Fungal Biology

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Biosynthesis and Molecular Genetics of Fungal Secondary Metabolites, Volume 2

Fungal Biology

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Fungal biology has an integral role to play in the development of the biotechnology and biomedical sectors. It has become a subject of increasing importance as new fungi and their associated biomolecules are identified. The interaction between fungi and their environment is central to many natural processes that occur in the biosphere. The hosts and habitats of these eukaryotic microorganisms are very diverse; fungi are present in every ecosystem on Earth. The fungal kingdom is equally diverse consisting of seven different known phyla. Yet detailed knowledge is limited to relatively few species. The relationship between fungi and humans has been characterized by the juxtaposed viewpoints of fungi as infectious agents of much dread and their exploitation as highly versatile systems for a range of economically important biotechnological applications. Understanding the biology of different fungi in diverse ecosystems as well as their interactions with living and nonliving is essential to underpin effective and innovative technological developments. This series will provide a detailed compendium of methods and information used to investigate different aspects of mycology, including fungal biology and biochemistry, genetics, phylogenetics, genomics, proteomics, molecular enzymology, and biotechnological applications in a manner that reflects the many recent developments of relevance to researchers and scientists investigating the Kingdom Fungi. Rapid screening techniques based on screening specific regions in the DNA of fungi have been used in species comparison and identification, and are now being extended across fungal phyla. The majorities of fungi are multicellular eukaryotic systems and, therefore, may be excellent model systems by which to answer fundamental biological questions. A greater understanding of the cell biology of these versatile eukaryotes will underpin efforts to engineer certain fungal species to provide novel cell factories for production of proteins for pharmaceutical applications. Renewed interest in all aspects of the biology and biotechnology of fungi may also enable the development of "one pot" microbial cell factories to meet consumer energy needs in the 21st century. To realize this potential and to truly understand the diversity and biology of these eukaryotes, continued development of scientific tools and techniques is essential. As a professional reference, this series will be very helpful to all people who work with fungi anduseful both to academic institutions and research teams, as well as to teachers, and graduate and postgraduate students with its information on the continuous developments in fungal biology with the publication of each volume.

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Preface

The introduction of penicillin in the 1940s not only revolutionized medicine but also triggered the screening of microbes for the production of bioactive secondary metabolites. Since then, respective attempts led to the discovery of tens of thousands of substances and revealed filamentous fungi as invaluable resources that produce a large number and diversity of chemical structures. Not only Ascomycetes like the well-studied *Aspergillus, Penicillium,* and *Fusarium* species but also Basidiomycetes are among the currently known highly prolific producers of secondary metabolites. During recent years, the potential of underexplored fungi, such as the plant-inhabiting endophytes or marine fungi, as a reservoir of novel biologically active substances has been recognized and the role of secondary metabolites in the interaction of fungi with their biotic environment came into focus.

The increasing availability of "omics" technologies opened up new avenues in fungal research. These approaches allow comprehensive system-level analyses and hence the identification of the complete genomic inventory of secondary metabolic gene clusters in a given fungus, the detailed study of their activation, and the global profiling of the resulting metabolites. Taking into account that fungi harbor large numbers of secondary metabolism-associated cryptic gene clusters, "omics"-guided approaches together with genetic engineering allow the exploitation of fungi for novel products. Furthermore, recent developments such as metagenomics and metatranscriptomics currently have found their way into research on fungal secondary metabolism. These techniques bear great potential by enabling to screen even unculturable fungi in their natural microbial communities and habitats for genes involved in the production of novel compounds and hence will contribute to natural product discovery from the large pool of the untapped fungal biodiversity.

In appreciation of the tremendous progress in the research on fungal secondary metabolism during recent years, we are pleased to present this book, the second volume on the *Biosynthesis and Molecular Genetics of Fungal Secondary Metabolites* within the Springer book series on Fungal Biology. This book aims to continue the compilation of the best-studied fungal secondary metabolites contained in the first volume by adding aspects on regulatory key players and epigenetic control of their biosynthesis, genomics- and metabolomics-guided approaches for a further unearthing of the potential of fungi as resources of novel biologically active

substances, the use of secondary metabolite profiles in fungal chemotaxonomy, less-exploited substances and their producers, and the biological roles of secondary metabolites in organismic interactions.

Fungal secondary metabolites significantly impact mankind as they comprise substances contributing to human well-being such as antibiotics, antivirals, immunosuppressives, antitumor, and anticholesterolemic agents, as well also toxins that act as virulence factors in their respective hosts and that may cause health problems by contaminating our food and indoor environment. For both, the use of beneficial substances in medicine and pharmaceutical industry and the risk reduction of fungal metabolites with adverse health effects, a detailed knowledge and understanding of fungal secondary metabolism are fundamental. The recent emergence of high-throughput "omics" techniques constitutes an important step in this regard and will further significantly contribute to the discovery of novel fungal metabolites.

We are grateful to all the authors who contributed to this book and we hope that this book will help the reader to obtain novel insights into the current status and future directions of this fascinating field.

Susanne Zeilinger
Juan-Francisco Martín
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Chapter 1
Fungal Secondary Metabolites in the "OMICS" Era

Susanne Zeilinger, Carlos García-Estrada and Juan-Francisco Martín

Definitions and Historical Perspective on Fungal Secondary Metabolites

Fungi produce an enormous array and variety of secondary metabolites. Several of these substances are of industrial and medical importance; hence fungal secondary metabolism represents an exciting topic of not only scientific but also commercial interest.

The definition "secondary metabolism" was coined by plant physiologists in the second half of the nineteenth century [1]. In 1873, the plant physiologist Julius Sachs made the following definition [2]:

> One can designate as by-products of metabolism such compounds which are formed during metabolism but which are no longer used in the formation of new cells. …Any importance of these compounds for the inner economy of the plant is so far unknown.

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Although this definition is still appropriate, it has been modified over time, also depending on the respective person/author. Frequently used current definitions of fungal secondary metabolites comprise the following: Secondary metabolites are usually compounds of low molecular weight, they are produced as families of related substances from a few precursors derived from primary metabolism, their production occurs during a limited stage of the cell cycle and often is correlated with a specific stage of morphological differentiation, they are—unlike metabolites from primary metabolism—unnecessary for growth, and they are of restricted taxonomic distribution [3–7]. However, secondary metabolites are not useless waste products for the producing fungus but serve survival functions. Fungi produce these substances, e.g., for self-protection and defense against predators, for inhibiting competing microorganisms, for communication purposes, for establishing interactions with their biotic environment, and as differentiation effectors [8].

A first report on a fungal secondary metabolite was published in the late nineteenth century [9]. Bartolomeo Gosio purified a crystalline compound from a *Penicillium* culture filtrate that showed antibiotic activity against anthrax bacteria and which was later identified as mycophenolic acid [10, 11]. During the 1920s and 1930s, by isolating more than a hundred new substances from fungi, Harold Raistrick and collaborators uncovered fungi as producers of a variety of secondary metabolites [12]. In 1928, the best-known antibiotic, penicillin, was discovered from *Penicillium notatum* by Sir Alexander Fleming and the development of a method for penicillin production by Howard Florey and colleagues in 1940 [13] opened up a new era in medicine and initiated extensive screening programs for bioactive microbial metabolites. The search continues today and has led to the discovery of tens of thousands of substances. These include on the one hand dozens of useful fungal secondary metabolites that are marketed as antibiotic, antiviral, antitumor, immunosuppressive or hypercholesterolemic agents, but on the other hand also mycotoxins with adverse effects to human, animal, and plant health as those with, for example, cytotoxic, mutagenic, carcinogenic, and teratogenic effects [4].

With the end of the twentieth century, research on fungal secondary metabolites has reached the "omics" era and high-throughput efforts to identify the genes and pathways in secondary metabolite biosynthesis were made possible with the sequencing of an increasing number of fungal genomes. Fungal genome analyses confirmed what has previously been evidenced from efforts to characterize the genes and pathways involved in the biosynthesis of well-explored secondary metabolites exemplified by penicillin [14–16] and cephalosporin [17], that is, the organization of a broad range of fungal secondary metabolism genes in clusters [4]. Most of these secondary metabolism-associated clusters of fungi contain genes encoding large, multimodular, multidomain enzymes such as polyketide synthases or nonribosomal peptide synthetases (NRPS), which build the general structural scaffolds of most secondary metabolites, and additional enzymes and transporters, which make modifications and are required for transport of the product [7]. Typical examples of the clustered organization of secondary metabolism genes also dealt with in volume I of this book [18] comprise those involved in the production of the aforementioned antibiotics—penicillin and cephalosporin C, the pharmacologically active lovastatin,

and of several mycotoxin families such as aflatoxins, ochratoxin A, gibberellins, fusarins, and fusaric acid.

Biosynthetic Routes Involved in Fungal Secondary Metabolite Production

The production of secondary metabolites is interconnected with primary metabolism as it requires energy and significant amounts of carbon and (sometimes) nitrogen. In contrast to the several hundred primary metabolites, microbial secondary metabolites comprise tens of thousands of known compounds and their number is rising every year. This wide range of products is achieved by slight variations of the biosynthesis pathways, whereas the backbones are originating from only a few key precursors derived from primary metabolism such as amino acids and acetyl-CoA [8]. Hence, defined by the key precursors, fungal secondary metabolites can be classified into major groups which are (1) nonribosomal peptides and amino-acid-derived compounds, (2) polyketides and fatty-acid-derived compounds, (3) terpenes, and (4) indole alkaloids [4] (Fig. 1.1):

1. Nonribosomal peptides are composed of both proteinogenic and nonproteinogenic amino acids and are biosynthesized independently of ribosomes function by multimodular NRPS enzymes. A minimal NRPS module consists of an adenylation domain, which activates the substrate via adenylation with adenosine triphosphate (ATP), a thiolation or peptidyl carrier protein domain, which binds the activated substrate to a 4′-phosphopantetheine cofactor and transfers it to a condensation domain for catalyzing peptide bond formation [19]. Nonribosomal peptides may be of various lengths and include substances such as the tripeptide beta-lactams [20, 21], the cyclic undadecapeptide immunosuppressive drug cyclosporine [22], the structurally variable siderophores [23], and the linear peptaibols, which are composed of up to 21 amino acids and typically contain α(alpha)-aminoisobutyric acid (Aib; Chap. 11).
2. Polyketides, which are synthesized by type I polyketide synthase (PKS) enzymes from acetyl-CoA and malonyl-CoA units, are the most abundant fungal secondary metabolites [4]. Fungal PKS contain at least a ketoacyl synthase, an acyl transferase, and a phosphopantetheine attachment site domain and most of these enzymes synthesize a polyketide by repeatedly adding a two-carbon unit to the growing chain [24]. The class of fungal polyketides comprises the mycotoxins aflatoxin [25] and fumonisin (Chap. 10), the pigments bikaverin and fusarubin [26], and the anticholesterolemic agents lovastatin and compactin [27].
   Recently, genome sequencing revealed PKS-NRPS hybrid genes in several fungi. These hybrid enzymes consist of a fungal type I PKS fused to a single, sometime truncated, NRPS module and lead to a larger diversity of structures [28]. PKS-NRPS are key enzymes in the biosynthesis of, for example, fusarin C [29], tenellin [30], and apsyridone [31].

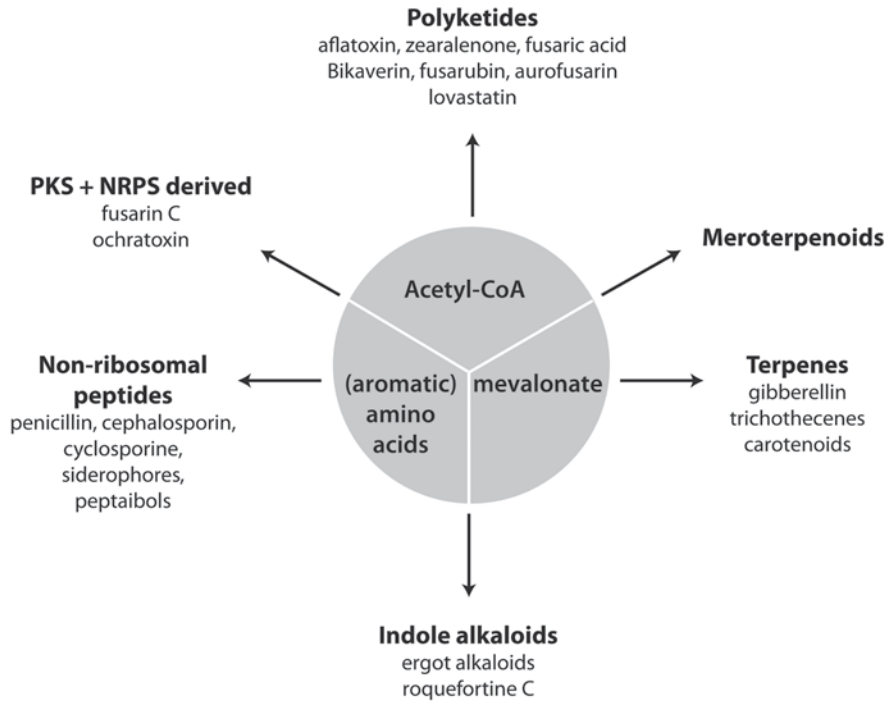

Fig. 1.1 Main classes of fungal secondary metabolites and their precursors. Most fungal secondary metabolites are derived from acetyl-CoA, mevalonate and amino acids and enzymes such as polyketide synthases (PKS), NRPS, hybrid PKS-NRPS, and terpene cyclases produce their respective backbones. Meroterpenoids are hybrid natural products that are produced from terpenoid and polyketide precursors

3. Terpenes such as trichothecenes and gibberellins [26] are composed of several isoprene units derived from the mevalonate pathway. The key enzymes in terpene biosynthesis are terpene cyclases including sesquiterpene cyclases and diterpene cyclases for the formation of complex cyclic terpenes, prenyl transferases for the synthesis of indole diterpenes and phytoene synthases for the formation of carotenoids [32].
4. Indole alkaloids comprise related compounds that are biosynthesized as complex mixtures by the contribution of the shikimic acid pathway and the mevalonate pathway as they are usually derived from the aromatic amino acid tryptophan and dimethlyallyl pyrophosphate [4]. Well-characterized metabolites of this class are the ergot alkaloids mainly produced by plant pathogenic *Claviceps* species [33] and roquefortine C, and related compounds such as meleagrin and glandicoline derived from *Penicillium* species [34].

Fungal Secondary Metabolic Gene Clusters and Their Regulation

Sequencing of fungal genomes has revealed an abundance of genes with a putative function in secondary metabolism and has made apparent that the clustering of these genes is common in fungi [4]. The coordinated activation or repression of the clustered genes frequently is achieved by the presence of a regulatory gene within the cluster that encodes a pathway-specific transcription factor. While this arrangement is exemplified by the *Aspergillus flavus* and *Aspergillus parasiticus* aflatoxin clusters and the *Aspergillus nidulans* sterigmatocystin cluster with *aflR* encoding a cluster-linked transcriptional activator, other clusters such as the penicillin clusters present in *Penicillium chrysogenum, Aspergillus oryzae*, and *A. nidulans* lack known cluster-encoded regulators (Chap. 2) [20, 25].

Besides pathway-specific regulators, fungal secondary metabolism genes are regulated by global, broad domain transcription factors that mediate the fungal response to environmental cues such as nutrient (mainly carbon and nitrogen) availability, pH, light, stress, etc. The Cys_2His_2 zinc finger transcription factors AreA, CreA, and PacC are involved in regulating several secondary metabolism-associated and nonassociated genes in response to nitrogen and carbon availability and pH, respectively, whereas the light-sensing heterotrimeric velvet complex links sexual and asexual development with secondary metabolite production (Chap. 2) [35]. The velvet complex consists of the velvet proteins VeA, VelB, and LaeA—the latter has been discovered through complementation of an *A. nidulans* mutant deficient in secondary metabolism [36, 37]. Deletion of *laeA* in *Aspergillus* blocks several secondary metabolic gene clusters such as those required for the biosynthesis of sterigmatocystin, penicillin, and lovastatin [36]. Similarly, *P. chrysogenum laeA* mutants are defective in the production of penicillin and several other metabolites [38]. Based on the findings that LaeA shows similarity to methyltransferases involved in histone modification and activates secondary metabolite gene expression in *A. nidulans* by counteracting the establishment of heterochromatin marks [39, 40], evidence accumulated that secondary metabolite biosynthesis clusters are epigenetically controlled and exhibit repressive chromatin domains during primary metabolism-favoring conditions (Chap. 3).

The Rise of Global Investigation Approaches in Fungal Secondary Metabolite Research

The substantial progress in the sequencing of fungal genomes made in the past years fueled the development of bioinformatic tools for discovering novel fungal secondary metabolites by genome mining (Chap. 4) [41]. However, as several of these predicted gene clusters remain silent (i.e., are not expressed under laboratory

conditions [42]), additional strategies based on genetic engineering (overexpression of regulators and biosynthetic genes, exchanging endogenous promoters by strong inducible promoters), chemical manipulation of chromatin modifications, and simulation of natural habitats through cocultivation of microorganisms from the same ecosystem have been applied. In several cases these approaches led to the activation of silent gene clusters and the production of hitherto unknown secondary metabolites (reviewed in Chap. 4) [4, 7, 43].

The development of high-throughput platforms initiated a movement away from single-gene analysis to a more global investigation of the whole (fungal) organism and increasingly makes visible the crucial importance of revealing the largely unknown direct relations among genes, proteins, and metabolites. In this context, it is important to be aware of the fact that there is not necessarily a one-to-one relationship between a gene and a (secondary) metabolite and that the metabolite levels are usually a result of many genes and their encoded enzymes [44]. Hence, genome sequencing and transcriptomic/proteomic screening approaches have to be complemented by metabolomics, which globally measures the systems' small molecule metabolites and reflects the phenotype of its underlying genomic, transcriptomic, and proteomic networks [45].

Similar to the genetic level, emerging technologies in mass spectrometry resulted in metabolomic analyses more and more shifting away from the investigation of single-compound classes toward comprehensive system-level analyses (Chap. 6) [46]. In fungal secondary metabolism research, comparative metabolomics is used for chemotaxonomical classification (Chap. 7) and for functional genomics studies, that is, for linking the effect of gene inactivation to a certain metabolic phenotype. The latter is exemplified by the comparative transcriptome and metabolome analyses of *Aspergillus fumigatus* wild-type and Δ(Delta)*veA* mutants revealing that *veA* not only controls the production of gliotoxin but also fumagillin, fumitromorgin G, fumigaclavine C, and glionitrin A [47].

Metabolomics-based approaches can further help to bridge the discrepancy between the genomic potential and the low numbers of actually identified compounds of a specific producer (Chap. 6) [46]. Recent achievements in this direction include the combination of nanospray desorption electrospray ionization (nano-DESI) and imaging mass spectrometry (IMS) for metabolic profiling of living bacterial communities containing multiple species directly from the petri dish without sample preparation [48, 49] and the combination of IMS with a peptidogenomic approach, which led to the identification of arylomycins and their biosynthetic gene cluster in *Streptomyces roseosporus* by monitoring metabolic exchange patterns during interaction with two bacterial pathogens [50]. IMS has also been applied to study inter-kingdom interactions between the bacterium *Pseudomonas aeruginosa* and the fungus *A. fumigatus* interacting on agar plates in which *A. fumigatus* was found to convert *P. aeruginosa* phenazine metabolites into other chemical entities with alternative properties [51]. These examples highlight the remarkable progress made in microbial metabolomics during recent years and the increasing application of the latest metabolomics tools such as IMS to fungi will result in unprecedented insights into secondary metabolism in these organisms.

The Exploitation of Underexplored Fungal Habitats and Lifestyles for New Metabolites

At least 1.5 million fungal species are estimated to exist on earth [52]. Of those, only about 10 % have been isolated or described while the rest may be associated with less explored habitats such as tropical forests, oceans, extreme environments, or other organisms that may harbor large numbers of understudied fungi, and/or they may be uncultivable.

As stated previously, the biosynthesis of several secondary metabolites depends on interactions in the natural environment, and fungi living in association not only with other microbes but also with higher organisms should be considered when hunting for novel substances with potential as biopharmaceuticals. Endophytic fungi that inhabit plant tissues without causing visible damage to their hosts are a promising, although still underexplored source [53]. Indeed, endophytes that produce host plant secondary metabolites with therapeutic value have been occasionally found including examples such as the anticancer drugs paclitaxel [54] and camptothecin [55], and the antidepressant hypericin [56, 57]. In addition, a comparison of 135 isolated metabolites showed that the production of novel structures produced by endophytes is considerably higher than that produced by soil isolates [58]. Chagas et al. (Chap. 8) provide a recent compilation of novel compounds isolated from endophytic fungi during recent years including polyketide and fatty acid, phenylpropanoid, and terpenoid derivatives, as well as nitrogen-containing compounds.

There is increasing evidence that fungi have the potential to adapt to the production of secondary metabolites in response to fungivore attack (Chap. 9). Examples include the specific expression of genes including the transcriptional regulator of sterigmatocystin biosynthesis *aflR* in *A. nidulans* upon attack by *Drosophila melanogaster* larvae [59]; the enhanced amounts of sterigmatocystin, emericellamides, and certain meroterpenoids in springtail-damaged *A. nidulans* colonies [60]; and the enhanced expression of the *easB* and *ausA* polyketide synthase genes in confrontation assays with fruit fly larvae [61, 62].

Certain fungal secondary metabolites may act as pathogenicity factors during plant infection. Among mycotoxins, this was clearly shown for fusaric acid and deoxynivalenol [29, 63]. In the rice blast fungus *Magnaporthe oryzae*, four PKS-NRPS encoding genes (*ace1, syn2, syn6, syn8*) are exclusively expressed during infection of host leaves, which suggests a role of the derived metabolites in pathogencity. However, as single-deletion mutants were still pathogenic on rice [64], multiple mutants and the respective metabolites produced by these PKS-NRPS hybrids have to be characterized in order to decipher the role in infection. The fact that due to functional redundancy, the testing by gene deletion rarely leads to clear-cut results about the role of mycotoxins in virulence is discussed by Adam et al. (Chap. 10), who also provide an integration of effector-like fungal secondary metabolites into the current model of plant–pathogen interaction.

Although only scratching on the surface of the multifaceted fungal secondary metabolite reservoir, these recent studies further illustrate the necessity to unravel the chemical ecological interactions of fungi for being able to fully exploit their potential of bioactive compound biosynthesis. This is of special importance when considering that complex communities exist in nature that involve not only one fungus and one interaction partner but also multiple—sometimes uncultivable—players exhibiting mutual interactions.

To understand the molecular interactions and cellular communication processes and to exploit the metabolic capacity of the involved (fungal) microorganisms, new experimental approaches such as metagenomics and metatranscriptomics bear great potential. The recent application of these methods to uncultured bacteria led to the discovery of bioactive small molecules and biosynthetic gene clusters involved in their synthesis [65]. In fungal research, these methods are still in their infancy (Chap. 5); however, they will be extremely useful for screening fungi for genes involved in the production of novel compounds in their natural microbial communities, during interaction with other organisms, or in less explored habitats (Fig. 1.2).

Conclusion

The capability of filamentous fungi to produce secondary metabolites with potential pharmaceutical, industrial, and agricultural applications is a current topic of scientific and industrial interest. Recent advances from genome mining studies revealed numerous cryptic secondary metabolic gene clusters and showed that even in well-studied fungal models such as *Aspergillus*, the fungus's full repertoire regarding diversity and amount of secondary metabolites largely remains unknown and hence unexploited. The already identified gene clusters, together with those of still under-explored fungi still awaiting their discovery, constitute virtually unlimited natural resources of useful products.

Increasing evidence suggests that fungal secondary metabolism is not a stand-alone property but is tightly interconnected with morphological differentiation, stress response, and biotic interactions. Hence, opening this treasure chest requires the integration of data originating from fungal genome sequencing and high-throughput transcriptomics, proteomics, and metabolomics in order to provide new holistic insights into the fundamental mechanisms of system control and regulation dynamics. Enhanced insights into the regulation of secondary metabolism in fungal systems will pave the way for metabolic engineering and synthetic biology approaches and will enable the economic utilization of fungal natural products for human well-being.

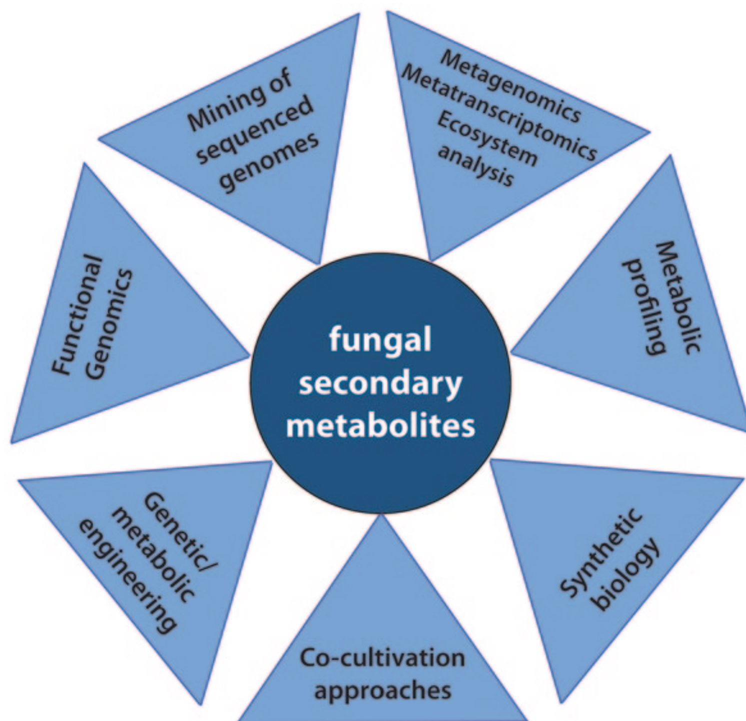

Fig. 1.2 Screening approaches for novel fungal secondary metabolites

References

1. Hartmann T (2007) From waste products to ecochemicals: fifty years research of plant secondary metabolism. Phytochemistry 68(22–24):2831–2846
2. Sachs J (1873) Lehrbuch der botanik. Leipzig: Wilhelm Engelmann
3. Martín JF, Gutiérrez S, Aparicio JF (2000) Secondary metabolites. In: Lederberg J (ed) Encyclopedia of microbiology. Academic, San Diego, pp 213–236
4. Keller NP, Turner G, Bennett JW (2005) Fungal secondary metabolism—from biochemistry to genomics. Nat Rev Microbiol 3(12):937–947
5. Bennett JW (1983) Differentiation and secondary metabolism in mycelial fungi. In: Bennett JW (ed) Secondary metabolism and differentiation in fungi. Marcel Dekker, New York, pp 1–34
6. Turgeon BG, Bushley KE (2010) Secondary metabolism. In: Borkovich KE, Ebbole D (eds) Cellular and molecular biology of filamentous fungi. ASM, USA, p 376–395
7. Brakhage AA (2013) Regulation of fungal secondary metabolism. Nat Rev Microbiol 11(1):21–32
8. Demain AL, Fang A (2000) The natural functions of secondary metabolites. Adv Biochem Eng Biotechnol 69:1–39
9. Gosio B (1893) Contributo all'etiologia della pellagra; ricerche chimiche e batteriologiche sulle alterazioni del mais. Giornale della Reale Accademia di Medicina di Torino 61:484–487

10. Abraham EP (1945) The effect of mycophenolic acid on the growth of *Staphylococcus aureus* in heart broth. Biochem J 39(5):398–408
11. Florey HW, Jennings MA et al (1946) Mycophenolic acid; an antibiotic from *Penicillium brevicompactum* Dlerckx. Lancet 1(6385):46–49
12. Anslow WK, Raistrick H (1931) Studies in the biochemistry of micro-organisms: 6-hydroxy-2-methylbenzoic acid, a product of the metabolism of glucose by *Penicillium griseo-fulvum* Dierckx. Biochem J 25(1):39–44
13. Chain E, Florey HW, Gardner AD, Heatley NG, Jennings MA, Orr-Ewing J et al (1940) Penicillin as a chemotherapeutic agent. Lancet 236(6104):226–228
14. Díez B, Gutiérrez S, Barredo JL, van Solingen P, van der Voort LHM, Martín JF (1990) The cluster of penicillin biosynthetic genes. J Biol Chem 265:16358–16365
15. Fierro F, Barredo JL, Díez B, Gutiérrez S, Fernández FJ, Martín JF (1995) The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. Proc Natl Acad Sci U S A 92:6200
16. Smith DJ, Burnham MK, Edwards J, Earl AJ, Turner G (1990) Cloning and heterologous expression of the penicillin biosynthetic gene cluster from *Penicillum chrysogenum*. Biotechnol (N Y) 8(1):39–41
17. Gutierrez S, Velasco J, Fernandez FJ, Martin JF (1992) The cefG gene of cephalosporium acremonium is linked to the cefEF gene and encodes a deacetylcephalosporin C acetyltransferase closely related to homoserine O-acetyltransferase. J Bacteriol 174(9):3056–3064
18. Martín JF, García-Estrada C, Zeilinger S (eds) (2014) Biosynthesis and molecular genetics of fungal secondary metabolites, vol I. Springer, New York.
19. Finking R, Marahiel MA (2004) Biosynthesis of nonribosomal peptides1. Annu Rev Microbiol 58:453–488
20. Garcia-Estrada C, Martin JF (2014) Penicillins. In: Martín JM, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 17–42
21. Bloemendal S, Kück U (2014) Cephalosporins. In: Martín JM, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 43–64
22. Velkov T, Lawen A (2014) Cyclosporines: biosynthesis and beyond. In: Martín JM, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 65–88
23. Sørensen JL, Knudsen M, Hansen FT, Olesen C, Romans Fuertes P, Lee TV et al (2014) Fungal NRPS-dependent siderophores: from function to prediction. In: Martin JF, Garcia-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 317–340
24. Kroken S, Glass NL, Taylor JW, Yoder OC, Turgeon BG (2003) Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. Proc Natl Acad Sci U S A 100(26):15670–15675
25. Linz JE, Wee JM, Roze LV (2014) Aflatoxin biosynthesis: regulation and subcellular localization. In: Martín JM, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 89–110
26. Studt L, Tudzynski B (2014) Gibberellins and the red pigments bikaverin and fusarubin. In: Martín JM, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 209–238
27. Dietrich D, Vederas JC (2014) Lovastatin, compactin, and related anticholesterolemic agents. In: Martín JM, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 263–288
28. Fisch KM (2013) Biosynthesis of natural products by microbial iterative hybrid PKS-NRPS. RSC Adv 3(40):18228–18247. doi:10.1039/C3RA42661K
29. Niehaus EM, Díaz-Sánchez V, von Bargen KW, Kleigrewe K, Humpf HU, Limón CM et al (2014) Fusarins and fusaric acid in fusaria. In: Martín JM, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 239–262

30. Eley KL, Halo LM, Song Z, Powles H, Cox RJ, Bailey AM et al (2007) Biosynthesis of the 2-pyridone tenellin in the insect pathogenic fungus *Beauveria bassiana*. Chembiochem 8(3):289–297
31. Bergmann S, Schumann J, Scherlach K, Lange C, Brakhage AA, Hertweck C (2007) Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. Nat chem biol 3(4):213–217
32. Ávalos J, Díaz-Sánchez V, García-Martínez J, Castrillo M, Ruger-Herreros M, Limón CM (2014) Carotenoids. In: Martín JM, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 149–186
33. Tudzynski P, Neubauer L (2014) Ergoit alkaloids. In: Martín JM, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 303–316
34. Martín JF, Liras P, García-Estrada C (2014) Roquefortine C and related prenylated indole alkaloids. In: Martín JM, García-Estrada C, Zeilinger S (eds) Biosynthesis and molecular genetics of fungal secondary metabolites. Springer, New York, pp 111–128
35. Hoffmeister D, Keller NP (2007) Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat Prod Rep 24(2):393–416
36. Bok JW, Keller NP (2004) Laea, a regulator of secondary metabolism in *Aspergillus* spp. Eukaryot Cell 3(2):527–535
37. Bayram O, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O et al (2008) Velb/vea/laea complex coordinates light signal with fungal development and secondary metabolism. Science 320(5882):1504–1506
38. Kosalková K, García-Estrada C, Ullán RV, Godio RP, Feltrer R, Teijeira F et al (2009) The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. Biochimie 91:214–225
39. Palmer JM, Keller NP (2010) Secondary metabolism in fungi: does chromosomal location matter? Curr Opin Microbiol 13(4):431–436
40. Reyes-Dominguez Y, Bok JW, Berger H, Shwab EK, Basheer A, Gallmetzer A et al (2010) Heterochromatic marks are associated with the repression of secondary metabolism clusters in *Aspergillus nidulans*. Mol Microbiol 76(6):1376–1386
41. Andersen MR, Nielsen JB, Klitgaard A, Petersen LM, Zachariasen M, Hansen TJ et al (2013) Accurate prediction of secondary metabolite gene clusters in filamentous fungi. Proc Natl Acad Sci U S A 110(1):E99–E107
42. Hertweck C (2009) Hidden biosynthetic treasures brought to light. Nat Chem Biol 5(7):450–452
43. Brakhage AA, Schroeckh V (2011) Fungal secondary metabolites-strategies to activate silent gene clusters. Fungal Genet Biol 48(1):15–22
44. Smedsgaard J, Nielsen J (2005) Metabolite profiling of fungi and yeast: from phenotype to metabolome by MS and informatics. J Exp Bot 56(410):273–286
45. Fiehn O (2002) Metabolomics-the link between genotypes and phenotypes. Plant Mol Biol 48(1–2):155–171
46. Krug D, Muller R (2014) Secondary metabolomics: the impact of mass spectrometry-based approaches on the discovery and characterization of microbial natural products. Nat Prod Rep 31(6):768–783
47. Dhingra S, Lind AL, Lin HC, Tang Y, Rokas A, Calvo AM (2013) The fumagillin gene cluster, an example of hundreds of genes under vea control in *Aspergillus fumigatus*. Plos One 8(10):e77147
48. Watrous J, Roach P, Alexandrov T, Heath BS, Yang JY, Kersten RD et al (2012) Mass spectral molecular networking of living microbial colonies. Proc Natl Acad Sci U S A 109(26):E1743–E1752
49. Traxler MF, Watrous JD, Alexandrov T, Dorrestein PC, Kolter R (2013) Interspecies interactions stimulate diversification of the *Streptomyces coelicolor* secreted metabolome. mBio 4(4):pii: e00459-e004513. doi:10.1128/mBio.00459-13
50. Liu WT, Kersten RD, Yang YL, Moore BS, Dorrestein PC (2011) Imaging mass spectrometry and genome mining via short sequence tagging identified the anti-infective agent arylomycin in *Streptomyces roseosporus*. J Am Chem Soc 133(45):18010–18013

51. Moree WJ, Phelan VV, Wu CH, Bandeira N, Cornett DS, Duggan BM et al (2012) Interkingdom metabolic transformations captured by microbial imaging mass spectrometry. Proc Natl Acad Sci U S A 109(34):13811–13816
52. Blackwell M (2011) The fungi: 1, 2, 3.. 5.1 million species? Am J Bot 98(3):426–438
53. Kusari S, Hertweck C, Spiteller M (2012) Chemical ecology of endophytic fungi: origins of secondary metabolites. Chem Biol 19(7):792–798
54. Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260(5105):214–216
55. Puri SC, Nazir A, Chawla R, Arora R, Riyaz-Ul-Hasan S, Amna T et al (2006) The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. J Biotechnol 122(4):494–510
56. Kusari S, Lamshoft M, Zuhlke S, Spiteller M (2008) An endophytic fungus from *Hypericum perforatum* that produces hypericin. J Nat Prod 71(2):159–162
57. Kusari S, Zuhlke S, Spiteller M (2009) An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues. J Nat Prod 72(1):2–7
58. Schulz B, Boyle C, Draeger S, Römmert AK, Krohn K (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol Res 106:996–1004
59. Caballero Ortiz S, Trienens M, Rohlfs M (2013) Induced fungal resistance to insect grazing: reciprocal fitness consequences and fungal gene expression in the Drosophila-Aspergillus model system. Plos One 8(8):e74951
60. Doll K, Chatterjee S, Scheu S, Karlovsky P, Rohlfs M (2013) Fungal metabolic plasticity and sexual development mediate induced resistance to arthropod fungivory. Proc Biol Sci 280(1771):20131219
61. Chiang YM, Szewczyk E, Nayak T, Davidson AD, Sanchez JF, Lo HC et al (2008) Molecular genetic mining of the *Aspergillus* secondary metabolome: discovery of the emericellamide biosynthetic pathway. Chem Biol 15(6):527–532
62. Lo HC, Entwistle R, Guo CJ, Ahuja M, Szewczyk E, Hung JH et al (2012) Two separate gene clusters encode the biosynthetic pathway for the meroterpenoids austinol and dehydroaustinol in *Aspergillus nidulans*. J Am Chem Soc 134(10):4709–4720
63. Jansen C, von Wettstein D, Schafer W, Kogel KH, Felk A, Maier FJ (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. Proc Natl Acad Sci U S A 102(46):16892–16897
64. Collemare J, Billard A, Bohnert HU, Lebrun MH (2008) Biosynthesis of secondary metabolites in the rice blast fungus *Magnaporthe grisea*: the role of hybrid pks-nrps in pathogenicity. Mycol Res 112(Pt 2):207–215
65. Banik JJ, Brady SF (2010) Recent application of metagenomic approaches toward the discovery of antimicrobials and other bioactive small molecules. Curr Opin Microbiol 13(5):603–609

Chapter 2
Key Players in the Regulation of Fungal Secondary Metabolism

Benjamin P. Knox and Nancy P. Keller

Introduction

Beyond their environmental ubiquity and critical roles in nearly every ecological niche as primary decomposers, fungi are well known for producing a wealth of low molecular weight molecules called secondary metabolites, which are also known as natural products. Although the true ecological function of most secondary metabolites (SMs) is still unknown, their roles as biotic and abiotic protectants or defensive metabolites is emerging [1–4]. Furthermore, their significant impact on human well-being, both positive and negative, makes them attractive study targets.

With a broad spectrum of biological activity, SMs can have major influences on human health. For example, subsets of SMs known as mycotoxins are responsible for millions of dollars in crop loss annually just in the USA alone [5]. When mycotoxin contamination of consumables goes undetected, the resulting mycotoxicoses have additional health and economic consequences, which has been documented throughout recorded history [6]. Crop losses and health consequences are especially devastating in developing countries where testing for toxin contamination is either not well established or is nonexistent [7]. Conversely, the diverse pharmacodynamics of medically relevant SMs such as the *β*(beta)-lactam antibiotics penicillin and cephalosporin, and the popular cholesterol-lowering agent lovastatin, are examples of how fungal SMs have had positive impacts on human well-being.

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At the genomic level, one of the defining hallmarks of SM is the grouping of biosynthetic genes into discreet clusters [8] and general localization to subtelomeric regions of the chromosome [9]. Many, but not all, clusters contain cluster-specific transcription factors that regulate expression of the biosynthetic genes for their respective metabolites. This clustering, coupled with unique chromosomal location, allows for multiple regulatory layers giving the producing fungus precise spatial and temporal control over metabolite expression and likely contributes to intra-, and possibly inter-kingdom, horizontal cluster transfer [10–12]. Additionally, SM production is often tightly correlated with growth and development [13], and oftentimes disruption of one process will have a significant effect on the other.

The study of fungal SMs has established several model fungal systems and particular metabolites with facile laboratory characterization allowing clear study of their expression and regulation. Yet, as an increasing number of fungal genomes become available, it has become clear that the number of metabolites observed under routine laboratory conditions is a paltry representation of the theoretic yield based on predicted *in silico* genomic regions containing signature SM biosynthetic cluster motifs [14]. Resulting from these observations, endeavors to unlock these "cryptic" gene clusters have shed light on SM regulators working at the level of individual metabolites or at a larger, global scale.

In this chapter, we review the general mechanisms of key players in the regulation of fungal SM. As will become evident in the following paragraphs, fungal SM regulation does not follow a strict hierarchical regime and is composed of overlapping and interconnected pathways making regulatory classifications of regulators less amenable to simple progressional delineations. As such, we will group and present key players based on the largely cluster-specific $Zn(II)_2Cys_6$ family of transcription factors, then investigate additional families that possess more pleiotropic regulatory characteristics, and finish with global regulators and multiprotein complexes that respond to environmental cues.

$ZN(II)_2CYS_6$

The grouping of SM biosynthetic genes into discreet and contiguous clusters is a distinguishing feature of SM in fungi [8]. In-cluster transcription factors are commonly found that exhibit regulatory control over their respective cluster's transcription of biosynthetic genes. As the production and regulation of SMs in fungi are replete with diversity, it is not surprising that multiple families of transcription factors regulate these processes. Therefore, we will begin with the largest cluster-specific family of fungal transcription factors (Table 2.1) [15–35].

Unique to fungi are the $Zn(II)_2Cys_6$ family of transcription factors. Some distinguishing features of these proteins are the binding of two zinc atoms to six cysteine residues and their ability to bind DNA as monomers, homodimers, or heterodimers [36]. Common nomenclatures for $Zn(II)_2Cys_6$ transcription factors include: C_6, Zn_2C_6, zinc binuclear cluster, as well as zinc cluster. Compared to other families of transcription factors, $Zn(II)_2Cys_6$ proteins are the most common family of dedicated cluster regulators for fungal SM.

Table 2.1 Examples of $Zn(II)_2Cys_6$ cluster-specific transcription factors (adapted from Yin and Keller 2011 [15])

Transcription factor	Target cluster	Species	Source
AflR	Aflatoxin/ sterigmatocystin	*Aspergillus nidulans* *Aspergillus flavus* *Aspergillus parasiticus*	Brown et al. 1996 [16] Woloshuk et al. 1994 [17] Chang et al. 1995 [18]
AfoA	Asperfuranone	*Aspergillus nidulans*	Chiang et al. 2009 [19]
ApdR	Aspyridone	*Aspergillus nidulans*	Bergmann et al. 2007 [20]
Bik5	Bikaverin	*Fusarium fujikuroi*	Wiemann et al. 2009 [21]
CTB8	Cercosporin	*Cercospora nicotianae*	Chen et al. 2007 [22]
CtnA	Citrinin	*Monascus purpureus*	Shimizu et al. 2007 [23]
CtnR	Asperfuranone	*Aspergillus nidulans*	Chiang et al. 2009 [19]
DEP6	Depudecin	*Alternaria brassicicola*	Wight et al. 2009 [24]
FapR	Fumagillin/pseurotin	*Aspergillus fumigatus*	Wiemann et al. 2013 [25]
FUM21	Fumonisin	*Fusarium verticillioides*	Brown et al. 2007 [26]
GIP2	Aurofusarin	*Gibberella zeae*	Kim et al. 2006 [27]
GliZ	Gliotoin	*Aspergillus fumigatus*	Bok et al. 2006b [28]
LovE	Lovastatin	*Aspergillus terreus*	Kennedy et al. 1999 [29]
MdpE	Monodictyphenone	*Aspergillus nidulans*	Chiang et al. 2010 [30]
MlcR	Compactin	*Penicillium citrinum*	Abe et al. 2002b [31]
MokH	Monacolin K	*Monascus pilosus*	Chen et al. 2010 [32]
ORFR	AK-toxin	*Alternaria alternata*	Tanaka and Tsuge 2000 [33]
SirZ	Sirodesmin PL	*Leptosphaeria maculans*	Fox et al. 2008 [34]
ZFR1	Fumonisin	*Fusarium verticillioides*	Flaherty and Woloshuk 2004 [35]

To date, the most well-characterized cluster-specific transcription factor is the aflatoxin (AF)/sterigmatocystin (ST) regulator AflR. As the paradigm for $Zn(II)_2Cys_6$-mediated cluster regulation, AflR has unequivocally established the importance of studying transcriptional regulators in the context of SM expression and regulation. Deletion of *aflR* in *Aspergillus nidulans* produced a condition in which ST production was suppressed at the transcriptional level, even under ST-stimulating conditions [37] and, conversely, increasing expression of *aflR* in normally non-AF producing conditions resulted in concomitant expression of AF biosynthetic genes [18].

Many of the SM clusters characterized in *A. nidulans* contain $Zn(II)_2Cys_6$ proteins including AfoA and CtnR, ApdR, and MdpE required for asperfuranone, aspyridone, and monodictyphenone gene expression, respectively [19, 20, 30].

Overexpression of Zn(II)$_2$Cys$_6$ proteins can be sufficient to activate silent SM clusters as was first reported for ApdR, thus representing a critical proof-of-principle for fungal genome mining in the postgenomic era [20]. This technique, however, does not always work, resulting in the development of alternative methods to bypass Zn(II)$_2$Cys$_6$ regulation altogether and target individual keystone SM genes [38]. Cluster-specific Zn(II)$_2$Cys$_6$ transcription factors regulating SM production in plant pathogenic fungi include CTB8 for cercosporin, DEP6 for depudecin, FUM21 and ZFR1 for fumonisin, GIP2 for aurofusarin, ORFR for AK-toxin, and SirZ for sirodesmin PL in *Cercospora nicotianae, Alternaria brassicicola, Fusarium verticillioides, Gibberella zeae, Alternaria alternata*, and *Leptosphaeria maculans*, respectively [22, 24, 26, 27, 33–35]. Additionally, Bik5 controls synthesis of the mycelial pigment bikaverin in *Fusarium fujikuroi* [21]. Understanding Zn(II)$_2$Cys$_6$ cluster regulation is also critical in biotechnologically relevant fungi given that this family of transcription factors can also control production of the important antihypercholesterolemic agents lovastatin and monacolin K, as well as the pravastatin sodium precursor compactin, regulated by LovE, MokH, and MlcR in *Aspergillus terreus, Monascus pilosus*, and *Penicillium citrinum*, respectively [29, 31, 32, 39]. Recently, an interesting new twist to the canonical view of a Zn(II)$_2$Cys$_6$ regulating a single SM came with the finding of the transcription factor FapR that simultaneously regulates biosynthetic genes of the intertwined fumagillin and pseurotin cluster in *Aspergillus fumigatus* [25]. Table 2.1 provides a few additional examples of these types of transcription factors [15–35].

CYS$_2$HIS$_2$

Common to fungi (Table 2.2) and all other eukaryotes are the Cys$_2$His$_2$ family of transcription factors [21, 40–69]. Defining features of this family include two or more of the conserved, repeating amino acid zinc finger units that bind a single zinc atom [36]. Cys$_2$His$_2$ proteins bind DNA as monomers and can also be referred to as C$_2$H$_2$ and classical zinc finger transcription factors.

For surviving a multitude of environmental challenges, fungi often require structural pigments for withstanding biotic and abiotic stresses [70]. A unique group of orthologous Cys$_2$His$_2$ transcription factors has been found in several plant pathogenic fungi, all positively regulating biosynthesis of the structural pigment melanin. The proteins Cmr1p, Pig1p, Cmr1, and BMR1 are found in *Colletrichum lagenarium, Magnaporthe grisea, Cochliobolus heterostrophus*, and *Bipolaris oryzae*, respectively [43, 45, 46]. In addition to two Cys$_2$His$_2$ motifs, these four proteins also possess a Zn(II)$_2$Cys$_6$ sequence. The earliest recorded examples of SM regulation by Cys$_2$His$_2$ proteins are MRTRI6 and Tri6 regulating trichothecene mycotoxin gene clusters in the plant pathogenic fungi *Myrothecium roridum* and *Fusarium sporotrichioides*, respectively [50, 58]. In one of the few cases of an SM-specific regulator gene lying outside of the cluster it controls, the Cys$_2$His$_2$ transcription factor ScpR on chromosome II was shown to activate the asperfuranone cluster in

Table 2.2 Transcription factor families and their regulated metabolite(s)

Regulator	Metabolite(s)	Species	Source
Cys_2His_2			
AreA	AF gibberellin fumonisin	*Aspergillus parasiticus* *Gibberella fujikuroi* *Fusarium verticiliioides*	Chang et al. 2000 [40] Mihlan et al. 2003 [41] Kim and Woloshuk 2008 [42]
BMR1	Melanin	*Bipolaris oryzae*	Kihara et al. 2008 [43]
BcYOH1	Botrydial/botcinic acid	*Botrytis cinerea*	Simon et al. 2013 [44]
Cmr1	Melanin	*Cochliobolus heterostrophus*	Eliahu et al. 2007 [45]
Cmr1p	Melanin	*Colletrichum lagenarium*	Tsuji et al. 2000 [46]
CreA	Flavipucine cephalosporin	*Aspergillus terreus* *Acremonium chrysogenum*	Gressler et al. 2011 [47] Jekosch and Kück 2000 [48, 49]
MRTRI6	Trichothecene	*Myrothecium roridum*	Trapp et al. 1998 [50]
PacC	Penicillin ST/AF cephalosporin bikaverin fumonisin gluconic acid	*Aspergillus nidulans* *A. nidulans*/*A. parasiticus* *Acremonium chrysogenum* *Fusarium fujikuroi* *Fusarium verticillioides* *Penicillium expansum*	Bergh and Brakhage 1998 [51] Keller et al. 1997 [52] Schmitt et al. 2001 [53] Wiemann et al. 2009 [21] Flaherty et al. 2003 [54] Barad et al. 2013 [55]
Pig1p	Melanin	*Magnaporthe grisea*	Tsuji et al. 2000 [46]
Sda1	Fumonisin B_1	*Fusarium verticillioides*	Malapi-Wight et al. 2013 [56]
ScpR	Asperfuranone	*Aspergillus nidulans*	Bergmann et al. 2010 [57]
Tri6	Trichothecene	*Fusarium sporotrichioides*	Hohn et al. 1999 [58]
bZip			
Aoyap1	Ochratoxin	*Aspergillus ochraceus*	Reverberi et al. 2012 [59]
AtfB	AF	*Aspergillus parasiticus*	Roze et al. 2011 [60]
HapX	Ferricrocin	*Aspergillus nidulans*	Eisendle et al. 2006 [61]
MeaB	Bikaverin AF	*Fusarium fujikuroi* *Aspergillus flavus*	Wagner et al. 2010 [62] Amaike et al. 2013 [63]
RsmA	ST/asperthecin gliotoxin	*Aspergillus nidulans* *Aspergillus fumigatus*	Yin et al. 2012 [64] Sekonyela et al. 2013 [65]
ToxE	HC-toxin	*Cochliobolus carbonum*	Bussink et al. 2001 [66]; Pedley and Walton 2001 [67]
Winged helix			
AcFKH1	Cephalosporin C	*Acremonium chrysogenum*	Schmitt et al. 2004 [68]
CPCR1	Cephalosporin C	*Acremonium chrysogenum*	Schmitt and Kuck 2000 [69]

A. nidulans, likely through binding the promoter region of *afoA*, embedded in the asperfuranone cluster located on chromosome VIII [57].

Recently, the characterization of two new Cys_2His_2 transcription factors has shown regulation of other physiological processes beyond SM. Exhibiting positive regulation over the toxins botrydial and botcinic acid in *Botrytis cinerea*, BcYOH1 exhibited a more global regulatory role as it also affects mechanisms in detoxification, virulence, and carbohydrate metabolism [44], whereas *sda1* knockout strains of *F. verticillioides* had excessive fumonisin B_1 biosynthesis, reduced capacity to form conidia, and an inability to grow on selected carbon sources [56].

bZIP

Found in all eukaryotic organisms, basic leucine zipper (bZIP) transcription factors are characterized by basic and leucine zipper regions. The basic region dictates sequence-specific DNA-binding whereas the leucine zipper region mediates dimerization of the protein. As dimers, bZIPs target palindromic DNA sequences. Many fungal bZIPs (Table 2.2 [21, 40–69]) have been characterized as stress response transcription factors, responding to a variety of environmental stresses that appears to link them to SM production [60, 71, 72]. bZIPs associated with SM regulation and stress include RsmA regulating sterigmatocystin and asperthecin in *A. nidulans* and gliotoxin in *A. fumigatus* [64, 65], AtfB regulating aflatoxin in *Aspergillus parasiticus* [60], and Aoyap1 regulating ochratoxin in *Aspergillus ochraceus* [59]. The bZIP protein MeaB, involved in nitrogen regulation, has also been associated with regulation of SMs including bikaverin in *F. fujikuroi* and AF in *A. flavus* [62, 63].

In the plant pathogen *Cochliobolus carbonum*, HC-toxin biosynthetic genes are regulated by the hybrid bZIP/ankyrin repeat transcription factor ToxE [73]. Despite having the basic region characteristic of bZIPS, ToxE lacks the leucine zipper sequence but possesses four ankyrin repeats. Both the basic region and ankyrin repeat region mediate DNA binding to promoter regions of all HC-toxin biosynthesis genes [67]. ToxE, together with the putative transcription factor Bap1 from the tomato pathogen *Cladosporium fulvum*, represent a potentially novel class of fungal-specific hybrid transcription factors possessing bZIP and ankyrin repeat characteristics [66].

Winged Helix

Winged helix proteins are found in all organisms and belong within the general helix-turn-helix structural group of proteins. Broadly speaking, the structure of a winged helix protein consists of two wings, three α(alpha) helices, and three β(beta) strands [74]. The industrially relevant fungus *Acremonium chrysogenum* is well

known for production of the antibiotic cephalosporin C. Cluster-specific regulation of cephalosporin C production in *A. chrysogenum* was first shown to be controlled by the transcription factor CPCR1, which belongs to the winged helix subfamily of regulatory factor X (RFX) proteins [69]. Another regulator of cephalosporin C, AcFKH1 belongs to a subfamily of winged helix proteins possessing a fork-head associated domain (FHA) and a forkhead DNA-binding domain (FKH) [68]. Consistent with the observation that SM production is often inextricably linked to morphological development, it was shown that CPCR1 is not only required for cephalosporin C production, but also for the formation of arthrospores and whereas *AcFKH1* deletion strains still retained the ability to form arthrospores despite possessing swollen and highly septate hyphae [75]. To the best of our knowledge, cephalosporin C is the only fungal SM currently known to be regulated by winged helix proteins (Table 2.2 [21, 40–69]).

Global Regulators

AreA—Nitrogen

So far we have reviewed transcription factors largely characterized as cluster-specific and will now move into global regulators that translate environmental cues into SM and concomitant physiological responses. In *A. nidulans* an increase in ST, and an increase in the rate of sexual development, is observed when grown on nitrate, whereas an opposite response for both phenotypes is observed on ammonium media [76]. Conversely, ammonium stimulates AF biosynthesis in *A. parasiticus* and nitrate inhibits its production [77]. Comparing the SM response to varying nitrogen sources between these two *Aspergillus* species suggests a highly dynamic interplay between environment, SM adaptation, and developmental regime.

Among fungi, the highly conserved global transcription factor AreA is responsible for repression of nitrogen metabolism in the presence of glutamine or ammonium, and is a member of the GATA family of transcription factors, which are conserved among eukaryotes and are characterized by their Cys_2Hys_2 zinc finger DNA binding domains [78]. Beyond its regulatory role in primary metabolism, AreA modulates morphological development and SM regulation in filamentous fungi and is likely responsible for the aforementioned species-specific mycotoxin responses based on the observation of multiple GATA sequences in the aflatoxin and sterigmatocystin regulatory genes *aflR* and *aflJ*, and subsequent AreA binding to these regions in *A. parasiticus* [40]. The rice pathogen *Gibberella fujikuroi* exhibits decreased transcript levels for nearly all structural genes for the SM gibberellin in an *areA* deletion mutant, showing direct regulation of this cluster by AreA [41]. Additionally, AreA in the maize pathogen *F. verticillioides* is a positive regulator of the toxic SM fumonisin [42].

PacC—pH

Beyond nutritional requirements, another environmental parameter essential for growth and development is pH. The global regulator PacC is a conserved Cis_2His_2 zinc finger transcription factor among fungi [79], capable of regulating a suite of physiological processes, including SM production, in response to environmental pH [80]. Studies investigating PacC regulation of SM have shown that this alkali-activated transcription factor dynamically controls metabolites to be expressed in a pH environment most suitable for compound bioactivity and maximum niche exploitation [81]. In *A. nidulans*, penicillin production was shown to be increased in an alkaline environment [51, 82], possibly as an ecological adaptation by the fungus to outcompete increased bacterial competition in high pH environments [81]. Also in *A. nidulans*, and in contrast to penicillin production, ST synthesis was repressed by alkaline pH as was AF synthesis in *A. parasiticus* [52]. Expression of another important *β*(beta)-lactam, cephalosporin, by *A. chrysogenum* was shown to be regulated by PacC through binding to promoter regions of structural genes [53]. Expression of the mycelial pigment bikaverin from the rice pathogen *F. fujikuroi* also exhibits PacC regulation through inhibition of its synthesis in a high pH environment [21]. Production of the mycotoxin fumonisin is also downregulated under elevated pH conditions by the maize pathogen *F. verticillioides* [54]. Interestingly, a recent study provides evidence for a dynamic role of PacC in tree fruit spoilage by the phytopathogen *Penicillium expansum* such that mechanisms of acidification after initial colonization, via secretion of organic acids such as gluconic acid, are mediated by PacC to control subsequent expression of the mycotoxin patulin [55]. Like other strains deficient in global regulators of SM, PacC deletion strains exhibit developmental phenotypes such as reduced conidiation [79] or growth inhibitions [21, 54], illustrating a pivotal role for pH sensing and homeostasis in proper growth, development, and SM expression.

CreA—Carbon

When grown in media rich in glucose, filamentous fungi downregulate genes required for metabolizing other carbon sources via a phenomenon called carbon catabolite repression [83], mediated largely by the Cys_2His_2 zinc finger transcription factor CreA [84]. Some examples of CreA-mediated regulation of SM include an overproducing cephalosporin strain of *A. chrysogenum*, obtained by classical mutagenesis techniques, which appeared to have deregulation of the CreA homologue Cre1 compared to wild-type strains [48]. Additionally, putative Cre1 binding sites found within the promoter regions of two cephalosporin biosynthetic genes suggest carbon catabolite repression of this metabolite [49]. In *A. nidulans*, glucose represses antibiotic production independent of a mutated CreA binding site in the penicillin biosynthetic gene *ipnA* promoter region [82] and was still unaffected by a CreA mutant background [85]. Although the role of CreA in regulating SM is less

clear-cut compared to AreA and PacC, it is important to acknowledge the role of this global regulator in environmental sensing and the concomitant physiological response and consider how SMs might be affected. For example, the stringency of glucose repression, likely mediated by CreA, on a cryptic SM cluster in *A. terreus* was recently shown to be insurmountable even by overexpression of a putative in-cluster transcription factor [47].

Velvet Complex—Light

Like most organisms, filamentous fungi sense and respond to light. Conserved throughout the filamentous and dimorphic ascomycetes and possibly basidiomycetes [86] is the light-sensing heterotrimeric velvet complex consisting of LaeA, and the velvet proteins VeA and VelB [87]. The velvet complex links sexual development with SM production in response to light and accomplishes this through tightly regulated spatial compartmentalization of velvet complex components. VelB has nuclear and cytoplasmic localization regardless of illumination status [87], whereas VeA is cytoplasmic under light conditions and migrates to the nucleus in the absence of light [88] as a heterodimer with VelB, and LaeA is constitutively nuclear [89]. Consequently, the three velvet complex proteins are only colocalized to the nucleus under dark conditions, allowing formation of the fully functional heterotrimeric velvet complex. The mechanism of velvet complex regulation of SMs is best studied in *A. nidulans* but increasingly well known in other fungi [90–97]. Once assembled in the nucleus of *A. nidulans*, the velvet complex drives sexual development and production of SMs, whereas these processes are repressed under illuminating conditions resulting from a dissociated velvet complex [81]. Consequently, when localization of members of this complex is compromised, such as with VeA via interacting with the newly described LaeA-like methyltransferase LlmF, neither sexual development nor SM production can proceed normally [98]. The LlmF mechanism of VeA control appears conserved in other ascomycetes as well [99].

The finding that LaeA exhibited some similarity to methyltransferases involved in histone modification, coupled with the observation that SM clusters were most frequently found in subtelomeric regions of the genome [9], led to the hypothesis that SM clusters could be under epigenetic regulation [100]. This indeed has proved to be the case and for detailed coverage of histone modifications and their influence on SM production, we direct the reader to Chap. 3 for chromatin-based regulation of secondary metabolism.

CBC—Iron

Beyond the velvet complex, which is required for coordinating sexual development and SM production in response to light, another well-characterized global regulatory complex in filamentous fungi is the CCAAT-binding complex (CBC). Unlike velvet

components, which are only conserved among filamentous fungi, CBC complexes are found in all eukaryotic organisms [101]. AnCF, the CBC in *A. nidulans* formerly known as PENR1 [101] is composed of the proteins HapB, HapC, and HapE. The AnCF is a positive regulator of genes required for penicillin biosynthesis including *ipnA* and *aatA* [102], is a negative autoregulator of *hapB* [103], and coordinates physiological processes in response to cellular redox state [104] and environmental iron [105]. With respect to iron depletion, CBC-mediated upregulation of *sidC* in *A. nidulans* is dependent upon physical interaction with the bZIP protein HapX [105] with SidC being a core enzyme for synthesis of ferricrocin, an SM siderophore that is essential for intracellular iron homeostasis and morphological development [61].

Conclusion

The study of SM in fungi originated primarily for two reasons: (1) to understand the regulation of mycotoxin gene clusters with the goal of using this knowledge to ameliorate the deleterious costs of crop contamination with mycotoxins and (2) to identify compounds with novel bioactivities applicable for pharmaceutical, medicinal, and agricultural uses. Since then, affecting all aspects of fungal biology, the integrated and critical roles of SMs have emerged as being greater than anticipated. SM regulators originally characterized as cluster-specific transcription factors have later been shown to have regulatory functions beyond their native cluster. For example, the canonical AF/ST regulator and $Zn(II)_2Cys_6$ transcription factor *aflR* was shown in *A. parasiticus* to regulate several genes beyond the defined boundaries of the cluster [64, 106]. Additionally, the Cys_2Hys_2 transcription factor Tri6 from *F. graminearum* was shown to regulate 192 genes, ranging from central metabolism to virulence, beyond the known targets within the trichothecene gene cluster [107], leading the authors to propose characterizing Tri6 as a global regulator much like the other known global Cys_2Hys_2 regulators AreA, PacC, and CreA.

Beyond expanding the roles of well-established regulators, research aimed toward elucidating new biosynthetic pathways and compounds has also benefited from exploiting the tight interplay of primary and SM. In one particular study, carbon catabolite repression was so stringent over a cryptic cluster, the authors utilized a suite of growth media containing various carbon sources to define nutrient conditions favorable for cluster activation, eventually identifying the compounds hydroisoflavipucin and dihydroisoflavipucin [47].

One of the greatest challenges in studying fungal SM will be to make sense of how key regulators fit into an ever-expanding network of global interactions linking primary and secondary metabolism with growth and development and how this modulates in response to nutrient availability, biotic and abiotic stresses, and niche exploitation. Taken together, it is apparent that ongoing and future studies will necessarily approach fungal SMs not from the standpoint that they are "accessory" molecules, but rather integrated members of a complex metabolic network affecting every facet of fungal biology.

References

1. Cho Y, Srivastava A, Ohm R, Lawrence CB, Wang K-H, Grigoriev IV et al (2012) Transcription factor Amr1 induces melanin biosynthesis and suppresses virulence in Alternaria brassicicola. PLoS Pathog 8:e1002974
2. Ortiz SC, Trienens M, Rohlfs M (2013) Induced fungal resistance to insect grazing: reciprocal fitness consequences and fungal gene expression in the Drosophila-Aspergillus model system. PLoS ONE 8:e74951
3. Mousa WK, Raizada MN (2013) The diversity of anti-microbial secondary metabolites produced by fungal endophytes: an interdisciplinary perspective. Front Microbiol 4:1–18
4. Subramani R, Kumar R, Prasad P, Aalbersberg W (2013) Cytotoxic and antibacterial substances against multi-drug resistant pathogens from marine sponge symbiont: citrinin, a secondary metabolite of Penicillium sp. Asian Pac J Trop Biomed 3:291–296
5. Robens J, Cardwell K (2003) The costs of mycotoxin management to the USA: management of aflatoxins in the United States. Toxin reviews. J Toxicol-Toxin Rev 22:139–152
6. Bryden WL (2012) Mycotoxin contamination of the feed supply chain: implications for animal productivity and feed security. Anim Feed Sci Technol 173:134–158
7. Gieseker KE and Centers for Disease Control and Prevention (2004) Outbreak of aflatoxin poisoning—eastern and central provinces, Kenya, January-July 2004. Public Health Fac Publ 53:790–792
8. Hoffmeister D, Keller NP (2006) Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat Prod Rep 24:393–416
9. Palmer JM, Keller NP (2010) Secondary metabolism in fungi: does chromosomal location matter? Curr Opin Microbiol 13:431–436
10. Khaldi N, Collemare J, Lebrun M-H, Wolfe KH (2008) Evidence for horizontal transfer of a secondary metabolite gene cluster between fungi. Genome Biol 9(1):1–10
11. Schmitt I, Lumbsch HT (2009) Ancient horizontal gene transfer from bacteria enhances biosynthetic capabilities of fungi. PLoS ONE 4:e4437
12. Slot JC, Rokas A (2011) Horizontal transfer of a large and highly toxic secondary metabolic gene cluster between fungi. Curr Biol 21:134–139
13. Calvo AM, Wilson RA, Bok JW, Keller NP (2002) Relationship between secondary metabolism and fungal development. Microbiol Mol Biol Rev 66:447–459
14. Andersen MR, Nielsen JB, Klitgaard A, Petersen LM, Zachariasen M, Hansen TJ, Blicher LH, Gotfredsen CH, Larsen TO, Nielsen KF, Mortensen UH (2013) Accurate prediction of secondary metabolite gene clusters in filamentous fungi. Proc Natl Acad Sci USA 110:E99–E107
15. Yin W, Keller NP (2011) Transcriptional regulatory elements in fungal secondary metabolism. J Microbiol 49:329–339
16. Brown DW, Yu JH, Kelkar HS, Fernandes M, Nesbitt TC, Keller NP, Adams TH, Leonard TJ (1996) Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in Aspergillus nidulans. Proc Natl Acad Sci USA 93:1418–1422
17. Woloshuk CP, Foutz KR, Brewer JF, Bhatnagar D, Cleveland TE, Payne GA (1994) Molecular characterization of aflR, a regulatory locus for aflatoxin biosynthesis. Appl Environ Microbiol 60:2408–2414
18. Chang P-K, Ehrlich KC, Yu J, Bhatnagar D, Cleveland TE (1995) Increased expression of Aspergillus parasiticus aflR, encoding a sequence-specific DNA-binding protein, relieves nitrate inhibition of aflatoxin biosynthesis. Appl Environ Microb 61:2372–2377
19. Chiang Y-M, Szewczyk E, Davidson AD, Keller N, Oakley BR, Wang CCC (2009) A gene cluster containing two fungal polyketide synthases encodes the biosynthetic pathway for a polyketide, asperfuranone, in Aspergillus nidulans. J Am Chem Soc 131:2965–2970
20. Bergmann S, Schümann J, Scherlach K, Lange C, Brakhage AA, Hertweck C (2007) Genomics-driven discovery of PKS-NRPS hybrid metabolites from Aspergillus nidulans. Nat Chem Biol 3:213–217

21. Wiemann P, Willmann A, Straeten M, Kleigrewe K, Beyer M, Humpf H-U, Tudzynski B (2009) Biosynthesis of the red pigment bikaverin in Fusarium fujikuroi: genes, their function and regulation. Mol Microbiol 72:931–946
22. Chen H, Lee M-H, Daub ME, Chung K-R (2007) Molecular analysis of the cercosporin biosynthetic gene cluster in Cercospora nicotianae. Mol Microbiol 64:755–770
23. Shimizu T, Kinoshita H, Nihira T (2007) Identification and in vivo functional analysis by gene disruption of ctnA, an activator gene involved in citrinin biosynthesis in Monascus purpureus. Appl Environ Microbiol 73(16):5097–5103
24. Wight WD, Kim K-H, Lawrence CB, Walton JD (2009) Biosynthesis and role in virulence of the histone deacetylase inhibitor depudecin from Alternaria brassicicola. Mol Plant Microbe Interact 22:1258–1267
25. Wiemann P, Guo CJ, Palmer JM, Sekonyela R, Wang CCC, Keller NP (2013) Prototype of an intertwined secondary metabolite supercluster. Proc Natl Acad Sci USA 110(42):17065–17070
26. Brown DW, Butchko R, Busman M, Proctor R (2007) The Fusarium verticillioides FUM gene cluster encodes a Zn(II)2Cys6 protein that affects FUM gene expression and fumonisin production. Eukaryot Cell 6:1210–1218
27. Kim J-E, Jin J, Kim H, Kim J-C, Yun S-H, Lee Y-W (2006) GIP2, a putative transcription factor that regulates the aurofusarin biosynthetic gene cluster in Gibberella zeae. Appl Environ Microbiol 72:1645–1652
28. Bok JW, Chung D, Balajee SA, Marr K, Andes D, Nielsen KF, Frisvad JC, Kirby KA, Keller NP (2006b) GliZ, a transcriptional regulator of gliotoxin biosynthesis, contributes to Aspergillus fumigatus virulence. Infect Immun 74:6761–6768
29. Kennedy J, Auclair K, Kendrew SG, Park C, Vederas JC, Hutchinson CR (1999) Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. Science 284:1368–1372
30. Chiang Y-M, Szewczyk E, Davidson AD, Entwistle R, Keller NP, Wang CCC, Oakley BR (2010) Characterization of the Aspergillus nidulans monodictyphenone gene cluster. Appl Environ Microbiol 76:2067–2074
31. Abe Y, Ono C, Hosobuchi M, Yoshikawa H (2002b) Functional analysis of mlcR, a regulatory gene for ML-236B (compactin) biosynthesis in Penicillium citrinum. Mol Genet Genomics 268:352–361
32. Chen Y-P, Yuan G-F, Hsieh S-Y, Lin Y-S, Wang W-Y, Liaw L-L et al (2010) Identification of the mokH gene encoding transcription factor for the upregulation of monacolin K biosynthesis in Monascus pilosus. J Agric Food Chem 58:287–293
33. Tanaka A, Tsuge T (2000) Structural and functional complexity of the genomic region controlling AK-toxin biosynthesis and pathogenicity in the Japanese pear pathotype of Alternaria alternata. Mol Plant Microbe Interact 13:975–986
34. Fox EM, Gardiner DM, Keller NP, Howlett BJ (2008) A Zn(II)2Cys6 DNA binding protein regulates the sirodesmin PL biosynthetic gene cluster in Leptosphaeria maculans. Fungal Genet Biol 45:671–682
35. Flaherty JE, Woloshuk CP (2004) Regulation of fumonisin biosynthesis in Fusarium verticillioides by a zinc binuclear cluster-type gene, ZFRI. Appl Environ Microbiol 70:2653–2659
36. MacPherson S, Larochelle M, Turcotte B (2006) A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol Mol Biol Rev 70:583–604
37. Yu J-H, Butchko RAE, Fernandes M, Keller NP, Leonard TJ, Adams TH (1996) Conservation of structure and function of the aflatoxin regulatory gene aflR from Aspergillus nidulans and A. flavus. Curr Genet 29:549–555
38. Ahuja M, Chiang Y-M, Chang S-L, Praseuth MB, Entwistle R, Sanchez JF, Lo H-C, Yeh H-H, Oakley BR, Wang CCC (2012) Illuminating the diversity of aromatic polyketide synthases in Aspergillus nidulans. J Am Chem Soc 134:8212–8221
39. Abe Y, Suzuki T, Ono C, Iwamoto K, Hosobuchi M, Yoshikawa H (2002a) Molecular cloning and characterization of an ML-236B (compactin) biosynthetic gene cluster in Penicillium citrinum. Mol Genet Genomics 267:636–646

40. Chang P-K, Yu J, Bhatnagar D, Cleveland TE (2000) Characterization of the Aspergillus parasiticus major nitrogen regulatory gene, areA. Biochim Biophys Acta 1491:263–266
41. Mihlan M, Homann V, Liu T-WD, Tudzynski B (2003) AREA directly mediates nitrogen regulation of gibberellin biosynthesis in Gibberella fujikuroi, but its activity is not affected by NMR. Mol Microbiol 47:975–991
42. Kim H, Woloshuk CP (2008) Role of AREA, a regulator of nitrogen metabolism, during colonization of maize kernels and fumonisin biosynthesis in Fusarium verticillioides. Fungal Genet Biol 45:947–953
43. Kihara J, Moriwaki A, Tanaka N, Tanaka C, Ueno M, Arase S (2008) Characterization of the BMR1 gene encoding a transcription factor for melanin biosynthesis genes in the phytopathogenic fungus Bipolaris oryzae. FEMS Microbiol Lett 281:221–227
44. Simon A, Dalmais B, Morgant G, Viaud M (2013) Screening of a Botrytis cinerea one-hybrid library reveals a Cys2His2 transcription factor involved in the regulation of secondary metabolism gene clusters. Fungal Genet Biol 52:9–19
45. Eliahu N, Igbaria A, Rose MS, Horwitz BA, Lev S (2007) Melanin biosynthesis in the maize pathogen Cochliobolus heterostrophus depends on two mitogen-activated protein kinases, Chk1 and Mps1, and the transcription factor Cmr1. Eukaryot Cell 6:421–429
46. Tsuji G, Kenmochi Y, Takano Y, Sweigard J, Farrall L, Furusawa I, Horino O, Kubo Y (2000) Novel fungal transcriptional activators, Cmr1p of Colletotrichum lagenarium and pig1p of Magnaporthe grisea, contain Cys2His2 zinc finger and Zn(II)2Cys6 binuclear cluster DNA-binding motifs and regulate transcription of melanin biosynthesis genes in a developmentally specific manner. Mol Microbiol 38:940–954
47. Gressler M, Zaehle C, Scherlach K, Hertweck C, Brock M (2011) Multifactorial induction of an orphan PKS-NRPS gene cluster in Aspergillus terreus. Chem 18:198–209
48. Jekosch K, Kück U (2000a) Glucose dependent transcriptional expression of the cre1 gene in Acremonium chrysogenum strains showing different levels of cephalosporin C production. Curr Genet 37:388–395
49. Jekosch K, Kück U (2000b) Loss of glucose repression in an Acremonium chrysogenum beta-lactam producer strain and its restoration by multiple copies of the cre1 gene. Appl Microbiol Biotechnol 54:556–563
50. Trapp SC, Hohn TM, McCormick S, Jarvis BB (1998) Characterization of the gene cluster for biosynthesis of macrocyclic trichothecenes in Myrothecium roridum. Mol Gen Genet 257:421–432
51. Bergh KT, Brakhage AA (1998) Regulation of the Aspergillus nidulans penicillin biosynthesis gene acvA (pcbAB) by amino acids: implication for involvement of transcription factor PACC. Appl Environ Microbiol 64:843–849
52. Keller NP, Nesbitt C, Sarr B, Phillips TD, Burow GB (1997) pH regulation of sterigmatocystin and aflatoxin biosynthesis in Aspergillus spp. Phytopathology 87:643–648
53. Schmitt EK, Kempken R, Kuck U (2001) Functional analysis of promoter sequences of cephalosporin C biosynthesis genes from Acremonium chrysogenum: specific DNA-protein interactions and characterization of the transcription factor PACC. Mol Genet Genomics 265:508–518
54. Flaherty JE, Pirttilä AM, Bluhm BH, Woloshuk CP (2003) PAC1, a pH-regulatory gene from Fusarium verticillioides. Appl Environ Microbiol 69:5222–5227
55. Barad S, Horowitz S, Kobiler I, Sherman A, Prusky DB (2013) Accumulation of the mycotoxin patulin in the presence of gluconic acid contributes to pathogenicity of Penicillium expansum. Mol Plant-Microbe Interact 27:66–77
56. Malapi-Wight M, Smith J, Campbell J, Bluhm BH, Shim W-B (2013) Sda1, a Cys2-His2 zinc finger transcription factor, is involved in polyol metabolism and fumonisin B1 production in Fusarium verticillioides. PLoS ONE 8:e67656
57. Bergmann S, Funk AN, Scherlach K, Schroeckh V, Shelest E, Horn U, Hertweck C, Brakhage AA (2010) Activation of a silent fungal polyketide biosynthesis pathway through regulatory cross talk with a cryptic nonribosomal peptide synthetase gene cluster. Appl Environ Microbiol 76(24):8143–8149

58. Hohn TM, Krishna R, Proctor RH (1999) Characterization of a transcriptional activator controlling trichothecene toxin biosynthesis. Fungal Genet Biol 26:224–235
59. Reverberi M, Gazzetti K, Punelli F, Scarpari M, Zjalic S, Ricelli A, Fabbri AA, Fanelli C (2012) Aoyap1 regulates OTA synthesis by controlling cell redox balance in Aspergillus ochraceus. Appl Microbiol Biotechnol 95:1293–1304
60. Roze LV, Chanda A, Wee J, Awad D, Linz JE (2011) Stress-related transcription factor AtfB integrates secondary metabolism with oxidative stress response in aspergilli. J Biol Chem 286:35137–35148
61. Eisendle M, Schrettl M, Kragl C, Müller D, Illmer P, Haas H (2006) The intracellular siderophore ferricrocin is involved in iron storage, oxidative-stress resistance, germination, and sexual development in Aspergillus nidulans. Eukaryot Cell 5:1596–1603
62. Wagner D, Schmeinck A, Mos M, Morozov IY, Caddick MX, Tudzynski B (2010) The bZIP transcription factor MeaB mediates nitrogen metabolite repression at specific loci. Eukaryot Cell 9:1588–1601
63. Amaike S, Affeldt KJ, Yin W-B, Franke S, Choithani A, Keller NP (2013) The bZIP protein MeaB mediates virulence attributes in Aspergillus flavus. PLoS ONE 8:e74030
64. Yin W-B, Amaike S, Wohlbach DJ, Gasch AP, Chiang Y-M, Wang CCC, Bok JW, Rohlfs M, Keller NP (2012) An Aspergillus nidulans bZIP response pathway hardwired for defensive secondary metabolism operates through aflR. Mol Microbiol 83:1024–1034
65. Sekonyela R, Palmer JM, Bok J-W, Jain S, Berthier E, Forseth R, Schroeder F, Keller NP (2013) RsmA regulates Aspergillus fumigatus gliotoxin cluster metabolites including cyclo(L-Phe-L-Ser), a potential new diagnostic marker for invasive aspergillosis. PLoS ONE 8:e62591
66. Bussink HJ, Clark A, Oliver R (2001) The Cladosporium fulvum Bap1 gene: evidence for a novel class of Yap-related transcription factors with ankyrin repeats in phytopathogenic fungi. Eur J plant Pathol 107:655–659
67. Pedley KF, Walton JD (2001) Regulation of cyclic peptide biosynthesis in a plant pathogenic fungus by a novel transcription factor. Proc Natl Acad Sci USA 98:14174–14179
68. Schmitt EK, Hoff B, Kück U (2004) AcFKH1, a novel member of the forkhead family, associates with the RFX transcription factor CPCR1 in the cephalosporin C-producing fungus Acremonium chrysogenum. Gene 342:269–281
69. Schmitt EK, Kuck U (2000) The fungal CPCR1 protein, which binds specifically to β-lactam biosynthesis genes, is related to human regulatory factor X transcription factors. J Biol Chem 275:9348–9357
70. Atanasova L, Knox BP, Kubicek CP, Druzhinina IS, Baker SE (2013) The polyketide synthase gene pks4 of Trichoderma reesei provides pigmentation and stress resistance. Eukaryot Cell 12:1499–1508
71. Hong S-Y, Roze LV, Linz JE (2013) Oxidative stress-related transcription factors in the regulation of secondary metabolism. Toxins 5:683–702
72. Temme N, Oeser B, Massaroli M, Heller J, Simon A, Collado IG, Viaud M, Tudzynski P (2012) BcAtf1, a global regulator, controls various differentiation processes and phytotoxin production in Botrytis cinerea. Mol Plant Pathol 13:704–718
73. Ahn JH, Walton JD (1998) Regulation of cyclic peptide biosynthesis and pathogenicity in Cochliobolus carbonum by TOXEp, a novel protein with a bZIP basic DNA-binding motif and four ankyrin repeats. Mol Gen Genet 260:462–469
74. Gajiwala KS, Burley SK (2000) Winged helix proteins. Curr Opin Struct Biol 10:110–116
75. Hoff B, Schmitt EK, Kück U (2005) CPCR1, but not its interacting transcription factor AcFKH1, controls fungal arthrospore formation in Acremonium chrysogenum. Mol Microbiol 56:1220–1233
76. Bayram O, Braus GH (2012) Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. FEMS Microbiol Rev 36(1):1–24
77. Feng GH, Leonard TJ (1998) Culture conditions control expression of the genes for aflatoxin and sterigmatocystin biosynthesis in Aspergillus parasiticus and A. nidulans. Appl Environ Microbiol 64:2275–2277

78. Wilson R, Arst H (1998) Mutational analysis of AREA, a transcriptional activator mediating nitrogen metabolite repression in Aspergillus nidulans and a member of the “streetwise” GATA family. Microbiol Mol Biol Rev 62:586–596
79. Tilburn J, Sarkar S, Widdick D, Espeso E, Orejas M, Mungroo J, Peñalva MA, Arst HN Jr (1995) The Aspergillus PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J 14:779–790
80. Trushina N, Levin M, Mukherjee PK, Horwitz BA (2013 Jan) PacC and pH-dependent transcriptome of the mycotrophic fungus Trichoderma virens. BMC Genomics 14(138):1–21
81. Brakhage AA (2013) Regulation of fungal secondary metabolism. Nat Rev Microbiol 11:21–32
82. Espeso EA, Tilburn J, Arst HN, Peñalva MA (1993) pH regulation is a major determinant in expression of a fungal penicillin biosynthetic gene. EMBO J 12:3947–3956
83. Ronne H (1995) Glucose repression in fungi. Trends Genet 11:12–17
84. Dowzer CEA, Kelly JM (1991) Analysis fo the creA gene, a regulator of carbon catabolite repression in Aspergillus nidulans. Mol Cell Biol 11:5701–5709
85. Brakhage AA, Browne P, Turner G (1992) Regulation of Aspergillus nidulans penicillin biosynthesis and penicillin biosynthesis genes acvA and ipnA by glucose. J Bacteriol 174:3789–3799
86. Ni M, Yu J-H (2007) A novel regulator couples sporogenesis and trehalose biogenesis in Aspergillus nidulans. PLoS ONE 2:e970
87. Bayram O, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O, Braus-Stronmeyer S, Kwon N-J, Keller NP, Yu J-H, Braus GH (2008) VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320:1504–1506
88. Stinnett SM, Espeso E, Cobeño L, Araújo-Bazán L, Calvo AM (2007) Aspergillus nidulans VeA subcellular localization is dependent on the importin alpha carrier and on light. Mol Microbiol 63:242–255
89. Bok JW, Keller NP (2004) LaeA, a regulator of secondary metabolism in Aspergillus spp. Eukaryot Cell 3:527–535
90. Chettri P, Calvo AM, Cary JW, Dhingra S, Guo Y, McDougal RL, Bradshaw RE (2012) The veA gene of the pine needle pathogen Dothistroma septosporum regulates sporulation and secondary metabolism. Fungal Genet Biol 49:141–151
91. Hoff B, Kamerewerd J, Sigl C, Mitterbauer R, Zadra I, Kürnsteiner H, Kück U (2010) Two components of a velvet-like complex control hyphal morphogenesis, conidiophore development, and penicillin biosynthesis in Penicillium chrysogenum. Eukaryot Cell 9:1236–1250
92. Kopke K, Hoff B, Bloemendal S, Katschorowski A, Kamerewerd J, Kück U (2013) Members of the Penicillium chrysogenum velvet complex play functionally opposing roles in the regulation of penicillin biosynthesis and conidiation. Eukaryot Cell 12:299–310
93. López-Berges MS, Hera C, Sulyok M, Schäfer K, Capilla J, Guarro J, Di Pietro A (2013) The velvet complex governs mycotoxin production and virulence of Fusarium oxysporum on plant and mammalian hosts. Mol Microbiol 87:49–65
94. Veiga T, Nijland JG, Driessen AJM, Bovenberg RAL, Touw H, van den Berg MA, Pronk JT, Daran J-M (2012) Impact of velvet complex on transcriptome and penicillin G production in glucose-limited chemostat cultures of a β-lactam high-producing Penicillium chrysogenum strain. OMICS 16:320–333
95. Wiemann P, Brown DW, Kleigrewe K, Bok JW, Keller NP, Humpf H-U, Tudzynski B (2011) FfVel1 and FfLae1, components of a velvet-like complex in Fusarium fujikuroi, affect differentiation, secondary metabolism and virulence. Mol Microbiol 77:972–994
96. Wu D, Oide S, Zhang N, Choi MY, Turgeon BG (2012) ChLae1 and ChVel1 regulate T-toxin production, virulence, oxidative stress response, and development of the maize pathogen Cochliobolus heterostrophus. PLoS Pathog 8(2):e1002542
97. Yang Q, Chen Y, Ma Z (2013) Involvement of BcVeA and BcVelB in regulating conidiation, pigmentation and virulence in Botrytis cinerea. Fungal Genet Biol 50:63–71
98. Palmer JM, Theisen JM, Duran RM, Grayburn WS, Calvo AM, Keller NP (2013) Secondary metabolism and development is mediated by LlmF control of VeA subcellular localization in Aspergillus nidulans. PLoS Genet 9:e1003193

99. Bi Q, Wu D, Zhu X, Gillian Turgeon B (2013) Cochliobolus heterostrophus Llm1– a Lae1-like methyltransferase regulates T-toxin production, virulence, and development. Fungal Genet Biol 51:21–33
100. Bok JW, Noordermeer D, Kale SP, Keller NP (2006a) Secondary metabolic gene cluster silencing in Aspergillus nidulans. Mol Microbiol 61:1636–1645
101. Brakhage AA, Andrianopoulos A, Kato M, Steidl S, Davis MA, Tsukagoshi N, Hynes MJ (1999) HAP-like CCAAT-binding complexes in filamentous fungi: implications for biotechnology. Fungal Genet Biol 27:243–252
102. Brakhage AA, Thön M, Spröte P, Scharf DH, Al-Abdallah Q, Wolke SM, Hortschansky P (2009) Aspects on evolution of fungal β-lactam biosynthesis gene clusters and recruitment of trans-acting factors. Phytochemistry 70:1801–1811
103. Steidl S, Hynes MJ, Brakhage AA (2001) The Aspergillus nidulans multimeric CCAAT binding complex AnCF is negatively autoregulated via its hapB subunit gene. J Mol Biol 306:643–653
104. Thön M, Abdallah Q A, Hortschansky P, Scharf DH, Eisendle M, Haas H, Brakhage AA (2010) The CCAAT-binding complex coordinates the oxidative stress response in eukaryotes. Nucleic Acids Res 38:1098–1113
105. Hortschansky P, Eisendle M, Al-Abdallah Q, Schmidt AD, Bergmann S, Thön M, Kniemeyer O, Abt B, Seeber B, Werner ER, Kato M, Brakhage AA, Haas H (2007) Interaction of HapX with the CCAAT-binding complex—a novel mechanism of gene regulation by iron. EMBO J 26:3157–3168
106. Price MS, Yu J, Nierman WC, Kim HS, Pritchard B, Jacobus CA, Bhatnagar D, Cleveland TE, Payne GA (2006) The aflatoxin pathway regulator AflR induces gene transcription inside and outside of the aflatoxin biosynthetic cluster. FEMS Microbiol Lett 255:275–279
107. Nasmith CG, Walkowiak S, Wang L, Leung WWY, Gong Y, Johnston A, Harris LJ, Guttman DS, Subramaniam R (2011) Tri6 is a global transcription regulator in the phytopathogen Fusarium graminearum. PLoS Pathog 7:e1002266

Chapter 3
Epigenetics of Fungal Secondary Metabolism Related Genes

Ming-Yueh Wu and Jae-Hyuk Yu

Fungal Secondary Metabolism and Epigenetics

Fungi produce a diverse array of low molecular weight, bioactive secondary metabolites are not essential for their survival. Secondary metabolism (SM) is defined as "the production of ancillary metabolites and 'useful' compounds, initiated after using preferred carbon and nitrogen sources" [1, 2]. Secondary metabolites are not necessary for normal growth, but are considered important for the producing fungus to flourishing in its niche [3–5], stress tolerance [6, 7], or defense against hostile and/or competing organisms [1, 8]. They are important for day-to-day human life as beneficial antibiotics, pharmaceuticals, and/or harmful mycotoxins [9]. However, the true biological functions of many fungal secondary metabolites in producing fungi are largely cryptic.

Fungal SM is a complex process, which is often tightly related with morphological development [10]. Due to the importance of fungal secondary metabolites, an increasing number of genes associated with SM have been identified and characterized. Furthermore, the availability of fungal genomes accelerates the identification of biosynthetic genes for secondary metabolites. However, the role and regulatory mechanisms of many of the newly defined genes remain to be investigated [11]. In fungi, secondary metabolite biosynthetic and regulatory genes are usually clustered and not evenly distributed across the genomes [12–14]. Many of the clusters are silent under the standard laboratory culture conditions, which makes it difficult to

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elucidate their functions and regulatory mechanisms. It is both time and resource consuming to find the appropriate conditions to express the gene clusters of interest. A promising strategy to investigate unknown SM cluster(s) is via modifying global epigenetic regulators to activate the silenced SM clusters [15, 16].

Epigenetic phenomena are defined as reversible and heritable changes in gene expression levels without altering the DNA sequences. Epigenetic phenomena can derive from DNA-, chromatin-, and RNA-based effects, and include DNA methylation, position effects, RNA silencing systems, centromere/telomere location, and chromatin structure changes. Many of the aforementioned phenomena occur in fungi throughout the life cycle [17] which makes fungi an excellent model system to understand the fundamental principles of epigenetics. During the life cycle, fungi regulate development by several epigenetic mechanisms. Most steps or cell types are known to be under control by DNA methylation, which is regulated by changes in the chromatin state. Methylation induced premeiotically (MIP) and repeat-induced point mutation (RIP) occur during dikaryon formation and conjugated nuclear division [18–25]. MIP is regulated by DNA methylation, and RIP may also be regulated by it. Moreover, filamentous fungi share conserved silencing systems with higher eukaryotes, such as RNA interference (RNAi) and DNA methylation [26–31]. However, it is uncertain whether RNAi, which regulates the parasexual cycle and germination, is related to DNA- or chromatin-mediated epigenetic phenomena [32]. In addition, meiotic silencing by unpaired DNA, also known as meiotic silencing (MSUD), is another RNA silencing mechanism, that occurs throughout meiosis [33, 34].

As mentioned, fungal secondary metabolite synthetic and regulatory genes tend to be clustered. Gene clusters may originate from the horizontal transfer of genes from bacteria to fungi [35–40]. However, some SM gene clusters—e.g., gibberellin (GB) gene cluster—are unlikely a result of horizontal transfer [41]. The clustered SM genes are likely subject to co-regulation by epigenetic changes. An emerging field, chemical epigenetics, has been evolving to stimulate expression of secondary metabolite gene clusters by altering epigenetic status such as DNA and/or histone modifications [1, 42].

Epigenetic Modifications that Affect Secondary Metabolism

The epigenetic regulation of fungal SM is mainly through histone modifications: methylation, acetylation, and sumoylation (Fig. 3.1) [43–47]. Histone proteins are the primary protein components of chromatin and function as a scaffold for the nucleosome formation. Histone octamer consisting of two each of H2A, H2B, H3, and H4 is wrapped by DNA and forms a nucleosome [48].

Histone modifications can affect chromatin conformation and recruited proteins that cause epigenetic changes by interacting with histones [49]. Most histone modifications involve histones H3 and H4 [44, 45, 50]. The N-terminus of H3 and H4 are

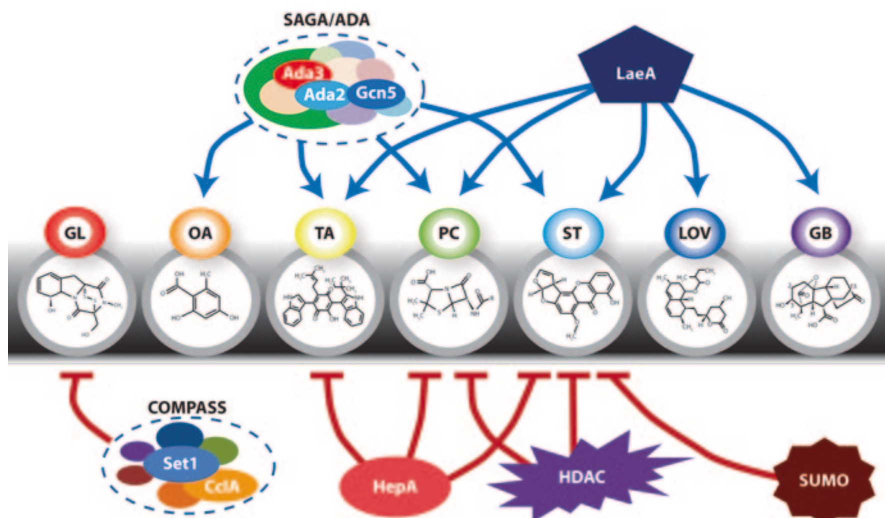

Fig. 3.1 Examples of fungal secondary metabolites and their epigenetic regulators. Certain fungal secondary metabolites regulated by one or more epigenetic regulators are shown. *PC* Penicillin, *LOV* Lovastatin, *GB* Gibberellin, *AT* Asperthecin, *GL* Gliotoxin, *OA* Orsellinic Acid, *TA* Terrequinone A, *ST* Sterigmatocystin

crucial to generate heterochromatin or euchromatin. In euchromatin, lysines in the H3 and H4 tails are hyperacetylated and H3K4 is trimethylated. In heterochromatin, in comparison, lysines in H3 and H4 are hypoacetylated and H3K9 is trimethylated [51]. By histone modifications, only a group of specific target genes inside of distinct regions of the chromosomes can be regulated, further supporting the advantage of SM genes being clustered [44, 45, 50].

Genes Affecting Histone Methylation

HepA HepA is the *Aspergillus nidulans* homolog of HP1 (the heterochromatin protein-1, SWI6 in *Schizosaccharomyces pombe*) [52–54]. Heterochromatin domains are silenced and have hypoacetylation of lysines in H3 and H4 [55] with different degrees of methylation of H3K9 (H3K9me) by a histone methyltransferase (Clr4 in *S. pombe*) [56, 57]. As a transcriptional repressor, HP1 recognizes H3K9me and directly binds to it, achieving both targeting and transcriptional repression by maintaining the heterochromatin structure [58–63]. Artificial recruitment of HP1 to a gene promoter region leads to gene repression, supporting that HP1 is essential in gene silencing [64, 65].

HepA acts as an epigenetic repressor in expression of secondary metabolite genes [52]. The deletion of *HepA* leads to derepression of secondary metabolite biosynthetic genes, including sterigmatocystin (ST), penicillin (PC), and terrequinone A (TA).

Biochemical analysis shows that the silent ST gene cluster is marked by H3K9me3 and recruits high levels of HepA, leading to repression of ST production during growth phase. Upon growth arrest and activation of SM, HepA, and H3K9me3 levels decrease while the acetylated histone H3 increases [52]. HepA occupancy and H3K9me3 levels are counteracted by the global SM regulator (LaeA) (Fig. 3.2).

LaeA (loss of aflR expression-A) is a global regulator of SM and development in filamentous fungi. This nuclear protein was first reported in *Aspergillus* spp. [44]. The lack of *laeA* blocks expression of several metabolic gene clusters, including ST, PC, and lovastatin (LOV). The overexpression of *laeA* contrarily increases expression of ST and LOV gene clusters and subsequent ST and LOV production [44]. In *Penicillium chrysogenum,* the overexpression of *laeA* increases PC production (~125 %) and the lack of *laeA* dramatically reduces PC gene expression levels and PC production [50]. Similarly, LaeA serves as a positive regulator of GB production in *Fusarium fujikuroi* [66]. In addition, microarray analysis indicates that LaeA regulates up to 9.5 % of the *Aspergillus fumigatus* transcriptome and up to 13 of its

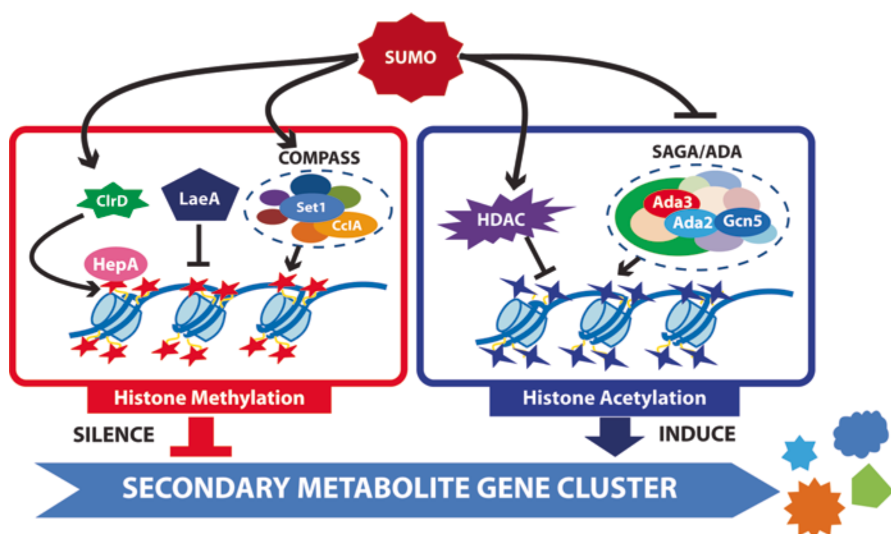

Fig. 3.2 Overview and the roles of the epigenetic regulators in fungal secondary metabolism. Many epigenetic regulators participate in fungal secondary metabolism. HepA, Clr4, COMPASS, and LaeA are involved in histone methylation (*red box, red stars indicate histone methylation*). Clr4 leads to H3K9 methylation, which enables HepA binding to histone. HepA binding stabilizes the heterochromatin structure and thus leads to silencing the secondary metabolic gene clusters. COMPASS methylates H3K4 and H3K9, and silences SM, while LaeA removes the histone methylation and HepA binding and induces SM. HDACs and SAGA/ADA complex play a role in controlling histone acetylation (*blue box, blue stars indicate histone acetylation*), which induces fungal SM. HDACs deacetylate the lysines of H3 and/or H4, while the SAGA/ADA complex acetylates them. SUMO (*the scarlet decagon*) conducts sumoylation of several epigenetic regulators, including Clr4, COMPASS, HDACs, and SAGA/ADA, and silences SM

22 secondary metabolite gene clusters, containing NRPS, PKS, and P450 monooxygenase genes [67].

LaeA forms a key heterotrimeric complex with the two *velvet* proteins, VelB and VeA. The VelB/VeA/LaeA trimeric complex coordinates light signals with fungal development and SM [68]. VeA physically interacts with VelB, and bridges it to LaeA. All three components in this complex are essential for sexual development and ST production in *A. nidulans*. Previous studies showed that LaeA and VeA interact in *P. chrysogenum* and *F. fujikuroi*, too [50, 66]. The successful cross-genus complementation between *Fusarium, Aspergillus*, and *Penicillium* indicates that the VelB/VeA/LaeA complex has undergone a divergence in specific functions mediating SM [66].

LaeA-mediated SM regulation primarily depends on histone methylation. LaeA contains a predicted and functionally necessary S-adenosyl-methionine (SAM) binding domain [68–70], which is present in all members of the methylase superfamily [71], and has sequence similarity to histone and arginine methyltransferase [44, 72]. The *laeA* gene is negatively regulated by AflR, a Zn_2/Cys_6 transcription factor located in the aflatoxin and ST gene clusters, in a feedback loop [44]. In *A. nidulans*, the ST gene cluster expression analysis shows that LaeA-mediated regulation of the cluster is location specific. The placement of *argB* in the ST cluster results in *argB* silencing in the *laeA* deletion background, whereas the genes bordering the ST cluster are unaffected [69]. Similar location-specific effects on SM gene regulation have been reported in other *Aspergillus* species as well [73–75]. Notably, the location specific effect is only reported in *Aspergillus* and *Neurospora* [13, 76],

These findings indicate that LaeA may differentially affect histone protein methylation, which in turn allows the cluster region to be more accessible to gene transcription [69]. Biochemical analyses of *laeA* and heterochromatin mutants (e.g., histone deacetylase and histone methyltransferase mutants) in *A. nidulans* demonstrate that LaeA activates SM gene expression by being involved in the removal of heteromatin marks like H3K9 methylation and HepA binding [13, 52, 77]; i.e., the LaeA-involved machinery reverses the heterochromatic signature and activates the gene expression inside the SM cluster.

COMPASS COMPASS (complex proteins associated with Set1) is a multi-subunit complex consisting of Set1, Bre2, Sdc1, Spp1, Swd1, Swd2, and Swd3 [78–80]. COMPASS is involved in H3K4 mono-, di-, and tri-methylation [77, 79–82], which is necessary for RNA Pol II binding and transcriptional activity in development and differentiation [79, 80, 83] in *Saccharomyces cerevesiae*. Three core components, Set1, Swd1, and Swd3 are essential for COMPASS [78]. Swd2, Bre2, Sdc1, and Spp1 affect the degree of Set1 methylation [84–86]. Set1 has the SET domain, which possesses histone or lysine methyltransferase (HMTase or KMTase) activity [87].

CclA (Bre2 in *S. cerevisiae*) is one of the eight members of COMPASS in *A. nidulans*. The lack of CclA leads to reduced levels of H3K4 and H3K9 di- and tri-methylation, as well as reduced H3 acetylation [88]. H3K4 di- and tri-methylation is associated with actively expressed genes and are required for telomere silencing in *S. cerevesiae* [79, 80, 89–91], and activating *A. nidulans* SMs, e.g.,

monodictyphenone, emodins, and the polyketides F9775A and F9775B [52, 77]. In *A. fumigatus*, loss of CclA results in slow fungal growth and increased SM production like gliotoxin [92]. Based on 6-azauracil (6AU) sensitivity test result, CclA plays a role in transcription elongation [92].

Genes Influencing Histone Acetylation

Histone Deacetylases (HDAC) Histone deacetylases (HDACs) and histone acetyltransferases (HATs) play critical roles in fungal epigenetic regulatory mechanism. Histone acetylation is reversible and controlled by HDACs and HATs [51]. HDACs are classified into three main groups based on their homology to yeast proteins: Class I HDACs have homology to yeast Rpd3; Class II HDACs have homology to yeast Hda1; Class III HDACs have homology to yeast Sir2. Both Classes I and II HDACs contain zinc in their catalytic site, and are known as epigenetic regulators in fungal SM. Class III HDACs do not have zinc in the catalytic site but require NAD^+ instead [93].

A. nidulans RpdA is a Class I HDAC and the homolog of the global repressor Rpd3 in *S. cerevisiae*. RpdA is necessary for growth, conidiation, and gene regulation. The lack of Rpd3 leads to increased acetylation of H4K5, H4K12, and H3K18 in derepressed genes [94]. The absence of RpdA is lethal in *A. nidulans* and *Neurospora crassa* [95]. Silencing of RpdA in *A. nidulans* reveals that RpdA is involved in normal growth and H3 and H4 deacetylation [96].

Histone deacetylase A (HdaA) is a Class II HDAC playing a counter role to LaeA in SM regulation in *A. nidulans*. Loss of *hdaA* causes precocious and increased expression of ST and PC biosynthetic genes. The deletion of *hdaA* causes derepression of SM gene clusters that are located close to the telomeres in *A. nidulans* [97]. In *A. fumigatus*, HdaA plays a similar role in SM regulation [98]. Inhibition of most HDACs induces the production of unknown SMs in Penicillium expansum [97]. Treating the fungus with HDAC inhibitors leads to overproduction of several secondary metabolites, suggesting that HDAC-mediated repression of certain SM gene clusters is conserved in fungi [97].

SAGA/ADA Complex The Spt-Ada-Gcn5-acetyltransferase (SAGA/ADA) coactivator complex regulates numerous cellular processes by posttranslational modifications of histones [99]. SAGA/ADA contains a HAT, Gcn5, and acetylates multiple lysine residues at the N-terminal tails of H3 and H2B. In *A. nidulans*, GcnE (Gcn5 homolog in *A. nidulans*) regulates PC biosynthesis gene cluster located on chromosome VI by histone acetylation [45, 100]. The Ada1–5 proteins (Alteration/deficiency in activation) are components of SAGA/ADA in *S. cerevisiae* [101]. Ada2/Ada3/Gcn5 complex is sufficient for robust histone and nucleosomal HAT activity in yeast [102].

Both GcnE and AdaB are required for induction of the orsellinic acid gene cluster in *A. nidulans*. Similarly, SAGA/ADA plays a major role in specific induction of

other SM gene clusters, such as ST, PC, and terrequinone [45]. Chromatin immunoprecipitation (ChIP) data shows that SAGA/ADA increases acetylation at H3K9 and H3K14 in *A. nidulans*. Interestingly, the increase of H3K14 acetylation is a global phenomenon of the whole genome, while the increase of H3K9 acetylation can be only observed within SM gene clusters [45].

Genes Impacting Sumoylation

*SUMO S*mall *u*biquitin-like *m*odifier (SUMO) is a small protein that has high structural similarity to ubiquitin, despite its low similarity at the level of the amino acid sequence [103–106]. SUMO covalently attaches to other proteins through the activities of an enzyme cascade (E1-E2-E3) similar to that of ubiquitination, and is known to play a role in histone modification like ubiquitin [105, 107–111]. Histone sumoylation mediates gene silencing through recruitment of HDAC and Hp1 both in vitro and in vivo in human cells [112, 113]. SUMO also modifies Gcn5, a member of the SAGA/ADA complex, and results in gene silencing in yeast [114].

In *A. nidulans*, SUMO represses sexual development and is involved in accurate induction and light stimulation of asexual development [104, 115]. CclA and SetA, two members of COMPASS, connects the SUMO network to histone modification. The interplay of the fungal sumoylation network controls temporal and spatial steps in cell differentiation [104].

SUMO is also essential for sexual fruiting body formation and SM in *A. nidulans* [47, 116]. Deleting *sumo* causes about 200-fold increase of asperthecin production but decreases production of austinol/dehydroaustinol and ST [47]. The effect of sumoylation on SM may occur at several levels, such as silencing the secondary metabolite gene clusters at the chromatin level or regulating TFs involved in the SM regulation [47]. Additional work needs to be done to elucidate how SUMO regulates specific secondary metabolite production.

Application of Epigenetic Regulators of Fungal Secondary Metabolites

Understanding the SM epigenetic regulators can accelerate fungal SM studies by activating certain SM gene clusters that are often silent and cryptic in lab culture conditions. Suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor, has been used to stimulate the production of new cladochromes and calphostin B, a known protein kinase C inhibitor [117], in *Cladosporium cladosporioides* [118]. Treatment with SAHA can boost nygerone A production in *Aspergillus niger* [119, 120] and orsellinic acid production in *A. nidulans* without coculturing with *Streptomyces rapamycincus* [45]. In addition, using a global SM regulator is a new approach to identify new secondary metabolic genes. For example, LaeA is an excellent ge-

nomic mining tool and has successfully been manipulated to uncover several novel secondary metabolites such as terrequinone A [15, 16]. Another way to alter expression of SM gene clusters is to manipulate histone modification, for example, by the deletion of *hepA* [52] or *hdaA* [97].

Conclusion

Fungi produce a wide range of secondary metabolites. These low molecular weight compounds are diverse in structure and perform important yet often cryptic biological functions. The scientific community shows great interest in fungal secondary metabolites due to their importance to humankind. However, sequencing data of the fungal genomes indicate that a large number of fungal secondary metabolites are yet to be uncovered and characterized. As most fungal secondary metabolic gene clusters are silent under standard laboratory conditions, the importance of global regulators and epigenetic regulatory mechanism has been increasingly recognized. Various proteins and their complexes play a role in the regulation of fungal SM gene clusters through histone modification. Some of these epigenetic regulators mediate modification at distinct sites, such as methylation, acetylation, and sumoylation, whereas others inhibit such alterations (Fig. 3.2).

In this chapter, we have reviewed several known epigenetic regulators that are involved in regulating fungal SM. Epigenetics is an emerging area for investigating fungal SM, and a better understanding of SM epigenetic regulation would lead to the discovery of new drugs.

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References

1. Brakhage AA, Schroeckh V (2011) Fungal secondary metabolites–strategies to activate silent gene clusters. Fungal Genet Biol 48(1):15–22
2. Brakhage AA, Schuemann J, Bergmann S, Scherlach K, Schroeckh V, Hertweck C (2008) Activation of fungal silent gene clusters: a new avenue to drug discovery. Natural compounds as drugs. Springer, Basel p. 1–12
3. Howard RJ, Valent B (1996) Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. Annu Rev Microbiol 50(1):491–512
4. Kimura N, Tsuge T (1993) Gene cluster involved in melanin biosynthesis of the filamentous fungus *Alternaria alternata*. J Bacteriol 175(14):4427–4435
5. Tsai H-F, Chang YC, Washburn RG, Wheeler MH, Kwon-Chung KJ (1998) The developmentally regulated *alb1* gene of *Aspergillus fumigatus*: Its role in modulation of conidial morphology and virulence. J Bacteriol 180(12):3031–3038

6. Leonard KJ (1977) Virulence, temperature optima, and competitive abilities of isolines of races T and O of *Biopolaris maydis*. Phytopathology 67(11):1273–1279
7. Klittich CJR, Bronson CR (1986) Reduced fitness associated with tox1 of *Cochliobolus heterostrophus*. Phytopathology 76(12):1294–1298
8. Yim G, Wang HH (2007) Antibiotics as signalling molecules. Philos Trans R Soc B Biol Sci 362(1483):1195–1200
9. Yu J-H, Keller N (2005) Regulation of secondary metabolism in filamentous fungi. Annu Rev Phytopathol 43:437–458
10. Calvo AM, Wilson RA, Bok JW, Keller NP (2002 Sep) Relationship between secondary metabolism and fungal development. Microbiol Mol Biol Rev 66(3):447–459
11. Fox EM, Howlett BJ (2008) Secondary metabolism: regulation and role in fungal biology. Curr Opin Microbiol 11(6):481–487
12. Ma L-J, Van Der Does HC, Borkovich KA, Coleman JJ, Daboussi M-J, Di Pietro A et al (2010) Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature 464(7287):367–373
13. Palmer JM, Keller NP (2010) Secondary metabolism in fungi: does chromosomal location matter? Curr Opin Microbiol 13(4):431–436
14. Fedorova ND, Khaldi N, Joardar VS, Maiti R, Amedeo P, Anderson MJ et al (2008) Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. PLoS Genet 4(4):e1000046
15. Bouhired S, Weber M, Kempf-Sontag A, Keller NP, Hoffmeister D (2007) Accurate prediction of the *Aspergillus nidulans* terrequinone gene cluster boundaries using the transcriptional regulator LaeA. Fungal Genet Biol 44(11):1134–1145
16. Bok JW, Hoffmeister D, Maggio-Hall LA, Murillo R, Glasner JD, Keller NP (2006) Genomic mining for *Aspergillus* natural products. Chem Biol 13(1):31–37
17. Freitag M, Selker EU (2005) Controlling DNA methylation: many roads to one modification. Curr Opin Genet Dev 15(2):191–199
18. Selker EU, Jensen BC, Richardson GA (1987) A portable signal causing faithful DNA methylation de novo in *Neurospora crassa*. Science 238(4823):48–53
19. Singer MJ, Marcotte BA, Selker EU (1995) DNA methylation associated with repeat-induced point mutation in *Neurospora crassa*. Mol Cell Biol 15(10):5586–5597
20. Miao VPW, Freitag M, Selker EU (2000) Short tpa-rich segments of the ζ-η region induce DNA methylation in *Neurospora crassa*. J Mol Biol 300(2):249–273
21. Tamaru H, Selker EU (2003) Synthesis of signals for de novo DNA methylation in *Neurospora crassa*. Mol Cell Biol 23(7):2379–2394
22. Rossignol JL, Faugeron G (1995) Mip: An epigenetic gene silencing process in *Ascobolus immersus*. Gene silencing in higher plants and related phenomena in other eukaryotes: Springer, Verlag, p. 179–191
23. Barry C, Faugeron G, Rossignol J-L (1993) Methylation induced premeiotically in *Ascobolus*: coextension with DNA repeat lengths and effect on transcript elongation. Proc Natl Acad Sci 90(10):4557–4561
24. Rhounim L, Rossignol J-L, Faugeron G (1992) Epimutation of repeated genes in *Ascobolus immersus*. EMBO J 11(12):4451
25. Faugeron G, Rhounim L, Rossignol J-L (1990) How does the cell count the number of ectopic copies of a gene in the premeiotic inactivation process acting in *Ascoborus immersus*? Genetics 124(3):585–591
26. Jorgensen RA, Que Q, Stam M (1999) Do unintended antisense transcripts contribute to sense cosuppression in plants? Trends Genet 15(1):11–12
27. Catalanotto C, Azzalin G, Macino G, Cogoni C (2000) Transcription: gene silencing in worms and fungi. Nature 404(6775):245–245
28. Romano N, Macino G Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. Mol Microbiol 6(22):3343–3353
29. Chen B, Choi GH, Nuss DL (1994) Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. Science 264(5166):1762–1764

30. Choi GH, Nuss DL (1992) Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science 257(5071):800–803
31. Nuss DL (2011) Mycoviruses, RNA silencing, and viral RNA recombination. Adv Virus Res 80:25
32. Hammond TM, Keller NP (2005) RNA silencing in *Aspergillus nidulans* is independent of RNA-dependent rna polymerases. Genetics 169(2):607–617
33. Aramayo R, Metzenberg RL (1996) Meiotic transvection in fungi. Cell 86(1):103–113
34. Shiu PKT, Raju NB, Zickler D, Metzenberg RL (2001) Meiotic silencing by unpaired DNA. Cell 107(7):905–916
35. Keller NP, Hohn TM (1997) Metabolic pathway gene clusters in filamentous fungi. Fungal Genet Biol 21(1):17–29
36. Rosewich UL, Kistler HC (2000) Role of horizontal gene transfer in the evolution of fungi. Annu Rev Phytopathol 38(1):325–363
37. Khaldi N, Collemare J, Lebrun M-H, Wolfe KH (2008) Evidence for horizontal transfer of a secondary metabolite gene cluster between fungi. Genome Biol 9(1):R18
38. Lawrence JG, Roth JR (1996) Selfish operons: horizontal transfer may drive the evolution of gene clusters. Genetics 143(4):1843–1860
39. Lawrence JG (1999) Gene transfer, speciation, and the evolution of bacterial genomes. Curr Opin Microbiol 2(5):519–523
40. Smith MW, Feng D-F, Doolittle RF (1992) Evolution by acquisition: the case for horizontal gene transfers. Trends Biochem Sci 17(12):489–493
41. Tudzynski B, Hedden P, Carrera E, Gaskin P (2001) The p450–4 gene of *Gibberella fujikuroi* encodes ent-kaurene oxidase in the gibberellin biosynthesis pathway. Appl Environ Microbiol 67(8):3514–3522
42. Wang X, Sena Filho JG, Hoover AR, King JB, Ellis TK, Powell DR et al (2010) Chemical epigenetics alters the secondary metabolite composition of guttate excreted by an atlantic-forest-soil-derived *Penicillium citreonigrum*. J Nat Prod 73(5):942–948
43. Shilatifard A (2006) Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annu Rev Biochem 75:243–269
44. Bok JW, Keller NP (2004) LaeA, a regulator of secondary metabolism in *Aspergillus spp*. Eukaryotic cell 3(2):527–535
45. Nützmann H-W, Reyes-Dominguez Y, Scherlach K, Schroeckh V, Horn F, Gacek A et al (2011) Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires saga/ada-mediated histone acetylation. Proc Natl Acad Sci 108(34):14282–14287
46. Strauss J, Reyes-Dominguez Y (2011) Regulation of secondary metabolism by chromatin structure and epigenetic codes. Fungal Genet Biol 48(1):62–69
47. Szewczyk E, Chiang Y-M, Oakley CE, Davidson AD, Wang CC, Oakley BR (2008) Identification and characterization of the asperthecin gene cluster of *Aspergillus nidulans*. Appl Environ Microbiol 74(24):7607–7612
48. Kornberg RD (1974) Chromatin structure: a repeating unit of histones and DNA. Science 184(4139):868–871
49. de la Cruz X, Lois S, Sánchez-Molina S, Martínez-Balbás MA (2005) Do protein motifs read the histone code? Bioessays 27(2):164–175
50. Kosalková K, García-Estrada C, Ullán RV, Godio RP, Feltrer R, Teijeira F et al (2009) The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. Biochimie 91(2):214–225
51. Smith KM, Phatale PA, Bredeweg EL, Pomraning KR, Freitag M (2012) Epigenetics of filamentous fungi. In: Meyers RA (ed) Epigenetic regulation and epigenomics (Current Topics from the Encyclopedia of Molecular Cell Biolo). Wiley-VCH Verlag GmbH & Co., pp 1063–1107
52. Reyes-Dominguez Y, Bok JW, Berger H, Shwab EK, Basheer A, Gallmetzer A et al (2010) Heterochromatic marks are associated with the repression of secondary metabolism clusters in *Aspergillus nidulans*. Mol Microbiol 76(6):1376–1386
53. Wang G, Ma A, Chow C-m, Horsley D, Brown NR, Cowell IG et al. (2000) Conservation of heterochromatin protein 1 function. Mol Cell Biol 20(18):6970–6983

54. Cryderman DE, Cuaycong MH, Elgin SC, Wallrath LL (1998) Characterization of sequences associated with position-effect variegation at pericentric sites in *Drosophila heterochromatin*. Chromosoma 107(5):277–285
55. Holbert MA, Marmorstein R (2005) Structure and activity of enzymes that remove histone modifications. Curr Opin Struct Biol 15(6):673–680
56. Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun Z-W, Schmid M et al (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406(6796):593–599
57. Noma K-i, Allis CD, Grewal SI (2001) Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science 293(5532):1150–1155
58. Fanti L, Pimpinelli S (2008) HP1: a functionally multifaceted protein. Curr Opin Genet Dev 18(2):169–174
59. Freitag M, Hickey PC, Khlafallah TK, Read ND, Selker EU (2004) HP1 is essential for DNA methylation in *Neurospora*. Mol Cell 13(3):427–434
60. Lewis ZA, Honda S, Khlafallah TK, Jeffress JK, Freitag M, Mohn F et al (2009) Relics of repeat-induced point mutation direct heterochromatin formation in *Neurospora crassa*. Genome Res 19(3):427–437
61. Sims RJ III, Nishioka K, Reinberg D (2003) Histone lysine methylation: a signature for chromatin function. TRENDS Genet 19(11):629–639
62. Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC et al (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature 410(6824):120–124
63. Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410(6824):116–120
64. Ayyanathan K, Lechner MS, Bell P, Maul GG, Schultz DC, Yamada Y et al (2003) Regulated recruitment of hp1 to a euchromatic gene induces mitotically heritable, epigenetic gene silencing: a mammalian cell culture model of gene variegation. Genes Dev 17(15):1855–1869
65. Li Y, Danzer JR, Alvarez P, Belmont AS, Wallrath LL (2003) Effects of tethering HP1 to euchromatic regions of the *Drosophila* genome. Development 130(9):1817–1824
66. Wiemann P, Brown DW, Kleigrewe K, Bok JW, Keller NP, Humpf HU et al (2010) FfVel1 and FfLae1, components of a velvet-like complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism and virulence. Mol Microbiol 77(4):972–994
67. Perrin RM, Fedorova ND, Bok JW, Cramer RA Jr, Wortman JR, Kim HS et al (2007) Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. PLoS Pathog 3(4):e50
68. Bayram Ö, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O et al (2008) VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320(5882):1504–1506
69. Bok JW, Noordermeer D, Kale SP, Keller NP (2006) Secondary metabolic gene cluster silencing in *Aspergillus nidulans*. Mol Microbiol 61(6):1636–1645
70. Hoffmeister D, Keller NP. (2007) Natural products of filamentous fungi: enzymes, genes, and their regulation. Natural Product Reports 24(2):393–416
71. Kozbial PZ, Mushegian AR (2005) Natural history of s-adenosylmethionine-binding proteins. BMC Struct Biol 5(1):19
72. Yin W, Keller NP (2011) Transcriptional regulatory elements in fungal secondary metabolism. J Microbiol 49(3):329–339
73. Chiou C-H, Miller M, Wilson DL, Trail F, Linz JE (2002) Chromosomal location plays a role in regulation of aflatoxin gene expression in *Aspergillus parasiticus*. Appl Environ Microbiol 68(1):306–315
74. Roze LV, Arthur AE, Hong SY, Chanda A, Linz JE. (2007) The initiation and pattern of spread of histone H4 acetylation parallel the order of transcriptional activation of genes in the aflatoxin cluster. Molecular Microbiol 66(3):713–726

75. Smith CA, Woloshuk CP, Robertson D, Payne GA (2007) Silencing of the aflatoxin gene cluster in a diploid strain of *Aspergillus flavus* is suppressed by ectopic aflR expression. Genetics 176(4):2077–2086
76. Smith KM, Kothe GO, Matsen CB, Khlafallah TK, Adhvaryu KK, Hemphill M et al (2008) The fungus *Neurospora crassa* displays telomeric silencing mediated by multiple sirtuins and by methylation of histone H3 lysine 9. Epigenetics Chromatin 1(1):5
77. Bok JW, Chiang Y-M, Szewczyk E, Reyes-Dominguez Y, Davidson AD, Sanchez JF et al (2009) Chromatin-level regulation of biosynthetic gene clusters. Nat Chem Biol 5(7):462–464
78. Roguev A, Schaft D, Shevchenko A, Pijnappel WWM, Wilm M, Aasland R et al (2001) The *Saccharomyces cerevisiae* Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4. EMBO J 20(24):7137–7148
79. Krogan NJ, Dover J, Khorrami S, Greenblatt JF, Schneider J, Johnston M et al (2002) Compass, a histone H3 (lysine 4) methyltransferase required for telomeric silencing of gene expression. J Biol Chem 277(13):10753–10755
80. Nagy PL, Griesenbeck J, Kornberg RD, Cleary ML (2002) A trithorax-group complex purified from Saccharomyces cerevisiae is required for methylation of histone H3. Proc Natl Acad Sci 99(1):90–94
81. Briggs SD, Bryk M, Strahl BD, Cheung WL, Davie JK, Dent SYR et al (2001) Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rdna silencing in *Saccharomyces cerevisiae*. Genes Dev 15(24):3286–3295
82. Santos-Rosa H, Bannister AJ, Dehe PM, Géli V, Kouzarides T (2004) Methylation of H3 lysine 4 at euchromatin promotes Sir3p association with heterochromatin. J Biol Chem 279(46):47506–47512
83. Eissenberg JC, Shilatifard A (2010) Histone H3 lysine 4 (H3K4) methylation in development and differentiation. Dev Biol 339(2):240–249
84. Nedea E, Nalbant D, Xia D, Theoharis NT, Suter B, Richardson CJ et al (2008) The Glc7 phosphatase subunit of the cleavage and polyadenylation factor is essential for transcription termination on snorna genes. Mol Cell 29(5):577–587
85. Dichtl B, Aasland R, Keller W (2004) Functions for *S. cerevisiae Swd2p* in 3’ end formation of specific mrnas and snornas and global histone 3 lysine 4 methylation. RNA 10(6):965–977
86. Cheng H, He X, Moore C (2004) The essential WD repeat protein Swd2 has dual functions in RNA polymerase ii transcription termination and lysine 4 methylation of histone H3. Mol Cell Biol 24(7):2932–2943
87. Shilatifard A (2012) The compass family of histone H3K4 methylases: mechanisms of regulation in development and disease pathogenesis. Annu Rev Biochem 81:65.
88. Meyers RA (2012) Epigenetic regulation and epigenomics: advances in molecular biology and medicine. Wiley-Blackwell, Chichester
89. Mueller JE, Canze M, Bryk M (2006) The requirements for COMPASS and Paf1 transcriptional silencing and methylation of histone H3 in *Saccharomyces cerevisiae*. Genetics 173(2):557–567
90. Nislow C, Ray E, Pillus L (1997) Set1, a yeast member of thetrithorax family, functions in transcriptional silencing and diverse cellular processes. Mol Biol Cell 8(12):2421–2436
91. Schneider J, Wood A, Lee J-S, Schuster R, Dueker J, Maguire C et al (2005) Molecular regulation of histone H3 trimethylation by compass and the regulation of gene expression. Mol Cell 19(6):849–856
92. Palmer JM, Bok JW, Lee S, Dagenais TRT, Andes DR, Kontoyiannis DP et al (2013) Loss of CclA, required for histone 3 lysine 4 methylation, decreases growth but increases secondary metabolite production in *Aspergillus fumigatus*. PeerJ 1:e4
93. Dokmanovic M, Clarke C, Marks PA (2007) Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res 5(10):981–989
94. Robyr D, Suka Y, Xenarios I, Kurdistani SK, Wang A, Suka N et al (2002) Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. Cell 109(4):437–446

95. Smith KM, Dobosy JR, Reifsnyder JE, Rountree MR, Anderson DC, Green GR et al (2010) H2B- and H3-specific histone deacetylases are required for DNA methylation in *Neurospora* crassa Genetics 186(4):1207–1216
96. Tribus M, Bauer I, Galehr J, Rieser G, Trojer P, Brosch G et al (2010) A novel motif in fungal class 1 histone deacetylases is essential for growth and development of *Aspergillus*. Mol Biol Cell 21(2):345–353
97. Shwab EK, Bok JW, Tribus M, Galehr J, Graessle S, Keller NP (2007) Histone deacetylase activity regulates chemical diversity in *Aspergillus*. Eukaryotic Cell 6(9):1656–1664
98. Lee I, Oh J-H, Keats Shwab E, Dagenais TR, Andes D, Keller NP (2009) Hdaa, a class 2 histone deacetylase of *Aspergillus fumigatus*, affects germination and secondary metabolite production. Fungal Genet Biol 46(10):782–790
99. Baker S, Grant P (2007) The saga continues: Expanding the cellular role of a transcriptional co-activator complex. Oncogene 26(37):5329–5340
100. Spröte P, Hynes MJ, Hortschansky P, Shelest E, Scharf DH, Wolke SM et al (2008) Identification of the novel penicillin biosynthesis gene *aatB* of *Aspergillus nidulans* and its putative evolutionary relationship to this fungal secondary metabolism gene cluster. Mol Microbiol 70(2):445–461
101. Barrios A, Selleck W, Hnatkovich B, Kramer R, Sermwittayawong D, Tan S (2007) Expression and purification of recombinant yeast Ada2/Ada3/Gcn5 and Piccolo NuA4 histone acetyltransferase complexes. Methods 41(3):271–277
102. Marcus GA, Silverman N, Berger SL, Horiuchi J, Guarente L (1994) Functional similarity and physical association between Gcn5 and Ada2: putative transcriptional adaptors. EMBO J 13(20):4807
103. Bayer P, Arndt A, Metzger S, Mahajan R, Melchior F, Jaenicke R et al (1998) Structure determination of the small ubiquitin-related modifier sumo-1. J Mol Biol 280(2):275–286
104. Harting R, Bayram Ö, Laubinger K, Valerius O, Braus GH (2013) Interplay of the fungal sumoylation network for control of multicellular development. Mol Microbiol 90(5):1125–1145
105. Pickart CM, Eddins MJ (2004) Ubiquitin: structures, functions, mechanisms. Biochim Biophys Acta 1695(1):55–72 ((BBA)-Molecular Cell Research)
106. Schwartz DC, Hochstrasser M (2003) A superfamily of protein tags: ubiquitin, SUMO and related modifiers. Trends Biochem Sci 28(6):321–328
107. Sampson DA, Wang M, Matunis MJ (2001) The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for sumo-1 modification. J Biol Chem 276(24):21664–21669
108. Bernier-Villamor V, Sampson DA, Matunis MJ, Lima CD (2002) Structural basis for E2-mediated sumo conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. Cell 108(3):345–356
109. Ohi MD, Vander Kooi CW, Rosenberg JA, Chazin WJ, Gould KL (2003) Structural insights into the U-box, a domain associated with multi-ubiquitination. Nat Struct Mol Biol 10(4):250–255
110. Swanson R, Locher M, Hochstrasser M (2001) A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matα2 repressor degradation. Genes Dev 15(20):2660–2674
111. Pichler A, Gast A, Seeler JS, Dejean A, Melchior F (2002) The nucleoporin RanBP2 has SUMO1 E3 ligase activity. Cell 108(1):109–120
112. Shiio Y, Eisenman RN (2003) Histone sumoylation is associated with transcriptional repression. Proceedings of the National Academy of Sciences 100(23):13225–13230
113. Shin JA, Choi ES, Kim HS, Ho JCY, Watts FZ, Park SD et al (2005) SUMO modification is involved in the maintenance of heterochromatin stability in fission yeast. Mol Cell 19(6):817–828
114. Sterner DE, Nathan D, Reindle A, Johnson ES, Berger SL (2006) Sumoylation of the yeast Gcn5 protein. BioChemistry 45(3):1035–1042

115. Harting R (2013) The sumoylation and neddylation networks in *Aspergillus nidulans* development. Dissertation, der Georg-August University
116. Wong KH, Todd RB, Oakley BR, Oakley CE, Hynes MJ, Davis MA (2008) Sumoylation in *Aspergillus nidulans: sumO* inactivation, overexpression and live-cell imaging. Fungal Genet Biol 45(5):728–737
117. Kobayashi E, Ando K, Nakano H, Iida T, Ohno H, Morimoto M et al (1989) Calphostins (UCN-1028), novel and specific inhibitors of protein kinase CI fermentation, isolation, physico-chemical properties and biological activities. J Antibiot (Tokyo) 42(10):1470–1474
118. Williams RB, Henrikson JC, Hoover AR, Lee AE, Cichewicz RH (2008) Epigenetic remodeling of the fungal secondary metabolome. Organic Biomol Chem 6(11):1895–1897
119. Fisch K, Gillaspy A, Gipson M, Henrikson J, Hoover A, Jackson L et al (2009) Chemical induction of silent biosynthetic pathway transcription in *Aspergillus niger*. J Ind Microbiol Biotechnol 36(9):1199–1213
120. Henrikson JC, Hoover AR, Joyner PM, Cichewicz RH (2009) A chemical epigenetics approach for engineering the in situ biosynthesis of a cryptic natural product from *Aspergillus niger*. Organic Biomol Chem 7(3):435–438

Chapter 4
Genome Mining for Fungal Secondary Metabolic Gene Clusters

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Introduction

The fungal kingdom represents a vast and largely untapped resource for the discovery of new natural products and their biosynthetic pathways. It is estimated that the number of fungal species (~ 1.5 million species) exceeds that of land plants by a ratio of 10:1. Only a fraction of this diversity (~ 100,000 species) has been described. Ascomycota (filamentous fungi) and Basidiomycota (including mushroom forming fungi) make up the vast majority of this diversity [1, 2]. However, despite this remarkable species diversity, relatively few fungi have been studied and even fewer species investigated for their ability to make natural products. Such studies are hindered by the complex life cycles of fungi, unknown or difficult to reproduce conditions for growth and natural products production, and genetic intractability of the majority of fungi [3]. Despite these challenges, natural products made by filamentous fungi such as *Aspergillus*, *Penicillium* have been used clinically as antibiotics, antifungals, immunosuppressants, and cholesterol-lowering agents (Fig. 4.1) [4–6]. Compared to filamentous fungi, Basidiomycota are a largely uncharted territory for natural products discovery, with only a very small fraction of the nearly 30,000 described species examined for their ability to produce secondary metabolites [7–10]. Even fewer studies have focused on the biosynthetic genes responsible for natural product production in this fascinating class of organisms [11–24].

Genome sequencing initiatives such as the US Department of Energy's Fungal Genomics Program [25] have resulted in a massive influx of fungal DNA sequence data over the course of the last 10 years. Driven by an interest in fungi as sources for lingocellulose-degrading enzymes [26] and affordable next-generation sequencing

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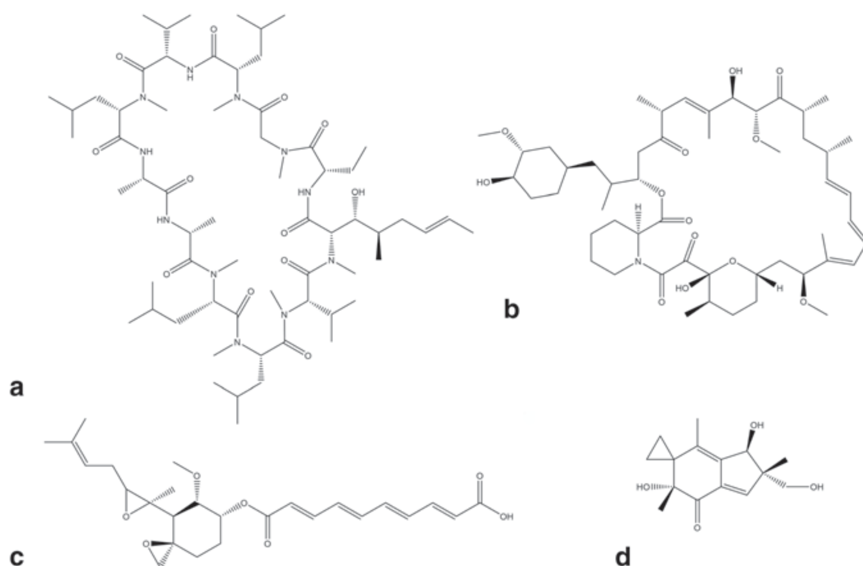

Fig. 4.1 Natural products from fungi have very complex structures and a wide range of biological activities. Many of these compounds have been used to make synthetic analogs with improved therapeutic applications. Some bioactive compounds isolated from fungi and representing different natural product classes include, **a** the immunosuppressant cyclosporine A (a nonribosomal peptide) [139], **b** the immunosuppressant sirolimus (rapamycin) (hybrid nonribosmal peptide and polyketide) [139], **c** the antimicrobial fumagillin [140] (meroterpenoid, hybrid terpenoid-polyketide), and **d** the antitumor compound illudin S (sesquiterpenoid). [141]

technologies [27], the number of Basidiomycota genomes alone has ballooned from less than 5 a few years ago to currently over 100 draft genomes listed at the Joint Genome Institute's (JGI) MycoCosm genome portal (http://genome.jgi.doe.gov/programs/fungi/index.jsf), of which only a select few have been annotated in detail [28–36]. About twice as many Ascomycota genome sequences are listed at this portal, and many more are deposited at the National Center for Biotechnology Information (NCBI). The large number of fungal genome data provides a tremendous opportunity for bioinformatics-guided approaches to assess and access the natural products potential of fungi independent from whether their biosynthetic pathways can be induced under laboratory conditions.

One of the hallmarks of fungal genetic organization, similar to bacteria, is the physical clustering of core secondary metabolic genes of natural products pathways in the genome. Genomic clustering is believed to facilitate efficient regulation of natural product biosynthesis through transcription factors and epigenetics [37–44]. Physical linkage between biosynthetic genes in a given pathway greatly aids in the characterization, discovery, and biotechnological exploitation of fungal natural biosynthetic pathways. Inexpensive DNA synthesis and the development of sophisticated synthetic biology approaches for heterologous biosynthetic pathway

assembly will eventually enable scientists to bypass current challenges such as finding conditions for growth and natural products biosynthesis, and tools for genetic manipulations.

In this chapter, we will provide an overview of fungal natural products biosynthesis with an emphasis on genomic- and bioinformatic-driven pathway discovery. Regulation of secondary metabolic pathways (including silent and cryptic pathways) will be discussed elsewhere in this book and will not be a focus of this chapter. We will begin by examining the different bioinformatics tools available for genome mining for biosynthetic gene clusters with an emphasis on open-source accessible algorithms and software. We will then discuss elucidation and characterization of gene clusters responsible for the biosynthesis of major fungal secondary metabolite classes, polyketides (PK)/nonribosomal peptides (NRP), and terpenes. Apart from representing major fungal natural product classes that have been characterized in some detail, differences in PK/NRP and terpenoid biosynthetic cluster abundance in Ascomycota and Basidiomycota genomes provide some insights into the natural products repertoire of these two fungal groups. Each section will provide key examples of the workflow required to identify and characterize the genes responsible for synthesizing the biologically active compounds introduced above.

The Bioinformatic Tools of Genome Mining for Natural Products in Fungi

The physical clustering of biosynthetic genes for a given natural product provides an elegant means of elucidating fungal biosynthetic pathways. Unlike in plants, whose genomes are more complex and lack clearly delineated biosynthetic gene clusters [37], in fungi, the identification of a gene encoding a key enzyme in a biosynthetic pathway may lead directly to most of the remaining genes in the pathway. This can be particularly important for the discovery and characterization of natural product scaffold-activating cytochrome P450 enzymes. Like in plants, this enzyme family has undergone extensive gene duplication in fungi, particularly in Basidiomycota [45]. This makes it difficult to determine specific P450 functions based on sequence homology alone, especially for novel, multifunctional fungal P450 gene families [46, 47]. The identification of biosynthetic gene clusters has led to the characterization of fungal P450s involved in statin [48], PK [49], and alkaloid biosynthesis [50, 51]. With the increase of genomic sequence data, initially for filamentous fungi and more recently for Basidiomycota, much effort has, therefore, been invested in the development of bioinformatics tools to mine fungal genomes for these clusters.

The identification of biosynthetic gene clusters has typically begun with an mRNA or genomic “anchor sequence” based on one or more known biosynthetic genes in a pathway. Such an “anchor gene”, typically (but not always, [52]) encodes the first key enzyme in the biosynthetic pathway—for example, a polyketide synthase (PKS), nonribosomal peptide synthase (NRPS) [18, 24], terpene synthase

(TPS), or other enzyme—depending on the type of natural product scaffold formed [12, 53–55]. Traditionally, clusters were identified through molecular genetic techniques. For example, the creation and sequencing of a cosmid library led to the identification of the first gene cluster responsible for the production of trichothecene, a sequiterpenoid mycotoxin, in *Fusarium graminearum* F15 [56]. In addition, when the anchor gene was cloned and sequenced, fungal biosynthetic gene clusters were identified through subsequent genome walking [54, 55, 57]. These molecular techniques, however, have only allowed for the identification of clusters up to a certain size (up to ~20 kb) and with the anchor gene fully sequenced. The more recent availability of fungal whole-genome sequence data has allowed for the identification of biosynthetic clusters of any size, provided the genome assembly data include sufficiently large scaffolds. Basic Local Alignment Search Tool (BLAST) searches of fungal genome sequences for classes of enzymes, such as PKS, NRPS, and TPS genes, typically yield several potential candidate genes for a target enzyme. Gene prediction algorithms, such as Augustus [58], can then be applied to predict open reading frames (ORFs) and encoding cDNAs of the putative anchor genes and of upstream and/or downstream located additional biosynthetic genes. Such an approach led to the discovery of multiple terpenoid biosynthetic gene clusters in *Coprinus cinereus* and *Omphalotus olearius* by our group [11, 12]. However, manual gene prediction and identification of cluster genes is tedious, and cluster boundaries can be hard to pin down.

As a result, several tools have been developed to more easily identify the putative fungal biosynthetic clusters (reviewed in [59–61]). The most extensive software tool available for the identification of biosynthetic gene clusters is antibiotics and Secondary Metabolite Analysis SHell (antiSMASH) [61]. When given a bacterial or fungal genome sequence data, antiSMASH identifies putative gene clusters by comparing all predicted gene products to a hand-curated set of models corresponding to gene families common to the two dozen types of secondary metabolic pathways it recognizes. Putative clusters are then further analyzed through sequence homology to identify types of multidomain enzymes such as PKSs or NRPSs, enzyme specificities, and potentially even a core structure of the natural product [61]. Originally released in 2011 [60] and updated in 2013 [61], antiSMASH now has the capability to analyze raw contig assemblies of entire genomes, although it should be noted that the assemblies must be relatively clean to avoid redundant identifications. The output visualizes cluster predictions (Fig. 4.2a), which shows homologs of clusters in other microbial species (i.e., fungi) (Fig. 4.2b), and allows for easy sequence extraction (Fig. 4.2c).

While antiSMASH is extremely powerful, we found that it has some notable limitations in its ability to correctly predict genes and their encoded proteins and identify full biosynthetic pathways. First, individual biosynthetic genes may be easily missed by antiSMASH. In *O. olearius*, our group identified 11 terpene synthases through manual BLAST searches, while antiSMASH was only able to identify four of those genes. Second, the rules for defining the boundaries of secondary metabolic clusters are not, as of yet, entirely clear. While the "core" cluster may be

Fig. 4.2 Sample output using antiSMASH 2.0 [61] to analyze the genome of the basidiomycete *O. olearius*. The raw scaffolds were uploaded to the antiSMASH server and analyzed for secondary metabolic genes/clusters. **a** The initial output lists all putative clusters and attempts to classify them by type. **b** Upon clicking on a cluster the view is expanded, and individual genes are shown within their genomic context. Additionally, homologous clusters from other fungi are shown below. **c** Each putative protein sequence can be quickly accessed and used to perform BLAST searches. (This figure was adapted from the output of antiSMASH 2.0)

predicted with ease, individual biosynthetic genes some distance away (10–20 kilobases) that may still be involved in late-pathway modifications are frequently missed. In some cases, satellite clusters of biosynthetic genes are located at distinct loci in the genome. For example, while most trichothecene biosynthetic genes in *Fusarium sporotrichioides* and *F. graminearum* exist in the *tri5* core gene cluster, two late pathway genes are clustered elsewhere [62]. Finally, accurate structural annotation of genome sequences is crucial for the identification of biosynthetic genes; unfortunately common gene prediction algorithms (such as Augustus [58]) trained on other eukaryotic genomes typically perform poorly for prediction in Basidiomycota, whose genomes are rich in small intron and exons. Furthermore, genes may be closely spaced and neighboring gene models may or may not be fused together and cryptic alternative splicing is not uncommon [63–67].

Our experience has shown that the gene prediction models used by most algorithms often lead to incorrect structural gene annotation in Basidiomycota, frequently requiring manual re-annotation using protein sequence alignment-guided identification of the most likely correct splice isoforms together with tedious attempts at obtaining correctly spliced cDNAs encoding functional proteins. Efforts to uncover the first biosynthetic gene of a biosynthetic pathway such as a terpenoid pathway can be arduous [68]. The presence of introns, some of which can be unusually small, complicates polymerase chain reaction (PCR) amplification [67]. In our

experience, many splice variants can be amplified from a given cDNA pool, though only one splice variant has ever been confirmed to produce an active enzyme after transcription. Even when a good splicing model is predicted, and the expected gene is amplified, the resultant protein may still be inactive, as was the case with Cop5, a sesquiterpene synthase our laboratory cloned and attempted to characterize from *Coprinus cinereus* [12]. We appear to be just beginning to understand the complex splicing, transcriptional regulation, and possibly posttranslational regulation that leads to active secondary metabolic genes.

In the future, deep RNA sequencing will be a key to improving computational prediction of fungal biosynthetic genes and gene clusters. Already, RNAseq data has been shown to improve the accuracy of gene prediction models [29, 65, 69, 70] and has shed light on differential splicing [71]. In addition, transcriptomic data has also been useful in the delineation of gene cluster boundaries [24]. Presently only some of the more recently sequenced Basidiomycota genomes have associated deep RNA sequence data [29, 70] useful for biosynthetic pathway identification. Advances in HT-RNA sequencing and continued cost reductions for sequencing now allow affordable rapid and deep profiling transcriptome analysis under a variety of conditions or for diverse genotypes to collect large data sets for one species. Such data can be used to create gene coexpression networks built on physical distance to a seed natural products biosynthetic gene (e.g., TPS, NRPS, PKS, P450s) (guilt by association) as a powerful tool for pathway discovery. The fact that NP pathway genes are generally co-regulated through levels of shared transcriptional control elements (e.g., transcription factors and upstream intergenic gene regions) [72] represents yet another approach for network analysis within and also across species. Significant advances have been made in understanding the regulatory control elements of NP pathways in filamentous fungi, including the velvet family of regulatory proteins that are conserved among Ascomycota and Basidiomycota [39, 73, 74]. Genome analysis of *Ganoderma* and *Schizophyllum* [29, 31, 75] suggests high conservation of regulatory networks among mushroom forming fungi, which can be exploited for network building. Yet, gene coexpression network analysis so far has been largely applied for the discovery of natural products genes in plant [76, 77]. Guilt by association-based analysis based on DNA expression arrays was only recently applied to natural product biosynthetic gene cluster analysis in *A. nidulans* [78].

Polyketide Synthases (PKS) and Nonribosomal Peptide Synthases (NRPS)

Polyketides (PK) and nonribosomal peptides (NRPs) are major, structurally diverse classes of natural products known to be produced by numerous filamentous fungi [79–81] and bacteria [82]. From an ecological perspective, these polyketide- and peptide-based secondary metabolites afford the host organism a wide array of largely cytotoxic or general antibiotic compounds, which effectively restrict the

growth and development of organisms that may compete for space and nutrients. Synthesis of these metabolites is achieved by a simple and highly conserved general mechanism involving the iterative elongation of either amino acid or carboxylic acid building blocks, for nonribosomal peptides and polyketides, respectively. In a similar manner as fatty acid synthases (FAS), the relevant enzymes that coordinate the production of these varied metabolites are multifunctional, mostly iterative enzymes, with a predictable set of core domains that repeatedly utilize the same active site to elongate peptide or polyketide chains. For PKSs, these include ketoacyl synthase (KS), acyltransferase (AT), acyl carrier protein (ACP), and thioesterase (TE) domains [83]. Utilizing this core domain set, the condensation of activated acetate units produces a polyketide scaffold, upon which a vast array of modifications can be imposed by one or more sparsely conserved ketoreductase (KR), dehydratase (DH), methyltransferase (MT), and enoyl reductase (ER) domains. The relative reduction status and product profile of a given PKS is linked to the presence or absence of these domains, with so-called "nonreducing PKSs" lacking these domains entirely and having a relatively limited product profile, while highly reducing PKSs contain all three of these domains and drastically alter the scaffold molecule into a wide array of alcohols, ketones, and other interesting chemical variants (reviewed in [79]).

Conservation of domains and reaction mechanisms suggest that PKSs and NRPSs share ancestral origins. It is, therefore, not surprising that NRPSs also contain a core set of domains similar to those in PKSs, including the peptidyl carrier protein (PCP), adenylation (A), condensation (C), and the thioesterase (TE) domains [84–86], which are required for the production of a scaffold peptide. This basal molecule can then be modified to varying degrees by a number of ancillary domains, including an epimerization or dual/epimerization domain (E and D/E, respectively), a reductase domain (R), and others involved in oxidation, cyclization, and methylation [85]. Additionally, genomic resources have revealed that many fungal PKSs and NRPSs cluster with cytochrome P450s (for example [87]) and that modifications of PKS and NRPS-derived scaffolds molecules by P450s have been implicated in the production of relevant secondary metabolites including mycophenolic acid, a grisan scaffold, tenellin, and the antifungal pneumocandin [88–91]. The propensity of fungi to cluster an array of modification enzymes such as P450s around core scaffold-producing enzymes such as PKSs and NRPSs is a widely conserved means to create vast libraries of products from simple PK, NRP, and terpenoid (discussed in next section) building blocks.

From an engineering perspective, a great deal of information has already been accumulated regarding the structure and function of PKS/NRPS enzymes, with numerous recent reports highlighting the potential for engineering PKSs and also NRPSs from Ascomycetes such as *Fusarium* and *Aspergillus* [92, 93] and Basidiomycetes such as *Ustilago maydis* and *Suillus grevillei* [15, 17]. Ma et al. [92] conducted a detailed characterization of the lovastatin nonaketide synthase LovB; a highly reducing PKS catalyzing the production of dihydromonacolin L. Extensive in vitro analyses, as well as production from *Saccharomyces cerevisiae* and

substrate feeding experiments provided the authors with a detailed understanding of LovB structure and function. The production of lovastatin from dihydromonacolin L is known to require LovB and the enoyl reductase (ER) domain of its partner enzyme, LovC [92]. Interestingly, in vitro experiments with LovB, LovC, and all required cofactors failed to release dihydromonacolin L, indicating that the action of another domain might be required. The authors successfully released dihydromonacolin L after coexpression of heterologous thioesterase-containing enzymes from *Gibberella zeae*, supporting the aforementioned claim. Moreover, the same can be accomplished with the ER domain protein LovC. Complementation of dihydromonacolin L release can be achieved via the heterologous expression of MlcG; an analogous ER domain containing protein from the compactin biosynthetic cluster of *Penicillium citrinum* [92]. These reports support the claim that the function of these enzymatic partners is more promiscuous than once believed and that engineering of designer pathways by swapping analogous domains from related PKSs and NRPSs is a viable strategy for production of novel chemistry as explained later in this chapter.

Despite the vast potential for isolation and production of valuable compounds from these metabolic clusters, there are significant gaps in our current level of understanding of NRP and PK biosynthesis in fungal systems. Indeed, a brief survey of the SciFinder returns fewer than 100 PKS- or NRPS-derived compounds from Basidiomycota, although recent work highlighting PKS diversity in Basidiomycota suggests that the number of biosynthetic genes grossly exceeds the number of reported PKs from these organisms [94]. This discrepancy most likely reflects a lack in the characterization of compounds produced by these enzymes.

A number of very recent reports have utilized multifaceted approaches to mine fungal genomes and increase our understanding of PK and NRP diversity. For example, a study by Lackner et al. [94] aimed to identify new PKSs in Basidiomycota probing the aforementioned genome resource at JGI with the KS domain of AflC of *Aspergillus parasiticus* and a selected group of related sequences. Thirty-five Basidiomycota genomes were queried, yielding more than 100 putative PKS genes [94], thus supporting the claim that the myriad of domain architectures presented by fungal PKSs represent an "*in silico* gold mine" for the discovery of new enzymes and possibly enzymes with variant domains. A similar approach has also been used with the well-conserved PCP, A, C, and TE domains of an NRPS to infer a great deal regarding the phylogeny and functional diversity of NRPSs [85] (reviewed in [84, 86]). Combining the information accrued from these reports, with greatly expanded genomic resources and the knowledge that a great deal of variation exists within the domain structure of PKSs, NRPSs, and also hybrid PK-NRPSs [94], it seems plausible to apply more advanced computational strategies for the isolation of novel metabolites produced from common scaffolds. In this scenario, known domain elements isolated from PKSs and/or NRPSs of interest could be used as queries against fungal genomic databases to isolate novel variants from a wide range of diverse genera (Fig. 4.3). From an engineering perspective, these variant domains represent modules that could be

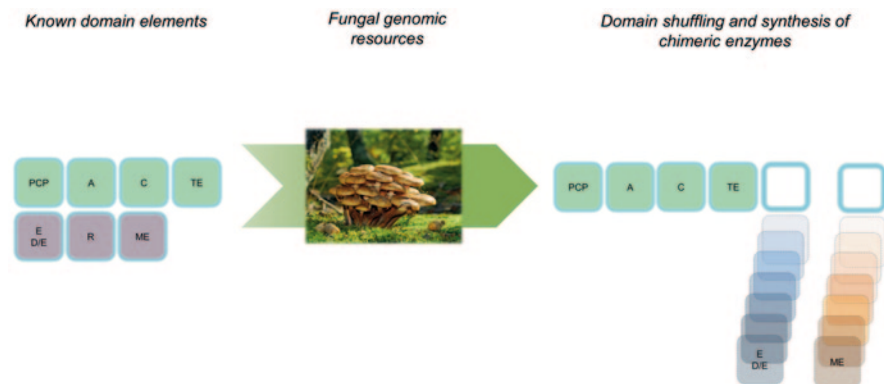

Fig. 4.3 Scheme for developing novel PKS- and NRPS-derived chemistries. Known domain elements of NRPSs (*green boxes*; *PCP*, *A*, *C*, and *TE*) as well as downstream, modification domains (*purple boxes*; *E D/E*, *R*, and *ME*) can be used to query vast fungal databases to isolate variants in domain of interest. Variants isolated in this way for the epimerization and methylation domains (*E D/E* and *ME*) are shown as examples in *dashed boxes*. Subsequent domain shuffling experiments would allow for the construction of novel, chimeric enzymes with the potential for producing novel metabolites. An NRPS is shown as an example, but the same scheme could be applied to PKSs and also hybrid PK-NRPSs

swapped interchangeably, yielding chimeric enzymes with the potential for novel chemistries (Fig. 4.3). This strategy has been implemented, albeit on a small scale, to engineer novel chemistry from an engineered hybrid PKS combining the asperfuranone and sterigmatocystin biosynthetic pathways of *A. nidulans* [95].

In addition to genome mining targeting PKSs and NRPSs themselves, another potential strategy for identifying biosynthetic gene clusters is to elicit and examine their transcription. Many biosynthetic clusters are transcriptionally inactive (a.k.a. cryptic gene clusters) under normal cultivation conditions, particularly in endophytic fungi reliant on small molecule signaling from another organism [96]. One way to combat this transcriptional repression is to alter the expression of transcriptional activators/repressors through global epigenetic regulators or with genetic knockouts. An excellent review of the function of LaeA and the velvet complex mentions the implication of these global regulators of secondary metabolism in more than half of the PKS and NRPS genes in *Trichoderma reesei* [41]. Both knockouts and overexpression of global regulators like LaeA and its homologs [38] (or other related machinery, such as histone acetyltransferases [97]) can allow for more detailed genome mining when examining RNA-sequencing datasets and searching for areas of the genome in which transcription is directly affected. A comprehensive discussion of fungal secondary metabolic pathway regulation is provided elsewhere in this book.

Terpene Synthases and Terpenoid Biosynthetic Clusters

Terpenoids are all derived from the five-carbon isoprene units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Prenyldiphosphate synthases then catalyze the head to tail condensation of two, three or four of these five-carbon units to produce 10-, 15- or 20-carbon (C_{10}, C_{15}, C_{20}) isoprenoid diphosphate molecules, which serve as the substrates for Class I terpene synthases that are dependent on the ionization of the allylic diphosphate to form a reactive carbocation and triggering a cascade of cyclization and rearrangement reactions in the enzymes active site [98]. Depending on their chain-length specificity, this class of terpene synthases utilize the 10-carbon substrate geranyl diphosphate (C_{10}, GPP) to form monoterpenes, farnesyl diphosphate (C_{15}, FPP) to generate sesquiterpenes, or geranylgeranyl diphosphate ($C_{20,}$ GGPP) to synthesize diterpenes [99]. Terpenoids with more than 20 carbons are typically formed by the head to head condensation of two FPP or GGPP molecules, yielding longer isoprene chains that are then modified into various C_{30} (sterols) and C_{40} (carotenoids) terpenoid structures. For example, sterols are formed by Class II terpene synthases that rely on a protonation-initiated cyclization mechanism that yields the scaffolds of bioactive triterpenoids isolated from many fungi [100, 101].

Identification of the first terpene synthases from filamentous fungi, such as the sesquiterpene synthases aristolochene [102, 103] and trichodiene synthase [53] required laborious efforts. Advances in sequencing and the increasing availability of sequences data has led to the discovery and characterization of a suite of novel fungal terpenoid biosynthetic enzymes in the past few years. For example, in the past 5 years more than two dozen new fungal sesquiterpene synthases have been cloned and characterized [104], [52], [12], [11, 105] [105–107]. Genome mining efforts not only enable the discovery of new types of terpene synthases and enzymes with new cyclization activities as discussed later but also comparative analysis of the natural production potential encoded by the genomes of the two major fungal phyla.

While polyketides and nonribosomal peptides are the major class of secondary metabolites discovered in filamentous fungi, terpenoids appear to be a predominant class of secondary metabolites in Basidiomycota. In contrast to the number of PKS and NRPS genes mentioned previously, Basidiomycota genomes contain large collections of sesquiterpenoid biosynthetic genes. In 2012 we identified more than 500 putative sesquiterpene synthases (TPS, see later) in only 40 Basidiomycota genomes, and many of the putative TPS appear to be part of a biosynthetic gene cluster [11]. As the number of publicly available genome datasets has doubled, so has the number of putative TPS genes, totaling nearly 1000. As a comparison, only 179 putative TPS genes were identified in close to 2000 bacterial genomes [108], and we identified only ~250 TPS genes in 80 Ascomycota genomes examined.

Conspicuously absent in sequenced Basidiomycota genomes are genes that could encode diterpene synthases, although some mushroom-forming fungi (including the pleuromutilin antibiotic producing fungus *Clitiopilus passeckerianus* [109]) have been reported to produce diterpenoids [110]. Ascomycota on the other hand are

known to be prolific producers of bioactive diterpenoids and several biosynthetic gene clusters have been characterized. These include the well-studied gibberellin pathways found in several fungi that use a bifunctional diterpene synthase that combines the domains of a class I and class II terpene synthases (which are separate enzymes in plant diterpene biosynthesis) to cyclize GGPP [111–113]. Mining of Ascomycota genomes followed by gene deletion studies and stepwise heterologous coexpression of pathway genes in *Aspergillus* has led to the elucidation of additional gene clusters involved in the biosynthesis of the diterpene compounds fusicoccin, brassicicene, aphidicolin and phomopsene [54, 55, 114–120], and a sesterterppenoid (C_{25}) [121]. Intriguingly, these terpenoid scaffolds are built by a novel-type of chimeric terpene synthases that combines the domains of a class I terpene synthase and a prenyldiphosphate synthase that provides the C_{25} isoprene substrate for the cyclase [55].

Genome sequences of several *Aspergillus* strains have recently enabled the discovery and heterologous reconstitution of a series of biosynthetic gene clusters involved in the biosynthesis of medicinally important meroterpenoids (e.g., pyripyropene [122], terretonin [123–125], fumagillin [126] (Fig. 4.1c), austinol, and dehydroaustinol [127]), which are polyketide-terpenoid hybrid compounds. Except for the fumagillin cluster, all of these biosynthetic pathways involve an iterative, nonreducing PKS and an aromatic prenyltransferase [125] that attaches a prenyl chain (typically C_{15}) to the polyketide moiety. The attached prenyl chain is then epoxidated by a flavin-dependent monooxygase to allow for cyclization by a novel type of membrane-bound Class II terpene synthase [122] [123–125] [127]. In fumagallin biosynthesis [126], however, a novel membrane-bound Class I TPS first generates the cyclized terpenoid scaffold that is then attached to a polyketide chain. Yet another novel membrane-bound type II terpene synthase has recently been proposed to catalyze the cyclization of the geranylgeranyl chain attached to the indole moiety in indole-diterpene biosynthesis [128]. Several indole-diterpene biosynthetic clusters have been identified in filamentous fungi [128–132]. Common to all clusters are genes that encode a putative GGPP synthase, aromatic prenyltransferase and a flavin monoxygenase and cyclase proposed to catalyze epoxidation and cyclization, respectively, of the GGPP chain[128–132].

The aforementioned studies illustrate that even with a genome sequence at hand, it may not be possible to assign function to biosynthetic gene clusters solely based on homology to known biosynthetic enzymes. The identification in filamentous fungi of different types of chimeric biosynthetic pathways and of novel enzyme folds that catalyze similar reactions as in the case of terpene cyclization indicates that we may have only just begun to scratch the surface of the fungal secondary metabolome.

While the abundance of putative TPS genes may appear daunting to characterize biochemically, we found that when focusing on sesquiterpene synthases in Basidiomycota a relationship between sequence and function could be uncovered. Specifically, we examined sequence conservation as it relates to the first committed bond-forming step in the cyclization of the terpene molecule. We discovered that,

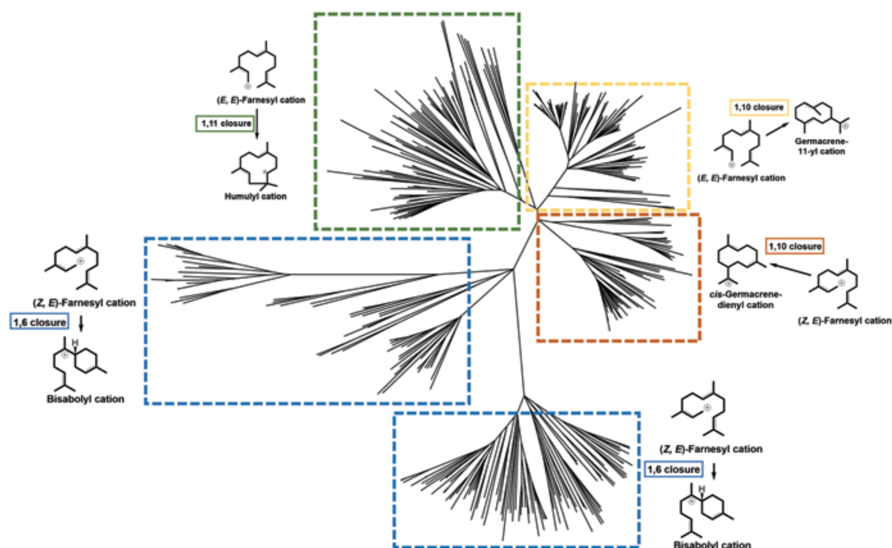

Fig. 4.4 Schematic representation of an unrooted neighbor-joining phylogenetic tree of sesquiterpene synthases identified in Basidiomycota genomes. We surveyed 42 Basidiomycota genomes and found 542 putative terpene synthase sequences, of which 392 were built into a phylogenetic tree to establish a link between sequence and function in this class of enzymes. By applying context to the tree through the inclusion of biochemical data, conservation of the initial cyclization reaction of farnesyl diphosphate (FPP) was identified. [11]

despite relatively poor automatic gene prediction in the publicly available databases, the TPS genes partitioned to five clades, which appear to segregate based on their cyclization mechanism (Fig. 4.4) [11]. These clades represent the four initial cyclizations of FPP known to be catalyzed by TPS. With this information it is now possible to sort through the large set of TPS sequences based on the initial cyclization believed to lead to the desired product. Our group recently carried out a study focused on validating the predictive framework by examining sesquiterpene biosynthesis in the crust fungus *Stereum hirsutum*. Not only did this fungus possess a large repertoire of terpene synthases, it also has been studied for its production of bioactive natural products. As many of the sesquiterpenoids reported are derived from a humulyl cation, we chose to target protoilludene synthase homologs, which we expected to go through the same cyclization mechanism. Using the framework described previously, it was possible to clone and characterize three novel protoilludene synthases [119]. While our work only focused on sesquiterpenes, similar studies can be carried out to examine other classes of terpenoids in order to provide the same genome mining roadmap.

Terpenoid biosynthetic gene clusters may be extremely difficult to characterize due to the complex nature of the clusters themselves. Clusters can range in size from only two genes (e.g., a terpene synthase and a P450 monooxygenase) to greater than a dozen. Complicating the matter further is the propensity for separate biosynthetic clusters to work together to form the same types of products.

For example, in searching for the enzymes responsible for illudin biosynthesis in the Jack O'Lantern fungus *Omphalotus olearius*, two protoilludene (the precursor to illudin compounds) synthases were identified (Omp6 and Omp7). Both were part of biosynthetic clusters, though one contained only three genes [11]. Varying kinetic values for the two terpene synthases indicate a possible mechanism for overcoming rate-limiting steps in the biosynthesis of illudins, though this has yet to be experimentally validated. A parallel example from the fungus *F. sporotrichioides* shows two clusters and an independent gene responsible for trichothecene biosynthesis. The larger cluster contains 12 genes, while the second smaller cluster and the independent gene are responsible for late-pathway reactions [62]. Additionally, very similar strains may contain orthologous biosynthetic genes, but some may be pseudogenes, inactivated by the accumulation of mutations [62, 113]. This sort of genomic segregation implies a need for tight transcriptional control on late-pathway genes, perhaps to minimize toxicity/reactivity of intermediates. This sort of variation in the genetic organization of biosynthetic clusters makes it particularly challenging to find all of the genes responsible for any given product.

After cluster gene identification, the next step is to characterize the function of individual genes. For a genetically tractable fungus, gene function can be determined in part by knockout and complementation studies. Additionally, if the clusters appear to be transcriptionally inactive, background mutations can be made to alter the level of transcription. In order to study the biosynthesis of botridal in *B. cinerea*, a knockout strain was engineered for the *bcg1* gene responsible for down-regulation of the pathway [52]. In another example studying tricothecene production in *F. graminearum*, the expression of the cluster proteins was too low, so a strain was engineered to contain an overexpression cassette with the *FgTri6* gene responsible for regulating the biosynthetic cluster [56].

When no genetic tools are available for the cluster's source organism, the biosynthetic cluster must be heterologously characterized. As expected, *Escherichia coli*is often the first prokaryotic chassis used for characterization of biosynthetic genes. While some enzymes, such as the terpene synthases discussed previously, express well and have high activity in *E. coli*, many of the other pathway enzymes prove difficult to characterize in this host [62]. This is, in part, due to the prevalence of P450 monooxgenases in many biosynthetic clusters, which are associated with the cell membrane and tend to express poorly in *E. coli*. Recent work, however, suggests different strategies to accommodate these enzymes, though the applicability across many different P450 homologs is unknown [133]. Several genes from the trichothecene biosynthetic cluster have been successfully characterized in *E. coli*, despite the difficulties described previously [62].

A more commonly used chassis for the expression and characterization of late-pathway biosynthetic enzymes is *S. cerevisiae.* Our laboratory characterized a terpene synthase and two associated P450 monooxgenases through standard plasmid-based expression [12]. While this is feasible for a small number of genes for characterization purposes, the assembly of an entire pathway for stable high-level production, as shown by Keasling's group in their efforts to produce artemisinin, is very laborious and requires extensive strain engineering [134]. Another consid-

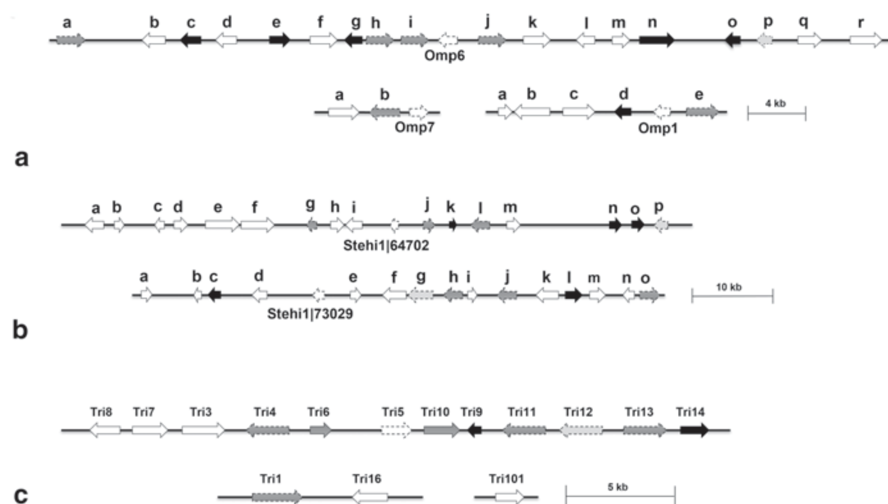

Fig. 4.5 Secondary metabolic clusters from three different fungi. Shown here are sesquiterpenoid biosynthetic cluster from **a** *Omphalotus olearius* [11]. **b** *Stereum hirsutum* [107], and **c** *Fusarium graminearium* [56]. Predicted ORFs are colored according to their putative function, with *gray arrows* with *dotted* outlines representing P450 enzymes, *white arrows* with *solid* outlines representing enzymes with predicted roles in sesquiterpene scaffold modification, *light gray arrows* with *dotted* outlines representing a transporter, and *white arrows* with *dotted* outlines representing the respective sesquiterpene synthases in each cluster. *Black* ORFs indicate hypothetical/unknown proteins and known transcriptional regulators are *gray arrows* with *solid* outlines in the trichothecene (Tri) biosynthetic cluster [142]

eration when producing secondary metabolites is their inherent toxicity and the absence of the machinery required to protect the host organism. For this reason and others stated previously, the development of genetically tractable and easy-to-manipulate fungal strains is the next step in studying biosynthetic pathways. Additionally, many fungal systems have developed transporters designed specifically to export or compartmentalize the toxic intermediates and products in order to maintain high levels of biosynthesis. Perhaps the most well-understood system is the “aflatoxisomes” in *Aspergillus*, which are responsible for the compartmentalization of aflatoxin in vesicles before export from the cell [135, 136]. Another analogous system in *Fusarium graminearium* involves the use of toxisomes to compartmentalize toxic compounds in tricothecene biosynthesis. Using colocalization experiments with GFP/RFP-tagged pathway enzymes, two P450s (P450-Tri1, P450-Tri4) and the HMG-CoA reductase (the rate-controlling enzyme of the mevalonate precursor pathway) were found to localize to toxisomes that interact with smaller vesicles. The smaller vesicles contain the MFS transporter Tri12 and are believed to accumulate toxic pathway products that are compartmentalized and then eliminated by fusion with the vacuole and plasma membrane. Interestingly, many biosynthetic clusters contain transporters believed to be similar in function to Tri12 (Fig. 4.5) [137, 138].

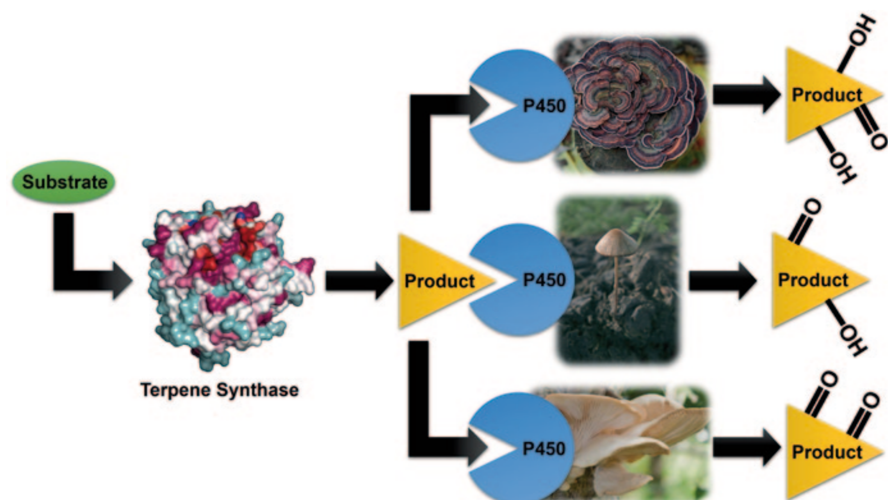

Fig. 4.6 Strategy for the biosynthesis of nonnatural products through combinatorial approaches. In the example shown here, a sesquiterpene synthase converts its substrate, farnesyl diphosphate to a sesquiterpene hydrocarbon scaffold. Such a hydrocarbon scaffold is typically activated through oxygenation catalyzed by P450 monooxygenases. In this example, P450s from three different fungal sources and sesquiterpene biosynthesis pathways are used to differentially oxygenate the product to different final sesquiterpenoid products. Further extension of these pathways with additional combinations of modifying enzymes could lead to a range of novel products

With the massive amount of sequence data available we are now limited by the speed by which we can biochemically characterize terpenoid biosynthetic genes for their function. The next step, after sufficient biochemical data has been collected, is to use this secondary metabolic enzyme toolbox to generate products not found in nature. Synthetic biology and metabolic engineering provide us with the tools to do something at a much faster rate than evolution. By combining interesting enzymes across many different fungi we will likely be able to generate terpenoids never seen before in nature (Fig. 4.6). For example, we may find multiple clusters in which the first committed step is for a specific sesquiterpene. While that step shows very little variance, the next step—the modification of that terpene scaffold—presents an opportunity for metabolic engineering of nonnatural pathways. These new pathways may contain P450 enzymes from a number of different fungi know to produce the precursor compound of interest, and known to modify that precursor to for a final product with interesting biological activity. These new compounds may have slightly different biological activities and serve as new starting compounds for useful pharmaceutical compounds.

Conclusion

Researchers have only begun to scratch the surface of the myriad of natural products and biosynthetic pathways that can be discovered through mining the genomes of higher fungi. The influx of fungal genome sequencing data in recent years, along with the development of bioinformatics tools, from BLAST to Augustus to antiSMASH, have allowed us to probe rapidly into the biosynthetic gene clusters abundant in this class of organisms. However, additional bioinformatic and genomic approaches need to be developed or adapted for improved gene prediction and functional annotation, and especially for the identification of biosynthetic clusters made up by novel types of enzymes, delineating cluster boundaries, and for finding satellite and chimeric clusters.

So far, sequencing data only exists for a tiny percentage of identified fungal species. As the amount of fungal genome sequencing data increases, fungi are bound to become a much greater source of new natural products and their biosynthetic enzymes. In addition to the development of genomic data and bioinformatic resources, a critical component of natural product discovery is the detailed characterization of the product and pathway enzymes. At this point in time, the biochemical characterization of biosynthetic gene clusters and heterologous refactoring of pathways is equally if not more challenging than their identification. To fully exploit the natural products potential of fungi, significant efforts are required that aim at developing synthetic biology approaches for high-throughput heterologous fungal pathway assembly; ideally facilitating direct translation of sequence information encoded in fungal genomes into biosynthetic output by a heterologous expression and production platform. The final, and arguably most interesting step, will be combinatorial biosynthetic approaches through the creation of novel biosynthetic assemblies for the production of an even greater diversity of potentially bioactive compounds.

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References

1. Stajich JE, Wilke SK, Ahren D, Au CH, Birren BW, Borodovsky M et al (2010 Jun 29) Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom *Coprinopsis cinerea (Coprinus cinereus)*. Proc Natl Acad Sci USA 107(26):11889–11894
2. Blackwell M (2011 Mar) The fungi: 1, 2, 3.. 5.1 million species? Am J Bot 98(3):426–438
3. Elisashvili V (2012) Submerged cultivation of medicinal mushrooms: bioprocesses and products (review). Int J Med Mushrooms 14(3):211–239
4. Hu F, Liu J, Du G, Hua Z, Zhou J, Chen J (2012 Aug) Key cytomembrane abc transporters of *Saccharomyces cerevisiae* fail to improve the tolerance to d-limonene. Biotechnol Lett 34(8):1505–1509

5. Kang J, Park J, Choi H, Burla B, Kretzschmar T, Lee Y et al (2011) Plant ABC transporters. *Arabidopsis* Book 9:e0153
6. Brase S, Encinas A, Keck J, Nising CF (2009 Sep) Chemistry and biology of mycotoxins and related fungal metabolites. Chem Rev 109(9):3903–3990
7. Zhong JJ, Xiao JH (2009) Secondary metabolites from higher fungi: discovery, bioactivity, and bioproduction. Adv Biochem Eng Biotechnol 113:79–150
8. Lindequist U, Niedermeyer THJ, Julich WD (2005) The pharmacological potential of mushrooms. Evid Based Complement Alternat Med 2(3):285–299
9. Alves MJ, Ferreira IC, Dias J, Teixeira V, Martins A, Pintado M (2012 Nov) A review on antimicrobial activity of mushroom (basidiomycetes) extracts and isolated compounds. Planta Med 78(16):1707–1718
10. Zjawiony JK (2004 Feb) Biologically active compounds from Aphyllophorales (polypore) fungi. J Nat Prod 67(2):300–310
11. Wawrzyn GT, Quin MB, Choudhary S, Lopez-Gallego F, Schmidt-Dannert C (2012 Jun 22) Draft genome of *Omphalotus olearius* provides a predictive framework for sesquiterpenoid natural product biosynthesis in basidiomycota. Chem Biol 19(6):772–783
12. Agger S, Lopez-Gallego F, Schmidt-Dannert C (2009 Jun) Diversity of sesquiterpene synthases in the basidiomycete *Coprinus cinereus*. Mol Microbiol 72(5):1181–1195
13. Lopez-Gallego F, Wawrzyn GT, Schmidt-Dannert C (2010 Dec) Selectivity of fungal sesquiterpene synthases: role of the active site's h-1 a loop in catalysis. Appl Environ Microbiol 76(23):7723–7733
14. Lopez-Gallego F, Agger SA, Abate-Pella D, Distefano MD, Schmidt-Dannert C (2010 May) Sesquiterpene synthases cop4 and cop6 from *Coprinus cinereus*: catalytic promiscuity and cyclization of farnesyl pyrophosphate geometric isomers. Chembiochem [Article] 11(8):1093–1106
15. Wackler B, Lackner G, Chooi YH, Hoffmeister D (2012 Aug 13) Characterization of the Suillus grevillei quinone synthetase grea supports a nonribosomal code for aromatic alpha-keto acids. Chembiochem 13(12):1798–1804
16. Schneider P, Bouhired S, Hoffmeister D (2008 Nov) Characterization of the atromentin biosynthesis genes and enzymes in the homobasidiomycete *Tapinella panuoides*. Fungal Genet Biol 45(11):1487–1496
17. Winterberg B, Uhlmann S, Linne U, Lessing F, Marahiel MA, Eichhorn H et al (2010 Mar) Elucidation of the complete ferrichrome a biosynthetic pathway in *Ustilago maydis*. Mol Microbiol 75(5):1260–1271
18. Welzel K, Eisfeld K, Antelo L, Anke T, Anke H (2005 Aug 1) Characterization of the ferrichrome a biosynthetic gene cluster in the homobasidiomycete *Omphalotus olearius*. FEMS Microbiol Lett 249(1):157–163
19. Quin MB, Wawrzyn G, Schmidt-Dannert C (2013 May) Purification, crystallization and preliminary x-ray diffraction analysis of omp6, a protoilludene synthase from *Omphalotus olearius*. Acta Crystallogr Sect F Struct Biol Cryst Commun 69(5):574–577
20. Bushley KE, Ripoll DR, Turgeon BG (2008 Dec 3) Module evolution and substrate specificity of fungal nonribosomal peptide synthetases involved in siderophore biosynthesis. BMC Evol Biol 8(328). doi:10.1186/1471-2148-8-328
21. Bushley KE, Turgeon BG (2010 Jan 26) Phylogenomics reveals subfamilies of fungal nonribosomal peptide synthetases and their evolutionary relationships. BMC Evol Biol 10(26). doi:10.1186/1471-2148-10-26
22. Condon BJ, Leng YQ, Wu DL, Bushley KE, Ohm RA, Otillar R et al (2013 Jan) Comparative genome structure, secondary metabolite, and effector coding capacity across *Cochliobolus* pathogens. Plos Genet 9(1):e1003233
23. Turgeon BG, Oide S, Bushley K (2008 Feb) Creating and screening *Cochliobolus* heterostrophus non-ribosomal peptide synthetase mutants. Mycol Res 112(Pt 2):200–206
24. Bushley KE, Raja R, Jaiswal P, Cumbie JS, Nonogaki M, Boyd AE et al (2013) The genome of *Tolypocladium inflatum*: evolution, organization and expression of the cyclosporin biosynthetic gene cluster. PLoS Genet 9(6):e1003496

25. Martin F, Cullen D, Hibbett D, Pisabarro A, Spatafora JW, Baker SE et al (2011 Jun) Sequencing the fungal tree of life. New Phytol 190(4):818–821
26. Gibson DM, King BC, Hayes ML, Bergstrom GC (2011 Jun) Plant pathogens as a source of diverse enzymes for lignocellulose digestion. Curr Opin Microbiol 14(3):264–270
27. Shendure J, Lieberman Aiden E (2012 Nov) The expanding scope of DNA sequencing. Nat Biotechnol 30(11):1084–1094
28. Morin E, Kohler A, Baker AR, Foulongne-Oriol M, Lombard V, Nagy LG et al (2012 Oct 23) Genome sequence of the button mushroom *Agaricus bisporus* reveals mechanisms governing adaptation to a humic-rich ecological niche. Proc Natl Acad Sci USA 109(43):17501–17506
29. Chen S, Xu J, Liu C, Zhu Y, Nelson DR, Zhou S et al (2012) Genome sequence of the model medicinal mushroom *Ganoderma lucidum*. Nat Commun 3:913
30. Nishida H, Nagatsuka Y, Sugiyama J (2011) Draft genome sequencing of the enigmatic basidiomycete *Mixia osmundae*. J Gen Appl Microbiol 57(1):63–67
31. Ohm RA, de Jong JF, Lugones LG, Aerts A, Kothe E, Stajich JE et al (2010 Sep) Genome sequence of the model mushroom schizophyllum commune. Nat Biotechnol 28(9):957–963
32. Martinez D, Challacombe J, Morgenstern I, Hibbett D, Schmoll M, Kubicek CP et al (2009 Feb 10) Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc Natl Acad Sci USA 106(6):1954–1959
33. Martin F, Aerts A, Ahren D, Brun A, Danchin EG, Duchaussoy F et al (2008 Mar 6) The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature 452(7183):88–92
34. Floudas D, Binder M, Riley R, Barry K, Blanchette RA, Henrissat B et al (2012 Jun 29) The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336(6089):1715–1719
35. Fernandez-Fueyo E, Ruiz-Duenas FJ, Ferreira P, Floudas D, Hibbett DS, Canessa P et al (2012 Apr 3) Comparative genomics of *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. Proc Natl Acad Sci USA 109(14):5458–5463
36. Bao D, Gong M, Zheng H, Chen M, Zhang L, Wang H et al (2013) Sequencing and comparative analysis of the straw mushroom (*Volvariella volvacea*) genome. PLoS ONE 8(3):e58294
37. Osbourn A (2010 Oct) Secondary metabolic gene clusters: evolutionary toolkits for chemical innovation. Trends Genet 26(10):449–457
38. Brakhage AA, Schroeckh V (2011 Jan) Fungal secondary metabolites—strategies to activate silent gene clusters. Fungal Gen Biol 48(1):15–22
39. Brakhage AA (2013 Jan) Regulation of fungal secondary metabolism. Nat Rev Microbiol 11(1):21–32
40. Yazaki K (2005 Jun) Transporters of secondary metabolites. Curr Opin Plant Biol 8(3):301–307
41. Prasad R, Goffeau A (2012) Yeast ATP-binding cassette transporters conferring multidrug resistance. Annu Rev Microbiol 66:39–63
42. Luzhetskyy A, Vente A, Bechthold A (2005 Jul) Glycosyltransferases involved in the biosynthesis of biologically active natural products that contain oligosaccharides. Mol Biosyst 1(2):117–126
43. Singh S, Phillips GN Jr, Thorson JS (2012 Oct) The structural biology of enzymes involved in natural product glycosylation. Nat Prod Rep 29(10):1201–1237
44. Lim FY, Sanchez JF, Wang CC, Keller NP (2012) Toward awakening cryptic secondary metabolite gene clusters in filamentous fungi. Meth Enzymol 517:303–324
45. Cresnar B, Petric S (2011 Jan) Cytochrome P450 enzymes in the fungal kingdom. Biochim Biophys Acta 1814(1):29–35
46. Podust LM, Sherman DH (2012 Oct) Diversity of P450 enzymes in the biosynthesis of natural products. Nat Prod Rep [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review] 29(10):1251–1266

47. Kelly DE, Krasevec N, Mullins J, Nelson DR (2009 Mar) The cypome (cytochrome P450 complement) of *Aspergillus nidulans*. Fungal Gen Biol [Research Support, Non-U.S. Gov't] 46(Suppl 1):53–61
48. Barriuso J, Nguyen DT, Li JW, Roberts JN, MacNevin G, Chaytor JL et al (2011 Jun 1) Double oxidation of the cyclic nonaketide dihydromonacolin l to monacolin j by a single cytochrome P450 monooxygenase, LovA. J Am Chem Soc [Research Support Non-US Gov't] 133(21):8078–8081
49. Artigot MP, Loiseau N, Laffitte J, Mas-Reguieg L, Tadrist S, Oswald IP et al (2009 May) Molecular cloning and functional characterization of two CYP619 cytochrome P450s involved in biosynthesis of patulin in *Aspergillus clavatus*. Microbiology [Research Support NIH Extramural Research Support Non-US Gov't] 155(5):1738–1747
50. Haarmann T, Ortel I, Tudzynski P, Keller U (2006 Apr) Identification of the cytochrome P450 monooxygenase that bridges the clavine and ergoline alkaloid pathways. Chembiochem [Research Support Non-US Gov't] 7(4):645–652
51. Kelly SL, Kelly DE (2013 Feb 19) Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us? Philos Trans R Soc Lond B Biol Sci [Research Support NIH Extramural Research Support Non-US Govt Review] 368(1612):20120476
52. Pinedo C, Wang CM, Pradier JM, Dalmais B, Choquer M, Le Pecheur P et al (2008 Dec 19) Sesquiterpene synthase from the botrydial biosynthetic gene cluster of the phytopathogen *Botrytis cinerea*. ACS Chem Biol 3(12):791–801
53. Hohn TM, Beremand PD (1989 Jun) Isolation and nucleotide sequence of a sesquiterpene cyclase gene from the trichothecene producing fungus *Fusarium sporotrichoides*. Gene [Article] 79(1):131–138
54. Toyomasu T, Nakaminami K, Toshima H, Mie T, Watanabe K, Ito H et al (2004 Jan) Cloning of a gene cluster responsible for the biosynthesis of diterpene aphidicolin, a specific inhibitor of DNA polymerase alpha. Biosci Biotechnol Biochem [Article] 68(1):146–152
55. Toyomasu T, Tsukahara M, Kaneko A, Niida R, Mitsuhashi W, Dairi T et al (2007 Feb) Fusicoccins are biosynthesized by an unusual chimera diterpene synthase in fungi. Proc Natl Acad Sci USA [Article] 104(9):3084–3088
56. Kimura M, Tokai T, O'Donnell K, Ward TJ, Fujimura M, Hamamoto H et al (2003 Mar 27) The trichothecene biosynthesis gene cluster of *Fusarium graminearum* F15 contains a limited number of essential pathway genes and expressed non-essential genes. FEBS Lett 539(1–3):105–110
57. Toyomasu T, Tsukahara M, Kenmoku H, Anada M, Nitta H, Ohkanda J et al (2009 Jul 16) Transannular proton transfer in the cyclization of geranylgeranyl diphosphate to fusicoccadiene, a biosynthetic intermediate of fusicoccins. Org Lett 11(14):3044–3047
58. Stanke M, Steinkamp R, Waack S, Morgenstern B (2004 Jul) Augustus: a web server for gene finding in eukaryotes. Nucleic Acids Res [Article] 32:W309–W312
59. Chen B, Ling H, Chang MW (2013) Transporter engineering for improved tolerance against alkane biofuels in *Saccharomyces cerevisiae*. Biotechnol Biofuels 6(1):21
60. Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA et al (2011 Jul) antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res 39(Web Server issue):W339–346
61. Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E et al (2013 June 3) antiSMASH 2.0-a versatile platform for genome mining of secondary metabolite producers. Nucleic Acids Res 41:W204–12
62. Kimura M, Tokai T, Takahashi-Ando N, Ohsato S, Fujimura M (2007 Sep) Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: pathways, genes, and evolution. Biosci Biotechnol Biochem 71(9):2105–2123
63. Csuros M, Rogozin IB, Koonin EV (2011 Sep) A detailed history of intron-rich eukaryotic ancestors inferred from a global survey of 100 complete genomes. PLoS Comput Biol 7(9):e1002150

64. Freitag J, Ast J, Bolker M (2012 May 24) Cryptic peroxisomal targeting via alternative splicing and stop codon read-through in fungi. Nature 485(7399):522–525
65. Larsen PE, Trivedi G, Sreedasyam A, Lu V, Podila GK, Collart FR (2010) Using deep RNA sequencing for the structural annotation of the *Laccaria bicolor* mycorrhizal transcriptome. PLoS ONE 5(7):e9780
66. Misiek M, Braesel J, Hoffmeister D (2011 Aug) Characterisation of the ArmA adenylation domain implies a more diverse secondary metabolism in the genus *Armillaria*. Fungal Biol 115(8):775–781
67. Misiek M, Hoffmeister D (2008 Feb) Processing sites involved in intron splicing of *Armillaria* natural product genes. Mycol Res [Article] 112:216–224
68. Wawrzyn GT, Bloch SE, Schmidt-Dannert C (2012) Discovery and characterization of terpenoid biosynthetic pathways of fungi. Meth Enzymol 515:83–105
69. Keller O, Kollmar M, Stanke M, Waack S (2011 Mar 15) A novel hybrid gene prediction method employing protein multiple sequence alignments. Bioinformatics [Research Support Non-US Gov't] 27(6):757–763
70. Yu GJ, Wang M, Huang J, Yin YL, Chen YJ, Jiang S et al (2012) Deep insight into the *Ganoderma lucidum* by comprehensive analysis of its transcriptome. PLoS ONE 7(8):e44031
71. McGettigan PA (2013 Feb) Transcriptomics in the RNA-seq era. Curr Opin Chem Biol [Research Support Non-US Govt Review] 17(1):4–11
72. Noble LM, Andrianopoulos A (2013 May 21) Fungal genes in context: genome architecture reflects regulatory complexity and function. Genome Biol Evol 5(7):1336–52
73. Yin W, Keller NP (2011 Jun) Transcriptional regulatory elements in fungal secondary metabolism. J Microbiol 49(3):329–339
74. Bayram O, Braus GH (2012 Jan) Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. FEMS Microbiol Rev 36(1):1–24
75. Ohm RA, de Jong JF, de Bekker C, Wosten HA, Lugones LG (2011 Sep) Transcription factor genes of *Schizophyllum commune* involved in regulation of mushroom formation. Mol Microbiol 81(6):1433–1445
76. Hur M, Campbell AA, Almeida-de-Macedo M, Li L, Ransom N, Jose A et al (2013) A global approach to analysis and interpretation of metabolic data for plant natural product discovery. Nat Product Rep 30(4):565–583
77. Higashi Y, Saito K (2013 Jan 21) Network analysis for gene discovery in plant-specialized metabolism. Plant Cell Environ 36(9):1597–606
78. Andersen MR, Nielsen JB, Klitgaard A, Petersen LM, Zachariasen M, Hansen TJ et al (2013 Jan 2) Accurate prediction of secondary metabolite gene clusters in filamentous fungi. Proc Natl Acad Sci USA 110(1):E99–E107
79. Chooi YH, Tang Y (2012 Nov 16) Navigating the fungal polyketide chemical space: from genes to molecules. J Org Chem 77(22):9933–9953
80. Evans BS, Robinson SJ, Kelleher NL (2011 Jan) Surveys of non-ribosomal peptide and polyketide assembly lines in fungi and prospects for their analysis in vitro and in vivo. Fungal Genet Biol 48(1):49–61
81. Boettger D, Hertweck C (2013 Jan 2) Molecular diversity sculpted by fungal PKS-NRPS hybrids. Chembiochem 14(1):28–42
82. Donadio S, Monciardini P, Sosio M (2007 Oct) Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. Nat Prod Rep 24(5):1073–1109
83. Paumi CM, Chuk M, Snider J, Stagljar I, Michaelis S (2009 Dec) ABC transporters in *Saccharomyces cerevisiae* and their interactors: new technology advances the biology of the ABCC (MRP) subfamily. Microbiol Mol Biol Rev 73(4):577–593
84. Wang X (2009 Oct 20) Structure, mechanism and engineering of plant natural product glycosyltransferases. FEBS Lett 583(20):3303–3309
85. Crouzet J, Roland J, Peeters E, Trombik T, Ducos E, Nader J et al (2013 May) Ntpdr1, a plasma membrane ABC transporter from *Nicotiana tabacum*, is involved in diterpene transport. Plant Mol Biol 82(1–2):181–192

86. Gupta RP, Kueppers P, Schmitt L, Ernst R (2011 Jan) The multidrug transporter Pdr5: a molecular diode? Biol Chem 392(1–2):53–60
87. Syed K, Yadav JS (2012 Nov) P450 monooxygenases (P450ome) of the model white rot fungus *Phanerochaete chrysosporium*. Crit Rev Microbiol 38(4):339–363
88. Hansen FT, Sorensen JL, Giese H, Sondergaard TE, Frandsen RJ (2012 Apr 16) Quick guide to polyketide synthase and nonribosomal synthetase genes in *Fusarium*. Int J Food Microbiol 155(3):128–136
89. Sa-Correia I, dos Santos SC, Teixeira MC, Cabrito TR, Mira NP (2009 Jan) Drug:H+ antiporters in chemical stress response in yeast. Trends Microbiol 17(1):22–31
90. Kretzschmar T, Burla B, Lee Y, Martinoia E, Nagy R (2011 Sep 7) Functions of ABC transporters in plants. Essays Biochem 50(1):145–160
91. Wendlandt S, Lozano C, Kadlec K, Gomez-Sanz E, Zarazaga M, Torres C et al (2013 Feb) The enterococcal ABC transporter gene lsa(E) confers combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics in methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. J Antimicrob Chemother 68(2):473–475
92. Ma SM, Li JW, Choi JW, Zhou H, Lee KK, Moorthie VA et al (2009 Oct 23) Complete reconstitution of a highly reducing iterative polyketide synthase. Science 326(5952):589–592
93. Rugbjerg P, Naesby M, Mortensen UH, Frandsen RJ (2013 Apr 4) Reconstruction of the biosynthetic pathway for the core fungal polyketide scaffold rubrofusarin in *Saccharomyces cerevisiae*. Microb Cell Fact 12(1):31
94. Lackner G, Misiek M, Braesel J, Hoffmeister D (2012 Dec) Genome mining reveals the evolutionary origin and biosynthetic potential of basidiomycete polyketide synthases. Fungal Genet Biol 49(12):996–1003
95. Proctor RH, Brown DW, Plattner RD, Desjardins AE (2003 Mar) Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. Fungal Genet Biol 38(2):237–249
96. Ro DK, Ouellet M, Paradise EM, Burd H, Eng D, Paddon CJ et al (2008) Induction of multiple pleiotropic drug resistance genes in yeast engineered to produce an increased level of anti-malarial drug precursor, artemisinic acid. BMC Biotechnol 8:83
97. Soukup AA, Chiang YM, Bok JW, Reyes-Dominguez Y, Oakley BR, Wang CC et al (2012 Oct) Overexpression of the *Aspergillus nidulans* histone 4 acetyltransferase EsaA increases activation of secondary metabolite production. Mol Microbiol 86(2):314–330
98. Christianson DW (2008 Apr) Unearthing the roots of the terpenome. Curr Opin Chem Biol 12(2):141–150
99. Davis EM, Croteau R (2000) Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. Biosynthesis: aromatic polyketides, isoprenoids, alkaloids. Springer, Berlin, p 53–95
100. Mitsuguchi H, Seshime Y, Fujii I, Shibuya M, Ebizuka Y, Kushiro T (2009 May 13) Biosynthesis of steroidal antibiotic fusidanes: functional analysis of oxidosqualene cyclase and subsequent tailoring enzymes from *Aspergillus fumigatus*. J Am Chem Soc 131(18):6402–6411
101. Kimura M, Kushiro T, Shibuya M, Ebizuka Y, Abe I (2010 Jan 1) Protostadienol synthase from *Aspergillus fumigatus*: functional conversion into lanosterol synthase. Biochem Biophys Res Commun 391(1):899–902
102. Cane DE, Kang I (2000 Apr 15) Aristolochene synthase: purification, molecular cloning, high-level expression in *Escherichia coli*, and characterization of the *Aspergillus terreus* cyclase. Arch Biochem Biophys 376(2):354–364
103. Proctor RH, Hohn TM (1993 Feb) Aristolochene synthase—isolation, characterization, and bacterial expression of a sesquiterpenoid biosynthetic gene (*Ari1*) from *Penicillium roqueforti*. J Biol Chem [Article] 268(6):4543–4548
104. Engels B, Heinig U, Grothe T, Stadler M, Jennewein S (2011 Mar 4) Cloning and characterization of an *Armillaria gallica* cDNA encoding protoilludene synthase, which catalyzes the first committed step in the synthesis of antimicrobial melleolides. J Biol Chem 286(9):6871–6878

105. McCormick SP, Alexander NJ, Harris LJ (2010 Jan) CLM1 *of Fusarium graminearum* encodes a longiborneol synthase required for culmorin production. Appl Environ Microbiol 76(1):136–141
106. Brock NL, Tudzynski B, Dickschat JS (2011 Oct 11) Biosynthesis of sesqui- and diterpenes by the gibberellin producer *Fusarium fujikuroi*. Chembiochem 12(17):2667–12
107. Quin MB, Flynn CM, Wawrzyn GT, Choudhary S, Schmidt-Dannert C (2013) Mushroom hunting using bioinformatics: application of a predictive framework facilitates the selective identification of sesquiterpene synthases in basidiomycota. Chembiochem 14(18):2480–91
108. Citron CA, Gleitzmann J, Laurenzano G, Pukall R, Dickschat JS (2012 Jan 23) Terpenoids are widespread in actinomycetes: a correlation of secondary metabolism and genome data. Chembiochem 13(2):202–214
109. Novak R (2011 Dec) Are pleuromutilin antibiotics finally fit for human use? Ann N Y Acad Sci 1241:71–81
110. Shen JW, Ruan Y, Ma BJ (2009 Jun) Diterpenoids of macromycetes. J Basic Microbiol 49(3):242–255
111. Toyomasu T (2008 May) Recent advances regarding diterpene cyclase genes in higher plants and fungi. Biosci Biotechnol Biochem 72(5):1168–1175
112. Troncoso C, Gonzalez X, Bomke C, Tudzynski B, Gong F, Hedden P et al (2010 Aug) Gibberellin biosynthesis and gibberellin oxidase activities in *Fusarium sacchari, Fusarium konzum and Fusarium subglutinans* strains. Phytochemistry 71(11–12):1322–1331
113. Bomke C, Tudzynski B (2009 Oct-Nov) Diversity, regulation, and evolution of the gibberellin biosynthetic pathway in fungi compared to plants and bacteria. Phytochemistry 70(15–16):1876–1893
114. Bromann K, Toivari M, Viljanen K, Vuoristo A, Ruohonen L, Nakari-Setala T (2012) Identification and characterization of a novel diterpene gene cluster in *Aspergillus nidulans*. PLoS ONE 7(4):e35450
115. Noike M, Liu C, Ono Y, Hamano Y, Toyomasu T, Sassa T et al (2012 Mar 5) An enzyme catalyzing O-prenylation of the glucose moiety of fusicoccin A, a diterpene glucoside produced by the fungus *Phomopsis amygdali*. Chembiochem 13(4):566–573
116. Noike M, Ono Y, Araki Y, Tanio R, Higuchi Y, Nitta H et al (2012) Molecular breeding of a fungus producing a precursor diterpene suitable for semi-synthesis by dissection of the biosynthetic machinery. PLoS ONE 7(8):e42090
117. Ono Y, Minami A, Noike M, Higuchi Y, Toyomasu T, Sassa T et al (2011 Mar 2) Dioxygenases, key enzymes to determine the aglycon structures of fusicoccin and brassicicene, diterpene compounds produced by fungi. J Am Chem Soc 133(8):2548–2555
118. Toyomasu T, Kaneko A, Tokiwano T, Kanno Y, Kanno Y, Niida R et al (2009 Feb 20) Biosynthetic gene-based secondary metabolite screening: a new diterpene, methyl phomopsenonate, from the fungus *Phomopsis amygdali*. J Org Chem 74(4):1541–1548
119. Toyomasu T, Niida R, Kenmoku H, Kanno Y, Miura S, Nakano C et al (2008 Apr) Identification of diterpene biosynthetic gene clusters and functional analysis of labdane-related diterpene cyclases in *Phomopsis amygdali*. Biosci Biotechnol Biochem 72(4):1038–1047
120. Fujii R, Minami A, Tsukagoshi T, Sato N, Sahara T, Ohgiya S et al (2011) Total biosynthesis of diterpene aphidicolin, a specific inhibitor of DNA polymerase alpha: heterologous expression of four biosynthetic genes in *Aspergillus oryzae*. Biosci Biotechnol Biochem 75(9):1813–1817
121. Chiba R, Minami A, Gomi K, Oikawa H (2013 Feb 1) Identification of ophiobolin F synthase by a genome mining approach: a sesterterpene synthase from *Aspergillus clavatus*. Org Lett 15(3):594–597
122. Itoh T, Tokunaga K, Matsuda Y, Fujii I, Abe I, Ebizuka Y et al (2010 Oct) Reconstitution of a fungal meroterpenoid biosynthesis reveals the involvement of a novel family of terpene cyclases. Nat Chem 2(10):858–864
123. Guo CJ, Knox BP, Chiang YM, Lo HC, Sanchez JF, Lee KH et al (2012 Nov 16) Molecular genetic characterization of a cluster in *A. terreus* for biosynthesis of the meroterpenoid terretonin. Org Lett 14(22):5684–5687

124. Matsuda Y, Awakawa T, Itoh T, Wakimoto T, Kushiro T, Fujii I et al (2012 Aug 13) Terretonin biosynthesis requires methylation as essential step for cyclization. Chembiochem 13(12):1738–1741
125. Itoh T, Tokunaga K, Radhakrishnan EK, Fujii I, Abe I, Ebizuka Y et al (2012 May 29) Identification of a key prenyltransferase involved in biosynthesis of the most abundant fungal meroterpenoids derived from 3,5-dimethylorsellinic acid. Chembiochem 13(8):1132–1135
126. Lin HC, Chooi YH, Dhingra S, Xu W, Calvo AM, Tang Y (2013 Mar 27) The fumagillin biosynthetic gene cluster in *Aspergillus fumigatus* encodes a cryptic terpene cyclase involved in the formation of beta-trans-bergamotene. J Am Chem Soc 135(12):4616–4619
127. Lo HC, Entwistle R, Guo CJ, Ahuja M, Szewczyk E, Hung JH et al (2012 Mar 14) Two separate gene clusters encode the biosynthetic pathway for the meroterpenoids austinol and dehydroaustinol in *Aspergillus nidulans*. J Am Chem Soc 134(10):4709–4720
128. Tagami K, Liu C, Minami A, Noike M, Isaka T, Fueki S et al (2013 Jan 30) Reconstitution of biosynthetic machinery for indole-diterpene paxilline in *Aspergillus oryzae*. J Am Chem Soc 135(4):1260–1263
129. Saikia S, Nicholson MJ, Young C, Parker EJ, Scott B (2008 Feb) The genetic basis for indole-diterpene chemical diversity in filamentous fungi. Mycol Res 112(Pt 2):184–199
130. Young C, McMillan L, Telfer E, Scott B (2001 Feb) Molecular cloning and genetic analysis of an indole-diterpene gene cluster from *Penicillium paxilli*. Mol Microbiol 39(3):754–764
131. Young CA, Felitti S, Shields K, Spangenberg G, Johnson RD, Bryan GT et al (2006 Oct) A complex gene cluster for indole-diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. Fungal Gen Biol 43(10):679–693
132. Young CA, Tapper BA, May K, Moon CD, Schardl CL, Scott B (2009 Apr) Indole-diterpene biosynthetic capability of *Epichloë* endophytes as predicted by *ltm* gene analysis. Appl Environ Microbiol 75(7):2200–2211
133. Teixeira MC, Godinho CP, Cabrito TR, Mira NP, Sa-Correia I (2012) Increased expression of the yeast multidrug resistance ABC transporter Pdr18 leads to increased ethanol tolerance and ethanol production in high gravity alcoholic fermentation. Microb Cell Fact 11:98
134. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D et al (2013 Apr 25) High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496(7446):528–532
135. Chanda A, Roze LV, Linz JE (2010 Nov) A possible role for exocytosis in aflatoxin export in *Aspergillus parasiticus*. Eukaryot Cell 9(11):1724–1727
136. Chanda A, Roze LV, Kang S, Artymovich KA, Hicks GR, Raikhel NV et al (2009 Nov 17) A key role for vesicles in fungal secondary metabolism. Proc Natl Acad Sci USA 106(46):19533–19538
137. Menke J, Weber J, Broz K, Kistler HC (2013) Cellular development associated with induced mycotoxin synthesis in the filamentous fungus *Fusarium graminearum*. PLoS ONE 8(5):e63077
138. Menke J, Dong Y, Kistler HC (2012 Nov) *Fusarium graminearum* Tri12p influences virulence to wheat and trichothecene accumulation. Mol Plant Microbe Interact 25(11):1408–1418
139. Bai J, Swartz DJ, Protasevich, II, Brouillette CG, Harrell PM, Hildebrandt E et al (2011) A gene optimization strategy that enhances production of fully functional P-glycoprotein in *Pichia pastoris*. PLoS ONE 6(8):e22577
140. Bernier SG, Lazarus DD, Clark E, Doyle B, Labenski MT, Thompson CD et al (2004 Jul 20) A methionine aminopeptidase-2 inhibitor, PPI-2458, for the treatment of rheumatoid arthritis. Proc Natl Acad Sci USA 101(29):10768–10773
141. Schobert R, Knauer S, Seibt S, Biersack B (2011) Anticancer active illudins: recent developments of a potent alkylating compound class. Curr Med Chem 18(6):790–807
142. Seong KY, Pasquali M, Zhou X, Song J, Hilburn K, McCormick S et al (2009 Apr) Global gene regulation by *Fusarium* transcription factors Tri6 and Tri10 reveals adaptations for toxin biosynthesis. Mol Microbiol 72(2):354–367

Chapter 5
Metagenomics and Metatranscriptomics for the Exploration of Natural Products from Soil Fungi

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Introduction

Microorganisms constitute rich sources of diverse biologically active metabolites. These metabolites have already found a broad spectrum of applications, for instance as antiparasitics, antibiotics, anticancer agents, immunosuppressants as well as agrochemicals [1]. A wide range of niches on Earth are occupied by microorganisms, ranging from deep rock sediments and marine environments to deserts, alpine, Arctic, and Antarctic regions, and even to thermal vents [1]. In terms of microbial diversity, soil is a remarkable site, which contains a hitherto largely unexplored microbiota. For instance, in as small as 1 g of soil, several thousands of bacterial species exist, the majority of which are uncultivable under standard microbiological conditions [2]. In parallel to prokaryotes, there is a substantial number of eukaryotic microorganisms hosted by soil, which contribute to the microbial biomass [3]. In the light of the enormous diversity of microorganisms in soil, only a handful of bacterial (less than 1 %) and fungal species (less than 5 %) are known at present. Hence, millions of microbial species out there need to be unearthed [4]. Isolation and in vitro growth of most prokaryotic and eukaryotic microorganisms is, however, difficult or impossible due to their general lack of cultivability. To address this obstacle, new experimental approaches, such as metagenomics, have been used to assess the true functional diversity and activities of microorganisms in soil. In the next sections, we will explore how the power of molecular tools can be harnessed to explore the wealth of genetic and functional information that exists right underfoot.

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Natural Product Exploration Using Metagenomics and Other "Omics" Tools

Natural environments, such as soil, are a great reservoir of genes involved in different biosynthetic pathways that are difficult to explore using cultivation techniques. This reservoir of genes can be unlocked using metagenomics; i.e., the study of the collective genomes of the microbial community. Metagenomics offers very powerful strategies that allow us to unearth both the functional potential and taxonomic diversity of microorganisms at the community level [5, 6]. This approach has already yielded a wealth of novel data. However, there are pitfalls in the approach, as will be discussed in the following section.

Analysis of Function of Soil—Amplicon Sequencing and Metagenomics

The function of soil can be studied using either a gene-centered or a genome-centered approach. In the former approach, the polymerase chain reaction (PCR) is used to amplify single target genes and the amplification products (amplicons) are used for sequencing to analyze the occurrence of the different orthologs of that gene in the whole community. In the second approach, random metagenomic sequencing is used in which total microbial community DNA is isolated from a soil sample and shotgun sequenced, resulting in an outline of all genes that are present in the community [7]. Next to these direct approaches, the DNA extracted from the sample can also be used to generate metagenomic DNA libraries, which are subsequently screened for function (Fig. 5.1).

The latter two approaches result in a wealth of information that is stored in the genomes of microorganisms, which occupy various niches in the soil environment [8]. The metagenomic libraries have potential applications in both applied and basic research. Several studies over the years have used metagenomics for purposes such as bioprospection for novel amylases [9], beta-agarases, cellulases and lipases [10]. Moreover, Schirmer et al. [11], Courtois et al. [12] and Gillespie et al. [13] reported on new polyketide synthase genes and their expressed compounds and two colored triaryl cation antibiotics. Other studies revealed information about important physiological processes of microorganisms after extensive sequencing of metagenomic libraries [14–16]. All these studies focused on prokaryotic microorganisms, thus excluding eukaryotic microorganisms, which was possibly due to their relative scarcity or because of their physical discrimination through filtration or centrifugation on density gradients before DNA extraction [3]. Eukaryotic metagenomics has faced certain constraints over the years, such as the giant genome sizes of most eukarya compared to the smaller bacterial genomes. (Micro)eukaryote genomes range from 13.8 Mbp for the yeast *Schizosaccharomyces pombe* [17] to 69 Mbp for the ciliate *Paramecium tetraurelia* [18]. The large sizes compromise seizing—to a sufficient extent—the eukaryotic microbial community gene content. Moreover, the

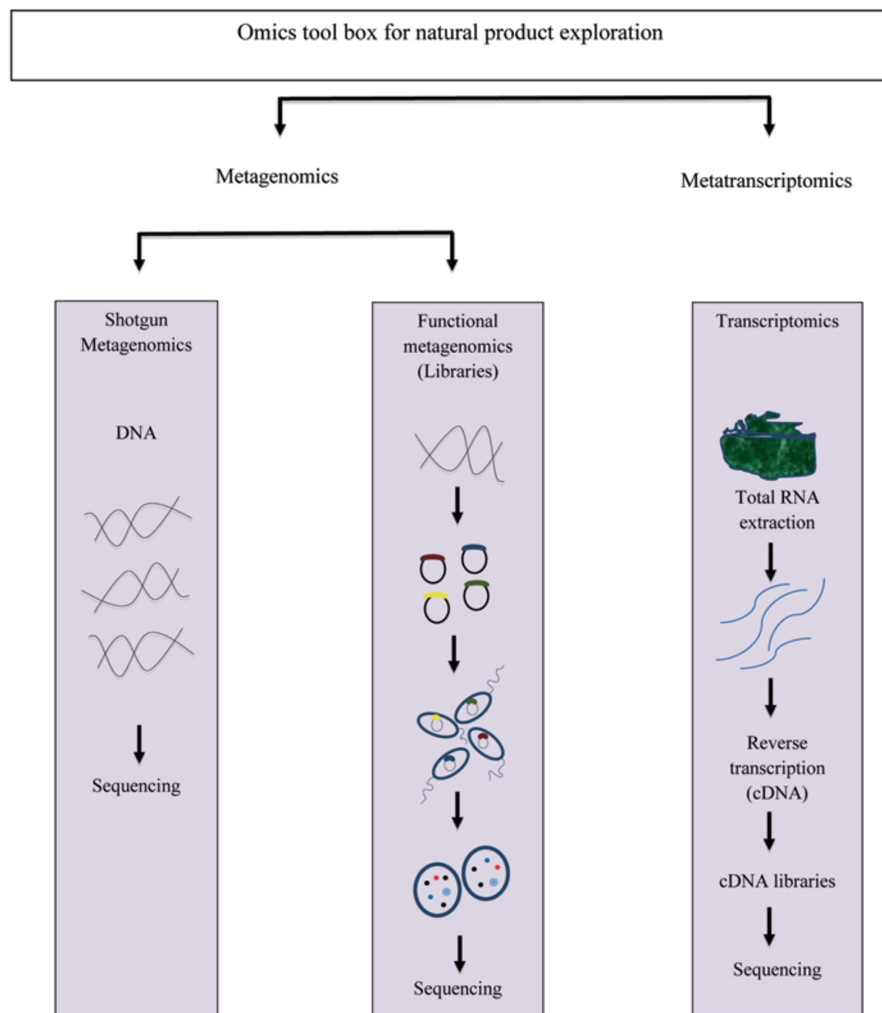

Fig. 5.1 A schematic workflow of 'omics' tools for screening of natural products

detection of the expression of eukaryotic protein-encoding genes is impeded by the existence of introns and the absence of a conserved motif in promoter sequences [3].

Construction and Screening of Metagenomic Libraries

Metagenomic libraries are most often constructed inside cloning vectors that are replicated in the common host *Escherichia coli*. Different cloning vectors can host DNA fragments ranging from up to 30 to 300 kb in size [19–23], which allows a wealth of possibilities in the cloning step. Single-gene traits can be picked up in

small-insert (up to several kb) vectors, whereas traits that are encoded by larger stretches of DNA (gene clusters) require larger insert vectors (see later). Gene clusters that encode natural products are mainly in the 30–300 kb range, which would allow cloning of whole intact gene clusters inside a single large-insert vector clone [24]. Recently, expression of whole gene clusters in suitable host organisms has been achieved, allowing biosynthesis of the natural product in question [25–27]. If gene clusters spread over multiple clones represent a target pathway, recombinogenic cloning could be used to streamline the whole metabolic pathway, as has been reported for heterologous expression of the tubulysin gene cluster [28].

A prerequisite for the construction of a metagenomic library is the selection of an appropriate vector, into which sheared and fragmented DNA isolated from an environmental sample can be inserted. For many gene clusters, vectors that can accommodate inserts up to 40 kb, such as fosmids [22] and cosmids [20], could be used. However, as discussed above, some gene clusters have sizes well above 40 kb. This has motivated researchers to use vectors that can harbor up to 300 kb fragments [19], such as P1-derived vectors [21] and bacterial artificial chromosomes (BACs) [23]. Consequently, an appropriate host organism should host vectors that carry random fragments of particular sizes. This results in a plethora of clones that are then screened to assess if DNA fragments of interest are successfully expressed or are detectable by genetic screens. Those screens that pinpoint interesting DNA fragments are often subjected to sequencing, resulting in information that is useful to mine the gene clusters of interest. This strategy has been used in a number of recent studies, i.e., to identify siderophore biosynthetic genes [26], to detect secondary metabolites from soil bacteria [29], and to discover novel natural products [30].

Shotgun Metagenomics or Direct Sequencing

The current affordability of next-generation sequencing (NGS) tools has revolutionized the direct shotgun metagenomics of the microbiota from environmental habitats. In this approach, microbial community DNA isolated from environmental samples is directly sequenced without first constructing clone libraries. Shotgun metagenomics has been applied in various studies to discover enzymes responsible for the biodegradation of lignocellulosic matter from sources such as cow rumen [31] and compost [32]. Furthermore, recent studies featured shotgun metagenomics and metagenomic library construction, exploring the microbiomes of the marine tunicate *Lissoclinum patella* as well as of coral reefs [33–35]. Other studies used pyrosequencing to investigate the marine sponge *Arenosclera brasiliensis* microbiome [36], and the *Ecteinascidia turbinata* tunicate metagenome [37]. The latter study identified a biosynthetic gene cluster encoding the chemotherapeutic natural product denoted 'ET-743'. However, there are potential constraints related to the data generated, as processing the data and extracting relevant information about the biosynthetic gene clusters of natural products is a daunting task. In particular, the massive data that we generate nowadays pose problems for downstream analyses such as cleaning up, binning and subsequent sequence-based and statistical analy-

ses. Computer power is increasingly limiting and so is bioinformatics. Clearly, one needs advanced (better) bioinformatics tools and pipelines to pave the way for improvement in the analysis of the data generated by shotgun metagenomics.

Metatranscriptomics

Metagenomics studies do not address questions regarding the expression state of genes, hampering conclusions about the functional role of particular genes in the environment under study. Therefore, new technology-coined metatranscriptomics strategies have come into being. Metatranscriptomics analyzes the collective set of messenger RNAs that are present in an environmental sample, in an all-at-once manner. However, RNA extracted from the natural microbiota is often dominated by ribosomal RNA (rRNA). Hence, in the early studies messenger RNA (mRNA) has been enriched by rRNA depletion (for bacteria) or poly-A tailed mRNA enrichment (for eukaryotes) to allow the investigation of overall gene expression profiles in the environments under study [3, 38]. Later on, massive parallel (pyro)sequencing, following a reverse transcription step, was adopted to analyze bacterial and archaeal mRNA from environmental (marine) samples, giving a much larger scale as compared to the previous studies. In one study, an in vitro amplification step was included to keep sample size small and preparation fast [39]. In another study in soil, the total RNA was analyzed all at once. This allowed the assessment of the limitations of earlier approaches (linking phylogeny to function) by the simultaneous determination of soil microbial community structure (rRNA) and function (mRNA) through metatranscriptomics [40]. However, the amount of mRNA that could be analyzed in the study was disappointingly low, and so the analysis of in situ gene expression was limited. On the other hand, in spite of the technical difficulties, analyses based on metatranscriptomics are very useful, as they provide clues concerning the in situ gene expression and point us to conditions under which key genes (e.g., those involved in the production of natural compounds) are expressed.

The Rhizosphere: A Potential Hotspot for Natural Products

The biologically active region in the immediate vicinity of plant roots, which is inhabited by soil microorganisms (in particular bacteria and fungi), is termed the rhizosphere [41–43]. The rhizosphere is under the direct influence of plant roots and their exuded products, such as secreted compounds, cell lysates, mucilage and gases such as respiratory CO_2 [44]. Although recalcitrant compounds are also present, the availability of easily degradable nutrients makes the rhizosphere a dreamland for microorganisms. It is also a playground for complex interactions among microorganisms, such as cooperation through cross-feeding or competition for nutrients, using, for instance, antagonism through chemical warfare [45–47]. The diversity and complexity of the rhizosphere in terms of microbial life, in addition to the selection

for interaction-proficient microbes, makes it a potentially important source of natural products. A number of natural products from fungi associated with plants have already been isolated and characterized, as described in the following examples. Penicillic acid and two new natural products (6-methoxy-5, 6-dihydropenicillic acid and 4*R*, 5*S* dihydroxy-3-methoxy-5-methylcyclohex-2-enone) have been isolated from *Aspergillus cervinus* associated with *Anicasanthus thurberi* [48]. Similarly, *Aspergillus terreus*, which is associated with the roots of *Opuntia versicolor*, is a producer of compounds such as betulinan, quadrone, terricyclic acid A, asterriquinone C-1, asterriquinone D and asterredione, among others [49, 50]. These few examples suggest that the rhizosphere is a rich source of natural products. The diverse microorganisms that inhabit the rhizosphere have apparently learned to deal with the ecology of the niches present and developed key genetic systems allowing survival by chemical warfare accordingly. On the other hand, the studies only reported on fungal strains that can be grown in vitro. In the light of the lack of culturability of many microorganisms, it is likely that there is much more potential for finding and exploring natural product producers. Thus, techniques such as metagenomics, metatranscriptomics and even metabonomics should be applied to overcome the limitation of microorganism culturing in vitro.

Endophytic Fungi as Sources of Natural Products

Microorganisms that reside inside plants without causing disease symptoms have been coined endophytes [51]. This unique plant–microbe interaction is established entirely inside plant tissues [52] and is defined by the fact that the two partners do not affect each other lethally. Endophytes offer great biotechnological potential in terms of the biosynthesis of natural products and bioactive metabolites for application such as therapeutics for a number of diseases [52–56]. Some studies have already reported the finding of key therapeutically important secondary metabolites produced by fungi, such as taxol [57], deoxypodophyllotoxin [58], podophyllotoxin [59, 60], hypericin and emodin [61, 62], azadirachtin [63] and camptothecin [64–67]. The production of these metabolites, as well as many others, makes endophytes very important microorganisms for studying from an ecological as well as biochemical standpoint. In order to investigate and explore new secondary metabolites, it is imperative that such plant-interactive microorganisms are exploited in the best possible way, which should include an assessment on how genes for the biosynthesis of key metabolites are regulated.

Bacterial–Fungal Interactions and Natural Product Discovery

Recently, microbial interactions were found to be drivers the of the production of particular metabolites in bacteria and fungi. The interactions between microorganisms, especially the bipartite ones (e.g., between bacteria and fungi) were deemed

important, as natural product formation can indeed be induced by bacteria that occur in the vicinity of fungi. For instance, the soil-dwelling bacterium *Streptomyces rapamycinicus* physically interacts with *Aspergillus nidulans*, and, in this interaction, activates a silent polyketide biosynthesis gene cluster [68]. Therefore, including microbial "neighbors" in studies for exploration of natural products is the way to go in further screens that eventually will include meta-omics techniques. The former study showed the production of polyphenols—i.e., cathepsin K inhibitors and lecanoric acid—derived from orsellinic acid [68]. In other studies on non-endophytic fungi, *Variovorax paradoxus* strain HB44 was found to be selected in the mycosphere of *Laccaria proxima* [69]. The organism was able to grow on compounds released by a close relative of *L. proxima*, i.e., *Lyophyllum* sp. strain Karsten, particularly glycerol. The study also reported the release of other compounds, i.e., acetic acid and formic acid, by the fungus [69]. Recent work in our laboratory shows that *Lyophyllum* sp. strain Karsten releases glycerol-rich exudates, which may be due to a stimulatory effect of the fungal-interactive *Burkholderia terrae* strain BS001. This mechanism may be of great significance for strain BS001 in an ecological context. The stimulation of the fungal release of glycerol could be considered as a strategy to acquire easily degradable carbonaceous food, allowing a better survival in the mycosphere [70].

With respect to bacterial–fungal interactions, complex interplays of events have been shown, in which the toxic compound rhizoxin was produced. Rhizoxin is the causative agent of rice seedling blight. Until recently, it was believed that the rice-pathogenic fungus *Rhizopus microsporus* was the producer of this rhizoxin. However, this turned out not to be the case, as the bacterium *Burkholderia rhizoxinica*, which inhabits the fungal cytosol, was revealed to be the producer [71]. Recently, it has been reported that *R. microsporus* also contributes to the potency of the (phytotoxic) rhizoxin, as the rhizoxin produced in co-cultures with *B. rhizoxinica* contained two bis-epoxide moieties compared to the one that is solely produced by the bacterium, clearly indicating that there is synergism in production of natural products [72].

In another study, fungi interacting with lichens were reported to produce natural products such as bis-naphtopyrones and lichenicolins A and B, both of which have activity against Gram-positive bacteria [73]. Co-culturing an unidentified bacterium with the fungus *Libertella* sp., diterpenoids- including libertellenone A–D—were discovered. The compounds were induced by the presence of the bacterium, as they did not show up in cultures without the bacterium [74]. The marine fungus *Emericella* sp., when grown in co-culture with *Salinispora arenicola*, produced two depsipeptides (emericellamides A and B) that were shown to exert antibacterial activity against methicillin-resistant *Staphylococcus aureus* [75], and are thus interesting as antibiotics against this dangerous bacterium. Similarly, formyl-xanthocillin analogs were shown to be synthesized when *Streptomyces peucetius* was grown with *Aspergillus fumigatus* [76].

All these studies on the biosynthesis of natural products have been carried out in relatively "simple" circumstances, in which basic (co-)culturing techniques were applied. However, the complexities of their natural environment, including fluctuating and often harsh circumstances, are rarely included in such experiments

and the full potential of natural compound production in nature is still cryptic. Therefore, to unlock the wealth of natural products hidden in the natural microbiota, it is imperative to dig deeply into natural habitats, focusing on the interacting microorganisms and to “eavesdrop” on the cross-talk between them using meta-omics tools. In such strategies, a combination of the current and newly emerging advanced molecular tools, including RNA sequencing and metabonomics, with traditional cultivation-based efforts needs to be applied as such an approach will take advantage of the complementary strong points of both types of analyses.

Conclusion

Outlook

There is a progressively increasing feeling that the scientific community, and society as a whole, is close to exhausting the capability of finding novel natural products that serve society, if we continue to bioexplore our natural environments by the traditional (cultivation-based) and advanced molecular methods. The natural products include the dearly needed novel antibiotics, which are often produced by fungi from soil or other natural environments, that allow us to treat dangerous bacterial or fungal diseases. The reasons for this contention of reaching the “limit” are the simple facts that (1) increasingly we reencounter organisms and the natural compounds they produce via the traditional way of cultivation and assessing bioantagonism, and (2) the novel molecular tools often stop short of telling us the complete story on the expression of genes/operons for natural products in the light of the absence of the suitable conditions that trigger gene expression.

To tackle both types of problems, it is imperative that a better focus is placed on mimicking the conditions that govern the life of the target microbiota in its natural environment. And, included in such conditions, we need to consider the biotic component of it (i.e., the presence of other organisms), as microorganisms such as fungi in nature have most likely “learned” to express their key antagonistic compounds when other organisms (that might present ecological threats) are in their vicinity. Hence, we here reviewed the available literature with respect to the effect of microbial “neighbors” on the expression of (otherwise silent) genes that underlie microbial antagonism and thus might yield novel antibiotic compounds. Moreover, we strongly advocate the inclusion of such organisms, or consortia of organisms, in screens for the production of novel compounds. Then, the power of meta-omics techniques might be harnessed to improve our screens and get at the natural products and their underlying genes in the most efficient way possible. This may include (in that order) metabonomics, metatranscriptomics and metagenomics, leading to the identification and isolation of genes/operons responsible for the biosynthesis.

However, in the end it might be very useful to also attempt to isolate the producer organism, allowing production by the natural organism, as incited by neighbors. In this isolation effort, the availability of molecular tools will be a great asset.

In other words, if fragments of interesting genes are discovered, probes and primers might be generated that enable the monitoring of the organisms in enrichments and allow the guidance of a directed isolation effort, leading to the availability of novel "nature-derived" producer organisms.

References

1. Gunatilaka AAL (2006) Natural products from plant-associated micro-organisms: distribution, structural diversity, bioactivity, and implications of their occurrence. J Nat Prod 69(3):509–526
2. Rappe MS, Giovannoni SJ (2003) The uncultured microbial majority. Annu Rev Microbiol 57:369–394
3. Bailly J, Fraissinet-Tachet L, Verner MC, Debaud JC, Lemaire M, Wésolowski-Louvel M, Marmeisse R (2007) Soil eukaryotic functional diversity, a metatranscriptomic approach. ISME J 1(7):632–642
4. Young P (1997) Major microbial diversity initiative recommended. ASM News 63:417–421
5. Cowan D, Meyer Q, Stafford W, Muyanga S, Cameron R, Wittwer P (2005) Metagenomic gene discovery, past, present and future. Trends Biotechnol 23(6):321–329
6. Schloss PD, Handelsman J (2003) Biotechnological prospects from metagenomics. Curr Opin Biotechnol 14(3):303–310
7. Gilbert JA, Dupont CL (2011) Microbial metagenomics: beyond the genome. Ann Rev Mar Sci 3:347–371
8. Rondon MR, August PR, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono KA, Lynch BA, MacNeil IA, Minor C, Tiong CL, Gilman M, Osburne MS, Clardy J, Handelsman J, Goodman RM (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl Environ Microbiol 66(6):2541–2547
9. Yun J, Kang S, Park S, Yoon H, Kim MJ, Hew S, Ryu S (2004) Characterization of a novel amylolytic enzyme encoded by a gene from a soil derived metagenomic library. Appl Environ Microbiol 70(12):7229–7235
10. Voget S, Leggewie C, Uesbeck A, Raasch C, Jaeger KE, Streit WR (2003) Prospecting for novel biocatalysts in a soil metagenome. Appl Environ Microbiol 69(10):6235–6242
11. Schirmer A, Gadkari R, Reeves CD, Ibrahim F, DeLong EF, Hutchinson CR (2005) Metagenomic analysis reveals diverse polyketide synthase gene clusters in micro-organisms associated with the marine sponge *Discodermia dissoluta*. Appl Environ Microbiol 71(8):4840–4849
12. Courtois S, Cappellano CM, Ball M Francou FX, Normand P, Helynck G, Martinez A, Kolvek SJ, Hopke J, Osburne MS, August PR, Nalin R, Guérineau M, Jeannin P, Simonet P, Pernodet JL (2003) Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. Appl Environ Microb 69:49–55
13. Gillespie DE, Brady SF, Bettermann AD, Cianciotto NP, Liles MR, Rondon MR, Clardy J, Goodman RM, Handelsman J (2002) Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. Appl Environ Microbiol 68(9):4301–4306
14. Rusch DB, Halpern AL, Sutton G, Heidelberg KB, Williamson S, Yooseph S , Wu D, Eisen JA, Hoffman JM, Remington K, Beeson K, Tran B, Smith H, Baden-Tillson H, Stewart C, Thorpe J, Freeman J, Andrews-Pfannkoch C, Venter JE, Li K, Kravitz S, Heidelberg JF, Utterback T, Rogers YH, Falcón LI, Souza V, Bonilla-Rosso G, Eguiarte LE, Karl DM, Sathyendranath S, Platt T, Bermingham E, Gallardo V, Tamayo-Castillo G, Ferrari MR, Strausberg RL, Nealson K, Friedman R, Frazier M, Venter JC (2007) The sorcerer II global sampling expedition: Northwest Atlantic through eastern tropical pacific. PLoS Biol 5(3):e77

15. Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH, Smith HO (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304(5667):66–74
16. Yooseph S, Sutton G, Rusch DB, Halpern AL, Williamson SJ, Remington K, Eisen JA, Heidelberg KB, Manning G, Li W, Jaroszewski L, Cieplak P, Miller CS, Li H, Mashiyama ST, Joachimiak MP, van Belle C, Chandonia JM, Soergel DA, Zhai Y, Natarajan K, Lee S, Raphael BJ, Bafna V, Friedman R, Brenner SE, Godzik A, Eisenberg D, Dixon JE, Taylor SS, Strausberg RL, Frazier M, Venter JC (2007) The Sorcerer II global ocean sampling expedition: expanding the universe of protein families. PLoS Biol 5(3):e16
17. Wood V, Gwilliam R, Rajandream MA, Lyne M, Lyne R, Stewart A, Sgouros J, Peat N, Hayles J, Baker S, Basham D, Bowman S, Brooks K, Brown D, Brown S, Chillingworth T, Churcher C, Collins M, Connor R, Cronin A, Davis P, Feltwell T, Fraser A, Gentles S, Goble A, Hamlin N, Harris D, Hidalgo J, Hodgson G, Holroyd S, Hornsby T, Howarth S, Huckle EJ, Hunt S, Jagels K, James K, Jones L, Jones M, Leather S, McDonald S, McLean J, Mooney P, Moule S, Mungall K, Murphy L, Niblett D, Odell C, Oliver K, O'Neil S, Pearson D, Quail MA, Rabbinowitsch E, Rutherford K, Rutter S, Saunders D, Seeger K, Sharp S, Skelton J, Simmonds M, Squares R, Squares S, Stevens K, Taylor K, Taylor RG, Tivey A, Walsh S, Warren T, Whitehead S, Woodward J, Volckaert G, Aert R, Robben J, Grymonprez B, Weltjens I, Vanstreels E, Rieger M, Schäfer M, Müller-Auer S, Gabel C, Fuchs M, Düsterhöft A, Fritzc C, Holzer E, Moestl D, Hilbert H, Borzym K, Langer I, Beck A, Lehrach H, Reinhardt R, Pohl TM, Eger P, Zimmermann W, Wedler H, Wambutt R, Purnelle B, Goffeau A, Cadieu E, Dréano S, Gloux S, Lelaure V, Mottier S, Galibert F, Aves SJ, Xiang Z, Hunt C, Moore K, Hurst SM, Lucas M, Rochet M, Gaillardin C, Tallada VA, Garzon A, Thode G, Daga RR, Cruzado L, Jimenez J, Sánchez M, del Rey F, Benito J, Domínguez A, Revuelta JL, Moreno S, Armstrong J, Forsburg SL, Cerutti L, Lowe T, McCombie WR, Paulsen I, Potashkin J, Shpakovski GV, Ussery D, Barrell BG, Nurse P (2002) The genome sequence of *Schizosaccharomyces pombe*. Nature 415(6874):871–880
18. Aury JM, Jaillon O, Duret L, Noel B, Jubin C, Porcel BM, Ségurens B, Daubin V, Anthouard V, Aiach N, Arnaiz O, Billaut A, Beisson J, Blanc I, Bouhouche K, Câmara F, Duharcourt S, Guigo R, Gogendeau D, Katinka M, Keller AM, Kissmehl R, Klotz C, Koll F, Le Mouël A, Lepère G, Malinsky S, Nowacki M, Nowak JK, Plattner H, Poulain J, Ruiz F, Serrano V, Zagulski M, Dessen P, Bétermier M, Weissenbach J, Scarpelli C, Schächter V, Sperling L, Meyer E, Cohen J, Wincker P (2006) Global trends of whole-genome duplications revealed by the ciliate *Paramecium tetraurelia*. Nature 444(7116):171–178
19. Alduina R, Gallo G (2012) Artificial chromosomes to explore and to exploit biosynthetic capabilities of actinomycetes. J Biomed Biotechnol. doi:10.1155/2012/462049
20. Collins J, Hohn B (1978) Cosmids: a type of plasmid gene-cloning vector that is packagable in vitro in bacteriophage lambda heads. Proc Natl Acad Sci U S A 75(9):4242–4246
21. Ioannou PA, Amemiya CT, Garnes J, Kroisel PM, Shizuya H, Chen C, Batzer MA, de Jong PJ (1994) A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. Nat Genet 6(1):84–89
22. Kim UJ, Shizuya H, de Jong PJ, Birren B, Simon MI (1992) Stable propagation of cosmid sized human DNA inserts in an F factor based vector. Nucleic Acids Res 20(5):1083–1085
23. Shizuya H, Birren B, Kim UJ, Mancino V, Slepak T, Tachiiri Y, Simon M (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc Natl Acad Sci 89(18):8794–8797
24. Schofield MM, David HS (2013) Meta-omic characterization of prokaryotic gene clusters for natural product biosynthesis. Curr Opin Biotechnol 24(6):1151–1158
25. Craig JW, Chang FY, Kim JH, Obiajulu SC, Brady SF (2010) Expanding small-molecule functional metagenomics through parallel screening of broad-host-range cosmid environmental DNA libraries in diverse proteobacteria. Appl Environ Microbiol 76:1633–1641
26. Fujita MJ, Kimura N, Sakai A, Ichikawa Y, Hanyu T, Otsuka M (2011) Cloning and heterologous expression of the vibrioferrin biosynthetic gene cluster from a marine metagenomic library. Biosci Biotechnol Biochem 75:2283–2287

27. McMahon MD, Guan C, Handelsman J, Thomas MG (2012) Metagenomic analysis of *Streptomyces lividans* reveals host-dependent functional expression. Appl Environ Microbiol 78(10):3622–3629
28. Chai Y, Shan S, Weissman KJ, Hu S, Zhang Y, Muller R (2012) Heterologous expression and genetic engineering of the tubulysin biosynthetic gene cluster using Red/ET recombineering and inactivation mutagenesis. Chem Biol 19(3):361–371
29. Feng Z, Kallifidas D, Brady SF (2011) Functional analysis of environmental DNA-derived type II polyketide synthases reveals structurally diverse secondary metabolites. Proc Natl Acad Sci U S A 108(31):12629–12634
30. Freeman MF, Gurgui C, Helf MJ, Morinaka BI, Uria AR, Oldham NJ, Sahl HG, Matsunaga S, Piel J (2012) Metagenome mining reveals polytheonamides as posttranslationally modified ribosomal peptides. Science 338(6105):387–390
31. Hess M, Sczyrba A, Egan R, Kim TW, Chokhawala H, Schroth G, Luo S, Clark DS, Chen F, Zhang T, Mackie RI, Pennacchio LA, Tringe SG, Visel A, Woyke T, Wang Z, Rubin EM (2011) Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science 331(6016):463–467
32. Allgaier M, Reddy A, Park JI, Ivanova N, D'haeseleer P, Lowry S, Sapra R, Hazen TC, Simmons BA, Vander Gheynst JS, Hugenholtz P (2010) Targeted discovery of glycoside hydrolases from a switchgrass-adapted compost community. PLoS ONE 5(1):e8812
33. Donia MS, Fricke WF, Partensky F, Cox J, Elshahawi SI, White JR, Phillippy AM, Schatz MC, Piel J, Haygood MG, Ravel J, Schmidt EW (2011a) Complex microbiome underlying secondary and primary metabolism in the tunicate-*Prochloron* symbiosis. Proc Natl Acad Sci U S A 108(51):E1423–E1432
34. Donia MS, Fricke WF, Ravel J, Schmidt EW (2011b) Variation in tropical reef symbiont metagenomes defined by secondary metabolism. PLoS ONE 6(3):e17897
35. Kwan JC, Donia MS, Han AW, Hirose E, Haygood MG, Schmidt EW (2012) Genome streamlining and chemical defense in a coral reef symbiosis. Proc Natl Acad Sci U S A 109(50):20655–20660
36. Trindade-Silva AE, Rua C, Silva GG, Dutilh BE, Moreira AP, Edwards RA, Hajdu E, Lobo-Hajdu G, Vasconcelos AT, Berlinck RG, Thompson FL (2012) Taxonomic and functional microbial signatures of the endemic marine sponge *Arenosclera brasiliensis*. PLoS ONE 7(7):e39905
37. Rath CM, Janto B, Earl J, Ahmed A, Hu FZ, Hiller L, Dahlgren M, Kreft R, Yu F, Wolff JJ, Kweon HK, Christiansen MA, Håkansson K, Williams RM, Ehrlich GD, Sherman DH (2011) Meta-omic characterization of the marine invertebrate microbial consortium that produces the chemotherapeutic natural product ET-743. ACS Chem Biol 6(11):1244–1256
38. Poretsky RS, Bano N, Buchan A, LeCleir G, Kleikemper J, Pickering M, Pate WM, Moran MA, Hollibaugh JT (2005) Analysis of microbial gene transcripts in environmental samples. Appl Environ Microbiol 71(7):4121–4126
39. Frias-Lopez J, Shi Y, Tyson GW, Coleman ML, Schuster SC, Chisholm SW, Delong EF (2008) Microbial community gene expression in ocean surface waters. Proc Natl Acad Sci U S A 105(10):3805–3810
40. Urich T, Lanzén A, Qi J, Huson DH, Schleper C, Schuster SC (2008) Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. PLoS ONE 3(6):e2527
41. Hiltner L (1904) Ueber neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brache. Arb Deut Landw Gesell 98:59–78
42. Lynch J (1982) The rhizosphere. In: Burns RG, Slater JH (eds) Experimental microbial ecology. Blackwell Scientific Publications, Oxford, pp 395–411
43. Singh BK, MillardP, Whiteley AS, Murrell JC (2004) Unravelling rhizosphere-microbial interactions: opportunities and limitations. Trends Microbiol 12(8):386–393
44. Lynch JM, Whipps M (1990) Substrate flow in the rhizosphere. Plant Soil 129:1–10

45. Kent AD, Triplett EW (2002) Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu Rev Microbiol 56:211–236
46. Morello JE, Pierson EA, Pierson LS III (2004) Negative cross-communication among wheat rhizosphere bacteria: effect on antibiotic production by the biological control bacterium *Pseudomonas aureofaciens* 30–84. Appl Environ Microbiol 70(5):3103–3109
47. Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Bot 52:487–511
48. He J, Wijeratne EM, Bashyal BP, Zhan J, Seliga CJ, Liu MX, Pierson EE, Pierson LS, Van Etten HD, Gunatilaka AA (2004) Cytotoxic and other metabolites of *Aspergillus* inhabiting the rhizosphere of Sonoran desert plants. J Nat Prod 67(12):1985–1991
49. Turbyville TJ, Wijeratne EMK, Whitesell L, Gunatilaka AA (2005) The anticancer activity of the fungal metabolite terrecyclic acid A is associated with modulation of multiple cellular stress response pathways. Mol Cancer Ther 4(10):1569–1576
50. Wijeratne EMK, Turbyville TJ, Zhang Z, Bigelow D, Pierson LS III, Whitesell L, Canfield LM, Gunatilaka AA (2003) Cytotoxic constituents of *Aspergillus terreus* from the rhizosphere of *Opuntia versicolor* of the Sonoran Desert. J Nat Prod 66(12):1567–1573
51. Bacon CW, White JF (2000.) Microbial endophytes. Marcel Deker Inc., New York
52. Kusari S, Spiteller M (2012) Metabolomics of endophytic fungi producing associated plant secondary metabolites: progress, challenges and opportunities. In: Roessner U (ed) Metabolomics. InTech, Rijeka, pp 241–66
53. Aly AH, Debbab A, Kjer J, Proksch P (2010) Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers 41(1):1–16
54. Kharwar RN, Mishra A, Gond SK, Stierle A, Stierle D (2011) Anticancer compounds derived from fungal endophytes: their importance and future challenges. Nat Prod Rep 28(7):1208–1228
55. Staniek A, Woerdenbag HJ, Kayser O (2008) Endophytes: exploiting biodiversity for the improvement of natural product-based drug discovery. J Plant Interact 3:75–93
56. Strobel GA, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic microorganisms. J Nat Prod 67(2):257–268
57. Stierle A, Strobel GA, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260(5105):214–216
58. Kusari S, Lamshoft M, Spiteller M (2009a) *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. J Appl Microbiol 107(3):1019–1030
59. Eyberger AL, Dondapati R, Porter JR (2006) Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. J Nat Prod 69(8):1121–1124
60. Puri SC, Nazir A, Chawla R, Arora R, Riyaz-Ul-Hasan S, Amna T, Ahmed B, Verma V, Singh S, Sagar R, Sharma A, Kumar R, Sharma RK, Qazi GN (2006) The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. J Biotechnol 122(4):494–510
61. Kusari S, Lamshoft M, Zuhlke S, Spiteller M (2008) An endophytic fungus from *Hypericum perforatum* that produces hypericin. J Nat Prod 71(2):159–162
62. Kusari S, Zuhlke S, Kosuth J, Cellarova E, Spiteller M (2009b) Light-independent metabolomics of endophytic *Thielavia subthermophila* provides insight into microbial hypericin biosynthesis. J Nat Prod 72(10):1825–1835
63. Kusari S, Verma VC, Lamshoft M, Spiteller M (2012) An endophytic fungus from *Azadirachta indica* A. Juss. that produces azadirachtin. World J Microbiol Biotechnol 28(3):1287–1294
64. Kusari S, Zuhlke S, Spiteller M (2009c) An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues. J Nat Prod 72(1):2–7
65. Kusari S, Zuhlke S, Spiteller M (2011) Effect of artificial reconstitution of the interaction between the plant *Camptotheca acuminata* and the fungal endophyte *Fusarium solani* on camptothecin biosynthesis. J Nat Prod 74(4):764–775
66. Puri SC, Verma V, Amna T, Qazi GN, Spiteller M (2005) An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. J Nat Prod 68(12):1717–1719

67. Shweta S, Zuehlke S, Ramesha BT, Priti V, Mohana Kumar P, Ravikanth G, Spiteller M, Vasudeva R, Uma Shaanker R (2010) Endophytic fungal strains of *Fusarium solani*, from *Apodytes dimidiate* E. Mey. ex Arn (Icacinaceae) produce camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin. Phytochemistry 71(1):117–122
68. Schroeckh V, Scherlach K, Nutzmann HW, Shelest E, Schmidt-Heck W, Schuemann J, Martin K, Hertweck C, Brakhage AA (2009) Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. Proc Natl Acad Sci U S A 106(34):14558–14563
69. Boersma FGH, Otten R, Warmink JA, Nazir R, van Elsas JD (2010) Selection of *Variovorax paradoxus*-like bacteria in the mycosphere and the role of fungal-released compounds. Soil Biol Biochem 42(12):2137–2145
70. Nazir R, Warmink JA, Voordes DC, van de Bovenkamp HH, van Elsas JD (2013) Inhibition of mushroom formation and induction of glycerol release—ecological strategies of *Burkholderia terrae* BS001 to create a hospitable niche at the fungus *Lyophyllum* sp. Strain Karsten. Microb Ecol 65(1):245–254
71. Partida-Martinez LP, Hertweck C (2005) Pathogenic fungus harbours endosymbiotic bacteria for toxin production. Nature 437(7060):884–888
72. Scherlach K, Busch B, Lackner G, Paszkowski U, Hertweck C (2012) Symbiotic cooperation in the biosynthesis of a phytotoxin. Angew Chem Int Ed Engl 51(38):9615–9618
73. He H, Bigelis R, Yang HY, Chang LP, Singh MP (2005) Lichenicolins A and B, new bisnaphthopyrones from an unidentified lichenicolous fungus, strain LL-RB0668. J Antibiot 58(11):731–736
74. Oh DC, Jensen PR, Kauffman CA, Fenical W (2005) Libertellenones A–D: induction of cytotoxic diterpenoid biosynthesis by marine microbial competition. Bioorg Med Chem 13(17):5267–5273
75. Oh DC, Kauffman CA, Jensen PR, Fenical W (2007) Induced production of emericellamides A and B from the marine-derived fungus *Emericella* sp. in competing co-culture. J Nat Prod 70(4):515–520
76. Zuck KM, Shipley S, Newman DJ (2011) Induced production of N-formyl alkaloids from *Aspergillus fumigatus* by co-culture with *Streptomyces peucetius*. J Nat Prod 74(7):1653–1657

Chapter 6
Metabolomics and Secondary Metabolite Profiling of Filamentous Fungi

Bernhard Kluger, Sylvia Lehner and Rainer Schuhmacher

Introduction

All fungi are heterotrophic organisms, and thus they depend on organic carbon. According to their nutritional needs, fungi can be found in intimate association with major natural carbon sources, i.e., plants, animals, and microbes. Therefore, it is not surprising that this huge group of eukaryotic organisms with an estimated number of 1.5 million species does occupy a myriad of ecological niches. Based on their life and nutritional styles, fungi can roughly be divided into saprotrophs, living on dead organic material (carbon recycling in the natural environment); biotrophs, which use nutrients from the living host-(cells); and necrotrophs, which first kill and then feed on dead host tissue [1].

The various ecological niches populated by filamentous fungi and different lifestyles are reflected by their capability to produce a great number of so-called secondary metabolites. The differentiation between basic and secondary metabolism had originally been suggested by Albrecht Koessel in 1891 [2]. Although a matter of debate since then, the classification in primary (nowadays also called central) and secondary metabolism/metabolites is still used today. According to the current concept, primary metabolism refers to the basic anabolic and catabolic processes required for respiration, nutrient assimilation, and growth, thus primary metabolites mainly comprise sugars, amino acids, fatty acids, and nucleosides, which form the

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building blocks of polysaccharides, proteins, lipids, and nucleic acids. Primary metabolites are needed for the basic functioning of life and hence are produced by every cell of a particular organism. Secondary metabolites are defined as all metabolites other than primary. Thus, fungal secondary metabolites are not essential for the regular growth and function in pure culture, for example, but their formation by the fungus is often restricted to specific parts of the life cycle, a specific physiological/developmental state or certain environmental conditions [3, 4]. Moreover, fungal secondary metabolites generally occur as families of related compounds that are only produced by a limited number of species or even strains [3, 5, 6]. Since fungal secondary metabolites show highly diverse and frequently complicated chemical structures (mainly polyketides, terpenoids, and nonribosomal peptides as well as mixed structures thereof [3]), their analytical determination, isolation, and detailed structure elucidation have remained a great technical challenge.

For a long time, the screening for secondary metabolites has almost exclusively been motivated by the search for novel bioactive ingredients of drugs, while the study of their biological function has largely been neglected [5]. Consequently, the role of most of these compounds remains largely unclear although it is generally acknowledged that fungi employ these secondary metabolites for intra- and inter-species communication and various interactions with their competitors and hosts.

Mainly driven by significant technical developments in analytical instrumentation and computing power as well as novel biological insights, a change in paradigm from reductionist to holistic approaches for the study of filamentous fungi can be observed currently. This development is reflected by the emergence of metabolomics as the latest of the so called -omics disciplines.

Metabolomics of Fungi—An Overview

The General Concept of Metabolomics

Similar to all biological low-molecular-weight molecules, fungal metabolites can be regarded as intermediates and end products of physiological regulatory processes. Their presence can be viewed as the ultimate response of a cell to genetic and environmental variations [7]. In analogy to genomics and other "-omics" disciplines, the total complement of all metabolites present in an organism like, for example, a filamentous fungus, is called the metabolome. The corresponding scientific discipline trying to determine the entirety of all low-molecular-weight metabolites is called metabolomics [8]. Thus, by definition, novel "holistic approaches" such as metabolomics aim at the comprehension of whole biological systems—looking at the biochemical changes taking place in living cells during metabolism. The general goal of most metabolomics studies is to generate a snapshot of the metabolic state of a biological sample and to characterize the changes in the abundances of the measured metabolites arising from natural fluctuations or external, experimental biotic or abiotic perturbations [9]. Due to this generic analytical concept, many different biological systems including filamentous fungi have been studied in various

metabolomics experiments with the aim to investigate various types of scientific questions such as the use of metabolite profiles for chemotaxonomy, biomarker and drug discovery, food safety, or host–fungus interactions (see later in this chapter).

Independent of the scientific question to be studied, most state-of-the-art metabolomics studies share a common workflow (see Fig. 6.1). Typically, a metabolomics study starts with a clear definition of the research question and design of the

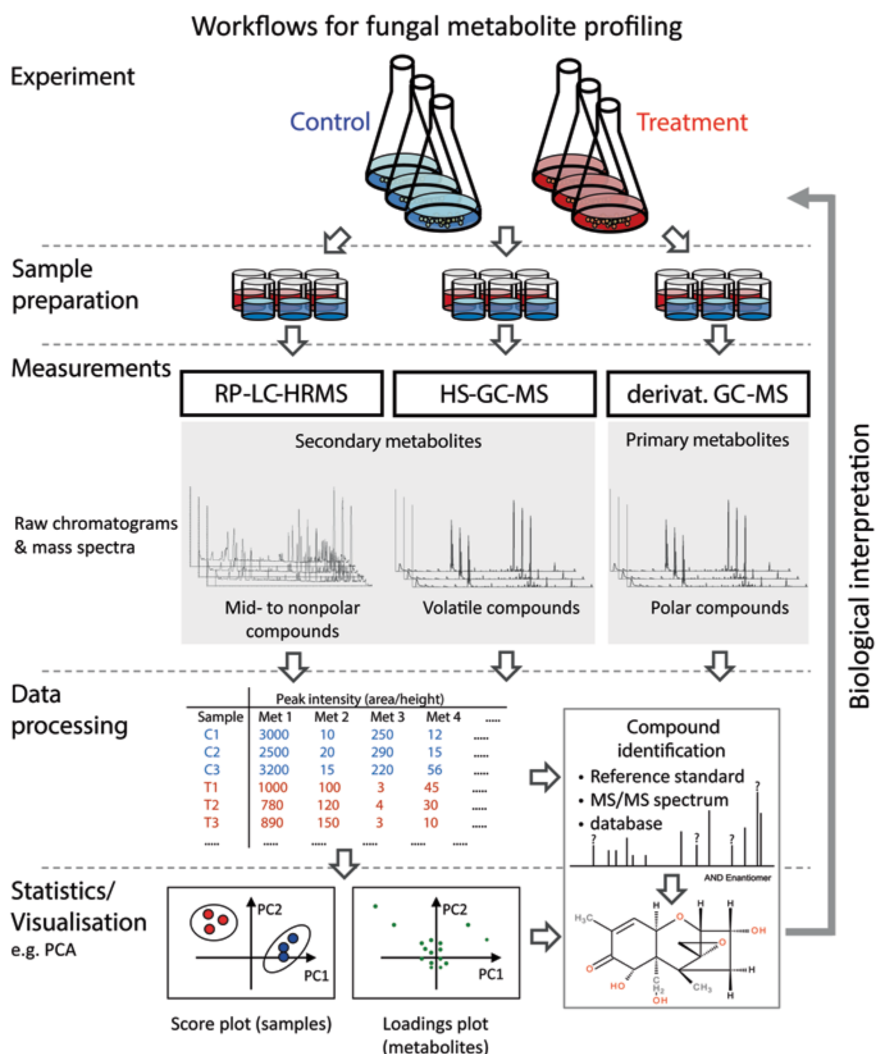

Fig. 6.1 Schematic overview of workflows used for metabolite profiling of filamentous fungi. For secondary metabolites, either reversed phase-liquid chromatography-high-resolution mass spectrometry (RP-LC-HRMS) for mid and nonpolar compounds or headspace-gas chromatography-mass spectrometry (HS-GC-MS) for volatile compounds are employed. Primary metabolites are analyzed after derivatization by GC-MS instrumentation

biological experiment. Separate experimental sample groups representing different experimental conditions (e.g., "control versus treatment" or "wild type versus mutant") are cultured in parallel and, after defined time periods, samples are taken according to the experimental scheme and stored until further analysis. While the sampling step requires immediate quenching of all metabolic processes without alteration of the metabolic state, the storage conditions shall also conserve metabolite levels without changing the biochemical composition of the biological samples. Subsequently, the samples have to be extracted and prepared for comprehensive analysis, typically by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) enabling the detection of a great number of metabolites simultaneously [10, 11].

Sample measurements with modern analytical instrumentation result in huge amounts of raw data that cannot be evaluated manually anymore. Instead, stepwise treatment of raw data with different methods and automated software tools is required and often comprises the bottleneck of metabolomics workflows. Data handling can be roughly divided in data processing and statistical analysis and has been summarized in several reviews (e.g., [12–14]). Data-processing steps include numerous tasks such as noise and background filtering, feature extraction, spectrum deconvolution (i.e., grouping of peaks that originate from the same metabolite), alignment of chromatograms, and internal standardization. Data processing finally results in the so-called data matrix, a simple-structured table that contains all samples and the abundances of the detected analytical features and is used for further data analysis. Once the data matrix has been prepared, data can be plotted and uni- and multivariate statistics can be carried out with the aim to further reduce data complexity and visualize metabolites, significantly differing between sample groups. The ultimate goal of every metabolomics study is to link the differentially expressed metabolites to the experimental factor, which had been varied to generate the different sample states. Thus, reliable annotation/identification of the detected metabolites is essential for a meaningful biological interpretation of the analytical results.

Analytical Approaches in Metabolomics

The metabolome of any fungus under investigation is highly complex and consists of a multitude of different primary and secondary metabolites. Due to the diverse chemical structures of the metabolites and their occurrence at a wide dynamic range from pico- to millimolar levels, global metabolome analysis cannot be achieved by a single analytical platform. Traditional analytical methods with a low number of predefined target analytes, for which authentic reference standards are available and thus allow absolute quantification, are not feasible in the field of metabolomics. Instead complementary system-wide approaches are applied with the aim to cover as many biochemical compounds as possible (see Fig. 6.1). They usually allow for comparative quantification of (partly) identified metabolites in different biological samples. The different analytical approaches used in metabolomics have been summarized and categorized in numerous well-received general (e.g., [15–17]) as well as microbe-focused review articles (e.g., [18–22]).

Metabolite profiling is one of the strategies applied in metabolomics and can be defined empirically as the (semi)quantitative analysis of a set of metabolites or derivative products (identified or unknown) of a sample, using a particular two-dimensional analytical technique. Most current state-of-the-art metabolomics studies use liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS) for metabolite profiling. LC-MS and GC-MS combine high sensitivity and detector linearity over 3–4 orders of magnitude with excellent selectivity (physical metabolite separation by chromatography and separation of co-eluting analytes according to *m/z* ratio of intact ionized molecule- or metabolite-specific fragment ions) and thus allow to determine hundreds of metabolites in a single analytical run. Other techniques such as liquid chromatography-ultraviolet (LC-UV) are used complementary for metabolite profiling (e.g., [23, 24]).

In addition, direct infusion MS, nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy are applied for *metabolite fingerprinting* (sometimes also called as metabolic fingerprinting) without prior metabolite separation. Fingerprinting techniques are mainly used for rapid sample classification without identification of individual metabolites. Chromatography-mass spectrometry based profiling methods result in a three-dimensional data structure (retention time, *m/z* value, and intensity). In contrast, fingerprinting approaches such as direct infusion MS lack the chromatographic separation step and therefore lead to two-dimensional data (*m/z* value and intensity) per sample (e.g., [15, 18]).

It should be noted that the term metabolite fingerprinting has also been used to describe the use of analytical methodologies (e.g., metabolite profiling) for the measurement of intracellular metabolites of a biological system in order to distinguish from the analysis of extracellular metabolites, for which the term metabolic footprinting had been introduced [18, 25, 26]. For the sake of convenience in this chapter, the terms metabolite profiling and metabolite fingerprinting will be used according to Fiehn [15], Goodacre et al. [16], and Dettmer [17].

Independent of the employed analytical technique, two complementary approaches are widely used for metabolite profiling: targeted and untargeted analyses. In the targeted approach, a set of predefined known substances is monitored, which usually allows absolute quantification and definite identification when limited to metabolites available as authentic reference standards. In contrast, untargeted profiling methods try to find analytical features of all detectable compounds and therefore show the potential of probing the entire metabolic space, including substances that are currently unknown (or at least unidentified) at the time of measurement. Thus, untargeted approaches are suitable to detect changes in unexpected parts of the metabolome and frequently lead to new scientific hypotheses.

Annotation and Identification of Metabolites

As already mentioned, meaningful biological conclusions can only been drawn from an (untargeted) metabolomics experiment if the detected and significantly differing analytical features can be reliably assigned to biological molecules. To annotate a compound's chemical structure, NMR is typically the technique of choice. However,

NMR is less sensitive compared to MS. Thus, for detailed chemical structure determination of non-predicted substances in biological samples by NMR techniques, the compounds of interest frequently need to be concentrated and isolated before the actual NMR measurements can be performed. Moreover, when analyzing complex mixtures, especially signals of less-abundant compounds can easily overlap with other signals producing complex spectra that are difficult to interpret [27]. Complementary to this, with LC-HRMS—the most frequently used technique for untargeted metabolomics experiments—compound annotation usually starts with the prediction of molecular formulas by matching accurately measured masses against chemical substance databases. In this respect, well-known databases are ChEBI (http://www.ebi.ac.uk/chebi/) [28], PubChem (http://www.ncbi.nlm.nih.gov/pccompound) [29], or AntiBase 2012 [30], with the latter currently containing more than 40,000 microbial metabolites with 15,220 entries assigned to the "Source [F] = fungus." While knowledge of the molecular formula can lead to putative compound annotation, the elucidation of chemical structures is further complicated by the fact that many structural isomers correspond to a single molecular formula. Consequently, according to the current state of the art, definitive substance identification by LC-HRMS can only be achieved by comparing two or more orthogonal properties such as retention time, accurate mass, or LC-MS/MS spectra with those obtained from an authentic reference standard under identical measurement conditions [31, 32]. The fact that many putatively identified compounds are not available as authentic standards poses a major limitation of current untargeted LC-HRMS-based metabolomics approaches. Another issue is lack of comprehensive LC-MS/MS spectrum databases.

Compared to LC-MS/MS, putative metabolite identification by GC-MS is generally more straightforward. Efficient software tools for chromatographic feature detection, deconvolution (i.e., "purification") of mass spectra, and calculation of retention indices are available and can freely be downloaded from the Internet (e.g., AMDIS, http://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis; Metabolite Detector, http://md.tu-bs.de/ [33, 34]). Compared to liquid chromatography tandem mass spectrometry (LC-MS/MS) , compound and structure annotation is largely facilitated by the use of standardized ionization conditions in the electron ionization (EI) source of the MS instrument (typically, 70 eV is used across different instruments and laboratories), which leads to reproducible and information-rich (i.e., fragment ion) mass spectra for a certain compound. Comprehensive MS libraries—such as, for example, the current Wiley Registry 10th Edition/NIST 2012 EI Mass Spectral Library [35] with 870,000 mass spectra—are available for similarity-based spectrum matching. Annotation of the detected compounds is usually achieved by comparison of experimental retention indices and mass spectra with those of library entries. Similar to LC-MS/MS approaches, definitive confirmation of the identity relies on the measurement of authentic standards in parallel [36–38].

This chapter will focus on the two techniques most frequently used in metabolomics experiments dealing with filamentous fungi: (1) GC-MS, which allows the study of both volatiles and -after sample derivatization- polar small metabolites; and (2) LC-MS, which is generally applied in reversed phase mode for the study of mid- to non-polar secondary metabolites.

Analysis of the Fungal Exo- and Endometabolome

Quenching and Sample Preparation for Exo- and Endometabolome Analysis of Nonvolatile Metabolites

The metabolite complement of a filamentous fungus consists of two components: the entirety of intracellular and extracellular metabolites, which have been named endo- and exo-metabolome respectively [19, 39]. Most of the endometabolome consists of primary metabolites that reflect the internal status and "regular" metabolic functioning of the fungus under investigation. Due to fast metabolite turnover rates of intracellular metabolites—in *Escherichia coli*, for example, isocitrate has been estimated to turn over 2.7 times per second [21]—instantaneous quenching of metabolic processes is crucial when the primary metabolome is studied. Moreover, metabolite leakage from quenched cells/fungal mycelium into the medium is of great concern and consequently has been addressed by many researchers. These studies generally revealed that fungi are less prone to leak intracellular metabolites into the medium compared to prokaryotic microbes. Among various quenching methods, the cold (−40 °C) methanol/water (60/40, v/v) method, liquid nitrogen treatment, or rapid filtration and immediate subsequent cooling with liquid nitrogen proved to be best suited for the rapid arrest for metabolism in filamentous fungi. For a detailed discussion of this topic, the reader may refer to review articles of Mashego et al. [21] and Xu et al. [22].

In contrast to the endometabolome, the exometabolome largely represents the secondary metabolites present under certain conditions at the timepoint of investigation. These metabolites can be regarded as being secreted by the organism to interact with its environment. Thus, compared to primary metabolites, secondary metabolites are more promising targets for investigating genus or species-specific traits as well as the interaction between different organisms. They may act as signaling molecules, attracting or repelling other organisms in fungus–fungus or fungus–insect interactions, induce metabolic responses in host organisms, inhibit or kill competitors such as bacteria and other fungi (antibiotics) or mediate susceptibility in host plants (small molecule effectors, typically toxins) [19]. Moreover, nutrition-related compounds such as organic acids and metal chelators (e.g., siderophores) may be produced and secreted into the extra-hyphal space to solubilize, bind, and assimilate inorganic nutrients (e.g., iron in the case of siderophores) . The secreted secondary metabolites do frequently reflect metabolic end points and are therefore less critical with respect to quenching of metabolism than primary metabolites. However, some secondary metabolites are chemically modified by a variety of enzymes (e.g., oxidases, methyltransferases, deacetylases) associated with cell walls or cellular organelles. It also has been argued that rapid quenching, which is necessary to measure intracellular metabolites, can be avoided since the metabolite turnover time is decreased by the dilution of secreted compounds into the larger extracellular culture volume [18].

For being able to distinguish between intracellular and extracellular metabolites, the mycelium and culture supernatant have to be separated prior to sample analysis. This can be achieved by centrifugation or filtration at low temperatures and subsequent washing of the pellet. With solid growth media, fungi can be cultured on cellophane membrane, for example, which enables the separation of the mycelium from the medium (e.g., [40]) before further analysis. Alternatively, it has been described that the mycelium can be carefully scraped from the culture plate [41]. The washed mycelium and/or solid medium can then be extracted, e.g., by (mixtures of) organic solvents, perchloric acid, or potassium hydroxide (reviewed in [21, 22, 25]). Liquid culture filtrates/centrifugates can be further concentrated or directly subjected to the measurement of secondary metabolites. Alternatively, if no separation of extracellular and intracellular metabolites is desired, the whole fungal culture can be extracted directly after quenching.

The Use of GC-MS and LC-MS for the Analysis of Fungal Culture Samples

With GC-MS and LC-MS/MS-based metabolite profiling techniques, which are most commonly used to study fungal exo- and endo-metabolomes, the simultaneous measurement of a group of related metabolites can be realized by single analytical methods. Typically, the simultaneously detected metabolites share certain physical/chemical properties or belong to related metabolic pathways. Using GC-MS under standard conditions (i.e., column temperature between ca. 30 and ≤350 °C) without chemical derivatization, for example, analytes with boiling points between approximately 40 and 450°C can be transported through the GC column by the mobile phase. Thus, metabolites that share the property of being volatile to semi-volatile, according to the World Health Organization (WHO) definition [42], can be determined by GC-MS in a single analytical run. Filamentous fungi have been described to produce a great variety of volatile metabolites belonging to saturated hydrocarbons, alcohols, aldehydes, ketones, lactones, linear esters, ethers, phenols, and terpenoids. Thus, a substantial part of their respective metabolomes can be covered by GC-MS analysis of the volatile organic compounds (VOCs) in liquid culture extracts or by directly probing the headspace above fungal samples (Figs. 6.1 and 6.2 [43]). For a detailed overview of VOC analysis in biological samples, the reader is asked to refer to published review articles [44–47].

Besides VOCs, also polar, non-volatile metabolite can be determined by GC-MS after chemical derivatization. In the area of metabolomics, this approach is still regarded as the gold standard for the comparative quantification of primary metabolites [48]. Derivatization is required to make a certain metabolite less polar, thus more volatile in order to enable proper separation on a GC column. Silylation and alkylation are the two major derivatization procedures that have been described for GC-MS-based profiling of primary metabolites, such as sugars, alcohols, amino acids, non-amino organic acids, and biogenic amines (Fig. 6.2) [43, 49]. A two-step derivatization protocol employing methoximation (to stabilize

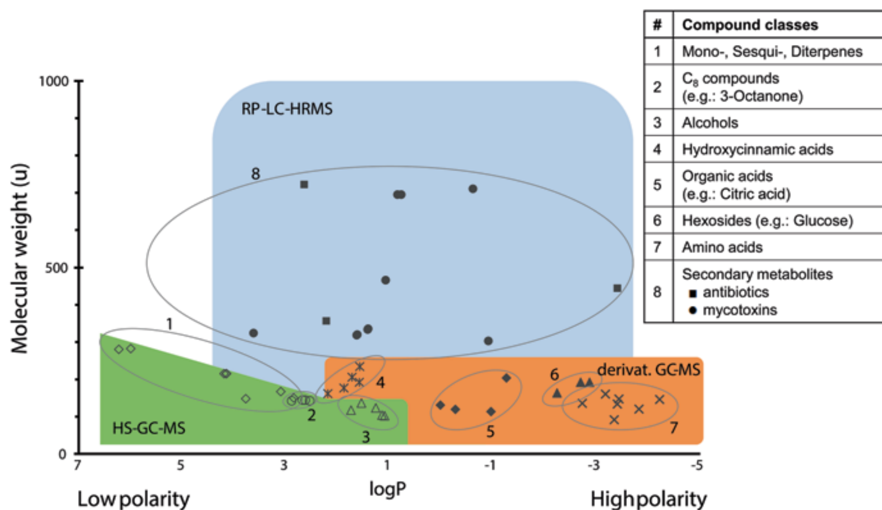

Fig. 6.2 Coverage of compound classes by commonly used GC-MS and LC-(HR)MS approaches in metabolomics experiments (based on Halket et al. 2005, modified [43]). Molecular weight of selected fungal metabolites is plotted against the predicted logP value (obtained from http://www.chemicalize.org/; accessed July 2014)

aldehyde and ketogroups) followed by silylation (most frequently using N-methyl-N-(trimethylsilyl) trifluoroacetamide [MSTFA]) has been used in microbial metabolomics [50, 51]. Smart et al. presented an alternative protocol that makes use of methyl chloroformate as a derivatization reagent [29]. The methyl chloroformate alkylation reaction converts primary and secondary amino groups into carbamate derivatives, whereas carboxylate groups are derivatized to form methyl esters, both of which can be analyzed by GC-MS. Although being less universal (as sugars, sugar alcohols, or amino sugars cannot be derivatized), the authors claimed that methyl chloroformate derivatization yields more stable derivatives and is less prone to matrix effects compared to silylation [49]. For the sake of completeness, it shall be noted that complementary to the GC-MS-based procedures, profiling of primary metabolites has also been achieved successfully by hydrophilic interaction chromatography (HILIC) MS [52, 53].

With respect to non-volatile fungal secondary metabolites, however, reversed phase (RP) LC-MS can certainly be regarded as the profiling technique of choice, which will be further discussed in this chapter. This can elegantly be exemplified with recently developed multi-mycotoxin methods, which involve RP stationary phases coupled to either electrospray ionization (ESI), triple quadrupole tandem mass spectrometers (TQMS), or ESI high resolution (HR)MS instrumentation. With the former technique, more than 300 mycotoxins and other fungal secondary metabolites can be measured (semi)quantitatively with a single analytical method [54]. The target analytes cover a broad range of chemical structures, typically produced across a number of different fungal genera and species. Complementary to such a targeted, low-resolution tandem MS approach, LC-ESI-HRMS has successfully

been used for untargeted profiling of fungal metabolites in the full scan mode. LC-ESI-HRMS-based methods are less suited for accurate quantification, however, they show the great advantage of allowing to inspect the presence of fungal metabolites by retrospective data analysis [23, 55, 56]. LC-ESI-HRMS-based untargeted metabolite profiling currently enables the most comprehensive, unbiased coverage of the secondary metabolome. Data processing and metabolite annotation are still considerably hampered by the fact, however, that the majority of the generated data points actually originate from noise and background ions, which should not be attributed to the metabolites contained in the samples under investigation. As a consequence, with the commonly used state-of-the-art LC-ESI-HRMS-based workflows, it is not possible to reliably describe the metabolic composition of individual biological samples, but with untargeted measurements data interpretation is rather restricted to analytical features that are significantly differing between control and treated samples. Novel stable isotope labeling (SIL)-assisted workflows have recently been developed (e.g., [11, 38, 57, 58]), which are excellently suited to circumvent major limitations associated to current LC-ESI-HRMS approaches (see next section).

Applications of Metabolomics Tools to Study Filamentous Fungi

The concept of metabolomics is clearly generic. Metabolomics is not only applicable to any type of biological system but can also be used for the study of various types of (biological) research questions. This young discipline has considerably matured over the last decade and has gained great popularity. In view of the steadily increasing use of metabolomics and metabolite profiling, a comprehensive description of its applications to filamentous fungi is far beyond the scope of this chapter. Instead, a few selected areas of fungal metabolomics and metabolite profiling shall be briefly presented.

Secondary Metabolite Profiling for Chemotaxonomical Classification The concept of metabolite profiling had already been well established and used successfully for medical and diagnostic purposes, long before the term metabolome was first introduced by Oliver et al. in 1998 [26, 59]. Already in 1983, Frisvad and colleagues reported about the "classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites" by thin layer chromatography (TLC) [60]. After 25-years, in 2008, the same author broadly defined the term chemotaxonomy as the use of chemical diversity for taxonomic classification of biological organisms [61].

Nowadays, chemotaxonomical classification approaches are used complementary to DNA-based techniques and are mainly based on primary and more frequently secondary metabolite profiles generated by GC-MS and LC-MS. However, it has to be stated that a limitation of metabolome-based taxonomy is that the expression of genes relevant for taxonomic purposes may not occur because of a variety of environment-dependent regulatory switches (regulatory genes) that are highly variable.

Basically, all fungi under investigation have to be evaluated in parallel in a standardized manner for the ease of comparability. Therefore, the respective fungal strains are cultivated under similar environmental conditions such as temperature, light cycle, and humidity on the same nutrition medium for a certain time period [61] before they are harvested and analyzed. Whereas initially, classification was based on the separation of mycotoxins TLC and HPLC followed by UV/visible (VIS) detection [60, 62], today, MS-based techniques are the methods of choice. These techniques enable a more comprehensive untargeted profiling of the complex metabolite mixtures produced by filamentous fungi. As mentioned, for each sample a metabolite profile is established using either GC-MS [63, 64] or LC-HRMS [65–67]. Both approaches in combination with chemometric analysis allow the distinct separation of fungal cultures into taxonomic classes. In order to evaluate the chemotaxonomic classification, molecular approaches based on the sequence analysis of ribosomal DNA internal transcribed spacers (ITS) for molecular phylogeny have been employed [63, 64, 67]. In the study of Kang and coworkers, which may serve as an example here, a total of 33 strains belonging to seven species of the saprophytic fungi *Trichoderma* were classified using an LC-HRMS and a GC-MS approach in parallel [63]. Metabolite-based chemotaxonomy was established based on principal component analysis (PCA) of LC-HRMS data and revealed most varying metabolite profiles in 16 days old cultures. However, comparison of GC-MS derived data with ITS-based classification showed less correlation compared to LC-HRMS-based metabolite profiles. In the study of Kang and co-workers, the LC-HRMS-derived secondary metabolite profiles seemed to be more reliable for chemotaxonomical classification than the GC-MS-based endometabolome profiles [63]. Despite the undisputable dominance of DNA-based methods for taxonomical classification, chemotaxonomical methods based on secondary metabolites still constitute a valuable complementary tool for the classification of filamentous fungi today.

Use of Metabolite Profiling for the Screening and Production of Fungal Natural Products

Ever since the “discovery” of the penicillin antibiotics, the diversity and bioactive potential of fungal natural products have inspired the pharmaceutical industry to search for lead structures of active drug ingredients [5]. It is obvious that modern MS-based profiling and metabolomics workflows, which are principally suited to screen for hundreds to thousands of natural products simultaneously, can be of great benefit for comprehensive exploration of the pool of putatively beneficial secondary metabolites. All approaches for the discovery of novel bioactive substances have in common that they rely on the existence of an observable phenotype or chemical property, such as biological activity, color, or a known mass, which can be tracked through successive rounds of detection and isolation [68]. Besides drug discovery, for the identification of fungal secondary metabolites, there is also the demand for the screening of active compounds for biological control agents or the discovery of currently unknown fungal metabolites putatively playing key roles in host–fungus interaction.

Many of these fungal compounds produced by fungi belong to one of the three major structural classes of nonribosomal peptides (NRPS), polyketides (PKS), and terpenoids [69–71]. The genes for biosynthesis of secondary metabolites are mostly organized in clusters, and interestingly for many species, the number of these gene clusters exceeds the number of known secondary metabolites produced by the respective fungal strains under laboratory conditions [72, 73]. It has been hypothesized that secondary metabolite formation has evolved for interaction with antagonists and fungal hosts and that therefore some of the metabolites are not produced under standard (axenic) culture conditions, which do not reflect the organism's native habitat.

Since the screening of natural products is often performed under laboratory conditions, the biosynthetic potential of filamentous fungi to produce interesting secondary metabolites has not been fully exploited yet. Various efforts are made to activate the production of these potentially valuable and bioactive metabolites and hence facilitate natural product discovery [73]. The nutrient composition of the cultivation medium can be varied to alter the secondary metabolite profile [74, 75]. In general, the production of secondary metabolite has been described to be higher if the fungi are grown on solid surface substrates. Also the cultivation on mineral-clay pellets coated with a semisolid agar substrate has been found to enhance the chemical diversity of the producing fungi [19]. Another methodology becoming increasingly popular for the activation of silent gene clusters is epigenetic remodeling. To this end, fungi have been treated with DNA methyltransferase and histone deacetylase inhibitors or other small molecule effectors (e.g., 5-azacytidine) with the aim to activate otherwise non-expressed gene clusters encoding secondary metabolite production [76]. Recent studies led to the discovery of a novel, not yet identified metabolite [77] or at least give a hint to the derepression of genes that are involved in the biosynthesis of secondary metabolites [78]. Epigenetic remodeling can be regarded as a promising tool to access biosynthetic pathways for novel, so far unknown secondary metabolites of fungi in the future.

Excessive exploration of the large diversity of fungal strains for the production of natural products, bear the risk of "rediscovering" already known secondary metabolites. Thus, a major task in natural products discovery is the development of efficient dereplication strategies for the identification of already known secondary metabolites during culture screening to avoid subsequent cost-intensive and time-consuming isolation and identification procedures [23, 79–81]. To this end, metabolic profiling using LC-HRMS, often in combination with UV/VIS detection, has evolved as a key technique in screening approaches. Thus, rapid and efficient comprehensive initial analysis of most of the secondary metabolites produced under certain laboratory conditions is of great help as has been recently reviewed by Breitling et al. [82]. As already discussed, current dereplication strategies emphasize a combination of different levels of identification based on authentic reference standards, tentatively identified compounds by LC-MS/MS spectra, UV/VIS spectra, and species-specific metabolite subsets from selected compound databases (e.g., Antibase 2012 [30]). Fungi of interest are cultivated under well-defined conditions and extracts are subsequently measured using LC-HRMS. The accurately measured

m/z value as well as LC-MS/MS and UV/VIS spectra of analytical features of interest are subsequently searched against available databases.

There are, however, still major challenges to be addressed, such as wrongly annotated metabolite ions due to the lack of suitable qualifier and/or fragment ions from literature data. The results described in current research studies demonstrate that still major efforts have to be undertaken to strengthen the reliability of metabolite profiling and metabolomics results, especially when it comes to structure elucidation of new compounds in natural product discovery.

Use of Metabolomics for the Study of Biological Interactions of Fungi

Although the number of metabolomics studies of filamentous fungi is steadily increasing, applications of this technology to investigate host–fungus interactions are still sparse. The intimate association between the fungus under investigation and its interaction partner (e.g., an antagonistic microbe or a plant) coupled with a general commonality of metabolites complicates the separation of the respective metabolomes [83, 84]. Whereas in fungus–fungus [85, 86] or fungus–bacteria interactions both partners can be assumed to contribute significantly to the overall biomass of the investigated samples, this is usually not the case in fungus–plant interactions. In both mutualistic as well as pathogenic plant–microbe interactions, there are several ways to minimize/circumvent the problem of properly dissecting the metabolomes:

1. The systemic response in the host plant can be studied by choosing a sampling site, distant from the physical interaction zone [87, 88].
2. The effect of a pure fungal protein- or fungal small molecule effector can be used to challenge the metabolism of the plant (or vice versa) [89, 90].
3. Depending on the study design, in many fungus–plant interactions it can be assumed that the biomass of the filamentous fungus is so low that its contribution to the sampled plant metabolome can be neglected (e.g., [91]). However, this should be verified by the measurement of fungus-specific metabolites such as mycotoxins or cholesterol [83].
4. An approach employing a plant cell—microbe coculture can be chosen as presented by Allwood and colleagues where plant cells and bacterial pathogen cells are separated by differential filtration after a defined time period of cocultivation [92]. The independent analysis of plant and bacterial cells allowed the metabolite changes within each interacting partner to be assessed [92]. This approach might be adapted for the study of fungus–plant interactions as well.

In recent years, several interesting plant metabolomics studies have provided novel fundamental insight into both mutualistic as well as host–fungus interactions. It has been shown that the combined application of different "-omics" disciplines such as proteomics and metabolomics (e.g., [89]) or metabolomics and transcriptomics (e.g., [91]) provides a more holistic and complementary view on the biochemical response of the organisms under investigation than either of the approaches alone.

Thereby, the combination of the complementary "-omic data" can greatly facilitate a meaningful biological interpretation of the generated study. Voll et al. used a combined transcriptomics and metabolomics approach to investigate the response of primary metabolism of barley leaves upon inoculation with three different biotrophic fungal pathosystems (*Blumeria graminis*, *Ustilago maydis*, and the hemibiotrophic pathogen *Colletrichum graminicola*) with the aim to study if common metabolic response motifs can be revealed for the three pathosystems [91]. Most interestingly, the authors found that common motifs in the response of cereal primary carbon and nitrogen metabolism to the different fungal pathogens were not based on similar transcriptional reprogramming [91]. In the study of Vincent et al. which investigated the effect of the effector protein SnToxA of the necrotrophic pathogen *Stagonospora nodorum* on wheat, the authors reported detailed conclusions on the molecular mechanisms by which necrotrophic pathogens appear to induce oxidative stress and cell death by disruption of photosynthesis and subsequent energy depletion in the effected host cells [89]. Vincent and coworkers emphasized the complementary nature of proteomics and metabolomics, which enabled them to draw general conclusions on how necrotrophs do most probably exploit host cell death mechanisms to promote its own growth and cause disease [89].

Elucidation of Low-Molecular-Weight Gene Products by Metabolite Profiling

Based on recent gene annotations, the number of nonribosomal peptide synthetase (NRPS), polyketide synthase (PKS), and terpene synthase (TPS) gene clusters encoding the enzymes for biosynthesis of secondary metabolites was estimated for different fungal species such as for *Fusarium graminearum* PH1 (NRPS: 20; PKS: 15; TPS: 17) [93] or *Trichoderma virens* (NRPS: 28; PKS: 18; TPS:4) [94]. To understand the function of specific gene clusters and their integration into metabolic pathways, commonly deletion mutants are generated by gene disruption. To this end, a gene cluster of interest is selected for deletion and the resulting phenotype is compared to the corresponding intact wild-type strain when cultivated under similar conditions.

Metabolic profiling approaches have emerged as powerful tools to study the effect of gene deletion on secondary metabolite formation and to draw conclusions on the functionality of the deleted gene cluster. This shall be exemplified with the following few studies. Metabolites associated with genes putatively encoding specific biosynthetic enzymes such the terpene cyclase *vir4* in *T. virens* [95] or VeA, a general regulator of the secondary metabolism in *Aspergillus fumigatus* [96], were successfully studied by GC-MS profiling of volatile compounds in the headspace of living fungal cultures. The authors of the latter study demonstrated that volatiles generated by a VeA disruption mutant are part of the complex regulatory machinery that mediates the effects of VeA on asexual conidiation and sclerotia formation [96]. In a further study, PKS gene products of *Aspergillus flavus* were successfully identified [97]. Comparative metabolomics, using ultra high performance liquid

chromatography (UHPLC) coupled to high resolution Orbitrap MS was used to detect metabolites differentially expressed in the *A. flavus* wild type and Δ(Delta) pks27 mutant strains [96, 97]. Four metabolites were identified that were only present in the wild-type cultures. These included asparasone A (358 Da), an anthraquinone pigment, and two related anthraquinones. The mentioned studies elegantly demonstrate that comparative metabolomics can provide novel insight to functional annotation of fungal secondary metabolite gene clusters either by identification of distinct (novel) metabolites, the production of which is encoded by the genes under investigation or complex metabolic shifts resulting from deletion of other secondary metabolism-associated genes.

Recent Developments for Improved Fungal Metabolomics

Despite considerable progress of metabolomics and its many successful applications to complex biological systems, there are still major challenges. In untargeted LC-HRMS-based approaches, the reliable annotation of truly sample-derived metabolites, their putative identification, accurate comparative metabolite quantification, and proper workflow validation can be considered substantial technical problems.

Stable isotope labeling-assisted techniques make use of the labeling of specific isotopic patterns obtained from the measurement of mixtures of labeled and native biological samples or metabolites. They show great potential to provide improved tools for untargeted GC-MS and LC-MS metabolomics [98, 99]. Heavy stable isotopes of carbon (^{13}C), nitrogen (^{15}N), or sulphur (^{34}S) can be used to enrich tracer metabolites or whole biological samples for metabolomics experiments. It should be noted that hydrogen (^{2}H) and oxygen (^{18}O) can also be used but are less suitable since they can be readily exchanged between individual metabolites or with non-labeled solvent molecules.

Most recent SIL-assisted applications involve the *in vivo* ^{13}C labeling of filamentous fungi for the untargeted metabolome annotation by LC-HRMS [11, 100]. For this purpose, fungi were grown in parallel on substrates containing either a native or labeled carbon source. Bueschl et al. cultured *F. graminearum* on U-^{13}C glucose [98], while Cano et al. used U-^{13}C (/^{15}N)-labeled wheat grains as culture substrate for *Aspergillus fumigatus* [100]. All fungal metabolites produced on these media had incorporated the ^{13}C (/^{15}N) label and thus when mixed with native samples were easily recognized by the corresponding characteristic isotopic patterns in the data. Signals originating from contaminants, solvent clusters, or artifacts were efficiently eliminated. While Bueschl et al. detected around 90 true metabolites originating from *Fusarium* [98], Cano et al. were able to find 21 secondary *Aspergillus* metabolites in the tested samples [100]. Moreover, the methodology offers several advantages over conventional approaches, such as the reduction of sum formula ambiguities by providing the exact number for atoms of the element used for labeling per metabolite ion or the correction of matrix effects by full metabolome internal standardization.

In a recent study, a mixture of the native and U-^{13}C-labeled *Fusarium* mycotoxin deoxynivalenol has been used as a tracer for the untargeted profiling and automated evaluation of its metabolization in flowering wheat ears [101]. The authors were able to find a total of nine wheat-derived toxin derivatives, among them are several novel glutathione-related deoxynivalenol conjugates.

Moreover, fungal metabolomics would benefit from improved temporal and spatial resolution of metabolite analysis, since fungal secondary metabolism is known to be both highly dynamic with respect to cultivation duration as well as age/developmental stage of individual hyphae. Compartmentation by hyphal septa further complicates data interpretation and meaningful biological conclusions.

The latest developments of ambient ionization as well as matrix-assisted laser desorption ionization (MALDI) MS now enable temporally and spatially resolved imaging of metabolite production by living fungal cultures [85, 102–104]. To mention just two of these studies, Moree and colleagues applied MALDI imaging MS directly to agar cultures with the aim to elucidate the bacterial metabolites produced by an antifungal *B. amyloliquefaciens* strain in a side-by-side interaction with an *A. fumigatus* and an *Aspergillus niger* strain [104]. The authors were able to demonstrate that the antifungal activity was mediated by lipopeptides of the iturin family. With such an assay, spatial distribution and relative MS detector intensities can be used to evaluate whether metabolites are secreted by the organisms into the media, confined to the colony, induced, or consumed by neighboring organisms [104].

Another interesting study was conducted by Hu and coworkers employing native and U-^{13}C-labeled glucose as a substrate to study the gradual incorporation and translocation of ^{13}C carbon in fungal hyphae [103]. To this end, the fungus *Neurospora crassa* was grown on glass slides and mass spectra were subsequently recorded after sample preparation using MALDI coupled to MS. Although only highly abundant intracellular metabolites could be mapped by this approach, lateral resolution was high enough to monitor cytoplasmic relocation of ^{13}C isotoplogs in the fungal hyphae.

Conclusion

It can be stated that the latest ambient MS imaging tools will enable a major step forward toward spatially and temporally resolved fungal metabolomics and will provide fascinating new insight into the metabolism of living fungal cultures. Furthermore, novel SIL-assisted approaches and data processing tools show great potential for improved untargeted metabolomics studies of filamentous fungi in the future.

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References

1. Carlile JM, Watkinson SC, Gooday GW (2001) The fungi. 2nd edn. Elsevier, London. ISBN-13: 978-0-12-738446-7
2. Kliebenstein DJ (2004) Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. Plant Cell Environ 27(6):675–684
3. Keller NP, Turner G, Bennett JW (2005) Fungal secondary metabolism—from biochemistry to genomics. Nat Rev Micro 3(12):937–947
4. Calvo AM, Wilson RA, Bok JW, Keller NP (2002) Relationship between secondary metabolism and fungal development. Microbiol Mol Biol Rev 66(3):447–459
5. Karlovsky P (2012) Secondary Metabolites in Soil Ecology. In: Karlovsky P (ed) Soil Biology, vol. 14. Springer, Berlin Heidelberg, pp 1–19
6. Martín JF, Gutiérrez S, Aparicio JF (2000) Secondary metabolites. In: Lederberg J (ed) En: encyclopedia of microbiology, vol. 4, 2nd ed. Academic Press, San Diego, pp. 213–236
7. Fiehn O (2002) Metabolomics—the link between genotypes and phenotypes. Plant Mol Biol 48(1):155–171
8. Goodacre R (2005) Metabolomics—the way forward. Metabolomics 1(1):1–2
9. Schuhmacher R, Krska R, Weckwerth W, Goodacre R (2013) Metabolomics and metabolite profiling. Anal Bioanal Chem 405(15):5003–5004
10. Patti GJ, Yanes O, Siuzdak G (2012) Innovation: metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol 13(4):263–269
11. Bueschl C, Kluger B, Lemmens M, Adam G, Wiesenberger G, Maschietto V, Marocco A, Strauss J, Bödi S, Thallinger GG, Krska R, Schuhmacher R (2014) A novel stable isotope labelling assisted workflow for improved untargeted LC-HRMS based metabolomics research. Metabolomics 10(4):754–769
12. Boccard J, Veuthey J-L, Rudaz S (2010) Knowledge discovery in metabolomics: an overview of MS data handling. J Sep Sci 33(3):290–304
13. Katajamaa M, Orešič M (2007) Data processing for mass spectrometry-based metabolomics. J Chromatogr A 1158(1–2):318–328
14. Sugimoto M, Kawakami M, Robert M, Soga T, Tomita M (2012) Bioinformatics tools for mass spectroscopy-based metabolomic data processing and analysis. Curr Bioinform 7(1):96–108
15. Fiehn O (2001) Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. Comp Funct Genomics 2(3):155–168
16. Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB (2005) Metabolomics by numbers: acquiring and understanding global metabolite data. Trends Biotechnol 22(5):245–252
17. Dettmer K, Aronov PA, Hammock BD (2007) Mass spectrometry-based metabolomics. Mass Spectrom Rev 26(1):51–78
18. Kell DB, Brown M, Davey HM, Dunn WB, Spasic I, Oliver SG (2005) Metabolic footprinting and systems biology: the medium is the message. Nat Rev Micro 3(7):557–565
19. Thrane U, Anderson B, Frisvad J, Smedsgaard J (2007) The exo-metabolome in filamentous fungi. In: Nielsen J, Jewett M (eds) Metabolomics. Springer, Berlin, pp 235–52
20. Werf MJvd, Overkamp KM, Muilwijk B, Coulier L, Hankemeier T (2007) Microbial metabolomics: toward a platform with full metabolome coverage. Anal Biochem 370(1):17–25
21. Mashego M, Rumbold K, De Mey M, Vandamme E, Soetaert W, Heijnen J (2007) Microbial metabolomics: past, present and future methodologies. Biotechnol Lett 29(1):1–16
22. Xu Y-J, Wang C, Ho WE, Ong CN (2014) Recent developments and applications of metabolomics in microbiological investigations. TrAC Trends Anal Chem 56(0):37–48
23. Klitgaard A, Iversen A, Andersen M, Larsen T, Frisvad J, Nielsen K (2014) Aggressive dereplication using UHPLC–DAD–QTOF: screening extracts for up to 3000 fungal secondary metabolites. Anal Bioanal Chem 406(7):1933–1943

24. Wehrens R, Carvalho E, Masuero D, de Juan A, Martens S (2013) High-throughput carotenoid profiling using multivariate curve resolution. Anal Bioanal Chem 405(15):5075–5086
25. Villas-Bôas SG, Mas S, Åkesson M, Smedsgaard J, Nielsen J (2005) Mass spectrometry in metabolome analysis. Mass Spectrom Rev 24(5):613–646
26. Villas-Bôas SG, Rasmussen S, Lane GA (2005) Metabolomics or metabolite profiles? Trends Biotechnol 23(8):385–386
27. Pan Z, Raftery D (2007) Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Anal Bioanal Chem 387(2):525–527
28. Degtyarenko K, Hastings J, de Matos P, Ennis M (2009) ChEBI: an open bioinformatics and cheminformatics resource. Current protocols in bioinformatics: John Wiley & Sons, Inc. Supplement 26, unit 14.9
29. Bolton E, Wang Y, Thiessen PA, Bryant SH (2008) PubChem: integrated platform of small molecules and biological activities. Chapter 12 IN Annual Reports in Computational Chemistry, vol 4. Elsevier, Oxford, pp 217–240
30. Laatsch H (2012) AntiBase 2012: The Natural Compound Identifier. Wiley-VCH Verlag GmbH & Co. KGaA, ISBN: 978-3527334063
31. Sumner L, Amberg A, Barrett D, Beale M, Beger R, Daykin C et al (2007) Proposed minimum reporting standards for chemical analysis. Metabolomics 3(3):211–221
32. Dunn WB, Erban A, Weber RJM, Creek DJ, Brown M, Breitling R et al (2013) Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics. Metabolomics 9(1):44–66
33. Stein SE (1999) An integrated method for spectrum extraction and compound identification from gas chromatography/mass spectrometry data. J Am Soc Mass Spectrom 10(8):770–781
34. Hiller K, Hangebrauk J, Jäger C, Spura J, Schreiber K, Schomburg D (2009) MetaboliteDetector: comprehensive analysis tool for targeted and nontargeted GC/MS based metabolome analysis. Anal Chem 81(9):3429–3439
35. Wiley Registry 10th Edition/NIST 2012 Mass Spectral Library. 2013. Wiley New York, ISBN: 978-1-118-61611-6
36. Jeleń HH (2003) Use of solid phase microextraction (SPME) for profiling fungal volatile metabolites. Lett Appl Microbiol 36(5):263–267
37. Stoppacher N, Kluger B, Zeilinger S, Krska R, Schuhmacher R (2010) Identification and profiling of volatile metabolites of the biocontrol fungus *Trichoderma atroviride* by HS-SPME-GC-MS. J Microbiol Methods 81(2):187–193
38. Kluger B, Zeilinger S, Wiesenberger G, Schoefbeck D, Schuhmacher R (2013) Detection and identification of fungal volatile organic carbons. In: Gupta VK, Tuohy MG, Ayyachamy M, Turner KM, O'Donovan A (eds). Laboratory protocols in fungal biology. Springer, New York, pp 455–465
39. Frisvad JC, Larsen TO, de Vries R, Meijer M, Houbraken J, Cabañes FJ et al (2007) Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins. Stud Mycol 59(0):31–37
40. Reithner B, Schuhmacher R, Stoppacher N, Pucher M, Brunner K, Zeilinger S (2007) Signaling via the *Trichoderma atroviride* mitogen-activated protein kinase Tmk1 differentially affects mycoparasitism and plant protection. Fungal Genet Biol 44(11):1123–1133
41. Gummer JA, Krill C, Du Fall L, Waters OC, Trengove R, Oliver R, et al (2012) Metabolomics protocols for filamentous fungi. In: Bolton MD, Thomma BPHJ (eds). Plant fungal pathogens—Methods in molecular biology, vol. 835. Humana Press, New York, pp 237–254
42. World Health Organisation Regional Office for Europe Copenhagen (1989) Indoor air quality: organic pollutants. Report on a WHO meeting Berlin (West) 23–27 August 1987
43. Halket JM, Waterman D, Przyborowska AM, Patel RKP, Fraser PD, Bramley PM (2005) Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. J Exp Bot 56(410):219–243
44. Tholl D, Boland W, Hansel A, Loreto F, Roese USR, Schnitzler J-P (2006) Practical approaches to plant volatile analysis. Plant J 45:540–560
45. Rubiolo P, Sgorbini B, Liberto E, Cordero C, Bicchi C (2010) Analysis of the plant volatile fraction. In: Herrmann A (ed) The chemistry and biology of volatiles. Wiley, Chichester, pp 49–93

46. Rowan DD (2001) Volatile metabolites. Metabolites 1(1):41–63
47. Zeilinger S, Schuhmacher R (2013) Volatile organic metabolites of *Trichoderma spp.*: biosynthesis, biology and analytics. In: Mukherjee PK, Horwitz BA, Shankar Singh U, Mukherjee M, Schmoll M (eds). *Trichoderma*—biology and Applications. CAB International, Wallingford, pp 110–127
48. Roessner U, Dias DA (2013) Plant tissue extraction for metabolomics. Methods Mol Biol 1055:21–28
49. Smart KF, Aggio RBM, Van Houtte JR, Villas-Boas SG (2010) Analytical platform for metabolome analysis of microbial cells using methyl chloroformate derivatization followed by gas chromatography-mass spectrometry. Nat Protocols 5(10):1709–1729
50. Koek MM, Muilwijk B, van der Werf MJ, Hankemeier T (2006) Microbial metabolomics with gas chromatography/mass spectrometry. Anal Chem 78(4):1272–1281
51. Madla S, Miura D, Wariishi H (2012) Optimization of extraction method for GC-MS based metabolomics for filamentous fungi. J Microbial Biochem Technol 4:005–009
52. Wu Z, Huang Z, Lehmann R, Zhao C, Xu G (2009) The application of chromatography-mass spectrometry: methods to metabonomics. Chroma 69(1):23–32
53. Klavins K, Drexler H, Hann S, Koellensperger G (2014) Quantitative metabolite profiling utilizing parallel column analysis for simultaneous reversed-phase and hydrophilic interaction liquid chromatography separations combined with tandem mass spectrometry. Anal Chem 86(9):4145–4150
54. Abia WA, Simo GN, Warth B, Sulyok M, Krska R, Tchana A, Moundipa PF (2013) Determination of multiple mycotoxins levels in poultry feeds from Cameroon. Jpn J Vet Res 61:S33–39
55. Lehner SM, Neumann NKN, Sulyok M, Lemmens M, Krska R, Schuhmacher R (2011) Evaluation of LC-high-resolution FT-Orbitrap MS for the quantification of selected mycotoxins and the simultaneous screening of fungal metabolites in food. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 28(10):1457–1468
56. Ates E, Godula M, Stroka J, Senyuva H (2014) Screening of plant and fungal metabolites in wheat, maize and animal feed using automated on-line clean-up coupled to high resolution mass spectrometry. Food Chem 142(0):276–284
57. Chokkathukalam A, Jankevics A, Creek DJ, Achcar F, Barrett MP, Breitling R (2013) mzMatch–ISO: an R tool for the annotation and relative quantification of isotope-labelled mass spectrometry data. Bioinformatics 29(2):281–283
58. Huang X, Chen Y Jr, Cho K, Nikolskiy I, Crawford PA, Patti GJ (2014) X13CMS: global tracking of isotopic labels in untargeted metabolomics. Anal Chem 86(3):1632–1639
59. Oliver SG, Winson MK, Kell DB, Baganz F Systematic functional analysis of the yeast genome. Trends Biotechnol 16(9):373–378
60. Frisvad JC, Filtenborg O (1983) Classification of *terverticillate penicillia* based on profiles of mycotoxins and other secondary metabolites. Appl Environ Microbiol 46(6):1301–1310
61. Frisvad JC, Andersen B, Thrane U (2008) The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycol Res 112(2):231–240
62. Scott PM, Lawrence JW, van Walbeek W (1970) Detection of mycotoxins by thin-layer chromatography: application to screening of fungal extracts. Appl microbiol 20(5):839–842
63. Kang D, Kim J, Choi JN, Liu KH, Lee CH (2011) Chemotaxonomy of *Trichoderma spp*. using mass spectrometry-based metabolite profiling. J Microbiol Biotechnol 21(1):5–13
64. Aliferis K, Cubeta M, Jabaji S (2013) Chemotaxonomy of fungi in the *Rhizoctonia solani* species complex performing GC/MS metabolite profiling. Metabolomics 9(1):159–169
65. Nielsen KF, Smedsgaard J (2003) Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography–UV–mass spectrometry methodology. J Chromatogr A 1002(1–2):111–136
66. Stadler M, Ju Y-M, Rogers JD (2004) Chemotaxonomy of *Entonaema, Rhopalostroma* and other *Xylariaceae*. Mycol Res 108(03):239–256
67. Abreu LM, Costa SS, Pfenning LH, Takahashi JA, Larsen TO, Andersen B (2012) Chemical and molecular characterization of Phomopsis and Cytospora-like endophytes from different host plants in Brazil. Fungal Biol 116(2):249–260

68. Deane C, Mitchell D (2014) Lessons learned from the transformation of natural product discovery to a genome-driven endeavor. J Ind Microbiol Biotechnol 41(2):315–331
69. Bode HB, Bethe B, Höfs R, Zeeck A (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. Chembiochem 3(7):619–627
70. Gross H (2007) Strategies to unravel the function of orphan biosynthesis pathways: recent examples and future prospects. Appl Microbiol Biotechnol 75(2):267–277
71. Lim FY, Sanchez JF, Wang CCC, Keller NP (2012) Toward awakening cryptic secondary metabolite gene clusters in filamentous fungi. In: David AH (ed). Methods in enzymology, vol 517. Elsevier, Amsterdam, pp. 303–324
72. Williams RB, Henrikson JC, Hoover AR, Lee AE, Cichewicz RH (2008) Epigenetic remodeling of the fungal secondary metabolome. Org Biomol Chem 6(11):1895–1897
73. Brakhage AA, Schroeckh V (2011) Fungal secondary metabolites—strategies to activate silent gene clusters. Fungal Genet Biol 48(1):15–22
74. Elias BC, Said S, de Albuquerque S, Pupo MT (2006) The influence of culture conditions on the biosynthesis of secondary metabolites by *Penicillium verrucosum* Dierck. Microbiol Res 161(3):273–280
75. Sørensen JL, Sondergaard TE (2014) The effects of different yeast extracts on secondary metabolite production in Fusarium. Int J Food Microbiol 170(0):55–60
76. Scherlach K, Hertweck C (2009) Triggering cryptic natural product biosynthesis in microorganisms. Org Biomol Chem 7(9):1753–1760
77. Wiemann P, Sieber CMK, von Bargen KW, Studt L, Niehaus E-M, Espino JJ et al (2013) Deciphering the cryptic genome: genome-wide analyses of the rice pathogen *Fusarium fujikuroi* reveal complex regulation of secondary metabolism and novel metabolites. PLoS Pathog 9(6):e1003475
78. Connolly LR, Smith KM, Freitag M (2013) The *Fusarium graminearum* Histone H3 K27 Methyltransferase KMT6 regulates development and expression of secondary metabolite gene clusters. PLoS Genet 9(10):e1003916
79. Lang G, Mayhudin NA, Mitova MI, Sun L, van der Sar S, Blunt JW et al (2008) Evolving trends in the dereplication of natural product extracts: new methodology for rapid, small-scale investigation of natural product extracts. J Nat Prod 71(9):1595–1599
80. Forner D, Berrué F, Correa H, Duncan K, Kerr RG (2013) Chemical dereplication of marine actinomycetes by liquid chromatography–high resolution mass spectrometry profiling and statistical analysis. Anal Chim Acta 805(0):70–79
81. Kildgaard S, Mansson M, Dosen I, Klitgaard A, Frisvad JC, Larsen TO et al (2014) Accurate dereplication of bioactive secondary metabolites from marine-derived fungi by UHPLC-DAD-QTOFMS and a MS/HRMS library. Mar Drugs 12(6):3681–3705
82. Breitling R, Ceniceros A, Jankevics A, Takano E (2013) Metabolomics for secondary metabolite research. Metabolites 3(4):1076–1083
83. Allwood JW, Heald J, Lloyd A, Goodacre R, Mur LJ (2012) Separating the Inseparable: The metabolomic analysis of plant–pathogen interactions. In: Hardy NW, Hall RD (eds). Plant metabolomics—methods in molecular biology, vol. 860. Humana Press, New York, pp. 31–49
84. Aliferis KA, Jabaji S (2012) Deciphering plant–pathogen interactions applying metabolomics: principles and applications. Can J Plant Pathol 34(1):29–33
85. Watrous J, Roach P, Alexandrov T, Heath BS, Yang JY, Kersten RD, et al (2012) Mass spectral molecular networking of living microbial colonies. PNAS 1743–1752
86. Jonkers W, Rodriguez Estrada AE, Lee K, Breakspear A, May G, Kistler HC (2012) Metabolome and Transcriptome of the Interaction between *Ustilago maydis* and *Fusarium verticillioides* in vitro. Appl Environ Microbiol 78(10):3656–3667
87. Balmer D, de Papajewski DV, Planchamp C, Glauser G, Mauch-Mani B (2013) Induced resistance in maize is based on organ-specific defence responses. Plant J 74(2):213–225
88. Brotman Y, Lisec J, Méret M, Chet I, Willmitzer L, Viterbo A (2012) Transcript and metabolite analysis of the *Trichoderma*-induced systemic resistance response to *Pseudomonas syringae* in Arabidopsis thaliana. Microbiology 158(1):139–146

89. Vincent D, Du Fall LA, Livk A, Mathesius U, Lipscombe RJ, Oliver RP et al (2012) A functional genomics approach to dissect the mode of action of the *Stagonospora nodorum* effector protein SnToxA in wheat. Mol Plant Pathol 13(5):467–482
90. Warth B, Parich A, Bueschl C, Schoefbeck D, Neumann NKN, Kluger B et al (2014) GC-MS based targeted metabolic profiling identifies changes in the wheat metabolome following deoxynivalenol treatment. Metabolomics (in press). doi. 10.1007/s11306-014-0731-1
91. Voll LM, Horst RJ, Voitsik AM, Zajic D, Samans B, Pons-Kühnemann J et al (2011) Common motifs in the response of cereal primary metabolism to fungal pathogens are not based on similar transcriptional reprogramming. Front Plant Sci 2:39
92. Allwood JW, Clarke A, Goodacre R, Mur LAJ (2010) Dual metabolomics: a novel approach to understanding plant–pathogen interactions. Phytochemistry 71(5–6):590–597
93. Cuomo CA, Güldener U, Xu J-R, Trail F, Turgeon BG, Di Pietro A et al (2007) The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. Science 317(5843):1400–1402
94. Kubicek C, Herrera-Estrella A, Seidl-Seiboth V, Martinez D, Druzhinina I, Thon M et al (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol 12(4):R40
95. Crutcher FK, Parich A, Schuhmacher R, Mukherjee PK, Zeilinger S, Kenerley CM (2013) A putative terpene cyclase, vir4, is responsible for the biosynthesis of volatile terpene compounds in the biocontrol fungus *Trichoderma virens*. Fungal Genet Biol 56(0):67–77
96. Roze L, Chanda A, Laivenieks M, Beaudry R, Artymovich K, Koptina A et al (2010) Volatile profiling reveals intracellular metabolic changes in *Aspergillus parasiticus*: veA regulates branched chain amino acid and ethanol metabolism. BMC Biochem 11(1):33
97. Roze L, Chanda A, Linz JE (Jan 2011) Compartmentalization and molecular traffic in secondary metabolism: a new understanding of established cellular processes. Fungal Genet Biol 48(1):35–48
98. Bueschl C, Krska R, Kluger B, Schuhmacher R (2013) Isotopic labeling-assisted metabolomics using LC–MS. Anal Bioanal Chem 405(1):27–33
99. Chokkathukalam A, Kim D-H, Barrett MP, Breitling R, Creek DJ (2014) Stable isotope-labeling studies in metabolomics: new insights into structure and dynamics of metabolic networks. Bioanalysis 6(4):511–524
100. Cano PM, Jamin EL, Tadrist S, Bourdaud'hui P, Péan M, Debrauwer L et al (2013) New untargeted metabolic profiling combining mass spectrometry and isotopic labeling: application on Aspergillus fumigatus grown on wheat. Anal Chem 85(17):8412–8420
101. Kluger B, Bueschl C, Lemmens M, Berthiller F, Häubl G, Jaunecker G et al (2012) Stable isotopic labelling-assisted untargeted metabolic profiling reveals novel conjugates of the mycotoxin deoxynivalenol in wheat. Anal Bioanal Chem 5031–5036
102. Hsu C-C, ElNaggar MS, Peng Y, Fang J, Sanchez LM, Mascuch SJ et al (2013) Real-time metabolomics on living microorganisms using ambient electrospray ionization flow-probe. Anal Chem 85(15):7014–7018
103. Hu J-B, Chen Y-C, Urban PL (2012) On-target labeling of intracellular metabolites combined with chemical mapping of individual hyphae revealing cytoplasmic relocation of isotopologues. Anal Chem 84(11):5110–5116
104. Moree W, Yang J, Zhao X, Liu W-T, Aparicio M, Atencio L et al (2013) Imaging mass spectrometry of a coral microbe interaction with fungi. J Chem Ecol 39(7):1045–1054

Chapter 7
Fungal Chemotaxonomy

Jens C. Frisvad

Introduction

Fungal chemotaxonomy can be based on qualitative or quantitative profiles of extrolites, outwards directed differentiation molecules, or quantitative profiles of introlites, all molecules used inside the cell for plain growth and cell metabolite economy [1]. Introlites, or in metabolic terms primary or general metabolites, primary or general proteins, and primary or general nucleotides, are in a constant state of flux, so chemical analytical quantitative snapshots of these molecules are not suited for chemotaxonomy. However, some of the internal metabolites are responses to ecophysiological factors from the external environment, and so they have a certain potential to be series- or species-specific [2]. Among these molecules, quantitative profiles of polyols or lipids, including fatty acids, have a certain potential, but they have not been used much in classification and identification of filamentous fungi [3–8] and large databases for carbohydrates or lipids have not been developed. MALDI-TOF (matrix-assisted desorption/ionization time-of-flight) analysis of primary or general proteins seems to be the most promising method for classification and identification of fungi, even though reproducibility and availability of sufficiently large databases are still a problem [9, 10].

Use of DNA sequences can be perceived as a chemotaxonomical method, but it is often simply called molecular classification (taxonomy), cladification (basis for phylogenetic hypotheses), or identification. This way of performing classifications, cladifications, and identifications is by far the most widespread and bar-coding techniques for identification have been based on it [11–13]. Despite the success of DNA sequencing in biosystematics, there are still some problems with these methods, especially misidentifications, unavailability of strains sequenced in DNA

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databases [14, 15], and some technical problems such as low DNA extraction yields, polymerase chain reaction (PCR) inhibitors, and the cost and time required for sequencing [10]. Furthermore, multigene or even genome sequences are often required for accurate species cladifications and identifications [16–18]. A polyphasic approach [19], using both phenotypic and genotypic characters [20, 21] would, of course, give the most accurate classification and cladification for a group of fungi, but it may be difficult to build standardized databases that can be used for identification based on such mixed characters.

The most widespread and useful chemotaxonomical methods have been based on chemical differentiation products (extrolites). Even though the exoglycome or exolipidome have shown some promise for chemotaxonomy [22, 23], these compounds have not been explored extensively in fungal taxonomy. All secondary or specific proteins, or the exoproteome, have been more useful in chemotaxonomy [24–26], and MALDI-TOF is one of the best methods to characterize the exoproteome [9, 27]. Exoproteins include hydrophobins, exoenzymes, bioactive proteins, and several other secreted proteins.

Secondary metabolites, on the other hand, have been used extensively in fungal taxonomy [28–45]. In principle, secondary metabolites can be used for classification, cladification, and identification, at least for isolates and species in several genera of filamentous fungi. However, in most cases secondary metabolites are used for classification, usually in conjunction with other methods in a polyphasic classification and anchored in a phylogenetic framework, as determined by multilocus gene sequences. In this review, only secondary metabolites will be treated with an emphasis on the genera *Penicillium* and *Aspergillus*.

Secondary Metabolites

In a metabolic sense, secondary metabolites have also been called specific metabolites, special metabolites [46], specialized metabolites [47], or exometabolites. Bennett and Bentley [46] argued for discontinuing calling all metabolites for primary versus secondary but rather use the terms general versus specific metabolites [48]. In a more functional sense, secondary metabolites have been called small molecules [49], natural molecules [50], bioactive molecules, natural products, idiolites [51], aristolites, and extrolites. Extrolites [52] should be defined as all outwards directed differentiation molecules that are fully secreted or deposited in the membrane or on the cell wall of organisms. Extrolites seem to play many roles in the interaction between species, as chemical signals in a broad sense. The term comes from “extro” = outwards directed and “ites” = a chemical compound. Since extrolites will include secreted carbohydrates, proteins, and lipids, aristolites could be used in a more restrictive sense for small molecules, if this needs to be specified. Aristo is based on “aristo” = the best and “ites” = chemical compounds. All microbial secondary metabolites have been called parvome [53] whereas they have also been called the secondary metabolome or exometabolome [54] in any one species or isolate. Some primary metabolites, such as citric acid, should still be regarded as such when they

are in a state of flux in the mitochondria in the citric acid cycle. However, it has been shown that when accumulated, secreted, and coded by a specific gene cluster, these small molecules act like secondary metabolites. The gene cluster found for kojic acid in *Aspergillus oryzae* [55] and the itaconic acid gene cluster in *A. terreus* [56, 57] show that these acids are important in an outwards directed functional way and are secreted first via the mitochondria to the cytosol and then outside the cell. For example, under neutral pH conditions *A. niger* will accumulate malate, which is transformed into oxaloacetate in the cytosol and then transformed into oxalic acid and secreted [58].

Secondary metabolites are extremely important for chemical communication between microorganisms and can therefore not be regarded as waste products. They are important molecules for ecology and evolution of living organisms [48, 50, 59–70]. Molecules that were first regarded as antibiotics, such as patulin and penicillic acid, have later been shown to have quorum sensing inhibitory activities as well [71], and, in general, smaller concentrations of secondary metabolites may have a strong sublethal activity [72] or together have the "Gulliver" effect; i.e., several small molecules can influence a target effectively [73]. There are examples of synergistic effect of secondary metabolites from two different biosynthetic pathways, for example kojic acid and aflatoxin B1 on caterpillars [74], showing the importance of all metabolites in the profile in any one isolate or species. Certain metabolites such as 1-octen-3-ol act as volatile self-inhibitors of conidium germination [75], and so may influence the later production of other secondary metabolites. In itself, 1-octen-3-ol may be one of the more common signals in fungi, but this volatile is a part of the total profile of secondary metabolites in many fungi. Many fungal secondary metabolites have several functions, some that the fungi make use of in nature, others can be the basis for development of new drugs. For example many original antifungal compounds have la.ter been shown to have anticancer effect [76].

For most known secondary metabolites, many derivatives are known. For example,*Penicillium crustosum* produces penitrem A-G [77, 78] in addition to the accumulation of precursors and possible shunt metabolites, such as thomitrems A and E, PC-M5′, PC-M6, paspaline, emindole SB, and secopenitrem D (Table 7.1) [3, 4, 20, 31, 34, 79–108]. This "molecular promiscuity" maybe an evolutionary advantage [109], but from a chemotaxonomic point of view the extra metabolites often give valuable information that can be used to classify and identify filamentous fungi. Most often one of the extrolites is produced in a much higher amount than the others. In the case of the penitrem biosynthetic family, penitrem A is always the major product [77, 80, 81]. *P. crustosum* also produces the diketopiperazine roquefortine C as the major end product, whereas *P. rubens* and *P. chrysogenum* transform roquefortine C into meleagrin, as the major end product [96]. Such biosynthetic differences are, of course, of taxonomic value, and appear to be consistent [20, 79, 110, 111].

Although yeasts have very few and uncomplicated secondary metabolites, such as ethanol, higher alcohols, small acids, esters, ketones, often derived directly from an amino acid [112], in addition to simple functional terpenes, lipids, and oxylipins [113–115], filamentous fungi can produce a mixture of complicated secondary

Table 7.1 Profile of secondary metabolites produced by *P. crustosum* [34, 79–81]. Data from Wu et al. [82] and da Silva et al. [83] were used as a basis to examine whether all isolates of *P. crustosum* also produced members of the clavatol biosynthetic family

Biosynthetic family	Secondary metabolite	Original (mis)identification, reference
Terrestric acid	*Cis* and *trans*-terrestric acid	*P. terrestre*, Birkinshaw and Raistrick 1936 [84]
	Viridicatic acid	*P. viridicatum*, Birkinshaw and Samant 1960 [85]
	"Lactone 4"	*P. griseoroseum*, da Silva et al. 2013 [83]
	"Lactone 5"	*P. griseoroseum*, da Silva et al. 2013 [83]
Clavatol	Clavatol	*P. griseoroseum*, da Silva et al. 2013 [83]
	Diclavatol	*P. griseoroseum*, da Silva et al. 2013 [83]
	2,4-dihydroxy-3-methoxymethyl-5-methylacetophenone, 2,4-dihydroxy-3-methylacetophenone, 2,4-dihydroxy-5-methylacetophenone	*P. commune*, Wu et al. 2012 [82]
	Communol A, C-G	*P. commune*, Wang et al. 2012 [86]
	1-O-(2,4-dihydroxy-6-methylbenzoyl)-glycerol	*P. commune*, Yan et al. 2012 [87]
Mix of terrestric acid and clavatol	Penilactone A and B	[a]Wu et al. 2012 [82]
Mix of clavatol and N-acetyltryptamin	Communol B	*P. commune*, Wang et al. 2012 [86]
Conidiogenones	Conidiogenone	*P. cyclopium*, Roncal et al. 2002 [88]
	Conidiogenol	*P. cyclopium*, Roncal et al. 2002 [88]
Andrastins	Andrastin A	[a]Sonjak et al. 2005 [80]
Hadacidin	Hadacidin	[a]Dulaney and Gray 1962 [89]
Roquefortine	Roquefortine C	[a]Kyriakidis et al. 1981 [90], [a]Frisvad and Filtenborg 1983 [31], *P. commune*, Wagener et al. 1980 [91]; *P. lanoso-coeruleum*, Wells and Payne, 1976 [92]
	Roquefortine D	[a]Frisvad and Samson 2004 [20]
	Roquefortine E	*P. verrucosum* var. *cyclopium*, Musuku et al. 1994 [93]
	16-hydroxyroquefortine C (R and S- forms)	[a]Trimble et al. 2012 [94]

Table 7.1 (continued)

Biosynthetic family	Secondary metabolite	Original (mis)identification, reference
	PF1– PF4	*P. farinosum*, Kozlovskii et al. 1989 [95]
	Histidyltryptophanyldiketopiperazine	Ali et al. 2013 [96], *ex P. chrysogenum*, but is also a precursor for roquefortine C in *P. crustosum*
	Dehydrohistidyltryptophanyldiketopiperazine	Ali et al. 2013 [96], *ex P. chrysogenum*, but is also a precursor for roquefortine C in *P. crustosum*
Penitrems	Penitrem A	[a]De Jesus et al. 1983 [77], *P. cyclopium*, Wells and Payne, 1976 [92], *P. lanosocoeruleum*, Wells and Payne, 1977 [97]
	Penitrem B-F	[a]De Jesus et al. 1983 [77]
	Penitrem G	[a]González et al. 2003 [78]
	Secopenitrem D	[a]Moldes-Anaya et al. 2011 [98]
	Thomitrem A and E	[a]Rundberget and Wilkins, 2002 [99]
	PC-M5' and PC-M6	[a]Hosoe et al. 1990 [100]
	Paspaline and emindole SB	[a]Sallam et al. 2013 [101]
	6-Bromopenitrem B and E	[a]Mantle et al. 1983 [102],[a]Sallam et al. 2013 [101]
Viridicatins	Cyclopeptin and dehydrocyclopeptin	[a]Frisvad and Filtenborg 1989 [34]
	Cyclopenin and cyclopenol	[a]Frisvad and Filtenborg 1989 [34]
	Viridicatin and viridicatol	[a]Taneguchi and Satomura, 1970 [103], [a]Frisvad and Filtenborg 1989 [34], *P. viridicatum*, Cunningham and Freeman 1953 [104]
Silvatins	*cis*-Bis(methylthio)silvatin = bis-(methylthio)dioxopiperazine, 6-epi-*cis*-Bis(methylthio)silvatin, fuzaperazine F	[a]Guimarães et al. 2010 [105], *P.commune*, Wang et al. 2012 [86]
Simple indole compounds	N-acetyltryptophan, 3-indolylacetic acid methyl ester, N-acetyltryptamine	*P. commune*, Yan et al. 2010 [87]; Wang et al. 2012 [86]
Sterols	Ergosterol, β-sitosterol, β-daucosterol, ergosta-7,22-diene-3β,5α,6β-triol	*P. commune*, Yan et al. 2010 [87]

Table 7.1 (continued)

Biosynthetic family	Secondary metabolite	Original (mis)identification, reference
Nucleotides	Uracil, thymidine, thymine	*P. commune*, Yan et al. 2010 [87]
Simple glycerides	1-O-acetylglycerol	*P. commune*, Yan et al. 2010 [87]
Phenylacetic acids	2-(2,5-dihydroxyphenyl)acetic acid	*P. commune*, Yan et al. 2010 [87]
Small lactones	(4R,5S)-5-hydroxyhexan-4-olide	*P. commune*, Yan et al. 2010 [87]
Esters	Ethyl acetate, ethyl propanoate, ethyl-2-methyl butanoate, ethyl isobutanoate, isobutyl acetate, ethyl butanoate, isobutyl acetate, ethyl isopentanoate, ethyl isopentanoate, iso pentyl acetate, ethylpentanoate, ethyl hexanoate, ethyl octanoate	[a]Larsen and Frisvad, 2005 [106]
Ketones	3-Octanone	[a]Larsen and Frisvad 2005 [106]
Dimethyldisulphide	Dimethyldisulphide	[a]Larsen and Frisvad 2005 [106]
Alcohols	Isobutanol, isopentanol, 4-ethylbutan-4-olide, 1-octen-3-ol	[a]Fischer et al. 1999 [107], [a]Larsen and Frisvad, 2005 [106]
Volatile terpenes	Geosmin, 2-methyl-isoborneol, limonene	[a]Fischer et al. 1999 [107], [a]Larsen and Frisvad 2005 [106]
Alkanes, alkenes and cycloalkenes	Styrene, dodecan	[a]Fischer et al. 1999 [107], [a]Larsen and Frisvad 2005 [106
Furans	2-ethylfuran, 2,5-diethylfuran, 2-ethyl-5-methylfuran, isopropylfuran, 2,3,5-trimethylfuran, furaneole	[a]Fischer et al. 1999 [107]
Acids	Linoleic acid	[a]Riley and Miller 1948 [108]
Sugar alcohols and trehalose	Glycerol, erythritol, arabinitol, mannitol, trehalose	[a]Frisvad 1986 [3], [a]Hendriksen et al. 1988 [4]

[a] Correctly identified as *P. crustosum*. Note that *P. terrestre* and *P. farinosum* are synonyms of *P. crustosum*

metabolites, derived from shikimic acid, polyketides, terpenes, modified nucleosides or nucleotides, amino acids, and even hybrid compounds based on two or more of these groups [116, 117]. However, even within the filamentous fungi the chemical inventiveness differs among ecological groups. Ruderal selected (R-selected) fungi are substrate pioneers, and apparently use more energy on growing fast, rather than on secondary metabolite production. This includes most Zygomycetous fungi, and in cases where secondary metabolites were found they, for example in *Rhizopus microsporus*, were actually produced by an endobacterium: a *Burkholderia* species [118]. Stress selected (S-selected) fungi, often extremotolerant or extremophile, have few competitors and in general produce few secondary metabolites. Competition selected (C-selected) fungi can often produce a large number of secondary metabolites and other extrolites [119]. These fungi include species in the ascomycetes *Aspergillus, Penicillium, Talaromyces, Byssochlamys, Alternaria, Fusarium Phoma, Phomopsis, Pestalotiopsis, Fusarium, Trichoderma*, and many other genera. Basidiomycete fungi are also very efficient producers of secondary metabolites [32].

Secondary Metabolite Profiles and (One Strains Many Compounds) OSMAC

Schiewe and Zeeck introduced the term one strains many compounds (OSMAC) in 1999 for secondary metabolite patterns in a *Streptomyces* strain [120]. However, this concept that one strain can produce many different compounds was already introduced in 1983 by Frisvad and Filtenborg for *Penicillium* species, where it was shown that the species examined had specific profiles of secondary metabolites, backed up by examining a large number of isolates of each species [31]. Furthermore, they used more than one medium for production of the different secondary metabolites to get the broadest possible profile, a concept also later recommended by Bode et al. [121], Bills et al. [122], Scherlach et al. [123], Kjer et al. [124], Nielsen et al. [125], Tormo et al. [126], and Frisvad [127, 128]. Media that has been very useful for screening filamentous fungi have included yeast extract sucrose (YES) agar, Czapek yeast autolysate (CYA) agar, malt extract agar, and oat meal agar. However, media based on 50% rice or white beans in water seem to be promising for the production of an impressive profile of secondary metabolites [124]. For example, as some secondary metabolites were formed by *P. steckii* on a rice medium, quite different metabolites were produced by the same strain on white beans. Some of the same compounds were found by Malmstrøm et al. [129] and Houbraken et al. [130] on the media CYA and YES, but not all of them. Similarly, *P. citrinum* produced nine secondary metabolites on white beans and six other secondary metabolites on rice [131]. Several of those were also produced on CYA and YES agar [130], but on white beans *P. citrinum* produced alternariol, never reported before from that species. A combination of CYA, YES, rice, and white beans seems to allow expression of many secondary metabolites in many species of fungi, but

special ecological groups of fungi may need modified media to be able to produce and accumulate their secondary metabolites. For example, marine-derived fungi are often grown on media with a sea salt mixture added [124].

Silent Gene Clusters for Secondary Metabolites

It has been a very clear result obtained after the bioinformatic studies of genomes sequenced from filamentous fungi that they have many apparently silent gene clusters potentially coding the production of "new" secondary metabolites [59, 123, 125, 132–140]. This is, of course, of great interest for the area of chemotaxonomy, as an expression of a more diverse sampling of secondary metabolites will help classifying the different species even better. However, in some cases the metabolites produced, for example, by *A. niger* only after the addition of epigenetic modifiers [134, 141, 142] to media like potato dextrose agar or minimal media, were after all produced efficiently on other media under other conditions [143].

Analytical Methods used in Chemotaxonomy

Originally, paper chromatography [144] and later thin layer chromatography (TLC) were used for lichen chemotaxonomy and some standardization was proposed for TLC methods by Culberson and coworkers [145–148]. TLC is still a good method for confirming results obtained by other chromatographic separation methods, and is occasionally used for chemotaxonomic studies in conjunction with high-pressure liquid chromatography (HPLC). For filamentous fungi, a very simple agar plug standardized TLC application technique was developed in 1980, allowing examination of a large number of isolates [149–154]. This standardized TLC method was later complemented with HPLC [155] and standardized HPLC with diode array detection (DAD). Later a direct inject electrospray method for mass spectrometric analysis of fungal extracts was introduced, allowing database search as an identification tool [156, 157], also using few agar plugs from fungal cultures [158]. For chemotaxonomic purposes, LC methods were later combined with both DAD and mass spectrometry (MS) detection, especially using electrospray MS [159–161]. The chemotaxonomic data obtained are often treated using multivariate data analysis such as principal component analysis, correspondence analysis, hierarchical cluster analysis, and fuzzy cluster analysis (e.g., [162, 163]), but recently network analysis has also been used successfully, in this case in conjunction with in situ nanospray desorption electrospray ionization MS [164]. Chemical image analysis has also been used on chemical data for different fungi in *Alternaria* and *Penicillium* [165].

Chemoconsistency

In general, filamentous fungi seem to contain a core secondary metabolome but reports of potential horizontal gene transfer have appeared [166]. If whole gene clusters for secondary metabolites can be transferred horizontally [167], the chemoconsistency in filamentous fungi may be less obvious. It has been shown that ancient horizontal gene transfer from bacteria to fungi may enhance the biosynthetic capability of the latter group of microorganisms [168]. Sterigmatocystin has been reported to be produced by fungi in several families of the ascomycetes [169], and the gene cluster has either evolved independently a large number of times or the gene cluster may have been transferred horizontally. Slot and Rokas [170] gave evidence for the hypothesis that the gene cluster for sterigmatocystin came from *Aspergillus* and was horizontally transferred to the unrelated, both phylogenetically and ecologically, *Podospora anserina*. In *Fusarium*, a pathogenicity minichromosome may have been transferred from an isolate in one species to an isolate in another species [171]. This points to the possibility of some variation in the secondary metabolite profile in species, but still it seems that most species examined have a rather constant secondary metabolite profile [172, 173].

There are many examples of consistent production of secondary metabolites in *Penicillium* [20, 34, 79] and this chemoconsistency in isolates from one species seems to be the rule among filamentous fungi [39, 174]. One example is *P. crustosum*, which has been isolated all over the world, from cold to warm climates [34, 80, 81]. Of 121 isolates examined by Sonjak et al. [80], 100 % produced penitrems, 100 % produced roquefortines, 100 % produced viridicatins, 99.2 % produced terrestric acids, and 73.5 % produced andrastin A. A re-examination of these 121 strains showed that 97.5 % produced clavatols. However, as mentioned previously, the media used, the incubation conditions, the extraction techniques for the metabolites, the detection methods, etc., will influence the result, and metabolites may not be produced or remain undetected in some cases. Filtenborg et al. [152] showed with *P. crustosum* as one of their examples, that this fungus produced the known secondary metabolites terrestric acid, roquefortine C and penitrem A on YES agar made of several yeast extract brands, but on one of them (Oxoid yeast extract) neither roquefortine nor penitrem A was produced. When magnesium sulfate was added, the isolate of *P. crustosum* produced both characteristic metabolites again.

As is seen from Table 7.1 [3, 4, 20, 31, 34, 79–108], *P. crustosum* is able to produce at least 107 metabolites, of which most can be regarded as secondary metabolites. Many of these were reported to be produced by *P. crustosum*, and some from its synonyms *P. farinosum* and *P. terrestre*, but a large number of extrolites were reported from species misidentified as *P. viridicatum, P. griseoroseum, P. commune, P. cyclopium, P. lanosocoeruleum*, and *P. verrucosum* var. *cyclopium*. Some of these latter fungi were identified using ITS sequences and searches in GenBank, but it is clear that ITS sequences are inadequate for identification in several cases [11]. In a “routine” analysis for secondary metabolites in a *P. crustosum* isolate, using

HPLC with diode array detection, only the most abundantly occurring extrolites are detected, including terrestric acid, viridicatic acid, clavatol, andrastin A, roquefortine C, penitrem A and B, thomitrem A, and all the six viridicatins, (and the unspecific ergosterol and linoleic acid), but the main secondary metabolites are sufficient to uniquely identify an isolate as *P. crustosum*. Using UHPLC-mass spectrometry many more compounds can be detected.

Rich Ecological Sources of Fungi Producing Secondary Metabolites

Fungi from certain kinds of habitats have been rich sources of new species and new secondary metabolites. One of the first broad habitats examined was soil and indeed many known compounds have been found in soilborne organisms (e.g., [175, 176]). However, other habitats such as dung [177], plants (endophytes) [178–182], marine environments [183], and cold environments [184, 185] are sources of metabolite-producing fungi. However, not all secondary metabolites from fungi may actually be produced by the fungus, but are rather plant products. The anticancer compound taxol was originally isolated from a *Taxus* tree, but was later claimed to be produced by endophytic fungi [179]. However, Heinig et al. [186] were able to show that taxol is not produced by fungi, even though there are approximately 160 patents and scientific papers claiming that fungi and other microorganisms can produce taxol. Another example of a plant natural product being confused with a fungal natural product was the report that *A. niger* NRRL 3122 can produce orobol, genistein, and other isoflavones [187]. However soy meal, which was used as growth medium for *A. niger*, is known to contain large amounts of genistein [188], and so the fungus may have deglycosylated or biotransformed the plant compounds [189, 190], but was not able to biosynthesize these isoflavones ab initio. However, the ability of many fungi to biotransform plant secondary metabolites could be a potential addition to the chemotaxonomy of fungi, in that the enzymes used for biotransforming, or their biotransformed products, could also be characterized.

Conclusion

Filamentous fungi, at least species in most genera of ascomycetes and basidiomycetes, produce species-specific profiles of secondary metabolites. However, there are cases—for example, in a field fungus like *Fusarium*—of what appears to be a horizontal transfer of minichromosomes containing gene clusters coding for secondary metabolites, which may make the extrolite profiles less consistent. Genome sequencing of many more isolates of filamentous fungi will show if these horizontal gene transfers are more common than we think. In *Penicillium, Aspergillus*, and *Talaromyces* there have been no direct or indirect evidence of horizontal transfer of

secondary metabolite gene clusters yet. Whether we will see such evidence or not, the core secondary metabolome in filamentous fungi seems to be rather constant in this slice of time. Of course, during evolution the core metabolomome may change, but that may follow speciation in those fungi. Even though chemotaxonomy in filamentous fungi seems to be a good basis for identification, it is recommended to use a polyphasic approach to both classification and identification in fungi.

References

1. Frisvad JC, Bridge PD, Arora DK (eds) (1998) Chemical fungal taxonomy. Marcel Dekker, New York, p 398
2. Andersen B, Frisvad JC (2002) Characterization of *Alternaria* and *Penicillium* species from similar substrata based on growth at different temperatures, pH and water activity. Syst Appl Microbiol 25:162–172
3. Frisvad JC (1985) Profiles of primary and secondary metabolites of value in classification of *Penicillium viridicatum* and related species. In: Samson RA, Pitt JI (eds) Advances in *Penicillium* and *Aspergillus* systematics. Plenum, New York, pp 311–325
4. Hendriksen HV, Mathiasen TE, Adler-Nissen J, Frisvad JC, Emborg C (1988) Production of mannitol by *Penicillium* strains. J Chem Technol Biotechnol 43:223–228
5. Blomquist GB, Andersson B, Andersson K, Brondz I (1992) Analysis of fatty acids. A new method for characterization of moulds. J Microbiol Meth 16:59–68
6. Stahl PD, Klug MJ (1996) Characterization and differentiation of filamentous fungi based on fatty acid composition. Appl Environ Microbiol 62:4136–4146
7. Pfyffer GE (1998) Carbohydrates and their impact on fungal taxonomy. In: Frisvad JC, Bridge PD, Arora DK (eds) Chemical fungal taxonomy. Marcel Dekker, New York, pp 247–261
8. Kock JLF, Botha A (1998) Fatty acids in fungal taxonomy. In: Frisvad JC, Bridge PD, Arora DK (eds) Chemical fungal taxonomy. Marcel Dekker, New York, pp 219–246
9. Croxatto A, Prud'hom G, Greub G (2012) Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS Microbiol Rev 36:380–407
10. Normand A-C, Cassagne C, Ranque S, L'Olliver C, Fourquet P, Roesems S, Hendrickx M, Piarroux R (2013) Assessment of various parameters to improve MALDI-TOF MS reference spectra libraries constructed for the routine identification of filamentous fungi. BMC Microbiol 13:76
11. Seifert KA, Samson RA, deWard JR, Houbraken J, Lévesque A, Moncalvo J-M, Louis-Seize G, Hebert PDN (2007) Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. Proc Natl Acad Sci U S A 104:3901–3906
12. Begerow D, Nilsson H, Unterseher M, Maier W (2010) Current state and perspectives of fungal DNA barcoding and rapid identification procedures. Appl Microbiol Biotechnol 87:99–108
13. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Lévesque A, Chen W, Fungal Barcoding Consortium (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc Natl Acad Sci U S A 109:6241–6246
14. Bidartondo MI, Bruns TD, Blackwell M, Edwards I, Taylor AFS, Horton T, Zhang N, Kõljalg U, May G, Kuyper TW et al (2008) Preserving accuracy in GenBank. Science 319:1616a
15. Taylor HR, Harris WE (2012) An emergent science on the brink of irrelevance: a review of the past 8 years of DNA barcoding. Mol Ecol Res 12:377–388
16. Shenoy BD, Jeewon R, Hyde KD (2007) Impact of DNA sequence-data on the taxonomy of anamorphic fungi. Fung Div 26:1–54
17. Cai L, Giraud T, Zhang N, Begerow D, Cai G, Shivas RG (2011) The evolution of species concepts and species recognition criteria in plant pathogenic fungi. Fung Div 50:121–133

18. Peterson SW (2012) *Aspergillus* and *Penicillium* identification using DNA sequences: barcode or MLST? Appl Microbiol Biotechnol 95:339–344
19. Vandamme P, Pot B, Gillis M, DeVos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 60:407–438
20. Frisvad JC, Samson RA (2004) Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Stud Mycol 49:1–173
21. Frisvad JC (2011) Rationale for a polyphasic approach in the identification of mycotoxigenic fungi. In: De Saeger S (ed) Determining mycotoxins and mycotoxigenic fungi in food and feed. Woodhead, Oxford, pp 279–297
22. Leal JA, Bernabé M (1998) Taxonomic applications of polysaccharides. In: Frisvad JC, Bridge PD, Arora DK (eds) Chemical fungal taxonomy. Marcel Dekker, New York, pp 153–181
23. Paterson RRM (1998) Chemotaxonomy of fungi by unsaponifiable lipids. In: Frisvad JC, Bridge PD, Arora DK (eds) Chemical fungal taxonomy. Marcel Dekker, New York, pp 183–217
24. Hennebert GL, Vancanneyt M (1998) Proteins in fungal taxonomy. In: Frisvad JC, Bridge PD, Arora DK (eds) Chemical fungal taxonomy. Marcel Dekker, New York, pp 77–106
25. Rosendahl S, Banke S (2008) Use of isozymes in fungal taxonomy and population studies. In: Frisvad JC, Bridge PD, Arora DK (eds) 1998. Chemical fungal taxonomy. Marcel Dekker, New York, pp 107–120
26. Notermans SHW, Cousin MA, De Ruiter GA, Rombouts FM (1998) Fungal immunotaxonomy. In: Frisvad JC, Bridge PD, Arora DK (eds) Chemical fungal taxonomy. Marcel Dekker, New York, pp 121–152
27. Brun S, Madrid H, van den Ende BG, Andersen B, Marinach_Patrice C, Mazier D, de Hoog GS (2013) Multilocus phylogeny and MALDI-TOF analysis of the plant pathogenic species *Alternaria dauci* and relatives. Fung Biol 117:32–40
28. Tyrrell D (1969) Biochemical systematics and fungi. Bot Rev 35:305–316
29. Benedict RG (1970) Chemotaxonomic relationships among basidiomycetes. Adv Appl Microbiol 13:1–23
30. Hawksworth DL (1976) Lichen chemotaxonomy. In: Brown DH, Hawksworth DL, Bailey RH (eds) Lichenology: problems and prospects. Academic Press, London, pp 139–184
31. Frisvad JC, Filtenborg O (1983) Classification of terverticillate Penicillia based on profiles of mycotoxins and other secondary metabolites. Appl Environ Microbiol 46:1301–1310
32. Moser M 1985. The relevance of chemical characters for the taxonomy of the Agaricales. Proc Indian Acad Sci (Plant Sci) 94:381–386.
33. Frisvad JC (1989) The use of high-performance liquid chromatography and diode array detection in fungal chemotaxonomy based on profiles of secondary metabolites. Bot J Lin Soc 99:81–95
34. Frisvad JC, Filtenborg O (1989) Terverticillate penicillia: chemotaxonomy and mycotoxin production. Mycologia 81:836–861
35. Frisvad JC (1994a) Classification of organisms by secondary metabolites. In: Hawksworth DL (ed) The identification and characterization of pest organisms. CAB International, Wallingford, pp 303–320
36. Whalley AJS, Edwards RL. 1995. Secondary metabolites and systematic arrangement within the Xylariaceae. Can J Bot 73:S802–S810
37. Frisvad JC, Thrane U, Filtenborg O (1998) Role and use of secondary metabolites in fungal taxonomy. In: Frisvad JC, Bridge PD, Arora DK (eds) Chemical fungal taxonomy. Marcel Dekker, New York, pp 289–319
38. Frisvad JC, Larsen TO, de Vries R, Meijer M, Houbraken J, Cabañes FJ, Ehrlich K, Samson RA (2007) Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins. Stud Mycol 59:31–37
39. Frisvad JC, Andersen B, Thrane U (2008) The use of secondary metabolite profiling in fungal taxonomy. Mycol Res 112:231–240

40. Andersen B, Dongo A, Pryor BM (2008) Secondary metabolite profiling of *Alternaria dauci, A. porri, A. solani* and *A. tomatophila*. Mycol Res 112:241–250
41. Nielsen KF, Smedsgaard J, Larsen TO, Lund F, Thrane U, Frisvad JC. (2004) Chemical identification of fungi—metabolite profiling and metabolomics. In: Arora DK (ed) Fungal biotechnology in agricultural, food and environmental applications. Marcel Dekker, New York, pp. 19–35
42. Stadler M, Hellwig V (2004) PCR-based data and secondary metabolites as chemotaxonomic markers in high-throughput screening for bioactive compounds from fungi. In: An Z (ed) Handbook of industrial mycology. Marcel Dekker, New York, pp 269–307
43. Andersen B, Sørensen JL, Nielsen KF, van den Ende BG, de Hoog S (2009) A polyphasic apporach to the taxonomy of the *Alternaria infectoria* species-group. Fung Genet Biol 46:642–656
44. Rank C, Larsen TO, Frisvad JC (2010) Functional systems biology of *Aspergillus*. In: Machida M, Gomi K (eds) *Aspergillus*. Molecular biology and genomics. Caister Academic Press, Norfolk, pp. 173–198
45. Polizzotto R, Andersen B, Martini M, Grisan S, Assante G, Musetti R (2012) A polyphasic approach for the characterization of endophytic *Alternaria* strains isolated from grapevines. J Microbiol Meth 88:162–171
46. Bennett JW, Bentley R (1989) What's in a name—Microbial secondary metabolism. Adv Appl Microbiol 34:1–28
47. Davies J (2013) Specialized microbial metabolites: functions and origins. J Antibiot 66:361–364
48. Raistrick H (1940) Biochemistry of the fungi. Annu Rev Biochem 9:571–592
49. Meinwald J (2009) The chemistry of biotic interactions in pespective: small molecules take center stage. J Org Chem 74:1813–1825
50. Clardy J, Walsh C (2004) Lessons from natural molecules. Nature 432:829–837
51. Walker JB (1974) Biosynthesis of the monoguanidinated inositol moiety of bluensomycin, a possible evolutionary precursor of streptomycin. J Biol Chem 249:2397–2404
52. Samson RA, Frisvad JC (2004) *Penicillium* subgenus *Penicillium*: new taxonomic schemes and mycotoxins and other extrolites. Stud Mycol 49:1–251
53. Davies J, Ryan KS (2012) Introducing the parvome: bioactive compounds in the microbial world. ACS Chem Biol 7:252–259
54. Thrane U, Andersen B, Frisvad JC, Smedsgaard J (2007) The exo-metabolome of filamentous fungi. In: Jewitt M, Nielsen J (eds) Metabolomics. A powerful tool in systems biology (Topics in current chemistry 276). Springer, Berlin, pp 235–252
55. Terabayashi Y, Sano M, Yamane N, Marui J, Tamano K, Sagara J, Dohmoto M, Oda K, Ohshima E, Tachibana K, Higa Y, Ohashi S, Koike H, Machida M (2010) Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from *Aspergillus oryzae*. Fung Genet Biol 47:953–961
56. Li A, van Luijk N, ter Brek M, Caspers M, Punt P, van der Werf M (2011) A clone-based transriptomics approach for the identification of genes relevant for itaconic acid production in *Aspergillus*. Fung Genet Biol 48:601–611
57. Liu J, Gao Q, Xu N, Liu L (2013) Genome-scale reconstruction and in silico analysis of *Aspergillus terreus* metabolism. Mol Biosyst 9:1939–1948
58. Poulsen L, Andersen MR, Lantz AE, Thykaer J (2012) Identification of a transcription factor controlling pH-dependent organic acid response in *Aspergillus niger*. PLoS ONE 7:e50596
59. Williams RB, Henrikson JC, Hoover AR, Lee AE, Cichewicz RH (2008) Epigenetic remodeling of the fungal secondary metabolome. Org Biomol Chem 6:1895–1897
60. Stone MJ, Williams DH (1992) On the evolution of functional secondary metabolites (natural products). Mol Microbiol 6:29–34
61. Chadwick DJ, Wheelan J (eds) (1992). Secondary metabolites: their function and evolution (Ciba Foundation Symposium 171). Wiley, Chichester, p 318
62. Christophersen C (1996) Theory of the origin, function, and evolution of secondary metabolites. In: Atta-ur-Rahman (ed) Studies in natural products chemistry 18. Stereoselective synthesis (part K). Elseveir, Amsterdam, pp 677–737

63. Firn RD, Jones CG (2000) The evolution of secondary metabolism—a unifying model. Mol Microbiol 37:989–994
64. Demain AL, Fang A (2000) The natural functions of secondary metabolites. Adv Biochem Eng/Biotechnol 69:1–39
65. Lineares JF, Gustafsson I, Baquero F, Martinez JL (2006) Antibiotics as intermicrobial signaling agents instead of weapons. Proc Nat Acad Sci U S A 103:19484–19489
66. Price-Wheelan A, Dietrich LEP, Newman DK (2006) Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. Nat Chem Biol 2:71–78
67. Fischbach MA, Walsh CT, Clardy J (2008) The evolution of gene collectives: how natural selection drives chemical innovation. Proc Nat Acad Sci U S A 105:4601–4608
68. Clardy J, Fischbach M, Currie C (2009) The natural history of antibiotics. Curr Biol 19:R437–R441
69. Meinwald J (2011) Natural products as molecular messengers. J Nat Prod 74:305–309
70. Stevens AM, Schuster M, Rumbaugh KP (2012) Working together for the common good: cell-cell communication in bacteria. J Bacteriol 194:2131–2141
71. Rasmussen TB, Skindersoe ME, Bjarnsholt T, Phipps RK, Christensen KB, Jensen PO, Andersen JB, Koch B, Larsen TO, Hentzer M, Eberl L, Hoiby N, Givskov M (2005) Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species. Microbiology (SGM) 151:1325–1340
72. Villa F, Villa S, Gelain A, Cappitelli F (2013) Sub-lethal activity of small molecules from natural sources and their synthetoic derivatives against biofilm forming nosocomial pathogens. Curr Top Med Chem 13:3184–3204
73. Bradley D (1996) Beating superbugs with the Gulliver effect. Drug Discov Today 1:361
74. Dowd P (1988) Synergism of aflatoxin B1 toxicity with the co-occurring fungal metabolite kojic acid to 2 caterpillars. Entomol Exper Appl 47:69–71
75. Chitarra GS, Abee T, Rombouts FM, Posthumus MA, Dijksterhuis J (2004) Germination of *Penicillium paneum* conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor. Appl Environ Microbiol 70:2823–2829
76. Bladt TT, Frisvad JC, Knudsen PB, Larsen TO (2013) Anticancer and antifungal compounds from *Aspergillus, Penicillium* and other filamentous fungi. Molecules 18:11338–11376
77. De Jesus AE, Steyn PS, van Heerden FR, Vleggaar R, Wessels PL (1983) Tremorgenic mycotoxins from *Penicillium crustosum*: Isolation of penitrems A-F and the structure elucidation and absolute configuration of penitrem A. J Chem Soc Perkin Trans 1:1847–1856
78. González MC, Lull C, Moya P, Ayala I, Primo J, Yúfera EP (2003) Insecticidal activity of penitrems, including penitrem G, a new member of the family isolated from *Penicillium crustosum*. J Agric Food Chem 51:2156–2160
79. Frisvad JC, Smedsgaard J, Larsen TO, Samson RA (2004) Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. Stud Mycol 49:201–241
80. Sonjak S, Frisvad JC, Gunde-Cimerman N (2005) Comparison of secondary metabolite production by *Penicillium crustosum* strains, isolated from Arctic and other various ecological niches. FEMS Microbiol Ecol 53:51–60
81. Sonjak S, Frisvad JC, Gunde-Cimerman N (2007) Genetic variation among *Penicillium crustosum* isolates from arctic and other ecological niches. Microbial Ecol 54:298–305
82. Wu G, Ma H, Zhu T, Li J, Gu Q, Li D (2012) Penilactones A and B, two novel polyketides from Antarctic deep-sea derived fungus *Penicillium crustosum* PRB-2. Tetrahedron 68:9745–9749
83. Da Silva JV, Fill TP, Da Silva BF, Rodrigues-Fo E (2013) Diclavatol and tetronic acids from *Penicillium griseoroseum*. Nat Prod Res 27:9–16
84. Birkinshaw JH, Raistrick H (1936) Studies in the biochemistry of micro-organisms. LII. Isolation, properties and constitution of terrestric acid (ethylcarolic acid), a metabolic product of *Penicillium terrestre*. Biochem J 30:2194–2200
85. Birkinshaw JH, Samant MS (1960) Studies in the biochemistry of micro-organisms. 107. Metabolites of *Penicillium viridicatum* Westling: viridicatic acid (ethyl carlosic acid). Biochem J 74:369–373

86. Wang J, Liu P, Wang Y, Wang H, Li J, Zhuang Y, Zhu W (2012) Antimicrobial aromatic polyketides from gorgonean-associated fungus *Penicillium commune* 518. Chin J Chem 30:1326–1342
87. Yan H-J, Gao S-S, Li C-S, Li X-M, Wanf B-G (2010) Chemical constituents of a marine-derived endophytic fungus *Penicillium commune* G2M. Molecules 15:3270–3275
88. Roncal T, Cordobës S, Ugalde U, He Y, Sterner O (2002) Novel diterpenes with potent conidiation inducing activity. Tetrahedron Lett 43:6799–6802
89. Dulaney EL, Gray RA (1962) Penicillia that make (N-formyl)-hydroxyaminoacetic acid, a new fungal product. Mycologia 54:476–480
90. Kyriakidis N, Waight ES, Day JB, Mantle PG (1981) Novel metabolites from *Penicillium crustosum*, including penitrem E, a tremorgenic mycotoxin. Appl Environ Microbiol 42:61–62
91. Wagener RE, Davis ND, Diener UL (1980) Penitrem A and roquefortine production by *Penicillium commune*. Appl Environ Microbiol 39:882–887
92. Wells JM, Payne JA (1976) Toxigenic species of *Penicillium, Fusarium* and *Aspergillus* from weevil-damaged pecans. Can J Microbiol 22:281–285
93. Musuku A, Selala MI, de Bruyne T, Clayes M, Schepens PJC (1994) Isolation and structure determination of a new roquefortine-related mycotoxin from *Penicillium verrucosum* var. *cyclopium* isolated from cassava. J Nat Prod 57:983–987
94. Trimble LA, Sumarah MW, Blackwell BA, Wrona MD, Miller JD (2012) Characterization of (16R) and (16S)-hydroxyroquefortine C; diastereomeric metabolites from *Penicillium crustosum* DAOM 215343. Tetrahedron Lett 53:956–958
95. Kozlovskii AG, Reshetilova TA, Sakharovskii VG, Adanin VM, Zyakun AM (1989) Metabolites of the alkaloids roquefortine and 3,12-dihydroroquefortine in the fungus *Penicillium farinosum*. Appl Biochem Microbiol 24:533–537
96. Ali H, Ries MI, Nijland JG, Lankhorst PP, Hankermeier T, Bovenburg RAL, Vreeken RJ, Driessen AJM (2013) A branched biosynthetic pathway is involved in production of roquefortine and related compounds in *Penicillium chrysogenum*. PLoS ONE 8:e65328
97. Wells JM, Payne JA (1977) Production of penitrem A and of an unidentified toxin from *Penicillium lanosocoeruleum* isolated from weevil-damaged pecans. Phytopathology 67:779–782
98. Moldes-Anaya A, Rundberget T, Uhlig S, Rise F, Wilkins AL (2011) Isolation and structure elucidation of secopenitrem D, an indole alkaloid from *Penicillium crustosum* Thom. Toxicon 57:259–265
99. Rundberget T, Wilkins AL (2002) Thomitrems A and E, two indole-alkaloid isoprenoids from *Penicillium crustosum* Thom. Phytochemistry 61:979–985
100. Hosoe T, Nozawa K, Udagawa S, Nakajima S, Kawai K (1990) Structures of new indoloterpenes, possible biosynthetic precursors of the tremorgenic mycotoxins, penitrems, from *Penicillium crustosum*. Chem Pharm Bull 38:3473–3475
101. Sallam AA, Houssen WE, Gissendanner CR, Orabi KY, Foudah AI, El Sayed KA (2013) Bioguided discovery and pharmacophore modeling of the mycotoxic indole diterpene alkaloids penitrems as breast cancer proliferation, migration, and invasion inhibitors. MedChemComm 4:1360–1369
102. Mantle PG, Perrera PWC, Maishman NJ, Mundy GR (1983) Biosynthesis of penitrems and roquefortine by *Penicillium crustosum*. Appl Environ Micorbiol 45:1486–1490
103. Taneguchi M, Satomura Y (1970) Isolation of viridicatin from *Penicillium crustosum*, and physiological activity of viridicatin sand its 3-carboxymethylene derivative on microorganisms and plants. Agric Biol Chem 34:506–509
104. Cunningham KG, Freeman GG (1953) The isolation and some chemical properties of viridicatin, a metabolic product of *Penicillium viridicatum* Westling. Biochem J 53:328–332
105. Guimarães DO, Borges WS, Vieira NJ, de Oliveira LF, da Silva CHTP, Lopes NP, Dias LG, Durán-Patrón R, Collado IG, Pupo MT (2010) Diketopiperazines produced by endophytic fungi found in association with two Asteraceae species. Phytochemistry 71:1423–1429

106. Larsen TO, Frisvad JC (1995) Characterization of volatile metabolites from 47 *Penicillium* taxa. Mycol Res 99:1153–1166
107. Fischer G, Schwalbe R, Möller M, Ostrowski R, Dott W (1999) Species-specific production of microbial volatile organic compounds (MVOC) by airborne fungi from a compost facility. Chemosphere 39:795–810
108. Riley RF, Miller DK (1948) The isolation and identification of an antibiotic substance present in the mycelium of *Penicillium crustosum* (Thom). Arch Biochem 18:13–26
109. Fischbach MA, Clardy J (2007) One pathway, many products. Nat Chem Biol 3:353–355
110. Houbraken J, Frisvad JC, Samson RA (2011a) Fleming's penicillin producing strain is not *Penicillium chrysogenum* but *P. rubens*. IMA Fungus 2:87–95
111. Houbraken J, Frisvad JC, Seifert KA, Overy DP, Tuthill DE, Valdez JG, Samson RA (2012) New penicillin-producing *Penicillium* species and an overview of section *Chrysogena*. Persoonia 29:78–100
112. Procopio S, Qian F, Becker T (2011) Function and regulation of yeast genes involved in higher alcohol and ester metabolism during beverage fermentation. Eur Food Res Technol 233:721–729
113. Surmacz L, Swiezewska E (2011) Polyisoprenoids—secondary metabolites or physiologically important superlipids? Biochem Biophys Res Commun 407:627–632
114. Ells R, Kock JFL, Albertyn J, Pohl CH (2012) Arachidonic acid metabolites in pathogenic yeasts. Lip Health Dis 11:100
115. Brodhun F, Feussner I (2011) Oxylipins in fungi. FEBS J 278:1047–1063
116. Turner WB (1971) Fungal metabolites. Academic Press, London, pp 446
117. Turner WB, Aldridge DC (1983) Fungal metabolites II. Academic, London, pp 631
118. Partida-Martinez LP, Hertweck C (2005) Pathogenic fungus harbours endosymbiotic bacteria for toxin production. Nature 437:884–888
119. Zac JC, Wildman HG (2004) Fungi in stressful environments. In: Mueller GM, Bills GF, Forster MS (eds) Biodiversity of fungi. Elsevier, Amsterdam, pp 303–315
120. Schiewe HJ, Zeeck A (1999) Cineromycins, gamma-butyrolactones and ansamycins by analysis of the secondary metabolite pattern created by a single strain of *Streptomyces*. J Antibiot 52:635–642
121. Bode HB, Bethe B, Höfs R, Zeeck A (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. ChemBioChem 3:619–627
122. Bills G, Platas G, Fillola A, Jiménez MR, Bur-Zimmerman J, Tormo JR, Peláez F (2008) Enhenacement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. J Appl Bacteriol 104:1644–1658
123. Scherlach K, Schuemann J, Dahse H-M, Hertweck C (2010) Aspernidine A and B, prenylated isoindolino alkaloids from the model fungus *Aspergillus nidulans*. J Antibiot 63:375–377
124. Kjer J, Debbab A, Proksch P (2010) Methods for isolation of marine-derived endophytic fungi and their bioactive secondary metabolites. Nat Protoc 5:479–490
125. Nielsen ML, Nielsen JB, Rank C, Klejnstrup ML, Holm DMK, Brogaard KH, Hansen BG, Frisvad JC, Larsen TO, Mortensen UH (2011a) A genome-wide polyketide synthase deletion library uncovers novel genetic links to polyketides and meroterpenoids in *Aspergillus nidulans*. FEMS Microbiol Lett 321:157–166
126. Tormo JR, Asensio FJ, Bills GF (2012) Manipulating filamentous fungus chemical phenotypes by growth on nutritional arrays. In: Keller NP, Turner G (eds) Fungal secondary metabolism: methods and protocols (Methods in molecular biology 944). Humana, New York, pp 59–78
127. Frisvad JC (2010) Metabolomics for the discovery of novel compounds. In: Baltz RH, Demain AL, Davies JE (eds) Manual of industrial microbiology and biotechnology, 3rd edn. ASM, Washington, DC, pp 73–77
128. Frisvad JC (2012) Media and growth conditions for induction of secondary metabolites. In: Keller NP, Turner G (eds) Fungal secondary metabolism: methods and protocols (Methods in Molecular Biology 944: 47–58). Humana, New York

129. Malmstrøm J, Christophersen C, Frisvad JC (2000) Secondary metabolites characteristic of marine and terrestrial isolates of *Penicillium citrinum, P. steckii* and related species. Phytochemistry 54:301–309
130. Houbraken J, Frisvad JC, Samson RA (2011b) Taxonomy of *Penicillium* section *Citrina*. Stud Mycol 70:53–138
131. Lai D, Brötz-Oesterhelt H, Müller WEG, Wray V, Proksch P (2013) Bioactive polyketides and alkaloids from *Penicillium citrinum*, a fungal endophyte isolated from *Ocimum tenuiflorum*. Fitoterapia 91:100–106
132. Bok JW, Hoffmeister D, Maggio-Hall LA, Murillo R, Glasner JD, Keller NP (2006) Genomic mining for Aspergillus natural products. Chem Biol 13:31–37
133. Schwab EK, Bok JW, Tribus M, Galehr J, Graessle S, Keller NP (2007) Histone deacetylase activity regulates chemical diversity in *Aspergillus*. Eukaryot Cell 6:1656–1664
134. Henrikson JC, Hoover AR, Joyner PM, Cichewicz RH (2009) A chemical epigenetics approach for engineering the in situ biosynthesis of cryptic natural products from *Aspergillus niger*. Org Biomol Chem 7:435–438
135. Zerikly M, Challis GL (2009) Strategies for the discovery of new natural products by genome mining. ChemBioChem 10:625–633
136. Brakhage AA, Schroeckh V (2011) Fungal secondary metabolites—strategies to activate silent gene clusters. Fung Genet Biol 48:15–22
137. Chiang Y-M, Chang S-L-, Oakley BR, Wang CCC (2011) Recent advances in awakening silent biosynthetic gene clusters and linking orphan clusters to natural products in microorganisms. Curr Opin Chem Biol 15:137–143
138. Davies J (2011) How to discover new antibiotics: harvesting the parvome. Curr Opin Chem Biol 15:5–10
139. Umemura M, Koike H, Nagano N, Ishii T, Kawano J, Yamane N, Kozone I, Horimoto JK, Shin-ya K, Asai K, Yu J, Bennett JW, Machida M (2013) MIDDAS: Motif-independent *de novo* detection of secondary metabolite gene clusters through the integration of genome and transcriptome data. PLoS ONE 8:e84028
140. Wiemann P, Sieber CMK, von Bargen KW, Studt L, Niehaus E-M, Espino JJ, Huss K, Michielse CB, Albermann S, Wagner D, Bergner SV, Conolly LR, Fischer A, Reuter G, Kleigrewe K, Bald T, Wingfileld BD, Ophir R, Freeman S, Hippler M, Smith KM, Brown DW, Proctor RH, Münsterkötter M, Freitag M, Humpf H-U, Güldener U, Tudzynski B (2013) Deciphering the cryptic genome: Genome-wide analyses of the rice pathogen *Fusarium fujikuroi* revel complex regulation of secondary metabolism and novel metabolites. PLoS Path 9:e1003475
141. Fisch KM, Gillaspy AF, Gipson M, Henrikson JC, Hoover AR, Jackson L, Najar FZ, Wägele H, Cichewicz RH (2009) Chemical induction of silent biosynthetic pathway transcription in *Aspergillus niger*. J Ind Microbiol Biotechnol 36:1199–1213
142. Henrikson JC, Ellis TK, King JB, Cichewicz RH (2011) Reappraising the structures and distribution of metabolites from black Aspergilli containing uncommon 2-benzyl-4H-pyran-4-one and 2-benzylpyridin-4(1H)-one systems. J Nat Prod 74:1959–1964
143. Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC (2009) Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Anal Bioanal Chem 395:1225–1246
144. Wachtmeister C (1956) Identification of lichen acids by paper chromatography. Bot Nor 109:313–324
145. Culberson CF, Kristinsson H-D (1970) A standardized method for the identification of lichen products. J Chromatogr 46:85–93
146. Culberson CF (1972) Improved conditions and new data for the identification of lichen products by a standardized thin-layer chromatographic method. J Chromatogr 72:113–125
147. Culberson CF, Johnson A (1982) Substitution of methyl tert. butyl ether for diethyl ether in standardized thin-layer chromatographic method for lichen products. J Chromatogr 238:438–487

148. Lumbsch HT (1998) Taxonomic use of metabolic data in lichen-forming fungi. In: Frisvad JC, Bridge PD, Arora DK (eds) Chemical fungal taxonomy. Marcel Dekker, New York, pp 345–385
149. Filtenborg O, Frisvad JC (1980) A simple screening method for toxigenic fungi in pure cultures. Lebensm Wiss Technol 13:128–130
150. Frisvad JC (1981) Physiological criteria and mycotoxin production as aids in identification of common asymmetric penicillia. Appl Environ Microbiol 41:568–579
151. Filtenborg O, Frisvad JC, Svendsen JA (1983) Simple screening method for moulds producing intracellular mycotoxins in pure cultures. Appl Envitron Microbiol 45:581–585
152. Filtenborg O, Frisvad JC, Thrane U (1990) The significance of yeast extract composition on metabolite production in *Penicillium*. In: Samson RA, Pitt JI (eds) Modern concepts in *Penicillium* and *Aspergillus* classification. Plenum, New York, pp 433–441
153. Frisvad JC, Thrane U (1987) Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone indices and UV-VIS spectra (diode-array detection). J Chromatogr 404:195–214
154. Frisvad JC, Thrane U (1993) Liquid column chromatography of mycotoxins. In: Betina V (ed) Chromatography of mycotoxins: techniques and applications. Journal of Chromatography Library 54. Elsevier, Amsterdam, pp 253–372
155. Frisvad JC (1987) High-performance liquid chromatographic determination of profiles of mycotoxins and other secondary metabolites. J Chromatogr 392:333–347
156. Smedsgaard J, Frisvad JC (1997) Terverticillate penicillia studied by direct electrospray mass spectrometric profiling of crude extracts: I. Chemosystematics. Biochem Syst Ecol 25:51–64
157. Smedsgaard J (1997a) Terverticillate Penicillia studies by direct electrospray mass spectrometric profiling of crude extracts. II. Database and identification. Biochem Syst Ecol 25:65–71
158. Smedsgaard J (1997b) Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J Chromatogr A 760:264–270
159. Nielsen KF, Smedsgaard J (2003) Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardized liquid chromatography-UV-mass spectrometry methodology. J Chromatogr A 1002:111–136
160. Nielsen KF, Månsson M, Rank C, Frisvad JC, Larsen TO (2011b) Dereplication of microbial natural products by LC-DAD-TOFMS. J Nat Prod 74:2338–2348
161. Klitgaard A, Iversen A, Andersen MR, Larsen TO, Frisvad JC, Nielsen KF (2014) Aggressive dereplication using UHPLC-DAD-QTOF—screening extracts for up to 3000 fungal secondary metabolites. Anal Bioanal Chem. doi: 10.1007/s00216-013-7582-x (Published online Jan 18, 2014)
162. Frisvad JC (1992) Chemometrics and chemotaxonomy: a comparison of multivariate statistical methods for the evaluation of binary fungal secondary metabolite data. Chemom Intel Lab Syst 14:253–269
163. Frisvad JC (1994b) Correspondence, principal coordinate, and redundancy analysis used on mixed chemotaxonomical qualitative and quantitative data. Chemom Intel Lab Syst 23:213–229
164. Nguyen DD, Wu C-H, Moree WJ, Lamsa A, Medema MH, Zhao X, Gavilan RG, Aparicio M, Atencio L, Jackson C, Ballesteros J, Sanchez J, Watrous JD, Phelan VV, van de Wiel C, Kersten RD, Mehnaz S, De Mot R, Shank EA, Charusanti P, Nagarajan H, Duggan BM, Moore BS, Bandeira N, Palsson B, Pogliano K, Gutiérrez M, Dorrestein P (2013) MS/MS networking guided analysis of molecule and gene cluster families. Proc Nat Acad Sci U S A 110:E2611–E2620
165. Hansen ME, Andersen B, Smedgaard J (2005) Automated and unbiased classification of chemical profiles from fungi using high performance liquid chromatography. J Microbiol Meth 61:295–304
166. Richards TA, Leonard G, Soanes DM, Talbott NJ (2011) Gene transfer into the fungi. Fung Biol Rev 25:98–110

167. Walton JD (2000) Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: an hypothesis. Fung Genet Biol 30:167–171
168. Schmitt I, Lumbsch TH (2009) Ancient horizontal gene transfer from bacteria enhances biosynthetic capabilities of fungi. PLoS ONE 4:e4437
169. Rank C, Nielsen KF, Larsen TO, Varga J, Samson RA, Frisvad JC (2011) Distribution of sterigmatocystin in filamentous fungi. Fung Biol 115:406–420
170. Slot JC, Rokas A (2011) Horizontal transfer of a large and highly toxic secondary metabolic gene cluster between fungi. Curr Biol 21:134–139
171. Ma L-J, van der Does HC, Borkovich KA, Coleman JJ, Daboussi M-J, Di Pietro A, Dufresne M, Freitag M, Grabherr M, Henrissat B et al (2010) Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature 464:367–373
172. Thrane U (1990) Grouping *Fusarium* section *Discolor* isolates by statistical analysis of quantitative high performance liquid chromatographic data on secondary metabolite production. J Microbiol Meth 12:23–39
173. Thrane U, Hansen U (1995) Chemical and physiological charcacterization of taxa in the *Fusarium sambucinum* complex. Mycopathologia 129:183–190
174. Larsen TO, Smedsgaard J, Nielsen KF, Hansen ME, Frisvad JC (2005) Phenotypic taxonomy and metabolite profiling in microbial drug discovery. Nat Prod Rep 22:672–695
175. Korzybski T, Kowszyk-Gindifer Z, Kuryłowicz W (1967) Antibiotics: origin, nature and propereties, vol I, II. Pergamon, Oxford, p 1651
176. Gottlieb D (1976) The production and role of antibiotics in soil. J Antibiot 29:988–1000
177. Bills GF, Gloer JB, An Z (2013) Coprophilous fungi: antibiotic discovery and functions in an underexplored arena of microbial defensive mutualism. Curr Opin Microbiol 16:549–565
178. Schulz B, Boyle C, Draeger S, Römmert A-K, Krohn K (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol Res 106:996–1004
179. Strobel GA, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic organisms. J Nat Prod 67:257–268
180. Wang L-W, Zhang Y-L, Lin, F-C, Hu Y-Z, Zhang C-L (2011) Natural products with antitumor activity from endophytic fungi. Mini Rev Med Chem 11:1056–1074
181. Kusari S, Hertweck C, Spiteller M (2012) Chemical ecology of endophytic fungi: origins of secondary metabolites. Chem Biol 19:792–798
182. Kusari S, Pandey SP, Spiteller M (2013) Untapped mutualistic paradigms linking host plant and endophytic fungal production of similar bioactive secondary metabolites. Phytochemistry 91:812–887
183. Blunt JC, Copp BR, Munro MHG, Northcote PT, Prinsep MR (2011) Marine natural products. Nat Prod Rep 28:196–268
184. Frisvad JC (2008a) Fungi in cold ecosystems. In: Margesin R, Schinner F, Marx J-C, Gerday C (eds) Psychrophiles: from biodiversity to biotechnology. Springer, Berlin, pp 137–156
185. Frisvad JC (2008b) Cold-adapted fungi as a source for valuable metabolites. In: Margesin R, Schinner F, Marx J-C, Gerday C (eds) Psychrophiles: from biodiversity to biotechnology. Springer, Berlin, pp 381–387
186. Heinig U, Scholz S, Jennewein S (2013) Getting to the bottom of taxol biosynthesis in fungi. Fung Div 60:161–170
187. Umezawa H, Tobe H, Shibamoto N, Nakamura F, Nakamura K, Matsuzaki M, Takeuchi T (1975) Isolation of isoflavones inhibiting DOPA decarboxylase from fungi and *Streptomyces*. J Antibiot 28:947–952
188. Fukutake M, Takahashi M, Ishida K, Kawamura H, Sugimura T, Wakabayashi K (1996) Quantification of genistein and genistin in soybeans and soybean products. Food Chem Toxicol 34:457–461
189. Miake Y, Minato K, Fukumoto S, Yamamoto K, Oya-Ito T, Kawakishi S, Osawa T (2003) New potent antioxidative hydroxyflavones produced with *Aspergillus saitoi* from flavanone glycoside in citrus fruit. Biosci Biotechnol Biochem 67:1443–1450
190. Esaki H, Watanabe R, Osawa T, Kawasaki S (2004) Transformation of genistein by the spores of *Aspergillus* spp. Nippon Shokuhin Kagaku Kogaku Kaishi 51:210–213

Chapter 8
Endophytic Fungi as a Source of Novel Metabolites

Fernanda O. Chagas, Andrés Mauricio Caraballo-Rodriguez and Mônica T. Pupo

Introduction

The term "endophytes" refers to microorganisms inhabiting plant tissues for at least a part of their life cycle without causing any visible damage to their host plant. Endophytic microorganisms can be fungi or bacteria, including actinobacteria, although the most frequently encountered endophytes are fungi [1]. Endophytic fungi are a highly diverse polyphyletic group of microorganisms [2, 3].

Most plants that have been previously studied harbor endophytic microorganisms [4, 5], and there is evidence, found in fossilized tissues of plants, that endophyte–host relationships have evolved from the time high plants first appeared on the earth [6, 7].

The asymptomatic colonization of plants by endophytic microorganisms is the result of a balance of antagonisms between endophytic virulence and plant defense [8]. If destabilization in this balance occurs, the fungal strain may perish or the plant may succumb. Recently, it was suggested that the plant–endophyte interaction might be much more complex than the balanced antagonism hypothesis, because in the same way that plants have to defend themselves against the fungal virulence factors, the endophytes also should have resistance mechanisms to counter the toxic secondary metabolites produced by the host plant [2].

While in a symbiotic relationship, both endophyte and host plant are benefited. The plant provides to endophytic microorganism protection, nutrients, and dissemi-

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nation to the next generation of hosts [9–11]. On the other hand, the endophytes are believed to be responsible for the adaptation of plants to abiotic stresses such as drought, high temperature and salinity, harmful effects of light, and metal toxicity, as well as to biotic factors such as herbivores, nematodes, insects, and pathogens [12–21]. The host protection is achieved mainly by natural products produced by endophytic microorganisms [3, 22]. Besides that, endophytes are also suggested to be capable of inducing host defense mechanisms [23].

The discovery of some endophytes producing biologically important secondary plant metabolites and their analogues—such as the anticancer drugs paclitaxel (**1**, Fig. 8.1) [24], camptothecin (**2**, Fig. 8.1), 9-methoxycamptothecin (**3**, Fig. 8.1), and 10-hydroxycamptothecin (**4**, Fig. 8.1) [25–28]; the anticancer drug lead compounds podophyllotoxin (**5**, Fig. 8.1) and deoxypodophyllotoxin (**6**, Fig. 8.1) [29–31]; the antidepressant hypericin (**7**, Fig. 8.1) along with emodin (**8**, Fig. 8.1) [32, 33]; and the natural insecticides azadirachtin A (**9**, Fig. 8.1) and B (**10**, Fig. 8.1) [34]—have fueled the investigation on these groups of microorganisms. Although endophytes capable of synthesizing plant compounds are continually being discovered, it has not yet been possible to utilize them for the sustained production of the desired plant compounds [35].

There are many hypothesized mechanisms proposed for the production of plant secondary metabolites by associated microorganisms. In some cases, it was suggested that the interactions between endophytes and their plant host contribute to the production of these bioactive molecules [36]. In others, it has been shown that

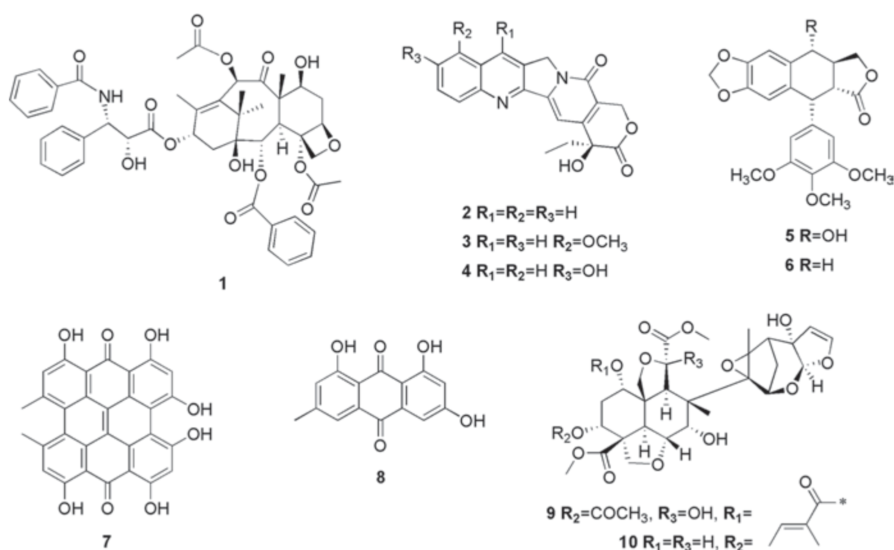

Fig. 8.1 Secondary metabolites common to endophytes and host plants

Table 8.1 Novel secondary metabolites from endophytic fungi

Compound	Endophytic fungus	Host	Biological activities	Reference
Polyketide and fatty acid derivatives (see Fig. 8.2)				
7-O-methyl neolambertellin (**11**)	*Coccomyces proteae*	*Disterigma humboldtii*	Anti-angiogenic activity	[109]
6,7-O, O-dimethyl neolambertellin (**12**)	*Coccomyces proteae*	*Disterigma humboldtii*	No anti-angiogenic activity	[109]
Photipyrone A (**13**)	*Pestalotiopsis photiniae*	*Roystonea regia* (H.B.K.) Cook	No tumor cell cytotoxicity	[110]
Photipyrone B (**14**)	*Pestalotiopsis photiniae*	*Roystonea regia* (H.B.K.) Cook	Tumor cell cytotoxicity	[110]
Phomopsinone A (**15**)	*Phomopsis* sp.	*Santolina chamaecyparissus*	Antifungal activity	[111]
Phomopsinone B (**16**)	*Phomopsis* sp.	*Santolina chamaecyparissus*	Antifungal activity, antibacterial, and algicidal activities	[111]
Phomopsinone C (**17**)	*Phomopsis* sp.	*Santolina chamaecyparissus*	Antifungal activity, antibacterial, and algicidal activities	[111]
Phomopsinone D (**18**)	*Phomopsis* sp.	*Santolina chamaecyparissus*	Antifungal activity	[111]
Pyrenocine J (**19**)	*Phomopsis* sp.	*Cistus salvifolius*	Antifungal, antibacterial,and algicidal activities	[112]
Pyrenocine K (**20**)	*Phomopsis* sp.	*Cistus salvifolius*	Antifungal, antibacterial,and algicidal activities	[112]
Pyrenocine L (**21**)	*Phomopsis* sp.	*Cistus salvifolius*	Antibacterial and algicidal activities, no antifungal activity	[112]
Pyrenocine M (**22**)	*Phomopsis* sp.	*Cistus salvifolius*	Antifungal, antibacterial,and algicidal activities	[112]
Seimatoporic acid A (**23**)	*Seimatosporium* sp.	*Epilobium hirsutum*	Strong antifungal activity in mixture	[113]
Seimatoporic acid B (**24**)	*Seimatosporium* sp.	*Epilobium hirsutum*	Strong antifungal activity in mixture	[113]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Pestalrone A (**25**)	*Pestalotiopsis karstenii*	*Camellia sasanqua*	No tumor cell cytotoxicity	[114]
Pestalrone B (**26**)	*Pestalotiopsis karstenii*	*Camellia sasanqua*	Tumor cell cytotoxicity	[114]
Mycoleptione (**27**)	*Mycoleptodiscus* sp.	*Tinospora crispa*	No antimicrobial activity	[115]
Helicascolide C (**28**)	*Daldinia eschscholzii*	*Gracilaria* sp.	Antifungal activity	[116]
Coryoctalactone A (**29**)	*Corynespora cassiicola*	*Laguncularia racemosa*	No tumor cell cytotoxicity, no antibacterial activity	[51]
Coryoctalactone B (**30**)	*Corynespora cassiicola*	*Laguncularia racemosa*	No tumor cell cytotoxicity, no antibacterial activity	[51]
Coryoctalactone C (**31**)	*Corynespora cassiicola*	*Laguncularia racemosa*	No tumor cell cytotoxicity, no antibacterial activity	[51]
Coryoctalactone D (**32**)	*Corynespora cassiicola*	*Laguncularia racemosa*	No tumor cell cytotoxicity, no antibacterial activity	[51]
Coryoctalactone E (**33**)	*Corynespora cassiicola*	*Laguncularia racemosa*	No tumor cell cytotoxicity, no antibacterial activity	[51]
2,4-dihydroxy-2′,6-diacetoxy-3′-methoxy-5′-methyl-diphenyl ether (**34**)	*Verticillium* sp.	*Rehmannia glutinosa*	Tumor cell cytotoxicity and antifungal activity	[117]
Pestalotiopyrone I (**35**)	*Pestalotiopsis virgatula*	*Sonneratia caseolaris*	No bacterial activity, no tumor cell cytotoxicity, and no insect larval toxicity	[118]
Pestalotiopyrone J (**36**)	*Pestalotiopsis virgatula*	*Sonneratia caseolaris*	No bacterial activity, no tumor cell cytotoxicity, and no insect larval toxicity	[118]
Pestalotiopyrone K (**37**)	*Pestalotiopsis virgatula*	*Sonneratia caseolaris*	No bacterial activity, no tumor cell cytotoxicity, and no insect larval toxicity	[118]
Pestalotiopyrone L (**38**)	*Pestalotiopsis virgatula*	*Sonneratia caseolaris*	No bacterial activity,no tumor cell cytotoxicity, and no insect larval toxicity	[118]
(6*S*,1′*S*,2′*S*)-hydroxypestalotin (**39**)	*Pestalotiopsis virgatula*	*Sonneratia caseolaris*	No bacterial activity, no tumor cell cytotoxicity, and no insect larval toxicity	[118]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
(*S*)-4-butoxy-6-(1-hydroxypentyl)-5,6-dihydro-2*H*-pyran-2-one (**40**)	*Phomopsis amygdali*	*Corylus avellana*	Tumor cell cytotoxicity	[119]
Tenuissimasatin (**41**)	*Alternaria tenuissima* (Nees & T. Nees: Fr.) Wiltshire	*Erythrophleum fordii* Oliver	No tumor cell cytotoxicity	[120]
Aspergillumarin A (**42**)	*Aspergillus* sp.	*Bruguiera gymnorrhiza*	Antibacterial activity	[121]
Aspergillumarin B (**43**)	*Aspergillus* sp.	*Bruguiera gymnorrhiza*	Antibacterial activity	[121]
Nigrosphaerin A (**44**)	*Nigrospora sphaerica*	*Vinca rosea*	No tumor cell cytotoxicity, no antileishmanial, antimalarial, antifungal, and antibacterial activities	[122]
Macrocarpon C (**45**)	*Fusarium tricinctum*	*Aristolochia paucinervis*	No antibacterial activity	[123]
(−)-Citreoisocoumarinol (**46**)	*Fusarium tricinctum*	*Aristolochia paucinervis*	No antibacterial activity	[123]
(3*S*,4a*R*,7*S*)-7,8-dihydroxy-3-methyl-3,4,10,5,6,7-hexahydro-1*H*-isochromen-1-one (**47**)	Related to the *Talaromyces*	*Cedrus deodara*	Tumor cell cytotoxicity. It induced apoptosis in HL-60 cells and caused significant microtubule inhibition in HL-60 cells	[124]
(1*S**, 3*R**, 5*R**)-3-methyl-2-oxabicyclo[3.3.1]nonan-7-one (**48**)	Related to the *Talaromyces*	*Cedrus deodara*	Tumor cell cytotoxicity. It induced apoptosis in HL-60 cells and caused significant microtubule inhibition in HL-60 cells	[124]
Pestaloficiol Q (**49**)	*Pestalotiopsis fici*	*Camellia sinensis*	No tumor cell cytotoxicity	[125]
Pestaloficiol R (**50**)	*Pestalotiopsis fici*	*Camellia sinensis*	No tumor cell cytotoxicity	[125]
Pestaloficiol S (**51**)	*Pestalotiopsis fici*	*Camellia sinensis*	No tumor cell cytotoxicity	[125]
(3a*R*,9*bR*)-6,9b-dihydroxy-8-methoxy-1-methylcyclopentene[c]isochromen-3,5-dione (**52**)	*Penicillium* sp.	*Riccardia multifida* (L.) S. Gray	Seed germination inhibitory activity	[126]
6-hydroxyl-deoxyfunicone (**53**)	*Penicillium* sp.	*Riccardia multifida* (L.) S. Gray	Seed germination inhibitory activity	[126]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
7-hydroxy-deoxytalaroflavone (**54**)	*Penicillium* sp.	*Ceriops tagal*	Antibacterial activity	[127]
Xylariacyclone A (**55**)	*Xylaria plebeja*	*Garcinia hombroniana*	No antifungal activity	[74]
Xylariacyclone B (**56**)	*Xylaria plebeja*	*Garcinia hombroniana*	No antifungal activity	[74]
Xylariaindanone (**57**)	*Xylaria* sp.	Garcinia hombroniana	No biological activity reported	[128]
Xylarellein (**58**)	*Xylaria* sp.	Garcinia hombroniana	No biological activity reported	[128]
Acremonide (**59**)	*Acremonium* sp.	*Rhizophora apiculata*	No antifungal activity	[129]
Acremonone A (**60**)	*Acremonium* sp.	*Rhizophora apiculata*	No antifungal activity	[129]
Acremonone B (**61**)	*Acremonium* sp.	*Rhizophora apiculata*	No antifungal activity	[129]
Acremonone C (**62**)	*Acremonium* sp.	*Rhizophora apiculata*	No antifungal activity	[129]
Acremonone D (**63**)	*Acremonium* sp.	*Rhizophora apiculata*	No antifungal activity	[129]
Acremonone E (**64**)	*Acremonium* sp.	*Rhizophora apiculata*	No antifungal activity	[129]
Acremonone F (**65**)	*Acremonium* sp.	*Rhizophora apiculata*	No antifungal activity	[129]
Acremonone G (**66**)	*Acremonium* sp.	*Rhizophora apiculata*	No antifungal activity	[129]
Acremonone H (**67**)	*Acremonium* sp.	*Rhizophora apiculata*	No antifungal activity	[129]
Embeurekol A (**68**)	*Embellisia eureka*	*Cladanthus arabicus*	No tumor cell cytotoxic, no antibacterial and antifungal activities	[130]
Embeurekol B (**69**)	*Embellisia eureka*	*Cladanthus arabicus*	No tumor cell cytotoxic, no antibacterial and antifungal activities	[130]
Embeurekol C (**70**)	*Embellisia eureka*	*Cladanthus arabicus*	No tumor cell cytotoxic, no antibacterial and antifungal activities	[130]
Microsphaerodiolin (**71**)	*Microsphaeropsis arundinis*	*Garcinia hombroniana*	No biological activities tested	[52]
Microsphaerophthalide A (**72**)	*Microsphaeropsis arundinis*	*Garcinia hombroniana*	Antifungal activity	[52]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Microsphaerophthalide B (**73**)	*Microsphaeropsis arundinis*	*Garcinia hombroniana*	No biological activities tested	[52]
Microsphaerophthalide C (**74**)	*Microsphaeropsis arundinis*	*Garcinia hombroniana*	No biological activities tested	[52]
Microsphaerophthalide D (**75**)	*Microsphaeropsis arundinis*	*Garcinia hombroniana*	No antifungal activity	[52]
Microsphaerophthalide E (**76**)	*Microsphaeropsis arundinis*	*Garcinia hombroniana*	Antifungal activity	[52]
Microsphaerophthalide F (**77**)	*Microsphaeropsis arundinis*	*Garcinia hombroniana*	No biological activities tested	[52]
Microsphaerophthalide G (**78**)	*Microsphaeropsis arundinis*	*Garcinia hombroniana*	No biological activities tested	[52]
(*S*)-8-Hydroxy-6-methoxy-4,5-dimethyl-3-methyleneisochroman-1-one (**79**)	*Xylaria* sp.	*Acanthus ilicifolius* L.	No tumor cell cytotoxicity	[131]
(*R*)-7-hydroxy-3-((*R*)-1-hydroxyethyl)-5-methoxy-3,4 dimethylisobenzofuran-1(3H)-one (**80**)	*Xylaria* sp.	*Acanthus ilicifolius* L.	No tumor cell cytotoxicity	[131]
4-(methoxymethyl)-7-methoxy-6-methyl-1(3*H*)-isobenzofuranone (**81**)	*Penicillium* sp.	*Avicennia* L.	Tumor cell cytotoxicity	[132]
Epicocconigrone A (**82**)	*Epicoccum nigrum*	*Mentha suaveolens*	Kinase and histone deacetylase inhibitory activities	[53]
Epicocconigrone B (**83**)	*Epicoccum nigrum*	*Mentha suaveolens*	No kinase inhibitory activity	[53]
3-Methoxyepicoccone B (**84**)	*Epicoccum nigrum*	*Mentha suaveolens*	Kinase inhibitory activity	[53]
3-Methoxyepicoccone (**85**)	*Epicoccum nigrum*	*Mentha suaveolens*	No kinase inhibitory activity	[53]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
2,3,4-Trihydroxy-6-(methoxymethyl)-5-methylbenzaldehyde (**86**)	*Epicoccum nigrum*	*Mentha suaveolens*	Kinase inhibitory activity	[53]
Epicoccolide A (**87**)	*Epicoccum* sp.	*Theobroma cacao*	Antibacterial and antifungal activities	[64]
Chaetosidone A (**88**)	*Chaetomium* sp.	*Zanthoxylum leprieurii*	Antibacterial activity and brine shrimp larvae cytotoxicity	[54]
4-hydroxy-5-methoxy-2-methylcyclo-pent-4-ene-1,3-dione (**89**)	*Aspergillus* sp.	*Cephalotaxus mannii*	No biological activities tested	[133]
New lactone (**90**)	*Aspergillus* sp.	*Cephalotaxus mannii*	No biological activities tested	[133]
9-dehydroxyeurotinone (**91**)	*Eurotium rubrum*	*Hibiscus tiliaceus*	Tumor cell cytotoxicity	[134]
Xestodecalactone D (**92**)	*Corynespora cassiicola*	*Laguncularia racemosa*	No tumor cell cytotoxicity, no antibacterial, antifungal and antitrypanosomal activities, and no protein kinase inhibitory activity	[135]
Xestodecalactone E (**93**)	*Corynespora cassiicola*	*Laguncularia racemosa*	No tumor cell cytotoxicity, no antibacterial, antifungal and antitrypanosomal activities, and no protein kinase inhibitory activity	[135]
Xestodecalactone F (**94**)	*Corynespora cassiicola*	*Laguncularia racemosa*	No tumor cell cytotoxicity, no antibacterial, antifungal and antitrypanosomal activities, and no protein kinase inhibitory activity	[135]
Corynesidone C (**95**)	*Corynespora cassiicola*	*Laguncularia racemosa*	No tumor cell cytotoxicity, no antibacterial, antifungal and antitrypanosomal activities, and no protein kinase inhibitory activity	[135]
Corynesidone D (**96**)	*Corynespora cassiicola*	*Gongronema latifolium*	No inhibitory activity on the Hsp90 chap-eroning machine, and no antiinflammatory activity	[55, 136]
Corynether B (**97**)	*Corynespora cassiicola*	*Gongronema latifolium*	No biological activities tested	[55]
Corynether lactone A (**98**)	*Corynespora cassiicola*	*Gongronema latifolium*	No biological activities tested	[55]
Wentiquinone A (**99**)	*Aspergillus wentii*	*Sargassum* sp.	No biological activities tested	[137]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Wentiquinone B (**100**)	*Aspergillus wentii*	*Sargassum* sp.	No biological activities tested	[137]
Wentiquinone C (**101**)	*Aspergillus wentii*	*Sargassum* sp.	No antioxidant activity	[138]
Excelsional (**102**)	*Phomopsis* sp.	*Endodesmia calophylloides*	Zoospore motility inhibitory and lytic effects	[56]
9-hydroxyphomopsidin (**103**)	*Phomopsis* sp.	*Endodesmia calophylloides*	Zoospore motility inhibitory and lytic effects	[56]
Xyolide (**104**)	*Xylaria feejeensis*	*Croton lechleri*	Antifungal activity	[139]
Mangiferaelactone (**105**)	*Pestalotiopsis mangiferae*	*Hyptis dilatata*	Antibacterial activity, no antimalarial nor antichagas activity, and no tumor cell cytotoxicity	[140]
Fusarone (**106**)	*Fusarium* sp.	*Melia azedarach* Linn.	No biological activities tested	[141]
Fusaroside (**107**)	*Fusarium* sp.	*Melia azedarach* Linn.	Brine shrimp larvae cytotoxicity	[57]
R-3-hydroxyundecanoic acid methylester-3-*O*-α-L-rhamnopyranoside (**108**)	*Guignardia* sp.	*Scyphiphora hydrophyllacea* Gaertn. F.	Antibacterial activity	[142]
Diaporthemin A (**109**)	*Diaporthe melonis*	*Annona squamosa*	No antibacterial activity	[58]
Diaporthemin B (**110**)	*Diaporthe melonis*	*Annona squamosa*	No antibacterial activity	[58]
Isorhodoptilometrin-1-methyl ether (**111**)	*Aspergillus versicolor*	*Halimeda opuntia*	Antibacterial activity, tumor cell cytotoxicity, and no hepatitis C virus protease inhibitory activity	[143]
(2*R*,3*S*)-7-ethyl-1,2,3,4-tetrahydro-2,3,8-trihydroxy-6-Methoxy-3-methyl-9,10-anthracenedione (**112**)	*Phomopsis* sp.	*Rhizophora apiculata*	Antibacterial activity and tumor cell cytotoxicity	[144]
Asperversin A (**113**)	*Aspergillus versicolor*	*Sargassum thunbergii*	No bacterial and antifungal activities, and no brine shrimp larvae cytotoxicity	[145]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Monodeacetylphomoxanthon B (**114**)	*Phomopsis longicolla*	Unknown	Antibacterial activity	[146]
3-O-(6-O-α-L-arabinopyranosyl)-β-D-glucopyranosyl-1,4-dimethoxyxanthone (**115**)	*Phomopsis* sp.	*Excoecaria agallocha*	Tumor cell cytotoxicity	[147]
Talaroxanthone (**116**)	*Talaromyces* sp.	*Duguetia stelechantha*	No biological activities tested	[148]
Dicerandrol D (**117**)	*Diaporthe* sp.	*Avicennia marina, Kandelia obovata, and Lumnitzera racemosa*	Antimalarial activity and tumor cell cytotoxicity	[149]
Diaporthochromone A (**118**)	*Diaporthe* sp.	*Avicennia marina, Kandelia obovata, and Lumnitzera racemosa*	No antimalarial activity	[149]
Diaporthochromone B (**119**)	*Diaporthe* sp.	*Avicennia marina, Kandelia obovata, and Lumnitzera racemosa*	No antimalarial activity	[149]
(2*E*,4*E*)-dimethyldeca-2,4-dienoic acid (**120**)	*Xylaria* sp.	*Avicennia marina, Kandelia obovata, and Lumnitzera racemosa*	No antimalarial activity	[149]
Sydoxanthone A (**121**)	*Aspergillus sydowii*	*Scapania ciliata* S. Lac	No immunosuppressive activity	[150]
Sydoxanthone B (**122**)	*Aspergillus sydowii*	*Scapania ciliata* S. Lac	No immunosuppressive activity	[150]
13-O-acetylsydowinin B (**123**)	*Aspergillus sydowii*	*Scapania ciliata* S. Lac	No immunosuppressive activity	[150]
Pestalotether A (**124**)	*Pestalotiopsis* sp.	*Rhizophora apiculata*	Antifungal activity	[59]
Pestalotether B (**125**)	*Pestalotiopsis* sp.	*Rhizophora apiculata*	Antifungal activity	[59]
Pestalotether C (**126**)	*Pestalotiopsis* sp.	*Rhizophora apiculata*	No biological activities tested	[59]
Pestalotether D (**127**)	*Pestalotiopsis* sp.	*Rhizophora apiculata*	No antifungal activity	[59]
Pestaloxanthone (**128**)	*Pestalotiopsis* sp.	*Rhizophora apiculata*	No antifungal activity	[59]
Pestalolide (**129**)	*Pestalotiopsis* sp.	*Rhizophora apiculata*	Antifungal activity	[59]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
4-hentriacontyl-dihydrofuran-2-one (**130**)	*Annulohypoxylon squamulosum*	*Cinnamomum* sp.	No biological activities tested	[151]
Annulosquamulin (**131**)	*Annulohypoxylon squamulosum*	*Cinnamomum* sp.	Tumor cell cytotoxicity	[60]
Pestalafuranone A (**132**)	*Pestalotiopsis besseyi*	Unknown	HIV-1 replication inhibitory activity and no antifungal activity	[152]
Pestalafuranone B (**133**)	*Pestalotiopsis besseyi*	Unknown	HIV-1 replication inhibitory activity and no antifungal	[152]
Pestalafuranone C (**134**)	*Pestalotiopsis besseyi*	Unknown	HIV-1 replication inhibitory activity and no antifungal	[152]
Pestalafuranone D (**135**)	*Pestalotiopsis besseyi*	Unknown	Antifungal activity, and no HIV-1 replication inhibitory activity	[152]
Pestalafuranone E (**136**)	*Pestalotiopsis besseyi*	Unknown	Antifungal activity, and no HIV-1 replication inhibitory activity	[152]
Pestalafuranone F (**137**)	*Nigrospora* sp.	*Saccharum arundinaceum* Retz.	No tumor cell cytotoxicity	[153]
Pestalafuranone G (**138**)	*Nigrospora* sp.	*Saccharum arundinaceum* Retz.	No tumor cell cytotoxicity	[153]
Pestalafuranone H (**139**)	*Nigrospora* sp.	*Saccharum arundinaceum* Retz.	No tumor cell cytotoxicity	[153]
Pestalafuranone I (**140**)	*Nigrospora* sp.	*Saccharum arundinaceum* Retz.	No tumor cell cytotoxicity	[153]
Pestalafuranone J (**141**)	*Nigrospora* sp.	*Saccharum arundinaceum* Retz.	No tumor cell cytotoxicity	[153]
Ficipyrone A (**142**)	*Pestalotiopsis fici*	*Camellia sinensis*	Antifungal activity	[154]
Ficipyrone B (**143**)	*Pestalotiopsis fici*	*Camellia sinensis*	No antifungal activity	[154]
Ficifuranone A (**144**)	*Pestalotiopsis fici*	*Camellia sinensis*	No antifungal activity	[154]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Ficifuranone B (**145**)	*Pestalotiopsis fici*	*Camellia sinensis*	No antifungal activity	[154]
Palmarumycin EG1 (**146**)	*Edenia gomezpompae*	*Callicarpa acuminata*	No biological activities tested	[61]
Preussomerin EG4 (**147**)	*Edenia gomezpompae*	*Callicarpa acuminata*	Phytotoxic activity	[61]
Aspergispiroketal (**148**)	*Asperilligus* sp.	*Huperzia serrata*	No biological activities tested	[155]
2,3-didehydro-19α-hydroxy-14-epicochlioquinone B (**149**)	*Nigrospora* sp.	*Pongamia pinnata*	Antibacterial activity and tumor cell cytotoxicity	[62]
6-O-desmethyldechlorogriseofulvin (**150**)	*Nigrospora* sp.	*Pongamia pinnata*	Antifungal activity	[62]
6′-hydroxygriseofulvin (**151**)	*Nigrospora* sp.	*Pongamia pinnata*	No antifungal activity	[62]
Ilanefuranone (**152**)	*Annulohypoxylon ilanense*	*Cinnamomum* sp.	Antibacterial activity	[156]
Annulofuranone (**153**)	*Annulohypoxylon* sp.	Unknown	No biological activities tested	[157]
Penicitriketo (**154**)	*Penicillium citrinum*	*Salicornia herbacea* Torr.	Antibacterial activity, and no antioxidant activity	[158]
Flavodonfuran (**155**)	*Flavodon flavus*	*Rhizophora apiculata*	No antibacterial activity	[159]
7-dehydroxyl-zinniol (**156**)	*Alternaria solani*	*Aconitum transsectum*	Anti-Hepatitis B virus activity	[160]
Epicolactone (**157**)	*Epicoccum nigrum*/*Epicoccum* sp.	*Saccharum officinarum*/ *Theobroma cacao*	Antibacterial and antifungal activities	[63, 64]
Biscogniazaphilone A (**158**)	*Biscogniauxia formosana*	*Cinnamomum* sp.	Antibacterial activity	[65]
Biscogniazaphilone B (**159**)	*Biscogniauxia formosana*	*Cinnamomum* sp.	Antibacterial activity	[65]
Cytosporin F (**160**)	*Pestalotiopsis theae*	*Turraeanthus longipes*	No tumor cell cytotoxicity, and no antibacterial activity	[161]
Cytosporin G (**161**)	*Pestalotiopsis theae*	*Turraeanthus longipes*	No tumor cell cytotoxicity, and no antibacterial activity	[161]
Cytosporin H (**162**)	*Pestalotiopsis theae*	*Turraeanthus longipes*	No tumor cell cytotoxicity, and no antibacterial activity	[161]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Cytosporin I (**163**)	*Pestalotiopsis theae*	*Turraeanthus longipes*	No tumor cell cytotoxicity, and no antibacterial activity	[161]
Cytosporin J (**164**)	*Pestalotiopsis theae*	*Turraeanthus longipes*	No tumor cell cytotoxicity, and no antibacterial activity	[161]
Cytosporin K (**165**)	*Pestalotiopsis theae*	*Turraeanthus longipes*	No tumor cell cytotoxicity, and no antibacterial activity	[161]
10′-deoxy-10′α-hydroxyascochlorin (**166**)	*Acremonium* sp.	*Ephedra trifurca*	Cancer cells migration inhibitory activity	[162]
Cephalanone A (**167**)	*Graphiopsis chlorocephala*	*Paeonia lactiflora*	No biological activities tested	[107]
Cephalanone B (**168**)	*Graphiopsis chlorocephala*	*Paeonia lactiflora*	No biological activities tested	[107]
Cephalanone C (**169**)	*Graphiopsis chlorocephala*	*Paeonia lactiflora*	No biological activities tested	[107]
Cephalanone D (**170**)	*Graphiopsis chlorocephala*	*Paeonia lactiflora*	No biological activities tested	[107]
Cephalanone E (**171**)	*Graphiopsis chlorocephala*	*Paeonia lactiflora*	No biological activities tested	[107]
Cephalanone F (**172**)	*Graphiopsis chlorocephala*	*Paeonia lactiflora*	No biological activities tested	[107]
Mycosphine A (**173**)	*Mycosphaerella* sp.	*Aloe arborescens*	No biological activities tested	[163]
Mycosphines B (**174**)	*Mycosphaerella* sp.	*Aloe arborescens*	No biological activities tested	[163]
Mycosphine C (**175**)	*Mycosphaerella* sp.	*Aloe arborescens*	No biological activities tested	[163]
Rhodostegone (**176**)	*Penicillium polonicum*	*Lysidice rhodostegia*	No tumor cell cytotoxicity	[164]
Tanzawaic acid G (**177**)	*Penicillium citrinum*	*Ceratonia siliqua* L.	No tumor cell cytotoxicity	[165]
Tanzawaic acid H (**178**)	*Penicillium citrinum*	*Ceratonia siliqua* L.	No tumor cell cytotoxicity	[165]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
6-methylcurvulinic acid (**179**)	*Penicillium citrinum*	*Ceratonia siliqua* L.	No tumor cell cytotoxicity	[165]
Ginsenocin (**180**)	*Penicillium melinii*	*Panax ginseng*	Tumor cell cytotoxicity	[166]
1-(2,6-dihydroxyphenyl)pentan-1-one (**181**)	*Cryptosporiopsis* sp.	*Clidemia hirta*	Antibacterial activity	[167]
(*Z*)-1-(2-(2-butyryl-3-hydroxyphenoxy)-6-hydroxyphenyl)-3-hydroxybut-2-en-1-one (**182**)	*Cryptosporiopsis* sp.	*Clidemia hirta*	Antibacterial activity	[167]
Chloropestolide B (**183**)	*Pestalotiopsis fici*	*Camellia sinensis*	Tumor cell cytotoxicity	[66]
Chloropestolide C (**184**)	*Pestalotiopsis fici*	*Camellia sinensis*	No tumor cell cytotoxicity	[66]
Chloropestolide D (**185**)	*Pestalotiopsis fici*	*Camellia sinensis*	No tumor cell cytotoxicity	[66]
Chloropestolide E (**186**)	*Pestalotiopsis fici*	*Camellia sinensis*	No tumor cell cytotoxicity	[66]
Chloropestolide F (**187**)	*Pestalotiopsis fici*	*Camellia sinensis*	No tumor cell cytotoxicity	[66]
Chloropestolide G (**188**)	*Pestalotiopsis fici*	*Camellia sinensis*	No tumor cell cytotoxicity	[66]
Cytosporone T (**189**)	*Phomopsis* sp.	*Scaevola hainanensis*	No neuraminidase inhibitory activity	[168]
Cytosporone U (**190**)	*Phomopsis* sp.	*Scaevola hainanensis*	No neuraminidase inhibitory activity	[168]
9*S*,11*R*-(+)-ascosalitoxin (**191**)	Unidentified strain	*Hintonia latif* lora	No calmodulin inhibitory activity	[169]
(*E*)-7-(2-hydroxy-4-(hydroxymethyl) phenyl)-2-methyloct-6-enoic acid (**192**)	*Phomopsis* sp.	*Daphniphyllum longeracemosum*	No biological activities tested	[170]
Dothideomycetide A (**193**)	*Dothideomycete* sp.	*Tiliacora triandra*	Tumor cell cytotoxicity and antibacterial activity	[67]
Dothideomycetone A (**194**)	*Dothideomycete* sp.	*Tiliacora triandra*	Tumor cell cytotoxicity	[67]
Dothideomycetone B (**195**)	*Dothideomycete* sp.	*Tiliacora triandra*	No cytotoxic activity	[67]
Paeciloside A (**196**)	*Paecilomyces* sp.	*Enantia chlorantha* Oliv.	Brine shrimp larvae cytotoxicity and bacterial activity	[171]
Expansol C (**197**)	*Penicillium expansum*	*Excoecaria agallocha*	Tumor cell cytotoxicity	[172]
Expansol D (**198**)	*Penicillium expansum*	*Excoecaria agallocha*	No biological activities tested	[172]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Expansol E (**199**)	*Penicillium expansum*	*Excoecaria agallocha*	Tumor cell cytotoxicity	[172]
Expansol F (**200**)	*Penicillium expansum*	*Excoecaria agallocha*	No biological activities tested	[172]
3-O-methyldiorcinol (**201**)	*Penicillium expansum*	*Excoecaria agallocha*	No tumor cell cytotoxicity	[172]
(3*S*)-3,6,7-trihydroxy-α-tetralone (**202**)	*Phoma* sp.	*Arisaema erubescens*	Antifungal activity	[173]
Coniothyrinone A (**203**)	*Coniothyrium* sp.	*Salsola oppostifolia*	Antibacterial and antifungal activity	[174]
Coniothyrinone B (**204**)	*Coniothyrium* sp.	*Salsola oppostifolia*	Antibacterial activity	[174]
Coniothyrinone C (**205**)	*Coniothyrium* sp.	*Salsola oppostifolia*	Antibacterial activity	[174]
Coniothyrinone D (**206**)	*Coniothyrium* sp.	*Salsola oppostifolia*	Antibacterial activity	[174]
Xylacinic acid A (**207**)	*Xylaria cubensis*	*Bruguiera parviflora*	No tumor cell cytotoxic and no antibacterial activities	[175]
Xylacinic acid B (**208**)	*Xylaria cubensis*	*Bruguiera parviflora*	No tumor cell cytotoxic and no antibacterial activities	[175]
Rubratoxin C (**209**)	*Penicillium* sp.	*Annonam muricata*	Tumor cell cytotoxicity	[176]
Diglucotol (**210**)	*Fusarium equiseti*	*Salicornia bigelovii* Torr.	Tumor cell cytotoxicity	[177]
Terpenoid derivatives (see Fig. 8.3)				
20-hydroxylergosta-4,6,8(14),22-tetraen-3-one (**211**)	*Alternaria solani*	*Aconitum transsectum*	No anti-Hepatitis B virus activity	[160]
3β-acetoxy-15α-hydroxylanosta-8,24-dien-21-oic acid (**212**)	*Ceriporia lacerate*	*Huperzia serrata*	No biological activities tested	[178]
3β-acetoxylanosta-7,9(11),24-trien-21-oic acid (**213**)	*Ceriporia lacerate*	*Huperzia serrata*	No biological activities tested	[178]
Norcyclocitrinol A (**214**)	*Penicillium chrysogenum*	*Huperzia serrata*	No tumor cell cytotoxicity	[179]
Erythro-11a-hydroxyneocyclocitrinol (**215**)	*Penicillium chrysogenum*	*Huperzia serrata*	No tumor cell cytotoxicity	[179]
Pesudocyclocitrinol A (**216**)	*Penicillium chrysogenum*	*Huperzia serrata*	No tumor cell cytotoxicity	[179]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
1α,7α-dihydroxyconfertifolin (**217**)	Unidentified strain	*Dracaena cambodiana*	No antibacterial activity	[180]
Diaporol A (**218**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[69]
Diaporol B (**219**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[69]
Diaporol C (**220**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[69]
Diaporol D (**221**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[69]
Diaporol E (**222**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[69]
Diaporol F (**223**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[69]
Diaporol D (**224**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[69]
Diaporol H (**225**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[69]
Diaporol I (**226**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[69]
Aspergiloid A (**227**)	*Aspergillus* sp.	*Ginkgo biloba*	No neuraminidase inhibitory activity	[181]
Aspergiloid B (**228**)	*Aspergillus* sp.	*Ginkgo biloba*	No neuraminidase inhibitory activity	[181]
Aspergiloid C (**229**)	*Aspergillus* sp.	*Ginkgo biloba*	No neuraminidase inhibitory activity	[181]
Aspergiloid D (**230**)	*Aspergillus* sp.	*Ginkgo biloba*	No neuraminidase inhibitory activity	[181]
Aspergiloid E (**231**)	*Aspergillus* sp.	*Ginkgo biloba*	No tumor cell cytotoxicity	[182]
Aspergiloid F (**232**)	*Aspergillus* sp.	*Ginkgo biloba*	No tumor cell cytotoxicity	[182]
Aspergiloid G (**233**)	*Aspergillus* sp.	*Ginkgo biloba*	No tumor cell cytotoxicity	[182]
Aspergiloid H (**234**)	*Aspergillus* sp.	*Ginkgo biloba*	No tumor cell cytotoxicity	[182]
Tricycloalternarene F (**235**)	*Guignardia mangiferae*	*Viguiera arenaria*	No biological activities tested	[183]
Guignardone D (**236**)	*Guignardia mangiferae*	*Viguiera arenaria*	No biological activities tested	[183]
Guignardone E (**237**)	*Guignardia mangiferae*	*Viguiera arenaria*	No biological activities tested	[183]
Guignardone F (**238**)	*Guignardia* sp.	*Scyphiphora hydrophyllacea* Gaertn. F.	No antibacterial activity	[184]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Guignardone G (**239**)	*Guignardia* sp.	*Scyphiphora hydrophyllacea* Gaertn. F.	No antibacterial activity	[184]
Guignardone H (**240**)	*Guignardia* sp.	*Scyphiphora hydrophyllacea* Gaertn. F.	No antibacterial activity	[184]
Guignardone I (**241**)	*Guignardia* sp.	*Scyphiphora hydrophyllacea* Gaertn. F.	Antibacterial activity	[184]
Coibanole A (**242**)	*Pycnoporus sanguineus*	*Desmotes incomparabilis*	No biological activities tested	[185]
Coibanole B (**243**)	*Pycnoporus sanguineus*	*Desmotes incomparabilis*	No biological activities tested	[185]
Coibanole C (**244**)	*Pycnoporus sanguineus*	*Desmotes incomparabilis*	No biological activities tested	[185]
Albican-11,14-diol (**245**)	*Aspergillus versicolor*	*Codium fragile*	Antibacterial and brine shrimp larvae cytotoxicity	[186]
Eremophilane sesquiterpene analogue (**246**)	*Xylaria* sp.	Acanthus ilicifolius L.	No tumor cell cytotoxicity, and no α-glucosidase inhibitory activity	[187]
Eremophilane sesquiterpene analogue (**247**)	*Xylaria* sp.	Acanthus ilicifolius L.	No tumor cell cytotoxicity	[187]
Eremophilane sesquiterpene analogue (**248**)	*Xylaria* sp.	Acanthus ilicifolius L.	No tumor cell cytotoxicity	[187]
Xylarenone F (**249**)	*Camarops* sp.	*Alibertia macrophylla*	Anti-inflammatory and antioxidant activities	[188]
Xylarenone G (**250**)	*Camarops* sp.	*Alibertia macrophylla*	Anti-inflammatory and antioxidant activities	[188]
MBJ-0011 (**251**)	*Apiognomonia* sp.	Unidentified host plant	Tumor cell cytotoxicity	[70]
MBJ-0012 (**252**)	*Apiognomonia* sp.	Unidentified host plant	Tumor cell cytotoxicity	[70]
MBJ-0013 (**253**)	*Apiognomonia* sp.	Unidentified host plant	Tumor cell cytotoxicity	[70]
Botryosphaerin F (**254**)	*Aspergillus terreus*	*Brugnieria gymnoihiza* (L.) Savigny	Tumor cell cytotoxicity	[189]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Pestalotiopen A (**255**)	*Pestalotiopsis* sp.	*Rhizophora mucronata*	Antibacterial activity	[71]
Pestalotiopen B (**256**)	*Pestalotiopsis* sp.	*Rhizophora mucronata*	No antibacterial activity	[71]
Arigsugacin I (**257**)	*Penicillium* sp.	*Kandelia candel*	Acetylcholinesterase inhibitory activity	[190]
Asperterpenoid A (**258**)	*Aspergillus* sp.	*Sonneratia apetala*	*Mycobacterium tuberculosis* tyrosine phosphatase B inhibitory activity	[191]
Xylarinonericin A (**259**)	*Xylaria plebeja*	*Garcinia hombroniana*	No antifungal activity	[74]
Xylarinonericin B (**260**)	*Xylaria plebeja*	*Garcinia hombroniana*	No antifungal activity	[74]
Xylarinonericin C (**261**)	*Xylaria plebeja*	*Garcinia hombroniana*	No antifungal activity	[74]
Perenniporin A (**262**)	*Perenniporia tephropora*	*Taxus chinensis* var. *mairei*	Tumor cell cytotoxicity and antifungal activity	[192]
Emericellene A (**263**)	*Emericella* sp.	*Astragalus lentiginosus*	No tumor cell cytotoxicity	[193]
Emericellene B (**264**)	*Emericella* sp.	*Astragalus lentiginosus*	No tumor cell cytotoxicity	[193]
Emericellene C (**265**)	*Emericella* sp.	*Astragalus lentiginosus*	No tumor cell cytotoxicity	[193]
Emericellene D (**266**)	*Emericella* sp.	*Astragalus lentiginosus*	No tumor cell cytotoxicity	[193]
Emericellene E (**267**)	*Emericella* sp.	*Astragalus lentiginosus*	No tumor cell cytotoxicity	[193]
10,11-dihydrocyclonerotriol (**268**)	*Trichoderma longibrachiatum*	*Azadirachta indica*	Antifungal activity	[194]
3α,3β,10β-trimethyl-decahydroazuleno[6, 7]furan-8,9,14-triol (**269**)	*Chaetomium* sp.	*Aquilaria sinensis* (Lour.) Spreng.	No antibacterial activity	[195]
1β,5α,6α,14-tetraacetoxy-9α-benzoyloxy-7β*H*-eudesman-2β,11-diol (**270**)	*Pestalotiopsis* sp.	*Sargassum horneri*	Tyrosinase inhibitory activity	[196]
4α,5α-diacetoxy-9α-benzoyloxy-7β*H*-eudesman-1β,2β,11,14-tetraol (**271**)	*Pestalotiopsis* sp.	*Sargassum horneri*	Tyrosinase inhibitory activity	[196]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Phenylpropanoid derivatives (see Fig. 8.4)				
4″-deoxy-3-hydroxyterphenyllin (**272**)	*Aspergillus* sp.	*Ginkgo biloba*	No neuraminidase inhibitory activity	[181]
4″-deoxy-5′-desmethylterphenyllin (**273**)	*Aspergillus* sp.	*Ginkgo biloba*	No neuraminidase inhibitory activity	[181]
5′-desmethylterphenyllin (**274**)	*Aspergillus* sp.	*Ginkgo biloba*	No neuraminidase inhibitory activity	[181]
4″-deoxycandidusin A (**275**)	*Aspergillus* sp.	*Ginkgo biloba*	Neuraminidase inhibitory activity	[181]
4,5-dimethoxycandidusin A (**276**)	*Aspergillus* sp.	*Ginkgo biloba*	No neuraminidase inhibitory activity	[181]
Terphenolide (**277**)	*Aspergillus* sp.	*Ginkgo biloba*	Neuraminidase inhibitory activity	[181]
Asperterone B (**278**)	*Aspergillus terreus*	*Malus halliana*	Tumor cell cytotoxicity	[197]
Asperterone C (**279**)	*Aspergillus terreus*	*Malus halliana*	Tumor cell cytotoxicity	[197]
Pestalochromone A (**280**)	*Pestalotiopsis* sp.	*Rhizophora apiculata*	No biological activities tested	[59]
Pestalochromone B (**281**)	*Pestalotiopsis* sp.	*Rhizophora apiculata*	No biological activities tested	[59]
Pestalochromone C (**282**)	*Pestalotiopsis* sp.	*Rhizophora apiculata*	No antifungal activity	[59]
3-hydroxy-4-(4-hydroxyphenyl)-5-methoxycarbonyl-5-(4-hydroxy-3-formylbenzyl)-2,5-dihydro-2-furanone (**283**)	*Aspergillus terreus* var. *boedijnii* (Blochwitz)	*Laurencia ceylanica* J. Agardh	β-glucuronidase inhibitory activity	[76]
Ilanenoid (**284**)	*Annulohypoxylon ilanense*	*Cinnamomum* sp.	No antibacterial activity	[156]
Chlorflavonin A (**285**)	*Aspergillus* sp.	*Ginkgo biloba*	No tumor cell cytotoxicity	[182]
N-containing compounds (see Fig. 8.5)				
Pullularin E (**286**)	*Bionectria ochroleuca*	*Sonneratia caseolaris*	No biological activities tested	[198]
Pullularin F (**287**)	*Bionectria ochroleuca*	*Sonneratia caseolaris*	No tumor cell cytotoxicity	[198]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Subenniatin A (**288**)	*Fusarium tricinctum*	*Aristolochia paucinervis*	No tumor cell cytotoxicity, and no antibacterial activity	[104]
Subenniatin A (**289**)	*Fusarium tricinctum*	*Aristolochia paucinervis*	No tumor cell cytotoxicity, and no antibacterial activity	[104]
12β-hydroxy-13α-methoxyverruculogen TR-2 (**290**)	*Aspergillus fumigatus*	*Melia azedarach*	Brine shrimp larvae cytotoxicity, antifungal activity, and no armyworm antifeedant activity	[199]
3-hydroxyfumiquinazoline A (**291**)	*Aspergillus fumigatus*	*Melia azedarach*	Brine shrimp larvae cytotoxicity, antifungal activity, and no armyworm antifeedant activity	[199]
Rel-(8*R*)-9-hydroxy-8-methoxy-18-epi-fumitremorgin C (**292**)	*Aspergillus fumigatus*	*Erythrophloeum fordii* Oliv.	No anti-inflammatory activity, and no tumor cell cytotoxicity	[200]
Rel-(8*S*)-19,20-dihydro-9,20-dihydroxy-8-methoxy-9,18-di-epi-fumitremorgin C (**293**)	*Aspergillus fumigatus*	*Erythrophloeum fordii* Oliv.	No anti-inflammatory activity, no tumor cell cytotoxicity	[200]
Rel-(8*S*,19*S*)-19,20-Dihydro-9,19,20-trihydroxy-8-methoxy-9-epi-fumitremorgin C (**294**)	*Aspergillus fumigatus*	*Erythrophloeum fordii* Oliv.	No anti-inflammatory activity, and no tumor cell cytotoxicity	[200]
(3*S*,8*S*,9*S*,18*S*)-8,9-dihydroxyspiro-tryprostatin A (**295**)	*Aspergillus fumigatus*	*Erythrophloeum fordii* Oliv.	No anti-inflammatory activity, and no tumor cell cytotoxicity	[200]
Verruculogen TR2-derivative (**296**)	*Aspergillus* sp.	*Cephalotaxus mannii*	No biological activities tested	[133]
12-demethyl-12-oxo-eurotechinulin B (**297**)	*Eurotium rubrum*	*Hibiscus tiliaceus*	Tumor cell cytotoxicity	[134]
9ξ-*O*-2(2,3-dimethylbut-3-enyl)brevianamide Q (**298**)	*Aspergillus versicolor*	*Sargassum thunbergii*	No bacterial and antifungal activities, and no brine shrimp larvae cytotoxicity	[145]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
3,1′-didehydro-3[2″(3‴,3‴-dimethyl-prop-2-enyl)-3″-indolylmethylene]-6-methyl pipera-zine-2,5-dione (**299**)	*Penicillium chrysogenum*	*Porteresia coarctata* (Roxb.)	Antibacterial activity	[79]
Phomazine A (**300**)	*Phoma* sp.	*Kandelia candel*	No tumor cell cytotoxicity	[201]
Phomazine B (**301**)	*Phoma* sp.	*Kandelia candel*	Tumor cell cytotoxicity	[201]
Phomazine C (**302**)	*Phoma* sp.	*Kandelia candel*	No tumor cell cytotoxicity	[201]
Secoemestrin D (**303**)	*Emericella* sp.	*Astragalus lentiginosus*	Tumor cell cytotoxicity	[193]
3-hydroxy-2-methoxy-5-methylpyridin-2(1H)-one (**304**)	*Botryosphaeria dothidea*	*Melia azedarach* L.	No tumor cell cytotoxicity, no antimicrobial and antioxidant activities, and no brine shrimp larvae cytotoxicity	[202]
3-hydroxy-N-(1-hydroxy-3-methylpen-tan-2-yl)-5-oxohexanamide (**305**)	*Botryosphaeria dothidea*	*Melia azedarach* L.	No tumor cell cytotoxicity, no antimicrobial and antioxidant activities, and no brine shrimp larvae cytotoxicity	[202]
Farinomalein C (**306**)	Unidentified strain	*Avicennia marina*	No tumor cell cytotoxicity, and no antibac-terial activity	[203]
Farinomalein D (**307**)	Unidentified strain	*Avicennia marina*	No tumor cell cytotoxicity, and no antibac-terial activity	[203]
Farinomalein E (**308**)	Unidentified strain	*Avicennia marina*	No tumor cell cytotoxicity, and no antibac-terial activity	[203]
(3R)-5,7-dihydroxy-3-methylisoindolin-1-one (**309**)	Unidentified strain	*Avicennia marina*	No tumor cell cytotoxicity, and no antibac-terial activity	[203]
Marinamide (**310**)	Unidentified strain	Unidentified host plant	Tumor cell cytotoxicity	[103, 204]
Methyl marinamide (**311**)	Unidentified strain	Unidentified host plant	Tumor cell cytotoxicity	[103, 204]
Trichalasin C (**312**)	*Trichoderma gamsii*	*Panax notoginseng* (BurK.) F.H.Chen	No tumor cell cytotoxicity	[205]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Trichalasin D (**313**)	*Trichoderma gamsii*	*Panax notoginseng* (BurK.) F.H.Chen	No tumor cell cytotoxicity	[205]
Trichalasin E (**314**)	*Trichoderma gamsii*	*Panax notoginseng* (BurK.) F.H. Chen	Tumor cell cytotoxicity	[86]
Trichalasin F (**315**)	*Trichoderma gamsii*	*Panax notoginseng* (BurK.) F.H. Chen	Tumor cell cytotoxicity	[86]
Trichalasin H (**316**)	*Trichoderma gamsii*	*Panax notoginseng* (BurK.) F.H. Chen	Tumor cell cytotoxicity	[86]
Chaetoglobosin Vb (**317**)	*Chaetomium globosum*	*Ginkgo biloba*	No antifungal and antibacterial activity	[87]
Aniquinazoline A (**318**)	*Aspergillus nidulans*	*Rhizophora stylosa*	Brine shrimp larvae cytotoxicity, no tumor cell cytotoxicity, and no antibacterial activity	[88]
Aniquinazoline B (**319**)	*Aspergillus nidulans*	*Rhizophora stylosa*	Brine shrimp larvae cytotoxicity, no tumor cell cytotoxicity, and no antibacterial activity	[88]
Aniquinazoline C (**320**)	*Aspergillus nidulans*	*Rhizophora stylosa*	Brine shrimp larvae cytotoxicity, no tumor cell cytotoxicity, and no antibacterial activity	[88]
Aniquinazoline D (**321**)	*Aspergillus nidulans*	*Rhizophora stylosa*	Brine shrimp larvae cytotoxicity, no tumor cell cytotoxicity, and no antibacterial activity	[88]
Cryptosporioptide (**322**)	*Cryptosporiopsis* sp.	*Viburnum tinus*	Antibacterial and lipoxygenase inhibitory activities, no antifungal and algicidal, and no acetylcholinesterase and butyrylcholinesterase inhibitory activities	[89]
Mycoleptodiscin A (**323**)	*Mycoleptodiscus* sp.	*Desmotes incomparabilis*	No biological activities tested	[206]
Mycoleptodiscin B (**324**)	*Mycoleptodiscus* sp.	*Desmotes incomparabilis*	Tumor cell cytotoxicity	[206]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Citriquinochroman (**325**)	*Penicillium citrinum*	*Ceratonia siliqua* L.	Tumor cell cytotoxicity, and no antibacterial activity	[165]
8-methoxy-3,5- dimethylisoquinolin-6-ol (**326**)	*Penicillium citrinum*	*Ceratonia siliqua* L.	No tumor cell cytotoxicity and no antibacterial activity	[165]
Podocarpiamide (**327**)	*Pestalotiopsis podocarpi*	*Podocarpus macrophyllus*	No biological activities tested	[207]
1-methoxy-1H-indol-3- ethanol (**328**)	*Pestalotiopsis podocarpi*	*Podocarpus macrophyllus*	No biological activities tested	[207]
Isochromophilone X (**329**)	*Diaporthe* sp.	*Rhizophora stylosa*	Tumor cell cytotoxicity	[208]
Isochromophilone XI (**330**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[208]
Isochromophilone XII (**331**)	*Diaporthe* sp.	*Rhizophora stylosa*	No tumor cell cytotoxicity	[208]
Bipolamide A (**332**)	*Bipolaris* sp.	*Gynura hispida*	No antifungal and antibacterial activities	[92]
Bipolamide B (**333**)	*Bipolaris* sp.	*Gynura hispida*	Antifungal activity, and no antibacterial activity	[92]
Campyrone A (**334**)	*Aspergillus niger*	*Zanthoxylum lemairei*	Brine shrimp larvae cytotoxicity, no antibacterial and antifungal activities	[209]
Campyrone B (**335**)	*Aspergillus niger*	*Zanthoxylum lemairei*	Brine shrimp larvae cytotoxicity, no antibacterial and antifungal activities	[209]
Campyrone C (**336**)	*Aspergillus niger*	*Zanthoxylum lemairei*	Brine shrimp larvae cytotoxicity, no antibacterial and antifungal activities	[209]
Embellicine A (**337**)	*Embellisia eureka*	*Cladanthus arabicus*	Tumor cell cytotoxicity and inhibitory effect on NF-κB transcriptional activity	[210]
Embellicine B (**338**)	*Embellisia eureka*	*Cladanthus arabicus*	Tumor cell cytotoxicity and inhibitory effect on NF-κB transcriptional activity	[210]
Ilanepyrrolal (**339**)	*Annulohypoxylon ilanense*	*Cinnamomum* sp.	Antimycobacterial activity	[156]
Xylariamide (**340**)	*Xylaria plebeja*	*Garcinia hombroniana*	No biological activities tested	[74]

Table 8.1 (continued)

Compound	Endophytic fungus	Host	Biological activities	Reference
Methyl 4-(3,4-dihydroxybenzamido) butanoate (**341**)	*Aspergillus wentii*	*Sargassum* sp.	Antioxidant activity	[138]
Pestalamine A (**342**)	*Pestalotiopsis vaccinii*	*Kandelia candel* (L.) Druce	Tumor cell cytotoxicity	[211]
Penicolinate A (**343**)	*Penicillium* sp.	Poaceae family (grass)	Tumor cell cytotoxicity, antimalarial and antitubercular activities, and no antibacterial and antifungal activities	[212]
Penicolinate B (**344**)	*Penicillium* sp.	Poaceae family (grass)	Tumor cell cytotoxicity, antimalarial, antitubercular, antibacterial and antifungal activities	[212]
Penicolinate C (**345**)	*Penicillium* sp.	Poaceae family (grass)	Tumor cell cytotoxicity, antimalarial, antitubercular, antibacterial, and antifungal activities	[212]
Penicolinate D (**346**)	*Penicillium* sp.	Poaceae family (grass)	No biological activities tested	[212]
Penicolinate E (**347**)	*Penicillium* sp.	Poaceae family (grass)	No biological activities tested	[212]

biosynthetic pathways of the same compounds evolve independently in fungi and plants [36–39], evidencing the unprecedented metabolic arsenal owned by fungal endophytic strains.

Indeed, endophytes are a remarkable reservoir of genetic diversity and their secondary metabolism may be activated by the metabolic interactions with the host, making the endophytic microorganisms a rich source of new biologically active natural products [6]. There are many specialized reviews covering the biological activities of the new compounds that have been isolated from endophytic and associated marine derived fungi [2, 3, 6, 15, 22, 23, 40–50].

Here, we reviewed the novel compounds isolated from these groups of microorganisms from 2012 to April 2014, mentioning their biological activities (Table 8.1). The novel metabolites were classified into four major groups based on their biosynthetic pathways, such as polyketide and fatty acid, phenylpropanoid and terpenoid derivatives as well as N-containing compounds. In the "N-containing compounds" group, compounds from nonribosomal peptide pathway and alkaloid derivatives were included, including those from polyketide pathway whose oxygen atom was substituted by nitrogen. Regarding other classification groups, compounds from mixed biosynthetic origin were not particularized, thus being included in one of the matching biosynthetic groups. In the text, only secondary metabolites containing any interesting structural novelty or relevant biological activity were highlighted.

Novel metabolites with biological or chemical relevance

Five new decalactone derivatives, coryoctalactones A–E (**29–33**, Fig. 8.2), were obtained from *Corynespora cassiicola* JCM 23.3, an endophyte of the mangrove plant *Laguncularia racemosa* (Combretacaeae). These new polyketides share a carbon skeleton containing an aromatic ring attached to an octalactone ring system, which have not been reported and are unusual. None of them were active in a panel of bioassays to evaluate cytotoxic activity against murine lymphoma cells, L5178Y cells, antimicrobial activity against several pathogenic microorganisms, and antitrypanosomal activity [51].

Several metabolites were obtained from *Microsphaeropsis arundinis* PSU-G18, endophytic fungus from the leaves of *Garcinia hombroniana*, including one new modiolin, microsphaerodiolin (**71**, Fig. 8.2), and seven novel phthalides, microsphaerophthalides A–G (**72–78**, Fig. 8.2). Interestingly, compounds **74–78**, which are 3-oxygenated phthalides, are rare natural products. Only compounds obtained in sufficient amount were submitted to biological tests. Thus, compounds **72**, **75,** and **76** were tested for antifungal activity. Compound **72** was moderately active against *Microsporum gypseum* SH-MU-4 with a minimum inhibitory concentration (MIC) value of 64 μg/mL, whereas compound **76** exhibited moderate antifungal activity against *Cryptococcus neoformans* with a MIC value of 64 μg/mL. Additionally, compound **76** showed mild activity against *M. gypseum* with a MIC value of 200 μg/mL [52].

Five new metabolites, epicocconigrones A and B (**82–83**, Fig. 8.2), 3-methoxyepicoccone B (**84**, Fig. 8.2), 3-methoxyepicoccone (**85**, Fig. 8.2), and 2,3,4-trihydroxy-6-(methoxymethyl)-5-methylbenzaldehyde (**86**, Fig. 8.2) were obtained from *Epicoccum nigrum*, an endophytic fungus isolated from the leaves of *Mentha suaveolens*. These compounds were evaluated for their inhibitory activity against a panel of 16 protein kinases. Compound **82** was active against all tested enzymes, except MEK1 wt. Compounds **84** and **86** inhibited only some kinases. A preliminary structure–activity relationship proposal suggested that a β-hydroxyl-α,β-unsaturated carbonyl moiety, present in **82**, **84,** and **86** but absent in inactive compounds (**83** and **85**), is necessary for the inhibition of protein kinases. Compound **82** showed also strong inhibition against histone deacetylase (HDAC), becoming a promising compound for the development of anticancer drugs [53].

Chaetosidone A (**88**, Fig. 8.2), a new depsidone, was isolated from *Chaetomium* sp., an endophytic fungus from the leaves of *Zanthoxylum leprieurii*. Interestingly, compound **88** is indeed the parent compound in the series of more than 60 orsellinic acid-derived depsidones. This compound exhibited moderate inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus* at a concentration of 40 μg per paper disk, and also moderate cytotoxicity towards brine shrimp larvae (*Artemia salina*) [54]. Compound **96**, named Corynesidone D, corresponds to the same natural product **88**, however, it was isolated from *C. cassiicola*, an endophytic fungus from *Gongronema latifolium*, and reported by the same time [55].

A new depsidone, excelsional (**102**, Fig. 8.2), and a new decaline derivative, 9-hydroxyphomopsidin (**103**, Fig. 8.2), were obtained from *Phomopsis* sp. CAFT69, an endophytic fungus from *Endodesmia calophylloides*. These two novel compounds exhibited strong motility inhibition and lysis of zoospores of grapevine pathogen *Plasmopara viticola*. Although further studies are necessary for understanding the biological mechanisms of motility inhibitory and lytic effects, it is suggested that these metabolites might play a role in the protection of the host plant [56].

The novel glycolipid fusaroside (**107**, Fig. 8.2) was obtained from *Fusarium* sp. LN-11, an endophytic fungus isolated from the fresh leaves of the tree *Melia azedarach* L. Based on 2D NMR experiments, the presence of two hexoses in their pyronose forms was revealed. Both of them were glucose residues having α anomeric configurations. Compound **107** is made of an unusual branched fatty acid and an α, α-trehalose, which is unique in nature since this family of glycolipids has not been reported previously in literature. This glycolipid exhibited moderate toxicity against brine shrimp larvae (*A. salina*), with the mortality rate of 47.6% [57].

Two new dihydroanthracenone derivatives, diaporthemins A and B (**109–110**, Fig. 8.2), were obtained from *Diaporthe melonis*, an endophytic fungus isolated from *Annona squamosa*. Compound **109** was identified as a heterodimer and the first compound possessing a C7–C5′ linkage between their monomeric subunits. Besides that, the planar structure of these two compounds was established suggesting they are stereoisomers. The mirror image of their circular dichroism (CD) spectra suggested that they were atropodiastereomers differing in the axial chirality. None of them showed antibacterial activities against the multi-resistant clinical isolate of

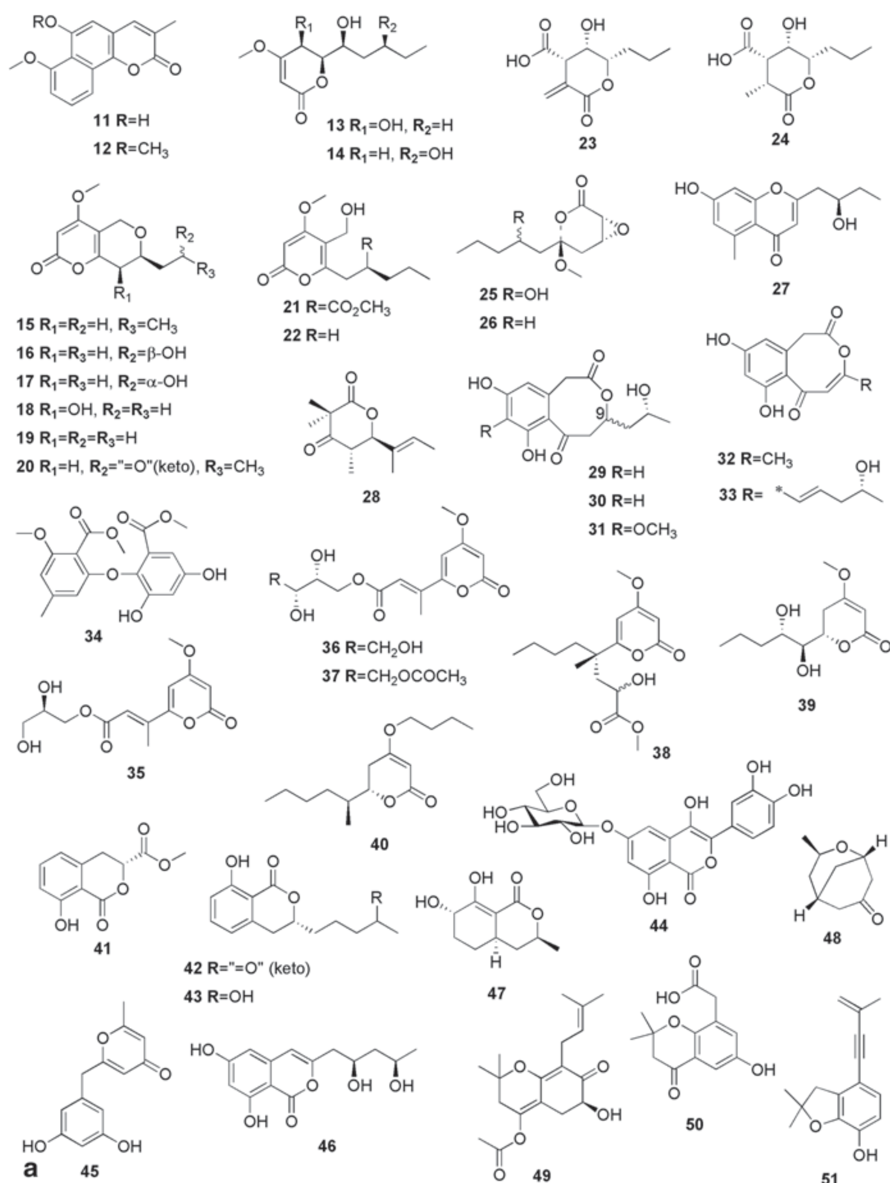

Fig. 8.2 **a**–**g** New polyketide and fatty acid derivatives from endophytic fungi

S. aureus 25697, a susceptible strain of *S. aureus* ATCC 29213, and *Streptococcus pneumoniae* ATCC 49619. Curiously, it was proposed that a C7–C5′ linkage present in these compounds seems to abolish antibacterial activity [58].

Chlorinated diphenyl ethers have seldom been found as fungal metabolites and three of them were isolated from *Pestalotiopsis* sp. PSU-MA69, an endophytic fun-

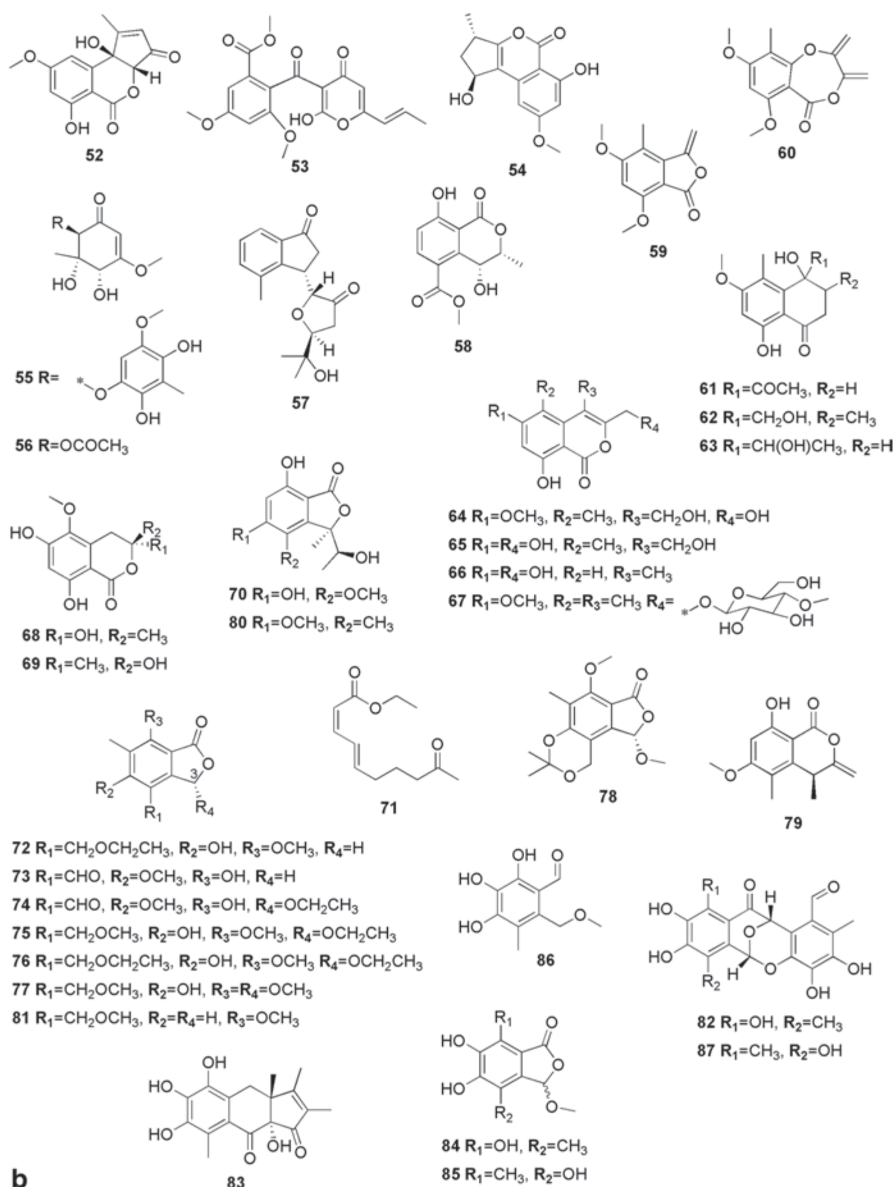

Fig. 8.2 (continued)

gus from a branch of the mangrove plant *Rhizophora apiculata*. Pestalotethers A–D (**124–127**, Fig. 8.2) were isolated and tested against *Candida albicans* NCPF3153 and *C. neoformans* ATCC90112 for antifungal activity. Compound **126** was not obtained in sufficient amount for biological tests. Compounds **124** and **125**, containing a chlorine atom, exhibited antifungal activity against *C. neoformans* (MIC value of

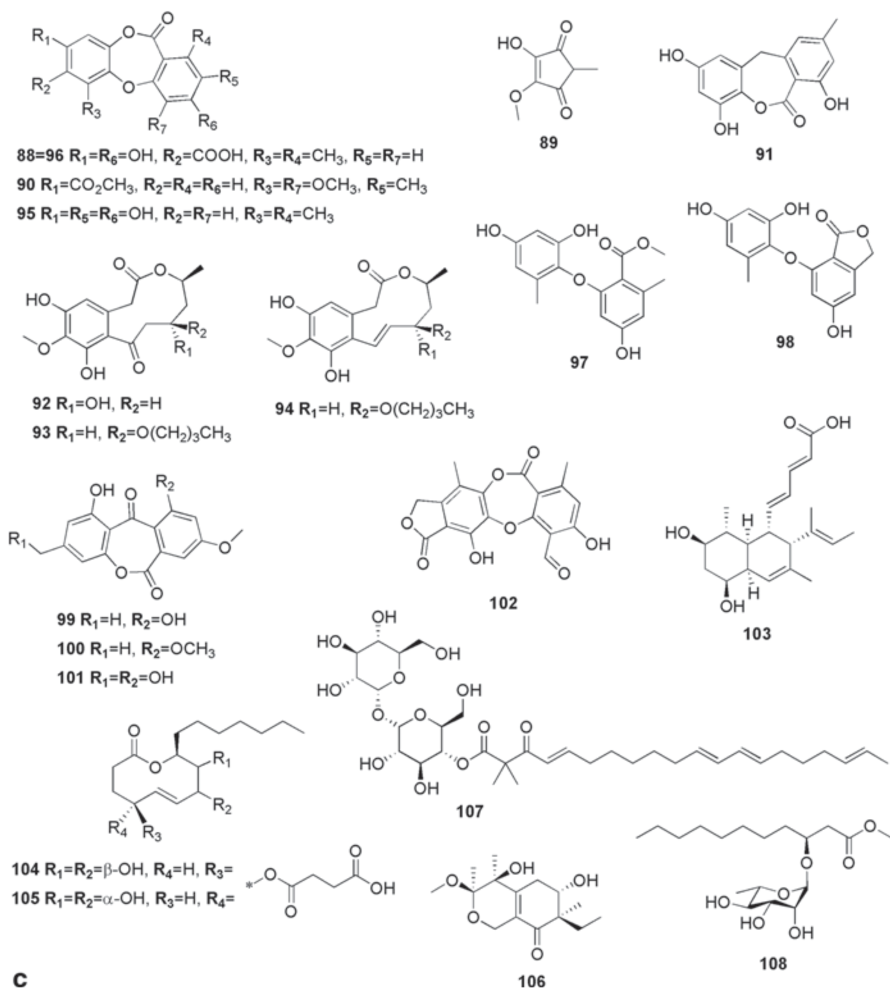

Fig. 8.2 (continued)

200 μ]g/mL) while **127** was inactive. These compounds were also inactive against *C. albicans* [59].

Annulosquamulin (**131**, Fig. 8.2), a dihydrobenzofuran-2,4-dione backbone possessing one alkyl side chain and a γ-lactone ring is rarely found when compared to other metabolites from the genus *Annulohypoxylon*. This compound was isolated from *Annulohypoxylon squamulosum* BCRC 34022, an endophytic fungus from the stem bark of medicinal plant *Cinnamomum* sp. Compound **131** exhibited significant cytotoxic activity against human breast adenocarcinoma (MCF-7), non-small cell lung cancer (NCI-H460), and glioblastoma (SF-268) cell lines with IC_{50} values <4 μg/mL [60].

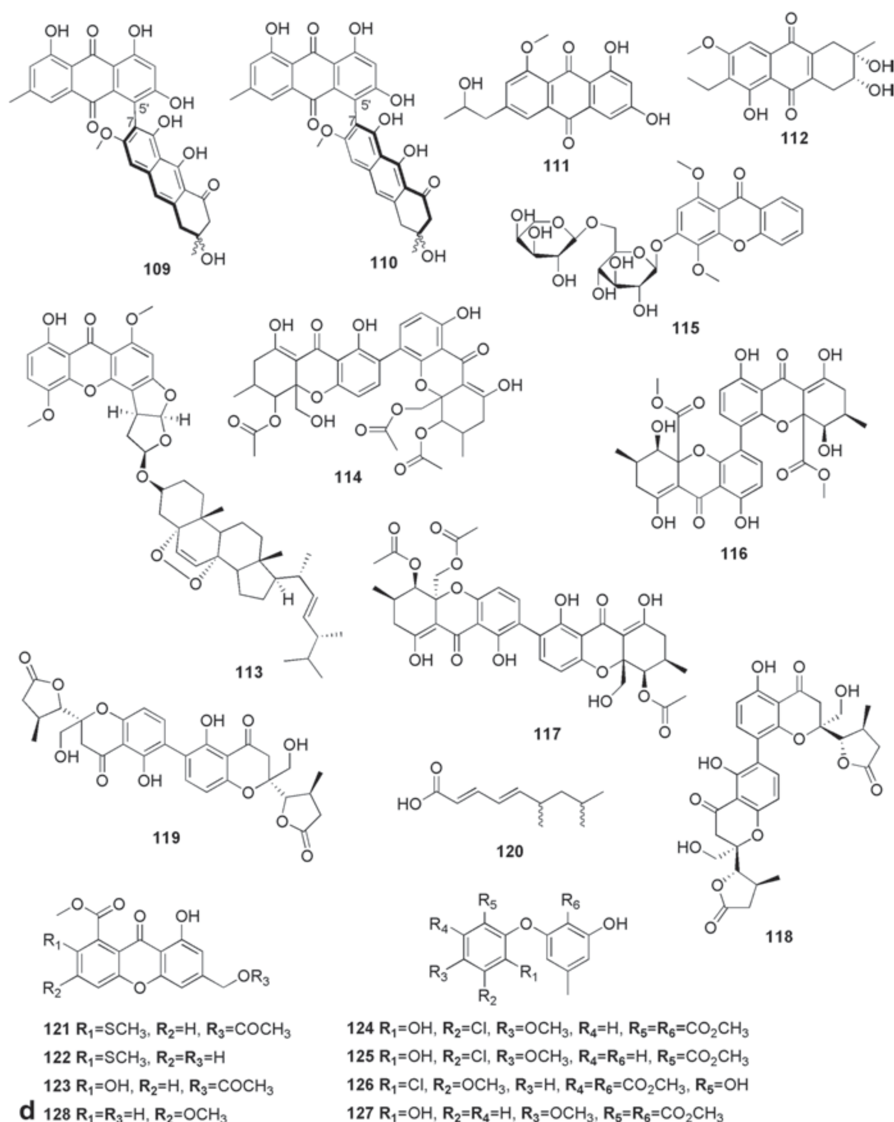

Fig. 8.2 (continued)

Two new members of the naphthoquinone spiroketal family, palmarumycin EG_1 (**146**, Fig. 8.2) and preussomerin EG_4 (**147**, Fig. 8.2), were obtained from *Edenia gomezpompae,* an endophytic fungus isolated from the leaves of *Callicarpa acuminata*. Compound **147** exhibited phytotoxic effect when evaluated for its ability to inhibit the seed germination, root elongation, and seedling respiration of *Amaranthus hypochondriacus, Solanum lycopersicum,* and *Echinochloa crus-galli* [61].

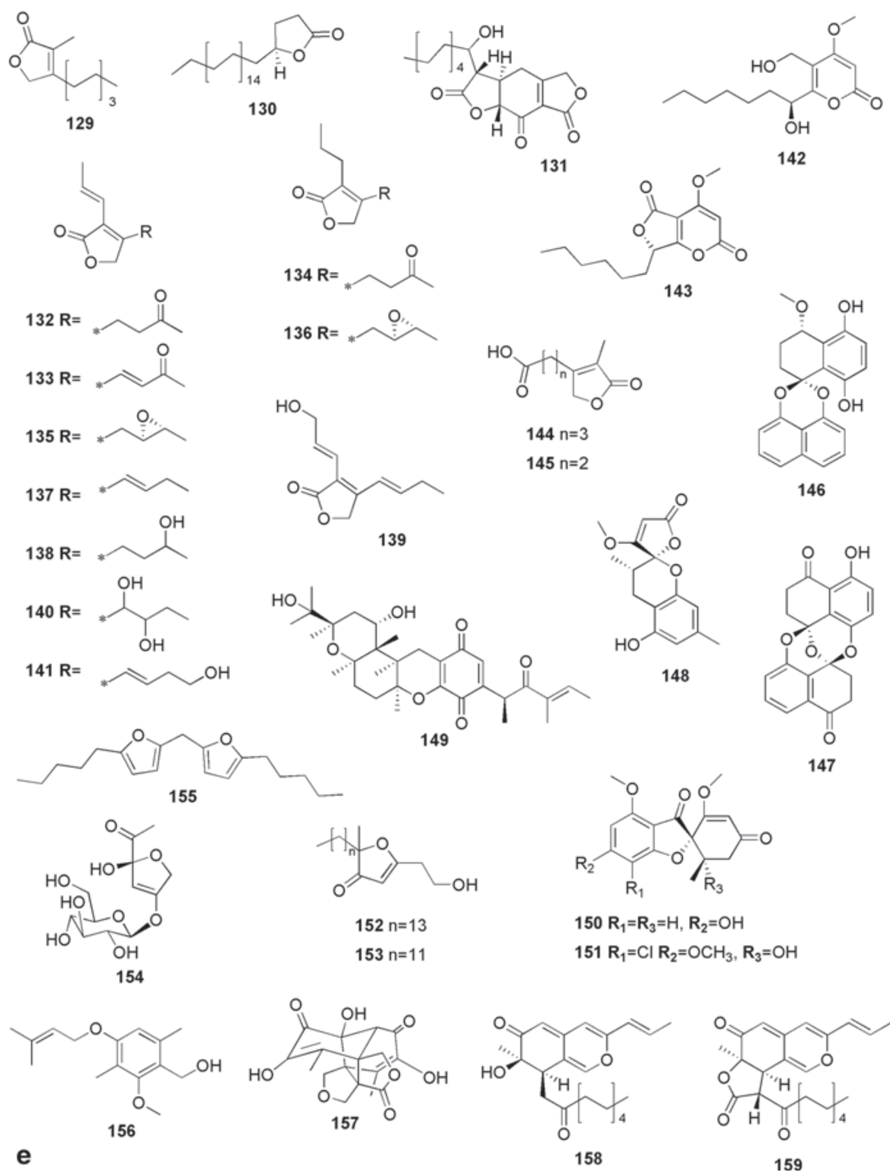

Fig. 8.2 (continued)

A novel compound, named as 2,3-didehydro-19α-hydroxy-14-epicochlioquinone B (**149**, Fig. 8.2), together with two new griseofulvin derivatives, 6-O-desmethyldechlorogriseofulvin and 6′-hydroxygriseofulvin (**150–151**, Fig. 8.2), were isolated from *Nigrospora* sp. MA75, an endophytic fungus from the stem of semi-mangrove plant *Pongamia pinnata*. Compound **149** showed antibacterial activity against methicillin-resistant *S. aureus* (MRSA, MIC 8 μg/mL), *Escherichia coli* (4 μg/mL),

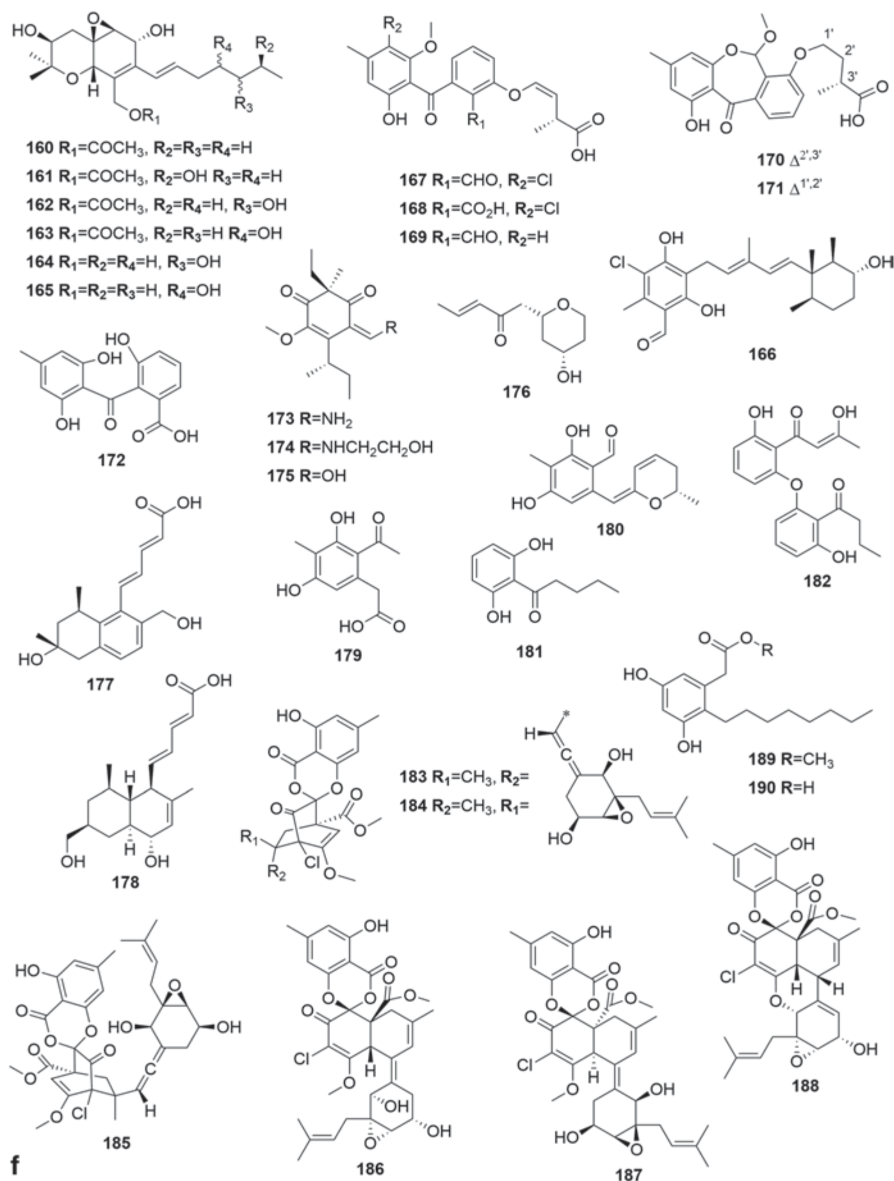

Fig. 8.2 (continued)

Pseudomonas aeruginosa (4 μg/mL), *Pseudomonas fluorescens* (0.5 μg/mL), and *Staphylococcus epidermidis* (0.5 μg/mL). Interestingly, the activity against *E. coli, P. fluorescens,* and *S. epidermidis* was stronger than ampicillin, used as positive control [62].

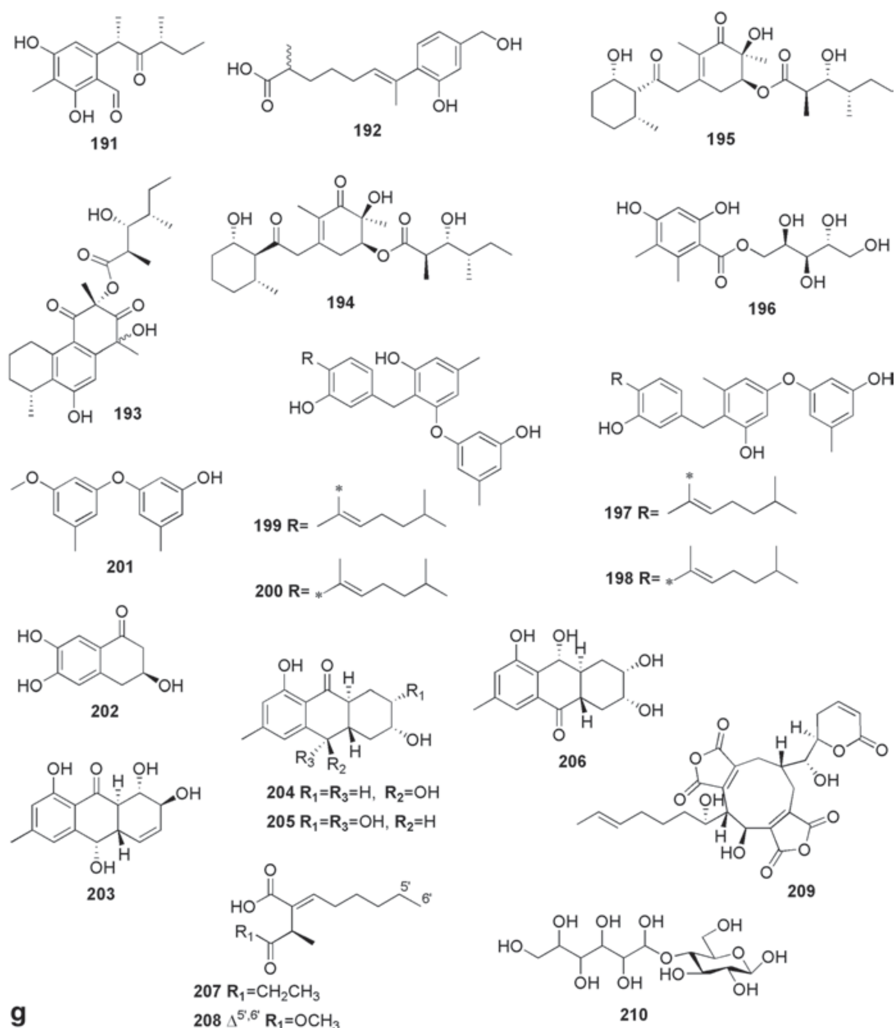

Fig. 8.2 (continued)

A new compound containing an inedited skeleton was isolated from an antimicrobial fraction of the ethyl acetate extract of *E. nigrum,* endophytic from *Saccharum officinarum* [63]. This unique natural product named epicolactone (**157**, Fig. 8.2) is a quasisymetrical molecule containing an unprecedented pentacyclic ring system exclude this final part. Analyses of X-ray crystallographic data showed that epicolactone may crystallize as a racemic mixture [63, 64].

Two new azaphilone derivatives, biscogniazaphilone A and B (**158–159**, Fig. 8.2), were isolated from *Biscogniauxia formosana* BCRC 33718, an endophytic fungus from the bark of medicinal plant *Cinnamomum* sp. Although both compounds exhibited antimycobacterial activity against *Mycobacterium tubercu-*

losis strain H37Rv, it was observed that **159**, possessing one γ-lactone group, was twofold (MIC ≤2.52 μg/mL) stronger than **158** (MIC ≤5.12 μg/mL), suggesting that the presence of that group plays a possible role in the antimycobacterial activity [65].

Six novel unique spiroketals, chloropestolides B–G (**183–188**, Fig. 8.2), were obtained from *Pestalotiopsis fici,* an endophytic fungus from the branches of *Camellia sinensis* (Theaceae). These new compounds are biosynthesized by naturally occurring Diels-Alder reactions, including reverse electron demand Diels-Alder (REDDA) for compounds **183–185** and normal electron demand Diels-Alder (NEDDA) for **186–188.** Despite their novelty, only compound **183** exhibited cytotoxic activity against the stable oncoprotein LMP1 integrated nasopharyngeal carcinoma cells (CNE1-LMP1), malignant melanoma cells (A375), and MCF-7 with IC_{50} values of 16.4, 9.9, and 23.6 μM, respectively [66].

Dothideomycetide A (**193**, Fig. 8.2), which is the first polyketide possessing a tricyclic 6,6,6 ring system, similar to that of a terpenoid, was isolated from *Dothideomycete* sp., an endophytic fungus from the roots of *Tiliacora triandra*. This compound was isolated together with two new compounds, dothideomycetone A and B (**194–195**, Fig. 8.2), which are diastereomers and probably derive from an azaphilone [67]. According to the biosynthetic proposal, compound **193** is likely derived from **194**. Compound **194** exhibited weak cytotoxicity against acute lymphoblastic leukemia cancer cell line (MOLT-3). Compound **193** exhibited weak cytotoxicity against human cholangiocarcinoma (HuCCA-1), human lung carcinoma (A549), human hepatocellular liver carcinoma (HepG2), and MOLT-3 cell lines. Besides that, compound **193** also exhibited moderate to weak antibacterial activity against *S. aureus* ATCC 25923 (MIC value of 128 μg/mL) and MRSA ATCC 33591 (MIC value of 256 μg/mL) [67].

Three new C_{25} steroids, named as norcyclocitrinol A (**214**, Fig. 8.3), erythro-11a-hydroxyneocyclocitrinol (**215**, Fig. 8.3), and pesudocyclocitrinol A (**216**, Fig. 8.3) were isolated from *Penicillium chrysogenum* P1X, an endophyte from *Huperzia serrate*. These compounds belong to a class of rare steroids featuring an unusual *A/B* bicyclic ring system, possibly originated from ergosterol by a carbon-skeleton rearrangement. Compound **214** possesses a tetracyclic C23-steroid skeleton, featuring a previously unreported bisnor C-atom side chain. Compounds were evaluated for their cytotoxic activities against HeLa (adenocarcinoma) and HepG2 cell lines. However, none exhibited a significant cytotoxicity at 20 μM [68].

A new sesquiterpenoid, diaporol A (**218**, Fig. 8.3), and eight new drimane sesquiterpenoids, diaporols B-I (**219–226**, Fig. 8.3), were isolated from *Diaporthe* sp., an endophytic fungus from the mangrove plant *Rhizophora stylosa*. Compound **218** possesses a new tricyclic framework, with an unusual six-membered lactone. All the compounds had their cytotoxicity against four cell lines assessed, but none of them was active at the concentration of 20 μM [69].

Three novel eremophilane sesquiterpenoids derivatives, MBJ-0011, MBJ-0012, and MBJ-0013 (**251–253**, Fig. 8.3) were produced by the endophytic fungus *Apiognomonia* sp., isolated from an unidentified Japanese plant. Compound **251** is an eremophilane derivative possessing an uncommon tetrahydro-α-methyl-5-oxo-2-

furanacetic acid moiety. The cytotoxic activities of all compounds against human ovarian adenocarcinoma SKOV-3 cells were assessed. After 72 h of treatment, compound **251** exhibited moderate cytotoxic activity with the IC_{50} value of 3.4 μM [70].

Pestalotiopens A and B (**255–256**, Fig. 8.3), produced by *Pestalotiopsis* sp., an endophyte from *Rhizophora mucronata,* are novel types of natural products with unprecedented hybrid carbon skeletons derived from a drimane-type sesquiterpene and a polyketide. Thus, compound **255** is a sesquiterpene and cyclopaldic acid-derived hybrid, whereas compound **256** also contains an additional triketide subunit linked through a cyclic acetal. In antimicrobial assays against a panel of six bacterial strains, compound **255** exhibited moderate antimicrobial activity against *Enterococcus faecalis* (MIC value between 125 and 250 μg/mL), whereas compound **256** was inactive [71].

Asperterpenoid A (**258**, Fig. 8.3) was isolated from a salt rice solid culture of the mangrove fungus *Aspergillus* sp., endophyte from *Sonneratia apetala,* and was identified as a novel sesterterpenoid with a new carbon skeleton, containing a planar 5/7/(3)6/5 pentacyclic structure. Its chemical structure was confirmed by single-crystal X-ray diffraction experiments. The inhibitory activity of compound **258** against *M. tuberculosis* protein tyrosine phosphatase B (*m*PTPB) was evaluated, showing it as a strong inhibitor, with an IC_{50} value of 2.2 μM [72].

New cyclohexenone–sordaricin derivatives xylarinonericins A–C (**259–261**, Fig. 8.3) were isolated from *Xylaria plebeja* PSU-G30, an endophytic fungus from *G. hombroniana*. In addition to peculiar tetracyclic moiety, which forms the known sordaricin structure [73], these compounds possess an unusual ester moiety at C6 of the sordaricin skeleton instead of a carboxylic acid. Besides that, compound **261** has a unique feature with an ester unit instead of an ether group at C19. All these compounds were tested against *C. albicans* ATCC 90028 and *C. neoformans* ATCC 90113, but none was active against both fungal strains at a concentration of 200 μg/mL [74].

Emericellenes A−E (**263–267**, Fig. 8.3) were obtained based on a bioactivity-guided fractionation of extracts of *Emericella* sp., an endophytic fungus from *Astragalus lentiginosus*. These new sesterterpenoids possess a scaffold similar to that of verticillane-type diterpenoids, with a 12-membered cyclic fused to another cyclic moiety. In addition, these compounds hold an isoprene unit, and compounds **264–267** hold a carboxylic acid, while **263** possesses a carbonyl moiety. Thus, the structures of these compounds represent a unique class of sesterterpenoid metabolites bearing a novel emericellane-type bicarbocyclic ring system. Their cytotoxic activities were evaluated against six tumor cell lines. All compounds were not cytotoxic up to a concentration of 5.0 μM [75].

The new butyrolactone derivative **283** (Fig. 8.4), isolated from *Aspergillus terreus* var. *boedijnii* (Blochwitz), an endophyte from red marine alga *Laurencia ceylanica* J. Agardh, displayed a considerable inhibitory activity against the enzyme β-glucuronidase (IC_{50} 6.2 μM), this activity being stronger than that provided by the positive control glucosaccharo-(1,4)-lactone (IC_{50} value of 48.4 μM) [76].

The novel cyclic depsipeptide pullularin E (**286**, Fig. 8.5) and the new linear peptide pullularin F (**287**, Fig. 8.5) were produced by *Bionectria ochroleuca*, an

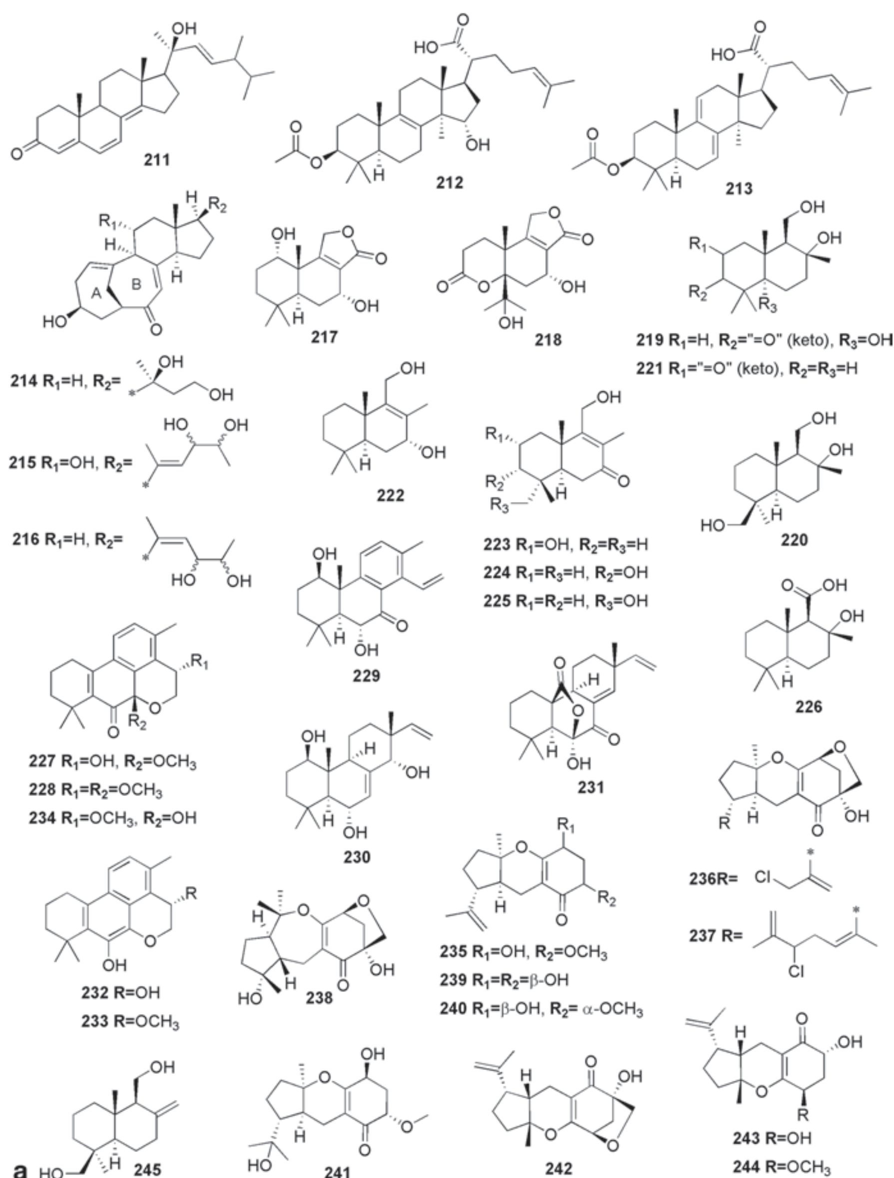

Fig. 8.3 **a**, **b** New terpenoid derivatives from endophytic fungi

endophytic fungus isolated from the mangrove plant *Sonneratia caseolaris*. The cytotoxicity assays in L5178Y cell line showed that antiproliferative properties were prevalent among some pullularin cyclic analogues, with IC_{50} values ranging between 0.1 and 6.7 µg/mL, whereas the linear compound **287** did not exhibit any cytotoxic activity at the tested dose of 10 µg/mL [77].

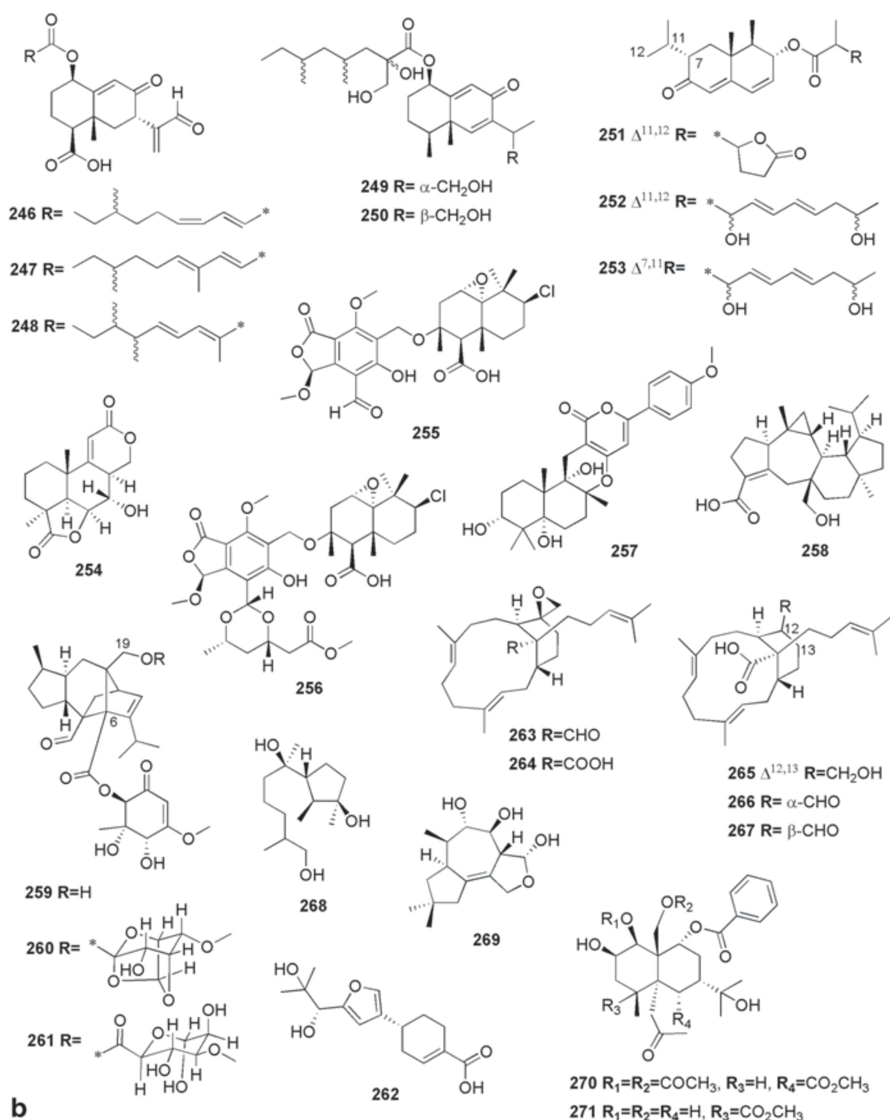

Fig. 8.3 (continued)

The new spirotryprostatin A derivative **295** (Fig. 8.5), produced by *Aspergillus fumigatus*, an endophyte from *Erythrophloeum fordii* Oliv., is related to diketopiperazines derived from amino acids proline and tryptophan. However, unlike those generally reported diketopiperazines, compound **295** possesses a spirocyclic moiety, typical in the spirotryprostatin class. Neither significant anti-inflammatory activity, nor detectable cytotoxicity ($IC_{50} > 10$ μM) toward five human tumor cell lines were displayed by this compound during the bioassay [78].

A novel diketopiperazine derivative containing an isopentenyl moiety attached (**299**, Fig. 8.5) was isolated from *P. chrysogenum* MTCC 5108, an endophytic fungus from the mangrove plant *Porteresia coarctata*. The crude extract of *P. chrysogenum* had been active against *Vibrio cholerae* MCM B-322, a pathogen causing cholera in humans. Then, the antibacterial activity of the pure compound against this pathogen was also tested, indicating that it is comparable to that of the standard antibiotic streptomycin [79].

The new thiodiketopiperazines phomazines A–C (**300–302**, Fig. 8.5) were produced by *Phoma* sp., endophyte from the mangrove plant *Kandelia candel*. Compounds **300** and **301** are biosynthesized from two phenylalanine residues with the uncommon oxidation of only one phenyl nucleus. In biological assays, compound **301** showed moderate to weak cytotoxicity against HL-60 (acute promyelocytic leukemia), HCT-116 (human colon carcinoma), K562 (chronic myelogenous leukemia), MGC-803 (human gastric cancer), and A549 tumor cell lines, while **300** and **302** were inactive [80].

The bioactivity-guided fractionation of extracts of *Emericella* sp., an endophytic fungus from *A. lentiginosus,* led to the isolation of secoemestrin D (**303**, Fig. 8.5). Compound **303** is a new epipolythiodioxopiperazine analogue harboring a tetrasulfide moiety. Compounds possessing disulphide bridge are recognized to their potentially toxic effects due to the reaction of thiol groups with proteins, and to the generation of reactive oxygen species [81]. Therefore, the potential anticancer activity of compound **303** was assessed using a panel of six human tumor cell lines: NCI-H460, SF-268, MCF-7, PC-3 M (metastatic prostate adenocarcinoma), MDA-MB-231 (breast adenocarcinoma), CHP-100 (neuroblastoma), and normal human fibroblast cells (WI-38). Compound **303** exhibited strong cytotoxicity against all the cancer cell lines with IC_{50} values of 0.15, 0.06, 0.14, 0.17, 0.06, and 0.10 μM, respectively. It was also toxic to normal cells (IC_{50} 0.24 μM), but showed a moderate selectivity for SF-268 and MDA-MB-231 cell lines [75].

The new farinomaleins C–E (**306–308**, Fig. 8.5), isolated from the fungus AMO 3-2 (unidentified), an endophyte from the mangrove plant *Avicennia marina,* are structurally related to the farinomalein class, of which only two compounds have been reported previously [82, 83]. Compounds **306–308** were not cytotoxic against L5178Y cells at the concentration of 10 μg/mL. Besides, none of them exhibited significant antimicrobial activities against *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 49619, and *E. coli* ATCC 25922, at the concentration of 64 μg/mL [84].

Three new aspochalasins, named trichalasin E, F, and H (**314–316**, Fig. 8.5), were isolated from the fungus *Trichoderma gamsii,* an endophyte from *Panax notoginseng* (BurK.) F.H. Chen. Compound **314** contains a unique hydroperoxyl group, which has not been reported before in the aspolachalasin class. Besides, compound **316** is stereoisomer of a known compound (aspergillin PZ) [85], possessing a rare 6/5/6/6/5 pentacyclic skeleton. Compounds **314–316** displayed weak cytotoxicity against three tumor cell lines (A549, MDA-MB-231, and PANC-1- human pancreatic carcinoma) [86].

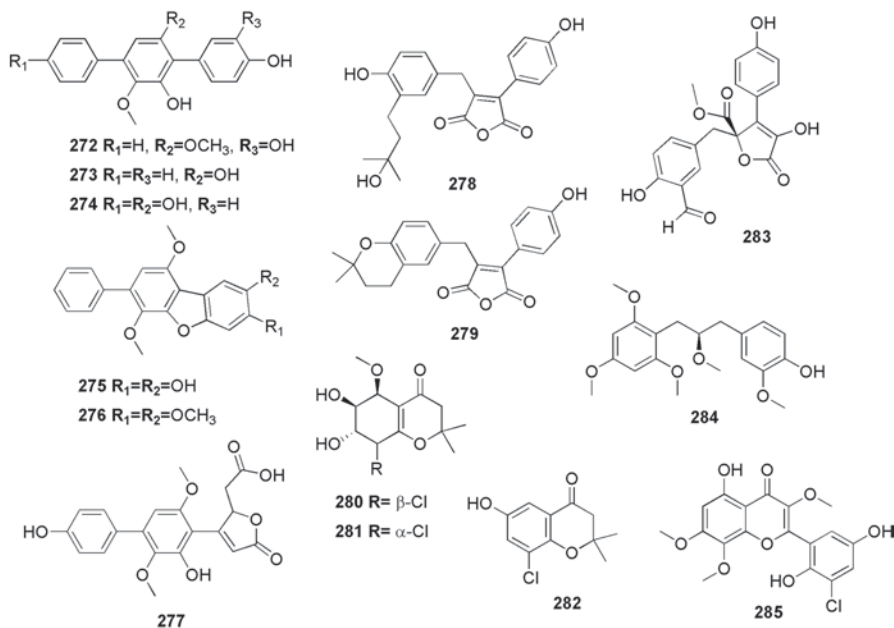

Fig. 8.4 New phenylpropanoid derivatives from endophytic fungi

Chaetoglobosin V_b (**317**, Fig. 8.5), isolated from a culture of *Chaetomium globosum,* an endophytic fungus from the medicinal plant *Ginkgo biloba,* possesses a very rare fusion into the macrocycle moiety forming a cyclopentenone ring. The absolute stereochemistry of this compound was determined based on CD spectrometry showing that it is a stereoisomer of a known compound (chaetoglobosin V, [87]). Compound **317** did not show antimicrobial activity against a panel of bacteria and fungi at the concentration of 100 µg/mL. Interestingly, its stereoisomer exhibited moderate to weak toxicity against *Alternaria solani, Bacillus cereus,* and *P. aeruginosa* [87].

New quinazolinone alkaloids were isolated from *Aspergillus nidulans* MA-143, an endophytic fungus from the marine mangrove plant *R. stylosa*. Those compounds, named aniquinazolines A–D (**318–321**, Fig. 8.5), own a remarkable structural diversity. The structure of **318** was confirmed by single-crystal X-ray diffraction analysis. All compounds were more toxic to brine shrimp larvae (LD_{50} values of 1.27, 2.11, 4.95, and 3.42 µM, respectively) than the positive control colchicine (LD_{50} values of 88.4 µM). However, none of those compounds displayed cytotoxic activity against four cell lines tested. Compounds were also inactive against *E. coli* and *S. aureus* [88].

The new polyketide-containing-nitrogen cryptosporioptide (**322**, Fig. 8.5) was produced by *Cryptosporiopsis* sp., an endophytic fungus from *Viburnum tinus*. Compound **322** possesses an unprecedented tetracyclic structure, holding a chro-

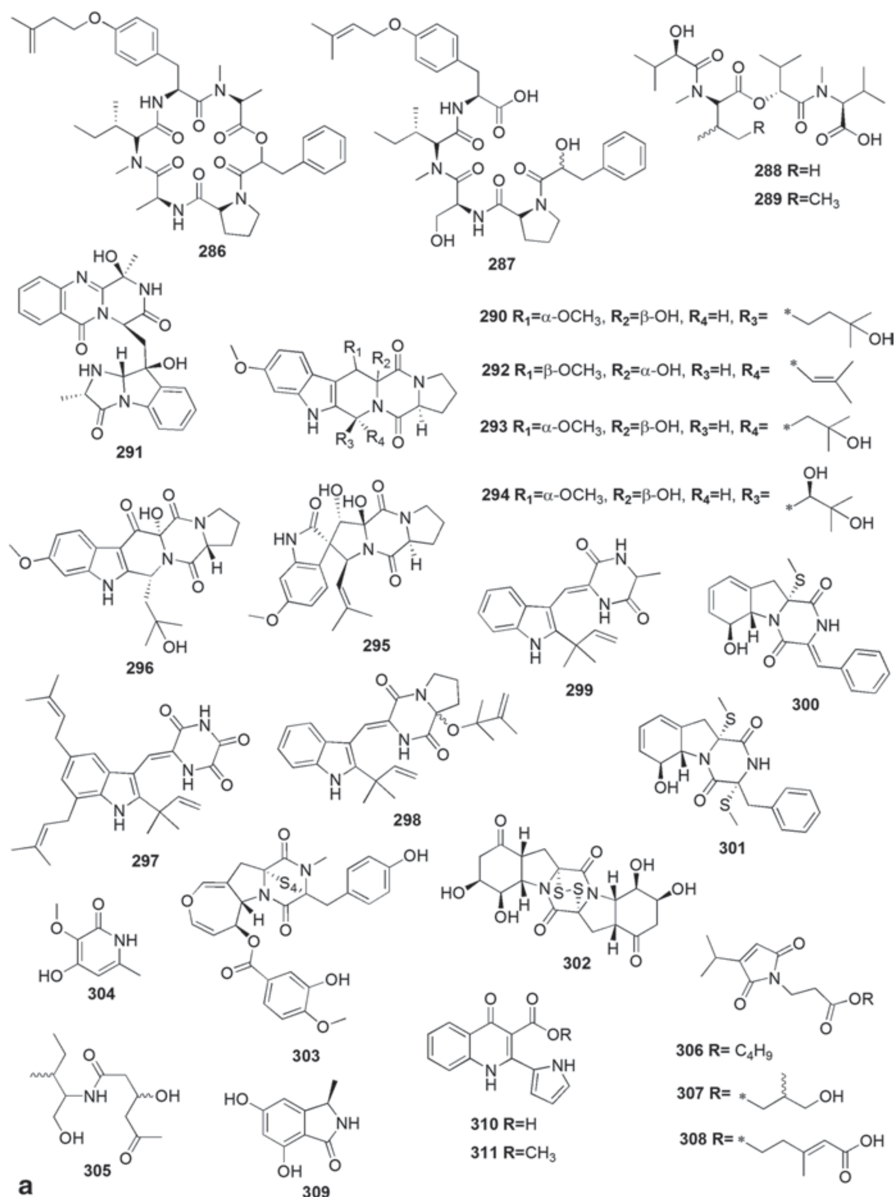

Fig. 8.5 **a**–**c** New N-containing compounds from endophytic fungi

mone nucleus merged with a five-membered cycle, in which an eight-membered cycle is attached. Its biological activities were assessed and compound **322** showed significant lipoxygenase inhibitory activity, while it was inactive against acetylcholinesterase and butyrylcholinesterase. In the antimicrobial assays, compound **322**

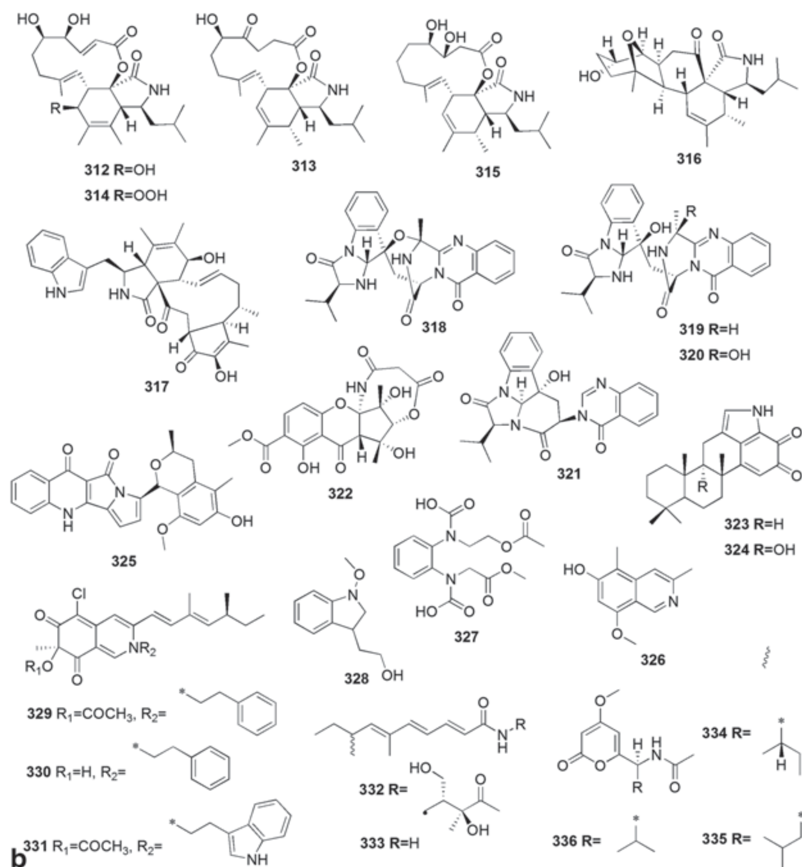

Fig. 8.5 (continued)

was active against *Bacillus megaterium,* but inactive against the bacteria *E. coli,* the fungi *Microbotyrum violaceum* and *Botrytis cinerea,* and the alga *Chlorella fusca* [89].

The new indole-terpenes mycoleptodiscins A and B (**323–324**, Fig. 8.5) were isolated from *Mycoleptodiscus* sp., an endophytic fungus from *Desmotes incomparabilis*. These compounds have new skeletons that are uncommon in nature. The terpenoid moiety is an indole ring forming a fused pentacyclic alkaloid. Compound **324** was tested against the four cancer cell lines H460, A2058 (human melanoma), H522-T1 (non-small cell lung cancer), and PC-3 (prostate cancer), showing strong cytotoxicity, with IC_{50} values ranging from 0.60 to 0.78 μM. However, this compound was also strongly cytotoxic against nonproliferating normal cells (IMR-90, IC_{50} 0.41 μM), indicating an indiscriminant cytotoxicity [90].

Citriquinochroman (**325**, Fig. 8.5), a novel compound with an unknown quinolactacide–isochroman skeleton, was isolated from rice cultures of *Penicillium citri-*

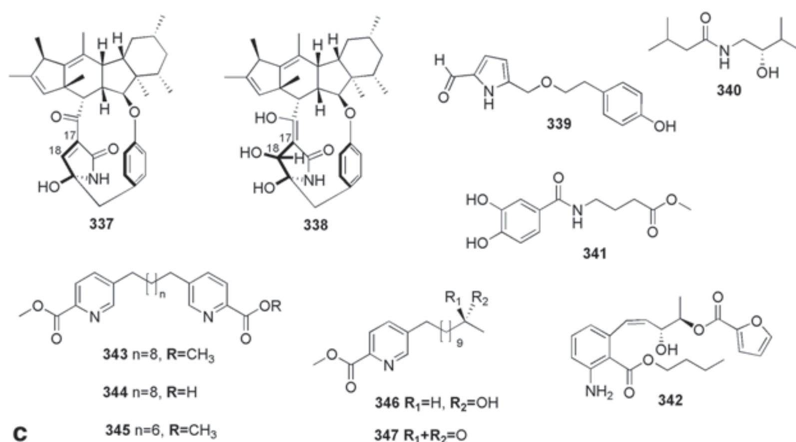

Fig. 8.5 (continued)

num, an endophytic fungus from *Ceratonia siliqua*. Compound **325** showed cytotoxicity against L5178Y cells (IC_{50} 6.1 μM) comparable with the positive control kahalalide F (IC_{50} 4.3 μM). In the antibacterial assays, it was not active against *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 49619, and *E. coli* ATCC 25922 at the concentration of 64 μg/mL [91].

Bipolamides A and B (**332–333**, Fig. 8.5) are new compounds and were isolated from *Bipolaris* sp., an endophyte from *Gynura hispida*. Compound **332** possesses an acyloin and triene fatty acid secondary amide moieties, neither of which is reported in nature. The last mentioned moiety is also present in compound **333**, making them rare natural products. Both compounds were inactive against four bacterial strains at the concentration of 512 μg/mL, and compound **333** showed only mild toxicity against some fungal strains tested [92].

Two new alkaloids named as embellicines A and B (**337–338**, Fig. 8.5) were obtained from *Embellisia eureka* CATS2, isolated from healthy stem tissues of *Cladanthus arabicus* (Asteraceae). It was observed that compound **338** was completely converted to **337** during storage, suggesting a direct intramolecular dehydration process from **338** to **337**. Besides, chemical correlation and biogenetic considerations suggest homochirality of these two compounds. Both compounds exhibited cytotoxicity against K562 cells, being able to induce cell death with an IC_{50} lower than 10 μM. It was also observed that compound **338** was highly active due to its cytotoxic/cytostatic potential. Interestingly, compound **338** was 5–10 times more active than compound **337** against K562 cells, indicating that the hydroxylation pattern in the pyrrolidinone ring is more important for its cytotoxicity than the presence of a C17-C18 double bond [93].

Triggering Biosynthesis of Novel Secondary Metabolites

Taking into account that endophytic microbes interact with their host plant and other associated microbes in the environment, all those interactions should trigger the production of secondary metabolites [2]. The lack of external stimulus under unnatural conditions may lead to a minimal or absent production of many interesting microbial natural products, which could be produced by the endophytes while interacting in their natural habitat.

Many strategies of cultivation have been used to stimulate the production of microbial secondary metabolites under laboratory conditions. These strategies include variations in media composition, pH, temperature, aeration, or shape of culturing flask; biotic elicitation by coculture of different strains; abiotic elicitation by physical or chemical stresses; and epigenetic modulation by chemical epigenetic modifiers [94–98].

Those approaches have been also applied to the endophytes cultures [99–106] and the following examples confirm that the secondary metabolism remodeling by cultivation-dependent approaches may yield new metabolites [103–105].

Six novel benzophenone derivatives, cephalanones A–F (**167–172**, Fig. 8.2), were obtained from *Graphiopsis chlorocephala,* an endophytic fungus from the leaves of *Paeonia lactiflora*. It was found that cultivation of this endophytic fungus in the presence of nicotinamide, HDAC inhibitor, yielded benzophenone production, including two uncommon chlorinated derivatives, **167** and **168**. This result is the first evidence that NAD^+-dependent HDAC inhibitors are an effective epigenetic strategy to access new natural products from endophytic fungi [107].

The new sesquiterpenes **270** and **271** (Fig. 8.3) were produced in response to abiotic stress elicitation when the endophytic fungus *Pestalotiopsis* sp. Z233, isolated from the alga *Sargassum horneri,* was grown in culture medium supplemented with $CuCl_2$. The tyrosinase inhibitory activities of those compounds were evaluated, showing that they are potent inhibitors, with IC_{50} values of 14.8 μM and 22.3 μM, respectively. The IC_{50} value of kojic acid, used as a control, was 21.2 μM [105].

The production of two new linear depsipeptides, subenniatins A and B (**288–289**, Fig. 8.5), was induced during the mixed culture of *Fusarium tricinctum* and *Fusarium begonia*, both endophytes from the plant *Aristolochia paucinervis*. Interestingly, these compounds are suggested to be biogenetic building blocks of cytotoxic enniatins produced by *F. tricinctum* in pure culture. The compounds were inactive against *E. coli, S. aureus,* and *P. aeruginosa* at 64 μg/mL, and did not display cytotoxicity against L5178Y (IC_{50} > 10 μg/mL) [104].

The cocultivation of two marine-derived mangrove endophytic fungi led to the isolation of the new compounds marinamide and methyl marinamide (**310–311**, Fig. 8.5), whose structures were revised, indicating that they are pyrrolyl 4-quinolone analogues [108]. Compounds **310–311** were active against *Pseudomonas pyocyanea* and *S. aureus,* and exhibited potent cytotoxicity against HepG2, 95-D (lung), MGC832 (gastric), and HeLa tumor cell lines [103, 108].

Conclusion

Undoubtedly, the endophytic fungi are a remarkable source of novel and biologically active compounds. Nevertheless, the huge potential of endophytes to produce new natural products is not fully exploited. The understanding of chemical ecology of these microorganisms with their natural environment will assist the human beings during this unraveling process.

References

1. Staniek A, Woerdenbag HJ, Kayser O (2008) Endophytes: exploiting biodiversity for the improvement of natural product-based drug discovery. J Plant Interact 3(2):75–93
2. Kusari S, Hertweck C, Spitellert M (2012) Chemical ecology of endophytic fungi: origins of secondary metabolites. Chem Biol 19(7):792–798
3. Aly AH, Debbab A, Kjer J, Proksch P (2010) Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers 41(1):1–16
4. Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN (2008) Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett 278(1):1–9
5. Kogel K-H, Franken P, Hueckelhoven R (2006) Endophyte or parasite—what decides? Curr Opin Plant Biol 9(4):358–363
6. Gutierrez RMP, Gonzalez AMN, Ramirez AM (2012) Compounds derived from endophytes: a review of phytochemistry and pharmacology. Curr Med Chem 19(18):2992–3030
7. Redecker D, Kodner R, Graham LE (2000) Glomalean fungi from the Ordovician. Science 289(5486):1920–1921
8. Schulz B, Boyle C (2005) The endophytic continuum. Mycol Res 109:661–686
9. Rudgers JA, Koslow JM, Clay K (2004) Endophytic fungi alter relationships between diversity and ecosystem properties. Ecol Lett 7(1):42–51
10. Schardl CL, Leuchtmann A, Spiering MJ (2004) Symbioses of grasses with seedborne fungal endophytes. Annu Rev Plant Biol 55:315–340
11. Muller CB, Krauss J (2005) Symbiosis between grasses and asexual fungal endophytes. Curr Opin Plant Biol 8(4):450–456
12. Saikkonen K, Gundel PE, Helander M (2013) Chemical ecology mediated by fungal endophytes in grasses. J Chem Ecol 39(7):962–968
13. Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M et al (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci U S A 102(38):13386–13391
14. Zhang HW, Song YC, Tan RX (2006) Biology and chemistry of endophytes. Nat Prod Rep 23(5):753–771
15. Gunatilaka AAL (2006) Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. J Nat Prod 69(3):509–526
16. Rodriguez R, Redman R (2008) More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. J Exp Bot 59(5):1109–1114
17. Bae H, Sicher RC, Kim MS, Kim S-H, Strem MD, Melnick RL et al (2009) The beneficial endophyte *Trichoderma hamatum* isolate dis 219b promotes growth and delays the onset of the drought response in *Theobroma cacao*. J Exp Bot 60(11):3279–3295
18. Sabzalian MR, Mirlohi A (2010) Neotyphodium endophytes trigger salt resistance in tall and meadow fescues. J Plant Nutr Soil Sci 173(6):952–957

19. Weyens N, van der Lelie D, Taghavi S, Vangronsveld J (2009) Phytoremediation: plant-endophyte partnerships take the challenge phytoremediation: plant-endophyte partnerships take the challenge. Curr Opin Biotechnol 20(2):248–254
20. Hartley SE, Gange AC (2009) Impacts of plant symbiotic fungi on insect herbivores: mutualism in a multitrophic context. Annu Rev Entomol 54:323–342
21. Rocha ACS, Garcia D, Uetanabaro APT, Carneiro RTO, Araujo IS, Mattos CRR et al (2011) Foliar endophytic fungi from *Hevea brasiliensis* and their antagonism on *Microcyclus ulei*. Fungal Divers 47(1):75–84
22. Aly AH, Debbab A, Proksch P (2011) Fungal endophytes: unique plant inhabitants with great promises. Appl Microbiol Biotechnol 90(6):1829–1845
23. Alvin A, Miller KI, Neilan BA (2014) Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. Microbiol Res 169(7–8):483–495
24. Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by taxomyces-andreanae, an endophytic fungus of Pacific yew. Science 260(5105):214–216.
25. Puri SC, Verma V, Amna T, Qazi GN, Spiteller M (2005) An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. J Nat Prod 68(12):1717–1719
26. Kusari S, Zuehlke S, Spiteller M (2009) An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues. J Nat Prod 72(1):2–7
27. Kusari S, Zuehlke S, Spiteller M (2011) Effect of artificial reconstitution of the interaction between the plant *Camptotheca acuminata* and the fungal endophyte *Fusarium solani* on camptothecin biosynthesis. J Nat Prod 74(4):764–775
28. Shweta S, Zuehlke S, Ramesha BT, Priti V, Kumar PM, Ravikanth G et al (2010) Endophytic fungal strains of *Fusarium solani*, from *Apodytes dimidiata* E.. Mey. ex Arn (Icacinaceae) produce camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin. Phytochemistry 71(1):117–122
29. Puri SC, Nazir A, Chawla R, Arora R, Riyaz-ul-Hasan S, Amna T et al (2006) The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. J Biotechnol 122(4):494–510
30. Eyberger AL, Dondapati R, Porter JR (2006) Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. J Nat Prod 69(8):1121–1124
31. Kusari S, Lamshoeft M, Spiteller M (2009) *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis L*. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. J Appl Microbiol 107(3):1019–1030
32. Kusari S, Lamshoeft M, Zuehlke S, Spiteller M (2008) An endophytic fungus from hypericum perforatum that produces hypericin. J Nat Prod 71(2):159–162
33. Kusari S, Zuehlke S, Kosuth J, Cellarova E, Spiteller M (2009) Light-independent metabolomics of endophytic *Thielavia subthermophila* provides insight into microbial hypericin biosynthesis. J Nat Prod 72(10):1825–1835
34. Kusari S, Verma VC, Lamshoeft M, Spiteller M (2012) An endophytic fungus from *Azadirachta indica* A. Juss. That produces azadirachtin. World J Microbiol Biotechnol 28(3): 1287–1294
35. Kusari S, Spiteller M (2011) Are we ready for industrial production of bioactive plant secondary metabolites utilizing endophytes? Nat Prod Rep 28(7):1203–1207
36. Heinig U, Scholz S, Jennewein S (2013) Getting to the bottom of taxol biosynthesis by fungi. Fungal Divers 60(1):161–170
37. Boemke C, Tudzynski B (2009) Diversity, regulation, and evolution of the gibberellin biosynthetic pathway in fungi compared to plants and bacteria. Phytochemistry 70(15–16): 1876–1893
38. Xiong Z-Q, Yang Y-Y, Zhao N, Wang Y (2013) Diversity of endophytic fungi and screening of fungal paclitaxel producer from Anglojap yew, Taxus x media. BMC Microbiol 13:71
39. Yang Y, Zhao H, Barrero RA, Zhang B, Sun G, Wilson IW et al (2014) Genome sequencing and analysis of the paclitaxel-producing endophytic fungus *Penicillium aurantiogriseum* NRRL 62431. BMC Genomics 15:69

40. Valachova M, Muckova M, Sturdikova M (2007) Metabolites of endophytic microorganisms as bioactive compounds. Chem Listy 101(6):486–494
41. Kharwar RN, Mishra A, Gond SK, Stierle A, Stierle D (2011) Anticancer compounds derived from fungal endophytes: their importance and future challenges. Nat Prod Rep 28(7):1208–1228
42. Debbab A, Aly AH, Proksch P (2012) Endophytes and associated marine derived fungi-ecological and chemical perspectives. Fungal Divers 57(1):45–83
43. Kusari S, Pandey SP, Spiteller M (2013) Untapped mutualistic paradigms linking host plant and endophytic fungal production of similar bioactive secondary metabolites. Phytochemistry 91:81–87
44. de Souza JJ, Vieira IJC, Rodrigues E, Braz R (2011) Terpenoids from endophytic fungi. Molecules 16(12):10604–10618 (Review)
45. Debbab A, Aly AH, Proksch P (2011) Bioactive secondary metabolites from endophytes and associated marine derived fungi. Fungal Divers 49(1):1–12 (Review)
46. Debbab A, Aly AH, Proksch P (2013) Mangrove derived fungal endophytes—a chemical and biological perception. Fungal Divers 61(1):1–27
47. Radic N, Strukelj B (2012) Endophytic fungi-the treasure chest of antibacterial substances. Phytomedicine 19(14):1270–1284
48. Wang LW, Zhang YL, Lin FC, Hu YZ, Zhang CL (2011) Natural products with antitumor activity from endophytic fungi. Mini Rev Med Chem 11(12):1056–1074
49. Kaul S, Gupta S, Ahmed M, Dhar MK (2012) Endophytic fungi from medicinal plants: a treasure hunt for bioactive metabolites. Phytochem Rev 11(4):487–505 (Article)
50. Kumar S, Kaushik N (2012) Metabolites of endophytic fungi as novel source of biofungicide: a review. Phytochem Rev 11(4):507–522 (Article)
51. Ebrahim W, Aly AH, Wray V, Proksch P, Debbab A (2013) Unusual octalactones from *Corynespora cassiicola*, an endophyte of *Laguncularia racemosa*. Tetrahedron Lett 54(48):6611–6614 (Article)
52. Sommart U, Rukachaisirikul V, Tadpetch K, Sukpondma Y, Phongpaichit S, Hutadilok-Towatana N et al (2012) Modiolin and phthalide derivatives from the endophytic fungus *Microsphaeropsis arundinis* psu-g18. Tetrahedron 68(48):10005–10010 (Article)
53. El Amrani M, Lai DW, Debbab A, Aly AH, Siems K, Seidel C et al (2014) Protein kinase and hdac inhibitors from the endophytic fungus *Epicoccum nigrum*. J Nat Prod 77(1):49–56 (Article)
54. Talontsi FM, Douanla-Meli C, Laatsch H (2013) Depsidones from an endophytic fungus *Chaetomium* sp. associated with *Zanthoxylum leprieurii*. Zeitschrift Fur Naturforschung (Section B–A Journal of Chemical Sciences) 68(11):1259–1264 (Article)
55. Okoye FBC, Lu S, Nworu CS, Esimone CO, Proksch P, Chadli A et al (2013) Depsidone and diaryl ether derivatives from the fungus *Corynespora cassiicola*, an endophyte of *Gongronema latifolium*. Tetrahedron Lett 54(32):4210–4214 (Article)
56. Talontsi FM, Islam MT, Facey P, Douanla-Meli C, von Tiedemann A, Laatsch H (2012) Depsidones and other constituents from *Phomopsis* sp. caft69 and its host plant *Endodesmia calophylloides* with potent inhibitory effect on motility of zoospores of grapevine pathogen *Plasmopara viticola*. Phytochem Lett 5(3):657–664 (Article)
57. Yang SX, Wang HP, Gao JM, Zhang Q, Laatsch H, Kuang Y (2012) Fusaroside, a unique glycolipid from *Fusarium* sp., an endophytic fungus isolated from melia azedarach. Org Biomol Chem 10(4):819–824 (Article)
58. Ola ARB, Debbab A, Kurtán T, Brötz-Oesterhelt H, Aly AH, Proksch P (2014) Dihydroanthracenone metabolites from the endophytic fungus *Diaporthe melonis* isolated from *Annona squamosa*. Tetrahedron Lett 55:3133–3136
59. Klaiklay S, Rukachaisirikul V, Tadpetch K, Sukpondma Y, Phongpaichit S, Buatong J et al (2012) Chlorinated chromone and diphenyl ether derivatives from the mangrove-derived fungus *Pestalotiopsis* sp. psu-ma69. Tetrahedron 68(10):2299–2305 (Article)
60. Cheng MJ, Wu MD, Yuan GF, Chen YL, Su YS, Hsieh MT et al (2012) Secondary metabolites and cytotoxic activities from the endophytic fungus *Annulohypoxylon squamulosum*. Phytochem Lett 5(1):219–223 (Article)

61. Macias-Rubalcava ML, Sobrino M, Melendez-Gonzalez C, Hernandez-Ortega S (2014) Naphthoquinone spiroketals and organic extracts from the endophytic fungus *Edenia gomezpompae* as potential herbicides. J Agri Food Chem 62(16):3553–3562 (Article)
62. Shang Z, Li XM, Li CS, Wang BG (2012) Diverse secondary metabolites produced by marine-derived fungus *Nigrospora* sp. ma75 on various culture media. Chem Biodivers 9(7):1338–1348 (Article)
63. Araujo FDD, Favaro LCD, Araujo WL, de Oliveira FL, Aparicio R, Marsaioli AJ (2012) Epicolactone—natural product isolated from the sugarcane endophytic fungus *Epicoccum nigrum*. Eur J Org Chem 2012(27):5225–5230 (Article)
64. Talontsi FM, Dittrich B, Schuffler A, Sun H, Laatsch H (2013) Epicoccolides: antimicrobial and antifungal polyketides from an endophytic fungus *Epicoccum* sp. associated with Theobroma cacao. Eur J Org Chem 2013(15):3174–3180 (Article)
65. Cheng MJ, Wu MD, Yanai H, Su YS, Chen IS, Yuan GF et al (2012) Secondary metabolites from the endophytic fungus *Biscogniauxia formosana* and their antimycobacterial activity. Phytochem Lett 5(3):467–472 (Article)
66. Liu L, Li Y, Li L, Cao Y, Guo LD, Liu G et al (2013) Spiroketals of pestalotiopsis fici provide evidence for a biosynthetic hypothesis involving diversified diels-alder reaction cascades. J Org Chem 78(7):2992–3000 (Article)
67. Senadeera SPD, Wiyakrutta S, Mahidol C, Ruchirawat S, Kittakoop P (2012) A novel tricyclic polyketide and its biosynthetic precursor azaphilone derivatives from the endophytic fungus *Dothideomycete* sp. Org Biomol Chem 10(35):7220–7226 (Article)
68. Ying Y-M, Zheng Z-Z, Zhang L-W, Shan W-G, Wang J-W, Zhan Z-J (2014) Rare c-25 steroids produced by *Penicillium chrysogenum* p1x, a fungal endophyte of *Huperzia serrata*. Helvetica Chimica Acta 97(1):95–101
69. Zang LY, Wei W, Guo Y, Wang T, Jiao RH, Ng SW et al (2012) Sesquiterpenoids from the mangrove-derived endophytic fungus *Diaporthe* sp. J Nat Prod 75(10):1744–1749 (Article)
70. Kawahara T, Itoh M, Izumikawa M, Sakata N, Tsuchida T, Shin-ya K (2013) Three eremophilane derivatives, MBJ-0011, MBJ-0012 and MBJ-0013, from an endophytic fungus *Apiognomonia* sp. f24023. J Antibiot 66(5):299–302 (Article)
71. Hemberger Y, Xu J, Wray V, Proksch P, Wu J, Bringmann G (2013) Pestalotiopens A and B: stereochemically challenging flexible sesquiterpene-cyclopaldic acid hybrids from *Pestalotiopsis* sp. Chemistry 19(46):15556–15564 (Article)
72. Huang X, Huang H, Li H, Sun X, Huang H, Lu Y et al (2013) Asperterpenoid A, a new sesterterpenoid as an inhibitor of Mycobacterium tuberculosis protein tyrosine phosphatase B from the culture of *Aspergillus* sp 16–5c. Org Lett 15(4):721–723
73. Liang H (2008) Sordarin, an antifungal agent with a unique mode of action. Beilstein J Org Chem 4:31
74. Rukachaisirikul V, Buadam S, Phongpaichit S, Sakayaroj J (2013) Amide, cyclohexenone, and cyclohexenone-sordaricin derivatives from the endophytic fungus *Xylaria plebeja* PSU-G30. Tetrahedron 69(50):10711–10717 (Article)
75. Xu Y-m, Espinosa-Artiles P, Liu MX, Arnold AE, Gunatilaka AAL (2013) Secoemestrin D, a cytotoxic epitetrathiodioxopiperizine, and emericellenes A–E, five sesterterpenoids from Emericella sp AST0036, a fungal endophyte of *Astragalus lentiginosus*. J Nat Prod 76(12):2330–2336
76. Haroon MH, Premaratne SR, Choudhry MI, Dharmaratne HRW (2013) A new-glucuronidase inhibiting butyrolactone from the marine endophytic fungus *Aspergillus terreus*. Nat Prod Res 27(12):1060–1066 (Article)
77. Ebrahim W, Kjer J, El Amrani M, Wray V, Lin W, Ebel R et al (2012) Pullularins E and F, two new peptides from the endophytic fungus *Bionectria ochroleuca* isolated from the mangrove plant *Sonneratia caseolaris*. Mar Drugs 10(5):1081–1091
78. Liu Y-X, Ma S-G, Wang X-J, Zhao N, Qu J, Yu S-S et al (2012) Diketopiperazine alkaloids produced by the endophytic fungus *Aspergillus fumigatus* from the stem of *Erythrophloeum fordii* Oliv. Helvetica Chimica Acta 95(8):1401–1408

79. Devi P, Rodrigues C, Naik CG, D'Souza L (2012) Isolation and characterization of antibacterial compound from a mangrove-endophytic fungus, *Penicillium chrysogenum* MTCC 5108. Indian J Microbiol 52(4):617–623
80. Kong F, Wang Y, Liu P, Dong T, Zhu W (2014) Thiodiketopiperazines from the marine-derived fungus *Phoma* sp OUCMDZ-1847. J Nat Prod 77(1):132–137
81. Gardiner DM, Waring P, Howlett BJ (2005) The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions and biosynthesis. Microbiology-Sgm 151:1021–1032
82. Putri SP, Kinoshita H, Ihara F, Igarashi Y, Nihira T (2009) Farinomalein, a maleimide-bearing compound from the entomopathogenic fungus *Paecilomyces farinosus*. J Nat Prod 72(8):1544–1546
83. Xu J, Kjer J, Sendker J, Wray V, Guan H, Edrada R et al (2009) Cytosporones, coumarins, and an alkaloid from the endophytic fungus *Pestalotiopsis* sp. isolated from the chinese mangrove plant *Rhizophora mucronata*. Bioorg Med Chem 17(20):7362–7367
84. El Amrani M, Debbab A, Aly AH, Wray V, Dobretsov S, Mueller WEG et al (2012) Farinomalein derivatives from an unidentified endophytic fungus isolated from the mangrove plant *Avicennia marina*. Tetrahedron Lett 53(49):6721–6724
85. Zhang Y, Wang T, Pei YH, Hua HM, Feng BM (2002) Aspergillin PZ, a novel isoindole-alkaloid from *Aspergillus awamori*. J Antibiot 55(8):693–695
86. Chen L, Liu Y-T, Song B, Zhang H-W, Ding G, Liu X-Z et al (2014) Stereochemical determination of new cytochalasans from the plant endophytic fungus *Trichoderma gamsii*. Fitoterapia 96:115–122
87. Xue M, Zhang Q, Gao JM, Li H, Tian JM, Pescitelli G (2012) Chaetoglobosin V–B from endophytic *Chaetomium globosum*: absolute configuration of chaetoglobosins. Chirality 24(8):668–674 (Article)
88. An CY, Li XM, Li CS, Wang MH, Xu GM, Wang BG (2013) Aniquinazolines A–D, four new quinazolinone alkaloids from marine-derived endophytic fungus *Aspergillus nidulans*. Mar Drugs 11(7):2682–2694 (Article)
89. Saleem M, Tousif MI, Riaz N, Ahmed I, Schulz B, Ashraf M et al (2013) Cryptosporioptide: a bioactive polyketide produced by an endophytic fungus *Cryptosporiopsis* sp. Phytochemistry 93:199–202
90. Ortega HE, Graupner PR, Asai Y, TenDyke K, Qiu D, Shen YY et al (2013) Mycoleptodiscins A and B, cytotoxic alkaloids from the endophytic fungus *Mycoleptodiscus* sp. F0194. J Nat Prod 76(4):741–744
91. El-Neketi M, Ebrahim W, Lin W, Gedara S, Badria F, Saad H-EA et al (2013) Alkaloids and polyketides from *Penicillium citrinum*, an endophyte isolated from the moroccan plant *Ceratonia siliqua*. J Nat Prod 76(6):1099–1104
92. Siriwach R, Kinoshita H, Kitani S, Igarashi Y, Pansuksan K, Panbangred W et al (2014) Bipolamides A and B, triene amides isolated from the endophytic fungus *Bipolaris* sp. MU34. J Antibiot 67(2):167–170
93. Ebrahim W, Aly AH, Wray V, Mandi A, Teiten M-H, Gaascht F et al (2013) Embellicines A and B: absolute configuration and NF-kappa B transcriptional inhibitory activity. J Med Chem 56(7):2991–2999
94. Bode HB, Bethe B, Hofs R, Zeeck A (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. Chembiochem 3(7):619–627
95. Cichewicz RH (2010) Epigenome manipulation as a pathway to new natural product scaffolds and their congeners. Nat Prod Rep 27(1):11–22
96. Pettit RK (2011) Small-molecule elicitation of microbial secondary metabolites. Microb Biotechnol 4(4):471–478
97. Marmann A, Aly AH, Lin W, Wang B, Proksch P (2014) Co-cultivation-a powerful emerging tool for enhancing the chemical diversity of microorganisms. Mar Drugs 12(2):1043–1065
98. Bertrand S, Bohni N, Schnee S, Schumpp O, Gindro K, Wolfender J-L (2014) Metabolite induction viamicroorganism co-culture: a potential way to enhance chemical diversity for drug discovery. Biotechnol Adv 32(6):1180–1204 (Epub)

99. Zhang Q, Wang S-Q, Tang H-Y, Li X-J, Zhang L, Xiao J et al (2013) Potential allelopathic indole diketopiperazines produced by the plant endophytic *Aspergillus fumigatus* using the one strain-many compounds method. J Agri Food Chem 61(47):11447–11452
100. Sun J, Awakawa T, Noguchi H, Abe I (2012) Induced production of mycotoxins in an endophytic fungus from the medicinal plant *Datura stramonium* l. Bioorg Med Chem Lett 22(20):6397–6400
101. Ul-Hassan SR, Strobel GA, Booth E, Knighton B, Floerchinger C, Sears J (2012) Modulation of volatile organic compound formation in the Mycodiesel-producing endophyte hypoxylon sp. CI-4. Microbiology-Sgm 158:465–473
102. Chagas FO, Dias LG, Pupo MT (2013) A mixed culture of endophytic fungi increases production of antifungal polyketides. J Chem Ecol 39(10):1335–1342
103. Zhu F, Lin Y (2006) Marinamide, a novel alkaloid and its methyl ester produced by the application of mixed fermentation technique to two mangrove endophytic fungi from the South China Sea. Chin Sci Bull 51(12):1426–1430
104. Wang J-p, Lin W, Wray V, Lai D, Proksch P (2013) Induced production of depsipeptides by co-culturing *Fusarium tricinctum* and *Fusarium begoniae*. Tetrahedron Lett 54(20): 2492–2496
105. Wu B, Wu X, Sun M, Li M (2013) Two novel tyrosinase inhibitory sesquiterpenes induced by CUCL2 from a marine-derived fungus *Pestalotiopsis* sp. Z233. Mar Drugs 11(8):2713–2721
106. Chen H, Awakawa T, Sun J, Wakimoto T, Abe I (2013) Epigenetic modifier induced biosynthesis of novel fusaric acid derivatives in endophytic fungi from *Datura stramonium* l. Nat Prod Bioprospecting 3(1):20–23
107. Asai T, Otsuki S, Sakurai H, Yamashita K, Ozeki T, Oshima Y (2013) Benzophenones from an endophytic fungus, *Graphiopsis chlorocephala*, from *Paeonia lactiflora* cultivated in the presence of an NAD(+)-dependent hdac inhibitor. Org Lett 15(8):2058–2061 (Article)
108. Zhu F, Chen G, Wu J, Pan J (2013) Structure revision and cytotoxic activity of marinamide and its methyl ester, novel alkaloids produced by co-cultures of two marine-derived mangrove endophytic fungi. Nat Prod Res 27(21):1960–1964
109. Cao SG, Cryan L, Habeshian KA, Murillo C, Tamayo-Castillo G, Rogers MS et al (2012) Phenolic compounds as antiangiogenic cmg2 inhibitors from *Costa rican* endophytic fungi. Bioorg Med Chem Lett 22(18):5885–5888 (Article)
110. Ding G, Qi YX, Liu SC, Guo LD, Chen XL (2012) Photipyrones a and b, new pyrone derivatives from the plant endophytic fungus *Pestalotiopsis photiniae*. J Antibiot 65(5):271–273 (Article)
111. Hussain H, Krohn K, Ahmed I, Draeger S, Schulz B, Pietro S et al (2012) Phomopsinones a–d: four new pyrenocines from endophytic fungus *Phomopsis* sp. Eur J Org Chem 2012(9):1783–1789 (Article)
112. Hussain H, Ahmed I, Schulz B, Draeger S, Krohn K (2012) Pyrenocines j–m: four new pyrenocines from the endophytic fungus, *Phomopsis* sp. Fitoterapia 83(3):523–526 (Article)
113. Hussain H, Krohn K, Schulz B, Draeger S, Nazir M, Saleem M (2012) Two new antimicrobial metabolites from the endophytic fungus, *Seimatosporium* sp. Nat Prod Commun 7(3):293–294 (Article)
114. Luo DQ, Zhang L, Shi BZ, Song XM (2012) Two new oxysporone derivatives from the fermentation broth of the endophytic plant fungus *Pestalotiopsis karstenii* isolated from stems of *Camellia sasanqua*. Molecules 17(7):8554–8560 (Article)
115. Siriwach R, Kinoshita H, Kitani S, Igarashi Y, Pansuksan K, Panbangred W et al (2012) Mycoleptione, a new chromone derivative isolated from the endophytic fungus *Mycoleptodiscus* sp. mu41. J Antibiot 65(12):627–629 (Article)
116. Tarman K, Palm GJ, Porzel A, Merzweiler K, Arnold N, Wessjohann LA et al (2012) Helicascolide c, a new lactone from an indonesian marine algicolous strain of *Daldinia eschscholzii* (xylariaceae, ascomycota). Phytochem Lett 5(1):83–86 (Article)

117. Peng W, You F, Li XL, Jia M, Zheng CJ, Han T et al (2013) A new diphenyl ether from the endophytic fungus *Verticillium* sp. isolated from *Rehmannia glutinosa*. Chin J Nat Med 11(6):673–675 (Article)
118. Ronsberg D, Debbab A, Mandi A, Wray V, Dai HF, Kurtan T et al (2013) Secondary metabolites from the endophytic fungus pestalotiopsis virgatula isolated from the mangrove plant *Sonneratia caseolaris*. Tetrahedron Lett 54(25):3256–3259 (Article)
119. Akay S, Ekiz G, Kocabas F, Hames-Kocabas EE, Korkmaz KS, Bedir E (2014) A new 5,6-dihydro-2-pyrone derivative from *Phomopsis amygdali*, an endophytic fungus isolated from hazelnut (*Corylus avellana*). Phytochem Lett 7:93–96 (Article)
120. Fang ZF, Yu SS, Zhou WQ, Chen XG, Ma SG, Li Y et al (2012) A new isocoumarin from metabolites of the endophytic fungus *Alternaria tenuissima* (nees & t. Nees: Fr.) wiltshire. Chin Chem Lett 23(3):317–320 (Article)
121. Li SD, Wei MY, Chen GY, Lin YC (2012) Two new dihydroisocoumarins from the endophytic fungus *Aspergillus* sp. collected from the South China Sea. Chem Nat Compd 48(3):371–373 (Article)
122. Metwaly AM, Kadry HA, El-Hela AA, Mohammad AEI, Ma GY, Cutler SJ et al (2014) Nigrosphaerin A a new isochromene derivative from the endophytic fungus *Nigrospora sphaerica*. Phytochem Lett 7:1–5 (Article)
123. Ola ARB, Thomy D, Lai D, Broetz-Oesterhelt H, Prolcsch P (2013) Inducing secondary metabolite production by the endophytic fungus *Fusarium tricinctum* through coculture with *Bacillus subtilis*. J Nat Prod 76(11):2094–2099
124. Kumar M, Qadri M, Sharma PR, Kumar A, Andotra SS, Kaur T et al (2013) Tubulin inhibitors from an endophytic fungus isolated from *Cedrus deodara*. J Nat Prod 6(2):194–199 (Article)
125. Liu SC, Guo LD, Che YS, Liu L (2013) Pestaloficiols q–s from the plant endophytic fungus *Pestalotiopsis fici*. Fitoterapia 85:114–118 (Article)
126. Jiao Y, Zhang X, Wang L, Li G, Zhou JC, Lou HX (2013) Metabolites from *Penicillium* sp., an endophytic fungus from the liverwort *Riccardia multifida* (l.) s. Gray. Phytochem Lett 6(1):14–17 (Article)
127. Jin P, Zuo W, Guo Z, Mei W, Dai H (2013) Metabolites from the endophytic fungus *Penicillium* sp. Fj-1 of *Ceriops tagal*. Acta Pharmaceutica Sinica 48(11):1688–1691
128. Rukachaisirikul V, Buadam S, Sukpondma Y, Phongpaichit S, Sakayaroj J, Hutadilok-Towatana N (2013) Indanone and mellein derivatives from the garcinia-derived fungus *Xylaria* sp. psu-g12. Phytochem Lett 6(1):135–138 (Article)
129. Rukachaisirikul V, Rodglin A, Sukpondma Y, Phongpaichit S, Buatong J, Sakayaroj J (2012) Phthalide and isocoumarin derivatives produced by an *Acremonium* sp. isolated from a mangrove *Rhizophora apiculata*. J Nat Prod 75(5):853–858 (Article)
130. Ebrahim W, Aly AH, Mandi A, Wray V, Essassi E, Ouchbani T et al (2013) O-heterocyclic embeurekols from *Embellisia eureka*, an endophyte of *Cladanthus arabicus*. Chirality 25(4):250–256 (Article)
131. Song YX, Wang J, Li SW, Cheng B, Li L, Chen B et al (2012) Metabolites of the mangrove fungus *Xylaria* sp. bl321 from the South China Sea. Planta Medica 78(2):172–176 (Article)
132. Yang JX, Huang RM, Qiu SX, She ZG, Lin YC (2013) A new isobenzofuranone from the mangrove endophytic fungus *Penicillium* sp. (zh58). Nat Prod Res 27(20):1902–1905 (Article)
133. Xue H, Lu CH, Liang LY, Shen YM (2012) Secondary metabolites of *Aspergillus* sp. cm9a, an endophytic fungus of *Cephalotaxus mannii*. Rec Nat Prod 6(1):28–34 (Article)
134. Yan HJ, Li XM, Li CS, Wang BG (2012) Alkaloid and anthraquinone derivatives produced by the marine-derived endophytic fungus *Eurotium rubrum*. Helv Chim Acta 95(1):163–168 (Article)
135. Ebrahim W, Aly AH, Mandi A, Totzke F, Kubbutat MHG, Wray V et al (2012) Decalactone derivatives from *Corynespora cassiicola*, an endophytic fungus of the mangrove plant *Laguncularia racemosa*. Eur J Org Chem 2012(18):3476–3484 (Article)
136. Okoye FBC, Nworu CS, Akah PA, Esimone CO, Debbab A, Proksch P (2013) Inhibition of inflammatory mediators and reactive oxygen and nitrogen species by some depsidones

and diaryl ether derivatives isolated from *Corynespora cassiicola*, an endophytic fungus of *Gongronema latifolium* leaves. Immunopharmacol Immunotoxicol 35(6):662–668 (Article)
137. Sun HF, Li XM, Meng LH, Cui CM, Gao SS, Li CS et al (2013) Two new secoanthraquinone derivatives from the marine-derived endophytic fungus *Aspergillus wentii* en-48. Helv Chim Acta 96(3):458–462 (Article)
138. Li X, Li XM, Xu GM, Li CS, Wang BG (2014) Antioxidant metabolites from marine alga-derived fungus *Aspergillus wentii* en-48. Phytochem Lett 7:120–123 (Article)
139. Baraban EG, Morin JB, Phillips GM, Phillips AJ, Strobel SA, Handelsman J (2013) Xyolide, a bioactive nonenolide from an amazonian endophytic fungus, *Xylaria feejeensis*. Tetrahedron Lett 54(31):4058–4060 (Article)
140. Ortega HE, Shen YY, TenDyke K, Ríos N, Cubilla-Ríos L (2014) Polyhydroxylated macrolide isolated from the endophytic fungus *Pestalotiopsis mangiferae*. Tetrahedron Lett 55:2642–2645
141. Yang SX, Gao JM, Laatsch H, Tian JM, Pescitelli G (2012) Absolute configuration of fusarone, a new azaphilone from the endophytic fungus *Fusarium* sp. isolated from *Melia azedarach*, and of related azaphilones. Chirality 24(8):621–627 (Article)
142. Zeng YB, Wang H, Zuo WJ, Zheng B, Yang T, Dai HF et al (2012) A fatty acid glycoside from a marine-derived fungus isolated from mangrove plant *Scyphiphora hydrophyllacea*. Mar Drugs 10(3):598–603 (Article)
143. Hawas UW, El-Beih AA, El-Halawany AM (2012) Bioactive anthraquinones from endophytic fungus *Aspergillus versicolor* isolated from red sea algae. Arch Pharm Res 35(10):1749–1756 (Article)
144. Klaiklay S, Rukachaisirikul V, Phongpaichit S, Pakawatchai C, Saithong S, Buatong J et al (2012) Anthraquinone derivatives from the mangrove-derived fungus *Phomopsis* sp. psu-ma214. Phytochem Lett 5(4):738–742 (Article)
145. Miao FP, Li XD, Liu XH, Cichewicz RH, Ji NY (2012) Secondary metabolites from an algicolous *Aspergillus versicolor* strain. Mar Drugs 10(1):131–139 (Article)
146. Choi JN, Kim J, Ponnusamy K, Lim C, Kim JG, Muthaiya MJ et al (2013) Identification of a new phomoxanthone antibiotic from *Phomopsis longicolla* and its antimicrobial correlation with other metabolites during fermentation. J Antibiot 66(4):231–233 (Article)
147. Huang ZJ, Yang JX, Lei FH, She ZG, Lin YC (2013) A new xanthone o-glycoside from the mangrove endophytic fungus *Phomopsis* sp. Chem Nat Compd 49(1):27–30 (Article)
148. Koolen HHF, Menezes LS, Souza MP, Silva FMA, Almeida FGO, de Souza AQL et al (2013) Talaroxanthone, a novel xanthone dimer from the endophytic fungus *Talaromyces* sp. associated with *Duguetia stelechantha* (Diels) R. E. Fries. J Braz Chem Soc 24(5): 880- + (Article)
149. Calcul L, Waterman C, Ma WS, Lebar MD, Harter C, Mutka T et al (2013) Screening mangrove endophytic fungi for antimalarial natural products. Mar Drugs 11(12): 5036–5050 (Article)
150. Song XQ, Zhang X, Han QJ, Li XB, Li G, Li RJ et al (2013) Xanthone derivatives from *Aspergillus sydowii*, an endophytic fungus from the liverwort *Scapania ciliata* s. Lac and their immunosuppressive activities. Phytochem Lett 6(3):318–321 (Article)
151. Cheng MJ, Wu MD, Chen IS, Hsieh SY, Yuan GF (2012) Chemical constituents from the endophytic fungus *Annulohypoxylon squamulosum*. Chem Nat Compd 48(2):218–220 (Article)
152. Liu HT, Liu SC, Guo LD, Zhang YG, Cui LJ, Ding G (2012) New furanones from the plant endophytic fungus *Pestalotiopsis besseyi*. Molecules 17(12):14015–14021 (Article)
153. Zhang HQ, Deng ZS, Guo ZY, Tu X, Wang JZ, Zou K (2014) Pestalafuranones f–j, five new furanone analogues from the endophytic fungus *Nigrospora* sp. bm-2. Molecules 19(1):819–825 (Article)
154. Liu SC, Liu XY, Guo LD, Che YS, Liu L (2013) 2h-pyran-2-one and 2h-furan-2-one derivatives from the plant endophytic fungus *Pestalotiopsis fici*. Chem Biodivers 10(11):2007–2013 (Article)

155. Chen G, Zhang L, Wang HF, Wu HH, Lu X, Pei YH et al (2013) A new compound along with seven known compounds from an endophytic fungus *Aspergillus* sp. hs-05. Rec Nat Prod 7(4):320–324 (Article)
156. Wu MD, Cheng MJ, Chen IS, Su YS, Hsieh SY, Chang HS et al (2013) Phytochemical investigation of annulohypoxylon ilanense, an endophytic fungus derived from *Cinnamomum species*. Chem Biodivers 10(3):493–505 (Article)
157. Cheng MJ, Wu MD, Chen IS, Chen JJ, Hsieh SY, Yuan GF (2013) A new furan-3-one derivative from the endophytic fungus *Annulohypoxylon* sp. Chem Nat Compd 49(3):446–449 (Article)
158. Wang X, Wang H, Liu T, Xin Z (2014) A pks i gene-based screening approach for the discovery of a new polyketide from *Penicillium citrinum* salicorn 46. Appl Microbiol Biotechnol (In press). doi:10.1007/s00253-014-5572-3
159. Klaiklay S, Rukachaisirikul V, Phongpaichit S, Buatong J, Preedanon S, Sakayaroj J (2013) Flavodonfuran: a new difuranylmethane derivative from the mangrove endophytic fungus *Flavodon flavus* psu-ma201. Nat Prod Res 27(19):1722–1726 (Article)
160. Ai HL, Zhang LM, Chen YP, Zi SH, Xiang H, Zhao DK et al (2012) Two new compounds from an endophytic fungus *Alternaria solani*. J Asian Nat Prod Res 14(12):1144–1148 (Article)
161. Akone SH, El Amrani M, Lin WH, Lai DW, Proksch P (2013) Cytosporins f–k, new epoxyquinols from the endophytic fungus *Pestalotiopsis theae*. Tetrahedron Lett 54(49):6751–6754 (Article)
162. Wanigesekara W, Wijeratne EMK, Arnold AE, Gunatilaka AAL (2013) 10′-deoxy-10′alpha-hydroxyascochlorin, a new cell migration inhibitor and other metabolites from *Acremonium* sp., a fungal endophyte in *Ephedra trifurca*. Nat Prod Commun 8(5):601–604 (Article)
163. Asai T, Otsuki S, Taniguchi T, Monde K, Yamashita K, Sakurai H et al (2013) Structures and absolute configurations of short-branched fatty acid dimers from an endophytic fungus of *Aloe arborescens*. Tetrahedron Lett 54(26):3402–3405 (Article)
164. Ding GZ, Liu J, Wang JM, Fang L, Yu SS (2013) Secondary metabolites from the endophytic fungi *Penicillium polonicum* and *Aspergillus fumigatus*. J Asian Nat Prod Res 15(5):446–452 (Article)
165. El-Neketi M, Ebrahim W, Lin WH, Gedara S, Badria F, Saad HEA et al (2013) Alkaloids and polyketides from *Penicillium citrinum*, an endophyte isolated from the moroccan plant *Ceratonia siliqua*. J Nat Prod 76(6):1099–1104 (Article)
166. Zheng CJ, Xu LL, Li YY, Han T, Zhang QY, Ming QL et al (2013) Cytotoxic metabolites from the cultures of endophytic fungi from *Panax ginseng*. Appl Microbiol Biotechnol 97(17):7617–7625 (Article)
167. Zilla MK, Qadri M, Pathania AS, Strobel GA, Nalli Y, Kumar S et al (2013) Bioactive metabolites from an endophytic *Cryptosporiopsis* sp. inhabiting *Clidemia hirta*. Phytochemistry 95:291–297
168. Wu Q, Guo Y, Guo ZK, Chu YL, Wang T, Tan RX (2013) Two new cytosporones from the culture of endophytic *Phomopsis* sp. Chem Nat Compd 48(6):938–941 (Article)
169. Leyte-Lugo M, Gonzalez-Andrade M, Gonzalez MD, Glenn AE, Cerda-Garcia-Rojas CM, Mata R (2012) (+)-ascosalitoxin and vermelhotin, a calmodulin inhibitor, from an endophytic fungus isolated from *Hintonia latiflora*. J Nat Prod 75(9):1571–1577 (Article)
170. Li JT, Chen QQ, Zeng Y, Wang Q, Zhao PJ (2012) A new phenol compound from endophytic *Phomopsis* sp. dc01. Nat Prod Res 26(21):2008–2012 (Article)
171. Talontsi FM, Kenla TJN, Dittrich B, Douanla-Meli C, Laatsch H (2012) Paeciloside a, a new antimicrobial and cytotoxic polyketide from *Paecilomyces* sp. strain caft156. Planta Medica 78(10):1020–1023 (Letter)
172. Wang JF, Lu ZY, Liu PP, Wang Y, Li J, Hong K et al (2012) Cytotoxic polyphenols from the fungus *Penicillium expansum* 091006 endogenous with the mangrove plant *Excoecaria agallocha*. Planta Medica 78(17):1861–1866 (Article)
173. Wang LW, Xu BG, Wang JY, Su ZZ, Lin FC, Zhang CL et al (2012) Bioactive metabolites from phoma species, an endophytic fungus from the chinese medicinal plant *Arisaema erubescens*. Appl Microbiol Biotechnol 93(3):1231–1239 (Article)

174. Sun P, Huo J, Kurtan T, Mandi A, Antus S, Tang H et al (2013) Structural and stereochemical studies of hydroxyanthraquinone derivatives from the endophytic fungus *Coniothyrium* sp. Chirality 25(2):141–148 (Article)
175. Klaiklay S, Rukachaisirikul V, Sukpondma Y, Phongpaichit S, Buatong J, Bussaban B (2012) Metabolites from the mangrove-derived fungus *Xylaria cubensis* psu-ma34. Arch Pharm Res 35(7):1127–1131 (Article)
176. Chen R-D, Yan Z, Zou J-H, Wang N, Dai J-G (2014) Rubratoxin c, a new nonadride derivative from an endophytic fungus *Penicillium* sp. F-14. Chin Chem Lett (In press) (http://dx.doi.org/10.1016/j.cclet.2014.03.040)
177. Wang H, Liu T, Xin Z (2014) A new glucitol from an endophytic fungus *Fusarium equiseti* salicorn 8. Eur Food Res Technol (In press). doi:10.1007/s00217-014-2230-z
178. Ying YM, Shan WG, Zhang LW, Chen Y, Zhan ZJ (2013) Lanostane triterpenes from *Ceriporia lacerate* hs-zjut-c13a, a fungal endophyte of *Huperzia serrata*. Helvetica Chimica Acta 96(11):2092–2097 (Article)
179. Ying Y-M, Z.-Z. Z, Zhang L-W, Shan W-G, Wang J-W, Zhan Z-J (2014) Rare c_{25} steroids produced by *Penicillium chrysogenum* p1x, a fungal endophyte of *Huperzia serrata*. Helvetica Chimica Acta 97:95–101
180. Chang DD, Zuo WJ, Mei WL, Dai HF (2012) Metabolites from endophytic fungus a12 of *Dracaena cambodiana*. J Asian Nat Prod Res 14(6):577–580 (Article)
181. Guo ZK, Yan T, Guo Y, Song YC, Jiao RH, Tan RX et al (2012) P-terphenyl and diterpenoid metabolites from endophytic *Aspergillus* sp. yxf3. J Nat Prod 75(1):15–21 (Article)
182. Yan T, Guo ZK, Jiang R, Wei W, Wang T, Guo Y et al (2013) New flavonol and diterpenoids from the endophytic fungus *Aspergillus* sp. yxf3. Planta Medica 79(5):348–352 (Article)
183. Guimaraes DO, Lopes NP, Pupo MT (2012) Meroterpenes isolated from the endophytic fungus *Guignardia mangiferae*. Phytochem Lett 5(3):519–523 (Article)
184. Mei WL, Zheng B, Zhao YX, Zhong HM, Chen XLW, Zeng YB et al (2012) Meroterpenes from endophytic fungus a1 of mangrove plant *Scyphiphora hydrophyllacea*. Mar Drugs 10(9):1993–2001 (Article)
185. Molinar E, Rios N, Spadafora C, Arnold AE, Coley PD, Kursar TA et al (2012) Coibanoles, a new class of meroterpenoids produced by *Pycnoporus sanguineus*. Tetrahedron Lett 53(8):919–922 (Article)
186. Liu XH, Miao FP, Li XD, Yin XL, Ji NY (2012) A new sesquiterpene from an endophytic *Aspergillus versicolor* strain. Nat Prod Commun 7(7):819–820 (Article)
187. Song YX, Wang JJ, Huang HB, Ma L, Wang J, Gu YC et al (2012) Four eremophilane sesquiterpenes from the mangrove endophytic fungus *Xylaria* sp. bl321. Mar Drugs 10(2):340–348 (Article)
188. Gubiani JR, Zeraik ML, Oliveira CM, Ximenes VF, Nogueira CR, Fonseca LM et al (2014) Biologically active eremophilane-type sesquiterpenes from *Camarops* sp., an endophytic fungus isolated from *Alibertia macrophylla*. J Nat Prod 77(3):668–672 (Article)
189. Deng CM, Huang CH, Wu QL, Pang JY, Lin YC (2013) A new sesquiterpene from the mangrove endophytic fungus *Aspergillus terreus* (no. Gx7–3b). Nat Prod Res 27(20): 1882–1887 (Article)
190. Huang XS, Sun XF, Ding B, Lin M, Liu L, Huang HR et al (2013) A new anti-acetylcholinesterase alpha-pyrone meroterpene, arigsugacin i, from mangrove endophytic fungus *Penicillium* sp. sk5gw1l of *Kandelia candel*. Planta Medica 79(16):1572–1575 (Article)
191. Huang XS, Huang HB, Li HX, Sun XF, Huang HR, Lu YJ et al (2013) Asperterpenoid a, a new sesterterpenoid as an inhibitor of *Mycobacterium tuberculosis* protein tyrosine phosphatase b from the culture of *Aspergillus* sp. 16–5c. Org Lett 15(4):721–723 (Article)
192. Wu LS, Hu CL, Han T, Zheng CJ, Ma XQ, Rahman K et al (2013) Cytotoxic metabolites from *Perenniporia tephropora*, an endophytic fungus from *Taxus chinensis* var. Mairei. Appl Microbiol Biotechnol 97(1):305–315 (Article)
193. Xu YM, Espinosa-Artiles P, Liu MPX, Arnold AE, Gunatilaka AAL (2013) Secoemestrin d, a cytotoxic epitetrathiodioxopiperizine, and emericellenes a-e, five sesterterpenoids

from *Emericella* sp. ast0036, a fungal endophyte of *Astragalus lentiginosus*. J Nat Prod 76(12):2330–2336 (Article)

194. Xuan QC, Huang R, Chen YW, Miao CP, Ma KX, Wang T et al (2014) Cyclonerol derivatives from *Trichoderma longibrachiatum* ym311505. Nat Prod Commun 9(3): 313–314 (Article)
195. Zuo WJ, Mn PF, Dong WH, Dai HF, Mei WL (2014) Metabolites from the endophytic fungus hp-1 of Chinese eaglewood. Chin J Nat Med 12(2):151–153 (Article)
196. Wu B, Wu XD, Sun M, Li MH (2013) Two novel tyrosinase inhibitory sesquiterpenes induced by cucl2 from a marine-derived fungus *Pestalotiopsis* sp. z233. Mar Drugs 11(8):2713–2721 (Article)
197. Gu W, Qiao C (2012) Furandiones from an endophytic *Aspergillus terreus* residing in *Malus halliana*. Chem Pharm Bull 60(11):1474–1477 (Article)
198. Ebrahim W, Kjer J, El Amrani M, Wray V, Lin WH, Ebel R et al (2012) Pullularins e and f, two new peptides from the endophytic fungus *Bionectria ochroleuca* isolated from the mangrove plant *Sonneratia caseolaris*. Mar Drugs 10(5):1081–1091 (Article)
199. Li XJ, Zhang Q, Zhang AL, Gao JM (2012) Metabolites from *Aspergillus fumigatus*, an endophytic fungus associated with *Melia azedarach*, and their antifungal, antifeedant, and toxic activities. J Agri Food Chem 60(13):3424–3431 (Article)
200. Liu YX, Ma SG, Wang XJ, Zhao N, Qu J, Yu SS et al (2012) Diketopiperazine alkaloids produced by the endophytic fungus *Aspergillus fumigatus* from the stem of *Erythrophloeum fordii* oliv. Helvetica Chimica Acta 95(8):1401–1408 (Article)
201. Kong FD, Wang Y, Liu PP, Dong TH, Zhu WM (2014) Thiodiketopiperazines from the marine-derived fungus *Phoma* sp. oucmdz-1847. J Nat Prod 77(1):132–137 (Article)
202. Xiao J, Zhang Q, Gao YQ, Tang JJ, Zhang AL, Gao JM (2014) Secondary metabolites from the endophytic *Botryosphaeria dothidea* of *Melia azedarach* and their antifungal, antibacterial, antioxidant, and cytotoxic activities. J Agri Food Chem 62(16):3584–3590 (Article)
203. El Amrani M, Debbab A, Aly AH, Wray V, Dobretsov S, Muller WEG et al (2012) Farinomalein derivatives from an unidentified endophytic fungus isolated from the mangrove plant *Avicennia marina*. Tetrahedron Lett 53(49):6721–6724 (Article)
204. Zhu F, Chen GY, Wu JS, Pan JH (2013) Structure revision and cytotoxic activity of marinamide and its methyl ester, novel alkaloids produced by co-cultures of two marine-derived mangrove endophytic fungi. Nat Prod Res 27(21):1960–1964 (Article)
205. Ding G, Chen L, Chen A, Tian XH, Chen XD, Zhang HW et al (2012) Trichalasins c and d from the plant endophytic fungus *Trichoderma gamsii*. Fitoterapia 83(3):541–544 (Article)
206. Ortega HE, Graupner PR, Asai Y, TenDyke K, Qiu DY, Shen YYC et al (2013) Mycoleptodiscins a and b, cytotoxic alkaloids from the endophytic fungus *Mycoleptodiscus* sp. f0194. J Nat Prod 76(4):741–744 (Article)
207. Luo DQ, Chen YP, Zhang J, Shi BZ, Yang ZQ, Chen C (2013) A new glycine derivative and a new indole alkaloid from the fermentation broth of the plant endophytic fungus *Pestalotiopsis podocarpi* isolated from the Chinese podocarpaceae plant *Podocarpus macrophyllus*. Helvetica Chimica Acta 96(2):309–312 (Article)
208. Zang L-Y, Wei W, Wang T, Guo Y, Tan R-X, Ge H-M (2012) Isochromophilones from an endophytic fungus *Diaporthe* sp. Nat Prod Bioprospect 2:117–120
209. Talontsi FM, Tatong MDK, Dittrich B, Douanla-Meli C, Laatsch H (2013) Structures and absolute configuration of three alpha-pyrones from an endophytic fungus *Aspergillus niger*. Tetrahedron 69(34):7147–7151 (Article)
210. Ebrahim W, Aly AH, Wray V, Mandi A, Teiten MH, Gaascht F et al (2013) Embellicines a and b: absolute configuration and nf-kappa b transcriptional inhibitory activity. J Med Chem 56(7):2991–2999 (Article)
211. Zhou XF, Lin XP, Ma WL, Fang W, Chen ZF, Yang B et al (2014) A new aromatic amine from fungus *Pestalotiopsis vaccinii*. Phytochem Lett 7:35–37 (Article)
212. Intaraudom C, Boonyuen N, Suvannakad R, Rachtawee P, Pittayakhajonwut P (2013) Penicolinates a–e from endophytic *Penicillium* sp. bcc16054. Tetrahedron Lett 54(8): 744–748 (Article)

Chapter 9
Fungal Secondary Metabolism in the Light of Animal–Fungus Interactions: From Mechanism to Ecological Function

Marko Rohlfs

Introduction

Research into the regulatory mechanisms of fungal secondary metabolite production has been propelled by the economic damage mycotoxigenic fungi cause to food and feedstuff and by the exciting perspective to discover new pharmaceutically relevant compounds. The biosynthetic machinery used for secondary metabolite formation and its molecular genetic checkpoints is particularly well investigated in some model fungi, such as *Aspergillus, Penicillium,* or *Fusarium* [1, 2]. From these and other fungi, numerous secondary metabolites have been isolated, their structure described, and biological activity evaluated. Manipulation of growth conditions and genomic mining approaches have revealed that filamentous microfungi harbor an extraordinarily diverse and apparently still underexplored repertoire of secondary metabolites fed from various biosynthetic pathways [3–6] (see Chap. 4). Despite the considerable progress in our mechanistic understanding of fungal secondary metabolite regulation, the reasons why natural selection has favored these mechanisms have largely been overlooked (e.g., [7, 8]). In this respect, there is, more than ever, a growing interest in giving fungal chemical diversity and its regulation a meaning in the context of fungal ecology and evolution.

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Recent Attempts to Integrate Fungal Secondary Metabolism into Ecology

In line with many other researchers, I argue that understanding the "natural" function of fungal metabolites is directly related to understanding the interactions of the metabolite producer with its environment. And that it is both the organismic *and* molecular mechanisms in a defined ecological context that matters if we wish to understand the "natural" function of one of the most distinctive properties of fungal organisms.

The following examples illustrate that manipulation of fungal culture conditions has led to interesting insights into not only the environment-dependent regulation of secondary metabolites but also the inherent difficulties in providing conceptually solid interpretations of variation in secondary metabolite production. Many studies demonstrate that visible electromagnetic radiation—light—is an important abiotic factor affecting the activity of the regulatory mechanisms for fungal secondary metabolite biosynthesis [9]. Perception of light is mediated by protein complexes that sense a broad range of wavelengths [10]. A notable feature of fungal light receptors is that they transmit their signals to other protein complexes that play a central role in light-dependent regulation of secondary metabolism [11]. For the model fungus *Aspergillus nidulans* (Fig. 9.1b), Bayram et al. [12] established the mechanistic basis for the negative relationship between light and production of the mycotoxin sterigmatocystin; yet, light in general does not always suppress secondary metabolite formation and the influence of light on mycotoxin production depends strongly on the nutritional resources available to the fungus (e.g., [13, 14]). Distinct metabolic profiles of *A. nidulans* cultivated on different media, for example, illustrate the tremendous variation in secondary metabolite formation as a function of the nutritional environment (e.g., [3]). Moreover, it has been observed that environment-dependent changes in secondary metabolite production is accompanied by changes in fungal growth characteristics and morphology (e.g., [3, 15]). In particular, formation of toxic metabolites is co-regulated with sexual development in facultative sexual *A. nidulans,* which requires the so-called velvet complex [12]. Beyond the joint influence of light and nutrients, further mutually not independent effects of other abiotic parameters, such as substrate pH, temperature, and water activity contribute to the chemical phenotype of fungi (e.g., [16, 17]).

In addition, biotic factors—that is, interactions with other organisms—determine a fungus' secondary metabolite profile. For example, in the field, fungi are assumed to interact with a multitude of other microorganisms [18, 19]). Recent studies have used confrontation assays with bacteria to determine influence of prokaryotes on fungal secondary metabolite formation. Intimate physical contact of bacteria with fungal hyphae, for example, appears to be an important stimulus that triggers the formation of previously unknown fungal secondary metabolites [20]. The effect of bacteria is mediated by fungal histone acetylation [21], suggesting that the induction of secondary metabolites involves epigenetic processes that require chromatin remodeling (see Chap. 3).

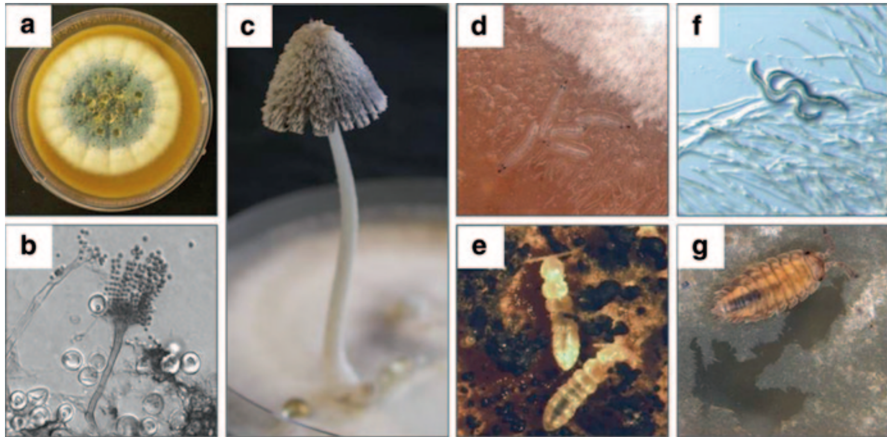

Fig. 9.1 Model organisms for investigating fungal secondary metabolite biosynthesis and regulation in relation to interactions with fungivorous animals. **a** *Penicillium expansum,* a notorious colonizer of decaying fruits and thus vital interaction partner of saprophagous insects, such as fruit fly larvae (**d**). **b** The genetic model fungus, *Aspergillus nidulans,* has been used in a number of insect-fungivore studies. **c** *Coprinopsis cinerea,* one of the few genetic model "mushrooms" that can be cultured in the lab and thus be used in carefully controlled fungus–fungivore experiments (image courtesy of U. Kües, University of Göttingen). **d** Fruit fly *Drosophila melanogaster* larvae exploit decaying plant material and feed facultatively on moulds but suffer extremely from fungal toxic secretion. **e** *Folsomia candida,* a springtail, representative of the large number of small (millimeters) fungivorous soil arthropods that have regularly been used in soil ecology studies. **f** Small (micrometers) fungivorous soil nematode worms. Here *Aphelenchoides saprophilus* pierce the hyphal membrane with a specialized stylet and suck the fungal cell content. **g** Isopods, *Oniscus assellus,* like springtails (**e**) chew on fungal tissue and due to their size (often > 1 cm) cause serious damage to mould colonies and probably larger basidiomycetes

These examples illustrate that fungi have evolved regulatory mechanisms that mediate the formation of secondary metabolites in response to changes in both abiotic and biotic environmental conditions. To date, however, it has remained difficult to relate this "ecology-driven" plasticity in secondary metabolite formation to its adaptive relevance. For example, what is the direct benefit of producing secondary metabolites in the dark or in interaction with bacteria to its producer? Rodriguez-Romero et al. [9] suggest that saprophagous soil fungi may experience more stressful conditions when growing on the soil surface (exposure to light) compared to growing within the soil matrix (darkness). From this argument follows that fungi, such as *A. nidulans,* would invest more in secondary metabolites in deeper soil layers than at the surface, just as investment in sexual reproduction would be higher in deeper soil layers than at the surface (as determined by the light-dependent activity of the velvet-complex). The consequences of aboveground versus belowground stress for fungal fitness have not been discussed further, but the argument is in contrast to results of recent evolution experiments. While Rodriguez-Romero et al. [9] suggest higher investment in a sexually reproducing phenotype under more benign

conditions in the soil, Schoustra et al. [22] demonstrate that real-time evolution of *A. nidulans* in a defined experimental setup favors a positive relationship between environmental stress (and hence fungal fitness decline) and the investment in sexual development. However, the latter study was not designed to provide information on whether fitness-associated sexual reproduction leads to intensified production of mycotoxins or other secondary metabolites.

Co-regulation of fungal secondary metabolite production and sexual reproduction has been suggested to be a defense strategy to resist fungivorous animals (Fig. 9.1d–g). For example, because the formation of a specific type of cells necessary for producing sexual fruiting bodies in *A. nidulans* (Hülle cells) and the synthesis of mycotoxins are controlled by the same protein complex, Sarikaya Bayram et al. [23] propose a specific role of secondary metabolites in providing protection to sexual fruiting bodies from fungivorous soil arthropods. While this is an appealing idea, it remains unclear why coordinated induction of sexual fruiting bodies and secondary metabolites is supposed to provide protection from fungivores in darkness (when fungi grow belowground) but not when fungi are exposed to light (when fungi grow aboveground). In fact, one would expect the reverse because the density of fungivorous microarthropods is highest in the (light-exposed) litter layer and decreases with increasing soil depth [24].

Despite these apparent conflicts between the attempts to interpret fungal secondary metabolite regulation in the light of animal grazing and the "real ecology" of fungus–fungivore interactions outside Petri dishes, it has widely been accepted that interactions with fungivorous animals have shaped fungal evolution, including fungal secondary metabolism [25–33]. In this review, I describe the results of the different approaches that have been used to test the idea of a chemical compounds-based defense of fungi against fungivores. I summarize and discuss novel evidence supporting the idea of inducible resistance against fungivores and its link to the mechanisms underlying secondary metabolite regulation.

Chemical Compound-Based Defense of Fungi Against Predators

What Do Pharmaceutical Studies Tell Us?

Numerous studies have used protocols for secondary metabolite isolation in combination with animal exposure assays to determine the toxicity of fungal chemicals (e.g., [34–42]). By using several insect systems, many metabolites turned out to have insecticidal properties; i.e., killed juveniles and adults or inhibited juvenile development (Fig. 9.2). The molecular mechanisms underlying toxicity to insects are not well-known but may be due, in part, to binding with DNA, inhibition of protein biosynthesis, neurotoxic, or anti-juvenile hormone effects [27, 43, 44]. Insects that feed on fungi or on plants infested with fungi, such as plant-parasitic *Fusarium*

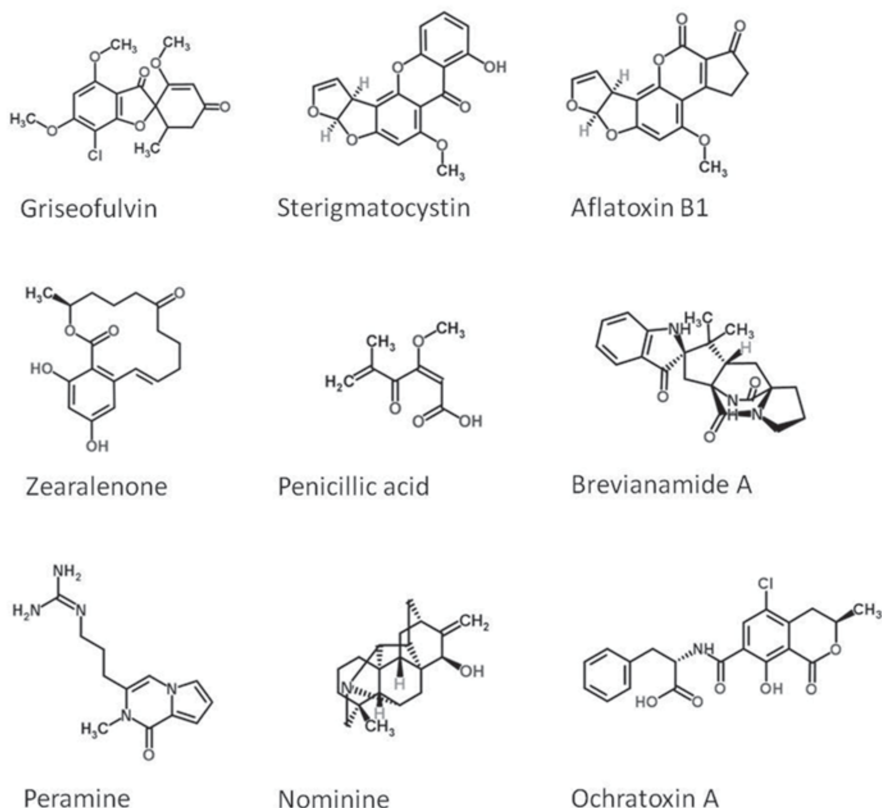

Fig. 9.2 Some fungal secondary metabolites with anti-fungivore properties

sp., do not encounter fungal toxins as isolated compounds but ingest a mixture of fungal secondary metabolites (plus plant metabolites), which may lead to synergistic effects associated with the simultaneous presences of specific compounds. Dowd [45] and Dowd et al. [35] provide evidence of the existence of synergistic effects of *Aspergillus* and *Fusarium* mycotoxins, respectively, that acted on herbivorous lepidopteran larvae, the fall armyworm, *Spodoptera frugiperda* and the corn earworm, *Helicoverpa zea*. Also, non-simultaneous but successive exposure of fruit fly *Drosophila melanogaster* larvae (Fig. 9.1d) to sterigmatocystin and aflatoxin B1 (Fig. 9.2) enhanced toxicity of the latter [46]. Dowd [45] discusses the possibility that synergistic effects of different chemicals in anti-fungivore defense may be favored by natural selection if the production of (lower) amounts of several compounds offsets the costs of producing high concentrations of one insecticidal metabolite. Because of the tremendous diversity of secondary metabolites that a single fungal colony is able to produce, synergistic effects in the chemical defense of fungi against insect grazers should receive more attention.

Most of the effects of secondary metabolites tested against fungivores have been linked to animal fitness consequences following the ingestion of the putative toxins. A few studies demonstrate the involvement of volatile organic compounds in repelling insects from fungi prior to the ingestion of fungal tissue. They found that 1-octen-3-ol, a common fungal volatile produced by the activity of lipoxygenase enzymes [47, 48], repels fungivorous springtails (small insect-like arthropods, Fig. 9.1e) [49] and has direct neurotoxic effects on *Drosophila melanogaster* fruit flies [50]. Nakamori and Suzuki [51] discuss the possibility that fungivore grazing causes wound-activated release of volatile organic compounds, which may play an important role in subsequent fungivore foraging decisions. Constitutive release of volatiles have repeatedly been demonstrated to influence fungivore feeding decisions, either as repellents or attractants [52–55]. Many fungal volatile organic compounds can be perceived by the insect's olfactory system (http://neuro.uni-konstanz.de/DoOR/default.html) and may lead to distinct avoidance reactions [56]. Volatile communication between fungi and fungivores deserves closer attention, in particular because the release of volatiles may be co-regulated with the formation of anti-fungivore toxins [57], so volatiles might have the potential to convey information on the capacity of a fungus to harm fungivores.

The impact of fungal secondary metabolites on insect fungivores is animal taxon-specific. For example, stored product beetles that prefer feeding on mould-infested over mould-free wheat flour are not or only little affected by various *Penicillium* toxins [58]; however, several *Penicillium* metabolites are highly toxic to *Drosophila* larvae [40]. The hairy fungus beetle, *Typhaea stercorea,* is able to complete its entire life cycle on *Aspergillus flavus,* a potent producer of aflatoxins, possibly due to its ability to tolerate high concentrations of aflatoxin B1 (Fig. 9.2, [59]). Animal taxon-specific sensitivity to mycotoxins may be due to variation in the employment of physiological resistance mechanisms. Recently, detoxification of aflatoxin B1 has been related to the activity of a cytochrome P450 monooxygenase in the corn earworm [60]. Feeding on maize, for example, frequently exposes these insect larvae to aflatoxin B1 if plants are infested with *A. flavus*. Interestingly, the plant allelochemicals induce the detoxification mechanism and hence improve insect fitness in the presence of aflatoxin B1 rather than the mycotoxin itself [61]. Low toxin sensitivity may also hold true for the ability of some drosophilid flies to exploit mushrooms of the genus *Amanita*. While larvae of mushroom-breeding species were able to develop in an artificial diet treated with different amounts of α-amanitin, species not breeding on mushrooms were highly susceptible to this compound [62]. Results of recent experiments indicate that the activity of cytochrome P450 monooxygenases may be involved in determining sensitivity of various *Drosophila* species to α-amanitin [63]. An evolution experiment that aimed at following the adaptation process of *D. melanogaster* during larval development to sterigmatocystin-producing *A. nidulans* revealed that insect populations harbor the genetic capacity to evolve reduced sensitivity to this mycotoxin [64]. The physiological mechanisms of this evolutionary adaptation, however, remain to be determined.

What Does Natural Variation in Secondary Metabolite Production Tell Us?

It is difficult to infer a defense function of secondary metabolites based on pharmaceutical tests alone, because description of a repellent or harmful effect of isolated compounds does not necessarily point to a causal link to resistance against a fungivore. That is, the benefit of producing a specific metabolite or blend of different metabolites cannot be quantified. Quantification of reciprocal fitness consequences, however, is of utmost importance for drawing conceptually solid conclusions regarding the protective function of fungal secondary metabolites.

Making use of the natural variation in secondary metabolite composition of fungi is one way to test whether a species/isolate that produces specific compounds achieves better protection against fungivory than those that do not produce these metabolites. Wicklow and Dowd [65] suggested that reduced performance of fall armyworm and corn earworm on maize kernels fermented with *A. flavus* compared to those fermented with *A. oryzae* may be due to the fact that the latter had lost the ability to produce aflatoxins during domestication (note that *A. oryzae* is thought to be a non-aflatoxigenic variant or ecotype of *A. flavus* [66]). Fungivore feeding experiments with more distantly related fungi revealed selective grazing behavior, which has been interpreted as avoidance of toxin-producing fungi [67, 68]. Less toxic fungi, in consequence, appear to suffer more grazing pressure by fungivores.

Secondary metabolites are not homogeneously distributed within fungal colonies or hyphal networks, but fungi allocate the production of specific compounds to certain structures. Several studies thoroughly investigated the occurrence of secondary metabolites in specific fungal tissues, sclerotia (reproductive bodies, which allow fungi to survive extended period of dormancy) and ascostomata (associated with the formation of sexual fruiting bodies). A major finding has been that several *Aspergillus* and *Eupenicillium* species produce high amounts of secondary metabolites exclusively in sclerotia and ascostomata, respectively [42, 69–74]. Many of these compounds have insecticidal properties and may thus contribute to a localized chemical defense. Because insects have been observed to avoid feeding on sclerotia [75], accumulation of secondary metabolites in these structures may support the hypothesis that organisms allocate defenses in direct proportion to the value of a specific tissue [76]. This idea is further corroborated by Cary et al. [77], who describe the gene cluster encoding a sclerotium-specific polyketide pigment in *A. flavus*. The targeted disruption of the polyketide synthase gene *pks27* within this cluster resulted in pigment-deficient sclerotia. The fungivorous nitidulid beetle *Carpophilus freemani* showed a significant feeding preference for pigment-deficient sclerotia over sclerotia from wild type fungi.

What Does Genetic Manipulation of Fungal Secondary Metabolism Tell Us?

The use of genetically manipulated fungi is an alternative approach to explore the role of secondary metabolites in mediating resistance to fungivores. This approach offers the opportunity to infer the fitness consequence for both the fungus and the fungivore when the expression of genes involved in secondary metabolite formation is impaired or enhanced but the genetic background remains unaltered. To date, however, only few studies have used mutant fungi in fungus–fungivore experiments (Table 9.1, [77–88]). A series of studies have demonstrated that knocking out the gene *laeA* in *Aspergillus* sp. leads to a distinct preference of fungal feeding springtails for the Δ*laeA* mutant strain relative to the corresponding wild type fungus. From the fungivore perspective, choosing the Δ*laeA* strain yields a distinct benefit in terms of increased survival and reproduction [79–82]. *LaeA,* a putative methyltransferase, involved in chromatin remodeling [89], is thought to play a central role in global regulation of secondary metabolite formation in several fungal taxa [90–97]. The effect of *laeA* deletion on fungus–fungivore interactions is, however, fungal species-specific. In *A. nidulans,* lack of *laeA* expression results in a substantial loss of resistance to fungivory. In particular, larvae of the fruit fly, *D. melanogaster* (Fig. 9.1d), which suffer 100 % mortality when exposed to wild type *A. nidulans* as the only fungal diet, were able to develop into adult flies when they were offered a Δ*laeA* strain [78]. In contrast, Δ*laeA A. fumigatus* and *A. flavus* still cause high mortality among *Drosophila* larvae and the fungi suffer only little from larval grazing [83]. *LaeA* couples with two other proteins, veA and velB, and thereby forms the nuclear velvet complex as previously explained. A VeA loss-of-function *A. nidulans* mutant has also been found to restore normal larval development of *D. melanogaster* [84]. This suggests that a functioning velvet complex is required for *A. nidulans* to be resistant to fungivores.

Cytotoxic and carcinogenic sterigmatocystin has been proposed to be the major secondary metabolite that protects *A. nidulans* from severe fungivore grazing. In agreement with this idea, a fungal mutant deficient in the production of oxylipins that regulate, through hormone-like signaling, sterigmatocystin production was found to be less detrimental to insect development than wild type *A. nidulans* [84]. Yin et al. [85] describe a positive relationship between activation of AflR (transcription factor for the sterigmatocystin biosynthetic pathway), through the transcriptional co-activator RsmA, sterigmatocystin formation and resistance of *A. nidulans* to springtail grazing. Together, these results indicate that sterigmatocystin, as controlled by global and pathway-specific regulators, LaeA and AflR, respectively, determines the capacity of *A. nidulans* to resist fungivory.

This conclusion, however, is in conflict with the finding that suppression of sterigmatocystin, by blocking the expression of *aflR* or biosynthetic genes of the sterigmatocystin pathway *(stcJ, stcE, and stcU),* does not improve fungivore fitness. In contrast, confrontation assays with such gene deletion strains even enhanced mortality of *D. melanogaster* larvae [84] and fungivorous springtails [98] relative to the influence of the wild type strain. This casts doubt on sterigmatocystin being the ultimate defensive metabolite for *A. nidulans* resistance to fungivory. These findings rather indicate that

Table 9.1 Fungal genes tested for their involvement in mediating resistance to fungivores

Gene name	Encoded molecular function	Fungal species	Manipulation	Fungivore fitness/ behavior	Fungal fitness	References
laeA	Methyltransferase activity	*A. nidulans* *A. fumigatus* *A. flavus*	ko ko ko	↑ ↑ ↑	↓ ↓ ↓	Caballero Ortiz et al.[78]; Janssens et al. [79]; Rohlfs et al. [80]; Staaden et al. [81]; Stötefeld et al. [82]; Trienens et al. [83]
veA	Velvet family protein (interacts with LaeA)	*A. nidulans*	ko	↑	n.q.	Trienens and Rohlfs [84]
aflR	Sequence-specific DNA binding (transcription factor for sterigmatocystin biosynthesis)	*A. nidulans*	ko	-,↓	n.q.	Trienens and Rohlfs [84]
hdaA	Histone deacetylase activity	*A. nidulans*	ko	-	n.q.	Trienens and Rohlfs [84]
rsmA	Sequence-specific DNA binding	*A. nidulans*	oe	↓	↑	Yin et al. [85]
stcJ *stcE* *stcU*	Biosynthetic enzymes of the sterigmatocystin pathway	*A. nidulans*	ko	-,↓	n.q.	Trienens and Rohlfs [84]
ppoA	Fatty acid dioxygenase	*A. nidulans*	ko	-	n.q.	Trienens and Rohlfs [84]
ppoA	Fatty acid dioxygenase	*A. nidulans*	oe	↓	↑	Trienens and Rohlfs [84]
ppoB	Fatty acid oxygenase	*A. nidulans*	ko	-	n.q.	Trienens and Rohlfs [84]
*ppoAC**	*ppoC* encodes a fatty acid oxygenase	*A. nidulans*	*Double ko	-	n.q.	Trienens and Rohlfs [84]
*ppoABC***	See above	*A. nidulans*	**Triple ko	↑	n.q.	Trienens and Rohlfs [84]
pksP	Polyketide synthase for melanin biosynthesis	*A. fumigatus*	Ko	↑	-	Scheu and Folger [86]; Scheu and Simmerling [87]
pks27	Polyketide synthase for asparasone biosynthesis in sclerotia	*A. flavus*	ko	n.q.	↓	Cary et al. [77]
AOL	*Arthrobotrys oligospora* lectin	*A. oligospora*	ko	-	n.q.	Balogh et al. [88]

ko candidate gene(s) was/were knocked out, *oe* candidate gene was overexpressed, -, ↑, ↓ neutral, positive, or negative influence on organismic traits, *n.q.* organismic traits were not quantified

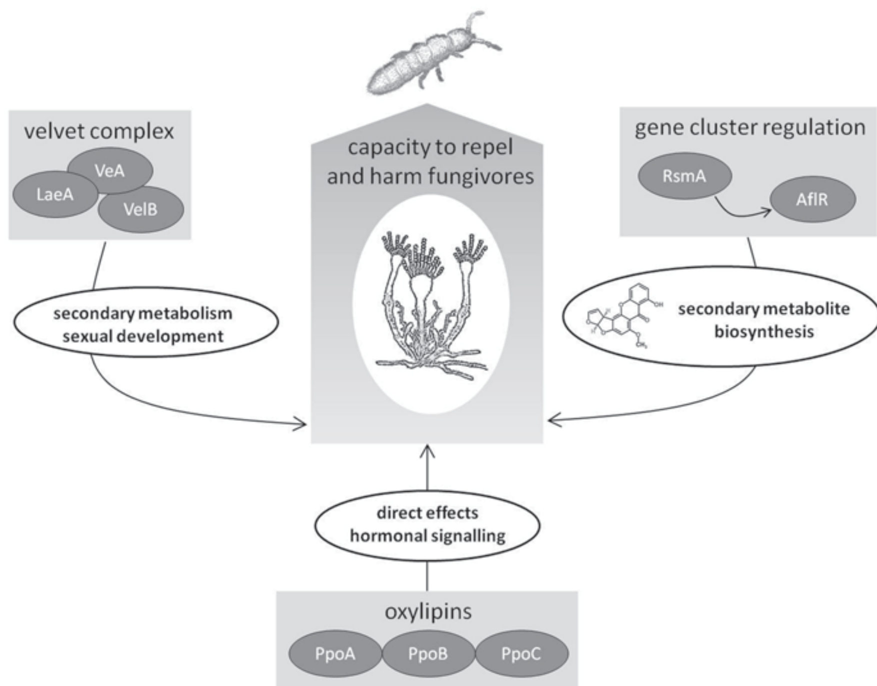

Fig. 9.3 Manipulation of fungus–fungivore interactions by means of genetically modified fungi (Aspergilli) revealed three fundamental molecular processes involved in determining a fungus' capacity to resist fungivores. *Left:* Global regulation of fungal secondary metabolism and sexual development by the *velvet* complex; *middle:* biosynthesis of oxylipins; *right:* secondary metabolite gene cluster regulation. A recent fungal gene expression analysis corroborates the involvement of these processes in building up resistance against fungivores (see Fig. 9.5, [78])

other metabolites or metabolite combinations are more efficient against fungivores, provided that sterigmatocystin biosynthesis is blocked. This would argue against synergistic effects involving sterigmatocystin as explained previously. One possible explanation for this counterintuitive phenomenon is that elimination of one biosynthetic pathway allows allocation of more energy and/or resources (e.g., coenzyme A activated acyl-precursor molecules for polyketide synthesis) to other pathways. The products of such pathways, alone or in combination, may be more efficient against fungivores but are, due to internal resource allocation conflicts, underrepresented in the wild type strain. If this turns out to be an adequate explanation for enhanced fungivore mortality when exposed to sterigmatocystin-deficient *A. nidulans,* chemical diversity might prove to impede optimal resistance to fungivores.

These examples illustrate two important aspects of using mutant fungi in the study of fungus–fungivore interactions. First, this approach helps in revealing the functional relevance of principle components of fungal secondary metabolism regulation in determining a fungus' capacity to resist fungivore grazing (Fig. 9.3, [78]). Second, this approach appears to have its limitations in pinpointing a specific de-

fensive pathway, possibly due to mechanistic constraints, such as resource allocation trade-offs. Despite the apparent complexity of *A. nidulans* chemical defense against fungivores, other studies have been able to identify the involvement of a single compound in deterring insect feeding. The first one is the aforementioned study by Cary et al. [77] on beetle-deterring pigments formed in *A. flavus* sclerotia. The second one investigated a tripartite plant–fungus–herbivore system [99]. By knocking out the biosynthesis of peramine (Fig. 9.2), the authors demonstrate that resistance of the plant–fungus *Neotyphodium lolii–Epichloë festucae* symbiosis to herbivorous insects is due to this specific compound produced by the endophytic fungus. In the context of fungal-plant symbioses, it is worth mentioning that arbuscular mycorrhizal fungi may also benefit from the transfer of plant defensive compounds to fungal tissue. Duhamel et al. [100] found the plant defensive compound catalpol to be transferred from *Plantago lanceolata* host plants to *Glomus* sp. fungal symbionts. Notably, the transfer is triggered by grazing of fungivorous springtails.

Induced Fungal Responses Toward Fungivore Grazers?

Both pharmaceutical and transgenic approaches have their specific pros and cons in convincingly demonstrating a causal relationship between secondary metabolite formation and resistance to fungal grazers. In combination, however, these approaches corroborate the general idea of a chemical compound-based defense against fungivory. Yet, this concept will be further substantiated by considering fungal phenotypic changes in response to fungivore attack. For example, fungivores have been reported to induce changes in fungal foraging strategies as characterized by alterations in mycelial networks and enzyme production [101–104]. Such responses may enable fungi to compensate for the fitness loss caused by fungivore grazing [105]. Compensatory foraging may thus be part of a defense strategy that leads to tolerance of, rather than resistance to, fungivores. While the former does not cause significant harm to fungivores the latter affects fungivores negatively.

The influence of animal grazing on the degree of anti-fungivore resistance is an underexplored aspect of fungal biology. Chemical compound-based resistance against fungivores can be “induced” and/or “activated” by animal feeding (Fig. 9.4). The latter comprises rapid formation of toxic compounds upon unspecific wounding of fungal tissue, which relies on the constitutive investment in a corresponding amount of precursor molecules. Tissue injury caused by animal grazers elicits enzyme-mediated oxidation processes and hence an immediate conversion of precursor compounds into defensive metabolites [33]. In contrast, inducible resistance does not involve preliminary investment in the biosynthesis of inactive precursors. Rather, the presence of the chemical shield depends on the presence of fungivores and is built *de novo* or secondary metabolites are synthesized to a significantly larger amount. Central to an inducible response is the activation of regulatory and biosynthetic genes involved in determining a fungus’ capacity to resist fungivory (see Table 9.1, [77–88]). Such changes in gene expression are usually mediated by

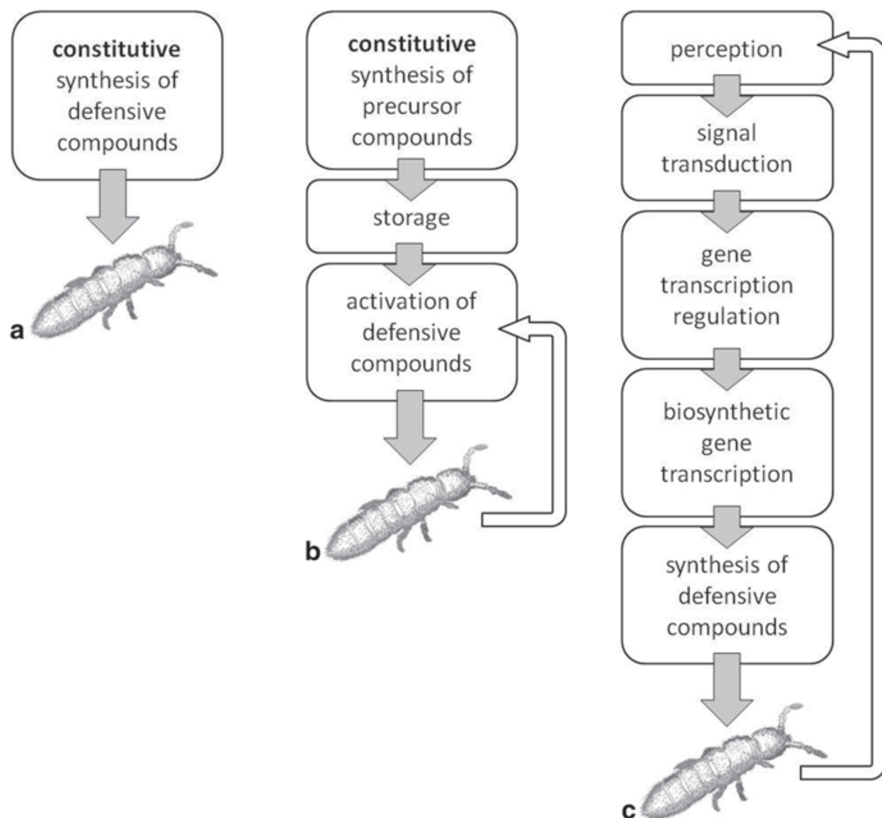

Fig. 9.4 Three different modes of the chemical compound-based resistance to fungivores. **a** Constitutive resistance: The presences of and hence the investment in defensive compounds is independent of fungivore damage. **b** Wound-activated resistance: Defensive compounds are (almost immediately) activated through fungivore damage and unspecific injuries. However, the investment in precursor compounds and storage mechanisms is independent of fungivore damage. **c** Inducible resistance: Defensive compounds are induced by fungivore damage. The formation of these compounds requires a coordinate course of up-stream molecular processes

the combined action of cell surface receptors and intra-cellular signaling cascades. Therefore, in inducible responses, investment in chemical defense starts upon perception of fungivory-specific signals (Fig. 9.4).

Only very few studies are available that used animal grazers to test for inducible changes in resistance to fungivores. Bleuler-Martínez et al. [106] propose a role of fungal lectins in inducible anti-fungivore defence of the mushroom *Coprinopsis cinerea*. Lectins, carbohydrate-binding proteins, appear to have diverse biological functions in organisms, including protection of plants from herbivores [107]. Fungal lectins have been suggested to be essential for fruiting body development but their explicit role in this process remains controversial [108]. Interestingly, when exposed to fungivorous nematodes, *Aphelenchus avenae,* vegetative mycelium of

C. cinerea (Fig. 9.1c) showed enhanced expression of fruiting body lectin-encoding genes, *cgl1* and *cgl2,* and the corresponding proteins [106]. This response was not triggered by unspecific wounding. Heterologous expression of various *C. cinerea* lectins in *Escherichia coli* and subsequent feeding of the modified bacteria to various organisms revealed toxicity of recombinant lectins against bacterial-feeding nematode worms *(Caenorhabditis elegans),* amoebozoa *(Acanthamoeba castellanii),* and mosquito larvae *(Aedes aegypti)*. These results are highly interesting, because they suggest that the fungal response possibly is an adaptive reaction to withstand fungivore attack. Unfortunately, this study missed in providing a link between enhanced production of lectins and its effect on "real" fungivores. Toxicity toward non-fungivorous animals and amoebozoa does not imply automatically a negative influence of fungal lectins on *A. avenae* or other invertebrate grazers. Despite toxic effects of *C. cinerea* lectins on non-fungivorous organisms, the fungus is a suitable host supporting growth and reproduction of fungivorous *A. avenae* [106]. It would thus be interesting to test whether *A. avenae* achieves higher fitness on lectin-deficient *C. cinerea;* i.e., when worms are not forced to invest in resistance to inducible lectin formation. Moreover, comparison of lectin-producer *versus* non-lectin producer would allow quantification of lectin-dependent feeding probability by fungivores. Balogh et al. [88] knocked out candidate lectin genes in the nematode-trapping fungus *Arthrobotrys oligospora*. Interestingly, both the efficiency of trapping and killing nematodes and the reproduction and survival of the fungivorous springtail *Folsomia candida* were not affected by the lack of lectins. It therefore remains to be tested if nematode-triggered expression of *C. cinerea* lectins is indeed an inducible response that enhances resistance to fungivores.

To test explicitly if insect grazing induces resistance to fungivory, Caballero Ortiz et al. [78] exposed *A. nidulans* to three different pretreatments: grazing by fruit fly larvae, artificial wounding, or no challenge. Subsequently, they followed the capacity of individual colonies to kill fruit fly *D. melanogaster* larvae. Approximately seven days after feeding on unchallenged or artificially wounded colonies 50 % of the larvae were found dead. In contrast, larvae exposed to colonies that were previously attacked by conspecific larvae died significantly earlier (50 % mortality was reached after less than four days). The expression of several genes that have previously been shown to be involved in determining the capacity of *A. nidulans* to resist fungivory (Table 9.1, [77–88]) was significantly enhanced in insect-challenged colonies (Fig. 9.5, [78]). Two aspects of the changes observed in the fungal transcriptome are worth highlighting: First, even though genes encoding transcriptional regulators for the sterigmatocystin pathway, *aflR* and *rsmA,* were significantly higher expressed, the polyketide synthase encoding gene *stcA* was not affected at that stage; however, biosynthetic genes for other pathways were significantly upregulated in insect-challenged colonies (Fig. 9.5). This finding supports the aforementioned assumption that sterigmatocystin is of subordinate relevance in the direct anti-fungivore defense response of *A. nidulans* and that other pathways are more important in shaping the inducible capacity to resist fungivores. Second, expression of genes, *pkaA, mpkA, mpkB,* and *hogA,* representative of signal transduction pathways [109], was significantly increased, which probably indicates a

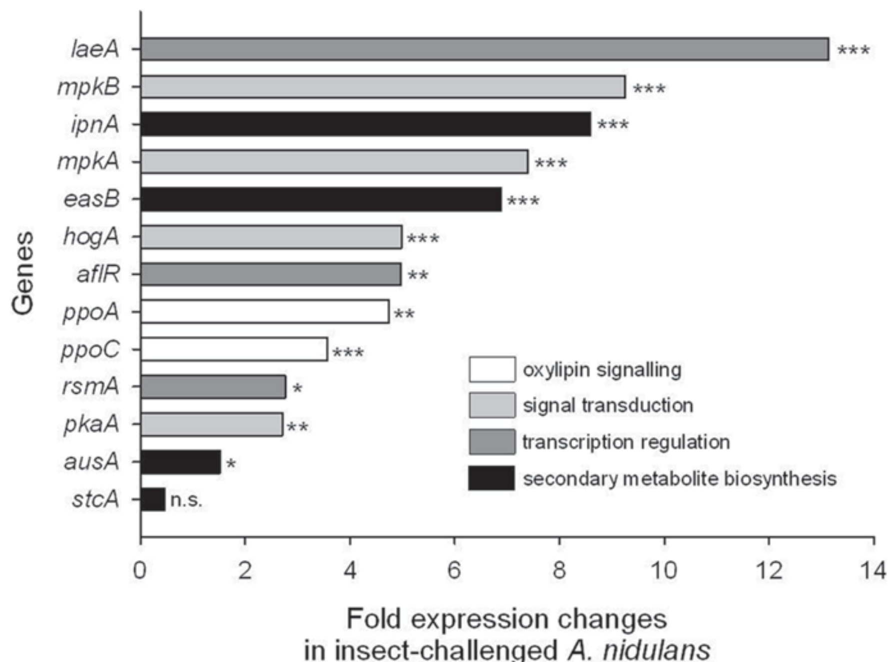

Fig. 9.5 *Aspergillus nidulans* gene expression changes in response to insect fungivory. The transcriptomic response indicates a fundamental phenotypic shift that involves various molecular pathways. $*P<0.05$, $**P<0.01$, $***P<0.001$ refer to differences in the amount of candidate messenger ribonucleic acid (mRNA) between insect-challenged and unchallenged fungi. (Data from Caballero Ortiz et al. 2013 [78], modified)

reshuffle of the intracellular signaling network. Similar to plants [110], accumulation of signal amplifiers may be a crucial step toward induced resistance against fungivores. Thus, a rather complex fungal phenotypic shift appears to underlie the observed changes in resistance to fungivores. A crucial aspect that remains to be investigated thoroughly is how the phenotypic shift on the gene expression level is related to changes in secondary metabolite formation and how the new chemical profile affects both fungivore and fungal fitness.

A chemical defense against fungivores may be particularly effective if it comprises the formation of metabolites that mimic the activity of compounds; e.g., insect hormones, relevant for fungivore development. Derangement of fungivore endocrine processes through the release of insect hormone analogues may have a direct impact on a fungivore's morphogenetic development and/or it might potentiate toxicity of other metabolites. Nielsen et al. demonstrate the potential of *A. nidulans* to produce sesquiterpenes, juvenile hormone III (JH III), JH-diol, and methyl-farnesoate (Fig. 9.6, [111]). These compounds have central hormonal function in the regulation of arthropod developmental and physiological processes [112]. Sesquiterpene production was achieved by expressing a putative binuclear

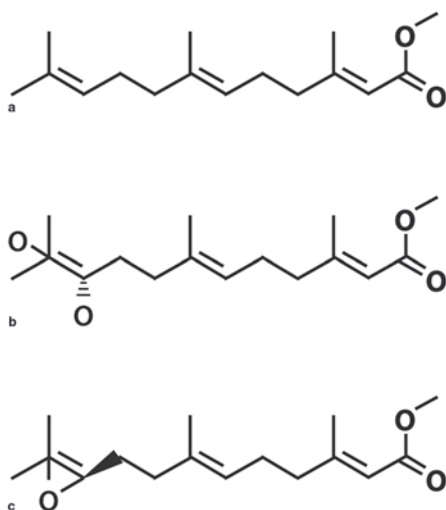

Fig. 9.6 Cryptic sesquiterpens of *A. nidulans* are induced by insect grazing: methyl farnesoate (**a**), juvenile hormone-diol (**b**), juvenile hormone III (**c**) [111]. These compounds have hormonal function in various arthropods

zinc finger transcription factor, st_fge1_pg_C_150220 (renamed as *smrA*), from *A. niger* in *A. nidulans*. Expression of *smrA* in *A. nidulans* significantly enhanced formation of JH production. In confrontation assays with *D. melanogaster* larvae, the JH overproduction strain had, however, relative to the reference strain, no dramatic influence on insect development. Interestingly, sesquiterpene formation was enhanced when *A. nidulans* was exposed to *D. melanogaster* larval grazing [111]. This insect-induced response possibly compensated for the constitutively enhanced formation of JHs in the *smrA*-expressing strain and might thus explain why constitutive overproduction of JHs had an only minor influence on the fungivores. To explore experimentally the exact contribution of inducible JH production in the chemical compound-based defense of *A. nidulans,* the molecular genetic and biosynthetic mechanisms underlying this response remain to be described.

The molecular genetic evidence connecting secondary metabolite formation with sexual reproduction has stimulated discussion on the putative role of mycotoxins in protecting sexual fruiting bodies as explained previously. Caballero Ortiz et al. [78] demonstrate insect-driven plasticity in the expression of genes involved in this versatile process of fungal biology (Fig. 9.5). Döll et al. [113] show changes in both morphology and chemistry of *A. nidulans* in response to grazing by the springtail *F. candida* (Fig. 9.1e). Springtail-damaged colonies that were found to repel later-arriving fungivores contained significantly higher amounts of sterigmatocystin and produced a significantly greater number of sexual fruiting bodies. Springtails appeared to be able to adapt in the long term to more toxic colonies and consumed vegetative hyphae and conidia, however, they avoided feeding on *cleistothecia* including the surrounding layer of Hülle cells [113]. This combined and flexible investment in secondary metabolites and sexual reproduction probably is a strategy for fungal survival and persistence in predator-rich niches. In addition to sterigmatocystin, further compounds—i.e., emericellamides and some me-

roterpenoids—were produced in excess when *A. nidulans* was exposed to springtail grazing. Interestingly, the formation of these or similar compounds is indicated by the enhanced expression of polyketide synthase genes, *easB* and *ausA* [114, 115], in confrontation assays with fruit fly larvae ([78], Fig. 9.5). It remains to be tested whether these compounds contribute to the direct chemical defense of *A. nidulans*. In summary, these four examples demonstrate not only the obvious importance of invertebrate fungivores in influencing fungal secondary metabolite production but also the complexities and uncertainties involved in elucidating the immediate relationship between inducible metabolite diversity, fungivore behavior, and fungal fitness.

Conclusion

The role of animal grazing in fungal secondary metabolite biosynthesis is a growing area of interest. Combination of mechanistic and eco-evolutionary analyses of fungal secondary metabolism in the light of fungus–fungivore interactions is a fundamental yet ambitious aim in current mycology. There is increasing evidence that fungi have the potential to regulate their chemical compound-based resistance in response to fungivore damage. However, there are numerous still undefined factors in fungus–fungivore interactions, ranging from the release and perception of reliable chemical signals to natural genetic variation in fungal chemical diversity [31]. The identity and relevance of these factors need to be tackled on the molecular and organismic level to fully understand how fungal secondary metabolism and its regulation have evolved in response to fungivory relative to other environmental influences. To achieve this goal, we need to select and establish suitable fungus–fungivore model systems (Fig. 9.1); i.e., simplified representations of more complex systems. Despite the intended simplification, model systems should be appropriate for providing conceptually solid conclusions. The extraordinary intra- and interspecific diversity of secondary metabolites produced by microscopic easy-to-culture mould fungi offers great opportunities to extend the investigation of fungus–fungivore interactions beyond these model systems. Fungus–fungivore confrontation assays coupled with fungal molecular genetics and biochemical analytics (see Chap. 6) will probably become routine in the future to improve our understanding of the mechanistic intricacies of fungal secondary metabolite regulation in interactions with animals.

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References

1. Brakhage AA, Schroeckh V (2011) Fungal secondary metabolites—strategies to activate silent gene clusters. Fungal Genet Biol 48:15–22
2. Magan N, Aldred D (2007) Post-harvest control strategies: minimizing mycotoxins in the food chain. Int J Food Microbiol 119:131–139
3. Nielsen ML, Nielsen JB, Rank C, Klejnstrup ML, Holm DK, Brogaard KH et al (2011) A genome-wide polyketide synthase deletion library uncovers novel genetic links to polyketides and meroterpenoids in *Aspergillus nidulans*. FEMS Microbiol Lett 321:157–166
4. Sanchez JF, Somoza AD, Keller NP, Wang CCC (2012) Advances in *Aspergillus* secondary metabolite research in the post-genomic era. Nat Prod Rep 29:351–371
5. Sarkar A, Funk AN, Scherlach K, Horn F, Schroeckh V, Chankhamjon P et al (2012) Differential expression of silent polyketide biosynthesis gene clusters in chemostat cultures of *Aspergillus nidulans*. J Biotechnol 160:64–71
6. Winter JM, Behnken S, Hertweck C (2011) Genomics-inspired discovery of natural products. Curr Opin Chem Biol 15:22–31
7. Deacon J (2006) Fungal biology. 4th ed. Blackwell, Oxford
8. Fox EM, Howlett BJ (2008) Secondary metabolism: regulation and role in fungal biology. Curr Opin Microbiol 11:481–487
9. Rodriguez-Romero J, Hedtke M, Kastner C, Müller S, Fischer R (2010) Fungi, hidden in soil or up in the air: light makes a difference. Annu Rev Microbiol 64:585–610
10. Bayram Ö, Braus GH, Fischer R, Rodriguez-Romero J (2010) Spotlight on *Aspergillus nidulans* photosensory systems. Fungal Genet Biol 47:900–908
11. . Bayram Ö, Braus GH (2012) Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins FEMS Microbiol Rev 36:1–24
12. Bayram Ö, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O et al (2008) VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320:1504–1506
13. Atoui A, Kastner C, Larey CM, Thokala R, Etxebeste O, Espeso EA et al (2010) Cross-talk between light and glucose regulation controls toxin production and morphogenesis in *Aspergillus nidulans*. Fungal Genet Biol. 47:962–972
14. Schmidt-Heydt M, Rüfer C, Raupp F, Bruchmann A, Perrone G, Geisen R (2011) Influence of light on food relevant fungi with emphasis on ochratoxin producing species. Int J Food Microbiol 145:229–237
15. Chanda A, Roze LV, Kang S, Artymovich KA, Hicks GR, Raikhel NV et al (2009) A key role for vesicles in fungal secondary metabolism. Proc Natl Acad Sci U S A 106:19533–19538
16. Schmidt-Heydt M, Magan N, Geisen R (2008) Stress induction of mycotoxin biosynthesis genes by abiotic factors. FEMS Microbiol Lett 284:142–149
17. Schmidt-Heydt M, Rüfer CE, Abdel-Hadi A, Magan N, Geisen R (2010) The production of aflatoxin B_1 or G_1 by *Aspergillus parasiticus* at various combinations of temperature and water activity is related to the ratio of *aflS* to *aflR* expression. Mycotoxin Res 26:241–246
18. Karlovsky P (2008) Secondary metabolites in soil ecology. In: Karlovsky P (ed) Secondary metabolites in soil ecology. Springer, Berlin Heidelberg, pp 1–22
19. Scherlach K, Graupner K, Hertweck C (2013) Molecular bacterial-fungal interactions with impact on the environment, food and medicine. Annu Rev Microbiol 67:375–397
20. Schroeckh V, Scherlach K, Nützmann H-W, Shelest E, Schmidt-Heck W, Schuemann J et al (2009) Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. Proc Natl Acad Sci U S A 106:14558–14563
21. Nützmann H-W, Reyes-Dominguez Y, Scherlach K, Schroeckh V, Horn F, Gacek A et al (2011) Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. Proc Natl Acad Sci U S A 108:14282–14287
22. Schoustra S, Rundle HD, Dali R, Kassen R (2010) Fitness-associated sexual reproduction in a filamentous fungus. Curr Biol 20:1350–1355

23. Sarikaya Bayram Ö, Bayram Ö, Valerius O, Park HS, Irniger S, Gerke J et al (2010) LaeA control of velvet family regulatory proteins for light-dependent development and fungal cell-type specificity. PLoS Genet. 6:e1001226
24. Illig J, Norton RA, Scheu S, Maraun M (2010) Density and community structure of soil- and bark-dwelling microarthropods along an altitudinal gradient in a tropical montane rainforest. Exp Appl Acarol 52:49–62
25. Bérdy J (2005) Bioactive microbial metabolites. J Antibiot 58:1–26
26. Demain AL, Fang A (2000) The natural function of secondary metabolites. In: Sheper T (ed) Advances in biochemical engineering/biotechnology. Springer, Berlin, pp 1–39
27. Dowd PF (1992) Detoxification of mycotoxins by insects. In: Mullin CA, Scrott JG (eds) Molecular mechanisms of insecticide resistance. American Chemical Society, Washington, pp 264–275
28. Gloer JB (1995a) The chemistry of fungal antagonism and defense. Can J Bot 73:1265–1274
29. Janzen DH (1977) Why fruits rot, seeds mold and meat spoils. Am Nat 111:691–713
30. Marmeisse R, Nehls U, Öpik M, Selosse M-A, Pringle A (2013) Bridging mycorrhizal genomics, metagenomics and forest ecology. New Phytol 198:343–346
31. Rohlfs M, Churchill ACL (2011) Fungal secondary metabolites as modulators of interactions with insects and other arthropods. Fungal Genet Biol 48:23–34
32. Sherratt TN, Wilkinson DM, Bain RS (2005) Explaining dioscorides' "double difference": why are some mushrooms poisonous, and do they signal their unprofitability? Am Nat 166:767–775
33. Spiteller P (2008) Chemical defence strategies of higher fungi. Chem A Eur J 14:9100–9110
34. Castillo M-A, Moya P, Cantín A, Miranda MA, Primo J, Hernández E et al (1999) Insecticidal, anti-juvenile hormone, and fungicial activities of organic extracts from different *Penicillium* species and their isolated active components. J Agric Food Chem 47:2120–2124
35. Dowd PF, Miller JD, Greenhalgh R (1989) Toxicity and interactions of some *Fusarium graminearum* metabolites to caterpillars. Mycologia 81:646–650
36. Gloer JB, Rinderknecht B, Wicklow DT, Dowd PF (1989) Nominine: a new insecticidal indole diterpene from the sclerotia of *Aspergillus nomius*. J Org Chem 54:2530–2532
37. Grove JF, Pople M (1981) The insecticidal activity of some fungal dihydroisocoumarins. Mycopathologia 76:65–67
38. Ondeyka JG, Dombrowski AW, Polishook JP, Felcetto T, Shoop WL, Guan Z et al (2003) Isolation and insecticidal activity of mellamide from *Aspergillus melleus*. J Ind Microbiol Biotechnol 30:220–224
39. Paz Z, Bilkis I, Gerson U, Kerem Z, Sztejnberg A (2011) Argovin, a novel natural product secreted by the fungus *Meira argovae*, is antagonistic to mites. Entomol Exp Appl 140:247–253
40. Paterson RRM, Simmonds MSJ, Blaney WM (1987) Mycopesticidal effects of characterized extracts of *Penicillium* isolates and purified secondary metabolites (including mycotoxins) on *Drosophila melanogaster* and *Spodoptora littoralis*. J Invertebr Pathol 50:124–133
41. Reiss J (1975) Insecticidal and larvicidal activities of the mycotoxins aflatoxin B1, rubratoxin B, patulin and diacetoxyscirpenol towards *Drosophila melanogaster*. Chem Biol Interact 10:339–342
42. Wicklow DT, Dowd PF, Gloer JB (1994) Antiisectan effects of *Aspergillus* metabolites. In: Powell KA, Renwick A, Peberdy JF (eds) The genus *Aspergillus*: from taxonomy and genetics to industrial applications. FEMS Symposium Series, vol 69. Plenum, New York, pp 93–114
43. Obana H, Kumeda Y, Nishimune T, Usami Y (1994) Direct detection using the *Drosophila* DNA-repair test and isolation of a DNA-damaging mycotoxin, 5,6-dihydropenicillic acid, in fungal culture. Food Chem Toxicol 32:37–43
44. Stark AA (1980) Mutagenicity and carcinogenicity of mycotoxins: DNA binding as a possible mode of action. Annu Rev Microbiol 34:235–262
45. Dowd PF (1988) Synergism of aflatoxin B1 toxicity with the co-occurring fungal metabolite kojic acid to two caterpillars. Entomol Exp Appl. 47:69–71

46. Chinnici JP, Gunst K, Llewellyn GC (1983) Effects of mycotoxin pretreatment on aflatoxin B1 post-treatment toxicity in *Drosophila melanogaster* (Diptera). J Invertebr Pathol 41:321–327
47. Brodhun F, Schneider S, Gobel C, Hornung E, Feussner I (2010) PpoC from *Aspergillus nidulans* is a fusion protein with only one active haem. Biochem J 425:553–565
48. Combet E, Eastwood DC, Burton KS, Henderson J (2006) Eight-carbon volatiles in mushrooms and fungi: properties, analysis, and biosynthesis. Mycoscience 47:317–326
49. Sawahata T, Shimano S, Suzuki M (2008) *Tricholoma matsutake* 1-octen-3-ol and methyl cinnamate repel mycophagous *Proisotoma minuta* (Collembola: Insecta). Mycorrhiza 18:111–114
50. Inamdar AA, Masurekar P, Bennett JW (2010) Neurotoxicity of fungal volatiles organic compounds in *Drosophila melanogaster*. Toxicol Sci 117:418–426
51. Nakamori T, Suzuki A (2006) Repellency of injured ascomata of *Ciborinia camelliae* and *Spathularia flavida* to fungivorous collembolans. Mycoscience 47:290–292
52. Bengtsson G, Hedlund K, Rundgren S (1991) Selective odor perception in the soil Collembola *Onychirus armatus*. J Chem Ecol 17:2113–2125
53. Hedlund K, Bengtsson G, Rundgren S (1995) Fungal odour discrimination in two sympatric species of fungivorous collembolans. Funct Ecol 9:869–875
54. Pierce AM, Pierce HD Jr, Borden JH, Oehlschlager AC (1991) Fungal volatiles: semiochemicals for stored-product beetles (Coleoptera: Cucujidae). J Chem Ecol 17:581–597
55. Wood WF, Archer CL, Largent DL (2001) 1-Octen-3-ol, a banana slug antifeedant from mushrooms. Biochem Syst Ecol 29:531–533
56. Stensmyr MC, Dweck HKM, Farhan A, Ibba I, Strutz A, Mukunda L et al (2012) A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. Cell 151:1345–1357
57. Tsitsigiannis DI, Keller NP (2007) Oxylipins as developmental and host-fungal communication signals. Trend Microbiol 15:109–118
58. Wright VF, Casas ED LAS, Harein PK (1980a) Evaluation of *Penicillium* mycotoxins for activity in stored-product Coleoptera. Environ Entomol 9:217–221
59. Tsai W-T, Mason LJ, Woloshuk CP (2007) Effect of three stored-grain fungi on the development of *Typhaea stercorea*. J Stored Prod Res 43:129–133
60. Niu G, Wen Z, Rupasinghe SG, Zeng RS, Berenbaum MR, Schuler MA (2008) Aflatoxin B1 detoxification by CYP321A1 in *Helicoverpa zea*. Arch Insect Biochem Physiol 69:32–45
61. Zeng RS, Wen Z, Niu G, Schuler MA, Berenbaum MR (2007) Allelochemical induction of cytochrome P450 monooxygenases and amelioration of xenobiotic toxicity in *Helicoverpa zea*. J Chem Ecol 33:449–461
62. Jaenike J, Grimaldi DA, Sluder AE, Greenleaf AL (1983) α-Amanitin tolerance in mycophagous *Drosophila*. Science 221:165–167
63. Stump AD, Jablonski SE, Bouton L, Wilder JA (2011) Distribution and mechanism of α-amanitin tolerance in mycophagous *Drosophila* (Diptera: Drosophilidae). Environ Entomol 40:1604–1612
64. Trienens M, Rohlfs M (2011) Experimental evolution of defense against a competitive mold confers reduced sensitivity to fungal toxins but no increased resistance in *Drosophila* larvae. BMC Evol Biol 11:206
65. Wicklow DT, Dowd PF (1989) Entomotoxigenic potential of wild and domesticated yellow-green Aspergilli: toxicity to corn earworm and fall armyworm larvae. Mycologia 81:561–566
66. Payne GA, Nierman WC, Wortman JR, Pritchard BL, Brown D, Dean RA et al (2006) Whole genome comparison of *Aspergillus flavus* and *A. oryzae*. Med Mycol 44:9–11
67. Shaw PJA (1988) A consistent hierarchy in the fungal feeding preference of the Collembola *Onychiurus armatus*. Pedobiologia 39:179–187
68. Wright VF, Harein PK, Collins NA (1980b) Preference of the confused flour beetle for certain *Penicillium* isolates. Environ Entomol 9:4

69. Belofsky GN, Gloer JB, Wicklow DT, Dowd PF (1995) Antiinsectan alkaloids: shearinines A-C and a new paxilline derivative from the ascostromata of *Eupenicillium shearii*. Tetrahedron 51:3959–3968
70. Gloer JB (1995b) Antiisectan natural products from fungal sclerotia. Acc Chem Res 28:343–350
71. Wang H, Gloer J, Wicklow D, Dowd P (1995) Aflavinines and other antiinsectan metabolites from the ascostromata of *Eupenicillium crustaceum* and related species. Appl Environ Microbiol 61:4429–4435
72. Whyte AC, Gloer JB (1996) Sclerotiamide: a new member of the paraherquamide class with potent antiisectan activity from the sclerotia of *Aspergillus sclerotiorum*. J Nat Prod 59:1093–1095
73. Wicklow DT, Shotwell OL (1983) Intrafungal distribution of aflatoxins among conidia and sclerotia of *Aspergillus flavus* and *Aspergillus parasiticus*. Can J Microbiol 29:1–5
74. Wicklow DT, Dowd PF, Alfatafta AA, Gloer JB (1996) Ochratoxin A: an antiinsectan metabolite from the sclerotia of *Aspergillus carbonarius* NRRL 369. Can J Microbiol 42:1100–1103
75. Wicklow DT, Dowd PF, Tepaske MR, Gloer JB (1988) Sclerotial metabolites of *Aspergillus flavus* toxic to a detritivorous maize insect (*Carpophilus hemipterus*, Nitidulidae). Trans Br Mycol Soc 91:433–438
76. Rhoades DF (1979) Evolution of plant chemical defense against herbivores. In: Rosenthal GA, Janzen DH (eds) Herbivores—their interaction with secondary plant metabolites. Academic, New York, pp. 3–54
77. Cary JW, Harris-Coward PY, Ehrlich KC, Di Mavungu JD, Malysheva SV, De Saeger S et al (2014) Functional characterization of a *veA*-dependent polyketide synthase gene in *Aspergillus flavus* necessary for the synthesis of asparasone, a sclerotium-specific pigment. Fung Genet Biol 64:25–35
78. Caballero Ortiz S Trienens M Rohlfs M (2013) Induced fungal resistance to insect grazing: reciprocal fitness consequences and fungal gene expression in the *Drosophila-Aspergillus* model system. PLoS One 8:e74951
79. Janssens TKS, Staaden S, Scheu S, Mariën J, Ylstra B, Roelofs D (2010) Transcriptional responses of *Folsomia candida* upon exposure to *Aspergillus nidulans* secondary metabolites in single and mixed diets. Pedobiologia 54:45–52
80. Rohlfs M, Albert M, Keller NP, Kempken F (2007) Secondary chemicals protect mould from fungivory. Biol Lett 3:523–525
81. Staaden S, Milcu A, Rohlfs M, Scheu S (2011) Olfactory cues associated with fungal grazing intensity and secondary metabolite pathway modulate Collembola foraging behaviour. Soil Biol Biochem 43:1411–1416
82. Stötefeld L, Scheu S, Rohlfs M (2012) Fungal chemical defense alters density-dependent foraging ehaviour and success in a fungivorous soil arthropod. Ecol Entomol 37:323–329
83. Trienens M, Keller NP, Rohlfs M (2010) Fruit, flies and filamentous fungi—experimental analysis of animal-microbe competition using *Drosophila melanogaster* and *Aspergillus* as a model system. Oikos 119:1765–1775
84. Trienens M, Rohlfs M (2012) Insect-fungus interference competition—the potential role of global secondary metabolite regulation, pathway-specific mycotoxin expression and formation of oxylipins. Fungal Ecol 5:191–199
85. Yin W-B, Amaike S, Wohlbach DJ, Gasch AP, Chiang Y-M, Wang CCC et al (2012) An *Aspergillus nidulans* bZIP response pathway hardwired for defensive secondary metabolism operates through *aflR*. Mol Microbiol 83:1024–1034
86. Scheu S, Folger M (2004) Single and mixed diets in Collembola: effects on reproduction and stable isotope fractionation. Funct Ecol 18:94–102
87. Scheu S, Simmerling F (2004) Growth and reproduction of fungal feeding Collembola as affected by fungal species, melanin and mixed diets. Oecologia 139:347–353
88. Balogh J, Tunlid A, Rosén S (2003) Deletion of a lectin gene does not affect the phenotype of the nematode-trapping fungus *Arthrobotrys oligospora*. Fung Genet Biol 39:128–135

89. Palmer JM, Keller NP (2010) Secondary metabolism in fungi: does chromosomal location matter? Curr Opin Microbiol 13:431–436
90. Bok JW, Keller NP (2004) LaeA, a regulator of secondary metabolism in *Aspergillus* spp. Eukaryot Cell 3:527–535
91. Kale SP, Milde L, Trapp MK, Frisvad JC, Keller NP, Bok JW (2008) Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus*. Fungal Genet Biol 45:1422–1429
92. Karimi-Aghcheh R, Bok JW, Phatale PA, Smith KM, Baker SE, Lichius A et al (2013) Functional analyses of *Trichoderma reesei* LAE1 reveal conserved and contrasting roles of this regulator. G3 3:369–378
93. Kim H-K, Lee S, Jo S-M, McCormick SP, Butchko RAE, Proctor RH et al (2013) Functional roles of FgLaeA in controlling secondary metabolism, sexual development, and virulence in *Fusarium graminearum*. PLoS One 8:e68441
94. Kosalková K, García-Estrada C, Ullán R V, Godio RP, Feltrer R, Teijeira F et al (2009) The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. Biochimie 91:214–225
95. Perrin RM, Fedorova ND, Bok JW, Cramer RA Jr, Wortman JR, Kim HS et al (2007) Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. PLoS Pathog 3:e50
96. Wiemann P, Brown DW, Kleigrewe K, Bok JW, Keller NP, Humpf H-U et al (2010) FfVel1 and FfLae1, components of a velvet-like complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism and virulence. Mol Microbiol 77:972–994
97. Wu D, Oide S, Zhang N, Choi MY, Turgeon BG (2012) ChLae1 and ChVel1 regulate T-toxin production, virulence, oxidative stress response, and development of the maize pathogen *Cochliobolus heterostrophus*. PLoS Pathog 8:e1002542
98. Albert M (2007) Der Einfluss des Sekundärmetabolismus von *Aspergillus nidulans* auf Reproduktion, Überleben und Nahrungswahl pilzfressender Collembolen. Christian-Albrechts Universität Kiel. p. 43
99. Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B (2005) A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Mol Microbiol 57:1036–1050
100. Duhamel M, Pel R, Ooms A, Bücking H, Jansa J, Ellers J et al (2013) Do fungivores trigger the transfer of protective metabolites from host plants to arbuscular mycorrhizal hyphae? Ecology 94:2019–2029
101. Crowther TW, Boddy L, Jones TH (2011a) Species-specific effects of soil fauna on fungal foraging and decomposition. Oecologia 167:535–545
102. Crowther TW, Jones TH, Boddy L, Baldrian P (2011b) Invertebrate grazing determines enzyme production by basidiomycete fungi. Soil Biol Biochem 43:2060–2068
103. Hedlund K, Boddy L, Preston CM (1991) Mycelial responses of the soil fungus, *Mortierella isabellina*, to grazing by *Onychiurus armatus* (Collembola). Soil Biol Biochem 23:361–366
104. Kampichler C, Rolschewski J, Donnelly DP, Boddy L (2004) Collembolan grazing affects the growth strategy of the cord-forming fungus *Hypholoma fasciculare*. Soil Biol Biochem 36:591–599
105. Bretherton S, Tordoff GM, Jones TH, Lynne Boddy (2006) Compensatory growth of *Phanerochaete velutina* ehavio systems grazed by *Folsomia candida* (Collembola). FEMS Microbiol Ecol 58:33–40
106. Bleuler-Martínez S, Butschi A, Garbani M, Wälti MA, Wohlschlager T, Potthoff E et al (2011) A lectin-mediated resistance of higher fungi against predators and parasites. Mol Ecol 20:3056–3070
107. Vasconcelos IM, Oliveira JTA (2004) Antinutritional properties of plant lectins. Toxicon 44:385–403
108. Lakkireddy KKR, Navarro-González M, Velagapudi R, Kües U (2011) Proteins expressed during hyphal aggregation for fruiting body formation in basidiomycetes. In: Savoie J-M,

Foulongne-Oriol M, Largeteau M, Barroso G (eds) Proceedings of the 7th international conference on mushroom biology and mushroom products; 4–7 Oct 2011, Arcachon, France. INRA, Bordeaux, pp. 82–94
109. May GS. Mitogen-activated protein kinase pathways in Aspergilli. In Goldman GH, Osmani SA (eds) The Aspergilli—genomics, medical aspects, biotechnology, and research methods. Taylor & Francis, Boca Raton, 2007. pp. 121–128
110. Conrath U (2011) Molecular aspects of defence priming. Trends Plant Sci 16:524–531
111. Nielsen MT, Klejnstrup M, Rohlfs M, Anyaogu DC, Nielsen JB, Gotfredsen CH et al (2013) *Aspergillus nidulans* synthesize insect juvenile hormones upon expression of a heterologous regulatory protein and in response to grazing by *Drosophila melanogaster* larvae. PLoS One 8:e73369
112. Jindra M, Palli SR, Riddiford LM (2013) The juvenile hormone behaviour pathway in insect development. Ann Rev Entomol 58:181–204
113. Döll K, Chatterjee S, Scheu S, Karlovsky P, Rohlfs M (2013) Fungal metabolic plasticity and sexual development mediate induced resistance to arthropod fungivory. Proc R Soc B Biol Sci 280:20131219
114. Chiang Y-M, Szewczyk E, Nayak T, Davidson AD, Sanchez JF, Lo H-C et al (2008) Molecular genetic mining of the *Aspergillus* secondary metabolome: discovery of the emericellamide biosynthetic pathway. Chem Biol 15:527–532
115. Lo H-C, Entwistle R, Guo C-J, Ahuja M, Szewczyk E, Hung J-H et al (2012) Two separate gene clusters encode the biosynthetic pathway for the meroterpenoids austinol and dehydroaustinol in *Aspergillus nidulans*. J Am Chem Soc 134:4709–44720

Chapter 10
Fusarium Mycotoxins and Their Role in Plant–Pathogen Interactions

Gerhard Adam, Gerlinde Wiesenberger and Ulrich Güldener

Role of Mycotoxins in Plant Disease?

Mycotoxins are by definition toxins produced by fungi. Yet, the term is normally used in a restricted sense for fungal metabolites that can cause toxic effects in animals and humans. Furthermore, poisons of mushrooms or toxins from molds growing on food commodities are generally excluded and the term is reserved for compounds contaminating plant-derived food commodities and feed due to infection with toxin-producing fungal pathogens in the field. There are exceptions, such as the *Penicillium* mycotoxin ochratoxin A, which is produced during storage of various plant commodities, and also directly on meat products [1]. Yet, in general, mycotoxins are secondary metabolites of plant pathogens. Only a limited number of fungal secondary metabolites are expected to have targets in mammals, and occur in such high concentrations in the infected crop plants that they are recognized as toxicologically relevant. In the case of *Fusarium* species, the following mycotoxins received most scientific attention: (1) members of the class of trichothecenes (in particular deoxynivalenol and T-2 toxin), which target primarily eukaryotic protein synthesis; (2) zearalenone and derivatives, which are resorcylic acid lactones with negligible acute toxicity but very high estrogenic activity; and (3) fumonisins,

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which affect ceramide biosynthesis and are possibly carcinogenic to humans. Based on a review of the toxicological hazard and assessment of exposure levels in Europe, maximum tolerated levels for these mycotoxins in various food commodities were enacted to protect consumers. Their toxicological properties are well characterized [2–4].

The main objective of this review is to summarize what is known about the role of these *Fusarium* mycotoxins, and other known secondary metabolites of *Fusarium graminearum* with respect to fungal virulence on host plants. Besides, an attempt is made to integrate secondary metabolites into a unified model of plant pathogen interactions.

The assumption that the quite costly production of secondary metabolites with multistep biosynthetic pathways should provide a benefit to the producing fungus seems reasonable. Yet, also notorious plant pathogenic fungi like *Fusarium* species do not only have to cope with the host plant but are in fact saprophytes during large parts of their life cycles. While the infection period can last till autumn in wild grasses and maize, *F. graminearum* infects wheat heads during anthesis and the interaction with the living host takes place only within the few weeks until harvest. Some metabolites may therefore rather provide protection against abiotic stress (e.g., ultraviolet light) or might also be directed against mites and insects consuming fungi in the natural environment [5]. Furthermore, they could play a role in the competition with other microbes co-occurring in soil and plant debris or provide protection against mycoparasitic fungi. But at least a few of the secondary metabolites produced by *Fusarium* are expected to play a role in the interaction of the pathogens with their host plants. While the assumption that certain fungal secondary metabolites act as virulence factors in various plant diseases [6] seems quite reasonable, there is surprisingly little solid evidence for this scenario. Therefore, the goal of this review is to reconsider the role of fungal secondary metabolites in the plant pathogen interaction by incorporating also the plant side into the picture, and to adjust the expectations accordingly.

The hypothesis that certain metabolites play a role as pathogenicity or virulence factors is initially often based on correlative evidence [7,8]. Toxins alone can often reproduce parts of the disease symptoms, and a correlation of virulence and the amount of toxin produced by strains *in vitro* may be observed. If genetically unrelated strains are compared, such data are typically very noisy, and often only weak or no correlations were found (e.g., between deoxynivalenol production and disease development [9]). The availability of molecular genetics tools for *Fusarium* allows cleaner experimental testing by creating targeted mutations and comparison of otherwise isogenic strains. The mainstream hypothesis is that if a particular compound has a role in the interaction, the disruption of the biosynthetic genes for such a metabolite should lead either to a complete loss of pathogenicity (pathogenicity factor) or at least to a severe reduction of disease symptoms at the end point of the observation or to changes in the dynamics of symptom development (area under the disease progression curve). Such metabolites are termed aggressiveness or virulence factors.

There are very few clear examples for metabolites functioning as pathogenicity factors, found primarily in the group of host selective toxins produced by

Cochliobolus and *Alternaria* species [10,11]. For instance, if both copies of the nonribosomal peptide synthase (NRPS) required for HC-toxin biosynthesis of *Cochliobolus carbonum* are inactivated, loss of the metabolite leads to loss of pathogenicity on corn [12]. On the other hand, if the plant contains a gene coding for an enzyme detoxifying the metabolite, resistance against the toxin leads to resistance to the pathogenic fungus [13]. Yet, such clear cases are rare and exceptional. One scenario is recent gain of the ability to produce a novel secondary metabolite by a formerly rather weak coevolved pathogen [14]. *Cochliobolus heterostrophus* race T, which seemingly acquired the ability to produce T-toxin by horizontal gene transfer, gained virulence specifically on corn with the Texas cytoplasm [15]. In other cases the removal of detoxification genes from coevolved plant hosts by plant breeding accidents may cause the specificity for certain host genotypes [16]. Homologs of the HC-toxin reductase occur not only in wild-type corn but also in other grasses. It has been shown that silencing of these genes in barley leads to the breakdown of nonhost resistance to *Cochliobolus carbonum*, only if the pathogen also produces HC-toxin [17]. So in many cases plants may have coevolved and possess the ability to antagonize toxins quite efficiently.

Excluding the case of a protein toxin (from the soybean pathogen *Fusarium virguliforme* [18]), we are not aware of cases of host-selective secondary metabolites of *Fusarium*. The analysis of the interaction between *F. graminearum* and wheat has revealed only quantitative differences in resistance. These are inherited in a polygenic fashion [19] with a rather weakly defined contribution of toxin resistance as a component of *Fusarium* resistance. Currently, such complex, polygenic interactions with fungal pathogens and the role of secondary metabolites are not well integrated in the prevailing picture of plant–pathogen interactions.

Secondary Metabolites and PAMP-Triggered Immunity

The innate plant defense response has been separated into two different phases. The first phase is known as microbe- or pathogen-associated molecular pattern (MAMP/PAMP)-triggered immunity [20]. Evolutionarily, highly conserved molecular patterns of the pathogen are recognized by cell surface receptors of plant cells, leading to the reprogramming of transcription and ultimately to a successful plant defense response [21]. In the older literature such compounds were called elicitors, most being either parts of highly conserved proteins or carbohydrate structures (e.g., fungal chitin fragments). There is limited evidence that secondary metabolites or small molecules from primary metabolism of microbes are also recognized. For ergosterol, a fungi-specific membrane sterol, it has been shown that nanomolar concentrations can elicit defense responses in tomato [22] and tobacco cells [23]. It is unclear whether fungal secondary metabolites also play a role as PAMPs and are recognized by cereal hosts. Candidates are the highly conserved siderophores, evolutionarily optimized for high iron binding capacity. Seemingly, bacterial siderophores can trigger defense responses in *Arabidopsis* [21]. Most pathogenic fungi

use reductive iron uptake and in addition produce extracellular siderophores that play a role to fetch the limiting iron from the environment, and also from the host. Triacetylfusarinin-type siderophores are widespread and conserved in fungi. While it is unclear whether plants can "smell" the presence of fungi by detecting such conserved siderophores, it is obvious that triacetylfusarinin (the product of the NRPS6 cluster) is required for full virulence of different pathogenic fungi, including *F. graminearum* [24]. The need for efficient iron acquisition from the host seems to outweigh the advantage of avoiding recognition by loss of siderophore biosynthesis. Yet, recognition could also be avoided by switching to another though less efficient siderophore. It is intriguing that symbiotic fungi (endophytes, mycorrhiza fungi) are an especially rich source of unconventional siderophores [25]. Also *F. graminearum* has an untypical siderophore, malonichrome [26]. We could show that the NRPS1 gene cluster, which is induced during infection, is responsible for synthesis of this metabolite [27].

Secondary Metabolites and Effector-Triggered Immunity

To overcome pattern-triggered immunity (PTI), successful pathogens have developed the means to introduce effectors into plant cells, which suppress the PAMP-triggered defense response in many different ways. Effector proteins are introduced, for instance, by type III secretion systems of Gram-negative bacteria. Many fungi, in particular biotrophs, can hijack still ill-characterized mechanisms of plants leading to active uptake of effector proteins, which are secreted by the fungus at the interaction interphase [28, 29]. The mechanisms of uptake are still controversial and a matter of intensive research [30–32].

To regain resistance, plants have evolved intracellular receptors, typically encoded by members of the large family of nucleotide-binding site leucine-rich repeat (NBS-LRR) class of disease-resistance genes [33–35], which can recognize the presence of specific effector proteins and trigger a strong defense response. In the case of biotrophs, a successful defense strategy of the plant is to trigger programmed cell death of infected cells—the classical "hypersensitive response." Often there is direct protein–protein interaction between the product of the resistance gene (R) and the product of the then so-called avirulence gene encoding a recognized effector. In other cases, the R-genes monitor the changes inflicted by the effector on the virulence target itself, or on a decoy [36].

Recently it has been discovered that *Botrytis cinerea* (in addition to effector proteins) uses small RNA effectors that are taken up by the host and which target conserved defense-signaling components [37, 38]. It seems likely that other fungi also employ RNA-based effectors.

Secondary metabolites also can act as effectors. A clear example for a small molecule effector from plant pathogenic bacteria is the *Pseudomonas* secondary metabolite coronatine. It has little phytotoxicity but suppresses several aspects of

plant defense [39], primarily by interacting with the F-box-protein COI1 (coronatine insensitive), which is involved in jasmonate signaling.

Another bacterial small molecule effector is syringolin A of *Pseudomonas*, which acts as a proteasome inhibitor. Its production clearly increases virulence on the compatible host [40], but it is recognized by many nonhosts and triggers a hypersensitive response in wheat [41]. The detection of effectors triggering defense is the basis of the classical gene-for-gene interaction. There is currently only limited evidence for specific genetic interactions of small molecule effectors with classical leucine-rich repeat (LRR) disease-resistance genes. The glycolipid syringolide, the enzymatic product of the *Pseudomonas syringae avrD* avirulence gene, triggers a hypersensitive response [42] depending on the presence of the soybean *RPG4* disease-resistance gene. In *Magnaporthe oryzae* (*grisea*) the avirulence gene *ACE1* turned out to encode a mixed polyketide/nonribosomal peptide synthase (PKS/NRPS) gene [43]. Most likely it is not the protein but the small molecule produced by gene products of this cluster that is recognized. The cluster is only expressed during the early stages of infection [44] and the metabolite produced is unknown. The metabolite is seemingly perceived by the product of the disease-resistance gene *Pi33*, which is also not yet identified but is assumed to be one of the classical resistance gene analogs found in the large interval introgressed from *Oryza rufipogon* [45].

While the molecular evidence for avirulence triggered by small molecules is still limited, the interaction of classical NBS-LRR disease-resistance genes with fungal metabolites triggering susceptibility is better characterized. The *Cochliobolus victoriae* toxin victorin triggers a hypersensitive response in certain *Arabidopsis thaliana* ecotypes. This response depends on the presence of an NBS-LRR "disease-resistance gene," which in this case confers susceptibility to the toxin and the toxin-producing pathogen [46]. Consistent with the guard model of disease-resistance genes, the product of the NBS-LRR disease-resistance gene *LOV1* seems to monitor the covalent modification of the virulence target of the toxin, a thioredoxin that covalently reacts with victorin [47]. In oats, the original *C. victoriae* host, the presumably analogous, not yet identified, Pc gene confers victorin susceptibility, and has been heavily used by plant breeders since it confers crown rust resistance.

Another example where an identified NBS-LRR disease-resistance gene confers susceptibility to a fungal metabolite is the case of Periconia toxin [48]. A gene (*Pc*) was identified in *Sorghum bicolor* that confers dominant susceptibility to the toxin of the pathogen *Periconia circinata* [49]. It also encodes an NBS-LRR-type "resistance gene."

The main role of secondary metabolite effectors could be to suppress defense responses activated by effector-triggered immunity—the recognition of effector proteins by R-gene products. With respect to *Fusarium* it is interesting to note that the trichothecene toxin diacetoxyscirpenol was identified in a screen for chemical suppressors of the *avrRpm1-RPM1* triggered hypersensitive cell death in *Arabidopsis* [50]. It was furthermore shown that this mycotoxin is also able to inhibit early PAMP-activated defense responses.

In summary, there is evidence that metabolites inflicting changes on their virulence targets can be recognized by plant resistance genes (guarding the target), and that production of such metabolites consequently may even cause avirulence. If the metabolite does not have a strong virulence function, one can expect that complete loss of such a metabolite would allow the fungus to regain virulence. If a high fitness price is associated with loss of such a metabolite (on hosts without the R gene or in the environment), irreversible inactivation of toxin biosynthesis may not be the most successful strategy of the pathogen. Silencing gene clusters and stochastic reactivation using epigenetic mechanisms would allow optimization (bet hedging [51]) and provide a selective advantage to progeny confronted with a genetically diverse host population.

Inactivation of the *F. graminearum* histone-methyltransferase gene required for correct chromatin structure leads to constitutive (over)expression of multiple genes that are normally not, or only very weakly, expressed in culture [52]. It was shown that this knockout strain had severely reduced virulence, consistent with the hypothesis that avirulence is triggered by one of the activated metabolites, instead of increasing virulence by production of higher amounts of metabolites. Yet, this result has to be considered preliminary, since the virulence testing was not done with cereals but on tomato as a surrogate host, and also the classical scenario that the mutant produces less of a toxin with a virulence function cannot be ruled out.

Plant Resistance to Small Molecule Effectors with a Role in Virulence

In order to regain resistance, plants have to counteract the small molecule effector. This is true, especially if the toxin targets not only the signaling components but an essential process like protein biosynthesis as in the case of trichothecenes. We therefore propose an extension of the ZIG-ZAG model for small molecules that can suppress R-gene mediated effector-mediated resistance (Fig. 10.1).

For a single compound, the simple situation may exist that one genetic difference (e.g., a single amino acid change in a plant protein leading to target insensitivity) could lead to a high-level resistance, as in the case of herbicide resistance [53]. An example for such a scenario may be *Alternaria alternata* AAL toxin in tomato. It has been show that AAL toxin is required for the ability of *A. alternata* [54] to cause the disease *Alternaria* stem cancer on tomato. Tomato plants resistant to the toxin due to the presence of the dominant *ASC-1* gene (encoding a presumably toxin-insensitive ceramide synthase subunit) are also resistant to the pathogen. This scenario was also reconstructed in *Arabidopsis*, where a loss of function of the *ASC-1* homolog leads to toxin sensitivity [55]. AAL toxin is structurally and mechanistically related to fumonisins [56], and interestingly the fumonisin gene cluster of *F. verticillioides* contains two genes (*LAG17* and *LAG18*) that are homologs of *ASC-1* and proposed to play a role in self-resistance of the producing fungus to its own toxin [57]. Cases with such a simple inheritance, caused by mechanisms such

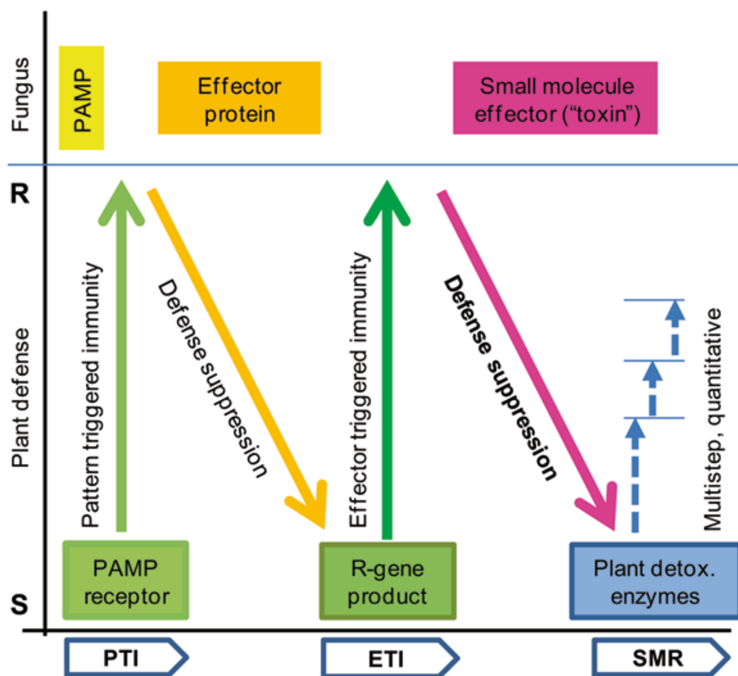

Fig. 10.1 Extended ZIG-ZAG model. The strength of the plant defense response is indicated by the Y-axis, ranging in the outcome from susceptible (*S*) to resistant (*R*). Fungal components affecting the interaction are shown on *top*, assuming the simplest (unrealistic) scenario that a single PAMP, effector protein, or small molecule effector is involved. The plant response can be divided into the classical phases (along X-axis): *PTI* pattern-triggered immunity, *ETI* effector-triggered immunity and the new addition, *SMR* small molecule resistance. SMR is mediated by multiple mechanisms and enzymes (symbolized by *dashed arrows*) and is effective only to provide a variable intermediate level of resistance. If a second small molecule effector is also suppressing defense and is not neutralized, the plant is highly susceptible

as target insensitivity or the complete detoxification as in the case of *Cochliobolus* HC-toxin by the maize HC-toxin reductase, are rather the exception. For most toxins, only quantitative differences in toxin resistance that are inherited in a polygenic fashion are observed. Obviously, such systems are more difficult to study and have attracted far less attention in basic science.

In the area of plant genomics, the underlying molecular mechanisms for quantitative polygenic toxin resistance mechanisms are increasingly understood. They seem to be predominantly based on the successive action of members of large gene families with different modes of action [58]. As a first line of defense, plasma membrane localized multidrug resistance transporters, such as members of the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), and the multidrug and toxic compound extrusion (MATE) family, can reduce the concentration at the target site by active efflux [59].

During phase I metabolization of toxins, either hydroxyl groups are introduced into molecules or become available for further conjugation; for instance, due to esterases removing acetyl- or other acyl-groups. In phase II, UDP-glucosyltransferases [60] and glutathione transferases [61] can reduce the toxicity by chemical modification of the toxin. Subsequently, these conjugates are either directly translocated across the plasma membrane or transported into the vacuole. In some cases conjugates are further modified, e.g., by malonyltransferases [62] in case of glucosides, or by processing of glutathione conjugates [63]. The sequestration in the vacuole and the incorporation of conjugates into cell wall material are effective strategies for removing toxins. Yet, it is conceivable that the interplay between toxin production by the fungus and detoxification by the plant is highly dynamic over time and depends on the concentration and distance from the site of toxin production. In any case, the outcome is typically not that the plant is fully resistant or susceptible, but quantitative and depending on the dose.

While the scenario is already highly complex for a single metabolite, it is worth considering the outcome of the still simple case that the fungus produces two metabolites, but only one is detoxified efficiently by the plant. In this case susceptibility will be dominant.

If both compounds are causing susceptibility, inactivation of the biosynthesis of only one metabolite by gene disruption therefore should lead to a still susceptible plant—no altered virulence is observed. Therefore, it is not too surprising that frequently results of such experiments fall short of the high expectations of many researchers. The analysis of fungal genomes is sobering. Due to the large capacity to produce secondary metabolites that could have a redundant function in virulence, the assumption that such a virulence function should be evident in most knockout strains seems highly unrealistic. Indeed, systematic inactivation of all polyketide synthase (*PKS*) genes in *F. graminearum* did not reveal a single gene disruption mutant with a virulence phenotype [64]. Likewise, a systematic knockout of all *NRPS* genes in *Cochliobolus*[1] only revealed one gene with a virulence function: *NRPS6*, the siderophore biosynthesis gene that is highly conserved in fungi and required also for full virulence of *F. graminearum* on wheat [65].

In essence, if single knockout strains have phenotypes, these are most likely subtle, and the main technical challenge is to reliably quantify small differences. The second technical challenge is that redundant effectors can only be revealed if both (or multiple genes) are simultaneously inactivated. For example, in *Botrytis* only the simultaneous inactivation of genes required for biosynthesis of the sesquiterpene botryoidal and the polyketide botcinic acid led to markedly reduced virulence [66], while single knockout strains showed unaltered virulence. The choice of well-functioning selection markers is limited, and marker recycling systems are still under development in filamentous fungi, so that construction of double and triple mutants in filamentous fungi is not trivial.

Full-genome sequences and transcriptome analysis indicate that pathogenic fungi, such as *F. graminearum*, have the potential to produce an array of secondary

[1] personal communication with Gillian Turgeon.

metabolites, and furthermore that a large portion of genes and clusters responsible for biosynthesis of (unknown) metabolites is not expressed under standard laboratory conditions. Some clusters are only expressed during plant infection [64,67], and even depending on the host plant species [68].

It is how many secondary metabolites can be produced in a single *Fusarium* species. Biosynthetic pathways of fungal secondary metabolites are typically highly complex, requiring multiple enzymatic steps. The genes encoding pathway enzymes tend to occur in gene clusters that are co-regulated in complex ways, often controlled by regulatory genes located in the cluster [69]. A classical hallmark of gene clusters, and therefore the starting point for functional analysis, is the presence of genes encoding molecular machines involved in biosynthesis of the carbon backbone of secondary metabolites: either a PKS [70], or NRPS [71], or hybrid *PKS-NRPS* gene [72], or terpenoid synthase (TPS [73]). Dimethylallyltryptophan synthases (DMATS) required for synthesis of prenylated indole-type metabolites are absent in *F. graminearum* but prominent in other fungi, especially *Aspergillus* [74]. Besides the classical secondary metabolite biosynthesis genes (*TPS, NRPS,* and *PKS*), cytochrome P450 enzymes (CYP), which introduce oxygen functions into the backbone, and other tailoring enzymes such as methyltransferases, acyltransferases, oxidoreductases, or glycosyltransferases are often parts of clusters. Enrichment of certain predicted functions is the basis of *de novo* prediction from whole-genome sequences using various tools, such as "secondary metabolite unique regions finder" (SMURF) [75] or "secondary metabolite biosynthetic" (SMB) [76], "antibiotics & secondary metabolite analysis shell" (AntiSMASH) [77], and others [78].

The number of gene clusters predicted by these methods is variable depending on the tool and underlying criteria and changes over time. A snapshot about the occurrence of classical secondary metabolite biosynthetic genes based on a recent survey [79] of sequenced *Fusarium* genomes is given in Table 10.1.

While the estimate from classical clusters (based on number of *PKS* + *NRPS* + *TPS* genes) seems already quite large, things may be even a lot more complex. Some clusters may be nonfunctional gene ruins, decreasing the number. On the other hand, some secondary metabolites may be derived from primary metabolism with

Table 10.1 Occurrence of selected *SMB* genes in the genomes of *Fusarium* species

SMB genes	*Fusarium graminearum*	*Fusarium verticillioides*	*Fusarium oxysporum*	*Fusarium solani*	*Fusarium circinatum*	*Fusarium mangiferae*
PKS	13	13	11	12	12	13
PKS/NRPS	2	3	3	1	3	3
NRPS	19	16	14	13	13	16
DMATS	0	2	2	0	4	4
TPS	7	8	6	0	9	10
CYP	114	130	168	156	145	116

PKS polyketide synthase, *NRPS* nonribosomal peptide synthase, *DMATS* dimethylallyltryptophan synthase, *TPS* terpenoid synthase, *CYP* cytochrome P450

no involvement of *PKS, NRPS,* or *TPS* genes but need, for instance, only action of cytochrome P450 or transferase genes. An example is butenolide [80]. A recent re-analysis [81] of the *F. graminearum* genome led to the proposal of 67 putative gene clusters based on clustering of protein features enriched for secondary metabolite biosynthetic functions. Co-regulation of expression was found for 42 clusters, and 26 showed correlated gene expression *in planta* in 4 different infection experiments. For 20 of these predicted clusters currently no corresponding metabolite is known. The known *F. graminearum* metabolites are summarized in Table 10.2.

Regarding known metabolites, we have to dampen expectations since we only have a skewed picture of the plant–fungus interaction. Most of the known secondary metabolites were identified since they are produced in large amounts in axenic cultures (after weeks in media preferred by natural product chemists). Most compounds were initially identified based on toxic effects on animals, and not due to plant-based bioassays. The situation is expected to change with the advances in

Table 10.2 Known *F. graminearum* metabolites and biosynthetic genes

Metabolite	Hallmark enzyme	Pharmacological property, role in plant disease
Trichothecene	TRI5	Protein biosynthesis inhibitor, virulence factor on wheat
Zearalenone	PKS4, PKS13	Powerful xenoestrogen in animals, no effect on virulence
Aurofusarin, rubrofusarin	PKS12	Golden yellow/red pigment of mycelium, low toxicity (can affect antioxidant levels in eggs). No effect on virulence
Triacetylfusarinine	NRPS6	Main extracellular siderophore, conserved role in virulence
Ferricrocin	NRPS2	Intracellular siderophore
Malonichrome	NRPS1	Extracellular siderophore, induced *in planta*
Butenolide	CYP	Low oral toxicity, depletes glutathione? No significant effect of gene disruption
Fusarin C	PKS10	Mutagen, possible carcinogen, instable compound. No effect on virulence
Culmorin	TPS	Antifungal, phytotoxic in high concentrations
Fusarielin	PKS9	Antifungal. Microtubule binding. No effect on virulence
Carotenoids	DTC1	Terpenoid pigments
Orcinol?	PKS14	Grain specific expression, orsellinic acid derivative? No effect on virulence

metabolomics, when more secondary metabolites will be recognized which occur at the right time at the right place. Exploring their mode of action will remain a challenging task.

Some “known unknowns” may be added to this list from work using genes as starting points. Inactivation of the *PKS3* gene of *F. graminearum* leads to unpigmented perithecia, hence this gene is involved in production of a precursor of the insoluble pigment [82]. Inactivation of NRPS4 leads to the loss of hydrophobicity of mycelia [83], but the nature of the compound that is involved either directly in water repelling or indirectly regulating this trait is still unknown.

Virulence Functions of Known *F. graminearum* Secondary Metabolites

Trichothecenes

The most prominent *F. graminearum* secondary metabolites are the trichothecenes deoxynivalenol (DON) and nivalenol (NIV). The initial bioassay that led to the identification of DON was induction of vomiting in pigs, which is still reflected in the older designation “vomitoxin.” The primary mode of action of trichothecenes is inhibition of eukaryotic protein synthesis. In animals (in lower concentrations), DON also triggers the ribotoxic stress response, leading to the induction of proinflammatory gene expression [84]. Also in plants, trichothecenes have defense-gene-activating (“elicitor-like”) effects. While deoxynivalenol in high concentrations is phytotoxic due to the inhibition of protein biosynthesis and presumably inhibits translation of induced defense transcripts, it has been shown that low concentrations (10 mg/L) of DON not only induce transcription of defense genes but actually increase production of the corresponding proteins with known antifungal function [85], such as β(beta)-1,3-glucanase (*PR2*) and chitinase (*PR3*). DON can trigger production of reactive oxygen and lead to programmed cell death [86] or have antiapoptotic activity [87], depending on the concentration. The role of DON in virulence was highly controversial for quite some time (reviewed in [88]), before near-isogenic lines were used both on the pathogen and plant side. The identification of the *TRI5* gene, the first step in the biosynthesis of trichothecenes (for review see [89]) allowed production of knockout strains. The *tri5* strain was less virulent in a wheat seed assay, but seemingly fully virulent on the maize cultivar tested [90]. DON in wheat turned out to be a virulence factor required for fungal spread. The inoculated spikelet of wheat was colonized and destroyed regardless of DON production, but the mutant strain was impaired in spreading from the infection site [91]. Spreading to the next spikelet is blocked by plant cell wall appositions in the rachis—a process that can obviously be suppressed by the toxin [92]. Interestingly, in barley both wild type and mutant are prevented from spreading, suggesting that DON is neutralized.

It is rarely considered that the outcome of virulence tests is strongly influenced by the plant genotype. While *tri5* mutants showed reduced spreading ability in many hexaploid wheat cultivars [93] and other cereals [94], still extensive spreading of the toxin deficient *tri5* mutant was observed in durum wheat (which seems to be highly susceptible to another virulence factor). While the genetics of *Fusarium* resistance is complex and polygenically inherited, one quantitative trait locus (QTL), Qfhs.ndsu-3BS—strongly contributing to *Fusarium* spreading resistance [95,96]—received particular attention. Lines containing this QTL, now mostly referred to as *Fhb1* resistance gene, show also pronounced toxin resistance, phenotypically evident, for instance, as resistance to bleaching of ears triggered by applied DON in susceptible lines [97,98]. The *Fusarium* spreading resistance, the toxin resistance phenotype, and the high ability to convert applied DON into DON-3-O-glucoside were found to co-segregate [97]. The conversion of DON into DON-3-O-glucoside by UDP-glucosyltransferase, first elucidated in the model systems yeast and *Arabidopsis* [99], clearly is a detoxification reaction. A highly DON-induced UDP-glucosyltransferase from barley capable of detoxifying DON was identified [100,101]. Presumably, the high spreading resistance of barley (where DON is only a weak virulence factor) is due to the high glycosylation capacity. In support of this hypothesis, constitutive overexpression of the barley UDP-glucosyltransferase HvUGT13248 in transgenic wheat caused high-level ability to detoxify DON and highly increased *Fusarium* resistance in greenhouse and field experiments [102].

DON is produced early in the interaction, in specialized infection structures [103] and *TRI* genes are transcribed in the moving infection front [104]. An immunohistochemistry study with antisera raised against DON showed that DON can move ahead of the fungus and presumably conditions susceptibility by interfering with translation. Yet, DON also triggers various resistance responses including enzymes involved in its degradation. In barley, DON application led to high upregulation of transcripts for efflux carriers and detoxification enzymes [100] such as UDP-glucosyltransferases. This study also provided the first evidence that glutathione-mediated detoxification of DON exists. Therefore, a very dynamic interaction seems to occur. If UDP-glucosyltransferases with the right specificity are rapidly transcriptionally induced and successfully translated (at lower DON concentrations due to diffusion) the plant can inactivate the toxin, while close to the source of the toxin, translation is most likely blocked to a large extent, so that the pathogen can suppress or delay the defense responses. Based on the *in vitro* translation data using wheat germ extracts [99,105], 50 % inhibition is reached already at a concentration of about 1.5 μM (400 μg/L), whereas DON concentrations in the inoculated spikelets and spikelets below the inoculation point may reach more than 1000-fold higher levels 4 days after inoculation [106].

It has recently been questioned that the mechanism underlying the *Fhb1* resistance locus is based in DON detoxification, since no significant difference in the DON/DON-glucoside ratio was found between Near isogenic lines (NILs) differing in *Fhb1* [107]. In this study, six spikelets per ear were inoculated with the

F. graminearum strain, and the inoculated spikelets were harvested 72 h post inoculation for metabolite extraction. In contrast, in the study by Lemmens et al. [97], two adjacent spikelets were treated with DON, and the whole ear was extracted after ripening. The very high detoxification observed in this experiment (up to 90 % converted to DON-glucoside in the resistant NIL) presumably occurs also after toxin transport in the tissue next to the application site during the 21 days. The *Fusarium*-infected spikelets are overwhelmed regardless of *Fhb1*, and only low conversion of DON to DON-3-O-glucoside is observed in susceptible and resistant lines (32 versus 34 %, respectively) if only this material is analyzed. A further complication is that the fungus has highly active β(beta)-glucosidase capable of hydrolyzing already formed D3G again, if the infection front moves on.

Trichothecenes are a structurally highly diverse class of toxins [89]. The review by Grove [108] lists 217 compounds, most of which are produced by plant pathogens. In case of *F. graminearum*, so-called chemotypes coexist, which produce either DON or NIV (for an extensive review see [109]). DON production is due to the inactivation of the *TRI13* gene encoding a C4-hydroxylating cytochrome P450 [110]. The pseudogene Ψ *tri13* in DON producers provides a useful chemotype marker. The DON producers can be further subdivided into 3-acetyl-DON and 15-acetyl-DON producers, based on which toxin is formed predominantly in axenic culture. The molecular basis for the difference is due to different alleles in the *TRI8* gene [111]. The Tri8 carboxylesterase removes one or the other acetyl group from the common biosynthetic precursor 3,15-di-acetyl-DON. The changes in the toxin structure impact toxicity. Acetylation of the C3–OH by the product of *TRI101* strongly reduces the toxicity at the ribosomal level [112], and is a mechanism of self-protection of the fungus. Yet, in animals or also in plants, depending on the species [109], 3-acetyl-DON is rapidly deacetylated. For instance in wheat, DON and 3-acetyl-DON have similar toxicity, while in *Chlamydomonas* toxins acetylated at C3 are far less toxic than their counterparts with free C3-OH [113]. Our group has recently identified the first carboxylesterases of the model plant *Brachypodium distachyon* capable of deacetylating trichothecenes (Schmeitzl et al., manuscript in preparation). A shift from the dominant 15-acetyl-DON producers to 3-acetyl-DON producers was observed in North America [114]. It is unclear what the selective advantage might be, but potentially changes in the host genetics, like increased use of wheat cultivars containing *Fhb1*, might be responsible [115].

Evidence has been provided that the genetic differences in the core *TRI* cluster responsible for the chemotypes predate speciation in *Fusarium* and are maintained by balancing selection [116]. Producing either DON or NIV may have an advantage on alternative host plant species or different host genotypes. NIV is more toxic than DON in animals (summarized in [109]), but the opposite is true for *Arabidopsis* [117] and other plants. One likely reason is that NIV in pigs is, in contrast to DON, not detoxified by the formation of glucuronides [118]. We have studied the UDP-glucosyltransferase gene family of the model plant *B. distachyon* and characterized a cluster of genes homologous to a barley DON-detoxifying gene [119]. Testing by heterologous expression in yeast revealed that one UGT gene confers high-level

NIV resistance but no relevant DON resistance. If this would be the only relevant UGT on such a hypothetical host plant it would be of advantage for *Fusarium* to inactivate *TRI13* and to produce DON instead of NIV, to escape glycosylation. Yet, another glucosyltransferase is encoded in the *Brachypodium* genome, which is able to detoxify both DON and NIV (Schweiger et al., manuscript in preparation).

Besides DON detoxification mediated by the very large gene family of UDP-glycosyltransferases consisting of roughly 180 genes in diploid plants [120], also glutathathione-mediated detoxification of trichothecenes exists. A DON-glutathione conjugate, and the processing products DON-Cys-Gly and DON-Cys are detected in DON treated wheat [121] but are presumably rather unstable. Currently, no resistance QTL of wheat or barley has been associated with increased glutathione-mediated detoxification. Recently for the first time, evidence was provided that methylthio-DON, a derivative with a much smaller substituent than present in DON-glutathione or DON-Cys, already has markedly reduced ability to inhibit translation *in vitro* [105]. Interestingly, a new population of *F. graminearum* (in the narrow sense) was discovered in North America, which produces a novel (type A) trichothecene [122], which lacks the conjugated keto-double bond that is necessary for the formation of Michael adducts with glutathione at C-10. It remains to be tested whether production of this new toxin provides a selective advantage.

Zearalenone

Zearalenone (ZEN) is called a mycotoxin, although its acute toxicity is negligible for animals. It is produced by *F. graminearum*, *F. culmorum*, *F. pseudograminearum*, *F. crookwellense*, *F. equiseti*, and *F. semitectum*, and potentially other species. In most of these other cases, the taxonomic status of strains analyzed by chemists is unclear or questionable. ZEN is formed on infected small grain cereals, albeit rather late during the infection compared to DON (reviewed in [123]). ZEN is therefore a problem in wheat if cool and humid weather delays harvesting [123]. ZEN contamination is also mainly a problem of maize, especially in late maturing varieties. Some *Fusarium* strains produce several g/kg substrate in axenic culture. In infected corn, high levels of ZEN also can be found, particularly in the cob, less in the kernels. Levels as high as 40–100 mg/kg were reported for infected corn cobs (reviewed in [124]). In comparison, due to the potent estrogenic activity, the maximum tolerated level in infant feed is more than 1000-fold lower, 20 μg/kg [125]. Plants do not have estrogen receptors, and the phytotoxicity of zearalenone is also low. Consequently, a role of zearalenone in virulence seems questionable. After the genome sequence of *F. graminearum* became available, the cluster containing two *PKS* genes responsible for ZEN biosynthesis was identified [126–128]. Gene disruption mutants were generated in two different *F. graminearum* strains and in one *F. pseudograminearum* strain [128]. Unaltered head blight symptoms on barley [126] and unchanged spreading on the highly sensitive wheat cultivar Wheaton was reported [127]. Likewise, both the wild-type and the ZEN-deficient mutant of

F. pseudograminearum showed barley root infection. This is not too surprising, since microarray studies [68] or reverse transcription polymerase chain reaction (RT-PCR) indicate lack or very low levels of *PKS4* and *PKS13* expression. Nevertheless, ZEN has interesting properties. In *A. thaliana* it is able to suppress the short root phenotype caused by a mutant with altered cell wall biosynthesis and constitutively activated ethylene production [129]. Microarray data revealed that ZEN in *Arabidopsis* rapidly triggered induction of genes encoding ABC transporters and glucosyltransferases. It was demonstrated by high-performance liquid chromatography-mass spectrometey (HPLC-MS) that ZEN is indeed rapidly inactivated first into a glucoside and further, partly unknown metabolites [130]. The conversion of ZEN into the conjugates ZEN-14-O-glucoside and ZEN-16-O-glucoside (according to a change in nomenclature [131]) by a DON-induced barley UGT was recently demonstrated [132]. The induction of small heat shock proteins and heat shock protein 90 (HSP90) in ZEN-treated *Arabidopsis*, and the structural similarity of ZEN with radicicol (produced by *Nectria radicicola* and other fungi) warranted testing of the hypothesis that ZEN might also be an inhibitor of HSP90 ATPase activity. We could show that indeed ZEN and more so β(beta)-zearalenol (a phase I metabolite in plants) inhibited the ATPase activity of purified HSP90 not only from yeast but also from *Arabidopsis* and *Brachypodium* (Torres-Acosta et al., in preparation). Compared to radicicol (IC50 of 1.5 μM), ZEN and β(beta)-zearalenol are weaker inhibitors (32x and 6x, respectively), but significant inhibition was observed at concentrations occurring *in planta*. HSP90 is a very prominent target with important roles in disease resistance. Several NBS-LRR resistance gene products are HSP90 client proteins requiring its activity for function (reviewed in [133,134]). Downregulation or pharmacological inhibition of Hsp90 leads to the loss of stability of multiple client proteins, including plant disease-resistance genes such as *F. oxysporum* resistance gene *I-2* from tomato [135]. Presumably, due to the rapid inactivation into glucosides, which are inactive as HSP90 inhibitors, ZEN can be considered to be a virulence factor already defeated by plants. Yet, potentially it has important roles outside of the interaction with the plant host. ZEN is toxic to fungi, and it has been shown that inactivation of ZEN by a lactone-esterase is relevant for mycoparasitism by *Gliocladium* [136].

Aurofusarin and Rubrofusarin

Aurofusarin is the typical pigment of *F. graminearum* and other closely related species. The color is dependent on the pH, ranging from yellow/orange at acidic pH to red/purple at more alkaline conditions. The naphthopyrone rubrofusarin, which can also be found in cultures, is similar to the monomer forming aurofusarin. The biosynthetic pathway, depending on the *PKS12* gene and proceeding via nor-rubrofusarin, which is dimerized by a laccase, was elucidated in *F. graminearum* and *F. pseudograminearum* [137–139]. It was recently partly reconstituted in yeast [140]. Extracellular reduction potential is necessary for the extracellular dimer formation [141].

Feed supplemented with the *Fusarium* pigment causes decreases in the concentrations of vitamins A, vitamin E, and carotenoids in quail egg yolk [142,143] indicating depletion of the antioxidant system. Aurofusarin occurs in naturally infected wheat sometimes at high levels [143,144], but it is unclear when it is actually produced during the infection, since microarray experiments indicate downregulation of the cluster during wheat and barley infection [64,68]. There is little evidence for phytotoxicity of aurofusarin or rubrofusarin. A related (*Aspergillus*) compound, rubrofusarin B, causes some inhibition of root growth [145]. The *F. pseudograminearum* knockout mutant showed more vigorous growth in culture, and also the virulence on barley roots and wheat heads was not impaired, or even somewhat higher than the wild-type [137]. Potentially, the pigment might be recognized as PAMP, but *pks12* mutants also overproduce zearalenone, thus any interpretations have to be met with caution. It has also been falsified that *PKS12*-mediated pigment production is involved in resistance to UV light [137].

Siderophores

Siderophore production is required for full virulence of plant and animal pathogens [24]. The first step of the biosynthesis of the extracellular siderophore is encoded by *NRPS6,* which is conserved in many fungi. It has recently been demonstrated that parts of the biosynthetic pathway for triacetyl-fusarinine C, which is also the main extracellular siderophore in *F. graminearum*, is partly localized in the peroxisomes in *Aspergillus* [146], which may, at least to some extent, explain why intact peroxisomes are required for virulence of various plant pathogenic fungi, including *F. graminearum* [147]. For iron homeostasis in most filamentous fungi an intracellular siderophore is employed. In *Fusarium* the *NRPS2* gene is required for ferricrocin biosynthesis [67]. In *Cochliobolus* it was recently demonstrated that *nrps6 nrps2* double mutants are less virulent [148] than single knockout mutants. *Magnaporthe* ferricrocin deficient mutants were also less virulent [149]. In *Fusarium* another extracellular siderophore, termed malonichrome, is induced during iron starvation [26]. We were able to show that malonichrome is missing in *F. graminearum nrps1* mutants[2]. *NRPS1* expression was induced *in planta* [67]. Triple mutants, *nrps1 nrps2 nrps6* have been made in *F. graminearum* and these are still virulent, although successively less[3]. Obviously, the reductive iron uptake system [148] is sustaining life and basal virulence. Reductive iron uptake has been shown to be relevant for virulence of *Ustilago maydis* [150].

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[2] Oide S, Berthiller F, Wiesenberger G, Adam G, Turgeon BG. Role of siderophores in *Fusarium graminearum* virulence and sexual development. Frontiers in Microbiology [27].

[3] Oide et al. [27].

Butenolide

Butenolide (4-acetamido-4-hydroxy-2-butenoic acid) production is reported for *F. graminearum* and *F. crookwellense*, and also other species like *F. sambucinum*, *F. poae*, and *F. sporotrichioides* can produce it (reviewed in [151]). It is not considered to be a relevant mycotoxin (oral LD50 of 275 mg/kg body weight in mice). Furthermore there seems the possibility of adaptation to even extremely high doses in mice [152]. Nevertheless, it has been suspected that butenolide may act synergistically with another unknown fungal or plant metabolite to cause the fescue foot symptoms in cattle. At least part of the symptoms could be reproduced by administering large amounts of pure butenolide [153]. Also phytotoxicity seems to be rather modest, 200 mg/l butenolide had no effect on pea seed germination [154]. Butenolide at 1 mM (141 mg/L) inhibited the growth of wheat coleoptile tissue segments in a cultivar dependent manner [155]. The concentrations in naturally contaminated plant material are comparably low (e.g., 1.4 mg/kg on average in positive feed samples [156]). The butenolide gene cluster was identified based on co-regulation of genes in a liquid medium triggering trichothecene production [80]. Inactivation of a cytochrome P450 encoding gene in this cluster abolished butenolide production. The genes in the cluster are in agreement with a postulated biosynthetic pathway starting from glutamic acid. Gene disruption mutants unable to produce butenolide showed unaltered virulence. No mode of action is known for butenolide *in planta*, but it can deplete glutathione in human cells [157] and glutathione supplementation antagonizes toxicity [158].

Fusarin C

Fusarin C, initially discovered in *F. verticillioides* extracts in South Africa, is a metabolite with strong mutagenic activity in the Ames test after microsomal activation. The history of its discovery, chemistry, and biology were also reviewed by [159]. Fusarin C is produced by many *Fusarium* species (*F. acuminatum*, *F. armeniacum*, *F. avenacium*, *F. crookwellense*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. sporotrichioides*, *F. tricinctum*, *F. venenatum, F. fujikuroi* and *F. verticillioides*) indicating that it may play an important role in plant pathogenesis. After the genome sequence of *F. graminearum* became available, the systematic functional testing revealed that a mixed PKS-NRPS (*PKS10*, *FgFUS1*) is necessary for biosynthesis [64]. In elegant work, the biosynthesis was elucidated in several *Fusarium* species [160,161] where its production is highly induced in liquid medium during nitrogen starvation.

Despite its mutagenic activity (and potential action as carcinogen) fusarin C is rather infrequently studied, presumably since it is already notoriously unstable in the laboratory [160]. It is surprising that its occurrence in food and feed [162,163] does not receive more attention. According to Zhu and Jeffrey [164], fusarin C is only partly inactivated during food processing.

Sondergaard et al. [165] reported growth stimulation in the estrogen-dependent MCF-7 cell line at very high concentrations of fusarin C (10 µM). In comparison, in the same assay the EC50 for zearalenone is about 1 nM [166]. The claimed estrogenic activity is, therefore, most likely irrelevant, especially in plants. Nothing is known about the fate of fusarin C *in planta*. Gene disruption mutants of *F. graminearum* showed unaltered virulence [64]. A potential explanation for the mutagenicity but lacking carcinogenicity in experimental animals is that fusarin C can be inactivated by reaction with glutathione, mediated by glutathione transferases but also nonenzymatically [167]. In this case the C13–C14 epoxide seems to be targeted. A hypothesis to be tested is that this reaction, potentially depleting the glutathione pool, could also occur *in planta*. The role of glutathione and redox-homeostasis in plant defense are increasingly recognized [168,169].

Culmorin

The terpenoid culmorin is another compound produced by many *Fusarium* species whose biosynthetic pathway has been elucidated [170]. Inactivation of the *CLM1 TPS* gene leads to loss of culmorin production. No effect on virulence was reported since then, suggesting a negative result. Culmorin and various hydroxy-culmorins co-occur with trichothecenes in infected cereals [171]. The toxicity to human and animal cells seems to be very low, but modest antifungal activity was reported [172]. Culmorin was found to be phytotoxic in the 1 mM range in a wheat coleoptile assay [155]. The fate of culmorin and derivatives, such as hydroxyl-culmorins and hydroxy-culmorones *in planta* is unknown.

Fusarielin

A reverse genetics approach led to the identification of the product of the *PKS9* gene cluster. Overexpression of the transcription factor located within the *PKS9* (FSL1) gene cluster led to the production of novel fusarielin type compounds [173], called fusarielin F, G, and H. In inoculated wheat, fusarielin H accumulates in the range from 392 to 1865 µg/kg [174], although the expression of *PKS9* was not observed in several microarray experiments [68]. The mode of action of fusarielins is rather unclear. Fusarielins showed low toxicity to a mammalian cell line. Interestingly, also for fusarielins at 25 µM growth stimulation of the estrogen-dependent MCF-7 cells was reported [175]. The first fusarielin, fusarielin A, was identified from an uncharacterized *Fusarium* strain based on its antifungal activity [176]. Fusarielin-type compounds are also produced in some *Aspergillus* species. Structural similarities of statins and fusarielins were noted [173], which would suggest a mode of action as inhibitor of plant or fungal terpenoid and sterol synthesis [177]. Yet, a biochemical approach to determine the mode of action of fusarielin A led to identification of binding to (highly abundant) cytoskeleton proteins [178], consistent with the hyphal curling effect observed in the antifungal activity test. Both proposed modes of ac-

tion, blocking (plant) mevalonate-dependent terpenoid synthesis and inhibition of plant vesicle transport, are consistent with a role in virulence. Yet, in the systematic *PKS* knockout screen, no virulence phenotype was observed for the single mutant.

Carotenoids

Like *Neurospora*, *Fusarium* is also able to synthesize carotenoids, in particular neurosporaxanthin. The functions of carotenoids in fungi are ill defined [179] but may range from functions in membrane stability, antioxidant activity, protection from excessive light, to light perception. Phytoene is synthesized by the bifunctional enzyme phytoene synthase/lycopene cyclase Al-2 in *Neurospora crassa* and by CarRA in *F. fujikuroi* [180,181]. A corresponding pathway has also been described in *F. graminearum*, where the carotenoid pigment is evident in *pks12* mutants [182]. The functions of carotenoids in plants are highly diverse [183], and in particular cleavage products of carotenoids play important roles in the biosynthesis of plant hormones, such as abscisic acid and strigolactones, which are also important for plant defense and interaction with mycorrhiza fungi [184–186]. It would be surprising if signaling occurs only in one direction, that solely fungi respond to plant-derived carotenoid compounds [187]. Yet, currently there is no evidence that fungal carotenoids play a role in the interaction with host plants. At least *Botrytis cinerea* and other fungi producing abscisic acid have developed a “short-cut” pathway [188].

Orcinol—the PKS14-Metabolite?

The *PKS14* cluster is expressed only *in planta* and on cereal grain substrates, but not on defined media [64, 68]. Recently, evidence was published regarding the identity of the product formed by this cluster: orcinol accumulates in a *F. graminearum* strain overexpressing the *PKS14* gene [189]. Most likely further metabolic reactions occur when the whole cluster is expressed in a coordinated fashion. The actual metabolite is unknown but should contain orsellinic acid or the (spontaneous) decarboxylation product orcinol. No virulence phenotype was detected for the *pks14* knockout strain, despite the suggestive expression pattern induction in wheat and barley ear infection but lack of expression in crown rot [64, 68].

Mycotoxins from Other *Fusarium* Species

Fusaric Acid

Fusaric acid is formed by *F. crookwellense, F. fujikuroi, F. nygamai, F. oxysporum, F. proliferatum, F. sacchari, F. sambucinum, F. solani, F. subglutinans, F. thapsinum, and F. verticillioides*. Some strains of the *Gibberella fujikuroi* complex pro-

duce very large amounts (up to 1 g/kg) in culture [190]. It occurs regularly in feed [191] and also in maize intended for human consumption [192]. Yet, the toxicity is rather low for mammals and there are no regulations in place. The pharmacological properties of fusaric acid were reviewed [193]. The main effect of fusaric acid is on neurotransmitters [194]; it is considered to act as an inhibitor of (copper containing) dopamine-β(beta)-monooxygenase [195]. Fusaric acid is one of the earliest discovered fungal metabolites with a proposed role in pathogenesis, since it causes wilting [196]. A large body of correlative evidence is available supporting a role in virulence (e.g., [197,198]), but due to a lack of correlation between loss of fusaric acid production in UV-mutagenized *F. oxysporum* and virulence this hypothesis has been largely dismissed [199]. A role of fusaric acid has been proposed again in recent literature, for instance in banana wilt [200] or vascular wilt of pea by *F. oxysporum* f. sp. *pisi* [201].

Induction of programmed cell death by fusaric acid was demonstrated in tobacco cells [202]. *A. thaliana* was used to study effects of low nontoxic concentrations. In the nM range, fusaric acid caused production of reactive oxygen species, and triggered calcium and other ion fluxes, and was able to induce the synthesis of phytoalexins. It was also reported to trigger ethylene production [203]. Thus it can be recognized as elicitor. At high concentrations fusaric acid is toxic, most likely due to the long known ability to chelate metal ions, in particular iron [204,205], but also copper [206,207]. Already in 1958 Gäumann hypothesized that "merely because of their ability to chelate heavy-metal ions they (i.e., fusaric acid molecules) can paralyze most varied enzyme systems of the host" [208].

Recently, the genes required for biosynthesis of fusaric acid were identified in *Fusarium verticillioides* [209] and a biosynthetic pathway was proposed. Likewise, the biosynthetic genes were characterized in *F. fujikuroi* [210]. Both groups produced knockout mutants deficient in fusarin production, but the results of "rigorous testing of the role in plant disease" are not yet reported. Interestingly it was discovered that in *F. fujikuroi* the structurally related metabolites fusarolinic acid and 9,10-dehydrofusaric acid are still produced in the knockout strain with abolished fusaric acid biosynthesis, indicating parallel pathways [210]. Hence, the virulence issue might be more complicated than anticipated. A first preliminary claim that fusaric acid production indeed plays a role in *F. oxysporum* f. sp. *vasinfectum* virulence has already been made at a conference [211]. According to the abstract, fusaric acid knockout mutants showed much weaker pathogenicity than their corresponding wild-type toward tomato seedlings in a germination bioassay on agar plates.

Fumonisins

Fumonisins are, based on the impact on human health, the most important class of "*Fusarium*" mycotoxins [212], which are also found in *Aspergillus* [213]. More than 30 structural variants of fumonisins have been described (for review see: [214]). The most prominent type B fumonisins contain a free amine group, while

in the A series the amine is acetylated. In the C-series the C1 methyl-group of B-type fumonisins is missing, leading to a shorter carbon backbone. The P-series is structurally identical to members of the B series except containing a 3-hydroxypyridinium functional group in place of the C-2 amine group. A more recent addition are esterified fumonisin B derivatives (EFBs), where long chain fatty acids, such as palmitic acid, form esters with hydroxyl-groups in the backbone [215,216]. In some strains these by-products have been reported to be present in culture material at levels as high as 30 % of the level of FB1 [217]. Yet, normally FB1 accounts for 70–80 % of the total fumonisins produced in culture, while FB2 usually makes up 15–25 %, plus still lower amounts of FB_3. Likewise other types make up less than 5 % in naturally contaminated corn samples, and therefore only the B-series fumonisins are of toxicological concern. A tolerable daily intake (TDI) for FB1, FB2, FB3, alone or in combination of 2 µg/kg body weight was established [218]. The highly increased incidence rate of human esophageal cancer in South Africa triggered research leading to the identification of fumonisins, followed by monitoring of the natural occurrence and investigation of fumonisin producing fungi (reviewed by [219,220]). Also the history of massive veterinary problems, such as equine leukoencephalomalacia in the USA (killing an estimated 5000 horses in Illinois) and swine pulmonary edema (killing more than 1100 swine in Iowa), most likely due to the contamination of corn with fumonisin, was summarized in these reviews. The toxicological properties of fumonisins were extensively reviewed [3]. The International Agency for Research on Cancer came to the conclusion that there is sufficient evidence in experimental animals for the carcinogenicity of FB1, but that still inadequate evidence in humans for the carcinogenicity exists [221]. FB1 was, therefore, classified as possibly carcinogenic to humans (Group 2B). It was also concluded that the greatest risk occurs in regions where maize products are the dietary staple. Fumonisin is non-genotoxic and acts primarily via inhibition of ceramide biosynthesis. Fumonisins are structurally similar to sphinganine, and inhibit dihydroceramide synthase [222]. The perturbation of sphingolipids directly impacts the membrane properties and consequently on the function of certain membrane proteins. The high risk of neuronal tube defects due to consumption of fumonisin-contaminated food, such as maize tortillas, has been attributed to dysfunctional folate receptors [223]. Besides the structural role in membranes, sphingolipids also have important functions as signaling molecules. Work in the model system *Saccharomyces cerevisiae* has revealed that they play an important role in response to stresses such as heat shock, osmotic stress, and in cell wall integrity signaling [224].

Fumonisin not only triggers cell death in mammalian cells, but also has effects in plant cells. *A. thaliana* has been used to elucidate some of the mechanisms. Production of reactive oxygen species, deposition of phenolic compounds and callose, accumulation of phytoalexin, and expression of pathogenesis-related (*PR*) genes are triggered by low concentrations of FB1 [225]. It was demonstrated that FB1 induces apoptosis-like programmed cell death in wild-type *Arabidopsis* protoplasts. However, it only marginally affects the viability of protoplasts from transgenic *NahG* plants, in which salicylic acid (SA) is metabolically degraded, from *jar1-1* mutants, which are insensitive to jasmonate, and from *etr1-1*, ethylene insensitive receptor

mutants [226]. A gene encoding a plasma membrane localized RING motif protein (*RING1*) was found to be transcriptionally upregulated by FB1 treatment. Knockdown of *RING1* caused increased FB1 resistance, while overexpression increased FB1 sensitivity, suggesting that the RING1 protein is involved in transmitting a signal from the membrane to induce the cell death program [227]. It was also discovered that FB1 is connected to extracellular ATP, which is a signaling molecule in plants. FB1 treatment of *Arabidopsis* triggered the depletion of extracellular ATP that preceded cell death, and exogenous ATP rescued *Arabidopsis* from FB1-induced cell death [228]. While hypersensitive cell death is often associated with effector-triggered immunity, it does not occur in PTI. It was recently shown that FB1 treatment activated the MAP kinases MPK3 and MPK6, but that this activation and the cell death triggered by fumonisin could be suppressed by treatment with the flg22 peptide [229]. FB1-induced programmed cell death was abolished in an *Arabidopsis* vacuolar processing enzyme (VPE) mutant lacking all four *VPE* genes in the genome [230]. The VPE seems to function as caspase in plants executing cell death [231]. The initial signal is derived from the disturbance of sphingolipid homeostasis. The first step of sphingolipid synthesis, performed by the enzyme serine palmitoyltransferase, is immediately upstream of the fumonisin inhibited step. Small subunits of the serine palmitoyltransferase, which stimulate enzyme activity, were recently also discovered in plants. Overproduction of the small subunit led to higher serine palmitoyltransferase activity and increased sensitivity to FB1, while downregulation of expression by RNA-interference reduced FB1 sensitivity [232]. The phytotoxicity of fumonisin and the responses in plants suggested that fumonisin production of the corn pathogenic *Fusarium* should be relevant for their virulence.

The first results showed a correlation between virulence in a seedling assay and fumonisin production, but genetic analysis revealed that rare progeny could be obtained from crosses that had high virulence but were lacking fumonisin production [233]. Likewise, naturally occurring fumonisin nonproducing strains were used to inoculate maize silk channels, and were fully capable of causing ear rot [234]. Rigorous testing was performed after identification of the *FUM* genes. Fumonisin-nonproducing mutants were generated by gene disruption of the polyketide synthase gene (*FUM1*) required for fumonisin biosynthesis. Two independent transformants were tested for 2 years in the field in two locations by silk-channel injection, by spraying on maize silks, injection into maize stalks, and application with maize seeds at planting. The results obtained showed that they caused ear and stalk rot similar to their fumonisin-producing progenitor strain regardless of the application method. It was, therefore, concluded that fumonisins are not required for virulence [235].

A different conclusion was reached when a naturally occurring strain of *F. verticillioides* virulent in banana but lacking *FUM* genes was investigated. This strain did not show symptoms—compared to a fumonisin-producing maize isolate that causes leaf lesions, developmental abnormalities, stunting, and sometimes death—on maize seedlings. When the fumonisin cluster was introduced into the banana strain, fumonisin production and virulence on maize seedling was gained. The authors also inactivated the *FUM1* gene in their corn isolate, and reported that the

gene was required to cause foliar symptoms on the seedlings [236]. Crosses were performed between a nonpathogenic stain lacking fumonisin production and a producing strain. It was observed that 13/13 progeny deficient in fumonisin production were nonpathogenic. The authors also found in the remaining progeny a correlation between the amount of fumonisin produced and seedling disease incidence ($R^2 = 0.66$) [236].

To reconcile these results one has to consider that different fungal isolates and different host plants were used and also different bioassays (seedling/ear infection) with different inoculum strengths were employed. The most important difference could be that corn-adapted strains may have a redundant set of virulence factors, making fumonisin dispensable, while the banana isolate was only missing fumonisin production to overcome plant defense and gain basic compatibility on corn. It should also be considered that the responses described for *Arabidopsis* occur between 1–10 µM, while some maize lines showed high-level resistance to fumonisin in a root growth assay (50 % inhibition at concentrations higher than 200 µM), other corn accessions were inhibited in root growth by 5 µM [237]. The molecular basis for the heritable difference is uncharacterized. Currently nothing is known about plant metabolites of fumonisins, although indirect evidence exists that a significant portion of bound fumonisin can be released by alkaline hydrolysis from plant matrix [238,239].

Conclusion

In summary, the task to prove a virulence function of a particular secondary metabolite, present in a background of a currently still largely unknown cocktail of small molecules, will be difficult. Methods for repeated gene disruptions in a single strain are needed to cope with the problem of redundancy. The hope that large effects are observed by single gene disruptions should be replaced by more realistic expectations. Results may also be strongly dependent on the plant cultivar used. A task for the future is the development of techniques allowing a more reliable quantification of disease symptoms and detection of small differences in virulence.

References

1. Jorgensen K (2005) Occurrence of ochratoxin A in commodities and processed food—a review of EU occurrence data. Food Addit Contam 22(Suppl 1):26–30
2. EFSA Panel on Contaminants in the Food Chain (CONTAM) (2004) Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to deoxynivalenol (DON) as undesirable substance in animal feed. EFSA J 73:1–42
3. EFSA Panel on Contaminants in the Food Chain (CONTAM) (2005) Opinion of the scientific panel on contaminants in food chain on a request from the commission related to fumonisins as undesirable substances in animal feed. EFSA J 235:1–32

4. EFSA Panel on Contaminants in the Food Chain (CONTAM) (2011) Scientific opinion on the risks for public health related to the presence of zearalenone in food. EFSA J 9(6):1–124
5. Rohlfs M, Churchill AC (2011) Fungal secondary metabolites as modulators of interactions with insects and other arthropods. Fungal Genet Biol 48(1):23–34
6. Mobius N, Hertweck C (2009) Fungal phytotoxins as mediators of virulence. Curr Opin Plant Biol 12(4):390–398
7. Yoder O (1980) Toxins in pathogenesis. Annu Rev Phytopathol 18:103–129
8. Graniti A (1991) Phytotoxins and their involvement in plant diseases (Introduction). Experientia 47(8):751–755
9. Gilbert J, Abramson D, McCallum B, Clear R (2002) Comparison of canadian *Fusarium graminearum* isolates for aggressiveness, vegetative compatibility, and production of ergosterol and mycotoxins. Mycopathologia 153(4):209–215
10. Walton JD, Panaccione DG (1993) Host-selective toxins and disease specificity: perspectives and progress. Annu Rev Phytopathol 31:275–303
11. Tsuge T, Harimoto Y, Akimitsu K, Ohtani K, Kodama M, Akagi Y et al (2013) Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*. FEMS Microbiol Rev 37(1):44–66
12. Panaccione DG, Scott-Craig JS, Pocard JA, Walton JD (1992) A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize. Proc Natl Acad Sci U S A 89(14):6590–6594
13. Johal GS, Briggs SP (1992) Reductase activity encoded by the HM1 disease resistance gene in maize. Science (New York NY) 258(5084):985–987
14. Mehrabi R, Bahkali AH, Abd-Elsalam KA, Moslem M, Ben M'barek S, Gohari AM et al (2011) Horizontal gene and chromosome transfer in plant pathogenic fungi affecting host range. FEMS Microbiol Rev 35(3):542–554
15. Turgeon BG, Baker SE (2007) Genetic and genomic dissection of the *Cochliobolus heterostrophus* Tox1 locus controlling biosynthesis of the polyketide virulence factor T-toxin. Adv Genet 57:219–261
16. Multani DS, Meeley RB, Paterson AH, Gray J, Briggs SP, Johal GS (1998) Plant-pathogen microevolution: molecular basis for the origin of a fungal disease in maize. Proc Natl Acad Sci U S A 95(4):1686–1691
17. Sindhu A, Chintamanani S, Brandt AS, Zanis M, Scofield SR, Johal GS (2008) A guardian of grasses: specific origin and conservation of a unique disease-resistance gene in the grass lineage. Proc Natl Acad Sci U S A 105(5):1762–1767
18. Brar HK, Swaminathan S, Bhattacharyya MK (2011) The *Fusarium virguliforme* toxin FvTox1 causes foliar sudden death syndrome-like symptoms in soybean. Mol Plant Microbe Interact 24(10):1179–1188
19. Buerstmayr H, Ban T, Anderson JA (2009) Qtl mapping and marker-assisted selection for *Fusarium* head blight resistance in wheat: a review. Plant Breed 128(1):1–26
20. Dodds PN, Rathjen JP (2010) Plant immunity: towards an integrated view of plant–pathogen interactions. Nat Rev Genet 11(8):539–548. ([10.1038/nrg2812]. 2010 08//print)
21. Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60:379–406
22. Granado J, Felix G, Boller T (1995) Perception of fungal sterols in plants (subnanomolar concentrations of ergosterol elicit extracellular alkalinization in tomato cells). Plant Physiol 107(2):485–490
23. Tugizimana F, Steenkamp PA, Piater LA, Dubery IA (2014) Multi-platform metabolomic analyses of ergosterol-induced dynamic changes in *Nicotiana tabacum* cells. PloS ONE 9(1):e87846
24. Haas H, Eisendle M, Turgeon BG (2008) Siderophores in fungal physiology and virulence. Annu Rev Phytopathol 46:149–187
25. Haselwandter K, Winkelmann G (2007) Siderophores of symbiotic fungi. In: Varma A, Chincholkar S (eds) Microbial siderophores. Springer, Berlin, pp 91–104

26. Emery T (1980) Malonichrome, a new iron chelate from *Fusarium roseum*. Biochim Biophys Acta 629(2):382–390
27. Oide S, Berthiller F, Wiesenberger G, Adam G, Turgeon BG (2014) Individual and combined roles of malonichrome, ferricrocin, and TAFC siderophores in Fusarium graminearum pathogenic and sexual development. Front Microbiol 5:759. http://www.ncbi.nlm.nih.gov/pubmed/25628608.
28. Giraldo MC, Dagdas YF, Gupta YK, Mentlak TA, Yi M, Martinez-Rocha AL et al (2013) Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. Nat Commun 4:1996
29. de Jonge R, Bolton MD, Thomma BP (2011) How filamentous pathogens co-opt plants: the ins and outs of fungal effectors. Curr Opin Plant Biol 14(4):400–406
30. Kale SD, Gu B, Capelluto DG, Dou D, Feldman E, Rumore A et al (2010) External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell 142(2):284–295
31. Wawra S, Djamei A, Albert I, Nurnberger T, Kahmann R, van West P (2013) In vitro translocation experiments with RxLR-reporter fusion proteins of Avr1b from *Phytophthora sojae* and AVR3a from *Phytophthora infestans* fail to demonstrate specific autonomous uptake in plant and animal cells. Mol Plant Microbe Interact 26(5):528–536
32. Tyler BM, Kale SD, Wang Q, Tao K, Clark HR, Drews K et al (2013) Microbe-independent entry of oomycete RxLR effectors and fungal RxLR-like effectors into plant and animal cells is specific and reproducible. Mol Plant Microbe Interact 26(6):611–616
33. Nimchuk Z, Rohmer L, Chang JH, Dangl JL (2001) Knowing the dancer from the dance: R-gene products and their interactions with other proteins from host and pathogen. Curr Opin Plant Biol 4(4):288–294
34. Martin GB, Bogdanove AJ, Sessa G (2003) Understanding the functions of plant disease resistance proteins. Annu Rev Plant Biol 54:23–61
35. Tan S, Wu S (2012) Genome wide analysis of nucleotide-binding site disease resistance genes in *Brachypodium distachyon*. Comp Funct Genomics 2012:418208
36. van der Hoorn RA, Kamoun S (2008) From guard to decoy: a new model for perception of plant pathogen effectors. Plant Cell 20(8):2009–2017
37. Weiberg A, Wang M, Lin FM, Zhao H, Zhang Z, Kaloshian I et al (2013) Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. Science (New York) 342(6154):118–123
38. Stower H (2013) Small RNAs: RNAs attack! Nat Rev Genet 14(11):748–749
39. Geng X, Cheng J, Gangadharan A, Mackey D (2012) The coronatine toxin of *Pseudomonas syringae* is a multifunctional suppressor of *Arabidopsis* defense. Plant Cell 24(11):4763–4774
40. Dudler R (2013) Manipulation of host proteasomes as a virulence mechanism of plant pathogens. Annu Rev Phytopathol 51:521–542
41. Waspi U, Schweizer P, Dudler R (2001) Syringolin reprograms wheat to undergo hypersensitive cell death in a compatible interaction with powdery mildew. Plant Cell 13(1):153–161
42. Atkinson MM, Midland SL, Sims JJ, Keen NT (1996) Syringolide 1 triggers Ca2+ influx, K+ efflux, and extracellular alkalization in soybean cells carrying the disease-resistance gene Rpg4. Plant Physiol 112(1):297–302
43. Bohnert HU, Fudal I, Dioh W, Tharreau D, Notteghem JL, Lebrun MH (2004) A putative polyketide synthase/peptide synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice. Plant Cell 16(9):2499–2513
44. Collemare J, Pianfetti M, Houlle AE, Morin D, Camborde L, Gagey MJ et al (2008) *Magnaporthe grisea* avirulence gene ACE1 belongs to an infection-specific gene cluster involved in secondary metabolism. New Phytol 179(1):196–208
45. Vergne E, Ballini E, Marques S, Sidi Mammar B, Droc G, Gaillard S et al (2007) Early and specific gene expression triggered by rice resistance gene Pi33 in response to infection by ACE1 avirulent blast fungus. New Phytol 174(1):159–171

46. Lorang JM, Sweat TA, Wolpert TJ (2007) Plant disease susceptibility conferred by a "resistance" gene. Proc Natl Acad Sci U S A 104(37):14861–14866
47. Lorang J, Kidarsa T, Bradford CS, Gilbert B, Curtis M, Tzeng SC et al (2012) Tricking the guard: exploiting plant defense for disease susceptibility. Science (New York) 338(6107):659–662
48. Macko V, Stimmel MB, Wolpert TJ, Dunkle LD, Acklin W, Banteli R et al (1992) Structure of the host-specific toxins produced by the fungal pathogen *Periconia circinata*. Proc Natl Acad Sci U S A 89(20):9574–9578
49. Nagy ED, Bennetzen JL (2008) Pathogen corruption and site-directed recombination at a plant disease resistance gene cluster. Genome Res 18(12):1918–1923
50. Serrano M, Hubert DA, Dangl JL, Schulze-Lefert P, Kombrink E (2010) A chemical screen for suppressors of the avrRpm1-RPM1-dependent hypersensitive cell death response in *Arabidopsis thaliana*. Planta 231(5):1013–1023
51. Veening JW, Smits WK, Kuipers OP (2008) Bistability, epigenetics, and bet-hedging in bacteria. Annu Rev Microbiol 62:193–210
52. Connolly LR, Smith KM, Freitag M (2013) The *Fusarium graminearum* histone H3 K27 methyltransferase KMT6 regulates development and expression of secondary metabolite gene clusters. PLoS Genet 9(10):e1003916
53. Lee H, Rustgi S, Kumar N, Burke I, Yenish JP, Gill KS et al (2011) Single nucleotide mutation in the barley acetohydroxy acid synthase (AHAS) gene confers resistance to imidazolinone herbicides. Proc Natl Acad Sci U S A 108(21):8909–8913
54. Akamatsu H, Itoh Y, Kodama M, Otani H, Kohmoto K (1997) AAL-toxin-deficient mutants of *alternaria alternata* tomato pathotype by restriction enzyme-mediated integration. Phytopathology 87(9):967–972
55. Egusa M, Miwa T, Kaminaka H, Takano Y, Kodama M (2013) Nonhost resistance of *Arabidopsis thaliana* against *Alternaria alternata* involves both pre- and postinvasive defenses but is collapsed by AAL-toxin in the absence of LOH2. Phytopathology 103(7):733–740
56. Abbas HK, Duke SO, Shier WT, Riley RT, Kraus GA (1996) The chemistry and biological activities of the natural products AAL-toxin and the fumonisins. Adv Exp Med Biol 391:293–308
57. Desjardins AE, Proctor RH (2007) Molecular biology of *Fusarium* mycotoxins. Int J Food Microbiol 119(1–2):47–50
58. Yuan JS, Tranel PJ, Stewart CN Jr (2007) Non-target-site herbicide resistance: a family business. Trends Plant Sci 12(1):6–13
59. Remy E, Duque P (2014) Beyond cellular detoxification: a plethora of physiological roles for MDR transporter homologs in plants. Front Physiol 5:201
60. Bowles D, Lim EK, Poppenberger B, Vaistij FE (2006) Glycosyltransferases of lipophilic small molecules. Annu Rev Plant Biol 57:567–597
61. Dixon DP, Lapthorn A, Edwards R (2002) Plant glutathione transferases. Genome Biol 3(3):Reviews 3004
62. Taguchi G, Ubukata T, Nozue H, Kobayashi Y, Takahi M, Yamamoto H et al (2010) Malonylation is a key reaction in the metabolism of xenobiotic phenolic glucosides in *Arabidopsis* and tobacco. Plant J 63(6):1031–1041
63. Brazier-Hicks M, Evans KM, Cunningham OD, Hodgson DR, Steel PG, Edwards R (2008) Catabolism of glutathione conjugates in *Arabidopsis thaliana*. Role in metabolic reactivation of the herbicide safener fenclorim. J Biol Chem 283(30):21102–21112
64. Gaffoor I, Brown DW, Plattner R, Proctor RH, Qi W, Trail F (2005) Functional analysis of the polyketide synthase genes in the filamentous fungus *Gibberella zeae* (anamorph *Fusarium graminearum*). Eukaryot Cell 4(11):1926–1933
65. Oide S, Moeder W, Krasnoff S, Gibson D, Haas H, Yoshioka K et al (2006) NPS6, encoding a nonribosomal peptide synthetase involved in siderophore-mediated iron metabolism, is a conserved virulence determinant of plant pathogenic ascomycetes. Plant Cell 18(10):2836–2853

66. Dalmais B, Schumacher J, Moraga J, LE Pechêur P, Tudzynski B, Collado IG et al (2011) The *Botrytis cinerea* phytotoxin botcinic acid requires two polyketide synthases for production and has a redundant role in virulence with botrydial. Mol Plant Pathol 12(6):564–579
67. Tobiasen C, Aahman J, Ravnholt KS, Bjerrum MJ, Grell MN, Giese H (2007) Nonribosomal peptide synthetase (NPS) genes in *Fusarium graminearum*, *F. culmorum* and *F. pseudograminearium* and identification of NPS2 as the producer of ferricrocin. Curr Genet 51(1):43–58
68. Lysoe E, Seong KY, Kistler HC (2011) The transcriptome of *Fusarium graminearum* during the infection of wheat. Mol Plant Microbe Interact 24(9):995–1000
69. Woloshuk CP, Shim WB (2013) Aflatoxins, fumonisins, and trichothecenes: a convergence of knowledge. FEMS Microbiol Rev 37(1):94–109
70. Hertweck C (2009) The biosynthetic logic of polyketide diversity. Angew Chem 48(26):4688–4716. (International ed in English)
71. Hur GH, Vickery CR, Burkart MD (2012) Explorations of catalytic domains in non-ribosomal peptide synthetase enzymology. Nat Prod Rep 29(10):1074–1098
72. Boettger D, Hertweck C (2013) Molecular diversity sculpted by fungal PKS-NRPS hybrids. Chembiochem Eur J Chem Biol 14(1):28–42
73. Wawrzyn GT, Bloch SE, Schmidt-Dannert C (2012) Discovery and characterization of terpenoid biosynthetic pathways of fungi. Methods Enzymol 515:83–105
74. Steffan N, Grundmann A, Yin WB, Kremer A, Li SM (2009) Indole prenyltransferases from fungi: a new enzyme group with high potential for the production of prenylated indole derivatives. Curr Med Chem 16(2):218–231
75. Khaldi N, Seifuddin FT, Turner G, Haft D, Nierman WC, Wolfe KH et al (2010) SMURF: genomic mapping of fungal secondary metabolite clusters. Fungal Genet Biol 47(9):736–741
76. Dionisio PM, Gurudu SR, Leighton JA, Leontiadis GI, Fleischer DE, Hara AK et al (2010) Capsule endoscopy has a significantly higher diagnostic yield in patients with suspected and established small-bowel Crohn's disease: a meta-analysis. Am J Gastroenterol 105(6):1240–1248 (quiz 1249)
77. Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E et al (2013) Antismash 2.0—a versatile platform for genome mining of secondary metabolite producers. Nucleic Acids Res 41(Web Server issue):W204–212
78. Fedorova ND, Moktali V, Medema MH (2012) Bioinformatics approaches and software for detection of secondary metabolic gene clusters. Methods Mol Biol 944:23–45
79. Wiemann P, Sieber CM, von Bargen KW, Studt L, Niehaus EM, Espino JJ et al (2013) Deciphering the cryptic genome: genome-wide analyses of the rice pathogen *Fusarium fujikuroi* reveal complex regulation of secondary metabolism and novel metabolites. PLoS Pathog 9(6):e1003475
80. Harris LJ, Alexander NJ, Saparno A, Blackwell B, McCormick SP, Desjardins AE et al (2007) A novel gene cluster in *Fusarium graminearum* contains a gene that contributes to butenolide synthesis. Fungal Genet Biol 44(4):293–306
81. Sieber CMK, Wong P, Münsterkötter M, Mewes H-W, Schmeitzl C, Varga E, Berthiller F, Adam G, Güldener U (2014) The *Fusarium graminearum* genome reveals more secondary metabolite gene clusters and hints of horizontal gene transfer. PloS ONE 9:e110311
82. Proctor RH, Butchko RA, Brown DW, Moretti A (2007) Functional characterization, sequence comparisons and distribution of a polyketide synthase gene required for perithecial pigmentation in some *Fusarium* species. Food Addit Contam 24(10):1076–1087
83. Hansen FT, Droce A, Sorensen JL, Fojan P, Giese H, Sondergaard TE (2012) Overexpression of NRPS4 leads to increased surface hydrophobicity in *Fusarium graminearum*. Fungal Biol 116(8):855–862
84. Pestka JJ (2010) Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Arch Toxicol 84(9):663–679
85. Desmond OJ, Manners JM, Stephens AE, Maclean DJ, Schenk PM, Gardiner DM et al (2008) The *Fusarium mycotoxin* deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat. Mol Plant Pathol 9(4):435–445

86. Arunachalam C, Doohan FM (2013) Trichothecene toxicity in eukaryotes: cellular and molecular mechanisms in plants and animals. Toxicol Lett 217(2):149–158
87. Diamond M, Reape TJ, Rocha O, Doyle SM, Kacprzyk J, Doohan FM et al (2013) The *Fusarium* mycotoxin deoxynivalenol can inhibit plant apoptosis-like programmed cell death. PloS ONE 8(7):e69542
88. McCormick S (2003) The role of don in pathogenicity. In: Leonhard KJ, Bushnell WR (eds) Fusarium head blight of wheat and barley. American Phytopathological Society, St. Paul, pp 165–183
89. McCormick SP, Stanley AM, Stover NA, Alexander NJ (2011) Trichothecenes: from simple to complex mycotoxins. Toxins 3(7):802–814
90. Proctor RH, Hohn TM, McCormick SP (1995) Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. Mol Plant Microbe Interact 8(4):593–601
91. Bai GH, Desjardins AE, Plattner RD (2002) Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. Mycopathologia 153(2):91–98
92. Jansen C, von Wettstein D, Schafer W, Kogel KH, Felk A, Maier FJ (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. Proc Natl Acad Sci U S A 102(46):16892–16897
93. Eudes F, Comeau A, Rioux S, Collin J (2001) Impact of trichothecenes on fusarium head blight [*Fusarium graminearum*] development in spring wheat *(Triticum aestivum)*. Can J Plant Pathol 23(3):318–322
94. Langevin F, Eudes F, Comeau A (2004) Effect of trichothecenes produced by *Fusarium graminearum* during fusarium head blight development in six cereal species. Eur J Plant Pathol 110(7):735–746
95. Liu S, Anderson JA (2003) Targeted molecular mapping of a major wheat QTL for *Fusarium* head blight resistance using wheat ESTs and synteny with rice. Genome 46(5):817–823
96. Buerstmayr H, Steiner B, Hartl L, Griesser M, Angerer N, Lengauer D et al (2003) Molecular mapping of QTLs for fusarium head blight resistance in spring wheat. II. Resistance to fungal penetration and spread. TAG Theor Appl Genet (Theoretische und angewandte Genetik) 107(3):503–508
97. Lemmens M, Scholz U, Berthiller F, Dall'Asta C, Koutnik A, Schuhmacher R et al (2005) The ability to detoxify the mycotoxin deoxynivalenol colocalizes with a major quantitative trait locus for fusarium head blight resistance in wheat. Mol Plant Microbe Interact 18(12):1318–1324
98. Horevaj P, Brown-Guedira G, Milus EA (2012) Resistance in winter wheat lines to deoxynivalenol applied into florets at flowering stage and tolerance to phytotoxic effects on yield. Plant Pathol 61(5):925–933
99. Poppenberger B, Berthiller F, Lucyshyn D, Sieberer T, Schuhmacher R, Krska R et al (2003) Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a udp-glucosyltransferase from *Arabidopsis thaliana*. J Biol Chem 278(48):47905–47914
100. Gardiner SA, Boddu J, Berthiller F, Hametner C, Stupar RM, Adam G et al (2010) Transcriptome analysis of the barley-deoxynivalenol interaction: evidence for a role of glutathione in deoxynivalenol detoxification. Mol Plant Microbe Interact 23(7):962–976
101. Schweiger W, Boddu J, Shin S, Poppenberger B, Berthiller F, Lemmens M et al (2010) Validation of a candidate deoxynivalenol-inactivating UDP-glucosyltransferase from barley by heterologous expression in yeast. Mol Plant Microbe Interact 23(7):977–986
102. Muehlbauer GJ, Shin S, Li X, Boddu J, Heinen S, Torres Acosta JA et al (2012) Developing *Fusarium* head blight resistant wheat. In: Canty S, Clark A, Anderson-Scully A, Van Sanford D (eds) Proceedings of the national *Fusarium* head blight forum, 2012 December 4–6, Orlando, Florida, USA. East Lansing, MI/Lexington, KY, USA: U.S. Wheat & Barley Scab Initiative, p 144
103. Boenisch MJ, Schafer W (2011) *Fusarium graminearum* forms mycotoxin producing infection structures on wheat. BMC Plant Biol 11:110

104. Hallen-Adams HE, Wenner N, Kuldau GA, Trail F (2011) Deoxynivalenol biosynthesis-related gene expression during wheat kernel colonization by *Fusarium graminearum*. Phytopathology 101(9):1091–1096
105. Fruhmann P, Weigl-Pollack T, Mikula H, Wiesenberger G, Adam G, Varga E et al (2014) Methylthiodeoxynivalenol (MTD): insight into the chemistry, structure and toxicity of thia-michael adducts of trichothecenes. Org Biomol Chem 12(28):5144–5150
106. Savard ME, Sinha RC, Lloyd Seaman W, Fedak G (2000) Sequential distribution of the mycotoxin deoxynivalenol in wheat spikes after inoculation with fusarium graminearum. Can J Plant Pathol 22(3):280–285
107. Gunnaiah R, Kushalappa AC, Duggavathi R, Fox S, Somers DJ (2012) Integrated metabolo-proteomic approach to decipher the mechanisms by which wheat QTL (Fhb1) contributes to resistance against *Fusarium graminearum*. PloS ONE 7(7):e40695
108. Grove JF (2007) The trichothecenes and their biosynthesis. Fortschr Chem Org Naturst 88:63–130
109. Desjardins AE (2006) *Fusarium* mycotoxins: chemistry, genetics and biology. (Chapter 1: Trichothecenes). American Phytopathological Society, St. Paul
110. Lee T, Han YK, Kim KH, Yun SH, Lee YW (2002) Tri13 and Tri7 determine deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae*. Appl Environ Microbiol 68(5):2148–2154
111. Alexander NJ, McCormick SP, Waalwijk C, van der Lee T, Proctor RH (2011) The genetic basis for 3-ADON and 15-ADON trichothecene chemotypes in *Fusarium*. Fungal Genet Biol 48(5):485–495
112. Kimura M, Kaneko I, Komiyama M, Takatsuki A, Koshino H, Yoneyama K et al (1998) Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. J Biol Chem 273(3):1654–1661
113. Alexander NJ, McCormick SP, Ziegenhorn SL (1999) Phytotoxicity of selected trichothecenes using *Chlamydomonas reinhardtii* as a model systemt. Nat Toxins 7(6):265–269
114. Ward TJ, Clear RM, Rooney AP, O'Donnell K, Gaba D, Patrick S et al (2008) An adaptive evolutionary shift in *Fusarium* head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. Fungal Genet Biol 45(4):473–484
115. McMullen M, Jones R, Gallenberg D (1997) Scab of wheat and barley: a re-emerging disease of devastating impact. Plant Dis 81(12):1340–1348
116. Ward TJ, Bielawski JP, Kistler HC, Sullivan E, O'Donnell K (2002) Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. Proc Natl Acad Sci U S A 99(14):9278–9283
117. Desjardins AE, McCormick SP, Appell M (2007) Structure-activity relationships of trichothecene toxins in an *Arabidopsis thaliana* leaf assay. J Agric Food Chem 55(16):6487–6492
118. Hedman R, Pettersson H, Lindberg JE (1997) Absorption and metabolism of nivalenol in pigs. Arch Tierernahr 50(1):13–24
119. Schweiger W, Pasquet JC, Nussbaumer T, Paris MP, Wiesenberger G, Macadre C et al (2013) Functional characterization of two clusters of *Brachypodium distachyon* UDP-glycosyltransferases encoding putative deoxynivalenol detoxification genes. Mol Plant Microbe Interact 26(7):781–792
120. Caputi L, Malnoy M, Goremykin V, Nikiforova S, Martens S (2012) A genome-wide phylogenetic reconstruction of family 1 UDP-glycosyltransferases revealed the expansion of the family during the adaptation of plants to life on land. Plant J 69(6):1030–1042
121. Kluger B, Bueschl C, Lemmens M, Berthiller F, Haubl G, Jaunecker G et al (2013) Stable isotopic labelling-assisted untargeted metabolic profiling reveals novel conjugates of the mycotoxin deoxynivalenol in wheat. Anal Bioanal Chem 405(15):5031–5036
122. Varga E, Wiesenberger G, Hametner C, Ward TJ, Dong Y, Schofbeck D, McCormick S, Broz K, Stuckler R, Schuhmacher R, et al (2014) New tricks of an old enemy: isolates of *Fusarium graminearum* produce a type A trichothecene mycotoxin. Environmental microbiology. http://www.ncbi.nlm.nih.gov/pubmed/25403493

123. Edwards SG (2011) Zearalenone risk in European wheat. World Mycotoxin J 4(4):433–438
124. Kuiper-Goodman T, Scott PM, Watanabe H (1987) Risk assessment of the mycotoxin zearalenone. Regul Toxicol Pharmacol 7(3):253–306
125. Commission regulation (ec) no 1881/2006 (2012) Setting maximum levels for certain contaminants in foodstuff—consolidated version 03.12.2012. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2006R1881:20121203:EN:PDF. Accessed 15 April 2015
126. Kim YT, Lee YR, Jin J, Han KH, Kim H, Kim JC et al (2005) Two different polyketide synthase genes are required for synthesis of zearalenone in *Gibberella zeae*. Mol Microbiol 58(4):1102–1113
127. Gaffoor I, Trail F (2006) Characterization of two polyketide synthase genes involved in zearalenone biosynthesis in *Gibberella zeae*. Appl Environ Microbiol 72(3):1793–1799
128. Lysoe E, Klemsdal SS, Bone KR, Frandsen RJ, Johansen T, Thrane U et al (2006) The PKS4 gene of *Fusarium graminearum* is essential for zearalenone production. Appl Environ Microbiol 72(6):3924–3932
129. Werner U (2005) Characterisation of the effect of the *Fusarium* mycotoxin zearalenone in *Arabidopsis thaliana*. Ph.D. thesis, BOKU-Universität für Bodenkultur
130. Berthiller F, Werner U, Sulyok M, Krska R, Hauser MT, Schuhmacher R (2006) Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) determination of phase II metabolites of the mycotoxin zearalenone in the model plant *Arabidopsis thaliana*. Food Addit Contam 23(11):1194–1200
131. Metzler M (2011) Proposal for a uniform designation of zearalenone and its metabolites. Mycotoxin Res 27(1):1–3
132. Kovalsky Paris MP, Schweiger W, Hametner C, Stuckler R, Muehlbauer GJ, Varga E et al (2014) Zearalenone-16-O-glucoside: a new masked mycotoxin. J Agric Food Chem 62(5):1181–1189
133. Shirasu K (2009) The HSP90-SGT1 chaperone complex for NLR immune sensors. Annu Rev Plant Biol 60:139–164
134. Kadota Y, Shirasu K (2012) The HSP90 complex of plants. Biochim Biophys Acta 1823(3):689–697
135. de la Fuente van Benten S, Vossen JH, de Vries KJ, van Wees S, Tameling WI, Dekker HL et al (2005) Heat shock protein 90 and its co-chaperone protein phosphatase 5 interact with distinct regions of the tomato I-2 disease resistance protein. Plant J 43(2):284–298
136. Utermark J, Karlovsky P (2007) Role of zearalenone lactonase in protection of *Gliocladium roseum* from fungitoxic effects of the mycotoxin zearalenone. Appl Environ Microbiol 73(2):637–642
137. Malz S, Grell MN, Thrane C, Maier FJ, Rosager P, Felk A et al (2005) Identification of a gene cluster responsible for the biosynthesis of aurofusarin in the *Fusarium graminearum* species complex. Fungal Genet Biol 42(5):420–433
138. Frandsen RJ, Nielsen NJ, Maolanon N, Sorensen JC, Olsson S, Nielsen J et al (2006) The biosynthetic pathway for aurofusarin in *Fusarium graminearum* reveals a close link between the naphthoquinones and naphthopyrones. Mol Microbiol 61(4):1069–1080
139. Frandsen RJ, Schutt C, Lund BW, Staerk D, Nielsen J, Olsson S et al (2011) Two novel classes of enzymes are required for the biosynthesis of aurofusarin in *Fusarium graminearum*. J Biol Chem 286(12):10419–10428
140. Rugbjerg P, Naesby M, Mortensen UH, Frandsen RJ (2013) Reconstruction of the biosynthetic pathway for the core fungal polyketide scaffold rubrofusarin in *Saccharomyces cerevisiae*. Microbe Cell Fact 12(1):31
141. Frandsen RJ, Albertsen KS, Stougaard P, Sorensen JL, Nielsen KF, Olsson S et al (2010) Methylenetetrahydrofolate reductase activity is involved in the plasma membrane redox system required for pigment biosynthesis in filamentous fungi. Eukaryot Cell 9(8):1225–1235

142. Dvorska JE, Surai PF, Speake BK, Sparks NH (2001) Effect of the mycotoxin aurofusarin on the antioxidant composition and fatty acid profile of quail eggs. Br Poult Sci 42(5):643–649
143. Dvorska JE, Surai PF, Speake BK, Sparks NH (2002) Antioxidant systems of the developing quail embryo are compromised by mycotoxin aurofusarin. Comp Biochem Physiol C Toxicol Pharmacol 131(2):197–205
144. Streit E, Naehrer K, Rodrigues I, Schatzmayr G (2013) Mycotoxin occurrence in feed and feed raw materials worldwide: long-term analysis with special focus on Europe and Asia. J Sci Food Agric 93(12):2892–2899
145. Macias M, Ulloa M, Gamboa A, Mata R (2000) Phytotoxic compounds from the new coprophilous fungus *Guanomyces polythrix*. J Nat Prod 63(6):757–761
146. Grundlinger M, Yasmin S, Lechner BE, Geley S, Schrettl M, Hynes M et al (2013) Fungal siderophore biosynthesis is partially localized in peroxisomes. Mol Microbiol 88(5):862–875
147. Min K, Son H, Lee J, Choi GJ, Kim JC, Lee YW (2012) Peroxisome function is required for virulence and survival of *Fusarium graminearum*. Mol Plant Microbe Interact 25(12):1617–1627
148. Condon BJ, Oide S, Gibson DM, Krasnoff SB, Turgeon BG (2014) Reductive iron assimilation and intracellular siderophores assist extracellular siderophore-driven iron homeostasis and virulence. Mol Plant Microbe Interact 27(8):793–808
149. Hof C, Eisfeld K, Welzel K, Antelo L, Foster AJ, Anke H (2007) Ferricrocin synthesis in *Magnaporthe grisea* and its role in pathogenicity in rice. Mol Plant Pathol 8(2):163–172
150. Eichhorn H, Lessing F, Winterberg B, Schirawski J, Kamper J, Muller P et al (2006) A ferroxidation/permeation iron uptake system is required for virulence in *Ustilago maydis*. Plant Cell 18(11):3332–3345
151. Desjardins AE (2006) *Fusarium* mycotoxins: chemistry, genetics and biology. (Chapter 5: Other selected metabolites). American Phytopathological Society, St. Paul
152. Burmeister HR, Grove MD, Kwolek WF (1980) Moniliformin and butenolide: effect on mice of high-level, long-term oral intake. Appl Environ Microbiol 40(6):1142–1144
153. Tookey HL, Yates SG, Ellis JJ, Grove MD, Nichols RE (1972) Toxic effects of a butenolide mycotoxin and of *Fusarium tricinctum* cultures in cattle. J Am Vet Med Assoc 160(11):1522–1526
154. Burmeister HR, Hesseltine CW (1970) Biological assays for two mycotoxins produced by *Fusarium tricinctum*. Appl Microbiol 20(3):437–440
155. Wang YZ, Miller JD (1988) Effects of *Fusarium graminearum* metabolites on wheat tissue in relation to *Fusarium* head blight resistance. J Phytopathol 122(2):118–125
156. Streit E, Schwab C, Sulyok M, Naehrer K, Krska R, Schatzmayr G (2013) Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. Toxins 5(3):504–523
157. Wang YM, Peng SQ, Zhou Q, Wang MW, Yan CH, Yang HY et al (2006) Depletion of intracellular glutathione mediates butenolide-induced cytotoxicity in HepG2 cells. Toxicology Lett 164(3):231–238
158. Liu JB, Wang YM, Peng SQ, Han G, Dong YS, Yang HY et al (2007) Toxic effects of *Fusarium* mycotoxin butenolide on rat myocardium and primary culture of cardiac myocytes. Toxicon 50(3):357–364
159. Desjardins AE (2006) *Fusarium* mycotoxins: chemistry, genetics and biology. (Chapter 4: Other selected mycotoxins). American Phytopathological Society, St. Paul
160. Kleigrewe K, Aydin F, Hogrefe K, Piecuch P, Bergander K, Wurthwein EU et al (2012) Structure elucidation of new fusarins revealing insights in the rearrangement mechanisms of the *Fusarium* mycotoxin fusarin C. J Agric Food Chem 60(21):5497–5505
161. Niehaus EM, Kleigrewe K, Wiemann P, Studt L, Sieber CM, Connolly LR et al (2013) Genetic manipulation of the *Fusarium fujikuroi* fusarin gene cluster yields insight into the complex regulation and fusarin biosynthetic pathway. Chem Biol 20(8):1055–1066

162. Kleigrewe K, Sohnel AC, Humpf HU (2011) A new high-performance liquid chromatography-tandem mass spectrometry method based on dispersive solid phase extraction for the determination of the mycotoxin fusarin C in corn ears and processed corn samples. J Agric Food Chem 59(19):10470–10476
163. Kleigrewe K, Niehaus EM, Wiemann P, Tudzynski B, Humpf HU (2012) New approach via gene knockout and single-step chemical reaction for the synthesis of isotopically labeled Fusarin C as an internal standard for the analysis of this *Fusarium* mycotoxin in food and feed samples. J Agric Food Chem 60(34):8350–8355
164. Zhu B, Jeffrey AM (1992) Stability of Fusarin C: effects of the normal cooking procedure used in China and pH. Nutr Cancer 18(1):53–58
165. Sondergaard TE, Hansen FT, Purup S, Nielsen AK, Bonefeld-Jorgensen EC, Giese H et al (2011) Fusarin C acts like an estrogenic agonist and stimulates breast cancer cells *in vitro*. Toxicol Lett 205(2):116–121
166. Shier WT, Shier AC, Xie W, Mirocha CJ (2001) Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. Toxicon 39(9):1435–1438
167. Gelderblom WC, Thiel PG, van der Merwe KJ (1988) The chemical and enzymatic interaction of glutathione with the fungal metabolite, Fusarin C. Mutat Res 199(1):207–214
168. Parisy V, Poinssot B, Owsianowski L, Buchala A, Glazebrook J, Mauch F (2007) Identification of PAD2 as a gamma-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of *Arabidopsis*. Plant J 49(1):159–172
169. Dubreuil-Maurizi C, Poinssot B (2012) Role of glutathione in plant signaling under biotic stress. Plant Signal Behav 7(2):210–212
170. McCormick SP, Alexander NJ, Harris LJ (2010) CLM1 of *Fusarium graminearum* encodes a longiborneol synthase required for culmorin production. Appl Environ Microbiol 76(1):136–141
171. Ghebremeskel M, Langseth W (2001) The occurrence of culmorin and hydroxy-culmorins in cereals. Mycopathologia 152(2):103–108
172. Pedersen PB, Miller JD (1999) The fungal metabolite culmorin and related compounds. Nat Toxins 7(6):305–309
173. Sorensen JL, Hansen FT, Sondergaard TE, Staerk D, Lee TV, Wimmer R et al (2012) Production of novel fusarielins by ectopic activation of the polyketide synthase 9 cluster in *Fusarium graminearum*. Environ Microbiol 14(5):1159–1170
174. Sorensen JL, Akk E, Thrane U, Giese H, Sondergaard TE (2013) Production of fusarielins by *Fusarium*. Int J Food Microbiol 160(3):206–211
175. Sondergaard TE, Klitgaard LG, Purup S, Kobayashi H, Giese H, Sorensen JL (2012) Estrogenic effects of *fusarielins* in human breast cancer cell lines. Toxicol Lett 214(3):259–262
176. Kobayashi H, Sunaga R, Furihata K, Morisaki N, Iwasaki S (1995 Jan) Isolation and structures of an antifungal antibiotic, fusarielin A, and related compounds produced by a *Fusarium* sp. J Antibiot 48(1):42–52
177. Sirtori CR (2014) The pharmacology of statins. Pharmacol Res 88:3–11
178. Noguchi-Yachide T, Dodo K, Aoyama H, Fujimoto H, Hori M, Hashimoto Y et al (2010) Identification of binding proteins of fusarielin A as actin and tubulin. Chem Pharm Bull 58(1):129–134
179. Vershinin A (1999) Biological functions of carotenoids—diversity and evolution. Biofactors (Oxford, England) 10(2–3):99–104
180. Arrach N, Schmidhauser TJ, Avalos J (2002) Mutants of the carotene cyclase domain of al-2 from *Neurospora crassa*. Mol Genet Genomics 266(6):914–921
181. Linnemannstons P, Prado MM, Fernandez-Martin R, Tudzynski B, Avalos J (2002) A carotenoid biosynthesis gene cluster in *Fusarium fujikuroi*: the genes carb and carra. Mol Genet Genomics 267(5):593–602
182. Jin JM, Lee J, Lee YW (2010) Characterization of carotenoid biosynthetic genes in the ascomycete *Gibberella zeae*. FEMS Microbiol Lett 302(2):197–202
183. Walter MH, Strack D (2011) Carotenoids and their cleavage products: biosynthesis and functions. Nat Prod Rep 28(4):663–692

184. Cao FY, Yoshioka K, Desveaux D (2011) The roles of ABA in plant-pathogen interactions. J Plant Res 124(4):489–499
185. Torres-Vera R, Garcia JM, Pozo MJ, Lopez-Raez JA (2014) Do strigolactones contribute to plant defence? Mol Plant Pathol 15(2):211–216
186. Akiyama K, Hayashi H (2006) Strigolactones: chemical signals for fungal symbionts and parasitic weeds in plant roots. Ann Bot 97(6):925–931
187. Dor E, Joel DM, Kapulnik Y, Koltai H, Hershenhorn J (2011) The synthetic strigolactone GR24 influences the growth pattern of phytopathogenic fungi. Planta 234(2):419–427
188. Siewers V, Kokkelink L, Smedsgaard J, Tudzynski P (2006) Identification of an abscisic acid gene cluster in the grey mold *Botrytis cinerea*. Appl Environ Microbiol 72(7):4619–4626
189. Jorgensen SH, Frandsen RJ, Nielsen KF, Lysoe E, Sondergaard TE, Wimmer R et al (2014) *Fusarium graminearum* PKS14 is involved in orsellinic acid and orcinol synthesis. Fungal Genet Biol 70c:24–31
190. Bacon CW, Porter JK, Norred WP, Leslie JF (1996) Production of fusaric acid by *Fusarium* species. Appl Environ Microbiol 62(11):4039–4043
191. Smith TK, Sousadias MG (1993) Fusaric acid content of swine feedstuffs. J Agric Food Chem 41(12):2296–2298
192. Mogensen JM, Sorensen SM, Sulyok M, van der Westhuizen L, Shephard GS, Frisvad JC et al (2011) Single-kernel analysis of fumonisins and other fungal metabolites in maize from South African subsistence farmers. Food Addit Contam Part A. Chem Anal Control Expo Risk Assess 28(12):1724–1734
193. Wang H, Ng TB (1999) Pharmacological activities of fusaric acid (5-butylpicolinic acid). Life Sci 65(9):849–856
194. Rimando AM, Porter JK (1999) Effects of fusarium mycotoxins on levels of serotonin, melatonin, and 5-hydroxytryptophan in pineal cell cultures. Adv Exp Med Biol 467:425–431
195. Nagasaka A, Hara I, Imai Y, Uchikawa T, Yamauchi K, Suzuki S et al (1985) Effect of fusaric acid (a dopamine beta-hydroxylase inhibitor) on phaeochromocytoma. Clin Endocrinol (Oxf) 22(4):437–444
196. Yabuta T, Kambe K, Hayashi T (1937) Biochemistry of the bakanae fungus. I. Fusarinic acid, a new product of the bakanae fungus. J Agric Chem Soc Jpn 10:1059–1068
197. Venter SL, Steyn PJ (1998) Correlation between fusaric acid production and virulence of isolates of *Fusarium oxysporum* that causes potato dry rot in South Africa. Potato Res 41(3):289–294
198. Gapillout I, Milat ML, Blein JP (1996) Effects of fusaric acid on cells from tomato cultivars resistant or susceptible to *Fusarium oxysporum* f. sp. *lycopersici*. Eur J Plant Pathol 102(2):127–132
199. Kuo MS, Scheffer JM (1964) Evaluation of fusaric acid as a factor in development of *Fusarium* wilt. Phytopathology 54:1041–1044
200. Dong X, Ling N, Wang M, Shen Q, Guo S (2012) Fusaric acid is a crucial factor in the disturbance of leaf water imbalance in fusarium-infected banana plants. Plant Physiology Biochem 60:171–179
201. Bani M, Rispail N, Evidente A, Rubiales D, Cimmino A (2014) Identification of the main toxins isolated from *Fusarium oxysporum* f. sp. Pisi race 2 and their relation with isolates' pathogenicity. J Agric Food Chem 62(12):2574–2580
202. Jiao J, Zhou B, Zhu X, Gao Z, Liang Y (2013) Fusaric acid induction of programmed cell death modulated through nitric oxide signalling in tobacco suspension cells. Planta 238(4):727–737
203. Wilson DM, Kays SJ, Etherton B (1978) The relationship between pathogenic fungal metabolities (fusaric and picolinic acid), endogenous ethylene evolution and the development of ethylene-like symptoms. Plant Soil 50(1–3):355–362 (1978/12/01)
204. Tamari K, Kaji J (1952) Studies on the mechanism of injurious action of fusarinic acid on plant-growth. J Agric Chem Soc Jpn 26(7):345–349

205. Malini S (1966) Heavy metal chelates of fusaric acid: in vitro spectrophotometry. J Phytopathol 57(3):221–231
206. Lakshminarayanan K, Subramanian D (1955) Is fusaric acid a vivotoxin? Nature 176(4484):697–698 (10/08/print, 10.1038/176697a0)
207. Pan JH, Lin YC, Tan N, Gu YC (2010) Cu(II): A “signaling molecule” of the mangrove endophyte *Fusarium oxysporum* ZZF51? Biometals 23(6):1053–1060
208. Gäumann E (1958) The mechanism of fusaric acid injury. Phytopathology 48:670–686
209. Brown DW, Butchko RA, Busman M, Proctor RH (2012) Identification of gene clusters associated with fusaric acid, fusarin, and perithecial pigment production in *Fusarium verticillioides*. Fungal Genet Biol 49(7):521–532
210. Brown DW, Lee SH, Kim LH, Ryu JG, Lee S, Seo Y, Kim YH, Busman M, Yun SH, Proctor RH, Lee T (2015) Identification of a 12-gene fusaric Acid biosynthetic gene cluster in fusarium species through comparative and functional genomics. Mol Plant Microbe Interact 28(3):319–332
211. Liu J, Bell A, Stipanovic R, Puckhaber L (2010) Fusaric acid production and pathogenicity of *Fusarium oxysporum* f. sp. *vasinfectum* [abstract]. Proceedings of Beltwide Cotton Conferences, January 4–7, 2010, New Orleans, Louisiana 2010 CDROM
212. Wu F, Groopman JD, Pestka JJ (2014) Public health impacts of foodborne mycotoxins. Annu Rev Food Sci Technol 5:351–372
213. Scott PM (2012) Recent research on fumonisins: a review. Food Addit Contam Part A. Chem Anal Control Expo Risk Assess 29(2):242–248
214. Rheeder JP, Marasas WF, Vismer HF (2002) Production of fumonisin analogs by *Fusarium* species. Appl Environ Microbiol 68(5):2101–2105
215. Bartok T, Tolgyesi L, Mesterhazy A, Bartok M, Szecsi A (2010) Identification of the first fumonisin mycotoxins with three acyl groups by ESI-ITMS and ESI-TOFMS following RP-HPLC separation: Palmitoyl, linoleoyl and oleoyl EFB(1) fumonisin isomers from a solid culture of *Fusarium verticillioides*. Food Addit Contam Part A. Chem Anal Control Expo Risk Assess 27(12):1714–1723
216. Falavigna C, Lazzaro I, Galaverna G, Battilani P, Dall’Asta C (2013) Fatty acid esters of fumonisins: first evidence of their presence in maize. Food Addit Contam Part A. Chem Anal Control Expo Risk Assess 30(9):1606–1613
217. Musser SM, Gay ML, Mazzola EP, Plattner RD (1996) Identification of a new series of fumonisins containing 3-hydroxypyridine. J Nat Prod 59(10):970–972
218. Scientific Committee on Food (2003) Updated opinion of HTE scientific comettee on food on fumonisin B1, B2 and B3 (expressed on 4 April 2003). European Commission, Brussels
219. Desjardins AE (2006) Fusarium mycotoxins: chemistry, genetics and biology. Chapter 3: Fumonisins. American Phytopathological Society, St. Paul
220. Nelson PE, Desjardins AE, Plattner RD (1993) Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry, and significance. Annu Rev Phytopathol 31:233–252
221. World Health Organization (International Agancy for Research on Cancer) (2002) Some traditional herbal medicines, some mycotoxins, naphthalene and styrene
222. Riley RT, Enongene E, Voss KA, Norred WP, Meredith FI, Sharma RP et al (2001) Sphingolipid perturbations as mechanisms for fumonisin carcinogenesis. Environ Health Perspect 109(Suppl 2):301–308
223. Marasas WF, Riley RT, Hendricks KA, Stevens VL, Sadler TW, Gelineau-van Waes J et al (2004) Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. J Nutr 134(4):711–716
224. Epstein S, Riezman H (2013) Sphingolipid signaling in yeast: potential implications for understanding disease. Front Biosci (Elite edition) 5:97–108
225. Stone JM, Heard JE, Asai T, Ausubel FM (2000) Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1-resistant (fbr) *Arabidopsis* mutants. Plant Cell 12(10):1811–1822

226. Asai T, Stone JM, Heard JE, Kovtun Y, Yorgey P, Sheen J et al (2000) Fumonisin B1-induced cell death in arabidopsis protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. Plant Cell 12(10):1823–1836
227. Lin SS, Martin R, Mongrand S, Vandenabeele S, Chen KC, Jang IC et al (2008) RING1 E3 ligase localizes to plasma membrane lipid rafts to trigger FB1-induced programmed cell death in *Arabidopsis*. Plant J 56(4):550–561
228. Chivasa S, Ndimba BK, Simon WJ, Lindsey K, Slabas AR (2005) Extracellular ATP functions as an endogenous external metabolite regulating plant cell viability. Plant Cell 17(11):3019–3034
229. Igarashi D, Bethke G, Xu Y, Tsuda K, Glazebrook J, Katagiri F (2013) Pattern-triggered immunity suppresses programmed cell death triggered by fumonisin B1. PloS ONE 8(4):e60769
230. Kuroyanagi M, Yamada K, Hatsugai N, Kondo M, Nishimura M, Hara-Nishimura I (2005) Vacuolar processing enzyme is essential for mycotoxin-induced cell death in *Arabidopsis thaliana*. J Biol Chem 280(38):32914–32920
231. Hara-Nishimura I, Hatsugai N, Nakaune S, Kuroyanagi M, Nishimura M (2005) Vacuolar processing enzyme: an executor of plant cell death. Curr Opin Plant Biol 8(4):404–408
232. Kimberlin AN, Majumder S, Han G, Chen M, Cahoon RE, Stone JM et al (2013) Arabidopsis 56-amino acid serine palmitoyltransferase-interacting proteins stimulate sphingolipid synthesis, are essential, and affect mycotoxin sensitivity. Plant Cell 25(11):4627–4639
233. Desjardins AE, Plattner RD, Nelsen TC, Leslie JF (1995) Genetic analysis of fumonisin production and virulence of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*) on maize (*Zea mays*) seedlings. Appl Environ Microbiol 61(1):79–86
234. Desjardins AE, Plattner RD (2000) Fumonisin B(1)-nonproducing strains of *Fusarium verticillioides* cause maize (*Zea mays*) ear infection and ear rot. J Agric Food Chem 48(11):5773–5780
235. Desjardins AE, Munkvold GP, Plattner RD, Proctor RH (2002) FUM1—a gene required for fumonisin biosynthesis but not for maize ear rot and ear infection by *Gibberella moniliformis* in field tests. Mol Plant Microbe Interact 15(11):1157–1164
236. Glenn AE, Zitomer NC, Zimeri AM, Williams LD, Riley RT, Proctor RH (2008) Transformation-mediated complementation of a FUM gene cluster deletion in *Fusarium verticillioides* restores both fumonisin production and pathogenicity on maize seedlings. Mol Plant Microbe Interact 21(1):87–97
237. Desjardins AE, Plattner RD, Stessman RJ, McCormick SP, Millard MJ (2005) Identification and heritability of fumonisin insensitivity in *Zea mays*. Phytochemistry 66(20):2474–2480
238. Dall'Asta C, Galaverna G, Mangia M, Sforza S, Dossena A, Marchelli R (2009) Free and bound fumonisins in gluten-free food products. Mol Nutr Food Res 53(4):492–499
239. Berthiller F, Crews C, Dall'Asta C, Saeger SD, Haesaert G, Karlovsky P et al (2013) Masked mycotoxins: a review. Mol Nutr Food Res 57(1):165–186

Chapter 11
Biosynthesis and Molecular Genetics of Peptaibiotics—Fungal Peptides Containing Alpha, Alpha-Dialkyl Amino Acids

Xiao-Yan Song, Bin-Bin Xie, Xiu-Lan Chen and Yu-Zhong Zhang

Introduction

In the past six decades, a unique group of nonribosomal peptides from filamentous fungi known as peptaibiotics has attracted increasing attention for their particular physicochemical properties and biological activities. Peptaibiotics are generally characterized by a high proportion of alpha-dialkyl alpha-amino acids (α [alpha], α [alpha]-dialkylated amino acids), rich in the marker amino acid α (alpha)-aminoisobutyric acid (Aib), and occasionally D-isovaline (Iva). The name peptaibiotic originated from the *pept*ides containing *Aib* and exerting a variety of (anti)*biotic* activities [1]. Peptaibiotics are usually composed of 5–21 amino acid residues with a molecular weight ranges from 500 to 2200 Da. The N-terminal amino acids are usually acetylated, while the C-termini of peptaibiotics are variable, including a free or methoxy-substituted 2-amino alcohol, amine, amide, free amino acid, diketopiperazine or sugar alcohol [2].

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Peptaibiotics have interesting physicochemical properties and have attracted much attention because of their biological activities and pharmacological properties, including antibacterial, antifungal, antiviral, antitumor, and immunosuppressive effects, in addition to elicitation of plant-systemic resistance activities [2–4]. The action mechanism of peptaibiotics is primarily related to channel formation in lipid membranes, which suggests a structure composed of a bundle of hydrophobic transmembrane helices surrounding a central pore [5]. Two special issues of the journal *Chemistry & Biodiversity* entitled "Peptaibiotics" and "Peptaibiotics II" were published in 2007 and 2013, respectively, to review the knowledge of peptaibiotics [6, 7]. The spectroscopic and molecular-dynamics methods for the structure analysis and the unique oxazolone/azirine approach for chemical synthesis are also introduced in these issues [8, 9]. The 2007 journal issue was compiled in a book entitled *Peptaibiotics—Fungal Peptides Containing Alpha-Dialkyl Alpha-Amino Acids* [10]. To learn more about the physicochemical activities, structural characteristics and biological activities, these two special issues and the book are highly recommended.

First identified in the 1960s, the number of peptaibiotics in filamentous fungi continues to increase, especially in the last two decades. More than 1000 peptaibiotics have been sequenced and compiled in the newly established peptaibiotics database (http://peptaibiotics-database.boku.ac.at; University of Natural Resources and Life Sciences Vienna [11]). Peptaibols, the largest group of peptaibiotics, sequences, and structures, that were found in the early stage are compiled in the peptaibols database (http://peptaibol.cryst.bbk.ac.uk), which is hosted by the School of Crystallography, Birkbeck College, University of London, UK [12]. Peptaibiotics are chemically diverse and microheterogenous. In an analogy to proteomics, peptidomics, and metabolomics, the term peptaibomics was introduced to study the entirety of peptaibiotics expressed by filamentous fungi under defined laboratory or fermentation conditions or in their natural habitat [1, 13, 14]. And an intact-cell matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) method used for single-cell analysis has also been developed to accelerate the study of peptaibomics [15].

As a class of peptide secondary metabolites, peptaibiotics are also produced by multifunctional enzymes, the nonribosomal peptide synthetases (NRPSs), which act in an assembly-line fashion. An excellent review has been provided by Kubicek on the biosynthesis of peptaibols from *Trichoderma* [16]. In this chapter, we summarize the current understanding of the biosynthesis and molecular genetics of peptaibiotics. In addition, a discussion of future research in peptaibiotics is provided.

The Biodiversity and Evolution of Peptaibiotics

Research in the past two decades has revealed that peptaibiotics are remarkably diverse and microheterogenous. The structural diversity of the peptaibiotics arises from the varying number of amino acid residues, the varying amounts of proteino-

genic and non-proteinogenic amino acids, and varying substitutions on the N- and C-termini.

Many genera of Ascomycetous fungi and their anamorphs have been recognized as producers of peptaibiotics. In the peptaibiotics database, peptaibiotic-producing strains are fungi from more than 20 genera, mostly from *Trichoderma*, *Hypocrea*, *Acremonium*, *Paecilomyces*, *Emericellopsis*, *Stilbella*, and *Geotrichum*, but occasionally from *Amycolatopsis*, *Ascomycet*, *Apiocrea*, *Boletus*, and *Chrysosporium,* etc. [11]. Of these, *Trichoderma* with teleomorphs in *Hypocrea* are the most abundant producers of peptaibiotics. Approximately 80% of the known peptaibiotics today have been found in *Trichoderma* and *Hypocrea* (756 from *Trichoderma* and 92 from *Hypocrea*), especially the species *T. viride*, *T. brevicompactum*, *T. virens*, *T. parceramosum*, and *T. harzianum*. Peptaibiotics produced by Basidiomycetes, such as *Boletus ssp.* and *Tylopilus*, are thought to have arisen from infections of the basidiomycete fruiting bodies by these fungi, which are frequently mycoparasites [15–17]. Bruckner et al. have analyzed 49 species and strains of filamentous fungi, and more than 30 genera containing the non-proteinogenic marker amino acid Aib of peptaibiotics [18]. An extensive survey of 28 *Trichoderma*/*Hypocrea* species with established species identity for peptaibol production by intact-cell MALDI-TOF mass spectrometry revealed that peptaibols are produced by all these strains, with some producing up to five peptide families of different sizes [15]. Moreover, Neuhof et al. assessed the peptaibol pattern formed by these 28 species of *Trichoderma* and found that the types of peptaibols do not correlate with the taxonomy of these species [15] .

Peptaibiotics, especially peptaibols, range from 5 to 21 residues in length. According to the structural characteristics, peptaibiotics have been grouped into six categories in the peptaibiotics database [11], including peptaibols, lipopeptaibols, lipoaminopeptides, cyclic peptaibiotics, other peptaibiotics, and all-Aib-replaced peptides. Peptaibols are the major class of peptaibiotics, and contain a 2-amino N-acyl (usually acetyl) terminus and a C- terminal alcohol, such as phenylalaninol or leucinol [19, 20]. Because all of the first peptaibols sequenced had a phenylalaninol at the C-terminus, they were originally defined as peptaibophols [21]. Lipopeptaibols are lipophilic peptaibiotics, with an N-terminus that is acylated by a short fatty acid chain such as octanoic acid, decanoic, or cis-dec-4-enoic acid instead of acetic acid [22, 23]. The third category is the lipoaminopeptides (also known as aminolipopeptides), in which the N-terminus is substituted by unbranched, α (alpha)- or γ(gamma)-methyl-branched, saturated or unsaturated C_4–C_{15} fatty acids. The N-terminal amino acid residue is an L-proline-, trans-4-hydroxy-L-proline, or cis-4-methyl-L-proline residue. The second residue is usually a lipoamino acid residue. 2-amino-6-hydroxy-4-methy-8-oxodecanoic acid (AHMOD) has been detected only in this subfamily [2]. Cyclic peptaibiotics are cyclic peptides, containing Aib and Iva from natural origins. The fifth category comprises all the other linear peptaibiotics that cannot be classified in any of the other categories. The sixth category comprises all-Aib-replaced peptides. Several examples of peptaibiotics are listed in Table 11.1 [17, 22, 24–35]. In the peptaibiotics database, of the 1062 sequences listed, 767 peptides represent peptaibols, 37 are lipopeptaibols, 64 are

Table 11.1 The peptaibiotics categories in peptaibiotics databases

Peptide category	Compound name	Typical sequence	Peptide subfamily	Producing strain	Reference
Peptaibol	Alamethicin F-30/1	Ac Aib Pro Aib Ala Aib Ala Gln Aib Aib Aib Gly Leu Aib Pro Val Aib Aib Glu Gln Pheol	SF1	*Trichoderma viride*	Degenkolb et al. 2003 [17]
	Alamethicin F-30/2	Ac Aib Pro Aib Ala Aib Ala Gln Aib Val Aib Gly Val Aib Pro Val Aib Aib Glu Gln Pheol			
	Alamethicin F-30/3	Ac Aib Pro Aib Ala Aib Ala Gln Aib Val Aib Gly Leu Aib Pro Val Aib Aib Glu Gln Pheol			
	Alamethicin F-30/4	Ac Aib Pro Aib Ala Aib Aib Gln Aib Val Aib Gly Val Aib Pro Val Aib Aib Glu Gln Pheol			
	Alamethicin F-30/5	Ac Aib Pro Aib Ala Aib Ala Gln Aib Val Aib Gly Leu Aib Pro Val Aib Val Glu Gln Pheol			
	Alamethicin F-30/6	Ac Aib Pro Aib Ala Aib Ala Glu Aib Val Aib Gly Leu Aib Pro Val Aib Aib Glu Gln Pheol			
	Alamethicin F-30/7	Ac Aib Pro Aib Ala Aib Aib Gln Aib Val Aib Gly Leu Aib Pro Val Aib Aib Glu Gln Pheol			
	Alamethicin F-30/8	Ac Aib Pro Aib Ala Aib Aib Gln Aib Leu Aib Gly Leu Aib Pro Val Aib Aib Glu Gln Pheol			
	Alamethicin F-30/9	Ac Aib Pro Aib Ala Aib Aib Gln Aib Val Aib Gly Leu Aib Pro Val Aib Val Glu Gln Pheol			
	Alamethicin F-30/10	Ac Aib Pro Aib Ala Aib Ala Gln Aib Aib Aib Gly Leu Aib Pro Val Aib Aib Glu Gln Pheol			
	Antiamoebin I	Ac Phe Aib Aib Aib Iva Gly Leu Aib Aib Hyp Gln Iva Hyp Aib Pro Pheol	SF2	*Stilbella erythro-cephala Stilbella fimetaria Gliocladium catenulatum*	Jaworski and Bruckner 2000 [24]
	Emerimicin II A	Ac Trp Ile Gln Aib Ile Thr Aib Leu Aib Hyp Gln Aib Hyp Aib Pro Pheol	SF3	*Emericellopsis salmosynnemata*	Rinehart jr. et al. 1981 [25]

Table 11.1 (continued)

Peptide category	Compound name	Typical sequence	Peptide subfamily	Producing strain	Reference
	Harzianin HB	Ac Aib Asn Leu Ile Aib Pro Iva Leu Aib Pro Leuol	SF4	*Trichoderma. harzianum*	Isabelle Augeven-Bour et al. 1997 [26]
	Ampullosporin A	Ac Trp Ala Aib Aib Leu Aib Gln Aib Aib Aib Gln Leu Aib Gln Leuol	SF6	*Sepedonium ampullosporum Sepedonium* sp.	Ritzau et al. 1997 [27]
	Peptaibolin	Ac Leu Aib Leu Aib Pheol	SF9	*Sepedonium ampullosporum*	Hulsmann et al. 1998 [28]
Lipopeptaibol	Trichogin A IV LP237 F5	Oc Aib Gly Leu Aib Gly Gly Leu Aib Gly Ile Leuol Oc Aib Pro Tyr Aib Gln Gln Aib EtNva Gln Ala Leuol	SF5 SF7	*T. longibrachiatum* *Tolypocladium geodes*	Auvin-Guette et al. 1992 [22] Tsantrizos YS et al. 1996 [29]
Lipoaminopeptide	Trichoderin A	MDA Pro AHMOD Aib Aib Ile Val Aib Aib AMAE		*Trichoderma* sp.	Pruksakorn et al. 2010 [30]
Cyclic peptaibiotic	Chlamydocin	Cyclo(Aoe D-Pro Phe Aib)		*Pochonia chlamydosporia*	Closse and Huguenin 1974 [31]; Degenkolb et al. 2008 [32]
Other peptaibiotic	Acrebol A Clonostachin	Ac Phe Vxx Gln Aib Ile Thr Leu Aib Pro Aib Gln Pro Aib X X X Serol Ac Aib Hyp Leu Iva Hyp Leu Iva Hyp Aib Iva Aib Hyp Iva Ile OCH(CH(OH)CH_2OH) CH(OH)CH(OH)CH_2OH	SF8	*Acremonium exuviarum* *Clonostachys* sp. F5898	Andersson et al. 2009 [33] Chikanishi et al. 1997 [34]
All-Aib-replaced peptide	Hypocompactin HCP-II	Gly Ala Lxx Vxx Gly Lxx Lxxol		*Hypocrea rodmanii*	(Degenkolb et al. 2008 [35])

Ac acetic acid; *Hyp* hydroxyproline; *Aib* alpha-aminoisobutyric acid; 2-methyl-alanine; *Iva* isovaline Lxx leucine or isoleucine (isobaric); *Vxx* valine or isovaline (isobaric); *Pheol* phenylalaninol; *Leuol* leucinol; *Serol* serinol; *Oc* octanoic acid; *Aoe* L-2-amino-9,10-epoxy-8-oxodecanoic acid; *AMAE* 2-[(2′-aminopropyl)-methylamino]-ethanol, trichodiaminol; *MDA* (2R)-methyl-decanoic acid; *AHMOD* (2S, 4S, 6S)-2-amino-6-hydroxy-4-methyl-8-oxodecanoyl(2S, 4S, 6S)-2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid; Other amino acids are indicated in three letter codes

lipoaminopeptides, 8 are cyclic peptaibiotics, 173 are other peptaibiotics, and 13 peptides are all-Aib-replaced peptides.

According to sequence identity and sequence length, Chugh and Wallace divided the peptaibols into nine subfamilies [36]. Each subfamily displays microheterogeneity based on small differences in their amino acid sequences at specific positions in the molecule. Subfamily 1 (SF1) is the largest SF, comprising about half of the known structures and sequences that contain 17–20 residues. All of these peptides have partial sequence identities or similarities. Characteristic of many members of this SF is the presence of a Gln near the middle, often at position 6 or 7. Additional Gln or Glu residues are found toward the C-terminus. In many cases, positions 18 and 19 are a Gln–Gln or Glu–Gln pair. The shorter members of this SF, however, usually only contain a single Gln residue in the C-terminal region. The Glu and Gln residues appear to be located in the pore lumen, and important for conductance. SF2 and SF3 are the most similar to SF1. They tend to range in size from 14 to 16 residues, and also to be classified as long peptaibols. SF4 is very different from the other families, consisting of peptaibols with either 11 or 14 residues. No aromatics or charged residues are present; however, these peptaibols still form channels. SF5 is a group of "short" peptaibols with either 7 or 11 residues. SF 6 to 9 have only a few members and also belong to the group of "short" peptaibols, with 15, 11, 14, and 5 residues, respectively. Although many peptaibiotics are beyond the nine subfamilies, this classification method is significant for understanding the microheterogeneity of peptaibiotics. Peptaibiotics/peptaibols may also be classified into groups according to peptide length: long-chain peptides with 17–21 residues, medium-chain peptides with 11–16 residues, short-chain peptides with 6–10 residues, and very short-chain peptides with 5 and fewer residues.

Peptaibiotics from one strain are not only distinguished by the exchange of single or multiple amino acids, but also compounds of shorter main-chain lengths. Amino acid-deletion peptides of major sequences have been detected [37]. An example is the Trichobrachins from *T. parceramosum* strain CBS936.69, which includes three groups of Trichobrachins I, II, and III (TB I, TB II, and TB III). Trichobrachins comprise ten 19-residue peptides with a free C-terminal Gln residue (TB I peptides), two 18-residue peptides with a free C-terminal Gln residue (TB II 1 and 2), seven 20-residue peptides with a C-terminal amide-bound phenylalaninol (TB II 3–10), and thirty-four 11-residue peptides with a C-terminal leucinol, isoleucinol or valinol (TB III 1–34). Moreover, the ten 19-residue peptaibiotics, trichobrachins I, lack the C-terminal Pheol residue, and the two 18-residue trichobrachins II exhibit a deletion of the C-terminal dipeptide [Gln_{19}-$Pheol_{20}$]. These two types of peptaibiotics were shown to originate from 20-residue trichobrachins II by enzymatic degradation [38] .

Peptaibiotics with chain lengths of 11–14 residues or 18–20 residues are the most common two groups, which constitute 70 % of all peptides compiled in the peptaibiotics database [11]. Kubicek et al. have separately aligned all 18–20- and 10–14-residue peptaibols from the peptaibol database [16]. However, due to broad substrate specificity of peptaibol synthetase and the involvement of genomic rearrangements in their evolution, the structures of the peptaibols were found to contain

no phylogenetic information. Analytical results of the first adenylation domains also confirmed the phylogenetic analysis results [16] .

Biosynthesis of Peptaibiotics

As a class of small molecule peptide secondary metabolites, peptaibiotics are also synthesized by large multidomain enzymes known as NRPSs, which act in an assembly-line fashion. The synthetases of peptaibiotics are important members of the NRPS family from fungi. To date, the biosynthetic knowledge for the peptaibiotics confined to the peptaibol-producing fungi.

The Biosynthetic Pathway

Similar to other NRPSs, the peptaibiotic synthetases are organized into modules that bind, activate, and condense each specific amino acid to form the peptide product. Each module possesses multiple conserved domains, including (1) an adenylation (A) domain that selects and activates the substrate molecule, (2) a thiolation (T) domain that serves as a carrier protein onto which the activated substrate of the upstream A domain is covalently tethered, and (3) a condensation (C) domain that catalyzes amide bond formation between adjacent T domain-tethered substrates [39]. Several excellent reviews are available for a comprehensive understanding of NRPSs and their enzymology [39–41]. Similar to other peptide synthases, each amino acid in a peptaibiotic is introduced by an A domain, which is covalently linked to the adjacent carrier domain. Peptide-bond formation takes place at the C domains, where adjacent and nonadjacent carrier domains deliver aminoacyl and peptidyl intermediates [16].

Significant early work used feeding experiments, time-course studies, isotope-labeling techniques, and enzymology to understand the biosynthesis of peptaibiotics. The first evidence for peptaibiotic synthesis was obtained by Reusser [42]. He found that alamethicin is synthesized at the end of the exponential growth phase of *T. viride* in culture. Cycloheximide did not interfere with the formation of alamethicin in vivo; therefore, it was proposed that alamethicin was not synthesized on ribosomes but by NRPSs [42]. Approximately 10 years later, Kleinkauf and Rindfleisch reported that in vitro synthesis of alamethicin is not influenced by RNase or puromycin [43]. The biosynthetase is a multienzyme complex with a molecular weight of approximately 480,000. The constituent amino acids are activated in the form of thioesters on the synthetase. The single amino acids are activated as thioesters at peripheral SH-groups of the synthetase by an adenosine triphosphate (ATP)-dependent reaction [43]. After purification of the synthetase by chromatography on hydroxyapatite, it was found that the synthetase consisted of two fractions. Using this synthetase, alamethicin was synthesized successfully in vitro. The results further revealed that the different amino acids are activated in the form of aminoacyl

adenylates and then bound to the synthesizing enzyme as thioesters [44]. Moreover, the site on alamethicin synthetase catalyzing the acetylation has a preference for Aib, and alamethicin formation on the synthetase is terminated by linkage of phenylalaninol to the carboxyl terminus of the peptide. They proposed that phenylalaninol was most likely the reaction product of a separate enzyme system [45].

In 2002, the first genetic evidence for the biosynthesis of peptaibiotics was provided by Wiest et al. [46], who cloned the peptaibol synthetase gene *Tex1* from *T. virens* GV29-8. To date, this is the best characterized gene. Mutation of the gene eliminated the production of all peptaibol isoforms with 11-, 14-, and 18-amino acids. However, it was confirmed subsequently that *Tex1* codes for the 18-module peptaibol synthetase is only responsible for the production of peptaibols with 18-amino acids [47, 48]. This gene contains a 62.8-kb continuous open reading frame and encodes a mature protein of 20,925 residues (approximately 2.3 MDa), which would specify the largest mRNA and the largest continuous coding region known. The predicted protein structure consists of 18 peptide synthetase modules, which are responsible for the incorporation of 18 amino acid residues with additional modifying domains at the N- and C-termini, the acetylation of the N-terminus and reducing the final amino acid to generate the C-terminal alcohol, respectively. How such large mRNAs and protein are processed in the cell is still unclear. The *psy1* gene from *T. virens* GV29-8, previously thought to be a peptide synthetase gene, is now known to be part of the peptaibol synthetase gene *Tex1* and not responsible for siderophore production [49].

Subsequently, multiple NRPS genes were found to determine peptaibol synthesis in *T. virens* and to be responsible for the synthesis of the shorter peptaibols [47]. However, not every putative peptide synthetase gene encoded for a protein involved in peptaibol biosynthesis. The putative peptide synthetase gene region of *salps1* cloned by Vizcaino et al. [50] contains four complete, and a fifth incomplete module, in which A, T and C domains are found, and also contains an additional epimerization domain at the C-terminal end of the first module. After analyzing the Salps1 protein sequence, Vizcaino suggested that it is neither a peptaibol synthetase nor a protein involved in siderophore biosynthesis. The presence of two breaks in the open reading frame and the expression of this gene under nitrogen starvation conditions suggest that *salps1* could be a pseudogene [51]. Other peptaibol synthetic genes were also partially cloned from *T. harzianum* and *T. asperellum* [50, 52].

The first genetic evidence for the synthesis of the shorter peptaibols was also obtained from *T. virens*. A single 14-module NRPS is responsible for the synthesis of two classes of peptaibols with 11- and 14-residue [53]. Based on the available sequence comparison of 11- and 14-residue peptaibols, Neuhof et al. predicted that SF4 11- and 14-residue compounds could be produced by a single peptaibol synthetase by the deletion of modules 3–5 (Leu3-Pro4-Aib5) [15]. However, after aligning the sequences of the 11- and 14-residue peptaibols, it was found that 11-residue peptaibols could be derived from 14-residue peptaibols by internal deletion of the residues in positions 4, 5, and 6. The only possibility by which the 14-module gene could be responsible for synthesis of both 11-residue and 14-residue peptaibols is

skipping of the three modules during the biosynthesis of the 11-residue peptaibol. Further study found that the 14-module NRPS type found in *T. virens*, *T. reesei* (teleomorph *H. jecorina*), and *T. atroviride* produces 11- and 14-residue peptaibols. The possible mechanism of module skipping was proposed [54]. Module-skipping during peptaibol synthesis has not been shown, but this phenomenon operates during the biosynthesis of many types of nonribopeptides, giving rise to enhanced chemodiversity [55, 56]. Analysis of NRPS genes from peptaibiotic-producing strains has much to offer in understanding the diversity of peptaibiotics.

Peptaibiotic Synthetic Genes in Trichoderma Genomes

The genomic information from peptaibiotic-producing strains provides a new approach to mine for peptaibol biosynthetic genes. Four genomes of *Trichoderma* species have been reported: *T. atroviride* (*H. atroviridis*), *T. virens*, *T. reesei* (*H. jecorina*), and *T. longibrachiatum*. The four sequenced *Trichoderma spp.* have three to seven chromosomes and genomes of 31–39 Mb, encoding 9129–12,427 proteins [57–59]. The 31.7-Mbp genome of *T. longibrachiatum*, which is the most recently sequenced *Trichoderma* genome, is the smallest of the four sequenced *Trichoderma spp.*, and is similar to that of *T. reesei* (33.9 Mbp), while the GC content of the assembly (54.0 %) is the highest among the sequenced *Trichoderma* [59]. The sequence analyses of fungal genomes have uncovered a large number of putative biosynthetic gene clusters, including polyketide synthase (PKS), NRPS, and/or PKS-NRPS hybrid genes. The peptaibol synthetase genes have been identified in the genomes of *T. reesei* and *T. atroviride*, *T. virens* and *T. longibranchiatum* [16, 57, 59, 60]. Genome analysis revealed that there are up to three types of NRPSs with 7, 14, or 18–20 amino acid incorporation modules in *Trichoderma* [53]. However, the NRPS for 7 amino acid incorporation has not been investigated in detail [53, 54] Other genome sequencing projects of *Trichoderma* are in progress, which will contribute to give insight into the NRPSs responsible for peptaibiotics biosynthesis [58].

The structural information for NRPS or PKS/NRPS for peptaibiotic production can be obtained from the pfam database (http://pfam.sanger.ac.uk). In the genome of *T. longibrachiatum*, several PKS, NRPS, and/or PKS-NRPS hybrid genes can be predicted for secondary metabolite production, although we identified only two NRPSs genes responsible for the production of peptaibols with 12- and 20-amino acid residues [59] (Fig. 11.1). Some gene clusters may be silent under the experimental conditions, and activating of these clusters could provide a new way to produce potentially novel peptaibiotics. In the genome of *T. virens*, three NRPS genes—*tex1* and *tex2*, and *tex3*, encoding 18, 14, and 7 modules, respectively—are responsible for the production of peptaibols with 18-, 11/14-, and 7- amino acid residues [46, 53, 57]. Degenkolb et al. found that *T. reesei* QM9414 generates 11, 14, and 20 amino acid residue peptaibols [54] In addition, only long-chain peptaibols with 20 amino acid residues from *T. reesei* have been sequenced and compiled

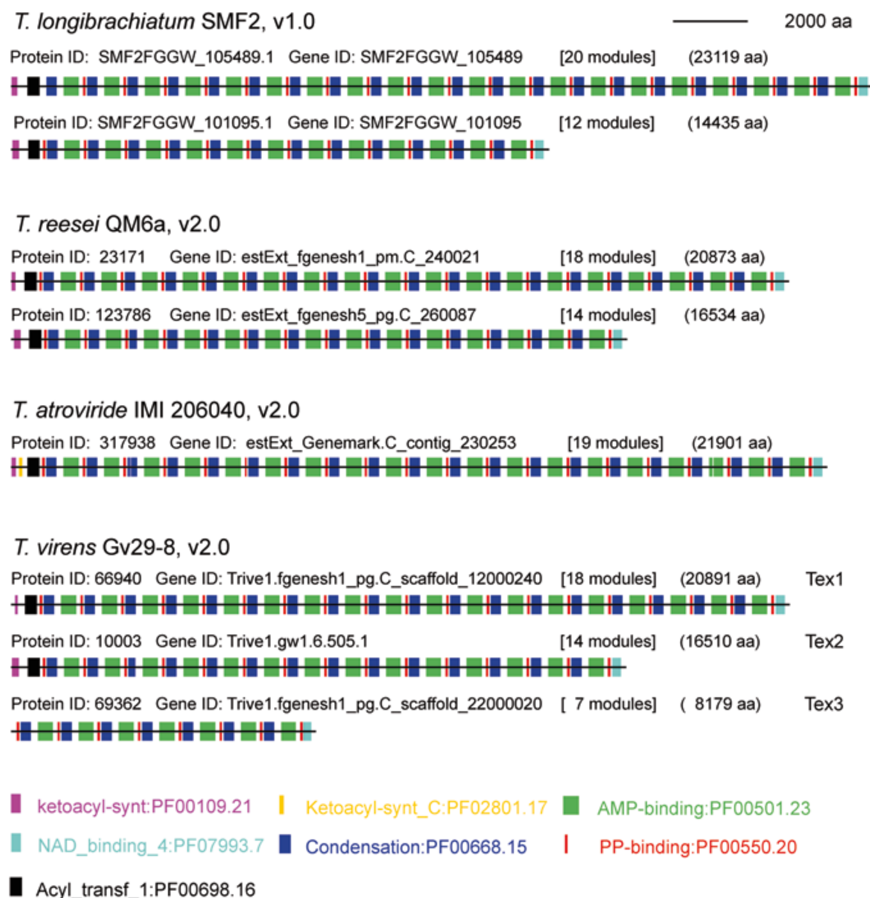

Fig. 11.1 Module structure of NRPSs putatively responsible for peptaibiotics synthesis extracted from the genome sequences of *Trichoderma* species. Protein sequences were annotated using Pfam database. Protein sequences (*fggw set*) for *T. longibrachiatum* SMF2 genome assembly v1.0 were available through anonymous ftp (ftp://222.206.24.193). Protein sequences were downloaded from the Department of Energy Joint Genome Institute (*JGI*) Genome Portal (http://genome.jgi.doe.gov/) for *T. reesei* QM6a genome assembly v2.0 (http://genome.jgi.doe.gov/Trire2/Trire2.home.html), *T. virens* Gv29-8 assembly v2.0 (http://genome.jgi.doe.gov/TriviGv29_8_2/TriviGv29_8_2.home.html), and *T. atroviride* IMI 206040 genome assembly v2.0 (http://genome.jgi.doe.gov/Triat2/Triat2.home.html)

in the peptaibiotics database [11]. However, in the *T. reesei* genome, there are also two NRPS genes encoding 14 and 18 typical peptide synthetase modules [60].

Komon-Zelazowska et al. deduced the *pbs1* gene from the *T. atroviride (H. atroviridis)* genome sequence [61]. It consists of 19 typical peptide synthetase modules with the required additional acetyltransferase and alcohol dehydrogenase domains at the N- and C-termini. Phylogenetic and similarity analyses of the individual A modules were consistent with its ability to synthesize atroviridins [61]. Moreover,

pbs1 is the only gene in the *T. atroviride (H. atroviridis)* genome responsible for the synthesis of peptaibols with more than 16 residues, including 19 and 20 residues. Atroviridins contain the sequence Aib5-Aib6-Gln7, while the structure of *pbs1* does not contain a second Aib domain, suggesting that the module responsible for the Aib incorporation is used twice [61]. This could be an additional mechanism contributing to peptaibol microheterogeneity, but should be verified by genetic evidence. Kubicek et al. found two peptaibol synthetases in the *T. atroviride* genome [57]. However, in the current versions (v1.0 and v2.0) of *T. atroviride* genome, the short NRPS gene was not modeled as one gene but as eight gene fragments [57].

Substrate Specificity of Peptaibiotics Synthetases

NRPSs exhibit significant substrate selectivity, but the molecular basis of substrate selectivity in these NRPS domains is not well understood [62]. In NRPSs, the A domains appear to be the primary determinants of substrate selectivity, which is responsible for the incorporation of the amino acid monomers that are to be incorporated into the final peptide product. Each A domain recruits a specific amino acid and the sequential order of A domains along the assembly line usually determines the primary sequence of the final peptide product.

Eight or ten residues present in the active site of peptide synthetases have been proposed to play a major role in defining substrate specificity for the incorporation of amino acids based on structural data [46, 50, 51]. These residues define the signature sequences specifying amino acid incorporation. However, the signature sequences alone do not allow the identification of the amino acid substrate for which they are specific. Wiest et al. noted that the signature sequences of the 1st, 9th, 12th, 15th, and 16th modules of Tex1 exhibit high similarity, which are likely responsible for Aib incorporation [46]. Modules 6 and 17 contain identical residues in the signature sequence that may be responsible for the incorporation of the glutamate residues in the position 6 and 17 of peptaibiotics [46]. Therefore, the selectivity of a module for amino acid incorporation in NRPSs is flexible, which is relevant to the availability of different amino acid substrates. Further study found that the specificity of the A domain may be predicted from nonribosomal codes, and substrate binding meets highly conserved binding pockets in case of Pro, Ala, or Gln [54].

Because of the low number of characterized NRPSs in peptaibiotic-producing fungi and because the fungal nonribosomal specificity codes are too limited, additional research is required to fully understand how the nonribosomal peptide synthases control substrate selectivity. Much more biochemical and genetics evidence and genomic data are needed to understand how synthetases control substrate specificity. It seems that fungi will be even more ingenious at creating structural diversity than we think they are, even after decades of research into their metabolic universe [39] .

Biosynthesis of Terminal Modified Peptaibol Residues

The N-terminal residue of peptaibols is largely acetylated and the C-terminal residues are amino alcohols. The first line of evidence for the origin of these terminal modified residues came from the study of alamethicin synthase [45]. It was concluded that neither of these residues are synthesized by other metabolic pathways nor are they direct substrates for the synthase.

This finding is substantiated by the domain structure of the peptaibol synthases. The module structure of Tex1 comprises an N-terminal PKS starter module, 18 NRPS modules, and a C-terminal reductase domain (R domain). The PKS starter module is composed of a ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) domain [41]. This PKS module is responsible for the acetylation of the N-terminal residue. The R domain is responsible for the release of the mature peptide product reductively, with the aid of an NADPH cofactor [46].

Recently, a three-dimensional model of the R domain was constructed based on the crystal structure of vestitone reductase (VR), which suggested that peptaibol biosynthesis incorporates a single R domain, that catalyzes the four-electron reduction reaction of a PCP-bound peptide to its corresponding primary alcohol [63]. However, Manavalan et al. also noted that there might be a structural difference between the alcohol- and aldehyde-forming R domains. The limitation at present is the lack of a suitable template for modeling aldehyde-forming R domains to explore their function. Additional studies are required to gain a complete understanding of the aldehyde-forming R domains [63] .

There are eight cyclic peptaibiotics or their analogues in the peptaibiotics database, but little is known about how these peptides are cyclized. Recently, Gao et al. proposed a universal macrocyclization strategy for fungal NRPSs [64]. The megaenzyme terminates with a condensation-like domain that may perform the macrocyclization reaction [64]. This may provide ideas for the cyclization of peptaibiotics.

The Biosynthesis of Non-Proteinogenic α (Alpha), α (Alpha)-Dialkyl Amino Acids

Peptaibiotics are peptides rich in unusual and non-proteinogenic amino acid residues α (alpha), α (alpha)-dialkyl amino acids, such as Aib and occasionally Iva. The function of these α (alpha), α (alpha)-dialkyl amino acids is to promote the formation of helical structures and to increase the resistance to proteolytic degradation [65]. However, the biosynthesis of these two special amino acids has not been studied extensively in peptaibiotic-producing strains.

Aib can be incorporated in peptaibols, and adding Aib to the culture medium stimulates peptaibol formation [66, 67]. Kubicek et al. [16] and Raap et al. [65] proposed two likely pathways for Aib formation. In one, Aib is formed by its ste-

reochemical neighbor, e.g., L-alanine, which would imply one or (in the case of l-isovaline) two methyltransferase reactions, using adenosyl-methionine as a methyl group donor [16, 65]. However, these hypotheses require confirmation by molecular genetic evidence.

Regulation of Peptaibiotics Biosynthesis

Unlike the well-characterized nonribosomal peptide biosynthesis, the regulatory mechanisms of peptaibiotic biosynthesis are largely unknown. However, several lines of evidence lend insight into the regulation of peptaibiotics biosynthesis.

Schirmbock et al. reported that peptaibol production can be detected only when *T. atroviride* is cultured in liquid minimal medium with *Botrytis cinerea* cell walls [68], which suggests a coregulation of peptaibol biosynthesis and the formation of cell wall-degrading enzymes. Subsequently, Leclerc et al. found that the formation of peptaibiotics is strongly influenced by supplementing the culture medium with specific amino acids [67]. *T. longibrachiatum* M-853431 has the ability to form 20-residue longibachins and *T. harzianum* M-902608 is capable of producing 18-residue trichorzins PA and 14-residue harzianins PC. When Aib, Glu, or Arg was added to the fermentation medium, the microheterogeneous mixtures of these peptaibiotics were simplified in the 2 *Trichoderma* strains. Moreover, the addition of Aib to the culture of a *T. harzianum* strain generated new peptaibol molecules, in which all Iva residues were replaced by Aib, while the Glu-supply favored the synthesis of acidic longibrachins of *T. longibrachiatum* [67]. When free Aib or Iva is added to culture medium, the ratio between the peptaibols of *Emericellopsis salmosynnemata* can be manipulated [65]. If Aib is added, *E. salmosynnemata* produces Zrv-IIA as the major secondary metabolite, whereas the addition of DL-Iva to the culture increases the production of Zrv-IIB.

Many studies also found that surface cultures had to be used to produce peptaibiotics [15, 54], while occasionally no peptaibiotics could be detected in submerged cultivation even if the medium was identical [46]. Moreover, it is necessary to add an insoluble component, such as cellulose, for peptaibiotic production in submerged cultivation. In the meanwhile, there are several reports on peptaibiotics produced by *Trichoderma* in liquid culture [11, 69]. Generally, the culture time for peptaibiotic production in liquid culture is longer than in solid culture. Solid state fermentation techniques might provide a better choice for peptaibol production [70]. The data also suggest that there is a link between conidiation and peptaibol biosynthesis. A line of evidence has verified that peptaibol-atroviridins are not formed during vegetative growth of *T. atroviride* (*H. atroviridis)*, but a microheterogenous mixture of atroviridins accumulates when the colonies begin to sporulate [61]. The correlation between sporulation and atroviridin accumulation is independent of the pathway inducing sporulation. In addition, it was observed that atroviridin formation was dependent on the functions of two blue light regulators, BLR1 and BLR2, under some but not all conditions of sporulation, and was repressed in a *pkr1* (regulatory sub-

unit of protein kinase A) antisense strain with constitutively active protein kinase A. Conversely, loss of function of the Galpha-protein GNA3, which is a negative regulator of sporulation and whose deletion leads to a hypersporulating phenotype, fully impairs atroviridin formation. Komon-Zelazowska et al. concluded that the formation of atroviridin by *T. atroviride* (*H. atroviridis)* occurs in a sporulation-associated manner but is uncoupled at the GNA3 stage [61] .

LaeA is a global regulator in several fungi, especially *Aspergillus nidulans*, and affects the expression of multiple secondary metabolite gene clusters by modifying heterochromatin structure [71]. Recently, Karimi-Aghcheh et al. found that the LaeA ortholog of *T. reesei* (LAE1) positively regulates the expression of 7 of 17 polyketide or nonribosomal peptide synthases including a peptaibiotic synthase responsible for paracelsin synthesis [72]. Moreover, *lae1* overexpression has a greater impact than *lae1* loss on secondary metabolite gene expression in *T. reesei*. However, the precise molecular function of LAE1 and its mechanism of action on peptaibiotic synthesis remain unknown [72].

Taken together, a more global regulation of secondary metabolism may occur in peptaibiotic-producing strains. The regulation of secondary metabolism biosynthesis pathways is complex and involves several interconnected networks [73]. Much work remains to uncover the regulation of peptaibiotic biosynthesis.

Conclusion

As a class of nonribosomal peptide secondary metabolites, peptaibiotics exhibit multiple biological activities, including antimicrobial effects, antiviral effects, antiprotozoan activity, elicitation of systemic plant-defense responses, tissue damage in insect larvae, and cytolytic activity toward mammalian cells. However, it is a long way before peptaibiotics are good candidates as new therapeutic agents [74].

The last decade has witnessed a tremendous advance in our knowledge of peptaibiotics, including the biosynthetic mechanism. But gaps remain in our knowledge, such as substrate selectivity and the regulation of peptaibiotic biosynthesis. With the rapid increase in the number of sequenced fungal genomes, systematic classification will greatly assist in obtaining an overview of the NRPS genes of peptaibiotics. And the genome, transcriptome, and metabolome analyses of peptaibiotics producing fungi will provide new insights into the biosynthesis and molecular genetics. Further experiments are required to draw a clear picture of the biosynthesis pathway of peptaibiotics.

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References

1. Krause C, Kirschbaum J, Bruckner H (2006) Peptaibiomics: an advanced, rapid and selective analysis of peptaibiotics/peptaibols by SPE/LC-ES-MS. Amino Acids 30:435–443
2. Degenkolb T, Kirschbaum J, Bruckner H (2007) New sequences, constituents, and producers of peptaibiotics: an updated review. Chem Biodivers 4:1052–1067
3. Kredics L, Szekeres A, Czifra D, Vagvoelgyi C, Leitgeb B (2013) Recent results in alamethicin research. Chem Biodivers 10:744–771
4. Shi M, Zhang T, Sun L, et al (2013) Calpain, Atg5 and Bak play important roles in the crosstalk between apoptosis and autophagy induced by influx of extracellular calcium. Apoptosis 18:435–451
5. Whitmore L, Wallace BA (2004) The peptaibol database: a database for sequences and structures of naturally occurring peptaibols. Nucleic Acids Res 32:D593–594
6. Kisakürek MV (Editor-in-Chief) (2007) Special issue: Peptaibiotics I. Chem Biodivers 4:1021–1412 (Wiley-VCH Verlag, Weinheim)
7. Kisakürek MV (Editor-in-Chief) (2013) Special issue: Peptaibiotics II. Chem Biodivers 10:731–961 (Wiley-VCH Verlag, Weinheim)
8. Altherr W, Linden A, Heimgartner H (2007) The 'azirine/oxazolone method' in peptaibol synthesis: preparation of a derivative of trichotoxin A-50 (G). Chem Biodivers 4:1144–1169
9. Blaser P, Altherr W, Linden A, Heimgartner H (2013) Attempts toward the synthesis of the Peptaibol antiamoebin by using the 'azirine/oxazolone method'. Chem Biodivers 10:920–941
10. Anke H (2009) Peptaibiotics: fungal peptides containing alpha-dialkyl alpha-amino acids [Edited by Claudio Toniolo and Hans Brückner]. WILEY-VCH Verlag, Weinheim
11. Stoppacher N, Neumann NKN, Burgstaller L, Zeilinger S, Degenkolb T, Brueckner H, Schuhmacher R (2013) The comprehensive peptaibiotics database. Chem Biodivers 10:734–743
12. Whitmore L, Wallace BA (2004) Analysis of peptaibol sequence composition: implications for in vivo synthesis and channel formation. Eur Biophys J Biophys Lett 33:233–237
13. Degenkolb T, Graefenhan T, Berg A, Nirenberg HI, Gams W, Brueckner H (2006) Peptaibiomics: screening for polypeptide antibiotics (peptaibiotics) from plant-protective *Trichoderma* species. Chem Biodivers 3:593–610
14. Degenkolb T, Bruckner H (2008) Peptaibiomics: towards a myriad of bioactive peptides containing C(alpha)-dialkylamino acids? Chem Biodivers 5:1817–1843
15. Neuhof T, Dieckmann R, Druzhinina IS, Kubicek CP, von Doehren H (2007) Intact-cell MALDI-TOF mass spectrometry analysis of peptaibol formation by the genus *Trichoderma/Hypocrea*: can molecular phylogeny of species predict peptaibol structures? Microbiol-SGM 153:3417–3437
16. Kubicek CP, Komon-Zelazowska M, Sandor E, Druzhinina IS (2007) Facts and challenges in the understanding of the biosynthesis of peptaibols by *Trichoderma*. Chem Biodivers 4:1068–1082
17. Degenkolb T, Berg A, Gams W, Schlegel B, Grafe U (2003) The occurrence of peptaibols and structurally related peptaibiotics in fungi and their mass spectrometric identification via diagnostic fragment ions. J Pept Sci 9:666–678
18. Brueckner H, Becker D, Gams W, Degenkolb T (2009) Aib and iva in the biosphere: neither rare nor necessarily extraterrestrial. Chem Biodivers 6:38–56
19. Benedetti E, Bavoso A, Di Blasio B, Pavone V, Pedone C, Toniolo C, Bonora GM (1982) Peptaibol antibiotics: a study on the helical structure of the 2–9 sequence of emerimicins III and IV. Proc Natl Acad Sci U S A 79:7951–7954
20. Bruckner H, Graf H, Bokel M (1984) Paracelsin; characterization by NMR spectroscopy and circular dichroism, and hemolytic properties of a peptaibol antibiotic from the cellulolytically active mold Trichoderma reesei. Part B. Experientia 40:1189–1197

21. Pandey RC, Meng H, Cook JC Jr, Rinehart KL Jr (1977) Structure of antiamoebin I from high resolution field desorption and gas chromatographic mass spectrometry studies. J Am Chem Soc 99:5203–5205
22. Auvin-Guette C, Rebuffat S, Prigent Y, Bodo B (1992) Trichogin A IV, an 11-Residue Lipopeptaibol from *Trichoderma longibrachiatum*. J Am Chem Soc 114:2170–2174
23. Toniolo C, Crisma M, Formaggio F, Peggion C, Epand RF, Epand RM (2001) Lipopeptaibols, a novel family of membrane active, antimicrobial peptides. Cell Mol Life Sci 58:1179–1188
24. Jaworski A, Bruckner H (2000) New sequences and new fungal producers of peptaibol antibiotics antiamoebins. J Pept Sci 6:149–167
25. Rinehart Jr KL, Gaudioso LA, Moore ML, Pandey RC, Cook JC, Barber M, Sedgwick RD, Bordoli RS, Tyler AN, Green BN (1981) Structures of eleven zervamicin and two emerimicin peptide antibiotics studied by fast atom bombardment mass spectrometry. J Amer Chem Soc 103:6517–6520
26. Augeven-Bour I, Goulard C, Rebuffat S, Prigent Y, Auvin C, Bodo B (1997) Harzianin HB I, an 11-residue peptaibol from *Trichoderma harzianum*: isolation, sequence, solution synthesis and membrane activity. J Chem Soc, Perkin Trans 1:1587–1594
27. Ritzau M, Heinze S, Dornberger K, Berg A, Fleck W, Schlegel B, Hartl AM, Grafe U (1997) Ampullosporin, a new peptaibol-type antibiotic from *Sepedonium ampullosporum* HKI-0053 with neuroleptic activity in mice. J Antibiot (Tokyo) 50:722–728
28. Hulsmann H, Heinze S, Ritzau M, Schlegel B, Grafe U (1998) Isolation and structure of peptaibolin, a new peptaibol from *Sepedonium* strains. J Antibiot 51:1055–1058
29. Tsantrizos YS, Pischos S, Sauriol F, Widden P (1996) Peptaibol metabolites of *Tolypocladium geodes*. Can J Chem 74:165–172
30. Pruksakorn P, Arai M, Kotoku N, Vilcheze C, Baughn AD, Moodley P, Jacobs WR Jr, Kobayashi M (2010) Trichoderins, novel aminolipopeptides from a marine sponge-derived *Trichoderma sp.*, are active against dormant mycobacteria. Bioorganic Med Chem Lett 20:3658–3663
31. Closse A, Huguenin R (1974) Isolation and structural clarification of chlamydocin. Helvetica chimica acta 57:533–545
32. Degenkolb T, Gams W, Bruckner H (2008) Natural cyclopeptaibiotics and related cyclic tetrapeptides: structural diversity and future prospects. Chem Biodivers 5:693–706
33. Andersson MA, Mikkola R, Raulio M, Kredics L, Maijala P, Salkinoja-Salonen MS (2009) Acrebol, a novel toxic peptaibol produced by an *Acremonium exuviarum* indoor isolate. J Appl Microbiol 106:909–923
34. Chikanishi T, Hasumi K, Harada T, Kawasaki N, Endo A (1997) Clonostachin, a novel peptaibol that inhibits platelet aggregation. J Antibiot 50:105–110
35. Degenkolb T, Dieckmann R, Nielsen KF, et al (2008) The *Trichoderma brevicompactum* clade: a separate lineage with new species, new peptaibiotics, and mycotoxins. Mycol Progress 7:177–219
36. Chugh JK, Wallace BA (2001) Peptaibols: models for ion channels. Biochem Soc Trans 29:565–570
37. Bruckner H, Toniolo C (2013) Towards a myriad of peptaibiotics. Chem Biodivers 10:731–733
38. Krause C, Kirschbaum J, Bruckner H (2007) Peptaibiomics: microheterogeneity, dynamics, and sequences of trichobrachins, peptaibiotics from *Trichoderma parceramosum* Bissett (*T. longibrachiatum* Rifai). Chem Biodivers 4:1083–1102
39. Kalb D, Lackner G, Hoffmeister D (2013) Fungal peptide synthetases: an update on functions and specificity signatures. Fungal Biol Rev 27:43–50
40. Fischbach MA, Walsh CT (2006) Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. Chem Rev 106:3468–3496
41. Evans BS, Robinson SJ, Kelleher NL (2011) Surveys of non-ribosomal peptide and polyketide assembly lines in fungi and prospects for their analysis *in vitro* and *in vivo*. Fungal Genet Biol 48:49–61

42. Reusser F (1967) Biosynthesis of antibiotic U-22,324, a cyclic polypeptide. J Biol Chem 242:243–247
43. Kleinkauf H, Rindfleisch H (1975) Non-ribosomal biosynthesis of the cyclic octadecapeptide alamethicin. Acta Microbiol Acad Sci Hung 22:411–418
44. Rindfleisch H, Kleinkauf H (1976) Biosynthesis of alamethicin. FEBS Lett 62:276–280
45. Mohr H, Kleinkauf H (1978) Alamethicin biosynthesis: acetylation of the amino terminus and attachment of phenylalaninol. Biochim Biophys Acta 526:375–386
46. Wiest A, Grzegorski D, Xu BW, Goulard C, Rebuffat S, Ebbole DJ, Bodo B, Kenerley C (2002) Identification of peptaibols from *Trichoderma virens* and cloning of a peptaibol synthetase. J Biol Chem 277:20862–20868
47. Wei X, Yang F, Straney DC (2005) Multiple non-ribosomal peptide synthetase genes determine peptaibol synthesis in *Trichoderma virens*. Can J Microbiol 51:423–429
48. Viterbo A, Wiest A, Brotman Y, Chet I, Kenerley C (2007) The 18mer peptaibols from *Trichoderma* virens elicit plant defence responses. Molecul Plant Pathol 8:737–746
49. Wilhite SE, Lumsden RD, Straney DC (2001) Peptide synthetase gene in *Trichoderma virens*. Appl Environ Microb 67:5055–5062
50. Vizcaino JA, Cardoza RE, Dubost L, Bodo B, Gutierrez S, Monte E (2006) Detection of peptaibols and partial cloning of a putative peptaibol synthetase gene from *T-harzianum* CECT 2413. Folia Microbiologica 51:114–120
51. Vizcaino JA, Sanz L, Cardoza RE, Monte E, Gutierrez S (2005) Detection of putative peptide synthetase genes in *Trichoderma* species: application of this method to the cloning of a gene from *T. harzianum* CECT 2413. FEMS microbiol lett 244:139–148
52. Chutrakul C, Peberdy JF (2005) Isolation and characterisation of a partial peptide synthetase gene from Trichoderma asperellum. Fems Microbiol Lett 252:257–265
53. Mukherjee PK, Wiest A, Ruiz N, Keightley A, Moran-Diez ME, McCluskey K, Pouchus YF, Kenerley CM (2011) Two classes of new peptaibols are synthesized by a single non-ribosomal peptide synthetase of *Trichoderma virens*. J Biol Chem 286:4544–4554
54. Degenkolb T, Aghcheh RK, Dieckmann R, Neuhof T, Baker SE, Druzhinina IS, Kubicek CP, Brueckner H, von Doehren H (2012) The production of multiple amall peptaibol families by single 14-module peptide synthetases in *Trichoderma/Hypocrea*. Chem Biodivers 9:499–535
55. Wenzel SC, Kunze B, Hofle G, Silakowski B, Scharfe M, Blocker H, Muller R (2005) Structure and biosynthesis of myxochromides S1-3 in *Stigmatella aurantiaca*: evidence for an iterative bacterial type I polyketide synthase and for module skipping in nonribosomal peptide biosynthesis. Chem biochem 6:375–385
56. Wenzel SC, Meiser P, Binz TM, Mahmud T, Muller R (2006) Nonribosomal peptide biosynthesis: point mutations and module skipping lead to chemical diversity. Angewandte Chemie 45:2296–2301
57. Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, et al (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol 12:R40
58. Mukherjee PK, Horwitz BA, Herrera-Estrella A, Schmoll M, Kenerley CM (2013) *Trichoderma* research in the genome era. Annu Rev Phytopathol 51:105–129
59. Xie BB, Qin QL, Shi M, et al (2014) Comparative genomics provide insights into evolution of *Trichoderma* nutrition style. Genome Biol Evol 6:379–390
60. Martinez D, Berka RM, Henrissat B, et al (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nature Biotechnol 26:553–560
61. Komon-Zelazowska M, Neuhof T, Dieckmann R, von Dohren H, Herrera-Estrella A, Kubicek CP, Druzhinina IS (2007) Formation of atroviridin by *Hypocrea atroviridis* is conidiation associated and positively regulated by blue light and the G protein GNA3. Eukaryot Cell 6:2332–2342
62. Lautru S, Challis GL (2004) Substrate recognition by nonribosomal peptide synthetase multienzymes. Microbiol-SGM 150:1629–1636

63. Manavalan B, Murugapiran SK, Lee G, Choi S (2010) Molecular modeling of the reductase domain to elucidate the reaction mechanism of reduction of peptidyl thioester into its corresponding alcohol in non-ribosomal peptide synthetases. BMC Structural Biol 10:1
64. Gao X, Haynes SW, Ames BD, Wang P, Vien LP, Walsh CT, Tang Y (2012) Cyclization of fungal nonribosomal peptides by a terminal condensation-like domain. Nat Chem Biol 8:823–830
65. Raap J, Erkelens K, Ogrel A, Skladnev DA, Bruckner H (2005) Fungal biosynthesis of non-ribosomal peptide antibiotics and alpha, alpha-dialkylated amino acid constituents. J Pept Sci 11:331–338
66. Brewer D, Mason FG, Taylor A (1987) The production of alamethicins by *Trichoderma* spp. Can J Microbiol 33:619–625
67. Leclerc G, Rebuffat S, Goulard C, Bodo B (1998) Directed biosynthesis of peptaibol antibiotics in two *Trichoderma* strains I. Fermentation and isolation. J Antibiot 51:170–177
68. Schirmbock M, Lorito M, Wang YL, Hayes CK, Arisan-Atac I, Scala F, Harman GE, Kubicek CP (1994) Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Appl Environ Microbiol 60:4364–4370
69. Roehrich CR, Vilcinskas A, Brueckner H, Degenkolb T (2013) The sequences of the eleven-residue peptaibiotics: Suzukacillins-B. Chem Biodivs 10:827–837
70. Song XY, Shen QT, Xie ST, Chen XL, Sun CY, Zhang YZ (2006) Broad-spectrum antimicrobial activity and high stability of Trichokonins from *Trichoderma koningii* SMF2 against plant pathogens. Fems Microbiol Lett 260:119–125
71. Butchko RA, Brown DW, Busman M, Tudzynski B, Wiemann P (2012) Lae1 regulates expression of multiple secondary metabolite gene clusters in *Fusarium verticillioides*. Fungal Genet Biol 49:602–612
72. Karimi-Aghcheh R, Bok JW, Phatale PA, et al (2013) Functional analyses of *Trichoderma reesei* LAE1 reveal conserved and contrasting roles of this regulator. G3 (Bethesda) 3:369–378
73. Brakhage AA (2013) Regulation of fungal secondary metabolism. Nat Rev Microb 11:21–32
74. Duclohier H (2007) Peptaibiotics and peptaibols: an alternative to classical antibiotics? Chem Biodivers 4:1023–1026

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