which amounts to 12% of the country's need.

Osmotic pressure increase was found to raise volumetric and specific production of the noncariogenic, noncaloric, and diabetic-safe sweetener erythritol but to decrease growth [5]. Production by a *Candida magnoliae* osmophilic mutant yielded 187 g/L, a rate of 2.8 g/L/hr and 41% conversion from glucose [6]. Previous processes were done with *Aureobasidium* sp. (48% yield) and the osmophile *Trichosporon* sp. (1.86 g/L/hr and 45% conversion). Erythritol can also be produced from sucrose by *Torula* sp. at 200 g/L after 120 hours, with a yield of 50% and a productivity of 1.67 g/L/hr [7].

2.2. Organic Acids

The market for citric acid amounts to \$1.4 billion per year, with a production level of 1.6 billion pounds [8]. Citric acid is easily assimilated, is palatable, and has low toxicity. Consequently, it is widely used in the food and pharmaceutical industry. It is employed as an acidifying and flavor-enhancing agent, an antioxidant for inhibiting rancidity in fats and oils, a buffer in jams and jellies, and a stabilizer in a variety of foods. The pharmaceutical industry uses approximately 16% of the available supply of citric acid.

Aspergillus niger has an inherent capacity to excrete organic acids during aerobic growth. In 4 to 5 days, the major portion (80%) of the sugar is converted to citric acid, titers reaching about 100 g/L. Another factor contributing to high citric acid production is the low pH optimum (1.7–2.0). Mutants of *A. niger* resistant to low pH (<2) are improved citric acid producers [9]. Another selective tool is resistance to high concentrations of citrate and sugar [10,11]. Citric acid exerts negative feedback regulation on its own biosynthesis in *A. niger* at concentrations above 10 g/L.

Other valuable organic acids include acetic, lactic, malic, gluconic, fumaric, itaconic, tartaric, pyruvic, and succinic acids. The global market for lactic acid is more than 100,000 tons per year [12], and its price is \$1.05 per pound [8]. *Rhizopus oryzae* is favored for production since it makes stereochemically pure L-(+)-lactic acid. Lactic acid was made at one time by chemical synthesis but this process has been totally eliminated in favor of fermentation. It is produced anaerobically with a 95% (w/w) yield based on charged carbohydrate, a titer of over 100 g/L, and a productivity of over 2 g/L/hr. Gluconic acid has a market of \$93 million, a price of \$0.85 per pound, and a production level of 40,000 tons per year [8]. Pyruvic acid production amounted to 69 g/L in a 56-hour fermentation, with a yield of 0.62 g/g of glucose using *Torulopsis glabrata* [13]. From 120 g/L of glucose, *Rhizopus arrhizus* produces 97 g of fumaric acid per liter [14]. Itaconic acid is used as a co-monomer in resins and synthetic fibers and is also used in coatings, adhesives, thickeners, and binders [15]. It is made by fungi (*Aspergillus* sp.) at 15,000 tons per year and sells for \$4 per kilogram. It is produced from raw corn starch at 1 g/L/hr and a concentration of 76 to 80 g/L with a 55% yield [16]. Synthetic processes are not competitive with the fungal process. *Aspergillus terreus* is the main producer of itaconic acid, but certain *Candida* sp. produce 42 g/L. Itaconic acid has an annual market of \$68 million [17].

2.3. Vitamins

Carotenoids are pigmented natural products with antioxidant activity, essential for all forms of life. They include vitamin A precursors, colorants of flowers, fruits, and vegetables, and are used in cosmetics and foods. The market for carotenoids was \$750 to \$800 million in 1999. About 100 tons of β-carotene are annually produced by chemical synthesis as well as fungal (*Blakeslea trispora*) and algal (*Dunaliella salina* and *Dunaliella bardawil*) fermentation [18]. Riboflavin (vitamin B₂) was produced commercially for many years by both fermentation and chemical synthesis [19], but today fermentation is the major route. Riboflavin overproducers include two yeastlike molds, *Eremothecium ashbyii* and *Ashbya gossypii* [20], which synthesize riboflavin in concentrations greater than 20 g/L. Annual production of riboflavin by the three most prominent producing companies (Roche, BASF, and ADM) is over 3000 tons per year [21]. The production of the vitamin F group (polyunsaturated fatty acids) is mainly by fermentation of different strains of the fungi *Mortierella isabellina* and *Mucor circinelloides*, which can accumulate up to 5 g/L of γ-linoleic acid [18]. Two related species, *Mortierella alpina* and *Mortierella alliacea*, are able to produce dihomo-γ-linoleic acid and, more importantly, arachidonic acid in concentrations of 7 to 11 g/L [22,23].

2.4. Other Applications

Candida utilis is the most widely used species for the production of feed yeast for animal diets. Its ability to grow on cheap substrates not suitable for most other yeasts has made it the organism of choice for this purpose. *C. utilis* requires no added vitamins, can utilize ammonium or nitrate nitrogen, can grow on many carbon sources (including cellobiose and D-xylose), and grows at 37°C. Crude substrates, such as sulfite waste liquor (a byproduct of the paper industry) or wood hydrolysate, are used for its propagation.

In the nutritional area, the content of methionine of the methylotrophic yeast *Candida boidinii* has been markedly increased (from 0.5 to 9 mg/g dry cell weight in the intracellular pool and from 6 to 16 mg/g as total cellular methionine) by mutation to ethionine resistance

[24]. A number of yeast products are useful in the flavor industry. Ethyl acetate and acetaldehyde are produced by *C. utilis*, esters and other volatiles are made by *Kluyveromyces lactis*, and monoterpenes are formed by *Ambrosiozyma monospora*. Terpenes and lactones are produced by *K. lactis*, *Saccharomyces fermentati*, and *Sporobolomyces adorus* [25].

A number of extracellular polymers from fungi have shown potential utility in foods or are used in industrial processes. The attractiveness of microbial polysaccharides is due in part to their differential stabilities and physical behaviors in solution. Several species of *Hansenula* produce copious quantities of extracellular phosphomannans [26] from glucose under aerobic conditions. In spite of attractive viscosity and gel properties for possible use as food additives, application of these water-soluble gums has been hampered because of their sensitivity to salts, shear, and heat, as well as their instability at low pH [27]. A second example is the production of the glucan pullulan by strains of the dimorphic yeastlike fungus *Aureobasidium pullulans*. Fermentation yields of pullulan from starch hydrolysates in 10% concentration have been reported to be as high as 75% [26]. Applications of pullulans include use as a flocculator of clay slimes in hydrometallurgical processes, in coating and packing material for foodstuffs and pharmaceuticals, in adhesives, and in the manufacture of special fibers and fabrics.

3. PRODUCTION OF ENZYMES

Enzymes are valuable in manufacturing because of their rapid and efficient action at low concentrations under mild pH values and temperatures, their high degree of substrate specificity (which reduces side-product formation), their low toxicity, and the ease of stopping their action by mild treatments. Some microbial strains produce very high concentrations of extracellular enzymes (e.g., strains of *Aspergillus* produce 20 g/L of glucoamylase [28]). Enzyme levels can be increased by environmental and genetic manipulations. Thousand-fold increases have been recorded for catabolic enzymes, and biosynthetic enzymes have been increased several hundred-fold.

Additional reasons for using microbial cells as sources of enzymes are as follows: (1) enzyme fermentations are quite economical on a large scale due to short fermentation cycles and inexpensive media; (2) screening procedures are simple and thousands of cultures can be examined in a reasonably short time; and (3) different species produce somewhat different enzymes catalyzing the same reaction, allowing flexibility with respect to operating conditions in the reactor.

In the last decade, fungal enzymes have been increasingly used for applications that traditionally employed plant and animal enzymes. These shifts include the partial replacement of (1) amylases of malted barley and wheat by amylases from *Aspergillus* in the beer, baking, and textile industries; (2) plant and animal proteases by *Aspergillus* protease for chill-proofing beer and meat tenderization; (3) pancreatic proteases by *Aspergillus* for leather tanning and in detergent preparations; and (4) rennet (chymosin) by *Mucor* for cheese manufacture.

The major application of microbial enzymes has been the use of bacterial glucose isomerase in conjunction with fungal α -amylase and glucoamylase to convert starch to mixtures of glucose and fructose known as "high-fructose corn syrup" [29]. Yeast enzymes extremely useful in industry include invertase from *K. fragilis*, *Saccharomyces carlsbergensis*, and *S. cerevisiae* for candy and jam manufacture, β -galactosidase (lactase) from *K. fragilis* or *K. lactis* for hydrolysis of lactose from milk or whey, and α -galactosi-

dase from *S. carlsbergensis* for crystallization of beet sugar. Inulinase from various yeasts has the capability to produce fructose in high concentration from inulin in Jerusalem artichokes and chikory.

The U.S. and European enzyme market—mainly made up of protease, amylase, glucoamylase, microbial rennet, and glucose isomerase—amounted to 700 million dollars in 1987, and microbial enzymes accounted for 400 million of that total. The main problem that retarded rapid development of enzyme technology in the 1980s was the poor availability of enzymes for large scale application testing. Only about 10% of the 2000 to 3000 enzymes described in the literature were commercially available, and over half of these sold for more than 1 million dollars per pound [30]. However, this situation improved, and the market reached \$1.6 billion in 1998 [31], divided into the following application areas: food, 45% (of which starch processing represented 11%); detergents, 34%; textiles, 11%; leather, 3%; and pulp and paper, 1.2%. The market continued to increase and reached \$2 billion in 2000, not even including diagnostic and therapeutic enzymes.

Fungi can be used for a number of bioconversions [32], including ketoreduction, oxidation of hydroxyl groups, ester hydrolysis and hydrogenation of double bonds in steroids, and conversions of alkaloids and xenobiotics. Also, L-lysine can be produced from L-aminocaprolic acid, and L-phenylalanine from *trans*-cinnamic acid. Other bioconversions include *n*-tetradecene to *t*-tetradecene and phenol degradation.

4. FUNGAL SECONDARY METABOLITES

Microbially produced secondary metabolites are extremely important to our health and nutrition [33]. As a group that includes antibiotics, other medicinals, toxins, pesticides, and animal and plant growth factors, they have tremendous economic importance. In batch or fed-batch culture, secondary metabolites are usually produced after growth has slowed down. They have no function in growth of the producing cultures, are produced by certain restricted taxonomic groups of organisms, and are usually formed as mixtures of closely related members of a chemical family. In nature, secondary metabolites are important for the organisms that produce them, functioning as (1) sex hormones; (2) ionophores; (3) competitive weapons against other bacteria, fungi, amoebae, insects and plants; (4) agents of symbiosis; and (5) effectors of differentiation [34]. A great many of these secondary metabolites are produced by fungi.

4.1. Antibiotics

Antibiotics are defined as low-molecular-weight organic natural products made by microorganisms that are active at low concentration against other microorganisms. Of the 12,000 antibiotics known in 1995, 22% could be produced by filamentous fungi [35,36]. The most important are the β -lactam antibiotics. These include the natural penicillin G and the biosynthetic penicillin V with a combined market of \$4.4 billion, many semisynthetic penicillins, and the semisynthetic cephalosporins, which have a market of \$11 billion. The search for new antibiotics continues in order to combat evolving pathogens, naturally resistant microbes, and previously susceptible bacteria and fungi that have developed resistance. Other desirable properties of new antibiotics include improved pharmacological properties, the ability to combat tumors, viruses, and parasites, and improved safety and potency. In the pursuit of effective antibiotics, many of the new products are made chemically by modification of natural antibiotics in a process called “semisynthesis.” The

antibiotic market includes about 160 antibiotics and derivatives, such as the β -lactam peptide antibiotics and others [36,37]. The global market for finished antibiotics reached \$35 billion in 2000.

4.2. Pharmacological Agents

In the early years, the major pharmaceuticals used for noninfectious diseases were strictly synthetic products. Similarly, major therapeutics for nonmicrobial parasitic diseases in animals came from the screening of chemically synthesized compounds followed by molecular modification. Despite the testing of thousands of synthetic compounds, only a few promising structures were uncovered. As new lead compounds became more and more difficult to find, microbial broths filled the void and microbial products increased in importance in therapy of nonmicrobial diseases. Many of the following fungal compounds were first isolated as poor or toxic antibiotics (e.g., cyclosporin A) or as mycotoxins (ergot alkaloids, gibberellins, zearelanone) before they were put to work for medicine and agriculture [38].

4.2.1. Immunosuppressive Agents

Cyclosporin A was originally discovered as a narrow-spectrum antifungal peptide produced by the mold *Tolypocladium nivenum* (previously *Tolypocladium inflatum*) [39]. Discovery of immunosuppressive activity led to its use in heart, liver, and kidney transplants and to the overwhelming success of the organ transplant field. Sales of cyclosporin A have reached \$1.5 billion. A very old broad-spectrum fungal antibiotic, mycophenolic acid, was never commercialized as an antibiotic, but its 2-morpholinoethyl ester was approved as a new immunosuppressant for kidney transplantation in 1995 and for heart transplants in 1998. The ester is called mycophenolate mofetil (CellCept[®]) and is a prodrug that is hydrolyzed to mycophenolic acid in the body.

4.2.2. Hypocholesterolemic Agents

Only 30% of the cholesterol in the human body comes from the diet. The remaining 70% is synthesized by the body, mainly in the liver. Many people cannot control their cholesterol at a healthy level by diet alone but must depend on hypocholesterolemic drugs. One huge success has been the fungal statins—including lovastatin (mevinolin), pravastatin and others [40]—which act as inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the regulatory and rate-limiting enzyme of cholesterol biosynthesis in liver. Statins have a very large market of \$15 billion. All members of the group are substituted hexahydronaphthalene lactones. Brown et al. [41] discovered the first member of this group, compactin (ML-236B) as an antibiotic product of *Penicillium brevicompactum*. Endo et al. [42] discovered compactin in broths of *Penicillium citrinum*, and later Endo [43] and Alberts et al. [44] independently discovered the more active methylated form of compactin known as lovastatin (monacolin K; mevinolin) in broths of *Monascus ruber* and *A. terreus*, respectively. Lovastatin exists in two forms. The majority (90%) is the acid (open) form, which is active; the minor part is the inactive lactone. Lovastatin was approved by the U.S. Food and Drug Administration (FDA) in 1987.

4.2.4. Antitumor Agents

Most natural anticancer agents are made by actinomycetes, but a recent successful nonactinomycete molecule is Taxol[®] (paclitaxel). This compound was originally discovered in

plants [45] but is also produced by the fungus *Taxomyces andreanae* [46]. It is approved for breast and ovarian cancer and is the only commercial antitumor drug known to act by blocking depolymerization of microtubules. In addition, Taxol promotes tubulin polymerization and inhibits rapidly dividing mammalian cancer cells [47]. In 2000, Taxol sales amounted to over \$1 billion for Bristol-Myers Squibb, representing 10% of the company's pharmaceutical sales and its third largest selling product [48].

4.4.5. Mycotoxins as Sources of Useful Agents

It is difficult to accept the fact that even poisons can be harnessed as medically useful drugs, yet this is true for the ergot alkaloids. These mycotoxins were responsible for fatal poisoning of humans and animals (ergotism) throughout the ages after consumption of bread made from grain contaminated with species of *Claviceps*. It is amazing but true that these "poisons" are now used for angina pectoris, hypertonia, migraine headache, cerebral circulatory disorder, uterine contraction, hypertension, serotonin-related disturbances, inhibition of prolactin release in agalactorrhea, reduction in bleeding after childbirth, and prevention of implantation in early pregnancy [49,50]. Among their physiological activities are the inhibition of action of adrenalin, noradrenalin, and serotonin and the contraction of smooth muscles of the uterus. Some of the ergot alkaloids also possess antibiotic activity.

Ergot alkaloids are not the only successful example. The genus *Gibberella* is responsible for two others, zearelanone and gibberellins. Zearelanone, produced by *Gibberella zaeae* (syn. *Fusarium graminearum*) [51], is an estrogen, and its reduced derivative zearanol is used as an anabolic agent in cattle and sheep, increasing both growth and feed efficiency. Gibberellic acid, a member of the phytotoxic mycotoxin group known as the gibberellins, is produced by *Gibberella fujikuroi* and is the cause of the "foolish rice seedling" disease of rice [52]. Gibberellins are used to speed up barley malting, improve malt quality, increase the yield of vegetables, and reduce by half the time required to obtain lettuce and sugar beet seed crops. They are isoprenoid growth regulators controlling flowering, seed germination, and stem elongation [53]. They are produced at a level of over 25 tons per year and have a market of \$100 million.

4.3. Pigments

Fermentation of *Monascus purpureus* on rice to prepare koji or ang-kak (red rice) has been used as a traditional Chinese food and medicine since 800 AD [54]. The water-soluble red pigments, monascorubramine and rubropunctamine, are produced by reaction of the orange pigments, monascorubrin and rubropunctatin, with amino acids present in the fermentation media [55]. The fungus is used for preparing red rice, wine, soy bean cheese, meat, and fish, and is authorized for food use in China and Japan.

The yeast *Phaffia rhodozyma* has become the most important microbial source for the production of the carotenoid astaxanthin [56]. This pigment is responsible for the orange to pink color of salmonid flesh and the reddish color of boiled crustacean shells. Feeding of pen-reared salmonids with a diet containing this yeast induces pigmentation of the white muscle [57,58]

5. REGULATION OF FUNGAL SECONDARY METABOLISM

The intensity of secondary metabolism can often be increased by addition of limiting precursors such as phenylacetic acid in the production of penicillin G. Most secondary

metabolites are formed via enzymatic pathways rather than by a ribosomal mechanism. The enzymes occur as individual proteins, free or complexed, or as parts of modules of large multifunctional polypeptides carrying out a multitude of enzymatic steps, such as in the cases of polyketide synthases and peptide synthetases. The genes encoding the enzymes involved in secondary metabolism are usually chromosomal. Whether chromosomal or plasmid-borne, the secondary metabolism genes are often clustered, but not necessarily as single operons. Clusters of fungal biosynthetic genes have been found encoding biosynthetic genes for production of penicillins and cephalosporins [59], sterigmatocystin [60] by *Aspergillus nidulans*, and trichothecenes [61] by *Fusarium sporotrichioides*.

5.1. Regulation by the Carbon Source

Glucose, usually an excellent carbon source for growth, often interferes with the formation of secondary metabolites (Table 1). Polysaccharides (e.g., starch), oligosaccharides (e.g., lactose), and oils (e.g., soybean oil, methyloleate) are often preferable for fermentations yielding secondary metabolites [34]. In media containing a mixture of a rapidly used and a slowly used carbon source, the former is used first to produce cells, but little to no secondary metabolite is formed. After the rapidly assimilated compound is depleted, the “second-best” carbon source is used for the production phase, known as the “idiophase.”

5.2. Regulation by the Nitrogen Source

Nitrogen regulation is of wide significance in microbiology, affecting both primary metabolism [62] and secondary metabolism [63]. The control of enzyme synthesis is generally exerted by the intracellular nitrogen pool. Many secondary metabolic pathways are negatively affected by nitrogen sources favorable for growth (e.g., ammonium salts). As a result, complex fermentation media often include a protein source and defined media include a slowly assimilated amino acid as the nitrogen source to encourage high production of secondary metabolites. Processes subject to nitrogen source regulation are shown in Table 2. Information concerning the mechanism(s) underlying the negative effect(s) of ammonium and certain amino acids on industrial processes is scarce.

There is also a more specific type of control in which a particular amino acid (or biosynthetic group of amino acids) represses and/or inhibits production of a secondary metabolite because the primary metabolite(s) and the idiolite are derived from the same branched pathway and the amino acid(s) exerts negative feedback regulation on the biosyn-

Table 1 Carbon Sources Interfering with Secondary Metabolism

Idiolite	Interfering Carbon Source	Noninterfering Carbon Source	Target
Benzodiazapine alkaloids	Glucose	Sorbitol, mannitol	
Cephalosporins	Glucose, glycerol	Sucrose, galactose	Expandase, acetylhydrolase
Ergot alkaloids	Glucose	Polyols, organic acids	
Penicillin	Glucose, fructose, galactose, sucrose	Lactose	

Table 2 Nitrogen Sources Interfering with Secondary Metabolism

Idiolite	Interfering Nitrogen Source	Noninterfering Nitrogen Source
Aflatoxin	Nitrate	NH_4^+
Alternariol	Nitrate, L-glutamate, urea	
Bikaverin	Glycine	
Cephalosporin	NH_4^+ , L-lysine	L-asparagine, L-arginine
Gibberellic acid	NH_4^+ , glutamine	
Macbecin	L-tryptophan, p-amino- benzoate, anthranilate	
Patulin	NH_4^+	
Penicillin	NH_4^+ , L-lysine	L-glutamate
Trihydroxytoluene	NH_4^+	

thetic pathway before the branch point. An example is the negative effect of lysine on penicillin synthesis, which is caused by lysine inhibiting homocitrate synthase [64], an enzyme involved in the formation of the penicillin precursor, L- α -amino adipic acid.

5.3. Regulation by the Phosphorus Source

Regulation by phosphorus sources, as occurs in the formation of bikaverin, ergot alkaloids, and cephalosporin C, includes both specific and general controls. A rather specific negative effect of inorganic phosphate arises from its ability to inhibit and/or repress phosphatases. Because biosynthetic intermediates of certain pathways are phosphorylated, whereas the ultimate product is not, phosphatases are sometimes required in biosynthesis. Although only little is known about the mechanism of general phosphate control of secondary metabolism, there is a strong possibility that phosphate regulation also works by affecting enzyme activities such as phosphorylation by protein kinases and dephosphorylation by phosphoprotein phosphatases [65]. Phosphate also appears to interfere in many secondary metabolic pathways not known to have phosphorylated intermediates. In general, phosphate-sensitive fermentations have to be conducted at low levels of free phosphate (usually below 10 μM), which are suboptimal for growth.

5.4. Regulation by Metals

Metals often exert control over secondary metabolism. Zinc stimulates aflatoxin production, and manganese increases patulin production via an effect on transcription [66]. Studies by Lin and Demain [67] have shown red pigment formation by *Monascus* to be increased by Zn^{2+} , Mn^{2+} , and Fe^{2+} via stimulation of synthase action rather than an effect on induction or enzyme stabilization. On the other hand, Mg^{2+} inhibited pigment synthase action. Optimal production of pneumocandins by *Zalerion arboricola* occurs under magnesium-limiting conditions [68], which slow down assimilation of the carbon source mannitol and allow it to remain for a longer period, thus extending the duration of antibiotic production.

5.5. Induction of Secondary Metabolite Synthases

In a number of secondary metabolic pathways, primary metabolites increase production of the final product. These effectors are often precursors, and one has to determine whether the effect is merely due to an increase in precursor supply or includes induction of one or more synthases of the biosynthetic pathway. Stimulatory precursors that are also inducers include tryptophan for dimethylallyltryptophan synthetase in ergot alkaloid biosynthesis [69], phenylalanine in benzodiazapene alkaloid formation [70], methionine for δ -(L- α -aminoadiphyll)-L-cysteine-L-valine synthetase (ACVS), cyclase and expandase in the cephalosporin pathway of *Acremonium chrysogenum* [71,72], and phenylacetate for the phenylacetate uptake system in *Penicillium chrysogenum* involved in penicillin G formation [73].

5.6. Control by Growth Rate

Growth rate control is important in secondary metabolism and may be the overriding factor in the cases where nutrient limitation is needed for production of secondary metabolites. In the production of patulin by *Penicillium urticae*, the shift from the growth phase (tropho-phase) to idiophase occurs at the point of nitrogen source depletion, when growth slows down and formation of the first enzyme, 6-methylsalicylic acid synthase, is derepressed. The resultant 6-methylsalicylic acid induces transcription of genes encoding later enzymes in the pathway. Derepression also occurs when the growth rate is reduced by other means. Thus, it appears that low growth rate is more important than nitrogen limitation [74]. *G. fujikuroi* produces bikaverin at a growth rate of 0.05 h^{-1} but forms gibberellin at 0.01 h^{-1} [75].

5.7. Fungal Regulatory Genes

Clustering of fungal genes is not common except in cases of assimilation of certain nutrients (e.g., proline, quinate, ethanol, nitrate) and production of secondary metabolites [76]. Regulation of pathways in fungi (mainly studied in *A. nidulans*) can be narrow or broad domain regulation [77]. Narrow domain regulation usually involves a positively acting pathway-specific regulatory protein containing a zinc binuclear cluster: $\text{CX}_2\text{CX}_6\text{CX}_6\text{CX}_2\text{CX}_6\text{CX}_2$. Broad domain control employs the positively acting nitrogen regulatory gene *areA* [78] and/or a negatively acting carbon repressor gene, *creA* [79].

5.8. Feedback Regulation

The role of feedback regulation in controlling secondary metabolism is well known. Many secondary metabolites inhibit or repress their own biosynthesis. Ergot alkaloids inhibit the first enzyme of their biosynthetic pathway, dimethylallyl tryptophan synthetase, whereas in the pathway to mycophenolic acid, the final enzyme, an O-methyltransferase, is inhibited.

5.9. Enzyme Decay

Production of secondary metabolites eventually stops due to feedback regulation (see above) and decay of the synthase(s). Cessation of patulin production by *P. urticae* is caused by decay of the initial enzyme, 6-methylsalicylic acid synthase, which has an *in vivo* half-maximal lifetime of 7 hours [80].

6. MOLECULAR FUNGAL BIOTECHNOLOGY

The development of molecular biology techniques has provided new ways to use yeasts and molds as microbial cell factories for the production of food ingredients and heterologous (especially mammalian) proteins. The choice of the host strain for protein production is made on the basis of production yields and regulatory issues, especially for fungi used in the food industry. Host strains are usually chosen from among those which have attained the so-called GRAS (Generally Recognized as Safe) status by the FDA. Several species of fungi have that status and are currently being used for large-scale production of recombinant proteins [81]. Four fungal genomes have been sequenced—*S. cerevisiae* [82], *Schizosaccharomyces pombe* [83], *Aspergillus niger* [84], and *Neurospora crassa* [85]—and sequencing of three others are in progress (*A. fumigatus*, *A. nidulans*, and *Candida albicans*) [86].

6.1. Production of Recombinant Pharmaceutical Polypeptides

Since it is a food organism, *S. cerevisiae* is considered to be a safe host for the production of pharmaceutical proteins. This yeast can be grown rapidly in simple media and to a high cell density, it can secrete heterologous proteins into the extracellular broth, and its genetics are more advanced than any other eukaryote [87]. Mammalian genes cloned and expressed in *S. cerevisiae* include human interferon [88,89], human epidermal growth factor [90], human hemoglobin [91], human superoxide dismutase [92], and interleukin-6 [81]. The most commercially important yeast recombinant process has been the production of the genes encoding surface antigens of the hepatitis B virus resulting in the first safe hepatitis B vaccine [93,94]. Despite these successful examples, *S. cerevisiae* is sometimes regarded as a less than optimal host for large-scale production of mammalian proteins because of certain drawbacks such as hyperglycosylation (which results in the addition of long chains of N-linked high-mannose oligosaccharides to the recombinant protein), presence of α -1,3-linked mannose residues that could cause antigenic response in patients, and absence of strong and tightly regulated promoters.

After the discovery in 1969 that some yeast species can grow on methanol [95], interest in using methanol as a pure, water-miscible, and relatively inexpensive substrate increased rapidly [96,97]. One of these species, *Pichia pastoris*, has become one of the most extensively used expression systems [87,98,99]. Among the advantages of this methyotrophic yeast over *S. cerevisiae* are (1) an efficient and tightly regulated methanol promoter (*AOX1*), which yields alcohol oxidase at 30% of soluble protein; (2) less extensive glycosylation, due to shorter chain lengths of N-linked high-mannose oligosaccharides, usually up to 20 residues lacking the terminal α -1,3-mannose linkages [100–102]; (3) integration of multicopies of foreign DNA into chromosomal DNA, yielding stable transformants [87,103]; (4) ability to secrete high levels of foreign proteins; and (5) high-density growth and straightforward scale-up [99,100]. Intracellular or extracellular recombinant products made in *P. pastoris* [87,98,100,104] include 6 to 10 g/L of tumor necrosis factor [101,105], 12 g/L of tetanus toxin fragment C [106], 1.25 g/L of the envelope protein of HIV-1 [107], 4 g/L of intracellular interleukin-2 [104], 4 g/L of secreted human serum albumin [104], and 0.5 g/L of human insulin-like growth factor [108]. However, one of the main drawbacks for this excellent expression system is the non-GRAS status of *P. pastoris*, although some products made by this yeast are being evaluated in phase

III clinical trials. One example is recombinant hirudin, a thrombin inhibitor from the medicinal leech *Hirudo medicinalis*, in which 1.5 g/L of secreted product was obtained [109].

Heterologous gene expression in the methylotrophic yeast *Hansenula polymorpha* is similar to that of *P. pastoris*. The promoter of the methanol oxidase gene is used to express foreign genes. As with *AOX1* in *P. pastoris*, the *MOX* gene in *H. polymorpha* is also highly expressed and tightly regulated, giving enzyme levels up to 37% of total cell protein [110]. One major difference is that expression of the *MOX* gene is significantly derepressed in the absence of glucose or during glucose limitation [111], and therefore tight regulation of the *MOX* promoter is lost in the high-glucose conditions usually used for high-biomass fermentations [112]. Expression levels of about 8 to 9 mg/L of intracellular hepatitis B middle surface antigen [113], 1 g/L of human serum albumin [114], and 1.5 g/L of secreted hirudin are achieved with this methylotrophic yeast.

The development of molecular techniques for production of recombinant heterologous proteins in filamentous fungi is laborious and contrasts markedly with the success achieved in yeasts. The ability to introduce or delete genes remains difficult although some advances in transformation have been recently reported, including restriction enzyme-mediated integration [115] or *Agrobacterium tumefaciens*-Ti plasmid-mediated transformation [116]. Levels of production of nonfungal proteins are low compared with those of homologous proteins. Several factors that influence production act at different levels (i.e., transcription, translation, secretion or extracellular degradation [81,117–119]). Different strategies have been developed to overcome these problems, including the construction of protease-deficient strains [120,121], introduction of a large number of gene copies [116,122], use of strong fungal promoters, efficient secretion signals [117,118,123], and gene fusions with a gene that encodes part of or an entire well-secreted protein [81,118]. The last-named strategy (i.e., gene fusion) remains the first choice in attempts to produce nonfungal proteins in fungal hosts. *A. niger* or *Aspergillus awamori* glucoamylase (*glaA*), *A. awamori* 1,4- β -endoxyylanase (*exlA*), *A. awamori* α -amylase (*amyA* and *amyB*), and *Trichoderma reesei* cellobiohydrolase I (*cbhI*) are genes cloned with this strategy [118]. Fusion has resulted in levels of secreted proteins of 5 mg/L of human interleukin-6 [124,125], 2 mg/L of human lysozyme [126], and 250 mg/L human lactoferrin [127]. Higher concentrations have been obtained for some of these proteins after mutagenic treatment of highly producing strains, such as human lactoferrin (5 g/L [128]) and chymosin (1 g/L) [129].

Several recent studies have shown the fungal secretory pathway to be a limiting factor in heterologous enzyme production. Studies on screening for mutant strains with altered secretion properties using green fluorescent protein as reporter [130], elucidation of the role of secretion-related chaperones and foldases [131–134], kinetic studies on protein secretion [135], and effect of hyphal branch frequency [136] are examples of the work being carried out to understand this complex process.

Almost all eukaryotic excreted polypeptides are glycosylated. Glycosylation is species, tissue, and cell-type specific [137]. For many proteins that have pharmaceutical applications, N-glycosylation is necessary for stability, proper folding (e.g., erythropoietin and human chorionic gonadotropin [hGC]), or pharmacokinetics [138]. Although O-linked glycosylation in yeast is quite different from that in higher eukaryotes, N-linked glycosylation is more conserved [87]. In yeast recombinant proteins, as well as in mammalian polypeptides, a core oligosaccharide unit (GlcNAc₂Man₉Glc₃) is present at the endoplasmic reticulum [139]. The three glucose residues and one mannose are removed, and

processing of the core oligosaccharide continues in the Golgi, where there is divergence between yeasts and higher eukaryotes. Recombinant yeast proteins usually show high-mannose side chains ($\text{GlcNAc}_2\text{Man}_{2-6}$), where elongation may take place in further addition steps. Mammalian proteins show two different types of oligosaccharide side chains: low-mannose residues ($\text{GlcNAc}_2\text{Man}_3$) plus additional residues of galactose, fucose, and sialic acid or a mixture of high-mannose and complex-type oligosaccharides [140]. Little research has been carried out on glycosylation in molds, although hyperglycosylation does not seem to occur and low-mannose side chains are formed [141–143]. The glycosylation of a protein can differ depending on factors such as the medium in which the cells are grown.

6.2. Recombinant Commercial Enzymes

Besides the production of some pharmaceutical heterologous proteins (see above), recombinant fungi are one of the main sources of enzymes used for industrial applications. Over 60% of the enzymes used in the detergent, food, and starch processing industries are recombinant products [144]. Although the number of heterologous fungal enzymes approved for food applications is not very large, the list is continuously increasing [145]. Because of the low yields achieved with nonfungal proteins (see above), many of the recombinant food-grade proteins are of fungal origin [119,146]. There is one exception in which the donor strain is not another fungus—calf rennin (chymosin) used for cheese making. Production of this bovine protein in recombinant *A. niger* var *awamori* amounted to about 1 g/L after nitrosoguanidine mutagenesis and selection for 2-deoxyglucose resistance [129]. Further improvement was done by parexual recombination, resulting in a strain producing 1.5 g/L from parents producing 1.2 g/L [147].

Microbial lipases have a tremendous potential in areas such as food technology, biomedical sciences, and chemical industries because they (1) are stable in organic solvents, (2) possess broad substrate specificity, (3) do not require cofactors, and (4) exhibit a high enantioselectivity [148–150]. In the food industry, lipases are commonly used in the production of different products (e.g., fruit juices and baked foods), in the production of desirable flavors in cheeses, and in the interesterification of fats and oils to produce modified acylglycerols. There are two fungal recombinant lipases currently used in this industry, one from *Rhizomucor miehi* and another from *Thermomyces lanuginosus*, both being produced in *A. oryzae* [146].

Lipases are also very important in the detergent industry. They are extensively used in household detergents, industrial cleaners, and leather processing, where they can be combined with proteases, oxidases, or peroxidases [151]. To be suitable, lipases should be alkalophilic and able to work at temperatures above 45°C and pH values of about 10. Besides, they should be capable of functioning in the presence of the various components of washing product formulations such as oxidants or surfactants. Novo-Nordisk was the first company to launch a product (Lipolase®) containing a recombinant fungal lipase. It was developed by genetic and protein engineering of the *Humicola lanuginose* lipase gene introduced into the *A. oryzae* genome for overproduction [151,152].

6.3. Brewing and Baking

Beer wort contains barley β -glucans that reduce the filtrability of beer and lead to precipitates and haze in the final product. The gene coding for endoglucanase was transferred

from *T. reesei* to brewer's yeast, and the engineered yeast strain efficiently hydrolyzed the β -glucans [153]. Similar technology has created starch-utilizing *S. cerevisiae* strains and wine yeast strains that produce lower acidity and enhanced flavor. Brewing yeasts have been modified by recombinant DNA technology so that they produce *A. niger* amyloglucosidase and break down unfermentable dextrins for light beer production [154]. The glucoamylase gene from *A. awamori* was cloned and expressed stably in polyploid distiller's yeast, a high level of glucoamylase being secreted. Ninety-five percent of the carbohydrates in the 25% starch substrate was utilized, and high levels of ethanol were produced. The engineered strain outperformed *Saccharomyces diastaticus* [155]. Brewing yeasts have been engineered to produce acetolactate decarboxylase from *Enterobacter aerogenes* or *Acetobacter aceti*. This enzyme eliminates both diacetyl and the requirement for a 3- to 5-week flavor maturation period, which normally follows a 1-week fermentation stage [156]. The resulting beer suffered no loss of quality and flavor. Lower acidity and enhanced flavor in wine has been achieved by transformation of wine yeast with the gene encoding the malolactic conversion enzyme from *Lactobacillus delbrueckii*. In 1990, a recombinant baker's yeast was approved for breadmaking in the United Kingdom; the genes amplified by recombinant DNA technology were melibiase and maltose permease. Despite these economic advancements, the recombinant yeasts were unfortunately not used because of fear of consumer reaction.

6.4. Production of Secondary Metabolites by Recombinant Fungi

Production of new fungal metabolites by application of recombinant DNA technologies is of great interest. Continued progress in the area of metabolic engineering has led to overproduction of limiting enzymes of important biosynthetic pathways, thereby increasing production of the final products. Major advances have been achieved in the antibiotic field, especially in the area of cephalosporins [157,158].

6.5. Other Products

Thaumatin, a protein from the plant *Thaumatococcus danielli* with an intense sweetness (about 3000 times sweeter than sucrose), has been recently approved as a food-grade ingredient (E-957). Successful expression of thaumatin was achieved in *Penicillium roqueforti* and *A. niger* var *awamori* [159] at yields of 2 to 7 mg/L. Recently, an impressive improvement in yield (up to 14 mg per liter) has been obtained in *A. niger* var *awamori* by use of stronger promoters and higher gene dosage [123]. Production of the sweetener xylitol has also been improved by transforming the *XYLI* gene of *Pichia stipitis* encoding a xylose reductase into *S. cerevisiae* [160].

Production of lactic acid in *S. cerevisiae* has been achieved by cloning and expression of a muscle bovine lactate dehydrogenase, reaching productivities of 11 g/L/hr [161]. Development of fermentation processes for the production of β -carotene in the food-grade yeast *C. utilis*, containing the carotenoid biosynthetic genes from the bacteria *Erwinia uredovora* and *Agrobacterium aurantiacum*, is in progress [162,163].

7. FUTURE PROSPECTS

The past few years have been a period of great progress using filamentous fungi as cell factories. There are four major fronts in which work is currently under way. The first is the development of alternative hosts, especially those which have already been given

GRAS status by the FDA and can be used in the food industry. Research has focused on species such as *Aspergillus sojae*, *Aspergillus japonicus*, *Mortierella alpine*, and *Fusarium venenatum*, among others [81]. The second front is the development of better molecular techniques to improve expression and secretion of nonfungal proteins. Recent studies on posttranslational modifications in filamentous fungi along the secretion pathway, mentioned in the previous section, have shown how complex the process is compared with that in yeast. The third major front involves the use of these molecular techniques to carry out metabolic engineering to modify and improve particular biosynthetic pathways. The final front will utilize the techniques dealing with the overall analysis of gene expression—that is, genomics, proteomics, and metabolomics. An initial step on filamentous fungal proteomics has recently been published [164,165], and undoubtedly much more will become available in the years ahead.

The future of fungal biotechnology is encouraging when one considers that all the contributions that have been made already by fungi have been done with less than 5% of the species present in nature. Soils and marine environments contain perhaps 10,000 unknown microbial species, many of them fungi. New methods are being used to harness “environmental DNA” and to bring about the cultivation of so-called unculturable microorganisms. These include growth as small microbial communities and supplying unknown nutrients from the organism’s natural environment. As new species are uncovered and cultivated, it is probable that new products will emerge for use in cure of human and animal diseases.

About 30% to 50% of known proteins have no known function. As more functions are revealed by functional genomics and bioinformatics, new targets will become available for screening of fungal products. These will be added to current targets, such as staphylococcal sortase, peptide deformylase, lipid A biosynthesis, drug efflux pumps, and signal transduction. Newly evolving diseases, acquired immunodeficiency syndrome, tuberculosis, prion diseases, and antibiotic resistance (especially in biofilms) will come under the influence of future fungal products. Fungal products will also become important for inhibition or stimulation of apoptosis and in complex diseases of humans. These include cancer, myocardial infarction, stroke, neurological degeneration (Parkinson’s disease, Huntington’s disease, Alzheimer’s disease), and schizophrenia. Lifestyle problems such as short stature, aging, wrinkles, male-pattern baldness, and sexual dysfunction will also be influenced by fungal products.

Fungal enzymes will be improved in activity, specificity, and stability by directed evolution of genes. Secondary metabolite pathways of fungi will be enhanced by directed evolution of whole cells in concert with metabolic engineering. New secondary metabolites will be created by combinatorial biosynthesis in fungi.

Fungi will also contribute to the environmental area in providing environmentally friendly pesticides, biodegradable plastics, and agents for bioremediation, energy production, and waste treatment, including hazardous waste.

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2

Phylogeny of the Fungal Kingdom and Fungal-like Eukaryotes

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1. INTRODUCTION

At the time of writing, high-quality draft genomic sequences of five ascomycete yeasts, *Saccharomyces cerevisiae*, *S. paradoxus*, *S. bayanus*, *S. mikatae*, and *Schizosaccharomyces pombe*, and the first filamentous fungus, *Neurospora crassa*, have been completed. Completion of the sequence of *Saccharomyces cerevisiae*, one of man's oldest domesticated partners, was a landmark in molecular biology and has enabled scientists to globally study eukaryotic gene expression and gene function. Sequencing projects for at least another dozen species of fungi, representing both the phylogenetic breadth of fungi and some of the most medically and economically significant species are in progress or in the planning stages [1]. The sequences of these fungi reveal organisms that are remarkably similar in genomic complexity, organization, and function to other eukaryotes. For example, *N. crassa* is estimated to have around 10,000 genes, roughly 70% as many as *Drosophila melanogaster* (14,000 genes), 50% as many as *Caenorhabditis elegans* (20,000 genes), and about 30% as many as *Homo sapiens* (21,000–39,000 genes). About 4000 of the estimated proteins of *N. crassa* are without obvious counterparts in public databases, which

may be a function of general lack of fungal genomic data and points to the enormous potential for new gene discovery in fungi. Among the 1400 genes that have counterparts with other eukaryotes are genes associated with a biological clock, light sensing, and many core signaling pathways for cellular functioning. Surprisingly, more than half the genes of *N. crassa* have no counterpart in *S. cerevisiae* or *Sch. pombe*, hinting at the potential genetic diversity in fungi, considering these are only three species among the estimated 150,000 species of ascomycetes [2]. Genomic analyses of *Neurospora crassa* have identified some seven polyketide synthases gene clusters, three nonribosomal peptide synthases, and several genes associated with diterpene biosynthesis. The complete or partial gene clusters of secondary metabolite biosynthetic pathways have now been characterized for only several dozen fungi. The finding of such extensive biosynthetic capacity in a fungus that is generally thought to be devoid of secondary metabolites, except for carotenoids and melanin pigments, reveals that our knowledge of secondary metabolites of fungi and how to elaborate and engineer their production is still in its infancy.

While genomic scientists have been opening the doorway to the realm and function of the fungal genome, mycologists have been deciphering the evolutionary histories within the fungal kingdom and other fungal-like organisms and defining their placement among the eukaryotes. Unparalleled progress has been made in the last decade toward developing a phylogenetic classification of fungi [3,4]. The basic backbone phylogeny based on phylo-

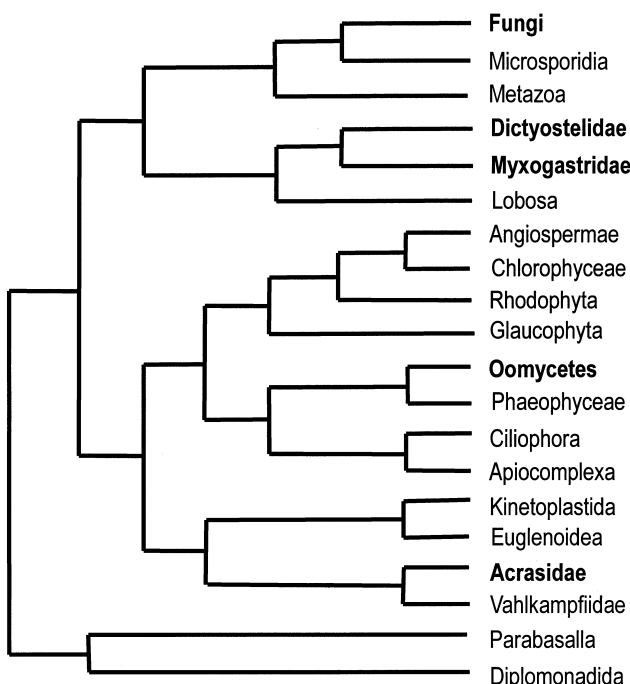


Figure 1 Phylogenetic position of the true Fungi, organisms formerly classified as fungi (bold text), and other major lineages of eukaryotic organisms. The tree was based on sequences of four protein-coding regions [α -tubulin, β -tubulin, actin, and EF-1 α] in addition to rDNA. (Adapted from Ref. 5.)

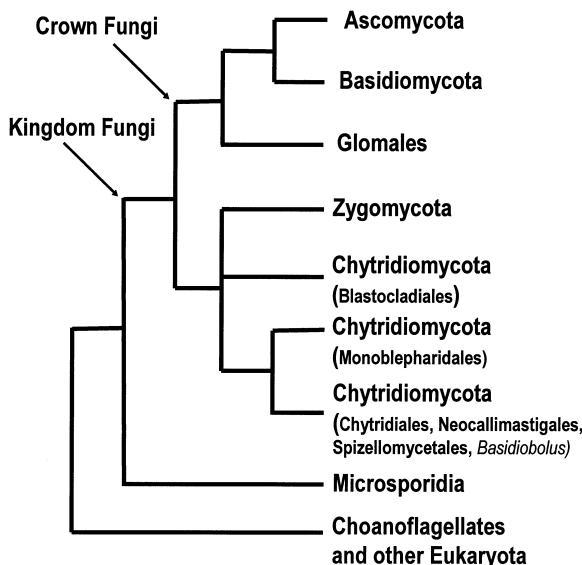


Figure 2 Relationships among the monophyletic grouping of the crown or terrestrial fungi (Gloales, Ascomycota, Basidiomycota), the Zygomycota, Chytridiomycota, and other Eukaryota.

genetic analyses using DNA characters was developed relatively early. One important finding has been that “fungi” are polyphyletic (Fig. 1), and their morphologies have converged, deriving from among several independent eukaryotic lineages. A well-defined and monophyletic group of “true fungi” (Figs. 1, 2), exclusive of slime molds and oomycetes, is considered a kingdom-level taxon [5–8].

Most phylogenetic studies to date have been performed on nucleotide data determined from the nuclear rDNA. The utilization of SSU rDNA has redefined fungal systematics for mycologists and allowed them to approach many long-argued questions in fungal systematics. The limitations of single gene phylogenies have been recognized, and fundamental questions regarding the fungal evolution will be solved by use of additional genes and taxa. What are the origins of the fungi? Are choanoflagellates the ancestors for fungi? Where is the origin of DAP lysine biosynthesis in the fungal ancestry? Can evolution of key structures (flagella, hyphae, etc.) be traced? Can the morphology and trophic relations of the “first fungus” be inferred? What were the earliest divergence and radiation events within the Kingdom Fungi?

The relationships among the terminal clades, the “crown fungi” (crown fungi Gloales, Basidiomycota, and Ascomycota) of the Kingdom Fungi, still are unclear (Fig. 2). The origin of the fungi seems to be correlated with the origin and diversification of land plants. However, the relationship of the Gloales with the ascomycete/basidiomycete clade is tenuous and sampling of plant-associated zygomycetes is incomplete. Furthermore, the early divergences within the filamentous ascomycota (Pezizomycotina) and macrosporocarp-producing basidiomycota (Hymenomycetes) are poorly understood. Could symbioses between green algae and fungi predate the evolution of land plants and their mycorrhizal symbioses? What is the origin of dikaryotic fungi? When do we find the first organized sporocarps and fungal tissues?

No single phylogenetic reconstruction to date has included more than about 800 fungi and fungal-like organisms [9], which represents less than 1% of the 120,000 currently known species [10]. Estimates of up to 1.5 million fungal taxa [11] are daunting, and the missing fungi must be discovered, identified, and incorporated in order to derive a definitive phylogenetic framework. In addition to the relatively sparse numbers of taxa included in the trees, taxonomic coverage across higher taxa has not been thorough. Until now, fungal phylogenetic studies relied heavily on ribosomal DNA, but an encouraging recent trend is the increasing use of additional genes and incorporation of biochemical and ultrastructural characters to help resolve conflicting trees based on single genes and analytical approaches (see Assembling the Fungal Tree of Life website <http://ocid.nacse.org/research/aftol>).

Referral to any two or more classification systems, as opposed to phylogenetic models, indicates that they are in a state of flux. Classifications should reflect phylogenetic relationships, but because the relationships of the fungi and their relatives are still unresolved or not included in all analyses, classification schemes may vary in their inclusiveness. To avoid the constant changing of formal names of higher taxa during ongoing phylogenetic research, the use of clade names, rather than formal taxa, has become a practical alternative [12], and this convention has been adopted for this discussion of higher fungal taxa (e.g., see section 3.4). The next section summarizes our current state of knowledge of the phylogeny of true fungi (Kingdom Fungi) and fungal-like organisms placed in several distinct eukaryotic clades (Fig. 1), as well as highlighting taxa that have produced historically important bioactive molecules, and fungi and fungal-like eukaryotes that are important pathogens or model organisms in biochemistry and genetics.

Another important realization during the last decade is that the dimensions of the fungal kingdom in terms of the diversity of species are enormous, easily rivaling the numbers of other speciose groups, such as the insects. The numbers of fungi have been consistently revised upward from the numbers of known described species, and it is generally suspected most fungi still await formal description and naming. The number of 1.5 million species is generally accepted as working estimate for the number of fungi [10,11], of which roughly 120,000 species have been validly named [10,13].

2. FUNGI AMONG THE EUKARYOTES

Multiple genes and data partitioning have been used to develop a hypothesis of the relationships of the large diverse group of eukaryotes, a group that includes fungi and fungal-like organisms (Fig. 1). Baldauf et al. [5] analyzed deduced amino acid sequences from four protein-coding regions [α -tubulin, β -tubulin, actin, and elongation factor 1-alpha (EF-1 α)] in addition to rDNA to improve resolution of the deep branches of the eukaryote tree, representing the earliest evolutionary divergences. The analyses identified and strengthened several branches, including the Fungi-Microsporidia link, the grouping of dictyostelid and plasmodial slime molds, and the remote basal position of the acrasid slime molds. In some cases, additional support has been derived from correspondence of nonmolecular traits (e.g., flagellation and arrangement of mitochondrial cristae). Baldauf et al.'s broad-view study of fungi and fungal-like organisms arrived at several important conclusions about the fungi (Fig. 1):

1. The fungi, comprising the traditional phyla Ascomycota, Basidiomycota, Zygomycota, and Chytridiomycota, are a monophyletic group of “true Fungi.”

2. The Microsporidia are a sister group to Fungi; these two taxa form a monophyletic lineage with the Metazoa (animals), linked by a choanoflagellate-like common ancestor.
3. The dictyostelid and plasmodial slime molds, although still sparsely sampled, form a monophyletic group that together with certain amoebae are the sister group to Fungi, Microsporidia, and Metazoa.
4. Oomycetes and other groups with a heterokont or derived flagellar condition (e.g., hyphochytrids, brown algae, diatoms, chrysophytes) form a clade; this large group is a sister to the clade made up of ciliates and Apicomplexa (sporozoans).
5. Myxomycetes, protostelids, and dictyostelids may constitute a monophyletic group; however, taxon sampling remains inadequate.
6. Plasmodiophorales belong to a clade of protozoans near alveolates [14].
7. Acrasid slime molds are distant from other slime mold groups, occurring in a clade with predaceous vahlkampfiid amoebae.

3. KINGDOM FUNGI

Historically, the monophyletic Fungi have been classified in four phyla, Chytridiomycota, Zygomycota, Basidiomycota, and Ascomycota [3,6,15]; however, current studies have shown that the classification is inadequate (Figs. 1, 2). Modern analyses of the SSU rDNA do not support the phyla Chytridiomycota and Zygomycota as monophyletic groups, and the two groups may intergrade or diverge at several points [16,17]. Phylogenetic analyses indicate that some chytrid lineages occupy the most basal branch of Kingdom Fungi, which is consistent with a choanoflagellate-like ancestor. The Chytridiomycota (estimated 800 species), currently defined by the primary ancestral character state of a smooth posterior flagellum, is not monophyletic.

The zygomycetes (approximately 1000 species), are defined by the presence of a meiospore known as a zygosporule and the lack of a flagellum. The traditionally defined zygomycetes are polyphyletic. A second traditional zygomycete group, the Glomales, is a sister clade to the Ascomycota/Basidiomycota clade [17–20]. Together, the Ascomycota, Basidiomycota, and the Glomales constitute the monophyletic “crown fungi” and are the most-derived clade within the Kingdom Fungi. The appearance of earliest members of the crown fungi on Earth likely coincided with the origin and diversification of terrestrial plants [19–22]. The Glomales are important in the evolutionary history of land plants; it is estimated that 80% of all plant species are associated with these arbuscular mycorrhiza-forming fungi. The Ascomycota and Basidiomycota are the two largest phyla of the Kingdom Fungi and together constitute over 95% of all known fungi [15]. The “crown” groups predominate in all terrestrial environments and are critical to the function of life on Earth.

3.1. Chytridiomycota (Zoosporic Fungi)

The Chytridiomycota (Figs. 2, Fig. 3) have been defined traditionally by the presence of a single posteriorly inserted smooth flagellum. In the past some mycologists doubted whether the Chytridiomycota were true fungi, but rDNA has clearly established their position among the fungi [7,23,24]. The monophyly of Chytridiomycota, however, has

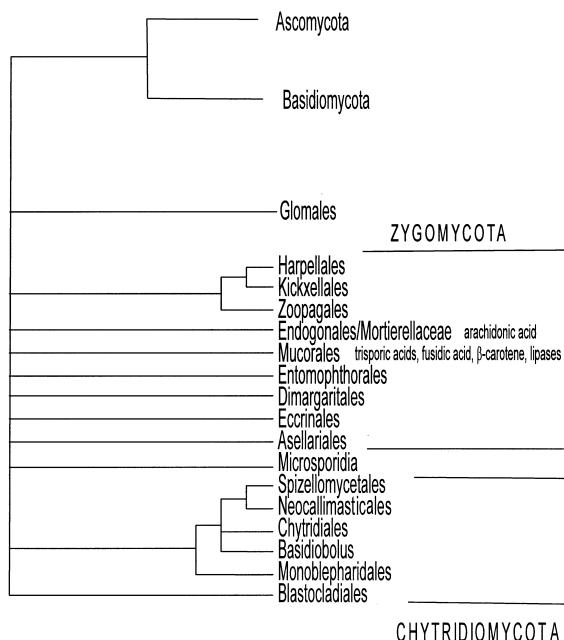


Figure 3 Major clades of the phyla Chytridiomycota and Zygomycota, excluding the Glomales, based on analyses of nuclear rDNA and EF-1 α data. The terminal clades (orders) of the Zygomycota and Chytridiomycota are generally well supported, but their relationships are poorly resolved. As a group, they represent the loss of the flagellated stage, possibly from a blastocladialean ancestor. Some important metabolites and enzymes of the Zygomycota are indicated to the right.

not been upheld by subsequent studies, including increased taxon sampling, although chytrid groups are basal in phylogenetic trees [16,25].

Five orders—Blastocladiales, Monoblepharidales, Neocallimastigales, Spizellomycetales, and Chytridiales—were established on the basis of zoospore ultrastructure [26]. Evolutionary models based on zoospore morphology are congruent with mitochondrial phylogenies [27]. More recently, the relationship of the clades to each other has been tested using SSU rDNA characters [16]. The phylogenetic analysis distinguished four monophyletic clades, consisting of the orders Blastocladiales, Monoblepharidales, Neocallimastigales, and Spizellomycetales, and four groups of Chytridiales in distinct clades, consistent with the groupings based on zoospore ultrastructure. However, the relationships among the orders and chytrid clades remain to be resolved [16]. Additional chytrid diversity likely will be uncovered when more chytrids with unique zoospore types are included in phylogenetic analyses [16].

Some chytrids with unique zoospore morphologies have not been classified in previously existing orders, but several of these, including *Batrachochytrium dendrobatis*, the chytrid associated with amphibian decline, do not fall within any of the defined groups on the basis of DNA characters. The complete genomic sequencing of *Batrachochytrium dendrobatis* has been proposed due to its implication in the worldwide amphibian decline [1]. The discovery of anaerobic rumen-inhabiting chytrids (Neocallimastigales) has been

considered one of most significant mycological discoveries of the 20th century. These true anaerobic fungi have been the most intensively studied chytrids because of their significant participation in rumen digestion and the potential for applications of their cellulolytic enzymes. *Blastocladiella emersonii* and *Allomyces* species (Blastocladiales) have been important model organisms for studying biochemical and cellular processes in zoosporic fungi.

3.2. Zygomycota

The Zygomycetes comprise a monophyletic group if the Glomales and, perhaps, *Basidiobolus ranarum* (Entomophthorales), are excluded. *Basidiobolus ranarum* commonly is associated with insects and amphibians in terrestrial habitats. Although this species lacks flagella, it has been placed within the core chytrid group in analyses using rDNA [17,28]. In trees based on analysis of α - and β -tubulin genes, however, *B. ranarum* falls within the zygomycetes (Fig. 3) [8]. The conflict could be caused by an accelerated rate of sequence evolution in SSU rDNA of zygomycetes relative to other fungi and resulting long-branch attraction in phylogenetic analyses employing the gene [16]. Compared with members of the Entomophthorales, *Basidiobolus* has a distinctive spindle pole body (nucleus-associated organelle) with microtubular structure reminiscent of centrioles that are known only among chytrids [3,29,30]. In addition, species of *Basidiobolus* have a rocketlike forcible spore release mechanism that differs from the remaining species of Entomophthorales, which have cannonlike mechanisms. However, most species of *Basidiobolus* share capilliconidia, distinctive animal-dispersed diaspores, with certain species in the Entomophthorales. The *Basidiobolus* example is worth highlighting as yet another persistent conflict between genetic and morphological data.

Several traditional zygomycete lineages are considered to be monophyletic. These include Entomophthorales, Trichomycetes (Harpellales, excluding Amoebidiales), Kickxellales (excluding *Spiromyces*), Mucorales, Mortierellales, Dimargaritales, and Zoopagales [16,25,29,31]. The relationships among the monophyletic lineages, however, have not been well supported in most cases (Fig. 3). O'Donnell et al. [31] used a greatly expanded molecular data set of SSU rDNA, LSU rDNA, EF-1 α gene exons, and 11 morphological traits to produce a tree providing a new view of the Mucorales but somewhat contradictory to morphology-based phylogenetic hypotheses. A monophyletic grouping of Mucorales was supported, and several members of the Mortierellales were identified as a basal outgroup. The striking result of this and subsequent studies [32,33], however, was the lack of support for the traditional families of the order. In particular, the larger families Mucoraceae, Thamnidiaeae, and Pilobolaceae were polyphyletic.

Some species of the Mucorales, especially *Rhizopus* and *Mucor* species, have been important in the food processing industry for their production of amylases, lipases, peptidases, rennins, organic acids, carotenoids, and alcohols. Their fast growth and high enzyme activity have made them popular for use as agents of organic molecule biotransformations, including transformations among intermediates in cortisone synthesis.

3.3. Glomales

The Glomales (Figs. 2, 3) comprise fungi with an arbuscular mycorrhizal (AM) habitat and, as mentioned above, the group has been estimated to be associated with 80% of the world's plant species. Their reduced morphological features have inaccurately portrayed the genetic diversity of the group. An early molecular study [20] placed the Glomales in

a monophyletic clade basal to the Ascomycota-Basidiomycota clade, and this placement has been reinforced by subsequent studies [17–19]. Two major lineages exist within the Glomales; one includes *Glomus* as the sister to *Gigaspora* and *Acaulospora* [19]. A surprising finding of the study was the discovery of two previously unrecognized lineages represented by the genera *Archaeospora* and *Paraglomus* [19,34] which, despite many superficial morphological similarities shared with Glomales, diverged outside of the Glomalean lineages. Differences in mycorrhizal morphology, fatty acid profiles, and immunological reactions against monoclonal antibodies are consistent with molecular evidence.

3.4. Ascomycota

The Ascomycota is the largest phylum of the Kingdom Fungi, with approximately 32,000 described species [15]. Ascomycetes are characterized by the production of meiospores (ascospores) within sac-shaped cells (asci). They are among the most commonly encountered fungi and have transformed human civilization through food (e.g., *Saccharomyces cerevisiae*), medicine (e.g., *Penicillium chrysogenum*), and disease (e.g., *Pneumocystis carinii*, *Candida albicans*, and *Trichophyton rubrum*). Most fungi used in industrial processes and as biotechnological and molecular biological tools belong to the phylum. The Ascomycota is composed of three major groups or classes, including Pezizomycotina (Euascomycetes, mostly filamentous, sporocarp-producing and mitosporic or conidial forms), Saccharomycotina (Saccharomycetes, the true yeasts), and Taphrinomycotina (Archiascomycetes, a paraphyletic assemblage of basal taxa) [35,36] (Fig. 4). Relationships of the major groups within each of these classes are still tenuous; most are represented by well-supported terminal clades that are linked by poorly supported more basal nodes [9,37–39].

3.4.1. Taphrinomycota

The Taphrinomycota (Archiascomycetes) are recognized almost solely on the basis of phylogenetic analyses of rDNA sequence data [35,36,40] and include yeastlike, filamentous, and possibly sporocarp-producing species. The Taphrinomycota are not strongly supported as a monophyletic group, but rather represent a series of basal lineages in phylogenetic trees based on nuclear SSU rDNA. Studies involving multiple independent loci are needed to test the validity of the Taphrinomycota concept. The largest order of the Taphrinomycota is the Taphrinales, which includes about 100 species of plant pathogenic fungi [41]. Other major taxa of the Taphrinomycota include *Pneumocystis carinii*, the causal agent of pneumocystis pneumonia, the fission yeasts (*Schizosaccharomyces pombe* and its relatives), and *Saitoella complicata*, an asexual yeast. The ascoma-producing earth tongue genus *Neolecta* also appears to be grouped among the Taphrinomycota [42,43].

3.4.2. Saccharomycotina

Most fungi that biologists consider as “true yeasts” are members of the Saccharomycotina (Saccharomycetes). Species of the class are ubiquitous in all environments and habitats. Certain species are inseparably linked to human civilization and have been domesticated for food beverage production (e.g., *Saccharomyces cerevisiae*, baker’s and brewer’s yeast), whereas others function as mammalian pathogens (e.g., *Candida albicans*, a facultative human pathogen) and mutualists and endosymbionts of arthropods and animals [44]. The Saccharomycotina are supported as a monophyletic clade and probably share a most recent

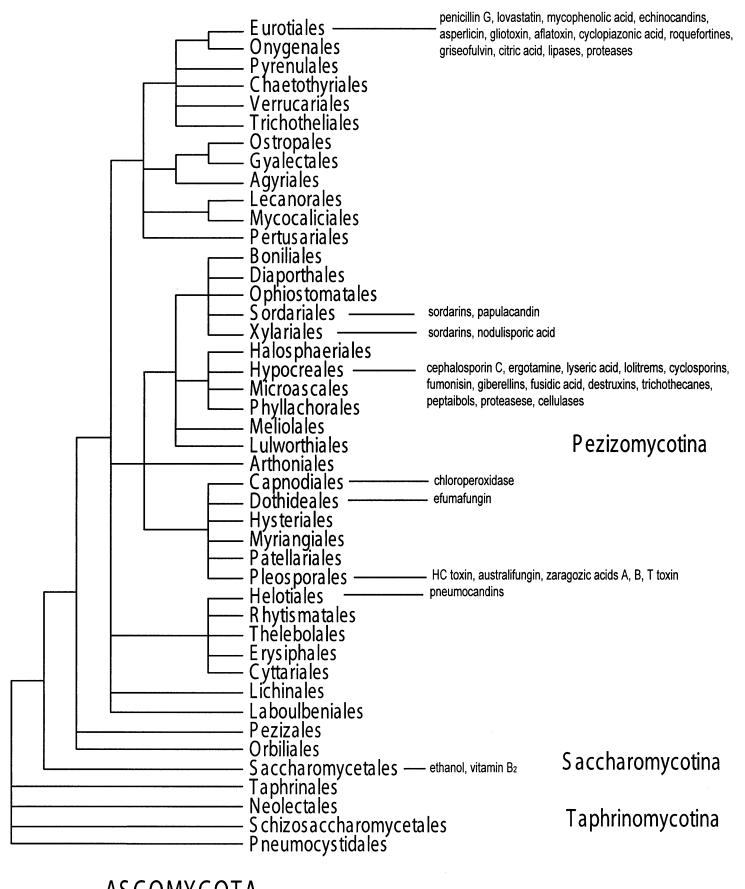


Figure 4 Major clades of the phylum Ascomycota, including the three classes Taphrinomycotina, Saccharomycotina, and Pezizomycotina, and the major lineages and orders of the Pezizomycotina polytomy, from analyses of nuclear rDNA and RNA polymerase II. Some important metabolites and enzymes are indicated to the right.

common ancestor with the Pezizomycotina [37,45]. The dominant phases of the lifecycle are yeast cells that lack the development of ascogenous hyphae and sporocarps. Numerous species exhibit dimorphism, however, alternating between filamentous and unicellular growth, whereas others, like *Ascoidea* spp., display predominantly filamentous growth [46] and species of *Cephaloascus*-producing ascophores, which have been interpreted as ascogenous hyphae [47]. The group shares a number of ultrastructural features, including unique details of nuclear division and ascospore delimitation.

3.4.3. Pezizomycotina

The Pezizomycotina (Euascomycetes) are the largest class of Ascomycota and include the major lines of filamentous, sporocarp-producing taxa and their equally diverse asexual lifecycle phases (Coelomycetes and Hyphomycetes). The Pezizomycotina perhaps are the

most successful group of fungi, with nutritional modes ranging from parasitic, pathogenic, and mutualistic with plants, algae, and animals, whereas saprobic lineages are able to decompose essentially all known organic substrates. These fungi include most of the most notorious mycotoxin-producing fungi, as well as the lion's share of fungi that produce bioactive metabolites. Traditional classifications have been based primarily on the morphology of sporocarps (ascomata) and asci. Molecular phylogenetic studies have shown that most traditional taxa based on ascromatal morphologies (e.g., apothecia, cleistothecia, and perithecia) do not represent natural groups. These morphologies have undergone repeated losses and gains during the evolutionary history of the fungi. Convergent evolution in ascus morphology has been apparently common, particularly among mechanisms of ascus dehiscence [48–51]. As with other higher groups of fungi, the Pezizomycotina are characterized by numerous well-supported terminal clades, but the relationships of the major clades still await definitive resolution. It has been postulated that the poorly resolved base of the Pezizomycotina clade was the result of a radiation event in which many of the major clades of the class originated over a relatively short time [37,39,52]. These hypotheses are based on a single gene, however, the nuclear SSU rDNA, and similar hypotheses for the major groups of eukaryotes [53] later proved erroneous, or at least resolvable, by analyses involving numerous independent loci [5].

In the past few years, the nomenclature for the major groups within the Pezizomycotina has been in constant flux; therefore, mycologists have tended to interchangeably use both formal and informal designators that refer to major groups [3]. Alternative classifications have been proposed [54] with the ultimate goal of accurately reflecting monophyletic clades of the Pezizomycotina. The primitive sporocarp morphology of the Pezizomycotina appears to be that of the apothecium [9,37,38], which is characterized by an exposed fertile layer of asci (hymenium). The most basal lineage of the apothecial fungi, as well as that of the Pezizomycotina, includes the genus *Orbilia*, an obscure clade of nematophagous ascomycetes [55,56]. The Pezizales are the next basal lineage of the class [37,38,56] and are possibly the best known and the largest group of apothecial fungi. They include numerous macroscopic forest fungi (e.g., *Morchella*, *Gyromitra*, *Helvella*, etc.). The Pezizales are characterized by the production of operculate asci; however, the order is not strongly supported as monophyletic, and numerous familial revisions have been proposed recently [57]. Moreover, it includes numerous independent lineages of truffles [57] and, as an order, probably contains the majority of ectomycorrhizal species of ascomycetes. In most phylogenetic analyses of the SSU rDNA, the remainder of the euascomycete diversity emanates from a large polytomy [52]. Analyses of nucleotide and predicted amino acid data from RNA polymerase II [58], however, along with other types of nucleotide sequence data (e.g., β -tubulin, EF-1 α), hold great promise toward resolving many components of the euascomycete polytomy [5,31].

The Helotiales are another major group of apothecial and anamorphic fungi and include endophytes, mycorrhizae, plant pathogens, and saprobes of litter and woody debris. Also found among the Helotiales is the fungus *Glarea lozoyensis*, which produces pnuemocandin B₀, the starting material for the antifungal drug Cancidas® (Merck) [59]. The Helotiales, sometimes called the inoperculate discomycetes, are distinguished from the operculate Pezizales because the asci lack the operculum. The apothecium in these fungi can vary from cup shaped to earth tongue to hysteriform, depending on the species. The order is grossly polyphyletic, with representatives found across the “backbone” of the Pezizomycotina tree [56,60,61].

The other major group of apothecial ascomycetes includes the lichenized species of the Lecanorales (some 7000 species); in general biology, they often are considered the quintessential mutualistic symbioses. Fewer phylogenetic studies have been performed on lichenized ascomycetes than nonlichenized species, and the integration of the two in phylogenetic analyses and classifications remains a major goal in fungal systematics [62]. In an analysis of nuclear SSU rDNA, two major clades of lichenized ascomycetes, indicating two independent origins of the lichen symbiosis, have been demonstrated [38], and lichenization has been independently lost more than once in both these lineages [62]. One lineage consisted of the Lecanorales sensu lato, and the second clade comprised the Arthoniales, which are possibly more closely related to the Pleosporales and Pyrenomycetes.

The loculoascomycetes are fungi that with “ascostromatic development,” in which ascogenous hyphae are produced in preformed locules within a stroma [63,64]. In addition, the asci of many species are bitunicate asci and display a “jack-in-the-box” mode of dehiscence [65]. Here we will focus on three orders—Pleosporales, Dothideales, and Chaetothyriales—because the majority of the species belong to these orders and most of molecular phylogenetic analyses have been performed with these taxa. Inclusion of all three orders in a monophyletic Loculoascomycetes is rejected by the data, with at least two independent origins of ascostromatic development [39,66,67].

The Pleosporales are supported as a monophyletic clade [66], a finding consistent with the synapomorphy of sterile cells (pseudoparaphyses) interspersed among the asci [63,68]. The Dothideales are largely characterized by the lack of paraphysoids [63,68], and their status as a weakly supported monophyletic group is sensitive to taxon sampling [66]. The Chaetothyriales, characterized by evanescent apical pseudoparaphyses [69,70], are supported as a monophyletic group; however, they are not supported as a member of the Dothideales [67]. Rather, the Chaetothyriales are more closely related to the plectomycetes in analyses of both SSU rDNA [39,71] and chitin synthetases [23].

Plectomycetes, an obsolete taxon formerly referring to fungi with a completely closed ascomata [48,72,73], have been limited to the clade of Eurotiales and Onygenales, which contain most taxa that have been considered plectomycetes. The Eurotiales and Onygenales include numerous species that produce closed ascomata (cleistothecia) and relatively simple, evanescent asci (protunicate asci). Furthermore, it has been hypothesized that the Plectomycetes are a highly derived lineage, whose origin was associated with the loss of a lichenization [62]. The plectomycete clade includes an unusually high concentration of fungi that are of human interest. Among them are important anamorphic fungi that produce important medicines (e.g., penicillin and lovastatin), life-threatening diseases (e.g., valley fever caused by *Coccidioides immitis*, aspergillosis caused by *Aspergillus fumigatus*, lung disease caused by *Histoplasma capsulatum*, and athlete’s foot caused by *Trichophyton rubrum*), and problem mycotoxins (e.g., aflatoxin and patulin). Phylogenetic analyses of nuclear SSU rDNA [48], chitin synthetases [23], and RNA polymerase II [58] have strongly supported the Eurotiales/Onygenales relationship. Furthermore, *Elaphomyces*, a genus of ectomycorrhizal false truffles, is also supported as a member of the plectomycete clade and is the only known ascomycete truffle lineage outside of the Pezizales [74].

Pyrenomycete (Sordariomycetes) is another outdated class name that is now used to designate a clade, rather than a specific ascatal morphology. The pyrenomycetes exhibit a wide breadth of macromorphology and micromorphology and include most ascomycetes that produce flask-shaped ascomata or perithecia with unitunicate asci. Within the pyrenomycetes are found the historically important fungi, *Tolypocladium inflatum*, which produces cyclosporine A, and *Claviceps purpurea*, the source of the ergot alkaloids.

Also, the class contains important genetic model organisms (e.g., *Neurospora crassa* and *Podospora anserina*); infamous forest pathogens, [e.g., chestnut blight (*Cryphonectria parasitica*) and dogwood blight (*Discula destructiva*)]; and virulent insect pathogens (e.g., *Metarhizium anisopliae* and *Beauveria bassiana*). Some groups of the Pyrenomycetes—especially the Hypocreales, Sordariales, and Xylariales—rival the Eurotiales in their number and complexity of their secondary metabolites, toxins, and degradative enzymes. The orders Diaporthales, Halosphaerales, Hypocreales, Lulworthiales, Microascales, Ophiostomatales, Phyllachorales, Sordariales, and Xylariales constitute the clade [48,50,51,75–80]. Although most species produce perithecial ascomata, the pyrenomycetes clade also possesses numerous lineages of cleistothecial fungi (formerly placed in the plectomycetes), which represent multiple and repeated losses of the perithecial neck and ostiole [48,51,81].

3.5. Basidiomycota

The Basidiomycota is the second largest phylum of Kingdom Fungi (approximately 23,000 described species) [15], including many of the common macroscopic forest fungi (e.g., mushrooms, shelf fungi, puffballs). The phylum is characterized by meiospores (basidiospores) that are borne upon club-shaped cells or basidia. Basidium morphology has been the focal point of past classifications of the Basidiomycota with fungi possessing septate basidia classified in the Phragmobasidiomycetes (Heterobasidiomycetes) and fungi with nonseptate basidia classified in the Homobasidiomycetes (Holobasidiomycetes) [82,83]. Phylogenetic studies have revealed that the Phragmobasidiomycetes are not monophyletic and that the septate basidium is probably an ancestral character state for the Basidiomycota [83–86]. Modern interpretations include the classes Urediniomycetes (the rusts and relatives), Ustilaginomycetes (the smuts), and Hymenomycetes (mushrooms and relatives) in the Basidiomycota (Fig. 5) [85,86]. Molecular analyses point to a greater degree of convergent evolution among macromorphology and micromorphology than was previously appreciated. The relationships of the three classes within the Basidiomycota are still unsettled, as are many of the ordinal relationships within each of them [83,85,87,88].

3.5.1. Ustilaginomycetes

Fungi of the Ustilaginomycetes (smuts) are characterized by the production of a diploid overwintering spore, the teliospore, and dimorphic lifecycles with a saprobic yeast phase and a pathogenic filamentous state (see section 3.5.2). Because of certain similarities in morphology and parasitic relationships with plants, members of this group often have been confused with some of the Urediniomycetes. Both groups have been the subjects of controversial and conflicting classifications [84]. Traditional classifications placed the two groups in the Teliomycetes, because of the teliospore [89,90], or in the Phragmobasidiomycetes, based largely on septate basidia [82,91]. However, neither the Phragmobasidiomycetes nor the Teliomycetes are supported as monophyletic taxa in molecular phylogenetic analyses [84,93,94]. Furthermore, the teliospore and the overall the “smut” morphology (i.e., basidiospores produced in a darkly pigmented sooty mass) are yet more examples of convergent evolution in morphology among fungi [93]. Ultrastructural studies and molecular phylogenetic analyses support the Ustilaginomycetes as possessing three major clades or subclasses with the following orders: the Entorrhizomycetidae includes the Entorrhizales; the Ustilaginomycetidae includes the Ustilaginales and Urocystales; and the Exobasidiomycetidae includes the Doassansiales, Entylomatales, Exobasidiales, Georgefish-

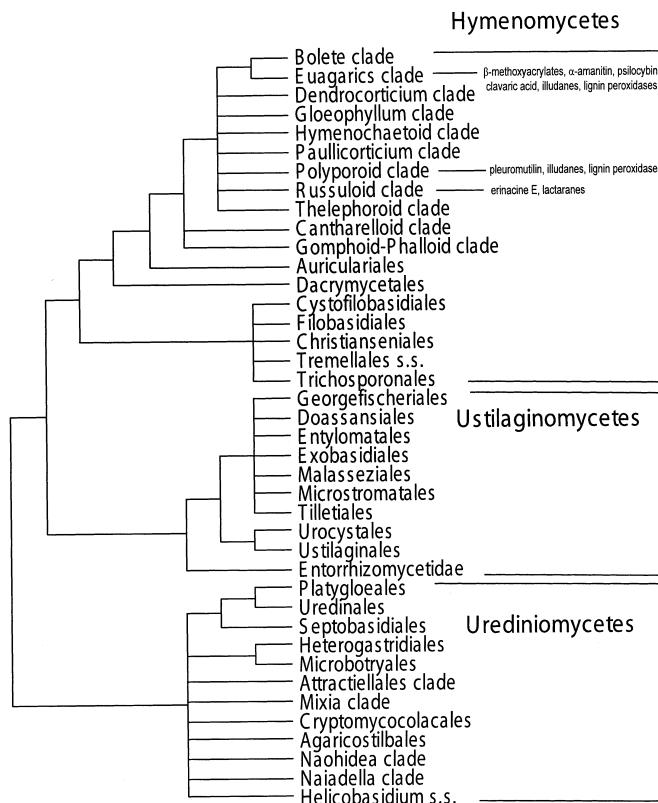


Figure 5 Major clades of the Basidiomycota, including the three classes Ustilaginomycetes, Urediniomycetes, and Hymenomycetes and their respective major orders from analyses of SSU and LSU rDNA sequences. Some important metabolites and enzymes are indicated to the right.

erales, Graphiolales, Malasseziales, Microstromatales, and Tilletiales [94]. Many taxa that were once thought to belong to the Ustilaginales clade (e.g., *Microbotryum*) have been convincingly demonstrated to be derived members of the Urediniomycetes [93,95].

3.5.2. Urediniomycetes

The Urediniomycetes is a large group of dimorphic and yeastlike fungi that includes the Uredinales and Septobasidiales of the Urediniomycetidae, Microbotryomycetidae, Agaricostilbomycetidae, Atractiellales, *Erythrobasidium*-clade, and the genus *Mixia* [92,96]. In the filamentous forms, Urediniomycetes share the trait of a septate mycelium with simple pores that lack any associated flaring of the cell wall surrounding the pore (dolipore septum) or associated pore membranes (parenthosomes) [84].

The Uredinales contain plant parasites that exhibit some of the more complex life-cycles known among fungi. The most elaborate lifecycles are those of the macrocyclic heteroecious rusts, requiring two distantly related plant hosts to complete their lifecycle, and possessing up to five distinct spore stages. More derived autoecious species exist on a single host with a reduced number of distinct stages. The Septobasidiales are an interest-

ing group of specialized symbionts of scale insects. The Microbotryomycetidae is a heterogenous group of species that is recognized mostly on molecular characters and includes smutlike species of *Microbotryum* and yeastlike species of *Rhodotorula*, *Rhodosporidium*, and *Sporidiobolus* [93,96]. However, basidiomycetous yeasts are polyphyletic with the species found in no fewer than three clades of the Urediniomycetes, including the *Erythrobasidium* clade and *Agaricostilbum* clade [96].

3.5.3. Hymenomycetes

The Hymenomycetes comprise the conspicuous fleshy fungi (e.g., mushrooms, jelly fungi, shelf fungi) familiar to most people. The clade is unified by a unique mycelial septum structure, the dolipore septum, which involves the flaring of the cell wall near the pore of the septum and a membrane structure, the parenthosome, on either side of the pore. The parenthosome may be perforated or not, depending on the clade, with the imperforated form being ancestral for the class; however, this character state is likely not without convergence [83]. The orders Ceratobasidiales and Tulasnellales, which include plant pathogenic (e.g., *Rhizoctonia*) and saprobic species, are members of the Hymenomycetes, perhaps closely related to the Auriculariales [92,97]. Also, in the Tremellales is found the human pathogen *Filibasidella neoformans* (anamorph *Cryptococcus neoformans*). Many of these taxa possess septate or deeply divided basidia and a dimorphic lifecycle with a yeast phases, a finding consistent with these characteristics being ancestral ones for the Basidiomycota.

The most derived clade of the Hymenomycetes includes those homobasidiomycetous taxa with nonseptate basidia and no yeast phase in their lifecycle. This clade includes the fungi with complex macroscopic fruiting bodies and the trophic modes ranging among mycorrhizae, litter and wood decay, plant pathogens, and insect symbioses. Traditional classifications of the homobasidiomycetous fungi relied largely on basidiocarp morphology, with particular emphasis on the spore-producing tissues or hymenophore. These classifications have been rejected by numerous phylogenetic analyses of molecular data from both the nuclear and mitochondrial genomes [88,98,99]. The general theme that has resulted from these studies is that the overall basidiocarp morphology has been subject to repeated episodes of convergent and divergent evolution and that it is not a phylogenetically informative trait at higher taxonomic levels. An estimated eight major clades exist within the Hymenomycetes, including polyporoid, euagaric, bolete, thelephoroid, russuloid, hymenochaetoid, cantharelloid, and gomphoid-phalloid clades, all of which contain multiple basidiocarp and hymenophore morphologies. For example, the crust (corticoid) morphology is found in all eight of the major clades and the cantharelloid clade contains four basidiocarp types, the fewest of any of the eight clades [83].

4. KINGDOM STRAMINIPILA (HETEROKONT ZOOSPORIC FUNGI)

The eukaryotes (Fig. 1) are divided into two large subgroups [5]. As mentioned already, one of these groups includes fungi, animals, and two groups of slime molds (see section 5). The other large group of eukaryotes is divided into two subgroups; one of these consists of the plants (including green algae) and the other subgroup, the Straminipila, contains algae with chlorophylls *a* and *c*, several groups of heterotrophs that previously were classified as fungi, and an assortment of “protozoan” groups. The organisms are characterized by zoospores with anteriorly or laterally biflagellate cells with one flagellum bearing tubular tripartite hairs and the other being smooth (whiplash); in some instances one (the

smooth one) or both flagella have been lost secondarily during evolution. Tubular hairs are sometimes found as ornamentation of certain zoospore cyst walls. In this kingdom level group are found not only photosynthetic organisms with chlorophylls *a* and *c* (e.g., brown algae, diatoms, golden brown algae, chrysophytes) but also heterotrophs (oomycetes, hyphochytrids, and labyrinthulids and thraustochytrids), which traditionally have been grouped with the fungi. For many years, mycologists recognized that these organisms were distinct from true fungi, emphasizing morphological and biochemical traits, such as flagellation, cell wall composition, and pesticide sensitivity that distinguished straminipiles from true fungi [100,101]. Therefore, confirmation of their distant phylogenetic position from fungi and similarity to certain algal groups based on molecular data was expected [102–105].

The name Straminipiles (also known by the variants “stramenopiles” and “straminopiles”) has undergone numerous changes and conflicting usages [102,104,106]. Chromista and Heterokonta, which in the past were roughly equivalent terms, were used before the widespread adoption of Straminipiles. Within the kingdom other name changes have occurred, most notably the use of Peronosporomycetes as a class name for the monophyletic grouping of Oomycota and Hyphochytriomycota [102,106]. In addition to the apparent sister taxa Oomycota and Hyphochytriomycota, the primarily marine labyrinthulids and thraustochytrids have been placed among the straminipiles, although it is unclear if the heterotrophic straminipiles are monophyletic [107,108].

4.2. Oomycota

The oomycetes are common in both terrestrial and aquatic environments. Their trophic modes range from saprobes to virulent pathogens of plants, algae and animals. Some members are causal agents of devastating crop diseases, such as *Phytophthora infestans* (late blight of potato), *Phytophthora cinnamomi* (damping off disease of many plants), and *Plasmopara viticola* (downy mildew of grapes). Phylogenetic hypotheses for oomycetes generally are in agreement with two major clades defined, Saprolegniomycetidae (water molds) and Peronosporomycetidae (plant and animal parasites). Recent trees have been based on morphology and biochemistry and three genes, small subunit rDNA, large subunit rDNA, and cytochrome oxidase (*cox II*) [109–112]. The Saprolegniales appear to be a monophyletic group within the oomycetes. One debatable point is the placement of *Sapromyces* and, therefore, the order Rhipidiales, a group of taxa with obligately fermentative respiration. Rhipidales are possibly an independent lineage within the oomycetes of equivalent rank to Saprolegniomycetidae and Peronosporomycetidae or, alternatively, may be included in Peronosporomycetidae [110].

5. MYXOMYCETES, PROTOSTELIDS, AND DICTYOSTELIDS: PLASMODIAL AND CELLULAR SLIME MOLDS

Many cell biologists and biochemists are familiar with common soil-dwelling cellular slime molds because of the extensive body of work carried with *Dictyostelium discoideum* [113–115]. The myxomycetes (Myxogastridae), or “true” or plasmodial slime molds, are common terrestrial organisms associated with decaying vegetation. Phylogenetic information on various slime mold groups indicates that plasmodial and cellular slime molds constitute a monophyletic group (Fig. 1) [5,116]. In an analysis of EF-1 α amino acid sequences, the myxomycete *Physarum polycephalum* was placed as a sister group to several

cellular slime molds (dictyostelids). This slime mold clade was the sister clade to a protostelid clade (*Planoprotostelium*) [116]. The monophyly of myxomycetes and dictyostelids is fairly well supported by analysis of protein-coding genes; data with rDNA genes were somewhat in conflict.

Spiegel [117] used life history and morphological and developmental characters in a phylogenetic analysis of a larger sampling of myxomycetes and protostelids. His results indicated that myxomycetes and some protostelids form a monophyletic group from which other protostelids were excluded, thereby indicating that the protostelid a group as originally circumscribed [114] is paraphyletic.

6. ACRASID SLIME MOLDS

Acrasid slime molds are another common terrestrial group of microscopic organisms associated with soil and decaying vegetation and fungi. They have been recognized as a distinct segregate from the other “slime molds” for the past three decades [114,118]. The animal-like movement and behavior of their amoebae caused a number of workers not only to separate the acrasids but also to suggest a close relationship to organisms with sluglike amoebae such as *Naegleria* and the vahlkampfids [114,118]. The mitotic apparatus of *Acrasis rosea* is strikingly different from those of other slime molds [119]. Furthermore, a phylogenetic analysis of slime molds and amoebae, including acrasids, dictyostelids, and *Naegleria* (Valkamfilidae), based on analysis of genes encoding glyceraldehyde-3-phosphate dehydrogenase, supported the grouping of acrasids in the Heterolobosa with Valkamfilidae (Fig. 1) [5,120].

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3

Biological Activities of Fungal Metabolites

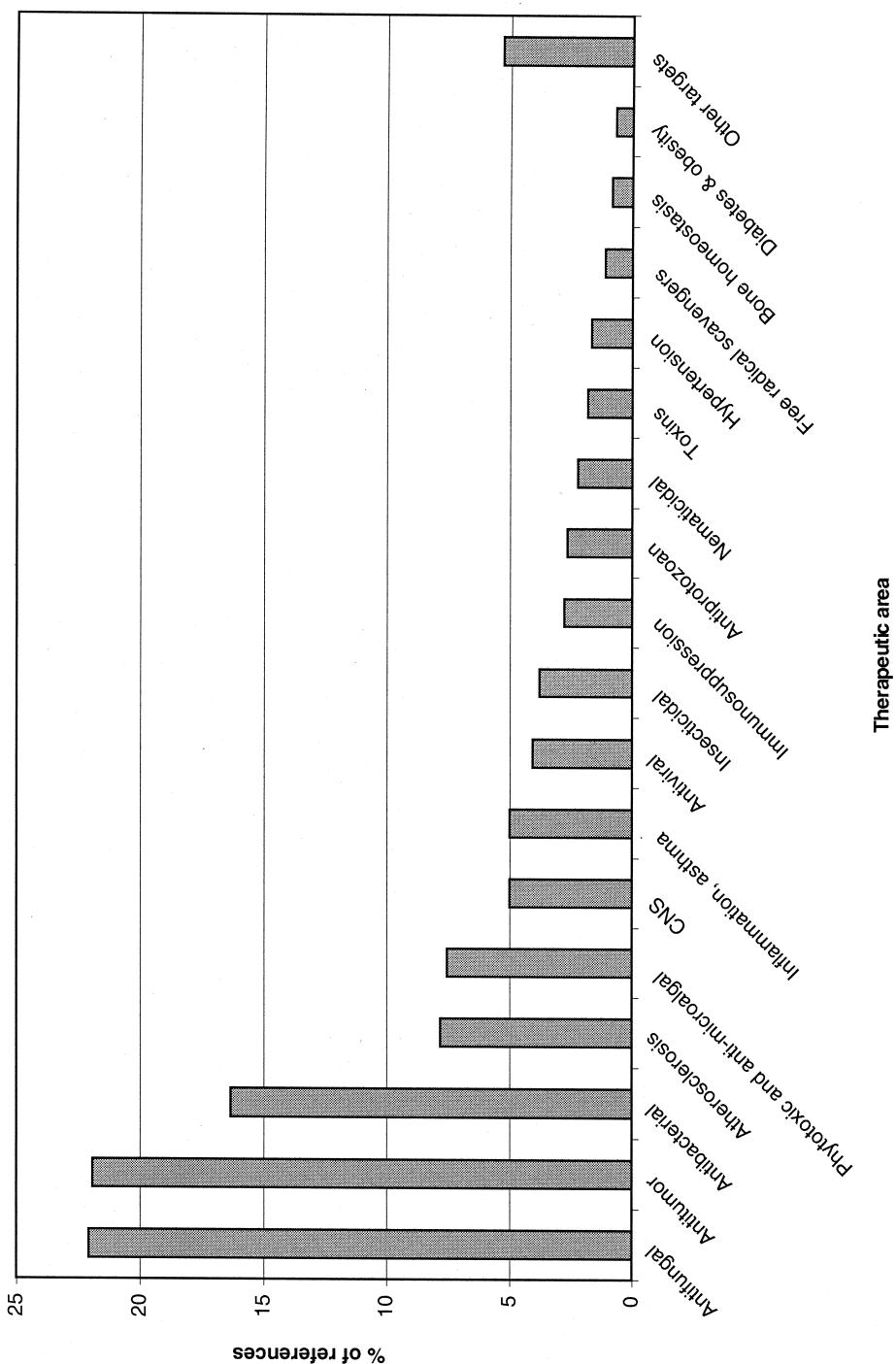
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1. INTRODUCTION

Fungal metabolites have had an extraordinary impact on the quality of human life during the 20th century. Antibiotics, antifungals, immunosuppressants, and cholesterol-lowering agents derived from fungal compounds have been used in the clinic during the past 50 years, contributing significantly to the welfare of mankind and to the spectacular rise in life expectancy observed in the second half of the century. The amazing range of chemical structures observed for fungal metabolites is derived from a relatively small number of basic metabolic pathways (mainly polyketides, nonribosomal peptides, and terpenoids, plus combinations of these), which have become extremely diversified during the course of evolution. In view of this remarkable structural diversity, it is not surprising that the number of targets hit by fungal compounds, as reported to date, is really astounding.

The results of a survey in the literature for fungal metabolites with biological activity reported from 1993 to 2001 are summarized in Fig. 1. A total of 718 nonredundant references were surveyed, accounting for more than 1500 metabolites or families of metabolites, displaying a broad array of biological activities. The vast majority of the references relate to newly discovered compounds; a few report new biological activities identified for previously known metabolites. Additionally, more than 60 references were found during the same period for fungal compounds without any reported biological activity; these references are not included in Fig. 1. Antifungal, antitumor, and antibacterial activities are reported most frequently from fungal metabolites. Overall, more than half of all the fungal metabolites described in this period showed one or more of these activities. However, fungal compounds also have been described as inhibitors of nearly 100 different



types of enzymes and receptors or as effectors of different biological activities measured in whole cell based assays. It seems plausible that one of the reasons why antibiotic activity (in its broader sense) stands out as the major type of biological activity reported has to do with the modest resources necessary to test for non-mode-of-action antibacterials, antifungals, or cytotoxic activities, when compared with the resources required to check for other biological activities needing more sophisticated techniques (enzymatic assays, etc.).

This chapter presents an overview of the most relevant biological activities with potential therapeutic relevance (mainly for human health) of fungal metabolites described during the last decade. For reasons of space, it is impossible to go over all the compounds discovered or biological activities reported even in this limited period of time. The selection has been based on criteria such as the number of compounds reported with a given biological activity, the scientific or medical impact, and the stage of development. Emphasis has been given to compounds that have recently progressed beyond discovery to subsequent phases of development (e.g., *in vivo* activity in animal models, medicinal chemistry, clinical development), as well as to those that have prompted additional research regarding their mechanism of action. The biological activities and metabolites are arranged by therapeutic areas, and these in turn have been sorted according to the number of references found for each, as shown in Fig. 1.

Mycotoxins, fungal metabolites that produce animal or human toxicity, have been studied extensively. Some of them, especially those produced by fungi growing on grains and food, represent important public health problems. They will not be treated in this chapter unless they show any additional biological activity of potential therapeutic interest. Likewise, phytotoxins, although also frequently reported as fungal metabolites (Fig. 1), will not be reviewed here.

2. ANTIFUNGAL

Fungal infections in humans range from superficial conditions of the skin (e.g., ringworm and athlete's foot) and nails (onychomycoses) to disseminated, life-threatening diseases. Serious invasive fungal infections caused by *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp., and other pathogens represent an increasing threat to human health. The prevalence of systemic fungal infections has risen significantly during the past decade, mainly due to the more frequent prescription of broad-spectrum antibiotics, marked increases in immunocompromised people (AIDS, cancer, and transplant patients), the use

Figure 1 Distribution of biological activities reported for fungal metabolites in the period 1993 to 2001. A total of 718 references were surveyed describing new metabolites with biological activities. When a metabolite was described in several different papers, these were counted as just one reference. The journals screened included *Journal of Antibiotics*, *Journal of Natural Products*, *Tetrahedron*, *Tetrahedron Letters*, *Journal of Organic Chemistry*, *Journal of the American Chemical Society*, *Bioorganic and Medicinal Chemistry Letters*, *Natural Products Letters*, *Organic Letters*, *Phytochemistry*, and the *Proceedings of the National Academy of Sciences*. References from these and other sources were also obtained by searching in the databases Biosis, Embase, and Current Contents.

of central venous catheters, and an aging patient population [1]. Only a limited number of antifungal agents (polyenes and azoles) currently are available for the treatment of life-threatening fungal infections. These antifungal agents show some limitations, such as the significant nephrotoxicity of amphotericin B and the emerging resistances to azoles. Although lipid formulations of polyenes and new triazoles have shown improved characteristics over the original compounds, the development of new antifungal agents, preferably with novel mechanisms of action, is an urgent medical need [1–3].

An astounding number of fungal metabolites with antifungal activity have been described in the literature over the last 10 years. Actually, the most recent drug based on a fungal metabolite that has reached the market at the time of preparation of this manuscript is the antifungal agent Cancidas® (caspofungin acetate; Merck). Most antifungal compounds described from fungi have been discovered by using non-mode-of-action based screens, that is, by simply looking for compounds able to inhibit the growth of a target yeast or filamentous fungus. Griseofulvin, a metabolite produced by *Penicillium* spp. discovered in 1939, which has been used in the clinic for several decades to treat skin infections by fungal dermatophytes, was discovered using this strategy, and its mode of action remains unclear even today [4]. Comparatively speaking, the number of antifungal compounds for which the mechanism of action is known is small. These will be the subject of the remainder of this section.

2.1. Cell Wall

One of the targets for novel antifungals under active investigation is the fungal cell wall. Antifungal agents acting on this target are expected to be inherently selective and should have fungicidal features that make them particularly attractive for clinical development. At present, glucan synthesis is the only process of cell wall synthesis that has been used successfully for the development of a new drug product. Glucan is a polysaccharide built of glucose monomers linked by (1,3)- β or (1,6)- β bonds, and it is an essential component of the cell wall, guaranteeing many of its physical properties [5]. Inhibitors of glucan synthesis have been shown to possess antifungal activity *in vitro* as well as *in vivo* in many different animal models. The most classic examples of inhibitors of glucan synthesis are the echinocandins, cyclic hexapeptides N-acylated with an aliphatic chain of different length [6]. Structural variants arise from different substitution patterns on the hexapeptide ring or in the fatty acid side chain. Since the first echinocandin was discovered in the early 1970s, many members of the family have been discovered in diverse fungi [6,7].

The pneumocandins, a type of echinocandins derived from the fermentation of the fungus *Glarea lozoyensis*, have been successfully used to develop an antifungal drug recently launched in the market. The semisynthetic echinocandin Cancidas is a substituted aza-derivative of pneumocandin B₀ (Fig. 2). The compound has been shown to be effective in animal models of disseminated candidiasis, aspergillosis, and *Pneumocystis carynii* pneumonia [7,8]. Clinical trials have demonstrated that caspofungin is well tolerated and is efficacious in the treatment of esophageal candidiasis [8,9] as well as in invasive candidiasis [10] and aspergillosis [7,8]. Cancidas has recently been approved in the United States and in several other countries for its use against invasive aspergillosis in patients refractory or intolerant to other therapies.

Other echinocandin-like antifungal agents in clinical trials are micafungin (FK463), an echinocandin derivative with a sulfate ester moiety in the hexapeptide nucleus, and

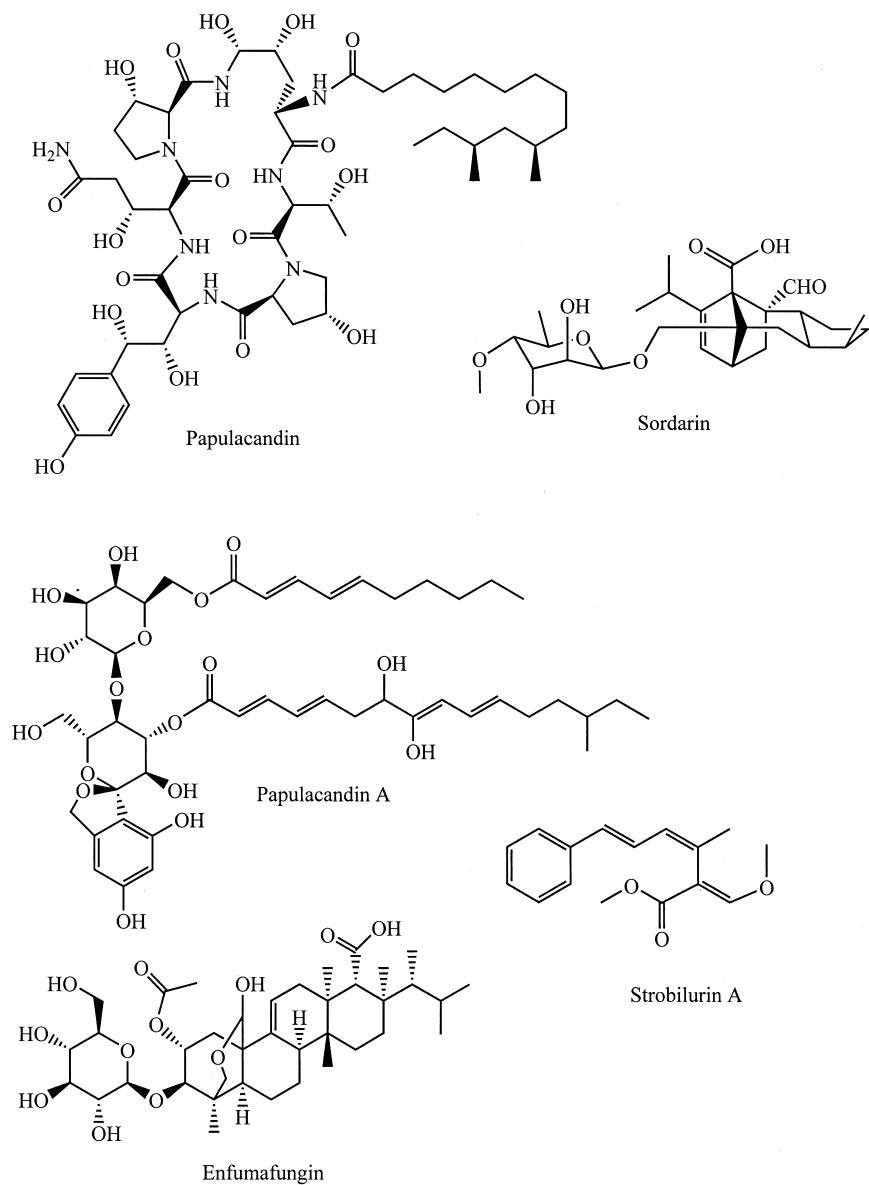


Figure 2 Antifungal compounds. Pneumocandin B₀, papulacandin A, and enfumafungin, glucan synthesis inhibitors. Sordarin, a protein synthesis inhibitor. Strobilurin, an inhibitor of respiratory chain.

anidulafungin (LY303366, V-echinocandin), which has a terphenyl head group and a C5 chain [3,7].

The success with the lipopeptide class of glucan synthesis inhibitors has prompted interest from the industry in the search for other structural types with improved features over the echinocandins (especially because of their lack of oral absorption). Besides echinocandins and the like, other cyclic peptides have been described as glucan synthesis inhibitors, such as the arborcandins, cyclodecapeptides with two lipophilic tails [11]; the compound FR901469, with 12 amino acids and a 3-hydroxypalmitoyl moiety [12]; and the clavariopsins, cyclic depsipeptides lacking a long lipophilic radical [13]. However, to date, besides cyclic lipopeptides, only two other types of glucan synthesis inhibitors are known: the papulacandins (and related compounds), and the acidic triterpenes. The papulacandins (Fig. 2) are glycolipids, discovered in the late 1970s. A series of related compounds have been discovered over the years, all of them produced by fungi. However, neither papulacandins nor any of their relatives have been developed as drugs, mainly due to their limited potency in animal models [14]. The most recently discovered class of glucan synthesis inhibitors are triterpenes containing a polar moiety [15]. This polar moiety can be a glycoside (as in enfumafungin and ascosteroside; Fig. 2), a succinate (as in arundifungin) or a sulfate-derivative aminoacid (as in ergokonin A). Although these compounds were inactive or only weakly active *in vivo* in a mouse model [7], they represent a new paradigm in the search for antifungal compounds with this mode of action, and they could be useful as the basis for the development of improved drugs.

2.2. Sphingolipid Synthesis

Although present in relatively small proportion in the fungal cytoplasmic membrane, sphingolipids are essential for cellular functions. Inhibition of sphingolipid synthesis results in growth inhibition and cell death [16]. Ceramide has been implicated as a component of an essential cell-signaling pathway in *Saccharomyces* [17]. Sphingolipids also are involved in the synthesis of glycosylphosphatidylinositol anchors in *Saccharomyces*, and they appear to be the major repository for very-long-chain fatty acids in fungi [18].

Although a number of steps in the human and fungal sphingolipid biosynthetic pathway are similar, there are several enzymes found exclusively in fungi, thus making sphingolipid synthesis a potential target for antifungal therapy. Three key enzymes in the pathway have been targeted in the search for novel antifungals: serine palmitoyltransferase, ceramide synthase, and inositol phosphoceramide synthase. Inhibitors of these three enzymes have been isolated from fungi [7,16,19]. Sphingofungins, viridiofungins, and an unnamed sphingosine-like metabolite isolated from *Colletotrichum acutatum* inhibit serine palmitoyltransferase. Fumonisin B1 and australifungin inhibit ceramide synthase, and aureobasidins and khafrefungin inhibit inositol phosphoceramide synthase. Furthermore, minimoidin was discovered as an inhibitor of the fatty acid elongation pathway. Although some of these compounds showed remarkable potency (e.g., khafrefungin and minimoidin), none of them have been subjected to further development due to issues of solubility, limited whole-cell or *in vivo* activity, and potential toxicity.

2.3. Protein Synthesis

Protein synthesis has always been considered to be one of the more attractive targets in the development of antimicrobial agents. However, application of this idea to antifungal therapy is not an easy task due to the eukaryotic nature of fungi and therefore the high

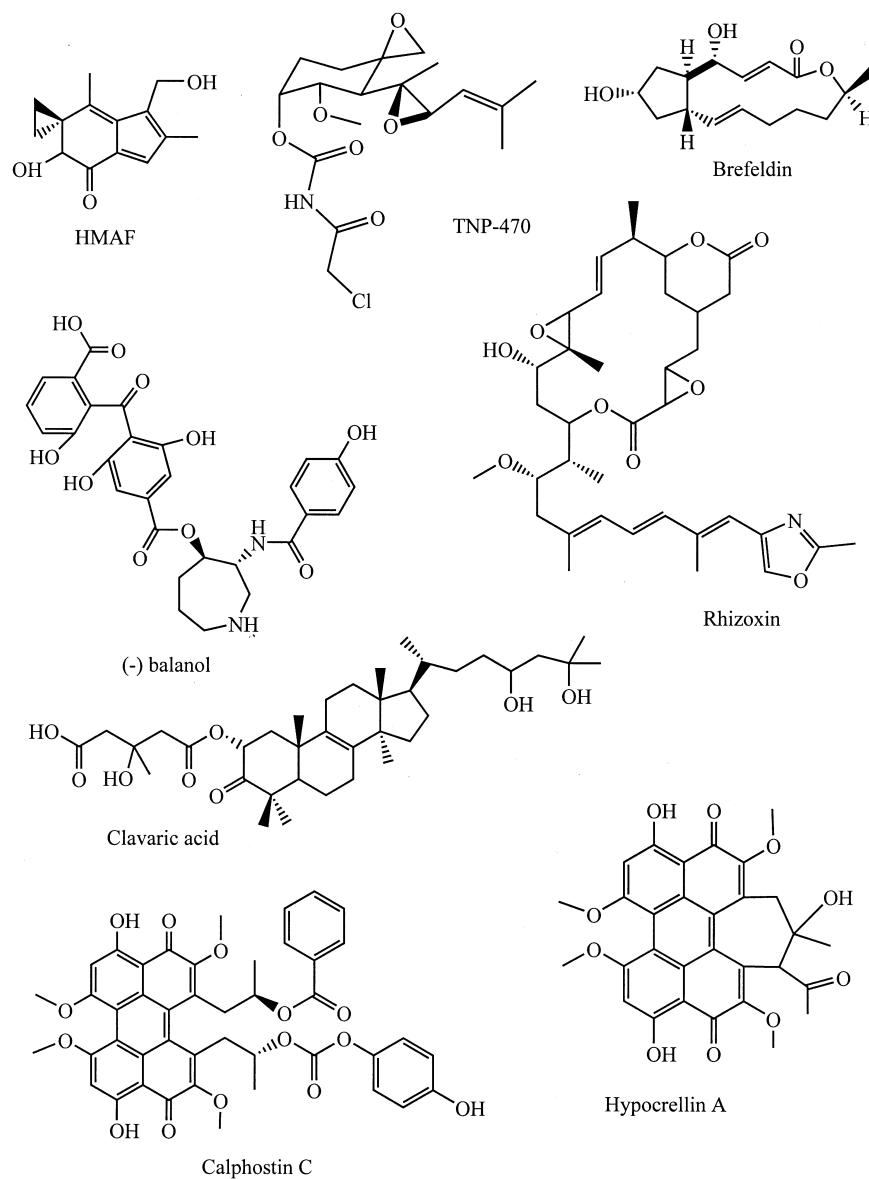


Figure 3 Antitumor compounds: irofulvene (HMAF), a cytotoxic compound; TNP-470, an angiogenesis inhibitor; brefeldin A, a protein secretion inhibitor; rhizoxin, an antimitotic agent; clavicular acid, an inhibitor of ras FPTase; hypocrellin A, a cell sensitizer; (–) balanol and calphostin C, PKC inhibitors.

similarity between the fungal and mammalian protein synthesis machineries. However, among the existing soluble translation factors, elongation factor 2 (EF-2) has been identified as a suitable antifungal target, because it is functionally distinct from its mammalian counterpart [20].

The most important family of antifungal agents acting at this level is the sordarins. Sordarin (Fig. 2) was isolated by scientists at Sandoz from fermentations of *Sordaria araeosa* [21]. Early studies revealed that sordarin targeted a protein involved in the translation of mRNA. Recent publications from the groups at Merck and Glaxo demonstrated that sordarins are potent and selective inhibitors of translation in fungi, acting by stabilizing the fungal EF-2–ribosome complex [20,22]. Compounds in this class inhibited *in vitro* translation in *C. albicans*, *C. tropicalis*, *C. kefyr*, and *C. neoformans* to varying degrees but were inactive against *C. krusei*, *C. glabrata*, and *C. parapsilosis*. Several structurally related compounds, sharing the aglycone moiety of sordarin, have been isolated from diverse fungal species [7].

The potent broad-spectrum *in vitro* activity and the fact that some of the sordarins have shown oral efficacy in animal models are significant advantages of these compounds. These advantages, together with additional pharmacokinetic and toxicological data, make the sordarins a promising series for clinical evaluation.

2.4. Antifungals in Agriculture

Outside the area of human health, mention must be made of the strobilurins (Fig. 2), fungal metabolites that have been used to develop several agricultural antifungal products (azoxystrobin, kresoxim-methyl, trifloxystrobin, etc.). This is an important market; sales of azoxystrobin (Heritage) alone amounted to \$415 million in 1999. The strobilurins and the related oudemansins are metabolites produced by many species of basidiomycetes belonging to the genera *Strobilurus*, *Oudemansiella*, *Mycena*, *Crepidotus*, and others, as well as by the ascomycete *Bolina lutea* [23,24]. These compounds are inhibitors of the respiratory chain by virtue of binding to mitochondrial cytochrome b. This target is not fungal-specific, and it has been reported that these compounds may also be phytotoxic, insecticidal, and/or cytotoxic to mammalian cell lines, depending on the derivative [24]. However, despite their mode of action against a ubiquitous target, they show very low oral toxicity in mammals, for reasons that are as yet unclear. This has allowed their development as commercially useful antifungal agents.

3. ANTITUMOR

Although there is a substantial number of antitumor agents derived from natural products in clinical use (doxorubicin, taxol, vinca alkaloids, etc.), none have been obtained from fungi. However, a substantial number of fungal metabolites have been reported to have either cytotoxic activity against tumor cell lines, or target signal transduction mechanisms mediating cell proliferation. Some of the most relevant compounds are reviewed in this section.

The illudins are a group of sesquiterpene antibiotics often reported as antibacterial and antitumor agents, produced by basidiomycetes from the genera *Omphalotus* and *Coprinus* [25]. A derivative of illudin S, irofulvene (6-hydroxymethylacylfulvene, HMAF, MG-114; Fig. 3), has shown good activity in animal models of cancer either alone or in combination with other antitumor agents [26]. This compound has been tested in Phase

II clinical trials in a variety of cancer models, but published results are not encouraging [27–29]. Although its mode of action is not clear, irofulvene has been shown to promote caspase-mediated apoptosis in tumor cells by a mechanism involving the activation of Erk 1/2 and JNK 1 kinases [30].

Another antitumor fungal compound that has been tested in humans is rhizoxin, a macrocyclic lactone produced by *Rhizopus chinensis*. The compound is an antimitotic agent that inhibits tubulin polymerization by binding to the same site as vinca alkaloids [31]. Rhizoxin showed good activity *in vivo* against various preclinical murine models, both leukemias and solid tumors models, as well as in vincristine- and doxorubicin-resistant leukemia lines [32]. The total synthesis of rhizoxin has been described recently [33]. Rhizoxin has been tested in clinical trials in phase II in breast cancer, melanoma, and other cancer types, but the results were disappointing [34,35]. Nonetheless, the compound showed some activity in a study on non-small-cell lung cancer [36]. More recent studies in humans have evaluated the use of rhizoxin in more prolonged dosing schedules, to test the hypothesis that the lack of clinical efficacy in early trials could be influenced by inadequate exposure to the drug [37]. Other fungal compounds have been described with the same mechanism of action as rhizoxin, such as phomopsin A from *Phomopsis leptostromiformis* [31], or the related compound ustiloxin A from *Ustilaginoidea virens* [38], but they have not been tested in humans.

Although most of the clinically useful compounds used to treat cancer have been discovered as simply cytotoxic compounds, extensive efforts have been made by a number of groups to identify potentially useful antitumor agents by using screens based on biochemical processes related to the cascade of events mediating cell proliferation. One of the enzymatic steps identified early on as essential in cell malignancy was prenylation of Ras protein. Normal and oncogenic Ras proteins are posttranslationally modified by a farnesyl group that promotes membrane binding. Inhibitors of farnesyl protein transferase (FPTase), the enzyme that catalyzes this prenylation, have been shown to inhibit growth of tumor cells. A number of inhibitors of this enzyme are in clinical trials [39]. In our own screening program of microbial natural products, we have identified as many as eight families of inhibitors of FPTase derived from fungal fermentations: chaetomellic acids, cylindrols, fusidiensols, barceloneic acid, oreganic acid, kampanols, clavaric acid, and some preussomerins [40].

The most interesting of all these compounds was clavaric acid [40,41] (Fig. 3). Clavaric acid was the only reversible inhibitor of FPTase competitive with Ras peptides found in our screening program, and one of the very few natural FPTase inhibitors reported with this mechanism of action. More importantly, the compound inhibited Ras-processing in NIH3T3 *ras*-transformed cells that express Ha-Ras, without manifesting any toxicity at the doses tested. Clavaric acid has been used as a template for obtaining more effective inhibitors. A series of related compounds derived from computer-based similarity searches using clavaric acid and an alkaline hydrolytic product, clavarinone, and subsequent rational chemical synthetic design, exhibited IC₅₀ against FPTase in a range from 0.04 to 100 μM, which were competitive with either Ras or farnesyl pyrophosphate. Modest changes in the structures of these derivatives dramatically changed their inhibitory activity [42]. It was clear from the analysis that the design and synthesis of more potent inhibitors can be achieved and selectively directed to either substrate binding site on the enzyme.

FPTase inhibitors reported by other groups include the andrastins and kurasoins [43], TAN-1813 [44], and several others. The compound TAN-1813, produced by an undetermined *Phoma* species, was shown to induce the morphological reversion of

NIH3T3 cells transformed with K-ras and has antitumor activity against human tumor models in mice [44].

One of the most promising antitumor targets studied in the recent years is histone deacetylase (HDAC). Inhibitors of HDAC are known to have detransforming activity—that is, they are able to induce morphological reversion from oncogen-transformed to normal cells [45,46]. A plethora of cultured tumor cell lines have been shown to be susceptible to HDAC inhibitors, and studies using rodent models for cancer have shown that these compounds reduce the growth of tumors and metastases *in vivo*, in some cases without noticeable side effects [47]. Acetylation and deacetylation of histones is known to play a critical role in the regulation of transcription in eukaryotic cells. Thus, inhibition of HDAC has been shown to result in upregulation of some genes and downregulation of others. The synthesis of p21^{WAF1} (an inhibitor of cyclin-dependent kinases) is the effect most consistently observed, and this could explain the G1 arrest and G2/M block frequently observed in cells treated with HDAC inhibitors, although there may be other factors involved in the mode of action of these compounds [47]. Besides their antitumor effect, it also has been reported that HDAC inhibitors have antiangiogenic activity in carcinoma mouse models, upregulating p53 and other tumor suppressor genes, and reducing the expression of angiogenic factors such as VEGF. This suggests that these compounds might be useful anticancer agents also due to their antiangiogenic properties [48]. The potential of some HDAC inhibitors as therapeutic agents is being tested in clinical trials [47].

Several fungal metabolites have been described as potent HDAC inhibitors, the most characteristic being cyclic tetrapeptides containing an epoxyketone moiety, such as trapoxins [49], chlamydocin, HC toxin, and others [50,51]. These compounds produce the irreversible inhibition of HDAC at nanomolar concentrations by covalently binding to the enzyme through the epoxide [49,52]. Structurally related fungal tetrapeptides lacking the epoxyketone also have been identified as HDAC inhibitors, such as apicidin [46], discussed below as an antiparasitic agent [50], diheteropeptin, and others [52]. Depudecin, a linear epoxide, is a less potent irreversible inhibitor [45]. Hybrid structures have been synthesized combining the tetrapeptide moiety of trapoxin and moieties similar to the hydroxamic acid of trichostatin A, a reversible HDAC inhibitor isolated from *Streptomyces hygroscopicus* [53]. The resulting compounds are also reversible inhibitors, with potencies in the subnanomolar range [54].

DNA topoisomerases are essential enzymes in the control of DNA topology and the multiple processes linked to DNA metabolism, including gene expression, replication, and recombination. These enzymes produce transient breaks in the DNA backbone, allowing the passage of DNA segments through these breaks before resealing. Type I topoisomerases produce single-strand breaks, whereas type II can simultaneously cleave both strands. Eukaryotic DNA topoisomerases are the target of several antitumor drugs already on the market or in advanced stages of development. For instance, doxorubicin targets type II topoisomerase, whereas topotecan and other derivatives of camptothecin, an alkaloid from *Camptotheca acuminata*, are selective for type I [55,56]. Several fungal metabolites have been described that target mammalian DNA topoisomerases, the most relevant being saintopin and related naphthacenediones. Saintopin is an antitumor antibiotic produced by *Paecilomyces* sp., discovered by screening using calf thymus DNA topoisomerase I. Saintopin induces both topo I– and II–mediated DNA breaks, showing a potency comparable to other inhibitors, such as camptothecin [57]. Structurally related molecules with the same mode of action also have been reported, such as UCE1022, also from *Paecilomyces* sp. [58], and the topopyrones, isolated from *Phoma* sp. and *Penicillium* sp. The latter are

very potent (comparable to camptothecin) and specific topoisomerase I inhibitors and also show antiviral activity against herpesvirus [59].

Hypocrellins (Fig. 3) are pigments derived from *Hypocrella bambusae* and *Shiraia bambusicola*, two fungi used in traditional medicine in China and which have been thoroughly studied for their ability to sensitize cells to light and ultrasonic waves [60]. Apparently, the effect of these polycyclic quinones is due to the formation of hydrogen peroxide in the cells after photoactivation, which leads to apoptosis via activation of caspase-3 [61]. Hypocrellin derivatives are being studied for the potential treatment of colorectal and breast cancers and nonmalignant diseases, including psoriasis, acne, and actinic keratoses.

Brefeldin A (Fig. 3) is one of the fungal metabolites that have been the subject of extensive research in the last decade (a simple Medline search for the word *brefeldin* from 1993 to date produced 1709 references). This compound, produced by species of *Penicillium* and *Ascochyta* [23], was originally isolated as an antibiotic in 1958, but later was established to have several other activities, including antifungal, antiviral, nematocidal, and antitumor effects [62]. Studies on its mode of action have revealed that brefeldin is an inhibitor of protein secretion, blocking traffic from the endoplasmic reticulum to the Golgi apparatus but not vice versa, resulting in the disassembly of Golgi complex. These effects appear to be mediated by the inhibition of guanine nucleotide-exchange proteins for ADP-ribosylation factors that are essential in vesicular trafficking ([63] and references therein). Furthermore, brefeldin A has been shown to affect the cell cycle and to induce apoptosis in cancer cells, and this has prompted interest in its development as an anticancer agent [64,65]. However, the compound shows pharmacokinetic properties that preclude its use in the clinics (poor oral bioavailability and rapid clearance from plasma), as well as low aqueous solubility [66]. A number of brefeldin A prodrugs have been synthesized to address these issues [62,67].

Protein kinases are a large family of proteins playing critical roles in signaling pathways regulating cellular functions such as cell growth, differentiation, and apoptosis. A number of oncogenes have been identified that encode for activated protein kinases, and this suggests that protein kinase inhibitors may have therapeutic value in the treatment of cancer. Small molecules or monoclonal antibodies inhibiting the tyrosine kinases associated with growth factor receptors such as EGF, VEGF, and PDGF have been already launched (e.g., Gleevec) or are in advanced stages of clinical development [39,68]. Inhibitors of cyclin-dependent kinases (CDKs), the kinases controlling the cell cycle, and of the protein kinases involved in the signaling cascade of the Ras pathway (RAF, MEK) also have reached clinical trials [39,68]. Several inhibitors of the protein kinase C (PKC) also are being tested in humans; some of these are derivatives of staurosporine, isolated from an actinomycete [69].

A number of fungal metabolites have been described in the last decade as protein kinase inhibitors. For instance, the paeciloquinones, emodin, and other related compounds [70] are inhibitors of EGF-TK, with potencies in the low micromolar range. These compounds also inhibited other tyrosine kinases such as vAbl and c-Src kinase, but were inactive or much more weakly active on serine/threonine kinases such as PKC [70]. The compound BE-23372-M, isolated from *Rhizoctonia solani*, is a submicromolar inhibitor of EGF-TK, competitive with respect to both ATP and the substrate peptide, showing selectivity for this kinase against other tyrosine and serine/threonine kinases [71]. A family of resorcylic acid lactones structurally related to the antifungal agent hypothemycin has been reported as potent and selective inhibitors of MEK (MAPK/ERK kinase). These compounds also inhibited the growth of several human epithelial cell lines and showed

antitumor activity in a mouse model [72]. Other hypothemycin analogues named as aigialomycins recently have been reported showing antimalarial activity and cytotoxicity against several tumor cell lines, but whether these other analogs are also protein kinase inhibitors has not been disclosed [73].

Several fungal metabolites have been reported as inhibitors of PKC. One of the most relevant ones is (–)balanol (Fig. 3), which was isolated from *Verticillium balanoides* in 1993 [74], and subsequently from *Fusarium merismoides* with the name azepinostatin [75], and from other fungi. The compound is an ATP competitive inhibitor exhibiting low nanomolar Ki values; it inhibits also other serine-threonine kinases such as PKA (cAMP-dependent protein kinase), but has no effect on tyrosine kinases. Crystal structures of PKA complexed with balanol have been elucidated [76]. A substantial number of analogs have been reported showing selectivity for PKC versus PKA or vice versa [77,78]. Another interesting PKC inhibitor derived from a fungus is calphostin C (Fig. 3). This compound has been used extensively as a research tool in an astounding number of publications. Calphostin C, produced by *Cladosporium cladosporioides*, inhibits PKC by competing at the binding site for diacylglycerol and phorbol esters, and this activity is dependent on exposure to light [79]. Calphostin C has been shown to induce light-dependent apoptosis in a number of tumor cell lines at nanomolar doses [80], an effect that resembles that of the hypocrellins, structurally related compounds discussed above as photosensitizers (Fig. 3). Again, the mechanism mediating this effect is not totally clear [81]. Besides being a PKC inhibitor, calphostin C has been reported to have several other effects, including directly inhibiting phospholipase D at nanomolar concentrations [82] and blocking L-type calcium channels [83]. Finally, secalonic acid D is an acutely toxic and teratogenic mycotoxin [84] produced by *Claviceps purpurea* and *Penicillium oxalicum*, which has been shown to be an inhibitor of PKC, PKA, and other kinases [85]. It has been suggested that the teratogenic effect of secalonic acid D could involve PKC inhibition [86].

Fumagillin, an old antibiotic used successfully to treat microsporidial infections in the clinic [87], as described in section 6, also has been used to develop an antitumor agent. Specifically, its semisynthetic derivative TNP-470 (Fig. 3) has been subjected to extensive studies in diverse types of cancer and also regarding its mechanism of action. These compounds are potent antiangiogenic agents [88], inhibiting neovascularization by arresting endothelial cell cycle in the late G1 phase. The target of fumagillin and its derivatives has been shown to be methionine aminopeptidase-2 (MetAP-2) [89]. The physiological role of this enzyme, which removes amino-terminal methionine residues from peptides, is still unclear. Its expression correlates with cell growth, but it is expressed in both endothelial and nonendothelial cells, so the selectivity of fumagillin and TNP-470 to inhibit the endothelial cell cycle may involve other factors. Recent work has revealed that TNP-470 produces the accumulation of p21^{CIP/WAF}, one of the cyclin-dependent kinases inhibitors, leading to cell cycle arrest at the G1 to S transition. This effect seems to involve activation of p53. This mechanism was observed only in endothelial cells and not in other cell types, such as fibroblasts [90]. TNP-470 is in clinical trials in Kaposi's sarcoma; renal cell carcinoma; and brain, breast, and other cancers [91].

4. ANTIBACTERIAL

Antibiosis against bacteria was the first described biological activity of fungal metabolites that resulted in a therapeutically useful drug. Obviously, the discovery and industrial development of penicillin from *Penicillium chrysogenum* stands out as a hallmark in the

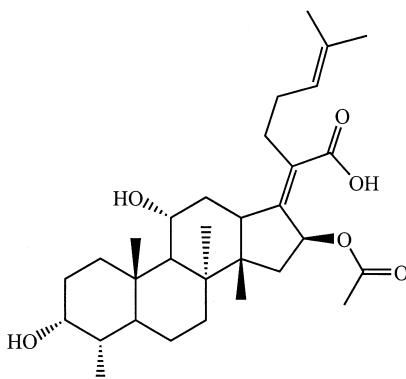


Figure 4 Fusidic acid, an inhibitor of protein synthesis in bacteria.

history of medicine. Since the discovery of penicillin, a few other fungal-derived antibiotics have been taken to the market, the most remarkable being cephalosporin and fusidic acid. Cephalosporin, like penicillin, is a β -lactam inhibitor of bacterial cell wall synthesis. Originally discovered from the species *Acremonium chrysogenum* in the 1940s, cephalosporin has been at the origin of several generations of semisynthetic derivatives (e.g., cefuroxime, cephalexin, cefaclor, cefotaxime, ceftriaxone, ceftazidime, cefixime), some of which are still among the best-selling antibiotics in the market today [92]. Fusidic acid (Fig. 4) is a bacteriostatic antibiotic, produced by *Fusidium coccineum* and other species [23], which inhibits protein synthesis in bacteria at the level of the elongation factor G [93]. It is active against gram-positive bacteria, including anaerobic species, but lacks activity against gram-negative bacteria [94]. It is most frequently used topically to treat skin [95] and ophthalmic infections [96], but it is also indicated for intestinal infection with *Clostridium difficile* [97] and for infections of different organs by *Staphylococcus aureus* and other gram-positive bacteria [98,99].

In the past 50 years, a huge number of fungal metabolites with antibacterial activity have been described, but none of them could be developed as clinically useful antibiotics. Nearly all the new antibacterial agents described from fungi have been discovered by using non-mode-of-action assays—that is, by looking for compounds able to inhibit the growth of the target bacteria. The few exceptions found in the literature in recent years include clerocidin and other fungal inhibitors of DNA gyrase [100,101], but these were not specific for the bacterial enzyme, because they inhibited eukaryotic topoisomerase II as well. Clerocidin, a metabolite isolated from *Fusidium viride* and other species, showed a broad spectrum of antibacterial activity, being as potent or more than ciprofloxacin against gram-positive and anaerobic bacteria [100].

5. ATHEROSCLEROSIS

Much of the prestige that fungi may have as an interesting source of pharmaceutically useful compounds has to be attributed to the statins, the inhibitors of 3-hydroxymethyl-glutaryl-CoA reductase that are the most widely used cholesterol-lowering drugs on the market. The first natural statin introduced in the market, lovastatin (Mevacor; Fig. 5), was

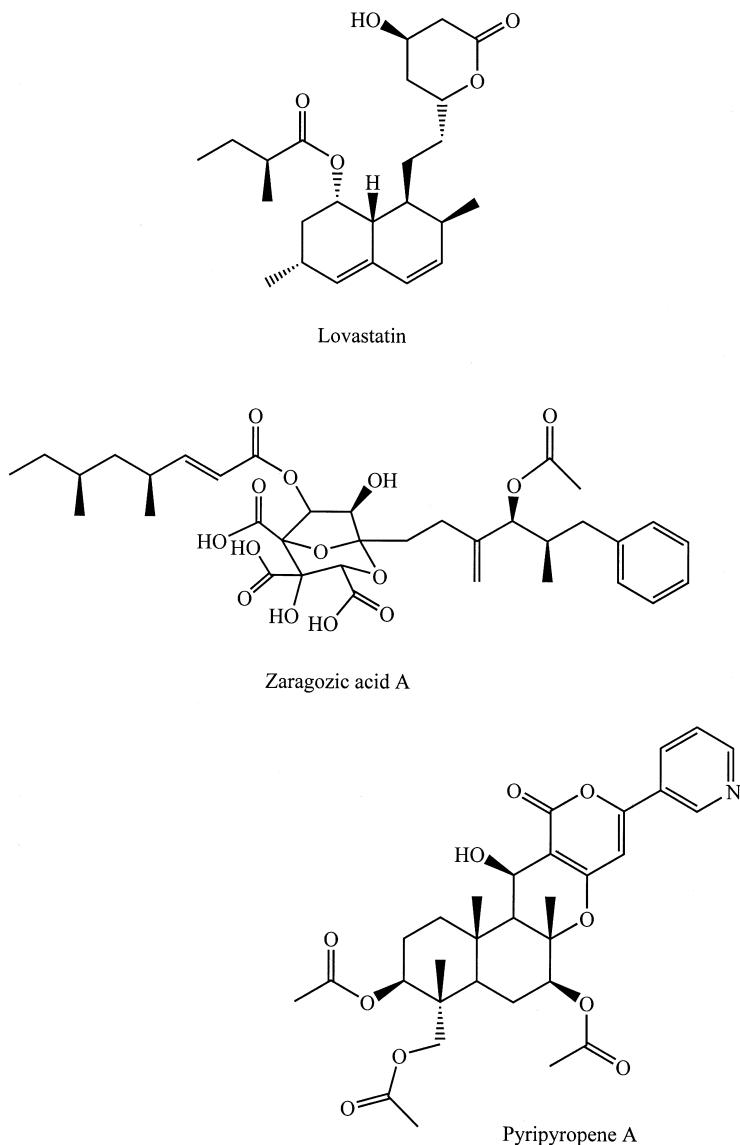


Figure 5 Cholesterol-lowering compounds: lovastatin, an HMG-CoA reductase inhibitor; zaragozic acid A, a squalene synthase inhibitor; pyripyropene A, an ACAT inhibitor.

discovered by Merck scientists in the late 1970s [102,103]. A semisynthetic derivative, simvastatin (Zocor), followed soon after. In the following years, other natural (pravastatin) or totally synthetic statins (atorvastatin, fluvastatin) from other companies reached the market. Several clinical studies have evidenced the benefits of statins in the prevention of coronary artery disease, in subjects with elevated or even average cholesterol levels. Thus, the therapeutic class of the statins has had a tremendous impact in the lives of

millions of patients at cardiovascular risk [104,105]. Moreover, their potential to treat other disorders, such as Alzheimer's disease, is being evaluated [106].

It is therefore no surprise that this has been an area in which extensive efforts have been made by many groups, and a number of targets have been used in industrial screening programs. One of the most exciting discoveries of the early 1990s in the area was that of zaragozic acids (also called squalenestatins; Fig. 5). These compounds are potent inhibitors of squalene synthase, the major regulatory enzyme for sterol biosynthesis, with IC₅₀s in the picomolar range. Squalene synthetase catalyses the first dedicated step in sterol biosynthesis, the head-to-head ligation of two molecules of farnesyl diphosphate. At least one synthetic inhibitor of the enzyme (BMS-187745) has been evaluated in humans in phase I [107]. The zaragozic acids were discovered simultaneously by groups at Merck and Glaxo [108]. A large number of natural analogs were directly isolated from many fungal species or produced by directed biosynthesis, and they were subjected to extensive medicinal chemistry studies regarding structure–activity relationships [109]. The zaragozic acids showed oral activity in animal models [108,110], but despite the initial excitement raised by these compounds, toxicity issues [102] precluded their development as drugs. Besides zaragozic acids, a few other inhibitors of squalene synthase have been isolated from fungi, such as schizostatin [111], the bisabosquats [112], and alkylcitrates such as the viridiofungins [113] and the related compounds CJ-13,981 and CJ-13,982 [114], among others, but all of these were much weaker inhibitors than zaragozic acids, with IC₅₀ values in the micromolar range.

Another enzyme that has been extensively used as a target for the discovery of cholesterol-lowering agents has been acyl-CoA:cholesterol acyltransferase (ACAT). This enzyme participates in the assembly of very low density lipoproteins, rich in triglycerides, as well as in the formation of cholesteryl ester storage droplets within cells residing in the vessel wall. ACAT inhibitors have been reported to lower cholesterol accumulation in animal models and some of them are being tested in clinical trials [104,115]. At least 10 families of compounds isolated from fungi have been reported as inhibitors of this enzyme, most of them showing IC₅₀s in the micromolar range. The most interesting are the pyripyropenes (Fig. 5), a large family of metabolites (18 members reported to date) isolated from *Aspergillus fumigatus*, some of which are submicromolar inhibitors of ACAT *in vitro*, and inhibit cholesterol absorption in a hamster model [116]. Epi-cochlioquinone A is a structurally related compound from *Stachybotrys bisbyi* that also shows activity *in vivo* in rats [117]. However, no further progress has been reported for any of them beyond the discovery step.

The cholesterol ester transfer protein (CETP) is another target that has been used to develop agents aimed at increasing high-density lipoproteins (HDL), which would be expected to have a protective effect against atherosclerosis. This protein plays a critical role in the process of reverse cholesterol transport from HDL to LDL. At least one CETP inhibitor is now in clinical trials, after showing efficacy in animal models [104]. Fungal inhibitors of CETP include a series of rosenonolactone derivatives isolated from *Trichothecium roseum* [118]; an apiosporamide-related metabolite reported from *Cytospora* sp. [119] and others, but all of them are relatively weak (IC₅₀ < 10 μM) and no further progress has been reported for any of them.

Other enzymes that have been suggested as potential targets for atherosclerosis, and for which fungal inhibitors have been reported, include ATP citrate lyase [120], 15-lipoxygenase [121], and diacylglycerol acyltransferase [122]. Also, fungal compounds have been reported that are inducers of LDL receptor gene expression [123].

6. ANTIPARASITIC

Fungi are known to produce a good number of compounds with antiparasitic activity (both for protozoan and metazoan endo- and ectoparasites). About 7.7% of the references surveyed for the period from 1993 to 2001 reported compounds with this type of activity. Some of the most interesting of these fungal metabolites are discussed here.

A number of human and animal parasitic diseases, including malaria, cryptosporidiosis, toxoplasmosis, and coccidiosis, are caused by protozoa of the subphylum *Apicomplexa*. The cyclic tetrapeptide apicidin (Fig. 6), produced by *Fusarium* species, was identified by Merck scientists as a broad-spectrum agent effective against a range of apicomplexan parasites [50]. It acts as a potent inhibitor (IC_{50} 1–2 nM) of the parasite histone deacetylase (HDAC). Apicidin exhibits a broad-spectrum *in vitro* activity against the *Apicomplexa*, as well as *in vivo* activity against *Plasmodium berghei* malaria in mice. Semisynthetic derivatives of apicidin with potencies in the picomolar range have been reported [124]. Other structurally related fungal tetrapeptides that were known inhibitors of mammalian HDAC, such as HC-toxin, also have potent antiprotozoal activity, comparable to apicidin, and they are able to inhibit the coccidial (*Eimeria tenella*) HDAC as well [50]. As already discussed, these HDAC inhibitors have been extensively studied for their additional potential as antitumor agents.

Fumagillin (Fig. 6) was discovered as an antibiotic in the 1950s. This compound has been used for the treatment of microsporidiosis, with satisfactory results in clinical trials [87]. Microsporidiosis is produced by *Enterocytozoon bieneusi*, and it causes chronic diarrhea, malabsorption, and wasting in immunocompromised (e.g., HIV-infected) patients. Currently, there is no effective treatment. Fumagillin also has been tested successfully for the control of infections in rainbow trout by *Loma salmonae*, a microsporidian gill pathogen [125]. In addition to its properties as an antiparasitic agent, fumagillin is an antiangiogenic agent, and some derivatives have been taken to clinical trials as antitumor agents, as previously discussed. The mechanism of action of fumagillin seems to be inhibition of methionine aminopeptidase-2 [89].

Paraherquamide (Fig. 6) is a broad-spectrum anthelmintic agent produced by *Penicillium paraherquei*. Its potency against a range of parasites evaluated in cattle models is lower than ivermectin, the gold standard of the area, but superior to other marketed products. Unfortunately, the compound showed toxicity in canine models, and this precluded its development as a drug. However, this toxicity is species-specific (it is not observed in ruminants), and it is still possible that further structural refinement could lead to a more promising candidate [126]. The mode of action of paraherquamide is not well understood, but it seems to be distinct from that of other major anthelmintic classes, given that the compound is very active against nematode strains resistant to ivermectin and benzimidazoles [126]. Other structurally related anthelmintic agents produced by *Aspergillus* spp. are the aspergillimides, which showed even higher potency than paraherquamide in rodent models [127], and sclerotiamide which, although lacking anthelmintic efficacy, shows remarkable insecticidal properties [128], whereas paraherquamide is a poor insecticidal agent. Other interesting anthelmintic agents derived from fungi found in the recent literature include the depsipeptide PF1022A, which appears to be a modulator of γ -aminobutyric acid receptors in nematodes, in a way different from ivermectin [126].

Nodulisporic acids (Fig. 6) are novel indole diterpenes that exhibit potent insecticidal properties, and were discovered by screening for activity against the larvae of the blowfly

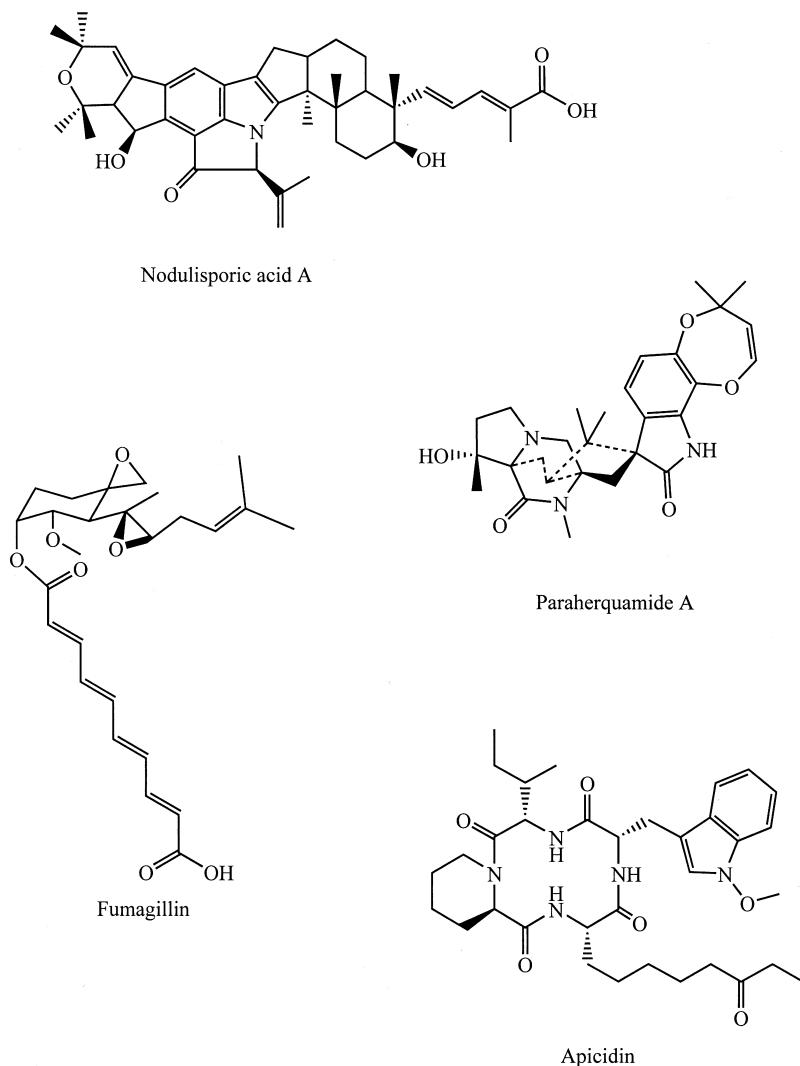


Figure 6 Antiparasitic compounds: fumagillin, a methionine-aminopeptidase-2 inhibitor with activity against microsporidia; apicidin, an HDAC inhibitor with antiprotozoan activity; paraherquamide, an anthelmintic agent; nodulisporic acid A, an activator of insect glutamate-gated chloride channels with insecticidal properties.

Lucilia sericata [129]. Nodulisporic acids are produced by a pantropical species of *Nodulisporium*, most likely the anamorph of a *Hypoxyylon* sp. [130]. Nodulisporic acids exert their action by activating the insect glutamate-gated chloride channels, in a similar fashion to ivermectin [126,131]. Nodulisporic acid A was about 10-fold more potent than ivermectin in a flea model. *In vivo* studies in dogs have shown that nodulisporic acid A exhibited potent systemic efficacy, with no adverse effects. Derivatives of these natural products with improved properties have been also reported [126].

Chitinases, enzymes able to hydrolyze linear chitin polymers, have been proposed as targets for the development of insecticidal and antimalarial drugs. Inhibitors have been reported to kill *Lucilia cuprina* blowfly larvae *in vitro*. Two fungal chitinase inhibitors have been reported recently, argifin and argidin, cyclic pentapeptides isolated from *Gliocladium* and *Clonostachys* spp., respectively [132]. The compounds are nanomolar inhibitors of the *L. cuprina* chitinase. The structure of the enzyme in complex with these two compounds recently has been disclosed [132], and this should prove useful in the design and synthesis of derivatives with greater therapeutical potential.

7. NEUROLOGIC DISORDERS

The most relevant clinically useful fungal compounds in the area of neurologic disorders are the ergot alkaloids. Ergotamine (Fig. 7), the main alkaloid of the ergot fungus *Claviceps purpurea*, was the cause of mass poisoning in Europe in the Middle Ages due to eating bread from rye contaminated with sclerotia of the fungus [133]. Because of its strong vasoconstrictor effects, it was used in traditional medicine to precipitate childbirth and control post-partum hemorrhage. The compound was isolated in 1918 and has been used for the treatment of migraine since 1926, without any specific alternatives for several decades thereafter. The mode of action of ergotamine has been the subject of a substantial

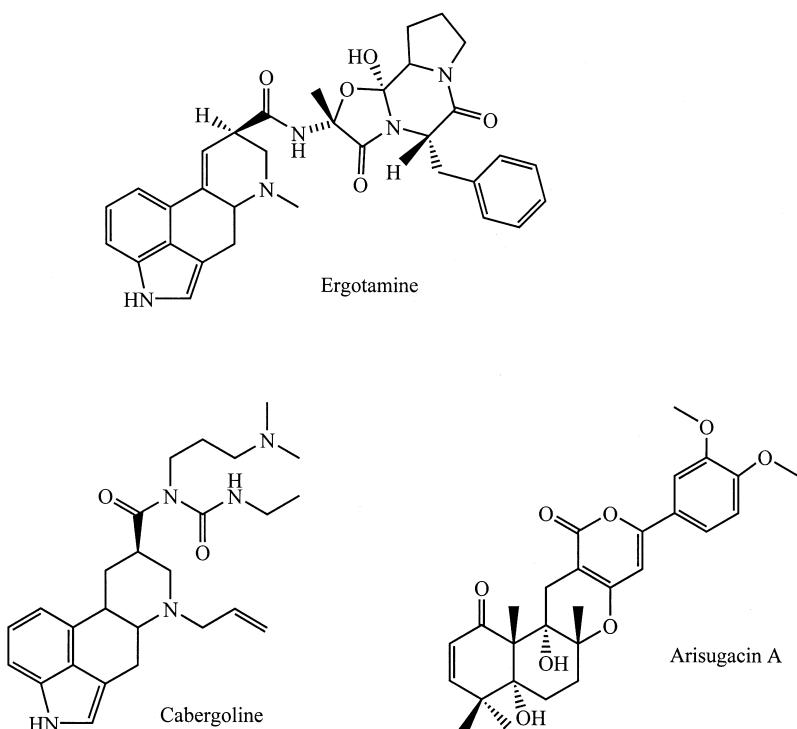


Figure 7 Central nervous system disorders: ergotamine, an antimigraine compound; arisugacin A, an inhibitor of acetyl cholinesterase.

number of studies [133]. The compound is able to bind a diversity of receptors, including several subtypes of the 5-HT, dopamine, and noradrenaline receptors. At therapeutically relevant doses, however, ergotamine appears to act mainly as an agonist of α -adrenoceptors, 5-HT_{1B/1D} and dopamine D₂ receptors. In contrast, the triptans, the new synthetic antimigraine agents, are more specific for 5-HT_{1B/1D} versus other receptors. The most obvious pharmacological effect of ergotamine is vasoconstriction, particularly marked in the carotid vascular bed. This has long been believed to be the basis for its antimigraine properties (although there are reports claiming that the effect is more due to inhibition of neurogenic inflammation and neuronal transmission). Although the drug has been used in the clinic for many years (as Cafergot, Ergostat, and other brand names), its usefulness is still controversial; reports have been published that both support and refute its efficacy [133]. Furthermore, due to its poor selectivity, a number of side effects have been reported that limit the applicability of this compound. Finally, with the introduction of the much more specific triptans, ergotamine has become a less preferable choice for clinicians and patients. However, ergotamine will keep its place in history as one of the most extensively used fungal compounds in the clinical practice.

Other structurally similar ergot alkaloids have been used in the clinics, such as ergotoxine (Hydergine), a vasodilator to treat disturbances in peripheral blood circulation [134]. Bromocriptine (2-bromo-ergocryptine) is a semisynthetic derivative that is a potent dopamine agonist specific for the D₂ receptor, and as such has been used to treat Parkinson's disease since the early 1970s. It is also a reducer of prolactin and growth-hormone secretion, and for this reason it has been used to stop the production of milk and to treat acromegaly [134]. Also, it has been used to treat hyperprolactinaemia due to prolactinomas, which represent 50% of all pituitary adenomas and 12% to 15% of all intracranial tumors [135]. Other analogs have been developed more recently, such as cabergoline (Fig. 7) and terguride, which are also dopamine agonists and are used for the treatment of Parkison's disease and hyperprolactinaemic disorders [102,136].

There is evidence that cholinergic functions decline in some brain areas in individuals affected by Alzheimer's disease and in a variety of afflictions in which memory and cognitive function are impaired. Therefore, enhancement of cholinergic neurotransmission by inhibiting acetylcholinesterase (AChE) has been considered as a therapeutical approach against Alzheimer's and other diseases. Inhibitors of the enzyme, such as tacrine, donepezil, rivastigmine, and metrifonate, have been reported to improve cognitive function in Alzheimer's disease patients [137]. However, some of the existing AChE inhibitors are known to have undesirable side effects. Tacrine, for instance, the first AChE inhibitor in the clinic, launched in 1995, is known to induce hepatotoxicity. All the inhibitors used in the clinic also inhibit butyrylcholinesterase (BuChE), an enzyme present in plasma. Therefore, more specific inhibitors of AChE would be expected to be attractive leads for the treatment of Alzheimer's disease [138]. Several fungal metabolites have been reported to be inhibitors of the enzyme, such as arisugacins, territremes [139,140], quinolactacins [138], and xyloketals [141]. Arisugacins A (Fig. 7) and B and the related territremes are especially potent, with IC₅₀ values in the nanomolar range, and highly specific for AChE versus BuChE [139], but none of them have progressed from the discovery step. However, a substructure of arisugacins has been used to prepare new synthetic analogs of tacrine that have improved selectivity for AChE vs. BuChE [142]. Arisugacins are structurally related to the pyripyropenes, inhibitors of acyl-CoA:cholesterol acyltransferase, discussed previously [116].

A number of fungal metabolites have been described that have some type of neurotrophic or neuritogenic activity. It is assumed that compounds with this activity could have an application in an array of neurological disorders, including dementia. For instance, stachybotrin C was demonstrated to induce differentiation of PC12 cells into neuronlike cells, much like nerve growth factor (NGF), at micromolar doses. The compound, produced by *Stachybotrys parvispora*, also showed a protective effect against neuronal damage in primary cultures of neurones [143]. Another compound, NG-061, isolated from *Penicillium minioluteum*, showed the same effect on PC12 cells, and the effect was additive to that of NGF, but it did not show any protective effect on neurone primary cultures [144]. Several other fungal compounds have been described as neuritogenic by screening for morphological changes in PC12 cells or other cell lines, but no details on the mechanism of action have been provided. An exception is the compound PS-990, produced by an *Acremonium* sp., which has been reported to be an inhibitor of calcium/calmodulin-dependent cyclic nucleotide phosphodiesterase (CaM-PDE), competitive with cyclic AMP at low micromolar levels, and inducing neurite formation in a neurally derived cell line [145].

8. INFLAMMATION

Anti-inflammatory drugs represent a very important sector within the pharmaceutical market, including blockbuster drugs such as the coxibs, the cyclooxygenase II inhibitors. Therefore, significant effort has been expended to find new anti-inflammatory compounds from fungal metabolites. However, this effort has not yet resulted in a marketable drug of fungal origin, or even in compounds moving beyond the early discovery phase. Nonetheless, a significant number of fungal metabolites have been reported with activity in targets related to inflammatory processes. For instance, fungal inhibitors of the production of the proinflammatory cytokines tumor necrosis factor α (TNF α) and/or interleukin-1 β (IL-1 β) induced by stimuli such as the bacterial lipopolysaccharide (LPS) in intact macrophages or peripheral blood monocytes (a typical inflammatory response) have been reported repeatedly. Examples include a series of phomalactone analogs produced by a *Phomopsis* sp. [146]; methyl-5-substituted pyridine-2-carboxylates from a basidiomycete, *Marasmiellus* sp. [147]; and diterpenes isolated from *Oidiodendron griseum* [148]; all of them with potencies ranging in the submicromolar to micromolar range. One of the *O. griseum* compounds (PR 1388, Fig. 8) has been shown to significantly attenuate TNF α levels in plasma when administered orally to LPS-challenged mice [149].

Other targets evaluated for the development of new anti-inflammatory agents include several phospholipases (PL), enzymes involved in the catabolism of phospholipids. PLA₂-II in particular, an extracellular phospholipase present in inflammatory regions, such as synovial fluid of patients with rheumatoid arthritis, has been shown to have proinflammatory activity. The most potent fungal inhibitors of PLA₂-II reported to date are the thiocolins (Fig. 8), isolated from *Thielavia terricola*, with IC₅₀ values in the nanomolar range [150], and much less potent against the pancreatic type PLA₂-I. Also, thiocolins A1 β and B3 showed activity in a rat carrageenan-induced pleurisy model [151].

Neutral sphingomyelinase (nSMase), an enzyme catalyzing the hydrolysis of sphingomyelin to ceramide and phosphocholine, also has been implicated in the inflammatory response. Ceramide production has been reported to increase in response to inflammatory stimuli, including TNF α and IL-1 β , and this subsequently triggers signaling pathways leading to cell proliferation and differentiation or to apoptosis. Inhibitors of nSMase would therefore be expected to interfere in the inflammatory response. Several fungal inhibitors

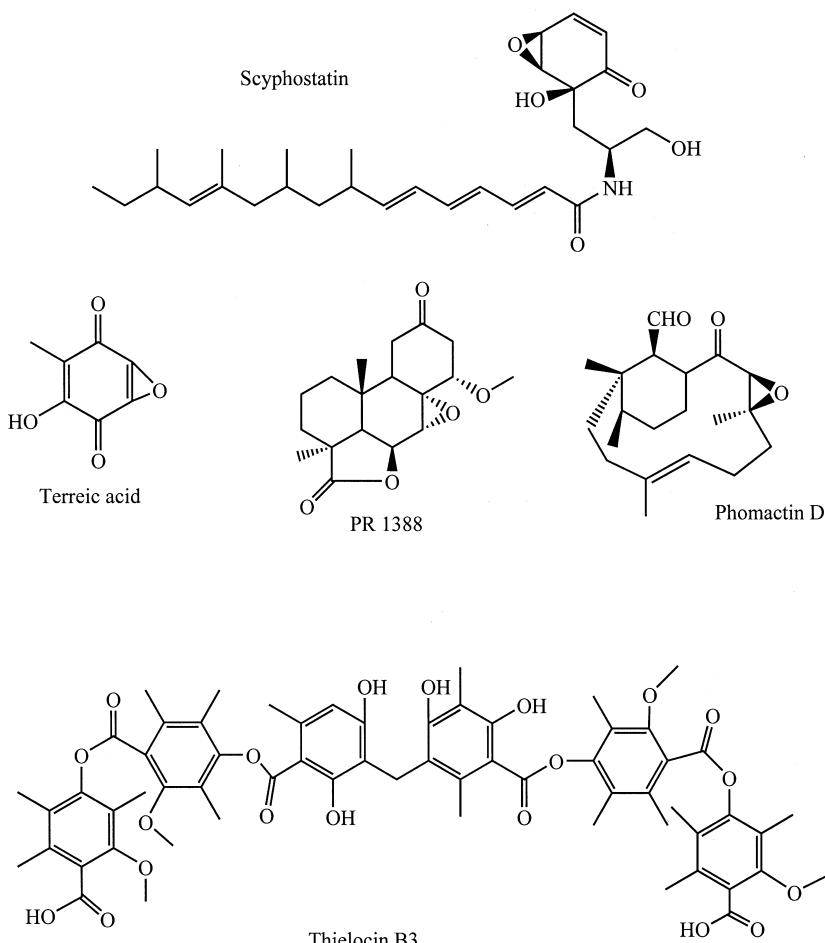


Figure 8 Antiinflammatory compounds: scyphostatin, a neutral sphingomyelinase inhibitor; phomactin D, an inhibitor of PAF; terreic acid, a Bruton's tyrosine kinase inhibitor; PR 1388, an inhibitor of TNF α production; thielocin B3, a phospholipase A $_2$ inhibitor.

of nSMase have been reported recently, the most interesting being scyphostatin (Fig. 8), produced by *Trichopeziza mollissima*. This compound is a selective micromolar inhibitor of nSMase, and much less potent versus acid SMase. It inhibited the production of prostaglandin E2 and IL-1 β induced by LPS in human peripheral monocytes and, when administered orally, it inhibited carrageenin-induced paw edema in rats [152].

There are several fungal metabolites reported that antagonize the platelet activating factor (PAF), a potential mediator of allergic and nonallergic inflammatory diseases. PAF binds to diverse cell types, inducing a series of biological responses, such as platelet aggregation, leukocyte activation, and hypotension. The most interesting PAF antagonists obtained from fungi are the phomactins, produced by *Phoma* sp. [153]. The most potent compound in the series, phomactin D (Fig. 8), showed an IC₅₀ in the submicromolar range in a platelet aggregation assay. Derivatives of this compound have been prepared with

enhanced potency [154]. Other fungal inhibitors have been described but were less potent and did not show activity in animal models [155].

Fungal inhibitors of other enzymes involved in the inflammatory process have been described, such as the interleukin-converting enzyme [156] or stromyelisin [157]. Terreic acid (Fig. 8) has been reported more recently as an inhibitor of Bruton's tyrosine kinase (Btk) in mast cells. Btk is activated by cross-linking of high-affinity IgE receptor, and is involved in the signaling cascades leading to exocytosis of pro-inflammatory cytokines in the allergic response [158]. Terreic acid was originally isolated in 1942 as an antibiotic from *Aspergillus terreus*. It inhibits protein synthesis by blocking the formation of leucyl-tRNA in sensitive bacteria. However, it also has shown to be a specific inhibitor of the interaction between Btk and PKC, and this results in the recapitulation of the phenotype of mutations of Btk, without blocking many other cellular functions. Although the compound cannot be used in the clinic because of its cytotoxicity, it could be used as a lead to generate Btk inhibitors with improved therapeutic profiles [158].

9. ANTIVIRAL

Considerable effort has been dedicated to the search of antiviral agents from natural products by several research groups. The HIV pandemic raised the urgent need for antiretroviral drugs. Two enzymes encoded by the viral genome, the reverse transcriptase and the protease, have been used to look for anti-HIV agents that have resulted in clinically useful drugs, but none of them originated from natural products. Although a number of fungal metabolites have been described as inhibitors of these two enzymes, none has progressed beyond early discovery. Examples of these are the mniopetals, metabolites produced by the basidiomycete *Mniopetalum* sp., weak inhibitors of reverse transcriptase of HIV and other viruses [159], and the semicochliodinols, metabolites related to the asterriquinones, produced by *Chrysosporium merdarium*, which are submicromolar and relatively specific inhibitors of HIV protease [160].

A promising target in the fight against HIV infection is the integrase, the enzyme that catalyzes the insertion of the viral DNA into the genome of the host cell. Inhibitors of the enzyme are able to inhibit viral replication in cells [161], and there are already some inhibitors in development as clinical candidates. Our group was involved in an intensive screening for HIV-1 integrase inhibitors from fungal natural products for several years. This effort resulted in a significant number of novel compounds that were active in the micromolar range, such as integrastatins [162], integramides [163], integracins [164], integrac acid, and phomasetin [165], plus some previously known compounds for which this biological activity had never been described, such as equisetin and oteromycin [165]. Some derivatives prepared by chemical and enzymatic modification of integrac acid (Fig. 9) have been described, although none of them showed improved properties compared to the parent molecule [166].

As for other viruses, several compounds inhibiting influenza virus replication in cells have been reported, such as 10-norparvulenone [167] and the compounds FR191512 and FR198248 (Fig. 9), which showed *in vivo* efficacy in a mouse model [168,169]. The mechanisms of action of these compounds have not been fully established, although at least the compound FR198248 seems to inhibit the stage of virus adsorption [169]. Another interesting anti-influenza compound is flutimide (Fig. 9), which was discovered in a screening program seeking inhibitors of cap-dependent transcription of influenza virus [170]. Flutimide is a substituted 2,6-diketopiperazine, structurally related to aspergillic acid. The

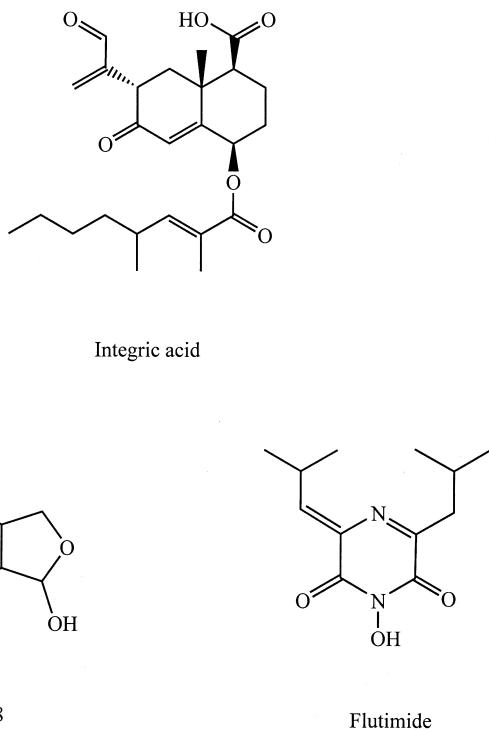


Figure 9 Antiviral compounds. Integric acid, an HIV integrase inhibitor. FR198248, an antiinfluenza agent. Flutimide, an inhibitor of cap-dependent transcription of influenza virus.

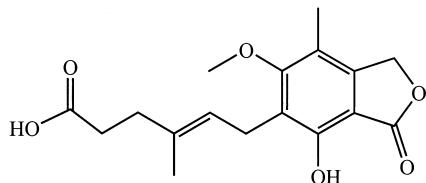
compound is produced by *Delitschia confertaspore*, a new fungal species isolated from dung of dassie collected in Namibia [171]. It specifically targets the endonuclease activity of the cap-dependent transcriptase of influenza A and B viruses, being ineffective versus other viral polymerases [170].

10. IMMUNOSUPPRESSION

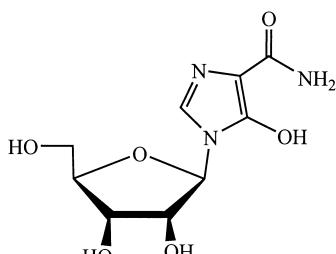
One of the major medical breakthroughs of modern medicine is undoubtedly the development of the organ transplantation techniques. The success of these approaches was possible only after the discovery of immunosuppressive agents. In this field, the discovery of cyclosporin A (Sandimmune, Neoral) and its application to renal transplantation in 1978 was an important milestone; cyclosporin soon became the most widely used immunosuppressive drug in the clinic [172]. Cyclosporin suppresses immune responses by blocking the calcium-dependent signal transduction pathway resulting from the activation of T-cell receptors, thereby inhibiting the activation of helper T-cells and the production of interleukin-2 (IL-2). The details on its mechanism of action have been extensively reviewed [173]. Numerous natural members of the cyclosporin family have been reported [174]. Although cyclosporin A is one of the few blockbuster drugs derived from a fungus, it has several important side effects, the most important of which is nephrotoxicity. These toxicity

issues are shared by other drugs with the same mode of action, such as tacrolimus, an actinomycete metabolite. This has prompted the search for safer immunosuppressants with a different mechanism of action.

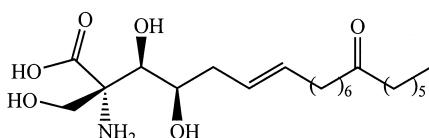
Some years after the development of cyclosporin, another immunosuppressant fungal metabolite reached the market, mycophenolic acid (Fig. 10) (see Bentley [175] for a comprehensive review on its history and properties). This compound, produced by *Penicillium* spp. and other fungi [23], had been discovered as an antibiotic in the first years of the 20th century (it was crystallized before penicillin), but its immunosuppressive properties were recognized only much later. The compound shows antibiotic, antifungal, antiviral,



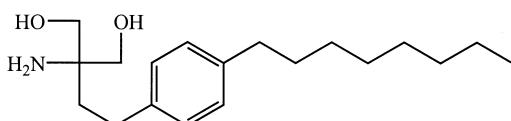
Mycophenolic acid



Mizoribine



ISP-I, myriocin



FTY720

Figure 10 Immunosuppressants: mycophenolic acid and mizoribine, inhibitors of purine biosynthesis; ISP-1, an inhibitor of serine palmitoyltransferase; FTY720, an agonist of S1P receptors.

and antitumor activities in animal studies, as well as activity against *Trichomonas*, *Eimeria*, and other parasitic protozoa. Clinical trials against several cancers showed only a poor response, and these studies were discontinued. However, mycophenolic acid was developed as a clinically useful immunosuppressant. A search for a derivative with improved oral availability led to the discovery of the 2-morpholinoethyl ester, which, as mycophenolate mophetil (CellCept), is a useful prodrug. It was approved by the U.S. Food and Drug Administration for prevention of rejection in renal allograft recipients in 1995 and for heart transplantation in 1998. The compound usually is coadministered with other immunosuppressants. Mycophenolic acid also has been used experimentally for the treatment of rheumatoid arthritis and other autoimmune diseases such as lupus, psoriasis, and others. Its potential in anti-HIV therapy has been recently suggested [176]. Furthermore, mycophenolic acid is less toxic than other immunosuppressants. The mechanism of action of mycophenolic acid is different from that of cyclosporin or tacrolimus. It is a nanomolar inhibitor of the enzyme inosine 5'-monophosphate dehydrogenase (IMPDH), an enzyme involved in the *de novo* biosynthesis of guanine. This inhibition results in impairment of DNA synthesis. T and B lymphocytes are especially sensitive to mycophenolic acid because, unlike other cell types, they seem to be dependent on the *de novo* pathway of guanine synthesis, almost lacking the capability to reutilize free purines generated in catabolic processes. The compound also prevents the glycosylation of adhesion molecules involved in the attachment and infiltration of lymphocytes and monocytes into sites of graft rejection, possibly as a result of the depletion of the GTP pool [175].

Mizoribine (bredinin; Fig. 10) is another fungal metabolite with the same mode of action as mycophenolic acid. This compound, produced by *Eupenicillium brefeldianum*, is a potent IMPDH inhibitor and has been extensively used in Japan for human renal transplantation and several autoimmune disorders [177,178].

More than a dozen publications have appeared since 1993 reporting fungal metabolites with immunosuppressive activity (or at least targeting anyone of the steps involved in the immune response). Examples include the stevastelins [179], which inhibited lymphocyte activation and proliferation, and the compound FR901483, which showed activity in a rat skin allograft model and is thought to be an inhibitor of purine nucleotide biosynthesis [180]. However, the most interesting of the fungal compounds described as immunosuppressants in this decade is the metabolite ISP-I (Fig. 10), the first of a family of fungal metabolites used for the development of a clinically useful immunosuppressant. ISP-I was discovered as a potent immunosuppressant produced by *Isaria sinclairii* [181], but it had earlier been isolated from other fungi as an antifungal agent, under the names of myriocin and thermozymocidin. The compound was more potent than cyclosporin A in suppressing lymphocyte proliferation in several models; unlike that compound, it did not inhibit production of IL-2 [181,182]. It was subsequently reported that the compound is a picomolar inhibitor of serine palmitoyltransferase, blocking sphingolipid biosynthesis and inducing apoptosis in an IL-2-dependent mouse cytotoxic T cell line [183]. Analogues with similar activity, the mycestericins, were described from one of the myriocin-producing strains [182]. All these compounds are structurally related to the sphingofungins, which have been reported to inhibit fungal serine palmitoyltransferase [7], as discussed in section 2. The antifungal activity of all these compounds seems to be due to the inhibition of this enzyme. Although the natural products were not suitable as clinical candidates, due to their toxicity and solubility problems, several synthetic analogs were reported with improved profiles [182,184]. One of these derivatives, FTY720 [184] (Fig. 10), showed good efficacy in animal models of transplantation as well as for several autoimmune disorders,

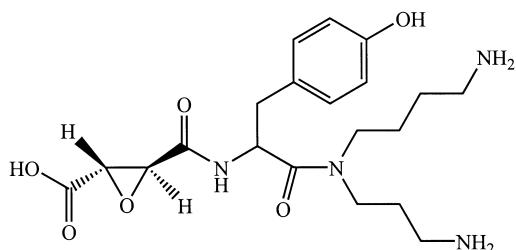
and has been tested successfully in clinical trials of renal allograft transplantation [185]. Interestingly, the mode of action of FTY720 seems to be different from that of the natural analogs, because this is not an inhibitor of serine palmitoyltransferase. Instead, the compound acts through the S1P (edg) receptors, G-protein coupled receptors that have sphingosine 1-phosphate as their natural ligand. Although FTY720 itself is only a weak ligand of some of these receptors, a phosphate ester metabolite of the compound that is naturally formed after administration in rats and mice has been shown to bind with high affinity (in the sub or low nM range) to at least four of the five members of the S1P receptor family. The activation of the S1P receptors has been correlated with the induction of lymphopenia in the animals. The retention of lymphocytes in the lymph nodes would inhibit the infiltration of lymphocytes into grafted organs [186].

11. HYPERTENSION

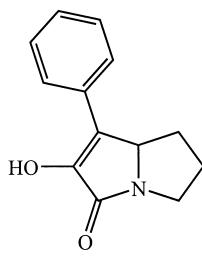
Endothelins (ETs) are peptidic hormones produced by endothelial cells that have been recognized as having a significant role in the development of hypertension and other cardiovascular diseases. The effects of endothelins are mediated through two receptors, ET_A and ET_B, which produce opposite effects, because ET_A is involved in vasoconstriction and ET_B in vasodilation. Several nonpeptidic antagonists of ET_A or dual ET_{A/B} antagonists have been developed and are in clinical trials for the treatment of vascular and pulmonary hypertension [187]. At least nine families of fungal compounds have been described as antagonists of ET_A, ET_B, or both. The list includes the spirodihydrobenzofuranlactams [188], azaphilones [189], oteromycin [190], RES-1214 [191], and others. However, all these antagonists showed only moderate affinity for the receptors, with Ki values in the micromolar range; no further progress has been reported.

12. FREE RADICAL SCAVENGERS

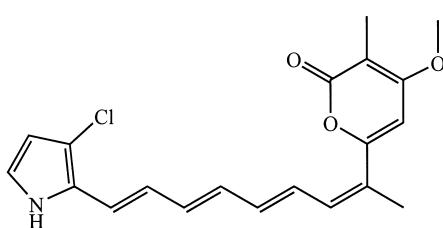
Peroxidative disintegration of cells and organellar membranes by free radicals has been implicated in the pathogenesis of a broad array of diseases, such as myocardial and cerebral ischemia, atherosclerosis, diabetes, rheumatoid arthritis, cancer initiation, and ageing processes. Therefore, free radical scavengers have been suggested to have a potential as protective agents against several diseases. Inhibition of lipid peroxidation activity in rat liver microsomes commonly has been used to search for compounds with free radical scavenging activity. A number of compounds isolated from basidiomycetes have been reported with this activity, such as the curtisians, *p*-terphenyls isolated from *Paxillus curtisii* [192]; the betulinans, from *Lenzitius betulina* [193]; and others. All of these compounds showed IC₅₀ values in the micromolar or submicromolar range. Phenopyrrozin (Fig. 11), a metabolite produced by *Penicillium* sp., reduced the chromosomal aberrations induced by paraquat, which are mediated by the generation of superoxide anions [194]. Rumbrin (Fig. 11), a metabolite produced by *Auxarthron umbrinum*, in addition to inhibiting lipid peroxidation, was able to prevent cell death caused by calcium overload in cells exposed to toxic concentrations of a calcium ionophore [195]. Finally, the ergot alkaloid derivative cabergoline (Fig. 7), which is used to treat Parkinson's disease (see section 7), has been described recently to have strong antioxidant activity by stimulating the production of glutathione and also by directly scavenging free radicals. This could help to explain its neuroprotective properties observed *in vivo* [196].



WF14861



Phenopyrrozin



Rumbrin

Figure 11 WF14861, a cathepsin B and L inhibitor; phenopyrrozin and rumbrin, free radical scavengers.

13. CATHEPSIN INHIBITORS

It has been suggested that cathepsins B, L, and K, lysosomal cysteine proteases, are involved in a number of physiopathological processes. Their participation in osteoclastic bone resorption in particular has been reported frequently, but they also play significant roles in the process of muscle fiber destruction in inflammatory myopathy and several other disorders, including rheumatoid arthritis, acute pancreatitis, and cancer progression [197]. Screening for cathepsin natural inhibitors has resulted in the discovery of several fungal metabolites, the most representative being several transepoxysuccinyl type inhibitors, such as the compound WF14861 (Fig. 11), produced by *Colletotrichum* sp. [198]. Compounds in this class inhibited cathepsins B and L at nanomolar concentrations *in vitro* and showed remarkable activity in a bone resorption mouse model [198–200].

14. DIABETES

One of the most exciting discoveries of the last decade concerning fungal metabolites was that of bis-demethyl-asterriquinone B1, DMAQ-B1 (Fig. 12). This compound was discovered as an orally available insulin mimetic agent through a screening assay designed to detect small molecules activating the human insulin receptor tyrosine kinase [101]. The compound, which was isolated from a *Pseudomassaria* sp., was shown to be selective for the insulin receptor versus other receptor tyrosine kinases, such as the insulin-like growth factor receptor or the EGF receptor. It mimicked insulin in several biochemical and cellular

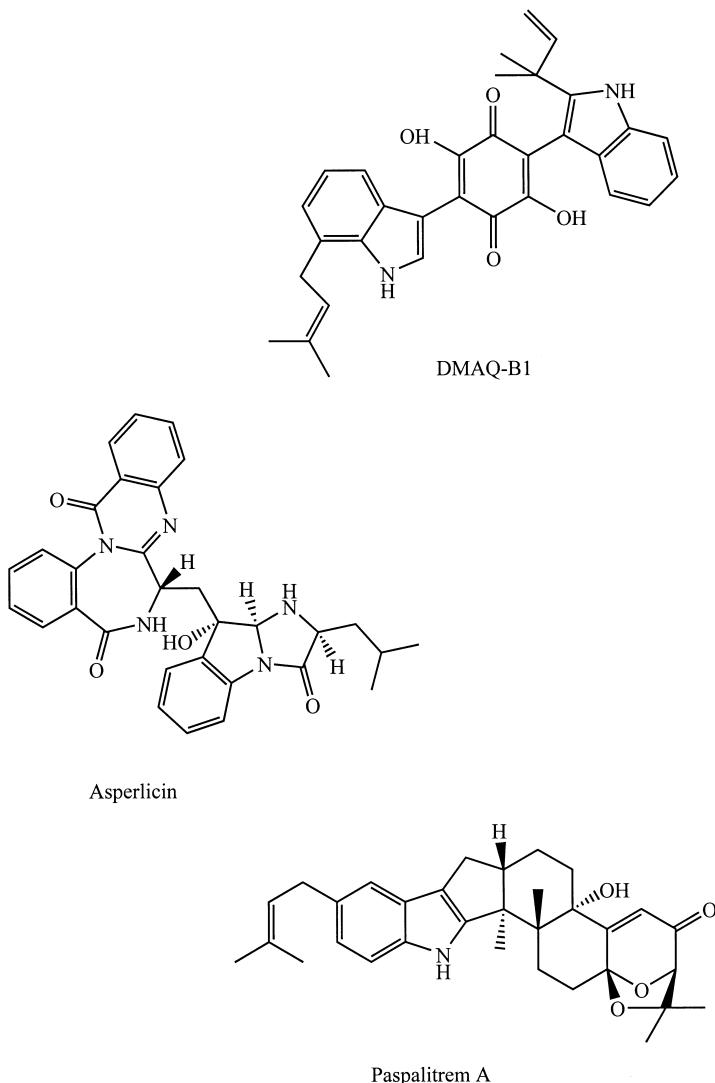


Figure 12 DMAQ-B1, an activator of the insulin receptor tyrosine kinase; asperlicin, an antagonist of CCK receptor; paspalitrem A, a potassium channel antagonist.

assays, including stimulation of glucose uptake in whole cells. Moreover, the oral administration of the compound in two mouse models of diabetes resulted in significant lowering of glucose levels and suppression of the elevated plasma insulin levels associated with one of these mouse models. This discovery was celebrated as a breakthrough in the field for the development of orally active antidiabetic agents. A synthetic derivative of DMAQ-B1 with improved characteristics subsequently has been reported [202,203]. The potential of these compounds as antiobesity agents also has been described recently. The administration of DMAQ-B1 via central intracerebroventricular resulted in a dose-dependent reduc-

tion of food intake and body weight in rats. Moreover, oral administration of the synthetic derivative in a mouse model of high-fat, diet-induced obesity reduced body weight gain, adiposity, and insulin resistance [104].

15. CHOLECYSTOKININ ANTAGONISTS

Cholecystokinins (CCKs) are peptide hormones and neurotransmitters widely distributed throughout the gastrointestinal tract and central nervous system that mediate a number of biological functions. Two receptor subtypes, CCK-A and CCK-B, have been identified: CCK-A is the predominant peripheral CCK receptor subtype, and CCK-B the predominant central CCK receptor [105]. Asperlicin (Fig. 12) was discovered in 1984 by Merck scientists as a CCK antagonist produced by *Aspergillus alliaceus* [106]. The discovery of asperlicin represented a major scientific breakthrough, this being one of the first nonpeptidic ligands described for a peptide receptor. This compound is a selective CCK-A antagonist, and it was used as a lead to develop other more potent antagonists [103], such as the clinical candidate MK-329 (devazepide) and other compounds that have been tested in humans. Devazepide was tested as a potential antilulcer therapy, but safety issues precluded further progress [107]. Both MK-329 and asperlicin were shown to inhibit the growth of CCK receptor-positive human pancreatic cancer in athymic mice. Based on these observations, the clinical candidate MK-329 was tested in patients with pancreatic cancer, but the compound failed to show any efficacy [208]. Interestingly, other asperlicin derivatives selective for the CCK-B receptor subtype have been developed more recently and proposed as development candidates for the treatment of anxiety and panic disorders and pain [102]. Some of these compounds have been tested in clinical trials, with inconclusive results [209]. Other derivatives that are CCK-B antagonists orally active in animal models recently have been studied for the treatment of gastroesophageal reflux disease [102]. Despite the lack of success in clinical trials, asperlicin derivatives have been very useful as research tools for understanding the physiological role of CCK.

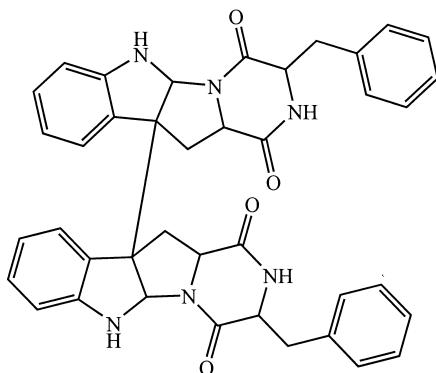
16. ION CHANNELS LIGANDS

Potassium channels are involved in the modulation of a number of physiological processes. Specifically, the high conductance calcium activated K⁺ (maxi-K or BK_{Ca}) channels, present in neurons and smooth muscle, are opened by increases in the intracellular concentration of calcium or by membrane depolarization, resulting in neurotransmitter release and smooth muscle contraction. Therefore, maxi-K channel agonists may be useful for treating neuronal and smooth muscle disorders, including hypertension and asthma. Peptidic channel blockers isolated from scorpion venoms, such as margatoxin, charybdotoxin, and iberiotoxin, have been useful tools to dissect the structure and physiological role of these channels. Some fungal metabolites also have been described that are very potent ligands of maxi-K channels [210]. These are the indole diterpenes commonly referred to as tremorgenic mycotoxins, such as paxilline, aflatrem, penitrem, or paspalitrem (Fig. 12), metabolites produced by a diversity of fungi, from the genera *Penicillium*, *Aspergillus*, *Claviceps*, and others [23]. Besides blocking the channels with high potency, these compounds modulate the binding of other ligands such as charybdotoxin by positive or negative

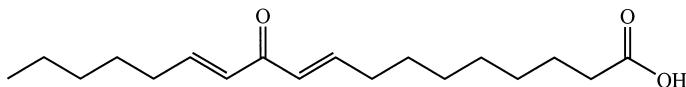
allosteric mechanisms [210] and stimulate the contractility of smooth muscle in models of guinea pig and rat urinary bladder and rat duodenum, among other effects [211].

17. NEUROKININ ANTAGONISTS

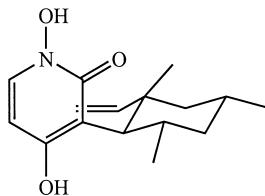
The tachykinin family of peptides, which includes substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), and other neuropeptides, are involved in a wide variety of biological functions, including smooth muscle contraction, secretion stimulation, and neuronal and immune system stimulation. They mediate their effects through three G-protein coupled receptors termed NK1, NK2, and NK3. Although the natural ligands bind to all



WIN64821



11-keto-9(*E*),12(*E*)-octadecadienoic acid (KOD)



8-methyl-pyridoxatin

Figure 13 WIN-64281, a neurokinin antagonist; KOD, a fibrinolytic enhancer; 8-methyl-pyridoxatin, an inducer of the EPO gene expression.

three, SP is more selective toward NK1, whereas NKA and NKB preferentially bind to the NK2 and NK3 receptors, respectively. Nonpeptidic antagonists of tachykinin receptors (mainly of NK1) have progressed to clinical trials for the treatment of diseases as diverse as depression/anxiety, emesis, pain, migraine, and asthma [212]. An extensive search by several industrial laboratories has led to the discovery of several fungal metabolites with activity as antagonists of NK1 and/or NK2. Most of the compounds isolated, however, showed weak affinities, with K_i in the micromolar range. The most interesting compounds reported to date are WIN-64281 (Fig. 13), isolated from *Aspergillus* sp., which showed submicromolar K_i values against both NK1 and NK2 and was active in functional assays such as the rat vas deferens model [213]; and several depsipeptides that are selective for NK2 with K_i values down to 27 nM [214,215].

18. FIBRINOLYTIC ENHancers

The plasminogen/plasmin system is involved in a variety of physiological and pathological conditions that require localized proteolysis, including fibrinolysis, inflammation, tissue remodeling, tumor metastasis, and others. In this system, plasminogen is proteolytically activated by several factors, each of which is regulated by specific inhibitors, originating in vascular endothelial cells. The balance between activators and inhibitors regulates fibrinolysis in the blood vessels. It is known that high levels of the plasminogen activator inhibitor-1 (PAI-1) are a risk factor for thrombosis, and that PAI-1 expression is increased in atherosclerotic arteries. The activation of plasminogen is enhanced on the surface of cells and fibrin, to which it is able to bind, localizing fibrinolytic activity on cell surfaces and fibrin clots. Agents able to promote the binding of plasminogen to fibrin and cells would be expected to be useful as fibrinolytic enhancers. Several fungal metabolites have been described with this activity, such as staphlabin and derivatives produced by *Stachybotrys microspora* [216], and the novel fatty acid KOD (11-keto-9(*E*),12(*E*)-octadecadienoic acid) [217]. The effect of KOD (Fig. 13) is mediated, at least partially, by inactivation of the inhibitor PAI-1.

19. ERYTHROPOIETIN GENE EXPRESSION INDUCERS

Erythropoietin (EPO) is the primary hormone regulating the proliferation and differentiation of immature erythroid cells. Recombinant EPO is in use as a treatment for anemia derived from chronic renal failure, cancer chemotherapy, and other conditions. An orally available alternative to recombinant EPO would present obvious advantages, and an extensive search has been made of fungal metabolites with this potential by using a screening assay looking for inducers of the expression of the EPO gene fused to a luciferase reporter gene. This effort has resulted in the discovery of at least three different families of compounds: namely, new destruxins [218], sesquiterpene tropolones [219], and a novel pyridone [220]. The most potent was the latter, 8-methyl-pyridoxatin (Fig. 13), which induced EPO gene expression by five-fold at a concentration of 0.3 μ M [220].

20. CONCLUSION AND OUTLOOK

As shown in this chapter, fungal metabolites have been described spanning a broad array of biological activities, from antibiotics, in the broadest sense, to inhibitors (and activators) of enzymes, receptors and other biological processes. In some cases, therapeutically useful

drugs have resulted, with significant impact on the history of modern medicine. Other compounds progressed beyond the discovery step but never reached the market, or are still in clinical development. Finally, some metabolites were never expected to become real drugs, but still were useful research tools to understand various biological processes.

The vast structural diversity of fungal metabolites, combined with their well-proved capabilities to interact with biological systems, as reviewed in this chapter, should make fungi an attractive source of leads for industrial drug discovery programs. Because of the strong competition from synthetic chemistry and to the inherent difficulties associated with natural products research, however, it is evident that these types of programs have lost favor in the pharmaceutical industry. The disadvantages commonly associated to natural products research include the significant costs and the length of time required to isolate and elucidate the structures of the active compounds from the original microbial strains. Additionally, there is a prevailing perception that complex natural products extracts are not amenable to some of the assay detection technologies used in ultra-high throughput screening laboratories. Although some of this is true, it remains that fungi are a viable source of novel chemical structures with biological potential, and this alone should maintain them a valuable resource for industry. Only by overcoming these disadvantages through the use of more efficient systems to shorten the time and cost required to get from the fungal isolated strains to the lead structures will fungal natural products eventually return to their rightful place at the core of industrial drug discovery programs.

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4

Cell Cycle Regulation in Morphogenesis and Development of Fungi

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1. INTRODUCTION

Cell growth and division are the most fundamental cellular activities of eukaryotic organisms. It thus is not surprising to find that regulatory mechanisms of the cell cycle are conserved largely through evolution from fungi to humans. Indeed, great insight into regulation of the cell cycle has come in the last three decades through a convergence of molecular genetic studies in model genetic systems of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus nidulans*, and biochemical studies in cell cycle extracts of activated frog and sea urchin eggs. In recognition for their pioneering work on cell cycle research using model systems, Paul Nurse, Lee Hartwell, and Tim Hunt together were awarded the 2001 Nobel Prize in Medicine.

This book deals with a range of scientific and industrial issues concerning fungal secondary metabolism and bioactive secondary metabolites. Although cell division is the cornerstone of biology, a role of cell cycle regulation in fungal secondary metabolism has not been investigated. However, it has long been known that production of secondary metabolites usually is associated with cell differentiation and development by organisms with filamentous growth and a complex morphology [1]. Moreover, a link between biosynthesis of secondary metabolites and asexual development recently was established by analysis of a G-protein mutant in *A. nidulans* [2]. It also was shown recently that an interaction between developmental regulators and cell cycle regulators is required for normal cell differentiation and morphogenesis during asexual development in *A. nidulans* [3]. Reinhard Fischer's laboratory has since provided further evidence supporting an impor-

tant role of cell cycle regulators in *Aspergillus* development [4,5]. Most intriguingly, a C-type cyclin-like gene, *FCC1*, is shown to play an important regulatory role in both fumonisin B₁ biosynthesis and asexual development in *Fusarium verticillioides* [6]. Thus, it is highly conceivable that cell cycle regulation plays an important role in fungal secondary metabolism, at least through its role in fungal development and morphogenesis.

In this chapter, I first summarize recent progress in cell cycle regulation in the model fungal system *A. nidulans*. Readers are referred to recent reviews on fungal cell cycle regulation for more comprehensive information [7,8]. I then more extensively discuss recent findings that help establish a key role of cell cycle regulation in fungal development and morphogenesis. In addition, recent work from my laboratory demonstrating an important regulatory role of sphingolipid metabolism in *Aspergillus* cell cycle, apoptosis, development, and morphogenesis also is discussed. I hope this chapter helps provide a framework by which future studies can be designed to demonstrate the direct involvement of cell cycle regulation in fungal secondary metabolism.

2. CELL CYCLE REGULATION IN ASPERGILLUS: A TALE OF TWO KINASES

The cell cycle was originally divided into interphase and mitosis based on cytological observations, as little cellular activities during interphase can be observed under a microscope [9]. The interphase occupies the majority of the cell cycle and is now functionally divided into G₁, S, and G₂ phases. DNA is replicated during S phase and segregated equally into two daughter nuclei during mitosis. Mitosis occurs very rapidly, ranging from about 5 minutes in *Aspergillus* to about 30 minutes in mammalian systems. The ensuing cytokinesis then separates each nuclei into two daughter cells. DNA replication occurs once and once only per cell cycle, and initiation of mitosis is coupled to completion of DNA replication [9]. Therefore, to ensure the faithful transmission of the genome, cells need to make a continuum of decisions about when to start and when to stop cell cycle-specific functions, especially when to start DNA synthesis, and when to enter into, and exit from, mitosis.

A great deal of cell cycle research has been directed to study the regulation of cyclin-dependent kinases (CDKs), because they play key roles in regulating major cell cycle transitions [10]. For instance, CDK1 containing the catalytic kinase subunit p34^{cdc2} and the regulatory subunit cyclin B is a key regulator of mitosis and is universally conserved [11]. In fact, the conservation of cell cycle regulators was first realized when the human Cdc2 homolog was cloned by functional complementation of a Cdc2 mutation in fission yeast [12] and also the determination of frog mitosis-promoting factor (MPF) as the CDK1 complex with cyclin B as the oscillating protein [13], which was first observed in sea urchin eggs to accumulate in abundance during interphase and is very rapidly degraded at each mitosis [14]. Indeed, initiation of mitosis in all eukaryotic cells requires activation of CDK1 [10,11].

Similar to budding and fission yeasts, cell cycle progression in *Aspergillus* requires the function of a single cyclin-dependent kinase p34^{cdc2} encoded by *nimX* [15]. The function of p34^{cdc2} is required for both initiation of DNA replication and entry into mitosis [15]. The p34^{cdc2} protein remains constant throughout the cell cycle and its kinase activity is regulated through its association with cyclins and by phosphorylation/dephosphorylation [10,11]. The kinase activity of p34^{cdc2} requires both binding to cyclins and subsequent activating phosphorylation at T161 upon cyclin binding by CDK activating kinase (CAK)

[16]. So far the mitotic cyclin B encoded by *nimE* is the only known partner of *Aspergillus* p34^{cdc2}. Whether there are also G1 cyclins as in budding yeast to regulate initiation of DNA replication in *Aspergillus* remains to be established.

The p34^{cdc2} and cyclin B become associated with each other to form an inactive CDK1 complex known as pre-MPF during interphase as cyclin B protein accumulates from late S-phase through G2 and peaks at G2/M transition. CDK1 is kept in the inactive state through tyrosine 15 phosphorylation by the Wee1 kinase (known as ANKA in *Aspergillus*) during S to G2 phase [17–19]. During G2/M transition, CDK1 becomes abruptly activated by tyrosine 15 dephosphorylation to initiate mitosis. Tyrosine 15 dephosphorylation is catalyzed by the tyrosine phosphatase Cdc25, known as NIMT in *Aspergillus* [17,20,21]. The rapidity of tyrosine 15 dephosphorylation of CDK1 is promoted by positive feedback activation of Cdc25 by activated CDK1 [22]. Thus, a key regulatory step to initiate mitosis is tyrosine 15 phosphorylation/dephosphorylation of CDK1 [10,11,17,22]. Thus, it is not surprising that tyrosine 15 phosphorylation of CDK1 plays key checkpoint roles in coupling initiation of mitosis to completion of S-phase and also in response to DNA damage to delay entry into mitosis until damaged DNA is repaired [10,19,22–25]. In addition, tyrosine 15 phosphorylation of CDK1 also plays a key role in linking cell cycle progression to morphogenesis and differentiation (see section 3). Then, as cells transverse through mitosis, cyclin B becomes rapidly degraded by ubiquitin-mediated proteolysis promoted by the anaphase-promoting complex (APC) upon exit from mitosis into G1 [22,26,27]. In fact, proteolysis of cyclin B is required for exit from mitosis [26].

Analysis of temperature-sensitive, cell cycle specific mutants in *Aspergillus*, originally isolated by Morris [28], however, demonstrates that activation of another protein kinase, NIMA, also is required to initiate mitosis [29,30]. Activation of CDK1 is necessary but not sufficient for entry into mitosis as inactivation of NIMA kinase through the temperature-sensitive *nimA5* mutation blocks cell cycle at G2 with fully activated CDK1 and no cytological signs of mitosis [7,8,29,30]. This indicates that unlike other cell cycle regulators, the requirement of NIMA kinase for mitotic entry does not involve regulation of CDK1 kinase activity. Interestingly, the NIMA protein oscillates once each cell cycle, very much like a mitotic cyclin [30]. The level of NIMA protein increases during G2 and peaks at early mitosis. As cells progress through mitosis, NIMA rapidly is proteolysed also in an APC-dependent manner [31]. Indeed, like cyclin B, proteolysis of NIMA also is required for exit from mitosis [32]. However, the NIMA protein does not contain a consensus destruction box motif required for APC-mediated ubiquitination and proteolysis by the proteosome pathway [7]. Whether APC-dependent proteolysis of NIMA is mediated through the ubiquitination-dependent proteolysis pathway like mitotic cyclinB remains to be determined.

Studies in *Aspergillus* have established that activation of both CDK1 and NIMA kinases is required for entry into mitosis and that their subsequent inactivation by proteolysis is required for progression through mitosis into G1 [7,8]. For CDK1 and NIMA to promote initiation of mitosis in a timely manner during the cell cycle and in response to checkpoint controls, they must thus functionally coordinate with each other or may even regulate each other's mitotic functions. Indeed, biochemical and cell biological studies show that CDK1 and NIMA kinases regulate each other's mitotic functions at multiple levels.

During G2 NIMA protein accumulates in a hypophosphorylated state but is active as a β -caseine kinase. Upon G2/M transition, NIMA becomes hyperphosphorylated and a MPM-2 antigen in a CDK1-dependent manner and its kinase activity is further activated

[30], suggesting that full mitotic function of NIMA kinase is regulated by CDK1. Indeed, NIMA is an excellent substrate of CDK1 *in vitro*, and CDK1 *in vitro* phosphorylation of NIMA also generates MPM-2 epitopes [30]. Furthermore, NIMA nuclear localization during initiation of mitosis requires CDK1 activation by Cdc25 dephosphorylation [33]. At Cdc25 G2 arrest point, NIMA protein is mainly cytoplasmic. Upon release from Cdc25 G2 block, NIMA is rapidly transported into nuclei, an event closely associated with initiation of mitosis [33].

As mentioned earlier, NIMA kinase is required for initiation of mitosis at a point after activation of CDK1 by tyrosine 15 dephosphorylation. Two lines of evidence indicate that NIMA kinase is required for nuclear localization of CDK1 in G2 [34]. First, indirect immunofluorescence analysis shows that CDK1 is localized predominantly to the nucleus during G2/M and CDK1 nuclear localization and its association with the nuclear-associated organelles and spindle pole body requires NIMA functions. Second, a suppressor of the *nimA1* mutation (*sonA*) is a homologue of the nucleocytoplasmic transporter GLE2/RAE1 and suppresses *nimA1* lethality at the restrictive temperature in an allele-specific manner [34]. Furthermore, this suppression of *nimA1* lethality is associated with the restoration of CDK1 nuclear localization. Together, recent studies provide strong evidence at the molecular level for a mechanism by which CDK1 and NIMA kinases coordinate their mitosis-promoting activities through interdependent regulation of their mitotic functions.

Enigmatically, NIMA can promote chromosome condensation in the absence of CDK1 function in many biological systems, at least when it is overexpressed [30,35], indicating a key role of NIMA in promoting this mitotic event. Phosphorylation of histone H3 serine 10 correlates with chromosome condensation and is required for normal chromosome segregation in *Tetrahymena* [36]. In *Aspergillus*, this phosphorylation is found to be dependent on NIMA function [33]. A direct role of NIMA in this phosphorylation is supported by the following observations. First, NIMA become associated with chromatins at initiation of mitosis, coincident with histone H3 serine 10 phosphorylation. Second, when untimely overexpressed, NIMA is able to promote this phosphorylation anywhere in the cell cycle. Third, NIMA directly phosphorylate this residue on histone H3 *in vitro* [33]. However, the function of NIMA is unlikely to just phosphorylate histone H3 during mitosis, because inactivation of NIMA prevents all aspects of mitosis and, additionally, NIMA proteolysis is also required for progression through mitosis [7,8]. Indeed, as discussed previously, one of the mitotic functions of NIMA kinase is to regulate CDK1 cytoplasmic to nuclear transport during G2/M transition [34]. Then upon entry into the nucleus, part of NIMA functions is to phosphorylate histone H3 serine 10 to promote DNA condensation. It remains to be determined, however, whether histone H3 serine 10 phosphorylation is essential for DNA condensation in this system. As cells transverse through mitosis, NIMA becomes transiently associated with the spindle microtubules and the spindle pole bodies, suggesting a role of NIMA at these sites during mitosis as well [33].

NIMA kinase is a founding member of an evolutionarily conserved family of protein kinases present from fungi to humans [7]. These kinases all contain an N-terminal catalytic domain and C-terminal regulatory domain with a coiled coil linker between the domains, and all have a very high isoelectric point (>10). In filamentous fungi, NIMA kinases particularly are highly conserved and also very likely are functionally conserved. The NIMA homologue (*nim-1*) isolated from *Neurospora crassa* can fully complement lack of NIMA function in *Aspergillus*, indicating the presence of a NIMA pathway of cell cycle regulation in other filamentous fungi [37]. Many NIMA-related kinases (NEK) have

been identified from humans and are implicated in human cell cycle regulation [7]. However, none of the human NEKs so far studied show all the characteristics of NIMA mitosis-promoting functions as observed in *Aspergillus*. Perhaps, just like the requirement for multiple CDKs, NIMA mitosis-promoting functions observed in the lower eukaryotic system may have been delegated to multiple NEKs due to the increased complexity of human cell cycle regulation.

3. DEVELOPMENTAL REGULATION OF THE CELL CYCLE IN MORPHOGENESIS AND CELL DIFFERENTIATION DURING CONIDIOPHORE DEVELOPMENT

Cell division must be carefully controlled during the development of multicellular organisms. First, cell division must be coupled to growth and morphogenesis to allow organs and tissues to reach their appropriate size. Second, cell division must also be coordinated with cell differentiation for the production of highly specialized cell types to perform specific physiological and cellular functions. How developmental programs orchestrate these different levels of controls over the cell cycle is a fundamental biological question and thus attracts intense scientific interest because of the enormous importance in understanding human pathological conditions caused by the deregulation of cell cycle and growth controls such as cancer.

A. nidulans has both a complex asexual and sexual lifecycle with highly defined morphogenic patterns [38]. Most important, extensive studies over the last several decades have established key regulators in both cell cycle control and development in this model experimental system [38–40]. Thus, *Aspergillus* is an attractive model to study the relationship between cell cycle regulation and developmental controls during morphogenesis and cell differentiation. Nuclear division and cytokinesis, or septation, are not strictly coupled in vegetative hyphal growth. In fact, first cytokinesis normally occurs after the third round of nuclear division of germinating spores, thus giving rise to multinucleate hyphal cells with up to 16 nuclei per cell [41]. Then during asexual development, *Aspergillus* elaborates several distinct cell types by both filamentous and budding growth to form the multicellular conidiophore structure from which the uninucleate conidiospores are derived by repeated asymmetric division. The first event of conidiophore development is the production of aerial stalks crowned with vesicles from specialized anchoring foot cells. Interestingly, many rounds of nuclear division take place in the stalk in the absence of cytokinesis. A series of cells, called metulae, are formed from the vesicle by budding and one nucleus from the stalk migrates into each of them. Metulae divide once to produce a second tier of cells, called phialides, which then produce a chain of uninucleate spores by repeated asymmetric mitotic divisions. These dramatic changes in modes of cell division and patterns of growth during conidiophore morphogenesis thus necessitate intimate interactions between the developmental program and cell cycle control mechanisms [42].

Recent studies have indeed established a key role of developmental regulation of CDK1 activity in coordinating cell cycle control with morphogenesis and cell differentiation. An important role of CDK1 in *Aspergillus* asexual development was first noticed with a strain bearing a mutant CDK1 whose inhibitory phosphorylation residues threonine 14 and tyrosine 15 are mutated to nonphosphorylatable alanine and phenylalanine, respectively [19]. This strain thus cannot negatively regulate CDK1 by Thre14 and Tyr15 phosphorylation and is shown to be deficient consequently in both the slowing S-phase and G2/M DNA damage checkpoint controls [19,23]. Remarkably, under laboratory conditions,

this mutant strain is viable and is able to grow vegetatively without significant cell cycle defects. However, this mutant strain conidiates very poorly [3]. Subsequent light and scanning electron microscopic examinations show multiple gross defects in conidiophore morphogenesis [3]. First, the mutant conidiophore stalks all have multiple septa. As mentioned above, nuclear division in the stalk normally occurs in the absence of septation. Second, normal conidiophores are highly symmetrical, whereas the mutant conidiophores display grotesque deformities and consequently lack symmetry. Third, many mutant conidiophores form multiple vesicles as a result of reiteration of the same cell type. These defects are apparently caused by inability of the mutant strain to develop the correct cell types sequentially [3]. Additionally, other mutant strains deficient in tyrosine phosphorylation of CDK1 also display similar morphogenic defects during asexual development. Furthermore, the lethality caused by a mutation of *nimT/CDC25* can be suppressed by multiple copies of *nimE/cyclin B* for vegetative growth, but the mutant strains still fail to develop the correct cell types of the conidiophore [20]. Together, these results clearly demonstrate that tyrosine phosphorylation of CDK1 plays a key role in coordinating cell cycle regulation with cell fate determination during conidiophore morphogenesis.

Interestingly, coordination between cell division and morphogenesis in *Drosophila* also is mediated through tyrosine phosphorylation of mitotic CDK regulated by *string*, the homologue of CDC25 tyrosine phosphatase [43]. Following fertilization, the fly embryo undergoes 13 rapid syncytial blastoderm divisions driven by maternally provided proteins and RNAs. Depletion of maternal *string* leads to a G2 cell cycle delay at cycle 14 to allow cellularization, a key morphogenic event [43]. Reinitiating mitosis is then elaborately controlled by regulated expression patterns of *string/CDC25* in time and space by developmental programs [43]. Two recent studies identified a new cell cycle regulator, *tribbles*, which coordinates mitosis and morphogenesis during fly ventral furrow formation by regulating *string/CDC25* proteolysis, thus providing further evidence supporting an important role for the regulation of CDK tyrosine phosphorylation in linking cell cycle regulation to morphogenesis in the fly [44,45]. Perhaps this mechanism coordinating the cell cycle with morphogenesis is conserved through evolution. Even in the unicellular budding yeast, a morphogenic checkpoint is mediated by tyrosine phosphorylation of CDC28, a homologue of CDK1, through the Swe1/Wee1 protein kinase activity to coordinate cell cycle with bud emergence [46,47].

In addition, CDK1 activity is highly induced during initiation of conidiophore development and this induction is dependent on *brlA*, which encodes a master transcriptional regulator in conidiophore development [3]. The central genetic pathway controlling asexual conidiophore development consists of five regulatory genes: *brlA*, *abaA*, *medA*, *stuA*, and *wetA*, with *brlA* functioning as the focal point for integration of developmental cues [38,40]. Ectopic expression of *brlA* or *abaA* under normally conidiation-suppressing conditions also markedly induces CDK1 activity [3]. This increase in CDK1 activity is closely correlated with the increase of both *nimX/Cdc2* mRNA and protein. The upstream regulatory domains of *nimX/Cdc2* contains multiple *brlA* regulatory elements (BRE). Thus, *brlA* is necessary and sufficient for induction of CDK1 activity during asexual conidiophore development; part of the functions of *brlA* is to regulate positively the expression of *nimX/Cdc2* and consequently increased activity of CDK1. This is the first observation showing developmental regulation of CDK1 activity through regulated Cdc2 transcription. As the mode of cell cycle changes from multinucleate to uninucleate cell division during conidiophore development, perhaps increased CDK1 activity is needed to couple each nuclear division to cytokinesis/septation. This is consistent with the observation that a slight de-

crease in CDK1 activity using the *nimT23/Cdc25* mutation at a semirestrictive temperature suppresses septation while nuclear division continues normally [41]. Conversely, CDK1 mutants deficient in inhibitory phosphorylation, consequently with higher kinase activity, promote septation [41]. These results again point to a key role of CDK1 regulation through tyrosine phosphorylation both in cell cycle regulation and development.

Recently, a genetic screen using restriction enzyme mediated integration (REMI) mutagenesis with *SmaI* for *Aspergillus* mutant defective in conidiophore morphogenesis identified a homologue of budding yeast Pcl cyclins, *pclA* [4]. The *pclA* expression also is developmentally regulated in a *brlA* dependent manner. Deletion of *pclA* markedly reduces spore production by three- to five-fold due to slower mitotic division in phialides. Subsequent biochemical analysis shows that the PCLA protein physically interacts with NIMX/Cdc2 kinase as demonstrated by coimmunoprecipitation experiment, and that the immunocomplex contains H1 kinase activity [5]. Perhaps part of the increased NIMX/Cdc2 during conidiophore morphogenesis is used to form complex with the Pcl-like cyclin to carry out yet unknown cell cycle functions required for spore production.

4. REGULATION OF CELL CYCLE PROGRESSION, APOPTOSIS, AND DEVELOPMENT BY SPHINGOLIPIDS

Sphingolipids are major components of eukaryotic cell membranes and are emerging as an important signaling mechanism [48]. Many metabolites derived from complex sphingolipids or from *de novo* synthesis, such as ceramide, sphingoid bases (sphingosine, dihydro-sphingosine, and phytosphingosine), and sphingoid base phosphate are highly bioactive molecules implicated as second messengers mediating a wide array of cellular activities, including stress response, apoptosis, inflammation, cell cycle, and cancer development [48–50]. Among these, ceramide and sphingosine-1-phosphate (S-1-P) are most extensively studied. It is now well documented in many biological systems that stress signals rapidly and transiently elevate the level of cellular ceramide, whereas growth factors stimulate rapid, transient generation of S-1-P catalyzed by sphingosine kinase [48–50]. S-1-P is a high-affinity ligand of the *edg* family of G-protein coupled receptors, and its ligation with the receptors is shown to promote cell survival and proliferation by antagonizing ceramide-mediated apoptosis [50]. It has been suggested that ceramide and S-1-P together act as a cellular rheostat, determining whether cells undergo apoptosis or continue to proliferate [50].

Because the basic chemical structure, biosynthesis, and metabolism of sphingolipids are largely conserved from fungi to humans, our laboratory has employed both genetics and pharmacological inhibitors to manipulate the sphingolipid biosynthesis pathway to gain new insight into cellular functions of sphingolipids in genetically tractable *A. nidulans* and has uncovered novel biological functions for sphingolipids. Establishment and maintenance of cell polarity is essential to the growth and development of multicellular organisms, and highly polarized hyphal growth is the most salient feature of fungi. Our research in *Aspergillus* shows that sphingolipids play a central role both in the establishment and in the maintenance of cell polarity via polarized organization of the actin cytoskeleton, perhaps mediated through functions of lipid rafts [51]. In normal growing cells, the level of cellular ceramide remains very low. Upon stress treatment such as heat shock, the level of cellular ceramide is rapidly and transiently elevated and the increase in cellular ceramide accompanies closely a transient inhibition of cell cycle progression and cell growth. We further find that inositol phosphorylceramide (IPC) synthase, which catalyzes the synthesis of

complex sphingolipids, plays a key role in mediating the level of cellular ceramide; accumulation of ceramide upon inhibition of IPC synthase activity causes cell cycle arrest at G1 [51]. Normally, cell cycle progression is tightly coupled to cell growth. Therefore, we suggest that IPC synthase activity plays an important role in coupling cell cycle to cell growth during stress response via its function in sphingolipid synthesis and regulation of the level of cellular ceramide, because sphingolipid biosynthesis is essential for cell growth and increases in cellular ceramide cause G1 arrest.

Dihydrosphingosine (DHS) and phytosphingosine (PHS) are the predominant sphingoid bases in fungi. Remarkably, DHS and PHS have potent antifungal activity against *Aspergillus*, which shows exquisite structural and stereo-chemical specificity [52]. In fact, only the naturally occurring DHS and PHS are biologically active in our assays. Further analysis reveals that DHS and PHS inhibit fungal growth via rapid induction of apoptosis [52]. Fungal apoptosis induced by DHS and PHS exhibits all major features characteristic of apoptosis originally described in animals, such as rapid DNA condensation independent of mitosis, DNA fragmentation, exposure of phosphatidylserine, and generation of reactive oxygen species (ROS) [53]. This apoptotic response is shown to be specific to the action of DHS and PHS, because several different chemical classes of potent antifungals all targeting fungal membrane functions do not induce apoptosis [52]. We further find that DNA condensation and DNA fragmentation in fungal apoptosis are in fact separate cellular events. DNA condensation can occur in the absence of DNA fragmentation, and DNA fragmentation but not DNA condensation requires protein synthesis [52]. Two lines of evidence demonstrate that apoptosis induced by DHS and PHS is very similar to caspase-independent apoptosis recently described in mammalian systems and in *Dictyostelium discoideum* [52,54–57]. First, DHS and PHS induce large-scale DNA fragmentation, but not the typical oligonucleosomal ladders associated with caspase-mediated apoptosis [53]. Second, apoptosis induced by DHS and PHS is independent of the highly conserved fungal metacaspase [54]. High structural and stereo-chemical specificity in induction of apoptosis by DHS and PHS suggests that their activities are physiologically relevant and must act on very specific molecular targets to bring about apoptosis.

At present, how cellular levels of endogenous DHS and PHS are normally regulated and under what biological conditions *A. nidulans* undergo DHS- and PHS-mediated apoptosis are not understood. In filamentous fungi, heterokaryon incompatibility results in cell death after anastomosis, presumably as a self-defense mechanism to prevent transmission of infectious elements [58]. Cell death associated with heterokaryon incompatibility shows microscopic and ultrastructural features—vacuolization of the cytoplasm, organelle degradation, and plasmolysis—consistent with some of the apoptotic features in animals [58,59]. In addition, in *A. nidulans* environmental stresses, such as nutrient limitation promote asexual sporulation, which is found to be accompanied by extensive hyphal death in the central portion of the colony, whose cellular contents are recycled for spore production [40]. Indeed, forced expression of *brlA*, the central regulator of asexual development of *Aspergillus*, during vegetative growth promotes conidiophore formation and also causes rapid cell death [60]. Perhaps sphingolipid metabolism plays a role in the cell death associated with heterokaryon incompatibility and conidiation. In support of this assumption, we found that *Aspergillus* mutant strains with compromised SPT activity are unable to conidiate, although they undergo vegetative growth normally as the wild type, indicating an essential role of sphingolipid metabolism in conidiation (Cheng et al., unpublished data).

An important role of sphingolipid metabolism in development also has been reported in *Drosophila* and *D. discoideum* [61,62]. *Drosophila* carrying the *lace* mutation that compromises SPT activity produce many developmental abnormalities. These developmental defects are shown to be caused by a considerable increase in cells undergoing apoptosis in the imaginal discs via activation of the JNK signaling pathway [61], suggesting that *de novo* synthesis of sphingolipids is required for the survival of various cell types during *Drosophila* development. In a REMI mutagenesis screen for increased resistance to anticancer drug cisplatin in *D. discoideum*, a S-1-P lyase mutant was isolated as a resistant mutant to cisplatin [63]. The function of S-1-P lyase is to break down S-1-P to phosphoethanolamine and hexadecanal. Interestingly, the S-1-P lyase mutant exhibits strong developmental defects and produces highly aberrant fruiting bodies [62]. Together, the studies in various biological systems support an important regulatory role of sphingolipid metabolism in development, although the underlying molecular mechanisms by which sphingolipids regulate development may vary in individual organisms.

5. CONCLUSION

Filamentous fungi are a remarkably diverse group of microorganisms of agricultural, medical, and industrial importance. They are distributed in a wide range of geographic regions, even found in extreme conditions, and contain a rich repertoire of pharmaceutically active agents. Production of secondary metabolites is shown to be linked to development in many fungi [1]. In this chapter, I discussed recent work establishing a key role of developmental regulation of the cell cycle in morphogenesis and cell differentiation during fungal development. Because cell cycle regulation is required for fungal development and fungal development is linked to secondary metabolism, by inference cell cycle regulation also likely plays an important role in fungal secondary metabolism. However, we currently do not have direct evidence supporting such a role of cell cycle regulation in fungal secondary metabolism. I believe that this void will be soon filled. Because cell cycle regulation, development, and secondary metabolism are all well established in *A. nidulans*, this fungus is well suited for studying the relationship between cell cycle regulation and secondary metabolism. A full understanding of the regulation of secondary metabolism within a broader context of fungal biology will enable us to devise novel methods to control harmful mycotoxins and to produce pharmaceutically beneficial agents.

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5

Agrobacterium-Mediated Transformation of Filamentous Fungi

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1. INTRODUCTION

Procedures to introduce exogenous DNA into a cell in a form that can be inherited and expressed for phenotypic change constitute a key technology, enabling the application of today's powerful gene manipulation strategies for purposes ranging from the elucidation of fundamental biological phenomena to the improvement of commercial biological processes. For two decades, the most commonly used transformation method for filamentous fungi has been the calcium ion–polyethylene glycol (PEG) procedure that requires first the conversion of the target organism to spheroplasts for the uptake of the DNA and subsequently the regeneration of cell walls for the resumption of hyphal growth. Other methods, including electroporation and particle bombardment, are less labor intensive and inherently faster, and they have been used successfully with certain fungi. In as much as comprehensive reviews detailing the use of these procedures with filamentous fungi have appeared recently [1,2], this chapter is devoted exclusively to a new method that offers the advantages of simplicity and general applicability. It makes use of *Agrobacterium tumefaciens*, a bacterium widely employed in the transformation of plant cells, and it stems from the 1998 report by de Groot et al. [3] that *A. tumefaciens* is capable of introducing DNA into fungal cells.

2. AGROBACTERIUM BACKGROUND

A mechanism for the mobilization of DNA has evolved in *A. tumefaciens* that allows the delivery of genetic information across the boundaries of biological kingdoms. The

pathogenic association of this Gram-negative prokaryote with certain plants, which manifests itself as a crown gall tumor, has its origin in large extrachromosomal elements, known as tumor-inducing or Ti plasmids, found in natural populations of *A. tumefaciens*. Establishment of a crown gall tumor requires the transfer of a specific segment of DNA, known as the T-DNA, from the Ti plasmid into the plant cell by a process that resembles bacterial conjugation [4]. Another portion of the Ti plasmid comprises a cluster of about 35 virulence (*vir*) genes that encode the proteins required to mobilize the T-DNA. The majority of the *vir* genes are quiescent until induced by phenolic compounds typically released by wounded plant cells. Many phenolics may act as elicitors; acetosyringone and coniferyl alcohol are two examples. The T-DNA is delimited by imperfect 25-base pair repeats called the right and left border sequences (RB and LB, respectively). Transport of the T-DNA into the plant cell is a polar process: the T-DNA is introduced, RB first, as a single-stranded molecule. Further mechanistic details on the establishment of this intriguing host-pathogen relationship can be found in a number of worthwhile reviews [4–10].

Because of their large size (>100 kb), Ti plasmids themselves are not readily manipulated. However, two characteristics of the DNA mobilization machinery have made it possible to harness *Agrobacterium* as a means of gene delivery, so that today the bacterium provides the preferred method for transforming cells of both dicotyledonous and monocotyledonous plants. The first feature is that the *vir* genes and the T-DNA need not reside on the same plasmid [11,12]. Ti plasmids that are *disarmed* by removal of the T-DNA segment propagate in *A. tumefaciens* but are incapable of producing crown gall tumors. Virulence is restored in an *A. tumefaciens* cell carrying a disarmed Ti plasmid when the Ti plasmid's T-DNA is introduced into the cell as part of a separate replicon [11]. The second significant characteristic concerns the nature of the T-DNA itself. The T-DNA region of a Ti plasmid encodes genes for the synthesis of both plant growth hormones and unusual amino acid derivatives known as opines. Examples of opines include octopine, histopine, nopaline, mannopine, and agrocinopine [9]. These derivatives serve as nutrients for the *A. tumefaciens* cells and constitute the benefit the bacteria derive from the expression of the opine genes within the plant. Although the hormone and opine genes play an integral part in crown gall tumor formation, they are not necessary for mobilization of the T-DNA. Any DNA element that is placed between the RB and LB can be mobilized by the *vir* gene products. These two properties have permitted the adaptation of the Ti plasmid into a binary plasmid system for DNA delivery (Fig. 1). A donor vector able to replicate in both *A. tumefaciens* and *Escherichia coli* carries the RB and LB sequences between which one or more genes of interest may be cloned. Following introduction of the donor plasmid into an *Agrobacterium* strain that carries a resident disarmed Ti plasmid, the genes that are flanked by the border sequences are subject to mobilization upon activation of the *vir* genes. Most often activation is achieved by the exogenous addition of acetosyringone.

As mentioned, the T-DNA portion of Ti plasmids encodes genes for opine production. In the older literature the Ti plasmids were classified according to the predominant opine associated with the crown gall tumor they cause. Thus, pTiT37 and pTiC58 have been considered nopaline plasmids, whereas pTiA6 and pTiAch5 have been placed in the octopine group. With the characterization of increasing numbers of Ti plasmids, it is now clear that this classification system is imperfect [6]. Despite the distinctions rooted in the opine genes, there is very little difference in the RB and LB sequences in the various Ti plasmids. Adjacent to the RB but outside of the T-DNA region there is an element (known as overdrive) that binds the *virC1* gene product and enhances T-strand production. Over-

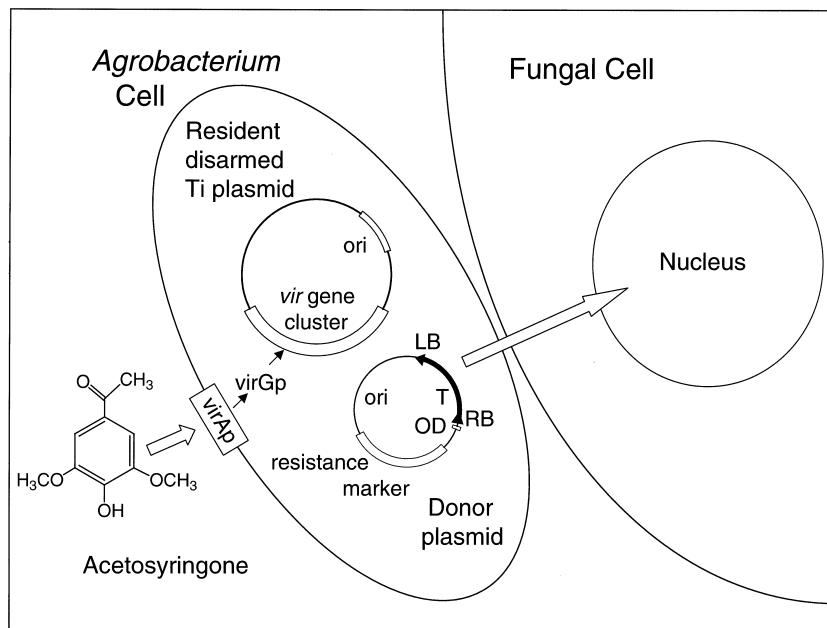


Figure 1 Binary plasmid system for *Agrobacterium tumefaciens*. A disarmed Ti plasmid resident in the bacterium carries a cluster of virulence genes (*vir*) encoding proteins that mobilize the T-DNA. Acetosyringone activates a cell surface receptor that is the product of *virA*, and this leads to activation of the *virG* protein, the transcriptional regulator of the *vir* cluster. T-DNA (T; shown as a black segment of the donor plasmid) flanked by right and left border sequences (RB and LB, respectively) is mobilized and transferred to the target cell where it enters the nucleus and integrates into genomic DNA. Replication of the donor plasmid in *E. coli* allows it to be manipulated by standard procedures before it is introduced into the *Agrobacterium* cell; a drug resistance marker is provided for selection in both bacteria. The donor plasmid has an overdrive (OD) sequence proximal to the RB to enhance T-strand formation.

drive sequences differ in the various types of Ti plasmids, but there appears to be functional equivalence among them [13].

3. FUNGI AS TARGETS FOR AGROBACTERIUM-MEDIATED DNA TRANSFER

Although the Ti plasmid system has evolved as a mechanism of plant pathogenesis, *A. tumefaciens* has the capability to deliver T-DNA to fungi under conditions that simulate those within a plant wound. The Dutch group led by P. J. J. Hooykaas reported the transformation of a wide variety of filamentous fungi (both ascomycetous and basidiomycetous) to hygromycin B resistance resulting from cocultivation of the fungi with *A. tumefaciens* (Table 1). Cocultivation was performed in the presence of acetosyringone to mobilize a T-DNA element containing the hygromycin B phosphotransferase gene driven by a fungal promoter [3]. Earlier reports from the same laboratory [14,15] and from E. W. Nester's group [16] had demonstrated the transfer of wildtype yeast *URA3* and *TRP1*

Table 1 Fungi That Have Been Transformed by the Agrobacterium Procedure

Organism	Donor Plasmid	Selectable Gene ^a	Features	Ref.
<i>Aspergillus awamori</i>	pBin19, pSDM14 pSDM14	<i>hph</i> (<i>gpdA</i> ^b) <i>pyrG</i>	Inducer-dependent transformation of conidia or protoplasts; single-copy ectopic insertions predominate Inducer-dependent transformation; frequency decreases as size of T-DNA increases	3,18 20
<i>Blastomyces dermatitidis</i>	pBIN19	<i>hph</i> (<i>gpdA</i> ^b) <i>Hc URA5</i>	Transformation of yeast and germinating conidia; higher efficiency with nutritional selection; cocultivation parameters explored; single-site insertions predominate	31
<i>Calonectria morgani</i>	pTASS5	<i>hph</i> (<i>gpdA</i> ^b)	Inducer-dependent transformation of conidia; first transformation of this organism	24
<i>Coccidioides immitis</i>	pAD1310	<i>hph</i> (<i>cpc1</i>)	Use of vector with N54D variant of <i>virG</i> for inducer-independent transformation of arthroconidia; cocultivation parameters explored	21
<i>Colletotrichum gloeosporioides</i>	pBin19	<i>hph</i> (<i>gpdA</i> ^b)	Inducer-dependent transformation of conidia	3,18
<i>Fusarium circinatum</i> <i>Fusarium oxysporum</i>	pZP201 pCAMBIA1300	<i>hph</i> (<i>gpdA</i> ^b) <i>hph</i> (<i>trpC</i>)	First transformation of this organism; single copy integration frequent Bacteria that had not been exposed to acetylxylose prior to cocultivation yielded a higher proportion of fungal transformants with single site insertions; inclusion of <i>ColE1 ori</i> and gene for chloramphenicol resistance in the T-DNA facilitated recovery of the integrated DNA by thermal asymmetric interlaced PCR	23 25
<i>Fusarium venenatum</i>	pBin19	<i>hph</i> (<i>gpdA</i> ^b)	Inducer-dependent transformation of conidia and rehydrated freeze-dried material	3
<i>Glarea lozoyensis</i>	pAgI	<i>hph</i> (<i>trpC</i>) Flanked by <i>pks1</i>	Inducer-dependent transformation of vegetative mycelia; efficient gene replacement	33
<i>Histoplasma capsulatum</i>		<i>hph</i> (<i>gpdA</i> ^b) <i>Hc URA5</i> <i>URA3</i>	Transformation of yeast form was more efficient with nutritional selection; single site insertions predominate	31
<i>Kluyveromyces lactis</i>	pBin19	Flanked by <i>TRP1</i>	Inducer-dependent transformation of yeast cells; <i>TRP1</i> gene replacement more efficient for ssDNA delivered as T-DNA than for dsDNA introduced by electroporation	19
<i>Magnaporthe grisea</i>	pBHT2	<i>hph</i> (<i>trpC</i>)	Inducer-dependent transformation of conidia; cocultivation parameters explored	26

<i>Mycosphaerella graminicola</i>	pBIN19 pCGN1589	<i>hph</i> (<i>gpdA</i> ^b) flanked by <i>MgAttr2</i>	Inducer-dependent transformation of cells or protoplasts at equivalent frequencies; efficient gene replacement	27
<i>Neurospora crassa</i>	pBin19	<i>hph</i> (<i>gpdA</i> ^b)	Inducer-dependent transformation of conidia.	3,18
<i>Saccharomyces cerevisiae</i>	pBin19	<i>URA3</i>	Inducer- and <i>vir</i> gene-dependent transformation of yeast cells; homologous integration of T-DNA into the genome; <i>in vivo</i> circularization of T-DNA containing the 2-μ ori	14
"	"	<i>TRP1</i>	Very high transformation rates with T-DNA containing an <i>ars</i> and telomere sequences (mini-chromosome); disarmed Ti plasmids compared	16
<i>Trichoderma reesei</i>	pBin19	<i>URA3</i>	Characterization of randomness and fine structure of ectopic integrants	15,17
<i>Verticillium dahliae</i>	pBin19	<i>hph</i> (<i>gpdA</i> ^b)	Inducer-dependent transformation of conidia	3,18
<i>Agaricus bisporus</i>	pBin19 pBin19 pCMBIA1300	<i>hph</i> (<i>gpdA</i> ^b) <i>hph</i> (<i>gpdA</i> ^b) <i>hph</i> (<i>gpdB</i>)	Cited as unpublished data Transformation of germinated basidiospores Transformation of germinated basidiospores and vegetative mycelia Transformation of gill tissue and fleshy tissue of the fruiting body; promoters and disarmed Ti plasmids compared	25 3 28 22
<i>Hebeloma cylindrosporum</i>	pCMBIA1300	<i>hph</i> (<i>gpdC</i>)	Transformation procedure optimized; 60% of transformants resulted from single-site integration	32
<i>Paxillus involutus</i>	pBin19	<i>Sh ble</i> (<i>gpdD</i>)	Transformation of an ectomycorrhizal basidiomycete	30
<i>Pleurotus ostreatus</i>	pBin19	<i>Sh ble</i> (<i>gpdD</i>)	Transformation of an ectomycorrhizal basidiomycete	30
<i>Suillus bovinus</i>	pBin19 pCMBIA1300	<i>hph</i> (<i>gpdA</i> ^b) <i>hph</i> (<i>gpdD</i>)	Inducer-dependent transformation of conidia and germlings ¹ Transformation of an ectomycorrhizal basidiomycete	18 30 29

^a Heterologous promoters are shown in parentheses.^b Glyceraldehyde-3-phosphate dehydrogenase promoter of *Aspergillus nidulans*.^c Glyceraldehyde-3-phosphate dehydrogenase promoter of *Agaricus bisporus*.^d Glyceraldehyde-3-phosphate dehydrogenase promoter of *Schizophyllum commune*.¹ Au: "germlings" correct or do you mean "germlines"?

genes from *A. tumefaciens* to *Saccharomyces cerevisiae* auxotrophs. Stable yeast prototrophs were obtained predominantly by homologous recombination, provided that yeast replication origins or telomeric sequences were absent from the T-DNA [14,16]. When incoming T-DNA is devoid of homologous sequences, stable transformation is established by ectopic insertion at random genomic loci, whether the recipient is a yeast [15,17], a filamentous fungus [3], or a plant cell.

The organisms transformed by de Groot et al. [3] included a broad range of fungi (Table 1). Successful use of the technique with other fungal species subsequently has been reported by several laboratories [18–33]. Significantly, the method does not require the target fungus to be converted to protoplasts; roughly equivalent transformation frequencies were obtained with either conidia or protoplasts in the case of *Aspergillus awamori* [3] and of *Mycosphaerella graminicola* [27]. In fact, conidia, germinated conidia, vegetative mycelia, a rehydrated lyophilized fungal preparation and, in the case of basidiomycetes, even fruiting tissue have been successfully transformed by means of *Agrobacterium*-mediated DNA transfer. The opportunity to use intact fungal cells as recipients is perhaps the most appealing aspect of the technique. Preparations of lytic enzymes and osmotically fortified solutions for protoplasting as well as knowledge of appropriate conditions for cell wall regeneration are not required; neither is specialized equipment for the ballistic delivery of DNA coated microparticles. The *Agrobacterium* method is far less complicated in execution than any of the earlier methods, with the possible exception of electroporation.

Another advantage of the *Agrobacterium* method is that the outcome of DNA integration is more predictable than it is with other transformation procedures. This is primarily because the T-DNA is delivered to the fungal cell and integrates into the genome predominantly as a single copy [3,21–27,31,32]. When an increase in gene dosage is the desired goal, the gene may be reiterated between the RB and LB in the donor vector before the plasmid is used to transform the fungus [20]. Targeted gene replacement is also readily achieved if the T-DNA contains sufficiently large stretches of homologous DNA. In *K. lactis*, for example, replacement of the *TRP1* gene with the *URA3* gene of *S. cerevisiae* was observed in 71% of the Ura⁺ transformants, when the *URA3* gene in the T-DNA was flanked with 0.8- and 1.4-kb segments homologous to the ends of the target *TRP1* gene [19]. Larger regions of homology are required to give high rates of gene replacement in filamentous fungi. Use of T-DNA comprised of a hygromycin B resistance cassette flanked by 2.6- and 4.1-kb segments from the *MgAtr2* gene provided null mutations at the *MgAtr2* locus of *Mycosphaerella graminicola* in 44% of the hygromycin B resistant transformants [27]. Similarly, a melanin-deficient phenotype was produced in 59% of hygromycin B-resistant transformants of *Glarea lozoyensis* when the resistance cassette in the T-DNA was flanked by 3.9- and 4.0-kb segments from opposite ends of *pks1*, a gene whose product catalyzes the formation of a polyketide precursor required for pigment synthesis [33]. In some cases, the single-stranded nature of the transforming DNA may favor homologous recombination. In *K. lactis*, ssDNA delivered by *Agrobacterium* was remarkably more efficient in producing gene replacements than a comparable DNA segment delivered as a double-stranded molecule via electroporation [27].

From these initial experiences with *Agrobacterium*-mediated transformation, it would appear that the technique will play an increasingly prominent role in the study of fungal biology and in the manipulation of commercially important fungal strains. Refinement of the currently available donor vectors and selection strategies may be expected.

4. DONOR VECTORS

A number of donor plasmids tailored for plant cell transformation have been described [34], but many of these contain segments that are inconsequential for fungal transformation. For example, the donor plasmid pBIN19 (Table 1) is a large (11.8-kb) plasmid with relatively few unique restriction sites located between the LB and RB. It was derived from the nopaline plasmid pTiT37 [12] and incorporates the Gram-negative broad host range RK2 origin of replication, which consists of the 700-bp *oriV* sequence and a *trans*-acting gene designated *trfA*. The *trfA* gene encodes two proteins required for initiation of DNA replication [35]. Less than 4 kb of pBin19 is required to encode its essential functional features, namely, the T-DNA flanked by the LB and RB, a drug-resistance marker (e.g., *nptIII*, a streptococcal kanamycin resistance gene that provides dominant selection in *A. tumefaciens* and *E. coli*), and the RK2 replicon [36]. We have made a streamlined version of pBin19 [33] by a PCR strategy similar to that employed by Xiang et al. [37]. The new plasmid, pAg1 (Fig. 2), has a size of only 4 kb and incorporates the generous multiple cloning site of pANT846 (C. DeSanti, personal communication) within the T-DNA region, as well as the overdrive element characteristic of octopine Ti plasmids [38].

A practical drawback of donor vectors based on the RK2 origin of replication is their propagation at low copy number in *E. coli*. To remedy this, several vectors have been developed that have two replication origins, the *ColE1 ori* of pBR322, for high copy number in *E. coli*, and a second *ori* for propagation in *A. tumefaciens*, such as the *ori* from the *Agrobacterium rhizogenes* Ri plasmid [39], the *Pseudomonas* plasmid pVS1 [40,41], or the wide-host range plasmid pSA [42]. It is important to note that in the interest of minimizing the size of these dual-origin donor vectors and providing for biocontainment, genes for obligatory *trans*-acting replication functions (e.g., *repA* in the case of SA and *trfA* of RK2) have been omitted from certain donor vectors, limiting their use to specific *Agrobacterium* host strains in which a copy of the replication gene is either incorporated into the genome or carried on a compatible companion plasmid.

Donor plasmids also may contain a mutant form of *virG* to alter the expression of the inducible *vir* genes. Transcriptional activation of these genes is under the control of a two-component regulatory system composed of constitutively expressed *vir* proteins (Fig. 1). One component is the *virA* product, a transmembrane protein that autophosphorylates in response to environmental stimuli (plant phenolics, sugars, acidic pH, and temperature) [8]. The activated form of the *virA* protein phosphorylates an aspartic acid residue (D52) in the second constitutively expressed component, the cytoplasmic *virG* protein, enabling it to become a transcriptional activator for the expression of the remaining *vir* genes, whose products then mobilize the T-DNA. A mutation within *virG* that converts a neighboring asparagine residue (N54) to aspartic acid allows the gene product to behave as a transcriptional activator without being phosphorylated [43–45]. Incorporation of this N54D mutant form of *virG* into a donor vector provides inducer-independent expression of the *vir* genes, and such a plasmid has been used in the transformation of *Coccidioides immitis* [21]. A derivative of pAg1 bearing the N54D form of *virG* has been constructed in our laboratory, and we found that, even though *A. tumefaciens* having this donor plasmid is no longer strictly dependent on acetosyringone for DNA mobilization, transformation frequency remains subject to further stimulation by addition of inducer. The extent of this stimulation, however, is only equivalent to that which could have been achieved with the inducer acting in concert with the wildtype *virG* gene of the resident disarmed Ti plasmid. Seeing no practical advantage over pAg1, we do not routinely make use of the pAg1 N54D*virG* derivative. Modeling of the 3D structure of the *virG* protein based upon its similarity with

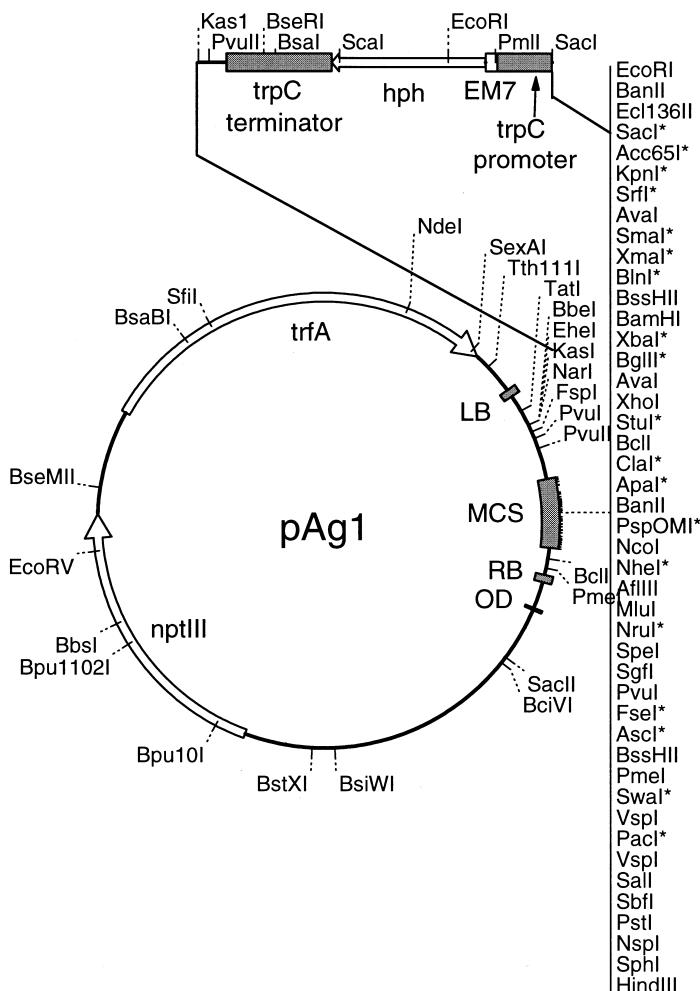


Figure 2 Map of donor plasmid pAg1, a 4-kb streamlined version of pBIN19 with a generous multiple cloning site (MCS) in the T-DNA segment delineated by the left and right border sequences (LB and RB, respectively). OD, overdrive element; *nptIII*, kanamycin resistance gene; *trfA*, a trans-acting gene of the RK2 replicon required for DNA replication. pAg-H4 was made by inserting a 2.35-kb hygromycin B resistance cassette at the *KasI* and *SacI* sites of pAg1. The *hph* gene in this cassette is under the control of tandemly arrayed promoters: the *trpC* promoter for expression in fungi and the *EM7* promoter for expression in *E. coli* and *A. tumefaciens*. Because transfer of T-DNA is polar beginning with the RB, selection for a marker placed near the LB ensures that each transformant will have received the entire T-DNA element. Restriction sites of the MCS that remain unique in pAg-H4 are marked by asterisks.

the *cheY* protein suggests that D52 is located within an “acidic pocket” [46], and changes in two amino acids (residues 7 and 106) near this pocket apparently account in large measure for the remarkably strong activity of the *virG* protein encoded by the hypervirulent plasmid pTiBo542. Piers et al. found that a donor plasmid used along with the disarmed form of pTiBo542 was more efficient in transforming *S. cerevisiae* than the same donor in combination with the disarmed version of pTiA6 [16]. Similarly for fruiting tissue of *Agaricus bisporus*, disarmed pTiBo542 supported transformation, whereas disarmed pTiC58 did not [22].

In certain areas of fungal biotechnology, the ability to transfer the genes for an entire biochemical pathway would offer tantalizing prospects. It is noteworthy, therefore, that the *Agrobacterium*-mediated transfer of 150 kb of DNA into plant cells has been achieved [47]. The efficiency of this process is improved by increasing the gene dosage for *virG* and providing for extra copies of the *virE2* protein [48]. Transfer of very large segments of DNA into filamentous fungi has not yet been reported, although without making provisions for augmented levels of *virG* and *virE2*, increasing the size of T-DNA from 3.5 to 15.5 kb has been found to decrease the efficiency of transformation [20].

Agrobacterium donor vectors have also been refined by inserting site-specific recombinase sites within the T-DNA to facilitate the subcloning of DNA fragments from other plasmids. Both the *loxP* site for the P1 bacteriophage Cre protein [49] and the site for the lambda phage *att* system (Gateway®; Invitrogen) [50] have been used. A vector with the *ColE1 ori* and a gene for chloramphenicol resistance in the T-DNA region has been used to allow recovery of fungal genomic DNA flanking the integration site by plasmid rescue [25].

5. DOMINANT SELECTION

Complementation of an auxotrophic mutation in a fungal recipient is a means of selecting transformants that is available only in the well-studied filamentous fungi [2]. For most strains of agricultural and industrial importance, selection based on dominant drug resistance provides a more generally applicable approach because it requires only that the target organism is sensitive to an agent for which a resistance gene is available. Three drugs that have proven useful in this context are hygromycin B, phleomycin (Zeocin), and phosphinothricin (glufosinate ammonium). Resistance is provided to hygromycin B by an *E. coli* hygromycin B phosphotransferase (*hph*) [51–53], to phleomycin by a drug binding protein from *Streptoalloteichus hindustanus* (*ble*) [54], and to phosphinothricin by an acetyltransferase from *Streptomyces hygroscopicus* (*bar*) [55,56]. Nourseothricin also may be useful in certain cases; resistance is given by an acetyltransferase encoded by *nat1* [57].

Expression of these bacterial genes in fungi under various growth conditions and at different stages of the life cycle requires that the genes be placed under the control of strong fungal promoters. Two from *Aspergillus nidulans* (*trpC* [58] and *gpdA* [59]) and one from *Neurospora crassa* (*cpc1* [60]) have been widely used in ascomycetous fungi. For basidiomycetes, the *gpd* promoters of *Schizophyllum commune*, *Phanerochaete chrysosporium*, *Agaricus bisporus*, and *Ustilago maydis* are available [61,62]; in *U. maydis*, hygromycin B phosphotransferase has been expressed under the control of the organism’s heat shock *hsp70* promoter [63]. The actin promoter from *Cryptococcus neoformans* has been used to express the genes for hygromycin B and nourseothricin resistance in this basidiomycetous human pathogen [64,65].

The recent commercial introduction of transposon systems that can be used to modify target DNA *in vitro* (e.g., the Tn7-based GPS; New England BioLabs) has prompted interest in drug resistance markers that may be selected in both bacterial and eukaryotic cells [66,67]. Fortuitous expression of the *hph* gene in a *trpC* promoter-*hph* construct [53] is apparently sufficient in *E. coli* [67] and *A. tumefaciens* [33] to allow growth of these bacteria in the presence of hygromycin B at 25 to 50 µg/mL, but *E. coli* cells containing the *hph* gene downstream of the fungal *hsp70* promoter do not express resistance to this drug [63]. In plasmid pTEF1/Zeo (Invitrogen), the *ble* gene for Zeocin resistance is under the control of two tandem-arrayed promoters, the *TEF1* promotor for expression in yeast, followed by the *EM7* promoter for expression in *E. coli*. Prompted by this example, we have constructed a tandem *trpC/EM7* promoter and placed *hph* under its control (unpublished). The new cassette was inserted into the T-DNA of pAg1 proximal to the LB to produce plasmid pAg-H4 (Fig. 2). In *E. coli*, pAg-H4 allows robust growth and overnight colony formation in the presence of hygromycin B at 100 µg/mL, despite its low copy number. It also appears that the *EM7* promoter is active in *A. tumefaciens*, because cells of this organism carrying pAg-H4 are resistant to hygromycin B at 50 µg/mL. As expected, *Agrobacterium*-mediated transfer of the T-DNA from pAg-H4 to either *Penicillium paxilli*, *Glarea lozoyensis*, or a *Nodulisporium* isolate results in hygromycin B resistant fungal transformants. This activity profile makes the new tandem-promoter cassette an ideal transposon marker, and it has been installed in pGPS3 (New England Biolabs) in place of the kanamycin marker (Fig. 2). The resulting plasmid, pTaH6, provides a transposon that is very convenient for generating insertional mutants of fungal genes cloned within the T-DNA region of pAg1. Following the random insertions of the transposon into the target DNA *in vitro* and destruction of the residual pTaH6, pAg1 derivatives containing the transposon can be recovered overnight in *E. coli* on the basis of hygromycin B resistance. Those with insertions in the gene (or region) of interest are identified by restriction analysis or sequencing and moved into *A. tumefaciens*. Transfer of the T-DNA bearing the insertionally inactivated gene into the fungus from which the gene was originally obtained results in replacement of the homologous gene by the inactive allele in a high proportion of the hygromycin B resistant transformants. Similar tandem promoter constructs could be prepared for other drug resistance genes to extend the utility of this gene knockout strategy for elucidating gene function in fungi [66,67].

6. GUIDELINES FOR AGROBACTERIUM-MEDIATED TRANSFORMATION

6.1. *Agrobacterium tumefaciens*

Preparations of *A. tumefaciens* LBA4404 ready for electroporation may be purchased from Life Technologies (catalog number 18313–015). These cells carry plasmid pAL4404, which is a disarmed derivative of the octopine plasmid pTiAch5 [11]. They can be used with a variety of donor plasmids. The strain also is available from the American Type Culture Collection (ATTC23341), as are strains A136 (carrying no Ti plasmid; ATTC51350) and AGL1 (with a disarmed hypervirulent succinamopaine plasmid derived from pTiBo542 [46]; ATCC BAA-101). General methods for handling and preserving *Agrobacterium* strains may be found elsewhere [68,69].

6.2. Donor Vectors

pBIN19 can be obtained from the American Type Culture Collection (ATCC37327). It is derived from the nopaline plasmid pTiT37 [12]. The dual-origin of replication vector,

pC22, is also in the ATCC collection (ATCC37493). Sources for other plasmids have been summarized in the vector guide compiled by Hellens et al. [34]. Vectors are propagated and manipulated in *E. coli* by standard methods making the accommodations necessary for those replicating at low copy number.

6.3. Culture Media for *A. tumefaciens*

The media specified here are those described by Cangelosi et al. [68], Lin [70], and Bélanger et al. [71].

LB broth (per liter; pH 7.5)

Bacto tryptone 10 g
Bacto yeast extract 5 g
NaCl 10 g

YM broth (per liter; pH 7.0)

Bacto yeast extract 0.4 g
Mannitol 10.0 g
K₂HPO₄ 0.5 g
MgSO₄·7H₂O 0.2 g
NaCl 0.1 g

LBYM broth

LB broth 1 volume
YM broth 1 volume

These broths may be converted to plating media by the inclusion of agar at 1.5% to 2% (w/v) and, after autoclaving and cooling to 55 °C, may be fortified with appropriate antibiotics.

Agrobacterium broth (AB) and glycerol induction (GI) media are defined media used for *A. tumefaciens* just before and during the cocultivation of the bacterium with the fungal recipient. Although they share common components, their differences are necessitated by the fact that induction of the *vir* genes is favored at pH values and phosphate concentrations lower than those optimal for the growth of the bacterium [72]. These media may be conveniently prepared from concentrated sterile stock solutions (Table 2). Except where noted, the stock solutions are sterilized by autoclaving. GI agar is cooled to 55 °C before acetosyringone is added.

6.4. Electroporation

Donor plasmids may be introduced into *A. tumefaciens* by electroporation [68,70,73–79]. The electrical pulse parameters appropriate for *Agrobacterium* in a specific electroporation apparatus can often be obtained from the equipment manufacturer. Immediately following the electrical pulse, the cells are diluted with 1 mL of YM broth and incubated for 3 hr at 28 to 30°C with gentle agitation for expression of the plasmid's resistance. Transformants are subsequently recovered by plating on YM agar containing the required selective agent. At 28 to 30°C, colonies appear after 2 to 3 days.

Table 2 Formulation of defined media.

Solution	Components	10×Stock (per L)	GI AB	GI Broth ^a	Agar ^b
AB salts 1	NH ₄ Cl	10.0 g	1 vol	1 vol	1 vol
	MgSO ₄ ·7H ₂ O	3.0 g			
	KCl	1.5 g			
AB salts 2 ^c	CaCl ₂ ·2H ₂ O	100.0 mg	1 vol	1 vol	1 vol
	FeSO ₄ ·7H ₂ O	25.0 mg			
AB phosphate (adjust pH to 7.0)	K ₂ HPO ₄	30.0 g	1 vol	—	—
	NaH ₂ PO ₄ ·H ₂ O	10.0 g			
AB glucose	Glucose	50.0 g	1 vol	—	—
GI phosphate	NaH ₂ PO ₄ ·H ₂ O	6.9 g	—	—	1 vol
GI carbon	Glucose	18.0 g	—	1 vol	1 vol
	Glycerol	50.0 g			
GI MES ^{c,d} (adjust to pH 5.2)	MES/NaOH	97.5 g	—	1 vol	1 vol
Sterile H ₂ O			6 vol	6 vol	—
Sterile 2× agar	3% agar		—	—	5 vol

^a Phosphate-free.^b To induce the *vir* genes during the cocultivation period, acetosyringone (3'-5'-dimethoxy-4-hydroxyacetophenone; Aldrich catalog number D-13440-6) is added to the GI agar at a final concentration of 200 µM, from a fresh 10³× stock solution prepared in ethanol or dimethylsulfoxide.^c Sterilized by filtration.^d 2-Morpholinoethanesulfonic acid monohydrate.

If an electroporator is unavailable, the older freeze-thaw method may be used for transformation [80,81].

6.5. Preparation of *A. tumefaciens* for Cocultivation

Two days before the fungal recipient is ready for cocultivation, *Agrobacterium* cells bearing the donor plasmid are grown in LBYM broth containing the appropriate selective agent. The culture is incubated at 28 to 30°C for 24 h with shaking at 220 rpm in a vessel that allows free transfer of oxygen, because the bacterium is an obligate aerobe. The resulting culture is diluted 1:100 into 50 mL of AB with the selective agent added. The new culture is incubated under the same conditions, and after 20 to 24 h the cells are collected by centrifugation and suspended in one-tenth the volume of phosphate-free GI broth. A dilution of this suspension is prepared in phosphate-free GI broth to achieve the desired cell density as measured by light scattering at 660 nm (discussed further in a subsequent section).

In our experience as well as that of Malonek and Meinhardt [24], it is unnecessary at this point to incubate the bacterial cells with inducer as described in other reports [14,16,18,19,22,27]; indeed, at the low pH of GI broth, certain strains have a propensity to clump upon incubation. Exposure to acetosyringone during the several days of cocultivation is sufficient to induce the T-DNA mobilization apparatus. Beyond these practical considerations, Mullins et al. reported that single site integration was more frequently found when bacteria were not exposed to acetosyringone prior to the cocultivation step [25].

6.6. Preparation of the Fungus for Cocultivation

Cocultivation is conveniently conducted on membrane filters that allow the cell mixtures to be kept for a period on GI agar and then moved to a selective agar medium. As discussed earlier, *Agrobacterium*-mediated transformation has been successfully employed with fungal recipients in various cellular forms. Because the morphology, lifecycle, and behavior of each fungus in culture will largely define the forms of a particular organism that could be used for cocultivation, only general protocol guidance can be provided here.

Perhaps the simplest situation is presented by a fungus capable of readily producing conidia that behave as propagules. The fungus is allowed to conidiate, and the conidia are harvested. Often spores need to be wetted with 0.1% (w/v) Tween 80 to allow their harvest as a suspension; they can then be washed in 0.05% (w/v) Tween 80 and stored at 4 °C. Their concentration should be determined. If the spores can germinate rapidly on GI agar, they can be washed free of detergent with phosphate-free GI broth and mixed directly with the *Agrobacterium* cells for placement on the filters. For other fungi, it may be necessary to place the spores on filters and allow germination on a different medium prior to transferring the filters to GI medium and applying the *Agrobacterium* cells. Detergents, even in trace amounts, appear to interfere with the transformation process, and the protocol should include measures to remove or minimize them.

For other fungi, the best form of recipient cells may be vegetative mycelia. In this case the objective is to obtain a suspension of dispersed mycelia. The organism is grown in a clear (nonparticulate) liquid medium with agitation. If the organism has a tendency to grow as pellets or balls in submerged culture, formulation of the growth medium with 0.4% agar may discourage aggregation of the hyphae. Often short lengths of mycelia may be separated from masses of tangled mycelia by mixing the culture with several volumes of sterile water in a conical tube, allowing larger clumps to settle to the bottom of the tube, and drawing off the suspended mycelia for collection by low-speed centrifugation. If the yield from this procedure is low, the culture might first be mildly homogenized to generate mycelial fragments (an Omni PCR Prep Homogenizer with disposable probes is convenient for this process). The mycelia are washed twice by suspension in sterile water and centrifugation. Following the last centrifugation, the volume of the pellet is estimated, and the cells are suspended in ten times that volume of GI broth (without phosphate).

6.7. Cocultivation and Selection of Fungal Transformants

Detergent-free membrane filters are placed on GI agar containing acetosyringone at 200 µM. TF series mixed cellulose ester filters or Durapore PVDF membrane filters are satisfactory (0.45 µm; Millipore Corp.). The fungal recipient and the *Agrobacterium* cells carrying the donor vector are applied to the filters and cocultivated at 20 to 25°C; T-DNA transfer is optimal at temperatures lower than those for the growth of the organism [32,82]. After the appropriate period, the filters are moved to plates of a fungal growth medium (e.g., potato dextrose agar) containing two selective agents, one to kill the *Agrobacterium* cells and the other to provide selection of the fungal transformants. For many *Agrobacterium* strains, carbenicillin at 500 µg/mL can be used, but for others, notably ALG1, cefotaxime at 100 µg/mL may be required. The plates are incubated at a temperature that is appropriate for the fungus, and once putative fungal transformants appear, they are purified by a second plating on the same type of selective medium. Cocultivation in the absence of acetosyringone is an important control to confirm that resistant fungal colonies arise only as a consequence of the induction of the T-DNA mobilization apparatus in the bacterium.

Two significant variables that are worth exploring in the first transformation experiment are the ratio of bacterial and fungal cells and the length of the cocultivation period [21,25,26,31,32]. It can be helpful in setting these parameters to be guided by the radial growth rate of the fungus on an agar medium. To illustrate, in our laboratory we have transformed *Penicillium paxilli*, a *Nodulisporium* isolate, and *Glarea lozoyensis* to hygromycin B resistance using pAg-H4. The radial growth rates of these fungi may be characterized as fast, moderate, and slow, respectively. For *P. paxilli*, a spore suspension (2×10^8 /mL) was mixed with an equal volume of an *Agrobacterium* cell suspension with an OD₆₆₀ of either 2.0 or 4.0. For *Nodulisporium* and *G. lozoyensis*, mycelia were employed as recipients and suspensions were mixed with bacterial preparations that had been adjusted to an OD₆₆₀ of 1.0. In each case, 100-μL portions of the mixtures were spread evenly on sterile, 47-mm membrane filters. The filters were incubated on GI agar containing acetosyringone for 1, 2, or 3 d for *P. paxilli*, *Nodulisporium*, and *G. lozoyensis*, respectively, before being transferred to selective agar. Hygromycin B resistant transformants were obtained under selective conditions after 4 to 5 days (*P. paxilli*), 6 to 7 days (*Nodulisporium*), or 9 to 10 days (*G. lozoyensis*).

6.8. Troubleshooting

When transformation of the organism of interest has already been achieved by another method, key variables such as the choice of promoter and resistance gene and the appropriate concentration of the selective agent are already known. For an organism that has never been transformed, its sensitivity to selective agents should be determined as a basis for choosing the most appropriate resistance gene, and careful consideration is needed in deciding which promoter is likely to drive expression of the resistance gene as discussed earlier in this chapter. If transformants are obtained in the initial experiment, a small number of subsequent trials exploring the variables identified in the previous section should optimize the procedure for routine laboratory use. Lengthening the cocultivation period may provide improved transformation rates, but excessive growth of either organism at this stage may confound subsequent efforts to select transformants. Overgrowth of either organism might be addressed by lowering the glucose in the GI agar from 10 mM to below 5 mM. When fungal mycelia are used as recipient, clumps that are not removed during the preparation of the suspension may eventually appear as small fungal colonies during selection. These are recognized by their failure to grow during the second plating on selective media. Experience with *Aspergillus nidulans* [18] demonstrates that a stringent selection procedure is a prerequisite for a successful outcome with this method.

7. CONCLUSION

The delivery of DNA through a binary plasmid approach mediated by *A. tumefaciens* offers a promising means of surmounting what has often been a significant obstacle in the study of many fungi. The method is simple and relatively rapid in execution and requires no lytic enzymes or specialized equipment. It has been successful with organisms throughout the fungal kingdom. Because T-DNA is transferred and integrated primarily as a single copy, analysis of the transformants tends to be uncomplicated. Bacterial cells carrying disarmed Ti plasmids are readily available, and donor vectors may be adapted or constructed to meet specific needs. *Agrobacterium*-mediated transformation is likely to become an indispensable experimental technique in many fields of mycology.

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6

Fungal Germplasm for Drug Discovery and Industrial Applications

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1. INTRODUCTION

The vision for this chapter stems from our experiences working in and managing industrial microbial screening programs and as professional mycologists. Many newcomers to microbial screening are as unacquainted with wild species of fungi as they are unknowing of what can be done with fungi in the context of the technology of pharmaceutical/industrial screening. Rather than exhaustively list methods for obtaining fungi from nature, we have attempted to set up a framework of the basic methods, tools, and organizational principles that we have found most useful for channeling fungal germplasm into the academic, pharmaceutical, or enzyme discovery laboratories. We start by reminding users of biodiversity of how the Convention on Biological Diversity (CBD) has altered the use of biological resources, and then follow with an analysis of recent organizational and technical trends of natural products discovery programs, especially in Japan [1]. We provide some step-by-step approaches for what we have found to be some of the most fruitful techniques for coaxing wild species into cultivation, especially filamentous ascomycetes and basidiomycetes, which are the most genetically apt to produce secondary metabolites. Finally,

we call attention to the need to be curious and critical and to continuously strive to evaluate and improve the contribution of the microorganism to the screening process.

The organisms that serve as the basis of screening are very much an essential infrastructure for the discovery program. Therefore, at the outset, several questions need to be carefully considered. What is the nature of products sought? Are they enzymes or small molecular weight molecules? What might be their potential targets? Where do we look for leads in realm of natural products and natural products pathways? In the past 10 to 15 years, much has been discovered about the magnitude and extent of bacterial and fungal diversity. Although much of the newly found bacterial diversity has been claimed to be inaccessible because of its unculturability, fungal biologists have long been aware of the inability to culture several major groups of fungi (e.g., Glomales and Uredinales). In fact, historically important fungal metabolites, like α -amanitin, muscarine, lysergic acid, ergotamine, and psilocybin, were discovered and elucidated before their source organisms were cultivated *in vitro*. However, after considering what would be the necessary genetic makeup of an organism that will produce the desired profiles of products, it is likely that only a small portion of the microbial universe is genetically equipped for producing the natural product metabolite or enzymes of interest. The fact that a wide cross-section of the usable microbial universe has already been mined heavily for their antimicrobials and other pharmaceutical agents during the past 50 years further clouds the discovery landscape [2–4]. Consequently, questions of whether the source has been exhausted of useful chemical structures or what types of natural products are likely to interfere with discovery assays need to be considered [4]. Reliable microbial phylogenies and easy access to marker gene sequences now provide a practical and facile means to map unknown, and even unseen, organisms, to their position in the phylogenetic universe (Tree of Life Web Project, <http://tolweb.org/tree/phylogeny.html>).

Historical, theoretical, and strategic considerations are thus essential in determining what kind of microbes should be examined as the *materia prima*. If the starting point is a known product and it is associated with a known gene family or gene cluster, starting with known organisms from culture collections may be sufficient. Elaborating from a known point with a phylogenetically or ecologically directed search may be a valid strategy. Other important considerations are whether to use preserved organisms from public service collections or from the company's in-house historical collections, collaborate with a third party to deliver strains of interest, or acquire fresh strains with in-house personnel. Consistently reproducing the product and its activity are equally critical; therefore, the means and facilities for strain preservation need to be factored in at the planning stages. All such plans should be closely integrated with decisions on how the organisms will be preserved and grown and how their cells and culture media will be treated to extract their products. Linking strain selection to growth and assay is critical because the growth forms and rates among the fungi are extremely heterogeneous, and product expression is likely to be very sensitive to how the organism is grown.

Consequently, our ability to exploit patterns of fungal diversity and the mechanisms that shape them is influenced by the approaches we use to evaluate the ecological and evolutionary structure of fungal communities. The parameters that characterize a community and that are central for assessing aspects of its biodiversity include species composition, along with its functional and genetic correlates; the types and intensities of interspecific interactions, and how they regulate species densities and species occurrences; and the dynamics of those attributes over time and space or as they change as a consequence of past or present human intervention (e.g., disturbance and fragmentation of the habitat,

transport, and storage). To truly take advantage of patterns of biodiversity and mechanisms contributing to those patterns, one must address all aspects of community organization and dynamics, plus the artifacts the nature-to-laboratory transition introduces into the system. Furthermore, the logistics of moving microorganisms, the regulatory environment governing their use and transport, personnel with their expertise and biases, equipment and automation, and space are apt to create bottlenecks in the discovery process and limit which kinds of microorganisms can be used. These latter considerations constrain the focus of screening programs to only a few aspects of microbial communities, thereby providing at best a superficial assessment of fungal biodiversity and its chemical complexity.

Fortunately for those interested in using biological diversity, the playing field for designing a screening program has broadened considerably in the past 10 years. A renaissance in biological diversity research has been spurred in part by the U.S. government's investment in biological sciences. The U.S. National Science Foundation has established programs for Biotic Surveys and Inventories, Microbial Observatories, Biological Complexity, and The Tree of Life that have supported basic studies in phylogenetics and ecology. Since 1993, the U.S. National Institutes of Health, the U.S. Agency for International Development, and Fogarty International Center have sponsored multidisciplinary, multi-institutional awards dubbed the International Cooperative Biodiversity Groups (ICBG). The goal of the ICBG program is to integrate and link the needs for improved human health products, drug discovery, incentives for conservation of biodiversity, and the development of new modes for sustainable economic activity [5]. Perhaps the most outstanding feature of the program from an industry viewpoint is that it has provided a training ground for future scientists in natural products management and discovery and has been instrumental in putting scientific infrastructure at the sources of biological diversity. Several large-scale, all-organism inventory projects [6] have been proposed, planned, or initiated in Costa Rica [7], the Great Smokey Mountains National Park (www.discoverlife.org), and the Hawaiian Islands (www.all-species.org). These projects have contemplated the human, technical, and financial resources required to observe and enumerate all the organisms in a complex ecosystem, including the fungal and bacterial kingdoms. The industrial microbiologist seeking to optimize the discovery opportunities from wild microbial species faces the same challenges of integrating limited resources as these landscape-scale inventories, although on a smaller multiple dimension.

2. CONVENTION ON BIOLOGICAL DIVERSITY AND FUNGI

As late as November 1983, the members of the Food and Agriculture Organization (FAO) adopted the International Undertaking (IU) on Plant Genetic Resources. Article 5 of the IU referred to the "universally accepted principle that plant genetic resources are the common heritage of mankind and consequently should be available without restriction." However, the concept has evolved along a totally different course during the subsequent 10 years. The CBD was opened for signature in June 1992, during the Rio Earth Summit (the United Nations Conference on Environment and Development) and entered into force in December 1993, after 30 countries ratified it. As of October 2002, 186 countries, including the European Union, have ratified the CBD.

Article 15 is probably the most relevant provision of the CBD with respect to use of microbial resources. Article 15.1 referred to the "Recognizing the sovereign rights of States over their natural resources, the authority to determine access to genetic resources

rests with the national governments and is subject to national legislation," and is interpreted to mean that in the transfer of biological resources from the country of origin to another country, the national government of the original country has the final decision about the material transfer. Therefore, in a case of using biological materials of another country, a user has to obtain appropriate permission for the use from the relevant government authority.

The Article 15 of the CBD referred to access to genetic resources and states:
Article 15.4 Access, where granted, shall be on mutually agreed terms and subject to the provisions of this Article.

Article 15.5 Access to genetic resources shall be subject to prior informed consent of the Contracting Party providing such resources, unless otherwise determined by that Party.

Article 15.7 Each Contracting Party shall take legislative, administrative, or policy measures, as appropriate ... with the aim of sharing in a fair and equitable way the results of research and development and the benefits arising from the commercial and other utilization of genetic resources with the Contracting Party providing such resources. Such sharing shall be upon mutually agreed terms.

Therefore, as illustrated in Fig. 1, a user who wants to use biological resources of another country, as the first step, inquires at an appropriate national authority the possibility of the utilization with Prior Informed Consent (PIC). And if the possibility exists, the user and the provider would negotiate a contract that includes a material transfer agreement and the benefit-sharing procedure under Mutually Agreed Terms (MAT).

Practical guidelines on the access and benefit-sharing process is described in "Bonn Guideline on Access to Genetic Resources and Fair and Equitable Sharing of the Benefits Arising Out of their Utilization," which was adopted at the Sixth Meeting of the Conference of the Parties (COP-6) held in the Hague, the Netherlands in April 2002 (<http://www.biodiv.org/>).

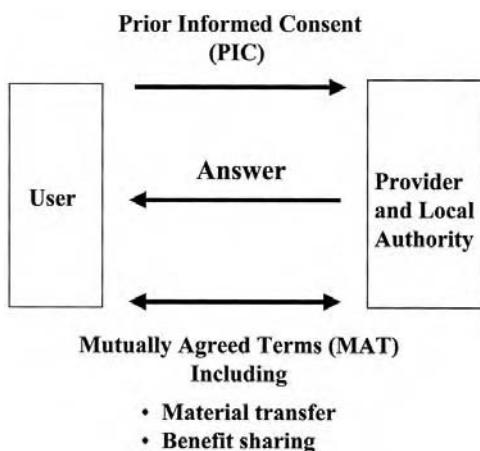


Figure 1 Simplified structure of an access and benefit sharing agreement for biological materials.

2.1. Fungal Acquisition and the Convention on Biological Diversity

Japan has maintained 23 collections of microorganisms and other biological resources that could function as national biological resource centers. In addition to these existing culture collections, Japan established a new national Biological Resource Center [BRC (NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, 2–5–8 Kazusakamatari, Kisarazu-shi, Chiba, 292-0818 Japan)] in April 2002, intended to meet the requirements of the life sciences and biotechnology in the 21st century. It was created to implement a basic plan, agreed upon by five ministries: the Ministry of International Trade and Industry; the Ministry of Agriculture, Forestry, and Fisheries; the Ministry of Education, Science, Sports, and Culture; the Ministry of Health and Welfare; and the Science and Technology Agency. Although industry research groups have had concerns that government involvement would introduce more bureaucratic hurdles and thereby impede the use of biological resources, the government may have an important role if it clarifies procedures and facilitates access. Poor understanding by government agencies of the difficulties and risks involved in biological innovation may exacerbate unrealistic expectations and interfere with exchange of biological resources on the open market. However, the goal of the BRC is to operate as a mediator between providing countries and the private sector for access to biological materials. In general, Japanese research organizations have bought into the concept and have adopted the view that a good relationship among providing countries, the Japanese government, industry, and academia is advantageous. Sustainable use of biological diversity and equitable sharing of derived benefits is desirable in the long run.

3. TRENDS IN ACQUISITION OF MICROBES IN INDUSTRY IN JAPAN

The Kanto Branch of the Mycological Society of Japan surveyed 48 organizations, mainly pharmaceutical, chemical, and biomedical industries in Japan and a few multinational companies [1]. The purpose of the survey was to investigate how these organizations run their current natural product-based drug discovery (NPDD) programs, and what were their objectives. The major issues addressed were (1) the general state of NPDD, (2) various technical aspects, and (3) effects of the CBD and the new BRC established in Japan in 2002. Thirty-seven (77.1%) of the 48 industrial organizations contacted responded. Most were Japanese, but several multinational companies were also polled.

3.1. General Aspects of Natural Product Screening Programs

Responding organizations fell into the following categories: pharmaceutical industry, 73%; other private companies, 19%; universities or colleges, 3%; and other research organizations or nongovernmental organizations, 5%. We considered the replies to reflect industry trends because the private sector comprised 92% of the respondents. Twenty-seven of 37 responding organizations (73%) ran NPDD screening programs. Opinions varied widely on different operational facets. Positive opinions are summarized here. The NPDD organizations consistently considered that such programs were capable of delivering lead structures beyond the imagination of the synthetic chemists, and 70% of the organizations thought that high-throughput screening (HTS) was an advantageous complement to the program. Obtaining leads based on a single, unique chemical structure was not the only important issue for screening natural products, because minor structural differences often revealed important structure–activity relationships. Although discovery of truly new chem-

ical entities from natural sources was difficult, it was also doubtful whether innovative compounds have been synthesized and identified using combinatorial chemistry. In fact, although many hits have been generated from combinatorial chemical libraries, none so far have reached advanced clinical development [8,9].

However, respondents made several negative statements. Statements such as “beyond synthetic chemists’ imagination” and “diverse fungi provide diverse compounds” have been so overused during the past 10 years that they are no longer a driving force, even for scientists involved in NPDD. The suggestion of “beyond synthetic chemists’ imagination,” although always a possibility in NPDD, can be counterproductive because the structural complexity of some natural products may be technically intractable for a medicinal chemistry program. Furthermore, the objective of NPDD is to find drug leads, not unusual chemical structures. Another negative opinion was that probably the majority of basic structural types and biosynthetic pathways have been found. Finally, paradigms for screening natural products differ among targets based on new genomic-derived and other newly developed targets. The same series of screenings protocols and various assay formats cannot be adopted for all targets with heterogeneous and complex natural products. Consequently, many HTS formats are often poorly suited for NPDD.

3.2. Screening Sources

Almost all organizations with NPDD programs used actinomycetes and fungi as screening sources. Unicellular bacteria, plants, and medicinal herbs were the next most frequently used organisms. Invertebrates and microalgae were screened by some, whereas insects were rarely exploited. Interestingly, a number of organizations have reduced or abandoned the use of plants, medicinal plants, microalgae, bacteria, or actinomycetes, whereas 33% of organizations have increased the proportion of fungi among their screening sources.

3.3. The Role of Systematics

More than 90% of organizations routinely isolated a broad range of microbes without targeting a specific taxon. Some sought microbes from unusual environments. About 40% have developed their own methods for optimizing microbial isolations when existing methods did not meet their needs [10–15].

If a company does not operate NPDD, taxonomic input is not necessary. Although some companies may screen natural products without any systematic guidance, Japanese industries conducting NPDD generally have skilled taxonomists on their staff. In Japan, taxonomists are either university educated or learn within the company through on-the-job training (OJT). The latter situation recently seems to have dominated in Japan, because Japanese universities have de-emphasized microbial systematics and students are less interested in the subject. Company-sponsored OJT is complemented through interaction with academic societies. For example, in recent years, the Kanto Branch of the Mycological Society sponsored a series of workshops for academic and industrial scientists on taxonomy of *Acremonium*, *Fusarium*, and aquatic fungi.

Some respondents said that they targeted specific ecological or taxonomic groups of fungi. Soil fungi (74%) and litter fungi (52%) were the ecological groups most frequently mentioned. Their regular use was no doubt a function of the convenience in obtaining high numbers of strains. When specific taxa were mentioned, mitosporic fungi and ascomycetes were most widely used. Other organizations simply reported they attempted to obtain as wide a range of taxa as possible. However, Oomycetes and Zygomycetes were men-

tioned infrequently, probably as a result of their limited history of secondary metabolite production [16,17].

3.4. Strain Management

When thousands of isolates are collected, strains and their corresponding biological activities, or lack thereof, are duplicated in the screening results. The strain duplication is either ignored or various strategies are invoked to overcome it. Most laboratories (67% to 70%) did not necessarily discard duplicate strains of the same species, and duplicate species were submitted to screening assays when there was some reason to suspect they could be genetically distinct (e.g., different geographical origin, cultural differences, or variations in genetic markers). Examination of infraspecific and population-level variations in metabolite production generally was thought to be valuable. Cosmopolitan species often were excluded when it was judged the species had been adequately represented in the screening collection. To ensure extract diversity for HTS, 37% organizations removed duplications based on complimentary genetic or chemical analyses [18–20]. Based on anecdotal evidence, some believed that the activity of fresh isolates is superior to that of strains preserved in culture collections. As a result, inactive isolates are not preserved and are discarded after being screened (30%). But most organizations (67%) preserved and reused strains, at least to some degree. Such recycled strains either served as the basis of a screening sample library to meet the demands of HTS or were used to pilot new assays. Construction of microbial extract collections for HTS therefore was thought to be worthwhile for certain applications.

3.5. Impact of the Convention on Biological Diversity

Fifty-six percent of the industrial groups collected at least part of their original samples themselves. About 41% obtained their materials within Japan, whereas some acquired raw materials from overseas with the following frequencies: microbial substrata, 48%; microbial strains, 26%; and extracts, 22%. Almost all pharmaceutical companies (96%) were conscious of the CBD and agreed with its principles. However, 41% of the organizations expressed concern that the CBD has hindered natural product screening activities or necessitated realignment their operation. In fact, some companies have abandoned use of biological materials from overseas, maintaining that useful microbes can still be discovered by carefully examining the Japanese microbial flora that spans from boreal to near tropical environments. Unreasonable expectations for financial investment will prevent many countries from participating in the drug discovery process. Seventy-four percent of organizations stated that complaining about the CBD was useless. Many have proactively contracted collaborative research agreements with a foreign partner to provide biological resources. However, 56% expected government organizations to help them gain access to foreign biological materials.

4. BRINGING FUNGI INTO THE LABORATORY

4.1. General Considerations for Collecting and Transporting of Fungi

The process of collection of and culturing of fungi generally can be delimited into three stages: obtaining and transporting the isolation source, cell separation or trapping tech-

niques for establishing in vitro growth, and manipulations of growth in isolation media. The three stages are interdependent and determine the success of the isolation. The factors that influence which components of fungal kingdom can be captured and brought in live into a screening program are illustrated in Fig. 2. The ensuing challenge is how to put what arrives at the laboratory into a usable form and then make the most of it.

Almost anything imaginable harbors fungi: materials range from desert rocks or high mountain snow with no apparent fungal growth to large, conspicuous fruiting bodies of mushrooms. The most often used materials are those that bear large numbers of culturable fungi and include soils, living and decomposing plants, invertebrates, other fungi, thalli of lichenized fungi, animal dung, and stored and processed food. Although finding natural substrata devoid of fungi is difficult, certain substrata are clearly more useful than others with respect to their numbers of species, their relative proportions of ubiquitous to substratum-specialized species, and their durability and survivability during handling and trans-

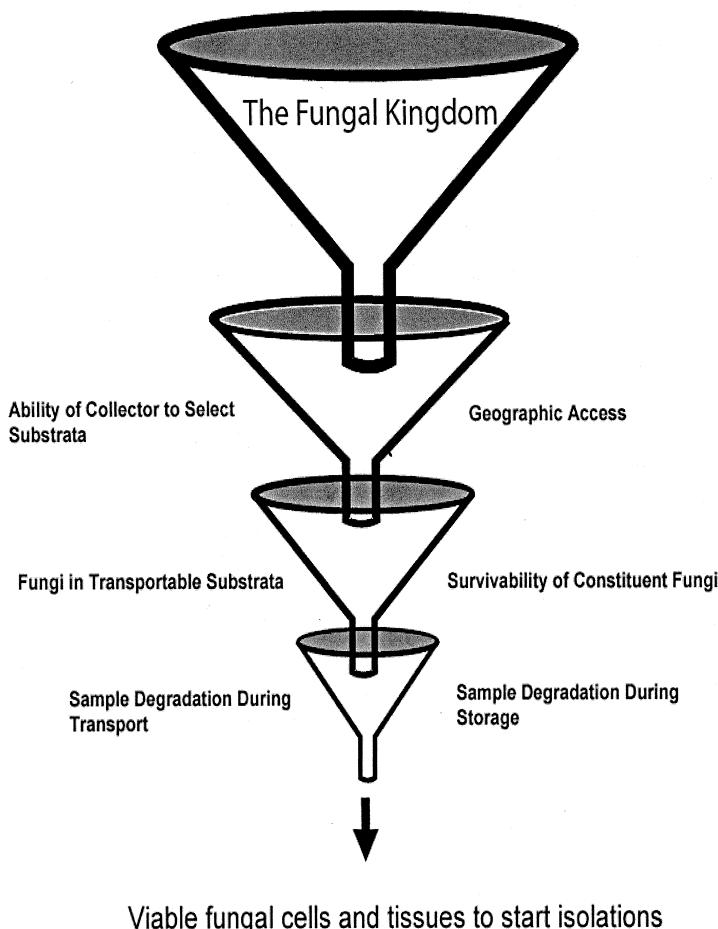


Figure 2 Effects of human intervention, transport, and storage that cause attrition and artifacts in the perception of fungal diversity.

port. Among the fungi of these materials, each harbors its characteristic core group of species; therefore, building a screening program based on soil isolates or endophytes ignores the vast majority of culturable fungi. After handling many isolates and becoming familiar with the mycota of a particular substratum, it becomes obvious that each type conveys its own set of omnipresent genera and species (e.g., *Phomopsis* species in woody plants, *Phoma* species in arid soils, or *Trichoderma* species in temperate forest litter). It cannot be emphasized enough that all fungi are not in one substratum, but some fungi appear to be in all substrata. In other words, certain cosmopolitan fungi, e.g., *Penicillium chrysogenum*, *Cladosporium cladosporioides*, *Aureobasidium pullulans*, and *Epicoccum nigrum*, seemingly can be recovered from any substratum anywhere.

Deciding what to culture and how much material is necessary to provide sufficient isolates needs planning. In cases where certain enzymatic properties are targeted, then the habitat relationships become especially crucial (see Chapter 10). Before going to the field, consider the following questions: (1) What kind of materials are to be collected for which targeted fungi? (2) Where do you need to go for those materials and do you have or need permission to take them? (3) How will the materials be packed and transferred to the laboratory? When planning a fungal inventory, the number of substratum samples and the number of isolates taken for examination need to be considered carefully. Even small numbers of soil, plant, or litter samples, examined intensively, can yield hundreds of taxa. If suspension plating or particle filtration is used to isolate fungi in small composite samples of temperate or tropical soils, asymptotes for species-isolate curves range from about 60 to 150 species for 600 to 1000 isolates [21]. Particle filtration applied to leaf litter may yield 80 to 400 species for 600 to 1000 isolates [22]. Arnold [23] estimated that approximately 350 genetically distinct species were recoverable from 100 leaves of a single tropical forest tree.

As a practical guide, the maximum number of isolates that can be adequately managed by two full-time experienced researchers using conventional microbiological techniques at a single time in a well-equipped laboratory is about 2000 to 3000. In contrast to mass-isolation techniques, isolations from field-collected fruiting bodies yield fewer taxa, but the organisms can be identified readily and often are obtained in no other way. A skilled mycologist may be able make up to two dozen isolates from single spore or tissue isolations in a day. Time constraints and costs of materials must be weighed against the priority of experimental questions or screening goals.

Fungi and fungi-containing materials should be processed soon after collection. Therefore, transportation is a major obstacle when the laboratory is distant from the collection sites. Abundant anecdotal evidence indicates that complex fungal communities rapidly deteriorate when separated from their habitat. Some fungi can quickly lose their forcible spore discharge. Obviously, fungal fruiting bodies with high water contents die, rot, and desiccate. However, some fungi, especially many perithecial, stromatic, or apothecial ascomycetes, and some Aphylophorales, can be carefully air-dried and packed for long-distance transport and later rehydrated. When materials arrive in the laboratory, they are kept at room temperature until processed or are stored at cool temperatures. Especially wet or humid material needs to be air-dried to prevent mold contamination in storage. Although some fungi may be lost during air-drying, we think reviving a dried sample is preferable to working with a wet and poorly handled sample that was stored in an anaerobic state, badly decomposed, infested with nematodes and mites, and inundated with yeast and bacterial films. Freezing usually is avoided, although some conidial fungi from temperate soils can survive freezer storage [24].

An alternative to basing the entire screening program only on fungi that survive long-distance transport is putting the laboratory near the habitat. The approach guarantees entry of sets of fungi that in no other way would ever be tested, especially fungi with fleshy or watery fruiting bodies that will not survive transport. Collaboration with a microbiology or phytopathology group near the site of interest is one way to gain access to these fungi. If no facility exists, investment in setting up a field laboratory in collaboration with local investigators or working with a university or government laboratory to upgrade its facilities may be worthwhile. The minimal requisites are a clean work area, preferably an air-conditioned room with a sink, a laminar flow hood or biosafety cabinet, microscopy equipment, an autoclave (or large pressure cooker), a refrigerator to store media and reagents, and a clean room or incubator for growing cultures. For example, during the Merck Research Laboratories' collaborative project with InBio (Instituto Nacional de Biodiversidad, Santo Domingo, Costa Rica), a semipermanent air-conditioned laboratory outfitted with incubator, refrigerators, and laminar flow hood was set up in an existing field laboratory at Santa Rosa National Park. Isolations and specimens were prepared at the remote laboratory, whereas media preparation and staging for shipment were carried out at InBio's main facilities in Santo Domingo.

Movement of fungi and their associated substrata, especially soils and plants, is highly regulated because a very small percentage of fungi potentially are plant pests or human pathogens. Furthermore, their substrata may contain harmful insects or invertebrates. It is imperative that, when working with materials from outside your country, that these materials be contained under the appropriate biosafety levels (P2 in the U.S. and E.U.). Consult with your local state, provincial, or national agricultural authorities (e.g., the U.S.D.A. Animal and Health Inspection Service in the U.S.) to learn what permits and certifications are needed to import and work with foreign biological materials. When importing fungi or other living materials from a foreign country, your collaborator likely will require phytosanitary export certificates and other commercial export licenses.

Mites and fungi coexist in nature, and mite infestation is a chronic problem in laboratories where large numbers of fungi are cultivated. Laboratory hygiene, physical separation of living cultures and natural materials, reduction of humidity, and a routine fumigation schedule can help prevent infestations. Guides on culture collection operation and maintenance recommend many mite control practices [25,26]. If all else fails, when mites have infested cultures and threaten to destroy them, media can be made lethal to mites by adding dieldrin at a concentration of 20 µg/mL. Dieldrin is extremely toxic to animals, but seems not to affect fungi. Avoid its inhalation and contact with skin. Glassware and used media need to be handled as toxic waste material. Add 1 mL of an acetone stock solution (200 mg dieldrin in 10 mL acetone) to a liter medium before or after autoclaving. A similar medium treatment can be made using lindane at a concentration of 75 µg/mL [25].

4.2. Useful Substrata for Fungi

4.2.1. Soils

Soils have been one of the most effective and popular materials for isolating large numbers of fungi. For natural products discovery, many of the most historically important and metabolite-rich genera (e.g., *Penicillium*, *Aspergillus*, *Fusarium*, and *Trichoderma*) are abundant in soils. There seems to be a misguided notion among natural products scientists that soil fungi have been exhausted as source of secondary metabolites. In fact, some

companies actually promote their collections as being superior because they avoided soil fungi. It is probably true that many species from temperate soils have been comprehensively surveyed, and that many soil fungi produce troublesome mycotoxins that interfere with assays. Nonetheless, informed selection of isolates, especially from the tropical and remote regions, or after application selective or baiting techniques, yields a wealth of taxa obtained from no other habitat.

Representatives of many orders of fungi can be found in soil. The number of soil-inhabiting fungi is unknown, and even within the known universe of soil fungi, the numbers of taxa are formidable. Domsch et al. [28] listed 153 common genera in their compendium, and Barron [29] included 202 genera of soil-dwelling mitosporic fungi. Even a set of exhaustive isolation of soil fungi on a small scale of a few hundred isolates may yield dozens of genera and upward of 50 to 100 species. Species in taxonomically complex, species-rich genera frequently are isolated, and specialized taxonomic references combined with ribosomal DNA sequence data is essential to cope with their identification. An understanding of the ecology of soil fungi and how soil fungal communities respond to geography, vegetation, and human intervention are therefore useful in devising collecting and isolation strategies [21,30–33]. However, some major groups of soil fungi, including some contributing the greatest biomass (e.g., the Glomales and many ectomycorrhizal basidiomycetes) have consistently defied attempts to isolate them into pure culture.

Almost any soil or sediment is potentially useful. The litter and humus layers that overlie mineral soil harbor distinct myotas, and they should be examined separately (see section 4.2.2). In temperate forests, the fungal communities also exhibit succession downward through the litter and to a lesser extent through soil horizons [34–37]. Diversity is usually highest in the uppermost horizons; therefore, collecting near the surface is more effective. In arid or semiarid regions, digging deeper may be necessary to reveal horizons with moisture or accumulated organic matter. Soil usually is collected with some kind of digging tool and packed in plastic, cloth, or heavy paper bags.

4.2.2. Plant Litter

Litter is an accumulated composite of plant parts in various stages of decay plus the organisms associated with those decay processes. The spatial-temporal heterogeneity and complexity of litter is the main cause of its extreme richness in fungal inhabitants. In most forests, the main fungi that colonize litter are saprobic basidiomycetes, a spectacular diversity of ascomycetous and anamorphic fungi that seem specifically adapted to decompose litter, plus their mycoparasites, and invertebrate associated fungi. Some *Aspergillus* and *Penicillium* spp. probably are specifically adapted to colonize seeds and other litter components, whereas their conidia reside in the soil's spore bank. Fungi that start their lifecycles in living plant leaves and stems can remain viable in the litter, and sporulate there to complete their lifecycles.

In temperate forests, the litter is often highly stratified. For example, conifer litter exhibits a classical vertical succession, and the species of fungi recovered varies with the depth of the litter layer. For example, *Cladosporium* sp. and *Aureobasidium pulullans* are dominant fungi in L-layer, *Thysanophora* and *Chaetopsina* spp. are in F1-layer, *Trichoderma* and *Mortierella* species are in the F2-layers, and soil fungi are isolated dominantly from the H-layer [34,38–41]. In humid tropical forests, litter decomposes very rapidly, usually persisting only a few months. Stratification is minimal and the litter–soil interface can be very abrupt.

Litter can be collected as a composite vertical section or segregated into its strata. Individual leaves, small-diameter woody debris, seeds, and other discrete components may be separated for direct examination or for preparation of moist chambers. Litter samples should be collected into paper bags or envelopes, and if wet and cannot be processed immediately, they should be air-dried. Extended storage of wet litter, especially in plastic, encourages contamination with bacteria, nematodes, abundant conidial fungi, or sometimes the sample can be consumed by a single basidiomycete mycelium. Air-dried samples that cannot be treated immediately should be kept in a dark, dry place. If possible, identify the plants and other components and make note of the degree of decomposition, because such data may aid in identification of the resultant fungi.

4.2.3. Dead Wood

Dead wood supports complex fungal communities, and the structure and the community composition varies with the stage of succession in the decay process, the physical location of the wood (standing, fallen, in service), and species of the tree or shrub. The involvement and interactions of fungi in mediating the wood-decay processes have been extensively reviewed [42,43]. Fungi associated with wood are generally assessed by visually inspecting the surfaces and collecting fruiting structures. For example, in one of the few studies ever to estimate ascomycete diversity on dead wood, pyrenomycetes were collected over a 1-year period on dead wood and bark of 31 woody plants along a 500-m transect in Puerto Rico [44]. A total of 157 species in 87 genera and 42 families (30% in the families Lasiosphaeriaceae, Xylariaceae, Hypocreaceae, and Tubeufiaceae) were found. In a lowland rainforest in Panama, 43 species of wood-decay basidiomycetes were observed along five 500-m transects [45]. Although wood-colonizing basidiomyceteous fungi usually are obtained by isolations from their fruiting bodies, they also may be rapidly extracted by indirectly plating colonized wood fragments onto an isolation medium (see section 4.4.1).

4.2.4. Living Plants

Fungi living internally in live plants, often loosely termed as endophytes [46], have become a mainstay of natural products screening programs. An extensive literature has accumulated on all aspects of the biology of the endophytes, including methods on how to optimize their isolations from nature [47,48]. The fungi of living plants are an extremely diverse and heterogeneous assemblage of ascomycetes and, to a lesser extent, basidiomycetes. Evidence from their study in tropical forests ranks them among the most species-rich assemblages of terrestrial organisms [23]. Many of the endophytic fungi belong to orders with a rich history of secondary metabolite production and, as now is being revealed, they are rich in biosynthetic gene clusters [49]. Some of these fungi are phytopathogens and their secondary metabolites have been selected as plant virulence factors.

A particularly elusive group of plant-associated fungi are the terrestrial aquatic fungi [50,51]. The terrestrial aquatic fungi grow slowly and their sporulation is very transient compared to other phylloplane fungi, plant pathogenic fungi, and saprophytic fungi; consequently, their isolation from living leaves and bark by usual isolation methods is very difficult. Sporulation of terrestrial aquatic fungi responds to water stimulus such as rain, fog, and mist. Short conidiophores develop rapidly and release conidia adapted for dispersion in water films. Therefore, the fungi are collected by sampling rainwater and water deposited from fog and mist on living leaves and barks and those draining from living leaves and barks [50,51]. Their conidia are concentrated by centrifugation and either separated manually or with a micromanipulator or plated directly.

4.2.5. Dung

Coprophilous fungi are those fungi that are adapted to live and reproduce on animal dung [52,53]. Specific groups among the zygomycetes, ascomycetes, and basidiomycetes have adapted to colonize dung. Coprophilous ascomycetous fungi have proven to be a surprisingly productive source of new bioactive metabolites, especially antifungal metabolites [54]. The dung most commonly used is that of herbivorous mammals, including domesticated and wild species. The fungi are usually obtained by incubating field-collected dung in humid chambers and observing the development of fruiting structures over time. Less elegant and precise methods of quickly extracting large numbers of strains from dung are based on solvent pasteurization [10] or particle filtration techniques. In addition to coprophilous fungi, these methods will recover fungi associated with the partially digested plant material and sometimes soil fungi that have colonized the dung after its deposition on the soil surface.

4.2.6. Invertebrates

Invertebrate-associated fungi are considered to be some of the most diverse and biologically attractive of all fungi. For example, about 3000 species of fungi in five orders spanning the Zygomycetes, Ascomycetes, and Basidiomycetes infest insects [55]. By and large, insect-associated fungi have been underused because they are often rare, occur in remote regions, are difficult to transport alive, are resistant to axenic culture, and are time consuming to collect in the field. Therefore, insect-associated fungi have not figured largely in the results of industrial screening, except for one important exception, certain species of the Clavicipitaceae with high saprobic capacity. Some species of the Clavicipitaceae, e.g., *Paecilomyces lilacinus*, *P. carneus*, *Metarrhizium anisopliae*, *Beauveria bassiana*, *Tolypocladium inflatum*, and *Chaunopycnis alba*, produce abundant conidia that are readily cultured from soil and plant litter, and therefore have received widespread attention from natural products researchers [2]. The most historically important soil-borne entomopathogenic fungus is *Tolypocladium inflatum*, the conidial stage of the coleopteran parasite *Cordyceps subsessilis* [56] that is used for commercial production of cyclosporine A [57].

Other important groups of invertebrate pathogens are the fungi that trap and infect nematodes, rotifers, and other microscopic invertebrates [58,59]. Only a few nematode-associated fungi have been thoroughly examined for secondary metabolites [60]. Unlike most insect-associated fungi, parasites of microinvertebrates generally are more accessible because they can be baited from soils and organic debris.

4.2.7. Freshwater Habitats

Several different assemblages of fungi and fungal-like protozoans complete part or all their lifecycles in freshwater habitats [61–63]. More than 300 genera of ascomycetes, 300 anamorph genera, and some basidiomycetes are known among these fungi. Conventional plating of water and sediments are inadequate to observe these fungi. They most commonly are observed by examining submerged plant debris or wood baits after incubation in humid chambers. Furthermore, the spores of aquatic hyphomycetes [64,65] can be collected from natural traps, such as river foam, or sporulation of aquatic hyphomycetes can be induced in the laboratory by aerating aquatic debris in water at cool temperatures. Relatively few of these fungi have been examined for secondary metabolites [66,67]. However, based on the fact that many belong to or are closely related to orders of ascomycetes with secondary metabolite biosynthetic pathways, they would appear to be a valid source for secondary metabolites.

4.2.8. Marine Habitats

Marine fungi are fungi that have evolved to complete their lifecycles in the oceans and their estuaries [68,69]. Although fungi isolated from marine habitats have been screened intensively in industrial programs, the actual contribution made by true marine fungi is unclear. Reports of metabolites from true marine fungi are relatively rare [70,71]. In recent years, many articles have appeared in natural products journals describing metabolites from “marine-derived” fungi that often belong to typical terrestrial genera or are not identified at all [72–74]. Such reports rarely have critically evaluated the nature of the organism and its relationship to its substratum; the suggestion that such fungi are somehow marine organisms may be misleading [69]. Various investigators have isolated filamentous fungi from soils, sands, and sediments of estuaries, mangroves, and marine and fresh water habitats [68,75,76]. Almost all taxa isolated from these habitats, with the exception of a few facultative marine mitosporic fungi (e.g., *Asteromyces cruciatus*, *Aspergillus insulicola*, *Dendryphiella arenaria*, and *D. salina*), some halophilic black yeasts, and some oligotrophic yeasts from open oceans appear to be terrestrial soil fungi [69]. In marine sediments, terrestrial fungal propagules are thought to accumulate because of the mycostatic effect of seawater and to form an inactive spore bank [68]. Many of these reports of new metabolites are as likely to reflect the novelty that the fungus was fermented in a seawater-based medium as they are a reflection of a metabolic adaptation to a marine habitat. Because marine fungi, especially the filamentous ascomycetes, belong to classes and orders that either are rich in secondary metabolite encoding genes or are sister groups, the rarity of reports may be due to the relative difficulty in collecting and culturing them and their often slow growth in agar and liquid culture, rather than lack of ability to produce secondary metabolites.

True marine fungi almost exclusively are associated with dead wood, seaweeds, seashore and estuary plants, animal exoskeletons, and sea sand [68,77]. The most species-rich materials seem to be the senescent and dead remains of mangrove trees and shrubs and intertidal monocots (e.g., *Spartina* and *Juncus* spp.). Seashore foam concentrates the appendaged conidia and ascospores of marine fungi, and therefore can provide material for starting cultures from single spores. Methods for collecting and culturing this challenging assemblage of fungi were reviewed by Kohlmeyer and Kohlmeyer [68] and Vrijmoed [77].

4.3. Isolation Strategies

4.3.1. Direct Isolations

Spores, mycelia, and tissues from fruiting bodies or substrata can be used to initiate cultures. Such methods are simple and usually do not require complex equipment or protocols. However, to make the most of fungal materials, the importance of knowledge of fungal biology, experience, manual dexterity, and adequate tools are worth considering. The main advantages of these methods for a screening program are that the organisms often can be identified with certainty, there is little doubt the organisms being cultured are distinct from each other and, for the most part, to provide access to fungi that are not cultivable by other means.

Tools for Dissection and Micromanipulation

Effective removal, separation, and manipulations of single spores and dissection of hyphae, microscopic colonies, and tissues require practice and adequate tools. A sewing needle or

a hypodermic needle mounted into a wooden or plastic handle often is sufficient and inexpensive (Fig. 3B). Larger needles may obliterate fine structures and are clumsy under higher magnifications. Very fine steel needles (e.g., minuten pins) need to be ethanol-sterilized and the ethanol removed by immersion in clean agar because they deteriorate when repeatedly sterilized by flame. Deteriorated needles can be sharpened with a very fine whetstone (Fig. 3D). A new needle also can be further sharpened or the tip can be modified into knifelike blade or a flat spatula. Insect pins are very good for microscopic manipulations and come in a variety of sizes (Figs. 3C, E). An especially useful pin for fine work is the minuten pin, normally used for mounting specimens of microscopic insects. The fine pins are glued into a glass or Teflon capillary, which in turn is fixed into a handle or pin vise.

Tungsten needles [41,78] can be made by cutting 0.2- to 0.5-mm diameter tungsten wire into pieces in about 5 to 10 cm long. The cut ends are sharpened to a point by electrolysis in a solution of 50 g NaOH and 150 g NaNO₃ dissolved in 900 mL distilled water. Two pieces of tungsten wire are connected to a variable transformer (e.g., a Slidacs variable transformer or a 6-V microscope transformer). The tips of the two pieces are dipped in the solution and electrolyzed in an alternating current at 5 to 10 V for several seconds. The pieces should be moved vertically into and out of the solution so that a finely sharpened needle is obtained. Too-high voltage and too-long electrolysis will shorten

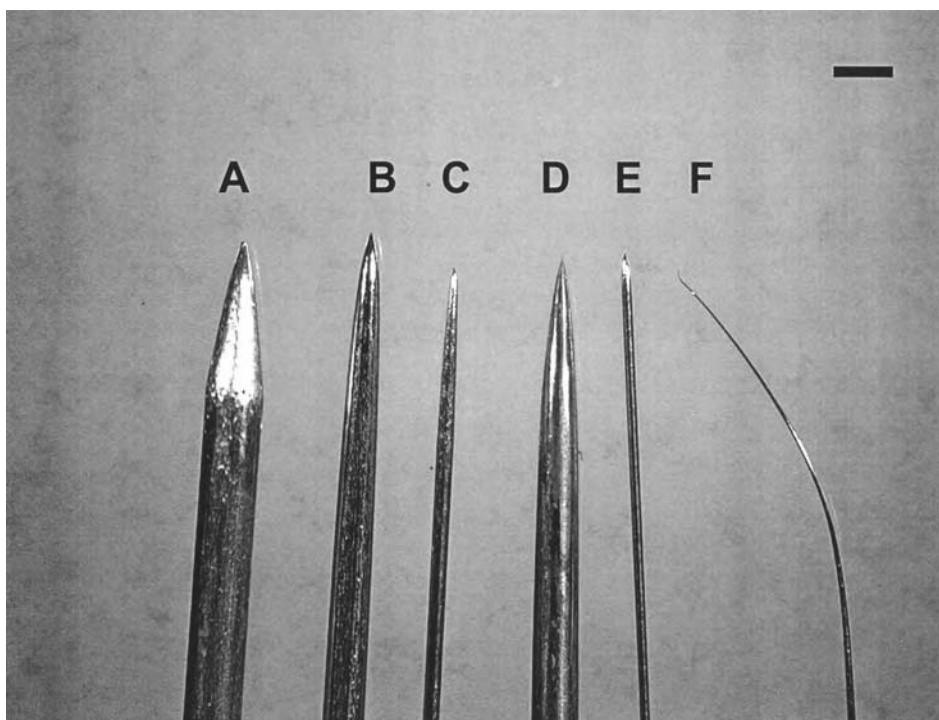


Figure 3 Assorted dissecting needles. A, Poor quality needle from a student's dissecting kit. B, Typical sewing needle. C, Insect pin (#2). D, Sharpened sewing needle. E, Extra fine insect pin (#000). F, Very fine Elgiloy needle. Scale bar = uals; 1 mm.

or curve the wire. After electrolysis, the alkaline solution is washed away in water. Two needles are obtained because alternating current electrolyses both ends simultaneously. Tungsten needles are hardened when sterilized in the flame of an alcohol lamp.

Elgiloy orthodontic wire, a cobalt-chromium-nickel-molybdenum-iron alloy, is also excellent for formation of microneedles because of its tensile strength and resiliency and ease of bending (G. Okada, unpublished). Wire 0.2 to 0.3 mm in diameter is cut into pieces about 5 to 10 cm long and electrolyzed in 50% H₂SO₄ as described above. Elgiloy wire is more easily straightened into needles than tungsten wire, which often bends or curls when electrolyzed. Elgiloy needles (Fig. 3F) should not be flame-sterilized, but rather simply dipped in ethanol and washed in the agar medium. Fine disposable scalpels are indispensable for dissecting fruiting bodies and substrata. If scalpels are unavailable, a scalpel or microcutter can be improvised by cutting a razor blade into small pieces and shaping them with a hammer and a file [79]. Additional techniques and tools for single-spore isolations have been described elsewhere [80,81].

Skerman's Micromanipulator

Micromanipulators are convenient tools for isolating single spores or separating individual cells and hyphae from natural substrata under a microscope. Although various kinds of micromanipulators are available on the market, the Skerman's micromanipulator may be one of the least known yet most useful and attractive devices for mycologists in terms of its reasonable price and simple handling [82,83]. Skerman's micromanipulator was first developed for separation of bacterial cells and later applied to fungal isolation. Its use has become very popular among Japanese mycologists, and a kit that includes a 10× objective lens designed to fit the manipulator is sold in Japan (Toyo Rikoki Co., Ltd, for about \$1000 U.S.). We have found this device indispensable for isolation of many kinds of fungi and for decontamination of fungal cultures. We believe that it is worthwhile to describe in detail its basic operation, especially because the manufacturer's operating instructions are in Japanese.

Set up of the Skerman's micromanipulator kit, the operation of the microforge to make fine glass needles, and the manipulation of spores are done with the same microscope. If the micromanipulator is used extensively, we recommend purchase of a dedicated microscope to avoid repeated assembly and disassembly. A compound microscope with a movable, vibration-free stage is needed. The operation of the microforge requires use of the transformer (Fig. 4A), the L-ring (Fig. 4B), the magnet (Fig. 4A, B), the microforge (Fig. 4B), and glass capillary tubes (about 0.8 mm in diameter). Cells are manipulated with a microtool mounted on the magnet attached to the V-ring (Fig. 4A, C).

Preparing Microtools with the Microforge:

1. A glass capillary tube (about 4 cm long) is inserted through the tube fixed on the base of the magnet and is fixed with melted paraffin between the capillary and the tube. Alternatively, a fine insect pin can be inserted through the tube, and the capillary fixed to the point of the pin with paraffin. The angle of the capillary can be adjusted by rotating the pin.
2. The L-ring is fastened to the objective lens(10×) of the microscope. The kit's 10× objective lens is made to fit the L- and V-rings perfectly. Other objectives may not fit as well, if at all.
3. The magnet with the capillary is set on the grooved steel slide of the L-ring.

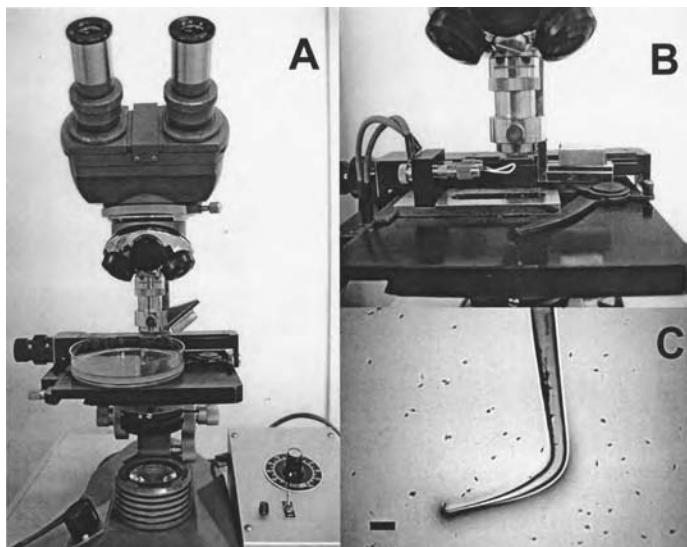


Figure 4 Skerman's manipulator kit and its set up. A, V-ring, sliding magnet, and microtool mounted on microscope. Note rheostat for microforge in the lower right. B, Microforge, sliding magnet, and L-ring set up on the microscope stage. C, Hooked microtool of a Skerman's manipulator with fungal conidia. Scale bar = uals; 100 μ m.

4. The magnet is moved forward in the groove slide until the glass capillary is focused in the center of the visual field.
5. The microforge connected to the transformer is placed in the slide caliper of the microscope stage (Fig. 4B).
6. The tungsten heating filament is bent into a tight, U-shaped tip and fastened into the vise clamp of the microforge. The tip of the heating filament of the microforge is moved to the center of the field of view with the microscope stage. The filament and glass capillary are then aligned along the same axis and moved together until they nearly touch each other (Fig. 4B). The filament is then raised or lowered into focus with the fine adjustment of the microscope stage (the capillary is already in focus). The tip of the heating filament should be near but not touching the capillary, because the wire filament expands when heated.
7. The transformer is turned on, and the power increased so that the heating filament redds and expands toward the glass. The filament is pushed into the glass tip with the mechanical stage forming a bead of melted glass. The filament is then retracted in a quick and smooth action that stretches the bead into fine tapered point.
8. The magnet is pulled back along the groove, retracting the tip of the tapered glass capillary until it is once again in the centered in the field of view.
9. The transformer's rheostat is lowered and the heated filament is moved carefully until it just meets and melts the tapered tip.
10. The mechanical stage and filament temperature are manipulated to forge the tip of tapered capillary into the desired shape (e.g., a microknob, microhook, or microloop). An L-shaped microhook is especially useful for dragging, pushing, or lifting fungal spores across the agar (Fig. 4C). Once the tip is forged into the desired tool, the magnet is slowly pulled out of the L-ring and set aside, where the microtool will not be broken.

Micromanipulating Single Spores:

1. The V-ring is secured to an objective lens (10 \times) of a microscope.
2. The magnet with a microtool is slowly inserted into the grooved steel channel of the V-ring.
3. The magnet is lowered by hand while observing through the microscope until the microtool is visible and centered in field of view.
4. Mark one or more circles on the bottom of Petri dish of agar isolation medium to facilitate location of the spores on the dish. Either 60- or 100- plates can be used, but 100-mm plates provide more area for movement. A small amount of spore suspension is pipetted on the agar surface above one or more of the marked circles, about 15 mm from the edge of the isolation plate (Fig. 4A). Alternatively, spores dissected from ascocarps, pycnidia, or conidiophores are placed above the marked circles.
5. The microscope stage is lowered and the Petri dish is set on the stage.
6. Using the marked circles as guides, spores targeted for separation are located and centered in the field of view.
7. At this point, the tip of the microtool also should be in focus and centered in the field of view.
8. The stage is slowly moved, either dragging or pushing the spores to a new clean surface (Fig. 4C). Sticky, viscous, or filamentous spores or cells may be gently lifted and moved to a new location.
9. Dragging and pushing spores scrubs bacteria and smaller spores from the target spores and therefore eliminates contamination from the spore surface.
10. The microtool can be pushed into the agar by raising the stage to mark the new location of the cleaned and separated spores.
11. The stage is lowered again and the Petri dish is removed from the stage.
12. The separated spores can be removed immediately with a needle, or the separated spores can be moved to fresh agar when they start to germinate (8 to 48 hours, depending on the species).

After the manipulator's initial setup, the procedure is very simple and, with practice, it is a very rapid and accurate. The level of confidence it provides in accurate establishment of strains and the ability to rescue contaminated cultures more than compensates for the initial expense and effort to learn its use.

Moist Chambers

Moist chambers are a mycological enrichment technique used to observe elusive fungi that may fruit sporadically or only under very specific conditions. Use of moist chambers increases the likelihood of observing nonfruiting, small, or inconspicuous forms. Moist chamber incubation also rehydrates desiccated specimens, permits maturation of immature specimens, or can break the dormancy of resting spores and sclerotia. A substratum is maintained moist in a chamber where ambient conditions can be manipulated so that fungal development and sporulation can be observed directly. The fungi, once developed, are removed for culturing and identification.

The substrata are placed inside a container lined with a clean absorbent material, such as filter paper or Perlite. The container should be transparent or translucent so that the contents are visible and illuminated from outside. Examples of useful moist chambers include 200- to 300-mL volume polystyrene cups with polyethylene snap lids, Petri dishes,

or lidded plastic food containers, such as Tupperware. Humidity adjustment requires some experience; too much moisture encourages growth of fast-growing mucoraceous fungi and nematodes and inundation with actinomycetes. If it is too dry, however, fragile fruiting bodies can dry out or never develop. Petri dishes give good aeration conditions, but materials are easily dried up in a few days, and its low profile may interfere with development of tall fungi from large substrata, such as agarics from dung. When Petri dishes are used, water should be added every few days. A lidded, airtight plastic container may better retain moisture while restricting movement of invertebrates associated with the substrata. Aeration usually is not a problem, because containers are opened frequently to examine the substrata. The containers are incubated at room temperature, although slightly cooler temperatures may help prevent overgrowth by aggressive fungi. Substrata should be examined every few days for newly emerged fungi for at least up to 1 month. Spores, sclerotia, hyphae, or other fruiting or vegetative structures are dissected from the substratum and planted onto fresh isolation media or further separated with a micromanipulator.

Baiting

Baiting is a nutritional enrichment technique whereby an organic substratum or living organism is placed in contact or mixed with the habitat sample (e.g., soil or river water) believed to contain the targeted fungi in the hope that they will preferentially colonize and reproduce on the substratum or host organism. The bait-substratum mixture is designed to mimic the natural differential colonization or infection process, such as, the decay and incorporation of a dead sheep's bones, hide, and wool into the soil or invasion of plant seedlings by damping-off fungi. As with selective media, the need to assay for difficult-to-isolate destructive plant pathogens has motivated the development of many baiting techniques [32,84,85]. Best known among baits for recovery of specialized saprobic or parasitic fungi from soils are cellophane and other cellulosic materials for isolation of cellulolytic fungi [86,87]; hair or feathers for keratinolytic fungi [87–89]; and insects [90], nematodes, and rotifers for their respective parasitic fungi [58,59,91]. Sterilized wood blocks and mesh bags containing sterilized leaves have been the preferred baits for trapping fungi from marine [68,77] and freshwater habitats [60].

Forcible Spore Discharge

Directly discharging spores onto an agar medium is an essential technique for culturing basidiomycetes or ascomycetes with large fruiting bodies [92,93], although spore discharge can be adapted to trap almost any forcibly ejected spore from microscopic fungi. Not all spores of all fungi germinate on agar media, and to best capitalize on the technique, it is necessary to know which types of fungi are unlikely to germinate on agar (e.g., basidiomycetes that form ectomycorrhizal symbioses or species of the Geoglossaceae). The best known form of the technique is to fix the hymenium (spore-bearing surface) of a basidiomycete or apothecial ascomycete onto the inside of a Petri dish lid with petroleum jelly. In a few minutes to several hours, basidiospores or ascospores are ejected and land on the agar. Apothecial or stromatal ascomycetes can be placed on the inside of the lid with agar surface inverted above to trap ascospores that are forcibly discharged upward. If no spores are observed on the agar, possibly the fungus is immature or spore discharge was arrested by transport of refrigeration. Once spores have been deposited on the agar, the fruiting bodies fastened to the cover should be removed to avoid contamination. Spores are observed periodically until they germinate; depending on the fungus, germ tubes emerge in a few hours to several days. As soon as possible after germination, at least several individual spores or small groups of spores should be transferred to new plates or tubes of media.

Leaves and stems infested with stromatic pyrenomycetes or loculoascomycetes can be used similarly to trap discharged spores [94]. Dead leaves or stems with black perithecia, stromata, or psuedothecia are soaked in water, cut to an appropriate size, and fixed on the lid of the dishes. If ascospores are discharged on to the agar, the relative positions of spores are used to locate the ascomata of origin so that the fungus can be identified. A variation on this theme is to use Petri dish lids with precut holes for spore discharge [79]. Square or rectangular holes of varying sizes (5×5 mm up to 10×30 mm) are cut into the lids of 100-mm plastic Petri dishes with a hot knife. A pre-cut lid is disinfected with 70% ethanol and placed over the isolation medium. The fertile part of fruiting body or substratum with a fruiting body is positioned over the hole so that the spores shoot onto surface. The lid is rotated gradually, dispersing and separating the spores on the medium. After spores are collected, the pre-cut lid is replaced with a new one. This variation avoids damage to scant, fragile, or small fruiting bodies (e.g., *Cordyceps*, *Epichloë*, and *Balansia* species), or when the fruiting body is very large and does not fit well inside the Petri dish.

Sometimes media and a convenient place to set up an isolation are unavailable. Discharged spores of larger fungi can be collected in a clean, empty Petri dish or on a clean paper surface (spore prints) for transport or mailing back to the lab. Success with preserved spore deposits may be erratic, because germination characteristics vary among fungi and viability can be negatively influenced by handling. Spore prints should be kept cool until they can be cultured by dilution or streaking onto fresh isolation medium.

Tissue, Rhizomorph, and Hypal Isolations

Basidiospores or ascospores often do not discharge or germinate on agar, and tissue isolations may be useful for such species [93]. The tissue should be fresh and free from other organisms. The thickest part of the fungus, e.g., the pileus, glebum, or stipe, is broken open to expose clean inner tissue. A small piece of the clean inner tissue is excised with a sterile scalpel or forceps and transferred to an agar medium. Freshly emerged hyphal strands from decaying wood, stems, leaves, or wood can be dissected and propagated onto agar as well. Once again, tissue transfer tends to be more successful with saprobic or decomposer fungi than with obligately symbiotic or parasitic fungi.

Bandoni's Method

Specialized aquatic or terrestrial aquatic fungi inhabit moist surfaces of leaf litter and woody debris, but observing them sometimes is difficult. Professor Robert Bandoni developed a method to stimulate sporulation and release of those spores of these fungi by incubation of the litter in water [50,95–97]. A handful of litter is soaked in 500 mL of sterile water in a beaker and gently mixed. After several minutes, conidia attached to the leaf surfaces float to the water's surface. The conidia accumulate on the meniscus layer of the water and are trapped with a fine screen or glass cover. Single conidia are separated with Skerman's manipulator. After the surface conidia are dislodged, the water is replaced. The beaker is aerated for 1 week with a submerged aquarium pump; the airflow stimulates conidia production and release of conidia from the leaf and debris surfaces. The foam forming on the surface is collected and pipetted onto a Petri dish of agar medium, and conidia are separated with Skerman's manipulator. Another variation is to place decayed vegetation or aquatic debris in a shallow container or Petri dish and partially cover it with sterile water. The containers are placed onto a rocking platform and incubated at cool temperatures (15° to 18°C) for several days to a week. Conidia released from plant debris and adapted for water dispersal will float to the surface, where they can be collected for separation or pipetted and spread onto an agar medium.

4.4. Indirect Isolation Methods

4.4.1. Isolation of Basidiomycetes from Decaying Wood

Wood-decay basidiomycetes, primarily Aphylophorales and Agaricales, can be selectively separated from wood by taking advantage of their relative resistance to the fungicide benomyl and their vigorous ability to decay sound wood of standing or recently fallen trees. Fresh, actively decaying wood is largely free of other fungi except for the fungus that is colonizing the wood. Small fragments of decayed wood can be dissected and placed on an agar medium to yield pure cultures of basidiomycetes.

Select recently dead standing stems, hanging branches, or recently fallen wood. Stems should be small enough to be cut and split with hand tools (3 to 15 cm diameter). Cut stems into manageable lengths (5 to 10 cm) that can be split easily with a hatchet or large knife. At each site, choose a variety of dead stems and branches from different tree species, ensuring that a wide variety of basidiomycetes will be encountered. Inspect the wood for signs of decay; usually the wood is discolored and loses strength and hardness, and the grain will be less apparent. Often, black or dark brown zone lines will be apparent where different fungal individuals meet inside the wood. Occasionally, white or light-colored mycelium of the basidiomycete will be visible on the wood surface underneath the bark or in cracks and fissures. Avoid highly porous, heavily fragmented wood, especially where insect activity is apparent. Tunneling insects introduce contaminating fungi and render the wood useless for this technique.

On return to the laboratory, have isolation media prepared and ready in the laminar flow hood. Split stem sections longitudinally with a quick blow with a hatchet or by prying the stem apart with a heavy knife. Splitting stems into quarters or eighths will yield many triangular edges from which it is easy to cut and remove wood fragments. Do not touch the inner surfaces of the wood with your fingers. Using a flamed, steel-handled scalpel or a sharp knife, cut a series of small fragments (less than 0.5 cm) along the triangular edge of the split stems. Remove about 8 to 15 fragments along the inner edges of each stem section. Insert 5 to 10 fragments on a plate of malt extract-benomyl media [98,99]. Label plates with collection data or collection code and incubate them at 15° to 25°C for 2 to 6 weeks.

Basidiomycetes will emerge slowly from fragments, appearing as light-colored mycelium. Basidiomycete mycelia tend to be colorless, white, cream-colored, yellow, pale orange, or light brown. Occasionally, some will produce chlamydospores, arthrosporic or, more rarely by holoblastic, conidia. Differentiated conidiophores are rare. Basidiomata or their primordial may form occasionally in older cultures. Many species will form clamp connections at the septa, verifying that it is a basidiomycete. The most troublesome contaminating fungi in wood are likely to be species of *Trichoderma*, *Cladosporium*, and *Penicillium*. The benomyl is omitted if Xylariales, Diatrypales, Diaporthales, or other ascomycetes characteristic of dead sapwood are sought. In this case, select only the most recently dead, insect-free stems and make isolations from the sapwood. Zone lines near the surface are a good indication of colonization by fungi of the Xylariales or other sapwood-colonizing ascomycetes.

Identifications of basidiomycetes from cultural features are difficult, if not impossible, but basidioma, if present on the stems, may provide clues. Several standard methods and protocols are used to describe and classify basidiomycetes based on their morphology in culture [93,100,101]. However, close familiar and generic approximations, or even a

species match, may be possible from ITS ribosomal DNA or near large subunit ribosomal DNA sequences [102,103].

Serial Dilutions

Serial dilutions are the classic method for isolation of soil fungi and fungi from other materials [31]. Soil samples often are sieved through a 2- to 5-mm mesh to remove large debris and to homogenize the sample. Soils are sometimes dried at room temperature, as is the case for actinomycetes isolations, but soil samples can be used immediately without drying. Anecdotal evidence indicates the isolation results can differ depending on the soil pretreatment. A typical dilution scheme suspends 200 to 500 mg of soil in 5 mL sterile water or 0.85% saline. A detergent, e.g., Tween 80, may be added to aid dispersal of soil aggregates. We often increase the viscosity of the suspension by adding 0.005% to 0.2% agar or carboxymethyl cellulose so that soil particles remain suspended. The suspension is mixed or vortexed vigorously and between 50 and 200 μ L 10^2 to 10^4 diluted suspensions are pipetted onto isolation medium. The suspension is evenly spread with a sterile glass rod. An alternative method is to mix the suspension with a pre-cooled agar medium. One mL of 10^3 to 10^5 diluted suspensions is poured into an empty plastic dish and about 19 mL of pre-cooled agar medium (45° to 55°C) is mixed with the suspension.

Soil Swabbing and Stamping

Very small quantities of soil can be directly applied to isolation plates. Soil is pulverized or sifted into a sterile Petri plate lid or plastic weighing pan. With a sterile cotton swab, touch the soil surface and streak particles, following a bacteriological purification pattern, onto the surface of the agar. The method works well with soils where propagule density is low, as in desert soils. With care and a light touch, swabbing or stamping can be applied to any soil and can even be inoculated in the field.

Cotton balls, sponge stoppers, or felt replicate pads also can be used to disperse soil particles across agar surfaces [104]. Place finely divided soil from each sample in a Petri dish lid. Place a row of 6 to 10 plates with the desired isolation medium to one side. Stamp a cotton ball or a felt pad lightly onto the soil. Then, starting at the first plate, stamp the entire surface of the agar in each plate in succession in a clockwise pattern. Usually the first two or three plates are overwhelmed by colonies, but the later plates yield a manageable number of colonies. Incubate plates at 15° to 20°C and review daily, transferring emerging hyphal tips to agar slants.

Heat and Chemical Pasteurization

Partial killing of a fungal population with dry heat, steam, or solvents has been employed for preferential isolation of species that have resistant propagules. Such soil treatments shift the population of isolates toward ascomycetes and basidiomycetes that have ascospores, thick-walled chlamydospores, or sclerotia. Ethanol pasteurization is a very effective method for extending the number of kinds of fungi recoverable from soils while lowering the total number of isolates of yeast species, as well as many species with thin-walled conidia, thus increasing isolation of ascosporic Eurotiales, *Chaetomium* species, *Xylaria* species, numerous genera of coprophilous fungi, and possibly some ascosporic yeasts. Some investigators have combined ethanol treatment with mild heat treatment, which is thought to stimulate ascospore germination [105]. Other researchers have researched isolates from ethanol- or phenol-treated soils for taxonomically novel fungi [106–111]. Formalin pasteurization [112], a variation of ethanol pasteurization, has effectively increased the frequency of isolations of ascomycetes from soils. Chemical pasteurization is useful

for recovery of large numbers of fungi from herbivore dung and reduces interference from contaminating yeasts [10].

Warcup Soil Plates

This method differs from other soil isolations because the particle structure of the soil is relatively undisturbed; therefore, the profile of isolates differs from that of suspension plating [31,113]. The method is fast and easy for culturing soil fungi, although obtaining optimal colony densities can be tricky. Molten isolation medium is prepared and maintained at about 45°C in a hot water bath. Add 0.2 to 5.0 mg of finely pulverized soil to the bottom of a sterile Petri dish. The quantity of soil will vary widely with propagule density and soil texture. The soil can be dispersed by agitation in 0.5 mL of sterile water or with a few milligrams of sterile sand grains. Overlay the soil particles with 8 to 10 mL of medium while gently agitating the plates to disperse particles. Young, submerged colonies need to be cut from beneath agar, and a thin layer of agar facilitates dissection of colonies embedded in the agar.

Serial Washing

Repeated washing of small substratum fragments removes surface spores. The effect is similar to, but less damaging than, surface sterilization, and generally is used with porous, deteriorated, or very thin materials in which disinfectants will kill cells, such as fine roots, bryophyte leaves, plant litter fragments, small invertebrates, and invertebrate parts [114]. Samples are cut into small pieces and put into tubes or vials. The material is added to a large volume of sterile water with 0.005% aerosol OT solution (di-iso-octyl sodium sulfosuccinate), Tween 80, or other surfactant, and vortexed. The wash solution is decanted and replaced for up to 10 times, enough times to remove surface propagules [40,41]. After air drying on a sterile filter paper overnight to reduce bacterial contamination, the washed fragments are plated on an agar medium and incubated for up to 1 month.

Density Gradient Methods

Differences in the specific gravity and size permit separation of mixtures of organic debris and inorganic soil particles containing spores and mycelia [13,115]. The Percoll pregradient with 0.15M NaCl is applied prior to the addition of soil suspension. The Percoll solution contained 7.0 mL Percoll (Pharmacia), 1.0 mL 1.5 M NaCl, and 2.0 mL distilled water. The solution is prepared in a tube and centrifuged at 60,000 × g at 20°C for 15 minutes to form the pregradient. Sieved soil samples (1 g) are added to 10 mL Percoll. The suspension is sonicated for 2 minutes and left to settle for 2 minutes while heavy soil particles sink. The supernatant (5 mL) is mixed with 45 mL distilled water and centrifuged at 2150 × g at 20°C for 10 minutes. The precipitate is suspended in 0.5 mL distilled water, which is then gently applied on the top of the Percoll pregradient. The solution is centrifuged at 240 × g at 20°C for 3 minutes and fractionated from the bottom up. Each fraction is diluted and spread on an agar medium. Fractionation of the gradient more thoroughly extracts fungal propagules from soil than a typical serial dilution.

A similar method using a sucrose density gradient was used to separate, concentrate, and directly enumerate basidiospores and sclerotia from soil in *Pinus contorta* forests [116]. Differential centrifugation also can separate large-spored predatory nematophagous fungi and soil microfauna infected with small-spored endoparasites [58].

Particle Filtration

In laboratories in Canada, Germany, and Sweden, particle-filtration techniques, also known as “soil washing,” were developed as an alternative to suspension plating for characteriza-

tion of soil fungal communities [30,114,117–120]. Particle-filtration techniques favor isolation of fungi from mycelial fragments immersed in the substratum while reducing recovery of fungal colonies initiated from spores. Isolation of species with chlamydospores embedded in organic particles may be favored, because their propagules probably are not entirely removed by washing [121]. Particle filtration is readily adaptable to many kinds of organic substrata. Theoretically, anything that can be broken into small particles can be used, and the method has been applied successfully to isolate terrestrial and aquatic litter, wood-decay, fungicolous, lichenicolous, and coprophilous fungi.

Good results usually are achieved only after considerable practice and careful preparation of materials. Despite the need for more preparation, the method often reveals numbers and kinds of fungi rarely seen by those acquired by conventional techniques. Prevention of intraparticle competition is the key to its success and is achieved by reducing particle size so that a colony:particle ratio of less than 1 results [117,122]. Choice of particle size depends on substratum texture [117,122]. Bååth [117] recommended particles in the range of 50 to 80 μm in diameter for conifer soils; Bills and Polishook [11,123] used 105- to 210- μm particles to study fungi in forest litter.

Simple vials, filtered syringes, nested sieves, and complex self-flushing/refilling devices have been used as washing devices [30,120,122,124]. Effectiveness of these devices depends on the ability to obtain well-washed particles, clearly separated into descending size categories. Choice of device seems to be a matter of personal preference, cost, and availability of materials.

Thorn et al. [125] used the particle filtration technique to remove spores of anamorphic fungi and Zygomycetes from soil, and plated the washed particles on a medium made selective for basidiomycetes by the incorporation of benomyl. The medium also contained alkali lignin to encourage growth of ligninolytic fungi, and guaiacol, which acts as a colorimetric indicator of the lignin-modifying enzymes laccase and peroxidase. The agar adjacent to a few of the 5 to 25 colonies that developed on each plate turned dark brownish red as guaiacol was oxidized to a quinone. These colonies are scanned under low magnification ($40\times$ to $100\times$) and putative basidiomycetes selected for transfer, whereas conidia of undesired anamorphic fungi (e.g., *Alternaria* and *Trichoderma* species) are avoided. A total of 67 basidiomycete isolates were recovered from 64 soil samples from 35 sample plots.

Surface Disinfection with Acidic Electrolyzed Water

Immersion in hypochlorite and ethanol solutions is probably the most widely used disinfection method for removal of superficial fungi from plants and other materials (Fig. 5) [126,127]. Recently, acidic electrolyzed water, prepared by the electrolysis of an aqueous sodium chloride solution and resulting in a dilute mixture of hypochlorite, hypochlorous acid, and chlorine, has been used for surface disinfection because of its flexibility and less corrosive and damaging effects on disinfected surfaces. Acidic electrolyzed water often is used for nonthermal disinfection of hospital instruments, food processing, and water purification, and it has been shown to be effective in controlling foliar fungi in greenhouses [128]. Recently, the technique was substituted for the traditional ethanol-hypochlorite disinfection for isolations of endophytic fungi [129]. The acidic electrolyzed water containing 40 mg/L sodium chloride showed similar antimicrobial activity to that of 0.1% hypochlorite solution. However, the activity of electrolyzed water decreases rapidly with increased contact with organic material, presumably due to nonselective reducing

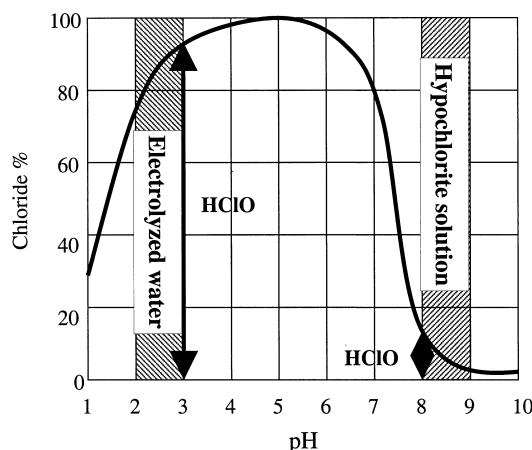


Figure 5 Effective chloride concentration as a function of pH in hypochlorite solution generated by acidic electrolysis.

agents. As a result, bacteria and fungal propagules on plant surfaces will be killed with a brief exposure, but endophytic fungi within the leaf are expected to survive.

A disinfection method was developed for fungal endophytes that used 2 L of 1% NaCl solution electrolyzed for about 20 minutes using electrolyzing apparatus (Amano Co., Yokohama, Japan; Fig. 6). Intact leaves were soaked in 1 L of the fresh electrolyzed solution at intervals of 10 minutes up to 1 hour. The treated leaves were washed thoroughly in sterile water with 0.5 g/L Tween 80, followed by air-drying on a sterile filter paper. They were then cut into small pieces (ca. 3 mm²) and plated onto a fungal isolation medium. In one experiment employing leaves of *Pieris japonica* soaked in electrolyzed solution for less than 10 minutes, *Pestalotiopsis versicolor*, *Cladosporium* spp., *Aureobasidium pullulans*, *A. pullulans* var. *melanogenum*, and *Tripospermum* sp., typical leaf-surface fungi, predominated. Longer treatments increased the ratios of *Phyllosticta* sp., *Phomopsis* sp., and *Colletotrichum gloeosporioides*, true endophytic fungi. Tests of the antifungal activity of the electrolyzed solution indicated that both conidia and mycelia of all these fungi were about equally susceptible to the water, and the differential isolation was not due to the differential susceptibility. Therefore, it is believed that isolations of fungi from plant tissues can be more precisely controlled by varying treatment times with acidic electrolyzed water than by immersion of tissues in with hypochlorite-ethanol solutions.

5. CULTURE MEDIA FOR ISOLATION, SPORULATION, AND MAINTENANCE

Appendix 1 provides formulae for some media that we have found useful in the laboratory for isolation of fungi into pure culture and for their cultivation, microscopical examination, and characterization. Additional recipes and procedures can be found among previous compilations of mycological methods [26,27,32,85,93,130,131].

Most culture media contain at least water, a simple or complex source of carbon (carbohydrates, lipids, peptides, amino acids, organic acids) and nitrogen (peptides, amino



Figure 6 Electrolyzing apparatus, Super Oxseed Labo (Amano Co., Yokohama, Japan).

acids, ammonium, nitrate), phosphate, sulfur, essential metal cations, and sometimes vitamins and essential amino acids. Nutrients can be supplied as pure reagents, when all media components need to be defined and reproducible or, as is often the case, as complex plant- or animal-derived additives (oatmeal, hay infusion, peptone, carrots). Fungal growth and development are extremely sensitive to the composition of the medium, to laboratory conditions, and to the physical environment (light, temperature, humidity). These factors are deliberately varied to influence the growth form, lifecycle, and metabolic products of fungi in culture. For example, dilute, low nutrient media with only polysaccharide carbon sources or plant-derived infusions often favor sporulation and suppress mycelial growth. Media with high concentrations of mono- or disaccharides tend to favor vegetative growth,

whereas extremely high carbohydrate or salt concentrations may inhibit growth by reducing water activity.

Addition of antibacterial antibiotics to fungal isolation media often is essential to prevent bacterial growth. Streptomycin sulfate, chlortetracycline, oxytetracycline, ampicillin, chloramphenicol, and vancomycin frequently are used. Most antibiotics are heat labile and are added to cooled media (45° to 50°C) just prior to pouring plates. An exception is chloramphenicol, which can be autoclaved and usually is used at a concentration of 100 mg/L medium. Most antibiotics are prepared in sterile water or filter-sterilized and stored as concentrated stock solutions. The concentrate is added to media to yield a final concentration of 30 to 100 mg/L of medium. Acidification of media to pH 4.5 with lactic acid can help to suppress bacterial growth. Rose bengal in isolation media may inhibit growth of many bacteria and reduce radial extension of fungal colonies. A good combination of antibiotics for isolation of most filamentous fungi is chlortetracycline (50.0 mg/L) and streptomycin sulfate (50.0 mg/L). These antibiotics specifically interfere with bacterial ribosome function, and we have never observed them to adversely affect establishment of basidiomycete or ascomycete cultures from direct plating of spores. Selectively toxicity toward certain fungi, particularly oomycetes and basidiomycetes, is possible; if selective toxicity is suspected, substitute other antibiotics.

5.1. Automation of Strain Isolation and Management

The evolution of HTS laboratories and their demands for increasingly larger chemical libraries and numbers of natural product samples for testing have challenged microbiologists to streamline and improve the manual processes of making microbial extracts. The precise challenge is to increase the numbers of samples, with as few as steps as possible, so that they are numerically competitive with synthetic chemical collections, while maintaining high levels of chemical complexity and uniqueness among the samples. Discussions of strategies for improving the productivity of microbial screening invariably lead to the suggestion that automated devices can supplement the traditional microbial processing techniques. The concept is based on building a set of interacting automated devices that carry out parallel handling of strains in a format compatible with current methods for automated liquid handling systems, rather than sequential handling of individual strains for flask or tube fermentations. Many of the necessary manipulations have been incorporated into automated devices developed by Olympus, Tomtec, Toyo-Sokki, Hitachi, Kühner AG, and others [132,133] for isolating homogenous colonies from clonal libraries of bacteria, yeasts, and certain actinomycetes. Tens of hundreds of colonies can be repetitively transferred from mother plates to daughter plates and arrayed as desired. The technology has been further elaborated for preparation and management of clone libraries through colony replication, cherry-picking, and macroarraying [134]. Such genomics tools are available from Genetix Ltd., GeneMachines, AutoGen Inc., and Bio-Rad Laboratories, among others. However, the pin-transfer tools employed in conventional colony pickers designed for transfer and array of bacterial or yeast genomic libraries are often incompatible with many kinds of filamentous fungal growth.

One of the only high throughput cultivation systems that was adapted for use with wild fungal and actinomycetes colonies was the CT4000 developed by Hitachi Electronics Engineering Company [135,136], used in the microbial screening program at Nippon Roche. The first phase of isolations, the preparation and application of soil dilutions to isolation plates, was still done manually. Automated strain manipulation started with ac-

tively growing soil isolation plates. The CT4000 had an automated colony recognition and picking system that replaced the largely manual and repetitive task of selecting and transferring microbial colonies from soil isolation plates to clean growth media. Images of colonies grown on agar were captured with CCD camera and the images were binarized so that the positions of the numerous randomly distributed colonies are recognized by a computer. The computer was programmed to recognize and select colonies and send instructions to a robot, which would pick them. The robot's picking needles accurately touched the selected colonies and transferred them onto an agar medium. The Hitachi CT4000 also inoculated liquid media with the isolated strains. The CT4000 usually worked very accurately without excessive errors from the time it was completed and installed. Despite its sophisticated features, however, the CT4000 was never well accepted by microbiologists, mainly because of reasons explained below and because manual selection of colonies for picking was still needed. The system was used in bacterial screening for a while and, in the long run, it is doubtful whether it saved human resources. Ultimately, its use for wild colony selection was abandoned, and it was used for manipulations in the strain improvement programs. We are aware that several other Japanese pharmaceutical laboratories operated robotic colony picking systems. Rarely were they operated for selection of wild strains for screening, but rather were employed in mutation and strain improvement screening in fermentation facilities.

Proposals for automated microbial isolation usually are based on the often-erroneous assumptions that the source of the organisms, for example soil, can be prepared in a homogeneous format, and that organisms are behaviorally and morphologically equivalent and therefore can be delivered to the system in a standardized format. That standardized format generally is assumed to be a Petri plate bearing wild colonies from soils. However, in the case of fungi, the underlying assumptions disregard differential growth rates, variable disseminated propagules, and the biased and narrow spectrum of the microbial flora that develop on soil isolation plates (Fig. 2). Implementation of such an automated system potentially could be counterproductive in terms of producing the diversity of metabolites, because reliance solely on large numbers of soil-derived strains compatible with surface growth on agar limits screening to a relatively restricted diversity of fungi and ignores the majority of the fungal kingdom.

Furthermore, design of an isolation system for wild fungal colonies needs to overcome obstacles inherent in the varied ways fungi grow and reproduce:

1. Fungal colonies from nature are very heterogeneous in terms of size, color, texture, and hardness, and those that produce spores do so in a myriad of ways, so that automating the recognition and discrimination of all colonies is difficult. Colonies of fungi may grow at very different rates, and some may not even appear on selective media for days, whereas others can inundate the plate in a few days. The inverse situation can limit the system; undifferentiated or immature colonies of different species may appear to be the same.
2. A single universal picking tool employing a single mechanical action is inadequate to dissect, lift, and transfer cells. Some fungi may grow appressed at the agar surface or submerged within the agar and, therefore, out of the range of a tool that operates at or above the surface. The range of motions that the human eye and hand match to each type of fungal colony in order to dissect and lift it is unlikely to be replicated by a robot.
3. Dry spores that interact with static electricity may scatter during the transferring process and cause contamination.

4. Liquid inocula of fungi are similarly heterogeneous. Not only do growth forms vary among organisms, but they are profoundly influenced by the vessel shape, rotation speed, addition of shearing devices, and the high viscosity of large mycelial pellets and submerged growth can obstruct or complicate pipeting.

Therefore, isolation of most fungi is likely to still rely on manual techniques and cognitive abilities of trained scientists.

An alternative route for parallel separation, isolation, and cultivation of microbes recently has been developed and relies on microencapsulation of microbial cells followed by cultivation under selective nutrient or environmental conditions and separation of encapsulated cells by fluorescence activated cell separation (FACS) [15,137]. Bacterial cells from soils, aquatic environments, or other materials are concentrated and then added to a nonpolymerized gelling agent (e.g., alginate or agarose). The gelling agent is then dispersed in an emulsifying agent with either a shearing device or by filtration through a microporous filter to form a homogeneous emulsion of microdroplets. If the volume of gelling agent is sufficiently high relative to the cell density, then the probability of entrapment of single cells inside individual microdroplets is high. The microdroplets are harvested and cultured briefly, and those that initiate cell growth are separated from those that are empty or dead by FACS. Parameters of the FACS are set to channel a subpopulation of living microdroplets to a collection vessel. FACS-separated microdroplets contain single-cell-derived microbial colonies that range from one to many cells. If the FACS is equipped with an automated cell sorter, cell-containing microdroplets can be distributed into wells of microculture plates for subsequent cultivation steps, replication plating, and downstream analyses.

The encapsulation-FACS technique has several obvious advantages over the use of colony pickers on soil plates. All cell types from the environment are equally likely to be encapsulated, and by differential application of enrichment cultures on the encapsulated population, different components of the population can be favored for growth, including cell types generally thought to be unculturable [15]. Encapsulated cells can be cultivated in a continuously fed open culture system that more likely simulates natural environments and thereby enables cultivation of microbes not normally seen on conventional agar plates. As a result, a much higher percentage of the microbial flora would be cultivated and assayed. Cell separation by FACS is achieved with commercially available (albeit costly) flow cytometry equipment that is often accessible at major research centers; therefore, engineering and programming of custom-built robotics are not required. Cell throughput of FACS is potentially orders of magnitude higher than could be achieved with mechanical colony separation. So far, the encapsulation-FACS technique has only been applied to bacteria, but it is easily imaginable that such a system could be adapted to entrapment, separation, and parallel cultivation of environmental fungi.

6. CODA: EVALUATING AND GUIDING THE OUTPUT

Success in microbial screening, as defined by immediate recognition of a drug lead, has always been an elusive event. There is no doubt that success ultimately rests in validity of the target and implementation of screening assay and on how they correlate with *in vivo* activity. Then in absence of discovery of a blockbuster active, how does one know that the goals established by the program are met, especially in industrial programs that may be providing natural products samples to multiple therapeutic target areas simultaneously?

Judgments based on one-parameter measurements, like number of strains isolated, numbers of extracts produced, and hit rates, are essentially meaningless because the units of measurement are undefined. Scrutiny and analysis of such numbers out of context can lead to bizarre predictions and conclusions. We believe that parameters with a genetic or physiological underpinning or those that lead to product formation should be emphasized, rather than attempting to maximize hypothetical probabilities from dimensionless measurements. The role of the microbiologist is the selection of organisms that have the best characteristics for the biosynthesis of the required products in quantities sufficient for detection. Environmental and nutritional conditions need to be identified that favor product formation, as well as the unique physiological and biochemical characteristics of the organisms responsible for their biosynthetic capabilities. However, each fermentation is unavoidably unique, and a natural tendency exists to focus on those unique features that lead to specific product formation. The challenge in screening large cross sections of microorganisms and fungi for metabolites is to focus on physiological and genetic components common to all fermentations while anticipating the recognition of a yet-defined product.

Several different parameters and outcomes that contribute to the overall goal of providing microbial chemical diversity can be evaluated. Phylogenetic or taxonomic diversity of the strains is not directly translatable into chemical diversity and alone is an imperfect surrogate for measuring the chemical complexity of the output. The reasons are simple: unrelated organisms often produce the same kinds of metabolites, and certain kinds of organisms are more genetically disposed to producing metabolites of different biosynthetic pathways than others [12]. Furthermore, just as certain synthetic chemical families are more useful for particular types of targets than others, certain microbial products will be more or less useful. The assumed equation between biological and chemical diversity becomes even more tenuous when one considers that strain selection must be tightly coupled with fermentation and extraction techniques to give a chemically meaningful result. However, phylogenetic breadth of the screening population, and whether it is targeted toward chemically relevant taxa, unknown taxa, or whether it is excessively repetitive, can be evaluated to some degree. An analysis of isolates at the generic and species level can confirm or reject whether isolation methods are achieving the claimed results. For example, do isolations from freshwater or marine habitats really obtain taxa adapted to those habits, or simply genera of terrestrial fungi whose propagules germinated on the isolation plates? Such confirmations need not be exhaustive, but can be carried out on subsets of screening populations by means of morphological and rDNA-sequence analyses. Maintaining records of diagnostic analyses of screening populations, and especially of strains that have yielded specific activities and compounds, can provide a valuable historical map of where the program has traveled and where it has not. Maintenance of historical records of molecules discovered and which organisms produced them discovered will reveal repetitive patterns and potential needs to shift focus in strain selection. Attention should be paid to types of metabolites with promiscuous activities and the qualitative differences between the discoveries of a totally new bioactive scaffold versus the discovery of new analogues within known scaffold series. Finally, it cannot be emphasized enough that quality control by spot-checking the identity of active strains in our screening programs has greatly improved reproducibility, confirmation, and scale-up of primary actives.

Bioactivity and chemical complexity of culture broths and extracts are more direct measurements of the utility of different fungi. Chemometric analyses and identification of extract components are detailed in other chapters of this volume. Direct probing and analyses of genes of biosynthetic pathways can confirm the genetic potential of target

organisms and likewise are described in other chapters of this volume. Evaluation of microbial extracts from their antimicrobial activity is another common method and is based the assumption that antibiosis is a primary or secondary effect of the production of one or more complex secondary metabolites [138]. In reality, detection of antibiotic activity is a test of the combination of the genetic capacity of an organism and its response to its cultural conditions, and production of antibiosis can be used to compare efficacy of production conditions for a given sets of strains. Therefore, negative results can mean either biosynthetic capacity is lacking or the fermentation parameters are inadequate.

For example, Hosoya [139] gathered comparative antimicrobial data on 350 strains of Helotiales tested against a panel of three bacteria (*Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*) and one of two yeasts (*Saccharomyces cerevisiae* or *Candida albicans*). Eighteen percent of the strains produced activity against at least one of the strains, although comparing strains at the family level, more than 30% of the strains the Dermataceae and Hyaloscyphaceae were active. The relative antibiotic activity of 317 isolates of basidiomycetes from different orders were surveyed with a single fermentation medium and compared to those of major groups of ascomycetes [140]. A high proportion of the basidiomycetes (45%) produced some type of antifungal or antibacterial activity. The levels of activity were comparable to those of some ascomycete orders, e.g., the Pezizales and Xylariales, but generally less than some of the better-known ascomycete orders (e.g., the Eurotiales and Hypocreales). Researchers at Sandoz submitted a set of endophytes from woody plants ($N = 400$) to a series of 14 varied assays, four of which were antimicrobial assays [12]. Results were contrasted to those of an undefined control group ($N = 764$). Performance, measured by detection of actives between the endophytes and the control set, was assay dependent, although overall a greater proportion of the actives were detected among the endophytes, especially in the antibiotic assays. Simple antibiotic readouts may not be able to guide the program to an optimal state of productivity, but they can help identify ineffective organism-medium-environment combinations.

Commercial products, to date, have only been derived from metabolites and enzymes of about two handfuls of different fungi. Mapping those few organisms onto the vast evolutionary tree of the fungi raises expectations that many new products await discovery. The selection of broad cross sections of different fungal taxa or the search for novel chemical structures [54] among poorly studied species by themselves are not enough ensure discovery success. Conversely, blind and arbitrary selection of strains or, worse, undifferentiated colonies from isolation plates, relegates discovery to an accident and would be analogous to screening unlabeled, arbitrarily selected, synthetic chemical libraries.

A more rational strategy for the discovery of fungal metabolites probably lies near the proposals of Dreyfuss and Chapela [12]. Programs need to be maneuvered toward biosynthetically creative pockets of organisms using logistical, ecological, and phylogenetic criteria. Some of these groups of fungi are well known, whereas others are yet to become apparent. The first step in setting the path is to carry out enough preliminary screening to be able to integrate ecological, phylogenetic, and pathway data to identify areas and correlate them with relevant chemical activities. The exploratory stage gradually progresses to a larger scale where parameters such as sample and organism selection, fermentations, and extractions are incrementally improved, while refining and redirecting the search as new leads are discovered.

Cloning and sequencing of specific pathways and comparative genomics of fungi and actinomycetes are uncovering cryptic, natural-product gene clusters and the genetic and regulatory basis of metabolic creativity and versatility. This new front in natural products research suggests that typical fermentation methods underestimate the production of antibiotics and other secondary metabolites, even by common model organisms, like *Neurospora crassa* [141]. With the ability to identify loci of secondary metabolism independent of expression, improved phylogenetic understanding, along with knowledge of the biochemistry of the targets, it should be possible to expand and better define paths to the most relevant creative pockets of the fungal kingdom.

APPENDIX. 1. FORMULAE FOR MEDIA USED TO ISOLATE AND CULTIVATE FUNGI

Bandoni's Sorbose–Yeast Extract–Tetracycline Medium [96]

L(–) sorbose, 4.0 g
Yeast extract, 0.5 g
Distilled water, 1000 mL

After autoclaving, cool to 50°C and add tetracycline (in 95% ethanol), 100 mg. Other antibacterial antibiotics may be substituted for isolations from natural substrata.

Use: Isolation and sporulation of freshwater hyphomycetes and ascomycetes, and microfungi from leaf litter.

Basidiomycete Isolation Medium (BDS) [99]

Malt extract, 15 g
Agar, 15–20 g
Benomyl, 2 mg
Dichloran, 2 mg
Water, 1000 mL

Antibacterial antibiotics of choice are added after autoclaving. To prepare a stock solution of the antifungal components, add 40 mg of benomyl to 50 mL of 95% ethanol, dilute the ethanol solution to 100 mL in warm water, and add 20 mg of dichloran. Refrigerate the stock solution, and add 10 mL to the malt extract medium, before or after autoclaving.

Use: Isolation of basidiomycetes from decaying wood, tissues of basidiomata, hyphal strands, and rhizomorphs.

Blakeslee's Malt Extract Agar (BMEA) [142,143]

Malt extract, 20 g
Peptone, 1 g
Glucose, 20 g
Agar, 20 g
Distilled water, 1000 mL

Use: Many fungi grow robustly on this medium. Recommended for identification of *Penicillium* and *Aspergillus* spp. Growth varies slightly depending on manufacturer of the malt extract and peptone, but Difco, Oxoid, or BDH products often are used.

Carboxymethyl Cellulose Agar (CMC) [35,144,145]

Sodium carboxymethyl cellulose, 10.0 g
Yeast extract, 0.5 g
 $(\text{NH}_4)_2\text{SO}_4$, 1.0 g
 NaNO_3 , 2.0 g
 KH_2PO_4 , 1.0 g
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g
KCl, 0.5 g
 CaCl_2 , 0.05 g
 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g
 CuSO_4 , 0.01 g
 $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.005 g
 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 g
Agar, 15.0 g
Water, 1000.0 mL

For convenience, the last five salts can be combined as a stock solution. Sodium carboxymethyl cellulose forms a highly insoluble gel, and some brands are easier to dissolve than others. Combine all dry ingredients in a flask. Slowly add hot water, stirring constantly. Allow mixture to stir for 15 to 30 minutes. Medium will probably be lumpy; most lumps disperse after autoclaving. Add antibacterial antibiotics after autoclaving.

Use: A low-nutrient medium for isolation of soil and litter fungi that supports only limited colony development. Minute hyphal growth is easily seen with a dissecting scope in this nearly transparent medium.

Czapek-Dox Agar (CZA)

Sucrose, 30.0 g
 NaNO_3 , 2.0 g
 K_2HPO_4 , 1.0 g
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g
KCl, 0.5 g
 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g
Agar, 15.0 g
Distilled water, 1000.0 mL

Antibacterial antibiotics should be added when used for isolations from natural substrata.

Use: Medium appropriate for isolation and cultivation of a wide variety of fungi, including animal pathogenic fungi, and fungi from extreme environments. It is also commercially available as a dehydrated liquid broth or agar medium.

Cornmeal Agar (CMA)

Yellow cornmeal, 40.0 g
Agar, 15.0–20.0 g
Distilled water, 1000.0 mL

Boil water and add cornmeal. Simmer for 15 minutes. Filter through three or four layers of gauze. Allow this infusion to rest 15 minutes. Slowly decant all but the bottom 200 mL. Bring volume back up to 1000 mL and add agar. Dehydrated cornmeal agar is available

from various distributors. Antibacterial antibiotics should be added after autoclaving if the medium is used for isolations from natural substrata.

Use: Often a good medium for induction of sporulation. It is an excellent medium for making single-spore isolations because of its transparency and because the main nutrient is starch, which limits mycelial growth.

Dichloran-Glycerol-18 Medium (DG18) [143]

Glucose, 10.0 g
Bacteriological peptone, 5.0 g
 KH_2PO_4 , 1.0 g
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g
Dichloran (0.2%, in 95% ethanol), 1.0 mL
Glycerol (analytical reagent grade), 220.0 g
Agar, 15.0 g
Distilled water, 1000.0 mL
Chlortetracycline (0.1%, aqueous) to give 5 $\mu\text{g}/\text{mL}$ (after autolaving)

Add all ingredients except glycerol, dichloran, and chlortetracycline to 800 mL distilled water. After heating to dissolve agar, add glycerol and dichloran solution, giving final concentrations of 18% (wt/wt) glycerol and 2 μg of dichloran/mL. Adjust final volume with distilled water to 1 L. Add filter-sterilized 0.1% aqueous solution of chlortetracycline to medium before pouring to give a concentration of 5 $\mu\text{g}/\text{mL}$. Final pH is 5.6; a_w is 0.955. Dichloran is highly toxic and should be used with extreme care and under proper ventilation.

Use: Isolation of osmotolerant, xerotolerant, and xerophilic fungi.

Dichloran-Rose Bengal-Chloramphenicol Agar (DRBC) [145]

Glucose, 10.0 g
Peptone, 5.0 g
 KH_2PO_4 , 1.0 g
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g
Dichloran, 0.002 g
Rose Bengal, 0.025 g
Chloramphenicol, 0.1 g
Agar, 15.0 g
Distilled water, 1000.0 mL

Dichloran has low water solubility. A convenient stock solution can be made by dissolving 0.2 g of dichloran in 100 mL ethanol; 1 mL is added to each liter of medium to attain a concentration of 2 mg/L. Dichloran can also be dissolved in a few milliliters of acetone and added to medium prior to autoclaving.

Chloramphenicol can be purchased as a supplement in ampoules or can be weighed and added directly to the medium. The manufacturer of DRBC recommends use of chloramphenicol, but other combinations of antibacterial antibiotics can be added after autoclaving.

Use: A good medium for isolation of soil fungi, food-associated fungi, and fungi from dried seeds and plant materials.

Glucose Ammonium Nitrate Agar (GAN) [146]

Glucose, 10.0 g
NH₄NO₃, 1.0 g
KH₂PO₄, 1.0 g
MgSO₄.7H₂O, 0.5 g
Rose Bengal, 0.03 g
Agar, 20.0 g
Water, 1000.0 mL

Add antibacterial antibiotics after autoclaving, and adjust pH if desired. Substitution of 2–10 mg cyclosporin A for the rose bengal is a useful variation of GAN.

Use: Isolation of soil fungi.

Malt Extract Agar

Malt extract, 20.0 g
Agar, 20.0 g
Water, 1000.0 mL

Use: The 2% version of malt extract agar is one of the widely of all media for cultivation and characterization of fungi.

Malt Extract Agar with Cyclosporin [11,147]

Malt extract, 2.0–20.0 g
Yeast extract (optional), 0.2–2.0 g
Agar, 20.0 g
Water, 1000.0 mL
Cyclosporin A, 2.0–10.0 mg

Quantity of malt extract is often varied. Yeast extract optional, depending on fungi to be isolated; produces malt-yeast agar (MYA). Antibacterial antibiotics and cyclosporin A (if control of colony expansion is desired, malt-cyclosporin A agar) added after autoclaving.

Use: A very versatile isolation medium, but especially for isolation of soil and leaf litter fungi.

Miru Agar (LCA) [148]

Glucose, 1.0 g
KH₂PO₄, 1.0 g
MgSO₄.7H₂O, 0.2 g
KCl, 0.2 g
NaNO₃, 2.0 g
Yeast extract, 0.2 g
Agar, 15.0 g
Distilled water, 1000.0 mL

Adjust pH to between 6.5 and 7.0. Add antibiotics and antifungal agents after autoclaving.

Use: Isolation and sporulation of freshwater hyphomycetes and ascomycetes.

Oatmeal Agar (OA)

Rolled oats, 30.0 g
Agar, 20.0 g
Tap water, to make 1 L

Boil oats for 15 to 30 minutes. Filter through cheesecloth or gauze. Bring filtrate volume to 1 L. Add agar. Autoclave 30–40 minutes at 15 psi. Alternatively, grind uncooked rolled oats in a blender; combine with agar and water, and autoclave for 45 minutes. Prepare this medium in an oversized flask, because the medium usually foams excessively when autoclaved. Commercially prepared versions of OA also are available.

Use: Very good medium for studying development of fungal morphology and for induction of sporulation in nonsporulating isolates.

Potato Carrot Agar (PCA) [27]**Potato-Carrot Extract**

Peeled carrots, 40.0 g
Peeled potatoes, 40.0 g
Tap water, 1000.0 mL

Shred carrots and chop potatoes into small pieces, add to water and boil for 15 minutes, filter the mixture through a layer of gauze. The extract maybe autoclaved for future use.

Potato-Carrot Agar

Potato-carrot extract, 500.0 mL
Tap water, 500.0 mL
Agar, 20.0 g

Use: This medium is useful for stimulation of sporulation in culture. It can be used as isolation medium for soil and litter fungi by adding antibacterial antibiotics after autoclaving.

Potato Dextrose Agar (PDA) [26]**PDA from Fresh Potatoes**

Potatoes, 200.0 g
Glucose, 20.0 g
Agar, 20.0 g
Distilled water, 1000.0 mL

Chop potatoes and place in water. Autoclave for 20 minutes at 15 psi. Strain autoclaved potatoes and liquid through several layers of gauze, squeezing the potatoes to extract all the liquid. Add enough water to bring the potato extract to 1000 mL. Add glucose and agar and autoclave 15 to 20 minutes at 15 psi.

PDA from Commercial Preparation

Potato dextrose agar, 39.0 g
Distilled water, 1000.0 mL

Antibiotics can be added to medium after autoclaving.

Use: PDA is one of the most commonly used culture media for fungi because of its simple formulation and its ability to support mycelial growth of a wide range of fungi. Several formulations are in the literature; one is provided here. The recipe for commercially prepared PDA is provided as well.

Soil Extract Agar (SEA) [27,149]

Soil extract, 100.0 mL
Glucose, 0.5 g
 K_2HPO_4 , 0.5 g
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g
Agar, 20.0 g
Distilled water, 900.0 mL

Soil extract: steam 1 kg soil (preferably sandy loam) in 1 L distilled water at 100°C for 1 hour. Filter first through cheesecloth; then centrifuge to clear. Autoclave extract for 1 hour on each of 2 consecutive days before preparing medium.

Use: Widely used for isolation of soil fungi and fungi associated with plant roots.

Spezieller Nährstofffarmer Agar, or Synthetic Low-Nutrient Agar (SNA) [27]

KH_2PO_4 , 1.0 g
 KNO_3 , 1.0 g
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g
KCl, 0.5 g
Glucose, 0.2 g
Sucrose, 0.2 g
Agar, 20 g
Distilled water, 1000.0 mL

Add antibacterial antibiotics after autoclaving if used as an isolation medium.

Use: Widely used for isolation, induction of conidia and identification of *Fusarium* and *Clonostachys* species and other hyphomycetes with slimy conidia.

Water Agar (WA)

Agar, 15.0–20.0 g
Distilled water, 1000.0 mL

Use: Isolation of endophytes, plant pathogens and soil fungi. Various combinations of antibiotics can be added to medium after autoclaving.

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7

Expression of Cosmid-Size DNA of Slow-Growing Fungi in *Aspergillus Nidulans* for Secondary Metabolite Screening

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INTRODUCTION

Filamentous fungi produce a vast array of bioactive secondary metabolites [1], including antibacterials (penicillin, cephalosporins, and fusidic acid), antifungals (pneumocandins, griseofulvin, and strobilurins), immunosuppressants (cyclosporin A and mycophenolic acid), antihypercholesterolemia agents (lovastatin and pravastatin), migraine and obstetrics pharmacologics (ergot alkaloids), plant growth hormones (gibberellins), and an animal growth promoter (zearalenone). Despite these successes, most efforts have been focused on fungi that can be easily isolated, cultured, and maintained on laboratory media. We expect to continue to discover new secondary metabolites from easily culturable fungi, but expansion of fungal natural products sources to unculturable and slow-growing fungi may lead to even more new structural classes. Improved cultivation techniques can help to take advantage of less manageable fungi, but some fungi may never be cultivated.

One way to capture the genetic diversity of unculturable or slow-growing organisms for secondary metabolite discovery is to introduce large segments of genomic DNA from these organisms into faster growing, genetically amenable hosts. In this approach, the host

is asked to express genes from the donor organism and to produce secondary metabolites that it does not naturally produce. This is possible because transcription and translation control sequences from one organism often function in closely related organisms, and sometimes even in distantly related organisms [2–7]. Furthermore, genes involved in the biosynthesis of major microbial secondary metabolites are often clustered. This tendency for genes to cluster allows us to gather most, if not all, of the genes for a particular secondary metabolite within one large fragment of DNA, which can then be introduced into a recipient host for potential production of recombinant metabolites.

In this chapter, we describe the heterologous expression of cosmid-size genomic DNA fragments from five strains of slow-growing cranberry endophytic fungi in *A. nidulans* for secondary metabolite screening. During the course of this study, we developed a reproducible procedure using 24-well microplates for high-throughput small-scale fermentation and a semi-automated mass spectroscopy protocol that quickly and sensitively characterized the sample strains. Because visual examination of the thousands of spectra to find those few that look different is both laborious and prone to error, we also have developed data analysis/pattern recognition procedures to assist in the prescreening. Filtering out the bulk of samples using these tools yielded a manageable number of samples that were individually examined and subjected to further chemical separation and biological testing. HPLC peak-guided isolation procedures resulted in the isolation of two compounds from extracts of two of the outlier transgenic strains that were not produced by either the expression host or the bulk of transgenic strains.

2. EXPERIMENTAL METHODS

2.1. Chemicals and Reagents

Restriction endonucleases and DNA modifying enzymes were from Gibco BRL (Rockville, MA), New England Biolabs (Beverly, MA), and Promega (Madison, WI). Media components and chemicals were from Difco Laboratories (Detroit, MI), Sigma (St. Louis, MO), and Fisher Scientific (Pittsburgh, PA). Ardamine PH was obtained from Champlain Industries, Inc. Soybean meal was from Centra Soy Co., tomato paste was from Beatrice/Hunt-Wesson, Inc., and reagents from other sources are identified in the text.

2.2. *A. nidulans* Transformations

A. nidulans strain MF5999 was grown in appropriately supplemented *A. nidulans* minimal medium [8]. *A. nidulans* transformations were carried out as previously described [9]. Transformants of *A. nidulans* strain MF5999 containing cosmid genomic DNA clones were selected for L-arginine prototrophy.

2.3. Genomic DNA Isolation

Fungal isolates from cranberry plants were grown on a layer of cellophane membrane (dialysis membrane, Bel-Art, Pequannock, NJ) on YME agar (per liter: yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; agar, 20 g). Total genomic DNA from fungal biomass ground to a powder in liquid nitrogen was isolated by a phenol-chloroform extraction procedure [10,11].

2.4. Construction of Cosmid Cloning Vector pANARGB2 and Cosmid Libraries

pANARGB2 was constructed as follows: the 1.8 kb *Xba*I/*Bam*HI fragment containing the *argB* gene of *A. nidulans* was isolated from plasmid pDC1 (FGSC, Kansas City, KS) and

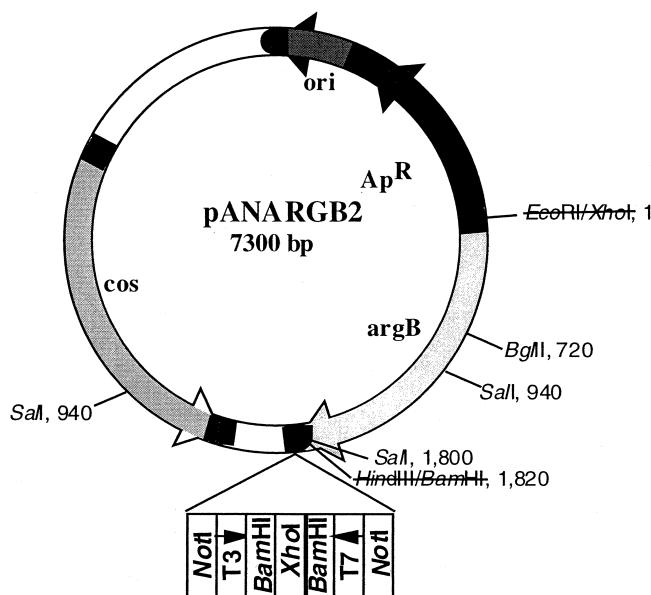


Figure 1 Features of pANARGB2. Only cloning and diagnostic restriction sites are shown. Ap^R , ampicillin resistance-encoding gene; argB , *A. nidulans* ornithine carbamoyltransferase-encoding gene; *ori*, origin of DNA replication; *cos*, cos site.

blunt-ended by Klenow fragment. The DNA fragment was then ligated to a 5.8-kb blunt-ended *Hind*III/*Eco*RI fragment containing the backbone of the cosmid cloning vector pMLF2 [12]. Fig. 1 shows the features of pANARGB2. Cosmid libraries were constructed for genomic DNA from five slow-growing strains of cranberry endophytic fungi (CR61, CR68, CR70, CR132, and CR133) using the Gigapack III XL packaging extract (Stratagene, La Jolla, CA). Briefly, 35- to 45-kb *Sau*3A1 partially digested genomic DNA fragments were isolated by sucrose gradient centrifugation and ligated into the *Bam*HI site of cosmid vector pANARGB2 [13]. Packaged cosmid clones were introduced into *E. coli* strain XL1-Blue MR.

2.5. Southern Blot Analysis

Genomic DNA from *A. nidulans* transgenic strains was isolated as previously described [9], digested with *Bam*HI, separated by electrophoresis on agarose gel, and transferred onto nylon membranes (Hybond N⁺, Amersham Corp.) following the manufacturer's protocol. Hybridization to ³²P-labeled random-primer DNA fragments (Multiprime DNA labelling systems, Amersham Corp.) was carried out at 65°C [14].

2.6. Generation of Transgenic Strains

About 1000 transformants were generated for each of the five cosmid libraries by transforming *A. nidulans* MF5999 using cosmid DNA isolated from pools containing ca. 4000

Table 1 Fungal Strains Used in this Study^a

Strain Name	Species	Genotype
MF5999	<i>Aspergillus nidulans</i>	biA1, argB2, methG1
CR61	Ascomycete	Wildtype
CR68	Ascomycete	Wildtype
CR70	Oomycete (?)	Wildtype
CR101	Ascomycete	Wildtype
CR132	Ascomycete	Wildtype
CR133	Ascomycete	Wildtype
MF5999/7-2-1	<i>A. nidulans</i>	biA1, methG1
MF5999/7-2-2	<i>A. nidulans</i>	biA1, methG1
MF5999/7-3-1	<i>A. nidulans</i>	biA1, methG1
MF5999/7-3-2	<i>A. nidulans</i>	biA1, methG1
MF5999/7-4-1	<i>A. nidulans</i>	biA1, methG1
MF5999/7-4-2	<i>A. nidulans</i>	biA1, methG1
MF5999/7-5-1	<i>A. nidulans</i>	biA1, methG1
MF5999/7-5-2	<i>A. nidulans</i>	biA1, methG1
A09T0943	<i>A. nidulans</i>	biA1, methG1
B07T0399	<i>A. nidulans</i>	biA1, methG1
B09T0391	<i>A. nidulans</i>	biA1, methG1
B09T0573	<i>A. nidulans</i>	biA1, methG1
A09T0567	<i>A. nidulans</i>	biA1, methG1

^a Source of all strains was this study, except for MF5999, which was from Larry Yager, Temple University, Philadelphia, PA.

clones of each library. Individual L-arginine prototrophic transformants were transferred into wells of 24-well plates to verify the L-arginine prototrophic phenotype. Transformants were stored at 4°C in 24-well plates on supplemented minimal media. Table 1 lists some of the fungal strains used in this study.

2.7. Fermentation Conditions

A. nidulans transformants were grown in either 24-well microplates or 250-mL flasks. The solid fermentation medium was yeast extract sucrose agar (YES) [15]. The medium used for liquid fermentations was LSF1 (per liter: glycerol, 75 g; glucose, 10 g; Ardamine PH, 5 g; soybean meal, 5 g; tomato paste, 5 g; sodium citrate, 2 g; (NH₄)₂SO₄; 2 g; and pH adjusted to 7.0 with NaOH). A total of 2 mL and 50 mL of YES and LSF1 media was placed into each well of 24-well microplates and into 250-mL flasks, respectively. Spores from *A. nidulans* (MF5999) strains were transferred from 24-well storage plates via replicating pins (V&P Scientific, Inc., San Diego, CA) or sterile cotton-tipped applicators (Hardwood Products Company, Guilford, MN) into wells of 24-well fermentation microplates (Whatman-Polyfiltronics, Milford, CT) and flasks, respectively. The cultures grown in LSF1 medium were incubated for 14 days at 25°C and 220 rpm on an orbital shaker with a 5-cm throw in Kuhner cabinets (Kuhner, Basel, Switzerland) at 85% relative humidity in the dark. The cultures grown on YES medium were incubated under static conditions under the same incubation parameters as stated above. After 14 days, cultures

in LSF1 medium were mixed with 1.5 volume of methyl ethyl ketone (MEK), homogenized for 30 seconds using a tissue homogenizer (Biospec Products, Inc.), and shaken for 1 hour on a reciprocal shaker at room temperature. Cultures grown on YES solid medium were extracted with 1.5 volume of MEK for 12 hours at room temperature. As an internal standard for FIA-ESI-MS and TLC analyses, griseofulvin at 10 mg/L was added to the MEK extracts. Sample extracts were prepared using an automated liquid dispensing system (Tecan US, Research Triangle Park, NC). A total of 1 mL of the top organic phase was removed and transferred to sealed glass vials (National Scientific Company) and stored at -20°C for downstream analyses.

2.8. Mass Spectrometry Instrument Conditions

Mass spectral data were collected on an Agilent-MSD mass spectrometer with an API-electrospray source. The instrument scanned from 1000 to 200 Da at a cycle time of 1.89 sec/cycle, with a peak width of 0.2 min and a ramped fragmentor voltage of 50 at m/z 200 to 120 at m/z 1500. Samples were introduced into the mass spectrometer by a flow injection analysis method and analyzed by electrospray (FIA-ESI-MS). An isocratic solvent system of 90% MeCN water solution with 1.3-mM NH₄-formate and 0.01% trifluoroacetic acid (TFA) at a flow rate of 400 $\mu\text{L}/\text{min}$ was used. A total of 5 μL of each extract was injected into the solvent stream after a 0.2-minute delay to allow for background determination. Time between each injection was 2 minutes.

2.9. Data Description

In all cases, background MS spectra were obtained from 0.12 to 0.2 minutes. The peak maximum of the total ion chromatogram was detected. An average spectrum at one-half height was determined and the background spectra were subtracted to produce a single representative spectrum per injection. This representative spectrum contained the abundance values reported as absolute abundance. The m/z values were converted to integers by using a rounding point of 0.8 mass units. The abundance values were zero-filled, generating a single corrected spectrum for each sample.

2.10. Notation

We denote the spectra of the n samples in the library by row vectors, $\mathbf{x}_I = (x_{I1}, \dots, x_{Ip})$, where $i = 1, \dots, n$; x_{Ij} is the absolute ion intensity (abundance) at the j -th m/z ratio; and p is the number of m/z ratios, 801 in our spectra. The combination of all spectra yields an n by p matrix X whose rows are the spectra of individual samples.

2.11. Identification of Novel Samples

Each spectrum \mathbf{x}_i could be represented as a point in a p -dimensional abundance space. Most of the samples not producing recombinant natural products should be similar and should yield similar spectra. These spectra should therefore cluster relatively close together in this space. The few novel samples will have distinct spectra that are far from the main cluster and appear as individuals or small groups of individuals. Therefore, the problem of identifying novel samples becomes a problem of detecting outliers in p -dimensional

space. While there are many methods for outlier detection in multidimensional data, none work satisfactorily in all situations. We have developed two procedures for our purpose: resistant Mahalanobis distances and a sequential clustering procedure (SCP). Because the two procedures are based on different models, they should be able to identify outliers containing different features.

2.12. Data Preprocessing

Before the outlier detection procedures were applied, the data were preprocessed by removing channels (m/z) where no sample has ionization intensity exceeding 1000 (the detection limit), since these channels would not contribute any useful information to the analysis. This step significantly reduced the dimension of the data by eliminating as many as 40% of the channels.

2.13. Outlier Detection by Mahalanobis Distances and MD-PCA

Mahalanobis distances and principal component analysis (MD-PCA) are widely used chemometrics methods for characterizing spectra and finding outliers [16,17]. However, a problem with using Mahalanobis distance for outlier detection is that the usual (least squares) estimate of the covariance matrix can be distorted by outliers so that the outliers are masked and therefore missed. We therefore computed an outlier-resistant estimate of the covariance matrix using the minimum covariance determinant (MCD) algorithm [18]. A simple plot of Mahalanobis distances based on this covariance matrix was then used to identify the outliers by plotting them in descending order. Data were also visualized by plotting the scores of the first few principal components of the MCD covariance matrix.

2.14. Outlier Detection by Sequential Clustering

The second method for determining novel extracts was based on cluster analysis [19]. We expected novel samples should be in small, isolated clusters (possibly having only one member). Novel samples are defined based on two assumptions: (1) there is at most a fraction, τ , of outliers in the data; and (2) these outliers belong to clusters that contain at most a fraction, ν , of the data. In other words, a sample must belong to a cluster that contain no more than $n\nu$ samples to be considered as an outlier, and there can be no more than $n\tau$ outliers in all. We refer to assumption 2 above as the ν -criterion, and clusters that satisfy this criterion as ν -clusters. We expected both τ and ν to be small. We used $\tau = 0.05$ and $\nu = 0.05$ in our analysis.

The procedure was performed as follows: (1) The Euclidean distances, Pearson correlations and rank correlation matrices were computed; (2) Agglomerative hierarchical clustering with average linkage was carried with each of the three matrices; (3) For each distance/similarity matrix and each sample spectrum, x_i , the smallest number of clusters, k_i^{\min} , was found such that x_i belongs to a cluster with fewer than $n\nu$ samples; (4) The $n\tau$ samples with the smallest k_i^{\min} (if there are ties in k_i^{\min} , the ties were considered as the outlying samples); and (5) The union of the three lists (one for each distance/similarity) was taken as the final list.

Perhaps the most important feature of this procedure is how it gets around the difficult problem of selecting the right number of clusters by using step (3) described

above. This step used the monotonicity property of hierarchical clustering methods. If the number of clusters is varied from $k = 2$ to n , then once a sample x_i becomes a member of a v -cluster at some $k = k_i^{\min}$, and hence appears as a potential outlier, it will be a member of some v -cluster at all k that are larger than k_i^{\min} . Another property of hierarchical clustering is that the more outlying a sample x_i , the smaller k_i^{\min} is. Thus we ranked the samples by their k_i^{\min} values, and step (4) above then yielded the outlier list.

2.15. Outlier Confidence Values

Once the list of novel samples is determined, confidence values of the samples' novelty were estimated. This confidence value is an estimate of the probability that a sample x_i would be called an outlier if the experiment that generated the data were repeated under the same conditions but with experimental variability yielding somewhat different data. Outliers with high confidence are thus more likely to represent real novelty. Therefore confidence values were used to prioritize samples for follow-up.

Because it is not feasible to repeat the experiment multiple times, we relied on the bootstrap method [20] to simulate what would happen if this were done. The details are as follows. Bootstrap matrices, X_b^* $b = 1, \dots, B$, were simulated by randomly selecting samples (rows) from the original matrix X with replacement. The SCP is applied to each matrix X_b^* , giving rise to B overall bootstrap outlier lists L_b , $b = 1, \dots, B$. The bootstrap confidence value λ_i^* is the ratio l_i/B_i , where l_i is the number of bootstrap outlier lists that included sample x_i and B_i is the number of bootstrap matrices containing that sample. We followed up on those samples with confidence values above 0.9.

2.16. TLC, HPLC, and Structural Identification

The MEK layer of the transgenic strain extraction was applied directly to pre-coated silica gel TLC plates and the plates were developed with toluene/ethyl acetate/formic acid (5:4:1) [15]. Secondary metabolites were visualized on TLC plates with UV and *p*-anisaldehyde spray reagent. For violaceol I isolation and identification, YES fermentations of culture B07T0391 were extracted with MEK, 100 mL total. The MEK extracts were filtered and concentrated to dryness *in vacuo*. The residue was dissolved in EtOAc, 20 mL, and this solution was extracted with 0.2% aq. H₃PO₄, 20 mL. The EtOAc layer was washed with H₂O and brine, and dried over anhydrous Na₂SO₄ to yield a red residue, 177 mg. The crude EtOAc extract was applied to a column of Sephadex LH-20, 1 × 80 cm, equilibrated with MeOH. The column was eluted with MeOH at 2 mL/min and 8.0-mL fractions collected. Fractions 19–23 were combined and concentrated to dryness. The material was further purified using preparative RP HPLC (Phenomenex Primesphere C8, 5 μm, 9.4 × 250 mm) using a mobile phase of 35% CH₃CN/65% H₂O containing 0.1% (v/v) TFA at 3.5 mL/min. Fractions 32 to 35, t_r = 16.5', were combined and concentrated *in vacuo* to yield violaceol I (1 mg). Spectroscopic data for this violaceol I isolate were consistent with that previously reported [21]. HRFT MS-ESI: found 263.0915 (M + H), calculated 263.0920 for C₁₄H₁₄O₅ + H; ¹H NMR (CD₃OD) δ 2.118 (s, 6H), 6.145 (brdd, 0.8, 1.2, 2H), 6.394 (brdd, 0.8, 1.6, 2H). For asperugin A, B isolation and identification, a portion (14 mg) of the MEK extract of a LSF1 fermentation of culture A09T0567 was partitioned between hexane: EtOAc:MeOH:H₂O (1:1:1:1). The upper layer was subjected to semi-preparative RP HPLC on C8 as described above eluted with a gradient of 10 to

90 CH₃CN containing 0.1% TFA. Peaks corresponding to asperugin A, B eluted at 29.0' and 26.5', respectively. The solvent was removed *in vacuo* to yield asperugin A (1.7 mg) and asperugin B (2 mg). NMR, MS, and UV data for these isolates were consistent with that previously reported [22].

3. RESULTS

3.1. Cosmid Vector and Libraries

An *A. nidulans*-specific cosmid-cloning vector pANARGB2 was constructed for this study. Briefly, the backbone and multiple cloning sites were from pMLF2, which has been described previously [12]. The *argB* gene complements arginine auxotrophic strains of *A. nidulans* that carry a defect in the ornithine carbamoyltransferase locus [23,24]. Transformation of *A. nidulans* MF5999 with pANARGB2 yielded arginine prototrophic transformants.

The five endophytic fungi (CR61, CR68, CR70, CR132, and CR133) grew slowly with colony diameters smaller than 5 mm in 1 month on YM agar. High-molecular-weight genomic DNA (>50–245 kb in size as determined by field inversion gel electrophoresis) was isolated from all five strains. DNA fragments of over 40 kb in size isolated from *Sau3AI* partial digestions by sucrose gradient centrifugation and fractionation were cloned into the *BamHI* site of pANARGB2. Digestions of ten randomly selected clones from each library with various enzymes showed that all clones contained an average insert size of approximately 35 to 45 kb.

3.2. Stability of Heterologous DNA in Transgenic Strains Grown on Selective and Nonselective Conditions

To test the stability of integrated DNA in heterologous hosts, two independent *A. nidulans* transformants generated with each of the four cosmids from library 7 (cosmids 7–2, 7–3, 7–4, and 7–5) were single-spore subcultured seven times at 5-day intervals in the presence and absence of L-arginine in minimally supplemented media. Genomic DNA was prepared from the eight cultures before subculturing and after the seventh subculturing. The *BamHI* digested genomic DNA was hybridized with the respective insert DNA isolated from the cosmids. The hybridization patterns of Southern blots of each transformant under both selective and non-selective conditions were the same and identical to the pattern obtained before the strain was subcultured, except transformant 7–3–2, in which an extra band was detected in the seventh transfer without selection (Fig. 2). Additionally, the hybridization patterns for the two independent transformants generated by the same cosmid were all different. For example, more copies of cosmid 7–2 were integrated in transformant 7–2–1 than in transformant 7–2–2 (Fig. 2).

3.3. Outlier Identification in Library 7

Extracts of 3,550 *A. nidulans* transgenic strains from four cosmid libraries (7, 10, 11, and 12) were processed and analyzed by FIA-ESI-MS (Table 2). Transgenic strains generated from library 7 were fermented on solid YES medium whereas all other transgenic strains were fermented in LSF1 liquid medium. The small percentage (~1.4% of the total samples) of wells with either no growth or contamination were excluded from subsequent analyses.

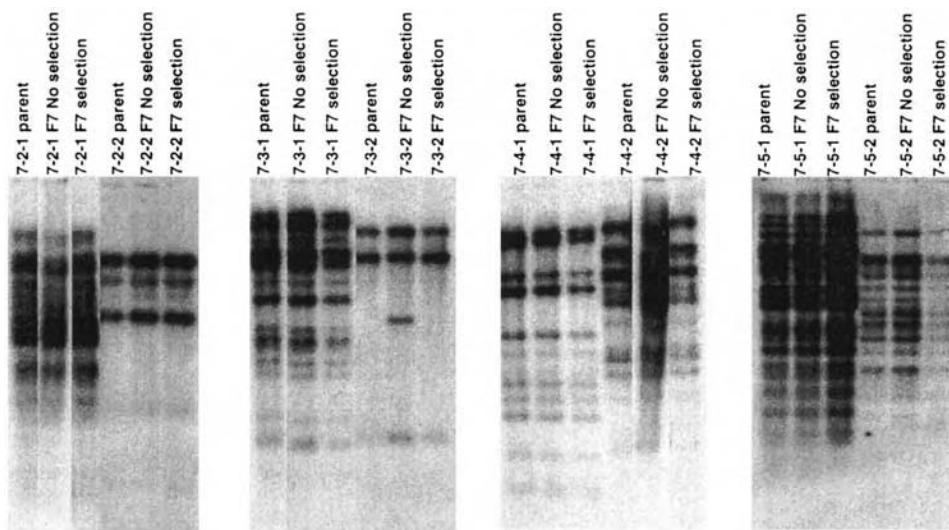


Figure 2 *Bam*HI-digested genomic DNA of *A. nidulans* transformants generated from four cosmid clones (p7-2, p7-3, p7-4, and p7-5) probed with insert DNA isolated from respective cosmids. Two independent transformants generated by each cosmid were included in the experiment (e.g., p7-2-1 and p7-2-2 are independent transformants generated from cosmid p7-2). Parent, original transformant without subculturing; F7 No selection, transformants were single-spore subcultured seven times on arginine supplemented medium at 5-day intervals; F7 selection, transformants were single-spore subcultured seven times on medium without arginine supplementation at 5-day intervals.

Table 2 Mass Spectrometry Outliers as Identified by MD-PCA and SCP in Four Fungal Libraries

	Library 7	Library 10	Library 11	Library 12
Donor fungus	CR61	CR101	CR132	CR133
Fermentation medium	YES	LSF1	LSF1	LSF1
Mass spectral analyses	764	980	970	836
MD-PCA outliers	48	51	56	127
% of outliers by MD-PCA	6.3	5.2	5.8	15.2
SCP outliers with >90% confidence value	54	50	63	43
% of outliers by SCP	7.1	5.1	6.5	5.1
Outliers by both MD-PCA and SCP	46	34	32	28
% of outliers by both MD-PCA and SCP	6.0	3.5	3.3	3.6

MD-PCA, Mahalanobis distance—principal component analysis; SCP, sequential clustering procedure.

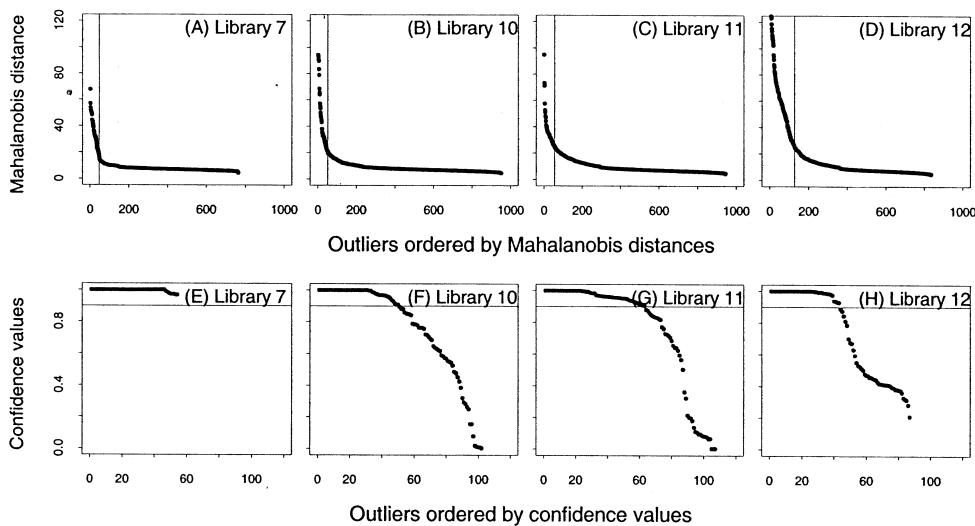


Figure 3 The Mahalanobis distances of all samples in libraries 7, 10, 11 and 12 (A–D) and the confidence values of outliers in libraries 7, 10, 11 and 12 that were detected by sequential clustering procedure (E–H). In A–D, vertical lines are drawn at the break of the curves to denote the cutoffs for outliers. Points to the left of the vertical lines have large Mahalanobis distances and are selected as outliers. In E–H, a horizontal line is drawn at 0.9 in each figure. Points above the line have confidence values of 90% or higher.

The Mahalanobis distance plot used to identify outliers in library 7 by Mahalanobis distance-Principal Component Analysis (MD-PCA) is shown in Fig. 3A. The vertical line in the figure indicates where a break occurred in the plot. The samples occurring before the break were selected as outliers. The library 7 SCP outliers are shown in Figs. 3E and 4 as filled dots and those with a 90% or higher confidence value were above the horizontal line in the figures. The difference between Figs. 3E and 4 is that the outliers were ordered by confidence values in Fig. 3E and the outliers were shown in sample numeric order in Fig. 4. Fig. 4 also shows that the outliers were randomly distributed throughout the entire library. MD-PCA and SCP identified 48 (6.3%) and 54 (7.1%) outliers, respectively, from the 764 strains in library 7 (Tables 2, 3). A total of 56 outliers were identified by either or both of two methods (Table 3), 46 (6.0%) of which were outliers identified by both methods (Tables 2, 3). Of the 10 nonoverlapping outliers, two were identified only by MD-PCA and remaining eight were identified by SCP (Table 3). All 54 outliers identified by SCP with the 5% cutoff criterion using three correlation measures have a bootstrap confidence value of at least 90% (Table 3, Fig. 3E). The two unique outliers identified by MD-PCA (B07T0414 and B07T0790) were ranked 47th and 48th among the 48 samples identified by this method and they are the two least significant outliers (Table 3). The difference between the median spectrum and the spectra of 8 representatives of the 56 outliers in library 7 is shown in Fig. 5.

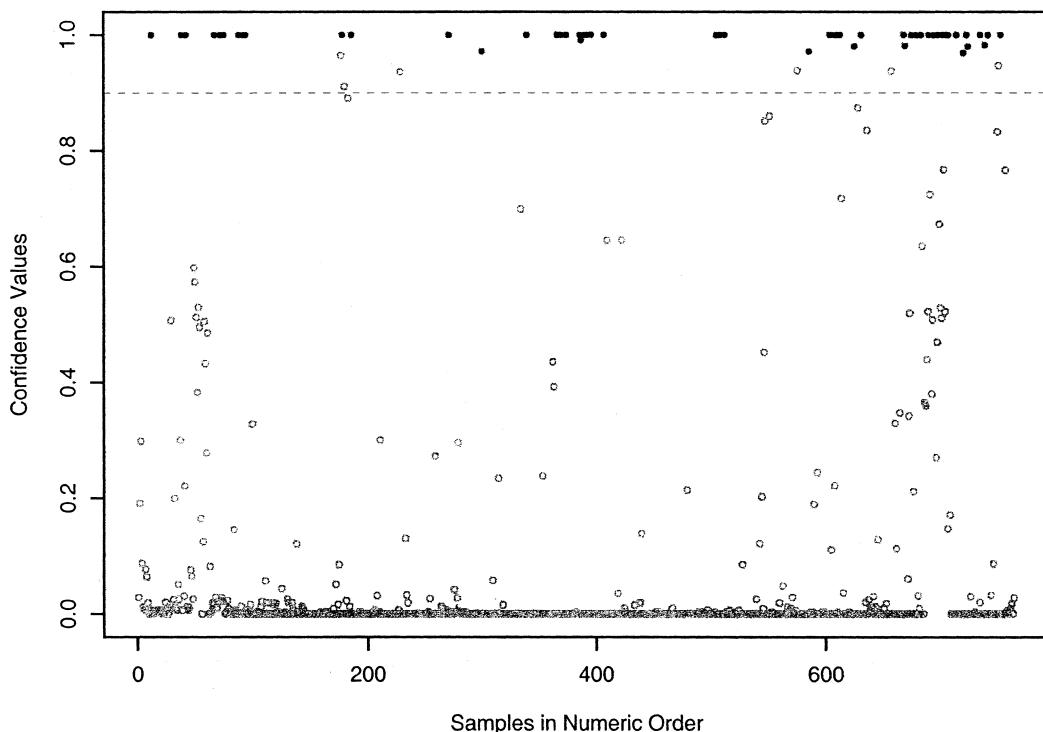


Figure 4 The distribution of the entire set of 764 samples in numeric order and based on their confidence values in library 7 as determined by the sequential clustering procedure. The outlying samples are marked with filled circles, and the remaining samples are marked with open circles. The horizontal dashed line shows the 0.9 cutoff.

3.4. Outlier Identification in Libraries 10, 11, and 12

The number of outliers identified by both methods from libraries 10, 11, and 12 were listed and shown in Table 2 and Fig. 3. The percentages of outliers as identified by MD-PCA for libraries 10, 11, and 12 were 5.2%, 5.8%, and 15.2%, respectively (Table 2, Fig. 3). The percentages of outliers with a confidence value of 90% or higher as identified by SCP for libraries 10, 11, and 12 were 5.1%, 6.5%, and 5.1%, respectively (Table 2). The numbers of overlapping outliers as identified by both methods were 34, 32, and 28 for libraries 10, 11, and 12, respectively (Table 2); these constitute 3.5%, 3.3%, and 3.6% of the total samples tested for libraries 10, 11, and 12, respectively (Table 2).

3.5. TLC Analysis of Recombinant Fungal Extracts

For TLC analyses, 604 strains (24 host cultures, 24 host/vector cultures, and 556 transgenic cultures) from library 9 (donor fungus CR70) were fermented in 2 mL of LSF1 medium in 24-well microplates and extracted with MEK. Fifteen of the 556 extracts showed distinctive TLC patterns as visualized with *p*-anisaldehyde/H₂SO₄ and UV. TLC pattern for one of the 15 outliers, A09T0567, is shown in Fig. 6. In addition to asperugin A, B, subsequent RP HPLC analysis of A09T0567 revealed several additional components not observed in the median extracts.

Table 3 Mass Spectrometry Outliers as Identified by MD-PCA and SCP in Library 7

Sample ID	$k^{\min a}$					
	Euclidean Distance	Pearson Correlation	Rank Correlation	Confidence Value	Mahalanobis distance	Method
B07T0211	5	6	5	1	67.76	Both
B07T0789	2	5	5	1	56.92	Both
B07T0691	2	2	2	1	53.56	Both
B07T0715	2	2	2	1	51.53	Both
B07T0791	2	2	2	1	51.04	Both
B07T0759	2	2	2	1	50.71	Both
B07T0767	2	2	2	1	50.39	Both
B07T0087	5	11	19	1	49.87	Both
B07T0763	2	2	2	1	49.40	Both
B07T0752	2	2	2	1	48.37	Both
B07T0447	5	11	19	1	44.27	Both
B07T0443	5	11	19	1	44.12	Both
B07T0786	5	9	17	1	42.87	Both
B07T0778	5	9	17	1	41.59	Both
B07T0779	2	2	2	1	41.19	Both
B07T0577	2	4	4	1	39.53	Both
B07T0774	2	2	2	1	39.38	Both
B07T0306	5	11	19	1	38.94	Both
B07T0012	2	2	2	1	36.14	Both
B07T0831	5	11	24	1	35.49	Both
B07T0391	4	7	16	1	34.83	Both
B07T0810	5	11	24	1	34.47	Both
B07T0782	5	9	17	1	33.29	Both
B07T0426	5	9	17	1	33.02	Both
B07T0573	2	7	5	1	32.23	Both
B07T0438	5	11	19	1	32.13	Both
B07T0451	5	9	19	1	31.92	Both
B07T0422	5	9	17	1	31.29	Both
B07T0462	5	11	24	1	30.88	Both
B07T0091	5	11	29	1	30.50	Both
B07T0200	5	15	18	1	29.62	Both
B07T0420	5	15	18	1	28.88	Both
B07T0079	5	11	29	1	27.59	Both
B07T0418	5	11	29	1	26.43	Both
B07T0115	5	11	29	1	24.05	Both
B07T0107	5	11	29	1	22.99	Both
B07T0042	5	11	29	1	22.80	Both
B07T0687	5	11	24	1	22.77	Both
B07T0847	5	11	24	1	22.54	Both
B07T0111	5	11	29	1	21.66	Both
B07T0038	5	11	29	1	20.81	Both
B07T0570	5	11	29	1	20.59	Both
B07T0679	5	11	24	1	19.58	Both
B07T0683	5	11	24	1	19.33	Both
B07T0439	5	11	29	0.99	18.16	Both
B07T0799	5	11	24	1	18.03	Both

(Continued)

Table 3 Continued

Sample ID	$k^{\min \text{ a}}$					
	Euclidean Distance	Pearson Correlation	Rank Correlation	Confidence Value	Mahalanobis distance	Method
B07T0414	92	54	>100	0.47	16.26	MD-PCA
B07T0790	51	36	>100	0.17	15.82	MD-PCA
B07T0347	5	24	29	0.97	13.88	SCP
B07T0822	5	11	24	1	13.01	SCP
B07T0827	5	24	81	0.98	12.72	SCP
B07T0753	5	24	57	0.97	11.88	SCP
B07T0707	5	24	>100	0.97	11.62	SCP
B07T0658	5	24	29	0.97	11.04	SCP
B07T0811	5	24	81	0.98	10.39	SCP
B07T0807	5	24	>100	0.97	9.68	SCP

^a k^{\min} in italics are not supported by the respective distance/correlation.

MD-PCA, Mahalanobis distance—principal component analysis; SCP, sequential clustering procedure.

Two outliers from library 7 based on their FIA-ES-MS profiles and statistical analysis, B07T0391 and B07T0573, were analyzed further with TLC. The two strains had different TLC patterns when compared with the typical background and host strains (Fig. 6). Both strains contained several new components not observed in extracts of the background strains (Figs. 5, 6). Reverse-phase HPLC analysis of the two cultures also showed clear differences from the typical transgenic background (data not shown). One of the major components from B07T0391 was identified as violaceol I (Fig. 6).

4. DISCUSSION

4.1. Donor Fungi

The secondary metabolites produced by the cranberry endophytic fungi are unknown, but the potential for producing novel bioactive chemical structures by these fungi exists because of their unique ecological niches. In a brief survey of evaluating the secondary metabolic potential of the cranberry endophytic fungi, we have cloned at least 12 ketosynthase domains of polyketide-encoding genes from 11 of the 23 isolates tested [39]. Because of the lack of morphological characteristics, these fungi were not readily identified. Phylogenetic analyses using SSU rDNA sequences suggests that CR61, CR68, CR132, and CR133 were unrelated ascomycetes and CR70 is most likely an oomycete.

4.2. Libraries and Transgenic Strains

Once high quality genomic DNA were obtained (50–245 kb), cosmid library construction was relatively straight forward. The Gigapack III cosmid packaging extracts from Stratagene were designed to preferentially package 47- to 51-kb recombinants. We found that it was still necessary to isolate DNA fragments larger than 40 kb by sucrose gradient centrifugation before packaging to obtain consistently high quality libraries. It is estimated that the genome size for most filamentous fungi is between 25 and 45 mb and with average size of about 35 mb [25]. Based on this estimation, we screened about 1000 transgenic

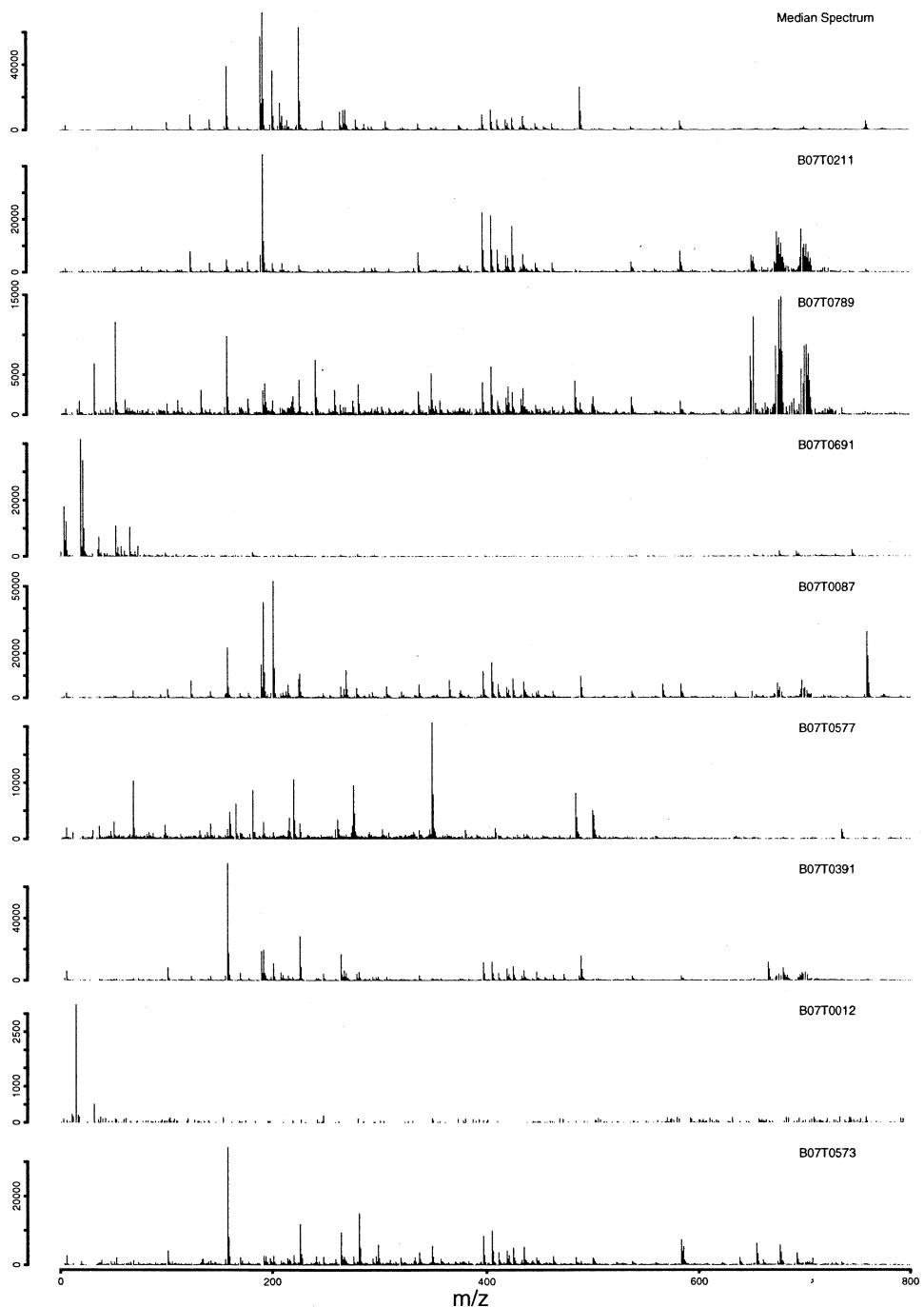


Figure 5 Mass spectra of 8 of the 56 outliers in library 7. For comparison, the median spectrum of what a typical sample should resemble is shown at the top of the column. The median spectrum is obtained by taking the median of each channel across all samples in the library.

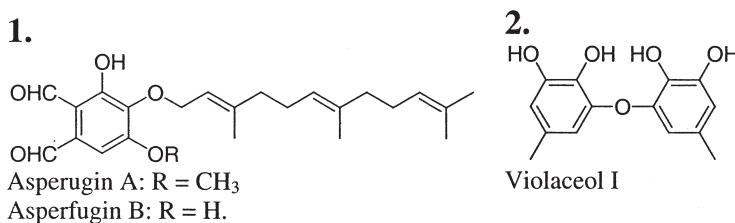
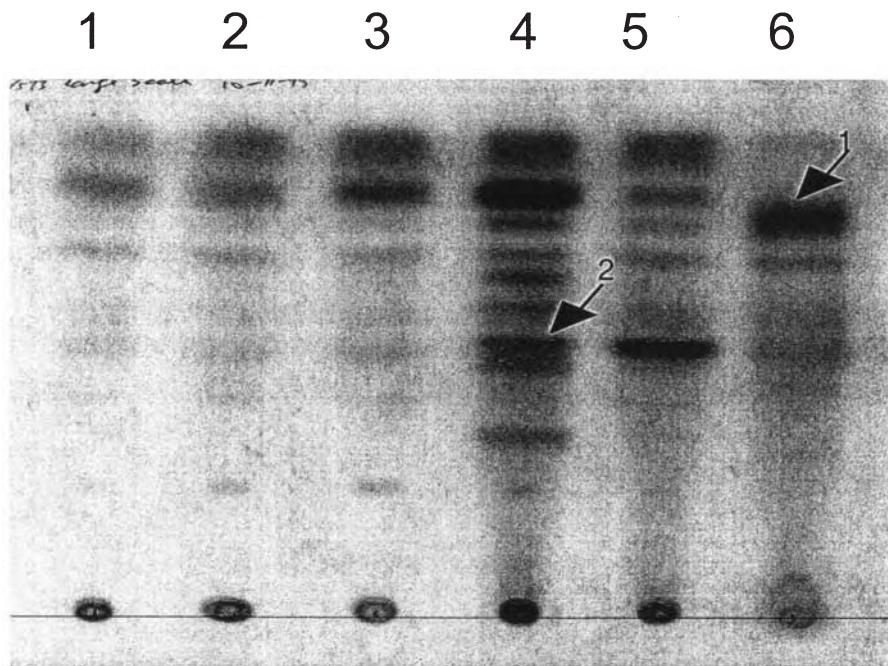


Figure 6 TLC patterns and structures of components from B07T0391 (lane 4), B07T0573 (lane 5), and A09T0567 (lane 6). Three controls were also included: the host (MF5999; lane 1), vector transformant (A09T00943; lane 2), and a background level transformant (B07T00399; lane 3). Two components were isolated from A09T0567 using preparative RP HPLC; the structures were elucidated from NMR and MS data as asperugin A (minor component) and B (major component). One of the major new components from B07T0391 was purified from a larger scale agar culture using Sephadex LH-20 and preparative RP HPLC. The isolated compound was identified as violaceol I.

strains for each library to give approximately 1X genomic coverage of the donor fungus. DNA integrated in the genome of *A. nidulans* and other fungi are, in general, mitotically stable [24,26–29]. This study further confirms that once DNA is integrated in the *A. nidulans* genome, it is not necessary to maintain selection during fermentation for stability of the heterologous DNA.

4.3. Fermentation and MS Analysis

Because of the large number of transformants generated in this experiment, a 24-well microplate fermentation method was developed. Microplate fermentation is relatively high

throughput and can be automated for downstream processing, such as addition of extraction solvent and other liquid transfer steps. One of major concerns with microplate fermentation is its reproducibility from experiment to experiment and from microwell to scale-up. A series of validation experiments, including cover design to prevent evaporation, well-to-well contamination testing with different fungi, and scale-up reproducibility testing with fungi producing known compounds, were conducted before it was used in this experiment. We found that the 24-well fermentation was highly reproducible, as evidenced by the number of spectra identical to controls, and with low cross-contaminations observed (<1%).

LC-MS tools have been found to be useful in taxonomy and secondary metabolite profiling of extracts. Smedsgaard and Frisvad [30] demonstrated that one could characterize the taxonomy and secondary metabolites of crude fungal extracts using FIA-ESI-MS methods similar to those described here. More recently, FIA-ESI-MS was used to measure the natural products productivity of actinomycetes under various fermentation conditions [31]. What was unique in our analysis was that we used the mass ion profiles to compare the overall similarities and differences among a large set of samples. Analyzing hundreds of genetically similar samples allowed us to define a background profile, and then we exquisitely detected differences with respect to this background using statistical tools.

4.4. Outliers in Library 7

An elbow existing in the Mahalanobis distance suggests that the small fraction of the samples deviating from the majority is not caused by random experimental variations. The usual approach for outlier detection using Mahalanobis distances is to use a critical value from the chi-squared distribution as the cutoff. This is based on the assumption that the data come from a multivariate normal distribution. We know, however, that the mass spectra data sets are far from this assumption. Therefore, we relied on the graphical approach, which is less exact but more useful. The vertical line defining the elbow in the Mahalanobis distance curve for this library can be readily recognized indicating that there was a clear boundary separating the small number of outliers (48) from the majority of background strains (716). Outliers identified by the two statistical models (MD-PCA and SCP) from library 7 overlapped significantly (82%). These results suggest that both methods are effective in identifying the most obvious outliers. Of the eight outliers identified only by SCP, five were supported by the Euclidean distance measure, one of the three measures used in the SCP analyses, suggesting they are probably marginal outliers. This is not surprising because Euclidean distance measure is more sensitive to experimental variations than that of the other two measures. Similarly, the two MD-PCA–unique outliers have the smallest Mahalanobis distance values among the 48 outliers identified by MD-PCA and they, therefore, are also marginal outliers. Outliers identified by both methods are very likely true outliers, but the so-called marginal outliers identified by one of the two methods may be rechecked in broad screening programs.

4.5. Outliers in Libraries 10, 11, and 12

The overlapping outliers identified by the two methods from libraries 10, 11, and 12 were 51%, 37%, and 20%, respectively. These numbers are much smaller than for library 7. It is not clear what caused the difference except that library 7 was fermented on a simple, defined, and solid medium under static conditions whereas the other three libraries were fermented in a complex liquid medium with agitation. Previous studies have shown that

fermentation media and culturing conditions play a major role in the morphology, physiology, and chemistry of fungi [32]. Using the same 5% cutoff criteria used for library 7, significantly more outliers were identified by the SCP method for libraries 10, 11, and 12 than found in library 7 (Fig. 3). However, the number of outliers that have a confidence value above 90% and the percentage of these outliers of the total samples was similar to the respective value from library 7. What we do not know is the importance of the outliers that have a confidence value higher than 90%. Elbows are also evident in the Mahalanobis distance curves for libraries 10, 11, and 12, but they are less defined than for library 7. This is particularly true for library 12. Consequently, 127 outliers were selected by the MD-PCA method. MD-PCA score plots of library 12 showed it to be a mixture of several—five or more—somewhat homogenous subgroups. There was a large overlap among subgroups; the data were scattered irregularly, however. Not surprisingly, with no clear pattern defining nonoutlying spectra, all the methods essentially found considerably different subsets that they considered unusual. This was also reflected in the relatively few points that passed the 90% bootstrap confidence criterion. In short, there was no typical pattern among the bulk of these data, and hence no clearly atypical patterns. There could be many reasons for this, ranging from biological variability to variations in fermentation conditions and sample storage and handling to MS measurement variations. We think it is prudent in such circumstances to apply a variety of methods that consider the data from several different perspectives.

4.6. TLC Analysis and Structure Isolation

As expected, TLC patterns for most of the cultures are similar because only a small fraction of the transgenic cultures are expected to have an altered chemical profile. A quick isolation of the dominant compound present in outliers B07T0391 and A09T0567 revealed structures that were not produced by the controls under the fermentation conditions used in this experiment. We believe that the production of these metabolites in the transgenic strains is most likely caused by the activation of silent biosynthetic pathways for these metabolites in *A. nidulans* by the transformation process, because both asperugin A, B and violaceol I are known metabolites of *Aspergillus* and *Emericella* species [21–23]. It is interesting that asperugin A and B were isolated from a mutant strain of *A. rugulosus* [34]. There are several possibilities how a silent pathway may be activated. For example, a transcriptional activator may have been introduced into the *A. nidulans* genome from the donor fungus and this transcription factor activated a silent pathway. It has been reported that some global transcriptional factors regulate the secondary metabolic pathways in fungi [35]. The structures of other HPLC fractions from these outliers have not yet been determined. One can expect that they might be structures encoded by secondary metabolite-encoding gene clusters from the donor fungus, or activation of additional silent host pathways, or hybrid compounds synthesized by genes from both the host and the donor fungus.

The approach described in this study generated a large number of transgenic strains. If a typical fungal genome is 35 mb [25], about 1000 cosmid clones are needed to have a 1X coverage of a given genome. This translates into about 1000 transgenic strains. It is difficult to estimate the number of secondary metabolic pathways for a given fungus, but a search of Chapman and Hall's *Dictionary of Natural Products* revealed more than 35 secondary metabolites produced by *A. nidulans* [36]. These metabolites represent at least 12 active distinct biogenic pathways. Assuming the average gene cluster size for

fungal secondary metabolic pathways is 34 kb and the *A. nidulans* genome is 30 mb, 12 biosynthetic gene clusters constitute about 1% to 2% of the fungal genome. The chances of having cosmid clones containing a complete large secondary metabolite-encoding gene cluster by screening libraries with only 1X genomic coverage is smaller than if libraries were large enough to cover the genome multiple times, but the increase of genome coverage would result thousands of more clones to screen. Libraries containing larger DNA inserts, such as BAC and YAC clones, would be preferable if available. If a typical BAC insert is 130 kb, one needs less than 1500 clones to cover a typical fungal genome five times. In this proof-of-principle study, we constructed libraries for individual fungi. In future discovery programs, it might be more desirable to construct a mega library that contains multiple fungal genomes. With the rapid sequencing of fungal secondary metabolite encoding genes and gene clusters, it is possible to prescreen libraries for clones that contain DNA fragments homologous to known natural product-encoding genes to create so called biased libraries. This biased library approach will significantly reduce the number of transgenic strains needed to evaluate.

While this study was being carried out, several similar experiments were being conducted by other investigators for heterologous expression of bacterial natural products encoding genes from environmental DNA. In one study, more than 1 Gbp of soil DNA was cloned in two BAC libraries and expressed in *E. coli*; some of the clones expressed antimicrobial activities [37]. In another study, BAC clones containing soil DNA fragments (5–120 kb) were expressed in *E. coli* and screened for antimicrobial activities; and screening of the library resulted in the identification of several clones that expressed antimicrobial activities [38]. In both cases, transgenic clones were screened for specific activities such as antimicrobial activities. In our approach, clones were first prescreened for novel chemical production potential based on their overall FIA-ESI-MS profiles. The novel chemistry outliers subsequently have been included in a library for screening against a broad set of therapeutic targets.

5. CONCLUSIONS

A transgenic approach was developed to capture the genetic diversity of slow-growing fungi for the production of secondary metabolites. Briefly, cosmid-size genomic DNA from slow-growing fungi was cloned and introduced into *A. nidulans* and the resultant transgenic strains were screened for altered secondary metabolite profiles. Our strategy included the following key steps: cosmid library construction; a 24-well microplate fermentation procedure; a semi-automated electrospray mass spectrometry (FIA-ESI-MS) protocol for characterizing the fermentation extracts; and two statistical methods for data analyses (MD-PCA and SCP). Between 3% and 6% of the mass spectra of transgenic strains were identified as significantly different from the background spectra. Isolation of dominant components from two outliers revealed natural products that were not produced by the controls under the fermentation conditions used in this experiment.

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8

The Isolation and Structure Elucidation of Fungal Metabolites

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The discovery of new fungal metabolites has been driven by the search for either novel chemical structures or for a desirable biological activity. The chemically guided discovery of new fungal metabolites relies on chromatographic and spectroscopic tools to detect new or unrecognized compounds. This approach is most useful from a chemotaxonomic perspective where the interest is in what metabolite(s) a particular fungus produces. In contrast, the focus of industrial natural products programs is almost entirely biological activity rather than interest in chemical novelty or chemotaxonomy. The power of the biological activity–driven approach is the detection of extremely low-titer metabolites, compounds that would be missed using the chemically guided approach. These low-titer and biologically active metabolites have a good probability of being new or even novel.

Industrial bioassay-guided natural products programs are multidisciplinary teams of scientists working in close association. Mycologists isolate and culture fungal strains from environmental substrates. The cultures are then passed to fermentation microbiologists for initial smaller scale culture. Extracts of these smaller-scale fermentations are prepared and screened in biochemical screens designed and executed by biochemists. On detection of a desired activity, natural products chemists employ chemical separation methodology to isolate the active component of the crude extract. The natural products chemist, in collaboration with specialists in organic spectroscopy, determines the structure of the metabolite. Finally, the large size of most industrial screening programs requires significant technical support from computer and automation specialists simply to generate the hundreds of thousands of extract samples and to track the data generated.

The purpose of this chapter is to provide a fungal metabolite centric overview of the natural products isolation and structure elucidation chemistry portion of this process.

The intended audience is the nonnatural products chemist with a need to either isolate a fungal metabolite/bioactivity present in a fungal extract or simply to understand the process better. A detailed description of each separation and structure determination method used by natural products chemists is beyond the scope of this chapter and would require volumes just for a meaningful overview. Neither is the goal to provide a general description of natural product isolation. Instead, an industrially biased, general three-step approach to the bioassay-guided isolation of natural products will be described as a framework to highlight aspects of natural products chemistry unique to fungal metabolites. This will be followed with a similar discussion of the dereplication and an overview of structure elucidation of the purified compounds. As will be evident, the bias of the chapter is toward the isolation rather than structure elucidation aspect of natural products chemistry. It is hoped that the nonnatural products chemist reader can use this information as an introduction to the fungal metabolite primary literature.

1. ISOLATION OF FUNGAL METABOLITES

Industrial natural products chemistry, more so than in academia, has been composed of three overlapping subdisciplines through much of its existence: isolation, structure elucidation, and perhaps synthetic modification of natural products. Today the most challenging of the three is the bioactivity-guided isolation of the pure compound. Rapid advances in the past 20 years in the spectroscopy of organic molecules, specifically nuclear magnetic resonance (NMR) and mass spectrometry, have decreased both the time and quantity of compound required for *de novo* natural product structure elucidation such that it is now more or less routine. For example, sufficient NMR data can now be acquired in a single overnight period on 1 or 2 mg of pure compound to allow proposal of a new structure. Often this is less material than required for an initial evaluation of the biological activity of the pure compound. In contrast, aside from the development and improvement of modern HPLC column materials, the bioactivity-guided isolation of natural products has not seen such major advances and in many cases is done the same way it was 20 or 30 years ago.

The isolation portion of the bioassay-guided isolation and structure elucidation process is often downplayed, misunderstood, and portrayed as time consuming. This perception is reinforced in the natural products literature. The bulk of most literature reports of new natural products describe the details of the structure elucidation with perhaps only one or two paragraphs describing the isolation if it is not simply relegated to the experimental section of the report. The difficulties encountered during the isolation of a new natural product or the strategy used for its bioassay-guided isolation are rarely discussed. The result is that those not skilled in the art have few sources of information about how to undertake the isolation of a fungal metabolite. The trial-and-error nature of the bioactivity-guided isolation can be reduced if the entire process, from crude extract to pure compound, is approached systematically. One such systematic approach is described below. This three-step approach is not novel and has many similarities to protein isolation. As will be shown from a literature survey of fungal metabolite isolations, it is a useful model to understand the fungal metabolite isolation process.

1.1. Extraction from Fermentation Broths

Fungal cultures are complex mixtures of mycelia, media components, and varying amounts of water. Disruption of the mycelia and solubilization of the compounds of interest is generally required for bioassay and secondary metabolite isolation. Usually this involves

treatment of the fungal culture with an organic solvent followed by clarification to yield an extract. The details of this process are determined by the physical properties of the compounds of interest and the type of fermentation, submerged liquid or solid substrate.

Submerged liquid fermentations can either be extracted whole or clarified and the resulting solids and culture broth separately extracted. Whole-broth extraction by addition of one to several volumes of a water miscible solvent such as acetone or an alcohol is common. The solids are removed by filtration or centrifugation resulting in a clarified aqueous extract. Since it is aqueous based, this extract is well suited to solid-phase extraction (SPE) on polymeric resins as described below. Alternatively, the organic solvent can be removed *in vacuo* and the resulting aqueous solution extracted with a water immiscible solvent chosen according to the polarity of the target compounds. The solids can be further extracted but this is rarely necessary. Alternatively, the whole broth can be clarified by filtration or centrifugation prior to extraction. This is particularly effective if the desired component(s) are associated with the mycelia and the fermentation media does not contain insoluble components.

Submerged liquid fermentations can also be directly extracted with a water immiscible organic solvent. Ethyl acetate (EtOAc) is commonly used for this purpose and efficiently extracts typical fungal secondary metabolites. The organic phase is removed, washed with water, dried, and the solvent removed *in vacuo* to yield a residue suitable for column chromatography. The only potential pitfall to this approach is emulsion formation or compound instability on concentration to dryness. The remaining aqueous layer can also be further extracted with a water miscible solvent for the recovery of polar metabolites as described above.

Solid substrate fermentations, such as those on grains, inorganic supports or agar, must be extracted whole since the mycelia and support cannot be separated. Solvent choice depends on the solubility of the compound of interest. After solvent addition, the mixture is stirred or shaken for several hours, the solids removed by filtration, and the filtrate further processed as required.

The pH of submerged liquid fermentations, and solid substrate fermentations extracted with an aqueous based solvent, can affect the recovery of fungal metabolites as well as their stability. The harvest pH of liquid fermentations should be noted prior to extraction and provides a reasonable starting point for component stability. Unbuffered fungal fermentations tend to be slightly acidic. The efficiency of extraction of ionizable components, especially into water-immiscible solvents, is pH dependent.

Extraction with a water-miscible solvent results in a mixture of cell debris, media components, and organic solvent that generally must be clarified before any chromatographic separation. Clarification can be accomplished using either filtration or centrifugation. The choice depends on the volume of the extract, the properties of the extract, and the available equipment and is summarized in Fig. 1. Vacuum filtration is suitable for extract volumes of 0.1 to 100 L. Centrifugation, using a commonly available laboratory centrifuge, is useful for volumes up to around 10 L. Laboratory scale continuous flow centrifuges are available and enable the rapid separation of solids from whole-broth volumes of tens to hundreds of liters. Analytical samples, volumes up to a few milliliters, can be clarified using a high-speed benchtop microfuge or using syringe filters.

1.2. Bioassay-Guided Isolation Process

Bioassay-guided isolation of fungal metabolites from fermentation samples has much in common with assay-guided protein purification [1]. Both begin with the detection of a biological activity in a crude preparation. The crude activity is evaluated for selectivity,

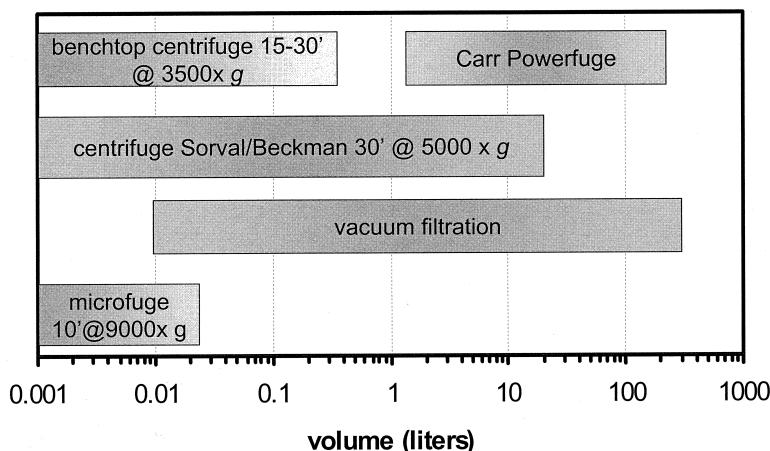


Figure 1 Useful sample volume ranges for common extract clarification methods.

potency, specific activity, etc. as required to determine interest. Fractionation of the crude preparation or extract is then guided by the assay. A sequence of separation methods for fractionation are chosen to maximize activity recovery while simultaneously increasing the specific activity of the preparation until a chemically or biochemically homogenous sample is obtained.

1.2.1. Metabolite Stability and pH Control

Protein purification and fungal metabolite isolation also share some frequently encountered difficulties (Table 1) that can result in loss of biological activity. The most common of these for fungal metabolites is pH-dependent instability/stability. Fortunately this source of instability can be controlled by conducting extract fractionation at the pH of optimum stability for the desired compound or activity using a buffer. A further advantage of the use of buffers, or at least some form of pH control, in the isolation process is that the ionic form of any ionizable metabolites should be understood. The disadvantage of using buffers for small molecule separations is that desalting of the final product is required prior to structure elucidation.

1.2.2. Biological Assay Interferences

Most of the reported fungal metabolites are generally soluble in organic solvents and aqueous-organic mixtures. The solubility of fungal extracts and fractions is not a general problem for chromatographic separations but it can be for bioassay. The lipophilic character of most fungal metabolites can cause solubility problems in an aqueous bioassay, especially if high concentrations of material are required for activity. It has recently been shown that the activity of some nonselective and moderately active pure compounds commonly found in industrial compound collections was due to physical aggregation phenomena rather than true biological activity [2]. Although this phenomena has not been studied specifically for natural products and natural product extracts its occurrence is plausible. Assay solubility problems can change as the active component is purified through the removal of impurities that can either aid solubility or themselves cause insolubility.

Crude fungal extracts may contain compounds that are not specifically active in an assay but interfere with bioactivity detection in some way. Free fatty acids are ubiquitous

Table 1 Common Problems of Protein Purification and Fungal Metabolite Isolation

Problem	Protein Purification	Fungal Metabolite Isolation
pH	Buffered for stability and optimum activity	No pH control Control of ionic form Buffered for stability
Solubilization	Detergents added to reduce hydrophobic interaction	Added agents not necessary—either organic or aqueous solvents can be used Solubility problems sometimes encountered when preparing samples for bioassay Aggregate effects?
Oxidation prevention	Reducing agents (e.g., DTT) added to prevent oxidation of thiols	Oxidation not limited to thiols
Metals	Chelating agents (EDTA) added to remove non-specific metals	Usually not a problem Metal complexes of fungal metabolites are known
Proteases	Protease inhibitors (e.g., PMSF) added to buffers	Not a common problem since nonribosomal peptides are generally less susceptible to nonspecific proteases Presence of proteases in fungal crude extracts can interfere with peptide-based bioassays (i.e., targeting protease inhibitors)
Concentration to dryness	Not commonly performed for proteins	Can be problem for reactive compounds (i.e., polyacetylenes)

and are found in titers ranging from less than 0.001 to 1 or 2 mg/L or more in fungal extracts. Depending on the assay system, unsaturated fatty acids may be real, but undesirable, actives that can mask or hinder the detection of active secondary metabolites. Strongly (UV) adsorbing and/or fluorescent fungal metabolites can quench or otherwise interfere with assay methodology involving detection of a fluorescent substrate. Fungal enzymes, particularly proteases, can lead to degradation of peptide substrates used for protease assays. A more detailed discussion of assay methodology as well as some of these issues is presented elsewhere in Chapter 10.

1.2.3. Activity Quantitation

Bioassay-guided isolation requires a system to quantitate activity. One such system is based on the IC₅₀ of the crude extract and its various subfractions. The total number of units of activity in the crude extract can be measured by titration of the sample and determination of an IC₅₀. One IC₅₀ unit is the amount, weight or volume, of a sample sufficient to be active at the measured IC₅₀. The total number of IC₅₀ units can be defined for a single active component, A, as follows:

$$\text{Total IC}_{50} \text{ units sample} = \left(\frac{V}{\text{IC}_{50}A} \right) \quad (1)$$

where

V = the total, initial, extract volume, in mL whole-broth equivalent (WBE)

$IC_{50}A$ = measured value for the extract in units of mL extract/mL assay volume

For example, if 0.010 mL of a crude extract, of total volume 1000 mL (1000 mL WBE), was sufficient to yield the an IC_{50} ($IC_{50} = 0.010$ mL WBE/mL), then the total IC_{50} units of the sample would be 100,000. Next, suppose the entire sample was subjected to column chromatography on silica gel. The activity recovery from the column can be determined by measuring the IC_{50} of the combined active fractions (rich cut) containing component A and comparing it with the total IC_{50} units of activity with that applied to the column. In the above example, if the combined active fractions exhibited an IC_{50} of 0.012 mL WBE/mL the total activity recovered would be 83,333 total IC_{50} units. The resulting activity recovery from the column would be 83.3%. The specific activity enrichment can be determined from total solids measurements of both the crude extract and the column rich cut.

Multiple active components, a common occurrence, can be treated by an extension of the same method. The total IC_{50} units present in the original extract is now the sum of the activity from each of the active components, A, B, etc.:

$$\text{Total } IC_{50} \text{ units sample} = \left(\frac{V}{IC_{50}A} \right) + \left(\frac{V}{IC_{50}B} \right) + \dots \quad (2)$$

As an extension of the above example, suppose the silica gel fractionation of the extract resulted in two rich cuts, containing components A and B, with IC_{50} s of 0.020 mL WBE/mL and 0.030 mL WBE/mL, respectively. The total activity recovered would be 50,000 and 33,333 IC_{50} units, respectively, for a total activity recovery of 83,333 IC_{50} units. Measurement of total solids of each rich cut can provide information about the relative potency of the two components.

The above equations can be extended to handle unstable components. As an approximation we can assume that an unstable component decomposes according to first order reaction kinetics and add a concentration term, at any given time, t , to the above equations. Thus, Eq. (1) becomes:

$$(\text{Total } IC_{50} \text{ units sample})_t = [A]_0 \exp(-kt) \left(\frac{V}{IC_{50}A} \right) \quad (3)$$

where

$[A]_0$ = the initial concentration of component A

k = the rate constant for the decomposition of component A

Similarly, Eq. (2) becomes:

$$(\text{Total } IC_{50} \text{ units sample})_t = [A]_0 \exp(-kt) \left(\frac{V}{IC_{50}A} \right) + [B]_0 \exp(-kt) \left(\frac{V}{IC_{50}B} \right) + \dots \quad (4)$$

In reality it is not possible to know the rate constants for the decomposition of individual components of an active mixture, at least in the early stages of characterization. Equation (4) is useful, however, to illustrate the difficulty of detecting and isolating unstable fungal metabolites, especially when coproduced with other metabolites. For example, consider a hypothetical mixture of a stable compound, A, and a relatively unstable compound, B,

with the following properties:

	A	B
Titer at $t = 0$	0.1 mg/mL	0.01 mg/mL
IC_{50}	0.01 mg/mL	0.001 mg/mL
Half-life	347 days	3.47 days
k	0.002	0.2
Volume	1000 mL	1000 mL

The titers and activities above were chosen so that A is present at a 10-fold higher titer than B but also so that B is 10-fold more active than A. The effect of the unstable activity of compound B on the potency and total activity of the initial mixture over time is shown in Figs. 2 and 3.

Complete decomposition of compound B only decreases the total activity units of the mixture by 2-fold (Fig. 2). The corresponding IC_{50} of the mixture increases, becomes less active, by only 2-fold (Fig. 1). This is particularly alarming because a 2-fold loss of activity in many bioassays might be attributed simply to assay variation resulting compound B being missed entirely. The solution to this dilemma is an effective initial fractionation of the crude extract (see section 1.3) that resolves the two activities. Once resolved from compound A the instability of compound B should be easier to detect and then ways to control it can be explored.

1.3. Fungal Metabolite Isolation as a Three-Step Process

1.3.1. The Art of Fungal Natural Product Isolation

As a general rule, fungal metabolites, or any natural product, cannot be purified to homogeneity in a single step. The isolation of any fungal metabolite is thus a multistep process

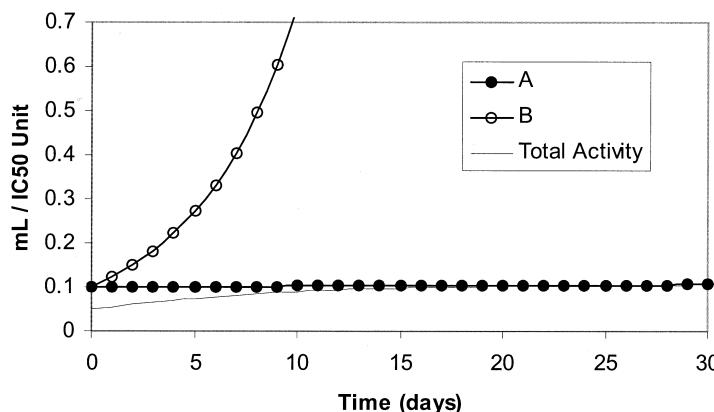


Figure 2 Observed time-dependent potency (in mL/ IC_{50} unit) of a hypothetical mixture of unstable (A) and stable (B) components. Details of component stability, activity, and relative concentration are given in the text.

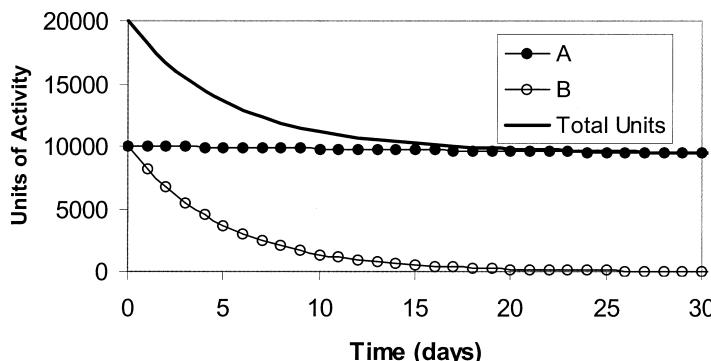


Figure 3 Observed time-dependent total activity (as measured in IC_{50} units) of a hypothetical mixture of unstable (A) and stable (B) components. Details of component stability, activity, and relative concentration are given in the text.

involving several different chemical separation methods. There is not a correct way to isolate a specific fungal metabolite but there are processes that are more efficient and elegant than others. Efficiency in natural product isolation, just as in natural product total synthesis, is a relatively objective thing that can be measured by the time required, the overall yield from the crude extract, or the number of total steps used. Elegance in isolation is also analogous to elegance in natural product total synthesis and is a largely subjective thing. An elegant natural product isolation might be one that employs two or three well designed, orthogonal (see below), and maybe even unique, separations resulting in a high yield of a relatively minor fungal metabolite.

Crude extracts are complex mixtures of primary and secondary metabolites as well as fermentation media components. The individual amounts of each can range from trace to 50% of the total solids. Analytical separations with chemical detection, such as UV adsorption, easily reveal the major, and even intermediate concentration, components of a crude extract. It is the low level and trace components that complicate purification of fungal metabolites. It is not uncommon for an apparently pure HPLC peak in a chromatogram of a crude extract, or other partially purified sample, to be a mixture of two or more components. Detection of the minor components of such a mixture depends on the methodology applied to the sample. For example, a chromatographic peak may be homogeneous by UV adsorption detection but a mixture if analyzed with mass spectroscopic detection. If the major, chemically detected component is the one of interest then the minor components are generally of little consequence. If, however, biological activity is the detector applied to such a mixture, the minor component(s) are as important as the major component until further resolved.

A systematic, three-step approach to the isolation of fungal metabolites is presented below. As previously noted, the isolation of fungal metabolites, as well as other natural products, is similar to the isolation of proteins. Biochemists have developed generally applicable, multistep approaches to protein isolation. Each of the steps has a predetermined purpose. If one accepts that a fungal metabolite cannot be purified in a single step then it is instructive to use the same framework to examine the entire fungal metabolite isolation process. Three distinct steps in the isolation of a fungal metabolite are described below:

step 1, recovery and resolution; step 2, purification; and step 3, polishing. The purpose, selection criteria for methodology, and commonly used separation techniques are discussed below and summarized in Table 2.

1.3.2. Step 1: Recovery and Resolution

Goal

The initial separation of a fungal extract is the most important step in the isolation process. This step has two purposes. First, a substantial increase in the specific activity of the active component(s) should result. Fungal fermentations on complex substrates or in undefined liquid media yield extracts that are usually high in total solids. The bulk of these solids are media components, partially fermented, which require removal prior to purification of the metabolites of interest. Second, the process should provide a biological resolution of multiple active components. Multiple active components, related and unrelated, are frequently produced by fungal fermentations. A biologically pure sample, containing only one active component, of each activity is extremely useful for characterization of an activity observed in a screening extract. Partially purified samples are generally more compatible with secondary evaluation assays than crude screening extracts. As seen above, resolution of an unstable activity from a stable coproduced one may be required to allow its initial detection. The degree to which these two goals are achieved often determines the difficulty of the remainder of the isolation process and sometimes, even more importantly, whether an active component is detected at all.

Methodology Constraints

The recovery and resolution step is more demanding of separation methodology than the downstream isolation steps. Thus the choice of generally useful techniques is restricted since at the crude stage relatively little is known about the physiochemical properties of the active component(s), the extract volume to be processed is usually large, solubility can be limited, and conditions for optimal stability may be unknown or poorly defined.

A general, first-step separation method should have the following characteristics:

1. Complete recovery of the desired biological activity. To ensure the highest recovery of active component(s), the separation methodology must be chemically gentle. Solid-phase adsorbants should be chosen for maximum solute recovery. For example, polymeric reverse-phase materials generally yield greater solute recovery than those based on silica. The separation methods should be effective at the extract pH, to allow isolation of native species, or be compatible with buffers to allow control of the ionic form of components or for optimal stability as necessary.
2. Moderate resolution. The goals of the recovery and resolution step require only moderate chromatographic resolution.
3. Scalability. Initial evaluation of bioactive fungal extracts is generally performed at a small probe scale (5–50 mL culture). This allows processing of relatively large numbers of active extracts to identify the smaller number of true interest for complete isolation and structure elucidation. Direct scaleup of the probe methodology to volumes of several liters of culture eliminates the need to pilot a new process for the initial large-scale isolation.
4. Reproducibility. Chromatographic retention behavior in a standardized initial fractionation process can be exploited to group extracts containing the same component. A typical primary screening assay yields tens to even hundreds of active extracts. To use this information effectively the process must be reproducible.

Table 2 Natural Product Isolation as a Three-Step Process

Step	Purpose	Selection Criteria		Methodology
			Commonly Used Techniques	
1. Recovery and resolution	Weight enrichment of activity	High recovery of solutes/activity	Sequential liquid partition	
	Removal of media components	Scalable	Differential pH partition	
	and primary metabolites	Moderate resolution	Adsorption/elution on polymeric adsorbants	
			Countercurrent chromatography (CCC)	
2. Purification	Biological resolution of multiple active components	Reproducible	Sephadex LH-20 chromatography	
	Purification of active component(s)	Automation potential	Normal phase column chromatography	
	Associate activity with extract component(s)	Orthogonal to step 1	Medium resolution reverse phase chromatography	
		Polarity, physical properties of active component(s)	Ion exchange	
3. Polishing	Preparation of final pure compound	Generally high resolution	CCC (all modes)	
			Sephadex LH-20 chromatography	
			Preparative reverse-phase HPLC	
			Crystallization	
			CCC	

5. Automation potential. Industrial screening programs typically yield tens to hundreds of active crude extracts depending on the size of the extract library. Repetitive execution of the initial isolation step for such large numbers of extracts is not only less tedious but also more robust and reproducible if it is automated.

Generally Useful Separation Techniques

SOLID-PHASE EXTRACTION. The typical aqueous methanol or aqueous acetone clarified fungal extract is suited to SPE. Polymeric adsorption resins fulfill all of the criteria for the recovery and resolution step listed above. The most useful for the general extraction of fungal metabolites from aqueous crude extracts are neutral polystyrenedivinylbenzene polymers. A wide range of particle sizes and polymer crosslinking is available. Particle size choice is based on the scale of the separation and the resolution desired during elution. Smaller particle sizes favor resolution and are generally used for smaller samples and combined with gradient elution. Larger particle sizes are typically used for larger scale processing of tens of liters or more. The industrial use of these resins for solid-phase metabolite capture has been described.

The separation mechanism on a high-quality resin is almost entirely adsorption. An aqueous alcoholic or acetone clarified extract is adjusted to a sufficiently low organic modifier strength to allow adsorption of the desired component(s) to the resin. This is accomplished by either dilution of the extract with water or removal of the organic solvent component *in vacuo*. After sample loading the resin is thoroughly washed to remove unadsorbed impurities. The desired component(s) or activity is then eluted with an increase in the organic solvent component. This can be done in steps or with a gradient.

LIQUID-LIQUID EXTRACTION. The lipophilic nature of most fungal metabolites allows their recovery from aqueous methanol or aqueous acetone fermentation extracts by extraction into an organic solvent. Typically, the organic extraction solvent, methanol or acetone, is removed by concentrating the crude extract *in vacuo* and then the mostly aqueous solution is extracted with an organic solvent. Ethyl acetate is a good general solvent for liquid-liquid extraction since it is easily dried, has a relatively low boiling point, and is relatively polar. A sequential series of solvents of increasing polarity and corresponding ability to extract metabolites can also be used [3]. The pH of the aqueous solution can be adjusted as necessary for more efficient extraction of ionizable metabolites.

Liquid-liquid extraction is not the perfect solution to the recovery and resolution step. Advantages to it are simplicity, high recovery, and the chemically gentle nature. Good increases in specific activity, when compared with the total solids of the aqueous methanol or acetone extract, are usually obtained. Concentration of the organic phase is easy and the resulting residue is ready for column chromatography. A disadvantage is that little or no resolution of multiple active components is obtained. Even the use of a sequence of increasing polar solvents (e.g., hexane, methylene chloride, followed by ethyl acetate), provides little resolving power and occasionally distributes a specific metabolite in more than one solvent.

The distribution coefficient of ionizable fungal metabolites subjected to liquid-liquid partition can be altered by varying the pH. For example, to favor the distribution of acidic compounds into an organic phase, the pH of the aqueous phase can be lowered a pH unit or more below the pKa of the acidic moiety of the compound. Similarly, the pH of the aqueous phase can be increased above the pKa of basic compounds to favor distribution into the organic layer. Controlling the pH of a liquid-liquid partition also allows control of the ionic form of a metabolite. Multiple extractions at different pHs, also known as

differential pH extraction, can be a highly effective way of increasing the specific activity of ionic compounds. This technique has been used for over a hundred years to prepare crude alkaloid preparations, generally secondary or tertiary amines, from plant crude extracts.

Countercurrent Chromatography and Sephadex LH-20. These (see methodology descriptions below) have also been effectively used for recovery and resolution although literature reports of their use for this purpose is rare. Both techniques are most effective when an organic extract of a crude fermentation is the feed to the separation since they require lower volume sample application. Both techniques fulfill most requirements for the recovery and resolution step with direct scaleup the only potential disadvantage. A specific form of CCC, dual-mode, was effectively used in a parallel fashion to fractionate small (< 100 mg) samples of 2-butanone extracts of fungal fermentations [4]. High specific activity gains were achieved and the retention information was useful for dereplication (see below). Sephadex LH-20 has also been used in a similar fashion.

1.3.2. Step 2. Purification

Goal

Successful execution of the recovery and resolution step results in a sample with improved specific activity, more defined solubility properties, a much smaller sample volume, and probably better characterized biological activity. The goal of step 2, the purification step, is to associate the biological activity of this active material with a specific component or components while simultaneously increasing its purity. A good portion of the art of bioactivity-guided natural product isolation lays in the careful selection of one or two separation methods for the purification step. Few absolute constraints exist for the choice of which separation method(s) to use but three factors can be useful when evaluating potential methods: (1) sample solubility/component polarity, (2) the presence of ionizable functional groups in the component of interest, and (3) the orthogonality of the separation method to the one used previously in the recovery and resolution step.

METHODOLOGY CONSTRAINTS. Sample solubility and component polarity are loosely associated. Sample solubility in a solvent suitable for applying the sample to the separation method is a general requirement and can guide method choice. For example, nonpolar samples soluble only in hydrocarbon solvents are poor candidates for reverse-phase separations and, conversely, samples soluble only in water or alcoholic solvents are poor feed samples for silica column chromatography. Thus, for nonpolar separations a normal phase mode technique such as silica gel column chromatography might be a reasonable choice. Similarly, for the polar sample, a reverse-phase separation method or perhaps ion exchange (see below) might be the first choice. Fortunately, such extremes are not the rule. Occasionally, components can have wildly different apparent polarity behavior in a separation method than might be expected from the gross solubility of the sample.

A bewildering number of chromatographic stationary phases exist for the purification of fungal metabolites. How does one choose a sequence of separations? To simplify method selection it is important to remember that this large number of stationary phases exploit only a limited number of actual separation mechanisms. Mechanistic interactions of solutes with neutral, solid supported stationary phases are either predominantly through partition or adsorption. Both operate in either normal (polar stationary phase) or reverse-phase (nonpolar stationary phase) modes. Ion exchange resins function predominantly through ionic equilibria between solute and stationary phase and are either anionic, cationic, or mixed mode.

Sequential combination of purification methods that exploit completely different separation mechanisms is the most effective strategy for fungal metabolite purification in the minimal number steps. Such combinations of separation techniques can be viewed as having orthogonal mechanisms. Examples of orthogonal pairs of separation methods are silica gel adsorption chromatography followed by reverse-phase partition chromatography or reverse-phase adsorption chromatography on polymeric resins followed by ion exchange.

The effect of using a purification step separation method orthogonal to that used for the recovery and resolution step is illustrated using the example shown in Figs. 4 and 5. Compound C is the target compound. Panel 1 of each figure illustrates a hypothetical rich cut obtained after a reverse-phase column chromatography Resolution and recovery step applied to a crude acetone fermentation extract (as analyzed using analytical reverse-phase HPLC). If this mixture is then subjected to reverse-phase HPLC, a nonorthogonal separation method, for the purification step, the resulting active rich cut is likely to be similar to that illustrated in Fig. 4, panel 2. Components A, B, F–H, and some of E were removed and the specific activity of rich cut is higher but the resulting rich cut still contains compounds D and some of E, the most difficult separation problem, making a second purification step, and third overall isolation step challenging. in contrast, if the same rich cut from the resolution and recovery step is further fractionated with an orthogonal separation method, such as normal phase adsorption chromatography on silica gel, the hypothetical rich cut mixture shown in Fig. 5, panel 2 might result. This mixture contains a broader polarity range of compounds including A, B, and E as well as C and is likely to be of lower specific activity than that obtained after the hypothetical RP HPLC step above. Importantly though, the most challenging separation problem for reverse-phase HPLC,

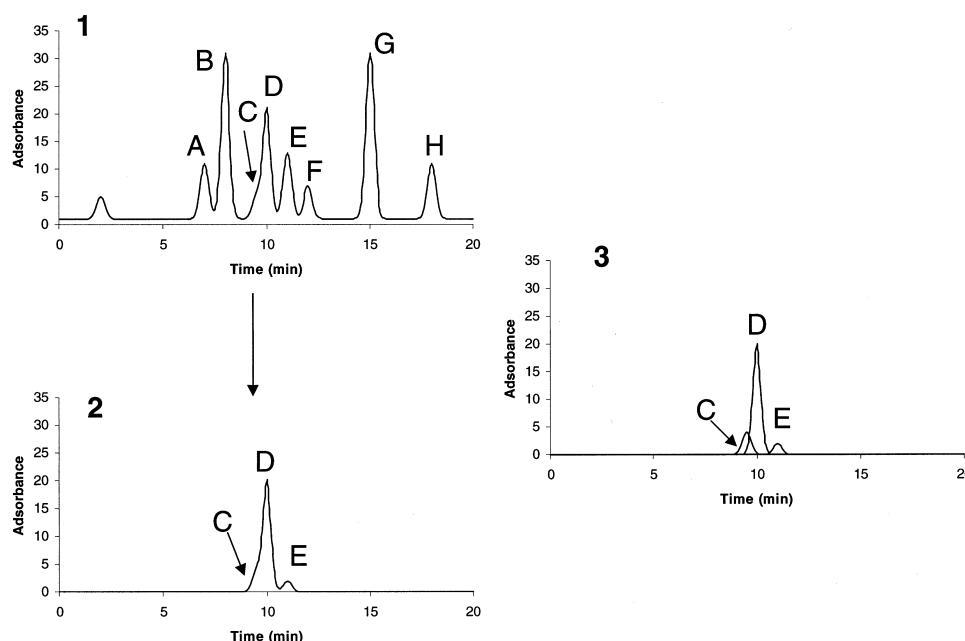


Figure 4 Separation of a hypothetical mixture of components A–G (panel 1) using a nonorthogonal separation method.

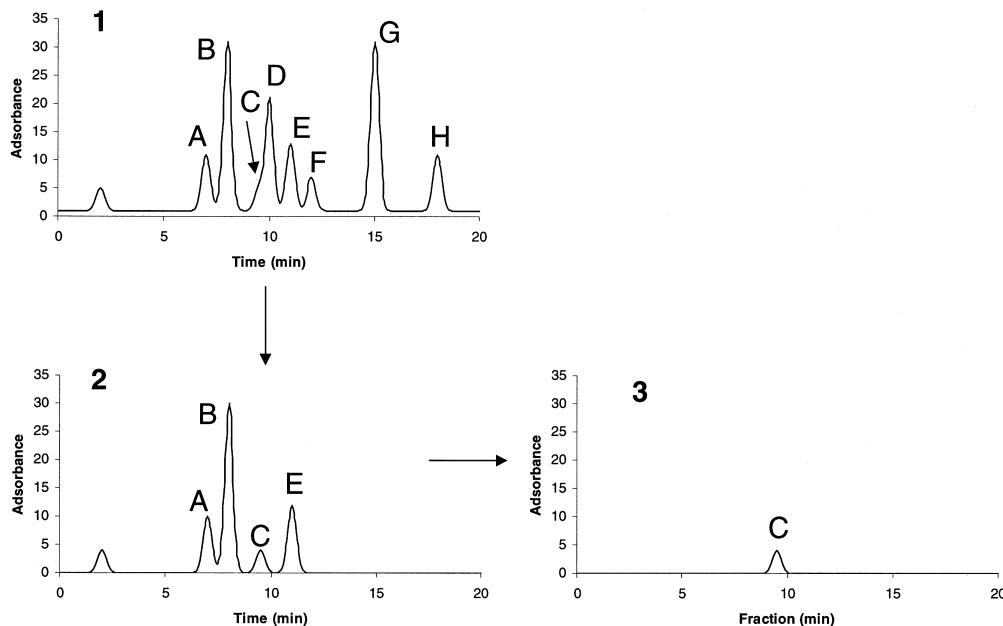


Figure 5 Separation of a hypothetical mixture of components A–G (panel 1) using an orthogonal separation method.

compounds C and D, has been eliminated since the silica gel separation completely removed compound D from the active rich cut. Thus, a second purification step and third overall isolation step using preparative reverse-phase HPLC should yield pure compound C.

Generally Useful Separation Techniques

NORMAL-PHASE ADSORPTION CHROMATOGRAPHY. Normal-phase adsorption chromatography on underivatized silica gel has been routinely employed for the isolation of fungal metabolites. For example, approximately 60% of the new fungal metabolite isolations reported in the *Journal of Antibiotics* between 1997 and 2001 used silica gel chromatography at some point during the purification sequence. Most forms of silica chromatography were represented and included classical, open-column, gravity-fed separation on 40- to 63- μm particles, medium pressure liquid chromatography (MPLC), preparative thin-layer chromatography (TLC), and HPLC. This general utility correlates with the nonpolar to moderately polar, organic soluble, small molecule character of the majority of reported fungal metabolites.

Normal-phase mode chromatography on polar stationary phases is perhaps the most commonly used separation method for fungal metabolites. Silica gel, or a polar derivative of silica gel, is the most frequently used stationary phase and is combined with organic mode phases ranging in polarity from hexane to methanol. Adsorption interaction of solutes is predominantly through hydrogen bonding with the silanol groups of the silica gel. Generally, the more polar the solute the stronger the adsorption. Exceptions are generally due to the presence of ionizable functional groups in the solute (carboxylic acids and amines). Retention of solutes is controlled by the mobile phase polarity and the relative elution strength of various organic solvents is known as an eluotropic series. The reader

is referred to the numerous literature descriptions of the mechanism and practice of normal phase adsorption chromatography for complete information [5].

Silica gel is slightly acidic in character due to the presence of particularly active silanol residues. This can lead to decomposition of acid sensitive metabolites and irreversible adsorption of solutes. The presence of ionizable functional groups (e.g., mono- or polycarboxylic acids, amines) in a molecule can result in stronger retention than expected from observed solubility. Diol silica can be an alternative for such problematic compounds. Its use has been reported for a small percentage of fungal metabolites (Table 4).

REVERSE-PHASE CHROMATOGRAPHY. In one or more of its forms, this is used in most fungal metabolite isolations. Reverse-phase chromatography media are either a silica or a polymer bead derivatized with a lipophilic bonded phase. Aqueous acetonitrile or methanol is typically used as a mobile phase with eluent strength increasing with increasing organic content. The separation mechanism on bonded phases such as octyl (C8) or octyldecy (C18) is generally partition. Underivatized neutral polymeric resins (e.g., Mitsubishi HP series, the Rohm and Haas XAD series, Hamilton PRP-1, and nonfunctionalized Amberchromes) are also used for reverse-phase chromatography. The same mobile phases are used as for the bonded phase materials, but the separation mechanism is adsorption rather than partition.

Reverse-phase chromatography can be performed in forms ranging from low-resolution open columns and vacuum bed separations to high-resolution HPLC. Resolution, and the corresponding equipment demands, increases with decreasing particle size of the packing material. For the three-step strategy described herein the highest possible resolution is not always necessary for the purification step. How the reverse-phase separation fits (its orthogonality) with the previous and subsequent steps in the isolation is of greater importance than resolution. The best combination of separation scale, speed, cost and resolution is often obtained using an intermediate particle size reverse-phase packing.

Solute retention in reverse-phase chromatography is determined by the percentage of organic cosolvent in the mobile phase. Separations can be performed isocratically (a fixed mobile phase composition), in step gradient mode (stepwise increases in percentage of organic solvent) or using a gradient (continuously, usually linear, increasing organic composition). The commonly used organic modifiers are acetonitrile and methanol, however, tetrahydrofuran and isopropanol are occasionally used. Selectively in reverse-phase separations can be controlled by changing organic modifier and the pH of the mobile phase. Separations of ionizable fungal metabolites are generally more reproducible if the pH of the mobile phase is controlled. This can be as simple as adding 0.01% to 1% of an organic acid, such as formic acid or trifluoroacetic acid to acidify the mobile phase, to use of a buffer such as potassium phosphate. Large selectivity changes within a crude or semipurified sample can be affected by pH modification.

Fungal metabolites present few unique problems for the application of typically practiced reverse-phase chromatography. The primary concern is the solubility of the crude or semipurified fungal extract. For typical preparative reverse-phase separations the sample is applied to the column in a solution of lower eluting strength than the initial mobile phase composition. For example, a separation employing a gradient from 30% to 90% acetonitrile requires the feed sample to be dissolved in a relatively small volume of an aqueous solution of 30%, or less, acetonitrile. Depending on the amount of sample, its purity, and the solubility of the mixture components, this may not be possible. Two strategies exist for sample application in solubility limited situations. The simplest is to dissolve the sample in a more nonpolar but water miscible organic solvent such as tetrahydrofuran or DMSO. This solution is then diluted with water or buffer to an aqueous composition equivalent to the starting column eluent. An alternative is to dilute the sample to be

separated in a large volume of a solution with an organic modifier content significantly below the initial column eluent. This solution is applied to the column using the column as a SPE, in effect, packing the sample onto the top of the bed. The column is then thoroughly washed to remove unretained material and then eluted normally.

SEPHADEX LH-20. Classical gel filtration is rarely employed for the isolation of fungal metabolites because their relatively small molecular weight, typically less than 1000 daltons, is below the fractionation range for such separations. The basic Sephadex polymer is a crosslinked polydextran typically used for gel filtration separation of proteins. However, the Sephadex LH-20 and LH-60 variants are unique in that they are hydroxypropyl ether derivatives of the basic Sephadex polymer to create a more lipophilic stationary phase suitable for use in organic solvents and have found wide application in natural product purification. Mobile phases of methanol, methylene chloride:methanol, or methanol:methylene chloride:hexane are typically used (Table 4, footnotes). The separation mechanism for small organic molecules on LH-20 is complicated and not well studied. Rather than size exclusion, a combination of partition and adsorption mechanisms dominate with the contribution of each mechanism for a particular compound loosely dependent on the number of polar functional groups and the mobile phase polarity [6].

LH-20 has two advantages for the isolation of fungal metabolites. First, it is an extremely gentle technique and high recovery of solutes is typical. Irreversible adsorption is uncommon and no reactive functionality is inherent to the polymer, as for silica based materials. Second, the magic separation, for want of a better terminology, is sometimes obtained. That is, compounds have occasionally been purified to near homogeneity from crude organic extracts after a single LH-20 fractionation. This reveals one of the significant problems with its use; these magic separations cannot be predicted. Just as frequently, maybe more so, a crude organic extract can be passed through LH-20 with little or no gain in specific activity of the active component(s). Sample loading is generally lower than with classical partition or adsorption methods but guidelines based on rigorous study are not available. Compound elution from LH-20 is typically broad as a result of the complex separation mechanism and the relatively large particle size. Nevertheless, many natural product chemists view LH-20 as a no lose technique.

ION EXCHANGE. Ion exchange-based separation methods can be exploited for fungal metabolites with a net anionic or cationic character. Commonly encountered mono-, and especially di- and tricarboxylic acids, can be retained on various anion exchange resins. Conversely, basic compounds are excellent candidates for retention on cation exchange resins. The relative rarity of cationic fungal metabolites makes this a powerful purification method if the target compound contains an amine.

This powerful technique is widely used for the isolation of classical, water-soluble antibacterial agents from bacterial sources but it is rarely used for the isolation of fungal metabolites (see below). The infrequent use of ion exchange for fungal metabolite isolation is likely due to the fact that it is regarded as a technique for water-soluble molecules and thus generally incompatible with the typical organic soluble fungal metabolite. The lack of water solubility can be overcome by the addition of an organic cosolvent to the ion exchange mobile phase. Ion exchange can be a highly selective tool for the enrichment of families of fungal metabolites as was demonstrated by the use of the weakly basic anion exchanger AG4 \times 4 to recover low levels of zaragozic acids from crude fungal fermentations [7,8].

COUNTERCURRENT CHROMATOGRAPHY. CCC has been referred to as the support-free liquid stationary phase [9]. CCC is true liquid-liquid partition chromatography. Almost

any biphasic mixture of two or more solvents can be used. One of the two phases is retained as a stationary phase using centrifugal force within a centrifuge-like apparatus. Two major types of instruments exist for this purpose: the multilayer coil planet centrifuge (MLCPC) and the centrifugal partition chromatograph (CPC). The column within the MLCPC consists of a long coil of tubing, typically Teflon 1.68 mm inside diameter \times 100 m long, wound in several layers on a spool. This coil is rotated in a planetary motion, hence the name, within the centrifuge like instrument generating the hydrodynamic fluid mixing responsible for the separation. In the CPC, a rotor containing numerous chambers interconnected by small channels is rotated in a centrifuge retaining one phase, hydrostatic, and the mobile phase is pumped through it [10,11].

CCC has many advantages for fungal metabolite isolation. It is extremely gentle because the problems of a solid stationary phase support are eliminated resulting in complete sample recovery. An almost infinite number of biphasic solvent systems can be conceived of and the partition behavior of the bioactivity or compound of interest can be characterized before separation in the instrument. A solvent system is selected that results in approximately equal distribution of bioactivity or compound between the two phases. Most biphasic solvent systems can be used in either normal or reverse-phase modes. Finally, three modes of operation are possible: classical continuous elution chromatography, dual-mode, and pH zone refining. The reader is referred to the references herein for a detailed description of these techniques.

CCC has some disadvantages, which are no doubt responsible for its uncommon use for fungal metabolite isolation. Specialized instrumentation is required and the learning curve is relatively steep when compared to more classical forms of chromatography. CCC has general utility for all three isolation steps and has been underused for fungal metabolite isolation.

1.3.3. Step 3. Polishing

Goal

The purpose of the polishing step is to provide pure compound for structure elucidation and biological evaluation. This step is generally the highest resolution or most selective step in the isolation process and provides relatively modest gains in specific activity. Usually the target compound is characterized by at least a chromatographic retention and UV spectrum. This aids in optimization of the polishing step separation.

Generally Useful Methodology

PREPARATIVE REVERSE-PHASE AND NORMAL-PHASE HPLC. Preparative reverse-phase HPLC is the highest resolution separation method available for fungal metabolite isolation and is unquestionably the most commonly used last isolation step. Hundreds of different reverse-phase HPLC bonded phases are commercially available, but the most commonly used for fungal metabolite isolation is either octadecyl (C18) or octyl (C8), as can be seen from Table 4. The variation in C8 and C18 bonded phases from different manufacturers derives from the physical characteristics of the silica used, the density of the carbon loading, the type of end-capping used to mask unreacted silanol residues, and the particle size [12]. This variation in surface chemistry can result in relative selectivity variations for a given mixture of components on different column materials. This selectivity variation can be exploited for the optimization of a particularly difficult separation. Particle sizes range from 3 to 5 μm spherical used for analytical (column diameters 2.1–4.6 mm) and small quantity preparative separations to 12 to 15 μm used for large scale preparative HPLC (column diameters ≥ 22 mm).

CRYSTALLIZATION. Crystallization is the method of choice for a polishing step in the isolation of a fungal metabolite. Product purities are high, the metabolite is obtained in a solid form, and the process can be scaled. Obtaining a crystalline product can be challenging and is especially difficult with the initial, few-milligram sample of a new compound. Crystallization is actively sought for process scale separations of fungal metabolites of high interest. Crystallization has the added benefit, assuming suitable crystals are obtained, of allowing structure elucidation using x-ray diffraction. Fungal metabolites do not require special treatment when compared with natural products from other sources.

COUNTERCURRENT CHROMATOGRAPHY. This has several different operational modes as described above. For use as a polishing step, since an analytical assay for the target compound presumably exists, the solvent system can be optimized using small scale partition experiments prior to actual separation of the sample in the column [4]. Advantages of CCC as a polishing step are the ability to use either normal or reverse-phase operational modes, ease of compound recovery from the mobile phase, and high recovery. Disadvantages of CCC include scaleup, if grams of compound are required, and the time required for development of a solvent system for the separation.

1.3.5. Case Study: Isolation of Sordarin and Related Derivatives

Sordarin is a novel diterpene glycoside with good *in vitro* antifungal activity against a variety of fungal pathogens [13]. Since its discovery in 1970, at least a dozen new sordarin analogues, all modifications of the sordarose carbohydrate portion of the molecule, have been reported. Members of this family are potent, highly selective inhibitors of protein synthesis in fungi (see Chap. 11). Pharmaceutical industry interest in this biological activity has lead to considerable medicinal chemistry effort to improve the potency and pharmacologic profile of this family. A wide range of natural product isolation chemistry has been described for this family encompassing the initial discovery isolation and structure elucidation of members of the family through the large scale production of sordarin for synthetic modification. As a case study in practical natural product purification, the reported isolation procedures for sordarin, 11-hydroxysordarin [14], SCH57404 [15], and three different 3'-sugar derivatives, GR135402 [16], neosordarin [17], and an unnamed 3'-oxoketal sordarin [18] (structures shown in Fig. 6) were analyzed in terms of the three-step isolation protocol described here (Table 3).

Sordarin is typically distributed to some degree between both the culture filtrate and mycelia at harvest. This is particularly true as a fermentation ages and some of the mycelial lysis occurs. The more lipophilic 3'-derivatives are typically associated to greater amount with the mycelia than sordarin itself. For sordarins, separation of the mycelia and culture broth prior to extraction is determined more by the initial isolation step than by the actual product distribution. Two water-immiscible solvents, methylethylketone or ethyl acetate, and one water-miscible solvent, methanol, were used for fermentation extraction in the eight examples. Both examples using a water immiscible solvent were followed by a normal-phase recovery and resolution step whereas the six water-miscible solvent extractions were all followed by a reverse-phase recovery and resolution step. This is more or less a typical workup for fungal natural products since most of the large scale capture methods rely on either polymeric or silica based reverse-phase materials compatible with aqueous alcoholic extracts. The use of various reverse- or normal-phase chromatography steps for both the purification and polishing steps of the eight compounds is also typical.

Atypical for fungal metabolite isolation is the exploitation of the ionic character of the sordarin aglycone. This was exploited in three ways in the eight examples: (1) differential pH extraction, (2) ion exchange, and (3) pH zone-refining CCC. The single carboxylic acid moiety is itself unremarkable but the highly pH-dependent solubility of sordarin has

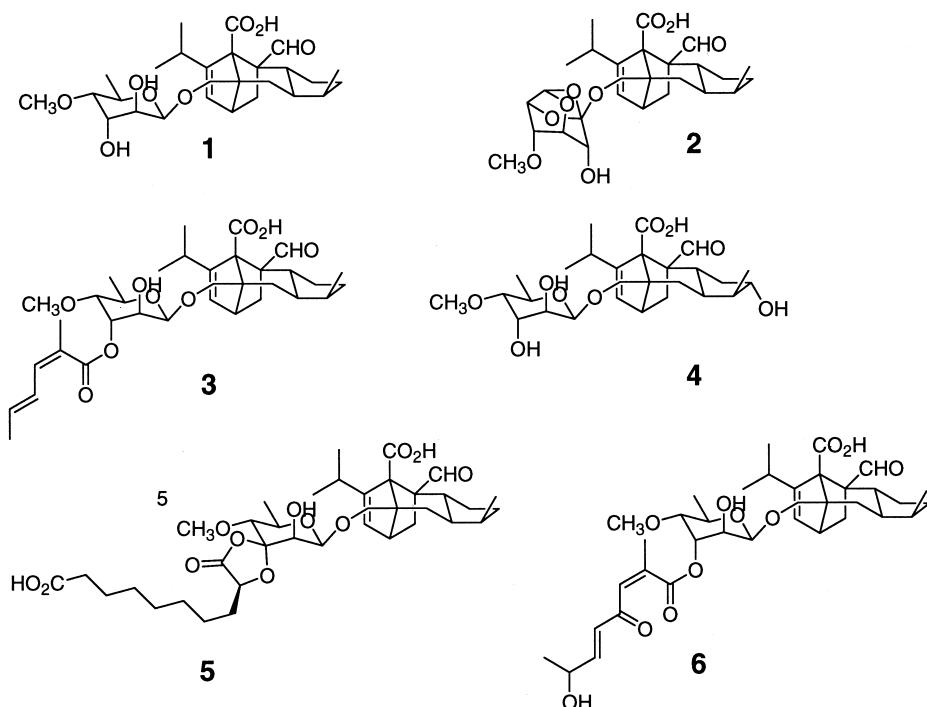


Figure 6 Structures of sordarin and related natural products: sordarin (1), SCH57404 (2), GR135402 (3), 11-hydroxsordarin (4), 3'-oxoketal sordarin derivative (5), neosordarin (6).

been exploited for its purification. The free acid form of sordarin, present under acidic conditions, is soluble in various organic solvents including dichloromethane, ethyl acetate, and methanol. The salt form, sodium or potassium for example, has much reduced solubility in the above organic solvents and exhibits significant water solubility. This pH-dependent solubility was recognized early and used in the initial isolation of sordarin. Differential pH extraction of the 11-hydroxy analogue of sordarin was also possible, although hydroxylation of the diterpene reduced the methylene chloride solubility and the more polar solvent ethyl acetate was substituted. Differential pH extraction was not useful for any of the 3'-ester derivatives due to the reduced aqueous solubility and stability of ester.

Ion exchange was extremely effective for the isolation of the 3'-oxoketal derivative, a dicarboxylic acid sordarin analog. The selectivity of the weak anion exchanger AG4×4 (formate cycle) was exploited to selectively retain and separate diacids from monocarboxylic acids. Finally, pH zone-refining CCC was effectively used to separate sordarin and 11-hydroxysordarin on a multigram scale [19]. This technique relies on the same pH-dependent solubility as differential pH extraction but in a zone-refining operational mode [20].

1.3.6. Literature Survey of Recent Fungal Metabolite Isolations

The isolation procedures reported for approximately 350 novel fungal metabolites described in the recent (1998–2002) literature were analyzed in terms of the three-step process of fungal metabolite isolation described above. This information is summarized in Table 4 and provides an unbiased sample of the actual methodologies currently being

Table 3 Comparison of the Reported Isolation Processes for Sordarin and Some Sordarin Analogs

	GR 135402	Neosordarin	SCH57404	Sordarin (original)
Fermentation volume (L)	500	100	1	Not reported
Product location	Mycelia	Culture filtrate	Mycelia	Mycelia
Extraction solvent	Methanol (2 × 200 L)		EtOAc	Methanol:water (9 : 1)
Misc. initial workup	Precipitation			
Step 1. Resolution and recovery	Reverse-phase chromatography Partisil BioPrep P40 ODS-3 (500 g)–batch adsorption, step gradient elution (CH ₃ CN:H ₂ O)	Reverse-phase capture Mitsubishi HP-21	Normal-phase adsorption chromatography Silica gel	Differential pH extraction
Step 2. Purification	Reverse-phase chromatography Partisil BioPrep P40 ODS-3 (500 g)–isocratic elution	Normal-phase adsorption chromatography Silica gel column chromatography	Sephadex LH-20 CH ₂ Cl ₂ :CH ₃ CN (1 : 1)	Normal-phase adsorption chromatography Kieselgel
Step 3. Polishing	Crystallization	Reverse-phase chromatography Preparative RP	HPLC–Nucleosil C18	
			<i>3'-Oxoketal derivative</i>	
			12	<i>3'-Oxoketal derivative</i>
			Whole broth	56
			Methylketone	Whole broth
				Methanol
Fermentation volume (L)			Countercurrent chromatography (CCC)	Mitsubishi SP207
Product location			Normal-phase, dual-mode	
Extraction solvent				
Misc. initial workup				
Step 1. Resolution and recovery		Reverse-phase capture Mitsubishi SP207, upflow mode		
Step 2a. Purification		Differential pH extraction		
Step 2b. Purification		Countercurrent chromatography pH zone-refining mode		
Step 3. Polishing		Reverse-phase chromatography Preparative RP HPLC–C8	Reverse-phase chromatography Preparative RP HPLC–C8	Reverse-phase chromatography Preparative RP HPLC–C8

Table 4 Survey of Reported Fungal Metabolite Isolations 1997–2002

Ref	Index	Type	Name	Genus	Species
[28]	1	1,3-dioxane	coruscol A	<i>Penicillium</i>	sp.
[29]	2	10-membered lactones	multiploide A, b	<i>Xylaria</i>	<i>multiplex</i>
[30]	3	12-membered macrolide	cladosorolide D	<i>Cladosporium</i>	
[30]	4	12-membered macrolide	cladosprolide D	<i>Cladosporium</i>	<i>byssoides</i>
[31]	5	16 member macrolide	macrophelide H, L	<i>Periconia</i>	sp.
[32]	6	16 member macrolide	integramide A, B	<i>Dendrodochium</i>	
[33]	7	16-mer peptide	cordypridone A, B, C, D	<i>Cordyceps</i>	<i>nipponica</i>
[34]	8	2-pyridones	TMC-205		<i>unident.</i>
[35]	9	3-carboxyindole	unnamed	<i>Clitocybe</i>	<i>catinus</i>
[36]	10	acetylene	rosellipins	<i>Gliocladium</i>	<i>roseum</i>
[37]	11	acyclic polyketide polyol esters	TMC-171 A-C	<i>Gliocladium</i>	
[37]	12	acyclic polyketide polyol esters	TMC-154	<i>Gliocladium</i>	
[38]	13	acyclic polyketide polyol esters	circumdatin G	<i>Aspergillus</i>	<i>ochraceous</i>
[39]	14	alkaloid	peniamidienone	<i>Penicillium</i>	sp.
[40]	15	alkyl amides	viriditdin	<i>Aspergillus</i>	<i>viridi-mutans</i>
[40]	16	alkyl amides	O-methylviriditdin	<i>Aspergillus</i>	<i>viridi-mutans</i>
[41]	17	alkyl amine	piptamine	<i>Piptoporus</i>	<i>betulinus</i>
[42]	18	alkyl citrate	CJ-15183	<i>Aspergillus</i>	<i>aculeatus</i>
[43]	19	alkyl nonenolide	microcarpalide		<i>unidentified</i>
[44]	20	alkyl phenol	none	<i>Chrysosporium</i>	
[45]	21	alkyl phenol	integracin A, B, C	<i>Cytonaema</i>	
[46]	22	anthracycline	seragakinone A	<i>unidentified</i>	
[47]	23	anthranilic acid	NII 15501A	<i>Penicillium</i>	
[48]	24	anthraquinone	BM2419-1,-2	<i>Paecilomyces</i>	
[49]	25	anthraquinone	neobulgarones	<i>Neobulgaria</i>	
[50]	26	anthraquinonepyrones	topopyrones A-D	<i>Phoma</i>	
[51]	27	anthraquinones	UCT1072M1-3	<i>Aspergillus</i>	
[52]	28	α -pyrone ester	phomalaactone ester	<i>Phomopsis</i>	
[53]	29	α -pyrones	11G219 a, b, c, d		<i>unidentified</i>

(Continued)

Table 4 Continued

Ref	Index	Type	Name	Genus	Species
[54]	30	aromatic polyketides	scytalols A, B, C, D aspochalasin F, G	<i>Scytalidium</i>	
[55]	31	aspochalasin		<i>Aspergillus</i>	<i>candidus</i>
[56]	32	asterriquinones		<i>Aspergillus</i>	<i>like</i>
[57]	33	azaanthraquinone	scorpinone	<i>Bispora</i>	<i>multicolor</i>
[58]	34	azaphilones		<i>Penicillium</i>	<i>multicolor</i>
[58]	35	azaphilones		<i>Penicillium</i>	<i>multicolor</i>
[58]	36	azaphilones		<i>Penicillium</i>	<i>multicolor</i>
[59]	37	azaphilones	RP-1551-M1, -M2	<i>Penicillium</i>	
[59]	38	azaphilones	RP-1551-7	<i>Penicillium</i>	
[59]	39	azaphilones	RP-1551-1, -2, -3, -4, -5, -6	<i>Zopfiella</i>	<i>inermis</i>
[60]	40	azaphilones	S-15183a	<i>Zopfiella</i>	<i>inermis</i>
[60]	41	azaphilones	S-15183b	<i>Neosartorya</i>	sp.
[61]	42	azaspirone	azaspirine	<i>Cladosporium</i>	<i>cladospoides</i>
[62]	43	benzofluoranthen-3-one	none	<i>Aspergillus</i>	<i>ochraceous</i>
[64]	44	benzodiazepine	circumdatin A, B, C	<i>Hypoxylon</i>	<i>truncatum</i>
[65]	45	benzofluoranthene	hypoxylonol A, B	<i>Acremonium</i>	
[66]	46	benzofuran	spirobenzofuran	<i>Microsphaeropsis</i>	
[67]	47	betaenone	10-hydroxy-18-methoxybetaenone	<i>Cordyceps</i>	<i>Pseudomildiis harrisii</i>
			none(11)	<i>Cytospora</i>	sp.
			cytoskytin B	<i>Cytospora</i>	sp.
			cytoskyrin A	<i>Gerronema</i>	sp.
			gerronemin A, B, C, D, E, F	<i>Sphaeropspidales</i>	<i>sclerotiorum</i>
			oxasterrinol D methyl ether	<i>Sphaeropspidales</i>	sp
			sphaerolone	<i>Mycena</i>	sp
			dihydrosphaerolone	<i>Botrytis</i>	
			9-hydroxyoudemansin A		<i>cinerea</i>
			breviane lactones		<i>brevicompactum</i>
			breviane spiroditerpenes		
[68]	48	bioanthrazenes			
[69]	49	bisanthraquinone			
[69]	50	bisanthraquinone			
[70]	51	biscatechol			
[71]	52	bisindolyl benzoid			
[72]	53	bisnaphthal			
[72]	54	bisnaphyl			
[73]	55	b-methoxyacrylate			
[74]	56	botryane lactones			
[75]	57	breviane			
			Brevione B, C, D, E		

[76]	58	carotane sesquiterpenes	<i>Trichoderma</i>	<i>virens</i>
[77]	59	carotenoid glycosyl ester	<i>Fusarium</i>	<i>albuminosus</i>
[78]	60	cerebrosides	<i>Termitomyces</i>	<i>extinguens</i>
[79]	61	chromone	<i>Tolyphocladium</i>	sp.
[80]	62	cinnamoylcyclopeptide	<i>Xylaria</i>	<i>anartia</i>
[81]	63	γ -lactones	<i>Bombardioidea</i>	<i>anartia</i>
[81]	64	cyclic depsipeptide	<i>Bombardioidea</i>	<i>roseum</i>
[82]	65	cyclic depsipeptide	<i>Trichothecium</i>	sp.
[83]	66	cyclic depsipeptide	<i>Fusarium</i>	
[84]	67	cyclic depsipeptide	<i>Gliomospora</i>	
[85]	68	cyclic depsipeptide	<i>Nigroscabulum</i>	
[85]	69	cyclic depsipeptide	<i>Nigroscabulum</i>	
[71]	70	cyclic peptide	<i>Aspergillus</i>	
[86]	71	cyclic peptide	<i>Ustilaginoidae</i>	
[87]	72	cyclic peptide	<i>Gliocladium</i>	
[88]	73	cyclic peptide	<i>Emericella</i>	
[89]	74	cyclic tetrapeptide	<i>Fusarium</i>	
[90]	75	cyclic tetrapeptide	<i>Diheteropora</i>	
[91]	76	cyclic tetrapeptide	<i>Fusarium</i>	
[92]	77	cyclodepsipeptide	<i>Beauveria</i>	
[93]	78	cyclodepsipeptide	<i>unident.</i>	
[94]	79	cyclohexadepsipeptide	<i>Hirsutella</i>	
[95]	80	cyclohexanone epoxide	<i>Pestalotiopsis</i>	
[95]	81	cyclohexanone epoxide	<i>Pestalotiopsis</i>	
[95]	82	cyclohexanone epoxide	<i>Pestalotiopsis</i>	
[96]	83	cyclopentabenzofuran	<i>Suillus</i>	
[97]	84	cyclopentanone	<i>Trichoderma</i>	
[98]	85	cyclopropenone	<i>Eupenicillium</i>	
[99]	86	cytochalasin	<i>Aspergillus</i>	
[100]	87	cytochalasin	<i>Phomopsis</i>	
[101]	88	cytochalasin	<i>Metarrhizium</i>	
[102]	89	cytochalasin	<i>Rhinocladiella</i>	sp.

(Continued)

Table 4 Continued

Ref	Index	Type	Name	Genus	Species
[103]	90	cytochalasin	cytochalasin Z1, Z2, Z3	<i>Pyrenopora</i>	<i>semenperda</i>
[104]	91	cytochalasin	cytochalasin X, Y, Z	<i>Pseuderotriatum</i>	<i>zonatum</i>
[105]	92	decalactone	Xestodecalactone A, B, C	<i>Penicillium</i>	<i>montanense</i>
[106]	93	decalin polyketide	decumbenone B	<i>Penicillium</i>	<i>decumbens</i>
[106]	94	decalin polyketide	decumbenone A	<i>Penicillium</i>	<i>decumbens</i>
[107]	95	decaline polyketide	phomopsidin	<i>Phomopsis</i>	
[108]	96	depside	anodepside A, B	<i>unidentified</i>	
[109]	97	depsipeptides	clavariopsin A, B	<i>Clavariopsis</i>	<i>aquatica</i>
[110]	98	depsipeptides	SCH378161(4)	<i>unident.</i>	
[111]	99	dihydroxanthones	F390B	<i>Penicillium</i>	
[111]	100	dihydroxanthones	F390B	<i>Penicillium</i>	<i>merdarium</i>
[112]	101	dihydroxybenzene quinones	semichloridinol A, B	<i>Chrysosporium</i>	
[113]	102	diketopiperazine	Sch56396	<i>Tolyptocladium</i>	
[114]	103	diketopiperazine	haematocin	<i>Nectria</i>	<i>haemalococa</i>
[115]	104	diketopiperazine	fellutanine A, B, C, D	<i>Penicillium</i>	<i>fellutanum</i>
[116]	105	diketopiperazine	CJ-17665	<i>Penicillium</i>	
[117]	106	diketopiperazine	sterin A, B	<i>Aspergillus</i>	<i>ochraceus</i>
[118]	107	dimethylchromene	polanrazine B, C, D, E, F	<i>Stereum</i>	<i>hirsutum</i>
[119]	108	dioxopiperazine	none	<i>Phoma</i>	<i>lingam</i>
[120]	109	diterrene	virescenoside M, N	<i>Oldiodendron</i>	<i>griseum</i>
[121]	110	diterpene glycoside	scabronine B, C, D, E, F	<i>Acremonium</i>	<i>striatissimum</i>
[122]	111	diterpenoid	atranone A-G	<i>Sarcodon</i>	<i>scabrosus</i>
[123]	112	diterpenoid	epicorazine	<i>Stachybotrys</i>	<i>chartarum</i>
[124]	113	epidothiopiperazine	leptosin M, M1, N, N1	<i>Stereum</i>	<i>hirsutum</i>
[125]	114	epipolysulfanylidioxopiperazine	Leptosphaeria	<i>Leptosphaeria</i>	
[126]	115	epoxylactone	Aspergillus	<i>niger</i>	
[127]	116	equisetin derivative	Pezicula	<i>sp.</i>	
[128]	117	equisetin derivative	CJ-17572	<i>niger</i>	
[129]	118	equisetin type	CJ-21058	<i>sp</i>	
			LL-49F233a		<i>unknown</i>

[130]	119	ergostane	<i>Agaricus blazei</i>
[131]	120	farnesyl hydroquinone	<i>Ganoderma pfeifferi</i>
[132]	121	fatty acids	<i>Clavipes</i>
[133]	122	furopyridine	<i>Cladoboytrum varium</i>
[134]	123	furosesquiterpene	<i>Laurilla tsugicola</i>
[135]	124	γ -lactone	<i>Talaromyces modonitum</i>
[136]	125	glycolipid	<i>Coryneum unidentified</i>
[137]	126	glycolipid	<i>Iplex</i>
[138]	127	glycosylated terpene	<i>Bovista</i>
[139]	128	hexacyclic illudane-illudane bis-sesquiterpene	sp.
	129	hexaketide lactone	<i>unidentified</i>
[140]	130	hexaketide lactone	<i>unidentified</i>
[140]	131	hexaketide lactone	<i>unidentified</i>
[140]	132	hexaketide lactone	<i>unidentified</i>
[141]	133	hydroxybenzaldehyde	<i>Aspergillus terreus</i>
[142]	134	ilicolin H related	<i>TCI-068</i>
[143]	135	ilicolin H like	<i>bassiana</i>
[143]	136	ilicolin H like	<i>bassiana</i>
[144]	137	ilicolin H like	<i>bassiana</i>
[144]	138	ilicolin H like	<i>bassiana</i>
[145]	139	illudane	<i>bassiana</i>
[146]	140	indole diterpenes	<i>Beauveria</i>
[47]	141	indole diterpenes	<i>Beauveria</i>
[148]	142	isobenzofuranone	<i>Beauveria</i>
[149]	143	isochroman	<i>Gloeophyllum</i>
[150]	144	isocyanoacyclopentanes	<i>Nodulisporium</i>
[151]	145	isonitrile	<i>Aspergillus ochraceous</i>
[151]	146	isonitrile	<i>Pestalotiopsis microspora</i>
	147	isoquinoline alkaloid	<i>Penicillium harzianum</i>
[152]	148	isoquinoline alkaloids	<i>Trichoderma ustus</i>
[153]	149	isoquinoline alkaloids	<i>Aspergillus ustus</i>
			<i>(Continued)</i>

Table 4 Continued

Ref	Index	Type	Name	Genus	Species
[153]	150	isoquinoline alkaloids	TMC-120B	<i>Aspergillus</i>	<i>ustus</i>
[154]	151	K-76 decalins	stachyflin	<i>Stachybotrys</i>	sp.
[154]	152	K-76 decalins	acetylstachyflin	<i>Stachybotrys</i>	sp.
[155]	153	lanostanoids		<i>Ganoderma</i>	<i>concinna</i>
[156]	154	linear lipohexapeptide	lipolexin	<i>Paecilomyces</i>	
[156]	155	linear lipohexapeptide	lipolexin	<i>Moesyzia</i>	
[157]	156	lipopeptides	FR901469	<i>unident.</i>	
[158]	157	lipopeptides	15G256c,d,e	<i>Hypoxyylon</i>	<i>oceanicum</i>
[158]	158	lipopeptides	15G256c,d,e	<i>Hypoxyylon</i>	<i>oceanicum</i>
[159]	159	lipopeptide	YM-170320	<i>deuteromycte unident</i>	
[160]	160	lipopeptide	arborandins A-F	<i>unident.</i>	
[161]	161	macrocyclic lactones	CJ-12950	<i>Mortierella</i>	<i>vericillata</i>
[161]	162	macrocyclic lactones	CJ-13357	<i>Mortierella</i>	<i>vericillata</i>
[162]	163	macrolide		<i>Cladosporium</i>	<i>herbarium</i>
[163]	164	meroterpenes		<i>Penicillium</i>	sp.
[164]	165	misc.		<i>Aspergillus</i>	<i>parasiticus</i>
[165]	166	misc.	Xylaria		
[166]	167	misc.	CP225917	<i>unidentified</i>	
[166]	168	misc.	CP263114	<i>Mollisia</i>	<i>benesuada</i>
[167]	169	misc.	PF1092A,B,C	<i>Penicillium</i>	<i>oblatum</i>
[168]	170	misc.		<i>Pterula</i>	
[169]	171	misc.		<i>Pterula</i>	
[169]	172	misc.		<i>Westerdykella</i>	
[170]	173	misc.		<i>Pseudosphaerotilus</i>	
[171]	174	misc.		<i>Pseudosphaerotilus</i>	
[171]	175	misc.		<i>Pseudosphaerotilus</i>	
[172]	176	misc.		<i>Corollospora</i>	
[173]	177	misc.		<i>Peniophora</i>	
[174]	178	misc.		<i>unidentified</i>	
		hongoquerin A, B			

175]	misc.	acremolactone A	<i>Acremonium</i>	<i>roseum</i>
176]	180	erinacine E	<i>resupinatus</i>	<i>ramosum</i>
177]	181		<i>Drechlera</i>	<i>avenae</i>
177]	182		<i>Pycnoporus</i>	<i>coccineus</i>
178]	183		<i>unidentified</i>	<i>minoluteum</i>
179]	184		<i>Penicillium</i>	<i>minoluteum</i>
179]	185		<i>Penicillium</i>	<i>flavus</i>
180]	186	Talaromyces	<i>Talaromyces</i>	
181]	187	Penicillium	<i>Penicillium</i>	
182]	188	Memnoniella	<i>echinata</i>	
182]	189	Memnoniella	<i>echinata</i>	
182]	190	Memnoniella	<i>echinata</i>	
183]	191	Penicillium	<i>griseofulvum</i>	
184]	192	Penicillium	<i>Penicillium</i>	
184]	193	Penicillium	<i>Penicillium</i>	
184]	194	Trichopezizella	<i>barbata</i>	
185]	195	kodaistatins A, B	<i>Aspergillus</i>	<i>terreus</i>
186]	196	kodaistatins A, B	<i>Aspergillus</i>	<i>terreus</i>
186]	197	10-hydroxy-18-N[2-naphthyl]- N-phenylaminobetaenone		
197]		TAN-1813	<i>Phoma</i>	
198]	199	cis-fumagillin	<i>Penicillium</i>	
199]	200	cis-fumagillin	<i>Penicillium</i>	
199]	201	SCH420789	<i>unidentified</i>	
200]	202	topopyrones A-D	<i>Penicillium</i>	
200]	203	phellinsin A	<i>Phellinus</i>	
201]	204	xanthoepocin	<i>Penicillium</i>	<i>simplicissimum</i>
201]	205	10-norparyleneone	<i>Microsphaeropsis</i>	
202]	206	waol B	<i>Myceliophthora</i>	<i>lutea</i>
203]	207	8-O-methylsclerotiorinamine	<i>Penicillium</i>	<i>moticolor</i>
203]	208	bisabosquals	<i>Stachybotrys</i>	
205]	209	bisabosquals	<i>Stachybotrys</i>	

(Continued)

Table 4 Continued

Ref	Index	Type	Name	Genus	Species
[195]	210	misc.	bisabosquals	<i>Stachybotrys</i>	
[195]	211	misc.	bisabosquals	<i>Stachybotrys</i>	
[196]	212	misc.	4'oxonacrophorim A	<i>Eupenicillium</i>	
[196]	213	misc.	4'oxonacrophorim D	<i>Eupenicillium</i>	
[197]	214	misc.	phenylpyropene C	<i>Pencillium</i>	
[198]	215	misc.	V214W	<i>unidentified</i>	
[199]	216	misc.	panepophenanthrin	<i>Panus</i>	
[200]	217	mixed terpenoid	pyrrocidine A, B	<i>unidentified</i>	
[201]	218	modified hydroxyquinone	polyozellin	<i>Polyozellus</i>	
[202]	219	modified octapeptide	MS-681a, b, c, d	<i>Myrothecium</i>	
[203]	220	mycophenolic acid deriv.	F13459	<i>Pencillium</i>	
[204]	221	naphthopyranone	unnamed	<i>Guanomyces</i>	
[39]	222	naphthopyranone	penidilamine	<i>Penicillium</i>	
[205]	223	naphthoquinone	aureoquinone	<i>Aureobasidium</i>	
[206]	224	nonenolide	herbarumin I, II	<i>Phoma</i>	
[207]	225	o-benzoquinone	laccardiones A, B	<i>herbarium</i>	
[208]	226	octaketide pyrone	cytopyrone D, E	<i>amethysteaa</i>	
[209]	227	ovalicin-like	Mer-f3	<i>Laccaria</i>	
[210]	228	paraherquamide-like	aspergillimides	<i>Cytospora</i>	
[211]	229	pentacyclic polyketides	UCS1025A, B	<i>Metarrhizium</i>	
[212]	230	pentaketide	CR377	<i>Aspergillus</i>	
[213]	231	pentaketide	epoxyquinol A	<i>Acremonium</i>	
[212]	232	pentaketide tricarbonyl	CR377	<i>Fusarium</i>	
[214]	233	pentanorlanostane	cladosporioide B-D	<i>Cladosporium</i>	
[215]	234	peptaibol	clonostachin	<i>Clonostachys</i>	
[216]	235	peptaibol	longibarchin LGB II, III	<i>Trichoderma</i>	
[217]	236	peptaibols	ampullosporin	<i>Sepedonium</i>	
[218]	237	peptaibols	peptaibolin	<i>Sepedonium</i>	
[219]	238	peptaibols	berofungins B-D	<i>Emericellopsis</i>	
					<i>donezii</i>

[219]	239	peptaibols	<i>Emericellopsis donezki</i>
[220]	240	peptaibols	<i>Tylopilus neofelleus</i>
[221]	241	Peptaibols	<i>tubakii</i>
[221]	242	Peptaibols	<i>tubakii</i>
[222]	243	Peptaibols	<i>atroviride</i>
[222]	244	Peptaibols	<i>atroviride</i>
[223]	245	peptide	<i>unidentified</i>
[224]	246	peptide derivative	<i>Apiospora montagnei</i>
[225]	247	peptide-aib	<i>unident.</i>
[226]	248	peptides	<i>Aspergillus oryzae</i>
[163]	249	peptides	<i>amisophiae</i>
[227]	250	phenalene	<i>niger</i>
[228]	251	phenalene	<i>sp</i>
[229]	252	phenolic tetralone	<i>Metarrhizium grisea</i>
[230]	253	phenopicolinic acids	<i>Aspergillus lecanii</i>
[230]	254	phenopicolinic acids	<i>Penicillium lecanii</i>
[230]	255	phenopicolinic acids	<i>lecanii</i>
[230]	256	phenopicolinic acids	<i>lecanii</i>
[230]	257	phenopicolinic acids	<i>lecanii</i>
[231]	258	phthalides	<i>lecanii</i>
[232]	259	polyacetylene	<i>velutina</i>
[233]	260	polyesters	<i>hepatica</i>
[233]	261	polyesters	<i>oceanicum</i>
[233]	262	polyesters	<i>oceanicum</i>
[234]	263	polyhydroxyisoprenoid	<i>oceanicum</i>
			<i>Acremonium</i>
[235]	264	polyketide+C4	<i>Massarina tunicata</i>
[236]	265	polyphenolic ester	<i>unident.</i>
[237]	266	prenylated hydroquinones	<i>Acremonium murorum</i>
[238]	267	prenylated indole	<i>caryae</i>
[239]	268	prenylated phenols+aa	<i>Stachybotrys parvispora</i>

(Continued)

Table 4 Continued

Ref	Index	Type	Name	Genus	Species
[239]	269	prenylated phenols#P1aa	parispiorin	<i>Stachybotrys</i>	<i>parvispora</i>
[240]	270	prenylated phenols+aa	SMTP-3, -4, -5, -6	<i>Stachybotrys</i>	<i>micropora</i>
[241]	271	prenylated quinone	clavilactone D	<i>Cliocybe</i>	<i>clavipes</i>
[242]	272	preussomerin	preussomerin J, K, L	<i>unidentified</i>	
[243]	273	p-terphenyl	curtisans A-D	<i>Paxillus</i>	<i>curtisi</i>
[244]	274	p-terphenyl	leucomentin-5, -6	<i>Paxillus</i>	<i>panuoides</i>
[244]	275	p-terphenyl	leucomentin-5, -6	<i>Paxillus</i>	<i>panuoides</i>
[245]	276	p-terphenyl	kynapen-12	<i>Polyozelles</i>	<i>multiplex</i>
[246]	277	pyridine	none	<i>Marasmiellus</i>	
[247]	278	pyridine	akanthomycin	<i>Akanthomyces</i>	<i>gracilis</i>
[248]	279	pyridone alkaloid	militarinone A	<i>Paecilomyces</i>	<i>militaris</i>
[249]	280	pyrone	sequioataone C, D, E, F	<i>Aspergillus</i>	<i>parasiticus</i>
[250]	281	pyrone	trichodion	<i>Trichosporiella</i>	
[251]	282	pyrone	actofuincone	<i>Talaromyces</i>	
[252]	283	pyrones		<i>Penicillium</i>	
[253]	284	pyrrolidinone	zopfiellamide A, B	<i>Zopfiella</i>	<i>waksmanii</i>
[253]	285	pyrrolidinone	zopfiellamide A, B	<i>Zopfiella</i>	<i>lateipes</i>
[254]	286	pyrrolizidine terpene	UCS1025A,B	<i>Acremonium</i>	<i>lateipes</i>
[255]	287	pyrrolizidinone	CJ-16264	<i>unident.</i>	
[255]	288	pyrrolizidinone	CJ-16367	<i>Penicillium</i>	<i>citrinum</i>
[256]	289	pyrroloquinolone	quinalactins	<i>Dicyophora</i>	<i>indusitata</i>
[257]	290	quinazoline	dicyoquinazol A, B, C	<i>Penicillium</i>	
[258]	291	quinolone	quinalactins A-C	<i>Penicillium</i>	sp.
[259]	292	quinolone	penillazine	<i>Penicillium</i>	<i>marine fungus</i>
[260]	293	quinone	chlorogenitisyquinone	<i>Aigialus</i>	<i>parvus</i>
[261]	294	resorcylic acid lactone	aigialomycin D	<i>Aigialus</i>	<i>parvus</i>
[261]	295	resorcylic acid lactone	aigialomycin C	<i>Aigialus</i>	<i>parvus</i>
[261]	296	resorcylic acid lactone	aigialomycin E	<i>Aigialus</i>	<i>parvus</i>
[261]	297	resorcylic acid lactone	aigialomycin F	<i>Aigialus</i>	

[261]	298	resorcylic acid lactone	<i>Aigialus</i>	<i>parvus</i>
[261]	299	resorcylic acid lactone	<i>Aigialus</i>	<i>parvus</i>
[261]	300	resorcylic acid lactone	<i>Aigialus</i>	<i>parvus</i>
[262]	301	resorcylic acid lactone	<i>Phoma</i>	
[263]	302	resorcylic acid lactone	<i>Chrysosporium</i>	<i>queenslandicum</i>
[264]	303	salicylic acid tetramer	<i>Chloridium</i>	sp.
[265]	304	sesquiterpene	<i>Xylaria</i>	<i>persicaria</i>
[266]	305	sesquiterpene	<i>Omphalotus</i>	<i>illudens</i>
[267]	306	sesquiterpene	<i>Phoma</i>	sp.
[267]	307	sesquiterpene	<i>Phoma</i>	sp.
[268]	308	sesquiterpene esters	<i>Armillariella</i>	<i>mellea</i>
[269]	309	sesquiterpene esters	<i>Polyporus</i>	
[270]	310	sesquiterpene ethers	<i>Polyporus</i>	
[270]	311	sesquiterpene ethers	<i>Trichoderma</i>	
[76]	312	sesquiterpene-carotane	<i>Resinipinatus</i>	
[271]	313	sesquiterpenes	<i>Collybia</i>	
[272]	314	sesquiterpenes		
		unnamed collybolides 4a–c,		
		5, 6		
		aspergilloxide	<i>Aspergillus</i>	
		mangicol A–G	<i>Fusarium</i>	<i>heterosporum</i>
		YW3699	<i>Codinaea</i>	<i>simplex</i>
		dihydrocarolic acid	<i>Aspergillus</i>	<i>niger</i>
		penitricin D	<i>Aspergillus</i>	<i>niger</i>
		diheteropeptin	<i>Diheterospora</i>	
		trichodenones A, B, C	<i>Trichoderma</i>	<i>harzianum</i>
		trichodenones A, B, C	<i>Trichoderma</i>	<i>harzianum</i>
		WF14861	<i>Colletotrichum</i>	
		CRM-51005	<i>unidentified</i>	
		WF14865B	<i>Aphanoascus</i>	<i>fulvescens</i>
		WF14865A	<i>Acremonium</i>	<i>fulvescens</i>
		1	<i>Sordaria</i>	sp.
		hydroxysordarin	<i>araneosa</i>	(Continued)

Table 4 Continued

Ref	Index	Type	Name	Genus	Species
[17]	329	sordarin	neosordarin	<i>Sordaria</i>	<i>araneosa</i>
[283]	330	sordarin type	GR135402	<i>Graphium</i>	<i>putredinus</i>
[16]	331	sordarin type	GR135402	<i>Graphium</i>	<i>putredinus</i>
[284]	332	sordarin type	BE-31405	<i>Penicillium</i>	<i>minioluteum</i>
[285]	333	squalene triterpene	concentriol B, C, D	<i>Daldinia</i>	<i>concentrica</i>
[285]	334	squalene triterpene	concentriol B, C, D	<i>Daldinia</i>	<i>concentrica</i>
[286]	335	strobilurins	tetrachloropyrocatechol	<i>Mycena</i>	
[286]	336	strobilurins	strobilurin M	<i>Mycena</i>	
[287]	337	strobilurins	strobilurin O, P	<i>Ganoderma</i>	<i>lucidum</i>
[288]	338	terpene	lucidinic acid O, lactone	<i>unidentified</i>	
[289]	339	terpene	guanacastepene	<i>Memnoniella</i>	
[290]	340	terpene peptide	memnopeptide	<i>Aspergillus</i>	
[291]	341	terphenyl	terpenins	<i>Aspergillus</i>	
[292]	342	terphenyl	asteric acid, 3-Cl and 3,5-diCl	<i>unidentified</i>	
[293]	343	terphenyl ether	sardonin	<i>Sarcodon</i>	<i>leucopus</i>
[294]	344	terphenyl-diketopiperazine hybrid	dicerandrol A, B, C	<i>Phomopsis</i>	<i>longicolla</i>
[295]	345	tetrahydroxanthone	cryptocin	<i>Cryptosporiopsis</i>	<i>querina</i>
[296]	346	tetramic acid	ascosalipyrrolidinone A, B	<i>Ascochyta</i>	<i>salicorniae</i>
[297]	347	tetramic acid	talaroconvolutin A, B, C, D	<i>Talaromyces</i>	<i>convolutus</i>
[298]	348	tetramic acid	talaroconvolutin A, B, C, D	<i>Talaromyces</i>	<i>convolutus</i>
[298]	349	tetramic acid	talaroconvolutin A, B, C, D	<i>Talaromyces</i>	<i>convolutus</i>
[298]	350	tetramic acid	talaroconvolutin A, B, C, D	<i>Fusarium</i>	<i>Penicillium</i>
[298]	351	tetramic acid	talaroconvolutin A, B, C, D	<i>Nectria</i>	<i>coccinea</i>
[299]	352	tetramic acid	lucilaetane	<i>Nectria</i>	<i>coccinea</i>
[300]	353	tetramic acid	ravenic acid		
[301]	354	tetramic acids	(7Z)-fusarin C		
[301]	355	tetramic acids	(5Z)-fusarin C		

[301]	356	tetrameric acids	
[302]	357	trichothecene	YM-47524, 47525
[303]	358	trichothecene	8-acetoxysyridin H
[303]	359	trichothecene	8-acetoxysyridin E
[304]	360	trichothecene	verrucarin M
[304]	361	trichothecene	rordin L,M
[305]	362	trichothecene-like	curvularol
[306]	363	tridepside	cytotoxic acid A, B
[307]	364	tridepside	colletotric acid
[307]	365	tridepside	colletotric acid
[308]	366	tripeptide	aspergillamide A, B
[309]	367	triprenyl phenol	SMTP-1,-2
[310]	368	triterpene	NF00659B1, B2
[310]	369	triterpene	NF00659A3
[310]	370	triterpene	NF00659A1, A2
[311]	371	triterpene	S19159
[312]	372	triterpene	S19159
[313]	373	triterpene	12 unnamed
[314]	374	triterpene	anicequol
[315]	375	triterpene	fuscoatoxide
[315]	376	triterpene	fuscoatramide
[316]	377	tropolone	cordytopolone
[317]	378	tyrosine lactone	PF1163A, B
[318]	379	xanthone dimers	phomoxanthone A, B

Table 4 Continued

Index ^a	F/T ^b	L/S ^c	Vol. ^d (L)	Location ^e	Extraction Solvent	Step 1 ^{f,g}	Step 2	Step 3	Step 4	Step 5	Step 6	Yield ^h (mg)
1	F	L	10	Mycelia	CHCl ₃ :MeOH (1:1)	SiO ₂	CC RP 4					12.4
2	F	L	6	Filtrate	EtOAc XT	LH20						22, 36
3	F	L	4	Mycelia	EtOAc							6.15–11.7
4	F	L	4	Filtrate	Acetone							5.7, 3.8
5	T	L	90	Filtrate	EtOAc XT	LH-20 (2)	RP HPLC 57					50, 23 mg/L
6	F	S	WB	MEK	MEK	LH-20 (1)	RP HPLC					85, 11, 11, 2, 2
7	F	L	15	Filtrate	EtOAc xtn	SiO ₂	LH-20 (1)	RP HPLC 39				3.3
8	F	L	7	WB	nBuOH	LH-20 (2)	CC RP	RP HPLC 72	RP HPLC 64			30, 70, 90
9	F	L			EtOAc	SiO ₂	Prep TLC					
10	F	L	4.8	WB	Acetone	EtOAc XT	CC RP 17	RP HPLC 57				2.1–10.1
11	F	S	3.5	WB	nBuOH	SiO ₂	LH-20 (3)	RP HPLC 56				7.5–148.6
12	F	S	3.5	WB	nBuOH	CC RP 5	LH20					62.4
13	F	L	2	WB	CHCl ₃	LH-20 (1)	RP HPLC					1.7
14	F	L	10	Filtrate	SiO ₂	Prep TLC						10
15	T	L	16	Filtrate	HP-21	SiO ₂	RP HPLC 42					108
16	T	L	16	Filtrate	HP-21	SiO ₂	RP HPLC 42					48
17	F	L	10	WB	EtOAc	LH20	SiO ₂					35
18	T	L	20	Filtrate	EtOAc	HP-20	RP HPLC 60	RP HPLC 60				32.2
19	F	L	4			Repeated normal phase chromatography						18
20	F	S		WB	EtOAc	SiO ₂						80
21	F	L	1	WB	MEK	LH-20 (1)	RP HPLC 71					260, 285, 1.5
22	F	L	10	Mycelia	CHCl ₃ :MeOH (1:1)	EtOAc XTn	RP HPLC 70					171
23	F	L	10	Filtrate	ODS	BuOH xtn	CC ODS	Gel filtration				2.2

24	T	L	20	Mycelia	Acetone	Hex: EtOAc xtn SiO ₂	nBuOH pptn	UV rxn	HPLC	3.5
25	T	L		Mycelia	Acetone: MeOH		RP HPLC 24			0.4-8.6
26	F	L	2.6	WB	nBuOH	RP HPLC 37	SiO ₂ —			0.5-23
27	T	L	1	Filtrate	HP20	CC RP 19	Lichroprep Si60			282, 2, 2
28	T	L	50	Mycelia	MeOH	EtOAc:hexane (1:1) XT	SiO ₂ — BiotopeFlash75	RP HPLC 28		31-7500
29	T	L	300	Mycelia	Acetone	EtOAc partition hexane:water partition	SiO ₂		RP HPLC 26	600, 28, 31, 18
30	T	L	19	Filtrate	HP21	EtOAc XT	SiO ₂	RP HPLC 24		46.8, 12.1, 4.8, 32.9
31	T	L	55	Mycelia	EtOAc XT EtOAc	hexane xtn CCC	SiO ₂			9, 0, 9.2
32	F	L	40	WB			RP HPLC 27	Prep TLC SiO ₂		97, 37, 2, 6, 4, 6, 6, 3.5
33	F	L	2	Mycelia	CHCl ₃ : MeOH (2:1)	Prep TLC		Crystallization		40
34	T	L	20	WB	EtOAc	SiO ₂	Toyopearl HW- 40	RP HPLC 51		3, 12.5
35	T	L	2	WB	EtOAc	SiO ₂	RP HPLC	RP or NP HPLC		24.7, 10.1, 3, 3
36	F	L	20	WB	EtOAc	SiO ₂	Xtal			2250
37	T	L	200	Mycelia	MeOH	HP-20	SiO ₂	RP HPLC	RP	47, 73
38	T	L	8	Mycelia	MeOH	HP-20	SiO ₂	RP HPLC	HPLC	57
39	T	L	8	Mycelia	MeOH	HP-20	SiO ₂	SiO ₂		787, 47, 22, 37, 20, 6
40	T	L	1	WB	Acetone	EtOAc XT	SiO ₂	RP HPLC 43		140
41	F	L	1	WB	Acetone	EtOAc XT	SiO ₂	RP HPLC 43		20
42	F	L	15	MMycelia	Acetone	EtOAc partition EtOAc XT	SiO ₂	RP HPLC 37	Xtal	83.5
43	T	L	50	Mycelia	Acetone: MeOH;	CC KP-Sil	RP HPLC 48	RP HPLC		12-1400

(Continued)

Table 4 Continued

63	T	L	4.8	Filtrate	EtOAc XT	SiO ₂	LH-20	SiO ₂	NP HPLC
64	F	L	4.8	Filtrate	EtOAc XT	SiO ₂	RP HPLC	RP HPLC 4	9.1
65	F	L	1.5	WB	HP-20	EtOAc xtn	SiO ₂	RP HPLC	46.1
66	F	L	20	Mycelia/ filtrate	EtOAc/ CH ₂ Cl ₂ :	CC RP 4	LH-20 (11)		61.7
67	F	S		WB	MeOH	EtOAc XT	nBuOH XT	RP HPLC	55, 4.1, 3.7
68	F	L	2.4	Filtrate	EtOAc XT	SiO ₂	RP HPLC 1	Crystallization	9.1
69	F	L	2.4	Filtrate	EtOAc XT	SiO ₂	RP HPLC 1		46.1
70	F	S		Sclerotia	CHCl ₃	LH-20 (3)	LH-20 (9)	RP HPLC 73	205
71			500g	False smut balls	Water	ODS	SiO ₂	CHP20P	9.6
72	F	L	8	Mycelia	MeOH	DiationSK1B	HP20	SiO ₂	1.2
73	F	S		WB	EtOAc: CHCl ₃ :	CIE	Dowex1	CHP20P	3.8
					MeOH	Hexane:80%Me	CC RP 4	RP HPLC 42	<0.5
					(3:2:1)	OH partition	RP HPLC 29	RP HPLC 42	18
74		L/S		WB	MEK	LH-20 (1)	SiO ₂	RP HPLC	20
75	F	L	2	Filtrate	EtOAc	SiO ₂	LH-20 (7)	RP HPLC	120
76	F	S		WB	EtOAc	Hexane:90%Me	SiO ₂	SiO ₂	18
77	F	L	10	WB	Acetone	OH partition	CC ODS	RP HPLC 45	20
78				WB	EtOAc	EtOAc XT	NP HPLC—	SiO ₂	120
						CCC-	YMC PVA-Sil	RP HPLC 45	15
79	F	L	5	Mycelia	CH ₂ Cl ₂	3 : 5:3:5	SiO ₂		?
80	F	L	1	Filtrate	CH ₂ Cl ₂	LH-20 (1)	SiO ₂		?
81	F	L	1	Filtrate	CH ₂ Cl ₂	SiO ₂	SiO ₂		?
82	F	L	1	Filtrate	CH ₂ Cl ₂	SiO ₂	Crystallization		?
83				FB	MeOH	seq Liq-liq partition	SiO ₂	LH-20 (1)	?

(Continued)

Table 4 Continued

Index ^a	F/T ^b	L/S ^c	Vol. ^d (L)	Location ^e	Extraction Solvent	Step 1 ^{f,g}	Step 2	Step 3	Step 4	Step 5	Step 6	Yield ^h (mg)
84	F	L	2	WB	EtOH	HP20	LH-20 (14)	CC RP 1	RP HPLC11			5, 13.9
85	F	S	WB	nBuOH	EtOAc XT	LH-20 (3)	CC RP 5	RP HPLC64	LH20			27
86	T	L	20	Acetone	EtOAc XT	CC RP 4	RP HPLC 64					22, 480
87	F	L	10	Filtrate	EtOAc	SiO ₂	LH-20 (1)	RP HPLC				12.3
88	F	L	1.5	Filtrate	EtOAc, CH ₂ Cl ₂	CC RP 4	RP HPLC 69	RP HPLC				2.4, 3.6,
89	F	S	WB	55% aq. MeOH	Hex,CH ₂ Cl ₂ seq liq-liq partition	SiO ₂	Prep TLC	Crystallization				11.8
90	F	L	0.6	Mycelia/ filtrate	EtOAc	CC RP 4	diol					1.1, 1.1, 1.3
91	F	L	10	Mycelia/ filtrate	MeOH/Et OAc	SiO ₂	RP HPLC 15					2.2, 2.5, 1.8
92	F	L	10	Filtrate	EtOAc	SiO ₂						5, 10.3, 19.2
93	F	L	1	WB?	OAc	SiO ₂	SiO ₂	Crystallization				70
94	F	L	1	WB?	EtOAc	SiO ₂	SiO ₂	RP HPLC				86
95	F	L	1	WB?	Acetone	SiO ₂	SiO ₂	RP HPLC 42				12
96	F	L	1	WB?	Freeze dried	CH ₂ Cl ₂ :MeOH xtn	Hex/MeOH partition	CH ₂ Cl ₂ xtn	RP HPLC			0.5, 20
97	F	L	6	Mycelia	Acetone	EtOAc XT	SiO ₂	CC RP 4	RP HPLC 4			2, 50
98	F	L	4	WB	EtOAc	LH20	RP HPLC 12					0.5–100mg
99	F	L	2.8	Filtrate		HP-20	EtOAc/H+ xtn	CC RP	LH-20 (19)	Prep TLC		1.9
100	F	L	1	Mycelia	Acetone	EtOAc xtn	SiO ₂	LH-20 (7)	CC RP 12	RP HPLC	55	0.5
101	T	L	200	WB	EtOAc	Fractogel TSK	CC RP 15	RP HPLC 34				70, 75
						HW-40						

102	F	L	8	WB	EtOAc	ppn-hexane	CHP20P	CHP20P	LH-20 (2)	YMC PVA- Sil	18			
103	F	L	5	Filtrate	EtOAc	SiO ₂	Prep TLC				12.5			
104	F	L	55.5	Filtrate	CHCl ₃	SiO ₂	Prep TLC				30, 11, 4.5, 6			
105	T	L	3	Mycelia	Aq. acetone	EtOAc XT	SiO ₂	Crystallization			5.5			
106	F	S	0.9	WB	EtOH	CC RP 8	SiO ₂		RPHPLC 35	Prep TLC	3, <1			
107	T	L	3	Filtrate	HP-20	EtOAc partition	SiO ₂	LH-20 (17)	RP HPLC 25		1, 1.5			
108	F	L	4	Filtrate	EtOAc	SiO ₂	Prep TLC	SiO ₂			3.5, 6.5, 2.5,			
109	T	L	12	WB	EtOH	EtOAc XT	RP HPLC 60				13.7-20.8			
110	F	S	WB	CHCl ₃ :MeOH (2:1)	SiO ₂	RP HPLC 42					22, 14			
111		1.4 kg	FB	MeOH	Hex:EtOAc:water partition	SiO ₂	RP HPLC 10					100, 18, 27,		
112	F	S	WB	CHCl ₃ :MeOH (1:1)	CHCl ₃ xtn	PEI silica	SiO ₂	RP HPLC 42				58, 190		
113	F	L	10	WB	EtOAc	SiO ₂	RP HPLC 33				14.7, 12.7, 4,			
114		L	90	Mycelia	MeOH	LH-20 (2)	SiO ₂	RP HPLC			5.1			
115	F	L	10	Mycelia	Acetone	Hexane XT	SiO ₂				trace-12			
116	F	L	0.9	Filtrate	HP-20	CC RP 8	Hex:MeOH:H ₂ O partition				50.8, 25.9,			
117	F	S	1.5	WB	EtOH	CC RP 8	RP HPLC 59				11.7, 12.1			
118	NR										18			
119	F	L		Mycelia	MeOH	CHCl ₃ xtn	SiO ₂	RP HPLC 43			41.7			
120		FB		CH ₂ Cl ₂	LH-20 (5)	LH-20 (13)	LH-20 (16)	LH-20 (20)			21.7			
											22, 20			

(Continued)

Table 4 Continued

Index ^a	F/T ^b	L/S ^c	Vol. ^d (L)	Location ^e	Extraction Solvent	Step 1 ^{f,g}	Step 2	Step 3	Step 4	Step 5	Step 6	Yield ^h (mg)
121		FB	85% EtOH	CHCl ₃ /H ₂ O partition	SiO ₂		SiO ₂	RP HPLC				60.9, 4.0, 4.4, 4.9, 3.5
122	F	S	3	WB	EtOH	HP-20	CC RP 8	LH-20 (1)	RP HPLC 59			700
123	S	S	WB	EtOAc	SiO ₂	CC RP 4	SiO ₂	RP HPLC 37				115, 105, 140
124	F	S	0.5	WB	Acetone	EtOAc XT	CPC					3.2
125	T	L	30	Mycelia	Acetone: CH ₂ Cl ₂	MeOH	trituration					3
126	A	3	MeOH:H ₂ O	Hexane, CHCl ₃ xtn	CC RP 4	RP HPLC						100,
127	T	L	20	Mycelia	Acetone: MeOH	SiO ₂	RP HPLC 23					20(70% pure)
128	T	L	20	Filtrate	EtOAc	SiO ₂	RP HPLC 34					1.1–100
129	F	L	WB	EtOAc	Hex, CHCl ₃ seq liq-liq	SiO ₂	RP HPLC 42					120 7.6
130	F	L	WB	EtOAc	Hex, CHCl ₃ seq liq-liq	SiO ₂	RP HPLC 42					1.6
131	F	L	WB	EtOAc	Hex, CHCl ₃ seq liq-liq	SiO ₂	RP HPLC 42					2.5
132	F	L	WB	EtOAc	Hex, CHCl ₃ seq liq-liq	SiO ₂	RP HPLC 42					0.6
133		1.2	Acetone	SP207								47
134	6.3	nBuOH	LH-20 (2)	HP20SS								43.3
				RP HPLC 67	CC RP 8							

135	F	L	18	Mycelia	Acetone	CEtOAc XT	SSiO ₂	SRP HPLC 44	RP HPLC 46
136	F	L	18	Mycelia	Acetone	EtOAc XT	SiO ₂	RP HPLC 44	RP HPLC 46
137	NR								
138	NR								
139	T	L	20	Filtrate	HP-21	SiO ₂	RP HPLC 34	5.2, 2.9, 18.2, 3.9	41
140	F	L	1	WB	MeOH	SP207	RP HPLC 68	22, 7, 1.5	
141	A			WB	MeOH	EtOAc xtn	LH-20	RP HPLC	
142	F	L	?	Filtrate	CH ₂ Cl ₂	SiO ₂	Crystallization		?
143	T	L	12	WB	70%	EtOAc XT	SiO ₂	LH-20 (1)	-180
144	T	L	6	Filtrate	Acetone	HP-20	EtOAc xtn	LH-20 (19)	Prep TLC SiO ₂
145	F	L	40	Filtrate		HP20		RP HPLC 58	0.7, 0.5
146	F	L	40	Mycelia	Acetone	SiO ₂	pptrn		
147									
148	T	L	44.5	WB	nBuOH	EtOAc XT	SiO ₂	LH-20 (3)	CC RP 4
149	T	L	44.5	WB	nBuOH	EtOAc XT	SiO ₂	LH-20(3)	RP HPLC Xtal
150	T	L	44.5	WB	nBuOH	EtOAc XT	SiO ₂	Xtal	22
151	F	S	2		Acetone	EtOAc XT	hex:90%aq. MeOH	SiO ₂	365.9
152	F	S	2		Acetone	EtOAc XT	partition hex:90%aq. MeOH	Crystallization ML	Crystalliz ation
153									4200
154									
155	T	L	40	Filtrate	EtOAc	LH-20 (1)	RP HPLC 50		
156	T	L	75	Mycelia	MeOH				
157	T	L	300	WB	Acetone	HP20	CC RP 7	CC RP 7	72
158	T	L	300	Filtrate	EtOAc XT	Hexane pptrn	RP HPLC 42	RP HPLC 42	2000, 62, 24
				Mycelia	MeOH	EtOAc XT	SiO ₂	SiO ₂	28000 c

(Continued)

Table 4 Continued

Index ^a	F/T ^b	L/S ^c	Vol. ^d (L)	Location ^e	Extraction Solvent	Step 1 ^{f,g}	Step 2	Step 3	Step 4	Step 5	Step 6	Yield ^h (mg)
159	F	S	3.5	F	lyophilized	50% Acetone	EtOAc xtn	SiO ₂	CC ODS flash	CPC	RP HPLC	3.9
160	T	L	600	T	Acetone: MeOH	HP20	HP20	RP HPLC	58	42		34-711
161	F	L	8	F	EtOH	HP-20	SiO ₂	RP HPLC	4			89
162	F	L	8	F	EtOH	HP-20	SiO ₂	RP HPLC	4			4.9
163	F	L	F	EtOAc	seq Liq-liq XTN	CC-Cl18	CC RP 4	RP HPLC	42			2.3, 3.16
164	F	S	F	MeOH	SiO ₂	SiO ₂	SiO ₂ —prep TLC					32, 6
165	F	L	1	F	MeOH	CHCl ₃ xtn Filtrate/MeOH xtn solids	CCC LH-20 (7)	CCC	NP	HPLC		1.3, 5.5
166	T	L	170	T	EtOAc	SiO ₂						1900, 2300, 23, 30, 50
167	T	L	15	T	EtOAc	Differential pH xt	Sequential liquid partition-hex, toluene,	LH-20 (7)	RP HPLC	2		31
168	T	L	15	T	EtOAc	Differential pH xt	CHCl ₃ Sequential liquid partition-hex, toluene, CHCl ₃	LH-20 (7)	RP HPLC	2		18.2

169	F	L	8	Filtrate		HP-20	EtOAc xtn	SiO ₂		RP HPLC 21	HPLC-LiChrogel PSI	51
170	F	S	6 kg medium	WB	EtOAc	SiO ₂		LH-20 (1)	xtal			60, 49, 30
171	T	L	16	Filtrate		HP-21	SiO ₂		NP HPLC—LiChrosorb			9.3
172	T	L	16	Mycelia	MeOH:acet one	SiO ₂		HPLC—Diol	RP HPLC 24			16
173	T	L	3	Filtrate		HP-20	EtOAc xtn	SiO ₂		LH-20 (1)	RP HPLC 57	4.6 mixture
174	T	L	600	Filtrate	EtOAc XT							
175	T	L	600	Mycelia	Acetone	EtOAc	SiO ₂	SiO ₂		MPLC SiO ₂	SiO ₂	90170
176	F	L	3	Filtrate	EtOAc	CCC	RRP HPLC			Prep TLC		
177			350g	FB	MeOH	EtOAc XT	SiO ₂			RP HPLC49		25, 15
178												
179	F	L	3	Filtrate	EtOAc	SiO ₂	Xtal					90
180	F	L	1	WB	Aq. EtOH	EtOAc xtn	SiO ₂		LH-20 (1)			30
181	A	A	1	Mycelia	Acetone	ETHER xtn	SiO ₂					20.4
182		1		Mycelia	Acetone	ether xtn	SiO ₂					
183				WB	MEK	SiO ₂	LH-20					
184				Filtrate		HP-20	EtOAc xtn	SiO ₂		RP HPLC 42	MeOH pptn	20.4
185		L	30	Mycelia	Acetone	HP-20	EtOAc xtn	SiO ₂				41
186	T	S		WB	67% aq. acetone	EtOAc XT	SiO ₂			RP HPLC 4		209
187	F	L	0.9	Filtrate	EtOAc	SiO ₂	Prep TLC			RP HPLC 36		16
188	F	S		WB	MeOH→CHCl ₃	PEI column	SiO ₂	CC—		(Chromatot Whatman LPS-1		90.2
	F							MeOH→CHCl ₃				

(Continued)

Table 4 Continued

Index ^a	F/T ^b	L/S ^c	V _{ol.} ^d (L)	Location ^e	Extraction Solvent	Step 1 _{f,g}	Step 2	Step 3	Step 4	Step 5	Step 6	Yield ^h (mg)
189	F	S	WB	MeOH→ CHCl ₃ ; MeOH	PEI column	SiO ₂ (Chromatot- ron)						22.8, 25.0
190	F	S	WB	MeOH→ CHCl ₃ ; MeOH→ CHCl ₃	PEI column	SiO ₂ (Chromatot- ron)						3.7
191			2	WB	EtOAc	CH ₂ Cl ₂ xtn	RP HPLC 62					100
192	T	L	36	Filtrate								3.4–109.3
193	T	L	36	Mycelia	MeOH	EtOAc	SiO ₂	RP HPLC 37				200
194	F	L	0.8	WB	Acetone	EtOAc XT	RP HPLC 9					
195	T		200	Filtrate	XAD 7							
196				Mycelia	30% MeOH	CHP20P	Fractogel HW	RP HPLC 22	RP HPLC 22			11–120
197	F	L		Mycelia/fil- trate	EtOAc	SiO ₂	CC RP 3					8.1
198	T	L	105	WB	EtOAc	SiO ₂	RP HPLC 66					2100
199	T	L	30	Filtrate	EtOAc	SiO ₂	CC RP 4					15
200	T	L	30	Mycelia	Acetone							
201				WB	EtOAc	CC	RP HPLC					
202												
203	F	L	10	Mycelia	EtOAc	SiO ₂	CC RP 4					6
204	F	L	5	WB	Acetone	EtOAc XT	LH 20 (6)	Prep TLC	RP HPLC 59			12
205	F	L	6	WB	EtOAc	SiO ₂	CC RP 4	RP HPLC 46				3.2

Table 4 Continued

Index ^a	F/T ^b	L/S ^c	Vol. ^d (L)	Location ^e	Extraction	Step 1 ^{f,g}	Step 2	Step 3	Step 4	Step 5	Step 6	Yield ^h (mg)
226	F	L	10	WB	EtOAc	Kupchan partition	CCR P4	SiO ₂	CCRP 4	RP HPLC 42	NR	
227	F	L		Filtrate	EtOAc XT	SiO ₂	LH20				10	
228	A	S	4.6	WB	Acetone	NP HPLC	HPLC-SiO ₂				11	
229												
230	F	L		WB	Hexane	SiO ₂						
231												
232	F	L		WB	Hexane	SiO ₂						
233	F	S	?	WB	CHCl ₃ - MeOH	SiO ₂						
234	F	L	2.5	Mycelia	Acetone	HP-20	SiO ₂					
235	F	L	20	Filtrate	nBuOH	LH-20 (1)	SiO ₂					
236	F	L	40	WB	EtOAc XT	SiO ₂	LH-20 (1)					
237	F	S	10	WB	EtOAc	SiO ₂	LH-20 (1)					
238	F	L	60	Filtrate	EtOAc XT	SiO ₂	RPHPLC 49					
239	F	L	60	Mycelia	EtOAc XT	SiO ₂	RPHPLC 38					
240				FB	MeOH	SiO ₂	Fractogel TSK					
241	T	L	70	Filtrate	CHP20		MW-40s					
242	T	L	70	Mycelia	MeOH							
243	F	L	12	Mycelia/fil trate	EtOAc	SiO ₂	LH-20 (1)					
244	F	L	12	Mycelia/fil trate	EtOAc	SiO ₂	LH-20 (1)					
245	F		10	Mycelia	CH ₂ Cl ₂ : MeOH	nBuOH	SiO ₂					
246						partition						
247	T	L	29.5	Filtrate	HP20	SiO ₂	RPHPLC 56					
			4	Filtrate	XAD-16	HPLC-	PLRP-S					

248	T	L	85	Filtrate	CH ₂ Cl ₂	HP-20	SiO ₂	CC RP 5	xtal or SiO ₂
249	F	L		Filtrate		Amberlite T RA-400 20	Amberlite IR 1 SiO ₂	RP HPLC	1600, 5, 4, 21, 4
250	F	L	3	Mycelia	Acetone	EtOAc XT	CC RP 16 LH-20 (1)	LH-20 (21)	RP HPLC 58
251	F	L	1	Filtrate	EtOAc	SiO ₂	NP HPLC		97.5 7.2, 6.4
252	F	L	?	Filtrate	EtOAc	SiO ₂	LiChrospher partition		2
253	F	S		WB	EtOAc	Hex:MeOH partition	LH-20 (15) SiO ₂	RP HPLC 73	7.8
254	F	S		WB	EtOAc	Hex:MeOH partition	LH-20 (15) SiO ₂	RP HPLC 73	2.1
255	F	S		WB	EtOAc	Hex:MeOH partition	LH-20 (15) SiO ₂	RP HPLC 73	2.7
256	F	S		WB	EtOAc	Hex:MeOH partition	LH-20 (15) SiO ₂	RP HPLC 73	18.1
257	F	S		WB	EtOAc	Hex:MeOH partition	LH-20 (15) SiO ₂	RP HPLC 73	3.4
258	T	L	~10	WB	EtOAc	EtOAc xtn EtOAc XT	LH-20 (1) Toyopearl HW-40	RP HPLC 8 MPLC C18	15.2 7.3, 0.6
259				FB	Acetone	SiO ₂	RP HPLC 54		420, 380, 560
260	?	L	5	Filtrate	EtOAc	SiO ₂	RP HPLC 54		420, 380, 560
261	?	L	5	Mycelia	85% aq acetone	SiO ₂	RP HPLC 54		420, 380, 560
262	?	L	5			SiO ₂	RP HPLC 54		420, 380, 560
263	F	L	1	WB	EtOAc	LH-20 (1)	CHP20P	RP HPLC 12	50, 10, 12, 10, 15
264			8		EtOAc	SiO ₂	LH-20 CC SILICAR CC-4	RP HPLC CC RP 8	5, 50, 0.2, 34, 70 960
265	T	L	20	WB	Acetone	EtOAc XT		CC RP 8	

(Continued)

Table 4 Continued

Index ^a	F/T ^b	L/S ^c	Vol. ^d (L)	Location ^e	Extraction Solvent	Step 1 ^{f,g}	Step 2	Step 3	Step 4	Step 5	Step 6	Yield ^h (mg)
266	F	L	0.7	Mycelia	Aq. acetone	EtOAc XT	SiO ₂	RP HPLC 9	NP HPLC— Senshu- Pak silica- 4251-N	7-34		
267	F	L	2	Filtrate	EtOAc	SiO ₂	Prep TLC SiO ₂				2, 4	
268	T	L	6	Filtrate	EtOAc	SiO ₂	LH-20 (12)	RP HPLC 44			9.2	
269	T	L	6	Filtrate	EtOAc	SiO ₂	LH-20 (12)	RP HPLC 44			3.7	
270	F	L	4.9	Filtrate	HP-20	SiO ₂	RP HPLC 16				7.3, 15.9, 12.5, 23.2	
271	F	S									20	
272	S	6	WB		EtOAc	SiO ₂	SiO ₂	Prep TLC SiO ₂			4.0, 8.2, 7.9,	
273				FB	MeOH	SiO ₂	LH-20 (8)	Prep TLC LH-20 (7)	RP HPLC 43		1.1-15	
274				FB	MeOH	EtOAc XT	SiO ₂	SiO ₂			3	
275				FB	MeOH	EtOAc XT	SiO ₂	LH-20	RP HPLC 38			
276				FB	MeOH	EtOAc XT	SiO ₂	CC RP 4			110	
277	F	L	4	WB	EtOH	nBuOH XT	SiO ₂	CC RP 4			16.7	
278	F	L	?	Filtrate	CH ₂ Cl ₂	Kupchan partition	LH20	RP HPLC			10.2-76.7	
279	F	L	?	Mycelia	MeOH	Solvent partition (HEMW) 1:1:1:1)	CCC (HEMW— partition 1:1:1:1)	CC RP 4	LH-20 (1)		1.6	
280	F	L		Mycelia	MeOH	CH ₂ Cl ₂ /H ₂ O partition	LH-20 (7)	SiO ₂	RP HPLC		4.6, 3.9, 15.5, 21.5	
281	T	L	20	Filtrate	HP21	SiO ₂ EtOAc	pptrn CC RP 10	RP HPLC			62	
282	T	L		Mycelia	Acetone	partition					17	

283	L	23	Filtrate	EtOAc	LH-20 (2)	SiO ₂ -MPLC	RP HPLC	5.2, 23.7, 418.9, 111, 3.85, 5.4, 3.8 25.7, 6.3 25.7, 6.3
284	T	16	Filtrate	EtOAc	SiO ₂	RP HPLC 31		
285	T	16	Mycelia	MeOH:	SiO ₂	RP HPLC 31		
			acetone	acetone				
286	T	30	Filtrate	HP20	SiO ₂	Xtal		131, 4.8
287	F	2	WB	EtOH	CC RP 8	LH20	RP HPLC 35	106.2
288	F	2	WB	EtOH	CC RP 8	LH20	RP HPLC 35	13.9
289	F	S	WB	Aq. acetone	SiO ₂	RP HPLC 58	Chiral	90
						HPLC—		
						Chirex—		
						NGY		
290			FB	MeOH	HP-20	EtOAc	LH-20 (17)	RP HPLC 61
291	S	5	WB	Acetone	HP20	partition		4, 1.5, 1.0
292	F	L	20	EtOAc	SiO ₂	SiO ₂	LH20	RP HPLC 52
293	F	L	5	Filtrate	EtOAc			9-138
294	F	L	14	Filtrate	EtOAc	LH-20 (2)	MeOH	12
								59-103
								33
295	F	L	14	Filtrate	EtOAc	LH-20 (2)	trituration	28
296	F	L	14	Filtrate	EtOAc	LH-20 (2)	SiO ₂	25
297	F	L	14	Filtrate	EtOAc	LH-20 (2)	SiO ₂	7.5
298	F	L	14	Filtrate	EtOAc	LH-20 (2)	MeOH	283
299	F	L	14	Filtrate	EtOAc	LH-20 (2)	trituration	95
300	F	L	14	Filtrate	EtOAc	LH-20 (2)	RP HPLC 29	101
301	T	L	?	WB	MEK	SiO ₂	RP HPLC	?
302	F	L	7.5	Filtrate	HP-20	SiO ₂	Prep TLC	16.5
303	F	L	1	Filtrate	EtOAc XT	LH-20 (1)	SiO ₂	23.5
							RP HPLC 59	

(Continued)

Table 4 Continued

322 T	L	300	Filtrate	EtOAc	LH-20 (2)	SiO ₂	SiO ₂	rep RP HPLC 40	7.3, 8.4
323 T	L	25	Mycelia	MeOH	Amberlite IRG-50	SP207	CC RP 9		120
324 T	L	10	Mycelia/ filtrate	EtOAc/ acetone	SiO ₂	CC RP 7	LH-20 (18)	RP HPLC 57	132
325 T	L	320	Mycelia	H ₂ O	HP20	CC RP 9	CC RP 9	CC RP 9	64.3
326 T	L	40	Mycelia	MeOH	HP20	CC RP 9	CC RP 9	HP20	11
327 F	L	7	WB	EtOAc	SiO ₂	CC RP 4	NP HPLC		14
328 T	L	100	Filtrate	HP-21	SiO ₂	SiO ₂	RP HPLC 34		16
329 T	L	100	Filtrate	HP-21	SiO ₂	RP HPLC 34			27
330									
331 F	L	500	Mycelia	MeOH	pptn	Batch BioPrep P40 ODS-3	RP HPLC 19	Xtal	1990
332 T	L	220	Mycelia	MeOH	HP-20	EtOAc:MEK xtn	Pptns	Xtal	41600
333									
334									
335 T	L	80	FB	EtOAc	SiO ₂	LH-20 (7)	SiO ₂	RP HPLC	28.6, 2.3
336 T	L	20	FB	EtOAc	SiO ₂	LH-20 (7)	SiO ₂	RP HPLC 24	95.9
			Filtrate	HP-20	SiO ₂	RP HPLC			12
			Mycelia	MeOH→ acetone	SiO ₂	NP HPLC LiChrosorb Diol			14
337 F	L		Mycelia	MeOH	EtOAc	SiO ₂	Prep TLC		20, 23
338			FB	Acetone	EtOAc XT	SiO ₂			5.8, 5.2
339 F	L		WB	Hexane	CC C18	RP HPLC			
340 T	L	200	Mycelia	MeOH	CHP20	Fractogel EMD SO ₃ ⁻	RP HPLC 32		140
341 F	L	2	Mycelia	Acetone	EtOAc	SiO ₂	RP HPLC		
342 T	L	50	Mycelia	Acetone	hex:CH ₂ Cl ₂	SiO ₂	RP HPLC 3	pptn	Xtal
				xtn					800
									2710

(Continued)

Table 4 Continued

Index ^a	F/T ^b	L/S ^c	Vol. ^d (L)	Location ^e	Extraction Solvent	Step 1 ^{f,g}	Step 2	Step 3	Step 4	Step 5	Step 6	Yield ^h (mg)
343	F	S	9	WB	Acetone hex, EtOAc	SiO ₂ polyamide	LH-20 (1) CC	SRP HPLC			7.5, 9	
344				FB		Kupchan partition	LiChroprep Diol	NP HPLC				
345	F	L	1.5	Filtrate	EtOAc	CH ₂ Cl ₂ xtn	SiO ₂	RP HPLC 42			220 5.8	
346	F	S	1	Filtrate	MeOH MeOH=> EtOAc	SiO ₂	SiO ₂	SiO ₂			14.6, 69.9, 63	
347	Agar			Mycelia/ filtrate	WB	CH ₂ Cl ₂	SiO ₂	NP HPLC 49			8.7, 0.8	
348	F	S			WB	CH ₂ Cl ₂	SiO ₂	NP HPLC			6	
349	F	S			WB	CH ₂ Cl ₂	SiO ₂	NP HPLC			4	
350	F	S			WB	CH ₂ Cl ₂	SiO ₂	Crystallization			72	
351	F	S			WB	CH ₂ Cl ₂	SiO ₂	NP HPLC			9	
352	T	L	30	Mycelia	Acetone	SiO ₂	RP HPLC37				50	
353	F	L	1.6	Mycelia	CH ₂ Cl ₂ : EtOH	CH ₂ Cl ₂ /H ₂ O part	LH-20 (1)				8.1	
354	T	L	19	Filtrate		HP-21	EtOAc xtn	SiO ₂	HPLC-LiCh rogel PS 1	HPLC- LiChrosorb	5.6	
355	T	L	19	Filtrate		HP-21	EtOAc xtn	SiO ₂	HPLC-LiCh rogel PS 1	HPLC- LiChrosorb	2.2	
356	T	L	19	Filtrate		HP-21	EtOAc xtn	SiO ₂	HPLC-LiCh rogel PS 1	HPLC-LiCh rogel PS 1	81	
357	F	L	2.5	Filtrate	EtOAc XT EtOAc	flash SiO ₂ Kupchan	Hexane xtnal SiO ₂	RP HPLC SiO ₂			6.5, 10.6	
358									NP	NP	2.85	
									HPLC-Nu cleosil			

359	L	10	Filtrate Mycelia	Acetone EtOAc XT	SiO ₂	HW40	RP HPLC 63	1.93
360	F	10	Filtrate Mycelia	SiO ₂	CCC	CC RP 10	CC RP 10	2.2-5.6
361	F	10	Filtrate WB	SiO ₂	RP HPLC 29	NP HPLC		46
362	T	1.5	CH ₂ Cl ₂ :MeOH (1:1)	EtOAc				7.9, 3.1
363	S			MeOH reflux?	SiO ₂			26
364	F	100	WB	EtOAc CH ₂ Cl ₂ :MeOH (1:1)	SiO ₂	LH-20 (7)		134
365	F	100	WB?	EtOAc CH ₂ Cl ₂ :MeOH (1:1)	SiO ₂			39.1, 3
366	F	20	Mycelia	CC RP 4	RP HPLC 14			
367		6	Filtrate Mycelia	HP-20	SiO ₂	RP HPLC 17		88, 18.7
368	F	5	Mycelia	MeOH:acetone MeOH:acetone MeOH:acetone 80% acetone	SiO ₂ SiO ₂ SiO ₂ EtOAc xin	CHP20 CHP20 CHP20 SiO ₂	SiO ₂ CHP20 CHP20 LH-20 (1)	49 22, 27 21, 9 6
369	F	5	Mycelia					
370	F	5	Mycelia					
371	T	14	Mycelia					
372								
373			FB	Hex, CH ₂ Cl ₂ , MeOH	SiO ₂			5-55
374	F	20	Mycelia	60% aq. acetone	HP-20	SiO ₂	Crystallization	55
375	F		WB	EtOAc	SiO ₂	RP HPLC 14		6.5
376	F		WB	EtOAc	SiO ₂	RP HPLC 14		2.2
377	F	5	Filtrate	EtOAc	EtOAc			382
378	S		WB	EtOAc	trituration	LH20		300830
379	F	5	Mycelia	MeOH	SiO ₂ EtOAc XTn	LH-20 (1)	RP HPLC 29	64, 3.7

(Continued)

^a Index	RP HPLC Column
1	Alltech Hyperprep 100 BDS
2	Bondpak RCM C18
3	C18 Bond-Elut
4	Capcell Pak C18
5	Capcell Pak UG
6	Capcell Pak
7	Carotenoil C30
8	Chemcosorb 5ODS-UH
9	Cosmosil 5C18-AR
10	Cosmosil 75C18-OPN
11	Daiso Pak C18 BP
12	DeltaPak C18
13	Develosil ODS-10
14	Dynamax
15	Eurospher C-18
16	Inertsil ODS
17	Inertsil PREP-ODS
18	J'spher ODS H80
19	Kromasil C8
20	LiChroprep RP-18
21	LiChrosorb
22	LiChrosorb RP-select B
23	LiChrospher WP300 RP18
24	LiChrosphere RP18
25	Metasil ODS
26	MODCol C18
27	NovaPak
28	NovaPak C18
29	NovaPakHR C18
30	Nucleosil 100-7 C18 HD
31	Nucleosil 100 C18
32	Nucleosil 100 C18AB
33	Nucleosil 100-5 C18
34	Nucleosil C18
35	ODS Fluofix IEW Z25
36	Pegasil C8
37	Pegasil ODS
38	PhenomenexMAXSIL C18
39	Prep Nova-Pak HRC18
40	Prep Shim-Pak PREPODS
41	RP HPLC
42	RP HPLC - ODS
43	Senshu Pak
44	Senshu Pak ODS H-4251
45	Senshu Pak PEGASIL 120-5
46	Senshu Pak PEGASIL ODS
47	Senshu-Pak ODS-N
48	Shandon hyperprep or Novaprep C18 60A
49	Spherisorb ODS-2
50	Spherisorb RP18
51	SSC ODS-SH
52	Supelcosil ABZ
53	Supelcosil C8
54	Whatman CCS/C8 or Kromasil C18
55	YMC AM312
56	YMC D-ODS-5-B
57	YMC D-ODS-AM
58	YMC ODS
59	YMC ODS AM SH-343-5-AM
60	YMC ODS AM-343
61	YMC ODS H80
62	YMC ODS S-5
63	YMC OSA AM-323
64	YMC-Pak D-ODS-5
65	YMC-Pak D-ODS-AM
66	YMC-Pak S-363
67	YMC-R-ODS-5B
68	Zorbax C8
69	Supelcosil SPLC -18
70	Cosmosil C18
71	Zorbax Rx C8
72	Varian Bond Elut C18
73	Ultrasphere C18

Index	Column Chromatography RP Packings
1	C18-Develosil
2	WhatmanLPS-1
3	Lobar RP-18
4	ODS
5	ODS-YMC-A60
6	RP
7	YMC ODS

^b F/T: F = flask fermentation, T = tank or fermentation, A = agar.

^c L/S: L = liquid fermentation, S = solid fermentation, FB = fruiting body.

^d Vol: fermentation volume harvested.

^e Location: portion of fermentation extracted to obtain reported compound; WB = whole broth, filtrate = clarified culture broth, mycelia = mycelium after clarification, filtrate/mycelia = components separately extracted and then extracts combined after extraction

^f Steps 1–6: isolation step; reverse phase column chromatography packings listed in footnote a (above), RP HPLC column used as reported in literature also listed in footnote a.

^g LH-20 solvents: LH-20 (1) = MeOH; LH-20 (2) = CH₂Cl₂:MeOH; LH-20 (3) = CH₂Cl₂:MeOH (1 : 1); LH-20 (4) = acetone; LH-20 (5) = hexane:CH₂Cl₂ (2 : 7) increasing to MeOH; LH-20 (6) = CH₂Cl₂:MeOH (2 : 1); LH-20 (7) = CHCl₃:MeOH (1 : 1); LH-20 (8) = CHCl₃:MeOH (2 : 1); LH-20 (9) = hexane:toluene:MeOH (3 : 1 : 1); LH-20 (10) = hexane:CH₂Cl₂:acetone; LH-20 (11) = isooctane:toluene:MeOH; LH-20 (12) = hexane:CH₂Cl₂:MeOH; LH-20 (13) = MeOH:H₂O (2 : 1); LH-20 (14) = 25% MeOH; LH-20 (15) = hexane:CH₂Cl₂ step gradient; LH-20 (16) = CH₂Cl₂:MeOH (4 : 1); LH-20 (17) = 70% aqueous MeOH; LH-20 (18) = hexane:CHCl₃:MeOH; LH-20 (19) = aqueous MeOH; LH-20 (20) = acetone:CH₂Cl₂ (2 : 1); LH-20 (21) = EtOH.

^h Yield: isolated yields of reported compounds

employed to isolate fungal metabolites in a random mix of industrial, academic and government laboratories. Three general types of information are included:

1. Source information—trivial name, fungal taxonomy (if identified), and a general description of the structural type
2. Extraction information—type of fermentation (solid or liquid, flask or tank), location of product (mycelium or filtrate), the volume of the fermentation, extraction solvent, and isolated yield
3. Isolation process—the reported sequence of isolation steps

Most of the individual isolation processes fit the three-step general model described, although it is doubtful that most were designed this way. The first reported isolation step was equated with recovery and resolution, the final step with polishing, and any in between as purification. Thirty percent of the isolations were accomplished in three steps, and 80% used between two and four steps (Fig. 7).

The inherent bias of practicing natural products chemists is that most fungal metabolites can be isolated using some combination of reverse-phase HPLC or column chromatography, silica gel chromatography, and LH-20. The data in Table 4 clearly support this. Silica gel, reverse-phase (HPLC and low-pressure columns combined) and LH-20 accounted for 73% of the total isolation steps reported for the 379 fungal metabolites (Fig. 8). The techniques used were further broken down to those reported for the first and last isolation steps to compare with the concepts of resolution and recovery and polishing steps, respectively (Fig. 9). Three separation methods—silica gel, liquid–liquid partition, and SPE (including low-pressure reverse-phase chromatography)—accounted for the first-step isolation method for 87% of the compounds. Silica gel is a commonly used first isolation step in the literature but is generally less commonly employed as such for industrial bioactivity-guided isolation. Most (56%) of the isolations used reverse-phase as the final, polishing, isolation step.

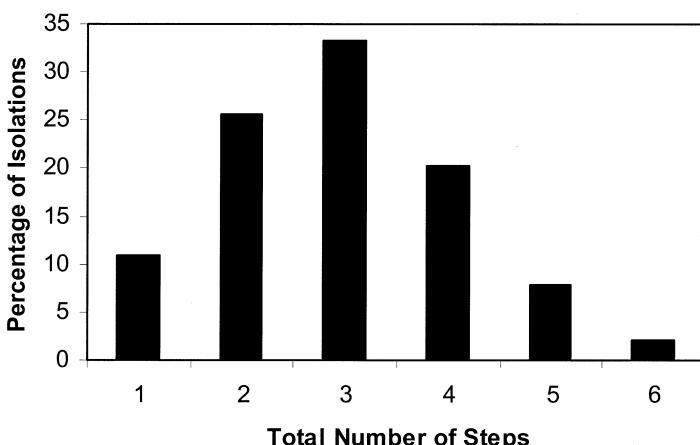


Figure 7 Frequency of the number of required purification steps for fungal metabolites described in Table 4.

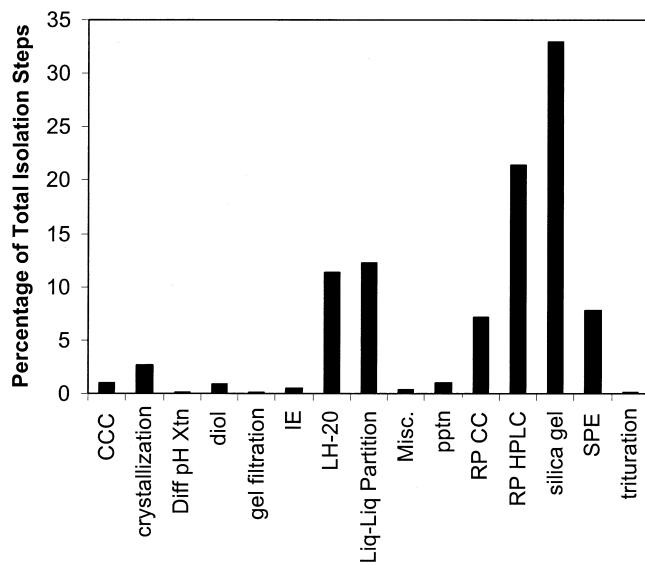


Figure 8 Frequency of usage of various separation methods reported for the isolation of fungal metabolites described in Table 4.

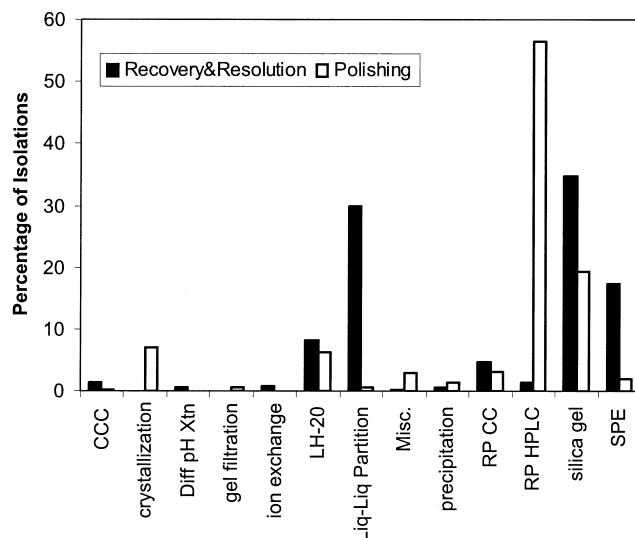


Figure 9 Comparison of the reported separation method usage for recovery and resolution step and for polishing step for fungal metabolites described in Table 4.

2. STRUCTURE ELUCIDATION

Structure elucidation is the process of proposing a chemical structure for an isolated fungal metabolite. Modern structure elucidation relies heavily on two techniques of organic spectroscopy, mass spectrometry and nuclear magnetic resonance spectroscopy (NMR). Advances in these two techniques currently allow routine structure determination of new natural products using only 0.5 to 1 mg of pure compound. This quantity of material is sufficient to allow the required datasets to be rapidly generated in 1 to 2 days or less. In many cases this is less material than required for evaluating the biological activity of the isolated compound. As a result of these advances, and as mentioned above, structure elucidation is no longer the most challenging or time consuming part of natural products chemistry.

The amount of time and effort required for the structure elucidation of a fungal metabolite depends on its degree of novelty. Thus, the first step for an isolated metabolite is to ascertain whether the compound is new or known—a process called dereplication. If it is known then a comparison of its physiochemical properties with those reported in the literature, or comparison with an authentic sample if available, is sufficient to establish the structure. For a new compound, it is helpful to determine just how novel it is. A new member of a known fungal metabolite family (e.g., trichothecenes, cytochalasins, sordarins, peptaibols) requires less time and effort than an entirely novel compound since interpretation of the spectroscopic data is aided by comparison with that reported for known analogs. Entirely novel metabolites require extensive spectroscopy to deduce the planar, stereochemical, and absolute structures. A general outline of the structure elucidation process is shown in Fig. 10 and will be further described below. Aside from dereplication, very little of this process is unique to fungal metabolites, but a general knowledge of the common families of fungal metabolites is useful.

2.1. Physiochemical Characterization

2.1.1. Establishment of the Molecular Weight and Molecular Formula

Establishment of the molecular weight and, ideally, a molecular formula for an isolated fungal metabolite is essential information for determining the novelty of an isolated compound. The molecular formula can be used to search natural products and other chemical databases to determine if the metabolite is known. Comparison of the physiochemical data for the isolated metabolite with the known structures of the same molecular formula leads to rapid recognition of a known or conversely, can strongly suggest that the isolated metabolite is novel.

Low resolution mass measurements are easily obtained using standard mass spectrometry methods or from HPLC mass spectrometry (LCMS) data. LCMS is typically used for low-resolution mass spectra of natural products because it allows correlation of the major chromatographic component of the sample with a mass spectrum. Electrospray ionization and atmospheric pressure chemical ionization (APCI) are the commonly used ionization techniques for LCMS. Both are chemically gentle and generally yield a strong molecular ion with little fragmentation. Both types of ionization can be used in positive ion mode, to measure a protonated molecular ion $[M + H]$, or other positively charged adducts such as $[M + Na]$, $[M - H_2O + H]$, or in negative ion mode to measure $[M - H]$. Positive ion mode is more general but the presence of a negative ion spectrum suggests the presence of an acidic or other negatively charged functional group.

physiochemical characterization

- MS
- 1H, 13C NMR spectra
- ultraviolet spectrum
- infrared spectrum



establish molecular weight / molecular formula

- exact mass measurement (HRMS) to suggest possible molecular formulae
- reconciliation with carbon number



dereplication

**known
compound**



- literature searches of chemical and natural product database



planar structure determination

- 2-dimensional NMR spectroscopy - 2 and 3 bond connectivity experiments (COSY, HMQC/HSQC, HMBC, TOCSY)
- MS-MS fragmentation



relative stereochemistry determination

- 1 and 2-dimensional NMR spectroscopy - atom proximity experiments (nOe difference, NOESY, ROSEY)



absolute stereochemistry determination

- X-ray crystallography
- chiroptical methodology (circular dichroism)

Figure 10 Outline of structure elucidation process.

Exact mass measurements obtained with high-resolution mass spectrometry are extremely valuable and are used to suggest or a possible molecular formula or support a proposed formula. Classically high-resolution electron impact or fast atom bombardment ionization techniques were used for high-resolution mass measurements. Fourier transform mass spectrometry is becoming increasingly available and allows accurate mass measurement to within 0.5 ppm. It also has the advantage of being compatible with the atmospheric pressure ionization techniques, electrospray ionization and APCI.

Exact mass measurements are used to suggest a potential molecular formula for isolated fungal metabolite. Greater accuracy in the measurement leads to a shorter list of potential molecular formulas. Comparison of the suggested molecular formula(s) with the other physiochemical data available for the compound is required to establish the correct formula for a novel metabolite. Most useful is a count of the total number of carbons present in the molecule. This can be directly determined from the number of resonances observed in the ^{13}C NMR spectrum if sufficient sample is available. An indirect carbon count is also possible from a combination of HMQC/HSQC and HMBC (see below) two-dimensional (2D) NMR data. It is not unusual, however, to begin structure elucidation of a new metabolite without a rigorously confirmed molecular formula.

2.1.2. Absorption Spectra and Other Physiochemical Data

Complete physiochemical characterization requires the recording of UV and infrared absorption spectra, optical rotation, melting point (if crystalline), and ^1H and ^{13}C 1D NMR spectra for the isolated metabolite. UV and infrared spectra reveal functional groups rather than the atom-to-atom connectivity derived from NMR spectra. The complexity and wavelength(s) of the absorption maxima in the UV spectrum indicate the extent of conjugation present in the metabolite. Structure elucidation relies predominantly on NMR and mass spectrometry, but this additional physiochemical data is useful for supporting or refuting the presence of certain functional groups in the unknown. For a recent isolate of a metabolite reported before the mid-1970s, this physiochemical data maybe the only way to establish a correlation between the two compounds.

2.2. Dereplication

Dereplication is the process of recognizing known natural products. Numerous, general dereplication strategies for natural product extracts have been reported and were recently reviewed [21]. These are commonly based on reverse-phase HPLC with diode array and mass spectrometry detection. Reference libraries of UV and mass spectra are constructed and used to recognize knowns from new sources. Dereplication is ideally applied to the crude extract or semipurified fraction as obtained from a well-designed recovery and resolution step (above). Early stage recognition of knowns saves the costly time and effort required for complete isolation and structure elucidation. It also allows the chemical examination of many more extracts than would otherwise be possible.

The known natural product(s) depends on the target of interest. For example, high-throughput screening of crude natural product extracts in a biological screening assay might yield tens of quality hits. If no natural products are known actives in the screen then dereplication is not necessary; the first natural product isolated from the active extracts is of interest. Following isolation of this first active component the remaining extracts require dereplication to rule out the presence of this compound. This is an iterative process as the number of known natural product actives increases. The tens of active extracts may actually contain only a few different active components.

Dereplication can also be a more general process. For example, a general standardized system has recently been described for fungal metabolites and mycotoxins based on liquid chromatography, UV, and mass spectrometry data [22]. The target for the system was simply the recognition of new fungal metabolites in a few genera of microfungi of interest to the authors. Data were reported for a reference collection of 476 fungal metabolites analyzed using the method. Thus, dereplication in this example had 476 knowns.

2.3. Structure Elucidation of New Metabolites

2.3.1. Planar Structure

Initial examination of the chemical shifts of the resonances present in the ^{13}C NMR spectrum of a new metabolite provides a general indication of the types of carbons (e.g., methyls, olefinic, aromatic, carbonyl, attached to a heteroatom) present in the molecule. Similarly, the chemical shifts of the protons in the ^1H NMR spectrum reveals the number of methyl groups, the presence of aromatic rings, olefins, and heteroatoms. The multiplicity, or the number of directly bonded protons, of each carbon is determined using various polarization transfer pulse sequences such as distortionless enhancement by polarization transfer (DEPT) or insensitive nuclei enhanced by polarization transfer (INEPT) [23].

Structure elucidation is the process of connecting and correlating the atoms of the compound. Obtaining this correlation information relies heavily on 2D NMR spectroscopy [24]. 2D NMR pulse sequences are designed to provide either ^1H - ^1H , ^1H - ^{13}C or ^{13}C - ^{13}C correlation information. One of the first and simplest of these sequences, ^1H - ^1H correlation spectroscopy (COSY), correlates protons that are directly coupled. In a COSY spectrum, a cross-peak is observed for each pair of directly coupled protons generally indicating that they are either geminal or vicinal in the molecule. From this correlation information the various spin systems of the metabolite can be established resulting in one to several partial structural fragments. Similar information can also be derived from the totally correlated spectroscopy (TOCSY) experiment. In this experiment, cross peaks are observed for all of the protons of a spin system.

Two general types of ^1H - ^{13}C correlation information can be obtained. The first type provides one bond ^1H - ^{13}C heteronuclear connection information (i.e., which protons or protons are attached to which carbon), which is derived from heteronuclear multiple quantum coherence (HMQC) or heteronuclear single quantum coherence (HSQC) experiments. The data from these experiments allow the correlation of every carbon-bonded proton with a carbon in the ^{13}C NMR spectrum. Quaternary carbons are not observed using these sequences since they do not have an attached proton. Thus, the carbons to which the protons of the spin systems established from COSY information can be determined.

The second type of ^1H - ^{13}C correlation information provides two-, three-, and occasionally four-bond correlation information. The most generally useful of these sequences is heteronuclear multiple bond correlation (HMBC). This pulse sequence is extremely useful for the connection of the isolated spin systems derived from COSY and HSQC data. In addition, HMBC spectra are able to see quaternary carbons if correlated with one or more protons in the molecule. In the HMBC spectrum, cross-peaks can be observed from a proton to carbons that are two and three bonds removed. In contrast to COSY and HMQC/HSQC spectra, the observation of a cross-peak depends on the scalar coupling, known as the J-coupling, between the ^1H and ^{13}C nuclei. Just as for ^1H - ^1H coupling, this coupling has an angular dependence so that just being separated by three bonds does not guarantee observation of a correlation. HMBC spectra are narrowly optimized for a particu-

lar multiple bond J_{HC} , usually ranging from 4 to 10 Hz, and it is typical to run two or three HMBC experiments, each optimized for a different multiplet bond J_{CH} .

A typical set of basic 1D and 2D NMR experiments for a new fungal metabolite includes DEPT, COSY, HSQC, and HMBC. In many cases, these experiments are sufficient to propose a planar structure for a new compound. Large and complex metabolites or those with extreme overlap of multiple proton and carbon resonances may require additional work or more selective pulse sequences. Variants of the COSY sequence—e.g., double quantum filtered COSY (DQCOSY), COSY-45, or long-range COSY (LR-COSY)—exist that allow better interpretation of complex cross-peaks or crowded regions of the spectrum. Combination pulse sequence experiments such as HSQC-TOCSY are particularly useful for triterpenes and peptides that can have a large amount of resonance overlap in a small region of the spectrum making unambiguous interpretation difficult. A guide to the selection of appropriate pulse sequences for such natural product structure elucidation problems has been presented [25].

2.3.2. Stereochemistry

Complete structure elucidation of a new fungal metabolite requires the determination of the relative 3D orientation of each atom with respect to the other atoms of the molecule and, ideally, the absolute chirality of the compound. These are known as relative and absolute stereochemistry, respectively. Stereochemical determination relies on NMR spectroscopy, chemical derivatization, x-ray crystallography, and occasionally circular dichroism spectroscopy. The general procedure is to first establish the relative stereochemistry followed by the absolute stereochemistry although if the compound is crystalline both can often be obtained using x-ray diffraction.

Determination of the relative stereochemistry of a fungal metabolite relies heavily on the analysis of the through-bond and through-space coupling between protons observed in 1D and 2D ^1H NMR spectra. Through-bond J -coupling of vicinal and geminal protons results in the splitting of the resonances observed for individual protons in a 1D ^1H NMR spectrum into multiplets. Analysis of these multiplets can reveal the magnitude of the J -coupling and thus their relative orientation in the molecule. The magnitude of the J -coupling, the coupling constant, is dependent on the relative angles of the C–H bonds of the two protons. For example, the geminal protons of a cyclic methylene exhibit a large, typically 10 to 12 Hz, coupling constant and vicinal proton coupling constant typical range from below 1 to 10 Hz. This J -coupling is also observed in some forms of 2D ^1H – ^1H spectroscopy such as double quantum filtered correlation spectroscopy (DQF-COSY) and J -resolved experiments.

Through-space interaction between the protons of a molecule also is observed. The magnitude of this interaction depends on the relative proximity of the protons within a molecule and occurs as a result of the nuclear Overhauser effect ($n\text{Oe}$). Since this is a through-space interaction the observation of a nuclear Overhauser enhancement is not limited to individual spin systems. Numerous 1D and 2D NMR pulse sequences have been described to obtain $n\text{Oe}$ information. The classic, and still useful, 1D method for the observation $n\text{Oes}$ is known as $n\text{Oe}$ difference spectroscopy. The most commonly used 2D NMR pulse sequences for observation of $n\text{Oes}$ are known as NOESY (nuclear Overhauser effect spectroscopy) and ROESY (and variants).

The above methodology for stereochemical assignment is most applicable to conformationally rigid fungal metabolites. Difficulties arise for acyclic molecules, such as polyketides, or molecules with an acyclic portion containing a chiral center. For example,

numerous fungal metabolites possess linear, 4- to 18-carbon aliphatic appendages containing one or more chiral centers, such as a secondary alcohol, attached to a cyclic core. Stereochemical assignment of the core is using the above methods is usually possible but assigning the stereochemistry of the chiral center in the acyclic portion can be difficult.

The absolute stereochemistry for a fungal metabolite of known relative stereochemistry can be determined by establishing the absolute chirality, R or S, of one of the chiral centers present in the molecule. Most methods require chemical derivatization followed by spectroscopic analysis of the resulting derivative. The Mosher ester method is the most widely applied and is based on formation of *R*- and *S*- α -methoxy- α -trifluoromethylphenyl acetic acid esters (MTPA) of *sec*-alcohols (or amines), which are then examined using NMR methods [26]. The effect of the MTPA ester on the chemical shifts of the neighboring protons of the alcohol (natural product) generally allows the determination of the absolute chirality of the starting alcohol. Analysis of exciton coupling in the circular dichroism spectra of a metabolite with distinct but coupled chromophores, or typically a di-benzoate ester derivative, can be used to determine the absolute orientation of the two chromophores and thus the absolute stereochemistry of the metabolite [27]. Finally, x-ray analysis of suitable crystals containing a heavy atom, such as a Br or Cl derivative, can be used to unequivocally establish the absolute stereochemistry, as well as the complete structure, of a fungal metabolite.

3. FUTURE PROSPECTS

Interdisciplinary research at the interface of biology and chemistry is an area of increasing interest to chemists. The power of collaboration between these two disciplines has long been evident to those involved in the bioassay-guided discovery of novel fungal metabolites. While industrial interest in fungal metabolites is cyclical, their potential as sources of new biological active compounds remains undiminished. Continued discovery of novel fungal metabolites depends on the ability of mycologists to isolate and culture the new fungi that produce them, the development of new biological assays to detect their presence, and the ability of natural products chemists to isolate them. The challenge to industrial natural products chemists is to increase the efficiency of the isolation of novel fungal metabolites. This will require the development of automated, parallel, and high-throughput separation methods and strategies that are as robust and as elegant as a single isolation.

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The author wishes thank his mentor, off-and-on supervisor over the years, and friend Dr. Ken Wilson who has been critical to the formulation of many of the ideas expressed herein. His rigorous, organic chemist approach to natural products isolation has continually demonstrated that the challenge and art of modern natural products chemistry is in the isolation of the compound. The author also wishes to thank past and present members of the Merck natural products chemistry group for countless valuable discussions over two decades.

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PCR-Based Data and Secondary Metabolites as Chemotaxonomic Markers in High-Throughput Screening for Bioactive Compounds from Fungi*

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1. INTRODUCTION

Even though natural products (NPs) in general and fungal metabolites in particular still play an important role in modern drug discovery, the introduction of high-throughput screening (HTS) technologies afforded considerable changes in paradigms to keep the workflow for NPs competitive with that of synthetic and combinatorial chemistry. As highly automated prefractionation and subsequent random testing have widely replaced the traditional extract screening, the reduction of redundancies in NP-screening libraries became more important than ever before. The application of chemotaxonomy and polymerase chain reaction (PCR)-based data in a preselection strategy for fungal strains to enhance the quality and structural diversity of the resulting sample pools is discussed. While classical chemotaxonomy constitutes a powerful tool to predict metabolic diversity in plants, alternative methods need to be established to reach this goal with fungi. Previous efforts of PCR fingerprinting to identify redundancies in collectives of morphologically similar fungi are summarized. Correlations between HPLC profiling, PCR-based applications, and

* Dedicated to Prof. Dr.(em) Wolfgang Steglich on the occasion of his 70th birthday.

classical morphology are exemplified by the outcome of a recent polyphasic study on *Daldinia* and allies (Xylariaceae). The relationships between chemotaxonomy and molecular phylogeny are illustrated by some examples of Boletales. Future perspectives for de-replication and screening of fungi in HTS are discussed in relation to the characterization of secondary metabolite gene clusters.

1.1. Change of Paradigms: Why Are Natural Products Compatible with High-Throughput Screening?

The current chapter will address the role of NPs—and fungal metabolites in particular—in the HTS procedures that were inaugurated in pharmaceutical and agricultural lead structure research [1]. Technologies relating to automated preparation and distribution of samples for random biological testing are being constantly refined and optimized. Robot-assisted miniaturized functional assays, involving reporter cell lines, target enzymes, or receptors, can now be performed in 384-well microtiter plates (or in so-called ultra-HTS using 1536-well microtiter plates). Alternative readouts such as fluorescence resonance energy transfer and fluorescence polarization are readily available for automated recording of test data [2–4]. Screening times and costs, as well as amounts of test samples and biological assay kits needed for primary screening, have decreased considerably. Microarrays appear promising to even further reduce the requirements for sample quantities [5]. Because of the developments in combinatorial chemistry and parallel synthesis, the synthetic pools available for testing drastically increased at the same time. These efforts were paralleled by adaptations of data management, endowing the revolutionary developments in computer technologies. Even though special NP-adapted screening approaches appear feasible for HTS, many companies regard screening NP and synthetic products in concert to be more efficient, making use of the same logistic prerequisites. The same capacities for generation of NP extracts, fractions, and pure compound libraries may even be shared by several business units, such as when a company is doing synergistic lead structure research in agricultural and in pharmaceutical indications.

Because of adjustments following the introduction of HTS, the process of NP screening underwent some major changes. The traditional workflow led from biologically active crude extracts via bioassay-guided fractionation to the identification of lead compounds. A large number of preliminary test data were needed during bioassay-guided fractionation before the active compounds were finally identified. This prolonged the time for evaluation in screens with high hit rates and/or low sample throughput. The identification of an appropriate lead structure from natural extracts therefore lasted several months or even years. Meanwhile, requirements for quantities and purity of NPs for structure determination have been steadily decreasing, due to further developments in the fields of nuclear magnetic resonance spectroscopy, mass spectrometry, and liquid chromatography coupled with spectral techniques. Structure elucidation of novel NP can now be done by using less than 1 mg of the enriched compound. High-performance liquid chromatography (HPLC) profiling [6,7] allows for the identification of known compounds from crude extracts in the nanogram range. Wherever possible, automated techniques were introduced for downstream processing of extracts as well (Fig. 1). Automation of extraction, chromatography, and sample preparation are additional features to accelerate the process of the screening workflow, rendering the work on NP highly compatible and competitive to HTS of substance pools from chemical synthesis or combinatorial chemistry. Even for assessment of preclinical *in vitro* structure–activity relationships, which usually precedes the large-scale preparation of lead compounds for evaluation in second-line test models (i.e., *in vivo* studies,

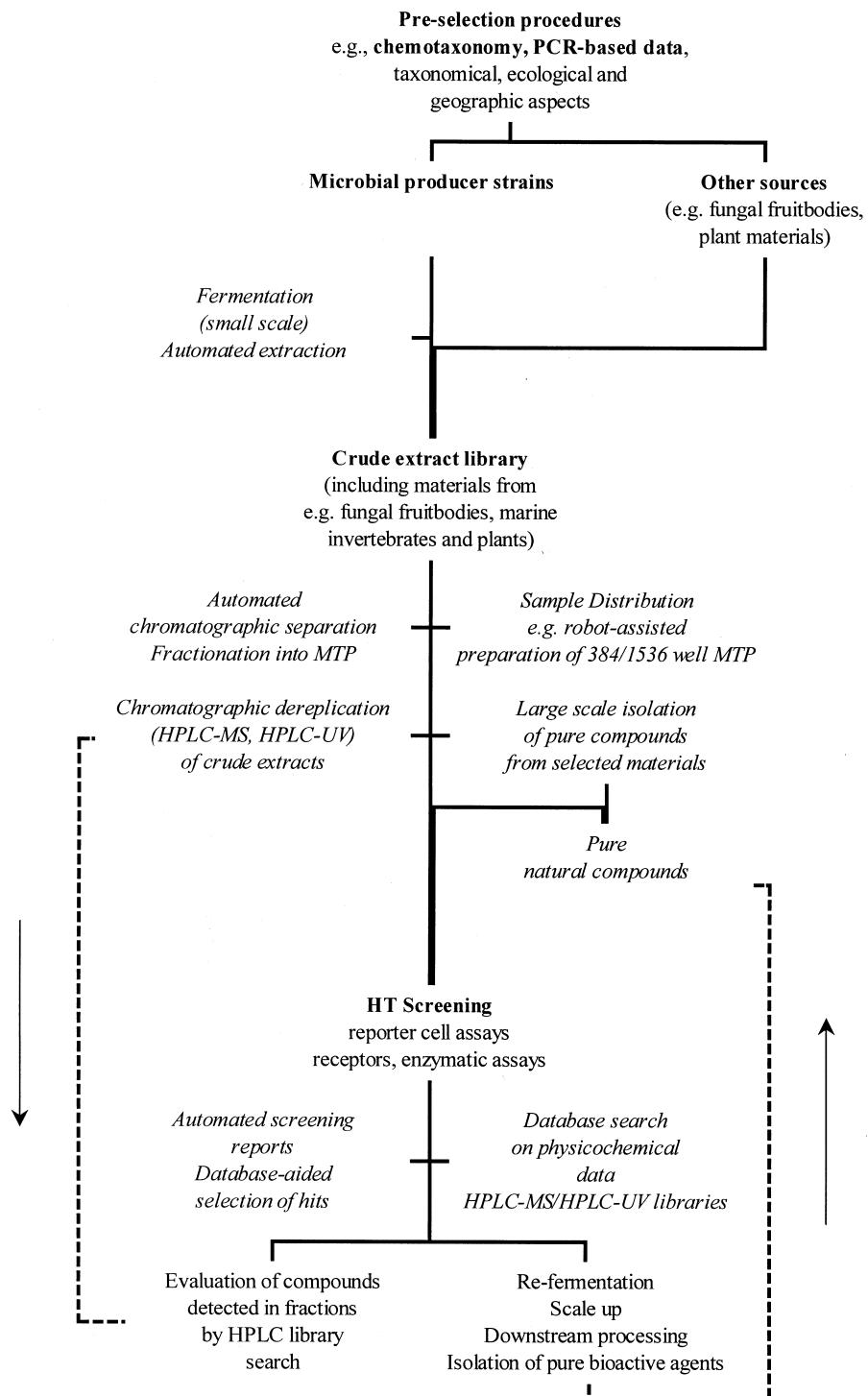


Figure 1 Schematic workflow involving the discovery of bioactive natural products in high-throughput screening.

etc.), the purity rather than the quantity of a given NP represents a limiting factor. Data on selectivity and potency can now be obtained using minimal amounts. Several industrial and academic groups specialized on the provision of technology platforms to create large libraries of prepurified NPs [8]. Such libraries of representative, easily accessible main metabolites appear helpful to complement the diversity of synthetic compound libraries. Nonetheless, this approach is not likely to replace the traditional and advanced extract screening. More than 50 years of experience with NPs clearly showed that the most interesting bioactive NPs are frequently formed as minor metabolites by particular organisms. Evidently, such chemical entities would be difficult to obtain from random isolation programs aimed at the purification of prominent compounds only.

On the other hand, many NPs are readily available in gram scale and can thus be utilized as synthons to be modified by directed or random chemical derivatization, resulting in “chimera” of natural and synthetic products with unprecedented biological properties. For example, a novel *N*-heterocycle (Fig. 2) resulted from the reactive fungal metabolite papyracillic acid as a side product of a conventional acetylation procedure. It did not share the reactivity of the parent compound but instead showed potent fibrinogen-lowering

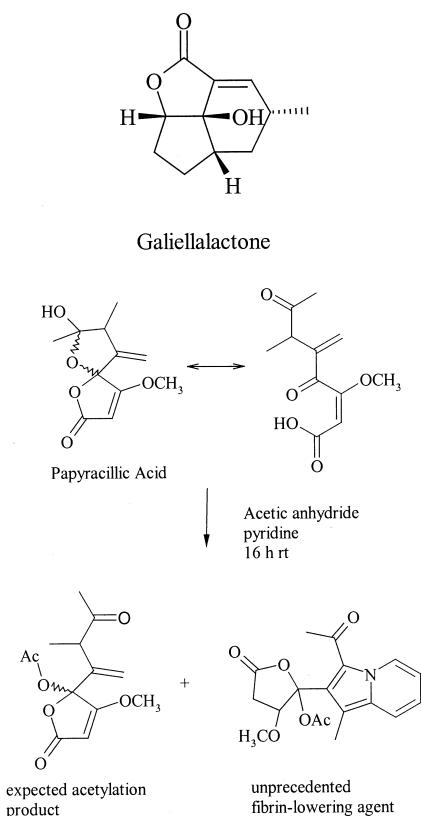


Figure 2 Compatibility of natural with combinatorial chemistry and high-throughput screening. Galiellalactone from *Galiella rufa* and papyracillic acid from *Lachnum papyraceum* and its unprecedented fibrinogen-lowering adduct. (From Refs. 9 and 23, respectively.)

activities [9,10]. The endowment of NP scaffolds and pharmacophores to produce large semisynthetic libraries for enhancement of the chemical diversity in synthetic pools is feasible [11,12]. Therefore, combinatorial chemistry and NP research should actually be seen as complementary rather than competitive approaches to increase the probability of success. However, HTS of NP extracts requires considerable adaptations and improvements of the quality and the quantity of samples. While test capacities are no longer limited, the time- and cost-intensive follow-up assays now have to be used in a second line of biological evaluation, that is, after identification of lead candidates from primary hits. Refermentation, optimization of production, scale-up, and, possibly, derivatization and mimetic syntheses to improve selectivity and potency of the lead compound and establish structure–activity relationship (SAR), all remain necessary. Therefore, it is important to focus the work-up procedure on those samples that contain novel compounds and to avoid redundancies within one screening project. This selection process is best performed prior to the screening. Because the initial sample amounts required for HTS decreased, only small-scale cultures are usually prepared for primary screening to enhance the throughput of fermentation. These are sufficient for testing over a period of several years. Still, this implies that cultures used for screening have to be preserved well. Even the best hit in HTS will be useless if the producer organism is no longer available. If large strain collectives of fungi are used in this process, strain preservation and fermentation (and refermentation of hits) will constitute the major bottlenecks. Parallel fermentation of a large number of fungal cultures will be less of a problem (e.g., with batches of randomly isolated soil isolates), which usually show similar growth characteristics and can be maintained easily. However, such strains are already well investigated on their secondary metabolites, and they frequently overproduce mycotoxins. In contrast, the handling of many untapped and “creative” but slow-growing fungi from which unprecedented leads may be expected in future (e.g., basidiomycete cultures) can hardly be automated but requires skilled staff and mycological, as well as microbiological know-how. These organisms sometimes need special culture media and may show highly varying growth rates. Some may produce secondary metabolites only after several weeks, and their evaluation is therefore difficult to synchronize, let alone automate.

HTS also means a high throughput of assays and lead evaluation projects. Screening laboratories, as well as microbiologists and NP chemists, are required to make use of the automated techniques by working simultaneously on several projects to keep pace with the steady flow of newly generated test data. Thus, the timelines have become rather narrow, usually not allowing for more than 6 months from reconfirmation of primary hits, until a decision about the most promising lead structure is made. These circumstances imply that false positives need to be excluded at an early stage, and only a few selected hits can usually be evaluated intensively.

Frequently, the number of hits from HTS is reduced by statistical approaches or dereplication by comparison of activities in several parallel biochemically related assays. Such a procedure is practical, such as in the course of a search for specific inhibitors of pharmacologically relevant enzymes, like kinases [13], or in parallel reporter cell assays using similar signal transduction cascades. Adequate database management will allow for exclusion of samples showing, for example, general cytotoxic effects in cellular screens. These will eventually be flagged as nonspecific (false-positives), thus prohibiting their further evaluation. Such information is very useful to establish selectivity profiles of screening libraries. In cases of synthetic compounds and pure NPs, information on chemical structures and previously noted bioactivities can be compiled in a database. Screening hits can thus be clustered according to their structural properties and selectivity. Hence, the

primary screen may already allow for the deduction of preliminary SAR, which is obviously not the case with NP extracts or their fractions. Several hundred thousands of chemicals may now compete with a fairly limited number of materials from natural sources. If screening results on natural and synthetic products are evaluated in concert by statistical methods, the probability of finding a follow-up lead in a NP extract will consequently increase with the overall percentage of NP samples in the screening. Moreover, crude extracts will sooner or later be flagged as nonspecific, even if they contain merely one nonselective or cytotoxic agent. In these cases, cometabolites with specific bioactivities present in the same extract may not easily be found, unless prefractionation techniques are employed. Fractionation, or other means of sample preparation, is also necessary for some commonly used HTS assay formats that are not practicable for crude extracts. Even ubiquitous primary metabolites and constituents of fermentation broths (fatty acids, ergosterol, phosphate) may at times interfere with particular readouts. Imperatively, some classes of secondary metabolites frequently disturb the screening as well. This applies to aforementioned cytotoxic agents and to those that disturb fluorescence-based readouts because of their physicochemical properties. If such compounds are present in abundance in the sample libraries, the identification of the potential lead structures will be prevented by unacceptable primary high hit rates.

Finally, the acquisition of NPs for bioprospecting has been rendered more complicated by the implementation of the Convention of Biological Diversity (CBD). The impact of these aspects was recently discussed in the course of a conclusive analysis as to the perspectives of the exploration of tropical microfungi in industrial screening [14] (see Chap.[Ando]). At times, NP research thus appeared less competitive with combinatorial and other synthetic approaches [15]. Therefore, several pharmaceutical companies substantially reduced or even terminated their efforts on microbial screening in a belief that newly evolving synthetic technologies would make the NP approach expedient. However, statistical evaluations using computerized comparisons of structural moieties of synthetic/combinatorial versus natural libraries showed that both pools are complementary, indicating that they should be used in concert to increase the chances of the lead finding process [16,17]. In fact, there are good reasons to continue with NP research, especially in the era of modern HTS. An increasing number of new “screenable” pharmaceutical targets are evolving from genomic approaches aimed at the identification of orphan genes encoding for pharmaceutically relevant receptors and proteins. The number of valid targets is thus expected to increase from the present 500 to several thousands, once the biological function of orphan genes have been identified [18]. Historically, there are countless examples where the elucidation of the biochemical mode of action of a particular natural compound—rather than a synthetic chemical—led to new targets, especially in the field of chemotherapeutics and antibiotics [19]. A recent overview further emphasized the importance of NPs as templates for drug discovery with focus on anticancer agents [20]. To our knowledge, no estimate of the percentage of currently accepted pharmaceutical targets that were found by NP screening approaches has ever been made. Nonetheless, the number of structurally unique biochemical tool compounds from nature that constitute potent modulators of enzymes and receptors is steadily increasing [21]. Powerful and selective natural inhibitors of cellular signal transduction were already found in fungi, employing the new generation of reporter cell screens. For instance, galillalactone (Fig. 2) from the sarcosomataceous ascomycete *Galiella rufa* inhibited the expression of an IL-6-responsive, element-driven reporter gene in the nanomolar range, interacting rather specifically with the IL-6-induced JAK/STAT pathway [22]. Interestingly, this compound was initially found as an inhibitor of a herbicidal target [23]. Such discoveries clearly constitute a benefit for the intellectual

property situation in pharmaceutical companies, since new targets and assays are patentable. Also, new target-based screens are easier to establish if a standard inhibitor is already available as biochemical tool. Hence, the adaptation of NPs to meet the requirements of HTS appears more promising once again. It is difficult to envision how modern pharmaceutical and agricultural lead structure research could do without them!

1.2. Chemotaxonomy of Plants and Fungi for Preselection

In agreement with the procedures used for synthetic chemicals, NP-adapted HTS can be performed in the following manner: the automated preparation of “standardized” and prefractionated samples from crude extracts replaces the traditional screening of extracts, and activity profiles of these fractions are stored in a database. This ultimately allows for comparison of hits in several assays in conjunction with a spectral library to verify the identity of the bioactive constituents already on the stage of the crude extracts by physicochemical dereplication [24–26]. Such information can be linked to proprietary and literature data on biological activities. The isolation and structure elucidation of known nonselective compounds becomes expedient if they are found by early-stage dereplication, and the efforts of the intensified evaluation can be focused on hits containing yet unknown components.

One challenge of microbial HTS is the deliberate increase of the metabolic diversity in a given screening library by adding new extracts from sources that are complementary to those already available in the NP pools. To meet the requirements of HTS, automated fractionation, along with HPLC-based dereplication of crude extracts, has become a valuable tool in most pharmaceutical and agricultural companies dealing with HTS of NPs. Still, even these powerful methods will fail if the redundancies in the sample libraries become too high. The full metabolic capabilities of microbial organisms are only expressed upon considerable variation of culture conditions. Then again, some secondary metabolites, including mycotoxins, may be formed in a broad range of culture media. As the individual metabolite patterns can hardly be predicted, several samples are prepared by different fermentations from each strain. It is therefore unavoidable that such samples cause undesired redundancy. This will even be enhanced by the increasing number of screening samples per strain, which multiply by HTS-adapted prefractionation. As many as 250 screening samples result from each strain if it is propagated in five-culture media and each of them is processed into 50 semipreparative HPLC fractions. The ratio of producers of nonselective agents and duplicate strains thus needs to be minimized at an early stage to increase the predictability and efficiency of the screening process. Capacities and manpower for fermentation, database management, handling of samples, and so forth, can thus be used more efficiently. Therefore, it appears crucial to reduce the surplus in microbial strain collections even prior to fermentation and extraction.

Databases containing information on the taxonomy, secondary metabolites and other features of the current pools constitute an important prerequisite for preselection of biological sources to be acquired. A literature survey for information on the taxonomy of species to identify taxa that are particularly interesting with regard to their secondary metabolism appears promising as a precondition to decide on their use in screening programs. Dreyfuss and Chapela [27] established a creativity index, relying on previously published information on fungal secondary metabolites. However, basidiomycetes and loculoascomycetes were not among the most creative taxa they identified because these groups had been widely neglected at that time. As discussed further below, just counting numbers of previ-

ously isolated metabolites from a given taxonomic group does not necessarily suffice to achieve sound preselection criteria. However, recent reviews on fungal metabolites include fungal pigments [28], compounds from loculoascomycetes and pyrenomycetes [29], basidiomycete cultures [30], or nematicidal and insecticidal agents [31]. The most comprehensive information of this kind is contained in commercially available NPs databases, such as Antibase [32], Bioactive Natural Products Database (BNPD), and Dictionary of Natural Products (DNP) [33]. In the current study, they were employed to search for entries on various taxa, in some cases including invalid and formerly misapplied synonyms. Antibase focuses on microbial metabolites, that is, it does not contain entries on compounds from higher plants or marine organisms. The BNPD was once the only comprehensive computer database on NP. The data contained in this free-format, text database were meanwhile incorporated to some extent into DNP, the most comprehensive database of this kind [34]. BNPD contained no chemical structures and was not compatible with modern software applications. It provided valuable features, however, such as the clustering of entries according to reported bioactivities, chemical types of secondary metabolites, and taxonomy of biological sources, which could be searched using well-defined identifiers. Moreover, the fact that BNPD contained entries only on biologically active compounds was of particular advantage for queries related to the process of drug discovery. In contrast, a search in DNP for entries on metabolites from a certain taxon frequently yields a lot of primary and other irrelevant metabolites (sugars, amino acids, volatiles). DNP and Antibase include chemical structures and provide various different search features and browsing functions. Metabolites may be sorted according to their chemical types. In contrast to BNPD, data on bioactivities and taxonomy are provided in DNP as comments in free-text format. Thus, a query for nematicides from fungi in DNP involves searching for “nematode,” “nematocide,” “nematocidal,” “nematicidal,” “anthelminthic,” “antiparasitic,” and various expressions circumscribing fungi before all entries have been found as desired.

For reasons discussed above, a kind of darwinistic chemotaxonomic approach appears feasible to achieve survival of the fittest taxa in the screening libraries. Several classes of NPs have proved to be suited for evaluation as lead structures, while others only interfere with the screening process. The value of chemotaxonomy for preselection of biological sources is best demonstrated with seed plants. Most of these have been studied thoroughly for secondary metabolites and chemotaxonomic affinities. Some examples (Fig. 3, Table 1) resulting from queries on entries for bioactive plant metabolites in BNPD are used to illustrate this preselection strategy at subclass and family levels. While particular subclasses of Angiospermae [35] are extremely rich in polyphenols (e.g., flavonoids, lignans), others are known to contain unique terpenes, polyketides, alkaloids, and metabolites of mixed biosynthetic origin (Fig. 3). The latter chemical types include numerous examples for lead structures, exemplified by the anticancer agents, taxol, camptothecin, and the *Vinca* alkaloids [36], respectively. In contrast, polyphenols and other aromatic compounds will usually show broad and nonspecific biological activity profiles. Many HTS readouts are sensitive to these nuisance compounds, which frequently are contained in the crude extracts as mixtures of congeners with varying polarity. It is virtually impossible to exclude them by prefractionation. Thus, their producer organisms are best to be avoided, and it should be more than sufficient to keep characteristic representatives in the screening libraries. In many cases, the patterns of biogenetic diversity may be restricted to the production of several congeners of the same type (rather than the simultaneous biosynthesis of manifold chemical types!), which also means a high degree of redundancy. Polycyclic triterpenes—and, among those, especially the steroids and the saponins—are other examples

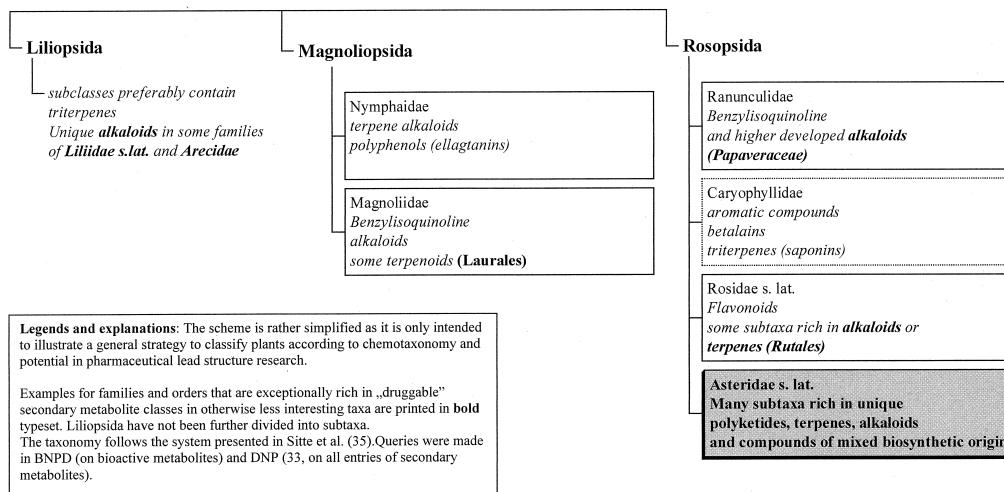


Figure 3 Classification of the major subclasses of Angiospermae (Magnoliophytina) according to quality of predominant secondary metabolites as lead structures. The scheme is simplified, since it is intended only to illustrate a general strategy to classify plants according to chemotaxonomy and potential in pharmaceutical lead structure research. Examples for families and orders that are exceptionally rich in "druggable" secondary metabolite classes in otherwise less interesting taxa are printed in bold. Liliopsida have not been further divided into subtaxa. The taxonomy follows the system presented in Sitte et al. [35]. Queries were made in BNPD (on bioactive metabolites) and DNP (on all entries of secondary metabolites) [33].

of NPs that from experience appear less promising as lead candidates. The same is true for some alkaloids (e.g., polycyclic aromatic benzylisoquinolines that are prevailing in Ranunculidae and Magnolidae except Laurales, and the betains, which are preferably found in Caryophyllales). In contrast, many taxa are included in Asteridae s. lat., in which a particularly high chemical diversity may be expected from the numbers and chemical types of previously reported bioactive secondary metabolites. Within this subclass, families such as Rubiaceae and Asclepiadaceae contain many structurally unique alkaloids, while the Asteraceae are among the richest sources of terpenes and polyketides. In comparison, the basic Asteridae such as Ericaceae (7 alkaloids and 60 aromatic compounds in BNPD) or the families of Cornaceae and Verbenaceae (no alkaloids but 25 aromatic compounds in BNPD) appear less interesting. Evidently, the number of species per family should be considered, and the criteria should be refined by searching for respective data at the generic and subgeneric levels. For instance, the Rosidae s. lat. (Table 1) contains several families that preferably produce alkaloids; while, in other groups, the polyphenols are predominant. The Leguminosae would be regarded as rather unsuited for HTS in the currently presented preselection process, considering the large amounts of flavonoids and other aromatic compounds known from this large superfamily. However, the results of Wink and coworkers [37] demonstrated the presence of metabolite families in various groups of Leguminosae. Their occurrence was in basic agreement with morphological features—and even with molecular data as inferred from analyses of the *rbcL* gene. Several taxa that preferably contain unique alkaloids or different flavonoid types were thus identified in evolutionary

Table 1 Results of a Database Search in Bioactive Natural Products Database

Family	Estimated No. of Species	No. of Alkaloids	No. of Aromatic “Nuisance” Compounds (e.g., flavonoids, polyphenols, quinones)	No. of Other Metabolites, (prevailing type)
Anacardiaceae	850	None	40	None
Burseraceae	540	None	25	12 (triterpenes)
Clusiaceae ^a	1,350	None	70	24 (aromatic terpenes)
Crassulaceae	1,500	2	1	5
Cucurbitaceae	760	None	None	32 triterpenes
Euphorbiaceae ⁴	7,950	2	86	153 (terpenoids)
Fagaceae	1,000	None	19	None
Geraniaceae	720	None	13	3 (triterpenes)
Leguminosae ^b	16,000	60	434	31 (terpenoids, polyketides)
Loranthaceae	940	3	7	15 (terpenoids)
Melastomataceae	4,000	None	7	None
Moraceae	1,280	2	84	18 (terpenoids)
Myrtaceae	3,000	1	28	20 (terpenoids)
Rhamnaceae	875	9	29	3 (macrolactams)
Rosaceae	3,100	None	53	13 (triterpenes)
Rutaceae	1,700	82	139	15 (terpenoids)
Salicaceae	435	None	28	5 (terpenoids)
Sapindaceae	1,300	None	5	8 (triterpenes)
Saxifragaceae	450	1	9	None
Simaroubaceae		21	7	144 (terpenoids)
Tiliaceae	725	2	3	2
Thymelaeaceae	720	None	25	26 (terpenoids)
Violaceae	830	None	10	7 (terpenoids)
Zygophyllaceae	250	5	17	None

⁴ AU: significance of bold in this table?

Previously reported bioactive secondary metabolites from some important families of Rosidae s. lat., sorted according to chemical types.

^aIncluding Hypericaceae.

^bBNPD allows for search only on the superfamily “Leguminosae,” actually consisting of the families now included in the order Fabales.

Source: M.S./BNPD; taxonomy according to Ref. [35].

basic and in advanced tribes of Leguminosae. From the outcome of these studies, it was concluded that the value of secondary metabolites as taxonomic markers may be limited by the reticulate nature of their metabolic expression.

Such criteria may be used for further acquisitions and for prioritization of stock materials to be used for screening and prefractionation. A continuous check of the current libraries against potentially interesting taxa to be newly attained can be done using chemotaxonomic criteria. New acquisitions should focus on those taxa for which many structurally unique metabolites are known, that are underrepresented in existing libraries, and that

have not been studied much for secondary metabolites. Even extraction protocols and prefractionation methods may be modified to enrich the basic alkaloids in a given plant and separate them from their undesired acidic phenolic co-metabolites prior to screening (the latter may be discarded). This practice facilitates selection and adequate handling of the most promising ones out of the approximately 422,000 plant species that are now believed to exist [38].

An analogous taxonomic preselection of fungi as sources for HTS is perhaps more difficult. In the first place, restrictions concern some groups of fungi or their respective life cycle stages that are to be excluded for practical reasons. For instance, ascomyceteous yeasts and zygomycetes (but certainly not basidiomycetes as stated by Dreyfuss and Chapela [27]) are indeed known to be rather poor in secondary metabolites, despite their importance in other biotechnological applications. Stable cultures of obligate plant and invertebrate pathogens and many mycorrhizal basidiomycetes can apparently not be established. At least the fruitbodies of some species may be made available for screening, or their saprophytic relatives can be used for screening of mycelial cultures. Perhaps because of changes in differential gene expression during morphogenetic events, mediated by signal transduction processes that are not yet fully understood [39], the fruitbodies of most basidiomycetes, as well as some ascomycetes [40], have been reported to contain different secondary metabolites. Fruitbodies and cultures should thus be regarded as complementary sources for fungal secondary metabolites, and it appears worthwhile to focus on both. However, fruitbodies of many species may not be easily collected in sufficient amounts to meet the requirements for stock samples necessary for follow-up work after HTS. Aside from these aspects of general availability and suitability, most taxa of higher fungi can still be used for screening purposes and can be assessed on their chemical diversity.

A comprehensive overview on the state of the art in fungal chemotaxonomy was given by Frisvad et al. [41]. Within the ascomycetes, chemotaxonomic surveys were mostly carried out in Xylariaceae [42] and in mycotoxin-producing taxa and important groups of plant pathogens such as the Trichocomaceae (see [6]), the genus *Fusarium* within the Hypocreaceae [43], and the pleosporaceous *Alternaria* species [44,45]. Chemotaxonomic relationships in basidiomycetes have been investigated for several decades. The results have been mainly based on their pigment chemistry, but studies on other secondary metabolites resulted in the discovery of a surprisingly high diversity of new carbon skeletons and other unprecedented chemical moieties. Several reviews [28,30,46–50] give further examples as to the potential taxonomic relevance of certain basidiomycete metabolites. Out of this context, only the Boletales are treated in detail further below. Table 4 gives some examples for the chemotaxonomic significance of fungal terpenes as judged from data presented in Antibase, DNP, and our own experience. HPLC profiling was carried out according to a previously described method [51], relying on samples from the Bayer extract pools. These examples clearly reveal the taxonomic significance of the unique terpene structures, all of which have rarely or never been encountered in other biological sources.

In Table 2, entries on metabolites from some genera of plant-associated and soil-inhabiting conidial fungi are compiled from Antibase. Several hundreds of compounds are known from each of the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma*, and more than a hundred were reported from *Alternaria* and fungi named *Verticillium*. The latter genus is now under revision and many species have been removed from it [52]. Many other genera appear underrepresented in these databases, and none or only a few of their secondary metabolites are known. Some chemotaxonomic correlations already

Table 2 Entries on Secondary Metabolites from 46 Anamorphic Genera of Plant-Associated Ascomyceteous Fungi

Genus	No. of Reported Metabolites	Genus	No. of Reported Metabolites
<i>Acremonium</i> (incl. <i>Cephalosporium</i> p.p.)	69	<i>Mycoleptodiscus</i>	0
Alternaria	110	<i>Mycosphaerella</i> ^a	28
<i>Aspergillus</i>	753	<i>Myrothecium</i>	72
<i>Botryosporium</i>	None	<i>Nectria</i> ^a	49
<i>Botrytis</i>	58	<i>Nigrospora</i>	8
<i>Cercospora</i>	50	<i>Paecilomyces</i>	42
<i>Chalara</i>	3	<i>Paracercospora</i>	None
<i>Cladosporium</i>	48	<i>Passalora</i>	None
<i>Clonostachys</i> (incl. <i>Gliocladium roseum</i>)	10	Penicillium	609
<i>Corynespora</i>	1	<i>Phaeoisariopsis</i>	None
<i>Cristulariella</i>	None	<i>Phaeoramularia</i>	0
<i>Curvularia</i>	63	<i>Phialophora</i>	17
<i>Cylindrocarpon</i>	28	<i>Phymatotrichopsis</i>	None
<i>Cylindrocladium</i>	11	<i>Pseudocercospora</i>	15
<i>Dendryphiella</i>	19	<i>Pseudoceropsporella</i>	None
<i>Dictyochaeta</i>	1	<i>Ramularia</i>	None
<i>Embellisia</i>	None	<i>Scolecobasidium</i>	2
<i>Epicoccum</i>	10	<i>Stachybotrys</i>	43
Fusarium	290	<i>Stemphylium</i>	20
<i>Geotrichum</i>	3	<i>Trichoderma</i> (incl. <i>Gliocladium virens</i>)	243
<i>Humicola</i>	18	<i>Trichothecium</i>	31
<i>Memnoniella</i>	1	<i>Ulocladium</i>	2
<i>Microdochium</i>	3	<i>Verticillium</i>	172
<i>Acremonium</i> (incl. <i>Cephalosporium</i> p.p.)	69	<i>Mycoleptodiscus</i>	0
<i>Alternaria</i>	110	<i>Mycosphaerella</i> ^a	28
<i>Aspergillus</i>	753	<i>Myrothecium</i>	72
<i>Botryosporium</i>	None	<i>Nectria</i> ^a	49
<i>Botrytis</i>	58	<i>Nigrospora</i>	8
<i>Cercospora</i>	50	<i>Paecilomyces</i>	42
<i>Chalara</i>	3	<i>Paracercospora</i>	None
<i>Cladosporium</i>	48	<i>Passalora</i>	None
<i>Clonostachys</i> (incl. <i>Gliocladium roseum</i>)	10	Penicillium	609
<i>Corynespora</i>	1	<i>Phaeoisariopsis</i>	None
<i>Cristulariella</i>	None	<i>Phaeoramularia</i>	0
<i>Curvularia</i>	63	<i>Phialophora</i>	17
<i>Cylindrocarpon</i>	28	<i>Phymatotrichopsis</i>	None
<i>Cylindrocladium</i>	11	<i>Pseudocercospora</i>	15
<i>Dendryphiella</i>	19	<i>Pseudoceropsporella</i>	None
<i>Dictyochaeta</i>	1	<i>Ramularia</i>	None
<i>Embellisia</i>	None	<i>Scolecobasidium</i>	2
<i>Epicoccum</i>	10	<i>Stachybotrys</i>	43
<i>Fusarium</i>	290	<i>Stemphylium</i>	20
<i>Geotrichum</i>	3	Trichoderma (incl. <i>Gliocladium virens</i>)	243
<i>Humicola</i>	18	<i>Trichothecium</i>	31
<i>Memnoniella</i>	1	<i>Ulocladium</i>	2
<i>Microdochium</i>	3	Verticillium	172

^aCorresponding teleomorphic genus.

Source: Ref. [33].

Those genera which have yielded more than 100 compounds are printed in bold.

become fairly evident at the generic level. For instance, most compounds previously obtained from *Trichoderma* are oligopeptides of the peptaibol type (>200 entries in Antibase). These substances cause membrane damage in mammalian cells and will thus disturb cellular reporter screens. *Myrothecium* is notorious for its macrocyclic trichothecenes (>50 entries in Antibase), which interfere with the biosynthesis of macromolecules. *Aspergillus*, *Penicillium*, and *Fusarium* species are well-known producers of mycotoxins, too. Still, the database entries from these genera and *Trichoderma* also include useful bioactive agents, such as cyclosporin, penicillins, mevinolin, compactin, and further unique chemical entities that were never obtained from other biological sources. It would be of special interest to focus on such species of these genera that do not or not only produce mycotoxins. With some exceptions reviewed further below, reports on novel biologically active agents do not necessarily include the taxonomy of the producers at species level. Sometimes, a given taxonomy can even be misleading because of morphological similarities that are not paralleled by secondary metabolite production. This problem of strain-specificity may be due to inadequate species concepts, which will be discussed in detail further below. In several other cases, not even the genus of the producer strains was reported along with new interesting metabolites. Some of them were obtained from fungi growing as sterile mycelia in culture, which, until recently, could not be classified at all. Evidently, not all researchers dealing with the discovery of new fungal metabolites are also experts in the taxonomy of the organisms they deal with. A database-aided retrospective chemotaxonomy becomes even more complicated because many secondary metabolites were reported several decades ago. Despite the fact that new evidence was obtained in the meantime to change the taxonomic position of the producers, neither the trivial names nor the respective database entries were updated according to the current knowledge. Some of the first antibiotics discovered in basidiomycetes are compiled in Table 3. Because of their pleurotoid habit, the fungi now classified under *Hohenbuehelia* and *Clitopilus* were still included in *Pleurotus* in the 1950s. Accordingly, their secondary metabolites were named pleurotin and pleuromutilin, respectively. It is now well-established that *Pleurotus* spp. in the current sense do not contain such compounds. Pleuromutilin and derivatives were repeatedly found in *Clitopilus* spp., including the nonpleurotoid *Clitopilus prunulus* (M.S., unpublished). The genus *Hohenbuehelia* and its anamorph, the nematophagous conidial basidiomycete *Nematoctonus robustus* [53], are the only organisms so far known to produce antibiotics of the pleurotin type. Albeit R. Singer already recognized in 1951 that the generic name *Drosophila* was an invalid synonym for *Psathyrella*; it had been in use by mycologists until 1975 [54]. Therefore, it is no wonder that NP chemists named several compounds from *Psathyrella* species “drosophilins.” Another example given in Table 3 (*Omphalotus* and illudins) is discussed further below.

Aside from such name changes, there are several cases of misidentifications of producer organisms in the literature, which are not discussed in detail here. Even if all reports on the taxonomy of producers ever published in the course of NP discoveries had been correct, drastic changes now inferred from molecular taxonomy and phylogeny would still have to be dealt with.

As mentioned before, many industrial companies have so far relied on large numbers of non-identified strains for screening. These may be selected from diverse ecological habitats and geographic localities in an attempt to increase the metabolic diversity in the screening pools by tapping the higher biodiversity to be expected from a broad range of isolation strategies and sampling techniques [55]. Such criteria should definitely be regarded as valid for preselection prior to HTS. Indeed, they relate to the taxonomic approach

Table 3 Some Antibiotics of Basidiomycetes: Current and Outdated Names of Their Producers

Structure	Trivial Name	Current (and originally reported) Taxonomy
	Pleuromutilin, drosophilin B	<i>Pleurotus</i> = <i>Clitopilus</i> spp. <i>Drosophila</i> = <i>Psathyrella</i> <i>subatrata</i>
	Pleurotin	<i>Pleurotus</i> = <i>Hohenbuehelia</i> spp. and associated anamorphs (<i>Nematoctonus</i>)
	Drosophilin A	<i>Drosophila</i> = <i>Psathyrella</i> spp. and other Basidiomycetes
	Drosophilin C	<i>Drosophila</i> = <i>Psathyrella</i> spp.
	Illudin M	<i>Clitocybe illudens</i> = <i>Omphalotus olearius</i> <i>Pleurotus japonicus</i> = <i>Lampteromyces japonicus</i>

because adaptive radiation of species is driven by ecological factors and geographic isolation. However, when isolated in large numbers from similar habitats, biotopes, or hosts, such strain contingencies will usually contain a high degree of redundancy as well. For instance, Cannon and Simmons [56] recently reported that of more than 2500 samples collected from 12 tree species in Guyana, almost as many endophytic fungal cultures were obtained, but those belonged to only 64 morphotypes, whereas a further 52 of their isolates were characterized as sterile mycelia. As prerequisite for HTS, the morphological dereplication of such large strain contingencies hardly appears feasible in a cost-efficient way. In the course of the isolation procedure from the natural habitat, duplicates among a series of cultures grown from the same plant or soil sample may be recognized by morphological or cultural characteristics. Assessing their novelty with respect to the existing strain contingencies—i.e., the collection to which they shall ultimately be added—will constitute a

problem. Predominantly, ecological groups like endophytes of seed plants and insect-associated fungi do not show many discriminative characters in culture. Anamorphs of creative secondary metabolite producers in the Clavicipitaceae, Hypocreaceae, Trichocomaceae, and Xylariaceae are difficult to discriminate by morphological characters. Other groups with high potential as untapped sources for novel bioactive agents (e.g., Helotiales [57,58], from which manifold unique compounds have already been reported) are strangely being neglected by taxonomists, and only few experts are still acquainted with them.

Some companies have actually employed mycologists and spend great efforts to enhance the taxonomic diversity in their collections [59], and the role of taxonomy and mycology as a core disciplines in NP screening is nowadays widely accepted [60]. However, it should be kept in mind that among other reasons, HTS was invented to reduce the costs of the screening process, including minimization of human capital. Hence, the number of mycologists involved is not likely to increase as rapidly as the number of newly obtained uncharacterized strains to meet the requirements of HTS. Complementary methods should be endowed to achieve a preselective classification similar to the one described above for the chemotaxonomy of higher plants.

1.3. PCR Fingerprinting: A Valuable Alternative

PCR fingerprinting and DNA sequencing have become state-of-the-art methodology in many disciplines of mycology and biotechnology. The number of sequences deposited in GenBank (<http://www.psc.edu/general/software/packages/genbank/genbank.html>) and related databases is growing rapidly. There is good reason to assume that the wealth of information on molecular data on the internet, along with further information relating to biodiversity [61], will once facilitate the discovery of new nature-derived therapeutic agents. Molecular methods frequently confirmed hypotheses that were based on ecological, morphological, and ultrastructural investigations. They proved especially helpful in differentiating groups of fungi that are poor in morphological characters. Even unculturable species such as obligate parasites can now be studied for molecular taxonomy as specific PCR-based methods allow for their *in situ* characterization using minimal amounts of material [62]. Such techniques have also found broad application in plant pathology and for indirect characterization of mycotoxin producing fungi [63]. Brief [64] and comprehensive [65,66] general overviews on these topics are readily available. The first applications of PCR fingerprinting in relation to industrial screening date back less than 10 years. Fujimori and Okuda [67] compared 74 strains of *Trichoderma* spp. on their ability to produce isonitrile antibiotics and their random amplified polymorphic DNA (RAPD) profiles. They noted correlations between secondary metabolite profiles and RAPD data but stated that only a part of their species that they identified by morphological methods as *T. harzianum* and *T. viride* were able to produce the characteristic antibiotics. Talbot et al. [68] studied 15 isolates of *Fusarium compactum* for metabolite profiles, RAPD patterns, and by restriction fragment-length polymorphism (RFLP) methodology. They used parsimony analyses of both the data on molecular characters and metabolite profiles and concluded that genetic features would be suited well to optimize the chances of identifying a wide range of metabolites from a given species. Another remarkable early application of RAPD and PCR primers for the cyclosporin synthetase gene was published by Möller et al. [69], who studied 64 isolates identified as *Chaunopycnis alba* for their secondary metabolite profiles in conjunction with growth characteristics and other characters. They found a broad diversity among the isolates of this species from different habitats and localities and suggested

the broader use of RAPD in selection for screening isolates as this method reflected high interspecific diversity. The affinities of *C. alba* to the Clavicipitaceae have only recently become evident: *C. pustulata*, a new species of this genus, which produced a new bioactive alkaloid, was characterized from a comparison of morphological, chemotaxonomic, and molecular data [70]. Interestingly, the taxonomy of this fungus did at first not become clear from morphological data but was ultimately deduced from a comparison of metabolite profiles and DNA sequences. In this context, the authors discussed the aforementioned results by Möller et al. [69]. They stated that *C. alba* would be likely to be split into several species, employing the phylogenetic species concept [71]. The other aforementioned examples of RAPD studies in conjunction with morphology and secondary metabolites dealt with anamorphic Hypocreaceae, whose taxonomy is about to change considerably. Recent evidence gained from molecular data suggests that the genus *Trichoderma* and the taxa studied by Fujimori and Okuda [67] will soon be segregated into several further species [72–74]. While none of the latter studies included chemotaxonomic parameters, this has already been accomplished with *Fusarium* (see below).

The characteristic metabolites of *Stachybotrys* and *Memnoniella* are satratoxin and other trichothecenes in addition to numerous spiro-drimane indene terpene alkaloids (Fig. 4) and structurally related aromatically substituted drimane terpenes. The distribution of secondary metabolites in these fungi was recently investigated in isolates from water-damaged buildings. This work resulted in the discovery of an undescribed species, as well as in the recognition of two different “chemotypes” within *St. chartatum* [75]. These chemotypes of drimane producers and trichothecene producers, respectively, were exam-

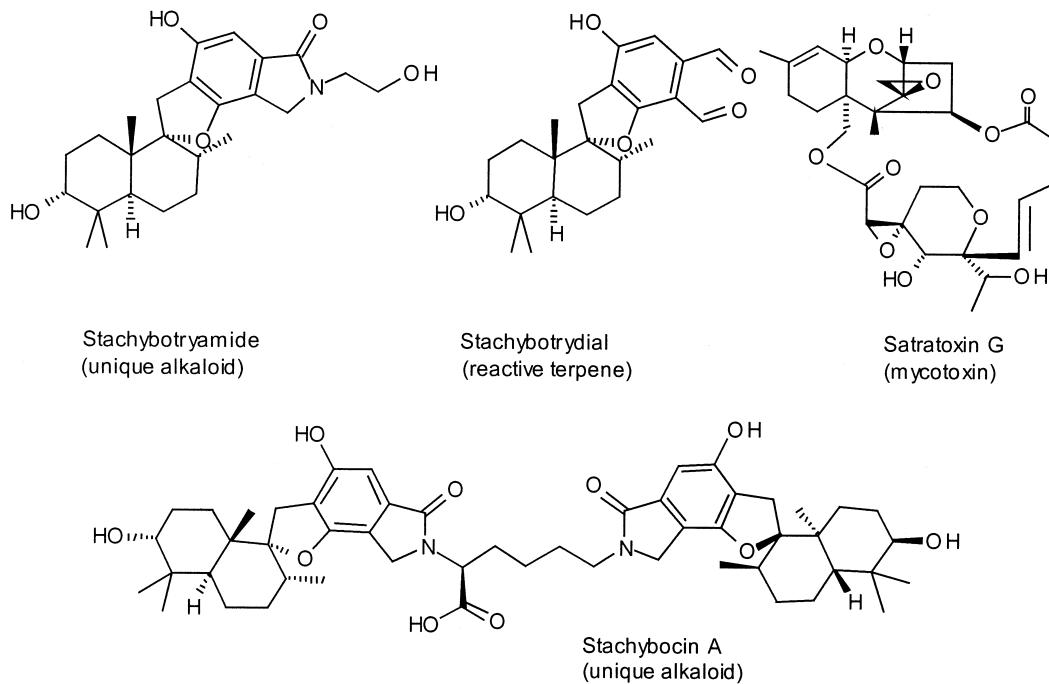
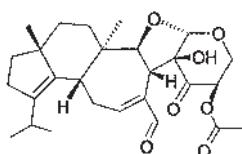
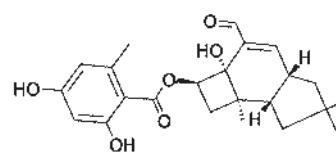
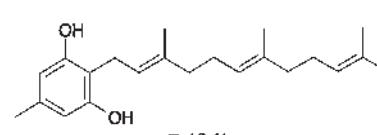
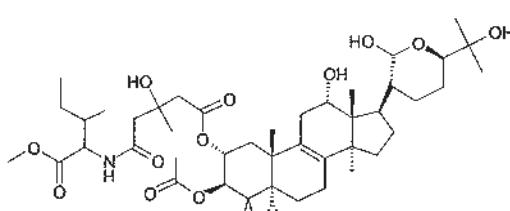
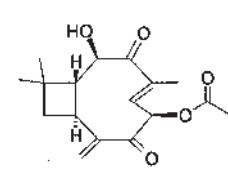


Figure 4 Some characteristic metabolites of *Stachybotrys* spp. [32,76,77].

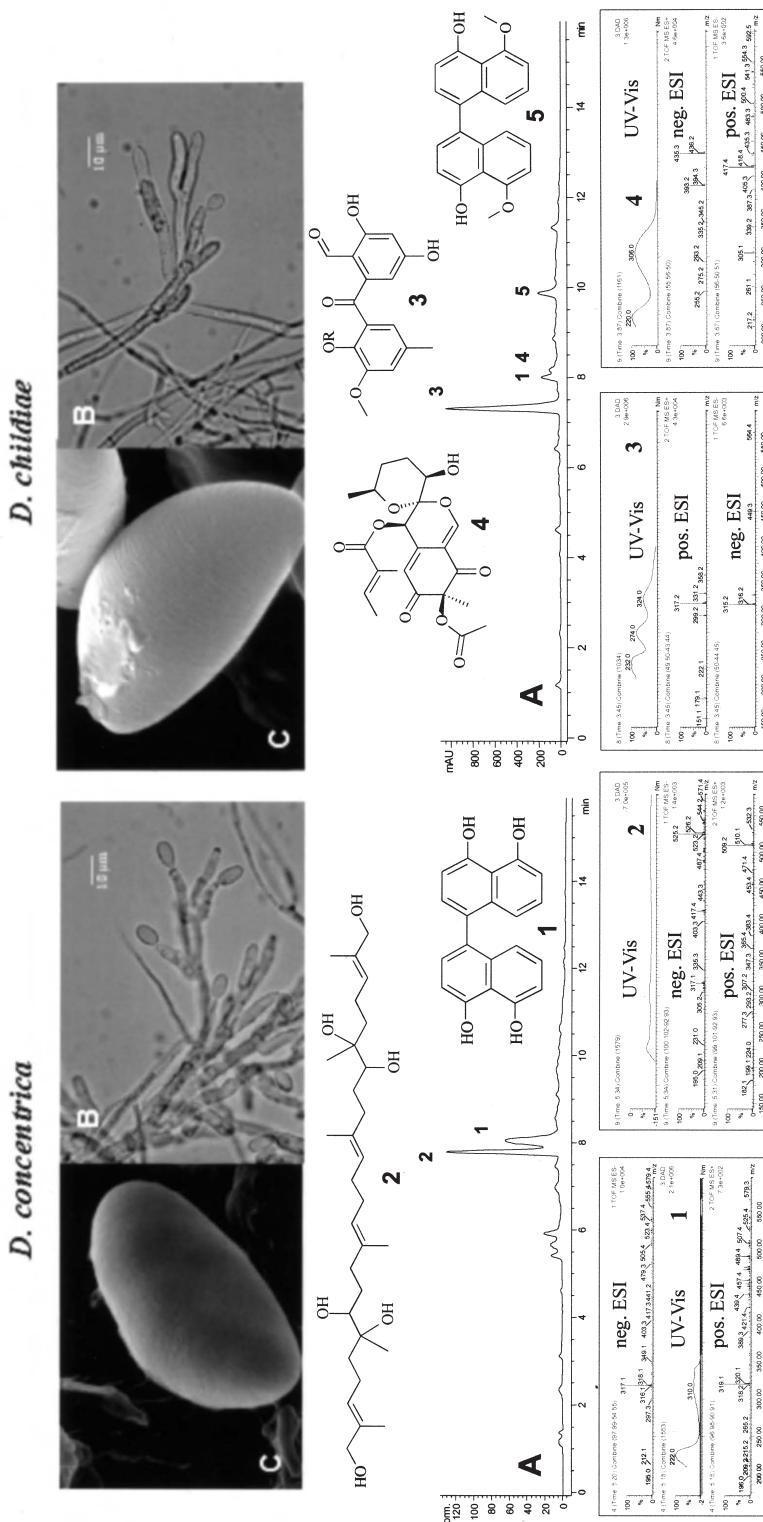
ined in concert employing RAPD and a specific PCR primer to detect *Tri 5*, a key biosynthetic enzyme of trichothecene biosynthesis [76]. The results revealed *St. chartarum* “chemotypes” to be in agreement with the PCR-based characterization. The data in Anti-base and DNP suggest that the production of the *spiro*-drimanes in Nature be restricted to *Stachybotrys* and *Memnoniella*. Obviously, these terpenoids are not mycotoxins, and, in contrast, some of them were reported to possess apparently selective bioactivities [77,78]. The role of cryptic species in *St. chartarum* was also recently discussed in a study involving molecular phylogenetic analyses of chitin synthase, beta-tubulin, and trichodiene synthase, the latter of which also constitutes a key enzyme of secondary metabolite biogenesis [79]. No relationship as to the correspondence of these chemotypes and cryptic species has so far been established. Correlations between molecular and chemotaxonomic features were also noted in *Alternaria* species from South Africa. These were subjected to ITS sequencing, and, concurrently, their secondary metabolite profiles were studied [80]. This methodology facilitated the recognition of species that are otherwise difficult to identify. Another striking example of the usefulness of molecular data in conjunction with HPLC profiling techniques is the identification of the pneumocandin-producing anamorphic fungus as *Glarea lozoyensis* [81] (see Chap. [Connors]). In this case, even a new genus was erected for the producer of a pharmaceutically important product, and in addition to PCR fingerprinting, DNA sequences were employed. (Table 5)

These examples show that in many groups of fungi the chemical diversity to be expected from their evaluation in screening programs can be deduced indirectly from their morphology or molecular taxonomy. In recent years, we have therefore attempted to find indirect methods to determine the metabolic diversity in particular groups of ascomycetes that have in common strong morphological similarity, along with unsolved problems as to their taxonomy, high “metabolic creativity” as revealed from the rather large number of previously reported metabolites, and frequent occurrence in strain contingencies that are usually provided for NP screening. For their characterization, HPLC profiling, morphological studies, and PCR fingerprinting methods were used. The most suitable PCR method, reflecting metabolic diversity, should qualify for general characterization of screening strains prior to HTS. The results so far published mainly involved the xylariaceous genus *Daldinia* and its allies. In the last world monograph [82], colors of stromatal pigments (i.e., characters relating to secondary metabolism) were first employed for segregation of species in addition to anamorphic and teleomorphic morphology. Thus, a basis for a polyphasic approach to their taxonomy had been provided. Only in 1999, the type species, *Daldinia concentrica* (Bolt.:Fr. Ces & de Not.) was recognized as an European taxon preferably associated with *Fraxinus*, while the fungus known as “*D. concentrica*” by American and Asian mycologists was reclassified as *D. childiae* [83]. Both species are otherwise quite similar but drastically differ in their secondary metabolite profiles (Fig. 5). In the following, some characteristic pigments of *Daldinia* spp. were identified and their distribution was evaluated in some 500 specimens and cultures from around the world. Concurrently, different PCR fingerprinting methods were compared on their discriminatory power [40,84]. In Fig. 6, several species and strains of *Daldinia* were compared by ARDRA (Amplified Ribosomal DNA Restriction Analysis) of the 18S nrDNA. Fragments obtained using three different restriction enzymes were compared on their similarity. Characteristic and specific patterns were only obtained with those species containing large introns in their 18S nrDNA (e.g., *D. loculata*, *D. fissa*, and a group consisting of *D. childiae* and *D. pyrenaica*). Several other species did not even differ in their ARDRA patterns from

Table 4 Occurrence of Some Characteristic Terpenes in Basidiomycete Cultures (C) and Fruitbodies (F)

Genus	Chemical Type	Structure of Characteristic Compound (usually one of several congeners produced)
<i>Cyathus</i> (C, F) ^a — 14 of 15 records examined	Cyathane diterpenes	 Striatal A
<i>Armillaria</i> (C)— 11 of 11 records examined	Protoilludane orsellinates (sesquiterpenes)	 Melleolide
<i>Albatrellus</i> (F)— 17 of 18 records examined	Farnesyl phenols	 Grifolin
<i>Hebeloma</i> (F)—14 of 15 records examined	Hebelomic acids (triterpenes)	 Hebelomic Acid I
<i>Hypholoma</i> (C) ^a — 17 of 21 records examined	Caryophyllane sesquiterpenes	 Naematalone

^aConfirmation of previous investigation cited by Erkel and Anke [30].



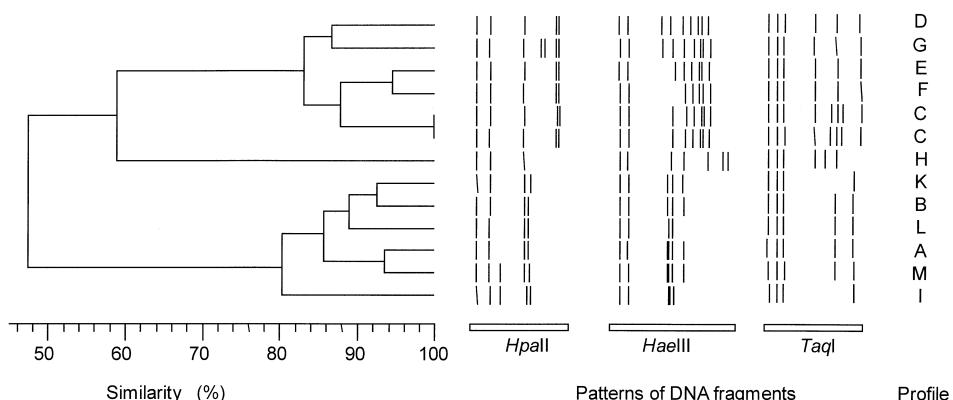


Figure 6 Diversity within *Daldinia* and allies as deduced from ARDRA profile types A through M (Table 5). The central part shows the profiles obtained after partial digestion of PCR products with *Hpa*II, *Hae*III, and *Taq*I, respectively. One example is shown for each profile type, except for type C. Here, the upper example is derived from *D. chilidiae*, and the other from *D. pyrenaica*. The close relationship between both species is reflected by their identical ARDRA patterns (which are otherwise unique within *Daldinia* spp.). (Modified from Ref. 40, with kind permission of Mycotaxon Ltd. and the Editor of *Mycotaxon*.)

the representatives of other genera, or the results were not species-consistent as deduced from morphological and chemotaxonomic results. A better resolution was obtained by using Minisatellite (MIS) PCR [85] as shown in Fig. 7. Species-specific data confirmed that *D. chilidiae* is indeed cosmopolitan and occurs besides *D. concentrica* in Europe, while *D. concentrica* is common only in Western and Northern Europe on *Fraxinus* but absent outside Europe and quite rare on other hosts. The MIS-PCR data also revealed the identity of some species of uncertain correspondence (e.g., strain “*D. vernicosa*” ATCC 36660 is an isolate of *D. caldariorum*). Moreover, they also pointed toward the existence of several new species that had been erroneously deposited under the names of known species, some of which were meanwhile formally described [86,87]. A comprehensive SEM study later confirmed these results [88]. Recently, further secondary metabolites were identified as minor or major components in the stromata of *D. concentrica* [89,90]. In summary, of the three morphologically similar *Daldinia* spp. thus far examined extensively on their stromatal constituents (*D. concentrica*, *D. chilidiae*, and *D. eschscholzii*), about 50 different compounds were obtained. Only four of those are present in more than one of the three examined species, and 80% of them constituted novel NPs at the time of their first discovery. From the data gathered so far, it was concluded that morphologically alike *Daldinia* spp. can be divided into several groups that strongly differ in their stromatal metabolite profiles; their metabolite profiles in culture differ from those of closely related genera such as *Hypoxylon*; the metabolite patterns observed in stromata were not closely correlated with the anamorphic branching patterns as defined by Ju and Rogers [91]; and, as exemplified by *D. chilidiae* and *D. concentrica*, care should be taken when assuming the conspecificity of these fungi, especially when records from different continents are compared. In Xylariaceae, stromatal metabolite profiles and SEM will be helpful to establish the identity of newly collected materials with previously described taxa, allowing for

Table 5 ARDRA Pattern Types (corresponding to Fig. 4) and Fragment Sizes of PCR Products^a

Profile Type	Species	Size of Amplified Fragment (kb)
A	<i>Daldinia bambusicola</i> , <i>D. caldariorum</i> (Mexico, UK), <i>D. albofibrosa</i> , <i>D. concentrica</i> , <i>D. eschscholzii</i> p.p. (Canary Islands), <i>D. grandis</i> , <i>D. steglichii</i> , <i>D. petriniae</i> , <i>D. decipiens</i> <i>Hypoxylon fragiforme</i> , <i>H. multiforme</i> , <i>H. howeanum</i> <i>Xylaria hypoxylon</i> , <i>X. longipes</i> , <i>X. polymorpha</i>	1.2
A'	<i>D. eschscholzii</i> p.p. (Cuba)	1.6
B	<i>Biscogniauxia nummularia</i>	1.2
C	<i>D. chilidiae</i>	1.8
	<i>D. pyrenaica</i>	
D	<i>D. eschscholzii</i> p.p. (Asia, USA)	1.8
	<i>D. clavata</i>	
E	<i>Daldinia</i> sp. "Russian Far East"	1.8
F	<i>D. fissa</i>	1.8
G	<i>D. caldariorum</i> ATCC 36660	1.4
H	<i>D. loculata</i>	2.8
I	<i>Entoleuca mammata</i>	1.2
K	<i>Kretzschmaria deusta</i>	1.2
L	<i>Nemania serpens</i>	1.2
M	<i>Rosellinia corticium</i>	1.2

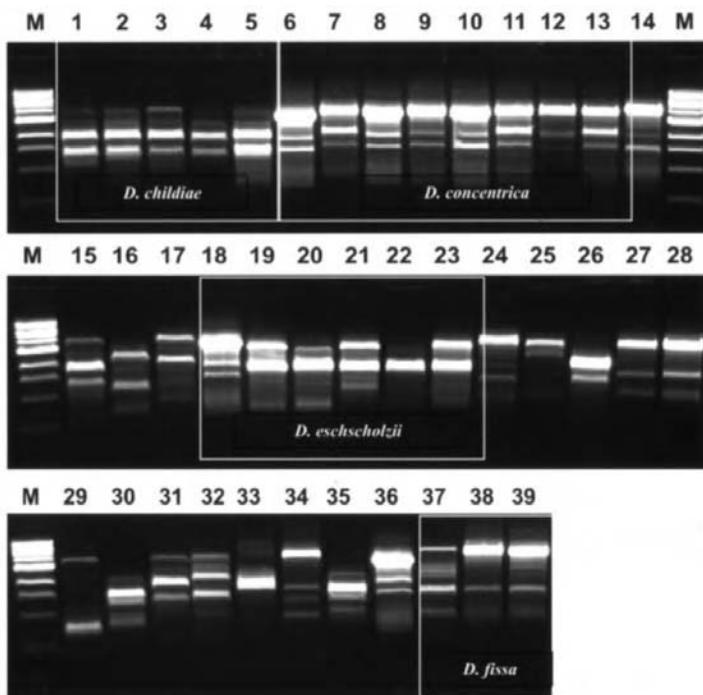
Taxa highlighted in bold showed specific patterns because of large introns contained in their rDNA.

^a Estimated from the electrophoretic mobility of the amplified, undigested 18s rDNA segments delimited by the primers NS1 and RDR116.

Source: Modified from Ref. [40] with kind permission of Mycotaxon Ltd. and the Editor of *Mycotaxon*.

comparison of type materials. Such complementary investigations also provide a good basis for studies on molecular phylogeny and may even be necessary to ensure concordance in this morphologically similar group of fungi. Recent findings also point toward the feasibility of this methodology for species discrimination in anamorphic Clavicipitaceae of the genus *Pochonia* [92,93], which had recently been segregated from the paraphyletic genus *Verticillium* [94]. Anamorphic Xylariaceae of the genus *Nodulisporium*, including producers of the important antiparasitic agent, nodulisporic acid, were also evaluated by a combination of various PCR-based methods (AP-PCR and sequencing of the ITS/5.8S nrDNA) in relation to their ability to produce the characteristic metabolite [95]. As inferred from these molecular data, the producers of this compound, which are present in several tropical countries, did not show similarities to any of several examined teleomorph species with *Nodulisporium*-like anamorphs. They appeared monophyletic and may therefore deserve the rank of a separate species, if not a genus of their own.

From the accumulating data gained on trichocomaceous Eurotiales, the extensive use of secondary metabolites in fungal classification is also strongly suggested. In this family, there are several examples for secondary metabolites or profiles of such compounds



No.	Species//Host/Origin	No.	Species//Host/Origin
1	<i>Daldinia childiae</i> (<i>Quercus</i> , USA)	21	<i>D. eschscholzii</i> (USA)
2	<i>D. childiae</i> (USA)	22	<i>D. eschscholzii</i> (<i>Bucida</i> , Cuba)
3	<i>D. childiae</i> (ex-type, France)	23	<i>D. eschscholzii</i> (<i>Jambosa</i> , Cuba)
4	<i>D. childiae</i> (<i>Fraxinus</i> , France)	24	<i>D. fissa</i> (<i>Fagus</i> , Germany)
5	<i>D. childiae</i> (<i>Quercus</i> , Japan)	25	<i>D. grandis</i> (New Zealand)
6	<i>D. concentrica</i> (<i>Fraxinus</i> , UK)	26	<i>D. steglichii</i> (ex-type, Papua New Guinea)
7	<i>D. concentrica</i> (ex-epitype, UK)	27	<i>D. petriniae</i> (<i>Alnus</i> , Switzerland)
8	<i>D. concentrica</i> (<i>Ulmus</i> , Italy)	28	<i>D. petriniae</i> (<i>Alnus</i> , Austria)
9	<i>D. concentrica</i> (<i>Fraxinus</i> , Germany)	29	<i>D. bambusicola</i> (ex-type, <i>Bambusa</i> , Thailand)
10	<i>D. concentrica</i> (<i>Fraxinus</i> , UK, ATCC)	30	<i>D. caldariorum</i> (Mexico)
11	<i>D. concentrica</i> (<i>Fraxinus</i> , Netherlands, CBS)	31	<i>D. childiae</i> (ex-type, France)
12	<i>D. concentrica</i> (<i>Fraxinus</i> , Germany)	32	<i>D. pyrenaica</i> (ex-type, <i>Quercus</i> , Spain)
13	<i>D. concentrica</i> (<i>Fraxinus</i> , France)	33	D. sp. "Russ. Far East" (Ref. Ju et al.)
14	<i>D. fissa</i> (ex-type, <i>Fagus</i> , Germany Ref. Ju et al.)	34	<i>D. decipiens</i> (<i>Betula</i> , Denmark)
15	<i>D. childiae</i> (ex-type, France)	35	<i>D. caldariorum</i> (<i>Ulex</i> , UK)
16	<i>D. clavata</i> (Mexico)	36	<i>D. concentrica</i> (<i>Fraxinus</i> , Germany)
17	<i>D. albofibrosa</i> (ex-type, Papua New Guinea)	37	<i>D. fissa</i> (<i>Corylus</i> , Germany)
18	<i>D. concentrica</i> (<i>Fraxinus</i> , Germany)	38	<i>D. fissa</i> (<i>Carpinus</i> , Germany)
19	<i>D. eschscholzii</i> (Guadeloupe)	39	<i>D. fissa</i> (<i>Fagus</i> , Germany)
20	<i>D. eschscholzii</i> (Thailand)	M	Standard basepair ladder

Figure 7 Agarose gel electrophoresis of amplified MIS regions of *Daldinia childiae*, *D. concentrica*, *D. eschscholzii* and further taxa of the genus using primer M13 core. Rather specific fingerprints were obtained with *D. childiae* and *D. concentrica*, while a higher degree of variability was found among the *D. eschscholzii* and *D. fissa* isolates. (Modified from Ref. 84, with kind permission of the Editor-in-chief of *Mycological Research*.)

that easily help to discriminate certain species from their morphologically similar allies [41,96–98]. Taken together, the value of chemotaxonomy alone or in conjunction with PCR-fingerprinting techniques for strain dereplication in screening is indisputable because it has been demonstrated in most of the fungal families that are well-known to contain unique chemical entities. In time, many fungal taxa now still regarded as “strains” or “chemotypes” will be recognized as good species from a combination of these methods. In conclusion, PCR-fingerprinting data are rather easy to obtain and can serve as prerequisite for the selection of “uniques” prior to more cost-and time intensive chemotaxonomic, morphological, and DNA sequencing studies if representative individuals among a large number of range of closely related strains shall be studied.

1.4. Chemotaxonomy and Molecular Phylogeny

Throughout the last decade, the endowment of molecular biology helped to solve various problems relating to fungal taxonomy. DNA sequences are now widely used not only to complement morphological and other data but even to establish phylogenetic relationships. Not even the most significant key publications in this field can all be discussed here in detail, albeit some are cited below and in the next chapter. There have been many discussions as to whether a phylogenetic species concept would stand in contradiction with phenetic or morphological data. However, it is the strong opinion of the authors that the final goal of fungal taxonomy and phylogeny should rather be a polythetic species concept. This especially applies to those fungi that are studied by nontaxonomists because of their value for biotechnological applications, including the production of secondary metabolites. The biosynthesis of secondary metabolites requires several specific genes, which remain to be recognized and studied in most cases. Evidently, the same applies to the morphogenetic genes that encode for particular morphological phenotypes. It will be a long way to go until such correlations are fully understood (e.g., from the wider application of functional genomics). For the time being, morphological and chemotaxonomic characters remain valuable, especially for segregation of fungi at generic and subgeneric levels. Secondary metabolites or indirect characters based on their presence or absence (color, odor, and taste) have been traditionally used in ascomycete and basidiomycete taxonomy and are also important cultural characters in conidial fungi [41]. On the contrary, secondary metabolite genes have so far been playing a secondary role as surrogate parameters for verification of molecular data. Especially nuclear or, less frequently, mitochondrial ribosomal DNA sequences (nrDNA and mrDNA, respectively) were widely used for practical reasons such as the availability of universal primers, and the rather frequent occurrence of template nrDNA copies, facilitating their PCR-based amplification. However, additional genes encoding for proteins are already being widely considered and studied on their significance. In recent years, the genetic background for secondary metabolism has been subject of several intensive studies. Even though these mainly dealt with economically important mycotoxins and their producers, the methodology available appears ready to find application for basic phylogenetic research on other fungi. As many secondary metabolite genes in fungi are organized in clusters, straightforward PCR-based methods can be developed to compare large numbers of individuals on their genetic properties by using, for example, specific primers.

Fusarium species are already being examined, using morphological, molecular, and chemotaxonomic data in concert [99,100]. “Conventional” molecular data were even

compared with the distribution of genes encoding for secondary metabolite biosynthesis. Multiple gene loci genealogies were found useful for segregation of species complexes such as *Gibberella fujikuroi* and *F. graminearum*, respectively [101–104]. For molecular taxonomy of the economically important *F. graminearum* complex, six nuclear genes were finally considered in a genealogical concordance. Here, the 5.8S/ITS nrDNA region (widely used for species delineation in other fungi) appeared less informative than other genes. Later, trichothecene production and genes encoding for those compounds were also taken into consideration. Neither the strain-specific differences in metabolite profiles (chemotypes) nor the sequences obtained from the trichothecene gene cluster were well-correlated with the concurrent results of the aforementioned genealogical concordance [105]. This was interpreted as “a transpecific polymorphism within the virulence-associated secondary metabolite genes, which has been maintained during evolutionary processes by balancing selection acting on chemotype differences that originated in the ancestors.”

It should be considered that the differences in the chemical structures and biosynthesis of the main *Fusarium* trichothecenes are fairly small. All are derived from a rather complicated carbon skeleton as a common biogenetic precursor. Also, while it is generally accepted that the possession of trichothecene biosynthesis genes significantly contributes to the fitness and virulence of *Fusarium* spp., the functions of some genes involved in trichothecene biosynthesis have only recently been clarified. For instance, the structure and organization of biogenetic genes appears to differ considerably in *Fusarium* and the macrocyclic trichothecene-producing *Myrothecium* species [106], pointing toward a convergent evolution of secondary metabolism in both genera. Lately, a main determinant of trichothecene diversity in *Fusarium* species, leading to either of the main trichothecene types was described as the activated stage of a single cytochrome P-450 [107]. Such functional but nonessential genes may easily be altered by mutation. Evidently, the production of trichothecenes should be regarded as specific for certain lineages instead of species in *Fusarium*. Similar investigations are under way in the *Gibberella fujikuroi* complex, where the evaluation of secondary metabolite genes concentrates on those encoding for the fumonisins [108] and the fungal phytohormones of the gibberellin type. Interestingly, the biosynthesis of the latter compounds in plants and fungi, respectively, appears to have arisen from convergent evolutionary processes, resulting in an analogous organization of gene clusters [109].

In *Aspergillus* sect. *Fumigati* and corresponding *Neosartorya* teleomorphs, morphological and chemotaxonomic data were compared with the sequences of partial β -tubulin and hydrophobin [110]. Here, protein-encoding genes were employed because of insufficient phylogenetically informative characters as inferred from the large subunit of the nrDNA. While evolutionary relationships between homothallic and heterothallic isolates were recognized, the data sets in combination showed that morphological and secondary metabolite characters used in taxonomy were not strongly correlated with phylogeny. As many as 59 out of 81 secondary metabolites were only present in one out of 18 species in the data set and therefore excluded from the phylogenetic analyses. In *Aspergillus flavus*, *A. oryzae*, and allies, the phylogenetics of mycotoxin and sclerotium production were studied by using partial sequences of a gene encoding aflatoxin biosynthesis gene, *omt12* [111]. As stated by the authors, the results “further demonstrated that *A. flavus* in the current sense constitutes a nonmonophyletic assemblage, including at least two major groups of strains that show evidence for a long history of geographic isolation”. They concluded that taxonomic changes in this paraphyletic group be necessary and found evidence that the nonaflatoxigenic *A. oryzae*, a species widely used in food technology,

is genetically distinct from *A. flavus*, differing from the latter in possessing a divergent class of non-functional alleles in the aflatoxin gene cluster (and in the general absence of aflatoxins). Thus, *A. oryzae* appears safe once again from being included as an infraspecific taxon in the toxigenic and pathogenic *A. flavus*, as suggested by some authors from the interpretation of nrDNA sequence data and other characters.

Remarkable efforts were also made with the elucidation of the molecular background of biosynthesis and expression of loline alkaloids in *Neotyphodium uncinatum*, the endophytic anamorph of *Epichloe* species [112]. Needless to say, the elucidation of the molecular genetic background of the biosynthesis of marketed drugs such as lovastatin [113] and cyclosporin [114] also made considerable progress, even allowing for production of new analogues by altering their chemical structures by site-directed mutagenesis.

The results obtained in the course of these studies may eventually facilitate screening approaches aimed at the discovery of additional producer strains for a given lead. Soon, such techniques will also be used to characterize large strain contingents on the presence of particular biosynthetic gene clusters. All such genes may be subjected to the design of specific PCR-primers, followed by a “genetic screening,” in which DNA preparations from large strain collection libraries may be employed. As a result of the introduction of capillary electrophoresis and other powerful sequencing equipment, not only PCR fingerprinting but even DNA sequencing nowadays can be done with a high throughput and at lower costs. It should be pointed out that the most work- and time-intensive step in PCR-based characterization of fungi is usually the extraction of DNA. Main hindrances are PCR inhibitors and DNAases, which may afford a dilution or purification step before employing standardized PCR protocols. Once the DNA has been extracted and purified adequately (e.g., for sequencing or PCR fingerprinting in the course of the above described dereplication procedure), the remainder may be stored until further use. Hence, the integration of such approaches into NP screening has been facilitated. However, more basic research on these matters is needed. Hopefully, the knowledge on the genetic background of fungal secondary metabolites is soon going to increase even in the case of those compounds that neither constitute mycotoxins nor developmental candidates of the pharmaceutical and agrochemical industry.

Naturally, the sequence data stored in the Internet can be compared by BLAST, FASTA or related software tools to those of metabolite producing strains found in the screening. In principle, this allows NP researchers to verify taxonomic relationships and to search for the most likely candidates among fungi showing similar sequences. These can subsequently be checked for related compounds or overproducers of a desired NP lead. However, several important groups of fungi known to produce unique secondary metabolites are still underrepresented in these Internet databases. Moreover, the correlations between the prevailing (rDNA) sequences and secondary metabolite production in fungi are still poorly understood. As discussed above, they may not even be informative enough for phylogenetic purposes. Finally, it should be kept in mind that these internet databases may occasionally contain sequences of misidentified strains.

1.5. The Boletales Example

The Boletales are a good example to demonstrate that fungal pigments and other secondary metabolites are relevant to the recognition of phylogenetic relationships and thus to the identification of chemical redundancies and of chemical diversity within a fungal order.

The traditional core group of these fungi comprises mycorrhizal species with fleshy fruitbodies and poroid hymenophore, which are now classified in the type genus *Boletus* and other genera with mostly or exclusively boletoid habit (*Leccinum*, *Suillus*, *Tylopilus*, and *Xerocomus*). While many of them are considered edible and are traditionally collected as food throughout the world, others bear close morphological resemblance to their edible counterparts but contain bitter, pungent, or even toxic principles. Most, if not all of these boletoid fungi are still included in the Boletaceae, while the Boletales nowadays comprise various fungi whose fruitbodies bear little or no morphological resemblance to the typical bolete. In recent years, not only the chemistry of their fruitbodies (and to some extent also of the corresponding cultures) but also the molecular phylogeny of the Boletales have been studied extensively. Therefore, the correlation of results from chemotaxonomy and from molecular phylogeny can now be evaluated, as exemplified below.

Traditionally, the taxonomic classification of species now assigned to the Boletales had been dependent on the importance associated with several criteria: morphology (e.g., macroscopic characteristics), ecology (e.g., mycorrhizal association, host-specificity of mycoparasites such as *Sepedonium*), and chemotaxonomic aspects (e.g., distribution and biosynthesis of pigments and colorless metabolites). Species of various subtaxa included in the Boletales have motivated research on their chemistry and biology for many decades. The pigments of the Boletales are well-investigated, mainly due to the efforts of Steglich et al. (Figs. 8,9 [28]). In addition, several colorless metabolites (e.g., bitter substances) were isolated from their toadstools (Fig. 10). These results served as a basis for interdisciplinary taxonomic research.

At an earlier stage of the chemical investigation of the Boletaceae, their pigmentation pattern was considered to be homogenous. Most important in this respect are the pulvinic acids, such as the yellow pigments variegatic acid and xerocomic acid (Fig. 8), which are responsible for the bluing reaction of many representatives of the Boletales [28]. A second group of relevant pigments are the terphenylquinones, which are derived from the dimerization of 4-hydroxyphenylpyruvic acid. Representatives are atromentin and the flavomentins and spiromentins from *Paxillus atrotomentosus*, as well as the biosynthetically closely related grevillins from the genus *Suillus* [28]. Some genera of the Boletales are characterized by the presence of cyclopentenones [28]. These include chamonixin, i.e., (+)-form from *Chamoniaxia caespitosa* and (−)-form from *Paxillus involutus* [115], and gyrocyanin from *Gyroporus cyanescens*, which are responsible for the bluing reaction in these species. Involutin, another cyclopentenone pigment from *Paxillus involutus* [28] induces a brown discoloration upon bruising of the fruitbodies.

However, soon other groups of substances were found in selected Boletaceae (Figs. 9, 10). This implicated that the common pigments like the pulvinic acids are useful to confirm the affiliation of controversially discussed taxa to the Boletales. On the other hand, it was essential to have a closer look at the other secondary metabolites of the various species, including pigments and colorless substances. These compounds often have a definite property, such as being formed during injury of the basidiocarps or causing bitter or pungent sensations. Such metabolites turned out to be important markers for the taxonomic relationships within particular Boletaceae subtaxa. In addition, these substances are more likely than the aromatic pigments to exhibit selective pharmacological activities because of their unique chemical structures. For investigations of the pigments, at that time TLC with reference standards were used as simple and efficient tool. Nowadays, HPLC and LC hyphenated spectral techniques allow for the fast detection and identification not only of pigments but also of colorless compounds with various characteristics.

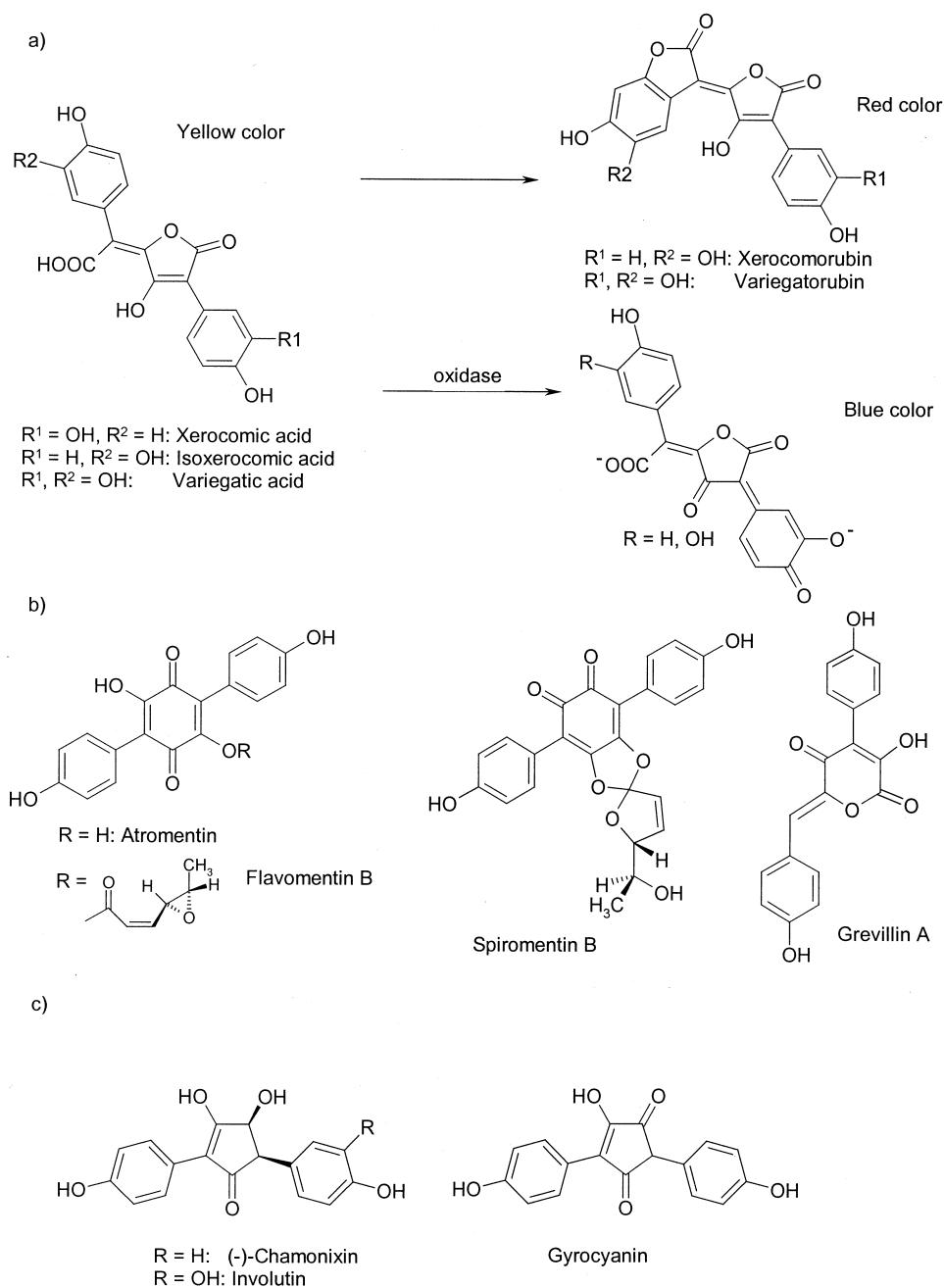


Figure 8 Some characteristic pigments of Boletales: (a) pulvinic acids, (b) terphenylquinones and grevillins, and (c) cyclopentenones.

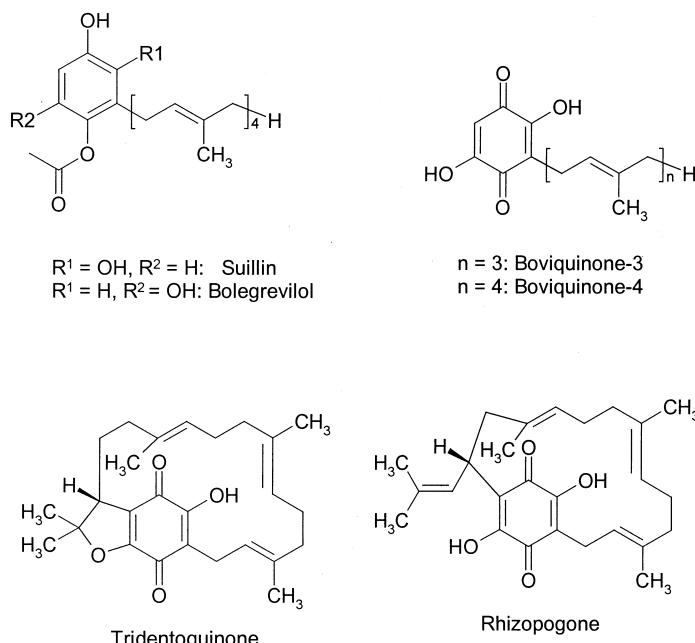


Figure 9 Some characteristic pigments of *Suillus* and related species.

1.5.1. *Suillus*

Among the Boletales, the recent taxonomic investigations of species within the genus *Suillus* and related genera were one of the first examples in which extensive chemotaxonomic investigations were compiled with molecular data. Characteristic pigments of various members of the Boletales are pulvinic acids, terphenylquinones and cyclopentenones. *Suillus* and *Gastroboletus*, *Gomphidius* and *Chroogomphus* are characterized by the occurrence of polyprenylated phenols and quinones as additional pigments. Examples are e.g., suillin, the boviquinones and tridentoquinone (Fig. 9) [28]. Such pigments are also found in *Rhizopogon*, (e.g., rhizopogone; Fig. 9) [116,117]. These genera mainly differ in the habit and anatomy of their basidiocarps. *Suillus* is boletoid, while *Gomphidius* and *Chroogomphus* are agaricoid and *Rhizopogon*, *Chamonixia*, and *Gastroboletus*. are gasteroid. Besl and Bresinsky [117] therefore erected the new family Suillaceae with the genera *Boletinus*, *Suillus* and *Gastrosuillus*, which is a member of the new suborder Suillenae, also containing the Gomphidiaceae and Rhizopogonaceae.

Interestingly, Mühlbauer et al. recognized that the structurally homologous fungal metabolites boviquinone-3 and boviquinone-4 are produced by two different pathways in the agaricoid *Chroogomphus rutilus* and the boletoid *Suillus bovinus* [118]. These metabolites were found to differ in the prenylation position of 3,4-dihydroxybenzoic acid during the biosynthesis.

Independent from these chemotaxonomic findings, molecular studies came to congruent results. DNA analyses confirmed the relationship between the genera *Suillus* and

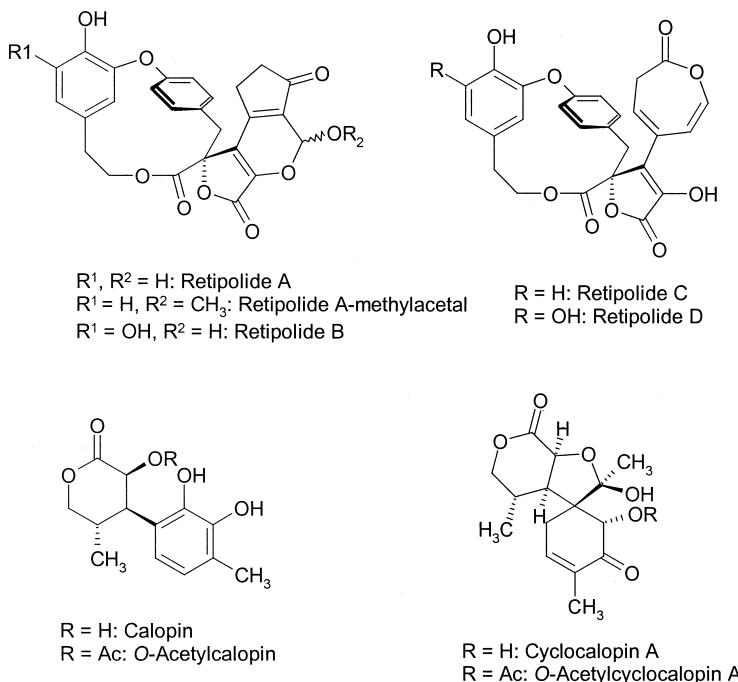


Figure 10 Some characteristic metabolites of selected Boletales: (a) retipolides and (b) (cyclo)calopins.

Rhizopogon [119] and demonstrated the close link of *Gomphidius* and *Chroogomphus* with *Suillus* [120]. The status of the Suillaceae as a sister family of the Boletaceae had not been recognized prior to these studies [121].

1.5.2. Sclerodermaceae

Due to chemotaxonomic investigations, the gasteroid genera *Scleroderma* and *Pisolithus* already were shown to have affinities to the Boletales. Three pigments – representing methylated and chlorinated derivatives of atromentic acid (methyl 4,4-di-*O*-methylatromentate, methyl per-*O*-methylatromentate and methyl 2',5'-dichloro-4,4'-di-*O*-methylatromentate), were isolated from the tropical species *Scleroderma sinnamariense* [122]. From *Pisolithus arhizus* the naphthalenoid pulvinic acid derivative pisoquinone was isolated, confirming its relationship to *Scleroderma* [123]. Chlorinated pulvinic acids had already been known before from the boletoid *Pulveroboletus auriflammeus* [124] and *Xerocomus chrysenteron* [125].

In the fruitbodies of the hypogaeous *Melanogaster broomeianus*, the cyclopentanoids chamonixin and involutin were detected. This pointed out a close relationship between the Melanogastraceae and the Boletales, which was further confirmed by the fact that *M. broomeianus* can be infected by *Sepedonium*, a mycoparasite genus generally known to parasitise Boletaceae and Paxillaceae. Thus, chemotaxonomic relations were reflected by

host-parasite relationships [126] and later proved by comparison of the 28S nrDNA sequences [127].

1.5.3. Omphalotaceae

Due to morphological features (e.g., lamellae, paxilloid habitat) members of the newly established Omphalotaceae were until recently included in the Paxillaceae [128]. Moreover, the detection of atromentin and pulvinic acids in cultures of *Omphalotus* and *Lampteromyces* had pointed toward their affinities to the Boletaceae [129]. Before, both genera have been included in *Clitocybe* and *Pleurotus*, respectively, and their characteristic illudane type sesquiterpenoids (Table 3) were named after the producing fungus, “*Clitocybe illudens* (=syn; *Omphalotus olearius*)” [130]. These sesquiterpenoids are neither found in the Paxillaceae nor in other Boletales. Recently, the nematicidal omphalotins were isolated from *O. olearius* [131–133]. Their production appears to be a rather constant feature of *Lampteromyces* and *Omphalotus* spp. [134].

Due to a comparison of 28S nrDNA data, Binder et al. placed the two genera in a separate family Omphalotaceae. This family forms a distinct clade within the Agaricales, while the sequence data clearly differ from the Boletales [135]. Another clear chemotaxonomic character to discriminate Omphalotaceae from Paxillaceae besides the occurrence of sesquiterpenoids is the presence of white rot (as opposed to brown rot in *Paxillus* and *Tapinella*, the Coniophoraceae and all other wood-destroying Boletales) and the occurrence of bioluminescence, which is not known from Boletales. Because the protoilludane and illudane carbon skeleton is frequently encountered in certain groups of Agaricales such as *Armillaria* and *Agrocybe*, the Omphalotaceae may be closely related to other gilled mushrooms. As inferred from phylogenetic studies of 28S nrDNA sequences, it was revealed that the pleurotoid *Nothopanus* is closely related to the Omphalotaceae [135]. This was confirmed when using a wider range of taxa [136]. The occurrence of pulvinic acids is unparalleled in agaricoid taxa, but the biosynthesis of these compounds may well have arisen independently several times in the course of the evolution of homobasidiomycetes. In contrast, the peptidic omphalotins and the terpenoid illudins may constitute determinants of a more advanced evolution of secondary metabolism.

1.5.4. *Retiboletus*

Since the first description of *Boletus ornatipes* and *B. retipes* it remained uncertain how to differentiate between these two species or whether they are distinct species at all. Investigations of their secondary metabolites guided by the bitter taste of these toadstools led Steglich et al. to the discovery of a unique group of macrocyclic butenolides, the retipolides A-E (Fig. 10), which may be derived biosynthetically from three molecules of 4-hydroxyphenylpyruvic acid [47,48,137]. A recent chemotaxonomic study of several collections from North America and Japan created three separate groups according to the occurrence of characteristic metabolites [138]. The first and the second group contain retipolide A and C. In addition, the species of the first group have a novel yellow pigment in common in contrast to the second group, which lacks this pigment. The third group was found to contain the retipolides A-D. Only in some species of the first group traces of xerocomic acid were detected.

A concurrent study of the 25S rDNA data of the same collections resulted in three major clades, which were congruent to the three groups distinguished in the chemotaxo-

nomic investigations [138]. Sequence data of the 28S nrDNA indicated that *B. retipes/B. ornatipes* are distinct from other groups in the Boletaceae, including *Boletus* itself. This was in agreement with the chemotaxonomic investigations, since only in one group of the *B. retipes/B. ornatipes* complex, xerocomic acid was detected as side metabolite, while other “typical” Bolete pigments were not found at all. Instead, the retipolides constitute the major pigments of this complex. Therefore, the new genus *Retiboletus* with *R. ornatipes*, *R. retipes* and *R. flavoniger* [139] was erected. However, there are still no morphological or anatomical characteristics available to distinguish *R. retipes* from *R. ornatipes*. The example of *R. retipes/ornatipes* reveals the importance of chemotaxonomic and PCR data to identify morphologically apparently similar or identical biological species producing different chemical substances.

1.5.5. *Boletus calopus*

Boletus calopus and closely related mushrooms of *Boletus* sect. *Calopodes* are characterized by their bitter taste, which is caused by *O*-acetylcyclocalopin A. Besides this bitter compound, several structurally related analogues, called calopins and cyclokalopins, are found in *Boletus calopus* and *B. radicans*, as well as in the North American species *B. coniferarum*, *B. rubripes* and *B. peckii* (Fig. 10) [140]. The common occurrence of the bitter principles in all species of the section, independent from their geographical distribution, confirmed their relationship. Up to now, the calopins with a new carbon skeleton seem to be produced only by species of sect. *Calopodes*. Again, molecular data agreed well with these results [139].

These examples suggest that also metabolites other than pigments may play an important role in chemotaxonomy within the Boletales. These compounds, e.g., terpenoids, are usually derived by a more complex biogenetic pathway and often are characteristic of one or only a few species, while some of the common pigments may be biosynthesized in a more convergent way and often represent precursors for more specialized metabolites. These findings can easily be interpreted in view of the chemotaxonomic preselection described above: In contrast to the more ubiquitous metabolites the species-specific compounds often possess unique chemical structures probably more likely to exhibit selective bioactivities, while other interesting, but not species-specific metabolites may also show nonselective reactions. Thus, they may disturb the HTS workflow by generating false positive hits and are probably not suitable for drug development. Important for the process of drug discovery are those compounds which exhibit selective bioactivities. To find these metabolites while avoiding handling of the unwelcome compounds, a careful preselection within the strain collections and the screening samples and hits is necessary. In conclusion, the Boletales example gives an idea of how to identify the producing strains of these desired compounds and how to avoid duplicates of other species. In Boletales, molecular data were actually used to validate these findings in retrospective [141] and chemotaxonomy probably helped to explain the molecular data, which were not always in clear accordance to morphological features. Not only concerning the Boletales, but also in many other areas of fungal taxonomy there is a lot of knowledge based on classical taxonomy and on chemotaxonomy. It is the challenge of NP research within the search for new lead structures to exploit and to extend this knowledge. This will facilitate rendering the search for new chemical entities from nature less complicated and less time- and cost-intensive. HPLC profiling at an early stage of the process will be rewarding not only in drug discov-

ery, but also for taxonomists, who should not concentrate on morphological methods in conjunction with molecular data alone.

1.6. Outlook

Amazingly, only a few interdisciplinary collaborations are currently ongoing to solve problems of basic fungal taxonomy also by including chemotaxonomic methods. Pharmaceutical and agrochemical companies are most likely to remain dependent on NPs as one source for new lead candidates. At least some of them have in house expertise in fungal taxonomy and will appreciate a renewal of chemotaxonomic and polyphasic approaches in order to increase the probability of success of their lead-finding process. A stronger focus on interdisciplinary education and training of young scientists, including classical morphology, as well as biochemical and molecular methods in combination with analytical chemistry—or at least stronger collaborations of expert groups on basic and applied research in these fields—are highly desirable to meet the future requirements for bioprospecting, using fungi as sources for novel lead compounds. In this sense, preserving the traditional knowledge on fungal taxonomy, ecology and “ethnomycology” may eventually help to find the future blockbuster drugs and agrochemicals.

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10

Screening for Biological Activity in Fungal Extracts

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1. INTRODUCTION

Many clinically important pharmaceuticals have been found by screening fungal extracts. These therapeutic agents, which are either the natural metabolite per se or derivatives of the natural products, include antibiotics, such as penicillins [1–4], cephalosporins [1–4], and griseofulvin [5]; cholesterol statins, such as mevinolin and simvastatin [4]; immunosuppressants, such as the cyclosporines [6,7]; and vasoconstrictors, such as syntomsetrine [8]. Initially, fungal extracts were tested for antimicrobial activity using whole-cell assays on agar plates. *In vivo* assays were then expanded to include multicellular organisms, such as free-living adult and larval insects and adult and larval parasitic worms both in and outside their hosts, to search for insecticides and anthelmintics. Mammalian cell cultures were employed to find anticancer and antiviral agents. As knowledge of the biochemistry of the targets advanced, *in vitro* assays were developed to detect inhibitors of critical enzymes and binding to crucial receptors. Molecular biology has provided techniques for creating designer cells with increased permeability that over-express or under-express the desired target (receptor or enzyme) and contain genes for reporter reactions, such as luciferase [9], β -galactosidase [10], and glutathione transferase [11]. These cells are more sensitive to inhibitors in low concentration and produce an easily measured signal. In addition, advances in assay technology, instrumentation, and robotics have provided more diversity in detection techniques, greater sensitivity, and smaller assay volumes. Assays in 384-, 1536-, and even higher-density well formats allow hundreds of thousands of samples to be screened in a day.

Adaption of fungal and other natural product extracts, which contain chromophores and fluorophores, to modern screening techniques is a continuing challenge. Sample prepa-

ration, assay validation, detection of activity, confirmation of activity, and assay support for isolating the active components are all crucial to the success of a program. In this chapter, we discuss a variety of these assays and related issues and also suggest some techniques and approaches that can be successfully employed.

2. GENERAL CONSIDERATIONS

The type of fungal extract has a significant impact on the screening program. Since fungal metabolites are both intracellular and extracellular [12], whole broth extracts of fungi grown in suspended aqueous culture contain all of the potentially active metabolites. If cells are grown on solid media, such as glass beads or vermiculite, extracts of the mycelia must suffice. Both polar (methanol:water) and nonpolar (2-butanone) extracts are needed for a broad screening program because most of the antimicrobials found to date have been polar compounds whereas most of the therapeutic agents have been nonpolar compounds. Fungal samples have been fractionated prior to screening in an attempt to increase the concentration of minor components, which might otherwise escape detection, and to decrease the amount of interference in assays (as discussed later in this chapter). Fractionation prior to screening dramatically increases both the number of samples to be screened and the cost. It should not be undertaken unless the assay has a high throughput and low cost. In experiments conducted at Merck Research Laboratories, screening of fractionated samples did not lead to discovery of any actives not found in crude extracts. Schering-Plough, however, reported one such discovery at the SIM meeting in 2000 [13].

Storage of extracts and samples for screening is an important issue. There is a paucity of experimental evidence concerning the stability of extracts; therefore, general rules for ensuring chemical stability should be applied. The common practice is to store the extracts at -20°C to -80°C or to dry the extracts at low temperature and store the dried sample at -20°C to -80°C . This supply of sample can then be aliquoted for screening, for fractionation prior to screening, and for isolation of active compounds. Comparison of HPLC patterns of fresh and stored extracts can provide a gross measure of change but is a poor indicator of the stability of individual components. Testing extracts periodically in a fixed number of assays would provide a better measure of component stability but is time-consuming and expensive. Samples for screening are generally prepared by dissolving a known amount of dried extracts in DMSO. Quantification of the screening samples is imperative because it provides the basis for determining the specific activity of an extract in a given assay and of the active components during isolation. Quantification can be based either on the dry weight of extract in the screening sample or on whole broth equivalents. Both methods have been successfully employed at Merck. Screening samples are kept frozen at -80°C , thawed for use, and then refrozen. Fresh samples should be prepared after several freeze-thaw cycles and at least every year.

Assay validation, screening concentration, interference, confirmation of activity, and assay support for isolation are related issues. Assay development is generally done using a pure active compound as the positive control and DMSO as the blank. An acceptable assay will have a signal-to-noise ratio of at least four-fold. This, however, does not ensure that the assay will perform well with fungal extracts, which often contain significant amounts of natural pigments including chromophores and fluorophores. To validate the assay for use with fungal extracts, one has to achieve an acceptable signal-to-noise ratio for the positive control in the presence of inactive extracts and demonstrate a proportional increase in activity with increasing concentration of the positive control in the presence

of the inactive extracts. It is important that this titration of activity yield incremental increases in activity with increasing concentration because a common type of interference encountered with fungal extracts results in an all or none response—that is, activity jumps from the 0% to 10% range directly to the 90% to 100% range. The number of inactive extracts to use is a matter of choice, but we found 10 to 20 to be a reasonable number to ensure a reliable assay. (Inactive extracts are found by limited screening of samples in the assay prior to this validation.) The titration curve of the positive control in the presence of the extract may be displaced from that obtained with the positive control plus DMSO. It will therefore yield a different IC₅₀ value (concentration of sample that yields 50% inhibition or activation, usually determined via nonlinear regression plots) but the shape of the curve should be similar. An EC₅₀ value (concentration of sample that yields activation or inhibition equal to 50% of the range of activity observed) can be used when the titration curves do not cover a full 0% to 100% range. The inactive extracts chosen should include those containing chromophores and fluorophores, depending on the assay detection methodology. Databases can be employed to compile lists of interfering extracts, which show up as actives in several unrelated assays. These are often highly pigmented and/or contain long-chain fatty acids, which act as detergents.

After validation of the assay, the screening concentration is determined empirically by testing several plates of samples at different concentrations and observing the frequency of actives (hit rate). Hits generally are defined either by using a fixed cutoff activity level or by statistical analysis. In the fixed cutoff method, the threshold of activity is set and all samples displaying activity above this threshold (% inhibition or activation) are considered active. In the statistical method, each plate is analyzed and outliers (generally >2 standard deviations) from the plate mean or median are considered hits. Statistical packages such as “SAS” and visualization programs such as “Spotfire” are often used to facilitate this approach. Figure 1 presents an analysis of a screen and indicates the top 1% actives by either percentage of activity or the number of standard deviations from the mean (score) [14]. Actives selected by both methods are in the lower left quadrant, those by percentage of inhibition are in the lower right quadrant and those chosen statistically are in the upper left quadrant. The statistically selected hits differ because selection is a function of the noise in the assay [i.e., the standard deviation of the mean of all samples in a plate (not just the controls)] and they are not subject to an arbitrary cutoff. Thus, a 40% inhibition may be selected statistically if the assay is not noisy. The desired hit rate is a function of the screening concentration and the threshold chosen for either activity or statistical outliers. These parameters are a matter of choice and are determined by the importance of the program and the support available for validation and isolation of actives. In screening fungal extracts that are crude mixtures, it is important to cast a wide net because the active compound may only be a minor component in the mixture. Many of the hits will be false positives due to interference and will not confirm.

Confirmation of actives is critical since it determines which extracts are to be pursued and which will be dropped. At Merck, we found this was best achieved by titration of the extract to determine an IC₅₀ or EC₅₀. This provided not only a repeat of the original assay but also a titration curve and a measure of potency. In the Natural Products program, we typically aimed for a 50% confirmation rate because it provided sensitivity and a manageable number of samples to pursue. Because some interfering compounds (false positives) may titrate nicely, it is important to check the titrated actives in assays with a different order of addition of reactants, a different format (nonhomogeneous), or in mock assays in which the target (enzyme, receptor) is omitted. This will eliminate extracts that appear

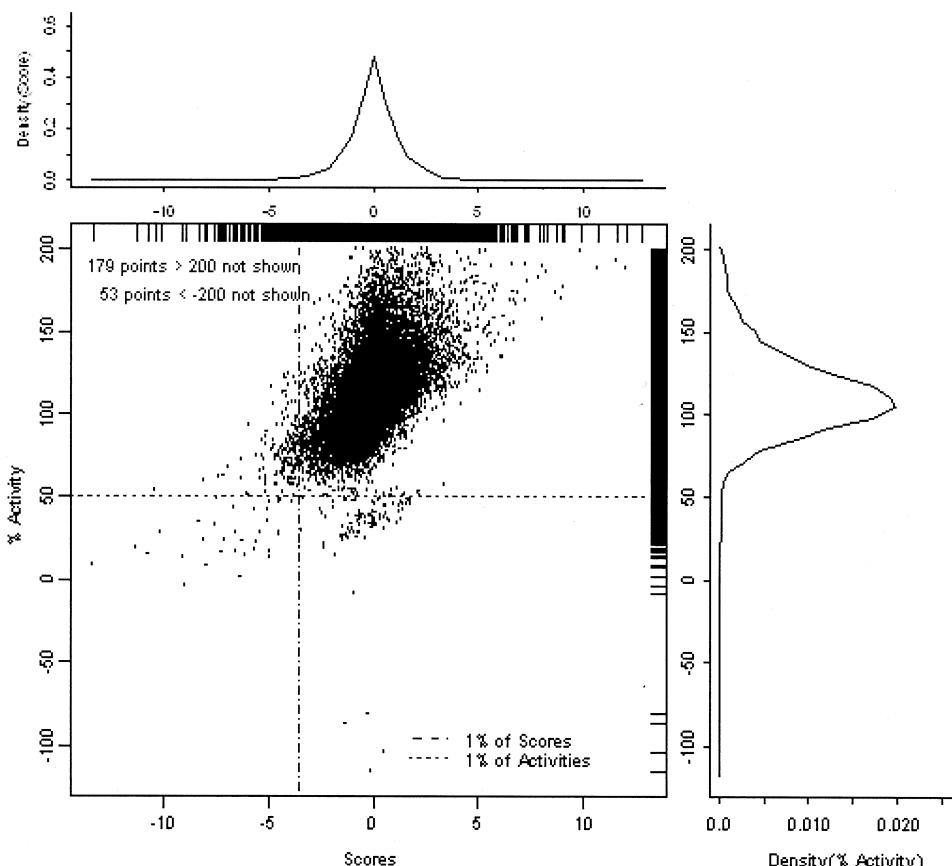


Figure 1 Statistical selection of actives. “Scores” is a statistical value related to the number of standard deviations from the mean.

active because they are interfering with the detection system employed. For example, there could be a detergent-like molecule that prevents antibody binding in an ELISA assay or a high concentration of biotin in a SPA assay using streptavidin-coated beads. This entails additional work but actually saves resources by limiting the number of samples selected for isolation. Counterscreens can be used to prioritize but not to eliminate actives since lack of specificity of the extract does not predict a lack of specificity of the purified component.

Assay support for isolation of the active component requires the largest commitment of time and resources because it entails titration (10 points) of each fraction produced in each step of the isolation procedure. Quantitation of activity during isolation is critical because it permits monitoring of the total activity applied and recovered in each step of the isolation procedure. This provides a measure of the specific activity of each component in the mixture and prevents the loss of potent minors. This information must be available in a timely fashion (within 2 to 3 days) to avoid potential loss of activity during the isolation due to instability of the active components. Broad counter-screening to establish

specificity of the active component is important once a high degree of purity (90%) is obtained.

3. ASSAY FORMATS

There are a wide variety of assay formats currently in use. Some rely on conventional technologies and are more labor intensive, and others rely on automated systems. The latter often require special reagents and detectors, which are expensive. A brief description and diagram of the commonly used assay formats is presented for the convenience of readers inexperienced in screening assays. More complete information on a given assay can be obtained from the references.

3.1. Filtration Assay

Figure 2 outlines a filtration assay for the binding of ligand to a generic cell-surface receptor. The assay steps include incubation of cells with radiolabeled ligand to allow binding. This can be done in small tubes or in 96- or 384-well microtiter plates. The reaction is then stopped by the addition of excess cold ligand and rapid filtration. The stopped reaction mixture is then filtered to separate unbound ligand from ligand-receptor complex. If the incubation is run in tubes, the reaction mixture is transferred to a millipore filter system, filtered, and then washed. The filters are then transferred to scintillation vials, fluid is added, and the amount of bound radioactivity is determined. If the incubation is in 96- or 384-well plates, samples can be filtered manually using microtiter filtration plates and a filtration manifold or automatically using a cell harvester. Comparison of the radioactivity detected in the control (no fungal extract added) with the samples (fungal extract present) is used to indicate binding inhibition or stimulation. This type of assay has been used for both receptors and enzymes [15,16].

3.2. Centrifugation Assay

A centrifugation assay successfully used to screen for inhibitors of the Hepatitis C NS3 protease is outlined in Figure 3 [17]. This reaction can be performed in small tubes or in 96-well plates. A synthetic substrate containing the CYS-ALA scissile bond and radiolabel

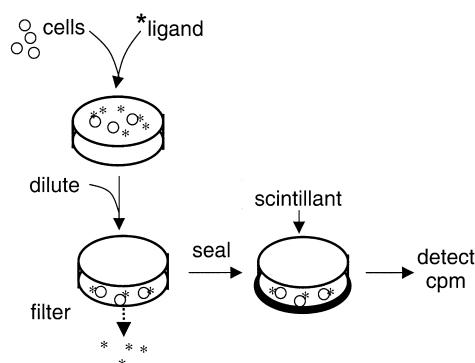


Figure 2 Filtration assay in microtiter format.

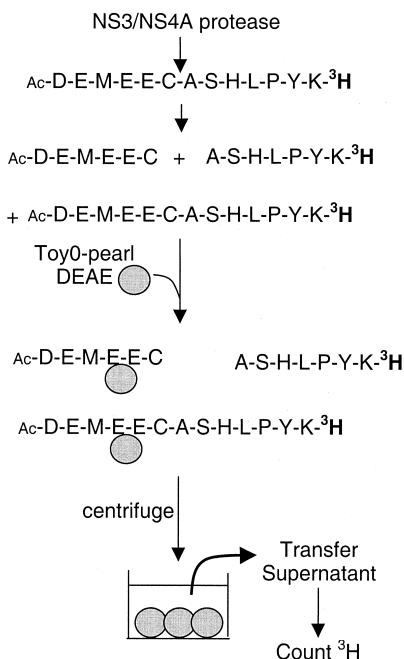


Figure 3 Centrifugation assay. Hepatitis C NS3 protease.

on the C-terminus is incubated with the active protease in the presence or absence of extract. The reaction is stopped by addition of Toyo-Pearl-DEAE beads, which bind the glutamate-rich region of the intact substrate and the N-terminal portion of the cleaved substrate. The reaction is centrifuged and an aliquot of the supernatant containing the cleaved C-terminal fragment of the substrate is transferred to a microtiter plate designed for scintillation counting or to a scintillation vial and counted. A comparison between the control and the sample indicates if the sample is an inhibitor (decrease in soluble radioactivity).

3.3. Enzyme-Linked Immunosorbant Assay

This assay has very broad application and is limited only by the availability of specific antibodies against the reaction product [14,18]. Enzyme-linked immunoassays (ELISAs) are generally run in microtiter plates with adherent surfaces. A generic ELISA is depicted in Figure 4, but for clarity, let us assume that this is a tyrosine kinase assay and the primary antibody recognizes the phosphotyrosine product. Before the assay is begun, the primary antibody is added to the wells and allowed to adhere. The excess antibody is then removed, and the reaction components are added. The reaction is terminated and the liquid is removed from the well, leaving the newly formed phosphotyrosine bound to the primary antibody. A second antibody raised against another portion of the peptide substrate and coupled to a reporter enzyme (in this case, horseradish peroxidase) is added and allowed to react. The liquid is removed and the plate is again washed to remove excess detection antibody. Finally, a nonfluorescent substrate, which is converted to a fluorescent

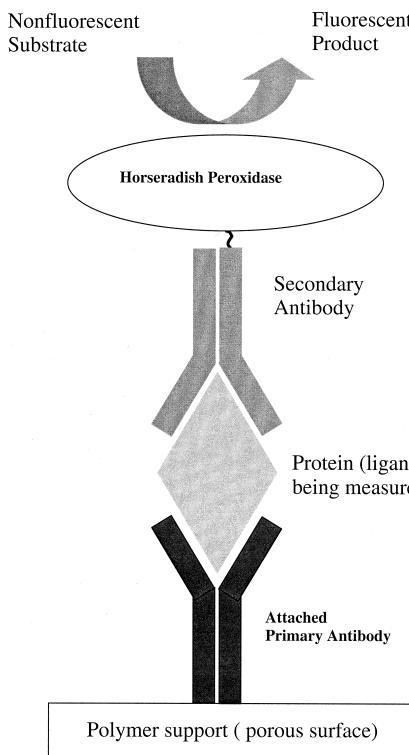


Figure 4 ELISA (*enzyme linked immunosorbant assay*).

product by horseradish peroxidase, is added and the amount of fluorescent product is determined in a fluorometer. This assay is very sensitive but is more qualitative than quantitative because the amount of phosphorylated product is amplified by the horseradish peroxidase reaction. A quantitative assay can be obtained using the Delfia technique [19], in which the second antibody is conjugated to Europium rather than a reporter enzyme. After the plate is washed, Europium fluorescence is then detected directly in a fluorometer (excitation at 337 nm, emission at 620 nm). All assays that use antibodies for detection are subject to interference by compounds that interfere with antibody binding, and it is important to validate them for use with fungal extracts.

3.4. Absorbance Assay

This assay measures a change in absorbance (generally an increase). The format is simple and is often used for proteases, peptidases, lipases, and esterases [20]. The substrate contains a chromophore that is liberated during the enzymatic reaction, causing a time-dependent increase in absorbance. Figure 5 provides an example of an absorbance assay for the protease papain [21]. The increase in absorbance, shown in the inset, is both time-dependent and proportional to enzyme concentration. This type of assay is subject to interference by chromophores and fluorophores, which interfere with absorbance measurements.

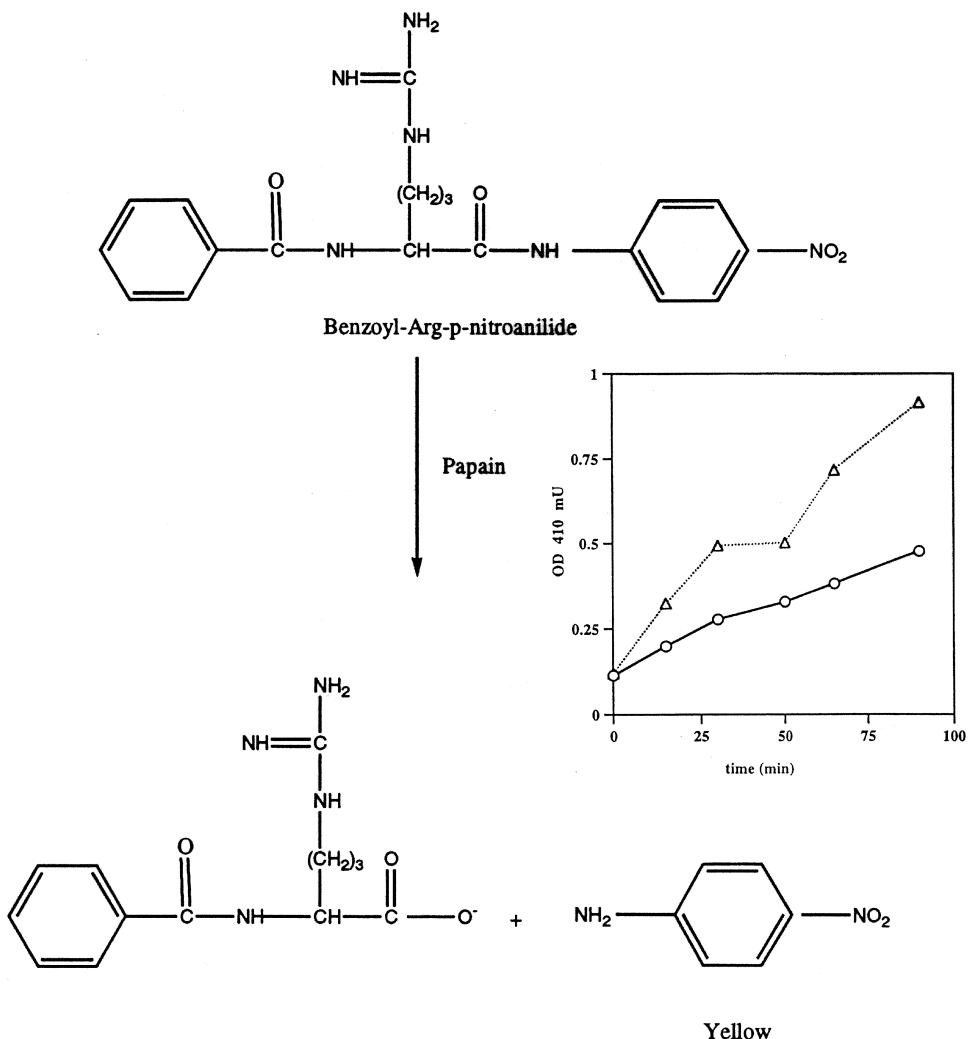


Figure 5 Absorbance assay.

3.5. Fluorescence Assay

This assay is similar to the absorbance assay except that a change (generally an increase) in fluorescence is measured. It is also often used for proteases, peptidases, lipases, esterases, and glycosidases. It is approximately 10 to 1000 times more sensitive than an equivalent absorbance assay because it is much easier to measure increased light emitted than to measure decreased light transmitted. In the example of a β -galactosidase assay depicted in Figure 6, the substrate contains the fluorophore 7-hydroxycoumarin-3-carboxylic acid bound to the β -D-galactose via a glycosidic bond [22]. Cleavage of the glycosidic bond liberates the fluorophore 7-hydroxycoumarin-3-carboxylic acid, which when excited at

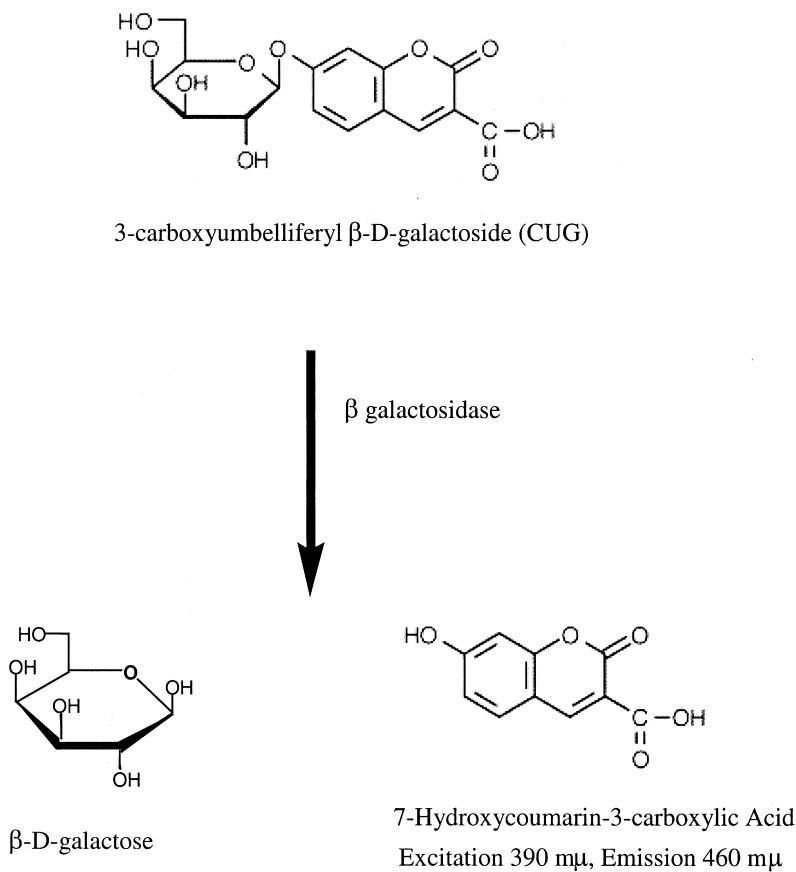


Figure 6 Fluorescence assay for β -galactosidase.

390 nm fluoresces at 460 nm. The increase in fluorescence is therefore a measure of substrate cleaved and will increase with both time and enzyme concentration.

3.6. Scintillation Proximity Assay

Scintillation proximity assay (SPA) provides a homogeneous format for conducting assays with radiolabeled ligands or substrates that eliminates the need to wash and remove bound ligand from free and eliminates the use of scintillation fluid. The basis for this assay is that the scintillant is locked into the SPA bead and is only excited by radioligands that are in proximity—that is, bound to the bead. Assays therefore are designed to allow the product but not the substrate to bind to the bead and be detected. Figures 7 and 8 outline two different SPA assays—one for a nuclear receptor, peroxisome proliferator-activated receptor gamma (PPAR γ) [23] and the other for a tyrosine kinase [24]. In the PPAR γ assay, the cloned PPAR receptor has been engineered as a fusion protein with glutathione transferase (GST). The added tritiated ligand (H) will either be free in solution or bound to the PPAR receptor. The bound ligand is detected using an anti-GST antibody and a

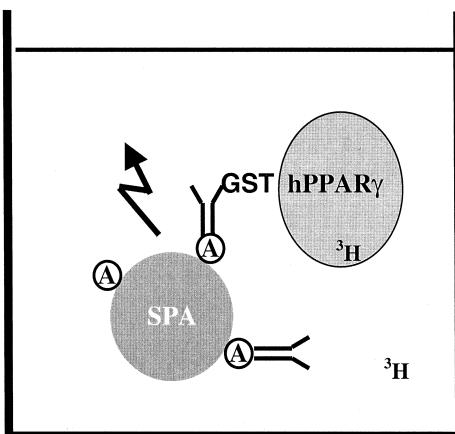


Figure 7 Scintillation proximity assay for peroxisome proliferator-activated receptor γ . Scintillation proximity bead coated with protein A (A), anti-GST Ab, GST-hPPAR γ fusion protein, tritiated ligand (^3H).

protein A-coated SPA bead. As depicted, binding of the protein A bead to anti-GST antibody that is bound to receptor brings the receptor-bound ligand in sufficient proximity to the bead to allow excitation of the scintillant and detection of bound ligand. Free ligand is not detected. In the tyrosine kinase assay [24], the peptide substrate is biotinylated and the SPA beads are coated with streptavidin. Radioactive γ -labeled ATP is used and detection of the reaction product, phosphorylated peptide, occurs via binding of the biotinylated, phosphorylated peptide to the streptavidin-coated bead. Beads with a wide variety of coatings are currently available, and more are under development to allow a wide variety of assays to be run in this format [24].

3.7. Flash Plate Assay

Flash plates are another means of conducting assays that use radiolabeled ligands without the addition of scintillation fluid [25]. In this case, special assay plates are used in which the scintillant is coated onto the walls of the wells. The walls are also coated with gelatin or other substances that allow receptors and proteins to adhere. Figure 9 depicts a generic flash plate assay in which a receptor is shown adhering to the wall of a well coated with scintillation fluid. After addition of radiolabeled ligand and incubation to allow binding, the well is washed and filled with appropriate buffer. Bound radioligand is detected because it is close enough to the scintillant to be detected. It is not imperative that the plates be washed to remove unbound ligand, but this generally is preferred since it lowers the background. A variety of flash plates are available [25], but this format is used less frequently than SPA beads.

3.8. Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) is a homogeneous assay technique based on energy transfer between two fluorophores within the same molecule [26]. Cleavage of

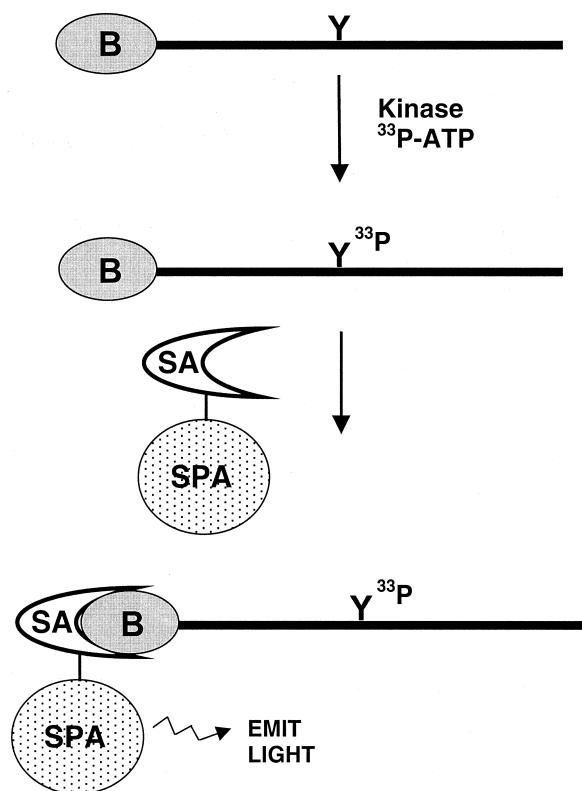


Figure 8 Scintillation proximity assay for tyrosine kinase. Biotin (B), tyrosine (Y), streptavidin (SA), scintillation proximity assay bead (SPA).

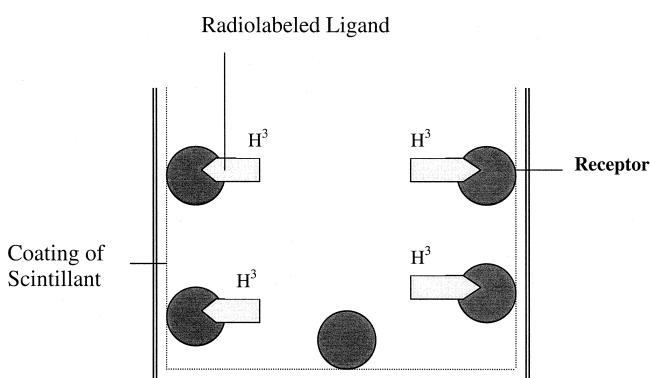


Figure 9 Flash plate assay.

the molecule separates the fluorophores and disrupts the energy transfer, thereby altering the wavelength of the emitted light. In the β -lactamase assay depicted in Figure 10, the cephalosporin has coumarin bound to the β -lactam ring and fluorescein bound to the dihydrothiazine ring. Excitation of this molecule at the coumarin absorption maximum (395 nm) yields the green fluorescence of fluorescein (535 nm) rather than the blue fluorescence of coumarin (460 nm) due to energy transfer from the coumarin to fluorescein. After cleavage of the molecule by β -lactamase, there is no longer any energy transfer, and excitation at 395 nm yields the blue fluorescence of coumarin. The ease of readout allows this assay to be run with either enzyme preparations or whole cells to detect and determine β -lactamase activity or to find inhibitors of β -lactamase [27]. This assay is most often used to detect β -lactamase when it is used as a reporter gene for activation or

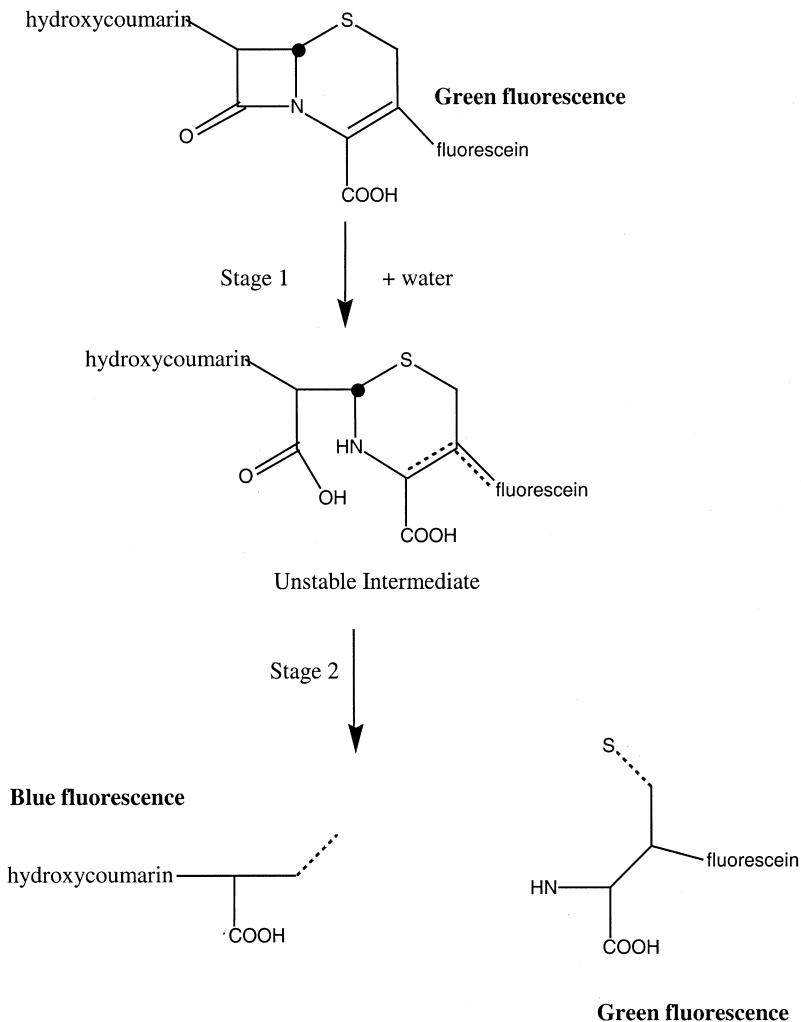


Figure 10 FRET (fluorescence resonance energy transfer).

inhibition of receptors. Because energy transfer requires close proximity between donor and acceptor dyes, FRET has also been used to measure protein–protein or protein–ligand interactions, by conjugating dyes directly to the proteins or to antibodies that recognize the proteins.

3.9. Homogeneous Time-Resolved Fluorescence

Homogeneous time-resolved fluorescence (HTRF) also involves energy transfer between fluorophores in close proximity, but in this case, the fluorescence emitted is long-lived thereby allowing measurements to be delayed sufficiently after excitation to avoid interference from normal (short-lived) background fluorescence [28,29]. This is achieved by excitation of lanthanides and energy transfer to XL-665 (allophycocyanin), a 105-kd protein that functions as an accessory pigment in cyanobacteria and red algae [30]. Figure 11 outlines the concepts of an HTRF assay [31]. Line 1 indicates a complete reaction in which there is energy transfer from the donor lanthanide (europium) excited at 337 nm to the acceptor XL-665, which then emits light at 665 nm. Lines 2 and 3 indicate events where there is no energy transfer. Europium absorbs light at 337 nm and emits long-lived fluorescence at 620 nm. XL-665 also absorbs at 337 nm and emits normal (short-lived) fluorescence at 665 nm. In a typical assay, long-lived fluorescence at both 665 nm (channel A) and 620 nm (channel B) are measured and the ratio of A/B is calculated. High-channel B readings are often an indication of interference [31]. Figure 12 is an HTRF tyrosine kinase assay in which the substrate is biotinylated, streptavidin is labeled with the acceptor

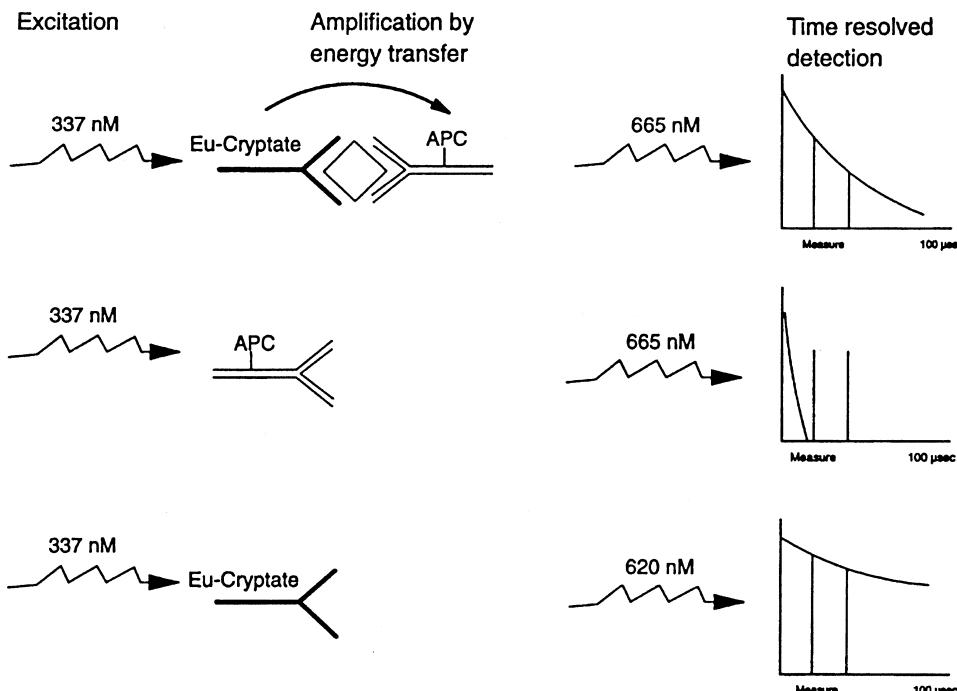


Figure 11 HTRF concepts.

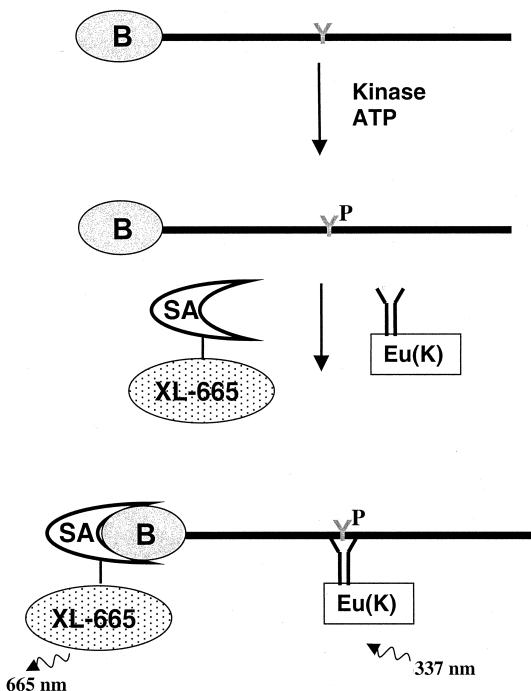


Figure 12 Tyrosine kinase assay in HTRF format.

dye XL-665, and an antibody against phosphotyrosine is labeled with europium cryptate [Eu(K)]. The XL-665-streptavidin binds to the biotinylated substrate and after phosphorylation, the europium-labeled anti-phosphotyrosine antibody binds to the phosphotyrosine enabling energy transfer, and an increase in fluorescence at 665 nm is measured.

3.10. Luminescence

Luminescence, a long-lived light emission produced by any one of a variety of luciferases acting on their corresponding substrate luciferins, is commonly used as readout in cell-based receptor assays. The luciferase gene is cloned into the cellular DNA downstream of the promoter of interest, and is used as a reporter gene. Figure 13 illustrates such an assay for a generic nuclear receptor. After binding of the ligand to the receptor's ligand-binding domain (LBD), the receptor–ligand complex enters the nucleus where the receptor's DNA-binding domain (DBD) binds to the DNA response element. After complexing with transcription factors, the promoter is activated and transcription of the luciferase reporter occurs. The cells are then lysed, luciferin is added, and resulting luminescence provides a measure of receptor activity. A comparison of luminescence in the control (no fungal extract) with those containing sample indicates antagonism or agonism. Also, luminescence is commonly used in assays for cytotoxicity in which luciferase and excess luciferin are added to measure levels of ATP [32,61].

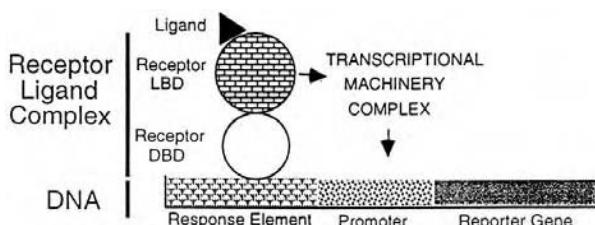


Figure 13 Nuclear receptor assay using luminescence as the reporter.

4. ASSAYS

4.1. Enzyme assays

Enzyme inhibition assays are probably the most common type of *in vitro* assay. They are rapid, require small amounts of material, and are easily adapted for high-throughput screening. The types of enzymes screened include proteases, peptidases, topoisomerases, kinases, oxidases, reductases, dehydrogenases, beta lactamases, RNA and DNA synthetases, and enzymes of bacterial and fungal cell-wall synthesis (e.g., transpeptidases, glucan synthetases). Enzymes have been successfully used as targets in a wide variety of therapeutic areas such as anti-microbials (i.e., anti-bacterials, anti-fungals and anti-virals), anti-cancer treatments, anti-inflammatories, anti-hypertensives, anti-obesity therapies, anti-parasitics, and cholesterol-lowering drugs. Because there are numerous assay types, this discussion focuses more narrowly on various assay formats and their applicability to screening of fungal extracts.

Nonhomogeneous formats are an older technology and rely on separation of unreacted tagged-reagents and macromolecular products via filtration, centrifugation, or binding to a surface. Protein kinases and polysaccharide synthetases (glucan, chitin) are examples of assays using filtration. In the kinase assays, γ -labeled ATP is separated from the phosphorylated protein product; in the glucan synthase or chitin synthetase assays, the radiolabeled glucose or N-acetylglucosamine is separated from the respective oligosaccharide products. Separation via centrifugation has been successfully employed in protease assays. In this case, the tagged unreacted protein substrate is either precipitated (by acid) or bound to ion exchange resin and then separated from the released radiolabeled amino acid or peptide by centrifugation. ELISA has been used for a wide variety of enzymes and is limited only by the availability of specific primary and secondary antibodies. In this technique, the immobilized primary antibody traps the reaction product, and detection occurs via binding of the secondary enzyme-linked antibody. In all nonhomogeneous assays, almost complete separation of reactants (including the screening sample) and products can be achieved by washing (filtration, ELISA) or centrifugation. This can be advantageous in screening fungal extracts because they contain fluorophores and chromophores, which may interfere with the signal detection. The extracts can quench a liquid scintillation assay and can quench or enhance (via autofluorescence) a fluorescence or absorbance assay signal. Assays employing ion exchange resins and ELISA assays are also subject to interference by compounds that inhibit binding of the ligand to the resin or antibody, respectively. If not employed in primary screening, nonhomogeneous assays are very useful for confirmation and should be used to verify all actives prior to beginning isolation.

Homogeneous assay formats are available for virtually all detection techniques and are commonly used in moderate- and high-throughput screening because they simplify the assay and decrease the time required. These assays, however, tend to be expensive due to the need to purchase specialized reagents and equipment, and they are more prone to interference because products are not separated from the reactants. Common types of homogeneous formats include measurement of increase in absorbance, increase/decrease in fluorescence, SPA, flash plate, FRET, HTRF, and luminescence. As mentioned earlier, the measurement of changes in absorbance and fluorescence is subject to interference by chromophores and fluorophores present in the fungal extract. Increases in absorbance can be masked by a high intrinsic absorbance of an extract, which can elevate the background sufficiently to decrease the sensitivity of the assay and lower the signal to noise ratio. False positives can result from a sample that fluoresces at the measuring wavelength. These difficulties have limited the use of absorbance assays with crude extracts. Increases in fluorescence can be masked by chromophores, which either absorb the excitation light or quench the emitted fluorescent light. Alternatively, a sample with autofluorescence would be a false positive. These difficulties in fluorescence assays can be overcome by use of time-resolved fluorescent assays employing lanthanides. These compounds emit a long-lived fluorescence that can be resolved from the background fluorescence produced by the extract. In these assays it is important to measure both the donor (lanthanide) and acceptor (XL-665) emissions and to use the ratio of these measurements to monitor changes in the donor emission due to changes in association between the donor and acceptor. SPA beads are a popular format for conducting radiolabeled assays in a homogeneous format. This technique relies on specific binding of the radioligand to the bead to allow detection by the scintillant embedded in the bead, and it is subject to interference by compounds that inhibit this binding, such as long-chain fatty acids, which are commonly found in fungal extracts. Once radioligand is bound to the bead, detection of radioactivity in SPA-based assays ultimately relies on light emission by the scintillant in the SPA bead and is therefore subject to the same types of interference as normal scintillation counting—i.e. color quench, chemical quench, and interference by fluorescence or luminescence. The scintillation counter must therefore be set to correct for luminescence, and quench curves must be prepared and used to correct the measured counts per minute (cpm) to disintegrations per minute (dpm). Luminescence assays using luciferase are also subject to interference by chromophores that may quench the emitted light, and an appropriate blank (containing the active extracts) must be used to confirm all actives. Interference from fluorescence is not significant because of the long life of the luminescent signal.

4.2. Receptor-Based Assays

Receptor–ligand binding is an important aspect of biology. There are two main types of receptors—cell-surface receptors and intracellular (nuclear) receptors [33,34]. The cell-surface receptors are divided into three families based on their mechanism of action: ligand-gated ion channel receptors, G-protein–coupled receptors, and enzyme-linked receptors [34]. Ligand-gated ion channel receptors open or close in response to ligand binding (acetylcholine, glutamate) and either allow or restrict passage of specific ions [35]. These receptors are commonly found on postsynaptic membranes. G-protein–coupled receptors undergo a conformational change upon ligand binding (epinephrine, glucagon). This in turn causes dissociation of the coupled G-protein, which induces downstream signaling [36]. Enzyme-linked receptors either have intrinsic enzyme activity (insulin receptor) or

can recruit proteins with catalytic function (tyrosine or serine-threonine kinases), which are activated by the conformational change elicited by ligand binding (insulin, interleukins, integrins) [37]. Nuclear receptors are transcription factors that reside in the cytoplasm or nucleus, and their ligands (testosterone, cortisol) consequently must pass through the membranes to reach these receptors. Ligand binding induces a conformational change that allows DNA binding and subsequent regulation of transcription [23,38].

Assays of ligand-receptor interactions are similar regardless of the receptor type. Binding to receptors can be measured in whole cells, cell membrane preparations, detergent-solubilized receptors, or with purified soluble receptor. In each case, the principle is the same: to measure the amount of specifically bound ligand. Irrespective of the assay format, the binding must be well-characterized prior to setting up a screen. Several textbooks provide a detailed discussion of receptor biochemistry [39–41], which is beyond the scope of this chapter. Briefly, conditions for saturable binding of ligand to receptor must first be determined, and the K_D calculated. The extent of nonspecific binding is measured by including an excess of unlabeled ligand to effectively compete for all the specific (reversible) binding sites in the preparation. The assay should then be designed to measure competition for binding of labeled ligand under equilibrium, at a concentration of labeled ligand below the K_D . Ideally, a ligand concentration of 10% to 20% of the K_D value should be used to obtain maximum sensitivity and a low ratio of nonspecific/specific binding.

Binding assays are most commonly used for primary screening, and whole-cell-based assays using reporter genes are generally used to confirm actives and demonstrate whole-cell functionality. Whole-cell functional assays have been used for primary screening. However, this is generally more time-consuming.

A variety of assay types are available for measuring ligand-receptor interactions. Traditionally, this is achieved by using radiolabeled ligand and removing any unbound ligand by filtration and washing [16,42]. Centrifugation binding assays are popular for measuring binding to receptors in membrane preparations. Here, the membranes bearing receptors and the bound radiolabeled ligand are sedimented, allowing unbound radioligand to be removed by aspirating the supernatant [15,43]. Charcoal adsorption and gel filtration are other methods that can be used to separate free radioligand from that bound to soluble or detergent-solubilized receptors [15,44]. Filtration, centrifugation assays, and gel filtration assays can be done in high-throughput format but are less desirable than homogeneous assays due to the radiolabel use and necessity for washing steps.

Homogeneous assays for ligand-receptor interactions include: SPA assay (PPAR receptor) in which receptor is immobilized on the bead and radioactive ligand interaction is detected (Fig. 7) [45]; fluorescence-based assays in which cells, membranes, or receptors are immobilized and bound fluorescent ligand is detected [46]; and HTRF in which receptor and ligand are in solution, each tagged with fluorophore (or fluorophore-linked antibody) and ligand binding is detected by long lived fluorescence at 655 nm [47]. Fluorescence polarization can be used for competitive assays to measure the capacity of a competitor compound to displace a high-affinity fluorescent ligand from either crude membrane preparations or purified receptors [48]. Because smaller molecules rotate faster than larger molecules in solution, fluorophores attached to smaller molecules yield a smaller polarization signal. The binding of fluorophore-labeled ligand to receptor results in an increase in the polarization signal compared to that of the labeled ligand alone. The polarization signal is linearly dependent on the extent of binding. In the presence of unlabeled competitor ligands, the polarization signal is reduced if the unlabeled ligand replaces the fluoro-

phore-labeled ligand at the receptor binding site. This technique requires a fluorescence polarization detector, which is available for microplate format. In addition to potential interference by fluorophores in the assay samples, polarization measurements are particularly sensitive to viscosity. Enzyme fragment complementation of β -galactosidase is a new technique for measuring displacement of ligands from solubilized receptors [49]. This technique uses β -galactosidase, genetically engineered into two inactive fragments that can recombine in solution to form active enzyme and cleave fluorescent or chemiluminescent substrate (see 3.5). To measure receptor binding, one enzyme fragment is conjugated to the ligand; the other is free in solution. Binding of the β -galactosidase-ligand-conjugate to receptor prevents recombination with the other fragment and thus results in reduced enzyme activity. Displacement of the conjugated ligand from the receptor due to competition from a component in the screening sample is detected by an increase in enzyme activity because the conjugated fragment is now free to recombine with the other fragment to form active β -galactosidase.

Receptor–ligand interactions can be assayed in whole cells genetically engineered to overexpress the receptor of interest and to contain reporter genes to provide convenient means of measurement. Whole-cell assays have been used for G-protein–coupled receptors and nuclear receptors. Such an assay for nuclear receptors using luciferase as a reporter was described earlier as an example of a luminescence assay (Fig. 13). β -galactosidase is another commonly used reporter gene that can be detected using the fluorescent substrates as described earlier (Fig. 6). In functional assays for the enzyme-linked receptors, the activity of the linked enzyme is measured directly following cell lysis. Because these are virtually all kinases, any of the assay formats described can be used. Ligand-gated ion channels are conveniently assayed using ionspecific fluorophores whose fluorescence intensity yield significantly increases (100-fold) on ion binding [50,51]. In a typical assay, cells are preloaded with the fluorophore and following stimulation, the influx of the requisite ion is monitored by measuring the increase in fluorescence.

5. SCREENING FOR ANTIMICROBIAL ACTIVITY

Natural products, including fungal metabolites, have yielded most of the clinically useful antimicrobial agents to date. Examples of useful antimicrobial drugs produced by fungi include penicillin, made by *Penicillium chrysogenum*, cephalosporin by *Acremonium*, and griseofulvin by *Penicillium griseofulvin*. In addition, active fungal metabolites that are unattractive as drugs themselves due to toxicity or low potency have been useful in identifying novel chemical classes, which have served as scaffolds for synthesis of successful antibiotics. Structural classes of natural product antimicrobial drugs have been reviewed by Silver and Bostian [52].

Identification of novel antibiotics can be achieved by screening fungal metabolites with various assay methodologies, including target-based screening, empiric growth assays, and the use of microbial genomics for discovery of novel antimicrobial targets. In target-based screening, enzymatic and receptor-binding assays are used to find inhibitors of specific biochemical targets. This approach requires extensive knowledge of the microbe and the difference in its biochemistry and physiology compared to mammalian cells in order to validate a given target. Current antimicrobial agents target members of macromolecular metabolism pathways, cell division, and nutrient transport. Tetracycline, streptomycin, chloramphenicol, and others inhibit key enzymes involved in bacterial protein synthesis [53]. Beta-lactams and glycopeptides are examples of antibiotics that target cell-wall

synthesis in bacteria [54], and echinocandin and nikkomycin target fungal cell-wall synthesis [55]. Bacterial fatty acid biosynthesis is inhibited by the hand soap additive triclosan, and RNA and DNA synthesis is inhibited by rifamycin and novobiocin, respectfully [52,53]. Therefore, once a target enzyme or pathway is validated, specific *in vitro* assays may be designed using purified enzymes and assay formats as discussed earlier in this chapter.

Cell-based assays may also be used to identify novel antimicrobials. Growth inhibition assays are advantageous in that they identify compounds that must be physically able to interact with their target protein in the cell (i.e., must be cell permeable if target is intracellular). Demonstration of cell-permeability at the initial hit identification stage eliminates the disappointment associated with the discovery of potent enzyme inhibitors that are later found to lack whole-cell activity because they cannot physically interact with their target in the cell. However, an enzymatic assay may identify a chemical class of potent inhibitors that can then be modified and made permeable, whereas a whole-cell assay would preclude finding such inhibitors. Another advantage of the cell-based growth screen is that it does not require knowledge of a particular cellular target. Such an empiric approach should allow for discovery of new antibiotics that act against novel targets.

Simple growth assays that measure the growth of bacteria or fungi in liquid culture are an inexpensive and straightforward means of screening for antimicrobial activity. Growth inhibition can be monitored in liquid culture by turbidity measurements (light absorbance at 600 nm) or by commercially available homogeneous chromogenic and fluorogenic assays. For example, metabolic activity can be measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] or Alamar Blue reduction. The MTT assay involves incubation of the bacterial culture with MTT, a soluble tetrazolium salt. The MTT is generally added after incubation of the culture with chemical compounds or fungal extract for several hours. In metabolically active viable cells, MTT is bioreduced by dehydrogenases into a colored formazan product. The quantity of formazan product produced is directly proportional to the number of living cells, and it is measured by light absorbance at 490 nm [56]. The MTT assay is highly reproducible and sensitive. Less is understood about the action of Alamar Blue (resazurin) although it has been widely used with success in high-throughput assays for antimicrobials [57,58]. A mitochondrial dehydrogenase is thought to reduce Alamar Blue to a pink product, which can be detected by absorbance or, for increased sensitivity, by fluorescence [59]. Other means of monitoring cell growth are commercially available. The Oxygen Biosensor is a fluorescence-based method that detects the amount of oxygen consumed over time, where oxygen consumption is proportional to the rate of cell respiration [60]. No chemicals are required for the oxygen detection because the system for measuring oxygen consumption is contained within the Oxygen Biosensor assay plate. In addition, bioluminescent detection of cellular ATP can be used to monitor cell toxicity [61]. Unlike the homogeneous assays described, ATP measurement requires the cells to be lysed at the end of the test period prior to the addition of a luciferase reporter to quantify cellular ATP. Since only viable cells at the time of lysis will contain ATP, and because the turnover of luciferin by firefly luciferase requires ATP, the luminescent intensity is directly proportional to the number of viable cells. These assays are all readily amenable to miniaturization to at least 384-well format. Because natural products contain chromophores and fluorophores, the same considerations mentioned earlier for enzyme assays relying on fluorescence and absorbance must be addressed. Bioluminescent assays are less prone to interference from the chromophores; fluorophores and turbidity commonly present in natural product extracts. For all liquid assays of growth

inhibition, confirmation of activity is generally achieved by demonstrating a dose-dependent inhibition of cell growth by the fungal extract. This confirmation of activity requires titration and retesting of the active extract and is used to determine the minimum inhibitory concentration.

Agar-based growth assays are also useful for screening for antimicrobial agents, but they require more sophisticated optical imaging systems and software for quantitation in high-density screening formats. To perform an agar diffusion assay, agar seeded with bacteria or fungi are poured into plates. Samples of interest are then spotted on the plates. Following incubation for sufficient time to establish a lawn of growth, zones of growth inhibition are measured as indication of antibiotic activity [62]. The size of the zone of inhibition is proportional to the concentration and potency of the drug. The clear zones of inhibition on an opaque lawn of growth can be measured manually. For higher density screening formats digital images of screening plates can be analyzed by software that recognizes pixel density differences and calculates the radii of the zones. Monitoring growth inhibition in agar is advantageous in that it allows a titration of drug to be achieved from a single spot due to the radial diffusion of the drug in agar. Thus, a dose titration is achieved at the same time as primary screening for active metabolites. Of course, different compounds in a complex mixture such as a fungal extract will have differing rates of diffusion. A zone of inhibition around a fungal extract may result from multiple weak antibiotics in the mixture or could be due to a single potent antibiotic present at very low concentration in the mixture. Either way, fractionation of extracts with confirmed activity will allow identification of the active component(s). Thus, an agar diffusion assay should facilitate discovery of antibiotics present as minor components in fungal extracts. Examples of antimicrobial agar diffusion screening assays are plentiful in the literature [63–67]. Caution must be taken when applying samples to agar. In particular, compatible solvents must be used to maintain the agar in a gel state and to maintain the metabolites in a soluble format to allow diffusion from the point of application. Samples may be spotted on top of agar plates in very small volumes, or a well may be formed in the agar to allow larger volumes of samples to be applied.

Whether monitoring growth in liquid or in agar, screening of fungal extracts is challenging because it is possible that a small concentration of a potent antibiotic could go undetected in an extract mixture because the presence of interfering compounds limits the concentration at which these natural product mixtures can be tested. Prefractionating the fungal extracts is one means of potentially enriching for the active metabolite. Fractionating the extracts may also help to identify and eliminate all the known antibiotic activities in the extracts, saving time and reagents by avoiding discovery of the same antibiotics repeatedly. Prefractionation of samples may not always be practical, however, because it drastically increases the number of samples to be screened. In an alternative approach, the cells can be rendered more sensitive to inhibitors. For example, gram-negative bacteria such as *Escherichia coli* are more resistant to antibiotics than gram positive bacteria due to limited entry of the drugs to the site of action. *E. coli* strains deficient in the envA gene product render the cells more permeable and therefore more sensitive to all antibiotics [68,69]. This will increase the chances of finding an antibiotic present at low concentration in an extract but would likely also increase the chances of finding nonspecific toxins. More specifically, microbes can be rendered more sensitive to inhibitors of particular pathways or enzymes, increasing the chances of finding a rare antibiotic. Microbial genomics provide the tools for engineering such particularly susceptible fungi and bacteria. In general, target specificity may be obtained by using genetically engineered strains, where

expression of particular genes are altered and sensitivity to fungal extracts is compared with the parent wildtype strain [70,71]. Here, the gene of interest is placed under the control of an inducible promoter to allow overexpression or underexpression of the protein. When putting a gene under the control of an inducible promoter, it is essential that the gene and promoter are chromosomally located for the construct to be inherited through the rapid divisions these cells undergo throughout the course of a growth assay. One could theoretically screen several targets in a single operon if it is behind an inducible promoter. This would allow screening for inhibitors of any stage of a given pathway, for example, rather than searching for inhibitors of one particular enzyme. This can be done both in liquid or agar but requires twice the screening (i.e., two-plate assays for comparisons of the overproducer or underproducer with the wildtype) [63]. With this strategy, it is possible to find leads that are active not only against whole cells but that are also specific for a biochemical target. Similar to the enzymatic and receptor-based screens, this approach requires extensive knowledge and validation of the biochemical targets.

Microbial genomics is useful to help guide target selection. With more than 60 complete genomic sequences of prokaryotes currently available, new strategies for discovering novel antibiotics are emerging [72]. Essential genes can be identified by high-throughput gene disruption analysis, in which genes are disrupted by various strategies and the ability of the strain to grow in the absence of the gene product is assessed [73–75]. Entire arrays of target-specific screening strains can be constructed in which each strain in the array has lowered expression of a single, essential gene product [76]. Inhibition of gene expression can be obtained through the use of antisense RNA. Elitra Pharmaceuticals has identified a large number of essential genes in *Staphylococcus aureus* by cloning DNA fragments coding for antisense RNA under control of a xylose-inducible promoter [77]. Expression of an antisense RNA complementary to the mRNA of an essential gene results in RNA–RNA duplexes that effectively prevent translation of that specific mRNA. This results in a reduction in the level of target protein and thereby confers specific sensitivity to compounds targeting that gene product. For example, an *S. aureus* clone expressing an antisense RNA to the gene encoding β-ketoacyl-acyl carrier protein synthase was found to have 12-fold increased sensitivity to the inhibitor cerulenin when the antisense RNA to the gene was induced with xylose, compared with the same clone grown in the absence of xylose. Such hypersensitivity to antibiotics specific to the gene product of interest should allow identification of antibiotics in complex mixtures. In Elitra's shotgun antisense approach, essential genes were identified after conditionally expressing random genomic fragments and screening the fragments for growth inhibition. Clones showing strong growth inhibition upon antisense induction were sequenced and the essential genes identified by BLAST analysis [77]. Conditional lethal mutants can also be used to identify essential genes as potential antibiotic targets. A conditional lethal mutation allows the gene product to function normally under one condition while being rendered inactive under another. Temperature-sensitive mutants are one example of conditional lethal mutants. Conditional mutants can also be generated by positioning a complementary copy of the essential gene of interest under the control of an inducible promoter, such as by positioning the gene at the ara locus of *E. coli*. This renders the expression of the gene and the growth of the *E. coli* dependent on arabinose. In the absence of arabinose, the gene product will be depleted and the organism will be more sensitive to inhibitors of that protein [75]. Parallel screening of multiple essential drug targets can be achieved using these methodologies. Pools or arrays of mutants can be screened together with each mutant distinguishable

by a molecular tag. The susceptible mutant can then be identified from the pool, indicating the target of the fungal metabolites [76].

With emerging technology for identifying novel targets, the chemical diversity of fungal metabolites can be exploited for discovery of novel antibiotics.

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Sordarins: Inhibitors of Fungal Elongation Factor-2

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1. INTRODUCTION

During the past two decades, the impact of systemic fungal infections associated with immunocompromised conditions has increased overwhelmingly. The AIDS pandemic as well as side effects of cancer chemotherapies have been responsible for the renewed emergence of these life-threatening pathologies. Consequently, many pharmaceutical companies initiated programs devoted to the discovery of new drugs that may help to improve their current antifungal arsenal. It is in this context that a family of natural products known as *sordarins* appeared in the antifungal arena in the mid-1990s. These molecules exert their antifungal effect by selectively impairing fungal, and not mammalian or bacterial, protein synthesis. This exquisite selectivity appeared as a remarkable and surprising property of sordarins given the high similarity among translational machineries in all living organisms. This became even more noticeable once it was shown that elongation factor-2 (EF-2) was the molecular target for sordarins.

This chapter provides a general perspective on the current knowledge about this family of compounds. After describing their chemical features, touching upon their chemical tractability including biotransformation procedures, we will summarize the more relevant features of their biological profile in order to envision their potential clinical applications. Finally, we will focus on the molecular mechanisms underlying sordarins' mode of action in the fungal cell with the aim of understanding the basis for their selectivity.

2. SORDARINS AS A CHEMICAL FAMILY

Sordarin, a tetracyclic diterpene glycoside that was originally isolated by workers at Sandoz in 1971 as a metabolite of *Sordaria araneosa* [1], is a key intermediate in the synthesis

of novel antifungal agents known as sordarins. Since sordarin's isolation, other producing microorganisms have been identified, such as *Zopfiella marina* (producer of Zofimarin by Sankyo) [2], *Penicillium mineoluteum* (producer of BE-31405 by Banyu) [3], *Rosellinia subiculata* (sordarins by Merck) [4], *Graphium putredinis* (producer of GR135402 by GlaxoSmithKline) [5] and the marine fungus *Hypoxyylon croceum* (producer of hypoxisordarin) [6]. Over the last few years, several pharmaceutical companies—such as GlaxoSmithKline, Merck, Banyu, and Schering-Plough—have reported the discovery of new members of this family as a result of their screening campaigns. All natural sordarin analogues contain a common aglycone moiety known as *sordaricin*, which bear as key groups in the putative pharmacophore a carboxylic function vicinal to a formyl group, not forming a cyclic hydroxylactone because of their rigid conformation in a high dihedral angle (Fig. 1). In fact, attempts to make a more flexible aglycone by cleaving ring bonds have led to compounds devoid of activity. The general lability of the aldehyde groups as a possible drawback for the development of sordarins as drugs has given rise to the search for more stable bioisosteres, especially to chemical or biological oxidation. Most chemical modifications of the aldehyde and carboxylic groups in these analogues abolished the activity of sordarins. Nevertheless, it has been possible to replace the aldehyde by nitrile group, the new analogues still having a remarkable antifungal activity [7]. The sugar moiety (i.e., *sordarose*) seems to play a role in enhancing the binding of sordarins to the active site. Although sordarin itself is considerably potent as a protein translation inhibitor in fungal cell – free systems, it shows only weak activity against whole yeast cells, and its aglycone, sordaricin shows no activity at all [7,8]. It is important to note that whereas sordarin derivatives containing nonsubstituted sordarose show a relatively modest antifungal activity, acylated sordarin derivatives such as Zofimarin and GR135402 exhibit a potent profile as inhibitors of the protein synthesis in yeast and fungi, both in cell-free

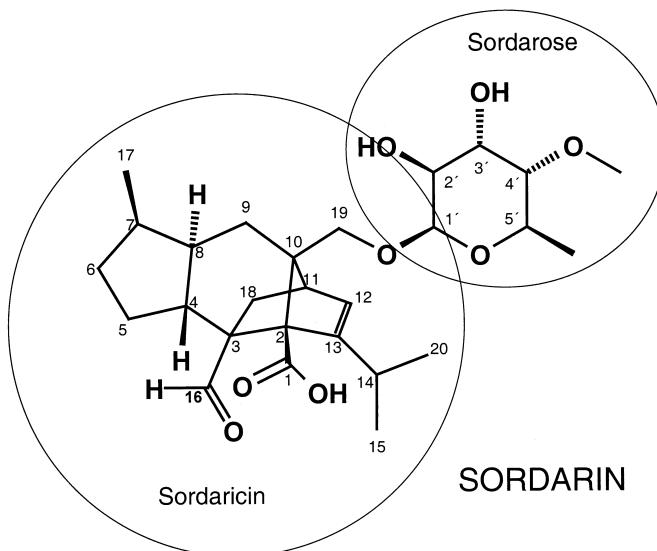


Figure 1 Chemical structure of sordarin. Sordaricin aglycone and sordarose sugar moieties are shown.

and whole-cell assays. An interesting development in the exploration of this natural product series was the discovery that when sordarinicin is functionalized with lipophilic side chains, the antifungal activity is markedly enhanced over that of sordarin and sordarinicin [7]. Hence, it seems that the lipophilicity of such an appendage is critical for the effective modulation of the cell uptake. Although most of the natural sordarins isolated so far only differ in the type of sordarose substitution, there are also sordarinicin derivatives bearing unusual sugar moieties that retain antifungal activity, such as BE-31405 [3], SCH57404 [9], and Xylarin [10]. Moreover, new semisynthetic sordarinicin derivatives with potent antifungal activity have been obtained in which sordarose has been replaced by alkyl-ethers (by Merck) [7], oximes (by Bristol-Myers Squibb) [11], or morpholines (azasordarins by GlaxoSmithKline) [12].

Although *de novo* total chemical synthesis of the methyl-ester of sordarinicin has been described [13], access to new sordarin derivatives has mostly been carried out by using natural product intermediates. Unfortunately, the biosynthesis of sordarins has not yet been studied in depth; therefore, it has not been possible to perform rational genetic engineering for new producing microorganisms. It is beyond the scope of the current chapter to present a comprehensive review of all the synthetic efforts made by the different groups involved in the chemistry of sordarins. In brief, we could distinguish the following approaches to new sordarin derivatives:

1. *Isolation of microbial metabolites.* Some of the compounds directly isolated are Sordarin, 4'-O-demethyl sordarin, GR135402, Zofimarin, SCH57404, xylarin, hypoxysordarin, neosordarin, and hydroxisordarin.
2. *Biotransformation of natural product intermediates with microbial organisms.* Some of the sordarin derivatizations accomplished by microbial transformations are hydroxylation of zofimarin aglycone at position 6 by *Streptomyces* [14], demethylation of sordarose in sordarin by *Streptomyces* [15], esterification of sordarinicin and sordarin by *Pithomyces*, oxidation of aldehyde to carboxylic acid by *Xylaria*, and reduction of aldehyde to alcohol by *Mortierella*. In general, the biotransformation products exhibited reduced antifungal activity compared with either sordarin, zofimarin, or GR135402. However, this approach has enabled access to other intermediates that expand the chemical tractability of sordarins.
3. *Chemical synthesis of new derivatives from natural product intermediates as starting materials.* This has been the most productive approach in terms of the number of new products generated, as well as in terms of the potency and pharmacological properties of the compounds made. Sordarinicin, sordarin, and 4'-O-demethyl sordarin have been extensively derivatized at the sordarose moiety (e.g., acylations, esterifications, substitutions of hydroxyls by nitrogen or carbon, etc.), including its replacement by nonsugar arrangements. Sordarin derivatives have also been made by using solid-phase chemistry in a combinatorial fashion (personal communication from Dr. S. Garcia-Ochoa, GSK). Semisynthetic derivatives starting from demethylsordarins, such as tetrahydrofurans and azasordarins, have undergone profuse preclinical investigations by GlaxoSmithKline [9,16,17].
4. *De novo chemical synthesis of sordarinicin derivatives* has been reported [13], and simplified aglycone analogues that resembled the pharmacophore of sordarinicin have also been pursued [18,19]. Unfortunately, the chemical synthesis of sordarin is too complex to make it exploitable in drug discovery programs, and

none of the simpler and more synthetically amenable structures has retained an interesting biological profile.

3. SORDARINS AS AN ANTIFUNGAL FAMILY

As mentioned, fungal infections caused by opportunistic microorganisms have emerged in recent decades as an important reason of morbidity and mortality in immunocompromised patients, such as those individuals affected by AIDS or receiving immunosuppressive therapy upon organ transplantation or oncological treatment. Present antifungal drugs on the market suffer from several drawbacks, such as resistance, toxicity, and limited spectrum of action. Thus, there is still an unmet need for new antifungal agents having a different mode of action from those currently used. For more than 15 years, polyenes, azoles, and allylamines have been the only three chemical classes of antifungals for the treatment of systemic fungal infections. They all interfere with the functionality on ergosterol in the fungal plasma membrane. The azoles and allylamines inhibit its biosynthesis, and polyenes physically interact with ergosterol and form pores that impair fungal membrane permeability.

Over the past 10 years, a considerable effort has been made in the pharmaceutical industry to come up with new chemical classes of antifungals through biased screening against novel molecular targets. As a result, Merck recently launched ® (caspofungin acetate). This is the first lipo-peptide antifungal in the market that belongs to the also known echinocandins or pneumocandins series of β -(1,3)-glucan synthetase inhibitors. On the other hand, GlaxoSmithKline has conducted advanced preclinical investigations on sordarins.

The most advanced of this company's sordarin derivatives can be grouped into two main chemical series represented by the four compounds included in Table 1. The first series includes compounds presenting different types of fused rings at positions C3' to C4' of the 4'-O-demethylsordarin [17]. GM193663 contains a dioxolane ring, whereas GM237354 contains a tetrahydrofuran ring. The second series includes compounds in which the sordarose sugar moiety in sordarin is replaced by a 6-methylmorpholin-2-yl group with different substitutions at N-4' position [9]. These derivatives, generically named *azasordarins*, have been more recently identified and have the additional advantage of an easier chemical synthesis. GW570009 and GW587270 may be considered as the best balanced and promising leads within this series in terms of antifungal potency, spectrum, and safety profile.

3.1. *In Vitro* Antifungal Profile

All the compounds showed low nanomolar potency to inhibit a cell-free translational system from *Candida albicans*, whereas no activity at all was exhibited at 100 μ g/mL against a similar counterpart system from rabbit reticulocyte [8]. Hence, the target selectivity profile against fungal EF-2 is retained. A summary of the antifungal profile of the four selected sordarin derivatives to inhibit the growth of key fungal microorganisms in culture is displayed in Table 1. The *in vitro* activity of sordarins has been extensively characterized by several workers, including clinical isolates of yeasts (*Candida* sp. and *Cryptococcus neoformans*), filamentous fungi (*Aspergillus* sp.), *Pneumocystis carinii*, dermatophytes (e.g., *Trichophyton*, *Epidermophyton*, *Microsporon*), dimorphic endemic fungal pathogens (e.g., *Histoplasma*, *Blastomyces*, *Coccidioides*), and other emerging, less common mold

Table 1 *In Vitro* Antifungal Activity of Sordarin Derivatives.

Microorganism	GM 193663 Chiral	GM 237354 Chiral	GW 570009 Chiral	GM 193663 Chiral
Yeast				
<i>Candida albicans</i>	0.001	0.008	0.015	0.004
<i>C. albicans</i> Flu ^r	0.001	0.001	0.03	0.008
<i>C. glabrata</i>	>32	1	0.25	0.25
<i>C. tropicalis</i>	0.25	0.06	0.06	0.03
<i>C. parapsilosis</i>	>32	8	2	0.5
<i>C. krusei</i>	>32	>32	>16	>16
<i>Cryptococcus neoformans</i>	4	0.12	>16	>16
Filamentous fungi				
<i>Aspergillus fumigatus</i>	>64	64	>16	>16
<i>A. flavus</i>	>64	4	>16	>16
Other fungal pathogens				
<i>Pneumocystis carinii</i>	<0.008	<0.008	NT	NT
<i>Histoplasma capsulatum</i>	<0.001	<0.001	NT	NT

MIC, minimum inhibitory concentration; NT, not tested; Flu^r, fluconazole-resistant strain

pathogens [9,16,17,20]. In terms of sensitivity to sordarins, we can distinguish the following groups of fungal microorganisms:

1. **Extremely sensitive (subnanomolar MIC):** Sordarins are extremely potent compounds against all strains of *C. albicans*, including fluconazole-resistant ones, with MIC values in the low- or subnanomolar range. Likewise, subnanomolar MICs have been reported for *P. carinii* and *Histoplasma capsulatum*.
2. **Highly sensitive (low-nanomolar MIC):** Sordarins are also active against *C. tropicalis* and *C. kefir*.
3. **Moderately sensitive (low- or submicromolar MIC):** Although less potent, they are also active against *C. glabrata* and *Cryptococcus neoformans*. Irregular responses are found for sordarin derivatives, and particular structure – activity relationships are found within members of the different chemical classes.
4. **Poorly sensitive or resistant (micromolar MIC or inactive):** Interestingly, *C. parapsilosis* and *C. krusei* are two *Candida* sp. reluctant to the action of sordarins. It has been shown that resistance resides in the nature of EF-2 rather than in the permeability or cellular stability of the compound [8,21]. Nevertheless, some compounds such as GM237354 have shown some activity against *C. parapsilosis*, which points to the possibility of more effective sordarins. Within this group of microorganisms we can also include *Aspergillus* sp. Some sordarins exhibit a marked slowness of *Aspergillus* growth, though the end-points were less sharp than those obtained with yeasts, and slight trailing growth is observed even in the highest concentrations.

As expected for protein synthesis inhibitors, sordarins are not intrinsically cidal drugs according to their primary effect. However, it has been shown that some sordarins produce a fungicidal effect (i.e., 3-log reduction in the number of viable cells) against *C. albicans* in the event of long exposure times (e.g., 48 hours) of the yeast cell to the compound.

Sordarin derivatives show negligible activity with respect to inhibiting the protein synthesis in intact mammalian cell lines cultured *in vitro*. Moreover, all the compounds tested showed no apparent evidence of genotoxicity in the Ames test, and they were not clastogenic in cultured human lymphocytes.

3.2. Therapeutic Efficacy in Animal Models

Sordarin derivatives have generally shown good activities in animal models for a wide range of fungal infections [16,17,22–25]. Given the high prevalence of *C. albicans* in fungal opportunistic infections, murine candidiasis models have been the most extensively studied. GM237354 has shown therapeutic efficacy against systemic or oral candidiasis after subcutaneous or oral administration. Likewise, sordarin derivatives (e.g., GM193663, GM211676, and GM237354) were effective in protecting immunosuppressed rats to develop *Pneumocystis carinii* pneumonia (PCP). Remarkably, sordarins showed superior activity over cotrimoxazole, which is the standard treatment for PCP. Sordarins have also turned out to be more effective than fluconazole, the gold standard antifungal agent, in the treatment of mice with disseminated histoplasmosis and coccidioidomycosis.

3.3. Pharmacokinetics and Pharmacodynamics

Pharmacokinetics of sordarin derivatives has been studied in mice, rats, dogs, and monkeys [16,17,25–28]. Short lifetimes and high clearance of sordarins in mice and rats have obligated to aggressive administration regimes in murine animal models. However, allometric relationships have been found among the species studied, which predict more favorable

pharmacokinetics as long as the body weight increases and the liver blood flow decreases. Hence, adequate administration regimes are foreseen for man (predicted $t_{1/2}$ for GM237354 in human is 8.9 hours) [28]. Although ADME has not been profusely investigated yet, preliminary studies point to phase II conjugation via glucuronidation as significantly responsible for the metabolic elimination of sordarins.

One of the most challenging problems in chemotherapy is how to ascertain the relationships between *in vitro* and *in vivo* activities that allow the prospective analysis of the therapeutic efficacy of compounds. GM237354 activity in a murine model of lethal candidiasis over a range of dosing intervals has been predicted by establishing a pharmacokinetics–pharmacodynamics (PK-PD) model in which both kidney burden and AUSTC (area under the survival time curve) correlate well with AUC (area under the curve), whereas neither C_{max} (maximum peak of compound concentration in serum) nor overall time over *in vitro* microbiological MIC were reliable PK predictors [26]. Moreover, by simulating different PK profiles in an *in vitro* dynamic system (i.e., bioreactor with growing *C. albicans*) it has been possible to correlate the efficacy of GM237354 only when the free, but not the total, *in vivo* levels in serum were reproduced *in vitro* [27].

3.4. Toxicology and Safety Pharmacology

Information on potential toxicological effects is still scarce. Sordarins are well-tolerated when orally administered [16,17]. Although high doses of GM237354 (i.e., 150 mg/kg) turned out to be lethal when intravenously infused in a rapid bolus due to cardiovascular effects, no significant effects were observed in arterial blood pressure heart rate, lead II ECG, and tracheal inflation pressure when the lower doses of compound are infused at a slower rate (i.e., 57 mg/kg in 2 hours). No significant subchronic toxicological effects were observed in clinical pathology and histopathology after 7 days of administration with GM193663 or GM237354.

4. MODE OF ACTION

The discovery of the precise mode of action of sordarins is an interesting story. The task was accomplished simultaneously by two different teams at Merck and GlaxoSmithKline, both using a double pronged approach with molecular biology and biochemistry tools. What follows is a summary of how these investigations evolved and how a nicely detailed picture describing the molecular mechanism by which sordarins exert their antifungal action could be devised.

4.1. Sordarins Are Real Protein Synthesis Inhibitors

Even though they were first described in 1971 [1], sordarins were rediscovered when screening for fungal protein synthesis inhibitors [5] using a radioactive cell-free protein synthesis assay as the one described by Tuite and Plesset [29]. However, the physiological significance of such an assay is rather questionable because of the presence of “artificial” components such as nonnatural mRNA (i.e., polyuridylic acid) or the use of high ionic strength to force initiation. Although there was good correlation among the arrest of cell growth and inhibition of the cell-free assay, those artificial components demand more evidence to support the notion that protein synthesis was the real target of sordarin derivatives within the fungal cell. This was achieved by testing the effect of a sordarin derivative in both protein and RNA synthesis *de novo* in *C. albicans* whole cells [8] and comparing

it to that of known inhibitors. While the effect of sordarins was almost identical to that of verrucarin (a known protein synthesis inhibitor), it was totally dissimilar to that of phenanthroline (a known RNA synthesis inhibitor). Moreover, this sordarin derivative caused a short-term stimulatory effect in RNA synthesis at low concentrations. Such effect, which disappeared at longer incubation times, had already been described for specific protein synthesis inhibitors added to cultures of amino acid – starved yeast cells [30].

4.2. Identification of the Molecular Target of Sordarins

Once protein synthesis was confirmed as the cellular process targeted by these drugs, the next step was to identify the biomolecules involved. Translation is a complex process, the machinery of which is well conserved among the eukaryotic kingdom. Indeed, elongation factor-3 (EF-3) is the only protein known to be different among fungal and mammalian translational systems. Given the exquisite selectivity of sordarins toward fungi, EF-3 was the most obvious candidate target. However, the results of further investigations were surprisingly different.

Protein synthesis comprises three sequential steps: initiation, elongation, and termination. The *in vitro* assay inhibited by sordarins includes only the elongation phase; therefore, it seemed plausible to deduce that the sordarin target might be one involved in that part of the translational process, which is depicted in Fig. 2. The biomolecules involved can be roughly divided into either ribosomal or soluble factors, depending on whether or not they constitute the ribosome particle and therefore precipitate or remain in the supernatant after ultracentrifugation. As a first approach to inquire into the target's nature, ribo-

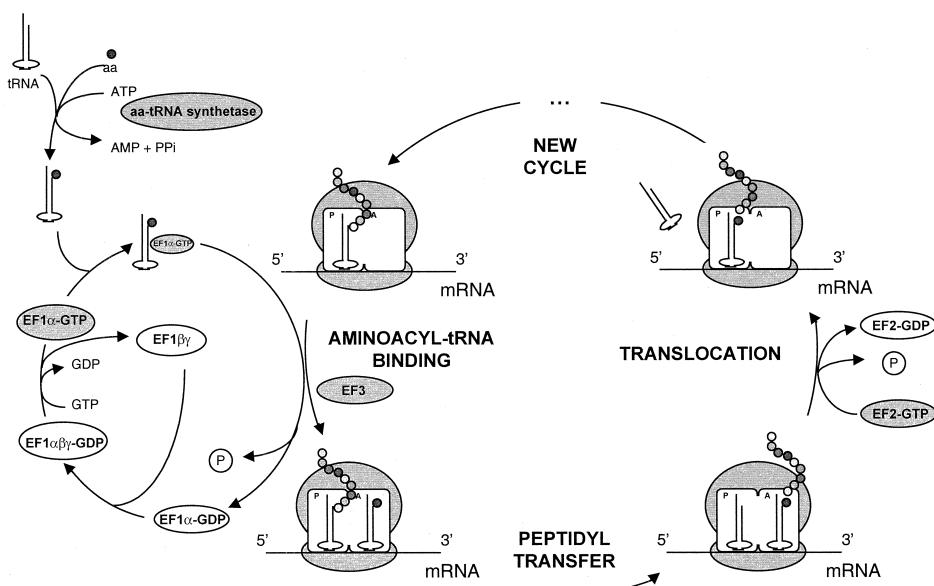


Figure 2 Overall scheme of elongation cycle in fungi. The ribosomal E-site, adjacent to the P-site but involving exclusively the large ribosomal subunit, is omitted in the interest of greater picture clarity.

somes of both sordarin-sensitive and sordarin-resistant *Candida* sp. were separated from their corresponding soluble factors. The ability to elongate poly-Phe was then recovered by mixing ribosomes and factors in homologous (components from the same species) and heterologous (components from different species) systems. The effect of sordarin on these systems was then examined under the rationale that their sensitive or resistant nature will be conferred by the target. Indeed, while the character of the homologous systems remained unchanged with respect to their sources, the sensitivity or resistance of the heterologous ones was linked to the source of the soluble factors [8]. After it was proved that resistance was not due to any sordarin-masking or sordarin-degrading activity, it was assumed that the target was one of the soluble factors involved in the elongation cycle.

Further experiments showed that aminoacyl-tRNA synthetases were not inhibited by these drugs [8]. The number of candidates was consequently restricted to the three fungal elongation factors: EF-1, EF-2, and EF-3. The availability of radiolabeled sordarin and the observation that ribosomes largely increase the binding affinity of sordarin to its target [31] suggested to afford the separation of the three *C. albicans* factors by using classical protein purification methods (as described by Uritani and Miyazaki [32]) and to track the sordarin target during the process by measuring the binding of radiolabeled drug. One single peak able to bind sordarin was identified after two chromatography steps. Analysis of this peak showed a rather pure protein as judged by SDS-PAGE (MW 98 kDa) that was not recognized by either anti-EF-1 α nor anti-EF-3 antibodies (deduced from Western blot analysis), but was susceptible to ADP-ribosylation by diphtheria toxin, which specifically modifies EF-2. Moreover, Edman analysis unequivocally rendered the sequence of *C. albicans* EF-2 as deduced by the high homology with the already known sequence of the same protein from *Saccharomyces cerevisiae*. Surprisingly, these extremely selective drugs were targeting a ubiquitous and well-conserved protein.

Almost simultaneously, two independent groups at Merck and GlaxoSmithKline afforded the identification of sordarin target through a molecular biology approach using *S. cerevisiae* as the working tool. [33,34]. Both teams isolated spontaneous sordarin-resistant mutants of the yeast and made a genetic analysis of them. The mutants fell within two complementation groups, the more numerous hitting *EFT2*, one of the two genes that encode for EF-2 in *S. cerevisiae*. Their results were thus in accordance with the biochemical evidences described earlier. However, analysis of the second less populated complementation group (which accounted for 4 of the 25 spontaneous mutants isolated at GlaxoSmithKline and for 5 of the 21 isolated at Merck) showed that the mutated gene in this group was *RPP0*, encoding the ribosomal protein rpP0 (formerly called L10e) located at the large ribosomal subunit stalk [35,36]. More interestingly, these works showed that sordarin-resistant mutants from this group still retained the ability to bind sordarin, although the drug was ineffective in impairing protein synthesis. That the ribosome was somehow involved in the mode of action of sordarins was expected because EF-2 functions by interacting with the ribosome. Additionally, as mentioned earlier, there were previous evidences of a significant increase in EF-2 affinity towards the drug when ribosomes were present [31]. “Crude” ribosomes from *C. albicans* [i.e., ribosomes isolated with elongation factors stuck to them] showed higher sordarin binding than the postribosomal supernatant (PRS), which contained the elongation factors but was devoid of ribosomes]. The same source of ribosomes lost the sordarin-binding ability after thorough washing with KCl, but such ability was completely recovered after further addition of PRS. However, this genetic approach was the first evidence of the direct involvement of ribosomes in the sordarin mode of action. Moreover, it was the first functional proof of a possible direct

interaction among rpP0 and EF-2, which was ultimately corroborated by direct visualization using cryoimages of the ribosome:EF-2:sordarin complex [36]. It was also noteworthy from these works that protein composition of the ribosome stalk affected sensitivity to sordarins. Indeed, deletions of either rpP1 α or rpP1 β (two acidic proteins that are part of a tetramer at the stalk base) decreased the level of resistance conferred by rpP0 mutations [37]. It must be emphasised at this point that the ribosome alone is unable to bind sordarin. However, EF-2 alone binds sordarin in a reversible mode ($K_d = 1.26 \mu\text{M}$ for *C. albicans* EF-2), and the affinity increases more than 1,000-fold in the presence of ribosomes. All of this evidence suggests EF-2 is the primary target for sordarins but that the EF-2:ribosome complex is the functional target.

4.3. Identification of the Sordarin-Binding Site and Implications on Sordarin Selectivity

EF-2 in eukaryotes, as EF-G in prokaryotes, promotes the translocation step within the elongation cycle in protein synthesis. Briefly, the step consists of the simultaneous movement of the newly formed peptidyl-tRNA and the recently deacylated tRNA from the ribosomal A site to the P site and from the P site to the E site respectively, while the ribosome moves ahead one codon on the mRNA being translated. Several events take place throughout this process in a precise and coordinated fashion to preserve translational accuracy. Though the structure of EF-2 has not been studied, detailed crystallographic studies have been performed with EF-G [38,39]. It is a tadpole-like molecule organized into five domains. The globular domain I is responsible for GTP binding and hydrolysis. It contains several structural motifs characteristic of the G-protein superfamily (indeed, it is referred to as *G-domain*) plus an extra insert called the G' subdomain. The role of G' subdomain remains unknown, although it has been suggested to act as a nucleotide-exchange factor. Interaction of the G-domain with the ribosome triggers GTP hydrolysis, and the energy released is transformed into mechanical movement. Eventually, domain IV (a fibrous domain at the other end of the molecule) moves away, stretching the EF-G shape and literally pushing the newly formed peptidyl-tRNA from the A to the P ribosomal site. It is still unclear how energy is transformed into movement, although it seems to be related to rearrangements within both EF-G and the ribosome that allow the complex to act as a molecular ratchet [40].

The eukaryotic system seems to be more sophisticated, as demonstrated by the larger number of proteins constituting the eukaryotic ribosome and by the ability to regulate EF-2 function by specific kinases and ADP-ribosylation. Nevertheless, the general features of the process are assumed to be the same. Accordingly, there is a substantial degree of homology between bacterial EF-G and eukaryotic EF-2. The more relevant differences are the greater length of EF-2 and the regulatory mechanisms described earlier. Likewise, it is remarkable that the G' subdomain has been replaced in EF-2 by another insert termed the G'' subdomain, which is 15 to 30 residues longer. Its position is displaced beyond in the sequence and shows no homology with the prokaryotic G' subdomain. On the other hand, EF-2 is a highly conserved protein within the entire eukaryotic kingdom. This fact makes especially striking the existence of EF-2 inhibitors such as sordarin, which exclusively impair fungal and not bacterial, mammalian, or plant protein synthesis machinery and which also discriminate between closely related fungal species.

Although the involvement of a diversity of ribosomal proteins may provide a clue for sordarins' selectivity, the role that EF-2 plays in such selectivity appears to be an

interesting mystery to explore. To this end, identification of the residues involved in sordarin binding to the protein might help. It is remarkable that nine of the 14 altered residues in *EFT2* from *S. cerevisiae* sordarin-resistant mutants mapped closely on a region thought to be domain III of EF-2 (by homology with EF-G). It was further proposed that a block of eight amino acids in this same domain may define a sordarin-specificity region [21]. Thus, declaring this region as the sordarin-binding pocket seems a very attractive hypothesis, but direct biochemical evidence is needed. As stated earlier, sordarin binding to EF-2 is reversible, thus the interaction is disrupted when using hazard conditions that affect protein conformation, such as those normally used when performing peptide mapping. Therefore, the use of radiolabeled sordarin for these purposes was ruled out. Instead, a radiolabeled photoactivatable aryl azide sordarin derivative was used to perform photoaffinity-labeling experiments on *C. albicans* EF-2. After the specificity of the labeling was proved and the conclusion made that the compound was covalently bound to a sordarin-binding site as a result of a photoactivatable process, it was possible to identify the labeled residue by running trypsin digestion of the labeled protein and subsequent purification of the labeled peptide by RP-HPLC followed by N-terminal sequencing [41]. The fragment Gln²²⁴-Lys²³² was identified as the modified peptide, Lys²²⁸ being the residue to which the photoprobe was linked. This residue is located in the G-domain of EF-2, more precisely at the beginning of the insert (the G" subdomain; see earlier discussion), far from the hypothetical pocket predicted from the resistant mutations. This can be explained in light of recent studies suggesting that domain III (where many of the mutations are) is closely related to the G-domain both topologically and functionally. It seems to influence the GTP binding center [42] and to participate in the transmission of conformational rearrangements from the G-domain to domain IV after GTP hydrolysis [43]. Therefore, these mutations in EF-2 may help to overcome the effect of the drug by affecting EF-2 function rather than sordarin binding to EF-2 alone, thus precluding the transition to a high-affinity sordarin-binding complex upon interaction with the ribosome. On the other hand, there are five more sordarin resistant mutations out of domain III—two of them located near the photolabeled peptide [33].

To gain more information from these photolabeling results, we should concentrate on and consider the topology of the G-domains. These GTP-binding domains are well-conserved structural cores composed of a six-stranded β -sheet surrounded by several α -helices. They are connected by five loops that are the most highly conserved elements within the domain and define the G-protein superfamily. These loops are designated G-1 to G-5 and are easily recognizable by their amino acid sequence. G-1 is also known as *Walker-A* and is thought to be involved in the accommodation of α - and β -phosphate from GTP. Analogously, G-3 is also known as *Walker-B* and is thought to interact with γ -phosphate. As already mentioned, prokaryotic EF-G contains an odd element within its G-domain that is called the G' subdomain. It is located before the G5 loop, between the fourth helix and the sixth strand of the domain. In EF-2, however, this insert is substituted by the one called G" subdomain, which is longer and placed after the G5-loop. Lys²²⁸, the residue to which the photoactivatable sordarin was linked, is located at the beginning of this G" subdomain, which is therefore involved in sordarin binding. Consequently, these differences among the G' and G" subdomains may provide a clue to explain the innocuousness of sordarin on bacterial protein synthesis. Moreover, alignment of the known G" subdomains (Fig. 3) show that (1) these subdomains are longer in higher animals than in the rest of eukaryotic species because of a 13-residue fragment at the beginning, and (2) two Walker motifs are recognizable in the G" subdomain of higher species, in

		Walker-A	Walker-B
FUN	<i>C.alb</i>	216 HGWAPFTVR QPAKTYSKKPGVD -----KEA-----W-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>S.cer</i>	216 HGWAPFTVR QPAKTYSKKPGVD -----KEA-----W-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>C.psi</i>	200 HGWAPFTVR QPAKTYSKKPGVD -----KEA-----W-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>C.tro</i>	202 HGWAPFTVR QPAKTYSKKPGVD -----KEA-----M-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>C.gla</i>	214 HGWAPFT LGRPKAKLYAKKGPGD -----EDKLMLGLWLGDGYD-----P-----A-----I-----M-----P-----K-----R-----E-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>D.dis</i>	214 HGWAPFT LGRPKAKLYAKKGPGD -----EDKLMLGLWLGDGYD-----P-----A-----I-----M-----P-----K-----R-----E-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>C.neo</i>	215 HGWAPFTLNQFAHGRYS KKKPGVD -----K-----A-----L-----P-----L-----W-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>C.lus</i>	200 HGWAPFTLNQFAHGRYS KKKPGVD -----K-----A-----L-----P-----L-----W-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>H.sap</i>	210 HGWAPFT LQAFDTRVYAKKFAAKGKGQD -----P-----A-----R-----A-----K-----V-----E-----M-----K-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
ANI	<i>R.nor</i>	219 HGWAPFT LQAFDTRVYAKKFAAKGKGQD -----P-----A-----R-----A-----K-----V-----E-----M-----K-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>C.gri</i>	219 HGWAPFT LQAFDTRVYAKKFAAKGKGQD -----P-----A-----R-----A-----K-----V-----E-----M-----K-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>G.gal</i>	219 HGWAPFT LQAFDTRVYAKKFAAKGKGQD -----P-----A-----R-----A-----K-----V-----E-----M-----K-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>D.mel</i>	222 HGWAPFT LQAFDTRVYAKKFAAKGKGQD -----P-----A-----R-----A-----K-----V-----E-----M-----K-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>C.ele</i>	230 HGWAPFT LQAFDTRVYAKKFAAKGKGQD -----P-----A-----R-----A-----K-----V-----E-----M-----K-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>L.maj</i>	230 HGWAPFT LQAFDTRVYAKKFAAKGKGQD -----P-----A-----R-----A-----K-----V-----E-----M-----K-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
PRO	<i>P.fal</i>	188 HGWAPFT EFTRVYAKKPGVE -----L-----S-----T-----R-----K-----Q-----S-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>C.par</i>	210 HGWAPTIEKFARLYAKKPGVE-----K-----S-----M-----Q-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>B.hom</i>	234 CQWGPFT KEARLYAKKPGD -----E-----T-----K-----R-----W-----K-----A-----K-----Q-----K-----P-----S-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>E.his</i>	214 HGWAPFT KEARLYAKKPGD -----R-----K-----R-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
PLA	<i>T.cru</i>	188 QAWAFSVTRFAKHYAKKPGVD-----E-----R-----K-----R-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>G.int</i>	188 HGWAPFT VTRFAKHYAKKPGVD -----L-----S-----T-----R-----K-----Q-----S-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
	<i>B.vul</i>	216 HGWAPTIEKFARLYAKKPGVE-----K-----S-----M-----Q-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
<i>C.kes</i>	<i>C.kes</i>	216 HGWAPFT VTRFAKHYAKKPGVD -----T-----K-----A-----M-----R-----E-----L-----W-----G-----Y-----P-----T-----N-----	D-----R-----P-----L-----E-----F-----P-----I-----L-----F-----G-----C-----G-----
		***	***

Figure 3 Sequence alignment of G" subdomains of EF-2 from different sources. The peptide identified by photoaffinity labeling with a sordarin derivative is highlighted in a black box. Regions identified as possible Walker A and B motifs are shaded. Asterisks denote conserved residues within all the sequences. FUN: fungi (*C.alb*, *Candida albicans*; *S.cer*, *Saccharomyces cerevisiae*; *C.psi*, *Candida parapsilosis*; *C.tro*, *Candida tropicalis*; *C.gla*, *Candida glabrata*; *D.dis*, *Dictyostelium discoideum*; *C.neo*, *Cryptococcus neoformans*; *C.lus*, *Clavispora lusitaniae*). ANI: animals (*H.sap*, *Homo sapiens* (human); *R.nor*, *Rattus norvegicus* (rat); *C.gri*, *Cricetulus griseus* (Chinese hamster); *G.gal*, *Gallus gallus* (chicken); *D.mel*, *Drosophila melanogaster* (fruit fly); *C.ele*, *Caenorhabditis elegans*). PRO: protists (*L.maj*, *Leishmania major*; *P.fal*, *Plasmodium falciparum*; *C.par*, *Cryptosporidium parvum*; *B.hom*, *Blastocystis hominis*; *E.his*, *Entamoeba histolytica*; *T.cru*, *Trypanosoma cruzi*; *G.int*, *Giardia intestinalis*). PLA: plants (*B.vul*, *Beta vulgaris*; *C.kes*, *Chlorella kessleri*). (From Ref. 41.)

addition to the ones already existing in the G-domain of EF-2 (i.e., outside the G" subdomain). Although the physiological significance of these observations is not known, they emphasize that despite the high degree of homology, significant differences in G" subdomains exist among species, and it does not seem absurd to suggest that these may be related to sordarin specificity.

As mentioned earlier, ribosomes increase the affinity of EF-2 towards sordarin by 1000-fold. Such an increase may result from conformational changes within EF-2 on interaction with the ribosome rather than from the creation of a new site, given that the latter seems unlikely in view of the notable affinity shown by EF-2 alone. The G" subdomain is thought to interact with the sarcin-ricin loop of the 26S rRNA in the ribosome stalk. It has been observed that sordarins increase the sensitivity of *S. cerevisiae* 26S rRNA to chemical modification, mainly affecting the sarcin–ricin loop that becomes more exposed [44]. Putting these data together, a combined binding site in the interface of the ribosome and EF-2, involving 26S rRNA, rpP0 and the G" subdomain seems plausible. This, together with the role played by rpP1 α and rpP2 β in modulating resistance to sordarin in rpP0 mutants, shows a diverse panel of biomolecules directly or indirectly involved in the sordarin mode of action, thus generating a complicated framework of interactions among them that ultimately may justify sordarin selectivity despite the fact that the individual elements involved are highly conserved.

4.4. Effect of Sordarin on EF-2 Function

On promoting ribosomal translocation, EF-2 (and the prokaryotic EF-G) catalyzes the hydrolysis of one GTP molecule. It was traditionally considered that EF-2 behaved as a typical G-protein and therefore GTP acted as a modulator of the affinity of the elongation

factor by the ribosome. According to these traditional models, the ribosome oscillates between two states that define two small-scale, but significantly different, conformational arrangements within the particle: pretranslocational (referred to here as PRE) and posttranslocational (referred to as POST). The EF-2:GTP complex would have high affinity for the PRE particle, whereas EF-2:GDP would have low affinity for the POST. Translocation would therefore precede GTP hydrolysis, and the resulting GDP molecule would trigger the release of EF-2 from the ribosome. However, in the late 1990s, a new theory was postulated that has been generally accepted: EF-2 acts like a mechanical protein that uses the energy released during GTP hydrolysis to promote the physical movement within the ribosome [45]. Consequently, GTP hydrolysis would precede translocation.

In any case, GTPase activity is a key element of EF-2 function. Therefore, testing the effect of sordarin on this hydrolytic activity of the factor appears indispensable. Justice et al. had already performed a related experiment in which they tested the effect of sordarin on the GDP/GTP turnover of the EF-2:ribosome complex by measuring exchange of unlabeled GTP with prebound [³H]-GTP to EF-2 plus ribosomes, the latter being in seven-fold excess over the factor [33]. Their result showed that sordarin behaved similarly to fusidic acid, preventing the dissociation of the EF-2:GDP complex from the ribosome. Dominguez et al. then performed the so-called uncoupled GTPase assay involving the measurement of GTP hydrolysis catalyzed by EF-2 and ribosomes in the absence of any other elements of the protein synthesis machinery [46]. When setting up the appropriate experimental conditions for this assay a 4:1 molar ratio of EF-2 to ribosomes was selected (i.e., completely opposite conditions to those used by the Merck group) since this yielded the optimum signal-to-background ratio. This apparently negligible detail would become quite significant. Fig. 4A shows the effect of sordarin in the uncoupled GTPase activity of this 4:1 EF-2:ribosomes mixture together with that of fusidic acid. While the latter rendered a typical dose-response inhibition curve, sordarin produced an odd inverted bell-shaped curve, showing some degree of inhibition at intermediate concentrations (around 0.1 μ M), which disappeared either at higher or at lower concentrations of the drug. As the effect of sordarin in whole-cell and in cell-free protein synthesis is a single dose-response curve (with higher inhibition at increasing concentrations of drug), this result can be considered as an artificial consequence of the nonphysiological conditions of this

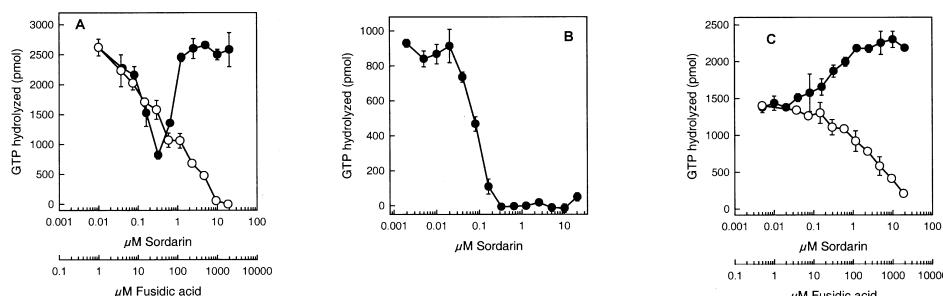


Figure 4 Effect of sordarin (black circles) and fusidic acid (white circles) on the uncoupled GTPase activity in mixtures of EF-2 and ribosomes from *C. albicans*. A: Excess of EF-2 over ribosomes. B: Equimolar mixtures. C: Equimolar mixtures, ribosomes previously treated with ricin. (From Ref. 46.)

in vitro assay, most notably the absence of any other components of the elongation cycle apart from EF-2 and ribosomes. Nevertheless, this result may still be indicative of some event that could give a clue to understanding the mode of action of the drug.

One of the classical models that fit the artificial uncoupled GTPase reaction to the physiological events that take place in the translocation process assumes that both ribosomal conformations (PRE and POST) are able to stimulate the intrinsic GTPase activity of EF-2 but with different efficiencies [47]. In addition, both conformations are able to interact with EF-2:GTP but with different affinities. Essentially, PRE shows high affinity for the EF-2:GTP complex but low efficiency in stimulating GTPase, whereas POST shows low affinity for the EF-2:GTP complex but high efficiency in stimulating GTP hydrolysis. This combination of opposite affinities and efficiencies could explain the unusual curve shown in Fig. 4A if we assume that the high-affinity/low-catalysis process (due to PRE) is inhibited by sordarin, while the low-affinity/high-catalysis process (due to POST) is stimulated by the drug. It should be noted that the ribosome preparation used in this assay is a heterogeneous mixture of PRE and POST ribosomes with predominance of the former. Consequently, in the absence of any inhibitor, the EF-2:GTP complex would interact mainly with PRE ribosomes because (1) they are present in excess over POST ribosomes and (2) they show higher affinity for the complex than POST ribosomes. The presence of sordarin would lead to the formation of a nonproductive PRE:EF-2:GTP:sordarin complex, thus explaining the descending half of the curve. On the other hand, the excess of EF-2 present in the assay medium may undergo interaction with the POST ribosomes. This interaction would be negligible in the absence of sordarin. It becomes significant, however, with increasing concentrations of the drug, overcoming the low affinity between POST and EF-2:GTP and giving rise to the second, ascending half of the curve. To sum up, increasing the amount of sordarin would inhibit the first process and stimulate the second.

This reasoning is based on the interpretation that the POST ribosomes are responsible for the recovery of GTPase activity at high sordarin concentrations, based on two assumptions: (1) that it can only happen when there is an excess of EF-2 in the system and (2) that sordarin stimulates the otherwise rare binding of EF-2:GTP to POST. The assay was repeated with equimolar mixtures of EF-2 and ribosomes (Fig. 4B) and sordarin clearly impaired the uncoupled GTPase reaction. It is worth noting that the IC₅₀ value deduced from Fig. 4B is 0.2 μM, which fits well with the corresponding value inferred from the inhibitory half of the curve in Fig. 4A (0.1 μM). On the other hand, testing the stimulatory effect of sordarin on the interaction between POST ribosomes and EF-2:GTP can be achieved by treating the ribosomal preparation with ricin. Ricin promotes selective depurination of one base in the larger rRNA molecule, destroying the dynamic properties of the ribosome and freezing it in a single conformation that has been identified with the POST state. As shown in Fig. 4C, sordarin stimulates the uncoupled GTPase activity of equimolar mixtures of EF-2 and ricin-treated ribosomes, whereas fusidic acid inhibits the reaction. Interestingly, the maximum stimulatory effect is reached at 1.0 μM sordarin, the same concentration at which maximum recovery of activity was observed (Fig. 4A).

All of these results correlate with the assumptions made. It can also be deduced that sordarin is not a direct inhibitor of the GTPase activity (Fig. 4C). Hence, the inhibition observed in Fig. 4A and B must be considered more as a consequence than as a primary effect. Since sordarin binds to EF-2:ribosome complexes with great affinity, it is unlikely that it may preclude the interaction between PRE ribosomes (the majority in such assay)

and EF-2:GTP. It follows that sordarin may directly impair the transition from PRE to POST, thus blocking the ribosome in the pretranslocational state.

To test this conclusion, the effect of sordarin on the so-called puromycin reaction was studied and compared to that of cycloheximide and fusidic acid. This is a classical tool for exploring the mode of action of protein synthesis inhibitors. It is based on the different accessibility of the ribosomal A-site to radiolabeled puromycin depending on whether the ribosome is in PRE (i.e., A-site inaccessible to puromycin) or in POST (i.e., A-site accessible). While fusidic acid did not show any effect on the puromycin reaction, a significant decrease in the incorporation of [³H]-puromycin was observed when poly-somes were preincubated with sordarin in a dose-dependent manner [46], a result similar to that obtained with cycloheximide. Consequently, the mode of action of sordarin seems to be closer to that of cycloheximide (described as an inhibitor of the translocation step) than to that of fusidic acid (which is known to inhibit the turnover of EF-2:GDP from the POST ribosome).

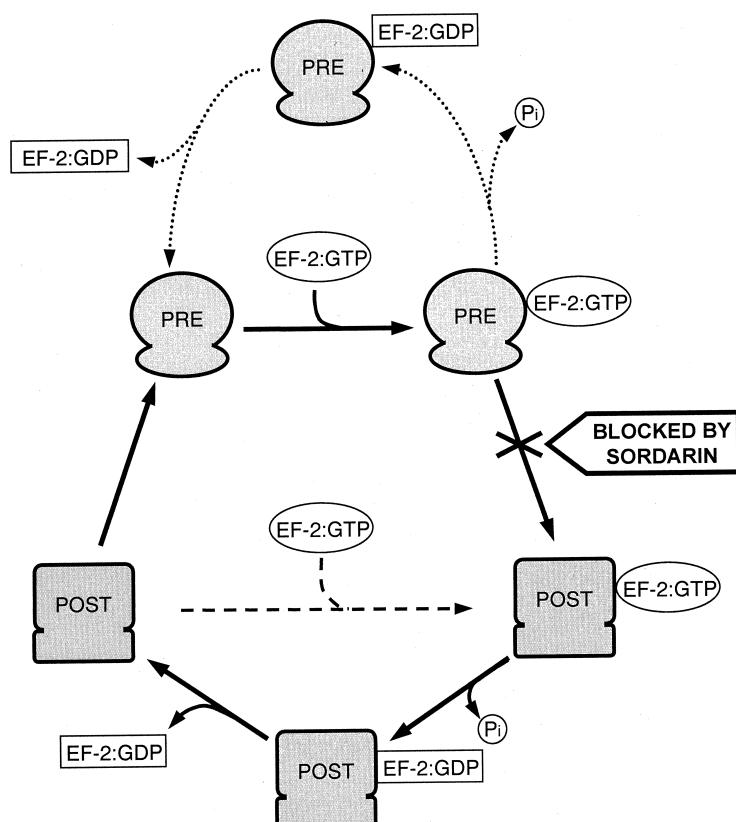


Figure 5 Proposed scheme for the uncoupled GTPase cycle and sordarin mode of action. *Dotted line*, rare process due to low catalytic efficiency. *Dashed line*, process negligible under physiological conditions due to low-affinity binding. The latter process can be stimulated *in vitro* either by a large excess of EF-2, by high sordarin concentration levels, or both. (From Ref. 46.)

The discrepancies in the results obtained by the two research groups might be justified according to their different experimental conditions: Justice et al. used a seven-fold excess of ribosomes over EF-2 that would lead to most EF-2 molecules' binding to the high-affinity PRE ribosomes, which would then be locked into a nonproductive PRE:EF-2:GTP complex by sordarin. This is depicted in Fig. 5, which shows a full cycle describing the molecular events taking place in the uncoupled GTPase assay according to the model for translocation [47]. The hypothetical role played by sordarin, which points to the transition from PRE:EF-2:GTP to POST:EF-2:GTP as the protein synthesis step inhibited by the drug, is indicated in this scheme. However, more detailed models to describe the translocation process are currently available. All are based on experimental data obtained with bacteria systems, although there are no apparent reasons not to apply them to eukaryotic systems. One of these is the "elaborated hybrid states" model [48], which incorporates the hypothesis that places GTP hydrolysis before translocation and considers GTP the source of energy that allows the mechanical movement of tRNAs and mRNA. The tRNAs move in alternate fashion with respect to ribosomal subunits, thus yielding the designated "hybrid states." The acceptor arms of the tRNAs are in the E or P sites of the large ribosomal subunit, while the anticodon arms remain in the P or A sites of the small subunit respectively. One of the main implications of this model is that the structural rearrangements that take place in the ribosome are subtle local changes rather than gross conformational changes. The model is composed of several intermediate states that are generated in the following sequence: (1) generation of a puromycin-unreactive hybrid state on the pretranslocated ribosome immediately after peptide bond formation; (2) binding of EF-2:GTP; (3) transition to a puromycin-reactive hybrid state; (4) GTP hydrolysis; (5) translocation of the anticodon arms; and (6) release of EF-2:GDP. This sequence of events is compatible with the proposed scheme in Fig. 5. Step 3 may be the one that shifts the PRE conformation of the ribosome into the POST. The subsequent events may exclusively affect tRNAs, mRNA, and EF-2:GTP. Accordingly, step 3 would be the one targeted by sordarin.

5. CONCLUSION

Sordarins are a promising family of novel antifungals. Their unique mode of action and their expanded chemical tractability are interesting properties that increase the clinical value deduced from their *in vitro* profile. Nevertheless, an expansion of their antifungal spectrum to include clinically relevant filamentous fungi like *Aspergillus fumigatus*, as well as some improvements in their pharmacokinetic properties to optimize their performance *in vivo*, would certainly be desirable. Hopefully, efforts in these directions will help to introduce these drugs for use in the clinic, providing physicians with a new and useful tool for the treatment of systemic fungal infections.

ADDED IN PROOF:

While this book was in press a new paper was published (Jorgensen, R.; Ortiz, P.A.; Carr-Schmid, A.; Nissen, P.; Kinzy, T.G.; Andersen, G.R. Two crystal structures demonstrate large conformational changes in the eukaryotic ribosomal translocase. *Nat. Struc. Biol.* 2003, 10, 379-385) showing crystals of yeast EF-2 with sordarin bound in a pocket between

domains III, IV and V. How these results match with the photoaffinity labeling data previously obtained, and whether or not it may imply significant rearrangements and movements within the protein molecule upon sordarin binding and ribosome interaction, will be an interesting matter of debate that will deserve deep study in the future.

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12

Secondary Metabolite Gene Clusters

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1. INTRODUCTION

Filamentous fungi display many unique characteristics that render them of great interest to the research community. Among these characteristics is the production of natural products, or secondary metabolites. These compounds often have obscure or unknown functions in the producing organism but have tremendous importance to humankind. Secondary metabolites display a broad range of useful antibiotic and immunosuppressant activities as well as less desirable phytotoxic and mycotoxic activities. The distribution of natural products is characteristically restricted to certain fungal taxa, particularly the Ascomycetes. Because of the great interest in these compounds, efforts have been expended in the last decade to clone and characterize the genes involved in their biosynthesis. Accumulating data from these studies support a model of fungal secondary metabolite gene clusters containing most if not all of the genes required for product biosynthesis.

A gene cluster can be defined as containing two or more closely linked genes participating in the same functional pathway. Although such a definition could include a description of several types of fungal gene clusters—including nutrient utilization clusters [1], pathogenicity islands [2], and mating-type clusters [3,4]—the focus of this chapter is on secondary metabolite gene clusters. Our goal is to summarize the existing descriptions of these clusters as well as to examine models that could explain the clusters' evolution.

2. NATURAL PRODUCT PATHWAYS

Based on biological activity, these compounds can be grouped according to their impact on humankind. In this section, we group these metabolites as either toxins (either mycotoxins exhibiting toxicity to animals or phytotoxins exhibiting toxicity to plants), pigments, growth hormones, or pharmaceuticals. It is important to keep in mind, however, that metabolites may exhibit more than one biological property.

A compilation of available data indicates that all of the gene clusters contain enzymatic genes and many clusters contain regulatory genes or genes associated with resistance to the metabolite (Table 1). In some cases, there are also several genes with no apparent role in production of the metabolite in question. In this chapter, we describe the basic organization of selected secondary metabolite gene clusters, some of which are examined in greater detail elsewhere in this book.

2.1. Mycotoxins and Phytotoxins

2.1.1. Ergot Alkaloids

Ergot alkaloids are widely known as fungal neurotropic mycotoxins and as important pharmaceuticals. They are produced by a wide range of filamentous fungi, primarily by members of the family Clavicipitaceae, including the ergot fungus *Claviceps purpurea*

Table 1 Physical Characteristics of Selected Fungal Secondary Gene Clusters^a

Cluster	Cluster Size (kb)	Number of Genes	Function of Genes ^b			
			Regulatory	Enzyme	Transport/Resistance	Unknown
Aflatoxin ^c	~75	24	2	17	1	4
AK-Toxin	?	6 ^d	2	4	0	0
Cephalosporin ^e	~17	4	0	2	1	1
	~3.5	2	0	2	0	0
Compactin ^c	~72	20	1	6	2	11
Ergot alkaloid ^c	~50	12	0	9	0	3
Fumonisin ^c	~75	~23	4	16	3	0
Gibberellins	17.2	7	0	7	0	0
HC toxin ^c	~600	>17 ^d	1	14	2	0
Lovastatin ^c	~64	~18	2	6	5	5
Melanin	~19	6	0	6	0	0
Paxilline ^c	~50	17	2	9	1	5
Penicillin ^c	~20	3	0	3	0	0
Sterigmatocystin ^c	~60	26	2	20	0	4
Trichothecene ^c	~29	12	2	7	1	2

^a References can be found in the text.

^b The function of genes was annotated based on their amino acid similarity with known proteins in the GenBank.

^c Cluster size (kb), number of genes/cluster, and function of each gene are not completely characterized.

^d Duplication of some of these genes in the cluster.

^e Genes are located on two clusters for cephalosporin production.

and the grass endophytes of the genera *Epichloe*, *Neotyphodium*, *Balansia* responsible for severe livestock intoxications [5,6]. Ergot alkaloids are also produced from the higher plants *Ipomoea*, *Rivea*, and related genera of the Convolvulaceae. The broad physiological effects of ergot alkaloids are mostly based on their interactions with neurotransmitter receptors on the cells [5,6].

The characteristic structural feature of most of the natural ergot alkaloids is the tetracyclic ergoline ring. The ring structure is derived from a hemiterpene unit, dimethylallyl diphosphate (DMAPP). The biosynthesis of ergot alkaloids begins with the condensation of L-tryptophan with DMAPP by the enzyme DMAT synthetase yielding 4-dimethylallyltryptophane (DMAT). Mixed function oxidases convert the DMAT to the corresponding hydroxy derivative. Several cyclase enzymes are involved in converting the intermediates into chanoclavine I, agroclavine, elymoclavine, and finally lysergic acid. The peptide alkaloid ergotamine is synthesized by the addition of amino acids (alanine, phenylalanine, and proline) to a lysergic acid precursor [5].

Tsai et al. [7] were the first to clone a gene of the ergot alkaloid pathway, *dmaW*, which encodes DMAT synthetase, the first enzyme of the pathway in *C. fusiformis*. The gene is induced under ergot alkaloid production conditions [8]. Using the *dmaW* gene of *C. fusiformis* as a probe, a putative DMAT synthetase gene (termed *cpd1*) was isolated from the strain P1 of *C. purpurea*, a strain capable of producing ergot alkaloids (mainly ergotamine) in axenic cultures. The *cpd1* gene served as a starting point leading to the detection of a putative ergot alkaloid gene cluster (Fig. 1) [9]. Another gene, *cpps1*, was localized downstream of *cpd1* and appears to encode a peptide synthetase required for the

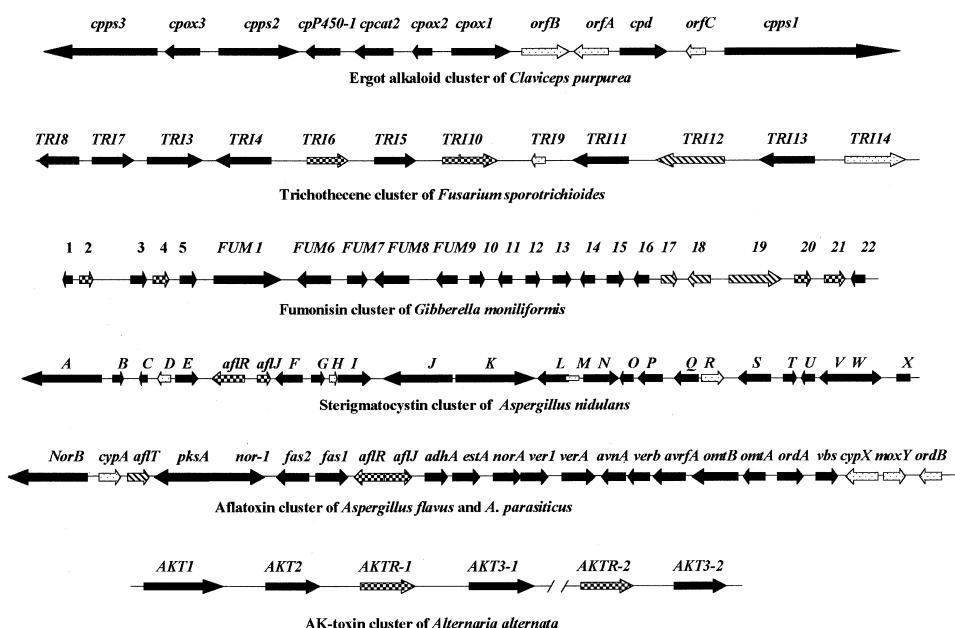


Figure 1 Secondary metabolite gene clusters. Size of genes is not representative. Genes were grouped according to putative function; however some of the genes have been disrupted with no apparent phenotype on metabolite production as discussed in text. (continues)

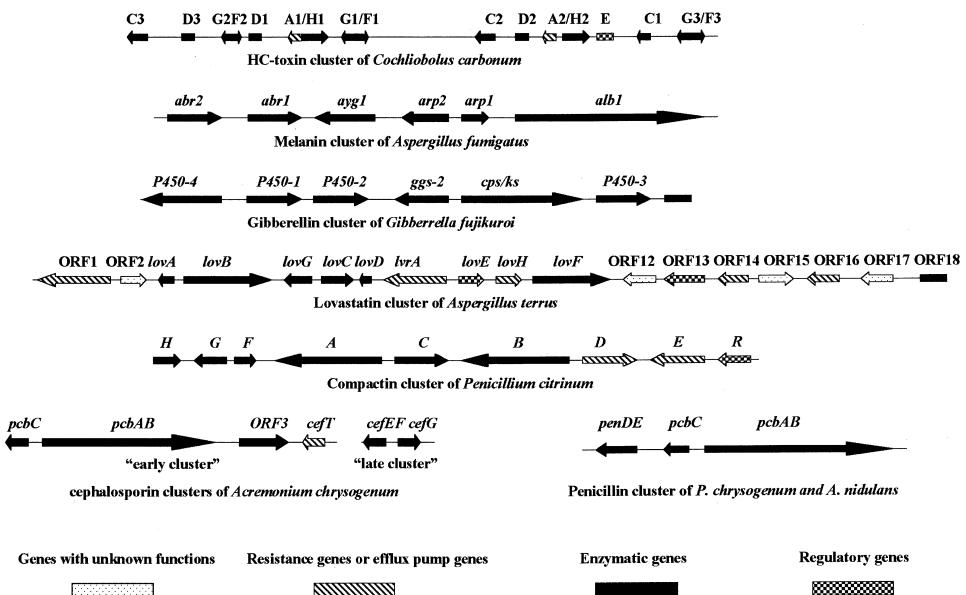


Figure 1 (continued)

penultimate step in alkaloid biosynthesis, that is the activation of the three amino acids of the peptide part of ergotamine linking them to the activated lysergic acid [6]. The *cpps1* encodes the previously characterized alkaloid biosynthetic enzyme LPS1 that was described by Riederer et al. [10]. Further sequencing of the upstream region of the *cpd1* gene in *C. purpurea* led to the identification of several genes that may be involved in alkaloid biosynthesis: (1) two putative monomodular peptide synthetase genes (*cpps2* and *cpps3*) that might encode the lysergic acid–activating enzyme; (2) one P-450-monooxygenase gene (*cp450-1*), which could catalyze the last steps of the lysergic acid biosynthesis and the last step of ergopeptine biosynthesis; and (3) several oxidases (*cpxo1*, *cpxo2*, *cpxo3*) that are good candidates for the early steps of biosynthesis. The presence of a housekeeping gene (isopropylmalate–dehydratase involved in amino acid biosynthesis) at the far left region of the available sequencing data may indicate the end of the cluster. Preliminary data show that all these genes of the cluster are induced in alkaloid-producing cultures (low phosphate) of strain P1 and repressed under high phosphate, conditions that do not favor the alkaloid production [6]. Progress has also been made in identifying ergot alkaloid genes in *Neotyphodium* spp. A peptide synthetase gene (*lpsA*) was cloned from *Neotyphodium lolii* and was insertionally mutated in *Neotyphodium* sp. Lp1. The *lpsA* loss-of-function endophyte did not produce any detectable quantities of the alkaloid ergovaline and retained full compatibility with its perennial ryegrass host plant [11].

Ergot alkaloid biosynthesis in axenic culture is strictly regulated in most strains. Tryptophan acts as both precursor and inducer, whereas phosphate, glucose, and ammonium repress synthesis. The presence of putative CreA (global regulator of carbon catabolite repression) and AreA (global regulator of nitrogen derepression) binding sites in the promoters of some of the alkaloid clustered genes may be indicative of alkaloid biosyn-

thesis regulation by C and N sources [12]. The involvement of these global regulators in gene cluster regulation is a recurring theme addressed in section 3 of this chapter.

2.1.2. Indole-Diterpene Alkaloids

Paxilline is representative of a type of alkaloid secondary metabolite sharing a common core structure composed of an indole and a diterpene skeleton [13]. Many of these metabolites exhibit mammalian tremorgenic or insecticidal activities [14–16]. The tremorgenic paxilline is synthesized by *Penicillium paxilli* [17] and is proposed to be an intermediate in the biosynthetic pathways of other indole-diterpenes [18]. The *pax* gene cluster has been identified in *P. paxilli*. The cluster is located within a 50-kb region and contains 17 genes. Twelve genes have significant similarity to genes of known function, four to genes of unknown function, and one gene has no significant similarity to genes in the databases (Fig. 1) [13]. The 12 genes are predicted to encode a geranylgeranyl pyrophosphate (GGPP) synthase (*paxG*), a prenyltransferase (*paxC*), a dehydrogenase (*paxH*), a metabolite transporter (*paxT*), an oxidoreductase (*paxO*), two FAD-dependent monooxygenases (*paxM* and *paxN*), two cytochrome P450 monooxygenases (*paxP* and *paxQ*), a dimethylallyltryptophan (DMAT) synthase (*paxD*), and two possible transcription factors (*paxR* and *paxS*), which contain a Cys6 DNA-binding motif [13]. Gene deletion analysis has confirmed the requirement of *paxG* for paxilline biosynthesis.

Although the involvement of the other 16 genes in paxilline production remains to be verified, gene expression studies have shown that *paxU*, *paxV*, *paxY*, *paxM*, *paxW*, and *paxP* are transcribed during the onset of paxilline production [13]. Furthermore, the expression profiles of *paxM*, *paxW*, and *paxP* correlate well with the initiation of paxilline biosynthesis [13].

Most recently a putative lolitrem B gene cluster has been identified in *Neotyphodium lolii* [19]. Lolitrem B is an analogue of paxilline and the cognate gene cluster appears to contain putative Pax orthologs which are expressed *in planta*.

2.1.3. Trichothecenes

The trichothecenes comprise a large family of sesquiterpenoid metabolites produced by a number of fungal genera, including *Fusarium*, *Myrothecium*, *Stachybotrys*, *Cephalosporium*, *Trichoderma*, and *Trichothecium* [20–22]. These compounds not only exhibit toxicity to vertebrates and plants but also are associated with virulence in specific plant-pathogen interactions [23–25]. The structurally diverse trichothecenes are classified as macrocyclic or nonmacrocyclic, depending on the presence of a macrocycle formed by esterification between C4 and C15 hydroxyl groups. Diacetoxyscirpenol (DAS), deoxynivalenol (DON), and T-2 toxin are the best studied nonmacrocyclic trichothecenes produced by *Fusarium* spp. Biochemical and genetic analyses of the T-2 toxin producer *F. sporotrichioides* led to the identification of the first trichothecene biosynthesis gene cluster. The gene cluster for DON production has also been identified in *F. graminearum*. The two clusters contain 10 to 12 ORFs and span about 29 kb (Fig. 1) [26–28]. The functions of 10 genes have been determined. Seven of them encode biosynthetic enzymes, including Tri3 (a 15-O-acetyltransferase), Tri5 (a trichodiene synthase), Tri8 (a c-3 esterase), Tri7 (required for acetylation of the oxygen on C-4 in T-2 toxin), as well as three cytochrome p450 monooxygenases, including Tri4, Tri11, and Tri 13 [1,27–30]. Tri6 and Tri10 are regulatory proteins and Tri12 is the efflux pump that is implicated to play a self-protection role [31,32]. The organization and transcription orientation of the genes in the two clusters are identical; however, Tri7 in *F. graminearum* is nonfunctional, consistent with the structural difference between T-2 toxin and DON [27]. Interestingly, one gene required for trichothe-

cene production, *Tri101* encoding a 3-*O*-acetyltransferase, resides outside of the cluster in both *F. graminearum* and *F. sporotrichioides* [33]. Most recently, a second mini-cluster has been described in *F. sporotrichioides* that contains two additional genes required for T-2 toxin formation [34].

Macrocyclic trichothecenes (e.g., roridin E, verrucarin A, and baccharinoid B7), which have similar toxic affects on vertebrates, are associated mostly with *Myrothecium* spp. Elucidation of the macrocyclic trichothecene biosynthetic pathway is less complete compared with that of the nonmacrocyclic trichothecenes. Genetic studies of *M. roridum* have identified three genes (e.g., *MRTRI4*, *MRTRI5*, and *MRTRI6*) involved in the biosynthesis of macrocyclic trichothecene [35]. *MRTRI5* encodes the trichodiene synthase and *MRTRI6* encodes a pathway specific transcription factor. The predicted *MRTRI4* product is a cytochrome P450 monooxygenase. Mapping data show that these genes are clustered within a 40-kb region, but their organization and orientation differ significantly from those of the cluster in *F. sporotrichioides* [35]. These data suggest that significant rearrangements have occurred during the evolution of gene clusters for the biosynthesis of these metabolites.

2.1.4. Fumonisins

Fumonisins are a group of polyketide mycotoxins that are produced by the maize pathogen *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) and several other *Fusarium* spp. These toxins can cause fatal animal diseases, including kidney and liver cancer in laboratory rodents [36,37]. Fumonisins resemble the sphingolipid intermediates sphinganine and sphingosine in structure, and they disrupt sphingolipid metabolism via inhibition of the enzyme ceramide synthase (sphinganine N-acyltransferase) [38]. *Fusarium verticillioides* is an economically important plant pathogen of maize and sorghum [39] and often contaminates maize kernels with fumonisins. B-series fumonisins (FB_1 , FB_2 , FB_3 , and FB_4), which are generally the most abundant fumonisins in naturally contaminated corn [39], consist of a linear 20-carbon backbone with an amine, one to three hydroxyl, two methyl, and two tricarboxylic acid moieties substituted at various carbon positions. Radio-labeling experiments suggest that the backbone is produced by a polyketide synthase [40]. The order in which the functional groups are attached to the polyketide backbone is obscure [41].

The genes involved in fumonisin biosynthesis are clustered (Fig. 1). Initially, Desjardins et al. [42] identified the tight linkage of three genetically defined *G. moniliformis* loci (Fum1, Fum2, and Fum3) required for fumonisin biosynthesis. Subsequent studies in the same fungus led to the discovery of a 75-kb region of DNA that consists of 23 genes thought to include the Fum1–3 loci [41]. The predicted functions of most of these proteins were consistent with enzyme activities expected to be required for fumonisin biosynthesis or self-protection [41,43]. Expression analysis indicated that 15 of these genes (*ORF1* and *ORF6–19*) are coregulated and exhibited patterns of expression that were correlated with fumonisin production. These ORFs are designated as *FUM* genes (*FUM1* and *FUM6–19*) and consist of an approximately 45-kb cluster of genes that is part of the initially characterized circa 75-kb region [41]. *FUM5* encodes the polyketide synthase gene that was shown to be required for fumonisin biosynthesis [44]. Disruption of *FUM6* and *FUM8* blocked production but did not lead to accumulation of detectable intermediates [43]. Complementation analysis results revealed that the Fum1 locus is equivalent to the *FUM1* gene [41]. Most recently, disruption of *FUM9*, which is predicted to encode a dioxygenase, produced a phenotype equal to the Fum3 locus, and sequence of *FUM9* in the Fum3 mutant showed a mutation in the coding region [45].

Considering the nonspecific toxicity of fumonisin to other organisms, researchers have speculated that *Fusarium* might contain a self-protection mechanism. Therefore, it was particularly interesting when two of the cluster genes, *FUM17* and *FUM18*, showed similarity to the tomato longevity assurance (LA) factor gene, *Asc-1*, which confers resistance to fumonisin B₁ and the structurally similar AAL toxins [41,46]. The FUM19 protein is similar to ABC transporters that act as efflux pumps transporting compounds from inside cells to the surrounding environment. However, disruption of *FUM17–19* did not lead to any obvious phenotype in *F. verticillioides*, and it is not known whether the fungus requires a self-protection mechanism against fumonisins [41].

Studies regarding the regulation of the fumonisin biosynthetic pathway are limited to evidence indicating that fumonisins are synthesized under nitrogen stress and acidic pH conditions [47,48]. None of the 15 genes within the cluster appears to be a regulatory gene. However, two ORFs upstream of *FUM1* (the left far end gene in the FUM cluster) appear to encode regulatory proteins: (1) a predicted WDR1 protein (*FUM2*) similar to several regulatory proteins with tryptophan-aspartic acid repeats and (2) a ZNF1 protein (*FUM4*) including regions similar to the cysteine-rich zinc finger domains of some transcription factors and kinases. Characterization of these genes has not been reported. Furthermore, another gene (*FCC1*) in *F. verticillioides* that does not seem to be clustered with the *FUM* genes plays a role in a putative signal transduction pathway that regulates fumonisin biosynthesis. FCC1 is closely related to UME3, the cyclin C of *S. cerevisiae* (cyclins are essential activating subunits of cyclin-dependent kinases [CDKs]) and regulates the expression of genes involved in conidiation and FB₁ biosynthesis when grown on cracked corn [48].

2.1.5. Aflatoxins and Sterigmatocystin

Aflatoxins and sterigmatocystin are polyketides derived from the same biosynthetic pathway. They are produced by several fungal genera, primarily by *Aspergillus* spp. Aflatoxins are the end-products in two agronomically significant fungi, *A. parasiticus* and *A. flavus*. Sterigmatocystin, the penultimate precursor to aflatoxin B₁, is the final product in the genetic model organism *A. nidulans* [49] and the building mold *A. versicolor* [50]. Both compounds are potent carcinogens and also exhibit mutagenic, teratogenic, and immunosuppressive properties. The aflatoxin cluster in *A. parasiticus* and *A. flavus* contains 25 genes that constitute a cluster spanning more than 70 kb (Fig. 2). Among the genes, 21 genes have been verified or appear to encode biosynthetic enzymes, including fatty acid synthases, a polyketide synthase, mono-oxygenases, reductases, dehydrogenases, methyltransferases, an esterase, a desaturase, and an oxidase [51,52]. One of the genes in the cluster, *aflR*, encodes a binuclear zinc cluster transcription factor regulating transcription of the aflatoxin biosynthetic genes [53]. Another cluster gene, *aflJ*, also seems to have a role in regulating aflatoxin production in *A. flavus* [54] by binding with AflR protein as a coactivator for biosynthetic gene expression [55]. In *A. nidulans*, the 60-kb sterigmatocystin cluster consists of 26 genes also regulated by *aflR* [56,57] (Fig.1). The function of most of the sterigmatocystin cluster genes has been determined: they are orthologs of aflatoxin cluster genes [58]. The roles of some genes remain elusive, however. For instance, disruption of *stcT*, *stcC*, *stcQ*, *stcI*, and *stcV* showed no effect on ST production (N. Keller et al., unpublished data). Deletion of *stcN* abolished ST biosynthesis but its function has not been assigned. Although most of the genes in the aflatoxin and sterigmatocystin clusters have the same functions, their order and transcription orientation are not well conserved between the two clusters (Fig. 2) [1]. In East Asian countries, *A. oryzae*, a nontoxic clade

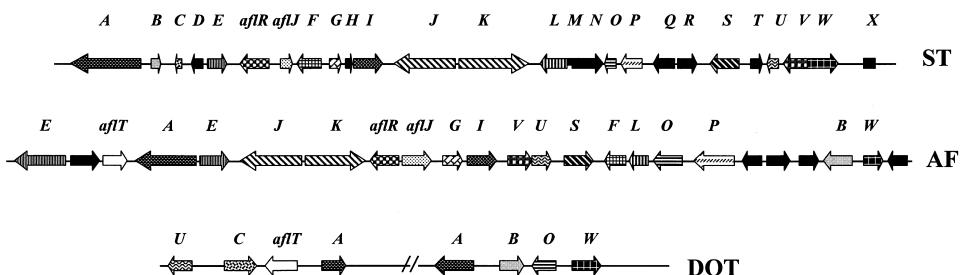


Figure 2 Order and direction of transcription of homologous genes in the sterigmatocystin (ST), aflatoxin (AF), and dothistromin (DOT) gene clusters. Homologous genes in the three clusters are indicated by the same bar pattern and the same letter that corresponds to the ST genes. Solid black bars represent ST, AF, DOT genes with no known homology among them. Size of genes is not representative.

of *A. flavus* [59], is traditionally used for fermented food and beverage production and does not produce aflatoxin or sterigmatocystin. Interestingly, studies have shown the presence of the entire or partial aflatoxin biosynthetic cluster in all of the *A. oryzae* strains tested [60–64]. The order of the genes in the cluster is identical to that of the cluster in *A. parasiticus*. Lack of *aflR* transcript is implicated as the reason for loss of toxin production in those strains containing an intact AF biosynthetic cluster [60].

Regulation of aflatoxin and sterigmatocystin biosynthesis has probably received more attention than any other mycotoxin. Several recent reviews address this topic [58,65,66] and show that regulation is complex involving pH, nitrogen, carbon, and signal transduction regulatory circuits. This is addressed in more detail in section 3 of this chapter.

2.1.6. Dothistromin

The difuranoanthraquinone polyketide dothistromin is produced by several plant pathogens, including *Dothistroma pini* and *Cercospora arachidicola* [67,68]. Studies have shown that dothistromin has a broad-spectrum toxicity to plant, animal, and microbial cells [69,70], and the compound is considered a virulence factor in needle blight of pines caused by *Dothistroma pini* [69]. ¹³C-NMR analysis has shown that dothistromin and aflatoxin share the same biosynthetic steps, in agreement with the substantial structural similarity between dothistromin and versicolorin B, a precursor of aflatoxin [71]. One gene, *dotA*, has been identified to be required for dothistromin biosynthesis. The accumulation of verisicolorin A in the *dotA* mutants and significant sequence similarity between DotA and *A. parasiticus* Ver-1 (= *A. nidulans* StcU) required for aflatoxin and sterigmatocystin biosynthesis indicates a ketoreducatase function for DotA [72]. Analysis of the genomic region beside *dotA* has identified three ORFs—*dotB*, *dotC*, and *dotD*—homologous to an oxidase, a toxin pump, and a thioesterase domain of a polyketide synthase associated with aflatoxin and sterigmatocystin production, respectively (Fig. 2) [72]. Unpublished data indicate the presence of additional biosynthetic genes exhibiting similarity to aflatoxin and sterigmatocystin cluster genes (Fig. 2) (R. Bradshaw, personal communication, 2003). Therefore, it appears that the genes for dothistromin biosynthesis also constitute a cluster. The identification of this cluster suggests that variations of the aflatoxin/sterigmatocystin

cluster exist in a wide distribution of genera and may provide clues toward the evolution of a gene cluster.

2.1.7. Alternaria Host-Specific Toxins

A number of plant pathogenic fungi produce a class of low-molecular-weight metabolites termed *host-specific toxins*. They are so-called because these toxins are the crucial determinants for the outcomes of specific host–pathogen interactions. Pathotypes of the fungus *Alternaria alternata* produce several structurally diverse host-specific toxins. Among them are AF-toxin produced by the strawberry pathotype, AK-toxin produced by the Japanese pear pathotype, and ACT-toxin produced by the tangerine pathotype. All contain a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structural moiety [73–77]. A mutagenesis study has led to identification of the first two genes, *AKT1* and *AKT2* essential for AK-toxin biosynthesis in the Japanese pear pathotype [78]. The product of *AKT1* is predicted to be a member of the carboxyl-activating enzyme superfamily. The predicted *AKT2* product has no homology to any proteins in the database. Both of the two genes have multiple copies not functional for AK-toxin production. Downstream of *AKT2* are two ORFs designated *AKTR-1* and *AKT3-1*. Attempts to disrupt these two ORFs revealed two other genes, *AKTR-2* and *AKT3-2*, which were shown to be essential for AK-toxin biosynthesis. Sequence comparison indicated that *AKTR-1* and *AKT3-1* share high similarity to *AKTR-2* and *AKT3-2*, respectively. *AKTR-1* and *AKTR-2* are predicted to encode a protein containing a zinc binuclear cluster DNA-binding domain, typical of a fungal transcription factor. The predicted products of *AKT3-1* and *AKT3-2* have similarity to members of the hydratase/isomerase enzyme superfamily. Both *AKT3-1* and *AKTR-1* are transcribed; however, their roles in AK-toxin production remain to be determined since neither has been successfully disrupted. Mapping analyses showed that *AKT1*, *AKT2*, *AKT3*, and *AKTR* and their paralogs are on a single chromosome [79] (Fig. 1).

The *AKT* paralogs have also been detected in the strawberry and tangerine pathotypes, in keeping with the fact that AK-toxin, ACT-toxin, and AF-toxin share a common core moiety. In one strain of the strawberry pathotype, three *AKT* homologs (*AFT1-1*, *AFR-1*, and *AFT3-1*) are present in multiple copies on a 1.05-Mb chromosome. Deletion of this chromosome resulted in loss of AF-toxin production and pathogenicity but did not affect saprophytic growth, suggesting that the chromosome is conditionally dispensable [80]. This is reminiscent of the discovery of a pathogenicity island located on a dispensable chromosome in the pea pathogen *Nectria haematococca* [2].

2.1.8. HC-Toxin

The cyclic tetrapeptide HC-toxin exhibits a cytostatic effect on plant and animal cells by inhibiting histone deacetylase [81]. Being a host-selective toxin, it is a critical virulence and specificity determinant for the interaction between maize and the toxin producer *Cochliobolus carbonum* race 1 [82]. An initial genetic study showed that HC-toxin production appeared to be controlled by a single locus, *TOX2* [83]. Recent molecular analyses indicated that *TOX2* consists of at least seven different types of genes that have been duplicated one or more times (Fig. 1). *HTS1* encodes a nonribosomal peptide synthetase, *TOXA* encodes a putative HC-toxin efflux carrier, *TOXC* encodes a fatty acid synthase beta subunit, *TOXD* encodes a putative dehydrogenase (its role in HC-toxin biosynthesis has not yet experimentally confirmed), *TOXE* encodes a pathway-specific transcription factor, *TOXF* encodes a putative branched-chain amino acid transaminase, and *TOXG* encodes an alanine racemase [84–88]. Arrangement of these genes in different isolates

of *C. carbonum* race 1 is slightly different and can be divided into two types. In the so-called type 1 pattern, one copy of *TOXE* is on a 0.7-Mb chromosome, whereas the second copy of *TOXE* and all copies of the other six genes are on a 3.5-Mb chromosome. In the type 2 pattern, the entire cluster, spanning about 600 kb, is on a 2.2-Mb chromosome. A mechanism of reciprocal translocation has been proposed to explain the phenomenon [89].

Gene disruption analyses indicated that most, if not all, copies of the *TOX2* genes are functional. One gene (encoding an exo-beta 1,3-glucanase) that has no role in HC-toxin biosynthesis has been located within the cluster [89]. The *TOX2* cluster in *C. carbonum* has several unique characteristics. For example, genetic analyses showed that the *TOX2* cluster is genetically unstable since about 5% of sexual progeny undergo spontaneous loss of one or more of their *TOX2* genes [90]. In type 1 strains, a large part (up to 1.4 Mb) of the 3.5-Mb chromosome is dispensable since deletion of this part affects only virulence and HC-toxin production but not fungal growth [90].

2.2. Pigments

2.2.1. Melanins

Melanin is a high-molecular-weight pigment produced by a wide range of fungi. Ascomycota and related Deuteromycota generally synthesize DHN melanin by oxidative polymerization of phenolic compounds via polyketide biosynthesis. 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) is the first polyketide intermediate, which is subsequently reduced to form scytalone. Scytalone is then dehydrated to produce 1,3,8-trihydroxynaphthalene (1,3,8-THN), which is then converted to 1,8-dihydroxynaphthalene (1,8-DHN) after additional reduction and dehydration cycles [91]. Finally, 1,8-DHN is polymerized to form DHN-melanin [91]. Melanin plays a crucial role in the survival and longevity of fungal propagules [91]. Particularly, DHN melanin is essential for the function of the rigidity of appressorium in penetration of host plants by *Colletotrichum* and *Magnaporthe* spp. [92]. Furthermore, melanin has been shown to be important for virulence in human pathogenic fungi, including *Cryptococcus neoformans* [93], *Aspergillus fumigatus* [94], and *Wangiella dermatitidis* [95].

The melanin biosynthesis genes are organized in clusters in some fungi and not in others. In *A. alternata*, a melanin pathway gene cluster contains at least three genes within a 30-kb region [96]. Characterization of the 30-kb region by complementation and gene disruption analysis led to the identification of genes encoding the polyketide synthase, 1,3,6,8-tetrahydroxynaphthalene synthase (*ALM*), the scytalone dehydratase (*BRM1*), and the 1,3,8-trihydroxynaphthalene reductase (*BRM2*). The three mRNA species accumulate in cultured mycelia of the wildtype strain synchronously with mycelial melanization [96]. Another developmentally regulated six-gene cluster spanning a region of 19 kb was identified in *A. fumigatus* and is involved in conidial pigment biosynthesis [97] (Fig. 1). DNA sequencing, gene disruption, expression, and biochemical analyses indicated that *A. fumigatus* synthesizes its conidial pigment through a pathway similar to the DHN-melanin pathway found in many brown and black fungi. The gene products of *alb1*, *arp1*, and *arp2* have high similarity to polyketide synthases, scytalone dehydratases, and hydroxy-naphthalene reductases, respectively. The *abr1* gene encodes a putative protein possessing two signatures of multicopper oxidases. The *abr2* gene protein has homology to the laccase encoded by the *yA* gene of *A. nidulans*. *Abr2* and *abr1* might polymerize and oxidize DHN into melanin. *Ayg1* catalyzes a novel biosynthetic step downstream of *Alb1* (heptaketide synthase) and upstream of *Arp2* (1,3,6,8-THN reductase). The protein *Ayg1* shortens the

heptaketide product of Alb1 to 1,3,6,8-THN, facilitating the participation of a heptaketide synthase in a pentaketide pathway via a novel polyketide-shortening mechanism in *A. fumigatus*. Involvement of the six genes in conidial pigmentation was confirmed by the altered conidial color phenotypes that resulted from disruption of each gene in *A. fumigatus* and the presence of a DHN-melanin pathway intermediate in *A. fumigatus* [97].

Conventional genetic analysis of melanin biosynthesis has also been performed with several other plant pathogenic fungi, such as *Cochliobolus heterostrophus* [98], *Cochliobolus miyabeanus* [99], *M. grisea* [100], and *Colletotrichum lagenarium* [101–103]. In *C. heterostrophus* and *C. miyabeanus*, the 1,3,6,8-tetrahydroxynaphthalene synthase and the 1,3,8-trihydroxynaphthalene reductase genes are closely linked but the scytalone dehydrogenase gene is segregated independently of the these two genes [104]. In contrast, the melanin biosynthetic genes are dispersed in genome of *M. grisea* [100,105] and *C. lagenarium* [104].

2.2.2. Carotenoids

Carotenoids are a class of fat-soluble terpenoid pigments found principally in plants, algae, and photosynthetic bacteria, where they play a critical role in the photosynthetic processes. They also occur in some nonphotosynthetic bacteria, yeasts, and filamentous fungi, where they may carry out a protective function against damage by light and oxygen or play a role in cell signaling [106]. Phytoene is the precursor in carotenoid biosynthesis and is produced from geranyl-geranyl pyrophosphate (GGPP) by the enzyme phytoene synthase. Further modifications of phytoene yield a variety of carotenoids accumulated by fungi. The synthesis of β-carotene from phytoene requires four consecutive dehydrogenations and two cyclizations. Oxygenated carotenoids (xanthophylls), such as neurosporaxanthin in *Neurospora crassa* and *F. fujikuroi* (formerly *G. fujikuroi* [107]) or astaxanthin in *Xanthophyllomyces dendrorhous*, require the activity of additional enzymes [106].

Genes that encode phytoene synthase and carotene cyclase are catalyzed by proteins encoded by different genes in plants and bacteria and by a single bifunctional gene in fungi [108]. Another structural gene of the pathway, phytoene dehydrogenase, has been cloned from many plants, bacteria, and fungi. In contrast to plants and bacteria, where the sequential dehydrogenations are performed by two enzymes, a single dehydrogenase is responsible for all the dehydrogenation reactions in fungi [109]. The linkage distance between the dehydrogenase and the phytoene synthase differs in the three fungi investigated. In the zygomycetes *P. blakesleeanus* [110] and *M. circinelloides* [111], the genes are organized in a gene cluster, whereas in the ascomycete *N. crassa*, they are located on the same chromosome but are not genetically linked. In *F. fujikuroi*, Linnemannstons et al. [112] reported the existence of a carotenoid biosynthesis gene cluster containing at least four genes. The gene *carB* is very similar to the genes that encode for phytoene dehydrogenases in other fungi [110,113,114], and its function was verified by mutational analysis [115]. The gene *carRA* encodes the bifunctional protein with phytoene synthase and carotene cyclase activities [112]. The expression level of *carRA* and *carB* is induced by light, and deletion of *carB* led to the enhanced expression of the *carRA* gene, suggesting the existence of a feedback regulatory mechanism.

2.3. Growth Hormones

2.3.1. Gibberellins

Gibberellins belong to a large family of tetracyclic diterpenoid carboxylic acids that occur in green plants, fungi, and bacteria. A total of 121 gibberellins have been identified from

these natural sources [116]. They were first identified as secondary metabolites of the rice pathogenic fungus *Gibberella fujikuroi* (mating population C) and some other fungal species [117]. Some members of gibberellins function as natural growth hormones in higher plants able to promote processes such as seed germination, stem elongation, leaf growth, flower development, and seed and pericarp growth [118]. Three groups of enzymes are involved in the gibberellin biosynthesis: terpene cyclases (*ent*-kaurene synthesis), P-450 monooxygenases (oxidation of *ent*-kaurene), and dioxygenases. The initial steps of the gibberellin biosynthetic pathway from transgeranylgeranyl diphosphate to GA₁₂-aldehyde are identical for plants and fungi, but the following steps diverge. Gibberellic acid (GA₃) is the major end-product of the pathway in *G. fujikuroi*, whereas it is a minor gibberellin component in most plant species. Gibberellins do not have a defined role in fungi, and strains of *G. fujikuroi* that lack the gibberellin biosynthetic genes grow normally in culture. However, their pathogenicity has not been assessed to date [119]. In contrast to plants, genes involved in gibberellin biosynthesis in *G. fujikuroi* were discovered to be organized in a cluster containing seven genes in a 17.2-kb DNA region (Fig. 1) [119]. Because about 18 steps are required for the formation of GA₃ from transgeranylgeranyl diphosphate (GGPP), it appears that not all of the genes have been identified or some genes encode multifunctional enzymes (Table 1). In *G. fujikuroi*, GGPP synthase is encoded by two genes, and one of these, *ggs2*, is specific for gibberellin biosynthesis [117]. Cyclization of GGPP is catalyzed by the bifunctional copalyl pyrophosphate (CPP)/*ent*-kaurene synthase (KS) enzyme [120]. The *ggs2* and *cps/ks* genes are clustered together in the gibberellin cluster. Four genes in the gibberellin biosynthesis cluster in *G. fujikuroi* encode cytochrome P450 monooxygenases. These genes are designated *P450-1*, *P450-2*, *P450-3*, and *P450-4*. The *P450-1* gene is closely linked to *P450-4* in the gene cluster, sharing the same promoter sequence but being transcribed in the opposite direction. *P450-4* encodes for a multifunctional *ent*-kaurene oxidase that catalyzes all three early oxidation steps between *ent*-kaurene and *ent*-kaurenoic acid [121]. *P450-1* catalyzes the next four oxidation steps in the main pathway from *ent*-kaurenoic acid to GA₁₄ via GA₁₂ aldehyde. *P450-1* and *P450-2* are classified as part of the CYP68 family [122]. *P450-2* encodes a 20-oxidase, and its product oxidizes the 3β-hydroxylated intermediate, GA₁₄, and its nonhydroxylated analogue GA₁₂ to GA₄ and GA₉, respectively [123]. This reaction (20-oxidation) in plants is catalyzed by dioxygenases and not monooxygenases as in *G. fujikuroi*. The characterization of the last two genes in the cluster, a fourth P450 monooxygenase (*P450-3*) and a desaturase gene that is thought to introduce the 1,2-double bond in the conversion of GA₄ to GA₇, is currently in progress (B. Tudzynski, personal communication, 2003).

Six of the seven genes of the gibberellin cluster are strongly induced under gibberellin production conditions (low nitrogen) indicating they may be under the control of the same regulatory gene(s) to ensure that gibberellin production occurs only at low nitrogen levels [117,123] (B. Tudzynski, personal communication, 2003). High nitrogen concentrations and specifically ammonium and glutamine repress gibberellin biosynthesis in *G. fujikuroi*. Disruption of the positive-acting nitrogen regulatory *areA-GF* gene in *G. fujikuroi* led to a 10% to 20% reduction of gibberellin production in gibberellin induction medium. In addition, the loss-of-function *areA-GF* strains were insensitive to ammonium-mediated gibberellin repression, supporting the conclusion that gibberellin biosynthesis is under the control of AreA-GF [124].

As is covered in section 4 of this chapter, the profound differences in gibberellin biosynthesis between *G. fujikuroi* and plants at the chemical, biochemical, and genetic

levels indicate that higher plants and fungi have evolved the gibberellin biosynthetic pathway independently and not by horizontal gene transfer [119].

2.4. Pharmaceuticals

2.4.1. Lovastatin

Lovastatin is an inhibitor of the enzyme (3S)-hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase that catalyzes the reduction of HMG-CoA to mevalonate during cholesterol biosynthesis. The compound is also toxic to fungi by inhibiting the same enzyme required for ergosterol biosynthesis [125]. This activity makes lovastatin a medicinally important compound with antihypercholesterolemic attributes [126] and antifungal properties [125]. Lovastatin is a secondary metabolite produced by *Aspergillus terreus* and is biosynthetically composed of two distinct polyketide chains joined through an ester linkage. One chain is the diketide 2-methylbutyrate and the other is a nonaketide that includes a distinctive conjugated hexahydronaphthalene ring system [127].

Kennedy et al. [126] recently sequenced the lovastatin biosynthetic cluster of *A. terreus* and identified 18 potential genes over a 64-kb genomic region, the functions of which were predicted by sequence comparisons and disruption analysis experiments (Table 1; Fig. 1). Of these genes, *lovB* and *lovF* encode two type I polyketide synthases (PKS). The *lovB* gene encodes the previously described lovastatin nonaketide synthase (LNKS) [128], which is required for the synthesis of the main nonaketide-derived skeleton. The *lovF* gene encodes the lovastatin diketide synthase (LDKS) that is probably responsible for the biosynthesis of the (2R)-2-methylbutyryl side chain of lovastatin. Lovastatin has two methyl groups derived from S-adenosyl-L-methionine (SAM), one on the nonaketide and the other on the diketide side chain. The presence of methyltransferase domains in LovB and the LovF protein indicates that in both cases, the methyl groups are likely to be added while the polyketide is being synthesized. The *lovC* gene is located adjacent to *lovB* and encodes a protein with high similarity to the product of the *Cochliobolus carbonum* *toxD* gene of unknown function from the HC-toxin biosynthesis cluster, to hormone and ripening-induced proteins from plants, and to ER domains of PKSs. Gene disruption analysis of *lovC* led to the conclusion that LovB and LovC proteins interact with each other to produce a polyketide of the correct length and with the correct reduction and cyclization pattern. The cooperation of *lovB* and *lovC* genes accomplish the approximately 35 steps necessary to generate dihydromonacolin L from acetyl-CoA, malonyl-CoA, NADPH, and SAM. Oxidative transformation of the PKS product dihydromonacolin L led to the formation of monacolin J. The 2-methylbutyryl side chain is produced by *lovF* and is further used by LovD to directly acylate monacolin J and yield lovastatin. The *lovD* gene is another gene of the cluster that is functionally associated with LovF and has similarity to β -lactamases, carboxypeptidases, lipases, and esterases. Disruption of *lovD* led to a strain that accumulated monacolin J, the immediate precursor to lovastatin. Kennedy et al. [126] proposed that *lovD* is responsible for the last step, the biosynthesis of the 2-methylbutyryl/monacolin J transesterase that joins together the two polyketide components of lovastatin. The function of LovA, a protein essential for formation of lovastatin, is not fully understood, but it has sequence homology to P-450 enzymes and its disruption leads to a very active β -oxidation system in *A. terreus* [129]. Among the rest of the ORFs in the biosynthetic gene cluster, two were annotated to encode regulatory proteins (LovE and ORF13), two belong to potential resistance genes, three encode putative transporter genes, and five genes have no known functions [126]. Interestingly, one of

the resistance genes encodes a putative HMG-CoA reductase [126], which is speculated to provide resistance to the fungal species containing the lovastatin cluster [130].

Despite the knowledge of the genes and the enzymes involved in the biosynthetic pathway, little is known about the regulation and the physiology of lovastatin biosynthesis. Some recent experiments [131] showed that lovastatin synthesis is dependent on the nitrogen source. Ammonium, nitrate, and urea inhibited the production of lovastatin, and only glutamate, histidine, and, to a lesser extent, glycine supported lovastatin biosynthesis. Experimental results from the same studies indicate also that carbon source starvation is required for the onset of lovastatin biosynthesis [131]. Analysis of the lovastatin biosynthetic cluster revealed that the motif of functional CreA (involved in carbon catabolite repression in *A. nidulans*) binding site *in vivo* is present in the putative promoters of ORF13 and in the putative promoter of the divergently transcribed ORF8 and *lovE*. Thus, the presence of putative functional CreA binding sites in two putative regulatory genes suggests that repression of lovastatin biosynthesis by glucose could be mediated by CreA [131].

2.4.2. Compactin

Several other fungi produce lovastatin-related structures, including *Monascus ruber*, which produces lovastatin, and *Penicillium citrinum* and *P. brevicompactum*, which produce compactin (ML-236B) [132]. Compactin is identical to lovastatin except that it is missing the methionine-derived methyl group on the nonaketide. Compactin, like lovastatin, inhibits the enzyme HMG-CoA reductase and is used as a substrate for microbial conversion to pravastatin sodium, a compound that has been widely used as a pharmaceutical drug in the treatment of hypercholesterolemia [133].

Genetic analyses in *P. citrinum* led to the discovery of an entire gene cluster related to compactin biosynthetic genes, spanning a 72-kb region that revealed the existence of 20 open reading frames (Table 1; Fig. 1) [133]. Nine genes were localized within a 38-kb region and were transcribed when compactin was produced. Nine genes, designated as *mlcA–mlcH* and *mlcR*, have predicted amino-acid sequences similar to those encoded by the genes for lovastatin biosynthesis. Two genes, *mlcA* and *mlcB*, encode putative novel multifunctional type I PKSs and share 59% and 61% identity with LovB and LovF, respectively. Disruption experiments provided evidence that *mlcA* and *mlcB* are required for the biosynthesis of the nonaketide and the diketide chains. *mlcC* encodes a putative P450 monooxygenase and shares 72% identity with LovA. *mlcF* encodes a putative oxidoreductase and shows some similarity to dihydrofolate reductases and also shares 57% identity with a putative polypeptide encoded by ORF5 in the lovastatin gene cluster. *mlcG* encodes a putative oxidoreductase and shows 70% identity to LovC, which has an enoyl reductase activity required for lovastatin biosynthesis. *mlcH* encodes a putative transesterase and displays 75% identity with LovD. Two other genes, *mlcD* and *mlcE*, encode putative polypeptides that may be involved in conferring resistance to compactin and in metabolite secretion [133].

Sequence of the *mlcR* gene suggests it is a Cys6 zinc binuclear cluster protein, and it exhibits 34% identity with LovE, indicating that it may be involved in the regulation of compactin biosynthesis in *P. citrinum* [134]. The induction of compactin production is correlating with the expression of *mlcR* and the biosynthetic genes *mlcA–H*, and it occurs mainly during the stationary phase. Introduction of additional copies of *mlcR* in *P. citrinum* showed increased transcription of *mlcR* and produced higher amounts of compactin. Constitutive expression of *mlcR* led to the production of compactin during the

exponential growth phase. Alterations in *mlcR* expression resulted in concomitant alterations in expression of some of the compactin biosynthetic genes, suggesting that *mlcR* may indeed be a transcriptional activator of some of the pathway-specific genes required for compactin biosynthesis. ORF1 is located next to *mlcR* and also encodes a putative Cys6 polypeptide, but its function still remains unknown [134].

2.4.3. β -lactams

The most commonly used β -lactams (β -cyclic amides) antibiotics are penicillins and cephalosporins. Their biosynthesis begins with nonribosomal condensation of three precursor amino acids to yield a tripeptide by ACV [delta-(L-alpha-amino adipyl)-L-cysteinyl-D-valine] synthetase. An IPN (isopenicillin N) synthetase catalyzes the cyclization of ACV to produce IPN. From IPN, different reactions lead to various penicillin and cephalosporin final products [135]. Cephalosporins are produced by both bacteria and fungi (e.g., *Acremonium chrysogenum*), whereas penicillins are produced only in several filamentous fungi (most notably *Aspergillus nidulans* and several *Penicillium* spp.). The three genes for penicillin biosynthesis—*pcbAB* (encoding ACV synthetase), *pcbC* (encoding IPN synthetase), and *penDE* (encoding acyltransferase)—form a cluster that spans approximately 20 kb identified in *A. nidulans*, *P. chrysogenum*, *P. nalgiovernse*, *P. notatum* and *P. griseofulvum* [136,137]. In these organisms, the three genes maintain the same order and transcription orientation (Fig. 1). In many industrial strains with high penicillin yield, the cluster is often amplified many times in tandem repeats [138]. A putative multidrug efflux pump encoded by *cefT* has been recently identified in *Acremonium chrysogenum*, along with a putative D-hydroxyacid dehydrogenase gene (*orf 3*) that is not required for cephalosporin biosynthesis [139]. *pcbAB*, *pcbC*, *orf 3*, and *cefT* form a so-called early cluster of about 17 kb on chromosome VI, while *cefEF* (encoding deacetylcephalosporin C synthetase/hydroxylase) and *cefG* (encoding an acetyl transferase) form a late cluster of about 3.5 kb on chromosome II (Fig. 1) [140].

Like several other gene clusters, expression of penicillin biosynthesis genes is under complex control by carbon and nitrogen source and ambient pH. It has been shown that pH effect is mediated by the global transcription factor PacC [141,142]. The major nitrogen regulatory protein AreA is implicated to regulate expression of penicillin biosynthetic genes, but it has not been verified by *in vivo* analysis [143]. The molecular basis of C-source regulation of *ipnA* expression remains to be elucidated [141,144] although data suggest carbon regulation of penicillin production differs to some degree between *Aspergillus* and *Penicillium* spp. [142]. No pathway-specific regulator has yet been identified to regulate the penicillin gene clusters. However, PENR1, a HAP-like transcriptional complex, has been shown to positively regulate penicillin cluster expression in *A. nidulans* [145]. For further discussion of regulation of penicillin gene expression, several review papers are available [135,146,147].

3. REGULATION OF GENE CLUSTERS

3.1. Transcriptional Regulation

In the preceding, mention was made of several modes of regulation of some of the gene clusters. Examination of several of the clusters shows the presence of genes encoding zinc-binding proteins [13,24,35,79,126,130,148], a major class of transcription factors that fungi employ to regulate secondary metabolism as well as development and nutrient

utilization. These proteins bind to the promotors of the target genes and control their transcription. Cys2His2 zinc finger proteins and Cys6 zinc binuclear cluster proteins are the most common types of zinc-requiring transcription factors, with the former protein found in many organisms but the latter found only in fungi. Evidence to date suggests that these transcription factors act positively to regulate the biosynthetic pathway genes in the cluster [1,24,56,57,134]. PAXR and PAXS for paxilline production, AKTR-1 and AKTR-2 for AK-toxin production, MlcR for compactin production, LovE for lovastatin production and AflR for AF/ST production are Cys6 zinc binuclear proteins, whereas Tri6 and MRTRI6 for trichothecene production are Cys2His2 zinc finger proteins. Another type of transcription factor found to specifically regulate secondary metabolism is the *C. carbonum* ToxE, which regulates HC-toxin biosynthesis. This protein has four ankyrin repeats and a basic region similar to those found in basic leucine zipper (bZIP) proteins, but it lacks any apparent leucine zipper [149]. PENR1 is a HAP-like transcriptional complex involved in regulating penicillin production in *A. nidulans* [145]. It is unknown, however, whether it is pathway specific.

Also novel is Tri10, a regulatory gene required for T-2 toxin production. The Tri10 protein does not contain any known DNA-binding motif, and as of yet, its precise function remains unknown [31]. AflJ, as mentioned in the preceding section, is a aflatoxin/sterigmatocystin cluster protein that appears to act as a coactivator by binding to *aflR*; however, the precise mechanism of how such binding influences *aflR* activation is unknown [55].

Numerous reports in the literature have shown that secondary metabolite biosynthesis is responsive to environmental cues like carbon and nitrogen source, ambient temperature, and pH. As mentioned earlier, the effect of these environmental signals is mediated through the global transcription factors CreA, AreA, and PacC, respectively, in *A. nidulans* [150–152]. Molecular studies have shown that these Cys2His2-type zinc finger proteins are important in the regulation of aflatoxin, sterigmatocystin, gibberellin, and penicillin [49,116,153,154]. It is likely they also play a role in the regulation of other, if not all, secondary metabolites. A thorough examination of the intergenic region between *aflR* and *aflJ* in several *A. flavus* isolates suggests that increasing numbers of AreA binding sites is positively correlated with degree of nitrate required to repress aflatoxin gene expression. This may indicate that AreA is a negative regulator of aflatoxin biosynthesis [154]. Interestingly, there are no AreA sites in the corresponding *aflR/aflJ* intergenic region in *A. nidulans* and sterigmatocystin biosynthesis is not repressed by nitrate.

3.2. Signal Transduction Regulation

It is known that secondary metabolism is linked with fungal development [155]. However, only recent molecular analyses (mostly conducted in *A. nidulans*) have begun to unravel the underlying mechanism connecting these two processes. This linkage of secondary metabolism and fungal development has been the topic of a recent review [65]. Through complementation of aconidial, nonsterigmatocystin-producing *A. nidulans* strains, it was determined that a G-protein signaling pathway negatively regulated both conidiation and sterigmatocystin biosynthesis [156]. The same study showed this regulation to be conserved in *A. parasiticus*. Further studies identified a protein kinase A catalytic subunit as partially mediating this repression [157]. The target of regulation in the sterigmatocystin/aflatoxin gene cluster is *aflR*, which is both transcriptionally and posttranscriptionally regulated by protein kinase A. Recent experimentation has identified a putative protein methyltransferase, LaeA, which mediates protein kinase A transcriptional regulation of

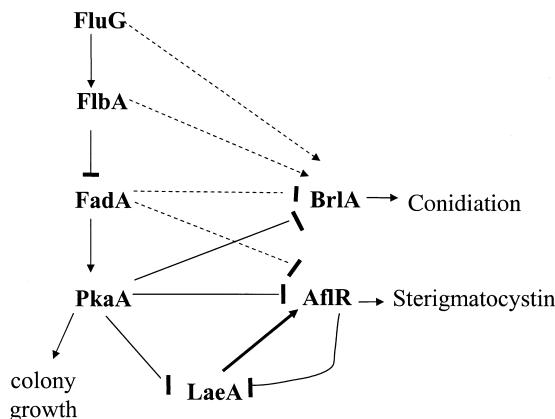


Figure 3 Proposed model of G-protein signal transduction regulating sterigmatocystin production and sporulation. Arrows indicate positive regulation, and blocked arrows indicate negative regulation. Solid lines indicate known pathways. Dashed lines indicate postulated pathways. FluG, early acting developmental regulator; FlbA, regulator of G-protein signaling; FadA, α subunit of G-protein; PkaA, catalytic subunit of PKA; LaeA, AflR regulator; AflR, sterigmatocystin-aflatoxin-specific transcription factor (From Ref. 65; copyright 2002, with kind permission from American Society for Microbiology.)

aflR (Fig. 3) [158]. LaeA also transcriptionally regulates penicillin and lovastatin cluster genes [158] raising the possibility of its global involvement in gene cluster regulation. This protein appears to be conserved in filamentous fungi.

Expanding studies on the role of G-protein signaling on fungal development suggests that signal transduction pathways will likely have significant impact on secondary metabolism as either a positive or negative regulator [65]. For example, while the *A. nidulans* G protein negatively regulates AF/ST biosynthesis, it positively regulates penicillin production in the same fungus and positively regulates trichothecene production in *Fusarium sporotrichioides* [159].

4. EVOLUTION OF GENE CLUSTERS

The evolution and maintenance of gene clusters have received a great deal of attention for both bacterial systems [160–162] and more recently for fungal gene clusters [1,163,164]. In this section, we discuss four models and how they may be used to explain either formation or maintenance of clusters [160,164] with consideration of current data.

4.1. Extant Models for Gene-Cluster Evolution

4.1.1. Natal Model

In the natal model, the cluster is a product of history. Duplication and subsequent divergence of genes result in clusters of genes within the same gene family. This model originated prior to a more modern understanding of molecular genetics and biochemistry. The reasoning was that because enzymes involved in a pathway would be making minor

changes to very similar substrates, the pathway would evolve based on gene duplications and minor changes in substrate specificity and enzymatic function [160]. Clearly this would be an adequate explanation for a limited set of fungal gene-cluster types, such as enzymatic genes involved in wood degradation [165]. However, because most functionally related gene clusters considered in this review represent assemblages of a variety of gene types (Table 1; Figs. 1, 2) and because the most closely related members of each of these gene families represented are frequently located in other unlinked gene clusters, this would argue against the natal model for the origin of most clusters considered here. Further, the natal model provides no mechanism for maintenance of a gene cluster once it is formed [160].

4.1.2. Coregulation Model

The coregulation model predicts that there is a selective benefit to clustering of functionally related genes due to more efficient regulation mechanisms associated with proximity of the genes. Presumably, this would either involve some sort of *cis*-acting element (as in bacterial operons) or an effect of the chromatin environment. Although Walton [164] dismissed the role of a common chromatin environment due to the idea that nonclustered fungal primary metabolism pathway genes are effective without a common chromatin environment, recent data hint that chromatin environment or gene proximity could be important in regulation of secondary metabolism. Chiou et al. [166] showed that aflatoxin biosynthetic genes are not transcribed normally when removed from the gene cluster, and the same has been observed for sterigmatocystin genes (N. Keller et al., unpublished data). It is intriguing to speculate that the recently described LaeA protein [158] could be involved in cluster regulation. The function of LaeA is not fully delineated but sequence analysis suggests it to be a protein methyltransferase with most similarity to the histone and arginine methyltransferases that play important roles in regulating gene expression. An interesting aspect of histone methyltransferases in regulating gene expression has been the recent discovery that histone methylation plays a role in defining boundaries of euchromatic and heterochromatic chromosomal domains, such as in the mating locus of yeast and the beta-globin locus in mice [167–170]. These findings suggest that histone methylation may be important in the regulation of gene cluster boundaries and may support a relationship with LaeA and histones. Also, studies of gene regulation in the *A. nidulans* penicillin gene cluster and the *A. nidulans* nitrate utilization gene cluster have shown that chromatin remodeling or DNA conformational changes are required for expression of genes in these clusters [145,171]. Clearly, coregulation would provide the selection pressure necessary to maintain a gene cluster; it is not clear, however, that it would be able to act to cause the formation of clusters [161].

4.1.3. Selfish Cluster Model

The selfish cluster model proposed recently by Walton [164] is an extension of the “selfish gene” [172] and the “selfish operon” [160–162] concepts. Basically, the idea is that the cluster is the unit on which selection is acting. The fitness of the cluster is enhanced by tight linkage of the genes because the probability of transfer via horizontal transmission is quite high. In the selfish operon model for bacteria, a great deal of emphasis is placed on the importance of horizontal gene transfer as a mechanism for the formation of clusters of genes. The horizontal transmission of an incipient cluster (fortuitous preexisting loose linkage between genes with related function separated by intervening sequences) would then provide for selection of the trait in absence of the need for the intervening sequences.

Thus, the horizontal transfer aspect of this model acts to make intervening sequences nonessential so that these sequences would experience strong selection to be lost. It could be argued that identification of secondary metabolite gene clusters located on dispensable chromosomes such as the *Alternaria* host-specific toxins [80] and the *Nectria haemato-cocca* pathogenicity island [2] may support this model.

Horizontal transmission has also been proposed as an explanation for why regulatory genes are present in many clusters of genes for dispensable functions in fungi. These transacting regulatory genes need not be proximate to influence the cluster function. However, were the cluster to be transferred to a recipient without the regulatory gene, the cluster would not provide a selectable phenotype.

The mechanisms and conclusive evidence for horizontal transfer in fungi is not nearly as well-established as in bacterial systems [163]. However, the advent of sufficient genome sequence data for fungi, bacteria, and plants will likely expand our understanding of the prevalence of horizontal gene transfer in fungi. To address gene cluster specifically, an obvious place to begin would be clusters with functions that are also present outside the fungal kingdom (e.g., penicillin or gibberellins).

Evidence from the penicillin cluster in bacteria and fungi provides the best support for horizontal transfer [163,173–175]. Many prokaryotes are capable of β -lactam production, whereas among eukaryotes, only a limited number of filamentous fungi possess this trait. Despite the length of time since divergence between bacteria and fungi, both the biochemical pathway and sequence of genes involved (e.g., IPNS) are similar [174,176], with the fungal genes showing codon usage more like prokaryotes than eukaryotes [176]. Buades and Moya [174] applied maximum likelihood statistics to examine the molecular clocks of the IPNS genes. This analysis revealed that fungal IPNS genes appear 1400 million years closer than expected to the bacterial genes [174].

Alternatively, evidence from gene cluster in *Gibberella fujikuroi* does not support horizontal gene transfer between plants and fungi [119]. Unlike *G. fujikuroi*, genes for gibberellin biosynthesis in plants are dispersed, not clustered. Furthermore, different types of enzymes perform the same function in the two different kingdoms. For example, the gibberellin 20-oxidase is a cytochrome P450 monooxygenase in *G. fujikuroi* and not a 2-oxo-glutarate-dependent dioxygenase like in plants. Finally, the difference in this gibberellin 20-oxidase has broader implications relative to gibberellin biosynthesis gene regulation. In plants, expression of this enzyme is regulated by negative feedback in the presence of biologically active gibberellins, thus maintaining homeostasis. This sort of negative feedback regulation does not seem to occur at this step in fungi [119].

4.1.4. Fisher Model

The Fisher model is an extension of the theory associated with linkage disequilibrium among coadapted gene complexes (177). Fisher's original idea was that fitness differences associated with variation in effectiveness of particular allele combinations from distinct genetic loci results in linkage of these alleles in the genotypes that persist in the population, despite the lack of physical linkage in the genome (linkage disequilibrium). Application of this idea to gene clustering requires the additional assumption that the selection is strong enough to favor actual physical clustering of these loci, thus, further ensuring decreased probability of breaking up the coadapted gene complexes. Both frequent recombination and the prerequisite genetic and phenotypic variation (polymorphism) among genotypes in the population would be required for the assembly of clusters. Once formed, the clusters would be more resilient to breakup by recombination. Thus, clustering would be both

selected for and presumably maintained in this model. This model is distinct from the idea of a selfish cluster in that selection acts at the level of individuals within the population, not on the cluster itself. The clusters form based on selection favoring gene conversion events that occur within the genome as opposed to invoking horizontal gene transfer.

One could argue that there is evidence to support this model available from possibly the most extensive study of secondary metabolite gene cluster diversity across a geographic sample of a fungus [178]. Initially, O'Donnell et al. [107] established seven biogeographic lineages within a worldwide collection of isolates of *Fusarium graminearum*. This robust result was based on concordance (reciprocal monophyly) across gene genealogies for six single-copy genes, including one gene trichothecene gene (*TRI101*; 3-O-acetyltransferase) that happens to be separate from the trichothecene biosynthesis cluster [179]. Since the three known trichothecene chemotypes (NIV, 3ADON, and 15ADON) proved not to be lineage specific, these researchers chose to investigate the phylogenies revealed by 19 kb of sequence from the trichothecene gene cluster of 39 isolates [178]. The combined gene genealogies from analysis of the TRI genes grouped isolates into chemotype-specific clades. Thus, the authors interpreted this to mean that the TRI-cluster haplotypes each have a single evolutionary origin. This was further supported by the nearly identical patterns exhibited between phylogenetic trees generated based on variation at synonymous sites and both the respective noncluster and TRI-cluster trees, indicating that the differences in the cluster and noncluster trees were not due to convergent evolution of the clusters. The authors' interpretation of these data was that the polymorphisms associated with chemotype were maintained by balancing selection despite divergence into separate species. These data are particularly consistent with an idea of coadapted gene complexes. In the absence of this sort of extensive study of genetic and phenotypic polymorphism among intraspecific or closely related interspecific lineages, it is difficult to know how prevalent this pattern may be.

4.2. Adopting a Unified Model for the Evolution of Gene Clusters

Because a variety of selection pressures likely interact to promote formation and maintenance of complex traits, a unified model may be the best explanation for evolution of fungal secondary metabolite gene clusters.

4.2.1. Formation of Gene Clusters

Selection acting on the function of the cluster (thus, its contribution to the organism) will favor combinations of alleles that provide the most benefit (or least cost) to the organism. In the presence of sufficient allelic variation and frequent recombination, selection will favor increased linkage among these coadapted gene complexes, thus favoring formation of (at least loosely) clustered functionally related genes. Because genes for essential functions experience strong purifying selection that will act to reduce allelic polymorphism [180], clusters of genes associated with essential functions will be rare.

At the level of the cluster itself, any factors that select against or obviate the need for intervening sequences (i.e., promote loss) within these loosely linked genes will also favor cluster formation. Although horizontal gene transfer is cited as the mechanism for this in selfish operon/cluster models, it is not the sole mechanism likely to promote selection against intervening sequences. Operating under the same idea that clusters are selfish (i.e., promote their own dissemination/fitness irrespective of organism's fitness), any factors that promote duplication of all or part of the incipient cluster (sensu the natal model)

within that genome would provide the same opportunity to select against the intervening sequences (which would then be redundant in the genome).

Furthermore, much like models for the evolution of novel traits after duplication and subfunctionalization of single genes [181], selection would act on duplicated clusters (or on partial clusters or incipient clusters) to favor new traits or they would be lost. Clearly, formation of clusters in this manner would not represent the same “ready-made” adaptation one might envision under a horizontal transfer scenario. However, because there is arguably a much higher probability of duplication of DNA within a fungal genome than horizontal transfer of DNA between genomes, seeding of new clusters from duplicated sequences seems an even more probable mechanism for these selfish units to proliferate.

4.2.2. Maintenance of Gene Clusters

Once formed, selection must act to favor maintenance of a gene cluster; otherwise, a variety of processes acts to disperse the cluster. Here again, selection favoring the function of the cluster acts to maintain (or continue to improve) particular coadapted gene complexes. Since any recombination at the locus that brings together nonnative combinations of genes results in reduced fitness for the organism, those lineages would not persist. In fact, under strong enough selection this might lead to evolution of reduced recombination in the region. Furthermore, if the trait encoded by the cluster was sufficiently phenotypically polymorphic relative to different native combinations of genes, then one might expect that ecological differentiation promoted might reduce the probability of recombination among lineages with different clusters (e.g., colonizing different niches within a heterogeneous environment resulting in fewer opportunities to mate).

Improvement of cluster function once clustered would be reasonable based on evolution of favorable protein–protein interactions or on substrate channeling associated with colocalization of the genes. Thus, selection would act against random factors acting to disperse genes over time. Furthermore, if there was any advantage to a common chromatin environment or via coregulation, these mechanisms could also help to maintain the cluster. Selection acting at the level of the selfish cluster will maintain the cluster so long as the factors that promoted its formation as a selfish unit are present (e.g., transposons). That is, factors that act to promote cluster duplication and/or horizontal transfer of clusters will maintain a prevalent pattern of functionally related dispensable gene clusters.

5. CONCLUSIONS AND FUTURE RESEARCH

Comparative and functional analyses of fungal genomes promise to be a tremendous resource for discerning the relative importance of these different factors (horizontal gene transfer, duplication, coadaptation of genes, coregulation) contributing to formation and maintenance of fungal gene clusters. Evidence for coadapted gene complexes will require comparisons within and across closely related species and subsequent tests for superior function. Ultimately, it will be of great interest to determine to what degree ecology and life history traits are associated with differences in the relative importance of all these factors. For example, will there be differences across asexual vs. sexual fungal lineages, or across pathogens vs. saprophytes.

In the rare cases in which genes for an essential function are associated with a cluster, it will be of interest to determine whether there is evidence for greater polymorphism or co-adaptation among the genes involved. Also, of interest will be the cases in which genes are clustered in some lineages but not others (e.g., melanin and carotenoid biosynthesis).

If such genes are known to be orthologous, then it will be of interest to determine whether there has been dispersal of a cluster or clustering of dispersed genes. Clearly, addressing this question will require ample access to data from many species with the trait. If there is evidence that it was a cluster that dispersed, it will be interesting to determine what sort of selection was relaxed.

Generally, across clusters, it will be of interest to assess whether genes in dispensable function pathways/clusters are more polymorphic than the paralogous members of the gene family involved in nondispensable functions. Is there phylogenetic evidence for coevolution of coadapted gene complexes? Is there biochemical/functional evidence for coadapted gene clusters? Are clusters associated with genome regions that are particularly likely or unlikely to recombine? Are clusters more likely than other pieces of DNA to be associated with genetic elements that promote movement of DNA segments?

Clustering of genes involved in fungal secondary metabolism is a clear trend uncovered during the decades of research to identify genes associated with particular natural products. On the horizon, we see a more reverse genetic approach in which predicted open reading frames identified in sequenced genomes might be targeted to determine whether or not they play a role in secondary metabolism. Furthermore, there will be a tremendous amount of data available to search for clusters of genes surrounding members of gene families associated with secondary metabolism. This will be the new frontier in prospecting for new natural products, based on predicting the products produced by this arrangement of genes. Also looming on the horizon is the need to determine the roles of the products for the producing organisms. This will assist in both applied use of the products and in deducing the ecological niches and evolutionary history of the organisms.

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Genomics and Gene Regulation of the Aflatoxin Biosynthetic Pathway

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1. INTRODUCTION

Fungi are both a blessing and a curse to mankind. On one hand, they have proved useful for millennia in the production of foods and other products. Indeed, the history of their use goes back at least as far as the production of wine and ale. However, they have also been responsible for some of the most insidious ailments we have faced. The process of ergot alkaloid contamination of grains by *Claviceps purpurea*, leading to the disease known as St. Anthony's Fire, is one prime example of the importance of mycotoxins in human health. Disease caused by this toxin was recorded as far back as the Middle Ages and was probably responsible for such traumatic social reactions as the Salem Witch Trials [1].

Today, the prospect of weaponizing microbes or their products is especially horrifying. Biological weapons have been fashioned using mainly bacteria and viruses as human or animal pathogens. Similarly, there is an increased interest in fungi—not as pathogens, but for the toxins they may produce. It has been documented that T-2 toxin (from *Fusarium* spp.) and aflatoxin (AF; from *Aspergillus* spp.) have been produced by Iraq and other countries for use in weapons programs [2,3].

Aflatoxin is a regulated mycotoxin that can occur in almost any poorly stored commodity or prepared food. It may also be produced in corn, peanuts, tree nuts, and cotton prior to harvest. Interstate trade in the United States is not permitted for food and feed containing greater than 20 ppb AF B1, the most toxic AF species. The allowable level in milk is less than 0.5 ppb (per the U.S. Food and Drug Administration). Many other countries have lower allowable levels of AF concentration (CAST, 2003). Contamination of corn by AF-producing fungi can be a problem in years when the summers are dry and

hot, conditions that favor the growth of the AF-producing fungi *A. flavus* and *A. parasiticus*. Although both of these species can contaminate crops, *A. flavus* is the most common of these pathogens on host crops preharvest and constitutes most of the AF contamination problem [4].

Due to these concerns over food safety and human health, it would seem logical that careful study of the AF pathway should be a research priority. A full understanding of the biochemistry and genetics of the pathway may yield new control strategies for abolition of AF contamination of food crops. To date, much has been discovered about the biochemistry of the AF pathway. Studies on a related pathway, the sterigmatocystin (ST) pathway in *A. nidulans*, have been instrumental in elucidating some of the genetics of AF/ST production. While the basic biochemical steps of the pathway are known and several regulatory factors have been identified [5–9], details of the molecular regulation of AF/ST production are complex and yet to be fully resolved. There have been numerous reviews covering the biosynthesis and ecological significance of this important secondary metabolite [10–16]. This review will cover the basics of the AF/ST pathway and will attempt to illuminate some of the molecular mechanisms behind AF/ST regulation.

2. BIOCHEMISTRY

2.1. Acetate to Hexanoic Acid

The first stable intermediate in the AF pathway is synthesized from acetate and malonyl units by the concerted efforts of a fatty-acid synthase (FAS) and a polyketide synthase (PKS) (Figure 1). The FAS synthesizes hexanoate, which is then elongated by the PKS through the addition of acetate units. The genes for both of these enzymes reside in the AF biosynthetic cluster. Evidence for hexanoate as the starter unit for AF biosynthesis was first provided by Townsend and colleagues [17], who showed that hexanoate provided to cultures could be converted into AF. Molecular studies support this hypothesis.

The gene identified as responsible for this assembly is *fas-1* [18]. Mahanti and colleagues performed UV mutagenesis on an *A. parasiticus* strain that accumulates norsolorinic acid (NA). Mutants were selected for the inability to accumulate NA. One of these mutants was then transformed with a cosmid that complemented both mutations, allowing AF production. Subsequent experiments led to the cloning of *fas-1*, which was shown to act upstream of the NA accumulation mutation.

Supporting biochemical evidence for *fas-1* encoding a hexanoate intermediate was obtained by Watanabe and coworkers [19]. Feeding of hexanoyLNAC to a *fas-1* disruptant yielded low quantities of NA. The researchers speculated that Fas-1 and PKS must be associated for efficient conversion of hexanoate to noranthrone.

Research in *A. nidulans* has also shed light on the relationship between primary and secondary metabolism. Two independent FAS complexes were described by Brown et al. [20]—one that is required for primary growth (FAS) and another that is distinct to ST biosynthesis (sFAS). In *A. nidulans*, there are two sFAS genes in the ST cluster: *stcJ* and *stcK*. They showed that ST production was inhibited in disruptants of the sFAS genes but could be restored with the addition of hexanoic acid. Disruptants of the primary FAS genes *fasA* and *fasB* were able to produce ST when supplemented with long chain fatty acids to allow for primary growth.

2.2. Hexanoic Acid to Norsolorinic Acid

Hexanoate appears to serve as the substrate for the AF polyketide synthase (Figure 1). Two PKS genes were cloned independently in *A. parasiticus*: *pksA* and *pksL* [21,22]. *pksA*

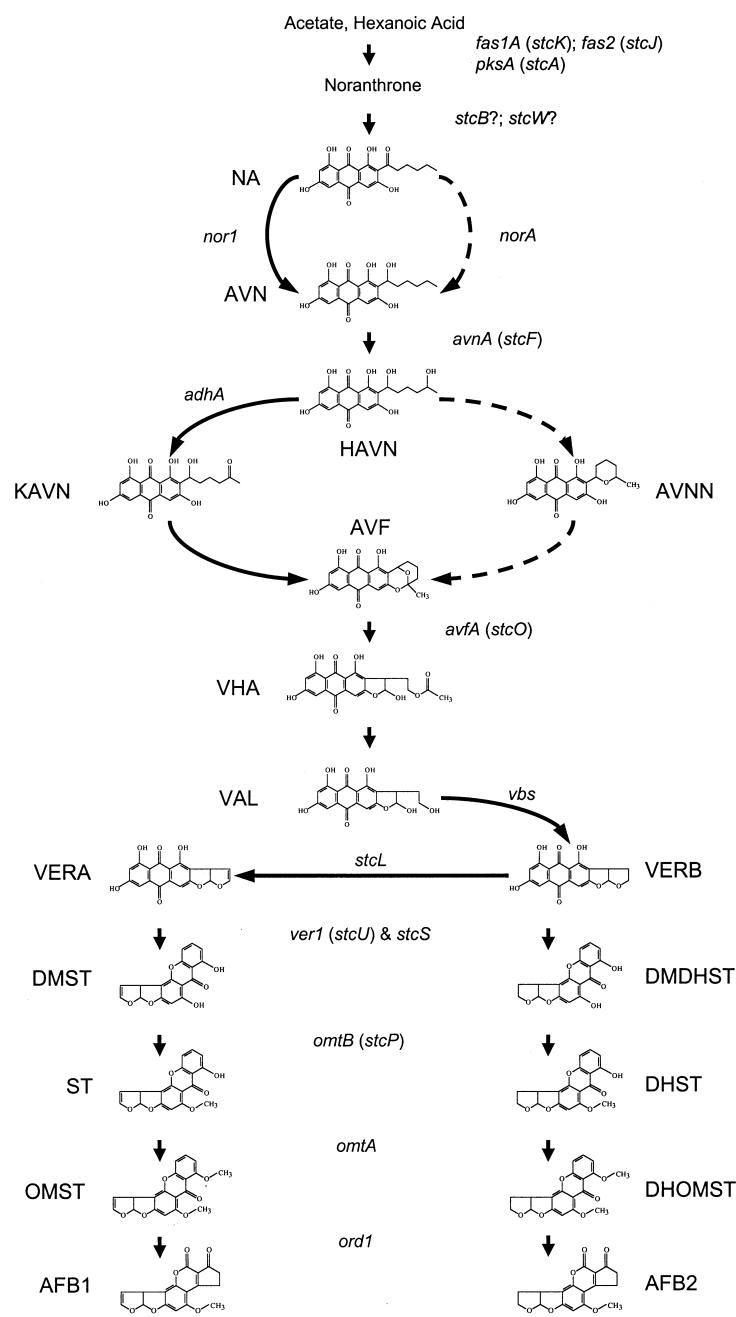


Figure 1 The aflatoxin/sterigmatocystin biosynthetic pathway. Gene names given in italics, with the genes from *A. nidulans* in parentheses. All other gene names are for *A. flavus/A. parasiticus*.

was identified based on sequence similarity to the *A. nidulans* *wA* gene, a PKS involved in spore pigmentation [23]. These two proteins each possess β -ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains, yet *WA* also has a thioesterase (TE) domain, which *PksA* lacks. Disruption of *pksA* in *A. parasiticus* led to inability of the fungus to produce AF and AF pathway intermediates but showed no effect on spore color [21]. Feng and Leonard [22] found that disruption of *pksL1* in *A. parasiticus* also yielded loss of AF and AF pathway intermediate production. In fact, *pksA* and *pksL1* are the same gene. BLAST analysis of the two genes reveals they are identical as was suggested in a previous review [15].

PKS activity leads to the production of noranthrone, presumably from a hexanoate starter unit [17]. The first stable intermediate in the AF pathway, however, is norsolorinic acid (NA; Fig. 1). It is as yet unknown how the conversion from noranthrone to norsolorinic acid occurs, and there are three competing hypotheses. The conversion may occur spontaneously, as suggested by Dutton [10]. Vederas and Nakashima [24] proposed that a noranthrone oxidase could be responsible for this conversion. To date, no such enzyme has been found in aflatoxigenic fungi. Others have proposed that a monooxygenase may be responsible [25]. This may be the most likely scenario as there are two known monooxygenases found in the ST biosynthetic cluster which have no assigned function at present: *stcB* and *stcW*.

2.3. Norsolorinic Acid to Versicolorin B

The steps from NA to versicolorin B (VERB) are fairly well established, with the only real controversy existing at the first step from NA to averantin (AVN; Fig. 1). Two genes have been identified with the capacity to carry out the reduction of NA to AVN: *nor-1* and *norA*. *nor-1* was the first AF biosynthetic gene cloned, and was able to complement the NA accumulation phenotype in *A. parasiticus* [26]. Computer analysis of the gene revealed a putative peptide of 29 kDa and indicated the peptide would be a ketoreductase [27]. Recently, Zhou and Linz [28] purified recombinant Nor1 protein using an *Escherichia coli* expression system and confirmed the ketoreductase activity of this enzyme.

The second ketoreductase gene found in the AF cluster is *norA*. The protein encoded by this gene was isolated and shown to possess ketoreductase activity [29]. Monoclonal antibodies were raised against this purified protein and used to screen a cDNA library to isolate the *norA* gene. Computational analysis of the open reading frame encoded by *norA* showed similarity to the *Phanerochaete chrysosporium* aryl-alcohol dehydrogenase gene. The relationship of these two enzymes within the AF pathway is unclear. It is known that NA-accumulating mutants of both *A. flavus* and *A. parasiticus* still produce low levels of AF [30–34]. Perhaps the seeming redundancy at this step in the pathway alludes to the fecundity of norsolorinic acid production or to a need for the fungus to rapidly synthesize AF at the appropriate time (Fig. 1).

The next step in the pathway involves the conversion of AVN to 5'-hydroxyaverantin (HAVN) by *avnA*. Bennett et al. [35] were first to place averantin in the AF biosynthetic pathway through the use of ^{14}C -labeled averantin in feeding studies. The gene responsible for the AVN–HAVN conversion was first described by Yu and colleagues and given the designation *ord-1* when transcripts were identified using DNA sequence flanking the *omtA* pathway gene as a probe [36]. Sequencing and analysis of the cDNA clone corresponding to the *ord-1* transcript revealed homology to cytochrome P-450 enzymes, in particular monooxygenases and dehydrogenases. Disruption mutants of *ord-1* fail to produce AF

and accumulate a yellow pigment identical to averantin [37]. Metabolite feeding studies using norsolorinic acid or averantin failed to support AF production, whereas 5'-hydroxy-averantin, averufannin, averufin, versicolorin A, sterigmatocystin, and O-methylsterigmatocystin supported AF production. *avnA* shows high similarity to the *stcF* gene found in the *A. nidulans* sterigmatocystin biosynthetic cluster, a gene linked to this same conversion [38].

Recently, *adhA*, a gene encoding a putative alcohol dehydrogenase, was implicated in the conversion of 5'-hydroxyaverantin to averufin [39]. This gene, found within the AF biosynthetic cluster, exhibits coordinated expression with other known pathway genes when the fungus is grown under AF conducive conditions. Disruption of *adhA* in *A. parasiticus* leads to an accumulation of 5'-hydroxyaverantin and a small amount of O-methylsterigmatocystin. This is most likely due to a second, minor route of averufin formation through an averufannin intermediate (Fig. 1). The authors postulated that *adhA* may oxidize HAVN to 5'-ketoaverantin (KAVN), which could then spontaneously cyclize to form AVF [39]. This route, utilizing AdhA, is probably the major route in the pathway as evidenced by the phenotype of the *adhA* disruptant.

The conversion of AVF to versiconal hemiacetal acetate (VHA) has been hypothesized to include multiple enzymatic reactions [25]. To date, only one enzyme has been characterized as responsible for this conversion. Using an AVF-accumulating mutant, Yu and colleagues were able to determine that the *avfA* gene product from *A. parasiticus* was responsible, at least in part, for the conversion of AVF to VHA [40]. Transformation of the *avfA* gene into the AVF-accumulation strain complemented the phenotype to restore AF production. Computational analysis suggested that the *stcO* gene in *A. nidulans* may be the homolog of *avfA*. These researchers were also able to recover *avfA* homologs from *A. flavus* and *A. sojae*. It is still unknown whether this gene is solely responsible for the AVF–VHA conversion.

Nothing is currently known about the conversion from VHA to versiconal (VAL; Fig. 1). This step involves removal of an acetaldehyde group, but no enzyme has yet been demonstrated as responsible for this process. Versicolorin B synthase (*vbs*) has been shown to produce versicolorin B (VERB) from either enantiomers of VAL [41]. This is an extremely important step in the synthesis of AF as the bisfuran ring, which is responsible for the DNA-binding property of AF, is formed. *vbs* resides in the AF gene cluster approximately 3.3 kbp upstream of *omtA*, [36,42]. An open reading frame approximately 1,400 bp long separates the two genes, and putatively encodes a cytochrome P-450 monooxygenase. However, no function has been experimentally determined for this putative gene.

2.4. Versicolorin B to Aflatoxin

Versicolorin B represents a major branch point in the AF pathway (Fig. 1). The two major AFs, B1 and B2, are delineated at this step. AFB1 is the more carcinogenic of the two and is suspected to be a major contributing factor for liver cancer in humans [43,44]. VERB is converted to versicolorin A (VERA), which eventually leads to AFB1 production. *stcL*, a putative cytochrome P-450 monooxygenase, has been shown to be important in the VERB to VERA conversion in the *A. nidulans* ST pathway [45]. Disruptants that were fed exogenous VERA were able to produce both ST and DHST (dihydrosterigmatocystin), whereas those not fed exogenous VERA could only produce DHST. This enzyme putatively carries out the desaturation of the bisfuran moiety in VERB to yield VERA, but

this has not been experimentally proven. Furthermore, the homolog of *stcL* has not yet been identified in AF-producing species.

VERB can also be used as a substrate for another enzyme to produce demethyl-dihydrosterigmatocystin (DMDHST). This reaction, as well as the VERA to demethylsterigmatocystin (DMST) conversion, have been hypothesized to require up to four different enzymatic steps including ketoreduction, oxidation, decarboxylation, and methylation [25]. One gene shown in *A. nidulans* to be partly responsible for this conversion is *stcU*. The *stcU* gene encodes a putative ketoreductase and has been demonstrated as necessary for the conversion of VERB to ST and DHST in a *stcL* background [45]. The homolog of *stcU* in *A. parasiticus* is *ver1* [46]. Two copies of *ver1* exist in *A. parasiticus*: *ver1A* and *ver1B* [47]. The second copy of this gene, *ver1B*, was recently demonstrated to encode a truncated, inactive protein [48]. *ver1B* is located elsewhere in the genome of the fungus, away from the AF cluster, in an apparently duplicated AF cluster region [48].

The conversion from VERB/VERA to DMDHST/DMST also requires the protein product of *stcS*. This gene in *A. nidulans*, first named *verB* [49], encodes a putative cytochrome P-450 monooxygenase and has been hypothesized as responsible for the oxidation component of this conversion [50]. *stcS* disruptants failed to produce ST under conducive conditions and were shown in metabolite feeding studies to be involved at this step. However, Keller and colleagues [50] were unable to demonstrate oxidation capability for this protein using cross-feeding experiments with a *stcU* disruptant. No new data have been published for *stcS* as of this review.

The next two reactions in the AF biosynthetic pathway involve the addition of methyl groups [51]. Only one of these reactions occurs in *A. nidulans*, as ST is the final product in this fungus. *stcP* is the gene identified as responsible for the conversion of DMST to ST in *A. nidulans* [52]. Kelkar and colleagues demonstrated that a *stcP* disruptant of *A. nidulans* accumulated demethylsterigmatocystin and produced no ST. *stcP* transcripts had been previously shown to be coregulated with other ST pathway gene transcripts [53]. Homologs of *stcP* have been found in *A. oryzae* (*dmtA*), *A. parasiticus* (*dmtA* or *omtB*), *A. flavus* (*omtB*), and *A. sojae* (*omtB*) [40,54]. In no case has the gene been disrupted.

The second methylation presumably occurs as the result of the activity of the putative methyltransferase *omtA* (previously *omt-1*), driving the conversions of ST/DHST to O-methylsterigmatocystin (OMST)/dihydro-O-methylsterigmatocystin (DHOMST) [55,56]. Antisera raised against the 40 kDa protein isolated by Keller et al. [55] was used to isolate a cDNA clone from an *A. parasiticus* expression library. Upon further analysis, recombinant protein expressed in *E. coli* was capable of converting ST to OMST [56]. However, *omtA* remains to be analyzed by gene disruption.

The final step in the AF pathway leading to the production of AFB1 and AFB2 from OMST and DHOMST respectively is catalyzed by *ord-1*. Prieto and colleagues [57] first identified this gene utilizing cosmid constructs containing different segments of the AF gene cluster. They were able to show that one construct conferred the ability to convert OMST to AFB1 on *A. flavus* strain 649, which is a mutant lacking the entire AF cluster. Further analysis narrowed the placement of *ord-1* to a 3.3 kb region of the cosmid. Subsequently, Prieto and Woloshuk [58] were able to identify the coding sequence of *ord-1*. This gene codes for a putative cytochrome P-450 monooxygenase, which is responsible for this final oxidoreduction step. Ord-1 protein was expressed in *S. cerevisiae* and demonstrated to convert exogenously fed OMST to AFB1.

3. REGULATION

The regulation of AF biosynthesis has been studied since 1965, when the role of kojic acid was investigated [59]. Since that time, four major nutritional and environmental stimuli affecting AF production have been characterized: nitrogen source, carbon source, temperature, and pH. Additionally, development plays a major role and may be an overriding factor in AF production. Indeed, the regulation of this mycotoxin appears to be highly complex (Fig. 2).

The AF/ST biosynthetic pathway is under the direct control of the pathway specific transcriptional regulator, *aflR* [60–64]. Another gene which shares a 737-bp stretch of DNA as a promoter region with *aflR* and is divergently transcribed is *aflJ* [65]. The exact function of this gene is unknown. Meyers et al. [65] did show that if *aflJ* is disrupted, no AF is produced yet the pathway genes are transcribed under AF conducive conditions. Further, the *aflJ* open reading frame does not appear to encode a transcription factor as does *aflR*. The predicted peptide exhibits three membrane-spanning motifs and a peroxisomal targeting signal [65].

It is also possible that *aflJ* is regulated by the global nitrogen regulatory gene *areA*. This possibility was first alluded to in a study focused on *aflR* overexpression [66]. While the effects of *aflR* overexpression on nitrate inhibition were being determined, a slight inhibition of *aflJ* transcription by nitrate was demonstrated. While not quantitative, the decrease in transcription seen on a Northern blot is noticeable. A later study by Ehrlich and Cotty [67] showed that the effect of nitrate on expression of *aflJ* is dependent on the genotypic background in which the experiment is conducted. These researchers demonstrated that S_B strains of *A. flavus* were much less inhibited by nitrate than were S_{BG} strains of the fungus. They also showed that transcript levels of *aflJ* were induced 2.6-fold in the S_B background, whereas they were repressed 2-fold in the S_{BG} background. The authors speculated that this difference in response to nitrate may be due to the amount and placement of AreA consensus binding sites in the *aflJ/aflR* intergenic region.

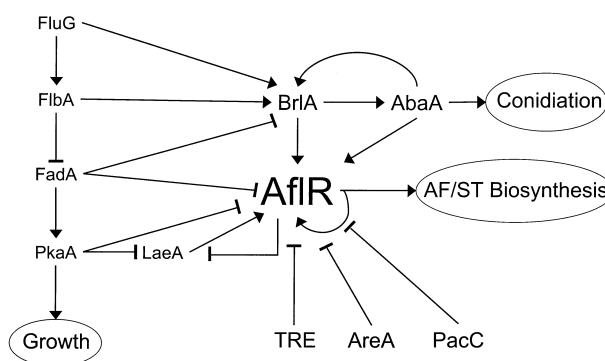


Figure 2 Representation of the regulatory apparatus impacting aflatoxin biosynthesis. The major inputs affecting the activity of the aflatoxin pathway specific regulator (*aflR*) are shown, including developmental (i.e., FadA), nutritional (i.e., AreA), and environmental (i.e., PacC) regulators. An unknown temperature response element (TRE) is inferred from the effect of temperature on the transcriptional and posttranscriptional control of *AflR*.

3.1. Nutritional and Environmental Factors

Aflatoxin is a complex carbon-containing molecule, and as such, it is reasonable to assume that certain carbon sources would be better substrates than others. Indeed, much research has been done that has identified supportive and inhibitive carbon sources [68–72]. Generally, simple sources of carbon, such as glucose, are supportive for AF production. More complex sources of carbon, such as peptone, lactose and oleic acid do not support AF production. The genetics of carbon source regulation have not yet been discovered.

Recently, a gene cluster was identified in *A. parasiticus* which is adjacent to the AF pathway [73]. This cluster of four genes includes a putative regulatory gene (*sugR*), a putative NADH oxidase (*nada*), a putative glucosidase (*glcA*), and a gene with homology to hexose transporters (*hxtA*). Yu and colleagues were able to show correlated expression of the *hxtA* gene with AF biosynthetic genes under conditions conducive for AF production. It is possible that this cluster is involved with the carbon regulation seen in AF-producing fungi, but as of yet no conclusive data have been shown.

Nitrogen source is a known influential factor for AF production. In general, organic sources support and inorganic sources do not support AF biosynthesis. Sources of nitrogen especially favorable to AF production are aspartate, glutamate, alanine, glutamine, proline, and ammonium sulfate [74–78].

The effect of nitrogen source on AF production is profound but not absolute. Kachholz and Demain [5] found that inorganic nitrogen sources, such as sodium nitrate, exhibited significant inhibition of averufin production in *A. parasiticus*. Sodium nitrate was the most inhibitory nitrogen source they tested, yet the fungus was still able to produce 65.9% of wildtype averufin levels on an mg/g dry weight scale. Further, these researchers found no effect of nitrogen source when fungal mycelia were resuspended in media containing either NH₄ or NO₃ as the nitrogen source [5]. One possible explanation for the nitrate effect involves NADPH consumption in the cell. NADPH is a common cofactor involved in enzyme reactions. It has been speculated that the mannitol cycle is upregulated in the presence of nitrate as the nitrogen source, and that this diverts NADPH away from AF pathway enzymes leading to their inactivity [79]. Preliminary data from our lab do not support this hypothesis as media amended with 100 μM NADPH were unable to ameliorate nitrate repression of AF biosynthesis (unpublished results).

Molecular evidence further supports the direct regulation of AF biosynthesis by nitrogen. The GATA-type transcription factor *areA* (*Neurospora crassa* homolog *nit2*) is the global transcriptional nitrogen regulator in *A. nidulans* [80]. This transcription factor has recently been isolated and characterized in *A. parasiticus* [81]. Chang and colleagues used electrophoretic mobility assays to show that AreA protein could bind GATA elements found in the 737 bp intergenic region between *aflR* and *aflJ*, suggesting a role in the regulation of one or both of these genes. The intergenic region between AF pathway genes *nor1* and *pksA* has also been investigated in *A. parasiticus* [64]. In this study, many potential regulatory elements were discovered, including putative binding sites for AflR and the nitrogen regulatory genes AreA and NirA (the pathway specific nitrogen regulatory gene in *A. nidulans*). Furthermore, at least one AreA-binding site was found in this region in three other AF producing fungi: *A. flavus*, *A. nomius*, and *A. pseudotamarii*. However, there was no AreA site found in the one AF-producing isolate of *A. bombycis*.

In some cases, there appears to be cooperation between factors repressive to AF biosynthesis. Katchholz and Demain [5], as well as studies in our lab (unpublished results) have shown nitrate inhibition in the absence of increasing pH. Flaherty and Payne [66]

demonstrated that nitrate inhibition of AF biosynthesis was due to a more complex phenomenon than simply repression of *aflR* transcription. Indeed, a strain of *A. flavus* expressing AflR constitutively was still unable to produce AF in media containing NO_3^- as the nitrogen source. The pathway gene *omtA* was expressed identically regardless of the nitrogen source used, but AF production was virtually nonexistent.

An association between AF repression by NO_3^- uptake and increased pH has been demonstrated [82]. Cotty showed that AF production decreased and number of sclerotia formed increased as pH increased in the presence of nitrate. The opposite was true for ammonia. However, when the ammonia cultures were buffered to maintain a pH above 4.4, sclerotia production was unaffected but AF levels dropped almost four-fold from the NH_4^+ -unbuffered levels.

Keller et al. [7] were able to show that increased pH is repressive to AF pathway gene transcription in its own right. In these studies, *A. nidulans* and *A. parasiticus* were grown under permissive nitrogen conditions, but the media were buffered in a pH range from 4–8. Transcripts of *stcU* and *ver1* were visualized by Northern blot using RNA harvested from tissue grown at pH 4, 5, 6, 7 or 8. Above pH 5, *stcU* transcripts were nonexistent; the same was true for *ver1* transcripts above pH 6. Production of AF and ST in these fungi paralleled the Northern blot data, dropping steadily as the culture pH rose.

Keller and colleagues data also link *pacC*, the global pH regulatory gene found in *A. nidulans*, to AF/ST pathway regulation. PacC is translated as an inactive pro-protein when the fungus is grown at acidic pH. As the ambient pH becomes more alkaline, the PacC pro-protein is proteolytically cleaved to yield the active transcription factor. This regulatory protein represses transcription of acidic-expressed genes and induces transcription of alkaline-expressed genes [83]. Keller et al. [7] found that *stcU* gene expression increased from 24–48 hours in a wild type background, but decreased over the same period in a *pacC* constitutive expression mutant. Furthermore, ST production also was lower as compared to wild type by as much as nine-fold.

The action of *pacC* expression on AF/ST biosynthesis is probably direct, as this transcription factor usually represses acid-expressed genes under alkaline conditions and AF/ST production is suppressed under these conditions. A putative PacC binding site has been reported in the promoter region of *aflR*, and has been shown to bind protein extracts from AF-induced mycelia of *A. parasiticus* [84]. While not conclusive proof, mutating the putative *pacC* binding site eliminated protein binding in the mobility shift assay performed by these researchers.

Temperature is also known to play a role in AF production, but the nature of the control it exerts on AF is still debated. Much early work was done to define the conducive temperatures for AF production [85–89]. Currently, it appears that temperature influences AF biosynthesis by affecting the transcription of pathway genes [8,90,91]. AF and ST production, in *A. parasiticus* and *A. nidulans* respectively, are regulated in opposite manners. In *A. parasiticus*, AF production was maximal at 27°C, and no AF was detected at 37°C [8]. Conversely, ST was generously produced at 37°C, and only small amounts were produced at 27°C. Feng and Leonard demonstrated that pathway genes, including *pksA*, *aflR*, *stcA*, *stcE*, and *stcD*, were not transcribed at the nonpermissive temperature in each species, which argues for transcriptional control of AF/ST biosynthesis. These data are in contrast to those by Liu and Chu [91], who saw low-level expression of *aflR* at restrictive temperatures in *A. parasiticus* and *A. nidulans* as well as lower AflR accumulation with no accumulation of the late-pathway protein OmtA at restrictive temperatures. This is consistent with the finding that AflR is under posttranscriptional control by PkaA, a protein

kinase responsible for regulation of development and secondary metabolism in *A. nidulans* [92]. Perhaps transcriptional profiling will assist in determining the identity of these (post)-transcriptional regulatory elements (TRE; Fig. 2).

3.2. Influence of Development

Fungal development and secondary metabolism are inextricably linked. Current knowledge points to a heterotrimeric G-protein coupled receptor as the starting point for a signaling cascade leading to conidiation and secondary metabolite production [93,94]. Understanding of the link between the two may yield improvements in human and animal health through the disconnection of fungal development and mycotoxin production.

The link between secondary metabolite production and development wasn't established until recently, when Hicks and colleagues showed that a G-protein signal was involved in both processes [6]. This research involved molecular characterization of a series of fluffy mutants, so-named because of their colony morphology due to a lack of conidiation [95]. Of the six loci/genes initially described as having the fluffy phenotype, two were found by Hicks and colleagues to act in a regulatory capacity: *fluG* and *flbA* (Fig. 2). Mutations in these two genes resulted in loss of both conidiation and sterigmatocystin production. *fluG* encodes a putative enzymatic protein believed to be responsible for producing an extracellular signal for development [96]. Later work by this group demonstrated that overexpression of *fluG* in submerged culture lead to conidiation but not sterigmatocystin production [97], suggesting other factors are involved.

Loss of function mutations in *flbA* share the *fluG* mutant phenotype. In addition, cultures with this mutation exhibit autolysis after a few days [6]. Interestingly, FlbA has been linked to G-protein signaling and contains a regulator of G-protein signaling domain [98]. FlbA interacts with FadA, a G-protein a subunit which represses development and secondary metabolism in *A. nidulans* [6,99]. Maintenance of vegetative growth is possible through FadA–GTP association. Once the GTPase activity of FadA is engaged, presumably through some action by FlbA, the fungus is permitted to continue development, and conidiation and secondary metabolism commences [6].

Other genes have been isolated from this G-protein signaling pathway in recent years. A cAMP protein kinase cascade has been inferred to interact with this system through the discovery of *pkaA*, a cAMP-dependent protein kinase which is believed to inhibit the pathway specific transcription factor AflR via phosphorylation [94] (Fig. 2). More recent evidence may also point to another gene, *leaA*, which may mediate the repression by *pkaA* [94]. *leaA* appears to be negatively regulated by PkaA and AflR. Further work must be done to link these early steps with the AF/ST pathway.

4. NEW TOOLS FOR DISCOVERY

4.1. Genomics Tools

Recent advances in the field of molecular biology have lead to an explosion of information. The new field of genomics is leading to breakthroughs in agriculture and medicine by allowing the study of cellular processes on a holistic level. Whereas regulatory pathways were traditionally studied one gene at a time, we can now view the entire transcriptional complement of a cell at once [100,101]. By combining two-dimensional gel electrophoresis and mass spectrometry, we can know if those transcripts are being translated into protein.

Advances in mass spectrometry are also making possible the elucidation of the entire metabolite profile of an organism. Clearly, these are exciting times.

Of these three main genomics technologies, transcriptional profiling (e.g. microarrays) will prove the most useful in exposing the regulatory networks involved in AF/ST production. Indeed, cDNA microarray technology has already been used to this end in other systems. Transcriptional profiles have been used to more accurately define cancer cell types by exposing the transcriptional programs inherent to those cell types [102]. Traditional methods of classifying lymphomas often group cancers into types that cannot be further divided due to irreproducibility of observations between diagnosticians.

Alizadeh and colleagues used a specialized microarray developed in-house (known as the Lymphochip) to analyze various B-cell lymphomas and determine distinct transcriptional profiles for them [103]. Using 96 normal and malignant cell types to generate 1.8 million data points, the researchers were able to resolve distinct subclasses of the diffuse large B-cell lymphoma cancer type. This was done by building a compendium of 128 array experiments using the 96 cell types. Hierarchical clustering of the data revealed common expression patterns of subsets of the 17,856 elements on the array, leading to distinct transcriptional fingerprints for the various cancer types.

Transcriptional profiling has also been used to reveal regulatory networks in *Saccharomyces cerevisiae*. When using a microarray representing all the open reading frames of yeast, DeRisi and colleagues found genes that changed in their expression pattern with time as the fungus shifted from anaerobic to aerobic metabolism [104]. Of particular interest were those genes with transcription profiles that changed in the same manner as genes known to be involved in fermentation and respiration. More than 400 genes of unknown function were discovered to fit this scenario, giving the first glimpse as to their function.

New methodologies utilizing cDNA microarray technology show even more promise for determining unknown gene function. In particular, a database (or compendium) of expression profiles can be utilized to narrow the focus of research to a few genes. Using 300 different growth conditions, including different nutritional and environmental cues, and various mutants, a compendium of expression profiles was created in an attempt to elucidate the function of unknown genes in *S. cerevisiae* [105]. Using a compendium approach, microarray fluorescence intensity values were clustered within tissue types to reveal similarly expressed genes. The tissue types were then clustered based on expression pattern. Using this approach in yeast, Hughes and colleagues were able to define the functions of eight previously undefined genes and also to discover a new drug target [105]. Perhaps application of this approach to the study of AF/ST pathway regulation will likewise illuminate the function of heretofore undefined genes.

4.2. Metabolic Pathway Analysis

Another approach that holds promise for further understanding the complex biochemistry of AF/ST biosynthesis is metabolic pathway analysis (MPA). This type of “genomic” analysis traces its theoretical roots to the 1980s and involves analyzing the stoichiometric properties of metabolic networks. While MPA is powerful for gaining insights into metabolic pathway regulation, it is highly mathematical in nature and beyond the scope of this review. Here, the overriding ideas behind MPA will be discussed and an example of using this type of analysis in studying primary metabolic pathways will be given. The reader is referred elsewhere for more indepth coverage of this topic [28,106].

From the biologist's perspective, MPA is basically involved with measuring the amount of metabolite flux through a system [28]. This property of metabolic pathways can be modeled mathematically, using the formula $S \cdot v = 0$, where S is a stoichiometric matrix made up of two vectors, m and n , that correspond to the number of metabolites and reactions in the network respectively. The vector v refers to the speed or activity of each reaction (n). Because of the nature of this design, the model is scaleable and could potentially encompass the entire metabolic network of a cell.

This kind of analysis has been used to study the metabolic control of the glycolytic pathway in *Trypanosoma brucei*, *Leishmania donovani*, and *Trichomonas vaginalis* [107]. Various genes involved in glucose metabolism, such as glyceraldehyde phosphate dehydrogenase (GAPDH) and phosphoglucose isomerase (PGI), and housekeeping genes, such as β -tubulin, were analyzed by using a gene regulation model: $1 = \rho_h + \rho_m$. Here ρ_h equals the change in enzyme concentration divided by the change in flux, and ρ_m equals the change in enzyme rate divided by the change in concentration of surrounding metabolites, multiplied by the change in concentration of surrounding metabolites divided by the change in flux. ρ_m is summed over all metabolites that change. Northern blot analysis was performed on all the genes being analyzed to track their expression levels throughout the experiment. Also, the various constituents of the model were measured, such as protein levels and enzymatic activities.

According to ter Kuile and Westerhoff, some genes in the glucose pathway are mostly metabolically regulated [107]. This was determined by plotting glucose consumption against enzyme activity, and comparing the results to the northern blots. For example, glycerol-3-phosphate dehydrogenase showed no increase in enzyme activity over nearly three orders of magnitude increase of glucose consumption, even though expression levels of the gene changed. This was also the case for PGI. Some genes showed a mix of transcriptional and metabolic regulation, such as GAPDH. In this case, enzyme activity was unchanged over one order of magnitude of glucose consumption. When the system reached a critical point between two and three orders of magnitude, enzyme activity increased as did the transcriptional component of the regulation.

ter Kuile and Westerhoff did note that mRNA levels for the genes studied did not correlate with enzyme activities, suggesting there can be differences in the conclusions drawn by analyzing a network with MPA versus transcriptional profiling. However, each method of pathway analysis has its place. MPA may serve a good purpose in illuminating fine-tuned regulation in the AF/ST pathway itself and lead to insights regarding why different cultures produce different amounts of mycotoxin even though they are grown under similar supporting conditions.

5. CONCLUSION

Fungi are amazing organisms, and can produce a wide array of secondary metabolites. Some are helpful, such as antibiotics and drugs used to control cholesterol levels in humans. Still, many secondary metabolites produced by fungi are toxic to plants and animals, and understanding of how they are produced and regulated will benefit society greatly.

Much has been learned in the past 40 years of studying AF production by *Aspergillus* spp., yet there is still much to be discovered about the regulation of this impressive toxin. New tools such as metabolic pathway analysis and transcriptional profiling hold promise for accelerating our understanding of AF production and for providing potential targets

for control. Hopefully these new technologies may even allow us to change these pathways in fungi and produce novel compounds that will benefit mankind for years to come.

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14

Indole-Diterpene Biosynthesis in Ascomycetous Fungi

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1. INTRODUCTION

The indole-diterpenes are a large, structurally diverse group of natural products principally found in filamentous fungi of the genera *Penicillium*, *Aspergillus*, *Claviceps*, and *Neotyphodium* [1–3]. These metabolites all have a common core structure comprising a cyclic diterpene skeleton derived from geranylgeranyl diphosphate (GGPP) and an indole moiety derived from either tryptophan or a tryptophan precursor [4–6]. Further complexity of the carbon skeleton is achieved by additional prenylations, different patterns of ring substitutions, and different ring stereochemistry. Many of these compounds are potent mammalian tremorgens [7] whereas others are known to have antiinsect activity [8].

Until recently, very little was known about the pathways for the biosynthesis of the indole-diterpenes, although putative biosynthetic schemes have been proposed on the basis of chemical identification of likely intermediates from the organism of interest and related filamentous fungi [9–11]. The recent cloning and characterization of a cluster of genes from *Penicillium paxilli* required for the biosynthesis of paxilline has provided the first insight into the genetics and biochemistry of indole-diterpene biosynthesis [12].

The structural complexity and potential biological use of these compounds have also attracted the attention of synthetic chemists. A number of paxilline-like indole-diterpenes have been successfully synthesized, principally by Smith and coworkers [13–15].

The combined chemical and biological approaches available to study the biosynthesis of these compounds will provide major insights into the molecular and genetic basis for the structural diversity and biological activities observed in nature.

2. TAXONOMIC DISTRIBUTION OF INDOLE-DITERPENES

Indole-diterpene metabolites have been reported in only two of the eight classes of ascomycetous fungi, namely the Eurotiomycetes (*Aspergillus* and *Penicillium*) and Sordariomycetes (*Claviceps* and *Epichloë*; Table 1). Even within these two taxonomic groupings, indole-diterpene biosynthesis is confined to just a few specific genera or species. This discontinuous taxonomic distribution is a common feature of other secondary metabolite biosynthetic pathways, and two hypotheses explain this pattern [16,17]. The first proposes acquisition of metabolite biosynthetic genes by a horizontal gene-transfer event [18]. While there is some supporting evidence for this hypothesis, there is no direct evidence for the mobility of these genes [19,20]. The second hypothesis proposes that secondary metabolite biosynthetic genes are ancestral in origin but have been lost in many groups of fungi as a consequence of rapid evolutionary processes [16]. Consistent with the latter hypothesis is the absence or dearth of these genes in the early diverging Archiascomycetes [21] and Hemiascomycetes [22] compared with the Euascomycetes [23]. As more fungal genomes are sequenced, key questions about the function, distribution, and evolution of these genes may be answered through comparative genomic and phylogenomic approaches.

Within the Eurotiomycetes, the ability to synthesize indole-diterpenes is commonly associated with species that form sclerotia—large reproductive bodies which aid the survival of these fungi [8]. This tissue-specific expression is analogous to the selective synthesis of ergot alkaloids in the ergot (sclerotium) of *Claviceps* spp. [8]. Sclerotia are a rich source of secondary metabolites, including several novel types of indole-diterpenes (Table 1) [24–27], and interestingly many of these compounds have potent antiinsect activity. The ability of these fungi to synthesize biologically active metabolites may aid the survival of these reproductive structures by protecting them from fungivorous insects. Synthesis of indole-diterpenes within the Sordariomycetes has been reported for several species of the Clavicipitales [28–31] and at least one species each of the Diaporthales [32] and the Xylariales [33,34] (Table 1). Many of the clavicipitaceous fungi are known parasites of plants, insects, and other fungi. Probably the best known group are the *Epichloë/Neotyphodium* endophytes, which form symbiotic associations (symbiota) with grasses [35–37]. The ability of endophytes to synthesize biologically active secondary metabolites confers a major ecological benefit to the symbiotum [35]. However, synthesis of these metabolites in forage grasses is detrimental to grazing livestock and can result in livestock disorders such as ryegrass staggers [38], a syndrome associated with elevated levels of the indole-diterpene, lolitrem B, in the grass. Consequently, there is considerable interest in identifying isolates that have either reduced synthesis of or a total lack of the ability to synthesize these mammalian toxins [39,40]. There is also interest in the molecular elucidation of the bisosynthetic pathways.

3. INDOLE-DITERPENE CHEMICAL DIVERSITY

The indole-diterpenes have been somewhat arbitrarily classified into six structural groups by Steyn and Vleggaar [3]. They are the penitrems, janthitrems, lolitrems, aflatrem, paxilline, and the paspaline/paspalinine/paspalitrems. The compounds of these six groups all possess the basic ring structure of paxilline. Paxilline is thought to be a pivotal biosynthetic precursor of many of these compounds [9,41]. To these six groups can be added the more recently discovered terpendoles, isolated from perennial ryegrass infected with *Neotyphodium lolii* [11] and *Albophoma yamanashiensis* (syn. *Chaunopycnis alba*) [42,43], the

Table 1 Distribution of Known Indole-Diterpenes Within the Filamentous Fungi

Taxonomy	Indole-Diterpene Class
Class: Eurotiomycetes; order: Eurotales; genera <i>Aspergillus</i> (teleomorph <i>Emericella</i>) and <i>Penicillium</i> (teleomorph <i>Eupenicillium</i>)	
<i>Emericella striata</i>	Emindole SA [45]; paxilline [94] ; paspaline, 13-desoxypaxilline, and emindole SB [46]
<i>E. desertorum</i>	Emindole DA [54]; emindole DB [55]; paxilline [54]
<i>E. nivea</i>	Emeniveol [95]
<i>Aspergillus flavus</i>	Aflatrem [96]; aflavazole [56]; four different aflavinines [27]; β -aflatrem [8]
<i>A. parasiticus</i>	Four different aflavinines [27]; aflavazole [56]
<i>A. sulphureus</i>	Sulpinines A-C, secopenitrem and penitrem B [25]; 10-oxo-11,33-dihydropenitrem B; 10,23-dihydro-24, 25-dehydroaflavinine [24]; radarins A-D [50]
<i>A. nomius</i>	Nominine [49]
<i>Eupenicillium crustaceum</i>	Two aflavinines, including 10,23-dihydro-24, 25-dehydroaflavinine [97,98]
<i>E. molle/E. reticulisperorum</i>	Two aflavinines, including 10,23-dihydro-24, 25-dehydroaflavinine [98]
<i>Eupenicillium shearrii</i>	Shearinines A-C [44]; 13-desoxypaxilline, paxilline, and three other paxilline-like metabolites [44]
<i>Penicillium paxilli</i>	Paspaline, paspaline B, and 13-desoxypaxilline [10]; paxilline [99]; 7 α -hydroxy paxilline, 7 α -hydroxy-13-desoxy paxilline, and 10 β -hydroxy-13-desoxy paxilline (PC-M6) [9]
<i>P. crustosum</i>	Penitrem A-F [5, 100]; Penitrem G [100]; PC-M6 [82]
<i>P. janczewskii</i>	Penitrem A, 19-desoxypaxilline-16 β -ol (PC-M6) [84]; penitrem A and E and pennigritrem [85]
<i>P. janthinellum</i>	Janthitrems B and C [85]; janthitrems A-D [72,101]
<i>P. thiersii</i>	Thiersinines, emindole SB [51]
<i>Petromyces muricatus</i>	Petromindole [47, 48]
Class: Sordariomycetes; order: Clavicipitales; family: Clavicipitaceae; genera: <i>Claviceps</i>, <i>Epichloë</i> (anamorph <i>Neotyphodium</i>), <i>Chaunopycnis</i>	
<i>Claviceps paspali</i>	Paspaline and paspalicine [102,103]; paspalinine [30]; paspalitrem A and B [61]; paspalitrem C [104]; emindole SB [4]
<i>Epichloë festucae</i>	Lolitrem B [52,105]; paxilline and lolitrem B [29]
<i>Neotyphodium lolii</i> ^a	Paspaline [11], 13-desoxypaxilline [11]; terpendole M [11]; paxilline [28,29]; 7 α -hydroxy paxilline, 7 α -hydroxy-13-desoxy paxilline, and 10 β -hydroxy-13-desoxy paxilline (PC-M6) [9]; lolitrem B [29,31]; lolitrem E [74]; lolitrem A [63]; lolilline [66]; lolitriol [62]; lolicine A and B [106]; lolitriol [106]; lolitrem N [106]; lolitrem F [65]
<i>Chaunopycnis pustulata</i>	Five compounds, including hydroxy paspalinic acid [107]
<i>C. alba</i>	Six compounds, including hydroxy paspalinic acid and nalanthalide (a diterpene pyrone) [107].
<i>Albophoma yamanashiensis</i> ^b	15 compounds, including terpendoles A-K and emindole SB [43]; terpendoles A-D, emindole SB, and paspaline [42]
Class: Sordariomycetes; order: Diaporthales; genus: <i>Phomopsis</i>	
<i>Phomopsis</i> spp.	Paspalitrem A and C [32]
Class: Sordariomycetes; order: Xylariales; genus: <i>Nodulisporium</i>	
<i>Nodulisporium</i> spp.	Nodulisporic acids A1 and A2 [33,34]

^a Previously named *Acremonium lolii* [108]; detection in almost all cases from *N. lolii*-infected perennial ryegrass.

^b Identical in morphology to *C. alba* [107].

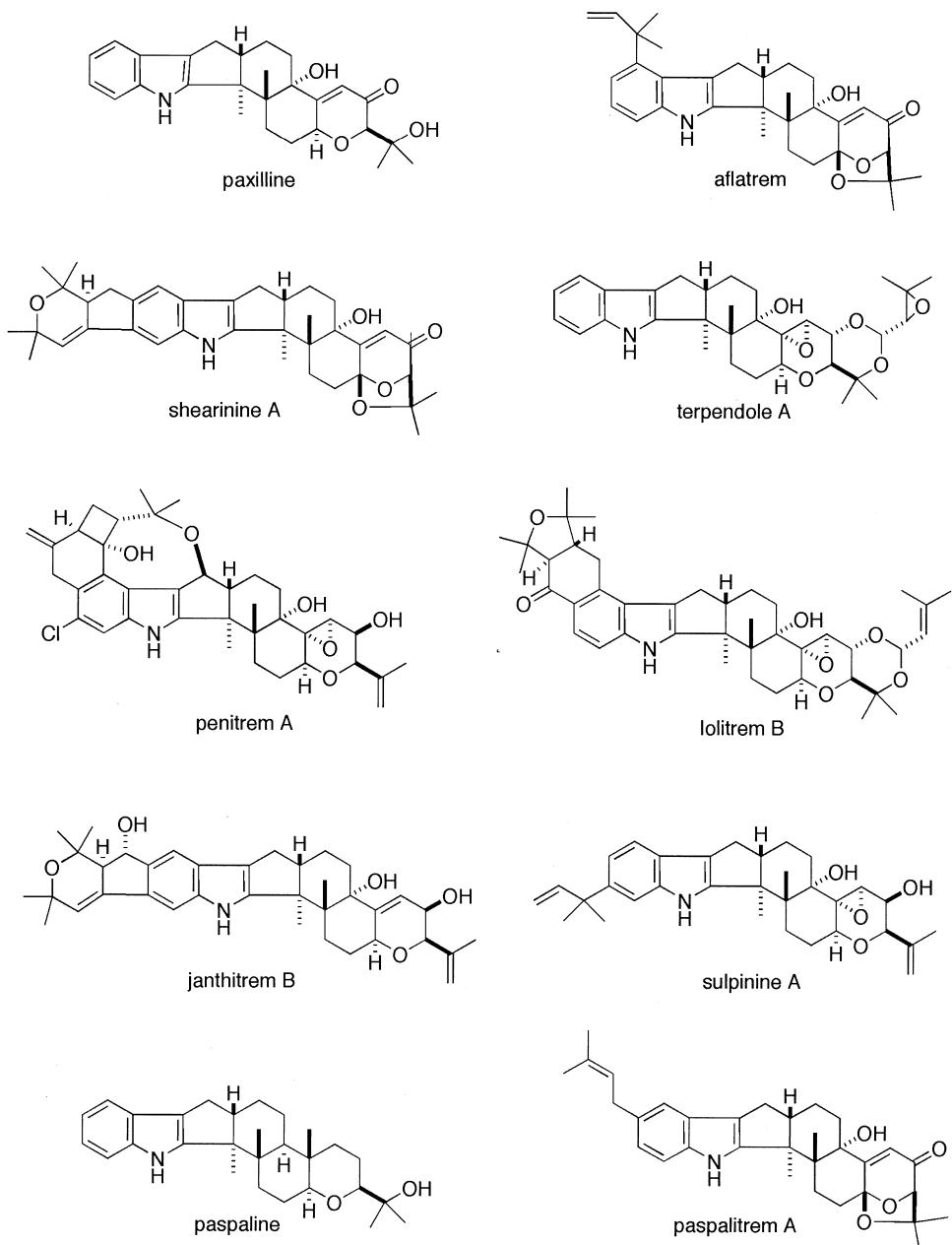


Figure 1 Paxilline-like indole-diterpenes

shearinines from *Eupenicillium shearrii* [44], and the sulpinines from *Aspergillus sulphureus* [25] (Table 1). These compounds also all possess a paxilline-like carbon skeleton (Fig. 1).

There is also a growing class of fungal indole-diterpenes with a skeletal framework that differs from that of paxilline (Fig. 2). This class of compounds includes the emindoles, isolated from *Emericella* spp. [45,46], nodulisporic acid from *Nodulisporium* sp. [33,34],

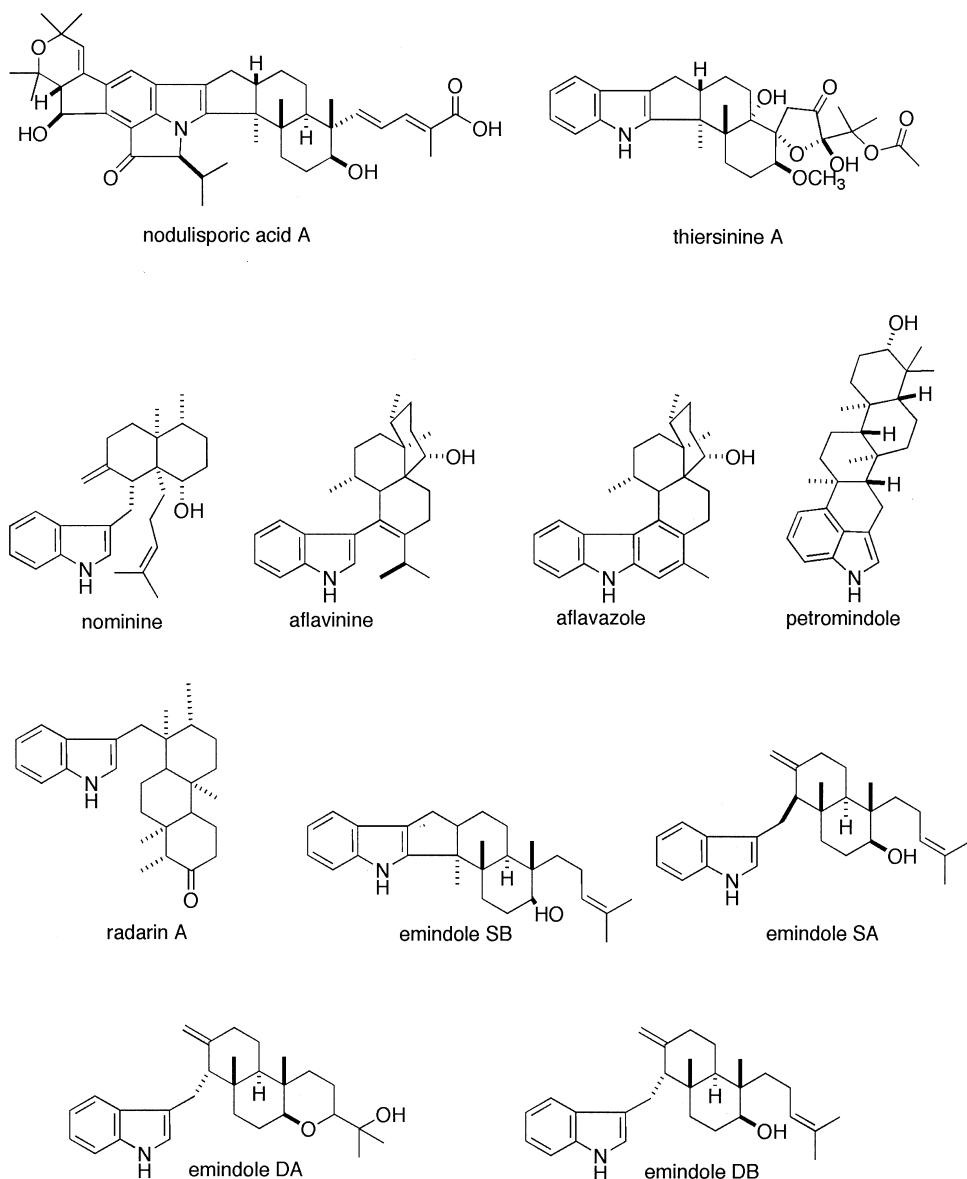


Figure 2 Other fungal indole-diterpenes

petromindole from *Petromyces muricatus* [47,48], nominine from sclerotia of *Aspergillus nomius* [49], the aflavinines from sclerotia of *A. flavus* [27], the radarins from sclerotia of *A. suphureus* [50], and the recently discovered thiersinines isolated from *Penicillium thiersii* which possess a unique spirocyclic structure [51] (Fig. 2).

3.1. Structural Diversity of the Paxilline-Like Indole Diterpenes

The paxilline-like compounds make up the majority of the more than 70 fungal indole-diterpenes isolated so far [52]. These compounds all have the common tetracyclic diterpene core fused to an indole moiety. For most of these compounds, including paxilline, the tetracyclic portion of the diterpene-derived skeleton has 19 rather than 20 carbons. However, there are a smaller number of compounds, including paspaline and paspaline B, in which all 20 carbons are present. Paspaline has been shown to be a biosynthetic precursor to paxilline [53]. The structures of the more complex members of this class of fungal indole-diterpenes can be considered in relationship to the paxilline skeleton (Fig. 3). The lolitrem, penitrem, janthitrems, and shearinines all have two additional isoprene units substituted onto the benzylic ring of the indole functionality leading to the formation of more complex cyclic structures. The paspalitrems, aflatrem, and the sulpinines have only a single additional benzylic isoprenoid substitution. The paspalitrems, paspalinine, paspalicine, and aflatrem have an additional ring structure through oxidation and acetalization at C7. The terpendoles and the lolitrem have an additional isoprene unit added to the C27 hydroxyl. The structures of the compounds within the groups exhibit different patterns of hydroxylations, epoxidations and acetylations, chlorinations, and ring stereochemistry.

3.2. Structural Diversity of Other Indole-Diterpenes

A number of different skeletal variations have been identified for the non-paxilline-like compounds (Fig. 2). Emindole SB [46], nodulisporic acid A [33,34], and thiersinines A

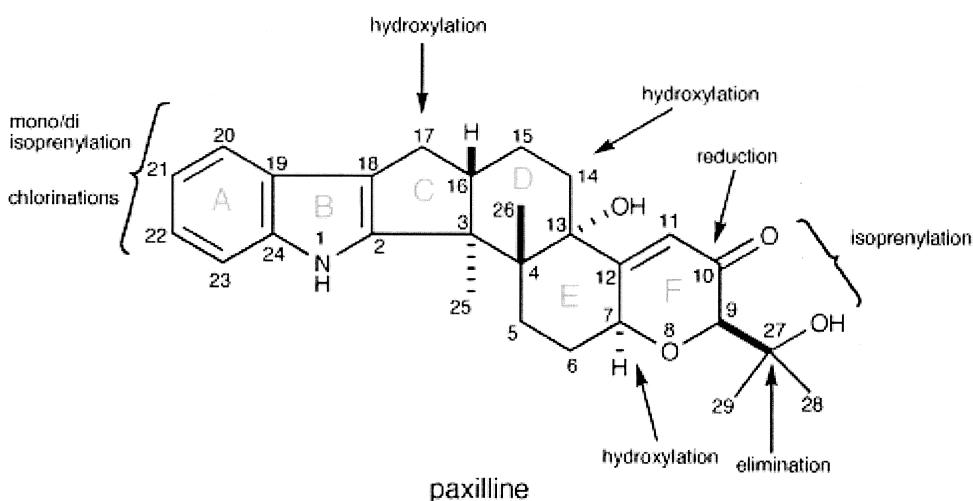


Figure 3 Summary of modifications/variations to the paxilline skeleton.

and B [51] are most paxilline-like with the C, D and E rings of paxilline present with the same stereochemistry. The thiersinines also are hydroxylated at C13, and the diterpene-derived skeleton is C19, as is found in paxilline, rather than C20. The less functionalized emindole SB has been proposed as an intermediate in paxilline/paspaline biosynthesis [10,46]. The other emindoles (DB, DA, and SA) are bicyclic or tricyclic structures attached to the indole unit at only one position [45,54,55].

The other compounds in this class have considerably different structures to paxilline. The aflavinines [27], nominine [49], and aflavazole [56] all appear to have a common core. Aflavazole also possesses a carbazole moiety [56]. In petromindole, the diterpenoid is fused to both the pyrrolic and benzylic parts of the indole unit [47,48].

4. BIOACTIVITY OF INDOLE-DITERPENES

Although indole-diterpenes possess a number of biological effects including insect feeding deterrence [8,57], modulation of insect [58] and mammalian ion channels [59,60], and inhibition of specific enzymes [42], their most dramatic effect is tremorgenic activity [31,61,62] (Table 2). Lolitrem B [62], lolitrem A [63], lolitrem C [64], lolitrem F [65], paxilline [62], terpendole C [66], aflatrem [67], paspalinine [61,68], paspalitrem A and B [61], penitrems A to D [69], and janithitrems B and C [70–72] are all potent mammalian tremorgenic agents [7,73] (Table 2). Paspalicine [30,61], α - and β -paxitriol [62], 13-desoxypaxilline [64], terpendoles D-I [64], lolitrem M [64], lolitrem E [74], lolitriol [62], and paspalicine [61], are all nontremorgenic in animal bioassays (Table 2). The clinical symptoms induced by these mycotoxins include diminished activity and immobility followed by hyperexcitability, convulsions, muscle tremor, ataxia, and tetanic seizures [75]. However, death seldom occurs and the animals recover if removed from the toxic feed source. The mechanism by which these fungal tremorgens act is not well defined, but biochemical and clinical studies indicate that these effects appear to be due in part to effects on receptors and interference with neurotransmitter release in the central and peripheral nervous systems [60,76]. In support of the proposal that some of these compounds act as surrogates of known activators of neurotransmitter release [73], aflatrem has been shown to potentiate chloride currents through GABA_A receptor chloride channels heterologously expressed in *Xenopus* oocytes [59].

Many indole-diterpenes have also been found to be potent inhibitors of high conductance Ca²⁺-activated K⁺ (maxi-K) channels [60]. The discovery of peptide toxins that bind to these channels, such as charybdotoxin (ChTX), has provided a powerful tool to study the action of indole-diterpenes on maxi-K channel activity. Paspalitrem A, paspalitrem C, aflatrem, penitrem A, and paspalinine inhibit binding of ChTX to maxi-K channels whereas paxilline and paspalicine enhance ChTX binding [60]. Despite these different allosteric interactions, all of these compounds are potent inhibitors of maxi-K channels [60]. Interestingly, paspalicine, a deshydroxy analogue of paspalinine lacking tremorgenic activity, is a potent inhibitor of maxi-K channels. This result suggests that tremogenicity may be unrelated to channel block [60]. Recent work showing that 13-desoxypaxilline has only a minor effect on maxi-K channels indicates that the C-13 OH group on paxilline is important for its ion channel activity [53]. The C-13 OH group is also important for tremogenicity [64].

Another important biological property of indole-diterpenes is their potent insecticidal activity [8]. Nodulisporic acid exhibits potent insecticidal activities against larvae of the mosquito (*Aedes aegypti*) [34], larvae of the blowfly (*Lucilia seracata*) [34], and larvae

Table 2 Biological Activities Associated with Indole-Diterpenes

Indole-Diterpene	Biological Activity
Paxilline	Potent tremogen in mice [9,62,66]; potent inhibitor of maxi-K channels and enhances ChTX ^a binding [60]; Ki of 30 nM [53]
13-Desoxypaxilline	Nontremogenic to mice [64]; very weak inhibitor of maxi-K channels; Ki of 730 nM [53]
7 α -Hydroxy-13-desoxy paxilline	Nontremogenic to mice [9]
α -Paxitriol	Nontremogenic to mice [62]
β -Paxitriol	Nontremogenic to mice [62]
Paspaline	Nontremogenic to mice [61]; no inhibition of maxi-K channels [53]; slight inhibition of acyl-CoA:cholesterol acyltransferase [42]
Aflatrem	Potent tremogen [67]; potent inhibitor of maxi-K channels and inhibitor of ChTX binding [60]
Paspalinine	Tremogenic [61,68]; potent inhibitor of maxi-K channels and inhibitor of ChTX binding [60]
Paspalicine	Nontremogenic [30,61]; potent inhibitor of maxi-K channels and enhancer of ChTX binding [60]
Paspalitrem A	Tremogenic [61]; potent inhibitor of maxi-K channels and inhibitor of ChTX binding [60]
Paspalitrem B	Tremogenic [61]
Paspalitrem C	Potent inhibitor of maxi-K channels and inhibitor of ChTX binding [60]
Penitrem A	Potent inhibitor of maxi-K channels and inhibitor of ChTX binding [60]
Penitrem B	Potent antifeedant activity against <i>Helicoverpa zea</i> [25]
Penitrem A–F	Tremogenic [69]; active against hemipteran <i>Oncopeltus fasciatus</i> and dipteran <i>Ceratitis capitata</i> [100]
Penitrem G	Inactive against <i>Oncopeltus fasciatus</i> and <i>Ceratitis capitata</i> [100]
10-oxo-11,33-dihydronitrem B	Antifeedant activity against corn earworm (<i>Helicoverpa zea</i>) and dried fruit beetle (<i>Carpophilus hemipterus</i>) [24]
Janthitrems B, C	Tremogenic [70–72]
Janthitrems E–G	Tremogenicity not known [101]
Lolitrem B	Potent tremogen in mice [62,63,66]
Lolitrem A	Tremogenic to mice [63]
Lolitrem E	Nontremogenic to mice [74]
Lolitrem F	Tremogenic to mice [65]
Lolitrem C	Tremogenic to mice [64]
Lolitrem M	Nontremogenic to mice [64]
Lolitriol	Nontremogenic to mice [62]
Lolilline	Nontremogenic to mice [66]
Terpendole C	Potent tremogen in mice [66]
Terpendoles A, B, D	Potent inhibitor of acyl-CoA:cholesterol acyltransferase [42]
Terpendole M	Potent inhibitors of acyl-CoA:cholesterol acyltransferase [42]
Terpendoles D–I	Mildly tremogenic in mice [11]
Terpendole E	Nontremogenic to mice [64]
Shearinines A–C	Inhibition of molecular motor kinesin [80] Significant antiinsect (<i>Helicoverpa zea</i> and <i>Carpophilus hemipterus</i>) activity [44]; shearinine B caused significant mortality against fall armyworm (<i>Spodoptera frugiperda</i>) [44]

Table 2 Continued

Indole-Diterpene	Biological Activity
Emindole SB	Slight inhibition of acyl-CoA:cholesterol acyltransferase [42]
Emeniveol	Pollen growth inhibitor [95]
Nodulisporic acid	Potent insecticidal activity against larvae of mosquito (<i>Aedes aegypti</i>) [34], larvae of blowfly (<i>Lucilia seracata</i>) [34], and cat flea (<i>Ctenocephalides felis</i>) [77]; potent inhibitor of insect (grasshopper and <i>Drosophila</i>) glutamate-gated chloride channels [58]
Aflavinines	Antifeedant activity against <i>Helicoverpa zea</i> and <i>Carpophilus hemipterus</i> [27]; some activity against <i>Bacillus subtilis</i> [27]
Aflavazole	Antifeedant activity against dried fruit beetle <i>Carpophilus hemipterus</i> [56]
Radarin A	Potent antifeedant activity against <i>Helicoverpa zea</i> [50]; active against human lung carcinoma and breast adenocarcinoma cells [79]
Radarin C	Antifeedant activity against <i>Helicoverpa zea</i> [50]
Radarins B, D	Inactive against <i>Helicoverpa zea</i> [50]
Thiersinines	Potent activity against the fall armyworm (<i>Spodoptera frugiperda</i>) [51]
Nominine	Potent antifeedant activity against <i>Helicoverpa zea</i> [49]
Sulpinines	Potent antifeedant activity against <i>Helicoverpa zea</i> [25]; sulpinine C also active against <i>Carpophilus hemipterus</i> [25]; sulpinine A also active against human lung carcinoma and breast adenocarcinoma cells [79]
Secopenitrem B	Potent antifeedant activity against <i>Helicoverpa zea</i> [25]

^a Charybdotoxin.

of the cat flea (*Ctenocephalides felis*) [77]. This compound is a potent inhibitor of insect glutamate-gated chloride channels, which may be one explanation for its toxicity [58]. Sclerotia of *Aspergillus* and *Penicillium* spp. have been shown to be a rich source of insect antifeedant compounds [8]. Active compounds include the aflavinines [27], nominine [49], penitrem B [25], a penitrem analogue [24], shearinines [44], radarins [50], sulpinines, and secopenitrem [25] (Table 2). The aflavinines are very active against the fungivorous beetle *Carpophilus hemipterus* and show some activity against the lepidopteran pest *Helicoverpa zea* [27] (formerly *Heliothis zea* [78]). Nominine exhibits potent activity against *H. zea*, resulting in significant mortality and weight reduction in feeding test assays [49]. The sulpinines are active against both *C. hemipterus* and *H. zea* [25] and are also active against human carcinoma cell lines [79]. Radarin A shows potent antifeedant activity against *H. zea*, but radarins B and D are inactive [50].

With the exception of terpendoles M and C, most of the terpendoles are nontremogenic in mouse bioassays [11,64]. However, terpendoles A through D are potent inhibitors of mammalian acyl-CoA cholesterol transferase (ACAT) [42]. A recent report demonstrating that terpendole E is also an inhibitor of a mammalian kinesin [80] highlights the broad range of biological effects associated with this group of compounds.

5. BIOSYNTHESIS OF INDOLE-DITERPENES

5.1. A. Biosynthesis of Paxilline-like Indole-Diterpenes

The diterpene and indole origins of these compounds have been clearly established by a series of labeling experiments. Acklin et al. [4] supplemented cultures of *Claviceps paspali* with [1-¹³C]-, [2-¹³C]-, and [1,2-¹³C]-acetate, and the enriched paspaline that was isolated showed that acetate was readily incorporated into the diterpenoid part of the molecule via conversion to mevalonate [4]. A similar pattern of incorporation was observed for paxilline and the penitrem [5]. Incorporation of the indole unit of tryptophan into penitrem A in *Penicillium crustosum* was demonstrated by the feeding of (2RS)-[benzene ring-U-¹⁴C]-tryptophan and (2RS)-[indole-2-¹³C,2-¹⁵N]-tryptophan [5]. Recent labeling studies performed to investigate the biosynthetic origin of nodulisporic acid in *Nodulisporium* have cast some doubt on whether the indole moiety is derived directly from tryptophan or a tryptophan-derived precursor [81]. Byrne et al. [81] found that radiolabeled anthranilic acid, but not tryptophan, was incorporated into nodulisporic acid and proposed that the indole precursor for nodulisporic acid is indole-3-glycerol phosphate.

Aside from the tryptophan (or tryptophan precursor) and diterpene origin of these paxilline-like compounds, very little is known about the pathways for their biosynthesis. Biosynthetic schemes have been proposed [9–11], but until recently, none of the proposed steps had been validated by biochemical or genetic studies. The most recent proposed pathway to paspaline and paxilline begins by combining tryptophan and geranylgeranyl diphosphate (GGPP) [10] (Fig. 4). This acyclic precursor then undergoes cyclization initiated by epoxidation of the two terminal alkenes to give paspaline. The skeletal rearrangement that occurs during this process has been verified by labeling experiments using both [1-¹³C]- and [2-¹³C]-acetate [5]. Products of partial cyclization have been also isolated from *Emericella* spp., leading to the inclusion of emeniveol and emindole SB in this scheme [10,46,82].

The cyclization to form paspaline appears to be under precise enzymatic control giving only the desired ring structure of the paxilline-related indole-diterpenes. A number of other bicyclic and tricyclic isomers of paspaline, including emindoles DA, DB, and SA, have been isolated from *Emericella* spp., indicating that alternative cyclization pathways of indole and GGPP adducts are possible without skeletal rearrangement [45,46]. A biomimetic chemical synthesis of emindole SA has been achieved [13], but the low yield obtained emphasizes the power of enzyme catalysis in the control of the correct backbone folding in the formation of paxilline-related indole-diterpene compounds. A more recently attempted biomimetic synthesis of an emindole SB analogue lead to the synthesis of a pentacyclic stereoisomer of the desired product [83].

The conversion of paspaline to PC-M6, 13-desoxypaxilline, or paxilline requires the loss of the methyl group at C12 of paspaline to produce the C27 unit on which all the complex indole-ditepenoid tremorgens are based. Formation of paspaline B, an oxidized form of paspaline isolated from *P. paxilli* cultures, may be the first step in the overall demethylation of paspaline [10]. It is suggested that loss of formaldehyde (or formic acid following further oxidation) from paspaline B produces an intermediate that is further oxidized to give PC-M6, a compound isolated as a minor fermentation product from *P. crustosum*, *P. janczewskii*, *P. paxilli*, and *N. lolii* cultures [9,82,84]. Following formation of PC-M6, the pathway to paxilline is proposed to bifurcate with two reactions required for paxilline formation—C10 oxidation and C13 hydroxylation occurring in either order. The isolation of 13-desoxypaxilline from cultures of *E. striata* [46], *P. paxilli* [10], and

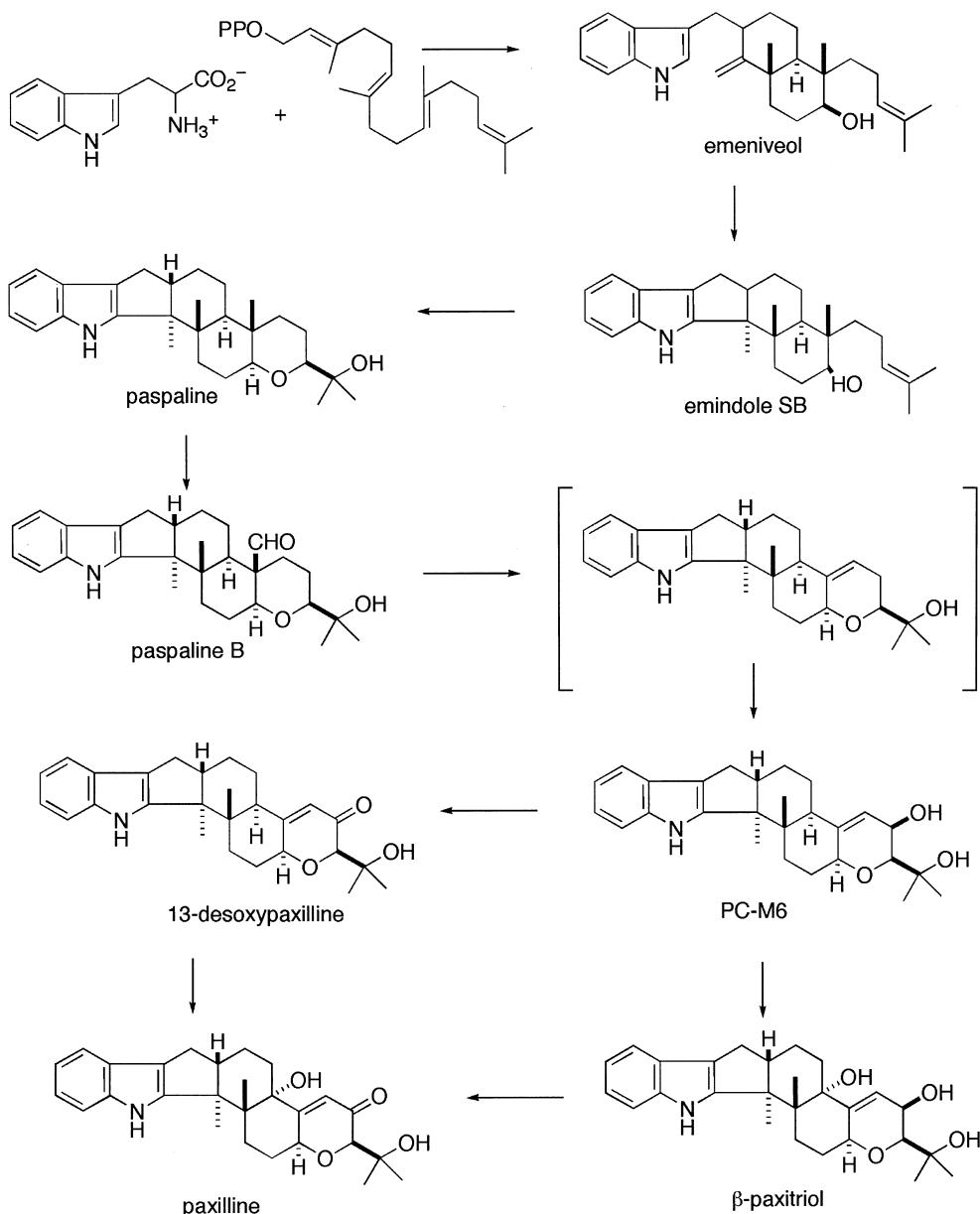


Figure 4 Biosynthetic pathway to paxilline as proposed by Munday-Finch et al. [10]

N. lolii infected perennial ryegrass [11] suggests that this compound is also a paxilline precursor. This was recently confirmed by isolating a mutant of *P. paxilli* that accumulates this compound [53]. The structure of β -paxitriol, a compound isolated in the acetate form from *P. crustosum* [82], would suggest that it is a possible penultimate intermediate in paxilline biosynthesis. Chemical feeding of putative intermediates to various paxilline-negative mutants (discussed later in this chapter) is consistent with the proposed bifurcation in the pathway between paspaline and paxilline.

Even fewer biochemical studies have been performed to unravel the biosynthetic pathways to the more functionalized compounds with paxilline-like skeletons. Radiolabeled paxilline and β -paxitriol (produced by chemical reduction of paxilline) fed to cultures of *P. janczewskii* and *P. janthinellum*, were shown to be incorporated into penitrem A and E as well as janthitrems B and C, respectively [84,85]. On the basis of these results, Penn and Mantle [85] suggested that paxilline may not be an obligate intermediate in the biosynthesis of those compounds with the β -stereochemistry. In these same experiments, α -paxitriol was not significantly incorporated into either the penitrems or the janthitrems, emphasizing the stereospecificity of the enzymes involved in these pathways. However, it may be an intermediate in the biosynthesis of the lolitrems and terpendoles, which all have the C10 α -stereochemistry. The testing of this hypothesis and further studies on the biosynthesis of the lolitrems has been considerably hindered by the inability of *N. lolii* to produce lolitrems in submerged culture. However, the link between lolitrem B and the occurrence of ryegrass staggers has encouraged a systematic analysis of the indole-diterpene profile in *N. lolii*—infected *Lolium perenne* (perennial ryegrass) seed and the proposal of a metabolic grid for the biosynthesis of the lolitrems [11,52,66] (Fig. 5).

From the range of compounds discovered it appears that the order in which a number of transformations occur can be varied. The terpendoles are a family of indole-diterpenes, isolated from *Albophoma yamanashiensis*, that are analogous to the lolitrems, lacking only the A/B ring structure [42,43,86]. The recent isolation of both lolitriol and terpendole M from *N. lolii* infected *L. perenne* indicates that the endophyte is capable of assembling the right-hand end of the lolitrem skeleton (attached to the diterpenoid) either before or after assembly of the A and B rings [11]. Additionally, isolation of terpendole E and lolicine A indicates that branching to the lolitrems may occur at paspaline as well as paxilline [10,43].

5.2. Biosynthesis of Other Indole-Diterpenes

It is apparent from the diversity of structures of the known fungal indole-diterpenes that the indole-diterpene precursor can cyclize in a number of ways. The pathways that have been proposed all involve epoxidation of at least one double bond for initiation of the cyclization cascade. Whereas emindole SB arises out of cyclization involving a bond migration as found in paspaline, emindoles DA and SA appear to arise out of an alternative cyclization without concomitant bond migration [45] (Fig. 6). Interestingly, the isolation of emindole SA along with paxilline from *E. striata* suggests that these alternative cyclization pathways can operate in a single species [45]. The labeling patterns obtained in precursor studies on nodulisporic acid A biosynthesis also reveal an emindole SB/paspaline type of bond migration in the biosynthesis of this compound [81]. Inspection of the structure of the thiersinines also suggests this type of rearrangement as part of the synthesis [51]. With isolation of emindole SB along with the thiersinines from *P. thiersii* [51], it is tempting

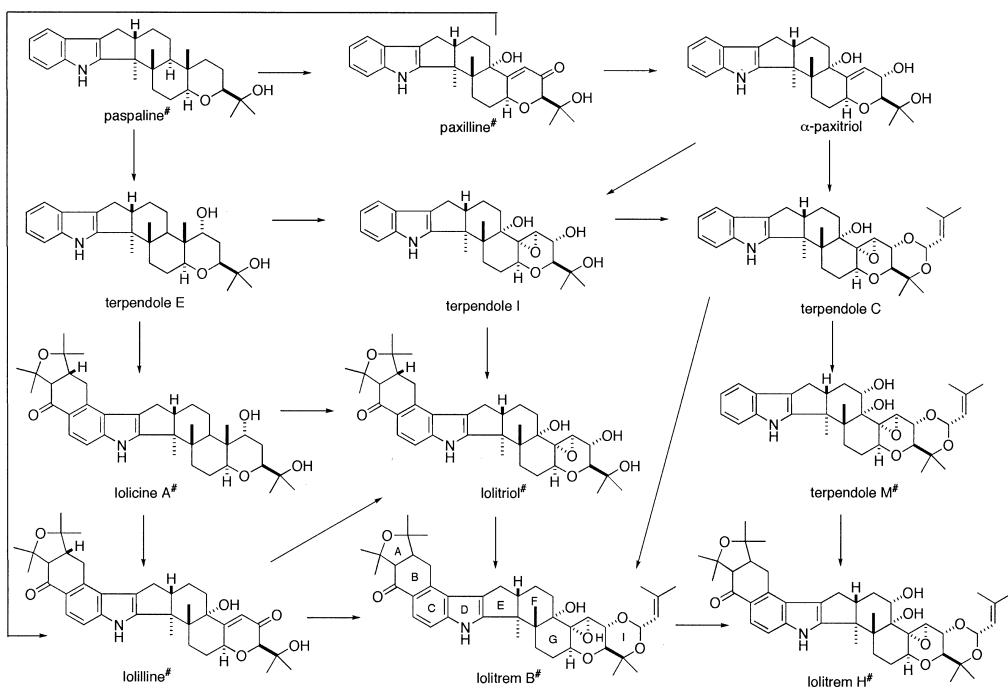


Figure 5 Metabolic grid to the lolitrems. Compounds marked with a hash have been isolated from endophyte-infected *L. perenne* (adapted from Lane et al. [52]).

to speculate that this emindole may be a precursor to these compounds. Nominine, the aflavinines, and aflavazole may also arise out of the same acyclic monoepoxidized species.

It has been proposed that the radarins and petromindole arise out of a cyclization pathway from acyclic indole-diterpene epoxidised at the terminal double bond [48]. This biosynthesis differs markedly from that of other indole-diterpenes in the regioselectivity of ring annulation and the formation of the rings most distal to the indole function. Formation of petromindole and the radarins is predicted to pass through a common intermediate with benzyl substitution yielding the former and backbone rearrangement giving the latter (Fig. 6). Based on the similarities between the structures of petromindole and pentacyclic triterpene lupeol, Xiong et al. [48] proposed that the putative petromindole cyclase is similar to a lupeol synthase. Using a lupeol synthase from *Arabidopsis thaliana*, these workers demonstrated the ability of this enzyme to catalyze the formation of petromindole and radarin C from the proposed acyclic precursor. As yet, no indole diterpene cyclases have been characterized.

5.3. Gene Cluster for Paxilline Biosynthesis

The recent cloning of a cluster of genes for paxilline biosynthesis in *Penicillium paxilli* (Fig. 7) has provided the first insight into the enzymes required for indole-diterpene biosynthesis [12]. Although the precise boundaries of the cluster must still be defined, gene inactivation studies have shown that at least five genes are required for paxilline biosyn-

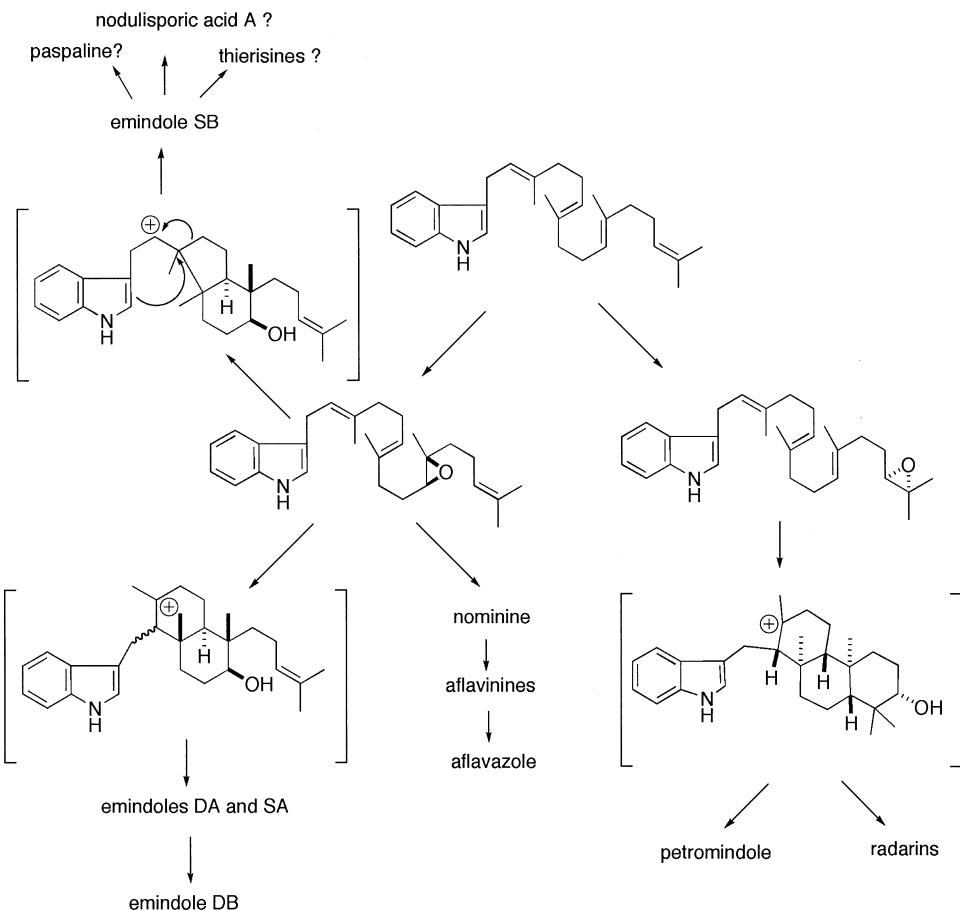


Figure 6 Alternative pathways for cyclization of a monoepoxidized acyclic indole-diterpene precursor

thesis [12,53]. The first committed step in the pathway is catalyzed by PaxG, a geranylgeranyl diphosphate synthase. *P. paxilli*, like *Fusarium fujikuroi* (teleomorph *Gibberella fujikuroi* [87]) which produces the gibberellins, contains two GGPP synthase enzymes—one for primary metabolism and one for secondary metabolism [12]. The recent discovery of two GGPP synthase genes in *N. lolii* (Young et al., unpublished results), an endophyte that synthesizes lolitrem B in perennial ryegrass, would suggest that a “signature” for identifying fungi that have the capability to produce diterpenes is the presence of two copies of the GGPP synthase gene. Deletion of *paxG* in *P. paxilli* resulted in a paxilline-negative phenotype, a result consistent with this gene being necessary for paxilline biosynthesis and the inability of the primary GGPP synthase to complement this mutation [12]. The complete absence of paxilline and other indole-diterpenes in a *paxG* deletion background would suggest that there is subcellular compartmentalization of the two GGPP synthases found in *P. paxilli* [12]. The presence of a specific GGPP synthase for paxilline biosynthesis suggests that IPP and DMAPP, rather than GGPP [10], are the carbon precur-



Figure 7 Paxilline biosynthesis gene cluster in *Penicillium paxilli*

sors from primary metabolism that support indole-diterpene biosynthesis. As discussed earlier, indole-3-glycerol phosphate is proposed to be the primary source of the indole group for this class of compounds [81].

Other genes required for the early steps in indole-diterpene biosynthesis in *P. paxilli* include *paxM*, encoding a FAD-dependent monooxygenase, and *paxC*, a diterpene cyclase. Mutants deleted for either *paxM* or *paxC* lack the ability to synthesize any identifiable indole-diterpene (Scott et al., unpublished results). On the basis of these results, it is conceivable that the first stable indole-diterpene product formed is paspaline, and that its synthesis requires the action of just three enzymes—PaxG, PaxM, and PaxC. Figure 8 outlines a proposed chemical scheme for the incorporation of indole-3-glycerol phosphate into the C20 terpenoid and cyclization (including skeletal rearrangement) of the product to form paspaline. To date, there is no evidence from gene disruption and radiolabeling experiments that emindole SB and emeniveol are intermediates in the biosynthesis of paxilline by *P. paxilli* (Scott et al., unpublished results). These compounds may represent a branch point rather than true biosynthetic intermediates.

At least three other genes are required for the biosynthesis of paxilline (Fig. 7). Deletion of *paxP* or *paxQ*, both coding for cytochrome P450 monooxygenase enzymes, gave rise to mutants that accumulated paspaline and 13-desoxypaxilline, respectively. This confirms that these two genes are essential for paxilline biosynthesis [53]. Furthermore, these results suggest that paspaline and 13-desoxypaxilline are the most likely substrates

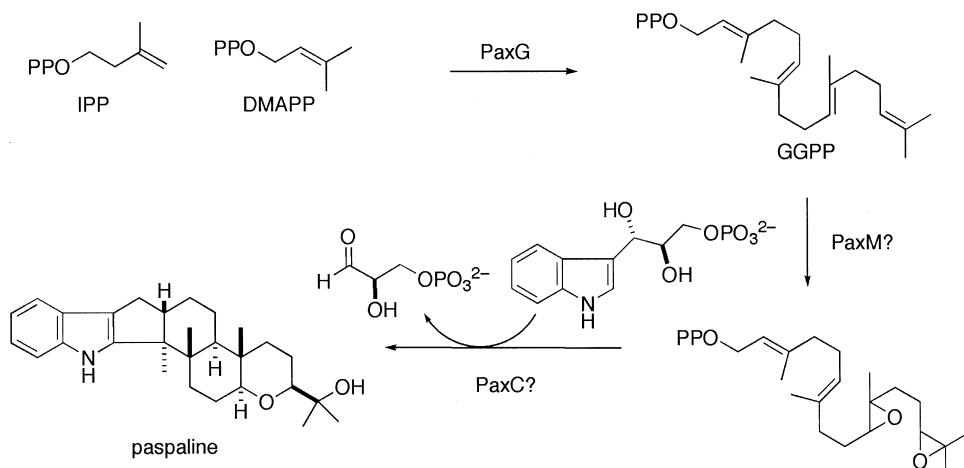


Figure 8 Possible biosynthetic scheme to paspaline from GGPP and indole-3-glycerol phosphate using only *paxG*, *paxM*, and *paxC*.

for the corresponding enzymes (Fig. 7). This hypothesis can be tested by feeding these substrates to mutants deleted for the entire cluster but containing functional copies of *paxP* and *paxQ*, respectively.

Given that a single cytochrome P450 enzyme is required to oxidatively demethylate the C-14 position of lanosterol in ergosterol biosynthesis [88,89], PaxP alone may catalyze the conversion of paspaline to PC-M6, via paspaline B. This hypothesis is supported by the demonstration that P450 enzymes required for gibberellin biosynthesis, in *F. fujikuroi*, are capable of multiple catalytic steps [90–92]. Thus, fewer genes/enzymes may be required for paxilline biosynthesis than originally proposed [12]. The metabolic grid as proposed suggests that the penultimate substrate for the formation of paxilline could be either 13-desoxypaxilline or β -paxitriol [10]. This is supported by the observation that β -paxitriol complements paxilline biosynthesis in a *paxQ* knockout mutant (Scott et al., unpublished results). Therefore, PC-M6 may also be a substrate for PaxQ, as the conversion from PC-M6 to β -paxitriol also involves a C-13 hydroxylation. Similarly, the conversion of β -paxitriol to paxilline and PC-M6 to 13-desoxypaxilline both involve oxygenation at position C-10, suggesting that a single dehydrogenase enzyme may perform both reactions. A further prediction from this scheme is that double mutants of *paxQ* and this dehydrogenase accumulate PC-M6.

The isolation of the paxilline gene cluster from *P. paxilli* now opens the way for the cloning of other fungal indole-diterpene clusters. As GGPP synthase is a relatively conserved gene, it is possible to design degenerate primers to conserved regions of *P. paxilli paxG* and other fungal GGPP synthases and to amplify *paxG* orthologues from other fungi. Given that the genes for the biosynthesis of most fungal secondary metabolites are organized in clusters [93], genes for other indole-diterpene pathways may be located by chromosome walking from the *paxG*-like gene locus.

Production of the more complex indole-diterpenes, such as the lolitremes, requires further functionalization of the paxilline skeleton, frequently by the addition of further isoprene units. We anticipate that fungi that have this extended biosynthetic capability will contain additional and unique genes to those found in *P. paxilli*.

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15

Loline and Ergot Alkaloids in Grass Endophytes

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1. INTRODUCTION

1.1. Alkaloids of the Clavicipitaceae

Many of the common grasses in woodlands and meadows, pastures, lawns, roadsides, and sport fields are aided in their survival by inconspicuous fungal symbionts, termed *endophytes*. The *Epichloë* spp. and *Neotyphodium* spp. (asexual counterparts of *Epichloë* spp.) are grass endophytes belonging to the fungal family Clavicipitaceae (phylum Ascomycota, order Hypocreales).

The family Clavicipitaceae is best known for the ergot fungi, *Claviceps* spp., which do not engage in such benign symbioses as do their endophytic cousins. Instead, *Claviceps* spp. infect florets on developing seed heads, where they replace ovaries with hard, dormant sclerotia. These “ergots” often contain high levels of alkaloids with neurotropic activities in vertebrates and insects. The term *ergot alkaloids* (also known as ergoline alkaloids, ergoalkaloids, or ergotoxins) refers to one class of such metabolites, especially those from *Claviceps purpurea*. Sclerotia of *C. purpurea* contain approx. 1% (dry mass) of the ergopeptines, ergotamine, and ergocristine [1]. In addition to the ergot alkaloid class, however, some *Claviceps* spp. also produce indole-diterpene tremorgens that are built from similar precursors but are structurally and pharmacologically quite different from ergot alkaloids. The tremorgens, which are also neurotropic and active against vertebrates and insects, are discussed in more detail in Chapter 14. Both ergot alkaloids and indole-diterpenes are produced by some of the grass endophytes, although the structures of the major alkaloids differ in some moieties from the counterparts in *Claviceps* spp.

Some grass endophytes produce either or both of two additional alkaloid classes—1-aminopyrrolizidines and a pyrrolopyrazine—in addition to, or instead of, ergot alkaloids and indole-diterpenes. These alkaloids are unknown from *Claviceps* spp. or any other fungi. The lolines are potent insecticidal alkaloids, composed of a saturated pyrrolizidine ring system with a 1-amino substituent and an oxygen bridge (Fig. 1). The pyrrolopyrazine alkaloid, peramine, is an insect-feeding deterrent and seems structurally to be derived from cyclic prolyl-arginine [2].

Recent developments have greatly advanced our understanding of the biosynthetic routes and underlying genetics for production of ergot alkaloids, lolines, and indole-diterpenes. This chapter reviews knowledge of the ergot alkaloids and lolines. The reader is referred to Chapter 14 for a review of the indole-diterpenes.

1.2. Endophyte Biology

Endophytic *Epichloë* and *Neotyphodium* spp. are common in many species in the grass subfamily Poöideae (cool-season grasses). Endophytism is particularly common in the tribe Poeae, species of which are extremely important in pastures and hay as well as turf. *Lolium* and *Festuca* grass species are best known for such endophytes, which occupy intercellular spaces (the apoplast) of the plants and survive there feeding on extracellular substrates [3–5]. The grass–endophyte symbioses range from pathogenic to mutualistic, based on the relative costs and benefits to the hosts. In the more pathogenic associations, the endophytes suppress maturation of some or all grass inflorescences (“choke disease”) so that seed production is reduced or eliminated. The endophyte then spreads contagiously (horizontally) by forcibly ejecting its meiotic spores (ascospores), which can then initiate infection of other grass plants during flowering. This yields infected seeds [6]. In the more mutualistic associations, the endophytes grow systemically within their host plants, infect

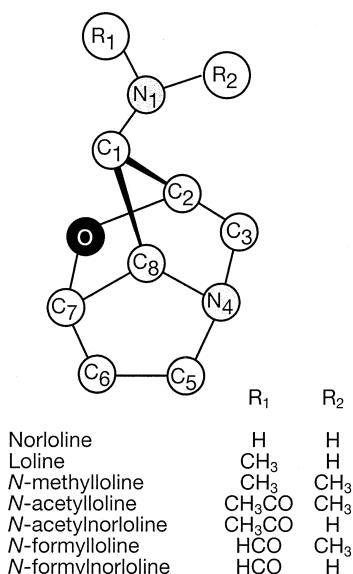


Figure 1 Generic structure of loline alkaloids with substitutions shown for common lolines.

Table 1 Typical Alkaloid Profiles for Some Natural Symbiota of *Lolium* and *Festuca* spp. with *Neotyphodium* and *Epichloë* spp.^a

Host Grass	Symbiont	Alkaloids			
		Ev	Lol	LmB	Pm
<i>Lolium arundinaceum</i>	<i>Neotyphodium coenophialum</i>	0.5	1100	ND	2
<i>Lolium pratense</i>	<i>Neotyphodium uncinatum</i>	ND	5600	ND	ND
<i>Lolium perenne</i>	<i>Neotyphodium lolii</i>	1.3	ND	4.7	19
<i>L. perenne</i>	<i>Epichloë typhina</i>	ND	ND	ND	53
<i>L. perenne</i>	<i>N. lolii</i> × <i>E. typhina</i>	4.8	ND	0.4	22
<i>Lolium giganteum</i>	<i>Epichloë festucae</i>	ND	300	ND	4
<i>Festuca longifolia</i>	<i>E. festucae</i>	0.9	ND	4.0	22
<i>Festuca rubra</i>	<i>E. festucae</i>	1.2	ND	ND	ND

^a Approximate concentrations given in µg/g dry weight.

Ev, ergovaline; Lol, total lolines; Lm, lolitrem B; Pm, peramine; ND; not detected

the developing seeds, and in this way are vertically seed-transmitted. Several endophyte species are capable of both means of transmission and are thus termed *pleiotropic* (or mixed-strategy) *symbionts* [7]. However, those that are only vertically transmitted are absolutely dependent for growth and dispersal on the survival and growth of the infected grass plants and on the seed progeny of such plants.

Vertical transmission is clearly advantageous to endophytes because essentially all seeds produced on the mother plant bear the endophyte [8]. The process is not problematic for the plants, which can produce as many or more seeds when infected compared to when they lack the endophytes [9]. The cost of carrying the symbiont is almost certainly outweighed by benefits to the host in at least some of the environments where these symbiota persist [10]. Such benefits often include the antiherbivore alkaloids. The fungal endophytes have been implicated in the production of all four of the aforementioned alkaloid classes, namely, ergot alkaloids, lolitrem, lolines, and peramine [2,11–14]. Although no grass-endophyte symbiotum that possesses all four alkaloid classes has yet been identified, tall fescue (*Lolium arundinaceum* = *Festuca arundinacea*) with the endophyte *Neotyphodium coenophialum* typically accumulates high levels of loline alkaloids, ergot alkaloid amounts sufficient to cause livestock toxicoses, and enough peramine to be a feeding deterrent against some major insect pests (Table 1) [15,16].

Although the endophyte alkaloids are well known and certainly relevant to grass ecology and livestock agriculture, the endophytes can have other effects on host plants, mechanisms for which are yet unknown. Such benefits include enhanced drought tolerance, increased ability to acquire mineral phosphate from soil, antinematode activity, and increased growth and competitiveness [17].

2. LOLINE ALKALOIDS

2.1. Distribution, Roles, and Activities of Lolines

The lolines (saturated 1-aminopyrrolizidines with an oxygen bridge between C-2 and C-7; Fig. 1) have a particularly broad spectrum of insecticidal activity [18–20] but no definitive

antimammalian activity [21,22]. The lolines are known from the following symbiota of fungal endophytes in grass hosts: *Epichloë festucae* in *Lolium giganteum* (= *Festuca gigantea*) [15], *Neotyphodium coenophialum* in *L. arundinaceum* [23], another *Neotyphodium* sp. (designated FaTG-3) in *L. arundinaceum* [24], *N. siegelii* in *Lolium pratense* (= *F. pratensis*) [25], *N. uncinatum* in *L. pratense* [26], *N. occultans* in *Lolium multiflorum* [27], *N. aotearoae* in *Echinopogon ovatus* [28,29], and in endophyte-infected *F. argentina* [30], *Poa autumnalis* [15], and *Achnatherum robustum* (= *Stipa robusta*) [27]. Among nongrass plants, lolines have been reported to occur in *Argyreia mollis* in the Convolvulaceae [31] and *Adenocarpus* spp. in the Fabaceae [32]. The loline alkaloids were determined to be of fungal origin in at least one of the grass-endophyte symbiota, namely *L. pratense* with *N. uncinatum*. Fermentation cultures of *N. uncinatum* produced three of the loline alkaloids found in the symbiont [14].

The loline alkaloids act as toxins and feeding deterrents to a broad spectrum of insects, including horn fly larvae (*Haematobia irritans*) [33], Japanese beetle grubs (*Popillia japonica*) [18]; fall armyworm larvae (*Spodoptera frugiperda*) [19]; European corn borer larvae (*Ostrinia nubilalis*) [19], oat–bird–cherry aphids (*Rhopalosiphum padi*) [20,34], and greenbug aphid (*Schizaphus graminum*) [20]. Wilkinson et al. [20] conducted matings of *Epichloë festucae* strains that produced lolines (*Lol⁺*) with loline nonproducing (*Lol⁻*) strains. In this and in a series of backcrosses and sib-crosses, the segregation ratio was significantly close to 1:1, which is expected for single-locus control of the phenotype in a haploid organism. After introducing the progeny into *L. pratense*, the researchers then demonstrated a genetic linkage between activity against the aphids and expression of lolines. Loline-alkaloid toxicity to insects appears to be neurotropic [35], but the mechanism has not been studied extensively.

Pyrrolizidine rings are featured in the more common plant-produced pyrrolizidine class, the senecio alkaloids [36], as well as in spiropyrrolizidines from poison-arrow frogs [37]. However, the loline alkaloids do not exhibit the cytotoxicity of the senecio alkaloids nor the potent nicotinic receptor blocking of the spiropyrrolizidines. Many studies of loline-alkaloid effects on mammals have used intravenous injection, but studies involving oral administration have failed to demonstrate toxic effects of the lolines [21,22]. In contrast, the hepatotoxicity of the senecio alkaloids [31] has been shown to be due to a macrocyclic ring structure associated with C-7 and C-1 via ester linkages. Such macrocyclic rings are absent in lolines, which instead have a 1-amine plus a simple oxygen bridge between C-7 and C-2. Kim et al. [38] have demonstrated senecio alkaloid toxicity by studying DNA cross-linking due to the plant pyrrolizidines. The α,β -unsaturation and the macrocyclic ring, characteristic of most plant pyrrolizidines, was involved in binding to DNA and proteins and in DNA crosslinking. These characteristics associated with toxicity of plant pyrrolizidines are also lacking in the lolines.

2.2. Loline Alkaloid Structures

The chemical structures of the lolines have been firmly established in the literature [39]. The first isolation of an alkaloid likely to be a loline was by Hofmeister in 1898, who named the alkaloid temuline because it was isolated from *Lolium temulentum*. In 1907, Hannig reported the presence of an alkaloid in the endophyte-infected seeds. In 1955, Yunosov and Akramov [40] isolated this compound from seeds of a ryegrass (*Lolium cuneatum*) and named it loline. In 1965, Yates and Tookey [41] isolated from tall fescue a compound they called festucine, but which was later demonstrated to be loline [42].

Structural studies by several groups have helped to determine the absolute configurations of the loline group of alkaloids, as shown in Fig. 1 [42–44]. Comprehensive ¹H and ¹³C nuclear magnetic resonance (NMR) was conducted by Petroski et al. [45].

Natural variants of lolines differ in substitutions on the 1-amine. The substituents that typically occur, singly or in combination, are methyl, formyl, and acetyl groups (Fig. 1). In many of the endophyte-grass symbiota, *N*-formylloline (NFL) and *N*-acetylloline (NAL) predominate as the major loline alkaloids with loline, norloline, and *N*-acetyl-norloline that possesses all four alkaloid classes (NANL) also as minor *N*-methylloline, *N*-formylnorloline constituents. In culture, NFL is most abundant but rivaled by NANL. Loline is also present in the production culture.

2.3. Extraction and Analysis of Lolines

Loline alkaloids from culture filtrates are analyzed as follows. Aliquots of medium from each culture are transferred into microcentrifuge tubes (a 2-day, 100°C pretreatment of these tubes removes contaminants that can interfere with the analysis). Culture filtrate (generally 0.5–1 mL) can be freeze-dried for later analysis. The freeze-dried samples are extracted by a modification of procedures from Yates et al. [46] and Robbins et al. [47]. Aqueous saturated sodium bicarbonate (0.25 g/mL) is added to each dried filtrate at one-tenth of the original volume, and the tubes are vortexed. To each suspension is added 1 mL chloroform (CHCl₃) with 14.2 mg/mL of quinoline or 25 mg/mL phenylmorpholine as internal standard. (Quinoline is preferred because a breakdown product of phenylmorpholine can interfere with NANL identification.) The suspension is vortexed, then shaken for 30 minutes. The CHCl₃ layer is then transferred to a capped glass vial for gas chromatographic (GC) analysis.

For larger scale analysis by GC or NMR, residue from 30 mL of culture filtrate is extracted with 1 mL saturated sodium bicarbonate and 30 mL CHCl₃. The organic layer is evaporated in a N₂(g) stream, leaving a brown oily residue. CHCl₃ (1 mL) is added to dissolve the residue, and the solution is spotted onto a silica gel TLC plate (K6F 60A, Whatmann Inc, Clifton, NJ). The plate is developed with a 50:50:1 (v/v/v) mixture of CH₃OH:CHCl₃:saturated NH₃OH. Plates are exposed to I₂ vapor, whereupon loline alkaloids can be visualized as dark brown spots. Rf values for loline, NANL, and NFL were measured by Blankenship et al. [14] at 0.26, 0.37, and 0.49, respectively. The spots are scraped from the plate and eluted with water. Then NFL and NANL are extracted with CHCl₃, and the solvent is removed in an N₂ gas stream. For loline, which is more easily volatilized, concentrated HCl is added to generate the loline salt, which is then recrystallized with ethanol.

The loline alkaloids can be isolated from plant or seed material as described by Robbins et al. [47], Petroski et al. [45], or Yates et al. [46], using either methylene chloride (CH₂Cl₂) or CHCl₃ under basic conditions. CHCl₃ extracts the loline alkaloids more thoroughly but can also extract from grass tissue contaminants that interfere with analysis. CH₂Cl₂ extraction presents less difficulty with contaminants. Grass tissue to be analyzed is harvested, freeze dried, and ground. To the powder (100 mg in a heat-treated microcentrifuge tube) is added 100 μL either of saturated sodium bicarbonate or 1M NaOH. A brief centrifugation can help wet the material. Then 1 mL CH₂Cl₂ (again, with quinoline as standard) is added, and the mixture is agitated for 30 minutes. The phases are separated by centrifugation at 15,000 × g for 5 minutes. The organic layer is harvested for GC analysis.

GC conditions are as published in Blankenship et al. [14] with slight modification. A Hewlett Packard (Avondale, PA) 5890 Series II Plus GC is equipped with a flame ionization detector and a fused silica capillary column, SE 30, 60 m × 0.30 mm internal diameter, with 0.25- μ m thick dimethylpolysiloxane film. The carrier gas is N₂ at 20.4 mL/min. The parameters are as follows: initial temperature = 110°C, injection temperature = 250°C, detector temperature = 325°C, purge time of 2.2 minutes, and ramp of 4°C/min to 220°C. The column is purged between runs at 280°C for 5 to 30 minutes.

The following ¹³C NMR spectra (in D₂O, 200 MHz, chemical shifts in ppm) and MS spectra (relative peak heights in parentheses) were determined for the more abundant loline alkaloids [14,45].

Loline-HCl: ¹³C NMR, 29.2 (C6), 34.0 (N-Me), 55.5 (C5), 61.8 (C3), 63.8 (C1), 70.1 (C8), 71.9 (C2), 80.9 (C7); EIMS m/z 154 [M]⁺ (0), 123(58), 110 (7), 95 (75), 82 (100).

N-Acetylnorloline: ¹³C NMR, 24.2 (MeC = O), 35.1 (C6), 54.8 (C5), 58.4 (C1), 61.4 (C3), 70.1(C 8), 74.1(C 2), 81.6 (C7), 171.9 (MeC = O); EIMS m/z 182 [M]⁺ (0), 153 [M-29]⁺ (8), 139 (0), 123 (9), 110 (0), 95 (30), 82 (100), 69 (22).

N-Acetylloline: ¹³C NMR, 22.3 (MeC = O), 33.0 (C6), 33.9 (N-Me), 54.7 (C5), 61.0 (C3), 64.1 (C1), 67.8 (C8), 73.4 (C2), 80.6 (C7), 171.5 (MeC = O); EIMS m/z 196 [M]⁺ (2.1), 167 [M-29]⁺ (5), 153 (8), 123 (23), 95 (43), 82 (100), 42 (42).

N-Formylloline: ¹³C NMR (D₂O, 200 MHz, major rotamer), 32.0 (C6), 34.8 (N-CH₃), 54.1(C5), 59.7 (C3), 65.5 (C1), 67.8 (C8), 74.3 (C2), 83.2 (C7), 165.1 (HC = O); ¹³C NMR (minor rotamer), 30.4 (N-CH₃), 31.7 (C6), 54.3 (C5), 60.6 (C3), 63.0 (C1), 68.6 (C8), 73.5 (C2), 81.4 (C7), 167.0 (HC = O); EIMS m/z 182 [M]⁺ (0), 154 [M-28]⁺ (25), 123 (13), 110 (13), 95 (28), 82 (100).

The ¹³C NMR spectrum of NFL exhibits two peaks for each of the ten carbons, as a result of two rotamers that are characteristic of this alkaloid [48]. The N-formyl group comprises a carbonyl linked to a tertiary 1-nitrogen. Rotation around this C-N bond is hindered due to its partial double-bond nature as shown in Fig. 2. The ratio of rotamers is consistent at a given temperature. NFL is the only natural loline that exhibits this rotamer effect because of the substituents on the tertiary 1-amine.

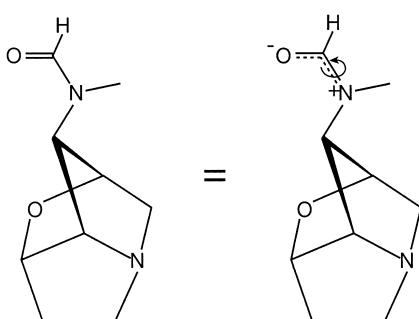


Figure 2 Rotamers of *N*-formylloline. The formyl substituent flips around the *N*-formyl partial double-bond to give the two rotamers.

2.4. Loline Alkaloid Biosynthesis

2.4.1. Expression In Symbio

Typical levels of loline alkaloids vary considerably among grass species–endophyte species symbiota. In several associations, the typical levels approach or exceed 1 mg/g dry mass [15]. Among these is tall fescue with *N. coenophialum*. The highest loline-alkaloid levels have been reported in *L. pratense* with either *N. uncinatum* or *N. siegelii*. Within 11 days following the clipping of leaves, total loline-alkaloid levels rose dramatically to nearly 20 mg/g dry mass [25]. This represents a three-fold rise for the *N. uncinatum* symbiotum and a 17-fold rise for the *N. siegelii* symbiotum. These results strongly suggest that loline production by the endophyte accelerates in response to plant injury. The removal of herbage from plants very likely induces strong shifts in the nitrogen and carbon allocation within the plants (e.g., for regrowth of leaves). Increased activity of the loline biosynthesis pathway could thus have to do with the form and amount of nitrogen and carbon sources available to the fungus. This is also evidenced by the results from *N. uncinatum* culture experiments in which differences in amino acids and sugars in the medium led to distinct differences in loline accumulation [14]. Levels and composition of nitrogen- and carbon-containing compounds as well as other molecules in the intercellular spaces (the local environment of the endophyte hyphae) might be important in the regulation of loline biosynthesis in the endophyte. These compounds could thereby function as precursors or as signal molecules in loline biosynthesis.

Young seedlings of *L. pratense* with *N. uncinatum* tend to have very high levels of lolines [49], possibly due to the high levels in seeds. During the plant's vegetative growth, lolines are distributed throughout the plant. They can even be found in the rhizosphere [17], where they may affect root-associated insects. Lolines in the rhizosphere might also reduce germination of seeds from competitor plants such as clover [17]. Since the endophyte does not grow appreciably in roots, lolines are likely phloem-transported. Antiaphid activity would imply that, indeed, there is some loline alkaloid in the phloem [20]. Given their high water solubility, lolines may also leach out of aerial plant tissues.

After panicle differentiation a loline-alkaloid gradient begins to form with the highest concentrations in the developing seed heads [49]. Levels in mature and imbibed seeds are particularly high. Justus et al. [49] report an average of 3.2 mg/g dry mass in embryos of imbibed seeds and 1.7 mg/g in the remaining seed tissue (excluding bracts).

2.4.2. Expression in Fermentation Culture

Unlike the other common alkaloids associated with endophyte infection (ergovaline, peramine, and indole-diterpenes), it remained unknown until recently if the fungal endophytes, the grass hosts, or a shared process generated the loline alkaloids. Endophytes are typically grown in complex medium, and their slow growth has discouraged studies of minimal defined media for growth. However, Kulkarni and Nielsen [50] showed that defined media can support endophyte growth. In tests of simple and complex sugars as carbon sources, mannitol and trehalose yielded maximal growth of *N. coenophialum*, and sucrose as the carbon source yielded about half the fungal dry mass. It was also determined that glutamine, asparagine, or proline as the sole nitrogen source yielded good growth of *N. coenophialum*. Thiamine was the only required vitamin.

Neotyphodium uncinatum was chosen for fermentation studies because it is known to produce exceptionally high levels of loline alkaloids *in symbio*; up to 20 mg/g plant dry mass [16,25]. Blankenship et al. [14] grew *N. uncinatum* on defined medium recipes

based on those of Kulkarni and Nielsen [50] and detected lolines in the culture filtrates. In contrast, complex media with yeast extract failed to support loline alkaloid production, although much greater biomass was produced. Lolines were consistently produced by *N. uncinatum* in a defined medium consisting of 30 mM (2-morpholino)ethanesulfonic acid (MES) buffer and 30 mM potassium phosphate, 16.7 mM mannitol as the carbon source, 15 mM asparagine as the nitrogen source, 2 mM MgSO₄, 0.6 µM thiamine, and trace elements (3.6 µM H₃BO₃, 1 µM CuSO₄, 0.7 µM KI, 0.8 µM Fe-Na-ethylenediaminetetraacetic acid, 1 µM MnSO₄, 0.5 µM NaMoO₄, and 0.4 µM ZnSO₄) [14]. The MES was substituted for half of the phosphate in the original recipe to permit a wide range of pH values to be tested for optimizing loline alkaloid production. There was little difference in loline alkaloid levels at culture pH ranging from 5.0 to 7.5. In both conditions the onset of loline production corresponded to a slight alkalinization, with an increase of approx. 0.3 pH units by the time loline levels plateaued. Given the buffer conditions, the alkaloid levels cannot account for more than a little of this pH change. It is possible that sufficient ammonium was generated by fermentation of the amino acids to cause the pH shift.

Still cultures in Petri plates and shaken cultures grown in the Erlenmeyer flasks did not generate levels of loline alkaloids and mycelium growth comparable to the shaking cultures in polystyrene Petri plates. Availability of oxygen may have played an important role in the amount of growth and subsequent production of loline alkaloids [14].

Effects of different carbon and nitrogen sources on loline-alkaloid production were investigated [14]. Among the carbon sources was sucrose (chosen because of its role in plants as the major transport sugar) and fructose and glucose, which are formed by invertase-catalyzed cleavage of sucrose. Invertase is secreted by grass endophytes [51]. Loline alkaloids were produced in cultures with all of the tested carbon sources, but the maximum production (reaching 4 mM total lolines) was evident in the sucrose-containing cultures.

Sources of nitrogen that were tested included NH₃NO₃, (NH₃)₂SO₄, and organic sources such as ornithine (Orn), arginine, asparagine, proline, methionine (Met), and urea. The amino acids were chosen as nitrogen sources because they are common in plant systems [52]. Although urea supported the highest levels of loline alkaloid production, Orn also supported high production. As discussed later in this chapter, feeding studies with proposed precursors positionally labeled with ¹³C indicated that aspartate (Asp) and Orn are loline alkaloid precursors (J.D.B. and C.L.S., unpublished data).

With the optimal defined medium being used, NFL, NANL, loline, and norlolaine were produced as the fungal cultures approached stationary phase. In microbial fermentation, the onset of secondary metabolism production with slowing of growth is well documented [53]. The cultures generated up to 4 mM total lolines (about 700 mg/L). This level approximates that produced *in symbio*. Levels of fungal biomass reached approximately 1.8 g/L, so up to 400 mg lolines were produced per gram of fungal dry mass.

2.4.3. Biosynthetic Precursors

Because of the similarity of the pyrrolizidine rings in the loline alkaloids and plant pyrrolizidines, it was originally suggested that the respective biosynthetic pathways could be related [54]. Therefore, we will briefly review what is known of plant pyrrolizidine biosynthesis; then we will discuss recent information on loline alkaloid biosynthesis.

Precursors have been identified for the pyrrolizidine ring of a common plant pyrrolizidine, retrorsine. Hydrolysis of retrorsine yields retronecine, which shares a similar ring structure with the loline alkaloids except in having an unsaturated bond between C-1 and

C-2 (α,β), lacking an oxygen bridge, and possessing a -CH₂OH substituent at C-1 (lolines have a C-1 amine). Feeding experiments with ¹⁴C- and ¹³C-labeled intermediates have indicated that homospermidine (Hspd) is a direct intermediate in the biosynthesis of the retronecine ring system [55,56]. Labeled Orn, putrescine (Put), spermidine (Spd), and Hspd were all incorporated into the pyrrolizidine ring, indicating that these plant pyrrolizidines originate from polyamines. Orn from the urea cycle is decarboxylated to yield Put, which is then conjugated to an aminopropyl group from decarboxylated S-adenosylmethionine (dcSAM) to produce Spd. Recent work has yielded two associated routes for the biosynthesis of Hspd: Put can obtain an aminobutyl group from another Put or from Spd [57]. Another plant pyrrolizidine, senecionine, is also derived from a polyamine-related pathway as evidenced by enzyme assays and isotope incorporation [58–61]. Again, Hspd is implicated as an intermediate. The oxidoreductase, Hspd synthase, is evolutionarily related to deoxyhypusine synthase [62], which is required in eukaryotes to posttranslationally activate translation initiation factor 5A.

Because of the arrangement of carbon and nitrogen atoms and their perceived structural similarity to plant pyrrolizidines, lolines were originally thought to be derived from spermidine and/or spermine [54]. However, precursor-feeding experiments (J.D.B. and C.L.S., unpublished data) have so far failed to support this hypothesis. When added to cultures, none of the ¹⁴C-labeled polyamines Put, Spd, and Spm incorporated into lolines. In Spd and Spm synthesis, Met contributes the aminopropyl groups. However, universally ¹³C-labeled Met did not chase into the loline ring system. However, feeding of positionally ¹³C-labeled Orn and Asp clearly indicated that these are precursors. Asp contributes carbons 1–3 and the 1-amine (based on ¹⁵N-Asp feeding and NMR analysis of NFL); Orn contributes carbons 5–8 and presumably (not yet tested) the ring N. Clues as to how these compounds feed into the biosynthetic pathway are also emerging from genetic studies, as discussed in section 2.5.

Precursor feeding studies also indicated that the formyl group is derived from the S-methyl group of Met (J.D.B. and C.L.S., unpublished data), suggesting that NFL may arise by oxidation of one of the N-methyl groups of *N*-methylolloline. The origin of the acetyl moiety in NAL and NANL is currently unknown.

2.5. Genetics of Loline Alkaloid Production

Alkaloid accumulation in fungi is often coordinated with upregulated transcription of the respective alkaloid biosynthesis genes [63,64]. Therefore, a working hypothesis is that loline alkaloid production is similarly controlled [24]. If this is correct, one may distinguish loline biosynthesis gene sequences from a pool of mRNA from loline-producing mycelium after eliminating those transcripts that are not upregulated in the producing cultures. This is the general strategy of various subtractive hybridization methods, which are routine practice in many molecular biology laboratories. We chose the PCR-based procedure, suppression-subtractive hybridization (SSH) [65], for enrichment of transcripts differentially expressed in the loline-producing *N. uncinatum* cultures.

The *N. uncinatum* culture system was ideally suited for SSH because loline production in the cultures could be readily induced or suppressed, depending on the growth medium used [14]. For suppression of loline-alkaloid accumulation, we selected a medium very similar to the defined minimal medium used for production but amended with half-strength potato dextrose broth [24]. Biomass accumulation of the endophyte under the loline-inducing conditions was relatively low in the cultures so the RNA yield was too

low for subtraction. The problem was overcome by using PCR to amplify full-length cDNAs (DNA made from the mRNAs) from the complete RNA mixture isolated from the cultures. It has been shown for several genes that the relative abundance of transcripts is maintained after the amplification step [66]. In our laboratory, when the amplified cDNA made from complete RNA was size-fractionated and analyzed by Southern blot, transcripts from several genes gave distinct bands with no detectable smearing when hybridized with labeled probes [24], indicating that most amplified transcripts likely had the full length of the original mRNAs. A cDNA library was constructed in a bacteriophage lambda vector (λ Triplex2, Clontech, Palo Alto, California). The amplified library was derived from 1.5×10^6 primary plaques.

The PCR-amplified cDNAs were also used in SSH to identify several gene sequences that were upregulated during loline alkaloid production [24]. The SSH fragments were cloned and sequenced, and the SSH-derived cDNA mixture was also labeled and used to probe the aforementioned cDNA library. Clones obtained by both procedures were sequenced, and the inferred sequences were compared to database sequences using BLAST algorithms. Two of the genes identified in this manner had highly significant similarities to enzymes in amino acid metabolism, namely to Asp kinases and *O*-acetylhomoserine sulfhydrolase (homocysteine synthase) [24]. These induced *N. uncinatum* genes were designated *lolA* and *lolC*, respectively, with the corresponding hypothetical proteins designated LolA and LolC. Both Asp kinase and homocysteine synthase are enzymes in the biosynthesis of Met and related amino acids [67]. Conceivably, LolA and LolC might be part of a dedicated Met biosynthesis pathway. However, precursor-feeding studies discussed earlier indicate that Met is not a precursor to the loline ring structure (although it contributes methyl groups to the 1-amine). Also, LolA cannot, by itself, be a functional Asp kinase because it lacks the portion corresponding to the substrate-binding and catalytic domains. Furthermore, an attempt to use *lolC* to complement Met auxotrophy in *Emericella nidulans* (*Aspergillus nidulans*) failed (M.J.S. and C.L.S., unpublished data). The auxotrophy was due to mutations in genes for both homocysteine synthase and cystathione- γ -synthase [68,69], evolutionarily related enzymes in alternative routes to Met. It appeared, therefore, that LolC has neither of these activities. These results suggest that LolA and LolC relationships may suggest an evolutionary link to polyamine biosynthesis enzymes rather than derivation of lolines from polyamines.

Notably, the LolC sequence also has similarity to an enzyme in biosynthesis of rhizobitoxine [24], a bacterial compound produced in some associations between *Rhizobium* spp. and plants [70]. So, it is a reasonable possibility that LolC possesses a catalytic activity related to, but distinct from, those of Met biosynthesis. Given that a pyridoxal phosphate binding motif was identified in the predicted LolC sequence [24], it is likely that the enzyme acts on one or more amino acid precursors or amino acid-like intermediates.

It is now evident that genes for secondary metabolites, such as mycotoxins and alkaloids, are frequently clustered in fungal genomes [63,71,72]. Consequently, if *lolA* and *lolC* are indeed loline biosynthesis genes, they might likewise be in close proximity in the genome of *N. uncinatum*. We tested this possibility by long-distance PCR [73]. PCR with primers specific to the *lolA* and *lolC* sequences amplified a DNA fragment approximately 8 kb in length (M.J.S. and C.L.S., unpublished data). The sequence of this DNA fragment verified the presence of *lolA* and *lolC* and revealed two additional putative genes, which we designated *lolD* and *lolO*. The predicted LolD and LolO sequences had highly significant similarity to oxidoreductases and Orn decarboxylase, respectively. Oxidoreductases are common among secondary metabolite genes [74], and Orn

decarboxylase catalyzes Put formation. The sequence information of the putative *LOL* cluster genes enabled further DNA cloning and sequencing in the adjacent 5' and 3' regions. So far we have identified 10 putative genes clustered within a region of approximately 25 kb (M.J.S. and C.L.S., unpublished data). Predicted functions include two monooxygenases, an epoxidase, an oxidoreductase (*LolO*), and three enzymes (including *LolC* and *LolD*) that use pyridoxal phosphate coenzymes. Possible functions of the other putative proteins are not apparent from their sequences.

In a survey of endophytes differing in their ability to produce lolines—including strains of *E. festucae* and *E. amarillans*, which are polymorphic for this phenotype—*lolA*, *lolC*, and the other genes with which they are clustered have been identified only among the loline alkaloid producers [24] (M.J.S. and C.L.S., unpublished data). This finding regarding the relationship of the predicted products to known biosynthetic enzymes and the expression of the genes under loline-producing conditions strongly suggest that the loline biosynthesis genes are clustered at the identified locus in *N. uncinatum*.

3. ERGOT ALKALOIDS

3.1. Ergotism and Fescue Toxicosis

Poisoning by the alkaloids of *Claviceps* spp. has been an important problem in human history. These fungi have an unusual host interaction that historically has made it difficult to avoid contamination of grains and feed. Infection of the host floret occurs during a brief period of susceptibility when the stigma is exerted and still hydrated [75]. Once the floret is pollinated, the stigma dries and the fungus is prevented from growing along the stigma to the ovary. Wheat is refractory to infection because it self-pollinates before the stigma is exerted, but the open-pollinated grain, rye, is very commonly infected. If the fungus manages to infect the susceptible tissue and to reach the ovary, it then proliferates a mass of mycelium that incorporates the ovary and eliminates it as a reproductive structure. Apparently, the mycelium thereby gains access to nutrients that would otherwise have been destined to fill the grain. The fungus forms its sclerotium, commonly called an *ergot*, within the floret. Ergots can be strikingly reminiscent of the seeds in appearance and, more importantly, in size and density. Modern mechanical processes can greatly reduce ergot contamination of grain, but during the Middle Ages, ergotism was so prevalent that major historical events are reputed to be associated with contaminated bread flour. These include frequent witch hunts in Europe and in the American colony of Massachusetts [76], the “great fear” before the French revolution [76], and the first Crusade [77]. Historical records suggest frequent dramatic episodes of mass ergot poisoning in medieval Europe.

Symptoms similar to ergot alkaloid poisoning can be suffered by livestock that graze on certain endophyte-infected grasses [78]. These symptoms characterize syndromes collectively known as fescue toxicosis, referring to their association with the tall fescue-*N. coenophialum* system [79]. The tendency for livestock to lose appetite and exhibit reduced weight gain is widely seen. Other problems include fat necrosis, rough hair coats, and elevated core body temperatures. Reproductive effects are especially problematic [80]. Cows grazed on ergovaline-containing grass have altered hormone balance, particularly low prolactin levels which are thought to contribute to agalactia and to a significant reduction in conception rates. Livestock, particularly horses, can also suffer increased frequencies of still-births [81,82]. Characteristically, these foals are delivered up to several weeks after normal gestation and show distortions in bone structure. Another severe ma-

lady, known as fescue-foot, involves gangrene of the feet and necessitates destruction of the animal.

The role of the ergopeptine, ergovaline, in fescue toxicosis is far from certain (Fig. 3). Administration of the alkaloid has caused some mild effects [83], but there has not been sufficient material available to carry out large, controlled experiments on livestock.

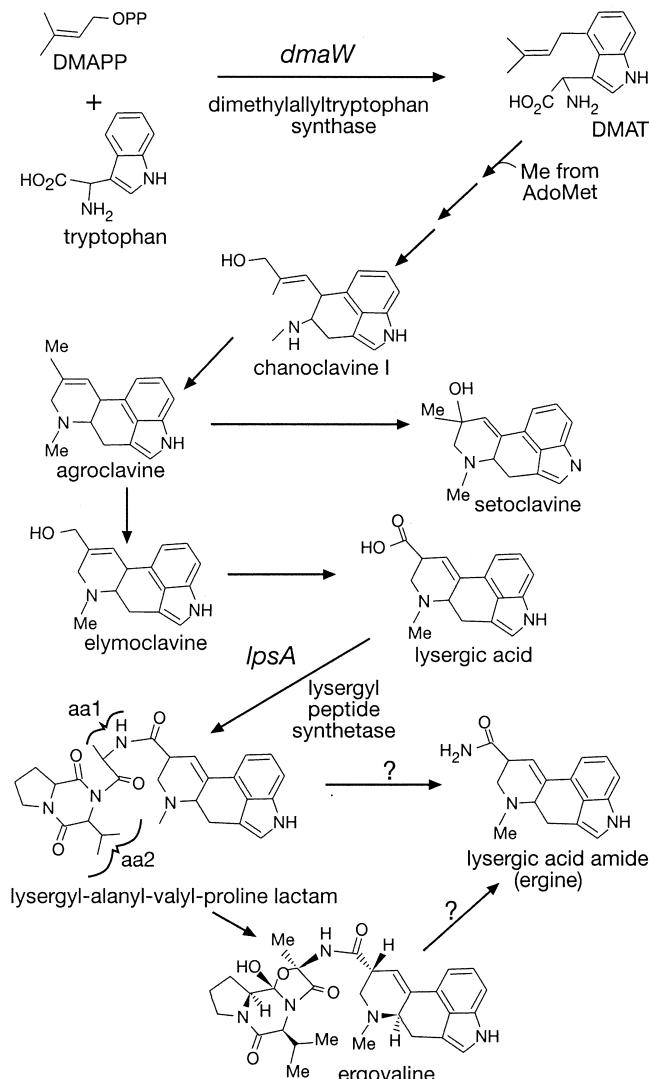


Figure 3 Summary of the proposed ergovaline biosynthesis pathway. Genes for DMAT synthase (*dmaW*) and lyseryl peptide synthetase (*lpsA*) are indicated. Most of the other steps are assumed to be similar to those of *Claviceps* spp. Of the three amino acids added by lyseryl peptide synthetase, the first two are labeled amino acid 1 (aa1) and amino acid 2 (aa2) on the lactam structure. These are the most variable among ergopeptides. DMAPP, dimethylallyl diphosphate; DMAT, dimethylallyltryptophan; AdoMet, S-adenosylmethionine.

It is also conceivable that other ergot alkaloids play an important role [84]. These alkaloids, including simple lysergyl derivatives as well as the clavines (Fig. 3), have not been routinely monitored, nor has their toxicity been well studied.

Fescue toxicosis is particularly problematic if fields are overgrazed or if the grass is permitted to flower before grazing or harvest of hay. Overgrazing forces animals to eat near the base of the plant. Ergovaline levels show dramatic gradients in distribution in the vegetative plant with the true stem, stem apex, and lower portions of leaf sheaths (which are bundled into the pseudostem structure) containing the highest levels [85]. Levels in the true stems (which contribute little biomass) are especially high, with approximately 4000 ng/g dry mass recorded from perennial ryegrass (*Lolium perenne*) with *N. lolii*. By comparison, the lower leaf sheaths have about half this concentration yet constitute the bulk of the tissue at the base of the shoot. Upper leaf sheaths and leaf blades have much lower levels of ergovaline. The problem associated with flowering plants is evident from the very high levels in panicles and seeds. Tall fescue panicles (with *N. coenophialum*) can have more than 2000 ng/g dry mass [86], and the seeds can have levels ranging from 1000 to 8000 ng/g [87].

3.2. Ergot Alkaloid Structures

Lysergic acid and simple derivatives (most infamously LSD, lysergic acid diethylamide) are known for their hallucinogenic effects. However, the main culprit in ergot poisoning by *C. purpurea* are ergopeptides such as ergotamine, which is biosynthesized from a cyclic peptide formed from lysergic acid, L-alanine, L-phenylalanine, and L-proline (Fig. 3). Once linked together by the enzyme lysergyl peptide synthetase [88], the latter two amino acids are cyclized to form a lactam, apparently by activity of the same enzyme. A second cyclization, probably by a monooxygenase, gives the ergopeptine.

Ergotamine is one of a group of ergopeptides known from *Claviceps* spp. and related fungi. Among the clavicipitaceous endophytes, *Balansia obtecta*, which is a systemic grass parasite, produces ergobalansine [89], and several *Neotyphodium* and *Epichloë* spp. produce ergovaline (Fig. 3). Ergovaline differs from ergotamine in possessing L-valine in place of the phenylalanine moiety, and ergobalansine is apparently built from lysergic acid, L-alanine, L-valine, and another L-alanine. Most other ergopeptides so far described from *Claviceps* spp. ergots contain the following in the order lysergic acid—amino acid 1—amino acid 2—L-proline, where amino acid 1 can be L-alanine, L-valine or L-2-aminobutyric acid; and amino acid 2 can be L-valine, L-phenylalanine, L-leucine, or L-isoleucine [2]. All of these combinations of amino acids 1 and 2 are known from ergots, and many endophytes produce ergovaline. However, the number of other ergopeptides produced by endophytes is unclear because some sources of material in which they were reported may have been contaminated with residues of *Claviceps* sclerotia. Shelby et al. [90], after taking care to avoid this problem, showed that tall fescue with *N. coenophialum* had ergovaline, ergosine (amino acid 1 = L-alanine, aa2 = L-leucine), ergonine (amino acid 1 = L-2-aminobutyric acid, amino acid 2 = L-valine), and two novel ergopeptides—didehydroergovaline and aci-ergovaline.

For each of the ergopeptides, an inine epimer is also known, but these are physiologically inactive and might be artifacts of extraction [2].

Simpler ergot alkaloids are also well known. These include ergine (lysergic acid amide = lysergylamide), which is found in *C. purpurea* sclerotia, as well as endophyte-infected tall fescue. Apparently, genetic disruption of *lpsA*, an endophyte gene for lysergyl

peptide synthetase, also eliminates ergine, supporting the proposition that ergine is derived by hydrolysis of more complex ergopeptides or ergopeptide lactams [91]. A simple ergoline alkaloid, ergonovine (lysergyl-3-hydroxyisopropylamide), was not identified in endophyte-infected tall fescue but was observed together with ergine in the endophyte-infected desert grass, *Achnatherum inebrians* [92]. In the latter, none of the multiple-amino acid ergopeptides was found. The origin of ergonovine remains unclear.

3.3. Analysis of Ergoline and Clavine Alkaloids

Methods to detect and quantify the major ergot alkaloids of the *Lolium* spp.–endophyte symbiota have recently been refined by Spiering et al. [85]. Leaf, leaf sheath (pseudostem), or stem material is freeze-dried then milled and extracted with 2-propanol–water–lactic acid (50:50:1) in which ergotamine tartrate (1.1 µg/mL) is included as internal standard. Samples are vortexed for 60 seconds then incubated 2 to 4 hours in the dark at room temperature. Debris is removed by centrifugation at 6000 × g for 10 minutes. The supernatant can then be analyzed by HPLC. The column used by Spiering et al. [85] is a 150 × 4.6-mm internal diameter, 5-µm film, Prodigy C18 (Phenomenex, Torrance, CA) with a RP-18 Brownlee Newguard precolumn (Perkin-Elmer, Norwalk, CT). The column is at 28°C with a flow rate of 1 mL/min. Sample extracts are injected with an autosampler and run with binary gradients formed from solvents A and B, where A is a 1:3 (v/v) mixture and B is a 3:1 mixture of acetonitrile:0.1 M ammonium acetate (aq.). The gradients are formed as follows: 0 minutes, 95% (v/v) A, 5% (v/v) B; 20 minutes, 80% A, 20% B; 35 minutes, 50% A, 50% B; 40 minutes, 30% A, 70% B; 47 minutes, 30% A, 70% B; 52 minutes, 95% A, 5% B. Ergovaline, ergotamine (standard), and their epimers (ergovalinine and ergotaminine) are quantitated by fluorescent detection with $\lambda_{\text{ex}} = 310$ nm and $\lambda_{\text{em}} = 410$ nm.

The propanol–water–lactic acid extraction protocol [85] requires less handling than previously published procedures. Furthermore, it is superior to acetic-acid extraction in recovery of ergotamine. This is important when ergotamine is used as standard to quantify ergovaline because the procedure avoids the overestimates that can occur with the acetic-acid method [85].

A slightly different HPLC protocol and a different detection system is required for the nonfluorescent clavine alkaloids [93]. The alkaloids have been separated with a Shimadzu LC-MS system equipped with a Phenomenex Synergi Polar-RP column (150 mm × 2-mm internal diameter, 4-µm film, flow rate = 0.2 mL/min), where the mobile phase is a gradient of solution A (5% acetonitrile + 95% aqueous 0.1 M ammonium acetate) and B (75% acetonitrile + 25% aqueous 0.1 M ammonium acetate). The gradient formed over 25 minutes is from 86% A + 14% B to 73% A + 27% B. Molecules are detected by MS using a QP8000α electrospray ionization detector in selected ion mode.

3.4. Ergot Alkaloid Biosynthesis

Most steps in ergot alkaloid biosynthesis have been elucidated [72] (Fig. 3), although only two of the enzymes have been purified. The first experiments were carried out with ergot growing parasitically on rye (*Secale cereale*). Mothes et al. [94] proposed a hypothesis for the origin of the ergoline ring system whereby the first step is condensation of tryptophan with an isoprenoid unit. Radioactively labeled (¹⁴C) tryptophan injected into host plants was incorporated into the lysergic acid moiety of ergonovine. These results were

confirmed with a fermentation culture of *Claviceps* spp., in which ^{14}C -tryptophan was incorporated into elymoclavine [95].

Evidence for the involvement of an isoprene unit (from mevalonic acid) as the second precursor molecule was demonstrated with incorporation of mevalonate into the ergoline ring [96,97]. This was the first demonstration of mevalonate involvement in the biosynthesis of alkaloids other than isoprenoids. Later, Heinstein et al [98] demonstrated synthesis of 4-(γ,γ)-dimethylallyltryptophan (DMAT) from dimethylallyl diphosphate and L-tryptophan, and proposed that this enzyme—dimethylallyl-pyrophosphate: tryptophan dimethylallyl transferase (DMAT synthase)—is the first pathway-specific step in ergot alkaloid biosynthesis (Fig. 3). DMAT *N*-methyl transferase activity was detected in cell-free extracts of *Claviceps* spp., providing evidence that *N*-methylation is the second step in ergot alkaloid biosynthesis [99,100]. The subsequent steps in closure of ring C and formation of chanoclavine I were demonstrated with intramolecular double-labeling of *N*-methyl DMAT [101]. Chanoclavine cyclase closes the D-ring (via a chanoclavine-I-aldehyde intermediate) to form agroclavine. This is oxidized to elymoclavine, which in turn is acted on by elymoclavine-17-monooxygenase. The product of this reaction spontaneously isomerizes to lysergic acid [72,102].

Studies of the later ergopeptide synthesis steps (Fig. 3) have provided an exciting convergence of the enzymatic and genetic investigations. The ergopeptide structures are reminiscent of many fungal metabolites that are composed of individual amino acids, both common and unusual, linked in peptide bonds. These are produced by nonribosomal peptide synthetases (NRPS), which have a modular configuration associated with the number of amino acid (and other) units of the final product [103]. Ergotamine is derived from lysergic acid, L-alanine, L-phenylalanine, and L-proline, which are sequentially linked and cyclized by an NRPS designated lysergyl peptide synthetase (LPS) [88]. However, the activity of such an NRPS yields a cyclic peptide lactam, which must be further oxidized to produce the ergopeptide [72]. The enzyme is unusual for an NRPS in that it has two subunits—one (LPS2) that activates lysergic acid and another (LPS1) that activates the other three amino acids [88]. The role of lysergyl peptide synthetase in ergovaline production has been demonstrated by site-directed mutagenesis of the gene *lpsA* in a ryegrass endophyte [104] as outlined in the next section.

3.5. Genetics of Ergot Alkaloid Production

Identification of genes for ergot alkaloid biosynthesis has occurred only very recently because *Claviceps* spp. have not been readily tractable genetic models. The inclusion of endophytes in these studies has provided the means to demonstrate the roles of particular genes in the pathway [93,104].

The first ergot alkaloid pathway gene to be identified, cloned, and sequenced was for the determinant step, DMAT synthase [105]. The gene was designated *dmaW* (W is the single-letter code for tryptophan). The gene was identified by employing sequences of peptide fragments from the protein, which was purified by Gebler and Poulter [106] from the *C. fusiformis* isolate ATCC 26245 (originally deposited in the American Type Culture Collection as *C. purpurea*). Polymerase chain reactions (PCR) with oligonucleotide primers based on the peptide sequences resulted in amplification of fragments that, when cloned and sequenced, revealed the expected open reading frame [105]. A cosmid (large-insert) clone of genomic DNA containing the gene, as well as the cDNA of *dmaW* mRNA, were then identified. Sequences of the gene and cDNA reveals a coding sequence

specifying a 455 a.a., 51,824 Da product in keeping with the size estimated for the purified enzyme monomer. The coding sequence is interrupted by two short introns. The full-length cDNA was placed under control of an inducible yeast promoter and introduced into *Saccharomyces cerevisiae*. Following induction (substituting galactose for glucose as the carbon source), DMAT synthase activity was detected in the extract. The activity directed synthesis of authentic DMAT, as evidenced by MS, ultraviolet spectroscopy, and comigration with labeled DMAT in HPLC analysis.

Based on the *dmaW* sequence, Arntz et al. [64] identified the counterpart in *C. purpurea* and showed that it was specifically expressed under ergot alkaloid production conditions. Further analysis indicated that this *dmaW* homolog (which they designated *cpd1*) was present in the genomic context of a cluster of genes whose relationships suggested biosynthetic function [107]. A strong indication that this was an ergot alkaloid biosynthesis gene cluster was that one of the genes (*cpps1*) was predicted to encode an NRPS with a match to the partial peptide sequence from purified LPS1.

A total of 12 genes have so far been identified in the cluster that includes *cpd1* and *cpps1* [72]. Two of the gene products are likely to be single-module peptide synthetases. One of these was recently shown to be responsible for activating lysergic acid [109]. Five other gene products are likely involved in redox reactions: a P450 monooxygenase, two oxidases, a dehydrogenase, and a catalase. The presence of a likely catalase gene is a surprise because such enzymes detoxify reactive oxygen. Whether the product of this gene is instead involved in the ergot alkaloid pathway is currently unknown. Finally, two open reading frames in the cluster predicted no significant match to proteins in the sequence databases.

Analysis of *dmaW* and *cpps1* homologs in a grass endophyte provided confirmation of the roles of these genes and the enzymes they encode in biosynthesis of ergot alkaloids. These studies employed a *Neotyphodium lolii* × *Epichloë typhina* hybrid endophyte, isolate Lp1, known for high ergot alkaloid production. The *lpsA* gene of *Neotyphodium* sp. Lp1 was identified by close similarity to *C. purpurea* *cpps1*. A gene-replacement approach resulted in substitution of an insertion mutation for wildtype *lpsA* in the Lp1 genome [104]. The mutated endophyte was then introduced into perennial ryegrass plants, and the resulting symbionts were tested for ergovaline production. As expected, there was no detectable ergovaline in the mutant. Also missing was lysergic acid amide (i.e., ergine), providing evidence that the simple amide arises from more complex ergopeptides [91]. Mutation and heterologous expression of the *C. purpurea* gene *ps2* demonstrated its role in activating lysergic acid [109].

A similar approach was taken to demonstrate the role of *dmaW* in *Neotyphodium* sp. Lp1 [93]. When the 5' portion of its coding region was replaced with a bacterial antibiotic resistance gene, the resulting mutant was unable to direct synthesis of ergovaline. Also undetectable was the intermediate, chanoclavine I. A further check was conducted by introducing the *C. fusiformis* *dmaW* cDNA under control of a β-tubulin gene promoter from *Epichloë typhina*. The resulting complemented strain produced ergovaline, confirming that the loss of ergovaline production had been due to the directed mutation of *dmaW*. These results confirmed the role of DMAT synthase as the likely determinant step in ergot alkaloid biosynthesis.

4. CONCLUSIONS

The *Epichloë* and *Neotyphodium* spp., mycoendophytes of grasses produce diverse alkaloids. Because the endophyte alkaloids are potent neurotoxins that affect insects, verte-

brates, or both, the symbioses in which they occur have been regarded as defensive mutualisms [108]. It is intriguing, however, that the endophytes vary widely in their alkaloid-production profiles [15,16,26]. With recent cloning of ergot-alkaloid and loline-alkaloid biosynthesis genes, it has become apparent that such variation is most often due to gene presence in producers and absence in nonproducers [20,24,93]. Furthermore, expression of alkaloids over time, throughout development, and in different host tissues suggests that the pathways—and probably the genes for those pathways—are highly regulated. Although these alkaloids appear entirely benign both to the host plants and to the fungi that produce them, the regulation of their synthesis and metabolic diversity among endophytes suggest that alkaloid production exacts a cost to the symbionts that is only sometimes counterbalanced by their protective qualities. It seems reasonable because different types of herbivores may be problematic in different parts of a grass species' range. Therefore, the genetic recombination among endophytes with different sets of alkaloid biosynthesis genes can provide an important means of adapting to different ecological contexts. Such recombination is readily accomplished by the sexual cycle of *Epichloë* spp., but the asexual (*Neotyphodium* spp.) counterparts cannot take advantage of this mechanism. However, many *Neotyphodium* spp. are interspecific hybrids, almost certainly arising by cell fusion between ancestral *Neotyphodium* spp. and *Epichloë* spp. [108]. Thus, even the asexual endophytes have the capability to diversify their metabolic capabilities and to fine tune their protective qualities.

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16

Biosynthesis of *N*-Methylated Peptides in Fungi

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1. INTRODUCTION

In biological systems, two different strategies are known for the biosynthesis of peptides: the complex ribosomal machinery and the RNA-independent multienzyme systems called *nonribosomal peptide synthetases* (NRPSs). The majority of cellular peptides and all proteins are of ribosomal origin. However, many bacteria and fungi nonribosomally produce numerous low-molecular-weight peptides, depsipeptides, peptidolactones, and lipopeptides with linear, branched, or cyclic structures as secondary metabolites. Among these substances are many veterinary agents, agrochemicals, and some of the most important therapeutic natural products in use today, including antiviral and antitumor agents as well as immunosuppressants [1]. The enormous structural diversity and complexity of these biomolecules is impressive. Although the actual biological roles of each of these metabolites in the producing organisms are still unclear, it was speculated rather early that the mechanism of synthesis must differ from that of protein synthesis because the vast majority of microbial peptides often contain unusual amino acids that are not found in proteins. Many of the nonproteinogenic amino acids have unique structures with respect to their carbon skeletons, presence of double bonds, unusual functional groups, or D-configuration at the α -carbon atom. The amino acid constituents can also undergo extensive modifications, including *N*-methylation, acylation, glycosylation, and covalent linkage to other functional groups. They can also introduce structural diversity into nonribosomal peptides and *N*-methylpeptides [2].

Several modifications such as racemization (e.g., D-Ala racemase in cyclosporin A biosynthesis) or hydroxylation (e.g., Omphalotin B, C, and D biosynthesis) occur before

substrate recognition or after drug biosynthesis [3,4]. These “tailoring enzymes” are thought to be in close “spatial” connection to the NRPS machinery and organized in large gene cluster [5,6].

Although diverse in structure, NRPSs have a common mode of biosynthesis. NRPSs are large, multifunctional enzymes that follow a common biosynthetic way to yield their products. Their molecular weights range from 100 kDa (PheA) to approximately 1700 kDa (CYSYN) [2]. Each of these peptide synthetases functions as a cellular factory representing the protein template for the construction of one defined peptide structure. NRPSs are organized in coordinated groups of active sites termed *modules* (Fig. 1). Each module is responsible for catalysis of one cycle of polypeptide chain elongation and associated functional group modifications [7–9]. The modules are dissected by functionally different domains, which are connected by flexible linker regions [10].

N-methylated peptides such as cyclosporins, enniatins, and PF1022A-related peptides constitute a class of pharmacologically interesting compounds (Fig. 2A–C). They are synthesized by a special class of enzymes representing hybrid systems of peptide synthetases and integrated *N*-methyltransferases (Fig. 3) [7–9]. These *N*-methyltransferases constitute a new family sharing high homology within prokaryotes and eucaryotes [11].

Like other NRPSs, *N*-methylcyclopeptide synthetases follow the multicarrier thiol template mechanism. According to this model, the substrate amino acids selected from the cellular pool are activated as acyl adenylates and then are covalently tethered to the adjacent carrier protein domains [7–9]. Peptide-bond formation is followed by step-by-step linkage of the activated substrates (elongation reaction) to give a peptide chain with a defined sequence [7–9], which is finally released from the multienzyme. During the synthesis, all substrates remain covalently attached to the multienzyme [12]. Similar organizational principles are also found in polyketide synthesis [13,14].

In this review, we focus on the current state of research on the enzymatic formation of fungal *N*-methylcyclopeptides. These molecular concepts are illustrated by selected examples of fungal peptide synthesis systems.

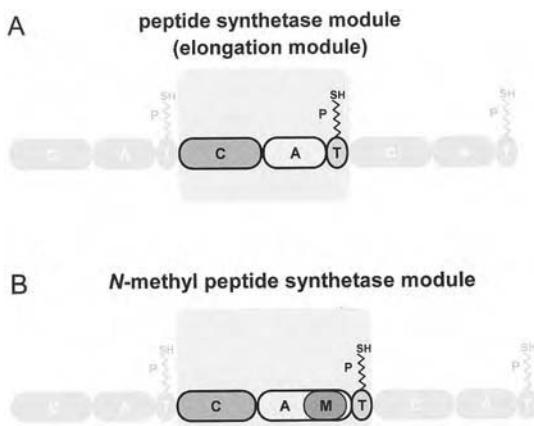


Figure 1 Module structure of NRPS. Dissection of a module of peptide synthetases (A) and *N*-methyl peptide synthetases (B). C, C domain; A, A domain; T, T domain; MT, *N*-MT domain; P, 4'-phosphopantetheine.

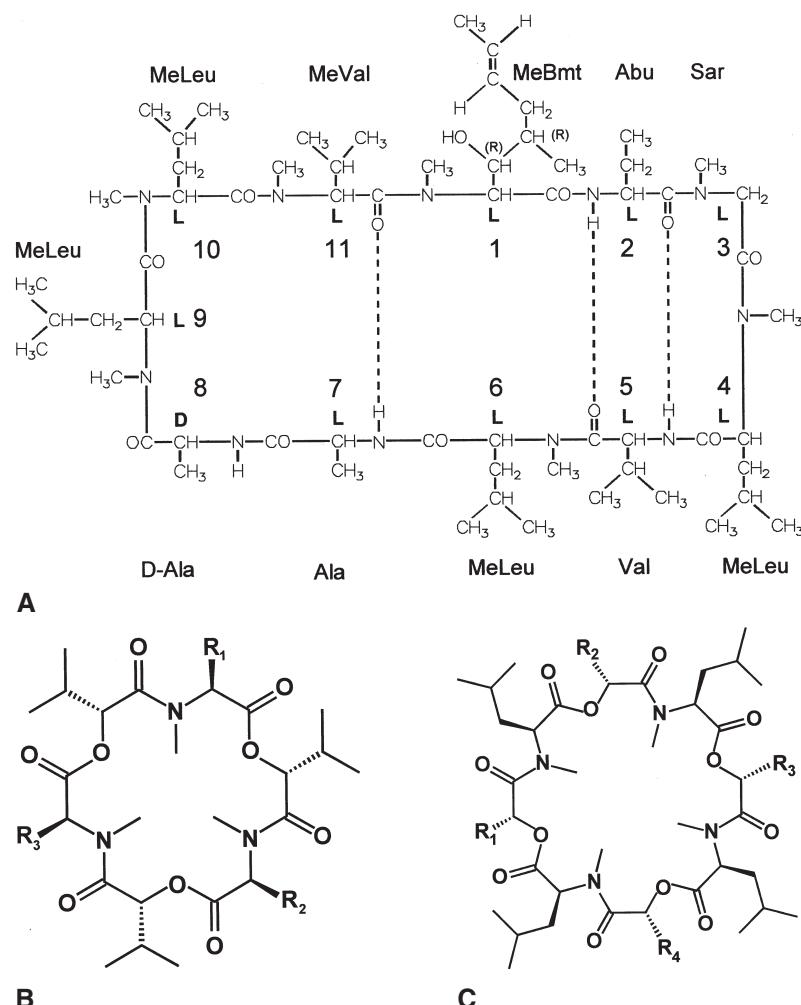
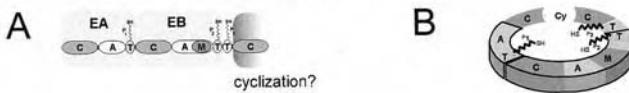


Figure 2 A. Structure of cyclosporin. A, Abu, L- α -aminobutyric acid; Bmt, 4-(E)-butenyl-4-methyl-L-threonine. B. Structure of enniatin. Enniatin A: R₁ = R₂ = R₃ = sec-butyl. Enniatin A₁: R₁ = isopropyl, R₂ = R₃ = sec-butyl. Enniatin B: R₁ = R₂ = R₃ = isopropyl. Enniatin B₁: R₁ = R₂ = isopropyl, R₃ = sec-butyl. Beauvericin: R₁ = R₂ = R₃ = benzyl. C. Structure of PF1022. PF1022A: R₁ = R₃ = benzyl; R₂ = R₄ = methyl. PF1022B: R₁ = R₂ = R₃ = R₄ = benzyl. PF1022C: R₁ = R₂ = R₃ = benzyl; R₄ = methyl. PF1022D: R₁ = benzyl, R₂ = R₃ = R₄ = methyl. PF1022E: R₁ = benzyl, R₂ = R₄ = methyl, R₃ = p-hydroxybenzyl; PF1022F: R₁ = R₂ = R₃ = R₄ = methyl. Bassianolide: R₁ = R₂ = R₃ = R₄ = isopropyl. PF1022-202: R₁ = R₃ = methyl, R₂ = R₄ = p-hydroxybenzyl.

1 N-Methyl Cyclodepsipeptide Synthetases

Enniatin Synthetase *esyn1*



PF1022A synthetase



2 Linear N-Methyl Cyclopeptide Synthetase

Cyclosporin Synthetase *simA*

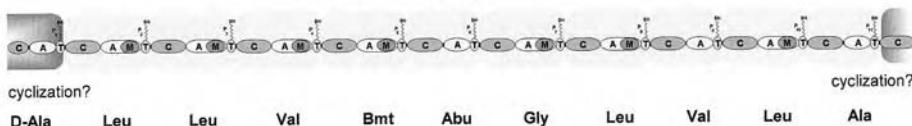


Figure 3 Schematic diagram of the module and domain organization of NRPS. Panel 1, *N*-methylcyclodepsipeptide synthetases: linear order (A) and functional protein (B). EA, EA module (activation of DHIV); EB, EB module (activation of L-Val). An insertion (compared to ESYN) of 63 amino acid residues in the first module of PF1022A synthetase is indicated as a bar. d-Hiv, D-hydroxy-isovaleric acid; d-Lac, D-lactic acid; d-PheLac, D-phenyllactic acid; C, condensation domain; A, adenylation domain; T, thiolation domain; M, *N*-methyltransferase domain. Panel 2, Linear *N*-methylcyclopeptide synthetase.

2. THIOL TEMPLATE MECHANISM AND MULTIMODULAR STRUCTURE OF PEPTIDE SYNTHETASES

The biochemistry of nonribosomal peptide synthesis by multifunctional enzymes has been studied since the 1960s. These studies established the multiple-carrier thiol template mechanism in which NRPSs catalyze peptide-bond formation without mRNA templates [15]. Isolation, sequencing, and characterization of genes encoding NRPSs of bacterial and fungal origin confirmed a multimodular arrangement of this class of enzymes (Fig. 1) [16,17]. Each module is responsible for activating and incorporating a single substrate residue of the final peptide or depsipeptide product [16,17]. In general, the modules are in colinear arrangement with the primary structure of the product [16,17]. Thus, the number of these units equals the number of residues serving as substrates of the corresponding enzymes [16]. There is only one exception with the syringomycin synthetase complex from *Pseudomonas syringae*. In this case, the SyrB subunit, which activates the last amino acid of syringomycin but is the first in the ORF in the gene cluster, has to interact specifically with the C-terminal end of SyrE in order to build a functional enzyme [18].

Elucidation of the highly conserved regions in NRPS systems from different origin revealed characteristic sequence motifs that are spread over the entire length of a module [19]. The modules can be further divided into single domains, each of which has a specific catalytic activity in the biosynthetic process (Fig. 1) [9].

A minimal module (elongation module) consists of an adenylation (A) domain, a thiolation (T) domain or peptidyl-carrier protein (PCP), and a condensation (C) domain in a linear order C-A-T in which the C domain is N-terminal (Fig. 1) [9,16]. The A domain is responsible for amino acid recognition and activation as an acyl adenylate under consumption of ATP [20–23]. In the second step of peptide biosynthesis, the activated substrate is attached by the thiol group of the flexible prosthetic 4'-phosphopantetheine residue (*thiol template*) under formation of active thioester [24]. Peptide-bond formation and elongation requires the adjacent positioning of one aminoacyl residue and one peptidyl residue (or two aminoacyl residues during initiation). During acyltransfer the growing aminoacyl (or peptidyl) chain bound to the upstream T domain is transferred to the attacking aminoacyl substrate thioesterified to the downstream T domain [24]. Formation of peptide bonds between each consecutive pair of substrates is catalyzed by C domains located at the N-terminal end of each module [25,26]. This leads to step-by-step elongation of the growing peptide chain in a N- to C-terminal direction [25]. In modules activating the first acyl constituent to be incorporated, so-called initiation modules, the C domain is usually absent (with the exceptions of CYSYN, ESYN, and PFSYN, and others in which C domains could be found in the initiation module; Fig. 3) [25]. In contrast to these starter modules, elongation modules cannot initiate peptide-bond formation [25]. Optional modifications (eg., *N*-methylation [10] or epimerization [26]) of amino acid residues, catalyzed by additional domains inserted in the basic module (Fig. 3), may occur before entrance to the catalytic peptide forming cycle [23]. This is prior to substrate activation, at the aminoacyl stage preceding elongation [27,28], or after elongation at the peptidyl intermediate stage [26]. The full-length peptide chain is finally released by hydrolysis, amidation, or cyclization (according to the specific product) catalyzed by a thioesterase-like or a cyclization domain which is located in the C-terminal activating module of NRPS systems [29–31].

2.1. Adenylation Domain

The A domain of NRPSs is today a well-understood unit for selection and activation of the appropriate substrate. Comparison of bacterial and fungal synthetases as well as biochemical studies have led to the identification of 10 core motifs within the A domains (A1–A10) [17,19]. The A domains belong to a superfamily of adenylate-forming enzymes, including luciferases, acyl- and aryl-CoA synthases, and plant 4-coumarate:coenzyme A ligases [17,32]. Shared among this group are an ATP-dependent carboxy activation and a homologous region of about 550 to 600 amino acids that exhibits homology in a range of 30% to 70% [32]. The specificity-determining region of A domains probably comprises about 100 amino acid residues at the N-terminus between the core motifs A4 and A5 [33]. Ten moieties in this region were found to constitute the selectivity-conferring code (signature sequence) of A domains, which facilitate recognition of the substrate sidechain [33,34].

To check the postulated selectivity code for different substrates, the substrate specificity Glu of the surfactin synthetase A (linear order of the domains C-A-T) from *Bacillus subtilis* was altered to Gln via a point mutation of one codon [35]. The recombinant

protein was catalytically active in recognizing and activating Gln. Alteration of the binding pocket of the recombinant A domain, AspA, derived from the second module of the surfactin synthetase B, was stepwisely (three amino acid changes) adapted for the recognition of L-Asn. A selectivity switch in the substrate recognition could be observed. One mutation was introduced back into the surfactin biosynthetic gene cluster. The mutational strain produced a new product lipoheptapeptide (Asn5) surfactin determined by LC/MS.

2.2. Thiolation Domain

Peptide synthetase modules that catalyze thioester formation possess an additional 80 amino acid-long stretch, the T domain or PCP (peptidyl carrier protein domain) of about 10 kDa, and C-terminal to the A domain, which is necessary for the translocation of the substrate [36]. T domains contain the core motif LGGXS with a highly conserved serine residue, and the sequence resembles the binding site of the 4'-phosphopantetheine cofactor in acyl carrier proteins of NRPSs, fatty acid- and polyketide synthases [24,37,38]. The structural core of the T domain can now be defined as a region spanning 37 amino acids in both directions from the conserved serine residue [36]. However, for NRPSs to be functional, each T domain must be converted to an active form by posttranslational modification with 4'-phosphopantetheine derived from coenzyme A which is covalently attached to the hydroxyl group of the invariant serine residue [24,39].

A special class of phosphopantetheinyl transferases is responsible for the transfer of the cofactor to the inactive form of the multienzyme. These are considered in some cases to be NRPS-specific [39].

2.3. N-Methylation Domain

A variety of NRPS sequences of Streptomycetes, *N*-methyltransferase domains of peptide synthetases from filamentous fungi, and cyanobacteria are available today. *N*-methyltransferases of NRPS share sequence similarities to each other with the exception of the *N*-methyltransferase domain of the Pyochelin synthetase complex [11,40]. *S*-adenosyl-L-methionine (AdoMet) is the commonly used *N*-methyl donor for the substrates.

In all peptide synthetases containing *N*-methyltransferase domains, the placement of the *N*-MT domain follows a strict order: C-A-[**N-MT**]-A(continued)-T whereas the *N*-MT domain is inserted in the A domain between the conserved motifs A7 and A8 (Fig. 4) [11,19,23]. The size of the domain is about 430 amino acids with a molecular weight of 45 kDa. At the N-terminus of the domains, four conserved methyltransferase motifs (motif I, II/Y [or post I], IV and V) are located in a linear order which may be involved in AdoMet binding in DNA and *N*-methyltransferases of different origin [11,23]. The mechanism of *N*-methylation is discussed in detail in sections 3.2 and 3.5.

2.4. Condensation Domain

Condensation domains are located between each consecutive pair of activation modules and show only low conservation [41,42]. The C domain represents an amide-bond-forming, chain-elongating catalytic domain of about 450 amino acids (50 kDa) in length that accepts acyl groups from the preceding module [42]. Each C domain contains the signature motif HHXXXDG (His motif), essential for translocation of the growing peptide chain [25]. It resembles the active-site motif of well-studied acyl transferases, such as the dihy-

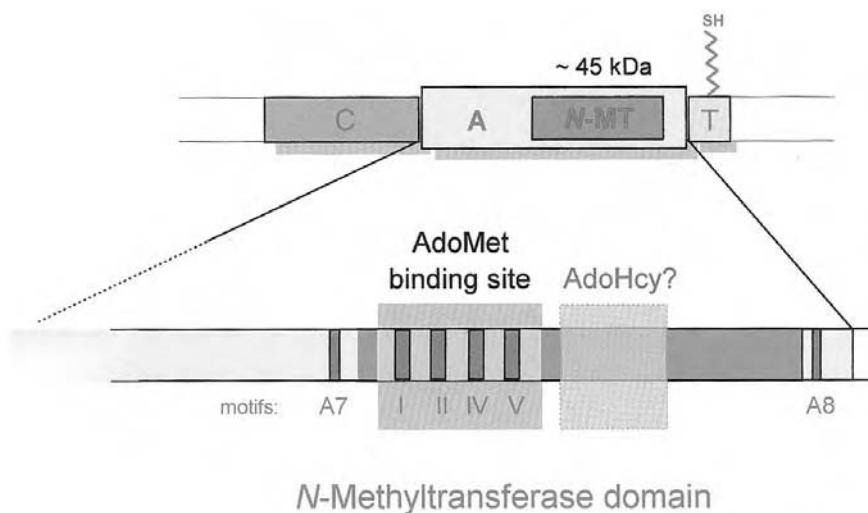


Figure 4 Structural features and organization of a *N*-methyl peptide synthetase module. C, C domain; A, A domain; T, T domain; N-MT, *N*-methyltransferase domain. Conserved motifs within the A and M domains are shown as black bars. Putative AdoMet and AdoHcy binding sites are highlighted.

drolipoamide acetyltransferase family and the chloramphenicol acetyltransferase family [43]. The His motif is located N-terminal to the 4'-phosphopantetheine attachment site (between each consecutive pair of A and T domains) and the distance between the motifs is nearly invariant (170–190 amino acids) [41].

In the case of the proline-activating tyrocidin synthetase B, a point mutation of the strictly conserved second histidine of the His motif abolished peptide-bond formation [25]. In a further report, the authors could identify the key residues in the C domain by site-directed mutagenesis and their proposed role in the catalytic mechanism. An additional residue (R62) of TycB in motif C2 is essential for the condensation reaction [44].

The C domains harbor two sites essential for acyltransfer: an upstream donor site and a downstream acceptor site [42,45]. After recognition and activation of the substrate amino acid, the C domain binds the formed aminoacyl-loaded 4'-phosphopantetheine of its module at the enantioselective acceptor site. In the condensation reaction with the preceding (upstream) aminoacyl- or peptidyl-loaded 4'-phosphopantetheine bound in the donor site, a peptidyl-loaded 4'-phosphopantetheine is formed, which is then handed over to the donor site of the following (downstream) C domain [42]. In the case of tyrocidin synthetase, evidence was presented that C domains are obviously not selective for the length and order of the incoming peptidyl chains, accepting both aminoacyl and peptidyl substrates [42]. A recent study described the discovery of an editing function of the C domains [46].

2.5. Termination Domain

Once the elongation process has produced a full-length peptide chain that has arrived at the T domain of the C-terminal module, the acyl chain has to be released from the enzyme.

A specialized final domain, located downstream to this carrier-protein domain, catalyzes release of mature NRPS products [29,30]. There are three alternative termination strategies in NRPS systems [29,30]. The C-terminal domain (300–500 amino acids of size) of the last module can be a thioesterase (catalyzing hydrolyzation or cyclization of the peptide chain), a reductase (releasing an aldehyde), or a C-domain variant heterocyclization (Cy) domain (catalyzing direct nucleophilic attack on the scissile thioester bond). This final domain recognizes only the mature peptide chain so that incomplete chains are not indiscriminately hydrolyzed [30]. In this domain, the conserved His motif of C domains is replaced by DXXXXD. In most NRPS systems, product release is done by a thioesterase domain [9,47] showing homology to thioesterases of fatty-acid metabolism [48]. C-terminal reductase domains that release aldehydes can be found, for example, in the yeast biosynthetic pathway [49] or in the biosynthesis of the antitumor drug saframycin [50]. In the case of CYSYN, a final C domain is proposed to catalyze amide bond formation to give the cyclic product cyclosporin A [30].

2.6. Structure of Peptide Synthetases

Several strategies including size exclusion chromatography, ultracentrifugation, crosslinking, electron microscopy and reconstitution experiments are described to elucidate the quarternary structure of NRPS. Analytical ultracentrifugation studies of four NRPSs have been carried out thus far: the dimodular 350-kDa *N*-methyldepsipeptide synthetase ESYN, the two-module tyrocidine synthetase TycB_{2–3} (238 kDa), and the 137-kDa enterobactin synthetase EntF. These NRPSs were shown to be functionally active as monomers [51,52]. For the six-domain VibF from the vibriobactin synthetase complex, ultracentrifugation data confirm a dimeric structure [53].

3. *N*-METHYLCYCLOHEXADEPSIPEPTIDES: ENNIATIN AND BEAUVERICIN

Enniatins belong to the class of *N*-methylated cyclodepsipeptides, which are produced by various strains of the genus *Fusarium* and *Alternaria kikuchiana* (enniatin A–F) [54]. Recently the isolation of an additional enniatin (G) from the mangrove fungus *Halosapheia* sp. was reported [55]. As shown in Fig. 2B, enniatins are composed of alternating residues of D-2-hydroxyisovaleric acid (DHIV) and a branched chain *N*-methyl-L-amino acid, linked by amide and ester bonds to form a hexadepsipeptide.

Enniatins exert antibiotic activities against various bacteria and fungi and exhibit immunomodulatory properties [56]. Recently, screening of naturally occurring enniatins revealed that enniatin A has a strong anthelmintic activity against *Nippostrongylus brasiliensis* and *Trichinella spiralis* [57]. Additionally, they are potent inhibitors of mammalian cholesterol acyl transferase [58]. Enniatins are also well known for their behavior as ionophores with high specificity for sodium and potassium ions [59]. They are able to form “sandwich” complexes across a bilayer membrane. The phytotoxic activity of the enniatins has been studied extensively. Toxin-producing *Fusaria* are non–host-specific plant pathogens [60]. Enniatins are postulated to play a role as wilt toxins during *Fusarium* infections of plants [61]. To test the hypothesis that enniatin contributes to phytopathogenicity, enniatin nonproducing mutants were constructed by gene disruption of the ESYN corresponding gene from a virulent strain of *F. avenaceum* and tested on potato tuber tissue. The virulence from seven transformants was significantly reduced; therefore, we

conclude that enniatin production contributes to virulence of *F. avenaceum* [62,63]. Interestingly, enniatins, like the structurally related cyclodepsipeptides beauvericin and bassianolide, exhibit insecticidal properties [64]. In the case of beauvericin, the branched-chain L-amino acid is substituted by L-phenylalanine (Fig. 2B). The mycotoxin beauvericin is also produced by some entomopathogenic and phytopathogenic *Fusarium* sp. [65] and is a specific cholesterol acyl transferase inhibitor [58].

3.1. Enniatin Synthetase: Structure and Function

Enniatin synthetase, the multifunctional enzyme responsible for the biosynthesis of enniatins from their primary precursors, has been isolated from *F. scirpi* (previously designated *F. oxysporum*) and studied in our laboratory [66–69]. ESYN was the first *N*-methylcyclopeptide synthetase that has been characterized. A 14 kb λ-clone containing the ESYN corresponding gene (*esyn1*) was isolated and sequenced [68–70]. This enzyme is expressed constitutively during fermentative growth of the producer *F. scirpi* [71]. Primer extension analysis of the RNA products showed that the start site of transcription is located 184 bp upstream of the start codon (A. Haese and R. Zocher, unpublished data). No additionally regulatory elements specific for secondary metabolism have been identified yet [72]. *Esyn1* encodes an open reading frame of 9393 bp and has been further characterized by heterologous expression in *E. coli* thus far (Fig. 3, panel 1) [11,23,70].

These studies revealed that the enzyme consists of a single polypeptide chain with a molecular mass of 347 kDa as can be maintained by analytical ultracentrifugation [51]. After disruption of the *esyn1* homologous gene locus in *F. avenaceum*, no enniatin biosynthesis could be detected indicating that indeed the *esyn1* gene is responsible for enniatin formation [62]. The biosynthesis of the depsipeptide enniatin proceeds via the thiol template mechanism on the multifunctional enzyme ESYN [68,69].

ESYN harbors all catalytic functions necessary for the synthesis of enniatins from the primary precursors D-HIV and a branched-chain L-amino acid such as leucine, isoleucine, or valine. In the *N*-methylation reaction, *S*-adenosyl-methionine acts as the methyl group donor. The *N*-methyltransferase domain catalyzes the methylation step from adenosylvaline to adenosyl *N*-methylvaline in enniatin biosynthesis [28,68]. Sequence analysis and biochemical characterization (expression studies, antibody mapping) of the *esyn1* gene indicated that the enzyme consists of two modules, EA and EB (Fig. 3, panel 1). Both modules show a high degree of similarity (39.5%) and identity (25%) to each other and to other multifunctional peptide synthetases (e.g., CYSYN) and to adenylate-forming enzymes, such as firefly luciferase and 4-coumarate ligase [11,32,73], with the exception of an insertion of 434 amino acid residues representing the integrated *N*-methyltransferase domain M [11]. Thus, ESYN can be considered a depsipeptide synthetase capable of forming ester and peptide bonds, joined with a *N*-methyltransferase. Three putative C domains of about 400 to 450 amino acid residues size each could be identified by sequence analysis (Fig. 3, panel 1). The second and third C domain exhibit high homology to C domains of other peptide synthetases [74]. The core catalytic motif HHXXXDG is present as ¹⁶²SHALVDS in the second C domain, ¹²²⁹HIIISDG in third C domain, and ²⁸⁴⁵SHALYDG in the first C domain [74]. All seven conserved motifs of C domains [19] can be found in the second and third C domain, whereas only four motifs (C_{1–3} and C₅) are present in the first C domain. The first C domain is not well conserved and may be needed for cyclization of the linear precursor peptide as discussed for the N-terminal C domain of CYSYN [6,14] and actinomycin synthetase II [75] as well as the C-terminal C domain

of HC toxin synthetase [76]. In the case of ESYN, however, the catalytical role of third C domain in condensation of dipeptidol units and condensation domain-mediated cyclization and product release as well as the function of the first C domain in cyclization and product release is still unknown. No experimental evidence has yet been presented.

Monoclonal antibodies directed to the multienzyme ESYN were used to map the catalytic sites of the enzyme [77]. The antibodies could be divided into three groups based on their influence on catalytic functions. Members of group one exclusively inhibited L-valine thioester formation, while members of group two interfered with DHIV thioester formation. Antibodies of group three inhibited both L-valine thioester and DHIV thioester formation as well as the *N*-methyltransferase. From these results, it was concluded that the two modules of ESYN containing the two catalytically binding sites are situated close to each other in the three-dimensional structure of the enzyme. Module EA, located at the N-terminal end of the enzyme, is responsible for the activation and binding of D-HIV, whereas the C-terminal module EB represents the L-amino acid-activating module [70,70,78].

The existence of two different sites in ESYN responsible for binding DHIV and the amino acid has also been deduced from inhibition studies using iodoacetamide and isovaleric acid [68,69].

These findings are in good agreement with results obtained from proteolytic digests of ESYN and expression studies of subclones derived from *esyn1* [67,70]. This result is also confirmed by the predicted ESYN protein sequence [70].

3.2. Molecular Structure of the *N*-Methyltransferase Domain

Amino acid sequence comparison of the *N*-methyltransferase domain of ESYN with other methyltransferases showed no significant sequence similarities except for the conserved glycine-rich motif I ²⁰⁸⁵VLEIGTGSGMIL (Fig. 4) [11,70].

A conserved phenylalanine residue in motif I was found to be crucial in positioning the adenine ring of AdoMet to the *HhaI* DNA methyltransferase [79]. The corresponding position of ESYNs is occupied by the first glycine of motif I (position 2089) [70]. In the case of γ DNA methyltransferases, the phenylalanine residue in motif I is also substituted [80]. A structure-guided sequence alignment using PSI-BLAST and the 3D-PSSM web-based method [81,82] revealed additional conserved motifs (II, IV, and V) that were not obvious from simple alignments (Fig. 4). This permits tertiary structure prediction of the *N*-methyltransferase domain of ESYN and other AdoMet-dependent methyltransferases (e.g., M.*TaqI* methyltransferase from *Thermus aquaticus* and glycine *N*-methyltransferase from rat [83,84]) from their amino acid sequences. Both ESYNs and group γ DNA methyltransferases contain a conserved phenylalanine in motif V downstream of motif IV. The phenylalanine in motif V of γ DNA methyltransferases makes van der Waals contact to the AdoMet adenine [85]. Kagan and Clarke [86] identified two motifs (II: G/P T/Q Y/F D/A V/I I/F, corresponding to motif IV in DNA methyltransferase nomenclature, and III: L K/R PGGXL) conserved in the sequences of AdoMet-dependent methyltransferases methylating small molecules. In Fig. 4, the regions containing the conserved motifs of *N*-methyltransferase domains of *N*-methyl peptide synthetases of eucaryotic and prokaryotic origin are shown.

In a previous work on enniatin biosynthesis, Pieper et al. [78] found a photolabeled nonapeptide after UV irradiation of ESYN in the presence of radiolabeled AdoMet and subsequent chymotryptic digestion. This peptide is located C-terminal to motif I and contains a conserved tyrosine. Therefore the included motif was named motif Y (Fig. 4) [87].

Motif Y shows high similarity to motif II in DNA methyltransferase nomenclature and small molecule *N*-methyltransferases (motif post I) [80], both in sequence and in location relative to motif I. From photolabeling experiments, a conserved tyrosine residue (¹³⁶Tyr) was also suggested to play a role in AdoMet-binding in rat guanidinoacetate methyltransferase [88]. Replacement of a conserved tyrosine residue (⁶⁸³Tyr) in vaccinia virus mRNA (guanine-7-) methyltransferase by Ala and Ser abolished the methyltransferase AdoMet-binding activity, whereas the specific activity of the Phe-mutant reached about 60% of that of wildtype [89].

Previous investigations indicated that deletion of motif I in the *N*-methyltransferase portion of ESYN resulted in a loss of the ability to bind AdoMet as measured by photoaffinity labeling [11].

Deletion of the first 21 N-terminal amino acid residues of the *N*-methylation domain did not affect AdoMet-binding. Further shortening of 16 amino acids close to motif I resulted in loss of binding activity. Complete loss of AdoMet-binding activity was associated both with truncation of a short portion (38 amino acids) from the C terminus and with deletions of internal sequences containing motif I, II/Y (or post-I), IV, and V [11] as well as only motif V, which contains an absolutely conserved Phe residue (with the exception of thaxtomin synthetase B from *Streptomyces acidiscabies* [90]). Point mutations converting the conserved ²¹⁰⁶Tyr in motif II/Y (close to motif I) into valine, alanine, and serine strongly diminished AdoMet-binding, whereas conversion of this residue to phenylalanine restored AdoMet-binding activity to about 70%. This indicated that ²¹⁰⁶Tyr is important for AdoMet-binding and that the aromatic residue of ²¹⁰⁶Tyr may be crucial for *S*-adenosyl-L-methionine binding in *N*-methyl peptide synthetases [11,91].

Today, sequence information of several *N*-methyltransferase domains of peptide synthetases of eucaryotic and prokaryotic origin is available. All domains are located between the motifs A7 and A8 and have an average size of 450 amino acid residues. In Fig. 5, an unrooted phylogenetic tree of *N*-methyltransferase domains of various peptide synthetases is shown. Sequence comparison revealed that the seven *N*-methyltransferase domains of the multifunctional enzyme CYSYN, the *N*-methyltransferase domain of PFSYN, and the *N*-methyltransferase domains of ESYN from *F. scirpi*, *F. sambucinum*, and *F. pallidoroseum* build one group.

The *N*-methyltransferase domains of the different *Fusaria* share 65% to 92% identity. The *N*-methyltransferase domain of PFSYN and the corresponding domain of ESYN from *F. scirpi* exhibit 59% identity. The CYSYN *N*-methyltransferase domains are 54% to 58% identical to each other. The identity of the CYSYN *N*-methyltransferase segments to the corresponding ESYN portions are 47% to 52%. The second group (prokaryotic origin) includes the *N*-methyltransferase domains from various *Streptomyces* and *Microcystis* spp. The *N*-methyltransferases from the different *Streptomyces* exhibit 33% to 68% identity to each other. The *N*-methyltransferases from *Microcystis* spp. and the different *Streptomyces* share 26% to 30% sequence homology. The overall sequence homology between the two major groups is in the range of 18% to 25%.

Recently, Walsh et al. characterized an *N*-methyltransferase activity in pyochelin synthetase PchF [40]. PchF contains an *N*-methyltransferase domain of 380 amino acids, which shows no sequence homologies to other *N*-methyltransferase domains of peptide synthetases [40]. Only the glycine-rich motif I could be identified by sequence analysis. From these findings and BLAST similarity search, we conclude that the PchF *N*-methyltransferase domain belongs to another group of methyltransferases found in peptide synthetases such as yersiniabactin synthetase HMWP2 and methyltransferases from polyketide synthase systems.

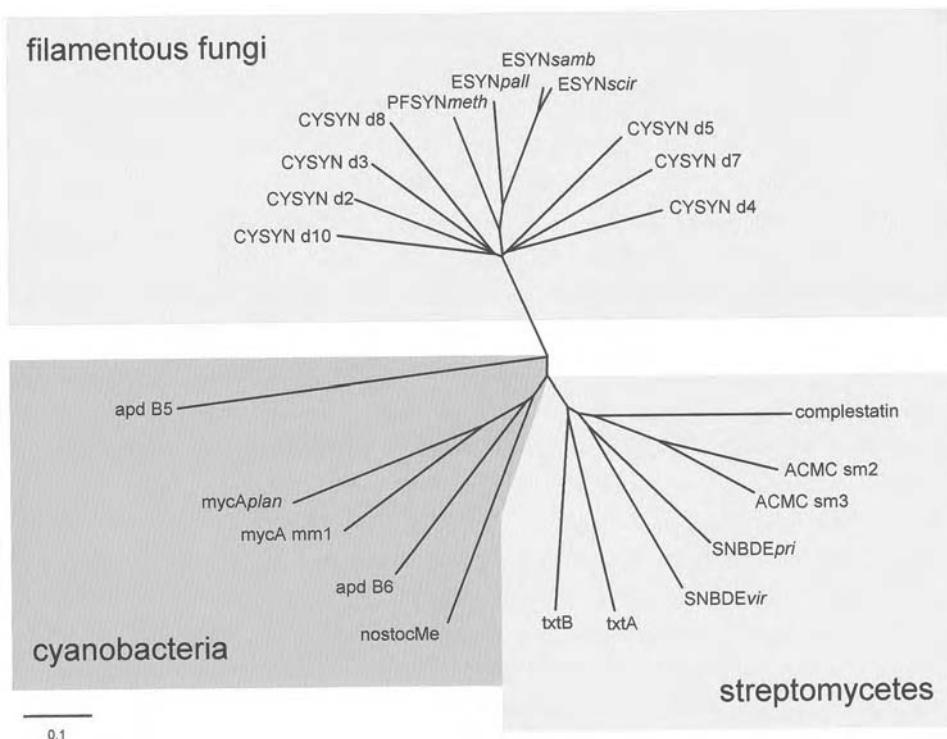


Figure 5 The tree was built by using the neighbor-joining analysis programs of PHYLIP (<http://evolution.genetics.washington.edu/phylip.html>). The branch length marker at the bottom of 0.1 gives the distance in which an amino acid substitution is found in every tenth position (10% difference). For example, ESYNscir/ESYNsamb with a distance of 0.05 has a sequence difference of 5%. The analysis was done by using a sequence alignment of a variety of *N*-methyltransferase domains of peptide synthetases. The selected sequences were aligned by using the ClustalW alignment program and GeneDoc package (<http://www.ebi.ac.uk/clustalw/>, <http://www.psc.edu/biomed/genedoc/>). Abbreviations for the *N*-methyltransferase domain sequences with their accession numbers are as follows: ESYNscir, enniatin synthetase from *Fusarium scirpi* Z18755; ESYNsamb, ESYN from *F. sambucinum* Z48743; ESYNpall, ESYN from *F. pallidoroseum* FPA345016; PFSYN, PF1022A synthetase from *Mycelia sterilia*; CYSYN, cyclosporin synthetase from *Tolypocladium niveum* Z28383; MCYA, microcystin synthetase A from *Microcystis* spp. AB019578; ACMC, actinomycin synthetase II from *Streptomyces crysomallus*; SNBDEpri, pristinamycin synthetase from *S. pristinaespiralis* Y11548 and SNBDEvir: virginiamycin synthetase from *S. virginiae* Y11547; TxtA and TxtB, thaxtomin synthetase A and B from *S. acidiscabies* AF255732; complestatin, complestatin synthetase comC from *S. lavendulae* AF386507; apdB5/6, anabaenopeptilide synthetase apdB from *Anabaena* strain 90 AJ269505; nostocMe, hypothetical protein (peptide synthetase) from *Nostoc punctiforme* ZP_00110697

3.3. Iterative Mechanism of Enniatin Biosynthesis

ESYN is a two-module enzyme but assembles three amino acids and three DHIV molecules in the final product enniatin. Studies on the mechanism of enniatin B formation revealed that the enniatin molecule is synthesized by three successive condensations of enzyme-bound dipeptidols with each other [69]. This implies that, besides the two T domains in the modules EA and EB, ESYN contains an additional T domain at the C terminus of module EB (waiting position) that picks up the intermediates of enniatin synthesis (i.e., the dipeptidol, tetrapeptidol, and hexapeptidol) to allow correct depsipeptide chain elongation (Fig. 3, panel 1). This agrees with the finding of three putative 4'-phosphopantetheine binding sites represented by the motif LGGXS [24,72,92]. Therefore, ESYN (together with beauvericin synthetase and PFSYN) belongs to the class of peptide synthetases that catalyze biosynthesis in an iterative manner. Dissecting the biosynthetic process in the individual steps catalyzed by the individual modules of ESYN has revealed the picture of reaction steps shown in Fig. 6. The reaction sequence leading to the cyclohexadepsipeptide includes the recognition and adenylation of DHIV and the L-amino acid by the corresponding A domains. Then the acyladenylates are covalently bound to the thiol groups of the 4'-phosphopantetheine arms P_1 and P_2 . 4'-Phosphopantetheine facilitates the ordered shift of carboxy thioester activated substrates between the two modules. Characteristically, the thioesterified L-amino acid is methylated with AdoMet, and thus *N*-methylation takes place prior to peptide-bond formation and subsequent cyclization reactions [28]. In a new bimodular domain-swapped actinomycin synthetase, a L-Val specific A domain was replaced by a L-MeVal A domain [93]. The recombinant protein catalyzed the formation of a dipeptide. In the presence of *S*-adenosyl-methionine, valine was converted to MeVal, but no peptide-bond formation was catalyzed. This result supports our findings that *N*-methylation occurred prior to peptide elongation. After *N*-methylation, the amino acid and DHIV form a dipeptidol. The peptide-bond formation is catalyzed by the N-terminal condensation domain of module EB (Fig. 3, panel 1). The dipeptidol unit remains covalently attached to the N-terminal T domain of module EB and is then transferred to the waiting position P_3 of the third T domain in order to reinitiate a new reaction cycle. The hydroxyl group of the newly formed dipeptidol then attacks the carboxy group of the dipeptidol in the waiting position in a nucleophilic reaction yielding a tetrapeptidol covalently bound to the waiting position. In a third condensation reaction, the next dipeptidol attacks the tetrapeptidol to form a hexapeptidol, which yields enniatin in the terminating cyclization reaction. The mechanism of dipeptidol transfer, cyclization and product release, however, is still unclear. The last C domain shares only weak sequence identities to crucial structural elements such as the His motif and so appeared to be a cyclization domain. Because the formation of cyclodepsipeptides larger than enniatin have been observed (e.g., PF1022A an octadepsipeptide, Fig. 2C), it is assumed that the size of a putative cyclization cavity apparently determines the length of the growing depsipeptide chain.

While this process is basically well established, no substantial insight into the three-dimensional arrangement of ESYN and NRPS and possible interactions exists to date. To find out whether enniatin synthesis is an intramolecular process or the result of three interacting Esyn molecules (intermolecular process), analytical ultracentrifugation equilibration studies were carried out [51]. The molecular mass of ESYN of 330 ± 40 kDa with no apparent concentration dependence determined by ultracentrifugation is in good agreement with that calculated from the open reading frame of the encoding gene of 347 kDa, indicating that ESYN exists in solution as a monomer [51]. This strongly suggests

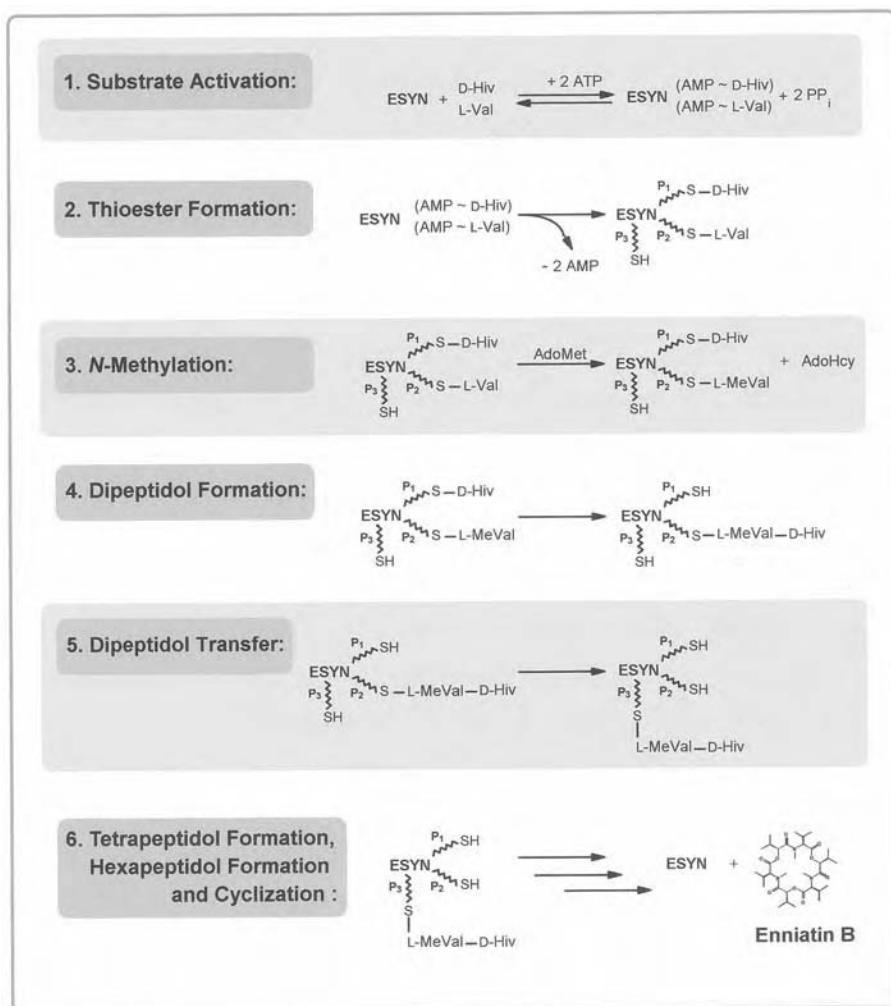


Figure 6 Scheme of partial reactions leading to enniatin B. P₁; P₂; P₃ : 4'-phosphopantetheine. P₃ represents the so-called waiting position.

that the synthesis of the cyclohexadepsipeptide enniatin follows an intramolecular reaction mechanism in which all three reaction cycles are catalyzed by a single ESYN molecule (Fig. 3, panel 1). This finding was supported by *in vitro* complementation studies in which [¹⁴C]-methylvalyl ESYN, upon incubation with the second substrate D-2-hydroxyisovaleric acid (DHIV) and ATP, did not yield radioactive enniatin. Furthermore, the sedimentation rate constant evaluated from analytical ultracentrifugation was lower ($S_{20,w} = 14.1$ S) than expected ($S_{20,w} = 16.9$ S) for a globular protein, indicating that Esyn has an extended structure.

3.4. Substrate Specificity of Enniatin Synthetases

Owing to the relatively broad substrate specificity of ESYN for amino and hydroxy acids, a variety of different enniatins can be synthesized by ESYN if appropriate concentrations

of substrates (depending on the various K_m values) are used [94]. Studies on the substrate specificity revealed that the enzyme is capable of synthesizing enniatins A–C and also mixed-type enniatins containing more than one species of amino acid [95]. Additions of isoleucine to an enniatin-producing culture of *F. scirpi*, for example, results in a rather complex mixture of homologues [95]. The multienzyme also synthesizes different enniatins depending on the amino acid present in the *in vitro* reaction mixture [67].

Nevertheless, ESYNs from different *Fusarium* strains may differ in their amino acid specificity as expressed by their V_{max}/K_m values. ESYN from the enniatin-B-producing fungi *F. lateritium*, for instance, exhibits high affinity for L-valine, the constituent amino acid of enniatin B, and therefore strongly resembles the ESYN from *F. scirpi* [94]. In contrast, the multienzyme from the enniatin-A-producer *F. sambucinum* preferably incorporates L-leucine and L-isoleucine. This difference may be due to mutations in the amino acid binding sites of the various ESYNs. Sequence analysis of the A domains of ESYNs from *F. scirpi* and *F. sambucinum* showed a high degree of sequence identity with the exception of three oligopeptide regions [74]. Previous reports on selectivity-conferring residues that mediate substrate specificity are controversial with respect to the amino acid residues involved in substrate recognition in ESYN [20,33,74,96,97]. Mapping of the key-residues on the three-dimensional model of firefly luciferase revealed that they codefine a putative-specific substrate binding pocket on the surface of luciferase, located near the ATP binding site between core A3 and A5 [73]. Amino acid sequence comparison between the 11 repeating modules of CYSYN led to the identification of site-specific amino acid binding pockets [97]. They were therefore suggested to provide a recognition code for the substrates of the CYSYN A domains.

The crystal structure of the N-terminal A domain of gramicidin S synthetase 1 (PheA) complexed with L-phenylalanine and AMP was solved [20]. Each domain of the enzyme has a similar topology to the corresponding domain of unliganded firefly luciferase. Based on this structure, eight amino acids lining the phenylalanine binding pocket have been implicated in substrate binding [20,96]. Table 1 shows the amino acid residues of the putative binding pockets of different peptide synthetase A-domains with L-Leu, L-Val, and L-Ile specificity and the A-domains from ESYN and PFSYN (responsible for the synthesis of PF1022A-related depsipeptides) corresponding to PheA-related positions.

Comparing these amino acids of the selectivity conferring code from ESYN (*F. scirpi* and/or *F. sambucinum*) with the other A domains, only low identities in the selectivity codes are found. So we suggest that further regions in the variable part of the A domains are involved in substrate recognition and specificity [74]. Additional topology data of A domains exhibiting different substrate specificities are needed to reveal more about the process of substrate recognition.

3.5. Mechanism of *N*-Methylation

A characteristic property of almost all *N*-methyltransferases that have been studied so far is their sensitivity to inhibition by the reaction product *S*-adenosyl-L-homocysteine (AdoHcy), which is formed from the methyl group donor AdoMet in the *N*-methylation step [86]. In addition, sinefungin, another compound structurally related to AdoMet, is known to act as a competitive inhibitor of various *N*-methylating enzymes [98]. Billich and Zocher [28] found that, similar to other methyltransferases, AdoHcy and sinefungin are potent inhibitors of the AdoMet-dependent reaction in enniatin synthesis. Kinetic analysis showed that AdoHcy exhibits an inhibition pattern typical for a partial competitive inhibitor, indicating that AdoHcy does not directly compete with AdoMet but binds to a discrete

Table 1 Critical Amino Acid Residues Lining the Substrate Binding Pockets of Adenylation Domains of Variant Peptide Synthetases^a

Activated Amino Acid	Amino Acid Position of PheA ^b										
	235	236	239	278	299	301	322	330	331	517	
Phe	D	A	W	T	I	A	A	I	C	K	PheA
Ile-1	D	G	F	F	L	G	V	V	Y	K	BacA, BacC, LicC, LchAC
Ile-2	D	A	F	F	Y	G	I	T	F	K	FenB, PPS5
Leu-1	D	A	W	F	L	G	N	V	V	K	BacA, LicA, LchAA, LicB, LchAB, SrfAA, SrfAB
Leu-2	D	A	W	L	Y	G	A	V	M	K	CYSYN
Leu-3	D	G	A	Y	T	G	E	V	V	K	GrsB, TycC
Leu-4	D	A	F	M	L	G	M	V	F	K	LicA, LchAA, SrfAA
Val-1	D	A	F	W	I	G	G	T	F	K	GrsB, FenE, LicB, LchAB, PPS3, SrfAB, TycC
Val-2	D	F	E	S	T	A	A	V	Y	K	AcvA
Val-3	D	A	W	M	F	A	A	V	L	K	CYSYN
Val	D	A	W	F	I	G	I	I	I	K	ESYNscir
Leu/Ile	D	A	W	F	A	G	V	M	I	K	ESYNsamb
Leu	D	A	W	L	V	G	A	V	I	K	PFSYN
D-Lac/	-	A	V	F	?	G	A	T	M	K	PFSYN
D-PheLac											
D-Hiv	-	A	L	F	?	G	G	S	I	K	ESYNscir

^a Selectivity-conferring code according to Stachelhaus et al. [33] derived from amino acid alignments of A domains using ClustalW (<http://www.ebi.ac.uk/clustalw/>).

^b Residue positions according to PheA numbering.

PheA, Gramicidin S synthetase 1; Bac, Bacitracin synthetase; Lic, Lichenysin A synthetase from *Bacillus licheniformis* ATCC 10716; Lch, Lichenysin A synthetase from *B. licheniformis* BNP29; PPS, Putative peptide synthetases from *B. subtilis*; Srf, Surfactin synthetase; CYSYN, Cyclosporin synthetase; GrsB, Gramicidin S synthetase 2; Tyc, Tyrocidin synthetase; Fen, Fengycin synthetase; Acv, ACV synthetase; Esynscir, Enniatin synthetase from *F. scirpi*; Esynsamb, ESYN from *F. sambucinum* and PFSYN, PF1022A synthetase from *M. sterilia*.

inhibitory site (Fig. 4). In addition, AdoHcy inhibited the formation of the unmethylated depsipeptide formed in the absence of AdoMet. In contrast, sinefungin blocked enniatin (methylated product) formation competitively, indicating direct competition for the AdoMet-binding site but that it does not exhibit an influence on the synthesis of desmethylenniatin even if present in excessive amounts. These results confirm the assumption that two different binding sites for the inhibitors must be present in the *N*-methyltransferase domain of ESYN. Thus, blocking of the *N*-methyltransferase function by AdoHcy results in inhibition of the peptide-bond formation (elongation reaction) and/or the cyclization ability of ESYN. This finding implies a dependence of the active sites for transacylation and *N*-methylation in the ESYN system.

3.6. Biosynthesis of Beauvericin

Beauvericin is a structural homologue of enniatins in which the branched-chain L-amino acid is substituted by the aromatic amino acid L-phenylalanine. Beauvericin synthetase,

which has been isolated from the fungus *Beauveria bassiana* [99] and various strains of *Fusaria* [65], strongly resembles ESYN with respect to its molecular size and the reaction mechanism. In contrast to ESYN, which is only able to incorporate aliphatic amino acids, beauvericin synthetase exhibits high substrate specificity for aromatic amino acids such as phenylalanine. This is obviously caused by mutational alterations in the A domain of this enzyme.

Recently, two new beauvericins were discovered [100]. These beauvericins are composed of L-phenylalanine, D-2-hydroxy-3-methyl-valerate, and D-2-hydroxyisovalerate, yielding mixed-type structures.

3.7. Role of D-2-Hydroxyisovalerate Dehydrogenase

In some fungal species, namely the enniatin producers of the genus *Fusarium*, there is a pathway leading from the primary precursor L-valine to DHIV via 2-ketoisovalerate [101,102]. The enzyme catalyzes the reversible reaction of 2-ketoisovalerate to DHIV, which is an intermediate in the biosynthetic pathway of enniatins in *Fusarium*. DHIV dehydrogenase consists of one polypeptide chain with a molecular mass of about 53 kDa. It is strictly dependent on NADPH and, in contrast to other NADPH-dependent oxidoreductases, exhibits a high substrate specificity with respect to 2-ketoisovalerate. This may explain the fact that DHIV is the exclusive hydroxy acid component in enniatins isolated from *Fusaria* [68]. DHIV dehydrogenase also has an important role in the biosynthesis of some other depsipeptides and peptolides, such as beauvericin [99], destruxin [103], valinomycin [104], bassianolide [105], and cyclosporin-like peptolide SDZ 214-103 [106], all of which contain DHIV.

4. *N*-METHYLCYCLOPEPTIDES: CYCLOSPORINS UND PEPTOLIDE SDZ-214-103

Cyclosporins, produced by the filamentous fungus *Tolypocladium niveum* and by numerous strains of *Fusaria* and *Neocosmospora*, represent a class of cyclic undecapeptides which are composed of hydrophobic aliphatic amino acids (Fig. 2A) [106–110]. They exhibit antiinflammatory, immunosuppressive, antifungal, and antiparasitic properties [108]. As a potent immunosuppressant agent, the main metabolite, cyclosporin A, profoundly represses the cellular immune response to foreign antigens and is therefore widely used in transplantation surgery and in the treatment of autoimmune diseases [111,112]. Besides cyclosporin A, there are at least 24 naturally occurring cyclosporins that differ in their amino acid composition in positions 1, 2, 4, 5, 7, and 11 and/or contain unmethylated peptide bonds in positions 1, 4, 6, 9, 10, or 11 [113–116]. Nevertheless, all naturally occurring cyclosporins have two amino acids in common: sarcosine in position 3 and the nonproteinogenic D-alanine in position 8 (Fig. 2A). Cyclosporin A contains two additional nonproteinogenic amino acids: α -aminobutyric acid in position 2 and the unusual amino acid (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine (Bmt) in position 2 [117]. All three nonproteinogenic amino acids have to be synthesized by a pathway independent of the primary metabolism. In addition, several peptide bonds of the cyclosporin molecule are *N*-methylated similar to the depsipeptides enniatin, beauvericin, and PF1022A-related peptides.

4.1. Mechanism of Cyclosporin Biosynthesis

Studies made by feeding experiments with ^{14}C -labeled precursors showed that the biosynthesis of cyclosporins proceeds nonribosomally by the thiol template mechanism, which

owing to the *N*-methylating steps, has strong resemblance to that of enniatin synthesis [118]. Responsible for cyclosporin biosynthesis is the multifunctional enzyme CYSYN (Fig. 3, panel 2) [119,120]. Further experiments on the mechanism of cyclosporin formation were reported by Dittmann et al. [121]. The authors were able to isolate four enzyme-bound intermediate peptides of cyclosporin biosynthesis from a complex mixture of unidentified peptides. All four peptides carried alanine as the N-terminal amino acid. From these findings, the authors conclude that D-alanine provides the “starter amino acid” of cyclosporin biosynthesis. Following this mechanism, no communication between the N-terminal and C-terminal module (1 and 11) is necessary. Evidence was obtained that the dipeptide L-Ala-D-Ala is formed upon incubation of the enzyme with L-Ala, D-Ala and ATP (results not shown). This supports our assumption that all 11 modules of the multienzyme CYSYN are organized in a circular equidistant arrangement. This is in agreement with the findings of de Crécy-Lagard et al. [41], who showed that a His motif is located in peptide synthetases between two consecutive modules, which is crucial in all condensation domains. Interestingly, CYSYN module 1 (D-Ala module) has an additional His motif at the N-terminus, whereas module 11 (L-Ala module) is lacking this motif (Fig. 3, panel 2). In a circular arrangement, module 11 and the additional His motif of module 1 get close and complete module 11. The (putative) N-terminal C domain, which is not well conserved, has been suggested to produce the cyclic product in the chain release step rather than initiation [31]. We therefore conclude that in the case of CYSYN, the N and C terminus of CYSYN are in proximity.

The biosynthesis of cyclosporin A includes at least 40 reaction steps, which can be divided into 11 substrate adenylation reactions, 11 thioester formations, 7 *N*-methylations, 10 elongations, and the final cyclization reaction. In this process, the *N*-methylation reactions occur prior to peptide-bond formation as described for enniatin, beauvericin, actinomycin, and PF1022A synthesis [28,99,93].

Besides the biosynthesis of cyclosporins, the formation of several diketopiperazines could be observed. Diketopiperazines, which represent a partial sequence of the cyclosporin molecule, are cyclic dipeptides formed in a side reaction of CYSYN. This reaction is the cyclization of neighboring substrate amino acids on the enzyme under the consumption of ATP (and AdoMet).

Like ESYN, the multienzyme CYSYN is unable to carry out racemization reactions due to a lack of an integrated racemase function [3,122]. Enzyme-bound L-alanine cannot be epimerized to D-alanine [3]. D-alanine is directly incorporated into the D-alanine position of cyclosporin. The D-alanine moiety is racemized from L-alanine by a separate specific alanine racemase, which plays a key role in cyclosporin biosynthesis [3]. The enzyme requires pyridoxal phosphate as the exclusive cofactor. Molecular mass determinations of the denatured racemase by SDS-PAGE gave a value of 37 kDa, whereas gel filtration calibration studies yielded a value between 120 and 150 kDa, indicating an oligomeric native structure [3]. A similar situation is found in the case of the peptide synthetase that synthesizes HC-toxin [61]. The authors conclude that the *toxG* gene encodes an alanine racemase whose function is to synthesize D-alanine for incorporation into HC-toxin [123]. Detailed incorporation experiments as well as enzymatic studies revealed the origin of the unusual amino acid Bmt backbone. The basic assembly reaction, performed by Bmt polyketide synthase, ends at the stage of 3(R)-hydroxy-4-(R)-methyl-6(E)-octenoyl-CoA [117], which then undergoes further methylation and reduction reactions to form the final precursor Bmt [124].

4.2. Molecular Structure of Cyclosporin Synthetase

The sequential order of the amino acid–activating modules and their accompanying domains in peptide synthetase systems as deduced from DNA sequences of the corresponding genes is also shown in the case of CYSYN (Fig. 3, panel 2). Cyclosporin biosynthesis is catalyzed by a single multienzyme, the cyclosporin synthetase [119]. CYSYN is encoded by an open reading frame of 45.8 kb [122]. Disruption of the CYSYN gene (*simA*) resulted in loss of the ability to produce cyclosporins [125]. The enzyme has an estimated molecular mass of 1.69 MDa containing 11 peptide synthetase modules, of which seven are homologous to the module EB of ESYN carrying the *N*-methyltransferase domains [122]. Based on the fact that modules in peptide synthetases are colinear to the arrangement of amino acids in the peptides to be synthesized, the authors conclude that the 5'-terminal module is responsible for D-alanine-activation (Fig. 3, panel 2). The C-terminal domain of CYSYN was shown to represent the L-alanine–activating peptide synthetase module. This assumption was supported by the finding that a CYSYN fragment of 130 kDa could be isolated, which is capable of activating L-alanine. Edman degradation of this protein yielded a sequence with an N-terminus in the position of amino acid 13601 of CYSYN [122].

4.3. *N*-Methylation Reaction

Because the overall reaction of the cyclosporin formation is a very complex process (at least 40 single reactions), to investigate the *N*-methylation reaction, the formation of the diketopiperazines cyclo(D-alanine-*N*-methylleucine) and cyclo(L-alanine-*N*-methylleucine) were used. In contrast to other *N*-methyltransferases, such as guanidinoacetate methyltransferase [126] or indoleethylamine methyltransferase [127] in which AdoHcy acts as a competitive inhibitor, in the case of the *N*-methyltransferase domains of CYSYN, AdoHcy interestingly exhibits a noncompetitive inhibition pattern [128]. Analogous to ESYN, the methyl group donor AdoMet and the inhibitor AdoHcy must therefore possess different binding sites on the *N*-methyltransferase domain. From the results, that neither the adenylation reaction of the substrate amino acids nor the thioester formation is influenced by AdoHcy, it was concluded that, as in the case of ESYN, the inhibitor blocks not only the *N*-methylation but also the elongation and/or cyclization reaction. Another similarity between CYSYN and ESYN is the effect of the antibiotic sinefungin on product formation. As in the case of enniatin formation, sinefungin inhibits the *in vitro* synthesis of cyclo(D-alanine-*N*-methylleucine) competitively, indicating a mutual binding site for the methyl group donor and the inhibitor, whereas the substrate activation reactions (adenylation and thioesterification) are not affected by sinefungin [128]. The organization of a peptide synthetase module (of ESYN, PFSYN and CYSYN) with an integrated *N*-methylation domain harbors a substrate amino acid binding site and a separate ATP-binding site. The methyl group donor and the inhibitor sinefungin occupy the same binding site on the *N*-methyltransferase domain, whereas AdoHcy binds to a discrete inhibitory site.

Recently Velkov et al. could isolate two AdoMet photolabeled peptides of *N*-methyltransferases domains of CYSYN—one tryptic fragment equivalent to module 8 *N*-methyltransferase domain and one chymotryptic fragment equivalent to module 2 [129]. In the tryptic peptide, one Glu residue, one Pro residue, and one Try residue could be identified by HPLC analysis and Edman degradation. In the chymotryptic fragment, one Glu and one Pro residue could be identified. Each of the peptide sequences of the proteolytic fragments fit with a predicted AdoMet binding site (motif II/Y) described for peptide synthetases by Hacker et al. [11].

4.4. Substrate Specificity of Cyclosporin Synthetase

From the spectrum of naturally occurring cyclosporins, it seems obvious that some of the peptide synthetase modules of CYSYN have a rather high substrate specificity and others, such as that responsible for Bmt, have a lower specificity, allowing incorporation of homologue substrates [113–116]. The only nonvariable positions in the natural cyclosporins are D-alanine in position 8 and sarcosine (*N*-methylglycine) in position 3 of the cyclosporin ring system (Fig. 2A). Exchange of one or more of the unmethylated constitutive amino acids of cyclosporin A by various amino acids gives a picture of the substrate specificity of CYSYN *in vitro* [130]. The amino acid Bmt in position 1 and L- α -aminobutyric acid in position 2 of the cyclosporin molecule can be exchanged by a large spectrum of amino acids, showing a great flexibility of these catalytic sites. Position 3 has a very high degree of substrate specificity, whereas the positions 4, 6, 7, 9, and 10 have marginally less. The variability of positions 5 and 11 is moderate, whereas position 8 shows only low substrate specificity *in vitro* [130].

Amino acid comparisons of cyclosporin synthetase activation domains with firefly luciferase sequence data and secondary structure information of the solved crystal structure led to the identification of three amino acid residues beginning at core 2 (A4). These positions in each A-domain of CYSYN are thought to define the putative amino acid specificity pocket [97]. Additionally, the “recognition triplet” does correspond to those forming the recognition pocket in the gramicidin synthetase PheA described by Conti and coworkers using X-ray data [20].

4.5. Structure Determination of CYSYN

CsCl density gradient centrifugation of purified CYSYN gave a molecular mass of 1.4 MDa for the single multienzyme indicating a monomer structure [131]. The molecular mass could be later calculated from the ORF *simA* as 1.7 MDa, which is in good agreement with the further findings. Sedimentation velocity runs in an analytical ultracentrifuge gave an average sedimentation coefficient of about 26. A reasonable interpretation of these data in structural terms by the authors indicates an oblate ellipsoid for the CYSYN [131]. Hoppert et al. presented data on electron microscopic imaging of the native enzyme [5]. The micrographs showed minor amounts of globular complexes of 25 to 30 nm diameter and smaller chainlike complexes of 7 nm. A globular protein of 25 nm diameter corresponds to a molecular weight of more than 7 MDa, which is not consistent with the molecular weight of 1.7 MDa for CYSYN. These preliminary results allow no further structural detail analysis.

4.6. Cyclosporin-Related Peptolide SDZ-214-103

Peptolide SDZ-214-103 is an undecapeptide lactone produced by the fungus *Cylindrotrichum oligospermum* [132]. It is closely related to cyclosporin A and has the main structural difference DHIV in ester linkage at position 8 instead of D-alanine in the cyclosporins. The peptolide is synthesized by the multifunctional SDZ 214-103 synthetase, of which the catalytic sites appear to be more specific than those of CYSYN [130,132]. Interestingly, the D-2-hydroxy acid position can be occupied by a large range of substrates varying from D-lactic acid to D-2-hydroxyisocaproic acid [130]. However, peptolide synthetase does not synthesize cyclosporin A [130,132]. The enzyme has been described to be a single polypeptide chain with a molecular mass of about 1400 kDa and strongly resembles

CYSYN [130]. This could be shown by cross-reaction of peptolide synthetase with antibodies directed specifically against CYSYN [132].

5. PF1022A AND RELATED CYCLOOCTADEPSIPEPTIDES

In the course of screening for new anthelmintic compounds, Sasaki et al. isolated an *N*-methylated cyclodepsipeptide, PF1022A, from the fungus *Mycelia sterilia* [133] (Fig. 2C). PF1022A exhibits strong anthelmintic properties in combination with low toxicity and is therefore one of the most outstanding anthelmintics [57,134,135]. PF1022A belongs to a new class of recently identified cyclooctadepsipeptides consisting of four alternating residues of *N*-methyl-L-leucine and four residues of D-lactate, D-phenyllactate or D-hydroxyphenyllactate (Fig. 2C). Structurally, the PF1022 anthelmintics are related to bassianolide, which is an insecticidal metabolite from the fungus *B. bassiana* [136], and to the enniatins.

5.1. Biosynthesis of PF1022A-Related Cyclooctadepsipeptides

Similar to enniatin biosynthesis, the molecular size in SDS-PAGE of the purified peptide synthetase (PFSYN) which is responsible for PF1022A formation is about 350 kDa [137]. The multienzyme is encoded by an ORF of 9633 nucleotides and has a calculated molecular mass of 354 kDa [138]. PFSYN and ESYN exhibit 54% identity on the amino acid level. Interestingly, the structural arrangement of both enzymes is nearly identical comprising two modules of which the second module has an integrated *N*-methyltransferase portion inserted in the second A domain of 47 kDa like in Esyn (Fig. 3, panel 1) [11,72]. In the second module, the L-leucine-activating domain of PFSYN has an insertion of 63 amino acids between motif A2 and A3, although PFSYN catalyzes the formation of a cyclooctadepsipeptide and therefore assembles eight residues instead of six in the case of ESYN to form the final product [137]. As in the case of ESYN, three C domains can be found in the sequence of PFSYN. The linear order of the domains is: C-A-T-C-A-[N-MT]-T-T-C (Fig. 3, panel 1).

Based on the assumption that PFSYN is a two-module enzyme like ESYN, catalyzing the biosynthesis of PF1022A-related compounds via an iterative mechanism, we suggest that one module of PFSYN activates L-leucine and is responsible for *N*-methylation. The other module activates D-lactate as well as D-phenyllactate (Table 1). The low substrate specificity of the latter module is also illustrated by the fact that the enzyme activates a wide range of different D-2-hydroxy acids [137–140]. The elongation reaction links the different combinations of the two possible dipeptidol units consisting either of D-lactate/*N*-methyl-L-leucine, D-phenyllactate/*N*-methyl-L-leucine, or D-hydroxyphenyllactate/*N*-methyl-L-leucine and therefore leads to the complete natural product spectrum of *M. sterilia*, including PF1022A–F and PF1022-202.

PFSYN is capable of synthesizing all known natural cyclooctadepsipeptides of the PF1022 type (A, B, C, D, E, F, and 202) differing in the content of D-lactate, D-phenyllactate, and D-hydroxyphenyllactate (Fig. 2C) [138,139]. In addition, the *in vitro* incubations produced di-, tetra-, and hexa-PF1022 homologues [137]. PF1022A, -B, -C, -D, and -F contain D-lactate and/or D-phenyllactate as the hydroxy acid constituent. PF1022E contains two D-lactate residues, one D-phenyllactate and one D-hydroxyphenyllactate. The PF1022 homologue PF1022-202 consists of D-lactate, D-phenyllactate and *N*-methyl-L-leucine. PFSYN strongly resembles the well-documented ESYN in size and mechanism. Taken together, these biochemical and genetic data suggest a close phylogenetic relationship between ESYN and PFSYN (Fig. 3, panel 1).

5.2. Role of D-Phenyllactate Dehydrogenase

The biosynthesis of cyclooctadepsipeptides of the PF1022 type *in vivo* is controlled by specific enzymes that are integrated in the biosynthetic pathway at key positions. They supply the precursors for the peptide synthetases [140] as in the case of DHIV in the biosynthesis of enniatins [101,102]. D-phenyllactate dehydrogenase (DPLDH) from the fungus *M. sterilia* stereospecifically catalyzes the reduction of phenylpyruvate to D-phenyllactate [140]. DPLDH exhibits a wide substrate specificity for other 2-ketoacids with a clear preference for phenylpyruvate and *p*-hydroxyphenylpyruvate as reflected by high k_{cat}/K_m values for these substrates. The enzyme requires NADPH as a cofactor. Molecular mass determination of the denatured enzyme by SDS-PAGE gave an estimated value of 38 kDa, which is in good agreement with the calculated molecular mass of 36.5 kDa for the amino acid sequence deduced from the gene (NCBI accession number BD105415). Sequence alignment of tryptic fragments of DPLDH shows that the purified enzyme is identical to the ORF. Gel filtration calibration studies yielded a value of about 80 kDa, indicating a dimeric structure. Because of substrate specificities, molecular properties, and sequence alignments of DPLDH, this enzyme can be classified as a D-isomer-specific 2-hydroxy acid dehydrogenase.

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17

Molecular Genetics of Lovastatin and Compactin Biosynthesis

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1. INTRODUCTION

Lovastatin and the closely related compactin (Fig. 1) are examples of the thousands of known microbial polyketide metabolites, so named because of a characteristic feature of their biosynthesis, the involvement of intermediates containing repeated β -carbonyl motifs [1,2]. These two fungal metabolites were discovered in the late 1970s, and they or drugs derived from them are taken daily by millions of people worldwide to inhibit cholesterol biosynthesis and thereby lessen the untoward effects of an overabundance of serum cholesterol on the cardiovascular system. Their importance in human medicine has resulted in

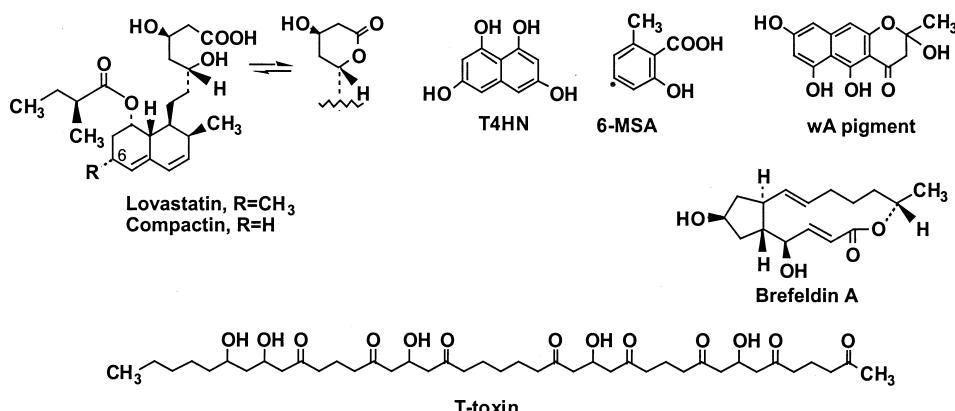


Figure 1 Typical aromatic and reduced fungal polyketides.

considerable knowledge about their pharmacology and medicinal chemistry [3]. This has in turn led to the development of methods for the large-scale production of lovastatin and compactin and, consequently, an interest in their biosynthesis [4]. This topic is reviewed in this chapter with a focus on the molecular genetics and biochemistry of lovastatin and compactin biosynthesis.

2. POLYKETIDE BIOSYNTHESIS IN BACTERIA AND FUNGI

The biosynthesis of polyketides is closely related to the formation of fatty acids, catalyzed by fatty acid synthases (FASs), but differs in that some of the reduction or dehydration reactions catalyzed by a polyketide synthase (PKS) can be suppressed at specific steps in the biosynthetic pathway [5]. Substrates besides acetyl-coenzyme A and malonyl-coenzyme A, the most common substrates of an FAS, can be used by PKSs to assemble the carbon chain. These attributes result in a much wider range of possible products than fatty acid metabolism which, together with post-PKS modifications, have resulted in a very large family of often biologically active secondary metabolites known generically as polyketides.

Microbial polyketides made by type I multifunctional PKSs are assembled in two different ways [5]. In a PKS such as that involved in the biosynthesis of erythromycin A (Fig. 2) [6,7], an antibacterial metabolite of *Saccharopolyspora erythraea*, one distinct group of active sites, called a module [8], on a single polypeptide is used to initiate and extend the carbon chain. The active sites present in each module are used only once during product assembly and determine the choice of starter and extender units, plus the level of reduction or dehydration for a particular extension cycle. The length of the polyketide carbon chain and the sequence of steps by which it is built are determined by the number and order of modules in the polypeptides constituting the PKS. This is illustrated for the polyketide portion of erythromycin in Fig. 2. Such enzymes are called modular PKSs and are involved in the biosynthesis of well-known antibiotics such as erythromycin A and rifamycin [9], as well as newer drugs such as the immunosuppressants rapamycin [10] and FK506 [11].

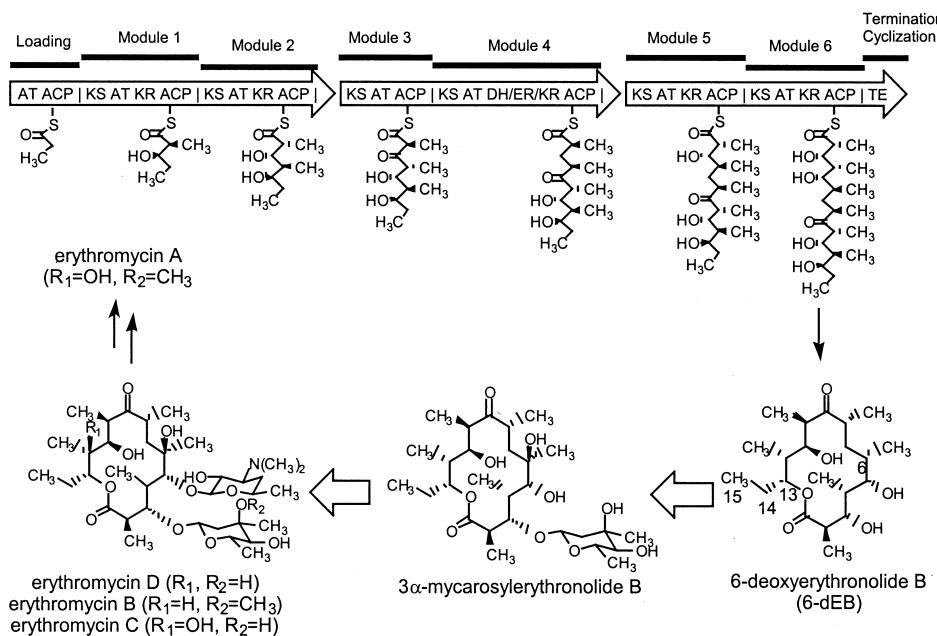


Figure 2 Form and function of 6-deoxyerythronolide B synthase (DEBS), a typical bacterial type I PKS. The DEBS1, DEBS2, and DEBS3 subunits are encoded by the genes *eryAI*, *eryAII*, and *eryAIII*, respectively. Each protein has two modules and each of these contains the activities needed for one cycle of polyketide chain elongation, as illustrated by the structures of the six enzyme-bound intermediates, following the propionate starter unit. Every module contains a ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) domain that together catalyze a two-carbon extension of the chain. The AT domains are specific for methylmalonylCoA except for the AT in the didomain loading module, which prefers propionyl-CoA but can use acetyl-CoA or butyryl-CoA. After each two-carbon unit condensation, the oxidation state of the β -carbon is either retained as a ketone (e.g., module 3), or modified to a hydroxyl, methenyl, or methylene group by the presence of a ketoreductase (KR) (e.g., module 2), a KR + a dehydrase (DH), or a KR + DH + an enoyl reductase (ER) (e.g., module 4), respectively. In effect, the AT specificity and the composition of catalytic domains within a module serves as a “code” for the structure of each two-carbon unit; the order of the modules in a PKS specifies the sequence of the distinct two-carbon units, and the number of modules determines the length of the polyketide chain. Polyketide assembly often is terminated by a thioesterase (TE) domain at the C-terminus of the final module (module 6) to release the product from the protein. For DEBS, release is coincident with the intramolecular cyclization that results in the characteristic macrolide product, 6dEB. Synthesis of 6dEB is followed by C6-hydroxylation (*eryF*) to yield erythronolide B. Addition of the sugar L-mycarose (via TDP-mycarose) yields 3- O - α -mycarosylerythronolide B, and the addition of desosamine (via TDP-desosamine) yields erythromycin D. The two sugars are produced by independent pathways not shown here but controlled by the genes designated *eryB* (mycarose) and *eryC* (desosamine). The final steps are hydroxylation of erythromycin D to yield erythromycin C by a second P450 enzyme (*eryK*) and *O*-methylation of the mycarosyl residue (*eryG*) to yield the cladinosyl moiety in erythromycin A. A side product is erythromycin B, which results from the methylation of erythromycin D and is only poorly converted to erythromycin A.

In contrast, iterative type I PKSs, which are commonly found in fungi and featured in this review, use each active site of the multifunctional PKS repeatedly during assembly of the carbon chain from acetate and malonate only. When these PKSs make aromatic compounds, such as 6-methylsalicylic acid (6-MSA), 1,3,6,8-tetrahydroxynaphthalene (T4HN) and the spore pigment precursor, wA (Fig. 1), the process usually involves a single type I enzyme that acts without any reductive steps or, at the most, only one such step. The dehydration steps, instead of being coincident with carbon chain assembly as in the modular PKSs, are believed to take place afterward, during the aldol reactions that form the aromatic ring(s).

Fungal PKSs also make nonaromatic, reduced compounds such as lovastatin (*syn.* mevinolin or monacolin K), compactin (*syn.* ML-236B) [4,12–15], brefeldin A [16], and T-toxin [17,18] (Fig. 1). All of these metabolites are derived from polyketide chains that vary in their state of reduction and dehydration as well as length. How this is accomplished is still a mystery, because it is not obvious how a single set of the activities typically found in an iterative type I PKS, having the same order of domains as mammalian and some microbial FASs, can make the choice of oxidation level at each chain-extension step.

Insight into the mechanistic basis of such enzymes recently has come from an investigation of lovastatin biosynthesis where the interaction of two type I PKSs with other enzymes of the pathway modulates their overall activity. One of the PKSs, while still acting iteratively, apparently has the ability to discriminate between several chemically distinct intermediates at different stages of carbon chain assembly. The other PKS, although of very similar architecture to the first one, seems to behave noniteratively, because each of its active sites are used only once during product formation.

3. LOVASTATIN AND COMPACTIN BIOSYNTHESIS

In the lovastatin pathway (Fig. 3), the nonaketide carbon chain is derived from one acetate and eight malonate molecules [12,13], and a proposed key intermediate may undergo an electrocyclic cyclization, as shown for the conversion of **5** to **6** [19]. Dihydromonacolin L, an established intermediate of lovastatin biosynthesis [20,21], has two hydroxyls, one double bond, and a methyl group at C-6 derived from the methyl of methionine. Most of these functionalities are created by the PKS while dihydromonacolin L is being synthesized, as illustrated in the boxed region of Fig. 3, consistent with the processive nature of fatty acid and polyketide biosynthesis. A nearly identical pathway has been proposed for compactin biosynthesis [22], but because the putative intermediate **2** (Fig. 3) does not undergo C-methylation, all the subsequent intermediates and the final product, compactin, lack the methyl group at C-6 of lovastatin.

Vederas et al. originally proposed that the hexaketide triene **5** or its nonaketide equivalent could undergo a Diels-Alder like cyclization to form a precursor octahydronaphthalene ring system [23,24]. This event has been modeled chemically, but a diastereomeric compound results because of the preference of the methyl group at C-6 to assume an equatorial geometry in the nonenzymatic reaction [19]. Access to the lovastatin biosynthesis genes recently has allowed the biochemical conversion of **5** to **6**, in the form of their *N*-acetylcysteamine thioesters [25], as noted below. If the PKS involved is able to utilize different sets of activities at different steps in the assembly of dihydromonacolin L, as specified in Fig. 3 for the formation of **1** to **6**, it must be quite remarkable because none of the microbial PKSs studied so far possess such discriminatory activity for the

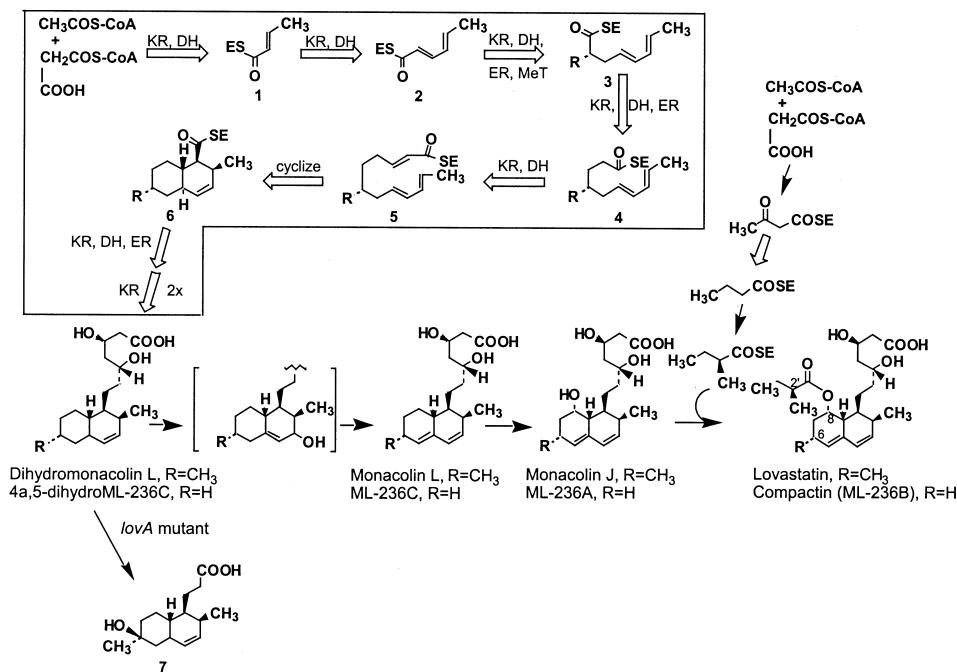


Figure 3 Lovastatin and compactin biosynthetic hypothesis. The boxed region shows the set and order of reactions thought to be necessary for the biosynthesis of dihydromonacolin L and dihydro-ML-236C, the first recognized intermediates in lovastatin and compactin biosynthesis, respectively. The intermediate in brackets following dihydromonacolin L has not been verified experimentally, but the products of the next two steps are bona fide intermediates of the respective pathways. The steps in the synthesis of 2-methylbutyrate are based on analogous reactions in fatty acid biosynthesis, although their exact order has not been determined.

biosynthesis of a single molecule. They simply make a largely unreduced polyketide of fixed length or, when different combinations of reduction and dehydration events are carried out, use each active site only once in accomplishing these changes.

4. LOVASTATIN BIOSYNTHESIS GENES

Pioneering genetic research on lovastatin biosynthesis by Hendrickson et al. [26] identified a type I PKS gene essential for lovastatin biosynthesis by *A. terreus*. Its 334,983-dalton deduced product, now called the lovastatin nonaketide synthase (LNKS), contains the six active sites—ketosynthase (KS), acyltransferase (AT), dehydratase (DH), enoyl reductase (ER), ketoreductase (KR), and acyl carrier protein (ACP)—found in type I FASs and many bacterial PKSs and arranged in the same order as in an animal FAS. Key differences are the presence of a methyltransferase (MeT) domain and replacement of the C-terminal thioesterase (TE) domain often found in FASs and PKSs with an approximately 500–amino acid extension of unknown function and similar to the condensation domain of nonribosomal peptide synthetases (NRPSs) [27]. As noted below, the ER domain of LNKS may

not be functional. The MlcA PKS of compactin biosynthesis is 59% identical to LNKS at the amino acid level and contains functionally equivalent domains, except for the presumed inactivity of its MeT domain [22].

Characterization of the LNKS (*lovB*) gene [26] sets the stage for understanding how the carbon skeletons of dihydromonacolin L and lovastatin are assembled. Moreover, because secondary metabolism genes invariably are clustered in microorganisms, this gene also provided a convenient entry into cloning and characterizing the other genes involved in lovastatin production. This was done as described by Kennedy et al. [28], who identified a cluster of 18 potential lovastatin production genes (Fig. 4), the functions of which largely could be predicted by sequence comparisons. Two of these genes, *lovB* and *lovF*, encode PKSs. The presence of MeT domains in LNKS and the LovF (LDKS) protein, as well as the corresponding MlcA and MlcB proteins [22] (see below), indicates that both of the methyl groups in lovastatin, which are derived from S-adenosyl-L-methionine (SAM), are likely to be added while the polyketide is being synthesized. In contrast, only the MlcB PKS of compactin biosynthesis is believed to contain a functional MeT domain [22]. Such domains are common among NRPSs, where N-methylation of amino acids can occur [27], but have only rarely been found as part of a PKS (e.g., in 4-but enylmethylthreonine synthase [29] and the epothilone PKS [30,31]). The role of the C-terminal region of LNKS and MlcA in the context of lovastatin and compactin biosynthesis is unclear, although they are likely to be involved in some of the unusual properties of these two PKSs.

By making targeted mutations of most of the genes shown in Fig. 4, we have established that the ones marked with an X are essential for lovastatin production by *A. terreus*. Besides *lovB*, the *lovA*, *lovC*, *lovD*, *lovF*, and *lovG* genes encode enzymes that catalyze steps in the biosynthetic pathway because their mutants do not produce lovastatin. The functions of the LovB, LovC, LovD, and LovF proteins are discussed below, whereas the role of the *lovG* gene remains unclear, apart from the fact that its mutant only makes a small amount of lovastatin. The deduced product of the *lovA* gene strongly resembles cytochrome P450 enzymes, suggesting that this gene could be responsible for a hydroxla-

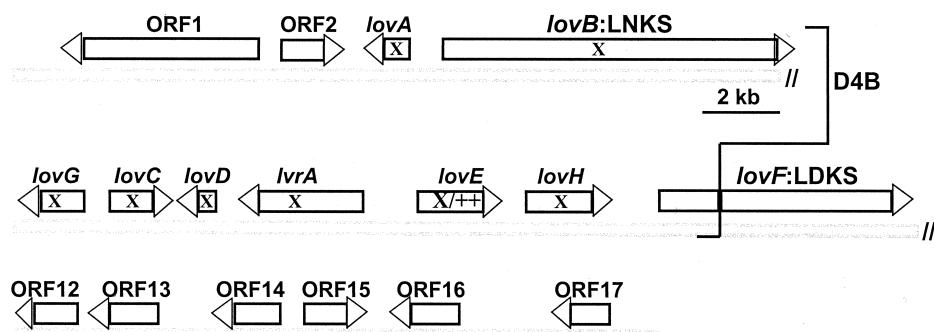


Figure 4 The lovastatin production genes, all of which occur in one contiguous region of the *A. terreus* genome. Broad-pointed arrows show the relative location and orientation of the genes, and the size of the arrows indicates the relative sizes of the gene products. Genes marked with an X have been mutated and are essential for lovastatin production. The ++ in the *lovE* gene indicates that extra copies cause enhanced lovastatin production. The bracket on the right hand side indicates the D4B cosmid that contains all of the genes above the top two lines except for *lovF*.

tion or oxidation step in lovastatin biosynthesis. Curiously, the *lovA* mutant has an unusually active β-oxidation system and accumulates the truncated compound **7** when fed dihydromonacolin L instead of some nonhydroxylated intermediate of lovastatin biosynthesis [32]. A *lovC* mutant, in contrast, efficiently converts dihydromonacolin L and monacolin J to lovastatin [33], or 6-desmethylmonacolin J to compactin [32].

Because the *lovE* and *ORF13* genes encode proteins with the binuclear Zn²⁺ finger motif characteristic of eukaryotic transcription factors such as GAL4 and HAP1 [34], we first assumed that they regulate lovastatin production at the transcriptional level. This idea is consistent with the facts that *lovE* and *ORF13* mutants do not produce any of the intermediates of lovastatin biosynthesis and that introduction of extra copies of *lovE* into the wild-type strain result in a 7- to 10-fold overproduction of lovastatin [28]. The latter behavior is typical of regulatory genes, because their products usually are produced in limiting amounts. Conversely, nonlovastatin-producing *ORF13* mutants were isolated infrequently (only 1 of 12 to 19 strains that appeared to be *ORF13* disruptants on the basis of Southern analysis were nonproducers) and lovastatin production was not restored by introduction of the w.t. *ORF13* gene (C. Park and C. R. Hutchinson, unpublished results). These results imply that the *ORF13* gene may not be involved in lovastatin biosynthesis after all. Furthermore, it is not obvious how lovastatin biosynthesis could use two regulatory genes; most other clusters of secondary metabolism genes contain only one gene of this kind [35].

Self-resistance to lovastatin is provided by the *lvrA* gene, which encodes a protein very much like known hydroxymethylglutaryl coenzyme A reductases [36], because introduction of *lvrA* into the lovastatin sensitive *Aspergillus nidulans* confers high level lovastatin resistance (K. Watts, J. Kennedy, and C.R. Hutchinson, unpublished results). No increase in lovastatin resistance was observed upon introduction of the *lovH* gene (formerly named *lovI* [37]) into the same host, even though its deduced product strongly resembles known transmembrane metabolite transport proteins of microorganisms, many of which provide resistance to toxins and naturally occurring antibiotics (e.g., [38]). Because the *lovH* mutant does not make lovastatin or its known precursors (J. Kennedy and C. R. Hutchinson, unpublished results), this gene may play a biosynthetic role or lack of the LovH protein may cause some type of feedback inhibition of other genes or enzymes.

Regardless of the current uncertainty about the roles of some of these genes, we believe that the minimum number required for lovastatin production in a heterologous fungal host are the ones marked with an X in Fig. 4 plus *lovF*. Introduction of the D4B cosmid bearing all of these genes except *lovF* (or without *ORF1*, *ORF2* and *lovF*; J. Kennedy and C. R. Hutchinson, unpublished results) conferred the ability to produce monacolin J on *A. nidulans* [28]. LovF is the PKS that makes the 2-methylbutyrate side chain of lovastatin (see below), and this enzyme plus LovD are the only enzymes believed to be required to convert monacolin J into lovastatin. These facts imply that the *ORF12* or the *ORF13* genes, both of which are not carried by the cosmid (Fig. 4), are nonessential. The *ORF14–17* genes also may not be involved because their disruption had no effect on lovastatin production (C. Park and C. R. Hutchinson, unpublished results).

A cluster of compactin biosynthesis genes from *P. citrinum* recently has been described [22] that contains orthologs of only the *lovA* to *lovH* and *lvrA* genes. Consequently, lovastatin and compactin biosynthesis seems to require nine enzymes: two multifunctional PKSs, self-resistance and export proteins, a cytochrome P450, a transesterase, and three other enzymes whose exact functions have not been established.

5. LOVASTATIN PKS AND RELATED ENZYMES

To further investigate the role of *lovB*, it was overexpressed in *A. nidulans* and a clone that clearly produced significant quantities of LNKS was selected for further study. This strain produced two new polyketides (Fig. 5): 4-hydroxy-6-[(1E,3E,5E)-1-methylhepta-1,3,5-trien-1-yl]-2-pyrone (**8**) and 4-hydroxy-6-[(1E,3E,5E,7E)-3-methylnona-1,3,5,7-tetraen-1-yl]-2-pyrone (**9**) [28]. The structures of these metabolites are consistent with their origin from acetate via the intermediate shown. These results are indicative of a malfunctioning PKS that has retained all activities (apart from the ER activity) but is apparently unable to coordinate them properly, resulting in shunt products that have a shorter carbon chain and lower degree of reduction than dihydromonacolin L. Both of these metabolites contain a methyl group derived from SAM at the seventh carbon from the methyl terminus, demonstrating that the integral MeT domain of LNKS is functional and methylates the correct position.

These data suggested that either LNKS was malfunctional or that some other enzyme might be needed. Introduction of the D4B cosmid that contains several other genes from the cluster (Fig. 4) into the *A. nidulans/lovB* transformant resulted in the production of monacolin J and a small amount of monacolin L [28]. Production of the yellow pigments **8** and **9** had stopped, or was much reduced, in such strains; furthermore, they were resistant to high levels of lovastatin. Hence, the genes introduced on the cosmid clearly are sufficient for the production of monacolin J and resistance to lovastatin and its precursors.

This result led us to look for an additional enzyme that might act with LNKS to produce the fully processed polyketide chain. When the properties of different *lov* mutants were studied, we discovered that the *lovC* mutant was blocked in lovastatin production and accumulated compound **8** [28]. Formation of the same product by overexpression of

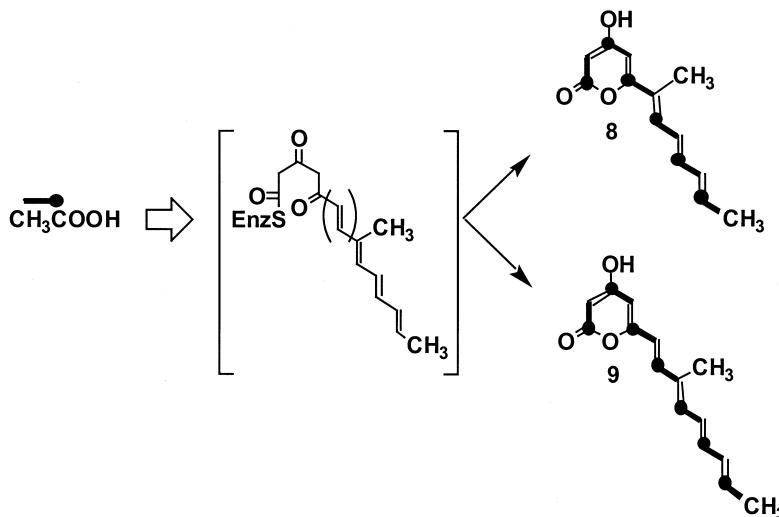


Figure 5 Proposed formation of compounds **8** and **9** by spontaneous closure of the β,δ -dicarbonyl acid in brackets to a 4-hydroxy-2-pyrone. The bracketed intermediate itself results from condensation of six or seven acetate-derived C₂ units [the () shows the additional C₂ unit used to form **9**], as indicated by the ¹³C-labeling pattern.

lovB in *A. nidulans* and by the *A. terreus* *lovC* mutant led us to study whether the products of these two genes interact functionally. Coexpression of *lovB* and *lovC* in *A. nidulans* resulted in the production of dihydromonacolin L [28]. Furthermore, cell-free extracts of this strain were found to make dihydromonacolin L in vitro and to incorporate ¹³C from ¹³C-labeled malonyl-coenzyme A at the expected positions [25] (K. Auclair and J. Vederas, unpublished results).

The fact that LNKS and LovC together make up a PKS capable of producing dihydromonacolin L was unexpected. LovC is highly similar to the products of genes for secondary or specialized metabolism in other fungi and some plants, and in particular to ER domains of PKSs [28]. Interaction of the LNKS and LovC proteins may help explain the remarkable substrate discrimination necessary for the biosynthesis of dihydromonacolin L. If LNKS does lack ER activity, as predicted [28], LovC could be responsible for the three enoyl reductions predicted to be necessary for dihydromonacolin L production and act at only the tetra-, penta-, and heptaketide stages (Fig. 2, boxed region). This enzyme probably acts upon the growing polyketide chain still bound to LNKS because, if it acted upon a free intermediate, we would expect the *lovB*-expressing strain to accumulate a full-length nonaketide. The two enzymes therefore are expected to be closely associated, which is consistent with the observation that his-tagged LovC and LNKS can be copurified from the cell extract of an *A. nidulans* strain carrying both of these genes (J. Kennedy and C.R. Hutchinson, unpublished). LNKS can clearly recognize when to methylate the polyketide intermediate, as in the formation of compounds **8** and **9**, but the formation of these compounds suggests that this PKS apparently is unable to catalyze further reactions correctly once one ER reaction has failed to occur. Moreover, in the correctly functioning system when LovC is present, LNKS must be able to discriminate the final two steps, when only the KR is believed to be used, from the rest when both KR and DH activities are believed to be used (Fig. 3).

Another property of LNKS that deserves special comment is the proposed conversion of compound **5** to **6** in Fig. 3. This is a biological equivalent of the Diels-Alder reaction, which has been speculated to be widespread in nature [39] but heretofore has been documented only to occur with solanapyrone synthase from *Alternaria solani* [40] and the closely related macrophomate synthase from *Macrophoma commeliniae* [41]. Thus, it is noteworthy that purified LNKS was able to convert the *N*-acetylcysteamine (NAC) ester of **5** (this ester mimics the role of the natural coenzyme A ester of the intermediate) into compound **10** (Fig. 6) preferentially [25]. Compounds **11** and **12** are formed spontaneously in the absence of the enzyme [19], and to some extent in its presence, but have the wrong relative stereochemistry for conversion into dihydromonacolin L. Solanapyrone synthase also converts its triene substrate preferentially into the stereochemically correct cyclic product, which is accompanied by additional cyclic products of incorrect stereochemistry [40]. It remains to be seen whether addition of LovC to LNKS will allow conversion of **10** to dihydromonacolin L, or whether a LNKS mutant that lacks the C-terminal domain can also carry out this Diels-Alder reaction. We do know, however, that the latter kind of mutant is still able to make **8** (J. Kennedy & C.R. Hutchinson, unpublished).

The *lovF* gene encodes lovastatin diketide synthase (LDKS) that contains the KS, AT, DH, ER, KR, and ACP domains of an FAS and PKS but no TE domain. It also has, like LNKS, a MeT domain but does not contain the unusual C-terminal domain, and its ER domain conforms closely to the consensus sequence for this type of activity [28]. A *lovF* mutant produced no lovastatin but instead accumulated monacolin J [28]. LDKS is

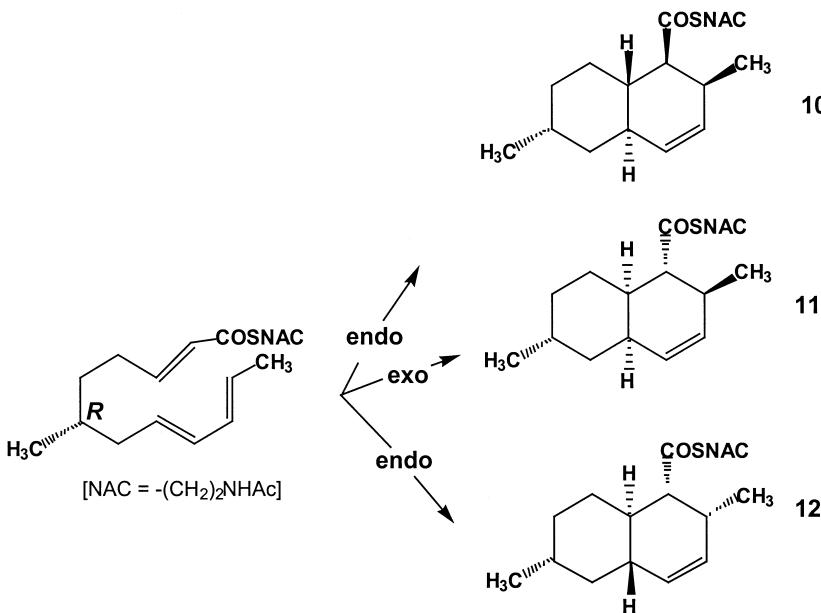


Figure 6 Formation of the Diels-Alder products. Compounds **11** and **12** are formed in approximately equal amounts by the thermally catalyzed reaction in nonaqueous or aqueous media. Compound **10** appears only in the presence of LNKS, accompanied by **11** and **12**. *R* = absolute stereochemistry at this position of the reactant.

therefore likely to be the enzyme responsible for the biosynthesis of the (2*R*)-2-methylbutyryl side chain at the C-8 position of lovastatin.

The absence of a TE domain at the C-terminus of LDKS led us to look for another protein that might release the 2-methylbutyric acid product. This is likely to be LovD, a protein of 413 amino acids that is similar to β -lactamases, carboxypeptidases, lipases, and esterases, because disruption of the *lovD* gene resulted in a strain that accumulated monacolin J [28]. LovD could be the 2-methylbutyryl/monacolin J transesterase that joins together the two polyketide components of lovastatin. An enzyme with this activity was partially purified from *A. terreus* and found by SDS-PAGE to have a molecular weight of 46 kDa, the same as that predicted for the LovD protein [26]. These researchers reported that the enzyme catalyzed the transfer of the 2-methylbutyryl side chain from lovastatin to monacolin J; neither methylbutyrate nor its coenzyme A derivative could substitute for the lovastatin in this reaction. Hence, we predict that LovD requires as substrates 2-methylbutyrate bound to the LovF protein and monacolin J. This idea accounts for the lack of a TE domain or equivalent product-releasing activity in LovF and also implies that LovD docks with LovF.

6. SUMMARY

The important hydroxymethyl glutaryl coenzyme A reductase inhibitors, lovastatin and compactin, are synthesized by PKS systems possessing a larger range of biosynthetic

activities than the typical fungal iterative PKS involved in the biosynthesis of aromatic polyketides. The dihydromonacolin L synthase consists of at least two enzymes, the iterative LNKS and LovC, a putative enoyl reductase. These two enzymes appear to be all that are necessary for the 35 separate reactions postulated to be necessary for the biosynthesis of dihydromonacolin L from acetyl-coenzyme A, malonyl-coenzyme A, NADPH, and SAM. The LNKS/LovC system must possess a remarkable discriminatory ability, the catalytic mechanism of which is very different from the linear programming found in bacterial modular PKS systems [5–7]. In contrast, LDKS appears to act more like the latter type of PKS in catalysing a single Claisen condensation followed by methylation, ketoreduction, dehydration, and enoyl reduction to produce (2R)-2-methylbutyrate (Fig. 3). This product thus results from a noniterative process and probably remains bound to the enzyme until transferred to monacolin J by LovD. LDKS consequently could be considered as an architecturally novel fungal FAS, consisting of a single protein whose active sites are ordered the same as in a mammalian FAS [42]. A typical fungal FAS consists of two separate proteins whose active sites are arranged differently from a mammalian FAS [43].

The widespread use of lovastatin and drugs derived from it and compactin to treat atherosclerosis [3] naturally has stimulated interest in using the cloned biosynthesis genes to increase production of either compound by the producing fungi. This has been achieved by introduction of extra copies of the *lovA* gene into *A. terreus* [44] and seven compactin genes into *P. citrinum* [45], although metabolite productivity was increased less than 2-fold in either case. Conversely, and clearly illustrated by the positive effect of adding additional copies of *lovE*, molecular genetic manipulation of the regulators involved in production of these compounds does seem to offer significant opportunity to increase process yields.

ACKNOWLEDGEMENTS

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18

The *Aspergillus Nidulans* Polyketide Synthase WA

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1. ASPERGILLUS NIDULANS

Aspergillus nidulans is a filamentous fungus belonging to the family of ascomycetes, which is one of the most ubiquitous worldwide. It grows rapidly on solid or in liquid media under a variety of nutritional conditions. It is normally haploid but also can be induced to grow as a heterokaryon or a vegetative diploid. It produces both asexual spores (conidia) and sexual spores (ascospores).

A. nidulans is one of the critical fungal systems in genetics and cell biology. It is important because it is closely related to a large number of other *Aspergillus* species of industrial and medical significance (e.g., *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus flavus*, and *Aspergillus fumigatus*) and serves as a model for understanding many biological questions. Unlike other asexual *Aspergillus* spp., *A. nidulans* has a well-characterized conventional genetic system. Its advantages include compact colonial morphology, uninucleate conidia, homothallism, meiotic genetics, haploid nature, and the ability to construct diploids and perform mitotic analysis. The relative ease of handling as a laboratory organism facilitates the investigation of physiology, genetics, and molecular biology of a lower, although multicellular, eukaryote; thus, *A. nidulans* has become a useful tool for fundamental research. Numerous auxotrophs, mutants in different metabolic pathways, and developmental mutants were isolated. Since transformation procedures were established for *A. nidulans* in the early 1980s [1–3], molecular genetic manipulation and reverse genetics have become standard techniques, and today all modern tools to study the regulation of gene expression or developmental processes can be used in *A. nidulans*.

The size of the *A. nidulans* genome is approximately 31 Mb. It has eight well-marked chromosomes, containing an estimated 11,000 to 12,000 genes. An *Aspergillus*

nidulans sequence project is underway as a part of the Whitehead Institute Fungal Genome Initiative [4]. The complete genome sequence data will soon become available to the public.

2. ASPERGILLUS NIDULANS WA AND YA LOCI

Asexual reproduction in *A. nidulans* involves the differentiation of complex, multicellular, spore-producing structures called conidiophores and spherical, uninucleate spores called conidia. The conidia of this species contain a dark green pigment in their walls, which is produced as the spores mature. It is known that this pigmentation confers resistance to ultraviolet light [5,6].

In the late 1940s, Pontecorvo et al. at the University of Glasgow were searching for an organism that would have characteristics suitable for detailed genetic analysis. Their search led them to the common soil fungus *A. nidulans* [7]. They found that spore color mutants of *A. nidulans* were autonomous in action, i.e., the color of the spore corresponded to the genotype. They designated two loci, *wA* and *yA*, involved in the spore pigment formation. *yA* mutants produce yellow spores and *wA* mutants produce white spores. It was demonstrated that *wA* mutations are epistatic to *yA* mutations, because *wA* and *yA* double mutants produce white spores [7,8]. Thus, it was assumed that the product of the *wA* gene mediates the synthesis of a yellow pigment intermediate from a colorless precursor [9], and that this yellow intermediate is subsequently converted to the mature green form in a reaction catalyzed by an enzyme encoded at the *yA* locus [8,10,11] (Fig. 1).

The protein product of the *yA* gene was identified and characterized [8–10]. Clutterbuck demonstrated that the wild-type showed a *p*-diphenol oxidase, or laccase, activity during conidiation, whereas the *yA* mutant did not. He also found that the *yA* gene is preferentially expressed when vegetative hyphae begin to differentiate conidiophores and to produce conidia [8]. Subsequently, the *yA* gene was cloned and its expression was shown to be regulated at the level of mRNA accumulation [12,13]. In contrast with the progress in *yA* gene studies, *wA* function was not approached until the molecular genetics was applied in late 1980s.

3. CLONING OF A. NIDULANS WA GENE

Timberlake et al. constructed an *A. nidulans* wild-type genomic DNA library based on their pKBY2 cosmid vector, which contains the bacteriophage lambda cos site to permit *in vitro* assembly of phage particles; a bacterial origin of replication and genes for resistance to ampicillin and chloramphenicol to permit propagation in *Escherichia coli*; the *A. nidulans trpC⁺* gene to permit selection in *Aspergillus* spp.; and a unique *Bam*H I restriction site to permit insertion of DNA [12]. The DNA library containing 35- to 40-kb DNA fragments from the wild-type strain *A. nidulans* FGSC4 (Glasgow wild type) was used to



Figure 1 Scheme of green spore pigment formation in *A. nidulans*.

transform *A. nidulans* *wA* strain NK002 (*paba1*, *ya2*; *wA3*; *veA1*; *trpC801*) into a tryptophan-independent state. Complementation of the *wA3* mutation led to yellow conidia because of the *ya2* mutation. Mayorga and Timberlake obtained one transformant that produced yellow spores out of 2950 *trp⁺* transformants [14]. Although the pKBY2 transformation is an integration system, the integrated cosmid could be recovered from the genomic DNA of the transformant by *in vitro* lambda packaging and subsequent transduction into *E. coli*. The cosmid DNA designated CosNK002 was recovered from the NK002 yellow transformant. This cosmid could restore the yellow conidia formation in NK002 by retransformation with over 50% efficiency.

Mayorga and Timberlake used a series of restriction enzyme fragments of the CosNK002 cosmid to locate the *wA* complementing activity to NK002 [14]. pNK3 containing the 13.8-kb *EcoRI* fragment and some of its subclones that restored yellow conidial color in *wA* mutants were identified. RNA blot analysis with pNK3 fragment as a probe indicated that the *wA* transcript was 7.5 k nucleotide-long and is present in conidiating cultures but absent from hyphae. Furthermore, disruption of the *wA* region of the *ya* mutant with the pNK3 derived fragments by homologous recombination gave white conidial transformants and thus confirmed that essential *wA* components reside within *ClaI-SmaI* 9-kb fragment of pNK3 insert, which is the *wA* region of *A. nudulans*.

Mayorga and Timberlake sequenced this region, and the introns involved were mapped by comparison with the corresponding sequences of cDNA fragments [15]. The predicted polypeptide product of *wA* has highly significant sequence similarities with fatty acid synthases (FASs) and polyketide synthases (PKSs). Thus, *wA* was considered to code for a PKS for yellow conidial pigment biosynthesis since fatty acids are unlikely to have the property of intense yellow pigmentation. The yellow polyketide product of WA PKS could be polymerized by *ya*-coded conidial laccase to form the mature green pigment. This *wA* was the second PKS gene cloned from fungi after the 6-methylsalicylic acid synthase (MSAS) gene of *Penicillium patulum* [16], which catalyzes the production of 6-methylsalicylic acid from acetyl-CoA and malonyl-CoA.

Fig. 2 shows the architecture of WA PKS deduced from the sequenced data reported by Mayorga and Timberlake [15], together with that of MSAS of *P. patulum* [16]. Very similar to MSAS, the predicted WA contained the β -ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains required for PKS basic function, although the ACP of WA is not single but tandem.

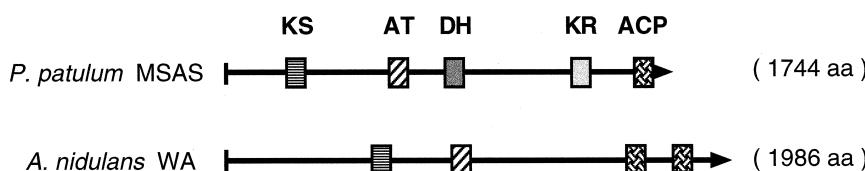


Figure 2 Architecture of *A. nidulans* WA polyketide synthase (PKS) and *P. patulum* 6-methylsalicylic acid synthase (MSAS). Active site positions on WA PKS and MSAS are shown schematically. KS, β -ketoacyl synthase; AT, acyltransferase; DH, dehydratase; KR, β -keto reductase; ACP, acyl carrier protein. The amino acid sequence of WA was deduced from the data reported by Mayorga and Timberlake [15]. MSAS data (accession number X55776) were reported by Beck et al. [16].

4. EXPRESSION OF WA POLYKETIDE SYNTHASE

The chemical structures of conidial green spore pigments of fungi, including that of *A. nidulans*, have not been identified because these pigments are considered to be insoluble polymerized substances. Several *yA* mutants were analyzed to identify the yellow pigment structure, but low accumulation and high background production of pigmented compounds prohibited its identification and characterization.

As mentioned above, sequence analysis of *wA* indicated that the gene encodes a multifunctional PKS [15]. Because polyketides are one of the most important group of natural products with huge structural variety and versatile biological activities [17,18], we conducted functional analysis of fungal PKSs. Before the cloning of the *wA* gene from *A. nidulans*, the MSAS of *P. patulum* was the sole example of a fungal PKS that was characterized at both the enzyme and gene level [16,19]. The other fungal PKS enzyme activity detected was the orsellinic acid synthase of *Penicillium cyclopium* [20]. Difficulties in fungal PKS enzymology were mostly attributed to enzyme instability and low expression level in fungal cells. To overcome these difficulties in fungal PKS functional analysis, we attempted to express fungal PKSs in heterologous fungal hosts and succeeded in expressing the *atX* gene cloned from *Aspergillus terreus* and confirmed that it coded for MSAS by product identification [21].

4.1. Fungal Polyketide Synthase Expression

As the first example of heterologous expression of fungal PKSs, MSAS was expressed in *Streptomyces coelicolor* [22] using actinomycete expression system that was successfully used in bacterial type I and type II PKS studies [23,24]. Although the expressed MSAS in actinomycete functioned and produced 6-methylsalicylic acid, its productivity was very poor.

We used the heterologous fungal expression system for fungal PKS. The fungal expression vector pTAex3 [25], which contains α -amylase promoter of *A. oryzae* [26] and auxotrophic selection marker *argB* [27], was used with the fungal host such as *A. oryzae*. In this expression system, it is not necessary to introduce 4'-phosphopantetheinyl transferase into the host for ACP modification of heterologous fungal PKSs. In addition, removal of introns from the PKS genomic DNA is not necessary to construct expression plasmid because maturation of PKS mRNA is expected to be carried out by fungal host machinery to excise introns correctly. Thus, we attempted to express the *A. nidulans wA* gene by this fungal expression system in order to identify the product of WA PKS.

4.2. Expression of WA Polyketide Synthase

Based on the sequence of *wA* gene reported by Mayorga and Timberlake [15], the WA expression plasmid pTA-wA was constructed as shown in Fig. 3 [28]. The *wA* gene was divided into two parts. The N-terminal region (start codon to *Kpn*I site; 2.2 kb) was amplified by PCR. The remaining region (4.3 kb) was cleaved from pNK3 with *Kpn*I and *Bam*HII. It was deduced that the stop codon was located just upstream from the *Bam*HII site in the sequence reported by Mayorga and Timberlake [15]. These two fragments were reconnected on a pBluescript and then introduced into pTAex3 to construct the WA expression plasmid pTA-wA.

This expression plasmid was used to transform the host fungus *A. oryzae* M-2-3 (*argB*) using the protoplast-PEG (polyethylene glycol) method [29]. Transformants were

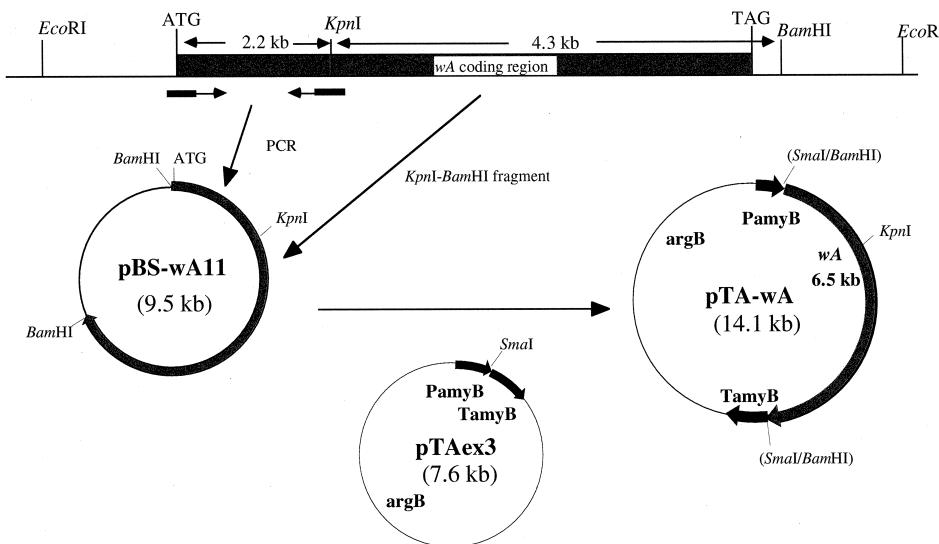


Figure 3 Construction of expression plasmid pTA-wA.

first cultured in glucose-containing Czapek-Dox medium then transferred into starch-containing Czapek-Dox induction medium. After 3-days induction culture, the compounds produced were extracted with ethyl acetate and separated on silica gel column. The main product was isolated as colorless crystals and identified to be (+)-citreoisocoumarin, which was previously reported from *Penicillium citreo-viride* B as a (−)-isomer [30]. The minor products were characterized to be a 4'-hydroxy-2'-keto isomer of citreoisocoumarin and a keto-enol equilibrium mixture of dehydrocitreoisocoumarin [28] (Fig. 4).

These isocoumarins were typical heptaketide compounds with oxygen atoms located at alternate carbons. Carbonyl carbons at 2' and 4' of dehydrocitreoisocoumarin might be reduced by the reductase activity of the host fungus to form citreoisocoumarins. This result indicated that the wA gene coded for a heptaketide synthase of *A. nidulans*. However, the

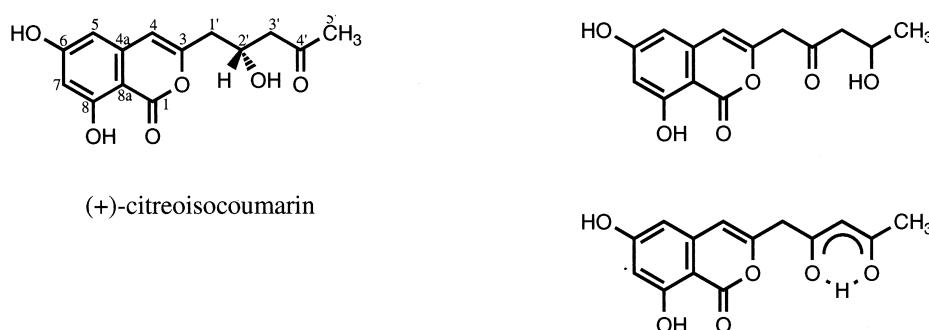


Figure 4 Products of *Aspergillus oryzae* pTA-wA transformant.

expected product of WA PKS was a yellow pigment, which could be polymerized by the γ A-coded laccase to green spore pigments [9]. Thus, it was doubtful whether these compounds are the true intermediates of conidial pigments and the correct product compounds of WA PKS function.

4.3. Expression of Full-Length WA Polyketide Synthase

As shown in Fig. 2, the deduced WA PKS has characteristic tandem ACPs at its C-terminus, which was rarely found in other PKSs, such as MSAS [16] and modular type I PKSs [31]. However, similar tandem ACPs were found in some fungal PKSs, *Colletotrichum lagenarium* PKS1 [32] and *A. nidulans* STCA [33], which are involved in 1,8-dihydroxy-naphthalene (DHN)-melanin biosynthesis and sterigmatocystin biosynthesis, respectively. Comparison of the active site organizations of these tandem ACP type fungal PKSs revealed that WA PKS alone lacked the thioesterase (TE) motif at its C terminal and had a shorter polypeptide length (deduced to be 1986 amino acids). To confirm this discrepancy, Watanabe resequenced the *wA* gene around its C-terminal region and found the error: one base just upstream from the stop codon was missing in the original sequence [34] (Fig. 5). With this correction, the WA PKS was found to have a longer polypeptide length (2157 amino acids) and possess a TE active site motif (-GWSAG-) at its C terminal. Thus, WA PKS has an organization of active sites similar to those of PKS1 and STCA, as shown in Fig. 6.

For the construction of the previous expression plasmid pTA-wA, the *wA* gene was cut at the *Bam*HI site, because the stop codon was reported to be located just upstream of this *Bam*HI site in the original sequence [28]. However, the actual open reading frame continues further through the *Bam*HI site. Thus, the previous expression plasmid pTA-wA lacked a part of C terminal and had additional extra amino acids derived from the ligated vector sequence. To express the correct full-length WA PKS, a new expression

7626	CGCCGCCAACCAACCGGCCGTACAACCTGGCGGCTGGTCCGCAGGCCGGATCTGCCCATACGACGCCAGCAGCTGACTGCAGCAGGGCAAATAAGTCGAGACCCCTTCTTCTT	7745
1954	R R Q P T G P Y N L G G W S A G G I C A Y D A A R K L V L Q Q G E I V E T L L L	1993
1954	R R Q P T G P Y N L G G W S Q A G S A H T T R H A S S Y C S R A K *	1986
7746	CTCGACACCCCCCTTCCCATTTGGCTTGAAGAACTCCGGCCCGCCTGACAGCTTCAACTCGATCGGCCCTTGGCGAAGGCAAGGCCGCCGCCGCCCTGGCTACTTCCCCT	7865
1994	L D T P F P I G L E K L P P R L Y S F F N S I G L F G E G K A A P P A W L L P H	2033
7866	TTTTGGCGTTATTGACTCCCTCGACCGCTACAAGGCCGTGCCCTTTCAACGAAACAGGAATGGAAGGGAAAGCTGCCAAAAGCTACCTTGTGGCTAAAGGATGGGCTGC	7985
2034	F L A F I D S L D A Y K A V P L P F N E Q E W K G K L P K T Y L V W A K D G V C	2073
7986	CCTAAGCCCTGGTATCCCTGGCTGAGCTGCCGAGGACGGAGCAAGGATCCACCGCAGATGGTTTACTCTAACCGGACGGATGGGTCGAATGGGATACCGCTTG	8105
2074	P K P G D P W P E P A E D G S K D P R E M V W L L S N R T D L G P N G W D T L V	2113
	(R G S V V A R E G W R V Y D G T A I Q S G I G	
8106	GGCAAGGAAAATATTGGCGGTATTACAGTGATTATGACGCCGAATCACTTTACTATGACAAGGCCAAAAGGCAAGGAGCTTGCATTTCTCATGAAAGAATGCGTGTGGGTTTGTGAG	8225
2114	G K E N I G G I T V I H D A N H F T M T K G E K A K E L A T F M K N A L G V C E	2153
	Q *)	
8226	CGCCGGTTGGCTGA	8240
2154	R R L V *	2157

Figure 5 Nucleotide and deduced amino acid sequence of the *A. nidulans* *wA* gene C-terminus. The nucleotide 7677 C (shown in boldface) was missing in the original report [15]. The second row shows the revised amino acid sequence, and the third row is the amino acid sequence deduced from the original data. The amino acid sequence in parentheses is the modification of the WA C terminal expressed by pTA-wA. The underlined amino acid sequence is the so-called thioesterase motif found in the revised amino acid sequence. The overlined nucleotide sequence is the *Bam*HI site, which was used for construction of the expression plasmid pTA-wA.

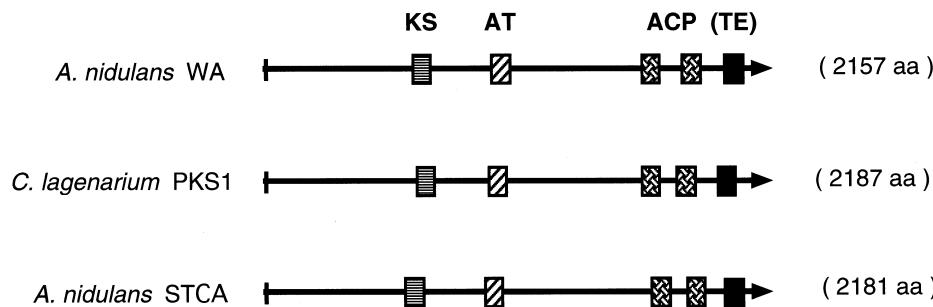


Figure 6 Architecture of fungal polyketide synthases *A. nidulans* WA, *Colletotrichum lagenarium* PKS1, and *A. nidulans* STCA. Active site positions are shown schematically. KS, β -ketoacyl synthase; AT, acyltransferase; DH, dehydratase; KR, β -keto reductase; ACP, acyl carrier protein; TE, thioesterase (so-called). *A. nidulans* WA, *wA* gene (accession number X65866); *C. lagenarium* PKS1, *PKS1* gene (D83643); *A. nidulans* STCA, *stcA* gene for sterigmatocystin biosynthesis (L39121).

plasmid pTA-nwA was constructed by replacing the C-terminal region of pTA-wA, as shown in Fig. 7 [34].

The *A. oryzae* transformant with pTA-nwA showed yellow pigmentation as expected, even on agar plates. This observation was in contrast to the pTA-wA transformant that developed white mycelia. Yellow compounds were isolated from the induction culture medium of the pTA-nwA transformant after silica gel column chromatography. The main compound was identified as a novel naphthopyrone compound named YWA1. The minor

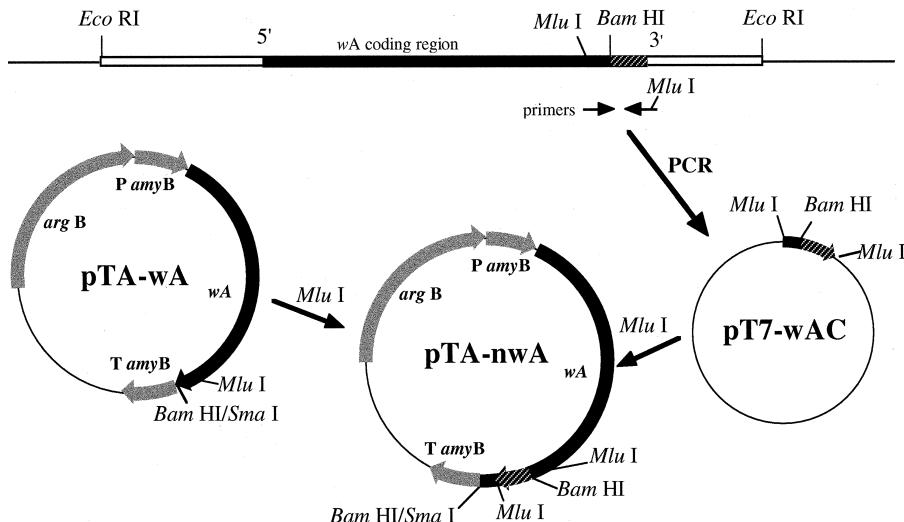


Figure 7 Construction of expression plasmid pTA-nwA.

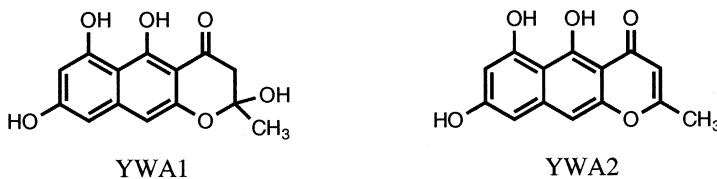


Figure 8 Products of the *A. oryzae* pTA-nwA transformant.

compound was identified as a dehydrated derivative of YWA1 and called YWA2 [34] (Fig. 8).

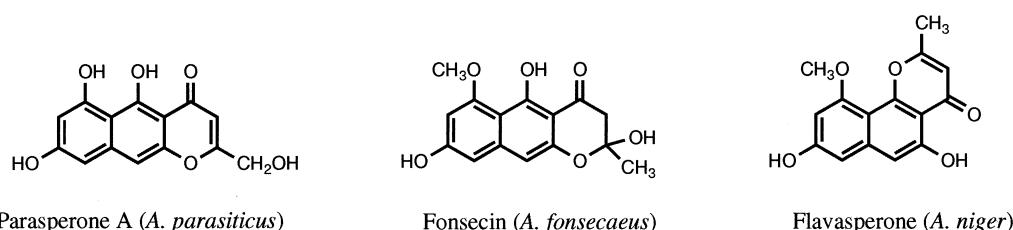
Naphthopyrone compounds such as rubrofusarin [35], fonsecin [36], parasperone A [37], etc. have been identified as fungal polyketides (Fig. 9). Among them, parasperone A was isolated from a laccase-deficient mutant of *Aspergillus parasiticus*, indicating it to be an intermediate of asexual spore pigment. Thus, naphthopyrone compound YWA1 is considered to be an actual intermediate of *A. nidulans* conidial pigments, and the wA gene was confirmed to code for a polyketide synthase of heptaketide naphthopyrone YWA1 [34].

5. FUNCTIONAL ANALYSIS OF WA POLYKETIDE SYNTHASE

In the two expression experiments of WA PKS, two different types of compound, isocoumarin and naphthopyrone, were produced. Apparently these are heptaketides, but their polyketide folding and/or cyclization patterns are different. This difference in polyketide products could be attributed to the differences in the C-terminal amino acid sequences because the first expression plasmid pTA-wA had a modified C terminal. Thus, it was assumed that condensation and chain elongation to form specific chain length are controlled by the KS, AT, and ACP regions of the PKS polypeptide, and the TE at C-terminus might have a specific role other than simple product release from the enzyme protein, because its modification caused a product change from naphthopyrone to isocoumarin [34].

5.1. Polyketide Folding Pattern in WA Polyketide Synthase Reaction

To understand the skeletal conversion in WA PKS products, it was important to determine polyketide folding patterns in these heptaketide products. The polyketide folding pattern



Parasperone A (*A. parasiticus*)

Fonsecin (*A. fonsecaeus*)

Flavasperone (*A. niger*)

Figure 9 Fungal naphthopyrones.

for citreoisocoumarin is obvious. Conversely, two contradictory folding patterns for heptaketide intermediates in naphthopyrone biosynthesis were reported for rubrofusarin [35] and fonsecin [36], as shown in Fig. 10.

A feeding experiment with $[1,2-^{13}\text{C}_2]$ sodium acetate established that the folding of the heptaketide intermediate for naphthopyrone YWA1 is pattern **c** (Table 1). Comparison of the folding patterns for naphthopyrone YWA1 and citreoisocoumarin indicated that the heptaketide chain first cyclizes to form the A ring and then second cyclization occurs to form the B ring to give naphthopyrone. Failure of second cyclization forces monocyclic precursor release to give isocoumarin. Hence, the C-terminal region of WA PKS, which is absent in the protein derived from pTA-wA, was suggested to be involved in the second B ring formation [38].

WA PKS first catalyzes the condensation of starter acetyl-CoA with six extender malonyl-CoAs to form a heptaketomethylene-ACP intermediate. When the chain length of growing polyketide becomes C_{14} -heptaketide, WA catalyzes aldol-type condensation to give a monocyclic intermediate. Then, ring B closure by Claisen-type cyclization must occur to form a naphthopyrone carbon skeleton. In case it is unable to cyclize the B ring, monocyclic carboxylic acid is released by hydrolysis and then converted to isocoumarin. Thus, it is deduced that the C-terminal region of WA is involved in Claisen-type cyclization to form ring B of naphthopyrone.

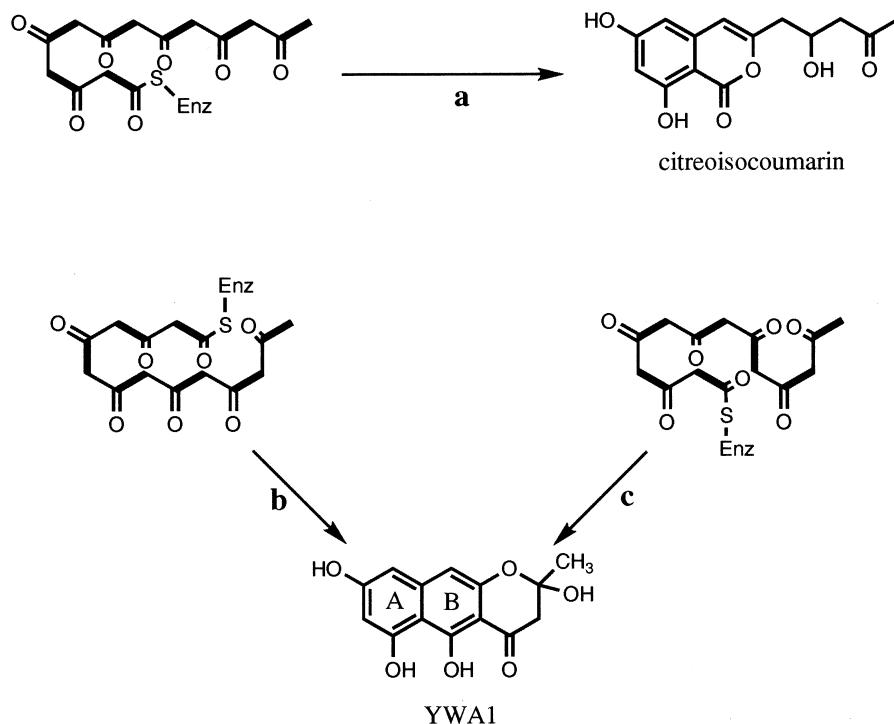


Figure 10 Polyketomethylene folding patterns for citreoisocoumarin and naphthopyrone YWA1.

Table 1 ^{13}C -NMR Data of Naphthopyrone YWA2 Fed with [1.2- $^{13}\text{C}_2$] Sodium Acetate

position	^{13}C δ ppm	^1J (^{13}C - ^{13}C) in Hz
2	169.5	50
3	105.6	58
4	183.1	58
4a	101.5	63
5	162.3	67
5a	105.3	67
6	158.3	73
7	100.6	73
8	160.7	69
9	100.7	69
9a	140.2	58
10	99.6	58
10a	151.8	63
CH ₃	20.1	50

Source: Ref. 38

5.2. Analysis of C-Terminal Deletion Mutants of WA Polyketide Synthase

To identify the essential region of the C terminal of WA PKS for ring B Claisen-type cyclization, C-terminal deletion mutants were expressed and their product compounds were analyzed. As shown in Fig. 11, the mutant WAC1 with 32 amino acids deletion from the C-terminus produced citreoisocoumarin instead of normal naphthopyrone YWA1, indicating the presence of amino acid residues essential for Claisen-type cyclization in the deleted region. WAC2 is the mutant with further deletion of 274 amino acids, including Ser residue of the so-called TE motif (-GWSAG-). This mutant also produced citreoisocoumarin and its productivity was not significantly affected. A comparable level of citreoisocoumarin production by WAC2 suggests that the TE domain of fungal aromatic PKSs does not function only as a simple TE [38].

5.3. Claisen-type Fungal Polyketide synthases

In fungal PKSs, the so-far-called TE motif is not always present, and MSAS that lacks this motif is a typical example [16]. TE motifs are found in PKSs such as PKS1 [32], STCA [33], PKSA (PKSL1) [39,40], etc. All fungal aromatic PKSs with TE motifs are assumed to involve Claisen-type cyclization in their PKS reactions, as shown in Fig. 12. Furthermore, their protein architectures are quite similar. Thus, TE motifs in these fungal PKSs are now considered to be Claisen cyclase motifs, as found in WA PKS.

Of the Claisen-type fungal PKSs other than WA PKS, PKS1 of *C. lagenarium* was expressed in a heterologous fungal host and its cyclization mechanism was proposed based on its PKS reaction byproduct [41]. Although the number of cloned fungal PKS genes is increasing, most of their functions have been assumed based on the results of gene disruption experiments and direct identification of their PKS products has rarely been reported.

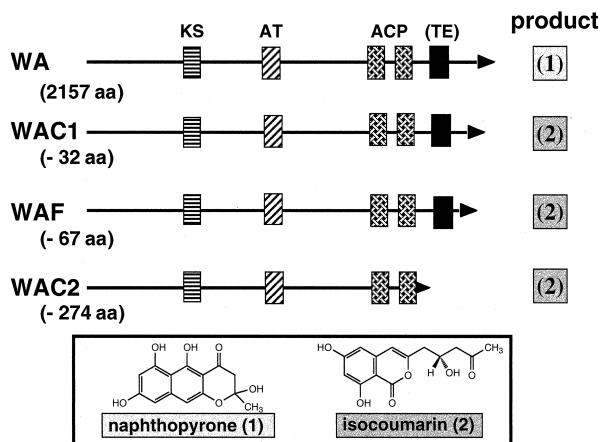


Figure 11 Architecture of WA PKS and its C-terminal-truncated mutants with their product compounds. TE indicates an active site position of the so-called thioesterase. (From Ref. 38.)

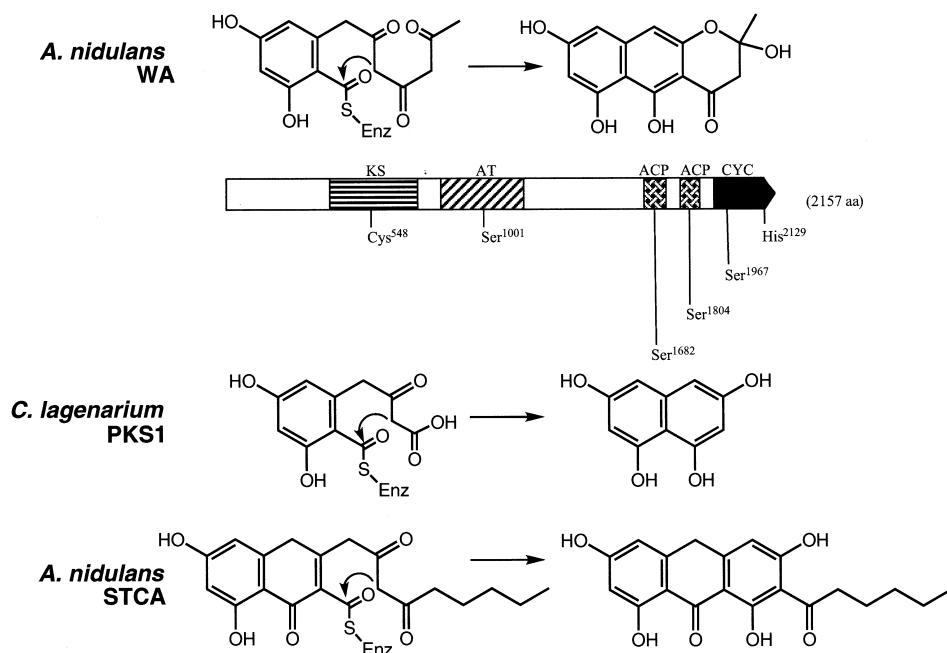


Figure 12 Claisen-type cyclization reactions involved in fungal PKS reaction and architecture of WA PKS with amino acid residues involved in its catalytic activity. CYC, Claisen cyclase domain.

5.4. Claisen Cyclase Motifs in Fungal Polyketide Synthases

Fig. 13 shows the alignment of Claisen cyclase motifs in fungal PKSs. This indicates the presence of several highly conserved amino acid residues (marked with asterisks in the figure). To identify the amino acid residue(s) essential for Claisen cyclization, site-directed mutagenesis was used to generate WA PKS mutants with mutations in the C terminal [38].

The mutant S1967A, in which Ser¹⁹⁶⁷ corresponding to the TE active site was mutated to Ala, did not produce naphthopyrone, but produced citreoisocoumarin instead. This is in accordance with the result of WAC2, which has a deletion up to this Ser residue. Also, the single mutation of His²¹²⁹ into Gln resulted in the production of citreoisocoumarin, which is also in accordance with the result that deletion mutant WAC1 lacks the amino acid residues after Asp²¹²⁶ just three residues upstream of His²¹²⁹. These results indicate that both Ser¹⁹⁶⁷ and His²¹²⁹ at least are essential for Claisen-type cyclization to form ring B of naphthopyrone YWA1 (Table 2).

To date, six TEs have been structurally characterized by x-ray crystallography. The *Vibrio harveyi* miristoyl ACP TE [42] and the mammalian palmitoyl protein TE [43] contain a classic Ser-His-Asp triad [44] in their active sites. In addition, the structure of the much more closely related TEs of modular PKSs, TEs of 6-deoxyerythronolide B synthase [45] and picromycin synthase [46], were determined. The active-site triad comprised of Asp-His-Ser preserved in these TEs could be operative in fungal PKS Claisen-type cyclization.

5.5. Role of Tandem ACP motifs

Most Claisen-type fungal PKSs including WA PKS possess tandem ACP motifs in which the Ser residue is known to be the site to which the 4'-phosphopantethein group is attached as an anchor for polyketomethylene chain elongation. Similar tandem ACP motifs are also found in type I modular PKSs in *Bacillus subtilis* [47].

The significance of this feature in WA PKS was probed by site-directed mutagenesis [38]. Ser¹⁶⁸² and/or Ser¹⁸⁰⁴ in two ACP motifs present in WA PKS were mutated to Cys or Ala. These mutant WA PKSs were expressed in *A. oryzae* and their products were analyzed. As shown in Table 3, either mutant with single mutation at Ser¹⁶⁸² or Ser¹⁸⁰⁴ produced naphthopyrone, except for the S1804A mutant, which produced isocoumarin instead. Double mutations at both Ser residues with Cys or Ala abolished the production of any compound as expected. Thus, presence of at least a single intact Ser residue, either Ser¹⁶⁸² or Ser¹⁸⁰⁴, is sufficient for WA PKS to function as naphthopyrone synthase. The production of isocoumarin by the S1804A mutant could be explained as being due to a secondary structural change of the C-terminus. Therefore, the characteristic feature of tandem ACPs in these PKSs is not related to Claisen cyclase function.

5.6. Role of N-Terminus of WA Polyketide Synthase

Compared with MSAS and reduced complex-type fungal PKSs, another conserved feature in these Claisen cyclization fungal PKSs is the relatively longer N-terminal region upstream from KS domains (Figs. 2 and 6). This region might have some important function in these PKSs. If these fungal PKSs have homodimeric subunit structures with head-to-tail interaction like mammalian FAs [48], C-terminal Claisen cyclase domains might interact with N-termini of complementary subunits. To assess this possibility, N-terminal deletion

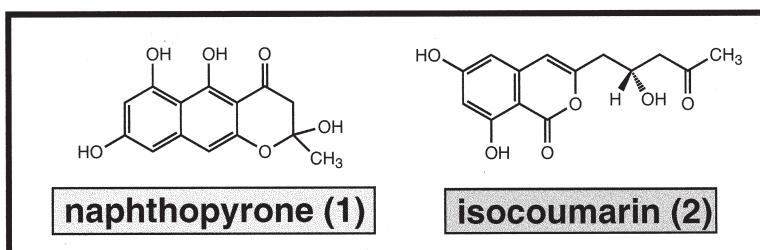
<i>A. nidulans</i> WA	ASKTLFLFPD GSGSATSYAT IPGVSP-NVA VYGLNCPYMK APEKLTCSLD SLTTPYLAEI	1952
<i>A. fumigatus</i> Alb1p	ATKKLFMFPD GSGSASSYAT IPALSP-DVC VYGLNCPYMK TPONLTCSDL ELTEPYLAEI	1947
<i>A. nidulans</i> STCA	AKQILFMLPD GGGSASSYLT IPRHLA-DVA IVGLNCPYAR DPENMNCTHQ SMIQSFCNEI	2013
<i>A. parasiticus</i> PKSL1	ARKTLFMLPD GGGSAFSYAS LPRLKS-DTA VVGLNCPYAR DPENMNCTHG AMIESFCNEI	1922
<i>C. lagenarium</i> PKS1	ATKNLWMVPD GSGCATSYTE ISQVSS-NWA VWGLFSPFMK TPEEYKCGVY GMAAKPIEAM	1994
<i>E. dermatitidis</i> WdPKS1	ATXNLFLFPD GSGSATSYVS IPALDSXNLA VYGLNCPFMK DPISYTCGIX SVSXLYLEKV	1978
<i>Nodulisporium</i> PKS1	ATRHFMPID GSGSATSYTE ISDLGS-DVA VWGMFSPFMK TPEEYKCGVY GMATKFIEEM	1971
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<i>A. nidulans</i> WA	RRRQPTGPYN LGGW SAGGIC AYDAARKLVL QQ-----GE IVETLLLLDT PFPIGLEKLP	2006
<i>A. fumigatus</i> Alb1p	RRRQPKGPYS FGGW SAGGIC AFDAARQLIL EE-----GE EVERLLLLDS PFPIGLEKLP	2001
<i>A. nidulans</i> STCA	KRRQPEGPYH LGGW SSGGAF AYVVAEALIN AG-----N EVHSLLIIDA PVPQVMEKLP	2066
<i>A. parasiticus</i> PKSL1	RRRQPRGPYH LGGW SSGGAF AYVVAEALVN OG-----E EVHSLLIIDA PIPQAMEQLP	1975
<i>C. lagenarium</i> PKS1	KARQSKGPYS LAGW SAGGVI AYEIVNQLTK AG-----E TVENLLIIDA PCPVTEPLP	2047
<i>E. dermatitidis</i> WdPKS1	LXRQPNPGPI LXGW SASGVF AYXITXQLXD LQXLHPDKNV TVEKLNLIIXS PCPIRLEPLP	2038
<i>Nodulisporium</i> PKS1	KRRQPEGPYA VSGW SAGGVI AYEIVNQLTK AG-----D EVSHLLIIDA PCPITIEPLP	2024
	* * . *** . *** : * : . * . * . * . : * . * . : * . * . : * . * . : *	
<i>A. nidulans</i> WA	PRLYQFFNSI GLFGEGKA-- ----APPAW LLPHFLAFID SLDAYKAVPL PFNEQEWKKG	2059
<i>A. fumigatus</i> Alb1p	PRLYKFNSI GLFGDGR-- ----APPDW LLPHFLAFID SLDAYKAVPL PFNDSKWAKK	2054
<i>A. nidulans</i> STCA	TSFYEYCNL GLFSNQPGGT TDGTAQPPY LIPHFQATVD VMLDYRVAPL KTN-----R	2120
<i>A. parasiticus</i> PKSL1	RAFYEHNSI GLFATQPGAS PDGSTEPPSY LIPHFATAVVD VMLDYKLAPL HAR-----R	2029
<i>C. lagenarium</i> PKS1	RSLHAWFASI GLLGEGDDE --AAKIPSW LLPHFAASVT ALSNYTAEPI PK-----EK	2098
<i>E. dermatitidis</i> WdPKS1	ARLHHHFDEI GLLGTGTG-- ----KTPNW LLPHFEYSIK ALTAYRPELK STRD----FN	2087
<i>Nodulisporium</i> PKS1	AGLHAWFAEI GLLGEGDG-- --EAKKIPSW LLPHFAASVT ALSNYTADPI PK-----DK	2074
	: . : * : . * : * : . * : * : . * : . * : . * : . * : . * : .	
<i>A. nidulans</i> WA	LPKTYLVWAK DGVCVKPGDP WPEPAEDGSK DPREMVWLLS NRTDLGPNGW DTLVGKENIG	2119
<i>A. fumigatus</i> Alb1p	MPKTYLIWAK DGVCVKPGDP RPEPAEDGSE DPREMQWLLN DRDLCGPWK DTLVGPQNIG	2114
<i>A. nidulans</i> STCA	MPKVGIIWAS ETVMDEDNAP ----- KMKGHMFPVQ KRWDGFPGDW DVVCPGAVFD	2170
<i>A. parasiticus</i> PKSL1	MPKVGIVWAA DTVMDERDAP ----- KMKGHMFPMIQ KRTEFGFPDW DTIMPAGASFD	2079
<i>C. lagenarium</i> PKS1	CPNVMAIWCE DGVCHLPTDP RPDPYPTG-- --HALPLLD NRTDFGPNRW DEYLDVNKFR	2153
<i>E. dermatitidis</i> WdPKS1	APPTLLIWAT DGVCVKPGDP RPPPQADDPK ---SMKWLL E NRTDFGPNGW DKLLGAEVCK	2144
<i>Nodulisporium</i> PKS1	CPKVTTIWCE DGVCVKLPDP RPDPYPTG-- --HALPLLD NRTDFGPNRW DEYLDIEKMT	2129
	* . : * . : * . * . : . * . : . * . : . * . : . * . : . * . : .	
<i>A. nidulans</i> WA	GITVIHDAN H FTMTKGEKAK ELATFMKNAL GVCERRLV	2157
<i>A. fumigatus</i> Alb1p	GIHVMEDAN H FTMTTGQKAK ELSQFMATAM SS-----	2146
<i>A. nidulans</i> STCA	ILRAEG- ANH LR-----	2181
<i>A. parasiticus</i> PKSL1	IVRADG- ANH FTLMQKEHVS IISDLIDRVM A-----	2109
<i>C. lagenarium</i> PKS1	TRHMPG-- NH FSMMHGDVVS QTTLSPYNNDD NLTRSF--	2187
<i>E. dermatitidis</i> WdPKS1	MVTVVG-- NH FTMMKPPVAK GVGQYIRESL SMXRA---	2177
<i>Nodulisporium</i> PKS1	FHHMPG-- NH FSMMHGDLAK QLGGFLKEGI HA-----	2159
	** :	

Figure 13 Alignment of C-terminal regions of Claisen-type fungal polyketide synthases. Fully or highly conserved amino acids are shown with * or : . below the alignment. Key amino acid residues Ser and His in Claisen cyclase domain are shown in boldface. *A. nidulans* WA, *wA* gene (accession number X65866); *A. fumigatus* Alb1p, *alb1* gene (AF025541); *A. nidulans* STCA, *stcA* gene for sterigmatocystin biosynthesis (L39121); *A. parasiticus* PKSL1, *pksL1* gene for aflatoxin biosynthesis (L42766); *C. lagenarium* PKS1, *PKS1* gene (D83643); *Exophiala dermatitidis* WdPKS1, *pks1* gene (AF130309); *Nodulisporium* sp. *PKS1*, *pks1* gene (AF151533).

mutants of WA PKS were expressed and analyzed. Surprisingly, the mutant WAN1 with only five amino acid deletions from its N terminal apparently lost PKS activity and no product was detected. None of mutants with further deletion from the N terminal produced any detectable polyketide products. These results indicated the important role of N-terminus of fungal Claisen type PKSs, which previously had not been recognized.

Table 2 Products of WA PKS and Its Mutants with Site-Directed Mutagenesis in the Possible Claisen Cyclase Domain

	(1)	(2)
native WA	+	-
H2129Q	-	+
S1967A	-	+



Source: Ref. 38

6. FUNCTIONAL ANALYSIS OF OTHER CLAISEN-TYPE POLYKETIDE SYNTHASES

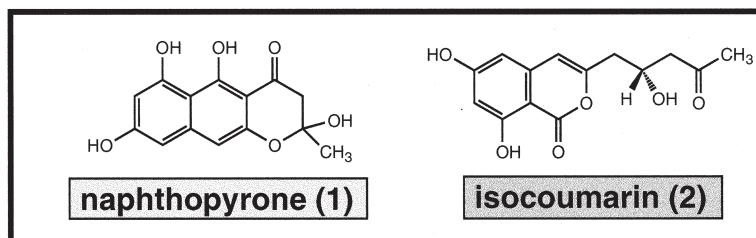
The *A. nidulans* WA PKS was found to be a typical Claisen-type PKS involved in naphthopyrone synthesis. For further characterization of this class of fungal PKSs, we carried out the heterologous expression of the PKS1 from *C. lagenarium* [41,49] and the Alb1p from *A. fumigatus* [50] using the pTAex3 and *A. oryzae* system.

6.1. PKS1 Polyketide Synthase from *Colletotrichum lagenarium*

The *PKS1* gene was cloned by Kubo et al. from *C. lagenarium*, which is a phytopathogenic fungus and a causal agent of anthracnose of cucumber [32]. In the biosynthesis of DHN-melanin, one of the determinants for the fungal infection, the *PKS1*-coded PKS was assumed to catalyze the formation of 1,3,6,8-tetrahydroxynaphthalene (T4HN), an established precursor for DHN-melanin. The function of *PKS1* was confirmed in the same heterologous expression system, in which the transformant produced T4HN as a main product along with byproducts [41]. Identification of a pentaketide monocyclic carboxylic acid as a byproduct led us to propose the folding pattern of the pentaketide intermediate and Claisen-type cyclization mechanism for the formation of the second aromatic ring of T4HN (Fig. 14).

Table 3 Products of WA PKS and Its Mutants with Site-Directed Mutagenesis in ACPs

	(1)	(2)
native WA	+	-
S1682C	+	-
S1804C	+	-
S1682C, S1804C	-	-
S1682A	+	-
S1804A	-	+
S1682A, S1804A	-	-



Source: [38]

The cell-free system prepared from the *A. oryzae* transformant with the PKS1 expression plasmid showed enzyme activity for the synthesis of T4HN solely from malonyl-CoA without incorporation of acetyl-CoA [49]. This *in vitro* result demonstrated that malonyl-CoA serves as the starter as well as extender units in the formation of T4HN by the PKS1 PKS and that decarboxylative Claisen cyclization functions to form the second aromatic ring of T4HN.

6.2. Alb1p Polyketide Synthase from *Aspergillus fumigatus*

Similar to the phytopathogenic fungus *C. lagenarium*, conidial DHN-melanin biosynthesis is an important virulence factor in *A. fumigatus*, a human pathogen [51]. The *alb1* gene was cloned from *A. fumigatus* and confirmed to be involved in DHN-melanin biosynthesis [52]. The *alb1*-coded protein, Alb1p showed a typical iterative-type I PKS architecture with Claisen cyclase motif. Thus, the Alb1p was assumed to be a T4HN synthase of *A. fumigatus* like the *PKS1* PKS of *C. lagenarium*. However, the Alb1p shows higher sequence similarity to the WA PKS of naphthopyrone synthase (67% identity, 80% similarity) than the *PKS1* PKS of T4HN synthase (43% identity, 60% similarity). To identify the actual function of this Alb1p PKS, we carried out its expression in *A. oryzae* using the pTAex3 expression system [50]. As suggested by its homology, the product of Alb1p was identified to be a heptaketide naphthopyrone YWA1, the same product of the WA

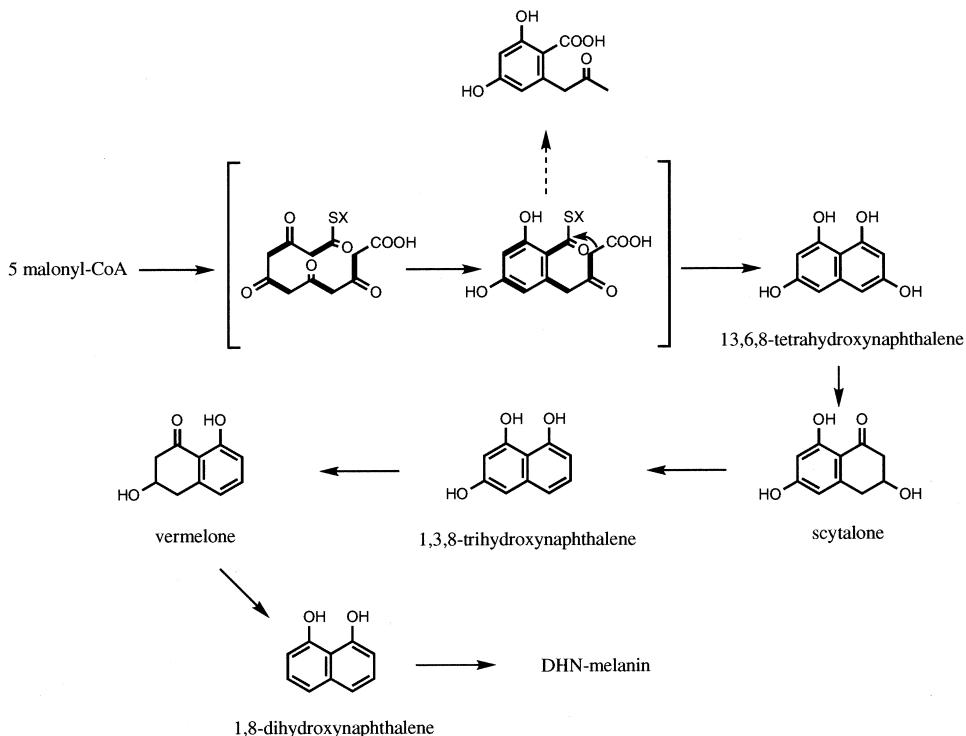


Figure 14 *C. lagenarium* PKS1 polyketide synthase reaction involved in DHN-melanin biosynthesis.

PKS. Involvement of naphthopyrone in DHN-melanin biosynthesis in *A. fumigatus* was solved by the functional identification of *Ayg1p*, which surprisingly converts naphthopyrone to T4HN by hydrolytic side-chain cleavage [53] (Fig. 15). Thus, *Alb1p* was identified as a functional homologue of WA PKS.

7. EXPRESSION OF CHIMERIC CLAISEN-TYPE POLYKETIDE SYNTHASES

Both *A. nidulans* WA and *C. lagenarium* PKS1 have quite similar enzyme architectures and are considered to be typical fungal PKSs, of which the reactions involve Claisen-type cyclizations. However, the chain lengths of their products are different: a heptaketide is produced by WA and a pentaketide by PKS1. The mechanism by which these iterative PKSs control polyketide chain length remains unknown. Because WA C-terminal deletion mutants still produce heptaketide products, citreoisocoumarin and its derivatives [38], the Claisen cyclase domain itself is not considered to be directly involved in chain length control. When the first aromatic ring is formed from the polyketide intermediate by aldol-type reaction, further chain elongation by condensation is terminated and then a product is released by TE and/or cyclase reaction. Therefore, chain-length control of polyketides is related to the aldol cyclase function of PKS. However, no such motif has been identified

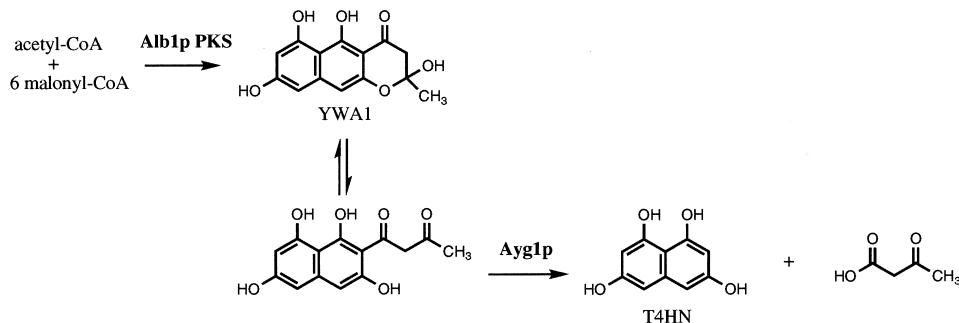


Figure 15 Biosynthesis of 1,3,6,8-tetrahydroxynaphthalene in *A. fumigatus*.

yet. The fact that *C. lagenarium* PKS1 produces tetraketide orsellinic acid as a byproduct (Y. Mori et al., unpublished data) indicates that its chain-length control is less strict than that of WA PKS, which does not yield such byproducts with different chain lengths.

To approach the mechanistic aspects of chain-length control by fungal iterative type I PKSs, chimeric PKSs between WA and PKS1 were constructed. Using conserved amino acid sequences, restriction enzyme sites were introduced by site-directed mutagenesis and interchanging regions of WA and PKS1 were connected each other. Seven chimeric PKSs were constructed and expressed in *A. oryzae* (Fig. 16). Of these, only SW-2 and SW-B produced polyketide products. Interestingly, both active chimeric PKSs are chimera of the N-half PKS1 and C-half WA and produced several compounds. SW-2 produced tetraketide orsellinic acid, pentaketide isocoumarin, hexaketide isocoumarin, and heptaketide naphthopyrone, but no original PKS1 product T4HN or heptaketide isocoumarin (A. Watanabe et al., unpublished data). SW-B produced the new hexaketide 2-acetyl-1,3,6,8-tetrahydroxynaphthalene as a main product, together with pentaketide carboxylic acid, pentaketide isocoumarin, and hexaketide isocoumarin [54] (Fig. 17).

These results indicated that both chimeric PKSs have much looser chain-length control, probably due to a less rigid active-site conformation. The Claisen cyclase of WA is found to be specific to hexaketide and heptaketide to form naphthalene with a C₂ or C₄ side chain and cannot catalyze pentaketide cyclization to form T4HN.

8. CONCLUSION

A. nidulans has been used as a model fungus to study genetics and cell biology, especially multicellular development, etc. Its draft genome sequence has been made available to the public [4]. Nearly 50 years have passed since Pontecorvo designated the *wA* locus in *A. nudulans* [7]. In early 1990s, Mayorga and Timberlake cloned the *wA* gene and indicated that it coded for a PKS [14,15]. Subsequently, we identified the function of the WA PKS as heptaketide naphthopyrone synthase and succeeded in characterizing the Claisen-type cyclase in its C-terminus [28,34,38]. Thus, the WA PKS is one of the most well-studied fungal PKSs.

The *wA* gene is expressed when vegetative hyphae begin to differentiate to produce conidia. Then WA PKS maturation occurs by 4'-phosphopantethein transfer to modify

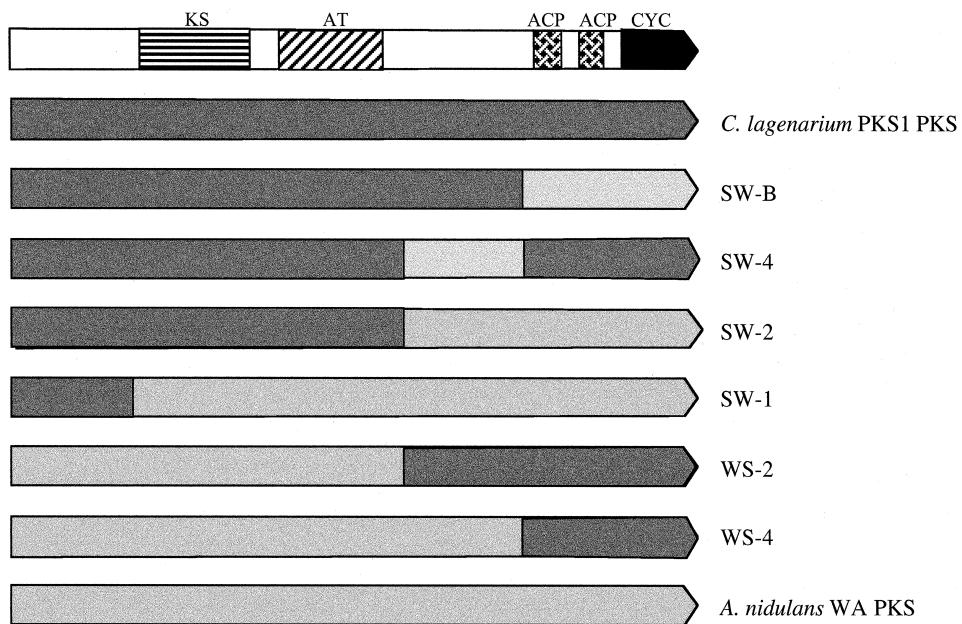


Figure 16 Constructs of chimeric polyketide synthases between *A. nidulans* WA and *C. lagenarium* PKS1.

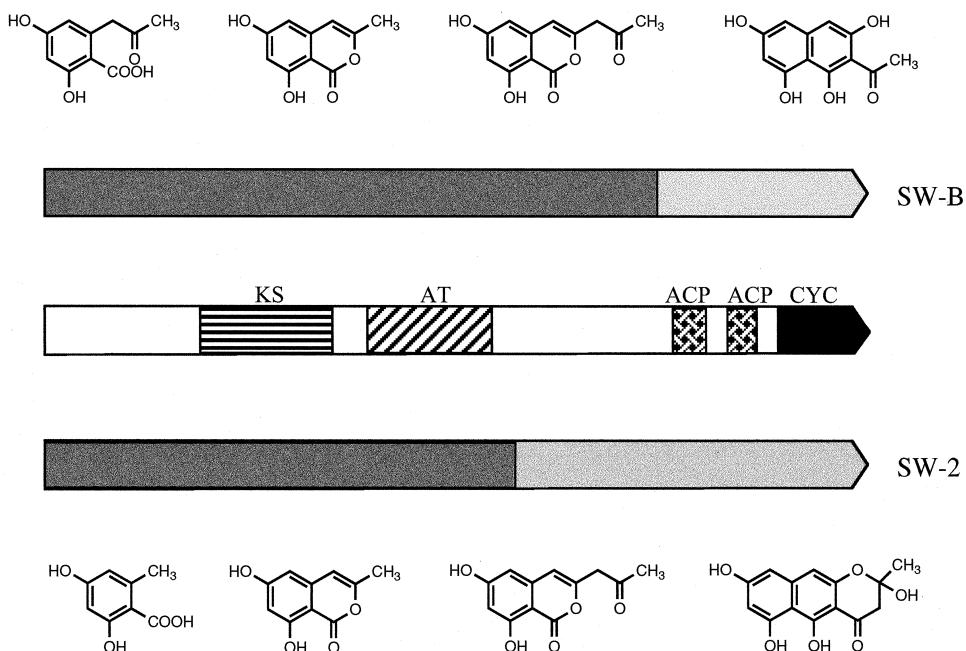


Figure 17 Products of chimeric polyketide synthases SW-B and SW-2.

either Ser residue in tandem ACP motifs. From the information on WA PKS obtained thusfar, the following reaction scheme could be proposed (Fig. 18).

The starter unit acetyl-CoA is loaded to the Cys residue of the KS domain by AT, and the extender unit malonyl-CoA is loaded onto the thiol group of phosphopantetheinyl ACP. Then chain elongation occurs by decarboxylative nucleophilic attack of malonyl active methylene to acetyl carbonyl. Iterative condensation continues until the β -polyketomethylene chain grows into a heptaketide. When the heptaketide intermediate is formed, the first aromatic ring is formed by aldol cyclization by an as yet unidentified domain. This cyclization prevents further chain extension. The monocyclic aromatic intermediate anchored on phosphopantetheinyl ACP thus formed is then transferred on to Ser¹⁹⁶⁷ with aid of proton transfer from Asp via His²¹²⁹. This step immobilizes the monocyclic intermediate from highly mobile ACP to facilitate the following second ring cyclization. Then, the His²¹²⁹ imidazole base abstracts the methylene proton at the position corresponding to C4a of YWA1 and aids its attack on the ester carbonyl to cyclize. Subsequent C–O bond cleavage releases the product naphthopyrone. The formation of hemiketal must proceed nonenzymatically, because its stereochemistry is not controlled.

Further analysis is necessary to confirm this WA PKS reaction mechanism. There are still many mechanistic issues to be resolved, including how fungal PKSs stabilize chemically active polyketomethylene intermediates, how they control the folding patterns of polyketomethylene intermediates, how they recognize chain lengths of growing polyketides, etc. No three-dimensional structures of iterative type I PKSs, including mammalian FASs, have yet been resolved. Information on steric interaction between PKS and polyketide intermediate will help us to understand how fungal iterative type I PKSs control their reactions.

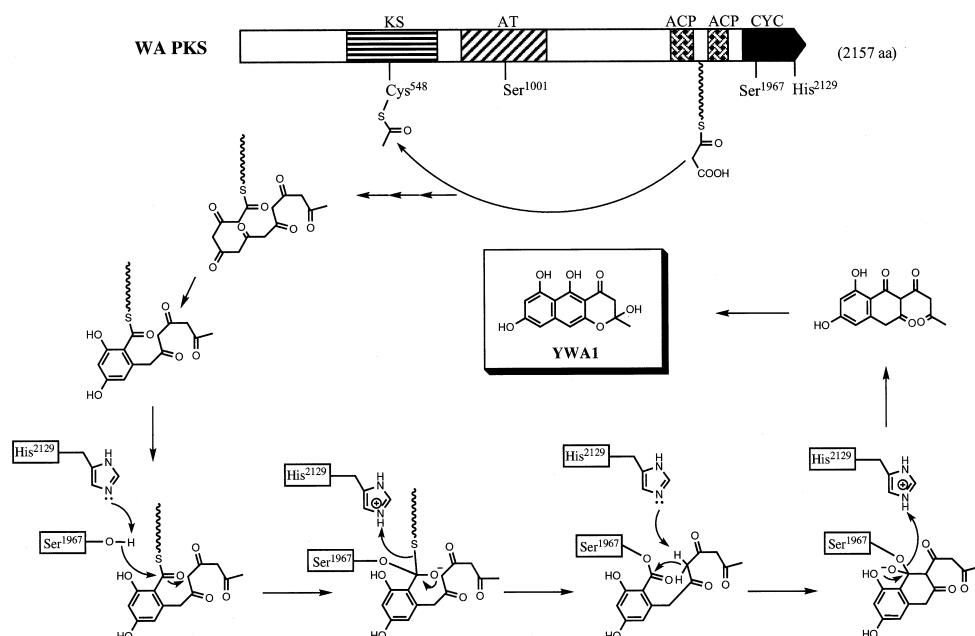


Figure 18 Proposed mechanism of *A. nidulans* WA polyketide synthase reaction.

ACKNOWLEDGMENTS

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19

Pneumocandin B₀ Production by Fermentation of the Fungus *Glarea lozoyensis*: Physiological and Engineering Factors Affecting Titer and Structural Analogue Formation

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1. INTRODUCTION

The world market for antifungal agents in 2002 was estimated to be \$4 billion with growth of this sector estimated at 10% annually [1]. Growth in the antifungal market is due to a dramatic increase in life-threatening fungal diseases (e.g., candidiasis and aspergillosis), mostly as a result of opportunistic fungal pathogens infecting immunocompromised patients. In addition, a number of the currently used antifungal drugs are acutely toxic (e.g., amphotericin B) or are “static” agents (e.g., azoles) that depend on the immune system to clear the existing fungal infection. Moreover, the azoles are ineffective against *Aspergillus* spp. because of natural resistance. These issues have motivated the discovery and development of antifungal agents that affect novel intracellular targets. The fungal cell wall is such a target, because it is essential for growth of the fungus and is absent from the mammalian host [2]. Cancidas® (Merck & Co.; caspofungin acetate, aka MK0991) has fungicidal activity against pathogens of clinical significance and recently has been approved for the treatment of life-threatening infections [3,4]. This “first-in-class” fungicidal agent is a potent inhibitor of glucan synthesis, a component of the fungal cell wall actively deposited at the hyphal ends during fungal growth. By comparison, the azoles,

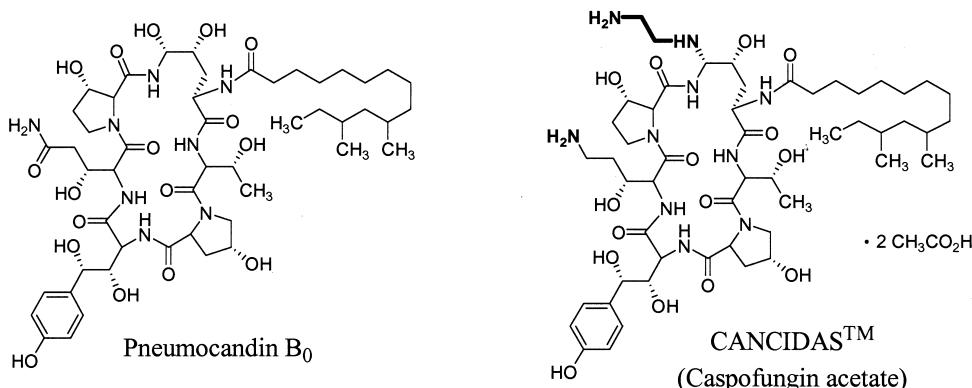


Figure 1 Structures of pneumocandin B₀ and Cancidas (represented as the acetate salt, i.e., caspofungin acetate). The chemical modifications (in bold) made to the natural product include the reduction of the primary amide of the hydroxyglutamine residue to an amine and the substitution of the hydroxyl group of the dihydroxyornithine residue with an ethylenediamine moiety.

which have been used since the 1960s to treat fungal infections, are static agents that inhibit a demethylation step in the synthesis of ergosterol, whereas amphotericin B disrupts fungal membrane function. Cancidas is produced by chemical conversion of pneumocandin B₀, one of a family of structurally related, acylated cyclic hexapeptides. This conversion involves chemically reducing the primary amide group of the hydroxyglutamine residue to an amine and substituting the hydroxyl group of the dihydroxyornithine residue, via a phenylsulfide intermediate, with an ethylenediamine moiety (Fig. 1) [5].

2. PNEUMOCANDIN DISCOVERY AND INITIAL DEVELOPMENT

The pneumocandins are produced by a dematiaceous hyphomycete isolated from pond water filtrates obtained from the Lozoya River valley near Madrid, Spain [6]. This hyphomycete was initially identified as *Zalerion arboricola* (ATCC 20868), but was later reclassified, based on morphological analysis and DNA fingerprinting, as *Glarea lozoyensis*, representing a new anamorph genus [7]. The pneumocandins produced by *G. lozoyensis* are similar to the echinocandin and aculeacin class of natural products in that the amino acids that compose the polar, hexapeptide nucleus each possess one or more hydroxyl groups [8,9]. Similarly, the pneumocandins also possess a nonpolar, amide-linked acyl side chain.

Pneumocandin A₀ is the major pneumocandin produced by wild-type *G. lozoyensis* (Fig. 2) [6]. The hexapeptide nucleus of pneumocandin A₀ was shown to consist of 3-hydroxy-4-methylproline, 4,5-dihydroxyornithine, threonine, 4-hydroxypoline, 3,4-dihydroxyhomotyrosine, and 3-hydroxyglutamine [10]. Anti-*Candida* activity for pneumocandin A₀ was comparable to aculeacin in vitro. Pneumocandin A₀ also showed good in vivo efficacy in the absence of acute or chronic toxicities employing a mouse model for systemic *C. albicans* infection [11].

Preliminary labeling studies with ¹³C amino acids verified the origins of the pneumocandin A₀ residues [12,13]. Glutamic acid enriched the 3-hydroxyglutamine, 4,5-dihydrox-

Pneumocandin	Position Affected	Residue Affected	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
B ₀	1		H	OH	OH	OH	CH ₃	OH
C ₀	1	<i>trans</i> 3-Hydroxyproline	OH	H	OH	OH	CH ₃	OH
D ₀	1	"	OH	OH	OH	OH	CH ₃	OH
E ₀	1	"	H	H	OH	OH	CH ₃	OH
A ₀	1	"	CH ₃	OH	OH	OH	CH ₃	OH
B ₅	2	Ornithine	H	OH	OH	H	CH ₃	OH
B ₆	2	"	H	OH	H	OH	CH ₃	OH
B ₂	2	"	H	OH	H	H	CH ₃	OH
B ₀ Serine Analogue	3	Threonine	H	OH	OH	OH	H	OH
B ₅ Serine Analogue	3	"	H	OH	OH	H	H	OH
B ₁	4	Homotyrosine	H	OH	OH	OH	CH ₃	H
D ₂	1,2	<i>trans</i> 3-hydroxyproline, Ornithine	OH	OH	H	H	CH ₃	OH

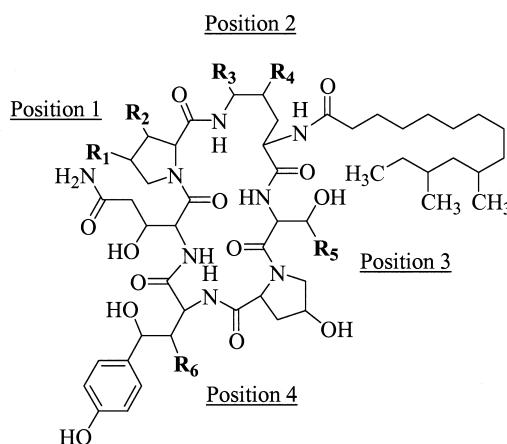


Figure 2 Structures of pneumocandins described in this chapter. Variability of the cyclic hexapeptide nucleus occurs primarily at four positions. (From Ref. 24.)

ornithine, and 4-hydroxyproline residues. Proline was shown to enrich the 4-hydroxyproline but not the 3-hydroxy-4-methylproline residue. The 3-hydroxy-4-methylproline residue is derived from leucine via sequential cyclization and hydroxylation reactions. Tyrosine is condensed with acetate to form the homotyrosine residue. In addition, the acyl side chain was determined to be a saturated C₁₄ unit derived from acetate units, with methyl groups at the C₁₀ and C₁₂ positions derived from methionine [10,12].

Pneumocandin B₀ was discovered in fermentations of wild-type *G. lozoyensis* grown in the presence of a number of amino acid analogues, with 3,4-dehydro-L-proline enhancing production to the largest degree [14]. Pneumocandin B₀, the metabolite chosen for development, possesses a 3-hydroxyproline in place of the 3-hydroxy-4-methylproline residue seen in pneumocandin A₀. The 3-hydroxyproline residue is derived from proline and not from leucine via a demethylation of 3-hydroxy-4-methylproline [13]. The pneumocandin A₀:B₀ ratio for the wild-type strain in the absence of any amino acid analogue was 7:1, but classical mutagenesis and production medium development shifted the ratio to 1:3, facilitating more thorough chemical and biological investigations [15].

3. PNEUMOCANDIN STRUCTURAL DIVERSITY

During the course of pneumocandin fermentation development at Merck Research Laboratories, over two dozen different pneumocandins have been identified. Alternate hydroxylation and amino acid incorporation patterns are observed at four of the six residues (i.e., positions 1 to 4; Fig. 2). It is unclear why the molecule is extensively hydroxylated, but one theory might be that these functional groups sterically hinder hydrolytic enzymes that could inactivate the molecule. Although strain improvement and production medium optimization beyond the initial development phase have focused on increasing the pneumocandin B₀ titer while reducing the levels of the structural analogues, the current fermentation process nevertheless displays a significant amount of pneumocandin structural diversity.

At position 1, all three alternate hydroxylation patterns have been observed. Pneumocandin C₀, the major and perhaps most troublesome analogue impurity in the current fermentation process, is a structural isomer of pneumocandin B₀ with a hydroxyl group at the C-4 of proline. Pneumocandin D₀ possesses hydroxyl groups at both C-3 and C-4 of proline, whereas pneumocandin E₀ has no hydroxyl groups on the proline at position 1.

Similarly at position 2, all four possible hydroxylation patterns have been observed for C-4 and C-5 of ornithine. Pneumocandin B₀ possesses hydroxyl groups at both C-4 and C-5, whereas pneumocandin B₂ has no functionality at these two positions. Pneumocandins B₅ and B₆ are structural isomers of each other with single hydroxyl groups present at C-5 and C-4, respectively. Pneumocandin D₂ represents an analogue with diversity at two positions; two hydroxyl groups on the proline at position 1 and no hydroxyl groups present on the ornithine at position 2.

Substitution of a threonine with a serine at position 3 results in two “serine analogues”—one an analogue of pneumocandin B₀ and the other an analogue of pneumocandin B₅, which represents another analogue with diversity at two positions. At position 4, loss of a hydroxyl group results in pneumocandin B₁.

Although never conclusively proven, pneumocandins are thought to be synthesized via a nonribosomal peptide synthetase similar to the mechanism described for the peptide antibiotics cyclosporin and gramicidin S [16]. Numerous reports in the literature describe how peptide antibiotic titer and analogue spectrum can be affected by amino acid supplementation, presumably due to broad substrate specificities of the active sites [17–21]. For example, the cyclosporin peptide synthetase possesses broad substrate specificity at most of its active sites, which results in the production of at least 25 cyclosporins [22]. The structural diversity of the pneumocandins is certainly comparable to what has been observed for cyclosporin production by *T. inflatum*, and the ability to manipulate analogue spectrum via directed biosynthesis also supports a peptide synthetase-based mode of biosynthesis.

4. KEY PHYSIOLOGICAL FACTORS AFFECTING TITER AND STRUCTURAL ANALOGUE FORMATION

Maximizing the production of pneumocandin B₀ while minimizing the levels of the structural analogues was the main goal of fermentation process development. Because of the rather large size of the pneumocandin molecule (MW >1000 daltons) and the subtle structural differences observed among the analogues, purification of pneumocandin B₀

could be challenging if the levels of the analogues were too high in the fermentation broth. It is important to recognize that for a commercially – viable, natural product manufacturing process, fermentation and isolation operations cannot be treated independently. Therefore, changes made to the fermentation process during the course of development had to be carefully evaluated from the standpoint of isolation. Ultimately, it is the final amount of isolated material that dictates overall productivity. In addition, the process needed to be reproducible to satisfy regulatory agencies. To accomplish these goals, it was important to identify which factors (i.e., chemical or physical) affected the production of pneumocandin B₀ or the analogue profile.

4.1. Pneumocandin Fermentation Process

The *Glarea lozoyensis* strain used for this work is a descendant of ATCC 74030 generated through a classical mutation/strain improvement program. Initial fermentation development efforts for the production of pneumocandin B₀ or other structural analogues employed a variety of complex production media. Variability, which can be seen with complex medium components, led to some variability in process performance, affecting both pneumocandin B₀ titers and the levels of key analogue impurities such as pneumocandin C₀. To eliminate the need for potentially inconsistent complex nutrients, a production medium was developed where the major carbon and nitrogen sources were defined entities and could be monitored (using standard analytical techniques) and fed appropriately to control fermentation performance and analogue spectrum.

Based on preliminary experiments, a medium consisting of fructose, proline, mono-sodium glutamate, yeast extract, salts, and trace elements was pursued. Simplex search methodology was used to optimize the nutrient concentrations, leading to the FGY production medium. The FGY production medium formulation, the LYCP-5 seed medium formulation, the shake-flask and lab-scale fermentor process descriptions, and analysis methods have been previously described [23,24]. Throughout this chapter, pneumocandin B₀ titers are given in “arbitrary units,” whereas the levels of the structural analogues are expressed as a percent of the amount of pneumocandin B₀ produced.

The semidefined medium developed in shake-flasks was scaled-up to 23-L fermentors; Fig. 3 illustrates typical growth kinetics, substrate utilization, and product formation. Synthesis of pneumocandin B₀ commenced after 100 hours and increased in a linear fashion (approximately 1.35 units/L/h), achieving a titer of 270 units/L after 300 hours. The continued increase in the titer was facilitated by two 25-g/L additions of fructose. Glutamic acid and proline were used sequentially, with glutamic acid being depleted after 150 hours. During the phase of proline consumption, the pneumocandin C₀ levels were maintained between 5.0% and 5.5%. Once the proline was exhausted, the pneumocandin C₀ level increased to 7% over the balance of the cycle. Additional development work demonstrated that midcycle additions of proline would attenuate this increase in pneumocandin C₀ levels.

Glarea lozoyensis grew satisfactorily between 22° and 26.5°C, with an optimal growth temperature between 23.5° and 25°C, as shown in Fig. 4. The growth rate was significantly slowed at 22° and 26.5°C with no measurable growth at 28°C, although culture viability was maintained at this higher temperature. The temperature optimal for growth (23.5° to 25°C) was also the optima for pneumocandin B₀ production. The unwanted analogues were proportional to the level of B₀ for the temperature range studied [25]. The temperature sensitivity demonstrated the need for robust control around 25°C. A greater

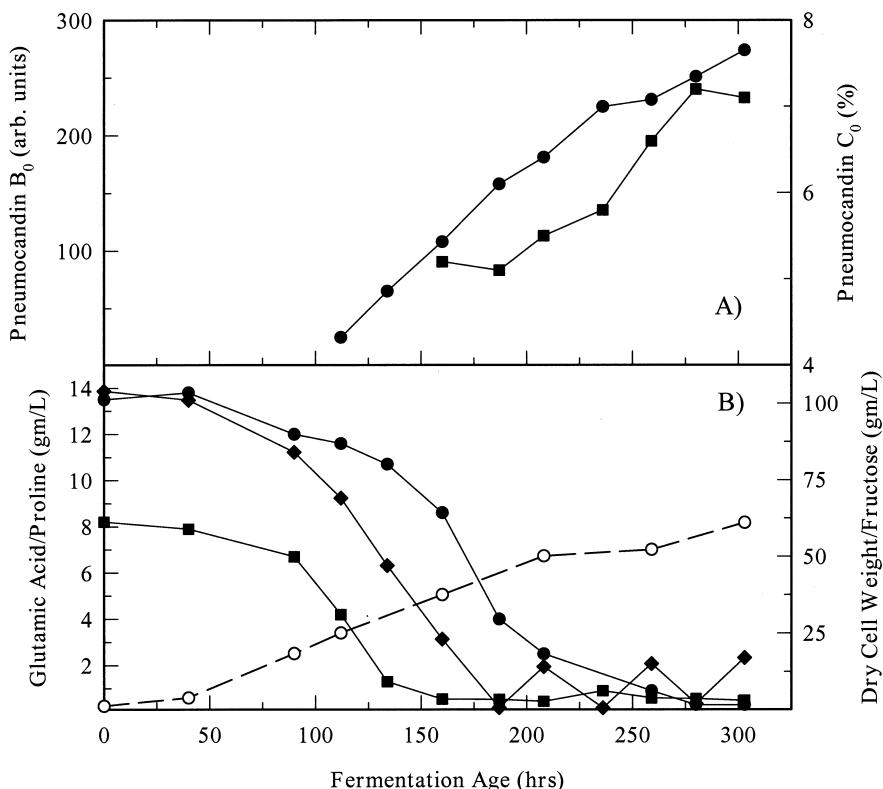


Figure 3 Scale-up of semidefined pneumocandin production medium. A, Pneumocandin B₀ (●) and pneumocandin C₀ (■) production. B, Kinetics of growth (as dry cell weight, ○) and substrate utilization (fructose, ♦; glutamic acid, □; and proline, ●).

understanding of the sensitivity of the cell physiology to temperature will aid the process transfer to manufacturing scale, where the concerns include the impact of the fermenter temperature control, impact of temperature gradients within the fermenter, and the impact of possible temperature excursions that may occur due to mechanical failures.

The pH had no significant impact on pneumocandin process as studied in the range of 4.5 to 6.5. The optimum pH for volumetric productivity was 5.2, and the growth kinetics (oxygen uptake rate, dry cell weight) showed no discernible trends with pH [26].

4.2. Intracellular Levels of *trans* 3- and *trans* 4-Hydroxy-L-proline Affect Pneumocandin Production

4.2.1. B₀ Titer and Levels of the Position 1 Analogue

Classical mutation and screening methods were successful at making B₀ the predominant pneumocandin produced by *Glarea lozoyensis*, nearly eliminating the production of pneumocandin A₀ [15,27]. High pneumocandin B₀-producing strains of *Glarea lozoyensis* nevertheless show significant structural diversity at position 1, and controlling the levels

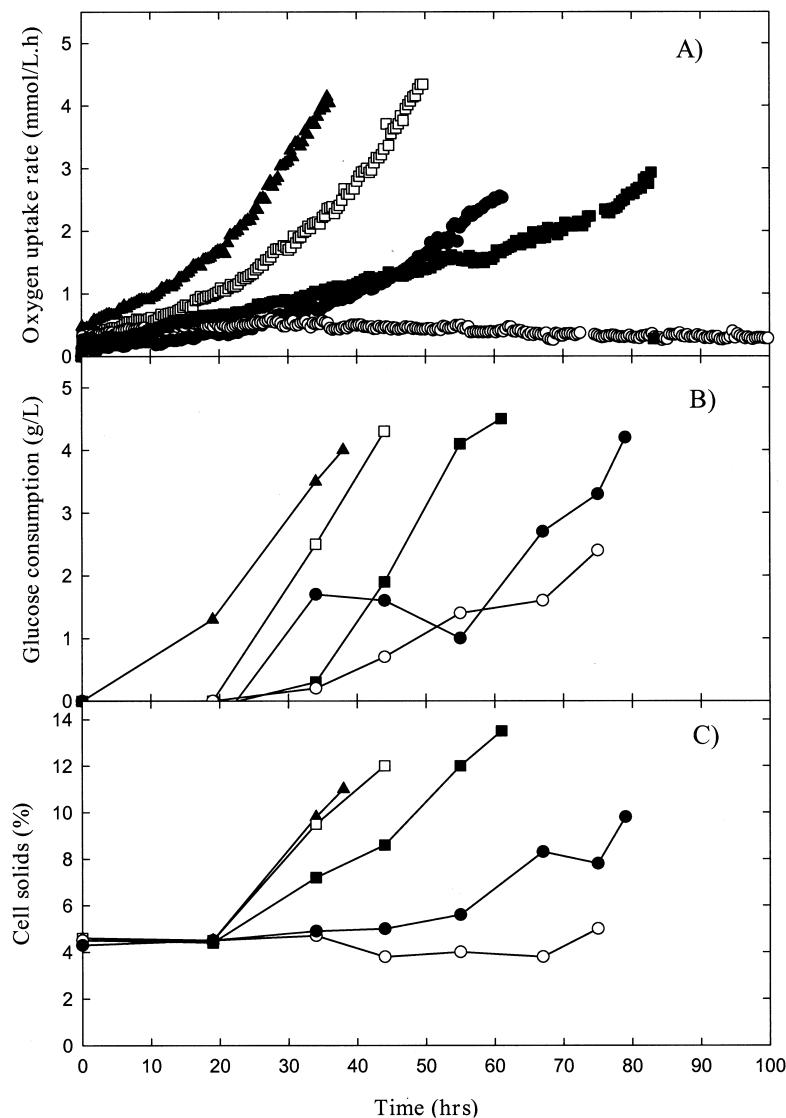


Figure 4 The effect of temperature on oxygen uptake rate (A), glucose consumption (B), and biomass formation (C) during growth of *G. lozoyensis* at the 0.8 m³ fermentor scale. Temperatures evaluated: 22°C (■), 23.5°C (▲), 25°C (□), 26.5°C (●), 28°C (○).

of the analogues marked by alternate hydroxylation patterns at this ring position was critical for a successful commercial fermentation process. Understanding the sensitivity of the pneumocandin C₀ analogue level to different process conditions was of critical importance, because the level of this analogue is a key determinant of isolation yield. Pneumocandin B₀ and C₀ are structural isomers possessing a *trans*-3-hydroxyproline or a *trans*-4-hydroxyproline residue, respectively, at position 1. This suggested that the extent

Table 1 Effect of Initial *trans*-3- and *trans*-4-Hydroxy-L-Proline on Pneumocandin B₀ Titer and Analogue Profile

	B ₀ (arbitrary units)	C ₀ (%)	D ₀ (%)	E ₀ (%)
Control (15 g/L proline)	233	4.8	0.5	2.7
<i>trans</i> -3-Hydroxy-L-proline ^a	324	0.1	0.2	0.7
<i>trans</i> -4-Hydroxy-L-proline ^a	253	9.4	0.7	2.4

Results are an average of duplicate flasks harvested on day 14 of the fermentation.

^a *trans*-3- and *trans*-4-hydroxy-L-proline were added at 0.13 M on day 6 of the fermentation.

Source: Ref. 24.

of incorporation of *trans*-3-hydroxyproline versus *trans*-4-hydroxyproline at position 1 would impact the pneumocandin B₀ titer and the levels of the position 1 analogues. Initially, it was not apparent whether the hydroxylation of proline occurred prior to or after incorporation into the peptide nucleus.

The effects of exogenously added *trans*-3- and *trans*-4-hydroxy-L-proline were examined by supplementing the medium at day 6 of the fermentation with each amino acid at a concentration equal to the amount of proline present (i.e., 0.13 molar; Table 1). *trans*-3-Hydroxy-L-proline supplementation increased the titer of pneumocandin B₀ and significantly reduced the levels of all the position 1 analogues. Conversely, supplementation with *trans*-4-hydroxy-L-proline had no impact on the pneumocandin B₀ titer but resulted in a doubling of the level of pneumocandin C₀. Supplementation with *trans*-4-hydroxy-L-proline had little impact on the levels of pneumocandins D₀ and E₀. Control experiments demonstrated that neither *trans*-3- nor *trans*-4-hydroxy-L-proline could serve as the sole nitrogen source in FGY medium. These results suggested that hydroxylation of proline occurred prior to incorporation and that the intracellular pool sizes of the hydroxyprolines is critical in determining titer and analogue levels.

Although directed biosynthesis using *trans*-3-hydroxy-L-proline would not be economically feasible on a commercial scale, precursor feeding studies carried out previously with L-[1-¹³C] proline demonstrated a 45-fold enrichment of both the *trans*-4-hydroxy-L-proline and the *trans*-3-hydroxy-L-proline residues in pneumocandin B₀ [13]. These results indicated that proline was the direct precursor of the two hydroxyproline residues, leading to the inclusion of proline in the production medium formulation based. Titration of proline in the production medium allowed an examination of the full impact of this nutrient.

Table 2 shows the results of an L-proline titration experiment. A dose-dependent increase in pneumocandin B₀ titer was observed while the levels of pneumocandins C₀

Table 2 Effect of Initial Proline Concentration on Pneumocandin B₀ Titer and Analogue Profile

Proline (g/L)	B ₀ (arbitrary units)	C ₀ (%)	D ₀ (%)	E ₀ (%)
0	103	14.0	4.0	0.9
5	161	6.0	2	1.5
10	208	3.0	0.7	2.0
15	201	3.0	0.5	2.0

Results are an average of duplicate flasks harvested on day 14 of the fermentation.

and pneumocandin D₀ (which possesses a dihydroxy proline residue) decrease as a function of initial proline concentration. Moreover, levels of pneumocandin E₀ (unmodified proline residue) increased as a function of initial proline concentration. The dose-dependency of the pneumocandin B₀ titer on proline is consistent with the fact that proline serves as a nitrogen source in the semidefined medium and as a precursor for hydroxyprolines. However, as the initial proline concentration is increased, its intracellular pool size is also likely to increase, resulting in more frequent misincorporations of unmodified proline and yielding higher levels of pneumocandin E₀.

The link between proline supplementation and levels of hydroxyprolines synthesized is illustrated in Fig. 5 [24]. Omitting proline from the medium resulted in a two-fold increase in the level of *trans*-4-hydroxyproline produced at day 8 of the fermentation compared to cultures supplemented with 15 g/L proline. However, after 14 days of fermentation, proline supplementation resulted in an enhanced, overall synthesis of *trans*-4-hydroxyproline. In contrast, the amount of *trans*-3-hydroxyproline produced at day 8 is not influenced by proline supplementation, but the overall synthesis at day 14 is increased by approximately 50%. In addition to the absolute amounts of each hydroxyproline formed, supplementation with 15 g/L proline reduces the *trans*-4-hydroxyproline:*trans*-3-hydroxyproline ratio at day 8 to 4:1, compared to a ratio of 8:1 in the absence of proline. Although the synthesis of *trans*-4-hydroxyproline is increased when proline is added to the medium (i.e., after 14 days of fermentation), the ratio of the hydroxyprolines is less than 4:1. Enhancing the overall amount of hydroxyprolines synthesized during the course of the fermentation was important for obtaining a good pneumocandin B₀ titer, whereas the hydroxyproline ratio appeared to be critical in determining the levels of pneumocandins

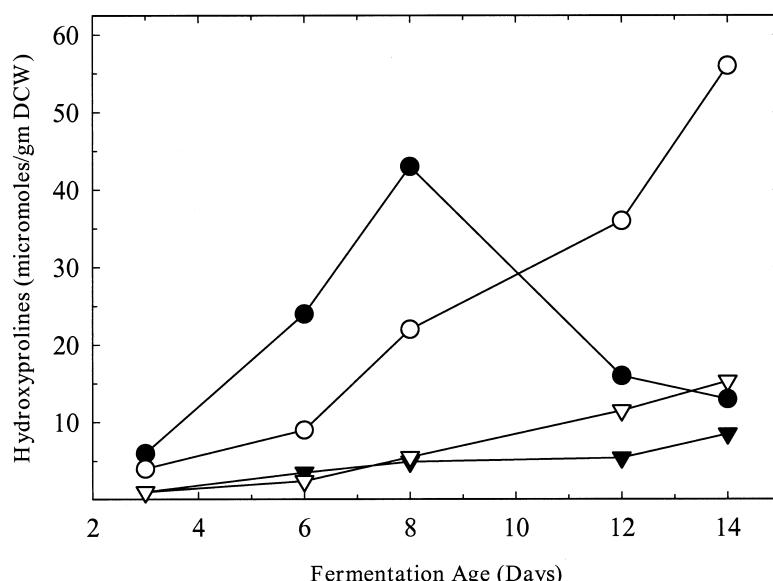


Figure 5 Production of *trans*-4-hydroxyproline (●,○) and *trans*-3-hydroxyproline (▼,▽) as a function of 0 g/L (filled symbols) or 15 g/L (open symbols) initial proline concentration. (From Ref. 24.)

C_0 and D_0 . However, when proline was supplemented at 15 gm/L, nonhydroxylated proline was incorporated directly into the hexapeptide forming pneumocandin E_0 .

Curious results were obtained when the fermentation was carried out with a lower initial fructose concentration (40 versus 125 g/L) combined with midcycle additions to avoid complete depletion of the carbon source [23]. Under this regime, pneumocandin B_0 titers increased, but with a doubling of the pneumocandin C_0 level—even though L-proline was present at 15 g/L (Table 3). Although the increase in the pneumocandin B_0 titer was welcome, the disproportionate increase in the level of pneumocandin C_0 made the product difficult to isolate. This result further underscores the importance of evaluating fermentation process changes on the isolation of the final product.

An osmotic pressure effect was demonstrated by adding 150 mM NaCl or 116 mM Na_2SO_4 to the “low-fructose” process on day 4, resulting in osmolalities between 900 and 1000 mmol/kg—comparable to osmotic pressures for the “high-fructose” process. The addition of the salts as “osmotic buffers” reduced the pneumocandin titers closer to those typically seen with the “high-fructose” process (Table 3). Importantly, the level of pneumocandin C_0 produced was reduced to levels more amenable to removal by chromatography.

Fig. 6 illustrates the underlying mechanism of how changes in osmolality affect the production of *trans*-3- and *trans*-4-hydroxyproline [23]. When the pneumocandin production fermentation is run at a low residual fructose concentration, the specific production of *trans*-4-hydroxyproline is almost double that of the fermentation run at a high residual fructose concentration. When NaCl at 150 mM or Na_2SO_4 at 116 mM is added to the low residual fructose process, the amount of *trans*-4-hydroxyproline formed is similar to the levels seen for the high residual fructose process. Furthermore, none of these fermentation conditions affects the levels of *trans*-3-hydroxyproline formed.

Similar to the results obtained for the titration of L-proline, the extent of pneumocandin production is contingent on an ample supply of both *trans*-4-hydroxyproline and *trans*-3-hydroxyproline. However, the hydroxyproline ratio is essential for determining the percentage of pneumocandin C_0 produced.

4.2.2. Enzymology of Hydroxyproline Formation

The importance of *trans*-3-hydroxyproline and *trans*-4-hydroxyproline to the production of pneumocandin B_0 and the formation of key analogues was the motivation for identifying and characterizing the enzyme activity responsible for the formation of these two hydroxy-L-prolines. 3-Hydroxyproline and 4-hydroxyproline as free amino acids are uncommon but have been found in some peptide antibiotics such as etamycin [28], telomycin [29],

Table 3 “Low-Fructose” Process with and without Added Salts for Osmotic Buffering

	High Fructose (125 g/L)	Low Fructose (40 g/L)	Low Fructose + 150 mM NaCl	Low Fructose + 116 mM Na_2SO_4
Pneumocandin B_0 (arbitrary units)	253	339	282	280
Pneumocandin C_0 (%)	4.3	8.1	5.5	6.2

Titer is given in arbitrary units and is the average of duplicate flasks assayed at 14 days.

Source: Ref. 23.

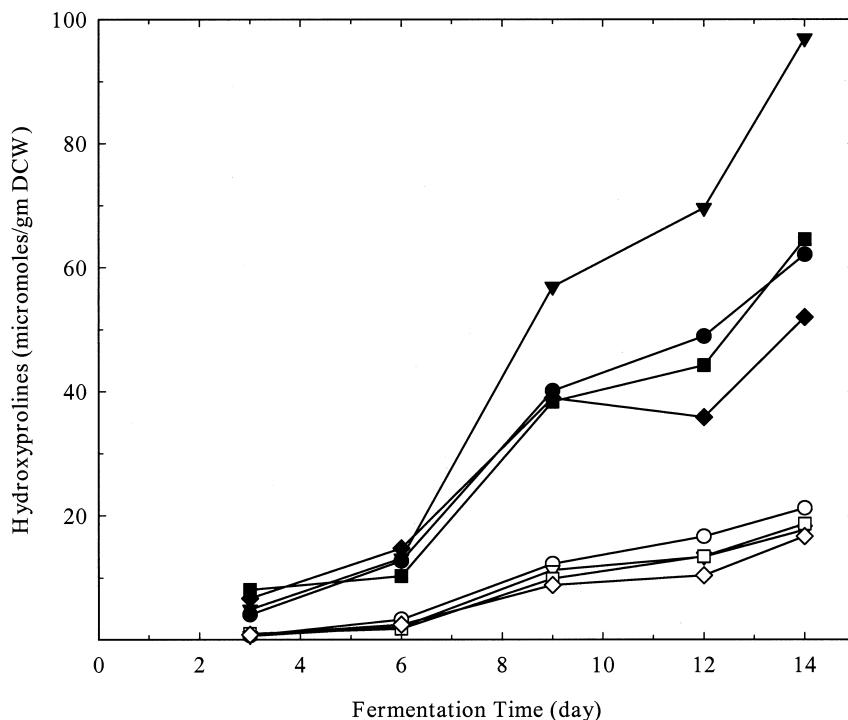


Figure 6 Production of *trans*-4-hydroxyproline (filled symbols) and *trans*-3-hydroxyproline (open symbols) at the shake-flask scale under different osmotic conditions: “high-fructose” process (\circ , ●); “low-fructose” process (\blacktriangle , \triangle); “low-fructose” plus 150 mM NaCl added on day 4 (\blacksquare , \square); and “low-fructose” plus 116 mM Na_2SO_4 added on day 4 (\blacklozenge , \lozenge). (From Ref. 23.)

plusbacin [30], and cryptocandin [31]. Like other prolyl and proline hydroxylases previously described, *trans* 3- and *trans* 4-hydroxyproline formation by *Glarea lozoyensis* is dependent upon α -ketoglutarate, iron, and ascorbate, which suggests that these enzymes belong to the family of α -ketoglutarate-dependent dioxygenases [32,33] (Table 4). The proline hydroxylase activities were also sensitive to EDTA and the divalent metal ions zinc, cobalt, copper, and nickel. Moreover, the relative amounts of the two hydroxyprolines formed in vitro are consistent with the amounts measured from fermentation whole broths (Table 4). Differences in the accumulation of *trans* 3- and *trans* 4-hydroxy-L-proline with respect to initial proline concentration (Fig. 5) and osmolality (Fig. 6) suggest the presence of two enzymes that are differentially regulated, rather than a single enzyme with relaxed regio-specificity [23,24].

4.3. Exogenously Added Serine and Threonine Affect the Position 3 Analogue Levels

In addition to the amino acid composition of the pneumocandins being dictated by the general substrate specificities of the putative NRPS, the metabolic pool size of the amino acids that could occupy a given position would also play a role. Exogenously added serine

Table 4 Substrate Specificity and Inhibitor Effects on Proline 3- and Proline 4-Hydroxylase Activities

	Specific Activity (pmoles min ⁻¹ mg ⁻¹ of protein)	
	Proline 3-Hydroxylase	Proline 4-Hydroxylase
Standard reaction ^a	31	231
– Ferrous sulfate	4	43
– Ascorbate	2	24
– DTT	20	153
– α -Ketoglutarate	ND	ND
+ Zinc sulfate	2	36
+ Copper chloride	6	99
+ Nickel chloride	0	5
+ Cobalt chloride	0	0
+ Magnesium sulfate	24	161
+ Manganese sulfate	13	88
EDTA (1 mM)	7	61

The reactions were done with a 35% to 75% ammonium sulfate fraction and analyzed using a dabsyl chloride derivatization procedure.

^a The standard reaction contained iron sulfate, dithiothreitol (DTT), ascorbate, proline, and α -ketoglutarate. The trace metal inhibitors were added at 0.4 mM; equimolar to FeSO₄.

ND = no activity detected.

Source: Ref. 32.

and threonine predictably affected the levels of the serine analogues of pneumocandins B₀ and B₅ (Table 5) [24]. Although it is likely that threonine is the preferred amino acid incorporated into the hexapeptide, exogenously added serine would likely increase the pool size of this amino acid, resulting in an increased rate of serine misincorporation. This misincorporation of serine, resulting in the formation of the two serine analogues, occurs in a dose-dependent manner. This directed-biosynthesis work was crucial in identifying the serine analogue of pneumocandin B₅, which is produced in small amounts under control conditions. Alternatively, exogenously added threonine reduces the levels of the two serine analogues, presumably by a similar mechanism. It was curious to observe that 5 g/L serine or threonine additions resulted in 25% to 30% reductions in pneumocandin B₀ titers.

Table 5 Effect of Exogenous Serine and Threonine on Pneumocandin B₀ Titer and Analogues

	Threonine		Serine		
	Control ^a	1 g/L	5 g/L	1 g/L	5 g/L
Pneumocandin B ₀ titer (arbitrary units)	472	453	344	446	364
Serine analogue of B ₀ (%)	2.2	1.3	0.3	3.7	22.3
Serine analogue of B ₅ (%)	0.5	0.3	ND	1.0	6.3

Serine and threonine were added on day 6 of the fermentation.

^a Results are an average of duplicate flasks harvested on day 14 of the fermentation.

ND = not detected.

Source: Ref. 24.

Table 6 Effect of Trace Metals on Pneumocandin B₀ Titer and Analogue Profiles

	Control ^a	Zn ²⁺	Co ²⁺	Cu ²⁺	Ni ²⁺
Pneumocandin B ₀ titer (arbitrary units)	629	312	421	605	546
E ₀ (%)	2.2	4.3	1.8	2.6	1.7
B ₅ (%)	4.8	3.9	7.2	5.2	4.4
B ₆ (%)	1.9	0.8	2.5	0.5	0.7
B ₂ (%)	2.8	1.9	6.0	2.7	3.1
B ₁ (%)	1.6	2.8	14.1	2.3	7.5

^a The base medium contains 36 μM Fe²⁺. Zinc, cobalt, copper, and nickel were added at 36 μM (equimolar to the iron in the base medium).

Source: Ref. 24.

4.4. Pleiotropic Influence of Divalent Metal Ions

Previous results suggested the involvement of one or two α-ketoglutarate-dependent dioxygenases that is/are responsible for the hydroxylation of proline [32]. Furthermore, the hydroxylation of proline in vitro showed varying sensitivities to zinc, cobalt, copper, and nickel. It is plausible that α-ketoglutarate-dependent dioxygenases are also responsible for the hydroxylations of the homotyrosine and ornithine residues, two of the four ring positions that show variability, and that differences in hydroxylation could be observed through altering divalent metal concentrations in the medium.

Pleiotropic effects were seen when cultures were supplemented with Zn²⁺, Co²⁺, Cu²⁺, and Ni²⁺ at 36 μM, equal to the Fe²⁺ concentration in the medium [24] (Table 6). Pneumocandin B₀ titer was reduced by the addition of zinc and cobalt and, to lesser degree, by nickel. Pneumocandin E₀ was the only position 1 analogue affected, an increase in the presence of zinc. Position 2 and position 4 analogues were increased to varying degrees by cobalt, but only the position 4 analogues were significantly affected by nickel.

Although the impact of divalent metal ions on pneumocandin B₀ titer and analogue formation provides presumptive evidence for the involvement of additional α-ketoglutarate-dependent dioxygenase, it is unclear if these enzymes would utilize the free amino acids as substrates or if the homotyrosine and ornithine are hydroxylated after incorporation into the hexapeptide.

5. EFFECTS OF THE PHYSICAL ENGINEERING ENVIRONMENT ON PNEUMOCANDIN PHYSIOLOGY

One approach to successful scale-up is to reproduce the environmental conditions that give the best results in the laboratory. This approach is hampered by the dependency of key parameters on scale (i.e., size of the fermenter). It may be possible to keep the chemical factors (such as oxygen solubility, pH, and temperature) sufficiently constant during scale-up. However, the physical factors, such as mixing (the time required to minimize concentration differences throughout the entire liquid volume), mass transfer, and power dissipation are dependent on fermenter size and change during the translation of scale [34]. High-power inputs and small mixing times achieved in laboratory fermenters are not attainable on a large scale. In practice, agitation power per unit volume (P/V) decreases with increas-

ing scale of operation, and the lower agitator P/V results in longer mixing times and lower oxygen transfer rates. Mixing times at the laboratory scale have been measured at less than 5 sec and reaching up to 100 sec on the 100-m³ scale [34]. At this large scale, it is likely that considerable heterogeneity exists within the fermentation broth. Liquid additions take time to disperse, and the nutrient concentration gradients may occur. These factors, coupled with high hydrostatic pressure and broth rheology, may create significant spatial variations of dissolved oxygen as measured by Oosterhuis et al. [35] at 19 m³ and Manafredini et al. [36] at 30 m³. For example, if the time constant for the rate of oxygen transfer is much shorter than the mixing time, then there will be vertical dissolved oxygen concentration gradients. This was observed by Manafredini et al. [36] for tetracycline production by *Streptomyces aureofaciens* on the 112-m³ scale. These effects, in combination with long mixing times, can result in gradients of nutrients, pH, and carbon dioxide at large scale, which may affect cell metabolism [37]. Therefore, the successful scale-up of a fermentation process requires a good understanding of the fermenter's environmental conditions, including pH, temperature, concentration of nutrients, dissolved gases, gas transfer, and liquid shear, and how these conditions may influence the behavior of the microorganism.

Because it is not possible to keep all chemical and physical parameters constant on a changing scale, it is important to understand what boundaries exist for successful implementation. This can be gained by characterizing the sensitivity of the process to the fundamental parameters such as agitation [38,39], dissolved gases (dO₂, dCO₂) [36,40,41], shear, pH, and temperature. This information allows operating boundary conditions to be defined and translated to scale. Fig. 7 illustrates the effects of the key parameters agitation and aeration. Changing one parameter can impact key factors such as bulk mixing, the dissolved carbon dioxide, and the level of foaming. For example, low agitation and aeration may not satisfy the oxygen requirement of the organism and lead to oxygen limitation. Using low aeration may limit the removal of dissolved carbon dioxide from the broth. This may cause accumulation of dissolved carbon dioxide, which in turn may lead to inhibition of growth and limit productivity. An understanding of the acceptable boundary limits will focus the scale-up effort and accelerate process development. This allows a product to be brought to market in a more timely, cost effective, and reliable manner. Establishing a defined process also satisfies regulatory requirements or the final clinical trials and process transfer to manufacturing.

5.1. Morphology and Rheology

A good understanding of the impact of the engineering on morphology and its relationship with productivity will enhance process development. The morphology of a mycelial fermentation can influence a process by affecting the broth rheological properties, which in turn influence the mixing (i.e., oxygen and heat transfer; Fig. 8). High-power inputs used to improve the oxygen transfer of highly viscous broths may result in high shear forces and, in turn, may fragment the mycelial structure and lead to poor productivity as shown for penicillin production [38]. The morphology of *G. lozoyensis* in production media [23] is predominately a dispersed form of mycelial clumps (i.e., aggregates of entangled hyphae) and freely dispersed hyphae [26], as shown in Fig. 9. Quantitative morphological analysis from 800-L fermentations has shown clumps with mean projected areas of $5600 \pm 1500 \mu\text{m}^2$ and mean total hyphal length of freely dispersed hyphae of $465 \pm 100 \mu\text{m}$ [26]. This morphology is similar to *Aspergillus* strains (400–500 μm) [39] and *Penicillium* (190 μm)

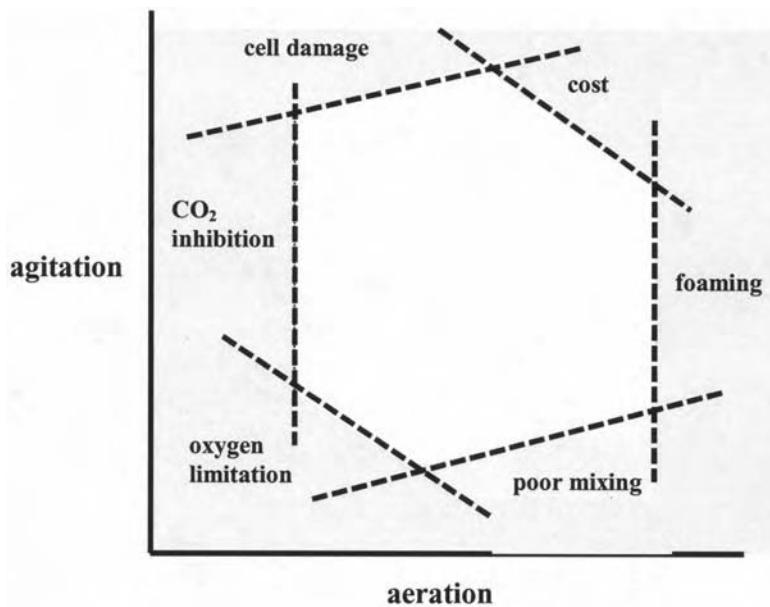


Figure 7 Agitation and aeration rates define the operating boundaries for a successful fermentation scale-up. Outside these boundaries exist a number of undesirable fermentation outcomes. From [34].

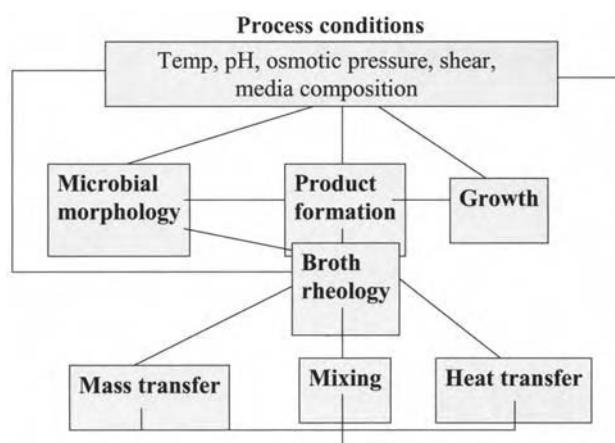


Figure 8 Relationship between morphology, broth rheology, and process engineering

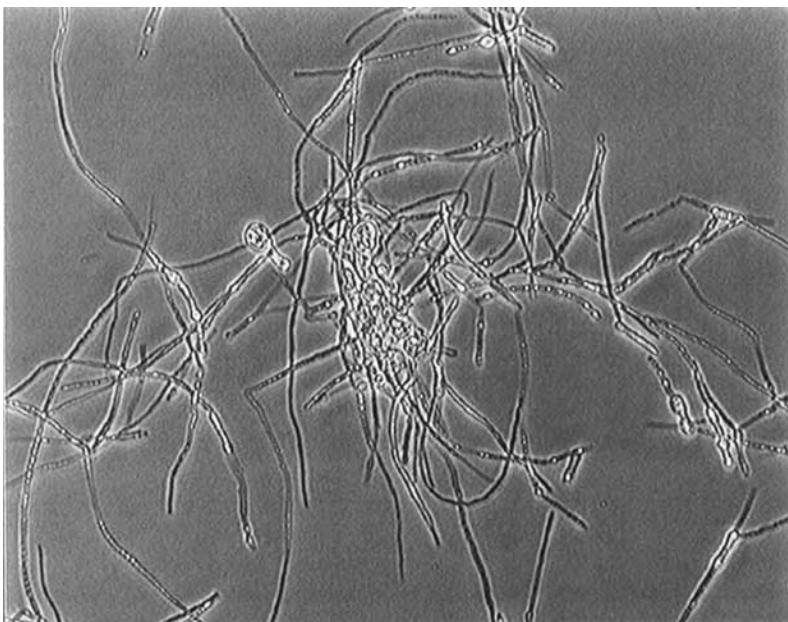


Figure 9 The morphology of *G. lozoyensis* (40 \times magnification).

[38], but much larger than the bacterium *S. erythraea* (30–70 μm) [41]. This mycelial morphology determines the viscosity of the fermentation broth. *G. lozoyensis* fermentations have been shown to change from initial water-like Newtonian rheology to high apparent pseudoplastic viscosity (1.5 Pa.s: 1500 times more viscous than water), as shown in Fig. 10. This has significant impact on oxygen transfer and the power requirements of the process. Therefore, the impact of agitation and low dissolved oxygen is a priority for process sensitivity studies.

5.2. Effect of Dissolved Oxygen

Scale-up success depends on satisfying the oxygen demand throughout the translation of scale. This is especially important for viscous, shear-sensitive filamentous broths, which have been associated with bulk mixing problems and oxygen transfer limitations at large scale. Low dissolved oxygen has a detrimental effect on pneumocandin production for both volumetric and specific productivity (Fig. 11). This was demonstrated by running multiple fermentations at a range of fixed dissolved oxygen levels (0.2% to 40% air saturation) using agitation as primary control, with aeration and backpressure as secondary control. The critical dissolved oxygen tension (C_{crit}) for production of pneumocandin B_0 was 20% air saturation with a significant reduction of the specific production rate below this value (Fig. 12). This effect was independent of growth, because the C_{crit} for growth was below 2%. For the pneumocandin analogues, the area ratio percentage of pneumocandin C_0 and the serine analogue compared with pneumocandin B_0 remained constant with dissolved oxygen setpoint. This showed the levels of all three pneumocandins were equally affected by dissolved oxygen. In contrast, low dissolved oxygen levels produced a substantial

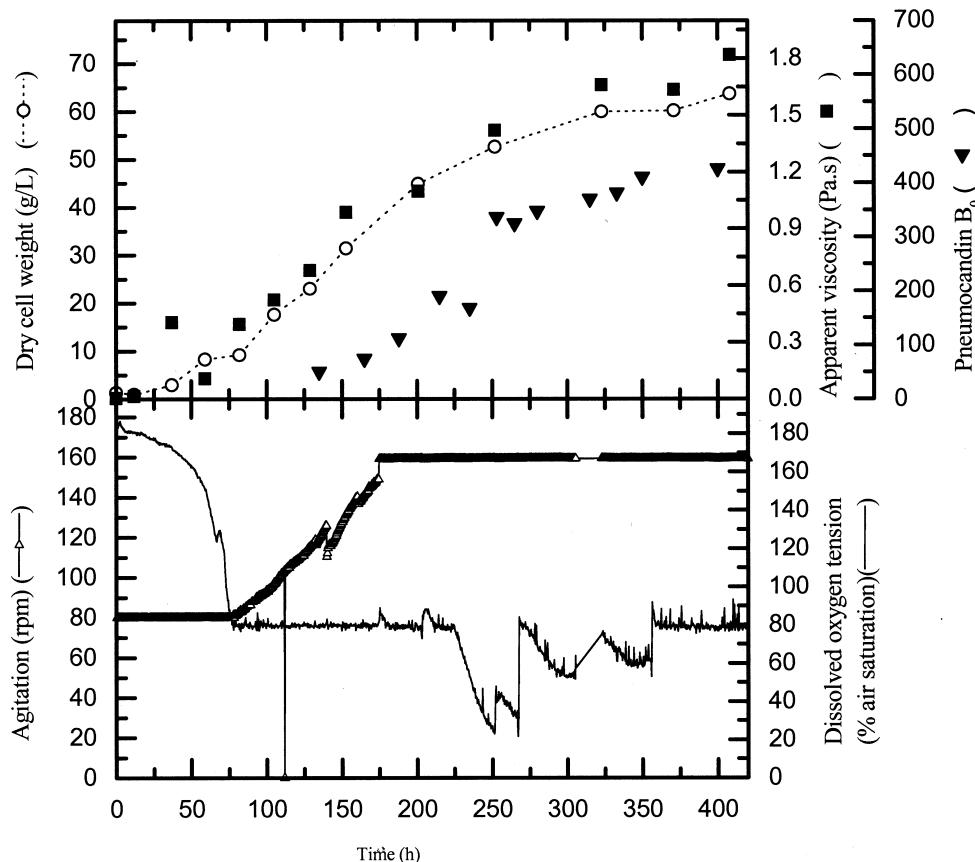


Figure 10 Pneumocandin fermentation at 19 m³. Agitation (Δ), dissolved oxygen (---), apparent viscosity (■), dry cell weight in g/L (○), and pneumocandin B₀ (▼).

increase of pneumocandins B₁, B₅, and E₀, whereas high dissolved oxygen levels produced a disproportionate increase in pneumocandin D₅ (Fig. 12).

5.3. Separating the Effects of Dissolved Oxygen from Agitation

The previous examples of dissolved oxygen effect on pneumocandin production used agitation to control dissolved oxygen. The difference in agitation rates among the fermentations (215 to 250 rpm) produced a difference in power dissipation of 4.5 to 7.0 kW/m³. The difference in mechanical forces and energy dissipation may influence the morphology of *G. lozoyensis*, which in turn may impact productivity and the response to dissolved oxygen. Agitation has been shown to influence the morphology of *Aspergillus sp.* [39] and *Penicillium chrysogenum* [38,41], as well as impacting penicillin production [38]. Gas blending of nitrogen into the inlet air can be used as a tool to separate the effects of dissolved oxygen from agitation. For the pneumocandin studies, dissolved oxygen levels of 5% and 10% were gas blended, with the agitation profile driven by a control tank at

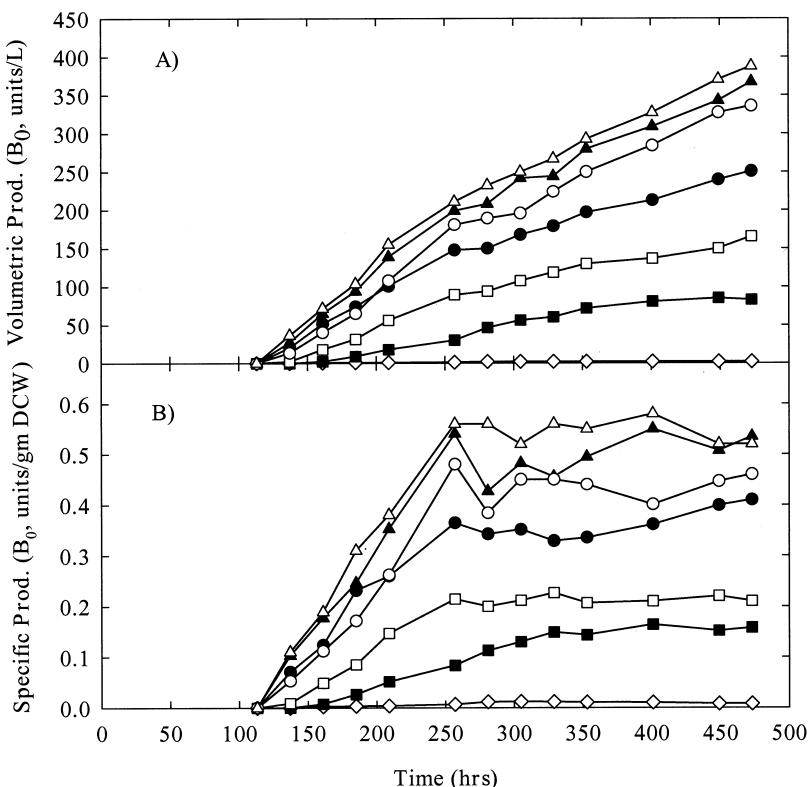


Figure 11 The effect of dissolved oxygen tension on the volumetric (A) and specific production (B) of pneumocandin B_0 at the 0.8 m^3 fermenter scale. Dissolved oxygen levels (% air saturation): 0.2 (\diamond), 2 (\blacksquare), 6 (\square), 12 (\bullet), 20 (\circ), 40 (\blacktriangle), and 80 (\triangle). (From ref. 26).

20% air saturation. The pneumocandin production produced a similar response to that shown to the previous experiment using agitation to control dissolved oxygen (Fig. 12). This indicates that the difference in power dissipation at 0.8 m^3 (2 to 7 kW/m^3) compared with 10 to 15 kW/m^3 at 0.07 m^3 had little impact on the productivity of the process. Amanullah et al. [39] showed similar effects with *Aspergillus* sp., where a decrease of agitation from 1000 to 500 rpm produced a rapid increase (less than 30 min) of the clump area (the major morphological form) of an *Aspergillus* sp. This morphological response to a change in energy dissipation (2.2 to 12 kW/m^3) was independent of the production of a recombinant protein. This is in contrast to the studies with *P. chrysogenum*, where the specific penicillin production rate and mean hyphal length inversely correlated with a power dissipation/circulation function, and where the dissolved oxygen remained above the critical 30% level [38].

The studies with pneumocandins concluded that the critical dissolved oxygen tension (C_{crit}) that impacted growth was below 2% air saturation. The C_{crit} for production of pneumocandin B_0 , as shown by the effect on q_{B_0} (specific production rate of pneumocandin B_0 , arbitrary units/L \times hours; Fig. 12), was 20% air saturation demonstrating that the C_{crit} for growth and production (pneumocandin B_0) were distinctly different. Vardar and

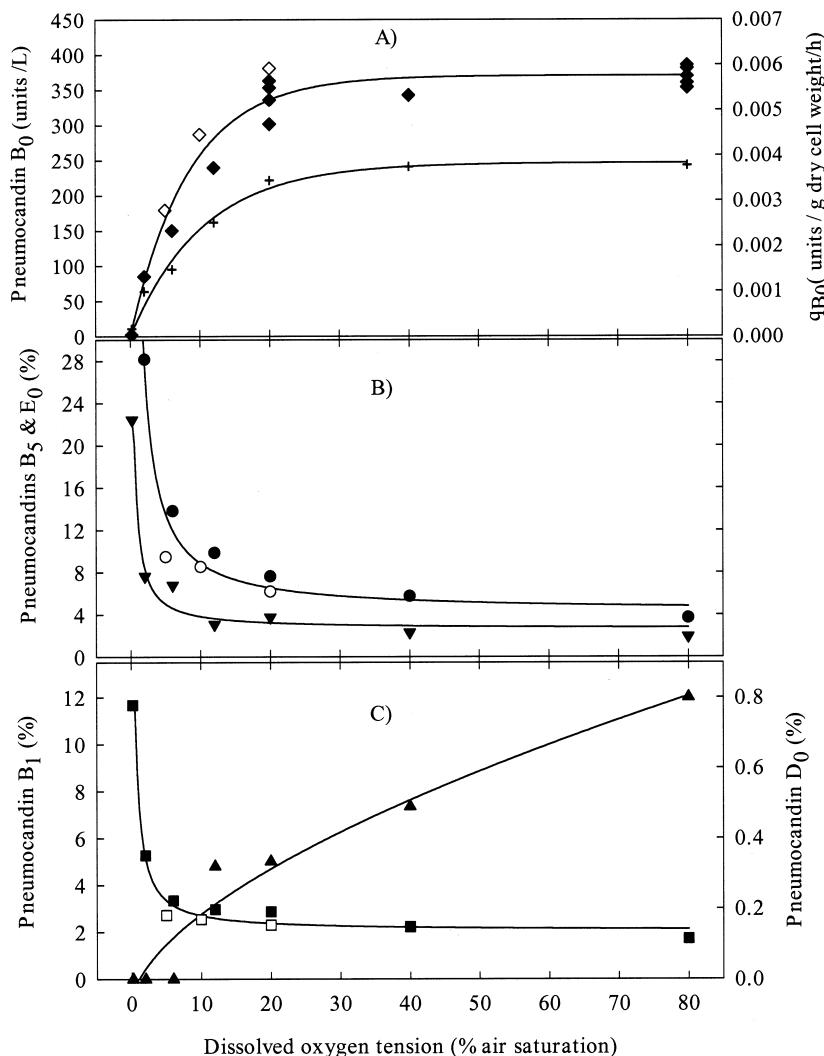


Figure 12 The effect of dissolved oxygen tension on pneumocandins B₀, B₁, D₀, B₅, and E₀ at the 0.8-m³ fermenter scale (closed symbols) and 0.07-m³ fermenter scale (open symbols; using gas blending). Data represent more than three experiments at 400 hours. A, Pneumocandin B₀ (□) and qB₀ (+). B, Pneumocandin analogues B₅ (●) and E₀ (▼). C, Pneumocandin analogues B₁ (■) and D₀ (▲). (From ref. 26).

Lilly [43] showed similar observations for penicillin production. The specific penicillin production rate was affected below 30% air saturation, whereas the OUR (oxygen uptake rate, mmoles/L × hour) was affected below 7%. The C_{crit} for antibiotic production from *S. lavendulae* and *S. griseus* also were found to be 20% air saturation, respectively [44,45]. The sensitivity of pneumocandin production to dissolved oxygen cannot be fully explained, because little is understood about the metabolism of *Glarea lozoyensis*. However, the

results show that increasing dissolved oxygen sensitivity occurs with increasing hydroxylation of the pneumocandin analogues. The largest number of hydroxylated groups is found with analogue D₀, which has the greatest dissolved oxygen sensitivity, whereas B₅ and B₁ have the least number of hydroxyl groups and are synthesized at higher levels with low dissolved oxygen tensions (see Fig. 2 for structures). Other examples have shown that dissolved oxygen can regulate the activities of biosynthetic enzymes involved in the production of cephalexin C [45]. Oxygen derepression of key pathway enzymes upon maintaining saturation dissolved oxygen levels was also demonstrated. The differing effect of dissolved oxygen on the production of pneumocandins was comparable to the effects on the production of difficidin and its hydroxylated derivative oxydifficidin by *Bacillus subtilis* [47]. The rate of difficidin synthesis was affected by dissolved oxygen below 40%, whereas oxydifficidin production was independent of dissolved oxygen.

5.4. Sensitivity to Carbon Dioxide

Dissolved carbon dioxide levels can have an inhibitory effect on growth, productivity, and morphology for a number of organisms, including *Aspergillus* [48], *S. erythraea* [49], and *Penicillium* [50]. In these examples, carbon dioxide levels greater than 5% (in the exhaust gas) were required before inhibitory effects were observed. Understanding the sensitivity of a fermentation process to carbon dioxide levels is important for determining

Table 7 Physical and Chemical Parameters That Affect Pneumocandin Titer and Structural Analogue Levels

Fermentation Parameter	B ₀ Titer	Effects on Pneumocandins Analogue(s)	Probable Mechanism
Proline	Increase	Decrease: C ₀ , D ₀ Increase: E ₀	Pool sizes and ratios of hydroxyprolines; pool size of proline
High residual fructose (osmolality)	Slight decrease	Decrease: C ₀	Pool sizes and ratios of hydroxyprolines
Low residual fructose (osmolality)	Increase	Increase: C ₀	Pool sizes and ratios of hydroxyprolines
Threonine	Slight decrease	Decrease: serine analogues of B ₀ and B ₅	Increase pool size of threonine favors incorporation
Serine	Slight decrease	Increase: serine analogues of B ₀ and B ₅	Increase pool size of serine favors incorporation
Dissolved O ₂ (<40% air saturation)	Decrease	Decrease: C ₀ , serine analogues, D ₀ Increase: B ₁ , B ₅ , and E ₀	Increase in analogue levels that lack hydroxyl groups
Dissolved CO ₂ (0.8%–2.1%)	No effect	No effect	NA
Temperature			
22 °–25 °C	Increase	Analogue levels changed in constant proportion of B ₀ titer	NA
25 °–28 °C	Decrease		
pH (4.5–6.5)	No effect	No effect	NA

the operating conditions for large-scale fermentations. Dissolved carbon dioxide levels can be controlled by manipulating the backpressure and airflow rate and agitation, as discussed with the boundary condition limits in Fig. 7. However, this strategy also impacts other parameters such as the control of dissolved oxygen, to which it has been shown that pneumocandin production is especially sensitive. The impact of dissolved carbon dioxide on the process was studied by manipulating the operating conditions to produce different carbon dioxide levels, as monitored by mass spectrometry of the exhaust gas. Fermenters at the 0.07 m^3 scale were operated with fixed backpressure between 5 and 20 Psi, airflow from 0.25 to 0.5 vvm, and dissolved oxygen at 20% by agitation cascade control. This range of operating conditions (including hydrostatic pressure) was similar to that expected at the manufacturing scale. The operating regimes resulted in a maximum achieved carbon dioxide, measured in the exhaust gas between 0.8% and 2.1%. This had no significant impact on any of the measurable process parameters: growth rates, biomass formation, and broth viscosity were similar between the operating conditions [26]. The pneumocandin B_0 and associated analogues were not significantly affected by the different operating conditions. This is similar to penicillin production, where carbon dioxide levels in the

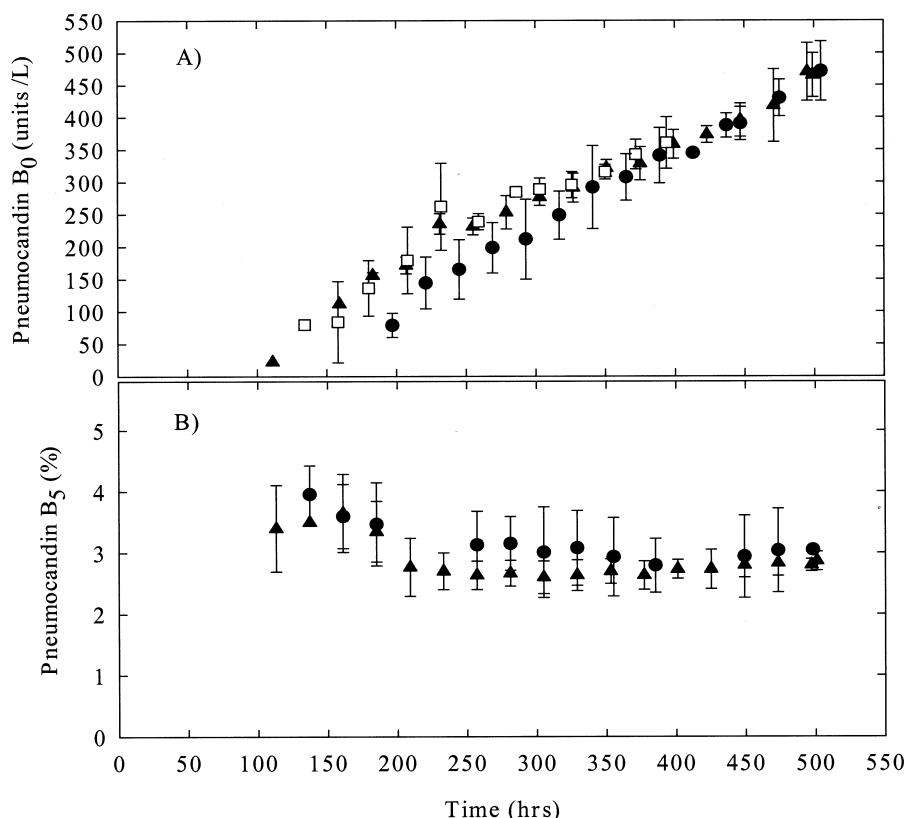


Figure 13 Consistency of pneumocandin B_0 (A) and B_5 (B) production at different fermenter scales: 0.07-m^3 fermenter scale (average data from four batches; \square), 0.8 m^3 (\bullet), and 19 m^3 (\blacktriangle). (From ref. 26).

exhaust gas of 4.2% to 4.8% had no impact on the specific penicillin production rate compared to control levels of 0.6% to 0.7% [42].

6. CONCLUSIONS

Table 7 summarizes the physical and chemical parameters that affect production of pneumocandins by the fungus *G. lozoyensis*. It is noteworthy that a fine balance must be struck when manipulating each of these parameters. For example, the addition of proline to the fermentation has proven necessary for reducing pneumocandin C₀ levels, but too high a concentration will lead to an increase in the level of the E₀ analogue. Similarly, controlling dissolved oxygen levels below 40% may be economically attractive; this would come at the expense of increased levels of pneumocandins B₁, B₅, and E₀. Characterization of these process sensitivities allowed the operating boundary conditions to be clearly defined. This physiological understanding was key to the control of unwanted pneumocandin analogues and the successful scale-up of pneumocandin production (Fig. 13).

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Strain Improvement for the Production of Fungal Secondary Metabolites

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1. INTRODUCTION

Secondary metabolites are produced when the rapid growth of the culture is completed. These have no obvious function in the growth of the producing organisms and often are produced as a family of structurally related compounds. Fungi are exceptionally prolific producers of secondary metabolites. It has been speculated that these compounds give some form of selective advantage to the producing organism in nature. The evidence in support of this hypothesis comes from the fact that the secondary metabolites possess wide range of biological activities. The secondary metabolites produced by fungi include antibiotics such as penicillins [1] and cephalosporins; the antifungals echinocandins [2,3] and pneumocandins [4]; cholesterol-lowering agents like lovastatin [5] and mevastatin [6]; the immunosuppressive agent cyclosporin [7]; alkaloids such as lysergic acid, ergotamine, and bromocriptine [8]; toxins like aflatoxin [9] and paraherquamide [10]; and asperlicin [11], a nonpeptide potent specific inhibitor of neurotransmitter cholecystokinin. Interestingly, mevastatin [12] and cyclosporin [13] were originally isolated as antifungal agents and paraherquamide was later shown to have anthelmintic activity [14]. For more discussion on secondary metabolites, see Chapter 1.

It has been observed generally that the original isolate from the nature does not produce very high levels of the active compound and more often produces a mixture of structurally related compounds. Both of these occurrences present problems in further studies of these active compounds and their subsequent development for medicinal purposes. Low concentrations of active compound produced necessitate generation of large volume of fermentation broths. This leads to a laborious isolation and purification process,

which becomes even more inefficient due to the presence of the mixture of related compounds. To shorten the time required to study and develop a newly discovered biologically active compound, it is essential to increase its yield and the purity. Thus, it is obvious that these have to be the major goals of the development of a fermentation process. Two approaches, optimization of medium composition, as well as of environmental conditions and genetic manipulation of the producing strain, are used in parallel for this purpose. The former approach falls out of the scope of this chapter; however, there are many reports in the literature that describe optimization of medium composition and environmental conditions, and the reader is referred to them. This chapter focuses on the use of genetic manipulation of the producing microorganism, often called strain improvement. Because there usually is little or no information available about the genetics of the producer organism, random mutation and screening for improved producer is often used for this purpose. This approach is referred to as the “classical” method. In recent years, with the advent of molecular biological techniques, attempts are being made to apply these for improvement in production. These two approaches are discussed in detail in the rest of this chapter, with emphasis on the application of the classical method.

2. CLASSICAL METHOD

2.1. DNA Damage, Repair, and Mutagenesis

Most of our understanding about DNA damage, repair, and mutagenesis has come from studies done with *Escherichia coli*. A substantial portion of this has been found applicable to other organisms. A short description of these phenomena is given here, as it is relevant to the discussion of the strain improvement process. For further information, the reader is referred to an excellent book by Friedberg et al. [15].

There are a number of ways DNA can be damaged. Apart from the errors that can occur during the replication, there are many physical and chemical agents, often referred to as “mutagens,” which react with DNA and cause chemical modifications. These changes can lead to misincorporation of bases or breaks in the DNA strands. Such a change, if not corrected, may disrupt a function essential for the survival of the cell and will cause the cell to become unviable. If it does not cause cell death, it may modify phenotypic behavior of the cell and will be identified as a mutation. Those mutations that do not exhibit any phenotype are called silent mutations. To ensure viability of the cell as well as to prevent mutations, which can be deleterious for the further survival of the organism, a number of DNA repair mechanisms has evolved. A partial list of mechanisms involved in mitigating DNA damage includes DNA mismatch correction, excision repair, recombination repair, and SOS repair. Methyl-instructed DNA mismatch correction in prokaryotes, which employs methylation of adenine in the sequence GATC to differentiate between the old and the new strand, recognizes small distortions in the DNA structure caused by incorporation of a wrong base during replication. The system removes a portion of the newly synthesized strand containing the wrong base and re-replicates the portion removed. Interestingly, however, the mismatch repair system in eukaryotic organisms does not use methylation to direct the repair, but rather uses the transient nicks in the newly synthesized strand. The excision repair system recognizes large distortions in DNA structure and hence is capable of repairing damage caused by many different agents. Mechanistically, it too excises the damaged part of the strand and re-replicates it. This system is very efficient. However, it requires one of the two strands of DNA to be undamaged so it can

be used as a template to replace the part that is removed. The recombination repair system allows the replication to proceed over the damage. Subsequently, the damaged strand undergoes recombination with the undamaged sister strand and that in turn allows repair of the damaged strand. Another important repair system is called the SOS system. The SOS system is induced when DNA is extensively damaged. It was found that there is a set of genes that is induced when DNA is damaged. These are named *din* (damage-induced) genes. At present there are more than 30 such genes known. A subset of the *din* genes is regulated by SOS response. The excision repair and recombination repair systems are not error prone; however, a repair system activated by SOS response is. This system replicates over the damage by misincorporating bases. Furthermore, this system is error prone and misincorporates bases even in the replication of the undamaged region. In summary, mutagens cause DNA damage and mutations are generated either by the failure of repair systems to remove the damage or by introduction of additional damage by the same.

2.2. Types of Mutagens

The strain improvement and process involves application of a mutagenic treatment followed by screening of the survivors for mutants with desired properties, such as improved yield or purity. Over the years many different mutagenic agents have been used. These can be divided into two classes: physical agents and chemical agents.

2.2.1. Physical Mutagens

This category includes ultraviolet (UV) light and ionizing radiation such as x-rays and gamma rays, as well as fast neutron exposure. Of these, UV light is the most commonly used mutagen because of ease of application and relative safety.

UV Light

As mentioned above, this is a commonly used mutagen. Short-wavelength UV light (i.e., below 300 nm) is very effective for this purpose because the bases in DNA strongly absorb the light in this region. Germicidal lamps that emit light at 254 nm are ideal for this purpose and are widely used. Because the penetration of UV radiation into aqueous medium is very limited, it is important to have a shallow layer of the cell suspension and agitate it during the exposure [16,17]. The effect of the absorbed energy is the formation of additional chemical bonds between adjacent atoms, such as those resulting in pyrimidine dimers. Thymine dimers (TT dimers) are formed by linking C-5 and C-6 of the adjacent pyrimidines. These linkages give rise to a cyclobutane ring, and therefore these dimers are also referred to as cyclobutane dimers. The second type of dimers is generated by reaction between C-6 of thymine and C-4 of cytidine [TC (6–4) dimmers]. The presence of both types of dimers causes distortion in the structure of DNA, which results in stalling of DNA polymerase and hence of replication. As mentioned above, a number of repair mechanisms have evolved to prevent interruption or errors in replication. In addition to those, a mechanism specific for repairing damage caused by UV irradiation has been observed. This system depends on the availability of light with wavelengths greater than 350 nm to remove the pyrimidine dimers and therefore is called photoreactivation. The enzyme responsible for this, termed photolyase, recognizes and binds to the dimers and uses the energy absorbed from light to separate the two pyrimidines. Therefore, for UV mutagenesis to be effective, it is important to keep the sample exposed to UV light in the dark for some time before plating for isolated colonies for screening. The dimers induce SOS response and, as mentioned earlier, in turn lead to mutations.

UV light at longer wavelengths (320 to 400 nm) by itself is not very effective; however, it was found that in combination with psoralen and its derivatives such as 8-methoxysoralen (MOP) it can be mutagenic. Psoralens are produced by more than 24 plants. They are planar molecules and used by the plants as defense against fungi and insects. These compounds are intercalating agents and upon photoactivation by the UV light of a longer wavelength, form adducts with pyrimidine bases commonly by addition across the 5,6 double bond in thymine. MOP is capable of forming adduct at both ends of the molecule (double adduct). The formation of the double adduct occurs in two steps and requires two independent UV absorption events. When the two bases are located on the two different strands of DNA, the double adduct crosslinks the two strands. This crosslinking in turn leads to significant distortion of the DNA structure and elicits response from the repair apparatus, including SOS response [18–20].

Ionizing Radiation

X-rays and gamma rays have been used in the past for mutagenesis. The radiation energy affects DNA both directly and indirectly. Not only does it interact with DNA per se, but it also interacts with other chemical entities that exist with DNA in the aqueous medium. Upon absorption of energy, these form reactive species (free radicals), which also react with DNA. It has been suggested that in mammalian cells the majority of the damage to DNA comes from these radicals [21]. The products of the reactions caused by ionizing radiation are damaged bases and, to a lesser extent, damaged sugars and strand breaks. The deleterious effects of ionizing radiation are enhanced in the presence of high concentrations of oxygen [22]. Although a wide range of types of damage to DNA is caused by the ionizing radiation, the exact relationship between individual lesions and lethality and mutagenesis is not known [15].

Practical guidance on the use of x-rays and gamma rays has been provided by Calam [16]. However, the logistics and safety considerations of using ionizing radiation and the extent of damage caused by it have limited its use.

2.2.2. Chemical Mutagens

Chemical mutagens are classified based on the type of chemical modification of DNA made by them. The chemical reactions include alkylation, deamination, or substitution of bases and intercalation in between bases. Some examples of chemical mutagens are listed below and in Fig. 1.

Alkylating Agents

These compounds react with nucleophilic centers in the bases. Although there are a number of these present in the structure of bases, it appears that there is a preference for the positions depending on the alkylating agent used (Fig. 1). For example, alkyl sulphates react exclusively with nitrogen, whereas N-nitroso compounds react primarily with oxygen in the bases. Alkylalkane sulfonates fall in between the two. Furthermore, those that react with nitrogen prefer N⁷ position of guanine and N³ position of adenine, and those that act on oxygen are selective for O⁶ position of guanine [23]. It was also noted that the alkylation is directed by both the base sequence and the physical structure of DNA [24]. Presence of adjacent guanines enhances negative potential of N⁷ of guanine and increases probability of alkylation at that position. Similarly, when DNA is in the right-handed helical form, N⁷ and O⁶ positions of guanine are in the major groove and more accessible than N³ of adenine [15]. To characterize this effect, further studies were done to determine the

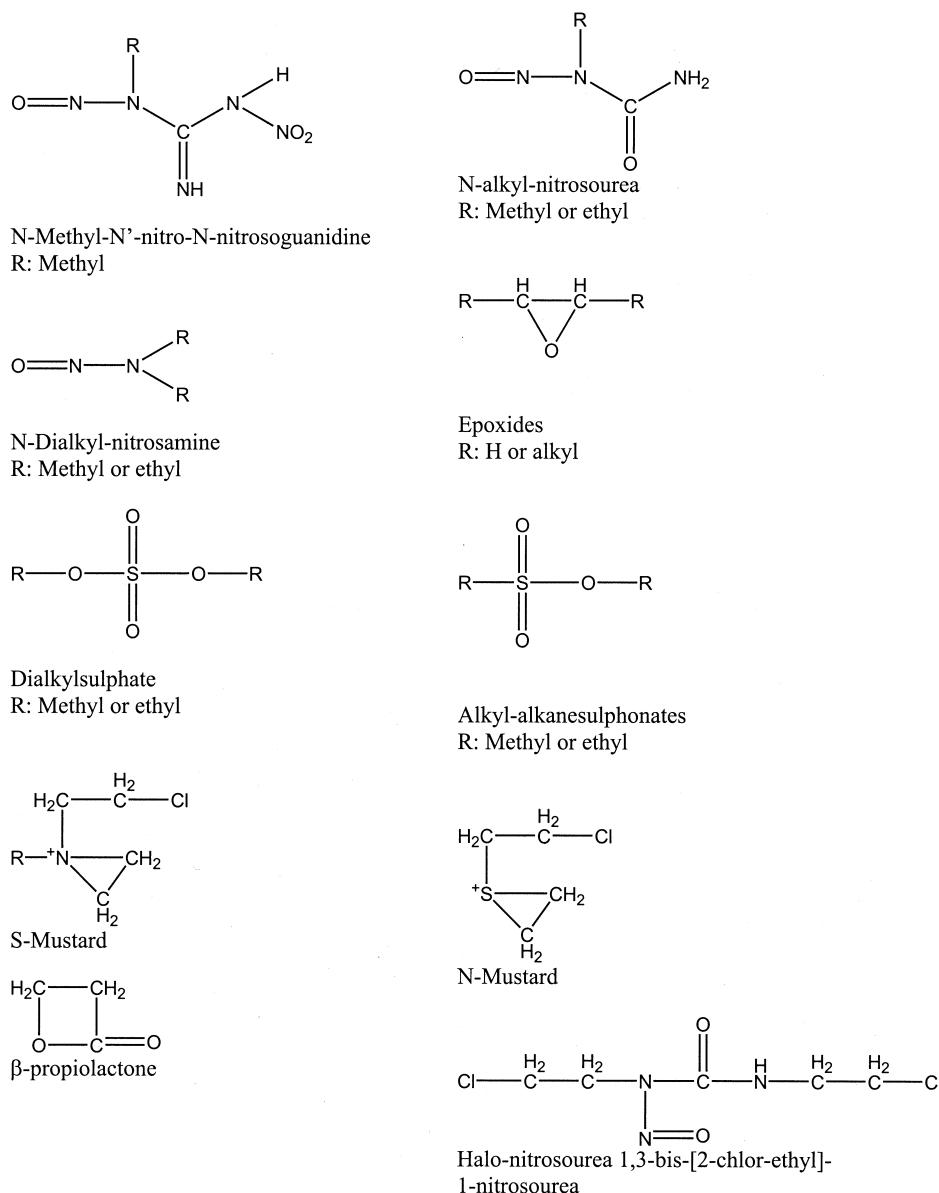


Figure 1 Structures of the alkylating agents.

cause–effect relationship between the modifications in the DNA bases and the mutagenesis. From these, Singer [23] concluded the following:

1. Bulky substituents at any position will interfere with replication. Some of these products can undergo rapid depurination and cause mutations via error-prone repair. The third possibility is the bypass by polymerase, which can lead to frame shift mutations.

2. Smaller alkyl derivatives are likely to cause misincorporation. O-alkyl derivatives generated by N-nitroso compounds fall in this group.

The repair mechanisms specifically induced by alkylation of DNA include specific N-glycosylase-dependent pathway and the system involving methyltransferases. In the case of the former, the N-glycosylase removes the alkylated base and apurinic/apyrimidinic (AP) endonuclease cuts the apurinic strand. This is followed by digestion of the cut strand by exonucleases and resynthesis of the removed strand by DNA polymerase I. In the case of the latter, the methyltransferases remove the alkyl groups from the alkylated bases by transferring these groups to themselves [25]. Furthermore, the alkylation of DNA also induces the error-prone repair mechanism.

Dialkylsulphates

The compounds commonly used are dimethyl- or diethylsulphate. As mentioned above, these preferentially react with the N³ position of adenine and N⁷ position of guanine. It has been observed that methylation agents are more effective mutagens than ethylating ones. The reason for this may be that the latter are considerably less reactive than the former. Overall, however, these compounds are not as good as the sulfonates or the N-nitroso compounds.

Alkyl Alkanesulfonates

The compounds commonly used in this category are methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), and ethyl ethanesulfonate (EES). These compounds alkylate the O⁶ position of guanine in addition to the nitrogen atoms in adenine and guanine mentioned above. It has been noted that alkylation of oxygen atoms results in more effective mutagenesis. Furthermore, these compounds, being liquid, are easy to use. Therefore, they are often the mutagens of choice.

N-Nitroso Compounds

N-Methyl-N'-nitro-N-nitrosoguanidine (NTG or MNNG) is probably the most popular mutagen used for strain improvement because it is a highly effective mutagen. The first careful development of protocol for the use of NTG was reported by Adelberg et al. [26]. Induction of auxotrophs in *E. coli* was used as a measure of mutagenesis. They found that it was possible to get high auxotroph frequency (50%) at relatively low reduction in viability. Subsequently, it was tested with other microorganisms [27,28] and the observations reported by Adelberg about the efficacy of NTG were confirmed. During their studies, Delic et al. [27] noted that in the case of *Streptomyces coelicolor*, higher mutation frequency was observed at the treatment pH of 9.0 than at pH 6.0 used by Adelberg [26]. At alkaline pH NTG decomposes with formation of diazomethane, which has been suggested to play a role in mutagenesis [29]. Another variable that can have a significant effect on the mutagenesis is temperature of treatment. Kasahara et al. found that for *S. cacaoi*, higher temperature, i.e., 42 °C, gave the highest frequency of mutation [30]. Another interesting characteristic of NTG mutagenesis is that it preferentially acts in the replicating region [31]. This observation opens up a possibility that a different spectrum of mutations can be obtained, depending upon the region of DNA being replicated during the exposure to NTG. An example of this was reported by Godfrey, who examined the response of *S. lipmanii* to NTG [32]. In this study, chromosome replication of the leu auxotroph of *S. lipmanii* was synchronized by amino acid starvation, followed by periodic exposure to NTG after addition of leucine. The examination of the mutants obtained

showed that the type of the mutation observed was dependent on the region of DNA being replicated.

Other N-nitroso compounds used are N-alkyl-N-nitrosourea [33], N-alkyl-N-nitrosouethane [34], and N-dialkyl-N-nitrosoamines [35]. The nitrosoureas react with oxygen in DNA, which makes them good mutagens. In contrast with other alkylating agents, nitrosoamines need to be metabolically activated for them to cause mutations. They react with DNA in the manner similar to nitrosoguanidine and nitrosourea.

Miscellaneous Alkylating Agents

In addition to those mentioned above, other alkylating compounds that have been used include lactones such as beta-propiolactone, epoxides such as ethylene oxide, N-mustard, S-mustard, and halo nitrosoureas. These predominantly act on nitrogens of adenine and guanine. S-mustard also reacts with nitrogen of cytosine. Both S-mustard and N-mustard also crosslink two strands of DNA. Halo-nitrosoureas, in addition to that mentioned above, react with nitrogens of cytosine and oxygen of guanine.

Deaminating Agents

Deaminating agents remove amino groups in adenine, guanine, and cytosine. Deamination reactions result in altered bases and lead to mispairing. This mispairing, if not corrected, will cause mutations. The specific repair system that has evolved to remove the deaminated bases involves specific glycosylases, which identify and remove the deaminated base and AP nucleases, which make a cut next to the position from where the modified base was removed. Next, the repair DNA polymerase synthesizes new DNA starting from the cut, using the other strand as a template, thus replacing the modified base with the correct one. 5-Methyl cytosine is sometimes present in DNA. Its deamination product is thymine, which cannot be removed by the glycosylases. A special mechanism called very short patch repair (10 nucleotides or fewer) corrects G:T mismatches such as result from the deamination of methylcytosine [36].

Nitrous Acid

This compound causes oxidative deamination. It is simple to use and mutagenesis can be easily controlled. Furthermore, the deamination of adenine, cytosine, and guanine changes the base pairing as expected. Therefore, it is one of the commonly used mutagens. Kinetics of deamination are faster at acidic pH and hence mutagenic treatment is done at pH 4.5. Nitrous acid is generated *in situ* by adding NaNO₂ solution to the acidic buffer. Nitrous acid is also used for *in vitro* mutagenesis.

Bisulfite

This compound is known to deaminate cytosine [37]. It also can deaminate 5-methylcytosine [38]. It was found to react with cytosine in single-stranded DNA and, therefore, was used as site-specific mutagen.

Intercalating Agents

Compounds with planar structures can intercalate into DNA, forcing the bases in the strand with the intercalated compound apart and causing slippage between the two strands. During replication, an extra base may be added to correct for the slippage or a base may be deleted. The mutations resulting from this type of DNA damage are called frame-shift mutations, because it changes triplet reading frame. Some acridines were noted to be mutagenic in certain systems. However, they were not very effective in bacteria. The

unpredictability of the efficacy of acridines has limited their use. A series of acridine derivatives was developed at the Institute for Cancer Research in Philadelphia, Pennsylvania. These are called ICR compounds. They were found to be very effective mutagens that caused frame-shift mutations [39].

2.3. General Considerations

The goal of strain improvement program is to increase the yield and the purity of the microbial product of interest. The term *purity* describes the proportion of the desired compound in the total metabolites produced or, more meaningfully, with respect to the related ones in the broth. Because the focus of this chapter is on the fungal secondary metabolites, the general aspects of strain improvement with respect to filamentous fungi are briefly discussed below. An excellent review of strain improvement for the production of microbial metabolites by Queener is recommended for interested readers [40].

One of the critical needs for efficient mutagenesis and isolation of mutants is the availability of single clones. Fungal spores that are true propagules are used for mutagenic treatment. However, some fungi do not form such spores; in those cases, the fragmented vegetative mycelium or protoplasts can be used. When using fragmented mycelium, it is important to remove the large pieces. This can be done by filtration or by low-speed centrifugation. Protocols for protoplasting have been published [41,42]. It has been noted that the regeneration frequency of fungal protoplasts is often low and optimization of the procedure to improve the regeneration frequency is needed. As described above, there are many mutagens available for use. The choice depends on the fungal culture used, experience of the worker, and the type of mutation desired. UV light and alkylating agents are more commonly used than other mutagens. Once the mutagen is selected, its effect on the survival of culture needs to be determined. The two variables studied are mutagen concentration and the time of treatment. Some idea about the efficiency of mutagenesis can be obtained by measuring the frequency of auxotrophs, of that of reversion of auxotrophy, of that of induction of resistance to a toxic metabolite or the morphological variation in the treated population. Although these results do not necessarily reflect the efficiency of mutation obtained in the actual strain improvement program, they do help in the design of the experimental protocol. Following the treatment, the survivors are plated for isolated colonies. In most cases, it is necessary to test a large number of colonies for the production of the desired metabolite, often referred to as “random screening.” To increase the probability of success, various statistical methods have been applied for the analysis of data. Sometimes it is possible by the examination of the structure of the product to infer potential mechanisms/metabolites that may regulate its synthesis. In these instances, it may be possible to design a medium that gives the improved mutants a selective advantage.

2.4. Strain Improvement

The undertaking of the strain improvement program for increasing the yield of penicillin in the early 1940s was the beginning of the modern industrial fermentation process development. Since then, many important fungal secondary metabolites were added to medicine’s arsenal for fighting disease. These include the antibiotic cephalosporin, hypocholesterolemic statins, and the immunosuppressant cyclosporin. The development of these therapeu-

tic compounds (Fig. 2) involved strain improvement programs; some aspects of these are discussed below.

2.4.1. Penicillins

The original culture, *Penicillium notatum*, which was observed by Fleming to produce penicillin, produced very low levels of the antibiotic and was not very useful for development. However, after the discovery of another culture, which produced penicillin and which was identified as *Penicillium chrysogenum*, the strain improvement program moved

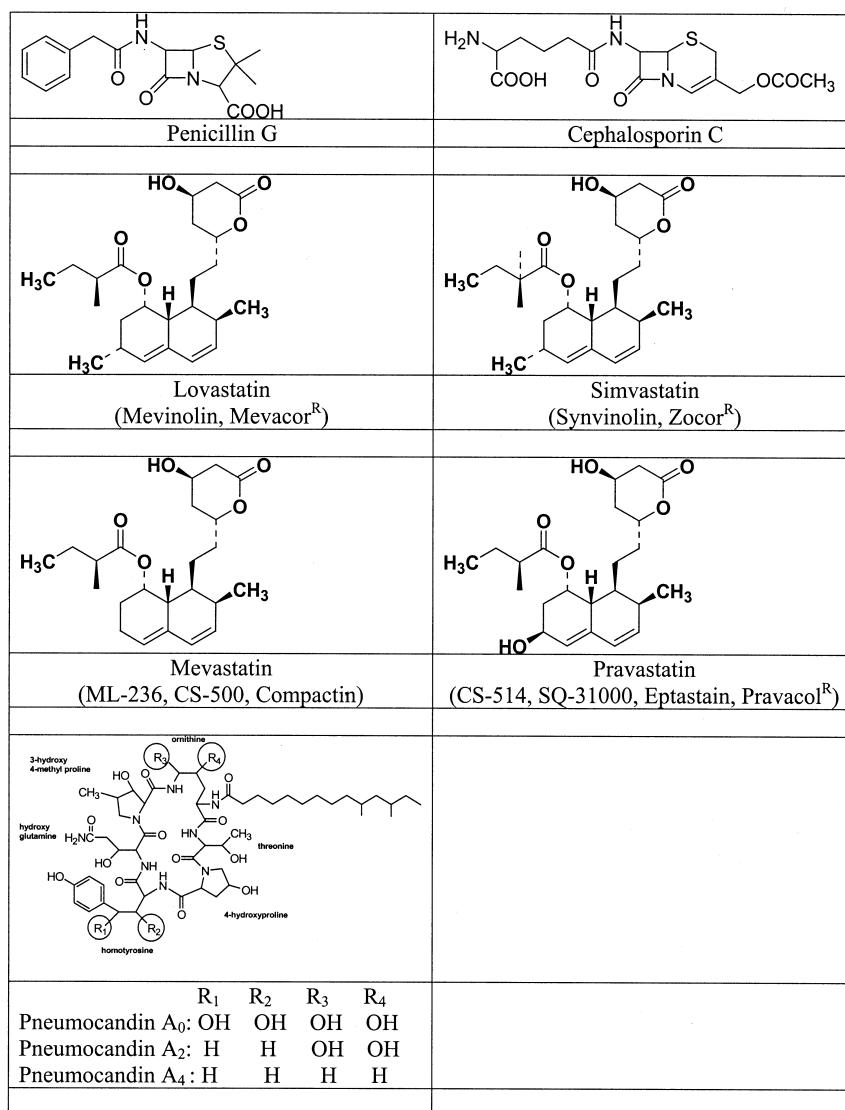
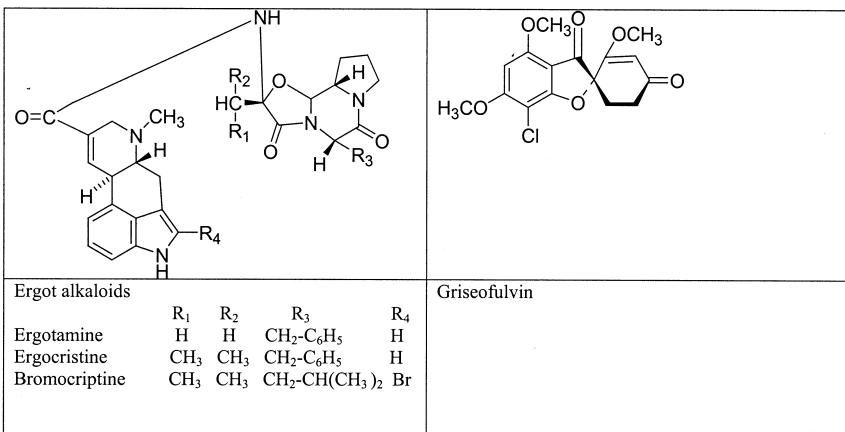
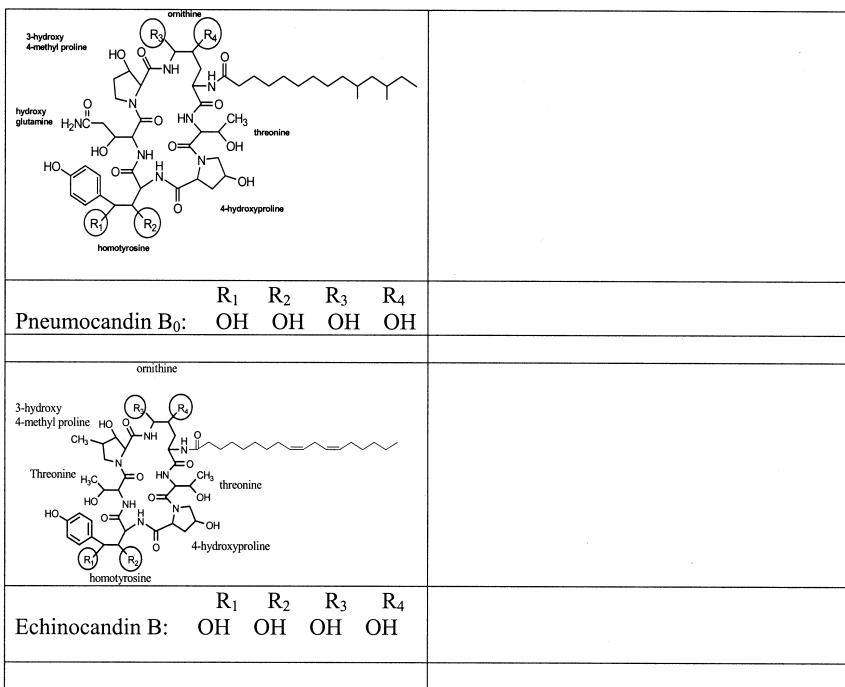


Figure 2a Structures of the secondary metabolites.

**Figure 2b**

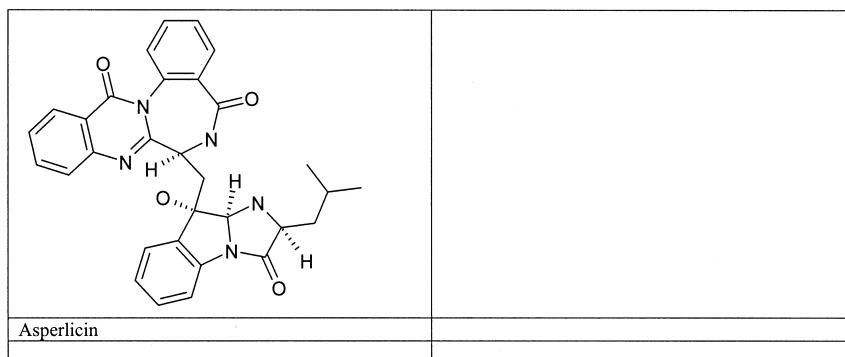


Figure 2c

forward. This strain was designated Northern Regional Research Laboratory strain 1951 (NRRL1951). Initial efforts involved single-spore reisolation of the culture. This approach resulted in increasing the yield from 60 to 150 mg/L. Interestingly, the lesson learned here has not been forgotten. Even today, the first step in the strain improvement program for a newly discovered active compound is the reisolation of the original isolate. In the early phases of a strain improvement program, when there was an absence of any type of selection, a very large number of colonies had to be tested to isolate improved producers. The first superior producer to be obtained with the use of mutagenic treatment was X-1612. It was isolated from the population exposed to x-rays by Demerec at the Carnegie Institute. This strain was then subjected to UV light at the University of Wisconsin to obtain the strain designated Q-176. This strain was given to commercial manufacturers and is the parent of many strains used for penicillin production since then [43]. The penicillin fermentation development program at the University of Wisconsin was initiated in 1943. It was expanded in 1955 to include the development of cephalosporin fermentation process and continued up to 1967. The original isolate of *P. chrysogenum*, NRRL 1951 and other improved strains derived from it, including Q-176, produced a yellow pigment, called chrysogenin, which could not be easily removed during isolation of penicillin. Therefore, a major effort at the University of Wisconsin was directed at isolation of pigmentless strains. This resulted in the isolation of pigmentless mutant BL3-D10 from Q-176 following UV mutagenesis [44]. Three parallel approaches were used in the program at the University of Wisconsin to isolate superior mutants. These included mutagenesis with UV, mutagenesis with nitrogen mustard, and reisolation. The Wisconsin strain improvement project started with culture X-1612, which gave a yield of 120 mg/L and produced the pigment. The last mutants isolated in the program were pigmentless and produced about 1.6 g/L. An excellent account of this effort was published by Elander [45].

In a study on the efficacy of treatment of *P. chrysogenum* Wis. 54-1255 with different chemical mutagens for isolation of auxotrophs, it was found that NTG at neutral pH was not effective. Conversely, when used at pH 9.0, it gave a frequency of auxotrophs of 13%. However, the mutagenesis was very severe and most of the auxotrophs had either multiple requirements or grew poorly. Upon treatment with EMS, auxotroph frequency of 3% was observed. In contrast to the auxotrophs isolated after the NTG mutagenesis at

pH 9.0, very few of those obtained with EMS treatment were either multiple auxotrophs or poor growers. This made EMS the mutagen of choice for further work [46].

Panlabs Inc. launched their contract project to increase the yield of penicillin in 1972. In this program, the mutagens used were dimethyl sulphate, EMS, and UV light. NTG was avoided due to the concern about potential multiple mutations, which is consistent with above-mentioned results. Two parental strains used were designated P-1 and P-2. The strain P-1 was the production strain of Toyo Jozo Co. (Japan) and the strain P-2 was an earlier production strain from Nippon Kayaku Co. (Japan). Selective and nonselective screening approaches were used with the P-1 line and the P-2 line, respectively. To accelerate mutation cycles, the highest producer obtained with a round of mutation and screening was used for the next round, without extensive testing to confirm the level of production, the rationale being that even if the yield of this strain was simply the highest end of the distribution of production observed, it may not be any worse than that of the parent [47].

The aim of the selection methods used were to disrupt the mechanisms that regulated penicillin biosynthesis. Addition of lysine was shown to inhibit the production of penicillin; however, high concentrations of lysine (10 to 20 mM) were required to affect the synthesis [48]. In light of this observation, it was necessary to determine the significance of lysine effect for production of penicillin in the absence of added lysine. For this purpose, lysine overproducing mutants were isolated by selecting for resistance to S-(2-aminoethyl)-L-cysteine, 5-hydroxylysine, and 4,5-dehydrolysine, which are toxic analogues of lysine. Comparison of production of penicillin and lysine by these resistant mutants showed a clear inverse relationship between the two. These results indicated that the lysine effect had practical significance [49]. To explore the possibility of disrupting the regulation by lysine, 2-amino adipic acid and lysine were used as selective agents. Because these are not toxic, they had to be used at very high concentrations. Other compounds used as selective agents were the valine analogue valine hydroxamate, sodium sulfide, and 2-aminobutyric acid. A combination of both approaches resulted in isolation of strains that produced 20 g/L penicillin at the end of the program [47].

It is important to note that the major manufacturers of penicillin had their own strain improvement programs, which continued through most of the period that penicillin was produced by them. Today there are a limited number of companies that produce penicillins, and they have continued to improve the process. It has been reported that the yield has been increased to 70 g/L in the strains used for manufacture currently [50,51]. The success of these strain improvement programs, coupled with environmental optimization studies, has almost converted penicillin from a fine chemical to a commodity one.

2.4.2. Cephalosporins

A strain of *Cephalosporium acremonium* was isolated by Brotzu in the Sardinian sea near a sewage outlet. He was able to show that this culture produced an unknown antibiotic [51]. He was not able to isolate the active compound. Later, three active compounds were isolated by Abraham et al. [52] from this strain: cephalosporin P, cephalosporin N, and cephalosporin C. Brotzu's strain was designated ATCC 11550.

Around the same period, the Michigan Department of Health reported the discovery of the antibiotic synnematin B from a culture initially classified as *Tilachlidium* [53]. It was later reclassified as *Cephalosporium salmosynnematum* [54]. Later, this antibiotic, along with cephalosporin N, was shown to be the penicillin N [51].

Of these, cephalosporin C was found to be the most effective, and an effort was launched to further increase its yields. Early work was done in England. An improved

producer, strain 8650, was developed at the Antibiotic Research Center in Clevedon, England from CMI 49137, which was isolated from Brotzu's strain [43]. In the United States, Eli Lilly and Co., along with other pharmaceutical companies and the University of Wisconsin, initiated similar programs. The program at the University of Wisconsin was started with strain M-8650 obtained from Eli Lilly and Co. This culture produced 0.3 g/L of cephalosporin C. In next 6 years, a number of superior mutants were isolated with the use of mutagenesis and spontaneous selection. The best mutant, OP-168-63-74, gave a yield of 1 g/L [45]. As was the case with the penicillin strain improvement programs, a number of selective agents were also used to increase the titer of cephalosporin C. These included amino acid analogues selenocysteine, selenomethionine, trifluoroleucine, 2-methylserine, trifluoromethionine, S-2-aminoethyl-L-cysteine, and DL-2-aminobutyric acid. Generation of precursor amino acid auxotrophs and their reversion is another method used; however, its success in the development of commercially useful strains is not clear [55].

Unfortunately, no reports on the strain improvement programs at various pharmaceutical industries have been published.

2.4.3. Statins

This class of compounds is hypocholesteremic and, therefore, has large economic value. Zocor, one of the number of statins available for treatment, had sales of almost \$6 billion in the year 2002. The natural products from which these drugs are derived are lovastatin, produced by *Aspergillus terreus*, and compactin, made by *Penicillium citrinum* [56]. Significant work on the strain improvement was done at the pharmaceutical companies where these compounds were originally discovered. However, there is no published information available about it. There was a mention of a manuscript in preparation on the strain improvement program at Sankyo Company (Japan) [57].

Vinci et al. reported their work on the enhancement of purity of lovastatin produced [58]. *Aspergillus terreus* produces sulochrin, a benzophenone, as a cometabolite of lovastatin. This compound is made by polyketide pathway and hence competes with lovastatin biosynthesis for intermediates. A surface fermentation process to screen the survivors of mutagenic treatment along, with high performance TLC method to analyze the products, was developed. They isolated two mutants. One of them, AH6, produced lovastatin equal to the parent, but no detectable levels of sulochrin. Another mutant, CB4, gave yields of lovastatin 16% higher than the parent, but produced 30% less sulochrin.

2.4.4. Pneumocandins and Echinocandins

These are cyclic hexapeptides with a fatty acyl side chain, which have potent anti-*Candida* activity [34,59]. Their potency and spectrum was enhanced by chemical modification. Cancidas (Merck and Co.), derived from pneumocandin B₀, was the first antifungal drug of this chemical class approved for clinical use. It was followed by Micafungin from Fujisawa Co. A third drug derived from Echinocandin B is in development.

A report on strain improvement for higher production and for altered product spectrum was published by Masurekar et al. [34]. Although it produced a family of related compounds, the major compound produced by the wild-type strain of *Glarea lozoyensis* (formerly classified as *Zalerion arboricola*) was named pneumocandin A₀. The wild-type strain was mutagenized with NMU and NTG. From the survivors of NMU treatment, a superior producer of pneumocandin A₀, designated MF5415, which gave yields 2.5-fold higher than the parent, was isolated. At the same time, the survivors were also examined for the changes in the product spectrum. Four mutants were isolated, which produced

a different pneumocandin as a major product instead of A₀. The major products were pneumocandin A₂ in mutant ATCC 20958, pneumocandin A₄ in mutant ATCC 20988, and pneumocandin B₀ in mutants ATCC 20957 and ATCC 74030. The level of pneumocandin A₀ was lower in mutant ATCC 74030 than in strain ATCC 20957 [34].

Echinocandin B is produced by *A. nidulans* A42355. The titer of echinocandin B was increased considerably by strain improvement [60]. However, there is no further information on this effort. One of the metabolites produced by this culture is sterigmatocystin, which is an intermediate in the biosynthesis of aflatoxin B1, a very potent carcinogenic mycotoxin [61]. It was noted that the improved mutants produced more sterigmatocystin, along with the higher levels of echinocandin B. After the failure to isolate a sterigmatocystinless mutant directly following mutagenesis and screening of survivors, which might have been due to the diploid nature of the strain, a two-step approach was used. In the first stage, a haploid strain of *A. nidulans* that retained the ability to synthesize echinocandin B was isolated. For this purpose, the parent was exposed to a gradient of spindle fiber poison, methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate in a Petri dish. Because haploids are more resistant to this compound than the diploids, colonies were obtained from the leading edge of the growth on this plate. Analysis of several isolates showed that they were indeed haploid. These were then mutagenized with UV light and screened for the resistance to 5-fluoro-orotic acid and loss of sterigmatocystin production. The resistance to 5-fluoro-orotic acid was used to ascertain the haploid nature of the strain tested for the loss of sterigmatocystin production. From the 9000 isolates screened, six mutants were found that did not produce sterigmatocystin. However, the titers of echinocandin B in these mutants were lower than those in the parent [62].

2.4.5. Ergot Alkaloids

Ergot alkaloids are produced by the ergot fungus (*Claviceps* sp.). These are characterized by the presence of nitrogen in heterocyclic ring. They contain a typical tetracyclic ergoline ring system. They have wide-ranging physiological effects and have found use in the treatment of migraine headaches, Parkinson's disease, senile dementia, disorders caused by hyperprolactinemia, and to modulate uterine contractions [56]. More than 40 alkaloids have been isolated from ergot fungus, and hundreds of derivatives have been prepared chemically. In the United States, about 50 proprietary and generic drugs derived from ergot alkaloids are available [8].

The ergot fungi that produce the alkaloids include *Claviceps purpurea*, *C. fusiformis*, *C. gigantea*, *C. paspali*, *C. sulcata*, *Spacelia sorghi*, *Penicillium sizovae*, and *Aspergillus fumigatus* [8,62]. A number of problems were faced in the initial phases of development, including lack of strain availability, inability of the cultures to produce lysergic acid derivatives on substrates other than rye, and instability of the cultures [8]. These problems were solved by minimizing the number of strain transfers and recycling them on the natural host, rye. This led to the selection of *C. paspali* isolates that were stable, improved producers. The strains subjected to this treatment gave yields as high as 3.5 g/L [63].

Mutation and selection was also used to isolate superior producers. There are very few papers published on this topic. An early report by Kobel et al. described the use of UV light for the isolation of improved mutants of *C. purpurea* [64]. Later, they reported on the use of mutation to alter the product spectrum [65]. In subsequent studies, they observed 180-fold increase in ergocristine production over that produced by the parent after eight rounds of mutagenesis [66].

To increase the screening throughput, a technique based on the use of the Salkowski reaction to measure alkaloids produced by colonies was developed [67]. This approach eliminated the laborious and time-consuming liquid fermentation step. These workers described the use of NTG in the two-step titer improvement program. The fungus studied was *Claviceps sp.* strain SD58. They isolated two superior mutants after one round of mutagenesis. These produced around 1.3 g/L. They were subjected to second round of mutation, and mutants that gave 30% higher yield than the parent were isolated. They also isolated amino acid auxotrophs, which did not produce as well as the parent. These were treated with NTG and revertant to prototrophy isolated. These gave 60% higher titers as compared to the parent. This approach was also used to isolate improved producers of cephalosporin C, as described above [55].

Protoplast fusion was successfully used to increase alkaloid production in *C. purpurea*. The standard protocol for this procedure involves the use of marked strains, often auxotrophs. It was found that when auxotrophic parents were used, the fused protoplast generated were low producers. Conversely, when the prototrophic parents were used, the fused protoplasts gave 25% higher yields [68].

2.4.6. Griseofulvin

This compound is produced by many species of *Penicillium*. It was first isolated from *Penicillium griseofulvum* [69]. It is used as an antifungal agent. A Rather unusual mutagen was used to obtain high producers of this compound. The parent culture was grown and sporulated on synthetic medium containing S³⁵. The conidia were then plated for isolated colonies and tested for production. The best mutant produced 3 g/L, as compared to the yield of 1.6 g/L obtained with the parent [70]. Other mutagens used include N-nitrosomethylurea, pretreatment with N-nitroso-N-methylbiuret or N-nitrosomethylurea followed by exposure to UV light. The culture used was *P. nigricans*. Titer increases of 30% to 40% were observed [69].

2.4.7. Asperlicin

Asperlicin is produced by *Aspergillus alliaceus*. It is a potent, competitive, nonpeptide inhibitor of neurotransmitter cholecystokinin (CCK), which is a 33—amino acid peptide found in gastrointestinal tract and in the central nervous system [56]. CCK is major stimulator of gall bladder contractions and pancreatic exocrine secretion and a regulator of gut motility. It is also known to produce satiety and, therefore, may be involved in the physiological regulation of appetite. In light of this, it is thought that asperlicin has the potential to be used to treat gastrointestinal disorders where CCK is involved.

Its structure showed that tryptophan is one of the precursors. Therefore, attempts were made to isolate tryptophan overproducers. The mutagenized spores were plated on toxic analogues of tryptophan to select resistant mutants. The culture was resistant to 20 analogues tested. Next, they were subjected to the selection for resistance to phenylalanine analogues. The culture was resistant to 8 out of 10 analogues tested, but was sensitive to either m- or p-fluorophenylalanine. Mutant resistant to m-fluorophenylalanine was isolated and was found to be a 20% improved producer of asperlicin [71].

3. APPLICATION OF MOLECULAR BIOLOGICAL METHODS

These methods, referred to as “recombinant DNA (rDNA) technology” or by more glamorous term “genetic engineering,” have been used for some time to produce proteins or

peptides heterologously. Examples of these are human insulin and interferons, which are already available for clinical use [72]. A very limited range of well-characterized hosts were used for this purpose. *Escherichia coli* was used more often as a host, followed by *Saccharomyces cerevisiae* and *Bacillus subtilis*. The application of these methods to filamentous fungi lagged behind because of technical difficulties. In order to transform filamentous fungi, it was necessary to generate protoplasts. It was found that the protocols available did not work well for these cultures. Considerable effort was devoted to developing procedures to protoplast filamentous fungi efficiently [41,73]. Other methods used to introduce DNA include electroporation and use of intact cells exposed to alkali cation such as Li^+ . Efficient transformation of protoplasts required the addition of PEG or CaCl_2 . Electroporation, although used only with the protoplasts, did not require either of these compounds; however, the efficiency of transformation was lower. Although in some cases, good transformation efficiency was observed with the used cells exposed to lithium acetate, it was found to be dependent on the fungus used [74,75]. Furthermore, although there have been reports of autonomously replicating plasmids in filamentous fungi, this has not been conclusively proven. Therefore, vectors developed for *E. coli* were modified for use in filamentous fungi. Both circular and linear plasmids have been used [76]. The fate of the transformed DNA in fungi is different from that in bacteria, in that it is integrated into the fungal genome. The type of integration is homologous in yeast and nonhomologous in filamentous fungi [75]. In the latter, the ectopic integration occurs without any significant sequence homology between the plasmid DNA and the chromosomal DNA [77]. The first report of transformation with recombinant plasmid was in *Neurospora crassa* [78]. Subsequent progress in identification of markers for selection of transformant accelerated the progress in application of molecular biological techniques to filamentous fungi. One such marker, resistance to the antibiotic hygromycin, produced by *Streptomyces hygroscopicus*, is used frequently. The mechanism of resistance is inactivation of hygromycin by phosphorylation. The phosphotransferase is coded by the hph gene [79]. This gene has been used with a filamentous fungal promoter upstream for its efficient expression in the fungal host [76].

A new procedure, known as the *Agrobacterium tumefaciens*-mediated transformation system, was recently developed for transformation of fungi. The new method offers the advantages of simplicity and general applicability. It is discussed elsewhere in this volume.

3.1. Metabolic Engineering

Ever since the beginning of the penicillin era, significant effort has been directed at elucidating the biosynthesis of commercially important secondary metabolites [43,80–85]. One of the goals of this endeavor was to develop understanding of the biogenesis that can lead to a rational approach to strain improvement. Examples of this have been given earlier in this chapter [47]. However, the lack of methodologies to identify and access rate-limiting steps have restricted the application of such techniques. As the molecular biological methods became available for filamentous fungi, this approach was revisited. There are many definitions of the term “metabolic engineering.” In this section, it is used to describe identification of a rate-limiting step in the biosynthesis, isolation of the gene encoding the enzyme that catalyzes this step, and improving the expression of the gene to eliminate the limitation. Because there was considerable information available on the biosynthesis of penicillin/cephalosporin (Fig. 3), their biosynthetic pathway was chosen for the applica-

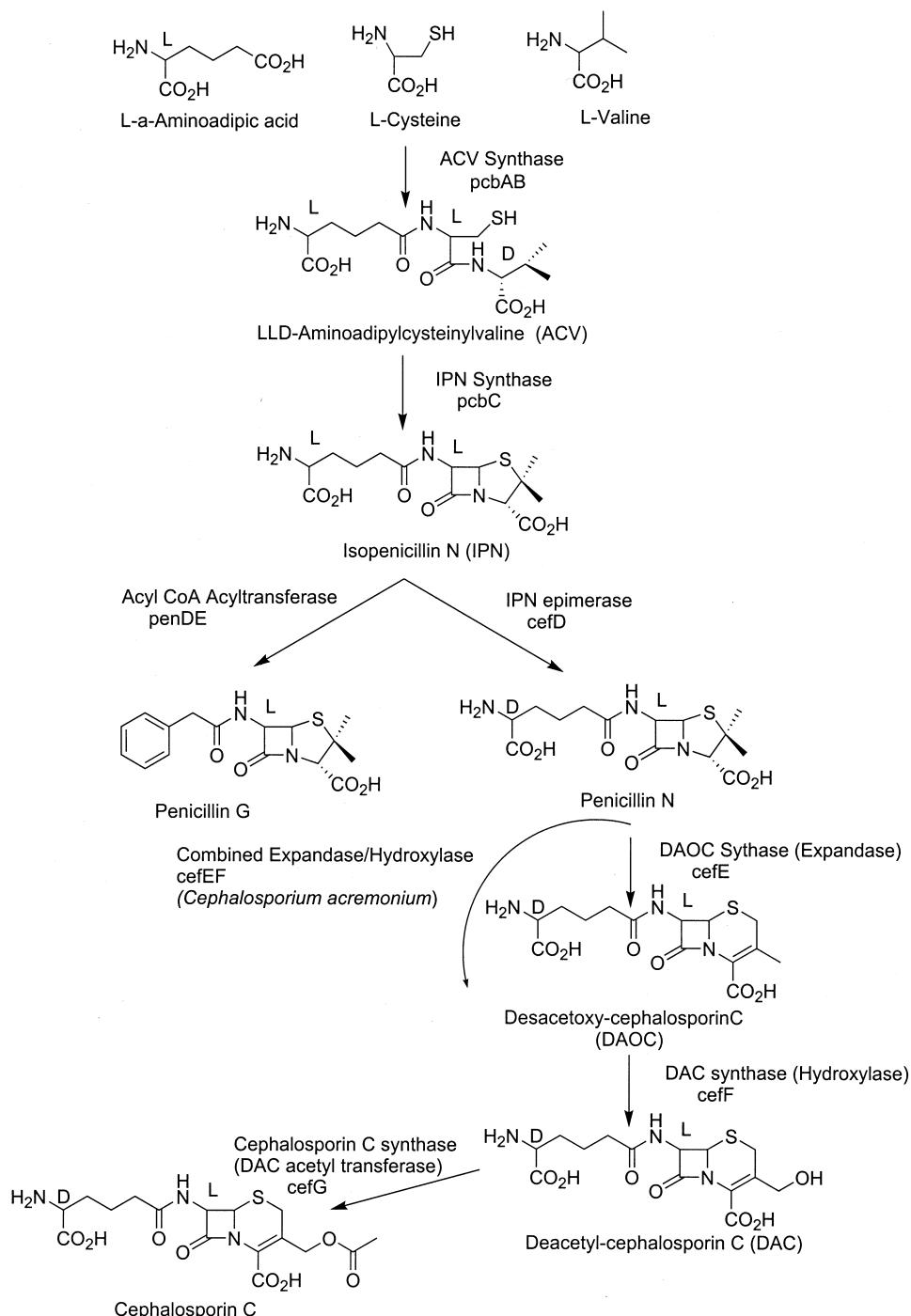


Figure 3 Pathway for the biosynthesis of cephalosporin C in *Cephalosporium acremonium*. In this fungus, expandase and hydroxylase are combined, whereas in *Streptomyces* and *Nocardia*, these are separate.

tion of the new technology and the biosynthetic genes were isolated and characterized [86]. It had been observed that during the cephalosporin fermentation, penicillin N was excreted in the broth [52,87]. The molar ratio between penicillin N and cephalosporin C in the industrial fermentation was found to be 0.3, which suggested that the conversion of penicillin N to deacetoxycephalosporin C (DAOC) was a rate-limiting step [87]. This step is catalyzed by DAOC synthase encoded by the gene *cefEF* in fungi [87]. To increase the copies of this gene, a strain of *Cephalosporium acremonium*, 394-4, which was used for the industrial production of cephalosporin C, was transformed with a plasmid containing *cefEF* and hygromycin B resistance genes. The transformants were selected for resistance to hygromycin B and tested for the production of cephalosporin C and penicillin N. A number of transformants were found to make more cephalosporin C and less penicillin N than the untransformed parent. One of them, strain LU4-79-6, produced 47% and 15% more cephalosporin C than the parent in the laboratory-scale fermentations and in the pilot plant fermenters, respectively. The Southern hybridization analysis of total DNA of the parent and of the transformant showed one additional copy of *cefEF* in the transformant. Similarly, the determination of DAOC synthase activity in the crude protein extract of the transformant showed 2-fold increase over the parent [87]. These observations showed that although the cephalosporin C production increased with the gene dosage, there was no one-to-one correlation between the number of gene copies, the DAOC synthase activity, and the yield. This suggested that another step in the biosynthesis had become rate limiting. A subsequent report on the cloning of *cefG* gene and its use in strain improvement, suggested that the last step in the biosynthesis of cephalosporin C, conversion of deacetyl cephalosporin C (DAC) to cephalosporin C, may be the rate-limiting step [50]. This reaction is catalyzed by cephalosporin C acetyltransferase, which is encoded by *cefG*. Wild-type *Cephalosporium acremonium* strain was transformed with plasmid containing *cefG* and phleomycin resistance genes, and phleomycin-resistant transformants were selected. Production of cephalosporin C by these was determined along with the number of copies of the gene *cefG*. Direct correlation between the two was noted [50]. The authors proposed that because the DNA construct used in the previous study contained in addition to the *cefEF* gene, *cefG* gene, the resultant increase in the production was not due to the increased levels of DAOC synthase but rather due to the increase in cephalosporin C acetyltransferase. Unfortunately, there are no data given on the level penicillin N to distinguish between the roles the two enzymes play in improved yields. Furthermore, the strains used were different and hence a direct comparison between the results of the two studies is not possible.

One of the difficulties in the application of metabolic engineering to strain improvement has been the determination of the rate-limiting step, as seen from the foregoing discussion. This can be done by measuring the metabolite flux through the pathway. Compounds labeled with ^{14}C or ^{13}C are used as a starting point, and the level of label present in the intermediates of the pathway is determined. It can be seen that this is a complex undertaking that requires substantial analytical resources. Furthermore, interpretation of the data obtained is not simple; this problem has limited the use of this approach.

3.2. Proprietary approaches

Recently, two biotechnology companies have reported development of proprietary technologies for strain improvement based on the molecular biological methods. These do not

require a priori knowledge of biosynthetic pathways or the rate-limiting steps. A brief description of these is given below.

3.2.1. Precision Engineering

Precision Engineering technology was developed by Microbia, which is located in Cambridge, Massachusetts. It is based on combination of metabolic and transcriptional profiling to identify the genes associated with desired product formation, use of global regulators, and high throughput genetically selective screening. The transcriptional profiling is done with microarrays. They have a library of more than 400 regulators associated with a variety of physiological regulatory pathways from diverse microorganisms [88]. They believe that this approach allows them to address the specific regulatory mechanisms directly and results in rapid strain development [www.microbia.com]. Their technology was developed for application to fungal strain improvement.

3.3.2. Molecular Breeding

The Molecular Breeding approach was developed by Maxygen and is licensed to Codexis Inc. (Redwood City, California). It involves gene shuffling or genome shuffling. The former is useful to improve the properties of a single enzyme, and the latter for increasing the yield of a microbial product. The genome shuffling combines multiparental crossing allowed by DNA shuffling with the recombination of entire genomes. In such a system, when a number of phenotypically selected strains are used, many of the progeny show improvement in that particular phenotype. To begin this process, the wild-type or the starting strain is mutagenized to generate clones with improved phenotype (i.e., higher titer than the parent). Few of these are combined, protoplasted, and fused, thus resulting in multiparent crosses. A number of progeny from this process are subjected to high-throughput screening to select further improved clones, and these are subjected to the multiparent crosses in the same manner as described before [89]. It was reported that when this technique was applied to *Streptomyces fradiae*, which produces the antibiotic tylosin, in two rounds of genome shuffling the yield was increased 6- to 8-fold [90]. It is important to note that the improved producers isolated by this method in 1 year were as good as or better than those generated by the classical strain improvement program in 20 years. Clearly, this approach shows great promise. However, there are no reports of application of genome shuffling to improve yields of fungal products.

4. FUTURE PROSPECTS

Fungal secondary metabolites have contributed significantly to humankind's fight against various diseases and disorders and will continue to do so in the future. Consequently, the economic value of these products is in billions of dollars annually and will remain so for a long time. Therefore, for cost-effective production of these metabolites, strain improvement will remain a major endeavor in the pharmaceutical industry. In the more than 60 years since the first commercial production of penicillins, mutagenesis and screening have remained the main approach for this purpose. This method has been incredibly successful in making the economics of producing these drugs attractive. However, the method is highly labor intensive and time consuming. To address these shortcomings, miniaturization of fermentations and application of automation, resulting in high-throughput screening methodologies, is being used. Furthermore, as our knowledge base about the biosynthetic pathways and their regulation expands, more selective methods will become available for

rapid and less labor-intensive strain improvement. Similarly, advances in the development of molecular biological methodologies and in bioinformatics and their application to strain improvement holds a great promise. It is clear that the fungal secondary metabolites will continue to play an important role in keeping mankind healthy for many years to come in a cost-effective manner.

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21

Fungal Bioconversions: Applications to the Manufacture of Pharmaceuticals

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1. INTRODUCTION

Recent investigations have clearly established that enantiomers of racemic pharmaceutical drug mixtures can present different pharmacokinetic properties. These can be attributed to the capacity of the desired enantiomer to exhibit higher binding affinity with its intended receptor or enzyme, to display a better bioavailability, or to present a more advantageous side-effect profile. A well-known example is ibuprofen, which has been manufactured as a racemate for several decades. Post-market-introduction studies, which were permitted by improved analytical technology, revealed that the (S) enantiomer was the active form of the drug [1]. Similarly, the bronchodilatory effects of the antiasthma agent albuterol were found to be associated with its (R) enantiomer, whereas (S) albuterol generally provides no therapeutic benefit [2]. Because of these vast differences in the therapeutic potential of enantiomers, it is not surprising that pharmaceutical development increasingly targets single enantiomer products. Consequently, the manufacture of only the active enantiomeric form of a drug has become a norm in the industry [3–6]. Most chiral syntheses are traditionally achieved through chemical methods employing an ever-expanding range of chiral catalysts and reagents [5]. Despite chirotechnology's swift technical advances, some chiral syntheses, especially those requiring both regioselectivity and stereoselectivity, remain difficult and can be extremely costly. This has substantial economic implications. In 2000, for instance, about 40% of all marketed pharmaceuticals were single enantiomers and accounted for about \$130 billions in sales, representing a nearly 100% increase since 1996 [6,7].

In addition to the extensive range of biochemical reactions that enzymes catalyze, most enzymes display regioselectivity and stereoselectivity with respect to their substrate. Considering the use of enzymes as catalysts for chemical syntheses has long been a logical extension of their activity realm. Microbial cells or their enzymes that are easily produced by fermentation are currently the most convenient, abundant, and economic source of biocatalysts. Regioselectivity and stereoselective biocatalysis, employing either whole microorganisms or isolated enzymes, are increasingly emerging as viable alternatives to difficult chemical syntheses that the pharmaceutical industry often faces [8–14]. In addition to solving some of chemistry's most difficult challenges, biocatalysis employs a renewable and natural source of reagents and offers the advantages of reducing or completely avoiding the use of solvents while operating at ambient temperature. This explains why biocatalysis has been recently named “green chemistry” [15]. The growing interest in the field of bioconversion is reflected by the recent publication of several textbooks and review articles that extensively cover its applications to the biosynthesis of pharmaceuticals and fine chemicals [11,16–34]. To provide some enzymatic and chemical background, Table 1 is an overview of the international enzyme classification system (EC) system. Tables 2 and 3 clarify the terms most commonly associated with enzyme specificity and stereoselectivity that are used in this chapter.

The pharmaceutical industry has always been keen on capitalizing on the abundance of microbial biochemical diversity as exemplified by the vast number of pharmaceuticals derived from microorganisms. Because of their extended biodiversity and metabolic richness, filamentous fungi are an important biochemical resource, and their capacity to mediate steps in organic syntheses has long been recognized. Consequently, filamentous fungi occupy a major place in all well-stocked microbial bioconversion collections. The first fungal bioconversion of steroids was reported more than a half century ago, with the regioselective and stereoselective hydroxylation of progesterone to 11-alpha-hydroxyprogesterone [35]. This breakthrough greatly simplified the synthesis of corticosteroids by considerably reducing the number of synthetic steps, which in turn lowered the production

Table 1 Enzyme Classification According to the Enzyme Class System

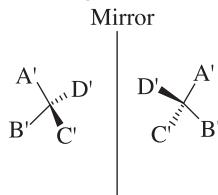
Enzyme Class	Reaction
Oxidoreductases	Oxidation reductions Oxygenation of C—H, C—C, and C=C bonds.
Transferases	Transfer of various groups such as acyl, sugars, methyl, etc.
Hydrolases	Hydrolysis of various bonds resulting in the formation of amides, esters, acids, etc.
Lyases	Additions to double bonds such as C=C, C=O, C=N, etc.
Isomerases	Racemization of cis-trans, bond migration, epimerizations
Ligases	Formation of C—C, C—N, C—O bonds

Detailed information on enzyme classification can be found at the following web site: http://www.genome.ad.jp/db/get-bin/get_htext?Ec table

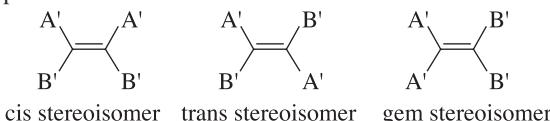
Table 2 Definitions of Some Commonly Used Bioconversion Terms

Chiral: Displaying handedness; mirror image is not superimposable

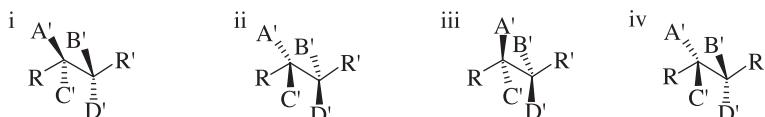
Enantiomers: Compounds that are mirror images of each other.



Stereoisomers: Compounds with identical formulas but dissimilar three-dimensional geometry.



Diastereomers: Relationship of some compounds with two or more chiral centers; stereoisomers that are not enantiomers. 2^n stereoisomers are possible where n is the number of chiral centers. In the example below, i and ii are enantiomers, whereas i and iii as well as ii and iv are diastereomers that share at least one chiral center



Enantiomeric excess: Quantitative relationship describing optical purity:

$$([{\text{major enantiomer}}] - [{\text{minor enantiomer}}])/([{\text{major enantiomer}}] + [{\text{minor enantiomer}}]) \times 100$$

Diastereomeric excess: Quantitative relationship describing ratio of related diastereomers in a sample:

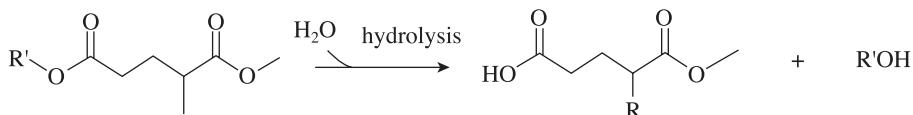
$$([{\text{major diastereomer}}] - [{\text{minor diastereomer}}])/([{\text{major diastereomer}}] + [{\text{minor diastereomer}}]) \times 100$$

cost by a factor of 20-fold which in turn resulted in a 95% reduction in production cost. Since this accomplishment, interest in the utility of this group of microorganisms has grown steadily. Bioconversions of many important classes of pharmaceuticals—such as antibiotics [36,37], steroids [38–40], alkaloids [41], and synthetic bio-active molecules [11,32]—by filamentous fungi or their isolated enzymes have been investigated extensively by the pharmaceutical industry. In addition to their extended range of potential targets, filamentous fungi present a diverse repertoire of enzymatic catalysis that allows them to perform a wide variety of chemical reactions. Tables 4 and 5 list generic reactions performed by filamentous fungi or their isolated enzymes. These are discussed later in this chapter in detail, including their applications to the synthesis of important pharmaceuticals. It should be noted that while there are currently few active manufacturing processes for pharmaceutical intermediate production, the utility of such biotransformations to pre-clinical compound development is very important. For example, fungal-mediated bioconversions can support the temporary production of a critical compound until an organic synthesis solution is finalized.

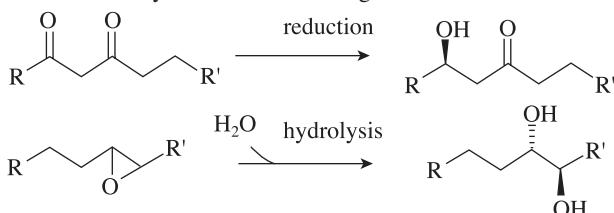
In addition to direct applications to the synthesis of pharmaceutical drugs, fungal bioconversions are useful in a variety of other contexts for the industry. These additional applications, a short list of which is provided in Table 6, highlight the ability of bioconver-

Table 3 Properties of Enzymatic Reactions

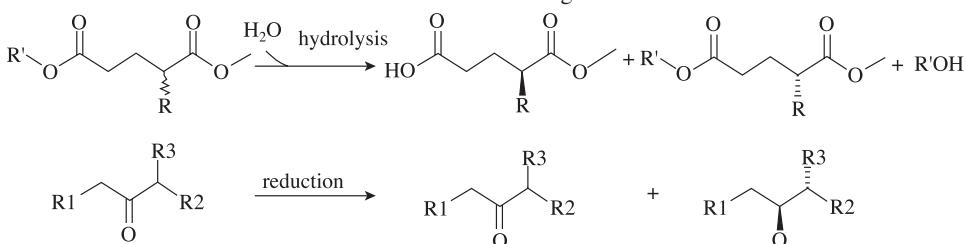
Regioselectivity: preferential attack of a functional group relative to an identical functionality



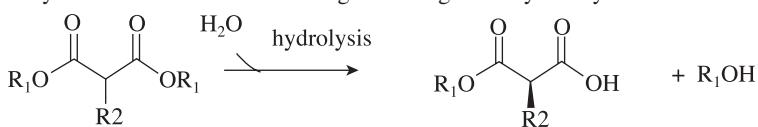
Stereoselectivity: Production of a single enantiomer from directional incorporation of functionality.



Resolution: Enantioselective chemical modification of single enantiomer of a racemic mixture.



Desymmetrization: Chemical change inducing chirality in a symmetrical molecule.



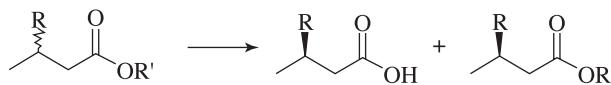
sions to affect multiple facets of pharmaceutical research spanning discovery, preclinical studies, and manufacturing.

As the complexity of pharmaceutical development compounds increases, opportunities to use filamentous fungi or fungal-derived enzymes should grow significantly. To maximize the potential for success, it is imperative to understand the biocatalytic potential of these microorganisms, to develop a cohesive strategy for organizing a collection of relevant fungi, and to plan for rapidly screening for the desired activities.

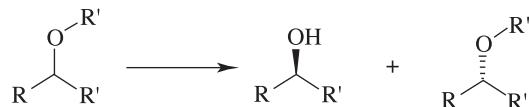
This chapter presents relevant examples of bioconversions mediated by filamentous fungi. Although their applications to the manufacture of pharmaceuticals are still modest, fungal bioconversions have played key roles in the synthesis of many experimental drug candidates. Consequently, the examples reviewed here will encompass both types of application. The later sections of this chapter focus on integrating filamentous fungi into screen-

Table 4 Diversity of Biotransformations Catalyzed by Free Enzymes of Fungi

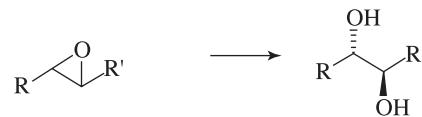
1 Ester resolution
(lipase, protease)



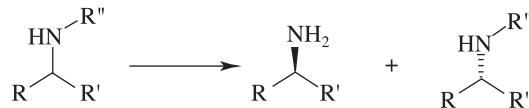
2 Epoxide hydrolysis
(epoxide hydrolase)



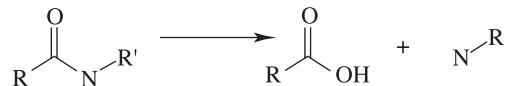
3 Alcohol resolution
(lipase, protease)



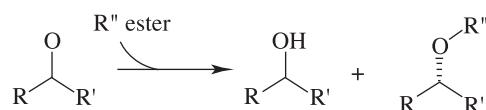
4 Amine resolution
(lipase, protease)



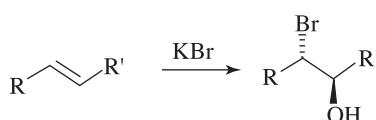
5 Amide hydrolysis
(lipase, protease)



6 Acylation
(lipase in organic solvent)



7 Halohydrin formation
(haloperoxidase)



8 Epoxidation
(haloperoxidase)

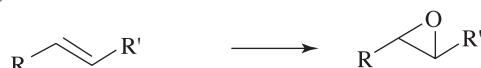


Table 5 Diversity of Cofactor-Dependent Reactions Catalyzed by Fungal Cells

1 Ketone reduction
(dehydrogenase)



2 Epoxidation
(cytochrome p450, haloperoxidase)



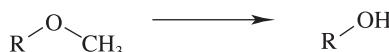
3 Hydroxylation
(cytochrome P450)



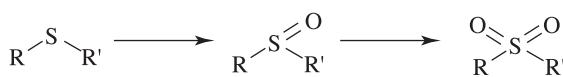
4 Baeyer-Villiger
(monooxygenase)



5 O-Demethylation
(cytochrome P450)



6 Sulfoxidation
(cytochrome P450)

**Table 6** Potential Applications of Fungal Biotransformations for the Pharmaceutical Industry

Model xenobiotic metabolism

Identify potential mammalian drug metabolism intermediates via liver enzyme mimicry. Provide small amounts of compound for metabolism inquiries.

Semisynthetic natural product

Modification of active natural product to facilitate medicinal chemistry can extend knowledge of structure activity relationship.

Green chemistry

Can lead to the potential elimination of solvent-intensive or hazardous reagent from synthetic route.
Lower overall environmental burden.

Remediation of pharmaceutical byproducts

Detoxify waste streams and reduce byproduct handling and treatment costs.

Basic research

Increase compound diversity by providing novel chemical entities. Participate in novel additions to chiral pool, facilitate route development, accelerate development timeline.

Manufacturing

Allow large-scale production of pharmaceutical intermediates.

ing programs aimed at identifying catalysts useful in pharmaceutical syntheses. Lastly, we attempt to provide practical guidance on the effective design and implementation of bioconversion screens.

2. APPLICATIONS OF FUNGAL BIOCONVERSIONS TO THE MANUFACTURE OF PHARMACEUTICALS

2.1. Review of Chemistries Mediated by Filamentous Fungi

Filamentous fungi and their enzymes are capable of catalyzing diverse chemical reactions of importance to the pharmaceutical industry. Tables 4 and 5 summarize the series of generic reactions that fungi can mediate. One should note that the R and R¹ groups in several instances (e.g., carbonyl reduction, epoxidation, epoxide hydrolysis, halohydrin formation, Baeyer-Villiger, and sulfoxidation) can be associated with the same cycloalkane or aryl ring systems. Among the enzyme classes listed in Table 1, oxidoreductases and hydrolases have been most widely employed in biocatalysis. This is because these enzymes accept a wide range of nonnatural substrates and are simple to use. While enzymatic biocatalysis of carbon–carbon bonds formation are not typically observed, the capacity to generate highly enantioenriched intermediates solidifies fungi bioconversions as an important element of synthetic chemistry.

In addition to bioconversion monographs [42], the following specialized reviews provide in-depth coverage of many specific classes of bioconversions, such as epoxidations [43–46], hydroxylations [47–50], Baeyer-Villiger [51], sulfoxidations [52], asymmetric reductions [53–55], haloperoxidations [44,45,56–58], uses of proteases and lipases [31,59], and epoxide hydrolases [46,60–65].

2.2. Comparison of Isolated Enzyme to Whole-Cell Biocatalysis

The first critical decision in exploiting the catalytic repertoire of filamentous fungi is whether to access the desired activity as an isolated enzyme or to use intact cells. The decision most often is driven by the chemistry targeted, availability and stability of catalysts known or suspected to mediate the reaction, practicality, and economics.

For hydrolytic reactions, employing proteases and lipases for instance, isolated enzymes are almost universally preferred to whole cells. Advantages include extended stability and ease of procurement, as many are commercially available. In addition, employing a purified enzyme in a bioconversion reaction can prevent unwanted side or competing reactions that may result from the presence of other enzymes when using whole cells. Some of the most commonly employed commercially available fungal enzymes are listed in Table 7. Although, the commercial availability of fungal hydrolases and similar enzymes from other microbes can obviate some up-front experimental work, it is still advantageous to complement these with other enzymes that can be easily produced in the laboratory. Most filamentous fungi secrete extracellular proteases and lipases, which can be readily accessed by screening culture supernatants. Many specific preparation procedures for secreted fungal enzymes are available from the literature. For example, a very useful source of practical information, which details all the laboratory scale production steps for several hydrolytic enzymes of fungal origin, can be found in Halpern's manual [66]. An added benefit of in-house enzyme production is that further enzyme purification and characterization may lead to cloning of its encoding DNA. Access to a cloned gene product presents a development program with the opportunity to improve catalyst performance through

Table 7 Commercially Available Fungal Enzymes

Enzyme Type/Source	Manufacturer
Lipases	
<i>Aspergillus niger</i>	Amano, Fluka, Novozymes
<i>Mucor javanicus</i>	Amano
<i>Mucor miehei</i>	Amano, Boehringer, Fluka, Novozymes
<i>Penicillium camemberti</i>	Amano
<i>Penicillium roqueforti</i>	Amano
<i>Rhizopus arrhizus</i>	Boehringer, Fluka
<i>Rhizopus delemar</i>	Amano, Fluka
<i>Rhizopus japonicus</i>	Amano, Fluka
Proteases	
<i>Aspergillus melleus</i>	Sigma
<i>Aspergillus oryzae</i>	Amano
<i>Aspergillus saitoi</i>	Sigma, ICN
<i>Mucor miehei</i>	Fluka
<i>Penicillium</i> spp.	Amano
<i>Rhizopus</i> spp.	Sigma
<i>Tritirachium album</i>	Sigma
Chloroperoxidase	
<i>Leptoxphium fumago</i>	Sigma

directed evolution or gene shuffling strategies [67–69]. The ability to produce an enantioselective enzyme in-house also can be an important economic driver for process specification.

Cell extracts can yield useful preparations of nonsecreted enzymes such as epoxide hydrolases. Once formulated, either as dry powders or mixed with cryopreservatives, these enzymes retain their activity for extended periods of time [70]. The approach of integrating enzymes from noncommercial sources presents the added benefit of accessing greater biodiversity since the screening can encompass microbes from various sources that have not been tapped for commercial enzyme production.

Isolated enzymes also can catalyze redox biotransformations with the caveat that this requires the addition of costly nicotinamide cofactors. Both glucose and formate dehydrogenases have been successfully employed to recycle NADPH or NADH, respectively (Fig. 1). Although cofactor recycling is possible, this technology still presents some economic and technical difficulties of implementation as it requires a second enzyme. In addition, fungal ketoreductases are not readily available from commercial suppliers; therefore, their use as isolated enzymes is further complicated by the need to purify the enzymes. Cell-free oxidoreductase enzymes do not necessarily need to be purified to homogeneity. Nonetheless, the purity required is dictated by the reaction profile, and even partial purification presents challenges. These enzymes may exhibit elevated instability when removed from the cellular environment. Similarly, since the recycling of cofactors is also required, the use of isolated enzyme for hydroxylation reactions triggers the same cofactor recycling challenges (Fig. 2). Partly due to these complications, hydroxylation and redox chemistries are typically implemented via whole-cell conversions because reduced nicotinamide cofac-

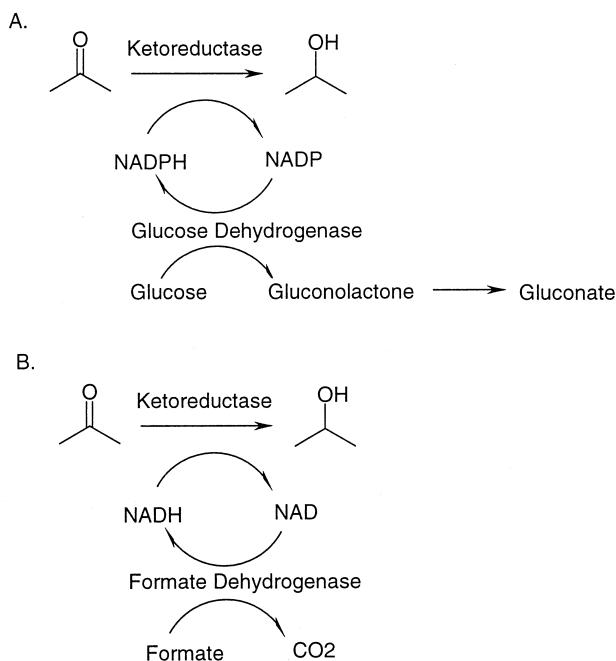


Figure 1 Nicotinamide cofactor recycling for reductions of ketones to chiral alcohols

tor pools for cytochrome P450 and dehydrogenase-mediated conversions can be easily regenerated through cellular metabolism with the addition of an inexpensive carbon source such as glucose.

Although the decision to pursue a whole-cell or enzyme-based redox route is typically driven by convenience and economics as mentioned, the latter approach can be

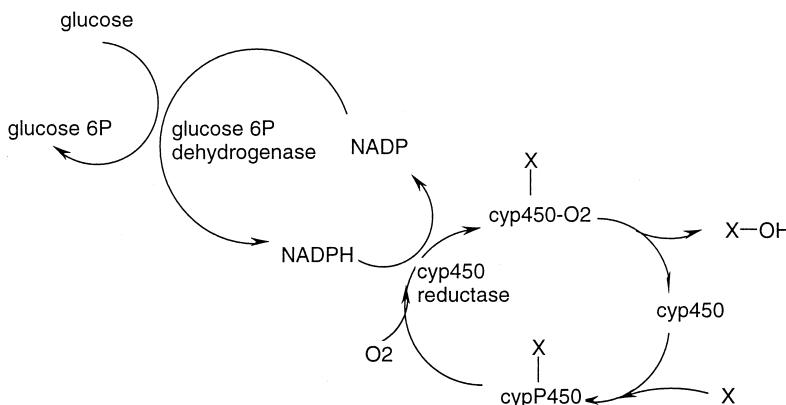


Figure 2 Recycling system for NADPH-dependent cytochrome P450-mediated hydroxylation

considered when a particularly toxic bioconversion substrate or product is encountered. For example, substrate or product toxicity may lead to failure of the whole-cell system to adequately supply reducing equivalents. Under these circumstances, one solution is to isolate the enzyme mediating the conversion and shift to a strategy in which the cofactor is regenerated through a coupled enzyme system as described earlier. Alternatively, the gene for the enzyme might be cloned into an expression host displaying more resistance to the substrate or product. Expression systems for fungal genes, such as the recently developed *Aspergillus* platforms, may increase access to these fungal enzymes [71,72].

Table 8 summarizes the key factors that influence the decision in selecting and developing an isolated enzyme or a whole cell-based biocatalysis process.

2.3. Generic Examples of Most Commonly Performed Isolated Enzyme-Based Biocatalysis

Mostly hydrolytic enzymes, EC class 3, have been used in their isolated or semipurified form for biocatalytic purposes. The most popular enzymes include lipases, proteases, amidases, and epoxide hydrolases. The most common applications are those of selective hydrolysis for the purpose of resolution and desymmetrization (Tables 3 to 5).

Enzymatic ester resolutions are usually cost effective and present a more scaleable alternative than chromatographic separation of the enantiomers. Emerging strategies to integrate metal catalysts for racemization of the undesired enantiomer may enable dynamic kinetic resolutions that overcome the 50% yield barrier [73]. Such methods may increase the pace of developing hydrolases in general and invigorate discovery efforts aimed at fungal-derived enzymes. In contrast to resolutions, which can afford a maximum theoret-

Table 8 Comparison of Isolated Fungal Enzymes vs. Whole-Cell Biocatalysts

Biocatalyst form	Pros	Cons
Isolated enzymes	<p>Procured easily (if commercially available)</p> <p>Processes are simple to implement and control</p> <p>Reactions can be performed in buffers and simplify purification</p> <p>Require only limited laboratory equipment</p>	<p>Some may require the use of cofactors</p> <p>Can be costly</p> <p>Limited choice as only a few are produced at the commercial scale</p>
Whole cells	<p>Almost unlimited choice of potential microorganisms</p> <p>Built-in cofactor recycling</p> <p>Usually yield robust processes</p>	<p>Require cultivation equipment (flasks and more likely fermentors)</p> <p>Potentially yield side reactions, resulting in byproduct formation</p> <p>May require a complicated purification scheme, involving cell removal and product extraction</p>

cal yield of only 50% for the production of any of the two possible enantiomers, enzymatic desymmetrizations of diesters present the potential to reach theoretical yields of 100%. Other hydrolytic resolutions, performed by isolated enzymes, include those performed by epoxide hydrolases yielding chiral diols, which constitute an important pool of chiral synthons in the syntheses of pharmaceuticals. Selective amide hydrolyses, employing isolated amidases, present definite advantages over chemical hydrolyses, which can not easily distinguish among several amide bonds in a large molecule such as an antibiotic.

In addition to their “natural” role, hydrolases can be used in the catalysis of bond formation. This is accomplished by performing the reaction in an organic solvent [74]. The absence of water results in a shift in the thermodynamic equilibrium, thus allowing the reaction to proceed in the direction of synthesis (Table 4).

In addition to hydrolytic enzymes, fungal haloperoxidases have been used as isolated enzymes (Table 4). Although there is only one, obtained from *Leptoxphium fumago* (formerly *Caldariomyces fumago*) and which is commercially available, they are easily produced by cultivation of many other fungal species [75]. As depicted in Table 4, their action on a carbon–carbon double bond yields chiral halo hydrins or epoxides. Both are very useful chiral synthons [58].

2.4. Generic Examples of Most Commonly Performed Whole-Cell-Based Biocatalysis

The most common reactions mediated by whole cells are oxidoreductions (Table 5). They include the reductions of carbonyls, epoxidations, hydroxylations, Baeyer–Villiger, O-demethylations, and sulfoxidations. The two most popular reactions are by far those of hydroxylations and carbonyl reductions.

Fungal P450s are key to many reactions that lead to the introduction of an oxygen atom. The introduction of a hydroxy group at a specific position in a regiospecific and stereospecific fashion can be a formidable chemical task when dealing with a large molecule. These chemistries are of prime importance as they lead to subsequent chemical steps at the activated carbon via hydroxylation. Fungal enzymes have proved to be very valuable catalysts for this class of chemical reactions. In addition, the formation of chiral epoxides by fungal P450 has also proven to be of great importance. Their ability to mediate oxidation of unactivated carbon–carbon bonds can provide chiral intermediates on synthetically valuable aromatic hydrocarbons such as naphthalene and indene [76–78]. P450 enzymes are very versatile as they also can introduce oxygen into ring systems via a Baeyer–Villiger type of reaction, generate sulfoxides and mediate O-demethylation reactions (Table 5). The physiological relevance of P540 enzymes is the introduction of oxygen that allows the subsequent utilization of aromatics as carbon sources by the microorganisms.

The second group of very useful filamentous fungal oxidoreductases stereoselectively reduces carbonyls to alcohols. To date, these fungal enzymes have supported the synthesis of many important pharmaceutical synthons because chiral alcohols are key to many syntheses. Although these enzymes have been recently well characterized for the yeast *Saccharomyces cerevisiae* [79], the filamentous fungal enzymes remain uncharacterized. It is safe to speculate, however, that as for *S. cerevisiae*, they are either alcohol dehydrogenases or various reductases involved in the central metabolism of the microorganism [79].

To appreciate the utility of these organisms to pharmaceutical intermediate syntheses, a series of examples is highlighted in the following section.

2.5. Examples of Fungal Biocatalysis Applied to the Manufacture of Pharmaceuticals

The use of fungal bioconversions in the pharmaceutical industry effectively demonstrates the biocatalytic repertoire of these organisms. In Figs. 3 to 5, various pharmaceutical examples have been compiled and sorted into broader areas of chemistry.

2.5.1. Asymmetric Bioreductions

As previously mentioned, asymmetric bioreduction to chiral alcohols is an extensively practiced bioconversion. Various applied research groups in the pharmaceutical industry have effectively used filamentous fungi as a source of catalysts for asymmetric bioreductions. In reaction **A**, for example, *Mortierella ramanniana* effectively reduced a prochiral ketone to its corresponding (R) chiral alcohol. This step provided the single chiral center of an antipsychotic drug substance with reaction yields of greater than 98% and enantiomeric excess (EE) of greater than 99% [80]. NADH cofactor recycling was achieved by a glucose feeding strategy. The cell mass could be used either immediately postfermentation or after a harvest step.

Utilizing another *Mortierella* sp., the beta ketoester in reaction **B**, an intermediate in the synthesis of a broad-spectrum carbapenem antibiotic was effectively converted to its corresponding (R) beta hydroxyester achieving a diastereomeric excess greater than 98% EE [81]. Hydroxyester titers in excess of 0.6 g/L were realized employing various carbon sources, and both diastereomeric excess and titer were essentially unaffected by the carbon source used for cofactor regeneration. In some instances, carbon source can impact the bioconversion stage. For example, glucose repression of the desired biocatalyst may adversely affect reaction metrics. The process was able to produce 0.55 g/L (R)-hydroxyester in 250 hours when employing laboratory scale bioreactors (23-L scale). Interestingly, a diverse set of yeast strains that had previously performed numerous asymmetric bioreductions was unable to catalyze this specific bioreduction. As discussed in the following section, this observation highlights the importance of screening filamentous fungi as an alternate to the more widely employed whole-cell yeast catalysts.

Sterically hindered diaryl ketones, as shown in reactions **C** and **D**, often present a difficult synthetic challenge [54,82]. *Rhizopus*, *Aspergillus*, and *Mucor* spp. harbor reductases capable of reducing these compounds to predominantly (S) or (R) alcohols. Such intermediates are useful in the synthesis of phosphodiesterase IV (PDEIV) inhibitors that can be used to treat asthma. A general difficulty in whole-cell reductions can be insolubility of the prochiral ketone, particularly with compounds that have multiple aryl functionalities, a common feature of selective PDEIV inhibitors. Approaches to meet this challenge include medium engineering for the bioconversion stage, including addition of cosolvents and surfactants. *Mucor* and *Rhizopus* spp. achieved substantially higher yields when diaryl ketone was solubilized in 20% Tween 80 acetone/solution [82].

Fungal reductions of sulfur-containing compounds have also been reported including reactions **E** and **F**, which are intermediates in the synthesis of Sotalol®, a beta receptor agonist used to treat ventricular tachiarhythmias, and of Trusopt®, a carbonic anhydrase inhibitor antiglaucoma agent, respectively [83,84]. The latter is a second example in which fungal-mediated reduction provides an alcohol in high diastereomeric excess as the desired *trans* (4S,6S) hydroxysulfone was obtained with diastereomeric excess of more than 98%. This example also highlights the capacity of fungal catalysts to produce chiral alcohols when the prochiral ketone is part of a ring system.

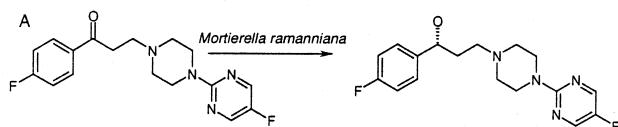
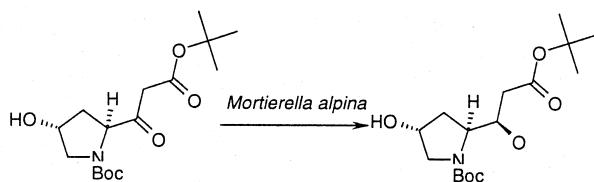
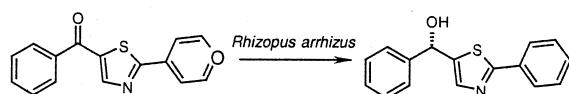
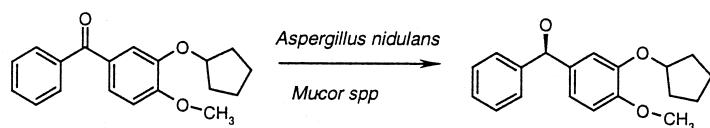
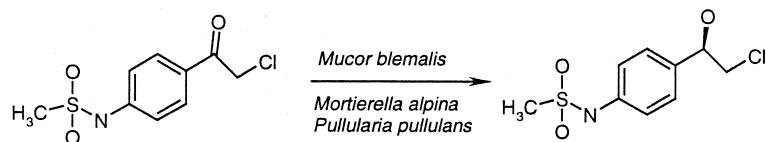
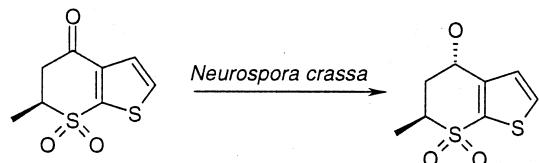
A**B****C****D****E****F**

Figure 3 Pharmaceutical industry examples of fungal asymmetric bioreductions

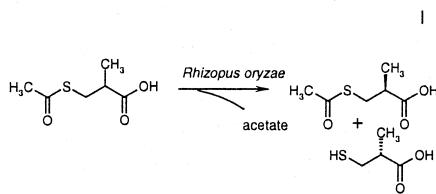
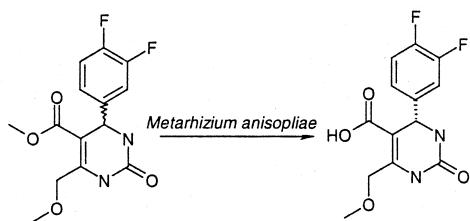
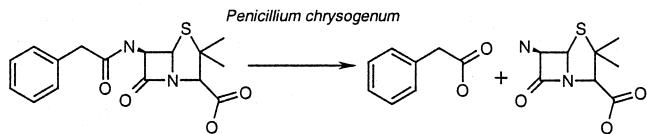
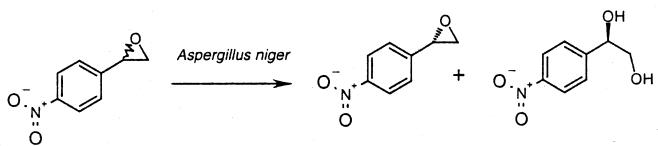
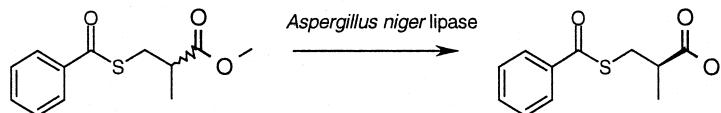
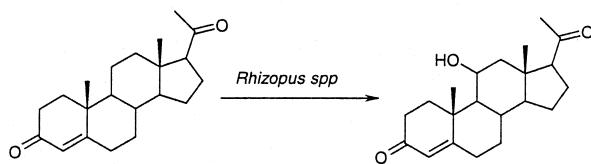
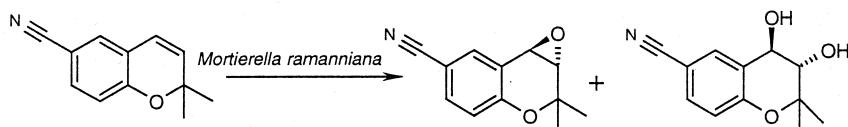
G**H****I****J****K**

Figure 4 Pharmaceutical examples of regioselective or stereoselective hydrolyses mediated by fungi or fungal derived enzymes

L



M



N

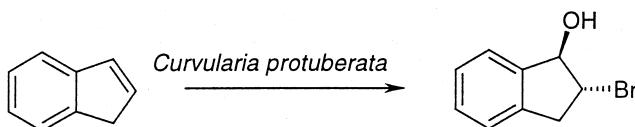


Figure 5 Pharmaceutical industry examples of fungal hydroxylations

The list of ketone reductions presented in Fig. 3 shows that filamentous fungi can often complement whole-cell bioreduction libraries which, by tradition, have been populated predominantly by yeasts.

2.5.2. Regioselective or Stereoselective Hydrolyses

Hydrolase-mediated chemistry is of prime importance for selective deprotections in the context of similar functional groups or the conversion of one of a set of enantiomers. The latter often is used as a means to upgrade optical purity through the conversion of the undesired enantiomer to an easily separable derivative (e.g., acid or ester). Examples of both strategies are detailed in Fig. 4.

It is not surprising that filamentous fungi harbor numerous hydrolases with biocatalytic utility and that these hydrolases can be integrated into organic synthesis schemes. Heat-dried *Rhizopus oryzae* cells have been used as a source of a lipase which enantioselectively hydrolyzes a thioester linkage of an intermediate in the synthesis of the antihypertensive agent Catopril® [85]. Resolution of the synthetically useful (S) enantiomer is depicted in reaction G, in which an EE greater than 95% is achieved as the undesired thioester is converted to mercaptoacid and acetate.

Proteases also are capable of similar enantioselective hydrolyses. For the synthesis of an alpha-1a adrenergic receptor antagonist, an experimental drug in the treatment of prostate enlargement, a protease from the entomopathogenic fungus *Metarhizium anisopliae* has been shown to be effective in resolving a DHP methyl ester (reaction H). This reaction is both regioselective and enantioselective [86,87]. In this instance, identification of the *Metarhizium* catalyst offered a viable and competitive alternative to a commercial catalyst.

The reaction depicted in **I** is useful for the synthesis of the 6-aminopenicillanic acid (6-APA). This convenient deacylation reaction represents a means to produce 6-APA from a benzylpenicillin precursor, a key precursor to semisynthetic penicillins [88]. Although the commercial process does not presently employ a fungal enzyme, this biotransformation, along with the later-described progesterone hydroxylation at the 11 position, represent the two most commercial successes of biotransformations in the pharmaceutical industry. Deacylation proceeds similarly to peptide hydrolysis and has also been used for regioselective synthetic problems [9].

Several resolutions mediated by *Aspergillus niger* cells or its isolated lipase(s), further delineate the spectrum of possible hydrolase chemistry. In reaction **J**, the indicated epoxide can be selectively opened to the desired diol intermediate required for synthesis of the beta adrenergic-receptor agonist (R) Nifenadol® which is used to treat asthma, bronchitis, and congestive heart failure [89]. The reaction yield was improved by coupling chemocatalysis, since acid treatment of the derived epoxide resulted in formation of additional diol. Although step yield was high (ca. 94%), the overall EE of 80% required an upgrade in subsequent chemical steps which achieved the production of (R) Nifenadol® with an overall yield of 58% and an optical purity of about 99% EE.

A lipase from *A. niger* was used to resolve a racemic methyl ester (reaction **K**) accessing the appropriate chiral configuration of Catopril® by an alternative means to that described in reaction **G** [90]. The resolution achieved 32% yield of a theoretical 50% maximum and produced the appropriate (R) acid with an EE greater than 98%.

2.5.3. Hydroxylations

The introduction of oxygen into ring systems by filamentous fungi offers an attractive route to certain alcohols and diols. Fig. 5 outlines a few pharmaceutically relevant examples.

Steroid compounds can be hydroxylated to alcohols, for example **L**, where progesterone is hydroxylated to 11-alpha-hydroxyprogesterone by *Rhizopus nigricans* [35]. The transformation produces the desired endproduct in high yield and is considered the single best example of fungal bioconversions to the manufacturing of pharmaceuticals. For many years, this fungal-mediated 11-hydroxylation of progesterone has been an exceptionally competitive route to the production of this key precursor. This bioconversion has allowed the mass production of corticosteroids at reasonable cost. The ability to introduce oxygen into an unactivated carbon is mediated by cytochrome P450 type enzymes (Fig. 2).

Diols can be accessed through epoxide intermediates, as shown for reaction **M**, in which *Mortierella ramanniana* produced a *trans* diol intermediate of a potassium channel activator (antihypertensive) with an optical purity greater than 90% EE [91]. As in the case of asymmetric bioreduction, the required reduced cofactor was supplied through the regenerating capacity of the fungus. This same species also mediates the bioreduction shown in example **A**.

A bromoperoxidase from *Curvularia protuberata* was found to produce (2S,1S) bromoindanol (Reaction **N**), which could be converted chemically through a methyl oxazoline intermediate to *cis* (1S,2R) aminoindanol, a key intermediate in the synthesis of the HIV-protease inhibitor Crixivan® [92]. In this specific example, the importance of an associated dehydrogenase was highlighted by the authors, as they indicated that the stereoselectivity achieved was due to a dehydrogenase-mediated resolution of the racemic *trans*-2-bromoindandiols. Alternatively, the (2S,1S)-bromoindanol could be treated with base to access the (1S,2R) epoxide also suitable for the synthesis of *cis* (1S,2R) aminoindanol.

These few relevant examples highlight the important role that filamentous fungi and their enzymes play in the application of biocatalysis to the synthesis of pharmaceuticals.

3. PRACTICAL BIOCONVERSIONS WITH FILAMENTOUS FUNGI

3.1. Overview

The extent of their biodiversity makes filamentous fungi de facto a key group of microorganisms that must be an integral part of any respectable bioconversion library. Dalboge recently remarked that the fungal kingdom is still largely untapped for the purpose of discovering biocatalysts [71]. As listed in Tables 4 and 5, filamentous fungi perform a wide range of biochemical reactions, and to date, fungal enzymes, either free or cell-associated, perform a wide variety of chemically difficult stereoselective or regioselective reactions of great importance in the synthesis of pharmaceuticals (Figs. 3 to 5). Considering both the large numbers of fungal species and fungal metabolic diversity, one can safely speculate that a biocatalyst that exhibits exquisite regioselectivity and stereoselectivity against a specific synthetic molecule is very likely to exist. The scientist's challenge is to design and implement an effective screening strategy to successfully match the synthetic molecule of interest and the best possible microbe.

The following sections provide practical guidance and highlight salient points that should be considered when designing and implementing a microbial bioconversion screening. Fig. 6 presents a sequential generic approach to a microbial bioconversion screening, which can be summarized as follows. When a specific reaction is desired (e.g., the asym-

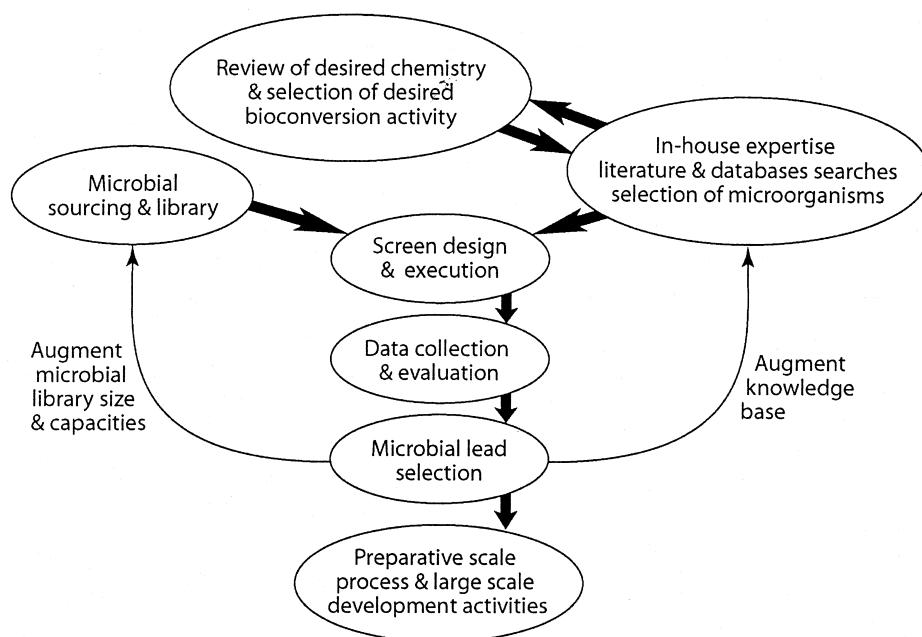


Figure 6 Bioconversion decision tree

metric reduction of a carbonyl group, a stereoselective hydrolysis, or a regioselective and stereoselective hydroxylation), the approach begins with the identification of the desired matching microbial enzymatic activity. For these specific examples, the investigator must evaluate microbial strains with known or suspected reducing, lipolytic, or hydroxylating activities, respectively. The second step is to search the published literature and external databases, and then review the in-house expertise repository. These searches will yield valuable information regarding the selection of the microbial strains likely to exhibit the desired activity. If readily available, the desired microbes can be extracted from a larger library based on their known or suspected activity. If such a library is not accessible, strains can be isolated from specific environments that are likely to harbor microbes with the desired activity. In most cases, the evaluation is conducted employing liquid cultivation media to which the substrate is added at a predetermined time. Following proper incubation and appropriate chemical extraction or purification, the presence of the desired product is detected via standard analytical procedures. Selection of the most appropriate isolate is made based on the data generated from these chemical analyses. Limited scale-up activities performed in either large flasks or laboratory fermenters will usually yield several grams (1–50 g) of the desired compound without resorting to extensive and lengthy process development activities. All of these steps are discussed in detail in the following sections.

It is possible for the aims of the screening effort to extend beyond the identification of a specific activity, and the production of a few grams of material, to include the production of large quantities of the desired molecule via bioconversion. Then, once the best candidate microbial strain is selected, further process development steps, such as the optimization of strain and cultivation medium and of process environmental conditions, will follow [93–95]. If the desired enzyme is isolated, overexpression via cloning and directed evolution can further be considered [67,68,96]. These activities extend beyond the scope of this chapter.

3.2. Practical Considerations

3.2.1. Biocatalysis Databases

Concurrently with the expansion of the field of biocatalysis, several databases have developed. They offer rapid access to an extensive and rather complete repository of bioconversion reactions that are complemented with the description of the biocatalyst. Additional information regarding the reaction conditions is also listed. All databases are web linked, and their access is listed in Table 9.

3.2.2. Biocatalyst Sourcing

Enzyme Sourcing

Many fungal enzymes are commercially available (Table 7). A reference book by Wiseman provides a very comprehensive database of all commercially available enzymes [97]. In addition, many fungal enzymes that are not commercially available can be relatively easily manufactured in the laboratory [66].

Table 9 Biocatalysis Databases and Enzyme Resources

Accelrys Biocatalysis Database (www.accelrys.com)

University of Minnesota Biocatalysis Biodegradation Database (umbbd.ahc.umn.edu)

Klotho (www.ibc.wustl.edu/moirai/klotho)

Brenda Enzyme Database (www.brenda.uni-koeln.de)

Table 10 Culture Collections for Accessing Fungal Biocatalysts

American Type Culture Collection (www.atcc.org)
Agricultural Research Service Culture Collection (nrrl.ncaur.usda.gov)
Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl)
Canadian Collection of Fungal Cultures (sis.agr.gc.ca/brd/ccc)
World Data Center for Microorganisms (www.wdcm.nig.ac.jp)

Microbial Sourcing

In practical terms, the best procedure for obtaining microbial strains is to order them from well-established cultures collections. Table 10 lists several large culture collections. For a matter of reference, the American Culture Type Collection (ATCC) collection also lists the strains with known bioconversion activity. If one would like to augment their collection with additional and potentially novel strains, direct isolation from the environment should be considered. Practically, one should try to encompass diversity in both the geographical sampling locations and material sampling sources. For example, Cagnon et al. found that among isolates from the rain forest, a wide range of activities and enantioselectivities existed among many of the strains of *Cunninghamella echinulata* that they isolated [98]. Before embarking on the isolation of microbes from the environment, it may be necessary to become familiar with basic techniques by consulting relevant references [99]. No established library is static; its size and diversity will grow with the experience of the owner, who should always be on the look out for the addition of novel activities.

3.2.3. Cultivation and Storage

Most fungal species will grow on standard solid or liquid media whose composition can be found in the literature. For referencing, the ATCC microbial sourcing manuals (Table 10) and textbooks [99] list the formulation of various cultivation media [74,100]. In addition, Table 11 provides the formulations of several commonly used fungal cultivation media that can be readily used in the laboratory.

It is now well established that most fungal cultures can be stored and preserved by deep-freezing at -65°C in the presence of a cryoprotectant such as glycerol [101,102].

Table 11 Commonly Used Growth Media for Fungi**YPD**

1% yeast extract

2% peptone

2% glucose

Sabouraud dextrose (SDB)

5 g/L meat peptone

5 g/L casein peptone

20 g/L glucose

YME

5 g/L yeast extract

5 g/L malt extract

20 g/L glucose

The most common procedure relies on the cultivation of the cells in liquid medium to midexponential growth, at which time a solution of glycerol is added to the flasks to give a final concentration of about 20%. The cell suspension is immediately aliquoted into small vials (1–4 mL) which are stored in a freezer. This methodology allows the preservation and handling of large microbial cultures to be simple and manageable. When using a relatively small collection of filamentous fungi, some investigators do prefer to preserve their microbial collection on solid medium. Usually, the cells are cultivated on solid medium slants, and once good surface growth has been achieved, the tubes are stored in cold rooms [19]. To preserve viability, the cultures need to be transferred on a regular basis (3–6 months).

3.2.4. Library Construction, Composition, and Size

The efficiency and potential outcome of a microbial screening for bioconversion activity will depend highly on the construction and composition of the library. As discussed earlier, substantial information regarding microbes possessing a specific bioconversion activity is available from both the scientific literature and bioconversion databases, and strain selection should rely heavily upon these bodies of information. For example, extensive information is available for microbial hydroxylations and asymmetric reductions (see Tables 4 and 5 for references to specific chemistries). When selecting microbial strains, one will try first to match the desired enzymatic activity with the type of bioconversion to be completed. If possible, priority should be given to microbes that have demonstrated the required activity against structurally similar molecules. One should be keenly aware, however, that a strain that has displayed hydroxylating activity against a specific molecule, for example, may not necessarily be active against another even closely related molecule. In addition to procuring the strains identified by specific literature and database searches, the species and strains with known bioconversion activities, which are listed in Table 12, constitute a good basis for establishing an initial bioconversion fungal library. In addition, several authors have published the compositions of their microbial libraries [82,103]. Commingling the microbes listed in Table 12 with those listed in the various publications can help to rapidly build an effective personal microbial library.

On a practical basis, a well-designed microbial library must be large enough to ensure a likely positive and timely outcome to the screening effort. It should not be so large that the evaluation of all strains becomes an extensively time-consuming activity. Unfortunately, no clear rules exist as to the number of strains that need to be evaluated because this will vary greatly from screen to screen. Success is directly related to the choice and number of microbes tested, as well as to the type of reaction considered and to the properties of molecule presented.

Although researchers in the pharmaceutical industry have access to very large libraries [80,83,104], screens employing in excess of 500 strains are usually rare [105]. Initially, limiting the size of the library to between 50 and 100 strains with known activity matching the desired bioconversion (e.g., hydroxylating activity) should lead to a successful outcome in most cases. Table 13 lists the size of the microbial libraries used, along with their associated frequency of success, for a few representative screens. From the data presented here, it is readily apparent that screening efforts have can range from rather simple, with less than 50 strains evaluated, to quite intensive, with the up to 1000 strains evaluated. If automated, high-throughput systems are used, the size of the microbial library evaluated in this fashion can be significantly larger than that employing more traditional and time-consuming manual methods. This is discussed in more detail in section 3.2.7.

Table 12 Examples of Fungal Species Mediating Biotransformations

<i>Absidia blakesleeana</i> ^a	<i>Mortierella alpina</i> ^{a,b,c}
<i>Alternaria alternata</i> ^{a,c}	<i>Mortierella isabellina</i> ^{a,b,c}
<i>Alternaria solani</i> ^{a,c}	<i>Mucor circinelloides</i> ^{a,b}
<i>Aspergillus flavus</i> ^{a,b,c}	<i>Mucor hiemalis</i> ^{a,b}
<i>Aspergillus fumigatus</i> ^{a,b,c}	<i>Mucor miehei</i> ^{a,b}
<i>Aspergillus niger</i> ^{a,b,c}	<i>Mucor racemosus</i> ^{a,b}
<i>Aspergillus nidulans</i> ^{a,b,c}	<i>Neurospora crassa</i> ^{a,b}
<i>Aspergillus ochraceus</i> ^{a,c}	<i>Penicillium camembertii</i> ^{a,b,c}
<i>Beauveria bassiana</i> ^{a,b,c}	<i>Penicillium chrysogenum</i> ^{a,b,c}
<i>Botrytis allii</i> ^a	<i>Penicillium digitatum</i> ^{a,b,c}
<i>Clonostachys rosea</i> ^a	<i>Penicillium pupureescens</i> ^{a,b,c}
<i>Cunninghamella echinulata</i> ^{a,b,c}	<i>Rhizopus arrhizus</i> ^{a,b,c}
<i>Cunninghamella elegans</i> ^{a,b,c}	<i>Rhizopus nigricans</i> ^{a,b,c}
<i>Curvularia lunata</i> ^{a,b,c}	<i>Rhizopus oryzae</i> ^{a,b,c}
<i>Curvularia protuberata</i> ^{a,b,c}	<i>Trametes</i> spp. ^a
<i>Fusarium oxysporum</i> ^{a,b}	<i>Trichoderma harzianum</i> ^a
<i>Fusarium graminearum</i> ^{a,b}	<i>Zopfiella</i> spp.
<i>Fusarium sambucinum</i> ^{a,b}	
<i>Gliocladium deliquescens</i> ^a	
<i>Glomerella cingulata</i> ^{a,c}	
<i>Leptoxphium fumago</i> ^{a,d}	
<i>Metarhizium anisopliae</i> ^a	

^a Resolutions.^b Reductions.^c Hydroxylations.^d Haloperoxidase.

See Tables 4 and 5.

Table 13 Examples of Library Sizes and Success Rate in Selected Bioconversion Screens^a

Screen Type	Number of Microbes Tested	% Positive	Reference
Bioreduction	260	<1%	81
	38	24%	82
Haloperoxidase	400	3%	92
Epoxide hydrolase	80	10%	126
Hydroxylations	224	1%	127
Hydrolyses	960	45%	106
		(primary lipase screen)	
		2.5%	
		(secondary bioconversion screen)	
	71	1.5%	86

^a For these selected examples, diverse microbial libraries were used, and the fungal isolates were found to be the most suitable biocatalysts.

3.2.5. Classical Screening Strategies

Various approaches can be envisioned when designing a specific bioconversion screen. Table 14 summarizes them and lists a few relevant references.

One approach is to rely on the use of existing databases. For example, Smith and Azerad recommend the use of about 30 to 60 microbes, including filamentous fungi, when screening for bioconversion of pharmacoeactive natural molecules [19,103]. These well-selected microbes have proved to yield the desired bioconversion products with very good success.

If the type of enzymatic activity desired is well defined, targeting microbes from a specific environment can yield positive outcomes. For example, based on the prior experience of Hunter-Cevera [75], when searching for haloperoxidase activity, Zhang et al. specifically tested fungal isolates sourced from desert environments [92]. A high success rate was achieved when employing this screening strategy, which yielded 13 producing strains out of 400 screened. A similar approach was used when trying to identify specific proteases. Based on preliminary data showing that one commercial enzyme, proteinase K, was a promising biocatalyst for resolution of a racemic ester, Sidler et al. found that preferentially screening filamentous fungi isolated from decaying insects greatly enhanced their ability to identify strains producing suitable proteases for this specific biotransformation [86]. Again, this targeted screening approach allowed the rapid identification of a suitable producing strain.

When production of a specific enzymatic activity (e.g., lipase) is desired from the microorganisms, a two-tiered screen can be implemented. The first iteration focuses on the enrichment and isolation of the microbes with the desired enzymatic activity. For example, if screening for lipase-producing microorganisms, the use of solid media containing a vegetable oil will rapidly identify those producing lipase. The second screening tier will evaluate the enzymatic activity against the molecule of interest. For example, Cardenas et al. reported the isolation of more than 400 microbial strains with lipase activity from various environments [106]. From this preselected panel of microorganisms, they identified several microorganisms, mostly filamentous fungi, that were capable of esterifying carboxylic acids or acylating various alcohols.

Similarly, filamentous fungi harboring cytochrome P450 enzymes can be isolated based on their capacity to degrade polycyclic aromatic hydrocarbons [78]. The ability of such fungi to mediate oxidation of unactivated carbon can provide chiral intermediates of synthetically valuable aromatic hydrocarbons, such as indene, which can be used as a precursor in the synthesis of HIV protease inhibitor [76,77].

Finally, screens of large collections of microorganisms also are conducted in some cases. This type of screening can be very useful when trying to build a large number of

Table 14 Various Microbial Screening Strategies

Screening type	Selected References
Use of existing microbial collections with proven activity	19, 82, 103 Robertson, 1982
Isolations from targeted environments	92, 104
Enrichments on specific substrates	77, 106
Nontargeted use of large microbial collection	77, 105

derivatives from a specific molecule. For example, Chen et al. screened up to 1000 strains for their bioconversion activity against two immunosuppressants. In conducting such a screen, they were able to generate a chemical library of 16 novel derivatives [107].

Several strategies, with the aim to refine the screen, also can be considered following initial screening. In some cases, the first round of screening may yield only a relatively poor performing strain. Conducting a secondary screen, focused more narrowly on a longer list of species from the genera producing the best leads, may prove to be a wise decision [108].

3.2.6. Experimental Considerations

Many choices and factors influence the outcome of a bioconversion screening. The following list, although not exhaustive, describes a generic experimental approach that includes some of the most important variables to consider. Fig. 7 presents an overview of the typical sequential phases of this procedure. Most screens rely on the aerobic cultivation of the fungi in test tubes or small Erlenmeyer flasks (250 mL) containing an appropriate cultivation medium. A seed stage, inoculated with cells from a frozen suspension or from a vegetative slant culture, is usually performed. Once reasonable growth is achieved, the cells are transferred to the screening stage. Timing of transfer can be based on either visual or quantitative evaluations, but is always strain dependent and must be experimentally determined. After a period of biomass production, the substrate for bioconversion is added and the flasks are returned to their prior incubation conditions. Sampling for detection

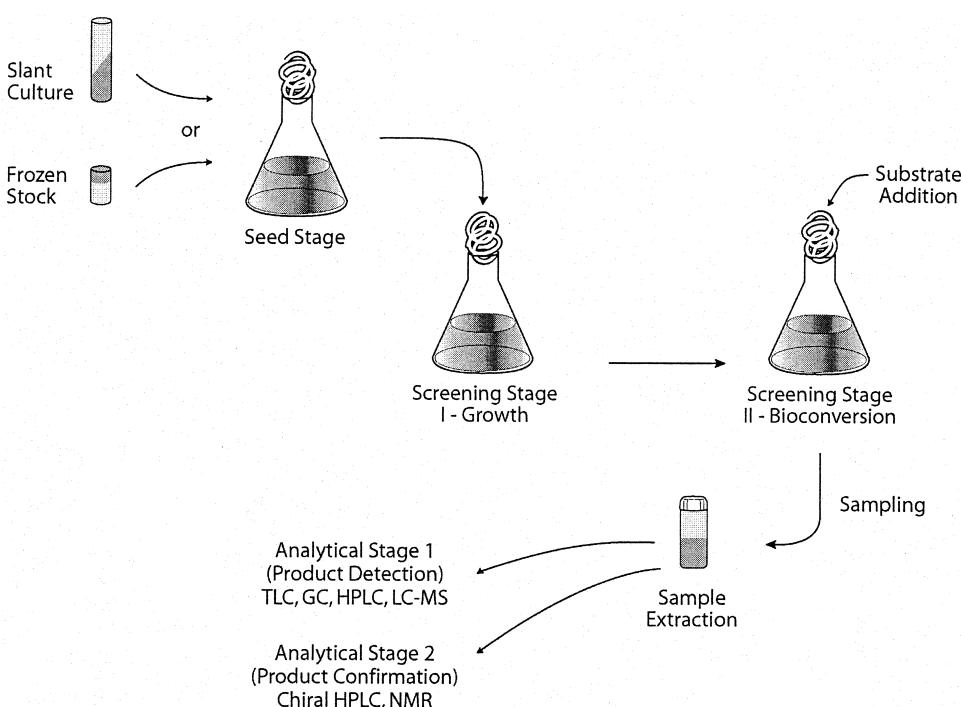


Figure 7 Various steps of bioconversion screening

activity is then performed at regular intervals. The broth is subjected to chemical extraction, and presence of the desired bioconversion product is detected by using standard analytical procedures.

Many of the factors and choices made during these operations influence the outcome of the bioconversion screening. They include the type of medium used, the time of substrate addition, the method used for substrate addition, and the cultivation conditions used.

Choice of Cultivation Medium

Unless the scientist is trying to mimic a preestablished bioconversion for which one can rely on the case-specific literature, the choice of cultivation medium should be primarily made on the basis of its support of adequate growth. Secondary considerations may include the absence of interferences with detecting bioconversion activity and the ease of preparation. Especially when a classical screening approach is used, most investigators will rely on the use of one or at most two media compositions for their screening operations in order to increase efficiency by limiting the number of flasks or tubes handled. It is important, however, to consider that the composition of the cultivation media can influence the induction of specific desired enzyme(s). For example, lipolytic activity can be easily induced by adding triglycerides. In addition to those listed in Table 11, additional media compositions can be easily found in the ATCC catalogs or web site (Table 10).

Cultivation and Substrate-Addition Methods

The addition of substrate for bioconversion can be the key to a successful screening. Of prime importance are the timing of addition, the methods of addition, and the final concentration of substrate employed. Because bioconversion substrates may present toxicity toward cell growth, additions performed too early in the growth phase may reduce biomass formation, thus limiting the amount of the desired catalyst. Investigators routinely add the substrate once ample microbial growth has been achieved in the tubes or flasks. Typically with most fungal cultures, this will translate in substrate additions made 2 to 4 days following inoculation. It is important, however, to keep in mind that certain bioconversion activities are induced by the presence of a specific substrate which may require the addition of a small amount of substrate early in the growth cycle followed by a larger bolus several days later.

A large fraction of the bioconversion substrates that are encountered exhibit limited water solubility. At first, for the purpose of simplifying substrate dispensing to the culture, dissolution into a biocompatible solvent is routinely performed. Ethanol, dimethylsulfoxide (DMSO), and dimethylformamide (DMF), at final concentrations ranging from 1% to 5% (vol/vol), are the carriers most often used [109]. Other methods aimed at increasing the solubility of the substrate have been employed. Surfactants (e.g., Tweens) that can emulsify water-insoluble substrates have been successfully used in steroid bioconversions [39]. Cyclodextrins, a doughnut-like molecular entity exhibiting a lipophilic core and a hydrophilic surface, also have been employed very successfully in many biotransformations [110]. Some investigators have successfully resorted to the fine milling of the substrate followed by its addition to the cultivation flasks as a fine powder [39,109]. When solvents are employed, once the substrate is added to the cultivation medium, it will form a fine amorphous precipitate that allows the compound to come into contact with the membrane, thus allowing for its transport inside the cell where the bioconversion reaction will take place. Substrates, unless they present a high toxicity to the cells, are added at final concentrations ranging from 250 to 1000 mg/L [111]. Such elevated substrate concentrations increase the chances of identifying the desired bioconversion activity, even if the initial yields are poor.

Cultivation methods are highly dependent on the species cultivated. Standard, aerobic cultivation methods that employ shaker flasks incubated under conditions that are permissive for the growth of microorganisms will generally support bioconversion activity as well.

It is worth mentioning that although nearly all bioconversion screens rely on the use of growing cells, several other methodologies have been successfully used, including those that employ either cell extracts [112], resting cells, fungal spores [113], immobilized cells [114], cell powders generated with acetone [115], lyophilized cells, and heat-dried cells. This is true especially in the context of process development activities. A review of these methodologies is available [109].

Detection of Product Formation

Once the substrate is added, intermittent sampling is required to evaluate if its bioconversion has taken place. Samples collected on a daily basis are usually sufficient. The samples usually require a simple workup, such as extraction with an equal volume of nonwater miscible solvent (e.g., methylene chloride) before analysis. The use of high-pressure liquid chromatography (HPLC) is highly recommended for quickly gathering screening data. Positive hits should be further confirmed by mass spectrometry analysis and, after product purification, by nuclear magnetic resonance (NMR).

3.2.7. High-Throughput Screening and Automation

Although cultivation in microtiter plates has been successfully applied to the cultivation of bacteria with bioconversion activities [16,117], the literature lacks published works specifically pertaining to the cultivation of fungi. This is perhaps due to the fact the filamentous fungi may not be so amenable to being cultivated in such types of vessels. The only relevant published example was that of the miniaturized and automated cultivation of yeasts by Stahl et al., who used a 24-well plate technology [118]. Each well contained a different strain, and the template plates were preserved at -65°C . When a screen was implemented, the screening plates were inoculated from the templates using multichannel pipetting devices. A cultivation volume of 2 mL was routinely used in the custom-made bioconversion plates, which had a well volume of 10 mL. To ensure proper aeration, the culture in each well was stirred with a small magnetic stirring bar. The use of a robotic system using an XYZ arm, coupled with a custom modified HPLC system, allowed for rapid and efficient automated sample extraction and analysis. Although not used specifically with filamentous fungi, this procedure supported the successful cultivation of several filamentous yeasts, and minor modifications should allow its use with filamentous fungi. Fig. 8 provides a pictorial of this high-throughput microbial screening.

3.2.8. Alternative Approaches

Use of Tagged Substrates

While classical microbial screening is very effective, it becomes fastidious and time-consuming if a desired catalyst is not found after evaluating a few hundred strains, in the absence of high-throughput and automation technology. However, it is sometimes possible to employ tools that simplify detection of the desired product. They include the use of substrates with fluorescent tags [119]. When applicable, these methodologies can greatly increase screening efficiency and throughput. If employed in combination with automation technology as described earlier, this type of screening has the potential to rapidly yield a biocatalyst candidate.

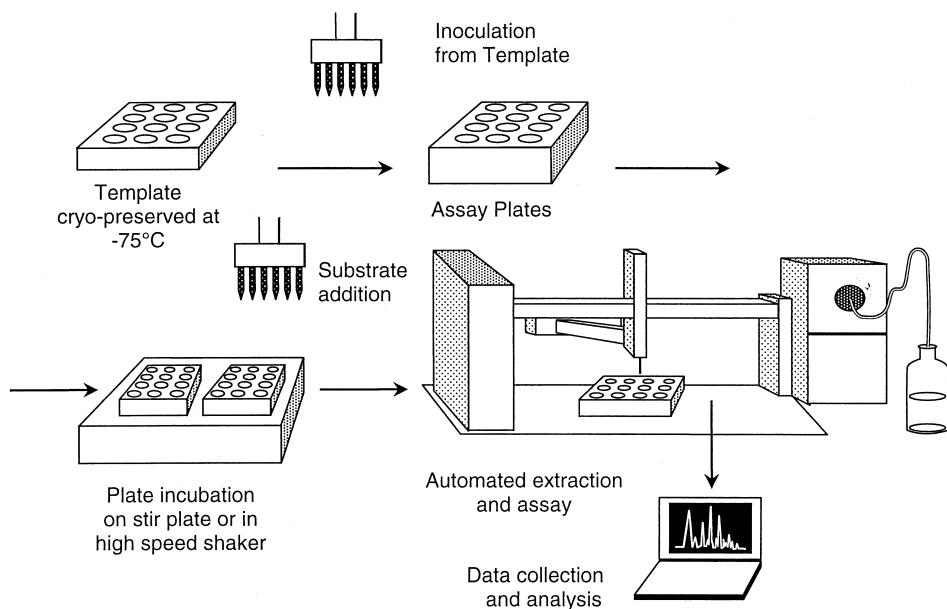


Figure 8 Automated high throughput bioconversion screening

Genetic Screening

Recently, several screening methods that employ genetic approaches, especially in the context of identifying novel cell-free biocatalysts such as lipases and proteases, and ketoreductases have been implemented [79,120,121].

One approach takes full advantage of the knowledge of the complete genome sequence of the microorganism. Using sequence homology with genes coding for already known enzymes, it is possible to construct PCR gene-specific primers that will rapidly identify the desired genes. This specific approach has been successful in cloning ketoreductase genes from the yeast *Saccharomyces cerevisiae* [79]. Although the diversity and size of microbial genomic sequences is still limited, these are expected to increase significantly in the coming years.

When the full genomic sequence information is not available, activity screenings have the potential to rapidly yield a desired novel enzyme. This approach relies on the use of consensus homology DNA probes that are constructed using available information from a specific class of enzymes (i.e., lipases). The unknown microorganisms are first cultivated under conditions permissive to the induction of the desired enzymatic activity. For example, cultivating unknown microorganisms in the presence of triglycerides will induce lipase activity. Secondly, using specific homology DNA probes to lipase coding consensus sequences, the mRNA is extracted from the cells and the corresponding cDNA synthesized. Further manipulations eventually lead to the construction of stable clones expressing large amounts of the desired novel enzyme [71,122].

Finally, the use of DNA obtained from environmental samples can also be considered. This random genetic approach is believed to address biodiversity to its fullest, as it potentially permits the expression of DNA obtained from “unculturable” microorganisms

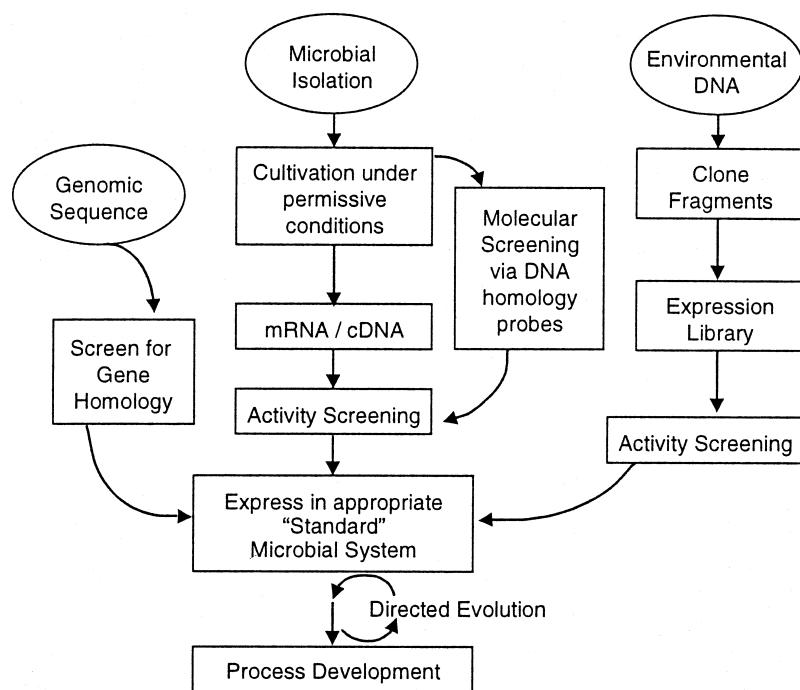


Figure 9 Genetic screening for bioconversion

[123]. Following isolation procedures, the obtained environmental DNA is reduced to a size amenable to cloning. The very large libraries generated by this approach are then evaluated for the presence of any desired biocatalytic activity. Once selected, the desired gene is then inserted into a standard microbial expression system amenable to large scale production system [124].

When coupled with high-throughput technology and the efficient use of microbial biodiversity, these molecular techniques and approaches offer the potential to be extremely powerful and successful. In addition, because the DNA of the identified catalyst is already cloned, directed evolution of the enzyme, which may yield a more efficient biocatalyst, can be performed almost seamlessly [71,125]. Fig. 9 provides a rapid survey of the various options described here. Although still time consuming, especially when dealing with rarer enzymatic activities, it is quite safe to speculate that the use of these type of molecular screening approaches to bioconversion activity will expand rapidly in the near future.

4. CONCLUSION

Filamentous fungi and their isolated enzymes have performed significant biocatalytic steps in the manufacture of commercial drugs and drug candidates. As opportunities to implement biocatalytic steps in pharmaceutical syntheses continue to emerge, one could predict that there will be many more instances in which filamentous fungi, or fungal-derived enzymes, could potentially be used. Focused screening initiatives to discover such biocata-

lysts will depend in large part on the interaction of mycologists, synthetic chemists, and biochemical engineers. The major bottleneck in most cases is the rapid identification of a suitable catalyst in a timeframe that can meaningfully impact either discovery or development programs. This chapter presented the background and rationale of a productive fungal-based biocatalyst screening program. Employing traditional screening methods has been very successful in the past and should continue to yield many useful microbes. However, it is eagerly anticipated that both uses of genetic approaches, up to the use of total genomic sequences, as well as high-throughput automation will greatly speed up biocatalyst discovery. In general, the relative success of efforts will be a function not just of one's understanding of the underlying physiology and enzymatic repertoire of the organisms constituting the *de facto* library of putative biocatalysts, but also the ability to integrate fungal biocatalysis into retrosynthetic analyses. The future growth of fungal biocatalysis applications depends on mycologists' creativity and willingness to direct their knowledge and experience of fungal biochemistry and biotechnology for the purpose of small molecule synthesis.

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22

Metabolomics

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1. INTRODUCTION

1.1. Metabolomics

Metabolomics is the computing of emergent properties of biological systems such as development, biological clocks, and infection processes from kinetic models of DNA, RNA, and proteins. These kinetic models are used to guide the process of gene-validated product discovery to transform medicine, industry, and agriculture. The ultimate challenge of genetics is to predict global properties of the organism, properties not necessarily manifested by individual subcomponents within the cell. Some of these properties only make sense with respect to the organism as a whole (e.g., pathogenicity lifestyle [1]) or life itself. These complex traits controlled by many genes represent the ultimate challenge in seeking an explanation in terms of detailed molecular mechanisms in the cell.

From the standpoint of human health, an explanation is sought for how an organism such as the opportunistic fungal pathogen, *Pneumocystis*, changes from a benign commen-

sal in the mammalian lung to the major killer of AIDS patients through a lethal pneumonia [2]. From the standpoint of agriculture, one of the major challenges of peanut production is controlling an opportunistic pathogen, *Aspergillus flavus*, that causes aflatoxin contamination in peanuts. Controlling aflatoxin biosynthesis has consequences for human health, for the quality of a major U.S. crop, and for domesticated animals ingesting contaminated peanuts [3,4]. From the standpoint of industry, fungi are producers of chemical feedstocks and biologicals, ranging from ethanol to taka-amylase and citric acid [5,6]. From the standpoint of fundamental questions in biology, an explanation is needed for how a fungus programs the development of a conidiophore [7] or captures the diurnal cycle within the cell [8]. One approach to explaining these global processes is through the identification of a biochemical and regulatory network that rationalizes these processes with a mechanistic model [9]. Unlocking these regulatory and biochemical networks provides an opportunity for their manipulation either through targeting of critical steps in metabolism for the discovery of antifungals or through manipulation of pathways to overproduce needed compounds like penicillin.

1.2. Paradigm Shift in Biology

Biology is currently going through a paradigm shift driven by microbial systems. The discipline is becoming data-driven through the avalanche of genomics information being released on a variety of fungal systems [10]. The discipline of fungal biology has become high-throughput, with vast amounts of data robotically generated through the use of automated sequencing machines [11] and the use of microarrays for analysis of gene expression [12] as well as mass spectrometers for protein–protein interaction mapping [13]. These data are highly structured and hierarchically organized [14]. At the center of any biochemical and regulatory network, whether it be the *lac* operon [15] or the biological clock [16], is the central dogma describing the most fundamental flow of information in the cell from DNA → RNA → protein. Within a cell, this dynamic flow of information is hierarchically arranged. Functionally, reaction networks have structure [17]. At their highest level, they are organized into broad functional categories, such as energy metabolism, nucleotide metabolism, recombination, and DNA repair. At a lower functional level, within any one of these functional categories, there is a finer definition of function in terms of genes and their products involved in, for example, the Embden-Meyerhof pathway, Krebs cycle, and oxidative phosphorylation. At the lowest level in the functional hierarchy, there is a particular pathway [18,19].

This information flow also has a structural hierarchy. Genes and proteins do not work in isolation within a reaction network [20,21]. Rather, proteins form complexes that carry out the work of the cell, such as signaling. Signaling cascades of proteins may work in a coupled fashion to connect the surface of the cell with the nucleus in order to respond to different environmental conditions [22]. These signaling wires themselves are made up of shared components to allow a coupled response to environmental signals. In other parts of the cell, proteins form smaller aggregates to carry out a specific function, such as transcription, which in turn aggregate to form a “some” like the transcriptosome composed of more than 100 proteins [23]. New tools are being developed to identify these molecular machines [24,13]. These subcellular structures within the cell may have arisen from simpler precursors, and the structure of these molecular machines may in part reflect their history [25].

The information in the cell is hierarchically organized through its history. The shared thread of the DNA links organisms into a reticulate structure, in which the history of genes traces out the organismal phylogeny linking all organisms in the tree of life. The appearance of each new mutation in the DNA can be viewed as the ticking of a molecular clock. This ticking can be used to link organisms into families, which can be in turn linked into pedigrees, which in turn give rise to genera, which in turn radiate into the larger taxonomic branches. This evolutionary organization is played out at different levels by comparing genes evolving at different rates [26]. Through the consideration of the detailed mechanics of the cell, biology has thus become an information science from a functional, structural, and evolutionary standpoint.

Because of the avalanche of information resulting from the genomics revolution, biology has changed into a mathematical discipline. Extensive automation is required to capture the data [27] through laboratory information management systems [28]. The information needs to be stored, managed, and retrieved in sophisticated databases [28–30]. The information needs to be integrated with new algorithms [31–37] and with new tools such as the semantic Web to make queries of the diverse resources now available for identifying reaction networks [38]. Models are being created to summarize the information [39,40]. This information must be analyzed to test hypotheses about the structure, function, and evolution of living systems [41]. Finally, the information needs to be visualized [42–44] to be understood and utilized. Computer scientists, mathematicians, and statisticians are engaged in all aspects of biology as an information science.

With the focus on complex traits involving many genes and their products, a new approach is needed that is more familiar to ecologists and neural biologists. This systems approach is at the heart of genomics. Measurements are taken on the system as a whole. The relative levels of all RNAs are measured [12]. The relative levels of all proteins are captured from crude protein extracts [45]. The response of the system as a whole is measured by capturing all RNA and protein levels in the cell. The ability to predict the global response of the system becomes the ultimate test of a biological hypothesis.

The promise is that by measuring the global response of the system, we can understand and predict complex traits [46]. Currently, this is only a promise by genomics, but it is a compelling challenge to move beyond *Mendelian* genetics. Most of the traits of interest, such as antibiotic production, pathogenicity, or clocks, are controlled by many genes and are tightly coupled to other processes [47].

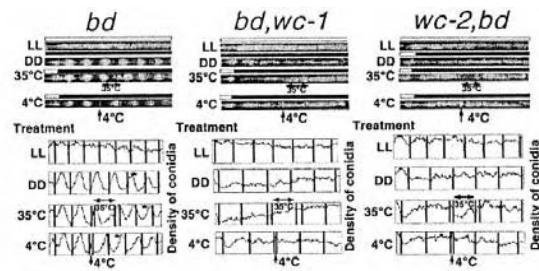
In this section, metabolomics as a discipline has been defined, and connections made with metabolic engineering. In section 2, the origin of metabolomics is explored. In section 3, the models or “biological circuits” behind metabolomics are sketched with their applications. In section 4, the process of discovery at the heart of metabolomics is considered. In section 5, the process of identifying biological circuits is considered along with the challenges. Lastly, in section 6, metabolomics is put in a larger context and summarized.

2. BIRTH OF METABOLOMICS AS A SYSTEMS SCIENCE

The challenge of genetics is formulating a detailed understanding of complex traits, particularly those that characterize the organism as a whole. Examples include high blood pressure, biological clocks, sex, development, and pathogenicity. Much of what we know about the biological clock, for example, comes from the study of a particular fungal system, *Neurospora crassa* [8]. Fig. 1 shows an example of this emergent property of *N. crassa*, the regular temporal sequence of conidiation by this organism growing in race tubes.

Complex Traits are controlled by many genes:

- Human Disease
- Biological Clocks
- Sex
- Development
- Pathogenicity



The focus of quantitative genetics has been on the interesting complex traits!

Figure 1 The promise of genomics is in understanding complex traits such as the circadian clock. An example of the effects of gene mutations (*wc-1* and *wc-2*) on circadian oscillations in *Neurospora crassa* is redisplayed. *N. crassa* is shown growing in race tubes with the regular pattern of conidia being formed displaying the clock under varied conditions. (From Ref. 148. Copyright 1997 American Association for the Advancement of Science.)

When transcriptional activators such as white-collar 1 (*wc-1*) and white-collar 2 (*wc-2*) are knocked out, the organism loses its ability to tell time. The extent of the circuit is unknown, but a number of genes are now implicated in the functioning of the circadian oscillator. One goal is to be able to predict the oscillatory response from a detailed biological circuit specifying the function of genes and their products.

Traditionally, the subject of quantitative genetics has focused on complex traits [48]. The approach has been model-based with the hypothesis of several loci on chromosomes contributing to a particular complex trait. Assumptions about dominance, penetrance, and epistasis are made, and then predictions about the inheritance of the trait are calculated. The subject has given rise to powerful methods for identifying quantitative trait loci (QTLs) that affect particular complex traits [49–52]. When QTLs are integrated with other kinds of genomic information, precise predictions of the location of genes can be made [52,53]. Unfortunately, this approach is divorced from a detailed understanding of genes and their products. In the end, the explanation of a complex trait is only a location on a chromosome.

Both genomics and quantitative genetics have a common goal, the understanding of complex traits. The challenge is how to transform genomics from a data-driven discipline to hypothesis-driven science. One approach is to cross genomics and quantitative genetics. The result is metabolomics. Metabolomics at its outset embraces the model-driven approach of quantitative genetics and combines this with the data-driven discipline of genomics. Metabolomics thus becomes hypothesis-driven genomics.

Metabolomics begins with the data-rich foundation of genomics. The starting point is the entire DNA sequence of an organism. This resource is used to capture RNA and protein profiles (i.e., the cellular state) under varied conditions. Models of the complex trait are introduced to explain the trait in terms of RNA levels, protein levels, and metabolite levels plus the organization of genes, their products, and substrates in the cell. The models serve to explain and predict, using the data-rich foundation of genomics. Predictions are made about the complex trait and the global state of the system from a detailed understand-

ing of DNA, RNA, and proteins. The success or failure of a scientific hypothesis can be judged in this wider genomic context.

2.1. System State

One of the remarkable advances of genomics has been in obtaining a fairly complete description of what the cell is doing. It is now possible to measure all relative RNA and protein levels in microbial systems [12,45].

Varied strategies can be used to examine gene expression, including differential display, subtractive hybridization, and restriction fragment differential display. In particular, two technologies have come to the fore—microarray analysis [54] and serial analysis of gene expression (SAGE) [55]. Some comparisons of these approaches have been made [56], and the result is that each method identifies different subsets of the total RNA population. With microarray analysis, varied implementations exist.

2.1.1. RNA Profiling by Microarraying

One illustration of the approach developed by DeRisi et al. [12] is described in Chu et al. [57], in which microarray analysis is used to analyze an emergent property of all living systems, reproduction. RNAs are isolated from 10 different time points in sporulation and the meiotic cell cycle, reverse transcribed, labeled with a red or green chromophore, and the cDNAs (red) from each time point mixed with cDNA derived from the 0 time point (green). This cocktail is then probed against all 6000 genes in the yeast genome (Fig. 2) [58]. The advantages of this approach are the linearity of signal response, the presence of an internal control (by mixing the cDNAs from different sources into *one* probe), and the simple approach to visualizing the transcriptosome.

One limitation has been an interaction between the source of the RNA and color label (i.e., the red or green chromophore), which has led others to radiolabeling cDNAs [23]. Seven clusters of genes are differentially regulated during sporulation [41], and the genes are clustered by the similarity of their profiles as shown in Fig. 2 [58]. This information then becomes a resource for detailed hypotheses about the cell cycle [59].

2.1.2. RNA Profiling by Serial Analysis of Gene Expression

An alternate approach and the one first used to characterize the yeast transcriptosome is simply to sequence efficiently the resulting cDNAs from different cellular states and to count the RNAs present (i.e., SAGE [55]). With the ability to quantify expression of all genes, the next step in the information flow of the central dogma is capturing relative protein levels in the cell.

2.1.3. Protein Profiling by Isotope-Coded Affinity Tagging

Isotope-coded affinity tagging (ICAT) has been used to characterize the *GAL* cluster in yeast [45]. Protein profiling can help us to identify genes that are under translational control [16,60] as well as provide a more complete description of the cellular state. The ICAT reagent contains a sulfhydryl-specific reaction group (iodoacetamide) to label cysteines, an affinity ligand (biotin) to capture the protein, and a linker region that contains either eight or zero deuterium atoms (D8 or D0) to label the cellular state. In the case of

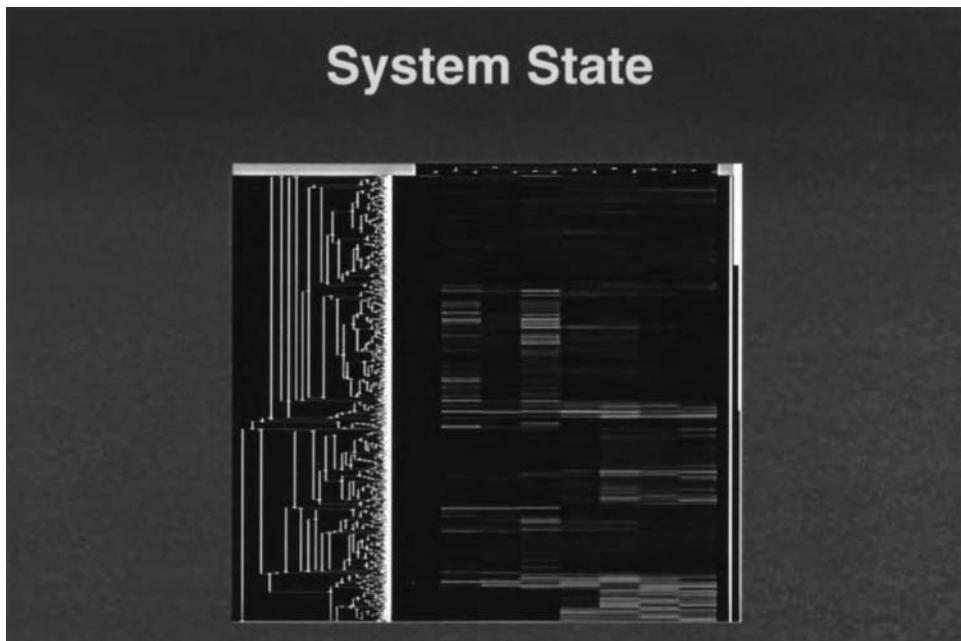


Figure 2 A display of relative RNA levels under 10 conditions (time points) during the cell cycle of *S. cerevisiae*. The 6000 genes are displayed vertically by the similarity in their RNA profiles using UPGMA as implemented in Wu [58]. Increasing red indicates increased expression relative to the beginning time point, and increasing green indicates decreased expression relative to the beginning time point.

yeast, Gygi et al. [45] compared proteins in cells grown on galactose or ethanol as a carbon source as the two cellular states.

Using the ICAT reagent, they were able extract and identify more than 800 proteins that responded differentially to change in carbon source. Data collection operated in two modes on a mass spectrometer. In one mode, peaks coming off the column were used to identify proteins from their BN-Y fragments. In the other mode, pairs of peaks were captured separating the two labeled forms (D0 vs. D8) of each protein to quantify the relative amounts of particular proteins in the two cellular states (labeled by D0 and D8). The use of the cysteine label decreased the complexity of the protein mixture and thus increased the opportunity to characterize more proteins in the cell. This is an attractive approach because of the use of an internal control, dual peaks as a form of replication (D0 and D8), and the ability to analyze insoluble proteins in contrast to other mass spectrometric methods, such as MALDI-TOF-MS/MS [13]. The major limitation is resolving all the proteins in a cell-free protein extract.

2.2. A Journey into the MudPIT

In multidimensional protein identification technology (MudPIT), the aim is to resolve all proteins in the proteome. One approach that has been successful is to combine multidimen-

sional chromatography with electrospray ionization on a mass spectrometer [61,62]. Peptides are systematically separated by charge in one dimension and by hydrophobicity in another dimension. An SCX cation exchange column was used to separate by charge and preceded by a prefractionation step on hydrophobicity prior to the dual liquid chromatography LC/LC step. Wolters et al. [62] estimated that up to 23,000 peptides could be separated using this approach. Using protein extracts from *S. cerevisiae*, they were able to separate 5540 unique peptides (approximately 1484 proteins) from a complex mixture. The estimated dynamic range for detection varied 10,000 to 1 with a lower detection limit of 100 copies of a protein per cell. The major advance MudPIT provides is reaching the insoluble protein fraction and more than doubling the number of resolvable proteins with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [13]. The major limitation is still the complexity of the protein mixture.

2.3. Who Counts the Small Molecules?

Having completed the review of large molecules, a segue to the characterization of the metabolite profiles is needed, and some initial efforts are reviewed [63,64]. Some of the new approaches to metabolite profiling are discussed in Chapter 14 [64]. A variety of separation procedures are being explored.

2.4. System Measurements

The basic measurements available on the system are the RNA and protein levels in different cellular states along with the levels of small molecules as available. These transcriptional and protein profiles become the resource to which the biological circuit is fitted.

2.5. Making Genomics Hypothesis-Driven

The process of making genomics hypothesis-driven is summarized in Fig. 3. The state of the system is captured in the RNA and protein profiles and whatever elements of metabolite profiles can be captured. These data are then used to identify a formal kinetics model to describe what genes and their products are doing. The classic elements of a biochemical reaction network are shown in Figure 3. The simplest kind of reactions are those that lead to Michaelis-Menten kinetics [65]. Once the table of reactions is specified, the profiling information can be used to identify the rate constants and initial conditions of the biochemical and gene regulatory reaction network.

One of the simplest kinds of reaction networks is a pair of coupled signaling cascades as shown in Fig. 3. A receptor protein (R or R^1) at the plasma membrane responds to an incoming signal, such as a pheromone or osmolarity [22]. The message is passed to a G-protein (G or G^1) which, through a signaling cascade, activates a transcriptional activator (E or E^1) to program the cell for an adaptive response. Even this simplest of systems can display emergent properties such as memory of the incoming signal, which the individual signaling wires by themselves do not manifest.

Once the kinetics model, or so-called biological circuit, is identified, familiar simulators like GEPASI, MIST, or SCAMP can be invoked to yield predictions about the *workflow process*, which is defined as an automated organizational process involving these tasks. Recently, laboratory information management systems (LIMS) systems have been introduced to design and manage these workflow processes [27]. An automated workflow management system (WfMS) is the collection of tools enabling workflow creation (which

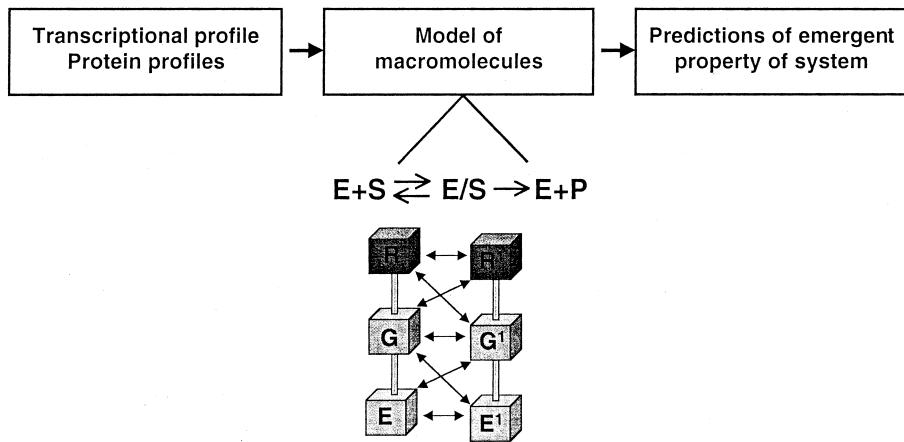


Figure 3 Genomics is made hypothesis driven by utilizing a chemical reaction network to integrate genomics information and to make predictions about emergent properties of the system of interest.

includes design), workflow enactment, and management of workflow processes. The goal of a workflow management system is to enforce intertask dependencies, scheduling, data management, and reliable execution. Workflow management systems can play a central role in monitoring and enforcing quality of service (QoS), such as sequence quality [66,67]. Genomics workflow systems require adaptability and ensured QoS.

Several workflow management systems are available [68–70]. One such system is METEOR, which provides four kinds of services: workflow builder, workflow repository, workflow enactment, and workflow manager. Enactment has been implemented in two versions. WebWork [71] is entirely web based and is suited for workflows that do not dynamically change their architecture, and OrbWork [72] supports dynamic modifications to a workflow.

METEOR has already been used to support portions of a workflow to identify reaction networks. A workflow like that in Figure 20 includes a subflow for sequencing, which has been implemented [73]. A larger subflow for protein–protein interaction mapping has also been created [28], which again is a subcomponent of circuit identification. Workflow management systems will be essential for integrating genomic and bioinformatic projects aimed at identifying biological circuits.

3. MODELS

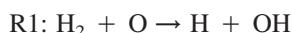
A variety of modeling approaches for biological circuits have been proposed. These include linear models with time as a factor [74], linear dynamic [75], Bayesian networks [76,77], neural networks [78], Boolean networks [79–81], and classic chemical-reaction networks satisfying mass balance [39]. At one extreme, Boolean networks are draconian simplifications of chemical reaction networks satisfying mass balance, but they may be informative

about crucial links in large reaction networks. At the other extreme are the stochastic formulations of reaction networks, in which the fate of individual molecules are tracked by applying a set of master equations summarizing the chemical reactions. Deterministic reaction networks strike a balance on this spectrum. The success of these competing approaches will ultimately be decided by the data. The focus here is on classic chemical-reaction network models because they are well grounded in physics and chemistry. These chemical reaction network models can either be deterministic [39] or stochastic [82,83].

For most reactions, enforcement of mass balance leads to specification of a system of differential equations to describe this reaction network [39]. Aleman-Meza et al. explain how the reaction network captured in the circuit of Fig. 4 is translated into a coupled system of nonlinear differential equations [40].

3.1. Water Models

Because most of the examples here are drawn from respiration, the modeling framework is illustrated with one of the simplest examples of combustion, the mixing of molecular oxygen and hydrogen, as shown in Fig. 4. This network diagram is the model. Circles denote reactions, and squares denote reactants or products. The arrows define the forward direction of a particular reaction. Incoming arrows lead from reactants, and outgoing arrows lead to reaction products. The end product is water, and we term this simplest of models, water model 1 (or the simple water model). Take reaction 1 as an example:



(with left to right as the forward reaction). Each such reaction has a pair of reaction constants: the forward reaction constant (k_f) and backward reaction constant (k_b). The net

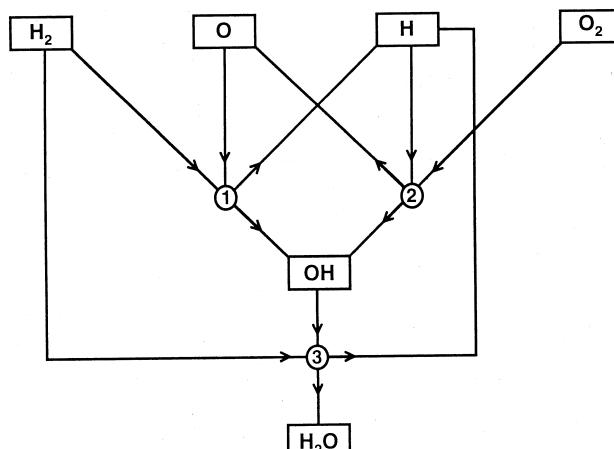


Figure 4 Water model 1 is a simple example of a chemical reaction network. Squares indicate chemical species, and circles indicate chemical reactions. Species with arrows pointing into a reaction are reactants, and species with arrows towards themselves and out of a reaction are products. In essence, the arrows define the forward reaction direction.

rate of production of species OH due to reaction R1 would be given by the simplest multiplicative kinetics by [65]:

$$\frac{d[OH]}{dt} = k_f [H_2][O] - k_b[H][OH]$$

where, for example, $[OH]$ denotes the concentration of OH at time t

The total rate of production of a species is then obtained by summing over reactions, containing OH for instance:

$$\frac{d[OH]}{dt} = \sum_r \frac{d[OH]}{dt}$$

The system of six differential equations characterizing the behavior of the reaction network can be found in [40], and the reader is encouraged to try the simulator KINSOLVER for this simple reaction network, found at <http://gene.genetics.uga.edu/stc>. With the advent of simulators like KINSOLVER, the focus for biologists then simply becomes to identify the biological circuit. This is the model.

As chemists have accumulated more kinetics data, they found the initial reaction network was an oversimplification of what makes some rockets go up (i.e., H_2 and O_2). The reaction network or circuit needed to be refined to that in Fig. 5 (water model 2). This inclusion of additional species and/or reactions is typical of building a model that fits a biological system.

3.2. Carbon Metabolism

A slightly more complicated biological circuit can be constructed for one of the two early paradigms for eukaryotic gene regulation [84,85] along with the *GAL* cluster in *S. cerevisiae*.

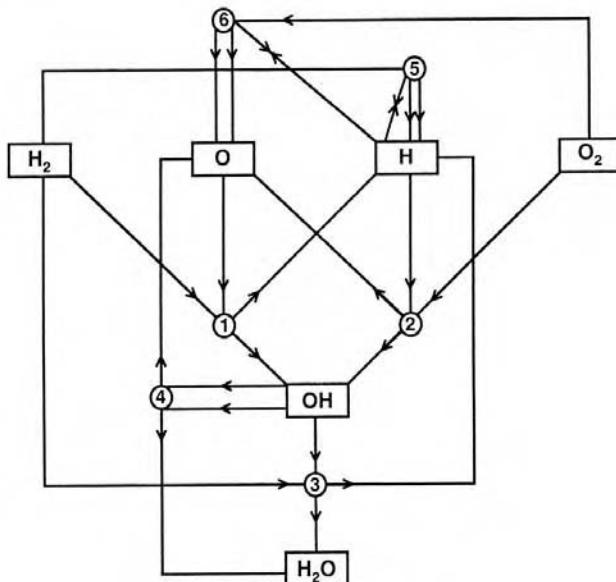


Figure 5 Water model 2 is an elaboration of water model 1, as dictated by being able to predict the kinetics of the reactions.

Network Model of *qa* Cluster

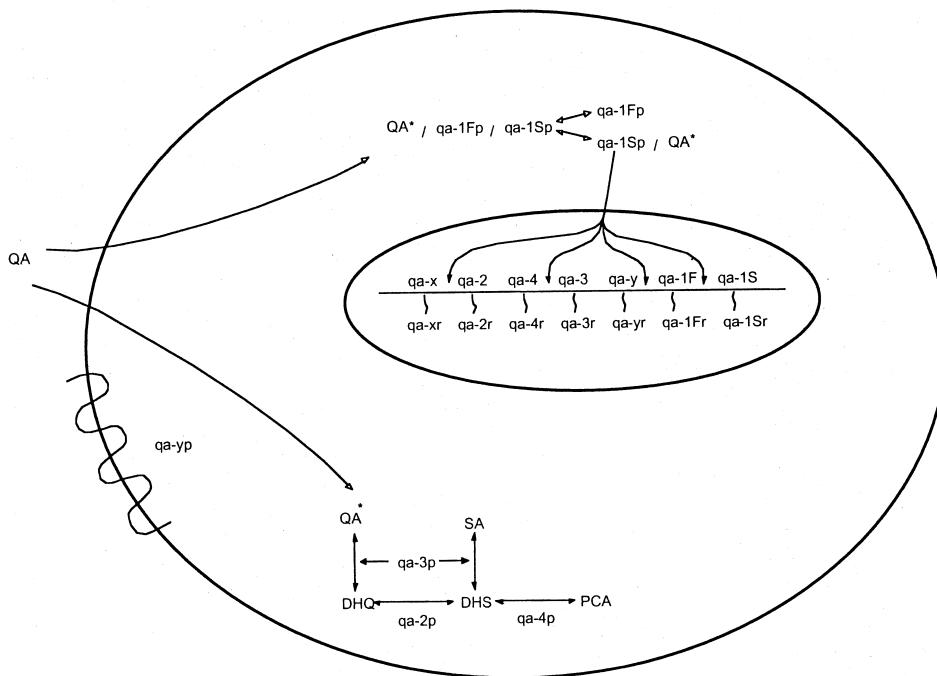


Figure 6 A pictorial summary of what is known about QA metabolism or informal biological circuit for QA metabolism. There are seven genes in the *qa* cluster that are coordinately regulated. Four of the genes are thought to participate in QA metabolism. Two of the genes are regulators of QA metabolism. The gene *qa-1F* is a transcriptional activator; the gene *qa-1S* is a repressor that is hypothesized to bind to the activator to shut down the genes in the cluster. The *qa-y* gene is thought to encode a permease, allowing QA into the cell.

iae [86,87]. Specifying the model in Fig. 6 begins by writing down the chemical reactions of the known participants in quinic acid (QA) metabolism. The circles on the wiring diagram denote reactions and boxes denote reactants. Arrows indicate the reactants entering a reaction, and outgoing arrows indicate the products of a reaction. Some reactions have no outgoing arrows, and they (the lollipops) are decay reactions. At the top of the circuit, reactants include the seven genes in the *qa* cluster (*qa-x*, *qa-2*, *qa-4*, *qa-3*, *qa-y*, *qa-1S*, *qa-1F*) [85]. These genes can be in either an unbound or a bound state with a transcription factor produced by the *qa-1F* gene as indicated by a superscript 0 or 1, respectively. These genes are, in turn, transcribed into messenger RNAs (superscripted with an r), which, in turn, are translated into proteins superscripted with a p (a slight departure from convention). A total of four of the seven protein products participate on a known biochemical pathway at the bottom of the diagram. In the circuit, there are two hypothesized cellular states for quinic acid, extracellular (denoted with an e) or intracellular quinic acid (QA). One of the genes, *qa-y*, is thought to encode a permease, *qa-y^p*, which may be involved in the transport of quinic acid into the cell. One hypothesized protein–protein interaction exists in the

model between the repressor, $qa\text{-}IS^p$, and the transcriptional activator, $qa\text{-}IF^p$. Quinic acid in the cell is hypothesized to be the cell signal that disrupts the bound complex of $qa\text{-}IS^p/qa\text{-}IF^p$ to favor induction by $qa\text{-}IF^p$ [85]. This story is summarized in Fig. 6.

This story is converted into a formal biological circuit in Fig. 7. The top structure to the circuit is the central dogma. At the bottom of the circuit is a piece of a biochemical pathway metabolizing QA. The pathway feeds into the Krebs cycle. The $qa\text{-}IF^p$ acts to create a feedback loop to activate the cluster and itself. When sucrose is added to the medium, a mechanism for catabolite repression is hypothesized, in which the presence of sucrose favors the binding of the repressor protein $qa\text{-}IS^p$ to the transcriptional activator $qa\text{-}IF^p$. At this time, the boxes in the third and fourth rows are the observables. The circuit can be simulated over the web at <http://gene.genetics.uga.edu/stc> as described by Aleman-

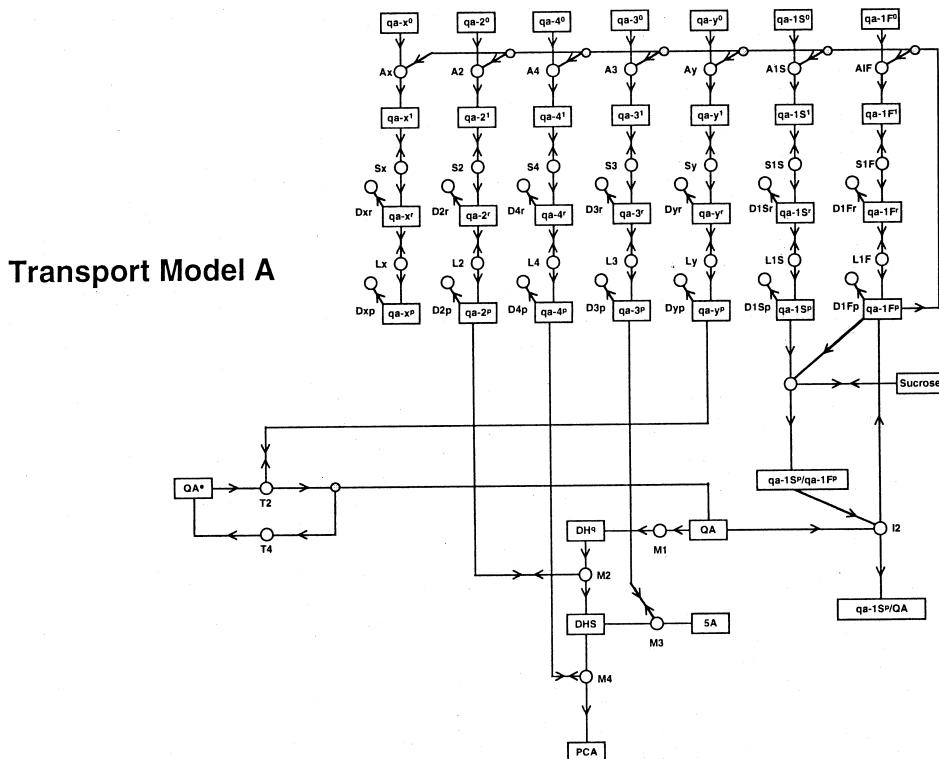
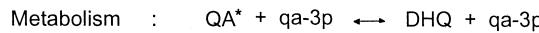


Figure 7 A formal biological circuit for the *qa* cluster is presented. The top part of the circuit represents the central dogma, while the bottom part of the circuit is the biochemistry. The top row of squares represents the transcriptionally inactive forms of the genes. The second row of squares from the top is the set of transcriptionally active genes bound to the activator protein $qa\text{-}IF^p$. The third row of squares is the set of the cognate RNAs, which are translated in the fourth row into polypeptides. In the bottom half of the diagram, the polypeptides are carrying out their biochemical functions. There is a feedback loop created by the transcriptional activator. The repressor $qa\text{-}IS^p$ is shown binding to $qa\text{-}IF^p$ to inactivate same. Sucrose acts to facilitate this repression reaction, acting as a catabolite repressor. The end metabolic product shown is protocatechui acid, which eventually leads into the Krebs cycle. The $qa\text{-}y^p$ polypeptide acts a transporter for QA.



$$\frac{d [\text{qa-2 / qa-1Fp}]}{dt} = +k_f [\text{qa-2}] [\text{qa-1Fp}] - k_b [\text{qa-2 / qa-1Fp}]$$



$$\frac{d [\text{qa-3p}]}{dt} = +k_f [\text{QA}^*] [\text{qa-3p}] - k_b [\text{DHQ}] [\text{qa-3p}]$$

Figure 8 The formal biological circuit specifies a system of ordinary differential equations describing the kinetics of all species. Each reaction contributes to the specification of the time rate of change of the species involved. In the first reaction, the transcriptional activator $qa\text{-1F}^p$ binds to the inactive gene to form the complex representing the transcriptionally active form of the gene. The forward reaction involving the collision of the $qa\text{-2}$ gene with $qa\text{-1F}^p$ occurs at a rate determined by the forward reaction rate k_f and the product of the molar concentrations indicated in brackets to produce the transcriptionally active complex $qa\text{-2/q-1F}^p$. In the backward reaction, the complex falls apart at a rate determined by the backward reaction constant k_b and the molar concentration of the complex. Similarly, the instantaneous change in the $qa\text{-3}^p$ protein from the reaction converting QA to DHQ can be computed. The reactants must collide as determined by the forward reaction constant and their concentrations, and the products must collide for the backward reaction to take place as determined by the backward reaction constant and the product of concentrations.

Meza et al. [40]. Some examples of the equations describing the 50 reactions can be found in Fig. 8. In this example transcriptional regulation is a reaction in which the transcriptional activator $qa\text{-1F}^p$ binds to the inactive gene like $qa\text{-2}$. One of the metabolic reactions is shown in which the enzyme $qa\text{-3}^p$ converts intracellular QA^* into dehydroquinate (DHQ).

3.3. The *lac* Operon

The classic example of a biological circuit and the first one to be worked out is the *lac* operon [15]. The top structure of the circuit reflects the central dogma in Fig. 9. The transition from inactive transcriptional state (i.e., $lacY^0$) to an active transcriptional state (i.e., $lacY^1$) is coupled to the transition from active transcriptional (i.e., $lacZ^1$) to an inactive transcriptional state (i.e., $lacZ^0$), as the RNA polymerase is handed from one gene to the next to form a polycistronic message. The $lacI^p$ protein binds to the operator in the absence of lactose. The catabolite repressor protein, crp^p , acts as a positive activator (like $qa\text{-1F}^p$) by stabilizing the recruitment of the RNA polymerase to the promoter site. The biological circuit differs from the usual story told in texts by inclusion of the internal signaling cascade linking PEP in glycolysis to the glucose transporter [87]. This particular circuit is about twice the size of the *qa* cluster circuit and still leaves out components of the Embden-Meyerhof pathway linking glucose-6-phosphate to PEP. Again, the circuit can be simulated over the web at <http://gene.genetics.uga.edu/stc>.

3.4. The *trp* Operon

One other classic circuit illustrating translational control is the *trp* operon [88]. As summarized in Fig. 10, there are two configurations of the RNA: one in which the ribosome is

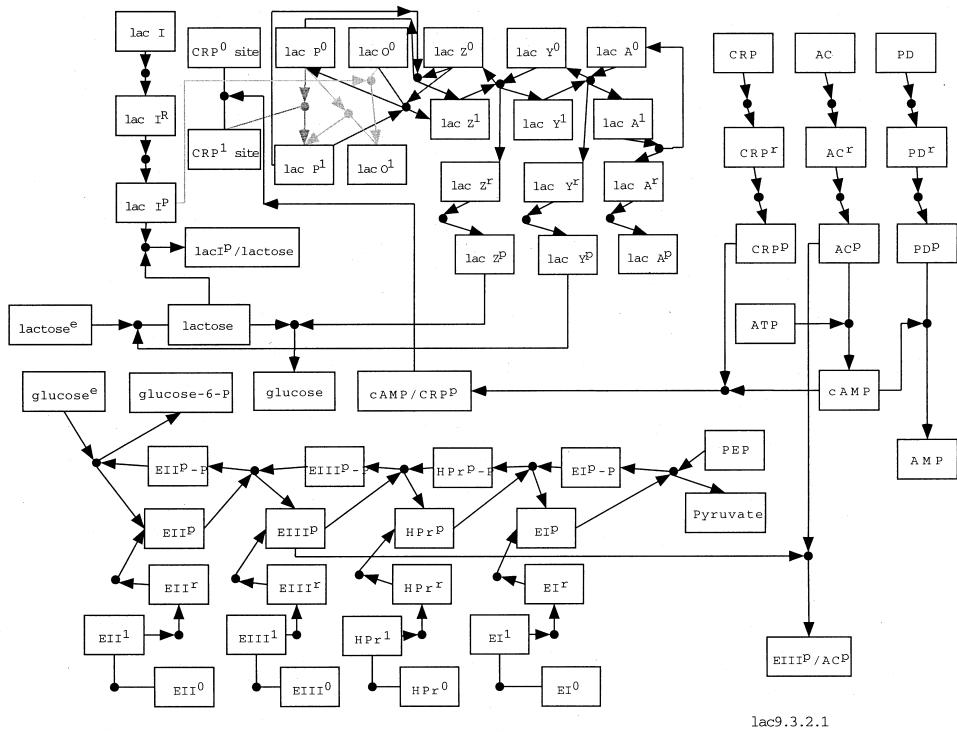


Figure 9 The biological circuit for the *lac* operon is more elaborate than that of the *qa* cluster. The *lacI^P* repressor can bind to the operator to shut down the cluster through a negative feedback loop unless lactose is present to bind to *lacI^P*, thereby titrating out the repressor. There is also a positive feedback loop provided by the catabolite repressor protein *crp^P*, which aids in the recruitment of RNA polymerase to the promoter (*lacP⁰*). The catabolite repressor protein is only active when bound to cAMP. The enzymes *ac^P* and *pd^P* make cAMP from ATP and convert cAMP to AMP, respectively. An internal signaling cascade including (*eI^P*, *eII^P*, *eIII^P*, and *HPr^P*) is included to take a phosphate on phosphoenolpyruvate (PEP) to glucose to pump glucose into the cell as glucose-6-phosphate.

stalled at a *trp* codon and one in which the ribosome is not stalled. When there is plenty of tryptophan, there is a feedback loop created in which the RNA forms a structure that leads to transcription termination such that no proteins are made. In the other configuration, the RNA polymerase transcribes the downstream structural genes, and the RNA gets translated. In addition, a feedback loop involving *trpR^P* which, in contrast to the *lac* operon, is activated in the presence of the metabolite to shut down the *trp* operon. The pathway is at the bottom of the circuit.

3.5. Examples of Biological Circuits Relevant to Agriculture, Industry, and Medicine

A preliminary circuit can be constructed for aflatoxin biosynthesis from the 25 known components of the sterigmatocystin cluster [89] and the identification of a positive regula-

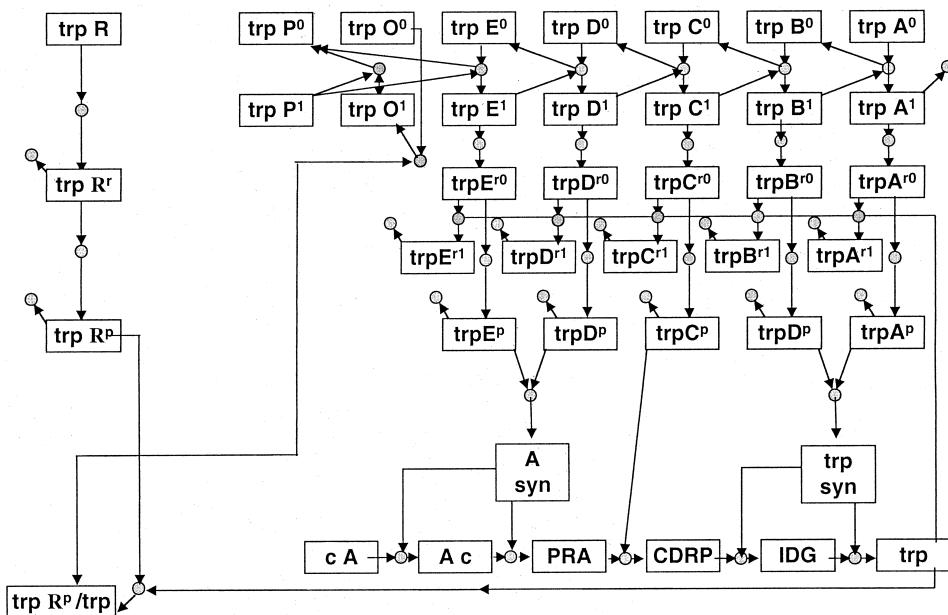


Figure 10 The *trp* operon differs from the *lac* operon in having translational control. Tryptophan synthesis provides feedback to attenuate translation. If tryptophan is rare in the cell, then the message assumes one configuration efficient for translation. If tryptophan is at high levels in the cell, the message assumes an altered conformation with the ribosome not conducive to translation. In addition, there is a repressor *trpR^P* acting on the operator to shut down the operon, and the repressor is activated in the presence of tryptophan as would be expected for a biosynthetic pathway.

tor of aflatoxin biosynthesis [90]. A preliminary circuit is available at <http://gene.genetics.uga.edu/stc>, but it is much larger than the other circuits described here. Mechanisms of negative regulation of the pathway are yet to be identified. This *A. flavus* system is one of the few approved for USDA piloting of release of competing strains to displace those strains producing aflatoxin. In this case, the circuit could help to identify which mutations are likely to be most effective in knocking out aflatoxin biosynthesis in genetically engineered strains and in determining how aflatoxin biosynthesis is triggered.

Another important example is the penicillin gene cluster in *Aspergillus nidulans* and *Penicillium chrysogenum* [47]. The cluster, with its approximately three genes, is conserved in prokaryotes and eukaryotes [91] with a partially specified regulatory system and may have arisen in fungi by horizontal transfer from a prokaryote such as *Streptomyces*. The regulation of the penicillin cluster at first sight appears more complicated than the paradigms like the *qa* cluster. The regulation of penicillin synthesis appears to be tied to biological circuits for carbon metabolism, pH sensing, and nitrogen metabolism as examples [47]. For example, Suarez and Penalva [92] present evidence that a *pacC* transcription factor involved in pH sensing may bind to an intergenic region between *acrA* and *ipnA* genes in the *P. chrysogenum* penicillin cluster. A hypothesis for the pathway describing biosynthesis of penicillin is well developed. A biological circuit is likely to contain several kinds of feedback loops to incorporate connections to other circuits. A genetic perturbation

of this circuit is likely to interact with a process of amplification of the penicillin cluster by sited directed homologous recombination mediated by a conserved hexanucleotide sequence [93]. Relevant environmental perturbations include the carbon source and pH. Some kinetics models have already been tried.

The final example is drug discovery for *P. carinii* (*Pc*), the major killer of AIDS patients [2]. An ATP bioluminescent assay for *in vitro* screening of anti-*Pc* drugs has been developed [94]. With the resources of the *Pneumocystis* Genome Project [37,95], more than 2,000 distinct cDNAs have been generated and partially sequenced. This cDNA collection includes genes such as *erg-9*, *erg-1*, and *erg-7* in sterol biosynthesis. A partial reaction network for sterol biosynthesis can be hypothesized by reference to KEGG [19]. The cDNA collection can be then exploited for transcriptional profiling to understand the mechanism of action of existing anti-*Pcs* (e.g., TMP-SMX, pentamidine, and atovaquone) for highlighting new potential drug targets in sterol biosynthesis and other critical pathways and for evaluating the proposed reaction network for sterol biosynthesis. The approach is to perturb the system with an array of potential protein inhibitors, observe the response with the ATP bioluminescent assay and transcriptional profiling, fit a hypothesized reaction network, evaluate the model, modify the model and perturbations, and repeat the cycle to discover drugs and their mechanism of action.

3.6. Simulating Arbitrary Reaction Networks Satisfying Mass Balance

A number of simulators now exist that simulate arbitrary reaction networks that satisfy mass balance. These include METAMODEL [96], GEPASI [97,98], SCAMP [99], KIN-SIM [100,101], MIST [102], E-CELL [103], and KINSOLVER [40]. These packages differ in the diversity and type of numerical solution methods for the systems of differential equations illustrated in Fig. 8. The simulators also differ in their ability to be used on different types of computers or over the web. Lastly, the simulators differ in their capability to examine many reaction networks at once relevant to a particular system [104].

3.7. Steady-State Approximations to Simplify Biological Circuits

A classic approach to simplifying the reaction network is to make steady-state approximations to obtain simplified kinetics [65]. The classic example is Michaelis-Menten (MM) kinetics derived from the reactions in Fig. 3 by making a steady-state approximation with respect to the level of enzyme complex (ES). With a general purpose simulator, this is not necessary and can in some cases be positively misleading. For example, the MM formulation tends to break down when there are multiple substrates for the enzyme.

With this caveat, it may be possible to simplify the kinetics model by steady-state approximations to reduce the number of parameters and to gain interpretability of the model (i.e., heuristic appeal). One example is shown in Fig. 11.

The deterministic model in Fig. 11 is a steady-state approximation to the full model in Fig. 7 in which the velocities for the concentrations of the bound-state of the genes are assumed approximately constant (i.e., $d[q_a - x^1]/dt = C$). In this model there are two sets of promoters: one set that is QA inducible and one set that is not QA inducible [105]. QA-independent rates of transcription of the activator (f), repressor (s), and structural genes (sg) are denoted by α_f , α_s , and α_{sg} . In contrast, the rate of production of message induced by QA is proportional to the level of inducer and activator protein. The rates of QA-inducible transcription of activator, repressor, and structural genes are denoted by δ_f ,

$$\begin{aligned}\frac{d m_f}{dt} &= -m_f + \frac{\delta_f p_f Q(t)}{1 + \gamma_f p_s^n} + \alpha_f \\ \frac{dp_f}{dt} &= -\beta_f p_f + m_f \\ \frac{d m_s}{dt} &= -m_s + \delta_s p_s Q(t) + \alpha_s \\ \frac{dp_s}{dt} &= -\beta_s p_s + m_s \\ \frac{d m_{sg}}{dt} &= -m_{sg} + \frac{\delta_{sg} p_f Q(t)}{1 + \gamma_{sg} p_s^n} + \alpha_{sg} \\ \frac{dp_{sg}}{dt} &= -\beta_{sg} p_{sg} + m_{sg}\end{aligned}$$

Figure 11 Steady-state approximations to the levels of some species can be used to reduce the number of model parameters in a biological circuit. After assuming that the levels of transcriptionally active genes are in steady state, the system of ordinary differential equations for the full biological circuit in Fig. 6 can be approximated by the reduced model specification below. It is enough to describe the message levels denoted by m and protein levels denoted by p . The α 's denote basal transcription rates, the δ 's, the QA inducible transcriptional rates, the γ 's, the repressor effects, and the β 's, the rates of protein decay. The subscripts f, s, and sg denote the *qa-IF*, *qa-IS*, and structural genes in the *qa* cluster.

δ_s , and δ_{sg} , respectively. The repressor interacts with the activator, and the effect of the repressor on transcriptional activation is captured in the repressor effects γ_f and γ_{sg} . Message levels (m_x) decay at the same rate in proportion to their level. The Hill Coefficient, n , is introduced as a shape parameter for the cooperative effect of repressor polypeptides on message levels. In this model, there is no posttranscriptional regulation. All messages are translated at the same rate, and protein levels (p_x) have different constant rates of decay of β_f for the activator, β_s for the repressor protein, and β_{sg} for the structural gene proteins. The number of parameters is reduced to 42, and the model in Fig. 11 is analytically tractable [146].

3.8. Stochastic Circuits

McAdams and Arkin [106] have presented evidence that stochastic factors play an important role in the λ switch response. Kepler and Elston [107] have also demonstrated that stochastic factors can play an important role in transcriptional control through the recruitment of RNA polymerase to the promoter. Abastado et al.[108] have made the case for stochastic factors in translational initiation by ribosome scanning of the uORFs upstream to *GCN4* [109]. The extent to which such stochastic factors play a role in most biological circuits is unknown. Gillespie [82] established a framework for stochastic kinetic models, which under certain regularity conditions, converge to the deterministic circuits satisfying mass balance described in the previous modeling sections.

The formulation of the model, as with deterministic models, begins by writing down the circuit diagram or, equivalently, the tables of hypothesized reactions as in Fig. 12. The formulation of a stochastic circuit is illustrated with the *qa* gene cluster circuit. From a microscopic point of view, binding of a free inducer molecule (i.e., quinic acid in the cell), activator, and repressor to the activator, gene, or activator, respectively, is very likely

to be a random process because of the low concentrations of the reactants in the cell [107,110].

In Fig. 12, time can be taken to advance in discrete steps due to random collisions of molecules participating in the reactions, where mF_{unl} and mS_{unl} are the basal number of mRNAs for *qa-IF* and *qa-IS*; mF_I and mS_I are the number of induced mRNAs for *qa-IF* and *qa-IS*; $qa-IF^p$ and $qa-IS^p$ are the number of protein molecules encoded by *qa-IF* and *qa-IS*; and mF_R is the number of transcriptional activators bound to a repressor protein. The quantities Z_i represent the numbers of product molecules, and the constants k_i are reaction rates. The sources A,B are the *qa* cluster DNA and assumed constant. As in Fig. 12, similar reactions can be written down to specify the role of the structural genes in the reaction scheme. The model is a discrete-time denumerable Markov chain [111]. A formal relation among the parameters in Fig. 12 and the reaction rates can be established following Gillespie [82]. For example, $\alpha_\phi = k_1 A \tau V / m_{fmax}$, where τ is the time-scaling

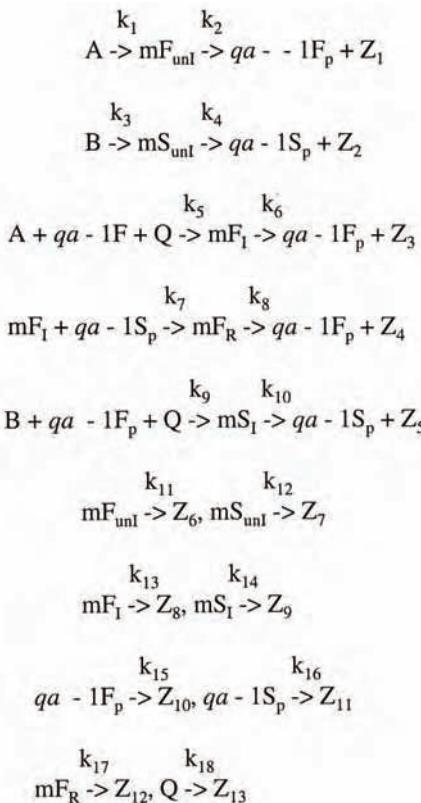


Figure 12 Part of the list of master equations for a stochastic circuit with the same structure as Fig. 7 is listed. Here mF_B and mS_B are the basal number of mRNAs for *qa-IF* and *qa-IS*; mF_I and mS_I are the number of induced mRNAs for *qa-IF* and *qa-IS*; and mF_R is the number of transcriptional activators bound to a repressor protein. The quantities Z_i represent the number of product molecules, and the constants k_i are reaction rates. The sources A,B are the *qa* cluster DNA and assumed constant.

parameter, $m_{f\max}$ is a concentration-normalization coefficient, and V is a volume factor. Recently, Kierzek [83] developed methodologies for simulating stochastic networks.

3.9. Limitations of Reaction Network Models

3.9.1. Too Many Parameters and Too Few Data

With each new species, a new parameter—its initial concentration—is added. With each new reaction, two new parameters—the forward and backward reaction constants—are added. In general, only a subset of the species are observed over time. The major problem is identifying one model that fits one reaction network with limited and noisy profiling data. To address this problem will require novel fitting procedures.

3.9.2. We May Not Have All the Pieces

To overcome this problem, any modeling, fitting, and model-evaluation framework must be general enough so that discovery of new species during profiling or new topological features during protein–protein interaction mapping can be included in the circuit. For example, a general purpose simulator KINSOLVER [40] is required.

3.9.3. Stochastic Factors May Play a Significant Role

Stochastic factors may play a significant role in the reaction network [106,112]. As a consequence, it is important to build on the work of Gillespie [82] and Kierzek [83] to generalize a deterministic simulator for an arbitrary reaction network satisfying mass balance, as Tomita et al. [103] have begun to do.

3.9.4. The Cell Is Not Well Stirred

That the cell is well stirred is a basic assumption of the family of models proposed. Weng et al. [113] point out that consideration needs to be given to cellular compartments, scaffolding, and reaction channeling. Compartmentalization can be handled in part by simulators like KINSOLVER by indexing the species by the compartment containing them [114]. Similarly, scaffolding and channeling can be represented by allowing for additional concentration variables and corresponding reactions for chemical species participating in a protein scaffold or reaction channel. Another option is the approach of E-CELL [103], which is to introduce another table describing the compartmentalization of reaction species.

3.9.5. Higher-Order Kinetics May Come into Play

The formal model is based on collision dynamics determining the right-hand side (RHS) of the coupled differential equations, like those in Fig. 8. Any number of reactants or products can be introduced into a particular reaction, allowing higher-order kinetics. The more standard nonmultiplicative MM kinetics can be derived as steady-state approximations to the full reaction network as in those based on collision dynamics [65], as was done in Fig. 11.

4. PERTURBATION, PREDICTION, AND OBSERVATION

4.1. Emergent Properties as a Predicted Response

Once a systems approach is embraced, an experimental framework is needed to study the global response of the system. One approach is to perturb the system experimentally and

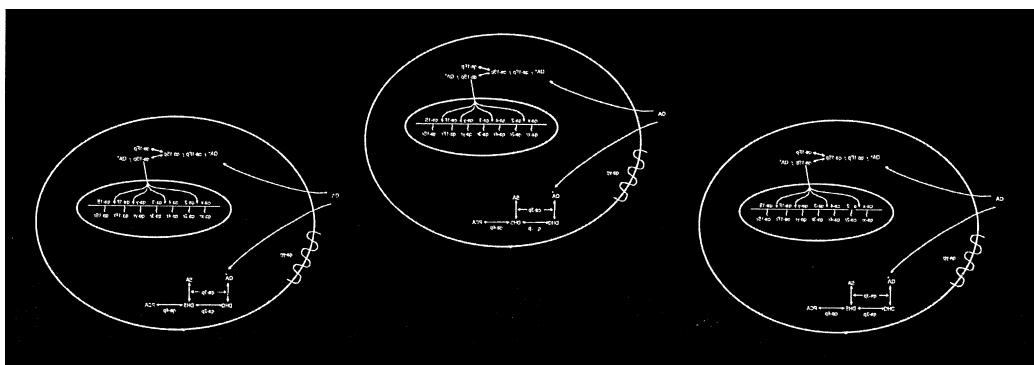


Figure 13 Three kinds of system perturbations are illustrated for the *qa* cluster: (1) genetic; (2) chemical, as in a drug; or (3) environmental.

then to measure the global system response. Predictions are made about the effects of various system perturbations and then compared to the observed state of the cell through profiling. Experiments are designed to test the predictions. In a systems approach, the goal is to understand and recover the behavior of the entire system. The system is not take-it-apart, but rather it is perturbed, and its total response measured. The hope is to be able to predict its system-wide behavior.

System perturbations can fall into three broad classes as illustrated with respect to the *qa* cluster in Fig. 13. They can be genetic in nature, such as gene mutations or more specifically, gene knockouts. A gene mutation in the *qa-2* gene removes its function in Fig. 13. Perturbations can be chemical in nature, such as adding a protein inhibitor to the medium to inhibit *qa-3P*. This kind of perturbation would characterize the search for drugs to inhibit essential activities in organisms such as *Pneumocystis*. Finally, a perturbation can be environmental in nature, such as a change in carbon source (i.e., sucrose for quinic acid). In each case, the response is predicted from the simulation and compared to that observed to validate the circuit.

4.2. Genetic Perturbations Can Be a Challenge

The major challenge for perturbation experiments is carrying out targeted gene knockouts in *N. crassa* and other fungi with a low rate of homologous recombination. High-throughput strategies for directed and random signature tagged mutagenesis (STM) using transposons have been developed in bacteria and yeast [115–121]. Recently Hamer et al. [121] have successfully utilized an STM strategy on a close relative of *N. crassa*, the rice blast fungus *Magnaporthe grisea*.

The STM strategy used by Hamer et al. [121] shares many of the common elements of all STM strategies originally developed by Hensel et al. [116] and Burns et al. [115]. Loss of function mutations are generated with a transposon. A tag is introduced into the mutation. The tag contains a marker that can be selected for in the target system after transformation. Strategies differ on whether or not the mutations are generated in a targeted way [119] or randomly [118] and whether or not they exploit homologous recombination present in the organism. They can also differ on the nature of the tag and whether or not

the collection of mutants is ultimately generated by a negative selection or screen. As knockout technology has progressed, there has also been a shift away from knockouts to conditional mutations and adding further functionality to the insertion cassette [122].

Hamer et al. [121] began the mutagenesis process with an engineered transposon cassette (containing a hygromycin resistance gene) that could be mobilized *in vitro* to mutagenize a large insert clone, such as a cosmid or BAC. The mutagenized cosmid or BAC is then transformed into the target organism such as *M. grisea* by selecting for hygromycin resistance. Polymerase chain reaction can then be used to screen for homologous recombination events. In this way, the researchers were able to generate 25,179 insertion mutants. A total of 33% of these insertion mutants were identified to have homologues in public databases. One example of an insertion mutant included insertions in the pathogenicity gene MAC1. The STM approach has also been used successfully to isolate pathogenicity islands in *Candida glabrata* [123] and *Cryptococcus neoformans* [124].

A simple example is shown in Fig. 14. The control perturbation involves growing wildtype *N. crassa* on quinic acid alone, and the main product, protocatechuic acid (PCA), is graphed using the simulator [40]. The system is perturbed by introducing a mutation into the *qa-2* gene. The predicted result in Fig. 14 is no PCA, a block in QA metabolism with no growth on QA alone.

With each perturbation, one of several responses might be observed. A transient response may be predicted. As in the case of the *qa* cluster, a transient response may be initiated by the environmental signal of QA, but once the signal is removed, the whole circuit may shut down again. In contrast, even relatively simple circuits can display emergent properties [39]. For example, Gardner et al. [125] built a simple toggle switch that may mimic many coupled signaling pathways. The product of gene A represses gene B, and the product of gene B represses gene A. Such a simple system has a biphasic response [125]—i.e., memory of a previous signal even after the signal is removed. Another example of an emergent property manifested by a circuit is an oscillatory response. The classic example is the biological clock [16], but a simpler circuit called the *repressilator* has been

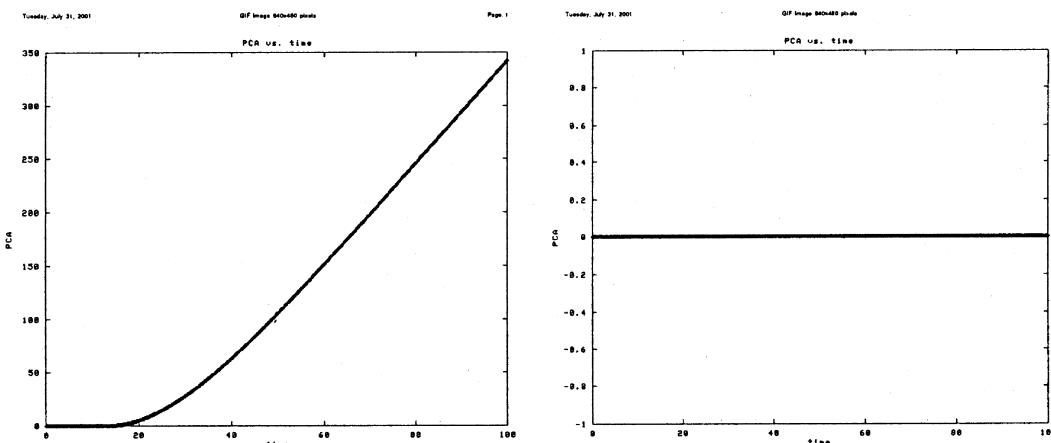


Figure 14 Response of PCA (protochatechuic acid) level with and without a *qa-2* gene knockout over time as simulated in KINSOLVER for the biological circuit in Fig. 6 [40].

engineered in *Escherichia coli* that oscillates [126]. Whatever the response of the biological circuit, if the model correctly predicts the emergent property, this serves as a validation of the model.

4.3. Observation by Profiling

An example of this prediction, perturbation, and observation process is given for the *qa* gene cluster. A quinic acid (QA)-inducible cDNA library was initially characterized. The QA-inducible cDNA library of 33 plates was robotically arrayed on one membrane [127]. Twelve replicates of the arrayed library were stamped, and one membrane was probed with an *AatII*-fragment of the H123E02 cosmid containing only the *qa* cluster [127]. Two of the positives in the cDNA library were sequenced and confirmed to be derived from messages of *qa-1F* and *qa-3*.

Transcriptional profiling allowed us to examine the outcome of an environmental perturbation and of a genetic perturbation [12]. The WT and mutant 246-89601-2A (a mutation in the *qa-2* gene) were shifted to 0.3% QA (with aromatic supplements only for the mutant) [128,129], and RNAs were isolated from WT and mutant 246-89601-2A at 4 time points after induction (30, 90, 120, or 240 minutes). These RNAs were reverse transcribed and radiolabeled to probe the cDNA arrays [127] as in Fig. 15.

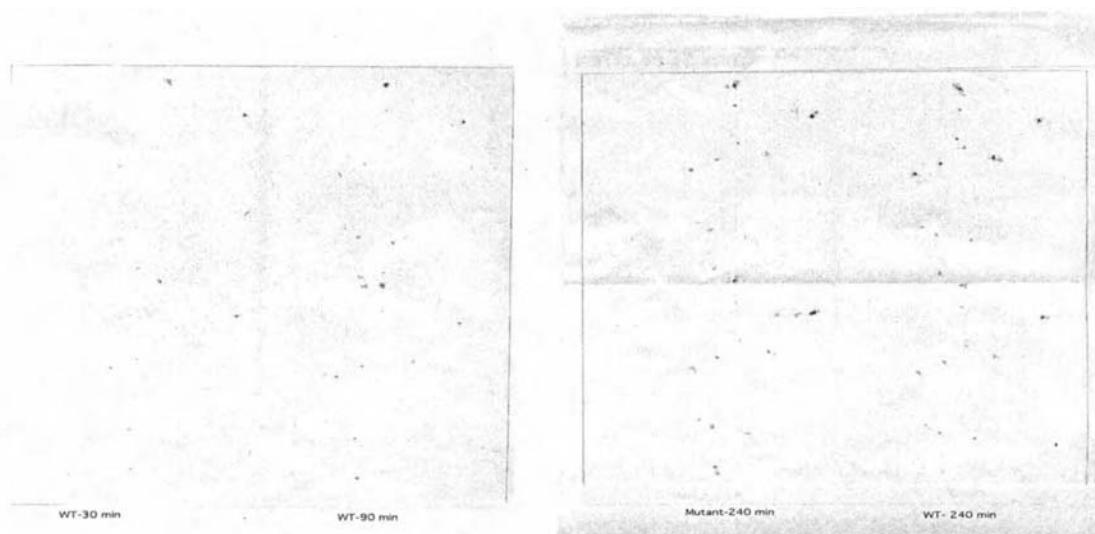


Figure 15 Transcriptional profiling: *N. crassa* was shifted from 1.5% sucrose to 0.3% quinic acid. A cDNA library derived from cells induced in quinic acid was robotically arrayed on nylon membranes (133). RNA was extracted from cells by grinding under liquid nitrogen with the High Pure RNA Isolation kit (Roche, Inc.). Simultaneous cDNA synthesis and ^{33}P radiolabeling were performed according to manufacturer directions. Unincorporated ^{33}P was removed by spin columns (Sigma, Inc.). Arrays were probed with ^{33}P -labeled cDNAs derived from three time points after the shift to quinic acid. Images were collected on a Packard Instant Imager over a 26-minute period. The *qa-2/aro-9* double mutant is also shown expressing the same transcripts at 240 minutes, but it does not grow.

As time progresses from left to right, more spots (genes) appear, and the intensities of the spots increase. The membranes are double-stamped so the spots appear symmetrically about the middle of each figure. A total of 12 genes (spots) appear to be QA-inducible by 240 minutes. Two of these genes were confirmed by end-sequencing to be *qa-1F* and *qa-3*. The remaining 10 genes did not hybridize to an *AatII* fragment of H123E02 (containing the entire *qa* cluster) at high stringency [127]. If so, this implies there are other genes outside the *qa* cluster that need to be considered in QA metabolism. For example, some of these 12 genes may be involved in a starvation response since QA is not a preferred carbon source. To distinguish these two hypotheses, the experiment needs to be repeated with a shift to a medium with no carbon source or starvation for an aromatic amino acid. The experiment was repeated with a *qa-2/aro-9* mutant, and the transcriptional profile at 240 minutes appeared identical to WT, although it did not grow when shifted to QA [128]. The experiment was replicated once with the same findings.

The profiling experiment was modified with a genetic perturbation. An *aro-9/qa-2* double mutant was transformed with the *qa-2 +* gene [130]. Transformant's RNA profile was obtained as previously described, except that the exposure time was increased to 1 hour on the Packard Instant Imager. The same 12 genes came up, but also an additional seven or eight cDNAs were positive. None of the additional positives matched to known *qa* cluster cDNAs.

The findings here are likely to be typical of studies that reexamine classic stories from a genomic perspective [9]. In Fig. 16, the expression of the 12 genes responding to

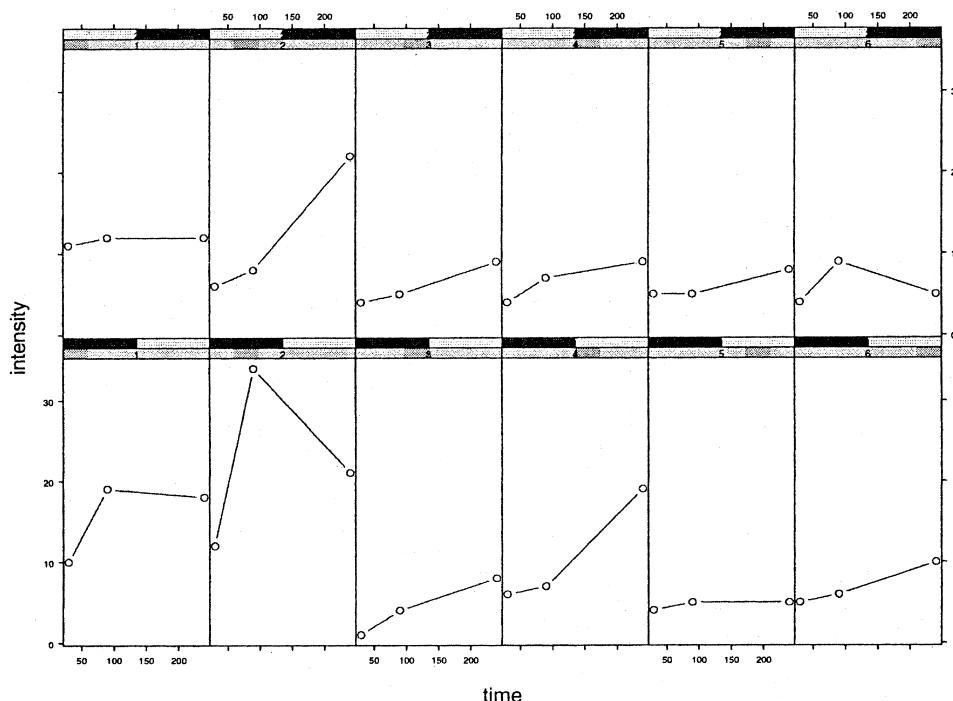


Figure 16 Twelve genes appear to respond to a shift from 1.5% sucrose to 0.3% quinic acid. The counts of 12 genes (from Fig. 15) recorded by the Packard Instant Imager are graphed as a function of time. Only two of these genes appear to be part of the *qa* cluster.

quinic acid, or possibly a starvation response, is shown. Only two of these genes appear to be part of the *qa* cluster. The remaining genes are unknown at this time. There are other genes that need to be included in the circuit in Fig. 7 because their response is not accounted for. New tools are being developed to permit scientists to explore relationships between genes uncovered in RNA profiling [131].

4.4. Protein–Protein Interactions: Observing the Links in a Circuit

Protein–protein interaction mapping is being pursued in a number of model systems [13,20,21,132]. Early approaches use the two-hybrid system to detect protein–protein interactions [20,21,132]. Two classes of strategies are being used to create the maps.

4.4.1. Clone-by-Clone Strategy

In a clone-by-clone strategy, one prey clone interrogates a robotically arrayed bait library by mating the yeast strain with the prey clone and each yeast strain with a bait clone. Interaction mating is achieved by robotically pinning the prey strain to all of the arrayed bait strains [21]. With yeast, this means that about 6000 potential interactions out of the 6000^2 can be examined at one time. While this approach is slow, the sensitivity to detect interactions is about three times that of the high-throughput screens.

4.4.2. High-Throughput Strategy

In a high-throughput strategy, some pooling scheme is employed. Ito et al. [20] pooled both bait and prey in pools of 96 and mated the pools. The resulting positives can be picked and sequenced to identify the interactors. Each interaction mating allowed the examination of 96^2 potential interactions. They found 183 interactions after scanning about 10 percent of the proteome. By extrapolation, about 2000 interactions are expected in the yeast proteome. Uetz et al. [21] created a 96-well plate of bait clones and then mated them with a strain containing a whole prey library. In this way, each experiment resulted in the examination of 96×6000 potential interactions. With their high-throughput strategy, they identified 957 potential protein–protein interactions out of about 2000 expected [20].

Their map can be visualized by tools like those of Fang et al. [44] as a protein mobile as shown in Fig. 17. In this protein mobile, nodes are proteins and edges are detected interactions. This protein mobile then becomes a search grid on which the scientist refines a biological circuit. Possible links in the circuit are, in part, guided by the links reported in the protein mobile presented in resources like BIND [149].

There are a number of limitations of two-hybrid screens. Numerous false positives and false negatives occur as evidenced by the lack of overlap between screens conducted by Ito et al. [20] and Uetz et al. [21]. Also, promiscuous proteins show up, repeatedly interacting with other proteins. Something may be missed in the original target system to make an interaction go in the *S. cerevisiae* or *E. coli* detection system. As a consequence, other approaches to building protein–protein interaction maps are being pursued.

Gavin et al. [13] describes how to use tandem affinity purification (TAP) in conjunction with MALDI-TOF mass spectrometry to characterize protein complexes in *S. cerevisiae*. By this method, they were able to identify 232 distinct protein complexes. A total of 58 of these complexes had not been previously reported. The major limitation of their approach was the use of two-dimensional protein gels to separate proteins, thereby setting aside insoluble proteins. Ho et al. [133] described a related approach.

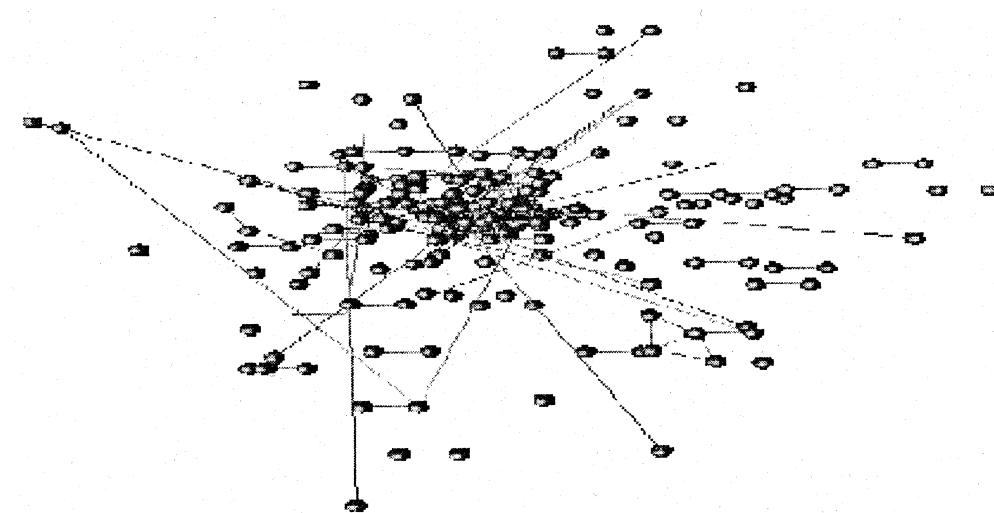


Figure 17 Protein mobile of protein–protein interaction map presented by Ito et al.[20]. Nodes represent proteins, and edges represent interactions. Graphic was generated by software described in part in Fang et al. [44].

4.5. Protein–DNA Interactions: Observing the Links in a Circuit

Other resources are needed for systematically reconstructing a biological circuit. One is a map of all protein–DNA interactions [134]. At this time, all that is available is the equivalent of a clone-by-clone screen of protein–DNA interactions.

4.6. Web Services to Unite the Bioinformatics Pool of Data Sources

Data sources for profiling data, protein–protein interaction, and protein–DNA interaction data, and metabolic pathway information for reaction network modeling have too many differences that inhibit unified access and interoperability of data sources [10,37]. For example, bioinformatics databases such as BIND, KEGG, NCBI, Ensembl, FlyBase, SGD, WormBase, and UCSC all provide relevant data, but they are use a wide range of different systems and formats [135]. Researchers wishing to integrate these data need to write hundreds and even thousands of different programs to carry out the integration of data sources without any assurance of correct enactment of bridging services. To unify these bioinformatics services, providers may adopt a Web services model [136].

A Web service is any piece of software that makes itself available over the Internet and uses a standardized XML messaging system. A Web service can have a public interface, defined in a common XML grammar. The interface describes all the methods available to clients and specifies the signature for each method. Currently, interface definition is accomplished via the Web Service Description Language (WSDL). Furthermore, if a web service is created, there should be a simple mechanism to publish it. There should also be a mechanism for interested parties to locate the service and its public interface. The most prominent directory of web services is currently available via Universal Description, Discovery, and Integration (UDDI). In this web services model, the data providers register their services in a formalized service registry, and researchers' scripts no longer need to

be concerned with the interface details of the different databases. This model may represent a unification platform needed in bioinformatics [135].

A number of bioinformatics services are currently available [137]. For example, the OmniGene project [138] from MIT aims to create an open source web services platform for bioinformatics. Additionally, the Distributed Annotation Service (DAS) provides a distributed platform for aggregating genome annotation data from multiple sources [139]. Lastly, the BioMOBY project aims to provide distributed access to multiple bioinformatics services and provides a centralized registry for finding new services. All of these projects are likely to see much growth in the near future.

5. FITTING BIOLOGICAL CIRCUITS

The profiling information together with protein–protein and protein—DNA interaction maps provide the information necessary to identify biological circuits. After system perturbation, the profiling information either agrees with the predictions of the circuit or does not. A figure of merit can then be used to guide the selection of a biological circuit that is consistent with the profiling data from the system in different cellular states. The information about links in the circuit can be used both to constrain the fitting process and to guide the comparison of new models evaluated for fit relative to the existing best model. We will describe the standard fitting approach for reaction networks:

Let the parameters in the biological circuit be denoted by the M-tuple, $\theta := (\theta_1, \dots, \theta_M)$. In the case explored here, the parameters are the rate constants k_f and k_r , for all reactions $r = 1, 2, \dots, M_R$ as in the reaction network of Fig. 11 and the initial concentrations $[s]_{t=0}$ for all intracellular species $s = 1, 2, \dots, M_S$. The number of parameters is $M = M_S + 2M_R$. For the deterministic model in Fig. 11, the rate constants and initial conditions are:

$$\theta = (\alpha_f, \alpha_s, \alpha_{qa-2}, \alpha_{qa-3}, \alpha_{qa-4}, \alpha_{qa-x}, \alpha_{qa-y}, \beta_f, \beta_s, \beta_{qa-2}, \beta_{qa-3}, \beta_{qa-4}, \beta_x, \beta_{qa-y}, \gamma_f, \gamma_{qa-2}, \gamma_{qa-3}, \gamma_{qa-4}, \gamma_{qa-x}, \gamma_{qa-y}, \delta_f, \delta_s, \delta_{qa-2}, \delta_{qa-3}, \delta_{qa-4}, \delta_{qa-x}, \delta_{qa-y}, \kappa_0, Q_0, m_{f,0}, m_{s,0}, m_{qa-2,0}, m_{qa-3,0}, m_{qa-4,0}, m_{qa-x,0}, m_{qa-y,0}, p_{f,0}, p_{s,0}, p_{qa-2,0}, p_{qa-3,0}, p_{qa-4,0}, p_{qa-x,0}, p_{qa-y,0})$$

with unit Hill coefficient

In the following, this parameter vector shall be referred to as “model θ ”:

Next, let $Y := (Y_1, \dots, Y_D)$ represent the D-tuple of all experimental observables which have been measured in one experiment or a series of time-dependent profiling experiments. Suppose that in a series of E experiments, labeled by $e \in E = \{1, \dots, E\}$ experiments, in each experiment the concentrations $[s]$ of certain species s are measured at time points t . Different experiments would be distinguished by externally controlled and quantitatively known experimental conditions which include, the carbon source and its concentrations, feeding/starvation schedules, choice of measurement time points, and functional presence or absence of certain genes or proteins, as controlled by mutations or protein inhibitors. The data vector Y would then comprise components:

$$Y_l := Y_{s,t,e} := ([s]_{t,e}/[s]_0) \quad [\text{with } l := (s,t,e)]$$

with some (known or unknown) reference concentration $[s]_e^{(\text{ref})}$, if for example, some linear measure of concentration is used or:

$$Y_l := Y_{s,t,e} := \ln ([s]_{t,e}/[s]_0) \quad [\text{with } l := (s,t,e)]$$

if log-induction ratios [12] are recorded. Here $l := (s,t,e)$ and $s \in S'$ labels the $M_{S'}$ different molecular species, with S' denoting the subset of all species whose time-dependent concen-

trations actually have been observed. Note that in general, S' is only a subset (generally a small one) of the set S of all M_S participating species in the biological circuit. With $t \in \{t_1, \dots, t_{M_t}\}$ labeling the M_t different time points at which species concentrations have been measured, the dimensionality of the data vector Y is then:

$$D = M_S' \times M_t \times E.$$

For the present mRNA profiling data set in Fig. 15 for the *qa* cluster, $E = 1$, $M_S' = 6$, $M_t = 7$, and $D = 42$.

Now, let $F(\theta) = [F_1(\theta), \dots, F_D(\theta)]$ denote the corresponding predicted values for these observables Y for a given model θ . For the previously described set of observables $Y_{s,t,e}$, the predicted values $F_l(\theta) = F_{s,t,e}(\theta)$ [with $l = (s,t,e)$] are calculated from θ by solving the network's system of rate equations for the rate constants and initial conditions comprised in θ using the simulator KINSOLVER [40] and then calculating from that solution the log-concentration ratio $\ln([s]_t/[s]_0)$ or the respective linear concentration measure for each observed species s at each observation time point t in each experiment e .

It is reasonable to assume that the probability distribution $P(Y; \theta)$ of the data is representable as a multivariate Gaussian, with error correlations only between data Y_l taken at the same time point. Hence, the following likelihood function will be used as the figure of merit:

$$P(Y; \theta) = \text{const} \times \exp [-\chi^2/2] \text{ with } \chi^2 = [Y - E(Y)]' \Sigma^{-1} [Y - E(Y)]$$

where

$E(Y)$ and Σ denote the mean and variance–covariance matrix of the observation vector Y for model θ

When multiple realizations of each profiling experiment are performed, then the variance–covariance matrix can be estimated. In the fitting reported in Fig. 18, a univariate

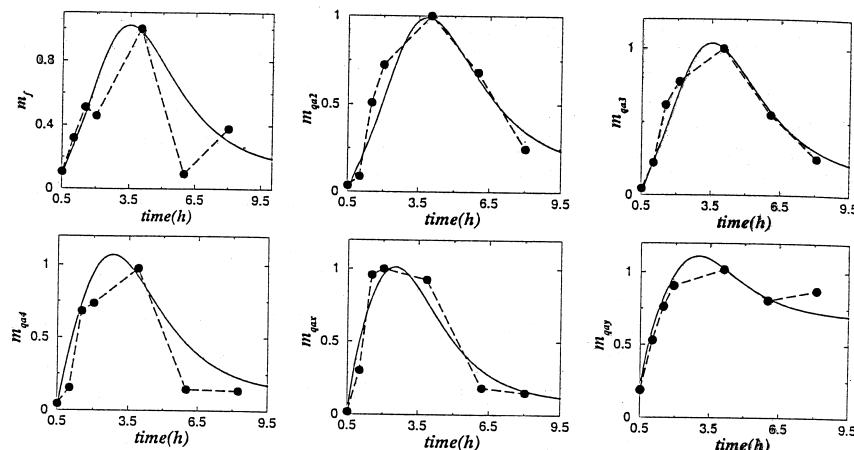


Figure 18 Measured trajectories over time of RNA levels for six of the seven *qa* cluster genes. Solid dots are the data [146]. Smooth curves are those of a fitted model as in Fig. 11, chosen to maximize the likelihood $P(Y; \theta)$

Gaussian with $\sigma/E(y_i) \times 100 = 20\%$ has been assumed and the observed single-experiment concentrations have been used as the data vector Y with the link function $E(Y) = F(\theta)$ [140]. To date, heteroscedasticity has been reportedly not an issue [141].

The fit is then obtained by maximizing the figure of merit $P(Y;\theta)$ with respect to the model parameters. A number of tools exist to carry out this fitting process [142,143]. A model from the family in Fig. 11 is displayed that fits the RNA profiling data of the *qa* cluster quite well in Fig. 18. Profiles were obtained for six out of seven of the *qa* genes. The RNA profile for *qa-1F* peaks at 4 hours and then drops after that point with another rise at 6 hours. The remaining profiles track that of *qa-1F* message levels. The simulator also yielded predictions about the protein profiles which are now testable [45].

5.1. Too Many Parameters, Too Few Data

The major limitation of current fitting procedures is that they do not address the major problem of too many parameters and too few data. In the example in Figure 11, after making steady-state assumptions, there were 42 parameters and 42 data points. This situation is not likely to change even with the availability of genome-wide RNA and protein profiling technologies. The reaction network in a cell is large and interconnected, and it is not clear at this time in studying a particular process such as carbon metabolism what other components of the reaction network need to be considered. For example, QA metabolism is intimately connected to aromatic amino acid metabolism through the *aro* cluster in *N. crassa* [144]. This raises the question of how QA metabolism is linked to general control [145]. Even in well-studied circuits involved in antibiotic production, it may not be safe to decouple secondary metabolism from, for example, energy metabolism. New fitting procedures are needed that directly address the problem of too many parameters and too few data [146].

5.2. A Stochastic Alternative

It is not clear at this time what role stochastic factors play in biological circuits. Patel and Giles [110] estimated that the number of *qa-1F* messages is on the order of 0.1 to 1 RNA per nucleus. This granularity within the cell may mean that a transcription factor finding a small 17 kbp stretch of DNA on the smallest chromosome in *N. crassa* may not be guaranteed. As a consequence, the stochastic formulation in Fig. 12 was simulated with the results for the *qa-1F* message shown in Fig. 19.

In this case, the four stochastic realizations have the same basic mountain shape observed for the real profile. The number of RNA molecules rises to about 400 molecules per cell. It is likely that the stochastic circuit will provide a description similar to the deterministic circuit. Either formulation leads to a similar story relative to the observed profiles. The challenge is comparing stochastic vs. deterministic circuits with the same circuit structure. Under some circumstances, deterministic circuits can be viewed as limits of the underlying master equations in Fig. 12 describing the stochastic circuit [82], but inference problems arise in distinguishing stochastic circuits vs. their limiting deterministic relative when one model (i.e., a deterministic one) lives on the boundary of the parameter space for a larger class of models (i.e., the stochastic ones).

6. CONCLUSION

Metabolomics is a process of discovery that promises a mechanistic understanding for interesting biological processes. This mechanistic understanding is captured in a kinetics

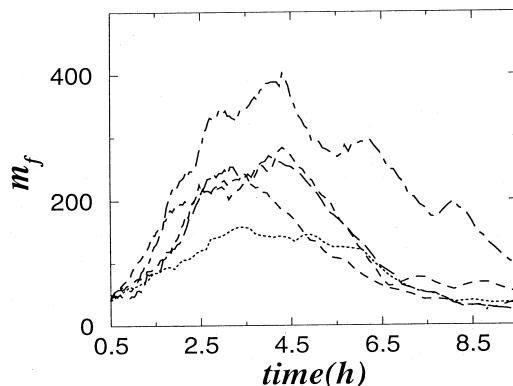


Figure 19 Counts of *qa-IF* message in a cell over time for five independent realizations of the stochastic alternative to the circuit in Fig. 7 (i.e., Fig. 12).

model well grounded in physics and chemistry. The discovery process itself is more akin to approaches used in systems ecology or neural biology. For 60 years, biologists have been taking biological systems apart to find their components. Now the process is about to reverse. With the complete genomic blueprint now in hand, the challenge is to reassemble the pieces. The adjectives describing metabolomics are hypothesis-driven, integrative, and reconstructive.

The most basic question in metabolomics is, What is a living system? [27]. One approach to answering this question is reconstructive and rooted in an approach originally adopted by Beadle and Tatum [147]: “From the standpoint of physiological genetics the development and functioning of an organism consist essentially of an integrated system of chemical reactions controlled in some manner by genes.” To identify this hypothesized reaction network requires an integrative approach.

The flow of the reconstruction process can be summarized simply in Fig. 20. The fungal system is perturbed. In the case of drug discovery, cells are treated with potential drugs, as an example, or in the case of industrial fermentation, genetically engineered strains are selected to increase production. The system is observed through RNA, protein, and metabolite profiling to compare the response with a control. The cells may die or may produce more of a desired product, such as penicillin. The profiling data are used to identify kinetics models or “biological circuits” to predict the response of the system. In many cases, the profiling step identifies additional genes and their products that must be included in the biological circuit. The fitted model then allows predictions about the total response of the system. The response of the system can sometimes be surprising when pathways are coupled and enlarged to explain the profiling data. Possible emergent properties include memory and a cyclical response. The model is reevaluated and tested for good fit. Current tests for better alternatives are limited and need to be developed.

A better model is selected and a new perturbation is selected. Choosing an informative perturbation is a challenging problem. The cycle completes and starts over. The result is a process of discovery and refinement. In each cycle the model serves to integrate available information on sequence, profiling, protein–protein interactions, protein–DNA

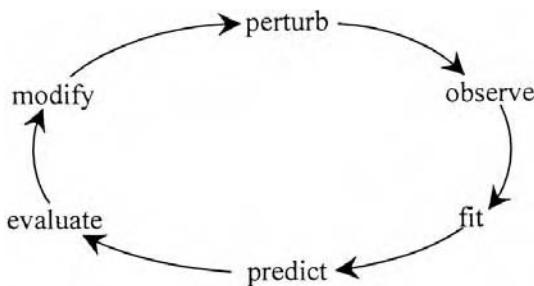


Figure 20 An example of hypothesis-driven genomics or the process of metabolomics.

interactions, and protein–lipid interactions. This discovery process ultimately will be automated into an adaptive control process to speed the process of gene-validated product discovery [28].

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Metabolic Engineering of Fungal Secondary Metabolite Pathways

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1. INTRODUCTION

Fungi are one of the richest sources of secondary metabolites of importance to humans [1–4]. Since the discovery and development of penicillin, tremendous effort has been directed toward production of, and screening for, fungal secondary metabolites. Traditional strain-improvement approaches usually include selecting strains with desired traits from mutagenesis programs and screening these strains for novel compounds [5]. These strategies have been invaluable for increasing production of fungal secondary metabolites, but are time consuming and labor intensive and cannot be relied on for rapid improvement of desired traits [6]. The development of recombinant DNA technologies and high-throughput transcript-, protein-, and metabolite-profiling technologies and the rapid accumulation of fungal genomic sequences provide new and important tools for redirecting metabolic pathways and obtaining superior yields of secondary metabolites.

The redirection of metabolic pathways for enhanced production of existing natural products (e.g., secondary metabolites), production of unnatural products, or degradation of unwanted molecules (e.g., environmental contaminants) is referred to as *metabolic engineering*. Metabolic engineering joins systematic and quantitative analysis of pathways using molecular biology, modern analytical techniques, and genomic approaches. Since Bailey [7] coined this definition for the emerging discipline of metabolic engineering, tremendous progress has been made in numerous fields and is summarized in many excellent reviews [8–26]. In this chapter, we review methods for metabolic engineering of filamentous fungi and their application to production of fungal secondary metabolites

(nonribosomal peptides, polyketides, and isoprenoids) using pathway analysis and modification.

2. TOOLS FOR METABOLIC ENGINEERING OF FILAMENTOUS FUNGI

In contrast to genetic engineering for protein production, metabolic engineering often involves the manipulation of two or more (and sometimes many) genes. Thus, it requires coordination of the expression of these heterologous genes with the cell's native genes. While many gene expression tools have been developed for filamentous fungi, engineering large, multigene pathways requires advanced tools not yet available, including improved methods for control over gene expression levels, segregationally stable autonomously replicating plasmids, and convenient systems for controlling the fate of transforming DNA [18]. In this section, we review the status of some of these tools and needs for metabolic engineering.

2.1. Control over Gene Expression Levels

A number of tools have been developed for expression of one-, two-, or three-gene systems under the control of inducible or constitutive promoters. The AlcR-inducible, CreA-repressible *alcA* promoter and the constitutive *gpdA* promoter of *Aspergillus nidulans* are examples of promoters used to engineer single- or multiple-gene overexpression systems. Their utility has been demonstrated for the heterologous production of interferon, epidermal growth factor, growth hormone, interleukin, superoxide dismutase, and lactoferrin [27–29]. The autoregulatory *A. nidulans* *alcR/alcA* system has also been shown to function in more industrially relevant organisms, such as *A. niger*, to drive production of the reporter gene β-glucuronidase [30].

Engineering metabolic pathways will require promoters of variable strengths, a problem that could be solved with artificial promoter engineering. Jensen et al. [31] have synthesized and used artificial promoters in bacteria and eukaryotes to express genes over a wide range of levels. While prokaryotic promoter engineering can result in promoters that express at higher levels than the original promoter, eukaryotic promoters have thus far shown only reduced activity as a result of the engineering efforts [32]. However, for the metabolic engineer, strong promoters capable of driving single genes may be of little use for more subtle adjustments to multiple components of a metabolic pathway. Efforts to better understand fungal promoter elements may improve our ability to control the expression of one or several genes simultaneously.

Another possible technique for controlling gene expression levels is to engineer translation initiation to allow expression of polycistronic mRNAs. The 5' Cap, Kozak sequence, and polyA tail are regulatory elements involved in eukaryotic mRNA translation. Required for translation of individual genes, these elements presumably cannot be used for expression of coding regions on polycistronic messages, a strategy that is commonly used to express multiple genes in prokaryotic hosts. The Cap is only functional at the 5' end of an mRNA and promotes translation machinery assembly and subsequent scanning for Kozak and start sequences. PolyA sequences are added posttranscriptionally to the 3' ends of transcripts and would not be available (nor presumably necessary) for internal cistrons of a polycistronic mRNA molecule. Further development of the highly disputed internal ribosome entry sequences (IRES) [33,34] for production of polycistronic mRNAs

holds considerable promise for pathway engineering in eukaryotes. IRES sequences have been reported to allow ribosome binding to transcripts and initiation of translation in the absence of the 5' Cap and Kozak sequences in a number of systems, including mammalian tissue, *Saccharomyces cerevisiae*, and *Drosophila melanogaster* [35,36]. Insertion of IRES sequences between a series of genes controlled by a single promoter provides internal translation start sites within a single polycistronic transcript. First identified in viruses, the IRES sequence is proposed to form a tertiary RNA structure that contacts the ribosomal 40S subunit and a number of additional ribosomal proteins [36]. Proteins necessary for IRES function may have to be included in constructs or engineered into strains in which the IRES is intended to be used [36]. The development of a fungal IRES would allow coordinated expression of multiple genes for reconstruction of entire metabolic pathways and may be significantly simpler than using independently controlled promoters for each gene.

2.2. Autonomous Plasmid Replication Systems

In bacteria and *S. cerevisiae*, the preponderance of genetic engineering has been accomplished using autonomously replicating plasmids. Plasmids are convenient vectors for introducing heterologous genes because (1) they are generally small enough to manipulate in vitro; (2) they can be transformed, tested, and reisolated for further manipulation; and (3) they do not suffer position-dependent expression or mutagenic effects that plague chromosomal integration. Unfortunately, most plasmid vectors are less segregatively stable than chromosomal integration vectors, a major problem for multinucleate fungal hosts.

Gene expression from autonomously replicating plasmids in *A. nidulans* was demonstrated by Alekseenko and Clutterbuck in the mid- to late 1990s. Their series of vectors incorporated the MATE and AMA1 elements. Two inverted repeats of a single MATE-enhancer element separated by a short spacer region compose a single AMA1 element. Enhancing transformation efficiency and allowing autonomous replication, respectively [37–40], neither the MATE element nor the AMA1 element provide permanent engineering solutions in a multinucleate host such as *A. nidulans*. The MATE and AMA1 elements increase transformation efficiency, but plasmid stability problems, resulting from the generation of heterokaryotic nuclei within successful transformants, remain unsolved.

Additional elements such as HTELS (human telomeric DNA sequences) have been successfully applied in *A. nidulans* and *Podospora anserina* and function to autonomously maintain linear DNA fragments [41,42]. The ability of telomeric sequences to maintain large (>20 kb) fragments extrachromosomally will aid in metabolic pathway construction and alteration. However, until self-replicating vectors can be homogeneously maintained in a multinucleate expression host, genomic integration will remain an essential technique for development of stable, isogenic expression strains, particularly for industrial production.

2.3. Controlling the Fate of Transforming DNA

Most often, DNA integration into the chromosome occurs by ectopic or nonhomologous integration. Selection conditions can ensure transforming DNA is present in the genome; however, the inability to control copy number and the potential for mutagenesis during integration make this integration method undesirable for engineering purposes. Currently, the most reliable heterologous expression vectors available for engineering filamentous fungi are those that integrate into the chromosome. DNA integration based on homology in

the filamentous fungi likely involves genes common to the better understood recombination systems in *S. cerevisiae* [43] and *Escherichia coli*. The *uvrC* gene, a homologue of *RAD51* from *S. cerevisiae* and *recA* from *E. coli*, is essential for homologous recombination in *A. nidulans* [44]. Both *RAD51* and *recA* have been demonstrated to function in scanning for homology between DNA sequences during recombination. Suggesting a similar function in *A. nidulans*, homologous recombination was eliminated in a *uvrC* null mutant, while multiple-site ectopic integration occurred at elevated frequencies [44]. There have been no reports to date of genes or mutations that increase recombination frequencies to 100% in *A. nidulans*.

Homologous recombination has the advantage that only one copy of the gene of interest could be integrated into a desired location on the chromosome, making expression more reproducible and predictable. To this end, a number of strategies have been developed to control the fate of transforming DNA. Longer regions of homology on either side of an integration cassette increase the likelihood of homologous recombination at the genomic target site [29,45]. Chaveroche et al. used *in vivo* recombination of a marker gene into *A. nidulans* genomic DNA (located on a cosmid maintained in *E. coli* expressing the λ proteins Red α , Red β , and Red γ to promote recombination) to generate a disruption cassette with flanking regions on the order of 10 Kb (Fig. 1, panel I) [46]. The *in vivo* cosmid recombination products demonstrated reduced transformation frequency in *A. nidulans*, but replacement efficiency was increased to 60% (compared with the 10% typically seen with 1-kb flanking regions). The usefulness of this technique for integrating large, multigene inserts (of approximately 10 kb), as might be necessary for metabolic engineering, was not addressed in this study.

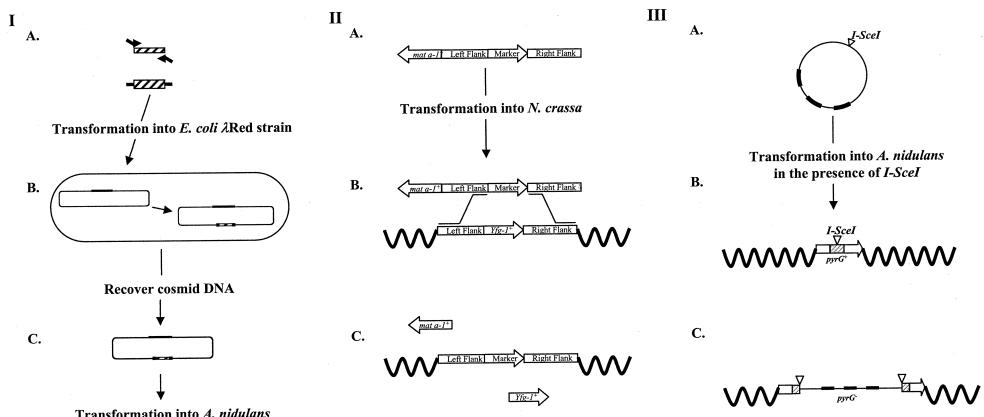


Figure 1 Strategies to increase site-specific recombination. Panel I, A bifunctional transformation marker is PCR amplified (A) and transformed into the λ Red *E. coli* strain (B) for *in vivo* recombination of the PCR fragment with an *A. nidulans* cosmid. The recombination product is transformed into *A. nidulans* (C). Panel II, A marker gene of interest is flanked by regions and the *mata-1* allele (A) prior to transformation into *N. crassa* (B). Loss of *mata-1* (transformant survival) increases the likelihood that a double-crossover event occurred (C). Panel III, Genes of interest are cloned into a plasmid with a rare-cutting endonuclease site (A) and transformed into an engineered strain of *A. nidulans* (B) prior to selection for transformants that have lost function of the target locus (C). (Panels I and II from Ref. 48.)

Techniques for systematically disrupting large numbers of genes have also been developed based on the thymidine kinase (TK) gene of the herpes simplex virus. TK catalyzes the formation of toxic thymidine dimers in the presence of acyclovir/gancyclovir. Sachs et al. demonstrated that the TK gene produces a functional TK enzyme when expressed in *Neurospora crassa* [47]. Building on the results of Sachs et al., Pratt and coworkers developed a method to take advantage of the TK gene's suicide nature to create a highly efficient system for systematically disrupting individual *N. crassa* genes on a genome-wide basis (Fig. 1, panel II) [48].

A potentially useful variation on the suicide-gene theme involves placement of enzyme-assisted integration target sites within introns of conditionally toxic genes to allow selection for site-specific integration of transformed DNA (Fig. 1, panel III). Enzyme-mediated recombination into an engineered intron sequence has been demonstrated, and the range of insert sizes tolerated using this approach is being determined (Laidlaw and Keasling, unpublished results) [49].

Regardless of the method used to insert DNA fragments into the chromosome, the location of the insert can have a significant impact on its level of expression. Positional influences on gene expression may involve heterochromatin structure, location-specific mutagenic effects, and possible expression interference by surrounding genes on the inserted construct. Liang et al. and Chiou et al. independently demonstrated the significance of positional effects on expression using *ver-1::GUS* and *pnor::GUS* reporter constructs, respectively, in *A. parasiticus* [50,51]. The groups integrated expression cassettes at both wildtype (*ver* and *nor*) and alternative (*niaD* and *pyrG*) loci. Construct integration at the *niaD* and *pyrG* loci resulted in a near elimination of gene expression; each construct expressed at normal levels when integrated at its wildtype locus. Similar effects have been documented in other organisms [52] and are a major concern for successful heterologous gene expression.

2.4. Laboratory Evolution Approaches to Metabolic Engineering

Discussion of metabolic engineering for single-gene or whole-strain improvement would be incomplete without considering the alternative to targeted, rational strain improvement—gene and genome shuffling technologies [53–63]. In contrast to rational metabolic pathway design, laboratory evolution offers a simple approach to pathway optimization as long as it is possible to screen or select for desired trait improvements. It has been demonstrated in a number of cases that successive rounds of shuffling and screening can produce desirable phenotypes at historically unparalleled rates [53,64–66]. Indeed, the limiting step for most laboratory evolution protocols is devising a screen to easily identify the desired phenotype among progeny. This technique may be particularly useful for filamentous fungi due to the lack of gene expression tools, the difficulty of applying traditional metabolic engineering principles, and the lack of a sequenced genome for most industrially important filamentous fungi.

3. FUNCTIONAL GENOMICS AND ITS APPLICATION TO METABOLIC ENGINEERING

The process of metabolic engineering involves successive iterations of genetic modification and analysis of the resulting metabolic changes [67]. In the past, analysis has been limited primarily to detailed characterization of the desired pathway and gross characteriza-

tion of the host (e.g., growth rate, substrate uptake, product formation). The recent development of the “omic” technologies (i.e., transcriptomics, proteomics, metabolomics, fluxomics, and interactomics) will continue to impact characterization of the host and the desired pathway(s) [68–72]. Until recently, however, sufficient information has not been available to make use of these technologies in filamentous fungi. The complete or partial sequencing of several fungal genomes has made it possible to apply DNA microarray analysis, proteome analysis, and metabolic flux analysis to the design and iterative improvement of industrially or pharmaceutically relevant fungal strains [73,74]. For detailed discussions of these technologies, the reader is directed to recent reviews on these topics and references therein [75,76]. The purpose here is to point out both the uses and the pitfalls of these techniques for metabolic engineering of the filamentous fungi. (The metabolome and fluxome are described in the Section 4.)

3.1. Application of DNA Microarray Data to Metabolic Engineering

Genomic PCR fragment, cDNA, EST, oligonucleotide, and randomly fragmented genome arrays are some of the DNA microarray technologies that have emerged since their development in the early 1990’s [77–83]. Early work demonstrated the utility of DNA microarrays for identifying genes involved in the response to such conditions as heat shock, cold shock, and available carbon source [84]. In addition, microarrays can be used to monitor cellular responses to single- or multiple-gene mutations and/or chemical agents [85–94]. With respect to metabolic engineering, it will be possible to use microarrays to develop more robust or optimal cultivation techniques and to analyze the effect of a heterologous metabolic pathway or the product of the pathway on global cellular gene expression [95]. Microarray data must be applied cautiously in engineering strain improvements. Identification of genes with increased or decreased expression levels may not accurately identify the enzymatic reactions most in need of alteration [96,97]. Transcriptome-response data provides insight to the state of the RNA when harvested but may not identify the dominant influence on phenotype or metabolism, or such data may represent a secondary (rather than primary) response to the condition in question [98]. Observed phenotype changes may be caused by any metabolite in a pathway, and changes in the transcriptome may result from increases or decreases in one or more other intermediate(s). The data provided by these experiments must be scrutinized to determine the true cause and effect [75,99].

Information provided by array experiments will only be as good as the controls included in the analysis [79,100–102]. High variability in reported expression levels for a single gene from one slide to the next and during dye-swapping experiments prepared under the same conditions highlights the need for replicate samples. Duplicate spots in a single array, secondary experimentation, and biological repetition of experiments are all necessary to validate the data from each array experiment [103–108]. Complete biological repetition of experiments, including slide preparation, sample cultivation, and preparation of target cDNA in triplicate, is the best filter for assigning statistical significance to data [109–114].

Regulatory themes developed from DNA microarray data have the limitation that they can only represent a cellular response at the RNA level. However, given that transcript profiling can be much easier to perform than the other “omic” technologies, DNA microarrays can diagnose potential stress responses due to changes in microbial metabolism. Understanding how the transcriptome relates to the proteome is the obvious next step [85,115–117].

3.2. Proteomics Emerges as a Tool for Pathway Analysis

Perhaps one of the most important tools to emerge for the metabolic engineer is the development of high-throughput proteome analysis technology [118–127]. Flux through any metabolic network is a result of the enzyme profile of that particular cell, now termed its *proteome* [67,128]. Two-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis (2-D SDS-PAGE) analysis has been used to analyze the proteome of *A. nidulans* in response to two macrolide secondary metabolites, bafilomycin and concanamycin (inhibitors of ATPases) [129]. Twenty proteins were found to be affected by the presence of the antibiotics in the medium. One of the main concerns with 2-D SDS-PAGE analysis is that due to its low sensitivity, it fails to detect proteins present in low abundance in a metabolic pathway. While not without its own limitations, the application of two-dimensional liquid chromatography coupled with high-resolution mass spectrometry (and variations thereof) can provide a wealth of more accurate information than traditional two-dimensional PAGE analysis [116,130–151]. As with DNA microarray technology, the statistical limitations of proteomics techniques have attracted considerable attention and must be addressed in any experimental design [120,126,127,152,153]. While proteomics is still at a very early stage of development, it is one step closer to metabolism than is transcriptomics and, as such, should have greater potential to diagnose potential problems in metabolic redirection.

4. QUANTITATIVE ANALYSIS OF METABOLISM

Quantitative analysis of metabolic pathways provides useful information to guide metabolic engineering. There are several types of analyses that have been applied to filamentous fungi: (1) *metabolic flux analysis* quantifies the fluxes through all pathways in the cell for a given set of conditions [154–159]; (2) *metabolic control analysis* uses kinetic information to identify the extent to which each enzyme in a given pathway controls the total flux [160–162]; and (3) *morphologically structured modeling* accounts for differences among cells within a hyphal filament in modeling growth and production of a compound of interest [163,164].

4.1. Metabolic Flux Analysis

Flux-based metabolic models quantify the metabolic fluxes through all reactions included in an organism's metabolic network and, as such, are important tools for metabolic engineering. The existence of pathways can be tested; theoretical yields can be determined; the rigidity of network branch points can be examined; pathways can be compared for their yield of product; and the effects of changes to genes or growth conditions on the entire metabolic network can be identified. Such information has been useful for the identification of candidate targets for genetic manipulation. Many references describe the theory and implementation of this technique [20,165–168].

Among filamentous fungi, metabolic flux analysis has been applied primarily to *Penicillium* and industrial *Aspergillus* spp. Jorgensen et al. constructed a stoichiometric model containing 61 internal fluxes and 49 intracellular metabolites for *Penicillium chrysogenum* grown in fed-batch mode [155]. Their model was used to predict the maximum theoretical yield of penicillin V on glucose. They also showed that the maximum yield of penicillin V on glucose would increase by 20% if cysteine was synthesized via direct sulfhydrylation rather than transsulfuration. This result makes cysteine biosynthesis

a potential target for metabolic engineering of *P. chrysogenum*. Using measurements of substrate uptake and biomass production rates, the model also indicates that approximately 40% of the glucose is shunted through the pentose phosphate pathway to provide the NADPH necessary for cysteine biosynthesis during the penicillin production phase.

Henriksen et al. used a modified version of this model to study *P. chrysogenum* grown under continuous conditions [154]. They determined that a maximum specific rate of production of penicillin V exists beyond which additional glucose is not used for penicillin, and therefore, the yield of penicillin on glucose decreases. Their result suggests that the bottleneck in penicillin production occurs somewhere other than the penicillin biosynthetic pathway, possibly in cofactor regeneration as a result of a thermodynamic upper limit for the relative flux through the pentose phosphate pathway.

Van Gulik et al. built upon these metabolic flux models for *P. chrysogenum* to predict the effect of penicillin G production on central metabolism [159]. Their model output was used to identify metabolic branch points most affected by penicillin production. Subsequent experiments targeting branch points identified as potential bottlenecks demonstrated that those associated with carbon metabolism were, in fact, unlikely to limit penicillin biosynthesis. Large changes in fluxes resulting from growth on different carbon sources had little effect on the amount of penicillin produced. Rather, the amount of available NADPH had a significant impact on penicillin production, suggesting that limitations were more likely associated with pathways involved in cofactor regeneration. Together with the results of Henriksen et al., these data support the hypothesis initially put forth by Jorgensen and coworkers that NADPH production via the pentose phosphate pathway is the limiting factor in penicillin production.

Pedersen et al. targeted α -amylase production in *A. oryzae* and used a metabolic model containing 69 reactions and 59 intracellular metabolites to compare a wildtype strain to a recombinant strain containing additional copies of the α -amylase gene [156]. Metabolic flux patterns at different growth rates and with different nitrogen sources were calculated. Analysis showed that the fraction of flux through the pentose phosphate pathway increased with specific growth rate due to increased demand for NADPH required for protein synthesis. Significant differences in the relative fluxes of the wildtype and recombinant strains were not found, although the absolute flux values were higher for the recombinant strain due to its higher specific glucose uptake rate.

In another body of work, Pedersen et al. used a similar metabolic model to characterize the effect on *A. niger* of a null mutation in the oxaloacetate hydrolase (*oah*) gene, which produces oxalic acid from oxaloacetate [157]. Because oxalic acid is an undesirable product in several industrial processes using *A. niger*, a strain incapable of making oxalic acid would be advantageous if the mutation did not have other deleterious effects. Metabolic flux values were not significantly affected by the mutation, suggesting that deletion of *oah* does not disrupt metabolism, and thus that this mutant strain could be industrially useful.

4.2. Metabolic Control Analysis

Metabolic control analysis (MCA) uses kinetic information to determine the extent to which each reaction in a given pathway controls the net flux through the pathway. With this technique, one can identify the rate-limiting step of a pathway and thus a potential target for metabolic engineering. Resources detailing the mathematical structure of MCA are readily available [20,169,170].

In filamentous fungi, MCA has been used to examine the pathway for penicillin-V production. In a series of three papers with increasingly complex kinetic models, it was demonstrated that control of flux to penicillin resides in the second reaction step, catalyzed by isopenicillin-N synthetase (IPNS) [160–162]. This conclusion was supported by the model result that increasing dissolved oxygen, a substrate for the second reaction, increases the flux through this pathway [160,171].

The related technique of biochemical systems theory [20,172–174] was used to develop a dynamic metabolic model for citrate production in *A. niger*. This model includes available kinetic data in addition to metabolic pathway information [175]. This model was used to determine optimal metabolic conditions for citric acid production and suggests that citric acid production could be increased to five to 12 times the basal rate depending on what constraints are used. However, the model also demonstrates that modifying a minimum of 13 enzymes would be necessary to achieve any significant increase, hence the need for tools that enable control of many genes simultaneously.

4.3. Morphologically Structured Modeling

Known differences between the metabolism of cells at the tip of hyphal fragments and those further back from the apical tip limit the accuracy of any model that treats biomass as homogeneous. For example, protein secretion occurs at the tips of extending hyphae, whereas increased penicillin production is associated with increased branching and the total number of subapical cells [164,176]. Models of varying complexity have been made, incorporating subpopulations of different types of cells with processes such as growth and product secretion limited to the appropriate cell types [163,164,177]. These models were successful at predicting levels of penicillin production in fed-batch fermentation of *P. chrysogenum* [164,177].

5. APPLICATIONS OF METABOLIC ENGINEERING

Metabolic engineering has been used to improve production of secondary metabolites in the native host organism or to produce secondary metabolites in a heterologous fungal host. In most cases, one or more of the tools described earlier were used to engineer the fungal host. In this section, we review applications of metabolic engineering to improve production of key secondary metabolites.

5.1. Metabolic Engineering of Nonribosomal Peptide Production

Nonribosomal peptides with diverse biological activities and structures are synthesized on large multifunctional enzymes called *peptide synthetases* [178]. The component moieties of these special metabolites are activated in the form of adenylate, acylphosphorylate, or coenzyme-A derivatives before they are linked together to form the specialized polypeptides. The best-known fungal nonribosomal peptides are the β -lactam antibiotics penicillins G and V and cephalosporin C. The genetics and biosynthesis of β -lactam compounds have been the subject of several reviews [179–185]. Here, we focus on metabolic engineering of β -lactam production in filamentous fungi.

5.1.1. Strain Improvements and Pathway Engineering for β -lactam Production

Penicillin and cephalosporin biosyntheses share the first two reactions and originate from three amino acid precursors: L- α -amino adipic acid (L- α -AAA), L-cysteine, and L-valine

(Fig. 2). L- α -AAA is a nonproteinogenic amino acid derived from the fungal-specific amino adipate pathway, which ultimately leads to the formation of L-lysine. Catabolic degradation of L-lysine is a second potential source of L- α -AAA in *A. nidulans* and *P. chrysogenum*, but whether this pathway contributes to biosynthesis of β -lactam compounds is not known [180]. Three enzymes— δ -(L- α -amino adipyl)-L-cysteinyl-D-valine synthetase (ACVS), isopenicillin N synthase (IPNS), and acyl coenzyme A:isopenicillin N acyltransferase (ACT)—encoded by three gene clusters—*pcbAB*, *pcbC*, and *penDE*—are responsible for biosynthesis of penicillin G in *P. chrysogenum*. In addition to the first two enzymes shared by penicillin biosynthesis, three enzymes—isopenicillin-N-epimerase (IPNE), deacetoxycephalosporin C synthetase (expandase)/deacetylcephalosporin C synthetase (hydroxylase; DAOCS/DACS), and cephalosporin C synthetase (acetyltransferase)—catalyze the biosynthesis of cephalosporin C in *Cephalosporum acremonium* (renamed *Acronymium chrysogenum*). These enzymes are produced from the *cefD*, *cefEF*, and *cefG* genes, respectively. All structural genes for β -lactam biosynthesis are clustered, transcribed separately, and expressed from different promoters [182,186,187].

Industrial strains of *P. chrysogenum* have been subjected to multiple rounds of mutagenesis and screening to produce higher penicillin titers [188]. Strain improvement via gene dosage using the *pcbC* gene in industrial strains of *P. chrysogenum* was unsuccessful. This led to the discovery that some industrial strains of *P. chrysogenum* carry several copies of the entire penicillin biosynthetic pathway [189–191] and that all genes in this

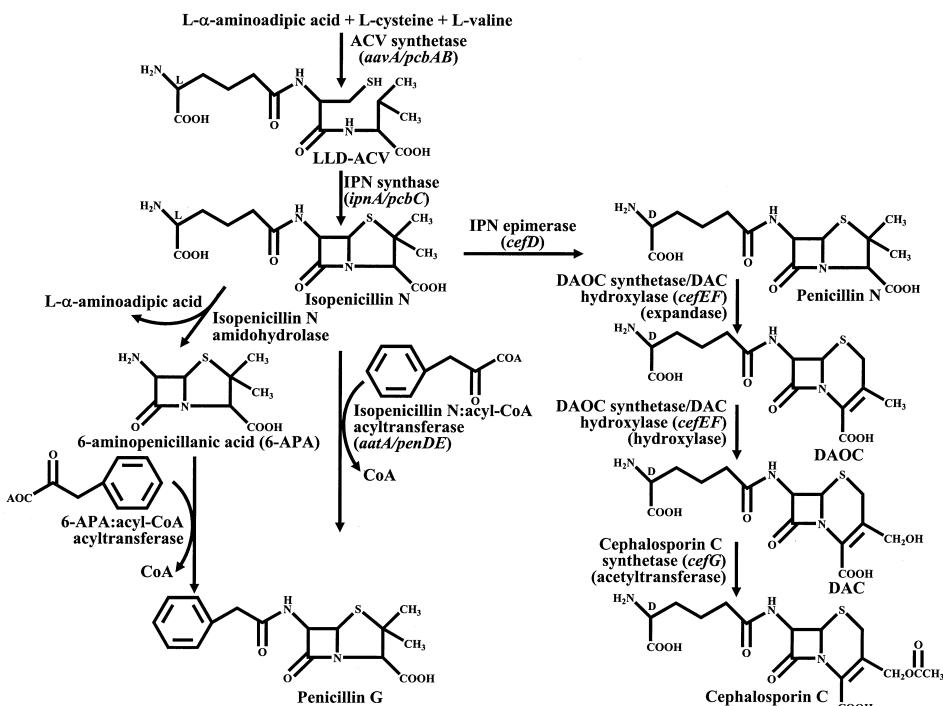


Figure 2 The biosynthesis pathways of penicillin G, cephalosporin C, and formation of the related products in filamentous fungi. See text for abbreviations.

pathway have higher mRNA levels relative to wildtype [187,189,192,193]. These studies indicated that high-yield production of penicillin in high-titer strains is closely related to the overexpression of the penicillin biosynthetic genes.

Increases in the penicillin gene cluster copy number have successfully increased the penicillin yield in low-titer production strains. Amplification of the *pcbC-penDE* gene cluster in *P. chrysogenum* Wis54-1255 led to 40% higher productivity than the wildtype strain [194]. Although overexpression of *pcbC-penDE* revealed that their products affect penicillin pathway flux, metabolic control analysis of the entire pathway indicated a shift in flux control from the first step to the second step as the fermentation proceeded [161,195]. This is consistent with the finding that the first intermediate— δ -(L- α -aminoadipyl)-L-cycteinyld-D-valin (LLD-ACV)—inhibits the first reaction in the pathway [196].

Additional work has been done in *A. nidulans* using the genes homologous to *pcbAB*, *pcbC*, and *penDE*: *acvA*, *ipnA*, and *aatA*, respectively. Overexpression of *acvA* in *A. nidulans* increased penicillin production by as much as 30-fold [188]. Thus, as in *P. chrysogenum*, ACVS is a rate-limiting enzyme for penicillin production in *A. nidulans*. Overexpression of *ipnA* (encoding IPNS) under the control of the *alcA* promoter caused a 40-fold increase in IPNS activity but only a 25% increase in penicillin production. An eight-fold increase in 6-aminopenicillanic acid acyltransferase (AAT) activity was observed when *aatA* was expressed under the control of *alcA*; however, penicillin production was reduced by 10% to 30% in this strain. The decrease in penicillin production can be explained by the isopenicillin-N amidohydrolase activity of AAT.

Finally, the non-penicillin-producing fungi *A. niger* and *N. crassa* have been engineered to produce penicillin following transformation with the penicillin gene cluster from *P. chrysogenum* [187], but the yield was very low compared with the industrial penicillin-production strains.

The first committed step in the biosynthesis of cephalosporin C is the isomerization of isopenicillin N to penicillin N catalyzed by IPN epimerase (Fig. 2). Penicillin N is converted to deacetoxycephalosporin C (DAOc) by DAOc synthetase. The methyl group at C-3 of DAOc is hydroxylated and oxidized to form deacetylcephalosporin C (DAC) in a reaction catalyzed by DAC hydroxylase. In *A. chrysogenum*, the product of *cefEF* catalyzes both reactions. Finally, the gene product of *cefG* converts DAC into cephalosporin C.

Flux analysis in the filamentous fungus *A. chrysogenum* C-10 (an industrial strain) and the actinomycete *Streptomyces clavuligerus* NRRL 3585 (a wildtype strain) indicated that the production of LLD-ACV is a rate-limiting step in β -lactam production and that ACVS is a potential rate-limiting enzyme [197–200]. This reaction has been shown to be sensitive to the supply of the amino acid precursor L- α -amino adipic acid. Overexpression of lysine ϵ -aminotransferase, a key enzyme in L- α -amino adipic acid biosynthesis in *Streptomyces*, increased cephalexin production by two to five times [101].

The gene product of *cefEF* was also identified as a rate-limiting enzyme because its substrate penicillin N accumulated in a cephalosporin-producing strain of *A. chrysogenum*. Homologous integration of an extra copy of the *cefEF* gene increased cephalosporin C production by 15% [102]. However, it was shown later that *cefG* was also present in the plasmid used for this transformation. Transformation of wildtype *A. chrysogenum* with additional copies of the *cefG* gene alone resulted in increased *cefG* mRNA levels and cephalosporin C titers [203,204]. These findings indicate that *cefG* might be a rate-limiting step in cephalosporin C production.

5.1.2. Rational Design of β -lactam Biosynthesis Through Pathway Engineering

Rational design of β -lactams has been pursued via several strategies using cloned β -lactam biosynthetic genes for the production of cephalosporin precursors as a starting point. Oral cephalosporins, such as cephalexin and cephadrine, are superior to penicillin and synthesized by derivatizing the 7-amino group of 7-aminodeacetoxycephalosporanic acid (7-ADCA) or 7-aminocephalosporanic acid (7-ACA) with appropriate side-chain moieties [105]. However, their use is limited because of the complexity of the 7-ADCA and 7-ACA production process [180].

There are several ways to generate these precursors. First, penicillin G from *P. chrysogenum* can be chemically converted into deacetoxycephalosporin G, which can be enzymatically deacylated to produce 7-ADCA [206–208] (Fig. 3). Second, deacetoxycephalosporin C (DAOC) and cephalosporin C can be enzymatically deacylated to form

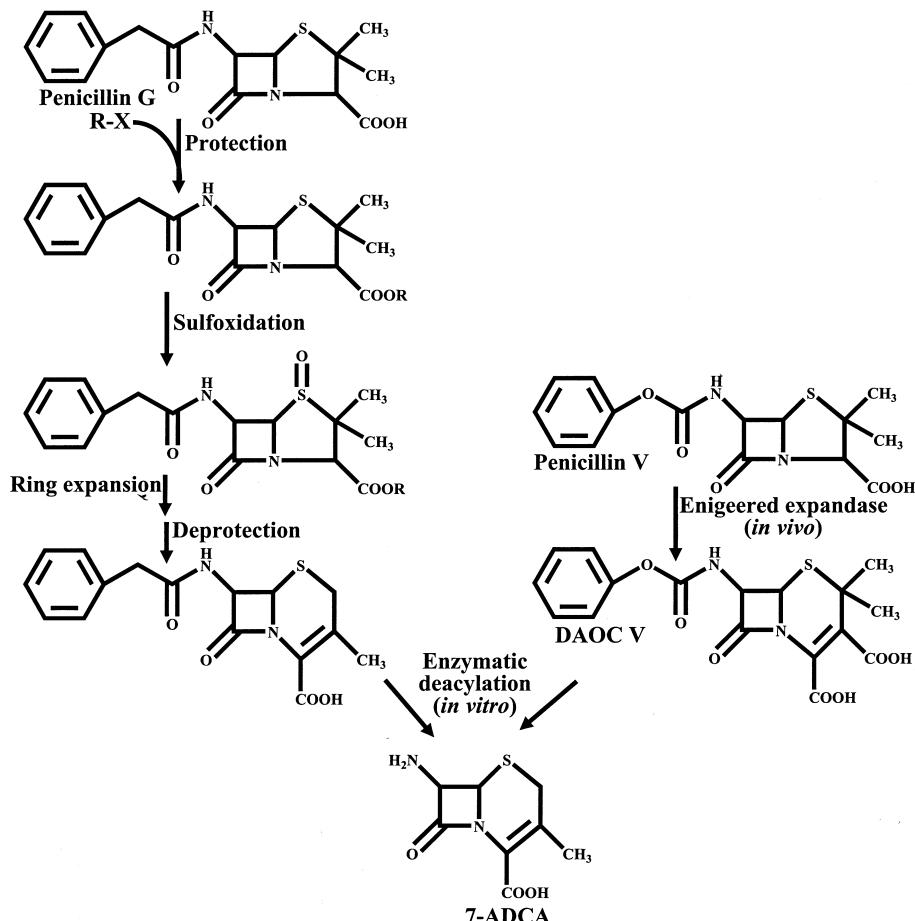


Figure 3 Schematic representation of routes for 7-ADCA production [5,206]. See text for abbreviations.

7-ADCA and 7-ACA, respectively [180,205,209] (Fig. 4). Finally, adipyl-cephalosporins can be used to produce 7-ADCA or 7-ACA by removing the adipyl side chain from the adipyl-cephalosporins [210]. Because removal of the natural adipyl side chain is inefficient [210,211], efforts have focused on isolation of superior enzymes to remove the side chain directly and development of alternative biosynthetic pathways to 7-ACA and/or 7-ADCA [180,210,211].

It has been proposed to use protein engineering to create an alternative pathway for 7-ADCA production [107]. Specifically, engineering the monofunctional *cefE* (encoding an expandase) gene of *S. clavuligerus* to produce an expandase that accepts penicillin V as a substrate would allow use of this easily producible fermentation product as a precursor. The product of this novel catalysis, deacetoxycephalosporin V (DAOC V), can be converted to 7-ADCA by penicillin acylase [107] (Fig. 3). As a first step toward this goal, the *S. clavuligerus cefE* gene was placed under the control of the *P. chrysogenum pcbC* promoter and terminator and was successfully expressed in *P. chrysogenum* [107]. In addition, DAOC was produced from penicillin in *P. chrysogenum* by coexpressing *cefE*

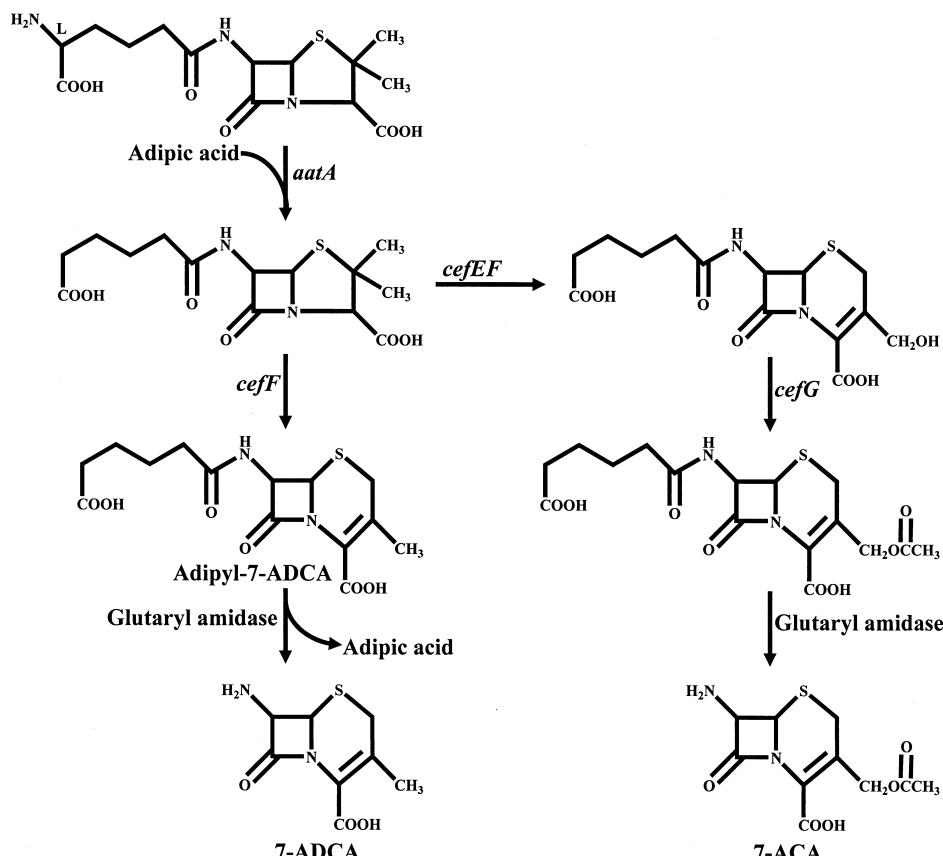


Figure 4 Engineered pathway for the production of the precursors of cephalosporin antibiotics in *P. chrysogenum* [210] and *A. chrysogenum* [212]. See text for abbreviations.

from *S. clavuligerus* and the *S. lipmanii* epimerase gene, *cefD*, using the *P. chrysogenum* regulatory elements from *penDE* and *pcbC* [208].

Another 7-ACA synthetic pathway (Fig. 4) was constructed in *A. chrysogenum* using genes encoding D-amino acid oxidase from *Fusarium solani* and cephalosporin acylase from *Pseudomonas diminuta*, both under the control of regulatory elements from the alkaline protease gene of *A. chrysogenum* [212]. Transformants containing the pathway from cephalosporin C to 7-ACA of *A. chrysogenum* BC2116, a high cephalosporin-producing strain, synthesized and secreted low levels of 7-ACA as the end product of fermentation. Although the 7-ACA titers were not commercially viable, the production of 7-ACA via microbial fermentation was shown to be possible.

Finally, in a series of experiments, *cefE* from *S. clavuligerus* and *cefEF* (encoding an expandase-hydroxylase) from *A. chrysogenum*, each under the control of the *P. chrysogenum ipnA* promoter or the β -tubulin gene promoter, were transformed separately into *P. chrysogenum* with and without the acetyltransferase gene (*cefG*) [210]. Feeding such transformants adipic acid led to the production of cephalosporins having an adipyl side chain. Transformants expressing *cefE* produced adipyl-7-ADCA, whereas transformants expressing *cefEF* produced both adipyl-7-ADCA and adipyl-7-aminodeacetylcephalosporanic acid (adipyl-7-ADAC). Transformants expressing *cefEF* and *cefG* (encoding an acetyltransferase) produced adipyl-7-ADCA, adipyl-7-ADAC, and adipyl-7-ACA (Fig. 4). The adipyl side chain of these cephalosporins was easily removed using a *Pseudomonas*-derived glutaryl acylase to yield cephalosporin intermediates [210].

5.2. Pathway Engineering of Polyketide Production

Polyketides comprise another large family of structurally diverse natural products. In the last decade, polyketides have been intensely studied because of their antitumor and antimicrobial activities. They are assembled from short-chain carboxylic acids by three types of polyketide synthases (PKSs) [213,214]. Fungi, particularly the Ascomycetes such as the well known *Penicillium* and *Aspergillus*, are among the most prolific sources of polyketides [215]. Fungal PKSs are type-I polyketide synthases, which are encoded by a single gene and have up to eight functional domains. These enzymes use acetate as starter units and malonate as extender units for the formation of poly- β -ketomethylene intermediates, which are subsequently modified/rearranged to form polyketides [215,216].

The best-studied fungal polyketide of pharmaceutical interest is lovastatin, an inhibitor of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and an antihypercholesterolemic agent. (For details of lovastatin biosynthesis, please refer elsewhere in this book.) Since the discovery of lovastatin from *A. terreus* [217] and *Monascus ruber* [218], tremendous efforts have been made to produce lovastatin analogues through strain isolation, chemical hydroxylation, and microbial biotransformation [219]. High-yield production of lovastatin was obtained mainly through optimization of fermentation conditions, changes in media formulation, and strain improvement via traditional mutagenesis [219–221] and engineering global regulators [222]. More recently, *A. nidulans* was used as heterologous host for lovastatin biosynthesis [223].

5.3. Pathway Engineering of Isoprenoid Production

Another important group of secondary metabolites is the isoprenoids. Isoprenoids are the most chemically and structurally diverse family of natural compounds known, with more than 30,000 individual molecules identified so far [224]. Fungi produce a number of

important isoprenoids (gibberellins, mycotoxins, and pharmaceutically important antibiotics) and isoprenoid precursors common to other valuable secondary metabolites. Fungi synthesize the universal isoprenoid precursor, isopentenyl pyrophosphate (IPP), via the mevalonate pathway. In the first step of this pathway, three molecules of acetyl-CoA form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA; Fig. 5) [225]. HMG-CoA is converted into mevalonate in an irreversible reaction catalyzed by HMG-CoA reductase and ultimately to IPP via three enzymes.

IPP is isomerized to dimethylallyl pyrophosphate (DMAPP) by IPP isomerase [226], and these two C₅ molecules combine to make geranyl diphosphate (GPP, C₁₀), which is the precursor to monoterpenes, and farnesyl diphosphate (FPP, C₁₅), which is the precursor to sesquiterpenes, squalene, cholesterol, and farnesylated proteins [227,228]. In fungi, FPP condenses with IPP to make geranylgeranyl diphosphate (GGPP, C₂₀) [228–231], a precursor to quinines, tocopherols [232,233], carotenoids, gibberellin plant hormones [234,235], cerebrovascular and cardiovascular drugs such as ginkolides [236], and anti-cancer drugs such as Taxol [232], sarcodictyin [237], and eleutherobin [238]. In some fungal species, GGPP serves as a precursor to gibberellins, paxilline, and mating pheromone biosynthesis [235,239,240].

The reduction of HMG-CoA to mevalonate, catalyzed by HMG-CoA reductase, is well documented as a rate-limiting step in the isoprenoid pathway [225]. Overexpression of the catalytic domain of *HMG* (encoding HMG-CoA reductase of *S. cerevisiae*) and *HMG* (a homologue of *HMG1* from *C. utilis*) in *C. utilis* increased isoprenoid accumulation [241]. Overexpression of the catalytic domain of *HMG1* also increased squalene accumulation [242]. In addition, overexpression of the catalytic domain of *HMG1* increased carotenoid accumulation in *N. crassa* [243].

Several lines of evidence suggest that IPP isomerase may be important in the regulation of isoprenoid biosynthesis [244,245] and subjected to allosteric regulation [245,246]. Although introduction of IPP isomerases from plant [247], algae [248,249], yeasts [248,250], or *E. coli* [229] has enhanced the accumulation of carotenoids in *E. coli* and algae, no study has yet shown that overexpression of this enzyme increases isoprenoid production in fungi.

Carotenoids have recently attracted attention because of their increasing commercial potential as nutraceutical food additives [251,252]. Fungal carotenoid biosynthesis has been studied extensively in the carotenogenic fungi *N. crassa* [253], *Phycomyces* spp. [254,255], *Mucor circinelloides* [256–260], and *A. giganteus* [261,262]. The carotenoid biosynthetic genes have been cloned and extensively characterized in *N. crassa* [253,263–267], *P. blakesleeanus* [255,268], and *M. circinelloides* [258,269,270]. A common feature of these fungal genes is that their products usually catalyze multiple carotenogenic reactions [268,271].

One challenge in engineering carotenoid production in fungi is that carotenoid production in these systems is tightly regulated by light and other environmental factors [253,256,257,261]. Recently, overexpression of a negative regulator, *crgA*, has been shown to abolish the light requirement for carotenoid biosynthesis in *M. circinelloides* [257]. The *crgA* null mutants overproduced β-carotene in dark and light conditions [256].

Another strategy for avoiding light regulation of carotenoid production is the use of heterologous hosts. The *Erwinia uredovora* carotenogenic genes have been used to produce lycopene and β-carotene in *S. cerevisiae* [272]; lycopene, β-carotene, and astaxanthin in the food yeast *Candida utilis* [273]; and lycopene in the noncarotenogenic fungus *A. nidulans* [229] (Figure 5).

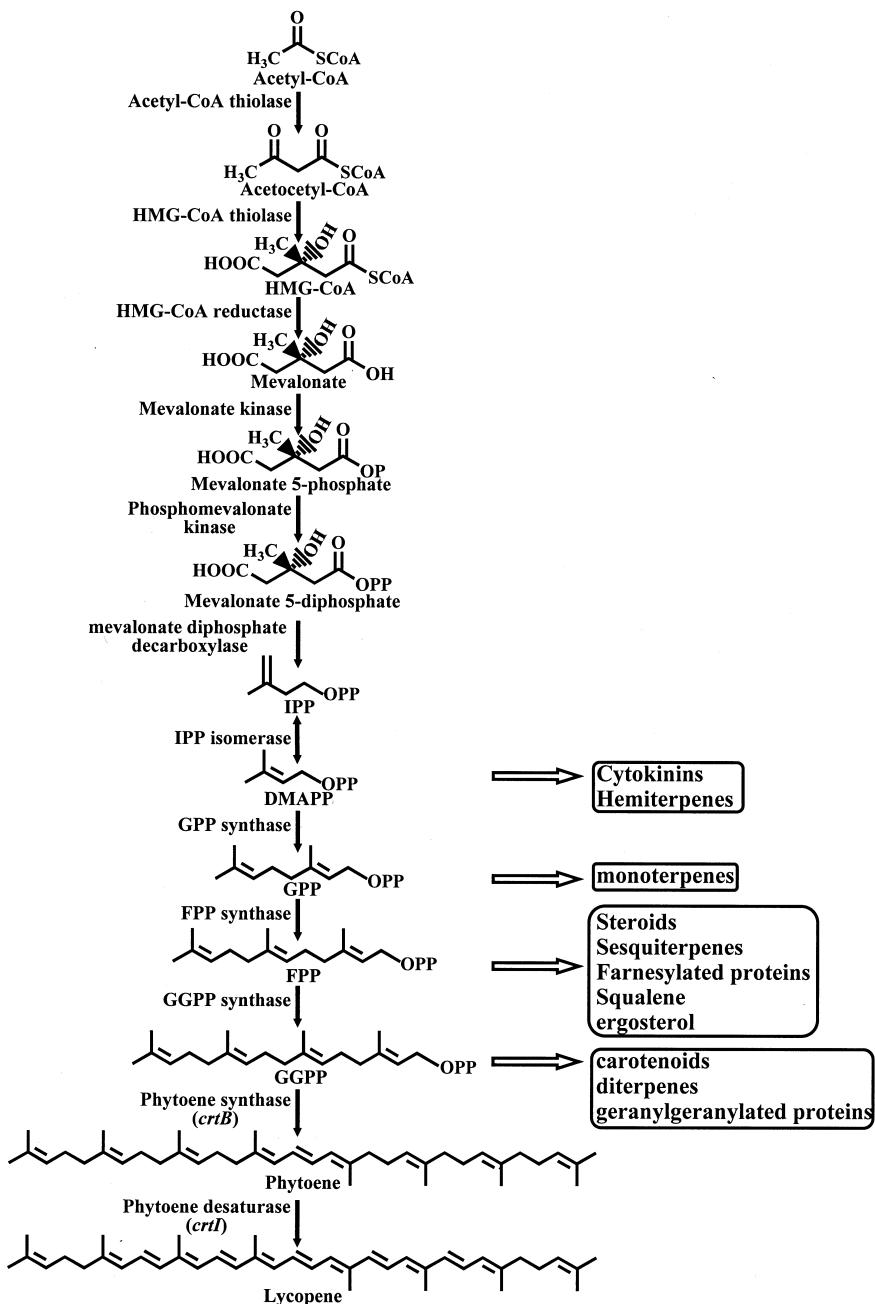


Figure 5 Isoprenoid biosynthesis through mevalonate pathway. See text for abbreviations.

6. CONCLUSION

Fungal secondary metabolite production is subject to regulation by carbon and nitrogen availability, environmental and fermentation conditions [180,253,256,257,274,275], and is often associated with fungal development [253,274]. In addition, fungal secondary metabolites are often synthesized in specific intracellular compartments. These factors complicate pathway engineering solutions for improving fungal secondary metabolite production. Nevertheless, engineered global regulators have been shown to increase production and abolish environmental regulations [222,256]. Indeed, engineering of global regulators could provide a short path to increase production of fungal secondary metabolites.

The production of modified natural products (or unnatural products) will expand the range of compounds available and is an important new area for metabolic engineering. Polyketide synthases and nonribosomal peptide synthetases have been shown to be amenable to domain shuffling and domain engineering [178,276–284]. Similar to these enzymes, the fungal carotenogenic enzymes also have multiple domains and catalyze multiple steps in carotenoid biosynthesis. Therefore, it is reasonable to assume that gene shuffling could be a powerful tool to engineer synthetic carotenogenic enzymes with superior and novel activity. Overall, we believe that fungal PKSs, NPSs, and carotenogenic genes with a diverse enzymology will be good candidates to be exploited in the generation of novel products or a library of analogues.

The ability to modify fungal secondary metabolic pathways is limited in part by available gene expression tools. For example, in the engineering of fungal secondary metabolite pathways, introduction of target genes has been achieved mainly via chromosomal integration. However, expression levels of integrated native or foreign genes in fungal genomes have been shown to be dependent on chromosomal location [50,52]. Discovery of those chromosomal locations in which the genes can be maximally expressed and guided integration of the gene of interest into these locations using engineered “smart” vectors will aid in fungal pathway engineering by improving gene expression predictability.

The development of autonomously replicating plasmids (or minichromosomes) would also enable predictable expression of heterologous genes and would allow introduction of several genes necessary to reconstruct an entire metabolic pathway. As evidenced in the examples given, rarely does a single step in a metabolic pathway limit production of the metabolite of interest, such that multiple genes encoding multiple steps in the pathway must be introduced to improve yields. Additionally, the development of new promoter systems that would allow coregulation of several genes simultaneously would greatly enhance our ability to manipulate fungal secondary metabolism.

Finally, changes in the relative level of one or multiple genes usually result in global responses in gene expression. How these global responses, in turn, affect secondary metabolite production is another important question to be addressed. Fortunately, the availability of integrative and functional genomic approaches (i.e., transcriptomics, proteomics, metabolomics, and fluxomics) along with the development of metabolic engineering tools will improve our ability to manipulate fungal secondary metabolic pathways to increase product titers and produce novel compounds.

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Heterologous Protein Expression in Yeasts and Filamentous Fungi

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1. INTRODUCTION

Heterologous protein expression was the impetus for the development of recombinant DNA technologies in the late 1970s. In the early 1980s, *Escherichia coli* and *Saccharomyces cerevisiae* expression systems were developed and used for foreign protein expression [1]. Several comprehensive reviews of these two expression systems are available [2–7]. Development of other expression systems, such as heterologous protein expression in methylotrophic yeasts [8,9] and filamentous fungi [10], followed quickly, and the broad utility of these systems is becoming increasingly apparent.

Heterologous protein expression technologies are widely used in both basic research and industrial applications. In discovery research, heterologous gene expression serves as an important tool to produce proteins and enzymes that are either at limited levels or difficult to purify in their native context. Many enzymes and proteins have important commercial and industrial value; thus, large-scale protein production is of great interest. Since different enzymes and proteins have various molecular and biochemical characteristics, different expression systems with different features are necessary to meet the growing needs of heterologous protein expression.

With the steady advance in gene-expression technology—such as new vectors for gene delivery, new recombinant selection methods, and modified expression hosts—production of recombinant proteins has become much easier and more efficient. In addition, introduction of fusion proteins such as the hexa-histidine (6X his) tag allows rapid detection and purification of recombinant proteins. Because the diverse nature of enzymes and proteins requires that expression systems be customizable and scalable for each application,

choosing the most suitable system for expression of the protein of interest becomes a major challenge. Some advantages and drawbacks for commonly used heterologous protein expression systems are listed in Table 1. *E. coli* expression systems provide quick and easy molecular manipulation and, in general, require only low-cost growth media. However, protein expression in *E. coli* becomes limited if foreign proteins require transcriptional and/or posttranslational processing and modifications. By contrast, fungal protein expression systems including yeast and filamentous fungi can provide high-level expression along with the posttranscriptional and posttranslational processes and modifications needed for eukaryotic proteins and enzymes. Similar to *E. coli* protein expression systems, fungal expression systems require only low-cost culture media, their scale-up fermentation

Table 1 Pros and Cons for Some Commonly Used Heterologous Protein Expression Systems

Expression Hosts	Pros	Cons
<i>E. coli</i>	Easy genetic manipulation, rapid growth, simple media requirements and low cost to grow, no human virus carried	Inclusion body, no secretion into media, lack of posttranslational process
<i>S. cerevisiae</i>	Low-cost culture and easy to grow, genetic regulation well known, secretion protein, no risk for human virus infection	Overyglycosylation at N-linked sites, cell difficult to break, expression level limited
<i>P. pastoris</i>	High-level expression, low cost and easy to grow, genetic regulation well known, secretion protein, no risk for human virus infection	Royalties can be expensive, potential for overglycosylation, cell difficult to break
Filamentous fungi	High level of expression, good secretion system, some level of posttranslational modification, culture medium is inexpensive	No good commercial vectors are available, longer culture time, spores are concerns for health problem, not well documented for production of therapeutic proteins
Insect cells	Easy to grow up to 400 L, easy to manipulate genetically for baculovirus, can grow in the same style tanks as mammalian cells	Relatively low expression, serum-free medium is expensive, royalty for the commercial available systems can be costly, few contractors have cGMP experience
Mammalian cell lines	Authentic protein expression and proper posttranslational processing and assembly for therapeutic proteins and other mammalian proteins of interest, extensively used, reasonable expression level, endotoxin is not a concern	Medium is expensive, special tank, require CO ₂ , potential risk of human virus in culture, longer expression time

Table 2 Some Yeast and Filamentous Fungi Species Used for Heterologous Protein Expression

Yeasts	Filamentous Fungi
<i>Schizosaccharomyces pombe</i> [13]	<i>A. niger</i> [20]
<i>Pichia pastoris</i> [15]	<i>A. awamori</i> [21]
<i>Pichia methanolica</i> [14]	<i>A. oryzae</i> [22]
<i>Kluyveromyces lactis</i> [16]	<i>A. nidulans</i> [23]
<i>Yarrowia lipolytica</i> [17]	<i>Trichoderma reesei</i> [24]
<i>Hansenula polymorpha</i> [18]	<i>N. crassa</i> [25]
<i>Candida boidinii</i> [19]	<i>Fusarium graminearum</i> [25]
<i>Saccharomyces cerevisiae</i> [6]	<i>F. oxysporum</i> [25] <i>A. chrysogenum</i> [25] <i>Tolypocladium geodes</i> [25] <i>Penicillium</i> spp. [25]

technologies have been developed, and the organisms are generally regarded as safe (GRAS) [11,12]. Other advantages of fungal expression systems include stable expression recombinants and controlled strain breeding. In contrast, insect and mammalian expression systems require special culture conditions and high-cost culture media, and they often have lower protein yields.

When choosing a heterologous protein expression system, the following general aspects should be considered: (1) translational modification and processing of proteins; (2) authenticity of the expressed proteins; (3) protein expression level and scale-up requirements; (4) subcellular localization (e.g., intracellular, membrane-bound, or secreted proteins); (5) cofactor requirement; and (6) cost, safety, and other regulatory issues. Each protein expression system offers a special set of properties. Even though successful examples can help in making the initial selection of an expression system, a good heterologous protein expression system often needs tailoring based on the properties of the individual protein of interest.

Table 2 lists some filamentous fungi and yeast species that have been used for heterologous protein expression [6,13–25]. Numerous reviews and articles have addressed foreign protein expressions in each of these fungal protein expression systems [6,25–30]. Most reviews focused on one specific system, and some addressed a specific aspect of the expression system, such as protein secretion in *S. cerevisiae* [31] and glycosylation of proteins in *P. pastoris* [32]. In this chapter, we review the most commonly used fungal expression systems (*S. cerevisiae*, *Pichia pastoris*, and *Aspergillus* spp.) with the focus on available vectors, promoter and leader sequences, posttranslational modifications, and fermentation scalability.

2. *S. CEREVISIAE* FOR HETEROLOGOUS PROTEIN EXPRESSION

S. cerevisiae, a unicellular yeast, is one of the most well-understood eukaryotic microorganisms. A tremendous amount of information on its genetics, physiology, biochemistry, metabolism, and fermentation is readily available. Thousands of genes from *S. cerevisiae* have been characterized [33], and its genome sequence has been determined [34]. In

addition, *S. cerevisiae* has been extensively used as a host for heterologous gene expression of numerous eukaryotic proteins for therapeutic and nontherapeutic applications [6,30].

2.1. Advantages and Disadvantages

S. cerevisiae has been used for centuries in brewing and baking industries and is a GRAS organism. Similar to *E. coli*, *S. cerevisiae* requires only simple culture media for rapid growth and is easily scaled up for large fermentations. However, as a eukaryotic organism, its subcellular organization, genetics, physiology, and biochemistry are different from bacteria and are more similar to higher eukaryotes. It is capable of accomplishing many posttranslational modifications, such as proteolytic processing, disulfide bond formation, glycosylation, and others. Numerous vector systems containing a variety of promoters and auxotrophic or dominant selectable markers have been developed that allow constitutive or regulated gene expression. Several methods for efficient high transformation of foreign DNA into *S. cerevisiae* have also been developed [35]. As a result, *S. cerevisiae* is one of the most widely used microbial hosts for heterologous gene expression [36]. There are some limitations to using *S. cerevisiae* for heterologous gene expression, however. The product yields can be low with maximum amounts of expressed proteins reaching only 1% to 5% of total cell protein. The use of episomal vector systems for heterologous protein expression in *S. cerevisiae* often results in plasmid instability during fermentation, which often leads to lower growth rate and reduced overall protein yields. Furthermore, because many proteins of therapeutic interest are secreted glycoproteins, the tendency of *S. cerevisiae* to hyperglycosylate proteins not only reduces the efficiency of protein secretion but also may lead to undesired changes in the immunogenic properties or biological activities of expressed proteins [37–39].

2.2. Vectors

S. cerevisiae expression vectors are usually shuttle plasmids that contain sequences for propagation and selection in both *S. cerevisiae* and *E. coli*. Table 3 [40–49] lists the common types of *S. cerevisiae* vectors. Two types of vectors have been described based on their mode of replication: episomal and integrating vectors.

Table 3 Vector Systems Used for Heterologous Protein Expression in *S. cerevisiae*

Vector	Copy Number per Cell	Reference
Episomal		
YEpl: 2μ-based	25–200	Futcher and Cox, 1984 [41]
YCp: centromere	1–2	Clarke and Carbon, 1980 [40]
YRp: replicating	1–20	Murray and Szostak, 1983 [42]
Regulated copy number	3–100	Chlebowicz-Sledziewska and Sledziewska, 1985 [43]
Integrating		
Yip	>1	Hinnen et al., 1978 [44]
rDNA-integrating	100–200	Lopes et al., 1989 [45]
Tyδ	<20	Sakai et al., 1991 [46], Shuster et al., 1990 [47]
Transplacement	1	Rothstein, 1983 [48]

Source: Adapted from Ref. 49.

Episomal vectors can be characterized according to their copy numbers, mode of replication, and stability. There are three major types of episomal vectors: the YRp type, the YCp type, and the YE_p type. YRp vectors contain an autonomous replication sequence (ARS) from the *S. cerevisiae* genome, and have an average copy number of 1 to 10 per cell. Higher copy numbers (up to 100) per cell have been reported. These vectors are unstable and easily lost in the absence of selection. YCp-type vectors, derived from the incorporation of *S. cerevisiae* centromeres into YRp plasmids, have improved plasmid stability but have lower copy numbers (1 to 2 copies per cell). The most commonly used episomal vectors are the YE_p type, derived from the naturally occurring plasmid called 2μ circle in *S. cerevisiae*. These vectors are present at an average of 40 copies per cell and exhibit higher stability than the YRp and YCp vectors. Consequently, YE_p vectors are the best-developed vectors for heterologous gene expression in *S. cerevisiae*. Other YRp- or YE_p-based vectors with regulated copy numbers have been described [43,50]. These vectors are especially useful for controlled expression of toxic proteins. More than 80 compact expression vectors have been developed based on the pRS series of centromeric and 2μ plasmids [51]. The GATEWAY systems (Invitrogen life technologies, Carlsbad, CA) were incorporated into some of these vectors in order to provide fast cloning and transfer of genes to different vectors for heterologous expression in most strains of *S. cerevisiae*. Other 2μ-based expression vectors are available from commercial sources such as YE_pFLAG-1 from Sigma (St. Louis, MO), the YES vectors from Invitrogen, and the pESC vectors from Stratagene (La Jolla, CA).

Integrative vectors contain selectable *S. cerevisiae* genes, and they lack sequences for autonomous replication. These vectors are highly stable but usually present at low copy numbers. They include the Yip-type vectors, which integration can be directed by homologous recombination between sequences carried on the plasmid and its homologous counterpart in the *S. cerevisiae* genome. Other integration systems use repetitive elements such as delta sequences, Ty elements, or tRNA genes for heterologous gene integration [52]. An integration system targeted to a ribosomal DNA cluster has also been described [45].

2.3. Promoters

An array of *S. cerevisiae* promoters has been used for heterologous gene expression. There are constitutive promoters derived from genes such as *CYC1*(cytochrome-c oxidase), *TEF2* (translation elongation factor), and *GPD* (glyceraldehyde-3-phosphate dehydrogenase) as well as regulated promoters such as *GAL1* (galactokinase/galactose epimerase 1), *ADH2* (alcohol dehydrogenase 2), *CUP1* (metallothionein), and *PHO5* (acid phosphatase). Hybrid promoters such as *GAP/GAL* and *GAL10/CYC1* have also been reported. Table 4 provides some examples of promoters and their use for heterologous gene expression in *S. cerevisiae* [53–63]. In addition, comprehensive reviews of *S. cerevisiae* promoters and their applications are available [49,64].

2.4. Transcriptional and Translational Regulation

For efficient heterologous gene expression in *S. cerevisiae*, it is important to tailor the expression plasmid to utilize the transcriptional, translational, and posttranslational regulatory machinery of *S. cerevisiae*. There have been many studies and extensive reviews on these aspects [65–67]. Generally, the use of strong *S. cerevisiae* promoters and robust *S. cerevisiae* transcriptional terminators are essential for maximal expression because most

Table 4 Common Promoters Used for Heterologous Gene Expression in *S. cerevisiae*

Promoter	Regulation	Reference
<i>GAL1</i>	Induction by galactose	Johnston et al., 1987 [53], Proudfoot et al., 1994 [54]
<i>ADH2</i>	Repression by glucose	Price et al., 1990 [55], Kaslow and Shiloach, 1994 [56]
<i>PHO5</i>	Induction by low temperature shift	Lau et al., 1998 [57]
<i>MFα-1</i>	Induction by low temperature shift	Brake et al., 1984 [58], Chen et al., 1994 [59]
<i>PGK</i>	Constitutive	Tuite et al., 1982 [60], Hitzeman et al., 1983 [61]
<i>CUP1</i>	Induction by Cu ²⁺	Karin et al., 1984 [62], Hottiger et al., 1995 [63]

prokaryotic or higher eukaryotic transcription terminators are generally not active in *S. cerevisiae*. More importantly, mRNA levels are controlled by the rate of transcription initiation and transcript turnover rate (mRNA stability).

In addition to upstream regulatory elements, evidence suggests that downstream activation sequences found in the *S. cerevisiae* genes are required for maximum transcription initiation. The expression levels of some foreign genes with high AT content (>60%) in *S. cerevisiae* can be low or absent due to incomplete transcript elongation [49].

Transcript-destabilizing sequence elements have been identified in the 5' untranslated region, coding region, and 3' untranslated regions of different mRNAs [67]. Therefore, if the yield and/or stability of mRNA are found to be problematic, it may be necessary to use a stronger promoter, to delete the destabilizing sequence elements, and to introduce alternated codons. It has been shown that secondary structure of mRNA is one of the most significant factors affecting the rate of initiation and translational elongation. For secreted proteins, posttranslational processes such as proteolytic cleavage, N-terminal modification, and glycosylation are critical to achieving properly folded proteins and high protein yields. Consequently, it is worthwhile to evaluate the effect of the posttranslational steps when the use of strong promoters and different strains fails to produce the desired product yield.

2.5. Heterologous Protein Secretion and Glycosylation

S. cerevisiae has been used for expression of many eukaryotic proteins in both intracellular and secreted forms. A secretion signal derived from the native protein signal sequence or from *S. cerevisiae* signal sequences is needed for efficient secretion of the heterologous proteins expressed in *S. cerevisiae*. With the limited studies available in the literature regarding the effects of different signal peptides on the yield of heterologous protein expression in *S. cerevisiae* [68], it is a good starting point to include the foreign protein's own signal sequence in the *S. cerevisiae* expression cassette. Alternatively, a *S. cerevisiae* signal from such genes as invertase (*SUC2*, 19 amino acids), acid phosphatase (*PHO5*, 17 amino acids), and the most widely used α-factor pheromone (*MFα1*, 20 amino acids) can be incorporated into the recombinant construct.

An important step during protein secretion in *S. cerevisiae* is the glycosylation of translated polypeptides. Heterologous glycoproteins expressed in *S. cerevisiae* are glycosylated at both N-linked and O-linked sites [69]. Little is known about O-linked glycosylation, but more information is available on the process of N-glycosylation. Similar to higher eukaryotes, in *S. cerevisiae* [70], a core sugar moiety consisting of two N-acetyl-glycosam-

ine (GlcNAc), nine mannose (Man), and three glucose (Glu) is added to the N-amide of asparagine (Asn) at the Asn-X-Ser/Thr sequence in endoplasmic reticulum (ER) in the early process of N-glycosylation. Three glucose and one mannose residue are subsequently removed. It is in the Golgi apparatus where further modification takes place and major differences between *S. cerevisiae* and higher eukaryotes in oligosaccharide structures are introduced. In higher eukaryotes, additional mannose residues are removed and several other sugars, such as galactose, sialic acid, and fucose, can be added. *S. cerevisiae* instead maintains the core mannose scaffold that is often extended with a large number of additional mannose residues. This often results in a hyperglycosylated outer chain containing more than 50 mannose residues with many branch chains. Many examples of hyperglycosylation of heterologous proteins in *S. cerevisiae* have been reported [37–39]. Because specific glycosylation patterns are important for the immunogenicities of mammalian proteins, *S. cerevisiae* may be less suitable for the production of therapeutic proteins with complex sugar structures.

2.6. Industrial Protein Production in *S. cerevisiae*

The first therapeutic recombinant protein expressed in *S. cerevisiae* was human α -interferon [1]. Since then, many other therapeutic proteins have been expressed in *S. cerevisiae*. These include the hepatitis-B surface antigen [71], human insulin [72], and many others. An excellent review by Vasavada details a comprehensive list of foreign proteins expressed in *S. cerevisiae* at small and large industrial scales [64]. Various strategies have been reported to improve the expression levels of different heterologous proteins in *S. cerevisiae*. These include screening of various host strains with different expression vectors, altering host genetics by molecular techniques, and changing recombinant *S. cerevisiae* fermentation conditions [64,73,74]. Since no general rules can be derived from these studies, it is worthwhile to examine different parameters for the development of an optimized process.

3. *P. PASTORIS* FOR HETEROLOGOUS PROTEIN EXPRESSION

The *Pichia* expression system has been widely used for expression of heterologous proteins from a diverse array of organisms, including bacteria, fungi, protists, plants, invertebrates, nonhuman vertebrates, and humans. *Pichia* protein expression systems have the following advantages. The systems have a tightly controlled and highly inducible promoter derived from the alcohol oxidase I gene (*AOXI*) of *P. pastoris*, similar molecular genetic manipulation to those of *S. cerevisiae*, a strong preference for respiratory growth, and availability of commercial vectors. Extensive studies have been published on the use of *Pichia* systems in heterologous protein expression. A recent review article by Cregg et al. [75] listed 241 individual studies of heterologous proteins expressed in *P. pastoris*, more than 80 of which were human proteins. Expression levels for heterologous proteins differ greatly, ranging from micrograms per liter to grams per liter [76]. For example, a plant hydroxynitrile lyase from *Hevea brasiliensis* was expressed at levels as high as 22 g/L [77]. Table 5 shows some of the more recent publications on using *P. pastoris* for heterologous protein expression [15,27,78–95].

3.1. Vectors, Leaders, and Host Strains

A fully developed *P. pastoris* expression system is commercially available (Invitrogen, Inc.). The *Pichia* vectors can be grouped into two categories based on the promoters used:

Table 5 Selected Publications on Heterologous Protein Production in *P. pastoris*

Protein	Secretion (S) or Intracellular (I)	Expression Level	Reference
Cation-dependent mannose 6-phosphate (CD-MPR), bovine	S	28 mg/L after purification	Reddy and Dahms, 2002 [27]
<i>Limulus</i> endotoxin-neutralizing protein (rENP)	S	5.46 mg/g dry cell weight	Paus et al., 2002 [15]
Equine lactoferrin	S	40 mg/L	Paramasivam et al., 2002 [80]
Porcine lactoferrin	S	12 mg/L	Wang et al., 2002 [81]
Equistatin from <i>Actinia equina</i>	S	1.66 g/L	Outchkourov et al., 2002 [82]
Monoamine oxidase A (MAO-A), human liver	I	330 mg/L	Li et al., 2002 [83]
Human μ -opioid receptor	I	16–100 pmol/mg	Sarramegna et al., 2002 [78]
Mouse muscle nicotinic acetylcholine receptor	S	3 mg/L	Yao et al., 2002 [84]
Human liver cytosolic beta-glucosidase	S	10 mg/L	Berrin et al., 2002 [85]
Human chitinase	S	144 mg/L	Schilling et al., 2001 [86]
Human chymotrypsinogen	S	480 mg/L	Curvers et al., 2001 [79]
Cysteine proteinase from adult <i>Clonorchis sinensis</i>	S	19.7 mg/L	Park et al., 2001 [88]
Human prostate-specific antigen (PSA)	S	SDS-PAGE and Western blot	Habeck et al., 2001 [89]
Human single-chain Fv antibody fragments	S	5–20 mg/L	Marty et al., 2001 [90]
<i>Rhizopus oryzae</i> lipase	S	12,888 units/L/hr	Minning et al., 2001 [91]
Human ETB endothelin	I	45 pmol/mg	Schiller et al., 2001 [92]
Merozoite surface protein 1 (MSP1) from <i>Plasmodium falciparum</i>	S	500 mg/L	Brady et al., 2001 [93]
Human bile salt-stimulated lipase (rh BSSL)	S	0.8–1 g/L	Murasugi et al., 2001 [94]
Anti-prostate-specific antigen single-chain antibody fragment	S	15–20 mg/L	Wang et al., 2001 [95]
Deacetoxyccephalosporin from <i>Streptomyces</i> spp.	S	120 μ g/mL	Adrio et al., 2001 [87]

(1) inducible promoters derived from the *AOX1* gene or the formaldehyde dehydrogenase gene (*FLD1*) [96], and (2) a strong constitutive promoter derived from the *P. pastoris* glyceraldehyde-3 phosphate dehydrogenase gene (*GAP*). The methanol-inducible *AOX1* promoter provides tighter control of gene expression. *FLD1* is required for growth of *P. pastoris* on either methanol as the sole carbon source or certain methylated amines, such as methylamine, as the sole nitrogen source. Those two conditions induce protein expres-

sion using *FLD1* promoter. Alternatively, the constitutive *GAP* promoter does not require cultures to be shifted from one carbon source to another for the induction of heterologous gene expression. If the foreign proteins to be expressed are toxic to the cells, however, constitutive expression of the enzyme or proteins may inhibit cell growth or result in loss of the expression vector from the cell.

Heterologous proteins expressed in *P. pastoris* can be retained in the cells or secreted into medium depending on the expression vectors used. Vectors commercially available for protein secretion carry a secretion leader sequence from α-MF of *S. cerevisiae*. Other leader sequences, such as the alkaline phosphatase (PHO) signal sequence from *P. pastoris* and some native protein leader sequences, have also been used for secretion of foreign proteins in *Pichia* [97]. To facilitate protein detection and purification, most vectors have 6X His (histidine) and *c-myc* epitope as C-terminal fusion tags.

A variety of selection markers are available for *Pichia* protein expression. Some can be used for selecting multicopy gene integration, such as zeocin and blasticidin, whereas others provide auxotrophic selection using his4⁻ mutant strains. Some of commercially available *Pichia* host strains include X-33, GS115, SMD1168, and KM71. Additional host strains for *P. pastoris* heterologous protein expression are reviewed by Cregg et al. [75].

3.2. Glycosylation in *P. pastoris*

P. pastoris, like *S. cerevisiae* and other fungi species, can potentially perform many protein posttranslational modifications, such as cleavage of signal sequences for secreted proteins, N- and O-linked glycosylation, and formation of disulfide bonds. Glycosylation is an important issue for heterologous protein expression because it affects not only proper folding of the proteins and biological activities, but also secretion and the final yield of expressed foreign proteins. Both N- and O-linked glycosylation have been reported using the *Pichia* systems for heterologous protein expression [75]. The consensus sequence for N-linked glycosylation in *Pichia* is the same as that in *S. cerevisiae* (Asn-X-Thr/Ser), in which Ser or Thr provides hydroxyl group for glycosylation. Similar to *S. cerevisiae* and other eukaryotic organisms, *Pichia* transfers a lipid-linked oligosaccharide unit, Glc₃Man₉-GlcNAc₂, to Asn in the ER. This oligosaccharide core is then trimmed to Man₈GlcNAc₂. Further modifications of the Man₈GlcNAc₂ polysaccharide units are unique to each organism. Unlike hyperglycosylation (>50 Man) occurring in *S. cerevisiae*, the most commonly found N-glycosylation polysaccharide in *P. pastoris* is Man₁₄GlcNAc₂. There has been no α-1,3 mannosyltransferase activity detected in *P. pastoris*. Only α-1,6 and α-1,2-linkages were found in glycosylated proteins in *Pichia* [32].

The information regarding glycosylation with O-linked oligosaccharides in *Pichia* is sketchy and no consensus sequence for O-linked glycosylation has been reported. The O-linked oligosaccharides in *P. pastoris* are generally short (fewer than five residues) and contain only α-1,2-linked mannose units. Phosphorylation of oligosaccharide units has been found in the *P. pastoris* expression system, but the exact sites of phosphate linkage on saccharides are unknown. Limited studies are available for other aspects of transcriptional and translational controls in *Pichia* for heterologous protein expression.

3.3. Scale-Up Fermentation and Limitations

Expression levels of heterologous proteins in *P. pastoris* vary greatly depending on the copy number of the foreign gene and the stability of both the mRNA and the protein.

Since expression levels are correlated with gene copy number, it is important to achieve a high copy number of gene integration. To obtain multiple copies of the expression gene integrated in *Pichia*, one can screen large numbers of transformants (>100) using high concentrations of the selecting agents (e.g., zeocin and blasticidin) or construct multiple copies of the gene in tandem repeats *in vitro*.

In addition to the high copy number transformants, optimization of expression process is also vital to the high-level expression of heterologous proteins in *Pichia*. Although some proteins can be expressed well in flask cultures, it is common for expression levels to be greatly enhanced in a controlled environment. Cultures in fermenter vessels offer several advantages over flask cultures, such as maintaining controlled pH and dissolved oxygen levels and continuous feeding of carbon and other components during cell growth and protein expression. Because *P. pastoris* cells have a preference for respiratory growth, cultures can be grown to very high cell densities (up to 500 absorbance units at 600 nm) [26,75]. This is particularly important for secreted proteins because the concentration of the expressed proteins is often proportional to the concentration of cells in culture. Common defined media for *P. pastoris* protein expression are composed of glycerol, biotin, salts, and trace elements. *Pichia* fermentation for heterologous protein expression can be separated into two phases. The first phase is growth using glycerol as the carbon source, and at this stage, the *AOX1* promoter is fully repressed. After depletion of glycerol, a transitional phase may be added to feed more glycerol at a growth-limiting rate. The second phase of feeding methanol starts induction of *AOX1* promoter and heterologous gene expression. Detailed protocols for fermentation of *P. pastoris* can be found in various articles [98–101].

Similar to other heterologous protein expression systems, proteolytic hydrolysis often causes instability of foreign proteins in *Pichia*. Several strategies can be applied to control proteolytic hydrolysis of foreign proteins. Some examples that have been used successfully to reduce hydrolysis of heterologous proteins in *Pichia* include the following: (1) adding amino acid-rich supplements, such as peptone or casamino acids; (2) culturing at a more acidic pH (*Pichia* culture grows well at pH 3 to 7); (3) using a protease- (*PEP4*, vacuolar protease gene) deficient strain for expression; and (4) controlling culture temperature. Another limitation of heterologous protein expression in *Pichia* is inefficient secretion of some mammalian proteins. In those cases, alternative protein-secretion sequences should be tested for high production of foreign proteins in *Pichia*.

4. FILAMENTOUS FUNGI FOR HETEROLOGOUS PROTEIN EXPRESSION

Filamentous fungi, most notably *Aspergillus* spp., can produce high levels of proteins and are capable of secreting proteins into the media. Several industrial enzymes have been successfully produced in filamentous fungi [102]. For some proteins, posttranslational modifications in *Aspergillus* spp., such as glycosylation, are less problematic than those documented for other expression hosts, such as *P. pastoris* and *S. cerevisiae*. In addition, some filamentous fungi have been regarded as GRAS organisms for production of enzymes important for the food and pharmaceutical industries [103]. However, there are several factors that can limit heterologous protein production in filamentous fungi, such as transcription and translation control, mRNA stability, secretion, and extracellular degradation. In the past 10 years, improvement in these areas has led to an increased number of diverse enzymes being produced in filamentous fungi [11,104].

4.1. Hosts Strains/Markers for Transformation

Unlike *Pichia* and *Saccharomyces*, there are no commercially available protein expression systems for *Aspergillus*. However, there are several web-accessible organizations from which the necessary tools for heterologous protein expression in filamentous fungi can be obtained. These include the ATCC (American Type Culture Collection), the FGSC (Fungal Genetics Stock Center), the NRRL (Agricultural Research Service Culture Collection), and the BCCP (Belgium Culture Collection).

For heterologous protein expression in *Aspergillus* spp., the first step is to select a suitable host strain. The expression host must be either auxotrophic or antibiotic-susceptible strains that can be used for efficient transformation and selection of transformants. The most genetically accessible strain is *A. nidulans*, but *A. niger*, *A. awamori*, and *A. oryzae* are extensively used because of high levels of commercial enzymes expressed in these strains. *A. niger* can efficiently secret many enzymes including food enzymes. Enzymes produced by *A. niger* are also considered GRAS by the U.S. Food and Drug Administration [103]. *A. oryzae* has become more amenable to molecular analysis, and current research is focused on the identification of strong promoters to improve expression of industrially valuable enzymes. Due to the limited number of studies on *Aspergillus* strains and patent restrictions on use of the strains and markers, it would be valuable to explore alternate *Aspergillus* host strains for heterologous gene expression.

Transformation of host strains can result in chromosomal integration of plasmids either through a random mechanism or through site-directed integration. Markers commonly used for transformation of auxotrophic strains are listed in Table 6 [105–112]. The *pyrG* gene (uracil/uridine auxotrophs) from *A. niger* is a common marker found on many useful plasmids. The *A. nidulans* *argB* gene is also used extensively for efficient transformation of a suitable auxotrophic host strain. For example, Buxton et al. [105] described

Table 6 Selectable Markers Used for Heterologous Gene Expression in *Aspergillus* spp.

Gene	Enzyme (Cellular Function)	Reference
Auxotrophic		
<i>argB</i>	Ornithine carbamoyl transferase (arginine biosynthesis) (<i>A. nidulans</i>)	Buxton et al., 1985 [105]
<i>pyr4</i>	Orotidine 5'-phosphate decarboxylase (uracil biosynthesis) (<i>N. crassa</i>)	Balance et al., 1983 [106]
<i>pyrG</i>	Orotidine 5'-phosphate decarboxylase (uracil biosynthesis) (<i>A. niger</i>)	Mattern et al., 1987 [107]
<i>trpC</i>	Phosphoribosyl anthranilate isomerase (tryptophan biosynthesis) (<i>A. nidulans</i>)	Yelton et al., 1984 [108]
<i>amdS</i>	Acetamidase (acetamide or acrylamide assimilation, N2 source) (<i>A. nidulans</i>)	Kelly and Hynes, 1985 [109]
<i>niaD</i>	Nitrate reductase (nitrate assimilation, N2 source) (<i>A. niger</i>)	Unkles et al., 1992 [110]
Dominant		
<i>hph</i> [HmB^R]	Hygromycin B phosphotransferase (<i>E. coli</i>)	Punt et al., 1987 [111]
<i>phleo</i> ^R	<i>E. coli</i> transposon Tn5 phleo ^R DNA	Punt and van den Hondel, 1992 [112]

the complementation of an auxotrophic *A. niger* strain with the *A. nidulans argB* gene. More recently, there was a published report on the complete disruption of the *argB* gene on chromosome I in *A. niger* [113]. The *A. niger argB* deletion strain can be complemented by the *argB* genes from both *A. niger* and *A. nidulans*, and it can serve as a useful host strain for heterologous protein expression in *Aspergillus*.

Although plasmids can be readily integrated into the chromosome (stable, multicopy integration) during transformation of *Aspergillus* sp., it is desirable to have a replicative plasmid for heterologous protein expression in filamentous fungi. In fact, sequences have been isolated that allow one to perform replicative transformation in *A. nidulans* [114].

4.2. Expression Vectors and Promoters

Promoters commonly used for heterologous protein expression in *Aspergillus* spp. are listed in Table 7 [102,115–120]. Constitutive promoters include the *trpC* and the *gpdA* promoters of *A. nidulans*. Other frequently used promoters are the starch-inducible promoters, *alpha*-amylase, and the glucoamylase promoters from *A. oryzae* and *A. niger*. The glucoamylase promoter responds to maltose as a carbon source and is repressed by glucose. More recently, the *TEF1-alpha* (translation elongation factor) promoter of *A. oryzae* has been studied for use as a strong constitutive promoter [120].

Another suitable promoter for the expression of heterologous proteins in filamentous fungi is the ethanol-regulated promoter *alcA*. Genes for ethanol utilization in *A. nidulans* are efficiently transcribed. The *alcA* promoter is tightly regulated through the *alcR*, a transactivating gene, and is subject to repression by the major carbon catabolite repressor, CreA [115]. In a recent report, expression of a portion of the *creA* antisense in *A. nidulans* increased levels of expression for glucose-repressible enzymes in the presence of glucose [121]. The derepression effect by the *creA* antisense did not alter cell morphology and growth parameters, as observed in a *creA* null mutant. The *alcR–alcA* from *A. nidulans* has been transformed into *A. niger*, which does not possess an inducible *alc* system [122]. The *alcR–alcA* transformed strain of *A. niger* showed similar levels of heterologous expression of the reporter enzyme, *beta*-glucuronidase, as in the *A. nidulans*.

Table 7 Promoters Used in Heterologous Gene Expression in *Aspergillus* Sp.

Gene	Enzyme (Cellular Function)	Reference
Strong inducible		
<i>alcA</i>	Alcohol dehydrogenase 1 (ADH1) (ethanol inducible) (<i>A. nidulans</i>)	Felenbok et al., 2001 [115]
<i>alpha</i> -amylase	Starch inducible (<i>A. oryzae</i>)	Christensen et al., 1988 [116]
<i>glaA</i>	Glucoamylase (starch and maltose inducible) (<i>A. nidulans</i>)	Gouka et al., 1997 [102]
Constitutive		
<i>gpdA</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>A. nidulans</i>)	Punt et al., 1990 [117]; Punt et al., 1991 [118]
<i>trpC</i>	Phosphoribosyl anthranilate isomerase (tryptophan biosynthesis) (<i>A. nidulans</i>)	Hamer and Timberlake, 1987 [119]
<i>TEF1-alpha</i>	Translation elongation factor 1 -alpha (<i>A. oryzae</i>)	Kitamoto et al., 1998 [120]

Ishida et al. [123] reported that tyrosinase gene promoter (*melO*) was four times stronger for the heterologous expression of β -glucuronidase from *E. coli* in a submerged culture as compared with other gene promoters, such as *amyB*, *glaA*, and *gpdA*, previously used for heterologous gene expression in *A. oryzae*. The *melO* promoter was used to produce the *glab*-type glucoamylase in a submerged culture of *A. oryzae* and achieved maximum yield of 3.3 g/L and 99% purity. This work establishes the *melO* promoter for high-level expression and high-purity production of heterologous proteins in *A. oryzae*.

4.3. Gene Fusions

Large numbers of mammalian enzymes and proteins have been expressed through genetic engineering; however, the secretion of heterologous proteins is often much less efficient than the secretion of fungal native proteins. During the past 10 years, the production levels of heterologous proteins in *Aspergillus* have been improved by fusing the protein of interest to the 3' end of a highly expressed gene of which the protein product is efficiently secreted. This gene-fusion strategy, or carrier approach, has been used with the *A. niger* and *A. awamori* glucoamylase genes. Increases of 5- to 1000-fold in protein production levels have been observed. Gouka et al. [124] extensively reviewed the production levels of different proteins fused to the glucoamylase. They also performed detailed experiments on the production levels of various proteins with carrier fusions to the glucoamylase protein in isogenic, single-copy strains of *A. awamori*. The glucoamylase gene can be divided into three distinct domains: the catalytic N-terminal domain, the C-terminal starch-binding domain, and a flexible linker region that separates the two domains. The C-terminal starch-binding domain can be replaced by a heterologous protein. These glucoamylase carrier proteins are believed to improve translocation in the ER, to enhance proper protein folding, and possibly to afford protection against proteolysis. In the secretory pathway, the carrier protein can be cleaved by a KEX2-like protease at a specific proteolytic cleavage site separating the glucoamylase carrier and the protein of interest. The amino acid sequence (NVISKR) of the cleavage site is derived from the pro-peptide of glucoamylase. The carrier approach appears to be the first choice in attempts to produce proteins in *Aspergillus* spp. There are many reports on the use of a carrier for heterologous protein secretion. A recent study reported a successful secretion of the laccase (*lac1*, an industrially useful enzyme for the oxidation of *p*-diphenols) from *Pycnoporus cinnabarinus* that used the *A. niger* glucoamylase pre-pro-sequence as a carrier for the heterologous enzyme expression. This allowed an 80-fold increase in laccase production compared with the levels observed with the laccase signal peptide sequence. Another advantage of using the glucoamylase pre-pro-sequence as a carrier for laccase in *A. niger* was the production of the 70-kDa protein, similar to that of the native protein, which suggests no hyperglycosylation as observed for the 110-kD protein produced in *P. pastoris*. Human interleukin IL-6 secretion was also improved using the native *A. niger* glucoamylase as a carrier. It has been reported [125] that the “unfolded protein response” (UPR), the deliberate upregulation of “foldases” and chaperones, can also improve the secretion of heterologous proteins.

4.4. Posttranslational Issues

Efficient heterologous protein production in fungi is not always achieved by alleviating the limitations of transcription. Problems with secretion and postsecretion play important roles. One of the major limiting factors in protein production in filamentous fungi is proteolytic degradation by the high levels of intracellular and extracellular proteases. A.

niger has four predominant extracellular proteases: two aspartyl proteases (PEPA and PEPB) and two serine carboxypeptidases (PEPF and PEPG). In *A. nidulans*, neutral and alkaline proteases seem to predominate. Efforts to eliminate specific protease activities through mutagenesis or gene disruption have improved protein expression levels. Van den Hombergh et al. [12] reviewed fungal proteases and described the development of a set of tester strains of *A. niger* which may be used to identify the appropriate genetic background for a particular protein.

Posttranslational modification by N-glycosylation is important for many pharmaceutically relevant proteins [126]. High-mannose-type glycosylation as experienced in proteins overproduced in *Saccharomyces* and *Pichia* spp. is not observed in filamentous fungi. Maras et al. [127] reviewed the fungal glycosylation pathway and approaches used for the production of mammalian proteins.

The successful expression of *Drosophila melanogaster* xanthine dehydrogenase in *A. nidulans* is a recent example of proper posttranslational modification of a heterologous enzyme in filamentous fungi [128]. In comparison to the baculovirus expression system, which produced only about 10% of active xanthine dehydrogenase enzyme due to incomplete incorporation of the molybdenum cofactor and two iron-sulfur clusters, *A. nidulans* produced 40% of active enzyme with relatively more complete cofactor incorporation [128].

4.5. *Aspergillus* Scaleup Fermentation

In addition to the molecular genetic approaches, a combination of host-strain selection, strain development, and production process development (including media optimization) have resulted in commercially attractive protein yields in *Aspergillus* protein expression [25]. For example, IL-6 gene fusion technique in *A. nidulans* in combination with host-strain development and growth optimization resulted in a 200-fold increase in production of active IL-6 [25]. A combination of gene dosage and growth condition optimization for the improvement of protein expression in *A. oryzae* was described for overproduction of phospholipase A1 (PLA1) at an industrial scale [129]. Transformants with 15 to 20 copies of the *PLA1* gene were integrated into chromosome and produced activity that was 60- to 7500-fold higher than that in the native host strain. In addition, improvement of the mycelial morphology from a pellet form to a filamentous form improved production of PLA1 in scaled up fermentations and resulted in production of PLA1 at 1.8 g/L consistently.

It has been recognized that fungal morphology plays an important role in determining process productivity during mycelial fermentations. This has led to investigations into alternative ways to conduct fermentations to optimize production yields. Pappagianni et al. investigated the effect of cell immobilization on protein production and secretion [130]. Cells of *A. niger* were immobilized by attachment to metal surfaces or spore entrapment and subsequently grown on Celite beads. They reported that cell attachment on metal surfaces decreased secretion of proteases and increased glucoamylase production, while growth in shake flasks on Celite beads reduced the secreted proteases by half and almost doubled glucoamylase-specific activity. In bioreactor cultures, these effects were greater.

5. FUTURE PROSPECTS

The demand for producing a variety of proteins and enzymes in heterologous hosts for research and therapeutic applications will remain strong. Diverse systems for heterologous

protein expressions must be continuously developed and perfected. Fungal expression systems offer many advantages over other expression systems and have been widely used for production of heterologous proteins from many organisms. Discovery of new promoters and the development of new vectors will provide more choices for different protein expression needs. More understanding of transcriptional control, mRNA stability, glycosylation, protein folding, and other posttranslational processes will allow us to overcome difficulties associated with heterologous protein expression in fungi, such as hyperglycosylation in yeasts and poor secretion associated with some mammalian proteins. It has been documented that the use of protease-deficient strains can increase the yield of recombinant protein production [131,132] and that the use of specific mutants may eliminate hyperglycosylation problems of some proteins expressed in *Saccharomyces* [73]. As more studies are making progress, heterologous protein expression in *Aspergillus* and other filamentous fungi has great potential to become a major player in heterologous enzyme production. Collectively, fungi protein expression systems will continue to be an alternative for production of heterologous proteins in small or large quantities.

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25

Toxigenic Fungi and Mycotoxins

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1. INTRODUCTION

Fungi are found in every environment and have been estimated to constitute about 25% of the earth's biomass [1]. Many fungi reproduce and disperse by forming billions of spores, and these fungal spores are present in the indoor and outdoor air. Thus, not surprisingly, our respiratory tract is constantly exposed to the aerosolized fungal spores. In addition, fungal spores may contain potent allergens to which certain people respond with strong hypersensitive (allergic) reactions. These allergenic fungal spores are particularly problematic for farmers. In fact, farmers can contract a lung disease called *farmer's lung* from breathing the billions of tiny fungal spores shaken out of bales of moldy hay. Over time, some people develop an allergic reaction to them. The symptoms are serious, and once the allergic reaction begins, the person will always have the potential for symptoms upon exposure to hay mold. Even worse, many fungi are toxigenic and produce toxic compounds called *mycotoxins*. These toxins can be present in fungal spores and elicit a response in the lungs. The presence of mycotoxins in the lungs can interfere with the cell-mediated immune responses. Fungal spores and associated mycotoxins are troublesome agents that raise a number of health and safety issues.

Fungi constitute major pathogenic organisms of plants and can cause extensive crop damage as a result of disease in the field and postharvest food spoilage that is often accompanied by production of mycotoxins. In reality, contamination of agricultural commodities by mycotoxins is more problematic than the fungal diseases themselves. Mycotoxins are highly toxic and cause serious adverse health effects. The economic costs of mycotoxins are impossible to accurately measure, but the mean annual cost of crop losses in

the United States due to three major mycotoxins (aflatoxins, fumonisins, and deoxynivalenol) is estimated to be more than \$900 million [2].

Mycotoxins are toxic secondary metabolites produced by filamentous fungi (molds). The name mycotoxin originated from the Greek word *mykes* (fungus) and the Latin word *toxicum* (poison). These low-molecular-weight compounds (usually <1000 Da) are naturally occurring and unavoidable. Mycotoxins can enter our food chain either by direct contamination resulting from the use of food components contaminated with mycotoxins or by indirect contamination resulting from the growth of toxigenic fungi on food [3]. Consumption of mycotoxin-contaminated food or feed causes acute or chronic mycotoxicoses to human and animals. Mycotoxins cause different symptoms and toxicities in various kinds of animals including cattle on the farm and human beings. In addition to concerns about the adverse effects of direct consumption of mycotoxin-contaminated foods and feeds, a public-health concern is also raised over the potential consumption of animal-derived food products (e.g., meat, milk, eggs) containing residues of mycotoxins or their metabolites [4–6]. These ingested mycotoxins can lead to deterioration of liver or kidney function, increase susceptibility to infections, and cause cancer and even death [7]. Diseases caused by ingested mycotoxins (intoxication) are called *mycotoxicoses*, whereas *mycosis* is a disease caused by fungal infection.

Historically, several outbreaks of mycotoxicoses have been reported [7]. In the Middle Ages, outbreaks of ergotism due to ergot alkaloids produced by *Claviceps purpurea* were responsible for the death of thousands of people in Europe [8], and recently, ergot toxicity has been reported in South Africa [9]. In the 1930s and 1940s, alimentary toxic aleukia was caused by trichothecenes produced by *Fusarium* spp. In 1960, Turkey X disease occurred in England and triggered modern mycotoxicology.

Members of genera *Aspergillus*, *Fusarium*, and *Penicillium* are the major mycotoxin producers in nature [10]. These fungi exist as saprophytes, plant pathogens of major crop plants, and rarely, human pathogens (certain species of *Aspergillus*) [11]. *Aspergillus* and *Penicillium* spp. contaminate foods and feeds under storage conditions with high humidity and temperature. *Fusarium* spp. are distributed worldwide as saprophytes and plant pathogens. *Fusarium* spp. have wide host ranges and infect crops growing in the field. When pathogenic *Fusarium* spp. infect crop plants such as wheat, barley, and corn, they propagate in plant tissues and produce mycotoxins [12]. Mycotoxins are not easily removed from foods or feeds during processing due to their stability against heat and physical/chemical processes.

This chapter describes the general characteristics of the important mycotoxins produced by the three major toxigenic fungal genera, the toxicity and modes of action of the major mycotoxins, current regulations and guidelines, some minor mycotoxins, and detection/analytical methods. In particular, the schematic illustrations of the modes of action of the major mycotoxins at the molecular level will help readers to understand the mechanisms of toxicity caused by major mycotoxins. Overall, this chapter provides basic knowledge and information regarding toxigenic fungi and mycotoxins.

2. MAJOR MYCOTOXINS

More than 300 mycotoxins have been identified to date, but only a few are regularly found in food and animal feedstuffs, such as grains and seeds. Six mycotoxins, or groups of mycotoxins, contaminate foods and feeds quite often and represent the major classes of mycotoxins: aflatoxins, trichothecenes (deoxynivalenol/nivalenol/T-2 toxins); zearalenone; fumonisins; ochratoxins; and patulin. Table 1 summarizes major mycotoxins, toxic-

Table 1 Major Mycotoxins, Toxigenic Fungi Producing Them, Modes of Action, and Toxic Effects

Mycotoxins	Fungal Species	Mode of Action	Toxic Effects	Food and Feed	Affected Species
Aflatoxins (B ₁ , B ₂ , G ₁ , G ₂ , M ₁ , M ₂)	<i>Aspergillus flavus</i> <i>A. parasiticus</i> <i>A. nomius</i>	Bioactivated AFB1-epoxide attacking macromolecules and causing tumor by forming AFB1-DNA adduct	Hepatotoxicity Carcinogenesis (liver tumors) Hemorrhage (intestinal tract, kidneys)	Peanuts, corn, nuts, rice, barley, wheat, milk, eggs, cheese, cottonseed	Cattle, human
Fumonisins (B ₁ , B ₂ , B ₃ , C ₁ , C ₃ , A ₁)	<i>F. verticillioides</i> <i>F. proliferatum</i>	Inhibition of ceramide synthase	Leukoencephalomalacia in horse, Pulmonary edema in swine, Human esophageal cancer	Corn, commercial corn products, mixed feed	Human, horse swine, rat, mouse
Ochratoxin A	<i>A. ochraceus</i> <i>P. verrucosum</i>	Inhibition of phenylalanine hydroxylase	Nephrotoxicity, Porcine nephropathy, Carcinogenesis	Cereals, coffee, dry beans, dried fruits	Duckling, swine, dog, rat, human
Patulin	<i>P. expansum</i> <i>P. coprobium</i> <i>A. terreus</i>	Inhibition of epithelial barrier function by inactivating protein tyrosine phosphatase	Capillary damage (liver, spleen, kidney) Carcinogenesis	Apple juice, rotted apple, moldy feed, fruits and nuts	Human, cattle
Trichothecenes (T-2, HT-2, DAS, DON, NIV, 4-ANIV)	<i>F. graminearum</i> <i>F. acuminatum</i> <i>F. avenaceum</i> <i>F. crookwellence</i> <i>F. poae</i> <i>F. sporotrichioides</i> <i>Stachybotrys</i> spp. <i>Myrotheicum</i> spp. <i>Trichothecium</i> spp.	Blocking protein synthesis by inhibiting to peptidyl transferase	Hemorrhage (stomach, intestine, lungs, bladder, kidney) Feed refusal,	Corn, wheat, barley, mixed feed	Human, cattle, chicken, turkey, rat, mouse, horse
Zearalenone	<i>F. graminearum</i> <i>F. sporotrichioides</i> <i>F. crookwellence</i> <i>F. semitecum</i>	Binding to estrogen receptors	Hyperestrogenism, infertility, abortion	Wheat, barley, sorghum, mixed feed, corn	Swine, dairy cattle, chicken, rat, mouse

genic fungi producing them, toxic effects observed in humans and animals, modes of action, and the staple food commodities they affect.

2.1. Aflatoxins

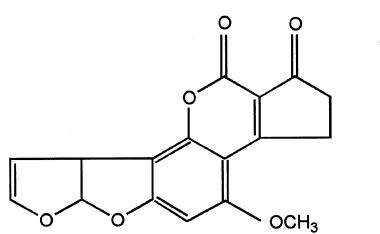
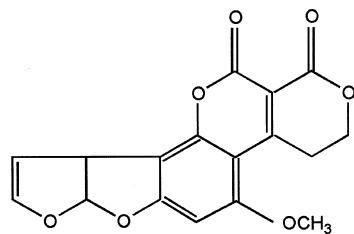
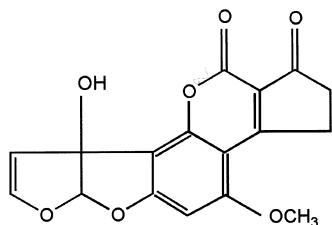
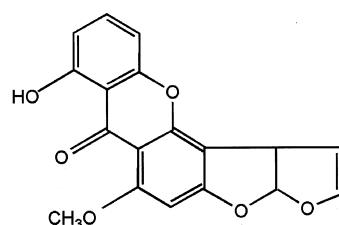
Aflatoxins were discovered as causative agents of Turkey X disease in Great Britain more than 40 years ago and have been subject to a great deal of research. They are potent human carcinogens and can interfere with immune system functions [13].

Aflatoxins are a group of structurally related toxic secondary metabolites produced by certain strains of four *Aspergillus* spp.: *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. pseudotamarii* [14–16]. However, *A. flavus* and *A. parasiticus* are major producers. In fact, the name *aflatoxin* originated from *A. flavus*. In 1960, an outbreak of Turkey X disease in Great Britain was traced to contaminated peanut meal imported from Brazil [17,18]. *A. flavus* was isolated from the peanut meal and found to produce causative toxic compounds, which were named as aflatoxins. Aflatoxins were identified as the cause of death for more than 100,000 young turkeys and some 20,000 ducklings, pheasants, and partridges. The isolation and identification of aflatoxins served as bases for modern mycotoxicology.

The first epidemic of aflatoxicosis affecting humans and dogs was reported in India [19]. The outbreak in 1974 was traced back to the consumption of maize contaminated with aflatoxins at levels up to 15 mg/kg (15 ppm). It has been reported that 994 people were affected and 97 people died. Aflatoxins caused adverse effects to all age groups, including children and to both sexes, and they even caused an almost 100% fatality rate in domestic dogs [20].

As mentioned, aflatoxins are produced mainly by *A. flavus* and *A. parasiticus*. *A. flavus* has a world-wide distribution and normally presents as a saprophyte in soil and many other different organic materials. This fungus is one of the most common species of *Aspergillus* and is often associated with aspergillosis (human disease). Under favorable conditions, particularly temperature and moisture, these fungi grow on certain foods and feeds and produce aflatoxins as secondary metabolites. While *A. flavus* strains produce only type B aflatoxins, *A. parasiticus* strains can produce aflatoxin B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁), and G₂ (AFG₂) [21]. Production of aflatoxins mostly takes place during harvest and storage, although field crops may be infested by *Aspergillus* before harvest. Aflatoxin-producing fungi grow on a variety of foods, including nuts and seeds (ground nuts, pistachios, almonds, walnuts, and cottonseeds [4]) and some cereals (maize, rice, barley, oats, and sorghum [22]). Surveys in several countries have shown that aflatoxin M₁ (AFM₁; M for milk) may be present in liquid or dried milk and in milk products. As AFM₁ is a major metabolite of AFB₁ in cows, levels of AFM₁ in milk are directly related to the daily intake of AFB₁ in dairy feeds [23].

Chemically, the aflatoxins are fluorescent heterocyclic compounds with molecular weights of 286 to 346 Da (Fig. 1). The chemical structures of AFB₂ (C₁₇H₁₄O₆, 314.3 Da) and AFG₂ (C₁₇H₁₄O₇, 330.3 Da) are the dihydroxy derivatives of AFB₁ (C₁₇H₁₂O₆, 312.3 Da) and AFG₁ (C₁₇H₁₂O₇, 328.3 Da), respectively. AFM₁ and AFM₂ are 4-hydroxy AFB₁ and AFB₂, respectively. The nomenclature B and G are derived from the blue and green fluorescent colors exhibited by these compounds under UV light on thin-layer chromatography (TLC) plates. AFM₁ was the first biotransformed metabolite identified, and its name is derived from its presence in milk. The toxicity of AFM₁ appears to be equivalent to that of AFB₁. Aflatoxins belong to the furofuran group of fungal

Aflatoxin B₁Aflatoxin G₁Aflatoxin M₁

Sterigmatocystin

Figure 1 Chemical structures of aflatoxins and sterigmatocystin. AFM₁ is the bovine metabolite of AFB₁ that is found in milk. Sterigmatocystin is a penultimate precursor of aflatoxins and is produced by more than 20 fungal species.

metabolites, which contain either an unsaturated 7,8-dihydrofuran (2,3-β) furan or the more reduced 2,3,7,8-tetrahydro-(2,3)-furan system [24]. Aflatoxins are soluble in moderately polar solvents (e.g., chloroform and methanol) and especially in dimethylsulfoxide (DMSO). They are very stable at high temperatures but relatively unstable when exposed to light and UV radiation. At present, ammoniation has been found to be the most effective way to reduce aflatoxin levels in foods and feeds [13,17].

The rank order of toxicity is AFB₁ > AFM₁ > AFG₁ > AFB₂ > AFM₂ > AFG₂. Aflatoxins cause liver damage, liver cancer, and decreased milk and egg production. Clinical signs of aflatoxicosis in animals include gastrointestinal dysfunction, reduced reproduction, reduced feed utilization and efficiency, and anemia [17]. The carcinogenicity of aflatoxin was first reported in 1961 by showing that rats developed hepatomas when fed the same peanut meal implicated in Turkey X disease [25]. AFB₁ has been identified as the most potent hepatocarcinogen known. Cancer induction by the aflatoxins AFB₁, AFG₁, and AFM₁ has been studied extensively because they show higher toxicity than any other aflatoxin derivatives. Three major aflatoxins cause various types of cancer in diverse animals. Presently, the International Agency for Research on Cancer (IARC) designates only AFB₁ as a carcinogen.

Aflatoxins cause adverse effects in animals (and presumably in humans), which can be categorized as follows:

Acute toxicity develops when moderate to high levels of aflatoxins are consumed.

High doses of aflatoxins, particularly AFB₁, inhibit protein and RNA synthesis, which is thought to contribute to the necrosis and fatty changes seen at high-dose levels. Symptoms may include hemorrhage, acute liver damage, malfunction of immune response, edema, interference with the digestion, absorption, and/or metabolism of nutrients, and possibly death [26,27].

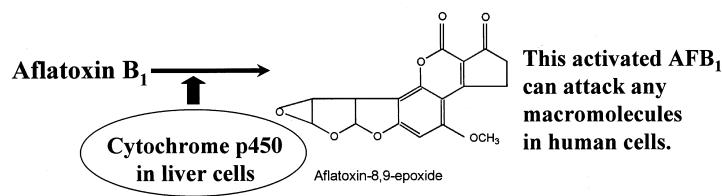
Chronic toxicity results from ingestion of low-to-moderate levels of aflatoxins.

Although the effects are usually subclinical and difficult to recognize, the major concern is cancer development [28]. Some of the visible symptoms caused by chronic toxicity may include impaired food conversion and slower rates of growth with or without the symptoms mentioned.

The mode of action of AFB₁ toxicity is well understood [29,30]. As presented in Fig. 2, when AFB₁ is consumed by animals (and humans), it is first metabolized by cytochrome P450 enzymes (CYPs), heme-containing monooxygenases, which catalyze the final step in the incorporation of oxygen into organic molecules. Such oxygenation usually makes foreign chemicals (xenobiotics) less toxic and more water-soluble, thereby facilitating elimination of xenobiotics from the body. The problem is that these enzymes can also transform nontoxic chemicals into reactive intermediates that are highly toxic and/or carcinogenic. To be toxic, AFB₁ must be bioactivated by CYPs into AFB₁-8,9-epoxide (also referred to as AFB₁-2,3-epoxide), the ultimate carcinogen and mutagen [30]. CYPs capable of activating AFB₁ in animals include members of the 1A, 2B, 2C, and 3A subfamilies. The toxic effects of AFB₁ are principally due to the binding of bioactivated AFB₁-8,9-epoxide to cellular macromolecules, particularly mitochondrial and nuclear nucleic acids and nucleoproteins, resulting in general cytotoxic effects [17]. AFB₁ has also been shown to induce lipid peroxidation in rat liver. AFB₁ is a very potent carcinogen in many species, including nonhuman primates, birds, fish, and rodents. AFB₁ has been shown to be 200 times more carcinogenic than benzopyrene (a major carcinogen in cigarette products). Carcinogenic effects are primarily caused by formation of AFB₁-⁷N-guanine (AFB₁-DNA adduct) resulting in G → T transversion. AFB₁-DNA adduct is formed by covalent bonding between C-8 of AFB₁-8,9-epoxides and ⁷N of guanine base in DNA (Fig. 2). Studies of the relationship between aflatoxin exposure and development of human hepatocellular carcinoma revealed that the p53 tumor-suppressor gene is a key player. In normal cells, p53 binds to DNA upstream of p21 (a gene encoding another protein) and activates transcription of p21, thereby increasing activity of p21. The p21 protein then interacts with cdk2 (cyclin-dependent kinase 2). The cdk2 protein is necessary for cell division, and binding of p21 to cdk2 blocks cell division and proliferation. Stoppage of cell division allows cells to check the condition and status of genetic materials and cellular functions and to repair any damage before the next stages of cell division. If damage cannot be repaired, p53 induces apoptosis (i.e., programmed cell death). AFB₁ causes mutations in p53, especially the arginine-to-serine substitution at the codon 249 position. Mutated p53 loses its function and can no longer activate p21 protein that, as described, acts as a “stop signal” for cell division. The absence of p21 thus results in uncontrolled cell division and proliferation and tumor development [31].

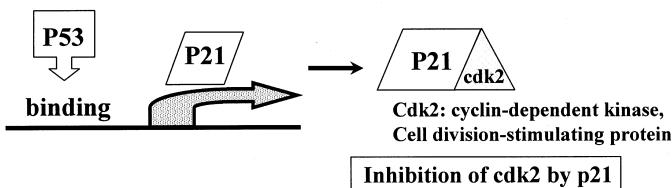
Although all animal species are susceptible to toxicity of aflatoxins, sensitivity varies considerably from species to species. For example, birds, fish, dogs, and swine appear to

A. Biotransformation of AFB₁



B. Tumor formation by AFB₁

1. Normal cell division: p53 activates p21, which inhibits cdk2



2. Uncontrolled cell division: non-functional p53, no p21, free cdk2

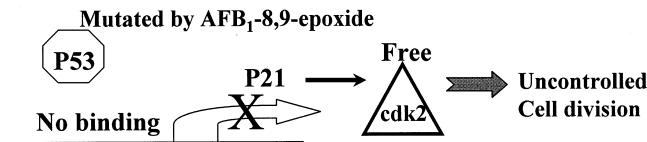


Figure 2 Mode of action of aflatoxin B₁. Aflatoxin B₁ is bioactivated to highly nucleophilic aflatoxin-8,9-epoxide in human and animal liver by cytochrome P450 (A). Carcinogenic effect of aflatoxin B₁ is primarily by the formation of AFB₁-⁷N-guanine (AFB₁-DNA adduct), resulting in G-to-T mutation (transversion). Mutations in the p53 tumor-suppressor gene that causes loss of function result in uncontrolled cell division.

be more susceptible than mature cattle. Environmental factors, exposure level, and duration of exposure, age, health, and nutritional status determine the susceptibility of individual species. For most species, the LD₅₀ value ranges from 0.5 to 10 mg/kg body weight [17]. Aflatoxins may cause vaccines to fail, increase birds' susceptibility to disease, and result in suppression of the natural immunity to infection.

Aflatoxins have been regulated by the U.S. Food and Drug Administration (FDA) since 1965 under Section 402(a)(1) of the Food, Drug and Cosmetic Act, which states that an additive is regarded as unsafe if it "may be injurious to health" or is "ordinarily injurious to health". Aflatoxins are the only mycotoxins for which action levels (regulation) have been established. Only advisory (recommended) levels are set for some other mycotoxins. The current action levels for aflatoxins in foods and feeds are as follows: (1) 20 parts per billion (ppb) in all products (except milk) for human consumption; (2) 0.5 ppb in dried milk and 0 ppb in liquid milk; (3) 20 ppb in corn for young animals and

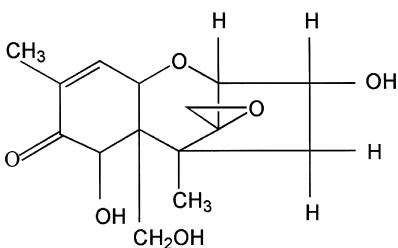
dairy cattle; and (4) 100 to 300 ppb in corn and peanut products in feeds, depending on kind and purpose of the animals (<http://vm.cfsan.fda.gov/~lrd/fdaact.html>).

2.2. Trichothecenes

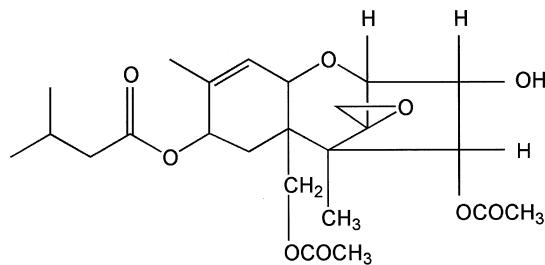
A disease called *alimentary toxic aleukia* (ATA) (meaning a lack of leukocytes or white blood cells) that arose from food poisoning occurred in the Soviet Union during World War II, particularly in the Orenburg District in 1944. Initial symptoms were nausea and vomiting from which patients appeared to recover. In many cases, the disease progressed to a stage where the bone marrow was damaged and failed to generate new red and white blood cells. There were no evident symptoms in the early stages. However, patient health declined and the disease progressed to a third stage manifested by subcutaneous hemorrhages, anemia, and up to 80% mortality [32]. The cause of this disease was found to be consumption of over-wintered cereal grains that were contaminated by *Fusarium poae* and *F. sporotrichioides*. These organisms were shown to be able to produce the actual causative agents, trichothecenes including T-2 toxin. Since then, more than 150 trichothecenes have been identified [33]. In 1934, in the Midwest, more than 5000 horses died because of “moldy corn disease,” and *Gibberella* ear rot caused extensive feed-refusal problems in swine in the Corn Belt of the United States in 1972 [34]. Red mold disease in Japan and Korea has been connected to *Fusarium* spp. producing deoxynivalenol (DON) and related trichothecene compounds [35,36].

Trichothecenes are composed of a tetracyclic sesquiterpene skeleton containing a six-membered oxane ring, a stable epoxide group in positions 12 and 13 and a 9,10 olefinic bond (Fig. 3). Of the more than 150 known trichothecenes, only a few are of importance because of their frequent presence and amount in agricultural commodities for human or animal consumption. There are four types of trichothecenes: type A, including diacetoxyscirpenol (DAS), monoacetoxyscirpenol, HT-2, neosolaniol, and T-2; type B, including DON (vomitoxin), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), 4-acetylvalenol (4-ANIV; fusarenon-X); and less important types C and D [33,34]. Type C trichothecenes (crotocin, baccharin) contain additional epoxide groups at C-7,8 or C-9,10, and type D toxins (satratoxin, roridin) contain a macrocyclic ring (structures not shown) between C-4 and C-15. Important ones are two highly toxic members of group A, which are DAS and T-2 toxin, as well as two toxins in group B, which are DON and NIV [33]. DON is the most common but least toxic trichothecene, and its common name, vomitoxin, came from the vomiting that generally accompanies trichothecene poisoning [37]. Trichothecenes are the most chemically diversified of all the mycotoxins.

Trichothecenes are produced as secondary metabolites by several common molds, including species in the genera *Acremonium* (*Cephalosporium*), *Cylindrocarpon*, *Dendrodochium*, *Myrothecium*, *Trichoderma*, *Trichothecium*, *Stachybotrys*, and most importantly in *Fusarium*. About 24 species of *Fusarium* are known to produce trichothecenes. The most common producers include *F. acuminatum*, *F. avenaceum*, *F. chlamydosporum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. moniliforme*, *F. nivale*, *F. proliferatum*, *F. sambucinum*, *F. solani*, *F. sporotrichioides*, *F. oxysporum*, and *F. tricinctum* [11,33,34]. Some *Fusarium* spp., particularly *F. graminearum*, can infect crop plants in the field during growth and can produce trichothecenes when they are propagating in plant tissues. *F. graminearum* and *F. culmorum* infect barley and cause *Fusarium* head blight called “scab,” which was first characterized in the 19th century. The most signifi-



DON (Vomitoxin)



T-2 toxin

Figure 3 Trichothecene structures. T-2 is the most toxic compound in the trichothecene type A group. Deoxynivalenol (DON; vomitoxin) is a type B toxin and causes feed refusal and vomiting when animals are fed with DON-contaminated feed.

cant mycotoxin associated with scab is DON. Based on the production of 8-ketotrichothecenes, *F. graminearum* is chemotaxonically divided into two groups: the NIV chemotype, which produces NIV and 4-ANIV, and the DON chemotype, which produces DON and 3-ADON [38]. While the NIV chemotype was reported in Asia and Italy [39,40], North American isolates of *F. graminearum* produce DON and 15-ADON as the major toxins, although grain contamination with NIV was also reported in Canada [41]. *F. sporotrichioides* and *F. poae* can infect crops and produce T-2 toxin and DAS [32]. *F. tricinctum*, *F. equiseti*, *F. sporotrichioides*, *F. avenaceum*, and *F. crookwellense* cause red ear rot and produce T-2 and DAS [34]. Collectively, the major *Fusarium* toxins that have been detected in agricultural commodities are DAS, monoacetoxyscirpenol, DON, 3-ADON, 15-ADON, NIV, 4-ANIV, T-2, HT-2, and neosolaniol.

The common symptoms of trichothecene toxicity are dermal irritation, feed refusal in animals, hemorrhage in intestines, and suppression of immune response [7,26,32–34]. Vomiting is also a significant symptom of trichothecene toxicosis in humans and animals. T-2 and DAS are potent trichothecenes for humans and animals and are naturally occurring food/feed contaminants [42]. NIV is less toxic, and DON is the least toxic of the four [34,43]. However, DON is the most widely distributed *Fusarium* mycotoxin, and it contaminates cereals of temperate regions of Japan, Korea, Europe, Southern Africa, and Australia [44]. Monoacetate derivatives, including 3-ADON, 15-ADON, and 4-ANIV, also occur as minor contaminants of DON and NIV [45–47].

Trichothecenes are highly toxic at the subcellular, cellular, and organ levels because they can easily penetrate cell-membrane lipid bilayers and react with DNA, RNA, and

cellular organelles [43]. The toxicity of these toxins at the subcellular level is largely due to the ability to inhibit protein synthesis [48]. Trichothecenes bind to peptidyl transferase and cause inhibition of initiation (NIV, T-2, and fusarenon X) or elongation/termination (DON and verrucarines) of protein synthesis (Fig. 4). Thus, the target organelle is the 60s subunit of eukaryotic ribosomes. Although protein synthesis is an essential function in all tissues and organs, the following tissues where cells undergo active multiplication are particularly susceptible to trichothecenes: intestinal mucosa, thymus, bone marrow, lymph nodes, spleen, and liver. Tissues such as the brain and muscle are less severely affected [33,34,43]. The trichothecenes are highly cytotoxic, causing cell lysis and inhibition of

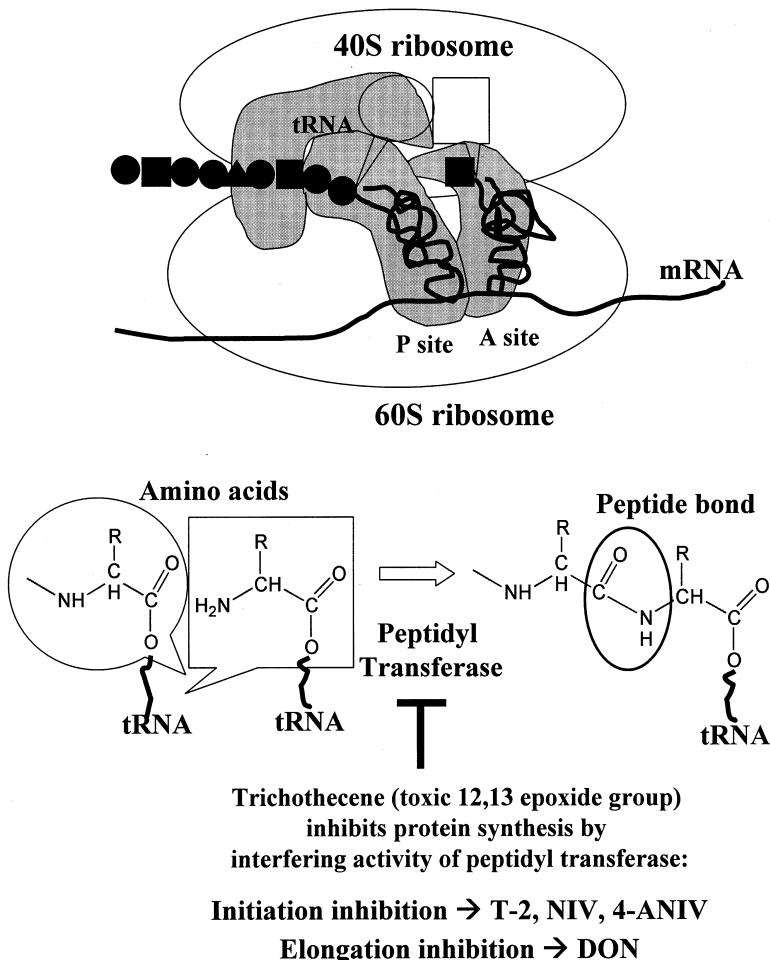


Figure 4 Mode of action of trichothecene toxicity. Trichothecenes inhibit (presented as —) protein synthesis by binding to 60S ribosomal subunit (upper illustration). Specifically, peptidyl transferase, which functions in generation of peptide bonds between amino acids carried by tRNAs (shown in lower left panel), is inhibited by trichothecenes causing the blockage of protein synthesis at the initiation or the elongation steps.

mitosis [43]. The immunosuppressive effects of trichothecenes result in decreased resistance to secondary infection by bacteria, yeasts, and viruses [49,50].

Swine are sensitive to DON, but dairy cattle and poultry are relatively insensitive to the dietary concentrations of DON likely to be found in feeds. Apparently, all domestic animals are susceptible to dietary levels of T-2, HT-2, and DAS in the range of a few parts per million [43]. In poultry, feed contaminated with 1 to 3.5 ppm of T-2 and 0.7 ppm of HT-2 may produce lesions at the edges of the beaks, abnormal feathering in chicks, a remarkable drop in egg production, eggs with thin shells, reduced weight gains, and mortality [51]. Trichothecenes-contaminated cattle feed results in feed refusal, slow growth, lowered milk production, and sterility. Trichothecenes, including DON and T-2 toxin, in amounts sufficient to cause toxic response have been found in corn that was still in the field, in silage, and in prepared feeds made in part from corn [52]. As trichothecenes can cause severe skin irritation, feed contaminated with these toxins must be handled carefully.

The FDA issued updated advisory levels for DON of 1 ppm ($\mu\text{g/g}$) for all finished wheat products (flour, bran and germ) that can be consumed by humans and of 5 to 10 ppm for all grain and grain byproducts destined for animal consumption (different levels depending on animals) (<http://www.cfsan.fda.gov/~dms/guidance.html#schem>).

2.3. Zearalenone

Zearalenone (ZEA) and zearalenol are estrogenic resorcylic acid lactone compounds produced almost exclusively by *Fusarium* spp. (Fig. 5) [53]. Although ZEA and its derivatives are structurally dissimilar to the steroidal estrogens, they induce estrogenic effects. When hydroxyl or potential hydroxyl groups of ZEA become appropriately orientated, ZEA acts like estrogen by binding to the cytosolic receptor proteins and then translocating a hormone-receptor complex into the nucleus, resulting in hyperestrogenic effects [29,54]. Similar binding affinities for ZEA have been determined for the estrogen receptor in sheep and calf uterus.

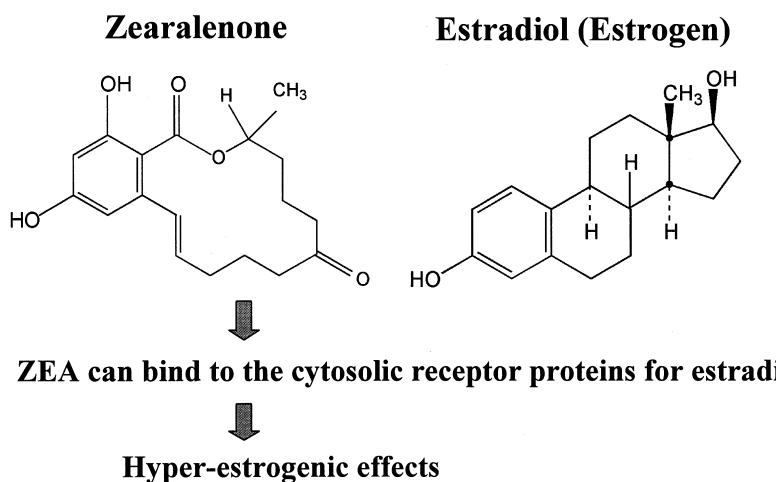


Figure 5 Zearalenone (ZEA) structure and mode of action. ZEA binds to the cytosolic receptor proteins for estrogen and causes hyperestrogenic symptoms, especially in swine.

The major fungal species producing ZEA are *F. graminearum* and *F. semitectum*, yet at least eight other species can produce variable amounts of ZEA [11,53]. Although ZEA is mostly found in corn, it is also found throughout the world in other important crops, such as wheat, barley, sorghum, and rye. ZEA naturally occurs with DON in most cases and sometimes with aflatoxins [55].

The typical symptoms caused by ZEA are hyperestrogenism, swelling of uterus and vulva, infertility, atrophy of ovaries, increased incidence of pseudopregnancy, and abnormal lactation. Swine are most significantly affected and considerably sensitive to ZEA [56]. Poultry show little response to ZEA ingestion. Although cattle are not as sensitive to ZEA as swine, infertility, reduced milk production, and hyperestrogenism in cows have been reported in association with a high concentration (approximately 10 ppm) of ZEA [57,58]. Holstein cows that consumed 25 to 200 ppm of ZEA for 42 consecutive days exhibited swollen and hyperemic external genitalia but had estrous cycles of normal lengths and normal ovulations. Dairy cattle fed a ration that contained 385 to 1925 ppb of ZEA for 7 weeks had normal milk production. No ZEA residues were found in milk, urine, serum, or tissues [51]. Although the critical level of ZEA contamination considered to be unsatisfactory is 0.5 ppm, no regulatory standards or guidelines have been established for ZEA.

2.4. Fumonisins

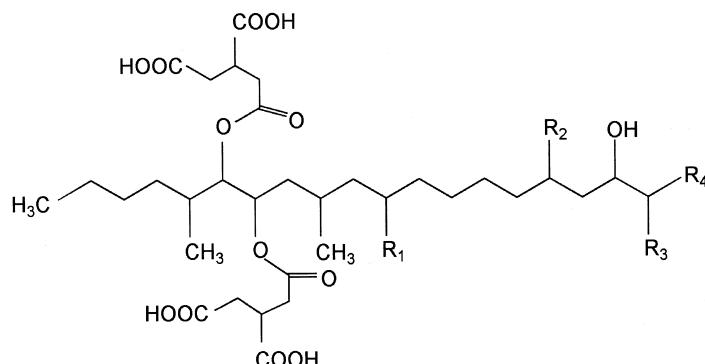
In 1988, a new group of mycotoxins, the fumonisins, were isolated and characterized by a group of South African researchers [59,60]. Fumonisins can cause blind staggers, technically known as *equine leucoencephalomalacia* (LEM), which occasionally occurs in horses, mules, or donkeys consuming feed heavily infected with *Fusarium verticillioides* [59]. In addition, *F. verticillioides* cause acute toxicity with pulmonary edema when fed to pigs [61]. In the eastern seaboard area of South Africa, a high incidence of human esophageal cancer was reported, and the *F. moniliforme* (now called *F. verticillioides*) strain was thought to be the causative agent because the *F. moniliforme* strain isolated from the areas with high rates of this cancer was very toxic and carcinogenic to rats [60]. A toxic compound, fumonisin B₁ (FB₁), had been isolated and characterized from the culture extract of *F. moniliforme* and given the name *fumonisin*. It has been reported that human esophageal cancer might correlate positively with high consumption of fumonisin-contaminated corn in Africa and China [62,63].

Fumonisins are produced by a number of *Fusarium* spp. within section Liseola and some isolates of *F. oxysporum* [64,65]. Two major fumonisin producers are *F. verticillioides* (synonym *F. moniliforme*, teleomorph *Gibberella moniliformis*, synonym *G. fujikuroi* mating population A) and *F. proliferatum*. While there are many different kinds of fumonisins, FB₁ is the most abundant in naturally infected maize kernels. Generally FB₁ makes up about 70% and both fumonisin B₂ (FB₂) and fumonisin B₃ (FB₃) make up 10% to 20 % of naturally occurring fumonisins [66]. Natural occurrence of fumonisins in animal feed and human food has been reported worldwide [67,68]. In the United States, it has been estimated that *F. verticillioides* contaminates between 80% and 100% of all corn harvested [68]. *F. verticillioides* infection of kernels occurs at or after flowering, often producing a starburst pattern on the kernels. Many kernels infected with this fungus show no symptoms. However, the visual absence of mold or symptoms does not mean that kernels are free from fumonisins. *F. verticillioides* overwinters in colonized plant residues, and the primary inoculum for seed infection is microconidia, which germinate on the ear.

Fusarium ear rot of maize is responsible for serious human and animal health concerns as well as significant economic loss due to low grain quality and decreased yield [68].

The basic structure of fumonisins is a diester of propane-1,2,3-tricarboxylic acid and a pentahydroxyicosane containing a primary amine group (Fig. 6). Fumonisins are soluble in polar solvents because of the four free carboxyl groups, the hydroxyl groups, and the amino group. Because of their polarity, fumonisins are not soluble in many organic solvents such as chloroform or hexane commonly used in mycotoxin analysis, and this partly explains the difficulty in their original identification. There are four different groups (A-, B-, C, and P-group) of fumonisins classified by chemical structures with several derivatives in each group. FB₁ consists of a linear carbon backbone with an amine group at C-2, methyl group at C-12 and C-16, and tricarballylic ester groups at C-14 and C-15. FB₂ and FB₃ have different numbers and positions of hydroxyl groups from FB₁. Group-A fumonisins have an acylated amine group at C-2, and group-C fumonisins have the same structures as B group except for the deletion of methyl group at C-1 (Fig. 6). The group-C fumonisins also occur in nature and their toxicities are similar to B-group fumonisins [69,70].

Toxicological studies on the fumonisins have focused on FB₁, and the mode of action of FB₁ is presented in Fig. 7. Fumonisins are structural analogues of sphinganine, the sphingolipid precursor. They are potent competitive inhibitors of ceramide synthase



Fumonisin	R ₁	R ₂	R ₃	R ₄
FB ₁	OH	OH	NH ₂	CH ₃
FB ₂	H	OH	NH ₂	CH ₃
FB ₃	OH	H	NH ₂	CH ₃
FC ₁	OH	OH	NH ₂	H
FA ₁	OH	OH	NHCOCH ₃	CH ₃

Figure 6 Structures of various fumonisins. Fumonisins are characterized by a 19- or 20-carbon aminopolyhydroxyalkyl chain, which is esterified with propane-1,2,3-tricarboxylic acid. Fumonisins include four types of derivatives (A-, B, C-, P-type). This group of mycotoxins is found in corn and corn-based products.

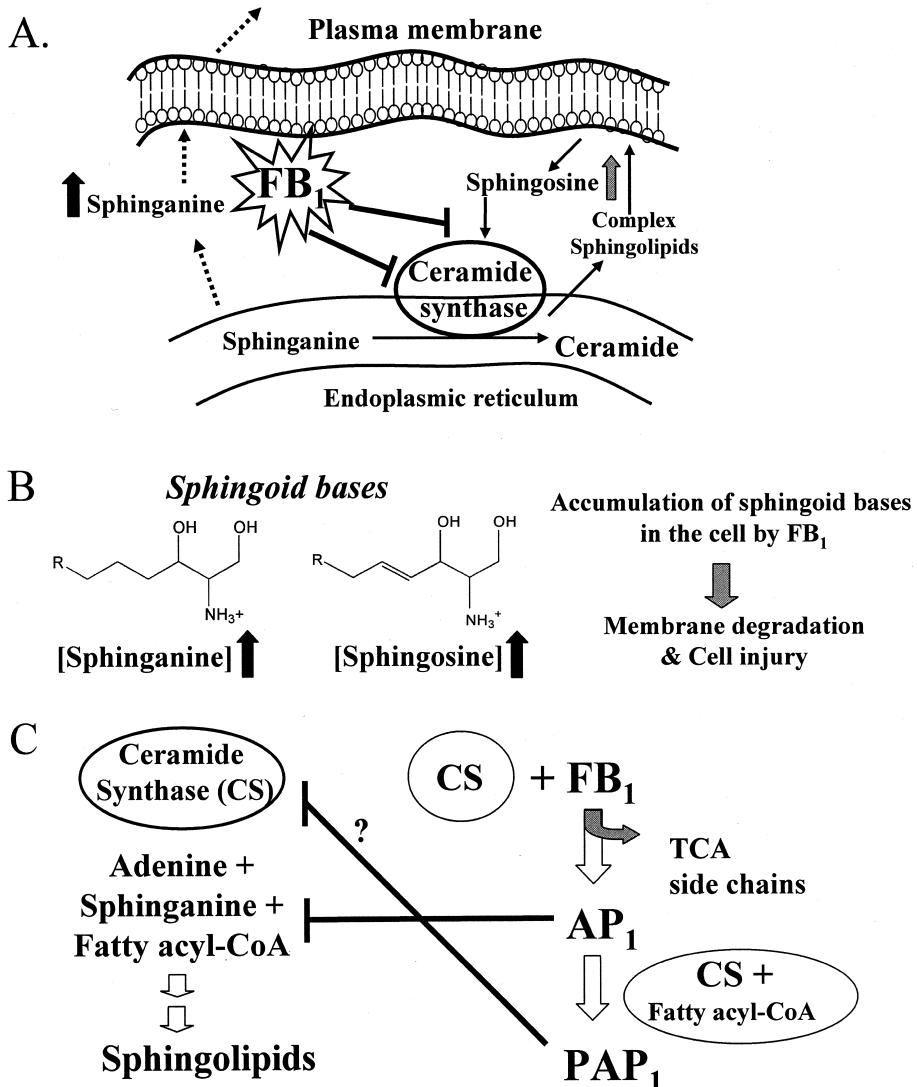


Figure 7 Mode of action of fumonisin B₁. Fumonisins are structurally similar to sphinganine and can attach to the binding site of sphinganine in ceramide synthase (CS). Inhibition (presented as —|) of CS by FB₁ results in increased sphingoid bases in the cell (A), which affect membrane stability and finally cause cell injury (B). In addition to inhibiting CS by FB₁, AP₁ and PAP₁, derivatives produced from the reaction between FB₁ (or AP₁) and CS also inhibit the critical CS enzymatic reaction and CS itself (proposed), respectively (C).

(CS), the enzyme that catalyses the acylation of sphinganine synthesis pathway, which is essential for the biosynthesis of sphingolipids [71]. Sphingolipids play an important role in membrane and lipoprotein structure and function as second messengers in cellular signaling. Due to their critical roles in cells, amounts of sphingolipids are tightly controlled. As shown in Fig. 7A, FB₁ competes for the binding sites of sphinganine and fatty acyl-CoA in CS and inhibits CS functions, which results in blockage of the biosynthesis of complex sphingolipids and in the accumulation of sphinganine and sphingosine (Fig. 7B) [72]. The accumulation of sphingoid bases causes growth inhibition and cytotoxicity and is thought to be a primary cause of the toxicity of FB₁. Alteration in sphingolipid base ratios occurs almost immediately after exposure, and this property is indicative of fumonisin exposure in a number of species, including horses and pigs. Additional toxic effects of FB₁ result from several biochemical events. The aminopentol backbone of FB₁ (AP₁) acts as both an inhibitor and a substrate for CS, and the resultant *N*-palmitoyl-AP₁ (PAP₁) is an even more potent inhibitor of CS (Fig. 7C).

F. verticillioides is common contaminant of corn, even the food-grade variety, and is often abundant in ground feeds and silage. Swine fed a ration containing 78% to 82% corn heavily colonized by *F. verticillioides* grow as well as control pigs fed a ration of high-quality corn [68]. Research on fumonisin toxins began only recently, and current thought is that concentrations of more than 5 to 10 ppm or 10 to 20 ppm are necessary for mycotoxicoses in horses and swine, respectively [73].

In November 2001, the FDA issued a guidance document (not action level) to recommend maximum fumonisin levels that FDA considers adequate to protect human and animal health and that are *achievable* in human foods and animal feeds with the use of good agricultural and manufacturing practices. FDA considers this guidance to be a prudent public health measure during the development of a better understanding of the human health risk associated with fumonisins and the development of a long-term risk (Table 2). (<http://vm.cfsan.fda.gov/~dms/fumongu2.html>)

2.5. Ochratoxins

Ochratoxins are a group of related compounds produced by *Aspergillus ochraceus* and related species, as well as *Penicillium verrucosum* and other *Penicillium* spp. [74,75]. These toxins have been found in a wide variety of agricultural commodities such as corn, wheat, barley, flour, coffee, rice, oats, rye, beans, peas, and mixed feeds [75] and are also found in wine, grape juice, and dried vine fruits [76]. Ochratoxins can also contaminate animal products, such as pork, poultry, and milk, and this can pose a serious problem to human health.

Because ochratoxins are very stable and can survive through processing, they can be found in cereal products, beer, and roasted coffee. Ochratoxin A (OTA), the most important toxin of the group, was first isolated in 1965 from *A. ochraceus*, from which its name originated [77]. The natural occurrence of OTA in foods and feeds is widespread, especially in temperate areas such as Canada, Denmark, Germany, Sweden, and the United Kingdom [78]. Furthermore, detectable amounts of OTA were even found in randomly collected human milk samples in Germany, Sweden, and Italy [79]. Because OTA is fat-soluble, it tends to accumulate in the fat stores of animals that consume OTA-contaminated feeds.

Table 2 Proposed Guidance Levels for Fumonisins in Foods and Feeds

<i>Product</i>	<i>Total Fumonisins (ppm)^a</i>
Human Food	
Degermed dry milled corn products (e.g., flaking grits, corn grits, cornmeal, corn flour with fat content of <2.25%, dry weight basis)	2
Whole or partially degemermed dry milled corn products (e.g., flaking grits, corn grits, cornmeal, corn flour with fat content of ≥2.25%, dry weight basis)	4
Dry milled corn bran	4
Cleaned corn intended for masa production	4
Cleaned corn intended for popcorn	3
Animal Feed	
Corn and corn byproducts intended for:	
Equids and rabbits	5 ($\leq 20\%$ of diet) ^b
Swine and catfish	20 ($\leq 50\%$ of diet) ^b
Breeding ruminants, breeding poultry, and breeding mink ^b	30 ($\leq 50\%$ of diet) ^b
Ruminants ≥ 3 months old being raised for slaughter and mink being raised for pelt production	60 ($\leq 50\%$ of diet) ^b
Poultry being raised for slaughter	100 ($\leq 50\%$ of diet) ^b
All other species or classes of livestock and pet animals	10 ($\leq 50\%$ of diet) ^b

^a $FB_1 + FB_2 + FB_3$.

^b Dry weight basis.

^c Includes lactating dairy cattle and hens laying eggs for human consumption.

OTA is chemically classified as a chlorine-containing pentaketide dihydroisocoumarin linked through the 12-carboxy group to phenylalanine. Since OTA contains phenylalanine (Phe), it can potentially act on all metabolic systems involving Phe (Fig. 8). Ochratoxin B differs in structure only by the absence of the chlorine atom and can occur naturally, but it is nontoxic.

OTA mainly affects the kidneys (nephrotoxic) and liver (hepatotoxic), causing both acute and chronic lesions [78]. The nephrotoxic effects have been demonstrated in all mammalian species that are exposed to naturally occurring levels of OTA [79]. In addition, turkeys and other poultry exhibit lower productivity levels during field outbreaks of ochratoxicosis. Symptoms include retarded growth and decreased feed conversion. OTA has also been known to affect egg production in laying hens.

OTA has also been suspected as a causal agent of endemic nephropathy in humans. Various studies have identified a correlation between the geographical distribution of Balkan endemic nephropathy (an irreversible and fatal human kidney disease) and a high incidence of OTA and mortality from urothelial urinary tract tumors [80]. OTA levels were found to be higher in the blood of patients with Balkan endemic nephropathy and/or urothelial urinary tract tumors than in unaffected people. Regardless, the presence of OTA in foodstuffs is clearly undesirable.

OTA is a potent teratogen in mice, rats, hamsters, and chickens but not apparently in pigs [43,78]. Teratogens are agents that can cause malformations of an embryo or fetus. Both teratogenic and reproductive effects by OTA have been demonstrated. OTA is also known to affect the immune system in a number of mammalian species [81]. OTA is

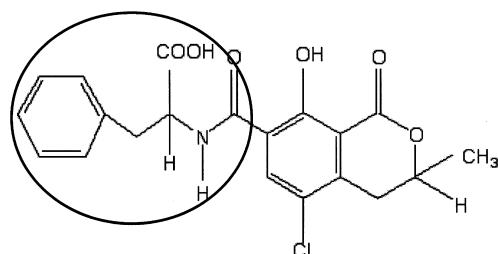
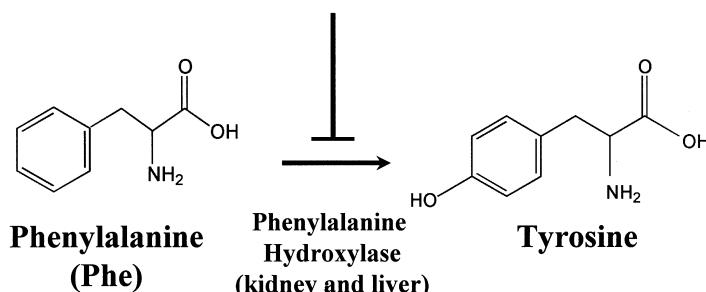
**Ochratoxin A (OTA) containing Phe**

Figure 8 Ochratoxin A (OTA) structure and mode of action. OTA inhibits (presented as ---) phenylalanine hydroxylase in kidney and liver. This enzyme catalyzes irreversible hydroxylation of phenylalanine (Phe) to tyrosine, the key regulation step in the catabolism of this essential amino acid. OTA is a structural analogue of Phe and can potentially act on all metabolic systems involving Phe.

genotoxic both in vitro and in vivo, but the mechanism of genotoxicity is still unclear. The European Scientific Committee for Food classified OTA as a genotoxic carcinogen in 1998.

For mode of action, OTA inhibits liver Phe hydroxylase, likely by acting as a substrate for the enzyme (Fig. 8). A tight control of Phe hydroxylase activity *in vivo* is required because too rapid degradation of its substrate will lead to depletion of Phe, whereas accumulation of Phe can lead to impaired production of dopamine and other compounds derived from Phe [82].

Currently, no advisory or regulatory levels for ochratoxins have been issued by the FDA. However, it is general consensus that levels between 10 to 20 ppb for commodities destined for human or animal consumption may cause health problems and economic losses. Some foreign markets have set regulation limits of OTA ranging from 5 to 50 ppb.

2.6. Patulin

Patulin is a polyketide mycotoxin produced by certain species of *Penicillium*, *Aspergillus*, and *Byssochlamys* growing on various fruit, including apples, pears, grapes, and others. *Penicillium expansum*, which is the principal causal agent on apples and a common pathogen in many fruits and vegetables, appears to be the mold usually responsible for patulin in apple juice [83,84]. Patulin has been found in damaged and rotting fruits and in fruit juices such as peach, pear, grape, and especially apple [85].

Initial studies of patulin started with the wide-spectrum antibiotic properties of this mycotoxin to evaluate whether patulin could be used to treat common colds. However, this compound was too toxic for use in humans, causing nausea, vomiting, and irritation to the stomach. Moreover, patulin is currently considered a suspect carcinogen by IRAC. In addition, patulin induces intestinal injuries, including epithelial-cell degeneration, inflammation, ulceration, and hemorrhage [83].

In a recent study of the cellular mechanisms associated with the intestinal toxicity of patulin, micromolar concentrations of patulin were found to induce a rapid and dramatic decrease of transepithelial resistance (TER) [86]. The inhibitory effect of patulin on TER was closely associated with its reactivity with SH groups. Moreover, the inhibitory effect of patulin on TER was mimicked and potentiated by phenylarsine oxide, a specific inhibitor of protein tyrosine phosphatase (PTP). These findings led the authors to propose that the toxicity of patulin for intestinal cells involves, among other potential mechanisms, an inactivation of the active site of PTP. This enzyme is a key regulator of intestinal epithelial barrier function, and the active site of PTP contains a cysteine (Cys215) residue that is essential for phosphatase activity. Patulin can react with the sulfide group of Cys215 and inhibit this enzyme activity (Fig. 9) [86].

Currently, FDA has issued only a compliance policy guide for its staff regarding patulin (http://www.fda.gov/ora/compliance_ref/cpg/cpgfod/cpg510-150.htm). However, the quality of fruit juice is controlled in some countries by a recommended maximum concentration in agreement with the apple-processing industry, which is commonly set at 50 µg/L (50 ppb, weight/volume). In the United Kingdom, regular monitoring of apple juice since 1992 has reduced the concentrations and incidence of patulin in juices to a very low level, chiefly through better storage of apples, the avoidance of damaged or poor-

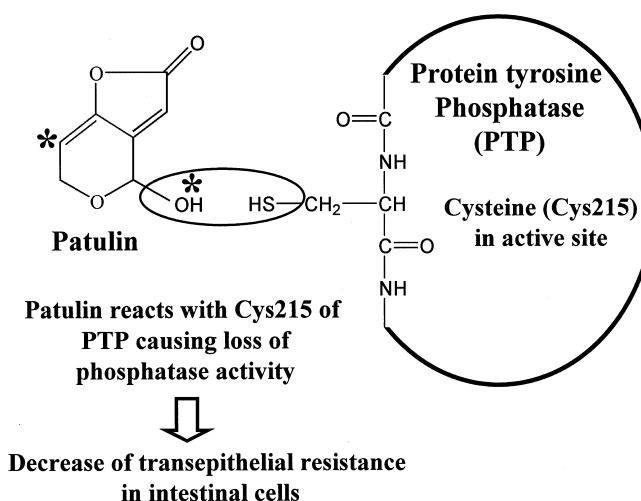


Figure 9 Patulin structure and mode of action. Protein tyrosine phosphatase (PTP) is a key regulator of intestinal epithelial barrier function that dephosphorylates protein tyrosine residues. The active site of PTP contains a cysteine residue (Cys215), which can react with two possible sites (*) in patulin. Inactivation of the active site of PTP by patulin results in the absence of phosphatase activity of PTP.

quality fruit, and better production protocols (European Mycotoxin Awareness Network at <http://www.lfra.co.uk/eman/fsheet8.htm>).

3. INDOOR FUNGI AND MYCOTOXINS

Recently, a short review describing mycotoxin production by indoor molds has been published [87]. Readers can obtain more information regarding air-borne fungi and associated mycotoxins from this and other reviews [88–90]. A wide range of toxigenic fungal species has been isolated from the indoor air of houses and buildings. These toxigenic molds include members of *Penicillium*, *Aspergillus*, *Stachybotrys*, and *Alternaria* [90]. *Stachybotrys* spp. have also been found in food and can degrade cellulose when they colonize on straw and other cellulose-rich materials. These toxigenic fungal species all reproduce by forming asexual spores that are the main means of dispersion. The problem is that these spores may also carry mycotoxins. Repeated or long-term exposure to airborne fungal spores and their toxic metabolites can result in various allergic and nonallergic responses in the human body.

Stachybotrys mycotoxicosis was first reported in 1930 in the Ukraine [91], and other cases have been reported. Many reports showed that *Stachybotrys* might be involved in the illness of humans occupying contaminated buildings, especially in water-damaged houses. Representative airborne fungus *S. chartarum* (*atra*) is capable of producing several toxins, including macrocyclic trichothecenes (satratoxins H, G, and F; roridin E; verrucarin J). Several strains of this fungus (*S. atra*, *S. chartarum*, and *S. alternans*, synonyms) may produce the trichothecene mycotoxin, satratoxin H, which is extremely toxic when inhaled [34,92]. *Stachybotrys atra* can grow and produce toxins in high-cellulose and low-nitrogen materials at a temperature range of 0° to 40°C. Adverse effects of stachybotrys toxins occur on the central nervous system, eyes, skin, and upper and lower respiratory tract and may include chronic fatigue. Other symptoms are immune suppression, bleeding, and adverse effects on the reproductive systems.

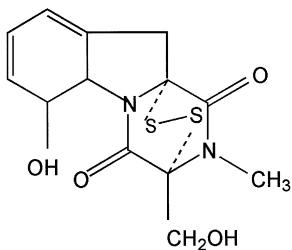
4. MINOR MYCOTOXINS

4.1. Gliotoxin

Gliotoxin is an antibiotic mycotoxin produced by a wide variety of yeast and fungi including *Trichoderma*, *Penicillium*, *Candida*, and *Aspergillus*, including *A. fumigatus*, a human pathogen. It was named after the species of fungus, *Gliocladium* (*Gladiocladium*) *fimbriatum*, in which it was first isolated. Gliotoxin is a lipid-soluble thiol-reactive agent and an antiphagocytic and immunosuppressive compound (Fig. 10) [93].

4.2. Citrinin

Citrinin is produced by several *Aspergillus* and *Penicillium* spp., including OTA-producing *P. verrucosum*. The name citrinin originated from *Penicillium citrinum*. Citrinin has been detected in cereal grains, such as wheat, barley, oats, rice, and corn. It often occurs with OTA simultaneously because most fungal isolates can produce both citrinin and OTA. Citrinin causes kidney damage. It affects domestic birds and causes watery diarrhea, increased water consumption, and reduced weight gain due to degeneration of tissues in chickens, turkeys, and ducklings [90,94].



Gliotoxin

Figure 10 Gliotoxin structure. A human pathogenic fungus, *A. fumigatus*, produces this immunosuppressive mycotoxin. Gliotoxin is lipid soluble.

4.3. Cyclopiazonic Acid

Cyclopiazonic acid (CPA) is produced by several fungal species that are either commonly found in agricultural commodities or used in fermented food production. These include *A. flavus*, *A. versicolor*, *A. tamari*, and several *Penicillium* spp. used in fermented sausage production in Europe [95]. *Penicillium camemberti*, used in the production of soy sauce, can also produce CPA. Other CPA-producing *Penicillium* spp. include *P. commune* and *P. griseofulvum*. “Kudoa poisoning” reported in India as resulting from ingestion of contaminated Kodo millet seeds has been linked to CPA. CPA has been detected in naturally contaminated mixed feeds, maize, peanuts, and other foods and feeds. CPA selectively inhibits Ca²⁺-ATPase, which results in decreased Ca²⁺ uptake and reduced Ca²⁺-dependent K⁺ current [96].

4.4. Wortmanin

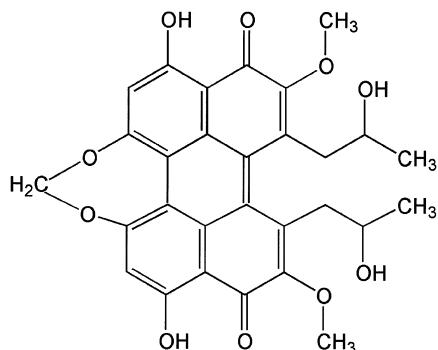
Wortmanin is a steroid fungal metabolite isolated from *Penicillium wortmanni*. It is also produced by *Myrothecium roridum*, *Fusarium oxysporum*, and *F. sambucinum* [97]. This toxin has been known to be a hemorrhagic factor. Experimentally, rats given crude wortmanin showed gastric and myocardial hemorrhages [98].

4.5. Sterigmatocystin

Sterigmatocystin is the penultimate precursor in the biosynthesis of aflatoxins (Fig. 1) and is produced by several species of *Aspergillus*, *Bipolaris*, and *Penicillium luteum* [99]. Sterigmatocystin possesses a bisdihydrofuran moiety like aflatoxins, and this moiety is believed to be crucial for the toxicity, mutagenicity, and carcinogenicity [100]. Sterigmatocystin is one of the major mycotoxins of concern that are present in cheese and has been detected in low concentrations in green coffee, moldy wheat, and the rind of hard cheese [101,102].

4.6. Cercosporin

Cercosporin is a photosensitizing perylenequinone toxin produced by the plant pathogenic *Cercospora* fungi (Fig. 11) [103]. This toxin generates highly toxic singlet oxygen (¹O₂) upon exposure to light. Singlet oxygen is an extremely toxic form of activated oxygen



Cercosporin

Figure 11 Cercosporin structure. Cercosporin is produced by the plant pathogenic fungi *Cercospora* and is classified as a photosensitizer because it generates the highly toxic singlet oxygen (${}^1\text{O}_2$) upon exposure to light.

species, and cells have few effective defense mechanisms against its effects. Thus, cercosporin shows broad-spectrum toxicity against bacteria, fungi, plants, and animals; however, *Cercospora* fungi are resistant to its effects. Cercosporin and other structurally diverse compounds are classified as photosensitizing compounds based on their common ability to absorb light energy and react with oxygen to produce activated oxygen species. In the presence of light, photosensitizers are converted to an activated triplet state, which in turn reacts with molecular oxygen, either by electron-transfer (radical) reactions through a reducing substrate (type I reaction) or directly by an energy-transfer mechanism (type II reaction). Type I reactions lead to the production of various free radicals, including lipid free radicals, and reduced oxygen species, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radicals (OH^\cdot). Type II reactions result in the production of the highly toxic, energetically activated singlet oxygen (${}^1\text{O}_2$). Cercosporin appears to exert its toxicity primarily by the formation of ${}^1\text{O}_2$. Production of ${}^1\text{O}_2$ by cercosporin in the colonized plants results in lipid peroxidation and membrane rupture, leading to leakage of nutrients from cells and thus allowing fungal growth.

5. DETECTION AND ANALYSIS OF MYCOTOXINS

Determination of the amounts of mycotoxins in field samples, such as corn ear and grains, is not a simple task. In many cases, concentrations of mycotoxins are in the parts-per-billion range. To achieve this goal, all analytical methods for mycotoxins need to consist of appropriate sample preparation, well-defined extraction and clean-up processes, and measurement of the toxins with sensitive analytical methods. Numerous analytical methods of mycotoxins have been published [104–107], and the Association of Official Analytical Chemists International is one of the organizations that coordinate and standardize the analytical methods for mycotoxins [108]. In this section various analytical methods will be briefly described, and in the Section 6, detailed protocols for detecting major classes of mycotoxins employing the simplest thin-layer chromatography will be presented.

5.1. Solid-Phase Extraction

All analytical procedures include extraction, purification, and determination. Recently, solid-phase extraction (SPE) has become the most commonly used preparation method for toxin analyses. SPE is an extraction method that uses a solid phase to isolate one or more of the analytes from mixed solutions and is used to clean up the samples before using liquid chromatography. SPE columns have different kinds of packing materials, such as C₁₈, C₈, and strong anion-exchanging material according to their polarities. The SPE column method is quick, solvent efficient, and economical and has been used commonly in the extraction of mycotoxins [109–111].

5.2. Thin-Layer Chromatography

This method is one of the most widely used separation techniques in mycotoxin analysis. TLC has been considered the AOAC official method for analyses of various kinds of mycotoxins and the detection limit is about 1 to 5 ng/g (ppb). The strong point of TLC is that the crude extracts, mixtures of other compounds with target mycotoxin, can be applied if standard toxins are available and chromatographed together. The appropriate solvent mixture for each compound migrates upward on the plate and separates chemical components that have different solubilities in the solvent system. For aflatoxin analysis, many different solvent systems can be used according to base materials, such as kinds of foods, feeds, or agricultural commodities. Most of the mycotoxins can be separated on TLC by varying solvent systems. Detailed TLC protocols for separation and detection of major mycotoxins are presented in Section 6.

5.3. Liquid Chromatography

High-performance liquid chromatography (HPLC) is commonly used for analyses of various mycotoxins including aflatoxins, fumonisins, and patulin [106,107,112,113]. HPLC operates on the same principles of TLC, except for using silica or other packing materials in a column. HPLC is much more expensive than TLC, but it is more sensitive and accurate for detecting each different compound. Detectors with different functions can be attached to HPLC. These include fluorescence detectors, ultraviolet/visible (UV/VIS) light detectors, and refractive index detectors (Fig. 12). A general reversed-phase HPLC analysis method has been developed for simultaneous separation and detection of 182 mycotoxins and other fungal metabolites [114]. Most known mycotoxins can be detected by the HPLC method. However, some recently identified mycotoxins (fumonisin and AAL toxins) lacking strong UV chromophores are not suitable for analysis by direct detection [115]. Fumonisins can normally be detected by HPLC using a fluorescence detector after derivatization with *o*-phthalaldehyde (OPA), which reacts with the primary amine group of fumonisins. Since fumonisins are highly polar, they can be eluted by solvent mixture of acetonitrile and water from HPLC or SPE columns [113].

Recently, sensitive and accurate analysis methods have been developed, including liquid chromatography/fast atomic bombardment/mass spectrometry (LC/FAB/MS) and electrospray LC/MS [107]. During HPLC, toxins are detected as a peak compared with standard toxin compound at the same retention time. However, with LC/MS, the exact compound can be detected by molecular ion pattern and molecular weight. Electrospray (ES) is an ionization method developed in response to the need for direct MS characterization of HPLC-separated components. A reverse-phase HPLC separation with ES/MS detection has been employed for the general analysis of fumonisins [107].

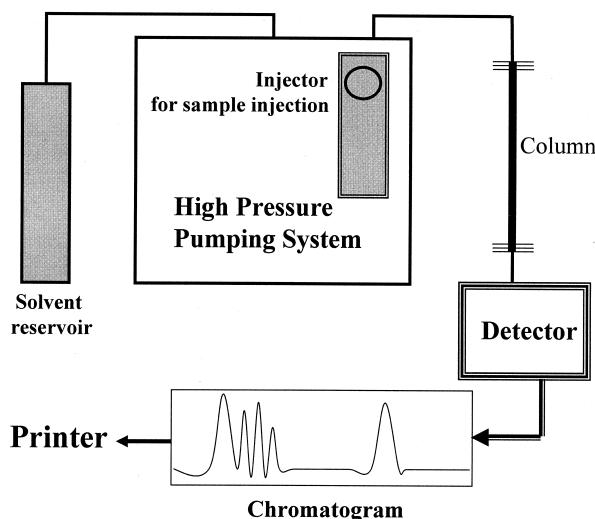


Figure 12 Schematic presentation of an HPLC instrument. Sample is injected through injector and separated based on chemical properties of compounds. Detector senses each separated compound and generates signals. Peaks shown in chromatogram represent certain compounds at a unique time point (retention time).

OTA are predominantly analyzed by HPLC with fluorescence detection [116]. However, recently, several immunoassay techniques for OTA have been described [117].

5.4. Immunochemical Methods

Enzyme-linked immunosorbent assay (ELISA) is an immunological assay tool that uses specific antibodies that react with mycotoxins for detection [118,119]. While the tests can be performed in several different ways, a competitive ELISA is the most commonly used method (Fig. 13). The extract and toxin labeled with an enzyme (conjugate) are added simultaneously to the microplate wells containing the precoated antibody. Competitive binding between the toxins in the extract and the toxin–enzyme conjugate for the antibodies occur in the well. After a sufficient period of time, the well is rinsed and a chromogenic substrate of the enzyme is added. The amount of antibody-bound toxin–enzyme conjugate will determine the level of color development. Thus, more toxin in the sample results in lighter color development in the wells and vice versa (Fig. 13). Simple detection kits for aflatoxins, T-2, DON, fumonisins, ochratoxins, and ZEA are commercially available.

5.5. Gas Chromatography and Mass Spectrometry

Gas liquid chromatography (GLC) has been applied to analyze several mycotoxins, such as trichothecenes, ZEA, and patulin [106,120]. Trichothecenes can be detected by TLC, gas chromatography (GC), and GC/mass spectrometry (GC/MS). On TLC plates, most trichothecenes show color reactions with spraying reagents, such as diluted sulfuric acid and *p*-anisaldehyde. The derivatization of purified extracts containing trichothecenes with vaporizing reagents such as trimethylsilylate (TMS) is necessary to analyze trichothecenes

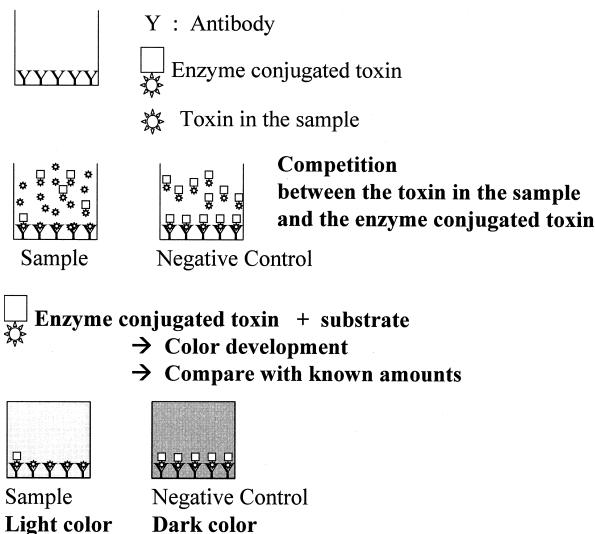


Figure 13 Competitive ELISA. Competition between the toxin in the sample and the enzyme-conjugated toxin determines the final amount of available enzyme and thereby color development.

when using GC or GC/MS. If unique molecular ions are put in a special program called SIM (selected ion monitoring) mode in GC/MS, the chromatogram and mass spectrometry can show the peaks of exact compounds corresponding to those ions. Other detection methods are also being developed, such as LC with fluorescence detector and LC/frit-FAB (fast atomic bombardment), high-resolution MS for identification and monitoring trichothecenes with derivatization. Development of ELISA methods for detection of trichothecenes is increasing, particularly for DON [121,122].

Methods of analysis for patulin in apple juice usually consist of multiple liquid-liquid partition steps, concentration, and separation and quantitation by GC [123], TLC, or LC [124].

6. PROTOCOLS FOR THIN-LAYER CHROMATOGRAPHY OF SELECTED MYCOTOXINS

Of the many analytical methods applied to mycotoxins, TLC is the most widely used and the easiest technique for mycotoxin detection, analysis, and characterization. The International Union of Pure and Applied Chemistry (IUPAC) and AOAC have standardized extraction, clean-up, and analytical methods for mycotoxins [104,106,108]. In this section, basic principles of TLC, solvent or solvent mixtures for extraction, clean-up, and development in TLC are described. The primary objective of this section is to present a quick and easy way to detect and characterize mycotoxins from fungal culture and naturally contaminated crops so that readers can analyze major mycotoxins in the laboratory. If needed, detailed and extensive information regarding TLC of mycotoxins (except fumonisin)s can be obtained from an excellent review by Betina [125].

6.1. Principles of Thin-Layer Chromatography

TLC consists of a stationary phase immobilized on a glass or plastic plate and a solvent as the mobile phase. The sample is spotted on the stationary phase and run in solvent reservoir. The constituents of a sample can be separated and identified by simultaneously running standard toxins. The different components move up the plate at different rates due to their chemical interactions between the stationary phase and the liquid mobile phase. The R_f (ratio of fronts) value of each spot is determined as follows (Fig. 14):

$$R_f = \frac{\text{Distance from origin to the center of spot (cm)}}{\text{Distance from origin to the solvent front (cm)}} = \frac{b}{a}$$

The most commonly used adsorbent (stationary phase) in mycotoxin analyses is silica gel with varying particle sizes. One of the popular products for mycotoxin analyses is Silica Gel 60 F-254 (20×20 cm, 0.25 mm; Merck) which contains a fluorescence indicator. Various adsorbents are commercially available and are chosen based on chemical characteristics of mycotoxins. For solvent systems as the mobile phase, the first choice can be various ratios of methanol in chloroform. Detection techniques are important after developing a sample because the mycotoxins need to be visualized under certain conditions. The general techniques for visualization of mycotoxins include examination under visible light; examination of fluorescence under a certain wavelength of UV light (e.g., aflatoxins, OTA, and sterigmatocystin); treating a fluorescent indicator and then observing under UV light (e.g., patulin); spraying a plate with a reagent and observing colored product (e.g., trichothecenes and fumonisins).

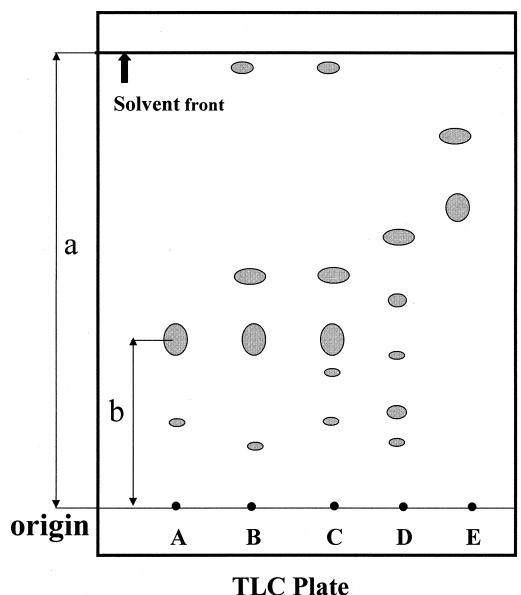


Figure 14 Examples of samples developed in TLC. The ratio of b/a represents the R_f value of the upper spot of sample A.

6.2. Detection of Aflatoxins

6.2.1. Extraction and Cleanup

Many food products contaminated with aflatoxins contain large amounts of lipids, pigments, and various natural products. To increase analytical sensitivity of final purified sample most of these natural products should be removed through extraction and cleanup procedures. There are several articles, reviews, and book chapters describing various procedures for detecting mycotoxins present in culture medium and/or diverse natural commodities [104–107,112–115,119,124,126] (http://ifa-tulln.boku.ac.at/AZ/english/eman_fs_aflatoxins.pdf). The best known extraction and clean-up methods for aflatoxins are as follows:

1. **Culture filtrate, mycelium, and agar medium:** chloroform extraction, no clean-up step or wash with petroleum or hexane
2. **Food or feedstuff and other agricultural commodities:**
 - a. **Extraction:** methanol in chloroform and addition of acetone or hydrochloric acid and dichloromethane for multimycotoxin method
 - b. **Cleanup:** SPE cartridge column with C₁₈, immunoaffinity columns (IACs)

6.2.2. Thin-Layer Chromatography Analysis of Aflatoxins

1. **Developing solvents:** toluene:ethylacetate (8:1), or chloroform:methanol (98:2)
2. **Detection:** Aflatoxins are fluorescent without any further treatment.

$$\lambda_{\text{excitation}} = 365 \text{ nm}; \lambda_{\text{detection}} = 430 \text{ nm}$$

3. **Detection limits:** ng/g (ppb) levels (http://ifa-tulln.boku.ac.at/AZ/english/eman_fs_tlc.pdf)

6.3. Detection of Trichothecenes and Zearalenone

6.3.1. Extraction and Cleanup

1. **From culture medium:** ethylacetate extraction and concentration to apply TLC (no necessary cleanup step)
2. **From grain, foods, and feeds:** acetonitrile:water (3:1) [120] or methanol:water (3:1)
3. **Cleanup:** silica gel, florisil column, C₁₈ SPE cartridges [20,127]

6.3.2. Thin-Layer Chromatography Analysis

Method I (good for both trichothecenes and ZEA analysis)

1. Trichothecenes and ZEA are separated using the developing solvent, chloroform:methanol (9:1).
2. The TLC plate is dried and the following reagent is sprayed onto the plate: 0.5% *p*-anisaldehyde solution (*p*-anisaldehyde, 0.5 mL; glacial acetic acid, 10 mL; sulfuric acid, 5 mL; methanol, 85 mL).
3. Bake the plate in a 110°C oven for 10 minutes. Brown spots will be visible.

Method II

1. Developing solvent: ethyl acetate:toluene (3:1)
2. Spray reagent: 1% 4-(4-nitrobenzyl) pyridine; heat in a 150°C oven for 30 minutes
3. Treat with 10% tetraethylenepentamine for enhanced visualization [128].

6.4. Detection of Fumonisins

6.4.1. Extraction and Cleanup

1. **Extraction:** from culture medium: acetonitrile:water (1:1); from corn samples: acetonitrile:water (1:1).
2. **Cleanup:** C₁₈ SPE cartridge [129], strong anion exchange (SAX) cartridge column [130].

6.4.2. Thin-Layer Chromatography Analysis

1. **Developing solvent:** chloroform:methanol:water:acetic acid (55:36:8:1) or chloroform:methanol:acetic acid (6:3:1)
2. **Spray reagent:** 0.5% *p*-anisaldehyde; heat in a 110°C oven for 10 minutes. Purple spots will become visible [130].

6.5. Detection of Ochratoxins

6.5.1. Extraction and Cleanup

1. **Extraction:** from barley, chloroform and H₃ PO₄; from green coffee: chloroform; from wheat: chloroform-ethanol:acetic acid (IUPAC/AOAC method, 991.44, 1992)
2. **Cleanup:** immunoaffinity column (IAC), SPE cartridge column and then IAC, silica gel cartridge

6.5.2. TLC Analysis

1. **Developing solvent:** toluene:ethylacetate:formic acid (6:3:1), benzene:acetic acid (4:1) [130], or benzene:acetic acid (3:1 or 25:1)
2. **Visualization:** blue-green under long-wave UV light

6.6. Detection of Patulin

6.6.1. Extraction and Cleanup

1. **Extraction:** ethyl-acetate or acetone [124]
2. **Cleanup:** silica gel column, florisil or celeite, SPE cartridge column

6.6.2. Thin-Layer Chromatography Analysis

1. **Developing solvents:** chloroform:methanol (97:3), ethylacetate:hexane (1:1), or ethanol:water (4:1)
2. **Spray reagent:** phenylhydrazinium chloride [131]
3. **Visualization:** yellow fluorescence under long-wave UV light after extraction

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Fungal Secondary Metabolites in Biological Control of Crop Pests

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1. INTRODUCTION

Biological control, which uses one or more organisms to maintain another organism below a level at which it is no longer an economical problem [1], offers an attractive alternative or supplement to chemical pesticides in agricultural crop protection. Microbial biocontrol agents are considered to be more compatible with the environment. Crop pests are less likely to develop resistance to a biopesticide due to the generally complex mode of action of biocontrol agents. To date, the most successful microbial control agent has been the insecticidal bacterium *Bacillus thuringiensis*, which accounts for more than 90% of the world biopesticide market [2]. However, there is an increasing interest in exploiting the use of fungi as biopesticides from various fungal taxonomic groups to control agricultural pests as shown in Table 1.

Fungi often suppress their competitors, including agricultural pests, via predation, parasitism, and antagonism. These suppressive mechanisms have evolved so that fungi may compete for food and space against their competitors. Such mechanisms may be very complicated and could be investigated from various aspects, such as pathology, ecology, epidemiology, and histology. General information on this topic can be found in two excellent books [3,4]. This chapter attempts to provide readers with an update on the role that secondary metabolites play in fungal biopesticides.

Table 1 Fungi Developed or Being Developed for Biological Control of Plant Pests

Fungus	Product	Target	Producer/Country
Entomogenous fungi			
<i>Beauveria bassiana</i>	Conidia	Coffee berry borer	Live Syst Tech, USA
	Ostrinil	Corn borer	Natural Pl Prot, France
	Mycotrol-WP	Grasshoppers, locusts	Mycotech, USA
	Naturalis	White flies, thrips, white grubs	Troy Biosciences, US
	Boverol	Colorado beetle	Czechoslovakia
	Boverosil	Colorado beetle	Czechoslovakia
<i>B. brongniartii</i>	Engerlingspilz	Cockchafer(s)	Andermatt
	Schweizer	Cockchafer(s)	Eric Schweizer, Switzerland
	Beauveria		
	Melocont	Cockchafer(s)	Kwizda, Austria
<i>Metarhizium anisopliae</i>	Biogreen	Scarab larvae on pasture	Biocare Tech, Australia
	Mataquino	Spittlebugs	Brazil
	Biologic	Black vine weevil	Bayer AG, Germany
	Bio-Payh	Cockroaches	EcoScience, USA
	Bio-Blast	Termites	EcoScience, USA
	Cobican	Sugarcane spittlebug	Probiagro, Venezuela
<i>M. flavoviride</i>	Green Muscle	Locusts, grasshoppers	CABI BioScience, UK
<i>Paecilomyces fumosoroseus</i>	PFR-21	Whitefly	W.R. Grace, USA
<i>Verticillium lecanii</i>	Pae-Sin	Whitefly	Agrobionsa, Mexico
	Mycotal	Whitefly and thrips	Koppert, The Netherlands
	Vertalec	Aphids	Koppert, The Netherlands
<i>Hirsutella thompsonii</i>	Mycar	Eriophid mites	Abbott Laboratories, USA
<i>Lagenidium giganteum</i>	Laginex	Mosquito larvae	AgraQuest, USA
Nematophagous fungi			
<i>Myrothecium verucaria</i>	DiTera	Plant-parasitic nematodes	Valent, USA, Japan
<i>Paecilomyces lilacinus</i>	Paecil (also known as Bioact)	Plant-parasitic nematodes	Technological Innovation Corporation Pty. Ltd., Australia
<i>Duddingtonia flagrans</i>	under development	Animal-parasitic nematodes	Christian Hansen, Denmark
<i>Pochonia chalmydospora</i>	under development	Plant-parasitic nematodes	DeCeuster, Belgium

(continued)

Table 1 Continued

Fungus	Product	Target	Producer/Country
Mycoparasites	AQ10 Biofungicide	Powdery mildew	Ecogen, Inc., USA
<i>Ampelomyces quisqualis</i> isolate M-10			
<i>Candida oleophila</i> I-182	Aspire	<i>Botrytis</i> spp., <i>Penicillium</i> spp.	Ecogen, Inc., USA
<i>Coniothyrium minitans</i>	Contans WG, Intercept WG	<i>Sclerotinia sclerotiorum</i> and <i>S. minor</i>	Prophyta, Germany
	KONI	As above	BIOVED, Ltd., Hungary
<i>Fusarium oxysporum</i> (nonpathogenic)	Biofox C	<i>Fusarium oxysporum</i>	S.I.A.P.A., Italy
	Fusaclean	As above	Natural Plant Protection, France
<i>Gliocladium catenulatum</i>	Primastop	Soilborne pathogens causing seed, root, and stem rot, and wilt disease	Kemira Agro Oy, Finland
<i>G. virens</i> GL-21	SoilGard	Damping-off and root-rot pathogens, especially <i>Rhizoctonia solani</i> and <i>Pythium</i> spp.	Certis, Inc., USA
<i>Phlebia gigantea</i>	Rotstop	<i>Heterobasidium annosum</i>	Kemira Agro Oy, Finland
<i>Pythium oligandrum</i>	Polyversum (formerly Polygandron)	<i>Pythium</i> spp., <i>Fusarium</i> spp., <i>Botrytis</i> spp., <i>Phytophthora</i> spp., <i>Alternaria</i> spp., <i>Gaeumannomyces graminis</i> , <i>Rhizoctonia solani</i> , <i>Sclerotium cepivorum</i> , and others	Biopreparaty Ltd., Czech
<i>Trichoderma</i> spp.	Bio-Fungus, Supresivit	Fungi causing wilt, take-all, root rot, and wood decay	BioPlant, Denmark
	Binab	As above	Binab, Sweden
<i>T. harzianum</i>	Root Pro (RootProtato)	Fungi causing wilt, take-all, root rot, and wood decay/foliar fungal diseases	Efal Agri, Israel
	T-22 and T-22HB	As above	Bioworks, Inc., USA
	Bio-trek, Rootshield		
	Trichopel	As above	Agrimm Technologies, Ltd., New Zealand
	Trichodex	As above	Makhteshim Chemical Works, Ltd., Israel

(continued)

Table 1 Continued

Fungus	Product	Target	Producer/Country
<i>T. harzianum</i> and <i>T. viride</i>	Trichodowels, Trichoject, Trichoseal & others	<i>Chondrosternum purpureum</i> & other soil & foliar pathogens	Agrimm Technologies, Ltd., New Zealand & others
<i>T. harzianum</i> and <i>T. polysporum</i>	Binab T	Fungi causing wilt, wood decay and take all	Bio-Innovation, Sweden
<i>T. viride</i>	Trieco	<i>Rhizoctonia</i> spp., <i>Pythium</i> spp., <i>Fusarium</i> spp., root rot, seedling rot, collar rot, red rot, damping-off, <i>Fusarium</i> wilt	Ecosense Labs Pvt. Ltd., India
Herbicidal fungi			
<i>Acremonium diospyri</i>	Under development	Persimmon (<i>Diospyros virginiana</i>) in Oklahoma rangeland	USA
<i>Alternaria zinniae</i> <i>A. cassiae</i>	Under development Casst	Noogoora burr (<i>Xanthium</i> Sicklepod (<i>Cassia obtusifolia</i>) and coffee senna (<i>C. occidentalis</i>) in soybeans and groundnuts	Italy USA
<i>Bipolaris setariae</i>	Under development	Goosegrass (<i>Eleusine indica</i>)	USA
<i>Cercospora rodmanii</i>	ABG 5003	Water hyacinth (<i>Eichhornia</i>)	Abbott Labs, USA, Canada
<i>Cochliobolus lunatus</i>	Under development	Barnyard-grass (<i>Echinochloa</i>)	The Netherlands
<i>Collectotrichum coccodes</i>	Velgo	Velvet-leaf (<i>Abutilon theophrasti</i>) in maize and soybeans	USA, Canada
<i>C. gloeosporioides</i> f. sp. <i>aeschynomene</i>	Collego	Northern joint vetch (<i>Aeschynomene virginica</i>) in rice	Encore Technologies, USA
<i>C. gloeosporioides</i> f. sp. <i>cuscutae</i>	Lubao 1	<i>Cuscutae chinensis</i> and <i>C. australis</i> in soybeans	PR China
<i>Chondrosterium purpureum</i>	Biocon	Black cherry (<i>Prunus serotina</i>) in forestry	Koppert, The Netherlands
<i>Fusarium laterium</i>	Under development	Giant ragweed (<i>Ambrosia trifida</i>)	The Netherlands
<i>Phytophthora palmivora</i>	DeVine	Milkweed vine (<i>Morrenia odorata</i>) in Florida citrus	Valent, USA
<i>Pyricularia grisea</i>	Under development	Large crabgrass	The Netherlands

Source: modified from ref. 3.

According to Griffin [5], most of the fungal secondary metabolites are from the following metabolic sources: (1) glucose-derived metabolites; (2) the mevalonic acid pathway from acetyl coenzyme A (CoA); (3) the shikimic acid pathway for biosynthesis of aromatic amino acids; (4) the polyketide biosynthetic pathway from acetyl CoA (e.g., patulin, cytochalasin, and zearalenone); and (5) amino acid–derived pathways. In addition to providing an overview on the production and bioactivity of fungal secondary metabolites involved in the biocontrol of agricultural pests, this chapter provides selected examples to illustrate the concepts and patterns for discovery of agrochemicals from fungal metabolites. This chapter is divided into sections covering plant diseases, weeds, nematodes, and insect pests as these are the most important factors confronting present agricultural production.

2. FUNGAL SECONDARY METABOLITES IN THE BIOCONTROL OF PLANT DISEASES

Biological control of plant diseases is defined as the reduction of inoculum density or disease-producing activity of a pathogen in its active or dormant state by using one or more organisms [6]. This may be achieved by direct or indirect approaches. Indirect approaches include the use of organic soil amendments to enhance the activity of indigenous microbial antagonists against specific pathogens and the use of nonpathogenic strains or their metabolic products to stimulate plant self-defense mechanisms. The latter approach is also known as cross protection or induced resistance. Direct approaches include the introduction of specific microbial antagonists into soil or on plant tissues. These antagonists must proliferate and establish themselves in the appropriate ecological niches in order to interfere effectively with the growth and/or survival of plant pathogens and thereby contribute to biological control. However, the two approaches may be deployed at the same time as is the case with biocontrol using some root-colonizing microbes [7–9].

Many fungal biocontrol products have been registered, although several problems still must be resolved, such as selection and development of superior strains, fermentation system for mass production, and formulation and delivery system (Table 1). Although elucidation of the mechanisms involved in the biocontrol activity is considered to be important in developing highly effective fungal biocontrol agents (BCAs), only a few have been subjected to a thorough analysis on the modes of action among the numerous fungal biocontrol products reported. Antibiosis, one of the most important mechanisms, involves a low-molecular-weight diffusible compound produced by a fungus to inhibit the growth of another microorganism. This mechanism has been recorded in the literature over the past few decades [10–15] and used as a vital criterion for selecting effective fungal BCAs. There is not enough evidence to prove the exact contribution of antibiotics to pathogen suppression and disease reduction *in situ* due to the complex conditions in soil or plant surface, but studies on the antibiotic compounds produced by fungal BCAs are essential in understanding their biocontrol mechanisms. In addition, antibiotics produced by fungal BCAs are a source of new fungicides.

Gliotoxin and peptaibols are the best understood examples to illustrate the roles played by secondary metabolites in plant disease biocontrol systems with fungal BCAs. Other fungal secondary metabolites have also been demonstrated to exhibit biocontrol properties against plant diseases. Gliovirin, originally isolated from *Gliocladium virens*, is a diketopiperazine antibiotic that appears to kill *Pythium ultimum* by causing coagulation of its protoplasm. Mycelium of *P. ultimum* that has been exposed to gliovirin does not

grow, even after washing and transferring the culture to fresh medium [16,17]. Furthermore, Howell and Stipanovic demonstrated that a gliovirin-deficient mutant of *G. virins* failed to protect cotton seedlings from *P. ultimum* damping-off when applied to seeds, whereas normal parent strain protect the seedlings from the disease [18]. This positive activity was also confirmed under practical conditions after introduction into soil at 10 ppm, but its performance against other oomycetes was insufficient. Argifin, a new chitinase inhibitor, produced by *Gliocladium* sp. FTD-0668, was recently identified [19,20], which may be of significance in development of fungicides to interfere with the chitin metabolic process of plant pathogenic fungi. Antifungal volatile alkyl pyrones produced by *Trichoderma harzianum* were identified by Claydon et al. [21]. These metabolites were inhibitory to a number of fungi *in vitro* and, when added to a peat soil mixture, they reduced the incidence of damping-off on lettuce caused by *Rhizoctonia solani*. Lin et al. described a novel antifungal protein from *T. viride* known as tricholin [22]. This ribosome-inactivating protein causes cessation of growth and uptake of amino acids and is active against *R. solani*. Corley et al. reported a novel trichotheccene produced by *T. harzianum*, known as harzianum, and the compound exhibited modest antifungal activities [23]. Other metabolites, mostly discovered in culture broths, include glisoprenin which inhibits *Magnaporthe grisea* appressoria formation on rice leaf surfaces [24,25]; cyclonerodiol and koninginins purified and characterized from strains of *T. koningii* [26]; harzianic acid produced by *T. harzianum* [27]; heptelidic acid produced by *G. virens* [28]; and terpenoid cyclonerodiol and its corresponding octaketide ketodiol [26] (Table 2).

Thus far, most of the antibiotic secondary metabolites of fungal BCAs are isolated from species of *Trichoderma* and *Gliocladium*, and it is not surprising that these are the two most widely used fungal genera in plant disease biocontrol. Even though it is likely that antibiotics produced by fungi in the two genera play a role in their biocontrol activities, antibiotics alone are not sufficient to explain their complete biocontrol mechanism. Mutants of *T. harzianum* with altered antibiotic production showed that strains with elevated levels of 6-n-pentyl pyrone, plus two additional antifungal compounds, did increase inhibition of hyphal growth of *R. solani* and *P. ultimum*, but there was no correlation between increased antibiotic production and their biocontrol activity [34]. In addition to studies on the antibiosis mechanism, more research is warranted to discover other biocontrol mechanisms conferred by fungal BCAs. The rest of this section is devoted to the two most studied antibiotics from plant disease fungal BCAs: peptaibols and gliotoxin.

2.1. Peptaibols

Peptaibols generally exhibit antimicrobial activities, and they are also known as antibiotic peptides. *Trichoderma* and *Emericellopsis* spp. are the main sources of peptaibols. They are usually short-chain peptides characterized by typically between 15 and 20 residues, a high proportion of nonstandard amino acid residues, a particular propensity for aminoiso-butyric acid (Aib), an alkyl N terminus, and a hydroxyl–amino acid at the C terminal. Along with Aib, nonstandard residues that have been observed include isovaline, hydroxyproline, and ethylnorvaline. Aib has a high tendency to form helices, and this is responsible for the helical structures of peptaibols.

Peptaibols are usually secreted as heterogeneous mixtures of peptides. Those secreted by *T. harzianum* include trichoziaines [35], trichokindins [36], trichozins, and harzianins [37–39]. *T. viride* produces alamethicins [40,41] and trichotoxin [42]. Paracelsin, originally described as a metabolite of *T. resei*, is produced by many *Trichoderma* spp. [43,44].

Table 2 Selected Fungal Secondary Metabolites Involved in Plant Disease Biocontrol Systems

Fungal Species	Compounds	Target Pathogens	References
<i>Coniothyrium minitans</i>	Macrosphelide A	<i>Sclerotinia sclerotiorum</i> <i>S. cepivorum</i>	29
<i>Gliocladium</i> sp. FTD-0668	Argifin	Fungi	19,20
<i>Gliocladium roseum</i>	Glisoprenin C, D and E	Fungi	510027–510029 ^a
<i>G. virens</i>	Gliovirin Gliotoxin Heptelidic acid	<i>Pythium ultimum</i> Bacteria	16,17 510131
<i>Pisolithus tinctorius</i>	Pisolithine A and B	Phytopathogenic and dermatogenic fungi	30
<i>T. harzianum</i>	Alkyl pyrones (6-pentyl-a-pyrone) Harzianum A (trichothecene) Trichorzin PA Trichorzin PAu 4 Harzianin PCu 4	Fungi	21,31 23
<i>T. longibrachiatum</i>	Longibrachin LGA and LGB	Bacteria	5,100,451,005
<i>T. viride</i>	Trichodecenins-I and -II Tichorovins Tichocellins Tricholin	Bacteria Fungi	32 22
<i>Verticillium biguttatum</i>	Bigultol Methylbigutol	<i>Rhizoctonia solani</i>	33

^a Series numbers in the Novel Antibiotics Data Base in the website <http://www.antibiotics.or.jp/journal/database>.

The antimicrobial activity of peptaibols is suggested due to their ability to form pores in lipid membranes. The pores so formed cause leakage of ionic elements across membranes, leading to the loss of osmotic balance and cell death. An example is that trichokindins, which are 18-residue peptides containing one to three isovaline residues, induce Ca^{2+} -dependent catecholamine secretion from bovine adrenal medullary chromaffin cells [36]. Furthermore, Lorito et al. reported that synergism between peptaibols and cell wall hydrolytic enzymes was involved in the antagonism of phytopathogenic fungi by *T. harzianum* [45]. β -Glucan synthase activity of isolated plasma membranes of *Botrytis cinerea* was inhibited in vitro by peptaibols trichorzianin TA and TB, but the inhibition was reversed by the addition of phosphatidylcholine. β -Glucan synthesis in vivo was inhibited by peptaibols, and this inhibition was synergized by exogenous *T. harzianum* β -1,3-glucanase. This synergism can be explained by the inhibition of the membrane-bound β -1,3-glucan synthase of the host by the peptaibols. Therefore, cell wall turnover is a major target of mycoparasitic antagonism.

2.2. Gliotoxin

Gliotoxin was discovered in 1934 as an antifungal agent and named after the species, *Gliocladium fimbriatum*, from which it was originally isolated [46]. It is an epidithiodiketo-

piperazine compound with the synonym [3R-3 α ,5 α β ,10a α]-2,3,5a,6-Tetrahydro-6-hydroxy-3-(hydroxymethyl)-2-methyl-10H-3, 10a-epidithiopyrazino[1,2a] indole-1, 4-dione and with a molecular weight of 326.4. The compound is sensitive to oxidation and heat. Its solubility at 7°C is 0.07, 4.7, 17, 20, and 77 mg/mL in water, ethanol, DMF, chloroform, and pyridine, respectively [[http://www.altcorp.com/AffinityLaboratory/Slide Shows/Gliotoxin.htm](http://www.altcorp.com/AffinityLaboratory/SlideShows/Gliotoxin.htm)]. In addition to *Gliocladium*, fungal species of *Trichoderma*, *Aspergillus*, *Penicillium*, and *Candida* have also been reported to produce gliotoxin.

In addition to antifungal activity, gliotoxin has also been reported to possess antiviral and immunomodulating activities [47]. The antifungal properties of gliotoxin were synergistically enhanced by cell wall-degrading enzymes from *T. harzianum* and *G. virens* in plant disease biocontrol systems [48]. Gliotoxin is also a toxic product of several molds that cause a serious respiratory disease of poultry and humans. Gliotoxin also inhibits several functions of the immune system and renders the host prone to disease-causing agents.

Its relatively short bioactive window limits the use of gliotoxin in certain applications. Wilhite and Straney examined the apparent transient accumulation of gliotoxin, a potential limitation in biocontrol activity [49]. ^{35}S -pulse labeling experiments indicated that *G. virens* strain G20-4VIB synthesizes gliotoxin within a short period of 16 hours during the replicative growth phase. An apparent lack of gliotoxin production in later growth phases was due to the cessation of synthesis rather than to increased gliotoxin catabolism. These results demonstrated that the regulation of gliotoxin biosynthesis is a major determining factor in the dynamics of gliotoxin production and illustrated the need for further study on the regulation of gene expression [49].

3. FUNGAL SECONDARY METABOLITES IN THE BIOCONTROL OF INSECT PESTS

Although application of entomopathogenic fungi has not always provided sustained suppression of any insect pests in any conditions, there are numerous reports on the efficacious control of insect pests with entomopathogenic fungi. Of the entomopathogenic fungi reported, *Metarhizium anisopliae* and *Beauveria bassiana* are the most widely and extensively studied. Other less studied but equally valuable species for commercial purposes are *Verticillium lecanii*, *Hirsutella* spp., and *Paecilomyces fumosoroseus* (Table 1).

Entomopathogenic fungi secrete various secondary metabolites. Some of these have been demonstrated to be important pathogenicity determinants (Table 3). Among the metabolites, some are produced by specific fungal genera, whereas others are ubiquitous. Here, we focus on a few representative examples produced by the well-studied and promising fungal species.

3.1. Destruxins

Destruxins were discovered in the early 1960s in a systematic study of toxin production by *M. anisopliae* *in vitro*. Two components, destruxins A and B, were identified [57]. Since then, 28 structurally different but related destruxins have been isolated from different sources but mostly from strains of *M. anisopliae* [65]. Destruxins are quite unique compounds presented as isomers or congeners. Their basic structural backbone consists of five amino acids and an α -hydroxy acid. However, little is known about the function of active groups in the different destruxin molecules. It has been suggested that an epoxy

Table 3 Selected Secondary Metabolites Produced by Important Entomogenous Fungi

Fungal Source	Compound	Target Insect	Mode of Action	Reference
<i>Beauveria bassiana</i> and other species	Beauvericin (type A and B)	Various insects	Increase permeability of cell membrane	50
	Bassianolide (cyclo-octadepsipeptide)	Silkworm larva	Interfere ionophore	51
	Beauverolides	Unknown	Unknown	52
	Bassianin	Unknown	Inhibit erythrocyte membrane ATPases	53
<i>Beauveria</i> spp. and other soil fungi	Tenellin			54
	Oosporein (dibenzuoquinone)	Various insects	React with proteins and amino acids, leading to enzyme malfunction	55,56
<i>Metarhizium anisopliae</i>	Destruxins (28 types)	Various insects	To block multicellular defense reactions, disturb macromolecular synthesis	57,58
<i>Tolypocladium cylindrosporum</i>	Linear peptidic efrapeptides (types C to G)	Mites, beetle, budworm, moth	Inhibitors of intracellular protein transport and mitochondrial ATPases	59–63
<i>T.geodes</i> <i>T.niveum</i> <i>T.parasiticum</i>			ATPases; Blockers of cell-surface expression of NDV-HN and VSV-G glycoproteins	
<i>Verticillium lecanii</i>	Vertilecanin A, B, and C and their methyl ester	<i>Helicoverpa zea</i>	Unknown	64

group in destruxin E increases its potency, while a COOH group may decrease potency, such as in destruxin D [66].

Destruxins have various effects on insects. It has been noted that the compounds may disrupt the calcium balance in cells [67] and inhibit vacuolar adenosine triphosphatases (ATPases) [68,69]. The insects attacked may exhibit tetanus, paralysis, and growth inhibition. For example, the larvae of mustard beetle (*Phaedon cochleariae*) and potato lady beetle (*Epilachna sparsa*) grew slowly when exposed to leaves treated with destruxins compared with those fed untreated leaves [57,70].

Toxicity of destruxin analogues varies against different insect species. LD₅₀ of destruxin A and B against silkworm larvae was 0.015 to 0.030 mg/g 24 hours after injection

[57,71], but these compounds were 10- to 30-fold less active against wax-moth (*Galleria*) larvae [72]. *Myzus persicae* is susceptible to destruxin E ($LD_{50} = 0.4 \text{ mg/cm}$) but not to the same degree as *B. brassicae* [73]. Destruxin E was more toxic to *Galleria* larvae than to *Musca domestica* [74]. Destruxins A and E appear to be the most toxic molecules and destruxin D the least toxic toward *Galleria* larvae [66,75]. It was found that destruxins A and E were equally toxic but more toxic than destruxin B when injected into or ingested by *Galleria* larvae [76].

Destruxin E may be conducted systemically in plants, as evidenced by the repelled cabbage aphid (*Brevicoryne brassicae*) in cabbage leaves soaked with an 8.8-ppm solution of destruxin E. Another report showed that cereal aphid *Rhopalosiphum padi* continued to feed on cereal leaves treated with destruxin E, even at a relatively high dosage, e.g., 6.6 mg/cm^2 [73]. Some researchers reported that contact toxicity may be one of the mechanisms of destruxins against insects [70,77], while others demonstrated no contact toxicity when applied to the integument [78]. It is not clear how these compounds cross the insect cuticle.

Destruxins are also toxic to small mammals. The LD_{50} of destruxins A and B following intraperitoneal injection in mice was 1 to 1.35 mg/kg and 13.2 to 16.9 mg/kg within 1 hour, respectively [57]. However, destruxins are less toxic to fish and amphibians. No lethal or teratogenic effect or postponement of emergence of the embryos was observed in the teleostean fish *Brachydanio rerio* [79]. The acute toxicity of destruxins on the amphibians *Xenopus laevis* and *Rana temporaria* was low [78].

3.2. Beauvericin and Bassianolide

Beauvericin is a hexadepsipeptide and it is isolated primarily from the *Beauveria* spp. and *Paecilomyces* spp. [80–82]. Two analogues, A and B, were described by Gupta et al. [50]. Beauvericin is a specific cholesterol acyltransferase inhibitor of certain cell lines. It induces programmed cell death similar to apoptosis and causes cytolysis, accompanied by internucleosomal DNA fragmentation into multiples of 200 base pairs [83]. It also forms Na^+ and K^+ complexes, leading to increased permeability of cell membrane [84]. Its insecticidal property ranges from moderate [50,51,85] to very weak [86], but it is highly toxic in vitro toward murine [83] and human cell lines [87]. It is also toxic to brine shrimp (*Artemia salina* L.) with a $LD_{50} = 2.8 \mu\text{g/mL}$ water and to *M. bahia* with a LD_{50} of 0.56 mg/L [88].

B. bassiana produces a cyclo-octadepsipeptide known as bassianolide [51]. This chemical induced atonic symptoms in silkworm larvae fed an artificial diet containing small amounts of the compound but was lethal at a high dosage of 13 ppm. Like beauvericin, bassianolide is an ionophore antibiotic, but it differs in its reaction to different cations [51,89].

3.3. Hirsutellin A

Hirsutellin A was discovered from culture filtrates of *Hirsutella thompsonii* [90]. Liu et al. monitored production of the compound and found that the peak level of hirsutellin A ($13\text{--}14 \mu\text{g/mL}$) during submerged fermentation of *H. thompsonii* occurred at the late exponential growth phase [91]. Insecticidal activity tests showed that 21-hour culture filtrates were highly toxic to wax-moth larvae. Pure hirsutellin A at a concentration of 40 pmol was highly toxic to *G. mellonella* larvae.

Hirsutellin A is an extracellular insecticidal protein. This toxic protein appears to be distinct from other known proteins. It is not glycosylated and does not show proteolytic activity. It is antigenic and thermostable. It can not be inactivated by treatments with proteolytic enzymes. Its gene encodes a precursor of 164 amino acids, which includes a 34-amino acid leader sequence. The mature 130-amino acid hirsutellin A, with a calculated $M_r = 14,159$ and $pI = 9.21$, is considered to be a stable hydrophilic protein [91a].

Pesticidal activity of hirsutellin A was determined by using contact/residual leaf bioassay at concentrations of 0, 10, 32, 56, and 100 $\mu\text{g}/\text{mL}$ against adult citrus rust mite (*Phyllocoptura oleivora*), the natural host of the parasitic fungus *H. thompsonii*. Mite mortality increased with an increase in hirsutellin-A concentration, reaching virtually 100% at 100 $\mu\text{g}/\text{mL}$. The number of eggs found on leaf discs within a 3-day period decreased significantly with increasing concentrations of the toxin, which suggested that fecundity was affected prior to host death [92]. Wax-moth larvae injected with hirsutellin A at 1 mg toxin per gram of body weight caused a high mortality rate. Hirsutellin A was also toxic to neonatal mosquito (*Aedes aegypti*) larvae [90].

It has been demonstrated that hirsutellin A inhibits protein translation and possesses biological features similar to the well-characterized ribosome-inhibiting proteins (RIPs), sarcin, mitogellin, and restrictocin. Liu et al. reported that hirsutellin A at 0.5- and 5.0- μM concentrations caused detectable cytopathic effects on *Spodoptera frugiperda* (Sf-9) cells within 2 to 4 hours and completely inhibited Sf-9 cell growth 4 days after treatment [93]. Observation under electronic microscope indicated that hirsutellin-A-treated Sf-9 cells became hypotrophied, with disrupted internal organelles and cell membranes. At the same concentration, it effectively inhibited brome mosaic virus protein synthesis in rabbit reticulocyte and wheat germ in *in vitro* translation systems.

3.4. Oosporein

Oosporein is a red dibenzoquinone compound produced by a large number of soil fungi including entomogenous *Beauveria* spp. [55,65]. Strasser et al. found that oosporein was the only major secondary metabolite produced by three commercial strains of *Beauveria brongniartii* in submerged cultures and on sterilized barley kernels. None of the other toxins, including bassianin, beauvericin, and tenellin, were detected by high-performance liquid chromatography and mass spectrometry techniques [94]. Laboratory experiments have shown that the maximum amount of oosporein produced in liquid batch reactors was 270 mg/L after 4 days. Production on sterilized barley kernels ranged between 2.0 and 3.2 mg/kg after 14 days. The maximum amount of oosporein detected in cockchafer (*Melolontha melolontha*) larvae infected with *B. brongniartii* was 0.23 mg per larva [94].

Oosporein is suggested to react with proteins and amino acids through redox reactions by altering SH groups, resulting in enzyme malfunction [56]. Oosporein, like tenellin and bassianin, inhibits erythrocyte membrane ATPase activity in a dose-dependent manner by as much as 50% at 200 $\mu\text{g}/\text{mL}$. These toxins inhibited Ca^{2+} -ATPases activity to a greater extent than Na^+/K^+ -ATPases activity. The ATPase-inhibitory activity of these toxins was not specific, but probably a consequence of membrane disruption, since they all caused alterations in erythrocyte morphology and promoted varying degrees of cell lysis [54].

Oosporein is effective in suppression of G^+ bacteria, but it has little effect on G^- bacteria [95–97]. No obvious effects on fungi and plants could be detected [95]. Melocont®-Pilzgerste, a commercial product based on *B. brongniartii*, was not toxic to

garden cress (*Lepidium sativum*), Hurd's grass (*Phleum pratense*), and potatoes; nor was oosporein detected in these plants and potato tubers [65]. However, oosporein has been reported to cause avian gout in broiler chicks and turkeys [98,99]. Furthermore, oosporein has been found to be toxic to 1-day-old male chickens with LD₅₀ value of 6 mg/kg [100]. Toxicity studies of oosporein in mice and hamsters indicated an LD₅₀ value of 0.5 mg/kg body weight, when injected intraperitoneally [97]. However, a daily oral administration of 7 mg/kg oosporein to mice over 47 days was nonlethal. Cytotoxicity tests on two different mammalian cell lines revealed that oosporein at 600 ng/mL had no adverse effect [101]. In conclusion, oosporein is safe to the environment, humans, and animals when used appropriately.

4. FUNGAL SECONDARY METABOLITES IN THE BIOCONTROL OF PLANT NEMATODES

As an important group of plant pathogen, nematodes reduce crop yield and quality not only by damaging plant tissue directly but also by transferring viruses and synergizing other pathogenic microorganisms. An alternative to chemical control of nematode pests is to use their natural enemies. Among these, nematophagous fungi are the most important and extensively studied. These fungi can be grouped into four types according to their mode of infection: (1) nematode-trapping fungi attacking free-living nematodes with adhesive or nonadhesive trapping devices; (2) endoparasitic fungi attacking free-living nematodes by zoospore encyst, adhesive, ingested and injected spores; (3) opportunistic fungi attacking nematodes in sedentary stages with hyphal tips; and (4) toxin-producing fungi attacking nematodes with toxins produced by special structure known as stephanocysts [102,103].

Many reports indicated that naturally occurring fungi sometimes maintain the populations of plant-parasitic nematodes well below the economic thresholds. The most documented examples are the control of *Heterodera avenae* Woll. in European cereal fields by *Verticillium chlamydosporium* and *Nematophthora gynophila* [104] and the control of root-knot nematodes in California peach orchards by *Dactylella oviparasitica* [105]. Numerous small-scale glasshouse and field experiments on biocontrol of plant parasitic nematodes with nematophagous fungi have yielded promising results for agricultural applications [106]. In fact, many commercial products have been developed or are under development (Table 1), although attempts to achieve the control level on a large field scale have not been as successful as hoped.

Early studies of relatively rapid immobilization of nematodes when attacked by nematophagous fungi suggested that toxic fungal metabolites might be involved [107,108]. Recently, it was found that the living culture of *Myrothecium verrucaria*, by which a new biological nematicide DiTera® was developed and introduced on the market in North America, did not control plant nematodes adequately. Extracted preparation of a sub-isolate fermentation, however, significantly reduced the galling by root-knot nematodes and promoted the mortality of other ectoparasitic nematodes in laboratory and greenhouse bioassays [109]. Thus far, many nematotoxins involved in the inactivation or killing of the nematodes have been observed in nematode-trapping fungi (e.g., *Arthrobotrys oligospora*) [110], endoparasitic fungi (e.g., *Nematoctonus haptocladius* and *N. concurrens*) [111], egg-parasitic fungi (e.g., *Paecilomyces lilacinus*) [112], and toxin-producing fungi such as *Pleurotus* [113]. Some of the nematicidal compounds have been isolated and well characterized. Examples are linoleic acid isolated from the liquid culture of *Arthrobotrys oligospora* [114,115], fatty acids such as trans-2-dodecenoic acid from *Pleurotus ostreatus*, linoleic and s-corilic acids from *Pleurotus pulmonarius* [116,117], acetic acid and phoma-

Table 4 Selected Secondary Metabolites Produced by Nematophagous Fungi

Fungal species	Compounds	Target Nematodes	References
<i>Arthrobotrys oligospora</i>	Oligosporon 4', 5'- Dihydrooligosporon	<i>Haemonchus contortus</i>	118
<i>Cylindrocarpon olidum</i>	Cannabiorcichromenic acid	<i>Heterorhabditis</i> nematodes	119
<i>Beauveria bassiana</i>	Beauvericin	<i>Meloidogyne incognita</i>	120
<i>Fusarium</i> spp.			
<i>Paecilomyces fumosoroseus</i>			
<i>F. roseum</i>	Trichothecolone	<i>Anguillula aceti</i>	121
<i>Trichothecium roseum</i>			
<i>Fusarium</i> spp.	Beauvericin Enniatin A Enniatin B	<i>Meloidogyne incognita</i> <i>Anguillula aceti</i> <i>Anguillula aceti</i>	120,121
<i>Gliocladium virens</i>	Viridin	<i>Anguillula aceti</i>	121
<i>Pleurotus ostreatus</i>	Trans-2-dodenedioic acid	<i>Panagrellus redivivus</i>	116
<i>P. pulmonarius</i>	S-Coriolic acid Linoleic acid p-Anisaldehyde p-Anisyl alcohol 1-(4-Methoxyphenyl)-1,2-propanediol 2-Hydroxy-(4'-methoxy)-propiophenone	<i>Caenorhabditis elegans</i>	117
<i>Verticillium sulfureum</i>	aurovertin	Helminths	122

lactone isolated from egg-parasitic fungi *Paecilomyces lilacinus* and *Pochonia chlamydospora* (i.e., *Verticillium chlamydosporium*), respectively (Table 4).

4.1. Linoleic Acid

Linoleic acid was isolated from mycelia of *Arthrobotrys oligospora* and *A. conoides* as the sole compound responsible for killing *Caenorhabditis elegans* [115]. In mycelia from submerged cultures, trap formation was induced by the addition of *C. elegans* or Phe-Val. Linoleic acid content increased with the number of traps formed [114], and it was demonstrated to be involved in the capturing of *C. elegans* by *Arthrobotrys* spp. [115]. Not all nematodes are sensitive to linoleic acid. For example, *Paragrellus redivivus* and *Aphelenchoides besseyi* were found to be sensitive to C8–C12 fatty acid but not to linoleic acid [116,123]. Plant parasitic *Meloidogyne incognita* was not affected by linoleic acid [115].

4.2. Acetic Acid

Culture filtrate of *Paecilomyces lilacinus*, the most prominent nematode egg parasite, inhibits egg hatching and immobilizes secondary-stage juveniles of *Meloidogyne* spp. [112,124] and *Heterodera glycines* [125]. It was demonstrated that the active substance

involved in these actions was acetic acid, which is produced abundantly by *P. lilacinus* in liquid medium [126]. Bioassay showed that acetic acid as a pure extract of *P. lilacinus* possessed selective toxicity to plant roots infecting nematodes. These nematodes—such as *Meloidogyne* spp., *Heterodera* spp., *Radopholus* spp., *Pratylenchus* spp., *Helicotylenchus* spp., and *Xiphinema* spp.—were significantly paralyzed by acetic acids. Other species, including leaf- and stem-infecting, mycophagous, saprophagous, entomophagous and animal parasitic nematodes were not significantly affected by acetic acid [126]. Acetic acid extracted from *P. lilacinus* was effective in paralyzing the juveniles of *Meloidogyne* spp. at low pH (<5.0), indicating that nematicidal activity was due to the acetic acid (CH_3COOH) and not to the acetate anion (CH_3COO^-) as the ionized form of acetic acid is few at lower pH in the solution. Secretion of acetic acid at low pH by *P. lilacinus* also explained the observation that this fungus was particularly active in controlling *Meloidogyne* spp. in the acidic soils [127].

4.3. Phomalactone

Phomalactone was first isolated from *Nigrospora* spp. in 1969, and its chemical structure was found to belong to the 6-substituted-5,6-dihydro-2H-pyran-2-one group [128]. Phomalactone has been reported to exhibit broad biological activities, including antibacterial, insecticidal, and herbicidal [128,129]. Recently, phomalactone was isolated with a bioassay-guided fractionation of culture liquid of *Pochonia chlamydospora*, which is another important egg-parasitic fungus of plant nematodes with great potential for nematode control. The phomalactone was demonstrated to be highly toxic to nematodes. Although the lethal activity of phomalactone against *Meloidogyne incognita* was not as strong as the chemical nematicide, aldicarb, their activity in suppressing root invasion was similar [130]. The result suggests that phomalactone is involved in the biocontrol of plant parasitic nematodes by *P. chlamydospora*.

4.4. Ostreatin

Some wood-rotting basidiomycetes capture and consume nematodes as a supplementary source of nitrogen and other nutrients [131]. Barron and Thorn described a mechanism by which *Pleurotus ostreatus* rapidly immobilize, penetrate, colonize, and digest host nematodes [113]. Nematitoxin in the tiny droplets produced by the minute spathulate secretory cells of *P. ostreatus* was found to be involved in nematode infection by the fungus. Kwok et al. purified a nematicidal toxin from *P. osreatus* NRRL 3526 grown on moistened, autoclaved wheat straw for 30 days at room temperature [116]. The toxin, named ostreatin and identified as trans-2-decenedioic acid, immobilized 95% of the tested nematode *Paragrellus redivivus* within 1 hour at 300-ppm concentration.

5. FUNGAL SECONDARY METABOLITES IN THE BIOCONTROL OF WEEDS

Use of fungal pathogens as weed-control agents has recently generated considerable research and commercial interests. *Chondrostereum purpureum*, a wood-rotting fungus that is sold as a water suspension of fungal mycelium under the trade name Biocon (Koppert) [132], has been used in forestry to prevent the regrowth of undesirable pest trees—such as American black cherry (*Prunus serotina*), yellow birch (*Betula lutea*), and poplar (*Populus* spp.)—with a 95% killing rate against tree stumps within 2 years of treatments. *Collectromphus gloeosporioides* f. sp. *aeschynomene*, sold as an aqueous suspension of conidia by

Valent BioSciences under the trade name Collego, is applied for weeds control in rice and soybean fields [132,133]. *Phytophthora palmivora* is used to control stangler vine (milkweed vine) in citrus orchards and other perennial crop fields, and it is sold as a liquid suspension of chlamydospores under the trade name DeVine by Valent BioSciences [132]. Other fungi, including *Cercospora rodmanii*, *Alternaria cassiae*, *Colletotrichum coccodes* and *Fusarium laterium*, are also being actively investigated or registered as biocontrol agents for weeds (Table 1).

Phytotoxic metabolites of fungi have been known to play a role in the process of weed biocontrol by causing necrosis, chlorosis, growth inhibition, wilting, and/or inhibition of seed germination of host weeds. Over the past few decades, production of toxins by fungal pathogens against some important weedy plants has been examined (Table 5). Some weed-control fungi, such as species of *Alternaria*, *Ascochyta*, *Cercospora*, *Fusarium*, and

Table 5 Some Semicommercialized and Promising Phytotoxins Produced by Herbicidal Fungi for Use as Natural Herbicides

Fungi	Phytotoxins	Target Weeds	Site of Action
<i>Alternaria alternata</i>	Tenuazonic acid Tentoxin	<i>Datura innoxia</i> Grasses, broad-leaved weeds	CF-1-ATPase
<i>A. alternata</i> f. sp. <i>maculosa</i>	Maculosins	Spotted knapweed	CF-2-ATPase
<i>A. alternata</i>	AAL toxin	Jimsonweed, duckweed, and northern jointwetch	CF-1-ATPase
<i>A. alternata</i> f. sp. <i>lycopersici</i>			
<i>A. zinniae</i>	Zinniol	Lettuce seedlings	Disruption of calcium-regulated cell processes
	Brefeldin A α,β - dehydrocurvularin	<i>Xanthium occidentale</i> (semi-selective)	Unknown
<i>Ascochyta caulina</i>	Trans-4-aminoproline	<i>Chenopodium rubrum</i>	Unknown
<i>A. hyalospora</i>	Ascochytine hyalopyrone	Lambsquarters, pricely sida	Electrolyte leakage and inhibition of root growth
<i>Bipolaris cyanodontis</i>	Bipolarotoxin	Velvetleaf and pigweed	Unknown
<i>Cephalosporium</i> sp.	1233 A	Weed in rice field	HMC CoA synthase
<i>Cercospora kikuchii</i>	Cercosporin	Higher plants (not defined)	Lipid peroxidation and photo- sensitizing action
<i>Fusarium oxysporum</i>	Fusaric acid	Jimsonweed and duckweed	Unknown
<i>Helminthosporium</i> <i>sativum</i>	Prehelminthosporal	Johnson grass	Unknown
<i>Irpep pachydon</i>	Irpepil	Johnson grass	Enzyme inhibitor
<i>Nigrospora sacchari</i>	Phomalactone	Higher plants (not defined)	Cellular disruption

Source: Modified from Ref. 135.

Bipolaris, have been demonstrated to produce a wide array of secondary metabolites toxic to plants [134,135]. These metabolites play an important role in the development of environmentally friendly herbicides or in control of weed in combination with plant pathogens [136]. Based on their chemical structures and biological properties, these phytotoxins are categorized as host-specific (HSPs) and non-host-specific (NHSPs) toxins [135]. NHSPs generally have a broader range of activities and applicabilities to weeds as exemplified by tentoxin and epoxydons, which are produced by *Alternaria alternata* and *Phoma* spp., respectively. These are toxic to both monocotyledonous and dicotyledonous weed species [137,138]. HSPs are produced by plant-specific pathogenic fungi, and some HSPs are so specific that they are only toxic to certain plant cultivars. Maculosin produced by *Alternaria alternata* and phomalairendone produced by black-leg fungus (*Phoma lingam*) are two examples of HSPs that are specifically toxic to spotted knapweed (*Centaurea maculosa*) [139,140]. The following is a detailed discussion on two fungal phytotoxins involved in biological control of weeds: ascaulitoxin and AAL toxins.

5.1. Ascaulitoxin

As an unusual phytotoxic bis-amino acid N-glucoside, ascaulitoxin was isolated from culture filtrate of *Ascochyta caulina*, a promising mycoherbicide for control of noxious weed *Chenopodium album* [141]. Preliminary experiments showed that culture filtrate of *A. caulina* increased the speed of disease onset and enhanced disease severity when used in combination with fungal spores [142]. Greenhouse experiments showed that use of ascaulitoxin solutions at 1 mg/mL¹ in conjunction with *A. caulina* spores at 10⁶/mL improved biocontrol efficacy of this fungus by more than 30% [143], indicating the involvement of ascaulitoxin in the fungal infection process. In addition to ascaulitoxin, two other minor toxins, trans-4-amino-D-proline and aglycone of ascaulitoxin, were also purified. These toxins exhibited herbicidal properties against host and nonhost weed plants, although their biocontrol mechanism remains unknown.

5.2. *Alternaria alternata* f. sp. *lycopersici* (AAL) Toxins

Alternaria spp. are widely distributed in nature, and some are highly pathogenic to plants. Some species have been demonstrated to produce phytotoxins that are involved in plant pathogenesis [144–146]. AAL toxins were isolated from *A. alternata* f. sp. *lycopersici* in 1981 by Bottini et al. [147]. They are long-chain alkylamines with one attached tricarboxylic acid moiety. Five types of AAL toxins have been described (i.e., TA, TB, TC, TD, and TE). Each type has two isomers [148,149]. Among the types, TA is the most active and is produced in greatest quantities by *A. alternaria* [150]. Initial studies suggested that AAL toxins were the active factors in stem canker disease of susceptible tomato cultivars with the genotype asc/asc [145]. Later tests indicated that AAL toxins were toxic to a number of weed and crop species [151,152]. Because AAL toxins are highly toxic to several noxious weeds (e.g., jimsonweed, black nightshade, prickly sida, and hemp sesbania) while not toxic to some important crops (e.g., cotton and maize), they are considered to have great potential as natural herbicides [153].

The similarity in structure between AAL toxins and fumonisins produced by several *Fusarium* spp. and *A. alternata* suggested they may have the same mode of action in killing plants. Fumonisins were known to inhibit ceramide synthase in animals. And both AAL toxin TA and fumonisin B1 induced disruption of sphingolipid metabolism and led

to phytotoxic injury and cell death. [153]. As fumonisins are highly toxic mycotoxins this mode of action is not suitable for an herbicide, and therefore it needs to be modified [154,155].

6. CONCLUSION

It is clear that fungal BCAs play an increasingly important role in agricultural and horticultural pest management. They represent a valuable management resource to be utilized within an integrated pest management framework and will contribute significantly in the reduction of chemical pesticide usage in agricultural, horticultural, and forest systems [156].

Secondary metabolites produced by fungal BCAs have different functions depending on the fungal species and their ecological niche. Some of the metabolites protect the ecological niches of their producing BCAs by inhibiting antagonistic microorganisms or saprophytic microbes on their plant hosts. Mycoparasites, in particular, may exploit this strategy to displace plant pathogens and suppress postharvest diseases [157]. Some bioactive metabolites may be important pathogenicity determinants [65]. Other secondary metabolites possess antifeedant repellent properties that presumably deter mycophagous organisms [157]. In certain cases, secondary metabolites enhance the pest-control efficacy of their producers [143].

Secondary metabolites have made great impacts on crop protection science in three ways: (1) as products in their own right; (2) as compounds with new modes of action, and (3) as structural skeletons for new synthesis strategies [132]. Microorganisms are the most important resources of secondary metabolites. Strobilurin fungicides are an example of successful fungal secondary metabolites [132]. Fungal BCAs are important resources for searching new compounds because fungi are closely associated with their hosts during coevolution. However, more work is necessary to understand the metabolites and their functions. For example, nematicidal products based on the fermentation extract of *Myrothecium verrucaria* have been demonstrated to be effective in controlling various nematodes, but their mechanisms are still unknown [109]. The metabolites involved in plant growth promotion by some fungal BCAs also need to be determined.

Concerns regarding the risks posed by metabolites of fungal BCAs to human, animal, and environment are increasing. However, it is believed that epizootics/epidemics induced naturally or artificially through inundative introductions of fungal BCAs do not pose real health risks, as most fungal BCAs are widespread soil inhabitants. Thus far, there are no documented accounts of increased toxin levels as a result of artificially induced or natural epizootics or epidemics. Similarly, there is no report of fungal BCAs' metabolites entering the food chain. Even so, it is important to develop simple and sensitive methods and tools for detecting, identifying, and quantifying secondary metabolites of fungal BCAs. These efforts will not only help public health authorities as well as registration and regulation bodies to monitor the safety of fungal BCAs, but they will also benefit other organizations associated with the research, development, and application of safe and effective fungal BCAs in agriculture, horticulture, and forest production systems.

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about the book . . .

This single-source reference provides a comprehensive overview of recent advances in industrial mycology—leading readers through the complex range of processes involved in the discovery, characterization, and profiling bioactive fungal metabolites.

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about the editor . . .

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