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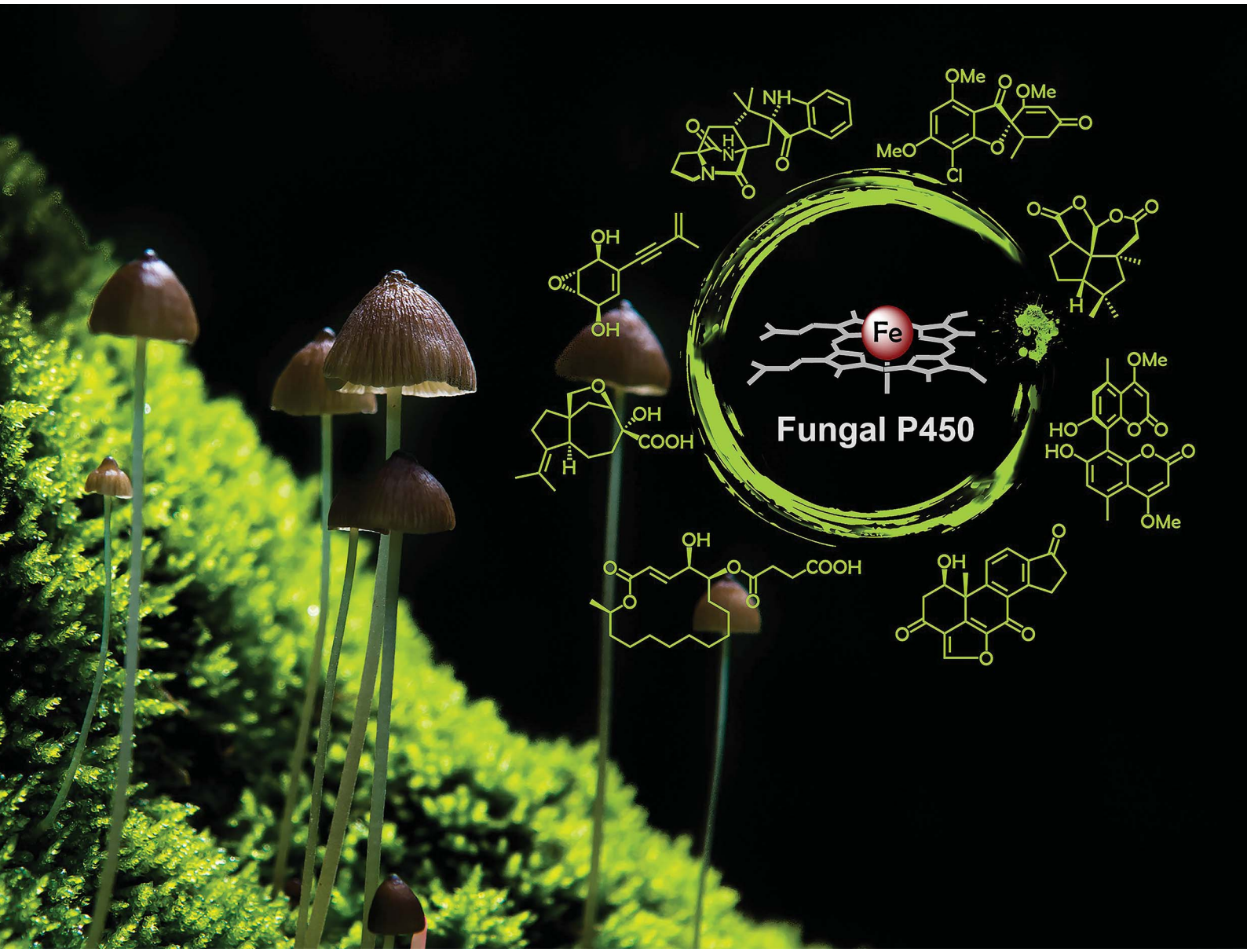

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REVIEW ARTICLE
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REVIEW

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Cytochrome P450 enzymes in fungal natural product biosynthesis

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Covering: 2015 to the end of 2020

Fungal-derived polyketides, non-ribosomal peptides, terpenoids and their hybrids contribute significantly to the chemical space of total natural products. Cytochrome P450 enzymes play essential roles in fungal natural product biosynthesis with their broad substrate scope, great catalytic versatility and high frequency of involvement. Due to the membrane-bound nature, the functional and mechanistic understandings for fungal P450s have been limited for quite a long time. However, recent technical advances, such as the efficient and precise genome editing techniques and the development of several filamentous fungal strains as heterologous P450 expression hosts, have led to remarkable achievements in fungal P450 studies. Here, we provide a comprehensive review to cover the most recent progresses from 2015 to 2020 on catalytic functions and mechanisms, research methodologies and remaining challenges in the fast-growing field of fungal natural product biosynthetic P450s.

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1 Introduction

Cytochrome P450 enzymes (P450s), a superfamily of heme-thiolate proteins, are ubiquitously distributed in all domains of life, including humans, animals, plants, protists, fungi, bacteria, archaea and viruses.[1–3] Up to now, more than 300 000 P450 genes have been discovered from the explosively growing genome sequencing data.[4] P450s are essential for a vast majority of organisms because they are extensively involved in both anabolism by participating in assembly of diverse endogenous biofunctional molecules, and metabolism by mediating degradation/detoxification of various xenobiotics.[5–9] For example, a majority of oxidative reactions are mediated by P450s in human steroidogenesis; meanwhile, P450s account for >70% of enzymes involved in drug metabolism in human body.[10–12]

In view of the innate chemistry, P450s belong to mono-oxygenases because the biocatalysts commonly insert a single O_2-derived oxygen atom into C–H, C=C, N–H, or S–H bonds in diverse substrates, giving rise to the hydroxylated or epoxidized products. The equation of a common hydroxylation reaction is

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as follow: R–H + O_2 + $2H^+$ + $2e^-$ → R–OH + H_2O. Besides these "common" reactions, a growing number of P450s have been found to catalyze various "uncommon" reactions, such as C–C, C–N and C–S bond formation and scission, ring formation, expansion and contraction, intramolecular and intermolecular aromatic coupling, and even more complex reactions that could dramatically reshape the initial structures.[13] Thus, the widespread P450s with great catalytic diversities have been named as the most versatile enzymes in nature.[14]

Particularly, P450s exhibit crucial catalytic abilities in microbial natural product (NP) biosynthesis.[5,15–17] NPs play essential roles in cell structure constructions, signaling networks, physiological regulations and chemical defenses of microorganisms.[18] Biosynthetically, NPs are mainly produced by special "assembly lines", in which different enzymes act as line workers to take different specific responsibilities.[19] For instance, polyketide synthases (PKSs) typically utilize C_2 (malonate) or C_3 (methylmalonate) units as building blocks to assemble the carbon skeletons during polyketide biosynthesis; non-ribosomal peptide synthetases (NRPSs) put together proteinic or non-proteinic amino acids to yield linear or cyclic peptides; and terpene cyclases (TCs) adopt diverse cyclization modes towards differently sized linear polyprenyl pyrophosphates.[19–21] After assembly of the central skeletons, different tailoring enzymes including oxidoreductases, methyltransferases, glycosyltransferases, halogenases,

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prenyltransferases and acylases, mediate highly diversified and sophisticated structural modifications.[19] Among them, P450s stand out because of their broad substrate scope, great catalytic diversity and high frequency of involvement during NP biosynthesis. The P450-catalyzed reactions enormously diversify the chemical structures and biological activities of NPs.

Most of microbial P450 catalytic systems require redox partner(s), in the form of either isolated protein(s) or fused domain(s) to P450, to deliver two electrons from NAD(P)H to the heme-iron reactive center of P450 for O_2 activation.[22–24] Fungal (eukaryotic) and bacterial (prokaryotic) P450 catalytic systems are distinct in that: (1) most bacterial P450s employ an iron–sulfur ferredoxin (Fdx) and an FAD-containing ferredoxin reductase (FdR) as their redox partners, while fungal P450s recruit a single FAD/FMN-containing flavoprotein, referred to as cytochrome P450 reductase (CPR), to shuttle reducing equivalents; and (2) the three components of a bacterial P450 system usually are soluble cytosolic proteins, while most of fungal P450s and CPRs are membrane-bound proteins (except for some mitochondrial ones).[22,23,25] These differences have led to thriving functional, structural and mechanistic studies on prokaryotic P450s, while greatly limited the corresponding researches on fungal P450s because: (1) the membrane-bound nature makes fungal P450s and CPRs difficult to be heterologously expressed, especially by prokaryotic hosts such as *Escherichia coli* for obtaining enough soluble and functional proteins, for *in vitro* mechanistic analysis and structural biology studies; and (2) the compatibility between a fungal P450 and a heterogenous CPR is far worse than that of a bacterial P450 and surrogate Fdx/FdR.[22,23,25] Fortunately, recent development of the CRISPR/Cas9-based gene editing techniques and the broader application of some filamentous fungi, such as several *Aspergillus* species, as heterologous expression hosts, have led to more functional and mechanistic studies on fungal NP biosynthetic P450s. However, the breakthrough in fungal P450 structural biology has yet to come due to some unsolved bottlenecks at present. In this review, we will comprehensively review the most recent advances in fungal P450s related to NP biosynthesis in the past six years.

2 Fungal P450 catalytic process

It is now clear that fungal P450s mediate a typical monooxygenation reaction *via* the canonical 'oxygen rebound' mechanism by going through the widely-accepted catalytic cycle (Fig. 1).[1,8] In this cycle, starting from the resting state (a), a water molecule coordinates to the heme-iron forming a hexacoordinated low-spin ferric adduct in cooperating with the absolutely conserved cysteine residue and the four nitrogen atoms of the porphyrin ring (Por). Upon substrate (R–H) binding to the active-site, the water molecule is displaced and the spin state shifts to a high-spin state of the ferric heme-iron (Fe^{III}) (b). The reactive heme-Fe^{III} initiates the first electron transfer from NADPH to CPR and then to P450, thereby being reduced to the ferrous heme-iron (Fe^{II}) species (c). Then, a dioxygen molecule binds to Fe^{II} forming a heme-Fe^{II}–O═O adduct (d), which is further reduced to the peroxo-ferric intermediate heme-Fe^{III}–O_2^- (e) by the secondly transferred electron. This intermediate is protonated to yield the first reactive species heme-Fe^{III}–O–OH (f) (compound 0, abbreviated as Cpd0). Cpd0 undergoes a second protonation to induce the heterolytic fission of the O–O bond to generate the high-energy porphyrin π radical cation ferryl intermediate [$Por^{\bullet+}$–Fe^{IV}═O] (CpdI; g) and release a water molecule. The highly reactive CpdI abstracts a hydrogen atom ($H^\bullet$) from the bound substrate leading to the ferryl-hydroxo heme-Fe^{IV}–OH species (CpdII; h) and yields a substrate radical ($R^\bullet$). In a typical hydroxylation reaction, the [$HO^\bullet$] in CpdII normally rebounds to $R^\bullet$ *via* a ferric heme-Fe^{III}–ROH complex (i), after which the heme-iron regenerates the resting state (a) by releasing the hydroxylation product and restores the H_2O coordination. Beyond the intact cycle, most of P450s could adopt the "peroxide shunt pathway" using H_2O_2 as the oxygen- and electron-donor, in which the high-spin substrate-P450 complex (b) could be directly transformed into Cpd0 (f) without involving NADPH, O_2 and redox partners.[1,8]

During the "uncommon" reactions, after the CpdI-triggered substrate radical ($R^\bullet$) is formed, instead of the common OH rebound, CpdII may abstract another hydrogen atom or an additional electron (e^-) to produce a diradical or carbocation intermediate, respectively, which could further undergo radical coupling, trapping, scavenging or rearrangement, thus giving rise to various "uncommon" products, such as substrate dimers (Fig. 1).[13,15,26] Notably, besides CpdI and Cpd0, CpdII and even the heme-Fe^{III}–O–O^- species have been proposed to be capable of taking different catalytic roles in some "uncommon" reactions.[13] These unusual findings may support more flexible usage of different P450 catalytic species to produce more unusual products in NP biosynthesis.

Since redox partners are essential for most P450s as electron shuttles, P450 catalytic systems have been categorized into different classes according to their cognate redox partners.[22] The fungal microsomal P450s belong to the two-component class II P450s, while the three-component mitochondrial P450s falling into class I as the majority of bacterial P450s.[15,22,27] The fungal NADPH-specific redox partner CPR, with an N-terminal FMN-containing domain and the C-terminal FAD- and NADPH-binding domains, serves as a membrane-bound electron shuttle to form a typical fungal microsomal P450 redox system (Fig. 1).[28] Briefly, NADPH first transfers a pair of electrons in the form of a hydride (H^-) to FAD, while FAD then transfers the two electrons one at a time to FMN and then to the heme-iron to drive the catalytic cycle (Fig. 1).[23] Of note, some fungal P450s could also receive the second electron from NADH through cytochrome b_5 reductase and cytochrome b_5 (Cytb5).[23]

3 P450s involved in fungal natural product biosynthesis

In this section, we summarize the recently discovered P450-mediated reactions in fungal NP biosynthesis, with emphasis on both catalytic functions and mechanisms for uncommon P450 reactions. For common P450 reactions including hydroxylation, epoxidation, and successive oxidation leading to

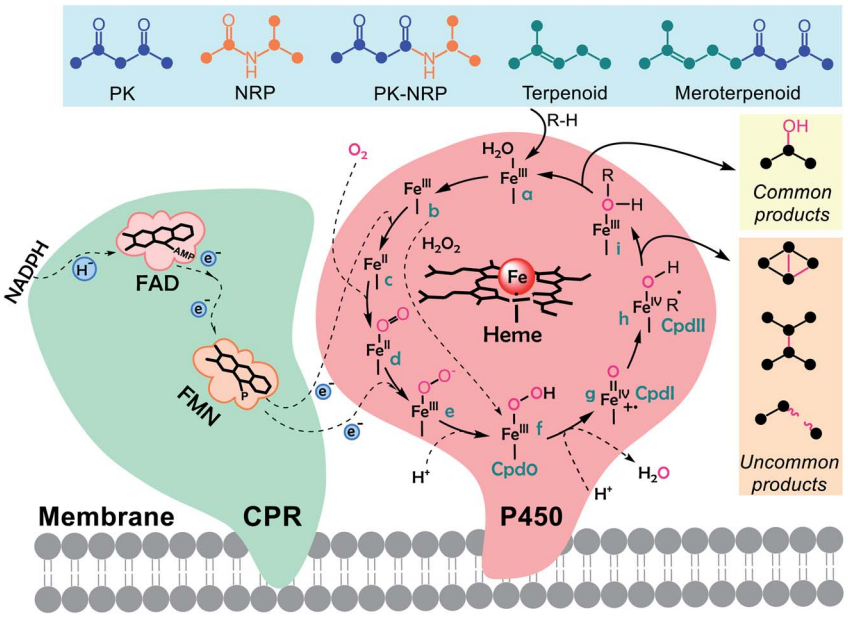


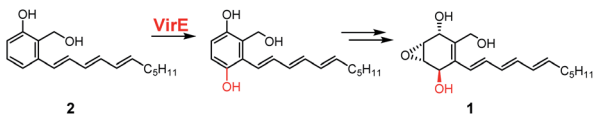
Fig. 1 The catalytic cycle of class II fungal P450.

aldehyde, ketone or carboxylic acid, we only introduce the substrate, product and reaction order, because the chemistry involved in these reactions largely comply with the typical catalytic cycle of P450s (Fig. 1). Specifically, we review these fungal NP biosynthetic P450s according to the structural classes of their substrates, including polyketides, non-ribosomal peptides, polyketide-non-ribosomal peptide hybrids, terpenoids and meroterpenoids.

3.1 Polyketide (PK) biosynthetic P450s

3.1.1 Common P450 reactions in PK biosynthesis. PKs encompass a family of structurally diverse NPs whose basic carbon-skeletons are assembled by different types of PKSs.[21] At the tailoring stages, P450s play important roles in oxo-functionalizing the PK skeletons, which not only optimize their aqueous solubility and biological/pharmaceutical activity, but also provide chemical handles for other downstream tailoring modifications.

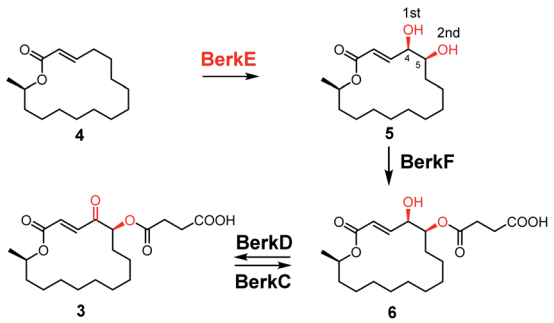
For example, during the biosynthesis of trichoxide (**1**), a newly isolated epoxycyclohexenol compound from the biocontrol fungus *Trichoderma virens*, P450 VirE was characterized to install the *p*-hydroxyl group onto the phenol substrate **2** (Scheme 1).[29] VirE's function was determined by the *in vivo* experiments based on its heterologous expression in *Aspergillus nidulans* combined with the precursor feeding experiment and the *in vitro* enzymatic reaction using the microsomal fraction of the recombinant *Saccharomyces cerevisiae* RC01 that contained VirE.[29] Similar *in vivo* and *in vitro* approaches have been widely used in fungal P450 functional characterization. It is worth noting that, unlike the typical sp^2 and sp^3 C–H bond hydroxylation, due to the special electronic property of the aromatic ring, the aromatic hydroxylation was also proposed to be possibly initiated by direct electron transfer from the benzene ring to CpdI or electrophilic attack of the benzene to CpdI. Alternatively, the typical hydrogen abstraction could occur at the substituent group on aromatic ring, such as the phenolic OH group in **2**, which is followed by radical relocation to the hydroxylation site.[30]



Scheme 1 The VirE catalysed hydroxylation during the biosynthesis of trichoxide (**1**).

For many antibiotic producing microorganisms, diverse self-resistance strategies are adopted to avoid self-harm during the production of the toxic NPs.[31,32] Recently, an interesting strategy for self-resistance was discovered during the biosynthetic study of the macrolide antibiotic A26771B (**3**) in the fungus *Penicillium egyptiacum*.[33] As reported, P450 BerkE catalyses successive hydroxylations of C4 and C5 towards **4** to yield **5**. The diol group in **5** is essential for both biological activity and self-resistance mechanism. Following the P450-mediated dihydroxylation, acyltransferase BerkF catalyses succinylation of **5** to form the pro-drug berkeleylactone E (**6**), which can be pumped out of fungal cells. Then, the extracellular flavin-dependent oxidase BerkD converts C4-OH into a carbonyl group, yielding the final bioactive product **3**. Furthermore, to avoid the self-toxicity caused by the cell-absorbed **3**, the intracellular short-chain dehydrogenase/reductase BerkC could reduce the toxic compound **3** back to nontoxic **6** (Scheme 2).[33]

Besides off-line enzymatic reactions, in a few cases, fungal P450s have also been proposed to mediate online oxidations, meaning P450s could oxidize the polyketide chain attached to

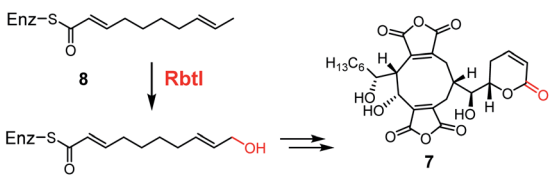


Scheme 2 The BerkE catalysed hydroxylation and the newly identified self-resistance strategy in the biosynthesis of A26771B (**3**).

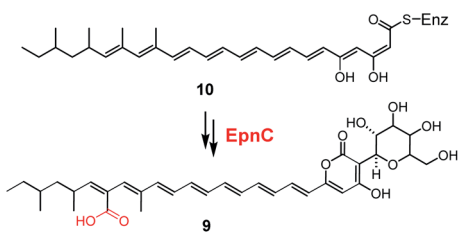
PKS at the elongation stage. For example, during the *in vivo* investigation of rubratoxin B (**7**)[34] biosynthesis in *P. dangeardii* Pitt, P450 RbtI was found to be the key enzyme involved in the δ-lactone ring formation. However, no product could be detected when using a yeast microsomal fraction containing RbtI to react with the isolated substrate analogs *in vitro*.[35] Thus, the authors attributed this negative result to the timing of the P450 reaction, and proposed that RbtI might catalyse an online hydroxylation of the PKS attached polyketide chain **8** (Scheme 3).

In another example, P450 EpnC involved in the biosynthesis of epipyrone-A (**9**)[36] was also proposed to perform online consecutive oxidations of the proposed substrate **10**, because no intermediate was observed in the *epnC*-deletion strain of *Epicoccum nigrum* when the production of **9** was abolished (Scheme 4).[37]

3.1.2 Uncommon P450 reactions in PK biosynthesis. Compared to common P450 reactions, uncommon P450 reactions are more attractive due to their intriguing catalytic mechanisms. Dimers of aromatic molecules stand for a large family of fungal-derived PKs that are usually biosynthesized *via* radical coupling reactions. Notably, the selective aromatic dimerization



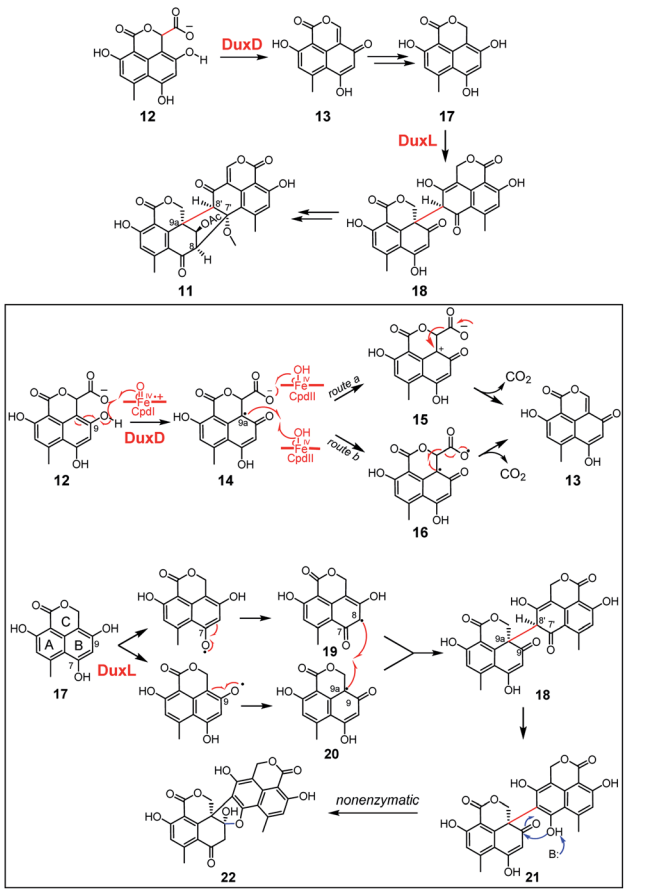
Scheme 3 The RbtI mediated online hydroxylation during the biosynthesis of rubratoxin B (**7**).



Scheme 4 The putative online successive oxidation reactions catalysed by EpnC.

is notoriously difficult to be accomplished by chemical synthesis owing to the challenges in simultaneously controlling of the regio- and stereoselectivity.[38–40] However, such radical coupling reactions can be readily achieved by some fungal P450s.

Duclauxins, such as duclauxin (**11**, Scheme 5), are a class of fungal NPs bearing an impressive [6-6-6-5-6-6-6] heptacyclic system.[41,42] Genome mining of *Talaromyces stipitatus* ATCC 10500 revealed the biosynthetic gene cluster (BGC) of **11**.[43] Through *in vivo* gene inactivation and heterologous gene expression in *A. nidulans* A1145 ΔSTΔEM, P450 DuxD was characterized to catalyse the oxidative decarboxylation of **12** to form **13**. Mechanistically (Scheme 5), it was proposed that CpdI of DuxD first abstracts a hydrogen atom from the C9 phenolic hydroxyl group in **12** to generate an oxygen radical that can be resonance delocalized to C9a forming **14**. The resulted CpdII further abstracts a single electron from C9a in **14** to form a carbocation intermediate **15**, which could trigger the cleavage of the carboxyl group to yield **13** (route a). Alternatively, CpdII abstracts a single electron from the carboxylate in **14** to form a diradical intermediate **16**, which could also undergo the decarboxylation reaction *via* electron rearrangement (route b).[43] Interestingly, these mechanisms are highly analogous to those of bacterial P450 fatty acid decarboxylases that use H_2O_2 as



Scheme 5 DuxD-catalysed decarboxylation and DuxL-mediated dimerization and the proposed catalytic mechanisms (in box).

cofactor, and stand for the classical mechanisms of P450-mediated C–C bond cleavage reactions.[44,45]

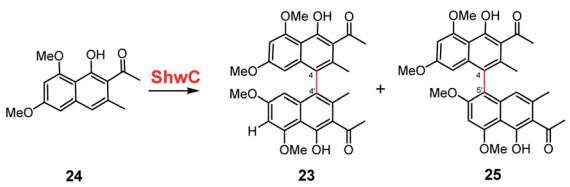
The enzyme responsible for the final dimerization was identified to be another P450 DuxL, which converts the monomer **17** into the dimer **18** (Scheme 5).[43] The mechanism for dimerization was proposed to be initiated by abstraction of two hydrogen atoms from the C7 and C9 phenolic OH groups in **17**, respectively, and followed by radical delocalization to yield two radicals **19** and **20**. Then, intermolecular radical coupling occurs at the active site of DuxL to form the C9a–C8′ bond in **18**. A further nonenzymatically nucleophilic addition of the tautomerization-derived intermediate **21** leads to the formation of the shunt product **22** (Scheme 5). This process was identified based on microsome-based enzymatic assays. However, the second C8–C7′ bond in **11** was not formed in the *in vitro* reconstituted reaction of DuxL, and it was proposed to be catalysed by another unknown enzyme *in vivo*.[43]

Rufoschweinitzin (**23**), isolated from the basidiomycete *Cortinarius rufoolivaceus*, is a symmetrically 4,4′-coupled dimer of torachrysone-8-O-methyl ether (**24**).[46] To explore the enzyme responsible for the key 4,4′-coupling step in the biosynthesis of **23**, the activity of P450 ShwC that is derived from the BGC of schweinitzin (a regioisomer of **23**) in the fungus *Xylaria schweinitzii* was investigated. It was revealed that, in a whole-cell approach using the *shwC*-expressed *A. aculeatus*, the proposed monomer **24** could be transformed into two dimers, **23** and its regioisomer alloschweinitzin (**25**) (Scheme 6). Thus, a ShwC-analogous P450 in *C. rufoolivaceus* may be responsible for the production of **23**.[46]

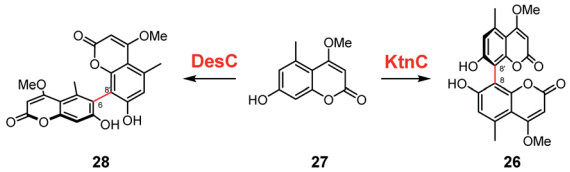
P-Orlandin (**26**) is another biaryl PK produced by *A. niger*.[47] KtnC, a P450 from the BGC of **26**, was disclosed to possess the capability of mediating a regio- and stereoselective aryl–aryl coupling reaction by gene knockout in *A. niger* and heterologous expression in *S. cerevisiae*.[48] Upon feeding 7-demethylsiderin (**27**) to the yeast fermentation culture, the recombinant *S. cerevisiae* cells were able to catalyse the C8–C8′ coupling of **27** to afford **26** of P-axial chirality (Scheme 7). Furthermore, using the sequence of KtnC as a probe, a homologous P450-encoding gene *desC* was mined from the M-desertorin A (**28**, the 6,8′-regioisomer of **26**) producing strain *Emericella desertorum*. As expected, DesC was shown to mediate a different regio- (C6–C8′) and stereoselective coupling of **27** to yield **28** of M-axial chirality (Scheme 7). Thus, the regio- and stereoselectivity of the dimerization of **27** is strictly controlled by the two P450s.[48]

Xanthone dimers are widespread in the kingdom of fungi and exhibit impressive antibacterial activities against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA).[49,50] Neosartorin (**29**) is such a xanthone dimer bearing a characteristic skeleton that consists of two different monomeric units. Gene-deletion results suggested that the challenging hetero-dimerization during the biosynthesis of **29** could be mediated by the P450 NsrP in *A. novofumigatus*.[51] The isolation of two monomers, **30** and **31**, from the *nsrP*-deleted strain, together with the discovery of two other dimers, **32** and **33**, from the *nsrL* (encoding an acetyltransferase that catalyses the subsequent step) knockout mutant, suggested that NsrP could dimerize at least three different xanthone monomers (**30**, **31** and **34**) to generate the corresponding heterodimerized or homodimerized products (**29**, **32** and **33**; Scheme 8). The regio- and stereoselectivity of the NsrP-mediated dimerization appears to be influenced by the structure of the monomer.[51]

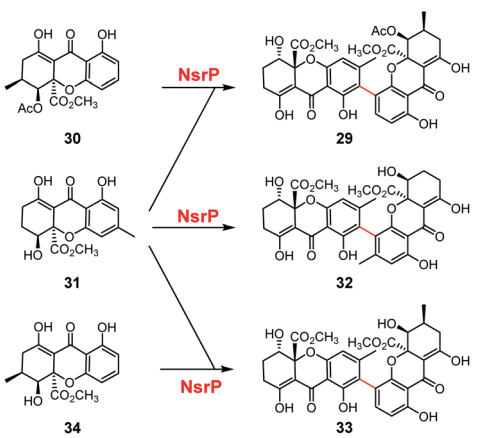
Besides C–C coupling, C–O bond formation is another important type of aromatic coupling reaction in fungal NP biosynthesis. Griseofulvin (**35**) is an ascomycete-derived PK that draws much attention due to its attractive spiro-cyclic structure and broad bioactivities.[52] In view of its biogenesis, the assembly of the spirocyclic scaffold in **35** is particularly intriguing. A P450 named GsfF from *P. aethiopicum* was revealed to catalyse the unusual spirocyclization.[53,54] As reported, the production of **35** was abolished in the *gsfF* knockout strain; meanwhile the *seco*-analog griseophenone B (**36**) was accumulated. By feeding compound **36** to the *gsfF*-expressed *S. cerevisiae* strain, which harbors the universal *A. terreus* derived CPR (*At*CPR), the spirocyclic scaffold bearing product desmethyl-dehydro grisefulvin A (**37**) was formed (Scheme 9). The classical diradical and epoxidation mechanisms were initially proposed to rationalize



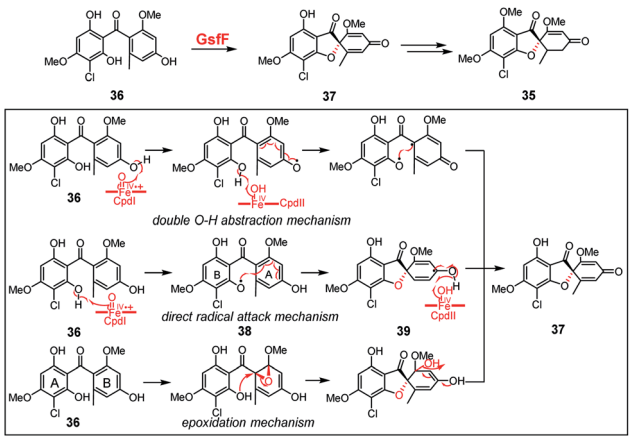
Scheme 6 The ShwC mediated aromatic dimerization reaction.



Scheme 7 Different regioselective dimerizations of the same substrate **27** by the two homologous P450s KtnC and DesC.



Scheme 8 NsrP-catalysed dimerizations of different monomers.



Scheme 9 The GsfF catalysed spiro-ring formation and the proposed mechanism (in box) during the biosynthesis of griseofulvin (**35**).

the spirocyclic formation (Scheme 9). In the following study, a new direct radical attack mechanism was proposed based on computational calculations of the reaction-energy barriers. Briefly, after hydrogen abstraction, the oxygen radical of **38** could directly attack the neighboring arene to form the spirocycle in **39**, which further undergoes another hydrogen abstraction to quench the new-born radical and yields **37** (Scheme 9).[53,54]

3.2 Non-ribosomal peptide (NRP) biosynthetic P450s

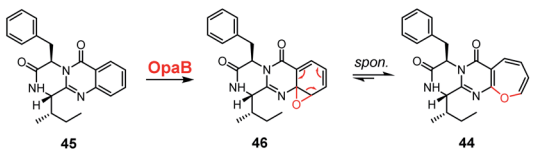
3.2.1 Common P450 reactions in NRP biosynthesis. NRPs are a class of amino acid derived NPs that are assembled by NRPSs shaping in linear or cyclic forms. Aspirochlorine (**40**) is an antifungal cyclopeptide with a diketopiperazine core and has been isolated from various *Aspergillus* species.[55] The study on the biosynthesis of **40** in *A. oryzae* revealed that two P450s, AclL and AclO, sequentially catalyse the C3-hydroxylation of **41** and N1-hydroxylation of **42** during the formation of the intermediate **43**. In the subsequent biocatalytic cascade, the N1-OH is further converted into an *N*-methoxyl group, setting the stage for the following C–C bond cleavage to remove the phenylalanine moiety (Scheme 10).[56]

3.2.2 Uncommon P450 reactions in NRP biosynthesis. Oxepinamides are fungal-derived, anthranilyl-containing tripeptides featuring a characteristic oxepin ring.[57,58] In the biosynthetic pathway of oxepinamide F (**44**) from *A. ustus* 3.3904, P450 OpaB was found to be responsible for oxepin-ring formation by mediating an oxidative benzene-ring expansion towards the substrate **45**.[59] Mechanistically, an epoxide intermediate **46** was proposed to be the direct product of OpaB. Then the unstable **46** could undergo spontaneous equilibrium with the more stable oxepin ring *via* electron rearrangement, giving rise to **44** (Scheme 11). This is the first example that a P450 converts a quinazolinone into a pyrimidinone-fused 1*H*-oxepin framework in fungal NP biosynthesis.

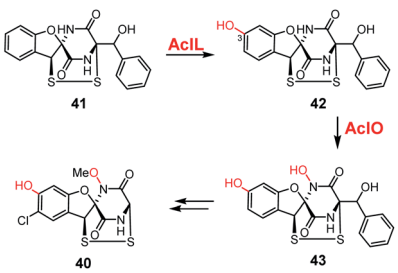
Herquline A (**47**) is a fungal alkaloid with an intriguing pentacyclic ring system [6-9-6-5-6] and shows inhibitory activity against influenza virus.[60] The biosynthetic enzymes of **47** were mined from *P. herquei*.[61] Among them, P450 HqlC was characterized to catalyse the intramolecular C3–C3′ coupling reaction of the substrate diphenyl piperazine **48** *via* the unstable intermediate **49**, as evidenced by isolation of the shunt product **50** from the HqlC-expressed *A. nidulans* recombinant strain (Scheme 12). This C–C coupling could be rationalized by a diradical mechanism as the P450-mediated aromatic coupling reactions during PK biosynthesis (Schemes 5 and 8).[43] Alternatively, the authors also proposed a single radical addition mechanism (Scheme 12), which is similar to the direct radical attack mechanism of GsfF (Scheme 9).[53,54,61]

3.3 Polyketide-nonribosomal peptide hybrid (PK-NRP) biosynthetic P450s

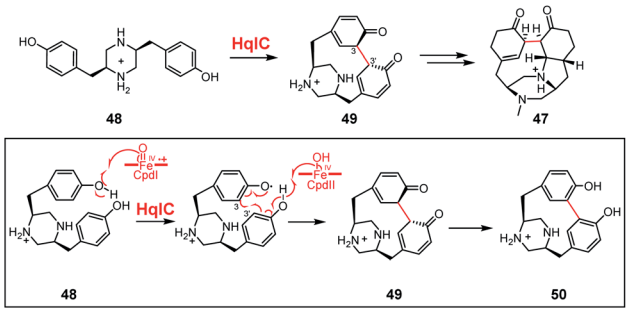
3.3.1 Common P450 reactions in PK-NRP biosynthesis. In addition to the pure PK and NRPS BGCs, in some cases, PK and NRPS genes are co-localized in a same BGC to cooperatively produce PK-NRP hybrid products, which contain both the PK-



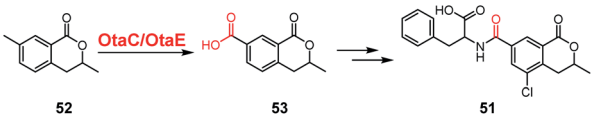
Scheme 11 The OpaB catalysed ring expansion in the biosynthesis of oxepinamide F (**44**).



Scheme 10 The hydroxylations co-mediated by P450s AclL and AclO set stage for the following C–C bond cleavage in the biosynthesis of aspirochlorine (**40**).



Scheme 12 The HqlC-catalysed C–C bond formation during the biosynthesis of herquline A (**47**) and the proposed single radical addition mechanism (in box).

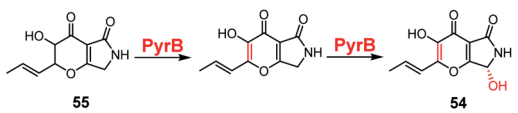


Scheme 13 The OtaC/OtaE catalysed successive oxidation during the biosynthesis of ochratoxin (**51**).

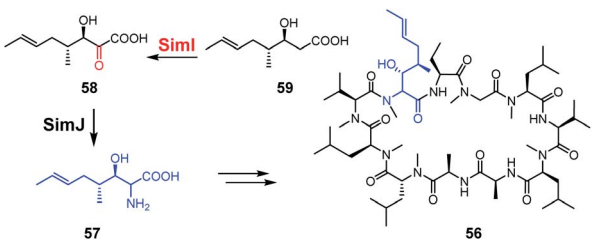
derived carbon skeleton and the NRP-derived peptide moiety. Ochratoxin A (**51**) is a structurally simple PK-NRP hybrid mycotoxin produced by *Aspergillus* and *Penicillium* species that is often found in moldy food and feed.[62] The BGC of **51** was identified from *A. ochraceus* fc-1 by comparative genomic analysis using several other sequenced producing strains.[63] Gene functional analysis revealed that P450 OtaC could transform the methyl group of 7-methylmellein (**52**) into a carboxyl group in **53** for further amidation in the later biosynthetic steps (Scheme 13).

Pyranonigrin A (**54**) is another PK-NRP hybrid produced by *A. niger*, which bears a pyrano[2,3-*c*]pyrrole bicyclic skeleton. Genome mining of the plant endophyte *P. thymicola* revealed a cryptic BGC, which was identified to be able to produce **54** by heterologous expression in *A. nidulans* A1145.[64] P450 PyrB was proposed to sequentially catalyse a dehydrogenation and a hydroxylation reaction towards **55** during the formation of **54** (Scheme 14).

Cyclosporin A (**56**), which was firstly isolated from the insect-pathogenic fungus *Tolypocladium inflatum* in 1973, has been used as an immunosuppressant for many years.[65–67] However, the biosynthetic details of **56** remain largely unknown until a recent study disclosing its BGC in the producing strain.[68] The cycloundecapeptide is assembled by a large NRPS (SimA) using ten proteinic amino acids and a non-proteinic amino acid (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine (**57**) as building blocks. Through individual gene deletions, PKS SimG, P450 SimI and transaminase SimJ were found to be responsible for the biogenesis of **57**. Based on gene-inactivation experiments, SimI



Scheme 14 The bifunctional PyrB mediated dehydrogenation and hydroxylation in the biosynthetic pathway of pyranonigrin A (**54**).



Scheme 15 The function of P450 SimI during the biosynthesis of the immunosuppressant cyclosporin A (**56**).

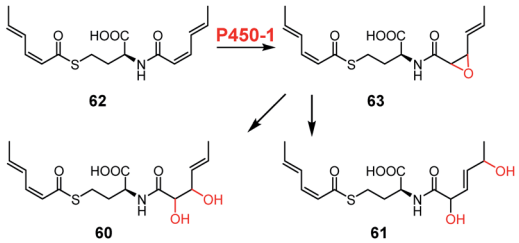
was believed to catalyse the formation of the α-keto group in **58** from the substrate **59**, thereby providing the reactive site for the following transamination reaction catalyzed by SimJ (Scheme 15).

Besides ascomycete fungi, the higher basidiomycete fungi (mushrooms) represent another important source of fungal NPs.[69–71] However, the difficulty in genetic manipulation of mushrooms and its slow growth-rate have limited the studies on their NP biosynthesis in native hosts. Thus, genomic approaches together with heterologous gene expression have become useful tools for exploring the cryptic NPs and their biogenetic mechanisms in these higher fungi.[69]

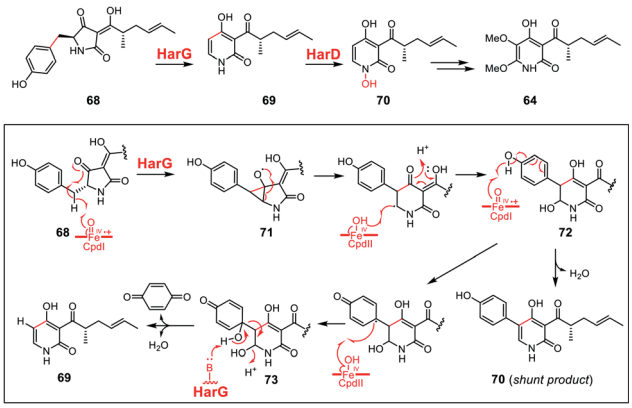
Genome mining of the basidiomycete fungal species *Punctularia strigosozonata* revealed a BGC containing a highly reducing PKS-NRPS (HR-PKS-NRPS) and three P450s (P450-1/2/3).[72] Co-expression of the HR-PKS-NRPS with P450-1 in an engineered *S. cerevisiae* strain (with its growth rate and expression level for heterologous proteins being improved and with integration of the expression cassettes for NpgA, a holo-acyl carrier protein (ACP) synthase from *A. nidulans* and the CPR from *A. terreus*)[73] led to the production of the PKS-NRPS hybrids **60** and **61**. P450-1 was proposed as an epoxidase to catalyse the epoxidation of the *cis* olefin in **62** (the product of HR-PKS-NRPS) to yield the unstable intermediate **63**, which could undergo ring-opening and rearrangement to give **60** and **61** (Scheme 16).[72]

3.3.2 Uncommon P450 reactions in PK-NRP biosynthesis. Pyridone-derived compounds are a class of fungal NPs possessing diverse bioactivities. For example, the *Trichoderma*-derived harzianopyridone (**64**, Scheme 17) is a nanomolar inhibitor of mammalian succinate ubiquinone oxidoreductase; while the *Penicillium*-derived citridones (**65–67**, Scheme 18) are capable of inhibiting the production of a key virulence factor staphyloxanthin in MRSA.[74–76] Tang and co-workers recently deciphered the biosynthetic details of **64** in *T. harzianum* and **65–67** in *Penicillium* FKI-1938.[77,78]

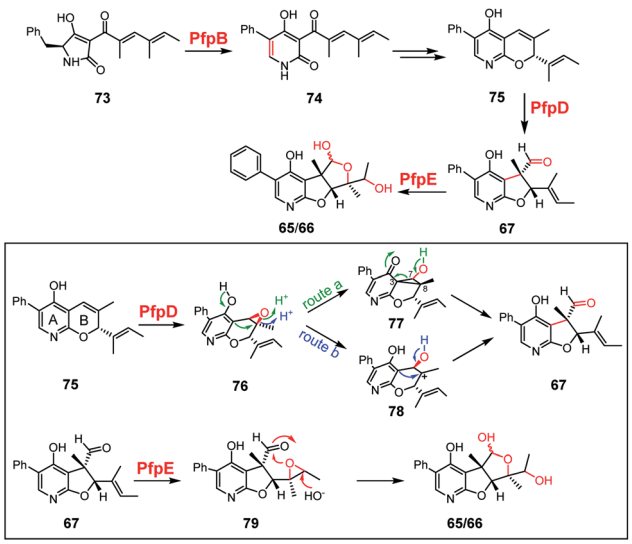
In the biosynthetic pathway of **64**, P450 HarG was identified to be a bifunctional enzyme that catalyses the successive ring expansion and phenyl-cleavage steps from **68** to **69** (Scheme 17). The second P450 HarD was characterized to catalyse an *N*-hydroxylation of **69** to form **70**.[77] Specifically, the *harG*-expressed *A. nidulans* could convert **68** into **69**. In addition, a shunt but mechanism-inspiring product **70** was observed (Scheme 17). Mechanistically, the ring expansion likely initiates from abstraction of a hydrogen atom from the benzyl CH_2 group in



Scheme 16 The function of P450-1 derived from the mined HR-PKS-NRPS BGC from the basidiomycete *P. strigosozonata*.



Scheme 17 The HarG/HarD catalysed C–C bond cleavage and N-hydroxylation reactions and the proposed mechanism of HarG (in box).



Scheme 18 Functions of the three P450s in the biosynthetic pathway of citridones (**65–67**) and the proposed mechanisms for PfpD and PfpE (in box).

68, and the resulted radical in **71** triggers electron rearrangement to yield the ring-expanded product **72** after [HO•] rebound from CpdII. If CpdI could be re-generated prior to the dehydration of **72**, which would otherwise generate the shunt product **70**, HarG would further cleave the quinone moiety to produce **69**. In this route, an unstable hydroxylated intermediate **73** was proposed to be formed after H-abstraction, radical resonance delocalization and [HO•] rebound steps. Finally, electron rearrangement results in C–C bond cleavage to yield **69** (Scheme 17).[77]

Produced by *Penicillium* sp. FKI-1938, citridones (**65–67**) are a group of potentiators of miconazole against *Candida albicans*. The BGC of these PK-NRP hybrids encodes three P450s including PfpB, PfpD and PfpE. These P450s play unusual roles in assembling the structures of **65–67**.[78] Specifically, PfpB mediates a ring-expansion of **73** to generate **74**; PfpD catalyses a ring-contraction of the pyrano[2,3-*b*]pyridine bearing substrate **75** to the furo[2,3-*b*]pyridine containing product **67**; and PfpE mediates a ring-formation reaction by converting **67** into **65/66** (Scheme 18). The catalytic mechanism of PfpB seems analogous to that of HarG (Scheme 17).[77] As for the transformation from **75** to **67**, an unstable epoxidation product **76** was proposed as the key intermediate which could induce a C–C bond migration *via* two possible routes. In route a, electron rearrangement in ring A opens the oxirane ring *via* an S_{N2}-like mechanism to generate a cyclopropyl alcohol intermediate **77** with inversion of the C8 configuration, and a second round of electron rearrangement starting from the new-born OH group cleaves the C3–C7 bond to form **67**. In route b, the unstable oxirane ring may directly open with the aid of a proton to yield a carbocation intermediate **78**, which could trigger a [1,2]-sigmatropic shift of the C3–C7 bond to yield the quaternary carbon and accomplish the formation of **67**. Furthermore, it was proposed that the PfpE-mediated ring D formation might occur *via* a stereospecific intermediate **79** by two steps of nucleophilic attacking with the aid of an exogenous OH^- (Scheme 18).[78]

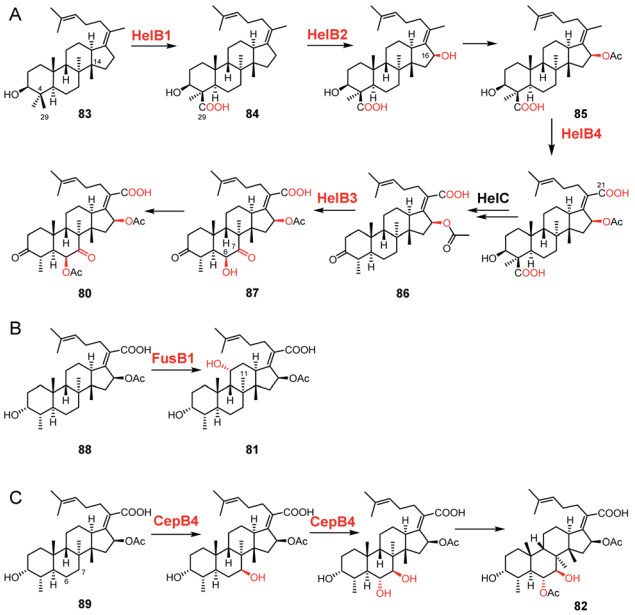
3.4 Terpenoid biosynthetic P450s

3.4.1 Common P450 reactions in terpenoid biosynthesis. Fungal terpenoids are a huge family of NPs that are originally derived from C_5 isoprene units. According to the number of incorporated prenyl motifs, terpenoids are divided into hemiterpenoids (C_5), monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}), tetraterpenoids (C_{40}), and other unusual variants.[79,80]

Fusidane-type antibiotics are a family of fungal-derived triterpenoids that share a steroid-like tetracyclic skeleton, and show potent anti-Gram-positive bacterial activities. The representative family members include helvolic acid (**80**), fusidic acid (**81**) and cephalosporin P1 (**82**), all of which have the same tetracyclic skeleton but differ at the degree of oxidation (Scheme 19).[81–83] Recently, using *A. oryzae* NSAR1 as a heterologous host, the biosynthetic pathways of **80** in *Acremonium fumigatus* Af293, of **81** in *A. fusidioides* ATCC 14700, and of **82** in *A. chrysogenum* ATCC 11550/*A. chrysogenum* CGMCC 3.3795 were elucidated one after another.[82–84]

During the biosynthesis of **80**, four P450s, namely HelB1-4 together accomplish the post-tailoring oxidative transformations (Scheme 19A).[84] HelB1 and a short-chain dehydrogenase/reductase (HelC) co-mediate an unusual 4β-demethylation of **83** to cleave Me-29. This progress is distinct from the 4α-demethylation during sterol biosynthesis. In such an unusual reaction, HelB1 first activates the Me-29 by oxidizing it to a carboxyl group in **84**.[84] HelB2 and HelB4 were found to catalyse the C16 hydroxylation and Me-21 successive oxidation towards **84** and **85**, respectively. HelB3 was identified to be a bifunctional enzyme catalysing both C6 hydroxylation and C7 successive oxidation to convert **86** into **87**.

As for **81**, the clinically used fusidane-type triterpenoid, P450 FusB1 was revealed to install the OH-11 group upon **88** (Scheme 19B).[83] In the biosynthetic pathway of **82**, CepB4 was



Scheme 19 The oxidative reactions catalysed by different P450s involved in the biosynthesis of helvolic acid (**80**; A), fusidic acid (**81**; B) and cephalosporin P1 (**82**; C).

characterized as a bifunctional P450 that sequentially hydroxylates C7 and C6 of **89** (Scheme 19C).[85]

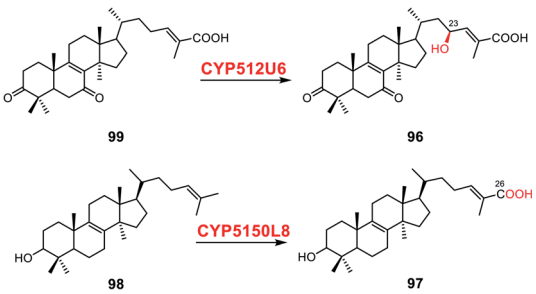
Demethoxyviridin (**90**) is a steroid-like bioactive NP having an extra furan ring fused with ring A and ring B.[86,87] Among the total 15 biosynthetic genes in the BGC of **90** from *Nodulisporium* sp. (no. 65-17-2-1), six of them encode P450s including VidA, VidD, VidE, VidG, VidK and VidR.[85] Functionally, VidE, VidG and VidR participate in the C20–C22 bond cleavage and the unusual furan ring formation during the conversion from **91** to **92**. However, the detailed functions for the three P450s remain unclear since no stable intermediates could be isolated from the gene-deletion mutants. VidD and VidA are responsible for aromatization of ring C in **93** likely by desaturating the C11–C12 bond in **94** and oxidative removal of the C13 methyl group in **95**, sequentially. Next, VidK catalyses the final C1 β-hydroxylation to form **90** (Scheme 20).[85]

Ganoderma lucidum (with the Chinese name of Ling Zhi) is a traditional medicinal mushroom in China and other East Asian regions. The triterpenoid ganoderic acids (GAs), such as hainanic acid A (**96**) and ganoderic acid Z (**97**), have been recognized as the main bioactive ingredients.[88] To mine the possible P450 enzymes in the biosynthesis of GAs from *G. lucidum*, 82 selected P450s and the native CPR were co-expressed in *S. cerevisiae* YL-T3. As a result, the P450 CYP05150L8 was found to mediate the successive oxidations of **98** to yield **97** (Scheme 21).[89] Additionally, in another study, the C23 hydroxylation of **96** was determined to be catalysed by P450 CYP512U6 towards **99**.[90]

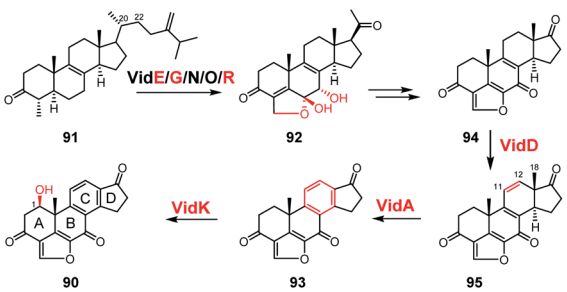
Fumihopaside A (**100**) is a hopane-type triterpenoid glycoside isolated from *A. fumigatus*.[91] In the biosynthetic pathway of **100**, a single P450 AfumB was found to convert **101** into **102** by catalysing the C24 hydroxylation and C30 successive oxidation reactions. Importantly, the formation of OH-24 provides a reactive site for the following glycosylation (Scheme 22).[91]

The polychrome filamentous fungus *Emericella variecolor* (the perfect state of *A. variecolor*) produces various terpenoids.[92,93] Genome analysis of this strain revealed a novel sesterterpene synthase and a P450 StI-P450. A new tricyclic sesterterpenoid stellatic acid (**103**) was produced upon co-expression of the two genes in *A. oryzae* NSAR1. Thus, StI-P450 was proposed to catalyse the Me-20 successive oxidation of **104**, giving rise to **103** (Scheme 23).[94] Similarly, a small gene cluster encoding a terpene synthase and a P450 (NfP450) was mined from the genome of *Neosartorya fischeri*.[95] Expression of this gene cluster resulted in production of the sesterterpene sesterfisheric acid (**105**), during which NfP450 could catalyse the Me-15 successive oxidation of **106** (Scheme 23).

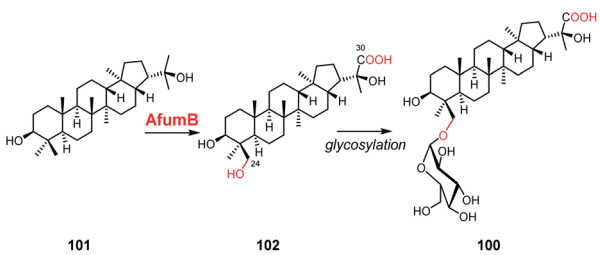
The carboxylated quiannulatene (**107**) is an impressive sesterterpenoid with a [5-6-5-5-5] fused ring system, which was



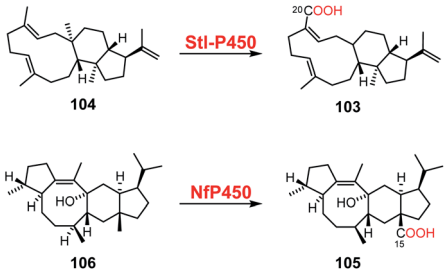
Scheme 21 Two P450s involved in the biosynthesis of ganoderic acids from *Ganoderma lucidum*.



Scheme 20 The P450 enzymes (in red) and their functions during the biosynthesis of demethoxyviridin (**90**).



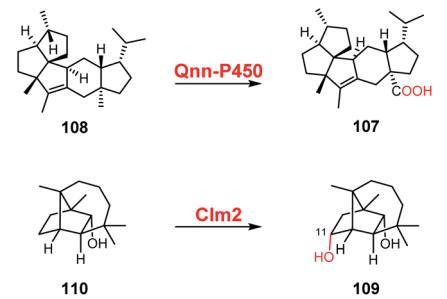
Scheme 22 The AfumB catalysed hydroxylation and successive oxidation in the biosynthesis of fumihopaside A (**100**).



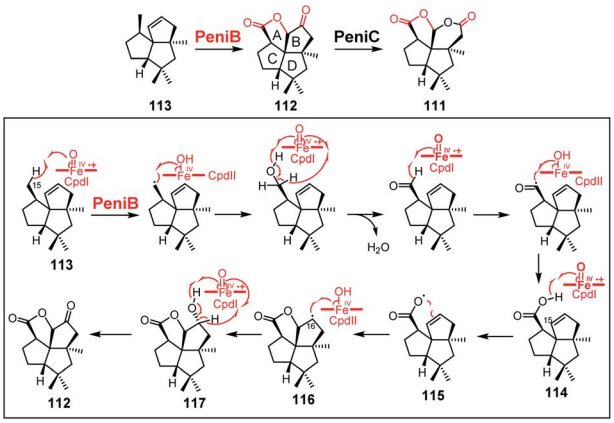
Scheme 23 Two P450s capable of successive oxidation identified from the biosynthetic pathways of stellatic acid (**103**) and sesterfisheric acid (**105**).

obtained by co-expression of an *E. variecolor* NBRC 32302 derived terpene cyclase EvQS and the adjacent P450 Qnn-P450 in *A. oryzae*.[96] Step-wise expression of the two genes revealed Qnn-P450 to be responsible for converting **108** to **107** (Scheme 24).[96] Culmorin (**109**) is a widely distributed sesquiterpene diol in *Fusarium* species, such as *F. graminearum*.[97,98] During the biosynthesis of **109**, P450 Clm2 was found to be responsible for installing the C11 hydroxy group onto the substrate **110** in a regio- and stereoselective manner (Scheme 24).[99]

3.4.2 Uncommon P450 reactions in terpenoid biosynthesis. Fenestranes are a specific class of fungal NPs bearing a fused [5-5-5-6] ring system with a shared central quaternary carbon.[100] Penifulvin A (**111**) is a representative fenestrane-type compound produced by *P. griseofulvum*.[101] The biogenesis of this intriguing skeleton was recently deciphered. It was found that a P450 PeniB and a flavin-dependent monooxygenase PeniC cooperatively accomplish the construction of the tetracyclic ring originated from a tricyclic carbon skeleton (Scheme 25).[102] PeniB could alone mediate the formation of ring A in **112** from **113**. Mechanistically, PeniB first catalyses the successive oxidation of Me-15 in **113** to yield **114**. Then CpdI of PeniB further abstracts a hydrogen from the new-born carboxyl group in **114** to generate a carboxyl radical **115**, which undergoes a ring closure reaction to form ring A *via* a radical addition process, giving rise to the intermediate **116**. After [HO•] rebound from CpdII to the C16 radical (to yield **117**) and a further oxidation at C16, the end product **112** is finally formed (Scheme 25).[102]

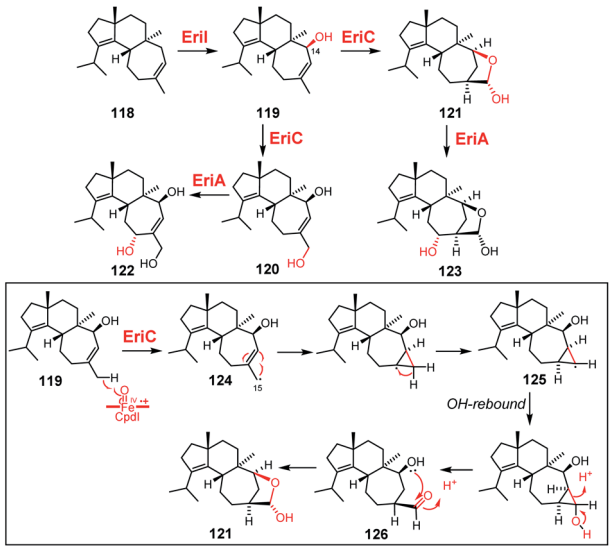


Scheme 24 The functions of Qnn-P450 and Clm2 involved in the biosynthesis of quiannulatene (**107**) and culmorin (**109**), respectively.



Scheme 25 The P450 PeniB mediated formation of the [5-5-5-6] ring system during the biosynthesis of penifulvin A (**111**) and the proposed catalytic mechanism (in box).

Basidiomycota fungi are an important resource of terpenoids, such as coriolin, melleolide and pleuromutilin.[103–105] In a recent study, the methodology of genome editing-based knock-in of a whole mushroom-derived BGC into the filamentous fungi *A. oryzae* was successfully developed by using the erinacine BGC from *Hericium erinaceus* as a practical example.[106] In the newly elucidated erinacine biosynthetic pathway, three P450s including EriA, EriC and EriI were found to be involved in the tailoring of the erinacine skeleton (**118–122**). Functionally, after the diterpene synthase EriG prepares the carbon skeleton **118**, EriI first installs OH-14 in **119**, then EriC takes it over and produces a C15 hydroxylated product **120** and an unexpected product **121**. EriA catalyses C11 hydroxylation of **120** and **121** to yield **122** and **123**, respectively (Scheme 26). With respect to the possible mechanism for the formation of **121**, briefly, the formed C15 radical in **124** reacts with the C12=C13 double



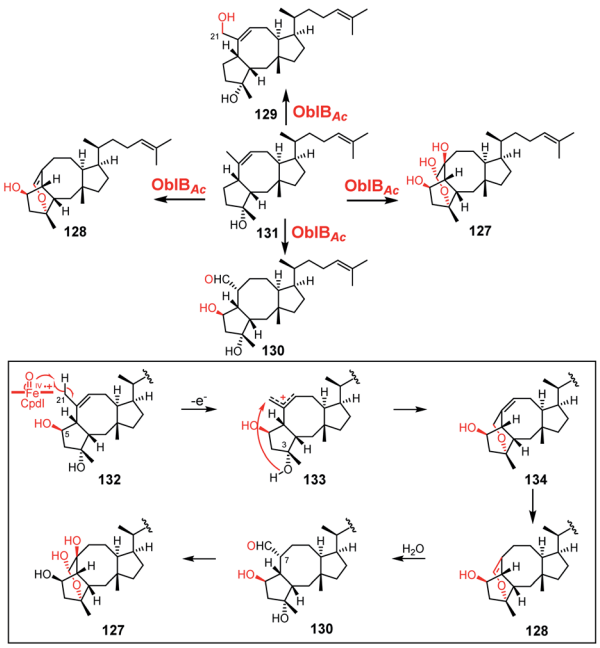
Scheme 26 The P450 catalysed reactions in the erinacine biosynthetic pathway and the proposed catalytic mechanism of EriC (in box).

bond to generate a cyclopropane ring with a rearranged radical being formed in **125**. Then [HO•] rebound from CpdII cleaves the cyclopropane ring to give a reactive C15 aldehyde group in **126**, which could induce an intramolecular nucleophilic attack from OH-14 to yield **121** (Scheme 26).[106]

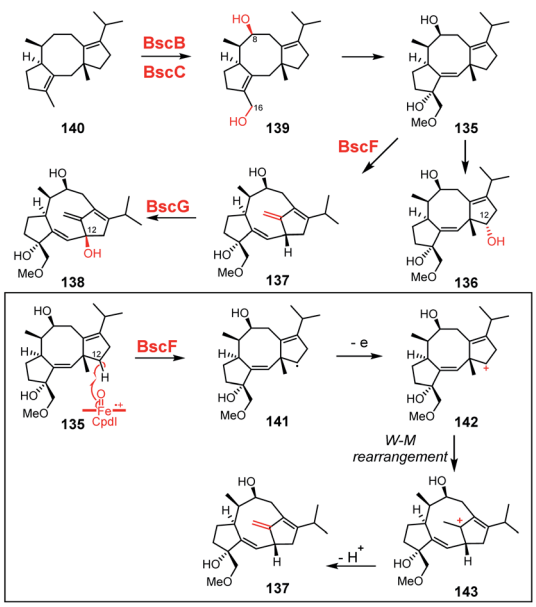
The sesterterpene ophiobolins are a class of phytotoxins isolated from *Bipolaris oryzae* (*Ophiobolus miyabeanus*).[107,108] A putative BGC of ophiobolins (*obl*) was mined from the genome of *A. clavatus*, although ophiobolins had not been isolated from this strain before.[109] When the only P450 $OblB_{AC}$ was co-expressed with the terpene synthase $OblA_{AC}$ in *A. oryzae* NSAR1, several new metabolites (**127–130**) were produced, including two unexpected products **127** and **128** bearing an additional ether ring (Scheme 27). The possible mechanism of the unexpected reaction catalysed by $OblB_{Ac}$ was proposed as shown in Scheme 27: $OblB_{Ac}$ first hydroxylates the substrate **131** at C5 to give **132**; then sequentially abstracts a hydrogen atom and an electron from C21 in **132** to yield a reactive carbocation intermediate **133**, which could induce a nucleophilic attack from OH-3 to give the unstable cyclic ether in **134**. The following 1,3-migration of the double bound generates a more stable enol ether in **128**, which could be further hydrolyzed to form the aldehyde **130**. Furthermore, $OblB_{Ac}$ could mediate the C7 hydroxylation of **130** to yield **127** in a hemiacetal form.[109]

Brassicicenes are a class of diterpenes originally isolated from the phytopathogenic fungus *Alternaria brassicicola*. These metabolites bear a fused [5-8-5] carbocyclic ring system (**135** and **136**) or a different tricyclo[9.2.1.0]tetradecane core containing a bridgehead double bond (**137** and **138**).[110,111] Genome mining and heterologous expression of a *Pseudocercospora fijiensis* derived BGC uncovered the biosynthetic pathway of brassicicenes, in which four P450s (BscB, BscC, BscF and BscG) are involved. Notably, BscF was identified as an unusual P450 that could transform the [5-8-5] skeleton of **135** into the tricyclo [9.2.1.0]tetradecane core in **137**, with the C12 hydroxylated product **136** being co-produced. Besides, BscB and BscC are the two hydroxylases to respectively install OH-8 and OH-16 in **139** from **140**, while BscG hydroxylates C12 in **137** to give **138** (Scheme 28).[112] With regard to the mechanism for the unusual skeleton transformation from **135** to **137**, the observation of **136** suggested that BscF might abstract H12 in **135** to initiate the reaction. The resulted radical intermediate **141** could undergo a further electron-abstraction to form the cationic intermediate **142**. Next, Wagner-Meerwein rearrangement may occur to give a new carbocation intermediate **143**, which could lose a proton to produce **137**.

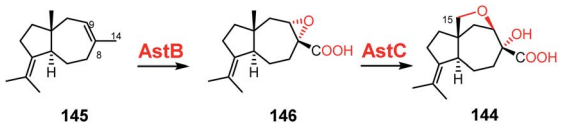
For many toxic NP producing strains, it is a common self-resistance strategy by harboring an additional copy of the functional target of the toxic product.[113,114] Interestingly, this kind of self-resistance genes have been used for orienting the mining of bioactive NPs, which could be further guided by the resistance-gene types. In a recent study, through searching for the self-resistance gene of a dihydroxyacid dehydratase, which is essential for plant growth, several BGCs containing a well-conserved set of four genes (containing the resistance gene and two P450 genes *astB* and *astC*) were identified from multiple fungal genomes, including *A. terreus*.[115] By expressing all the four genes in *S. cerevisiae* RC01, the compound aspterric acid (**144**), a previously reported inhibitor of pollen development in the plant *Arabidopsis thaliana*, was produced. Stepwise expression of the three biosynthetic genes revealed the functions of the two P450s. Specifically, AstB catalyses the C8=C9 epoxidation and Me-14 successive oxidation of **145** to yield **146**, and AstC reshapes the structure of **146** by forming a new oxo-



Scheme 27 The non-specific reactions catalysed by $OblB_{Ac}$ and the proposed catalytic mechanism (in box).



Scheme 28 The P450-catalysed reactions involved in the biosynthetic pathway of brassicicenes (**135** and **136**) and the proposed catalytic mechanism for the BscF-mediated skeleton transformation (in box).



Scheme 29 The AstB-catalysed epoxidation/successive oxidation and the AstC-mediated structure-reshaping in the biosynthesis of aspterric acid (**144**).

bridged ring in **144** (Scheme 29). In the latter process, the C15 hydroxylation product was proposed to be an intermediate to trigger the intramolecular epoxide opening to generate **144**. As predicted, **144** was validated to be an herbicide with a new mode of action.[115]

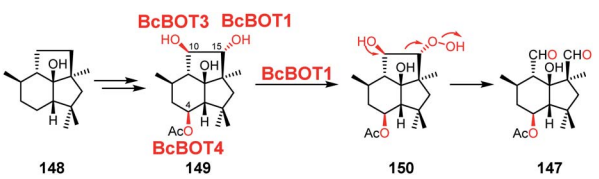
Phytopathogenic fungi could cause great damages and economic loss in agricultures. Some phytopathogenic fungi often produce phytotoxins to kill their plant hosts, such as botrydial (**147**), a heavily oxidized sesquiterpenoid produced by *Botrytis cinerea*.[116,117] Among the five genes in the BGC of **147**, three of them encode P450s (BcBOT1, BcBOT3 and BcBOT4). By gene inactivation and corresponding metabolites identification, BcBOT1, BcBOT3 and BcBOT4 were shown to catalyse the regio- and stereoselective hydroxylations at C15, C10 and C4 in **148**, respectively, to give **149**. Particularly, BcBOT1 could further mediate the bond cleavage of C10–C15 in **149** to yield **147** *via* a C15 hydroperoxide intermediate **150** (Scheme 30).[118,119]

It could be learned from the above examples that, during biosynthesis of fungal terpenoids, a single P450 catalysing multi-site and/or successive oxidation reactions often occurs. This presents a simple but effective strategy for P450-mediated biosynthetic cascade to expand the structural diversity of fungal NPs.

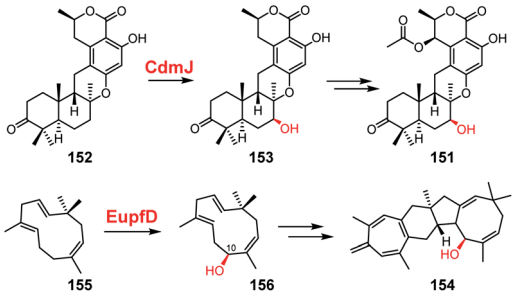
3.5 Meroterpenoid biosynthetic P450s

3.5.1 Common P450 reactions in meroterpenoid biosynthesis. Fungal meroterpenoids are a superfamily of NPs, whose skeletons are partially originated from the terpenoid biosynthetic systems. Regardless the non-terpenoid structural moiety, these special terpenoids usually contain different numbers of the C_5 isoprene units.

Chrodrimanin B (**151**) is a polycyclic meroterpenoid, whose skeleton is produced from the cooperation of a PKS and a pair of prenyltransferase/terpene cyclase in the fungus *P. verruculosum* TPU1311.[120] Elucidation of **151** biosynthetic pathway in *A. oryzae* revealed a P450 CdmJ functioning as the C7 β-hydroxylase to convert **152** into **153** (Scheme 31).[121] Neosetophomone B (**154**) is a representative meroterpenoid of tropolone-sesquiterpene



Scheme 30 The three P450s and their functions in the biosynthetic pathway of botrydial (**147**).



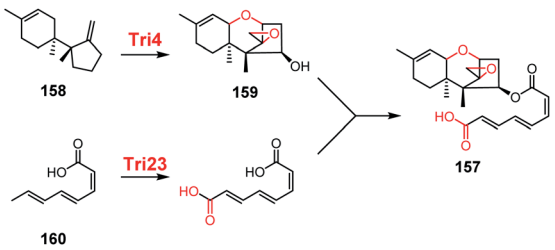
Scheme 31 The reactions catalysed by P450 hydroxylases CdmJ and EupfD during the biosynthesis of chrodrimanin B (**151**) and neosetophomone B (**154**), respectively.

family that has been isolated from several fungal species and shows potent antitumor activity at nanomolar level.[122] The enzymatic cascade for the biosynthesis of **154** from *P. janthinellum* was reconstituted in *A. nidulans* and the single P450 EupfD was found to catalyse the C10 hydroxylation at the early biosynthetic stage from **155** to **156** (Scheme 31).[123]

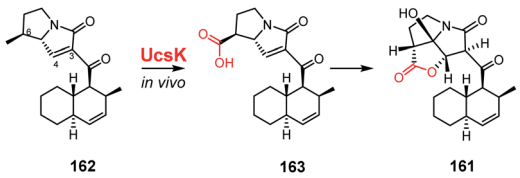
Trichothecenes are a class of sesquiterpene toxins produced by many fungal species.[124] Among them, harzianum A (**157**) is a rare meroterpenoid consisting of the trichothecene core and a linear polyketide-derived substituent *via* an ester linkage.[124] In 2007, P450 Tri4 was identified to catalyse multiple oxidation reactions against the sesquiterpene core at multiple sites by converting **158** into **159** (Scheme 32).[125] In a more recent biosynthetic study of **157**, the successive oxidation of the terminal carbon in the polyketide chain **160** was characterized to be accomplished by a P450 Tri23 from *T. arundinaceum* (Scheme 32).[126]

The telomerase inhibitor UCS1025A (**161**) is a fungal meroterpenoid containing an impressive tricyclic furopyrrolizidine core.[127,128] The molecular basis for assembly of the tricyclic system was discovered from *Acremonium* sp. KY4917. According to the *in vivo* gene inactivation and *in vitro* biochemical results, UscK was found to catalyse the C9 successive oxidation of **162** to form **163**. Then an *oxa*-Michael cyclization from the new-born COOH to the C3=C4 double bond occurs, thus furnishing the furopyrrolizidine core (Scheme 33).[129]

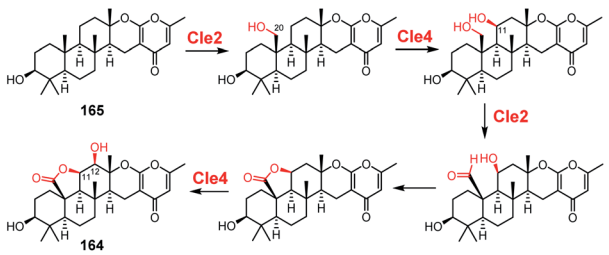
In another study, genome mining of the endophytic fungus *A. versicolor* 0312 uncovered the BGC of a new chevalone family NP **164**, which bears an additional γ-lactone ring compared with a typical chevalone-skeleton (**165**).[130] According to the decoded biosynthetic machinery of **164**, two multifunctional



Scheme 32 The two P450s Tri4 and Tri23 accomplish multi-oxidation reactions in the biosynthesis of harzianum A (**157**).



Scheme 33 The P450 UcsK mediated lactone-ring formation during the biosynthesis of UCS1025A (**161**).



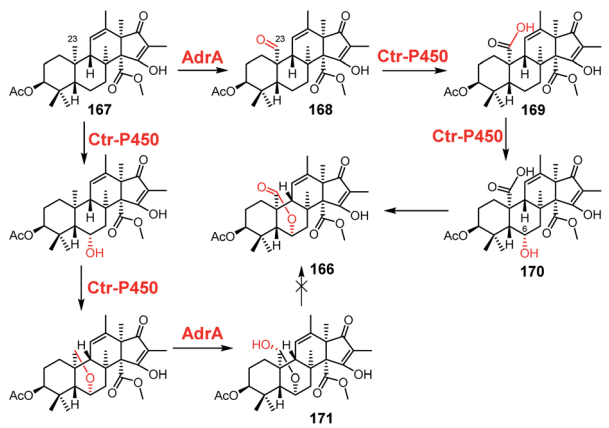
Scheme 34 The oxidative cascade co-mediated by multi-functional P450s Cle2 and Cle4 in the biosynthetic pathway of **164**.

P450s, Cle2 and Cle4, cooperate to accomplish the formation of the lactone ring. In brief, Cle2 catalyses the successive oxidation of C20 and Cle4 mediates hydroxylation at C11 and C12 with an alternate catalytic sequence (Scheme 34). Then, an intramolecular lactonization between COOH-20 and OH-11 spontaneously occurs to form the γ-lactone ring (Scheme 34).[130]

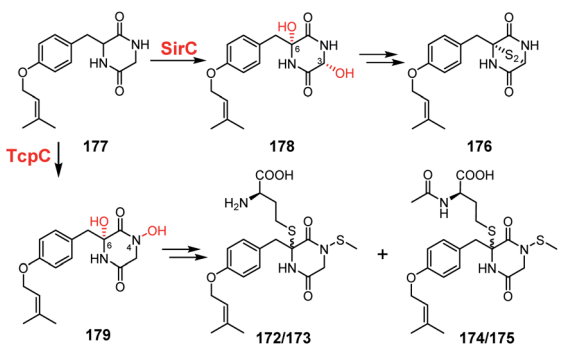
Citreohybridonol (**166**) is an andrastin type, polyketide derived meroterpenoid.[131] Genome mining of *E. variecolor* NBRC 32302 revealed the **166** BGC (*ctr*),[132] which includes an extra P450 gene *ctr-P450* comparing with the andrastin BGC in *P. chrysogenum* (*adr*).[133] Gene functional analysis revealed that Ctr-P450 and P450 AdrA co-mediate the lactone ring formation in **166**. AdrA oxidizes C23 in **167** to an aldehyde group to form **168**, then the new-born aldehyde group is oxidized to a carboxyl group by Ctr-P450, yielding **169**. Ctr-P450 further hydroxylates the C6 position to give **170**, which would trigger a spontaneous lactonization to generate **166**. This process is similar to the consecutive oxidations in gibberellin biogenesis.[129,130] Alternatively, if Ctr-P450 first hydroxylates the C6 position, a different product **171** with an ether ring would be produced (Scheme 35), which could not be converted into **166** by the two P450 enzymes, thus demonstrating a strict catalytic sequence of these two P450s.[132]

Epipolythiodiketopiperazines (ETPs) are a family of fungal toxins bearing a diketopiperazine backbone. ETPs show toxicity against a broad range of organisms, including viruses, bacteria and fungi, thus having been considered as potential drug candidates.[134,135] By overexpressing a positive transcriptional factor, an ETP BGC (*tcp*) in *Claviceps purpurea* was activated to produce ETP-like compounds.[136] Unexpectedly, the products of *tcp* were a class of unusual N–S bond bearing compounds (**172**/**173** and **174**/**175**) (Scheme 36), instead of the characteristic disulfide bridge containing products, such as dithiociaperine (**176**). Biosynthetic analysis suggested that the dysfunctional P450 TcpC might prevent the production of typical ETPs. It was proposed that, in a typical ETP biosynthetic pathway, such as the one from the ETP-producing strain *Leptosphaeria maculans*, the normal P450 SirC likely catalyses the hydroxylation of C3 and C6 in **177**, then the formed OH-3 and OH-6 in **178** are replaced by cysteine-derived thiols *via* a cascade of unknown enzymatic reactions. By contrast, the dysfunctional TcpC hydroxylates N4 and C6 in **177**, and the two resulted hydroxyl groups in **179** would be replaced by cysteines and subjected to late-stage tailoring reactions to yield the unexpected products **172**/**173** and **174**/**175** (Scheme 36).[136]

Ascofuranone (**180**) and ascochlorin (**181**) are PK-terpene hybrid meroterpenoids produced by various filamentous fungi.[134,135] This class of compounds show various biological functions, including anti-viral, anti-tumor, anti-inflammatory, and hypolipidemic activities.[137,138] The biosynthetic pathways for **180** and **181** were identified from *Acremonium egyptiacum* recently.[139] It was found that **180** and **181** share the early biosynthetic pathway, which leads to the formation of ilicicolin A-epoxide (**182**), with the epoxide ring in **182** being introduced by P450 AscE towards **183**. Then, **181** biosynthesis continues with the same BGC, in which AscG is responsible for the formation of the C12=C13 double bond in **181** *via* an oxidative



Scheme 35 The catalytic functions of Ctr-P450 and AdrA and their strict catalytic sequence in the biosynthesis of citreohybridonol (**166**).



Scheme 36 The different reactions catalysed by the homologous P450 SirC and TcpC result in distinct end-products in the biosynthesis of ETPs.

desaturation. The biosynthesis of **180** branches from the intermediate **182** and accomplishes the later steps using another distantly located cluster. Specifically, AscH from the separate cluster hydroxylates C16 of **182**, thereby guiding the metabolic flux to the production of **180** (Scheme 37).[139]

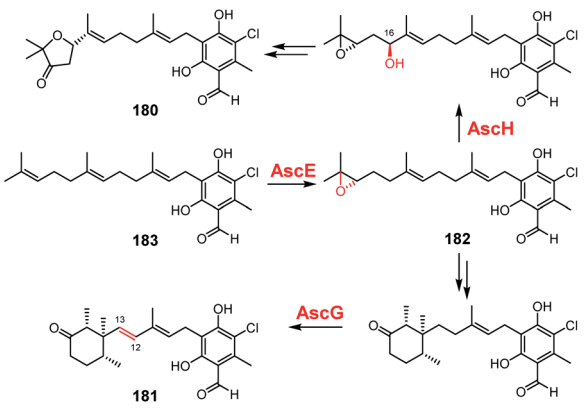
It is noteworthy that AscE is a multi-domain P450 and shares 36% protein sequence identity with the bacterial P450 alkene epoxidase/alkane hydroxylase $P450_{BM-3}$ from *Bacillus megaterium*.[140,141] Homology modeling indicated that AscE adopts a fused structure containing a P450 domain and an additional flavin-$NADP^+$ binding domain, suggesting that AscE could directly use NADPH as electron donor without the requirement of a separate redox partner protein. Most fungal-derived biosynthetic P450s have an N-terminal transmembrane region. However, TMHMM (prediction of transmembrane helices in proteins) analysis of AscE revealed no transmembrane region in its sequence, indicating AscE to be a rare soluble fungal-derived P450 for NP biosynthesis. This was confirmed by expressing AscE in *Escherichia coli* using its cDNA sequence. *In vitro* assay using the purified AscE further validated its epoxidation functionality.[140] Even though several soluble fungal P450s have been reported, such as P450nor,[142,143] they usually catalyse the oxidative reactions of alkanes/fatty alcohols/fatty acids within mitochondria.[144,145] Therefore, AscE represents a rare soluble fungal-derived P450 that participates in NP biosynthesis. However, it remains unclear whether AscE is a mitochondrial P450.

3.5.2 Uncommon P450 reactions in meroterpenoid biosynthesis. The 1,3-enyne group is often found in bioactive molecules and pharmaceutical agents.[146–148] Although several strategies have been developed to chemically build 1,3-enyne, the biogenesis of 1,3-enyne had remained largely unknown until recently.[146,149–151] Asperpentyn (**184**) and biscognienyne B (**185**) are fungal-derived meroterpenoids containing the special 1,3-enyne moiety.[152] In 2020, two laboratories independently revealed the 1,3-enyne synthases of **184** (from *Aspergillus* sp. PSURSPG185) and **185** from *Biscogniauxia* sp. (71-10-1-1), both of which are P450s.[150,151]

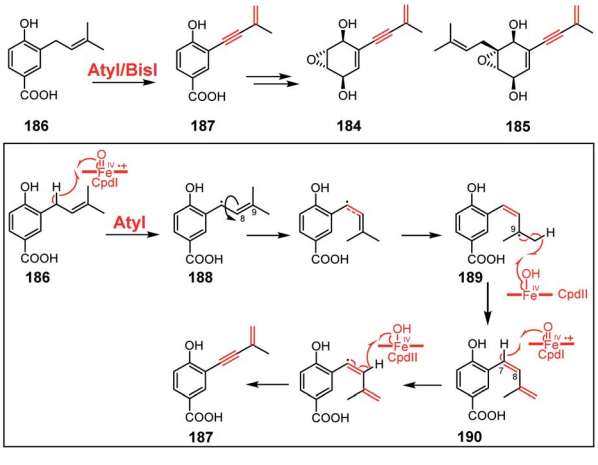
In the work from Chen *et al.*, *in vivo* and *in vitro* experiments both confirmed that P450 AtyI could convert the precursor eutypinic acid **186** into the 1,3-enyne bearing product **187**. To explore the catalytic mechanism, the activities of AtyI were examined towards a number of synthesized substrate-analogs. As results, the **186**-to-**187** transformation was attributed to a four-electron oxidation process (Scheme 38): AtyI CpdI first abstracts a hydrogen atom from the allyl position in **186** to yield a radical intermediate **188**, and the radical could re-locate to C9 to give **189** *via* a 1,2-shift of the C8=C9 double bond; then CpdII abstracts the second hydrogen atom from the terminal methyl group in **189**, and the following radical-pairing leads to a terminal double bond in **190**; after that, in a refreshed catalytic cycle, CpdI and CpdII successively abstract another two hydrogen atoms from the new-born C7=C8 *cis* double bond in **190**, and the resulted two radicals would pair to form a new bond, thus giving rise to the alkynyl group in **187**.[150] In a back-to-back article from Gao and co-workers, another P450 BisI was characterized to catalyse the same alkynylation during the biosynthesis of **185** (Scheme 38).[151]

15-Deoxyoxalicine B (**191**) is a PK-derived meroterpenoid bearing two characteristic asymmetric spiro carbons.[153] P450 OlcB from the BGC of **191** in *P. canescens* was found to catalyse the final oxidative rearrangement of decaturin A (**192**) to generate the C27 spiro center in **191** (Scheme 39).[154] Such a transformation presumably occurs *via* a C32 or C33 hydroxylated intermediate (**193**/**194**), in which an intramolecular hydrogen bond could induce a favorable conformation to trigger electron rearrangements for the C27–C28 bond cleavage (Scheme 39).

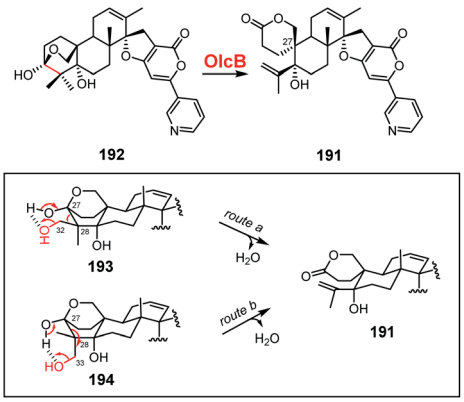
Communesins including calycanthine,[155] chaetocin,[156] perophoramidine[157] and communesin,[158] represent a group of fungal meroterpenoids that are derived from dimerization of mono-indole moieties. The defining feature of these meroterpenoids is the presence of a vicinal quaternary carbon stereocenter, which is fascinating from both synthetic and biosynthetic perspectives. Intriguingly, a single P450 was identified to be the sole enzyme to mediate the mechanistically complicated transformation (Scheme 40).[159] Specifically, P450



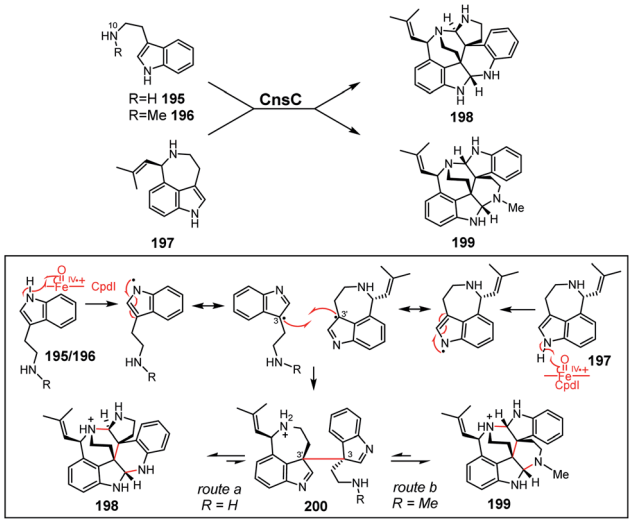
Scheme 37 The three P450s including AscE, AscH and AscG play different roles in the biosynthesis of ascofuranone (**180**) and ascochlorin (**181**).



Scheme 38 The P450 AtyI/BisI catalysed 1,3-enyne group formation during the biosynthesis of asperpentyn (**184**) and biscognienyne B (**185**), and the proposed catalytic mechanism (in box).



Scheme 39 The C–C cleavage reaction catalysed by P450 OlcB in the biosynthesis of 15-deoxyoxalicine B (**191**) and the proposed catalytic mechanism (in box).

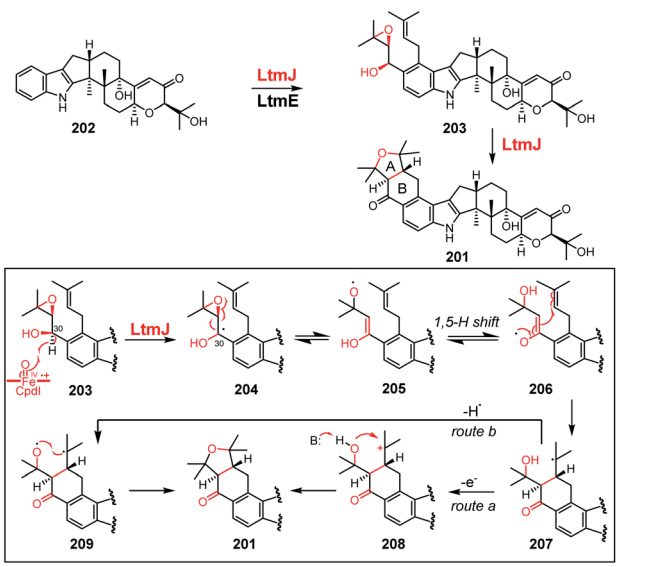


Scheme 40 The P450 CnsC mediated coupling reactions in the formation of communesin (**198**) and isocommunesin (**199**) scaffolds, and the proposed mechanisms for the complex transformations (in box).

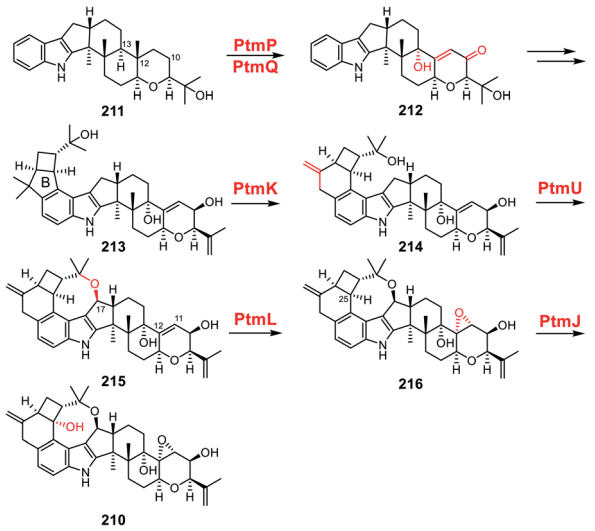
CnsC could mediate the heterodimeric coupling between two different indole-containing monomers, tryptamine (**195**)/*N*-methyltryptamine (**196**) and aurantioclavine (**197**), leading to the heptacyclic communesin (**198**) or isocommunesin (**199**) skeletons. A plausible mechanism for the formation of the highly interconnected ring system was proposed according to sophisticated computational modeling studies (Scheme 40). After the two radicals of **195**/**196** (at C3) and **197** (at C3′) are generated by CpdI of CnsC, further radical relocation and intermolecular radical coupling lead to the first C3–C3′ bond formation to give the key intermediate **200**. Then the amine/amino groups in the two halves of **200** subsequently attack the nucleophilic C=N carbon to form the pair of aminal linkages in communesin alkaloids **198** (route a). Alternatively, an N-methylated substrate (**196**) could lead the pathway to route b, in which CnsC may control the regioselectivity of the two aminal bonds, thus yielding a different scaffold **199**, in accordance to the computational calculation results.[159]

Lolitrems are a family of tremorgenic mycotoxins produced by the fungal endophyte *Epichloe festucae* var. *lolii*.[160,161] Structurally, lolitrems, such as lolilline (**201**), bear a *trans*-fused [5-6] bicyclic system (A/B rings). The biogenesis of the bicyclic ring was revealed to be accomplished by the cooperation of a prenyltransferase LtmE and a P450 LtmJ. As reported, LtmE and LtmJ together transform terpendole I (**202**) into lolilline (**201**) through an epoxyalcohol-containing intermediate **203** (Scheme 41), which was co-isolated from the recombinant *A. oryzae* in a precursor feeding experiment.[162] P450 LtmJ was proposed to be solely responsible for mediating the formation of the characteristic bicyclic ring in lolitrems. A radical-induced cyclization mechanism for LtmJ was proposed as follow (Scheme 41): after formation of the epoxyalcohol moiety in **203**, LtmJ abstracts H30 to generate a radical intermediate **204**, which could induce epoxide opening to form alkoxy radical **205**. Then, the 1,5-H shift occurs to yield the isomeric alkoxy radical **206**, which triggers an intramolecular addition to generate the first ring and gives **207**. The resulted tertiary radical in **207** could be further oxidized into a tertiary cation in **208**, which would be readily trapped by the tertiary hydroxyl group, thereby furnishing the A/B rings in **201** (route a). In an alternative pathway (route b), the later oxidation occurs at the tertiary hydroxyl group in **207** with a hydrogen atom being abstracted, and the tertiary radical in **209** could couple with the new-born alkoxy radical to form the second ring, yielding **201**.[162]

Penitrems, such as penitrem E (**210**), are a class of fungal neurotoxins isolated from *P. simplicissimum* that possess an unusual octacyclic ring system.[163,164] During the biosynthetic pathway of **210**, six P450s including PtmP, PtmQ, PtmK, PtmU, PtmL and PtmJ were determined to be involved in elaboration of the complex structures (Scheme 42).[165] Specifically, PtmP and



Scheme 41 The P450 LtmJ catalysed ring formation in the biosynthesis of lolilline (**201**) and the proposed catalytic mechanism (in box).

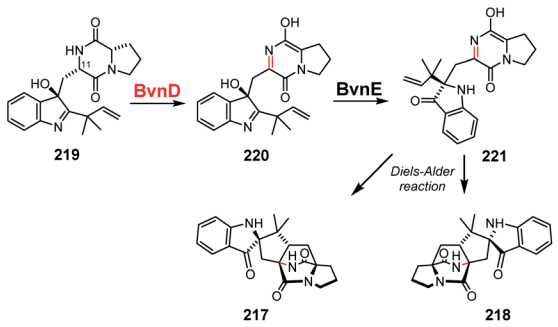


Scheme 42 The six P450s and their catalysed reactions during the biosynthesis of penitrem E (**210**).

PtmQ co-mediate the Me-12 elimination, C10 successive oxidation and C13 hydroxylation of **211** to form **212**. PtmK catalyses the oxidative B-ring expansion of **213** to produce **214**, likely *via* a mechanism similar to those in the oxidative ring expansions in tenellin biosynthesis and penicillin metabolism.[166,167] PtmU catalyses the sequential C17 hydroxylation and dehydration of **214** to afford the octatomic ether ring in **215**. In addition, PtmL and PtmJ respectively mediate the C11=C12 epoxidation of **215** and the C25 hydroxylation of **216** to produce the final product **210** (Scheme 42).

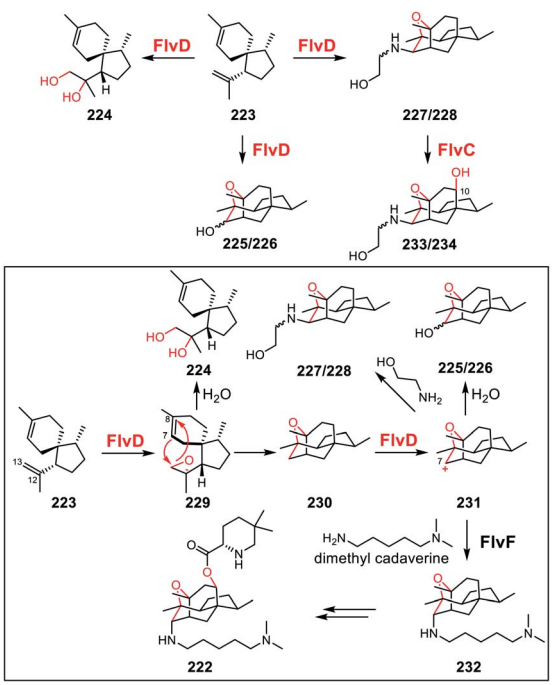
Brevianamides A and B (**217** and **218**) belong to the class of fungal indole alkaloids bearing an unusual bicyclo[2.2.2]diazaoctane core.[168] The biogenesis of such a spiro-cyclized skeleton has long drawn much attention from both synthetic and biological chemists.[169–172] In the newly identified brevianamide biosynthetic pathway from *P. brevicompactum* NRRL 864, P450 BvnD was found to set the stage for the following spontaneous intramolecular [4 + 2] *hetero*-Diels–Alder cycloadditions by mediating a C–N bond desaturation towards **219**, thus forming the required diene moiety in the dioxopiperazine ring of **220**, which could undergo spontaneous non-selective Diels–Alder reactions to generate multiple brevianamide isomers. Through bond dissociation energy calculations and comparative analysis of its homologous proteins, BvnD was proposed to catalyse the C11 hydroxylation, then the unstable intermediate undergoes spontaneous dehydration/tautomerization to yield **220** (Scheme 43).[173] Interestingly, BvnE, an isomerase/semi-pinacolase, was revealed to catalyse a semipinacol rearrangement towards **220** to produce **221**, thus stereoselectively controlling the outcome of the spontaneous Diels–Alder reaction, giving rise to the predominant product brevianamide A and a minor product brevianamide B upon spontaneous Diels–Alder reactions (Scheme 43).[173]

BGCs containing multiple skeleton-assembling genes usually have the potential to produce structurally complex



Scheme 43 The P450 BvnD mediated C–N bond desaturation sets the stage for the following spontaneous intramolecular [4 + 2] Diels–Alder cycloaddition.

NPs.[174] Flavunoidine (**222**) is such a NP discovered from *A. flavus* by genome mining using this strategy.[175] The tetracyclic, cage-like and oxygenated meroterpenoid is particularly intriguing with respect to its biosynthetic mechanisms. P450 FlvD was proposed to catalyse the essential formation of the cage-like tetracyclic ring from the substrate **223** based on identification of a series of intermediates and side products (**224–228**) (Scheme 44). Putatively, FlvD first epoxidizes the C12=C13 double bond in **223** to give the unstable intermediate **229**, which could undergo an epoxide opening reaction with a molecule of water to yield the side product **224**. Alternatively, an intramolecular [3 + 2] cycloaddition may occur between C7=C8 double bond and the epoxide ring to form the cage-like system in **230**. Next, FlvD mediates a further oxidation of C7



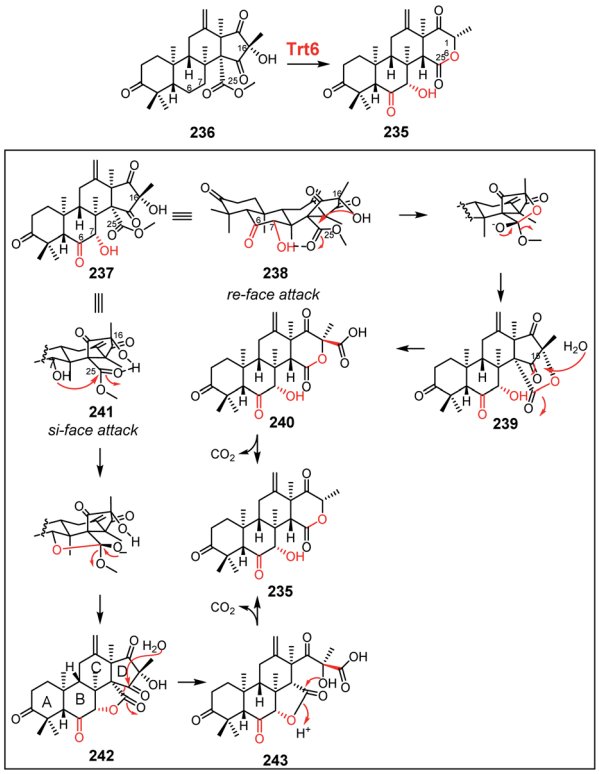
Scheme 44 The catalytic functions of P450s FlvD and FlvC and the proposed mechanism for the FlvD-mediated uncommon reactions (in box).

in **230** to form a reactive carbocation (**231**), which could be quenched by a nucleophile such as water (to yield **225**/**226**) or an ethanolamine motif (to yield **227**/**228**). With the assistance of FlvF, a terpene cyclase, the dimethyl cadaverine could be attached to **231** and yield **232**, the precursor of **222** (Scheme 44). Besides, another P450 FlvC in the pathway could further hydroxylate **227**/**228** to **233**/**234**, thus installing OH-10 for the final esterification step leading to **222** (Scheme 44).[175]

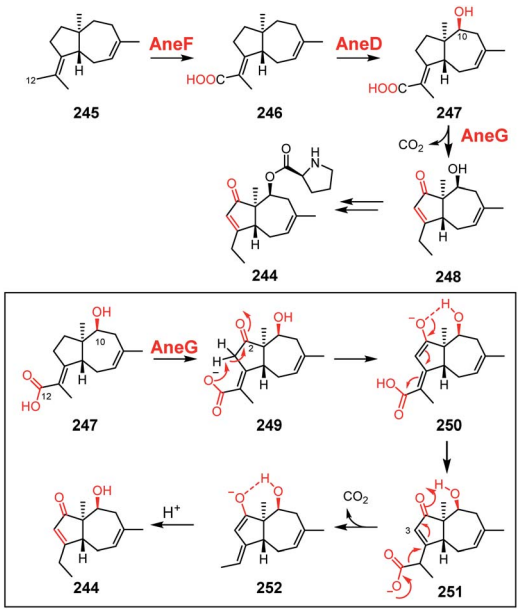
Terretonins, such as terretonin H (**235**), are highly oxygenated tetracyclic meroterpenoids isolated from *A. terreus*.[176] During the biosynthesis of **235**, P450 Trt6 was discovered to be a multi-functional enzyme to catalyse the oxidation, D-ring expansion and decarboxylation reactions by converting terrenoid (**236**) into **235** (Scheme 45).[177] Two conformation-dependent mechanisms were proposed with both involving a key nucleophilic attack from either the *re*-face or the *si*-face (Scheme 45). Specifically, after Trt6 oxidizes **236** to **237**, which bears the C6 carbonyl and C7 hydroxyl groups, the hydrogen bond between the α-oriented OH-7 group and C25 carbonyl oxygen could induce a favourable conformation (**238**) for nucleophilic attack from OH-16 to C25 (*re*-face attack). The resulted γ-lactone intermediate **239** could trigger a further nucleophilic attack from a water molecule to C15, then a *retro*-Claisen type cleavage occurs to expand the D-ring, giving rise to the δ-lactone in **240**. The unstable intermediate could be transformed into **235** by cleaving the carboxyl group. Alternatively, if the hydrogen bond is formed between the α-oriented OH-16 and the C25 carbonyl oxygen (**241**), the OH-7 would first attack C25 (*si*-face attack) to produce a different lactone in **242** that is fused with ring B and ring C. Subsequently, the above mentioned *retro*-Claisen type cleavage happens, resulting in a new lactone (in **243**), which could be further opened to form the D-ring in **235**.[177]

Aculenes are a rare type of meroterpenoids from *A. aculeatus* with a nordaucane skeleton linked to a proline moiety.[178,179] During the biosynthesis of aculene A (**244**), the degradation of the isopropenyl group to an ethyl side chain was revealed to be catalysed by three P450s including AneD, AneF and AneG.[180] Specifically, AneF firstly successively oxidize Me-12 in **245** to form **246**; AneD installs the OH-10 in **247** as an inducing group for the following rearrangement; and AneG is directly responsible for Me-12 cleavage during the formation of aculene D (**248**) (Scheme 46). The mechanism for the subtly stepwise demethylation was proposed as follow: after OH-10 and COOH-12 are installed in **247**, AneG further oxidizes C2 to a keto group to form **249**, which could tautomerize to give the hydrogen bond stabilized enol form (**250**). Then, isomerization of the enol group of **250** to the keto form (**251**) sets a conjugated state to trigger further electron rearrangement from the terminal carboxylate, leading to C–C cleavage to produce the unstable intermediate **252**; and rearrangement of the double bonds with the aid of a proton finally affords **244** (Scheme 46). Such a complex but delicate mechanism shed light on how nature employs P450s to perform a demethylation process.[180]

In fungal meroterpenoid biosynthesis, many P450s could mediate multiple and complex oxidation steps. This may be attributed to the constant existence of electrophilic olefinic bond, nucleophilic hydroxy/carbonyl group or conjugated system in the structures of meroterpenoids. These reactive



Scheme 45 The multi-site oxidation reactions catalysed by P450 Trt6 and the proposed mechanisms for the C–C bond cleavage and ring formation (in box).



Scheme 46 The three P450s AneF, AneD and AneG cooperatively accomplish the demethylation reaction in the biosynthesis of aculene A (**244**) and the proposed mechanism for AneG (in box).

moieties could readily react with the P450-induced radical, carbocation or rebound HO$^{\bullet}$ to yield the uncommon P450 products.

4 Methodologies for functional studies on fungal P450s

To explore diverse P450 functions involved in fungal NP biosynthesis, gene-inactivation in the native producer strain is usually the first choice. By analysing the change in metabolite profiles before and after gene-inactivation, the P450's function could be deduced. However, even though new gene-editing tools (*e.g.* CRISPR/Cas9 technology) have been invented and increasingly applied in recent years, gene-deletion efforts in coenocytic fungi and many higher fungal species remain impractical, less-effective or time-consuming. Thus, an appropriate heterologous gene expression system for fungal P450s is crucial not only for their functional and mechanistic studies, but also for their application development.

4.1 *Escherichia coli* as a heterologous expression host

E. coli is an excellent host for expressing the high-level recombinant proteins due to its innate advantages including fast growth rate, high cell density and the highest availability of genetic tools.[185–187] *E. coli* has long been used to express prokaryotic P450s and their redox partners for protein preparation, whole-cell catalysis, biotransformation and practical application.[185–187] However, for eukaryotic P450s and CPRs, in consideration of their membrane-bound nature, it is highly challenging to express these proteins in *E. coli* cells. Despite abundant efforts, such as addition of a solubility tag, N-terminal sequence modification, vector screening, chaperon co-expression, codon optimization, expression condition optimization and any combinations of these strategies, expression of eukaryotic P450 proteins in *E. coli* remains to be a tedious trial-and-error process, especially for the filamentous fungal-derived P450s.[17,188,189]

One of the biggest challenges to express a fungal P450 in a bacterial host is how to deal with the N-terminal anchor-membrane region (NAR) that contains a conserved proline-rich region prior to the catalytic domain, because the NAR may negatively affect proper folding of fungal P450s in the distinct membrane system of bacterial host.[188–190] According to previous studies, sequence mutagenesis, truncation and replacement for the NAR region sporadically worked for very few fungal P450s.[188–190] However, it lacks a general rule for each fungal P450 to guide the modification of NAR in order to enable the functional expression in *E. coli*. In an extensive study of 304 fungal P450s from *Phanerochaete chrysosporium* and *Postia placenta*, only 27 of them could be expressed as active P450 proteins with/without NAR optimizations in the membrane-bound protein tolerant *E. coli* C41(DE3) strain,[189] and some of them may be mitochondrial or nonmembrane-associated proteins. Of note, among all the fungal P450s reviewed above, only a single P450, AscE, was successfully expressed in *E. coli* (Table 1 and Fig. 2A). However, AscE, as a redox partner-fused and cytoplasm soluble protein, may also be located in mitochondria.[140] Thus, *E. coli* has yet to be developed into a suitable expression host for fungal P450s, and more efforts should be made in order to gain much more knowledge on the sequence-expression-activity relationship of the membrane-bound fungal P450s.

4.2 *Saccharomyces cerevisiae* as a heterologous expression host

Single cell yeast *S. cerevisiae* is a widely used expression host for fungal-derived proteins.[191–193] As a eukaryotic counterpart of *E. coli*, *S. cerevisiae* is a more preferable expression host for fungal P450s due to its ability of expressing membrane-bound proteins without any requirement for protein sequence modification, owing to its eukaryotic nature. Moreover, in consideration of the electron transport system for fungal P450s, the native CPR in *S. cerevisiae* could serve a heterogeneous fungal P450. However, preparation of the cDNA sequence of target P450-encoding gene is often necessary for a yeast host in order to avoid miss-splicing of the introns contained in the P450 sequences from higher filamentous fungi. Upon construction of a recombinant yeast, precursor biotransformation experiments could readily affirm the proposed function of the target P450. Alternatively, an *in vitro* enzymatic assay using the microsomal fraction containing the expressed fungal P450 could be carried out to reconstitute the enzymatic activity.

Among the above reviewed 82 heterologously expressed P450s, 21 of them were studied using the *S. cerevisiae* system, 10 of which were characterized by running *in vitro* assays (Table 1 and Fig. 2A). However, not all the fungal P450 genes could be successfully expressed in *S. cerevisiae*. For instance, when studying 120 P450s from *P. chrsosporium*, only 70 of them were actively expressed in *S. cerevisiae*.[194,195] Nonetheless, yeast is still a better expression system than *E. coli* for fungal P450 expression. In particular, the yeast system is more viable for practical applications, attributing to its potency in high-density culture, the advantages in fast and easy genetic manipulations and resistance to phages.

4.3 *Aspergillus* spp. as heterologous expression hosts

Higher fungi, namely, basidiomycetes and ascomycetes, are the main sources of fungal NPs.[196–198] Thus, an evolutionarily closer fungal strain, which can maturate mRNA precursors (*i.e.*, splicing introns) and handle post-translational modifications for higher fungal-derived P450 genes, is a preferable host. Several filamentous fungi, such as several *Aspergillus* and *Trichoderma* species, have been developed to heterologously express recombinant proteins of higher fungal origins.[199,200] *A. nidulans* and *A. oryzae* are the most widely used hosts for functional characterization of fungal NP biosynthetic P450s.[200] Among the reviewed 82 heterologously expressed P450s, 56 of them were expressed using either *A. nidulans* (17) or *A. oryzae* (39), and four P450s were characterized *in vitro* using the *Aspergillus* microsomal fractions containing the target P450s (Table 1 and Fig. 2A). In addition, other filamentous fungi host, including *A. aculeatus*,[46] *A. sojae*[139,140] and *C. pupurea*,[175] were also successfully used for heterologous expression of fungal P450s.

Table 1 The methodologies for functional characterization of fungal P450s in recent years

P450 name	NP biosynthetic pathway	Function(s)	P450 source	Gene-deletion (Yes/No)	Heterologous gene expression host/CPR source[a]	Gene source[b]	Ref.
GsfF	Griseofulvin A	C–O formation	*P. aethiopicum*	Y	*S. cerevisiae*/*At*CPR	cDNA	53 and 54
KtnC/DesC	Orlandin/desertorin A	C–C formation	*A. niger*/*E. desertorum*	Y	*S. cerevisiae*/native	cDNA	48
RbtI	Rubratoxin B	Hydroxylation	*P. dangeardii* Pitt	Y	*S. cerevisiae*/native	cDNA	35
DuxD	Duclauxins	C–C cleavage	*T. stipitatus*	Y	*A. nidulans*/native	cDNA	43
DuxL	Duclauxins	C–C formation	*T. stipitatus*	Y	*A. nidulans*/native	cDNA	43
NsrP	Neosartorin	C–C formation	*A. novofumigatus*	Y	N	N	51
BerkE	A26771B	Hydroxylation	*P. egyptiacum*	N	*A. nidulans* and *S. cerevisiae*/native	gDNA/cDNA	33
VirE	Trichoxide	Epoxidation	*T. virens*	N	*A. nidulans* and *S. cerevisiae*/native	cDNA	29
EpnC	Epipyrone-A	Successive oxidation	*E. nigrum*	Y	N	N	37
ShwC	Rufoschweinitzin	C–C formation	*C. rufoolivaceus*/*X. schweinitzii*	N	*A. aculeatus*/native	cDNA	46
AclO	Aspirochlorine	Hydroxylation	*A. oryzae*	Y	*S. cerevisiae*/native	cDNA	56
HqlC	Herquline A	C–C formation	*P. herquei*	N	*A. nidulans*/native	cDNA	61
SimI	Cyclosporin A	Successive oxidation	*T. inflatum*	Y	*N*	N	68
HarG	Harzianopyridone	Ring expansion/C–C cleavage	*T. harzianum*	N	*A. nidulans*/native	cDNA	77
HarD	Harzianopyridone	Hydroxylation	*T. harzianum*	N	*A. nidulans* and *S. cerevisiae*/native	cDNA	77
OpaB	Oxepinamide F	Ring expansion	*A. ustus*	Y	*A. nidulans*/native	gDNA	59
PyrB	Pyranonigrin A	Dehydrogenation/hydroxylation	*P. thymicola*	N	*A. nidulans*/native	cDNA	64
OtaC	Ochratoxin A	Successive oxidation	*A. ochraceus* fc-1	Y	N	N	63
PfpB	Citridone A	Ring-expansion	*Penicillium* FKI-1938	N	*A. nidulans*/native	cDNA	78
PfpD	Citridone A	Ring-extraction	*Penicillium* FKI-1938	N	*A. nidulans*/native	cDNA	78
PfpE	Citridone A	Ring-formation	*Penicillium* FKI-1938	N	*A. nidulans*/native	cDNA	78
P450-1	Aminoacylated product-1	Epoxidation	*P. strigosozonata*	N	*S. cerevisiae*/native	cDNA	72
Stl-P450	Stellatic acid	Successive oxidation	*E. variecolor*	N	*A. oryzae*/native	gDNA	94
NfP450	Sesterfisheric acid	Successive oxidation	*N. fischeri*	N	*A. oryzae*/native	gDNA	95
Clm2	Culmorin	Hydroxylation	*F. graminearum*	Y	N	N	99
BcBOT1	Botrydial	Hydroxylation	*B. cinerea*	Y	N	N	119 and 181
BcBOT3	Botrydial	Hydroxylation	*B. cinerea*	Y	N	N	119 and 181
BcBOT4	Botrydial	Hydroxylation	*B. cinerea*	Y	N	N	119 and 181
$OblB_{Bm}$/$OblB_{Ev}$	Ophiobolin A	Hydroxylation/successive oxidation	*B. maydes*/*E. variecolor*	N	*A. oryzae*/native	cDNA	109
$OblB_{AC}$	Ophiobolin A	Ring formation	*B. maydes*/*E. variecolor*	N	*A. oryzae*/native	cDNA	109
Qnn-P450	Quiannulatene	Successive oxidation	*E. variecolor*	N	*A. oryzae*/native	gDNA	96
VidA	Demethoxyviridin	Desaturation	*Nodulisporium* sp.	Y	N	N	85
VidD	Demethoxyviridin	C–C cleavage	*Nodulisporium* sp.	Y	N	N	85
VidK	Demethoxyviridin	Hydroxylation	*Nodulisporium* sp.	Y	N	N	85
VidE/VidG/VidR	Demethoxyviridin	C–C cleavage/ring formation	*Nodulisporium* sp.	Y	N	N	85
HelB2	Helvolic acid	Hydroxylation	*A. fumigatus* Af293	N	*A. oryzae*/native	gDNA	84

Table 1 (*Contd.*)

P450 name	NP biosynthetic pathway	Function(s)	P450 source	Gene-deletion (Yes/No)	Heterologous gene expression host/ CPR source[a]	Gene source[b]	Ref.
HelB4	Helvolic acid	Hydroxylation	*A. fumigatus* Af293	N	*A. oryzae*/native	gDNA	84
HelB3	Helvolic acid	Hydroxylation/successive oxidation	*A. fumigatus* Af293	N	*A. oryzae*/native	gDNA	84
HelB1	Helvolic acid	Successive oxidation	*A. fumigatus* Af293	N	*A. oryzae*/native	gDNA	84
FusB1	Usidic acid	Hydroxylation	*A.fusidioides*ATCC 14700	N	*A. oryzae*/native	cDNA	83
CepB4	Cephalosporin P1	Hydroxylation/successive oxidation	*A.chrysogenum*ATCC 11550/*A.chrysogenum* CGMCC 3.3795	N	*A. oryzae*/native	gDNA	82
CYP5344B1	Protoilludene	Hydroxylation	*P. placenta*	N	*S. cerevisiae*/native	cDNA	182
CYP5348E1	Protoilludene	Hydroxylation	*P. placenta*	N	*S. cerevisiae*/native	cDNA	182
CYP5348J3	Protoilludene	Hydroxylation	*P. placenta*	N	*S. cerevisiae*/native	cDNA	182
BscB	Brassicicenes	Hydroxylation	*P. fijiensis*	N	*A. oryzae*/native	gDNA	112
BscC	Brassicicenes	Hydroxylation	*P. fijiensis*	N	*A. oryzae*/native	gDNA	112
BscF	Brassicicenes	C–C bond rearrangement	*P. fijiensis*	N	*A. oryzae*/native	gDNA	112
BscG	Brassicicenes	Hydroxylation	*P. fijiensis*	N	*A. oryzae*/native	gDNA	112
$BscI_{Ab}$	Brassicicenes	Hydroxylation	*A. brassicicola*	N	*A. oryzae*/native	gDNA	112
CYP5150L8	Ganoderic acids	Successive oxidation	*G. lucidum*	N	*S. cerevisiae*/*Gl*CPR	cDNA	89
AstB	Aspterric acid	Epoxidation/successive oxidation	*A. terreus*	N	*S. cerevisiae*/native	cDNA	115
AstC	Aspterric acid	Ring formation	*A. terreus*	N	*S. cerevisiae*/native	cDNA	115
CYP512U6	Ganoderic acids	Successive oxidation	*G. lucidum*	N	*S. cerevisiae*/native	cDNA	90
EriI	Erinacine	Hydroxylation	*H. erinaceus*	N	*A. oryzae*/native	gDNA	106
EriC	Erinacine	Hydroxylation/ring formation	*H. erinaceus*	N	*A. oryzae*/native	gDNA	106
EriA	Erinacine	Hydroxylation	*H. erinaceus*	N	*A. oryzae*/native	gDNA	106
AfumB	Fumihopaside A	Hydroxylation/successive oxidation	*A. fumigatus*	N	*A. nidulans*/native	gDNA	91
PeniB	Penifulvin A	Ring formation/successive oxidation	*P. griseofulvum*	Y	*A. nidulans*/native	gDNA	102
UscK	UCS1025A	Successive oxidation	*Acremonium* sp. KY4917	Y	*S. cerevisiae*/*At*CPR	cDNA	129
EupfD	Neosetophomone B	Hydroxylation	*P. janthinellum*	N	*A. nidulans*/native	gDNA	123
AneF	Aculene A	Successive oxidation	*A. aculeatus*	Y	*S. cerevisiae* and *A. oryzae*/native	cDNA	180
AneD	Aculene A	Hydroxylation	*A. aculeatus*	Y	*S. cerevisiae* and *A. oryzae*/native	cDNA	180
AneG	Aculene A	C–C cleavage	*A. aculeatus*	Y	*S. cerevisiae* and *A. oryzae*/native	cDNA	180
AtyI	Asperpentyn/eutypinic acid	Enyne formation	*Aspergillus* sp. PSURSPG185	N	*S. cerevisiae*/*At*CPR	cDNA	150
BisI	Biscognienyne B	Enyne formation	*Biscogniauxia* sp. (71-10-1-1)	N	*A. oryzae*/native	gDNA	151
CnsC	Communesins	C–C/C–N formation	*P. expansum* NRRL 976/*P. rivulum*	Y	*S. cerevisiae*/*Pe*CPR	cDNA	159
Trt6	Terretonin	Ring expansion/C–C cleavage	*A. terreus*	N	*A. oryzae*/*At*CPR	gDNA	177
PtmP/PtmQ	Penitrems	C–C cleavage/successive oxidation	*P. simplicissimum*	N	*A. oryzae*/native	gDNA	165
PtmK	Penitrems	Ring expansion	*P. simplicissimum*	N	*A. oryzae*/native	gDNA	165
PtmU	Penitrems	Hydroxylation/dehydration	*P. simplicissimum*	N	*A. oryzae*/native	gDNA	165
PtmL	Penitrems	Epoxidation	*P. simplicissimum*	N	*A. oryzae*/native	gDNA	165
PtmJ	Penitrems	Hydroxylation	*P. simplicissimum*	N	*A. oryzae*/native	gDNA	165
LtmJ	Lolitrems	Ring formation	*E. festucae* var. *lolii*	N	*A. oryzae*/native	gDNA	162

Table 1 (*Contd.*)

P450 name	NP biosynthetic pathway	Function(s)	P450 source	Gene-deletion (Yes/No)	Heterologous gene expression host/CPR source[a]	Gene source[b]	Ref.
Ctr-P450	Citreohybridonol	Ring formation	*E. variecolor* NBRC 32302	N	*A. oryzae*/native	gDNA	132
AdrA	Andrastin A	Successive oxidation	*E. variecolor* NBRC 32302	N	*A. oryzae*/native	gDNA	132
OlcB	15-Deoxyoxalicine B	C–C bond rearrangement	*P. canescens*	Y	N	N	154
CdmJ	Chrodrimanin B	Hydroxylation	*P. verruculosum* TPU1311	N	*A. oryzae*/native	cDNA	121
SpdJ	Sespendole	Epoxidation/hydroxylation	*P. terrestris* FKA-25	N	*A. oryzae*/native	gDNA	183
AscE	Ascofuranone/ascochlorin	Epoxidation	*A. egyptiacum*	Y	*A. sojae* and *E. coli*/fused CPR domain	Synthesized	139 and 140
AscG	Ascofuranone	Desaturation	*A. egyptiacum*	Y	*A. sojae*/native	Synthesized	139
AscH	Ascochlorin	Hydroxylation	*A. egyptiacum*	Y	*A. sojae*/native	gDNA	139
Tri23	Harzianum A	Successive oxidation	*T. arundinaceum*	Y	N	N	126
Cle2	Chevalone E	Successive oxidation/ring formation	*A. versicolor* 0312	N	*A. oryzae*/native	gDNA	130
Cle4	Chevalone E	Hydroxylation	*A. versicolor* 0312	N	*A. oryzae*/native	gDNA	130
DpfgJ	Decalin-containing meroterpenoids	Successive oxidation	*F. graminearum* PH-1	N	*A. oryzae*/native	gDNA	184
DpmpJ	Decalin-containing meroterpenoids	Hydroxylation	*M. phaseolina* MS6	N	*A. oryzae*/native	gDNA	184
FlvD	Flavunoidine	Ring formation	*A. flavus*	N	*A. nidulans* and *S. cerevisiae*/native	gDNA/cDNA	175
FlvC	Flavunoidine	Hydroxylation	*A. flavus*	N	*A. nidulans*/native	gDNA	175
TcpC	Epipolythiodiketopiperazines	Hydroxylation	*C. purpurea*	N	N	N	136
SirC	Epipolythiodiketopiperazines	Hydroxylation	*L. maculans*	N	*C. purpurea*/native	gDNA	136
BvnD	Brevianamides	Desaturation	*P. brevicompactum* NRRL 864	Y	*A. oryzae*/native	cDNA	173

[a] "Native" indicates that the native CPR of the expression host was used. [b] Gene source indicates the source of DNA sequence for construction of the P450 gene expression vectors (gDNA: genomic DNA; cDNA: complementary DNA).

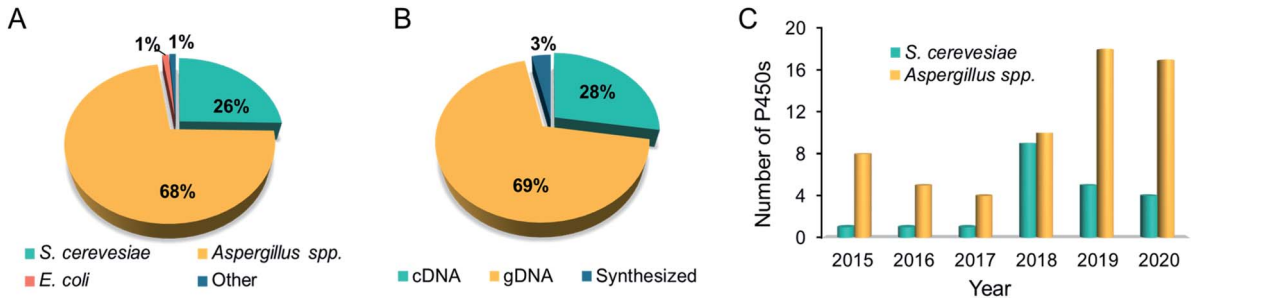


Fig. 2 (A) The ratio of different heterologous gene expression hosts used for the studies on the reviewed fungal P450s in this article ("Other" indicates the fungus *Claviceps purpurea* used in the study of P450 SirC); (B) the ratio of P450-gene sequence sources used for heterologous P450 expression in any *Aspergillus* host; (C) the change of the popularity of the heterologous gene expression hosts of the reviewed fungal P450s in 2015–2020.

Aspergillus spp. were also found to be effective expression hosts for P450 genes from higher basidiomycota fungi (mushrooms). By genome-editing-based knock-in of the gDNA sequence of the erinacine biosynthetic genes from *Hericium erinaceus* into the high expression loci of *A. oryzae*, more than 90% of the introns were correctly spliced.[106] When the cDNA sequences were further introduced, the end product was successfully produced in *A. oryzae*. Among the above-reviewed P450s that were expressed in an *Aspergillus* host, 69% of them were directly cloned from gDNA sequences (Table 1 and Fig. 2B). Thus, the high splicing-rate and higher possibility of successful functional expression are the major advantages of *Aspergillus* hosts when compared with yeasts as the heterologous expression hosts for fungal P450s. This explains why *Aspergillus* spp. have become a more preferred gene expression host for fungal NP biosynthetic studies in recent years (Fig. 2A and C).

5 Outstanding questions and challenges

Although fungal P450 studies have gained remarkable achievements and advances in the past decade, there still lies several key bottlenecks when compared with prokaryotic P450s: (1) the poor expression level and low activity of fungal P450s when being expressed in a heterologous host have greatly limited their functional characterization and practical application. Unavailability of enough proteins also makes it impractical for structural biology studies of fungal P450s, which is critical for mechanistic elucidation, especially for those "uncommon reactions". Currently, these mechanisms are proposed largely based on the knowledge gained from bacterial and mammalian P450s as well as the results of computational simulations. (2) It could be learned from Table 1 that most of functional characterizations of fungal P450s were performed by feeding the proposed substrate/intermediate to the heterologous expression host that harbouring the P450 gene(s) of interest. However, during the precursor feeding experiments, various native enzymes in the heterologous host may directly or indirectly disturb the expected P450-mediating reactions. For example, in an unsuccessful but inspiring study of usnic acid biosynthesis, the putative substrate of the lichen (a symbiont of fungi and algae/cyanobacteria) derived fungal P450 MPAO was metabolized by unknown enzymes in the heterologous host *A. oryzae*, and the PKS gene (*mpas*) was transcribed but the mRNA could not be successfully translated.[201] (3) The CPR limitation: comparing to bacterial P450s and their redox partners,[202] very limited studies have been conducted towards the fungal P450-CPR relationship. Although it has been showed that fungal P450s could work with a heterologous CPR, the catalytic efficiency, substrate specificity, and product profile could be influenced by the choice of reductase.[22,23,25] At present, in most cases, the heterologously expressed fungal P450s in yeast or other fungal hosts were functionalized using the native CPR of the heterologous host. In a few studies, the *A. terreus* originated CPR (*At*CPR)[53,54,129,150,177] or the BGC host derived CPR[89,159] was co-expressed, aiming to improve the electron transfer efficiency and hence the catalytic activity. (4) The ambiguity exists when predicting the 5′-end of a fungal P450 gene. Since the N-terminal protein sequence is crucial for translation initialization and correct protein-folding, the wrong gene annotation could fail the functional expression of the target P450. Taken together, these bottlenecks have significantly limited the functional and mechanistic studies and practical application of fungal P450s so far.

6 Conclusions and prospects

Nature creates a tremendous number of NPs to support all kinds of organisms in the living world. Fungi are one of the most important sources of NPs. With regard to biosynthesis, fungi are also a huge arsenal of biosynthetic enzymes. P450s stand for the most versatile biocatalysts during fungal NP biosynthesis. In the post-genomic era, with the development of high-throughput sequencing technologies, a fast-growing number of fungal P450s have been discovered and functionally characterized. As summarised in this review, fungal P450s catalyse diverse reactions far beyond the classical hydroxylation and epoxidation reactions. Diverse pericyclization, bond formation and scission, ring expansion and contraction, site- and stereoselective aromatic coupling and other unusual bond rearrangement reactions have continuously been discovered, which greatly expand the chemical space for fungal NPs.

Interestingly, compared with prokaryotic P450s, the membrane-associated fungal P450s seem more inclined to catalyse multi-step oxidations and some unusual structural transformations. However, the influence of membrane on the multi-functionality of fungal P450s remains elusive and unexplored.

Accumulation of knowledge on fungal P450 enzymology will provide more mechanistic understandings for these previously underexplored enzymes, with which we would be able to harness the catalytic versatility of fungal P450s to create more "unnatural" compounds of diverse utilities. Notably, several fungal P450s have successfully been applied for production of valuable chemicals (*e.g.*, ganoderic acid, hydrocortisone, psilocybin and ω-hydroxyfatty acids) on the basis of enzyme and metabolic engineering.[89,203–205] However, some significant limitations still need to be overcome in the future for both scientific researches and applications. With a better understanding of the sequence-splicing-translation-folding-function relationship, we envision that more and better heterologous P450 gene expression systems will arise, and the application of fungal P450s will become more feasible in the future.

7 Author contributions

Li S. and Zhang X. conceived and wrote the manuscript. Zhang X., Guo J. and Cheng F. drew figures and schemes.

8 Conflicts of interest

There are no conflicts to declare.

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