Polyketide Synthase–Nonribosomal Peptide Synthetase Hybrid Enzymes of Fungi

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CONTENTS

I. Introduction

People have been long attracted to the bioactivity of natural products that are found in various organisms in nature. Usefulness of such compounds often surpasses that of the chemicals that can be artificially synthesized de novo in the laboratory. Thus, it is important for us to continue discovering new bioactive natural products in the present age for our academic, clinical, and industrial interests. However, discovery of new compounds, especially those with new bioactivities, has become progressively difficult, because natural products that can be obtained easily from the huge number of organisms examined to date have been isolated exhaustively. Recently, development of cheaper and faster sequencing technologies has accelerated dramatically the process of uncovering genomic information of many microbes. Results from genome sequencing studies revealed that there were many more biosynthetic gene clusters that are potentially capable of producing natural products in the genome of a single microorganism than the number of compounds that can be isolated from that microorganism (Brakhage and Schroeckh 2011). This tendency is particularly pronounced in streptomycetes and fungi, two families of microorganisms that are known to produce a wide array of natural products. This finding suggested that many of the biosynthetic genes encoded in the genome of a microbe are not activated to produce natural products under the conventional culture conditions used in the laboratory. More recent studies have indicated that epigenetic control of gene expression in those microorganisms is partly responsible for the lack of activation of silent natural product biosynthetic gene clusters (Bok et al. 2009). What this finding implicates is that our past effort of screening microorganisms for useful compounds may have missed identifying microbes that are capable of producing compounds that exhibit unique or potent bioactivities. However, this also poses an opportunity to discover natural products having unprecedented structural framework or high bioactivities from already known microorganisms, if we could control the expression of natural product biosynthetic genes in those organisms. More-

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over, once biosynthetic gene clusters are identified in various organisms, such gene clusters can be transferred from the original microbes to other more conventional microorganisms that are amenable to genetic modifications and readily grow under a laboratory setup. Once the genes can be transferred to a convenient heterologous host, it becomes feasible to examine and engineer the biosynthetic pathways for the production of those novel natural products and their analogs. Such heterologous biosynthetic systems would also allow production of those compounds at a large scale for commercial distribution. Here, we review the construction of model fungi *Chaetomium globosum*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus nidulans*, and *Aspergillus oryzae* that can serve as platform organisms having high versatility to allow production of new natural products and their analogs. Those organisms can also serve as a very powerful tool for identifying biosynthetic intermediates or examining the activity of specific enzymes for elucidating the detailed mechanisms of how complex natural products are biosynthesized.

II. Engineering of Fungi for Making Them Amenable to Molecular Genetics Manipulations

A. *Chaetomium globosum*

To date, many biosynthetic gene clusters have been found in various different organisms through recent genome and metagenome sequencing efforts, and this is especially true for fungi (Primm and Franzblau 2007). Those clusters carry genes encoding enzymes responsible for the production of the backbone structure of secondary metabolites, such as polyketides (PKs), nonribosomal peptides (NRPs), and mixed PK–NRPs. Those clusters also contain genes for multiple auxiliary enzymes that are responsible for the modification of the backbone structure. However, many fungi are often difficult to culture at a large scale (Ezaki et al. 2008). Furthermore, despite the existence of an upward of 40 biosynthetic gene clusters in a fungus, oftentimes only a few compounds can be isolated from a fungal culture that is grown under typical growth conditions (Brakhage and Schroeckh 2011). This is exactly the case for *C. globosum*, where the genome sequencing shows the presence of approximately 33 polyketide biosynthetic genes despite the fact that only 11 polyketide products can be isolated from the culture grown under typical growth conditions (Tsunematsu et al. 2012). One way of forcing the fungus to turn on a silent biosynthetic gene cluster is to artificially activate the expression of a transcription factor gene present within the target gene cluster. For example, analysis of the *C. globosum* genome sequence identified a gene cluster named *cgs* containing seven genes, including a PK synthase (PKS) gene *cgsA* and a GAL4-like transcription factor gene *cgsG*. Upon introduction of a copy of *cgsG* under the control of a constitutively active actin promoter, the *cgs* gene cluster was activated. This resulted in the identification that the cluster was responsible for the biosynthesis of a small aromatic compound called shanorellin (Tsunematsu et al. 2012).

While the kind of "brute force" method of activating a silent gene cluster described above can be successful, a more direct modification of the genome, such as gene knockout or substitution, would allow more efficient analyses of the functions of unknown biosynthetic gene clusters and the genes found within those clusters. Such genome modifications can be effectively performed by homologous recombination between the exogenous DNA and a specific locus within the genome of the target organism. However, many fungi, including *C. globosum*, carry out high level of nonhomologous random recombination with foreign DNA molecules, making site-specific genome modifications practically impossible (Ishibashi et al. 2006). Therefore, to engineer the wild-type *C. globosum* into a homologous recombination-compatible strain, the *CgligD* gene, a homolog of a Lig4-type DNA ligase responsible for the nonhomologous random recombination found in *C. globosum*, was deleted from the genome (Nakazawa et al. 2013). This *ΔCgligD* strain, which was designated as CGKW10 (Fig. 1), was shown to be able to perform targeted

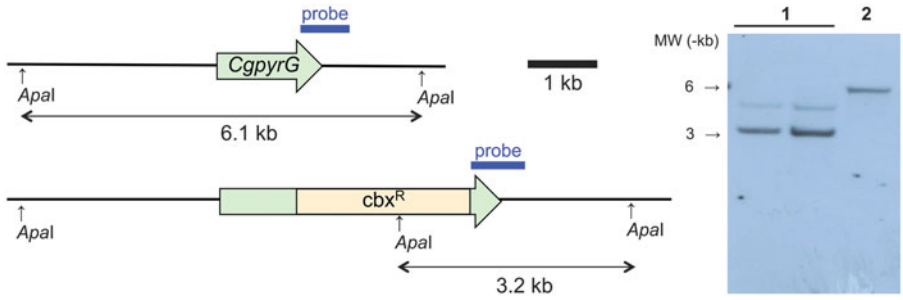


Fig. 1 Strategy for deleting the orotidine-5′-phosphate decarboxylase gene *CgpyrG* by inserting a carboxin resistance gene (*cbx*^{R}) in the *ΔCgligD C. globosum* strain CGKW10 for developing a high-efficiency gene-targeting system. The carboxin resistance gene was integrated into the *CgpyrG* locus in CGKW10 to generate the *CgpyrG*-mutated strain of *Chaetomium globosum*, CGKW12. Southern blotting analyses were performed on the *Apa*I-digested genomic DNA from the desired transformant CGKW12 and the parent strain CGKW10 using probes designed to anneal to regions indicated by blue bars. Lane 1, *CgpyrG*::cbxR/*CgligD*::*hph* (CGKW12); Lane 2, *CgligD*::*hph* (CGKW10) as a control

homologous recombination efficiently. Furthermore, a convenient positive and negative selection system, equivalent of the *URA3* gene of *Saccharomyces cerevisiae* (Boeke et al. 1984), that is based on nutritional (uridine) requirement and metabolite toxicity (5-fluoroorotic acid), respectively, was also established by deleting *CgpyrG* (Weidner et al. 1998), an orotidine-5′-phosphate decarboxylase gene, from the genome of CGKW10. The resulting *ΔCgligD*/*ΔCgpyrG* strain named CGKW12 (Fig. 1) made it possible to prepare various gene knockout strains, which accelerated dramatically the investigation into natural product biosynthesis by *C. globosum*. Later, the hygromycin resistance gene *hph* used to disrupt *CgligD* in CGKW10 and CGKW12 was eliminated to yield another *ΔCgligD*/*ΔCgpyrG* strain, CGKW14. Using CGKW14, knockout of several transcriptional regulator genes associated with epigenetic silencing of secondary metabolite biosynthetic pathways was performed successfully. Those knockout mutants were shown to produce a total of 11 compounds from *C. globosum* grown on a simple oatmeal agar medium, including 2 compounds mollipilin A and B that had not been isolated from *C. globosum* previously (Nakazawa et al. 2013). Those results confirmed the usefulness of CGKW14 for studying natural product biosynthesis in *C. globosum*. CGKW14 was also used extensively for deciphering the mechanism of biosynthesis of natural products, including PK–NRP hybrid compounds chaetoglobosin A and Sch 210972. Details of those studies are discussed in detail below.

B. *Aspergillus fumigatus*

It is well known that *A. fumigatus* is capable of biosynthesizing PKs, NRPs, and PK–NRP hybrid compounds, such as fumagillin (Lin et al. 2013, 2014), spirotryprostatin B (Tsunematsu et al. 2013), and pseurotin A (Tsunematsu et al. 2014; Wiemann et al. 2013; Zou et al. 2014) (Fig. 2). However, it is frequently difficult to resolve the biosynthetic pathway that employs multiple enzymes to generate various intermediates for the formation of a family of complex final products. For deciphering a complex biosynthetic pathway, preparation of knockout strains lacking each of the genes in the target biosynthetic gene cluster is indispensable. By identifying missing intermediates in the knockout mutants and performing biochemical assays with the enzyme coded by the deleted gene, details of the biosynthetic pathway can be elucidated.

As discussed earlier, the ability to perform targeted homologous recombination is crucial for performing gene knockout, where a specific gene can be inactivated in the genome of the target producer organism. However, as is the

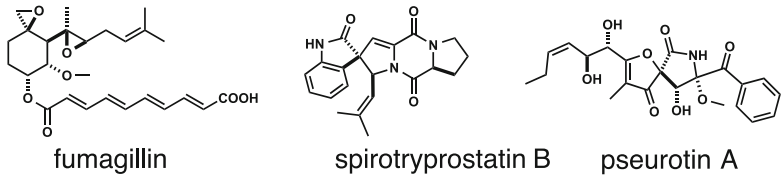


Fig. 2 Chemical structures of natural products isolated from *A. fumigatus*

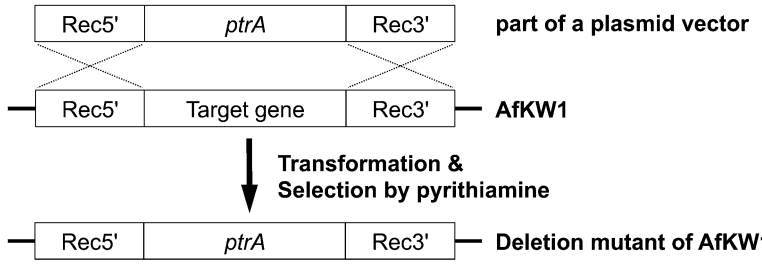


Fig. 3 A schematic diagram showing the construction of a disruption cassette-containing plasmid using the yeast-based homologous recombination method (Kubodera et al. 2002) for preparation of a mutant of AfKW1 in which the target gene is deleted

case with *C. globosum*, *A. fumigatus* also possesses a native random DNA recombination activity. To suppress non-specific DNA integration and improve the efficiency of targeted gene disruption significantly in *A. fumigatus*, it was necessary to eliminate *ku70* gene, which codes for a protein involved in the nonhomologous end-joining pathway (Ninomiya et al. 2004). Therefore, the *ku70*-deficient *A. fumigatus* strain A1159 (Krappmann et al. 2006) was chosen as a parent strain for detailed investigations of secondary metabolite biosynthesis in this fungus. However, two additional techniques needed to be applied to make this strain more useful for the study. Firstly, like in *C. globosum*, the orotidine-5′-phosphate decarboxylase gene *pyrG* was deleted in *A. fumigatus* A1159 to generate a *ΔpyrG/Δku70* strain, which was named AfKW1 (Weidner et al. 1998; Tsunematsu et al. 2014). This strain allowed the same negative/positive selection described earlier for *C. globosum* to be applied to *A. fumigatus*. In addition, to facilitate the selection of desired deletion mutants, a pyrithiamine resistance gene *ptrA* (Kubodera et al. 2002) was also used as a selection marker in the gene disruption cassette (Fig. 3). The use of AfKW1 and *ptrA* selection system facilitated our identification of biosynthetic genes and investigation of the detailed mechanisms of the biosynthesis of the pseurotin-type complex natural products (Tsunematsu et al. 2014).

C. *Aspergillus niger*

A. niger, like other fungi discussed here, is known to produce a number of natural products. One such class of compounds is pyranonigrins, which is a group of antioxidative natural products that has a characteristic fused γ-pyrone core (Hiort et al. 2004; Schlingmann et al. 2007). The backbone structure of pyranonigrins is biosynthesized by PynA, a megaenzyme that is a hybrid of a PKS and an NRP synthetase (NRPS). Previously, Abe et al. attempted activation of the pyranonigrin biosynthetic gene cluster by expressing the pathway-specific transcriptional regulator *pynR* under the control of an *aga* (arginase) promoter from a plasmid in *A. niger* ATCC 1015 (Awakawa et al. 2013). This study is another example of a study, where activation of a silent or a poorly expressed biosynthetic gene cluster is accomplished by inducing the expression of a pathway-specific transcription factor. However,

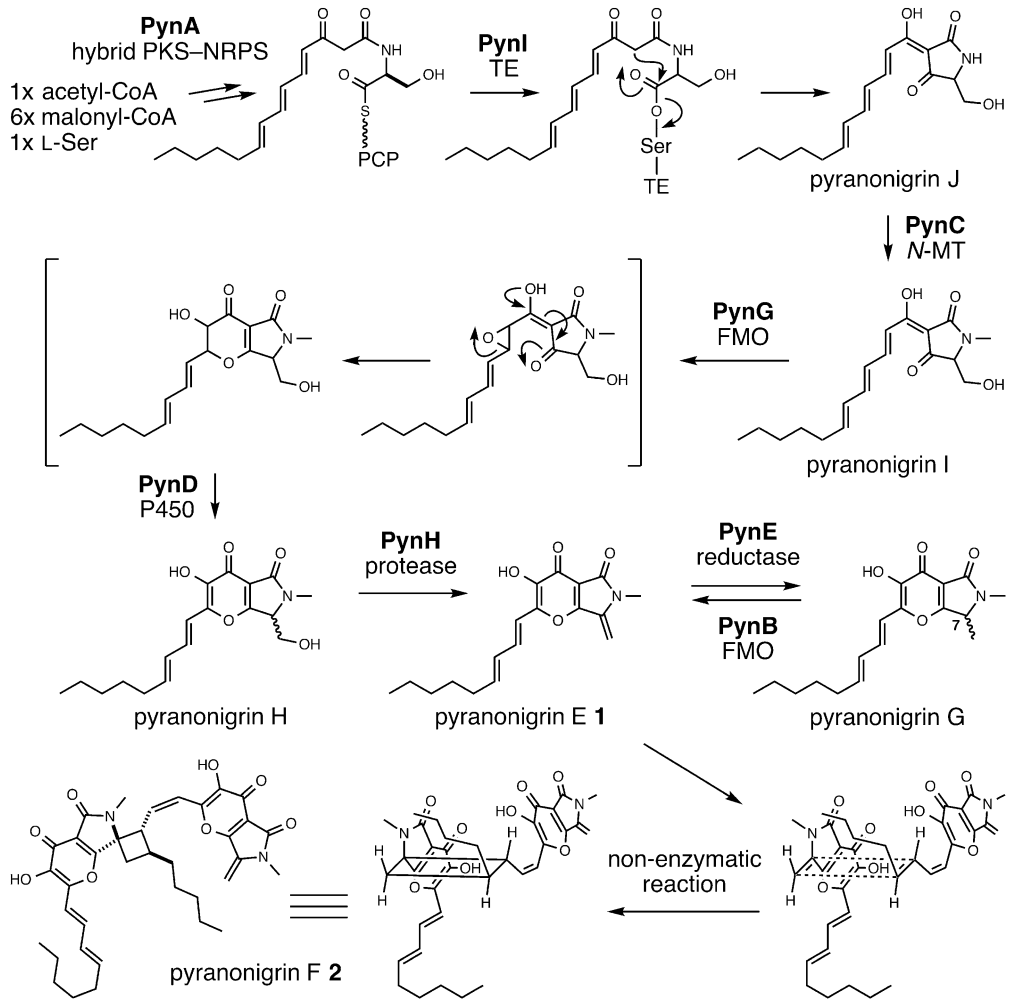


Fig. 4 A proposed pathway for the biosynthesis of pyranonigrin F **2**. *TE* thioesterase, *N-MT N*-methyltransferase, *FMO* flavin-containing monooxygenase

it resulted in only a modest induction of the production of pyranonigrin E **1** (Fig. 4). To effect a greater activation of the *pyn* gene cluster, Watanabe et al. took advantage of the *kusA*- and *pyrG*-deficient *A. niger* A1179 strain that allowed efficient targeted chromosome modification (Meyer et al. 2007). Just like CGKW14 and AfKW1 described earlier, *A. niger* was modified to be suppressed in the random integration of DNA into its chromosome and efficient in performing targeted homologous recombination by knocking out *kusA*, an ortholog of the *ku70* described above. In addition, transformant selection was made more reliable by the use of the *pyrG*-based selection system. This *kusA*- and *pyrG*-deficient *A. niger* strain was used in deciphering the mechanism of biosynthesis of spirotryprostatins (Tsunematsu et al. 2013). Furthermore, the original promoter for *pynR* was replaced with a strong *glaA* promoter (Ganzlin and Rinas 2008) in *A. niger* A1179 to generate AnKW2 (Yamamoto et al. 2015). AnKW2 was able to produce **1** at a yield of 1 g/L. In addition, AnKW2 was able to produce a new compound, which was named pyranonigrin F **2**. This compound had an unusual spiral cyclobutane core structure, which was predicted to be formed through dimerization of **1** (Fig. 4). This high-level activation of the gene cluster, combined with specific gene dele-

tion via site-specific homologous recombination, provided an opportunity to conduct a detailed study of the biosynthesis of pyranonigrins. Through the study, the complete mechanism of how pyranonigrins are produced was revealed successfully. For instance, the γ-pyrone core formation, which was previously proposed to take place spontaneously, was shown to be catalyzed by the flavin-containing monooxygenase PynG and the cytochrome P450 PynD, highlighting the importance of the ability to induce sufficient transcription of necessary biosynthetic genes and to perform deletion of specific genes in the fungus being studied when attempting to unravel the mechanism of the biosynthesis of complex natural products in detail (Yamamoto et al. 2015).

D. *Aspergillus nidulans* and *Aspergillus oryzae*

As illustrated earlier, the ability to generate knockout strains is vital to conducting studies on natural product biosynthesis in fungi. Another way to exploit the potential of fungi and their genomic information for drug discovery and development is to establish a heterologous production system using a convenient host organism capable of expressing the exogenous biosynthetic genes and producing the corresponding compounds. Typical host organisms used in this type of efforts are *Escherichia coli* and *Saccharomyces cerevisiae*. However, reconstitution of a fungal secondary metabolite biosynthetic pathway in those hosts, especially in *E. coli*, often encounters difficulty in producing fungal proteins in their active forms, leading to lack of formation of desired products. Naturally, expression of fungal genes is handled more reliably by fungi. In particular, fungi are more competent in producing massive enzymes like PKSs, NRPSs, and their hybrid enzymes, as well as redox enzymes like cytochrome P450s and flavin-containing monooxygenases. These properties make fungal model hosts particularly suitable for heterologous reconstitution of natural product biosynthetic pathways of interest. Heterologous reconstitution of a biosynthetic pathway is a very effective way of deciphering how a natural product is formed. Reconstitution allows extraction of the target pathway from the background of complex metabolic pathways and easy modification of the pathway, making it easier to identify and characterize each step of the biosynthesis of the target compound. For such studies, engineered *A. nidulans* (Chiang et al. 2013) and *A. oryzae* (Pahirulzaman et al. 2012) are frequently used. Among other examples, those heterologous host systems are successfully used for studying the biosynthesis of Sch 210972 (Sato et al. 2015) and cytochalasin (Fujii et al. 2013; Song et al. 2015) as discussed below in depth.

III. Chemical Structures of PK–NRP Hybrid Natural Products

A. Natural Product Biosynthesis Involving Enzymes Catalyzing Diels–Alder Reactions

Despite the fact that Diels–Alder (DA) reaction is one of the most important transformations employed in chemical synthesis (Corey 2002), biogenic and biocatalytic DA reactions are still recognized poorly. However, since proteins (Xu et al. 2004; Preiswerk et al. 2014) and nucleic acids (Jäschke and Seelig 2000) have been engineered to catalyze DA reactions, biomolecules are expected to be competent catalysts for this type of cycloaddition reaction. While DA reaction has been implicated as a key transformation during the biosynthesis of a growing number of natural products (Oikawa 2010), the identification of enzymes, Diels–Alderases (DAases), for the cycloaddition has proven challenging. Thus far, only a handful of enzymes has been identified as DAases. Many of the DAases are found in fungi, including lovastatin nonaketide synthase LovB (Auclair et al. 2000) and solanapyrone synthase (Kasahara et al. 2010). However, only the bacterial DAases, SpnF involved in the biosynthesis of spinosyn A (Kim et al. 2011) and VstJ responsible for the formation of the spirotetronate framework of versipelostatin (Hashimoto et al. 2015), have the specific rate for accelerating the

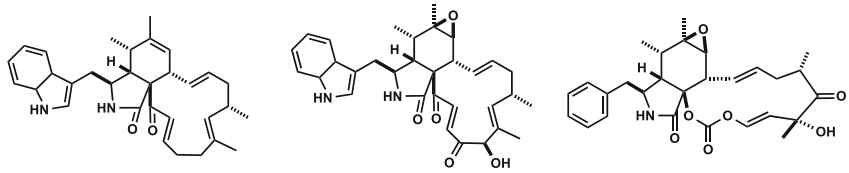


Fig. 5 Chemical structures of the representative members of the cytochalasan family of natural products

[4 + 2] cycloaddition reaction determined experimentally. Nevertheless, the limited understanding of enzymatic DA reactions and the potential synthetic utility of DAases warrant further studies for establishing the existence and catalytic modes of natural DAases. Below, we will discuss fungal PK–NRP hybrid compounds whose biosynthetic process is considered to employ a DA reaction.

B. Chaetoglobosins

Fungal natural products often exhibit biological activities of medicinal importance. Among them is chaetoglobosin A **3** (Fig. 5), which has a unique inhibitory activity against actin polymerization in mammalian cells (Löw et al. 1979; Scherlach et al. 2010). The first discovery of the gene cluster responsible for the biosynthesis of **3** in *Penicillium expansum* was accomplished using an siRNA technology (Schümann and Hertweck 2007). Based on this study, the core structure of **3** was predicted to be formed by a PKS–NRPS hybrid enzyme [CheA in *P. expansum* and CHGG_01239 in *C. globosum* (Ishiuchi et al. 2013)] and a stand-alone enoyl reductase (ER) (CheB in *P. expansum* and CHGG_01240 in *C. globosum*) found within the gene cluster. This stand-alone ER is proposed to work in trans with the PKS–NRPS for reduction of olefins in the polyketide backbone. It is commonly found in gene clusters responsible for the biosynthesis of similar fungal metabolites and has been shown to be essential for the formation of the final products (Boettger and Hertweck 2013; Ma et al. 2009; Qiao et al. 2011). How the straight PK–NRP chain off-loaded from the PKS–NRPS remains poorly established for the biosynthesis of **3**. However, for a related compound cytochalasin E **4** from *A. clavatus*, the C-terminal reductase (R) domain of the PKS–NRPS was considered to perform a reductive release of the PK–NRP intermediate as an aldehyde. Then, the aldehyde is thought to undergo a cyclization to form a 2-pyrrolidinone moiety via a Knoevenagel condensation (Qiao et al. 2011; Fujii et al. 2013). The same mechanism is thought to apply for the biosynthesis of **3**. Once the 2-pyrrolidinone moiety is formed, it is thought to act as a dienophile for the DA reaction that achieves cyclization of the released intermediate to form prochaetoglobosin I 5 (Ishiuchi et al. 2013). However, the detailed reaction mechanism involving the 2-pyrrolidinone formation and the DA reaction is yet to be uncovered. Further investigation is currently ongoing in our laboratory to reveal the mechanism of chaetoglobosin biosynthesis by using the genetically modified *C. globosum* strain CGKW14 described above.

C. Sch 210972

Sch 210972 **6** is another PK–NRP compound produced by *C. globosum* whose biosynthetic process is thought to involve a DA reaction (Fig. 6). This compound is characterized by a tetramic acid moiety, which is also found in a number of similar natural products, such as equisetin **7** (Fig. 6). In those compounds, the tetramic acid moiety is attached to a decalin core. This bicyclic core structure is also found in other fungal secondary metabolites, such as lovastatin **8**, and it is proposed to be formed via a DA reaction (Ma et al. 2009; Kakule et al. 2013; Sato et al. 2015). To be able to examine the biosynthesis of **6** in detail, the corresponding biosynthetic genes were pursued. A BLASTP

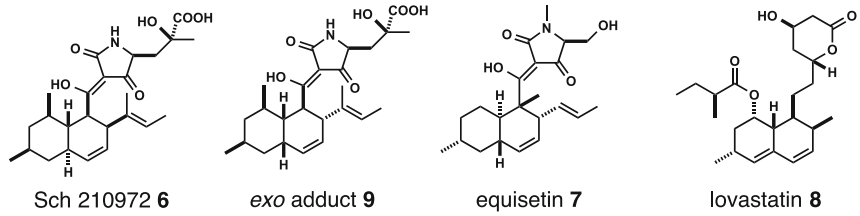


Fig. 6 Natural products with a decalin core whose biosynthesis is proposed to proceed via a Diels–Alder reaction

(Johnson et al. 2008) search of the genome of *C. globosum* identified three recognizable hybrid PKS–NRPS genes: CHGG_01239, CHGG_05286, and CHGG_02374–CHGG_02378. Inspection of the sequence of CHGG_02374 through CHGG_02378 suggested that the five annotated open reading frames (ORFs) were actually a single ORF coding for a hybrid PKS–NRPS. As discussed above, CHGG_01239 codes for the PKS–NRPS responsible for the formation of **3** (Ishiuchi et al. 2013). In addition, deletion of CHGG_05286 did not affect the production of 6. Therefore, the reassigned ORF CHGG_02374–CHGG_02378, which was named *cghG*, was predicted and later confirmed by homologous recombination-mediated targeted gene disruption to code for the PKS–NRPS responsible for the biosynthesis of **6**. Functionally very similar PKS–NRPSs, CheA (Schümann and Hertweck 2007)/CHGG_01239 (Ishiuchi et al. 2013), CcsA (Qiao et al. 2011), and EqxS (Kakule et al. 2013, 2015), are also found in the biosynthetic gene clusters responsible for the production of **3**, **4**, and **7**, respectively (Fig. 7). As mentioned earlier, this class of PKS–NRPSs is often accompanied by a stand-alone ER, and they are CghC (CHGG_02368), CheB/CHGG_01240, CcsC, and EqxC for the biosynthesis of **6**, **3**, **4**, and **7**, respectively. Disruption of this ER abolishes the assembly of the PK–NRP intermediate, such as **5**, by the PKS–NRPS (Sato et al. 2015). For **3**, **4**, and **7**, the extender unit for the NRPS portion of the hybrid enzyme is a natural amino acid; it is L-tryptophan, L-phenylalanine, and L-serine for **3**, **4**, and **7**, respectively. For **6**, however, it was predicted to be an unusual amino acid, (2*S*,4*S*)-4-hydroxy-4-methylglutamic acid. Knockout strains prepared with the use of the engineered strain CGKW14 allowed determination of the function of CghB, a predicted aldolase, as the enzyme responsible for the production of (2*S*,4*S*)-4-hydroxy-4-methylglutamic acid through dimerization of two molecules of pyruvic acid (Fig. 8). As in the case of chaetoglobosin biosynthesis, the amino acid portion of the PK–NRP intermediate for **6** undergoes a cyclization upon release from the PKS–NRPS, although a tetramic acid moiety, not a 2-pyrrolidinone moiety, is formed. Unlike for the biosynthesis of **3**, the heterocyclic moiety is not predicted to be involved in the intramolecular DA reaction for the formation of the decalin core of **6** (Fig. 9).

During the examination of the involvement of DA reaction in the biosynthesis of the natural products discussed here, it became apparent that the biosynthetic gene cluster for **3**, **4**, **6**, and **7** all contained a gene that coded for a small protein of unknown function. One such gene CHGG_01241 (CheC in *P. expansum*) found in the gene cluster responsible for the biosynthesis of **3** was knocked out in CGKW14 to investigate its role in the biosynthesis of **3**. However, deletion of CHGG_01241 completely abolished the formation of **3** despite the high titer of 100 mg or more of **3** per liter of culture attained with the wild-type strain. The straight chain form of **5**, i.e., the intermediate before undergoing an intramolecular DA reaction, could not be observed, either (Ishiuchi et al. 2013). Thus, insight into the role of DA reaction during the biosynthesis of **3** could not be obtained from this study. Likewise, the role of the proteins coded in other clusters, CcsF for **4**, which was suggested to play a role in the formation of the isoindolone core via a DA reaction (Qiao et al. 2011), and Eqx3 for the formation of the decalin core of **7** (Kakule et al. 2013, 2015) have not been investigated in

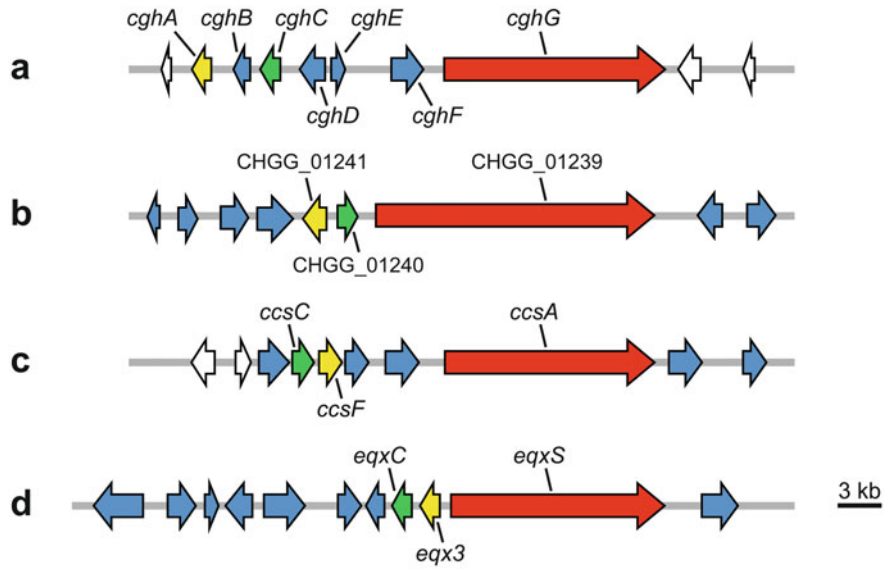


Fig. 7 The gene clusters proposed to contain a gene coding for a Diels–Alderase. The overall organization of (**a**) the *cgh* gene cluster for the biosynthesis of Sch 210972 **6** from *Chaetomium globosum*; (**b**) the CHGG gene cluster for the biosynthesis of chaetoglobosin A **3** from *C. globosum*; (**c**) the *ccs* gene cluster for the biosynthesis of cytochalasin E **4** from *Aspergillus clavatus*; and (**d**) the *eqx* gene cluster for the biosynthesis of equisetin **7** from *Fusarium heterosporum*. Predicted Diels–Alderase gene is colored in yellow, while the PKS–NRPS and its associated stand-alone ER genes are shown in red and green, respectively

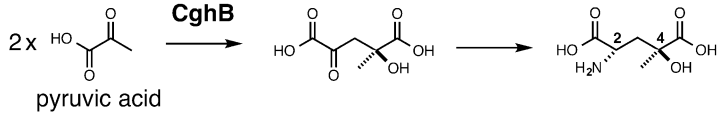


Fig. 8 Formation of an unusual amino acid, (2*S*,4*S*)-4-hydroxy-4-methylglutamic acid, via dimerization of two molecules of pyruvic acid catalyzed by an aldolase CghB

details. However, a very interesting result was obtained during the examination of the biosynthetic gene cluster for **6**. When *cghA*, the proposed DAase-coding gene, was knocked out in CGKW14 (Fig. 9), the deletion strain produced the original *endo* adduct **6** and its diastereomeric exo adduct **9**, both being expected products of the proposed DA reaction, albeit at a much lower overall yield (2 mg/L of **6** and 1 mg/L of **9**) than in the wild-type strain (60 mg/L of **6** only). The stereochemistry of the products was determined by NMR spectroscopy for both compounds and X-ray crystallography for **9**. In addition, reconstitution of the biosynthetic pathway for **6** in an engineered *A. nidulans* strain confirmed that four genes, *cghG* (PKS–NRPS), *cghC* (ER), *cghA* (speculative DAases), and *cghB* ((2*S*,4*S*)-4-hydroxy-4-methylglutamic acid-forming aldolase), were sufficient for the formation of **6**, with *cghA* being required for the strict stereocontrol over the product formation (Sato et al. 2015). Without CghA in the reaction, the decalin-forming [4 + 2] cycloaddition reaction proceeds in a non-stereoselective manner, suggesting that CghA is a DAase responsible for controlling the stereoselectivity of the intramolecular DA cycloaddition of the PK–NRP straight chain intermediate. Furthermore, processing of the straight chain intermediate by CghG appears to be diminished significantly in the absence of CghA, presumably because CghA-catalyzed decalin formation in the inter-

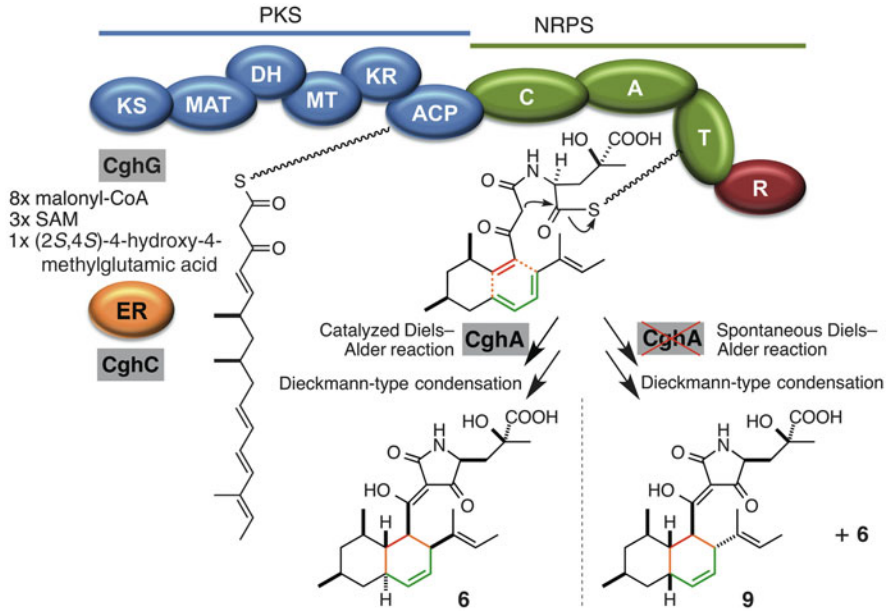
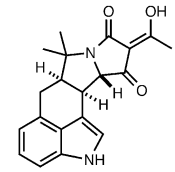


Fig. 9 Proposed biosynthetic pathway of **6**. CghG-catalyzed synthesis of a linear intermediate and subsequent cyclization for the formation of a tetramic acid moiety are shown. Two diastereomeric products Sch 210972 **6** and **9** can be formed via a Diels–Alder reaction in this pathway, depending on the presence of the proposed Diels–Alderase, CghA. *KS* ketosynthase, *MAT* malonyl-CoA acyltransferase, *DH* dehydratase, *MT* methyltransferase, *KR* ketoreductase, *ACP* acyl carrier protein, *C* condensation, *A* adenylation, *T* thiolation, *R* reductase, *ER* enoyl reductase, *SAM* *S*-adenosyl-L-methionine

mediate that is covalently bound to the thiolation (T) domain of CghG is required for subsequent processing for an efficient release from the PKS–NRPS. The release of the intermediate from the PKS–NRPS is thought to be catalyzed by the C-terminal R domain of the PKS–NRPS through a Dieckmann-type condensation that forms the tetramic acid moiety in the final product. This aspect of the biosynthesis of **6** and related compounds is discussed in greater depth below. Again, the engineered *C. globosum* strain CGKW14 is being used effectively for a further investigation of the biosynthetic mechanism of **6**.

D. Equisetin

Equisetin **7**, which is already mentioned in the previous section, is a PK–NRP compound produced by various *Fusarium* fungi, including *Fusarium equiseti* (Burmeister et al. 1974),



cyclopiazonic acid **10**

Fig. 10 Chemical structure of cyclopiazonic acid **10**

Fusarium heterosporum (Sims et al. 2005), and *F.* sp. FN080326 (Kato et al. 2015), that is highly similar to **6** in its structure (Fig. 6). Another compound, cyclopiazonic acid **10** (Fig. 10), isolated from various *Aspergillus* and *Penicillium* fungi (Liu and Walsh 2009), is also related to **6** and **7** in that it has a tetramic acid moiety in its structure. However, **10** is comprised of a tryptophan-containing PK–NRP core with a prenyl side chain that undergoes multiple cyclization reactions to form a fused pentacyclic framework. All of the PKS–

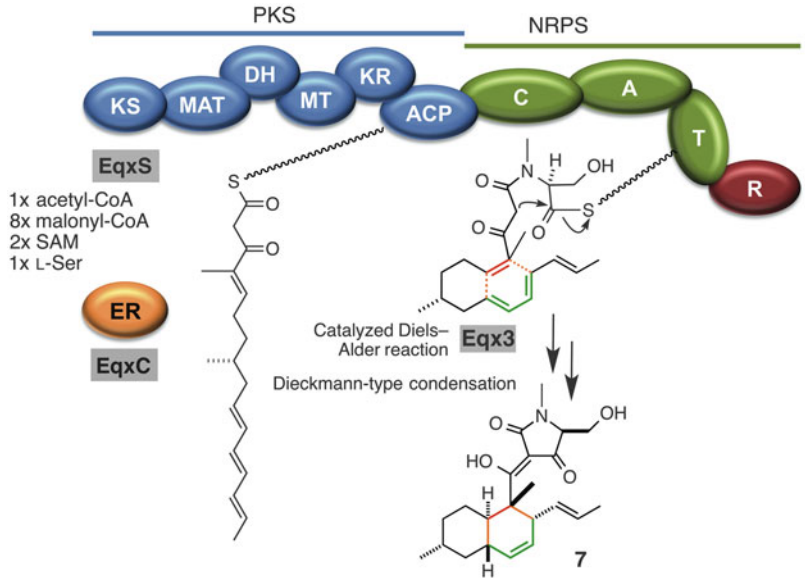


Fig. 11 Proposed biosynthetic pathway of **7**

NRPS responsible for the biosynthesis of **3**, **4**, **6**, and **7** contain a C-terminal R domain. As described for the biosynthesis of **3** and **4**, the R domain can perform a reductive cleavage of the PK–NRP intermediate from the T domain to release the product as an aldehyde. However, the R domain found in EqxS responsible for the biosynthesis of **7** was shown not to bind NAD(P)H or perform a reduction to form an aldehyde but to form a tetramic acid via a Dieckmann-type condensation (Fig. 11) (Sims and Schmidt 2008; Scherlach et al. 2010). The same transformation was proposed to be involved in the biosynthesis of a fungal PK–NRP product tenellin (Halo et al. 2008) and **6** (Sato et al. 2015). A possible explanation of why certain R domains perform a reductive cleavage of a thioester linkage while other R domains perform a Dieckmann-type condensation for concomitant tetramic acid formation and product release is discussed below. As to the involvement of a DA reaction in the biosynthesis of **7**, an investigation into the function of the uncharacterized protein Fsa2 [Eqx3 in *F. heterosporum* (Kakule et al. 2015)], a homolog of CghA, through deletion of *fsa2* in *Fusarium* sp. FN080326 identified it to be a likely DAase (Kato et al. 2015).

E. Pyrrolocins

Pyrrolocin C **13** and its diastereomer pyrrolocin B **12**, along with an *N*-methylated analog of **13** pyrrolocin A **11** (Jadulco et al. 2014), comprise a group of antituberculosis agents that is structurally related to **6** and **7** (Fig. 12). Pyrrolocins are produced by a strain of endophytic fungus designated as NRRL 50135 that was identified as a phylogenetically new strain. This strain mainly produced **11**, while only a negligible amount of the desmethylated compounds **12** and **13** were isolated. During the isolation and characterization of this series of compounds, NRRL 50135 stopped producing **11** under the standard culture conditions examined. To salvage the production of **11** for examining its chemical structure and bioactivity, the biosynthetic locus for the formation of **11** was isolated from NRRL 50135 and transferred to a filamentous fungus *F. heterosporum* ATCC 74349, which was known to produce **7** at a very high titer (Kakule et al. 2015), for heterologous production of **11**. For the production of **11**, an engineered *F. heterosporum* ATCC 74349 strain was used. In this modified strain, the equisetin biosynthetic gene cluster was knocked out, and the expression of the equisetin biosynthetic

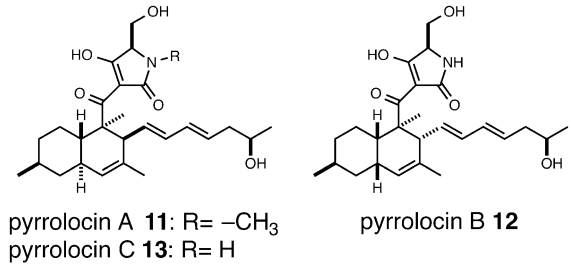


Fig. 12 Chemical structures of pyrrolocin A **11**, B **12**, and C **13**

gene regulator gene *eqxR* was placed under the control of an inducible *A. nidulans alcA* promoter. Furthermore, like CGKW14 and AfKW1 described earlier, the *pyrG* gene was also knocked out for auxotrophic selection (Kakule et al. 2013). Two biosynthetic genes, *prlS* (PKS–NRPS) and *prlC* (ER), were successfully cloned from the genome of NRRL 50135. However, the third gene coding for a methyltransferase (MT) presumably necessary for the *N*-methylation to form **11** could not be located in the vicinity of the *prl* gene cluster. Thus, *prlS* and *prlC* were cloned into a plasmid and placed under the control of *PeqxS*, the promoter for the equisetin PKS–NRPS gene *eqxS*. In place of the missing *prl* MT gene, the gene coding for the equisetin MT *eqxD* was provided in the engineered *F. heterosporum* ATCC 74349 strain. This heterologous system only produced less than 10 mg of **11** per kilogram of culture medium. However, it produced roughly 800 mg/kg of **12** and **13** in an approximately 2-to-1 ratio (Kakule et al. 2013, 2015). Loss of production of **11**, the only *N*-methylated member of pyrrolocins, is likely due to lack of correct MT in the system. The compound **12** contains a cis-decalin, which is a more favored endo adduct of a DA cycloaddition, whereas **13** has a trans-decalin formed through a less favored exo transition state. Thus the higher yield of **12** over **13** is consistent with the notion that the decalin formation during the pyrrolocin biosynthesis occurs through an intramolecular DA reaction. When only the PKS–NRPS and the stand-alone ER but not the presumed DAase like CghA for the biosynthesis of Sch 210972 are provided to the heterologous host, the system produces more of the chemically more favored endo product, **12**. In contrast, **12** is not produced in the original producer where the presumed DAase would be present. The fact that products are formed efficiently by the pyrrolocin-forming enzymes in the absence of a DAase suggests that the biosynthetic mechanism, especially the mechanism involving the decalin formation, might be somewhat different between pyrrolocins and other related compounds like **6**. For the biosynthesis of **6**, knockout of the presumed DAase-coding gene *cghA* abolishes the production of any products (Sato et al. 2015). It is possible that the C-terminal R domain of the PKS–NRPS, which is responsible for the chain release and tetramic acid formation, requires the decalin to be present in the substrate. Whether products are formed efficiently by a DAase gene-knockout strain may depend on the relative efficiency of the spontaneous decalin core formation.

Oftentimes, identification of different intermediates formed during the biosynthesis of a target natural product provides valuable insight for deciphering how the final product is biosynthesized. Unfortunately, the straight chain PK–NRP intermediate has not been isolated from the biosynthetic systems responsible for the production of **6**, **7**, or **11** to date. Interestingly, however, isolation of a possible intermediate was reported for the cytochalasin biosynthetic system by Oikawa et al. (1995) (Fig. 13a). For cytochalasin biosynthesis, the straight chain intermediate is thought to be off-loaded from the PKS–NRPS by the C-terminal R domain as an aldehyde. The isolated compound was not an aldehyde **14** but a primary alcohol **15** (Fig. 13b) (Fujii et al. 2013). For this study, the auxotrophic *A. oryzae* strain

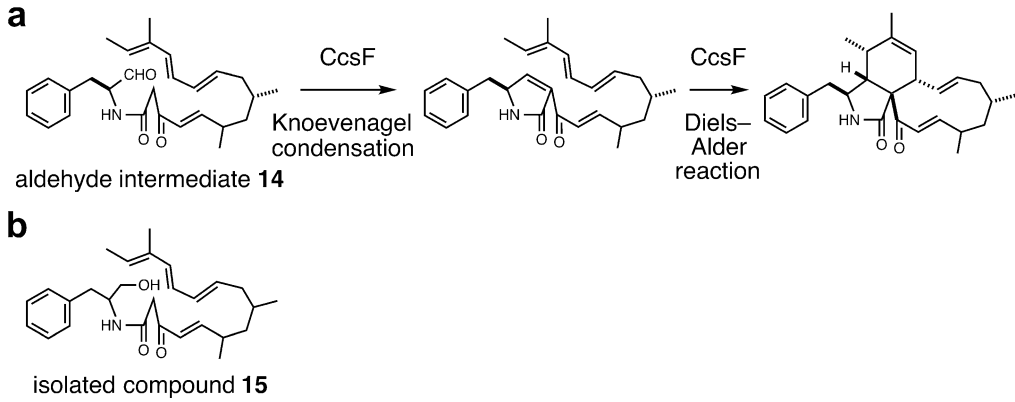


Fig. 13 Proposed biosynthetic pathway of cytochalasins. (**a**) Speculative functions of CcsF, catalyzing a Knoevenagel condensation on a straight-chain aldehyde intermediate **14** to form a 2-pyrrolidinone moiety and a Diels–Alder reaction to form a decalin core. (**b**) Isolated alcohol **15** as a shunt product produced by an unexpected reduction in *A. niger*

NSAR1 (*niaD*$^{-}$, *sC*$^{-}$, *ΔargB*, *adeA*$^{-}$) (Jin et al. 2004) was used as the heterologous host for the plasmid-based expression of the PKS–NRPS gene *ccsA* and the stand-alone ER gene *ccsC* without including the gene for the presumed DAase *ccsF*. Isolation of **15** from this heterologous system is a good indication that the C-terminal R domain of CcsA actually catalyzes off-loading of the PK–NRP intermediate via a reduction of the thioester linkage between CcsA and the bound intermediate. It is not clear whether the R domain is actually catalyzing only a two-electron reduction only to form an aldehyde or a four-electron reduction (Li et al. 2008; Song et al. 2015) to form the primary alcohol found in **15**. Nonetheless, this observation suggests that the pyrrolinone moiety of **4** is formed through a Knoevenagel condensation, not through a Dieckmann-type cyclization as proposed for **6**, **7**, and **11**. This observation is consistent with the amino acid sequence of the CcsA R domain having an intact NAD(P)H-binding motif GXSXXG and the Ser–Tyr–Lys catalytic triad conserved among the short-chain dehydrogenase/reductase family of proteins (Liu and Walsh 2009; Qiao et al. 2011). The reason the R domain of the PKS–NRPS for the biosynthesis of **4** performs a reductive cleavage of the thioester linkage while the R domain for the biosynthesis of **6**, **7**, and **11** catalyzes a Dieckmann-type condensation for concomitant tetramic acid formation may have to do with the subsequent transformation required for the biosynthesis of the final product. For the formation of **4**, 3-pyrrolin-2-one is thought to act as a dienophile for the DA reaction that forms the six-membered ring in the core structure. A Dieckmann-type condensation would result in the formation of a pyrrolidine-2,4-dione, which would have to undergo additional reduction and dehydration of the 4-carbonyl to be converted into 3-pyrrolin-2-one. However, a Knoevenagel condensation of the aldehyde intermediate would lead to direct formation of 3-pyrrolin-2-one. Thus, it makes sense for the R domain of the chaetoglobosin- and the cytochalasin-forming PKS–NRPSs to possess a reductase activity. On the other hand, the tetramic moiety of **6**, **7**, and **11** does not participate in a DA reaction. Thus, the R domains of the PKS–NRPSs that form those compounds could gain a Dieckmann-type condensation activity. Based on this idea, the R domain of CHGG_01239 for the biosynthesis of **3** is predicted to catalyze a reduction, not a Dieckmann-type cyclization. The engineered *C. globosum* strain CGKW14 combined with in vitro biochemical assays can be used to characterize the activity of the CHGG_01239 R domain.

IV. Conclusions

In this review, we mainly summarized the findings from recent studies that focused on engineering of important fungi, primarily *C. globosum*, *A. fumigatus*, *A. niger*, *A. nidulans*, and *A. oryzae*. Creation of those engineered fungal strains has enabled many of the studies described in this review. The engineering typically focuses on eliminating the fungi's native ability to randomly recombine exogenous DNA into their genome. Abolishing the random recombination activity in those fungi leaves the site-specific homologous recombination activity intact, allowing targeted modifications of the genome. Another modification focuses on establishing selection schemes that improve the efficiency of isolating correctly manipulated strains. Those engineered strains make knockout and other genome editing experiments, such as promoter exchange studies, reliable. Those strains also make heterologous reconstitution of biosynthetic pathways of interest straightforward and efficient. Those experiments allow isolation and characterization of biosynthetic pathway intermediates that provide critical insight into what genes are involved in the formation of certain natural products and how such complex natural products are biosynthesized.

Among the interesting chemical transformations found in fungal secondary metabolite biosynthesis, we focused on the biosynthetic steps that are considered to involve enzyme-catalyzed DA reactions. Among them, we paid a particular attention to those proposed for the biosynthesis of chaetoglobosin, Sch 210972, equisetin, and pyrrolocin. There is a shared aspect in the biosynthesis of these compounds, namely, the scaffolding formed by a PKS–NRPS hybrid enzyme with a stand-alone ER, and the cyclization of the linear intermediate by a DA reaction. The DA reaction is thought to be catalyzed by a small protein whose function is unknown due to lack of amino acid sequence homology to other known proteins. Use of engineered fungal strains discussed in the first part of this chapter made it possible to perform targeted gene deletion and heterologous reconstitution of the biosynthetic pathways. Investigation into the biosynthesis of chaetoglobosin, Sch 210972, equisetin, and pyrrolocin with those engineered fungal strains provided strong indication of the involvement of enzyme-catalyzed DA reaction and identification of the uncharacterized proteins as a bona fide DAase. Detailed biochemical analyses and X-ray crystallographic characterizations of the DAases are currently ongoing to understand how those proteins catalyze DA reactions. Those studies are combined with theoretical calculations that can model the reaction and compute transition states of the DA reaction leading to the formation of each of the four compounds discussed in this chapter. Experimentally determined structural information of the DAases will be combined with the results from the computational studies using quantum-mechanical calculations to gain thorough insight into the mechanism of enzyme-catalyzed DA reactions. Those efforts will expand our understanding of how nature biosynthesizes complex chemical structures found in various natural products.

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