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Expansion of chemical space for natural products by uncommon P450 reactions



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Expansion of chemical space for natural products by uncommon P450 reactions

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Cytochrome P450 enzymes (P450s) are the most versatile biocatalysts in nature. The catalytic competence of these extraordinary hemoproteins is broadly harnessed by numerous chemical defenders such as bacteria, fungi, and plants for the generation of diverse and complex natural products. Rather than the common tailoring reactions (e.g. hydroxylation and epoxidation) mediated by the majority of biosynthetic P450s, in this review, we will focus on the unusual P450 enzymes in relation to new chemistry, skeleton construction, and structure re-shaping *via* their own unique catalytic power or the intriguing protein–protein interactions between P450s and other proteins. These uncommon P450 reactions lead to a higher level of chemical space expansion for natural products, through which a broader spectrum of bioactivities can be gained by the host organisms.

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1 Introduction

Natural products (NPs) are powerful weapons for chemical defenders, including archaea,¹ bacteria,² fungi,³ plants,⁴ and lower animals,⁵ to survive in the ever-changing environments and ecosystems.⁶ The whole NP pool consists of countless natural compounds falling into different classes including polyketides (PKs), non-ribosomal peptides (NRPs), ribosomally synthesized and post-translationally modified peptides (RiPPs), saccharides, terpenoids, alkaloids, and the hybrids thereof.^{7–9} Structural diversity and complexity of NPs are not only the intrinsic requirement for gaining various biological or ecological activities by their native producers,¹⁰ but also are one of the central motivations for people to study NPs in order to develop new drugs as well as biomimetic synthetic methodologies.^{11–13} Over the past two decades, the pharmaceutical industry has shifted its lead compound discovery efforts, to different extents, from the screening of NP libraries toward the screening of synthetic chemical libraries. However, NPs, semi-synthetic NP derivatives, or NP inspired chemical entities continue to account for the majority of approved drugs since NPs still hold great advantages over synthetic compounds with their better biocompatibility, much higher ‘hit rates’, and broader chemical space (*i.e.* scaffold diversity and structural complexity).^{11,12}

From the point of view of NP biosynthesis, the structural diversity and complexity of NPs are generated at three different stages including the pre-assembly stage, assembly stage, and post-assembly stage.^{14–16} At the first stage, simple primary metabolites such as acyl–CoAs, amino acids, and mono-saccharides are converted into various secondary building

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blocks by dedicated enzymes.^{17,18} At this hierarchical level of structural diversification, the current synthetic prowess has outperformed biosynthetic capabilities. However, only a very limited number of synthetic building block compounds can be recognized by native biosynthetic enzymes.¹⁹ At the assembly level (mainly for PKs and NRPs), the structural diversity is derived from the variety of starter and extender units, the rounds of elongation, and the timing and modes of chain release/cyclization.^{20,21} Although great efforts and significant progress have been made in the field of combinatorial biosynthesis, big challenges remain in (1) maintaining correct protein folding, productive modular interactions, and overall functional integrity of multiple mega-synthases during the deletion, insertion, swapping, or modification of an individual domain or a whole module, (2) enabling all involved catalytic domains to tolerate the structural modification when unnatural building blocks are incorporated, and (3) achieving a practical overall yield of the target product upon the multi-step compromised activities of a large number of participating enzymes in the assembly.^{22–24}

The post-assembly tailoring enzymes are arguably responsible for the most diverse and sophisticated structural modifications of NPs. Many NP tailoring enzymes override chemical catalysts in terms of their regio- and stereoselectivity towards complex substrates.^{15,25} Practically, it is more feasible to engineer a stand-alone tailoring enzyme than a multi-modular mega-synthase. Among diverse tailoring modifications such as oxidation, methylation, glycosylation, halogenation, prenylation and acetylation,²⁶ oxidative modifications are of particular significance because they not only diversify chemical structures, but also shape/mature the bioactivity and bioavailability of the resultant products.²⁷

Cytochrome P450 (CYP) enzymes (EC 1.14.x.x) are the major players in oxidative tailoring of NPs. Since this super-family of

hemoproteins can catalyze a great variety of reactions and their substrate spectra are extraordinarily broad, they have been called the most versatile biocatalysts in nature.²⁸ Thus, the catalytic competence of these talented biocatalysts is widely harnessed by numerous organisms throughout four biological kingdoms (archaea, bacteria, eukarya, and viruses), of course including by human beings.²⁹

Instead of the typical tailoring reactions dedicated to decorating the core NP structures (*e.g.* hydroxylation and epoxidation) mediated by the majority of biosynthetic P450s,³⁰ in this review, we will focus on the unusual P450 enzymes in relation to new chemistry, skeleton construction, structure re-shaping, and intriguing protein–protein interactions. These uncommon P450 reactions lead to a higher level of chemical space expansion for NPs.

2 The P450 catalytic cycle

Although CYPs have undergone significant divergent evolution, a vast majority of P450 enzymes share a common catalytic cycle (Scheme 1) to generate highly reactive species for the catalysis of normal mono-oxygenation and other unusual reactions.^{31,32} Specifically, a canonical P450 catalytic cycle starts from the resting state (A), where one water molecule serves as the sixth ligand to the ferric heme-iron (Fe^{III}) that is coordinated to the absolutely conserved cysteine (Cys) residue and four nitrogen atoms of the porphyrin (Por) ring. Upon substrate (R–H) binding to the water-bound, hexa-coordinated, low-spin ferric CYP enzyme, displacement of the water ligand from the substrate binding pocket induces a subtle change in the iron position relative to the Por plane and the spin state shift, leading to a substrate-bound, high-spin, penta-coordinated ferric CYP complex (B). The spin state shift is concomitant with the change of redox potential of the heme-iron, which



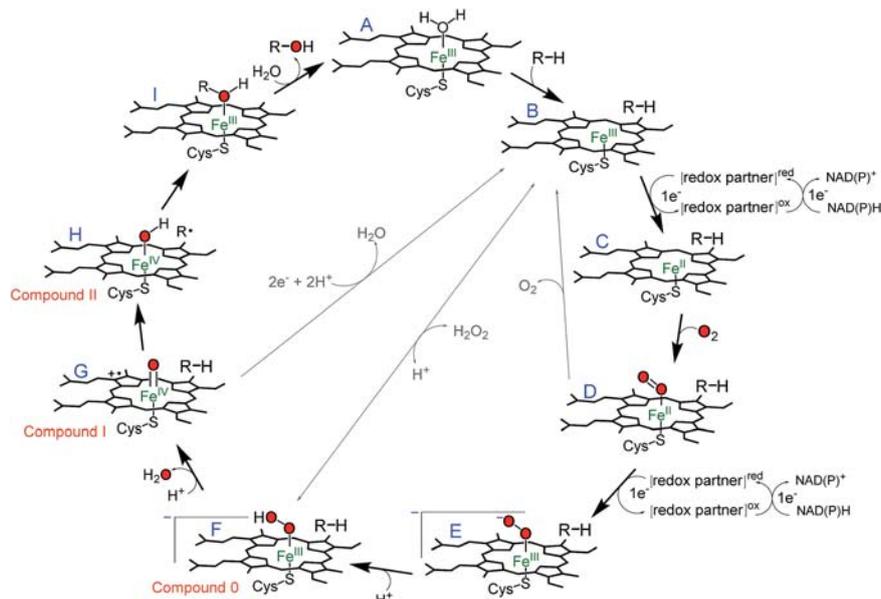
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Scheme 1 The P450 catalytic cycle (the grey arrows illustrate the peroxide shunt pathway and P450 uncoupling).

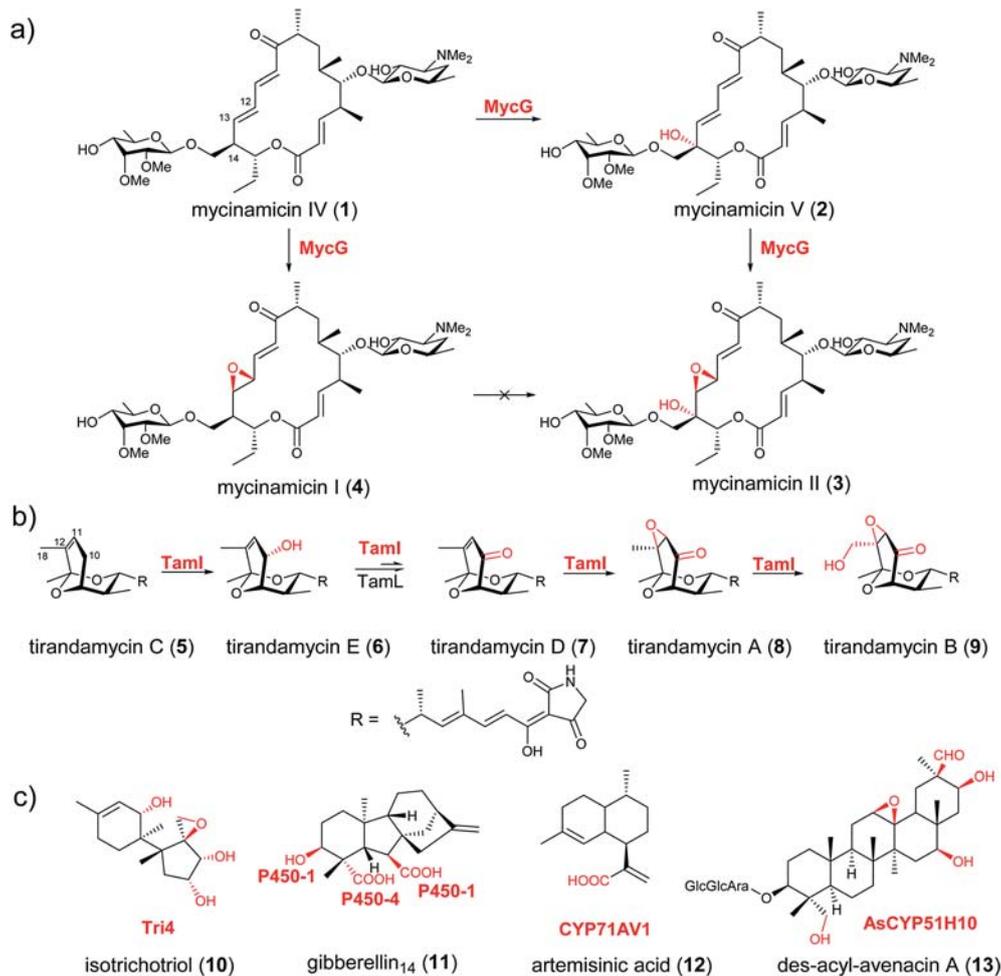
initiates the first electron transfer for reduction of the ferric (Fe^{III}) CYP to the ferrous (Fe^{II}) state (C). The electron originates from NAD(P)H and is shuttled by redox partner protein(s). Next, ambient O_2 binds to the ferrous heme-iron (Fe^{II}) to form a dioxygen adduct [$\text{Cys-Fe}^{\text{II}}\text{-O}_2$] (D). The second electron reduction produces a super-nucleophilic, dinegatively charged, and ferric-peroxy intermediate $^-\text{[Cys-Fe}^{\text{III}}\text{-OO}^-]$ (E) with one negative charge on the distal oxygen atom and the other delocalized over the Cys thiolate ligand. This intermediate is quickly protonated, giving rise to the ferric hydroperoxy species $^-\text{[Cys-Fe}^{\text{III}}\text{-OOH]}$ (F), which is referred to as compound 0 (Cpd0). The following second protonation and heterolytic cleavage of the O–O bond with concurrent loss of a water molecule lead to the formation of the porphyrin π radical cation ferryl species [$\text{Por}^+\text{Fe}^{\text{IV}}\text{=O}$] (G), *i.e.* compound I (CpdI). This highly reactive species abstracts a hydrogen atom from the substrate, generating a substrate radical and the ferryl-hydroxo compound II (CpdII, H). The radical rebound to the hydroxyl group of CpdII results in the apparent insertion of an oxygen atom into the substrate (R–H) (I). The mono-oxygenated product (R–OH) is finally released from the active site, and the rebound water molecule restores the resting state (A) of the CYP enzyme.

In addition to the typical catalytic cycle, most CYPs can also adopt the peroxide shunt pathway,^{31,33} by which the substrate–P450 complex (B) is directly converted into Cpd0 (F) with the activation of H_2O_2 , avoiding the need for O_2 , NAD(P)H, and a redox partner system. However, except for the P450 peroxxygenase (*e.g.* CYP152) sub-family,^{32,34} the peroxide shunt pathway is normally inefficient due to the low binding affinity of H_2O_2 and quick enzyme inactivation by peroxides. Notably, during the P450 catalytic cycle, premature decomposition of the reactive species could occur, leading to P450 uncoupling.

3 Common P450 reactions in natural product biosynthesis

In this review, C–H or N–H bond hydroxylation and C=C double bond epoxidation are considered as common P450 reactions because: (1) they represent the most ubiquitous, recognizable, and studied tailoring reactions mediated by CYPs during NP biosynthesis; (2) they do not significantly alter the skeleton structure of their substrates; and (3) the mechanisms for these minor structural modifications are clear. P450 enzymes responsible for these usual reactions have been studied mainly regarding their substrate specificity and reaction selectivity, based on which the order of biosynthetic steps could be established, and the biotechnological potential of these CYPs would be assessed.³⁵

Recently, an emerging group of biosynthetic P450s that catalyze normal but consecutive hydroxylation/epoxidation has attracted much attention.^{27,36,37} For example, the bacterial P450 mono-oxygenase MycG^{38,39} from the rare actinomycete *Micromonospora griseorubida* catalyzes consecutive hydroxylation and epoxidation of the 16-membered ring macrolide mycinamicin IV (1) to form mycinamicin V (2) and mycinamicin II (3) sequentially. MycG also directly epoxidizes 1 to mycinamicin I (4), which cannot be further hydroxylated to 3 (Scheme 2a). The TamI P450 enzyme from *Streptomyces* sp. 307-9 demonstrates even greater versatility.^{40,41} It first hydroxylates C10 of tirandamycin C (5) to generate tirandamycin E (6), which is next dehydrogenated by an FAD-dependent enzyme TamL to produce tirandamycin D (7). Interestingly, 7 is taken over by TamI again to be sequentially epoxidized at C11=C12 and hydroxylated at C18, giving rise to tirandamycin A (8) and B (9), respectively (Scheme 2b). If taking into account the minor activity of TamI to mediate the conversion of 6 to 7 *via* the



Scheme 2 Examples of multifunctional P450 enzymes that mediate consecutive hydroxylation/epoxydation reactions. (a) MycG from the rare actinomycete *Micromonospora griseorubida* for the biosynthesis of mycinamicins; (b) TamI from the bacterium *Streptomyces* sp. 307-9 for the biosynthesis of tirandamycins; and (c) other representative multifunctional CYPs including Tir4, P450-1, and P450-2 from fungi, and CYP71AV1 and AsCYP51H10 from plants.

unstable geminal diol intermediate, this multifunctional P450 enzyme is able to catalyze four consecutive oxidation steps. Furthermore, a growing number of fungal and plant P450s, such as Tri4 (for the biosynthesis of trichothecenes, **10**) from the fungus *Fusarium graminearum*,⁴² P450-1 and P450-4 (for gibberellin biosynthesis, **11**) from the rice pathogen *Gibberella fujikuroi*,⁴³ CYP71AV1 (for artemisinic acid biosynthesis, **12**) from the medicinal plant *Artemisia annua*,⁴⁴ and the oat enzyme AsCYP51H10 (for the biosynthesis of antimicrobial triterpenes, **13**), have also been found to be multifunctional enzymes (Scheme 2c).⁴⁵

It is intriguing to elucidate the mechanisms that underlie the changing substrate recognition/localization and oxidation selectivity of these multifunctional P450 enzymes. Another important but untouched issue is whether or not these P450s are processive enzymes, which catalyze consecutive reactions without releasing substrates. To answer these important mechanistic questions, more intensive functional and structural studies are required to gain significant insight into the

molecular basis for the unusual combinations of common P450 reactions.

4 Unusual P450 reactions in natural product biosynthesis

Excluding the common P450 reactions of C–H/N–H bond hydroxylation and C=C double bond epoxidation, other types of reaction, such as aromatic/phenolic coupling, bond cleavage or migration, and ring opening, closure, and rearrangement, are defined as unusual ones in this section. Important characteristics of unusual P450 reactions involved in NP biosynthesis include: (1) these reactions may cause dramatic structural modifications; (2) catalysis of the structural transformations by P450 enzymes is often unrecognizable without detailed biochemical analysis; and (3) the catalytic mechanisms remain unknown or debatable. According to the above criteria, unusual P450 reactions are further categorized into reactions with new chemistry, for skeleton construction (C–C bond formation or

cleavage), and for structure re-shaping (*e.g.* the topology change arising from ring formation and rearrangement by C–O coupling).

4.1 New chemistry

In this subsection, a number of unique P450 reactions *via* non-canonical catalytic cycles or unclear mechanisms will be discussed.

Thaxtomin A (**14**) is a phytotoxin produced by plant-pathogenic *Streptomyces* species that contains an infrequent nitro group in the structure (Scheme 3), and the nitro group is essential for its phytotoxicity.⁴⁶ Typically, nitro groups in NPs result from oxidation of amino groups.⁴⁷ Alternatively, peroxidases and globins are capable of catalyzing the nitration of *L*-tryptophan (**15**) in the presence of NO₂[−]/H₂O₂ or NO/O₂.⁴⁸ However, the biosynthetic origin of the non-proteinogenic amino acid *L*-4-nitrotryptophan (**16**) remained elusive until a unique P450 enzyme TxtE was identified within the thaxtomin biosynthetic pathway from *S. turgidiscabies*.⁴⁹

TxtE catalyzes the nitration of **15** to afford **16** using O₂ and NO generated from *L*-arginine by the nitric oxide synthase TxtD. Following the formation of complex **D** (Scheme 1), an atypical ferric superoxide intermediate is proposed to react with NO, resulting in a ferric peroxynitrite complex. The subsequent homolytic cleavage gives rise to a NO₂[•] radical and an Fe^{IV}=O species. The latter abstracts a hydrogen atom from the substrate, leading to NO₂ addition and the formation of an Fe^{III}–OH species, which can be further protonated for regeneration of the resting state of the enzyme.⁴⁹

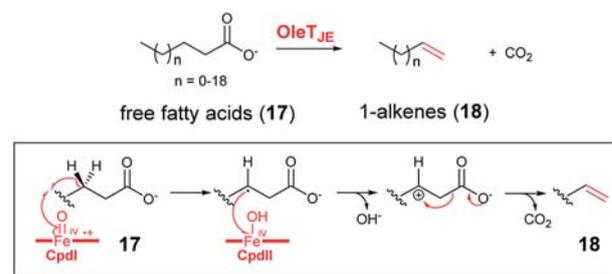
Biological terminal olefins are special NPs with unknown physiological function. Nonetheless, these biohydrocarbons hold significant application potential as either biofuels or biomaterials. Recently, a growing number of CYP152 peroxxygenase family members, including OleT_{JE} (CYP152L1) from *Jeotgalicoccus* sp. ATCC 8456,^{50,51} P450_{BSB} (CYP152A1) from *Bacillus subtilis*,⁵² CYP-MP (CYP152P1) from *Methylobacterium populi*,⁵³ CYP-Aa162 (CYP152A8) from *Alicyclobacillus acidocaldarius*, and LAA1 and CYP-Sm46 (CYP152L2) from *Staphylococcus massiliensis* S46,⁵⁰ have been identified to be capable of catalyzing the decarboxylation of free fatty acids (**17**) to form 1-alkenes (**18**). These P450s can also mediate the hydroxylation of fatty acids at different positions. Taking OleT_{JE} as an example, it

employs H₂O₂ as the sole oxygen and electron donor. Firstly, the substrate-aided H₂O₂ binding and heterolytic cleavage of the O–O bond directly produces CpdI *via* the peroxide shunt pathway (Scheme 1). Next, CpdI abstracts a hydrogen atom from the β-position of the fatty acid, forming a substrate radical and CpdII. Finally, an additional abstraction of a single electron from the substrate radical results in the formation of a substrate carbocation, which readily removes a molecule of CO₂ and forms the C_α=C_β double bond (Scheme 4). Concomitantly, the ferric-hydroxo species is protonated to restore the aqua-ferric resting state of the enzyme. If the canonical oxygen rebound event occurs, the normal hydroxylation product would be generated.⁵⁰

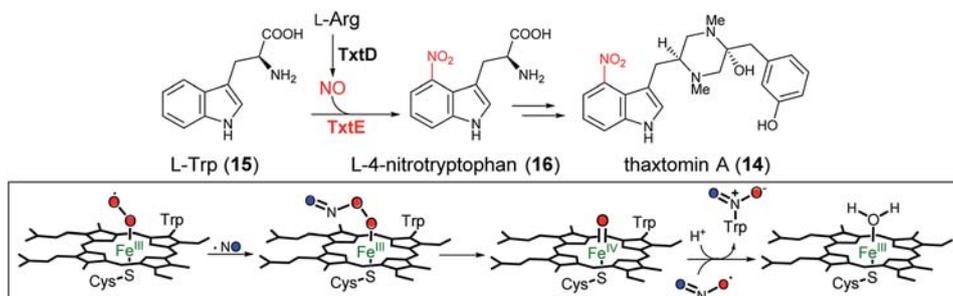
CYP154A1 from *S. coelicolor* A3(2) is known to have a unique heme orientation that is flipped 180° relative to that of most other P450s based on its crystal structure.⁵⁴ Through a metabolomic approach, dipentaenone (**19**) was identified as an endogenous substrate of CYP154A1, which was converted into a Paternò–Büchi-like product (**20**) *via* an unprecedented intramolecular cyclization between the C5 carbonyl group and the C11=C12 double bond (Scheme 5). Remarkably, this transformation without net oxidation–reduction does not require any reducing equivalents and redox partners. The unique catalytic property of CYP154A1 might be associated with the abnormal heme orientation. However, the underlying mechanism of this unusual P450 enzyme still requires further exploration.^{54,55}

4.2 Skeleton construction

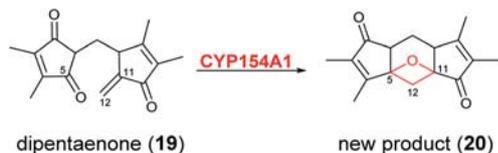
In this subsection, P450-mediated aromatic coupling, ring formation, ring expansion, ring contraction, C–C bond



Scheme 4 The free fatty acid decarboxylation by OleT_{JE} from *Jeotgalicoccus* sp. ATCC 8456, and the plausible mechanism (boxed).



Scheme 3 The nitration of *L*-tryptophan by TxtE from the bacterium *S. turgidiscabies* in the biosynthesis of thaxtomin A, and the putative mechanism (boxed).



Scheme 5 The unique cyclization of dipentaenone catalyzed by CYP154A1 from the bacterium *S. coelicolor* A3(2).

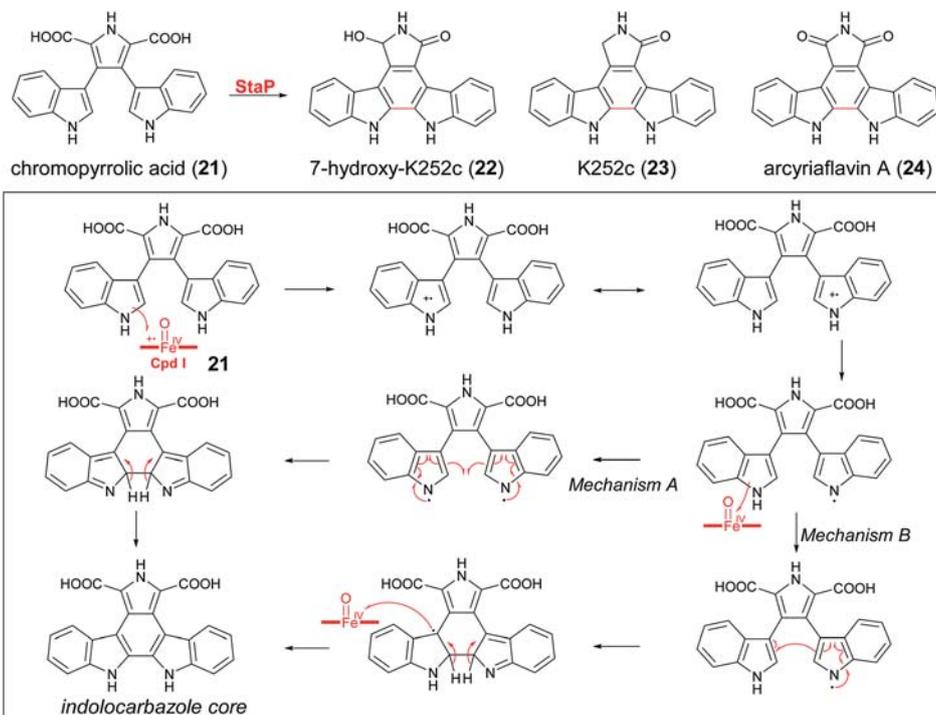
cleavage, and C–C bond migration that lead to dramatic changes of carbon skeletons will be reviewed.

4.2.1 Intramolecular aromatic coupling. Chemically, NPs bearing biaryl groups are notoriously difficult to synthesize because it is highly challenging to control the linkage of the two molecular halves (regioselectivity) and the configuration of the biaryl axis (stereoselectivity) at the same time. Biocatalytically, P450 enzymes have been found to catalyze many aromatic coupling reactions during NP biosynthesis. For instance, staurosporine isolated from *Streptomyces* sp. TP-A0274 is an indolocarbazole alkaloid that exhibits strong anti-tumor activity.⁵⁶ A key step in staurosporine biosynthesis is the generation of the indolocarbazole core within the staurosporine congeners (21–24) by intramolecular C–C bond formation, which is catalyzed by the P450 enzyme StaP (CYP245A1, as shown in Scheme 6).⁵⁷

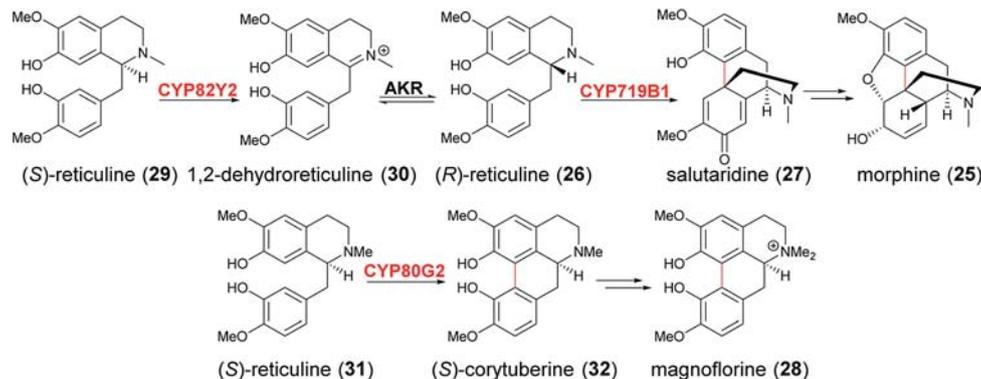
Based on biochemical and structural analyses, the aryl–aryl coupling between the two indolyl moieties was proposed to adopt an indole cation radical mechanism as follows: CpdI in the StaP–21 complex first removes one electron from the indole

ring proximal to the heme iron reactive center to generate an indole cation radical and a ferryl-oxo species ($\text{Fe}^{\text{IV}}=\text{O}$). This acidic indole cation radical ($\text{p}K_{\text{a}} = 4.3$) then loses a proton to form a neutral substrate radical. The remaining $\text{Fe}^{\text{IV}}=\text{O}$ species of StaP again removes one electron from the substrate to produce a biradical intermediate. The following intramolecular radical coupling forms the C–C bond between the two indole rings (mechanism A in Scheme 6). Finally, tautomerization of the six-membered ring gives rise to the aromatized ring system of the indolocarbazole core. Alternatively, the indole-neutral radical could directly create the C–C bond. An additional one-electron oxidation and deprotonation of the proximal indole ring could also lead to the production of the indolocarbazole core (mechanism B in Scheme 6). The following oxidative decarboxylation and variant oxidation steps yield 7-hydroxy-K252c (22), K252c (23), and arcyriflavin A (24), with 23 being the direct precursor for staurosporine.⁵⁸

Morphine (25), the well-known analgesic alkaloid NP, possesses an intriguing tetracyclic scaffold. In the ingenious morphine biosynthetic pathway identified from opium poppy (*Papaver somniferum*), the P450 enzyme CYP719B1 is responsible for catalyzing the intramolecular C–C phenyl coupling of (*R*)-reticuline (26) to yield salutaridine (27), likely using radical chemistry.⁵⁹ A similar aromatic coupling reaction also occurs during the biosynthesis of magnoflorine (28), an aporphine-type alkaloid produced by *Coptis japonica* (Scheme 7).⁶⁰ Interestingly, the enigmatic *S*-to-*R* epimerization of reticuline (29 → 26) was recently elucidated by two research groups independently to be mediated by a natural fusion protein containing



Scheme 6 The intramolecular aryl–aryl coupling reaction catalyzed by StaP from the bacterium *Streptomyces* sp. TP-A0274 in the biosynthesis of staurosporine. The two alternative mechanisms are shown in the box.



Scheme 7 The intramolecular aryl–aryl coupling reactions catalyzed by plant P450s.

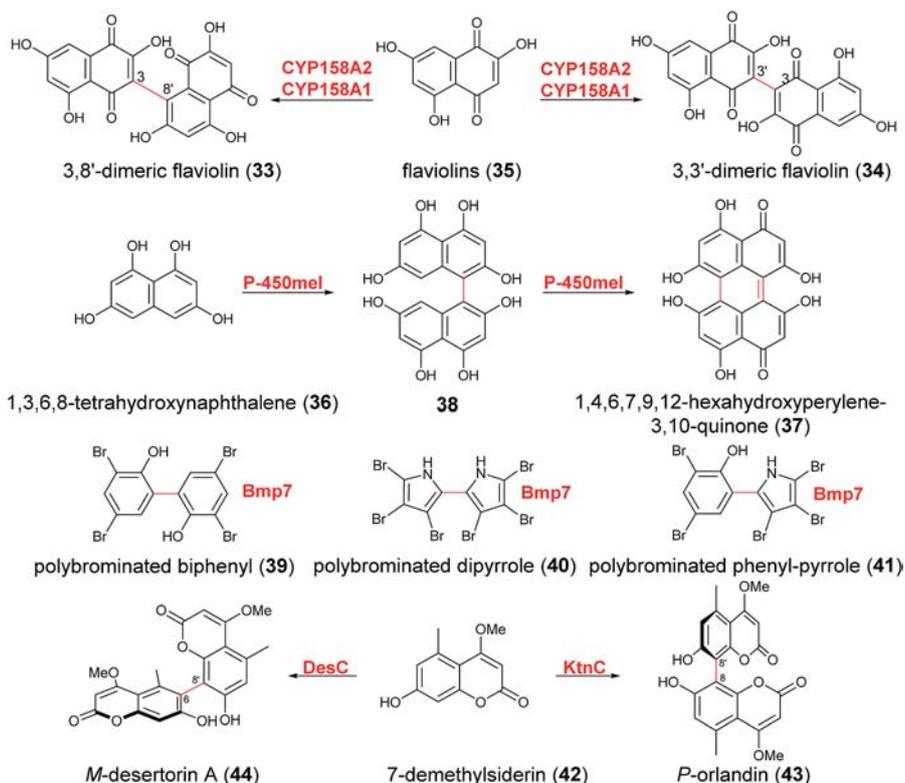
a CYP domain (CYP82Y2) and an aldo-keto reductase (AKR) domain. The P450 domain catalyzes the conversion of (*S*)-reticuline (29) to 1,2-dehydroreticuline (30), while the AKR domain converts 30 into 26 rather than functioning as a P450 redox partner.^{59,61,62} The transformation of (*S*)-reticuline (31) to (*S*)-corytuberine (32) in the biosynthesis of 28 is supposed to adopt a similar biradical mechanism with CYP719B1.

4.2.2 Intermolecular aromatic coupling. Besides intramolecular aromatic coupling, CYPs also catalyze intermolecular aromatic coupling reactions *via* similar biradical mechanisms (Scheme 8). Melanins and many pigments can physically protect the producing organisms from UV radiation.⁶³ A significant property of these pigments is the existence of aromatic conjugation systems in their structures, which are sometimes formed *via* dimerization of aromatic rings. Dimeric flaviols (33 and 34) are such red and brown pigments produced by *S. coelicolor* A3(2). The key step of their biosynthesis, oxidative C–C coupling between C3/C3' or C3/C8' of the type III polyketide synthase (PKS)-derived flaviols (35),⁶⁴ is achieved by the catalytic activity of two P450s (CYP158A1 and CYP158A2) from the same CYP sub-family.^{65,66} Similarly, P-450mel from *S. griseus* was found to catalyze the intermolecular dimerization of another type III PKS compound 1,3,6,8-tetrahydroxynaphthalene⁶⁷ (36) by two rounds of oxidative biaryl couplings to generate the highly conjugated 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone (37) *via* the intermediate 38.⁶⁸ Additional examples of P450-catalyzed biaryl coupling occur in the biosynthesis of polybrominated biphenyls (39), polybrominated dipyrroles (40), and polybrominated phenyl-pyrroles (41) from marine bacteria *Pseudalteromonas luteoviolacea* 2ta16 and *P. phenolica* O-BC30. It has been elucidated that the P450 enzyme Bmp7 is responsible for the central aryl–aryl coupling at different carbon sites of related mono-subunits.⁶⁹

Recently, two homologous fungal P450 enzymes, KtnC and DesC, were functionally identified to possess the capability of mediating aryl–aryl coupling reactions.⁷⁰ In brief, KtnC from *Aspergillus niger* catalyzes the C8/C8' coupling of 7-demethylsiderin (42) to afford *P*-orlandin (43), which is a precursor of kotanin. In contrast, DesC from *Emericella desertorum*, which shows 44% protein sequence identity to KtnC, demonstrates distinct regioselectivity by catalyzing the C6/C8' coupling in 42, leading to *M*-desertorin A (44) as a regioisomer of 43.

Dimeric indole alkaloids are a large subset of plant and fungal NPs that are usually derived from dimerization of mono-indole moieties. Examples include the tryptamine-derived calycanthaceous alkaloids such as calycanthine,⁷¹ chaetocin,⁷² perophoramidine,⁷³ and communesin.⁷⁴ A defining feature of these alkaloids is the presence of vicinal quaternary carbon stereocenters, which are fascinating from both synthetic and biosynthetic perspectives (Scheme 9). The enzyme responsible for the vicinal quaternary stereocenter formation remained a mystery for a long time until P450 CnsC from *Penicillium expansum* was recently identified to catalyze the heterodimeric coupling between two different indole-containing compounds, tryptamine (45)/*N*-methyltryptamine (46) and aurantioclavine (47), leading to the unique heptacyclic communesin or isocommunesin scaffolds (48 and 49).⁷⁵ Through elegant biochemical characterization of the activity of recombinant CnsC, together with sophisticated computational modelling by Tang and co-workers, a plausible mechanism for the formation of the highly interconnected ring system was proposed. Specifically, CnsC CpdI first generates the C3 radical of 45/46 and the C3' radical of 47, and next mediates the radical coupling to form the C3–C3' coupling intermediate with two vicinal quaternary stereocenters. Then, the two 3*H*-indoles in the two halves of the molecule are subjected to intramolecular nucleophilic attack from the two amine groups, thus forming the pair of aminal linkages in the communesin alkaloids. The computational calculations suggest that CnsC also controls the regioselectivity of the two aminal bonds, leading to alternative production of the communesin scaffold (48) or isocommunesin scaffold (49) depending on the identity of the N10 substituent of tryptamine.⁷⁵

An even more challenging aromatic coupling task is fulfilled by a P450 enzyme involved in the biosynthesis of himastatin (50), a novel dimeric cyclohexadepsipeptide antibiotic with striking structural features (Scheme 10).^{76,77} From the biosynthetic pathway of himastatin in *S. hygroscopicus* ATCC 53653, three P450 enzymes, namely HmtN, HmtS, and HmtT, have been functionally characterized.^{78,79} In turn, HmtT acts as an oxidative cyclase, which converts the indole ring of the Trp residue in the cyclohexadepsipeptide substrate (51) into a pyrroloindole moiety in 52, putatively through epoxidation of the indole ring and the subsequent electron rearrangement and



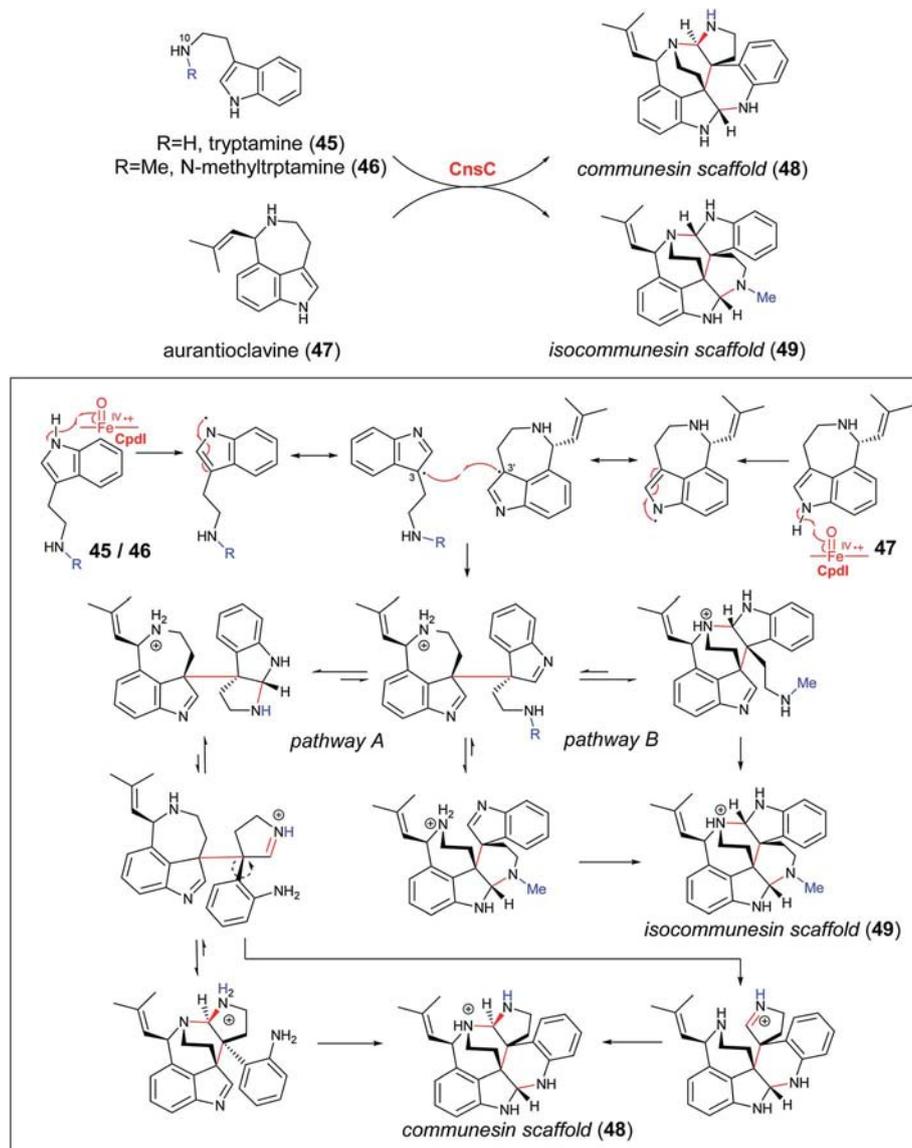
Scheme 8 The intermolecular aryl–aryl coupling reactions mediated by microbial P450s (DesC and KtnC are of fungal origin, while the other P450s are from bacteria).

cyclization. Next, HmtN is responsible for a common hydroxylation at the C3 position of the piperazine residue of 52 to yield 53. Finally, HmtS catalyzes the symmetrical and regioselective biaryl coupling of the two monomers of 53.^{78,79} Mechanistically, it would be fascinating to solve the structure of the P450–substrate complex to visualize how the two bulky monomers could be accommodated by the catalytic pocket of HtmS in order for a probable diradical coupling reaction to occur.

4.2.3 Ring formation. In the skeleton construction of NPs, ring forming reactions play fairly important roles since they can significantly change the shape, configuration, rigidity, and hence the bioactivity of NPs. In recent years, a growing number of P450s have been reported to catalyze intramolecular ring formation by varying mechanisms. Viridicatumtoxin (54) is a fungal meroterpenoid with a unique monoterpene-derived spirobicyclic ring fused to a PKS-derived tetracycline-like scaffold as shown in Scheme 11.⁸⁰ The very last step of its biosynthesis in *P. aethiopicum* is the cyclization of the geranyl moiety in previridicatumtoxin (55). Strikingly, a cytochrome P450 enzyme VrtK, rather than a terpene cyclase, was identified as the sole enzyme required for construction of the spirobicyclic ring system.⁸¹ Based on biochemical evidence and corresponding computational quantum chemistry calculations, the newly proposed mechanism for the P450-catalyzed terpene cyclization involves an initial dehydrogenation of the allylic C17 position of 55 to afford a carbon radical intermediate, which is followed by electron transfer to the heme-iron center to form a key

carbocation intermediate. The cyclopentane intermediate is then generated by the C–C bond formation between C15 and C19. Next, a ring expansion (1,2-alkyl shift) and 1,3-hydride shift through a single-step reaction, in which these two events are combined asynchronously, give the penultimate intermediate. The final product 54 is produced by further C7/C15 cyclization and H7 dehydrogenation coupled with the electron rearrangement for rearomatization.^{81,82} As the first CYP capable of catalyzing terpene cyclization, VrtK is functionally analogous to a class II terpene cyclase, which does not require the presence of the allylic diphosphate ester, but promotes cyclization by protonating a terminal double bond. Interestingly, the albaflavone synthase CYP170A1 from *S. coelicolor* A(3)2 was discovered to have a second distinct active site responsible for the moonlighting class I terpene synthase activity, which can convert farnesyl diphosphate into farnesene isomers.⁸³ Together, these unexpected discoveries highlight the distinctive ability of P450s to explore novel chemical reactions.

4.2.4 Ring expansion. P450-mediated ring expansion is another important type of skeleton construction reaction in NP biosynthesis (Scheme 12). The biosynthesis of tenellin (56), a 2-pyridone NP produced by the entomopathogenic fungus *Beauveria bassiana*,⁸⁴ involves a representative type of P450-catalyzed ring expansion.⁸⁵ In the iterative PKS–NRPS hybrid biosynthetic pathway of 56, the P450 enzyme TenA catalyzes an unprecedented oxidative ring expansion of pretenellin-A (57) to form pretenellin-B (58). A likely mechanism for such a unique



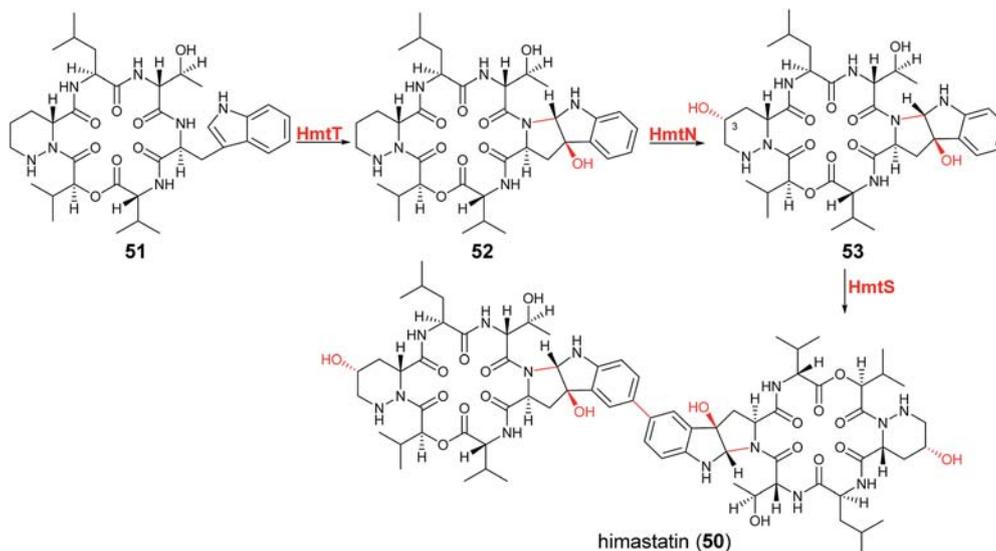
Scheme 9 The vicinal quaternary stereocenter formation mediated by CnsC from the fungus *P. expansum* in the biosynthesis of communesin, and the hypothetical mechanisms (boxed).

rearrangement initiates from hydrogen atom abstraction by Cpdl at the benzylic position of 57. Next, the benzylic radical induces a cascade of electron arrangements for ring expansion, and a second round of hydrogen abstraction results in the formation of a 2-pyridone moiety.⁸⁵ This mechanism could be widely adopted by an array of fungal P450s, leading to the production of fischerin,⁸⁶ leporine-B,⁸⁷ PF1140,⁸⁸ sambutoxin,⁸⁹ aspyridone-A,⁹⁰ and so on.

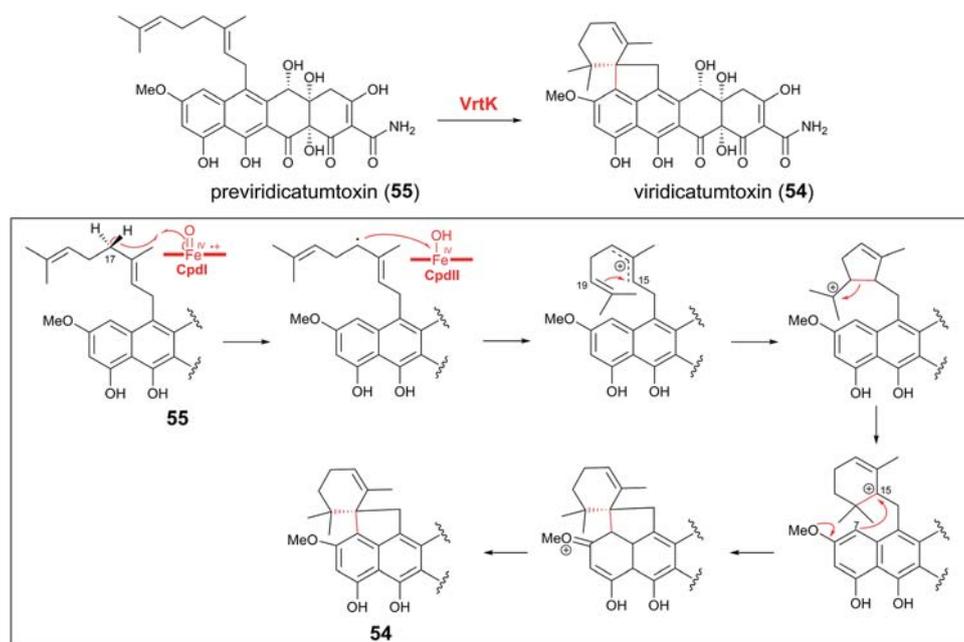
Penitremes are a class of fungal neurotoxin that possesses an unusual tricyclic system merged to the paxilline core structure (Scheme 12).⁹¹ According to a study on the biosynthetic pathway of penitremes in *A. oryzae* NSAR1,⁹² the structures of these fungal indole diterpenes are heavily elaborated by six P450 enzymes including PtmP, PtmQ, PtmK, PtmU, PtmL, and PtmJ. Among these CYPs, PtmK and PtmU involved in the unique tricyclic skeleton construction are particularly interesting. PtmK

catalyzes the oxidative ring expansion of PC-M4 (59) perhaps via a TenA-like mechanism.⁸⁵ Alternatively, it may adopt a similar ring expansion process during cephalosporin biosynthesis, which is mediated by a non-heme iron/ α -ketoglutarate-dependent dioxygenase.⁹³ As for the octatomic ether ring formation of secopenitrem D (60), it is rather difficult to achieve this transformation using chemical synthesis due to non-bonding transannular repulsion. However, it seems that PtmU readily catalyzes the sequential hydroxylation and dehydration to afford penitrem D (61).

Brassinosteroids have been recognized as the sixth class of plant hormone.⁹⁴ Some of these polyhydroxysteroids, such as brassinolide (62), structurally feature a 7-membered lactone ring instead of the normal hexatomic B-ring in common steroids (Scheme 13). Biosynthetically, 6-deoxocastasterone (63) derived from campesterol is firstly ketonized at C6 by CYP85A1/



Scheme 10 The post-NRPS tailoring pathway of himastatin co-mediated by three P450 enzymes including HmtT, HmtN, and HmtS from *S. hygroscopicus* ATCC 53653.

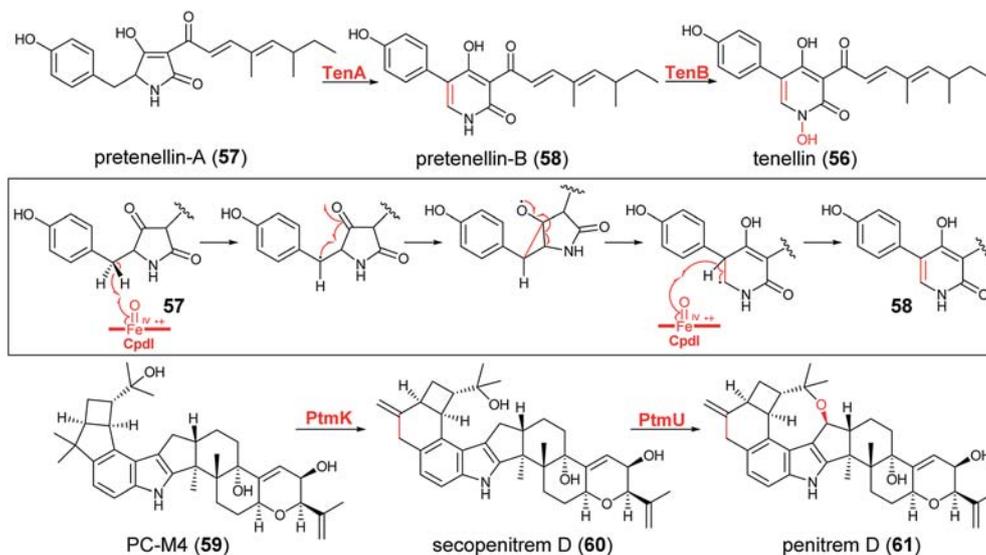


Scheme 11 The unique terpene cyclase-like activity of VrtK from the fungus *P. aethiopicum* in the biosynthesis of viridicatumtoxin, and the proposed mechanism (boxed).

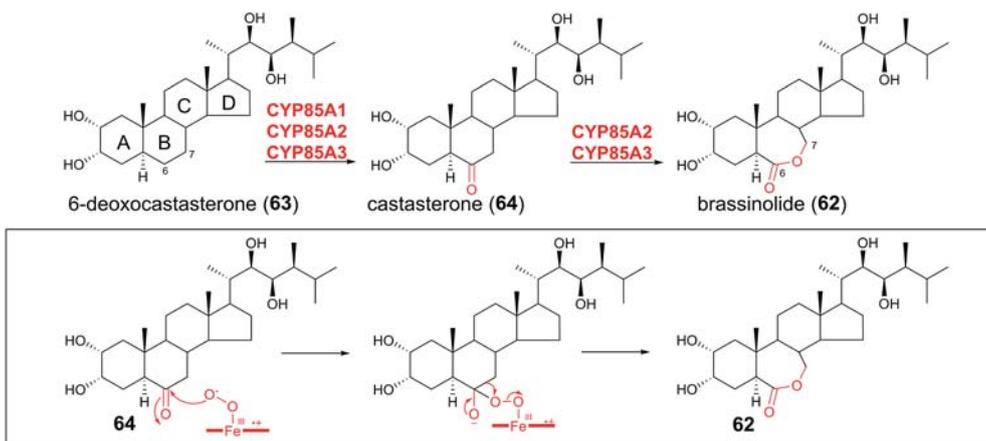
CYP85A2/CYP85A3 to form the intermediate castasterone (**64**), and the key Baeyer–Villiger-type mono-oxygenation leading to **62** is catalyzed by either CYP85A2 from *Arabidopsis thaliana* or CYP85A3 from tomatoes.^{95–98} Mechanistically, the peroxy-iron anion ($\text{Fe}^{\text{III}}\text{--O--O}^-$) nucleophilically attacks the C6 carbonyl carbon of **64**. The following B-ring expansion is driven by a series of electron rearrangements. This and the example shown in Scheme 11 clearly indicate that it could be misleading to speculate the type of biocatalyst responsible for a specific

biosynthetic reaction simply based on the structural feature of a given NP.

Similar oxidative ring expansions *via* oxygen atom insertion were also observed during the biosynthesis of austinols and terretonin H (**65**) (Scheme 14). Briefly, the P450 AusI from *A. nidulans* is able to transform iso-austinone (**66**) into austinolide (**67**) by inserting an oxygen atom between C3' and C4' of **66** to create the δ -lactone ring in **67**.⁹⁹ Trt6 from *A. terreus* is responsible for converting terretonin (**68**) into terretonin H (**65**).¹⁰⁰ Two conformation-dependent routes involving a key nucleophilic



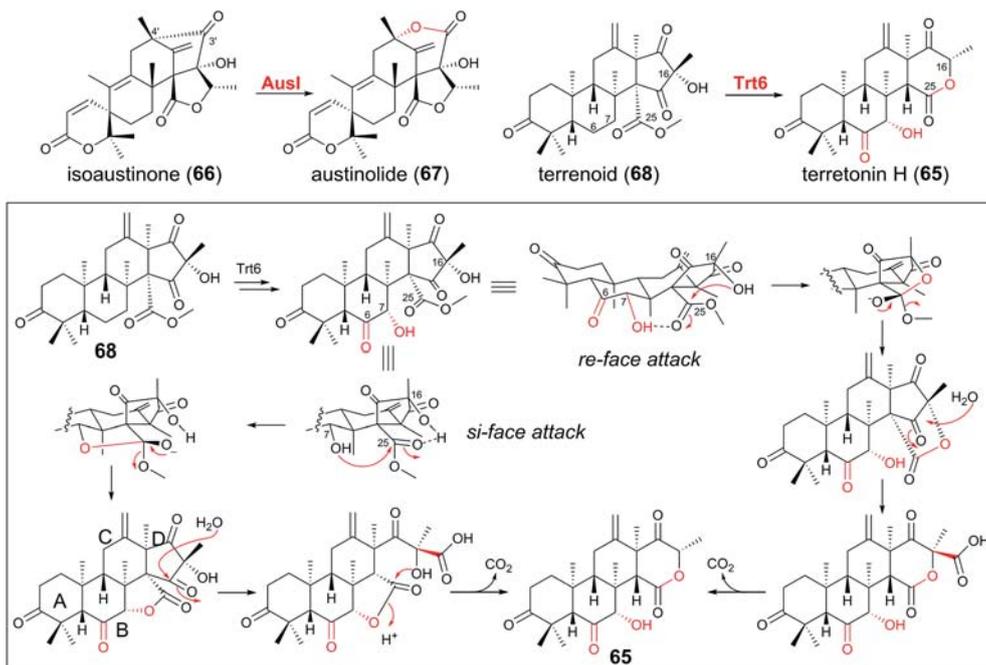
Scheme 12 The ring expansion reactions catalyzed by different fungal P450s, and the related mechanism (boxed).



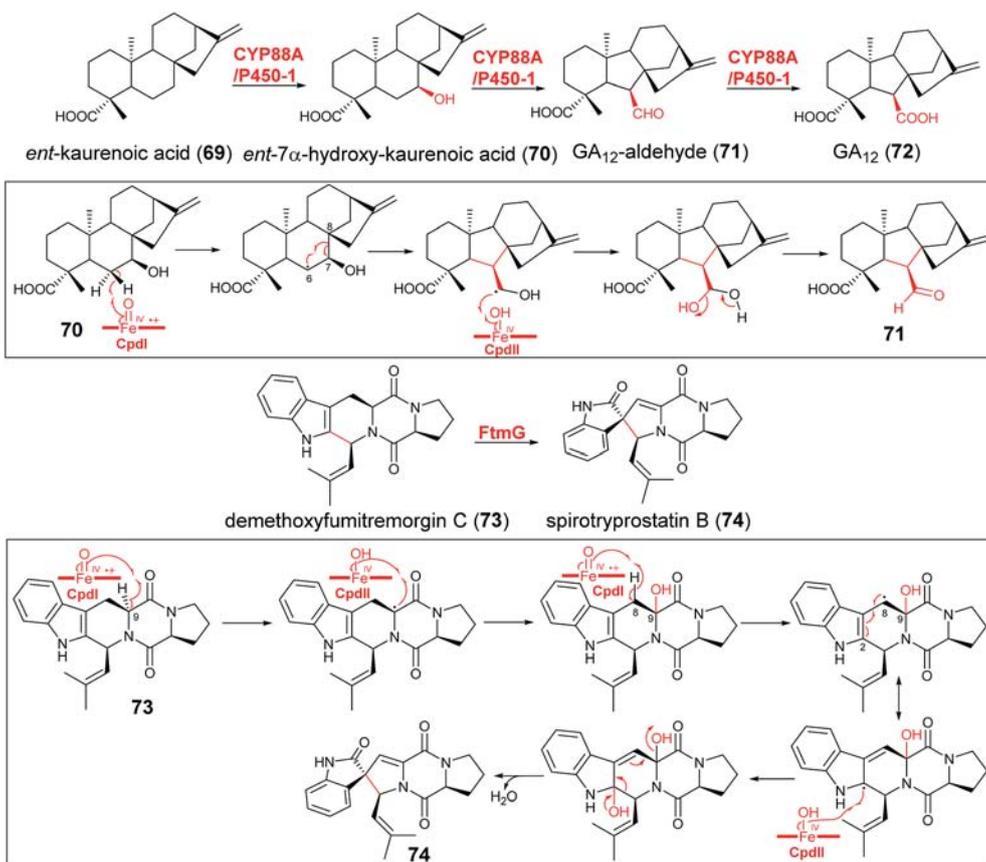
Scheme 13 The Baeyer–Villiger-type ring expansion catalyzed by plant CYP85A sub-family enzymes, and the proposed mechanism (boxed).

attack from either the *re*-face or the *si*-face were proposed by Matsuda *et al.* In both processes, Trt6 initially oxidizes **68** to form the intermediate bearing the C6 carbonyl and the C7 hydroxyl groups. The hydrogen bond between the α -oriented C7 hydroxyl group and C25 carbonyl oxygen creates a favourable conformation to induce the lactonization *via* the nucleophilic attack by the C16 alcohol (*re*-face attack). Then, after the formation of the unstable γ -lactone, retro-Claisen-type cleavage happens at the β -ketoester moiety to yield the ring-expanded β -keto acid. Finally, a spontaneous decarboxylation occurs to yield **65**. Alternatively, if the hydrogen bond is formed between the α -oriented C16 hydroxyl group and the C25 carbonyl oxygen, the C7 alcohol would first attack the C25 carbonyl carbon (*si*-face attack) to produce a different γ -lactone that is interconnected with ring B and ring C. Subsequently, the above-mentioned retro-Claisen-type cleavage occurs and transforms the γ -lactone to a more stable one *via* lactonization between the C16 alcohol and C25 carbonyl.¹⁰⁰

4.2.5 Ring contraction. Other than ring expansion, P450-guided oxidation can also induce ring contraction through different mechanisms (Scheme 15). Gibberellins are important plant hormones that regulate various developmental processes.¹⁰¹ These tetracyclic diterpenoids are derived from *ent*-kaurenoic acid (**69**).¹⁰² Based on recent studies,¹⁰³ in *A. thaliana*, CYP88A first hydroxylates **69** in a regioselective manner, giving rise to the intermediate *ent*-7-hydroxykaurenoic acid (**70**). Then, the key ring contraction of **70** and the following consecutive oxidations, namely **70** \rightarrow GA₁₂-aldehyde (**71**) \rightarrow GA₁₂ (**72**), are mediated by the same P450 CYP88A. The ring contraction is proposed to follow a carbon radical mechanism, which involves the generation of the C6 radical followed by electron rearrangements resulting in the extrusion of C7. The further C7 hydroxylation forms a geminal diol intermediate, which is unstable and undergoes spontaneous dehydration to give GA₁₂-aldehyde (**71**).⁹⁸ Interestingly, the same biosynthetic pathway can also be achieved by a microbial P450 enzyme P450-



Scheme 14 The ring expansion with oxygen insertion catalyzed by fungal P450s AusI and Trt6, and the putative Trt6 mechanism (boxed).



Scheme 15 The ring contraction reactions catalyzed by CYP88A (plant origin), and P450-1 and FtmG (fungal origin). The proposed mechanisms are shown in the boxes.

1 from the plant-pathogenic bacterium *Gibberella fujikuroi*, which suggests an event of horizontal gene transfer between plants and microorganisms.¹⁰⁴

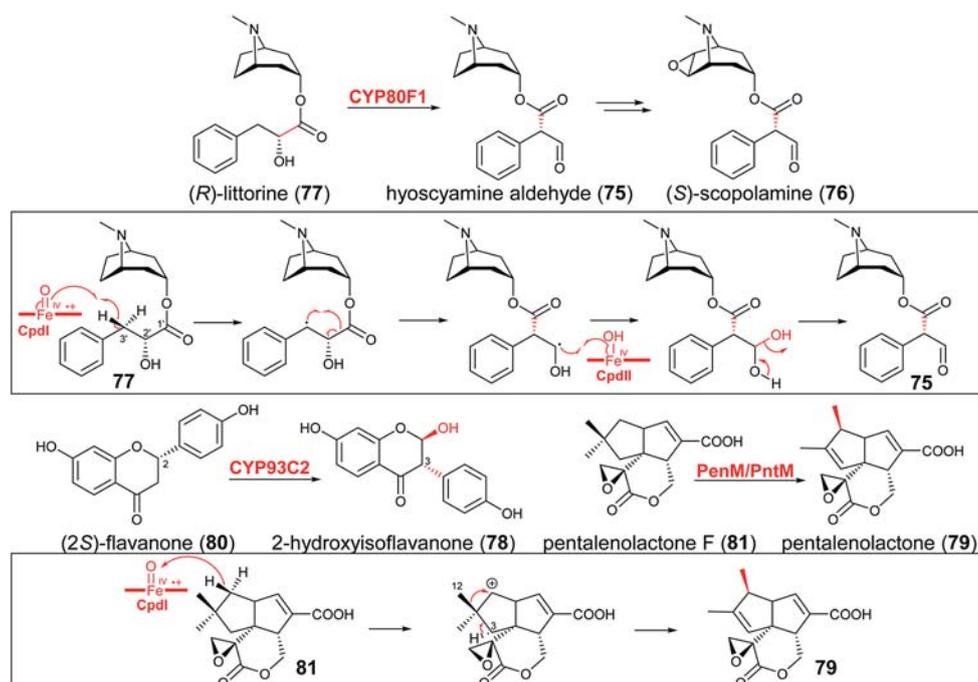
The fungal NPs spirotryprostatins have drawn much attention due to their chemically complex spiro-carbon-bearing scaffold and their anti-mitotic activity towards tumor cells.¹⁰⁵ Recently, Watanabe and co-workers¹⁰⁶ elucidated the cryptic transformation from demethoxyfumitremorgin C (73) to spirotryprostatin B (74) in *A. fumigatus*, which is mediated by a P450 enzyme FtmG (Scheme 15). Specifically, FtmG first introduces a hydroxyl group at the C9 position of the diketopiperazine containing substrate 73. Next, the C8 radical formation and the C8-to-C2 radical migration induces the second hydroxylation at the C2 position, which further undergoes a semipinacol-type rearrangement involving a concomitant spiro-ring formation with dehydration to yield 74. Intriguingly, a highly similar conversion from fumitremorgin C to spirotryprostatin A in the same microorganism was revealed not to be fulfilled by FtmG or another P450 enzyme. Instead, an FAD-dependent monooxygenase FqzB is responsible for this transformation *via* an epoxidation-initiated pinacol-like rearrangement.^{106,107}

4.2.6 Group migration. Hyoscyamine aldehyde (75) is the direct precursor of (*S*)-scopolamine (76), a drug used to treat motion sickness and post-operative nausea and vomiting.¹⁰⁸ It is biosynthesized by carbon skeleton rearrangement of the tropane ring group in (*R*)-littorine (77, Scheme 16).¹⁰⁹ Such a group migration is mediated by CYP80F1, a plant P450 enzyme involved in the biosynthetic pathway of 76 in *Hyoscyamus niger*. The unusual rearrangement is putatively initiated by CpdI-mediated hydrogen abstraction at C3' and followed by 1,2-ester carbonyl migration from C2' to C3'. The provisional

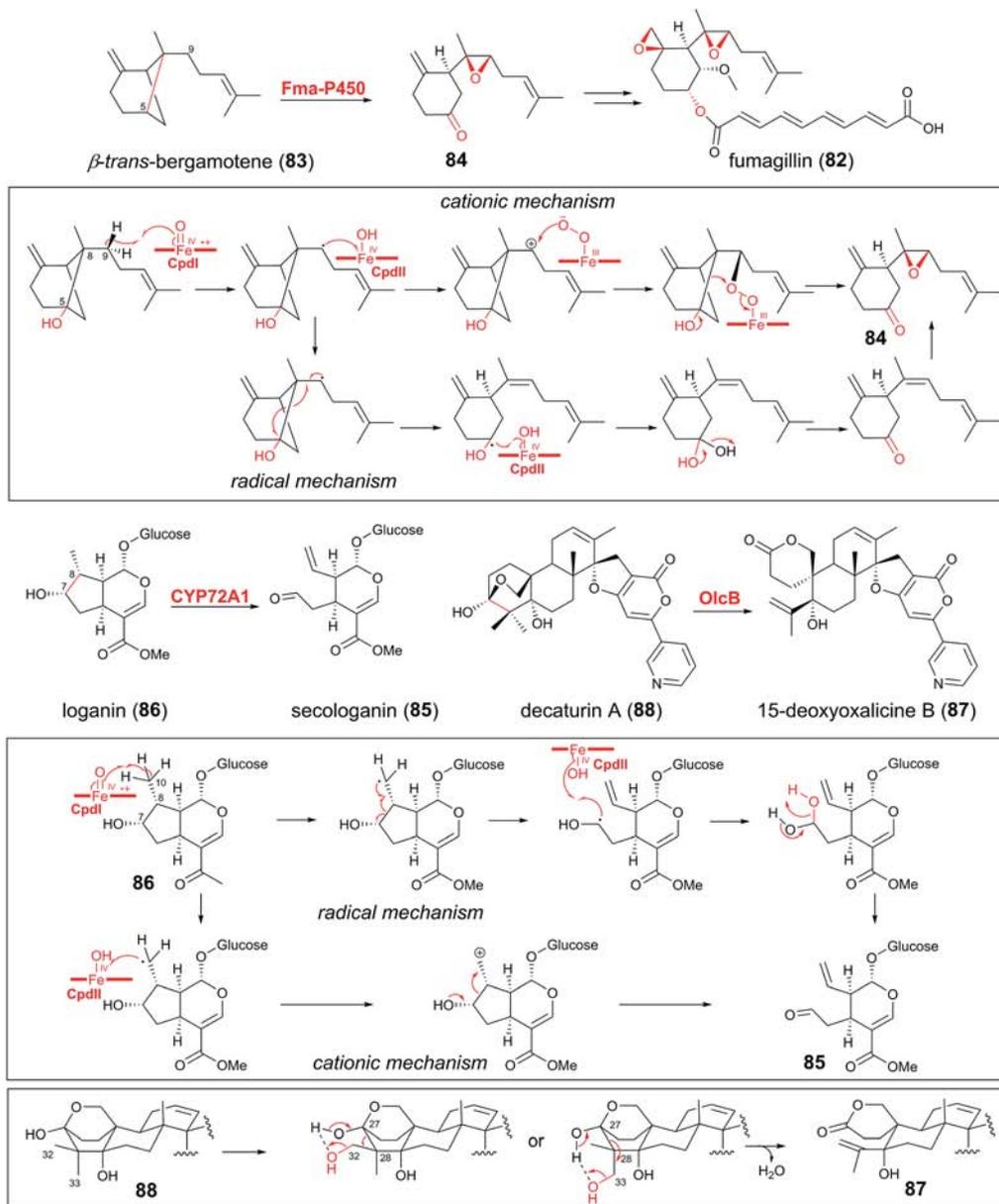
radical at C2' is then hydroxylated by CpdII, followed by spontaneous dehydration, giving rise to 75.^{110,111}

Similar C–C bond migrations are also involved in the biosynthesis of plant NP 2-hydroxyisoflavanone (78) and the bacterial sesquiterpenoid antibiotic pentalenolactone (79) as shown in Scheme 16. Compound 78 is a chemical defence agent mainly found in leguminous plants, and originates from (2*S*)-flavanone (80) *via* rearrangement of the phenolic ring from the C2 to C3 position.¹¹² CYP93C2 from licorice *Glycyrrhiza echinata* L. was identified as a 2-hydroxyisoflavanone synthase by catalyzing the phenolic ring migration *via* a similar mechanism as that with CYP80F1.^{112,113} Pentalenolactone (79) is a broadly occurring bacterial NP that has been isolated from more than 30 species of *Streptomyces*.^{114–118} The final step in the biosynthetic pathway of 79 is the 1,2-methyl migration in pentalenolactone F (81), and can be mediated by either the P450 enzyme PenM from *S. exfoliatus* UC5319 or PntM from *S. arenae* TŪ469. To explain the methyl group migration, a Wagner–Meerwein-type carbocation mechanism was proposed, in which a hydride abstraction by CpdI leads to the formation of a carbocation. Subsequently, successive *syn*-migration of the C12 methyl group and coupled *anti*-deprotonation of H3 results in the formation of 79. However, the usual P450-catalyzed radical rearrangement could not be definitively ruled out.¹¹⁹

4.2.7 C–C bond cleavage. The above-described ring expansion, ring contraction, and group migration all involve C–C bond cleavage. In this subsection, several examples of C–C bond scission resulting in ring opening are discussed. Fumagillin (82) is a highly oxygenated meroterpenoid produced by *A. fumigatus* (Scheme 17).¹²⁰ The multifunctional CYP Fma-P450 was shown to play a central role in both the construction and tailoring of



Scheme 16 The group migration reactions catalyzed by CYP80F1 and CYP93C2 (plant origin) and PenM/PntM (bacterial origin) *via* two distinct mechanisms (boxed).



Scheme 17 The ring opening reactions resulted from C–C bond cleavage that are mediated by Fma-P450 and OlcB (fungal origin) and CYP72A1 (bacterial origin) via different mechanisms (boxed).

the sesquiterpenoid core skeleton.¹²¹ The first oxidative C–C bond cleavage of the bicyclic sesquiterpene *β*-trans-bergamotene (83) is mechanistically attractive. A plausible cationic mechanism was recently proposed by Tang and co-workers that initiates from an early normal hydroxylation of C5, and is followed by CpdI-mediated hydrogen abstraction and CpdII-directed one-electron oxidation to give a C9 carbocation. The unstable hydroxy cyclobutylcarbanyl cation is then attacked by the nucleophilic peroxy-iron anion ($\text{Fe}^{\text{III}}\text{-O-O}^-$) to form a transient complex, which induces the central ring cleavage between C5 and C8 via bond migration to give 84. Nonetheless, an alternative radical mechanism could not be excluded, in which the C–C bond cleavage might occur in the earlier C9 radical.¹²¹

Secologanin (85), a precursor of monoterpene indole alkaloids such as ajmaline, vincristine and vinblastine, is produced by CYP72A1 through direct cleavage of the cyclopentane ring between C7 and C8 of loganin (86) in *Catharanthus roseus* (Scheme 17).¹²² The ring cleavage mechanism likely involves the abstraction of hydrogen at the C10 methyl group followed by electron rearrangement and the key C–C bond cleavage between C7 and C8. The newly generated carbon radical at C7 is then converted into a geminal diol, finally yielding an aldehyde group by spontaneous dehydration.^{123,124} An alternative cationic mechanism was also proposed: the C10 radical could undergo further one-electron oxidation to give the C10 cation, which is able to induce the C–C bond cleavage directly.¹²⁴

15-Deoxyoxalicine B (**87**) is another fungal meroterpenoid consisting of a unique pyridinyl- α -pyrone PKS subunit and a diterpenoid subunit connected through a characteristic asymmetric spiro-carbon (Scheme 17). From the **87** biosynthetic pathway in *P. canescens*, a predicted P450 enzyme OlcB was proposed to catalyze the final oxidative rearrangement of the direct precursor decaturin A (**88**).¹²⁵ The ring cleavage presumably occurs *via* the formation of either a C32- or C33-hydroxylated intermediate, in which an intramolecular hydrogen bond would form between the two hydroxyl groups, thus providing a favourable conformation to facilitate electron rearrangement for the C–C bond cleavage between C27 and C28.

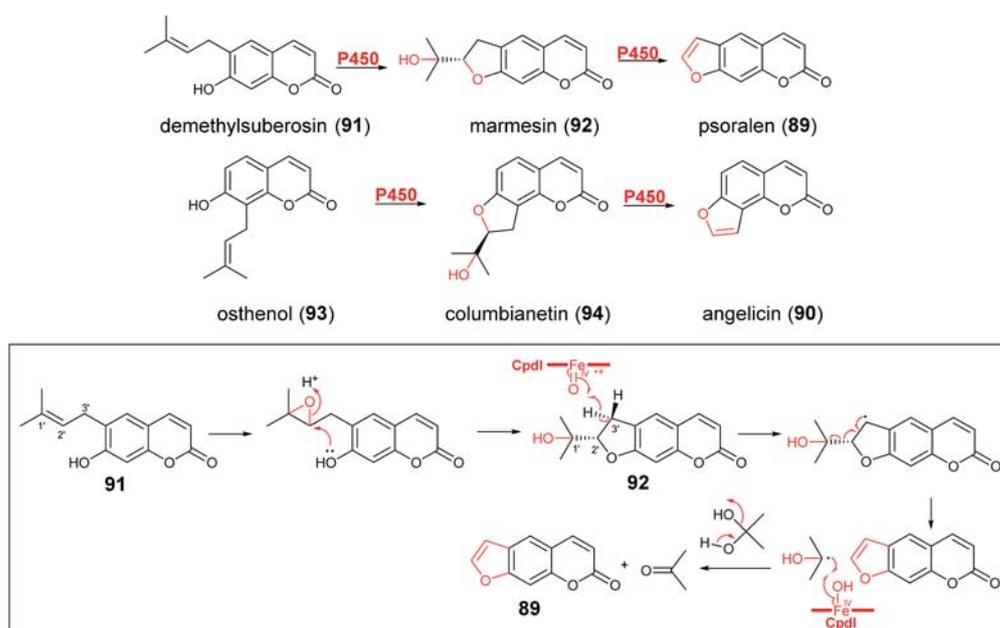
Furanocoumarins are a group of plant NPs structurally featuring a furan moiety fused to benzopyran-2-one, of which psoralen (**89**, a linear furanocoumarin) and angelicin (**90**, an angular furanocoumarin) are two representative compounds (Scheme 18).¹²⁶ Regarding the biogenesis of **89**, an uncharacterized P450 marmesin synthase from *Ammi majus* is hypothesized to catalyze the initial conversion from demethylsuberosin (**91**) to marmesin (**92**) *via* oxidative cyclization.¹²⁷ Subsequently, CYP71AJ1 is responsible for converting **92** into **89** through C–C bond cleavage.^{128,129} With respect to the catalytic mechanism of P450 marmesin synthase, it might go through a classical epoxidation process, in which the C1' = C2' epoxidation would induce the intramolecular nucleophilic attack by the phenolic OH group, giving rise to **92**. As for the following C–C bond cleavage catalyzed by CYP71AJ1, it is likely initiated by abstraction of a hydrogen atom from the C3' position of **92**, followed by the key electron rearrangement resulting in the sp³ carbon bond cleavage. The final oxygen rebound to the 2-propanol radical would produce a molecule of acetone and reset the resting state of CYP. The biosynthesis of the angular furanocoumarin **90** from osthenol (**93**) *via* columbianetin (**94**) is

likely mediated by other P450s with mechanisms similar to those for **89**.¹³⁰

4.2.8 Other uncommon P450 reactions for skeleton construction. Camalexin (**95**) is a phytoalexin produced by the model plant *Arabidopsis thaliana*.¹³¹ It is fantastic that **95** can be completely assembled by P450 enzymes (Scheme 19).^{132–135} The cascade of reactions begin with the CYP79B2/CYP79B3-catalyzed oxidative decarboxylation of tryptophan (**15**) to indole-3-acetaldoxime (**96**), followed by the CYP71A13-mediated formation of Cys-indole-3-acetonitrile (**97**) *via* indole-3-acetonitrile (**98**) from **96**. After that, CYP71B15 takes over the final thiazoline ring closure and decarboxylation steps to yield camalexin *via* the intermediate **99**.

Mechanistically, all these P450-catalyzed reactions, especially the CYP71A13-catalyzed C–S bond formation and the CYP71B15-catalyzed ring closure, appear to be intriguing. It was reported that the C–S bond is formed *via* an intermolecular nucleophilic reaction between cysteine and an intermediate derived from hydroxylation and dehydration of **98**, leading to **97**. The ring closure may be achieved by two rounds of intramolecular nucleophilic attack from the Cys nitrogen atom.^{132–135} By co-incubation of three P450s, CYP79B2, CYP71A13, and CYP71B15, the substrates Trp and Cys, and the necessary redox partners and cofactors, the Sattely group successfully reconstituted the camalexin biosynthetic pathway *in vitro*.¹³⁴

In some cases, P450s can cause drastic structural transformations through very complicated rearrangements. CypX, AflN, and OrdA are such amazing P450s among the fifteen enzymes involved in aflatoxin biosynthesis.¹³⁶ Aflatoxins, a group of PKS-derived NPs produced mainly by strains of the fungal genus *Aspergillus*, are highly poisonous and carcinogenic to animals and humans.^{137,138} Exposure to a trace amount of aflatoxins could result in hepatotoxicity, teratogenicity,



Scheme 18 The P450-catalyzed C–C bond cleavage in the biosynthesis of plant furanocoumarins, and the proposed mechanism (boxed).

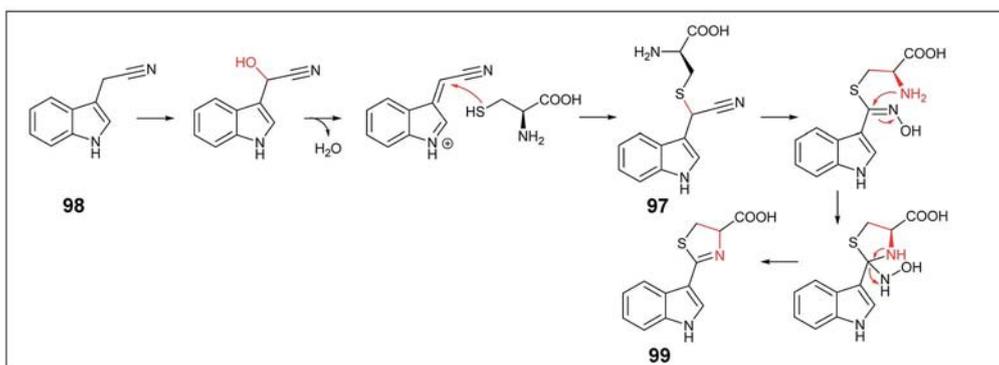
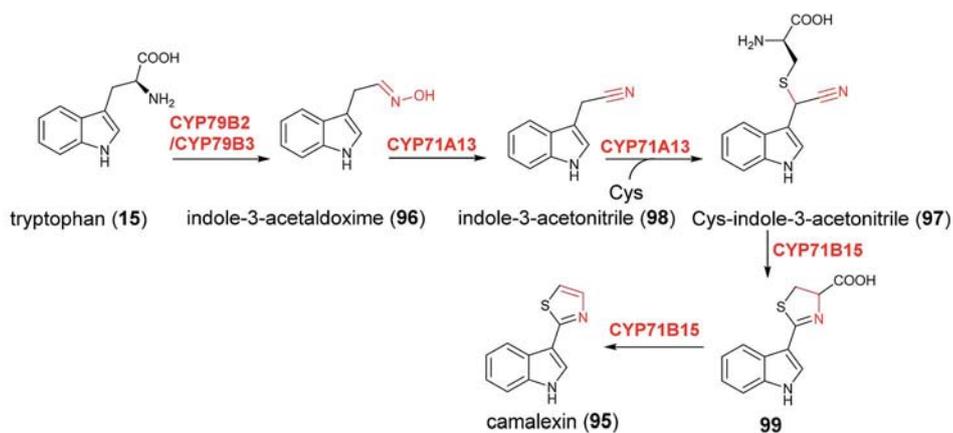
immunotoxicity, or even death. Aflatoxin B₁ (**100**) is the most common and toxic derivative, whose biogenesis requires several key P450s.¹³⁶ In the early benzobisfuran formation step in *A. parasiticus* NRRL 2999, the conversion from averufin (**101**) to hydroxyversicolorone (**102**) is catalyzed by the P450 enzyme CypX (Scheme 20a).^{136,139} The drastic rearrangement is initialized by hydrogen atom abstraction, followed by dearomatizing electron rearrangements, giving rise to an unstable cyclopropane intermediate. Driven by rearomatization and tension release, the cyclopropane ring is immediately broken to give the oxy-methylene radical, to which the hydroxyl group of CpdII is rebound, inducing a second round of intramolecular bond rearrangements to yield a provisional oxonium ion. The oxonium ion intermediate then undergoes a hydrolyzed ring cleavage reaction and a further intramolecular nucleophilic attack to finally form the simplified tetracyclic scaffold of **102**.^{136,139}

Another essential oxidative transformation during the biosynthesis of aflatoxins is the conversion of the linear-shaped versicolorin A (**103**) to the angle-shaped demethylsterigmatocystin (**104**), which is co-mediated by a cytochrome P450 enzyme AflN and an NADPH-dependent oxidoreductase AflM (Scheme 20b).^{136,140} Hypothetically, AflN first catalyzes a normal epoxidation of **103**, then the opening of the unstable oxirane ring leads to a hydroxylated intermediate. After the AflM-mediated reductive step of the new-born C3 carbonyl group and a further dehydration, a second round of AflN-catalyzed

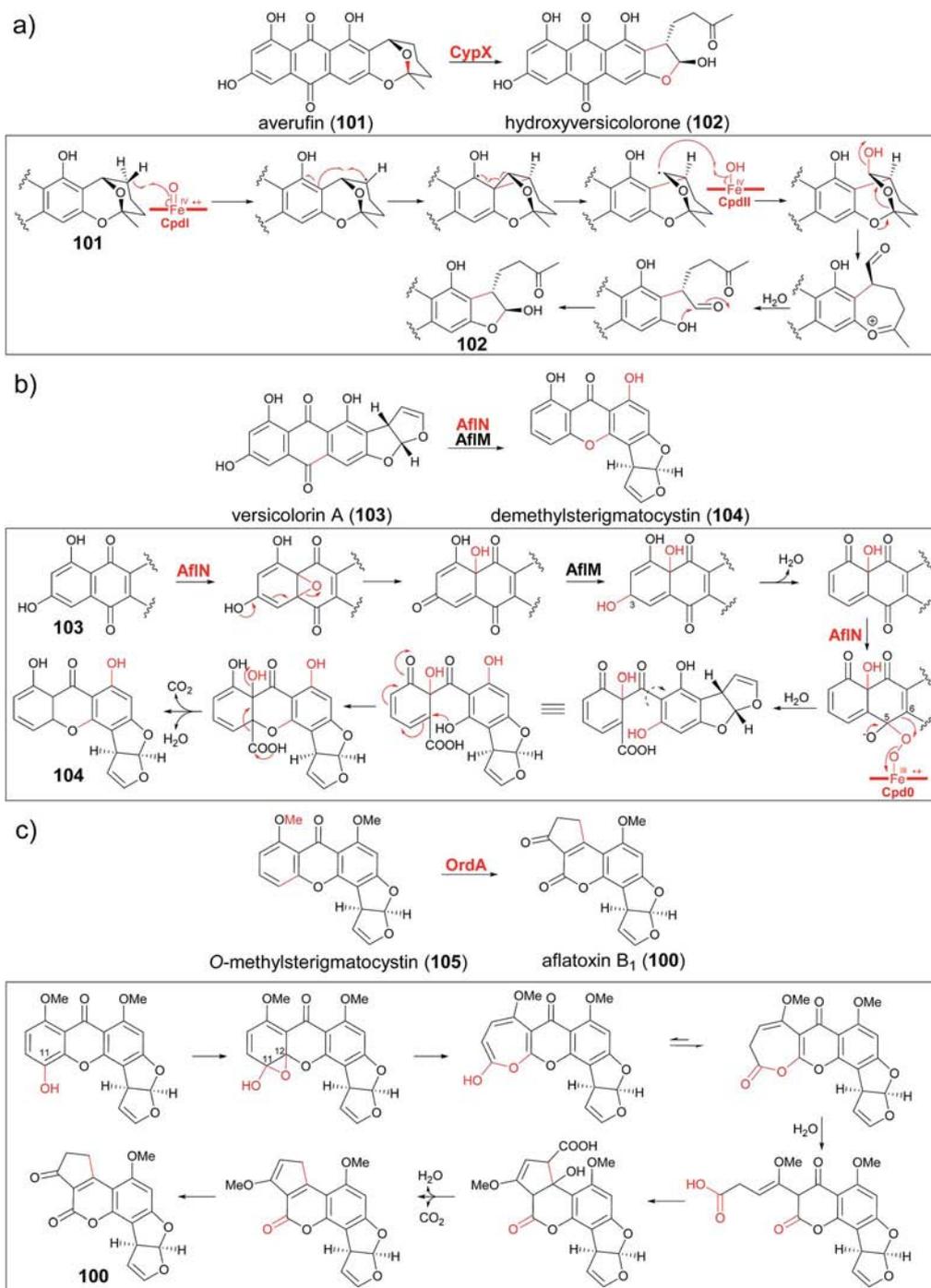
oxidation would occur through a Baeyer–Villiger-type cleavage of C5–C6, presumably by Cpd0. This ring opening event restores the conformational freedom of the connecting C–C bond, which enables the re-formation of a distorted ring system. Finally, **104** is formed by coupled decarboxylation and dehydration reactions.^{140,141}

In the final post-PKS tailoring step of the aflatoxin B₁ (**100**) biosynthetic pathway, P450 OrdA is responsible for transforming xanthone *O*-methylsterigmatocystin (**105**) into **100** (Scheme 20c).^{142,143} Again, the rearrangement cascade starts from the OrdA-mediated common hydroxylation of C11 in **105**, which is followed by a further epoxidation of C11=C12 to induce the Baeyer–Villiger-like ring expansion. The newly generated 7-membered lactone ring then undergoes a series of hydrolytic ring cleavage, ring reclosure, decarboxylation, and demethylation reactions to yield the final product aflatoxin B₁.¹⁴³ Overall, the whole post-PKS modification process is like an interesting game of snake cube.

One of the most famous meroterpenoids, paclitaxel (marketed as Taxol®) isolated from yew, is a clinically effective anticancer drug.^{144,145} Structurally, it contains a fused tricyclic skeleton, and taxadiene (**106**) has been identified as the initial precursor of the tricyclic skeleton.¹⁴⁶ In the yet to be completely elucidated biosynthetic pathway of paclitaxel, a number of P450s have been identified as important tailoring enzymes. 5 α CYP (CYP725A4) from *Taxus cuspidata* is one of such P450s that was reported to catalyze the first C5- α -hydroxylation of **106**



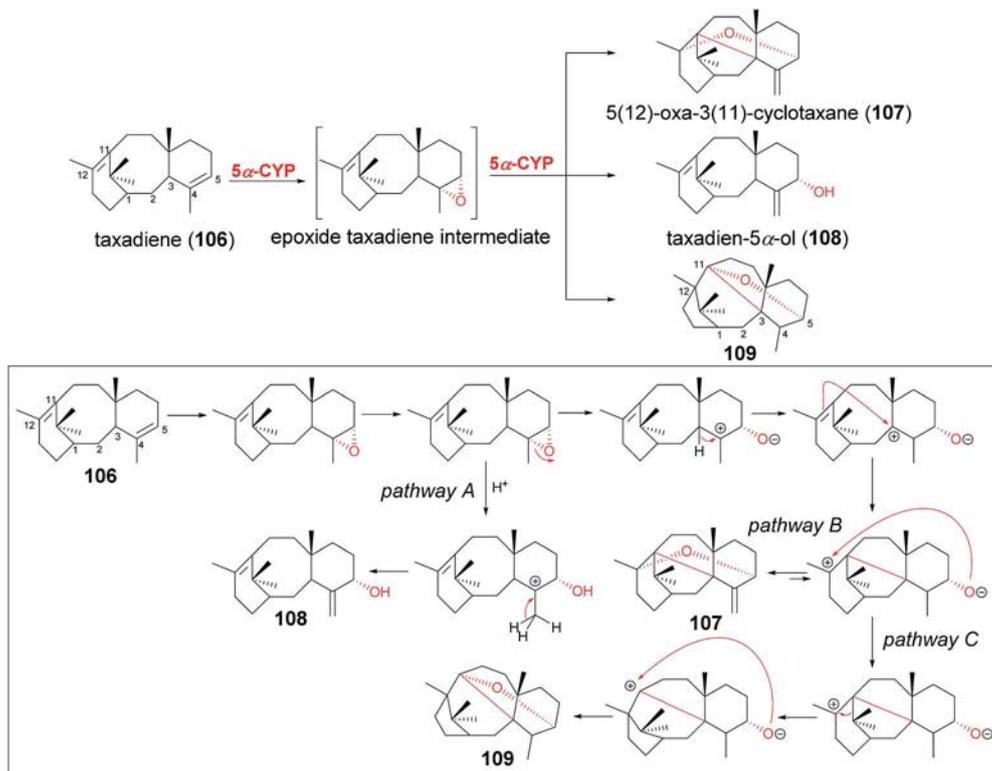
Scheme 19 The camalexin biosynthesis completely mediated by multiple P450s from *Arabidopsis* species, and the proposed mechanisms for the key steps including the intermolecular C–S bond formation and the subsequent ring closure (boxed).



Scheme 20 The drastic structural transformations catalyzed by CypX, AfIN, and OrdA in the biosynthesis of aflatoxins from the fungus *A. parasiticus*: (a) the CypX-catalyzed conversion of averufin to hydroxyversicolorone, (b) the conversion from the linear-shaped versicolorin A to the angle-shaped demethylsterigmatocystin co-mediated by P450 AfIN and the NADPH-dependent oxidoreductase AfIM, and (c) the final OrdA-catalyzed formation of aflatoxin B₁ from O-methylsterigmatocystin. The proposed mechanisms are shown in the boxes.

(Scheme 21).¹⁴⁷ However, a subsequent *in vitro* study showed that the primary enzymatic product of 5 α CYP was 5(12)-oxa-3(11)-cyclotaxane (**107**), instead of the expected taxadiene-5 α -ol (**108**), which challenges the initial finding and suggests that 5 α CYP is a P450 with poor selectivity.¹⁴⁸ More recently, based on the detection of multiple products, including a novel ring-

expanded product **109**, from both the *in vitro* assay and the native plant host, the Stephanopoulos group proposed an unstable epoxidized taxadiene to be the key intermediate.¹⁴⁹ The alleged poor selectivity of 5 α CYP should arise from non-selective degradation of the epoxide intermediate produced upon the selective oxidation of **106**. Specifically, three



Scheme 21 The oxidative transformations of taxadiene catalyzed by 5 α CYP from the plant *Taxus cuspidata* in paclitaxel biosynthesis, and the hypothetical mechanisms (boxed).

alternative pathways were proposed to explain the formation of **107–109** by 5 α CYP (Scheme 21). In pathway A, **108** is formed *via* oxirane ring opening and a following carbocation elimination. In pathway B, **107** is produced *via* charge transfer and the subsequent two rounds of nucleophilic attack by the C11=C12 olefin and the C5–oxygen anion, respectively. As for the newly observed **109**, it could be generated through an interposed ring expansion step of the imminent intermediate of **107** prior to the occurrence of the oxygen anion-initiated nucleophilic attack (pathway C).^{148,149}

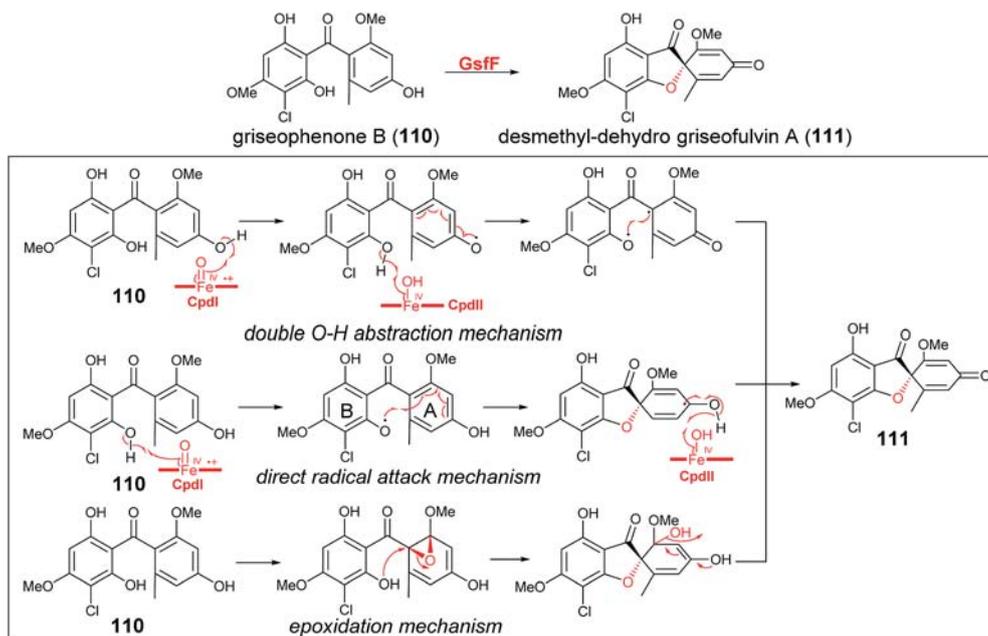
4.3 Structure re-shaping

Although some P450s do not change the carbon skeleton of NPs since no C–C bond formation or scission is involved, they still significantly modify the structural ‘phenotype’ of the given NPs. Here, such a group of P450 enzymes is named the structure re-shaping P450s. In this section, we will focus on these CYPs that mediate intra- or intermolecular C–O bond formation, which could result in an array of unusual structural transformations including ring closure, dimerization, intramolecular etherification, and others.

Griseofulvin, of interest in terms of its antifungal, anti-cancer, and antiviral activities, bears a structurally impressive and biosynthetically intriguing spirocyclic scaffold that is essential for its biological activities.¹⁵⁰ Recently, the P450 enzyme GsfF from *P. aethiopicum* was reported to catalyze the conversion from the benzophenone precursor griseophenone B

(**110**) to desmethyl-dehydro griseofulvin A (**111**) *via* intramolecular oxidative coupling between the orcinol and the phloroglucinol rings (Scheme 22).^{151,152} Three different mechanisms were proposed to rationalize the spirocyclic formation. Firstly, the classical biradical mechanism could be used to interpret such an intramolecular C–O coupling. Secondly, a new direct radical attack mechanism was proposed by Houk and co-workers based on density functional theory. Briefly, after hydrogen abstraction, the oxygen radical of ring B might directly attack the neighbouring arene to form the spirocycle. Thirdly, an epoxide intermediate could also be generated for further intramolecular nucleophilic attack. Through computational calculation of energy barriers, it was concluded that the phenolic C–O coupling in **111** prefers the direct radical attack mechanism to other mechanisms.^{151,152}

NPs containing a xanthone moiety are widely distributed in higher plants.¹⁵³ The biosynthesis of the xanthone scaffold is achieved by P450 enzymes belonging to the CYP81AA sub-family (Scheme 23a).^{154,155} After the common 3'-hydroxylation of 2,4,6-trihydroxybenzophenone giving rise to 2,3',4,6-tetrahydroxybenzophenone (**112**), CYP81AA1 continues to catalyze a cyclization of **112** at the *para* position to form 1,3,7-trihydroxyxanthenes (**113**), whereas the homologous enzyme CYP81AA2 catalyzes cyclization at the *ortho* position, yielding the isomeric 1,3,5-trihydroxyxanthone (**114**). Homology modeling and reciprocal mutagenesis revealed that the distinct regioselectivity of C–O phenol coupling by CYP81AA1 and

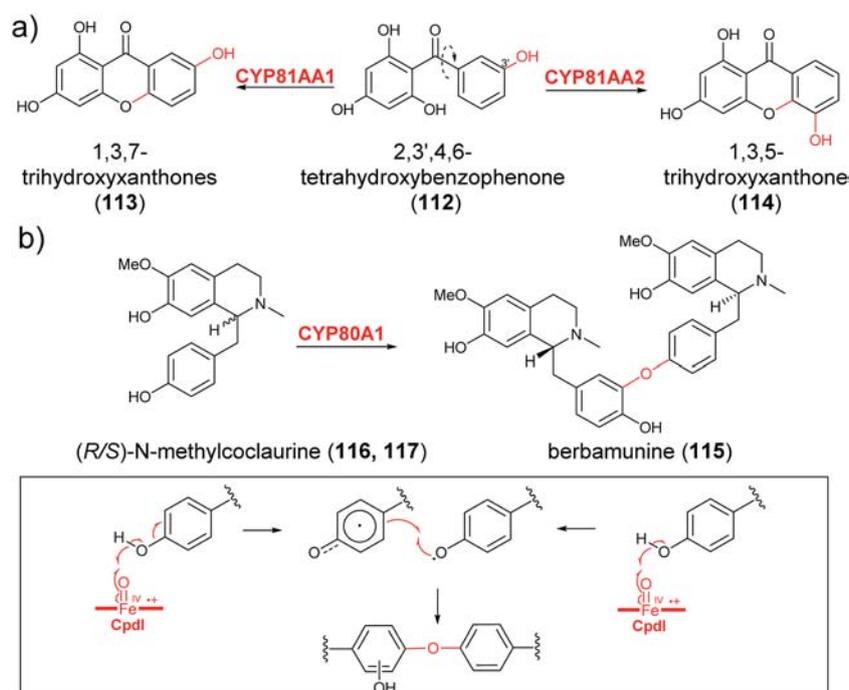


Scheme 22 The intramolecular C–O phenol coupling catalyzed by GsfF from the fungus *P. aethiopicum* in the biosynthesis of desmethyl-dehydro griseofulvin A, and the three hypothetical mechanisms (boxed).

CYP81AA2 is accurately controlled by specific amino acid residues (S375, L378, and A483) in the catalytic pocket.^{154,155}

Beyond intramolecular C–O coupling, P450s are also capable of catalyzing intermolecular phenol polymerization *via* the biradical mechanism that is also adopted by C–C coupling reactions. Berbamunine (**115**) is an unsymmetrical dimer

derived from a pair of epimers of *N*-methylcoclaurines (**116** and **117**), which are bisbenzylisoquinoline alkaloids. In the biosynthetic pathway of **115** from *Berberis stolonifera*, CYP80A1 was identified as the polymerase, catalyzing the key intermolecular C–O phenolic coupling step between **116** and **117** *via* the biradical mechanism as shown in Scheme 23b.¹⁵⁶



Scheme 23 The intramolecular (a) and intermolecular (b) C–O phenolic coupling catalyzed by different plant P450s, and the plausible biradical mechanism (boxed).

The intriguing methylene dioxy-bridge in many plant-derived isoquinoline alkaloids is another great aspect of CYPs, and it is produced *via* a ring closure reaction between a phenolic methoxyl and the *ortho*-phenolic hydroxyl group in the substrate (Scheme 24).^{157–159} It has been reported that several P450s within the same sub-family of CYP719A from Japanese goldthread *Coptis japonica* are methylene dioxy-bridge synthases. Specifically, CYP719A1 catalyzes the formation of (*S*)-tetrahydroberberine (**118**) from (*S*)-tetrahydrocolumbamine (**119**), CYP719A5 directs the conversion of (*S*)-scoulerine (**120**) to the monomethylene dioxy-bridge-containing (*S*)-cheilanthifoline (**121**), while CYP719A2 and CYP719A3 mediate further oxidative cyclization of **121** to yield the dimethylene dioxy-bridge-containing product (*S*)-stylopine (**122**). In addition, CYP719A9 from *Eschscholzia californica* is able to convert (*S*)-reticuline (**123**) into *N*-demethylation-escholinine (**124**). Similarly, CYP81Q1 from *Sesamum* sp. is responsible for the production of (+)-sesamin (**125**) from (+)-pinoresinol (**126**) *via* (+)-piperitol (**127**).^{157–159} A plausible oxonium ion mechanism was proposed as follows: firstly, a hemiacetal intermediate is formed by hydroxylation of the methoxy group. Then, the unstable hemiacetal initiates nucleophilic attack from the phenolic oxygen atom, leading to the key oxonium ion intermediate. This transient oxonium ion induces a second nucleophilic attack. Importantly, in the final step of methylene dioxybridge formation, the *ortho*-phenolic hydroxyl serves as the electron donor of the nucleophilic reaction.^{160,161}

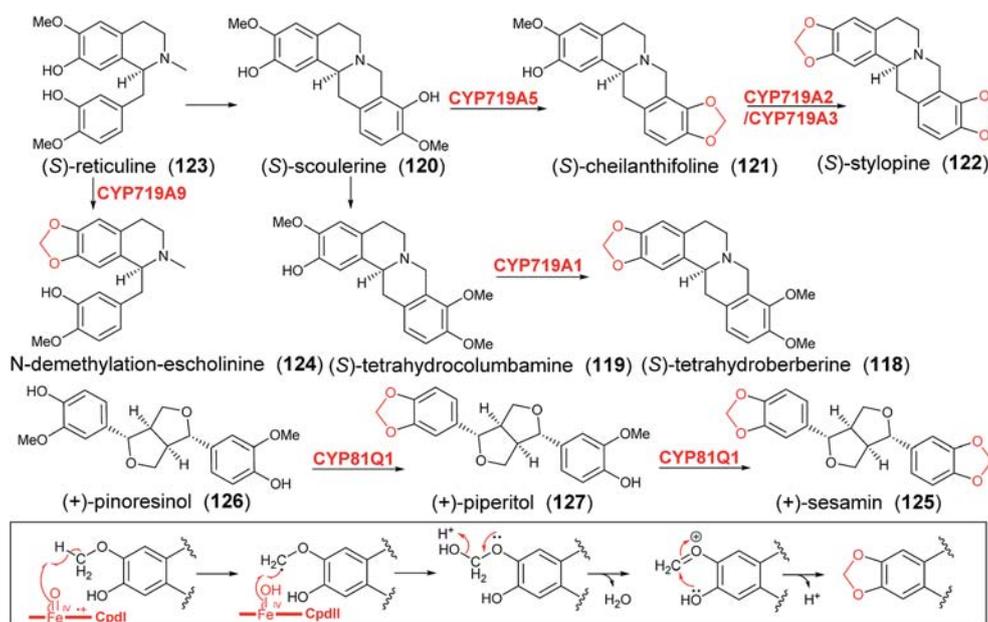
Aureothin (**128**) is a PKS-derived antibiotic possessing a unique tetrahydrofuran ring in its structure, and this ring is a vital component for its bioactivity (Scheme 25).¹⁶² In the aureothin biosynthetic pathway, AurH was identified to be a P450 enzyme responsible for the formation of the tetrahydrofuran ring when using deoxyaureothin (**129**) as the substrate.^{163–166}

Such a structural fine-tuning originates from the stereospecific C7(*R*) hydroxylation of **129**, a common P450 reaction. In a second P450 catalytic cycle, AurH sequentially abstracts a hydrogen atom from the C9 methyl group and an electron from the new-born carbon radical by CpdI and CpdII, respectively, giving rise to a carbocation. Eventually, **128** is produced *via* heterocyclization between the 7-OH group and the C9 carbocation. Alternatively, the ring closure might occur through nucleophilic substitution *via* a bis-hydroxylated intermediate (Scheme 25).

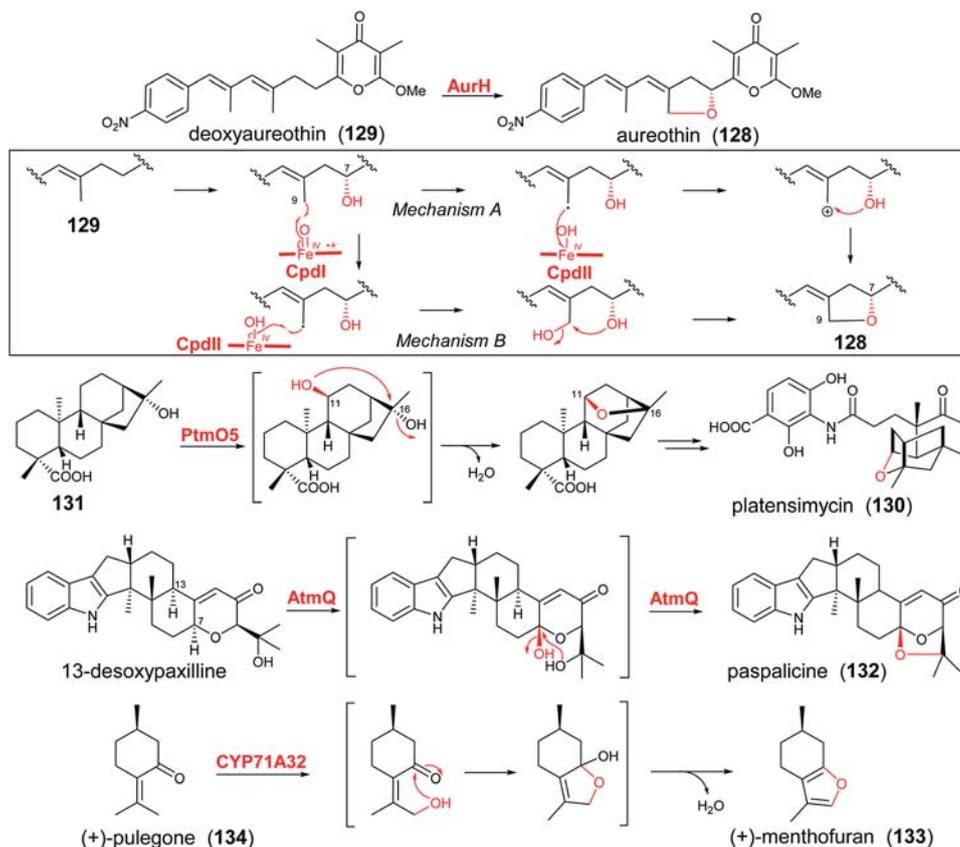
Platensimycin (**130**) is a bacterial meroterpenoid bearing an elegantly fused tetracyclic system.¹⁶⁷ The exclusive ether ring is the catalytic outcome of the P450 enzyme PtmO5 from *S. platensis* SB12029, *via* hydroxylation and nucleophilic ring formation in the initial substrate **131**.¹⁶⁸ Similar reactions also occur during the biosynthesis of paspalicine (**132**), a potent tremorgenic toxin produced by the soil fungus *A. flavus*,¹⁶⁹ as well as in the production of (+)-menthofuran (**133**) from (+)-pulegone (**134**) in peppermint *Mentha piperita* (Scheme 25).¹⁷⁰

5 Unique reactions derived from protein–protein interactions between P450s and other proteins

For almost all P450 enzymes, protein–protein interactions (*i.e.* P450–redoxin interactions for prokaryotic CYPs and P450–cytochrome P450 reductase (CPR) interactions for eukaryotic CYPs) are required for their catalytic activities.¹⁷¹ However, in this section, we will focus on a number of special interactions between P450s and other third-party proteins, with which the functionality of the involved P450s would be enabled or expanded.



Scheme 24 The methylene dioxy-bridge formation reactions mediated by variant plant P450s, and the plausible mechanism (boxed).



Scheme 25 The intramolecular ether ring formation reactions catalyzed by AurH, PtmO5 (bacterial origin), AtmQ (fungal origin), and CYP71A32 (plant origin) via different mechanisms (boxed or in brackets).

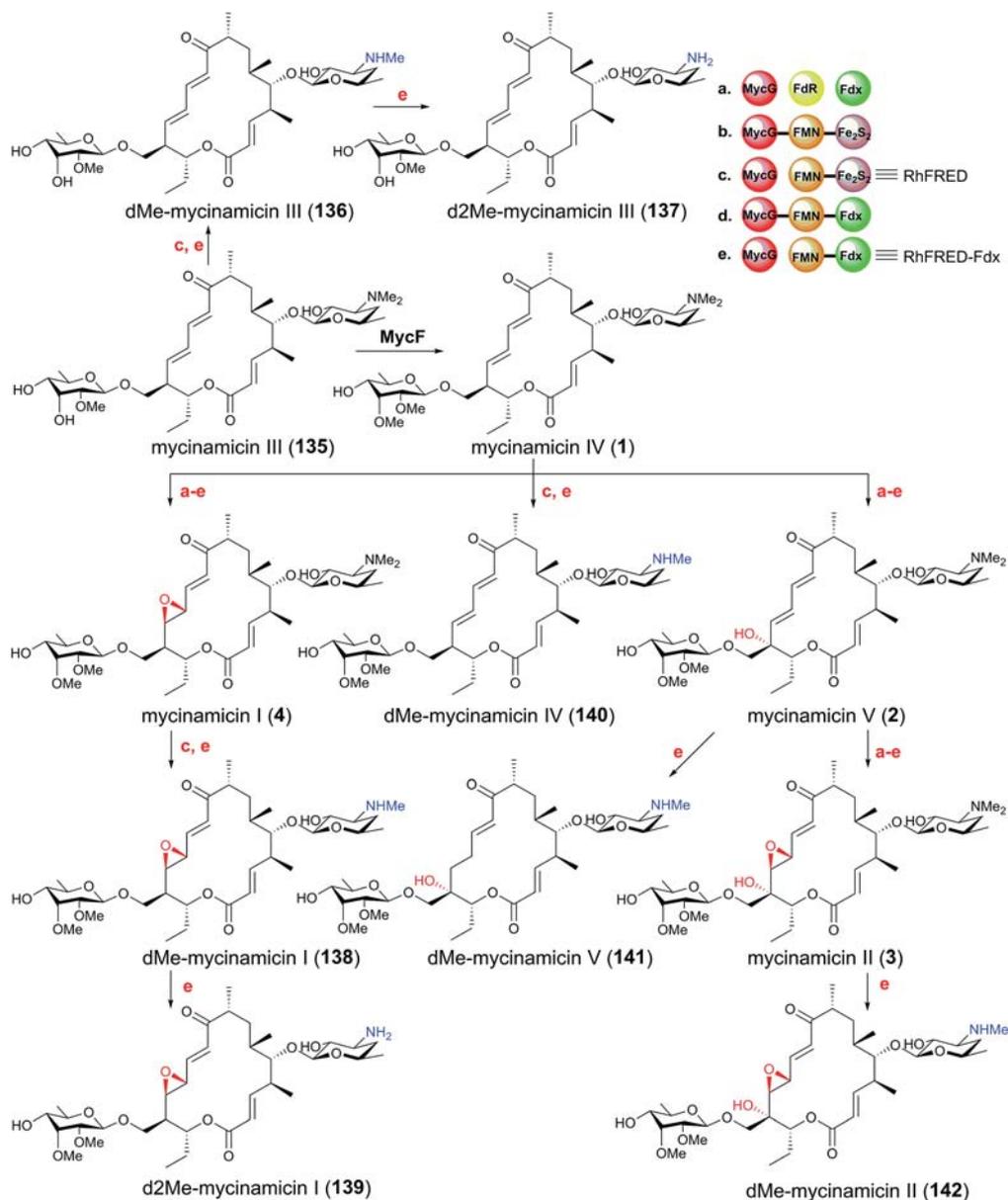
Surrogate redox partners, acting either in isolation or as artificially fused protein complexes, are often used in functional characterization or synthetic application of P450 enzymes.¹⁷² It is almost always taken for granted that the choice of alternative redox partners or their mode of action would not affect the type and selectivity of reactions catalyzed by P450s.

Recently, an accidental observation made by our laboratory on the MycG-catalyzed reactions started to challenge this broadly accepted postulate.¹⁷³ As shown in Schemes 2 and 26, MycG acts as a common P450 mono-oxygenase capable of hydroxylating and epoxidizing mycinamicin macrolides (**1** and **135**) when partnered with the native unknown redox proteins *in vivo* or surrogate spinach ferredoxin and spinach ferredoxin reductase *in vitro*. Strikingly, when using the free *Rhodococcus* reductase domain RhFRED or the engineered *Rhodococcus*-spinach hybrid reductase RhFRED-Fdx, MycG demonstrated a novel *N*-demethylation activity towards mycinamicin I–V, by which seven new ‘unnatural’ NPs with mono- or di-*N*-demethylated mycinamicins (products **136–142**) were generated. Notably, the MycG-RhFRED and MycG-RhFRED-Fdx fusion P450 proteins only mediated the usual hydroxylation and epoxidation reactions. This finding highlights the greater potential of P450–redox partner interactions in modulating the activities of P450 enzymes. Considering that multiple redox partner encoding genes exist in many organisms, P450s could

be even more versatile under physiological conditions than previously thought, since these biocatalysts probably interact with a variety of redox partners to gain alternative activities. From the viewpoint of NP biosynthesis, this behavior may impart evolutionary advantages to the host organisms through synthesizing a broader variety of NPs to adapt to the ever-changing environments.¹⁷³ Biotechnologically, this phenomenon could be utilized to diversify NP structures for new drug development.

Polyketides, fatty acid-derived compounds, and non-ribosomal peptides account for a significant proportion of NPs. Their biosynthetic machinery including polyketide synthases (PKSs), fatty acid synthases (FASs), and non-ribosomal peptide synthetases (NRPSs) all employ carrier proteins (*i.e.* acyl carrier proteins (ACPs) for PKSs and FASs, and peptidyl carrier proteins (PCPs) for NRPSs) to covalently carry starter/extension units, and elongate intermediates to interact with other functional domains or proteins.¹⁷⁴ Thus, P450 enzymes that deal with modifications during assembly also need to interact with those carrier proteins.

For example, P450_{BioI} (CYP107H1) is involved in the biotin biosynthesis of *Bacillus subtilis*. Although the activity of P450_{BioI} could be reconstituted with free fatty acid substrates, its native substrate is believed to be myristyl-ACP (**143**).¹⁷⁵ To form the key biotin precursor pimeloyl-ACP (**144**), P450_{BioI} catalyzes

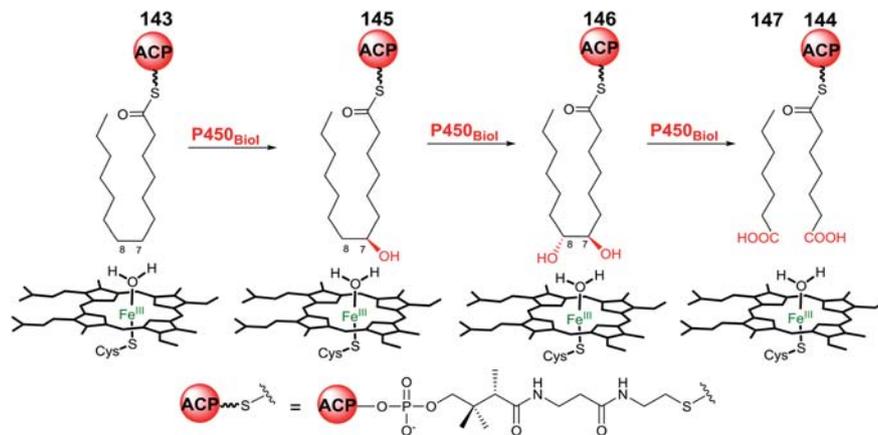


Scheme 26 Common oxidations (in red) and uncommon demethylations (in blue) of mycinamicins mediated by MycG when interacting with alternative redox systems: (a) MycG + spinach FdR + spinach Fdx, (b) MycG-RhFRED, (c) MycG + RhFRED, (d) MycG-RhFRED-Fdx, and (e) MycG + RhFRED-Fdx. Abbreviations: dMe = mono-*N*-demethylated and d2Me = di-*N*-demethylated.

consecutive oxidation of the sp^3 aliphatic chain in **143** to generate the alcohol (**145**) and *threo*-diol (**146**) intermediates, with the latter being in-chain cleaved between C7 and C8 by P450_{BioI} to yield **144** and heptanoic acid (**147**), as shown in Scheme 27.¹⁷⁶ Based on the co-crystal structure of the P450_{BioI}-fatty acyl ACP substrate,¹⁷⁵ the acyl chain is bound to the P450 active site in a *U*-conformation, thereby presenting the C7 and C8 carbons closest to the heme-iron. Of particular importance is that the ACP forms significant protein-protein interactions with P450_{BioI} mainly through the polar contacts between the acidic residues of ACP and the basic residues of P450_{BioI}, as well as the interactions of backbone amide nitrogen and carbonyl oxygen atoms. The prosthetic phosphopantetheine arm of ACP, to

which the fatty acid is bound, forms a number of direct or H₂O-mediated hydrogen bonds with various P450_{BioI} backbone and side chain atoms. Interestingly, it is not uncommon that some P450 enzymes interact with different PCP-tethered amino acid substrates for the production of β -hydroxylated tyrosine, tryptophan, valine, or histidine at the pre-assembly stage.¹⁷⁵ However, simple hydroxylating decoration is not within the scope of this review.

The structurally complicated glycopeptides vancomycin (**148**) and teicoplanin (**149**) are considered as the last resort agents for the treatment of severe infections caused by *Enterococci*, methicillin-resistant *Staphylococcus aureus* (MRSA), and other drug-resistant pathogens.¹⁷⁷ These antibiotics exert their



Scheme 27 The in-chain cleavage of the ACP-tethered tetradecanoic acid directed by the ACP–P450_{Biol} interaction.

functions by forming a non-covalent complex with the peptidoglycan precursor terminated with D-Ala–D-Ala, which is necessary for cell wall biosynthesis in Gram-positive bacteria.¹⁷⁸ As shown in Scheme 28, it is biosynthetically intriguing that all interconnections of aromatic side chains of the NRPS-derived linear heptapeptide precursor are mediated by P450 enzymes including OxyA, OxyB, OxyC, and OxyE.

Extensive *in vivo* gene disruption experiments have suggested the catalytic role for each of the four P450s.^{179–182} The sequence of oxidative cross-linking during the biosynthesis of **148/149** has also been established. Specifically, in the case of **148** biosynthesis, OxyB and OxyA sequentially introduce the aromatic C–O cross-link between residues 4/6 and 2/4, respectively. OxyC next mediates the last C–C coupling between residues 5/7, leading to the matured scaffold to be released by the thioesterase (TE) domain.^{179–184} As for biosynthesis of **149**, a further phenolic C–O coupling reaction catalyzed by OxyE between residues 1/3 is required, and the acting order of the above P450s becomes OxyB, OxyE, OxyA, and OxyC.¹⁸⁵

In view of P450 catalysis, C–O or C–C coupling reactions are likely achieved by a biradical mechanism as discussed earlier. However, the mechanism underlying the recruitment of different P450 enzymes to interact with the NRPS-bound heptapeptide had remained largely unknown until recent functional elucidation of the conserved X-domain that is embedded in the final module of all glycopeptide antibiotic NRPSs.¹⁸⁶ Functionally, the X-domain is responsible for recruiting each individual P450 enzyme to the NRPS for the essential amino acid side chain cross-linking, without which the PCP domain alone is not sufficient to generate a competent substrate for external CYPs. Structurally, the X-domain interacts with OxyB *via* a rigid body docking mode using an interface other than those for PCP–P450 and ferredoxin–P450 interactions. Functional and structural insight into the X-domain has shed the first light on the fascinating interactions among alternative P450s, the PCP domain and X-domain from the same NRPS, and a so far untouched redox partner protein. To completely understand the details underlying the most complicated P450-based catalytic system, much more effort involving full

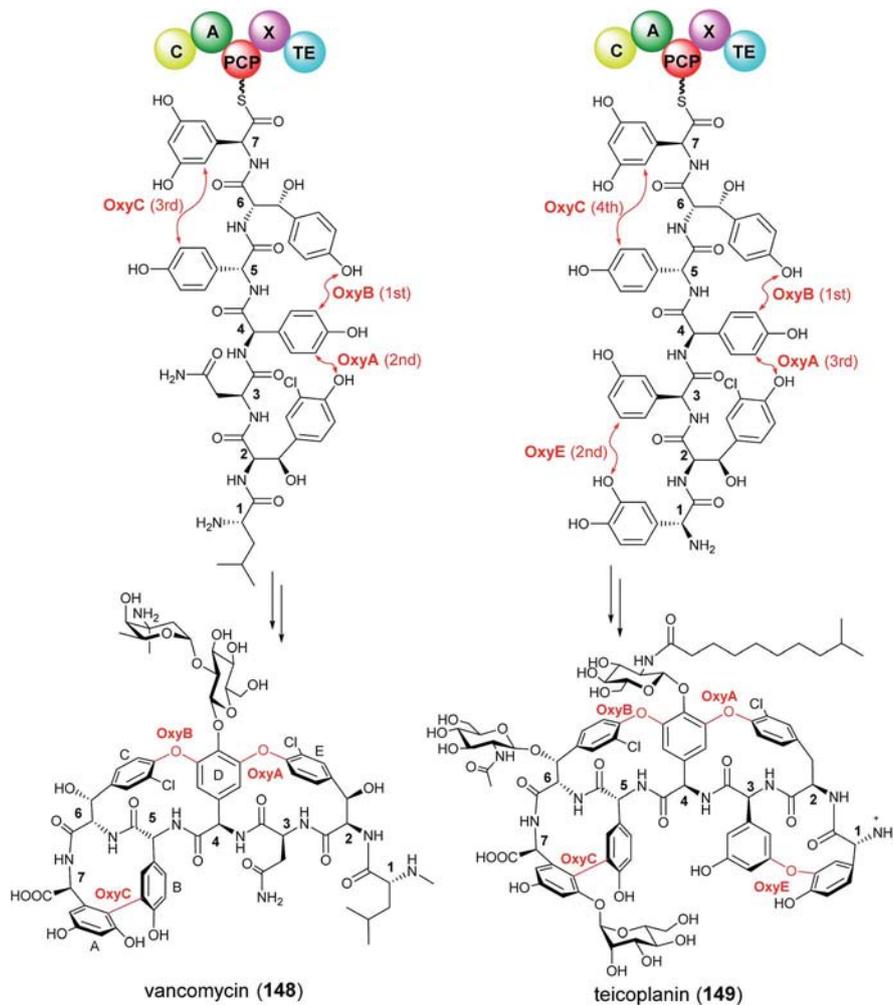
reconstitution of the *in vitro* biosynthesis of **148/149**, structural elucidation of the whole NRPS module, and direct observation of the OxyA–NRPS, OxyB–NRPS, OxyC–NRPS, and OxyE–NRPS complexes perhaps by cryo-EM technology is required.¹⁸⁶

6 The logic of P450-mediated expansion of chemical space for natural products

Countless NPs, which are also referred to as secondary metabolites in many cases, constitute the immense chemical arsenal available for diverse organisms that implement chemical defence to combat ever-changing ecological environments. Thus, the intrinsic evolutionary logic for NP-producing machinery, *i.e.* biosynthetic enzymes, is to diversify their own functionalities, thereby expanding the chemical space of NPs. This logic also happens to be beneficial for human beings to develop new pharmaceuticals, cosmetics, and dietary supplements.

As a super-family of NP biosynthetic enzymes, cytochrome P450 enzymes also adhere to this logic. Due to the functional versatility of CYPs, these heme-thiolate proteins are extensively involved in the pre-assembly, assembly, and post-assembly structural modification of diverse NPs. Essentially, P450 enzymes act as both skeleton constructors and decorators. The extraordinary ability of P450s to catalyze numerous common and uncommon reactions is mainly, if not totally, due to the following reasons: (1) the C–H bond as the predominant target of P450s represents the most ubiquitous chemical bond in organic compounds; (2) the size of a P450 substrate binding pocket determined by the amino acid composition and protein dynamics could go from rather small to very spacious to accommodate all kinds of substrate; and (3) the highly reactive CpdI or Cpd0 is able to react with almost any adjacent groups presented by the bound substrate.

The uncommon P450 reactions discussed in this review largely originate from the variable fate of substrate radicals. Other than the classical oxygen rebound mechanism leading to common P450 reactions, the chemical property of the radical's



Scheme 28 The C–O and C–C cross-linking sequentially mediated by OxyB, OxyE (optional), OxyA, and OxyC P450s during the biosynthesis of vancomycin and teicoplanin through PCP–X-domain–P450 interactions.

neighbouring atoms, as well as the surrounding functional groups from both the P450 enzyme and substrate, could result in stabilization, trapping, scavenging, or rearrangement of the substrate radical, thus leading to diverse uncommon P450 reactions. Furthermore, the redox partners and other third-party proteins that could interact with P450s would bring in more variables for the determination of P450 functions. Taken together, the outcome of a P450-mediated reaction is very much an interplay of substrate–P450–redox partner–other protein(s).

7 Conclusions and prospects

In addition to the well-understood common hydroxylation and epoxidation reactions catalyzed by P450 enzymes, herein, we have reviewed a select number of uncommon P450 reactions including nitration, decarboxylation, C–C bond formation and scission, ring opening and closure, ring expansion and contraction, as well as some unique P450-mediated biotransformations resulting from special protein–protein interactions. These unusual transformations not only result in the wealth of structural diversification, but also frequently endow the

resultant NPs with improved bioactivities.²⁷ It is important to note that the examples discussed above do not cover all uncommon P450 reactions involved in NP biosynthesis, but instead highlight the representative unusual reactions by juggling bacterial, fungal, and plant P450s. A larger number of unusual examples can be found in other recent reviews.^{27,98,187–189} Interestingly, one may have noticed that the majority of examples in this work are derived from bacterial P450s. However, this does not necessarily suggest that the prokaryotic P450s hold higher catalytic diversity than their eukaryotic counterparts. One important reason could be that it is much easier at present to study the soluble bacterial P450s than the membrane-bound P450s from fungi and plants. We envision that much more unusual P450 reactions will be uncovered from filamentous fungi and higher plants along with the technical advancement on functional and structural analysis of membrane-bound proteins. After all, the number of P450 genes contained by fungi and plants is an order of magnitude greater than that contained by bacteria.¹⁹⁰

Due to limited understanding on the complex dynamic interactions between the substrate, P450, redox partner, and other optional protein(s), it is highly challenging, at least for now, to predict, design, and engineer uncommon P450 reactions that are chemically impractical. Unlike the engineering of usual P450 mono-oxygenation, which often only requires projecting the bond to be oxidized towards the heme-iron reactive center and within a certain distance, the change of any factor in an uncommon P450 reaction, such as an active site residue, a sub-structure of a substrate, or a partner protein, could dramatically impact the outcome of the reaction. For instance, saturation mutagenesis of two active site residues in the P450 fatty acid decarboxylase OleT_{JE} unanimously abolished the unique decarboxylation activity of this enzyme, while the normal hydroxylation activity was maintained to varying extents.¹⁹¹ Nonetheless, with more comprehensive understanding of the catalytic mechanisms of P450s, we believe that the uncommon P450 reactions will eventually become manipulable. More new chemistries of P450 enzymes,¹⁹² such as olefin cyclopropanation *via* carbene transfer¹⁹³ and C–Si bond formation,¹⁹⁴ will be created in the future.

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