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Unusual chemistries in fungal meroterpenoid biosynthesis

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Meroterpenoids are polyketide and terpenoid hybrid natural products with remarkable biological activities. Recent progress in fungal meroterpenoid biosynthesis has revealed several unusual enzyme reactions and novel enzymes, including unique terpene cyclization reactions by a novel family of membrane-bound terpene cyclases and post-cyclization modification reactions by oxygenases, such as non-heme iron-dependent dioxygenases, flavin adenine dinucleotide-dependent monooxygenases, and cytochrome P450 monooxygenases. They contribute to the structural diversification and increase in complexity of fungal meroterpenoids. Structure-function studies of these enzymes provide strategies for engineering the biosynthetic machinery to create novel molecular scaffolds for drug discovery.

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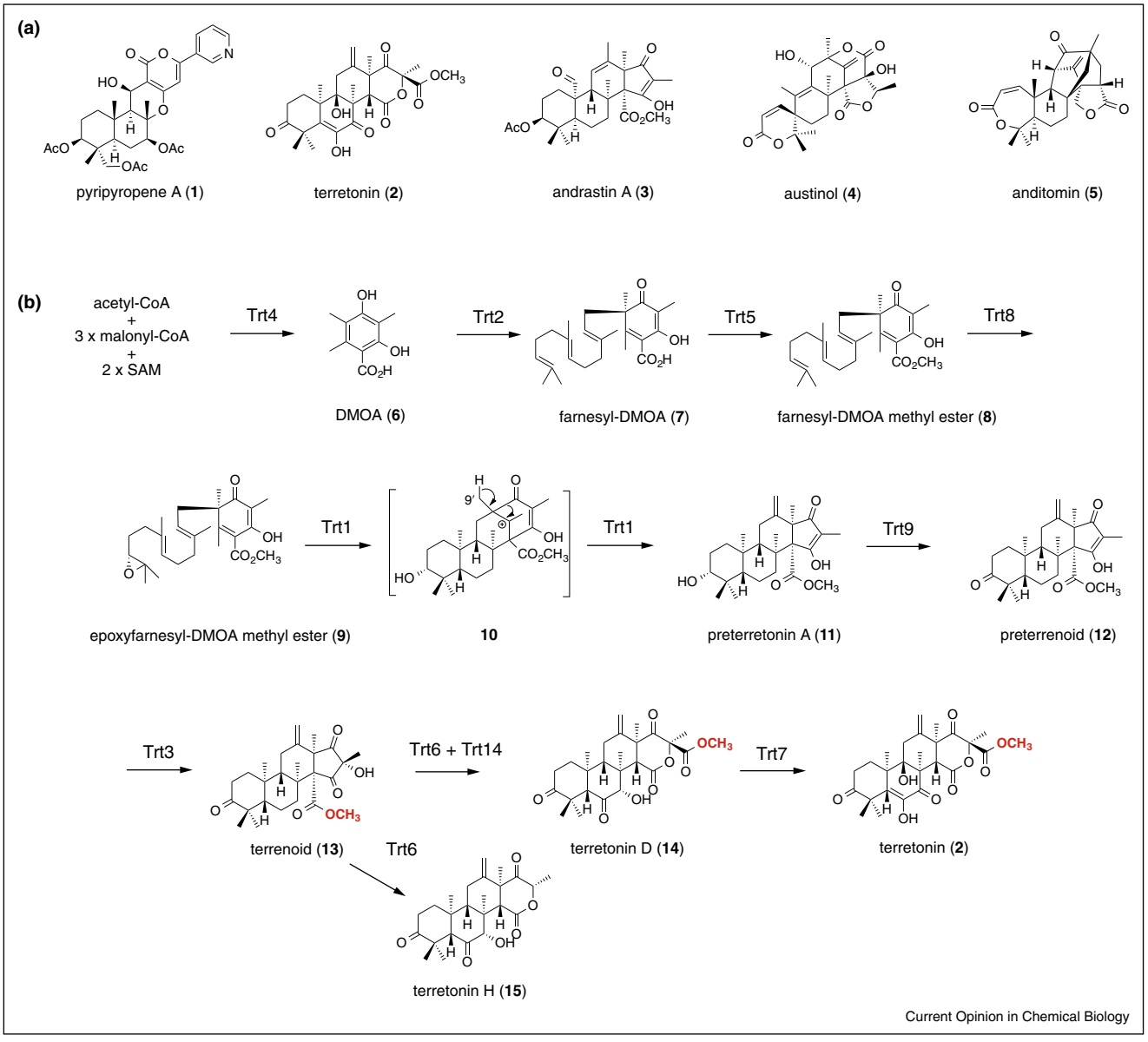
Introduction

Remarkable structural diversity and complex molecular scaffolds are the attractive features of fungal meroterpenoids, polyketide and terpenoid hybrid natural products with important biological activities [1,2••]. The biosynthetic pathways for these complex natural products include a diverse range of fascinating chemistries, and are therefore rich sources of novel enzymes catalyzing unusual enzyme reactions. Indeed, since our first report on the identification and characterization of the biosynthetic gene cluster of pyripyropene A (**1**) from *Aspergillus fumigatus* in 2010 [3], remarkable advances in the studies of fungal meroterpenoid biosynthesis have revealed many interesting enzymes that catalyze quite unique chemical conversions, as well as novel families of enzymes [2••]. This short review summarizes the very recent research progress on the biosyntheses of the fungal meroterpenoids terretonin (**2**) [4–6,7•], andrastin A (**3**) [8], austinol (**4**) [9,10••], and anditomin (**5**) [11••] (Figure 1a). In particular, we will focus on the remarkable terpene cyclization reactions catalyzed by a novel family of membrane-bound terpene cyclases (TPCs) and the post-cyclization modification reactions by oxygenases, including non-heme iron-dependent dioxygenases, flavin adenine dinucleotide-dependent monooxygenases (FMOs), and cytochrome P450 monooxygenases, which significantly contribute to the structural diversification and increase in complexity of fungal meroterpenoids.

Terpene cyclization reactions

The biosynthesis of meroterpenoids starts with the assembly of the polyketide moiety, which is followed by prenylation of the polyketide, stereospecific epoxidation of the olefin of the prenyl chain, and cyclization of the terpenoid moiety to generate the diverse meroterpenoid core scaffolds [1,2••]. For example, the fungal meroterpenoids terretonin (**2**), andrastin A (**3**), austinol (**4**), and anditomin (**5**), are all biosynthesized from a simple aromatic polyketide, 3,5-dimethylorsellinic acid (DMOA) (**6**), but they have different cyclic terpenoid scaffolds [1,2••]. The structural diversity is due to the differences in the mechanisms of the cyclization reactions, which are catalyzed by a novel family of membrane-bound terpene cyclases (TPCs). The cyclization reaction is thus one of the key steps to generate the structural diversity of the fungal meroterpenoids. Remarkably, the stereochemistry of the cyclization reaction, which usually affords a single product, is strictly controlled by each enzyme. These novel TPCs are quite small (ca. 25 kDa) integral membrane proteins with seven transmembrane helices, and share very low sequence similarity to the known TPCs [3,5,11••]. Notably, homologous proteins are widely distributed in the biosynthetic pathways of terpenoid-bearing secondary metabolites from fungi and actinomycetes, including indole diterpenoids [3]. A sequence comparison of these homologous proteins indicated the presence of several conserved motifs, but none of the aspartate-rich motifs (DDXXD or DXDD motifs) normally found among known TPCs [3]. Therefore, the detailed mechanism for the catalysis still remains to be elucidated, but a mutational study of Pyr4, involved in the biosynthesis of pyripyropene A (**1**) in *A. fumigatus*, revealed that two conserved acidic amino acid residues, Glu63 and

Figure 1

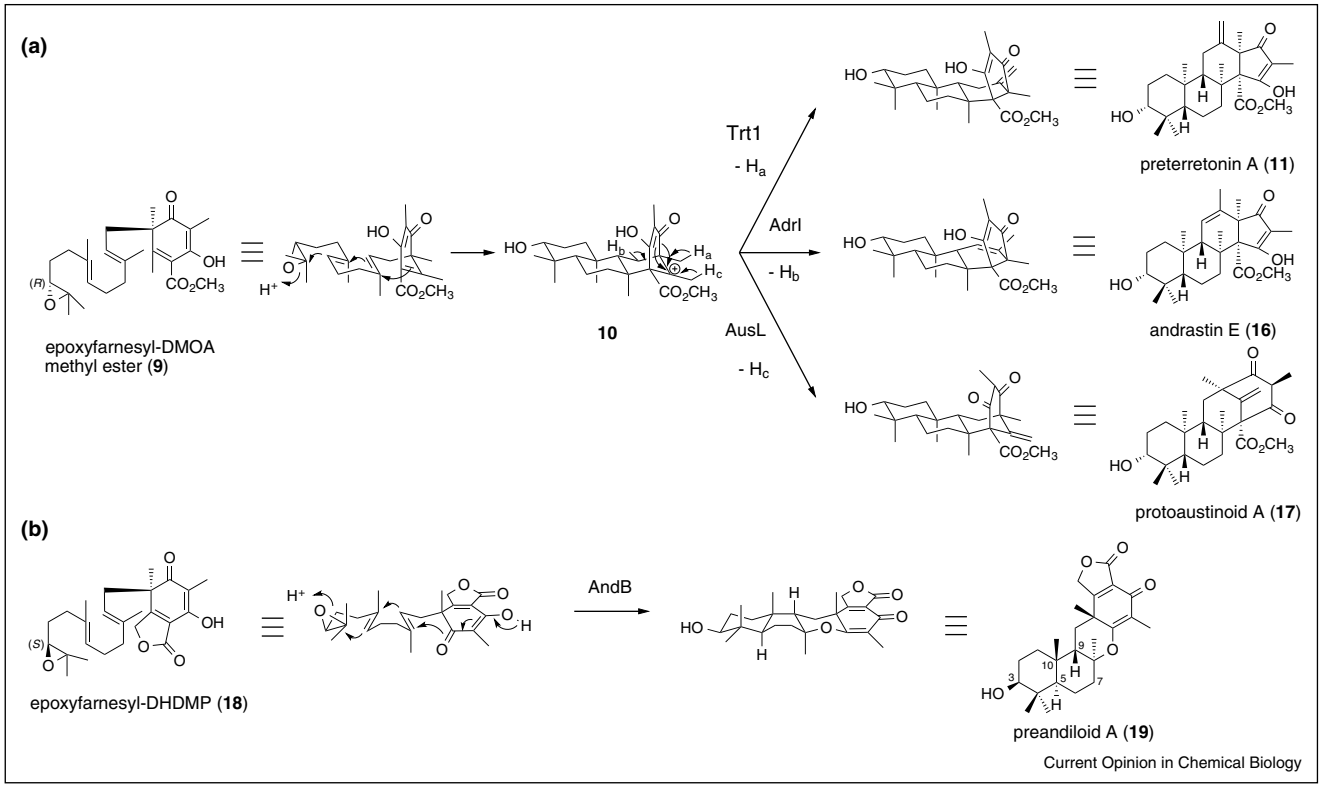


(a) Structures of fungal meroterpenoids. **(b)** Biosynthetic pathway of terretonin (**2**) in *Aspergillus terreus*.

Asp218, are essential for the catalytic activity, suggesting that these residues serve as general acids that protonate the terminal epoxide to initiate the sequential ring-forming reaction [3].

Terretonin (**2**), from *Aspergillus terreus*, is one of the DMOA-derived meroterpenoids, with a unique tetracyclic scaffold [4–6,7•]. Interestingly, our recent studies revealed that, in terretonin biosynthesis (Figure 1b), the methylation of the carboxyl group of farnesyl-DMOA (**7**) is essential for the substrate recognition by the novel transmembrane TPC, Trt1 [5]. Without the methylation of the carboxyl group, the cyclization reaction does not occur. Interestingly, this is also the case for the biosyntheses of other DMOA-derived fungal meroterpenoids, including andrastin A (**3**) and austinol (**4**) [5]. The cyclase Trt1 is thought to bind (10'*R*)-epoxyfarnesyl-DMOA methyl ester (**9**) in the '*chair-chair*' conformation, and initiate the sequential ring-forming reaction by protonating the terminal epoxide (Figure 2a) [5]. The sequential C–C bond-forming reaction first produces the 6.6.6.6-fused tetracyclic tertiary carbocationic intermediate (**10**), which then undergoes carbon skeletal rearrangement (D-ring contraction) with proton abstraction from the methyl group at C-9′ (H_a) to generate preterretonin A (**11**) with a 5-membered D-ring. In contrast, in the

Figure 2



Comparison of the terpene cyclization reactions in the DMOA-derived meroterpenoid biosynthesis.

biosyntheses of andrastin A (**3**) in *Penicillium chrysogenum* and austinol (**4**) in *Aspergillus nidulans*, AdrI and AusL, the two homologous proteins to Trt1, eliminate the proton from C-11 (H_b) and C-1′ (H_c), of the common tetracyclic cation (**10**), to yield the 6.6.6.5-fused tetracyclic andrastin E (**16**) and the 6.6.6.6-fused tetracyclic protoaustinoid A (**17**), respectively (Figure 2a) [5]. Thus, the three novel TPCs, Trt1, AdrI, and AusL, generate different molecular scaffolds as a single product, via a common tetracyclic tertiary carbocationic intermediate, according to the position where the deprotonation occurs.

In contrast, in the biosynthesis of anditomin (**5**), another DMOA-derived meroterpenoid from *Emericella variecolor*, DMOA is first converted to 5,7-dihydroxy-4,6-dimethylphthalide (DHDMP) by a P450 monooxygenase and hydrolase fusion protein, AndK [11••]. After farnesylation by a UbiA-like membrane-bound prenyltransferase (PT), and FMO-mediated stereo-specific epoxidation of the terminal double bond, another novel TPC, AndB, catalyzes the cyclization of (10′*S*)-epoxyfarnesyl-DHDMP (**18**) to produce the 6.6.6.6.5-fused unique pentacyclic preandiloid A (**19**) (Figure 2b) [11••]. Here it should be noted that the absolute configuration of the substrate of AndB is a (10′*S*)-epoxide, and the protonation initiated cyclization reaction proceeds with the epoxyfarnesyl moiety folded in the '*chair-boat*' conformation to yield the product with the 3β-hydroxy group [11••]. In contrast, Trt1, AdrI, and AusL catalyzes cyclization of (10′*R*)-epoxide folded in the '*chair-chair*' conformation, to produce the 3α-hydroxy scaffolds [5] (Figure 2a). Interestingly, AndB shares relatively low sequence identity (~30% identity) with Trt1, AusL, and AdrI, but high similarity with AtmB (47% identity), which is involved in the biosynthesis of the indole diterpenoid aflatrem, from *Aspergillus flavus* [12]. It is remarkable that, in all of these cases, the stereochemistry of the cyclization reactions is rigidly controlled and a single product is obtained from the novel family of transmembrane TPCs. The structure-function relationships of these novel TPCs are very interesting, and to understand the intimate three-dimensional structural details of the enzyme-mediated cyclization reactions, crystallographic studies are now in progress in our laboratories.

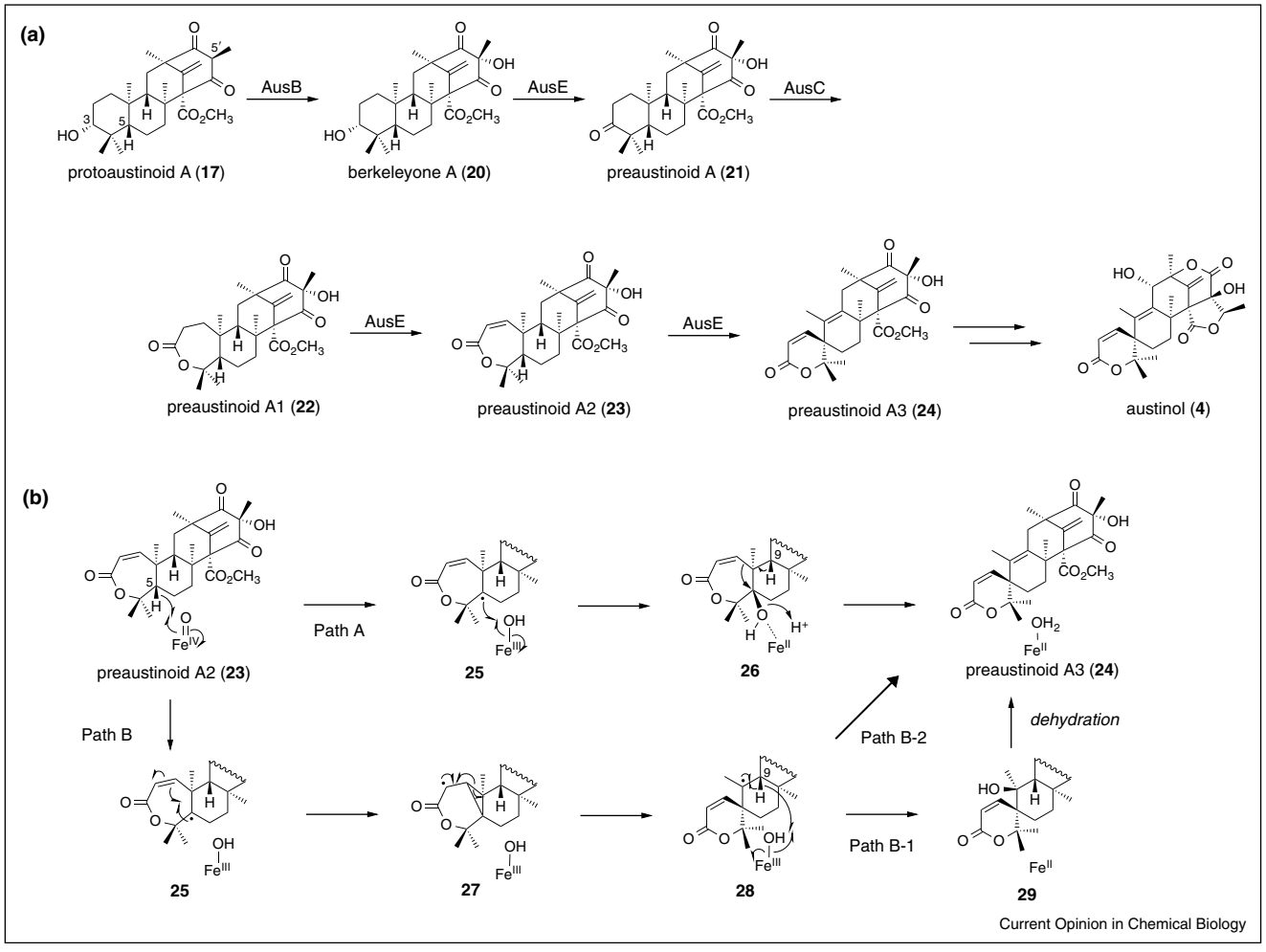
Post-cyclization modification reactions

The structural diversity of the fungal meroterpenoids is produced by the differences in the polyketide starter moieties, prenyl chain lengths, and cyclization reactions [1,2••]. In addition, various post-cyclization modifications

dramatically increase the structural complexity. In particular, oxygenases, such as cytochrome P450 monooxygenases, FMO monooxygenases, and non-heme iron/α-ketoglutarate (Fe(II)/α-KG)-dependent dioxygenases, significantly contribute to the structural complexification reactions. Indeed, for the biosyntheses of fungal secondary metabolites, numerous interesting reactions have recently been reported, including a variety of oxidative rearrangements [13–17], a carbonate-forming reaction [18], and the cyclization of a terpenoid moiety [19–21].

An interesting example of fungal meroterpenoid biosynthesis is the unusual spiro-lactone ring-forming reaction in the austinol biosynthetic pathway in *A. nidulans*, which is catalyzed by an Fe(II)/α-KG-dependent dioxygenase, AusE, as well as two FMOs, the 5′-hydroxylase AusB and the Baeyer-Villiger monooxygenase AusC (Figure 3a) [10••]. Remarkably, AusE is a multifunctional enzyme that catalyzes sequential oxidation reactions, including oxidative carbaspirocycle formation, to generate the austinol scaffold. Thus, in the austinol pathway, after the cyclization reaction of (10′*R*)-epoxyfarnesyl-DMOA methyl ester by AusL, the 6.6.6.6-fused tetracyclic protoaustinoid A (**17**) is oxidized into berkeleyone A (**20**) by the 5'-hydroxylase FMO AusB. This reaction is followed by C-3 dehydrogenation by the Fe(II)/α-KG-dependent dioxygenase AusE, to generate the preaustinoid A (**21**). Then, the Baeyer-Villiger FMO AusC inserts one oxygen atom between C-3 and C-4 to yield the preaustinoid A1 (**22**), with a seven-membered lactone A-ring. Subsequently, AusE again accepts **22** as a substrate to catalyze two consecutive oxidations, in which **22** undergoes dehydrogenation to the preaustinoid A2 (**23**) and oxidative rearrangement to the preaustinoid A3 (**24**). It should be noted that the dioxygenase AusE catalyzes oxidation reactions 'before' and 'after' the Baeyer-Villiger reaction by AusC, to generate the spiro-lactone ring system of **24**. The Fe(II)/α-KG-dependent dioxygenases are known to

Figure 3

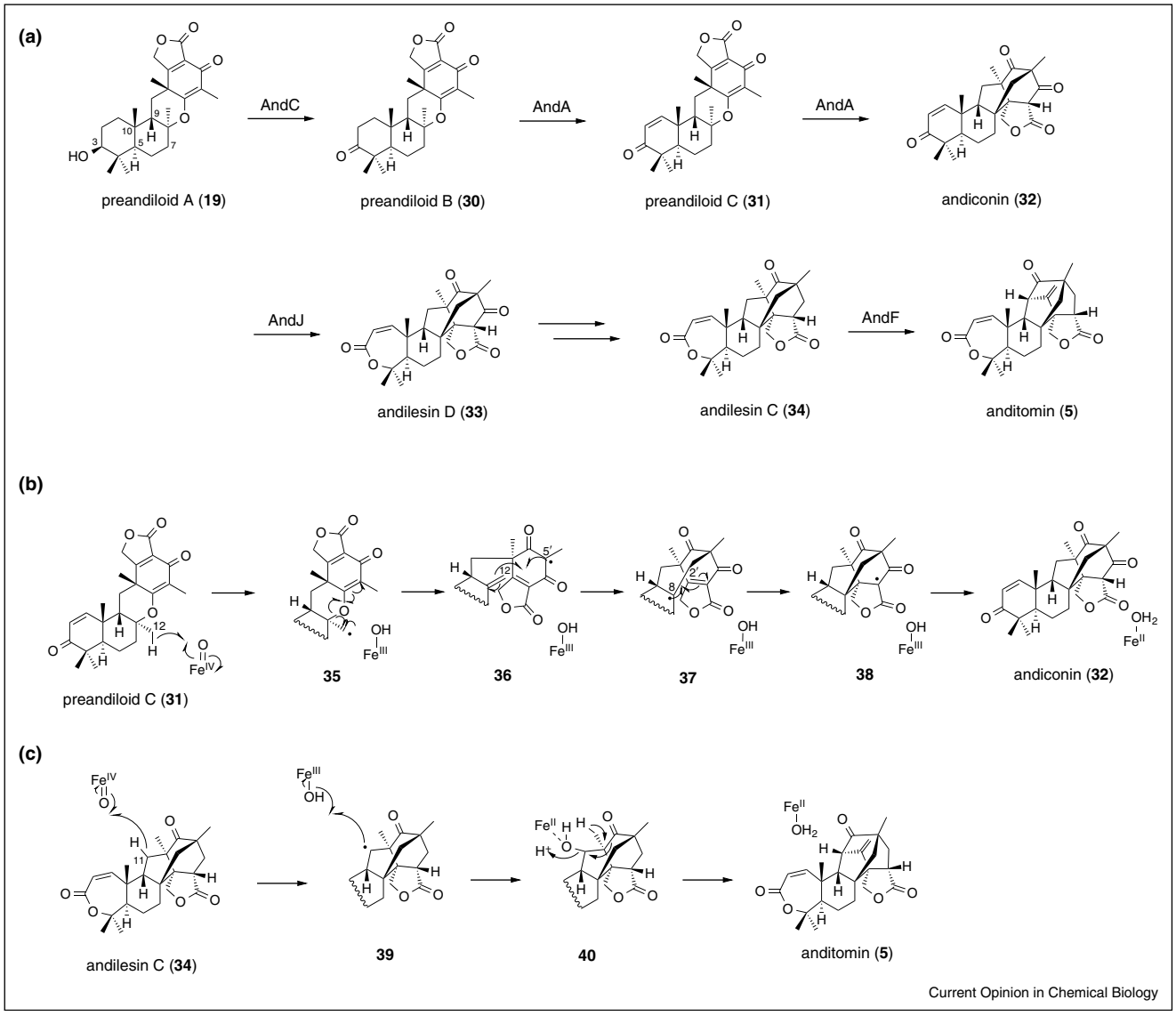


(a) Biosynthetic pathway of austinol (**4**) in *Aspergillus nidulans*. **(b)** Proposed mechanism for the AusE-catalyzed spiro-lactone ring-forming reaction.

catalyze versatile reactions, including the ring expansion reaction in the bioconversion of penicillin to cephalosporin [22], and the tropolone ring-construction in stipitatic acid biosynthesis [23]. However, this is the first example of the oxidative formation of a spiro-lactone ring system by an Fe(II)/α-KG-dependent dioxygenase.

Like other Fe(II)/α-KG-dependent dioxygenases, AusE maintains a conserved 2-His-1-carboxylate iron-binding facial triad (H130, D132, H214) [24]. We propose that AusE initiates the reaction by abstracting the C-5 hydrogen of the substrate preaustinoid A2 (**23**) to yield the radical **25**, using the active site Fe(IV)-oxo species produced by the oxidative decarboxylation of α-KG (Figure 3b) [10••]. The radical species **25** would then be hydroxylated by the enzyme to generate the hydroxylated intermediate **26** bound to an Fe(II) species. Upon the elimination of the C-5 hydroxyl group as a water molecule, the carbon skeletal rearrangement and deprotonation from C-9 would generate the spiro-lactone preaustinoid A3 (**24**) (Path A). An alternative mechanism would involve the formation of the cyclopropane ring **27**, which is further rearranged to the spiro-lactone radical **28**. The enzymatic reaction is hereby completed by the rebound of the hydroxyl radical to **28** to yield **29**, which undergoes spontaneous dehydration to produce the preaustinoid A3 (**24**) (Path B-1) [13]. It is also possible that the abstraction of the C-9 hydrogen atom of **28** directly yields **24** (Path B-2).

Figure 4



(a) Biosynthetic pathway of anditomin (**5**) in *Emericella variecolor*. **(b)** Proposed mechanism for the AndA-catalyzed rearrangement reaction. **(c)** Proposed mechanism for the AndF-catalyzed rearrangement reaction.

The multifunctional Fe(II)/α-KG-dependent dioxygenases are widely distributed in the biosynthetic pathways of fungal meroterpenoids, and another unusual fascinating enzyme reaction is catalyzed by AndA, an AusE homologue, in the biosynthesis of anditomin (**5**) in *E. variecolor* [11••]. In this case, AndA accepts the 6.6.6.6.5-fused pentacyclic preandiloid B (**30**) as a substrate and catalyzes multistep reactions to generate the bicyclo[2.2.2]octane ring system of andiconin (**32**), by an unprecedented carbon skeletal reconstruction (Figure 4a) [11••]. The dioxygenase AndA also maintains the conserved iron-binding facial triad (H135, D137, H213), and after the initial oxidation to the enone preandiloid C (**31**), AndA abstracts the hydrogen atom of the C-12 methyl group of **31** to produce the radical species **35** (Figure 4b). The C–O bond at C-8 would then be cleaved to generate the resonance-stabilized radical **36**, followed by the first C–C bond formation between the electrophilic C-5′ and the electron-rich C-12 to generate the radical **37**. Then, the second C–C bond formation between the resulting nucleophilic C-8 and the electron-deficient C-2′ generates the radical **38**. Notably, the AndA reaction requires ascorbate as an essential factor in vitro, for the reduction of the radical **38** and the ferric ion in the active site of the enzyme, to generate the final product andiconin (**32**).

Interestingly, the anditomin biosynthetic pathway contains another multifunctional Fe(II)/α-KG-dependent dioxygenase, AndF, which catalyzes the very last step of the biosynthesis to generate the highly congested molecular skeleton of anditomin (**5**) (Figure 4c) [11••]. Thus, the dioxygenase AndF accepts andilesin C (**34**), with a seven-membered lactone A-ring, as a substrate, and initiates the reaction by abstracting the C-11 hydrogen of the substrate to produce the radical species **39**, which is then hydroxylated by the enzyme to generate the hydroxylated intermediate **40** bound to an Fe(II) species. Subsequent elimination of the hydroxyl group as a water molecule, and carbon skeletal rearrangement of the resulting carbocationic intermediate with deprotonation from C-9′ would finally lead to the formation of anditomin (**5**).

In addition to the dioxygenases, the recently reported unusual D-ring construction at the late stage of terretonin biosynthesis involves the unprecedented cooperation of a multifunctional P450 monooxygenase and a novel isomerase (Figure 1b) [7•]. In this case, the P450 Trt6 catalyzes multistep oxidation reactions on the B-ring to transform the 6.6.6.5-fused tetracyclic terrenoid (**13**) into an unstable intermediate, which is followed by the D-ring expansion and the methoxy group rearrangement to yield terretonin D (**14**) with the 6-membered D-ring. Remarkably, the novel isomerase Trt14, a small protein with fewer than 150 amino acids, catalyzes this unprecedented rearrangement reaction [7•]. In the absence of Trt14, the multifunctional P450 Trt6 converts terrenoid (**13**) into the D-ring expanded terretonin H (**15**), but without the methyl ester group at the C-16 position. A feeding experiment with [^{13}C]-labeled methionine indicated the involvement of the intramolecular transfer of the methoxy group, and excluded the possibility that Trt14 is a methyltransferase [7•]. The detailed catalytic function of Trt14 remains to be elucidated, but Trt14 is likely to be involved in the intramolecular methoxy group rearrangement to construct the terretonin scaffold. The novel isomerase Trt14 shares almost no sequence similarity with any other characterized proteins, but appears to be structurally related to some isomerases as well as epoxide hydrolases [25–28]. Interestingly, several Trt14 homologues are present in the biosynthetic pathways of other fungal meroterpenoids, including AusJ, AusH, and AusF, in austinol biosynthesis [9,10••]. A sequence alignment of these homologous enzymes indicated several highly conserved aspartate and glutamate residues, suggesting that these residues play crucial roles in the Trt14-catalyzed reaction.

Conclusions

The biosynthetic pathways for the structurally diverse complex fungal meroterpenoid molecules are rich in unique enzyme reactions as well as novel enzymes. Notably, 'unusual' reactions are often catalyzed by 'usual' enzymes. Indeed, as described, several unprecedented reactions are mediated by common oxygenase enzymes, including P450 and FMO monooxygenases, and a family of Fe(II)/α-KG-dependent dioxygenases. This suggests that the peculiar features of the substrate and enzyme recognition are also crucial for the unique chemical reactions to occur. In addition, completely new families of enzymes, such as membrane-bound TPCs and unique isomerases, also contribute to the structural diversification and increase in complexity of fungal meroterpenoids. Structure-function studies of these intriguing enzymes will reveal the intimate structural details of their fascinating chemistries, and provide strategies for engineering the biosynthetic machinery to create novel meroterpenoid scaffolds for drug development.

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