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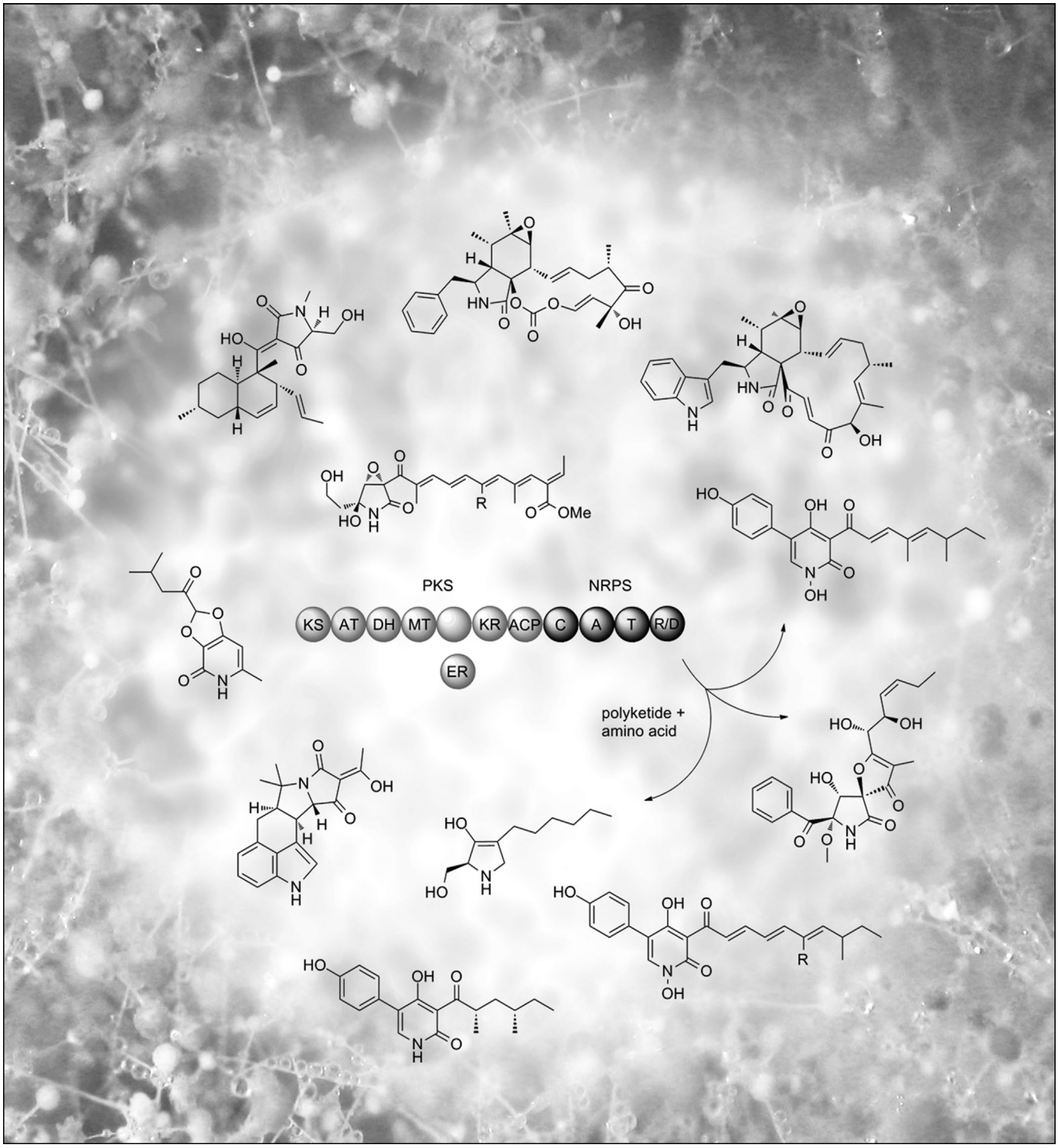
Molecular Diversity Sculpted by Fungal PKS–NRPS Hybrids

Daniela Boettger[a] and Christian Hertweck*[a, b]



Fungal polyketide synthase–nonribosomal peptide synthetase (PKS–NRPS) hybrids manufacture a wide range of structurally diverse secondary metabolites that play an eminent role in the environment, as molecular tools and leads for therapeutic development. To date, a dozen PKS–NRPS megasynthetases can be linked to the corresponding secondary metabolites, which stand out because of their structural complexity. The diversity of their structures, biological activities, and biosynthetic routes are particularly intriguing considering the iterative use of the catalytic domains of the biosynthetic enzymes—implying an enigmatic biosynthetic code. This review provides an overview of the characterized fungal PKS–NRPS hybrids, their manifold functionalities, and the diversity of the resulting secondary metabolites, as well as molecular engineering attempts that highly improved the understanding of their cryptic programming.

Introduction

The immense number of secondary metabolites produced by microorganisms and plants evolves from surprisingly simple biosynthetic building blocks. Small acetyl-CoA units form polyketides,[1] lipids,[2] and terpenes,[3] whereas amino acids constitute the main chemical source for ribosomal and nonribosomal peptides[4] and alkaloids.[5] The great diversity of the natural products results from different types and numbers of condensed subunits and the extent of further processing reactions, such as cyclization, aromatization, alkylation, glycosylation, hydroxylation, and epoxidation.[5–7] Further variety is added through the marriage of biosynthetic pathways, as in the well-known example of aflatoxin, where a polyketide synthase incorporates a fatty acid as a starter unit.[8] From a structural point of view, however, the fusion of polyketide and nonribosomal peptide biosynthetic pathways is particularly intriguing, and widespread among microorganisms.[9]

Important examples of bacterial polyketide–amino acid (PK-AA) hybrid molecules include bleomycin,[10] epothilone,[11] yersiniabactin,[12] and rapamycin.[13] Their biosynthesis is performed by bacterial type I polyketide synthase–nonribosomal peptide synthetase (PKS–NRPS) hybrids with a modular domain arrangement (one catalytic module for each incorporated subunit). This colinear modularity has facilitated comprehensive studies on the functioning of these multidomain enzymes. In contrast, the function and biosynthetic programming of fungal hybrid synthetases is much less understood. Not only is the iterative action of the PKS module enigmatic, handling and genetic manipulation of fungi often poses a challenge.

Nonetheless, fungal metabolites assembled by PKS–NRPS hybrids constitute a large group of structurally complex molecules that are often endowed with highly relevant biological activities. Perhaps the best-known representatives are the actin-filament-destroying cytochalasins,[14] the neuritogenic and immunosuppressive agent pseurotin A,[15] and the infamous mycotoxins fusarin C,[16] cyclopiazonic acid,[17] and flavipucine[18] (Table 1). Insights into the molecular basis of their biosynthetic pathways and the function of the multifarious enzymes involved could greatly benefit in analyzing the pathobiology of fungal pathogens. Furthermore, from a pharmacological perspective, engineering of PKS–NRPS hybrid pathways could grant access to new chemical entities that are difficult to yield by chemical synthesis.

Table 1. Fungal polyketide–amino acid hybrid compounds, producers, biological activities, and known biosynthetic enzymes.

Compound	Producer(s)	Biological activity	PKS–NRPS
aspyridone	*A. nidulans*	cytotoxic	ApdA
chaetoglobosin A	*P. expansum*	actin filament capping	CheA
cyclopiazonic acid	*A. oryzae, A. flavus*	mycotoxin, inhibition of Ca^{2+}-ATPase	CpaS
cytochalasin E	*A. clavatus*	anti-angiogenic	CcsA
equisetin	*F. heterosporum*	inhibition of HIV-1 integrase	EqiS
fusarin C	*G. moniliformis, G. zeae*	mycotoxin, mycoestrogenic	FUSS, FusA
isoflavipucine	*A. terreus*	mycotoxin	ATEG_00325
NG-391	*M. robertsii*	mutagenic	NGS1
pseurotin A	*A. fumigatus*	neuritogenic, immunosuppressive	PsoA
tenellin, desmethyl-bassianin	*B. bassiana*	entomopathogenic, membrane ATPase inhibition	TenS, DmbS
xyrrolin	*Xylaria* spp.	moderate cytotoxicity	PKS3
unknown	*M. grisea*	avirulence factor	Ace1
unknown	*Trichoderma* spp.	host defense response	Tex13

Recent advances in molecular cloning, functional analysis, and heterologous expression of fungal PKS genes[19] led to the identification of many fungal PKS–NRPS hybrids in the last decade. To date, a dozen different polyketide–amino acid (PK–AA) hybrid molecules have been connected to the gene cluster(s) encoding the responsible PKS–NRPS hybrids and tailoring enzymes (Table 1). Mutations in two further genes coding for PKS–NRPS hybrids altered biological characteristics of the producer strains; however, the corresponding compounds await identification.[20–21]

This review provides an overview of all characterized PKS–NRPS hybrids from fungi, their genetic background, and the proposed biosynthetic routes of the corresponding compounds. Furthermore, it highlights distinctive features of par-

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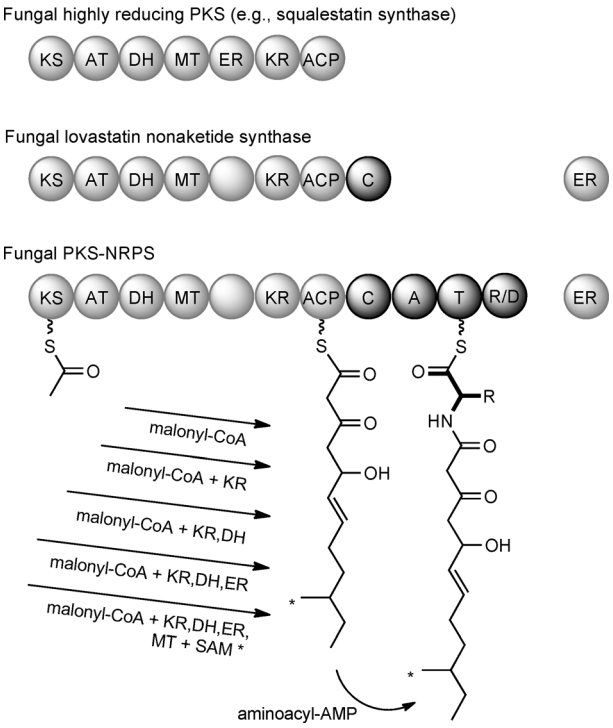
ticular gene clusters, enzymes, and metabolites, and introduces primary molecular engineering approaches.

Basic PKS–NRPS Enzymology

Fungal type I PKS–NRPS build up PK–AA hybrid molecules by using a set of catalytically active domains in an iterative manner,[22] unlike those in bacteria. Phylogenetic analyses of fungal NRPS have shown that the known and putative fungal PKS–NRPS hybrids fall into a well-characterized monophyletic group, suggesting a common origin.[23] Moreover, all hybrids identified so far are restricted to the subclass of Euascomycetes, and possess a domain organization that is common for fungal highly reducing PKS (hrPKS, Scheme 1).[24] In the course of biosynthesis, the ketosynthase domain (KS) elongates an acetyl-CoA starter unit by malonyl-CoA extenders that are loaded onto the acyl carrier protein (ACP) with support from the acyl transferase domain (AT). After each elongation, ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains mediate β-keto processing steps.[1] A prominent feature of fungal PKS–NRPS hybrids is the lack of an intact ER domain within the PKS module (Scheme 1).[25] Its catalytic function is either not essential for product formation, or is complemented by a *trans*-acting ER encoded in the biosynthetic gene cluster or elsewhere in the genome. In contrast to many bacterial modular type I PKSs, which can incorporate branched extender units,[6] to date no fungal PKS that utilize extender units other than malonyl-CoA are known. Chain branches are usually introduced by *S*-adenosylmethionine (SAM)-derived α-methylation of the growing polyketide chain by designated C-methyltransferase (MT) domains.

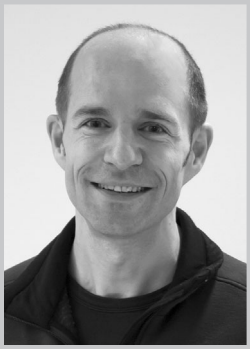


Scheme 1. Domain organization of fungal PKS and PKS–NRPS hybrids, and biosynthesis of polyketide–amino acid hybrids.

The NRPS module selects and activates a specific amino acid by means of the adenylation domain (A), which loads the aminoacyl residue onto the thiolation domain (T). How the NRPS selects the correct amino acid to load is still ambiguous; however, the identification of a selectivity signature in the A and condensation domains (C) provides clues about the incorporation of aromatic amino acids.[26] After completion of the full polyketide substructure, the C domain catalyzes fusion of the polyketide chain to the activated amino acid to yield an amide.[24]

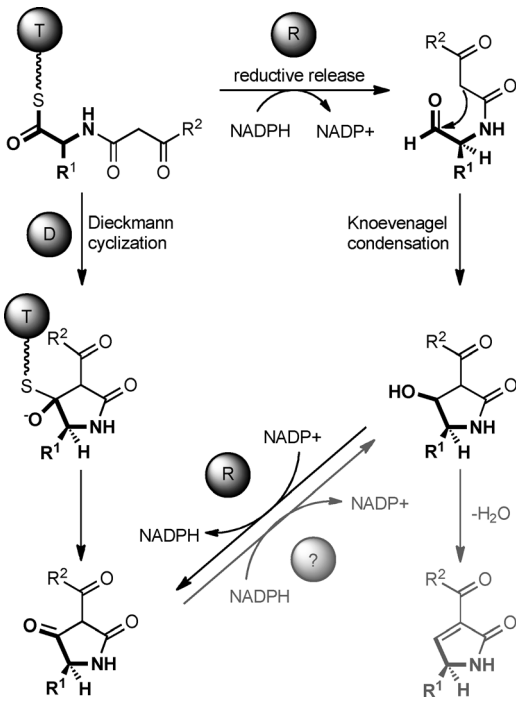
There are two conceivable routes to release the amide resulting from the fusion of the polyketide chain with an amino acid (Scheme 2). Sequence similarities of the terminal reductive domain (R) to short-chain dehydrogenases/reductases (SDR) suggest a reductive release, thereby generating a free aldehyde that could undergo a Knoevenagel condensation to form a pyrrolinone.[27] Particular protein motifs, such as an NADPH-binding motif and a catalytic triad Ser-Tyr-Lys, are indispensable for this reduction step.[28–29] In an alternative route, a tetramic acid derivative can be released directly through Dieckmann cyclization (DKC), catalyzed by the terminal domain (D). This reaction does not necessarily require the characteristic motifs of a reductase-like R domain, but was shown to depend on the presence of a specific aspartate residue.[30] Irrespective of the type of cyclization, typically, tetramic acids (2-pyrrolidones) are the primary products of the hybrid synthetase.

To yield the final product, the polyketide amino acid hybrid molecules can pass through a set of tailoring reactions and



Christian Hertweck gained a Ph.D. in organic chemistry at the University of Bonn and at the MPI for Chemical Ecology. From 1999 to 2000 he was Feodor Lynen postdoctoral fellow at the University of Washington, Seattle, before starting his own research group at HKI Jena. Since 2006 he has been full professor at the Friedrich Schiller University, Jena, and head of the Department of Biomolecular Chemistry at the Leibniz Institute for Natural Product Research and Infection Biology (HKI). His research interests encompass bacterial and fungal natural products, with a focus on biosynthesis, pathway engineering, and microbial interactions.

Daniela Boettger received her diploma in biochemistry and molecular biology from the Friedrich Schiller University of Jena in 2009. She is currently working on her Ph.D. thesis in the Hertweck laboratory at the Leibniz Institute for Natural Product Research and Infection Biology, focusing on the biosynthesis of polyketides, nonribosomal peptides, and hybrid metabolites in fungi.



Scheme 2. Possible release mechanisms of polyketide–amino acid hybrids to yield tetramic acids (black) and pyrrolinones (gray).

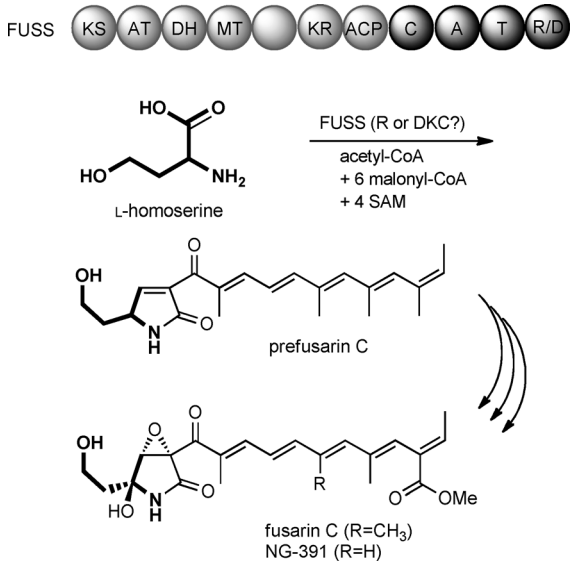
structural rearrangements. Genes for the required enzymatic activities are usually clustered around the PKS–NRPS-encoding gene.[31] For example, an enzymatic ring expansion of the tetramic acid may give rise to pyridine alkaloids (2-pyridones), and macrocycles can result from intramolecular Diels–Alder reactions.

From an evolutionary point of view, fungal PKS–NRPS hybrids are intriguing because it appears that they likely evolved from a bacterial donor through horizontal gene transfer in an early evolutionary event.[32] Their A domains form a distinct clade among fungal[23] and bacterial NRPS[32] A domains in phylogenetic analyses, and the PKS and NRPS components of full-length known and putative fungal hybrids show highly similar arrangements when compared in phylogenetic tanglegrams.[26] Of particular interest is the strong homology of fungal PKS–NRPS to lovastatin nonaketide synthase (LNKS, Scheme 1) involved in the biosynthesis of the blockbuster drug lovastatin.[33] Phylogenetic and sequence analyses and molecular engineering attempts suggest that LNKS shares a common ancestor with fungal hybrid enzymes but has developed different functional activities for its catalytic domains.[26]

Fusarin C and NG-391

The first fungal PKS–NRPS hybrid was not identified until 2004, when the two fungal strains *Fusarium moniliforme* and *F. venenatum* were investigated for the presence of fungal PKS C-methyltransferase domains.[16] The PCR-based identification and cloning of the *fusA* gene (encoding the fusarin synthetase, FUSS or FusA) allowed the construction of a knockout strain deficient in fusarin C production.[16] Later analysis of the polyketide synthase genes in the plant pathogen *Gibberella zeae* identified another locus, the *GzFUS1* gene (encoding PKS10), which was also associated with fusarin C production.[34] Fusarin C is a potent mycotoxin that has been suggested to cause human esophageal cancer in South Africa and China due to its high presence in *Fusarium*-infected grain.[35–36] This is supported by recent findings that fusarin C constitutes a new structural class of estrogenic agonists (mycoestrogens) that promote growth of breast cancer cells in vitro.[37]

Biosynthetically, fusarin C is composed of a tetramethylated heptaketide connected to L-homoserine (Scheme 3).[38] The FUSS hybrid synthetase is closely related to LNKS, with a similar domain organization, including an MT domain and a nonfunc-



Scheme 3. Proposed biosynthesis of fusarin C.

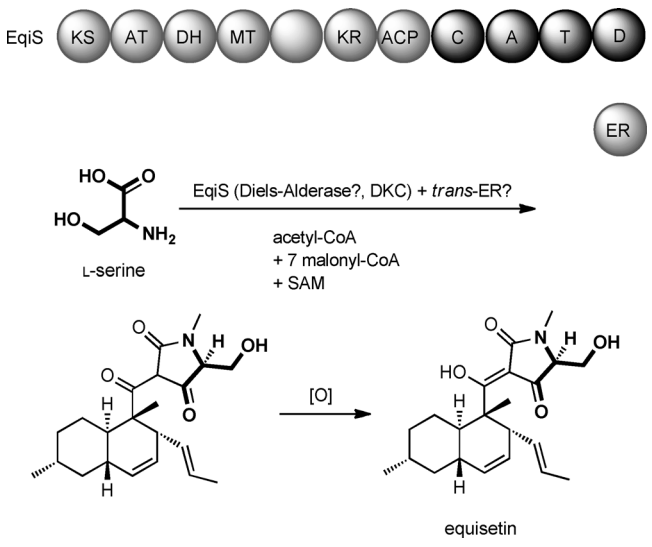
tional ER domain.[39] Other than in lovastatin synthesis, the function of the ER does not need to be substituted by an in *trans* enzyme, as the fusarin C structure suggests it does not require any enoyl reduction step. After amide bond formation between the polyketide chain and the amino acid, the terminal reductase domain is believed to release a free aldehyde ready to undergo an intramolecular aldol condensation to form the pyrrolidone ring of prefusarin C. Although the presence of an intact NADPH-binding domain and the full catalytic triad supports reductive release, chain off-loading by an intramolecular cyclization cannot be excluded.[30] However, the resulting tetramic acid has to undergo several further modifications, including carboxylation, epoxidation, and hydroxylation, to form fusarin C. Since the required enzymatic activities are not encoded in the biosynthetic gene cluster, it is suggested that similar enzymes, encoded elsewhere in the genome, perform the transformation of prefusarin C into fusarin C.[16]

Several years after the identification of the FUSS biosynthetic enzyme, the highly similar hybrid synthetase NGS1 from the entomopathogen *Metarhizium robertsii* was reported.[40] The NGS1 biosynthetic gene cluster is involved in the biosynthesis

of NG-391, a desmethyl analogue of fusarin C.[41] *NGS1* deletion mutants had no impact on the virulence of the insect pathogen compared to wild type.[40] Nonetheless, the mutagenic properties of NG-391 strongly question the use of the fungus *M. robertsii* as a biocontrol agent against insect pests.[42] Interestingly, NG-391 possesses neurotrophic activity, which could be helpful in the treatment of neurodegenerative diseases.[43] NG-391 and fusarin C are likely to involve an almost identical biosynthetic route, as their structures differ by only one methyl group. Furthermore, the biosynthetic gene clusters around *fusA* and *NGS1* are highly similar in sequence and open reading frame (ORF) arrangement, thus suggesting a common evolutionary origin of these gene clusters.[40]

Equisetin

Simultaneously with investigations on the FUSS PKS-NPRS, the *eqi* biosynthetic gene cluster, responsible for the production of equisetin in *F. heterosporum*, was identified.[44] Among the numerous mycotoxins from *Fusarium* species,[45] equisetin attracts special attention because of its broad toxicity[46] and its inhibition of HIV-1 integrase.[47] Structurally, equisetin represents a tetramic acid with a decalin component that is reminiscent of the structure of lovastatin. These structural homologies led to the PCR-based identification of the *eqi* biosynthetic gene cluster including the *eqiS* gene, which encodes the PKS–NRPS hybrid responsible for equisetin production. The domain organization of EqiS closely resembles that of LNKS. Moreover, the structure of equisetin suggests the necessity of a *trans*-acting ER for correct product formation.[44] Thus, a lovastatin-like biosynthetic mechanism is likely for equisetin: EqiS forms a methylated octaketide chain that is likely to undergo Diels–Alder cyclization at the heptaketide stage, in analogy to the lovastatin biosynthesis.[48] Subsequent attachment of a serine is followed by R-domain-catalyzed off-loading, thereby creating the free tetramic acid (Scheme 4).



Scheme 4. Putative biosynthetic pathway of equisetin.

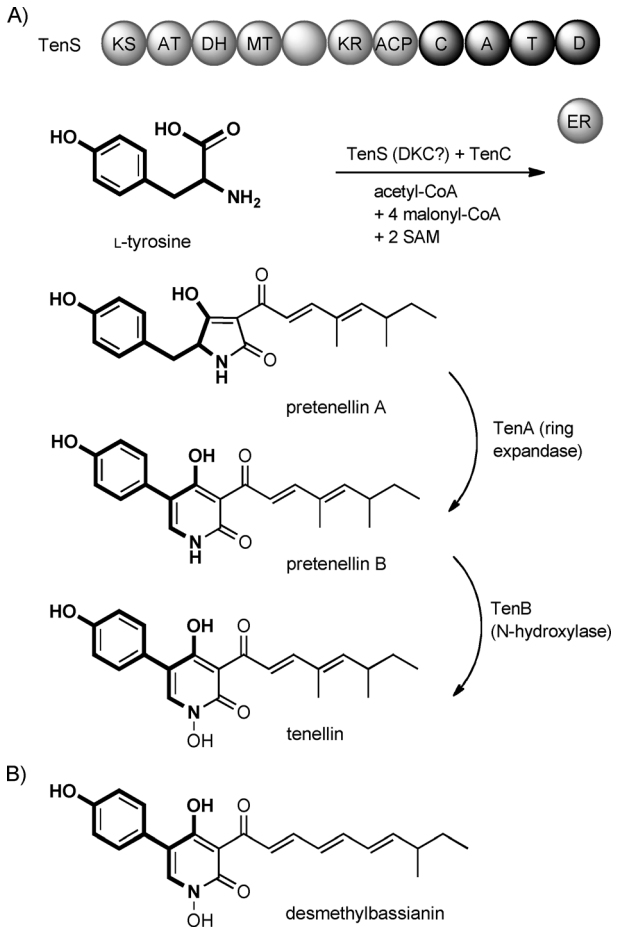
Although sequence analysis revealed the presence of an intact catalytic triad and NADPH-binding motif in the terminal R domain,[30] EqiS does not perform the typical NADPH-dependent reductive release.[27] Instead, it acts as a Dieckmann cyclase (D), directly releasing the tetramic acid.

Tenellin and desmethylbassianin

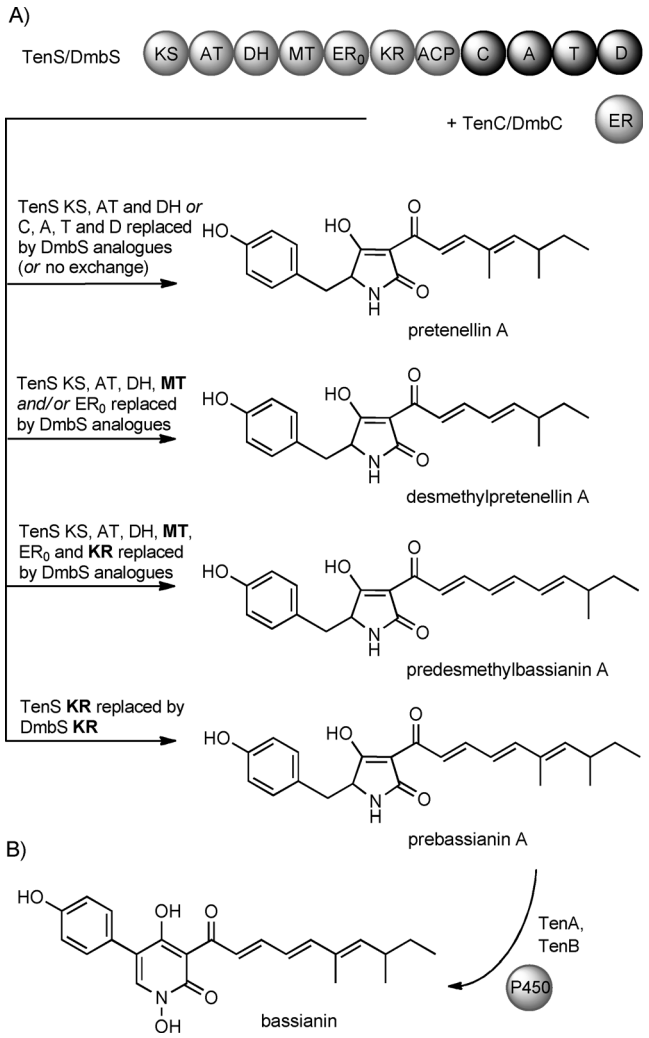
The synthetase TenS is responsible for the production of the toxic compound tenellin.[49] Its entomopathogenic producer strain *Beauveria bassiana* is well known as a biocontrol agent in a number of agricultural applications.[50] However, tenellin does not participate in its pathogenicity, as *tenS* knockouts showed no effect on the virulence of *B. bassiana* towards larvae of *Galleria mellonella*.[49] In an independent study it was shown that tenellin generates morphological changes and lytic effects on erythrocytes, and inhibits membrane ATPase activity.[51]

To date, TenS is probably the most intensively studied PKS–NRPS hybrid. A wide range of experiments have been used to investigate the role, functioning, and programming of TenS. The identification of the *ten* gene cluster was based on the presence of the unique MT domain in fungal PKS. Through heterologous expression of *tenS* in *Aspergillus oryzae*, the requirement of a *trans*-acting ER (*tenC*, ORF3) for correct production of pretenellin A was proven.[25] Without coexpression of the *tenC* ER, TenS produces tetra- and pentaketides. Moreover, the timing of the second C-methylation is delayed by one cycle (after the third instead of the second extension cycle). This is analogous to lovastatin biosynthesis, where hexa- and heptaketides instead of nonaketides are produced in the absence of the LovC ER.[52] Coexpression of only *tenS* with *tenC* results in the production of completed tetramic acids with retained enol functionality at C-4. This implies Dieckmann cyclase activity of the terminal domain rather than R-domain-mediated release.[25]

The *ten* multigene cluster was the first biosynthetic locus to be fully reconstructed heterologously, showing that only four genes are necessary to produce tenellin.[53] For the assembly of the pyridine alkaloid tenellin, tyrosine and a dimethylated pentaketide chain are fused by the TenS hybrid synthetase and released as pretenellin A (Scheme 5).[54] To convert pretenellin A into tenellin, two oxidative reactions are necessary: ring expansion of the tetramic acid pretenellin A into the 2-pyridone pretenellin B by a ring expandase, and N-hydroxylation to yield tenellin. The *ten* locus contains genes for two P450 oxygenases shown to catalyze these reactions: *tenA* (ORF1) and *tenB* (ORF2).[55] TenA promotes the formation of pretenellin B, most likely through the extraction of hydrogen and a radical-induced ring expansion.[55] Homology searches using the TenA sequence uncovered other putative ring expandases present in the vicinity of PKS–NRPS-like genes. Thus, this unusual enzymatic activity might lead to the discovery of unknown biosynthetic gene clusters. The timing of N-hydroxylation in tenellin biosynthesis could be fixed to the 2-pyridone stage, as TenB was shown to hydroxylate the nitrogen of pretenellin B but not the acyltetramic acid pretenellin A.[55] P450 enzymes are



Scheme 5. A) Proposed biosynthetic route of tenellin, and B) structure of desmethylbassianin.



Scheme 6. Domain-swap experiments of TenS with DmbS and production of bassianin. A) Chimeric hybrid synthetase genes were heterologously expressed in *A. oryzae*. TenS PKS–NRPS served as the core hybrid, and single or multiple domains were exchanged with the appropriate counterpart from DmbS. Only predominant products are shown. Domains with crucial programming influence are bold. B) Production of bassianin through coexpression of *tenS*(ΔKR:*dmbS*-KR) with *tenC* or *dmbC* and the P450 genes *tenA* and *tenB*.

rarely involved in N-hydroxylation; other enzymes take over this role, such as the FAD-dependent monooxygenases in the biosynthesis of δ-*N*-hydroxyornithine in fungi.[56]

A gene cluster associated with the production of a compound that is highly similar to tenellin, desmethylbassianin (Scheme 5), was identified in the same producer, *B. bassiana*.[57] This 2-pyridone consists of a hexaketide portion with only one SAM-derived methyl group and tyrosine. The tremendous structural similarity between tenellin and desmethylbassianin, as well as the high homology of the producing enzymes (TenS and DmbS), permitted insightful studies on domain activities and remodeling of fungal PKS–NRPS hybrids. By heterologous coexpression of *tenS* and the *dmbC trans*-ER gene (or the complementary pair: *dmbS* and *tenC*) in *A. oryzae*, the ER domains were shown to be interchangeable.[57] In both cases, the hybrids worked as normal and produced pretenellin A and predesmethylbassianin A, respectively. Furthermore, the generation and heterologous expression of a chimeric fusion protein consisting of the PKS part of TenS and the NRPS portion of DmbS also resulted in the production of pretenellin A.[57] Both results illustrate the independence of the PKS and the NRPS portions of hybrid enzymes. The PKS is programmed for polyketide chain assembly while the NRPS is mainly responsible for amino acid selection and amide bond formation. The *trans* ER domain influences the fidelity of the PKS–NRPS hybrid.

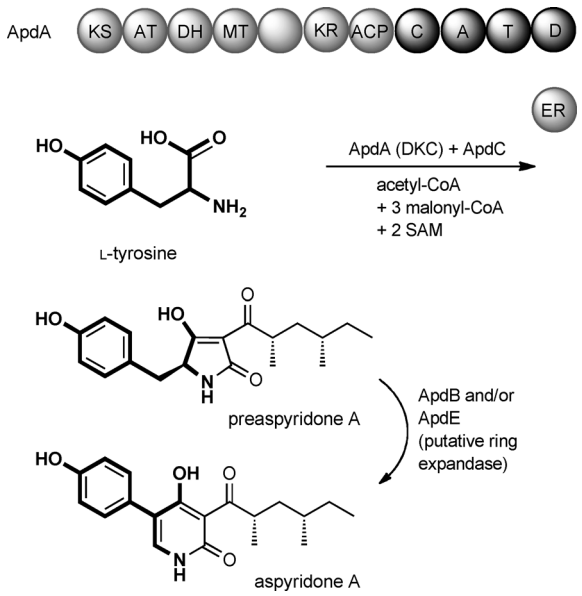
The step-by-step exchange of TenS domains with their DmbS analogues revealed the influence of the single PKS domains on PKS programming (Scheme 6 A).[58] The heterologous expression of the chimeras in *A. oryzae* revealed structural differences in the produced metabolites. Replacement of the KS-AT and the KS-AT-DH domains of TenS with the appropriate DmbS counterpart showed no influence on programming and resulted in the production of pretenellin A. Additionally, exchange of MT and ΨKR (a structural domain of KR) led to the production of mainly desmethylpretenellin.[58] This metabolite possesses the core structure and chain length of tenellin and the methylation pattern of desmethylbassianin, indicating the self-programming role of MT domains. Also, replacing the nonfunctional ER region had no impact on the resultant com-

pound, whereas the additional exchange of the KR domain or the entire PKS portion caused complete disruption of the biosynthetic pathway, and only predesmethylbassianin A was extracted from the *A. oryzae* culture (Scheme 6 A).[58] Thus, in the case of TenS and DmbS, the KR domain appears to represent the chain-length-determining factor. To prove this hypothesis, the TenS KR domain alone was replaced with the KR domain of DmbS, showing that all possible combinations of chain length and methylation degree could be detected, with a surplus of hexaketides over pentaketides. Heterologous expression of *tenS*(ΔKR:*dmbS*-KR) with the other *ten* cluster genes (*tenA–C*) gave rise to the "extinct" metabolite bassianin. (This compound had previously been isolated from *Beauveria* spp.,[59] but could not be traced in existing *Beauveria* strains.)[58] Bassianin is composed of the hexaketide chain of desmethylbassianin, and doubly methylated like tenellin (Scheme 6 B).[58] These studies showed for the first time the programming role of a KR domain in fungal hrPKS.

Finally, ways to modify the tenellin structure were explored. In particular, individual genes of the *ten* cluster were silenced in *B. bassiana*, and the transcriptional activity was challenged with epigenetic modifiers such as 5-azacytidine and suberoyl *bis*-hydroxamic acid. Alone or in combinations, these methods led to the detection of 22 structurally diverse compounds, including randomly oxidized compounds and shunt metabolites. The ability to produce a wide range of structural variations was believed to allow quick adaption of the producer strain to environmental changes.[60] Nevertheless, these experiments also question the reliability of results from awakening silent gene clusters through the application of epigenetic modifiers, as they might simulate a metabolic profile that does not reflect the true biosynthetic aim.

Aspyridone

Aiming at decryption of a silent biosynthetic gene cluster in *Aspergillus nidulans*, the aspyridone metabolic pathway was activated.[61] A novel strategy of overexpressing the putative pathway regulator gene *apdR* under control of an inducible alcohol dehydrogenase promoter (*alcA*p), led to the identification of two new metabolites: aspyridones A and B. These cytotoxic pyridine alkaloids were not produced by the *A. nidulans* wild-type under standard fermentation conditions. The gene *apdA* encodes a fungal PKS–NRPS hybrid that assembles one acetyl-CoA and three malonyl-CoAs with the support of a *trans*-ER protein (ApdC) and attaches it to the aromatic amino acid tyrosine.[61] As in vitro reconstruction of preaspyridone biosynthesis depended only on ApdA and ApdC activity and not on additional oxidative enzymes, reductive off-loading could be excluded, suggesting the involvement of a Dieckmann cyclase (D) domain (Scheme 7).[62] To yield aspyridone A or B, further oxidative reactions are required, and most likely performed by P450 oxygenases encoded in the *apd* gene cluster. Similar to tenellin and desmethylbassianin biosynthesis, aspyridones require a ring expansion reaction to achieve a 2-pyridone (Scheme 7). By comparing the sequence of the Apd P450 enzymes with the TenA ring expandase from tenellin biosynthesis, ApdE was identified as the putative ring expandase for aspyridone production.[55]

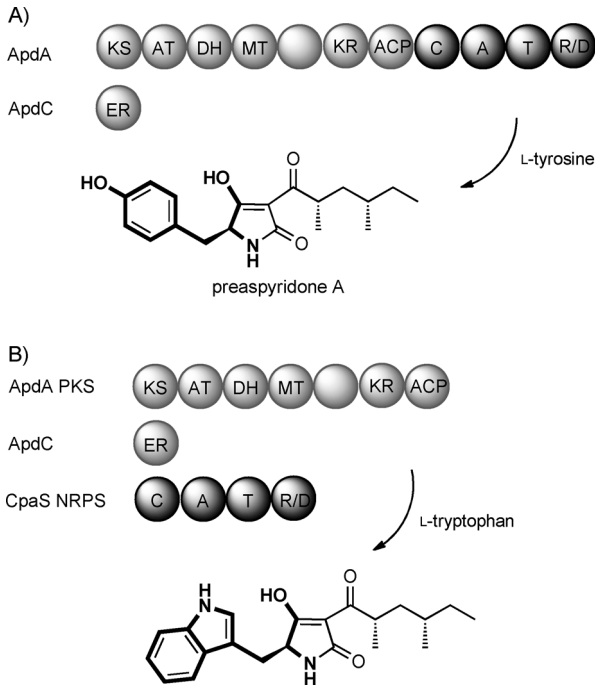


Scheme 7. Proposed biosynthesis of aspyridone A.

With the expression and in vitro characterization of the full and dissected ApdA hybrid synthetase, the flexibility of a fungal PKS—NRPS was tested. ApdA had a higher tolerance for amino acid incorporation, and that stand-alone modules can act in *trans*. Full-length ApdA and ApdC, heterologously produced in *Saccharomyces cerevisiae,* incorporated tyrosine in an in vitro assay, thus reconstituting the genuine pathway.[62] Interestingly, under these conditions, ApdA also accepted other aromatic amino acids, albeit with lower affinity. When L-phenylalanine, L-4-fluorophenylalanine, or L-tryptophan were presented to ApdA and ApdC, the corresponding metabolites were produced (not shown).[62] Moreover, the combination of the dissected PKS portion of ApdA with the separate NRPS component of another fungal hybrid, CpaS from the cyclopiazonic hybrid synthetase (see below), led to the attachment of tryptophan to the polyketide chain (Scheme 8).[62] Thus, the native amino acid selectivity of the CpaS NRPS is not affected when used in conjunction with ApdA.

Pseurotin A

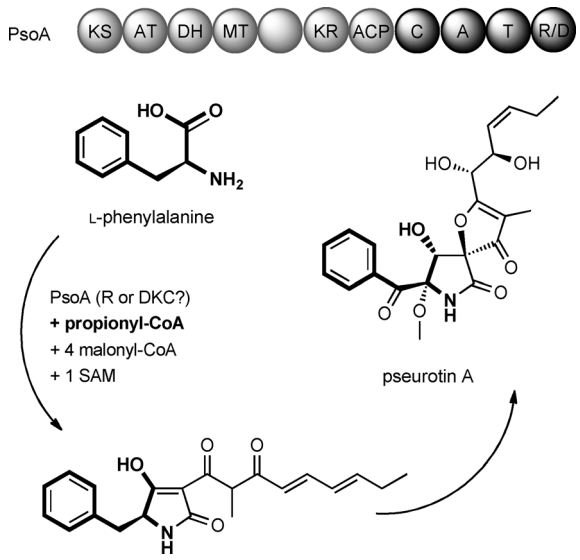
The human pathogenic fungus *Aspergillus fumigatus* has the potential to produce a large set of distinct secondary metabolites.[63] However, according to the genome sequence, the production of only one PK-AA hybrid, pseurotin A, is encoded in the genome.[63–64] This compound possesses neuritogenic and immunosuppressive properties, as well as antifungal activity through competitive inhibition of chitin synthase.[15,65–66] Structurally, pseurotin A represents a tetramic acid with an unusual heterospirocyclic system. Labeling studies suggested a PKS–NRPS pathway behind pseurotin A biosynthesis, as ^{13}C-labeled propionate, malonate, and the amino acids L-methionine and L-phenylalanine were shown to be incorporated.[67]



Scheme 8. A) Coexpression of full *apdA* and *apdC*, and B) coexpression of genes encoding dissected ApdA PKS, CpaS NRPS, and ApdC. In vivo reconstruction in *Saccharomyces cerevisiae* led to the production of preaspyridone A (A) and the appropriate tryptophan derivative (B), respectively.

The putative biosynthetic gene cluster for the production of pseurotin A consists of five ORFs, encoding the PKS–NRPS hybrid, a methyl transferase, a P450 oxidative enzyme, and two hydrolases.[68] The involvement of this gene cluster in pseurotin A biosynthesis was shown by deletion and overexpression of the PKS–NRPS gene *psoA*. The hybrid synthetase has the common domain arrangement of an hrPKS and is thought to lack a functional ER. Based on the structure of pseurotin A, ER activity does not appear to be required. During the assembly of pseurotin A, the PKS–NRPS attaches four malonyl-CoA units to the propionate starter to form a monomethylated pentaketide that is further fused to phenylalanine (Scheme 9).[68] Whether the hybrid molecule is released through reductive off-loading or by catalyzed cyclization remains to be clarified. A set of subsequent processing reactions, including several oxidations and epoxidations, is required to build up the spiro compound. The presence of only one P450 gene within the gene cluster suggests the involvement of other tailoring enzymes encoded elsewhere in the genome.[68]

Recently, it was shown that during the adaption of *A. fumigatus* to hypoxia, the expression of pseurotin A cluster genes was upregulated significantly.[69] Adaption to altered environmental conditions is an important feature of fungal virulence. The proposed role of pseurotin A during hypoxia involves the promotion of survival of *A fumigatus* against competitors in hypoxic environments, and a possible function in its pathogenicity in mammals, for example, due to an inhibitory effect on immunoglobulin E (IgE).[69]



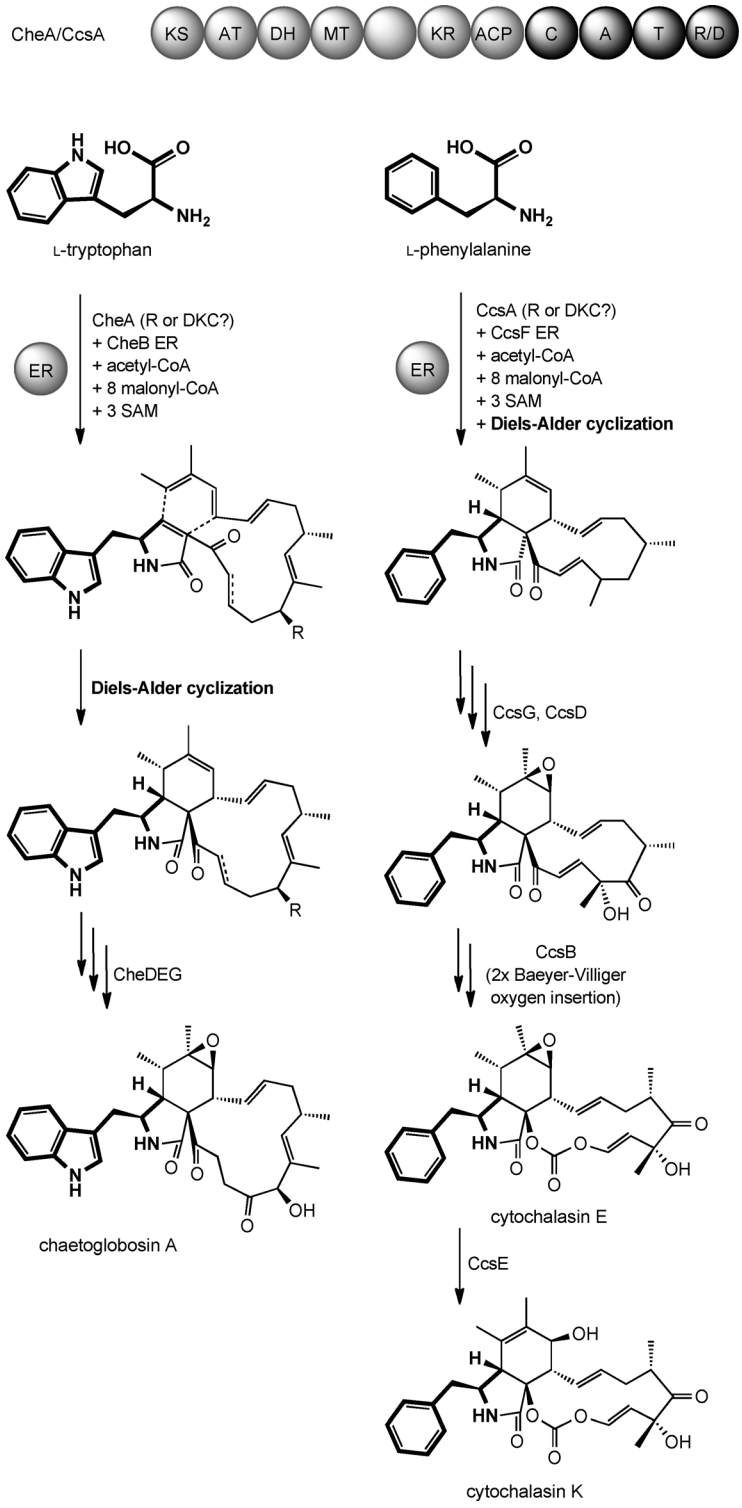
Scheme 9. Putative biosynthesis of pseurotin A.

Cytochalasins (Chaetoglobosin A and Cytochalasin E/K)

Cytochalasins (or cytochalasans) comprise a large and diverse group of fungal PK-AA hybrids.[14] This well-studied compound class exhibits a wide range of biological activity, including its most-studied property: capping actin filaments.[70] The broad diversity of cytochalasins results from the variety of assembled polyketide chains and from the range of incorporated amino acids. Compounds of the subgroups cytochalasins and zygosporins mainly involve phenylalanine, whereas chaetoglobosins incorporate tryptophan, pyrichalasins tyrosine, and aspochalasins leucine.[14]

Molecular investigations of the chaetoglobosin-A-producing plant pathogen *Penicillium expansum* led to the discovery of the first cytochalasin biosynthetic gene cluster.[71] The *che* gene locus codes for a PKS–NRPS hybrid synthetase with the typical fungal domain organization: hrPKS (CheA), a stand-alone enoyl reductase (CheB), and various oxidoreductases (CheD, E, and G), which are likely involved in tailoring reactions. The construction of *P. expansum che* knockout mutants as well as overexpression of *che* cluster genes in *Aspergillus* spp. proved to be cumbersome. Eventually, the involvement of *cheA* in chaetoglobosin A biosynthesis was proven by RNA silencing experiments, which resulted in a strong reduction of the tetramic acid production.[71]

During the biosynthesis of chaetoglobosin A, an acetyl-CoA starter unit and eight malonyl-CoA units are assembled by the CheA PKS to form a nonaketide which is then attached to the NRPS-bound tryptophan (Scheme 10). The product is released as a pyrrolinone, either through a catalyzed Dieckmann cyclization or by reductive off-loading with subsequent Knoevenagel condensation. This dienophile is predetermined to undergo a Diels–Alder [4+2] cycloaddition that results in the characteristic tricyclic core structure in which a macrocycle is fused to an isoindolone.[14] The involvement of a true "Diels–Alderase" is still questionable,[48,72–73] but possible in the case of cytochala-



Scheme 10. Suggested biosynthetic routes of cytochalasins.

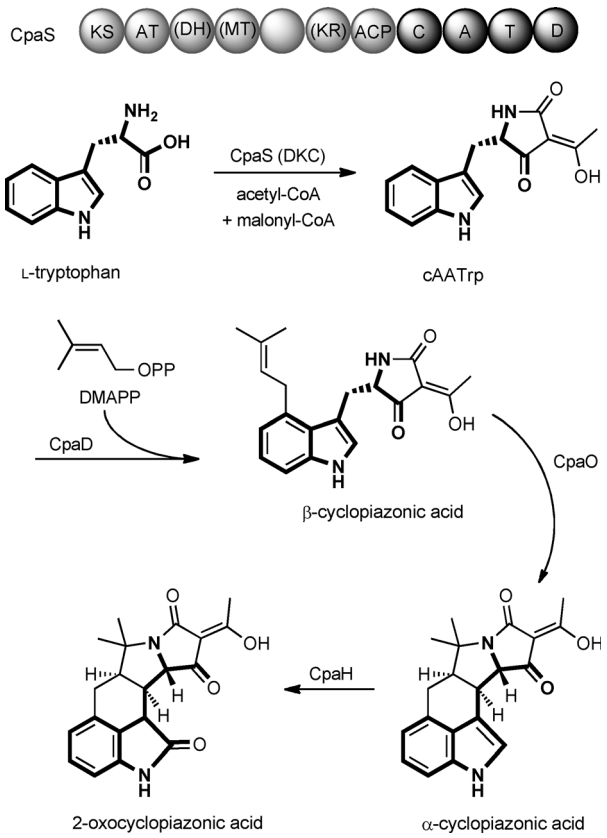
sins, as in chemical synthesis the Diels–Alder [4+2] macrocyclization requires fierce conditions.[74–75] However, enzymatic support by a "Diels-Alderase" would greatly facilitate this ring closure reaction. A set of oxidative tailoring reactions, putatively performed by CheD, CheE, and CheG, completes the biosynthetic route to chaetoglobosin.

Only recently, the biosynthetic gene cluster for two other cytochalasins, cytochalasins E and K, was identified in *Aspergillus clavatus*.[76] The *ccs* gene cluster contains eight genes, including *ccsA*, coding for the hybrid synthetase. It is thought that CcsA lacks a functional ER domain and that this is likely replaced by a *trans*-acting enzyme encoded in the cluster (CcsC). Cytochalasins E and K exemplify trimethylated octaketides attached to phenylalanine and principally their biosynthesis resembles that of chaetoglobosin A.[76] A reductive release of the hybrid molecule from its synthetase is most likely, as the terminal R domain contains the complete NADPH-binding motif and an intact catalytic triad. Moreover, a Dieckmann release would require further ketoreduction and dehydration, and these enzymatic activities are not encoded in the *ccs* gene cluster.[76] The processing of the compound after formation of the tricyclic core includes epoxidation (CcsD) and double oxygen insertion (Scheme 10). The latter reaction is likely performed by an unusual putative Baeyer–Villiger monooxygenase flavoprotein (BVMO, CcsB), and creates in a two-step reaction a unique vinyl carbonate moiety that is rare in cytochalasins.[76]

Cyclopiazonic acid

Cyclopiazonic acid probably draws the most attention among fungal PK-AA hybrids due to its ability to contaminate food, such as maize and peanuts.[77–78] This mycotoxin is produced by several *Penicillium* and *Aspergillus* species, including those employed in the production of fermented food, such as *Penicillium camemberti* (camembert cheese) and *A. oryzae* (sake and soy sauce).[79–80] In combination with aflotoxin,[81] it is believed to be involved in Turkey X disease.[82] The toxicity of cyclopiazonic acid (CPA) arises most likely from its inhibitory effects on sarcoplasmic reticulum Ca^{2+}-ATPases.[83]

The first description of CPA reaches back to 1968, but it was not until 40 years later that the responsible *cpa* gene cluster was identified in the CPA-producing *A. oryzae* strain NBRC 4177.[17,84] Three proteins encoded within the cluster catalyze the biosynthesis of the prenylated indole alkaloid. *cpaS*, *cpaD*, and *cpaO* encode a PKS–NRPS hybrid, a prenyl transferase, and a putative flavoprotein oxidocyclase, respectively.[85] The CpaS PKS–NRPS hybrid promotes the generation of the cycloacetoacetyl tryptophan (cAATrp) tetramic acid through assembly of a diketide and tryptophan that are released through a Dieckmann cyclization (Scheme 11).[30,86] An assay with heterologously produced T-R didomain of *A. flavus* CpaS demonstrated NADPH-independent release/cyclization of a hybrid molecule that needed to be tethered to the phosphopantetheine residue of the thiolation domain.[30] This is in contrast to the EqiS R domain, which also accepts free *N*-acetylcysteamine (SNAC)



Scheme 11. Proposed biosynthesis and inactivation of cyclopiazonic acid.

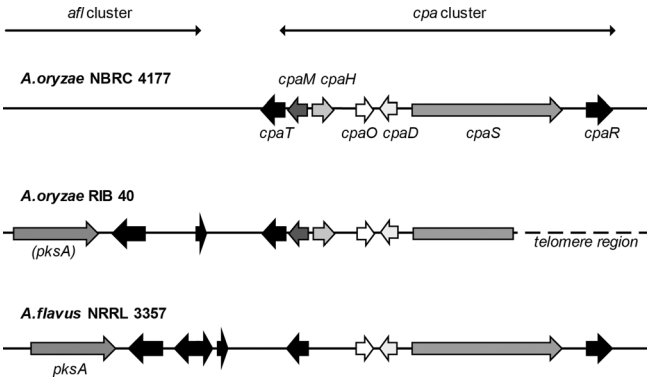


Figure 1. *CpaS/afl* gene cluster arrangement in different *Aspergillus* strains. Gene arrangements are shown in the adjacent *afl* and *cpa* gene clusters for the production of aflatoxin and cyclopiazonic acid, respectively. The depicted strains are the 2-oxocyclopiazonic acid producer *A. oryzae* NBRC 4177, the non-producing strain *A. oryzae* RIB40, and the toxic strain *A. flavus* NRRL 3357, which produces cyclopiazonic acid and aflatoxin.

thioesters and coenzyme A (SCoA) thioesters to form tetramic acid.[27] Furthermore, the lack of the second tyrosine in the catalytic triad of the R domains (SYK) also refutes the possibility of reductive release and suggests that the R domain possesses Dieckmann cyclase activity.

After off-loading of cAATrp from CpaS, the dimethylallyl transferase (DMAT) CpaD prenylates the indol moiety at the C-4 position to yield β-CPA (Scheme 11).[87] In contrast to other fungal Trp-DMATs, CpaD does not transfer dimethylallyl pyrophosphate onto free L-tryptophan. An in vitro assay showed that CpaD prenylates with acceptable conversion rates tryptophan-containing tetramic acids, cyclic peptides, and even dipeptides. Surprisingly, CpaD can act as an O-dimethylallyl transferase when tyrosine-containing tetramic acids are provided.[87] Finally, after prenylation of cAATrp, a final oxidative cyclization catalyzed by CpdO converts β-CPA into cyclopiazonic acid (Scheme 11).[86]

Interestingly, not all strains of the same species produce CPA. Comparison of the *cpa* gene cluster of the CPA producer *A. oryzae* NBRC 4177 with that of the non-producing strain *A. oryzae* RIB40 revealed deletion of one half of the *cpaS* gene through telomeric replacement in RIB40 (Figure 1).[88] However, stable-isotope-labeling experiments for RIB40 with the CpaS product cAATrp led to the production of 2-oxo-CPA and proved that the remaining genes of the cluster, *cpaD* and *cpaO*, were still functional. Further conversion of CPA into the less toxic 2-oxo-CPA is catalyzed by the putative P450 enzyme CpaH (Scheme 11).[88] Surprisingly, this additional enzymatic activity is not present in the *cpa* clusters of toxic CPA-producing species like *A. flavus* (Figure 1).[89] Thus, it can be speculated that self-detoxification through CpaH developed as an alternative strategy to regulate the production of toxic compounds, and that biosynthetic gene clusters can not only evolve from clustering but also from deletion of single genes.[89]

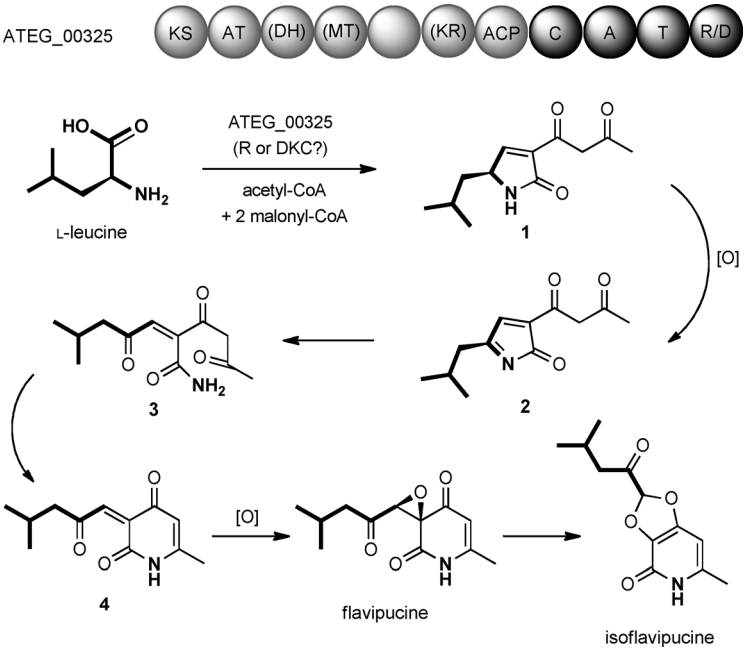
The occurrence of CPA is often accompanied by aflatoxin production, for example in *A. flavus*. This observation can be attributed to the close proximity of the two appropriate gene clusters and their location in a telomere-adjacent region (Figure 1). Deletions in this region represent the main difference between producing and nonproducing strains and also explain the co-occurrence of the two metabolites in certain strains.[17] Furthermore, the aflatoxin and the cyclopiazonic acid gene clusters are both suspected to be regulated by the global fungal regulator LaeA, which influences the production of numerous secondary metabolites.[90]

Flavipucine derivatives

Activating of the silent and sole PKS–NRPS gene cluster from *Aspergillus terreus* resulted in the production of isoflavipucine and dihydroisoflavipucine.[18] These mycotoxins were previously only known to be produced by *A. flavipes* (or accessible through semisynthetic approaches).[91–92] Their induction in *A. terreus*, however, demanded a complex strategy, including overexpression of cluster regulator genes, gene deletions, and a reporter assay to investigate the influence of varying cultivation conditions. Pathway regulator overexpression successfully unraveled the function of the silent aspyridone biosynthetic gene cluster in *A. nidulans*.[61] In contrast, overexpression of the putative regulator gene in *A. terreus* under control of the glucose-induced *gpdA* promoter failed to activate expression of the PKS–NRPS gene.[18] A strong negative impact of glucose on the activation of the pathway genes was observed instead. This effect is suspected to be mediated by the global carbon catabolite repressor CreA,[93–94] which occupies binding sites in

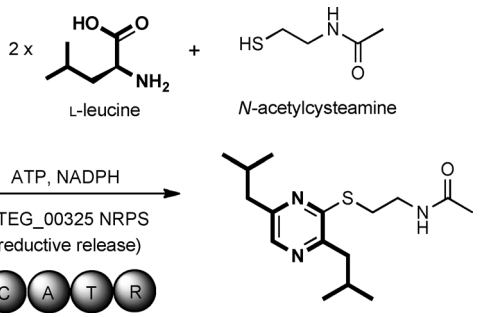
the promoter regions of the (dihydro)isoflavipucine PKS–NRPS gene.[18] Thus, the production of these PK–AA hybrids appears to be controlled by diverse global regulators, whose impacts likely overcome the influence of the cluster-specific counterparts.

Under the inducing conditions, the PKS–NRPS ATEG_00325 assembled an unreduced poly-β-keto triketide and fused it to L-leucine (Scheme 12). The nonreduced nature of the polyketide portion was supported by domain analysis of the hybrid enzyme, revealing the absence of functional domains for β-processing (KR, DH, and ER) and C-methyl transfer.[18] The terminal R domain of the hybrid includes all residues required for reductase activity. Thus, a reductive release and subsequent cyclization of the triketide-leucine hybrid is most likely. An intriguing set of subsequent reactions, including a putative transamination and unusual rearrangements, results in the final products, isoflavipucine and dihydroisoflavipucine (Scheme 12).[18]



Scheme 12. Proposed biosynthetic route of isoflavipucine. After release of a putative tetramic acid intermediate (1), oxidation (2) and hydrolysis (3) are likely followed by a transamination step (4). Subsequent oxidation forms flavipucine and further rearrangements form isoflavipucine.

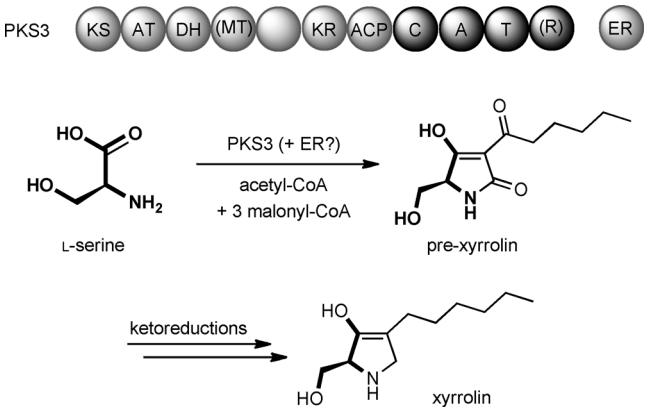
The reductive-release model is also supported by functional analyses of the NRPS component of the (dihydro)isoflavipucine PKS–NRPS. Surprisingly, the A domain appears to accept a variety of aliphatic and aromatic amino acids, and the stand-alone NRPS is able to synthesize a range of unusual thiopyrazines from non-natural substrates.[95] In an in vitro assay, the NRPS module produced as many as 63 diverse compounds in the presence of different amino acids and free thiols (e.g., DTT, *N*-acetylcysteamine). Each thiopyrazine assembly involved two L-amino acids and one free thiol and required reductive release accomplished by the R domain (Scheme 13). Mutations in the NADPH-binding site of the R domain completely abolished thiopyrazine formation.[95]



Scheme 13. Thiopyrazine formation catalyzed by the NRPS module of the ATEG_00325 PKS–NRPS. Product formation is exemplified by the reaction of two L-leucines with *N*-acetylcysteamine.

Xyrrolin

While originally searching for the gene cluster responsible for the production of 19,20-epoxycytochalasin Q from the wood-decay fungus *Xylaria* sp. BCC 1067,[96] the novel PK–AA hybrid xyrrolin was identified.[97] Deletion of *pks3*, the only gene reported to encode a PKS–NRPS hybrid in the producer strain, abolished xyrrolin production. This pyrroline compound is similar to anticancer pyrrolidines that have been rationally designed to interfere with the formation of metastases.[98] During xyrrolin biosynthesis, a tetraketide is assembled by PKS3, most likely with the support of a putative *trans*-acting ER, and subsequently fused to the aliphatic amino acid serine (Scheme 14). Unlike other PKS–NRPS hybrids, a terminal R/D domain is absent in PKS3, and the exact product-release mechanism has not been determined. However, a Dieckmann-type release would theoretically require one more keto group in the polyketide chain than that for a reductive release.[97]



Scheme 14. Suggested biosynthesis of xyrrolin.

Unknown Hybrid Metabolites Involved in Plant–Fungus Interactions

Investigations on the PKS–NRPS genes *ace1* and *tex13* from the plant-associated fungi *Magnaporthe grisea* and *Trichoderma virens*, respectively, uncovered their ecological importance but failed to identify the metabolites they produce.

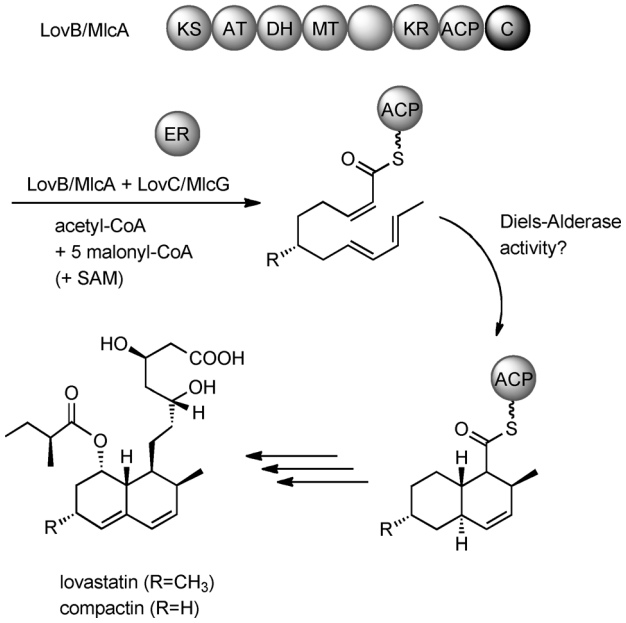
M. grisea ace1 is expressed solely during fungal penetration of rice plant leaves.[20,99] The putative PKS–NRPS gene appears together with another PKS gene in a large (15 ORF) gene cluster.[100,101] Deletion of individual genes within the cluster failed in most attempts, likely because of limited accessibility to the respective genomic region. Disruption mutants of *syn2* and a putative ER gene showed no influence on *M. grisea* virulence.[100] Nevertheless, mutations of the KS catalytic domain in the ACE1 hybrid led to inability of Pi33 resistant rice cultivars (those containing the *Pi33* resistance gene) to recognize the invading fungus.[20] Therefore, ACE1 likely mediates avirulence of *M. grisea*. In contrast to other avirulence factors, the PKS–NRPS protein is not recognized by the host plant, but the metabolite it produces is.[20,102–104]

Trichoderma spp. are the most widely applied biocontrol fungi and are known to protect plants from pathogenic microorganisms.[105–106] With mutations in the putative PKS–NRPS *tex13* gene from *T. virens*, the usual upregulation of the plant defense response gene *pal* (phenylalanine ammonia lyase) could not be observed.[21] Thus, the metabolic product of the Tex13 hybrid synthetase is suspected to communicate the presence of other microorganisms to the host plant, and is thus involved in the development of plant resistance.

Lovastatin—A Truncated Hybrid?

The fungal metabolite lovastatin (also mevinolin or monacolin K) represents a pure polyketide and does not strictly belong to the PK–AA hybrid molecules group. Nevertheless, a discussion of the biosynthesis of lovastatin and its desmethyl analogue compactin (or mevastatin) is important in the context of fungal PK–AA hybrids.[107] Lovastatin is produced by *A. terreus* and higher fungi and is a celebrity among natural products.[108] As an efficient inhibitor of hydroxymethylglutaryl (HMG)-CoA reductase, the key enzyme in cholesterol biosynthesis, it is widely applied to treat hypercholesterolemia in order to prevent cardiovascular disease.[109] Lovastatin is composed of two polyketide chains, a nona- and a diketide chain, each containing one methionine-derived methyl group[110–112] and each assembled by a distinct PKS: the iterative lovastatin nonaketide synthase (LNKS) LovB, and the noniterative lovastatin diketide synthase (LDKS) LovF.[33]

Sequence comparison and phylogenetic analysis of the biosynthetic enzymes demonstrates the remarkable similarity of the LNKS to reported fungal PKS–NRPS hybrids.[24,26] The domain organization highly resembles that of a fungal hrPKS, including an MT that is common among NRPSs. Its presence in a PKS was reported for the first time in LNKS and LDKS.[52] Furthermore, the LNKS enzyme also depends on the enoyl reductase activity of the *trans*-acting monofunctional protein LovC.[113] For the nonaketide assembly catalyzed by the LNKS, a Diels–Alder reaction is proposed to be responsible for ring closure at the hexaketide stage (Scheme 15).[48,114–115] The full nonaketide chain is released from its synthase to yield dihydromonacolin L.

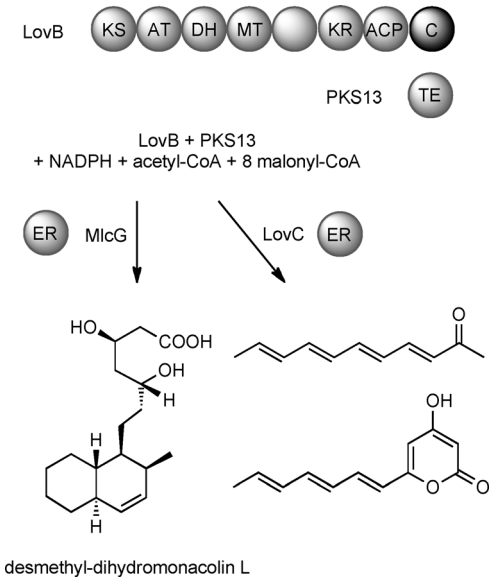


Scheme 15. Biosynthesis of lovastatin and compactin.

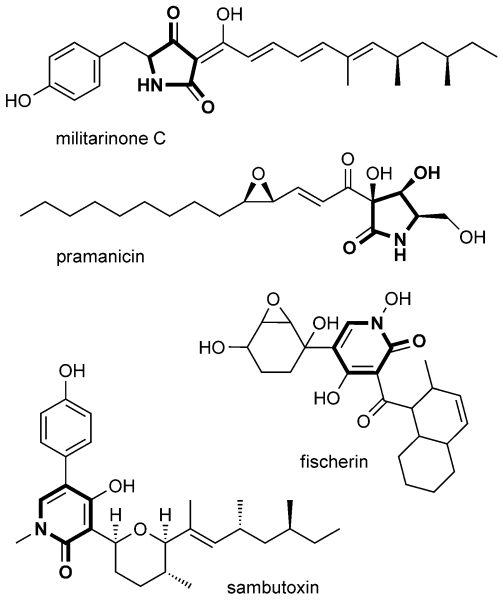
Following further processing, the transesterase LovD promotes the fusion to the methylated diketide that is assembled simultaneously by the noniterative LDKS.[33,116] Several studies have focused on the programming rules of the LNKS. Investigation of *lovC* mutants revealed the high impact of this ER on correct nonaketide formation, as truncated polyketides with a lower degree of reduction than dihydromonacolin L accumulated.[52] Moreover, an in vitro assay has shown that in the absence of the methyl group donor of *S*-adenosylmethionine (SAM), LovB and LovC solely produce shunt products (Scheme 16).[117]

Exchanging LovC with the MlcG enoyl reductase of the compactin biosynthetic pathway can overcome the methylation-dependent truncation and produce full desmethyldihydromonacolin L in the absence of SAM.[117] It remains a mystery as to how LNKS releases dihydromonacolin L after assembly. An in vitro assay with LovB and LovC failed to generate free dihydromonacolin L without the addition of the PKS13 thioesterase domain (TE) from *G. zeae*. Thus, it is likely that another TE domain, present elsewhere in the genome, off-loads the full polyketide chain.[117]

As they normally appear only in NRPS, the presence of a C-terminal condensation domain in LNKS suggests that this enzyme inhabits a space between hrPKS and PKS–NRPS hybrids. To date it is not clear whether this domain is a remnant of an evolutionary cut-off or if it has any influence on correct lovastatin formation.[24] The latter hypothesis is supported by the inability of LovB to produce dihydromonacolin L after dele-



Scheme 16. In vitro assay with purified enzymes in the absence of SAM. LovB interacts with enoyl reductases LovC and MlcG (lovastatin and compactin pathways, respectively).



Scheme 17. Fungal tetramic acids with unknown biosynthetic pathways. The tetramate core (militarinone C, pramanicin) and the 2-pyridone unit (fischerin, sambutoxin) are shown in bold.

tion of the C domain.[26,117] Attachment of the CheA A, T, and R domains onto the full LovB sequence (including the inherent LovB C domain) to obtain a full PKS–NRPS hybrid chimera only resulted in the abolishment of lovastatin production, and not in the production of PK–AA hybrids or other new metabolites.[26] Sequence comparisons between the C domains of fungal NRPS, PKS–NRPS hybrids, and LovB-like enzymes revealed the lack of the catalytically important second His in the active site motif HHXXXDG of the truncated enzymes.[26] Furthermore, in phylogenetic analyses, LovB-like synthases gather in one distinct clade among fungal PKS–NRPS hybrids, thus suggesting their early evolutionary separation.[26] Hence, it is conceivable that the condensation domain of LNKS has not lost, but rather has changed, its function during evolution.

A genetic approach to fuse the LovB PKS domains with the NRPS domains of the chaetoglobosin A synthetase CheA (replacing the LovB C domain) also did not result in the production of new metabolites.[26] Thus, fungal hrPKS (LovB) and the PKS–NRPS hybrid (CheA) appear to be incompatible for molecular engineering approaches, possibly a consequence of their evolutionary distance.[26]

However, the intriguing similarity between the lovastatin nonaketide synthase and fungal PKS–NRPS hybrids will continue to raise questions about their evolutionary relationship and will likely promote further functional and phylogenetic analysis.

Summary

Fungi produce a highly diverse set of polyketide–amino acid hybrid metabolites. However, they all arise from tetramic acid derivatives due to their common biosynthetic origin. Numerous tetramic acids and 2-pyridones are described from fungi, among them militarinone A,[118] pramanicin,[119] fischerin,[120] and sambutoxin (Scheme 17).[121] Their structures suggest the involvement of fungal PKS–NRPS hybrids in their biosynthesis. To date, only a dozen PK–AA hybrids have been linked to their respective PKS–NRPS biosynthetic enzymes. Surprisingly, these megasynthetases share strong homology with a fungal pure PKS, the lovastatin nonaketide synthase (LovB). It is very likely that fungal hybrids and pure PKS possess a common ancestor, and diverged from other fungal PKS in an early evolutionary event. The generated PK–AA hybrid molecules show a wide range of remarkable biological features, such as inhibition of HIV-1 integrase (equisetin), actin capping (chaetoglobosin A), or food spoilage (cyclopiazonic acid). The underlying structural diversity, however, only provides a glimpse of the possible variety of fungal hybrid metabolites.

The genetic potential of fungal strains to produce novel PKS–NRPS hybrids promises exciting future discoveries. With the sequencing of more fungal genomes and the application of bioinformatic tools, new PKS–NRPS biosynthetic enzymes will inevitably be identified and characterized. Moreover, improved understanding of the biosynthetic machinery will provide a strong basis for future molecular engineering approaches.

Keywords: cytochalasin · lovastatin · nonribosomal peptides · polyketides · pseurotin A

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