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<th>Journal:</th>
<th><em>Natural Product Reports</em></th>
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<td>Manuscript ID:</td>
<td>NP-REV-04-2014-000046.R1</td>
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<tr>
<td>Article Type:</td>
<td>Review Article</td>
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<tr>
<td>Date Submitted by the Author:</td>
<td>05-Jul-2014</td>
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New insights into the echinocandins and other fungal non-ribosomal peptides and peptaibiotics

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Non-ribosomal peptide synthetases (NRPSs) are a primary modality for fungal peptidic natural product assembly and are responsible for some of the best known, most useful, and most destructive fungal metabolites. Through genome sequencing and computer-assisted recognition of modular motifs of catalytic domains, one can now confidently identify most NRPS biosynthetic genes of a fungal strain. The biosynthetic gene clusters responsible for two of the most important classes of NRP fungal derived drugs, cyclosporine and the echinocandins, have been recently characterized by genomic sequencing and annotation. Complete biosynthetic gene clusters for the pneumocandins and echinocandins have been mapped at the genetic level and functionally characterized to some extent. Genomic sequencing of representative strains of most of the variants in the echinocandin family, including the wild-type of the three fungal strains employed for industrial-scale production of caspofungin, micafungin and anidulofungin, has enabled characterization of the basic architecture of the echinocandin NRPS pathways. A comparative analysis of how pathway genes cause variations in lipoinitiation, biosynthesis of the non-proteinogenic amino acids, amino acid substitutions, and hydroxylations and sulfonations of the core peptide and contribute to the molecular diversity of the family is presented. We also review new information on the natural functions of NRP, the differences between fungal and bacterial NRPSs, and functional characterization of selected NRPS gene clusters. Continuing discovery of the new fungal nonribosomal peptides has contributed new structural diversity and potential insights into their biological functions among other natural peptides and peptaibiotics. We therefore provide an update on new peptides, depsipeptides and peptaibols discovered in the Fungi since 2009.

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Abbreviations

aa amino acid(s)

A adenylation

C condensation

E Epimerization

HDAC histone deacetylase

HPLC high performance liquid chromatography

HST host-specific toxin

MW molecular weight

NRP nonribosomal peptide

NRPS nonribosomal peptide synthetase

PCP peptidyl carrier protein

ROS reactive oxygen species

TE thioesterase

T thiolation

1 Introduction and scope of the review

Non-ribosomal peptide synthetases are a primary modality for fungal peptidic natural product assembly and are responsible for some of the best known, most useful, and most destructive fungal metabolites. Fungal peptides, depsipeptides, and peptaibiotics have been comprehensively reviewed by Degenkolb\textsuperscript{1} and by Anke and Antelo in the \textit{The Mycota}.\textsuperscript{2} An in-depth general review of recent discoveries and breakthroughs in the
structural elucidation, molecular mechanism, and chemical biology underlying the discrete domains within NRPSs appeared in 2012, while a synthesis focusing on the consequences of: 1) absence of C domains in NRPSs; and 2) predictions of aa specificity has recently appeared. A recent review on fungal cyclooligomer depsipeptides focussed primarily on the biosynthetic mechanisms involved in assembly of beauvericin, enniatins, hirsutellide A, bassianolide, PF1022, verticilide and the diketomorpholines, bassianatin and lateritiam. The biosynthesis of complex Aspergillus and Penicillum peptidyl alkaloids from anthranilate and tryptophan monomers via iterative bimodular and trimodular gene clusters has been recently reviewed, and will be further covered in a companion review by Tang (ref). NRPs are one of the main chemical weapons of entomopathogenic fungi, and new data on NRPS biosynthesis and functions in these fungi appear in the review by Gibson in this issue (ref).

Our understanding about how fungi biosynthesize non-protein peptides is rapidly expanding, and new insights from newly characterised genomes and biosynthetic gene clusters continually modify previous viewpoints. Through comprehensive genome sequencing and computer-assisted recognition of modular motifs of catalytic domains one can now confidently identify most NRPS biosynthetic genes of a fungal strain, and occur as partial NRPSs embedded as single modules in hybrid biosynthetic genes with polypeptide or prenyl transferases. The established methods of cloning and genome walking for characterisation of each cluster region have largely become obsolete. Those methods have been superseded by genome sequencing technologies that enable facile and comprehensive analysis of an organism’s core biosynthetic gene clusters while including identifications of previously unknown genes in the vicinity of these loci. The biosynthetic gene clusters responsible for two of the most important classes of NRP fungal derived drugs have been recently characterised by genomic sequencing and annotation. The pathway for echinocandin B in Aspergillus rugulosus has recently been identified through genome scanning, while our group recently published the results of the genomic sequencing of Glarea lozoyensis, the fungus producing pneumocandin B, the starting metabolite for the three-step manufacturing synthesis of the antifungal drug CANCIDAS. Complete biosynthetic gene clusters for the pneumocandins and echinocandins have been mapped at the genetic level, and functionally characterised to some extent, although many questions remain regarding the regulatory and mechanistic processes at the enzymatic level and as well as the evolutionary origins of the echinocandin gene clusters from within the Fungi. The genomic sequence of Tolypocladium inflatum and RNA-Seq transcriptome analysis has recently defined the complete gene cluster for cyclosporine biosynthesis. Unlike the echinocandins, the formidable cyclosporine NRPS simA seems to have evolved via duplication of simpler NRPSs that are also found among other fungi of the Hypocreales. A domain from several other NRPSs responsible for smaller cyclic depsipeptides in Hypocrealean fungi, such as enniatin, beauvericin and bassianolide synthetases, the aureobasidin A NRPS and two modules of destruxin synthetase appear to form a monophyletic lineage within the SimA clade. We will also review the recent characterisations of some NRPS gene clusters, e.g., the pathways responsible for apicidin and trichovirin. Discovery of the new fungal nonribosomal peptides continue to contribute new structure diversity and potential insights into their biological functions among other natural peptides and peptaibiotics. We provide an update on new peptides and depsipeptides from the Fungi since 2009.

2 Natural functions of non-ribosomal peptides in fungi

Ribosome-independent synthesized peptides and depsipeptides likely mediate a multitude of functions in the microbial cell and its environment, but most functions remain obscure. Some of the best studied functions in fungi are those of extracellular and intracellular siderophores involved in iron transport, storage and homeostasis and plant and animal virulence. Fungi generally produce hydroxamate-type siderophores. Siderophore-encoding NRPSs do not follow the typical NRPS co-linearity rule, but instead iteratively use of A domains, activating an identical aa more than once in an NRPS catalytic cycle, and employ additional T-C domains as partial modules to extend the NRPS products beyond the number of complete A-T-C modules in the NRPSs. Their synthesis starts with the N5-hydroxylation of L-ornithine catalysed by an L-ornithine-N5-monoxygenase and then is followed by an acylation to form the hydroxamate group, an NRPS assembles the final siderophore by linkage of hydroxamate groups, or other aa in the case of ferrichrome-type siderophores. Fungal siderophores are categorized into four structural types, ferrichromes, fusarines, coprogens and rhodotorulic acid. Fungal siderophores are believed to have arisen before the Ascomycete and Basidiomycete lineages diverged. Aligned A domains of siderophore NRPSs form a well-defined clade that includes both basidiomycete and ascomycete ferrichrome synthetases.

Other non-ribosomal cyclic depsipeptides, e.g., beauvericin, enniatins, isariins, pithionomycyolide, and PF1022A also have structures that enable release of amino acids, and are general iron chelating and ionophore acyltransferases. Siderophores can be virulence factors in animal pathogenesis. Silencing of SidA (L-ornithine N5-oxogenase) in Aspergillus fumigatus prevents infections in murine models of pulmonary aspergillosis. Aspergillus fumigatus produces four siderophores: fusarine C encoded by NRPS SidD and used for extracellular iron acquisition; triacetyl fusarinine C which is derived via fusarine C via SidG (N5-hydroxyornithine-acetylase) and used for extracellular iron acquisition; ferricrocin which is used for intracellular iron storage, and hydroxyferricrocin which is used for conidial iron acquisition and storage during infection, and both are encoded by SidC. Another A. fumigatus peptide synthetase gene, sidE, encodes a bimodular NRPS with 2,109 aa that is located near the sidG locus. Thus SidE was anticipated to be involved in siderophore production; however, unlike other siderophore encoding genes, its expression depended on LaeA, a positive regulator of virulence and secondary metabolism in A. fumigatus. Expression of sidE was upregulated during iron depletion, high-iron conditions, exposure to H2O2, and high temperatures. The sidE expression was up-regulated 16-fold in nascent Aspergillus infections in the neutropenic murine lung and therefore, was believed be involved in virulence. Both in vitro and comparative in vivo studies under heat-stressed growth determined that the iron source was the pathway product. SdE orthologs appear to form a unique lineage of fungal NRPSs that are common among other fungi. The authors suggested that the iron source was the pathway product. SdE orthologs appear to form a unique lineage of fungal NRPSs that are common among other fungi. The authors suggested that the iron source was the pathway product. SdE orthologs appear to form a unique lineage of fungal NRPSs that are common among other fungi. The authors suggested that the iron source was the pathway product. SdE orthologs appear to form a unique lineage of fungal NRPSs that are common among other fungi. The authors suggested that the iron source was the pathway product. SdE orthologs appear to form a unique lineage of fungal NRPSs that are common among other fungi. The authors suggested that the iron source was the pathway product.
transporter HasB, an O-methyltransferase HasC, a NRPS HasD, a 7-dimethylallyltriptophan synthase HasE, a putative FAD-binding protein HasG, and a cytochrome P450 HasH. The pathway’s product was identified as a tryptophan-derived iron(III)-complex, hexahydro-astechrome (2). The authors speculated that the precursor of this metabolite could function as a siderophore, or compound 2 could act as oxidative virulence factor by releasing astechrome monomers into the host tissue.

Structures 1 to 18 here

Siderophores play a role in fungus-plant interactions. Coprogens encoded by NPS6 in *Cochliobolus heterotrophus* enhance virulence to maize and resistance to oxidative stress.30 Orthologues of these genes with conserved functions appear to be widely distributed among filamentous Ascomycetes. AaNPS6 of *Altenaria alternate* is responsible for dimethyl coprogen siderophores and was necessary for siderophores production and virulence on citrus.31 *Magnaporthe grisea* excretes coprogen-type siderophores for iron acquisition while employing ferricrocin for intracellular iron storage. Thus, positively affecting iron nutrition of the host plant. New evidence comes from newly discovered tris-hydroxamate siderophores and was necessary for siderophores excretion ferrirhodin (trans-AMHO) moieties. This extracellular siderophore is essential for maintenance of the mutualistic endophytic growth of *Epichloë festucae* within tissues of perennial ryegrass (*Lolium perenne*). In addition to SidN’s role in the grass-fungus mutualism, this protein is significant because its third A domain was heterologously expressed and was the first eukaryotic NRPS domain to be purified and characterised at the structural level.39 Substrate activation of the SidN A domain 3 was modelled with cis-AMHO obtained by alkaline hydrolysis of fusigen. X-ray crystallography and ligand-docking experiments determined that a large 17-residue binding pocket is needed to accommodate the bulky aa substrate, cis-AMHO. The specific germination of cis-AMHO was confirmed biochemically, and an AMHO moiety was unequivocally identified as a constituent of the fungal siderophore. This case clearly illustrated the divergence of A domain signature sequences between prokaryotes and eukaryotes and why substrate prediction methods based on bacterial NRPS A domains generally fail for eukaryotes. Thus, the structure of the E. festucae SidN A domain will contribute to improved prediction methods for eukaryotic NRPS enzymes.

Host-specific toxins (HSTs) mediate virulence and pathogenicity among certain plant pathogenic species of the Dothideomycetes. HSTs encompass about 20 different structurally diverse metabolites, including peptides and depsipeptides (AM-toxin, HC-toxin (8), victorin, phomalide, depsilairdin, BZR-cotoxins).40-42 Other non-host specific fungal peptides, e.g., destruxins and roseotoxins, also have been implicated as plant pathogenicity or necrosis factors.43-44 Some widespread NRPSs may structurally support fungal cell walls and contribute to their hydrophobicity. FnGRPS4 in *F. graminearum* is a five-module NRPS and is conserved throughout four genome-sequenced *Fusarium* species, *F. graminearum*, *F. oxysporum*, *F. solani*, and *F. verticillioides*. Disruption of fgrps4 in *F. graminearum* resulted in loss of mycelial water repellency and led to altered conidial morphology.45 ChNRP4 of *Cochliobolus heterotrophus* has a similar five-module structure and appears to be an orthologue of FnGRPS4; its disruption also abolishes mycelial surface hydrophobicity and water repellency.46 NRP2 in *Alternaria brassicicola* appears to be another orthologue, and deletion mutants show a similar decreased hydrophobicity, distorted conidial cell wall morphology, poor sporulation and germination, and thus, reduced infection capability.47 BLAST searches with the protein sequence of FnGRPS4 retrieve a wide range of five-module NRPSs with similarity scores >40%, including GLNRPS6 from *G. lozoyensis* (45% similarity).48 Despite persist attempts at identification,49 the products of these NRPSs have remained elusive. The eventual identification of these putative pentapeptides will be of great interest to phytopathologists and fungal biologists.

Peptaibols and other peptaibiotics, as defined by Degenkolb & Brückner, are characteristic of mycoparasitic species of the Hypocreales, and are occasionally detected in other Ascomycete fungi. Their biology and chemistry have been reviewed extensively.49-51 These specialized peptides have structural properties that induce spontaneous formation of voltage-gated channels in bilayer lipid membranes, and have been observed to have antibacterial, antifungal, and a myriad of other biological activities. A comprehensive database with 1043 entries of peptaibiotics listing compound name, aa sequence, sequence length, producing organism, subfamily classification, molecular formula, monoisotopic masses, was recently made available for download.52 Their producing fungi, particularly *Trichoderma (=Hypocreum) and Clonostachys* species, have
been intensively exploited as biological control agents for plant pathogenic fungi, with over 950 different peptide sequences known from the Trichoderma lineage alone.\textsuperscript{51}

Two recent studies have investigated the question of whether peptaibiotics are produced in natural ecosystems. The mycoparasitic Trichoderma phellinicolus naturally attacks the polypore, Phellinus ferruginosus. LC-ESI-HR-TOF analysis of field-collected T. phellinicolus specimens and naturally infected P. ferruginosus basidomata revealed that a family of 20-residue peptaibiotics, named the hypophellins \((\text{9})\), were biosynthesized in natural mycelia and stromata and were subsequently released into the host fungus.\textsuperscript{52} The structures of naturally occurring peptaibiotics were independently confirmed by analysing agar cultures of \(T. \text{phellinicolus} \). In vitro, this strain produced 39 new 17-, 18-, and 19-residue hypophellins that displayed the same biosynthetic scheme as the 20-residue hypophellins found in natural specimens. Two of the 19-residue peptaibiotics were thought to carry tyrosinol, a novel C-terminus residue, as predicted by HR tandemMS. A parallel investigation of biosynthetic scheme as the 20-residue hypophellins found in plant fungal pathogens. A recent study presented the first in-depth investigation of antibiotic mechanism of peptaibiotics against fungal plant pathogens. Trichokinon VI (TK VI) \((\text{18})\) induced extensive apoptotic programmed cell death in plant fungal pathogens.\textsuperscript{57} To better understand the apoptotic mechanism involved in the effects of TK VI, cells of the mycoparasitic host, \(Fusarium \text{oxysporum} \), were observed. When cells of \(F. \text{oxysporum} \) were treated with TK VI, they exhibited apoptotic morphological symptoms, e.g., phosphatidylerine exposure, the appearance of ROS, chromosome condensation, and nuclear DNA fragmentation. Moreover, TK VI-treated cells accumulated cytoplasmic vacuoles and lost mitochondrial transmembrane potential. Cell death was metacaspase independent, and therefore TK VI induced metacaspase-independent apoptotic cell death in \(F. \text{oxysporum} \).

### 3 Differences between fungal and bacterial non-ribosomal peptide synthesis

NRPSs synthesize peptides by employing modular sets of core domains that minimally consist of three domains: 1) an A domain that recognizes and activates the substrate via adenylation with ATP, 2) a T- or PCP-domain which binds the activated substrate to a 4′-phosphopantetheine cofactor via a thioester bond and transfers the substrate to 3) a C-domain which catalyses peptide bond formation between tethered activated substrates on the megasynthase complex. Major features that set fungal NRPSs apart from bacterial NRPSs are their aa compositions. Fungal and bacterial NRP composition was compared based on an analysis of the Norine database,\textsuperscript{58, 59} that catalogues 1000 peptides representing more than 10,000 monomer occurrences and >500 different monomer types.\textsuperscript{60} A total of 34% of the database was comprised of fungal peptides. Correlation coefficients between fungi and bacteria were low for peptide size distribution (0.236) and for monomer composition (0.253) which supported phylogenetic inferences of an independent fungal- and Ascomycete-specific expansion of NRPSs.\textsuperscript{22} The database’s most frequent monomer was 2-aminoisobutyric acid (Aib) which is a hallmark of fungal peptaibiotics that can occur multiple times in the same peptaibol (mean = 6) and explained why Aib was the database’s most frequent monomer. Other monomers are specific to fungal NRPs, especially those in peptaibols, e.g., isovaline, phenylalaninol, leucinol, and valinol.

Structures 19 and 20 here (Figs for NPR-part3)

Bacterial NRPSs employ thioesterase domains to catalyse peptide cyclization, while fungal NRPSs use an alternative enzymatic mechanism.\textsuperscript{61} The folds of fungal C\(_T\) domains and bacterial TE domains are completely different and utilize distinct mechanisms to cyclise the same reaction. To date, all fungal NRPSs that assemble macrocyclic peptides terminate with a condensation-like C\(_T\) domain that catalyses the cyclization reaction, and thus, is a near universal macrocyclization strategy used by fungal NRPSs. Fungal C\(_T\)
domains are phylogenetically distinct from chain-extending C domains, and in fact, genome sequencing data indicated that a majority (60–90%) of the NRPSs found in filamentous fungi have a terminal C7 domain.61 The biochemical mechanism of the cyclization reaction was recently investigated by comparing the in vitro reconstitution of the Penicillium aethiopicum trimodular NRPS TqaA, responsible for transforming the linear anthranilate-D-tryptophan-L-alanyl tripeptide into fumiquinazoline F (19) with that of the bimodular NRPS AnaPS that produces 3-indolylmethyl-3,4-dihydrobenzo-1,4-diazepine-2,5-dione precursor R-2 of acetylazosalenalin (20) in Nannotoxia fischeri. The first two modules of TqaA are identical to AnaPS, however the addition of the third module radically alters the structure of the final cyclic alkaloid products. Both gene clusters were expressed in Saccharomyces cerevisiae. Extensive biochemical and mutational studies confirmed that differences in the cyclization patterns are likely due to the precise control of the reactive dipeptide D-4 intermediate, and the use of a C-like cyclization domain that regioselectively directs the intramolecular attack of the anthranilic acid amine nucleophile. A catalytic histidine at the C7-active site deprotonates the amine nucleophile, thus, facilitating its attack on the thioester carbonyl leading to cyclization and product release. The C7 domain also differs mechanistically by its requirement for a specific companion T domain in the C7 domain catalysed cyclization reaction. This study showed that only peptide substrates tethered to the natural T domain portion of the C7 domain are efficiently recognized; peptidyl-SNAC or peptidyl-Co substrates were not recognized. Such a mechanism differs from the usual bacterial TE domains where small-molecule mimics of the peptidyl-thioester can be used to probe the cyclization functions.

4 New understanding of the biosynthesis of pneumocandins and echinocandins

4.1 Pathway discovery

The echinocandin family of lipopeptides has been investigated for over three decades and has formed the basis of a successful new class of antifungal drugs. Their chemistry, biology, and distribution among different kinds of Ascomycete fungi have been reviewed extensively.62-66 Until recently, knowledge of their biosynthesis was largely inferred from the patterns of incorporation of the eight different biosynthetic precursors molecules into the hexapeptide core and acyl side chain of the pneumocandins and subsequent decorations with hydroxyl and methyl groups.67 During the last two years, genomic sequencing of representative strains of most of the important variants in the family, including the parent wild types of three fungal strains employed for industrial scale production of caspofungin, micafungin and anidulofungin, has enabled characterisation of the basic architecture of the echinocandin NRPS pathways. Thus, an analysis of how pathway genes produce variations in lipoinitiation, biosynthesis of the non-proteinogenic aa, aa substitutions, hydroxylations, and sulfonations of the core peptide contribute to the molecular diversity of the family is now possible.

Figure 1 here

Fig. 1. Representation of the pneumocandin gene cluster from Glarea lozoyensis ATCC 20868 and the echinocandin B gene cluster, including the disjunct L-homotyrosine gene cluster, from Aspergillus rugulosus NRRL 111440.

The gene cluster responsible for the synthesis of echinocandin B (21) of Aspergillus rugulosus (ecd) (Fig. 1, 2) was characterised via genome sequencing and bioinformatic analysis and was confirmed by functional knock-out experiments and heterologous expression of selected pathway genes.18,19 The ecd gene cluster spans 12 genes (ecdA to ecdL). The core NRPS gene ecdk encodes for a six-module megasynthetase with each module consisting of a C, A, and T domain. An extra T0 domain initiates the first module at the N-terminus, while the terminal module ends with a C0 domain. Gene ecdl encodes a fatty-acyl-AMP ligase responsible for activating lineolate and loads it onto the first thiolation domain of ecdA. EcdA loads L-Orn as the first module. Iron oxygenases EcdG, EcdH and EcdK mediate hydroxylations of the core hexapeptide.68 Other genes flanking ecdA include a transcription factor gene edcB, and three transporter proteins, edcC, ecdD and ecdL.19 Furthermore, the hty gene cluster for L-homotyrosine biosynthesis was discovered on another scaffold, and its disruption silenced echinocandin production.69 The hty gene cluster consists of an isopropyl malate synthase gene (htyA), a transaminase (htyB), an isopropyl malate dehydrogenase (htyC), and a aconitase gene (htyD), along with two oxygenase genes (htyF, htyE) for further hydroxylation of homotyrosine (Fig. 1).

Structures 21 to 29 here (Figs for NPR- part4)

Pneumocandin A0 (22) is the major antifungal product of wild-type Glarea lozoyensis and of certain Peticula (=Cryptosporiopsis) species. Like echinocandin B (21), five of the same six aa in the cyclic hexapeptides are incorporated in the same order with the same hydroxylation pattern, the only difference being hydroxy-L-glutamine in position 5 in G. lozoyensis instead of L-threonine. Hydroxylation of the two proline residues in pneumocandin A0 are catalysed by a proline-3-hydroxylase and a proline-4-hydroxylase.68 The enzyme responsible for hydroxylation of 4-methyl-proline derived from leucine in pneumocandin A0 may also be a proline-3-hydroxylase because 4-methyl-proline is an analogue of L-proline.68 Shortly after the publication of the echinocandin B gene cluster, our group published the genomic sequence of the Ascomycete G. lozoyensis and annotated and functionally confirmed the biosynthetic gene cluster (Fig. 1) responsible for the antifungal lipohexapeptides pneumocandins A0 (22) and B0 (23).

Figure 2 here

Fig. 2. Comparative organization of the principal echinocandin gene clusters. Genes in black are of unknown function or believed not to participate in the biosynthesis.

The commonalities of pneumocandin and echinocandin B pathways are striking (Fig. 1) because most of clusters’ genes appear to be orthologs despite some organizational differences. Pneumocandin’s core NRPS, GLNRPS4, and EcdA (T4CATCATACTCATACTC) are equivalent with same direction of transcription and sharing a high nucleic acid identity (60.8% identity over 22.7 kb, 55.2% identity over 7218 aa). However, unlike ecdA, glnrps4 sits downstream of, and
adjacent to, a highly reducing polyketide synthase gene, glpks4. These two core genes are centrally located in the pneumocandin gene cluster and are independently transcribed and translated. Instead of the pneumocandin acyl side chain originating from cytosolic fatty acids as proposed in ecd, GLPKS4 synthesizes 10,12-dimethylmyristate. The 10,12-dimethylmyristoyl side chain is released from GLPKS4 as a carboxylic acid and subsequently converted to a CoA thioester during elongation of the cyclic hexapeptide. Threonine, 4-hydroxy-proline, 4,5-dihydroxy-homotyrosine, 3-hydroxy-glutamine and 3-hydroxy-proline or 3-hydroxy-4-methyl-proline are sequentially added to the growing chain in the same sequence as in EcdA. Like EcdA, the last C domain of GLNRP5 is a C\(\delta\) and is responsible for cyclization by condensation to form the peptide bond between 4,5-dihydroxy-ornithine and 3-hydroxy-4-methyl-proline.

Other gene downstream of the glpks4 (Fig. 1) involved in the biosynthesis is GLAREA10043 encoding an acyl-CoA ligase which shares 43% identity with EasD which converts a polyketide carboxylic acid to a CoA thioester during emericellamide A (24) and B (25) biosynthesis in A. nidulans.\(^{20, 69}\) This gene’s existence suggested that the polyketide intermediate was first synthesized by GLPKS4, and then transferred to the T\(\delta\) domain of GLNRP5, which would be mechanistically equivalent to the emericellamide biosynthetic pathway.\(^{69}\) Further contributing to the autonomy of pneumocandin gene cluster, and unlike the echinocandin B pathway (Fig. 1), a set of genes orthogonal to the echinocandin hty pathway for the homotyrosine residue of the pneumocandin hty pathway contains the homotyrosine residue sits downstream of the NRPS.\(^{19}\) Both pathways also contain a number of oxygenases that tailor the multiple hydroxyl or diol groups of the peptide core, but their physical linkage is rearranged.

DNA sequences for the gene clusters of additional echinocandin-type pathways recently have been made available at the NCBI GenBank and at the US Department of Energy’s Joint Genome Institute. These sequences include the gene cluster for aculeacin A (26)\(^{70, 71}\) from A. aculeatus (ATCC 16872 = JCM 22898) and the sulfonated homotyrosine echinocandin-pneumocandin variants: FR190293 (27) from a Leotiomycete fungus related to Phialophora hyalina\(^{72}\) (referred to from here on as P. cf. hyalina, it previously was misidentified as Tolypocladium parasiticum\(^{73}\)), which is essentially a sulfonated pneumocandin A\(\delta\); FR901379 (WF11899A) (28) from Coleophoma empetri,\(^{74}\) the starting molecule for micafungin; and FR209602 (29) from C. crateriformis.\(^{75}\) The availability of these sequences of the gene clusters responsible for almost the entire range of echinocandin complexity now enables development of hypotheses about the functions of specific pathway genes where their presence or absence in certain pathways can be correlated with specific differences in the pathway products (Fig. 2). The sequences also present the opportunity to trace the evolutionary history of the echinocandin-pneumocandin gene clusters, as well as investigate the significance of these potent cell wall-modifying metabolites to the fungi that produce them.

These newly characterized gene clusters are distributed among fungi from two major evolutionary lineages, Eurotiomycetes (A. rugulosa, A. nidulans var. echinulatus, A. aculeatus) and Leotiomycetes (G. lozoyensis, C. empetri, C. crateriformis, P. cf. hyalina) that diverged around 290 to 390 million of years ago.\(^{76}\) Explanation of this pattern based on independent parallel evolution seems improbable. Echinocandin-producing fungi vary in their ecology\(^{62, 66}\) and range from soil and litter saproples (Aspergillus species, possibly G. lozoyensis, P. cf. hyalina) and endophytes and weak pathogenic fungi, plants (Pezicula and Coleophoma species. All these strains were discovered because of their fermentations were potently active in antifungal screens; therefore, the pathways are operative. In our experience, echinocandin biosynthesis is not tightly regulated in the laboratory.\(^{66, 77, 78}\) The natural function of echinocandins remains uninvestigated; however, the ability to produce null pathway mutants\(^{19, 20}\) opens up interesting possibilities for experimentation in fungal microcosms.

### 4.2 Structure and gene order conservation of the echinocandin gene clusters

The functions determined to date for enzymes in the echinocandin pathway\(^{18-20}\) are consistent with the presence or absence of specific genes each strain and their specific pathway end products. Genes in those pathways lacking experimentally determined roles can be linked with hypothesized steps on the basis of the functions predicted from their sequences and their presence or absence in clusters among the various strains compared to those of G. lozoyensis and A. rugulosis where some pathway genes have been functionally characterised (Fig. 1). For example, clusters that incorporate a highly reducing PKS (G. lozoyensis, P. cf. hyalina, Fig. 2) have a branched acyl side chain. Other genes responsible for structural differences can be inferred, such as in FR209602 (29) from C. crateriformis, where the single hydroxylation of the homotyrosine is likely due to the absence of one of the oxygenases of the hty pathway, i.e. the orthologue of htyF and GLAREA10030. Likewise, the absence of orthologues to oxygenase GLAREA10042 in the Aspergillus echinocandin pathways points to their function in the hydroxylation of hydroxyl-glutamine at position 4. Finally, orthologues of GLAREA10045 and ecd\(\delta\) are in all echinocandin pathways (Fig. 2). Its consistent presence in all pathways suggests an important function that remains unknown. In summary, by comparing common pathway elements, the minimal elements needed to biosynthesize a functional echinocandin would include a six-module NRPS, an acyl-AMP ligase, the four orthologs of HtyA-D of the hty pathway to synthesize L-homotyrosine, and the three orthologs of oxygenases EcdG, K, and H which hydroxylate the L-ornithine, L-homotyrosine, and leucine precursor of the fifth 4-methyl-L-proline residue.

The most obvious dichotomies between pathways are those that incorporate a dedicated highly reducing PKS for biosynthesis of a methylated, branched acyl side (G. lozoyensis, P. cf. hyalina) and pathways that incorporate linear fatty acid-derived acyl side chains from the cytosol fatty acids, and those pathways where the C4 of the homotyrosine residue is sulfonated (Coleophoma spp. and P. cf. hyalina). Thus, the most compact pathway architectures would correspond to that of the aculeacin pathway where the hty genes sit immediately downstream of, and separated by, two unknown genes and a cytochrome P450, from the core NRPS gene (Fig. 2). In contrast, the most elaborate cluster is assumed to be that of FR190293 (27) which not only recruits a highly reducing PKS for the side chain synthesis, but also has a sulfonated
homotryosine residue. To date, the enzyme(s) responsible for the sulfonation reaction remains unknown. An obvious candidate sulfotransferase gene responsible for the homotryosine sulfonation is not evident in the clusters. Therefore, sulfonation is assumed to occur post-synthesis of the core hexapeptide and that the responsible gene(s) is located at some distance from the cluster. The vincinal dihydroxy groups in hydroxymethotryosine, like the hydroxyl group of phenol, would have a strong activating effect on the aromatic ring because of its high electron density within the ring. Chemically this directing influence is strong enough that ortho sulfonation of phenols in the presence of sulphuric acid occurs at room temperature without a catalyst.79

Predicted protein sequence similarities of the core NRPS range from near 100% between the two most similar clusters, the two Coleophoma species, and the two echinocandin B NRPSs from the different species of the A. nidulans complex, while the most dissimilar NRPSs are those of A. aculeatus and A. rugulosus and A. nidulans var. echinulatus versus G. lozoyensis (71%). Furthermore, a dichotomy between the Leotiomycetes and Eurotiomycete gene clusters is consistently evident across all pairwise comparisons between the proteins sequences of G. lozoyensis and those of the other fungi (Chen, Bills, unpublished).

4.3 Evolution of the echinocandin gene clusters
BLAST and HMMER searches with the GLNRPS4 and EcdA across the NCBI and the Joint Genome Institute databases retrieved only homologues of echinocandin gene clusters. BLAST searches with individual A domains of Ecd4 and GLNRPS4 also indicated that uncharacterised dipeptides NRPS AN3496 (ImpB) from A. nidulans, and its orthologues As12236 in A. sydowii and Av13511 in A. versicolor were related (Fig. 3). The top 50 BLAST hits to EcdA and GLNRPS4 from the NCBI database failed to retrieve any bacterial sequences. More targeted BLAST searches with individual catalytic domains (A, T, C) of the NRPSs only retrieved other Ascomycete NRPS domains. These searches suggested that the echinocandins likely evolved by internal duplication of modules of an ancestral module originating within the Ascomycete fungi rather than through HGT from bacteria.

Figure 3 here

3. Phylogeny of selected fungal NRPS A domains, including A domains of the principal echinocandin-type NRPSs (see Fig. 2). Data were partially resampled from Bushley and Turgeon22. Protein sequences were annotated by using the HMMER analytical tool. The A domain aa sequences were aligned with M-Coffee. A maximum likelihood tree was constructed by applying WAG plus gamma model using RAxML analytical software with 100 replicates. Bootstrap values greater than 50% are shown above branches. The A domains corresponding compounds that indicated by different symbols are listed on the left side. A domains of selected fungal metabolites (left) were color-coded and mapped on the tree (right).

To infer phylogenies of the NRPSs components, the deduced aa sequences for all the echinocandin A domains were aligned with homologues throughout the ascomycetes by resampling and updating a previously published data set of fungal NRPS A domains.22 A maximum likelihood tree (Fig. 3) constructed from the A domain protein alignments indicated all the echinocandin A domains and the A domains of the predicted dipeptides NRPS AN3496 (InpB) and its orthologs As12236 and Av13511 formed a distinctive clade (59% bootstrap value). The echinocandin A domain clade clearly nested within an Ascomycete-exclusive subfamily of NRPSs previously designated as the euascomycete only subclade22 (Fig. 3). The same analysis22 detected the singularity of the AN3496 NRPS where it appeared as a weakly supported branch and a sister group to the A domains of the emericellamide (EAS, AN2545) NRPS.66 Thus, the echinocandin NRPSs along with the AN3496 NRPS appear to form a novel lineage within the Ascomycete-exclusive subclade of fungal NRPSs.

In addition to the echinocandin A domain genes forming a monophyletic clade, the intra-clade relationships among the six individual A domains were consistent. Remarkably, the internal topology of the clade clade was resolved into six subclades each consisting of the corresponding A domain modules for each aa positions in the echinocandin nucleus (Fig. 3). This topology was supported by strong internal bootstrap values and indicated that the A domains for each aa position from each fungus were more similar to each other than the individual A domains from a single given echinocandin NRPS from any one species. This one-to-one correspondence between modules and aa specificity of different species is consistent with models of tandem duplication and subfunctionalization prior to divergence from a hypothetical ancestor, as has been hypothesized to occur during the evolution of cyclosporine and peptabiol NRPSs.21,22

We have constructed gene phylogenies for rest of the dedicated pathway genes, including the cytochrome p450s, oxygenases, enzymes of the hty pathway, the acyl-ligase, and the ABC transporter. When examined in the context of their respective fungal orthologs, each group of the echinocandin genes form well-supported monophyletic groups with tree topologies similar to that of the core NRPSs (unpublished). In a few cases, e.g., some NRPS A domains, acyl-ligases from close neighbours of the genus Aspergillus (A. nidulans and A. sydowii) were sister groups to the clade formed by the clustered echinocandin genes. The presence of these gene relatives in related Aspergilli suggests an origin of the pathway in this genus, possibly in common ancestors of sections Nidulantes and Nigri.83 This high degree of functional similarity conflicts with established phylogenies of the Eurotiomycetes, and Leotiomycetes.82 The echinocandin pathways among these divergent fungal species from distinct evolutionary lineages appear to have shared a common ancestor, and a minimal ancestral echinocandin pathway likely existed before radiation into or between the two fungal lineages.

5 Other recently characterised NRPS gene clusters
5.1 The apicidin gene cluster and its relationship to HC-toxin.
The cyclic tetrapeptide apicidin A (30), produced by various Fusarium species, was identified as a broad-spectrum in vitro and in vivo agent effective against a range of apicomplexan parasites, including Plasmodium berghei malaria in mice83, 84 and been used as molecular template for synthesis of HDAC inhibitors.85 Apicidin and related fungal tetrapeptides, e.g., traxopin, HC-toxin (8), and chlamydacin are also potent inhibitors of other protozoans and affect the HDACs of many eukaryotic organisms. Unlike apicidin, similar cyclic peptides including HC-toxin (8), traxopins and others contain an epoxyketone moiety on the 2-amino-decanoic acid residue. These compounds irreversibly inhibit HDAC at nanomolar
concentrations by covalently binding to the enzyme through the epoxide group. The apicidins produced by plant-inhabiting Fusaria may function as a phytotoxin in a manner similar to the HST, HC-toxin (8) produced by Cochliobolus carbonum.86 The apicidin gene cluster (ASP1) has been characterised in F. semitectum by the sequencing of three overlapping cosmids clones covering a 63 kb genomic region with 19 ORFs of which 10 genes (ASP1-ASP11) appear to be involved in synthesis and regulation.23 The APS1 gene consists of four peptide synthase modules with an E domain in module 1. The modules sequentially incorporate L-pipeolic acid, L-isoleucine, N-methoxytryptophan, and L-2-amino-8-oxodecanoic acid (AODA). ASP2 has significant homology to HC-toxin synthase HTS1 (43%) (Fig. 3).

Structures 30 to 51 here (Figs for NPR-part5)

Mutation of APS3 gene resulted in apicidin B (31), i.e. substitution of D-pipeolic acid by proline at position 1, thus leading to the conclusion that the APS3 may be involved in reducing Δ-pyrroline-6-carboxylate into L-pipeolic acid, while the E domain of APS1 converts the latter to D-pipeolic acid. The first A domain of ASP1 was sufficiently flexible to accept proline as a substrate. The ASP2 gene was predicted to be a transcription factor that was essential for pathway expression. APS5 is an α-subunit of a fatty acid synthase dedicated to the production of the lipid chain of AODA. It was thought that β-subunit from cytosolic fatty synthase complemented the biosynthesis. Mutants of the cytochrome P450 ASP7 accumulated apicidin E (32) indicating that this gene is responsible for the conversion of S-2-amino-decanedioic acid to 2S,8S-2-amino-hydroxydecanedioic acid. Further side-chain oxidations are mediated by the oxygenase ASP9 resulting in AODA; loss of ASP9 resulted in production of apicidin D (33). ASP8, another cytochrome P450, was also thought to be involved in the sequential oxidative formation of AODA. ASP4 was found to be an aminotransferase, and orthologue to TOXF. ASP6, an O-methyltransferase, was believed to methylate tryptophan. Viable deletion mutants of the predicted efflux pump ASP11 could not be obtained, indicating that efflux of apicidin out the cells was essential to the fungus.

The genomic sequence of the causal agent of bakanae disease of rice,88 F. fujikuroi, revealed an apicidin-like gene cluster (NRPS31) largely syntenic to the organization of the APS cluster in F. semitectum. Transcriptome analysis revealed expression of the F. fujikuroi APS cluster homologue during growth in acidic high-nitrogen conditions, and possible gene silencing under the low-nitrogen growth conditions. Overexpression of the pathway-specific transcription factor-encoding gene led to increased accumulation of an unknown metabolite with the molecular formula (C$_{33}$H$_{43}$N$_{8}$O$_{8}$) similar to that of 30 (C$_{45}$H$_{48}$N$_{9}$O$_{8}$). NMR, MS and acid hydrolysis revealed a new apicidin, apicidin F (34), consisting of N-methoxytryptophan, D-pipeolic acid, and L-phenylalanine and L-2-amino-8-oxodecanoic acid.89 It was about three-fold less potent than apicidin A (30) in a Plasmadium falciparum inhibition assay.

HC-toxin (8) was unexpectedly detected in extracts of Alternaria jesenskae while screening for new HDAC inhibitors.91 Genomic sequencing of A. jesenskae revealed that the major genes (designated AJTOX2) involved in the biosynthesis of A. jesenskae HC-toxin were orthologous to those of TOX2, and their proteins shared 75-85% aa identity (Fig. 3). Similarly in both fungi, the genes for HC-toxin biosynthesis were duplicated. The genomic organization of TOX2 clusters in the two fungi showed a similar but not identical partial clustering arrangement.

5.2 Other NRPS biosynthetic gene clusters

Monomodular NRPSs with an A-T-E domain structure have been found to synthesize 1,4-benzoquinone compounds from symmetric condensation of two aromatic 2-oxo acids; they include metabolites such as terrequinoine A (35) encoded by the Tda cluster.17,92 Recently an efficient expression of TdiA in E. coli was reported with the objective of making enzymatic preparations of bi-3,4-indolylquinone metabolites.93 The pigment atromentin (36) from various mushrooms in the Boletus group is a terphenylquinone arising from the adenylation and dimerization of 4-hydroxyphenylpyruvic acid. The biosynthesis is mediated by atromentin synthetase AtrA, encoding a quinone synthetase, and an aminotransferase, AtrD, that adenylates and dimerizes 4-hydroxyphenylpyruvic acid.94 One of these enzymes, GreA, has been identified in Sulfuris grevillei and was heterologously expressed and biochemically characterised.95 Atromentin biosynthesis was reconstituted in vitro. Another A-T-E NRPS-like enzyme, AN3936.4 from A. nidulans has also been heterologously expressed in A. niger to confirm its function and was shown to produce a furanone compound, designated microperfurane (37).96 Cosmid libraries of the mushroom Armillaria mellea were genetically screened for genes encoding natural product biosynthetic pathways, thus leading to the recognition of a tdiA-like gene (armh) encoding a A-T-E domain structure.97 This locus lacked a typical neighbouring aminotransferase indicating affinity for substrates other than 2-oxo acids. Two genes coding for flavin-dependent halogenases, armh1 and armh2 were located nearby to armh. The cDNA of armh was heterologously expressed to determine the substrate specificity of its A domain employing an ATP-pyrophosphate exchange assay. Surprisingly, considering that Armh lacks a C domain, the preferred substrates were leucine, threonine, and other hydrophobic aa, leading to the conclusion that Armh may represent a new alternative route to diketopiperazine formation.

WYK-1 (38) from Aspergillus oryzae is an isooquinoline derived from a tripeptide consisting L-tryptophan, L-phenylalanine or L-tyrosine, and L-leucine.98 The A. oryzae genome contained four NRPS genes that were approximately 9 to 16 kb long. Comparative analysis of expression levels of these NRPS genes by RT-PCR under WYK-1 producing or non-producing conditions detected that only expression of locus AOR1-1360164 was correlated with WYK-1 production. This locus encoded a trimeric peptide synthase WykN, and transcription analysis identified 10 more genes that were coexpressed with WykN. The proposed biosynthesis commenced with the tripeptide synthesis, followed by methylation by WykI. Cyclization of the phenylalanine or tyrosine residue by the oxidase, WykG, would form an isoquinoline ring, followed by methylation by N-methyltransferase, WyKH, and hydroxylation by phenol hydroxylase WykA to yield WYK-1 (38).

Genome sequencing projects of three representative Trichoderma strains from the genus’ major evolutionary lineages indicated the existence of as many as three kinds of NRPSs with 7, 14, or 18–20 modules. The major peptoids of biocontrol strain of T. viride were determined to be trichovirins of two classes, 11- and 14-residues99. Of the 35 11-residue peptoids sequenced, 18 were new, and all of the 53 14-residue sequences were new. A 14-module NRPS, trichovirin A
Pochonia bulbinosa peptides (40-tetraEmodular NRPS, designated HcpA, in intermediates. synthetase. characteristic, such as antiplasmodial, antiviral, insecticidal, fungal cell wall inhibition assay measuring differential cell wall polymerase enzyme complex. A new cyclic hexapeptide (52) mutant of 6 New peptides and depsipeptides and their bioactivities the same series of metabolites in leading to the conclusion that deletion of HcpA was 54% identical with an orthologous protein from domain towards different aa monomers resulted in a range of 39 domain. Furthermore, the NEtermini of these NRPSs appeared adjacent A2 and A4 domains flanked by a single C and PCP domain. Furthermore, the N-termini of these NRPSs appeared to be incomplete, lacking an adjacent A domain, leading to an incomplete module C4-PCP. HcpA was confirmed to be responsible for encoding the biosynthesis of the metabolite fungisorpin (39) along with a series of new cyclic and linear peptides (40-51). Microheterogeneity of each adenylation domain towards different aa monomers resulted in a range of cyclic tetrapeptides produced by this single NRP synthetase. HcpA was 54% identical with an orthologous protein from A. niger that had the same module organization. Furthermore, all cyclic products (39-48) present in P. chrysogenum could be detected in the culture broth of an A. niger strain. Parallel deletion of HcpA in this strain abolished their production leading to the conclusion that HcpA encodes for the production the same series of metabolites in A. niger.

6 New peptides and depsipeptides and their bioactivities

New peptides and depsipeptides continue to be discovered either by empirical spectral probing of fermentation extracts or because of detection of interesting in vitro biological characteristics, such as antiplasmodial, antiviral, insecticidal, cytotoxic, and antiproliferative properties. In a few cases, in-depth investigation of their biology has led to the discovery of unique modes of action hypotheses.

6.1 Peptides

A fungal cell wall inhibition assay measuring differential response in a two-plate assay with a wild-type and a wall-less mutant of Neurospora crassa led to the isolation of a linear hexapeptide (52) (L-threonine, D-alanine, L-alanine, L-alanine, D-tyrosine, L-valine) with a β-hydroxyxymyric acid amide substituted N-terminus on the threonine residue from a strain of Pochonia bulbinosa. Although the peptide strongly inhibited fungal growth and caused protoplast formation in some fungi, it failed to inhibit chitin synthase or β-1,3-glucan synthetase, suggesting that its mechanism may be via a regulatory unit of cell wall polymerase enzyme complex. A new cyclic heptapeptide, unguisin E (53), comprised of cyclo alanyl-tryptophyl-L-aminobutanyloyl-alanyl-valyl-β-methyl-phenylalaninyl-valyl, was isolated from the fermentation an unidentified Aspergillus strain. Fermentation extracts of A. sclerotiorum yielded 11 new aspochracin-type cyclic tripeptides, sclerotiotides A-K. Sclerotiotide A (54) consisted cyclic N-Me-L-alanine, L-valine, NR-octa-2,4,6-trienoyl-L-ornithine. Sclerotiotides A, B, F, and I selectively inhibited growth of Candida albicans. A strain of Penicillium canescens yielded the linear tetrapetide D-phenylalanine, L-valine, D-valine, L-tyrosine (55). The peptide inhibited growth of Fusarium virguliforme and Bacillus subtilis.

An unidentified vegetative Xylaria strain produced two new cyclopentapeptides (56, 57). These pentapeptides had an aa sequence of cyclo N-methyl-L-phenylalanine, L-valine or L-isoleucine, D-isoleucine, L-leucine, L-proline. Although not specifically antifungal, the peptide enhanced the activity of ketoconazole. A novel cyclic octapeptide, epichlicin (58) was found from Epichloe typhina, a biotrophic symbiont of timothy (Phleum pratense). The cyclic peptide sequence consisted of 3-amino-tetradecanooic acid, L-asparagine, L-tyrosine, L-asparagine, L-glutamine, L-serine, L-asparagine, and L-proline. Although its role in the symbiosis remains unknown, the peptide inhibited conidial germination of Cladosporium phlei, a pathogen of timothy.

The family of fellutamide lipopeptide-aldehyde proteasome inhibitors has continued to expand. Fellutamides C (59) and D (60) were discovered by screening with the genome-wide Candida albicans fitness test and analysis of the haploinsufficient heterozygote test set indicated that their mode of action was mediated through inhibition of the eukaryote 20S proteasome complex. The new fellutamides were produced from an undescribed species of Metulocladosporiella. The specific active extract was found by screening microfermentations and standard flask fermentations in which cytotoxicity-guided fractionation of an extract from A. versicolor.

Pseudallescheria boydii, a common environmental fungus and invasive fungal pathogen, produced a family of cyclic peptides designated pseudacyclins A-E. The peptide sequence of pseudacyclin A (62) was comprised of three isoleucine residues (one N-acetylated) and one residue each of phenylalanine, ornithine, and proline. The cyclopeptide could also be detected in the fungal conidia by semiquantitative MS. Besides the pseudacyclin family, cyclic peptide of phenylalanyln-proline was found to be a major metabolite in one of investigated strains of P. boydii. Pseudacyclins A was weakly cytotoxic to activated lymphocytes, but not resting lymphocytes. Onychochola sclerotica is a rare keratinophilic fungus. Arrays of microfermentation extracts from the type strain were examined to survey its metabolic capacity. In one of the eight media, LC-UV-MS analysis detected three major compounds unrecognized by an in-house microbial natural products dereplication database. The most abundant was determined to be three cyclotetrapeptides (63-65) with an aa sequence of L-N-methyl-phenylalanine, L-valine or L-isoleucine, L-N-methyl-phenylalanine, and L-valine or L-isoleucine. The compounds were moderately active in cardiac calcium channel assay, but were not cytotoxic or antibiotic.

Structures 52 to 73 here (Figs for NPR-part6.1) An investigation of a strain of Asteromyces cruciatus is a rare example of a new metabolite from an obligate marine
fungus. The fungus was grown in a battery of different media conditions intended to diversify the production of low abundance metabolites; UV-spectral analysis led to the isolation and purification of a novel pentapeptide molecule, lajollamide A (66), consisting of valine, N-methylleucine, and three leucine residues.\textsuperscript{112} The compound was weakly antibacterial towards Gram-positive bacteria. During isolation of cytotoxic cytochalasins from an unidentified *Penicillium* sp., *penilumamide* (67), a novel lumazine peptide was also isolated.\textsuperscript{113} *Penilumamide* consisted of a unique combination of a 1,3-dimethylumazine-6-carboxylic acid, a methionine sulfoxide group and an anthranilic acid methyl ester. No biological activity was identified for the molecule.

**Secondary metabolite phenotypes of* Hamigera* were analysed and correlated with phylogeny.\textsuperscript{114} HPLC-UV-based chemical analysis revealed distinctive intrageneric patterns of secondary metabolite production. Fifteen of 19 strains among 9 species produced one or more of the peptides, avellanins A (68) and B (69). Avellanins A and B consist of five aa: alanine, phenylalanine, proline and anthranilic acid as common monomers and isoleucine for avellanin A and valine for avellanin B. *Hamigera paravellanea* and *H. ingelheimensis* produced a new pentapeptide avellanin C (70) in which the isoleucine residue of avellanin A was replaced by phenylalanine. A biosynthetically related series of peptides, PF1171B (71), the leucine-version of the pentapeptide avellanin A, and PF1171A (72) and PF1171C (73) were also variably present among several species.

### 6.2 Depsipeptides

Verticilide A1 (74) is a cyclodepsipeptide related to bassianolid and enniatin.\textsuperscript{115, 116} Acyl-CoA:cholesterol acyltransferase (ACAT) has been proposed as a target for a new type of anti-atherosclerotic agent. Screening for ACAT2-selective inhibitors with a CHO cell-based assay discovered a new series of verticilide cyclodepsipeptides, A2 (75), A3 (76) and B1 (77).\textsuperscript{117} Extracts obtained from mixed fermentation of *Fusarium tricinctum* and *Fusarium begonia* on white-bean medium were analysed by HPLC, and compared to those from fermentation of monocultures on the same medium. HPLC-UV spectra indicated extracts of the mixed culture fungi differed significantly.\textsuperscript{118} These induced metabolites were determined to be new non-cyclic forms of enniatins and were designated, subenniatins A (78) and B (79). Unlike, the cyclic enniatins, the linear forms were determined to inactive in antimicrobial and cytotoxicity assays.

Structures 74 to 91 here (Figs for NPR-part6.2-J)

Pullularin A (80) was isolated as the most abundant cyclohexadepsipeptide from an ethyl acetate extract of an *Aureobasidium* sp.\textsuperscript{119} In addition to pullularins, C (81) and D (82), new pullularins, E (83) and F (84), were isolated from a strain of the ubiquitous *Bionectria ochroleuca* (previously named *Gloeosporium roseum*), from the mangrove *Sonneratia caseolaris*.\textsuperscript{120} Pullularin E (83) had an aa sequence of D-3-phenyl-lactate, L-proline, L-alanine, N-methyl-L-isoleucine, O-prenyl-L-tyrosine, N-methyl-L-alanine. Subsequent testing of the pullularins demonstrated they were cytotoxic towards a murine lymphoma cell line. F04W2166A, a proteasome inhibitor from a *Verticillium* strain is apparently equivalent to pullularin C (81), and therefore proteasome inhibition may be responsible for the cytotoxicity observed in previous studies.\textsuperscript{121}

Fusaristatins A (85) and B (86) are cyclic lipotetradepsipeptides consisting of glutamine or 2-aminoacidic acid, dehydroalanine and β-aminoisobutyric acid and a β-hydroxy C24 fatty acid isolated from an unidentified *Fusarium* strain.\textsuperscript{122} Although the fusaristatins were not antibiotic to common human pathogenic yeasts and bacteria, their similarity to the cyclic lipopeptide topotatin\textsuperscript{123} prompted testing of their activity towards human DNA topoisomerases I and II by employing relaxation assays with supercoiled plasmid DNA. Fusaristatin B was found to be weakly active against calf thymus topoisomerase I and human topoisomerase II. The *Candida albicans* genome-wide fitness test revealed two mechanistically related antifungal cyclic lipodepsipeptides,\textsuperscript{124, 125} The first of these, phomafungin (87), was observed in multiple strains of a widespread *Phoma*-like fungus.\textsuperscript{124} Phomafungin is an octapeptide linked to a β-hydroxy-γ-methyl-hexadecanoic acid. Phomafungin-containing extracts and purified phomafungin produced a complex and unique response in the population of *C. albicans* haploinsufficient heterozygotes resulting in four hypersensitive strains that corresponded to genes involved in sphingolipid biosynthesis (LCB2, RTA2/RSB1, MIT1/SUR1/CSH1, AUR1) along with strains involved in the regulation of Ca\textsuperscript{2+}, e.g. calmodulin (CMD1), Ca\textsuperscript{2+}-binding protein (FRQ1) and a vacuolar Ca\textsuperscript{2+}-ATPase involved in ion regulation (PMC1).\textsuperscript{124} Later, during investigations of the mechanism of action of phoeafungin (88), phomafungin (87) was observed to potentiate the effects of cyclosporin A, indicating it may exert its antifungal activity through the calcineurin pathway. Phoeafungin (88), from a *Phaeosphaeria*-like fungus, consists of seven aa and a β3-dihydroxy-γ-methyl-hexadecanoic acid. Response of heterozygote strains to phoafungin in the *Candida* fitness test partially overlapped with that of phomafungin, in particular, affecting heterozygotes deficient in genes involved sphingolipid biosynthesis. It also induced hypersensitivity of two GSK1\textsuperscript{1+/−} strains and a group of double heterozygous deletion strains implicated in the action of inhibitors of fungal glucan synthase. Consistent with these effects on heterozygotes for sphingolipid and glucan synthase genes, phoafungin synergized the activity of aureobasidin A, a sphingolipid biosynthesis inhibitor, and caspofungin, a glucan synthase inhibitor. The authors speculated that phoafungin’s and phoeafungin’s antifungal effects might be differentially exerted through the *C. albicans* cell membrane resulting in changes in sphingolipid content, Ca\textsuperscript{2+} concentration, and cell wall integrity.

Organic extracts of a rice-cultivation of *Eupenicillium javanicum* were highly and specifically inhibitory to growth of *A. fumigatus*. Bioassay-guided purification yielded a cyclic depsipeptide consisting of nine aa linked by a lactic acid residue that was named eujavanic acid (89).\textsuperscript{126} A fungal isolate (MSX 51320), tentatively identified as a *Trichothecium* sp. by sequence homology of LSU rDNA gene and by production of some known *Trichothecium*-associated metabolites, produced cyclodepsipeptides test partially overlapped with that of phomafungin, in particular, affecting heterozygotes deficient in genes involved sphingolipid biosynthesis. It was also induced hypersensitivity of two GSK1\textsuperscript{1+/−} strains and a group of double heterozygous deletion strains implicated in the action of inhibitors of fungal glucan synthase. Consistent with these effects on heterozygotes for sphingolipid and glucan synthase genes, phoafungin synergized the activity of aureobasidin A, a sphingolipid biosynthesis inhibitor, and caspofungin, a glucan synthase inhibitor. The authors speculated that phomafungin’s and phoeafungin’s antifungal effects might be differentially exerted through the *C. albicans* cell membrane resulting in changes in sphingolipid content, Ca\textsuperscript{2+} concentration, and cell wall integrity.
Structures 92-95 here (Figs for NPR-part6.2-2)

New cyclic depsipeptides, JBI-113 (93), JBI-114 (94) and JBI-115 (95) were isolated from a nutrient-supplemented rice culture of Penicillium sp. based on spectral analysis. An attempt to associate the compounds with a biological function, found that the compounds were not cytotoxic to a human cell line, nor inhibited growth of a Gram-positive or a Gram-negative bacterium.

6.3 Peptaibols

Although the chemical diversity of peptaibols and peptabiotics are among the best-studied of all fungal NRPs, new peptide sequences continue to be reported. Three strains of Trichoderma atroviride were found to produce typical 19-residue peptaibols, however, three of them exhibited unusual peptidic sodium-adduct \([M + 2Na]^{2+}\) ions. Peptide sequencing revealed two series of unprecedented 17-residues with an atypical C-terminus (e.g., 96) and, thus, they were recognized as a new peptaibiotic family. Comparison of sequences for 17- and 19-residue peptides showed that positions 1 to 16 were similar, but differences occurred at position 17 where the typical glutamine was replaced with an unknown residue of a MW of 129 Da. The authors hypothesized that this new unexpected residue, possibly dihydro-glutamine or \(N^2\)-hydroxornithine, may have prematurely terminated peptide elongation. These 17-residue peptaibiotics were weakly toxic towards human oral epidermoid carcinoma cells. Trichoderma citrinoviride, associated with Quercus suber (cork oak), has been observed to antagonize various fungal stem pathogens involved in oak decline. Fermentation extracts were screened for the presence of antagonistic secondary metabolites. From liquid culture, a mixture of peptaibols of the paracelsin family was characterized. Twenty-eight 20-mer peptides with C-terminal phenylalaninol and N-terminal acetylation were identified, and the major group was characterized. As expected, the peptide mixture was strongly inhibitory to growth of the oak bark pathogen Biscogniauxia mediterranea.

Structures 96 to 103 here (Figs for NPR-part6.3.)

Suzukacillins are one of oldest known peptaibol families. The classic suzukacillin-producing strain Trichoderma viride strain 63 C-1 produced two microheterogeneous groups of suzukacillin peptaibols in fermentations. Both groups were easily distinguishable by TLC, and the major group was designated suzukacillin-A and the minor group suzukacillin-B. Fourteen peptides comprised of the C-terminal sequence Pro-\(^2\)-Lxx-Lxx-Aib-Pro-Vxxol/Lxxol were identified among the latter group, typical for 11-mer peptaibols and one peptide tentatively identified as a ten-residue sequence, where the C-terminal 1,2-amino alcohol was missing, thus terminating in free proline. Trichoderin A (98), A1 (99) and B (100) are new aminolipopeptaiobiotics from a Trichoderma sp. and are related to leucinostatins, trichopolyns, helioferins, and roseofersins. They were discovered with a screening system against dormant mycobacteria and demonstrated potent antimycobacterial activity against Mycobacterium smegmatis, M. bovis and M. tuberculosis under both aerobic conditions and latency-inducing hypoxic conditions. Rice-culture extracts of two strains of fungi of the Hypocreales were investigated for the cause of cytotoxicity to a human non-small cell lung carcinoma cell line. Extract fractionation yielded series of 12 peptaibols, eight of which represented new sequences (e.g., atoviridin D 101). The set of peptaibols exhibited a range of bioactivities against tumour cell lines, Gram-positive bacteria, and Haemonchus contortus larval motility. Screening for molecules inhibiting Trypanosoma brucei led to the discovery of new trichosporins B-VIIa (102) and B-VIIb (103) from a Trichoderma polysporum.

7 Potential for novel peptides in newly sequenced and annotated fungal genomes

A global overview of NRPS evolution and diversification in the Fungi is now emerging, and significant progress has been made in developing a predictive framework for classifying fungal NRPSs. Few NRPSs are fully conserved across ascomycete fungi. As a result, the high diversity of domain structures, and the patchy distribution of equivalent A domains complicates ortholog recognition among different fungal species. As illustrated above, NRP biosyntheses in model fungi, major human, animal, and plant pathogens has received considerable attention because of its involvement in pathogenicity and the origin of drug-like peptides. However, genome sequencing projects covering the whole spectrum of environmental fungi are providing fertile ground for directed peptide discovery through pathway prediction, activation, heterologous expression, and pathway engineering.

Figure 4 here

Fig. 4. Frequency of putative NRPS gene clusters in selected genome sequenced species of the Basidiomycota. NRPS gene clusters with >5 modules are absent.

Recently one of the first evaluations of NRPS complexity in a lower filamentous ascomycete, Pyronema confluens, has confirmed that typical Ascomycete secondary metabolites are underrepresented in the Pezizales. This fungus possessed seven putative NRPS genes, and one PKS gene, substantially fewer than in the genomes of the kinds of Ascomycetes described above. PCON_02859 was predicted to encode an NRPS which had the typical domain structure of siderophore NRPSs and was part of a cluster of gene homologues which are involved in siderophore biosynthesis in other fungi. A second putative NRPS gene (PCON_07777) was not part of cluster and lacked homology to NRPSs with known functions. The remaining five NRPS genes all had a domain structure that is typical for alpha-aminoadipate reductase (AAR)-type NRPSs, and (with the exception of PCON_04030) all have high sequence similarity to aminoadipate semialdehyde dehydrogenase, an enzyme for lysine biosynthesis that is conserved in fungi. Curiously, most fungi usually have only a single AAR-type NRPS, therefore the multiplicity of AAR-type NRPS genes in P. confluens presently appears to be an anomaly. Possible reason for this duplicity may be selective amplification of the specific gene family or the loss of other NRPS genes leaving only AAR-type NRPS genes. One of these AAR-type NRPS genes, PCON_04030 was located adjacent to the single PKS gene (PCON_04029) in a gene cluster that also contained other genes encoding enzymes that might participate in the biosynthesis of a hybrid PK-NRP, rather than in lysine biosynthesis. The existence of such structured gene clusters encoding separate PKS and NRPS proteins that act in a common biosynthetic pathway is reminiscent of the cluster responsible for the production of emericellamide and the
echinocandins. Such examples are fertile targets for new metabolite discovery through genome mining techniques.

Figure 5 here

Fig. 5. Frequency of putative NRPS gene clusters in selected genome sequenced species of the Ascomycota. NRPS gene clusters with >5 modules are indicated in maroon.

Many single modular NRPS-like proteins do not consist of the typical A-T-C module architecture, but often consist of an A-T bidomain followed by a variety of C-terminal domains. These monomodular NRPS-like proteins may or may not be involved in secondary metabolic biosynthesis; for example, the aminoadipate reductase LYS2 in lysine biosynthesis pathway which terminates with a thioester reductase domain. Macrocyclic peptides and peptaibiotics have a high affinity for biological targets and would be desirable objectives for genome mining and gene cluster activation. The Norine and The Comprehensive Peptaibiotics Database projects have plotted size distributions for published fungal peptides. As a simple illustration, we analysed the frequency and species distribution of large peptide synthases (>5 A domains) and in a selection of recently sequenced fungal genomes as an example for future exploration, and as a retrospective analysis of whether natural products discovery paradigms have been effective in unearthing these generally highly bioactive molecules (Figs. 4, 5).

Because the correlation of NRPS genes with their products has become straightforward, the focus now will shift to the question of how best to obtain the products of these pathways in sufficient quantities and channel them into screening libraries where their chemical biology can be explored. Although vast numbers of predicted NRPS genes have been annotated in fungal genomes, still only a few dozen pathways have been identified and characterised experimentally, including those for cryptic or silent gene loci. Several strategies have been developed for discovering the products of these biosynthetic gene clusters. As illustrated in the above examples, gene knockouts are probably the most direct way. For example, Chiang et al. randomly deleted six NRPS genes in A. nidulans, and compared the metabolic profiles of the wild-type strain with the six deletion mutants. They found five compounds disappeared in the AN2545.3 deletion mutant. However, four new compounds appeared. After isolation and structural characterisation, they were found to be emericellamides C-F. In some cases, biosynthetic gene clusters will be silent or their expression levels will be very low, making metabolites difficult to detect in typical fermentation conditions, and particularly if many other metabolites are present. For this situation, other strategies would be needed, for example induced expression of transcriptional activator and heterologous gene expression. A PKS-NRPS hybrid gene cluster (apdA, apdB, apdC, apdD, apdE, apdG and apdR) in A. nidulans was activated by induced expression of transcriptional activator ApdR, and two new compounds were found and named aspyridones A-B. Xu et al. expressed the ApdA (PKS-NRPS hybrid) and ApdC (ER) in Saccharomycetes cerevisiae, reconstituted their functions in vitro and in S. cerevisiae, and isolated a new compound named preaspyridone 7. Wasil et al. heterologously expressed genes from the aspyridone biosynthetic cluster in A. oryzae, and detected eight different compounds in addition to aspyridone A. Moreover, Halo et al. expressed tens, a PKS-NRPS hybrid gene responsible for the production of tenellin, with orf3 (trans-acting ER) in A. oryzae and obtained three new compounds. These successful experiments have demonstrated that heterologous gene expression is an efficient method to attain the target gene’s product. However, many fungal NRPSs contain more than five A domains which means their genes exceed 15 kb, and the largest ones can consist of 19 A domains encoded by a 66 kb gene (Fig. 5). Such large genes are very difficult to amplify and heterologously express with conventional molecular biology methods. Gressler et al. outlined a strategy that might work around this limitation. They searched for the compound biosynthesized by PKS-NRPS hybrid ATEG00325 in A. terreus. They constructed a lacZ reporter strain of A. terreus to reveal conditions for expression, which finally led to the isolation of three compounds, isoflavipucine, dihydroisoflavipucine, and a phytotoxin, under specific physiological conditions.

Finally, in any other natural products discovery process, after overcoming the technical challenges of activation, expression, detection, and production of the products, one needs to seek biological functions. Therefore, a major challenge for the field as a whole remains overcoming the logistical, infrastructural and administrative barriers that prevent flow of compounds from the laboratories of individual research groups into the appropriate screening centres where they can be efficiently accessed by biologists. Taking full advantage of these new sources of molecules will require the building of cohesive scientific teams with creative know-how in exploiting sources of natural chemical novelty and biological functions, along with those seeking new molecular entities.

Acknowledgements

This work was sponsored by the National Basic Research Program of China (2013CB127505), National Natural Science Foundation of China (U1036602, 31328001 to ZA), the National High Technology Research and Development Program of China (2011AA10205), a scholarship awarded to XN (2012070305) from China Scholarship Council, the University of Texas Health Science Centre at Houston new faculty start-up funds (to GFB), the Texas Emerging Technology Fund and the Welch Foundation (AU00024 to ZA).

Notes and references


