Personal Account

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THE CHEMICAL RECORD

Elucidation of biosynthetic pathways of natural products

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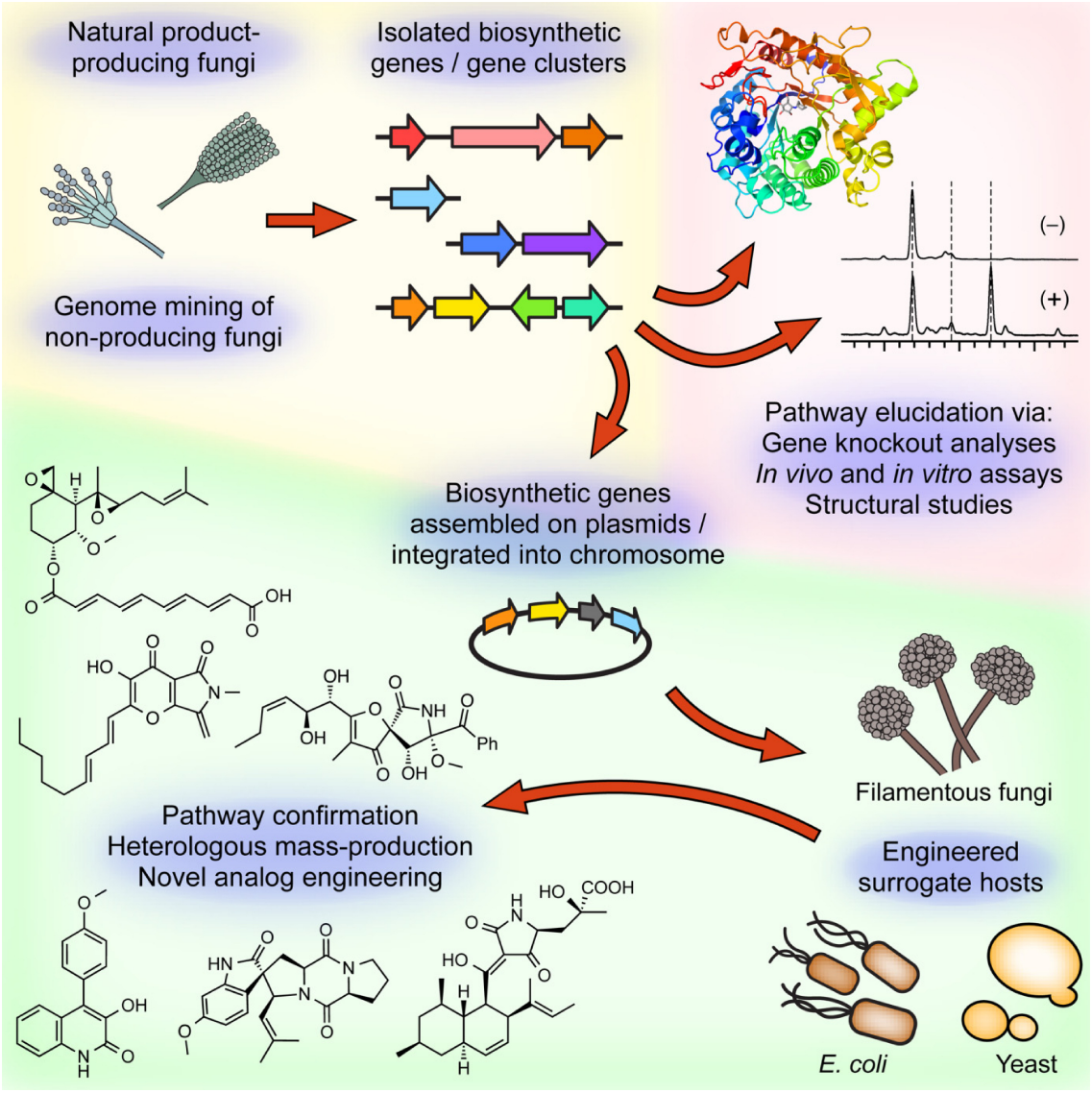

Abstract: During the last decade, we have revealed biosynthetic pathways responsible for the formation of important and chemically complex natural products isolated from various organisms through genetic manipulation. Detailed in vivo and in vitro characterizations enabled elucidation of unexpected mechanisms of secondary metabolite biosynthesis. This personal account focuses on our recent efforts in identifying the genes responsible for the biosynthesis of spirotryprostatin, aspoquinolone, Sch 210972, pyranonigrin, fumagillin and pseurotin. We exploit heterologous reconstitution of biosynthetic pathways of interest in our study. In particular, extensive involvement of oxidation reactions is discussed. Heterologous hosts employed here are *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *A. niger* that can also be used to prepare biosynthetic intermediates and product analogs by engineering the biosynthetic pathways using the knowledge obtained by detailed characterizations of the enzymes. (998 char.)

Keywords: natural product, biosynthesis, enzymatic reaction, engineered biosynthesis, target-guided genome mining

1. Introduction

Nature produces a variety of chemical structures and biologically active molecules whose design is beyond human ingenuity, and many have been turned into valuable pharmaceutical agents for commercial distribution or exploited as lead compounds in drug discovery. Those compounds are frequently produced by plants and microbes as their secondary metabolites, and securing large quantities of such compounds for clinical and industrial applications has been a persisting problem.[1] This is part because a considerable number of organisms are not amenable to artificial cultivation that would allow mass-production of the compounds of interest.[2] Low and inconsistent production level of desired products and slow growth of the producing hosts can add to the difficulty.[3] Native natural products can also be too toxic or have poor bioavailability to be used as drugs.[4] Therefore, semisynthetic techniques, which employ biologically produced compounds as starting materials for chemical synthesis of desired final products, have been used as one of the methods for supplying large quantities of drug substances and installing more desirable pharmacological properties into naturally available compounds.[5] However, technological progress made in multiple fronts in recent years, including genome sequencing efforts and development of genetic and molecular biological techniques for manipulating microbes, is transforming how we study and engineer biosynthesis of valuable natural products.[6]

In this personal account, we will reflect on our recent progress in isolating the genes responsible for the biosynthesis of interesting natural products and uncovering the detailed mechanisms of how those compounds are formed by the concerted efforts of various enzymes. We frequently use techniques, such as gene knockout, heterologous reconstitution of biosynthetic pathways of interest, bioconversion of fed substrates and in vitro assays with recombinantly produced enzymes, to characterize the enzymes involved in the biosynthesis in order to determine the sequence and the mechanism of transformations leading to the formation of interesting chemical structures found amongst the secondary metabolites. We will focus our discussion on the following fungal secondary metabolites: spirotryprostatins, viridicatins, Sch 210972, pyranonigrins, fumagillin and pseurotins. In particular, the use of *Saccharomyces cerevisiae*, *Aspergillus niger* and *A. nidulans* as the heterologous hosts of choice for detailed characterizations of the biosynthetic enzymes is discussed. Also, extensive involvement of oxidation reactions catalyzed by cytochrome P450s (P450s) and flavin-containing monooxygenases (FMOs) for complexity generation in fungal secondary metabolites is highlighted.

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2. Detailed Biosynthetic Pathways of Natural Products

2.1. Spirotryprostatin Biosynthesis

The spiro-carbon-containing fungal secondary metabolites spirotryprostatin A (**1**) and B (**2**) from *A. fumigatus* have interesting chemical structures and exhibit a cell-cycle arresting activity that is useful in anticancer drug development.[7] Those compounds were thought to be formed from the tryprostatin–fumitremorgin–verruculogen biosynthetic pathway intermediates. While the biosynthetic steps leading to the formation of many of the compounds derived from the pathway have been established (Scheme 1),[8] no spiro-carbon-forming enzyme responsible for the biosynthesis of **1** or **2** has been identified during earlier detailed studies. Thus,

we set out to identify the enzyme by initially developing heterologous production systems capable of producing the pathway intermediates through incorporation of the fumitremorgin biosynthetic genes into suitable hosts. For this study, we chose to use both *S. cerevisiae* strain BY4705[9] and *A. niger* strain A1179[10] as the heterologous hosts of choice.

Our search for the spiro-carbon-forming enzymes uncovered a surprising involvement of FqzB, an FMO from unrelated fumiquinazoline biosynthetic pathway encoded by AFUA_6G12060 that is located on a chromosome separate from where the fumitremorgin biosynthetic gene cluster is located.[11] This FMO catalyzed the spiro-carbon formation in fumitremorgin C to form **1** via an epoxidation–pinacol-type rearrangement route (Scheme 2). On the other hand, bioconversion and in vitro studies revealed that FtmG, one of three P450s from the fumitremorgin biosynthetic pathway, actually catalyzed the spiro-ring formation in **2** from demethoxyfumitremorgin C via a radical-mediated hydroxylation–pinacol-type rearrangement route in two steps (Schemes 2 and 3).

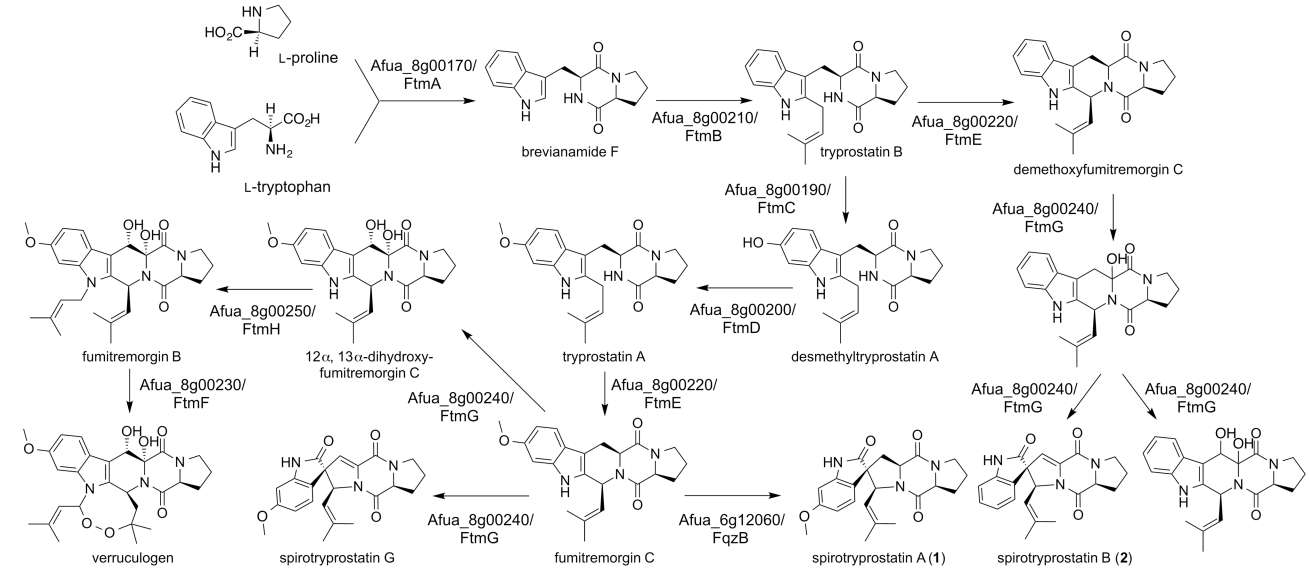
Extraction of the culture of *A. niger* transformed with the six necessary genes (*ftmA*, *C*, *D*, *B*, *E* and *fqzB*) yielded approximately 1.0 mg of **1** per liter of culture. Since **1** was isolated at 0.003 mg per liter of culture from the original *A. fumigatus* producer strain,[7] the heterologous system enabled us to not only elucidate the reaction mechanism of spiro-carbon formation but also achieve over 330-fold improvement in the yield of this compound with our *A. niger*-based *de novo* production system.[12] We used both *S. cerevisiae* and *A. niger* as the heterologous host for the production of **1** and biosynthetic pathway intermediates to augment each other's shortcomings. *S. cerevisiae* worked well in producing the

Dr. Shinji Kishimoto received his Ph.D. in 2015 in chemical biology from Kyoto University, Japan. In 2015, he joined the department of pharmaceutical sciences at University of Shizuoka as a designated assistant professor. His interest lies in mechanistic and structural characterization of enzymes involved in natural product biosynthesis.

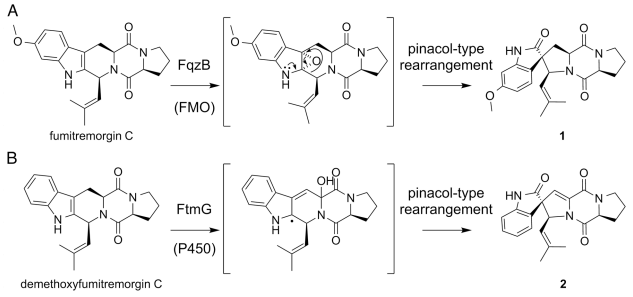
Dr. Yuta Tsunematsu received his B.Sc. degree in chemistry from Nagoya University in 2006 and Ph.D. in organic chemistry from Kyoto University, Japan in 2011. His postdoctoral fellowship from JSPS included a year at University of Shizuoka and he joined the department of pharmaceutical sciences at University of Shizuoka as a designated assistant professor in 2012. He was promoted to assistant professor at University of Shizuoka in 2014. His postdoctoral fellowship included a year at Leibniz Institute for Natural Product Research and Infection Biology in 2015. He is studying the regulatory and biosynthetic mechanisms of natural products in fungi.

Dr. Michio Sato received his Ph.D. in 2011 in bioorganic chemistry from Hokkaido University, Japan. His postdoctoral fellowship from JSPS included a year at University of Shizuoka and he joined the department of pharmaceutical sciences at University of Shizuoka as a designated assistant professor in 2012. His postdoctoral fellowships included a year at University of California, Berkeley and a two-year appointment at University of California, Los Angeles. In 2016, he returned to University of Shizuoka as an assistant professor. His research is on the establishment of new methodology for genome mining and isolating new natural products from fungi.

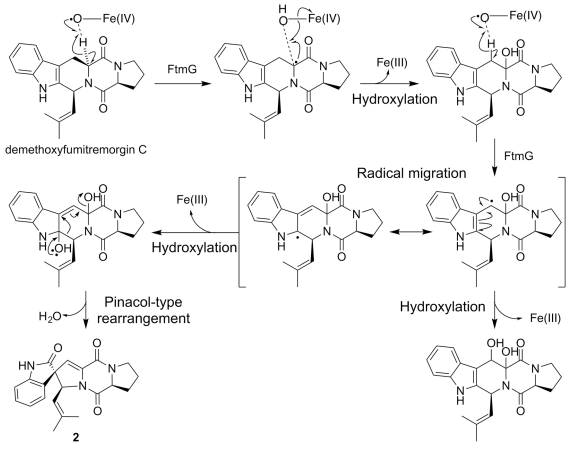
Kenji Watanabe received his Ph.D. in 2000 in bioorganic chemistry from Hokkaido University, Japan. His postdoctoral fellowships included a year at University of Wisconsin-Madison and a two-year appointment at Stanford University. In 2003, he returned to Hokkaido University as an assistant professor. In 2004, he joined the department of pharmaceutical sciences at University of Southern California as a research assistant professor. In 2008, he received a tenure-track position at Okayama University. He was promoted to associate professor in 2009 and professor in 2016 at University of Shizuoka. His research interest has been focused on developing a heterologous production system for natural products from PKS, NRPS and other important molecules of interest. Also, he has placed much effort into the mechanistic and structural characterization of enzymes involved in natural product biosynthesis.



Scheme 1. Proposed biosynthesis of tryprostatins, fumitremorgins and spirotryprostatins in *Aspergillus fumigatus.*



Scheme 2. Distinct mechanism of the formation of spiro-carbon in spirotryprostatin A and B. (A) Spirotryprostatin A biosynthesis proceeds *via* an epoxidation formation catalyzed by the flavin-containing monooxygenase FqzB. (B) Spirotryprostatin B biosynthesis proceeds *via* a radical formation catalyzed by the cytochrome P450 FtmG.

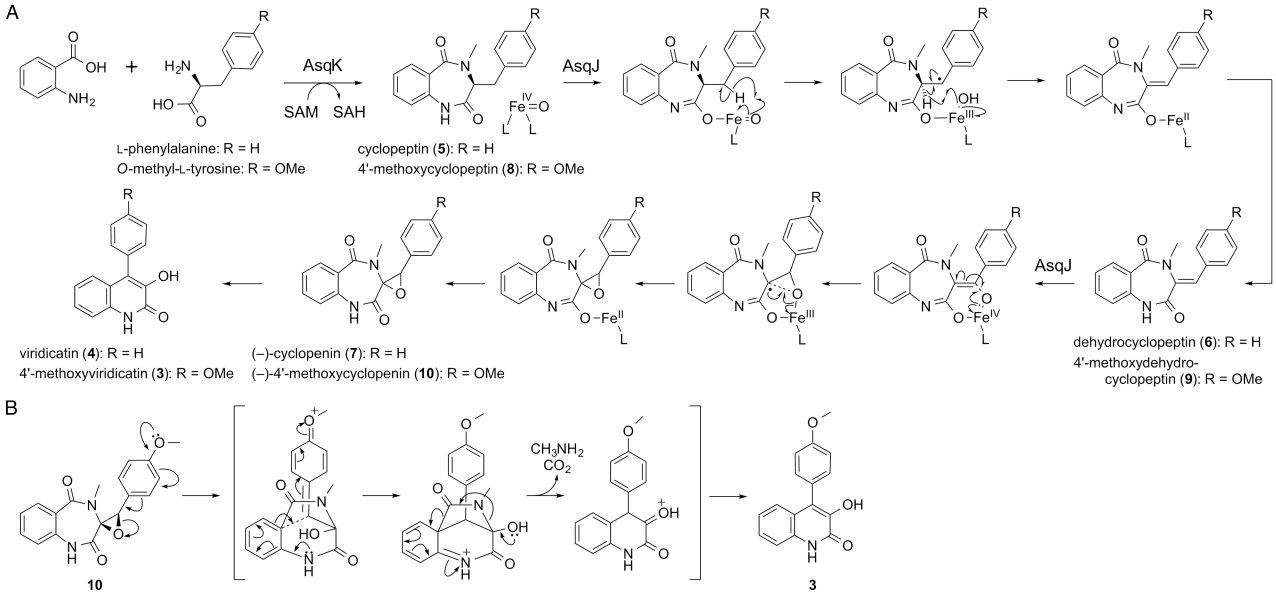


Scheme 3. The proposed mechanism for the formation of spirotryprostatins B catalyzed by FtmG.

initial pathway intermediates. For instance, tryprostatin B, which was essential for the in vitro characterization of FqzB for the biosynthesis of **2**, was obtained at approximately 100-fold greater yield from *S. cerevisiae* (35.6 mg per liter of culture) than the original producing host, *A. fumigatus* BM939.[13] However, *A. niger* was required for the production of **1.** Weak activity of FtmC (P450) and inactivity of FtmD (*O*-methyltransferase) for the formation of later-stage intermediates appear to be the hurdle for *S. cerevisiae* to produce later intermediates at sufficiently high level. Although the copy number of the *S. cerevisiae* cytochrome P450 reductase *NCP1*[14] was raised to improve regeneration of P450s, product yield remained suboptimal. Further optimizations, such as boosting the intracellular heme concentration through modulation of the heme biosynthetic pathway in yeast,[15] may be necessary to allow foreign P450s to attain their full activity in yeast. Nevertheless, our results revealed the versatile role of oxygenating enzymes, such as P450s and FMOs, in the biosynthesis of structurally complex natural products. The results also highlighted the potential of crosstalk among different biosynthetic pathways in facilitating product diversification in natural product biosynthesis.[12]

2.2. Viridicatin Biosynthesis

The structurally interesting and pharmaceutically attractive viridicatin scaffold[16] is found in natural products, such as 4′-methoxyviridicatin (**3**) isolated from *A. nidulans*, which is the



Scheme 4. Biosynthesis of 4′-methoxyviridicatin (**3**). (A) Proposed biosynthetic pathway of **3** involving two consecutive oxidations that are catalyzed by a single α-ketoglutarate-dependent dioxygenase, AsqJ. L: ligand comprised of two histidine and one aspartic acid residues.[24] (B) Proposed mechanism of formation of the quinolone framework of **3** from (−)-4′-methoxycyclopenin.

compound characterized by us for the first time,[17] viridicatin (**4**) isolated from *Penicillium* sp.,[18] and aspoquinolones from *A. nidulans.*[19] Those compounds are considered to be derived from the benzodiazepinedione class of fungal secondary metabolites, such as cyclopeptine (**5**),[20] dehydrocyclopeptine (**6**)[20] and cyclopenin (**7**),[16a] that can be generated by condensation of anthranilic acid and L-phenylalanine[21] by a bimodular NRPS[22] (Scheme 4A). The epoxy-6,7-bicyclic compound **7** can then go through a rearrangement to form the phenyl-substituted 6,6-quinolone core of the viridicatin scaffold. However, the mechanism of the rearrangement or the enzyme that is capable of catalyzing such a transformation has been only partially identified.[20,23] Hence, we set out to isolate the catalysts that can produce the epoxy-6,7-bicyclic framework and convert it to the 6,6-bicyclic quinolone to generate the viridicatin group of alkaloids.

We searched the *A. nidulans* genome for the genes involved in the biosynthesis of **3** based on the report of *A. nidulans* strains, including an endophytic variant, producing a group of compounds having the viridicatin scaffold.[19] Of the gene clusters containing a bimodular NRPS gene, we focused on a cluster containing a total of 14 genes, including an NRPS gene, which we named *asqK.* We found that the genes were only partially expressed under conventional culturing conditions, indicating that this cluster was an example of a silent biosynthetic gene cluster.[6a] Thus, *asqK* was overexpressed in *A. nidulans* A1149 from a plasmid by placing it under the control of an inducible promoter. This strain produced **5** and 4′-methoxycyclopeptin (**8**), supporting the notion that the *asq* gene cluster was responsible for the formation of **3.** Since the yield of **8** was approximately 10 times higher than that of **5**, we surmised that AsqK accepts *O*-methyl-L-tyrosine as the actual substrate while tolerating L-phenylalanine as an alternate substrate.[17] To ascertain the involvement of the predicted redox enzymes AsqE, F, G, I, J and L in the steps leading to the formation of the quinolone core structure, we performed bioconversion of **8** by a series of *A. nidulans* A1149 strains expressing each of the six genes from a plasmid. The result identified that **8** was transformed to 4′-methoxydehydrocyclopeptin (**9**) by the strain carrying *asqJ.* Thus, we hypothesized that AsqJ, a predicted α-ketoglutarate-dependent dioxygenase, could catalyze the dehydrogenation of the Cα–Cβ bond of the *O*-methyl-L-tyrosine side chain of **8** to form the double bond in **9** (Scheme 4A). To confirm this idea and to look into a possible reaction mechanism, we performed an in vitro assay using recombinant AsqJ produced in *E. coli* and observed the conversion of **8** to **9.** However, to our surprise, AsqJ converted **9** further to also form (−)-4′-methoxycyclopenin (**10**) via an epoxidation of the olefin (Scheme 4B). This observation was completely unexpected, since epoxidation is not a typical reaction catalyzed by this class of dioxygenases.[24] Lastly, all of the products were converted to **3** after a prolonged incubation of the reaction mixture. Because the spontaneous transformation of **10** to **3** proceeded quickly in an aqueous buffer at 30 °C, the conversion is likely non-

enzymatic. Thus, we proposed a mechanism of formation of **3** from **10** that was similar to the previously proposed mechanism of formation of **4**.[22,25] Epoxide opening initiated by donation of the methoxy oxygen lone pair followed by elimination of carbon dioxide and methylamine in the form of methyl isocyanate can lead to the transformation of the 6,7-bicyclic system of **10** into the 6,6-bicyclic quinolone of **3**.[17]

Considering that AsqK was capable of producing **5**, we examined whether the Asq enzymes are capable of biosynthesizing the non-4′-methoxylated series of products. When AsqJ was provided with **5**, we were able to observe the formation of **6** and **7** in a sequential fashion.[17] However, conversion of **7** to **4** was not observed. While it is known that **7** can be converted into **4** non-enzymatically,[16b,25–26] the rate of conversion is far slower than the conversion of **10** into **3.** This slow rate of conversion of **7** to **4** is the likely reason viridicatin-producing fungi carry an enzyme called cyclopenase. Interestingly, cyclopenase has only been prepared as a lipid fraction and eluded isolation to date.[18b,26–27] Through this study, AsqK was shown to be a new NRPS that is capable of condensing anthranilic acid with aromatic amino acids other than L-tryptophan.[28] Furthermore, AsqJ was identified as a very unique bifunctional dioxygenase that can catalyze two different types of reactions, a dehydrogenation and an unusual epoxidation reaction, in the consecutive steps of the viridicatin biosynthetic pathway.

2.3. Sch 210972 Biosynthesis

A decalin-containing natural product Sch 210972 (**11**) (Figure 1) isolated from filamentous fungus *Chaetomium globosum* has an inhibitory activity against a cell surface receptor CCR-5.[29] This activity can be exploited for developing novel antiviral agents against HIV-1 infection that work by blocking viral entry into cells. In addition, the biosynthesis of the cyclic core segment of **11** and other related compounds was suggested to proceed through an enzyme-catalyzed intramolecular Diels–Alder (DA) reaction.[30] Thus, we took interest in investigating the mechanism of biosynthesis of **11** in detail.

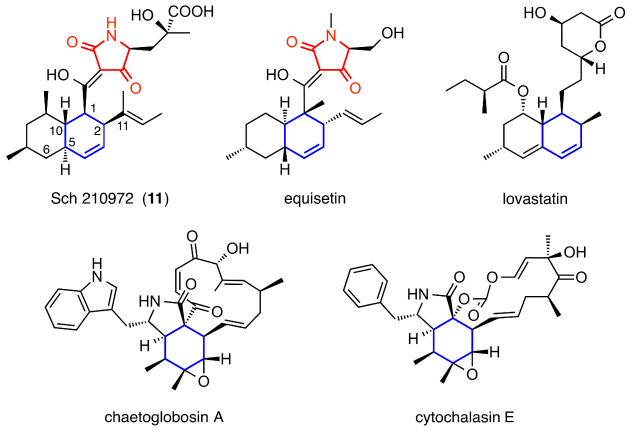
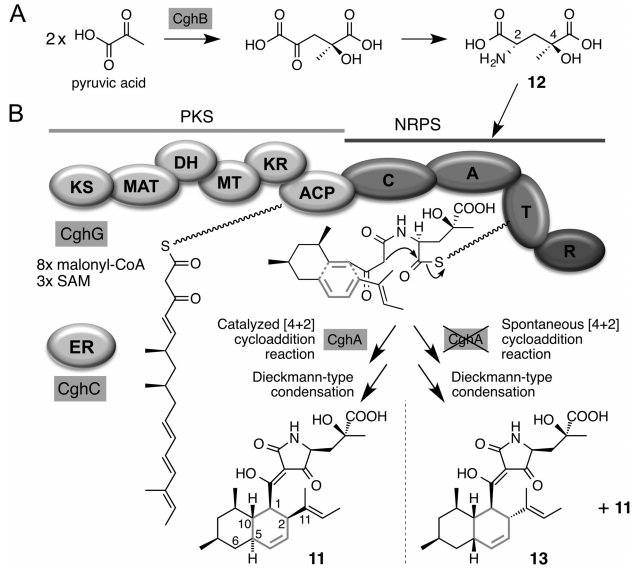


Figure 1. Natural products carrying a ring structure considered to be biosynthesized by a Diels–Alder reaction (shown in blue). Tetramic acid moieties are highlighted in red.

The presence of a decalin core and a tetramic acid moiety in **11** suggested that its framework is formed by a PKS–NRPS hybrid enzyme. In fact, previous studies have revealed that the tetramic acid moiety in other compounds, such as equisetin from *Fusarium heterosporum*[31] (Figure 1) is formed *via* a Dieckmann-type cyclization reaction that is catalyzed by the reductase-like domain located at the C-terminus of the NRPS module of a PKS–NRPS enzyme. Search of the *C. globosum* genome found three genes potentially coding for a PKS–NRPS hybrid enzyme. Upon deletion of one of the genes, which was originally annotated in the genome as five separate open reading frames (ORFs) CHGG_02374 through CHGG_02378, we were able to observe the loss of production of **11**, confirming its involvement in the biosynthesis of **11.** This gene was later re-annotated and named *cghG* (Scheme 5B).[32] The type of hybrid PKS–NRPSs examined in this study often accompanies a stand-alone enoyl reductase (ER) that works *in trans* with its corresponding



Scheme 5. Proposed biosynthetic pathway of **11**. (A) Formation of an unusual amino acid, γ-hydroxymethyl-L-glutamic acid **12**, from two molecules of pyruvic acid dimerized by an aldolase, CghB. (B) CghG-catalyzed synthesis of the linear substrate and a tetramic acid moiety, and the two final products that can be formed by the proposed Diels–Alder reaction. Abbreviations: KS: ketoreductase, 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.

PKS–NRPS for reduction of a nascent polyketide backbone double bond to install desired geometry into the chemical structure of the product. The initial discovery of this *trans*-acting ER was made in the biosynthetic pathway for another decalin-containing fungal polyketide lovastatin from *A. terreus*, where the ER LovC works with the PKS–NRPS LovB to generate the actual product (Figure 1).[33] The same arrangement is found in the chaetoglobosin biosynthetic pathway from *Penicillium expansum* and *C. globosum*, with the ER CheB cooperating with the PKS–NRPS CheA in *P. expansum*[30c] and CHGG_01241 with CHGG_01239 in *C. globosum* (Figure 1).[30e] Other examples are found in the cytochalasin E biosynthetic pathway from *A. clavatus*,[30d] the equisetin biosynthetic pathway from *F. heterosporum*[34] and *Fusarium* sp. FN080326[30f] (Figure 1). Since CghC showed high amino acid sequence homology to CheB, we proposed CghC to be the ER involved in the biosynthesis of **11**, and confirmed it by a knockout experiment.[32]

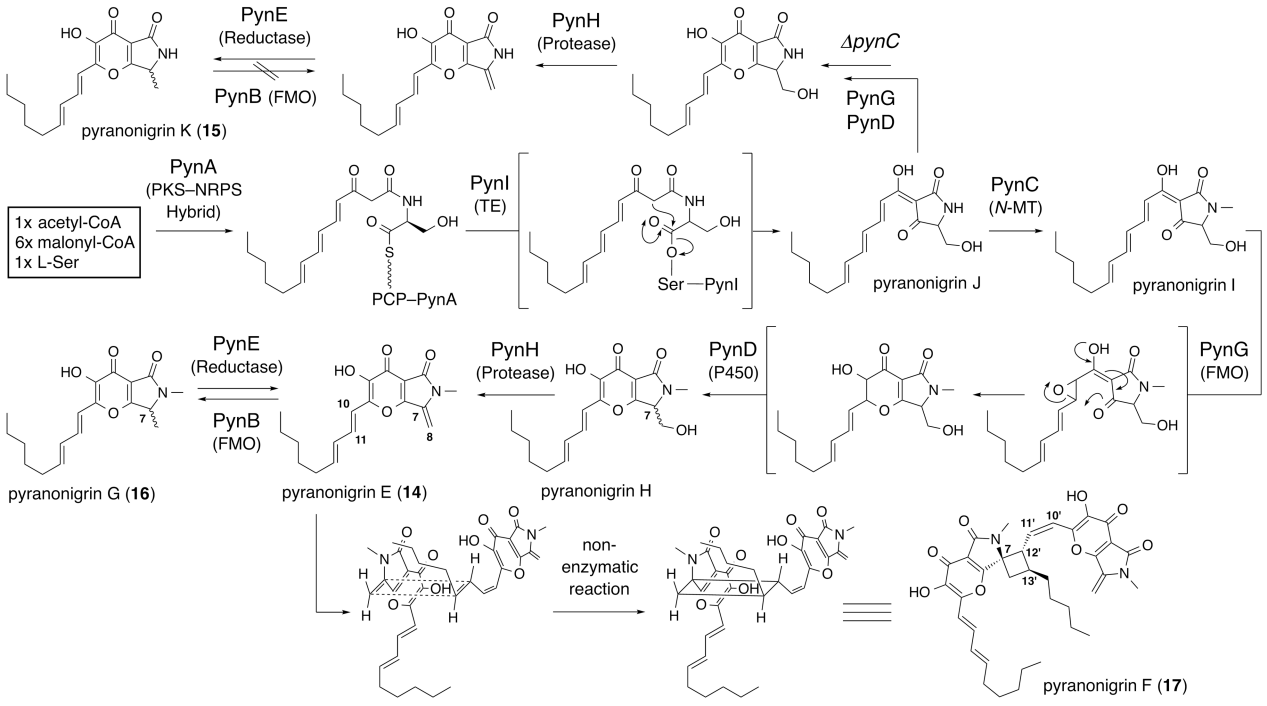
Inspection of the chemical structure of **11** suggested the involvement of an unusual amino acid, γ-hydroxymethyl-L-glutamic acid (**12**), as the substrate for the NRPS portion of CghG (Scheme 5A). We predicted that the enzyme that forms this non-proteinogenic amino acid would be encoded within the *cgh* gene cluster. One of the genes, *cghB*, was predicted to be an aldolase. Since the α-keto acid precursor of **12** can be obtained by an aldol condensation of two molecules of pyruvic acid, we speculated that CghB might be involved in the formation of **12.** To test this hypothesis, we synthesized **12** chemically by dimerizing pyruvic acid under a basic condition in aqueous solution, then transaminated the product with a glutamic oxalacetic transaminase using cysteinesulfinic acid as an amino group donor to obtain **12** and its diastereomers.[35] Then, those compounds were fed to the *cghB* knockout strain of CGKW14, an engineered *C. globosum* strain capable of site-specific homologous recombination.[36] While the *ΔcghB*/CGKW14 strain lost the ability to produce **11**, it produced **11** when **12** was supplemented to the culture medium. On the other hand, feeding of the diastereomer (2*S*,4*R*)-4-hydroxy-4-methylglutamic acid did not recover the production of **11.** These results indicated strongly that CghB was responsible for initiating the biosynthesis of **12** to be incorporated into **11** by CghG. It is not clear, however, which transaminase is responsible for converting the α-keto acid precursor into **12** in *C. globosum.*

Lastly and most interestingly, we attempted to identify the catalyst responsible for the proposed intramolecular DA reaction that completes the formation of the decalin core of **11.** There is a gene in the *cgh* cluster without a predicted function whose homologs are seen in other gene clusters responsible for the biosynthesis of the previously mentioned compounds that are thought to be formed via a DA reaction. Those genes are *cghA* for **11**,[32] CHGG_01241 for chaetoglobosin A,[30e] *ccsF* for cytochalasin E[30d] and *eqx3*[37] and *fsa2*[30f] for equisetin. Using the CGKW14 strain, we prepared a *ΔcghA*/CGKW14 strain. This knockout strain exhibited a significant reduction of production of **11.** More detailed analysis of the extract from the knockout strain revealed that it was producing **11** and its diastereomer **13** (Scheme 5). Furthermore, we were able to establish that **11** and **13** corresponded to the *endo* and *exo* adduct of the proposed decalin-forming [4+2] cycloaddition reaction, respectively. Upon complementing the *ΔcghA*/CGKW14 strain with a functional copy of *cghA*, **11** became the sole product, and formation of **13** was no longer observed. To eliminate possible interference from factors present natively in *C. globosum*, we reconstituted the *cgh* biosynthetic pathway by introducing *cghA*, *B*, *C* and *G* into *A. nidulans* A1145. This engineered *A. nidulans* strain produced **11** exclusively, while reduced formation of **11** and reemergence of **13** were observed when *cghA* was excluded. Therefore, we speculate that CghA binds to and locks the straight-chain polyketide substrate in a specific conformation to promote and direct the stereoselectivity of the DA reaction.[32] Along with the detailed in vitro characterization of LovC[30a] and recent identification of other proposed Diels–Alderases (DAases),[38] such as SpnF for spinosyn A biosynthesis, VstJ for versipelostatin biosynthesis and TclM for thiocillin biosynthesis, as well as crystal structure determination of PyrI4 from the pyrroindomycin biosynthetic pathway and AbyU from the abyssomicin C biosynthetic pathway,[39] our studies are building the case toward the existence of natural DAases. Nevertheless, further investigations are warranted to understand how DAases catalyze asymmetric DA reactions.

2.4. Pyranonigrin Biosynthesis

Pyranonigrins are a family of antioxidative compounds[40] isolated from *A. niger* that are characterized by the pyrano [2,3-c]pyrrole core structure.[41] Previously, activation of a biosynthetic gene cluster containing a PKS–NRPS hybrid enzyme gene was attempted by expressing the predicted transcriptional regulator gene *pynR* found within the cluster. This approach has successfully activated silent gene clusters previously.[42] A plasmid harboring the *pynR* gene under the control of an active promoter was introduced to *A. niger* ATCC 1015, and the expression of the *pyn* biosynthetic genes was accomplished. However, only a modest formation of the pathway product, pyranonigrin E (**14**), was achieved.[43] Since insufficient activation of the *pyn* biosynthetic cluster prevented thorough characterization of the pyranonigrin biosynthetic pathway, we decided to take a more comprehensive approach in examining this biosynthetic pathway.

First, using the *kusA*- and *pyrG*-deficient *A. niger* strain A1179 that allowed efficient targeted chromosome modifica-



Scheme 6. Proposed biosynthetic pathway for the formation of the pyranonigrin family of natural products in *Aspergillus niger.* Abbreviations: TE, thioesterase; *N*-MT, *N*-methyltransferase; FMO, flavin-containing monooxygenase; P450, cytochrome P450; PCP, peptidyl carrier protein.

tion,[10] we engineered the chromosome to replace the native promoter of *pynR* with a strong *glaA* promoter[44] to achieve high-level production of **14.**[45] Subsequent deletion of *pynA*, which encodes a PKS–NRPS hybrid enzyme, or *pynI*, a predicted thioesterase, abolished the production of all pyranonigrins, confirming that both enzymes are indispensable to the biosynthesis of pyranonigrins. As discussed in Section 2.3, formation of a tetramic acid moiety is typically catalyzed by the terminal reductase-like domain of the PKS–NRPS responsible for the formation of the straight-chain intermediate.[31–32] Pyranonigrin biosynthesis also involves a tetramic acid formation, and PynA does have a terminal reductase domain. However, it may be non-functional and require the assistance of PynI for the release of the straight-chain intermediate pyranonigrin J from PynA via the formation of the tetramic acid (Scheme 6).[45] While thioesterase-mediated tetramic acid formation and chain release has been observed before,[46] our finding here is the first example suggesting the occurrence of a stand-alone thioesterase that is responsible for a tetramic acid formation and a chain release from PKS–NRPS in a fungal biosynthetic system. Combined results from in vitro assays on recombinant PynG prepared in *E. coli* and in vivo bioconversion assays using *S. cerevisiae* expressing *pynD* and *pynG* showed that those enzymes are responsible for the γ-pyrone formation. We proposed that the cyclization proceeds through an epoxidation of the polyketide chain catalyzed by PynG, an FMO, and the subsequent oxidation by PynD, a P450, completes the formation of the γ-pyrone core of pyranonigrins (Scheme 6).[45]

Deletion of *pynC*, which encodes an *N*-methyltransferase, revealed that PynC is responsible for methylating the nitrogen atom of the tetramic acid moiety. Moreover, the *pynC* knockout strain also revealed that PynG and PynD tolerate absence of the methyl group to produce demethylated products such as pyranonigrin K (**15**).[45] Another interesting discovery from our study was that PynH, a predicted aspartic protease-like protein, turned out to be a dehydratase enzyme that catalyzes the dehydration of the serine side chain for the formation of the *exo*-methylene structure in **14.** While *exo*-methylene formation by dehydration of a serine side chain group is well documented in the lantibiotic biosynthesis,[47] PynH is the first enzyme of the kind that is involved in the modification of polyketide–nonribosomal peptide products.[45] An in vitro assay of recombinant PynE prepared in *E. coli* showed that PynE, a predicted reductase, can catalyze the reduction of **14** to form pyranonigrin G (**16**) (Scheme 6). On the other hand, **16** was converted to **14** by *S. cerevisiae* expressing *pynB.* PynB is predicted to be an FMO. The same reduction was observed to form **15** when the demethylated analog of 14 was treated with PynE. However, PynB failed to act on **15** to regenerate the *exo*-methylene group. Further

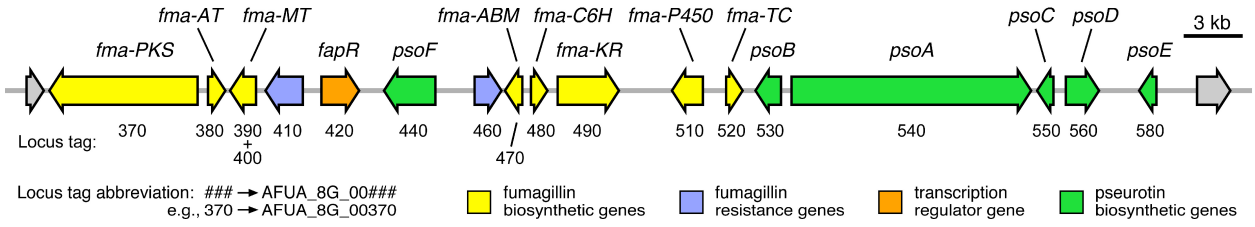
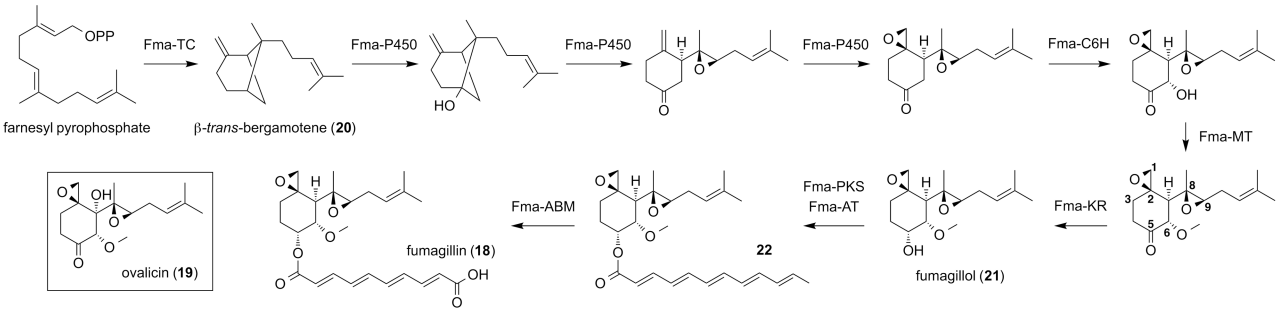


Figure 2. The intertwined fumagillin and pseurotin biosynthetic gene clusters.[49a, 50–51]



Scheme 7. The proposed fumagillin (**18**) biosynthetic pathway.

investigation is required to clarify the role of PynB and PynE in the pyranonigrin biosynthetic pathway.[45] Lastly, our study also identified that a dimeric product pyranonigrin F (**17**) can be generated from **14** non-enzymatically. We speculate that the dimerization of **17** might proceed *via* an intermolecular [2+2] cycloaddition between the *exo*-methylene and the C-10,11 olefin of **14** (Scheme 6).[45]

In this work, combination of targeted gene deletion, heterologous in vivo bioconversion and in vitro assays allowed us to identify the main pathway for the biosynthesis of pyranonigrins along with six of the intermediates formed and interesting activities of the enzymes responsible for the multistep transformation leading to the formation of **14.** Again, this study reiterated the notion[30e, 48] that fungal natural product biosynthesis relies heavily on redox enzymes to generate complex chemical structures that can give rise to unique bioactivities. Such knowledge will be valuable in further characterization and engineering of natural product biosynthetic systems for bioproduction of novel compounds. While we did not observe the formation of pyranonigrin analogs other than those discussed in this study, one could imagine, as was the case for the pseurotin biosynthesis discussed in the next section,[49] that possible combination of enzymatic and nonenzymatic isomerization of the conjugated side chain could further widen the structural diversity of the natural products being generated.

2.5. Fumagillin and Pseurotin Biosyntheses

Last section will look at the fumagillin and the pseurotin biosynthetic pathways. Apart from their chemical complexity, what is interesting about these compounds is that their biosynthetic gene clusters are encoded in the genome of *A. fumigatus* not side by side but intermingled with each other (Figure 2). The $Zn(II)_2Cys_6$ transcription factor FapR controls the expression of both clusters, indicating that the two pathways are also interwoven at the transcriptional level as well.[50] In our study, we attempted to uncover the mechanism of biosynthesis of two specific natural products, fumagillin[48b, 51] (**18**) and pseurotin A[49] (**23**).

Initially isolated from *Aspergillus* sp. in the 1950s,[52] **18** (Scheme 7) is a meroterpenoid known for its antiangiogenic activity exerted by binding irreversibly to human type 2 methionine aminopeptidase (MetAP-2).[53] Because a structurally related compound ovalicin (**19**)[54] was proposed to be derived from a sesquiterpene called β-*trans*-bergamotene[55] (**20**), the biosynthesis of **18** was presumed to involve a terpene cyclase for the formation of the cyclohexyl core moiety from farnesyl pyrophosphate (FPP) (Scheme 7). However, examination of the *A. fumigatus* genome sequence did not reveal the existence of terpene cyclase homologs in the genome, despite the known existence of atypical terpene cyclases in other fungal meroterpenoid gene clusters.[56] This suggested that yet another unknown type of terpene cyclase might be involved in the biosynthesis of **18**, and prompted us to look for the corresponding gene cluster. Also, **18** has a

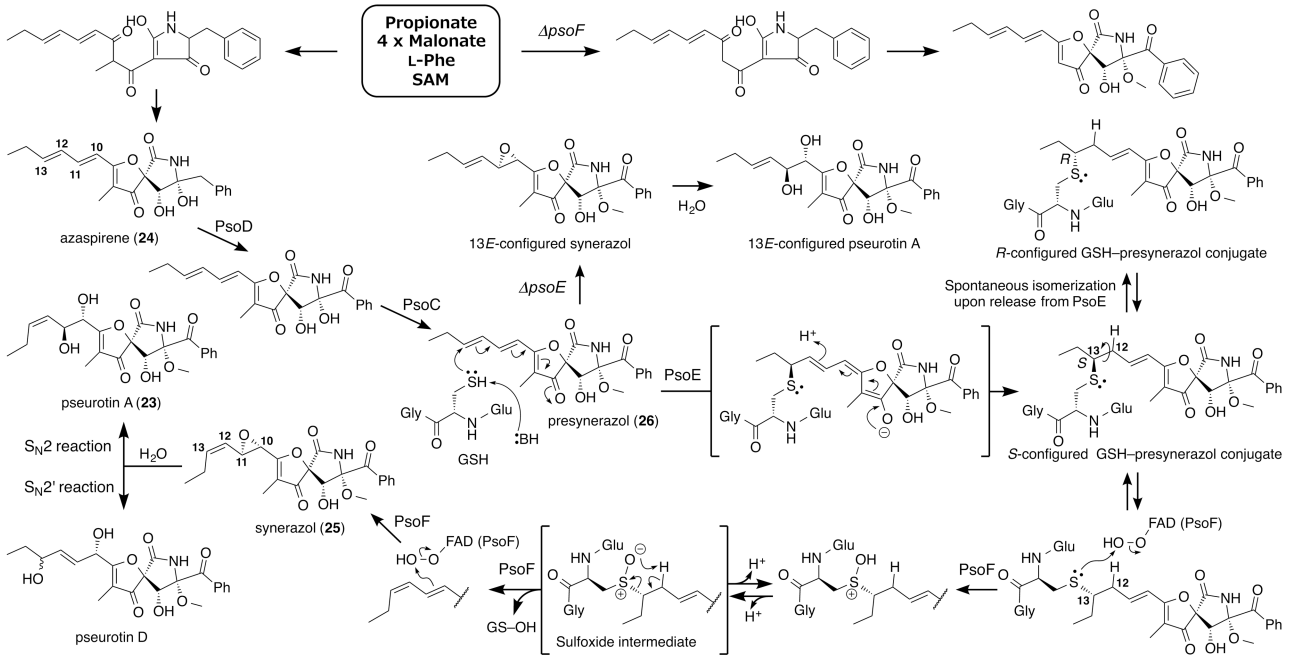
penta-substituted cyclohexane core that is highly oxygenated. However, the biochemical mechanism or the order of all the oxygenative tailoring reactions were unknown. Thus, we decided to investigate the fumagillin biosynthetic pathway in detail.

Analysis of known *A. fumigatus* secondary metabolite biosynthetic gene clusters identified a cluster containing two genes AFUA_8G00410 and AFUA_8G00460 predicted to code for a type 1 and type 2 methionine aminopeptidase, respectively (Figure 2). Since **18** was known to bind to human MetAP-2, these genes were suspected to be resistance genes against **18.** Upon knocking out AFUA_8G00370, the only gene encoding a highly reducing PKS (HR-PKS)[57] in the cluster, production of **18** was abolished, indicating that this gene cluster, which we named *fma*, was responsible for the biosynthesis of **18.**[51] As to the formation of the FPP-derived **20**,[54] the *fma* gene cluster was searched for a gene encoding a possible terpene cyclase. Although AFUA_8G00520, which we named *fma-TC*, had no homology to terpene cyclases, deletion of *fma-TC* resulted in the loss of production of **18** in *A. fumigatus.* Heterologous expression of *fma-TC* in *S. cerevisiae* led to *de novo* production of **20**, and the microsomal fraction from this yeast converted FPP to **20** in vitro, confirming that Fma-TC was the new, membrane-bound class II terpene cyclase responsible for the formation of **20** (Scheme 7).[51] *In vitro* assays using recombinant Fma-PKS and Fma-AT revealed that Fma-PKS is responsible for the formation of dodecapentaenoic acid, while Fma-AT transfers the unsaturated fatty acid onto **21** to form **22.**

The most interesting finding from this study came from the investigation into the mechanism of extensive oxidations and structural rearrangement that the intermediates undergo for the formation of **18.** In particular, conversion of **20** to **18** requires opening of a strained cyclobutane bridge of **20** to form the cyclohexane core having six contiguous stereocenters involving two epoxide and two hydroxyl groups. Combination of genetic knockout, bioconversion, heterologous reconstitution in *S. cerevisiae*, and in vitro biochemical assays successfully revealed the sequence of redox reactions and the involvement of the three redox enzymes, Fma-P450, Fma-C6H (a non-heme Fe(II) and α-ketoglutarate-dependent dioxygenase) and Fma-KR (a ketoreductase). Specifically, Fma-P450 was found to be responsible for three oxidation reactions leading to the opening of the strained cyclobutane bridge and installation of the ketone and two epoxide groups in **20.** Fma-C6H installs the hydroxyl group at C6 and Fma-KR reduces the ketone at C5. Prior to the ketoreduction, however, the C6 hydroxyl group is methylated by Fma-MT before the formation of **21** is completed (Scheme 7).[48b] Lastly, AFUA_8G00470 named *fma-ABM* was predicted to code for a oxygenase belonging to the cofactor-independent antibiotic biosynthesis monooxygenase (ABM) superfamily.[58] Bioconversion experiment using *S. cerevisiae* expressing *fma-ABM* successfully formed the terminal carboxylic acid in **22** to generate **18** (Scheme 7).[48b]

The second group of fungal secondary metabolites described here is the pseurotin family of compounds represented by **23** (Scheme 8). Pseurotins[59] comprise a family of structurally related Aspergillal natural products having interesting hetero-spirocyclic γ-lactam core structure and bioactivities, such as induction of neural cell differentiation.[60] The PKS–NRPS gene responsible for the production of **23** was identified earlier,[61] but little remained understood about the biosynthetic steps involved in the formation of the complex chemical features. Systematic deletion of the pseurotin biosynthetic genes in *A. fumigatus* (Figure 2) resulting in disappearance or accumulation of specific pathway intermediates allows a rough outline of the biosynthetic pathway to be drawn. Then we performed *S. cerevisiae in vivo* bioconversion and in vitro assays with recombinantly produced enzymes to characterize the activities of the enzymes in detail.[49a] Results from those experiments allowed us to elucidate the main biosynthetic steps leading to the formation of **23**, starting from the universal precursor azaspirene[62] (**24**) through the immediate precursor synerazol[63] (**25**) (Scheme 8). Our study illuminated the combinatorial nature of the pseurotin biosynthetic pathway, leading to the formation of multiple closely related compounds that varied mainly in the hydroxylation and methylation patterns, as well as *trans–cis* isomerization and reduction of the conjugated diene side chain.

The most notable discovery from the study was the identification of PsoF, a fusion protein having a methyltransferase and an FMO domain, that performs a *C*-methylation of the nascent polyketide backbone carbon atom *in trans.*[49a] This was the first report of such a type of methylation in polyketide biosynthesis. Our search for the enzymatic mechanism behind the *trans*-to-*cis* isomerization of the C12–C13 olefin focused on *psoE* that was predicted to encode a glutathione-*S*-transferase (GST) which is known to catalyze isomerization reactions.[64] However, we found that PsoF catalyzing the epoxidation step immediately following the *trans*-to-*cis* isomerization step was also required for the isomerization to be completed. Detailed biochemical and structural studies indicated that the main function of PsoE is to perform a stereospecific conjugation of the 12,13*E*-configured intermediate called presynerazol (**26**) with GSH.[49b] Substitution of C13 with a bulky GSH residue in **26** drives the C12–C13 bond to assume a pro-*cis* conformation. The crystal structure also revealed that PsoE has an unusually open substrate-binding cleft that presumably facilitates the GSH conjugate of **26** to depart from the enzyme readily (Figure 3). Upon release from PsoE, the GSH–**26** conjugate is transferred to the FMO domain of



Scheme 8. The proposed pseurotin (**23**) biosynthetic pathway.

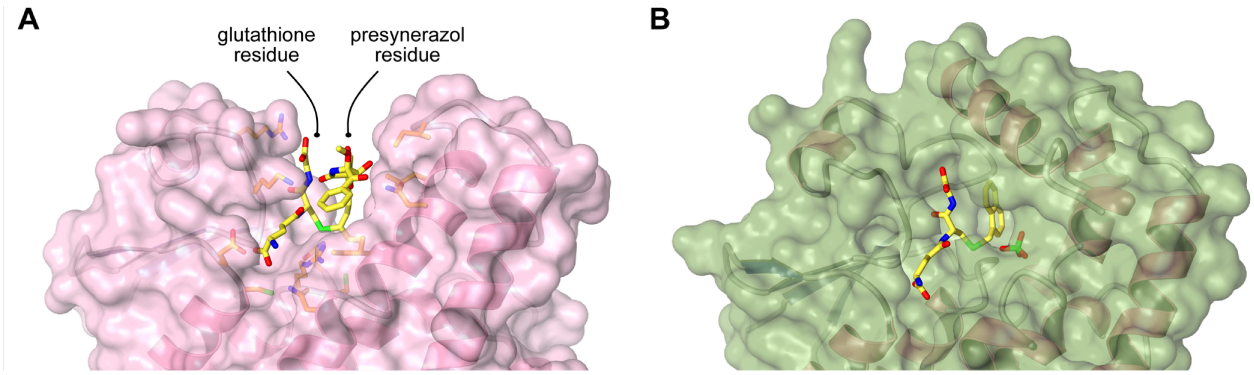


Figure 3. Surface representation of (A) PsoE in complex with the glutathione conjugate of presynerazol (PDB ID: 5F8B)[49b] and (B) human T2-2, a Theta glutathione-*S*-transferase, with bound ligands 1-menaphthyl, glutathione and sulfate (PDB ID: 3LJR).[66]

PsoF where the sulfur atom of the GSH conjugate is oxidized by flavin hydroperoxide. Following a pericyclic *syn*-elimination of an oxidized GSH, 12,13*Z*-configured stereoisomer of **26** remaining in the PsoF active site is epoxidated to form **25** in succession by another molecule of flavin hydroperoxide that is regenerated at the expense of NADPH (Scheme 8). While successive oxidations of a substrate by an FMO has been observed previously,[65] it is uncommon to see two different types of oxidation reactions, namely a sulfur oxidation and an epoxidation, carried out on a single substrate in succession. This finding made PsoF a trifunctional enzyme that is key to the complexity generation in pseurotin biosynthesis.

3. Conclusion

In this account, we have highlighted the value of using different heterologous hosts for studying the biosynthesis fungal secondary metabolites. As far as the choice of heterologous host is concerned, it depends on the aim of the experiments to be conducted and the original production hosts being examined. For instance, *S. cerevisiae* is the most convenient host to work with, but its ability to produce

certain secondary metabolites seems to be inferior to filamentous fungi, such as *A. niger*. Filamentous fungi appear to be able to produce heterologous redox enzymes, P450s in particular, and maintain their activity more effectively than *S. cerevisiae*, as was observed in the study of the spirotryprostatin biosynthesis.[12] Since fungal natural products are often heavily oxidized, and oxidation reactions are frequently used to introduce complex chemical structures into the carbon skeleton,[30e] the ability of filamentous fungi to produce functional redox enzymes more effectively seems to confer an advantage in generating the type of fungal secondary natural products discussed here. While *A. niger* and *A. nidulans* are used relatively interchangeably, clearly we would use *A. nidulans* when studying biosynthetic pathways from *A. niger*, and *vice versa*. It is also worth mentioning that *Escherichia coli* is still a very useful organism, especially when preparing purified enzymes for *in vitro* assays (FtmD and FqzB for the spirotryprostatin biosynthesis,[12] AsqJ for the viridicatin biosynthesis,[17] PynE and PynG for the pyranonigrin biosynthesis[45] and PsoC and PsoF for the pseurotin biosynthesis[49a]) or structural characterization (PsoE for the pseurotin biosynthesis[49b]). However, *E. coli* is notoriously poor at heterologous production of P450s. Instead, preparation of P450s as a microsomal fraction from *S. cerevisiae* works better (FtmG for the spirotryprostatin biosynthesis[12] and PsoD for the pseurotin biosynthesis[49a]). Similarly, for heterologous production of massive multidomain enzymes, such as PKSs and NRPSs, *S. cerevisiae* is the host organism of choice (Fma-PKS for the fumagilin biosynthesis[51]).

Accumulation of genome sequence information and development of various experimental techniques have made deciphering of the complex mechanisms of natural product biosynthesis a more tractable task in recent years. As illustrated throughout this account, manipulating the genome of a target organism for unraveling the biosynthetic pathway of interest is becoming a routine work. When such genetic modifications are difficult in the target organism, biosynthetic genes can be transferred to a more convenient host for a heterologous reconstitution of the target biosynthetic pathway for detailed examinations. Furthermore, combination of in vitro assays and in vivo bioconversion experiments is a powerful method of establishing the functions of biosynthetic enzymes and elucidating the fine details of the biosynthetic pathway. However, we are still mainly concerned with microbes that can be cultivated. To further our efforts in the discovery of novel natural products and biosynthetic enzymes, we must tap into microbes that are uncultivable under standard laboratory conditions, which are thought to constitute 99 % of microbes present in the environment.[67] The metagenomic approaches of extracting genetic information from environmental samples have seen success in discovering novel natural products and their corresponding biosynthetic gene clusters.[68] Pairing the growing genetic and enzymological knowledge of secondary metabolite biosynthesis with the streamlined synthetic biological methodologies of engineering microbes could facilitate the development of bio-based technological platform that can allow efficient screening and scalable production of natural products and their analogs in the near future.

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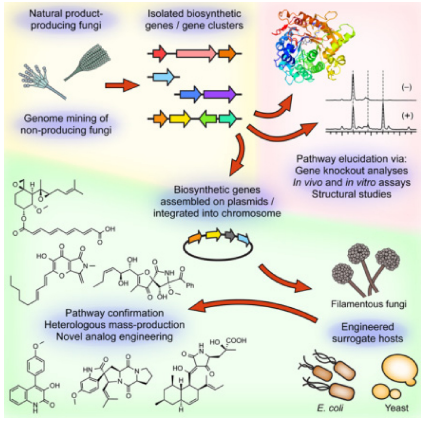
PERSONAL ACCOUNT

Biosynthetic pathways of fungal natural products having interesting chemical structures and valuable biological properties have been elucidated. Biosynthetic genes are identified through genome mining and genetic manipulation of the producing organism. Those genes are used for heterologous reconstitution of the biosynthetic pathway being studied in yeast or engineered fungi for preparing pathway products and intermediates. Lastly, detailed in vivo, in vitro and structural characterizations of the enzymes result in elucidation of unexpected mechanisms of secondary metabolite biosynthesis.



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Elucidation of biosynthetic pathways of natural products