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MINI-REVIEW

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Biotechnology and genetics of ergot alkaloids

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Abstract Ergot alkaloids, i.e. ergoline-derived toxic metabolites, are produced by a wide range of fungi, predominantly by members of the grass-parasitizing family of the *Clavicipitaceae*. Naturally occurring alkaloids like the D-lysergic acid amides, produced by the "ergot fungus" *Claviceps purpurea*, have been used as medicinal agents for a long time. The pharmacological effects of the various ergot alkaloids and their derivatives are due to the structural similarity of the tetracyclic ring system to neurotransmitters such as noradrenaline, dopamine or serotonin. In addition to "classical" indications, e.g. migraine or blood pressure regulation, there is a wide spectrum of potential new applications of this interesting group of compounds. The biotechnology of ergot alkaloids has a long tradition, and efficient parasitic and submerse production processes have been developed; the biochemistry of the pathway and the physiology of production have been worked out in detail. The recent identification of a cluster of genes involved in ergot alkaloid biosynthesis in *C. purpurea* and the availability of molecular genetic techniques allow the development of strategies for rational drug design of ergoline-related drugs by enzyme engineering and by biocombinatorial approaches.

Introduction

Ergot alkaloids are fungal metabolites that have a long history as mycotoxins. While their effects on the central nervous system (CNS) are deleterious, they also have a long biotechnological tradition, with manifold applications in the therapy of human CNS disorders. Chemically the ergot alkaloids are 3,4-substituted indol derivatives having a tetracyclic ergoline ring structure (Fig. 1). Based on their complexity, they can be divided into two families of compounds. In the D-*lysergic acid derivatives*, a simple amino alcohol or a short peptide chain (e.g. ergotamine) is attached to the ergoline nucleus in amide linkage via a carboxy group in the 8-position. In the simpler *clavine alkaloids* (e.g. agroclavine) that carboxy group is replaced by a methyl or hydroxymethyl to which attachment of side groups such as in the amide-type alkaloids is not possible. Nearly always, the natural ergoline-derived alkaloids have a double bond in ring D of the tetracyclic ring system, either as $\Delta^{9,10}$ or $\Delta^{8,9}$ (the ring system termed therefore ergolene), and the nitrogen in ring D is always methylated (Fig. 1). They are produced by the ergot fungus *Claviceps purpurea*, an ubiquitous pathogen of cereals, and related species of the Clavicipitaceae, also endophytes. In *C. purpurea*, the alkaloids are contained in the so-called sclerotia formed on infected grass flowers. In the past, contamination of cereals with alkaloid-containing sclerotia has led to severe disease symptoms in humans, which in the Middle Ages resulted in vast epidemics called St. Anthony's fire. Today, cereals are mechanically cleaned of sclerotia, which – due to a growing number of grain-sized sclerotia – does not provide complete protection. Therefore contamination of flour with ergot alkaloids is still a problem, and has even increased during the past several years, e.g. due to *C. purpurea*-susceptible new hybrid rye varieties.

Already in the Middle Ages, midwives knew of the therapeutic potential of the ergot alkaloids and used them to aid in childbirth or abortion, hence the common name "Mothercorn". However, impurities and non-reproducibility in alkaloid content as well as in the composition of the alkaloid mixture of the sclerotia precluded its use in modern medicine until the pure ergot alkaloids became available. Investigation of these compounds facilitated clarification of the various toxic and pharmacological effects of ergot alkaloids which are caused by the structural homology of their tetracyclic ring system to

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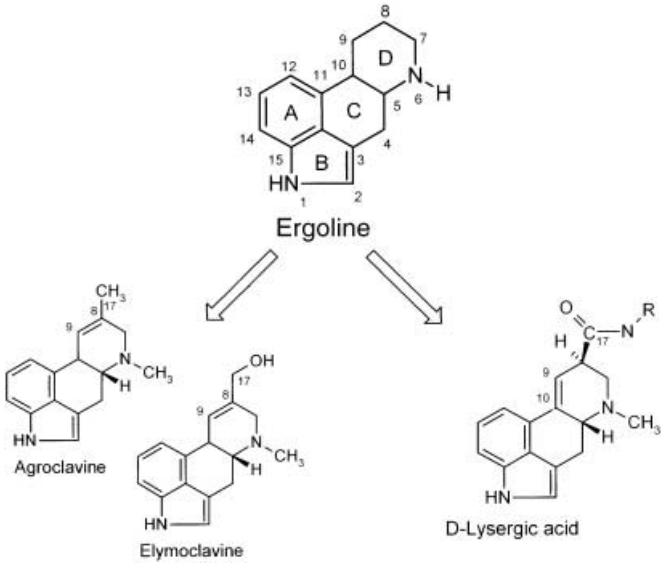


Fig. 1 Structures of the ergoline ring system, of two representative clavine alkaloids, and of D-lysergic acid amides

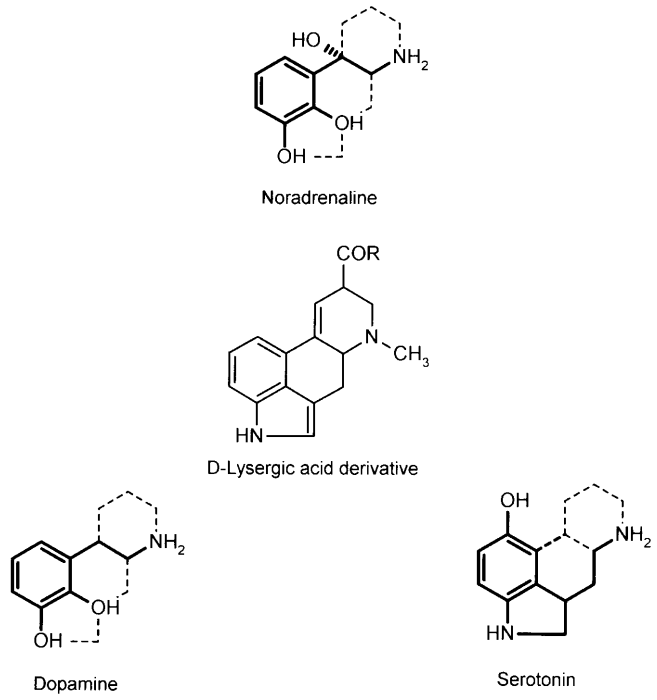


Fig. 2 Structural analogy between the ergoline ring system and different neurotransmitters (dopamine, noradrenaline, serotonin). Peptide ergot alkaloids usually have high affinity for α-adrenergic receptors. Derivatives of D-lysergic acid amidated with small amino-alcohols show high affinity for serotonin receptors. Bromination of ergocryptine in the 2-position strongly increases dopamine agonist activity

neurotransmitters like noradrenaline, dopamine, or serotonin. These different homologies explain the multitude of actions of this class of compounds, which, however, also leads to a broad specificity and therefore can provoke undesired side effects (for review, see, e.g. Berde and Stürmer 1978).

Approaches to improve ergot alkaloids as therapeutic agents therefore involve narrowing the specificity of compounds by chemical modifications of natural ergolines. As yet, engineering of enzymes of the biosynthesis pathway to achieve biotechnological production of new ergoline compounds is not possible. However, with the advent of the isolation of genes of the ergot alkaloid biosynthesis pathway, future gene manipulations of the biosynthesis pathways for targeted alteration of biosynthesis enzymes are imaginable. This review will report on the present state of ergot alkaloid research, including a discussion of the most recent advances in the biochemistry and genetics of ergot alkaloid biosynthesis pathway, biotechnological production of ergot alkaloids and current approaches to further develop ergot alkaloids for safer and more specific therapeutic applications.

Pharmacology

D-Lysergic acid-derived compounds have a wide spectrum of activities. Depending on its substituents attached to the carboxy group at C-8 of the ergoline ring system, the D-lysergic acid pharmacophore has different affinities towards the various receptors of the noradrenaline, dopamine and serotonin neurotransmitters (Fig. 2). It can behave as agonist or antagonist or play a dual role as partial-agonist and antagonist. The peptide ergot alkaloids exert vasoconstrictive and sympatholytic–adrenolytic effects due to their affinity for adrenergic receptors (Berde and Stürmer 1978; Hofmann 1978; Stadler and Giger 1984). By contrast, clavines and the simple D-lysergic acid amides with a small side chain such as ergometrine (Fig. 3) have much less adrenolytic activity and show strong anti-serotoninergic action due to elevated affinity for serotonin (5-HT) receptors. Structural alterations of the ergoline-ring as well as the introduction of unnatural side chains at the C-8 substituent have drastic effects. Thus, in the case of ergotamine the hydrogenated derivative has a much more dominant adrenolytic effect, with concomitant reduction of the vasoconstrictive effect (Hofmann 1964). Dihydroergotamine is used in the treatment of migraine, and dihydroergotoxine finds application as an anti-hypertensive agent and in the treatment of cerebral dysfunction in the elderly (Wadworth and Crisp 1992). The adrenolytic action of the dihydroergolene ring-derived compounds was further elaborated by the synthesis of various derivatives such as nicergoline (Fig. 3), a superior blocking agent for α_1-adrenoreceptors (Bernardi 1979). Nicergoline is used as an anti-hypertensive agent.

The ergopeptine ergotoxine, which is a mixture of several compounds including ergocryptine, was shown to have an inhibitory effect on the release of peptide hormones from the pituitary, such as prolactin. This led to the systematic search for new derivatives by chemical modification. 2-Bromo-ergocryptine (Fig. 3) was the first of the clinically important prolactin inhibitors used

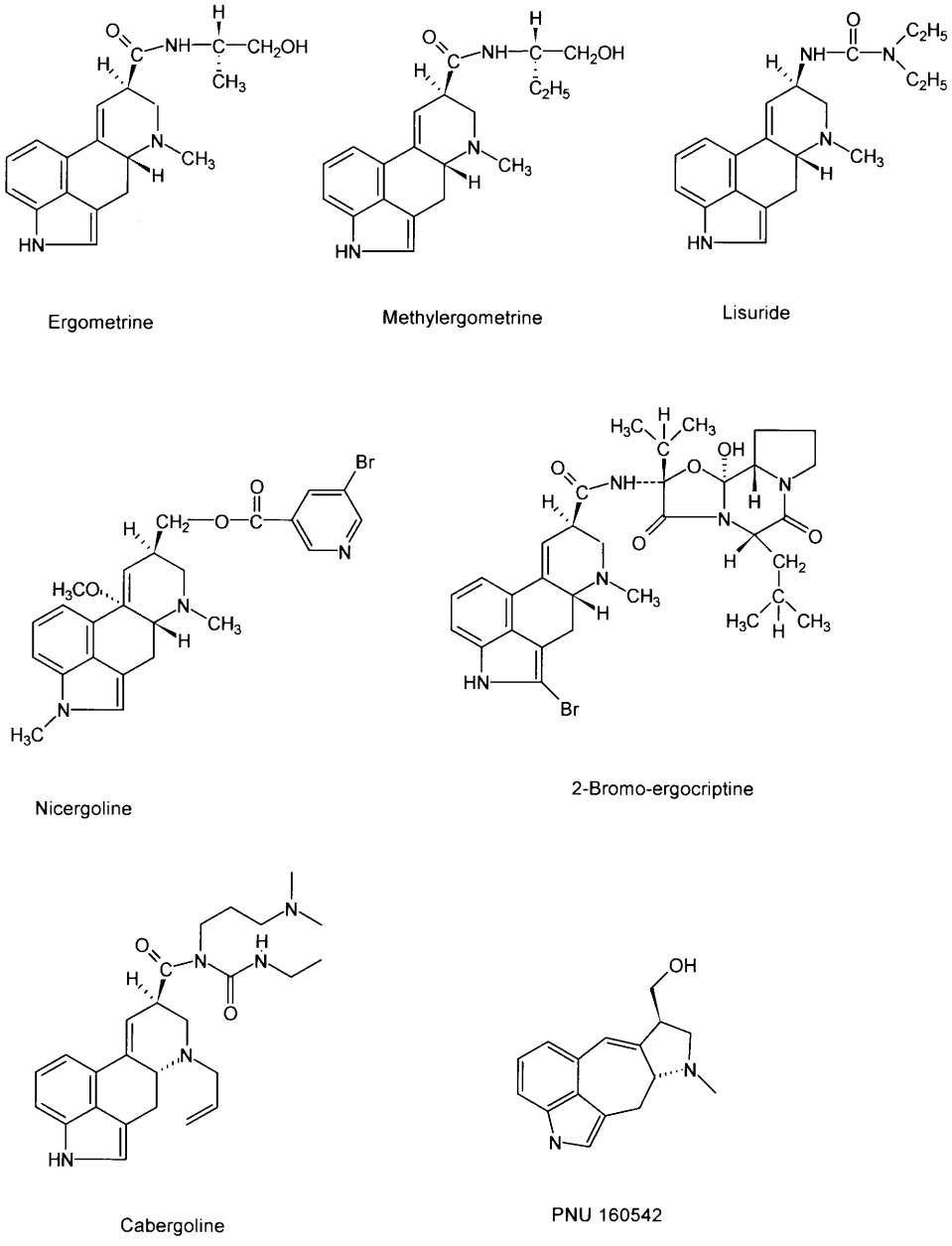


Fig. 3 Structures of some natural and semisynthetic D-lysergic acid-derived compounds used in therapy. PNU 160542 is an abeo-ergoline with a ring structure different from that of ergolines. It is obtained by chemical rearrangement of the ergoline ring system (Mantegani et al. 1999)

to treat prolactin-related disorders such as galactorrhea, prolactin-dependent mammary carcinoma, amenorrhea and acromegaly (Schneider et al. 1977). Bromocryptine also has dopamine-like activity due to its interaction with dopaminergic receptor sites, which became the basis for its use in the treatment of Parkinson's disease (Keller and Da Prada 1979). Other ergolines obtained by chemical synthesis, such as lisuride (Fig. 3) and its dihydroanalogues terguride and pergolide, have been introduced into therapy as anti-prolactin and anti-Parkinson agents (Calve et al. 1983). Cabergoline (Fig. 3), an ergolinyl-urea of characteristic structure, is a further example of a dopamine receptor (D2) agonist that is much more active than ergocryptine and which has much fewer side effects than the latter (Rabey et al. 1994). Ergoline ring chemistry has also revealed anti-dopaminergic compounds with antagonistic behavior to D2 receptors as well as compounds with mixed dopaminergic/anti-dopaminergic behavior which have potential as psychopharmacological agents, such as *d*-lysergyl-dioxopyrimidines or 3,5-dioxopiperazines (Buonamici et al. 1991).

Despite the non-selectivity of most ergolines against the heterogeneous family of 5-HT receptors, some ergolines have been shown to possess activity as agonists or antagonists against the important 5-HT_{1A} and 5-HT_{A2} receptor sites, which most likely are involved in psychiatric disorders such as depression, schizophrenia and depression (Zifa and Fillon 1992). The introduction of bulky substituents into the benzene moiety of the ergoline ring system, such as alkyl groups at the C-13 or C-14 position (see Fig. 1), strongly reduces interaction of ergolines with the dopaminergic receptor but leads to se-

lectivity for 5-HT_2 receptors (Mantegani et al. 1998). Serotoninergic effects with higher affinity and selectivity for 5-HT_{1A} receptors are also displayed by a another group of ergolines, the abeo-ergolines (Mantegani et al. 1999). These are obtained by a rearrangement of rings C and D of the tetracyclic ergoline ring skeleton as in PNU160542 (Fig. 3). For all of these interesting and valuable compounds, from which a considerable number are firmly commercially established, D-lysergic acid serves as the starting material in their chemical synthesis. Whether new natural ergot compounds can be obtained by biotechnological production depends on whether the ergoline biosynthesis pathway can be genetically engineered in order to create enzymes with novel substrate specificities or to introduce additional chemical conversions.

Biosynthesis of ergot alkaloids

The pathway of ergoline ring formation

The main steps of the pathway of ergoline ring formation are shown in Fig. 4. They were primarily established by in vivo precursor studies in alkaloid-forming *Claviceps purpurea*, *Claviceps fusiformis*, or *Claviceps paspali* (for reviews, see Floss 1976; Floss and Anderson 1980; Gröger and Floss 1998; Keller 1999). By contrast, the enzymology of ergoline ring formation has been a slowly progressing field apparently because of the instability of most of the involved enzymes in cell-free extracts from *Claviceps* sp. The only exception to this is dimethylallyltryptophan (DMAT) synthase, which catalyzes the condensation of L-tryptophan with dimethylallylpyrophosphate (step 1, Fig. 4) (Heinstein et al. 1971). Only limited information is available for a few other enzyme activities (e.g. steps 4, 5, 6, Fig. 4) involved in the ergoline pathway; these were investigated in partially purified protein fractions. DMAT synthase, which produces dimethylallyltryptophan, has been purified to homogeneity and was found to be an α_2 dimer of 105 kDa (Gebler and Poulter 1992; Lee et al. 1976). The DMAT-forming reaction is the committed step of alkaloid synthesis in ergot fungi and delivers the carbon skeleton of the ergoline ring system. Moreover, expression of the enzyme is genetically controlled by tryptophan, which induces alkaloid synthesis and relieves repression by phosphate, a most important issue in the regulation of ergot alkaloid biosynthesis (Krupinski et al. 1976; Robbers et al. 1972). The DMAT synthase gene has been cloned from both *Claviceps fusiformis* and *Claviceps purpurea* (Tsai et al. 1995; Tudzynski et al. 1999). The *C. fusiformis* gene was expressed in *Saccharomyces cerevisiae* and shown to produce a catalytically active enzyme. Interestingly, the DMAT synthase gene displayed almost no similarity to other prenyltransferase sequences, with the exception of a possible prenyl diphosphate motif (DDSYN) at position 113–117 of the amino acid sequence. This motif is also conserved in other farnesyl diphosphate and geranylgeranyl diphosphate synthases (Song and Poulter 1994).

All of the enzymatic steps after DMAT formation concern modifications and rearrangements leading to formation of rings C and D. *N*-methylation of the amino nitrogen of DMAT (step 2, Fig. 4) is catalyzed by a methyltransferase, which was analyzed with respect to its kinetic constants and substrate specificity (Otsuka et al. 1979, 1980) The methylation step is followed by decarboxylation and closure of ring C (step 3), a reaction in which chanoclavine-I cyclase is involved (Erge et al. 1973; Gröger and Sajdl 1972). Except for its substrate requirements, chanoclavine-I-cyclase was not further characterized and it is not clear which particular step of the mechanism of chanoclavine-I cyclization is catalyzed. Another known enzyme activity of the pathway is that of agroclavine-17-monooxygenase (Kim et al. 1981), which exclusively converts agroclavine to elymoclavine (step 6). Elymoclavine in turn is converted to paspalic acid (step7) by elymoclavine-17-monooxygenase (Kim et al. 1983; Maier et al. 1988). Both oxygenases are dependent on NADPH and molecular oxygen and therefore most probably are cytochrome P450-monooxygenases. Interestingly, elymoclavine-17-monooxygenase was not detected in a *Claviceps* strain that produces as end products agroclavine and elymoclavine. By contrast, the enzyme was present in a strain that produces D-lysergic acid amides and peptides (Kim et al. 1983; Maier et al. 1988). This may indicate that the clavine-producing *Claviceps* strain lacks the enzyme that converts elymoclavine to paspalic acid. Paspalic acid itself is spontaneously isomerized to D-lysergic acid (step 8).

Its is most noteworthy that the pathway of ergot alkaloid formation may end at different stages, such as at chanoclavine-I, a representative of the so-called bis-seco ergolenes, formed as the main compound in a number of strains of *C. fusiformis*. In other *C. fusiformis* strains the pathway ends at the stage of agroclavine or elymoclavine, the two main representatives of the clavine group of alkaloids. In strains of *C. paspali* or *C. purpurea*, the alkaloid pathway continues through elymoclavine to the next intermediate, paspalic acid, the immediate precursor of D-lysergic acid from which the next step leads to the simple D-lysergic acid amides or to the ergopeptines, the classical ergot peptide alkaloids of *C. purpurea* (Flieger et al. 1997). The different editions of the alkaloid biosynthesis pathway found in nature may be the result of natural mutations in the downstream genes of the clavine assembly genes or a complete lack of the relevant genes.

Assembly of ergot peptides and simple D-lysergic acid amides

A major issue of ergot alkaloid biosynthesis research is investigation of the formation of the various amide-derivatives of D-lysergic acid. To these belong the ergopep-

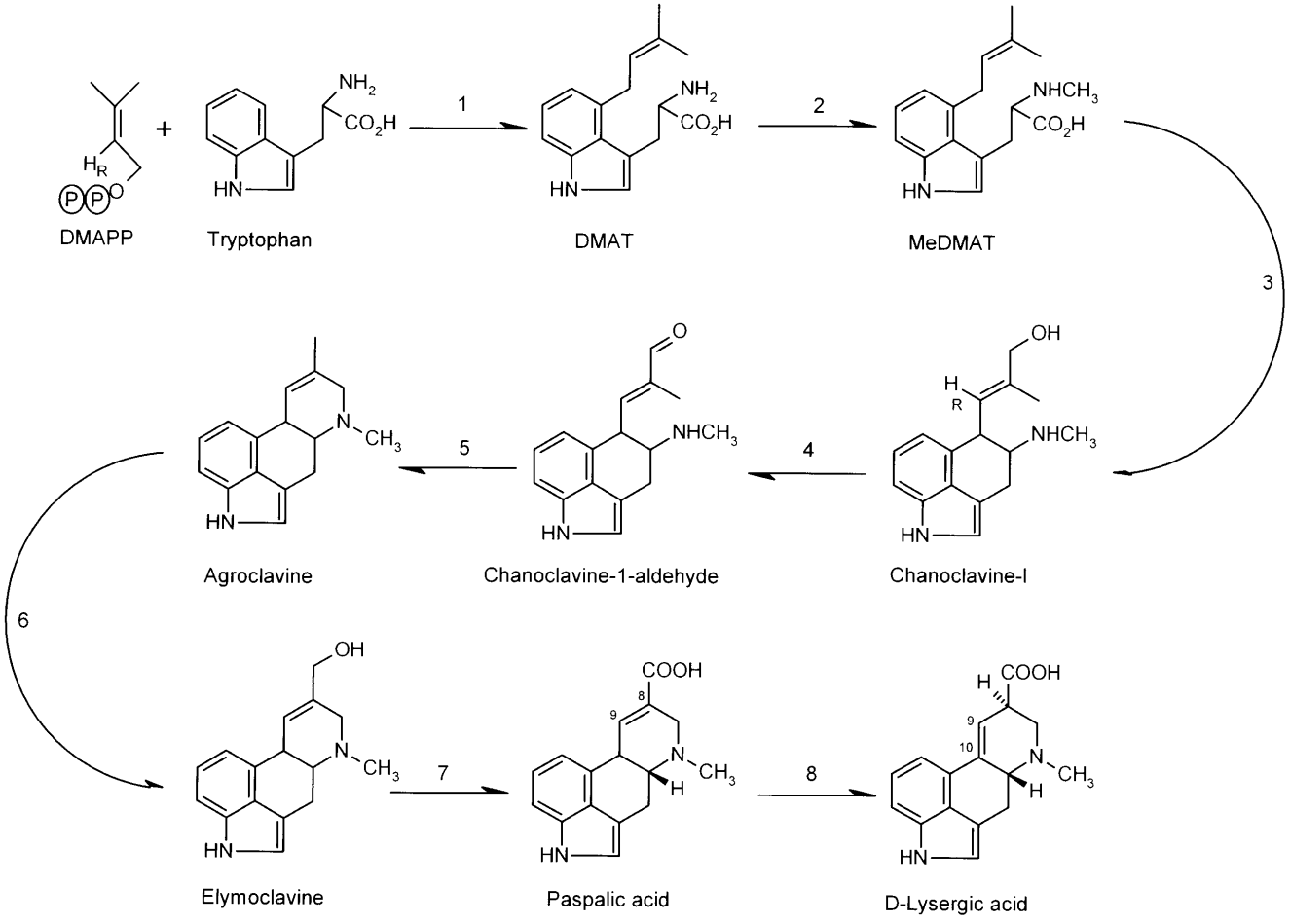


Fig. 4 Pathway of ergoline ring synthesis up to the stage of D-lysergic acid. The various biosynthetic steps are described in the text

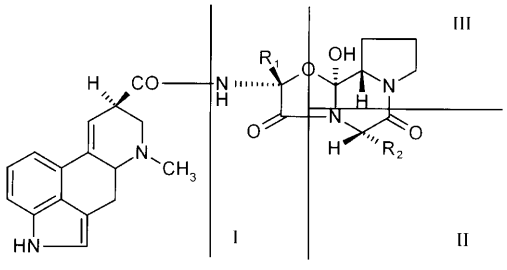


Fig. 5 General formula of ergopeptines. The various ergopeptines differ by substitutions in positions I and II with L-amino acids characterized by side chains R1 and R2. The amino acid in position III is always L-proline

tines, in which D-lysergic acid is attached to the bicyclic cyclol-lactam structure formed from three amino acids (Hofmann 1964). The numerous ergopeptines differ from each other by amino acid substitutions in the first two amino acid positions (i.e. the ones adjacent to D-lysergic acid) of the cyclol-lactam chain while the third (containing proline) is invariable (Fig. 5). The variable amino acids are aliphatic ones with linear and branched side chains (alanine, valine, isoleucine, leucine, aminobutyric acid) and the amino acid phenylalanine. Ergopeptines found in nature are classified into the ergotamine, ergoxine, and ergotoxine groups depending on their amino acid composition.

The ergopeptines are derived from D-lysergyltripeptide lactam precursors (Fig. 6). The latter are enzymatically converted to the corresponding ergopeptines by a cytochrome-P450-related activity which hydroxylates the first amino acid of the peptide chain (Quigley and Floss 1981). The D-lysergyltripeptide lactams are assembled from D-lysergic acid and the amino acids of the peptide lactam chain by a non-ribosomal mechanism (Keller et al. 1988). The enzyme D-lysergyl peptide synthetase (LPS) has been purified from crude extracts of *C. purpurea* (Riederer et al. 1996). It is a typical non-ribosomal peptide synthetase (NRPS) which activates D-lysergic acid and the three amino acids of the peptide portion of the alkaloid as adenylates and binds them as thioesters (Marahiel et al. 1997). The enzyme consists of four modules each housing domains for adenylation, thiolation, and condensation of the constituents of the ergot peptide alkaloid backbone. Interestingly, LPS consists of two large subunits, which is unusual because, as yet, all described fungal NRPSs consist of a single polypeptide chain. The larger (370 kDa) polypeptide (LPS1) acti-

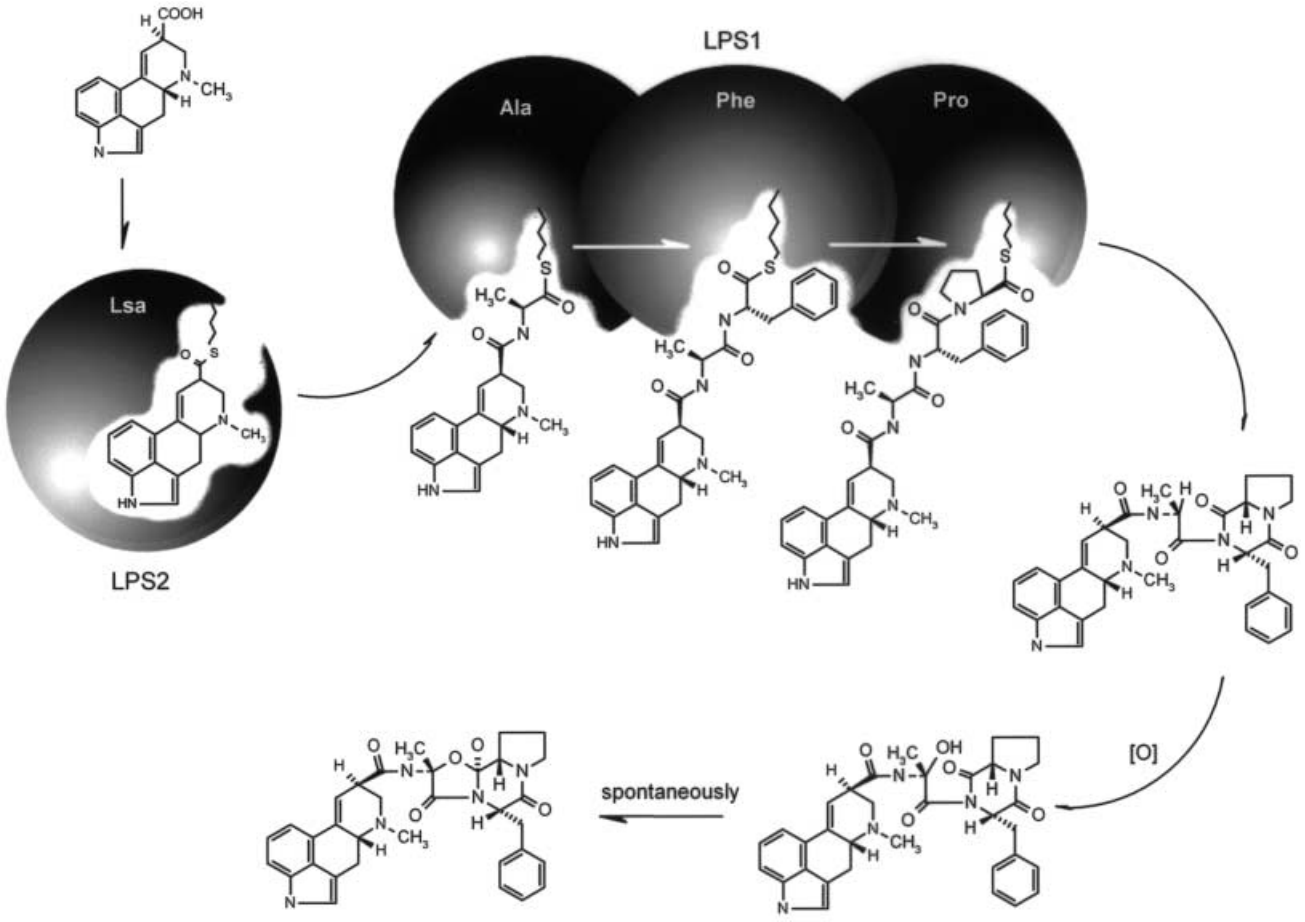
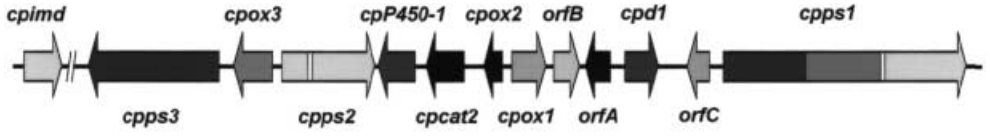


Fig. 6 D-Lysergyl peptide lactam formation catalyzed by the D-lysergyl peptide synthetases (*LPS*). LPS2 contains the module for D-lysergic acid (*Lsa*) activation and binding. LPS1 contains three modules responsible for the activation and binding of the amino acids of the peptide portion of the D-lysergyl peptide lactam. The mechanism of synthesis is by successive addition of the amino acids (e.g. alanine, phenylalanine, and proline in the case of ergotamine) to D-lysergic acid, resulting in peptide chain growth from the amino terminus to the carboxy terminus. The resultant D-lysergyl-tri-peptide is cyclized and released from LPS1 presumably by the action of a thioesterase domain

vates the amino acids of the peptide portion. The smaller (140 kDa) subunit is responsible for D-lysergic acid activation (LPS2). Analysis of enzyme-bound reaction intermediates accumulating on LPS in the course of D-lysergylpeptide lactam synthesis revealed that peptide synthesis starts when D-lysergic acid binds to the LPS2 subunit (Fig. 7). By condensation of D-lysergic acid with the amino acid bound to the first module of LPS1, a D-lysergyl-monopeptide is formed as the first intermediate and sits in the first module of LPS1. LPS1 then catalyzes the successive condensation of the D-lysergylmonopeptide with the next two amino acids bound to modules 2 and 3 of LPS1. Finally, the D-lysergyltripeptide is released from LPS1 by cyclization to the lactam (Figure 7) (Riederer et al. 1996; Zocher and Keller 1997).

In contrast to the D-lysergyl-tripeptides, the mechanism of formation of the simple D-lysergic acid amides D-lysergic acid α-hydroxyethylamide and ergometrine is unknown. Precursor studies indicate that they are formed from D-lysergyl-alanine (Agurell 1966; Castagnoli et al. 1970). This indicates a specific D-lysergyl peptide synthetase operating in the case of the simple D-lysergic acid amides. This NRPS may contain two modules, one responsible for D-lysergic acid activation and the other for alanine activation. As yet, neither this enzyme nor the reaction leading from D-lysergylalanine to ergometrine or *d*-lysergic acid α-hydroxyethylamide is known (Keller 1999).

Fig. 7 The genomic region of *Claviceps purpurea* strain P1, including genes of the ergot alkaloid biosynthetic pathway. The orientation of the genes is indicated by *arrowheads*, the three modules of *cpps1* are indicated by different *shading*, positions of introns are shown only for the peptide synthetase modules (*open boxes*). For explanation of gene symbols, see text



Biotechnology

Alkaloid-producing fungi

For biotechnological applications, mainly members of the genus *Claviceps* have been used, especially isolates of the species *C. purpurea*, *C. paspali*, and *C. fusiformis*. The species differ considerably with respect to their potential to synthesize specific alkaloids: peptide alkaloids are produced by *C. purpurea* only, lysergic acid and its simple derivatives also by *C. paspali*, whereas the other species produce various ergoline alkaloids (see Flieger et al. 1997). However, the capacity to synthesize ergolines is not restricted to the genus *Claviceps*. Other members of the Clavicipitales, several non-related fungi, and even some higher plants have been shown to produce (or contain) ergot alkaloids (see compilation by Flieger et al. 1997; Kozlovski 1999):

- Ergot alkaloids are synthesized by some clavicipitaeous grass endophytes of the genera *Epichloe* (*Acremonium*) and *Balansia*, which have been the reason for severe livestock intoxications worldwide (see http://www.caf.wvu.edu/~forage/fescue_endophyte/Story.htm). Since these endophytes often increase the fitness of their hosts, attempts are being made to create alkaloid -non-producing mutants, e.g. in *Neotyphodium* sp. (C. Schardl, personal communication).
- Some members of the *Aspergillus* and *Penicillium* groups have been shown to produce clavine alkaloids (reviews: Flieger et al. 1997; Reshetilova and Solovyeva 1996). The role of these alkaloids especially in food-contaminating fungi has been the subject of increasing attention (see e.g. Reshetilova et al. 1995). Isolates of the potential human pathogen *Aspergillus fumigatus* were found to produce considerable amounts of clavine alkaloids (festucaclavine, fumigaclavines, and others) on molded silage; crude extracts of *A. fumigatus* cultures induced severe diarrhea, irritability, enteritis and lung damages in calves (Cole et al. 1977).
- In higher plants, ergot alkaloids have been detected, especially in members of the family Convolvulaceae; in *Ipomoea argyrophylla* the ergopeptide β-ergosine was even found (Friedmann et al. 1989). The question is, however, whether these alkaloids are indeed produced by the plants, or whether they stem from microorganisms (endophytes?). Molecular techniques now provide the tools to answer such questions (see below).

Production in parasitic culture

The first large-scale technology for the parasitic production of ergot on rye was developed (and patented) by Bekesy already in 1934 (see the recent review on the history of ergot by Minghetti and Crespi-Perellino 1999). For a long time, this type of field cultivation has been the major production technology in eastern Europe, but also in Germany and Switzerland, mainly due to severe problems in early fermentation processes. More recently, cultivation, inoculation and harvesting methods have been improved (see Németh 1999). Today, hybrid rye based on cytoplasmic-male-sterile (CMS) lines (Geiger and Bausbach 1979) is used as host, because the infection rate and the alkaloid yield is significantly higher than with normal rye cultivars (probably because the florets stay "open" longer); also, a major advantage is that infection can be performed by spraying conidial suspensions instead of pricking, i.e. standard chemical spraying devices can be used instead of specific pricking machinery. The average yield by this parasitic culture technology can reach 1,000–2,000 kg sclerotia/ha (Németh 1999). Since the alkaloid content in sclerotia of commercial strains averages 1% and more, an alkaloid yield of 8–10 kg per hectare can be achieved, even if losses during the purification process are taken into account.

An important benefit of this production technology is that a large set of strains producing specific alkaloids is available; these strains usually are more stable than those used for submerse production, and selection procedures are easier since these strains produce conidia and are, in general, fitter than the submerse producing "crippled" strains. The main technical problem is the generation and storage of high-quality inocula; this is mostly achieved by using deep-frozen conidial suspensions. However, several disadvantages of this method have led to a significant reduction of parasitic alkaloid production: (1) production is too dependent on environmental parameters; (2) the areas in which the climate is adequate for such production (and where it is allowed: protection of other cereals!) are limited; (3) protection of the valuable production strains is difficult; and (4) the purification procedures are expensive. Therefore the present proportion of alkaloids produced in parasitic culture worldwide is low, although exact figures are not available.

Saprophytic culture

The industrial production of the ergot peptide alkaloids in submerged culture is nowadays preferred over field cultivation. Successful submerged fermentations for the production of D-lysergic acid, the simple D-lysergic acid amides, and D-lysergic acid peptides were described in the survey by Malinka (1999). Chemical synthesis of the natural D-lysergic-acid-related compounds, such as ergometrine and ergopeptines, as well as of new semisynthetic derivatives used in therapy (methylergometrine, methysergide, or cabergoline, Fig. 3), has led to D-lysergic acid fermentations being preferred to that of its derivatives. Since D-lysergic acid rarely occurs in free form, it is obtained indirectly either via paspalic acid or via the simple D-lysergic acid amides such as D-lysergic acid α-hydroxyethylamide, ergine/erginine, and ergometrine. In some cases, ergopeptines themselves have been reported as a source of D-lysergic acid. All of the D-lysergic acid amides can be hydrolyzed by alkaline hydrolysis to D-lysergic acid in good yields (Rucman 1976).

To achieve good productivity of *Claviceps* strains in submerged cultures, one has to take into account that the formation of ergot alkaloids in nature is concomitant with that of the sclerotia during the life cycle of *Claviceps* sp. By contrast, mycelium from the sphacelial stage never produces alkaloids. Sphacelium is characterized by the presence of long slim hyphae with a narrow cell wall, several nuclei, small vacuoles and a low fat content (Lösecke et al. 1980, 1982). Good alkaloid-producing cells in submerged cultures have a different morphology: They are short and thick (polyedrical), and have thick walls, a reduced number of nuclei and mitochondria, and large vacuoles and numerous lipid droplets in the cytoplasm. The structure of these cells greatly resembles the hyphal structure seen in the plectenchymatic tissue of the sclerotia and they are thus called sclerotia-like cells (Spalla 1973).

The sclerotia-like cell type as a prerequisite for good productivity is distinct from the genetic predisposition of a strain, i.e. natural isolate or strain-selected by mutagenesis for overproduction, and is a result of highly specific environmental conditions which are attained by the choice of characteristic media. Media suitable for growing ergot fungi with regard to alkaloid production have been described in the literature (Kobel and Sanglier 1978, 1986). These media are characterized by the presence of a sugar in high concentration (e.g. 300 g sucrose/l or 100 g glucose/l) (Amici et al. 1969). The high osmotic pressure (10–20 bar) of the medium is a prerequisite for induction of sclerotia-like cells while inhibiting conidiation (Puc and Socic 1977). Moreover, alkaloid production requires a non-inhibitory, slowly metabolized carbon source such as mannitol, sorbitol or – as mostly used – sucrose (Abe and Yamatodani 1964; Amici et al. 1966). Sucrose is the principal sugar in phloem sap and serves as a nutrient of the fungus in parasitic conditions (Bassett et al. 1972; Mower and Hancock 1975). *Claviceps* strains readily convert sucrose to fructofuranosyl-oligosaccharides with glucose as the reducing end (Arcamone 1977). These oligosaccharides are used as a glucose source in the late stage of the fermentation, which is the most active stage for the production of D-lysergic acid derivatives.

Characteristically, production media contain an organic acid of the TCA cycle as carbon source, usually as its ammonium salt (asparagine or aspartate have sometimes been used as a readily consumed nitrogen source) (Amici et al. 1969; Mantle and Nisbet 1976). The effect of the organic acid is that it saturates the TCA cycle, which lastly results in the accumulation of large amounts of acetyl-CoA, from glycolysis and from citrate (Didec-Brumec et al. 1996; Glund et al. 1981). Acetyl-CoA is the key substrate for both alkaloid and lipid synthesis. The resulting overflow of the glycolytic pathway (by carbohydrate utilization) leads to enhancement of the glyoxylate cycle and the pentosephosphate cycle (Spalla et al. 1978). The latter provides the NADPH necessary for the mevalonate and lipid biosynthesis pathways. The glucose used is either derived from sucrose as long as available or from the oligosaccharides during the later stage of fermentation. From the unusual high sugar content of the production media a high C/N ratio, often exceeding 50, results. With the limitation of phosphate, this medium virtually promotes the high level of oxidative metabolism in the *Claviceps* cells necessary for secondary product synthesis (Paźoutová et al. 1980; Spalla et al. 1978). Characteristically, alkaloid synthesis starts after depletion of phosphate from the medium (Arcamone et al. 1960; Paźoutová and Rehacek 1984). Additions of excess phosphate leads to undesired growth-linked repression of alkaloid synthesis (Dierkes et al. 1993).

In summary, the various media have in common that they induce the sclerotial-cell like morphology by substrate limitation and by favoring oxidative metabolism. Nevertheless, it is necessary to use strains that have been preselected in strain improvement programs (with or without mutagenic treatments) for good alkaloid production as well as for obtaining the desired sclerotia-like cell morphology and pigmentation.

Selection procedures are also necessary for the maintenance of the alkaloid-producing capacity of industrial strains. Strain degeneration is quite frequent in *Claviceps* and is characterized by loss of sclerotia-like cell morphology and lowered alkaloid production. Without constant surveillance and appropriate selection after streaking, strains have the tendency to assume a sphacelial-like morphology accompanied by irreversible loss of alkaloid production even in alkaloid production media (Malinka 1999).

Genetics of ergot alkaloid biosynthesis

Breeding and selection of producing strains

Most field isolates of *C. purpurea* produce significant amounts of ergot alkaloids only in the parasitic phase, not in axenic culture. Thus the screening of different ergot strains from field surveys or various culture collections over the years has yielded interesting strains producing different, specific alkaloids (or desired mixtures) at moderate rates *in planta*, but they were only suited (if at all) for parasitic production, not for submerse production of alkaloids. Therefore beginning in the early days of ergot biotechnology *strain improvement programs* were launched, aimed at an increased capacity of strains to produce high amounts of a specific alkaloid.

This was mainly achieved – as in other strain improvement programs of secondary-metabolite-producing fungi – by a *stepwise mutagenesis selection process*. However, due to the lack of antibiotic or other activity that can be tested in quick bioassays, the screening is more time-consuming than normal and requires a great deal of analytical work in order to select among the numerous clones the desired ones. Preselection methods to narrow down the number of interesting isolates a priori involve growing natural isolates or mutated clones under culture conditions that enforce sclerotia-like cell growth, such as by the addition of salt to the media (Udvardy 1980) or by strictly using media imitating the natural physiology of parasitic culture (Strnadova et al. 1986).

Further selection among prescreened clones can be made, e.g. on agar media based on the morphology and/or pigmentation. Other approaches, albeit time-consuming, may involve biochemical characterization of clones such as for the presence of elevated levels of tryptophan synthase or other enzymes of the alkaloid biosynthesis pathway (Schmauder and Gröger 1983).

Mutagenesis as a means to improve the selection procedures for obtaining alkaloid high-producers has been broadly applied in many laboratories (Didec-Brumec et al. 1987; Kobel and Sanglier 1978). Most suitable for treatment with various mutagens such as radiation (UV, X-ray) or chemicals (nitrous acid, *N*-nitrosoguanidine, nitrosourea, ethylmethansulfonate, ethyleneimine) are uninucleate conidia, which after development to colonies can be tested for improved alkaloid production in liquid and solid media (Didec-Brumec et al. 1987; Kobel and Sanglier 1973; Srikai and Robbers 1983; Strnadova 1964). In the case of strains unable to form conidiospores, protoplasts have been used as unicellular units for mutagenesis (Spalla and Marnati 1978). Protoplasting of hyphae previously grown in the presence of a mutagen such as MNNG or EMS followed by regeneration to single-cell-based colonies is another method to obtain mutants with an improved capacity to synthesize ergot alkaloids (Keller 1983).

Though *C. purpurea* is a perfect fungus, *sexual genetics* cannot be used on a broad scale for strain improvement, only for parasitic production strains (see Tudzynski 1999). Most submerse-producing mutant strains that produce alkaloids are imperfect (and have often even lost the ability to sporulate, see above). However, most *C. purpurea* field isolates are highly heterokaryotic, and heterokaryosis can be easily induced in laboratory strains by protoplast fusion or anastomoses; therefore, *parasexual genetics* offer an alternative strategy for strain improvement. The importance of heterokaryosis for the production of alkaloids is controversially discussed in the literature: Esser and Tudzynski (1978) demonstrated that homokaryotic mycelia can produce alkaloids, in contrast to earlier observations (e.g. Amici et al. 1967) that only heterokaryotic strains were good producers. It was shown by several groups that formation of heterokaryons between strains of different genetic background indeed had a significant impact on alkaloid biosynthesis; here, effects like heterosis, complementation, and gene dose are probably important. Spalla et al. (1976) showed that a forced heterokaryon between *C. purpurea* strains producing ergochristine and ergocornine/ergocryptine, respectively, produced all three alkaloids. Also, protoplast-mediated fusions between species have been successfully used to obtain different spectra of alkaloids, e.g. between an ergotamine-producing *C. purpurea* strain and a clavin-producing *C. fusiformis* strain (Nagy et al. 1994; Robbers 1984) and strains of *C. purpurea* and *C. paspali* (Spalla and Marnati 1981).

Brauer and Robbers (1987) demonstrated recombination of genetic markers in heterokaryotic strains due to parasexual processes. Therefore, fusion of strains and generation of defined heterokaryons represent a powerful tool for improvement even of imperfect submerse production strains.

Molecular genetic approaches

Molecular genetic techniques have recently been successfully applied to *Claviceps* sp. (for review see Tudzynski 1999; Paźoutová and Parbery 1999) and have considerably widened the options for genetic research. They have been used for detailed characterization of strains (for "typing" of strains and for the evaluation of crosses), for evolutionary studies, and for a molecular analysis of the biosynthetic pathway; also, detailed regulatory studies have been initiated. These molecular approaches will allow the design of recombinant strains with improved production properties (e.g. by enhancing expression of genes of interest or by creating defined deletion mutants) or that produce new compounds which are not accessible by chemical synthesis.

Molecular characterization of strains

Intraspecific variation was analyzed in *C. purpurea* by restriction fragment length polymorphism (RFLP), PCR-based techniques such as random amplified polymorphic DNA (RAPD), and separation of chromosomes by pulsed-field gel electrophoresis (PFGE). Jungehülsing and Tudzynski (1997) tested 58 different random decamer primers using as template genomic DNA from 29 field isolates of *C. purpurea* from various host plants; nine of 16 primers yielding a reproducible RAPD pattern were used for comparative analysis since they showed significant differences. With some of these primers, each of the 29 isolates showed an unique RAPD pattern, confirming that this technique can be used for the identification of *C. purpurea* strains. A preliminary tree analysis based on these nine RAPD primers gave indications for some degree of host specificity or beginning sub-species formation; PFGE data supported this view (Hüsgen et al. 1999). A similar but broader approach by Paźoutová and Parbery (1999) confirmed these findings and extended them to interspecies variation; it was shown that specific RAPD primers could be used to distinguish several *Claviceps* species unequivocally. The relevance of the application of these techniques also for ergot alkaloid studies was confirmed by Paźoutová and Tudzynski (1999). Using RAPD and RFLP analyses, they showed that strain ATCC 26245, which previously had intensively been used for biochemical and genetic studies of the alkaloid pathway, actually is not a *C. purpurea* isolate but most probably a *C. fusiformis* strain.

Molecular dissection of the ergot alkaloid biosynthetic pathway

Schardl and coworkers were the first to clone a gene of the alkaloid pathway (Tsai et al. 1995): *dmaW* was shown to encode DMAT synthase, the first enzyme of the specific part of the pathway (see Fig. 2). A cDNA clone of the same gene was obtained during a differential

cDNA screening approach (Arntz and Tudzynski 1997), using a cDNA library from an alkaloid-producing culture of strain ATCC 26245 (the same strain as used by Tsai et al. 1995) and cDNA preparations from an alkaloid-producing and non-producing culture as probes. Northern analysis confirmed that the gene was induced concomitant with ergot alkaloid production, the first evidence for transcriptional control of alkaloid biosynthesis.

The main disadvantage of strain ATCC 26245, which has turned out to be a *C. fusiformis* isolate (see above), in the molecular analysis of the alkaloid pathway is that it produces no peptide alkaloids, i.e. it probably lacks the final part of the pathway. In a recent study, Tudzynski et al. (1999) initiated a detailed analysis of genes involved in the alkaloid biosynthesis in *C. purpurea* using strain P1, a derivative of strain 1029 (obtained after two rounds of mutagenesis; Keller 1983) which produces peptide alkaloids in axenic culture (mainly ergotamine). Using the *dmaW* gene of *C. fusiformis* as probe, two putative DMAT synthase genes were cloned from strain P1, one of which obviously was an inactive copy (containing a frameshift mutation due to an internal 7-bp duplication, Arntz 1999). The derived amino acid sequence of the active copy, termed *cpd1*, showed about 70% homology to the amino acid sequence from the corresponding gene of strain ATCC 26248, confirming the distant relationship of these two strains.

A chromosome walking approach starting from *cpd1* led to the detection of a *putative ergot alkaloid gene cluster* in this strain: a gene for a putative trimodular peptide synthetase (*cpps1*) was located close to *cpd1*. Internal peptides obtained by Keller and coworkers from the LPS1 from *C. purpurea* (Riederer et al. 1996; Keller, unpublished results) matched exactly to parts of the gene-derived amino acid sequence. Therefore, *cpps1* obviously encodes this enzyme, which catalyzes activation of the three amino acids of the peptide part of ergotamine and links them to the activated lysergic acid (see above). In close proximity of the genes *cpd1* and *cpps1*, encoding the first and one of the final steps of the pathway, further potential genes were detected: *cpox1*, a good candidate for the chanoclavine cyclase, and *cpox2*, a putative dehydrogenase, as well as several yet-unidentified open reading frames (Tudzynski et al. 1999).

Extensive sequencing of the genomic region "left" of the initially found part of the ergot alkaloid gene cluster (Correia, Lübbe and Tudzynski, unpublished results) led to the identification of several new candidates for alkaloid biosynthesis genes (see Fig. 3): two putative monomodular peptide synthetase genes (*cpps2, 3*), one of which might code for the lysergic acid activating enzyme (LPS2, Riederer et al. 1996; see above); one potential P450-monooxygenase gene (*cp450-1*), which could be involved in the last steps of lysergic acid biosynthesis and the final step of ergopeptine biosynthesis (see Fig. 2); and several oxidases (*cpox1, 2, 3*) that could have functions in the early steps of biosynthesis. At the left border of the so far available sequence, a putative "housekeeping" gene encoding an enzyme of the amino acid biosynthesis (isopropylmalate-dehydratase) indicates the "end" of the cluster.

The idea that all these genes might indeed be part of an alkaloid cluster was recently substantiated by expression studies. Northern analysis showed that all of the genes (also the peptide synthetases) are induced in alkaloid-producing cultures of strain P1 (low phosphate) and repressed under high phosphate conditions; preliminary analyses confirm this co-regulation also for pH effects (Lübbe, Correia, Tudzynski, unpublished results). However, gene disruption studies and heterologous expression in *E. coli* or yeast will be necessary to confirm specific functions of these genes in ergot alkaloid biosynthesis.

Functional analysis by targeted gene inactivation

Transformation of *C. purpurea* is now a well-established process and several selection systems are available (see compilation in Tudzynski 1999). Though homologous integration of transforming DNA in the genome is a rare event in *C. purpurea* (~1–2%), gene inactivation by transformation has been achieved by Tudzynski and coworkers in several cases. So far, genes for a cellulase (*cpcel1*; Müller 1996), two xylanases (*cpxyl1/2*; Giesbert et al. 1998), two polygalacturonases (*cppg1/2*; Oeser, Müller, Tudzynski, unpublished results), two catalases and a superoxidedismutase (*cpcat1, 2; cpsod1*; Garre et al. 1998; Moore and Tudzynski, unpublished results); and two MAP kinases (*cpmk1/2*; Mey and Tudzynski, unpublished results) have been successfully knocked out. In most of these cases a gene replacement approach was used. Protoplasts were transformed with a linear DNA fragment containing a selection cassette flanked by 5′ and 3′ parts of the gene, respectively; homologous integration was easily checked by diagnostic PCR. Functional analysis of genes of the ergot alkaloid pathway using this approach is under way (Correia and Tudzynski, unpublished results).

Perspectives

Ergot alkaloids have biotechnological relevance due to their use as medicinal agents and to their role as toxins in agricultural industry. Future research on biotechnological aspects of ergot fungi will therefore most probably focus on the following trends:

Analysis of the biosynthetic pathway at both the enzymatic and genetic level

The recent cloning of the ergot peptide alkaloid gene cluster will allow a functional analysis of all genes involved in ergoline ring synthesis and identification of the corresponding steps by enzymatic analysis of the gene products. The characterization of the various enzymes in

terms of substrate specificity and structure will facilitate the development of strategies for rational drug design of ergoline-related drugs by enzyme engineering and by biocombinatorial approaches, e.g. by introducing unnatural precursors into the ergoline ring system which as yet have failed to become incorporated into the ergoline ring backbone. Improvement of the efficiency of the pathway could also be achieved by the targeted alteration of some of the enzymatic steps in the processing of DMAT to D-lysergic acid. Alternatively, construction of recombinant peptide synthetases containing the D-lysergic acid activating module should be a means to produce recombinant ergot drugs. Preferably, in these compounds D-lysergic acid should be combined with substituents that were difficult to obtain by chemical synthesis, thus leading to new or improved ergoline-derived compounds.

Evaluation of the regulation of ergot alkaloid production

With the availability of the ergot alkaloid biosynthesis gene cluster from *C. purpurea*, it will become possible to determine the regulatory mechanisms directly at the molecular level. This will be of importance for the improved biotechnological production of alkaloids in genetically engineered, alkaloid high-producing strains of *Claviceps*.

Evolutionary aspects

There is considerable interest in the evolution of the ergot alkaloid biosynthesis pathway, which in its complete state leads from tryptophan to the ergopeptines. In many fungi, this pathway is incomplete, ending at the stage of clavine alkaloids, e.g. agroclavine, while in others – predominantly *C. purpurea* – the pathway is complete, i.e. the full set of genes is operative. Molecular techniques will allow questions such as whether the genes involved in ergot alkaloid assembly were acquired by lateral gene transfer (e.g. from fungus to plant or vice versa) to be addressed. Furthermore, analysis for the presence of alkaloid genes would allow determination of whether the ability of higher plants, such as the Convolvulaceae, to synthesize ergoline alkaloids is a true characteristic of these plants or whether these compounds are produced by as yet unknown endophytes.

Ergot alkaloids have gained considerable agricultural relevance not only due to contamination of cereals with sclerotia of the genus *Claviceps*, but also because of ergopeptine-producing endophytes that are involved in the intoxication with ergot drugs of animals held on pasture grassland in many parts of the world. These endophytes, mainly of the genus *Acremonium* (living intercellularly on grasses such as *Festuca*), cause serious problems in livestock breeding. It is therefore of great importance to understand the regulation patterns of alkaloid biosynthesis in such fungi as well as the environmental factors governing the outbreak of these toxins. Strategies for the control of these fungal epidemics could be developed on the basis of the molecular genetics of alkaloid biosynthesis.

In summary, detailed biochemical and molecular genetic research covering the various aspects of ergot alkaloid-based drug development and control of toxin production in the environment will continue to be of great importance.

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