

Reinhard Jetter *Editor*

# The Formation, Structure, and Activity of Phytochemicals



 Springer

# The Formation, Structure, and Activity of Phytochemicals

# Recent Advances in Phytochemistry

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Reinhard Jetter  
Editor

# The Formation, Structure, and Activity of Phytochemicals

Volume 45



Springer

*Editor*

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# Preface to the 45th Volume of the Recent Advances in Phytochemistry Series

Welcome to the fifth volume since the reintroduction of the *Recent Advances in Phytochemistry (RAP)* series, an annual journal of the Phytochemical Society of North America. *RAP* is dedicated to publishing both review and primary research articles for a broad audience of biologists, chemists, biochemists, pharmacologists, clinicians, and nutrition experts, especially those interested in the biosynthesis, structure, function, and/or bioactivity of plant natural products. Recurring themes include the evolution and ecology of specialized metabolites, the genetic and enzymatic mechanisms for their formation and metabolism, the systems biology study of their cell/tissue/organ context, the engineering of plant natural products, as well as various aspects of their application for human health. In addition, also new developments in the techniques used to study plant natural products are presented and discussed, for example, for structure elucidation and quantification, for “omic” (genomic/proteomic/transcriptomic/metabolomics) profiling, or for microscopic localization. In short, this series combines chapters from researchers that explain and discuss current topics in the most exciting new research in phytochemistry.

Two main types of articles are published in *RAP*: Perspectives and Communications. The *RAP* Perspectives aim to give a general introduction to a field and an overview of the pertinent literature, as a background for understanding new results from the primary literature and (in many cases) previously unpublished results. These articles may be similar to review articles, but also are intended to present important ideas and hypotheses, and may put forward proposals for interesting new research directions in the field. It is the hope of the Editorial Board that these articles will be of great value to a large audience. The *RAP* Communications focus more on primary data and synthesize only a small number of papers, to showcase particular new advances in a field that will be of interest to a large audience. Articles of both types are typically solicited from prominent members of the Phytochemical Society of North America, based on the content of presentations at the annual meeting. However, the Editorial Board also invites additional Perspectives and/or Communications from selected authors beyond the society’s meeting to give a rounded picture of all “Recent Advances in Phytochemistry.”

All submissions to *RAP* go through a rigorous, external peer review process, overseen by the Editorial Board. *RAP* is indexed together with all other journals published by *Springer*. All *RAP* papers are available not only in the published volume form, but also electronically through *Springer's* online literature services. This marks a significant change from past volumes of *RAP*, and it is the hope of the Editorial Board that this will lead to broader dissemination and greater interest in *RAP*.

This 45th volume of *RAP* includes a total of eight chapters, many, but not all, based on talks presented at the 52nd annual meeting of the Phytochemical Society of North America. As was seen in *RAP* volumes 41–44, these papers span the breadth of plant (bio)chemistry research in North America, which is also indicative of the state of the field worldwide. The first article presented here reviews the 50-year history of the Phytochemical Society of North America, thereby highlighting research milestones of the past decades. The other seven chapters describe the integration of several different approaches to ask and then answer key questions regarding the function of interesting plant metabolites, either in the plant itself or in chemical ecology or human health application.

Two perspectives focus on chemical structure elucidation: Nikolić et al. summarize new findings on the nitrogen-containing compounds found in Black Cohosh (*Actaea racemosa* syn. *Cimicifuga racemosa*), while Ling et al. give an overview of natural products in various *Jatropha* species along with other Euphorbs.

Two other chapters give updates on the biosynthesis of selected plant natural products. In one of them, Umezawa et al. summarize recent progress on *O*-methyltransferases involved in the formation of lignans. Next, Kumar et al. review the biosynthesis and functions of the plant hormone salicylic acid and its derivatives.

Two more perspectives focus on phytochemicals involved in interactions between plants and pathogens or insects. In particular, Chezem and Clay review recent literature on the regulation of enzymes involved in formation of phenylpropanoids and aromatic alkaloids, whereas Jeschke et al. summarize the current understanding of phytochemical metabolism in insect herbivores.

Finally, a communication by Sumarah et al. provides primary data on the isolation of novel fungal endophytes from Eastern white pine (*Pinus strobus*), and on their secondary metabolites enhancing pathogen tolerance of the host tree.

Overall, we are excited to present this broad set of review papers on various aspects of modern Phytochemistry. We hope you will find them to be interesting, informative, and timely. It is our goal that *RAP* will act not only as the voice of the Phytochemical Society of North America, but that it will serve as an authoritative, up-to-date resource that helps to set the standard for thought and research in plant natural products. Enjoy the read!

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# **Chapter 1**

## **A Half-Century of the Phytochemical Society of North America: 1961–2011**

**Stewart A. Brown, Constance Nozzolillo, and Tom J. Mabry**

**Abstract** In this chapter, the history of the Phytochemical Society of North America (PSNA) is reviewed on the occasion of its 50th anniversary. The formation of the PSNA's precursor organization is described, and its transformation to the present society, whose scope and coverage are discussed. The main body of this chapter consists of summaries of the meetings in each of the PSNA's five decades held annually at diverse sites in the USA, Canada, and Mexico, and twice in Europe jointly with the Phytochemical Society of Europe. Other joint meetings have been held with four other cognate societies. The format of the meetings is organized around a symposium topic discussed by several invited speakers, and has usually included contributed papers. The talks have been published first as individual books by the PPGNA and later by the PSNA in the annual series entitled *Recent Advances in Phytochemistry (RAP)*, sometimes replaced more recently by a special issue of *Phytochemistry*. Other publications are briefly discussed: the newsletter, meeting programs, and the website maintained this century by technically knowledgeable volunteer members. Executive functions have also been the responsibility of senior volunteer members. Membership, mostly from the USA, Canada, and Mexico, also includes many foreign phytochemists. A student membership has aimed from earliest days to encourage participation in the Society's activities by young scientists through lower membership and meeting registration fees, competitions with prizes awarded for best oral or poster presentation, assistance with travel expenses to attend meetings, and, in 2007, formation of a Young Members Committee. Awards

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by the Society have been Life Memberships, since 2006 the Phytochemical Pioneer Award to senior North American and foreign phytochemists, and since 1995 the Arthur Neish Young Investigators Award tied to a minisymposium by student members at many of the annual meetings. Tribute is paid to those whose efforts have been responsible for the success and survival of the Society for so many years.

**Keywords** Plant Phenolics Group of North America (PPGNA) • Annual meetings • Publications of the PPGNA • Phytochemical Society of Europe (PSE) • American Society of Pharmacognosy (ASP) • Awards • Officers • Presidents

## 1.1 The Early Days as the Plant Phenolics Group of North America

The last day of August 1961 saw the beginning of a 2-day symposium at Colorado State University, Fort Collins, entitled *Biochemistry of Plant Phenolic Substances*. Organized by Gestur Johnson of the host university and Ted Geissman of the University of California, Los Angeles, it had seven invited speakers, including two from Japan, covering topics on the chemistry, biosynthesis, and animal metabolism of this group of compounds. Although certainly timely and important in furthering interest in this nascent but growing field, the major historical significance of the Fort Collins meeting was that on the second day the assembled specialists in plant phenolics from the USA and Canada held the organizational meeting of the Plant Phenolics Group of North America (PPGNA). An executive committee was formed at this organizational meeting, with Simon Wender of the University of Oklahoma as the first President, Leonard Jurd of the United States Department of Agriculture, Albany, California, as Vice-President, and Victor Runeckles of Imperial Tobacco Company of Canada, Ltd., Montreal, Quebec, as Secretary-Treasurer.

In retrospect, the run-up to the founding of the PPGNA is worth noting. It was not the first such organization globally, as a plant phenolics group had been formed several years earlier in Britain that aroused interest across the Atlantic. In fact, as one of us has outlined in a history of the Phytochemical Society of North America (PSNA) published on the occasion of the Society's 30th anniversary in 1991 (S.A. Brown, *RAP* Vol. 26 pp. 377–393), it was the attendance of Neil Towers of McGill University at one of their meetings and his resulting enthusiasm that was a major factor leading to informal discussions at the Ninth Botanical Congress in Montreal in 1959 about the possibility of forming a counterpart in North America. This idea was explored further by Neil Towers, Runeckles, and Ted Geissman. Concurrently, Eric Conn of the University of California, while on sabbatical leave at Cambridge in the spring of 1960, attended a meeting of the British society with Tony Swain and Jeffrey Harborne. He also thought it would be great to have a similar organization in the USA and Canada, and then when he returned he found that steps were already being taken to start such an organization. When funds became available to Gestur Johnson from the National Science Foundation 2 years later for

a symposium on plant phenolics, it was decided that it should be the venue for launching a plant phenolics organization on this side of the Atlantic. This led to the 2-day meeting discussed at the beginning, with further details to be found in the abovementioned history (pp. 378–381), at which the PPGNA was formally launched.

## 1.2 Evolution to the Phytochemical Society of North America

The transition from the Plant Phenolics Group to the PSNA occurred in three major stages between 1965 and 1967. First, it was formally proposed to the membership. Although these symposia and the associated sessions of original presentations were deemed to have been largely successful, there was some sentiment in the PPGNA almost from the outset that the Society would benefit from extending its coverage to other classes of plant secondary metabolites. This view was by no means universal, but what tipped it in the direction of expansion was a formal proposal adopted at the 1965 meeting that the PPGNA become a phytochemical society. Because of its basic importance and long-term implications the proposal to form such a society was approved by a majority of the membership in a mail vote.

Next, an executive committee and constitution were established. To get the ball rolling Bernie Finkle of the United States Department of Agriculture in Albany, one of the organizers of the 1965 meeting, approached Tom Mabry of the Botany Department at the University of Texas in Austin on behalf of the PPGNA executive about the possibility of his hosting the following year's meeting at that institution. Mabry, an organic chemist who had been engaged to set up a plant chemistry program in that department, had an interest in phytochemicals that extended well beyond phenolics. He had quite recently finished a postdoctoral research project on the identification of betanidin under André Dreiding at the University of Zürich. He concurred with the request, but only if the meeting were to cover different classes of natural products and not just phenolics. This condition, which was in accordance with the decision to expand the scope of the Society, was accepted by the executive.

Finally, a new format for the meetings had to be established. Mabry's initial task was to obtain funding to bring European phytochemists to Austin. Fortunately, W. Gordon Whaley, a Botany Professor and Dean of the College of Natural Sciences at Austin, agreed to provide most of the funding needed for the meeting. The Department also undertook to fund two European visiting professors during the spring of 1966: W.D. Ollis of the University of Sheffield and Dreiding, both of whom agreed to speak at the April 1966 symposium. Holger Erdtman of the Royal Institute of Technology, Stockholm, Sweden, Anders Kjaer and Martin Ettlinger, Royal Veterinary and Agricultural College, Copenhagen, Denmark, N.A. Sørenson, Norway Institute of Technology, Trondheim, Norway, Hans Grisebach, University of Freiburg, Germany, and G. Ponsinet, Institute of Chemistry, Strasbourg, France, provided a further international complement to the list of speakers that also included Frank Stermitz, Colorado State University, Werner Herz, Florida State University,

and Ralph Alston, The University of Texas. The talks were presented in four parts: (1) The role of chemistry in modern biology, (2) nitrogen and sulfur compounds, (3) acetate- and mevalonate-derived compounds, and (4) flavonoids, and they were published as Volume 1 of *Recent Advances in Phytochemistry (RAP)* by Appleton Century Crofts Press. The papers were edited by Mabry, Alston, and Runeckles. Sadly, Dr. Alston (1925–1967) did not live to see this volume in print. It is dedicated to his memory and his many publications in the field of genetics and chemistry of plant secondary compounds.

At this meeting the PSNA was formally established, with appropriate changes to the constitution. On January 1, 1967, it came into legal existence, with Mabry serving as its first President. The post of Secretary-Treasurer of the former PPGNA had been split, with A.J. Merritt becoming the first Secretary and Howard Wright Treasurer.

### 1.3 Scope and Coverage of the PSNA

A definition of the scope and coverage of the Society was offered soon after its formation. In the preface to a 1963 symposium Runeckles described the PPGNA as “*an informal association of scientists of various disciplines, which aims to promote the furtherance of phenolic and related plant constituents as regards their chemistry, biochemistry, and physiological and pathological effects, and the application of such knowledge to industry and agriculture.*” Deletion of “informal” and “phenolic and related” would still today produce a fair description of the PSNA. Since its inception it has concentrated its attention on secondary plant products, as opposed to those of mainstream anabolic and catabolic reactions involving substances not unique to plants, but usually found in the metabolic pathways of animals and microbes.

At that time, secondary plant products were largely ignored and even scorned by many in biochemistry and botany/plant biology, and plant physiology texts made little or no mention of them. They were regarded as of no significance in comparison to the essentiality of the “primary” compounds such as amino acids and sugars, or the intermediates and products of photosynthesis that may be considered to represent mainstream plant biochemistry. But of course, it is the vast array of secondary products that, in addition to the photosynthesis-related metabolites, make the metabolism of plants, these incredibly complex chemical factories, unique and distinctive. Those who published in this field at that time frequently complained about the reluctance of mainstream biochemical journals to accept manuscripts based on research in this field, and of granting agencies to support such research. The founding of *Phytochemistry* as a specialty journal catering to studies of secondary metabolism helped greatly in correcting this bias, but there is no doubt that the existence of the PSNA and its European counterpart, the Phytochemical Society of Europe (PSE), have also made a major contribution to greatly increased recognition of research on plant secondary metabolism in recent decades.

## 1.4 Relations with Cognate Societies

The Society was well into its second decade before it moved formally into relationships with cognate organizations. No scientific society exists in a vacuum, and interaction with cognate organizations leads to very desirable cross-fertilization, often including collaborative research. Recognizing this, the PSNA has met jointly on several occasions with related groups, most prominently twice with the PSE, on both sides of the Atlantic. These meetings have been adjudged generally successful in bringing together phytochemists from two continents, and probably only the factors of distance and expense, together with greater organizational problems, have rendered such joint meetings relatively infrequent. It is clearly desirable to hold more of them in future if possible.

There have also been joint meetings with other cognate societies on several occasions. These have included two meetings each with the American Society of Pharmacognosy and the International Society of Chemical Ecology (ISCE), as well as one joint meeting with the American Society of Plant Physiologists (now the American Society of Plant Biologists), and one with the Mid-Atlantic Plant Molecular Biology Society, all generally regarded as productive and worthwhile. Further mention of these joint meetings can be found below in the accounts of the individual meetings.

## 1.5 Annual Meetings and Publication of Symposia in *Recent Advances in Phytochemistry*

It is, of course, in the interests of a society to hold its meetings in as diverse locations as circumstances permit. A varied environment is thus assured, and members in different localities who might be financially or otherwise constrained from attending meetings far afield are encouraged to participate in more of them. The PSNA has had much success in such diversification. With the great majority of its members resident in the USA the annual scientific and business meetings have naturally been held predominantly in that country. Through 2011 fifteen states have been host to the annual gatherings, several of them more than once (Table 1.1). Four Canadian provinces and three Mexican states have also been meeting sites, in harmony with the North American scope implied in the Society's name. The meeting sites have ranged from the Atlantic at Boston to the Pacific at Vancouver, from the sprawling metropolis of Mexico City to the small community of Cullowhee, North Carolina, in the foothills of the Appalachians, from the Canadian Rockies to the tropical island of Hawaii and the plains of Oklahoma, and from the Cajun atmosphere of New Orleans to the French-Canadian ambiance of Quebec City. But the fact remains that lower population density in most of North America compared to Europe has not permitted meetings more often than annually, and this has imposed some restrictions on meeting site diversity.

**Table 1.1** Meeting sites, symposium titles, and corresponding volume numbers of the recent advances in phytochemistry (*RAP*)<sup>a</sup>

Year	Location	Symposium title	RAP Vol.
1961	Fort Collins, CO	Biochemistry of plant phenolic substances	
1962	Corvallis, OR	Plant phenolics and their industrial significance	
1963	Toronto, ON	Aspects of plant phenolic chemistry	
1964	Norwood, MA	Phenolics in normal and diseased fruits and vegetables	
1965	Albany, CA	Phenolic compounds and metabolic regulation	
1966	Austin, TX	Recent advances in phytochemistry	1
1967	Madison, WI	Phytochemical techniques	2
1968	Tucson, AZ	Phytochemistry and the plant environment	3
1969	Banff, AB	Enzymology and biochemistry of phenolics	4
1970	Beltsville, MD	Structural aspects of phytochemistry	5
1971	Monterrey, Nuevo Leon	Terpenoid chemistry and biochemistry	6
1972	Syracuse, NY	Chemistry and biochemistry of plant hormones	7
1973	Pacific Grove, CA	Metabolism and regulation of secondary plant products	8
1974	Cullowhee, NC	Phytochemistry in relation to disease and medicine	9
1975	Tampa, FL	Biochemical interaction between plants and insects	10
1976	Vancouver, BC	The structure, biosynthesis and degradation of wood	11
1977	Gent, Belgium	Biochemistry of plant phenolics	12
1978	Stillwater, OK	Topics in the biochemistry of natural products	13
1979	De Kalb, IL	Resource potential in phytochemistry	14
1980	Pullman, WA	Phytochemistry of cell recognition and cell surface interactions	15
1981	Ithaca, NY	Cellular and subcellular localization in plant metabolism	16
1982	Ottawa, ON	Mobilization of reserves in germination	17
1983	Tucson, AZ	Phytochemical adaptation to stress	18
1984	Boston, MA	Chemically mediated interactions between plants and other organisms	19
1985	Pacific Grove, CA	The shikimate pathway: recent developments	20
1986	College Park, MD	The phytochemical effects of environmental compounds	21
1987	Tampa, FL	Opportunities for phytochemistry in plant biotechnology	22
1988	Iowa City, IA	Plant nitrogen metabolism	23
1989	Vancouver, BC	Biologically active products of the mevalonic acid pathway	24
1990	Quebec City, QC	Modern phytochemical methods	25
1991	Fort Collins, CO	Phenolic metabolism in plants	26
1992	Miami Beach, FL	Phytochemical potential of tropical plants	27
1993	Pacific Grove, CA	Genetic engineering of plant secondary metabolism	28

(continued)

**Table 1.1** (continued)

Year	Location	Symposium title	RAP Vol.
1994	Mexico City, DF	Phytochemistry of medicinal plants	29
1995	Sault Ste. Marie, ON	Phytochemical redundancy in ecological interactions	30
1996	New Orleans, LA	Food phytochemicals: flavors, stimulants and health promoters	31
1997	Leiden, The Netherlands	Plant communication with the environment	32
1998	Pullman, WA	Phytochemicals in human health protection, nutrition, plant defense	33
1999	Montreal, QC	Evolution of metabolic pathways	34
2000	Beltsville, MD	Regulation of phytochemicals by molecular techniques	35
2001	Oklahoma City, OK	Phytochemistry in the genomics and post-genomics eras	36
2002	Mérida, Yucatan	Integrative phytochemistry; from ethnobotany to molecular ecology	37
2003	Peoria, IL	Secondary metabolism in model systems	38
2004	Ottawa, ON	Chemical ecology and phytochemistry in forest ecosystems	39
2005	La Jolla, CA	Integrative plant biochemistry as we approach 2010	40
2006	Oxford, MS	(No specific topic)	
2007	St. Louis, MO	(No specific topic)	
2008	Pullman, WA	(No specific topic)	
2009	Towson, MD	Biologically active phytochemicals	41
2010	St. Petersburg Beach, FL	Natural solutions to 21st century problems	42
2011	Hilo, HI	The biological activity of phytochemicals	43

<sup>a</sup>Beginning in 1966 the symposia were published as *Recent Advances in Phytochemistry* by the following publishers: Vols. 1–4, 1966–1969 Appleton Century Crofts; Vols. 5–8, 1970–1973 Academic; Vols. 9–33 1974–1998 Plenum; Vols. 34–40 1999–2005 Pergamon; Vol. 41 2009 Springer

Although a society with small membership faces not a few challenges, in one respect it has a distinct advantage. In marked contrast to the mega-meetings of larger societies that can be accommodated only in major cities with attendant high expense, the small size of the PSNA has usually permitted its annual meetings to be held at smaller locations, often on the campuses of universities in small or moderately large communities. Although Boston, Toronto, Vancouver, Tampa, Mexico City, and Montreal have all been sites for meetings, they have been more typically held in such places as Cullowhee in North Carolina, Stillwater in Oklahoma, Pacific Grove in California; Banff in Alberta; Pullman in Washington, and Ithaca in New York. A fairly relaxed and intimate atmosphere has thus typically prevailed.

As can be seen below, the symposia from most of the meetings over the years have also been published in the *RAP* by several publishing firms (Table 1.1). These published volumes have proved to be an important resource for researchers seeking to keep apace of the latest developments in phytochemistry.

### 1.5.1 The First Decade

Beginning in 1962 more symposia were scheduled by the PPGNA: *Plant Phenolics and their Industrial Significance* at Corvallis, Oregon, in August of 1962, *Aspects of Plant Phenolic Chemistry* in Toronto in September 1963, *Phenolics in Diseased Fruits and Vegetables* at Norwood, Massachusetts, in July 1964, and *Phenolic Compounds and Metabolic Regulation* at Albany, California, in August 1965. The first three of these were published by the Society in soft cover, and the 1965 symposium in Albany was the first to appear in hard cover, in a book entitled *Phenolic Compounds and Metabolic Regulation*.

The second meeting of the fledgling PSNA was in August of 1967 at Madison, Wisconsin, to hear six speakers on the symposium topic *Phytochemical Techniques*. These included instrumental methods such as nuclear magnetic resonance, mass spectrometry and gas chromatography, plant tissue culture and chemical methods applied to lignin structure elucidation. The talks were published as Volume 2 of *RAP* by the same press, again under only the general title of *Recent Advances in Phytochemistry* with no mention of the specific subject. The papers were edited by Margaret Seikel and Victor Runeckles.

The third meeting of the PSNA was preceded by the tenth anniversary meeting of the British group at Cambridge University in the spring of 1968, attended by about 130, mostly locals but also by a large contingent of assorted Europeans and at least two members of the PSNA, Conn and Mabry. Participants stayed in almost luxurious quarters: brand new residences of Emmanuel College vacated by the students during their holiday break, where most of the very good meals were provided, and in whose common room the annual dinner, preceded by a sherry reception, was held. The cost of registration was, yes, £4! The scientific sessions were held in the anatomy building down the road from the college. It was at this meeting that one of the present authors (C.N.), who was not yet a PSNA member, was converted from plant physiologist to phytochemist. The revelation of the fundamental role of phenylalanine not only in "primary metabolism" such as protein synthesis but also in "secondary metabolism" such as lignin synthesis and flavonoid pigments in the life of a plant was confirmed by Conn, her tablemate at dinner, discoverer with his student Jane Koukol of the enzyme phenylalanine ammonia lyase (PAL). Later in 1968 the PSNA met at the University of Arizona in Tucson with the topic *Phytochemistry and the Plant Environment*. The 11 talks covering subjects such as stress factors, fungal and insect resistance were published as *RAP* Volume 3 edited by Steelink and Runeckles.

The following year the PSNA met at the Banff School of Fine Arts in Alberta, in a meeting organized by Victor Runeckles and John Watkin that must surely be considered one of the most successful ever. Its symposium title was *Enzymology and Biochemistry of Phenolics*. Advantage was taken of the spectacular backdrop of the Canadian Rockies by adoption of a format analogous to that of the Gordon Conferences, in which the meetings were scheduled morning and evening, with the afternoons left open for informal discussions but also allowing participants to mount

excursions to the many points of interest within easy reach of the meeting site. The talks were later published as *RAP* Volume 4, edited by the organizers, with the first chapter devoted to aromatic amino acid metabolism, the second to PAL, the third to cinnamic acid metabolism, the fourth to flavonoid evolution, the fifth to furanocoumarins, the sixth to the possible role of phenolics in regulation of germination, the seventh and eighth on lignin, and the ninth on major frontiers in phytochemistry.

In October 1970, the meeting organized by Tien Tso in Beltsville, Maryland, was attended by all three of the present authors. Nozzolillo recalls presenting a talk on anthocyanins, whose colors were matched by beautiful red and blue bruises on her face that had resulted from an encounter with a post at the airport! The 11 invited talks were published as *RAP* Volume 5 by Academic Press and edited by Runeckles and Tso, but with the title of the symposium predominant on the cover: *Structural and Functional Aspects of Phytochemistry*. Since the speakers included the eminent plant physiologists Winslow Briggs and Arthur Galston, primary metabolism such as photosynthesis and phytochrome action predominate in the first four chapters. Mabry et al. begin discussion of secondary metabolism in Chap. 5 with betalains. The remaining six chapters are then devoted to various other aspects of phytochemistry.

October was again the month selected for the 1971 meeting in Monterrey, Mexico, on the topic of terpenoids. The nine invited talks were published by *RAP* as Volume 6, edited by Runeckles and Mabry under the title *Terpenoids: Structure, Biogenesis, and Distribution*. The first two chapters are contributions from the Universidad Nacional Autónoma de Mexico, the third from CNRS in France, the fourth, and seventh to ninth from universities in the USA with contribution to one of them by Ernst von Rudloff of the National Research Council of Canada. The sixth by A. Ian Scott et al. of Yale departs somewhat from the theme by discussing indole alkaloids.

### 1.5.2 *The Second Decade*

*Chemistry and Biochemistry of Plant Growth Regulators* was the topic of the symposium organized by Ernest Sondheimer et al. at the 1972 meeting in Syracuse, New York, and the title of *RAP* Volume 7. As was the case for the 1970 meeting, the topic chosen overlaps with the “primary” aspects of plant physiology. These growth hormones, although not essential to basic metabolism and present in vanishingly small amounts, were recognized as nevertheless playing a very important role in plant development. Two chemists from England updated information on structure of the terpenoid gibberellins and abscisic acid, respectively, while an American reported on the purine-derived cytokinins. Three plant physiologists discussed how indoleacetic acid, gibberellin, and ethylene, respectively, function in the plant. R.M. Silverstein’s banquet address on chemical communication among insects, or sex and the single insect, was an indication of the ecological interests of many members that would culminate in the formation of the ISCE a decade later. The

Syracuse meeting was the final one held in the autumn. Volume 7 is dedicated to Dr. Sondheimer (1923–1973), organizer of the symposium, who died before it appeared in print. Fellow member of the organizing committee D.K. Walton aided in the editing process, hence the editors are listed as Runeckles, Sondheimer, and Walton.

The following year, the Society met in August at Pacific Grove, California, on the topic of *Metabolism and Regulation of Secondary Plant Products*. This beautiful site on the shores of the Pacific Ocean was most conducive to immersion in matters phytochemical, since there are no distractions other than its natural beauty. Those attending were saddened by news of the untimely death of Dr. Milton Zucker (1928–1973), who was to have presented the first symposium paper, on PAL, and to whom Volume 8 is dedicated. Le Creasy presented Zucker's paper and completed the manuscript for publication in *RAP*, Volume 8. Reports from three European laboratories are included in the volume: those of Hans Grisebach on flavonoid biosynthesis, Leslie Fowden on nonprotein amino acids from plants, and Terry Galliard on unusual fatty acids in plants. From laboratories in the USA Helen Stafford talked of multienzyme complexes in phenolic biosynthesis, Heinz Floss of alkaloid biosynthesis, Clarence (Bud) Ryan of proteinase inhibitors, and Frank Loewus of biosynthesis of myoinositol. Neil Towers reported on the effect of light on phenylpropanoid biosynthesis in the fungus *Polyporus*. Volume 8, edited by Runeckles and Conn, is the first one published by Academic Press and bears the title of the symposium on the cover.

Mountains, this time the peaceful and enchanting Appalachians, were again the backdrop for a meeting in 1974 organized by James Wallace, a former student of Mabry, in Cullowhee, North Carolina. The symposium dealt with *Phytochemistry as Related to Disease and Medicine*. The star attractions at that meeting were Richard Evans Schultes and Koji Nakanishi, the former for his account of his experiences in the Amazon with the use of hallucinogenic plants by the indigenous peoples, and the latter, in addition to his analysis of azadirachtin, for his skill in magic illusions demonstrated at the annual banquet. James Duke presented extensive data on use of plants in folk medicine. Munroe Wall talked of cannabinoid chemistry and Dietrich Hoffman of the cancer-inducing effects of smoking them. Allergic reactions, teratogenic constituents of potatoes, neurotoxins of certain legumes, tumor-inhibiting compounds, antimicrobial agents from higher plants, and biogenesis of indole alkaloids completed the list of subjects. Plenum Press was responsible for *RAP* Volume 9, the only title given to the book, and the editor was Runeckles. But the show at this meeting was almost stolen by events at the highest level: during the annual banquet all eyes were glued to the television screen for President Richard Nixon's long anticipated address announcing his resignation.

In 1975, the meeting was organized by Dick Mansell and held in Tampa, Florida, with the symposium on *Biochemical Interaction Between Plants and Insects*. The distinguished alkaloid chemist Kurt Mothes from what was then East Germany, to whom the volume is dedicated, was the key speaker at this meeting. He talked of the potential of breeding plants for presence of desired compounds such as morphine. The seven other speakers concentrated on varied aspects of plant–insect interactions. *RAP* Volume 10 was again published by Plenum, but this time the symposium title is featured on the cover. Editing was done by Wallace and Mansell.

Across the continent again to Vancouver for the 1976 meeting that highlighted *The Structure, Biosynthesis and Degradation of Wood*. The 11 symposia, as reported in *RAP* Volume 11, edited by Loewus and Runeckles, first provided an outline of wood structure, followed by two talks on biosynthesis of cell wall polysaccharides and glycoproteins, respectively. Two other speakers then discussed the structure and biosynthesis of lignin and were followed by a talk on chemistry and biosynthesis of lipid polymers such as cutin and their role in pathogenesis. W.E. Hillis from Australia outlined the changes that occur in wood as the tree ages, while the next two speakers described microbial breakdown of polysaccharides and lignin, respectively. One presentation examined defense against insects and pathogens, while the final speaker looked at the utilization of wood chemicals. The annual Society banquet was unforgettable, being held at the most outrageously far-out restaurant those attending had ever seen: the Medieval Inn. With Runeckles presiding in an extravagant period costume, the guests ate their food with only the knife provided and their bare fingers, off plates that were not changed after each “remove” (course), the remnants thereon being scraped into a garbage pail carried around from diner to diner.

The first joint meeting with the PSE mentioned above was organized by Chris Van Sumere of Ghent, Belgium, and was held in that picturesque old city in 1977. The symposium topic was *Biochemistry of Plant Phenolics*, with publication as *RAP* Volume 12, edited by Swain, Harborne, and Van Sumere. A stellar group of 17 speakers from North America and Europe discussed a wide range of topics, including analytical methods, enzymology, biosynthesis, degradation, and medicinal uses of phenolic substances over the 5 days of the meeting. There was no time allotted for contributed oral presentations, but a poster session was held, in which there was notably a participant from behind the Iron Curtain, Alicja Zobel from Warszawa University in Poland. This is of special interest to one of the current authors (S.A.B.) because 9 years later, after a period as a visiting scientist in his laboratory, she became his wife, and a research collaborator until his retirement, as well as serving later as Secretary of the PSNA for 5 years.

The delicious 3-h annual banquet served at the Rector’s reception in St. Pieter’s Abbey near the university, featuring multiple courses and three kinds of wine, may have been the all-time high for this feature of the meetings. Participants had to choose between two equally memorable all-day tours to Bruges or to the Ardennes, the latter with a dramatic boat ride on the river Lesse through the huge caverns of Han featuring artfully illuminated groupings of stalactites (*qui tombent*) and stalagmites (*qui montent*), and a visit on the return trip to the historic battlefield of Waterloo.

Back to the PSNA’s home territory for George Waller’s 1978 meeting in Stillwater, Oklahoma, organized around *Topics in the Biochemistry of Natural Products*. Participants endured the extreme summer heat in that part of the continent, but fortunately air conditioning in the buildings provided a more comfortable environment for the eight speakers and their audience. The symposium was published as *RAP* Volume 13, edited by Swain and Waller. The topics covered included stereochemistry of enzymatic reactions, modes of alkaloid synthesis and the terpenoids. The final two chapters introduce a topic new to the PSNA, that of natural products from

marine algae. The annual banquet consisted of an outdoor barbecue featuring an entire steer on a spit, with entertainment provided by aboriginal singers and dancers. One afternoon and evening was devoted to a cross-state visit to Tsa-La-Gi, a recreated Cherokee village near Taliquah. Performance in the outdoor theatre of "The Trail of Tears," a drama depicting the forced march of the Cherokee from Florida to Oklahoma, was a highlight of the meeting.

A pleasant contrast in temperatures, this time in the pleasant 20 °C+ range, greeted those arriving for the 1979 meeting organized by Robert Kleiman at Northern Illinois University, De Kalb, Illinois, just west of Chicago in the heart of corn and soybean country, the original home of barbed wire! The eight speakers reviewed the potentials of various crops for production of phytochemicals for uses other than as food under the topic: *The Resource Potential in Phytochemistry*. Whereas most speakers approached the subject from a chemical point of view, the dynamic Julia Morton took the ethnobotanical approach to her search for antitumor agents. The symposium was published as *RAP Volume 14*, which is dedicated to founding member Dr. Theodore (Ted) Geissman (1908–1978), and was edited by Swain and Kleiman. The annual banquet was again an outdoor barbecue on the campus grounds. A visit to a mushroom-producing facility nearby provided the additional evidence that corn and soybeans are not the only products of the area!

In 1980, the first and only joint meeting with the American Society of Plant Physiologists was held at Pullman, Washington, with the topic *The Phytochemistry of Cell Recognition and Cell Surface Interactions*. The 11 symposium papers, comprising three categories, are published in *RAP Volume 15* edited by Loewus, newly appointed as the first official Editor-in-Chief, and Ryan. The first chapter provides an overview of the structures and properties of lipopolysaccharides and glycoproteins. Then five chapters discuss specific systems in plants and microorganisms. The third group provides examples of cell recognition and cell surface interactions. The Pullman campus was covered by the grey ash from a recent explosion of nearby Mount St. Helens, a sample of which is still in Nozzolillo's possession. She recalls that the all too common problem of slides jammed in the Kodak Carousel projector in the middle of a precisely timed presentation plagued the speakers—ancient history now!

Back east again in 1981 to the beautiful campus of Cornell University in Ithaca, New York, with symposium presentations featuring *Cellular and Subcellular Localization in Plant Metabolism*. A strong disagreement among some of the participants as to the presence or not of phenolics in the plastids was abundantly evident! The talks were published as *RAP Volume 16*, edited by Creasy and Hrazdina. Primary functions of metabolism in guard cells and in leaves of C<sub>4</sub> plants, and in various cellular structures such as microtubules, plastids, mitochondria, peroxisomes, and vacuoles predominated in the talks by seven of the speakers, and only one discussed the metabolism of a natural product, the cyanogenic glycosides. Geza Hrazdina not only organized the meeting, but he and his wife Helga also acted as gracious hosts to the Society at their beautiful home with spacious grounds on the shore of Lake Seneca at nearby Geneva. Through his involvement in the local wine industry those present were treated to a tour of a winery, complete with sampling, followed by an excursion to scenic Watkin's Glen.

### 1.5.3 *The Third Decade*

In 1982 it was Connie Nozzolillo's turn to host the annual meeting at the University of Ottawa in Canada's picturesque capital. She says that no doubt her background and interests in plant physiology influenced the symposium topic: *Mobilization of Reserves in Seedlings*. Ten leading plant physiologists/phytochemists from Canada, the USA, Ireland, and England, including editor Peter Lea, described changes in membranes, interconversions of seed reserves, and auxin mobilization in germinating seeds, and one speaker detailed the nutritional benefits of eating sprouted seedlings. The talks were published as *RAP* Volume 17, edited by Nozzolillo, Lea, and Loewus. A notable outcome of the meeting, the subsequent formation of the International Society for Chemical Ecology, was strongly influenced by the plant-insect interaction research interest of Nozzolillo's colleagues, entomologist Bernard Philogene and phytochemist John Thor Arnason, and the presence of Gerry Rosenthal. Despite the initial fear that the PSNA would lose members to this upstart group, both societies have prospered ever since.

Heat was again a notable feature at the 1983 meeting in Tucson, Arizona, organized by Cornelius Steelink and Barbara Timmerman. The symposium title was *Phytochemical Adaptation to Stress*, and stress there was aplenty for the visitors from northern climes: Brown remembers all too vividly the record-tying 114 °F (45 °C) reached the day the participants gathered. Widely divergent topics were discussed by the nine speakers, but all were based on the desire to increase yields of both food and medicinal crop plants despite unfavorable environmental conditions. The talks were published in *RAP* Volume 18 edited by Timmerman, Steelink, and Loewus. The annual business meeting and banquet were held at Old Tucson, site of the filming of many western movies. At this event, Neil Towers and Eloy Rodriguez sang a duet, much to the delight of the audience. During an accompanying excursion up Mt. Lemon the dramatic difference in temperature between downtown Tucson and the summit, with its more northern vegetation startlingly different from the cacti below, was a welcome relief.

The 1984 meeting in Boston was organized by the late incomparable Tony Swain and his wife, Gillian Cooper-Driver, with the topic *Chemically Mediated Interactions Between Plants and Other Organisms*. Nine symposium speakers discussed the various aspects of plant responses to pathogenic organisms and herbivores. The final speaker described how plants may communicate with each other when faced with such attacks. The symposium talks were published as *RAP* Volume 19 edited by Cooper-Driver, Swain, and new Editor-in-Chief Conn. The accompanying excursion to the aquarium and tour of the harbor provided a pleasant break. Outstanding memories of the meeting are the security accompanying access to the student residence at the downtown campus of Boston University, and Jonathon Poulton's proficiency at the piano during the annual banquet.

The notable 25th anniversary of the PPGNA and the PSNA, organized by PSNA Secretary George Wagner and Bock Chan, was celebrated in 1985 in Asilomar at Pacific Grove on the California coast, with the symposium topic *The Shikimate*

*Pathway: Recent Developments.* After an opening overview of the pathway by Heinz Floss, ten additional speakers examined various aspects of the biosynthesis of the many substances to which the shikimate pathway and its products give rise. The talks were published as *RAP* Volume 20 edited by Conn. As part of the anniversary celebrations at the annual banquet, founding member Gestur Johnson engaged the audience with his remembrances of the history of the Society and how his interest in phenolic substances arose. Robert Horowitz then spoke on his recollections of a second founding member, the late Ted Geismann. These talks are recorded in the Newsletter of September 1985. To complete the evening's entertainment, Connie Nozzolillo presented a slide show of photos taken at PSNA meetings since 1970.

In 1986, the annual meeting was held for the second time in the Washington DC area, this time at the University of Maryland, College Park, with the topic *Phytochemical Effects of Environmental Compounds*. Housing was available either in the university residence or in nearby hotels and motels. The problems addressed by the ten invited symposium speakers included the effects of aerial pollution by sulfur dioxide, ozone, or polychlorinated biphenyls (PCBs), water and soil pollution by salts, and acidic precipitation on plant growth and development. The role of plants and soil bacteria as remediaters of pollution by herbicides, phenols, and heavy metals was also discussed. The symposium papers are published in *RAP* Vol. 21, edited by two members of the organizing committee, Saunders and Kosak-Channing, and Editor-in-Chief Conn. One afternoon was left free for either a tour of Washington DC or a visit to Baltimore inner harbor. Contributed oral papers and posters were presented by the registrants, including those by five students competing for the two \$250 travel awards. The annual banquet was held during a 3-h cruise on the Potomac River.

In 1987, the first recognition of the new era of genetic engineering was evident in the symposium topic: *Opportunities for Phytochemistry in Plant Biotechnology*. Dick Mansell had again invited the Society to meet at Tampa and shared his enthusiasm for the art of Salvador Dalí with those present. Discussions of the state of the art of biotechnological manipulations of plants showed how secondary compounds, especially the flavonoids, (may) play crucial roles in plant defense against insects and pathogens, whether fungal, bacterial, or viral. Resistance of crop plants to herbicides such as glyphosate (Roundup<sup>®</sup>) may be obtained by manipulation of the genes of the shikimate pathway. Understanding of cell-cell recognition as in pollination is facilitated by the molecular approach. Such studies may use cell cultures, selected mutants, or whole plants. The symposium, published in *RAP* Volume 22 and edited by Conn, was organized by Tsune Kosuge, to whom the volume is dedicated. Dr. Kosuge died before the book appeared in print and a copy was presented to his widow.

The Midwest was again the site of the 1988 meeting at the University of Iowa in Iowa City. The topic, although in an important area, *Plant Nitrogen Metabolism*, was unusual for the Society, but the program also included developments in biotechnology. It began with an overview of the topic and then examined the interactions of symbiotic nitrogen fixation, molecular biology of nitrate reductase, ammonia

assimilation, ethylene production, polyamine metabolism, and alkaloid biosynthesis. The talks are published in *RAP* Volume 23, edited by Poulton, Romeo, and Conn. A dinner cruise on the Mississippi River provided a pleasant contrast to the meeting's prairie setting some 95 km to the west.

The University of British Columbia hosted the Society for a second occasion in 1989, with a symposium focused on the terpenoids: *Biologically Active Products of the Mevalonic Pathway*. Nine speakers detailed the state of current knowledge in various products of the pathway: mono-, di-, and sesquiterpenes, and the enzymes and intermediates involved in their synthesis. One speaker described the use of molecular techniques in the form of cloning of one of the genes involved in the pathway. A marine invertebrate was introduced as a novel source of terpenoids, those important natural products otherwise associated with the plant kingdom. The final speaker discussed biosynthesis of the complex group of plant growth hormones called gibberellins. The symposium is published as *RAP* Volume 24, edited by Towers and Stafford, newly installed as Editor-in-Chief. The salmon barbecue at the elegant faculty club was undoubtedly a highlight of the meeting. Late-departing registrants were treated to a post-meeting tour of the noted TRIUMF (Tri-University Meson Facility) on campus. This facility contains the largest cyclotron in the world and was very much involved in the discovery of the Higgs boson in 2012. Its co-founder and director for 13 years, Dr. Erich Vogt (1929–2014), was a distinguished Canadian-born physicist.

Next year the meeting site stayed north of the border, but this time in francophone Canada at Laval University in St. Foy, a suburb of Quebec, one of North America's oldest cities. This 1990 meeting was held jointly with the ISCE, a society with roots in the 1972 Syracuse meeting of the PSNA, as mentioned above. There were two symposia each with eight speakers, but with no overlap in time of presentations. That of the PSNA, organized by Klaus Fischer and President-Elect Murray Isman, was *Modern Phytochemical Methods*. The latest developments in analytical techniques: various types of column chromatography, electrophoresis, mass spectrometry, nuclear magnetic resonance and separations using artificial membranes, as well as techniques for isolation from specialized plant structures such as trichomes were described. The talks are published in *RAP* Volume 25, edited by Fischer, Isman, and Stafford. Registrants were taken by bus from the Laval campus into the old walled city of Quebec for the annual banquet at the historic Chateau Frontenac Hotel. No other excursions were planned for registrants, since facilities for experiences such as cruises on the St. Lawrence River or tours of the area are well provided by the local tourist industry.

Coming full circle, the 1991 meeting returned after 30 years to Fort Collins with the symposium organized by Ragai Ibrahim on the same topic, *Phenolic Metabolism in Plants*, as that at the first annual meeting of the group at that same site. Eleven speakers to the topic detailed advances in determining localization in the plant and in knowledge of the enzymatic activities of phenolic metabolism together with advances in molecular biology and genetics. New understanding of the complex chemistry of lignin and of hydrolysable and condensed tannins was also described. The talks are published in *RAP* Volume 26 edited by Stafford and Ibrahim. This volume is dedicated to Hans Grisebach (1926–1990) for his outstanding contribu-

tions to the study of phenolics chemistry, especially that of the flavonoids. It includes in addition the first published history of the Society since its beginnings 30 years before, written by Stewart Brown.

#### **1.5.4 The Fourth Decade**

A posh hotel on Miami Beach, Florida, was the site of the second joint meeting with the PSE in 1992, with *Phytochemical Potential of Tropical Plants* as the topic, and organized by Kelsey Downum and John Romeo. Eleven symposium papers with emphasis on the chemical properties of various tropical species, in relation to medicinal use for human ailments or in providing resistance to plant pathogens and pests such as insects, were presented by authorities from both sides of the Atlantic. The talks, published in RAP Volume 27, edited by Downum, Romeo, and Stafford, were a delight for the classical chemist, but molecular aspects received minimal attention. As Eloy Rodriguez reported, the human use of such plants by indigenous peoples may have resulted from observation of wild apes as they sampled plants for uses other than their nutritional value. This meeting had a catastrophic postscript: Hurricane Andrew, which struck the area shortly after the meeting finished and will live in the memory of all those who attended. Devastation of the Fairchild Botanic Garden, where a 1-day session had been held, was especially severe, with almost total destruction of invaluable and possibly irreplaceable collections of palms and cycads. The home of incoming President Downum in nearby Homestead was severely damaged, and he had no electric power or telephone contact for weeks afterwards.

Compensation for the deficiency in biotechnology at the 1992 meeting was provided at the 1993 Asilomar meeting, where *Genetic Engineering of Plant Secondary Metabolism* was the order of the day. It was the third time the Society had met on this section of the beautiful California coast. Eleven speakers described their work on the biotechnology of alkaloid, glucosinolate, lignin, or monoterpene biosynthesis, anthocyanin coloration of flowers and introduction of novel shades, antibiotic production in *Streptomyces*, and modification of phenolase. Equally important was a discussion of evaluation of the environmental impact of genetically engineered plants. The papers are published in RAP Volume 28, edited by Stafford.

After a previous meeting elsewhere in Mexico, its capital Mexico City hosted the PSNA in 1994. The symposium topic was in line with the interests of the Mexican membership and the increasing interest in herbal science in North America: *Phytochemistry of Medicinal Plants*. Aspects covered in the 13 presentations by four speakers from Mexico, three each from Europe and the USA, two from Canada and one from Chile were methodology, phytochemistry, pharmacognosy, and pharmacology of plants with known medicinal uses. The talks are published in RAP Volume 29 edited by PSNA president Arnason, newly appointed Editor-in-Chief Romeo, and future President Mata. Two poster sessions were needed to accommodate the 71 posters brought by the registrants, but conference time was allowed for

only 17 oral contributions. Two afternoons were left free, the first for a conducted tour of the campus and the second for a tour of the botanic garden followed by the annual banquet in the historic Hotel de Genève. This hotel was the mailing address given by Russell Marker, co-founder of Syntex, when he submitted his famous papers on the semisynthesis of progesterone from Mexican *Dioscorea* phytosteroids that led to development of the birth control pill.

For the 1995 meeting at the Canada–USA border in Sault Ste. Marie, Ontario, the forestry interests of the region were close in the background in selection of the topic: *Phytochemical Diversity and Redundancy in Ecological Interactions*. The aim was to point out the variety of plant chemical defenses against biological stressors such as insects, fungi, and large herbivores. To this end, 12 speakers discussed bioassay techniques, and described the roles of terpenoids, phenolics, and glucosinolates, among other diverse phytochemicals, in plant resistance to herbivory or disease, or postulated on the role of such compounds in evolution. The talks are published in RAP Volume 30. At this meeting also, the idea proposed by Kelsey Downum of instituting a mini-symposium to be presented by young scientists was first realized. The topic selected, *Current Aspects of Plant Molecular Biology*, provided the contrast necessary to draw as many participants as possible to the meeting. Six invited speakers gave an insight into the possibilities of genetic engineering of the pathways of synthesis of important natural products. Talks from both symposia are published in RAP Volume 30 edited by Romeo et al. The handsome logo for the meeting, created by local First Nations artist Zoey Wood-Salomon, symbolized the strategic position of the Sault at the juncture of Lakes Michigan, Huron, and Superior. An evening cruise through the canals separating Canada from the USA, complete with buffet on the ship, was enjoyed by all on board. Several participants also took advantage of the invitation of the local organizer, Mamdouh Abou-Zaid, to tour the forestry building in which his laboratory was situated. The annual banquet was held in the hotel and featured speeches by President Arnason, incoming President Fischer and James Saunders. Here the Pergamon Medal, presented for only the second time to a North American, life member Eric Conn, nominated by the PSNA, was awarded by Dr. Helen McPherson who had come from England expressly for that purpose.

More French flavor, this time of the Cajun variety, was experienced in New Orleans, as the 1996 meeting continued in a predominantly “classical” tradition with the topic *Food Phytochemicals: Flavors, Stimulants and Health Promoters*, a most fitting topic in view of the many celebrated local restaurants, which the registrants enjoyed sampling. Twelve invited speakers examined the topic from many perspectives: chemical structure before and after consumption, as promoters of health and disease prevention, as natural food colorants, as flavor constituents, their localization, and as subjects of genetic manipulation. A mini-symposium by established phytochemists Neil Towers, David Seigler, John Arnason, and Jim Saunders was chaired by incoming President Rachel Mata. It was followed by a round-table discussion chaired by Kelsey Downum examining the future of phytochemistry. The talks were published in RAP Volume 31, edited by Romeo et al. and entitled simply *Functionality of Food Phytochemicals*. The colorful logo for the meeting, featuring

a New Orleans Mardi Gras mask and local fruits and vegetables, was designed by graduate student Steven Robbs. Following the annual business meeting the usual banquet was held on the *Creole Queen*, a paddle-wheeler, as it cruised the Mississippi.

The second joint meeting with the PSE in Leiden, The Netherlands, the following year, 1997, again primarily catered to the classical chemical approach with the topic *Phytochemical Signals and Plant-Microbe Interactions*. Ten invited speakers, four from the USA, discussed conventional studies of antifeedants, antipathogens, allelopathy, plant signaling, and *Agrobacterium tumorigenesis*, but two others introduced the use of molecular techniques to the problems of insect resistance and *Striga* parasitism. There was no time allotted for oral contributions, but 64 posters were set up for examination by the registrants. The talks are published as *RAP* Volume 32, with royalties equally shared with the PSE. The meeting was very poorly attended by PSNA members, probably because of the prohibitive cost, and as a result there was no annual business meeting.

The return to Pullman in 1998 with the topic: *Phytochemicals in Human Health Protection, Nutrition, and Plant Defence* continued in the primarily “classical” mode with the exception of talks by Richard Dixon and Rodney Croteau on molecular controls and pathway engineering. Sixteen invited speakers from seven countries discussed a range of natural products from bryophytes over coniferous and deciduous trees to herbaceous flowering plants. Presentation of the talks was divided into sessions coordinated with 49 accompanying oral presentations, plus ten in the best student oral presentation category. Three poster sessions were necessary to enable presentation of the 71 posters, 15 in the student best poster contest. Registrants were disappointed by the absence due to illness of the 17th speaker, flavonoid pioneer Jeffrey Harborne. The talks are published in *RAP* Volume 33 edited by Romeo et al. The conference began, in the words of Secretary Dennis Clark, “with a wonderful salmon barbecue accompanied by an excellent selection of wines and microbrew beers.” At the annual banquet held in Spokane high tribute was paid to Phytochemical Pioneer Neil Towers in honor of his 75th birthday, for which members of his family had driven from Vancouver. He also delivered the final symposium talk after the dinner, and his youngest daughter entertained the diners with a dance performance.

Further in accordance with the Society’s policy of diverse meeting sites, the 1999 meeting was held across the continent in the cosmopolitan city of Montreal in the middle of a jazz festival. The topic *Evolution of Metabolic Pathways* encompassed the molecular interests of the organizers Ragai Ibrahim and colleagues, as well as the more classical approach. The first of the regular Arthur Neish mini-symposia on the topic of biochemistry and molecular biology of brassinosteroid hormones was also presented by four young investigators. The 12 more senior invited speakers explored a wide range of subjects, including the role of secondary metabolites in evolution, the evolutionary origin of polyketides and terpenes, oxidative reactions and evolution of secondary metabolites, the evolutionary origin of substitution reactions and structure–function relationships of steroid sulfonation. Papers from both symposia can be found in *RAP* Volume 34 edited by Romeo and the organizers

of the symposia, which was published for the first time by Pergamon Press. The volume is dedicated to the memory of Arthur Neish (1916–1973), a renowned Canadian scientist and a pioneer in the synthesis and use of radioactive tracers in biosynthetic studies.

In 2000, the annual meeting returned to Beltsville, Maryland, where it was held jointly with yet another cognate group, the Mid-Atlantic Plant Molecular Biology Society. Not surprisingly, the symposium topic, organized by PSNA member Saunders and M-APMBS President Matthews, was entitled *Regulation of Phytochemicals by Molecular Techniques*. The four Neish mini-symposium young investigator speakers were invited to address the more specific topic of *Regulation of Terpenoid, Monoterpene Indole Alkaloid, Flavonoid and Anthocyanin Pathways*. The talks explored various aspects of the topic, including development of tools for the study of gene expression in plants, genetic modifications of secondary metabolites and the effect of such modifications on the environment, pharmaceutical production in plants (“farmacy”) and the use of plant viruses to modify plant gene expression. The papers are published by Pergamon Press (Elsevier) in *RAP* Volume 35, edited by the meeting organizers and Editor-in-Chief Romeo. Virginia Walbot was the invited after-dinner speaker at the annual banquet.

Meetings over the next few years continued to emphasize a wide range of phytochemical subjects. The 2001 meeting in Oklahoma City, organized by Richard Dixon et al., continued the molecular aspect with a symposium entitled: *Phytochemistry in the Genomics and Post-Genomics Era*, a topic intended to provide information on how best to handle the massive amount of data obtained by molecular means in order to solve phytochemical problems. Over the course of 4 days, 24 speakers, including 4 from Europe, presented their studies in six specialized sessions. One afternoon was left free for an organized tour prior to the annual business meeting and banquet, but after-dinner speaker Joanne Chory continued discussion of the topic by describing genetic approaches to brassinosteroid biosynthesis. One evening was devoted to presentations by the three invited young researchers in the Neish mini-symposium on the non-molecular subject of *Phytochemical Synthesis and Analysis*. Posters were presented in two afternoon sessions, but time permitted only five contributed oral talks.

Symposium talks from this meeting are published in *RAP* Volume 36 edited by Romeo and Dixon. In the words of the Elsevier advertisement in the October 2002 PSNA Newsletter, “Contributors to this timely volume explore a wide range of topics that include:

- Bioinformatics and computational biology
- Metabolomics as a component in functional genomic studies
- Metabolic profiling
- Biopanning by activation tagging
- Functional genomics of cytochromes P450 and their role in biosynthesis
- Sequence-based approaches to alkaloid biosynthesis gene identification
- Structurally guided alteration of biosyntheses”

### 1.5.5 *The Fifth Decade*

The theme of the 2002 meeting in Merida on Mexico's Yucatan peninsula returned to an emphasis on the chemistry and biology of the major groups of "secondary" compounds with the view that it is essential to maintain a perspective on their function and the ability to analyze them. Hence the title of the symposium was *Integrative Phytochemistry: from Ethnobotany to Molecular Ecology*. The meeting opened with a classical guitar concert provided by nine young men. The symposium was divided into four sessions devoted, respectively, to phenolics, terpenes and glucosinolates (each five speakers), and alkaloids (four speakers) plus an evening session devoted to presentations by four young researchers invited for the Neish mini-symposium, on this occasion on the same topic. To quote from President Loyola Vargas' letter in the October 2002 Newsletter, "The subject of the talks ranged from ethnobotany and drug discovery through biological diversity and molecular biology of secondary metabolites. The mini-symposium provided an example of how new lines of research can provide solutions to the search for new compounds with biological activity." The talks are published by Plenum Press in *RAP* Volume 37 edited by Romeo. A break from air-conditioned lecture rooms came in the form of a guided tour of the local botanic garden one afternoon, with a return to the hotel for the annual banquet that evening.

The following year, 2003, the meeting was held in Peoria, Illinois, with the symposium organized by Mark Berhow under the topic *Secondary Metabolism in Model Systems*. It entailed five such systems: *Arabidopsis*, maize, legumes, rice, and fungi. In addition, there was a symposium on new techniques, and the mini-symposium focused on the chemistry of insect control and how secondary metabolites affect plant-insect interaction. There were three speakers for each category of the major symposium and five young researchers. Their talks are published in *RAP* Volume 39 edited by Romeo and Berhow. Registrants stayed in the historic Hotel Pere Marquette and attended sessions in the nearby Peoria Community Centre. For relaxation, all were invited prior to the annual banquet to watch the local baseball team inaugurate their brand-new stadium.

In 2004, the PSNA returned to the University of Ottawa to meet with the ISCE, with the theme *Chemical Ecology and Phytochemistry of Forests*. The same Ojibway artist was again commissioned to produce a symbol for the program cover and the souvenir shopping bag, complete with an explanatory text. The mini-symposium given by five young researchers dealt with the wine grape, and a tasting of Ontario's Niagara wines, hosted by sommelier Debra Inglis from McMaster University, followed during the evening poster session. The main symposium centered on the interactions of plants with their insect herbivores, with 12 speakers comprising a mix of phytochemists and chemical ecologists. The talks are published in *RAP* Volume 39 edited by Romeo. A cruise on the Ottawa River provided a pleasant break before the annual banquet held at the venerable Chateau Laurier Hotel, at which Neil Towers gave his usual inspiring, and sadly his last account of his adventures in the field (literally) of phytochemistry. The ISCE program was run

concurrently, and after-dinner presentations were made to ISCE members at the same dinner following Neil's talk.

The topic for the 2005 meeting at the Salk Institute in La Jolla, California, was *Integrative Plant Biochemistry* and was published as *RAP* Volume 40, the final one with Elsevier as publisher and edited by Romeo. There were six symposium sessions into which the talks of the invited Neish Award young investigators were also integrated. The sessions were entitled: Metabolic Networks, Temporal and Spectral Regulation of Metabolism, Biosynthesis and Signaling of Secondary Metabolites, Translational Opportunities in Plant Biochemistry, and Lipids, Fatty Acids and Related Metabolites. As a relief from such intense mental activity, registrants were given an afternoon off to go to the zoo and/or the beach, and afterwards to attend the annual banquet. Travel awards to students had been presented at an earlier buffet.

The 2006 meeting at the University of Mississippi, "Ole Miss," in Oxford was the first not to have a major symposium topic for later publication in the *RAP* series. Instead, there were five major themes with a limited number of invited speakers. Presentations of contributed papers were mixed in with those of the invited speakers, including invited young investigators. The intention was to make the meeting as attractive as possible to satisfy as wide a variety of interests as possible. By rearrangement, the proceedings of the meeting were published in a special edition of *Phytochemistry* with no financial benefit to the Society. The numerous problems it faced, especially the question of low membership, were discussed at length at the business meeting, and several new committees were set up to relieve the load of duties for the traditional members of the Executive Committee. It was also proposed to lengthen the 1-year term of the incoming president to 2 years. At the annual banquet, the society recognized the contributions of the late Neil Towers as well as the very much alive Stewart Brown and Nikolaus Fischer by bestowing on each the newly established title of Phytochemical Pioneer. It may be noted here that an *in memoriam* recognition of devoted service to the Society on the part of Past President J.W. (Jerry) McClure (1933–2006) was published in the November issue of the Newsletter.

A 2-year term for incoming President Norman Lewis was approved at the 2007 meeting, held in St. Louis, Missouri. There were 18 invited speakers from 12 countries for a symposium on a broad range of subjects again designed to attract as wide an audience as possible. In addition, six young investigators were invited to participate in the mini-symposium. The sessions were held in the Donald Danforth Plant Science Centre opened in 2001, and Dr. William Danforth, chairman of the board of Directors of the Centre, gave the opening remarks. A new policy of having poster presenters give a short talk was introduced at this meeting and has been continued at following meetings. At the annual banquet three additional members were designated Phytochemical Pioneers: Frank Loewus, Eric Conn, and Helen Stafford, but only Frank and his wife Mary could attend. Frank wrote a full description of his long career for the February 2008 edition of the *Newsletter* including the fact that his wife joined his research program in 1971 once their children were in school and stayed until retirement in 1990. For this reason both Frank and Mary were called to the microphone when the award was presented.

The 2008 meeting was held for the third time on the campus of Washington State University at Pullman, organized by President Lewis with a wide variety of topics, but with no plans for publication. Discussions were initiated at the business meeting for a return to a PSNA publication to replace or revive *RAP*. Again, a wide range of subjects was discussed as indicated by session headings such as: Phytochemicals and Human Health, Plant Cell Wall Assembly, Metabolomic Biochemistry, Bioinformatics, Metabolic Engineering, Natural Products for Pest Management. In addition, the Tannin Group formerly associated with the ACS had been invited to participate, and so a session on Plant Phenol/Tannin biochemistry was included. An afternoon was devoted to brief oral presentations by those presenting posters in two evening sessions. The annual banquet was held in downtown Pullman at the Old Post Office Wine Cellar and Gallery, followed by presentation of several awards: five students received a Frank and Mary Loewus Travel Award, two students received Best Poster Presentation Awards, John Romeo became a Life Member of the PSNA, Nancy Terrier from France was recipient of the Arthur Neish Young Investigator Award, Takashi Yoshida from Japan received the Tannin Award, and Helen Stafford (in absentia) and Ulrich Matern from the University of Marburg, Germany, were recipients of the Phytochemical Pioneer Award.

The 2009 meeting was held across the continent on the campus of Towson University in Towson, a suburb of Baltimore, Maryland. The meeting topic, and title of the regenerated series, *RAP* Volume 41, was *Biologically Active Phytochemicals*, published by Springer and the final volume with Romeo as Editor-in-Chief. Four young scientists were invited as mini-symposium speakers, although there was no mini-symposium per se. Four senior scientists were also invited speakers, including James Duke as keynote speaker. Dr. Duke kindly invited the audience to a tour of his herbal garden at Fulton, asking a friend to provide a background of classical guitar for that occasion. It seems worthy of mention that this was the third PSNA meeting organized by James Saunders, surely a record! Jim has been a faithful member, secretary, and past president of the PSNA since his early '70s days as graduate student of Jerry McClure at Miami University, Ohio.

The meeting moved southward to Florida in 2010 and was held jointly for the second time with the American Society of Pharmacognosy at the elegant Tradewinds Grand Resort in St. Petersburg. The meeting theme was *Natural Solutions to 21st Century Problems—from Discovery to Commercialization*. Symposia topics such as Natural Products in Agriculture, Biodiversity, Drug Discovery-Problematic Diseases, Natural Products: Ecological Roles and Tritrophic Interactions, Metabolism and Metabolomics, and Metabolomic Engineering and Biotechnology indicate the wide diversity of interests of the two societies. Altogether there were over 500 registrants, 50 oral presentations in six sessions and over 300 posters in two sessions. At the annual banquet the ASP presented awards to three of its members, and the PSNA presented the Phytochemical Pioneer title to Meinhart Zenk. Four young researchers who had been invited to deliver talks in the mini-symposium were also honored at the banquet. Relaxation from the busy schedule was provided by a beach party on the resort decks and a free afternoon for touring local sights.

Celebration of the 50th anniversary of the founding of the Society was held at the Fairmont Orchid Hotel in Hawaii in December 2011, an unusual time given that most previous meetings had been held in the summer or autumn months, but always

in more temperate climates. In light of the unusual topography of the islands and their distance from mainland North America, for the first time a pre-conference field trip was offered as well as mid-conference tours. The local host was John Pezzuto, who was also a part of the eight-member organizing committee headed by Lewis and aided by over 30 additional scientific advisors of widespread global distribution. Over 500 attended the meeting, including over 60 researchers invited to speak on various aspects of phytochemistry. Immediately after the traditional welcome Eric Conn, a PSNA Pioneer, gave an address: "PSNA: Some reflections," which included his memories of the founding of the PSNA and the thrust of his own research. He was followed by H.R.H. Princess Chulabhorn Mahidol of Thailand on that nation's phytochemical diversity and promise, Mansukh C. Wani, co-discoverer of taxol and camptothecin, Koji Nakanishi who did seminal work on ginkgolides, and Daneel Ferreira on proanthocyanidin/polyphenols. As the program stated, "together, they provide wonderful examples of the importance of our remarkable phytochemical treasures, much of which has only been discovered in the last 50 years."

Over 50 awards, 40 of them for travel thanks to the grant-obtaining successes of President Cecilia McIntosh and Lewis, were presented at the annual banquet. Six of the awards were for best posters, of which 80 had been entered in competition. One award each was presented to the undergraduate and master's students, and two awards each to doctoral and postdoctoral students. As well, four young investigators received the Neish Award and Richard Hemingway was inaugurated as a Phytochemical Pioneer. As Editor-in-Chief of *Phytochemistry*, Norman Lewis arranged for a special issue of that journal based on papers presented at the meeting. As well, a new volume of the resuscitated *RAP* was published by Springer, *RAP* Volume 42 entitled *The Biological Activity of Phytochemicals* and edited by David Gang with assistance from six associate editors.

The authors hope that this rather extensive summary of the annual meetings will give a fair indication of the scope of the subjects that have occupied the PSNA's attention since its original inception as the PPGNA. The evolving nature of the discipline over the Society's history is evident from the fact that some of the later areas of research covered were unknown or only in their infancy in the 1960s. We read of cellular and subcellular localization of phytochemicals, plant biotechnology, genomics, genetic engineering, integrative phytochemistry and allelopathic interactions, all of them subjects that would have scarcely been envisaged 45 years ago. There can be no question that currently unheard-of topics will be symposium titles over the next half-century.

## 1.6 Publications of the PSNA Other Than *RAP*

### 1.6.1 *Newsletter*

From early in its history and continuing to this day, a Society Newsletter has been of major importance in maintaining contact with the membership, especially in view of the wide geographical distribution of its members and the prospect of

meetings no more frequently than annually. The first of these was published in about 1970. It had very humble early beginnings under its first editor, Secretary Helen Stafford, and its publication was rather irregular, but it succeeded in its cohesive effects, and these were enhanced under subsequent editors, usually the Society Secretary. The Newsletter has been published without interruption except for an hiatus about 2005, when there was no issue for over a year. It has been particularly useful in providing advance information about the annual meetings and subsequent reports thereof, and also carried the program for each meeting, complete with abstracts. As time passed and postal rates increased, the Newsletter no longer carried full details of the program which thus was no longer circulated to every member of the Society. It is currently also available in pdf form on the PSNA website.

### **1.6.2 Meeting Programs**

Programs distributed in advance of a meeting are always essential in encouraging attendance. Like other scientific societies, the organizing committees of the Society's meetings prepare a printed program, originally as part of the Newsletter as mentioned above, but later as a cost-saving measure, for distribution only to registrants attending the meetings. However, with the development of electronic communication, meeting programs can again be published in advance of the meeting on the Web. The printed programs have evolved in sophistication, but as there is unfortunately no archival collection the abstracts of the contributions, whether oral in the early days, or more as posters today, can be found only in private collections or, for more recent meetings, on the Society website.

### **1.6.3 Special Issues of *Phytochemistry***

In recent years there have been several special editions of *Phytochemistry* devoted to papers presented at annual meetings, no doubt facilitated by Past-President Lewis' position as editor of this journal.

### **1.6.4 Website**

The advent of the Internet has of course brought about a major change in communication with both the Society's membership and other interested parties. For over a decade now there has been a website at <http://psna-online.org/> for those seeking information about its organization, meetings, current activities, and other aspects. The registration and securing of the domain name was done by Peter Facchini of the

University of Calgary in 2000 while serving as the Secretary for the PSNA. David Bird, while a student of Dr. Facchini, created the first website to go public by the PSNA. It currently provides a link to the Old PSNA website with additional material from the earlier site, including the Society's constitution and bylaws, names of all past officers, and past meetings and symposia, an electronic newsletter archive back to 1996, publications including *RAP*, and other material. Those seeking further material about the Society are directed to this source.

## 1.7 The Executive Committee

Throughout the history of the Society its functions have been managed by unpaid members elected or appointed by the membership. At the beginning of the PPGNA, the Executive Committee (EC) consisted only of three positions: the President, the Vice-President, and a Secretary-Treasurer. After the first year, the post of Past-President was added. Since the earliest days there has been a provision in the constitution that the Vice-President automatically assumes the office of President the following year, and since 1986 the President has been restricted to one term (of 1 year) of office. Upon formation of the PSNA, the dual duties of Secretary-Treasurer were separated and, in 1980, an Editor-in-Chief was appointed for the *RAP* Volumes; thus the executive committee increased to the present six members. For a listing of the editors up to 2011 see Table 1.2. The first Editor-in-Chief, Frank Loewus, died in 2014. He and his wife Mary lived into their nineties and spent many productive years together as researchers. In addition to editing the symposia papers, usually assisted by the organizer(s) of each conference, the duties of the Editor-in-Chief include negotiating a contract for publication of the *RAP* Volumes. To date, following previous publishers Appleton Century Crofts and Academic Press, Plenum Press (Elsevier) and Springer have been added to the list. The series was temporarily suspended at Volume 40 following the 2005 meeting in La Jolla, but resumed with Volume 41 following the 2009 meeting. To provide a broader perspective of phytochemistry, the format of the volume has been changed from the previous one, which contained only chapters on the symposia delivered at the meeting. Volume 42 and subsequent volumes now consist of two aspects: perspectives or reviews of selected phytochemical topics and presentation of research results on a specific topic.

Later, an Advisory Committee was formed to advise on such matters as the annual meetings and the membership. Currently there are several standing committees established to lighten the work load of the unpaid EC: PSNA Membership and Society Advancement, PSNA Website, PSNA Newsletter, PSNA Awards, PSNA Editorial and Proceedings, PSNA Future Meetings, PSNA Young Members, PSNA Guidelines and Procedures, and PSNA Fundraising. The reader is again directed to the Society's website for details of past officers of the PSNA and the PPGNA, and other aspects of its administration and organization.

**Table 1.2** Officers of the society,<sup>a</sup> 1961–2011

Plant phenolics group of North America			
Year	President	Secretary-treasurer	
1961	Simon Wender	Victor Runeckles	
1962	Leonard Jurd	Victor Runeckles	
1963	Stewart Brown	Victor Runeckles	
1964	Margaret Seikel	Victor Runeckles	
1965	Bernard Finkle	Victor Runeckles	
Phytochemical society of North America			
Year	President	Secretary	Treasurer
1966	Tom Mabry	A.J. Merritt	Howard Wright
1967	Victor Runeckles	Sarah Clevenger	Howard Wright
1968	Bruce Bohm	Cornelius Steelink	Howard Wright
1969	Peyton Teague	Cornelius Steelink	Howard Wright
1970	Tien Tso	Helen Stafford	Jerry McClure
1971	Eric Conn	James Wallace	Jerry McClure
1972	Kenneth Hanson	James Wallace	Jerry McClure
1973	Neil Towers	James Wallace	Richard Mansell
1974	Heinz Floss	Constance Nozzolillo	Richard Mansell
1975	Frank Loewus	Constance Nozzolillo	Richard Mansell
1976	Jerry McClure	Constance Nozzolillo	Richard Mansell
1977	Helen Stafford	Constance Nozzolillo	Richard Mansell
1978	George Waller	Constance Nozzolillo	John Romeo
1979	Le Creasy	Constance Nozzolillo	John Romeo
1980	Cornelius Steelink	James Saunders	John Romeo
1981	Constance Nozzolillo	James Saunders	John Romeo
1981	Geza Hrazdina	James Saunders	John Romeo
1983	Ragai Ibrahim	James Saunders	Jonathan Poulton
1984	Richard Mansell	George Wagner	Jonathan Poulton
1985	David Loomis	George Wagner	Jonathan Poulton
1986	Neil Towers	George Wagner	Jonathan Poulton
1987	John Romeo	Helen Habermann	Jonathan Poulton
1988	David Seigler	Helen Habermann	Jonathan Poulton
1989	Jonathan Poulton	Helen Habermann	Kelsey Downum
1990	Brian Ellis	Helen Habermann	Kelsey Downum
1991	Murray Isman	Helen Habermann	Kelsey Downum
1992	James Saunders	Helen Habermann	Susan McCormick
1993	Kelsey Downum	Alicja Zobel	Susan McCormick
1994	John Arnason	Alicja Zobel	Susan McCormick
1995	Nikolaus Fischer	Alicja Zobel	Susan McCormick
1996	Rachel Mata	Alicja Zobel	Susan McCormick
1997	<sup>b</sup>	Alicja Zobel	Susan McCormick
1998	Vincent De Luca	Dennis Clark	Cecilia McIntosh
1999	Susan McCormick	Dennis Clark	Cecilia McIntosh
2000	Richard Dixon	Peter Facchini	Cecilia McIntosh

(continued)

**Table 1.2** (continued)

Plant phenolics group of North America			
Year	President	Secretary-treasurer	
2001	Hector Flores	Peter Facchini	Cecilia McIntosh
2002	Victor Loyola-Vargas	Peter Facchini	Charles Cantrell
2003	Daneel Ferreira	Peter Facchini	Charles Cantrell
2004	Clint Chapple	Mark Berhow	Charles Cantrell
2005	Clint Chapple	Mark Berhow	Charles Cantrell
2006	Norman Lewis	Mark Berhow	Franck Dayan
2007	Norman Lewis	Mark Berhow	Franck Dayan
2008	Mark Bernards	Soledade Pedras	Franck Dayan
2009	David Gang	Soledade Pedras	Franck Dayan
2010	Charles Cantrell	Soledade Pedras	Franck Dayan
2011	Cecilia McIntosh	Eric Johnston	Daniel Owens

<sup>a</sup>Beginning in 1980 the Editor of the *RAP* was included as an officer in the Executive Committee: 1980–1983 Frank Loewus, 1984–1987 Eric Conn, 1988–1994, Helen Stafford, 1995–2009 John Romeo, 2010 Mark Bernards, 2011 David Gang

<sup>b</sup>No business meeting

## 1.8 Membership

Membership in the Society has always been open to anyone interested in phytochemistry. In the early days of the PPGNA, there were about 100 members, a number which gradually grew to at most 500. Membership has, of course, been predominantly North American, but there has always been a substantial number of members from outside the continent, mostly from Europe and Japan, and these have frequently participated in the annual meetings, both as invited speakers in the symposia and as authors of contributed papers. Fees have always been held at the lowest level necessary to cover expenses such as mailing costs. Advantages of membership include a reduced registration fee at the annual meeting, a reduced cost of *RAP* volumes, and a reduced subscription rate to the flagship journal *Phytochemistry*.

A worrisome decline in membership began in the early 1980s. Successive Newsletters contained annual pleas of the President addressed to the membership that they should each spread the word of the Society's worthy mission and encourage their colleagues and students to join and present the results of their research at the annual meetings. A gradual increase in numbers of paid-up members in recent years and the ongoing success of the annual meetings indicate that their efforts have not been in vain.

The Society in 2011 had a total of 442 members. Of these, 190 were from the USA, 53 from Canada and 12 from Mexico. Of those outside North America, it is notable that no fewer than 129 were from the Asia-Pacific region. Forty-four were Europeans. There were 123 student members.

Undergraduate and graduate students have been encouraged to join at an early stage in their careers. Student fees are low, and further inducement was provided by the early institution of travel assistance awards, and best poster and best oral presentation

awards, further to encourage their participation in the annual meetings. Students must apply and compete for these awards. To help attract younger scientists to the Society, a Young Members Committee was set up at the business meeting in 2007 and had its first event in 2008 in the form of an “Ask the Editor” panel discussion with advice on how to get a paper published.

To encourage the development of the younger generation of phytochemists, the Arthur C. Neish Young Investigator Awards, to which we have referred earlier, are given by the PSNA each year. Such an award was first proposed by Kelsey Downum in 1993. Selected young investigators present their research by invitation only at the annual meeting, frequently as part of a mini-symposium. As mentioned above, there was a preliminary trial, organized by Vince de Luca, in 1995 at the meeting in Sault Ste. Marie. The problem of funding had to be addressed, however, and donations towards a fund sufficiently large that interest payments would sustain the program were sought. This objective was partially reached by 2000. Despite the funding difficulties, the first regular Neish mini-symposium was held in 1999 and has been held every year since, except at La Jolla in 2005 and at Towson in 2009. Funding is still being sought to reach the desired capital goal of \$50,000. Over 40 young phytochemists, whose names can be found by visiting the Society’s website, have been so honored through 2011. The theme of the mini-symposium always differs from that of the main symposium, in order to enhance the attractiveness of the program to prospective participants. Recently, the custom of presenting these young speakers with a Neish Award at the annual banquet has been established.

## 1.9 Awards to Members of the PSNA

The PSNA has established, at different times, two categories of awards for its members recognizing outstanding contributions to the field and to the Society. Over the course of its existence, 15 members of the PSNA have been awarded life memberships in recognition of outstanding contributions to the Society and to phytochemistry: Stewart Brown, Eric Conn, Ragai Ibrahim, Gestur Johnson, Leonard Jurd, Norman Lewis, Frank Loewus, Tom Mabry, Constance Nozzolillo, John Romeo, Victor Runeckles, Helen Stafford, Neil Towers, Tien Tso, and Simon Wender.

Later, in 2006, the practice was initiated of making Phytochemical Pioneer Awards to North American and foreign phytochemical pioneers, nine of whom have been honored through 2011. In the first year, Neil Towers (posthumously), Stewart Brown and Nikolaus (Klaus) Fischer were the recipients, and they were followed in 2007 by Eric Conn, Frank Loewus (since deceased), and Helen Stafford (since deceased), in 2008 by Ulrich Matern, in 2010 by Meinhart Zenk (since deceased), and in 2011 by Richard Hemingway.

Moreover, the Phytochemical Society Pergamon award has also been presented on occasion to PSNA members. The first such award was presented to the late Edward Leete in 1992. It was presented again in 1994 to Eric Conn, in 1998 to Tom Mabry and later, posthumously, to Neil Towers.

## 1.10 An Appreciation

In conclusion, the authors would be remiss to refrain from expressing their appreciation for the tireless and devoted work that so many of their colleagues in the PSNA have contributed to the success of its first half-century. The present authors themselves have all served on the executive committee at some time in the course of those 50 years, and have also been involved in other aspects of the Society's operations. At those times and during the intervals, they have continually witnessed the invaluable contributions of many among the membership toward making the PSNA work and make its contribution to phytochemistry. In addition to their paid duties, usually as university professors or government research officers with the attendant "Publish or Perish" atmosphere, these individuals spent untold hours in deliberations aimed at devising means to keep the Society viable. Without the devoted volunteer service of successive Executive Committees the Society would not have survived. These dedicated workers deserve the lasting gratitude of the Society. A complete listing of the officers of the PPGNA and the PSNA in the first half-century is presented in Table 1.2.

## Chapter 2

# Nitrogen-Containing Constituents of Black Cohosh: Chemistry, Structure Elucidation, and Biological Activities

Dejan Nikolić, David C. Lankin, Tamara Cisowska, Shao-Nong Chen,  
Guido F. Pauli, and Richard B. van Breemen

**Abstract** The roots/rhizomes of black cohosh (*Actaea racemosa* L. syn. *Cimicifuga racemosa* [L.] Nutt., Ranunculaceae) have been used traditionally by Native Americans to treat colds, rheumatism, and a variety of conditions related to women's health. In recent years black cohosh preparations have become popular dietary supplements among women seeking alternative treatments for menopausal complaints. The popularity of the plant has led to extensive phytochemical and biological investigations, including several clinical trials. Most of the phytochemical and biological research has focused on two abundant classes of compounds: the triterpene glycosides and phenolic acids. A third group of phytoconstituents that has received far less attention consists of the alkaloids and related compounds that contain nitrogen. This chapter summarizes the current state of knowledge of the chemistry and biological activities associated with this group of constituents and provides some perspective on their significance for future research on this interesting plant.

**Keywords** Black cohosh • *Cimicifuga racemosa* • Triterpene glycosides • Alkaloids • MassBank • Guanidino alkaloids • Cimipronidine • Arginine •  $\gamma$ -Guanidinobutyric acid • Acylated arginines • Isoquinoline alkaloids • Salsolinol • Benzylisoquinoline alkaloids • Reticuline • Aporphine alkaloids • Magnoflorine • Protoberberine alkaloids • Protopine • Indole alkaloids •  $\beta$ -Carbolines • Monoamine oxidase •  $N_{\omega}$ -Methylserotonin • 5-HT<sub>7</sub> receptor • Pictet-Spengler reaction • Methylcimetrypazepine • Cinnamides • *N*-Feruloyl arginine • *N*-Feruloyl tyramine glucoside • Choline • Betains • Trigonelline

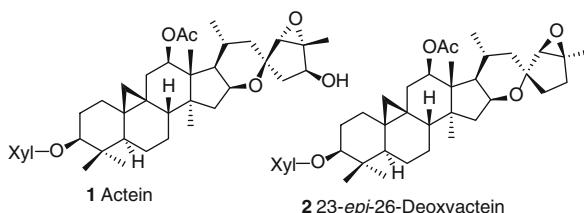
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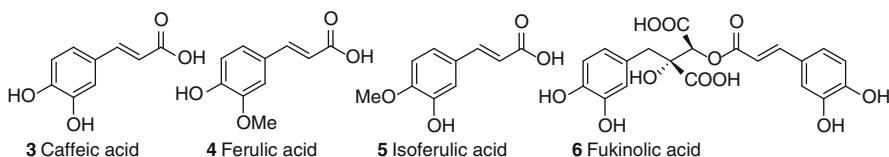
## 2.1 Introduction

Black cohosh (*Actaea racemosa* L. syn. *Cimicifuga racemosa* [L]. Nutt., Ranunculaceae) is a flowering plant native to North America. The roots/rhizomes have been used traditionally by Native Americans to treat colds, rheumatism, and a variety of conditions related to women's health [1]. Because of the risks associated with hormone replacement therapy, black cohosh preparations have become popular dietary supplements among women seeking alternative treatments for menopausal complaints [2, 3]. The most recent sales figures place black cohosh as seventh best-selling herb with annual sales of approximately \$17 million [4]. The popularity of the plant has led to extensive phytochemical and biological investigations, including numerous clinical trials, some of which date back to the 1950s. Despite such extensive research, the clinical efficacy of black cohosh products remains controversial [2]. Early studies suggested that black cohosh extracts were effective in reducing the frequency and intensity of hot flashes among premenopausal and postmenopausal women [5–8], while several recent trials including a Phase II double-blind placebo-controlled trial conducted at the authors' UIC/NIH Center for Botanical Dietary Supplements Research demonstrated no vasomotor symptoms benefits [9–12]. Clinical efficacy is not the only controversy surrounding this plant. The question of long-term safety of black cohosh came to light after initial reports of liver failure allegedly associated with the use of black cohosh had appeared in the literature [13]. The concern was serious enough to warrant two workshops organized by the NIH Office of Dietary Supplements to discuss issues related to safety of black cohosh. A recent review sponsored by the US Pharmacopoeia summarized 30 cases of liver damage associated with black cohosh use and recommended that black cohosh products carry a cautionary statement that they may adversely affect the liver [14]. In contrast, several recent reviews of randomized controlled clinical trials concluded that there is no evidence of hepatotoxicity [15–18], and recent in vitro and animal studies seem to support this conclusion [19, 20].

When it comes to phytochemical investigations, the past several decades of research have focused almost exclusively on two abundant classes of compounds: triterpene glycosides and phenolic acids. Triterpene glycosides represent the major constituents of all hydroalcoholic black cohosh extracts and have been extensively studied from both the phytochemical and biological side. More than 40 triterpenes have been isolated and structurally characterized to date [21, 22]. They represent a particular analytical challenge due to their close structural similarities and overall complexity of distinguishing these structures, which include numerous stereocenters. The most abundant triterpenes, particularly actein (**1**) and 23-*epi*-26-deoxyactein (**2**), are often used as markers for the standardization of black cohosh preparations [23–25]. Extensive reviews on the chemistry, rational naming system, and biological activities of the triterpenes have been published recently [22, 26, 27].



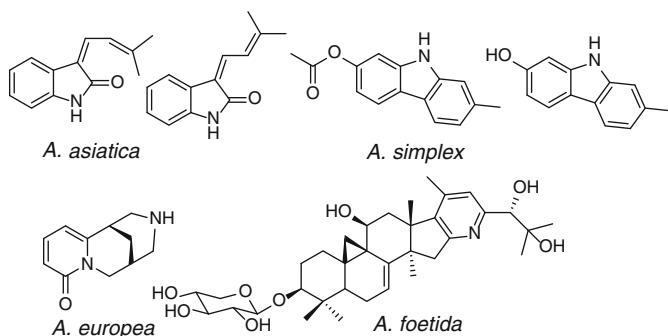
The major phenolic constituents of black cohosh are the hydroxycinnamic acids, caffeic acid (**3**), ferulic acid (**4**), and isoferulic acid (**5**), as well as their condensation products with glycoloyl phenylpropanoids, commonly known as cimicifugic acids (e.g., fukinolic acid (**6**)). Numerous members of this class have also been isolated and fully characterized, and a rational naming system has been proposed [22, 28, 29].



A third group of phytoconstituents that has received far less attention consists of the alkaloids and related compounds that contain nitrogen. These constituents represent a largely unexplored part of the black cohosh metabolome, and most of the work in this area has been confined to our laboratories in the UIC/NIH Center for Botanical Dietary Supplements Research. This review summarizes the current state of knowledge of the chemistry and biological activities associated with this group of diverse constituents and provides some perspective on their significance for future research on this interesting and widely used medicinal plant.

## 2.2 Historical Overview

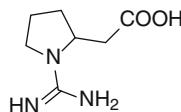
It is somewhat surprising that the alkaloid metabolome of black cohosh has been overlooked for so long, considering that from a chemotaxonomic perspective the Ranunculaceae family is a well-known producer of alkaloids. The authoritative review on alkaloids written by Cordell et al. [30] lists Ranunculaceae as the third-most producing family, with more than 1,500 alkaloids isolated. Sporadic and inconclusive reports of the presence of alkaloids in black cohosh can be traced back to the beginning of the last century [27, 31]. However, due to changing taxonomic classification and varying degrees of documentation for some of those reports, it was not always clear whether the tested species was indeed *A. racemosa*. One of the first notable clues that black cohosh may contain alkaloids can be found in a report by Crum et al. who tested different parts of the plant using five alkaloid detection reagents and concluded that seeds contained alkaloids but not roots/rhizomes [32].



**Fig. 2.1** Reported alkaloids from *Actaea* species

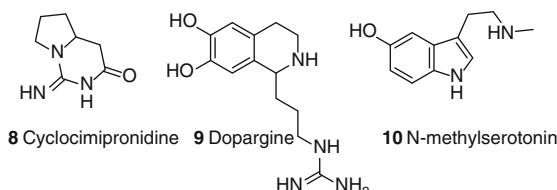
No identification of a specific compound was mentioned in that early report. Scattered reports of the presence of alkaloids in other *Actaea* species have been published over the years, and some examples of reported alkaloids and their sources are shown in Fig. 2.1 [33–35].

The first report on the isolation and full structural characterization of an alkaloid in black cohosh was published in 2005 by Fabricant et al., who reported on the identification of a new cyclic guanidine-type alkaloid named cimipronidine (**7**) to reflect its source (*Cimicifuga racemosa*), the(homo)proline base structure, and the occurrence of a guanidine group [36].



**7** Cimipronidine

This was an interesting discovery not only because of the novelty of the structure (new chemical entity with seven carbon atoms), but also because guanidine alkaloids are relatively rare in higher plants. Furthermore, cimipronidine was the first reported guanidine alkaloid from the Ranunculaceae family. It turned out that cimipronidine was not an odd occurrence, as it was subsequently revealed that guanidine alkaloids and other compounds containing a guanidino group represent a significant part of the nitrogenous metabolome of black cohosh. Subsequently, two other guanidine alkaloids cyclocimipronidine (**8**) and dopargine (**9**) were isolated and structurally characterized [37].



Importantly, the identification of highly basic guanidino compounds led to modifications in the separation procedure which allowed the effective separation of the complexes of the alkaloids with abundant phenolic acids and enabled effective dereplication procedure to be carried out [28]. This process led to the identification of *N<sub>ω</sub>*-methylserotonin (**10**) as the active serotonergic principle [38]. Most recently, extensive mass spectrometric investigations led to conclusive or tentative identification of 73 nitrogenous primary or secondary metabolites [39]. This study unequivocally confirmed that alkaloids are well represented in the black cohosh metabolome.

## 2.3 Structure Elucidation by Mass Spectrometry

Of all the currently known nitrogenous metabolites of black cohosh, only **7**, **8**, and **9** were isolated and fully characterized using full sets of mass spectrometry and 1D and 2D NMR spectroscopy data; the rest was identified or tentatively identified by a mass spectrometry-based dereplication approach that will be described here in more detail.

Detection of alkaloids by mass spectrometry is relatively straightforward. The presence of basic nitrogen allows for sensitive detection using positive ion electrospray ionization. In addition, the presence of a basic center fixes the initial charge site, which allows for easier interpretation of the tandem mass spectra. Another benefit of the basic nitrogen is that molecular species observed upon electrospray ionization are typically protonated molecules. However, one should be mindful that some alkaloids contain quaternary nitrogen. Thus, if the analyst routinely subtracts a proton to arrive at the molecular formula, quaternary alkaloids can be easily missed. Indeed, we have encountered many such cases in black cohosh, which were easily solved once a possibility of quaternary nitrogen was considered.

The dereplication process starts with the determination of the elemental composition by accurate mass measurement. The determination of the correct formula is the critical step in the process, since an incorrect formula will ultimately lead to a wrong assignment. The validity of the elemental composition can be established using additional criteria such as the isotope pattern, the elemental composition of fragment ions, as well as general plausibility based on general knowledge of natural product chemistry and biosynthetic relationships. The latter point should not be underestimated as the analyst ultimately has to reconcile the mass spectrometry data with what is realistically possible (or probable) in nature. There is ample discussion in the literature regarding determination of elemental composition using accurate mass measurement, and the interested reader is referred to those publications for more details [40, 41].

The molecular formula and the product ion spectra can be searched in the SciFinder and Reaxys databases as well as in the MassBank ([www.massbank.jp](http://www.massbank.jp)) database of tandem mass spectra. If a match is obtained in the MassBank database, the final confirmation of compound identity can be achieved by comparing the retention time and fragmentation pattern with those of authentic standards. This

cautious approach considers the well-known fact that different types of instruments often generate different product ion spectra of the same compound, which is one of the main obstacles in the development of the standardized databases similar to those obtained by electron ionization [42]. During our work on black cohosh, the MassBank database was of limited utility and was useful mostly for identification of primary metabolites. For compounds for which there were no spectra in the MassBank database, the hits obtained in the SciFinder or Reaxys databases provided clues on possible structures. As alkaloids are comparatively rare natural products, database searches usually return only a handful of possible structures that need to be considered. We observed that SciFinder provided a somewhat broader coverage since it revealed compounds that have been identified or putatively identified in plants, but not necessarily derived from formal isolation. Based on the interpretation of product ion spectra and taking into account all available information, a proposed structure can be tested by comparison with an authentic standard. This iterative process is repeated until a conclusive assignment can be made. One challenging aspect of elucidating compounds from black cohosh was that database searches in many instances returned no hits, suggesting an entirely new structure. This necessitated a tedious process of de novo interpretation of tandem mass spectra to come up with a plausible structure that could be compared with authentic standards, most of which had to be synthesized.

As mass spectrometry does not have the ability of de novo structure elucidation of unknown compounds, the proposed structural assignment always comes with a degree of uncertainty [43]. In other words, the assignment can be made with various degrees of confidence. How to best grade the level of confidence in the assignment is a subject of active discussion in the metabolomics community [44]. The most commonly used nomenclature is that proposed by Sumner et al. [45], which assigns three levels of confidence. Identification at level 1 is established by comparing the retention time and fragmentation pattern of an unknown with those of an authentic standard. This level of evidence provides the highest degree of confidence in the assignment and is a widely accepted criterion for positive identification of compounds not only in the research domain but also in forensic and regulatory areas. Some compounds were identified at level 2 by comparing their product ion tandem mass spectra either with published spectra or with tandem mass spectra of close structural analogs. Comparison of tandem mass spectra of close chemical analogs is a viable approach commonly used in drug metabolism or chemical degradation studies. The basic idea behind this approach is the long-established “shift technique” pioneered by Klaus Biemann [46, 47]. This approach relies on the correct interpretation of tandem mass spectrum of a template molecule and tries to assign where the structural difference(s) is(are) with the unknown. Perhaps fittingly, the original approach was developed to determine the structure of alkaloids.

Finally, for level 3 characterization, only a chemical class of the unknown compound can be ascertained based on the similarity of its tandem mass spectrum relative to known compounds belonging to the same class. Because direct comparison with the authentic standard is not accomplished, identifications at level 2 and 3 should always be considered tentative. Even if the measured spectrum of the

unknown matches a published spectrum, without comparison of retention times under identical conditions, there is always a possibility that the two compounds may just be close structural analogs. Indeed, there are many classes of compounds where fragmentation pattern is dominated by ions from one portion of molecule producing nearly identical spectra for compounds with different structures. It is also important to point out that the strength of analytical evidence is not related to the novelty of the compound. Indeed, many of the compounds discussed in this review identified at level 1 are new natural products, for which synthetic standards were prepared after interpretation of the mass spectrometric data. If a natural product is identical to the synthetic standard regarding retention time and mass spectral characteristics, it may still not be identical due to differences in stereochemistry. Table 2.1 lists the mass spectrometric data for all of the compounds discussed in this review. The table is adapted from Nikolic et al. [39] and includes data for new compounds reported here for the first time. For easier referencing, the compounds are listed in the order in which they are discussed in the text. In the following sections, the black cohosh alkaloids with guanidine (2.4), isoquinoline (2.5), indole (2.6) structures will be discussed, together with the accompanying cinnamides (2.7), cholines and betains (2.8), as well as other primary and secondary products (2.9).

## 2.4 Guanidino Alkaloids

The presence of a substantial number of guanidino compounds is one of distinguishing features of the black cohosh metabolome. Cimipronidine (7), a cyclic guanidine alkaloid, was the first isolated alkaloid from black cohosh [36]. Guanidine alkaloids are quite rare in nature, and it is interesting to find a plant with such extensive diversity of this class of compounds.

Guanidino compounds are readily detected by mass spectrometry. The highly basic guanidine moiety allows for very sensitive detection using electrospray ionization. A molecular formula with three or more nitrogens is a strong indicator for the presence of a guanidino group, as there are few other possibilities that can account for so many nitrogens in the molecule, particularly if the molecular mass is modest (<400 Da). During collision-induced dissociation, acyclic guanidines display a characteristic loss of neutral guanidine ( $-59$  Da;  $\text{CH}_5\text{N}_3$ ). Accurate mass measurements are particularly useful to distinguish this loss from other isobaric losses of 59 Da, such as elimination of acetamide ( $\text{CH}_3\text{CONH}_2$ ) originating from an acetylated amino group or trimethylamine originating from a quaternary nitrogen. In addition to the loss of neutral guanidine, protonated guanidine ( $\text{CH}_6\text{N}_3$ ) of  $m/z$  60 may be observed, albeit only at low collision energies. Formation of protonated guanidine is thought to proceed via an ion-neutral complex. When collision energy is low, the ion-neutral complex survives long enough for proton transfer to occur; otherwise the complex simply dissociates without proton transfer [48]. It should also be noted that, for many small guanidine molecules, no useful tandem mass spectra could be obtained with collision energies above 25 eV using a quadrupole/time of flight instrument.

**Table 2.1** Mass spectrometry data for compounds identified in 75 % ethanolic extract of roots/rhizomes of black cohosh

No.	$m/z$ [M+H] <sup>+</sup>	Formula	Error (ppm)	Major fragments <sup>a</sup>	Identification	Level
<b>7</b>	172.1086	$C_7H_{13}N_3O_2$	0.0	172.1086(100); 154.0972(64); 137.0704 (18); 130.0863(70); 119.0611(15); 112.0762(25); 95.0568(21); 94.0538(16); 70.0657(60)	Cimipronidine	1 <sup>b</sup>
<b>8</b>	154.0978	$C_7H_{11}N_3O$	-1.3	154.0978(100); 112.0768(20); 95.0547(6); 94.0656(16); 70.0676(16); 67.0512(10)	Cyclocimipronidine	1 <sup>b</sup>
<b>9</b>	265.1673	$C_{13}H_{20}N_4O_2$	3.0	206.1178(100); 137.0613(18); 70.0560(10); 60.0448(5)	Dopargine	1 <sup>b</sup>
<b>10</b>	191.1192	$C_{11}H_{14}N_2O$	4.2	160.0745(100); 148.0725(18); 132.0824(22); 115.0588(20)	$N_o$ -Methylserotonin	1
<b>11</b>	175.1191	$C_6H_{14}N_4O_2$	-1.5	175.1191(12); 158.0938(12); 130.0978(75); 116.0693(35); 112.0840(18); 71.0493(25); 70.0653(100); 60.0565(10)	Arginine	1
<b>T12</b>	132.1127	$C_5H_{13}N_3O$	-7.6	132.1127(100); 90.0960(15); 73.0617(12); 60.0570(7); 55.0575(8)	$\gamma$ -Guanidino butanol	2 <sup>c</sup>
<b>13</b>	146.0925	$C_5H_{11}N_3O_2$	-3.4	146.0969(100); 128.0856(20); 111.0585(12); 104.0720(12); 87.0440(40); 86.0601(35); 69.0307(7); 60.0570(6)[15 eV]	$\gamma$ -Guanidino butyric acid	1
<b>T14</b>	130.0971	$C_5H_{11}N_3O$	-6.9	130.0971(60); 112.0872(30); 71.0502(12); 70.0660(100); 60.0570(7) [15 eV]	$\gamma$ -Guanidino butyraldehyde	2
<b>15</b>	160.1081	$C_6H_{13}N_3O_2$	-3.1	160.1098(70); 128.0857(5); 118.0834(5); 101.0598(100); 8 6.0602(5); 59.0504(9)	$\gamma$ -Guanidino butyric acid methyl ester	1
<b>16</b>	174.1244	$C_7H_{15}N_3O_2$	0.6	174.1244(100); 146.0953(9); 132.1046(9); 128.0857(11); 1 5.0753(73); 87.0432(85); 86.0611(12)[15 eV]	$\gamma$ -Guanidino butyric acid ethylester	1
<b>T17</b>	160.1077	$C_6H_{13}N_3O_2$	-5.4	160.1077 (10); 101.0026(100); 100.0532(88)	$\delta$ -Guanidinovaleric acid	2
<b>T18</b>	174.1243	$C_7H_{15}N_3O_2$	0.0	174.1244 (100); 132.1034(8); 115.0762 (78); 100.0771(8); 73.0659(18); 55.0568(15)	$\delta$ -Guanidinovaleric acid methyl ester	2
<b>19</b>	203.1144	$C_7H_{15}N_4O_3$	0.0	203.1144(100); 186.0820; 175.1172 (50); 158.0940(30); 144.0661(45); 143.0809(10); 130.1028(10); 116.0690(20); 112.0845(15); 98.0640(18); 71.0493(10); 70.0653(30); 60.0660(6) [15 eV]	$\alpha$ -N-Formyl arginine	1

<b>20</b>	217.1297	C <sub>8</sub> H <sub>16</sub> N <sub>4</sub> O <sub>3</sub>	-1.8	217.1297(9); 175.1200(22); 158.0935(100); 130.0978(17); 116.0693(60); 115.0881(35); 112.0840(50); 74.0231(18); 71.0505(32); 70.0653(90); 60.0565(5)	α-N-Acetyl arginine	1
<b>21</b>	279.1461	C <sub>13</sub> H <sub>18</sub> N <sub>4</sub> O <sub>3</sub>	1.4	279.1465(43); 262.1194(34); 220.0967(74); 219.1130(72); 216.1135(10); 201.1028(19; 175.1249(9); 174.0921(11); 173.1082(19); 159.0781(8); 122.0607(16); 113.0716(13); 105.0342(100); 70.0659(30)	α-N-Benzoyl arginine	1 <sup>e</sup>
<b>T22</b>	168.1128	C <sub>8</sub> H <sub>13</sub> N <sub>3</sub> O	-5.4	168.1128(100); 112.0768(13); 94.0658(6); 70.0678(12); 67.0512(4)	N-Methyl cyclocimproniidine	2
<b>23</b>	180.1017	C <sub>10</sub> H <sub>13</sub> NO <sub>2</sub>	-4.4	180.1017(3); 163.0752(12); 151.0727(10); 145.0651(46); 117.0698(100); 115.0540(95); 105.0962(8); 91.0541(20)	Salsolinol	1
<b>24</b>	166.0869	C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>	0.6	166.0869(18); 149.0608(18); 137.0618(100); 121.0641(17); 121.0649(90); 103.0530(28); 91.0560(37); 77.0390(12)	Norsalsolinol	1
<b>25</b>	178.0882	C <sub>10</sub> H <sub>11</sub> NO <sub>2</sub>	7.9	178.0882(100); 176.0704(5); 163.0645(44); 162.0566(54); 160.0765(5); 137.0614(11); 135.0453(16); 117.0354(8); 115.0569(8); 89.0410(4)	1,2-Dehydrosalsolinol	1
<b>26</b>	314.1749 [M <sup>+</sup> ]	C <sub>19</sub> H <sub>24</sub> NO <sub>3</sub> <sup>+</sup>	-2.2	314.1749(100); 271.1338(10); 269.1193(50); 239.1040(10); 237.0931(46); 211.1048(15); 209.0978(28); 192.1030(15); 175.0763(35); 151.0752(10); 145.0646(18); 143.0534(18); 137.0611(18); 107.0484(40)	Magnocurarine	1
<b>T27</b>	272.1287	C <sub>16</sub> H <sub>17</sub> NO <sub>3</sub>	0.0	255.0926(20); 237.0890(40); 161.0591(30); 143.0712(30); 115.0583(37); 107.0503(100); 77.0449(18)	Norcoclaurine	2 <sup>d</sup>
<b>28</b>	330.1719	C <sub>19</sub> H <sub>24</sub> NO <sub>4</sub>	4.2	330.1720(6); 299.1310(6); 267.1067(6); 192.1037(100); 177.0803(11); 175.0783(20); 143.0502(20); 137.0599(24); 115.0526(10)	Reticuline	1

(continued)

Table 2.1 (continued)

<b>T29</b>	314.1750 [M <sup>+</sup> ]	C <sub>19</sub> H <sub>24</sub> NO <sub>3</sub> <sup>+</sup>	-1.9	314.1767(100); 271.1332(10); 269.1196(50); 239.1010(1) 0; 237.0925(25); 211.1153(5); 209.0938(20); 192.1030(18) ; 175.0801(15); 145.0646(15); 143.0513(14); 137.0603(10); 115.0569(20); 107.0506(40)	Isoner of magnocurarine (oblongine)
<b>30</b>	342.1705 [M <sup>+</sup> ]	C <sub>20</sub> H <sub>24</sub> NO <sub>4</sub> <sup>+</sup>	0.0	342.1725(28); 299.1301(11); 297.1129(84); 282.0892(34); 279.1039(8); 265.0876(100); 237.0920(30); 222.0700(10); 219.0804(14); 209.0981(8); 207.0806(8); 191.0882(10)	Magnoflorine
<b>T31</b>	342.1702 [M <sup>+</sup> ]	C <sub>20</sub> H <sub>24</sub> NO <sub>4</sub> <sup>+</sup>	-0.9	297.1237(28); 282.1075(10); 265.1015(100); 237.0991(40); 250.0738(10); 237.0991(42); 233.0677(22); 205.0789(20)	Laurifoline
<b>32</b>	314.1392	C <sub>18</sub> H <sub>19</sub> NO <sub>4</sub>	1.3	297.1128(18); 282.0860(25); 265.0874(78); 237.0914(100); 222.0710(15); 205.0646(50); 177.0729(15)	Laurolisine
<b>33</b>	356.1861 [M <sup>+</sup> ]	C <sub>21</sub> H <sub>26</sub> NO <sub>4</sub> <sup>+</sup>	-0.3	356.1865(30); 313.1440(8); 311.1273(38); 296.1041(35); 281.0831(17); 279.1023(100); 280.1116(30); 265.0888(15); 251.1107(21); 264.0786(42); 248.0844(40); 236.0838(16)	Menisperine
<b>T35</b>	356.1874 [M <sup>+</sup> ]	C <sub>21</sub> H <sub>26</sub> NO <sub>4</sub> <sup>+</sup>	3.4	356.1865(6); 311.1273(40); 296.1041(60); 281.0831(16); 280.1116(100); 265.0868(13)	Xanthoplanine
<b>T36a/T36b</b>	342.1705 [M <sup>+</sup> ]	C <sub>20</sub> H <sub>24</sub> NO <sub>4</sub> <sup>+</sup>	0.0	342.1710(5); 192.1018(100); 177.0802(10)	Phellodendrine or cyclanoline
<b>T37</b>	356.1870 [M <sup>+</sup> ]	C <sub>21</sub> H <sub>26</sub> NO <sub>4</sub> <sup>+</sup>	2.2	356.1870(5); 192.1016(100); 177.0803(10)	N-Methyl tetrahydrocolumbamine or isomer
<b>38</b>	354.1359	C <sub>20</sub> H <sub>19</sub> NO <sub>5</sub>	5.1	354.1359(90); 336.1168(20); 323.0955(9); 275.0661(20); 247.0757(15); 206.0807(15); 189.0783(64); 188.0702(85); 149.0609(25); 119.0476(6); 91.0582(6)	Protopine
					1

<b>39</b>	370.1653	C <sub>21</sub> H <sub>23</sub> NO <sub>5</sub>	-0.3	370.1653(100); 352.1522(42); 290.0963(42); 206.0807(34); 189.0783(38); 188.0702(100); 165.0932(10); 149.0609 (10)	Alloryptopine	1
<b>40</b>	217.0981	C <sub>12</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	1.8	144.0806(100); 143.0723(12); 130.0654(6); 117.0685(8)	1,2,3,4,-Tetrahydro- $\beta$ -carboline-3-carboxylic acid	1
<b>41</b>	233.1136	C <sub>13</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	0.9	231.1136(7); 214.0850(31); 188.0695(31); 168.0788(40); 1; 58.0945(100); [54.0654(12); 146.0593(30); 144.0792(10); 143.0718(16); 130.0647(15); 118.0653(7)](15 eV)	Harmane, 1,2,3,4-tetrahydro-3-carboxylic acid	1 <sup>e</sup>
<b>42</b>	203.1185	C <sub>12</sub> H <sub>14</sub> N <sub>2</sub> O	0.5	160.0758(100); 159.0696(6); 132.0865(6); 117.0614(6)	N(2)-Methyl-6-hydroxy- 1,2,3,4-tetrahydro- $\beta$ -carboline	1
<b>43</b>	203.1185	C <sub>12</sub> H <sub>14</sub> N <sub>2</sub> O	0.5	188.0953(5); 174.0938(100); 162.0894(30); 160.0758(20); 159.0696(50); 147.0688(46); 146.0600(22); 131.0760(12); 130.0651(10); 129.0706(6)	Cimiltrypazepine	1
<b>44</b>	217.1338	C <sub>13</sub> H <sub>16</sub> N <sub>2</sub> O	-1.4	217.1338(86); 188.1071(100); 186.0913(70); 176.1099(20) ; 174.0920(16); 171.0657(14); 160.1110(8); 159.0771(11); 1; 58.0914(5) [15 eV]	Methylcimiltrypazepine	1 <sup>e</sup>
<b>45</b>	201.1031 [M <sup>+</sup> ]	C <sub>12</sub> H <sub>13</sub> N <sub>2</sub> O <sup>+</sup>	1.5	201.1031(100); 186.0808(29); 185.0730(6); 172.0771(11); 171.0578(40); 170.0608(52); 160.0777(12); 142.0562(12); 115.0558(15)	N(2)-Methyl-6-hydroxy- 3,4-dihydro- $\beta$ -carboline	1
<b>46</b>	337.1514	C <sub>13</sub> H <sub>20</sub> N <sub>4</sub> O <sub>5</sub>	0.6	337.1514(6); 278.1035(6); 175.1211(15); 163.0399(100); 1; 58.0932(10); 145.0291(30); 135.0449(20); 117.0340(19); 8 9.0396(10); 70.0672(6)	$\alpha$ -N-Caffeoyl arginine	1
<b>47</b>	351.1664	C <sub>16</sub> H <sub>22</sub> N <sub>4</sub> O <sub>5</sub>	-1.1	351.1678(6); 292.1192(5); 177.0554(100); 175.1200(16); 158.0932(8); 149.0603(8); 145.0282(83); 130.0983(5); 117 .0342(32); 116.0710(5); 89.0396(11); 70.0671(7); 60.0572(5)	$\alpha$ -N-Feruloyl arginine	1

(continued)

Table 2.1 (continued)

<b>48</b>	351.1664	C <sub>16</sub> H <sub>22</sub> N <sub>4</sub> O <sub>5</sub>	-1.1	351.1678(6); 292.1192(5); 177.0554(100); 175.1200(11); 163.0394(8); 158.0932(8); 149.0603(17); 145.0282(24); 130.0983(5); 117.0342(18); 116.0710(5); 89.0396(11); 70.0671(5); 60.0572(4)	α-N-Isoferuloyl arginine	1
<b>49</b>	265.1543	C <sub>14</sub> H <sub>20</sub> N <sub>2</sub> O <sub>3</sub>	-3.4	177.0558(90); 149.0595(18); 145.0300(100); 117.0347(90); 89.0390(55)	Feruloyl putrescine	1
<b>50</b>	265.1543	C <sub>14</sub> H <sub>20</sub> N <sub>2</sub> O <sub>3</sub>	-3.4	177.0553(100); 163.0356(4); 149.0590(22); 145.0289(31); 134.0355 (7); 117.0343(30); 89.0385(25)	Isoferuloyl putrescine	1
<b>T51</b>	332.1257	C <sub>16</sub> H <sub>18</sub> N <sub>3</sub> O <sub>5</sub>	3.3	177.0551(100); 163.0395(3); 149.0597(16); 145.0283(21); 117.0346(20); 110.0744(5); 89.0395(10)	α-N-Isoferuloyl histidine	2
<b>T52</b>	338.1234	C <sub>16</sub> H <sub>19</sub> NO <sub>7</sub>	-1.8	338.1234(20); 177.0558(100); 163.0390(6); 149.0600(21); 145.0286(30); 117.0350(40); 89.0401(25)	α-N-Isoferuloyl glutamic acid	2
<b>T53</b>	476.1929	C <sub>24</sub> H <sub>29</sub> NO <sub>9</sub>	1.7	314.1398(100); 177.0558(90); 149.0607(6); 145.0282(45); 121.0617(3); 117.0363(10); 89.0380(6)	N-Feruloyl tyramine hexoside	2
<b>T54</b>	492.1878	C <sub>24</sub> H <sub>29</sub> NO <sub>10</sub>	1.6	330.1343(24); 177.0533(100); 149.0657(4); 145.0320(52); 117.0320(12)	N-Feruloyl dopamine hexoside	2
<b>T55</b>	506.2026	C <sub>25</sub> H <sub>31</sub> NO <sub>10</sub>	2.4	344.1525(12); 177.0558(100); 149.0609(5); 145.0282(62); 117.0336(15); 89.0414(6)	N-Feruloyl-methoxytyraminehexoside	2
<b>T56</b>	492.1878	C <sub>24</sub> H <sub>29</sub> NO <sub>10</sub>	1.6	321.0980(15); 177.0558(100); 163.0390(3); 149.0657(10); 145.0328(17); 137.0606(22); 119.0501(5); 117.0375(10); 91.0558(7); 89.0421(6)	Dopamine-O-(isoferuloyl)-hexoside	3 <sup>f</sup>
<b>57</b>	104.1068 [M <sup>+</sup> ]	C <sub>5</sub> H <sub>14</sub> NO	-6.7	104.1068(100); 60.0818(14)	Choline	1
<b>58</b>	280.1341 [M <sup>+</sup> ]	C <sub>12</sub> H <sub>18</sub> NO <sub>2</sub> <sup>+</sup>	1.4	208.1341(6); 149.0584(90); 105.0357(100); 77.0374(18)	Benzoyl choline	1
<b>59</b>	280.1554 [M <sup>+</sup> ]	C <sub>15</sub> H <sub>22</sub> NO <sub>4</sub> <sup>+</sup>	1.8	221.0815(100); 206.0581 (20); 177.0550(60); 149.0604(8); 145.0325(30); 117.0372(20); 89.0386(8)	Feruloyl choline	1
<b>60</b>	280.1554 [M <sup>+</sup> ]	C <sub>15</sub> H <sub>22</sub> NO <sub>4</sub> <sup>+</sup>	1.8	221.0813(100); 206.0578(20); 177.0544(60); 163.0358(5); 162.0318(5); 149.0605 (10); 145.0320(14); 134.0369(6); 117.0341(10); 89.0388(10)	Isoferuloyl choline	1
<b>T61</b>	266.1617 [M <sup>+</sup> ]	C <sub>11</sub> H <sub>23</sub> NO <sub>6</sub> <sup>+</sup>	4.9	266.1617(95); 104.1073(100); 60.0838(13)	Choline hexoside	3

<b>62</b>	118.08772 [M <sup>+</sup> ]	C <sub>5</sub> H <sub>12</sub> NO <sub>2</sub> <sup>+</sup>	3.4	118.08772(100); 59.0711(10)	Glycine betaine	1
<b>63</b>	144.1018 [M <sup>+</sup> ]	C <sub>7</sub> H <sub>14</sub> NO <sub>2</sub> <sup>+</sup>	-4.9	144.1018(100); 84.0823(10); 58.0672(10)	Proline betaine	1
<b>64</b>	162.1122 [M <sup>+</sup> ]	C <sub>7</sub> H <sub>16</sub> NO <sub>3</sub> <sup>+</sup>	-4.9	162.1122(100); 103.0406(20); 102.0930(8); 85.0300(7); 60.0829(8) [15 eV]	L-Carnitine	1
<b>T65</b>	198.1237 [M <sup>+</sup> ]	C <sub>9</sub> H <sub>16</sub> N <sub>3</sub> O <sub>2</sub> <sup>+</sup>	-3.0	198.1237(14); 154.1338(100); 95.0619(60); 68.0517(6); 60.0829(8) [15 eV]	Histidine betaine	2
<b>66</b>	138.0547 [M <sup>+</sup> ]	C <sub>7</sub> H <sub>8</sub> NO <sub>2</sub> <sup>+</sup>	-5.8	138.0547(100); 136.0394(5); 110.0587(6); 94.0647(30)92. 0494(24); 65.0377(6)	Trigonelline	1
<b>67</b>	130.0860	C <sub>6</sub> H <sub>11</sub> NO <sub>2</sub>	-6.1	84.0823(100)	Pipeolic acid	1
<b>68</b>	130.0503	C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub>	-0.8	84.0449(100)	Pyroglutamic acid	1
<b>70</b>	332.1340	C <sub>14</sub> H <sub>21</sub> NO <sub>8</sub>	-1.5	332.1340(6); 314.1237(29); 152.0704(100); 136.0765(16); 134.0602(28); 124.0764(26); 108.0816(55); 106.0651(10)	5'-O-(β-D-glucopyranosyl) pyridoxine	3
<b>71</b>	220.1178	C <sub>9</sub> H <sub>17</sub> NO <sub>5</sub>	-3.2	220.1178(8); 142.0858(10); 124.0712(37); 103.0721(12); 98.0217(51); 90.0529(100); 85.0547(35); 72.0417(69); 70.0295(18)	Pantothenic acid	1
<b>T72</b>	282.1202	C <sub>11</sub> H <sub>15</sub> N <sub>5</sub> O <sub>4</sub>	2.1	150.0783(100)	N-Methyladenosine	2
<b>T73</b>	282.1183	C <sub>11</sub> H <sub>15</sub> N <sub>5</sub> O <sub>4</sub>	-6.7	136.0622(100); 119.0360(10)	2'-O-Methyladenosine	2
<b>T74</b>	178.0875	C <sub>10</sub> H <sub>11</sub> NO <sub>2</sub>	3.9	178.0879(20); 119.0476(40); 91.0547(100)	N-Phenylacetyl acetamide	3
<b>T75</b>	206.1550	C <sub>13</sub> H <sub>19</sub> NO	2.4	107.0504(100); 100.1135(5); 79.0544(18); 77.0405(16)	N-Cyclohexyl-4-hydroxybenzylamine	3

<sup>a</sup>Instrument: waters SYNAPT quadrupole/time-of-flight. Spectra taken at 25 eV except where noted

<sup>b</sup>Isolated compound

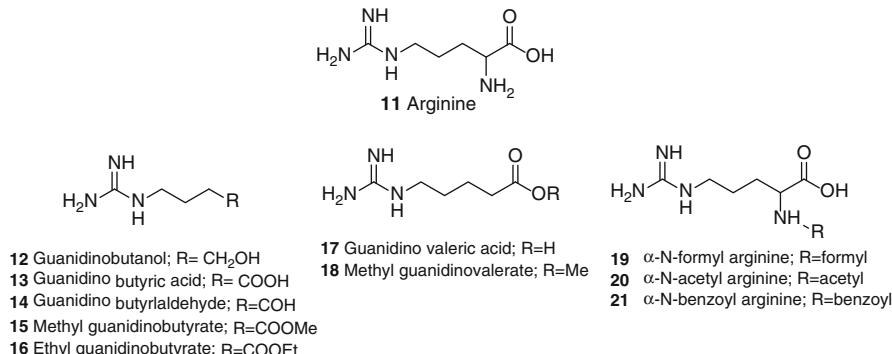
<sup>c</sup>Most compounds annotated at levels 2 or 3 were tentatively (T) identified by comparison of their fragmentation patterns with those of structural analogs

<sup>d</sup>Identification based on published tandem mass spectra

<sup>e</sup>Compound reported here for the first time

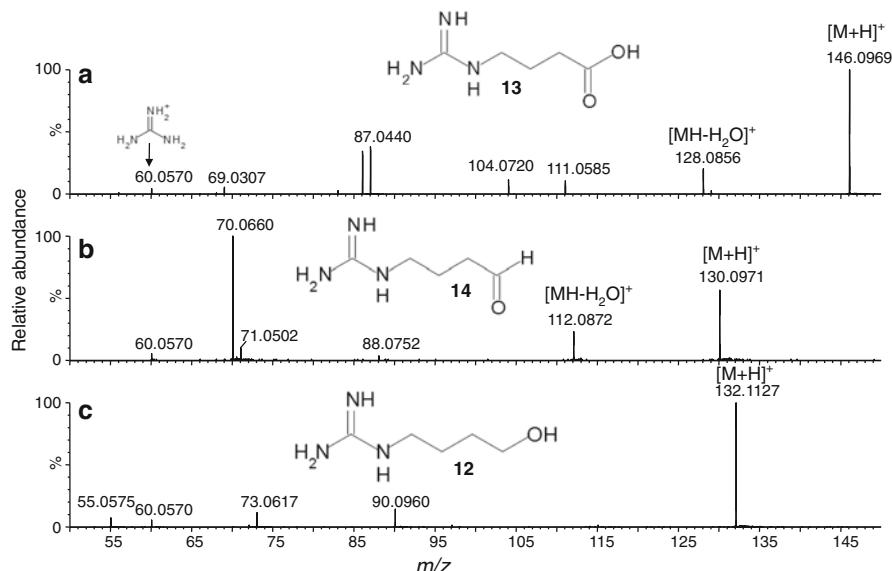
<sup>f</sup>1.5 eV spectrum of **56** is shown in Fig. 2.13c. Adapted from Nikolic et al. [39]

The prototype acyclic guanidino compound and a likely biosynthetic precursor of the guanidine alkaloids is the amino acid arginine (**11**). Fragmentation pathways of arginine have been extensively studied [48–50] and have been used as a template to discover numerous analogs (**12–21**).

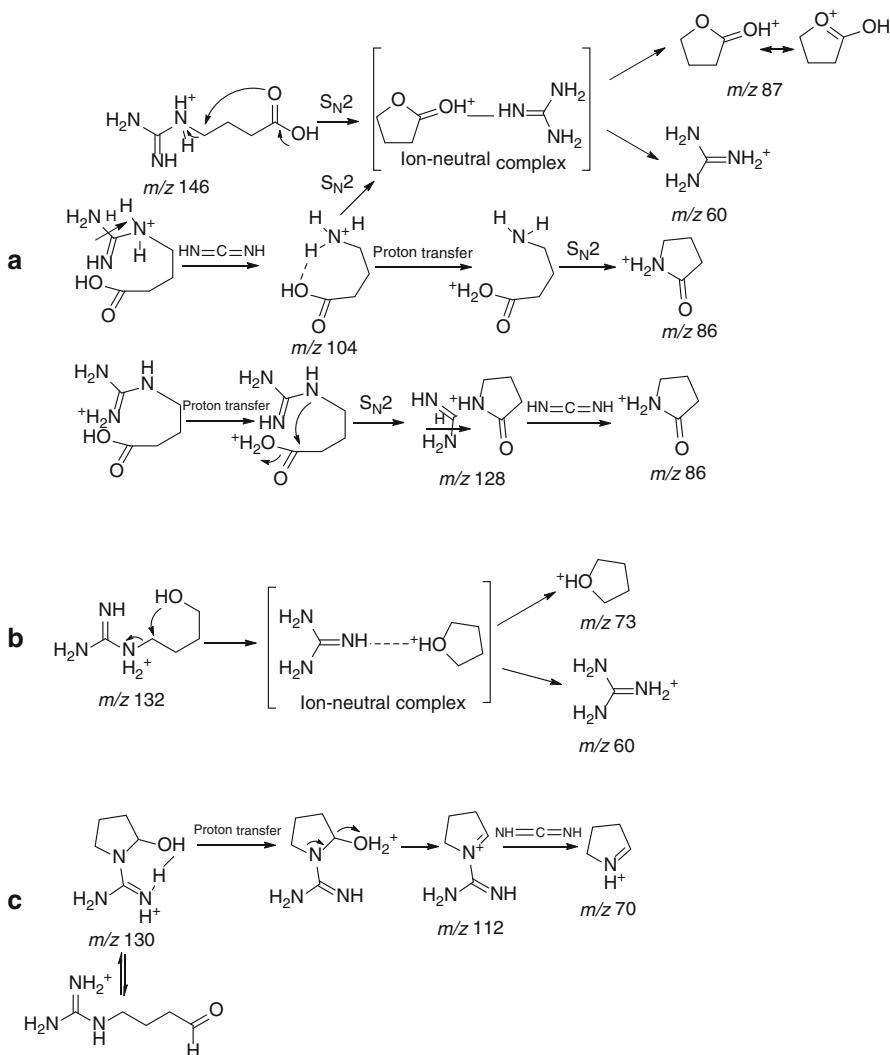


As an example of the identification strategy, Fig. 2.2 shows tandem mass spectra of  $\gamma$ -guanidinobutyric acid (**13**) and its oxidation analogs  $\gamma$ -guanidinobutanol (**12**) and  $\gamma$ -guanidinobutyraldehyde (**14**).

In the tandem mass spectrum of **13** the ion of  $m/z$  87 is likely to be protonated butyrolactone, and its formation can be rationalized by an S<sub>N</sub>2 attack of the carbonyl oxygen on the carbon atom bearing the guanidino group (Fig. 2.3). Direct attack of

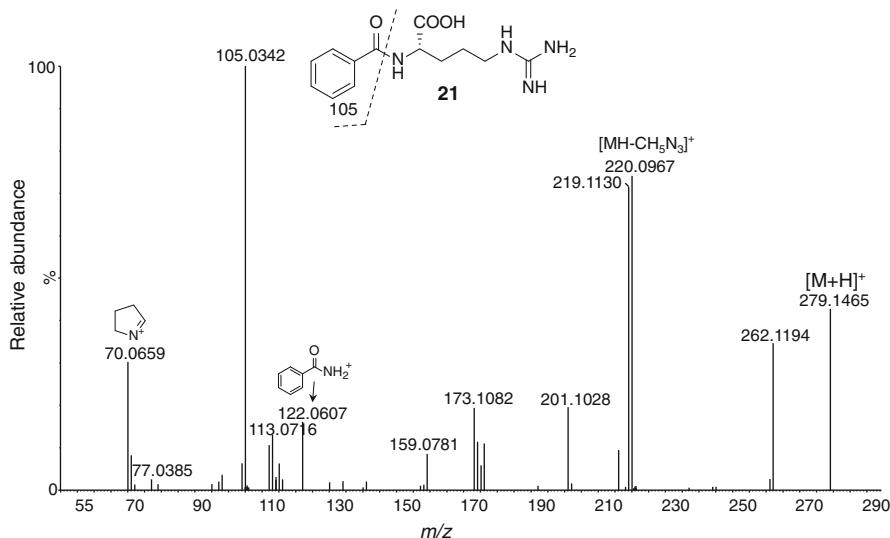


**Fig. 2.2** Tandem mass spectra of (a)  $\gamma$ -guanidinobutyric acid (**13**), (b)  $\gamma$ -guanidinobutyraldehyde (**14**), and (c)  $\gamma$ -guanidinobutanol (**12**). Reproduced from Nikolic et al. [39] with permission



**Fig. 2.3** Proposed fragmentation pathways for **(a)**  $\gamma$ -guanidinobutyric acid (**13**); **(b)**  $\gamma$ -guanidinobutanol (**12**); and **(c)**  $\gamma$ -guanidinobutyraldehyde (**14**)

the carbonyl oxygen is supported by observation of protonated guanidine at  $m/z$  60, which is formed by proton transfer in the ion-neutral complex between guanidine and protonated butyrolactone. An additional minor pathway for formation of the ion of  $m/z$  87 is by elimination of ammonia from protonated  $\gamma$ -aminobutyric acid (GABA) ( $m/z$  104), as determined in separate ion-trap experiments. Similarly, the product ion of  $m/z$  86 has an elemental composition of  $C_4H_8NO$  corresponding to protonated butyrolactam. Ion-trap experiments indicated that the main pathways for formation of this ion are a loss of methylenediamine ( $NH=C=NH$ ) from the ion of



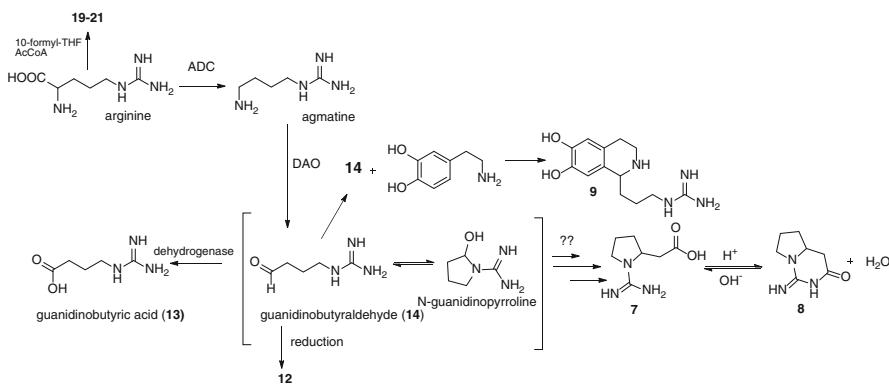
**Fig. 2.4** Tandem mass spectrum of  $\alpha$ -N-benzoyl arginine (**21**)

$m/z$  128  $[\text{MH}-\text{H}_2\text{O}]^+$  and a loss of water from protonated GABA. Similarly, in the tandem mass spectrum of **12** the hydroxyl group participates in the  $S_{\text{N}}2$  attack to form protonated tetrahydrofuran at  $m/z$  73. The spectrum of **14** can be explained as being derived from both the cyclic and acyclic forms of this aldehyde (Fig. 2.4c). The presence of the cyclic form can explain the ready loss of water, as well as the base peak of  $m/z$  70 which has the structure of protonated dihydropyrrrole.

Using similar spectral arguments, other analogs such as  $\delta$ -guanidinovaleric acid and its methyl ester (**17–18**) and acylated arginines (**19–21**) could be identified. The identification of *N*-benzoyl arginine (**21**) is reported here for the first time. Its tandem mass spectrum (Fig. 2.4) has all the characteristic features described above for acyclic guanidines.

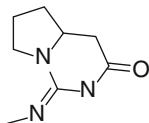
In addition, the ions of  $m/z$  105 and 77 suggested the presence of a benzoyl derivative. Combined with the molecular formula of  $\text{C}_{13}\text{H}_{18}\text{N}_4\text{O}_3$ , the most plausible structure consistent with the data is that of  $\alpha$ -N-benzoyl arginine which was confirmed by comparison with authentic synthetic standard. Interestingly, database searches revealed that this compound has not been reported in plants even though the ethyl ester of  $\alpha$ -N-benzoyl arginine is a widely studied compound. Its most common use is as a trypsin substrate to measure enzymatic activity of trypsin preparations [51]. As ethyl ester it has also been used as a model for arginine-containing peptides, and at least one study found that it induced relaxation of blood vessels independent of its properties as a substrate for nitric oxide synthase [52].

In contrast to acyclic guanidines, the predominant fragmentation pathway of cyclic guanidines proceeds via loss of methylenediamine ( $-42 \text{ Da}$ ;  $\text{NH}=\text{C}=\text{NH}$ ). Using spectra of **7** and **8**, various esters of cimipronidine could be identified in the



**Fig. 2.5** Possible biosynthetic pathways for formation of guanidino compounds in black cohosh. As a central intermediate, **14** could be formed via action of arginine decarboxylase (ADC) and diaminopropene amine oxidase (DAO). A candidate gene for an unspecified amine oxidase has been identified by Spiering et al. [53]. **14** may be further oxidized into **13** by a  $\gamma$ -guanidinobutyraldehyde dehydrogenase. One such dehydrogenase has been identified in leaves of *Vicia faba* [130], and Spiering et al. identified a candidate gene for an aldehyde dehydrogenase in black cohosh roots/rhizomes. In addition, the same study identified an alcohol dehydrogenase which could convert **14** into **12**. **14** can also serve as the coupling aldehyde for biosynthesis of **9** via Pictet-Spengler condensation. Arginine could also serve as a substrate for acyl transferases that produce a number of acylated arginine derivatives (**19–21**). Adapted from Gödecke et al. [37]

black cohosh extracts depending on the solvent used to extract the plant or to redissolve the sample prior to injection. In addition, *N*-methylcyclocimipronidine (**22**) could be tentatively identified.



**22** *N*-methylcyclocimipronidine

The biosynthetic origin of cimipronidine has not yet been established, but is most certainly linked to arginine metabolism. A possible biosynthetic scheme for production of guanidino alkaloids is shown in Fig. 2.5. The proposed central intermediate *N*-guanidinopyrrolidine is a cyclic hemiacetal form of **14**, which could be formed by action of an amine oxidase on agmatine, a decarboxylation product of arginine.

A candidate gene encoding an unspecified amine oxidase has been identified by Spiering et al. [53]. *N*-Guanidinopyrrolidine may through yet to be identified steps be converted into cimipronidine. Cimipronidine easily cyclizes into cyclocimipronidine under acidic conditions, which implies that cyclocimipronidine may be an isolation artifact. The acid–base interconversion between **7** and **8** in aqueous medium was demonstrated by Gödecke et al. [37].

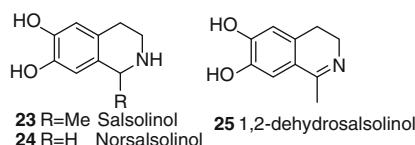
Since the guanidine alkaloids were identified in black cohosh only recently, it cannot yet be assessed if any of these compounds are unique to *A. racemosa* or whether they are more widespread in the genus *Actaea*, but in any event they are certainly strong candidates for further investigation as possible novel standardization markers.

## 2.5 Isoquinoline Alkaloids

The isoquinoline alkaloids represent a large group of alkaloids that are biosynthetically derived from Pictet-Spengler condensation of dopamine with various aldehydes. Among all the alkaloids identified from various Ranunculaceae, isoquinolines represent the second-most common class [30]. Thus, it is not surprising that a significant diversity of this class was identified in black cohosh.

### 2.5.1 Dihydro- and Tetrahydroisoquinoline Alkaloids

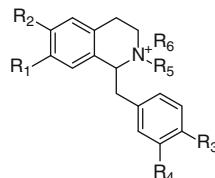
The simplest structures within the isoquinoline alkaloids are the tetrahydroisoquinoline alkaloids. Dopargine (**9**), mentioned above, was the first alkaloid from this class isolated from black cohosh [37]. This alkaloid is likely derived from condensation of dopamine with  $\gamma$ -butyraldehyde (see Fig. 2.5). Salsolinol (**23**), a condensation product of dopamine and acetaldehyde, can be considered a prototype molecule of this class. It was initially identified based on spectral database searches and subsequently confirmed by comparison with an authentic standard [39]. Salsolinol is widely distributed in the plant kingdom and can be found in many dietary sources including alcoholic beverages, bananas, cheese, beef, milk, and cocoa [54–56]. Importantly, salsolinol can be synthesized endogenously in dopaminergic neurons of mammals including humans [57]. Pharmacologists have extensively studied salsolinol for its neuropharmacological effects, such as modulation of catecholaminergic transmission, as well as for a possible role in the etiology of alcoholism [58]. Dopaminergic activity of salsolinol is often invoked as a mechanism for addictive effects of chocolate. However, recent studies showed that salsolinol does not cross the blood brain barrier [59]. Although it is unlikely that exogenously administered salsolinol can exhibit activities on the central nervous system (CNS), it still may show peripheral activities mediated by dopamine D2 receptors [60]. Salsolinol may be at least partially responsible for the observed in vitro dopaminergic activity of black cohosh extracts [61].



In addition to salsolinol, norsalsolinol (**24**) and 1,2-dehydrosalsolinol (**25**) have been identified at level 1. Both of these compounds have been identified as dopamine metabolites in the brain [62] and in urine [63]; however, they have not been reported in other higher plants. At this point it is not clear whether **24** is an isolation artifact or a genuine natural product. Traces of formaldehyde might have been present in the extraction solvent, which may have led to a nonenzymatic condensation with dopamine [57].

### 2.5.2 Benzylisoquinoline Alkaloids

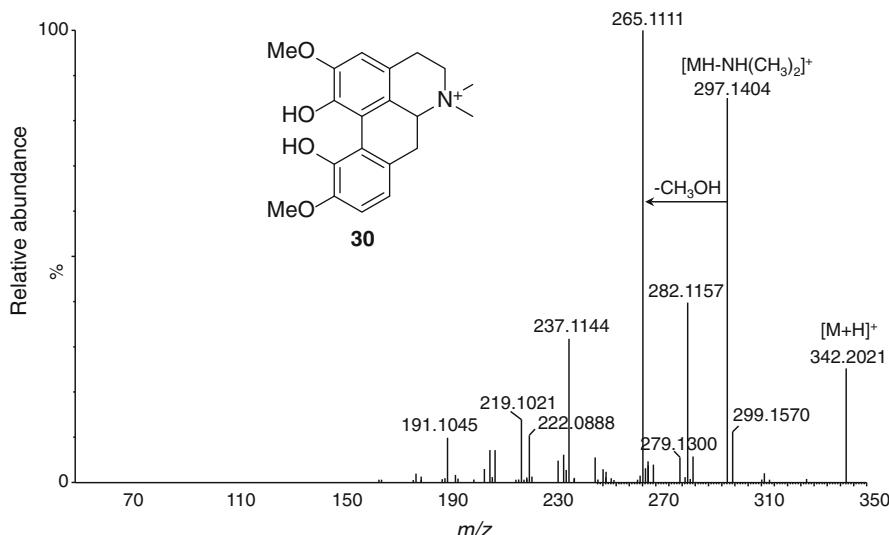
A large group of isoquinoline alkaloids is derived from condensation of dopamine and 4-hydroxyphenylacetaldehyde to form a benzyl tetrahydroisoquinoline skeleton that can be further coupled into a plethora of alkaloids including aporphines, protoberberines, and protopines [64]. Norcoclaurine (**26**), the prototype molecule of this group of alkaloids, was tentatively identified based on comparison with the published product ion tandem mass spectra [65, 66]. Another critical molecule in the biosynthetic pathways of isoquinoline alkaloids is reticuline (**27**), which was identified at level 1 by comparison with authentic standard. The study by Spiering et al. identified a candidate gene for *N*-methylcoclaurine 3-hydroxylase, an enzyme involved in the biosynthesis of reticuline, providing a mechanistic basis for the origin of this group of alkaloids in black cohosh [53].



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>	
<b>26</b>	OH	OH	OH	H	H	H	Norcoclaurine
<b>27</b>	OH	OMe	OMe	OH	H	Me	Reticuline
<b>28</b>	OH	OMe	OH	H	Me	Me	Magnocurarine
<b>29</b>	OMe	OH	OH	H	Me	Me	Oblongine

Reticuline is an interesting molecule, not only because it represents a branching point in the synthesis of complex isoquinoline alkaloids in plants, but also because recent studies provide evidence that it can serve as a biological precursor for the biosynthesis of endogenous morphine via action of the cytochrome P4502D6 [67–70]. In addition, reticuline has biological activities of its own, as a dopamine antagonist [71] and a mild activator of the mu opioid receptor [72].

Compounds **28** and **29** produced nearly identical product ion spectra (Table 2.1) but had different retention times, suggesting two isomeric structures. Loss of dimeth-



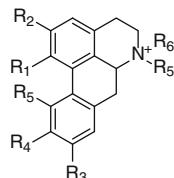
**Fig. 2.6** Tandem mass spectrum of magnoflorine (**30**). Note the presence of numerous radical cations in the spectrum ( $m/z$  284, 282, 222). Such radicals are stabilized by delocalization of the free electron onto the extended aromatic system

ylamine ( $m/z$  271) suggested that these compounds contain quaternary nitrogen (see below). Database searches with this structural constraint revealed that these compounds are likely analogs of the alkaloid magnocurarine. Comparison with authentic magnocurarine led to assignment of **28** as magnocurarine, whereas **29** is likely one of the known positional isomers of magnocurarine such as lotusine or oblongine.

### 2.5.3 Aporphine Alkaloids

Aporphine alkaloids represent one of the largest groups of the isoquinoline alkaloids, with more than 600 known compounds. They are biosynthetically derived from oxidative *o*, *p* coupling of benzylisoquinolines. In order to identify aporphines in black cohosh extracts, we relied on the previous studies that examined fragmentation behavior of this class of compounds [73, 74]. Due to their rigid structure, tandem mass spectra of aporphines are characterized by losses of small molecules such as water, CO, or CO<sub>2</sub>, with cation radical fragment ions frequently present. The degree of substitution on the nitrogen can be easily distinguished based on the loss of nitrogen in the form of ammonia (secondary nitrogen), methylamine (tertiary nitrogen), or dimethylamine (quaternary nitrogen). For example, in the product ion spectrum of magnoflorine (**30**) (Fig. 2.6) loss of dimethylamine (−45 Da) was the second most abundant peak, typical for a quaternary aporphine. Interestingly, most of the aporphines identified in black cohosh (**30–35**) were quaternary alkaloids.

Aporphine alkaloids have been extensively studied for their wide range of biological activities and have been subjected to extensive medicinal chemistry modifications to produce more active analogs for a specific indication. The activities relevant for medicinal uses of black cohosh include vasorelaxing, antinociceptive, antispasmodic, or neuroleptic effects [75–77]. Mechanistically, these effects can be explained by strong affinity of aporphinoids for  $\alpha$ -adrenergic, dopamine, and serotonin receptors. Both agonistic and antagonistic activities have been described, depending on the particular aporphinoid. An excellent compilation of activities of natural and synthetic aporphinoids against dopamine receptors has been provided by Zhang et al. [78]. Laurolitsine (32), for example, displayed IC<sub>50</sub> values of 2.6 and 6.8  $\mu\text{M}$  against dopamine D1 and D2 receptors, respectively. Among serotonergic activities, most interest has focused on 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors, and natural aporphinoids have been used as model pharmacophores to develop novel synthetic analogs [79, 80]. For example, *N*-methyllaurotetanine, a methyl analog of laurotetanine identified in black cohosh, is a potent ligand against 5-HT<sub>1A</sub> [81]. Interestingly, a recent study by de la Peña et al. claims that magnoflorine possesses sedative and anxiolytic effects mediated by the GABAergic system [82]. Aporphinoids also act as  $\alpha$ -adrenergic antagonists [83], and those with quaternary nitrogen have affinity for neuronal nicotinic receptors. For example, xanthoplanine (35) bound to the  $\alpha 4\beta 2$  subtype of nicotinic receptors with a K<sub>i</sub> of 0.91  $\mu\text{M}$  [84]. Overall, as a class, aporphinoids are likely to contribute significantly to the overall pharmacological profile of black cohosh and are strong candidates for inclusion in the standardization process.

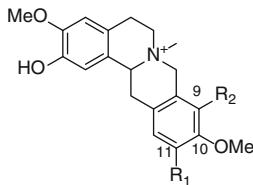


	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>	R <sub>7</sub>	
<b>30</b>	OH	OMe	H	OMe	OH	Me	Me	Magnoflorine
<b>31</b>	OH	OMe	OH	OMe	H	Me	Me	Laurifoline
<b>32</b>	OMe	OH	OH	OMe	H	H	H	Laurolitsine
<b>33</b>	OMe	OMe	H	OMe	OH	Me	Me	Menisperine
<b>34</b>	OMe	OMe	OH	OMe	H	H	H	Laurotetanine
<b>35</b>	OMe	OMe	OH	OMe	H	Me	Me	Xanthoplanine

### 2.5.5 Protoberberine Alkaloids

Protoberberines are a widely distributed class of alkaloids, with some of the most prolific producers in the Ranunculaceae. Based on limited mass spectrometric evidence, three alkaloids from this class were tentatively identified at level 3 in black cohosh. The

main difficulty in assigning the structures is due to the limited fragmentation that does not allow differentiation between 9,10- and 10,11-substitution pattern.



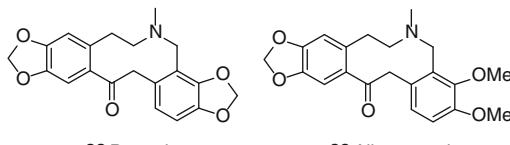
**36a**  $R_1=OH, R_2=H$  Phellodendrine

**36b**  $R_1=H, R_2=OH$  Cyclanoline

**37**  $R_1=H, R_2=OMe$  N-methyl tetrahydrocolumbamine

### 2.5.6 Protopine-Type Alkaloids

Two members of the protopine subclass of the isoquinoline alkaloids, protopine (**38**) and allocryptopine (**39**), were identified in black cohosh based on MassBank database searches and confirmed by comparison with authentic standards. These two alkaloids were among the few for which public databases were useful for identification.



**38** Protopine

**39** Allocryptopine

Protopine (**38**) and allocryptopine (**39**) are common in the genera *Corydalis* and *Fumaria* of the Papaveraceae. In the Ranunculaceae they have been reported only in the genus *Thalictrum*. Biological activities of protopine and allocryptopine have been extensively studied (for a review see [85]). Of relevance to black cohosh medicinal use, protopine has been reported to have benzodiazepine-like, analgesic, antidepressant, and anticholinergic activities in vitro and in animal studies [86–88]. The presence of this alkaloid might explain anecdotal reports of “vivid dreams” and opioid-like activities observed by patients taking black cohosh [89], as well as the observed in vitro opioid activity [90]. Li et al. found that protopine and allocryptopine were potent competitive inhibitors of the cytochrome P4502D6, with  $K_i$  values of 78 nM and 122 nM, respectively. This inhibition was studied in the context of potential drug-herb interactions with tamoxifen [91]. One of the side effects of tamoxifen therapy is occurrence of hot flashes, and patients often use black cohosh to alleviate such symptoms. However, since tamoxifen is a prodrug that is metabolically activated by the cytochrome P4502D6, there is concern that inhibition of this enzyme may render tamoxifen therapy less effective. Based on the measured content of protopine and allocryptopine (0.0063 % and 0.0088 %, respectively), it was

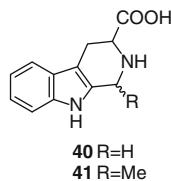
concluded that these two compounds alone accounted for approximately half of the total activity of the active fraction [91].

## 2.6 Indole Alkaloids

Indole alkaloids are a large group of alkaloids biosynthetically derived from the amino acid tryptophan. Several compounds belonging to the subclasses of indole alkylamines and tetrahydro- $\beta$ -carbolines have been detected in black cohosh.

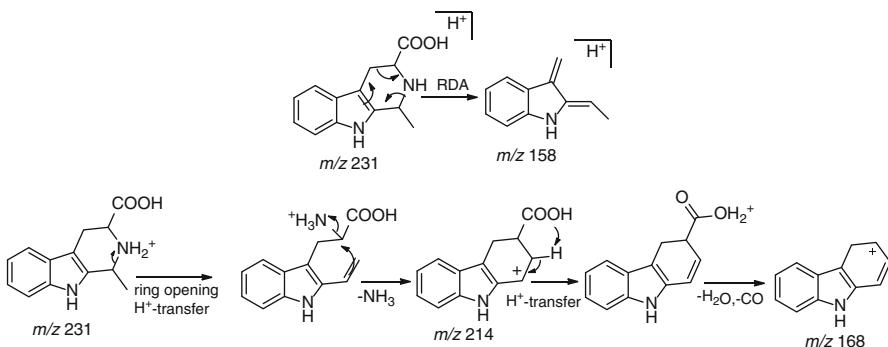
### 2.6.1 Tryptophan-Derived Pictet-Spengler Adducts

Compounds **40** and **41** are Pictet-Spengler adducts formed by condensation of tryptophan with formaldehyde and acetaldehyde, respectively. Compound **40** was initially identified based on its mass spectrum characterized by an abundant fragment ion of  $m/z$  144, with the elemental composition of  $C_{10}H_{13}N_2$  corresponding to protonated tryptamine. In-source fragmentation followed by MS-MS product ion analysis of  $m/z$  144 showed a fragmentation pattern identical to authentic tryptamine, suggesting that this compound is a tryptamine derivative. The neutral loss of iminoacetic acid ( $C_2H_3NO_2$ ) combined with database searching suggested that **41** might be a tetrahydro- $\beta$ -caroline carboxylic acid. Subsequent synthesis of two possible positional isomers (1- and 3-substituted) identified **40** as 1,2,3,4-tetrahydro- $\beta$ -caroline-3-carboxylic acid [39]. By similar reasoning, **41** was identified as 1-methyl-1,2,3,4-tetrahydro- $\beta$ -caroline-3-carboxylic acid and confirmed by comparison with authentic standard. The presence of **41** in black cohosh is reported here for the first time.



The main fragmentation pathway of these adducts involves retro Diels-Alder cleavage to eliminate the neutral iminoacetic acid mentioned above. At low collision energies, loss of ammonia is also observed, which can be rationalized by the mechanism proposed in Fig. 2.7.

Both **40** and **41** are widespread alkaloids found in many dietary products such as fruits, wine, beer, chocolate, soy sauce, cheese, smoked meat products, and raisins [92–95]. **40** has also been detected in the human brain, which has generated interest in its contribution to the pathogenesis of alcoholism and psychiatric disorders [96].

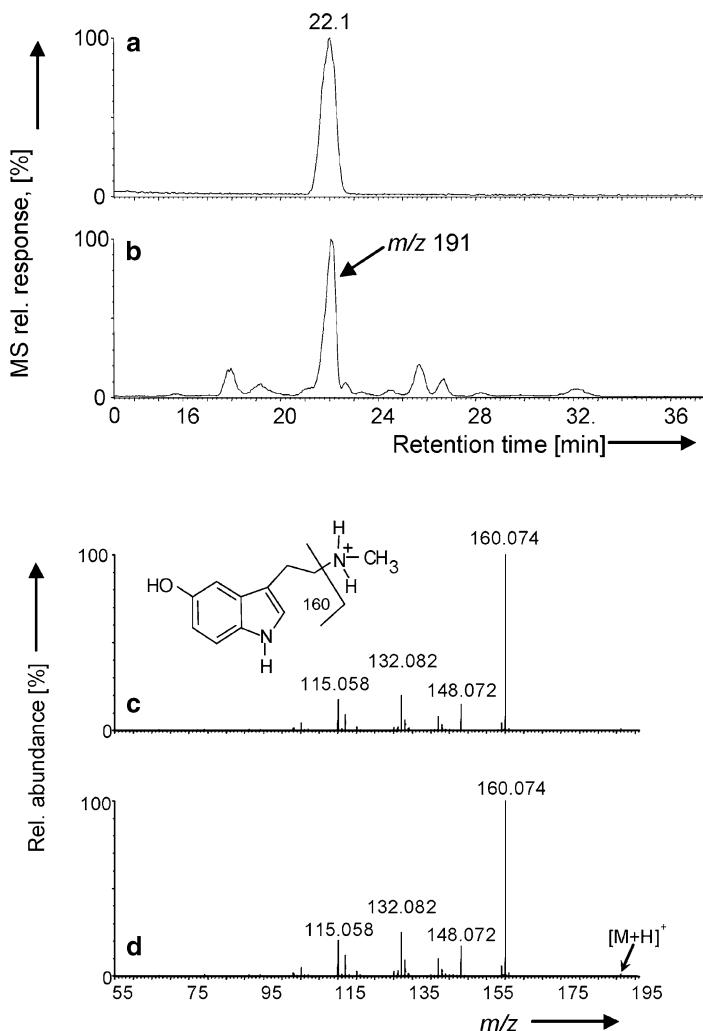


**Fig. 2.7** Proposed origins of abundant fragment ions of **41**

There are scarce data on the potential biological activities of these compounds.  $\beta$ -Carboline is a common structural motif for monoamine oxidase (MAO) inhibition; however, **40** and **41** do not inhibit MAO [97]. There have been some speculations that **41** is a causative substance of eosinophilia-myalgia syndrome associated with ingestion of L-tryptophan [98, 99]. However, definitive proof for this has yet to be provided. Since **40** and **41** are common dietary ingredients, it would be difficult to assign any particular significance of these compounds to the overall clinical effects of black cohosh. Women are likely to ingest significantly more of these alkaloids through diet than from consuming black cohosh extracts

### 2.6.2 *N<sub>ω</sub>-Methylserotonin and Corresponding Pictet-Spengler Adducts*

Identification of *N<sub>ω</sub>*-methylserotonin (**10**) represents an important chapter in the black cohosh alkaloid story. The serotonergic activity of black cohosh extracts was first described by Burdette et al. [100]. In that study, black cohosh extracts prepared by extraction with different solvents (methanol, 75 % ethanol, and 40 % isopropanol) were screened for binding to multiple serotonin receptor subtypes. The highest activity was observed for 5-HT<sub>1A</sub>, 5-HT<sub>1D</sub>, and 5-HT<sub>7</sub> receptors and, given the involvement of 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors in thermoregulation, these two receptor subtypes were pursued further. In the radioligand displacement assay, the methanolic extract was found to inhibit binding of receptor probes 8-OHDPAT (5-HT<sub>1A</sub>) and LSD (5-HT<sub>7</sub>) with IC<sub>50</sub> values of 2.5 and 2.2  $\mu\text{g}/\text{mL}$ , respectively. The 75 % ethanolic extract was somewhat more selective, with IC<sub>50</sub> values of 13.0 and 3.1  $\mu\text{g}/\text{mL}$  for the 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors, respectively. These initial results could not be fully reproduced in a subsequent study which found that methanolic and 75 % ethanolic extracts inhibited binding of LSD to 5-HT<sub>7</sub> receptor with IC<sub>50</sub> values of 87 and 55  $\mu\text{g}/\text{mL}$ , respectively [38]. The bioassay-guided fractionation and identification of active principle(s) proved to be quite a challenge, and only development of an



**Fig. 2.8** Identification of  $N_{\omega}$ -methylserotonin (**10**) in black cohosh. (a) LC-MS chromatogram of  $N_{\omega}$ -methylserotonin standard; (b) LC-MS chromatogram of 5-HT<sub>7</sub>-active fraction; (c) and (d) tandem mass spectra of  $N_{\omega}$ -methylserotonin standard and the compound eluting at the same retention time in the active fraction, respectively. Reproduced from [38] with permission

elaborate fractionation scheme involving use of pH-zone refined centrifugal partition chromatography in combination with mass spectrometric dereplication enabled identification of the active principle as  $N_{\omega}$ -methylserotonin (Fig. 2.8).

$N_{\omega}$ -Methylserotonin is a rare indole alkylamine that occurs both in plants and in animals. In plants, it has been reported in *Ranunculus sceleratus* (Ranunculaceae) [101] and, interestingly, in several *Citrus* species [102]. In its glucosidated form, it has been identified in *Zanthoxylum piperitum* seeds [103]. The biosynthetic pathway has

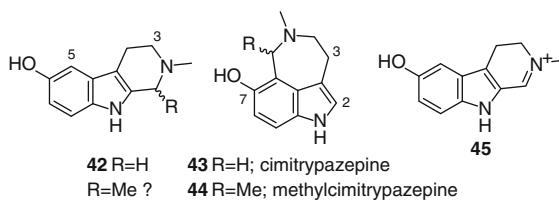
not been yet established, but may involve either methylation of serotonin by an amine *N*-methyltransferase or hydroxylation of *N*-methyltryptamine by tryptamine 5-hydroxylase. Interestingly, a gene discovery study by Spiering et al. [53] identified two tryptophan decarboxylase-related genes. However, enzyme expression was found limited to immature flowers and young leaves, and no expression was detected in roots/rhizomes. The authors speculate that *N*<sub>ω</sub>-methylserotonin translocates to the roots from aerial parts. In support of this hypothesis, we did detect *N*<sub>ω</sub>-methylserotonin in aerial parts (submitted manuscript). *N*<sub>ω</sub>-Methylserotonin is one of the few alkaloids in black cohosh for which some data on concentrations in plant material exist. The reported content in the study of Powell et al. is 0.0031 % which would translate into a daily dose of 3.7 µg under typical 120 mg extract/day dosing regimen.

Biological activities of *N*<sub>ω</sub>-methylserotonin were evaluated in a battery of bioassays designed to evaluate its properties as a serotonergic compound. In the radioligand displacement assay, *N*<sub>ω</sub>-methylserotonin showed greatest selectivity for 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors [38]. The affinity for 5-HT<sub>7</sub> was particularly strong, with an IC<sub>50</sub> of 23 pM, which makes *N*<sub>ω</sub>-methylserotonin one of the most potent known ligands to any receptor, not just to 5-HT<sub>7</sub>. For comparison, the natural ligand serotonin showed an IC<sub>50</sub> value of 0.86 nM for the same receptor. In the functional assay using production of cAMP as a marker of receptor stimulation, *N*<sub>ω</sub>-methylserotonin acted as agonist (EC<sub>50</sub>=22 nM). Unfortunately, insufficient data were presented to determine whether it is a full or partial agonist. However, given the structural similarity to serotonin, it is reasonable to assume that *N*<sub>ω</sub>-methylserotonin is a full agonist.

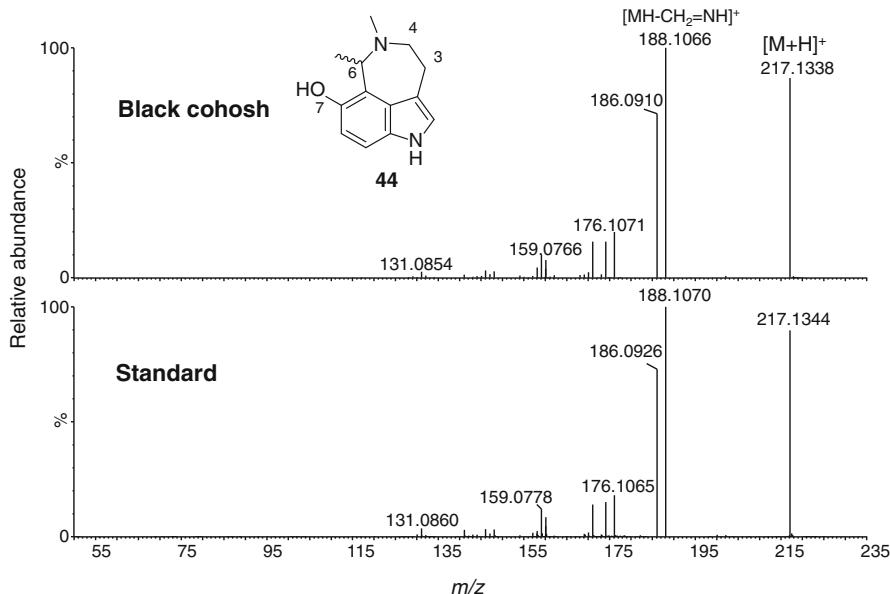
Two separate studies have determined that *N*<sub>ω</sub>-methylserotonin binds to the serotonin transporter and thus may be regarded as selective serotonin reuptake inhibitor (SSRI). Chang et al. reported an IC<sub>50</sub> of 1 µM [104], while Powell et al. reported an IC<sub>50</sub> of 0.49 µM [38]. Direct comparison between these values is difficult, since the two studies used very different concentrations of radiolabeled serotonin (100 vs. 20 nM). However, in both studies the observed inhibition was likely an apparent one, since it is possible that *N*<sub>ω</sub>-methylserotonin acted as a substrate for the serotonin transporter and not necessarily as an inhibitor like the classical therapeutic SSRIs. This is reminiscent of the situation in enzymatic assays where alternative enzyme substrates are sometimes erroneously interpreted as inhibitors.

To gain more insights into its potential in vivo activity, we recently studied the metabolism of *N*<sub>ω</sub>-methylserotonin using human liver microsomes [105]. The experiments showed that this compound is predominantly metabolized by MAO-A. The kinetic parameters ( $K_m$  and  $V_{max}/K_m$ ) were very similar to those of serotonin, suggesting that orally taken *N*<sub>ω</sub>-methylserotonin is unlikely to reach the CNS in any appreciable amount. Thus, *N*<sub>ω</sub>-methylserotonin shares the same fate as other biogenic amines, most of which are not orally active due to degradation by MAO. Overall, these data, combined with the very low content in black cohosh, make it unlikely that *N*<sub>ω</sub>-methylserotonin contributes to the pharmacological activity of black cohosh extracts.

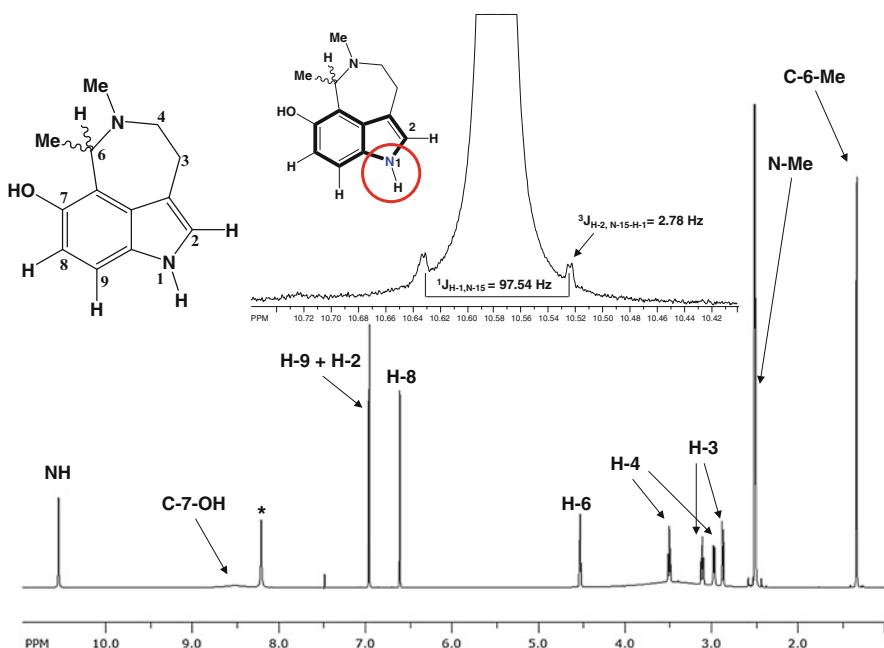
Once the presence of *N*<sub>ω</sub>-methylserotonin was established, it was possible to identify a number of compounds that can be regarded as Pictet-Spengler adducts of *N*<sub>ω</sub>-methylserotonin with various aldehydes.



Previously, we reported the identification of the formaldehyde-derived adducts 6-hydroxy-2-methyl-1,2,3,4-tetrahydro- $\beta$ -carboline (**42**) and 3,4,5,6-tetrahydro-7-hydroxy-5-methyl-1H-azepino[5,4,3-cd]indole, trivially named cimitrypazepine (**43**). In addition, a dihydro- $\beta$ -carboline compound *N*(2)-methyl-6-hydroxy-3,4-dihydro- $\beta$ -carboline (**45**) was also identified. Here we report the identification of the acetaldehyde-derived azepine adduct 3,4,5,6-tetrahydro-7-hydroxy-5,6-dimethyl-1H-azepino[5,4,3-cd]indole (**44**), trivially named methylcimitrypazepine, as a new natural product. The presence of **44** was confirmed by comparison of retention times and fragmentation pattern with the authentic standard (Fig. 2.9). In contrast to the  $\beta$ -carbolines, azepine structures cannot produce retro Diels-Alder fragments and fragment instead by opening of the azepine ring followed by elimination of methylene imine to produce the base peak (*m/z* 188 in **44** and 174 in **43**).



**Fig. 2.9** Identification of methylcimitrypazepine **44**, a new azepine from black cohosh. The stereochemistry at the chiral center is unknown



**Fig. 2.10** 900 MHz  $^1\text{H}$  NMR spectrum of synthetic methylcimitrypazepine (**44**), a new azepine identified in black cohosh. The Asterisk indicates the presence of a trace of formic acid used in the purification procedure

Further confirmation of the structure of **44** was derived from NMR analysis. A synthetic sample [106] was examined by  $^1\text{H}$  NMR (qHNMR, gCOSY, 900 MHz, DMSO- $d_6$ /0.5–0.5 mg/600  $\mu\text{L}$ ), giving a relatively clean proton NMR spectrum (Fig. 2.10) with evidence for the presence of all the expected structural elements. An isolated 4-spin system  $\text{A}_3\text{X}$ , ( $\text{CH}_3-\text{CH}$ ) is present, with the methyl resonance appearing at  $\delta$  1.318 ppm and the CH at  $\delta$  4.526 ppm ( $^3\text{J}_{\text{H,H}}=6.95$  Hz). An ethyl linkage ( $-\text{CH}_2-\text{CH}_2-$ ), contained in the 7-membered ring, exhibits an NMR pattern consistent with protons occupying pseudoaxial and pseudoequatorial orientation, suggesting that the 7-membered ring assumes a puckered (non-planar) conformation. The presence of the aromatic AB pattern ( $\delta$  6.619 and  $\delta$  6.977 ppm;  $^3\text{J}_{\text{H,H}}=8.37$  Hz) confirms that cyclization to **44** has proceeded as desired.

Table 2.2 summarizes the  $^1\text{H}$  NMR data acquired in DMSO- $d_6$  together with those previously reported by Somei et al. [106] for comparison. Two points of particular note relate to the  $^1\text{J}_{\text{H,C}}$  coupling constants, which are measured from the  $^{13}\text{C}$ -satellites in the  $^1\text{H}$  spectrum. From the magnitude of the one-bond C,H couplings to the aliphatic carbons, it is clear which carbons are attached to the nitrogen in the 7-membered ring, especially the methylene carbons, and these are consistent with the indicated structure. Furthermore, a detailed examination of the signal from the indole proton ( $\delta$  10.579 br. s), which is attached to both  $^{14}\text{N}$  (99.64 % isotopic abundance, spin=1) and  $^{15}\text{N}$  (0.36 % isotopic abundance, spin= $\frac{1}{2}$ ), reveals the presence of a  $^{15}\text{N}$

**Table 2.2**  $^1\text{H}$  NMR data for methylcimitypazepine (**44**)

Proton	DMSO-d6 (900 MHz)					MeOH-d4 [106] (500 MHz)	
	$\delta$ Chemical shift (ppm)	$^2\text{J}$ (Hz)	$^3\text{J}$ (Hz)	$^4\text{J}$ (Hz)	$^1\text{J}_{(\text{H,C})}$ (Hz)	$\delta$ Chemical shift (ppm)	$^3\text{J}$ (Hz)
H-1	10.579 s, br, 1H		2.78			Not observed	
H-2	6.975 s, br, 1H		2.78	1.31	178.45	6.95 s	
H-3	2.868 dt, 1H	-16.1	3.03		126.2	3.19–3.27 m, 1H	
	3.107 dddd, 1H	-16.1	4.77,5.75	1.31	123.2	2.97–3.04 m, 2H	
H-4	2.971 ddd, 1H	-13.73	4.72,2.33		n.m. a		
	3.491 td, 1H	-13.73	3.47		135.71	3.60–3.68 m, 1H	
C-6 Me	1.318 d, 3H		6.95		126.6	1.44 d	6.8
H-6	4.526 q, 1H		6.95		137.1	4.73 q	6.8
H-8	6.619 d, 1H		8.37		157.01	6.65 d	8.6
H-9	6.977 d, 1H		8.37		158.95	7.03 d	8.6
N-Me	2.55 s, br, 3H				137.81	2.59 s	
7-OH	8.547 s, br, 1H					Not observed	

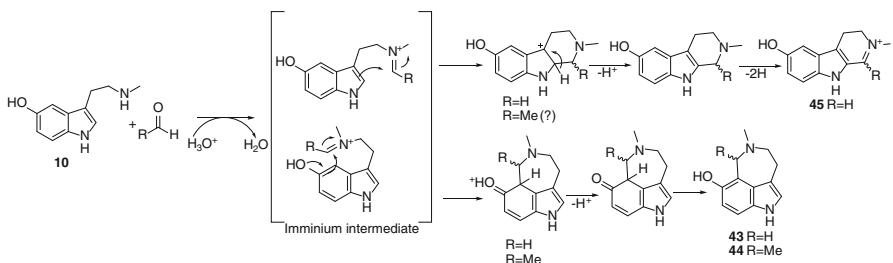
<sup>a</sup>The  $^{13}\text{C}$  satellites for the H-4 signal appearing at 82.971 ppm were heavily overlapped

satellite at natural abundance appearing at the base of the broad  $^1\text{H}$ – $^{14}\text{N}$  resonance (see inset in Fig. 2.10). From the  $^{15}\text{N}$ ,  $^1\text{H}$  satellites, values for  $^1\text{J}_{\text{N}-15\text{N},\text{H}-1}=97.26$  Hz and  $^3\text{J}_{\text{H}-1,\text{H}2}=2.78$  Hz may be extracted and are consistent with the indole structure. The  $^{15}\text{N}$  satellite information is typically observed when the NMR spectrum of the N–H compound is acquired in DMSO-d<sub>6</sub>, where chemical exchange, in this case of the indole H-1 proton, is slow, and the associated resonance of the proton attached to  $^{14}\text{N}$  is not so broad (<  $^1\text{J}_{\text{N},\text{H}-1}$ ) as to completely obscure the  $^{15}\text{N}$  satellite.

While  $\beta$ -carbolines are known natural products, the azepine structures found in black cohosh have not been reported before. As noted by Somei et al. [106], Pictet-Spengler condensation of aldehydes with serotonin or  $N_{\omega}$ -methylserotonin can produce both the carboline and azepine products (see Fig. 2.11).

The azepine cyclization occurs due to activation by the 7-hydroxyl group. Which cyclization pathway will predominate depends both on the experimental conditions and on the type of aldehyde. Typically, acidic conditions favor  $\beta$ -carbolines, while basic conditions favor azepine. Interestingly, we could not conclusively demonstrate the presence of a  $\beta$ -carboline adduct with acetaldehyde, although there were indications that this adduct might also be present. The main difficulty arose due to co-elution of another compound with the same molecular formula, resulting in a tandem mass spectrum that had the same ions as the  $\beta$ -carboline standard but in a slightly different ratio. In any case, the abundance of the  $\beta$ -carboline product was much lower compared to the azepine adduct.

Given that **42–44** can be formed by nonenzymatic condensation, it is unclear whether they represent isolation artifacts or genuine natural products. Both formaldehyde and acetaldehyde may be present as impurities in organic solvent used for extraction, thus this reaction could conceivably occur during sample processing. Alternatively, it is possible that formation of the azepine ring can also be catalyzed



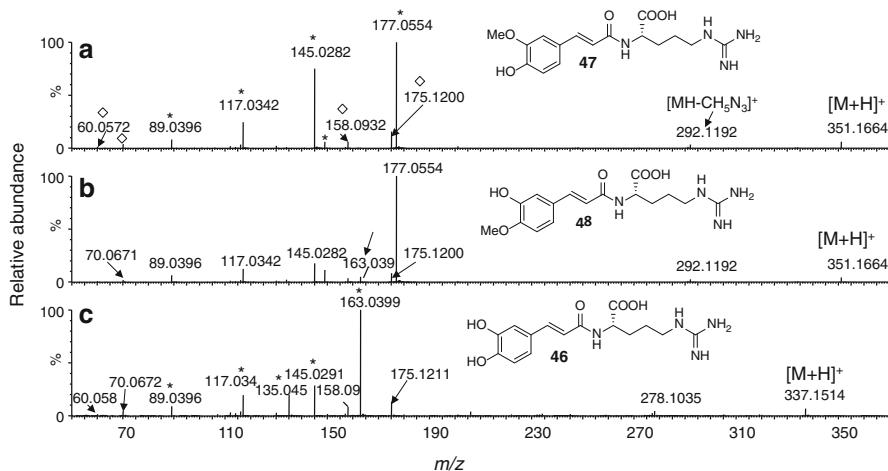
**Fig. 2.11** Proposed mechanism for formation of  $\beta$ -carboline and azepine adducts of *N,N*-dimethylserotonin (**10**) and aldehydes (adapted from [39])

by enzymes, as Pictet-Spengler reactions are proven steps in biosynthetic pathways leading to other natural products. Tests for such a reaction leading to the formation of **42–44** may represent an interesting area of future research.

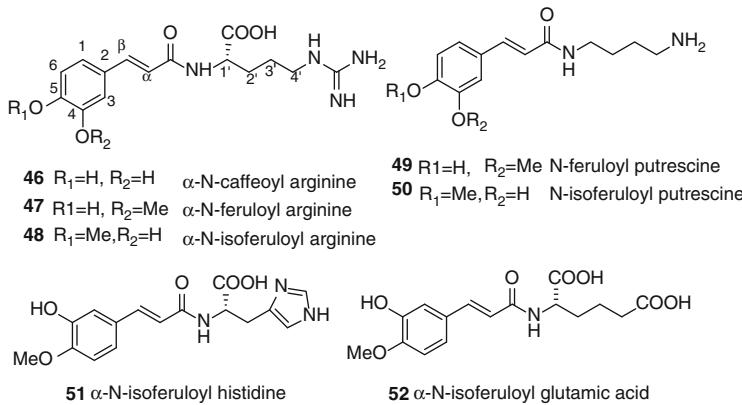
Compound **45** had an elemental composition containing two hydrogens less than **42** and **43** ( $\text{C}_{12}\text{H}_{12}\text{N}_2\text{O}$ ), suggesting a dihydro- $\beta$ -carboline structure. The ready loss of a methyl radical ( $m/z$  186), along with the fragment ion of  $m/z$  170 corresponding to the loss of methylamine ( $\text{MH}-\text{CH}_3\text{NH}_2^+$ ), indicated that the  $N(2)$  nitrogen on the  $\beta$ -carboline ring was methylated. Biosynthetic considerations were used to deduce the position of the double bond on the  $\beta$ -carboline ring. Accordingly, the most likely position of the double bond is 1,2 which was confirmed by comparison of retention time and fragmentation pattern with authentic  $N(2)$ -methyl-6-hydroxy-3,4-dihydro- $\beta$ -carboline. Biosynthetically, this compound is likely formed by dehydrogenation of **42** and represents a new natural product. It should be noted that dihydro- $\beta$ -carbolines are often by-products of Pictet-Spengler condensation [107]. Thus, it is possible that **45** is an isolation artifact. Since **42–45** are rare natural products, their biological activities are largely unexplored. In our laboratory, **42** and **43** did not bind to the 5-HT<sub>7</sub> receptor, nor did they inhibit CYP2D6 or CYP3A4.

## 2.7 Cinnamides

Alkamides are a large group of natural products occurring in at least 33 plant families. They possess a broad range of biological activities including analgesic, cannabinomimetic, and immunomodulatory properties. Interestingly, two recent extensive reviews on alkamides did not list Ranunculaceae as an alkamide-producing family [108, 109]. Cinnamides can be considered a subclass of alkamides in which the acid portion is derived from hydroxycinnamic acids such as caffeic, ferulic, or isoferulic acid, and the amine part is derived from amino acids or biogenic amines. The identification of black cohosh cinnamides, many of which are new natural products, was accomplished by a simple strategy that will be described here using the unique black cohosh cinnamides caffeooyl (**46**) and feruloyl/isoferuloyl arginine (**47,48**) as exemplary compounds.



**Fig. 2.12** Product ion tandem mass spectra of amides of hydroxycinnamic acids; (a)  $\alpha$ -N-feruloyl arginine (**47**); (b)  $\alpha$ -N-isoferuloyl arginine (**48**); and (c)  $\alpha$ -N-caffeooyl arginine (**46**). Ion series corresponding to the acid portion are labeled with “asterisks,” while those corresponding to the amine portion are labeled with a “diamond.” Note the low abundance but diagnostic fragment ion of  $m/z$  163, which is formed by amides of isoferulic but not ferulic acid. Adapted from reference [39], with permission



In general, tandem mass spectra of cinnamides are dominated by the ions originating from the acid part of the molecules. Tandem mass spectra of many cinnamic acids have been published, thus the coupling acid can be identified by comparing the ion series in the unknown amide with referenced spectra of free acids in positive ion mode. Fragment ions corresponding to the protonated amine may also be observed, but their abundance is usually low and strongly depends on the type of amine and the applied collision energy. As illustrated in Fig. 2.12, the fragmentation pattern of  $\alpha$ -N-feruloyl/isoferuloyl arginine (**47**, **48**) is dominated by fragment ions of  $m/z$  177, 149, 145, 117, and 89 originating from the ferulic/isoferulic acid portion of the amide. Similarly,  $\alpha$ -N-caffeooyl arginine (**46**) produces a similar ion series at

**Table 2.3**  $^1\text{H}$  NMR data (900 MHz, DMSO-d<sub>6</sub>) for arginine derivatives **47** and **48**

Proton	Feruloyl ( <b>47</b> )		Isoferuloyl ( <b>48</b> )	
	$\delta$ Chemical shift (ppm)	$^3\text{J}(\text{Hz})$	$\delta$ Chemical shift (ppm)	$^3\text{J}(\text{Hz})$
H-2	7.085 s, br, 1H		6.947 s, br, 1H	
H-5	6.741 d, 1H	7.998	6.9068 d, br, 1H	8.42
H-6	6.901 d, 1H	7.998	6.891 d, br, 1H	8.42
H- $\alpha$	7.213 d, 1H	15.72	6.582 d, 1H	15.66
H- $\beta$	6.626 d, 1H	15.72	7.176 d, 1H	15.66
NH	7.907 d, 1H	7.51	7.907 d, br, 1H	6.75
MeO	3.787 s, 3H		3.779 s, 3H	
NH <sub>2</sub>	9.223 s, br, 2H		8.343 s, br	
NH <sub>2</sub>	9.390 s, br, 2H		9.140 s, br	
H-1'	4.101 dt	7.51, 5.64	4.069 dt, 1H	6.75, 4.07
H-2'	1.730 m, 1H		1.698 m, br, 1H	
	1.640 m, 1H		1.618 m, br, 1H	
H-3'	1.512 m, 1H		1.504 m, br, 1H	
	1.458 m, 1H		1.459 m, br, 1H	
H-4'	3.084 m, 1H		3.066 m, br, 2H	
	3.047 m, 1H			

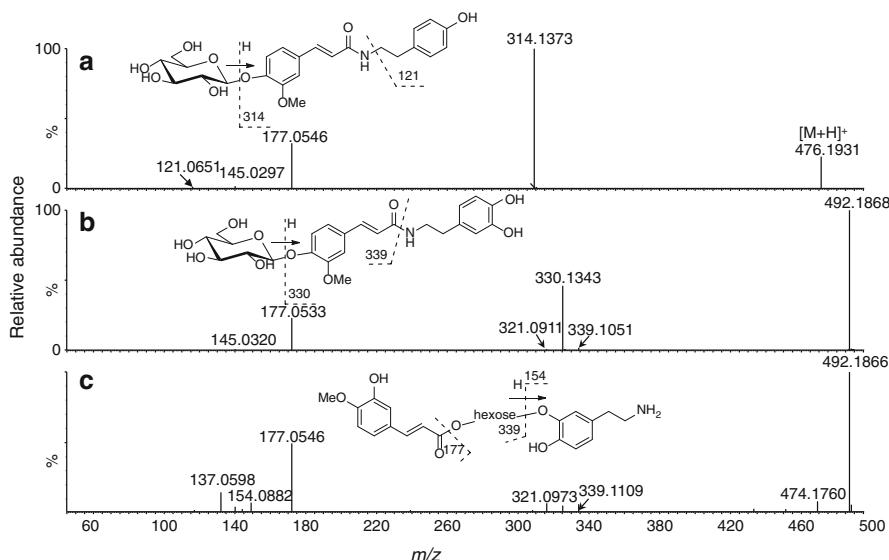
*m/z* 163, 145, 135, 117, and 89. Differentiation between ferulic and isoferulic acid amides can be accomplished based on two characteristic spectral features. The first is that amides of isoferulic acid tend to produce less of the secondary fragment ions (*m/z* 149, 145 and 117) originating from elimination of small neutrals (H<sub>2</sub>O, MeOH, and CO) from the primary acylum ion (*m/z* 177) (compare Fig. 2.12a, b). Another distinguishing feature is the presence of a small, but diagnostic fragment ion of *m/z* 163 with the elemental composition of C<sub>9</sub>H<sub>7</sub>O<sub>3</sub>. This ion was observed only during fragmentation of protonated isoferulic acid, but not ferulic acid ([110] and our own observations). Our investigations on both quadrupole/time-of-flight and ion trap instruments revealed that this fragment ion originates from an ion-molecule reaction in the collision cell and is formed by addition of water to the ion of *m/z* 145.

Once the diagnostic ions from cinnamic acid are observed in the product ion spectrum of an unknown compound, the amine portion of the amide can be deduced based on database searching of the elemental composition of the remainder of the molecule. For example, the molecular formula of feruloyl arginine is C<sub>16</sub>H<sub>22</sub>N<sub>4</sub>O<sub>5</sub>. After subtracting ferulic acid (C<sub>10</sub>H<sub>10</sub>O<sub>4</sub>) and adding a water molecule to account for the fact that water is lost during formation of the amide, the formula of the amine is predicted as C<sub>6</sub>H<sub>14</sub>N<sub>4</sub>O<sub>2</sub>. A database search of this formula yielded only one reasonable hit, arginine. The presence of arginine can then be ascertained by observation of *m/z* 175 (protonated arginine) along with other ions originating from the fragmentation of arginine, such as *m/z* 158, 70, and 60. The presence of the latter two ions, along with the low-abundance ions of *m/z* 278 (**46**) or *m/z* 292 (**47**, **48**) which originate from the loss of neutral guanidine, indicates a free guanidino group and confirms that acylation occurred at the  $\alpha$ -amino group. These structural assignments were confirmed by synthesis of authentic standards (see Table 2.3 for the summary

of 900 MHz  $^1\text{H}$  NMR data). The NMR spectra obtained consist of two spectral elements: (1) the ferulic acid/isoferulic acid components and (2) the arginine component. The spectral elements of the former component consists of signals (an AMX pattern in the case of the ferulic acid moiety and an ABX pattern in the case of the isoferulic acid moiety) associated with the tri-substituted aromatic ring and an AM pattern (first order analysis) associated with the double bond. In all cases, the geometry of the double bond was shown to be *trans* as characterized by the large coupling constant ( $^3J_{\text{H},\text{H}} = \sim 15.7$  Hz, average) between the olefinic protons. The signals of the arginine component were complex, exhibiting non-first order behavior upon resolution enhancement, and consisted of a signal from the CH proton  $\alpha$  to the carboxyl group of the amino acid, which appears as a doublet of triplets (dt) with a pattern arising from spin coupling to the diastereotopic protons H-2' and an amide NH proton. This represents the key bonding link between the two structural components in the molecule. The proton pairs H-3' and H-4' are also diastereotopic, as a result of the chiral center at C-1'. Because the molecules exist with the free acid in the arginine residue, it may be presumed that both the carboxyl and the guanidino group are largely ionic at physiological pH, and thus zwitterions will predominate in solution.

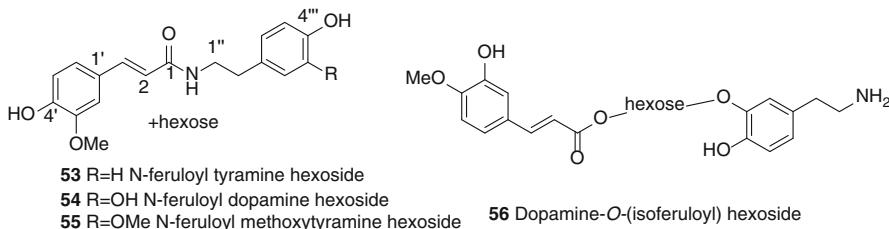
The above-described mass spectrometric identification strategy also led to identification of feruloyl (**49**) and isoferuloyl (**50**) putrescine, and to the tentative identification of the two new cinnamides  $\alpha$ -*N*-isoferuloyl histidine (**51**) and  $\alpha$ -*N*-isoferuloyl glutamic acid (**52**). Although mass spectrometry cannot distinguish between the *cis* and *trans* configurations of the double bonds in these compounds, based on co-elution with the synthetic standards the configuration was determined to be *trans* in all cases. It is interesting to note that in all the cinnamoyl amino acids identified here isoferulic acid analogs were more abundant than the corresponding ferulic or caffeic acid analogs.

Another part of the cinnamide metabolome consists of glycosidated analogs. Interestingly, glycosidation was observed only for cinnamides where the coupling amine originates from the tyrosine metabolites tyramine and dopamine. Identification of glycosidated analogs can in principle be accomplished using the same basic strategy described above. In general, identification of glycosidated cinnamides involves assignment of three structural components: the amide moiety, the type of sugar, and the position of glycosidation. All glycosides detected in black cohosh were hexosides which can be ascertained based on tandem mass spectra characterized by a common loss of 162 units ( $\text{C}_6\text{H}_{10}\text{O}_5$ ); however, the type of hexose or its configuration ( $\alpha$  or  $\beta$ ) cannot be determined based on this information. After subtracting the hexose from the elemental composition of the entire molecule, the cinnamide core can be identified based on database searches. Using this strategy, hexosides of *N*-feruloyltyramine (**53**), *N*-feruloyldopamine (**54**), and *N*-feruloyl methoxytyramine (**55**) were tentatively identified. The identity of the hexose at this point is unknown as it cannot be determined based on the mass spectrometric data alone. Interestingly, a recent publication by Yim et al. described identification of *cis* and *trans* *N*-feruloyl tyramine-4'-*O*- $\beta$ -D-allopyranoside and *trans* *N*-feruloylmethoxytyramine-4'-*O*- $\beta$ -D-allopyranoside from *Actaea* (syn. *Cimicifuga*) *heracleifolia* [111]. Similarly, *N*-feruloyl methoxytyramine-4'-*O*-galactoside has been identified in *Actaea* (syn. *Cimicifuga*) *dahurica* [112]. The possibility that black cohosh produces allosides is intriguing, as



**Fig. 2.13** Product ion tandem mass spectra of (a) synthetic *N*-feruloyl tyramine-4'-*O*- $\beta$ -D-glucopyranoside; (b) synthetic *N*-feruloyl tyramine-4'-*O*- $\beta$ -D-glucopyranoside; and (c) a putative dopamine-*O*-(isoferuloyl)-hexoside from black cohosh (**56**). Note the absence of protonated amine in the spectra of *N*-feruloyl tyramine glucoside (*m/z* 138) and *N*-feruloyl dopamine glucoside (*m/z* 154). In the spectrum of the unknown compound **56**, fragment ions originating from dopamine (*m/z* 154 and 137) are clearly visible suggesting that it is not a cinnamide, but most likely a dopamine hexoside where the hexose unit is acylated with ferulic/isoferulic acid. The site of hexose attachment to dopamine was arbitrarily drawn to position 3 for clarity. Spectra were taken at 15 eV collision energy on a Waters SYNAPT quadrupole/time-of-flight mass spectrometer

these hexosides are quite rare and detailed mass spectrometric investigations of this type of compounds are lacking to date.



When it comes to establishing the position of glycosidation, we initially proposed that the presence of the fragment ion corresponding to the hexosidated acylum ion of ferulic/isoferulic acid at *m/z* 339 and the secondary fragment formed by a loss of water (*m/z* 321) is an indication that the sugar is attached to the acid portion [39]. To obtain experimental confirmation for this hypothesis, we synthesized standards of *N*-feruloyl dopamine-4'-*O*- $\beta$ -D-glucopyranoside and *N*-feruloyl tyramine-4'-*O*- $\beta$ -D-glucopyranoside. Interestingly, only the dopamine analog fragmented to produce the glucosidated feruloyl ion in low abundance (Fig. 2.13b). In both cases,

loss of glucose ( $m/z$  314 and 330) was the dominant fragmentation pathway. From these limited studies we can conclude that the *presence* of  $m/z$  339 and 321 may be used as an indication of glycosidation on the acid part, but the *absence* of such ions does not exclude glycosidation on the ferulic acid, particularly if a weak precursor ion signal precludes detection of these weak fragments. Another important observation was that fragment ions corresponding to the protonated amine ( $m/z$  138 and  $m/z$  154 for tyramine and dopamine, respectively) were not observed (Fig. 2.12a, b). This observation can be explained by a lack of readily available acidic hydrogen that needs to be transferred to the amine during fragmentation.

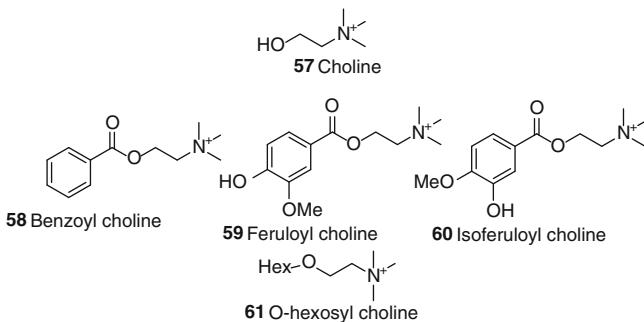
In contrast, the tandem mass spectrum of a metabolite whose molecular formula nominally corresponds to a glycosidated feruloyl/isoferuloyl dopamine analog (**56**) (Fig. 2.13c) clearly shows the dopamine-related ions at  $m/z$  154 and 137. The presence of protonated dopamine suggests that the amino group of dopamine is free, thus this compound is not a cinnamide. The spectrum also shows an ion of  $m/z$  339 representing feruloyl/isoferuloyl hexose [113, 114]. These spectral features are consistent with a dopamine hexoside where the hexose unit is acylated with ferulic/isoferulic acid. Based on the presence of a weak, but diagnostic fragment ion of  $m/z$  163 we can ascertain that the hexose is acylated with isoferulic acid [39]. However, neither the site of attachment of hexose to dopamine nor the position of acylation of the hexose can be determined based on the mass spectrometric data, although position 6 on the hexose part appears to be the most common acylation site [114, 115]. In general, cinnamate conjugates with amines and amino acids are widely distributed throughout the plant kingdom, with coffee being the major dietary source [116, 117]. Biosynthetically, they are formed by transfer of an acyl group from hydroxycinnamoyl-SCoA onto the corresponding amine, catalyzed by hydroxycinnamoyl-CoA acyltransferases. A gene for benzoyl/hydroxycinnamoyl transferase was identified by Spiering et al. and found expressed in all tested tissues including roots/rhizomes [53]. A glycosyl transferase gene was also detected that could be involved in the synthesis of glycosidated analogs. Although cinnamides in general are ubiquitous compounds, the metabolome found in black cohosh is unique. The abundance of arginine amides in particular is quite distinctive. Along with observation of numerous guanidine alkaloids, this implies that arginine processing represents a significant component of the plant's metabolic activity. Since many of the cinnamoyl amino acids are new natural products, it is still early to say whether they are unique to the genus of *Actaea*, but they are certainly good candidates for inclusion as new standardization markers. In contrast to their alkyl counterparts, which display a number of interesting biological activities, hydroxycinnamoyl amides have been mostly studied for their antioxidant activity [118]. However, given the uniqueness and novelty of the compounds identified in black cohosh, there is a lot to be learned about their biological activities.

## 2.8 Cholines and Betains

Compounds derived from amino alcohol choline (**57**) and containing a quaternary ammonium cation are generally designated as cholines. Choline itself is an essential nutrient present in egg yolks, meats, milk, and other animal products. It is also sold

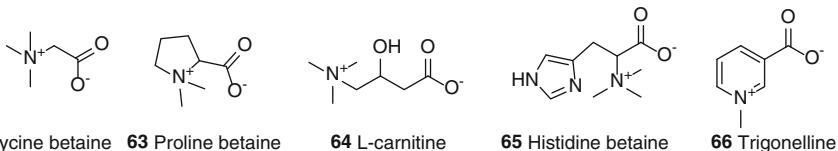
as dietary supplement in free form or as phosphatidylcholine. Choline was identified in black cohosh by mass spectra database searching and by comparison with an authentic standard. A number of choline esters with aromatic acids such as benzoic, ferulic, and isoferulic acid (**58–60**) were also identified by comparison with authentic standards. During CID, cholines display a characteristic loss of trimethylamine from the precursor ion, which can be used for selective detection of this class of compounds. For example, in the spectrum of benzoyl choline (**58**), the precursor ion of *m/z* 208 loses trimethylamine to form an ion at *m/z* 149, which can further fragment to lose CO<sub>2</sub> and produce an acylium ion of *m/z* 105, typical for benzoyl derivatives (see Table 2.1). A more detailed discussion of the fragmentation pathways of choline esters can be found in Böttcher et al. [119].

Aromatic choline esters have been predominantly reported from seeds of plants from the Brassicaceae. Choline esters and other compounds with quaternary nitrogen are prototype agents acting on the cholinergic system. Benzoyl choline, for example, has been of interest to pharmacologists since the early 1950s, primarily as a model substrate for butyrylcholinesterase [120] and as a pharmacological tool to investigate various actions of acetyl choline. In the cholinergic system, benzoyl choline shows weak muscarinic and strong nicotinic activity [121]. Similar cholinergic activities have not been investigated for ferulic and isoferulic acid choline esters. However, the biological activity of choline esters after oral ingestion is low due to rapid hydrolysis into acid and choline catalyzed by butyrylcholinesterase.



Another interesting compound (**61**) from this class was detected in the highly polar water fraction of black cohosh. The elemental composition and product ion tandem mass spectrometric analysis of **61** indicated that it also contains a choline moiety, but the remainder of the molecule corresponded to that of a hexose sugar, which suggests a tentative assignment of this compound as *O*-hexosyl choline. This identification is also consistent with late elution of **61** under HILIC conditions, indicative of a highly polar molecule. There have been no reports of this type of compound occurring in plants.

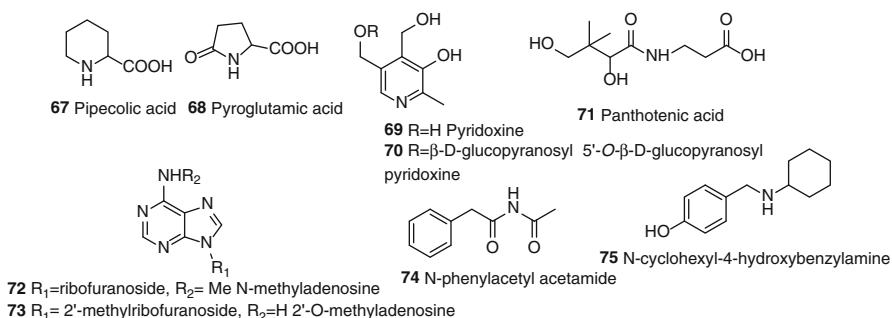
In addition to choline derivatives, several betaines (**62–66**) have been identified in black cohosh. Chemically, betains can be considered permanent zwitterions. In plant cells, betains serve important roles as osmoprotectors balancing the osmotic pressure difference between cells and their surroundings.



Among the identified betains, L-carnitine (**64**) and trigonelline (**66**) have been most extensively studied. L-carnitine is widely distributed throughout the plant and animal kingdoms. In living cells, it plays an important role in energy production, since it helps transport fatty acids from the cytoplasm into the mitochondria, where their degradation takes place. Although many plants contain L-carnitine, meat products are the main source of L-carnitine in the human diet. L-Carnitine is also sold as a dietary supplement for its purported beneficial roles in the contexts of cardiovascular disease, diabetes, or weight loss. Trigonelline is a widespread plant alkaloid formed by methylation of nicotinic acid. Among other plants, it has been identified in coffee [122], and it has been extensively investigated for a number of purported beneficial properties such as antioxidant, neuroprotective, or hypoglycemic (reviewed in [123]). Interestingly, a recent study by Allred et al. identified trigonelline as a novel phytoestrogen capable of stimulating growth of MCF-7 cells in very low doses [124]. This is a potentially significant finding, given that this is such a common compound. However, these results have not been confirmed by others, and black cohosh is not estrogenic as determined in numerous in vitro and in vivo studies [11, 125–128].

## 2.9 Miscellaneous Primary and Secondary Metabolites

Mass spectrometric dereplication has also been used to identify a number of primary metabolites in black cohosh extracts. Those include the nonessential amino acids pipecolic acid (**67**) and pyroglutamic acid (**68**), vitamins (**69–71**) and methylated nucleosides (**72, 73**). Two unusual compounds **74** and **75** were tentatively identified at level 3 based on interpretation of their tandem mass spectra. The origin of these constituents is unclear, and it is possible that they are impurities from extraction solvents.



## 2.10 Concluding Remarks and Future Directions

As the number of identified nitrogenous compounds in black cohosh approaches one hundred, the overall picture of this important part of the *A. racemosa* metabolome is gradually taking shape. Nitrogenous metabolites show great chemical diversity, including the presence of some unique and rare compounds. In particular, this plant appears to extensively process arginine, as manifested in the presence of numerous acylated derivatives of arginine, as well as in the abundance of guanidine-type alkaloid diversity. Guanidine alkaloids are rare in the plant kingdom, and we think that new compounds and even chemical templates are likely to be discovered from this part of the metabolome in the future. Another interesting observation is the presence of a significant number of quaternary amines. These, along with highly basic and essentially permanently positively charged guanidines, offer a potential explanation for the biological relevance of phenolic acids which might act as counter ions for positively charged alkaloids. Formation of strong ion pairs between abundant phenolic acids and alkaloids should be taken into account, not only during the isolation of both the acids and the alkaloids, but also when interpreting biological observations related to both classes of phytoconstituents.

The presence of glycosidated cinnamides is also an interesting discovery. This type of compounds is, again, not very common in the plant kingdom. While the identity of the specific hexose remains to be shown, based on chemotaxonomic considerations it seems plausible that allose is the coupling sugar. This hypothesis is very intriguing, as such compounds have not been explored in detail either chemically or biologically.

When it comes to biological relevance, the discovery of alkaloids represents an important breakthrough towards a better understanding of the pharmacological properties of black cohosh. Alkaloids are flagship natural products with proven biological activities, particularly as CNS agents, and as such they are likely the active principles responsible for the observed CNS activities of black cohosh. It is important to note that, as alkaloids are minor but potent constituents, small differences in their quantity may lead to large differences in the observed activities of crude extracts. In this regard, the alkaloids may indeed be a missing link that can explain at least some of the variability in various in vitro studies or clinical trials of black cohosh. As all clinical trials to date were carried out without consideration and/or knowledge of the existence of these alkaloids, there is no way of knowing how much (if any) of these compounds were present in the tested extracts.

In an indirect way, the discovery of alkaloids in black cohosh is also relevant for a better understanding of the biological activities of other classes of *Actaea/Cimicifuga* compounds, particularly phenolic acids. As noted above, these acids tend to form relatively strong ion pairs with alkaloids, making the isolation of pure acids free from alkaloids a demanding task. If the *N*<sub>ω</sub>-methylserotonin case is any guide, even traces of a very potent compound may produce positive results in a bioassay, which may then be erroneously attributed to the major compound. This is particularly true if compounds are tested at high concentration, as is frequently the case. For example, at 100 µM of the test compound, even 99 % purity would result

in a 1 µM concentration of impurity. At this level, many alkaloids described here exhibit significant biological activity. Therefore, the present findings of a prominent alkaloid metabolome in black cohosh make it important to check any isolated compound, especially phenolic acids, for possible alkaloid impurities. A prominent example of a minor alkaloid impurity being responsible for the observed prominent biological effect has recently been reported by Fitch et al. [129].

Future research in this area is promising and is likely to produce exciting results. Even though a large number of nitrogenous compounds have already been identified, it is likely that the diversity reported here is still only the tip of the iceberg. Many more new compounds or even chemical templates will likely be discovered. As many of the identified compounds are either new to black cohosh or even new natural products, there is plenty to learn about them. Potential new insights may include biosynthetic pathways, chemotaxonomic relationships and, most certainly, a more comprehensive understanding of their biological properties. Finally, given the known broad range of biological activities of alkaloids in general and the already large variety of alkaloids in black cohosh in particular, it is reasonable to propose that the inclusion of some of these compounds will improve future standardization protocols for black cohosh preparations.

## References

1. McKenna DJ, Jones K, Humphrey S, Hughes K (2001) Black cohosh: efficacy, safety, and use in clinical and preclinical applications. *Altern Ther Health Med* 7:93–100
2. Borrelli F, Ernst E (2008) Black cohosh (*Cimicifuga racemosa*) for menopausal symptoms: a systematic review of its efficacy. *Pharmacol Res* 58:8–14
3. Mahady GB, Parrot J, Lee C, Yun GS, Dan A (2003) Botanical dietary supplement use in peri- and postmenopausal women. *Menopause* 10:65–72
4. Lindstrom A, Ooyen C, Lynch ME, Blumenthal M (2013) Supplement sales increase 5.5% in 2012. *HerbalGram* 99:60–65
5. Osmer R, Friede M, Liske E, Schnitker J, Freudenstein J, Henneicke-von Zepelin HH (2005) Efficacy and safety of isopropanolic black cohosh extract for climacteric symptoms. *Obstet Gynecol* 105:1074–1083
6. Wuttke W, Seidlova-Wuttke D, Gorkow C (2003) The *Cimicifuga* preparation BNO 1055 vs. conjugated estrogens in a double-blind placebo-controlled study: effects on menopause symptoms and bone markers. *Maturitas* 44(Suppl 1):S67–S77
7. Frei-Kleiner S, Schaffner W, Rahlf VW, Bodmer C, Birkhäuser M (2005) *Cimicifuga racemosa* dried ethanolic extract in menopausal disorders: a double-blind placebo-controlled clinical trial. *Maturitas* 51:397–404
8. Kronenberg F, Fugh-Berman A (2002) Complementary and alternative medicine for menopausal symptoms: a review of randomized, controlled trials. *Ann Intern Med* 137:805–813
9. Jacobson JS, Troxel AB, Evans J, Klaus L, Vahdat L, Kinne D, Lo KM, Moore A, Rosenman PJ, Kaufman EL, Neugut AI, Grann VR (2001) Randomized trial of black cohosh for the treatment of hot flashes among women with a history of breast cancer. *J Clin Oncol* 19:2739–2745
10. Newton KM, Reed SD, LaCroix AZ, Grothaus LC, Ehrlich K, Guiltinan J (2006) Treatment of vasomotor symptoms of menopause with black cohosh, multibotanicals, soy, hormone therapy, or placebo: a randomized trial. *Ann Intern Med* 145:869–879
11. Liske E, Hanggi W, Henneicke-von Zepelin HH, Boblitz N, Wüstenberg P, Rahlf VW (2002) Physiological investigation of a unique extract of black cohosh (*Cimicifugae racemosae* rhi-

- zoma): a 6-month clinical study demonstrates no systemic estrogenic effect. *J Womens Health Gend Based Med* 11:163–174
- 12. Geller SE, Shulman LP, van Breemen RB, Banuvar S, Zhou Y, Epstein G, Hedayat S, Nikolic D, Krause EC, Piersen CE, Bolton JL, Pauli GF, Farnsworth NR (2009) Safety and efficacy of black cohosh and red clover for the management of vasomotor symptoms: a randomized controlled trial. *Menopause* 16:1156–1166
  - 13. Whiting PW, Clouston A, Kerlin P (2002) Black cohosh and other herbal remedies associated with acute hepatitis. *Med J Aust* 177:440–443
  - 14. Mahady GB, Low Dog T, Barrett ML, Chavez ML, Gardiner P, Ko R, Marles RJ, Pellicore LS, Giancaspro GI, Sarma DN (2008) United States Pharmacopeia review of the black cohosh case reports of hepatotoxicity. *Menopause* 15:628–638
  - 15. Naser B, Schnitker J, Minkin MJ, de Arriba SG, Nolte KU, Osmers R (2011) Suspected black cohosh hepatotoxicity: no evidence by meta-analysis of randomized controlled clinical trials for isopropanolic black cohosh extract. *Menopause* 18:366–375
  - 16. Teschke R, Bahre R, Gentner A, Fuchs J, Schmidt-Taenzer W, Wolff A (2009) Suspected black cohosh hepatotoxicity—challenges and pitfalls of causality assessment. *Maturitas* 63:302–314
  - 17. Teschke R (2008) Questions regarding causality in presumed black cohosh hepatotoxicity. *Del Med J* 80:233–234, author reply 235
  - 18. Teschke R (2010) Black cohosh and suspected hepatotoxicity: inconsistencies, confounding variables, and prospective use of a diagnostic causality algorithm. A critical review. *Menopause* 17:426–440
  - 19. Lude S, Torok M, Dieterle S, Knapp AC, Kaeufeler R, Jaggi R, Spornitz U, Krahenbuhl S (2007) Hepatic effects of *Cimicifuga racemosa* extract in vivo and in vitro. *Cell Mol Life Sci* 64:2848–2857
  - 20. Huang Y, Jiang B, Nuntanakorn P, Kennelly EJ, Shord S, Lawal TO, Mahady GB (2010) Fukinolic acid derivatives and triterpene glycosides from black cohosh inhibit CYP isozymes, but are not cytotoxic to Hep-G2 cells in vitro. *Curr Drug Saf* 5:118–124
  - 21. Fabricant DS, Farnsworth NR (2005) Black cohosh (*Cimicifuga racemosa*). In: Coates PM, Blackman MR, Cragg GM, Levine M, Moss J, White JD (eds) Encyclopedia of dietary supplements. Marcel Dekker, New York, pp 41–54
  - 22. Li JX, Yu ZY (2006) Cimicifugae rhizoma: from origins, bioactive constituents to clinical outcomes. *Curr Med Chem* 13:2927–2951
  - 23. Avula B, Wang YH, Smillie TJ, Khan IA (2009) Quantitative determination of triterpenoids and formononetin in rhizomes of black cohosh (*Actaea racemosa*) and dietary supplements by using UPLC-UV/ELS detection and identification by UPLC-MS. *Planta Med* 75:381–386
  - 24. Ciciek SS, Aberham A, Ganzena M, Stuppner H (2011) Quantitative analysis of cycloartane glycosides in black cohosh rhizomes and dietary supplements by RRLC-ELSD and RRLC-qTOF-MS. *Anal Bioanal Chem* 400:2597–2605
  - 25. He K, Pauli GF, Zheng B, Wang H, Bai N, Peng T, Roller M, Zheng Q (2006) Cimicifuga species identification by high performance liquid chromatography-photodiode array/mass spectrometric/evaporative light scattering detection for quality control of black cohosh products. *J Chromatogr A* 1112:241–254
  - 26. Qiu F, Imai A, McAlpine JB, Lankin DC, Burton I, Karakach T, Farnsworth NR, Chen SN, Pauli GF (2012) Dereplication, residual complexity, and rational naming: the case of the Actaea triterpenes. *J Nat Prod* 75:432–443
  - 27. Qiu F, McAlpine JB, Krause EC, Chen S, Pauli GF (2014) Pharmacognosy of black cohosh: phytochemical and biological profile of a widely used botanical. In: Kinghorn A, Falk H, Kobayashi J (eds) Progress in the chemistry of organic natural products. Springer, New York/Vienna
  - 28. Godecke T, Nikolic D, Lankin DC, Chen SN, Powell SL, Dietz B, Bolton JL, van Breemen RB, Farnsworth NR, Pauli GF (2009) Phytochemistry of cimicifugic acids and associated bases in *Cimicifuga racemosa* root extracts. *Phytochem Anal* 20:120–133

29. Kruse SO, Löhning A, Pauli GF, Winterhoff H, Nahrstedt A (1999) Fukiic and piscidic acid esters from the rhizome of *Cimicifuga racemosa* and the in vitro estrogenic activity of fukinolic acid. *Planta Med* 65:763–764
30. Cordell GA, Quinn-Beattie ML, Farnsworth NR (2001) The potential of alkaloids in drug discovery. *Phytother Res* 15:183–205
31. Finnemore H (1909) Constituents of rhizome of *Cimicifuga racemosa*. *Pharm J* 83:145
32. Crum JD, Cassady JM, Olmstead PM, Picha NJ (1965) The chemistry of alkaloids. I. The screening of some native Ohio plants. *Proc West Va Acad Sci* 37:143–147
33. Dan C, Zhou Y, Ye D, Peng S, Ding L, Gross ML, Qiu SX (2007) Cimicifugadine from *Cimicifuga foetida*, a new class of triterpene alkaloids with novel reactivity. *Org Lett* 9:1813–1816
34. Gao JC, Zhang JC, Chen Y, Yang MS, Xiao PG (2007) Study on chemical constituents from rhizomes of *Actaea asiatica*. *Zhongguo Zhong Yao Za Zhi* 32:2256–2258
35. Gao JC, Zhang JC, Zhu GY, Yang MS, Xiao PG (2007) Chromones and indolinone alkaloids from *Actaea asiatica* Hara. *Biochem Syst Ecol* 35:467–469
36. Fabricant DS, Nikolic D, Lankin DC, Chen SN, Jaki BU, Krunic A, van Breemen RB, Fong HH, Farnsworth NR, Pauli GF (2005) Cimipronidine, a cyclic guanidine alkaloid from *Cimicifuga racemosa*. *J Nat Prod* 68:1266–1270
37. Godecke T, Lankin DC, Nikolic D, Chen SN, van Breemen RB, Farnsworth NR, Pauli GF (2009) Guanidine alkaloids and Pictet-Spengler adducts from black cohosh (*Cimicifuga racemosa*). *J Nat Prod* 72:433–437
38. Powell SL, Gödecke T, Nikolic D, Chen SN, Ahn S, Dietz B, Farnsworth NR, van Breemen RB, Lankin DC, Pauli GF, Bolton JL (2008) In vitro serotonergic activity of black cohosh and identification of N(omega)-methylserotonin as a potential active constituent. *J Agric Food Chem* 56:11718–11726
39. Nikolic D, Gödecke T, Chen SN, White J, Lankin DC, Pauli GF, van Breemen RB (2012) Mass spectrometric dereplication of nitrogen-containing constituents of black cohosh (*Cimicifuga racemosa* L.). *Fitoterapia* 83:441–460
40. Kind T, Fiehn O (2006) Metabolomic database annotations via query of elemental compositions: mass accuracy is insufficient even at less than 1 ppm. *BMC Bioinformatics* 7:234
41. Kind T, Fiehn O (2007) Seven Golden Rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. *BMC Bioinformatics* 8:105
42. Bristow AW, Webb KS, Lubben AT, Halket J (2004) Reproducible product-ion tandem mass spectra on various liquid chromatography/mass spectrometry instruments for the development of spectral libraries. *Rapid Commun Mass Spectrom* 18:1447–1454
43. Cech NB, Kate Y (2013) Mass spectrometry for natural products research: challenges, pitfalls, and opportunities. *LCCG North America* 31:938–947
44. Schymanski EL, Jeon J, Gulde R, Fenner K, Ruff M, Singer HP, Hollender J (2014) Identifying small molecules via high resolution mass spectrometry: communicating confidence. *Environ Sci Technol* 48(4):2097–2098
45. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, Fan TW, Fiehn O, Goodacre R, Griffin JL, Hankemeier T, Hardy N, Harnly J, Higashi R, Kopka J, Lane AN, Lindon JC, Marriott P, Nicholls AW, Reily MD, Thaden JJ, Viant MR (2007) Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* 3:211–221
46. Biemann K (2002) Four decades of structure determination by mass spectrometry: from alkaloids to heparin. *J Am Soc Mass Spectrom* 13:1254–1272
47. Biemann K, Grossert JS, Hugo JM, Occolowitz J, Warren FL (1965) The indole alkaloids. IV. The structure of henningsamine. *J Chem Soc* 46:2814–2818
48. Dookeran N, Yalcin T, Harrison A (1996) Fragmentation reactions of protonated alfa-amino acids. *J Mass Spectrom* 31:500–508
49. Csonka IP, Paizs B, Suhai S (2004) Modeling of the gas-phase ion chemistry of protonated arginine. *J Mass Spectrom* 39:1025–1035

50. Shek PY, Zhao J, Ke Y, Siu KW, Hopkinson AC (2006) Fragmentations of protonated arginine, lysine and their methylated derivatives: concomitant losses of carbon monoxide or carbon dioxide and an amine. *J Phys Chem A* 110:8282–8296
51. Stewart JA, Dobson JE (1965) Trypsin-catalyzed hydrolysis of N-benzoyl-L-arginine ethyl ester at low pH. *Biochemistry* 4:1086–1091
52. Fasehun OA, Gross SS, Rubin LE, Jaffe EA, Griffith OW, Levi R (1990) L-arginine, but not N alpha-benzoyl-L-arginine ethyl ester, is a precursor of endothelium-derived relaxing factor. *J Pharmacol Exp Ther* 255:1348–1353
53. Spiering MJ, Urban LA, Nuss DL, Gopalan V, Stoltzfus A, Eisenstein E (2011) Gene identification in black cohosh (*Actaea racemosa* L.): expressed sequence tag profiling and genetic screening yields candidate genes for production of bioactive secondary metabolites. *Plant Cell Rep* 30:613–629
54. Riggan RM, Kissinger PT (1976) Letter: identification of salsolinol as a phenolic component in powdered cocoa and cocoa-based products. *J Agric Food Chem* 24:900
55. Riggan RM, McCarthy MJ, Kissinger PT (1976) Identification of salsolinol as a major dopamine metabolite in the banana. *J Agric Food Chem* 24:189–191
56. Duncan MW, Smythe GA, Nicholson MV, Clezy PS (1984) Comparison of high-performance liquid chromatography with electrochemical detection and gas chromatography-mass fragmentography for the assay of salsolinol, dopamine and dopamine metabolites in food and beverage samples. *J Chromatogr* 336:199–209
57. Dostert P, Benedetti MS, Bellotti V, Allievi C, Dordain G (1990) Biosynthesis of salsolinol, a tetrahydroisoquinoline alkaloid, in healthy subjects. *J Neural Transm Gen Sect* 81:215–223
58. Mravc B (2006) Salsolinol, a derivate of dopamine, is a possible modulator of catecholaminergic transmission: a review of recent developments. *Physiol Res* 55:353–364
59. Lee J, Ramchandani VA, Hamazaki K, Engleman EA, McBride WJ, Li TK, Kim HY (2010) A critical evaluation of influence of ethanol and diet on salsolinol enantiomers in humans and rats. *Alcohol Clin Exp Res* 34:242–250
60. Melzig MF, Putscher I, Henklein P, Haber H (2000) In vitro pharmacological activity of the tetrahydroisoquinoline salsolinol present in products from *Theobroma cacao* L. like cocoa and chocolate. *J Ethnopharmacol* 73:153–159
61. Jarry H, Metten M, Spengler B, Christoffel V, Wuttke W (2003) In vitro effects of the *Cimicifuga racemosa* extract BNO 1055. *Maturitas* 44(Suppl 1):S31–S38
62. Musshoff F, Lachenmeier DW, Kroener L, Schmidt P, Dettmeyer R, Madea B (2003) Simultaneous gas chromatographic-mass spectrometric determination of dopamine, norsalsolinol and salsolinol enantiomers in brain samples of a large human collective. *Cell Mol Biol* 49:837–849
63. Musshoff F, Daldrup T, Bonte W, Leitner A, Lesch OM (1997) Salsolinol and norsalsolinol in human urine samples. *Pharmacol Biochem Behav* 58:545–550
64. Facchini PJ (2001) Alkaloids biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. *Annu Rev Plant Physiol Plant Mol Biol* 52:29–66
65. Schmidt J, Raith K, Boettcher C, Zenk MH (2005) Analysis of benzylisoquinoline-type alkaloids by electrospray tandem mass spectrometry and atmospheric pressure photoionization. *Eur J Mass Spectrom* (Chichester, Eng) 11:325–333
66. Schmidt J, Boettcher C, Kuhtz C, Kutchan TM, Zenk MH (2007) Poppy alkaloid profiling by electrospray tandem mass spectrometry and electrospray FT-ICR mass spectrometry after [ring-13C6]-tyramine feeding. *Phytochemistry* 68:189–202
67. Zhu W, Cadet P, Baggerman G, Mantione KJ, Stefano GB (2005) Human white blood cells synthesize morphine: CYP2D6 modulation. *J Immunol* 175:7357–7362
68. Zhu W, Mantione KJ, Stefano GB (2004) Reticuline exposure to invertebrate ganglia increases endogenous morphine levels. *Neuro Endocrinol Lett* 25:323–330
69. Grobe N, Zhang B, Fisinger U, Kutchan TM, Zenk MH, Guengerich FP (2009) Mammalian cytochrome P450 enzymes catalyze the phenol-coupling step in endogenous morphine biosynthesis. *J Biol Chem* 284:24425–24431

70. Neri C, Ghelardini C, Sotak B, Palmiter RD, Guarna M, Stefano G, Bianchi E (2008) Dopamine is necessary to endogenous morphine formation in mammalian brain in vivo. *J Neurochem* 106:2337–2344
71. Banning JW, Uretsky NJ, Patil PN, Beal JL (1980) Reticuline: a dopamine receptor blocker. *Life Sci* 26:2083–2091
72. Nikolaev VO, Böttcher C, Dees C, Bunemann M, Lohse MJ, Zenk MH (2007) Live cell monitoring of mu-opioid receptor-mediated G-protein activation reveals strong biological activity of close morphine biosynthetic precursors. *J Biol Chem* 282:27126–27132
73. Stevigny C, Jiwan JL, Rozenberg R, de Hoffmann E, Quetin-Leclercq J (2004) Key fragmentation patterns of aporphine alkaloids by electrospray ionization with multistage mass spectrometry. *Rapid Commun Mass Spectrom* 18:523–528
74. Zhang Y, Shi Q, Shi P, Zhang W, Cheng Y (2006) Characterization of isoquinoline alkaloids, diterpenoids and steroids in the Chinese herb Jin-Guo-Lan (*Tinospora sagittata* and *Tinospora capillipes*) by high-performance liquid chromatography/electrospray ionization with multi-stage mass spectrometry. *Rapid Commun Mass Spectrom* 20:2328–2342
75. Chen KS, Ko FN, Teng CM, Wu YC (1996) Antiplatelet and vasorelaxing actions of some aporphinoids. *Planta Med* 62:133–136
76. Zhao Q, Zhao Y, Wang K (2006) Antinociceptive and free radical scavenging activities of alkaloids isolated from *Lindera angustifolia* Chen. *J Ethnopharmacol* 106:408–413
77. Zetler G (1988) Neuroleptic-like, anticonvulsant and antinociceptive effects of aporphine alkaloids: bulbocapnine, corytuberine, boldine and glaucine. *Arch Int Pharmacodyn Ther* 296:255–281
78. Zhang A, Zhang Y, Branfman AR, Baldessarini RJ, Neumeyer JL (2007) Advances in development of dopaminergic aporphinoids. *J Med Chem* 50:171–181
79. Leopoldo M, Lacivita E, Berardi F, Perrone R, Hedlund PB (2011) Serotonin 5-HT7 receptor agents: structure-activity relationships and potential therapeutic applications in central nervous system disorders. *Pharmacol Ther* 129:120–148
80. Cannon JG, Flaherty PT, Ozkutlu U, Long JP (1995) A-ring ortho-disubstituted aporphine derivatives as potential agonists or antagonists at serotonergic 5-HT1A receptors. *J Med Chem* 38:1841–1845
81. Gafner S, Dietz BM, McPhail KL, Scott IM, Glinski JA, Russell FE, McCollom MM, Budzinski JW, Foster BC, Bergeron C, Rhyu MR, Bolton JL (2006) Alkaloids from *Eschscholzia californica* and their capacity to inhibit binding of [<sup>3</sup>H]8-Hydroxy-2-(di-N-propylamino)tetralin to 5-HT1A receptors in vitro. *J Nat Prod* 69:432–435
82. Dela Pena IJ, Lee HL, Yoon SY, Dela Pena JB, Kim HK, Hong EY, Cheong JH (2013) The ethanol extract of *Cirsium japonicum* increased chloride ion influx through stimulating GABA(A) receptor in human neuroblastoma cells and exhibited anxiolytic-like effects in mice. *Drug Discov Ther* 7:18–23
83. Cassels BK, Asencio M (2008) Monoaminergic, ion channel and enzyme inhibitory activities of natural aporphines, their analogues and derivatives. *Nat Prod Commun* 3:643–653
84. Iturriaga-Vasquez P, Perez EG, Slater EY, Bermudez I, Cassels BK (2007) Aporphine metho salts as neuronal nicotinic acetylcholine receptor blockers. *Bioorg Med Chem* 15:3368–3372
85. Vacek J, Walterova D, Vrablova E, Simanek V (2010) The chemical and biological properties of protopine and allocryptopine. *Heterocycles* 81:1773–1789
86. Xu LF, Chu WJ, Qing XY, Li S, Wang XS, Qing GW, Fei J, Guo LH (2006) Protopine inhibits serotonin transporter and noradrenaline transporter and has the antidepressant-like effect in mice models. *Neuropharmacology* 50:934–940
87. Ustunes L, Laekeman GM, Gozler B, Vlietinck AJ, Ozer A, Herman AG (1988) In vitro study of the anticholinergic and antihistaminic activities of protopine and some derivatives. *J Nat Prod* 51:1021–1022
88. Xu Q, Jin RL, Wu YY (1993) Opioid, calcium, and adrenergic receptor involvement in protopine analgesia. *Zhongguo Yao Li Xue Bao* 14:495–500
89. Gurley B, Hubbard MA, Williams DK, Thaden J, Tong Y, Gentry WB, Breen P, Carrier DJ, Cheboyina S (2006) Assessing the clinical significance of botanical supplementation on

- human cytochrome P450 3A activity: comparison of a milk thistle and black cohosh product to rifampin and clarithromycin. *J Clin Pharmacol* 46:201–213
90. Rhyu MR, Lu J, Webster DE, Fabricant DS, Farnsworth NR, Wang ZJ (2006) Black cohosh (*Actaea racemosa*, *Cimicifuga racemosa*) behaves as a mixed competitive ligand and partial agonist at the human mu opiate receptor. *J Agric Food Chem* 54:9852–9857
91. Li J, Gödecke T, Chen SN, Imai A, Lankin DC, Farnsworth NR, Pauli GF, van Breemen RB, Nikolic D (2011) In vitro metabolic interactions between black cohosh (*Cimicifuga racemosa*) and tamoxifen via inhibition of cytochromes P450 2D6 and 3A4. *Xenobiotica*
92. Herranz T, Galisteo J (2003) Tetrahydro-beta-carboline alkaloids occur in fruits and fruit juices. Activity as antioxidants and radical scavengers. *J Agric Food Chem* 51:7156–7161
93. Herranz T (2000) Tetrahydro-beta-carboline-3-carboxylic acid compounds in fish and meat: possible precursors of co-mutagenic beta-carbolines norharman and harman in cooked foods. *Food Addit Contam* 17:859–866
94. Herranz T (1999) 1-Methyl-1,2,3,4-tetrahydro-beta-caroline-3-carboxylic acid and 1,2,3,4-tetrahydro-beta-caroline-3-carboxylic acid in fruits. *J Agric Food Chem* 47:4883–4887
95. Herranz T (2000) Tetrahydro-beta-carbolines, potential neuroactive alkaloids, in chocolate and cocoa. *J Agric Food Chem* 48:4900–4904
96. Matsubara K, Fukushima S, Akane A, Hama K, Fukui Y (1986) Tetrahydro-beta-carbolines in human urine and rat brain—no evidence of formation by alcohol drinking. *Alcohol Alcohol* 21:339–345
97. Herranz T, Chaparro C (2006) Analysis of monoamine oxidase enzymatic activity by reversed-phase high performance liquid chromatography and inhibition by beta-caroline alkaloids occurring in foods and plants. *J Chromatogr A* 1120:237–243
98. Adachi J, Yamamoto K, Ogawa Y, Ueno Y, Mizoi Y, Tatsuno Y (1991) Endogenous formation of 1-methyl-1,2,3,4-tetrahydro-beta-caroline-3-carboxylic acid in man as the possible causative substance of eosinophilia-myalgia syndrome associated with ingestion of L-tryptophan. *Arch Toxicol* 65:505–509
99. Ogawa Y, Adachi J, Tatsuno Y (1993) Accumulation of 1-methyl-tetrahydro-beta-caroline-3-carboxylic acid in blood and organs of rat. A possible causative substance of eosinophilia-myalgia syndrome associated with ingestion of L-tryptophan. *Arch Toxicol* 67:290–293
100. Burdette JE, Liu J, Chen SN, Fabricant DS, Piersen CE, Barker EL, Pezzuto JM, Mesecar A, van Breemen RB, Farnsworth NR, Bolton JL (2003) Black cohosh acts as a mixed competitive ligand and partial agonist of the serotonin receptor. *J Agric Food Chem* 51:5661–5670
101. Bhargava KP, Kishor K, Pant MC, Saxena PR (1965) Identification of tryptamine derivatives in *Ranunculus sceleratus* L. *Br J Pharmacol Chemother* 25:743–750
102. Servillo L, Giovane A, Balestrieri ML, Casale R, Cautela D, Castaldo D (2013) Citrus genus plants contain N-methylated tryptamine derivatives and their 5-hydroxylated forms. *J Agric Food Chem* 61:5156–5162
103. Yanase E, Ohno M, Harakawa H, Nakatsuka S (2010) Isolation of N, N-dimethyl and N-methylserotonin 5-O- $\beta$ -glucosides from immature *Zanthoxylum piperitum* seeds. *Biosci Biotechnol Biochem* 74:1951–1952
104. Chang AS, Chang SM, Starnes DM (1993) Structure-activity relationships of serotonin transport: relevance to nontricyclic antidepressant interactions. *Eur J Pharmacol* 247:239–248
105. Nikolic D, Li J, van Breemen RB (2014) Metabolism of N $\omega$ -methylserotonin, a serotonergic constituent of black cohosh (*Cimicifuga racemosa*, L. Nutt.), by human liver microsomes. *Biomed Chromatogr* 28(12):1647–1651
106. Somei M, Teranishi S, Yamada K, Yamada F (2001) The chemistry of indoles. CVII. A novel synthesis of 3,4,5,6-tetrahydro-7-hydroxy-1H-azepino[5,4,3-cd]indoles and a new finding on Pictet-Spengler reaction. *Chem Pharm Bull (Tokyo)* 49:1159–1165
107. Bjorklund A, Falck B, Lindvall O, Svensson LA (1973) New aspects on reaction mechanisms in the formaldehyde histofluorescence method for monoamines. *J Histochem Cytochem* 21:17–25
108. Boonen J, Bronselaer A, Nielandt J, Veryser L, De Tre G, De Spiegeleer B (2012) Alkamid database: chemistry, occurrence and functionality of plant N-alkylamides. *J Ethnopharmacol* 142:563–590

109. Rios MY (2012) Natural alkamides: pharmacology, chemistry and distribution. In: Vallisuta O (ed) Drug discovery research in pharmacognosy. InTech, Rijeka, pp 107–144, [www.intechopen.com](http://www.intechopen.com)
110. Kuhnert N, Jaiswal R, Matei MF, Sovdat T, Deshpande S (2010) How to distinguish between feruloyl quinic acids and isoferuloyl quinic acids by liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 24:1575–1582
111. Yim S, Kim H, Jeong N, Pak K, Lee Y, Cho S, Lee I (2012) Structure-guided identification of noel phenolic and phenolic amide allokaloids from the rhizomes of *Cimicifuga heracleifolia*. *Bull Korean Chem Soc* 33:1253–1258
112. Li C, Chen D, Xiao P, Hong S, Ma L (1994) Chemical constituents of traditional Chinese drug “Sheng-ma” (*Cimicifuga dahurica*). *Acta Pharm Sin* 52:296–300
113. Cuyckens F, Claeys M (2004) Mass spectrometry in the structural analysis of flavonoids. *J Mass Spectrom* 39:1–15
114. Yang Z, Nakabayashi R, Okazaki Y, Mori T, Takamatsu S, Kitanaka S, Kikuchi J, Saito K (2014) Toward better annotation in plant metabolomics: isolation and structure elucidation of 36 specialized metabolites from (rice) by using MS/MS and NMR analyses. *Metabolomics* 10:543–555
115. Lin LZ, He XG, Lindenmaier M, Nolan G, Yang J, Cleary M, Qiu SX, Cordell GA (2000) Liquid chromatography-electrospray ionization mass spectrometry study of the flavonoids of the roots of *Astragalus mongolicus* and *A. membranaceus*. *J Chromatogr A* 876:87–95
116. Clifford M (2000) Chlorogenic acids and other cinnamates—nature, occurrence, dietary burden, absorption and metabolism. *J Sci Food Agric* 80:1033–1043
117. Clifford M, Knight S (2004) The cinnamoyl-amino acid conjugates from green Robusta coffee beans. *Food Chem* 87:457–463
118. Shahidi F, Chandrasekara A (2010) Hydroxycinnamates and their in vitro and in vivo antioxidant activities. *Phytochem Rev* 9:147–170
119. Böttcher C, von Roepenack-Lahaye E, Schmidt J, Clemens S, Scheel D (2009) Analysis of phenolic choline esters from seeds of *Arabidopsis thaliana* and *Brassica napus* by capillary liquid chromatography/electrospray-tandem mass spectrometry. *J Mass Spectrom* 44:466–476
120. Ormerod WE (1953) Hydrolysis of benzoylcholine derivatives by cholinesterase in serum. *Biochem J* 54:701–704
121. Akcasu A, Sinha YK, West GB (1952) The pharmacology of benzoylcholine. *Br J Pharmacol Chemother* 7:331–337
122. Stennert A, Maier HG (1994) Trigonelline in coffee. II. Content of green, roasted and instant coffee. *Z Lebensm Unters Forsch* 199:198–200
123. Zhou J, Chan L, Zhou S (2012) Trigonelline: a plant alkaloid with therapeutic potential for diabetes and central nervous system disease. *Curr Med Chem* 19:3523–3531
124. Allred KF, Yackley KM, Vanamala J, Allred CD (2009) Trigonelline is a novel phytoestrogen in coffee beans. *J Nutr* 139:1833–1838
125. Liu J, Burdette JE, Xu H, Gu C, van Breemen RB, Bhat KP, Booth N, Constantinou AI, Pezzuto JM, Fong HH, Farnsworth NR, Bolton JL (2001) Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. *J Agric Food Chem* 49:2472–2479
126. Overk CR, Yao P, Chen S, Deng S, Imai A, Main M, Schinkovitz A, Farnsworth NR, Pauli GF, Bolton JL (2008) High-content screening and mechanism-based evaluation of estrogenic botanical extracts. *Comb Chem High Throughput Screen* 11:283–293
127. Ruhlen RL, Haubner J, Tracy JK, Zhu W, Ehya H, Lamberson WR, Rottinghaus GE, Sauter ER (2007) Black cohosh does not exert an estrogenic effect on the breast. *Nutr Cancer* 59:269–277
128. Ruhlen RL, Sun GY, Sauter ER (2008) Black cohosh: insights into its mechanism(s) of action. *Integr Med Insights* 3:21–32
129. Fitch RW, Sturgeon GD, Patel SR, Spande TF, Garraffo HM, Daly JW, Blaauw RH (2009) Epiquinamide: a poison that wasn’t from a frog that was. *J Nat Prod* 72:243–247
130. Matsuda H, Suzuki Y (1984) gamma-Guanidinobutyraldehyde Dehydrogenase of *Vicia faba* Leaves. *Plant Physiol* 76:654–657

## Chapter 3

# *Jatropha* Natural Products as Potential Therapeutic Leads

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**Abstract** Natural products (NPs) are nature's evolutionarily product, containing complex and diverse molecular structures. For the past two decades, pharmaceutical companies have focused on developing targeted therapeutic agents by screening libraries of small synthetic molecules. However, recent development of new technologies that improve the extraction/characterization process as well as new synthetic methodologies that render NP derivatization possible have ignited a revival in the field of NPs. NPs are now being used as a rich repository for drug discovery. Here we review recent work on the bioactive NPs derived from *Jatropha isabelli* along with their corresponding derivatives. Most of these NPs are sesquiterpenoids with unique molecular architectures. They display activity against various disease models and therefore may offer early compound leads for drug discovery programs.

**Keywords** Natural products • *Jatropha* • *Euphorbia* • Jatropheone • *Plasmodium falciparum* • Malaria • Leishmania • Antiviral • Chikungunya virus (CHIKV) • Anticancer • Parasite • Vector • Antioxidant • Mitochondria • Oxidation • Diterpene • Sesquiterpene • Lignans • High throughput screening • Multidrug resistance (MDR) • Infectious agents • Mammalian cell • 1,4-Michael addition reaction • Mukaiyama aldol reaction • Nucleophilic • Glutathione (GSH) • Gram-positive bacterium • Macrocycles • Plant extracts

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### 3.1 Introduction

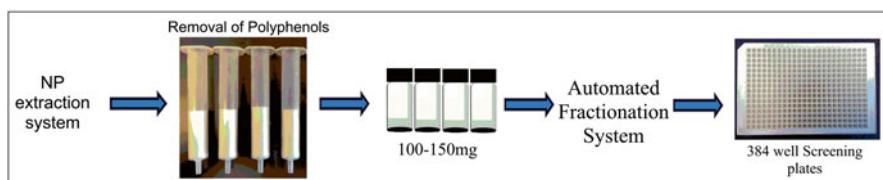
The Euphorbiaceae or spurge family is a large group of flowering plants comprising 300 genera and approximately 7500 species [1–3]. These terrestrial plants typically produce a milky sap and small unisexual flowers surrounded by a cup-like structure composed of fused bracts. The term “spurge” is derived from the Latin *expurgare*, meaning “to cleanse,” referring to early observations of these plants’ purgative properties [2]. This brief perspective focuses on two genera of this family, *Jatropha* and *Euphorbia*, both of which produce biologically relevant sesquiterpene natural products (NPs), including jatrophe.

The genus *Jatropha* comprises approximately 175 species of succulent herbs, shrubs, and trees [3]. Aqueous and organic extracts from this family have historically been used worldwide as purgatives, styptics, emetics, and therapeutics for warts, tumors, rheumatism, herpes, pruritus, toothache, scabies, eczema, and ringworm [3–6]. Similarly, the genus *Euphorbia* is one of the largest genera of flowering plants and has multiple reported medicinal uses. The scope of this perspective will limit our discussion to only a selected number of species that produce NPs related to jatrophe.

### 3.2 Advances in Natural Product Fractionation Systems

NPs serve as valuable tools to investigate biological processes and as therapeutic agents. However, the discovery of potential NP leads is hampered by the need for dereplication and the cost of isolation and structure elucidation. Primary screening of crude plant extracts or microbial fermentations, followed by bioassay-guided fractionation, purification, and structure elucidation of novel bioactive compounds can take several years to yield a suitable lead compound [7]. Therefore, over the past two decades many pharmaceutical companies have discontinued this type of research. However, academic groups are becoming more proactive in developing methods to maximize drug discovery efforts.

Figure 3.1 diagrams the current NP extraction/fractionation protocol at St. Jude Children’s Research Hospital. The crude plant extracts are filtered on polyamide



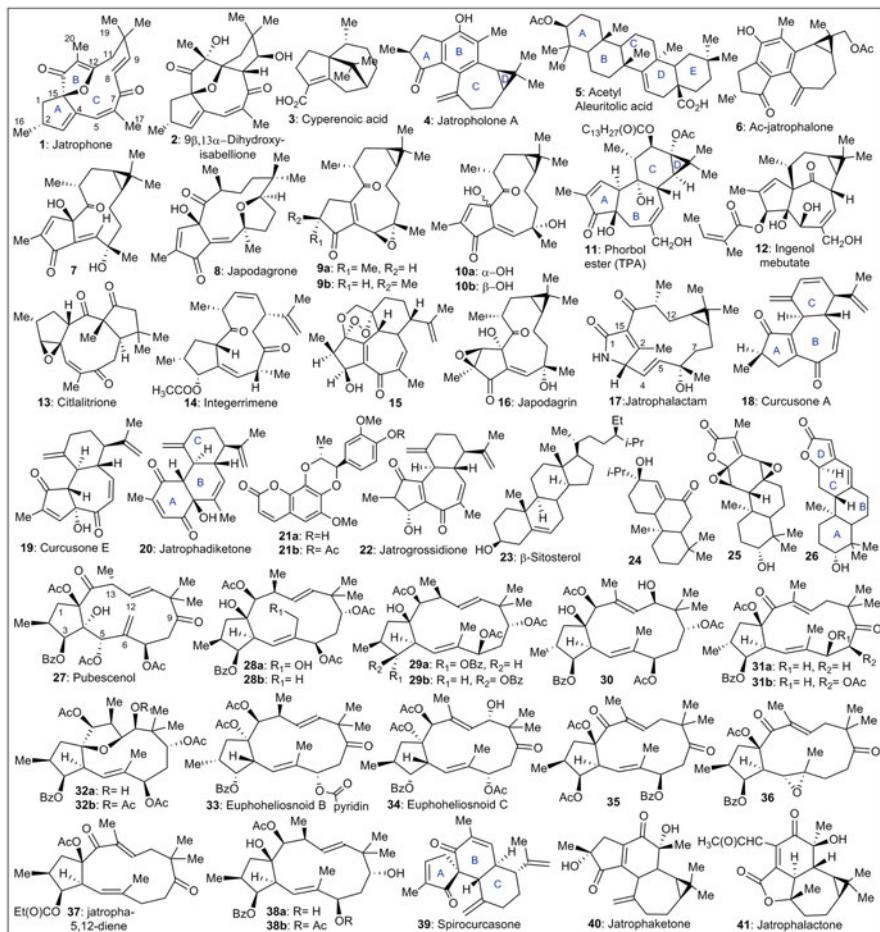
**Fig. 3.1** High throughput NP fractionation system for Hit-to-Lead drug discovery platform [8]. Crude plant extracts are filtered to remove polyphenols, fractionated by high performance liquid chromatography, dried, weighed, and screened for biological activity in 384-well plates. In parallel, spectroscopic data are collected to elucidate the structures of compounds in each fraction

solid-phase extraction cartridges to remove polyphenols (tannins), which can cause false-positive results in enzymatic and cellular screening assays due to nonselective inhibition and alteration of cellular redox potential [8]. The resulting filtrate is fractionated by an automated high performance liquid chromatography system. The fractions are dried, weighed, and reformatted for screening in 384-well plates. Mass spectrometry data are collected for each fraction, complemented by information from photodiode array and evaporative light scattering detectors to facilitate structure elucidation. The process allows rapid evaluation and dereplication of the compound leads [8]. Our group recently utilized this automated high-throughput fractionation system for NP screens against infectious agents and cancer, and identified promising compounds [9].

The Guy research group and the National Center for Natural Product Research at the University of Mississippi developed an automated, high-throughput fractionation method to prepare and analyze natural product libraries for drug discovery screening, utilizing new analytical tools to minimize cost [8]. We anticipate that further advancements in high-throughput screening, fractionation systems, and dereplication programs will lead to the identification of new NP leads and their biological targets. Below we will summarize some of the major NP structures isolated from *Jatropha* and *Euphorbia* species by different laboratories using various high-throughput screening approaches, and will discuss their biological activities and potential biosynthetic relationships.

### 3.3 Biosynthesis and Biological Activity of *Jatropha* and *Euphorbia* Natural Products

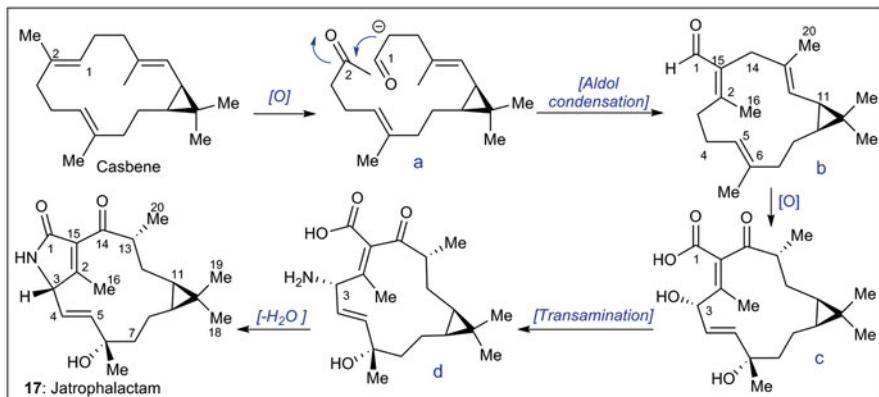
The recent discovery and rediscovery of NPs from various *Jatropha* species, ranging from complex terpenoids to symmetrical polyphenols (**1–41**, Fig. 3.2), has raised substantial interest in the medicinal chemistry community. These NPs represent a broad spectrum of chemical composition, from large macrocycles such as compounds **1** and **41** to small macrocycles (e.g., cyperenoic acid, compound **3**). A fascinating feature of many of these NPs is their highly electrophilic functionality, which allows conjugated systems, epoxides, and cyclopropyl groups that render them susceptible to nucleophilic attack. These molecules are diverse, but careful evaluation of their carbon backbone suggests a parental origin in their biosynthesis, with extensive variation in oxidation state (Fig. 3.2). The proposed biosynthetic pathways that form these NPs, based on synthetic work and isolation of NPs resembling intermediates, suggest that the rhamnofolane core could be derived from either the lathyrane or cembrane scaffold [10, 11]. The potent biological activity of jatropheone (compound **1**, Fig. 3.2) has prompted great attention to this compound, including various ethnopharmacological studies [4–6, 10–14]. Jatropheone is a macrocyclic diterpene featuring a unique oxaspiro core and several electrophilic centers. Jatropheone can be isolated from multiple *Jatropha* species, but our own isolation efforts have derived larger quantities of jatropheone from *Jatropha isabelli*



**Fig. 3.2** A large number of structurally diverse NPs are produced by the Euphorbiaceae plant family; some of these NPs have recently been discovered and re-discovered

than from *Jatropha gossypifolia* (Florida, USA) or *Jatropha curcas* (Paraguay, South America). *J. isabelli* produces various NPs (**1–5**, Fig. 3.2), but jatropheone has received the most attention because of its biological activity [14].

Jatropheone (25 mg/kg) demonstrated gastroprotection against HCl/EtOH-induced gastric lesions in rats [14]. Jatropheone also showed antiproliferative effects in several human cancer cell lines, with the most promising results in U251 (glioma) cells, NCI-ADR/RES (drug-resistant ovarian cancer) cells, and K562 (a myelogenous leukemia cell model) [15]. Other biological properties of jatropheone (antimalaria and antileishmania) will be described below. The jatropholone **4**, its epimer, and NPs **6–10** (Fig. 3.2) were isolated from *J. isabelli* in much greater quantities than jatropheone, and they displayed activity against cancer cell lines [14].



**Fig. 3.3** Proposed biosynthesis of jatrophalactam **17** from the casbene core through a series of linear chemical transformations [20]

The phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate [TPA], compound **11**, Fig. 3.2) was isolated from *Croton tiglium*, which is commonly used to treat various diseases in traditional Chinese medicine, although it frequently serves as a cancer-inducing agent in basic research [16].

The jatropholones are promising lead compounds for further development because of their potent bioactivity and their reported physical chemical properties [15]. One of the leading members of this compound family is ingenol mebutate (**12**, Fig. 3.2). This NP has already been approved for topical treatment of actinic keratosis, a pre-malignant condition that can progress to squamous cell carcinoma [17]. The NP citellalitrione (**13**, Fig. 3.2) was isolated from *J. dioica* var. *sessiliflora* (Hook), a plant used by the Aztecs to treat toothache and skin lesions [6]. Jatropholone and β-sitosterol (**4** and **23**) were isolated from the plant's roots and elucidated via 2D nuclear magnetic resonance (NMR) and X-ray crystallographic methods [6]. *J. integerrima*, a common plant in Thailand also known as *J. pandurifolia* Andr., produces endoperoxide rhamnolane scaffolds such as compound **15** and the macrocyclic diterpene integrermene **14**, which may be a precursor of compound **15** (Fig. 3.2) [10, 11]. *J. podagraria* (Hook) produces japodagrin **16**, japodagrone **8**, and similar isomers (**7**, **9**, and **10**; Fig. 3.2). These compounds have shown antibacterial activity in standard disk assays [18, 19]. Both japodagrone **8** and japodagrin **16** exhibited promising activity against the gram-positive bacterium, *Bacillus subtilis*. In addition, japodagrin showed potent activity against the gram-positive bacterium, *Staphylococcus aureus* [19].

Recently, jatrophalactam (**17**, Fig. 3.3) isolated from *J. curcas* was found to feature a 5/13/3 tricyclic framework, as confirmed by extensive 2D NMR and X-ray diffraction analysis [20]. Jatrophalactam was evaluated against the human cancer cell lines A549 (lung cancer), HT-29 (colon cancer), and A431 (epidermal squamous-cell carcinoma), but it showed no bioactivity under the experimental conditions [20]. Jatrophalactam has a core structure similar to that of jatrophe, with the exception of the unsaturated enone system, which may be responsible for its bio-

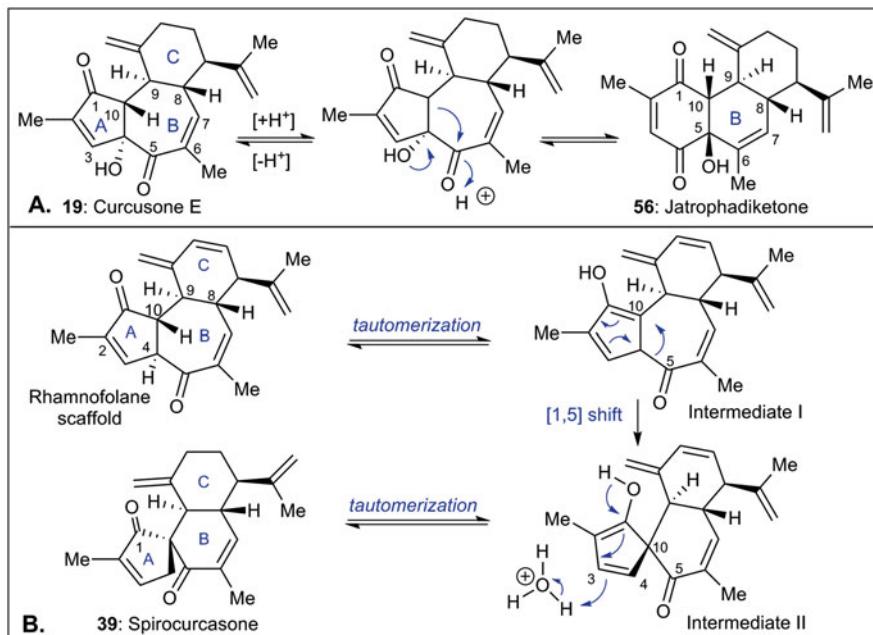
logical activity. The proposed biosynthetic pathway of this novel lactam requires only a few chemical transformations from the casbene core, namely oxidative cleavage across C1–C2 followed by an aldol condensation to afford the intermediate b, which could undergo further oxidation at C1, C3, and C6 to afford intermediate c. Transamination at C3 and closing of the ring would then yield the NP (Fig. 3.3). This proposed biosynthetic pathway is consistent with previously reported chemical transformations, but further mechanistic studies are required [20].

*J. grossidentata* is a species native to El Chaco, Paraguay, and it is rich in secondary metabolites such as NPs **8–10**, **18–19**, and **22**, along with the lignans (**21**, Fig. 3.2) [21, 22]. This plant is widely used in traditional Paraguayan medicine [21]. Extracts of *J. isabelli* and *J. grossidentata* have been tested in vitro and in vivo against *Leishmania* and *Trypanosoma cruzi* strains [13]. The main active components of these extracts are jatropheone **1** and jatrogrossidione **22**, which are unique compounds appropriate for mechanistic studies. The in vitro inhibitory effect of jatropheone against leishmania was promising ( $IC_{100}=5.0\text{ }\mu\text{g/mL}$ ); jatrogrossidione also showed a potent effect against leishmania ( $IC_{100}=0.75\text{ }\mu\text{g/mL}$ ) and trypanosomes ( $IC_{100}=1.5\text{--}5.0\text{ }\mu\text{g/mL}$ ) [13]. These results were validated by in vivo efficacy in BALB/c mice infected with *L. amazonensis*. Jatrogrossidione showed modest activity in comparison to glucantime (positive control), and jatropheone significantly reduced the diameter of leishmania lesions. However, half of the cohort treated with jatropheone (25 mg/kg/day for 13 days) did not survive, indicating a need to reduce the compound's off-target effects [13].

The *Euphorbia* family is the source of the rhamnofolane NPs. These NPs share a similar core structure that has three rings and five chiral centers, whereas the lathyrane-type compounds share an 11-member macrocycle and are oxidized to various degrees (**18**, **27–36**; Fig. 3.2). The latter NPs are polyoxygenated, and their biological activities are under investigation [23]. *Euphorbia fischeriana* produces a broad array of compounds ranging from the abietane diterpenoids (**24–26**, Fig. 3.2) to phorbol compounds (**11**, Fig. 3.2) [24]. The crude plant extracts are widely used in Chinese medicine, and compounds **11** and **25** have shown potent activity against human Burkitt lymphoma cells [24].

Pubescenol (**27**, Fig. 3.2) was isolated from *Euphorbia pubescens* and has shown promising biological properties [23]. Both **27** and the related compound **30** showed activity against the human cancer cell lines MCF-7 (breast adenocarcinoma), NCI-H160 (non-small-cell lung cancer), and SF-268 (central nervous system cancer), with potency similar to that of doxorubicin ( $EC_{50}<100\text{ }\mu\text{M}$ ) [23]. The NP **26** was also isolated from *E. pubescens*, and it showed significant activity against *Staph. aureus* [23].

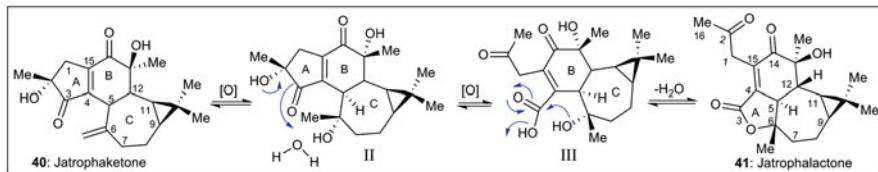
The jatropheone diterpenes **30–34** (Fig. 3.2) were isolated from *E. helioscopia* L. and elucidated via extensive 2D NMR studies [24, 25]. More than 30 diterpenoids with a similar structural core have been isolated from this plant [25]. The crude extract of *E. helioscopia* L. was active against P388 cells (murine leukemia model), but the purified individual constituents showed no activity under the same conditions, suggesting synergy between the constituents [24, 25]. The recently isolated compounds **28** and **26** were active against HeLa (cervical carcinoma) cells and MDA-MB-231 (triple-negative breast cancer) cells in the low micromolar range [26].



**Fig. 3.4** (a) Proposed biosynthesis of jatrophadiketone from curcusone E via contraction of the B ring [20]. (b) Proposed biosynthesis of spirocurcasone from a rhamnofolane scaffold via contraction of the B ring [20]

*J. curcas* is recognized primarily for its seeds' high content of oil, which can be converted to biodiesel fuel; it also produces a broad range of diterpenes, triterpenes, lignans, and coumarins (e.g., **1**, **4**, **7–9**, **10**, **16**, and **17–19**; Fig. 3.2) [21]. The curcasones **18–19** (Fig. 3.2) were first elucidated by the Clardy group by 2D NMR spectroscopy and X-ray diffraction studies [27]. The curcasones have shown antiproliferative activity against the mouse lymphoma cell line L5178Y [28]. The structure of jatrophadiketone (**20**) was elucidated via 2D NMR and X-ray crystallographic studies. It was postulated that jatrophadiketone (**20**) could arise from protonation at C5 and further oxidation at C4 with concurrent [1,2]-alkyl shift of C10 to C5 of curcusone E (**19**, Fig. 3.4) [28].

The diterpenoid spirocurcasone (**39**, Fig. 3.4) recently isolated from *J. curcas* features an unprecedented quaternary spirocenter [20]. Spirocurasone was isolated as a colorless solid. Because it was unsuitable for X-ray analysis, its assigned absolute configuration was obtained through extensive 2D NMR, electronic circular dichroism analysis, and quantum mechanical time-dependent density functional theory calculations [20]. From a biosynthetic perspective, spirocurcasone could be derived from a rhamnofolane core (Fig. 3.4). Tautomerization of the A ring could afford intermediate I, which could undergo a [1,5]-sigmatropic alkyl shift of the C5 bond to the C10, leading to the spiro intermediate II. The intermediate II could finally undergo tautomerization to produce the NP [20].



**Fig. 3.5** Proposed biosynthesis of jatrophalactone **41** from jatrophaketone **40** through a series of transformations that rearrange the A ring [20]

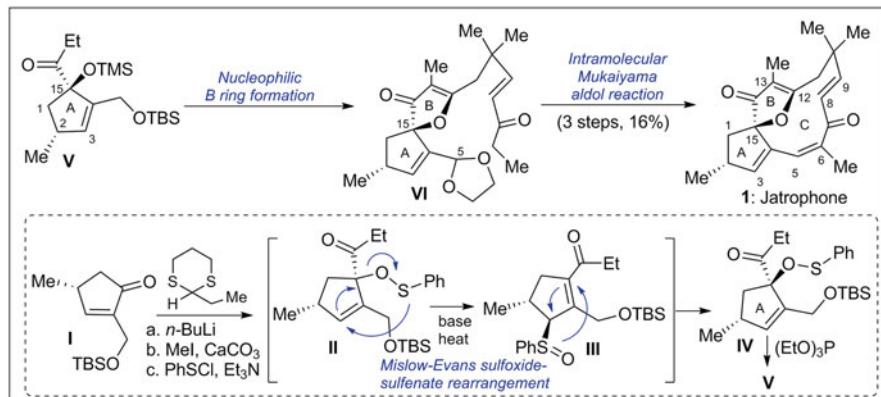
The anticancer activity of individual chemical constituents of *J. curcas* extracts (jatrophadiketone, jatrophaketone, and jatrophalactone) were evaluated against HL-60, SMMC-7721, A-49, and MCF-7 human cancer cell lines. Jatrophadiketone and jatrophaketone showed no bioactivity ( $EC_{50} > 40 \mu\text{M}$ ), but jatrophalactone showed cytotoxic activity against all of the cell lines in the low micromolar range and is a promising candidate for further studies [20]. Jatrophaketone (**40**, Fig. 3.5) could undergo acid-catalyzed electrophilic addition at C6 and oxidative cleavage of the C2-C3 bond, followed by intramolecular lactonization to afford jatrophalactone (**41**, Fig. 3.5).

### 3.4 Synthetic Studies of Jatropheone

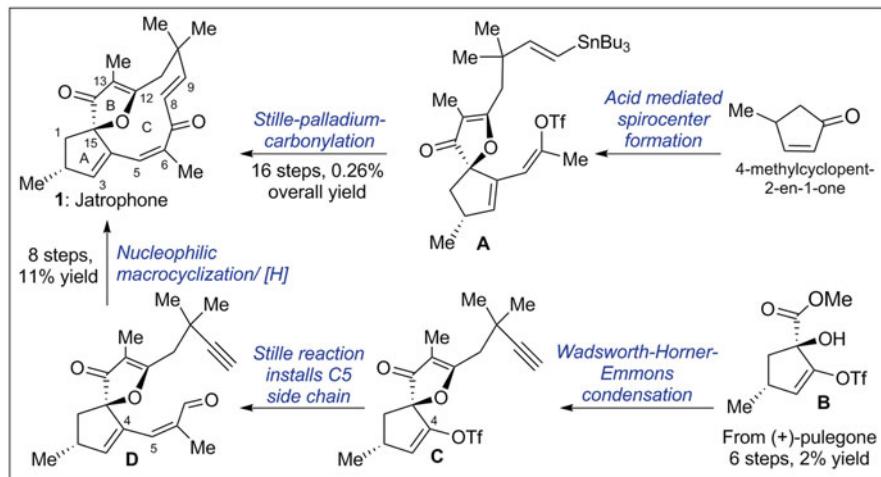
The rhizomes of *J. isabelli* have the highest content of jatropheone. Although this NP is naturally abundant, synthetic approaches would afford multigram quantities of the NP and possibly synthetic derivatives for biological studies. The synthetic NP derivatives provide structure–activity relationship studies.

The Smith group completed the first total synthesis of racemic jatropheone and related NPs of this family [29–31]. They used a streamlined synthetic approach, utilizing a dithiane-based Umpolung strategy to establish the chiral centers of the A ring. The Umpolung strategy involves a dipole inversion, as depicted in Scheme 3.1, and the 1,3-dithiane serves as an acyl anion equivalent, allowing full functionalization of the A ring of jatropheone [29]. Compound I was treated with lithiated dithiane anion, followed by removal of the thiol-protecting group with MeI in the presence of base. The resulting tertiary alcohol was subjected to phenyl sulfur chloride treatment to afford intermediate II. This intermediate was treated with base under heat, leading to the desired thermal Mislow–Evans sulfoxide sulfene rearrangement, and ultimately providing compound V. Spirofuranone formation by nucleophilic macrocyclization provided compound VI, concluding with a successful intramolecular Mukaiyama aldol reaction to yield compound 1, with a 16 % overall yield from intermediate VI (Scheme 3.1) [30].

An alternative total synthesis of ( $\pm$ )-jatropheone was completed in 16 linear steps with an overall yield of 0.26 % (Scheme 3.2). The synthesis of the A ring of jatropheone was uneventful, with the exception of control of the C15 center. Unfortunately,



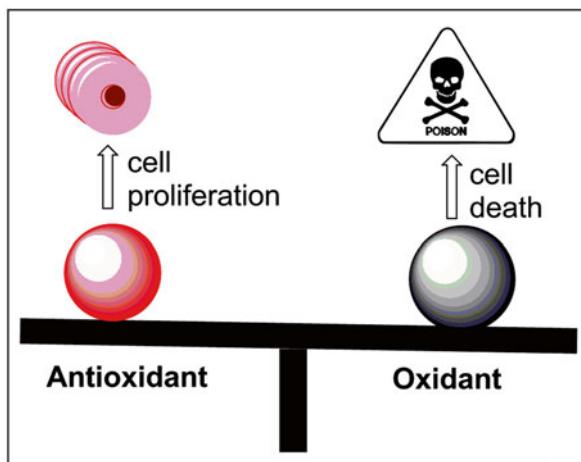
**Scheme 3.1** Total synthesis of jatropheone using an Umpolung strategy to establish the two stereocenters (C2, C15) of the A ring [30–32]



**Scheme 3.2** Total synthesis of jatropheone featuring palladium-mediated reactions to either generate the macrocycle or install part of the macrocycle system [32, 33]

the undesired diastereoisomer was favored (9:1). However, the isomers could be separated during purification. The remaining synthetic approach was robust, and the reactions provided good chemical yields. The successful palladium-catalyzed carbonylation reaction of intermediate **A** to close the macrocycle was a key element in the synthetic strategy (Scheme 3.2) [32].

The first enantioselective total synthesis of jatropheone was reported by Wiemera [33]. The synthetic approach commenced with enantiomerically pure intermediate **B**, which was synthesized from (R)-pulegone in six linear steps, with an overall yield of 2 % (Scheme 3.2). The spirofuranone ring was then generated via an intra-

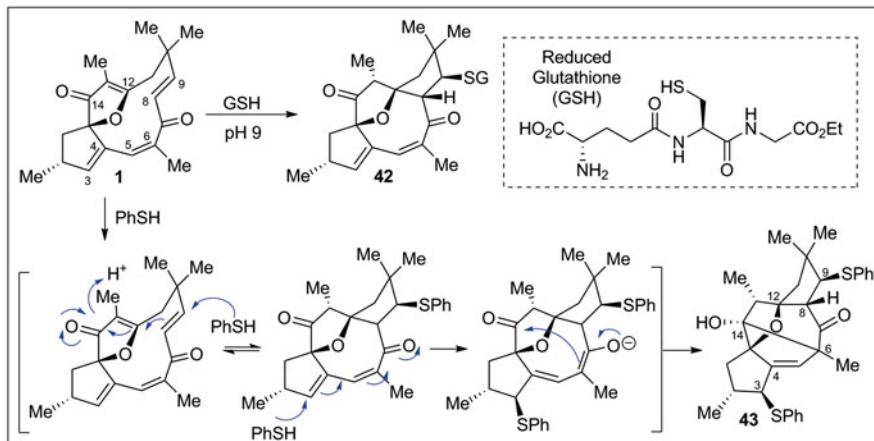


**Fig. 3.6** Variables are carefully regulated to maintain cellular homeostasis. Internal cellular conditions are stable and relatively constant. Any chemical imbalance can cause cell death [35]

molecular Wittig reaction to produce intermediate **C**, followed by Stille coupling and Swern oxidation to afford the advanced intermediate **D** (*Z*-aldehyde). The synthetic efforts concluded with a robust intramolecular Mukaiyama aldol reaction followed by standard oxidation reactions to afford the enantiomerically pure jatrophone (Scheme 3.2).

Mechanistic studies support the hypothesis that NP-containing electrophilic functionalities sequester regulatory macromolecules (peptides, nucleic acids, etc.) via Michael addition reactions [5, 34]. Jatrophone has been reported to interact with both proteins and nucleic acids. The reaction of jatrophone with *Escherichia coli* sRNA showed that the carbonyl groups of jatrophone (C7, C14) interacted with the RNA phosphate groups through hydrogen bonding [34]. Jatrophone improved the stability of this sRNA, as measured by its melting temperature ( $T_m$ ). Further NMR studies indicated the formation of two jatrophone species in solution when combined with sRNA, but their structures were not determined [34].

Reactive molecules such as hydrogen peroxide can indiscriminately damage carbohydrates, lipids, proteins, and nucleic acids, causing loss of their native function. Accordingly, a critical balance in pH and chemical composition must be maintained to support cellular homeostasis (Fig. 3.6). Eukaryotic cells have evolved protective mechanisms to avoid damage by chemical reagents such as electrophiles. The cells typically utilize thiol groups to protect themselves from electrophilic agents because thiol groups have a high affinity for such reactive species. Glutathione (GSH) is the most abundant nonprotein thiol in eukaryotic cells, and it is found in high intracellular concentrations in the human body [36]. One of the main roles of GSH is to scavenge free radicals, among its other roles in DNA synthesis and cell proliferation [35, 37, 38]. Cellular redox state usually refers to the balance of  $\text{NAD}[\text{P}] + / \text{NAD}[\text{P}] \text{H}$ , but recent findings suggest that the GSH/GSSG redox state is crucial to overall cell fate [35]. GSH serves as a protective antioxidant, and when GSH is depleted,

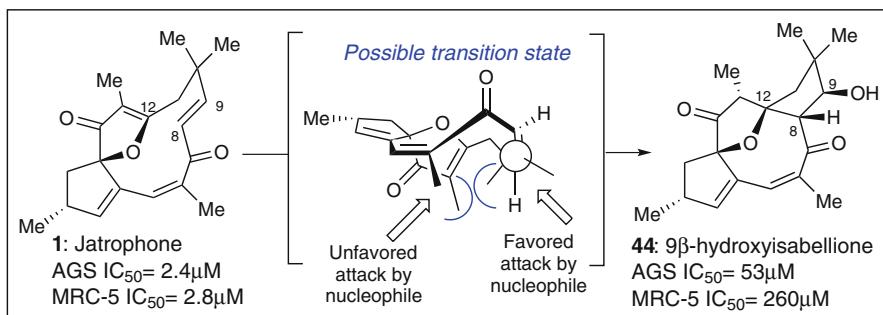


**Fig. 3.7** Jatropheone reacts with either thiophenol or glutathione to produce a stable conjugate system via a 1,4-Michael addition [9]

irreversible cell damage can lead to cell death [35, 37–39]. Hence, GSH can be modulated by chemical reagents (NPs) for therapeutic purposes. Preliminary studies suggest that jatropheone promotes cell death by disrupting the cell's redox balance via GSH depletion [3, 4, 9].

To evaluate jatropheone's mode of action, our group and others have tested and confirmed the hypothesis that jatropheone functions as a Michael acceptor and depletes glutathione. Jatropheone, when treated with a mild base at a low glutathione concentration, affords compound 42 (Fig. 3.7) and traces of a double Michael addition product. To capture potential double adducts, we replaced glutathione with the smaller thiophenol to create a model system for elucidation of product structures [9]. Jatropheone was treated with a mild base and excess thiophenol, yielding compound 43 as the sole product under the experimental conditions [9]. The most accessible double bond at C8–C9 undergoes nucleophilic attack to form a 5-membered ring, followed by a second nucleophilic Michael addition at the C3–C4 double bond (Fig. 3.7). The reaction culminates with the formation of a carbon–carbon bond between C6 and C14 to generate a 5-membered ring [9]. Extensive 2D NMR studies support the C8 and C9 stereochemistry illustrated in Fig. 3.7, which is consistent with a report of the X-ray analysis of a jatropheone derivative [3, 4].

The biotransformation of jatropheone by *Aspergillus niger* provided additional information about the general reactivity of jatropheone [40]. The *Aspergillus niger* culture produced the new compound 9β-hydroxyisabellione (44, Fig. 3.8), a Michael addition reaction product. A single diastereoisomer was observed, presumably due to favorable access to the C8–C9 double bond from the β-face. Upon the addition, a new bond forms with C12 due to close proximity, forming a new ring (Fig. 3.8). The same product can be obtained by chemical treatment of jatropheone with MeOH or other nucleophiles [9]. Treatment of jatropheone with hydroxylamine salt in MeOH provides compound 44 as a single diastereomer [9]. Jatropheone and 44 showed



**Fig. 3.8** Incubation of jatrophe with the fungus *Aspergillus niger* produces 9β-hydroxyisabellione. The nucleophile attacks at the most accessible face, providing a single diastereomer [40]

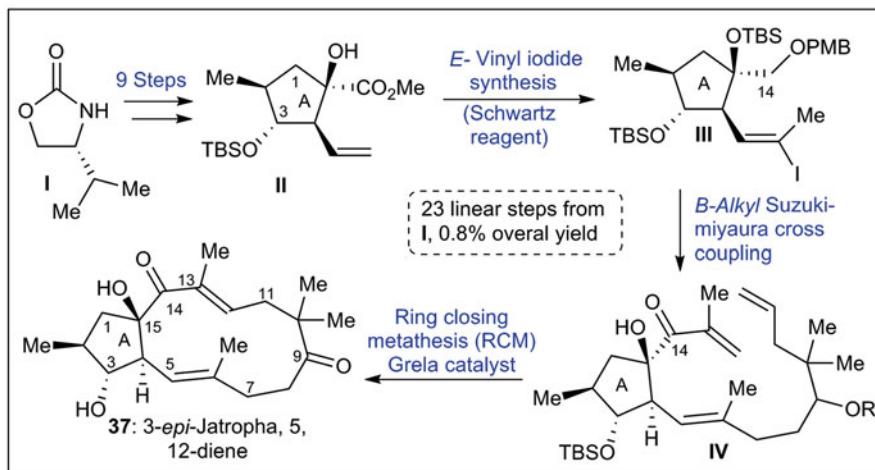
promising biological activity against human AGS (gastric epithelial) and MRC-5 (lung fibroblast) cancer cell models. However, jatrophe exerted the greatest potency in the low micromolar range [40].

### 3.5 Synthetic Analogs of Jatrophe: Potential Therapeutic Applications

#### 3.5.1 Potential Application in Cancer

Jatrophane diterpenes isolated from *Euphorbia characias* possess unusual molecular architecture, having a *trans*-bicyclo[10.3.0]pentadecane core and a set of double bonds at C5/C6 and C12/C13 within the macrocyclic system (e.g., 37, Fig. 3.9) [42–44]. These NPs have demonstrated promising biological properties, and some are selective P-glycoprotein (P-gp) modulators [42]. Transport proteins are extremely relevant to both infectious disease and cancer therapies, as they provide a mechanism of multidrug resistance (MDR) that develops during treatment [41, 43–45]. Therefore, potent and selective P-gp inhibitors are potential targeted agents to combat chemotherapy drug resistance. The synthetic chemistry community is attracted to this family of NPs because of their unusual biological and structural properties, and various campaigns have worked toward their total synthesis [41, 43, 44]. Jatrophane-5,12-diene inhibitors of the drug efflux transporters ABCB1 P-gp and ABCG2 (ATP-binding cassette transporter, also referred to as breast cancer resistance protein) have recently been developed [41].

The enantioselective total synthesis of the jatrophane diterpene (37, Fig. 3.9) started by using the advanced cyclopentane building block **II**, created from the simple asymmetric system **I**. The advanced intermediate, compound **III**, was synthesized by using Schwartz reagent to install the *E*-vinyl; then, after a few manipulations at C14, it was subjected to a *B*-alkyl Suzuki-Miyaura cross-coupling reaction to form compound **IV**. The synthesis was completed by ring-closing metathesis to



**Fig. 3.9** Total synthesis of 3-*epi*-jatropho-5,12-diene in 23 linear steps from (R)-4-isopropylloxazolidin-2-one **I** to introduce chirality into the A ring [41]

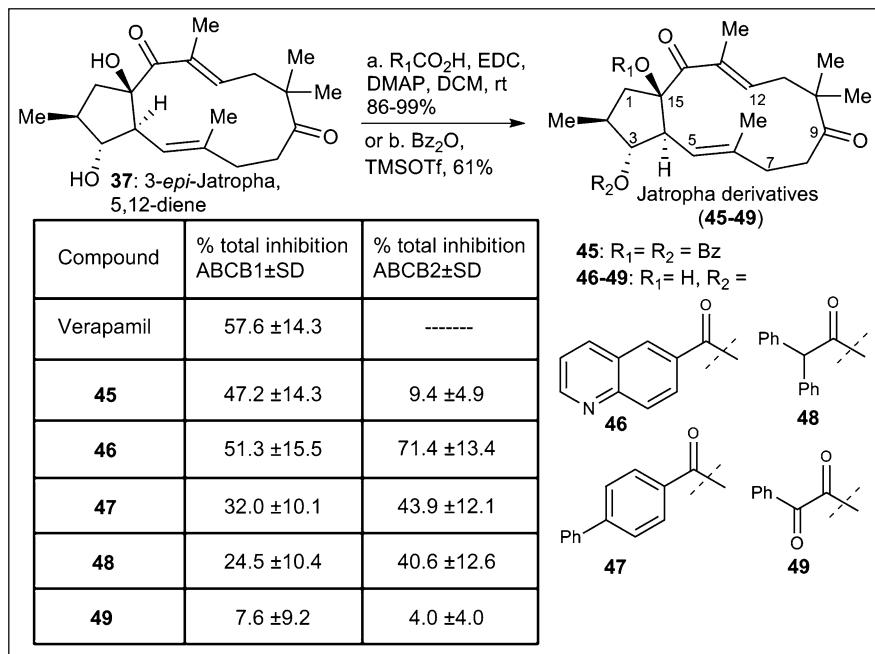
form the rigid 12-membered ring. The synthesis of *epi*-jatropho-5,12-diene consisted of 23 linear steps, with good overall chemical yield (Fig. 3.9 describes robust reactions that allow the scale-up of these reagents).

Synthesis of the 3-*epi*-jatropho-5,12-diene derivatives commenced with C3-hydroxyl group acylation of **37** with commercially available carboxylic acids in the presence of EDC and catalytic DMAP, to afford good chemical yields of compounds **45–49** (Fig. 3.10). Alternatively, treatment of **37** with benzoic anhydride in the presence of catalyst TMSOTf provided compound **45** in 61 % yield. When the compounds were evaluated against ABCB1 and ABCG2 [41], compound **46** showed the most promise with 51 % and 71 % inhibition of ABCB1 and ABCG2, respectively, as compared to the control agent verapamil [41].

The combined findings indicate that a lipophilic aromatic ester substituent at the hydroxyl of C3 group of compound **37** enhances biological activity. In addition, Schnabel and coworkers found that the absolute configuration of the C3-hydroxyl group is important for activity. The 3*R*-configuration (**45**, Fig. 3.10) was more active than the naturally occurring 3*S*-diastereomer, demonstrating the value of synthetic efforts [41].

### 3.5.2 Potential Application in Viral Infections

The chikungunya virus (CHIKV) is an arthropod-borne virus that can cause morbidity in immunocompromised individuals. It can also cause epidemics; there had been 914,960 suspected cases in the Americas (primarily Central and South America) as of November 2014 [46], and El Salvador alone reported 123,000



**Fig. 3.10** Biological activity of 3-*epi*-jatrophane-5,12-diene derivatives **45–49** against transport proteins involved in multidrug resistance [41]

suspected cases, of which only 157 were clinically confirmed [47]. This viral infection causes fever and joint pain lasting several days. There is currently no vaccine or therapeutic agent for CHIKV infection, but the search for potential therapies is under way [46, 47]. Of similar concern is the sindbis virus (also arthropod-borne), which causes sindbis fever and is more common in South and East Africa.

A bioassay-guided screen of *Euphorbia amygdaloides* ssp. *semiperfoliata* extracts against the CHIKV, SINV, and HIV-II viruses led to the isolation of several jatrophane ester derivatives (Fig. 3.11) [48]. Their structures were determined by extensive spectroscopic and X-ray analyses. Some of these compounds displayed promising inhibition activity, and representatives are shown in Fig. 3.11. Jatrophane ester III was the most potent compound against both CHIKV and HIV, with activity in the low micromolar range. Studies of its mode of action and identification of its target(s) are necessary to advance this drug discovery program [48].

### 3.5.3 Potential Application in Protozoan Infections

Malaria is the third most prevalent cause of death from infectious disease [49–53]; the World Health Organization (WHO) estimates that more than 2.19 million malaria cases worldwide in 2010 resulted in more than 700,000 deaths [49]. Human

**Jatrophane ester derivatives**

	R <sub>1</sub>	R <sub>2</sub>	EC <sub>50</sub> CHIKV (μM)	EC <sub>50</sub> SINV (μM)	EC <sub>50</sub> HIV-II (μM)
I	Ac	H	>164	n.d.	n.d.
II	iBu	H	>196	>157	>79
III	Tig	H	0.76	44	0.043± 0.05
IV	Bz	H	4.3	>165	10.9± 1.2
V	Ac	Ac	60	>154	5.9± 0.8
VI	Tig	Ac	17.4	>145	2.0± 0.2

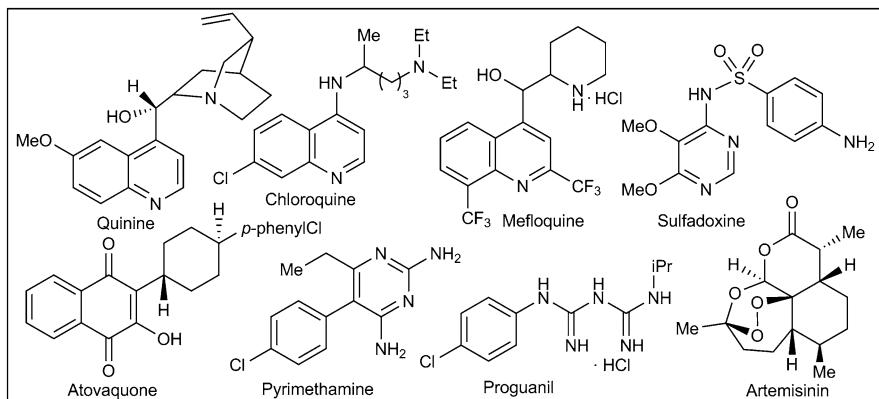
**Jatrophane ester derivatives**

	R <sub>1</sub>	R <sub>2</sub>	EC <sub>50</sub> CHIKV (μM)	EC <sub>50</sub> SINV (μM)	EC <sub>50</sub> HIV-II (μM)
VII	Ac	H	19.5	30.1	5.2± 0.05
VIII	iBu	H	21.0	28.8	>39
IX	MeBu	H	111	51.0	>76
X	H	Ac	80	>163	32.5± 3.5
XI	H	H	10	11	>15

**Fig. 3.11** Antiviral activity of jatrophane ester derivatives **I–XI** against chikungunya (CHIKV), sindbis (SINV), and HIV-II virus. Some of these compounds show promising biological activity in the low micromolar range [48]

malaria can be caused by the *Plasmodium* parasite, *P. malariae*, *P. vivax*, *P. ovale*, and *P. falciparum* (the most virulent) [51]. Symptoms range from fever, muscle weakness, anemia, and jaundice to seizures, kidney failure, and ultimately death, if untreated [52–54]. Despite the great public health impact of malaria, only a few therapeutic agents are available, and fewer are under development [52, 53]. The current antimalarial arsenal is composed of very few compound classes, and some are chemically related (Fig. 3.12). These include the quinolones (chloroquine, amodiaquine, mefloquine, and atovaquone) inspired by the NP quinine (Fig. 3.12), and the NP-based artemisinin compounds.

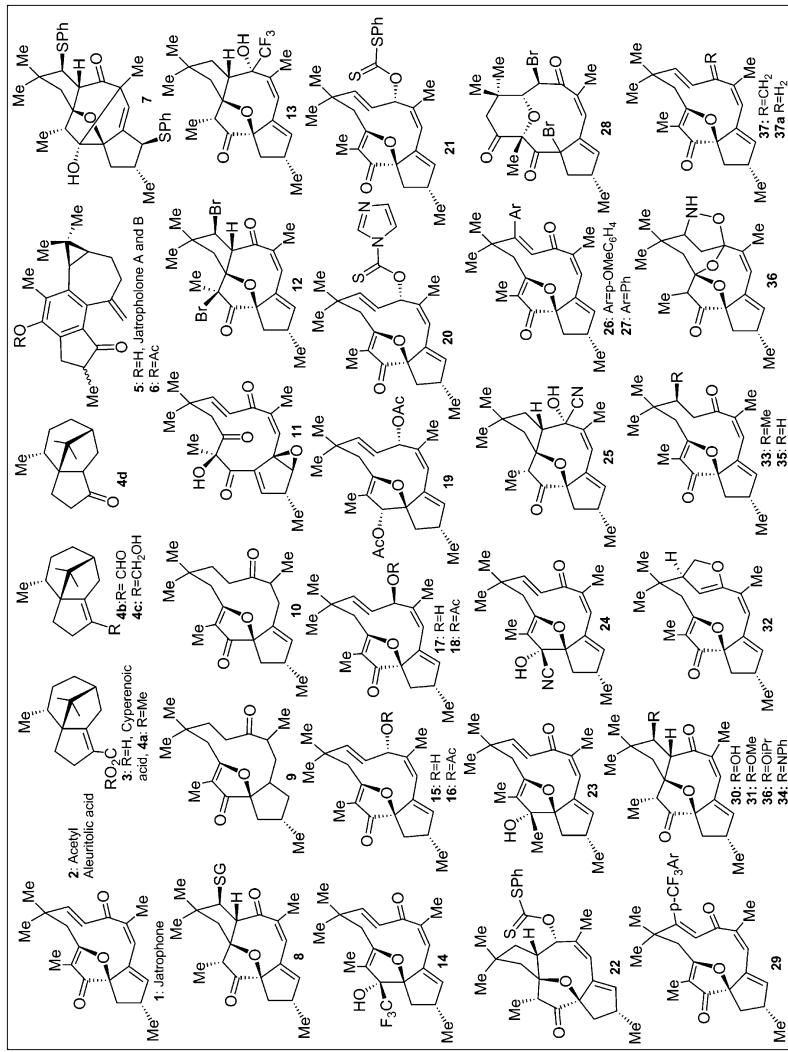
The mode of action of most antimalarial compounds remains poorly understood, but some of their biological targets have been identified [52, 53]. For example, atovaquone targets the *Plasmodium* mitochondria by depleting their membrane potential, whereas pyrimethamine is a potent dihydrofolate reductase inhibitor [52, 53]. The main biological target of artemisinin-based compounds remains unclear, but studies suggest that multiple biological processes of the parasite are targeted, including protein synthesis, mitochondrial function, and the sarco/endoplasmic reticulum Ca<sup>+</sup> ATPase channel [51]. Resistance has been reported against all classes of antimalarial therapy, increasing potential mortality [55, 56]. Further, the lack of scaffold diversity will pose a threat once multidrug-resistant strains emerge, and new antimalarials are urgently needed to overcome drug resistance [52, 53].



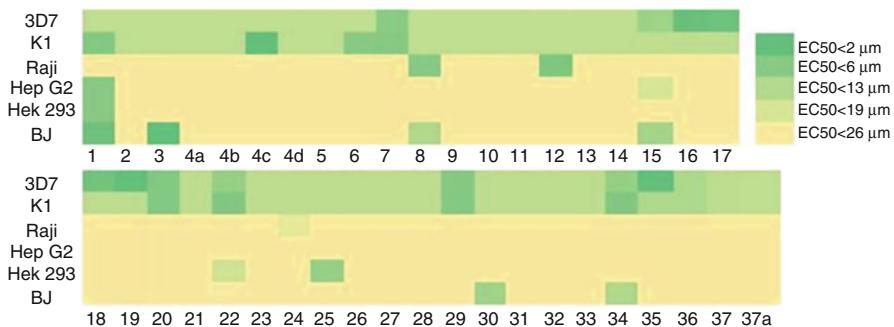
**Fig. 3.12** Current antimalarial therapeutic agents used in combination therapy. As shown, some of these compounds share the same molecular core [52]

In search of new antimalarial agents, we evaluated a library of terrestrial plant extracts against two malaria strains, 3D7 (chloroquine-sensitive *P. falciparum*) and K1 (chloroquine- and pyrimethamine-resistant *P. falciparum* expressing the chloroquine efflux transporter and upregulating MDR efflux pump expression) [57]. Jatropheone was identified as a potent and promising lead compound. This NP represents a new class of antimalarials as it possesses a core structure different from those of previous agents. A focused chemical library based on jatropheone was synthesized to evaluate its structure–activity relationship (Fig. 3.13) [9]. The synthetic plan included 1,4-Michael addition reactions at C7–C9 or C14–C12 conjugated systems. Grignard reagents were also employed to evaluate the carbonyl groups (at C7 and C14). Various electrophilic addition reactions were carried out to evaluate the overall electronic effects of heteroatoms in the biological activity of jatropheone. Heck-mediated cross-coupling reactions of jatropheone afforded compounds **26**, **27**, and **29**. Addition of soft non-carbon nucleophiles ( $\text{H}_2\text{O}$ ,  $\text{MeOH}$ ,  $i\text{PrOH}$ ) to the C9 of jatropheone afforded compounds **30**, **31**, and **36**.

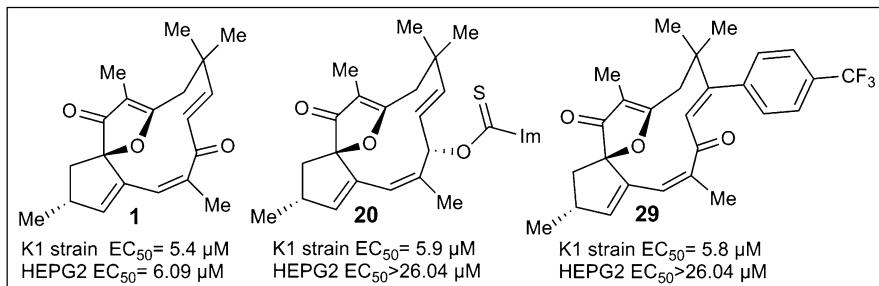
The compound library was tested against the 3D7 and K1 strains of *P. falciparum* and against a panel of mammalian cell lines to evaluate cytotoxicity; these included the HepG2 (human liver carcinoma stable cell line), HEK293 (transformed human embryonic kidney), Raji (Burkitt lymphoma), and BJ (human foreskin fibroblast) cell lines. Many jatropheone derivatives exerted antimalarial activity against the 3D7 and K1 strains (Fig. 3.14), but few compounds were comparable to jatropheone [9]. The heat map shows that some analogs are cytotoxic to BJ cells, but only jatropheone showed cytotoxicity to HepG2 cells (a stable human liver carcinoma cell model also used to evaluate cytotoxicity). Electron-rich aromatic groups at C9 (compounds **26**–**27**) led to weak antimalarial activity. Compounds **20** and **29** were the most promising leads, displaying good activity against the drug-resistant K1 strain ( $\text{EC}_{50}>7 \mu\text{M}$ ) with no detectable cytotoxicity at the tested concentrations (Fig. 3.15). The combined findings suggest that jatropheone analogs have potential for further development as antimalarial agents.



**Fig. 3.13** Jatrophe focused compound library synthesized to evaluate jatrophe's structure-activity relationship against the 3D7 and K1 malaria strains [9]



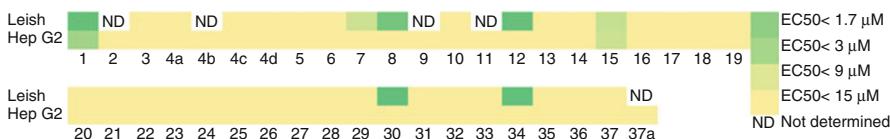
**Fig. 3.14** Heat map of jatrophe and its synthetic derivatives against strains of *P. falciparum*, also showing the compounds' biological activity against mammalian cell lines [9]



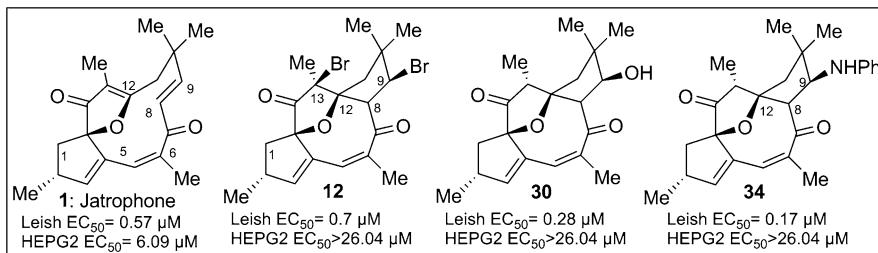
**Fig. 3.15** Activity of jatrophe and two of its most promising derivative leads **20**, **29** against the drug-resistant K1 malaria strain [9]

Leishmaniasis is caused by the protozoan parasites *Leishmania* spp. Leishmaniasis is commonly found in the context of population displacement, limited housing, and overall lack of resources to combat the vector (the sandfly) [58, 59]. An estimated 1.3 million new cases are reported yearly, and 20,000–30,000 deaths occur annually in at least 88 countries [60]. Leishmaniasis can cause disfiguring skin lesions, and its advanced stage can cause fatal organ damage. The antimonial [Sb(III)] agents are the first line of therapy and have been in clinical use since the 1930s. A rise in drug resistance has aroused global concern, particularly in India [59]. The past decade has seen progress in understanding the disease, but no new drug has moved forward to clinical trials. In an effort to identify new potential leads, we screened several NP extracts containing jatrophe analogs (Fig. 3.16), using a transgenic *Leishmania mexicana* model developed by the Landfear group [61, 62]. Jatrophe and its synthetic derivatives **12**, **30**, and **34** (Fig. 3.17) showed promising activity against leishmania in the low micromolar range.

Compound **34** showed the greatest potency against Leishmania ( $EC_{50}=0.17 \mu M$ ) and no observable cytotoxicity against HepG2/Hek293/BJ. Interestingly, the active jatrophe derivatives do not have the most accessible double bond (C8–C9), and



**Fig. 3.16** Heat map of jatropheone and its synthetic derivatives against the *Leishmania mexicana* LMGT2TX model developed by the Landfear group (unpublished data, Rivas group)



**Fig. 3.17** Jatropheone and its most promising synthetic derivatives, with activity against the HEPG2 cell model system. No activity was observed against the BJ cell line (data not shown)

possess heteroatoms at C9 (Fig. 3.17). These findings warrant further mechanistic studies to fully evaluate their potential as therapeutic agents against infectious agents.

## 3.6 Conclusion

Diverse species in the genera *Jatropha* and *Euphorbia* produce a broad range of NP compounds, which inhabit a unique chemical space. These NPs possess complex molecular architecture and show promising biological activity against cancer, viruses, and protozoan parasites. NP research (isolation/fractionation/screening/elucidation) in combination with medicinal chemistry efforts (structure-activity exploration in Hit-to-Lead and Lead Optimization programs leading to candidate selection for pre-clinical development) provide the basis for the next generation of therapeutic agents.

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## References

- Chopra RN, Nayar SL, Chopra IC (1956) Glossary of Indian medicinal plants. Council of Scientific and Industrial Research, New Delhi, p 145
- Burkill HM (1994) The useful plants of west tropical Africa, (families E-J), 2nd edn. Royal Botanical Gardens, Kew, pp 90–94

3. Torrance ST, Wiedhopf RM, Cole JR, Arora SK, Bates RB, Beavers WA, Cutler RS (1976) Antitumor agents from *Jatropha macrorhiza* (Euphorbiaceae). II. Isolation and characterization of jatrophatrione. *J Org Chem* 41:1855–1857
4. Kupchan SM, Sigel CW, Matz MJ, Gilmore CJ, Bryan RF (1975) Structure and stereochemistry of jatrophe, a novel macrocyclic diterpenoid tumor inhibitor. *J Am Chem Soc* 98(8): 2296–2300
5. Lillehaug JR, Kleppe K, Sigel CW, Kupchan SM (1976) Reactions of biological thiols with the tumor inhibitor jatrophe. Synthesis and structure-activity relationship of disubstituted benzamides as a novel class of antimalarial agents. *Biochim Biophys Acta* 327:92–100
6. Villarreal AM, Dominguez XA, Williams HJ, Scott AI, Reibenspies J (1988) Citlalitrione, a new diterpene from *Jatropha dioica* var. *sessiliflora*. *J Nat Prod* 51:749–753
7. Eldridge GR, Vervoort HC, Lee CM, Cremin PA, Williams CT, Hart SM, Goering MG, O’Neil-Johnson M, Zeng L (2002) High-throughput method for the production and analysis of large natural product libraries for drug discovery. *Anal Chem* 74:3963–3971
8. Tu Y, Jeffries C, Ruan H, Nelson C, Smithson D, Shelat AA, Brown KM, Li X-C, Hester JP, Smillie T, Khan IA, Walker L, Guy K, Yan B (2010) Automated high-throughput system to fractionate plant natural products for drug discovery. *J Nat Prod* 73:751–754
9. Hadi V, Hotard M, Ling T, Salinas YG, Palacios G, Connelly M, Rivas F (2013) Structure activity relationship of *Jatropha isabelli* natural products and their synthetic analogs as potential antimalarial therapeutic agents. *Eur J Med Chem* 65:376–380
10. Sutthivaiyakit S, Mongkolvisut W, Ponsitipiboon P, Prabpais S, Kongsaeree P, Ruchirawat S, Mahidol C (2003) A novel 8, 9-seco-rhamnofolate and a new rhamnofolane endoperoxide from *Jatropha integerrima* roots. *Tetrahedron Lett* 44:3637–3640
11. Jakupovic J, Grenz M, Schmeda-Hirschmann G (1988) Rhamnofolane derivatives from *Jatropha grossidentata*. *Phytochemistry* 27:2997–2998
12. Duarte DFP, Sant’Ana AEG, Calixto JB (1992) Analysis of the vasorelaxant action of jatrophe in the isolated aorta of the rat: influence of potassium channel blockers. *Eur J Pharmacol* 215:75–81
13. Schmeda-Hirschmann G, Razmilic I, Sauvain M, Moretti C, Munoz V, Ruiz E, Balanza E, Fournet A (1996) Antiprotozoal activity of jatrogrossidione from *Jatropha grossidentata* and jatrophe from *Jatropha isabelli*. *Phytother Res* 10:375–378
14. Pertino M, Schmeda-Hirschmann G, Rodriguez JA, Theoduloz C (2007) Gastroprotective effect and cytotoxicity of terpenes from the Paraguayan crude drug “yagua rova” (*Jatropha isabelli*). *J Ethnopharmacol* 111:553–559
15. Fernandez ES, Rodrigues FA, Tofoli D, Imamura PM, Carvalho JE, Ruiz ALTG, Foglio MA, Minguzzi S, Silva RCL (2013) Isolation, structural identification and cytotoxic activity of hexanic extract, cyperenoic acid, and jatrophe terpenes from *Jatropha ribifolia* roots. *Rev Bras Farm* 23:441–446
16. Goncalves-Moraes VL, Rumjanek VM, Calixto JB (1996) Jatrophe and 12-O-tetradecanoyl phorbol-13-acetate antagonism of stimulation of natural killer activity and lymphocyte proliferation. *Eur J Pharmacol* 312:333–339
17. American Academy of Dermatology. Retrieved January 12, 2013, from <http://www.aad.org/>
18. Aiyelaagbe OO, Adesogan EK, Ekundayo O, Adeniyi BA (2000) The antimicrobial activity of roots of *Jatropha podagrica* (Hook). *Phytother Res* 14:60–62
19. Aiyelaagbe OO, Adesogan EK, Ekundayo O, Gloer JB (2007) Antibacterial diterpenoids from *Jatropha podagrica* Hook. *Phytochemistry* 68:2420–2425
20. Wang X-C, Zheng Z-P, Gan X-W, Hu L-H (2009) Jatrophalactam, a novel diterpenoid lactam isolated from *Jatropha curcas*. *Org Lett* 11:5522–5524
21. Devappa RK, Makkar HPS, Becker K (2011) *Jatropha* diterpenes: a review. *J Am Oil Chem Soc* 88:301–322
22. Schmeda-Hirschmann G, Tsichritzis F, Jakupovic J (1992) Diterpenes and a lignan from *Jatropha grossidentata*. *Phytochemistry* 31:1731–1735
23. Valente C, Pedro M, Duarte A, Nascimento MSJ, Abreu PM, Ferreira MJU (2004) Bioactive diterpenoids, a new jatrophane and two ent-abietanes and other constituents from *Euphorbia pubescens*. *J Nat Prod* 67:902–904

24. Wang Y, Huang R, Wang H-B, Jin H-Z, Lou L-G, Quin G-W (2006) Diterpenoids from the roots of *Euphorbia fischeriana*. *J Nat Prod* 69:967–970
25. Zhang W, Guo YW (2005) Three new jatrophe-type diterpenoids from *Euphorbia helioscopia*. *Planta Med* 71:280–283
26. Lu Z-Q, Guan S-H, Li X-N, Chen G-T, Zhang J-Q, Huang H-L, Liu X, Guo D-A (2008) Cytotoxic diterpenoids from *Euphorbia helioscopia*. *J Nat Prod* 71:873–876
27. Naengchomnong W, Thebtaranonth Y, Wiriyachitra P, Okamoto KT, Clardy J (1986) Isolation and structure determination of four novel diterpenes from *Jatropha curcas*. *Tetrahedron Lett* 27:2439–2442
28. Liu JQ, Yang YF, Wang CF, Li Y, Qiu MH (2012) Three new diterpenes from *Jatropha curcas*. *Tetrahedron* 68:972–976
29. Smith AB III, Adams CM (2004) Evolution of dithiane-based strategies for the construction of architecturally complex natural products. *Acc Chem Res* 37:365–377
30. Smith III AB (1984) In: Lindberg T (ed) Evolution of a synthetic strategy: total synthesis of jatrophe. In: *Strategies and tactics in organic synthesis*. T. Lindberg (Ed.). New York: Academic Press, 1984; pp 223–274.
31. Smith AB III, Guaciaro Schow SR, Wovkulich PM, Toder BH, Hall TW (1981) A strategy for the total synthesis of jatrophe: synthesis of normethyljatrophe. *J Am Chem Soc* 103:219–222
32. Gyorkos AC, Stille JK, Hegedus LS (1990) Total synthesis of ( $\pm$ )-*epi*-jatrophe and ( $\pm$ )-jatrophe using palladium-catalyzed carbonylative coupling of vinyl triflates with vinylstannanes as the macrocycle-forming step. *J Am Chem Soc* 112:8465–8472
33. Han Q, Wiemer DF (1992) Total synthesis of (+)-jatrophe. *J Am Chem Soc* 114:7692–7697
34. D'Alagni M, De Petris M, Marini-Bettolo GB, Temusi PA (1983) Study of the binding of jatrophe to *Escherichia coli* s-ribonucleic acid. *FEBS Lett* 164:51–56
35. Wu JH, Batist G (2012) Glutathione and glutathione analogues, therapeutic potentials. *Biochim Biophys Acta* 1830:3350–3353
36. Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronzato MA, Marinari UM, Domenicotti C (2013) Role of glutathione in cancer progression and chemoresistance. *Oxid Med Cell Longev* 2013:972913
37. Calvert P, Yao KS, Hamilton TC, O'Dwyer PJ (1998) Clinical studies of reversal of drug resistance based on glutathione. *Chem Biol Interact* 111–112:213–224
38. Estrela JM, Ortega A, Obrador E (2006) Glutathione in cancer biology and therapy. *Crit Rev Clin Lab Sci* 43:143–181
39. O'Brien ML, Tew KD (1996) Glutathione and related enzymes in multidrug resistance. *Eur J Cancer* 32A:967–978
40. Pertino M, Schmeda-Hirschmann G, Santos LS, Rodriguez JA, Theoduloz C (2007) Biotransformation of jatrophe by *Aspergillus niger* ATCC 16404. *Z Naturforsch* 62b:275–279
41. Schnabel C, Sterz K, Muller H, Rehbein J, Wiese M, Hiersemann M (2011) Total synthesis of natural and non-natural  $\Delta^{5,6}\Delta^{12,13}$ -jatrophane diterpene and their evaluation as MDR modulators. *J Org Chem* 76:512–522
42. Barile E, Borriello M, Barile E, Borriello M, Di Pietro A, Doreau A, Fattorusso C, Fattorusso E, Lanzotti V (2008) Discovery of a new series of jatrophane and lathyrene diterpenes as potent and specific P-glycoprotein modulators. *Org Biomol Chem* 6:1756–1762
43. Schnabel C, Hiersemann M (2009) Total synthesis of jatrophe diterpenes from *Euphorbia characias*. *Org Lett* 11:2555–2558
44. Lentsch C, Rinner U (2009) General synthesis of highly functionalized cyclopentane segments for the preparation of jatrophane diterpenes. *Org Lett* 11:5326–5328
45. Sutherland CJ, Polley SD (2011) Genomic insights into the past, current and future evolution of human parasites of the genus *Plasmodium*. In: *Genetics and Evolution of Infectious Diseases* (Tibayrenc, M., ed.), pp. 607–627, Elsevier

46. Centers for Disease Control and Prevention (2014). <http://www.cdc.gov/chikungunya/>. Accessed 1 Dec 2014
47. Pan American Health Organization (2013–2014) Health topics: chikungunya. <http://www.paho.org/>. Accessed 1 Dec 2014
48. Nothias-Scaglia LF, Retailleau P, Paolini J, Pannecouque C, Neyts J, Dumontet V, Roussi F, Leyssen P, Costa J, Litaudon M (2014) Jatrophane diterpenes as inhibitors of chikungunya virus replication: structure activity relationship and discovery of a potent lead. *J Nat Prod* 77:1505–1512
49. World Health Organization (2011) World Malaria Report 2011, Geneva. [http://www.who.int/malaria/world\\_malaria\\_report\\_2011/en/index.html](http://www.who.int/malaria/world_malaria_report_2011/en/index.html). Accessed 1 Oct 2013
50. Mitachi K, Salinas YG, Connelly M, Jensen N, Ling T, Rivas F (2012) Synthesis and structure-activity relationship of disubstituted benzamides as a novel class of antimalarial agents. *Bioorg Med Chem Lett* 22:4536–4539
51. Cupit M, Ling T, Rivas F (2013) New molecular scaffolds as potential therapeutic agents to combat malaria drug-resistance. In: Antimalarial drug research and development. Nova Science, New York, p 49
52. Schlitzer M (2008) Antimalarial drugs—what is in use and what is in the pipeline? *Arch Pharm (Weinheim)* 341:149
53. VanSchalkwyk DA, Egan TJ (2006) Quinoline-resistance reversing agents for the malaria parasite *Plasmodium falciparum*. *Drug Resist Updat* 9:211
54. Sinka ME, Bangs JM, Manguin S, Coetzee M, Mbogo CM, Hemingway J, Patil PA, Temperley WH, Gething PW, Kabaria CW, Okara RM, Boekel TV, Godfray HCJ, Harbach RE, Hay SI (2010) The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. *Parasit Vectors* 3:117
55. Lakshmanan V, Bray PG, Verdier-Pinard D, Johnson DJ, Horrocks P, Muhle RA, Alakpa GE, Hughes RH, Ward SA, Krogstad DJ, Sidhu AB, Fidock DA (2005) A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. *EMBO J* 24:2294–2305
56. Henry M, Alibert S, Orlandi-Pradines E, Bogreau H, Fusai T, Rogier C, Barbe J, Pradines B (2006) Chloroquine resistance reversal agents as promising antimalarial drugs. *Curr Drug Targets* 7:935
57. Smilkstein M, Sriwilajaroen N, Kelly JX, Wilairat P, Riscoe M (2004) Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob Agents Chemother* 48:1803–1806
58. Croft SL, Coombs GH (2003) Leishmaniasis—current chemotherapy and recent advances in the search of novel drugs. *Trends Parasitol* 19:502–508
59. Yasinzai M, Khan M, Nadhman A, Shahnaz G (2013) Drug resistance in leishmaniasis: current drug delivery systems, and future perspectives. *Future Med Chem* 5:1877–1888
60. World Health Organization Report (2013). <http://www.who.int/leishmaniasis/en/>
61. Feistel T, Hodson CA, Peyton DH, Landfear SM (2008) An expression system to screen for inhibitors of parasite glucose transporters. *Mol Biochem Parasitol* 162:71–76
62. Rodriguez-Contreras D, Feng X, Keeney KM, Bouwer HG, Landfear SM (2007) Phenotypic characterization of a glucose transporter null mutant in *Leishmania mexicana*. *Mol Biochem Parasitol* 153:9–18
63. Martin RE, Marchetti RV, Cowan AI, Howitt SM, Bröer S, Kirk K (2011) Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science* 325:1680

# Chapter 4

## O-Methyltransferases Involved in Lignan Biosynthesis

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and Masaomi Yamamura

**Abstract** Lignan structures show diverse oxidation levels and substitution patterns, and *O*-methylation is often crucial in determining the final product distribution. In the secondary metabolic processes of plants, *O*-methylation is usually catalyzed by *S*-adenosyl-L-methionine-dependent *O*-methyltransferases (OMTs). Recently, we have isolated six cDNAs encoding lignan OMTs: *Carthamus tinctorius* matairesinol OMT (CtMROMT), *Sesamum indicum* OMT1 (SiOMT1), *Sesamum radiatum* OMT1 (SrOMT1), *Anthriscus sylvestris* matairesinol OMT (AsMROMT), *A. sylvestris* thujaplicatin OMT (AsTJOMT), and *Forsythia koreana* matairesinol OMT (FkMROMT). CtMROMT and AsMROMT formed a small clade with plant OMTs including caffeic acid *O*-methyltransferase from *Rosa chinensis* var. *spontanea* (RcOMT3) and reticuline 7-*O*-methyltransferase from *Papaver somniferum* (PsOMT1). In contrast, AsTJOMT, FkMROMT, SiOMT1, and SrOMT2 were found in a distinct clade together with caffeic acid OMTs

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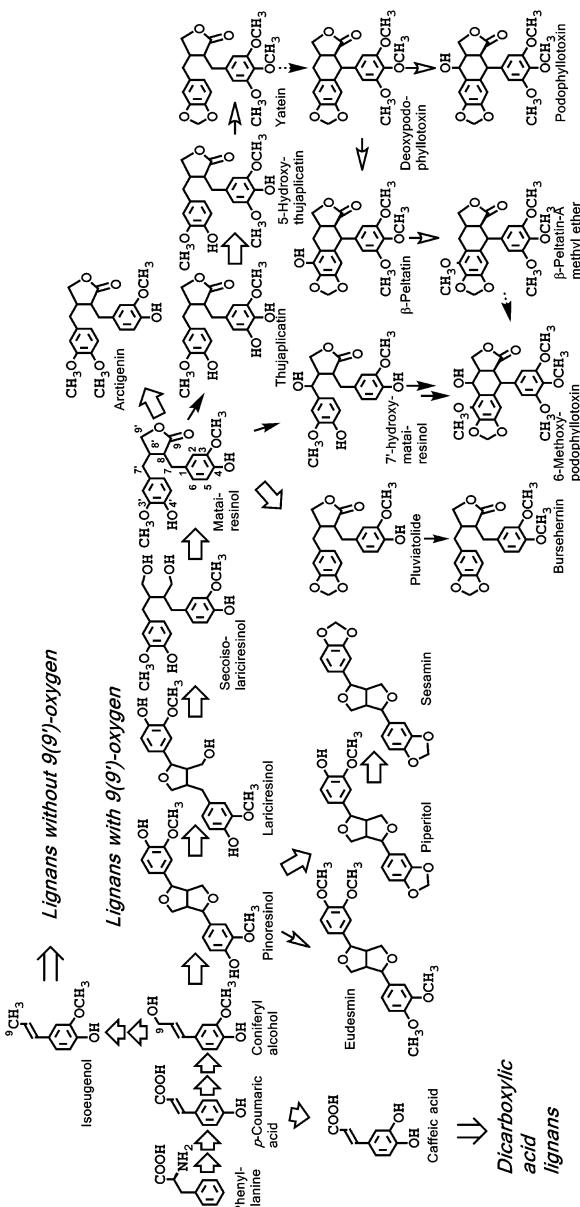
(5-hydroxyconiferaldehyde OMTs). The phylogenetic relationship suggested that the lignin OMTs arose independently in various lignan-producing plants via acquisition of substrate specificity for lineage-specific lignan structures. Biochemical characterization of their recombinant proteins indicated that they are highly regioselective and selective in terms of substrate enantiomers; CtMROMT and AsMROMT methylated matairesinol to give 4'-*O*-methylmatairesinol (arctigenin), while FkMROMT methylated matairesinol to give 4-*O*-methylmatairesinol (isolarctigenin). These reactions were also found to be highly selective in terms of substrate enantiomers. In addition, AsTJOMT catalyzed the selective methylation of thujaplicatin to give 5-*O*-methylthujaplicatin. SiOMT1 and SrOMT2 catalyzed *O*-methylation for furofuran lignans. These findings on lignan OMTs provide useful information for further identification of cDNAs encoding other biologically active lignan OMTs, as well as clues to understand diversity and plasticity of plant secondary metabolism beyond lignan biosynthesis.

**Keywords** Lignan • *O*-Methyltransferase • Regioselective • Enantiomer-selective • Matairesinol • Thujaplicatin

## 4.1 Introduction

Lignans are dimers of phenylpropanoids that are linked at the central carbons (C8 and C8') of their propyl side chains [1, 2]. Lignans can be classified into three categories depending on the oxidation state of their C9 (C9') position, which is located at the terminus of the propyl side chain. These categories include (1) lignans with 9(9')-oxygen, (2) lignans without 9(9')-oxygen, and (3) dicarboxylic acid lignans [2]. Figure 4.1 outlines the biosynthetic pathways for the three different categories of lignans. Methoxyl groups present in lignan molecules can be classified into two subgroups on the basis of their biosynthetic pathways, including (1) 3(3')-methoxyl groups and (2) others (Fig. 4.1). The 3(3')-methoxyl groups are introduced into phenylpropanoid monomers in the cinnamate/monolignol pathway [3], and are retained in lignan molecules. Most lignans bearing a 9(9')-oxygen are formed via the dimerization of coniferyl alcohol [2, 4, 5]. In addition, lignan structures show diverse oxidation levels, and substitution patterns and *O*-methylation is often crucial in determining the final product distribution [2, 6].

Methoxylated lignans possess a range of biological activities that are useful for human health and play important physiological roles in plants [2, 7, 8]. For example, the trimethoxylated aryltetralin lignan podophyllotoxin (Fig. 4.1) is an important antitumor compound that has been used as a starting material to prepare semisynthetic antitumor agents for use in cancer chemotherapy [9, 10]. Another lignan with antitumor activity is the trimethoxylated dibenzylbutyrolactone arctigenin (4'-*O*-methylmatairesinol) that shows selective toxicity towards cancer cells under glucose starvation conditions [11–13]. Arctigenin also exhibits a variety of other biological activities, such as antiproliferative [14, 15], hepatoprotective [16],



**Fig. 4.1** Outline of the lignan biosynthetic pathways. *Broad open arrow:* Reaction step where cDNA encoding the responsible enzyme was isolated. *Narrow open arrow:* Reaction step evidenced by an enzymatic experiment. *Narrow closed arrow:* Reaction step evidenced by a feeding experiment. *Dashed arrow:* Reaction step without experimental evidence

anti-inflammatory, and analgesic activities [17, 18]. Furthermore, arctigenin has been reported to suppress the heat shock response in mammalian cells [19] to inhibit mitogen-activated protein kinases [20] and cyclic AMP phosphodiesterase [21] and to have a relaxation effect on the histamine-induced contraction of tracheal muscles [22]. Arctigenin and its acid form, arctigenic acid, have also been reported to play important roles in the stimulatory allelopathy of *Arctium lappa* seeds during the seed germination stage [23]. Arctigenin [24, 25] and the related trimethoxylated dibenzylbutyrolactone lignan yatein (Fig. 4.1) [26] are typical examples of lignans deposited in the heartwood region of trees, where they probably prevent fungal heart rot [4, 27–29].

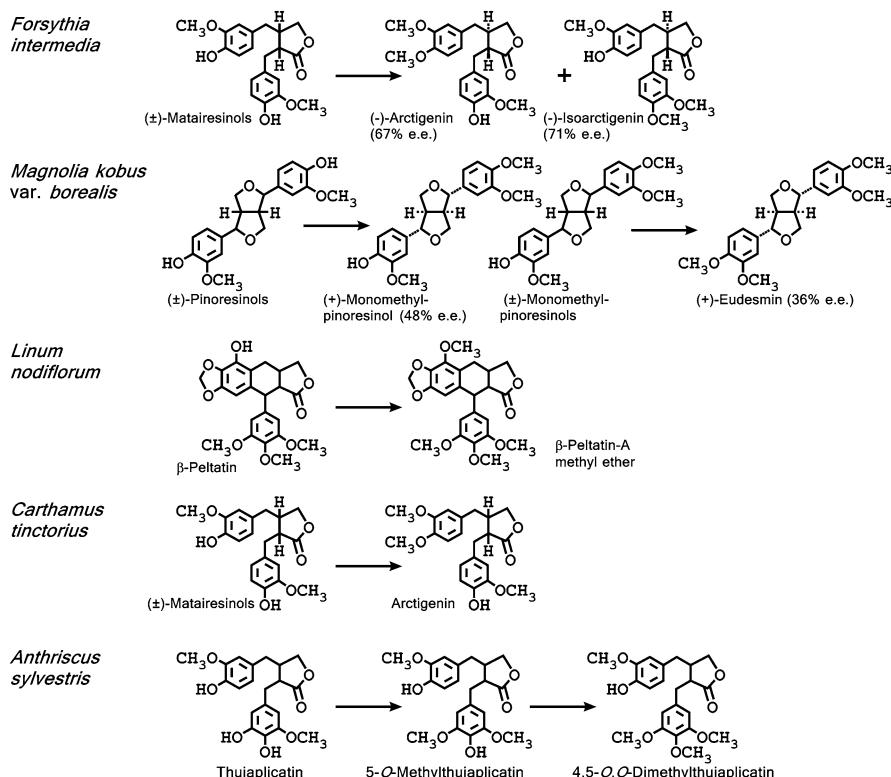
In the secondary metabolic processes of plants, *O*-methylation is usually catalyzed by *S*-adenosyl-L-methionine-dependent *O*-methyltransferases (OMTs). During the last two decades, OMTs involved in lignin biosynthesis, as well as many other plant OMTs involved in the methylation of flavonoids, stilbenes, coumarins, phenylpropenes, and alkaloids have been characterized in great detail [30–33]. The *O*-methyl group of the coniferyl alcohol involved in lignin biosynthesis is introduced by the caffeoyl CoA OMT (CCoAOMT)-catalyzed methylation of caffeoyl CoA [3]. The 5-hydroxyconiferaldehyde OMT [CAldOMT or caffeic acid OMT (CAOMT)]-catalyzed methylation of caffealdehyde to give coniferaldehyde has also been reported in *Medicago sativa* (alfalfa) [34, 35], as well as being implicated in *Arabidopsis thaliana* (thale cress) [36].

In contrast, until recently no studies have been reported pertaining to the genes encoding the OMTs involved in the methylation of lignans (lignan OMTs), even though lignans represent an abundant class of plant phenolic compounds. It thus remained unclear whether coniferyl alcohol as the lignan precursor is synthesized by those CCoAOMT or CAldOMT isoenzymes involved in lignin biosynthesis, by other CCoAOMT or CAldOMT isoenzymes, or by some other OMTs. Sakakibara et al. suggested that the coniferyl alcohol used in lignan biosynthesis may be formed via ferulic acid, which may not require CCoAOMT [37]. The other methoxyl groups in lignans, e.g., the 4(4'), 5(5'), and 6(6')-methoxyl groups on the aromatic rings, are introduced by lignan-OMT-catalyzed post-coupling methylation of the corresponding 4(4'), 5(5'), and 6(6')-hydroxylignans [2, 4] (Fig. 4.1). The methylenedioxy groups of the lignans may then be formed from the methoxyl groups [38, 39].

We recently reported the isolation of cDNAs encoding OMTs catalyzing methylation of lignans (lignan OMTs) [40–44]. Herein, we will provide a summary of the characteristics of these lignan OMTs.

## 4.2 Plant Lignan OMT Activity

There have been several reports in the literature to date pertaining to plant enzyme-mediated lignan methylation processes. Ozawa et al. reported the OMT activities of cell-free extracts from *Forsythia intermedia* [45] and found that the cell-free extracts catalyzed the methylation of the dibenzylbutyrolactone lignan matairesinol to give



**Fig. 4.2** Lignan methylation by plant proteins

both 4'-*O*-methylmatairesinol (arctigenin) and 4-*O*-methylmatairesinol (isoarctigenin) (Fig. 4.2) [45]. Isoarctigenin was not detected in *F. intermedia*, and it was consequently suggested that the selective formation of the arctigenin moiety of arctiin (4-*O*-glucoside of arctigenin) in the plant occurred via the selective glycosylation of matairesinol to give matairesinoside (4-*O*-glucoside of matairesinol), followed by methylation by the non-regioselective OMT to afford arctiin [45].

In contrast, the cell-free extracts of *Carthamus tinctorius* (safflower) (Fig. 4.3) catalyzed the regioselective methylation of matairesinol to give arctigenin exclusively, with no isoarctigenin being formed (Fig. 4.2) [42]. Some other examples of the regioselective methylation of lignans were reported, including the use of the cell-free extracts of *Anthriscus sylvestris* (cow parsley) (Fig. 4.3) to methylate thujaplicatin and 5-*O*-methylthujaplicatin to afford 5-*O*-methylthujaplicatin and 4,5-*O,O*-dimethylthujaplicatin, respectively (Fig. 4.2) [43].

Pinoresinol and its dimethyl ether (eudesmin) were isolated from *Magnolia kobus* var. *borealis*, and cell-free extracts from the plant catalyzed the methylation of pinoresinol in a stepwise manner to afford eudesmin via monomethylpinoresinol (Fig. 4.2) [46]. Lignan OMT activity has also been reported for *Linum nodiflorum*;



*Carthamus tinctorius*  
cv. Round-leaved White



*Forsythia koreana*



*Anthriscus sylvestris*



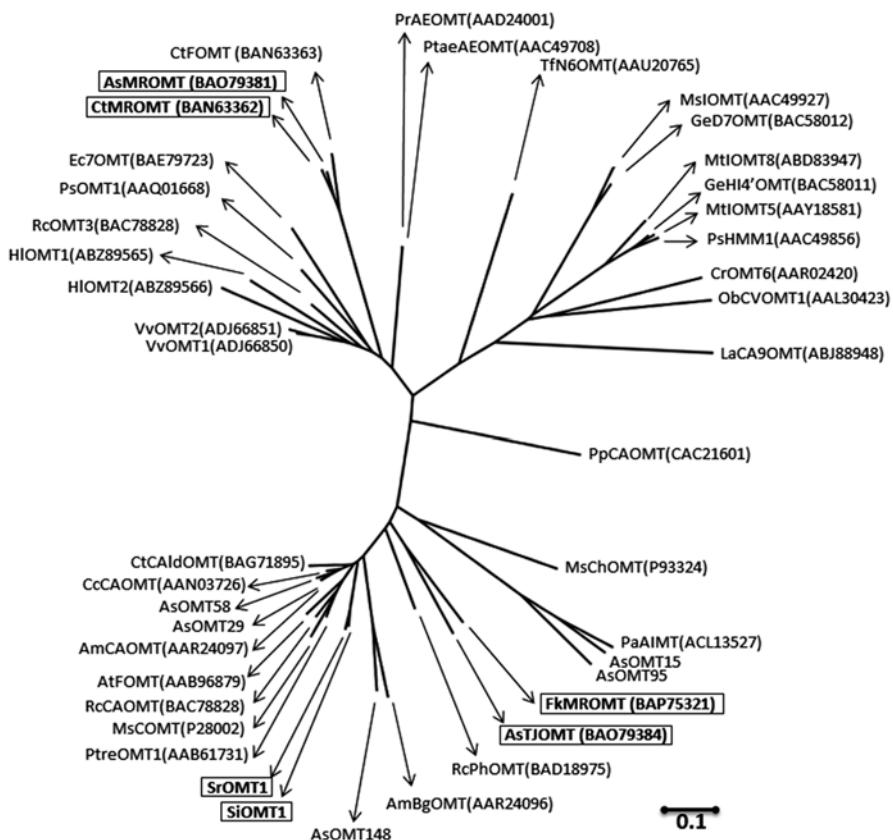
*Sesamum indicum*

**Fig. 4.3** Lignan-producing plants

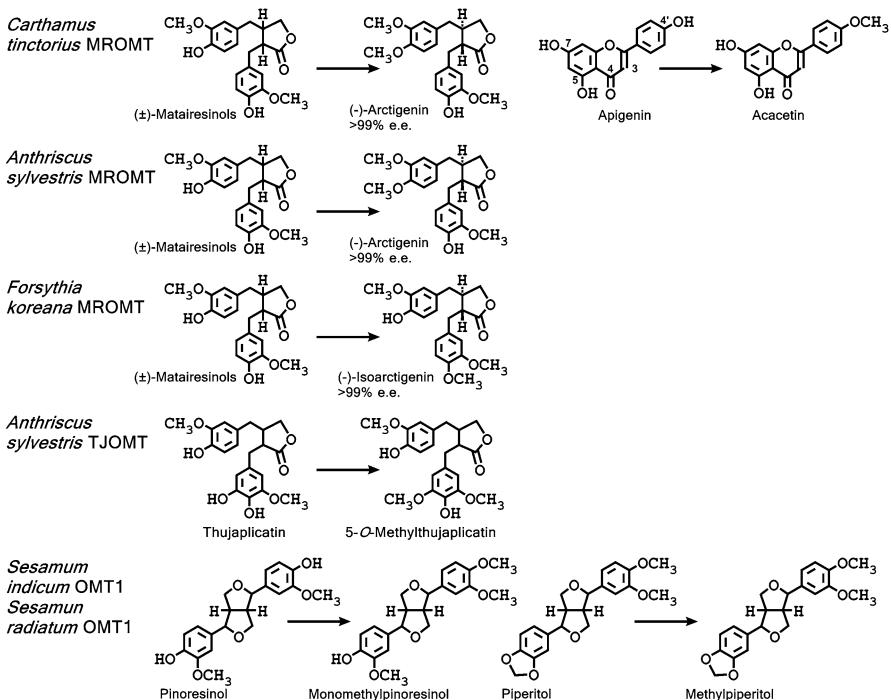
$\beta$ -peltatin 6-O-methyltransferase was isolated and characterized from the cell suspension cultures of the same species (Fig. 4.2) [47]. This reaction has been reported to be one of the putative biosynthetic steps in the biosynthesis of 6-methoxypodophyllotoxin from deoxypodophyllotoxin (Fig. 4.1) [47].

### 4.3 cDNAs Encoding Lignan OMTs

To date, only six cDNAs encoding lignan OMTs have been reported (Fig. 4.4), with the first of these being the cDNA encoding matairesinol-methylating OMT [matairesinol OMT (MROMT)] obtained from *C. tinctorius* seeds [40, 42]. The second example involved CAOMT homologs from sesame plants [41], SiOMT1 from *Sesamum indicum* (sesame) (Fig. 4.3) and SrOMT1 from *Sesamum radiatum* (black



**Fig. 4.4** Phylogenetic tree of lignan OMTs with related OMTs by the neighbor-joining method from amino acid alignment generated by MAFFT. Lignan OMTs are surrounded with frames. GenBank accession numbers are in parentheses after protein names



**Fig. 4.5** Lignan methylation by recombinant lignan OMTs

sesame). The two cDNAs were structurally highly similar to each other (76 % amino acid sequence identity), and were shown to catalyze *O*-methylation for furofuran lignans (pinoresinol and piperitol) (Fig. 4.5) that are precursors for sesamin, the most abundant lignan in sesame seeds [41]. Two more MROMT-encoding cDNAs were subsequently isolated from *A. sylvestris* (*AsMROMT*) [43, 44] and *Forsythia koreana* (Fig. 4.3) (*FkMROMT*) [44], respectively. The last of these cDNAs was isolated from *A. sylvestris* and encoded a thujaplicatin OMT (*AsTJOMT*) [43].

Figure 4.4 shows the phylogenetic tree of the amino acid sequences of the lignan OMTs and related plant OMTs. It is clear from the figure that the lignan OMTs are grouped in two distinct clades. CtMROMT and AsMROMT form a small clade with caffeic acid *O*-methyltransferase from *Rosa chinensis* var. *spontanea* (RcOMT3) [48], reticuline 7-*O*-methyltransferase from *Papaver somniferum* (opium poppy) (PsOMT1) [49], methoxypyrazine-forming *O*-methyltransferases from *Vitis vinifera* (common grape) (VvOMT1 and VvOMT2) [50], *O*-methyltransferases from *Humulus lupulus* (hop) (HIOMT1 and HIOMT2) [51], *Eschscholzia californica* (California poppy) reticuline 7-*O*-methyltransferase (Ec7OMT) [52] and two conifer hydroxycinnamic acids/hydroxycinnamoyl CoA esters *O*-methyltransferases (AEOMTs) [53, 54].

In contrast, AsTJOMT, FkMROMT, SiOMT1, and SrOMT2 were found in a distinct clade together with the caffeic acid OMTs [CAOMTs, or 5-hydroxyconferaldehyde OMTs (CAlgOMTs)]. Phloroglucinol OMT (PhOMT,

BAD18975) from *R. chinensis* var. *spontanea* [48] showed high sequence homology to AsTJOMT (50.7 %) and FkMROMT (52.9 %). In addition, this clade was composed of *Ammi majus* (bishop's flower) bergaptol 5-*O*-methyltransferase (AmBgOMT, AAR24096 [55]) as well as the CAOMTs (CAldOMTs) of various plant species including *Populus tremuloides* (quaking aspen) (AAB61731 [56, 57]), *M. sativa* (P28002 [58, 59]), *A. thaliana* (AAB96879 [60], AAM10127 [36]), *C. tinctorius* (BAG71895 [61]), *M. sativa* isoliquiritigenin 2'-*O*-methyltransferase (MsChOMT, P93324 [62, 63]), and *Pimpinella anisum* (anise) *t*-anol/isoeugenol *O*-methyltransferase 1 (PaAIMT1, ACL13527 [64]).

Thus, the primary structures of plant OMTs (Fig. 4.4) revealed that the lignan OMTs do not form a monophyletic clade in plant OMTs, indicating that the lignan OMTs are unlikely to be derived from a common ancestral OMT. Instead, it appears likely that they arose independently in various lignan-producing plants via acquisition of substrate specificity for lineage-specific lignan structures. Therefore, it is difficult to predict their substrate specificities based on their phylogenetic relationship. In the phylogenetic tree (Fig. 4.4), SiOMT1 and SrOMT1 are closely related to CAOMTs that are involved in aromatic modification of phenylpropanoid monomers. Considering that the monomers are precursors for lignans, CAOMTs likely have evolved prior to lignan OMTs, suggesting that SiOMT1 and SrOMT1 differentiated from CAOMT or a related OMT.

#### 4.4 Substrate Specificity of the Lignan OMTs

The recombinant lignan OMTs (Fig. 4.5) and closely related OMTs showed distinct substrate specificities. CtMROMT catalyzed the regioselective methylation of matairesinol to give arctigenin, with no isoarctigenin being detected. Furthermore, CtMROMT efficiently methylated the *C. tinctorius* flavonoid apigenin to afford acacetin with none of the other regioisomers (i.e., genkwanin and thevetiaflavone) being formed [42]. Regioselective flavonoid methylation has also been reported to be catalyzed by another *Carthamus* OMT, CtFOMT, which shared 73.7 % amino acid sequence identity with CtMROMT. Despite its high homology with CtMROMT, however, CtFOMT did not exhibit any matairesinol OMT activity [42]. Collectively, the enzymatic bifunctionality of CtMROMT for apigenin and matairesinol, in addition to structural similarity to CtFOMT, suggests that CtMROMT and CtFOMT arose from gene duplication followed by alteration of substrate specificity in *Carthamus*.

AsMROMT, which has 85.5 % amino acid sequence identity with CtMROMT, showed different substrate specificity to those of CtMROMT and CtFOMT. The *Anthriscus* OMT (Fig. 4.5) catalyzed the regioselective methylation of matairesinol to give arctigenin, but not isoarctigenin, whereas it did not methylate apigenin [43], although this flavonoid was detected in *A. sylvestris* [65, 66].

AsTJOMT, which has 29.7 % amino acid sequence identity with CtMROMT, efficiently catalyzed the methylation of thujaplicatin to give 5-*O*-methylthujaplicatin, although matairesinol, 5-*O*-methylthujaplicatin, and apigenin did not serve as sub-

strates for this OMT (Fig. 4.5) [43]. This methylation proceeded at the position *meta* to the propyl side chain, and was therefore in accordance with the regioselectivity of the plant CAldOMTs that are located in the same clade as AsTJOMT (Fig. 4.4). In contrast, FkMROMT, which is also located in the same clade as AsTJOMT (Fig. 4.4), catalyzed the *para*-methylation of matairesinol [44]. These results indicated differences in the activities of lignan OMTs belonging to this clade, in terms of their regioselectivity towards the *meta* and *para* positions relative to the propyl side chains. The methylation product of incubations of matairesinol with FkMROMT was isoarctigenin, but not arctigenin. Considering that isoarctigenin was not detected in *Forsythia* plants [44, 45], the physiological function of the OMT remains elusive. Ozawa et al. [45] reported isoarctigenin-forming OMT activity in the cell-free extracts of *F. intermedia*, suggesting that they included a matairesinol-methylating OMT with the same regioselectivity as FkMROMT (Fig. 4.2). In addition, FkMROMT did not catalyze the methylation of thujaplicatin or apigenin.

To explore the additional FkOMTs responsible for arctigenin biosynthesis, we conducted RNA-Seq analysis of *F. koreana* using next generation sequencing. The results of this analysis, however, did not reveal clear homologs for *CtMROMT* or *AsMROMT*, and the highest amino acid identity of any putative FkOMTs with the MROMTs was only 35 %, suggesting that the FkOMTs responsible for the formation of arctigenin in *F. koreana* were likely to be phylogenetically distant from CtMROMT and AsMROMT [44].

## 4.5 Enantiomer Selectivity of Lignan OMTs

The conversion of coniferyl alcohol to matairesinol has been observed in a variety of different plant species, suggesting quite strongly that this transformation is a general biosynthetic pathway for lignans bearing 9(9')-oxygen (Fig. 4.1) [2]. Many other subclasses of lignans with 9(9')-oxygens are formed from the lignans on the general pathway, and modifications to the aromatic substituents, such as the *O*-methylation of the phenolic hydroxyls, also start from these lignans (Fig. 4.1) [2].

Most naturally occurring pinoresinol and lariciresinol exist as mixtures of both enantiomers [2, 67, 68], whereas all of the dibenzylbutyrolactone lignans analyzed by HPLC on chiral stationary phases so far were found to be optically pure [2, 67]. Furthermore, the predominant enantiomers of pinoresinol, lariciresinol, and the dibenzylbutane lignans vary considerably depending on the plant species from which they are derived [2, 67, 68].

The enantiomeric control for the general pathway towards matairesinol is well understood. Thus, production of optically pure matairesinol not only requires dirigent proteins to mediate enantioselective formation of pinoresinol [2, 4, 5, 69–74], but also enzymes such as pinoresinol(lariciresinol) reductase that are involved in reaction steps following pinoresinol formation [5, 75, 76]. For example, it was demonstrated that differential expression of pinoresinol reductase isoforms that have distinct selectivities of substrate enantiomers plays a significant role in determining

enantiomeric compositions of the product, lariciresinol, as well as dirigent protein, in *A. thaliana* [5, 75].

In contrast, until recently the stereochemical control of the further conversion of the lignans of the general pathway remained unknown [2, 4, 5, 75]. Several enantiomer-selective OMT activities have been reported, although none of these have been lignan OMTs. For example, an OMT from *Berberis* cell cultures showed enantiomer selectivity towards the (*S*)-enantiomer of the alkaloid scoulerine to give (*S*)-tetrahydrocolumbamine, but showed no activity towards the corresponding (*R*)-enantiomer [77]. In a separate report, 2-hydroxyisoflavanone 4'-OMT from *Glycyrrhiza echinata* (licorice) and (+)-6a-hydroxymaackiain 3-OMT from *Pisum sativum* (pea) were shown to exhibit enantiomer selectivity towards the (+)-enantiomers instead of the (-)-enantiomers of their substrates [78]. With regard to lignan OMTs, the cell-free extracts of *F. intermedia* catalyzed the monomethylation of matairesinol to give both arctigenin and isoarctigenin. This methylation, however, occurred without strict selectivity for the enantiomers of the substrate, although the plant produced optically pure levorotatory arctigenin (Fig. 4.2) [45]. Furthermore, the eudesmin isolated from *M. kobus* var. *borealis* was the optically pure (+)-enantiomer, whereas pinoresinol existed as a mixture of (+)- and (-)-enantiomers with the former being predominant (77.1 % e.e.) [46]. This result suggested that the enantiomer-selective methylation of pinoresinol could occur during the formation of eudesmin in the plant. Cell-free extracts from the same species, however, did not show strict enantiomer selectivity; the methylation of (+)- and (-)-pinoresinols afforded both enantiomers of eudesmin, with the naturally occurring (+)-enantiomer being formed as the major enantiomer (Fig. 4.2) [46].

Ragamustari et al. [44] have recently reported enantiomer-selective lignan OMT activities. Chiral LC-MS analysis of the product obtained by incubation of matairesinol with CtMROMT indicated that the product was optically pure (-)-arctigenin (Fig. 4.5), and this result corresponded well with the optically pure (-)-arctigenin isolated from the plant species. AsMROMT, which shared a high amino acid sequence homology (85.5 % identity) with CtMROMT, also catalyzed the regioselective methylation of matairesinol to give arctigenin. Once again the resulting arctigenin was optically pure (-)-enantiomer (Fig. 4.5), although it was not possible to determine the predominant enantiomer of this lignan occurring in *A. sylvestris*, because it was formed in such small quantities that it could not be isolated [79].

These results clearly indicated that the MROMTs involved in the conversion of matairesinol to arctigenin were highly selective in terms of the different enantiomers of the substrates. In contrast, *F. intermedia* crude enzyme preparations showed MROMT activity generating both enantiomers of the two regioisomers, arctigenin and isoarctigenin, when incubating ( $\pm$ )-matairesinols, even though the plant produced optically pure (-)-arctigenin but not isoarctigenin (Fig. 4.2) [45]. Similar to *F. intermedia*, *F. koreana* produced optically pure (-)-matairesinol and (-)-arctigenin [80].

FkMROMT, which catalyzed the regioselective methylation of matairesinol to give isoarctigenin, also exhibited strict selectivity for the substrate enantiomer, with isoarctigenin being formed as the optically pure (-)-enantiomer (Fig. 4.5) [44]. This

MROMT activity most likely corresponds to the isoarctigenin-forming activity previously observed in the crude *F. intermedia* enzyme [45].

## 4.6 Conclusions

Several cDNAs encoding lignan OMTs that catalyze the methylation of the phenolic hydroxyls of lignans to the corresponding methyl ethers have been isolated. Biochemical characterization of their recombinant proteins indicated that they are highly regioselective and selective in terms of substrate enantiomers. Lignan OMTs are structurally and biochemically diverse, and they probably evolved independently in each plant lineage. The findings on lignan OMTs described herein present useful information for further identification of cDNAs encoding other lignan OMTs that are involved in the biosynthesis of biologically active and valuable lignans. In addition, these findings provide clues to understand the diversity and plasticity of plant secondary metabolism beyond lignan biosynthesis.

## References

1. Moss GP (2000) Nomenclature of lignans and neolignans. Pure Appl Chem 72:1493–1523
2. Umezawa T (2003) Diversity in lignan biosynthesis. Phytochem Rev 2:371–390
3. Umezawa T (2010) The cinnamate/monolignol pathway. Phytochem Rev 9:1–17
4. Suzuki S, Umezawa T (2007) Biosynthesis of lignans and norlignans. J Wood Sci 53:273–284
5. Umezawa T, Yamamura M, Nakatsubo T, Suzuki S, Hattori T (2011) Stereoselectivity of the biosynthesis of norlignans and related compounds. In: Gang D (ed) The biological activity of phytochemicals (recent advances in phytochemistry, 41). Springer, New York, pp 179–197
6. Umezawa T (2003) Phylogenetic distribution of lignan producing plants. Wood Res 90:27–110
7. Harmatha J, Dinan L (2003) Biological activities of lignans and stilbenoids associated with plant-insect chemical interactions. Phytochem Rev 2:321–330
8. MacRae WD, Towers GHN (1984) Biological activities of lignans. Phytochemistry 23: 1207–1220
9. You Y (2005) Podophyllotoxin derivatives: current synthetic approaches for new anticancer agents. Curr Pharm Des 11:1695–1717
10. Srivastava V, Negi AS, Kumar JK, Gupta MM, Khanuja SPS (2005) Plant-based anticancer molecules: a chemical and biological profile of some important leads. Bioorg Med Chem 13:5892–5908
11. Awale S, Lu J, Kalauni SK, Kurashima Y, Tezuka Y, Kadota S, Esumi H (2006) Identification of arctigenin as an antitumor agent having the ability to eliminate the tolerance of cancer cells to nutrient starvation. Cancer Res 66:1751–1757
12. Kim J-Y, Hwang J-H, Cha M-R, Yoon M-Y, Son E-S, Tomida A, Ko B, Song S-W, Shin-ya K, Hwang Y-I et al (2010) Arctigenin blocks the unfolded protein response and shows therapeutic antitumor activity. J Cell Physiol 224:33–40
13. Sun S, Wang X, Wang C, Nawaz A, Wei W, Li J, Wang L, Yu DH (2011) Arctigenin suppresses unfolded protein response and sensitizes glucose deprivation-mediated cytotoxicity of cancer cells. Planta Med 77:141–145

14. Ryu SY, Ahn JW, Kang YH, Han BH (1995) Antiproliferative effect of arctigenin and arctiin. *Arch Pharm Res* 18:462–463
15. Matsumoto T, Hosono-Nishiyama K, Yamada H (2006) Antiproliferative and apoptotic effects of butyrolactone lignans from *Arctium lappa* on leukemic cells. *Planta Med* 72:276–278
16. Kim SH, Jang YP, Sung SH, Kim CJ, Kim JW, Kim YC (2003) Hepatoprotective dibenzylbutyrolactone lignans of *Torreya nucifera* against CCl<sub>4</sub>-induced toxicity in primary cultured rat hepatocytes. *Biol Pharm Bull* 26:1202–1205
17. Fan C-Q, Zhu X-Z, Zhan Z-J, Ji X-Q, Li H, Yue J-M (2006) Lignans from *Saussurea conica* and their NO production suppressing activity. *Planta Med* 72:590–595
18. Kang HS, Lee JY, Kim CJ (2008) Anti-inflammatory activity of arctigenin from *Forsythiae fructus*. *J Ethnopharmacol* 116:305–312
19. Ishihara K, Yamagishi N, Saito Y, Takasaki M, Konoshima T, Hatayama T (2006) Arctigenin from *Fructus fructii* is a novel suppressor of heat shock response in mammalian cells. *Cell Stress Chaperones* 11:154–161
20. Cho MK, Jang YP, Kim YC, Kim SG (2004) Arctigenin, a phenylpropanoid dibenzylbutyrolactone lignan, inhibits MAP kinases and AP-1 activation via potent MKK inhibition: the role in TNF- $\alpha$  inhibition. *Int Immunopharmacol* 4:1419–1429
21. Nikaido T, Ohmoto T, Kinoshita T, Sankawa U, Nishibe S, Hisada S (1981) Inhibition of cyclic AMP phosphodiesterase by lignans. *Chem Pharm Bull* 29:3586–3592
22. Fujimoto T, Nose M, Takeda T, Ogihara Y, Nishibe S, Minami M (1992) Studies on the Chinese crude drug “Luoshiteng” (II) on the biologically active components in the stem part of luoshiteng originating from *Trachelospermum jasminoides*. *Shoyakugaku Zasshi* 46:224–229
23. Higashinakasu K, Yamada K, Shigemori H, Hasegawa K (2005) Isolation and identification of potent stimulatory allelopathic substances exuded from germinating burdock (*Arctium lappa*) seeds. *Heterocycles* 65:1431–1437
24. Chang S-T, Wang S-Y, Su Y-C, Kuo Y-H (1999) Structural elucidation of three dibenzyl- $\gamma$ -butyrolactone type lignans isolated from Taiwania (*Taiwania cryptomerioides* Hayata) heartwood: 7-oxohinokinin, sventenin and arctigenin. *Q Jour Chin For* 32:121–129
25. Chang S-T, Wang DS-Y, Wu C-L, Shiah S-G, Kuo Y-H, Chang C-J (2000) Cytotoxicity of extractives from *Taiwania cryptomerioides* heartwood. *Phytochemistry* 55:227–232
26. Erdtman H, Harmatha J (1979) Phenolic and terpenoid heartwood constituents of *Libocedrus yateensis*. *Phytochemistry* 18:1495–1500
27. Suzuki S, Umezawa T, Shimada M (2001) Norlignan biosynthesis in *Asparagus officinalis* L.: the norlignan originates from two non-identical phenylpropane units. *J Chem Soc Perkin Trans 1*:3252–3257
28. Suzuki S, Nakatsubo T, Umezawa T, Shimada M (2002a) First *in vitro* norlignan formation with *Asparagus officinalis* enzyme preparation. *Chem Commun (Camb)* 1088–1089
29. Suzuki S, Yamamura M, Shimada M, Umezawa T (2004) A heartwood norlignan, (*E*)-hinokiresinol, is formed from 4-coumaryl 4-coumarate by a *Cryptomeria japonica* enzyme preparation. *Chem Commun (Camb)* 2838–2839
30. Ibrahim RK, Bruneau A, Bantignies B (1998) Plant *O*-methyltransferases: molecular analysis, common signature and classification. *Plant Mol Biol* 36:1–10
31. Joshi CP, Chiang VL (1998) Conserved sequence motifs in plant S-adenosyl-L-methionine-dependent methyltransferases. *Plant Mol Biol* 37:663–674
32. Schröder G, Wehinger E, Schröder J (2002) Predicting the substrates of cloned plant *O*-methyltransferases. *Phytochemistry* 59:1–8
33. Zubietta C, He X-Z, Dixon RA, Noel JP (2001) Structures of two natural product methyltransferases reveal the basis for substrate specificity in plant *O*-methyltransferases. *Nat Struct Biol* 8:271–279
34. Guo D, Chen F, Inoue K, Blount JW, Dixon RA (2001) Downregulation of caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase in transgenic alfalfa: impacts on lignin structure and implications for the biosynthesis of G and S lignin. *Plant Cell* 13:73–88
35. Parvathi K, Chen F, Guo D, Blount JW, Dixon RA (2001) Substrate preferences of *O*-methyltransferases in alfalfa suggest new pathways for 3-*O*-methylation of monolignols. *Plant J* 25:193–202

36. Nakatsubo T, Kitamura Y, Sakakibara N, Mizutani M, Hattori T, Sakurai N, Shibata D, Suzuki S, Umezawa T (2008) At5g54160 gene encodes *Arabidopsis thaliana* 5-hydroxyconiferaldehyde O-methyltransferase. *J Wood Sci* 54:312–317
37. Sakakibara N, Nakatsubo T, Suzuki S, Shibata D, Shimada M, Umezawa T (2007) Metabolic analysis of the cinnamate/monolignol pathway in *Carthamus tinctorius* seeds by a stable-isotope-dilution method. *Org Biomol Chem* 5:802–815
38. Ono E, Nakai M, Fukui Y, Tominori N, Fukuchi-Mizunati M, Saito M, Satake H, Tanaka T, Katsuta M, Umezawa T, Tanaka Y (2006) Formation of two methylenedioxy bridges by a *Sesamum* CYP81Q protein yielding a furofuran lignan, (+)-sesamin. *Proc Natl Acad Sci U S A* 103:10116–10121
39. Marques JV, Kim K-W, Lee C, Costa MA, May GD, Crow JA, Davin LB, Lewis NG (2013) Next generation sequencing in predicting gene function in podophyllotoxin biosynthesis. *J Biol Chem* 288:466–479
40. Umezawa T, Li L, Suzuki S, Sakakibara N, Nakatsubo T, Chiang VL (2004) A novel O-methyltransferase catalyzing a regioselective methylation of lignan. In: Proceedings of the 49th Lignin Symposium, Tsukuba, Japan, pp 33–36
41. Ono E (2007) PCT/JP2007/057363. <http://patent.ipexl.com/WO/WOZZSLASHZZ2007ZZSLASHZZ119639.html>
42. Umezawa T, Ragamustari SK, Nakatsubo T, Wada S, Li L, Yamamura M, Sakakibara N, Hattori T, Suzuki S, Chiang VL (2013) A lignan O-methyltransferase catalyzing the regioselective methylation of matairesinol in *Carthamus tinctorius*. *Plant Biotechnol* 30:97–109
43. Ragamustari SK, Nakatsubo T, Hattori T, Ono E, Kitamura Y, Suzuki S, Yamamura M, Umezawa T (2013) A novel O-methyltransferase involved in the first methylation step of yatein biosynthesis in *Anthriscus sylvestris*. *Plant Biotechnol* 30:315–326
44. Ragamustari SK, Yamamura M, Ono E, Hattori T, Suzuki S, Suzuki H, Shibata D, Umezawa T (2014) Substrate-enantiomer selectivity of matairesinol O-methyltransferases. *Plant Biotechnol* 31:257–267
45. Ozawa S, Davin LB, Lewis NG (1993) Formation of (−)-arctigenin in *Forsythia intermedia*. *Phytochemistry* 32:643–652
46. Miyauchi T, Ozawa S (1998) Formation of (+)-eudesmim in *Magnolia kobus* DC. var. *borealis* Sarg. *Phytochemistry* 47:665–670
47. Kranz K, Petersen M (2003) β-Peltatin 6-O-methyltransferase from suspension cultures of *Linum nodiflorum*. *Phytochemistry* 64:453–458
48. Wu S, Watanabe N, Mita S, Ueda Y, Shibuya M, Ebizuka Y (2003) Two O-methyltransferases isolated from flower petals of *Rosa chinensis* var. *spontanea* involved in scent biosynthesis. *J Biosci Bioeng* 96:119–128
49. Ounaroon A, Decker G, Schmidt J, Lottspeich F, Kutchan TM (2003) (*R*, *S*)-Reticuline 7-O-methyltransferase and (*R*, *S*)-norcooclaurine 6-O-methyltransferase of *Papaver somniferum*—cDNA cloning and characterization of methyl transfer enzymes of alkaloid biosynthesis in opium poppy. *Plant J* 36:808–819
50. Dunlevy JD, Soole KL, Perkins MV, Dennis EG, Keyzers RA, Kalua CM, Boss PK (2010) Two O-methyltransferases involved in the biosynthesis of methoxypyrazines: grape-derived aroma compounds important to wine flavour. *Plant Mol Biol* 74:77–89
51. Nagel J, Culley LK, Lu Y, Liu E, Matthews PD, Stevens JF, Page JE (2008) EST analysis of hop glandular trichomes identifies an O-methyltransferase that catalyzes the biosynthesis of xanthohumol. *Plant Cell* 20:186–200
52. Fujii N, Inui T, Iwasa K, Morishige T, Sato F (2007) Knockdown of berberine bridge enzyme by RNAi accumulates (*S*)-reticuline and activates a silent pathway in cultured California poppy cells. *Transgenic Res* 16:363–375
53. Li L, Popko JL, Zhang X-H, Osakabe K, Tsai CJ, Joshi CP, Chiang VL (1997) A novel multi-functional O-methyltransferase implicated in a dual methylation pathway associated with lignin biosynthesis in loblolly pine. *Proc Natl Acad Sci U S A* 94:5461–5466
54. Moyle R, Moody J, Phillips L, Walter C, Wagner A (2002) Isolation and characterization of a *Pinus radiata* lignin biosynthesis related O-methyltransferase promoter. *Plant Cell Rep* 20:1052–1060

55. Hehmann M, Lukačin R, Ekiert H, Matern U (2004) Furanocoumarin biosynthesis in *Ammi majus* L. Cloning of bergaptol *O*-methyltransferase. *Eur J Biochem* 271:932–940
56. Bugos RC, Chiang VLC, Campbell WH (1991) cDNA cloning, sequence analysis and seasonal expression of lignin-bispecific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase of aspen. *Plant Mol Biol* 17:1203–1215
57. Li L, Popko JL, Umezawa T, Chiang VL (2000) 5-Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. *J Biol Chem* 275:6537–6545
58. Gowri G, Bugos RC, Campbell WH, Maxwell CA, Dixon RA (1991) Stress responses in alfalfa (*Medicago sativa* L.) X. Molecular cloning and expression of *S*-adenosyl-L-methionine: caffeic acid 3-*O*-methyltransferase, a key enzyme of lignin biosynthesis. *Plant Physiol* 97:7–14
59. Zubietta C, Kota P, Ferrer J-L, Dixon RA, Noel JP (2002) Structural basis for the modulation of lignin monomer methylation by caffeic acid/5-hydroxyferulic acid 3/5-*O*-methyltransferase. *Plant Cell* 14:1265–1277
60. Zhang H, Wang J, Goodman HM (1997) An *Arabidopsis* gene encoding a putative 14-3-3-interacting protein, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase. *Biochim Biophys Acta* 1353:199–202
61. Nakatsubo T, Li L, Hattori T, Lu S, Sakakibara N, Chiang VL, Shimada M, Suzuki S, Umezawa T (2007) Roles of 5-hydroxyconiferaldehyde and caffeoyl CoA *O*-methyltransferases in monolignol biosynthesis in *Carthamus tinctorius*. *Cell Chem Technol* 41:511–520
62. Maxwell CA, Harrison MJ, Dixon RA (1993) Molecular characterization and expression of alfalfa isoliquiritigenin 2'-*O*-methyltransferase, an enzyme specifically involved in the biosynthesis of an inducer of *Rhizobium meliloti* nodulation genes. *Plant J* 4:971–981
63. Ichimura M, Furuno T, Takahashi T, Dixon RA, Ayabe S-I (1997) Enzymic *O*-methylation of isoliquiritigenin and licodione in alfalfa and licorice cultures. *Phytochemistry* 44:991–995
64. Koeduka T, Baiga TJ, Noel JP, Pichersky E (2009) Biosynthesis of *t*-anethole in anise: characterization of *t*-anol/isoeugenol synthase and an *O*-methyltransferase specific for a C7-C8 propenyl side chain. *Plant Physiol* 149:384–394
65. Kurihara T, Kikuchi M (1979) Studies on the constituents of *Anthriscus sylvestris* Hoffm. II. On the components of the flowers and leaves. *Yakugaku Zasshi* 99:602–606
66. Milovanovic M, Picuric-Jovanovic K, Vucelic-Radovic B, Vrbaski Z (1996) Antioxidant effects of flavonoids of *Anthriscus sylvestris* in lard. *J Am Oil Chem Soc* 73:773–776
67. Umezawa T, Okunishi T, Shimada M (1997) Stereochemical diversity in lignan biosynthesis. *Wood Res* 84:62–75
68. Smeds AI, Eklund PC, Willför SM (2012) Content, composition, and stereochemical characterisation of lignans in berries and seeds. *Food Chem* 134:1991–1998
69. Umezawa T, Davin LB, Lewis NG (1991) Formation of lignans, (–)-secoisolariciresinol and (–)-matairesinol with *Forsythia intermedia* cell-free extracts. *J Biol Chem* 266:10210–10217
70. Davin LB, Wang H-B, Crowell AL et al (1997) Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. *Science* 275:362–366
71. Halls SC, Lewis NG (2002) Secondary and quaternary structures of the (+)-pinoresinol-forming dirigent protein. *Biochemistry* 41:9455–9461
72. Pickel B, Constant M-A, Pfannstiel J, Conrad J, Beifuss U, Schaller A (2010) An enantio-complementary dirigent protein for the enantioselective laccase-catalyzed oxidative coupling of phenols. *Angew Chem Int Ed Engl* 49:202–204
73. Finefield JM, Sherman DH, Kreitman M, Williams RM (2012) Enantiomeric natural products: occurrence and biogenesis. *Angew Chem Int Ed Engl* 51:4802–4836
74. Pickel B, Schaller A (2013) Dirigent proteins: molecular characteristics and potential biotechnological applications. *Appl Microbiol Biotechnol* 97:8429–8438
75. Nakatsubo T, Mizutani M, Suzuki S, Hattori T, Umezawa T (2008) Characterization of *Arabidopsis thaliana* pinoresinol reductase, a new type of enzyme involved in lignan biosynthesis. *J Biol Chem* 283:15550–15557
76. Hemmati S, Schmidt TJ, Fuss E (2007) (+)-Pinoresinol/(-)-lariciresinol reductase from *Linum perenne* Himmelszelt involved in the biosynthesis of justicidin B. *FEBS Lett* 581:603–610

77. Muemmler S, Rueffer M, Nagakura N, Zenk MH (1984) S-adenosyl-L-methionine: (S)-scoulerine 9-O-methyltransferase, a highly stereo- and regio-specific enzyme in tetrahydroprotoberberine biosynthesis. *Plant Cell Rep* 4:36–39
78. Akashi T, VanEtten HD, Sawada Y, Wasmann CC, Uchiyama H, Ayabe S (2006) Catalytic specificity of pea *O*-methyltransferases suggests gene duplication for (+)-pisatin biosynthesis. *Phytochemistry* 67:2525–2530
79. Koulman A, Bos R, Medarde M, Pras N, Quax WJ (2001) A fast and simple GC MS method for lignan profiling in *Anthriscus sylvestris* and biosynthetically related plant species. *Planta Med* 67:858–862
80. Umezawa T, Isohata T, Kuroda H, Higuchi T, Shimada M (1992) Chiral HPLC and LC-MS analysis of several lignans. In: Kuwahara M, Shimada M (eds) Biotechnology in pulp and paper industry. Uni, Tokyo, pp 507–512

## Chapter 5

# Hormone Signaling: Current Perspectives on the Roles of Salicylic Acid and Its Derivatives in Plants

Dhirendra Kumar, Imdadul Haq, Danda Chapagai, Diwaker Tripathi, David Donald, Mir Hossain, and Shivakumar Devaiah

**Abstract** Salicylic acid (SA) is an important plant hormone with a wide range of effects on plant growth and metabolism. Plants lacking SA exhibit enhanced susceptibility to pathogens. SA plays important signaling roles in resistance against biotrophic and hemi-biotrophic phytopathogens. It is synthesized in plastids along two pathways, one involving phenylalanine ammonia lyase (PAL) and the other isochorismate synthase (ICS). In *Arabidopsis*, during immune response most SA is synthesized through the ICS-dependent pathway, but clearly an ICS-independent pathway also exists. Several SA effector proteins have been identified and characterized which mediate downstream SA signaling. This includes SABP, a catalase, SABP2, a methyl salicylate esterase, SABP3, a carbonic anhydrase, NPR1 (non-expressor of pathogenesis-related 1), NPR3 (a NPR1 paralog), and NPR4 (another NPR1 paralog). NPR3 and NPR4 regulate the turnover of NPR1, a process which plays a key role in activating defense gene expression. The role of SA in abiotic stress signaling is gradually becoming clearer. Various components of SA signaling in biotic stress also appear to impact abiotic stress signaling.

**Keywords** Salicylic acid (SA) • Isochorismate synthase (ICS) • Phenylalanine ammonia lyase (PAL) • Non-expressor of pathogenesis-related 1 (NPR1) • NPR3 • NPR4 • Methyl salicylate (MeSA) • Systemic acquired resistance (SAR) • Salicylate

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hydroxylase (*NahG*) • Glucosylated SA (SAG) • SA carboxyl methyltransferase 1 (BSMT1) • Tobacco mosaic virus (TMV) • Abscisic acid (ABA) • Enhanced disease susceptibility 1 (EDS1) • Phytoalexin-deficient 4 (PAD4) • Senescence-associated gene 101 (SAG101) • Glutathione S-transferases (GST) • Thimet oligopeptidases (TOP) • SA-binding protein 2 (SABP2) • SA-binding proteins 3 (SABP3) • Carbonic anhydrase (CA) • Reactive oxygen species (ROS) • Histone deacetylases (HDAs) • Sirtuin-type deacetylase • SIR2 • Heat shock proteins (HSPs) • Jasmonic acid (JA) • Benzothiadiazole S-methyl ester (BTH) • Gibberellic acid (GA) • Cytokinins (CK)

## 5.1 Introduction

Salicylic acid (SA), a small phenolic compound, is known for its beneficial role in reducing pain and fever. The SA derivative aspirin, first chemically synthesized by the Bayer Pharmaceutical Company, relieves pain and is one of the most-used pharmaceutical drugs [1, 2]. In humans, SA and its derivatives are known to inhibit synthesis of prostaglandins and to affect immunity through modulation of NF- $\kappa$ B [3]. Recently, SA was shown to activate AMP kinase, a key regulator of cellular ATP levels [4, 5]. SA and its derivatives are also known to help prevent cancer, reduce the risk of heart attack, and improve diabetic conditions [6–8].

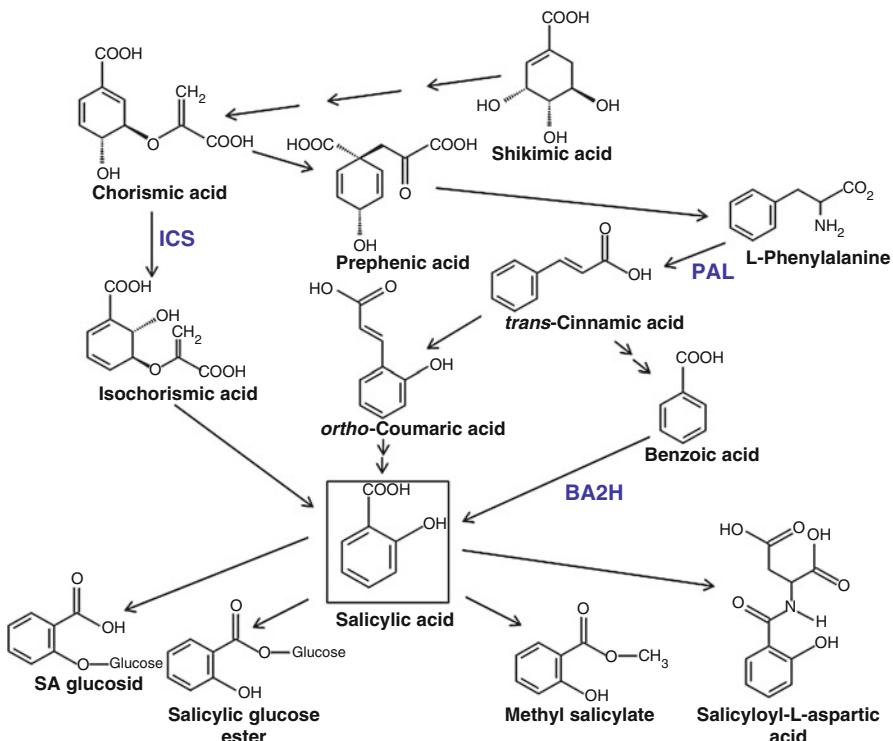
Although the beneficial role of SA and its derivatives in curing various ailments were known to humankind for a very long time, their roles in plants have come to attention only recently: tobacco plants treated with aspirin exhibited enhanced immunity to tobacco mosaic virus (TMV) [9], and plants exhibiting enhanced resistance to pathogen infection accumulated high levels of SA [10–13]. Conversely, transgenic tobacco plants failing to accumulate significant levels of SA upon expression of bacterial salicylate hydroxylase (*NahG*) were more susceptible to pathogens [14]. Finally, it was shown that SA is also required for inducing systemic acquired resistance (SAR), a heightened state of resistance in secondary tissues induced by pathogen infection of primary tissues [14, 15]. Based on these initial discoveries, tremendous progress has been made in our understanding of the role of SA signaling in plant immune responses [16, 17]. The best-studied role of SA is in mediating disease resistance signaling. Various synthetic analogs of SA have been developed and used for crop protection against microbial pathogens [18]. Currently SA-oriented thiourea derivatives are being developed as potential herbicidal as well as plant growth promoting compounds [19].

Besides immunity, SA also affects various other aspects in plants, including thermogenesis [20], stomatal response [21, 22], growth [23], leaf senescence [24], senescence [25, 26], cell growth [27], trichome growth [28], respiration [29], thermotolerance [30], seed germination [31], nodulation [32], seedling establishment [33, 34], photosynthesis [35]. This review surveys some of the new developments in our understanding of SA signaling in both biotic and abiotic stress response in plants, with special emphasis on SA biosynthesis (Sect. 5.2), its roles in biotic and abiotic stress responses (Sects. 5.3 and 5.4, respectively), and its interactions with other plant hormones (Sect. 5.5).

## 5.2 Biosynthesis of SA and Its Derivatives

SA is a small phenolic compound synthesized in plastids of plants along two parallel pathways. Both pathways use chorismate, the end product of the shikimate pathway, and they thus compete with the biosynthesis of other compounds including the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Fig. 5.1). The first pathway discovered to synthesize SA in plants involves phenylalanine ammonia lyase (PAL). Later it was shown that another pathway involving isochorismate synthase (ICS) is the major route for SA biosynthesis in *Arabidopsis* plants. Mutant plants lacking functional *ICSI* accumulate very low levels (~5 %) of SA, indicating the importance of this pathway in SA biosynthesis in plants [36]. The ICS-involving SA biosynthetic pathway is now known to occur in other plants including *Nicotiana benthamiana* and tomato [37, 38]. Homologs of *ICS* genes have been identified in many other plants including tobacco, tomato, pepper, soybean, rice, grapevine, and *Medicago truncatula* [37–43].

Synthesis of SA via the PAL-involving pathway is through the synthesis of *trans*-cinnamic acid, which also serves as a precursor for many important molecules includ-



**Fig. 5.1** The proposed pathway for SA biosynthesis. From chorismate, SA is synthesized through pathways involving ICS (isochorismate synthase) or PAL (phenylalanine ammonia lyase). (Adapted from Dempsey et al. 2011)

ing flavonoids, lignin, lignans, and volatile benzenoid esters [16, 44, 45]. From *trans*-cinnamic acid, SA is synthesized via two potential routes through *ortho-coumaric* acid or benzoic acid [46]. It was shown that radiolabeled SA accumulated via *ortho*-coumaric acid after feeding *Primula acaulis* and *Gaultheria procumbens* leaves with [<sup>14</sup>C]-Phe [47, 48]. In tomato plants infected with *Agrobacterium tumefaciens*, SA was synthesized through hydroxylation of cinnamic acid to *ortho*-coumaric acid, followed by  $\beta$ -oxidation [49]. In contrast, SA was synthesized primarily from cinnamic acid via the benzoic acid route in healthy tomato plants [49].

The other major pathway used by plants to synthesize SA is through isochorismate synthase 1 (ICS1) and ICS2. T-DNA insertion mutation in the *ICS1* gene (*sid2: SA induction deficient 2*) caused significant reduction of SA levels and compromised the induction of SAR [50, 51]. Interestingly, *ICS2* mutation did not result in a significant decrease of SA levels but had moderate impact on SA levels beyond what was observed in *sid2* mutants [52]. Analysis of SA in an *ics1/ics2* double mutant also confirmed the existence of another SA biosynthetic pathway in *Arabidopsis* [52]. ICS enzyme activity was characterized and found to be localized in plastids [53]. Overall, these studies suggested that multiple pathways for SA biosynthesis exist in plants.

The SA synthesized in plant cells is readily converted into various derivatives, likely to avoid the toxic effects of higher cellular SA levels. Some of these modifications lead to degradation, while other derivatives are stored in the vacuole and may serve as reserve in case of need by the cell. One of the most common SA derivatives is the glucosylated SA (SAG), known to accumulate, for example, in *Arabidopsis* and tobacco. TMV-infected tobacco plants accumulate high levels of the SAG (SA 2- $\beta$ -D-glucoside) near the site of hypersensitive response [54, 55], and a tobacco glucosyltransferase (UDP glucose: SA glucosyltransferase) was found to catalyze the formation of SAG [56]. It has been suggested that SAG could be converted back to SA by  $\beta$ -glucosidase [57–59].

Four other minor derivatives are known to accumulate in plants. First, the SA glucose ester (SGE) [60] has been detected, for example, in the leaves of tobacco and *Arabidopsis* [60, 61]. However, not much is known about the role of SGE either in biotic or in abiotic stress. Second, SA may also be methylated to generate methyl salicylate (MeSA), a volatile ester derivative [62]. Tobacco plants infected with TMV synthesize large amounts of MeSA, in contrast to healthy plants with almost undetectable levels [62, 63]. In *Arabidopsis*, a benzoic acid/SA carboxyl methyl-transferase (BSMT) 1 catalyzes the synthesis of MeSA from SA [64]. *BSMT1* expression is induced during both biotic and abiotic stress [64–67]. MeSA also serves as a phloem-mobile signal for the induction of SAR in tobacco plants [68]. Third, SA is known to occur in many plants, including grapes and French beans, as amino acid-conjugated derivatives such as salicyloyl aspartic acid [69, 70]. Salicyloyl aspartic acid accumulates at high levels in pathogen-infected *Arabidopsis* plants [71, 72]. Finally, sulfonation is another important modification affecting several plant hormones including SA [73]. Sulfotransferase (SOT) has been shown to sulfonate SA in vitro [73]. Accordingly, an *Arabidopsis* mutant defective in SOT12 exhibited hypersensitivity to SA, salt, and abscisic acid (ABA) treatments [73].

### 5.3 Salicylic Acid in Biotic Stress Responses

Tremendous progress has been made in our understanding of SA involvement in biotic stress signaling. The *Arabidopsis* genome sequence as well as the availability of T-DNA insertion mutants and their large-scale analysis has led to the discovery of several new SA-dependent stress response components.

#### 5.3.1 Upstream Components of the SA Response

Two upstream components of the SA response, Phytoalexin-deficient 4 (PAD4) and Enhanced Disease Susceptibility 1 (EDS1), were identified during screening of mutant *Arabidopsis* plants [74]. Close cooperation between PAD4 and EDS1 is required for the activation of SA-dependent plant response [75]. A positive feedback loop regulated by SA involves both EDS1 and PAD4 [76]. Accordingly, SA is known to activate defense responses in *Arabidopsis eds1* and *pad4* mutants.

EDS1 is a lipase-like protein localized in both cytoplasm and nucleus, and required for SA- but not the jasmonic acid (JA)-mediated response [77]. It plays important role in basal resistance by interacting with PAD4 and SAG101 (Senescence-Associated Gene 101). EDS1 is targeted by the *Pseudomonas syringae* effector protein, AvrRPS2 (Avirulent RPS2), to dampen basal resistance. Involvement of EDS1 in connecting RPS4 (Resistance to *Pseudomonas syringae* 4), a TIR-like receptor in *Arabidopsis*, to AvrRPS4 has recently been shown [78, 79]. Other TIR receptor proteins, RPS6 (Resistance to *Pseudomonas syringae* 6) and SNC1 (Suppressor of NPR1 Constitutive 1), are also known to interact with EDS1. These findings establish a clear role for EDS1 in SA-mediated responses activated by Toll-interleukin-1 receptor-nucleotide binding-leucine-rich repeats (TIR-NB-LRR) proteins.

#### 5.3.2 Salicylic Acid-Interacting Proteins

The exact role of SA in inducing resistance against pathogens has been unclear until recently. One hypothesis was that SA may interact with cellular protein/s to mediate downstream signaling and thus activate responses leading to resistance. Following this hypothesis, several candidate SA-interacting/-binding proteins have been identified, including a catalase, a carbonic anhydrase, an ascorbate peroxidase, a methyl salicylate esterase, NPR1, NPR3, and NPR4, the E2 subunit of  $\alpha$ -ketoglutarate dehydrogenase, several glutathione S-transferases (GSTF2, GSTF8, GSTF10, and GSTF11), and thimet oligopeptidases (TOP1 and TOP2) [17]. These thimet oligopeptidases are zincin-like metalloendopeptidases [80, 81].

In most cases, biochemical approaches using radiolabeled SA ( $^{14}\text{C}$ -SA or  $^3\text{H}$ -SA) were used to identify and characterize SA-binding proteins [82, 83]. The first SA-binding protein (SABP) to be identified using  $^{14}\text{C}$ -SA from tobacco leaves was

a catalase-like protein [82, 84]. Catalases help the cell to detoxify of H<sub>2</sub>O<sub>2</sub> (into water and oxygen) and reactive oxygen species (ROS). Binding of SA inhibited the catalytic activity of catalase, resulting in increased accumulation of H<sub>2</sub>O<sub>2</sub> [84]. On the other hand, increased levels of H<sub>2</sub>O<sub>2</sub> in the cell resulted in enhanced expression of defense-related genes and activation of SAR. It was therefore concluded that SA may exert its function by binding to SABP and inhibiting its catalase activity, resulting in accumulation of H<sub>2</sub>O<sub>2</sub> and hence activation of defense responses. This conclusion was supported by several studies reporting inhibition of catalase activity by SA [85]. However, the role of SA in binding and inhibiting catalases was subsequently questioned by several other results [86, 87], and further studies using <sup>3</sup>H-SA with higher specific activity led to discovery of new SA-binding proteins.

Using <sup>3</sup>H-SA, a ~29 kDa soluble protein, SABP2, was identified from tobacco leaves. It bound <sup>3</sup>H-SA in vitro with high affinity ( $K_D = 90 \text{ nM}$ ; [83]). SABP2 was purified from tobacco leaves and characterized to be a putative esterase/lipase-like protein belonging to the  $\alpha/\beta$ -hydrolase superfamily of proteins [88]. SABP2 has conserved active-site residues typical of  $\alpha/\beta$ -hydrolases and exhibited in vitro activities with commercially available short- and long-chain ester substrates [88]. Interestingly, substitutions of only two amino acids in SABP2 changed its enzymatic activity from an esterase to mandelonitrile lyase [89]. Transgenic tobacco plants with silenced SABP2 expression exhibited enhanced susceptibility to TMV and failed to mount SAR [88]. Crystal structure analysis showed that SA was bound in the active site of SABP2 [90]. SABP2 catalyzed the conversion of MeSA to SA with high affinity. High levels of SA (i.e., the product of the enzymatic reaction) inhibited the esterase activity of SABP2, providing a mechanism for fine tuning of SA levels inside the cell [90]. Further biochemical analysis, coupled with grafting experiments using transgenic SABP2-silenced tobacco plants, showed that MeSA is the phloem-mobile signal required for SAR in plants [68]. These studies also showed that active SABP2 is required to recognize and process the SAR signal (generated in primary infected tissues) in distal tissues [68, 91]. Subsequent studies revealed the presence of SABP2 homologs in other species including *Arabidopsis* [92], potato [93], tomato [94], and poplar [95].

Another SA-binding protein is SABP3, a chloroplastic carbonic anhydrase (CA) which also exhibits antioxidant activity [96, 97]. CA catalyzes the interconversion of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>. It binds SA with relatively moderate affinity ( $K_D = 3.7 \mu\text{M}$ ) and is localized in the stroma of tobacco chloroplasts. Virus-induced gene silencing of CA in tomato plants suppressed their hypersensitive response to *pto:avrpto*, implicating it in plant defense response [96]. *Nicotiana benthamiana* plants lacking CA showed enhanced susceptibility to *Phytophthora infestans* [98]. An *Arabidopsis* mutant lacking CA exhibited reduced levels of seedling establishment, likely due to poor photosynthetic performance [99].

Another group of plant proteins reported to have critical effects on SA-mediated signaling are NPR1 and its paralogs NPR3 and NPR4 [100, 101]. Plants lacking NPR1 are susceptible to both biotrophic and hemi-biotrophic pathogens [102, 103]. It was suggested that NPR1 functions as a transcription co-activator for SA-responsive genes, and thus activation of disease resistance signaling [104]. Interestingly, earlier studies had found no significant affinity of NPR1 for SA or its active analogs and derivatives [105]. However, in a new study using a highly sensitive SA-binding

**Table 5.1** SA-binding affinities of SA-interacting proteins

SA-interacting protein	$K_D$ ( $\mu\text{M}$ )	Source
SABP	14.0	Tobacco
SABP2	0.09	Tobacco
SABP3	3.70	Tobacco
NPR1	0.14	<i>Arabidopsis</i>
NPR3	0.98	<i>Arabidopsis</i>
NPR4	0.046	<i>Arabidopsis</i>

assay, it was shown that NPR1 only binds to SA ( $K_D=140 \text{ nM}$ ) and not to its inactive analogs [106]. NPR1 was localized primarily to the cytoplasm in a multimeric form which is destabilized upon changes in cytoplasmic redox potential, likely due to elevated levels of SA [107]. It was suggested that SA-binding may lead to conformational changes in NPR1 to release its C-terminal transactivation domain from the auto-inhibitory N-terminal domain. Upon release, the monomeric NPR1 travels to the nucleus to activate transcription of SA-responsive defense genes [104, 107, 108].

In another study, NPR3 and NPR4 were also shown to mediate SA-dependent signaling [105]. NPR3 and NPR4 acted as CUL3 adapters to bind NPR1, leading to its degradation via ubiquitination and the 26S proteosome. NPR3 and NPR4 have different binding affinities for NPR1, which are further affected by SA levels. Interestingly, both NPR3 ( $K_D=0.98 \mu\text{M}$ ) and NPR4 ( $K_D=0.046 \mu\text{M}$ ) bind SA with different affinities, suggesting differential roles for both proteins depending on the cellular levels of SA (Table 5.1).

Some of the other newly identified SA-binding proteins include an E2 subunit of  $\alpha$ -ketoglutarate dehydrogenase and glutathione S-transferases (GSTF2, GSTF8, GSTF10, and GSTF11) [109]. Further *Arabidopsis* SA-binding proteins are the thio-met oligopeptidases (TOP1 and TOP2) [81], identified using protein microarrays. SA inhibits the enzymatic activity of TOP1 and TOP2, and both are known to play a role in plant immunity against avirulent pathogens [81]. A recent X-ray diffraction analysis using *Arabidopsis* TOP2 did not support its binding to SA [110].

### 5.3.3 Epigenetic Effects of Salicylic Acid-Mediated Signaling

Various posttranslational chromatin modifications, e.g., methylation, acetylation, histone variants replacements, and ATP-dependent chromatin remodeling, have been shown to regulate plant defense mechanisms [111, 112]. Histone acetylation catalyzed by acetylases is associated with transcriptional up-regulation of gene expression, while the deacetylation mediated by histone deacetylases (HDAs) is linked with downregulation of transcription. HDA19, a histone deacetylase is involved in the SA-mediated plant defense response [113]. Loss of HDA19 resulted in increased accumulation of SA, leading to enhanced expression of SA-responsive genes including *PR* genes and increased resistance against *Pseudomonas syringae* [113]. Thus, HDA19 appears to be a negative regulator of SA-signaling involved in modifying chromatin to a repressive state to ensure lower basal expression of defense-related genes and to prevent unwanted expression of defense responses

[113]. HDA19 interacts with the transcriptional activators WRKY38 and WRKY62, known negative regulators of plant basal defenses [114]. SRT2, a sirtuin-type deacetylase, lowers the expression of both SA-biosynthetic (*SID2*) and SA-responsive (*PAD4*, *EDS5*) genes [115]. *Arabidopsis* plants infected with pathogens exhibit repression of *SRT2* expression, with a simultaneous increase in accumulation of SA and expression of SA-responsive genes [115].

Changes in chromatin modifications are involved in SAR and could have a long-lasting trans-generational effect [116]. A recent study suggested that *Arabidopsis* plants infected with *Pseudomonas syringae* DC3000 showed a priming trans-generational effect [117]. These primed plants did not show increased SA/SAG levels but exhibited enhanced resistance to *Pseudomonas syringae* DC3000 and other pathogens. Molecular analysis of these primed plants showed enhanced acetylation of histone proteins associated with the promoters of *PR-1*, *WRKY6*, and *WRKY 53*. In contrast, mutant *npr1* plants did not show enhanced trans-generational defense responses, underlining the important role of this gene in trans-generational SAR mediated through reduced DNA methylations at non-CpG sites [118]. DNA methylation at these sites is likely controlled by enzymes of RNA-directed DNA methylations. Accordingly, mutants defective in RNA-directed DNA methylations (e.g., *ago4-3*, *clys1-1*, *nrp2d2a-1*, *drd1-6*, and *dcl3-1*) failed to exhibit trans-generational SAR [118].

### 5.3.4 Stomata Opening and Closing

Many plant pathogens gain entry into plants through stomata, and some even force entry through these natural pores by regulating their opening/closing. SA-mediated regulation of stomatal aperture opening is important for entry of plant pathogens like *Pseudomonas syringae* DC3000 [119, 120]. Accumulation of SA results in closure of stomatal aperture mediated through reactive oxygen species (ROS) [121, 122]. Only the ROS generated via peroxidase-catalyzed reaction, and not by NADPH oxidase, is involved in stomatal closure [121]. Several *Arabidopsis* mutants, including *cpr5* and *acd6*, are known to accumulate increased levels of SA, exhibit smaller stomatal aperture and enhanced drought tolerance. Conversely, introduction of *NahG* reverses stomatal closure and drought tolerance [121]. These studies clearly show effect of SA accumulation on stomatal aperture. Finally, involvement of lipoxygenases (*LOX1*) in SA-dependent stomatal closure was also shown [123].

## 5.4 Salicylic Acid in Abiotic Stress Response

An increasing number of studies are suggesting an additional role for SA in mediating abiotic stresses:

1. Plants failing to accumulate SA (e.g., *NahG* transgenics) showed increased sensitivity to ozone exposure [124]. This effect of SA is likely mediated through the antioxidant defense mechanism in plants [125].

2. SA has protective effects against metal toxicity [126–128], and, for example, treatment with aluminum and cadmium increased endogenous SA levels [128, 129].
3. Heat tolerance was improved by treatments of plants with SA [130–133], again likely mediated by effects of SA on antioxidant system. Accordingly, levels of SA were shown to increase in heat-acclimatized mustard seedlings [133, 134], and *Arabidopsis npr1* mutant plants showed reduced tolerance to heat [30].
4. SA appears to help provide protection against chilling-induced damages (low temperatures above freezing; [135–137]). Enhanced SA-mediated chilling tolerance was observed in shoots and roots of cucumber, rice, and corn [135].
5. A role of SA in freezing (sub-zero) tolerance is observed in some plants (e.g., wheat), while not in others (e.g., winter rye; [135, 138–141]).
6. A large number of species are affected by salt stress and osmotic stress. SA application enhances ability of some plants to cope with salt stress. For example, tomato plants treated with SA through root-drench provided protection against 0.2 M NaCl [142]. Endogenous levels of SA are known to increase in plants treated with NaCl. Pretreatment of tomato plants with SA improved its acclimation to salt stress [143]. Interestingly, expression of a tomato homolog of *SABP2* was induced in a NaCl-treated tolerant tomato cultivar but not in a sensitive cultivar, indicating a *SABP2*-mediated role of SA in salt stress tolerance [94].
7. The effect of exogenous SA on drought tolerance of various species depends on the concentration of SA applied. High concentrations of SA (2–3 mM) suppressed drought tolerance in wheat seedlings [144], while treatment of muskmelon seedlings with low concentrations (0.1–1 mM) of acetyl SA enhanced drought tolerance [145]. Treatment of tomato and bean seeds with SA (0.1–0.5 mM) prior to sowing resulted in enhanced drought tolerance of these species [146, 147]. Treatment of 2-week-old corn plants with SA resulted in increased sensitivity to drought [148]. In contrast, treatment of barley plants with SA prior to drought stress exposure reduced its damaging action [149].

Thus, most abiotic stress signals led to increases in endogenous SA levels, while effects of exogenous SA on abiotic stress were dependent on various conditions, e.g., the mode of treatment, status of plant, or timing of treatment. One possible mechanism to explain the protective role of exogenous SA treatment is through its effect on oxidative damage. Transgenic *NahG* rice plants which fail to accumulate SA are increasingly sensitive to treatment with chemicals known to cause oxidative damage [150]. Treatments with higher concentration of SA likely increase levels of oxidative stress beyond control of the SA-induced antioxidant system. Another mode of action of SA in abiotic stress may be through activation of protein kinases [151]. Accordingly, calcium-dependent protein kinases involved in mediating abiotic stress signals are known to be modulated by SA [152, 153]. Besides the above-mentioned mechanisms, SA also likely works through modulating expression of various other genes/proteins, e.g., topoisomerase [154], heat shock proteins, dehydrins [155, 156], or osmotins [30, 157]. Various Heat Shock Proteins (HSPs), e.g., Hsp70, Hsp17.6, and HSP101, are known to be modulated by SA and likely play a role in basal and acquired thermotolerance [30]. More targeted studies are needed to decipher role of SA in abiotic stress.

## 5.5 Interaction of SA with Other Plant Hormones

While the role of SA in inducing resistance against biotrophic pathogens is well known, the other phytohormones, e.g., auxin, ethylene, and ABA, have been shown to promote virulence in plants [158, 159]. Jasmonic acid (JA) is well known for its role in wound responses induced by herbivory and in resistance against necrotrophic pathogens. JA is also known to have an antagonistic effect to SA signaling, but the existence of synergistic effects is also well documented [160–163]. NPR1 is known to mediate interaction between the SA and JA responses. Interestingly, the nuclear localization of NPR1, which is required for SA-mediated signaling, is not required for JA-mediated signaling [164]. The interaction between both signaling pathways therefore likely occurs in the cytoplasm rather than the nucleus [164, 165].

Ethylene, a plant hormone best studied for its role in fruit ripening, is also known to play an important role in plant immunity [159, 166, 167]. It can either positively or negatively regulate plant immunity, and exhibits extensive cross talk with SA and JA in modulating plant immunity [168, 169]. Ethylene plays an important role in determining the role of NPR1 in SA-mediated suppression of the JA response [170]. Full activation of the ethylene and JA response prior to activation of the SA response shields the JA response from SA-mediated repression. This effect is likely due to ethylene, and the presence of ethylene thus gives the plant flexibility to induce the JA response over the SA response.

ABA is another plant hormone well known for its role in abiotic stresses including cold stress, salinity, and water stress. Increasing body of evidence suggest its role in biotic stress especially during pathogen infection [166]. Levels of ABA change during pathogen infections [171–173]. Treatments with exogenous ABA prior to or during infections increase plant susceptibility to pathogens [174]. Enhanced susceptibility is likely mediated via ABA-mediated suppression of the SA response [171, 175]. This cross talk between ABA and SA occurs at multiple steps starting with biosynthesis [176]. ABA treatment inhibited induction of SAR and conversely SAR activation suppressed/inhibited expression of ABA biosynthetic and ABA-responsive genes [176].

Auxins, i.e., indole-3-acetic acid (IAA) derivatives, are widely studied for their diverse role in various aspects of plant growth from early development to senescence [177, 178]. Additionally, recent studies have suggested their involvement in plant–microbe interactions. For example, *Puccinia graminis* ssp. *tritici*, a wheat stem rust fungus, induces its host to accumulate auxin [179, 180]. This modulation of host auxin levels is likely required for full pathogenicity of the pathogen [179, 180]. Various studies have shown that many plant pathogens either manipulate the host auxin biosynthetic pathway to accumulate auxin or that they produce auxin themselves [181–184]. Many of the mutant plants accumulating high levels of SA show phenotypes similar to auxin-deficient mutants [185]. To study the underlying mechanisms, Wong and colleagues analyzed the global gene expression in *Arabidopsis* plants treated with benzothiadiazole S-methyl ester (BTH), a synthetic SA analog [181]. Auxin-related genes were in general repressed, indicating involve-

ment of SA in modulating auxin levels in host-pathogen interactions [181]. Treatment of plants with bacterial flagellin, an inducer of host immunity, triggers downregulation of auxin signaling, supporting the hypothesis that auxin plays an important role in plant immunity [158]. Accumulation of a conjugated form of auxin (IAA-Asp) in *Arabidopsis* plants infected with *Botrytis cinerea* and *Pseudomonas syringae* DC3000 was the leading cause of increased disease development [186]. However, a recent study suggested that in auxin-overproducing *Arabidopsis* plants increased pathogen growth was independent of SA-mediated defenses [187].

Gibberellic acid (GA)-mediated degradation of DELLA proteins promotes resistance via modulation of JA-SA signaling [188]. This is achieved by suppressing JA-responsive genes and thereby allowing full activation of SA signaling. *Arabidopsis della* mutant plants show enhanced and earlier expression of SA marker genes, while the expression of JA/ethylene marker genes was significantly delayed [188]. DELLA proteins are known to regulate ROS levels, which are thought to effect cellular SA levels. This shows a likely interaction between SA and GA in plant defense signaling. GA is also known to modulate SA-mediated plant responses to abiotic stress [34].

Cytokinins (CKs) are well known and widely studied for their role in growth and development of plants. However, recently they have also been implicated in modulating plant defense signaling [189–192]. CKs directly modulate SA signaling by interaction between ARR2 (a CK-activated transcription factor) and TGA3, leading to enhanced immunity to *Pseudomonas syringae* and expression of the *PR-1* gene [193]. High levels of CKs lead to enhanced SA-mediated resistance, while lower levels result in increased susceptibility [190]. SA is also known to feedback-inhibit CK signaling [190].

Most investigations into the effects of SA involved adult plants; however, some studies also focused on its role in seed germinations. For example, SA showed an inhibitory effect on corn seed germination, while wheat and pea seed germination exhibited enhanced seed vigor [194–197]. More detailed studies using *Arabidopsis* showed positive effects of SA treatments on seed germination, including re-induction of late maturation program allowing germinating seedlings to better deal with water stress [31]. SA treatment also affected protein translation, priming of seed metabolism, synthesis of antioxidant enzymes, and mobilization of seed storage proteins leading to increased seed vigor [31]. Similar positive effects of SA treatment on seedling growth were also reported in bean [198]. SA is now implicated in promoting *Arabidopsis* seedling germination by modulating antioxidant activity [199]. *Arabidopsis sid2* mutant seeds (defective in SA biosynthesis) showed decreased tolerance to high salinity. Under high salinity, endogenous H<sub>2</sub>O<sub>2</sub> levels were elevated [199], whereas treatment with low concentrations of SA reduced the H<sub>2</sub>O<sub>2</sub> levels under high salinity [199]. Interestingly, germinating *NahG* transgenic seeds exhibited less severe effects of salinity due to accumulation of catechol (converted from SA by NahG enzyme; [199, 200]). Catechol, due to its antioxidant activity, is likely responsible for protection against high salinity in *NahG* transgenic plants [201, 202].

## 5.6 Conclusions

Clearly, SA is a versatile hormone with important roles in various physiological processes in plants. Its role in plant immunity is particularly important, and has received global attention from academic to industrial research. SA concentrations are under tight cellular control. The discovery of SABP2, NPR1, NPR3, and NPR4 as key players in mediating SA signaling has enhanced our understanding. It is important to note that SA-binding affinities for these important players are in a relatively narrow range from 0.046 μM (NPR4) and 0.09 μM (SABP2) to 0.14 μM (NPR1) (Table 5.1).

The primary site of SA synthesis in plastids raises the question how it is delivered to specific downstream effector molecules and cell compartments. Accumulation of SA to high levels in any one location within the cell is lethal and therefore carefully avoided by plants. Studies localizing SA inside cell needs more attention. A recent study using transgenic plants over expressing *NahG* gene in chloroplasts showed that these plants failed to accumulate SA in response to biotic or abiotic factors [203]. This suggests that SA is primarily synthesized inside the chloroplast and is transported out of chloroplast for signaling in biotic and abiotic stresses. How is SA transported out of chloroplast? Is it through EDS5, or is it transported in an inactive form which is converted to active form at its destination? A recently published report suggested that SA is transported out of chloroplast through EDS5, a multidrug and toxin extrusion transporter [204]. There is increasing evidence to support the role of SA in various abiotic stress signaling processes, which raises the possibility of its direct use in mitigating these stresses. More detailed biochemical and genetic studies in this important area will likely answer some of these questions in future.

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## References

1. Weissmann G (1991) The actions of NSAIDs. Hosp Pract 26(8):60–76
2. Weissmann G (1991) Aspirin. Sci Am 264(1):84–90
3. Kopp E, Ghosh S (1994) Inhibition of NF-kappa B by sodium salicylate and aspirin. Science 265(5174):956–959
4. Hardie DG, Ross FA, Hawley SA (2012) AMP-activated protein kinase: a target for drugs both ancient and modern. Chem Biol 19(10):1222–1236
5. Hawley SA, Fullerton MD, Ross FA, Schertzer JD, Chevtzoff C, Walker KJ et al (2012) The ancient drug salicylate directly activates AMP-activated protein kinase. Science 336(6083):918–922

6. Giannini EG, Kane SV, Testa R, Savarino V (2005) 5-ASA and colorectal cancer chemoprevention in inflammatory bowel disease: can we afford to wait for ‘best evidence’? *Dig Liver Dis* 37(10):723–731
7. Wu R, Laplante MA, de Champlain J (2005) Cyclooxygenase-2 inhibitors attenuate angiotensin II-induced oxidative stress, hypertension, and cardiac hypertrophy in rats. *Hypertension* 45(6):1139–1144
8. Nilsson S (1959) Treatment of diabetes mellitus with a preparation containing salicylic acid, para-amino benzoic acid, and ascorbic acid (PASCON): a therapeutic experiment. *Acta Med Scand* 165:273–278
9. White RF (1979) Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology* 99(2):410–412
10. Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250(4983):1002–1004
11. Metraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M, Gaudin J et al (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250(4983):1004–1006
12. Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S et al (1992) Acquired resistance in *Arabidopsis*. *Plant Cell* 4(6):645–656
13. Vernooij B, Uknes S, Ward E, Ryals J (1994) Salicylic acid as a signal molecule in plant-pathogen interactions. *Curr Opin Cell Biol* 6(2):275–279
14. Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S et al (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261(5122):754–756
15. Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic acid in plant disease resistance. *Science* 266(5188):1247–1250
16. Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic acid, a multifaceted hormone to combat disease. *Annu Rev Phytopathol* 47:177–206
17. Yan S, Dong X (2014) Perception of the plant immune signal salicylic acid. *Curr Opin Plant Biol* 20C:64–68
18. Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H et al (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J* 10(1):71–82
19. Li S, Li H, Cao X, Chen C (2014) Synthesis and bio-evaluation of novel salicylic acid-oriented thiourea derivatives with potential application in agriculture. *Lett Drug Des Discov* 11:98–103
20. Raskin I, Ehmann A, Melander WR, Meeuse BJ (1987) Salicylic acid: a natural inducer of heat production in arum lilies. *Science* 237(4822):1601–1602
21. Manthe B, Schulz M, Schnable H (1992) Effect of salicylic acid on growth and stomatal movements of *Vicia faba*: evidence for salicylic acid metabolism. *J Chem Ecol* 18:1525–1539
22. Lee JS (1998) The mechanism of stomatal closing by salicylic acid in *Commelina communis* L. *J Plant Biol* 41:97–102
23. Rate DN, Cuenca JV, Bowman GR, Guttmann DS, Greenberg JT (1999) The gain-of-function *Arabidopsis* acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. *Plant Cell* 11(9):1695–1708
24. Morris K, MacKerness SA, Page T, John CF, Murphy AM, Carr JP et al (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant J* 23(5):677–685
25. Rao MV, Lee HI, Davis KR (2002) Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. *Plant J* 32(4):447–456
26. Rao MV, Davis KR (2001) The physiology of ozone induced cell death. *Planta* 213(5):682–690
27. Vanacker H, Lu H, Rate DN, Greenberg JT (2001) A role for salicylic acid and NPR1 in regulating cell growth in *Arabidopsis*. *Plant J* 28(2):209–216

28. Traw MB, Bergelson J (2003) Interactive effects of jasmonic acid, salicylic acid, and gibberellin on induction of trichomes in *Arabidopsis*. *Plant Physiol* 133(3):1367–1375
29. Norman C, Howell KA, Millar AH, Whelan JM, Day DA (2004) Salicylic acid is an uncoupler and inhibitor of mitochondrial electron transport. *Plant Physiol* 134(1):492–501
30. Clarke SM, Mur LA, Wood JE, Scott IM (2004) Salicylic acid dependent signaling promotes basal thermotolerance but is not essential for acquired thermotolerance in *Arabidopsis thaliana*. *Plant J* 38(3):432–447
31. Rajjou L, Belghazi M, Huguet R, Robin C, Moreau A, Job C et al (2006) Proteomic investigation of the effect of salicylic acid on *Arabidopsis* seed germination and establishment of early defense mechanisms. *Plant Physiol* 141(3):910–923
32. Stacey G, McAlvin CB, Kim SY, Olivares J, Soto MJ (2006) Effects of endogenous salicylic acid on nodulation in the model legumes *Lotus japonicus* and *Medicago truncatula*. *Plant Physiol* 141(4):1473–1481
33. Alonso-Ramirez A, Rodriguez D, Reyes D, Jimenez JA, Nicolas G, Lopez-Climent M et al (2009) Cross-talk between gibberellins and salicylic acid in early stress responses in *Arabidopsis thaliana* seeds. *Plant Signal Behav* 4(8):750–751
34. Alonso-Ramirez A, Rodriguez D, Reyes D, Jimenez JA, Nicolas G, Lopez-Climent M et al (2009) Evidence for a role of gibberellins in salicylic acid-modulated early plant responses to abiotic stress in *Arabidopsis* seeds. *Plant Physiol* 150(3):1335–1344
35. Mateo A, Funk D, Muhlenbock P, Kular B, Mullineaux PM, Karpinski S (2006) Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. *J Exp Bot* 57(8):1795–1807
36. Nawrath C, Metraux JP (1999) Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* 11(8):1393–1404
37. Uppalapati SR, Ishiga Y, Wangdi T, Kunkel BN, Anand A, Mysore KS et al (2007) The phytoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with *Pseudomonas syringae* pv. *tomato* DC3000. *Mol Plant Microbe Interact* 20(8):955–965
38. Catinot J, Buchala A, Abou-Mansour E, Metraux JP (2008) Salicylic acid production in response to biotic and abiotic stress depends on isochorismate in *Nicotiana benthamiana*. *FEBS Lett* 582(4):473–478
39. Ogawa D, Nakajima N, Sano T, Tamaoki M, Aono M, Kubo A et al (2005) Salicylic acid accumulation under O<sub>3</sub> exposure is regulated by ethylene in tobacco plants. *Plant Cell Physiol* 46(7):1062–1072
40. Sawada H, Shim IS, Usui K (2006) Induction of benzoic acid 2-hydroxylase and salicylic acid biosynthesis-modulation by salt stress in rice seedlings. *Plant Sci* 171:263–270
41. Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A et al (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature* 449(7161):463–467
42. Tsai CJ, Harding SA, Tschaplinski TJ, Lindroth RL, Yuan Y (2006) Genome-wide analysis of the structural genes regulating defense phenylpropanoid metabolism in *Populus*. *New Phytol* 172(1):47–62
43. Zhang H, Franken P (2014) Comparison of systemic and local interactions between the arbuscular mycorrhizal fungus *Funneliformis mosseae* and the root pathogen *Aphanomyces euteiches* in *Medicago truncatula*. *Mycorrhiza* 24(6):419–430
44. Dempsey DA, Vlot AC, Wildermuth MC, Klessig DF (2011) Salicylic acid biosynthesis and metabolism. *Arabidopsis Book* 9, e0156
45. Dempsey DA, Klessig DF (1994) Salicylic acid, active oxygen species and systemic acquired resistance in plants. *Trends Cell Biol* 4(9):334–338
46. Yalpani N, Leon J, Lawton MA, Raskin I (1993) Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. *Plant Physiol* 103(2):315–321
47. Ellis BE, Amrhein N (1971) The “NIH shift” during aromatic hydroxylation in higher plants. *Phytochemistry* 10:3069–3072

48. El-Basyouni SZ, Chen D, Ibrahim RK, Neish AC, Towers GH (1964) The biosynthesis of hydroxybenzoic acids in higher plants. *Phytochemistry* 3:485–492
49. Chadha KC, Brown SA (1974) Biosynthesis of phenolic acids in tomato plants infected with *Agrobacterium tumefaciens*. *Can J Bot* 52:2041–2047
50. Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414(6863):562–565
51. Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. *Plant Cell* 8(10):1809–1819
52. Garcion C, Lohmann A, Lamodiere E, Catinot J, Buchala A, Doermann P et al (2008) Characterization and biological function of the ISOCHORISMATE SYNTHASE2 gene of *Arabidopsis*. *Plant Physiol* 147(3):1279–1287
53. Strawn MA, Marr SK, Inoue K, Inada N, Zubieta C, Wildermuth MC (2007) *Arabidopsis* isochorismate synthase functional in pathogen-induced salicylate biosynthesis exhibits properties consistent with a role in diverse stress responses. *J Biol Chem* 282(8):5919–5933
54. Enyedi AJ, Yalpani N, Silverman P, Raskin I (1992) Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc Natl Acad Sci U S A* 89(6):2480–2484
55. Malamy J, Hennig J, Klessig DF (1992) Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. *Plant Cell* 4(3):359–366
56. Enyedi AJ, Raskin I (1993) Induction of UDP-glucose:salicylic acid glucosyltransferase activity in tobacco mosaic virus-inoculated tobacco (*Nicotiana tabacum*) leaves. *Plant Physiol* 101(4):1375–1380
57. Chen Z, Malamy J, Henning J, Conrath U, Sanchez-Casas P, Silva H et al (1995) Induction, modification, and transduction of the salicylic acid signal in plant defense responses. *Proc Natl Acad Sci U S A* 92(10):4134–4137
58. Kawano T, Tanaka S, Kadono T, Muto S (2004) Salicylic acid glucoside acts as a slow inducer of oxidative burst in tobacco suspension culture. *Zeitschrift fur Naturforschung* 59(9-10):684–692
59. Kawano T, Furuchi T, Muto S (2004) Controlled salicylic acid levels and corresponding signaling mechanisms in plants. *Plant Biotechnol* 21(5):319–335
60. Lee HI, Raskin I (1998) Glucosylation of salicylic acid in *Nicotiana tabacum* Cv. Xanthi-nc. *Phytopathology* 88(7):692–697
61. Dean JV, Delaney SP (2008) Metabolism of salicylic acid in wild-type, ugt74f1 and ugt74f2 glucosyltransferase mutants of *Arabidopsis thaliana*. *Physiol Plant* 132(4):417–425
62. Shulaev V, Silverman P, Raskin I (1997) Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature* 385(6618):718–721
63. Seskar M, Shulaev V, Raskin I (1998) Endogenous methyl salicylate in pathogen-inoculated tobacco plants. *Plant Physiol* 116:387–392
64. Chen F, D'Auria JC, Tholl D, Ross JR, Gershenson J, Noel JP et al (2003) An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J* 36(5):577–588
65. Song JT, Koo YJ, Park JB, Seo YJ, Cho YJ, Seo HS et al (2009) The expression patterns of AtBSMT1 and AtSAGT1 encoding a salicylic acid (SA) methyltransferase and a SA glucosyltransferase, respectively, in *Arabidopsis* plants with altered defense responses. *Mol Cells* 28(2):105–109
66. Liu PP, von Dahl CC, Park SW, Klessig DF (2011) Interconnection between methyl salicylate and lipid-based long-distance signaling during the development of systemic acquired resistance in *Arabidopsis* and tobacco. *Plant Physiol* 155(4):1762–1768
67. Attaran E, Zeier TE, Griebel T, Zeier J (2009) Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in *Arabidopsis*. *Plant Cell* 21(3):954–971
68. Park SW, Kaimoyo E, Kumar D, Mosher S, Klessig DF (2007) Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318(5847):113–116

69. Steffan H, Ziegler A, Rapp A (1988) N-Salicyloyl-aspartic acid: a new phenolic compound in grapevines. *Vitis* 27:79–86
70. Bourne DJ, Barrow KD, Milborrow BV (1991) Salicyloylaspartate as an endogenous component in the leaves of *Phaseolus vulgaris*. *Phytochemistry* 30:4041–4044
71. Chen Y, Shen H, Wang M, Li Q, He Z (2013) Salicyloyl-aspartate synthesized by the acetyl-amido synthetase GH3.5 is a potential activator of plant immunity in *Arabidopsis*. *Acta Biochim Biophys Sin* 45(10):827–836
72. Zhang Z, Li Q, Li Z, Staswick PE, Wang M, Zhu Y et al (2007) Dual regulation role of GH3.5 in salicylic acid and auxin signaling during *Arabidopsis*-*Pseudomonas syringae* interaction. *Plant Physiol* 145(2):450–464
73. Baek D, Pathange P, Chung JS, Jiang J, Gao L, Oikawa A et al (2010) A stress-inducible sulphotransferase sulphonates salicylic acid and confers pathogen resistance in *Arabidopsis*. *Plant Cell Environ* 33(8):1383–1392
74. Parker JE, Holub EB, Frost LN, Falk A, Gunn ND, Daniels MJ (1996) Characterization of eds1, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different RPP genes. *Plant Cell* 8(11):2033–2046
75. Rietz S, Stamm A, Malonek S, Wagner S, Becker D, Medina-Escobar N et al (2011) Different roles of enhanced disease Susceptibility1 (EDS1) bound to and dissociated from Phytoalexin Deficient4 (PAD4) in *Arabidopsis* immunity. *New Phytol* 191(1):107–119
76. Shah J (2003) The salicylic acid loop in plant defense. *Curr Opin Plant Biol* 6(4):365–371
77. Falk A, Feys BJ, Frost LN, Jones JD, Daniels MJ, Parker JE (1999) EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc Natl Acad Sci U S A* 96(6):3292–3297
78. Bhattacharjee S, Halane MK, Kim SH, Gassmann W (2011) Pathogen effectors target *Arabidopsis* EDS1 and alter its interactions with immune regulators. *Science* 334(6061):1405–1408
79. Heidrich K, Wirthmueller L, Tasset C, Pouzet C, Deslandes L, Parker JE (2011) *Arabidopsis* EDS1 connects pathogen effector recognition to cell compartment-specific immune responses. *Science* 334(6061):1401–1404
80. Barrett AJ, Brown MA, Dando PM, Knight CG, McKie N, Rawlings ND et al (1995) Thimet oligopeptidase and oligopeptidase M or neurolysin. *Methods Enzymol* 248:529–556
81. Moreau M, Westlake T, Zampogna G, Popescu G, Tian M, Noutsos C et al (2013) The *Arabidopsis* oligopeptidases TOP1 and TOP2 are salicylic acid targets that modulate SA-mediated signaling and the immune response. *Plant J* 76(4):603–614
82. Chen Z, Ricigliano JW, Klessig DF (1993) Purification and characterization of a soluble salicylic acid-binding protein from tobacco. *Proc Natl Acad Sci U S A* 90(20):9533–9537
83. Du H, Klessig DF (1997) Identification of a soluble, high-affinity salicylic acid-binding protein in tobacco. *Plant Physiol* 113(4):1319–1327
84. Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* 262(5141):1883–1886
85. Kawano T, Sahashi N, Takahashi K, Uozumi N, Muto S (1998) Salicylic acid induces extracellular generation of Superoxide followed by an increase in cytosolic calcium ion in tobacco suspension culture: the earliest events in salicylic acid signal transduction. *Plant Cell Physiol* 39:721–730
86. Bi YM, Kenton P, Mur L, Darby R, Draper J (1995) Hydrogen peroxide does not function downstream of salicylic acid in the induction of PR protein expression. *Plant J* 8(2):235–245
87. Ruffer M, Steipe B, Zenk MH (1995) Evidence against specific binding of salicylic acid to plant catalase. *FEBS Lett* 377(2):175–180
88. Kumar D, Klessig DF (2003) High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. *Proc Natl Acad Sci U S A* 100(26):16101–16106
89. Padhi SK, Fujii R, Legatt GA, Fossum SL, Berchtold R, Kazlauskas RJ (2010) Switching from an esterase to a hydroxynitrile lyase mechanism requires only two amino acid substitutions. *Chem Biol* 17(8):863–871

90. Forouhar F, Yang Y, Kumar D, Chen Y, Fridman E, Park SW et al (2005) Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. *Proc Natl Acad Sci U S A* 102(5):1773–1778
91. Kumar D, Klessig DF (2008) The search for the salicylic acid receptor led to discovery of the SAR signal receptor. *Plant Signal Behav* 3(9):691–692
92. Vlot AC, Liu PP, Cameron RK, Park SW, Yang Y, Kumar D et al (2008) Identification of likely orthologs of tobacco salicylic acid-binding protein 2 and their role in systemic acquired resistance in *Arabidopsis thaliana*. *Plant J* 56(3):445–456
93. Manosalva PM, Park SW, Forouhar F, Tong L, Fry WE, Klessig DF (2010) Methyl esterase 1 (StMES1) is required for systemic acquired resistance in potato. *Mol Plant Microbe Interact* 23(9):1151–1163
94. Sun W, Xu X, Zhu H, Liu A, Liu L, Li J et al (2010) Comparative transcriptomic profiling of a salt-tolerant wild tomato species and a salt-sensitive tomato cultivar. *Plant Cell Physiol* 51(6):997–1006
95. Zhao N, Guan J, Forouhar F, Tschaplinski TJ, Cheng ZM, Tong L et al (2009) Two poplar methyl salicylate esterases display comparable biochemical properties but divergent expression patterns. *Phytochemistry* 70(1):32–39
96. Slaymaker DH, Navarre DA, Clark D, del Pozo O, Martin GB, Klessig DF (2002) The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response. *Proc Natl Acad Sci U S A* 99(18):11640–11645
97. Clark D, Rowlett RS, Coleman JR, Klessig DF (2004) Complementation of the yeast deletion mutant Delta NCE103 by members of the beta class of carbonic anhydrases is dependent on carbonic anhydrase activity rather than on antioxidant activity. *Biochem J* 379(Pt 3):609–615
98. Restrepo S, Myers KL, del Pozo O, Martin GB, Hart AL, Buell CR et al (2005) Gene profiling of a compatible interaction between Phytophthora infestans and *Solanum tuberosum* suggests a role for carbonic anhydrase. *Mol Plant Microbe Interact* 18(9):913–922
99. Ferreira FJ, Guo C, Coleman JR (2008) Reduction of plastid-localized carbonic anhydrase activity results in reduced *Arabidopsis* seedling survivorship. *Plant Physiol* 147(2):585–594
100. Fu ZQ, Dong X (2013) Systemic acquired resistance: turning local infection into global defense. *Annu Rev Plant Biol* 64:839–863
101. Cao H, Li X, Dong X (1998) Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. *Proc Natl Acad Sci U S A* 95(11):6531–6536
102. Delaney TP, Friedrich L, Ryals JA (1995) *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc Natl Acad Sci U S A* 92(14):6602–6606
103. Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88(1):57–63
104. Spoel SH, Mou Z, Tada Y, Spivey NW, Genschik P, Dong X (2009) Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* 137(5):860–872
105. Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N et al (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486(7402):228–232
106. Wu Y, Zhang D, Chu JY, Boyle P, Wang Y, Brindle ID et al (2012) The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep* 1(6):639–647
107. Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113(7):935–944
108. Zhang Y, Fan W, Kinkema M, Li X, Dong X (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc Natl Acad Sci U S A* 96(11):6523–6528
109. Tian M, von Dahl CC, Liu PP, Friso G, van Wijk KJ, Klessig DF (2012) The combined use of photoaffinity labeling and surface plasmon resonance-based technology identifies multiple salicylic acid-binding proteins. *Plant J* 72(6):1027–1038

110. Wang R, Rajagopalan K, Sadre-Bazzaz K, Moreau M, Klessig DF, Tong L (2014) Structure of the *Arabidopsis thaliana* TOP2 oligopeptidase. *Acta Cryst* 70(Pt 5):555–559
111. Walley JW, Rowe HC, Xiao Y, Chehab EW, Kliebenstein DJ, Wagner D et al (2008) The chromatin remodeler SPLAYED regulates specific stress signaling pathways. *PLoS Pathog* 4(12), e1000237
112. Berr A, Menard R, Heitz T, Shen WH (2012) Chromatin modification and remodelling: a regulatory landscape for the control of *Arabidopsis* defence responses upon pathogen attack. *Cell Microbiol* 14(6):829–839
113. Choi SM, Song HR, Han SK, Han M, Kim CY, Park J et al (2012) HDA19 is required for the repression of salicylic acid biosynthesis and salicylic acid-mediated defense responses in *Arabidopsis*. *Plant J* 71(1):135–146
114. Kim KC, Lai Z, Fan B, Chen Z (2008) *Arabidopsis* WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. *Plant Cell* 20(9):2357–2371
115. Wang C, Gao F, Wu J, Dai J, Wei C, Li Y (2010) *Arabidopsis* putative deacetylase AtSRT2 regulates basal defense by suppressing PAD4, EDS5 and SID2 expression. *Plant Cell Physiol* 51(8):1291–1299
116. van den Burg HA, Takken FL (2009) Does chromatin remodeling mark systemic acquired resistance? *Trends Plant Sci* 14(5):286–294
117. Luna E, Bruce TJ, Roberts MR, Flors V, Ton J (2012) Next-generation systemic acquired resistance. *Plant Physiol* 158(2):844–853
118. Luna E, Ton J (2012) The epigenetic machinery controlling transgenerational systemic acquired resistance. *Plant Signal Behav* 7(6):615–618
119. Melotto M, Underwood W, He SY (2008) Role of stomata in plant innate immunity and foliar bacterial diseases. *Annu Rev Phytopathol* 46:101–122
120. Underwood W, Melotto M, He SY (2007) Role of plant stomata in bacterial invasion. *Cell Microbiol* 9(7):1621–1629
121. Miura K, Okamoto H, Okuma E, Shiba H, Kamada H, Hasegawa PM et al (2013) SIZ1 deficiency causes reduced stomatal aperture and enhanced drought tolerance via controlling salicylic acid-induced accumulation of reactive oxygen species in *Arabidopsis*. *Plant J* 73(1):91–104
122. Mori IC, Pinontoan R, Kawano T, Muto S (2001) Involvement of superoxide generation in salicylic acid-induced stomatal closure in *Vicia faba*. *Plant Cell Physiol* 42(12):1383–1388
123. Montillet JL, Leonhardt N, Mondy S, Tranchimand S, Rumeau D, Boudsocq M et al (2013) An abscisic acid-independent oxylipin pathway controls stomatal closure and immune defense in *Arabidopsis*. *PLoS Biol* 11(3), e1001513
124. Sharma YK, Leon J, Raskin I, Davis KR (1996) Ozone-induced responses in *Arabidopsis thaliana*: the role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. *Proc Natl Acad Sci U S A* 93(10):5099–5104
125. Rao MV, Davis KR (1999) Ozone-induced cell death occurs via two distinct mechanisms in *Arabidopsis*: the role of salicylic acid. *Plant J* 17(6):603–614
126. Krantev A, Yordanova R, Janda T, Szalai G, Popova L (2008) Treatment with salicylic acid decreases the effect of cadmium on photosynthesis in maize plants. *J Plant Physiol* 165(9):920–931
127. Popova LP, Maslenkova LT, Yordanova RY, Ivanova AP, Krantev AP, Szalai G et al (2009) Exogenous treatment with salicylic acid attenuates cadmium toxicity in pea seedlings. *Plant Physiol Biochem* 47(3):224–231
128. Yang ZM, Wang J, Wang SH, Xu LL (2003) Salicylic acid-induced aluminum tolerance by modulation of citrate efflux from roots of *Cassia tora* L. *Planta* 217(1):168–174
129. Metwally A, Finkemeier I, Georgi M, Dietz KJ (2003) Salicylic acid alleviates the cadmium toxicity in barley seedlings. *Plant Physiol* 132(1):272–281
130. Larkindale J, Knight MR (2002) Protection against heat stress-induced oxidative damage in *Arabidopsis* involves calcium, abscisic acid, ethylene, and salicylic acid. *Plant Physiol* 128(2):682–695

131. Larkindale J, Hall JD, Knight MR, Vierling E (2005) Heat stress phenotypes of *Arabidopsis* mutants implicate multiple signaling pathways in the acquisition of thermotolerance. *Plant Physiol* 138(2):882–897
132. Dat JF, Lopez-Delgado H, Foyer CH, Scott IM (1998) Parallel changes in H<sub>2</sub>O<sub>2</sub> and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. *Plant Physiol* 116(4):1351–1357
133. Dat JF, Foyer CH, Scott IM (1998) Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. *Plant Physiol* 118(4):1455–1461
134. Larkindale J, Huang B (2004) Thermotolerance and antioxidant systems in *Agrostis stolonifera*: involvement of salicylic acid, abscisic acid, calcium, hydrogen peroxide, and ethylene. *J Plant Physiol* 161(4):405–413
135. Kang HM, Saltveit ME (2002) Chilling tolerance of maize, cucumber and rice seedling leaves and roots are differentially affected by salicylic acid. *Physiol Plant* 115(4):571–576
136. Kang GZ, Wang ZX, Xia KF, Sun GC (2007) Protection of ultrastructure in chilling-stressed banana leaves by salicylic acid. *J Zhejiang Univ Sci* 8(4):277–282
137. Cai G, Wang G, Wang L, Pan J, Liu Y, Li D, ZmMKK1, a novel group (2014) A mitogen-activated protein kinase kinase gene in maize, conferred chilling stress tolerance and was involved in pathogen defense in transgenic tobacco. *Plant Sci* 214:57–73
138. Tasgin E, Atici O, Nalbantoglu B, Popova LP (2006) Effects of salicylic acid and cold treatments on protein levels and on the activities of antioxidant enzymes in the apoplast of winter wheat leaves. *Phytochemistry* 67(7):710–715
139. Janda T, Szalai G, Lesko K, Yordanova R, Apostol S, Popova LP (2007) Factors contributing to enhanced freezing tolerance in wheat during frost hardening in the light. *Phytochemistry* 68(12):1674–1682
140. Wang R, Li R, Sun Z, Ren Y, Yue W (2006) [Anti-freezing proteins and plant responses to low temperature stress]. *Ying Yong Sheng Tai Xue Bao* 17(3):551–556
141. Yu XM, Griffith M, Wiseman SB (2001) Ethylene induces antifreeze activity in winter rye leaves. *Plant Physiol* 126(3):1232–1240
142. Stevens J, Senaratna T, Sivasithamparam K (2006) Salicylic acid induces salinity tolerance in tomato (*Lycopersicon esculentum* cv. Roma): associated changes in gas exchange, water relations and membrane stabilization. *Plant Growth Regulation* 49:77
143. Szepesi A, Csiszar J, Gemes K, Horvath E, Horvath F, Simon ML et al (2009) Salicylic acid improves acclimation to salt stress by stimulating abscisic aldehyde oxidase activity and abscisic acid accumulation, and increases Na<sup>+</sup> content in leaves without toxicity symptoms in *Solanum lycopersicum* L. *J Plant Physiol* 166(9):914–925
144. Kang G, Li G, Xu W, Peng X, Han Q, Zhu Y et al (2012) Proteomics reveals the effects of salicylic acid on growth and tolerance to subsequent drought stress in wheat. *J Proteome Res* 11(12):6066–6079
145. Korkmaz A, Uzunlu M, Demirkiran A (2007) Treatments with acetyl salicylic acid protects muskmelon seedlings against drought stress. *Acta Physiol Plant* 29:503–508
146. Hamada AM (2001) Salicylic acid versus salinity-drought-induced stress on wheat seedlings. *Rostl Vyr* 47:444–450
147. Senaratna T, Touchell D, Bunn E, Dixon K (2000) Acetyl Salicylic acid (aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. *Plant Growth Regul* 30:157–161
148. Nemeth R, Janda T, Horvath E, Paldi E, Szalai G (2002) Exogenous salicylic acid increases polyamine content but may decrease drought tolerance in maize. *Plant Sci* 162:569–574
149. Bandurska H, Stroinski A (2005) The effect of Salicylic acid on barley response to water deficit. *Acta Physiol Plant* 27:379–386
150. Yang Y, Qi M, Mei C (2004) Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. *Plant J* 40(6):909–919
151. Jonak C, Okresz L, Bogre L, Hirt H (2002) Complexity, cross talk and integration of plant MAP kinase signalling. *Curr Opin Plant Biol* 5(5):415–424

152. Chung E, Park JM, Oh SK, Joung YH, Lee S, Choi D (2004) Molecular and biochemical characterization of the *Capsicum annuum* calcium-dependent protein kinase 3 (CaCDPK3) gene induced by abiotic and biotic stresses. *Planta* 220(2):286–295
153. Leclercq J, Ranty B, Sanchez-Ballesta MT, Li Z, Jones B, Jauneau A et al (2005) Molecular and biochemical characterization of LeCRK1, a ripening-associated tomato CDPK-related kinase. *J Exp Bot* 56(409):25–35
154. Hettiarachchi GH, Reddy MK, Sopory SK, Chattopadhyay S (2005) Regulation of TOP2 by various abiotic stresses including cold and salinity in pea and transgenic tobacco plants. *Plant Cell Physiol* 46(7):1154–1160
155. Yang Y, He M, Zhu Z, Li S, Xu Y, Zhang C et al (2012) Identification of the dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress. *BMC Plant Biol* 12:140
156. Shen Y, Tang MJ, Hu YL, Lin ZP (2004) Isolation and characterization of a dehydrin-like gene from drought-tolerant *Boea crassifolia*. *Plant Sci* 166:1167–1175
157. Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA et al (1987) Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. *Plant Physiol* 85(2):529–536
158. Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M et al (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312(5772):436–439
159. van Loon LC, Geraats BP, Linthorst HJ (2006) Ethylene as a modulator of disease resistance in plants. *Trends Plant Sci* 11(4):184–191
160. Gupta V, Willits MG, Glazebrook J (2000) *Arabidopsis thaliana* EDS4 contributes to salicylic acid (SA)-dependent expression of defense responses: evidence for inhibition of jasmonic acid signaling by SA. *Mol Plant Microbe Interact* 13(5):503–511
161. Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC et al (2000) Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proc Natl Acad Sci U S A* 97(21):11655–11660
162. van Wees SC, de Swart EA, van Pelt JA, van Loon LC, Pieterse CM (2000) Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* 97(15):8711–8716
163. Mur LA, Kenton P, Atzorn R, Miersch O, Wasternack C (2006) The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol* 140(1):249–262
164. Spoel SH, Koornneef A, Claessens SM, Korzelius JP, Van Pelt JA, Mueller MJ et al (2003) NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15(3):760–770
165. Yuan Y, Zhong S, Li Q, Zhu Z, Lou Y, Wang L et al (2007) Functional analysis of rice NPR1-like genes reveals that OsNPR1/NH1 is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. *Plant Biotechnol J* 5(2):313–324
166. Pieterse CM, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SC (2012) Hormonal modulation of plant immunity. *Annu Rev Cell Dev Biol* 28:489–521
167. Broekaert WF, Delaure SL, De Bolle MF, Cammalleri BP (2006) The role of ethylene in host-pathogen interactions. *Annu Rev Phytopathol* 44:393–416
168. Sato M, Tsuda K, Wang L, Coller J, Watanabe Y, Glazebrook J et al (2010) Network modeling reveals prevalent negative regulatory relationships between signaling sectors in *Arabidopsis* immune signaling. *PLoS Pathog* 6(7), e1001011
169. Glazebrook J, Chen W, Estes B, Chang HS, Nawrath C, Metraux JP et al (2003) Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J* 34(2):217–228
170. Leon-Reyes A, Spoel SH, De Lange ES, Abe H, Kobayashi M, Tsuda S et al (2009) Ethylene modulates the role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in cross talk between salicylate and jasmonate signaling. *Plant Physiol* 149(4):1797–1809

171. Cao FY, Yoshioka K, Desveaux D (2011) The roles of ABA in plant-pathogen interactions. *J Plant Res* 124(4):489–499
172. Ton J, Flors V, Mauch-Mani B (2009) The multifaceted role of ABA in disease resistance. *Trends Plant Sci* 14(6):310–317
173. Whenham RJ, Fraser RS, Brown LP, Payne JA (1986) Tobacco-mosaic-virus-induced increase in abscisic-acid concentration in tobacco leaves: intracellular location in light and dark-green areas, and relationship to symptom development. *Planta* 168(4):592–598
174. Ward EW, Cahill DM, Bhattacharyya MK (1989) Abscisic acid suppression of phenylalanine ammonia-lyase activity and mRNA, and resistance of soybeans to phytophthora megasperma f.sp. *Glycinea*. *Plant Physiol* 91(1):23–27
175. Jiang CJ, Shimono M, Sugano S, Kojima M, Yazawa K, Yoshida R et al (2010) Abscisic acid interacts antagonistically with salicylic acid signaling pathway in rice-*Magnaporthe grisea* interaction. *Mol Plant Microbe Interact* 23(6):791–798
176. Yasuda M, Ishikawa A, Jikumaru Y, Seki M, Umezawa T, Asami T et al (2008) Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in *Arabidopsis*. *Plant Cell* 20(6):1678–1692
177. Dharmasiri S, Jayaweera T, Dharmasiri N (2013) Plant hormone signalling: current perspectives on perception and mechanisms of action. *Ceylon J Sci* 42(1):1–17
178. Santner A, Estelle M (2009) Recent advances and emerging trends in plant hormone signalling. *Nature* 459(7250):1071–1078
179. Chague V, Maoz R, Sharon A (2009) CgOpt1, a putative oligopeptide transporter from *Colletotrichum gloeosporioides* that is involved in responses to auxin and pathogenicity. *BMC Microbiol* 9:173
180. Yin C, Park JJ, Gang DR, Hulbert SH (2014) Characterization of a Tryptophan 2-Monoxygenase Gene from *Puccinia graminis* f. sp. *tritici* Involved in Auxin Biosynthesis and Rust Pathogenicity. *Mol Plant Microbe Interact* 27(3):227–235
181. Wang D, Pajerowska-Mukhtar K, Culler AH, Dong X (2007) Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. *Curr Biol* 17(20):1784–1790
182. Manulis S, Haviv-Chesner A, Brandl MT, Lindow SE, Barash I (1998) Differential involvement of indole-3-acetic acid biosynthetic pathways in pathogenicity and epiphytic fitness of *Erwinia herbicola* pv. *gypsophilae*. *Mol Plant Microbe Interact* 11(7):634–642
183. Glickmann E, Gardan L, Jacquet S, Hussain S, Elasri M, Petit A et al (1998) Auxin production is a common feature of most pathovars of *Pseudomonas syringae*. *Mol Plant Microbe Interact* 11(2):156–162
184. Chen Z, Agnew JL, Cohen JD, He P, Shan L, Sheen J et al (2007) *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. *Proc Natl Acad Sci U S A* 104(50):20131–20136
185. Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog* 2(11), e123
186. Gonzalez-Lamothe R, El Oirdi M, Brisson N, Bouarab K (2012) The conjugated auxin indole-3-acetic acid-aspartic acid promotes plant disease development. *Plant Cell* 24(2):762–777
187. Mutka AM, Fawley S, Tsao T, Kunkel BN (2013) Auxin promotes susceptibility to *Pseudomonas syringae* via a mechanism independent of suppression of salicylic acid-mediated defenses. *Plant J* 74(5):746–754
188. Navarro L, Bari R, Achard P, Lison P, Nemri A, Harberd NP et al (2008) DELLA proteins control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. *Curr Biol* 18(9):650–655
189. Naseem M, Kunz M, Ahmed N, Dandekar T (2013) Integration of boolean models on hormonal interactions and prospects of cytokinin-auxin crosstalk in plant immunity. *Plant Signal Behav* 8(4), e23890
190. Argueso CT, Ferreira FJ, Epple P, To JP, Hutchison CE, Schaller GE et al (2012) Two-component elements mediate interactions between cytokinin and salicylic acid in plant immunity. *PLoS Genet* 8(1), e1002448

191. Choi J, Choi D, Lee S, Ryu CM, Hwang I (2011) Cytokinins and plant immunity: old foes or new friends? *Trends Plant Sci* 16(7):388–394
192. Robert-Seilaniantz A, Grant M, Jones JD (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu Rev Phytopathol* 49:317–343
193. Choi J, Huh SU, Kojima M, Sakakibara H, Paek KH, Hwang I (2010) The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR1-dependent salicylic acid signaling in *Arabidopsis*. *Dev Cell* 19(2):284–295
194. Guan L, Scandalios JG (1995) Developmentally related responses of maize catalase genes to salicylic acid. *Proc Natl Acad Sci U S A* 92(13):5930–5934
195. Shakirova FM, Sakhabutdinova AR, Bezrukova MV, Fatkhutdinova RA, Fatkhutdinova DR (2003) Changes in the hormonal status of wheat seedlings induced by salicylic acid and salinity. *Plant Sci* 164(3):317–322
196. Gunes A, Inal A, Alpaslan M, Eraslan F, Bagci EG, Cicek N (2007) Salicylic acid induced changes on some physiological parameters symptomatic for oxidative stress and mineral nutrition in maize (*Zea mays* L.) grown under salinity. *J Plant Physiol* 164(6):728–736
197. McCue PA, Zheng Z, Pinkham JL, Shetty K (2000) A model for enhanced pea seedling vigour following low pH and salicylic acid treatments. *Process Biochem* 35(6):603–613
198. Agostini EA, Machado-Neto NB, Costodio CC (2013) Induction of water deficit tolerance by cold shock and salicylic acid during germination in the common bean. *Acta Scientiarum Agronomy* 35(2):209–219
199. Lee S, Kim SG, Park CM (2010) Salicylic acid promotes seed germination under high salinity by modulating antioxidant activity in *Arabidopsis*. *New Phytol* 188(2):626–637
200. Borsani O, Valpuesta V, Botella MA (2001) Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. *Plant Physiol* 126(3):1024–1030
201. Zhou YC, Zheng RL (1991) Phenolic compounds and an analog as superoxide anion scavengers and antioxidants. *Biochem Pharmacol* 42(6):1177–1179
202. Zhu JK (2001) Plant salt tolerance. *Trends Plant Sci* 6(2):66–71
203. Fragniere C, Serrano M, Abou-Mansour E, Metraux JP, L'Haridon F (2011) Salicylic acid and its location in response to biotic and abiotic stress. *FEBS Lett* 585(12):1847–1852
204. Serrano M, Wang B, Aryal B, Garcion C, Abou-Mansour E, Heck S et al (2013) Export of salicylic acid from the chloroplast requires the multidrug and toxin extrusion-like transporter EDS5. *Plant Physiol* 162(4):1815–1821

# **Chapter 6**

## **Regulators and Pathway Enzymes That Contribute to Chemical Diversity in Phenylpropanoid and Aromatic Alkaloid Metabolism in Plant Immunity**

**William R. Chezem and Nicole K. Clay**

**Abstract** Plant secondary or “specialized” metabolites play an important role in plant survival, often as defense chemicals, and are the predominant natural sources of drugs for human health because of their chemical diversity and biosynthetic complexity. As a consequence, the biosynthesis and regulation of plant specialized metabolism have been intensively studied in a number of model and non-model plant species. Here, we review what is currently known about the transcriptional regulators and pathway enzymes that contribute to the chemical diversity and biosynthetic complexity of two of the three major groups of plant specialized metabolites, the phenylpropanoids and the nitrogen-containing alkaloids. For the latter, we will focus on aromatic amino acid-derived alkaloids that have been extensively studied for their regulation and biosynthesis. They include the amine-derived terpenoid indole alkaloids, the free indole-derived benzoxazinoids, and the oxime-derived aromatic compounds, such as the tyrosine-derived cyanogenic glycosides, indole glucosinolates, and sulfur-containing indole alkaloids. In addition, we will discuss the possible phylogenetic conservation of the transcriptional regulators of phenylpropanoid metabolism in defense.

**Keywords** Cytochrome P450 • Basic-helix-loop-helix (bHLH) proteins from subgroup 3e • R2R3-MYBs from subgroup 2 • R2R3-MYBs from subgroup 12 • ERFs from subgroup 9 • WRKY • Phenylpropanoid • Local acquired resistance (LAR) • Systemic acquired resistance (SAR) • Nodulation • Cell wall-reinforcement • Phytoanticipins • Phytoalexins • Amine-derived aromatic alkaloids • Indole-derived aromatic alkaloids • Aldoxime-derived aromatic alkaloids • Terpenoid indole alkaloids • Cyanogenic glycosides • Indole glucosinolates • Sulfur-containing indole alkaloids • Jasmonates • Salicylates

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## 6.1 Introduction

Plants rely heavily on highly diversified and “specialized” chemical defenses, both constitutive and inducible, to protect themselves against microbial and herbivore attacks and from their abiotic environment (e.g., UV irradiation). They are highly diversified because closely related compounds may differ in their bioactivity profiles, and may serve as insect deterrents, phytoalexins (i.e., inducible antimicrobials), phytoanticipins (i.e., preformed antimicrobials) [1], cell wall reinforcements, and chemical signals mediating, for example, nodulation and cell wall reinforcement. They are “specialized” because they are adaptive products to specific ecological challenges. Due to selection pressures from different pathogens, herbivores and/or competitor plant species [2–5], plant specialized metabolites can vary widely in their range of distribution from rare or species-specific to genus-, family- or order-specific, as differentiated from the primary metabolites that are present in all plant species. For example, the glucosinolates ( $\beta$ -thioglucosides) make up the largest known group of specialized metabolites in *Arabidopsis* but are generally restricted to species in the order Brassicales [6, 7]. In contrast to prokaryotes or animals, plants probably produce hundreds of thousands of specialized metabolites [8–10], suggesting that there is a wealth of bioactive plant chemicals yet to be discovered in even the best characterized plant species.

A major contributing factor to the evolution of chemical diversity in plant specialized metabolism is the number of highly diversified, mid-to-large-sized families or superfamilies of evolutionarily ancient, skeleton-forming enzymes, e.g., type-III polyketide synthases (including chalcone synthases), terpene synthases, and cytochrome P450s, encoded in the genomes of all land plants [11–14]. They are highly diversified because closely related enzymes within a family may differ in their product profiles, reaction mechanisms, subcellular localizations, or *in planta* substrates. For example, cytochrome P450 enzymes are grouped into families and subfamilies based on amino acid sequence identity (P450 proteins with  $\geq 40\%$  identity and  $\geq 55\%$  identity grouped into the same family and subfamily, respectively) [13], and some possess the capacity to manipulate molecular scaffolds, catalyzing transformations beyond the canonical oxygen-dependent hydroxylation. Cytochrome P450 genes are estimated to make up  $>1\%$  of the total number of genes for a given plant species [14, 15]. However, the vast majority remain uncharacterized. For example, the model plant *Arabidopsis* has  $\sim 286$  P450 genes, but only a few dozen of them have been characterized to any extent. The discrepancy between the number of known P450-catalyzed reactions and the number of P450-encoding genes suggests that plants produce a relatively large number of specialized metabolites via P450-catalyzed reactions.

The expansion of certain transcription factor families in plants (e.g., basic helix-loop-helix (bHLH), R2R3-type MYELOBLASTOSIS (MYB), ethylene response factor (ERF), and WRKY proteins) may be another major factor in the rapid evolution of chemical diversity in plant specialized metabolism. Upon pathogen infection, plants undergo dramatic transcriptomic reprogramming to shift from normal growth and development to an immune response [16–18]. This rapid process is

orchestrated by a number of defense-regulated transcription factors from subfamilies that are typically conserved in sequence and function among different plant families. These transcription factors receive external signals (both biotic and abiotic) as well as internal signals, such as defense hormones, and translate them into a functional immune response by (1) binding to sequence-specific *cis*-regulatory elements in target promoters of metabolic genes, (2) activating or repressing metabolic gene expression by recruiting and/or modulating the activity of the core RNA polymerase II transcriptional machinery, and (3) regulating the cross talk between different hormone signaling pathways [19, 20].

The extraordinary chemical diversity of defense-regulated plant specialized metabolites is thought to be the result of gene duplication of pathway member genes followed by neo-functionalization [21]. This mechanism typically produces novel, simple, and mutually exclusive pathways, where the enzymes catalyzing the first committed steps of a pathway are more ancient than their corresponding transcriptional regulators. In some cases, the pathway enzymes are genetically clustered to facilitate their common regulation, but even then their genomic arrangement reflects the inherent simplicity in their pathways. However, for the majority of the specialized metabolic pathways, the nonhomologous gene constituents are not genetically clustered [22], suggesting a different evolutionary mechanism for pathway gene recruitment that involves more ancient transcriptional regulators.

Pathway member genes that are dispersed in the genome but are transcriptionally clustered into a regulon (a collection of genes under the control of the same regulatory protein) typically share a common promoter element that is bound by a specific subfamily of transcription factors. This pathway organization allows for a limited set of key regulators to combinatorially trigger defined transcriptional responses throughout an entire regulon. While it is still unclear how evolutionary pressures for pathway diversification can lead to gene recruitment by specific transcription factor subfamilies, certain plant transcription factors have been shown to coordinate the expression of relevant gene networks to redirect metabolic flux between competing pathways.

In this review, we will focus on four transcription factor subfamilies: the subgroup 3e (SG3e) bHLHs, the subgroup 2 (SG2) R2R3-MYBs, the subgroup 9 (SG9) ERFs, and the WRKYs. Their member proteins have been found to regulate the biosynthesis of two groups of plant specialized metabolites, the aromatic alkaloids and the phenylpropanoids, from primary products of the shikimate pathway for a number of different plant species.

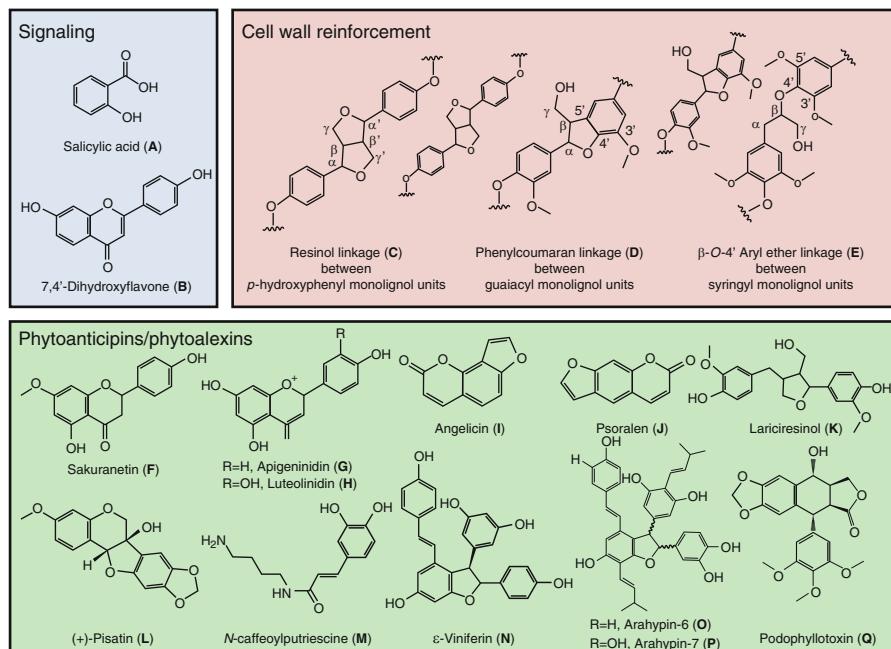
## 6.2 Phenylpropanoids Involved in Defense Responses

The phenylpropanoids represent one of the largest and most ancient families of specialized metabolites involved in chemical defense of terrestrial as well as marine plants, and are synthesized by a metabolic sequence that consists of the general pathway followed by a number of major branch and subbranch pathways. The first step in the general pathway is the deamination of phenylalanine by phenylalanine-ammonia

lyase (PAL) to form *trans*-cinnamic acid and its 4-hydroxylated product, *p*-coumaric acid (**R**, Fig. 6.2). Downstream of these acid intermediates, the general pathway branches into the lignin, flavonoid, and stilbene pathways. In addition, intermediates in the lignin pathway can be diverted to the formation of coumarins, phenolamides, and lignans. Moreover, the end products from each pathway within phenylpropanoid metabolism are associated with a distinct core structure and function, including benzoic acids (**A**, Fig. 6.1) for defense signaling, lignin (**C–E**, Fig. 6.1) for vascularization and stem rigidity, lignans (**K, Q**, Fig. 6.1), coumarins (**I–J**, Fig. 6.1; **U**, Fig. 6.2), and stilbenes (**N–P**, Fig. 6.1; **T**, Fig. 6.2) for protection against microbial pathogens, phenolamides (**M**, Fig. 6.1) for protection against herbivory, and flavonoids (**F–H, L**, Fig. 6.1; **S**, Fig. 6.2) for protection against UV-irradiation and microbial pathogens, and for mediating plant–microbe interactions.

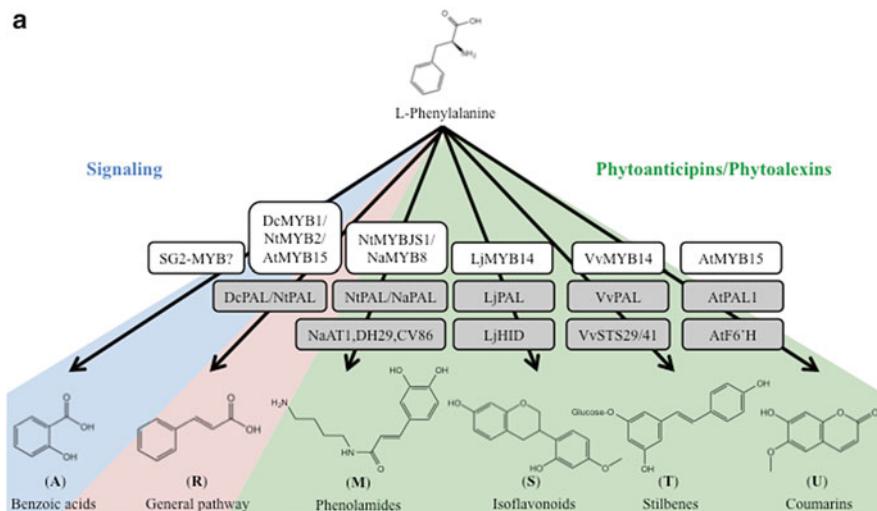
### 6.2.1 Signaling Phenylpropanoids

Salicylic acid (**A**, Fig. 6.1) is a ubiquitous signaling regulator of local acquired resistance (LAR) in pathogen-infected cells, and systemic acquired resistance (SAR) in non-infected cells at a distance from the initial infection site [23, 24]. Two pathways

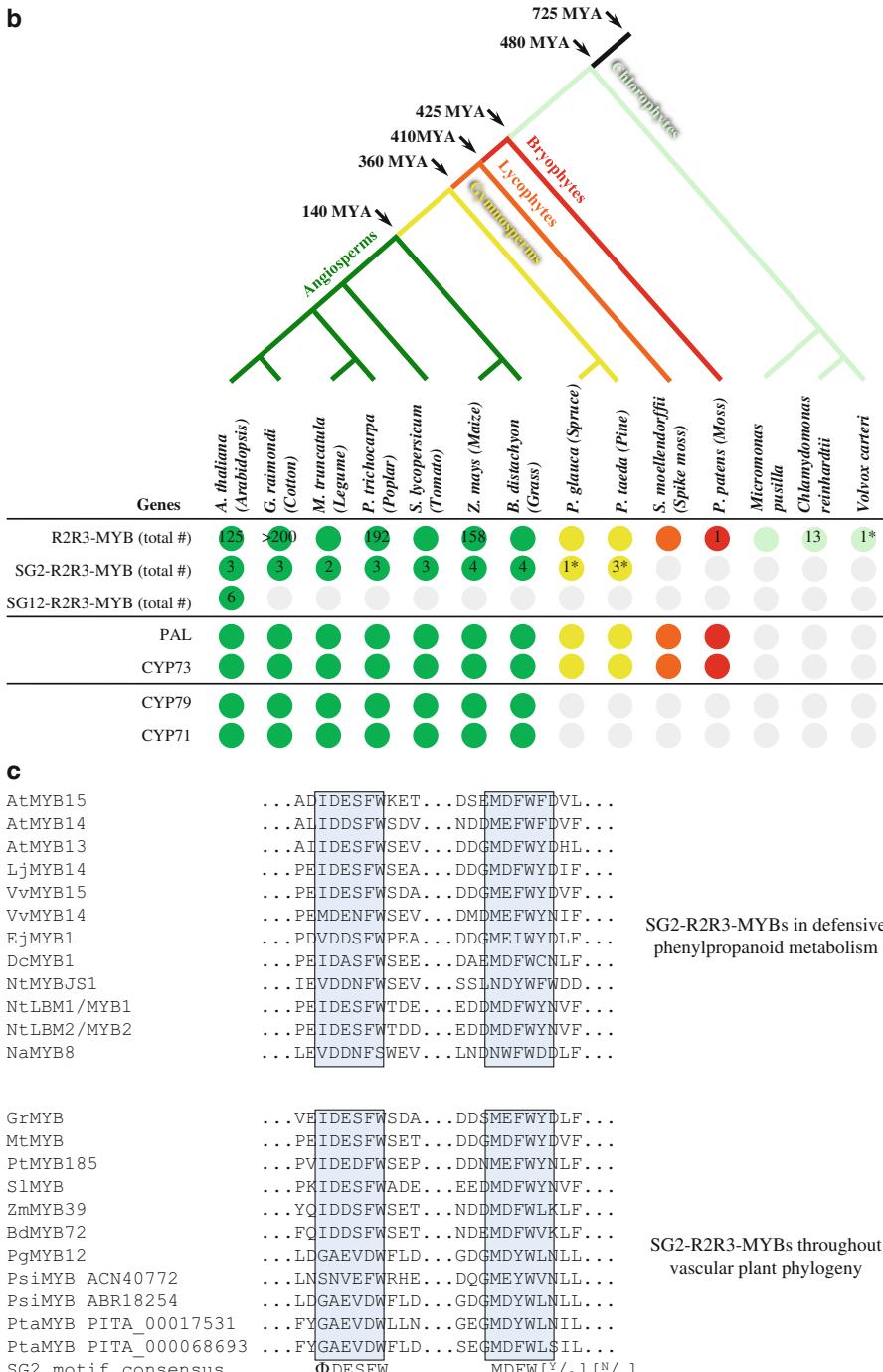


**Fig. 6.1** Defense-regulated phenylpropanoid metabolites. Metabolites **A**, **C–E** are produced in most vascular plants. *Poaceae*-specific metabolites: **F–H**. *Fabaceae*-specific metabolites: **B, L, O–P**. *Vitaceae*-specific metabolites: **N**. For lignin substructures, ring and side-chain carbons were labeled for clarity

of salicylic acid biosynthesis have been proposed in plants; the first is derived from isochorismate from the shikimate pathway, and the second is derived from cinnamic acid from the general phenylpropanoid pathway. While salicylic acid can be synthesized from the latter pathway in a pathogen-induced manner [23, 25, 26], genetic approaches thus far have failed to ascribe a protective role for this pathway, or identify any of its downstream pathway enzyme constituents or regulators. Other signaling



**Fig. 6.2** The SG2 motif-containing R2R3-MYB transcription factors regulate most known branches of defense-regulated phenylpropanoid metabolism in vascular plants. (a) Transcription factors (white boxes) and committed pathway enzymes (gray boxes) are arranged in order along the pathway. At *Arabidopsis thaliana*, Dc *Daucus carota*, Gm *Glycine max*, Lj *Lotus japonicus*, Nt *Nicotiana tabacum*, Vv *Vitis vinifera*. Pathway enzymes: AT1/DH29/CV86, hydroxycinnamyl-CoA: polyamine transferases; PAL phenylalanine-ammonia lyase, HID 2-hydroxyisoflavanone dehydratase, STS stilbene synthase, and F6'H ferulyl-CoA 6'-hydroxylase. Phenylpropanoids (left to right): salicylic acid, *trans*-cinnamic acid, *N*-caffeoyleputrescine, vestitol, *trans*-piceid, and scopoletin. (b) Simplified cladogram illustrating gene conservation of SG2 motif-containing R2R3-MYB transcription factors, PAL, and CYP73/79/71 P450s in the plant kingdom. Colored circles indicate presence of homologous genes. Homologous genes were identified using Phytozome v9.1 (<http://www.phytozome.net>), Plant Transcription Factor Database (<http://planttfdb.cbi.pku.edu.cn/>), Cytochrome P450 Homepage (<http://drnelson.uthsc.edu/CytochromeP450.html>), NCBI Genbank (<http://www.ncbi.nlm.nih.gov>), and TreeGenes (<http://dendrome.ucdavis.edu/treegenes>). MYA million years ago. (c) Amino acid sequence alignment of the SG2 motif. φ denotes hydrophobic residues. At *Arabidopsis thaliana*, Bd *Brachypodium distachyon*, Lj *Lotus japonicus*, Vv *Vitis vinifera*, Dc *Daucus carota*, Gr *Gossypium raimondii*, Mt *Medicago truncatula*, Nt *Nicotiana tabacum*, Na *Nicotiana attenuata*, Pg *Picea glauca*, Psi *Picea sitchensis*, Pta *Pinus taeda*, Pt *Populus trichocarpa*, Sl *Solanum lycopersicum*, Zm *Zea mays*. Genbank or Uniprot identifiers: AtMYB15, AEE76741; VvMYB15, KC514110; VvMYB14, EU1814240; DcMYB1, AB218778; NtMYBJS1, AB236951; NtLBM1, AB028649; NtLBM2, AB028650; NaMYB8, GU451752; MtMYB, XP\_003590028; PtMYB185, XP\_002311670; SiMYB, X98308; ZmMYB39, AFW63053; BdMYB72, XP\_003575392; PgMYB12, A5JYF6; PsMYBs, ABR18254 and ACN40772. Phytozome v9.1 identifiers: GrMYB, Gorai.011G173900; LjMYB14, chr5.CM0071.380.r2.d; PITA\_00017531 PITA\_000068693, [http://dendrome.ucdavis.edu/treegenes/protein/prot\\_summary.php](http://dendrome.ucdavis.edu/treegenes/protein/prot_summary.php).

**Fig. 6.2** (continued)

phenylpropanoids are the nodulation-inducing flavonoids involved in plant–*Rhizobium* spp. symbiosis. They include the flavone luteolin (3',4',5,7-tetrahydroxyflavone) from alfalfa (*Medicago sativa*) [27], the flavones apigenin (4',5,7-trihydroxyflavone) and 7,4'-dihydroxyflavone (**B**, Fig. 6.1) from pea and clover, respectively, the isoflavones daidzein and genistein from soybean (*Glycine max*) [28], and the flavanones naringenin (4',5,7-trihydroxyflavanone) and hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone) from pea (*Pisum sativum*) and lentil (*Lens culinaris*) [29].

### 6.2.2 Cell Wall-Reinforcing Phenylpropanoids

Lignin is synthesized through the polymerization of three different monomers, the monolignols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, which in the context of lignin make up the *p*-hydroxyphenyl (H; **C**, Fig. 6.1), guaiacyl (G; **D**, Fig. 6.1) and syringyl (S; **E**, Fig. 6.1) units, respectively. In higher plants, lignin biosynthesis is most commonly known to be developmentally regulated, and predominantly utilizes G units (although a significant portion of the angiosperm lignin is also composed of S units) [30]. However, lignin biosynthesis is also induced by wounding, pathogen ingress [31, 32] and the cell-surface perception of a variety of microbial elicitors, both insoluble (fungal cell wall preparations) and soluble (e.g., bacterial flagellin) [33–35]. However, in contrast to developmentally regulated lignin biosynthesis, the transcription factors and pathway enzyme isoforms involved in defense-regulated lignin biosynthesis have not yet been identified. It is believed that the composition of defense-regulated lignin may be different from that of developmentally regulated lignin. For example, the rust-induced lignin in the angiosperm wheat (*Triticum aestivum*) was characterized to have an increase in S lignin units, and hypothesized to be more resistant to fungal degradation [36]. By contrast, the fungal cell wall preparation-induced lignin in the gymnosperm Norway Spruce (*Picea abies*) was characterized to have a substantial increase in H-lignin units and resolin ( $\beta$ -O- $\beta'$ ) linkages [33]. Although only two plant species were examined thus far, the findings suggest that gymnosperms and angiosperms may utilize different strategies in producing defense-regulated lignin, and that both S- and H-lignins may contribute to pathogen resistance.

### 6.2.3 Phenylpropanoid Phytoanticipins and Phytoalexins

Many phenylpropanoids function as phytoanticipins or phytoalexins. For example, the phenolamides are mono- and bis-acylated spermidine phytoanticipins that are derived from hydroxycinnamic acid intermediates in the lignin branch pathway, and are widely distributed throughout the plant kingdom. They are thought to require an additional activating factor to have a protective function against herbivores [37, 38]. Similarly, coumarins are widely distributed heterocyclic metabolites that are derived from hydroxycinnamyl-CoA intermediates in the lignin branch pathway and can

function as phytoalexins. For example, the bicyclic coumarin phytoalexin scopoletin (7-hydroxy-6-methoxychromen-2-one; **U**, Fig. 6.2) has been shown to be synthesized in tobacco and *Arabidopsis* plants in response to treatments with microbial elicitors (e.g., fungal cell wall preparations and bacterial flagellin) or pathogenic infections by tobacco mosaic virus (TMV) and the vascular wilt-causing fungus *Fusarium oxysporum*, respectively [39–42]. Once synthesized, it has significant antiviral [43] and antifungal activities [44, 45] that increase synergistically when combined with other phenylpropanoids [46]. There are also tricyclic coumarin phytoalexins such as psoralen (**J**, Fig. 6.1) and angelicin (**I**, Fig. 6.1).

Compared to coumarin phytoalexins, the flavonoid phytoalexins are not widely distributed in plants, but are found in cereal crops and legumes. For example, the major rice phytoalexin is the flavanone sakuranetin (**F**, Fig. 6.1), which is induced by pathogen infection and UV irradiation and inhibits the spore germination of the rice blast disease-causing *Magnaporthe oryzae* [47]. Other flavonoid phytoalexins include the sorghum 3-deoxyanthocyanidins, apigeninidin (**G**, Fig. 6.1), and luteolinidin (**H**, Fig. 6.1), which have been shown to contribute to localized resistance to the anthracnose disease-causing fungus *Colletotrichum graminicola* as well as to the southern leaf blight-causing fungus *Cochliobolus heterostrophus* [48–50]. In addition, there is a large number of legume-specific isoflavanoid phytoalexins [51–53], such as the pterocarpans (+)-pisatin (**L**, Fig. 6.1), phaseollin, and glyceollin I [54–57], as well as the isoflavone wighteone from *Glycine wightii* [58].

Related to the flavonoid phytoalexins, the grapevine stilbene phytoalexins resveratrol and the resveratrol dimer ε-Viniferin (**N**, Fig. 6.1) have been shown to confer resistance to the grey mold-causing fungus *Botrytis cinerea* and the grapevine downy mildew-causing oomycete *Plasmopara viticola* [59–62]. Similar to the grapevine stilbene phytoalexins, the peanut stilbene phytoalexins arahypins (**O–P**, Fig. 6.1) are induced by wounding, pathogen attack, and UV irradiation [63–65].

Finally, the lignan phytoanticipins are structurally related to lignin, except that they are dimers, not polymers, of monolignols. Like lignin, lignans are widely distributed in plants, and many different lignans may be present in a single plant species. Unlike lignin, the biosynthesis of lignan requires the dirigent (DIR) proteins to align the two monolignol precursors for a stereospecific linkage [66]. The expression of several *Arabidopsis* DIR genes involved in lignan biosynthesis has been shown to increase dramatically in response to the bacterial elicitor flagellin [67]. Additionally, the production of the lignan phytoanticipins piperitol and sesamolinol is induced in *Arabidopsis* plants infected with the *Verticillium* wilt-causing fungus *Verticillium longisporum* [68]. Similarly, the synthesis of the lignan phytoanticipins lariciresinol and podophyllotoxin is induced in *Juniperus* and *Linum* cell cultures treated with the fungal elicitor chitin and fungal cell wall preparations [69, 70]. However, no regulators of lignan metabolism have been identified to date.

Lignans are known to have diverse biological activities; the lignan phytoanticipins pinoresinol, lariciresinol, and secoisolariciresinol have been shown to have broad antimicrobial activity, weakly inhibiting the growth of *Bacillus*, *Micrococcus*, and *Citrobacter* bacteria, and strongly inhibiting the growth of wood-rot fungi [71], while other studies have shown anti-herbivory effects [72, 73].

### 6.3 Regulators of Defense-Related Phenylpropanoid Metabolism

The R2R3-MYB family of transcription factors is one of the largest families of regulatory proteins known, having expanded early in the history of land plants (ca 250–400 million years ago) [74]. In addition, the R2R3-MYBs are closely associated with phenylpropanoid metabolism. For example, the R2R3-MYB proteins from subgroups 6 and 7 are known regulators of the production of anthocyanins and flavonols, respectively [75], while the R2R3-MYBs from subgroup 3 regulate lignin biosynthesis by binding to AC elements present in the target promoters of most lignin biosynthetic genes [76, 77]. Recently, a number of R2R3-MYB proteins from subgroup 2 (SG2-R2R3-MYBs) have been identified in diverse plant species to regulate the production of defense-regulated phenylpropanoids. For example, the tobacco NtMYB2 and the carrot DcMYB1 have been shown to activate general steps in the phenylpropanoid pathway in response to wounding, microbial elicitors, and UV-B irradiation [78, 79]. Similarly, the herbivory-inducible and JA-regulated NtMYBJS1 and NaMYB8 in *Nicotiana* spp. have been shown to activate the first committed steps in the general and phenolamide-specific pathways in order to redirect the metabolic flux towards phenolamide biosynthesis during insect herbivory [45, 80, 81]. Moreover, the stress-inducible SG2-R2R3-MYBs lotus (*Lotus japonicus*) LjMYB14, grapevine VvMYB14 and VvMYB15, and *Arabidopsis* AtMYB15 have been shown to regulate the first committed steps in various branch pathways leading to the production of the isoflavone phytoalexin vestitol (Fig. 6.2a), the stilbene phytoalexin *trans*-piceid (Fig. 6.2a), and the coumarin phytoalexin scopoletin (Fig. 6.2a), respectively [45, 82, 83]. AtMYB15 has also been shown to activate multiple enzymes in the shikimate pathway to synthesize phenylalanine [84].

The SG2-R2R3-MYBs are characterized by the presence of two 50- to 52-residue-long imperfect repeats as the common DNA-binding MYB domain [85] and a short conserved SG2 motif  $\Phi$ DESFW-MDFW[Y/ $\Phi$ ][N/D] (where  $\Phi$  is a hydrophobic residue) in the longer, divergent C-terminal region (Fig. 6.2c) [86, 87]. While the SG2 motif appears to be conserved among the angiosperms and gymnosperms (Fig. 6.2b), only a handful of SG2-containing R2R3-MYB genes are present in a given plant species, compared to the 125 and 158 total R2R3-MYB genes that are present in the *Arabidopsis thaliana* and maize (*Zea mays*) genomes, respectively (Fig. 6.2b) [87–89]. Finally, although individual phenylpropanoid metabolites may differ among plant species, the SG2-R2R3-MYBs are likely to be functionally redundant, displaying very similar target gene specificities, as was found for R2R3-MYB proteins from subgroups 6 and 7 in *Arabidopsis* and maize [75, 90, 91]. It is very likely that the SG2-R2R3-MYBs are important factors involved in the rapid evolution of chemical diversity in phenylpropanoid metabolism.

### 6.4 Aromatic Alkaloids Involved in Defense Responses

Alkaloids are a class of low-molecular-weight nitrogen-containing molecules, usually with a heterocyclic structure, that are largely derived from the decarboxylation or *N*-hydroxylation to form amine or oxime intermediates, respectively, of the amino

acids tryptophan, tyrosine, phenylalanine, lysine and ornithine. More than 16,000 alkaloid structures have been characterized in plants [92]. Here, we will focus on several classes of aromatic alkaloids that have been extensively studied for their regulation and biosynthesis, including the amine-derived terpenoid indole alkaloids, the free indole-derived benzoxazinoids, and the oxime-derived aromatic cyanogenic glycosides, indole glucosinolates and sulfur-containing indole alkaloids.

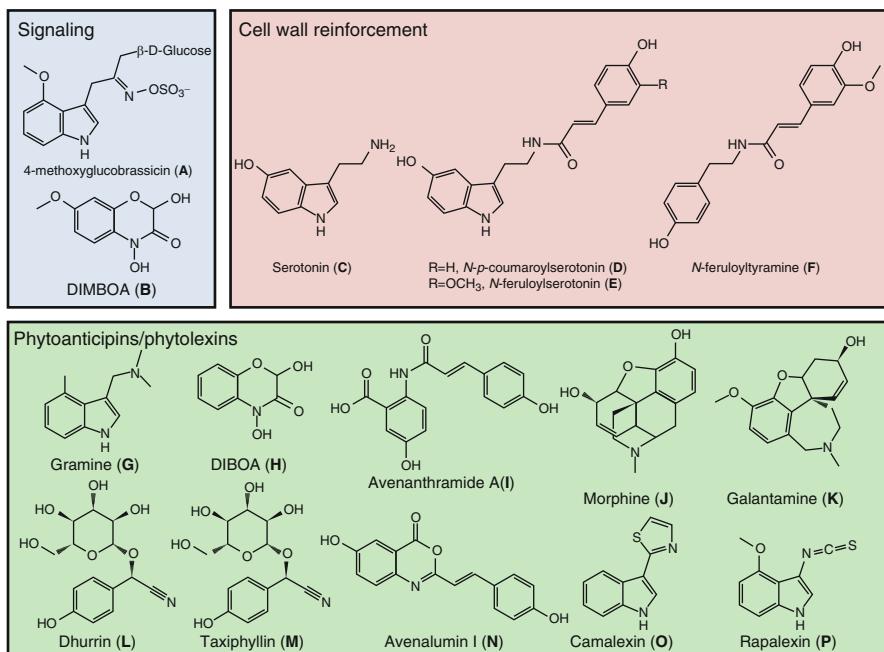
#### 6.4.1 Amine-Derived Aromatic Alkaloids

The terpenoid indole alkaloids (TIAs) and the benzylisoquinolines are synthesized by the decarboxylation of tryptophan and tyrosine by the aromatic amino acid decarboxylase enzymes TDC and TyDC, respectively, to form their respective primary amine intermediates tryptamine and tyramine. In addition, TIAs have a mixed biosynthetic origin, requiring the subsequent condensation of tryptamine to the terpenoid precursor secologanin by the strictosidine synthase (STR) enzyme. The TIAs are restricted to plant species within the *Rubiaceae*, *Loganiaceae*, *Nyssaceae*, and *Apocynaceae* families, and the *Apocynaceae* species Madagascar periwinkle (*Catharanthus roseus*) has become the model plant for understanding TIA biosynthesis and regulation [93, 94]. There are over 1800 different pharmacologically active TIAs [95], of which the primary TIAs of pharmaceutical interest in *Catharanthus* are ajmalicine (Fig. 6.4a), serpentine, vinblastine (Fig. 6.4a) and vincristine. Ajmalicine and serpentine are used in the treatment of hypertension [96], while vinblastine and vincristine are used in the treatment of cancers [97].

Plant TDCs and TyDCs are also involved in the synthesis of other aromatic amine-derived alkaloids, such as the cell wall-reinforcing serotonin (also known as 5-hydroxytryptamine, C, Fig. 6.3) and hydroxycinnamic acid amides in monocots (D–F, Fig. 6.3) [98], theavenanthramide phytoalexins in oat (I and N, Fig. 6.3) [99–101], and the simple alkaloid phytoalexin hordenine in barley (*Hordeum vulgare*) [102], as well as a number of widely prescribed pharmaceuticals, such as the analgesic morphine (J, Fig. 6.3), the microtubule disrupter colchicine and the acetylcholine esterase inhibitor galantamine (K, Fig. 6.3) [95].

#### 6.4.2 Indole-Derived Aromatic Alkaloids

The benzoxazinoids are a class of cyclic hydroxamic acids that are derived from the cleavage of indole-3-glycerol-phosphate by the tryptophan synthase alpha subunit (TSA) homolog BX1 to form indole and glyceraldehyde-3-phosphate. The free indole is then metabolized by four closely related but highly substrate-specific cytochrome P450s of the CYP71 family, each of which catalyzes a different step in the modification of the free indole to a benzoxazinoid [103, 104]. The benzoxazinoid glucosides represent a major class of phytoanticipins widely distributed in the *Poaceae* as broad-spectrum antifungals, insecticides and allelochemicals [105,



**Fig. 6.3** Defense-regulated aromatic alkaloid metabolites. *Poaceae*-specific metabolites: **B, G–I, L–N**. *Brassicaceae*-specific metabolites: **A, O–P**

[106]. While the glucosides are constitutively expressed, the aglycones accumulate in response to microbial elicitors [107]. The major benzoxazinoids in wheat, maize, rye and wild barley are 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA; **B**, Fig. 6.3) and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA; **H**, Fig. 6.3). Recently, DIMBOA was shown to act as a bifunctional defense chemical, serving both as a phytoanticipin and as a signaling molecule responsible for triggering the deposition of the cell wall-reinforcing  $\beta$ -glucan polymer callose to localized sites of pathogen ingress in maize [107]. The other related major defense-regulated indole phytoanticipin in gramineous plants is gramine (**G**, Fig. 6.3) [108]. Although no regulatory and biosynthetic genes have been identified for gramine, the biosynthetic pathways for gramine and the benzoxazinoids were found to be mutually exclusive in wild barley species, indicating strong selection against simultaneous expression of both pathways [109].

#### 6.4.3 Aldoxime-Derived Aromatic Alkaloids

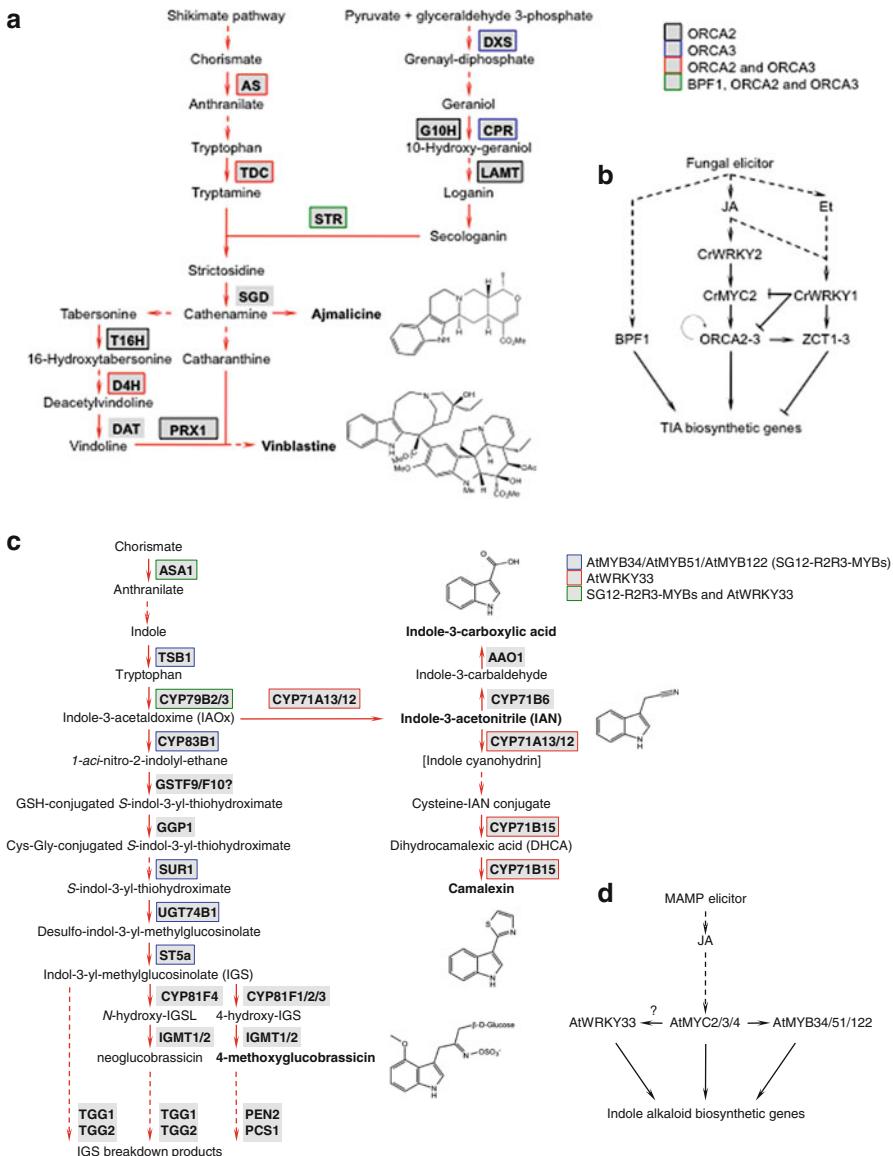
The aromatic cyanogenic glycosides, indole glucosinolates and the major sulfur-containing indole alkaloids in *Arabidopsis* are all derived from aldoximes, which are generated and metabolized by cytochrome P450s of the CYP79 and CYP71

families, respectively [110, 111], both of which are only found in angiosperms. Aldoximes are well-known precursors to several classes of defense compounds that act directly (e.g., toxins and feeding deterrents) and indirectly (e.g., volatiles) against microbial pathogens and herbivores.

The cyanogenic glycosides are  $\beta$ -glycosides of aldoxime-derived  $\alpha$ -hydroxynitriles. They represent the largest group of nitride glycoside phytoanticipins and are widely distributed throughout the plant kingdom, present in the pteridophytes, gymnosperms and angiosperms, as well as in a few arthropod species [112–114]. Aromatic cyanogenic glycosides, such as dhurrin (**N**, Fig. 6.3), taxiphyllin (**O**, Fig. 6.3) and triglochinin, require tissue disruption by chewing herbivores for their activation by  $\beta$ -glycosidases to form the aglycone insect deterrents and the rapidly acting poison hydrogen cyanide [80, 115, 116]. By contrast, the indole glucosinolates are  $\beta$ -thioglucosides of aldoxime-derived sulfonated thiohydroximates, and are found mainly in the order Brassicales [117, 118] and in the genus *Drypetes* within the order Malpighiales [119]. Indole glucosinolates are well-known insect deterrents against generalist herbivores and aphids [120, 121], and can also be precursors of some cruciferous phytoalexins formed in response to pathogen infections and microbial elicitors [122, 123]. In addition, similar to DIMBOA, the unidentified aglycone(s) of the indole glucosinolate metabolite 4-methoxyglucobrassicin (**A**, Fig. 6.3) may act as a signaling molecule to activate the deposition of the cell wall-reinforcing  $\beta$ -glucan polymer callose to localized sites of pathogen ingress [122]. The glucosinolate pathway most likely has evolved from the more ancient cyanogenic glycoside pathway by the appearance of the CYP83 (CYP71 is the correct nomenclature) family of cytochrome P450 enzymes to convert the aldoximes to reactive *aci*-nitro compounds instead of  $\alpha$ -hydroxynitriles [112] (Fig. 6.4). Despite sharing a common biosynthetic origin, the pathways of glucosinolates and cyanogenic glycosides are mutually exclusive [111] and coexist in only a few species in the order Brassicales (e.g., papaya

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**Fig. 6.4** (continued) acid *O*-methyltransferase, *PRX1* vacuolar class III peroxidase, *SGD* strictosidine  $\beta$ -d-glucosidase, *SLS* secologanin synthase, *STR* strictosidine synthase, *TDC* tryptophan decarboxylase, *T16H* tabersonine 16-hydroxylase. **(b)** Regulatory network of TIA biosynthesis. Unbroken black arrows indicate direct regulation, and broken black arrows indicate indirect regulation. **(c)** Indole alkaloid biosynthetic pathway in *Arabidopsis thaliana*. Blue boxes indicate enzymes upregulated by the SG12 motif-containing R2R3-MYB transcription factors AtMYB34/AtMYB51/AtMYB122 transcription factors. Red boxes indicate enzymes upregulated by the AtWRKY33 transcription factor. Green boxes indicate enzymes upregulated by the SG12-R2R3-MYBs and AtWRKY33. *AAO1* indole-3-aldehyde oxidase, *ASA1* anthranilate synthase  $\alpha$ -subunit, *CYP71A13/12* indole-3-acetaldoxime dehydratase and indole-3-acetonitrile  $\alpha$ -hydroxylase, *CYP71B6* indole-3-acetonitrile  $\alpha$ -hydroxylase and indole-3- $\alpha$ -hydroxy-acetonitrile  $\alpha$ -hydroxylase, *CYP71B15* dihydrocamalexate synthase and dihydrocamalexic acid decarboxylase, *CYP79B2/3* tryptophan *N*-hydroxylases, *CYP81F1-3* indol-3-yl-methylglucosinolate 4-hydroxylase, *CYP81F4* indol-3-yl-methylglucosinolate *N*-hydroxylase, *CYP83B1* indole-3-acetaldoxime *N*-hydroxylase, *GST* glutathione transferase, *GGPI*  $\gamma$ -glutamyl peptidase 1, *IGMT2* and *IGMT3* indole glucosiminate *O*-methyltransferases, *PEN2* 4-glucobrassicin thioglucosidase, *PCSI* phytochelatin synthase, *STS/SOT16* desulfoglucosinolate sulfotransferase, *SURI* S-alkyl-thiohydroximate lyase, *TGG* thioglucosidase, *TSB1* tryptophan synthase b-subunit, *UGT74B1* UDP-glucose:thiohydroximate *S*-glucosyltransferase. **(d)** Regulatory network of indole alkaloid biosynthesis. Unbroken black arrows indicate direct regulation, and broken black arrows indicate indirect regulation



**Fig. 6.4** Multiple transcription factors are involved in defense-regulated aromatic alkaloid metabolism. Unbroken red arrows indicate single enzymatic conversions, and broken red arrows indicate multiple enzymatic conversions. **(a)** Terpenoid indole alkaloid (TIA) biosynthetic pathway in *Catharanthus roseus*. Black boxes indicate enzymes upregulated by the SG9 motif-containing ERF transcription factor ORCA2. Blue boxes indicate enzymes upregulated by the SG9 motif-containing ERF transcription factor ORCA3. Red boxes indicate enzymes upregulated by both ORCA2 and ORCA3. Green boxes indicate enzymes upregulated by the BPF1, ORCA2 and ORCA3 transcription factors. Adapted from [178]. AS anthranilate synthase, CPR cytochrome P450 reductase, D4H deacetoxyvinodoline 4-hydroxylase, DAT acetyl-CoA:4-O-deacetylvinodoline 4-O-acetyltransferase, DXS D-1-deoxyxylulose 5-phosphate synthase, G10H geraniol 10-hydroxylase, LAMT loganic

(*Carica papaya*) and garlic mustard (*Alliaria petiolata*)) and the *Drypetes* genus [119, 124, 125].

The majority of the phytoalexins identified from the *Brassicaceae* are sulfur-containing indole alkaloids, which are derived from tryptophan with the sulfur atom typically derived from cysteine. At present, more than 44 cruciferous phytoalexins from at least six different structural classes have been identified [126], including camalexin (3-thiazol-2'-yl-indole; **O**, Fig. 6.3) and rapalexin A (**P**, Fig. 6.3). Camalexin is the major phytoalexin that accumulates in *Arabidopsis* in response to pathogens [127–129] and microbial elicitors [44, 130–132], and confers resistance to several necrotrophic pathogens, such as the black spot disease-causing fungi *Alternaria brassicicola* and the grey mold-causing *Botrytis cinerea* [128, 133, 134], and to the biotrophic, powdery mildew-causing fungus *Erysiphe pisi* [121]. In addition to camalexin, the phytoalexin rapalexin A (**P**, Fig. 6.3) has been detected in *Arabidopsis* [135].

The camalexin biosynthetic pathway in *Arabidopsis* has been fully elucidated recently [136] and involves three cytochrome P450 enzymes, CYP79B2/3, CYP71A13, and CYP71B15 (also known as PAD3), to transform tryptophan to camalexin [137] (Fig. 6.4c). CYP71A13 is a multifunctional cytochrome P450 that metabolizes the aldoxime to indole-3-acetonitrile (IAN) and then to a cysteine-IAN conjugate [134, 136]. CYP71B15 is also a multifunctional cytochrome P450 [138, 139] that catalyzes the conversion of the cysteine-IAN conjugate to dihydrocamalexin acid (DHCA) and then to camalexin (Fig. 6.4c) [140]. The paralog to the *CYP71A13* gene, the *CYP71A12* gene is induced highly in roots by the bacterial flagellin elicitor and is responsible for root-specific camalexin biosynthesis [132].

## 6.5 Regulators of Defense-Related Aromatic Alkaloid Metabolism

### 6.5.1 Jasmonates and Subgroup 3e-bHLHs in Plant Specialized Metabolism

Jasmonates (JAs) are plant defense signaling molecules that act as functionally conserved elicitors of a number of specialized metabolic pathways across the plant kingdom, including the TIA pathway in *Catharanthus roseus* and the indole alkaloid pathway in *Arabidopsis thaliana* (Fig. 6.4a, c) [141]. The core signaling module underlying JA-mediated regulation of specialized metabolism contains the Skp-Cullin-F-box-type (SCF) E3 ubiquitin ligase complex, its substrate the COI1 F-box protein and its degradation target proteins, the JA ZIM domain (JAZ) family of repressor proteins. The JAZ proteins also interact with COI1 and a broad array of transcription factors. In the presence of the bioactive JA-isoleucine (JA-Ile) conjugate, the JAZ and COI1 proteins directly interact and ultimately trigger the degradation of the JAZ proteins by the 26S proteasome pathway [142, 143]. JA-triggered

JAZ degradation then releases the bound transcription factors for subsequent regulation of specific sets of JA-responsive genes and specialized metabolites.

The bHLH transcription factors from subgroup 3e (SG3e) are the best known targets of the JAZ proteins and have been shown to directly and indirectly regulate transcriptional regulators in specialized metabolism. For example, the SG3e bHLH CrMYC2 has been shown to act upstream of the transcriptional activators ORCA2 and ORCA3 to positively regulate terpenoid indole alkaloid production in *Catharanthus roseus* (Fig. 6.4b) [144]. In addition, the SG3e bHLHs AtMYC2/AtbHLH06, AtMYC3/bHLH05/ATR2 and AtMYC4/bHLH04 have been shown to interact directly with all five SG12-R2R3-MYBs in *Arabidopsis thaliana* and redundantly activate the production of both aliphatic glucosinolates and indole glucosinolates in response to JA signaling (Fig. 6.4d) [145–147]. The same SG3e bHLHs are also likely to redundantly activate camalexin biosynthesis in response to JA signaling, possibly via the WRKY-type transcription factor AtWRKY33, so that a knockout of any one of the three SG3e bHLH genes upregulates the expression of the other two (Fig. 6.4d) [145–147].

### 6.5.2 Subgroup 9-ERFs in Terpenoid and Alkaloid Metabolism

In addition to the R2R3-MYBs and bHLHs [19, 149], other transcription factor classes have been characterized in the coordinated activation of specialized metabolic pathways in plant defense, such as the WRKYs [20], the NACs [148] and the ERFs. Among these, the ERF proteins from subgroup 9 (also known as subgroup B3) are most closely associated with JA-responsive terpenoid and alkaloid metabolism in plants [141, 150].

Characterized members of this subgroup (SG9-ERFs) include the JA-responsive *Catharanthus* ORCA proteins in synthesis of TIAs (see below; [151, 152]), the JA-responsive tobacco NtERF189 and the NtERF221/ORC1 proteins in the synthesis of the alkaloid nicotine [153, 154], and the JA-responsive *Artemisia* AaERF1 and AaERF2 proteins in the synthesis of the sesquiterpene artemisinin [155]. In addition to the possible conserved function in alkaloid and/or terpenoid biosynthesis, SG9-ERFs are also involved in the regulation of defense signaling pathways and the cross talk among the defense signaling hormones salicylic acid (A, Fig. 6.1), JA and ethylene [150, 156, 157]. For example, the tomato SG9-ERFs Pti4, Pti5, and Pti6 have been shown to interact with the tomato disease resistance protein Pto to induce transcriptional reprogramming of the plant cell [158]. In addition, they differentially integrate defense hormone signals in this transcriptional response [159]. Furthermore, chromatin immunoprecipitation (ChIP) experiments with Pti4 indicate that this transcription factor not only binds to a conserved target sequence (i.e., the GCC box) but also to non-GCC box elements, either directly or through interaction with other transcription factors, to regulate salicylic acid-induced genes [160], suggesting that some SG9-ERFs may bind to different *cis*-elements in the target promoters.

### 6.5.3 Regulators of the Terpenoid Indole Alkaloid Pathway

Terpenoid indole alkaloids (TIAs) are synthesized by a metabolic sequence that consists of two branch pathways, indole and monoterpenoid, that merge to form a single pathway with subsequent downstream branches (Fig. 6.4a). TIA biosynthesis in *Catharanthus roseus* is a complex, multi-step process that is regulated by at least twelve transcription regulators: seven putative activators (ORCA2, ORCA3, BPF1, CrMYC1, CrMYC2, CrWRKY1 and CrWRKY2) and five putative repressors (ZCT1, ZCT2, ZCT3, GBF1 and GBF2) (Fig. 6.4b). ORCA2 and ORCA3 are JA-responsive SG9-ERF transcription factors that are proposed to activate the same genes in the indole branch, different genes in the monoterpenoid branch, and partially overlapping genes in the downstream branches of the TIA pathway [151, 161] (Fig. 6.4a). In addition, both ORCA2 and ORCA3 are proposed to activate *Strictosidine synthase* (*STR*) gene expression by binding to the JA- and elicitor-responsive element (JERE) in the *STR* promoter [162, 163]. Overexpression of the *ORCA2* transgene leads to increased expression of the endogenous *ORCA2* and *ORCA3* genes, but not vice versa with the *ORCA3* transgene [161, 164]. BPF1 is also proposed to activate *STR* transcription by binding to a separate element in the *STR* promoter [151] (Fig. 6.4a). CrMYC1 and CrMYC2 are bHLH transcription factors that bind to G-box sites in the *STR* promoter region [144, 165], possibly competing with the G-box-binding transcriptional repressor proteins, GBF1 and GBF2 [166]. In addition, CrMYC2 has been shown to act upstream of ORCA2 and ORCA3, activating their transcription [144]. CrWRKY1 and CrWRKY2 are JA-responsive WRKY transcription factors that positively regulate expression of several genes involved in TIA biosynthesis [167, 168]. Overexpression of CrWRKY1 also leads to increased transcript levels of the TIA transcriptional repressors *ZCT1*, *ZCT2* and *ZCT3*, and decreased transcript levels of the TIA transcriptional activators *ORCA2*, *ORCA3* and *CrMYC2* [168]. Similarly, ORCA2 regulates *ZCT1*, *ZCT2* and *ZCT3*, but not *GBF1* or *GBF2* [161]. In contrast, overexpression of CrWRKY2 leads to increased expression of both specific TIA transcriptional activators (*ORCA2*, *ORCA3* and *CrWRKY1*) and repressors (*ZCT1* and *ZCT3*) [167]. The three zinc finger proteins, *ZCT1*, *ZCT2* and *ZCT3*, have been found to bind specifically to the *Tryptophan decarboxylase* (TDC) and *STR* promoters in vitro, inhibiting their activities [169].

### 6.5.4 Regulators of the Indole Glucosinolate and Camalexin Pathways

The proteins involved in the transcriptional regulation of indole glucosinolate biosynthesis in *Arabidopsis* are three R2R3-MYB transcription factors from subgroup 12 (SG12), AtMYB34/ATR1, AtMYB51/HIG1 and AtMYB122 [170–172]. Of the three, AtMYB51 has been shown to be primarily responsible for indole

glucosinolate production in shoots [171], by activating committed steps in the primary shikimate pathway prior to the formation of the aldoxime, and nearly every step in the indole glucosinolate biosynthetic pathway [172]. In addition, AtMYB51 expression is defense regulated and required for the synthesis of the indole glucosinolate metabolite 4-methoxyglucobrassicin (**A**, Fig. 6.3) in response to the bacterial flagellin elicitor [122] (Fig. 6.4c). Unlike the SG2-R2R3-MYBs, AtMYB51 orthologs and related SG12-R2R3-MYBs are present only in cruciferous plants (Fig. 6.2b).

Although a number of transcription factors from several different classes have been characterized as regulators of camalexin biosynthesis in *Arabidopsis*, the main defense-regulated transcription factor in camalexin biosynthesis is the WRKY transcription factor AtWRKY33. Similar to JA-mediated specialized metabolism, the core signaling module underlying AtWRKY33-mediated synthesis of camalexin involves the MAP kinase 4 (AtMPK4)-AtMKS1-AtWRKY33 trimeric complex in the nucleus. In response to pathogen infection and microbial elicitors, AtWRKY33 is directly phosphorylated by MAP kinases AtMPK3 and AtMPK6 and subsequently released from the trimeric complex in order to bind to the target promoters of camalexin biosynthetic genes [173–175]. Interestingly, a MPK3 homolog in *Catharanthus* was recently reported to function in the regulation of TIA accumulation [176]. In addition, AtWRKY33 has been shown to positively regulate the JA signaling pathway [177], by directly activating the expression of the JA-responsive SG9-ERF transcription factor AtORA59 and inhibiting the expression of a number of JAZ repressor proteins [175].

## 6.6 Conclusions

The study of defense-regulated plant specialized metabolism has a rich, decades-long history, originally focused on the characterization of each species' arsenal of compounds. Thanks to advances in mass spectrometry, crystallography, and functional genomics, researchers are finally able to study specialized metabolism as a whole, from defense signaling to transcription factors to the pathway enzymes. Now more than ever, the study of plant specialized metabolism offers nearly limitless avenues of inquiry, with the potential to engineer specialized metabolism for the benefit of both plants and humans.

## References

1. VanEtten HD, Mansfield JW, Bailey JA, Farmer EE (1994) Two classes of plant antibiotics: phytoalexins versus “phytoanticipins”. *Plant Cell* 6:1191–1192
2. Bjarnholt N, Rook F, Motawia MS, Cornett C, Jorgensen C, Olsen CE, Jaroszewski JW, Bak S, Boller BL (2008) Diversification of an ancient theme: hydroxynitrile glucosides. *Phytochemistry* 69:1507–1516

3. Chan EK, Rowe HC, Kliebenstein DJ (2010) Understanding the evolution of defense metabolites in *Arabidopsis thaliana* using genome-wide association mapping. *Genetics* 185:991–1007
4. Kuzina V, Nielsen JK, Augustin JM, Torp AM, Bak S, Andersen SB (2011) *Barbarea vulgaris* linkage map and quantitative trait loci for saponins, glucosinolate, hairiness and resistance to the herbivore *Phylloptreta nemorum*. *Phytochemistry* 72:188–198
5. Kleine S, Müller C (2011) Intraspecific plant chemical diversity and its relation to herbivory. *Oecologia* 166:175–186
6. Hogge LR, Reed DW, Underhill EW, Haughn GW (1988) HPLC separation of glucosinolates from leaves and seeds of *Arabidopsis thaliana* and their identification using thermospray liquid chromatography-mass spectrometry. *J Chromatogr Sci* 165:551–556
7. Kliebenstein DJ, Lambrix VM, Reichelt M, Gershenson J, Mitchell-Olds T (2001) Gene duplication in the diversification of secondary metabolism: tandem 2-oxoglutarate-dependent dioxygenases control glucosinolate biosynthesis in *Arabidopsis*. *Plant Cell* 13:681–693
8. Dixon RA (2001) Natural products and plant disease resistance. *Nature* 411:843–847
9. Wink M (1988) Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor Appl Genet* 75:225–233
10. Mohanta TK (2013) Plant metabolomics: missing link in next generation functional genomics era. *J Appl Biol Biotechnol* 1:1–10
11. Chen F, Tholl D, Bohlmann J, Pichersky E (2011) The family of terpene synthases in plants: a mid-sized family of genes for specialized metabolism that is highly diversified throughout the kingdom. *Plant J* 66:212–229
12. Abe I, Morita H (2010) Structure and function of the chalcone synthase superfamily of plant type III polyketide synthases. *Nat Prod Rep* 27:809–838
13. Nelson DR (2006) Cytochrome P450 nomenclature, (2004). *Methods Mol Biol* 320:1–10
14. Nelson DR, Schuler MA, Paquette SM, Werck-Reichhart D, Bak S (2004) Comparative genomics of rice and *Arabidopsis*. Analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and dicot. *Plant Physiol* 135:756–772
15. Nelson DR, Ming R, Alam M, Schuler MA (2008) Comparison of cytochrome P450 genes from six plant genomes. *Trop Plant Biol* 1:216–235
16. Maleck K, Levine A, Eulgem T, Morgan A, Schmid J, Lawton KA, Dangl JL, Dietrich RA (2000) The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat Genet* 26:403–410
17. Tao Y, Xie Z, Chen W, Glazebrook J, Chang HS, Han B, Zhu T, Zou G, Katagiri F (2003) Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* 15:317–330
18. Katagiri F (2004) A global view of defense gene expression regulation—a highly interconnected signaling network. *Curr Opin Plant Biol* 7:506–511
19. Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell* 15:165–178
20. Dong JX, Chen CH, Chen ZX (2003) Expression profiles of the *Arabidopsis* WRKY gene superfamily during plant defense response. *Plant Mol Biol* 51:21–37
21. Weng JK, Philippe RN, Noel JP (2012) The rise of chemodiversity in plants. *Science* 336:1667–1670
22. Chu HY, Wege E, Osbourn A (2011) From hormones to secondary metabolism: the emergence of metabolic gene clusters in plants. *Plant J* 66:66–79
23. Sticher L, Mauch-Mani B, Metraux JP (1997) Systemic acquired resistance. *Annu Rev Plant Pathol* 35:235–270
24. Dangl JL, Jones JD (2001) Plant pathogens and integrated defence responses to infection. *Nature* 411:826–833
25. Meuwly P, Molders W, Buchala A, Metraux JP (1995) Local and systemic biosynthesis of salicylic acid in infected cucumber plants. *Plant Physiol* 109:1107–1114
26. Coquoz JL, Buchala A, Metraux JP (1998) The biosynthesis of salicylic acid in potato plants. *Plant Physiol* 117:1095–1101

27. Peters NK, Frost JW, Long SR (1986) A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science* 233:977–980
28. Rolfe BG (1988) Flavones and isoflavones as inducing substances of legume nodulation. *Biofactors* 1:3–10
29. Begum AA, Leibovitch S, Migner P, Zhang F (2001) Specific flavonoids induced nod gene expression and pre-activated nod genes of *Rhizobium leguminosarum* increased pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.) nodulation in controlled growth chamber environments. *J Exp Bot* 52:1537–1543
30. Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W (2010) Lignin biosynthesis and structure. *Plant Physiol* 153:895–905
31. Vance CP, Kirk TK, Sherwood RT (1980) Lignification as a mechanism of disease resistance. *Annu Rev Phytopathol* 18:259–288
32. Freudenberg K (1968) In: Neish AC, Freudenberg K (eds) Constitution and biosynthesis of lignin. Springer, New York, pp 47–116
33. Lange BM, Lapierre C, Sandermann H Jr (1995) Elicitor-induced spruce stress lignin (structural similarity to early developmental lignins). *Plant Physiol* 108:1277–1287
34. Adams-Phillips L, Briggs AG, Bent AF (2010) Disruption of poly(ADP- ribosylation) mechanisms alters responses of *Arabidopsis* to biotic stress. *Plant Physiol* 152:267–280
35. Kishi-Kaboshi M, Okada K, Kurimoto L, Murakami S, Umezawa T, Shibuya N, Yamane H, Miyao A, Takatsujii H, Takahashi A, Hirochika H (2010) A rice fungal MAMP-responsive MAPK cascade regulates metabolic flow to antimicrobial metabolite synthesis. *Plant J* 63:599–612
36. Menden B, Kohlhoff M, Moerschbacher BM (2007) Wheat cells accumulate a syringyl-rich lignin during the hypersensitive resistance response. *Phytochemistry* 68:513–520
37. Bassard JE, Ulmann P, Bernier F, Werck-Reichhart D (2010) Phenolamides: bridging polyamines to the phenolic metabolism. *Phytochemistry* 71:1808–1824
38. Kaur H, Heinzel N, Schottner M, Baldwin IT, Gális I (2010) R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates, which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*. *Plant Physiol* 152:173–1747
39. Costet L, Fritig B, Kauffmann S (2002) Scopoletin expression in elicitor-treated and tobacco mosaic virus-infected tobacco plants. *Physiol Plant* 115:228–235
40. Schenke D, Böttcher C, Scheel D (2011) Crosstalk between abiotic ultraviolet-B stress and biotic (flg22) stress signalling in *Arabidopsis* prevents flavonol accumulation in favor of pathogen defence compound production. *Plant Cell Environ* 34:1849–1864
41. Chezem WR, Clay NK (2015) Novel gene regulatory complexes in phenylpropanoid metabolism for inducible defense metabolites and pathogen resistance. In preparation.
42. Kai K, Shimizu B, Mizutani M, Watanabe K, Sakata K (2006) Accumulation of coumarins in *Arabidopsis thaliana*. *Phytochemistry* 67:379–386
43. Chong J, Baltz R, Schmitt C, Beffia R, Fritig B, Saindrenan P (2002) Downregulation of a pathogen-responsive tobacco UDP-Glc:phenylpropanoid glucosyltransferase reduces scopoletin glucoside accumulation, enhances oxidative stress, and weakens virus resistance. *Plant Cell* 14:1093–1107
44. Shukla YN, Srivastava A, Kumar S, Kumar S (1999) Phytotoxic and antimicrobial constituents of *Argyreia speciosa* and *Oenothera biennis*. *J Ethnopharmacol* 67:241–245
45. Carpinella MC, Ferrayoli CG, Palacios SM (2005) Antifungal synergistic effect of scopoletin, a hydroxycoumarin isolated from *Melia azedarach* L fruits. *J Agric Food Chem* 53:2922–2927
46. Sun H, Wang L, Zhang B, Ma J, Hettenhausen C, Cao G, Sun G, Wu J, Wu J (2014) Scopoletin is a phytoalexin against *Alternaria alternata* in wild tobacco dependent on jasmonate signalling. *J Exp Bot* 65:4305–4315
47. Kodama O, Miyakawa J, Akatsuka T, Kiyosawa S (1992) Sakuranetin, a flavanone phytoalexin from ultraviolet-irradiated rice leaves. *Phytochemistry* 31:3807–3809

48. Nicholson RL, Kollipara SS, Vincent JR, Lyons PC, Cadena-Gomez G (1987) Phytoalexin synthesis by the sorghum mesocoyl in response to infection by pathogenic and nonpathogenic fungi. *Proc Natl Acad Sci U S A* 84:5520–5524
49. Snyder BA, Nicholson RL (1990) Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. *Science* 248:1637–1639
50. Zuther K, Kahnt J, Utemark J, Imkampe J, Uhse S, Schirawski J (2012) Host specificity of *Sporisorium reilianum* is tightly linked to generation of the phytoalexin luteolinidin by *Sorghum bicolor*. *Mol Plant Microbe Interact* 25:1230–1237
51. Dixon RA, Lamb CJ, Masoud S, Sewalt VJ, Paiva NL (1996) Metabolic engineering: prospects for crop improvement through the genetic manipulation of phenylpropanoid biosynthesis and defense responses—a review. *Gene* 179:61–71
52. Dixon RA, Steele CL (1999) Flavonoids and isoflavonoids—a gold mine for metabolic engineering. *Trends Plant Sci* 4:394–400
53. Grayer RJ, Kokubun T (2001) Plant-fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants. *Phytochemistry* 56:253–263
54. Cruickshank IAM, Perrin DR (1960) Isolation of a phytoalexin from *Pisum sativum* L. *Nature* 187:799–800
55. Cruickshank IAM, Perrin DR (1961) Studies on phytoalexins III: the isolation, assay, and general properties of a phytoalexin from *Pisum sativum* L. *Australian J Biol Sci* 14:336–348
56. Perrin DR (1964) Structure of phaseollin. *Tetrahedron Lett* 5:29–35
57. Burden RS, Bailey JA (1975) Structure of the phytoalexin from soybean. *Phytochemistry* 14:1389–1390
58. Ingham JL, Keen NT, Hymowitz T (1977) A new isoflavone phytoalexin from fungus-inoculated stems of *Glycine wightii*. *Phytochemistry* 16:1943–1946
59. Langcake P, Pryce RJ (1977) A new class of phytoalexins from grapevines. *Experientia* 33:151–152
60. Langcake P (1981) Disease resistance of *Vitis* spp and the production of the stress metabolites resveratrol, e-viniferin, a-viniferin, and pterostilbene. *Physiological Plant Pathol* 18:213–226
61. Coutos-Thevenot P, Poinsot B, Bonomelli A, Yean H, Breda C, Buffard D, Esnault R, Hain R, Boulay M (2001) *In vitro* tolerance to *Botrytis cinerea* of grapevine 41B rootstock in transgenic plants expressing the stilbene synthase *Vst1* gene under the control of a pathogen-inducible PR 10 promoter. *J Exp Bot* 52:901–910
62. Malacarne G, Vrhovsek U, Zulini L, Cestaro A, Stefanini M, Mattivi F, Delledonne M, Velasco R, Moser C (2011) Resistance to *Plasmopara viticola* in grapevine segregating population is associated with stilbenoid accumulation and with specific host transcriptional responses. *BMC Plant Biol* 11:114
63. Al R-P, Lamuela-Raventos RM, Andres-Lacueva C, de La Torre-Boronat MC (2001) Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. *J Agric Food Chem* 49:210–215
64. Vannozzi A, Dry IB, Fasoli M, Zenoni S, Lucchin M (2012) Genome-wide analysis of the grapevine stilbene synthase multigene family: genomic organization and expression profiles upon biotic and abiotic stresses. *BMC Plant Biol* 12:130
65. Sobolev VS (2013) Production of phytoalexins in peanut (*Arachis hypogaea*) seed elicited by selected microorganisms. *J Agric Food Chem* 61:1850–1858
66. Davin LB, Wang HB, Crowell AL, Bedgar DL, Martin DM, Sarkanen S, Lewis NG (1997) Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. *Science* 275:362–366
67. Denoux C, Galletti R, Mammarella N, Gopalan S, Werck-Reichhart D, DeLorenzo G, Ferrari S, Ausubel FM, Dewdney J (2008) Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. *Mol Plant* 1:423–445
68. König S, Feussner K, Kaever A, Landesfeind M, Thurow C, Karlovsky P, Gatz C, Polle A, Feussner I (2014) Soluble phenylpropanoids are involved in the defense response of *Arabidopsis* against *Verticillium longisporum*. *New Phytol* 202:823–837

69. Muranaka T, Miyata M, Ito K, Tachibana S (1998) Production of podophyllotoxin in *Juniperus chinensis* callus cultures treated with oligosaccharides and a biogenetic precursor. *Photochemistry* 49:491–496
70. Esmailzadeh Bahabadi S, Sharifi M, Behmanesh M, Safaei N, Murata J, Araki R, Yamagaki T, Satake H (2012) Time-course changes in fungal elicitor-induced lignan synthesis and expression of the relevant genes in cell cultures of *Linum album*. *J Plant Physiol* 169:487–491
71. Cespedes CL, Avila JG, Garcia AM, Becerra J, Flores C, Aqueveque P, Bittner M, Hoeneisen M, Martinez M, Silva M (2006) Antifungal and antibacterial activities of *Araucaria araucana* (Mol) K Koch heartwood lignans. *Z Naturforschung C* 61:35–43
72. Kozawa M, Baba K, Matsuyama Y, Kido T, Sakai M, Takemoto M (1982) Components of the root of *Anthriscus sylvestris* Hoffm. II Insecticidal activity. *Chem Pharm Bull* 30:2885–2888
73. Miyazawa M, Fukuyama M, Yoshi K, Kato T, Ishikawa Y (1999) Biological active components against *Drosophila melanogaster* from *Podophyllum hexandrum*. *J Agric Food Chem* 47:5108–5110
74. Rabinowicz PD, Braun EL, Bowen B, Grotewold E (1999) Maize R2R3 Myb genes: sequence analysis reveals amplification in higher plants. *Genetics* 153:427–444
75. Stracke R, Ishihara H, Huep G, Barsch A, Mehrrens F, Niehaus K, Weisshaar B (2007) Differential regulation of closely related R2R3-MYB transcription factors control flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. *Plant J* 50:660–677
76. Raes J, Rohde A, Christensen JH, Peer YY, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in *Arabidopsis*. *Plant Physiol* 133:1051–1071
77. Zhou J, Lee C, Zhong R, Ye ZH (2009) MYB58 and MYB63 are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in *Arabidopsis*. *Plant Cell* 21:248–266
78. Sugimoto K, Takeda S, Hirochika H (2000) MYB-related transcription factor NtMYB2 induced by wounding and elicitors is a regulator of the tobacco retrotransposon Tto1 and defense-related genes. *Plant Cell* 12:2511–2528
79. Maeda K, Kimura S, Demura T, Takeda J, Ozeki Y (2005) DcMYB1 acts as a transcriptional activator of the carrot phenylalanine ammonia-lyase gene (DcPAL1) in response to elicitor treatment, UV-B irradiation and the dilution effect. *Plant Mol Biol* 59:739–752
80. Gális I, Simek P, Narisawa T, Sasaki M, Horiguchi T, Fukuda H, Matsuoka K (2006) A novel R2R3 MYB transcription factor NtMYBJS1 is a methyl masmonate-dependent regulator of phenylpropanoid-conjugate biosynthesis in tobacco. *Plant J* 46:573–592
81. Onkokesung N, Gaquerel E, Kotkar H, Kaur H, Baldwin IT, Gális I (2012) MYB8 controls inducible phenolamide levels by activating three novel hydroxycinnamoyl-coenzyme A:polyamine transferases in *Nicotiana attenuata*. *Plant Physiol* 158:389–407
82. Shelton D, Stranne M, Mikkelsen L, Pakserescht N, Welham T, Hiraka H, Tabata S, Sato S, Paquette S, Wang TL, Martin C, Bailey P (2012) Transcription factors of lotus: regulation of isoflavanoid biosynthesis requires coordinated changes in transcription factor activity. *Plant Physiol* 159:531–547
83. Höll J, Vannozzi A, Czermel S, D'Onofrio C, Walker AR, Rausch T, Lucchin M, Boss PK, Cry IB, Bogs J (2013) The R2R3-MYB transcription factors MYB14 and MYB15 regulate stilbene biosynthesis in *Vitis vinifera*. *Plant Cell* 25:4135–4149
84. Chen Y, Zhang X, Wu W, Chen Z, Gu H, Qu LJ (2006) Overexpression of the wounding-responsive gene AtMYB15 activates the shikimate pathway in *Arabidopsis*. *Acta Bot Sinica* 48:1084–1095
85. Ogata K, Morikawa S, Nakamura H, Sekikawa A, Inoue T, Kanai H, Sarai A, Ishii S, Nishimura Y (1994) Solution structure of a specific DNA complex of the Myb DNA-binding domain with cooperative recognition helices. *Cell* 79:639–648
86. Kranz HD, Denekamp M, Greco R, Jin H, Leyva A, Meissner RC, Petroni K, Urzainqui A, Bevan M, Martin C, Smeekens S, Tonelli C, Paz-Ares J, Weisshaar B (1998) Towards functional characterisation of the members of the R2R3-MYB gene family from *Arabidopsis thaliana*. *Plant J* 16:263–276

87. Stracke R, Werber M, Weissshaar B (2001) The R2R3-MYB gene family in *Arabidopsis thaliana*. *Curr Opin Plant Biol* 4:447–456
88. Reichmann JL, Ratcliffe OJ (2000) A genomic perspective on plant transcription factors. *Curr Opin Plant Biol* 3:432–434
89. Du H, Feng BR, Yang SS, Huang YB, Tang YX (2012) The R2R3-MYB transcription factor gene family in maize. *PLoS One* 7, e37463
90. Gonzalez A, Zhao M, Leavitt JM, Lloyd AM (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. *Plant J* 53:814–827
91. Fornalé S, Shi X, Chai C, Encina A, Irar S, Capellades M, Fuguet E, Torres JL, Rovira P, Puigdoménech P, Rigau J, Grotewold E, Gray J, Caparrós-Ruiz D (2010) ZmMYB31 directly represses maize lignin genes and redirects the phenylpropanoid metabolic flux. *Plant J* 64:633–644
92. Cordell GA, Quinn-Beattie ML, Farnsworth NR (2001) The potential of alkaloids in drug discovery. *Phytother Res* 15:183–205
93. Memelink J, Gantet P (2007) Transcription factors involved in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. *Phytochem Rev* 6:353–362
94. Facchini PJ, De Luca V (2008) Opium poppy and Madagascar periwinkle: model non-model systems to investigate alkaloid biosynthesis in plants. *Plant J* 54:763–784
95. Facchini PJ, Huber-Allanach KL, Tari LW (2000) Plant aromatic L-amino acid decarboxylases: evolution, biochemistry, regulation and metabolic engineering applications. *Phytochemistry* 54:121–138
96. Vakil JR (1949) A clinical trial of *Rauwolfia serpentina* in essential hypertension. *Br Heart J* 11:350–355
97. Holland JF, Scharlau C, Gailani S, Krant MJ, Olson KB, Horton J, Shnider BI, Lynch JJ, Owens A, Carbone PP, Colsky J, Grob D, Miller SP, Hall TC (1973) Vincristine treatment of advanced cancer: a cooperative study of 392 cases. *Cancer Res* 33:1258–1264
98. Kang S, Kang K, Lee K, Back K (2007) Characterization of rice tryptophan decarboxylases and their direct involvement in serotonin biosynthesis in transgenic rice. *Planta* 227:263–272
99. Mayama S, Tani T, Matsuura Y, Ueno T, Fukami H (1981) The production of phytoalexins by oat in response to crown rust, *Puccinia coronata* f sp *avenae*. *Physiol Plant Pathol* 19:217–226
100. Collins FW (1989) Oat phenolics: avenanthramides, novel substituted N- cinnamoylanthrani- late alkaloids from oat groats and hulls. *J Agric Food Chem* 37:60–66
101. Crombie L, Mistry J (1990) The phytoalexins of oat leaves: 4H-3,1-benzoxazin-4- ones or amides? *Tetrahedron Lett* 31:2647–2648
102. Leete E, Marion L (1953) The hydrogenolysis of 3-hydroxymethylindole and other indole derivatives with lithium aluminium hydride. *Can J Chem* 31:775–784
103. Frey M, Schullehner K, Dick R, Riessmann A, Gierl A (2009) Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants. *Phytochemistry* 70: 1645–1651
104. Melanson D, Chilton MD, Master-Moore D, Chilton WS (1997) A deletion in an indole synthase gene is responsible for the DIMBOA-deficient phenotype of bxbx maize. *Proc Natl Acad Sci U S A* 94:13345–13350
105. Niemeyer HM (1988) Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones) defense chemicals in the Gramineae. *Phytochemistry* 27:3349–3358
106. Niemeyer HM (2009) Hydroxamic acids derived from 2-hydroxy-2H-1,4- benzoazin-3(4H)- one: key defense chemicals of cereals. *J Agric Food Chem* 57:1677–1696
107. Ahmad S, Veyrat N, Gordon-Weeks R, Zhang Y, Martin J, Smart L, Glauser G, Erb M, Flors V, Frey M, Ton J (2011) Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. *Plant Physiol* 157:317–327
108. Gross D, Lehmann H, Schütte HR (1974) Zur biosynthese des Gramins. *Biochem Physiol Pflanz* 166:281–287

109. Grün S, Frey M, Gierl A (2005) Evolution of the indole alkaloid biosynthesis in the genus *Hordeum*: distribution of gramine and DIBOA and isolation of the benzoxazinoid biosynthesis genes from *Hordeum lechleri*. *Phytochemistry* 66:1264–1272
110. Nelson D, Werck-Reichhart D (2011) A P450-centric view of plant evolution. *Plant J* 66:194–211
111. Hamberger B, Bak S (2013) Plant P450s as versatile drivers for evolution of species-specific chemical diversity. *Philos Trans R Soc Lond B Biol Sci* 368:20120426
112. Bak S, Paquette SM, Morant M, Morant AV, Saito S, Bjarnholt N, Zagrobelny M, Jørgensen K, Osmani S, Simonsen T, Perez RS, Heeswijk TB, Jørgensen B, Møller BL (2006) Cyanogenic glycosides: a case study for evolution and application of cytochrome P450. *Phytochem Rev* 5:309–329
113. Zagrobelny M, Bak S, Rasmussen AV, Jørgensen B, Naumann CM, Lindberg Møller B (2004) Cyanogenic glucosides and plant-insect interactions. *Phytochemistry* 65:293–306
114. Zagrobelny M, Bak S, Møller BL (2008) Cyanogenesis in plants and arthropods. *Phytochemistry* 69:1457–1468
115. Ito T, Kumazawa K (1995) Precursors of antifungal substances from cherry leaves (*Prunus yedonensis* Matsumura). *Biosci Biotechnol Biochem* 59:1944–1945
116. Morant AV, Jørgensen K, Jørgensen C, Paquette SM, Sanchez-Perez R, Møller BL, Bak S (2008) β-glucosidases as detonators of plant chemical defense. *Phytochemistry* 69:1795–1813
117. Fahey JW, Zalcmann AT, Talalay P (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5–51
118. Halkier BA, Gershenson J (2006) Biology and biochemistry of glucosinolates. *Annu Rev Plant Biol* 57:303–333
119. Saupe SG (1981) Cyanogenesis and angiosperm phylogeny. In: Young DA, Siegler DS (eds) *Phytochemistry and angiosperm phylogeny*. Praeger, New York, pp 80–116
120. Kim JH, Jander G (2007) *Myzus persicae* (green peach aphid) feeding on *Arabidopsis* induces the formation of a deterrent indole glucosinolates. *Plant J* 49:1008–1019
121. Müller R, de Vos M, Sun JY, Sønderby IE, Halkier BA, Wittstock U, Jander G (2010) Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *J Chem Ecol* 36:905–913
122. Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, Molina A, Schulze-Lefert P (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum anti-fungal defense. *Science* 323:101–106
123. Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate metabolites required for an *Arabidopsis* innate immune response. *Science* 323:95–101
124. Bennett RN, Kiddie G, Walls-grove RM (1997) Biosynthesis of benzylglucosinolate, cyanogenic glucosides and phenylpropanoids in *Carica papaya*. *Phytochemistry* 45:59–66
125. Frisch T, Møller BL (2011) Possible evolution of alliarinoid biosynthesis from the glucosinolate pathway in *Alliaria petiolata*. *FEBS J* 279:1545–1562
126. Pedras MS, Yaya EE, Glawischnig E (2011) The phytoalexins from cultivated and wild crucifers: chemistry and biology. *Nat Prod Rep* 28:1381–1405
127. Tsuji J, Jackson EP, Gage DA, Hammerschmidt R, Somerville SC (1992) Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive reaction to *Pseudomonas syringae* pv *syringae*. *Plant Physiol* 98:1304–1309
128. Thomma BP, Nelissen I, Eggermont K, Broekaert WF (1999) Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. *Plant J* 19:163–171
129. Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM (2003) *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J* 35:193–205
130. Qutob D, Kemmerling B, Brunner F, Kufner I, Engelhardt S, Gust AA, Luberacki B, Seitz HU, Stahl D, Rauhut T, Glawischnig E, Schween G, Lacombe B, Watanabe N, Lam E, Schlichting R, Scheel D, Nau K, Dodt G, Hubert D, Gijzen M, Nurnberger T (2006)

- Phytotoxicity and innate immune responses induced by Nep1-like proteins. *Plant Cell* 18:3721–3744
131. Gust AA, Biswas R, Lenz HD, Rauhut T, Ranf S, Kemmerling B, Gotz F, Glawischnig E, Lee J, Felix G, Nurnberger T (2007) Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in *Arabidopsis*. *J Biol Chem* 282:32338–32348
132. Millet YA, Danna CH, Clay NK, Songnuan W, Simon MD, Werck-Reichhart D, Ausubel FM (2010) Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. *Plant Cell* 22:973–990
133. Kliebenstein DJ, Rowe HC, Denby KJ (2005) Secondary metabolites influence *Arabidopsis/Botrytis* interactions: variation in host production and pathogen sensitivity. *Plant J* 44:25–36
134. Nafisi M, Goregaoker S, Botanga CJ et al (2007) *Arabidopsis* cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. *Plant Cell* 19:2039–2052
135. Pedras MSC, Adio AM (2008) Phytoalexins and phytoanticipins from the wild crucifers *Thellungiella halophila* and *Arabidopsis thaliana*: rapalexin A, wasalexins and camalexin. *Phytochemistry* 69:889–893
136. Klein AP, Anarat-Cappillino G, Sattely ES (2013) Minimum set of cytochromes P450 for reconstituting the biosynthesis of camalexin, a major *Arabidopsis* antibiotic. *Angew Chem Int Ed Engl* 52:13625–13628
137. Rahut T, Glawischnig E (2009) Evolution of camalexin and structurally related indolic compounds. *Phytochemistry* 70:1638–1644
138. Schuhegger R, Nafisi M, Mansourova M, Petersen BL, Olsen CE, Svatos A, Halier BA, Glawischnig E (2006) CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. *Plant Physiol* 141:1248–1254
139. Zhou N, Tootle TL, Glazebrook J (1999) *Arabidopsis PAD3*, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. *Plant Cell* 11:2419–2428
140. Böttcher C, Westphal L, Schmotz C, Prade E, Scheel D, Glawischnig E (2009) The multi-functional enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) converts cysteine-indole-3-acetonitrile to camalexin in the indole-3-acetonitrile network of *Arabidopsis thaliana*. *Plant Cell* 21:1830–1845
141. De Geyter N, Gholami A, Goormachtig S, Goossens A (2012) Transcriptional machineries in jasmonate-elicited plant secondary metabolism. *Trends Plant Sci* 17:349–359
142. Fonseca S, Chico JM, Solano R (2009) The jasmonate pathway: the ligand, the receptor and the core signaling module. *Curr Opin Plant Biol* 12:539–547
143. Pauwels L, Goossens A (2011) The JAZ proteins: a crucial interface in the jasmonate signalling cascade. *Plant Cell* 23:3089–3100
144. Zhang H, Hedhili S, Montiel G, Zhang Y, Chatel G, Pre M, Gantet P, Memlink J (2011) The basic helix-loop-helix transcription factor CrMYC2 controls the jasmonate-responsive expression of the ORCA genes that regulate alkaloid biosynthesis in *Catharanthus roseus*. *Plant J* 67:61–71
145. Dombrecht B, Xue GP, Sprague SJ, Kirkegaard JA, Ross JJ, Reid JB, Fitt GP, Sewelam N, Schenk PM, Manners MJ, Kazan K (2007) MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. *Plant Cell* 19:2225–2245
146. Schweizer F, Fernández-Calvo P, Zander M, Diez-Díaz M, Fonseca S, Glauser G, Lewsey MG, Ecker JR, Solano R, Reymond P (2013) *Arabidopsis* basic helix-loop-helix transcription factors MYC2, MYC3, and MYC4 regulate glucosinolate biosynthesis, insect performance, and feeding behavior. *Plant Cell* 25:3117–3132
147. Frerigmann H, Berger B, Gigolashvili T (2014) bHLH05 is an interaction partner of MYB51 and a novel regulator of glucosinolate biosynthesis in *Arabidopsis*. *Plant Physiol* 166:349–369
148. Delessert C, Kazan K, Wilson IW, van der Straeten D, Manners J, Dennis ES, Doferus R (2005) The transcription factor ATAF2 represses the expression of pathogenesis-related genes in *Arabidopsis*. *Plant J* 43:745–757

149. Anderson JP, Badruzaufari E, Schenk PM, Manners JM, Desmond OJ, Ehler C, Maclean DJ, Ebert PR, Kazan K (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modelates defense gene expression and disease resistance in *Arabidopsis*. *Plant Cell* 16:3460–3479
150. Gutterson N, Reuber TL (2004) Regulation of disease resistance pathways by AP2/ERF transcription factors. *Curr Opin Plant Biol* 7:465–471
151. van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. *Science* 289:295–297
152. van der Fits L, Memelink J (2001) The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. *Plant J* 25:43–53
153. Shoji T, Kajikawa M, Hashimoto T (2010) Clustered transcription factor genes regulate nicotine biosynthesis in tobacco. *Plant Cell* 22:3390–3409
154. De Sutter V, Vanderhaeghen R, Tillemen S, Lammertyn F, Vanhoutte I, Karimi M, Inzé D, Goossens A, Hilson P (2005) Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. *Plant J* 44:1065–1076
155. Yu ZX, Li JX, Yang CQ, Hu WL, Wang LJ, Chen XY (2012) The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in *Artemisia annua* L. *Mol Plant* 5:353–365
156. McGrath KC, Dombrecht B, Manners JM, Schenk PM, Edgar CI, Maclean DJ, Scheible WR, Udvardi MK, Kazan K (2005) Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of *Arabidopsis* transcription factor gene expression. *Plant Physiol* 139:949–959
157. Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiol* 140:411–432
158. Zhou J, Tang X, Martin GB (1997) The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. *EMBO J* 16:3207–3218
159. Gu YQ, Wildermuth MC, Chakravarthy S, Loh YT, Yang C, He X, Han Y, Martin GB (2002) Tomato transcription factors pti4, pti5, and pti6 activate defense responses when expressed in *Arabidopsis*. *Plant Cell* 14:817–831
160. Chakravarthy S, Tuori RP, D'Ascenzo MD, Robert PR, Despres C, Martin GB (2003) The tomato transcription factor Pti4 regulates defense-related gene expression via GCC box and non-GCC box *cis* elements. *Plant Cell* 15:3033–3050
161. Li CY, Leopold AL, Sander GW, Shanks JV, Zhao L, Gibson SI (2013) The ORCA2 transcription factor plays a key role in regulation of the terpenoid indole alkaloid pathway. *BMC Plant Biol* 13:155
162. Menke FLH, Champion A, Kijne JW, Memelink J (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. *EMBO J* 18:4455–4463
163. van der Fits L, Zhang H, Menke FLH, Deneka M, Memelink J (2000) A *Catharanthus roseus* BPF-1 homologue interacts with an elicitor-responsive region of the secondary metabolite biosynthetic gene Str and is induced by elicitor via a JA-independent signal transduction pathway. *Plant Mol Biol* 44:675–685
164. Peebles CAM, Hughes EH, Shanks JV, San KY (2009) Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time. *Metab Eng* 11:76–86
165. Chatel G, Montiel G, Metal P (2003) CrMYC1, a *Catharanthus roseus* elicitor- and jasmonate-responsive BHLH transcription factor that binds the G-box element of the strictosidine synthase gene promoter. *J Exp Bot* 54:2587–2588
166. Sibéry Y, Benhamra S, Memelink J, Giglioli-Guivaré N, Thiersault M, Boisson B, Doireau P, Ganet P (2001) *Catharanthus roseus* G-box binding factors 1 and 2 act as repressors of strictosidine synthase gene expression in cell cultures. *Plant Mol Biol* 45:477–488

167. Suttipanta N (2011) Characterization of G10H promoter and isolation of WRKY transcription factors involved in *Catharanthus* terpenoid indole alkaloid biosynthesis pathway, Ph.D. thesis. University of Kentucky, Plant Physiology Department, Lexington
168. Suttipanta N, Pattanaik S, Kulshrestha M, Patra B, Singh SK, Yuan L (2011) The transcription factor CrWRKY1 positively regulates the monoterpenoid indole alkaloids biosynthesis in *Catharanthus roseus*. *Plant Physiol* 157:2081–2093
169. Pauw B, Hilliou FA, Martin VS, Chatel G, de Wolf CJ, Champion A, Pré M, van Duijn B, Kijne JW, vand der Fits L, Memelink J (2004) Zinc finger proteins act as transcriptional repressors of alkaloid biosynthesis genes in *Catharanthus roseus*. *J Biol Chem* 279:52940–52948
170. Celenza JL, Quiel JA, Smolen GA, Merrikh H, Silvestro AR, Normanly J, Bender J (2005) The *Arabidopsis* ATR1 Myb transcription factor controls indolic glucosinolate homeostasis. *Plant Physiol* 137:253–262
171. Frerigmann H, Gigolashvili T (2014) MYB34, MYB51, and MYB122 distinctly regulate indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. *Mol Plant* 7:814–828
172. Gigolashvili T, Berger B, Mock HP, Müller C, Weisshaar B, Flügge UI (2007) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. *Plant J* 50:886–901
173. Andreasson E, Jenkins T, Brodersen P, Thorgrimsen S, Petersen NHT, Zhu S, Qiu JL, Micheelsen P, Rocher A, Petersen M, Newman MA, Björn Nielsen H, Hirt H, Somssich I, Mattsson O, Mundy J (2005) The MAP kinase substrate MKS1 is a regulator of plant defense responses. *EMBO J* 24:2579–2589
174. Qiu JL, Fül B, Petersen K, Nielsen HB, Botanga CJ, Thorgrimsen S, Palma K, Suarez-Rodriguez MC, Sandbech-Calusen S, Lichota J, Brodersen P, Grasser KD, Mattsson O, Glazebrook J, Mundy J, Petersen M (2008) *Arabidopsis* MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. *EMBO J* 27:2214–2221
175. Birkenbihl RP, Diezel C, Somssich IE (2012) *Arabidopsis* WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection. *Plant Physiol* 159:266–285
176. Raina S, Wankhede D, Jaggi M, Singh P, Jalmi S, Raghiram B, Sheikh A, Sinha A (2012) CrMPK3, a mitogen activated protein kinase from *Catharanthus roseus* and its possible role in stress induced biosynthesis of monoterpenoid indole alkaloids. *BMC Plant Biol* 12:134
177. Zheng Z, Qamar SA, Chen Z, Mengiste T (2006) *Arabidopsis* WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. *Plant J* 48:592–605
178. Memelink J, Verpoorte R, Kijne JW (2001) ORCANization of jasmonate-responsive gene expression in alkaloid metabolism. *Trends Plant Sci* 6:212–219

# **Chapter 7**

## **Metabolism of Glucosinolates and Their Hydrolysis Products in Insect Herbivores**

**Verena Jeschke, Jonathan Gershenson, and Daniel Giddings Vassão**

**Abstract** The glucosinolates produced by plants of the order Brassicales are part of a potent activated chemical defense system. These nitrogen- and sulfur-containing glucosides are hydrolyzed by myrosinases upon tissue damage, forming a toxic mixture of compounds consisting mostly of the corresponding isothiocyanates and nitriles. While humans find these compounds pleasantly spicy and beneficial to health, many of them are noxious and deterrent towards microorganisms and insect herbivores. Nonetheless, ingenious and efficient biochemical mechanisms employed by several insect herbivores enable these to feed on glucosinolate-producing plants, circumventing the effects of these plant defenses. Here, we summarize some of the counteradaptations utilized by insects to overcome the defense imposed by these compounds and their hydrolysis products. Insects can divert hydrolysis to less toxic products or desulfate the parent glucosinolates to preclude them from being hydrolyzed by myrosinases. Once hydrolysis occurs, toxic electrophilic hydrolysis products can be conjugated to glutathione and various amino acids. Another insect strategy is the rapid sequestration of ingested glucosinolates to prevent hydrolysis and allow them to be used in their own defense.

**Keywords** Activated plant defense • Brassicales • Herbivory • Glucosinolate • Myrosinase • Isothiocyanate • Mustard oil bomb • Isothiocyanate toxicity • Insect development • Specialized herbivore • Chewing insect • Nitrile specifier protein • Nitrile • Cyanide • Sulfatase • Desulfo-glucosinolate • Generalist herbivore • Xenobiotic detoxification • General detoxification • Glutathione-S-transferase • Dithiocarbamate • Mercapturic acid pathway • Sucking insect • Indole-3-carbinol • Sequestration • Hemolymph • Insect defense

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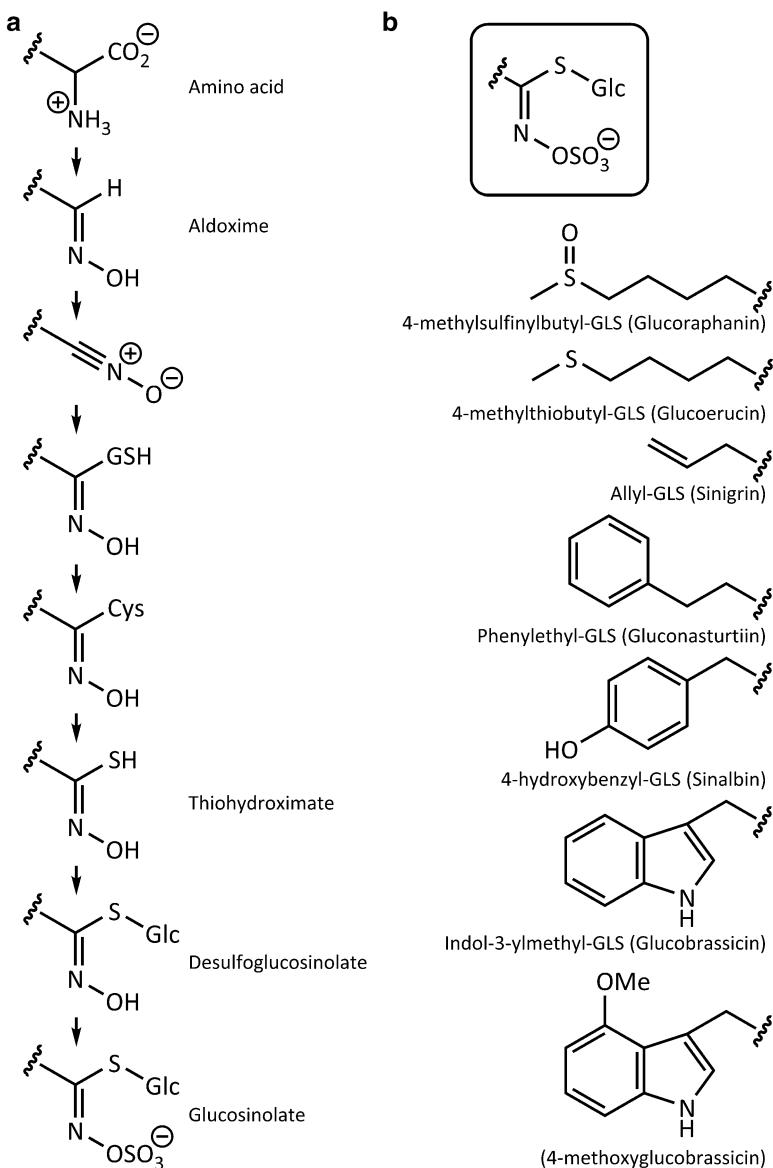
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## 7.1 Introduction

Plant species are often under pressure from herbivores causing extensive damage and loss of biomass. Alongside the production of physical defensive structures such as trichomes, thorns, and waxy surfaces, an extraordinary diversity of plant secondary metabolites (PSM), also known as specialized metabolites, is crucial to the ability of plants to resist herbivory. Many PSM either show toxic properties and/or act as feeding deterrents against herbivores [1–5]. For example, the chemical defenses of the well-studied herbaceous species *Arabidopsis thaliana* comprise an array of structurally different PSM with varying putative targets, including sulfur-containing glucosinolates, phenolics, and terpenoid volatiles [1, 6]. Many plant defenses are present constitutively before an attack has taken place, while others are produced in large quantities only after initial pathogen or herbivore contact. Furthermore, activated plant chemical defenses are produced and accumulate as pro-toxins in the intact plant material, and only fulfill a defensive role after being converted into the corresponding toxic components. These compounds, also referred to as phytoanticipins, include benzoxazinoids, cyanogenic glucosides, and glucosinolates that can be stored at high concentrations *in planta* without ill effects, but can be quickly activated to generate formidable toxic chemical weapons on demand.

The glucosinolate-myrosinase system found in plants of the order Brassicales is one of the best-studied activated plant chemical defense systems. It is present in oilseed rape (Canola) and agriculturally important vegetable crops such as cabbage, Brussels sprouts, and broccoli, and well known for conferring the characteristic pungency to horseradish, wasabi, and mustard. The glucosinolates comprise a very large and diverse chemical family of metabolites [7]. The most recent review reports 132 structures [8], although another review from 2010, with less rigorous standards of proof, lists a larger number [9]. Glucosinolates share a common chemical backbone consisting of an S-glycosylated thiohydroximate sulfate ester with a variable side chain (Fig. 7.1) typically deriving from one of eight proteinogenic amino acids [7, 10, 11]. Thus, glucosinolates are often broadly grouped according to the characteristics of their parental amino acids: benzenic (derived from tyrosine or phenylalanine), aliphatic (derived from methionine, leucine, isoleucine, valine, or alanine), and indolic (derived from tryptophan) glucosinolates. The diversity of structures is amplified by modifications of the amino acid side chain prior to the core structure biosynthesis (e.g., chain elongation), or later by further functionalization (e.g., hydroxylation, oxygenation, benzoylation). The enzymes and genes involved in the biosynthesis of glucosinolates are mostly elucidated [10–14], which has allowed the engineering and use of transgenic plants with altered and defined glucosinolate profiles for controlled feeding studies and bioassays [15–18]. The biosynthesis of glucosinolates has arisen twice during plant evolution, i.e., in the mustard clade and the unrelated genus *Drypetes* (Euphorbiaceae) [19], possibly via recruitment of biosynthetic precursors and enzymes involved in formation of cyanogenic glucosides. Based on phylogenetic analyses within the Brassicales, it has been proposed that benzenic and branched-chain amino acid-derived glucosinolates appeared first during the evolution of glucosinolate biosynthesis (with these amino acids being also

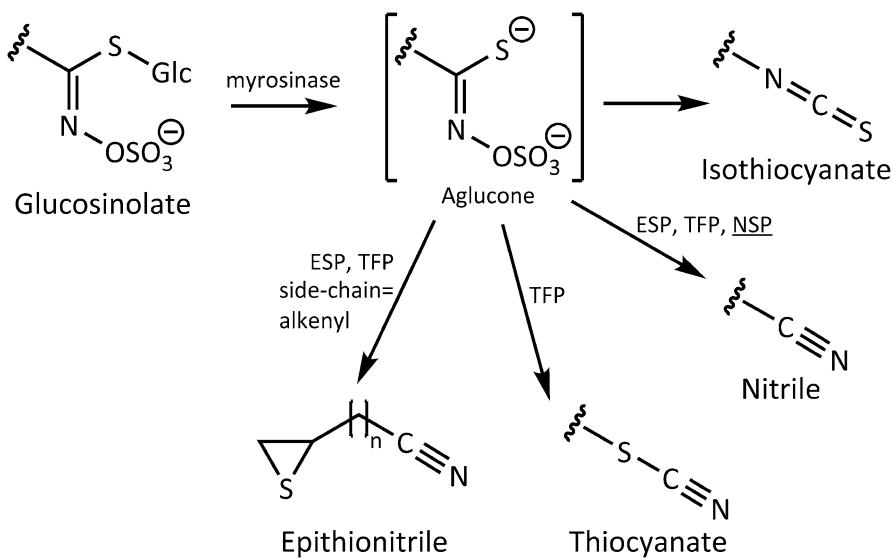


**Fig. 7.1** (a) Simplified scheme of the biosynthesis of glucosinolates from proteinogenic amino acids. Further steps of chain elongation, oxidation, and esterification contribute to the chemical diversity reported within this compound class. (b) Examples of side chains of major glucosinolates present in several Brassicaceae species

precursors of most cyanogenic glucosides), followed by Trp-derived indolic glucosinolates, and more recently by Met-derived glucosinolates [20]. Interestingly, indolic glucosinolates can also serve as precursors and share biosynthetic steps with a series of crucifer phytoalexins that act independently from myrosinase and have been implicated in defensive interactions against fungi [21–23].

The resulting glucosinolate profile is characteristic for each plant species. However, the glucosinolate content and composition can vary among different accessions of the same species, and among individuals, plant organs, developmental stages, and with environmental factors [7, 10, 24–27]. Additionally, herbivory (or other mechanical damage) can lead to dramatically increased levels of glucosinolate accumulation, especially of indolic glucosinolates [28–30]. Glucosinolates can be unevenly distributed within individual plant organs [31], and this can also influence how these compounds are perceived and what their effects are. For example, surface glucosinolates are thought to influence ovipositing female moths [25, 32–35] and act as feeding stimulants [36–39] in some specialist species, while young generalist caterpillars avoid feeding on leaf regions containing highest constitutive or induced glucosinolate levels [31, 40]. Nevertheless, most of the purported biological activities of glucosinolates emerge only after enzymatic activation by plant myrosinases, a class of  $\beta$ -thioglucoside glucohydrolases (EC 3.2.1.147). The glucosinolate-myrosinase system is a two-compartment system that is activated upon disruption of the natural tissue organization and compartment separation of intact plant tissues, such as by chewing herbivores [41, 42]. In *A. thaliana*, sulfur-rich cells (S-cells) in close proximity to the phloem are thought to be the sites of in vivo glucosinolate storage [43], whereas myrosinase is stored in idioblasts known as myrosin cells and in guard cells [44, 45]. Upon cell disruption, the “mustard oil bomb” is detonated: myrosinases come into contact with their substrate and cleave the glucose moiety of glucosinolates, yielding an unstable aglucone which rearranges spontaneously into an array of toxic hydrolysis and breakdown products. The composition of this toxic cocktail depends on the presence of specifier proteins, as well as pH, metal cofactors, plant organ, developmental stage, and environmental factors (Fig. 7.2) [42, 46–53]. The most characteristic hydrolysis products are the very reactive isothiocyanates (ITCs) that are readily formed by the spontaneous Lossen rearrangement of the unstable aglucone intermediate. Alternative breakdown products include epithionitriles, simple nitriles, thiocyanates, and others, depending in part on the side chain of the corresponding parent glucosinolate [46, 54, 55]. Alone or in combination, these hydrolysis products are responsible for most of the reported anti-herbivory effects of the mustard oil bomb.

Glucosinolates and their corresponding hydrolysis products have purported defensive and deterrent properties against birds, rabbits, gastropod mollusks, non-adapted insects, and plant pathogens [15, 16, 22, 41, 56–63]. The ITCs formed upon hydrolysis are thought to be the compounds most responsible for the toxic effects observed in a variety of organisms after ingestion of glucosinolate-containing plants, such as delays in growth and development, and even death [6, 15, 16, 63–68]. The toxicity of ITCs is attributed to their electrophilic and relatively lipophilic natures. These characteristics allow them to cross cellular membranes and reach the



**Fig. 7.2** Hydrolytic activation of glucosinolates by myrosinases (“mustard oil bomb”). Rearrangement of the unstable aglucone is under the influence of several additional factors that lead predominantly to formation of the corresponding isothiocyanates and nitriles

intracellular environment, and they also make them reactive towards nucleophiles such as GSH and some amino acid residues of proteins (mostly cysteines and lysines) at most physiological pHs [69, 70], but not towards DNA or RNA [71]. Covalent reaction with ITCs can then result in disturbances to the function and structure of those proteins, leading to loss/alteration of enzymatic capabilities or initiation of stress-related signaling cascades [70, 72–75]. The non-ITC hydrolysis products do not seem to play such an obvious role in direct defense, but their biological functions are not yet fully understood. Simple nitriles, for example, typically have lower direct toxicity than the corresponding ITCs [6, 51, 76]. There is increasing evidence that these may be involved in the indirect defense of Brassicaceae, attracting parasitoids of lepidopteran larvae [6, 46, 76, 77]. However, nitriles and epithionitriles do not seem to have a strong signaling function for the aphid parasitoid *Diaeretiella rapae* that reacts more strongly to the corresponding ITCs when searching for aphid-infested plants [78, 79]. Furthermore, the resulting biological activity can also depend on the rate of release of the hydrolysis products [64], and also in part on the side chain structure leading to alternative hydrolysis products [16, 80]. For example, glucosinolates containing allylic side chains have been reported to form epithionitriles after hydrolytic activation. The formation of thiocyanates has only been observed with benzylglucosinolate, allylglucosinolate, and 4-methylthiobutylglucosinolate in the presence of thiocyanate-forming proteins (TFP) [47, 49, 81]. In most cases, however, the biological activities of these more unusual glucosinolate hydrolytic products have not yet been thoroughly investigated.

The diversity of glucosinolates and their corresponding hydrolysis products limits the growth of a rather wide range of herbivores [16, 26, 80, 82, 83], but various glucosinolate types are reported to have different effects depending on the herbivore. It is thought that generalist chewing herbivores (such as the tobacco hornworm *Manduca sexta* and the cabbage looper *Trichoplusia ni*) are primarily affected by aliphatic glucosinolates, which form mostly ITCs upon hydrolysis; nevertheless, both aliphatic and indolic glucosinolates affect the development and growth of the generalist beet armyworm *Spodoptera exigua* [16]. Other studies showing the differential effects of aliphatic and indolic glucosinolates using transgenic plants demonstrate that growth and development of the generalists *Spodoptera littoralis* (African cotton leafworm) and *Mamestra brassicae* (cabbage moth) improved when feeding on plants devoid of indolic glucosinolates [58] and aliphatic glucosinolates [15], respectively. The lack of aliphatic glucosinolates was also reported to similarly affect feeding by the specialist diamondback moth *Plutella xylostella* and the generalist cotton bollworm *Helicoverpa armigera* [36]. Thus, both types of glucosinolates play a role in the defense against generalist and specialist chewing herbivores, with ITC-forming aliphatic and benzenic glucosinolates possibly having stronger effects. In contrast, sucking insects such as aphids do not cause as much tissue damage while feeding as chewing insects, and are consequently thought to rarely come into contact with the “mustard oil bomb” *per se*. Indolic glucosinolates, which are less stable and form breakdown products even without myrosinase activity, may thus play particularly important roles in the defense against aphids [28, 29, 82]. These feeding behaviors of both chewing and sucking herbivores additionally reflect the specific distribution and induction of the types of glucosinolates as mentioned above [26, 31, 40, 84]. Taking all of this into account, the glucosinolate-myrosinase system can be seen as a dynamic defensive apparatus responding to various endogenous and exogenous signals and offering strong and complex protection against insect herbivores. The effectiveness of the system has been shown in numerous studies using artificial diets supplemented with pure ITCs in bioassays as well as by comparing wild-type and glucosinolate-deficient transgenic plants [16, 18, 29, 64], with the toxicity of some isothiocyanates being equivalent to that of commercial insecticides [65].

Despite the presence of glucosinolates and other PSM, herbivores can be observed to feed readily on chemically defended plants without ill effects. Several counteradaptation strategies of insect herbivores to plant defense metabolites are known [85–89], and have been intensively studied since they often underlie the resistance of insects to commercial insecticides [90–92]. Based on the efficiency of its biochemical and/or behavioral adaptations towards toxins, a phytophagous insect may be able to cope and thus feed on a wide range of host plants (a generalist herbivore). These insects can be expected to rely more on general detoxification mechanisms when faced with glucosinolates and their hydrolysis products, including differentially inducible detoxification enzymes and nonspecific responses. In contrast, insects whose adaptations allow them to circumvent one group of toxins may become specialized (specialist herbivores) to plant families or species that are defended by these toxins.

The complexity of the glucosinolate-myrosinase system as an activated defense allows for different strategies for avoiding the toxic effects of the hydrolysis products: (a) the activation of the glucosinolates can be stopped by preemptively diverting the glucosinolate substrate to a compound that cannot be activated or by interfering with the myrosinase activity; (b) if toxic isothiocyanates are formed by hydrolysis, their harmful effects can be relieved by spontaneous chemical or enzymatic metabolism of the hydrolysis products; (c) herbivores can feed in a way that avoids the “detonation” of the mustard oil bomb. Once herbivores can feed on glucosinolate plants without being poisoned, they may even exploit parent glucosinolates or hydrolysis products as kairomones (signals) for feeding and oviposition [36, 38, 39, 77, 93–95]. Some specialist glucosinolate-feeding herbivores take further advantage of this defense system by selectively accumulating glucosinolates for their own defense [96–99].

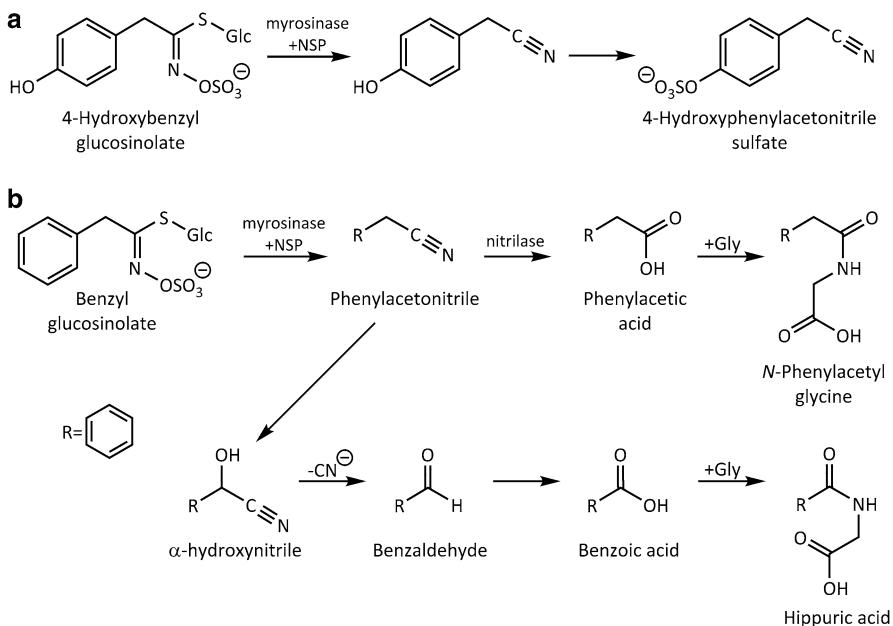
In the next sections, we will discuss the known biochemical strategies of insect herbivores in avoiding or adapting to the glucosinolate-myrosinase system. These include specialized biochemical reactions that alter (Sect. 7.2.1) or even prevent (Sect. 7.2.2) glucosinolate hydrolysis, or that mediate the metabolism of the toxic products formed after hydrolysis (Sects. 7.2.3 and 7.2.4). Additionally, we will examine the sequestration and use of these compounds by insects (Sect. 7.3).

## 7.2 Biochemical Detoxification of Glucosinolates

While crucifer-feeding specialists are typically not negatively affected by host plant glucosinolates [16], and may even prefer to feed on glucosinolate-containing plants [35–37, 84, 100–106], they are indeed sensitive to isothiocyanates [64, 67, 107]. Nevertheless, these insects feed successfully on important crops such as cabbage, broccoli, cauliflower, rapeseed, and mustard and represent major agricultural pests, suggesting the presence of mechanisms to avoid formation of the toxic isothiocyanates upon ingestion. In the next two sections, we discuss two well-studied specialized adaptations to feeding on glucosinolate-containing plants [108–111]: a protein that interferes with the hydrolysis of glucosinolates by redirecting the breakdown of the aglucone and an enzyme that modifies the glucosinolate yielding a product that is not a myrosinase substrate.

### 7.2.1 *Specifier Proteins: Deflecting the Toxic Mustard Oil Bomb*

The close relationship between herbivorous Pierid butterflies and plants of the order Brassicales is a prime example of the so-called *coevolutionary arms race* between lepidopterans and angiosperms leading to increased speciation and chemical diversity [112]. It has been estimated that ancestral Pierinae developed biochemical



**Fig. 7.3** Nitrile-specifier protein (NSP)-assisted detoxification of glucosinolates in *Pieris rapae* via redirection of the aglucone to less toxic nitriles. **(a)** The phenolic moiety of 4-hydroxyphenylacetonitrile is sulfated. **(b)** Phenylacetonitrile, on the other hand, is converted into the corresponding carboxylic acid (presumably by a nitrilase) and undergoes conjugation to glycine. Alternatively, oxygenation of its benzylic carbon leads to elimination of a C<sub>1</sub> unit as cyanide, with the resulting benzaldehyde being oxidized to benzoic acid and conjugated, e.g., to glycine to form hippuric acid

adaptations that enabled them to feed on glucosinolate-containing plants approximately 10 million years after the evolution of the glucosinolate-myrosinase system in plants of the Brassicales [110, 113]. The key evolutionary innovation that enabled such host usage was a nitrile-specifier protein (NSP) that is active in the larval gut [108]. This protein allows Pierinae larvae to circumvent the release of the mustard oil bomb by redirecting the myrosinase-catalyzed glucosinolate breakdown to the less toxic nitriles and thus avoid the production of noxious isothiocyanates (Fig. 7.3). These nitriles are then excreted in the feces, either unchanged or after further metabolism [108]. To date, larval NSPs have only been identified in crucifer-feeding Pierinae species, and this enzyme activity may have enabled an adaptive radiation of these lepidopterans and promoted speciation within this herbivore clade [110].

Analysis of the metabolites present in *P. rapae* larval frass revealed that aliphatic nitriles are excreted unmodified, whereas nitriles derived from benzenic glucosinolates may undergo further metabolism of the nitrile group and/or of the benzenic side chain in vivo prior to excretion [100, 108, 114–116]. For example, the phenolic group of the nitrile derived from 4-hydroxybenzyl glucosinolate is metabolized to its corresponding sulfate ester (4-phenylacetonitrile sulfate, Fig. 7.3a) [108, 116, 117]. In contrast, hydrolysis of benzylglucosinolate (Fig. 7.3b) gives the corre-

sponding phenylacetonitrile, which is further metabolized in *P. rapae* to hippuric acid and smaller amounts of *N*-phenylacetylglycine and *N*-benzoylisoserine [114]. Hippuric acid, as well as a malonyl conjugate of benzylamine, were also found in frass of some benzylglucosinolate-fed generalist caterpillars (*S. exigua*, *S. littoralis*, and *T. ni*) where, in the absence of an insect NSP, these compounds presumably originate from natural glucosinolate hydrolysis products in *A. thaliana* leaves [118]. Analogously, the glycine and isoserine conjugates of indole-3-carboxylic acid presumably derived from indol-3-ylmethyl glucosinolate were also observed [114]. These results imply the action of a nitrilase that converts phenylacetonitrile into phenylacetic acid, with the latter then subsequently becoming conjugated to glycine to form *N*-phenylacetylglycine [114]. However, formation of the major benzoic acid derivative hippuric acid and its minor analog *N*-benzoylisoserine from phenylacetonitrile could not be directly explained by a nitrilase reaction, as this would require loss of a C1 unit. Stauber and coworkers later provided evidence in *P. rapae* that these nitriles undergo an enzymatic  $\alpha$ -hydroxylation resulting in an unstable  $\alpha$ -hydroxynitrile, which further spontaneously decomposes to an aldehyde with loss of a C1 unit as cyanide [119]; oxidation of the resulting aldehyde would then account for benzoic acid formation.

Turning the “mustard oil bomb” into a “cyanide bomb” [119] is not without danger. Cyanide is a potent inhibitor of cytochrome c oxidase in cellular respiration and thus universally toxic to aerobic cells [120, 121]. Stauber *et al.* thus investigated the development of *P. rapae* in comparison to the generalist *S. littoralis* on transgenic *A. thaliana* lines with ectopic production of the cyanogenic glucoside dhurrin. Larvae of *P. rapae* tolerated high levels of dhurrin with no negative effects, due to efficient detoxification of cyanide by  $\beta$ -cyanoalanine synthase and rhodanese in the gut. Furthermore, these larvae may be able to utilize the glucosinolate-derived cyanide in amino acid metabolism, thus compensating for the loss of nitrogen from glycine during formation of hippuric acid [119]. This ability to detoxify cyanides derived from benzenic nitriles possibly allowed Pieridae to feed on cyanogenic Fabales as their ancestral host plants [119, 122], and later facilitated the shift to Brassicales.

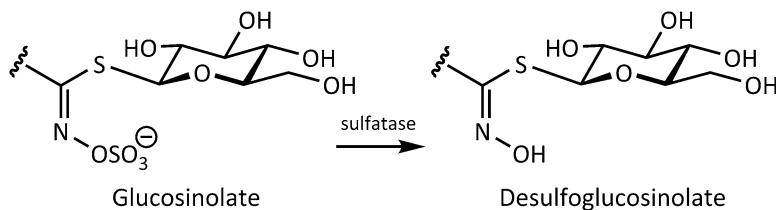
In addition to the discussed routes, Phe-derived glucosinolates can undergo other complex metabolism in *P. rapae* including *O*-demethylation prior to *O*-sulfation [115, 116]. Interestingly, plant-derived enzymatic activities were also shown to contribute to glucosinolate metabolism, including potentially in the conversion of nitriles to the arguably more toxic carboxylic acids [116]. This suggests that some plant-derived enzymes work in the insect gut extending and contributing to the complexity of metabolism of benzenic glucosinolates [115–117].

Metabolism of Phe-derived benzenic glucosinolates in *P. rapae* is thus considerably more complex than that of the Met-derived aliphatic glucosinolates, whose corresponding nitriles are apparently the only end product of myrosinase-catalyzed glucosinolate hydrolysis in the presence of NSP [108]. Phe-derived glucosinolates occur in a wider range of families than Met-derived glucosinolates, and are so thought to be ancestral to them [7, 20]. Thus this higher metabolic complexity may simply be a result of the long coevolution between Pierid herbivores and plants producing these glucosinolates. On the other hand, the especially reactive benzylic carbon of benzenic glu-

sinolates and their hydrolysis products is involved in many of the downstream reactions described above, thus providing an alternative biochemical reason for this added metabolic complexity. This may also help explain the inherent instability and the wide range of products formed upon hydrolysis and further metabolism of indolic glucosinolates, where the methyl group at position 3 is similarly particularly reactive.

In addition to insect NSP, plant proteins have been described to mediate similar reactions: An epithiospecifier protein (ESP) was identified in several species of Brassicaceae, and a thiocyanate-forming protein (TFP) has been purified from *Lepidium sativum* [49, 51, 55, 123, 124]. These specifier proteins are thought to interact with the unstable aglucone formed after hydrolysis of the thioglucosidic bond, but have no inherent hydrolytic activity towards the intact glucosinolates. Despite the functional similarity of plant-based epithiospecifier proteins and the insect-derived nitrile-specifier proteins, biochemical analysis revealed that they do not share sequence similarity [125]. Burow and coworkers compared the biochemical properties of these proteins using purified preparations, determining that both proteins functioned as enzymes rather than allosteric cofactors of myrosinases. Besides primary sequence, other fundamental biochemical differences were found: Using allylglucosinolate as a substrate, ESP promoted the formation of the corresponding epithionitrile, whereas NSP led only to the simple nitrile (allyl cyanide) [125]. Furthermore, larval NSP had low substrate specificity among different glucosinolates and did not depend on a metal ion as cofactor, unlike the *A. thaliana* ESP that required either Fe<sup>2+</sup> or Fe<sup>3+</sup> [125]. Although they are functionally related, the activity of both proteins seems to involve different catalytic mechanisms, which fits their different biological roles in plant–insect interactions.

The biological function of the ESP is yet unknown, but *A. thaliana* nitrile-specifier proteins, AtNSP1 and AtNSP2, were identified that promote constitutive and herbivore-induced nitrile formation in rosette leaves [50]. Feeding studies showed that simple nitrile formation upon glucosinolate hydrolysis in *A. thaliana* rosette leaves increases in response to herbivory independently of the action of the ESP, but at the expense of isothiocyanate formation [50, 51]. This supports the notion that the plant applies specifier proteins as a switch between direct (isothiocyanate) and indirect (nitrile) defenses. An increase in nitrile content has been reported to lower the attractiveness of a plant as a choice for ovipositing females [77, 126], possibly because nitriles are interpreted as indicating the presence of competitors and of induced defensive secondary metabolites that would negatively influence larval development [57, 67, 77, 127]. However, studies comparing feeding behavior on *A. thaliana* wild-type plants and *tgg1tgg2* myrosinase-deficient mutants suggest that breakdown products serve as feeding stimulants, rather than the intact glucosinolates [128]. On the other hand, nitriles derived from aliphatic glucosinolates have been shown to act as a signal attracting parasitoids of Pierid species such as the parasitic wasp *Cotesia rubecula* [77]. Furthermore, the benzylglucosinolate breakdown product phenylacetonitrile plays a role in indirect plant defense, (a) acting as an anti-aphrodisiac preventing the repeated mating of female moths and (b) attracting the generalist egg parasitoid *Trichogramma brassicae* to mated females that will soon oviposit [129–131]. These biological activities serve to further illus-



**Fig. 7.4** Sulfatase-catalyzed metabolism of glucosinolates to desulfo-glucosinolates. The latter compounds are not substrates of plant myrosinases, and are therefore not hydrolytically activated

trate the intricate coevolutionary processes involving glucosinolate-containing plants: while a specialist insect herbivore detoxifies glucosinolates by converting them to nitriles, plants employ the same nitriles as deterrents to oviposition by this herbivore and attractants for its parasitoids.

### 7.2.2 Glucosinolate Sulfatases: Eluding the Myrosinase System

A glucosinolate sulfatase (GSS) has been identified in insect herbivores of different taxa that feed on cruciferous plants, including the specialist lepidopteran *P. xylostella* (diamondback moth) [109] and the generalist herbivores, *S. gregaria* (desert locust) [111] and *Helix pomatia* (Burgundy snail) [132]. Sulfatases are not part of general detoxification mechanisms in animals, suggesting this to be a specific biochemical counteradaptation allowing the use of glucosinolate-containing plants by these herbivores [100]. The activity of a GSS converts an intact glucosinolate into a desulfo-glucosinolate (Fig. 7.4), in essence reversing the last step of glucosinolate biosynthesis. Thies first discovered this enzymatic activity in the digestive tract of *H. pomatia*, and this sulfatase is now commonly used in the quantitative analysis of glucosinolates from plant tissues by converting these to desulfo-glucosinolates [132].

More than 20 years later, we have gained a better understanding of how specialist herbivores use GSS to counteract the glucosinolate-myrosinase system. A glucosinolate sulfatase was identified by Ratzka and coworkers in 2002, after desulfo-glucosinolates were detected in aqueous extracts of the feces of *P. xylostella* [109]. These desulfo-glucosinolates are no longer substrates for myrosinases, which recognize glucosinolates in their binding site via the sulfate group [42]. Furthermore, the released sulfate competitively inhibits myrosinase activity [133]. Thus, GSS not only eliminates the substrates of myrosinase, but also inhibits its activity [109]. The GSS is secreted into the gut lumen, where it very efficiently outcompetes the ingested myrosinase for glucosinolate substrates. Smaller amounts of GSS activity could also be observed in the gut tissues, but not in the remainder of the body. Besides tissue-specific activity and expression profiles, RT-PCR revealed that the glucosinolate sulfatase gene is constitutively expressed in all larval stages when *P.*

*xylostella* actively feeds on glucosinolate-containing plants, but is not expressed in eggs, pupae, or adults [109]. A fourth-instar larva contains approximately 5–7 µg GSS protein, equaling a total GSS activity of 20–28 nmol/min. Thus, if one *P. xylostella* larva would take several hours to eat 100 mg of fresh leaf tissue (approximately 50 h according to observations by Ratzka and coworkers), its GSS content would be capable of detoxifying the glucosinolates contained therein (approximately 150 nmol in *A. thaliana* Col-0) in a matter of minutes. Furthermore, GSS has low substrate specificity, rapidly desulfating glucosinolates with widely differing side chain structures [109]. As a result, *P. xylostella* larvae are not affected by variation in glucosinolate content [64, 134, 135]. Only elevated myrosinase activity, which presumably out-competes the GSS, was shown to negatively affect herbivory by *P. xylostella* [64].

Interestingly, although *H. pomatia* is a major herbivore of *A. thaliana*, its GSS does not seem to be an important factor in the detoxification of glucosinolates in vivo [136]. Desulfo-glucosinolates could not be detected in feces of *H. pomatia* fed on cruciferous plants, but were present after ingestion of filter paper soaked with sinigrin [109], suggesting *H. pomatia* GSS cannot outcompete the plant myrosinases. Investigating the role of glucosinolates in the natural defense of crucifers against molluskan herbivores, Falk *et al.* found that some species of snails and slugs (*Arion lusitanicus*, *H. pomatia*, and *Deroberas laeve*) detoxify the isothiocyanates resulting from glucosinolate breakdown via conjugation to glutathione [136].

To date, GSS activity could not be identified in other lepidopteran insects feeding on glucosinolate-containing plants. No glucosinolate sulfatase activity could be detected in gut extracts from eight glucosinolate-specialist Pierid species as well as several generalist species [100, 109, 110]. The only other herbivore known to take advantage of GSS activity is the generalist locust *S. gregaria* [111] that occasionally feeds on the glucosinolate-rich, west African annual *Schouwia purpurea* without detrimental effects [137, 138]. This species of the Brassicaceae has a tenfold higher glucosinolate content than the average member of this family [7, 137] with the short-chain aliphatic 3-butetyl glucosinolate being the most abundant (64–92 %), followed by (S)-2-hydroxy-3-butetyl and indol-3-ylmethyl glucosinolates [111]. *S. purpurea* seems to possess an epithiospecifier protein [51, 76] that leads to formation of epithionitriles as the main glucosinolate breakdown product (56 % and more). Thus, the hydrolysis of the main metabolite 3-butetyl glucosinolate results most dominantly in the corresponding epithionitrile, 3-epithiobutyl cyanide (=3-epithio pentanenitrile), with only minor amounts of 3-butetyl isothiocyanate (7 %) [6, 111]. HPLC-UV analysis of aqueous extracts of guts and feces revealed that 99 % of the total glucosinolate is converted to desulfo-glucosinolates by *S. gregaria*. Its GSS protein showed optimum activity at pH 6.5, close to the physiological gut pH of 6.2, was present at similar levels throughout the whole digestive system in all developmental stages, and utilized all glucosinolates from *S. purpurea* [111]. In contrast to the *P. xylostella* GSS which is constitutively expressed [109], the *S. gregaria* GSS is induced fourfold within 3 days of feeding on a glucosinolate-containing diet, rising up to tenfold within 9 days of continuous

feeding [111]. The inducibility of this enzyme is a very efficient/economic strategy to minimize the metabolic costs of detoxification, and is considered to be a common characteristic in generalist feeders that encounter a variety of different toxins in their diet [139–141]. Additionally, the locust may adapt its feeding behavior eating first on older leaves that have lower glucosinolate content, similarly to what has been observed in small lepidopteran herbivores [31, 111].

### 7.2.3 General Detoxification Strategies

Glucosinolate hydrolysis products, like most xenobiotics, are potentially toxic due to their reaction with cellular components. As these molecules are not typically actively imported into the cell, most of them can only reach the intracellular environment via passive non-controlled diffusion through cell membranes, a process favored by lipophilic character. Therefore, xenobiotic detoxification systems generally target nonpolar compounds non-specifically, employing a number of biochemical strategies to generate more polar products that are more easily excreted and not readily able to re-enter the cell. These enzymatic reactions most often follow a series of broad steps (phases): (a) modification or activation of the xenobiotic molecule (phase I) by the introduction of polar group(s); (b) conjugation of these reactive molecules (phase II) with charged or strongly polar molecules; and (c) active excretion of the resulting conjugates (phase III) to the extracellular milieu. Phase I enzymes include several esterases/hydrolases and oxidoreductases (e.g., cytochrome P450s), while phase II metabolism involves several classes of transferases (e.g., sulfo-, acetyl-, glucosyl- or glucuronosyl-, and glutathione-transferases) that add relatively large polar groups, which then aid in phase III cross-membrane excretion (e.g., by ABC transporters).

Among the strategies described above, glutathione-S-transferase (GST)-catalyzed conjugation to glutathione is a common phase II reaction in the nonspecific metabolism of exogenous molecules containing electrophilic centers, such as isothiocyanates. Glutathione (GSH) is an unusual tripeptide containing Glu, Gly, and most importantly Cys, where Glu is linked by its gamma side chain carbon. The thiol group of GSH is one of the most abundant and important biological nucleophiles and reducing agents. GSH is present intracellularly in mM concentrations, and its oxidized disulfide form (GSSG) is metabolized by glutathione reductase to regenerate GSH; indeed, the intracellular ratio of GSH to GSSG is a good indicator of cellular redox equilibrium or stress [142]. After addition to electrophiles, the resulting covalent glutathione conjugates are then often further processed by peptidases via the so-called *mercapturic acid pathway*. This pathway involves the hydrolysis of Glu and Gly by gamma-glutamyl transpeptidases and dipeptidases, respectively, to yield cysteine conjugates, which can be then N-acetylated before excretion.

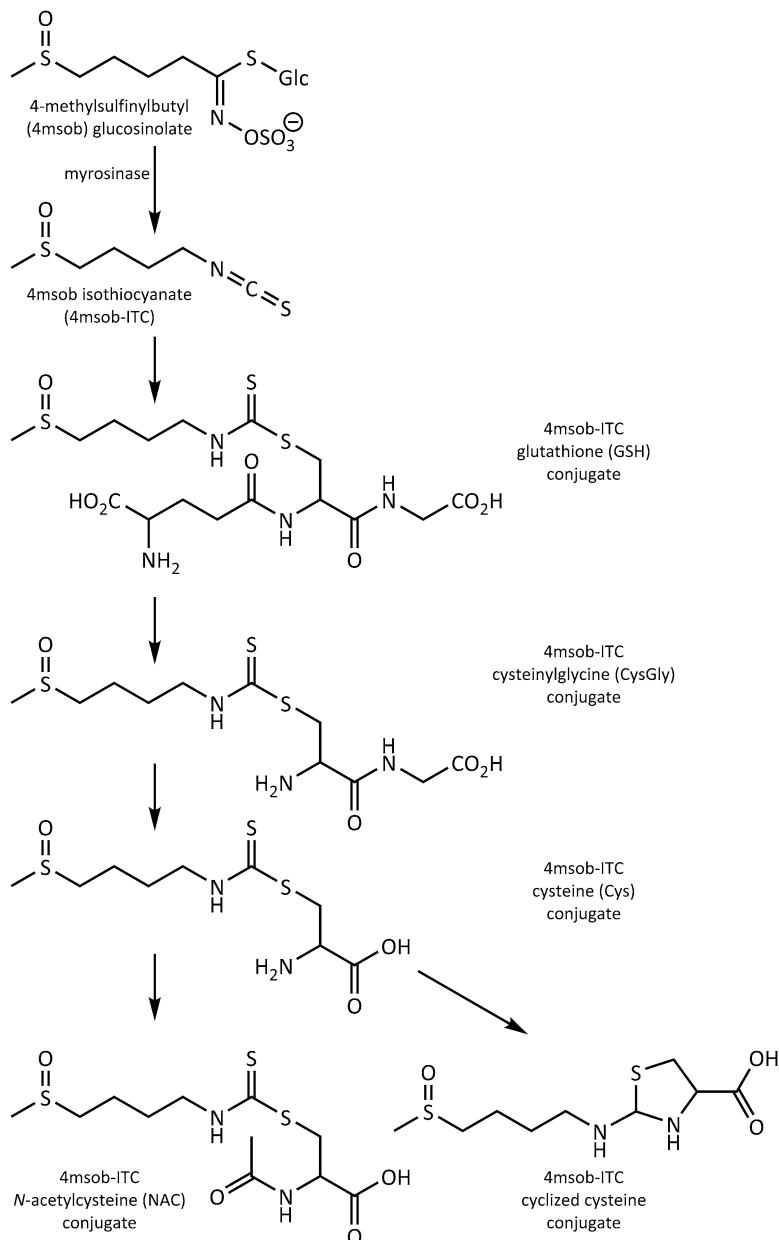
Several lepidopteran insects have been suggested or demonstrated to utilize glutathione conjugation for the detoxification of glucosinolate-derived isothiocy-

anates [18, 68, 90]. After entering cells, the isothiocyanates react readily with the thiol group of GSH under intracellular pH conditions, leading to formation of semi-stable dithiocarbamates (Fig. 7.5). While this reaction can proceed spontaneously even in the absence of GST activity, insect GSTs have the potential to greatly increase the rate of conjugation in vivo. Nitriles, on the other hand, do not possess a comparable electrophilic potential and are thus not metabolized in a similar way. The isothiocyanate-GSH conjugates, or their mercapturic acid derivatives, are drastically more polar than the parental isothiocyanate and can be actively excreted.

The GSH pathway of isothiocyanate metabolism was shown to be operative in several mollusks and in generalist lepidopteran larvae including *S. littoralis*, *S. exigua*, *H. armigera*, *M. brassicae*, and *T. ni* [18, 118, 136]. These species have varying preferences towards glucosinolate-containing plants, from species that are only rarely observed to feed on glucosinolate-containing plants in nature to the two latter species that are pests on various Brassicaceae crops. Interestingly, products of this pathway were also detected in species of *Scaptomyza* (Diptera), whose larvae are leaf-mining specialists on glucosinolate-containing plants. Unlike many glucosinolate specialists, these larvae do not employ a specific extracellular biochemical mechanism to prevent isothiocyanate formation, and instead metabolize these compounds intracellularly after exposure [143]. However, biochemical evidence suggests that particular GST enzymes in these species may have been modified by evolution to better utilize isothiocyanates as substrates, in comparison to orthologous enzymes in related non-herbivorous dipterans. Interestingly, the mercapturic acid pathway is also active in isothiocyanate metabolism in mollusks [136] and mammals [144, 145]. Humans and *Scaptomyza flava* larvae further process the cysteine conjugates to the corresponding *N*-acetylcysteine derivatives, a reaction not detected in the generalist lepidopterans and mollusks. In the latter, however, a downstream metabolite formed by intramolecular cyclization of the cysteine conjugate has been characterized [136]. This reaction can also occur spontaneously in solution and had been previously observed after conjugation of other isothiocyanates to cysteine [70, 146]. The formation of isothiocyanate conjugates with variable side chains by a range of herbivores reflects the wide application of this substrate-versatile detoxification strategy across the animal kingdom.

#### 7.2.4 Generalist Aphids and Indolic Glucosinolate Effects

Generalist aphids (in contrast to the glucosinolate-sequestering aphids discussed later) have also been suggested to use well-known biochemical adaptations, such as the induction of GSTs, to cope with the glucosinolate-myrosinase system [147]. The green peach aphid *Myzus persicae* is one of the most extreme generalist aphid

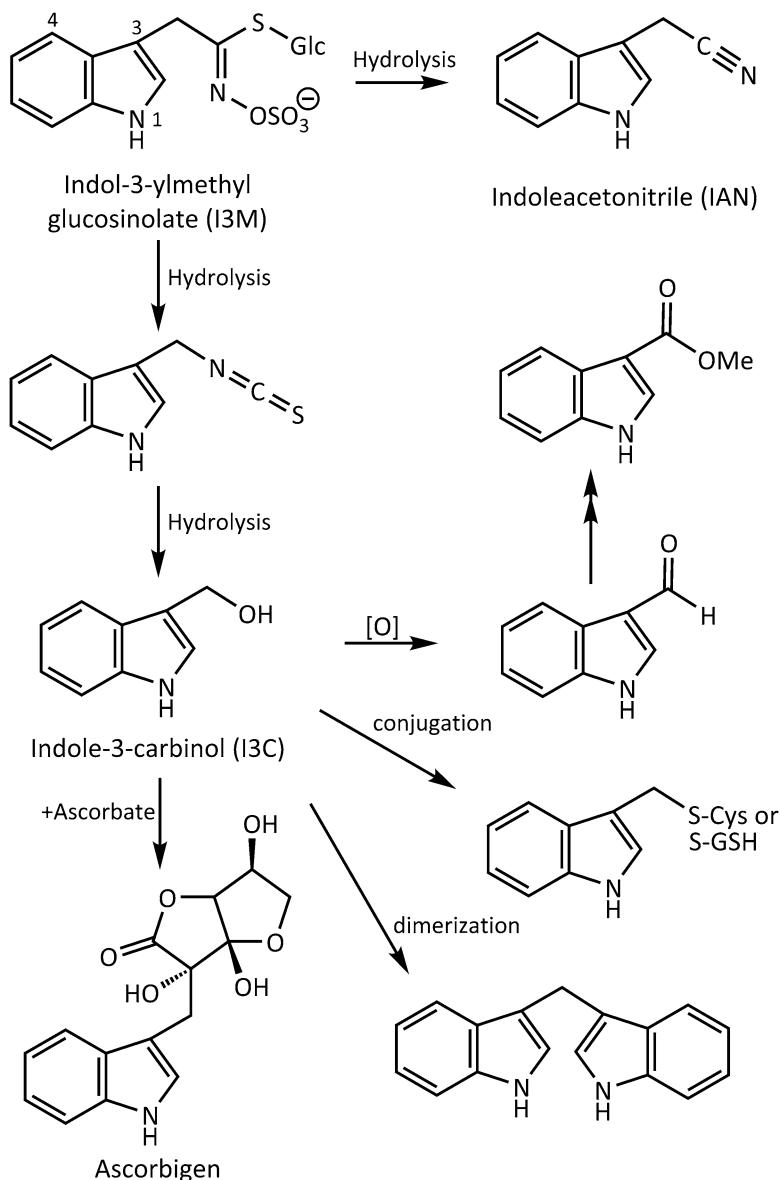


**Fig. 7.5** Detoxification of glucosinolate-derived isothiocyanates via conjugation to glutathione (GSH). The electrophilic isothiocyanate group, here exemplified by 4-methylsulfinylbutyl isothiocyanate, reacts with the nucleophilic thiol group of GSH to form a dithiocarbamate. Further hydrolytic metabolism via the mercapturic acid pathway leads to the corresponding cysteinylglycine and cysteine conjugates. The latter can be *N*-acylated or undergo spontaneous intramolecular cyclization

species, infesting almost 400 host plant species in temperate regions. Such a highly polyphagous behavior suggests that this aphid species employs a broad set of detoxification enzymes to cope with various chemical defenses from its host plants. The basic aphid feeding mode itself may help avoid the glucosinolate-myrosinase system. *M. persicae* and other aphids insert stylets between cells to reach phloem sieve elements while feeding [148], and therefore avoid bringing glucosinolates into contact with myrosinases stored in S-cells adjacent to the phloem [43, 45, 149, 150]. Accordingly, intact aliphatic glucosinolates are excreted by *M. persicae* in its honeydew. In contrast, indolic glucosinolates are mostly degraded between ingestion and excretion, even in the absence of plant myrosinase activity [82], indicating a selective breakdown process and suggesting that these compounds may play an active role in plant–aphid interactions.

The major indolic glucosinolate in *A. thaliana* and most other crucifers is indol-3-ylmethyl glucosinolate (I3M), and hydrolytic cleavage of its thioglucose generates (a) indoleacetonitrile (IAN) or (b) the corresponding isothiocyanate, which is quickly and spontaneously converted into indole-3-carbinol (I3C) via substitution of the isothiocyanate moiety in aqueous medium. This latter compound is also unstable, and is further modified via oxidation to the aldehyde and carboxylic acid [151], as well as dimerization and coupling, e.g., to GSH and ascorbate, to generate a number of end products (Fig. 7.6) [82]. Indolic glucosinolates are inducible upon aphid herbivory [29] and by jasmonates [135, 152], further supporting a role in defense against aphids and other insects. Indeed, the breakdown products of indolic glucosinolates seem to act as antifeedant compounds towards aphids [29]. As a result, aphid reproduction is negatively correlated with the host plant's indolic glucosinolate content [82], and this effect is independent of the presence of myrosinases [128].

The factor(s) controlling the breakdown of indolic glucosinolates are not yet fully elucidated. However, analyses of *M. persicae* cDNA sequences led to identification of a gut-expressed gene that has similarity to a Brassicales myrosinase [153]. *M. persicae* excretes a variety of glucosinolate-derived breakdown products in the honeydew, including conjugates with cysteine, proline, isoleucine, glutathione, as well as coupling products that are thought to be detoxification products but still show negative effects on the insect [82]. Furthermore, aphid feeding leads to *in planta* conversion of I3M to methoxyindole glucosinolates that may also significantly contribute to aphid deterrence, with some of the corresponding breakdown products exerting even stronger toxicity than those from the parent I3M [29, 154, 155]. Some of these effects may be explained by differences in the rates of degradation of these compounds, as spontaneous degradation of 4-methoxyindol-3-ylmethyl glucosinolate is significantly slower than that of its 1-methoxy- and nonmethoxylated analogs [29, 128]. These observations raise questions about how plants that produce indolic glucosinolates avoid auto-toxicity, interesting aspects that should be addressed in future research.

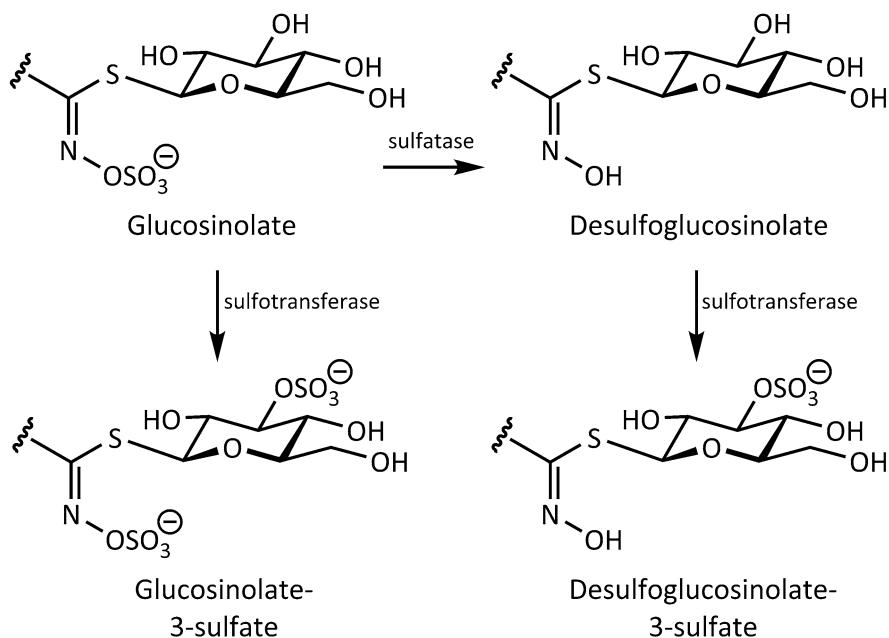


**Fig. 7.6** Breakdown of indol-3-ylmethyl glucosinolate (I3M) in aphids. The glucosinolate can undergo myrosinase-independent hydrolysis to give the nitrile or isothiocyanate, with the latter then undergoing a series of further hydrolytic, oxidative, and conjugation reactions

### 7.3 Sequestration

Some insects feeding on glucosinolate-producing plants have been shown to selectively and actively accumulate glucosinolates in specific body tissues, and use these compounds for their own benefit [25]. This process is called sequestration, and has also been observed with many other PSM such as alkaloids, cardenolides, and flavonoids [96, 98, 156–164]. The sequestration of glucosinolates requires efficient physiological adaptations to avoid self-poisoning by unintended activation of the glucosinolate-myrosinase system [156, 157]. A very efficient uptake mechanism must also exist that captures the glucosinolates before they can be hydrolyzed by myrosinases, and the myrosinases themselves may also be inhibited by insects. Hydrolytic breakdown could also be prevented by a feeding strategy such as that used by aphids and other phloem-feeders in which the compartmentalization of the glucosinolate-myrosinase system is not disturbed. Several insect species, including sucking and chewing insects from different genera (e.g., *Athalia*, *Brevicoryne*, or *Murgantia*), have been reported to sequester glucosinolates, thus preventing their breakdown to toxic products and allowing storage for the insects' own defense against predators [98, 99, 156, 165–168].

An illustrative and relatively well-studied example is the sawfly *Athalia rosae* which sequesters intact glucosinolates in its hemolymph, using them in defense against predators [98, 166, 169]. The hemolymph of *A. rosae* larvae is readily released from the integument to the outer environment when the insect is touched or injured, in the so-called *easy bleeding* phenomenon [169, 170], with the droplets of hemolymph having a deterrent effect to predators [171–173]. Studies suggest that *A. rosae* does not biosynthesize these defensive compound de novo, since the composition of glucosinolates found in the larval hemolymph is restricted to those compounds present in its host plant [98]. However, not all glucosinolates seem to be sequestered with the same efficiency [98] and intact indolic glucosinolate, for example, cannot be detected in the insect [174], perhaps having undergone spontaneous degradation as discussed above [82]. Analysis of different insect tissues using [<sup>14</sup>C]-labeled compounds indicated that the glucosinolates are stored exclusively in the hemolymph, and total amounts can reach around 5 µmol/g fresh weight [174]. This phenomenon can be observed in all larval developmental stages as well as in adults; however, depending on natural variations in plant glucosinolate levels and the individual efficiency of sequestration, the final concentrations of glucosinolates in the insect can fluctuate [98, 174, 175]. The uptake of the glucosinolate from the plant material is very efficient. Newly offered glucosinolates are first detected in the hemolymph after 30 min, and then increase steadily in concentration during the first 20 h until reaching a stable level maintained during continuous feeding. Yet, these glucosinolates are not stored indefinitely, but decrease rapidly within the first 20 h after a diet switch [174]. Therefore, *A. rosae* larvae need to feed continuously on cruciferous plants in order to maintain their defensive arsenal. Only traces of intact glucosinolates were detected in the feces of *A. rosae* over their larval development, and minor concentrations of the corresponding desulfo-glucosinolates could be found in hemolymph and feces [174]. The fast uptake and excretion of these



**Fig. 7.7** Metabolism of intact glucosinolates in the glucosinolate-sequestering sawfly *Athalia rosae*. In addition to sulfatase activity, the action of a sulfotransferase has also been described in this insect

compounds in the insect must be then coupled to an efficient transport of the intact glucosinolate to and from the hemolymph, with subsequent metabolism, e.g., to desulfo-glucosinolates prior to excretion [174, 176].

The metabolism and rapid redistribution and excretion of ingested glucosinolates in *A. rosae* were studied in more detail with feeding experiments employing isotopically labeled compounds [176] and demonstrated with MALDI-MSI (Matrix Assisted Laser Desorption Ionization-Mass Spectrometry Imaging) [177]. [<sup>14</sup>C]-labeled benzylglucosinolate was topically applied to leaves of *A. thaliana* and fed to *A. rosae* larvae. By monitoring the metabolites in larval hemolymph and feces, degradation of benzylglucosinolate to one or more metabolites was observed within 30 min after uptake. The main metabolite in the larval hemolymph was found to be desulfovbenzylglucosinolate-3-sulfate, with trace amounts of desulfovbenzylglucosinolate, presumably the first metabolite in the degradation of benzylglucosinolate, being also detected (Fig. 7.7). As discussed before in Sect. 7.2.2, glucosinolate desulfation is a detoxification strategy known in other species of the orders Orthoptera and Lepidoptera feeding on brassicaceous plants, and very efficient glucosinolate sulfatases have been identified [109, 111]. Thus, it seems likely that *A. rosae* possesses a glucosinolate sulfatase; however, this enzyme remains to be characterized. After desulfation, desulfovbenzylglucosinolate-3-sulfate is formed by the addition of a sulfate moiety to the glucose residue, presumably catalyzed by

a sulfotransferase enzyme located in the hemolymph or gut cells. Sulfotransferases are involved in the detoxification of a broad range of plant secondary metabolites [178, 179], including the metabolism of glucosinolates and their breakdown products [114, 116]. The *O*-sulfation at C-3 of the glucose moiety likely aids in excretion by making the molecule even more hydrophilic, and desulfobenzylglucosinolate-3-sulfate is not a substrate for plant myrosinases making it a nontoxic compound for the larvae. In agreement, desulfobenzylglucosinolate-3-sulfate is the main metabolite detected in the feces. In addition, benzylglucosinolate-3-sulfate is excreted with the feces, suggesting a sulfotransferase activity in the gut epithelia or gut lumen that promotes detoxification and excretion of non-sequestered glucosinolates. The sequestration of the glucosinolates into the hemolymph and the excretion of the final desulfoglucosinolate-3-sulfate into the gut lumen both require the action of a transporter. Although transporters of conjugated plant metabolites are known in insect systems [180–184], the identity of the involved transporter(s) in *Athalia* sawflies remains elusive [176]. Moreover, the fate of the plant myrosinase in the insect gut is unknown, and its possible inhibition has not yet been well investigated.

Since no myrosinase activity could be detected in protein extracts of the *A. rosae* larval hemolymph, gut tissues, or body samples [174], the sequestered glucosinolates are presumably not hydrolytically activated by the sawfly during their use as defenses. This is in remarkable contrast to other glucosinolate-sequestering specialist herbivores (e.g., *Brevicoryne brassicae* and *Lipaphis pseudobrassicae* aphids), which produce their own myrosinase that is kept in crystalline microbodies spatially separated from the glucosinolates sequestered in the hemolymph. These aphids have therefore created their own insect mustard oil bomb defensive system that is activated when they suffer tissue damage [168, 185]. Several studies showed that the glucosinolates are actively sequestered and not biosynthesized by the aphid itself [167, 185–187]. Interestingly, the hoverfly *Episyphus balteatus*, which is a predator of these aphids, is biochemically adapted to its prey's defense system and induces glutathione-S-transferases as detoxification enzymes when feeding on *B. brassicae* [186]. Nonetheless, the survival and the development of these aphidophagous hoverflies and of the ladybug *Adalia bipunctata* are dependent on the glucosinolate content of the plant on which the aphids were reared [97, 167]. When the survival of two polyphagous ladybugs, *A. bipunctata* and *Coccinella septempunctata*, was compared while feeding on *B. brassicae* aphids reared on plants with different sinigrin concentrations, *A. bipunctata* survival decreased significantly with increased sinigrin, whereas *C. septempunctata* tolerated up to 1 % sinigrin without decreasing survival, although this ladybug experienced decreased larval growth and prolonged developmental time [187]. Thus, sequestration of glucosinolates by aphids can have significant defensive value. Survival rates of *A. bipunctata* rose 90 % when predating on the generalist *M. persicae* reared on *B. nigra*, demonstrating that these generalist aphids are not capable of sequestering glucosinolates [187]. In a more recent study, *B. brassicae* reared on a plant with higher glucosinolate content contained more glucosinolates and showed shorter generation times and greater fecundity than when reared on a plant with lower glucosinolate content [188]. Correspondingly, aphid predators of the Syrphidae family (hoverflies) had lower

feeding and higher mortality rates (in the laboratory), and lower densities (in the field) when feeding on aphids with high vs. low glucosinolate content [188]. The parasitization rate of *Diaeretiella rapae* on *B. brassicae* was also influenced by the quality of the aphid host plant, probably due to the nutritional status of the aphids or to toxic compounds ingested from the plant [189].

The differences in glucosinolate accumulation between the specialist *B. brassicae* and the generalist *M. persicae* were measured more precisely by feeding both on *B. nigra* (which mainly contains sinigrin). After 1 week, one *B. brassicae* aphid contained between 102 and 148 ng sinigrin, tenfold the amount detected in *M. persicae* [168]. In the specialist, sinigrin accumulated mainly in the hemolymph, and was not detectable in the remainder of the body [185]. Interestingly, glucosinolate accumulation is three to four times higher in wingless aphids than in winged individuals, which excrete higher glucosinolate amounts in the honeydew [185]. Wingless aphids may need to arm themselves more heavily to compensate for their relative inability to flee an approaching predator. Indeed, the higher amounts of sinigrin in wingless individuals had a significant negative impact on the survival of the ladybird predator *A. bipunctata* [185].

The *B. brassicae* myrosinase has been studied in detail [165, 190–192]. The enzyme is localized in the non-flight muscles and does not show high sequence similarity to plant myrosinases; however, a homology to plant  $\beta$ -glucosidases could be inferred [190, 192] and accordingly a low *O*- $\beta$ -glucosidase activity was demonstrated using standard substrates [192]. Expression of this myrosinase increases approximately twofold throughout the developmental stages in both wingless and winged aphid forms [185]. Large differences in activity and kinetics could be observed towards pure glucosinolates with different side chains [165]. In general, aliphatic glucosinolates were more readily hydrolyzed than benzenic glucosinolates, with glucoerucin (4-methylthiobutyl glucosinolate) having the highest activity. For all tested glucosinolates, the main hydrolysis product was the corresponding isothiocyanate, except for epiprogoitrin, which was more readily converted into an oxazolidinethione. Unfortunately, Francis and coworkers did not test indolic glucosinolates [165]; however, other studies have shown that these can also be degraded in a myrosinase-independent fashion [82, 193].

Another glucosinolate-sequestering specialist insect is the harlequin bug, *Murgantia histrionica* [99]. This sucking insect also benefits from its feeding mode in avoiding the detonation of the mustard oil bomb, and additionally sequesters the intact glucosinolates. The glucosinolate content of the insect mirrors that of the host plant, at concentrations 20–30 times higher in the hemolymph than in the gut [99]. Earlier investigations revealed the presence of isothiocyanates and nitriles in the prothoracic fluid of the bugs, with these being emitted when the bug is squeezed [194]. A direct defensive function of these compounds has not been proven, but feeding studies with *M. histrionica* predators have shown that the bugs are unpalatable to several species of birds [99]. Although the isothiocyanates and nitriles are likely derived from the sequestered glucosinolates, the harlequin bug myrosinase remains to be characterized. Additionally, the transport processes of glucosinolates in these sequestering species are also an area that demands further attention.

Besides serving as defensive compounds, glucosinolate-derived isothiocyanates have other functions in insect herbivores. For example, in the specialist glucosinolate-sequestering turnip aphid *Lipaphis erysimi*, isothiocyanates synergize the action of the aphid alarm pheromone *E*- $\beta$ -farnesene, which promotes the dispersion of the aphid colony after enemy attack [195]. In *Phylloptreta striolata* flea beetles, they promote pheromone-induced aggregation behavior [196]. This insect also selectively sequesters glucosinolates from its host plants and utilizes a myrosinase to activate these compounds [197].

## 7.4 Conclusions

Although plants of the order Brassicales produce a considerable diversity of defensive glucosinolate structures that result in an even larger number of toxic breakdown products, some insects have developed efficient biochemical means enabling them to utilize these plants as food sources and even benefit from these compounds. Some of these tactics are specialized for glucosinolate defenses, while others are recruited from generalized processes of xenobiotic transport and metabolism. Although only a relatively small number of different cases have been investigated to date, these examples already serve to illustrate how inventive and exquisite the adaptations of crucifer herbivores are. As the number of investigated insect species grows, it seems inevitable that further novel biochemical processes aimed at escaping or exploiting these defenses will be described.

Future research will be facilitated by recent advances in insect molecular methods that have greatly improved the ability to isolate genes and manipulate their expression. For instance, the ease with which insect genomes and transcriptomes can be sequenced enhances the ability to identify genes controlling glucosinolate detoxification, excretion, or sequestration, and will allow us to gain a needed evolutionary perspective on the development of these strategies. Phylogenetic analyses may explain, for example, why GSS activities have evolved multiple times in unrelated lineages, and what genes and enzymes have served as progenitors for this activity. New methods to routinely silence the expression of insect genes have recently been developed for species of many different orders. These techniques enable us not only to determine the participation of specific genes in glucosinolate metabolism, but also to measure the metabolic costs associated with different detoxification strategies. Such manipulations can also be applied to elucidate unresolved mechanisms of glucosinolate transport and sequestration in insects and may also shed light on the major modes of action of glucosinolates.

Finally, new information on how insects process different glucosinolates may also reveal why plants synthesize such a large variety of glucosinolate structures with an accompanying variety of hydrolysis products. As different types of glucosinolates are processed in different ways among various groups of insects, this may explain the need for plants to possess such a diverse library of glucosinolates for effective defense.

## References

1. Kliebenstein DJ (2004) Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. *Plant Cell Environ* 27:675–684
2. Kliebenstein DJ, Rowe HC, Denby KJ (2005) Secondary metabolites influence *Arabidopsis/Botrytis* interactions: variation in host production and pathogen sensitivity. *Plant J* 44:25–36
3. Wink M (1988) Plant-breeding—importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor Appl Genet* 75:225–233
4. Hartmann T (2007) From waste products to ecochemicals: fifty years research of plant secondary metabolism. *Phytochemistry* 68:2831–2846
5. Fränkel GS (1959) Raison d'être of secondary plant substances. *Science* 129:1466–1470
6. Wittstock U, Kliebenstein DJ, Lambrix V, Reichelt M, Gershenzon J (2003) Glucosinolate hydrolysis and its impact on generalist and specialist insect herbivores. *Recent Adv Phytochem* 37:101–125
7. Fahey JW, Zalcman AT, Talalay P (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5–51
8. Agerbirk N, Olsen CE (2012) Glucosinolate structures in evolution. *Phytochemistry* 77:16–45
9. Clarke DB (2010) Glucosinolates, structures and analysis in food. *Anal Methods* 2:310–325
10. Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. *Annu Rev Plant Biol* 57:303–333
11. Sønderby IE, Geu-Flores F, Halkier BA (2010) Biosynthesis of glucosinolates—gene discovery and beyond. *Trends Plant Sci* 15:283–290
12. Halkier BA, Du LC (1997) The biosynthesis of glucosinolates. *Trends Plant Sci* 2:425–431
13. Benderoth M, Pfalz M, Kroymann J (2009) Methylthioalkylmalate synthases: genetics, ecology and evolution. *Phytochem Rev* 8:255–268
14. Geu-Flores F, Olsen CE, Halkier BA (2009) Towards engineering glucosinolates into non-cruciferous plants. *Planta* 229:261–270
15. Beekwilder J, van Leeuwen W, van Dam NM, Bertossi M, Grandi V, Mizzi L, Soloviev M, Szabadó L, Molthoff JW, Schipper B, Verbocht H, de Vos RCH, Morandini P, Aarts MGM, Bovy A (2008) The impact of absence of aliphatic glucosinolates on insect herbivory in *Arabidopsis*. *PLoS One* 3, e2068
16. Müller R, de Vos M, Sun JY, Sønderby IE, Halkier BA, Wittstock U, Jander G (2010) Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *J Chem Ecol* 36:905–913
17. Brader G, Mikkelsen MD, Halkier BA, Palva ET (2006) Altering glucosinolate profiles modulates disease resistance in plants. *Plant J* 46:758–767
18. Schramm K, Vassão DG, Reichelt M, Gershenzon J, Wittstock U (2012) Metabolism of glucosinolate-derived isothiocyanates to glutathione conjugates in generalist lepidopteran herbivores. *Insect Biochem Mol Biol* 42:174–182
19. Rodman JE, Soltis PS, Soltis DE, Sytsma KJ, Karol KG (1998) Parallel evolution of glucosinolate biosynthesis inferred from congruent nuclear and plastid gene phylogenies. *Am J Bot* 85:997–1006
20. Mithen R, Bennett R, Marquez J (2010) Glucosinolate biochemical diversity and innovation in the Brassicales. *Phytochemistry* 71:2074–2086
21. Pedras MSC, Okinyo DPO (2008) Remarkable incorporation of the first sulfur containing indole derivative: another piece in the biosynthetic puzzle of crucifer phytoalexins. *Org Biomol Chem* 6:51–54
22. Pedras MSC, Yaya EE, Glawischnig E (2011) The phytoalexins from cultivated and wild crucifers: chemistry and biology. *Nat Prod Rep* 28:1381–1405
23. Pedras MSC, Yaya EE, Hossain S (2010) Unveiling the phytoalexin biosynthetic puzzle in salt cress: unprecedented incorporation of glucobrassicin into wasalexins A and B. *Org Biomol Chem* 8:5150–5158

24. Brown PD, Tokuhisa JG, Reichelt M, Gershenzon J (2003) Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochemistry* 62:471–481
25. Hopkins RJ, van Dam NM, van Loon JJ (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Annu Rev Entomol* 54:57–83
26. Kliebenstein DJ, Kroymann J, Brown P, Figuth A, Pedersen D, Gershenzon J, Mitchell-Olds T (2001) Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation. *Plant Physiol* 126:811–825
27. Textor S, Gershenzon J (2009) Herbivore induction of the glucosinolate-myrosinase defense system: major trends, biochemical bases and ecological significance. *Phytochem Rev* 8:149–170
28. De Vos M, Jander G (2009) *Myzus persicae* (green peach aphid) salivary components induce defence responses in *Arabidopsis thaliana*. *Plant Cell Environ* 32:1548–1560
29. Kim JH, Jander G (2007) *Myzus persicae* (green peach aphid) feeding on *Arabidopsis* induces the formation of a deterrent indole glucosinolate. *Plant J* 49:1008–1019
30. Koritsas VM, Lewis JA, Fenwick GR (1991) Glucosinolate responses of oilseed rape, mustard and kale to mechanical wounding and infestation by cabbage stem flea beetle (*Psylliodes chrysoccephala*). *Ann Appl Biol* 118:209–221
31. Shroff R, Vergara F, Muck A, Svatoš A, Gershenzon J (2008) Nonuniform distribution of glucosinolates in *Arabidopsis thaliana* leaves has important consequences for plant defense. *Proc Natl Acad Sci U S A* 105:6196–6201
32. Städler E, Renwick JAA, Radke CD, Sachdevgupta K (1995) Tarsal contact chemoreceptor response to glucosinolates and cardenolides mediating oviposition in *Pieris rapae*. *Physiol Entomol* 20:175–187
33. Marazzi C, Patrian B, Städler E (2004) Secondary metabolites of the leaf surface affected by sulphur fertilisation and perceived by the diamondback moth. *Chemoecology* 14:81–86
34. Marazzi C, Städler E (2004) *Arabidopsis thaliana* leaf-surface extracts are detected by the cabbage root fly (*Delia radicum*) and stimulate oviposition. *Physiol Entomol* 29:192–198
35. Renwick JA, Radke C, Sachdev-Gupta K, Städler E (1992) Leaf surface chemicals stimulating oviposition by *Pieris rapae* (Lepidoptera: Pieridae) on cabbage. *Chemoecology* 3:33–38
36. Badenes-Perez FR, Reichelt M, Gershenzon J, Heckel DG (2013) Interaction of glucosinolate content of *Arabidopsis thaliana* mutant lines and feeding and oviposition by generalist and specialist lepidopterans. *Phytochemistry* 86:36–43
37. Huang XP, Renwick JAA (1993) Differential selection of host plants by two *Pieris* species—the role of oviposition stimulants and deterrents. *Entomol Exp Appl* 68:59–69
38. Hicks KL (1974) Mustard oil glucosides—feeding stimulants for adult cabbage flea beetles, *Phylloptreta cruciferae* (Coleoptera-Chrysomelidae). *Ann Entomol Soc Am* 67:261–264
39. David WAL, Gardiner BO (1966) Mustard oil glucosides as feeding stimulants for *Pieris brassicae* larvae in a semi-synthetic diet. *Entomol Exp Appl* 9:247–255
40. Perkins LE, Cribb BW, Brewer PB, Hanan J, Grant M, de Torres M, Zalucki MP (2013) Generalist insects behave in a jasmonate-dependent manner on their host plants, leaving induced areas quickly and staying longer on distant parts. *Proc R Soc Lond B* 280:20122646
41. Bones AM, Rossiter JT (1996) The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiol Plantarum* 97:194–208
42. Matile P (1980) The mustard oil bomb—compartmentation of the myrosinase system. *Biochem Physiol Pflanz* 175:722–731
43. Koroleva OA, Davies A, Deeken R, Thorpe MR, Tomos AD, Hedrich R (2000) Identification of a new glucosinolate-rich cell type in *Arabidopsis* flower stalk. *Plant Physiol* 124:599–608
44. Zhao ZX, Zhang W, Stanley BA, Assmann SM (2008) Functional proteomics of *Arabidopsis thaliana* guard cells uncovers new stomatal signaling pathways. *Plant Cell* 20:3210–3226
45. Andreasson E, Jorgensen LB (2003) Localization of plant myrosinases and glucosinolates. *Recent Adv Phytochem* 37:79–99

46. Wittstock U, Burow M (2010) Glucosinolate breakdown in *Arabidopsis*: mechanism, regulation and biological significance. *Arabidopsis Book* 8, e0134
47. Wittstock U, Burow M (2007) Tipping the scales—specifier proteins in glucosinolate hydrolysis. *IUBMB Life* 59:744–751
48. Burow M, Rice M, Hause B, Gershenzon J, Wittstock U (2007) Cell- and tissue-specific localization and regulation of the epithiospecifier protein in *Arabidopsis thaliana*. *Plant Mol Biol* 64:173–185
49. Burow M, Bergner A, Gershenzon J, Wittstock U (2007) Glucosinolate hydrolysis in *Lepidium sativum*—identification of the thiocyanate-forming protein. *Plant Mol Biol* 63:49–61
50. Burow M, Losansky A, Müller R, Plock A, Kliebenstein DJ, Wittstock U (2009) The genetic basis of constitutive and herbivore-induced ESP-independent nitrile formation in *Arabidopsis*. *Plant Physiol* 149:561–574
51. Lambrix V, Reichelt M, Mitchell-Olds T, Kliebenstein DJ, Gershenzon J (2001) The *Arabidopsis* epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. *Plant Cell* 13:2793–2807
52. Wentzell AM, Kliebenstein DJ (2008) Genotype, age, tissue, and environment regulate the structural outcome of glucosinolate activation. *Plant Physiol* 147:415–428
53. Kissin R, Bones AM (2009) Nitrile-specifier proteins involved in glucosinolate hydrolysis in *Arabidopsis thaliana*. *J Biol Chem* 284:12057–12070
54. Kissin R, Rossiter JT, Bones AM (2009) The ‘mustard oil bomb’: not so easy to assemble? Localization, expression and distribution of the components of the myrosinase enzyme system. *Phytochem Rev* 8:69–86
55. Tookey HL (1973) Crambe thioglucoside glucohydrolase (EC3.2.3.1)—separation of a protein required for epithiobutane formation. *Can J Biochem* 51:1654–1660
56. Mithen R, Raybould AF, Gihamoustaris A (1995) Divergent selection for secondary metabolites between wild populations of *Brassica oleracea* and its implications for plant-herbivore interactions. *Heredity* 75:472–484
57. Mewis I, Appel HM, Hom A, Raina R, Schultz JC (2005) Major signaling pathways modulate *Arabidopsis* glucosinolate accumulation and response to both phloem-feeding and chewing insects. *Plant Physiol* 138:1149–1162
58. Schlaeppi K, Bodenhausen N, Buchala A, Mauch F, Reymond P (2008) The glutathione-deficient mutant pad2-1 accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore *Spodoptera littoralis*. *Plant J* 55:774–786
59. Blau PA, Feeny P, Contardo L, Robson DS (1978) Allylglucosinolate and herbivorous caterpillars—contrast in toxicity and tolerance. *Science* 200:1296–1298
60. Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, Llorente F, Molina A, Parker J, Sommerville S, Schulze-Lefert P (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. *Science* 310:1180–1183
61. Lipka U, Fuchs R, Lipka V (2008) *Arabidopsis* non-host resistance to powdery mildews. *Curr Opin Plant Biol* 11:404–411
62. Bednarek P, Piślewska-Bednarek M, Svatoš A, Schneider B, Doubský J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, Molina A, Schulze-Lefert P (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* 323:101–106
63. Tierens KFMJ, Thomma BPHJ, Brouwer M, Schmidt J, Kistner K, Porzel A, Mauch-Mani B, Cammue BPA, Broekaert WF (2001) Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of *Arabidopsis* to microbial pathogens. *Plant Physiol* 125:1688–1699
64. Li Q, Eigenbrode SD, Stringham GR, Thiagarajah MR (2000) Feeding and growth of *Plutella xylostella* and *Spodoptera eridania* on *Brassica juncea* with varying glucosinolate concentrations and myrosinase activities. *J Chem Ecol* 26:2401–2419

65. Lichtenstein EP, Morgan DG, Strong FM (1962) Naturally occurring insecticides—identification of 2-phenylethylisothiocyanate as an insecticide occurring naturally in edible part of turnips. *J Agric Food Chem* 10:30–33
66. Seo ST, Tang CS (1982) Hawaiian fruit-flies (Diptera: Tephritidae)—toxicity of benzyl isothiocyanate against eggs or 1st instars of three species. *J Econ Entomol* 75:1132–1135
67. Agrawal AA, Kurashige NS (2003) A role for isothiocyanates in plant resistance against the specialist herbivore *Pieris rapae*. *J Chem Ecol* 29:1403–1415
68. Wadleigh RW, Yu SJ (1988) Detoxification of isothiocyanate allelochemicals by glutathione transferase in three lepidopterous species. *J Chem Ecol* 14:1279–1288
69. Hanschen FS, Brüggemann N, Brodehl A, Mewis I, Schreiner M, Rohn S, Kroh LW (2012) Characterization of products from the reaction of glucosinolate-derived isothiocyanates with cysteine and lysine derivatives formed in either model systems or broccoli sprouts. *J Agric Food Chem* 60:7735–7745
70. Kawakishi S, Namiki M (1982) Oxidative cleavage of the disulfide bond of cysteine by allyl isothiocyanate. *J Agric Food Chem* 30:620–622
71. Xiao Z, Mi L, Chung FL, Veenstra TD (2012) Proteomic analysis of covalent modifications of tubulin by isothiocyanates. *J Nutr* 142:1377–1381
72. Kawakishi S, Kaneko T (1987) Interaction of proteins with allyl isothiocyanate. *J Agric Food Chem* 35:85–88
73. Cross JV, Rady JM, Foss FW, Lyons C, Macdonald TL, Templeton DJ (2009) Nutrient isothiocyanates covalently modify and inhibit the inflammatory cytokine macrophage migration inhibitory factor (MIF). *Biochem J* 423:315–321
74. Hu CQ, Eggler AL, Mesecar AD, van Breemen RB (2011) Modification of Keap1 cysteine residues by sulforaphane. *Chem Res Toxicol* 24:515–521
75. Mi L, Di Pasqua AJ, Chung FL (2011) Proteins as binding targets of isothiocyanates in cancer prevention. *Carcinogenesis* 32:1405–1413
76. Burow M, Müller R, Gershenson J, Wittstock U (2006) Altered glucosinolate hydrolysis in genetically engineered *Arabidopsis thaliana* and its influence on the larval development of *Spodoptera littoralis*. *J Chem Ecol* 32:2333–2349
77. Mummm R, Burow M, Bukovinszky Kiss G, Kazantzidou E, Wittstock U, Dicke M, Gershenson J (2008) Formation of simple nitriles upon glucosinolate hydrolysis affects direct and indirect defense against the specialist herbivore, *Pieris rapae*. *J Chem Ecol* 34:1311–1321
78. Kissen R, Pope TW, Grant M, Pickett JA, Rossiter JT, Powell G (2009) Modifying the alkyl-glucosinolate profile in *Arabidopsis thaliana* alters the tritrophic interaction with the herbivore *Brevicoryne brassicae* and parasitoid *Diaeretiella rapae*. *J Chem Ecol* 35:958–969
79. Pope TW, Kissen R, Grant M, Pickett JA, Rossiter JT, Powell G (2008) Comparative innate responses of the aphid parasitoid *Diaeretiella rapae* to alkenyl glucosinolate derived isothiocyanates, nitriles, and epithionitriles. *J Chem Ecol* 34:1302–1310
80. Bidart-Bouzat MG, Kliebenstein DJ (2008) Differential levels of insect herbivory in the field associated with genotypic variation in glucosinolates in *Arabidopsis thaliana*. *J Chem Ecol* 34:1026–1037
81. Lüthy J, Benn MH (1977) Thiocyanate formation from glucosinolates: a study of autolysis of allylglucosinolate in *Thlaspi arvense* L. seed flour extracts. *Can J Biochem* 55:1028–1031
82. Kim JH, Lee BW, Schroeder FC, Jander G (2008) Identification of indole glucosinolate breakdown products with antifeedant effects on *Myzus persicae* (green peach aphid). *Plant J* 54:1015–1026
83. Mauricio R (1998) Costs of resistance to natural enemies in field populations of the annual plant *Arabidopsis thaliana*. *Am Nat* 151:20–28
84. Siemens DH, Mitchell-Olds T (1996) Glucosinolates and herbivory by specialists (Coleoptera: Chrysomelidae, Lepidoptera: Plutellidae): consequences of concentration and induced resistance. *Environ Entomol* 25:1344–1353
85. Holzinger F, Frick C, Wink M (1992) Molecular basis for the insensitivity of the monarch (*Danaus plexippus*) to cardiac glycosides. *FEBS Lett* 314:477–480
86. Self LS, Hodgson E, Guthrie FE (1964) Metabolism of nicotine by tobacco-feeding insects. *Nature* 204:300–301

87. Ivie GW, Bull DL, Beier RC, Pryor NW, Oertli EH (1983) Metabolic detoxification: mechanism of insect resistance to plant psoralens. *Science* 221:374–376
88. Hartmann T (1999) Chemical ecology of pyrrolizidine alkaloids. *Planta* 207:483–495
89. Dussourd DE, Eisner T (1987) Vein-cutting behavior: insect counterploy to the latex defense of plants. *Science* 237:898–901
90. Yu SJ (1984) Interactions of allelochemicals with detoxication enzymes of insecticide-susceptible and resistant fall armyworms. *Pest Biochem Physiol* 22:60–68
91. Iqbal M, Wright DJ (1997) Evaluation of resistance, cross-resistance and synergism of abamectin and teflubenzuron in a multi-resistant field population of *Plutella xylostella* (Lepidoptera: Plutellidae). *Bull Entomol Res* 87:481–486
92. Furlong MJ, Wright DJ (1994) Examination of stability of resistance and cross-resistance patterns to acylurea insect growth-regulators in-field populations of the diamondback moth, *Plutella xylostella*, from Malaysia. *Pestic Sci* 42:315–326
93. Bartlet E, Parsons D, Williams IH, Clark SJ (1994) The influence of glucosinolates and sugars on feeding by the cabbage stem flea beetle, *Psylliodes chrysocephala*. *Entomol Exp Appl* 73:77–83
94. Nault LR, Styler WE (1972) Effects of sinigrin on host selection by aphids. *Entomol Exp Appl* 15:423–437
95. Lankau RA (2007) Specialist and generalist herbivores exert opposing selection on a chemical defense. *New Phytol* 175:176–184
96. Nishida R (2002) Sequestration of defensive metabolites from plants by lepidoptera. *Annu Rev Entomol* 47:57–92
97. Francis F, Lognay G, Wathelet JP, Haubruge E (2001) Effects of allelochemicals from first (Brassicaceae) and second (*Myzus persicae* and *Brevicoryne brassicae*) trophic levels on *Adalia bipunctata*. *J Chem Ecol* 27:243–256
98. Müller C, Agerbirk N, Olsen CE, Boevé JL, Schaffner U, Brakefield PM (2001) Sequestration of host plant glucosinolates in the defensive hemolymph of the sawfly *Athalia rosae*. *J Chem Ecol* 27:2505–2516
99. Aliabadi A, Renwick JAA, Whitman DW (2002) Sequestration of glucosinolates by harlequin bug *Murgantia histrionica*. *J Chem Ecol* 28:1749–1762
100. Winde I, Wittstock U (2011) Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system. *Phytochemistry* 72:1566–1575
101. Giamoustaris A, Mithen R (1995) The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus* ssp. *oleifera*) on its interaction with specialist and generalist pests. *Ann Appl Biol* 126:347–363
102. Hilker M, Meiners T (2002) Induction of plant responses to oviposition and feeding by herbivorous arthropods: a comparison. *Entomol Exp Appl* 104:181–192
103. Isidoro N, Bartlet E, Ziesmann J, Williams IH (1998) Antennal contact chemosensilla in *Psylliodes chrysocephala* responding to cruciferous allelochemicals. *Physiol Entomol* 23:131–138
104. Roessingh P, Städler E, Baur R, Hurter J, Ramp T (1997) Tarsal chemoreceptors and oviposition behaviour of the cabbage root fly (*Delia radicum*) sensitive to fractions and new compounds of host-leaf surface extracts. *Physiol Entomol* 22:140–148
105. Nielsen JK, Hansen ML, Agerbirk N, Petersen BL, Halkier BA (2001) Responses of the flea beetles *Phyllobretha nemorum* and *P. cruciferae* to metabolically engineered *Arabidopsis thaliana* with an altered glucosinolate profile. *Chemoecology* 11:75–83
106. Sarfraz M, Dosdall LM, Keddie BA (2006) Diamondback moth-host plant interactions: implications for pest management. *Crop Prot* 25:625–639
107. Åhman I (1986) Toxicities of host secondary compounds to eggs of the *Brassica* specialist *Dasineura brassicae*. *J Chem Ecol* 12:1481–1488
108. Wittstock U, Agerbirk N, Stauber EJ, Olsen CE, Hippler M, Mitchell-Olds T, Gershenson J, Vogel H (2004) Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proc Natl Acad Sci U S A* 101:4859–4864

109. Ratzka A, Vogel H, Kliebenstein DJ, Mitchell-Olds T, Kroymann J (2002) Disarming the mustard oil bomb. *Proc Natl Acad Sci U S A* 99:11223–11228
110. Wheat CW, Vogel H, Wittstock U, Braby MF, Underwood D, Mitchell-Olds T (2007) The genetic basis of a plant-insect coevolutionary key innovation. *Proc Natl Acad Sci U S A* 104:20427–20431
111. Falk KL, Gershenzon J (2007) The desert locust, *Schistocerca gregaria*, detoxifies the glucosinolates of *Schouwia purpurea* by desulfation. *J Chem Ecol* 33:1542–1555
112. Ehrlich PR, Raven PH (1964) Butterflies and plants—a study in coevolution. *Evolution* 18:586–608
113. Beilstein MA, Nagalingum NS, Clements MD, Manchester SR, Mathews S (2010) Dated molecular phylogenies indicate a Miocene origin for *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* 107:18724–18728
114. Vergara F, Svatoš A, Schneider B, Reichelt M, Gershenzon J, Wittstock U (2006) Glycine conjugates in a lepidopteran insect herbivore—the metabolism of benzylglucosinolate in the cabbage white butterfly, *Pieris rapae*. *Chembiochem* 7:1982–1989
115. Agerbirk N, Olsen CE, Topbjerg HB, Sørensen JC (2007) Host plant-dependent metabolism of 4-hydroxybenzylglucosinolate in *Pieris rapae*: Substrate specificity and effects of genetic modification and plant nitrile hydratase. *Insect Biochem Mol Biol* 37:1119–1130
116. Agerbirk N, Olsen CE, Poulsen E, Jacobsen N, Hansen PR (2010) Complex metabolism of aromatic glucosinolates in *Pieris rapae* caterpillars involving nitrile formation, hydroxylation, demethylation, sulfation, and host plant dependent carboxylic acid formation. *Insect Biochem Mol Biol* 40:126–137
117. Agerbirk N, Müller C, Olsen CE, Chew FS (2006) A common pathway for metabolism of 4-hydroxybenzylglucosinolate in *Pieris* and *Anthocaris* (Lepidoptera: Pieridae). *Biochem Syst Ecol* 34:189–198
118. Winde IB (2011) Entgiftung des Glucosinolat-Myrosinase-systems durch generalistische Herbivoren der Lepidoptera. Doctoral Thesis, Technical University Braunschweig
119. Stauber EJ, Kuczka P, van Ohlen M, Vogt B, Janowitz T, Piotrowski M, Beuerle T, Wittstock U (2012) Turning the ‘mustard oil bomb’ into a ‘cyanide bomb’: aromatic glucosinolate metabolism in a specialist insect herbivore. *PLoS One* 7, e35545
120. Ballhorn DJ, Kautz S, Heil M, Hegeman A (2009) Cyanogenesis of wild lima bean (*Phaseolus lunatus* L.) is an efficient direct defence in nature. *PLoS One* 4, e5450
121. Gleadow RM, Woodrow IE (2002) Constraints on effectiveness of cyanogenic glycosides in herbivore defense. *J Chem Ecol* 28:1301–1313
122. Conn EE (1980) Cyanogenic compounds. *Annu Rev Plant Phys* 31:433–451
123. Petroski RJ, Kwolek WF (1985) Interactions of a fungal thioglucoside glucohydrolase and cruciferous plant epithiospecifier protein to form 1-cyanoepithioalkanes: implications of an allosteric mechanism. *Phytochemistry* 24:213–216
124. Foo HL, Grønning LM, Goodenough L, Bones AM, Danielsen BE, Whiting DA, Rossiter JT (2000) Purification and characterisation of epithiospecifier protein from *Brassica napus*: Enzymic intramolecular sulphur addition within alkenyl thiohydroximates derived from alkenyl glucosinolate hydrolysis. *FEBS Lett* 468:243–246
125. Burow M, Markert J, Gershenzon J (2006) Comparative biochemical characterization of nitrile-forming proteins from plants and insects that alter myrosinase-catalysed hydrolysis of glucosinolates. *FEBS J* 273:2432–2446
126. Rothschild M, Schoonhoven LM (1977) Assessment of egg load by *Pieris brassicae* (Lepidoptera: Pieridae). *Nature* 266:352–355
127. Mewis I, Tokuhisa JG, Schultz JC, Appel HM, Ulrichs C, Gershenzon J (2006) Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry* 67:2450–2462
128. Barth C, Jander G (2006) *Arabidopsis* myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defense. *Plant J* 46:549–562

129. Fatouros NE, Broekgaarden C, Bukovinszke' Kiss G, van Loon JJA, Mumm R, Huigens ME, Dicke M, Hilker M (2008) Male-derived butterfly anti-aphrodisiac mediates induced indirect plant defense. *Proc Natl Acad Sci U S A* 105:10033–10038
130. Fatouros NE, Huigens ME, van Loon JJA, Dicke M, Hilker M (2005) Chemical communication—butterfly anti-aphrodisiac lures parasitic wasps. *Nature* 433:704
131. Andersson J, Borg-Karlsson AK, Wiklund C (2003) Antiaphrodisiacs in pierid butterflies: a theme with variation! *J Chem Ecol* 29:1489–1499
132. Thies W (1979) Detection and utilization of a glucosinolate sulfohydrolase in the edible snail, *Helix pomatia*. *Naturwissenschaften* 66:364–365
133. Shikita M, Fahey JW, Golden TR, Holtzclaw WD, Talalay P (1999) An unusual case of ‘uncompetitive activation’ by ascorbic acid: purification and kinetic properties of a myrosinase from *Rapshanus sativus* seedlings. *Biochem J* 341:725–732
134. Sarosh BR, Wittstock U, Halkier BA, Ekbom B (2010) The influence of metabolically engineered glucosinolates profiles in *Arabidopsis thaliana* on *Plutella xylostella* preference and performance. *Chemoecology* 20:1–9
135. Kliebenstein D, Pedersen D, Barker B, Mitchell-Olds T (2002) Comparative analysis of quantitative trait loci controlling glucosinolates, myrosinase and insect resistance in *Arabidopsis thaliana*. *Genetics* 161:325–332
136. Falk KL, Kästner J, Bodenhausen N, Schramm K, Paetz C, Vassão DG, Reichelt M, von Knorre D, Bergelson J, Erb M, Gershenson J, Meldau S (2014) The role of glucosinolates and the jasmonic acid pathway in resistance of *Arabidopsis thaliana* against molluscan herbivores. *Mol Ecol* 23:1188–1203
137. Ghaout S, Louveaux A, Mainguet AM, Deschamps M, Rahal Y (1991) What defense does *Schouwia purpurea* (Cruciferae) have against the desert locust—secondary compounds and nutritive value. *J Chem Ecol* 17:1499–1515
138. Mainguet AM, Louveaux A, El Sayed G, Rollin P (2000) Ability of a generalist insect, *Schistocerca gregaria*, to overcome thioglucoside defense in desert plants: Tolerance or adaptation? *Entomol Exp Appl* 94:309–317
139. Terriere LC (1984) Induction of detoxification enzymes in insects. *Annu Rev Entomol* 29:71–88
140. Yu SJ, Hsu EL (1993) Induction of detoxification enzymes in phytophagous insects: roles of insecticide synergists, larval age, and species. *Arch Insect Biochem Physiol* 24:21–32
141. Hoy CW, Head GP, Hall FR (1998) Spatial heterogeneity and insect adaption to toxins. *Annu Rev Entomol* 43:571–594
142. Noctor G, Queval G, Mhamdi A, Chaouch S, Foyer CH (2011) Glutathione. *Arabidopsis Book* 9, e0142
143. Gloss AD, Vassão DG, Hailey AL, Dittrich ACN, Schramm K, Reichelt M, Rast TJ, Weichsel A, Cravens MG, Gershenson J, Montfort WR, Whiteman NK (2014) Evolution in an ancient detoxification pathway is coupled with a transition to herbivory in the Drosophilidae. *Mol Biol Evol* 31:2441–2456
144. Al Janobi AA, Mithen RF, Gasper AV, Shaw PN, Middleton RJ, Ortori CA, Barrett DA (2006) Quantitative measurement of sulforaphane, iberin and their mercapturic acid pathway metabolites in human plasma and urine using liquid chromatography-tandem electrospray ionisation mass spectrometry. *J Chromatogr B* 844:223–234
145. Kassahun K, Davis M, Hu P, Martin B, Baillie T (1997) Biotransformation of the naturally occurring isothiocyanate sulforaphane in the rat: Identification of phase I metabolites and glutathione conjugates. *Chem Res Toxicol* 10:1228–1233
146. Eklind KI, Morse MA, Chung FL (1990) Distribution and metabolism of the natural anticarcinogen phenethyl isothiocyanate in A/J mice. *Carcinogenesis* 11:2033–2036
147. Francis F, Vanhaelen N, Haubrige E (2005) Glutathione S-transferases in the adaptation to plant secondary metabolites in the *Myzus persicae* aphid. *Arch Insect Biochem Physiol* 58:166–174
148. Tjallingii WF, Hogen Esch T (1993) Fine structure of aphid stylet routes in plant-tissues in correlation with EPG signals. *Physiol Entomol* 18:317–328

149. Husebye H, Chadchawan S, Winge P, Thangstad OP, Bones AM (2002) Guard cell- and phloem idioblast-specific expression of thioglucoside glucohydrolase 1 (myrosinase) in *Arabidopsis*. *Plant Physiol* 128:1180–1188
150. Thangstad OP, Gilde B, Chadchawan S, Seem M, Husebye H, Bradley D, Bones AM (2004) Cell specific, cross-species expression of myrosinases in *Brassica napus*, *Arabidopsis thaliana* and *Nicotiana tabacum*. *Plant Mol Biol* 54:597–611
151. Pedras MS, Nycholat CM, Montaut S, Xu Y, Khan AQ (2002) Chemical defenses of crucifers: elicitation and metabolism of phytoalexins and indole-3-acetonitrile in brown mustard and turnip. *Phytochemistry* 59:611–625
152. Bodnaryk RP (1994) Potent effect of jasmonates on indole glucosinolates in oilseed rape and mustard. *Phytochemistry* 35:301–305
153. Ramsey JS, Wilson ACC, de Vos M, Sun Q, Tamborindeguy C, Winfield A, Malloch G, Smith DM, Fenton B, Gray SM, Jander G (2007) Genomic resources for *Myzus persicae*: EST sequencing, SNP identification, and microarray design. *BMC Genomics* 8:423
154. Agerbirk N, Olsen CE, Sørensen H (1998) Initial and final products, nitriles, and ascorbigens produced in myrosinase-catalyzed hydrolysis of indole glucosinolates. *J Agric Food Chem* 46:1563–1571
155. Pfalz M, Vogel H, Kroymann J (2009) The gene controlling the indole glucosinolate modifier1 quantitative trait locus alters indole glucosinolate structures and aphid resistance in *Arabidopsis*. *Plant Cell* 21:985–999
156. Opitz S, Müller C (2009) Plant chemistry and insect sequestration. *Chemoecology* 19:117–154
157. Duffey SS (1980) Sequestration of plant natural-products by insects. *Annu Rev Entomol* 25:447–477
158. Abe F, Yamauchi T, Honda K, Omura H, Hayashi N (2001) Sequestration of phenanthroindolizidine alkaloids by an Asclepiadaceae-feeding danaid butterfly, *Ideopsis similis*. *Phytochemistry* 56:697–701
159. Dobler S, Daloz D, Pasteels JM (1998) Sequestration of plant compounds in a leaf beetle's defensive secretion: cardenolides in *Chrysocinus*. *Chemoecology* 8:111–118
160. Dobler S, Haberer W, Witte L, Hartmann T (2000) Selective sequestration of pyrrolizidine alkaloids from diverse host plants by *Longitarsus* flea beetles. *J Chem Ecol* 26:1281–1298
161. Schittko U, Burghardt F, Fiedler K (1999) Sequestration and distribution of flavonoids in the common blue butterfly *Polyommatus icarus* reared on *Trifolium repens*. *Phytochemistry* 51:609–614
162. Scudder GGE, Moore LV, Isman MB (1986) Sequestration of cardenolides in *Oncopeltus fasciatus*: morphological and physiological adaptations. *J Chem Ecol* 12:1171–1187
163. Rothschild M, Edgar JA (1978) Pyrrolizidine alkaloids from *Senecio vulgaris* sequestered and stored by *Danaus plexippus*. *J Zool* 186:347–349
164. Trigo JR (2000) The chemistry of antipredator defense by secondary compounds in neotropical lepidoptera: facts, perspectives and caveats. *J Brazil Chem Soc* 11:551–561
165. Francis F, Lognay G, Wathelet JP, Haubruge E (2002) Characterisation of aphid myrosinase and degradation studies of glucosinolates. *Arch Insect Biochem Physiol* 50:173–182
166. Müller C (2009) Interactions between glucosinolate- and myrosinase-containing plants and the sawfly *Athalia rosae*. *Phytochem Rev* 8:121–134
167. Francis F, Haubruge E, Gaspar C (2000) Influence of host plants on specialist/generalist aphids and on the development of *Adalia bipunctata* (Coleoptera: Coccinellidae). *Eur J Entomol* 97:481–485
168. Bridges M, Jones AME, Bones AM, Hodgson C, Cole R, Bartlet E, Wallsgrove R, Karapapa VK, Watts N, Rossiter JT (2002) Spatial organization of the glucosinolate-myrosinase system in brassica specialist aphids is similar to that of the host plant. *Proc R Soc Lond B* 269:187–191
169. Müller C, Brakefield PM (2003) Analysis of a chemical defense in sawfly larvae: easy bleeding targets predatory wasps in late summer. *J Chem Ecol* 29:2683–2694

170. Boevé JL, Schaffner U (2003) Why does the larval integument of some sawfly species disrupt so easily? The harmful hemolymph hypothesis. *Oecologia* 134:104–111
171. Ohara Y, Nagasaka K, Ohsaki N (1993) Warning coloration in sawfly *Athalia rosae* larva and concealing coloration in butterfly *Pieris rapae* larva feeding on similar plants evolved through individual selection. *Res Popul Ecol* 35:223–230
172. Schaffner U, Boevé JL, Gfeller H, Schlunegger UP (1994) Sequestration of *Veratrum* alkaloids by specialist *Rhadinoceraea nodicornis* Konow (Hymenoptera, Tenthredinidae) and its ecoethological implications. *J Chem Ecol* 20:3233–3250
173. Heads PA, Lawton JH (1985) Bracken, ants and extrafloral nectaries. III. How insect herbivores avoid ant predation. *Ecol Entomol* 10:29–42
174. Müller C, Wittstock U (2005) Uptake and turn-over of glucosinolates sequestered in the sawfly *Athalia rosae*. *Insect Biochem Mol Biol* 35:1189–1198
175. Müller C, Zwaan BJ, de Vos H, Brakefield PM (2003) Chemical defence in a sawfly: genetic components of variation in relevant life-history traits. *Heredity* 90:468–475
176. Opitz SEW, Mix A, Winde IB, Müller C (2011) Desulfation followed by sulfation: metabolism of benzylglucosinolate in *Athalia rosae* (Hymenoptera: Tenthredinidae). *Chembiochem* 12:1252
177. Abdalsamee MK, Giampà M, Niehaus K, Müller C (2014) Rapid incorporation of glucosinolates as a strategy used by a herbivore to prevent activation by myrosinases. *Insect Biochem Mol Biol* 52:115–123
178. Yang RSH, Wilkinson CF (1973) Sulfotransferases and phosphotransferases in insects. *Comp Biochem Physiol* 46:717–726
179. Smith JN (1955) Comparative detoxication. 4. Ethereal sulphate and glucoside conjugations in insects. *Biochem J* 60:436–442
180. Homolya L, Várádi A, Sarkadi B (2003) Multidrug resistance-associated proteins: export pumps for conjugates with glutathione, glucuronate or sulfate. *Biofactors* 17:103–114
181. Liu S, Zhou S, Tian L, Guo E, Luan Y, Zhang J, Li S (2011) Genome-wide identification and characterization of ATP-binding cassette transporters in the silkworm, *Bombyx mori*. *BMC Genomics* 12:491
182. Discher S, Burse A, Tolzin-Banasch K, Heinemann SH, Pasteels JM, Boland W (2009) A versatile transport network for sequestering and excreting plant glycosides in leaf beetles provides an evolutionary flexible defense strategy. *Chembiochem* 10:2223–2229
183. Kuhn J, Pettersson EM, Feld BK, Burse A, Termonia A, Pasteels JM, Boland W (2004) Selective transport systems mediate sequestration of plant glucosides in leaf beetles: a molecular basis for adaptation and evolution. *Proc Natl Acad Sci U S A* 101:13808–13813
184. Strauss AS, Peters S, Boland W, Burse A (2013) ABC transporter functions as a pacemaker for sequestration of plant glucosides in leaf beetles. *eLife* 2, e01096
185. Kazana E, Pope TW, Tibbles L, Bridges M, Pickett JA, Bones AM, Powell G, Rossiter JT (2007) The cabbage aphid: a walking mustard oil bomb. *Proc R Soc Lond B* 274:2271–2277
186. Vanhaelen N, Haubruege E, Lognay G, Francis F (2001) Hoverfly glutathione S-transferases and effect of Brassicaceae secondary metabolites. *Pest Biochem Physiol* 71:170–177
187. Pratt C, Pope TW, Powell G, Rossiter JT (2008) Accumulation of glucosinolates by the cabbage aphid *Brevicoryne brassicae* as a defense against two coccinellid species. *J Chem Ecol* 34:323–329
188. Chaplin-Kramer R, Kliebenstein DJ, Chiem A, Morrill E, Mills NJ, Kremen C (2011) Chemically mediated tritrophic interactions: opposing effects of glucosinolates on a specialist herbivore and its predators. *J Appl Ecol* 48:880–887
189. Bayhan SÖ, Ulusoy MR, Bayhan E (2007) Is the parasitization rate of *Diaearetiella rapae* influenced when *Brevicoryne brassicae* feeds on *Brassica* plants? *Phytoparasitica* 35:146–149
190. Husebye H, Arzt S, Burmeister WP, Härtel FV, Brandt A, Rossiter JT, Bones AM (2005) Crystal structure at 1.1 Å resolution of an insect myrosinase from *Brevicoryne brassicae* shows its close relationship to β-glucosidases. *Insect Biochem Mol Biol* 35:1311–1320

191. Jones AME, Bridges M, Bones AM, Cole R, Rossiter JT (2001) Purification and characterisation of a non-plant myrosinase from the cabbage aphid *Brevicoryne brassicae* (L.). Insect Biochem Mol Biol 31:1–5
192. Pontoppidan B, Ekbom B, Eriksson S, Meijer J (2001) Purification and characterization of myrosinase from the cabbage aphid (*Brevicoryne brassicae*), a brassica herbivore. Eur J Biochem 268:1041–1048
193. Agerbirk N, Vos M, Kim JH, Jander G (2008) Indole glucosinolate breakdown and its biological effects. Phytochem Rev 8:101–120
194. Aldrich JR, Avery JW, Lee CJ, Graf JC, Harrisons DJ, Bin F (1996) Semiochemistry of cabbage bugs (Heteroptera: Pentatomidae: *Eurydema* and *Murgantia*). J Entomol Sci 31:172–182
195. Dawson GW, Griffiths DC, Pickett JA, Wadhams LJ, Woodcock CM (1987) Plant-derived synergists of alarm pheromone from turnip aphid, *Lipaphis (Hyadaphis) erysimi* (Homoptera, Aphididae). J Chem Ecol 13:1663–1671
196. Beran F, Mewis I, Srinivasan R, Svoboda J, Vial C, Mosimann H, Boland W, Büttner C, Ulrichs C, Hansson BS, Reinecke A (2011) Male *Phyllotreta striolata* (F.) produce an aggregation pheromone: identification of male-specific compounds and interaction with host plant volatiles. J Chem Ecol 37:85–97
197. Beran F, Pauchet Y, Kunert G, Reichelt M, Wielsch N, Vogel H, Reinecke A, Svatoš A, Mewis I, Schmid D, Ramasamy S, Ulrichs C, Hansson BS, Gershenzon J, Heckel DG (2014) *Phyllotreta striolata* flea beetles use host plant defense compounds to create their own glucosinolate-myrosinase system. Proc Natl Acad Sci U S A 111:7349–7354

# **Chapter 8**

## **Screening of Fungal Endophytes Isolated from Eastern White Pine Needles**

**Mark W. Sumarah, Allison K. Walker, Keith A. Seifert, Adrian Todorov, and J. David Miller**

**Abstract** Foliar endophytes of white pine were isolated from trees at a site in New Brunswick (Canada) and grown in liquid culture. Oxford disk bioassays of the resulting extracts against yeast showed that, of the 86 strains tested, 22 produced antifungal extracts. The resulting extracts were screened by LC-MS and showed variability in metabolite production, but the majority produced pyrenophorol and a number of related derivatives, all of which are known to be antifungal. DNA sequencing of these strains determined that the majority were *Lophodermium nitens*. A strain of white pine blister rust was isolated in liquid culture from young seedlings infected with the pathogen for toxicity testing. Bioassays performed with 5 µM pyrenophorol showed that it significantly reduced the cell dry weight of the pathogen compared to controls. These data support the hypothesis that pyrenophorol-producing *L. nitens* endophytes may increase the tolerance of the host tree to white pine blister rust.

**Keywords** White pine blister rust • Needle endophytes • Pyrenophorol • *Lophodermium* • White pine

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## 8.1 Introduction

Eastern white pine (*Pinus strobus*) is a valuable species for lumber and finished wood products, and it is typically viewed as a species with many value-added opportunities. It was among the first tree species to be commercially harvested on a large scale in Canada, particularly for the ship-building industry [1]. The fungal pathogen *Cronartium ribicola* (white pine blister rust) was introduced separately to British Columbia in 1910 and to Ontario and Quebec in 1911 on nursery stocks [2]. Blister rust spreads by alternating between currant/gooseberry bushes (*Ribes* spp.) and pine trees. In the spring, white pine blister rust spores infect currants and, after growing on this host over the summer, produce spores in the autumn that infect white pine trees through the needles. The disease then spreads into the branch and down the stem until it reaches the trunk, where it kills the infected tree within a few years (Fig. 8.1). Infections are most severe during cool, damp summers, making northern latitudes particularly susceptible to infection. Although there was optimism for breeding and selection programs to produce resistant western white pine (*P. monticola*) lines, these did not meet early expectations for success in either BC [3–5] or the western USA [6–8]. Instead, *C. ribicola* damage has virtually eliminated white pine as a commercial species in the Pacific Northwest of the USA and British Columbia and is a threat in Ontario, Quebec, and the Maritime Provinces. We are studying the alternative strategy of using endophytic fungi to aid in white pine resistance; this idea was inspired by precedence in cool-season fescues.

Over the past 25 years, there was a major investment in research on the role of foliar endophytes of white and red spruce in relation to increased tolerance to spruce budworm (*Choristoneura fumiferana*). Trees infected with natural populations of



**Fig. 8.1** White pine branch infected with *C. ribicola*

toxigenic fungal endophytes of spruce suffer reduced levels of insect damage. Different fungal endophytes collected in the Acadian forests of New Brunswick, eastern Quebec, and Maine produce a wide array of metabolites toxic to insects [1, 9]. Some of these compounds showed modest antifungal activity against yeast [9–11].

Cool-season fescues have a very well understood mutualism with alkaloid-producing endophytes comprising a few species in the Clavicipitaceae. Endophyte-enhanced grasses are favored for use in lawns and golf courses because less pesticide is required for insect control [12]. In vitro studies demonstrated the production of antifungal compounds by these grass endophytes [13–15]. Endophyte-positive grasses have reduced frequency of infection by foliar diseases, including rusts [16, 17]. These results led to our exploration of whether white pine endophytes also produced antifungal compounds.

In our previous work to test this hypothesis, we isolated a small test collection of 35 endophytes from surface-sterilized white pine needles. These were cultured, and cell extracts screened for activity against *Saccharomyces cerevisiae*. In the initial screening, five strains yielded extracts with potent antifungal activity using the Oxford disk assay [18]. The major components were characterized and included the known antifungal compounds pyrenophorol, dihydropyrenophorin, and pyrenophorin. These compounds had previously been reported from an endophyte of *Lycium intricatum* (Solanaceae) collected in the Canary Islands [19]. This plant occurs around the Mediterranean Sea and northwest Africa. These compounds are active against both *S. cerevisiae* and the easily cultured *Microbotryum violaceum* (formerly *Ustilago violacea*) [19]. We confirmed the antifungal activity of pyrenophorol, and the new compounds were also shown to be antifungal. Preliminary DNA sequence analysis indicated that all five strains were *Lophodermium* species [18].

Based on the encouraging results from the first phase of the work, the study was expanded to include more strains and to investigate the taxonomy of these endophytes. This chapter provides the species identities of pyrenophorol-producing white pine endophytes from a larger collection from a site in New Brunswick. Individual trees at this site included grafted branches originating across the range of the Acadian forest in Canada. In addition, we isolated suspension cultures of white pine blister rust and tested the effect of pyrenophorol on growth in vitro.

## 8.2 Materials and Methods

### 8.2.1 Sample Collection and Analysis

White pine needles were collected from 446 trees located in a breeding archive of grafted trees, developed and maintained by J.D. Irving Limited in Sussex, NB, Canada. These trees included branches collected from superior phenotypes (straight and taller compared to the rest of the stand) in New Brunswick, Nova Scotia, Prince Edward Island, and Quebec. The branches were grafted onto rooting stock, i.e., the lower portions of individual small trees with tops cut off. The resulting tree and the

seeds it produces reflect the tree from which the branch was obtained. The needles were surface-sterilized and plated on malt extract agar plates as previously described for spruce needles [10]. From this, 86 isolates were recovered as pure cultures. These isolates were fermented, extracted, and analyzed as described in [18]. Briefly, all 86 isolates were grown in individual 1 L Glaxo bottles containing 2 % malt extract medium. After 3 months, the mycelium was filtered to remove cells, and the filtrate was extracted with two equal volumes of ethyl acetate. The resulting extracts were concentrated by rotary evaporation and then screened for antifungal activity against yeast in disk diffusion assays. Each extract was further analyzed by liquid chromatography-high resolution mass spectrometry (LC-HRMS) on an Agilent 1200 series HPLC, equipped with an Agilent 1100 series binary pump, connected to an Agilent G1969A LC/MSD TOF HRMS. An aliquot (5  $\mu$ L) of each extract was analyzed in both positive and negative electrospray ionization (ESI+ and ESI-) mode with a ZORBAX Eclipse Plus C-18 column using a 1 mL/min flow of CH<sub>3</sub>CN/H<sub>2</sub>O + 0.1 % formic acid (gradient 10:90–95:5 over 5 min). The MS was operated with the following settings: drying gas temperature 350 °C, nebulizer pressure 60 psi, drying gas flow of 13 L/min, and a spray voltage of 4000 V.

The fungal cultures were identified using DNA sequence data from the internal transcribed spacer (ITS) region of rRNA. The ITS region is the formally acknowledged DNA barcode marker for fungal species identification [20]. DNA was extracted from fungal cultures using an UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA). The ITS region (ITS1-5.8S-ITS2) was amplified using Illustra Ready-To-Go™ PCR Beads (GE Healthcare, Waukesha, WI) with ITS5 (5'-TCCTCCGCTTATTGATATGC-3') and ITS4 (5'-GGAAGTAAAAGTCGTAACAAGG-3') primers [20] using the following thermocycler conditions: initial denaturing at 95 °C for 3 min, 34 cycles of denaturing at 95 °C for 60 s, annealing at 56 °C for 45 s, extension at 72 °C for 90 s, and a final extension step of 72 °C for 10 min. Amplification was confirmed using gel electrophoresis, and forward and reverse ITS sequences were obtained by the dideoxy chain-terminating method using sequencing primers ITS5 and ITS4 and ABI PRISM 3100 or ABI PRISM 3130xl automated DNA sequencers (Applied Biosystems, Foster City, CA). Sequences were edited and aligned using Geneious 6.0.4 software (Biomatters). Consensus sequences were compared against the reference NCBI sequence database GenBank using the BLAST search algorithm. The top BLAST matches were subsequently downloaded and a maximum likelihood (ML) analysis was performed in PHYML using 516 informative characters (bp) from the ITS region [21, 22].

### 8.2.2 White Pine Blister Rust Cultures

To determine if *Cronartium ribicola* was sensitive to pyrenophorol, isolation and in vitro culture of the pathogen was required. *C. ribicola* was isolated by strictly following the methods of Kinloch and Duper ([23]; personal communications,

G. Dupper, USDA Forest Service, 2009). To this end, young white pine seedlings (J.D. Irving, Limited) were cultivated in growth chambers and infected with *C. ribicola* collected from the alternative host. As soon as symptoms appeared, seedlings were transferred to the lab under cool conditions, and needles were harvested, surface-sterilized and plated on the complex agar medium of [24] as modified by [23], and incubated at 10 °C. Emerging colonies of *C. ribicola* were isolated into pure culture on the same medium and allowed to grow for up to a few weeks. As reported by [23], the cultures did not develop particularly well on solid media and were subsequently maintained as liquid cultures in 2 % malt extract (50 mL in 250 mL Erlenmeyer flasks) under stationary conditions. These were maintained by serial transfer into fresh medium every 4–6 weeks at 10 °C.

The bioassay method was developed with cultures of *M. violaceum* to determine dose-response relationships for both pyrenophorol and the positive control, nystatin (Sigma, Canada). Cultures were diluted (7:3) in fresh media, and 10 mL transferred to sterile 50 mL Erlenmeyer flasks. Nystatin was dissolved in DMSO to deliver doses of 0.05, 5, and 50 µM in the 10 mL cultures in two experiments each done in triplicate. Controls were performed in four sets of triplicates. Cultures were placed on a rotary table shaker and incubated for 3–4 days at 25 °C with slight agitation. Cells were extracted from the cultures pooled in triplicate using a Millipore 250 mL filter holder equipped with a 47 mm diameter 0.45 µm pore size Teflon™ filter. Suction was applied for approximately 5 min following full filtration of the medium. Cells were removed from the membrane using a spatula and transferred to pre-weighed 1.5 mL plastic tubes. Wet cells were frozen and freeze-dried (NESLAB CC-100) for 48 h. Dry cells were re-suspended in PBS buffer and sonicated for 2 h at 4 °C. Cells were solubilized with 1 mL of cold PBS buffer (11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The tubes were agitated gently at room temperature for 10 min and then sonicated for 2 h at 4 °C. The lysed mixture was centrifuged at 17 000 g for 2 min and the supernatants transferred to new tubes. Protein content was determined using the Bradford method. Diluted supernatant was combined with the Bradford reagent (Bio-Rad) in a 1:1 ratio. BSA was used as a standard.

Experiments with *M. violaceum* determined the minimum effective dose of nystatin for testing with the available culture material of *C. ribicola*. Previous studies demonstrated that the toxicity of pyrenophenol was similar to nystatin to this fungus [18, 19]. The experiments with *C. ribicola* were conducted as above with a single dose of pyrenophorol. Pyrenophorol and nystatin were dissolved in DMSO to deliver 5 µM in 10 mL cultures at least in triplicate. Replicate cultures were also tested with 10 µM nystatin and were incubated in stationary culture for 10–12 days at 25 °C. After incubation, cells were extracted from individual cultures by high-speed centrifugation (20,000 rpm, 20 min). Pellets were washed with Milli-Q water, frozen, and freeze-dried. The remaining protocol was as above. Pyrenophorol (with purity greater than 98 % based on <sup>1</sup>H NMR) was isolated from fungal strain CBS 127941 following the methods of Sumarah et al. [18]. ANOVA followed by a Tukey's test were used to evaluate differences in dry weight (SYSTAT v.13).

### 8.3 Results and Discussion

The initial screening of the 86 extracts against *S. cerevisiae* identified 22 strains producing metabolites that showed zones of inhibition ranging from 0.5 to 4 mm in diameter (Table 8.1). DNA sequencing showed that the majority of the endophytes isolated were species of the fungal genus *Lophodermium* (Ascomycota; Leotiomycetes; Rhytismatales; Rhytismataceae; Fig. 8.3). The majority of the

**Table 8.1** List of active strains including species identification, metabolite production, and results of Oxford disk assays

strain #	Species identification (ITS sequence)	Closest GenBank ITS match	Metabolite production	Antifungal disk test (mm)
3664-1A	<i>Lophodermium nitens</i> 98/99	AY465520.1	Produces a large amount of pyrenophorol only	0.5
829-1A	<i>Lophodermium nitens</i> 95/99	AY465520.1	Produces mostly pyrenophorol in addition to dihydropyrenophorin and some minor related derivatives	1
3658-1A	<i>Lophodermium nitens</i> 95/99	AY465520.1	Produces pyrenophorol, dihydropyrenophorin, and some minor related derivatives	2.5
820-1A	<i>Lophodermium nitens</i> 95/99	AY465520.1	Produces pyrenophorol, dihydropyrenophorin, and some minor related derivatives	0.5
931-1A	<i>Lophodermium nitens</i> 98/99	AY465520.1	Produces mostly pyrenophorol in addition to dihydropyrenophorin and some minor related derivatives	1
84581-2A	<i>Lophodermium nitens</i> 98/99	AY465520.1	No pyrenophorol production, extract contains a few unidentified compounds	1.5
1058-2A	<i>Phaeomoniella niveniae</i> 96/100	JQ044435.1	Two unidentified peaks in extract	1
886-1A	<i>Celosporium laricicola</i> 98/100	FJ997287.1	Two unidentified peaks in extract	2
831-2A	<i>Lophodermium nitens</i> 97/99	AY465520.1	No pyrenophorol production, extract contains a number of compounds reported in Sumarah et al. (2011)	0.5
3601-1A	<i>Sydiowia polyspora</i> 100/100	AM921728.1	Two unidentified peaks in extract	1
804-1A	<i>Lophodermium nitens</i> 100/100	AY465520.1	Produces mostly pyrenophorol and a few related derivatives	0.5

(continued)

**Table 8.1** (continued)

strain #	Species identification (ITS sequence)	Closest GenBank ITS match	Metabolite production	Antifungal disk test (mm)
928-2A	<i>Lophodermium nitens</i> 97/99	AY465520.1	Produces pyrenophorol, dihydropyrenophorin, pyrenophorin, and one unknown derivative	1.5
920-2A	<i>Lophodermium nitens</i> 99/100	AY465520.1	Produces pyrenophorol, dihydropyrenophorin, pyrenophorin, and one unknown derivative	1
837-1A	<i>Lophodermium nitens</i> 99/100	AY465520.1	Produces pyrenophorol, dihydropyrenophorin, and one unknown derivative	1
3632-1B	<i>Lophodermium nitens</i> 99/100	AY465520.1	No pyrenophorol production, extract contains compounds reported in Sumarah et al. (2011) and a few unknowns	4
3609-2B	<i>Lophodermium nitens</i> 99/100	AY465520.1	Produces a large amount of pyrenophorol only	0.5
847-2A	<i>Lophodermium nitens</i> 99/99	AY465520.1	Produces pyrenophorol, dihydropyrenophorin, pyrenophorin	1.5
828-2A	<i>Lophodermium nitens</i> 97/99	AY465520.1	Produces a small amount of pyrenophorol and two unidentified compounds	1.5
84581-2B	<i>Lophodermium nitens</i> 98/99	AY465520.1	Produces pyrenophorol, dihydropyrenophorin, and two related derivatives	2
3619-2B	<i>Celosporium laricicola</i> 99/100	FJ997287.1	Produces a few unidentified peaks	1.5
1991-2A	<i>Fomitopsis rosea</i> 99/100	DQ491410.1	Produces one major unidentified peak and a number of smaller ones	0.5
849-2A	<i>Lophodermium nitens</i> 99/99	AY465520.1	Produces a small amount of pyrenophorol	1.5
CBS127942	<i>Lophodermium piceae</i> 99/95	AY971734.1	[18]	2.5
CBS127941	<i>Lophodermium nitens</i> 98/99	AY465520.1	[18]	1.5
CBS127940	<i>Lophodermium nitens</i> 100/78	AF426058.1	[18]	1.5
CBS127938	<i>Lophodermium nitens</i> 97/99	AY465520.1	[18]	2
CBS127939	<i>Lophodermium nitens</i> 98/99	AY465520.1	[18]	3

pyrenophorol-producing strains had high (>97 %) ITS sequence homology with a vouchered isolate of *Lophodermium nitens* collected from the type locality (BPI842077/GenBank AY465520, Muskoka, Ontario). The ML analysis placed fungal strains identified in the BLAST search as *L. nitens* within a well-supported clade containing sequenced vouchered specimens examined during this study (99 % ML bootstrap support; Fig. 8.3). Based on our BLAST results and ML phylogenetic analysis, the majority of fungal strains producing pyrenophorol (18 out of 19 strains) formed a monophyletic group with sequences of *L. nitens*, with one pyrenophorol-producing isolate (CBS 127942) sister to *L. piceae* (Fuckel) Höhn. LC-MS analysis indicated that 14 out of 17 strains of *L. nitens* isolated in this study produced pyrenophorol. Sumarah et al. reported that three out of the four *L. nitens* white pine strains produced pyrenophorol in addition to its production by the single strain identified as *L. piceae*. In addition to pyrenophorol, several strains produced dihydropyrenophorin, pyrenophorin, related derivatives, and new sesquiterpenes reported in [18] (Table 8.1). Of note is the great variability in secondary metabolite production from these strains identified as *L. nitens* by ITS sequencing. The secondary metabolites from the five antifungal non-*Lophodermium* strains were analyzed by LC-MS but were not further characterized.

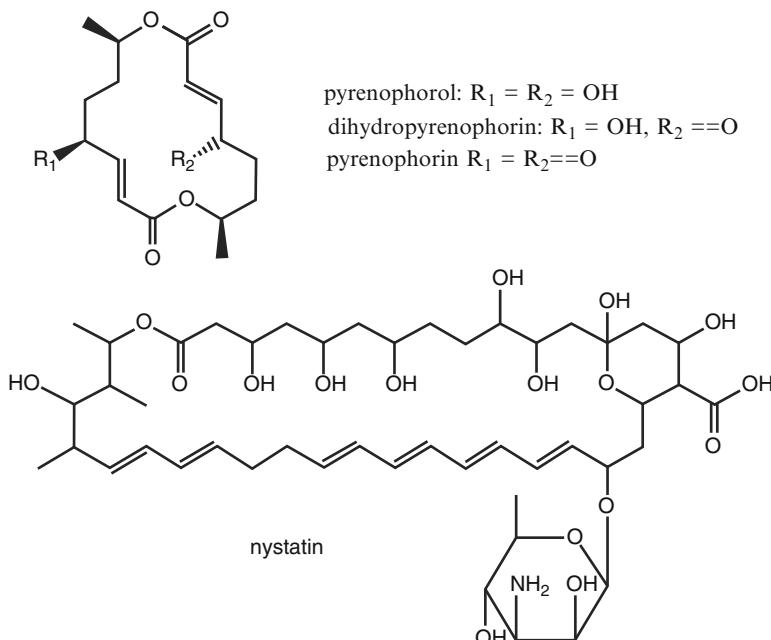
Deckert et al. suggested that *L. nitens* was the most commonly isolated needle endophyte of *P. strobus* in stands north of Toronto, Ontario [25]. In our collections from the Acadian forest, it was a commonly isolated needle endophyte of *P. strobus*. Sixteen species were recognized from pine in the most recent monograph of the genus *Lophodermium* [26]. A molecular phylogeny analysis including pine species revealed that *Lophodermium* is a polyphyletic genus, with pine isolates forming a monophyletic sister group to *Lophodermium* species isolated from more distantly related host plants (Poaceae, Ericales) from the Southern Hemisphere [27]. The genus has a worldwide distribution, and its species are not known to be host specific [26]. However, high levels of genetic variation were found in Mexican populations of *L. nitens* corresponding to host phylogeography [28]. The amount of ITS variation visible in Fig. 8.3 is evidence that *L. nitens* as it is currently understood may represent a species complex that requires further taxonomic work and multigene phylogenies to unravel. *Lophodermium piceae* is known from both pine and spruce, and requires further taxonomic study as it may also represent a species complex [29]. Specimens of *Lophodermium* on North American pines are often identified as *L. pinastri* (Schrad.) Chevall. However, Minter suggested that reports of *L. pinastri* from outside Europe were based on misidentifications of other *Lophodermium* species [26].

Toxicity testing showed that a dose of 5 µM of both pyrenophorol and nystatin reduced the cell dry weight of the *C. ribicola* suspension cultures (Fig. 8.4). Although the relative impact of pyrenophorol on growth was similar to that of nystatin, there was no change to the protein content/cell (data not shown). Pyrenophorol has anthelmintic, nematicidal, and antibiotic properties but is not very phytotoxic [30, 31]. The primary toxic effect of nystatin is binding to the membrane sterol of fungi, ergosterol which causes the formation of pores in the membrane and thus the death of the fungus. However, nystatin is also known to inhibit protein synthesis in yeast [32], which was not observed for pyrenophorol in our experiments. The potency of nystatin is dependent on the nystatin-sterol affinity in the organism. The membranes

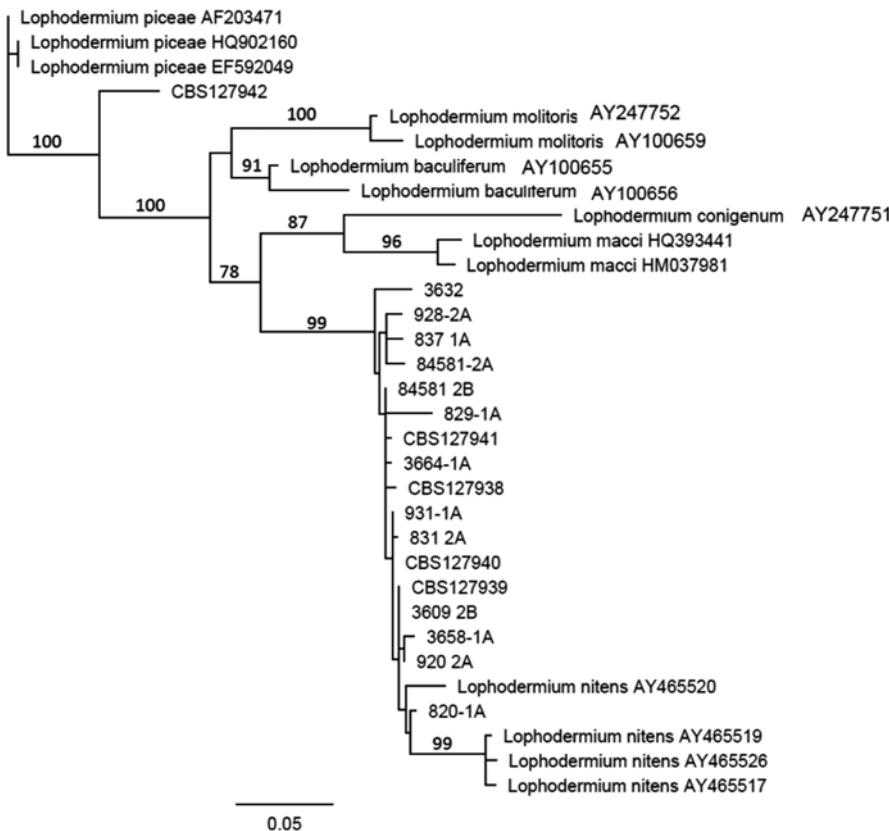
of *C. ribicola* have a high lipid content [33] but rust fungi do not accumulate ergosterol, but rather 24-ethyl-cholest-7,24(28)-dienol [34]. Although pyrenophorol and nystatin share some structural features, the mode of action is apparently different.

## 8.4 Conclusions

The antifungal macrolide pyrenophorol (Fig. 8.2) was produced as the major secondary metabolite by the majority of the bioactive fungal endophyte strains isolated from this site. As noted, the source of these isolates was a breeding plantation comprising grafted branches from mature trees in Eastern Canada. Conifer endophytes are known to be horizontally transmitted [35]. Our data over the past 15 years suggests that, if a tree is infected under optimal conditions either in the laboratory or in nature, the original endophyte strain continues to dominate in all branches in that tree (e.g., [36]). The source of the endophytes discussed here was likely the original tree from the field. The branches used as the genetic source of the trees at the Sussex location were collected by professional foresters from mature trees that were superior. We hypothesize that the strains we tested originated in these superior trees from the field around the region. These strains produced pyrenophorol as the major antifungal constituent, a compound known to be equally active against to *S. cerevisiae* (an Ascomycetous yeast) and the rust *C. ribicola* and while not being very phytotoxic [30].

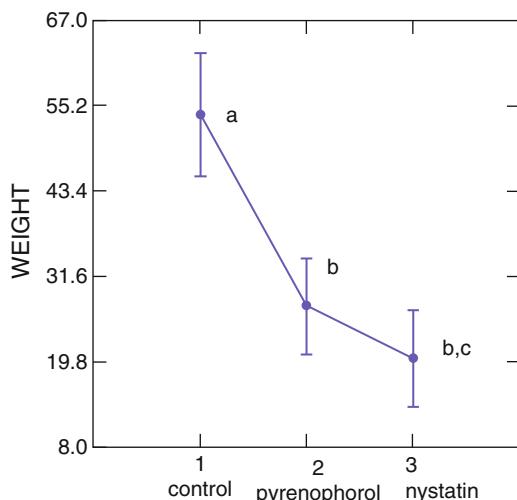


**Fig. 8.2** Structures of the antifungal compounds pyrenophorol, dihydropyrenophorin, and pyrenophorin, with the positive control nystatin for comparison



**Fig. 8.3** Phylogenetic relationships of selected pyrenophorol-producing strains. Tree represents a PHYML maximum likelihood analysis of 32 taxa based on ITS nrDNA sequence data (516 bp). Branch labels refer to PHYML bootstrap support values >70 % based on 1000 replicates. Bar indicates 0.05 nucleotide substitutions per site

**Fig. 8.4** The effect of pyrenophorol on *C. ribicola*; weight is in mg. The concentration of nystatin or pyrenophorol was 5  $\mu$ M. Points with different letters are significantly different by ANOVA followed by a Tukey test ( $p < 0.000$ )



After we began our work, Ganley et al. [6] reported that mixed inoculations of uncharacterized endophytes in western white pine seedlings resulted in tolerance independent to white pine blister rust. These authors ruled out the production of antifungal compounds as a mechanistic basis. They suggested that the tolerance was a form of induced resistance. Our data continues to support competitive exclusion as an important mechanism [18]. *L. nitens* CBS 127941 along with some related strains have been successfully inoculated in white pine seedlings [37]. Studies are underway in several locations to attempt to infect seedlings infected with pyrenophorol-producing strains with *C. ribicola* as well as with other species of pine endophytes found to produce potently antifungal compounds [38].

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## References

1. Miller JD (2011) Foliar endophytes of spruce species found in the Acadian forest: basis and potential for improving the tolerance of the forest to spruce budworm. In: Pirttil AM, Frank AC (eds) Endophytes of forest trees: biology and applications, forestry sciences, vol 80. Springer, Berlin, pp 237–249
2. Allen EA, Humble LM (2002) Nonindigenous species introductions: a threat to Canada's forests and forest economy. *Canad J Plant Pathol* 24:103–110
3. Hunt RS (2009) History of western white pine and blister rust in British Columbia. *For Chronicle* 85:516–520
4. Geils BW, Hummer KE, Hunt RS (2010) White pines, *Ribes*, and blister rust: a review and synthesis. *For Pathol* 40:147–185
5. Meagher MD, Hunt RS (1996) Heritability and gain of reduced spotting vs blister rust on western white pine in British Columbia, Canada. *Silvae Genetica* 45:75–81
6. Ganley RJ, Snieszko RA, Newcombe G (2008) Endophyte-mediated resistance against white pine blister rust in *Pinus monticola*. *For Ecol Manage* 255:2751–2760
7. Kearns HJS, Jacobi WJ (2007) The distribution and incidence of white pine blister rust in central and southeastern Wyoming and Northern Colorado. *Can J Forest Res* 37:462–472
8. Kinloch BB Jr, Davis DA, Burton D (2008) Resistance and virulence interactions between two white pine species and blister rust in a 30-year field trial. *Tree Genet Genome* 4:65–74
9. Sumarah MW, Miller JD (2009) Anti-insect secondary metabolites from fungal endophytes of conifer trees. *Nat Prod Commun* 4:1497–1504
10. Sumarah MW, Puniani E, Blackwell BA, Miller JD (2008) Characterization of polyketide metabolites from foliar endophytes of *Picea glauca*. *J Nat Prod* 71:1393–1398
11. Sumarah MW, Puniani E, Sørensen D, Blackwell BA, Miller JD (2010) Secondary metabolites from anti-insect extracts of endophytic fungi isolated from *Picea rubens*. *Phytochemistry* 71:760–765
12. Kuldau G, Bacon C (2008) Clavicipitaceous endophytes: their ability to enhance resistance of grasses to multiple stresses. *Biol Control* 46:57–71
13. Christensen MJ (1996) Antifungal activity in grasses infected with *Acremonium* and *Epichloë* endophytes. *Australias Plant Path* 25:186–191

14. Clay K, Schardl C (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *Am Nat* 160:S99–S127
15. Clay K (1989) Clavicipitaceous endophytes of grasses: their potential as biocontrol agents. *Mycol Res* 92:1–12
16. Li C-J, Gao J-H, Nan Z-B (2007) Interactions of *Neotyphodium gansuense*, *Achnatherum inebrians* and plant-pathogenic fungi. *Mycol Res* 111:1220–1227
17. Siegel MR, Latch GCM (1991) Expression of antifungal activity in agar culture by isolates of grass endophytes. *Mycologia* 83:529–537
18. Sumarah MW, Kesting JR, Sørensen D, Miller JD (2011) Antifungal metabolites from fungal endophytes of *Pinus strobus*. *Phytochemistry* 72:1833–1837
19. Zhang W, Krohn K, Egold H, Draeger S, Schulz B (2008) Diversity of antimicrobial pyrenophorol derivatives from an endophytic fungus, *Phoma* sp. *Eur J Org Chem* 25:4320–4328
20. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W (2012) Fungal barcoding consortium. *Proc Natl Acad Sci U S A* 109:6241–6246
21. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52:696–704
22. Schmitt I, Barker FK (2009) Phylogenetic methods in natural product research. *Nat Prod Rep* 26:1585–1602
23. Kinloch BB, Dupper GE (1996) Genetics of *Cronartium ribicola* 1. Axenic culture of haploid clones. *Can J Bot* 74:456–460
24. Gresshoff PM, Doy CH (1972) Development and differentiation of haploid *Lycopersicon esculentum* (tomato). *Planta* 107:161–170
25. Deckert RJ, Hsiang T, Peterson RL (2002) Genetic relationships of endophytic *Lophodermium nitens* isolates from needles of *Pinus strobus*. *Mycol Res* 106:305–313
26. Minter DW (1981) *Lophodermium* on pines. *Mycological Papers* 147:1–75
27. Ortiz-García S, Gernandt DS, Stone JK, Johnston PR, Chapela IH, Salas-Lizana R, Alvarez-Buylla ER (2003) Phylogenetics of *Lophodermium* from pine. *Mycologia* 95:846–859
28. Salas-Lizana R, Santini NS, Miranda-Pérez A, Piñero DI (2012) The Pleistocene glacial cycles shaped the historical demography and phylogeography of a pine fungal endophyte. *Micol Prog* 11:569–581
29. Müller MM, Valjakka R, Hantula J (2007) Genetic diversity of *Lophodermium piceae* in South Finland. *For Pathol* 37:329–337
30. Chrysaiyi-Tokousbalides M, Machera K, Kyriakopoulou K, Aliferis KA, Schrader KK, Tsoutsanis I, Anastasiadou P (2007) Comparative toxicity of the phytotoxins (8R,16R)-(-)-pyrenophorin and (5S,8R,13S,16R)-(-)-pyrenophorol on aquatic organisms. *Bull Environ Contam Toxicol* 79:499–503
31. Shiomii K, Omura S (2004) Antiparasitic agents produced by microorganisms. *Proc Jpn Acad Ser B Phys Biol Sci* 80:245–258
32. Alonso MA, Vázquez D, Carrasco L (1979) Compounds affecting membranes that inhibit protein synthesis in yeast. *Antimicrob Agents Chemother* 16:750–756
33. Tulloch AP, Ledingham GA (1962) The component fatty acids of oils found in spores of plant rusts and other fungi. Part II *Canad J Microbiol* 8:379–387
34. Weete JD, Abril M, Blackwell M (2010) Phylogenetic distribution of fungal sterols. *PLoS One* 5(5), e10899
35. Miller JD, Cherid H, Sumarah MW, Adams GW (2009) Horizontal transmission of the *Picea glauca* foliar endophyte *Phialocephala scopiformis* CBS 120377. *Fungal Ecol* 2:98–101
36. Frasz SL, Walker AK, Nsiamia TK, Adams GA, Miller JD (2014) Distribution of the foliar fungal endophyte *Phialocephala scopiformis* and its toxin in the crown of a mature white spruce tree as revealed by chemical and qPCR analyses. *Can J Forest Res* 44:1138–1143
37. Frasz SL (2014) The development and comparison of quantitative PCR assays and enzyme-linked immunosorbent assays as rapid detection methods for specific foliar endophytes. Department of Biology, Carleton University, Ottawa, Ontario, MSc thesis
38. Richardson SN, Walker AK, Nsiamia TK, McFarlane J, Sumarah MW, Ibrahim A, Miller JD (2014) Griseofulvin-producing *Xylaria* endophytes of *Pinus strobus* and *Vaccinium angustifolium*: evidence for a conifer-understory species endophyte ecology. *Fungal Ecol* 11:107–113

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