reviews

molecular pharmaceutics

In Vivo and In Vitro Production Options for Fungal Secondary Metabolites

Patrick Schneider,[†] Mathias Misiek,[†] and Dirk Hoffmeister*[,‡]

Pharmaceutical Biology and Biotechnology, Albert-Ludwigs-Universität, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany, and Department of Plant Pathology, University of Minnesota—Twin Cities Campus, 1991 Upper Buford Circle, St. Paul, Minnesota 55108

Received December 26, 2007; Revised Manuscript Received February 25, 2008; Accepted February 25, 2008

Abstract: Microbial natural products, among them a vast diversity of fungal origin, represent a major source for new drug candidates. Focusing on fungal metabolites, our review covers recent advances in the field of biotransformation, heterologous expression, *in vivo* production approaches, genomics, and the metabolism of unexplored fungal groups as options to generate and identify new compounds or optimize known ones.

Keywords: Natural products; fungi; secondary metabolism; pharmaceutical biotechnology; biotransformation; heterologous expression; genomics

Introduction

Natural products continue to serve the pharmaceutical sciences as an abundant source of new drug leads. The ingenuity of natural product assembly and their structural complexity inspires chemists, the bioactivities of natural products fascinate pharmacists, and their relevance in ecosystems, if known at all, intrigues biologists, thus making natural product research truly and intrinsically interdisciplinary. We would like to refer our readers to recent review articles for the bird's eye view on the various facets of natural products from fungal and other sources.[1–7] The share of clinically used natural products, mainly of microbial origin, or compounds directly derived from those varies between medical indications. Yet, the overall picture shows that natural products—including semisynthetic derivatives as well as synthetic compounds mimicking naturally occurring pharmacophores—account for approximately 50% of new chemical entities approved for medicinal use in the years 1981–2006.[8,9] However, the currently known chemical diversity

* To whom correspondence should be addressed. Mailing address: University of Minnesota, Plant Pathology, 1991 Upper Buford Circle, St. Paul, MN 55108. E-mail: dirkh@umn.edu. Phone: 612-624-2291. Fax: 612-625-9728.

[†] Albert-Ludwigs-Universität.

[‡] University of Minnesota.

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10.1021/mp7001544 CCC: $40.75
Published on Web 03/11/2008

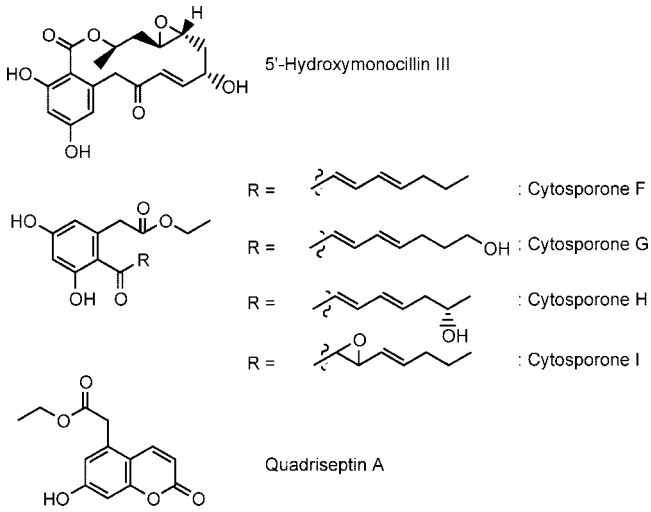

Figure 1. Chemical structures of cytosporones F−I, quadriseptin A, and 5′-hydroxymonocillin III, isolated from the cultures of two plant-associated fungi, *Paraphaeosphaeria quadriseptata* and *Chaetomium chiversii,* after a change in the fermentation protocol.

which filamentous fungi provide to pharmaceutical chemists represents only the tip of the iceberg: completed genome sequences of fungi suggest that the biosynthetic abilities for natural products reach far beyond what was expected by chemical analysis prior to the genome projects, as recently demonstrated, *e.g*., for various *Aspergillus* species.[10,11]

In the past, much effort was directed toward optimizing the production of those drugs which have already been introduced into clinical practice. Researchers primarily relied on classical approaches like chemical or UV-mutagenesis[12] or varied fermentation parameters, as described for β-lactam antibiotics[13] and pneumocandins in great detail.[14] Eliciting the production of unknown compounds was successfully accomplished by the one-strain-many-compounds approach (OSMAC), which systematically varies the fermentation parameters,[15] or by adding plant exudates to cultures.[16] A recent validation of how fermentation parameters impact upon the secondary metabolite spectrum was reported by Gunatilaka et al.,[17] who discovered six new metabolites, the cytosporones F−I, quadriseptin A, and 5′-hydroxymonocillin III (Figure 1), after replacing tap water with distilled water for the culture broth of two plant-associated fungi, *Paraphaeosphaeria quadriseptata* and *Chaetomium chiversii*. All of these approaches anticipate the genetic and physiological abilities of fungi to produce a broader range of small molecules than evident from previous chemical analyses.

Following recent estimates, only about 6–7% of fungal species are known and have been described.[18] Even when we take some redundancy into account, the biggest portion of the fungal natural product pool whose wealth seems practically limitless remains to be discovered. Also, it is generally accepted that evolution of a given natural product pathway coincided with a phenotypical biological validation of the compound, which means the diversity we see at present does very likely not merely cover random areas in the chemical space.[19,20]

Making headway toward production of fungal secondary metabolites relies on more than one route. We structure our article by approaches and cover recent developments in (i) biotransformation, (ii) heterologous expression and *in vitro* approaches, (iii) genomics, and (iv) new compounds from uninvestigated fungi.

Biotransformation

Filamentous fungi have widely and successfully been used for biotransformations ranging from food production and bioremediation by breakdown of environmentally hazardous compounds to the catalysis of particular steps from a (in most cases synthetic) drug lead to the final product. Eminent examples, such as the 11-α-hydroxylation of progesterone by the fungus *Rhizopus nigricans,* have been reviewed by Chartrain and Sturr.[21] With regard to natural product derivatization, biotransformations by either precursor-directed biosynthesis (using a wild-type producer) or mutasynthesis (using a biosynthetically deficient strain) emerged as a valuable production and modification option. Very recently, a review article covering both microbial and plant metabolites appeared which we recommend for a comprehensive overview.[22] For a detailed description of the textbook example

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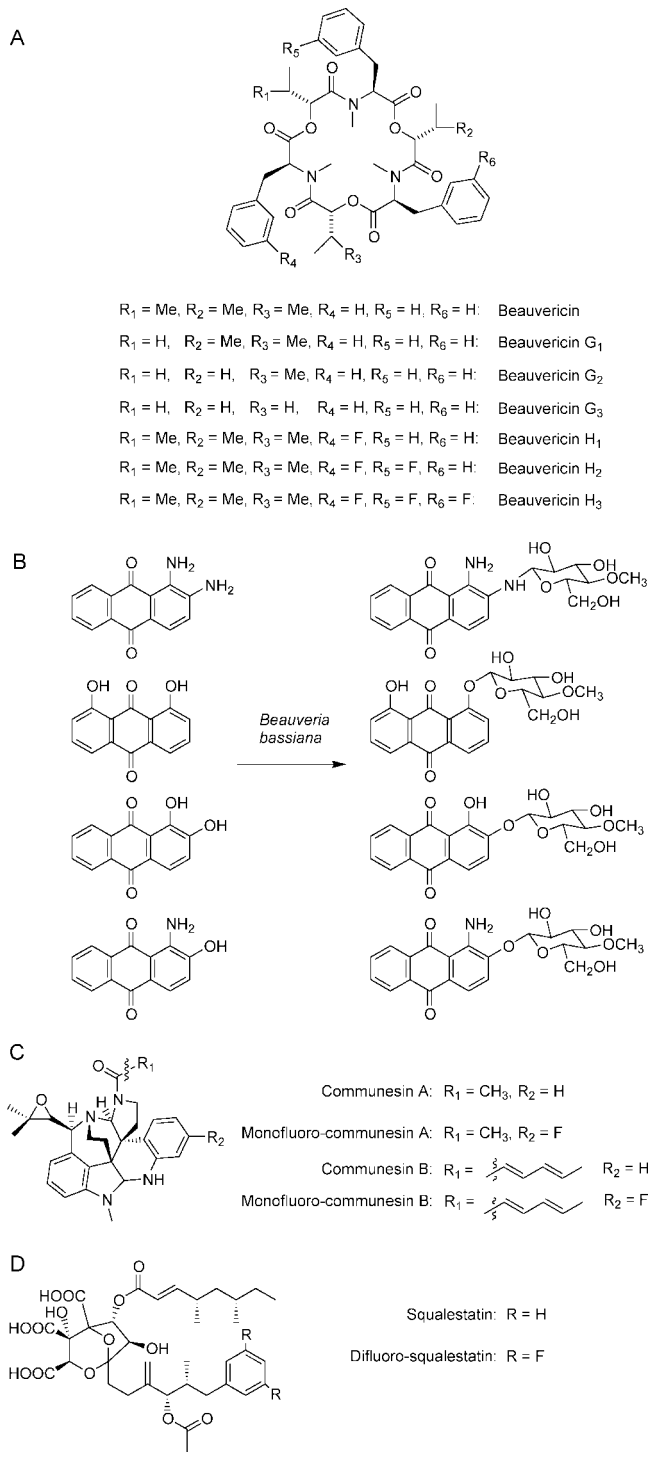

Figure 2. Fungal biotransformations: (A) the structures of new beauvericins; (B) anthraquinone glycosylations by *Beauveria bassiana* cultures; (C) chemical structures of fluorinated communesin analogues; (D) one example of fluorinated squalestatins, obtained after feeding of fluorinated benzoic acid to cultures of an unidentified *Phoma* species.

for biotransformation, the β-lactam antibiotics, we refer the reader to reviews, *e.g.*, by Demain and Elander[23] and Elander.[13] Beauvericin (Figure 2 A) is a cyclic (2*R*)-2-hydroxy-3-methylbutanoyl-*N*-methyl-L-phenylalanine trimer, synthesized by a nonribosomal peptide synthetase. Beauvericin exhibits insecticidal and antimicrobial activities, yet received most attention for its cytotoxic effects. In two parallel approaches Molnar and co-workers generated modified derivatives *in vivo* by offering substrate mimics of either monomeric building block (D-hydroxyisovalerate or L-phenylalanine) to submer cultures of *Beauveria bassiana* ATCC 7159:[24] The substitution of one, two, or all three D-hydroxyisovalerate units by 2-hydroxybutyrate yielded beauvericins G_{1-3} whereas feeding 3-fluorophenylalanine led to the corresponding fluorinated analogues, the beauvericins H_{1-3} (Figure 2 A). Besides creation of modified beauvericins, which would have been very difficult to accomplish chemically, these bioconversions also shed more light on structural requirements of the pharmacophore, as beauvericin of the G-series showed decreased antihaptotactic properties, while the beauvericins H were somewhat increased in cytotoxicity. The biosynthetic potential of *Beauveria* was also utilized for biotransformations of anthraquinones. Feeding 1-amino-2-hydroxyanthraquinone, 1,2-diaminoanthraquinone, 1,8-dihydroxyanthraquinone, and 1,2-dihydroxyanthraquinone to submers cultures of *Beauveria bassiana* ATCC 7159 resulted in selective glycosylation of the 2-amino group of 1,2-diaminoanthraquinone and remarkable yields (46–72%) of other *N*- and *O*-4′-*O*-methyl-glucosylated anthraquinones (Figure 2 B).[25] Similar approaches included antileukemic communesins and antihypercholestaemic squalestatins (Figure 2 C): A *Penicillium* species known to produce communesins accepted 6-fluorotryptophan as building block for secondary metabolism, yielding the corresponding monofluoro analogues of communesins A and B.[26] A *Phoma* species fed with fluorinated benzoic acids incorporated those into the squalestatins (Figure 2 D).[27]

Heterologous Expression and *In Vitro* Approaches

Filamentous fungi are established eukaryotic expression systems for industrial enzymes and therapeutic proteins.[28] Here, we highlight pertinent reports on manipulated production of compounds in fungal systems, yet also overexpression of fungal genes in bacteria for *in vivo* or *in vitro* use. While

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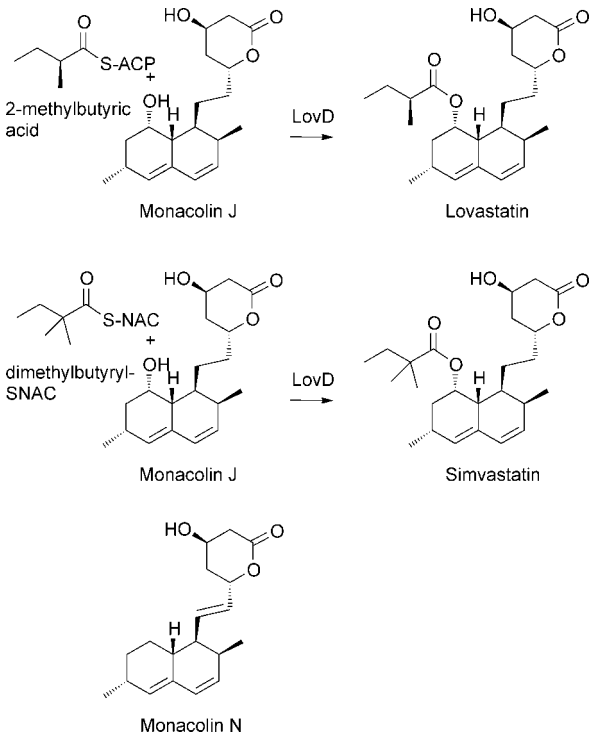

Figure 3. Production of statins: *Aspergillus terreus* uses the enzyme LovD to form an ester bond between monacolin J and 2-methylbutyric acid, which is provided as carrier protein (ACP)-bound thioester. Heterologously overexpressed LovD accepted an *N*-acetylcysteamine (SNAC)-thioester of dimethylbutyric acid as substrate to convert monacolin N into simvastatin. Monacolin N was found as a new statin after heterologous overexpression of the lovastatin biosynthesis enzymes LovB and LovC in *Aspergillus nidulans*.

the above examples reflect work on wild-type strains, the statins illustrate how bacterial cells overexpressing a fungal enzyme have successfully been used for biotransformations as well: during lovastatin biosynthesis in *Aspergillus terreus*, the ultimate biosynthetic step is the LovD-catalyzed acyl transfer to the C-8 hydroxyl of monacolin J, to yield the final product, lovastatin. Using LovD, heterologously overexpressed in *E. coli*, Tang and colleagues demonstrated that LovD transferred, albeit with different efficiencies, about 20 CoA-thioesters or *N*-acetylcysteamine (SNAC) -thioesters to monacolin J, among them α-dimethylbutyryl-SNAC (Figure 3). As an elegant strategy to create new analogues, this heterologous expression/biotransformation approach represents a shortcut for simvastatin synthesis as it can be produced from monacolin J and α-dimethylbutyryl-SNAC in a single, LovD-catalyzed step.[29]

The production of β-lactams in genetically engineered aspergilli represents a textbook example of this category of production options. An overview over early *in vivo* and *in vitro* attempts to enhance β-lactam production is provided by Skatrud.[30] One of the key enzymes during penicillin biosynthesis, the δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACV-synthetase, Figure 4), is encoded by the *Aspergillus nidulans* gene *acvA*. Replacing the native promoter with the inducible alcohol dehydrogenase promoter (p)*alcA* increased titers about 30-fold when the *acvA* gene was induced with cyclopentanone.[31] A very recent example shows that heterologous gene expression is still a viable option for β-lactam production *in vivo*.[32] A *Penicillium chrysogenum* strain blocked in the final step of penicillin G biosynthesis served as host to overexpress the "late" cephalosporin-specific genes *cefEF* and *cefG*, *i.e.*, the bifunctional expandase/hydroxylase and deacetylcephalosporin-acetyltransferase, respectively, along with *cefD1* and *cefD2* (isopenicillin N-CoA synthetase and isopenicillin N-CoA epimerase) from *Acremonium chrysogenum* (= *Cephalosporium acremonium*) to epimerize isopenicillin N into penicillin N and then extend the metabolic flow further to deacetylcephalosporin C (Figure 4).

Genetic engineering of fungi was successfully used to produce polyketides as well. Elucidating the biosynthetic events for lovastatin assembly in *Aspergillus terreus*, Hutchinson and co-workers overexpressed the genes for the key enzymes LovB and LovC in *A. nidulans*, which then produced dihydromonacolin L, the first recognized intermediate en route to lovastatin.[33] Follow-up work on this engineered strain identified a new derivative, monacolin N (Figure 3).[34]

Recent examples for heterologously expressed fungal proteins utilized for *in vitro* syntheses of natural compounds

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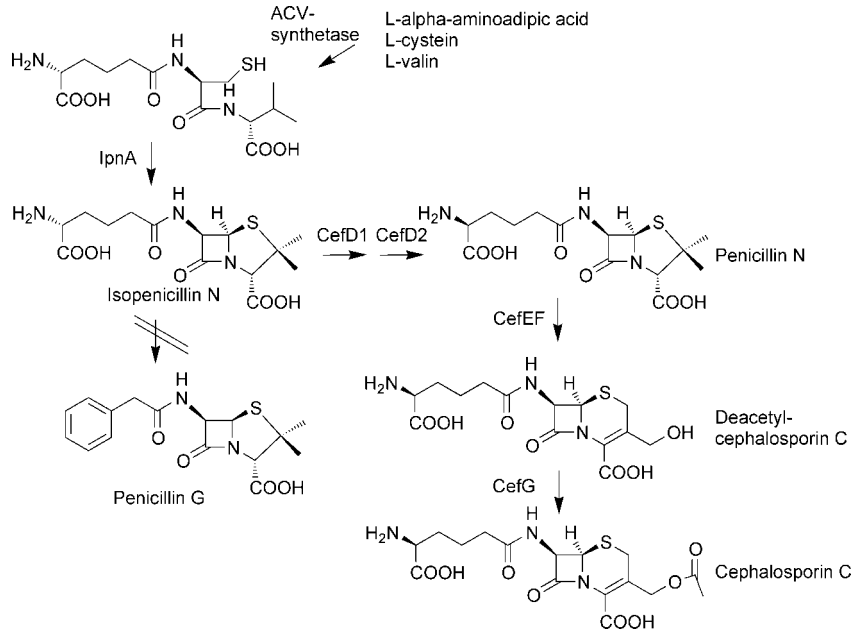

Figure 4. Heterologous expression of β-lactam biosynthesis genes in an engineered *Penicillium chrysogenum* strain blocked in the step from isopenicillin N to penicillin G diverted its production to deacetylcephalosporin C and cephalosporin C. The cephalosporin-specific enzymes isopenicillin N-CoA synthetase (CefD1), isopenicillin N-CoA epimerase (CefD2), expandase/hydroxylase (CefEF) and deacetylcephalosporin-acetyltransferase (CefG) are encoded by genes taken from *Acremonium chrysogenum*. Cephalosporin C was produced by the transformants, yet not secreted into the culture broth.

include bikaverin-related polyketides, the amino acid derived terrequinone A, and the nonribosomally synthesized peptide enniatin.

After expression of the polyketide synthase PKS4 from the plant pathogen *Gibberella fujikuroi* in *E. coli* and the subsequent purification of this protein the compound SMA76a (Figure 5A), which is the unmethylated and nonoxidized nonaketide precursor of bikaverin, was synthesized. Intriguingly, PKS4 preferred the unnatural starter unit octanoyl-CoA over malonyl-CoA. Feeding of octanoyl-CoA led to the formation of the bicyclic aromatic penta- and hexaketides SMA76b and SMA76c (Figure 5 A).[35] An engineered version of PKS4 which underwent inactivation of its thioesterase/cyclase domain led to SMA93 (Figure 5 A), a new, differently cyclized poyketide with two distinct ring systems (a benzopyrone and dihydroxyphenyl system). Upon coincubating this engineered enzyme with streptomycete PKS-enzymes, such as a ketoreductase from the actinorhodin pathway, bikaverin-unrelated polyketides were produced.[36]

In an attempt to diversify the fungal enniatin cyclodepsipeptide family, the purified multimodular enniatin synthetase (ESyn) was used to incorporate mimics of one of its natural substrates, α-D-hydroxyisovaleric acid, *in vitro*. The intrinsic isopropyl side chain could be replaced, at varied efficiencies, by ethyl, chloromethyl, bromomethyl, fluoromethyl, propargyl and other groups (Figure 5 B).[37]

Asterriquinones are tryptophan-derived fungal quinone-alkaloids. Using biosynthetic enzymes encoded in the *Aspergillus nidulans tdi*-gene cluster which were overexpressed in *E. coli*, the *in vitro* syntheses of didemethylasterriquinone D, the generic precursor for all asterriquinones, and terrequinone A, an asymmetrically diprenylated derivative, have been reported (Figure 5 C).[38,39] The asterriquinones show multiple bioactivities and spawned particular interest as orally available antidiabetes agents and, more recently, as nerve growth factor receptor agonists, which may hold potential for the treatment of neurodegenerative health conditions.

Genomics

The availability of entire genomes is an exciting means to identify and decipher cryptic biosynthetic abilities.

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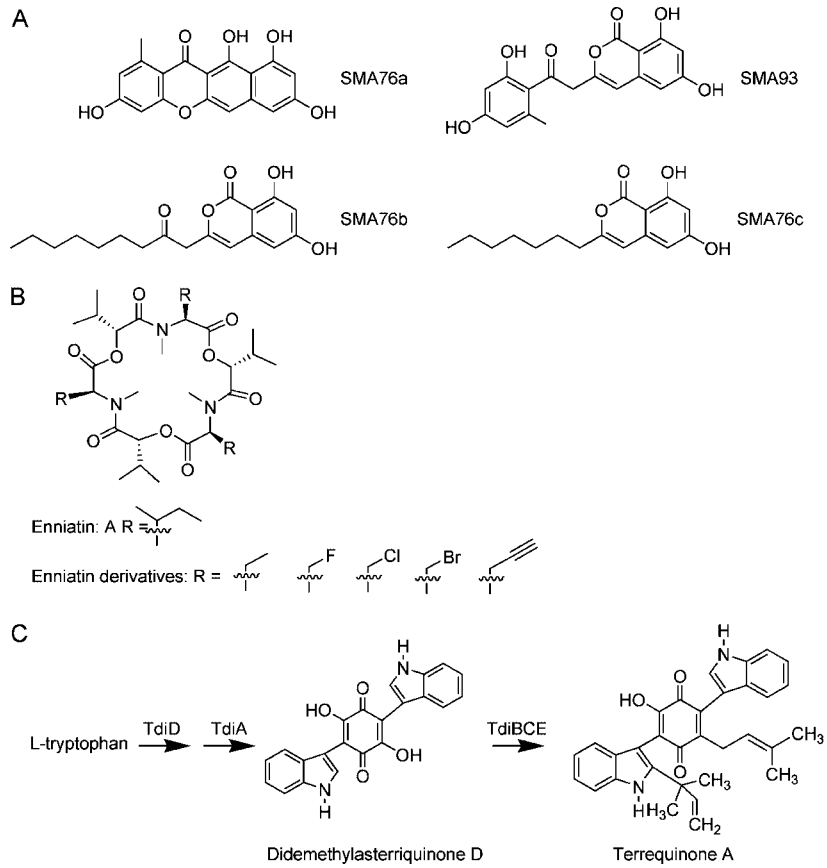

Figure 5. Heterologous expression: (A) New polyketides SMA76a, SMA76b, SMA76c, and SMA93 produced by the *Gibberella fujikuroi* bikaverin polyketidsynthase PKS4; (B) chemical structure of enniatin and new derivatives, generated *in vitro*; (C) *Aspergillus nidulans tdi*-genes, overexpressed in *E. coli*, were used for *in vitro* synthesis of the bisindolylquinones didemethylasterriquinone D and terrequinone A.

Completed genomes provide us with an intimation of the true chemical diversity that is waiting to be discovered. Among the most intriguing genomes, seen from the drug discovery angle, are those from the aspergilli, *e.g.*, the recently published genomes from *Aspergillus nidulans*, *A. niger*, and *A. oryzae*.[10,40,41] Using the numbers of genes for polyketide synthases and nonribosomal peptide synthetases as indicators, aspergilli are unexpectedly rich in genetic loci dedicated to secondary metabolism as they harbor between 30 and 50 distinct clusters per species.[11] Comparative genomics demonstrates that the majority of pathways identified in aspergilli is neither redundant in different species nor duplicated within a given genome. Similar estimates of the metabolic wealth can also be made for other fungi.

Using a microarray which covered the full genome of the lovastatin (Figure 3) producer *Aspergillus terreus*, Madden and co-workers correlated transcriptional profiles with metabolic trends (*i.e.*, decreased or increased titers of lovastatin and other natural products) to identify sets of genes encoded within or outside biosynthesis gene clusters associated with the de or increase.[42] The authors found a positive correlation of *lovF* expression and lovastatin titers. *lovF* is a biosynthetic gene in the lovastatin pathway, which integrates multiple physiological cues to regulate lovastatin production. Using the *lovF* promoter to drive the expression of the phleomycin resistance gene, the authors presented a simple selection system to identify improved producer strains.

A strategy to mine the genome for unknown biosynthetic capabilities in *A. nidulans* is based on the global transcriptional regulator LaeA. It regulates selectively and a substantial number of secondary metabolite genes.[43,44] The notion of LaeA being a selective regulator has recently been

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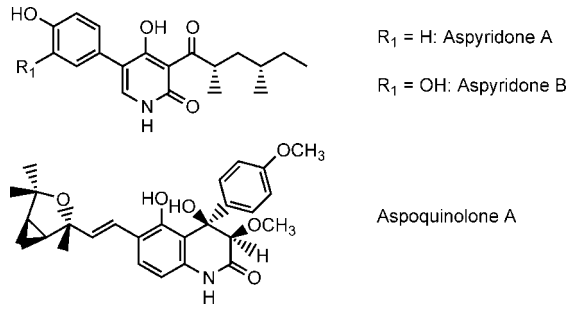

Figure 6. Chemical structures of *Aspergillus nidulans* natural products aspyridones A and B, and aspoquinolone A whose biosynthetic genes were identified (or production was anticipated) through genomics.

confirmed in the pathogen *Aspergillus fumigatus*.[45] The proof of principle for LaeA-based identification was provided by the discovery of the terrequinone A (Figure 5 C) gene cluster, without prior evidence that *A. nidulans* produces asterriquinones at all.

Recently, the corresponding metabolite for another *Aspergillus* natural product gene locus in search of function has been identified: Hertweck and co-workers elegantly varied the genomic approach by homologously overexpressing a Zn_2Cys_6-regulator gene from a cluster of natural product biosynthesis genes which codes, among others, for an unknown NRPS-PKS hybrid enzyme. Via this approach hitherto unknown compounds now referred to as aspyridones (Figure 6) were identified.[46] Also, as the *A. nidulans* genome codes for multiple anthranilate synthases, biosynthetic abilities for quinoline or quinazoline alkaloids were anticipated and verified by isolation of aspoquinolone A (Figure 6) and three derivatives, however without assigning their biosynthesis to a particular gene.[47]

New Compounds from Uninvestigated Fungi and Organismic Interactions

The majority of examples presented in this review included either established classes of pharmaceuticals like statins or β-lactams, or familiar organisms, such as the penicilli and aspergilli. While these still hold enormous potential, as shown in the previous section, much opportunity lies in unexplored fungal taxonomic groups or fungal habitats, such as marine species, endolichenic, or insect-associated fungi, and others. In recent years new drugs based on fungal natural products have entered clinical practice or trials.[5] Most of them were developed as antiinfective or anticancer agents, among them two metabolites from conspicuous fruiting body-forming basidiomycetes *e.g.*, the topical antibiotic retapamulin (Altabax, Figure 7) to treat infections with methicillin-susceptible streptococci and staphylococci.[48] Retapamulin is derived from pleuromutilin (Figure 7), a terpene metabolite from *Clitopilus scyphoides* (= *Pleurotus mutilus*). Irofulvene, a potent semisynthetic anticancer agent and derivative of the sesquiterpene illudin S (Figure 7) from the basidiomycete *Omphalotus illudens*, is currently investigated in phase II trials against prostate, ovarian, and other cancers.[49,50]

About one hundred natural products from marine fungi have been described so far.[51] NPI-2358 (Figure 7), a derivative of the diketopiperazine (−)-halimide which was isolated from a marine *Aspergillus* sp., has potent tubulin depolymerizing activity and has entered phase I of clinical trials for treatment of advanced solid tumor malignancies or lymphoma.[52]

Lichens are symbiotic associations of a fungus (the mycobiont) and algae and/or cyanobacteria, the photobiont. In addition, lichens can be inhabited by further endolichenic fungi which are not part of the actual symbiosis. Very recently, the first investigation ever on the secondary metabolites of endolichenic fungi was reported. New polyketides (the heptaketides corynesporol and 1-hydroxydehydroherbarin, Figure 7 A) were purified from cultures of a *Corynespora* species isolated from the beard lichen *Usnea cavernosa*.[53]

Very little is known about mycophilic fungi, *i.e.*, those which live closely associated with other fungal species. From an *Epicoccum* species taken from the inside of conspicuous fruiting bodies of the mushroom *Pholiota squarrosa* ("Shaggy

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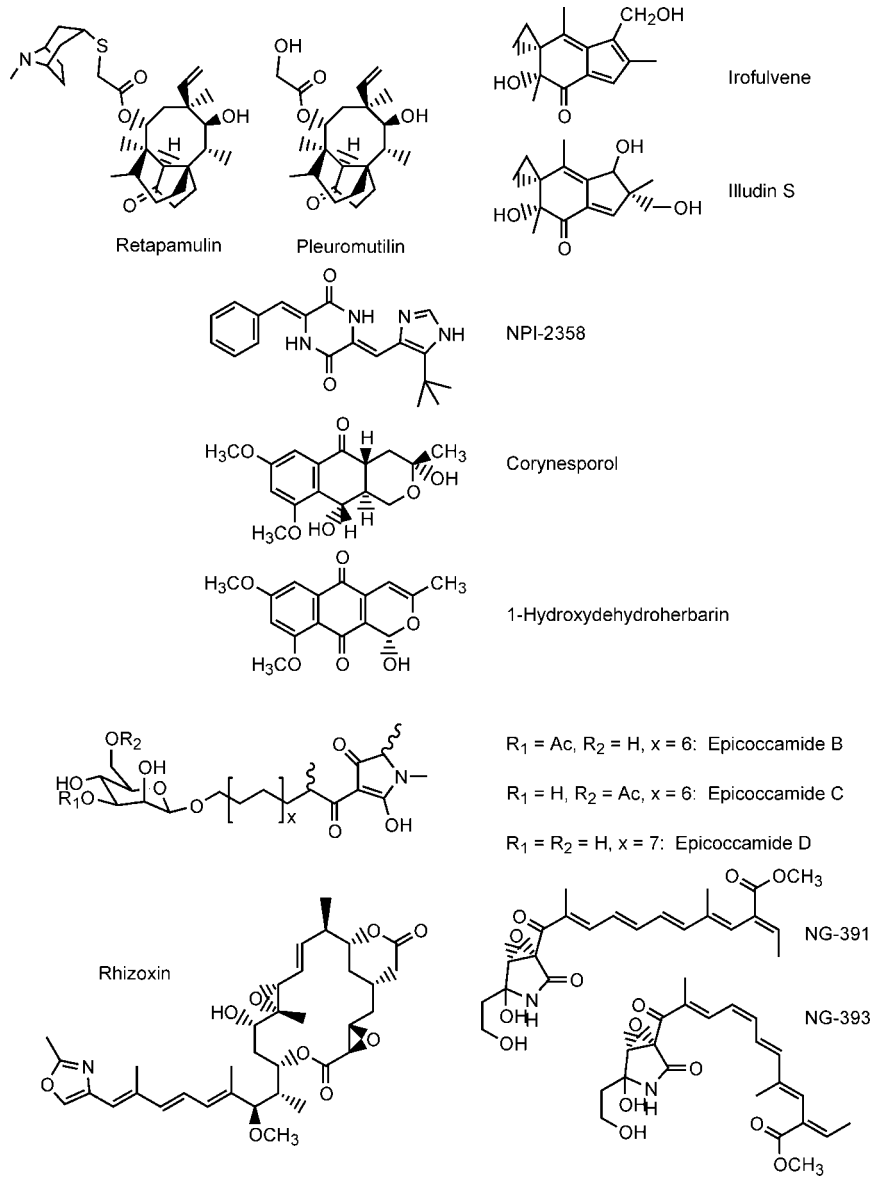

Figure 7. Miscellaneous fungal secondary metabolites and semisynthetic derivatives.

Scalycap") the new epicoccamides B−D (Figure 7), moderately cytotoxic glycosylated tetramic isolated acids, were isolated.[54]

Insect associated clavicipitaceous fungi represent another emerging group for natural product discovery. In the past these fungi were mainly explored by invertebrate pathologists for biocontrol purposes. Yet, the prolific secondary metabolism of these fungi also provided such prominent compounds as cyclosporine, produced by *Tolypocladium inflatum*, the anamorph (asexual state) of the insect pathogen *Cordyceps subsessilis*. A PCR-based survey on the occurrence of polyketide synthases in 157 fungi sampled from insects or nematodes identified 76 unique PKS-gene fragments.[55] *Metarhizium anisopliae* is an entomopathogenic fungus, perhaps the asexual stage of a *Cordyceps* species, which is commercially used as a biological insecticide to control insects like grasshoppers and termites. Nerve-cell growth stimulating secondary metabolites, NG-391 and NG-393 (Figure 7), were isolated from this fungus. However, these compounds also showed a potent cytotoxic activity.[56]

The term endophyte describes microorganisms—among them countless fungi—which occupy a special ecological niche in that they live inside plants in the intracellular space, however do not cause overt symptoms or exert malicious effects. As the closely interacting mutualistic or symbiotic

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lifestyle has considerable impact on secondary metabolism, these organisms can be expected to represent a rich source of secondary metabolites.[57,58] In fact, many fungi in plant−fungal symbioses produce bioactive metabolites, *e.g.*, as a chemical defense against herbivores exemplified best by the grass-endophyte *Neotyphodium uncinatum* which produces the strongly insecticidal loline alkaloids.[59] The lolines also tell us about a potential drawback when dealing with such highly specialized organisms, as the cultivation of the isolated fungus outside its natural environment may lead to significantly reduced metabolite titers. Much optimization is then required to stimulate secondary metabolism the way natural symbiosis does.[60]

Another pertinent example includes the morning glory plants and their ergot alkaloids produced by seed-transmitted endophytic clavicipitaceous fungi thereby conferring drought resistance on and deterring pests from their hosts.[61]

One intriguing development evolved around *Muscodor albus*, an endophytic fungus isolated from a cinnamon tree (*Cinnamomum zeylanicum*) in Honduras. The fungus was found to produce about 30 volatile organics compounds, among them various alcohols, esters, ketones, and others, which, as a synergistic mixture, have strong microbicidal effects on bacteria and fungi.[62] The mixture has been developed into a commercial product to treat—"mycofumigate"—fruits in transit or storage and seeds to control microbial contamination.

In return, not all potent fungal metabolites are truly fungal ones: the plant-pathogenic zygomycete *Rhizopus microsporus* has long been attributed with the production of rhizoxin (Figure 7). Derivatives thereof rank among the most potent antimitotic agents. Recently, *Burkholderia rhizoxina*, a bacterial endosymbiont inhabiting the inside of the fungal mycelium, has been identified as the true producer of this secondary metabolite.[63]

Acknowledgment. D.H.'s research is supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG), by the Foundation of the Chemical Industry (Fonds der Chemischen Industrie, FCI), by a seed grant of the University of Minnesota, and by the Minnesota Agricultural Experiment Station (MAES). M.M. gratefully acknowledges a predoctoral fellowship of the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt, DBU).

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