REVIEW

Biosynthesis of fungal meroterpenoids†

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Meroterpenoids are hybrid natural products that partially originate from the terpenoid pathway. The meroterpenoids derived from fungi display quite diverse structures, with a wide range of biological properties. This review summarizes the molecular bases for their biosyntheses, which were recently elucidated with modern techniques, and also discusses the plausible biosynthetic pathways of other related natural products lacking genetic information. (Complementary to the coverage of literature by Geris and Simpson in *Nat. Prod. Rep.*, 2009, 26, 1063–1094.)

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† Dedicated to Professor Ushio Sankawa on the occasion of his 80th birthday.
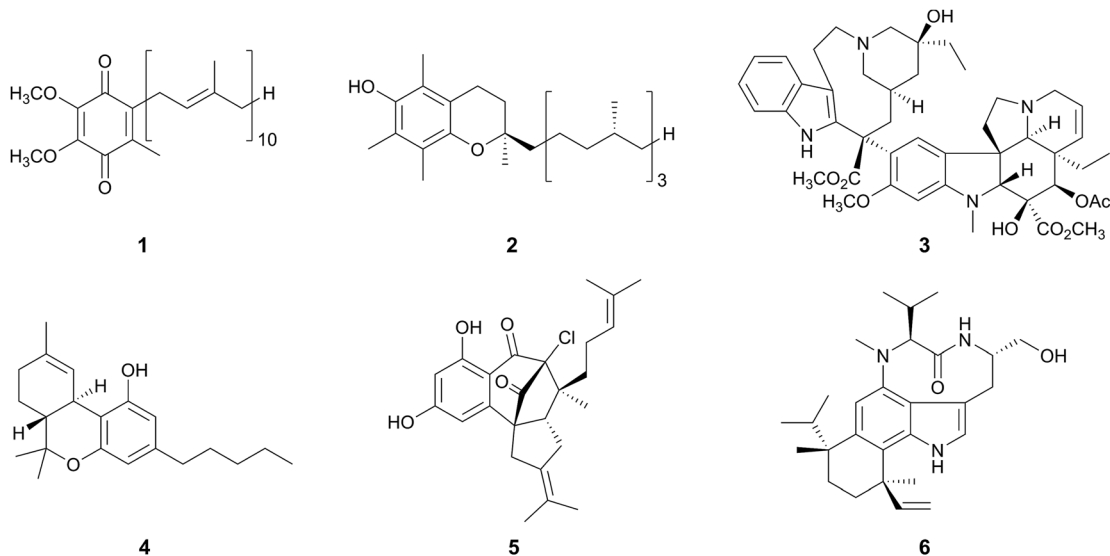
1　Introduction

Meroterpenoids are hybrid natural products partially derived from terpenoid pathways as the prefix "mero-" means "part, partial, and fragment".[1] On the basis of their definition, numerous compounds derived from animals, plants, bacteria, and fungi can be grouped into this class of natural products, which range from widely distributed molecules to species-specific secondary metabolites. Ubiquinone-10 (**1**, "coenzyme Q10")[2] and α-tocopherol (**2**, "vitamin E")[3] are examples of commonly occurring meroterpenoids, whereas the anti-cancer chemotherapy drug vinblastine (**3**),[4] and a component of marijuana, tetrahydrocannabinol (**4**),[5] are produced by specific plants. Merochlorin A (**5**)[6] and teleocidin B-4 (**6**)[7] are generated by the actinomycetes in the genus *Streptomyces*, and thus represent the bacterial meroterpenoids.

Among the meroterpenoid producers, fungi synthesize molecules with diverse chemical structures and a wide range of biological activities (Fig. 1).[1] Mycophenolic acid (**7**), produced by several *Penicillium* species, is a strong inhibitor of inosine 5′-monophosphate dehydrogenase (IMPDH),[8] and its derivative mycophenolate mofetil is clinically used as an immunosuppressant drug.[9] Fumagillin (**8**), isolated from *Aspergillus fumigatus*,[10] is used for the treatment of microsporidiosis and amebiasis,[11] and has also been investigated as an anti-angiogenesis agent, since **8** inhibits methionine aminopeptidase-2 (MetAP2).[12] Pyripyropene A (**9**),[13] territrem B (**10**),[14] and ascofuranone (**11**)[15] are highly potent inhibitors of acyl-coenzyme A:cholesterol acyltransferase-2 (ACAT-2), acetylcholinesterase (AChE), and trypanosome alternative oxidase, respectively. Berkeleyacetal C (**12**) was originally isolated from *Penicillium rubrum*, based on its inhibitory activities against matrix metalloproteinase-3 (MMP-3) and caspase-1.[16] Subsequently, **12** was found to exhibit anti-inflammatory activity by inhibiting nitric

oxide (NO) production and inducible NO synthase (iNOS), in which the target molecule of **12** was elucidated to be interleukin-1 receptor-associated kinase-4 (IRAK4).[17]

Although the above-described fungal meroterpenoids are all polyketide–terpenoid hybrid molecules, several other meroterpenoids are also derived from non-polyketide starters. For example, the kinesin Eg5 inhibitor terpendole E (**13**)[18] contains an indole ring instead of a polyketide moiety as the non-terpenoid part, and thus is classified as an "indole-diterpene (-diterpenoid) alkaloid". Compound **13** also serves as a biosynthetic intermediate of lolitrem B (**14**), which is produced by *Neotyphodium lolii* and is a potent tremorgenic neurotoxin.[19]

Besides their biological activities, structural diversity and complexity are important characteristics of the fungal meroterpenoids, as depicted in Fig. 1. These intriguing structures

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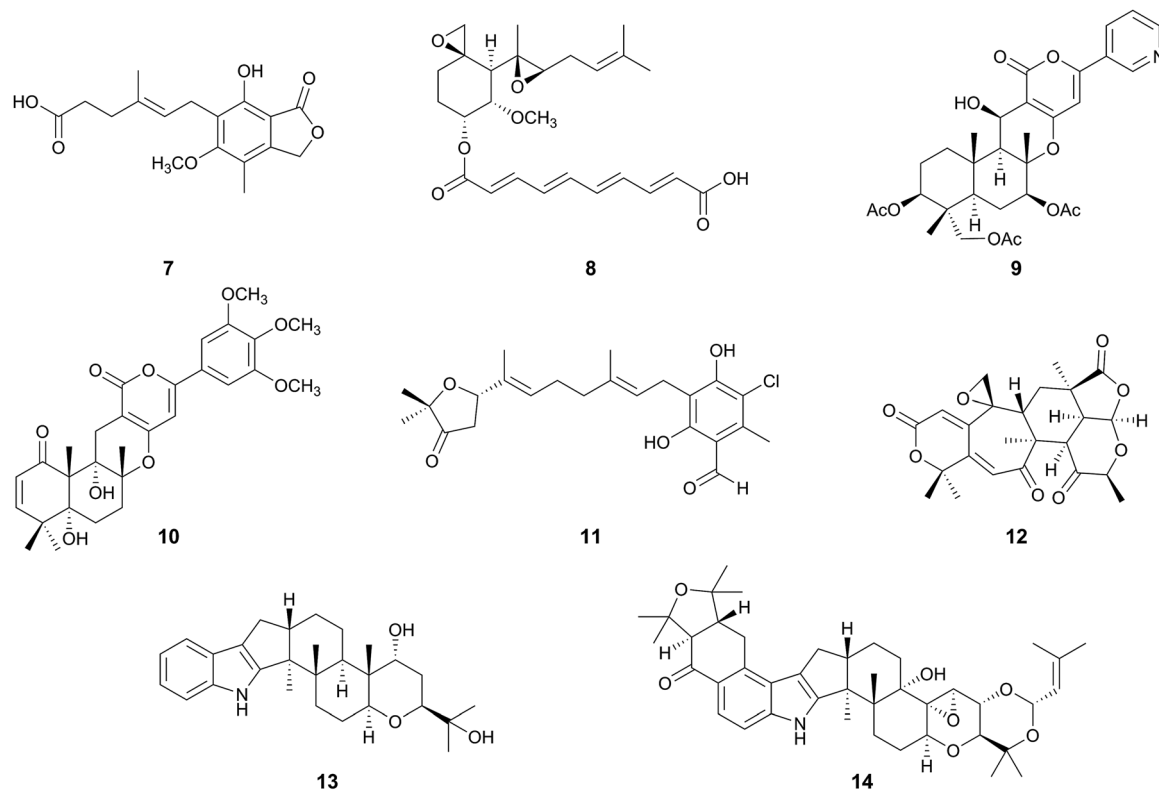Nat. Prod. Rep., 2016, **33**, 26–53 | 27

Fig. 1 Representative fungal meroterpenoids.

have attracted scientists for elucidation of their biosynthetic pathways, as well as the key reactions involved in their biosyntheses. Significantly, recent advances in genome-sequencing technologies and the development of tools for biosynthetic studies, as well as intensive researches at genetic and enzymatic levels, have allowed the discovery of many biosynthetic gene clusters for fungal meroterpenoids. This review is intended to continue the coverage of the literature previously reviewed in this journal by Geris and Simpson.[1] It will mainly focus on the recent biosynthetic studies of fungal meroterpenoids for which the biosynthetic genes/enzymes have been elucidated, and also discuss the plausible biosynthetic mechanisms of some related meroterpenoids for which genetic information is lacking. Fungal meroterpenoids are quite often generated *via* the addition of a C_5 isoprene unit, catalyzed by dimethylallyltryptophan synthases (DMATSs), but we will limit this review to the meroterpenoids originating from relatively long prenyl chains (more than C_5) as the functions of DMATSs have been well summarized elsewhere.[20,21]

2 Pyripyropene A: a model case of fungal meroterpenoid biosynthesis

2.1 Biosynthesis of pyripyropene A

Due to their structural diversity, it is difficult to generalize the biosynthetic machinery of fungal meroterpenoids, but the

biosynthesis of pyripyropene A (**9**), which has been recently fully elucidated (Fig. 2A), exemplifies one common strategy for the generation of this class of natural products. Basically, the biosynthesis can be divided into several phases: preparation of the non-terpenoid starter unit, prenylation of the non-terpenoid moiety, epoxidation of the olefin of the prenyl group, proton-ation-initiated cyclization of the prenyl portion, and various decorations of the cyclized intermediate.

Earlier studies of pyripyropene A biosynthesis, using isotope-labeled substrates, allowed an outline of the pathway to be proposed, albeit without genetic information.[22] Subsequently, the biosynthetic gene clusters for **9** have been individually identified from two different fungal species, *Aspergillus fumigatus* Af293 (the *pyr* cluster) and *Penicillium coprobium* PF 1169 (the *ppb* cluster), and the biosynthesis was investigated, mainly by heterologous expression of the genes in *Aspergillus oryzae*.[23–25] The two clusters indeed share the same gene organization, with high sequence identity with each corresponding gene in the other cluster. The first half of the biosynthesis of **9** was investigated using the *pyr* genes,[23] whereas the latter half was elucidated using the *ppb* cluster.[24,25] Considering the high similarity between the two clusters, it is reasonable that each corresponding enzyme plays the same role in the biosynthetic scheme.

In the pyripyropene A pathway, the non-terpenoid portion is synthesized by two enzymes, the coenzyme A (CoA)-ligase Pyr1/Ppb1 and the iterative type I polyketide synthase (PKS) Pyr2/Ppb2, from an unusual nicotinic acid (**15**) starter. In the first

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(A)

(B)
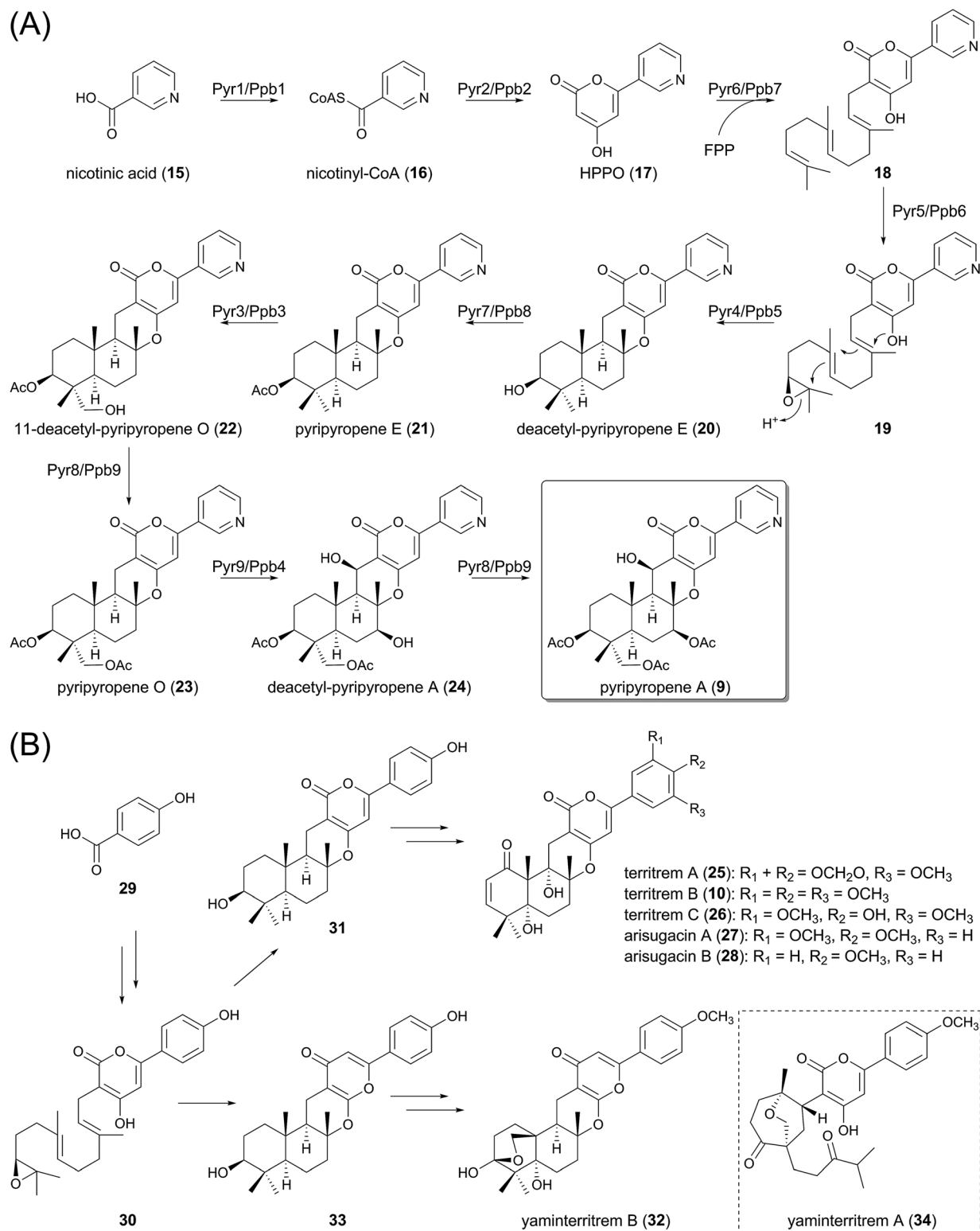
Fig. 2 Biosynthesis of (A) pyripyropene A and (B) related compounds.

committed step, **15** is transformed into nicotinyl-CoA (**16**) by Pyr1/Ppb1, which is then used as the starter substrate for the PKS Pyr2/Ppb2, with ketosynthase, acyltransferase, and acyl carrier protein domains, but presumably no other functional domains,

to yield 4-hydroxy-6-(3-pyridinyl)-2*H*-pyran-2-one (HPPO, **17**) by condensation with two molecules of malonyl-CoA.

The α-pyrone **17** is subsequently farnesylated by the trans-membrane prenyltransferase (PTase) Pyr6/Ppb7, using farnesyl

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pyrophosphate (FPP) as a prenyl donor, to provide **18**. This reaction is followed by the epoxidation of the terminal olefin, catalyzed by the flavin-dependent monooxygenase (FMO) Pyr5/Ppb6. Interestingly, Pyr6/Ppb7 is homologous to UbiA, the 4-hydroxybenzoate polyprenyltransferase involved in ubiquinone biosynthesis,[26] whereas Pyr5/Ppb6 shares sequence similarity with UbiF, UbiH, and UbiI, which are also oxidative enzymes required for ubiquinone biogenesis.[27] This observation suggests that the gene cluster evolved from the ubiquinone pathway.

The resultant epoxide **19**, synthesized by Pyr5/Ppb6, undergoes cyclization of its terpenoid moiety to yield deacetyl-pyripyropene E (**20**). The cyclization reaction, initiated by an acid-catalyzed epoxide ring-opening with participation by a neighboring π-bond, is catalyzed by Pyr4/Ppb5. These enzymes are membrane-bound proteins with seven-trans-membrane helices, and therefore the enzyme can also serve as a transporter or has been evolved from transport proteins.[28] Intriguingly, they share no sequence homology with any previously characterized terpene cyclases (TPCs). Thus, the detailed mechanism for the catalysis still remains unclear, but a mutational study has revealed that two conserved acidic amino acid residues, Glu63 and Asp218 (numbering in Pyr4), are essential for the cyclization, suggesting that these residues might serve as general acids that protonate the epoxide ring of **19**.[23] Importantly, a protein homologous to Pyr4 is also present in the actinomycete biosynthetic pathways, such as that for xiamycin A.[29]

The scaffold of the pyripyropenes is thus prepared, and is then subjected to the tailoring reactions. The acetyltransferase Pyr7/Ppb8 acetylates the hydroxyl group of deacetyl-pyripyropene E (**20**) to yield pyripyropene E (**21**), which is hydroxylated by the cytochrome P450 monooxygenase Pyr3/Ppb3 to produce 11-deacetyl-pyripyropene O (**22**). The other acetyltransferase Pyr8/Ppb9 introduces an acetyl group to this newly introduced hydroxyl group, thus producing pyripyropene O (**23**). Two distinct positions of **23** undergo hydroxylation reactions catalyzed by the other P450 Pyr9/Ppb4 for conversion into deacetyl-pyripyropene A (**24**), and the acetyltransferase Pyr8/Ppb9 again accepts **24** to complete the pathway for pyripyropene A (**9**).

As discussed later in this review, the fungal meroterpenoid pathways often employ biosynthetic enzymes similar to those for the production of **9** in their early biosynthetic stage. For example, the iterative type-I PKS, UbiA-like PTase, flavin-dependent epoxidase, and transmembrane TPC are often utilized as common components for meroterpenoids biosyntheses, thus facilitating the rapid discovery of putative biosynthetic gene clusters for meroterpenoids from the available genomic data.

2.2 Biosynthesis of territrems, arisugacins, and yaminterritrem B

The pyripyropene A pathway provides plausible schemes for the biosynthesis of molecules lacking genetic information, such as territrems A–C[30] (**25**, **10**, **26**) and arisugacins A (**27**) and B (**28**)

(Fig. 2B),[31,32] which are all strong inhibitors of AChE, although they have similar carbon skeletons to that of **9**. If 4-hydroxy-benzoic acid (**29**) is used instead of nicotinic acid (**15**), and undergoes the same reactions utilized in the first five steps of the pyripyropene A pathway, then the cyclized compound **31** harboring the scaffolds of **10** and **25–28** would be obtained, via the pre-cyclization intermediate **30**. Further modifications, including oxidations and methylations, should consequently generate **10** and **25–28**. Meanwhile, yaminterritrem B (**32**) has a quite similar backbone structure to those of **10** and **25–28**, but differs in that it contains the γ-pyrone moiety instead of the α-pyrone.[33] Compound **32** also appears to be derived from **30**, but the cyclization occurs in a different manner to provide **33**, an isomer of **31**, which undergoes several oxidations and ether linkage formation to generate **32** (Fig. 2B). Interestingly, the fungal strain producing **32** also synthesizes another meroterpenoid, yaminterritrem A (**34**), which harbors the α-pyrone motif.[33]

The biosyntheses of **9**, **10**, **25–28**, and **32** provide important insights into fungal meroterpenoid pathways that generate their structural diversity: the same intermediate can be cyclized into different compounds, and furthermore, the same or highly similar scaffolds can be tailored in versatile manners.

2.3 Biosynthesis of 15-deoxyoxalicines

Although HPPO (**17**) is farnesylated in the pyripyropene A biosynthesis, **17** can also undergo geranylgeranylation in other pathways. Very recently, the biosynthetic gene cluster for 15-deoxyoxalicine A (**35**) and B (**36**) was discovered from the genome of *Penicillium canescens* (the *olc* cluster), and their biosyntheses were investigated by targeted gene deletions.[34] In the proposed pathway (Fig. 3), after the formation of **17** in the same way as that for pyripyropene A (**9**), **17** is geranylgeranylated by the UbiA-like PTase OlcH to yield **37**. Interestingly, unlike the biosynthetic machinery for **9**, the prenyl donor of the pre-nylation, geranylgeranyl pyrophosphate (GGPP), seems to be prepared by the GGPP synthase OlcC encoded by the *olc* cluster, whereas the *pyr* cluster possesses no corresponding gene. Subsequently, the FMO OlcE epoxidizes **37** to **38**, which is cyclized into predecaturin E (**39**) by the transmembrane TPC OlcD.

The multicyclic intermediate **39** is then oxidized by the P450 OlcG to provide decaturin E (**41**) probably via **40**, which is the presumable product of OlcG. Two further rounds of oxidations by the P450 OlcJ and the short-chain dehydrogenase/reductase (SDR) OlcF transforms **41** into a predicted intermediate **43** via **42**, which is followed by the hemiacetal formation to give decaturin C (**44**). Afterward, **44** undergoes hydroxylation to generate decaturin A (**45**) by the Fe(II)/α-ketoglutarate (α-KG)-dependent dioxygenase OlcK and the transmembrane protein OlcL. Finally, **45** is converted into the end product **36** with the aid of the P450 OlcB. Multicyclic **44**, the deoxy analogue of **45**, can also be accepted by OlcB, generating **35**.

The mechanism for the spiro-lactone formation in the last step of the biosynthesis is proposed as follows.[34] OlcB hydroxylates either of the methyl groups bound to C-28 to yield
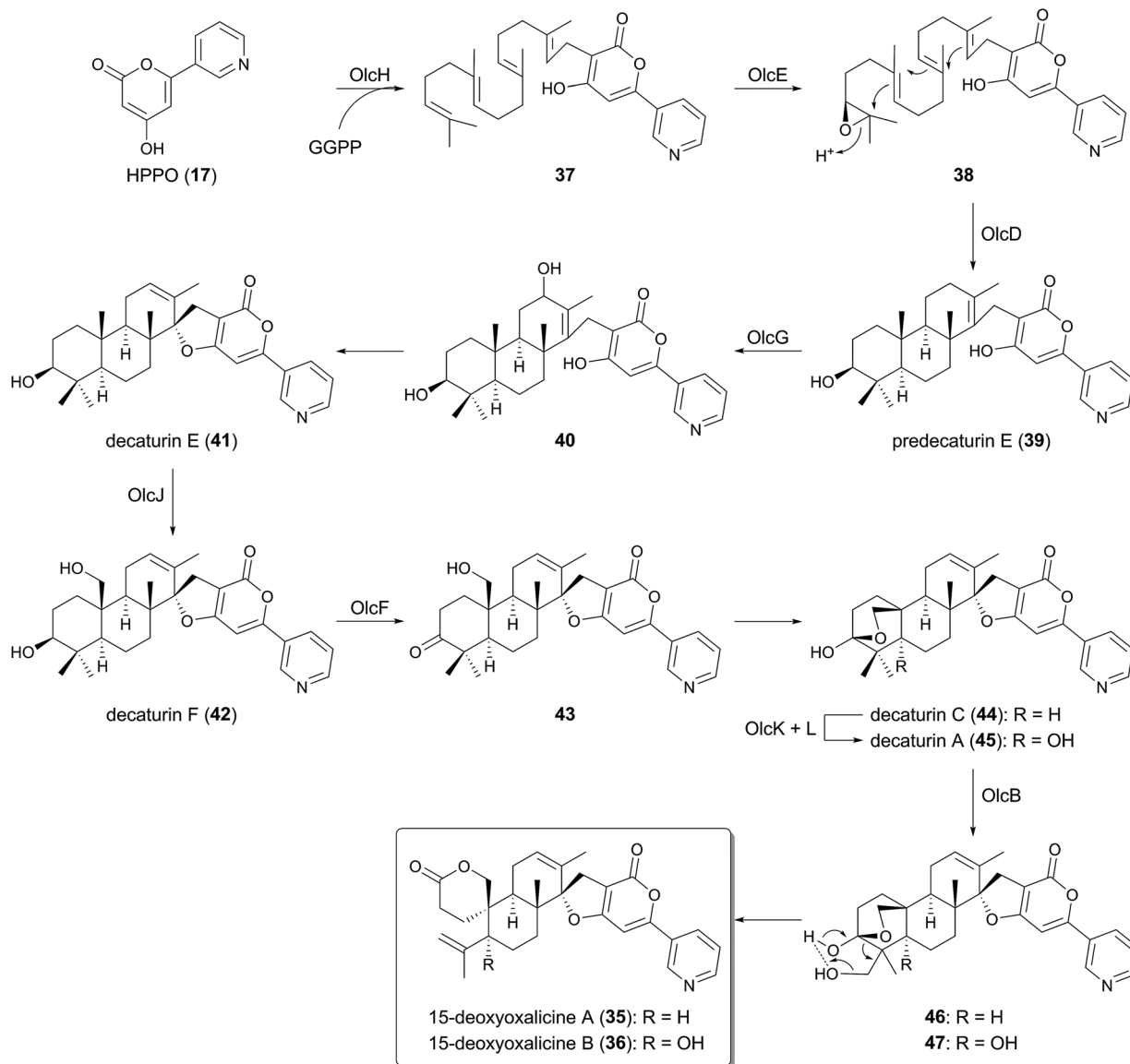
Fig. 3 Biosynthesis of 15-deoxyoxalicines.

46 or **47**, which could induce the elimination of a water molecule and the oxidative rearrangement to form **35** or **36** (Fig. 3).

3 Meroterpenoids with an uncyclized terpenoid moiety

Although many biosynthetic pathways for fungal meroterpenoids involve the cyclization of their prenyl side chains, those with linear terpenoid moieties are also widely found in nature, including mycophenolic acid (**7**), yanuthone D (**48**),[35] and aspernidine A (**49**).[36] Thus, the gene clusters for these compounds do not encode the enzymes for the terpene cyclization. This section summarizes the molecular bases for the biosyntheses of **7**, **48**, and **49**, which have recently been investigated.

3.1 Biosynthesis of mycophenolic acid

The biosynthetic gene cluster of **7** was first identified in the genome of *Penicillium brevicompactum* IBT23078 (the *mpa* cluster),[37] and subsequently was also discovered in *P. brevicompactum* NRRL 864 (the *mpa′* cluster).[38] The two clusters are almost identical, and each consists of seven open reading frames, with the same gene organization.

The first three steps of the pathway were investigated using the *mpa* genes, by gene deletion and heterologous expression in *Aspergillus nidulans* (Fig. 4).[37,39,40] The initial reaction is catalyzed by the type-I non-reducing PKS (NR-PKS) MpaC, which synthesizes 5-methylorsellinic acid (**50**, 5-MOA) from one molecule of acetyl-CoA, three molecules of malonyl-CoA, and one molecule of *S*-adenosylmethionine (SAM). In the next step, the unique chimeric protein of P450 monooxygenase and the hydrolase, MpaDE, transforms **50** into 5,7-dihydroxy-4-

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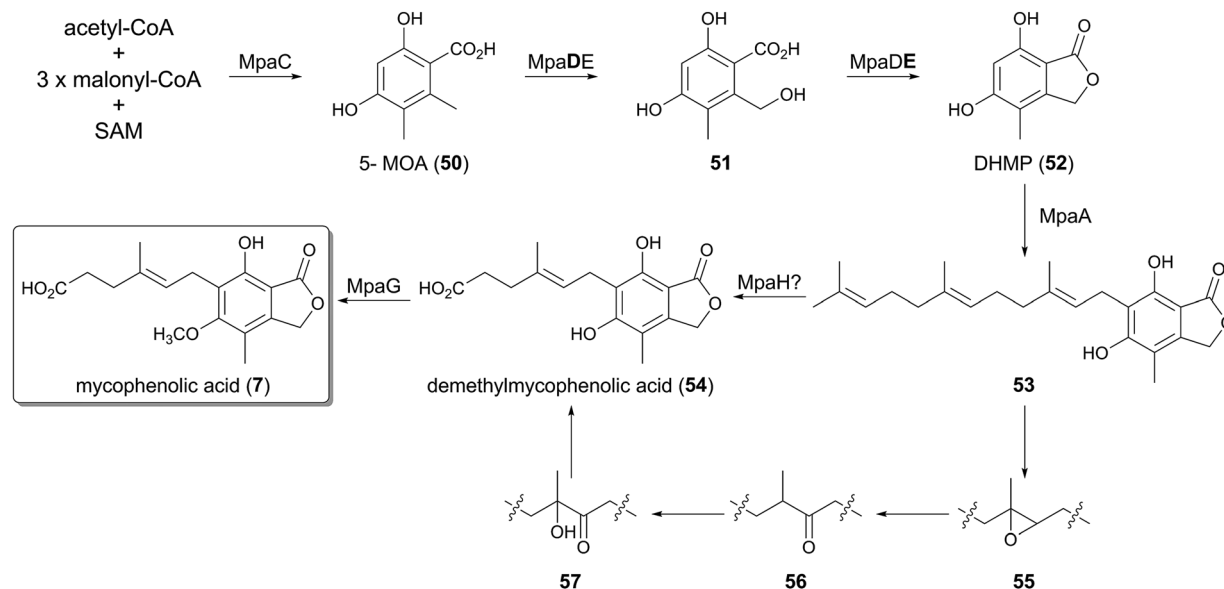*Nat. Prod. Rep.*, 2016, **33**, 26–53 | 31

Fig. 4   Biosynthesis of mycophenolic acid.

methylphthalide (**52**, DHMP), in which the P450 domain (MpaD) is responsible for the hydroxylation of the methyl group, whereas the hydrolase domain (MpaE) performs the lactonization to yield the phthalide **52**. The bifunctionality of MpaDE was confirmed by the observation that the heterologous expression of MpaC and only the MpaD part of the chimeric enzyme resulted in the accumulation of **51**. A similar fusion protein is also utilized to form a phthalide derivative in the biosynthesis of anditomin, as described later.[41]

The mid-stage of the mycophenolic acid pathway after DHMP (**52**) still remains to be elucidated, since there is no experimental evidence to date. The phthalide **52** would then be accepted by the PTase MpaA, which is homologous to Pyr6/Ppb7, and converted into its farnesylated form **53**. The next step should be the oxidative olefin cleavage of the prenyl side chain to synthesize demethylmycophenolic acid (**54**), in a reaction that may be catalyzed by MpaH, which exhibits sequence homology to α/β-hydrolases.[37] An isotope-feeding study suggested the mechanism for the oxidative cleavage, in which the olefin of **53** is transformed into the epoxide **55**, the ketone **56**, and the α-hydroxy ketone **57** in this order.[42] Subsequent C–C bond cleavage would finally produce **54**, but the actual intermediates for this conversion have yet to be identified.

Intriguingly, MpaH shares weak amino acid sequence identity (*ca.* 15% identity) with 1-*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase (HOD) and 1-*H*-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (QDO).[43] HOD and QDO both have the α/β-hydrolase fold, but these enzymes serve as cofactor-independent dioxygenases that cleave the C–C double bond of their substrate quinoline derivatives, resulting in the formation of a carboxyl group. The reactions by HOD and QDO are similar to those in the mycophenolic acid pathway, in terms of the olefin cleavage followed by the carboxylate formation. Thus, MpaH could also perform its catalysis in a similar manner to that of HOD and

QDO, but this should be confirmed by the future biochemical characterization of MpaH.

The last reaction in the biosynthetic scheme was established by biochemical experiments using the purified MpaG′ protein,[38] in which the phenolic hydroxyl group of demethylmycophenolic acid (**54**) is methylated (Fig. 4). Investigations of the substrate tolerance of MpaG′ revealed that the enzyme is promiscuous, as it accepts several unnatural substrates.

3.2   Biosynthesis of yanuthone D

The farnesylated epoxy cyclohexenoids, yanuthones, are known as antifungal compounds targeting *Candida albicans*, and among them yanuthone D (**48**) is the most potent agent.[44] The biosynthetic gene cluster for **48**, consisting of ten open reading frames, was identified in the genome of *Aspergillus niger*, and was designated as the *yan* cluster.[45] The yanuthone D pathway was studied by gene disruption, heterologous expression, and isotope-feeding experiments (Fig. 5).

The partially reducing PKS (PR-PKS) YanA initiates the pathway to form 6-methylsalicylic acid (**58**, 6-MSA). Compound **58** is subsequently transformed to *m*-cresol (**59**) by the decarboxylase YanB, and is further converted to toluquinol (**60**) by the P450 YanC. The involvement of **58** and **59** in the biosynthesis of yanuthone D (**48**) was validated, as the feeding of **58** or **59** restored the production of **48** upon the deletion of the PKS gene *yanA*. These results excluded the previous hypothesis that **48** is derived from shikimic acid.[35]

The next several steps still remain ambiguous since no biosynthetic intermediate has been obtained from the targeted gene deletions. It was proposed that toluquinol (**60**) undergoes epoxidation to generate **61**, in a reaction that might be catalyzed by YanD, a putative dehydrogenase, and/or YanE with unknown function. Compound **61** is then farnesylated by the UbiA-like PTase YanG to yield 7-deacetoxyyanuthone A (**62**). The
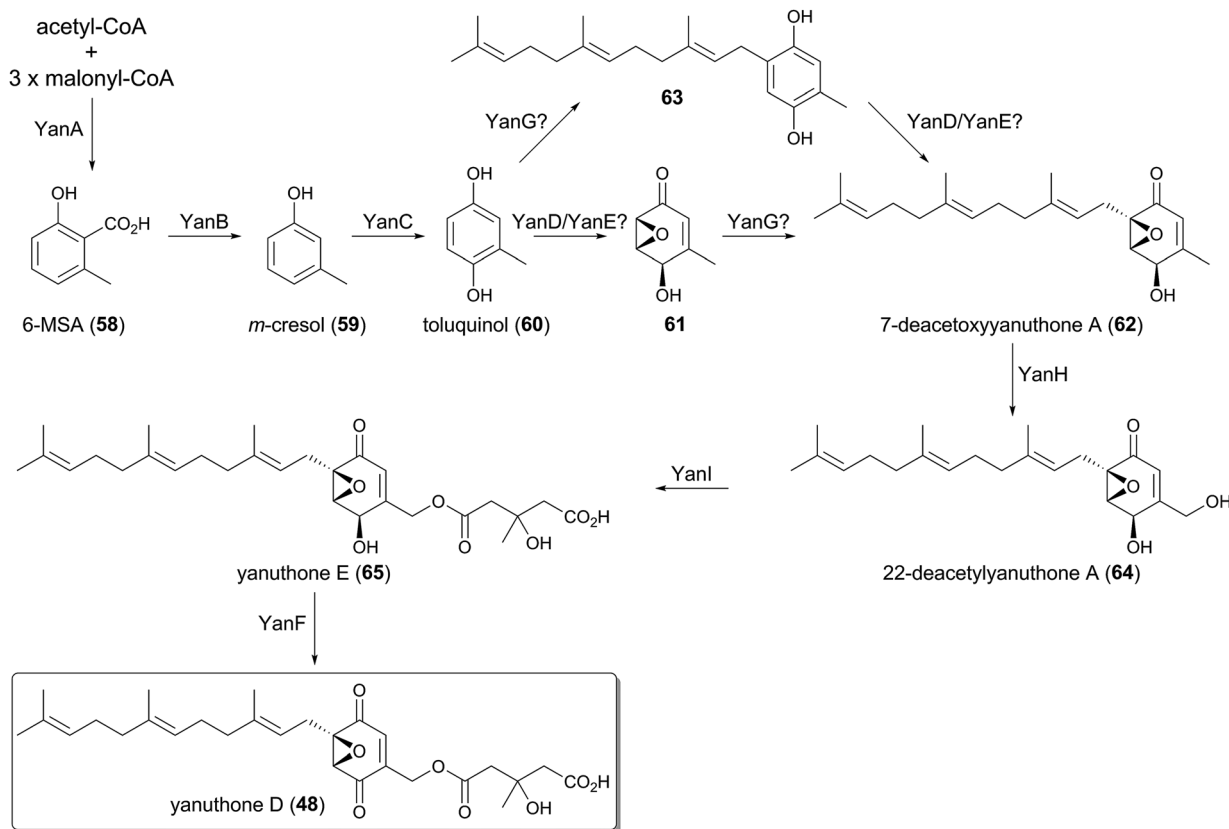
Fig. 5 Biosynthesis of yanuthone D.

epoxidation is predicted to precede the farnesylation, since the prenylated toluquinol has never been isolated from the yanuthone D-producing strain or its mutants.[45] However, considering that the *C*-prenylation reaction generally requires the π-electron of a prenyl acceptor to attack the allylic cation generated on the prenyl donor, **60** may also be accepted by YanG to yield **63** before the epoxidation occurs, as **61** loses a π-electron at the prenylation site due to the epoxide formation.

The late-stage biosynthesis of yanuthone D (**48**) is well established, since the precursors of **48** were successfully obtained by the gene disruptions. The P450 YanH hydroxylates **62** to 22-deacetylyanuthone A (**64**), and the introduced hydroxyl group is then mevalonylated by the membrane-bound *O*-acyltransferase YanI to generate yanuthone E (**65**). Lastly, the flavin-containing dehydrogenase YanF oxidizes the secondary alcohol of **65**, to produce the ketone functionality and complete the biosynthesis.

3.3   Biosynthesis of aspernidine A

The prenylated isoindolinone alkaloid, aspernidine A (**49**), has been isolated as a bioactive compound with moderate anti-proliferative activity.[36] The *pkf* cluster, responsible for the biosynthesis of **49**, was subsequently identified in the genome of *A. nidulans*.[46] The cluster is relatively small with six genes altogether, and the functions of each gene were investigated by gene deletion experiments (Fig. 6).

In the biogenesis of aspernidine A (**49**), PkfA, an NR-PKS with a reductase (R) domain at its C-terminus, first synthesizes orsellinaldehyde (**66**). Compound **66** is subsequently converted into aspernidine D (**67**), in which the farnesylation should be performed by the UbiA-like PTase PkfE, whereas the other enzymes participating in the remaining transformations (hydroxylations and methylation) have yet to be discovered. The P450 PkfB introduces a hydroxyl group to yield aspernidine E (**68**), which is proposed to be further oxidized to the dialdehyde **69** by the dehydrogenase PkfF. The last several steps leading to **49** are still enigmatic since no enzyme for this scheme has been identified.

4   Meroterpenoids derived from 3,5-demethylorsellinic acid

Among the fungal meroterpenoids, those derived from a simple aromatic tetraketide 3,5-dimethylorsellinic acid (**70**, DMOA) display huge structural diversity. After the isolation of the first member of this group in 1976,[47] >100 compounds have been described as DMOA-derived meroterpenoids. These molecules include andrastin A (**71**),[48–50] austinol (**72**),[51] terretonin (**73**),[52] anditomin (**74**),[53] novofumigatonin (**75**),[54] tropolactone A (**76**),[55] paraherquonin (**77**),[56] and emervaridione (**78**)[57] (Fig. 7). It is noteworthy that they often possess complex and intriguing skeletons, as exemplified by the spiro-lactone of **72**, the

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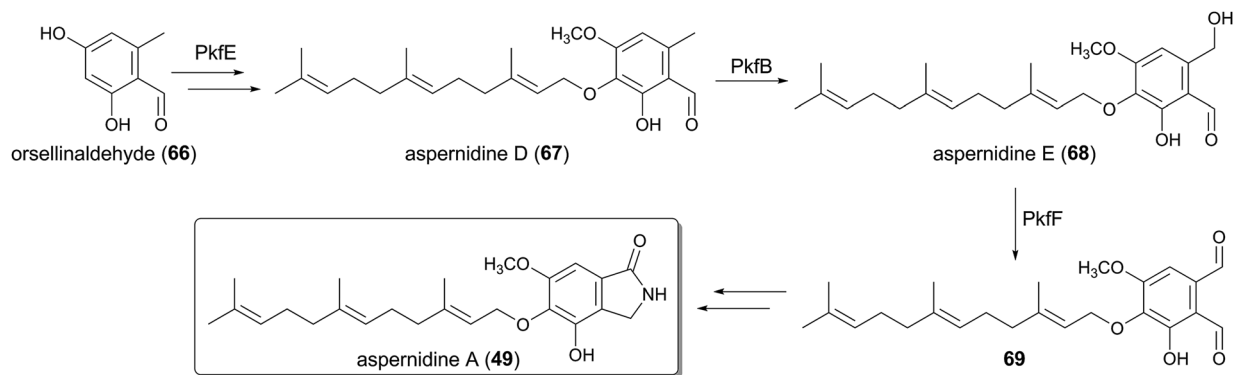Nat. Prod. Rep., 2016, 33, 26–53 | 33

Fig. 6 Biosynthesis of aspernidine A.

intramolecular bridged-ring systems of **74** and **78**, the orthoester of **75**, and the tropone moiety of **76**.

The biosyntheses of these fascinating molecules have been investigated for decades, and recently the molecular bases for four DMOA-derived meroterpenoids (**71–74**) have been reported,[41,58–65] enabling more in-depth predictions of the biosynthetic mechanisms for other related meroterpenoids. One of the major contributors to the structural diversity of DMOA-derived meroterpenoids is the presence of many cyclization patterns of the terpenoid moiety, as well as the divergence of the pathway before the cyclization. The other important factor is the existence of a variety of tailoring reactions that often are responsible for dynamic structural rearrangements. This section explains how a simple aromatic precursor DMOA (**70**) can be transformed into diverse and complex compounds.

4.1 Diversity in early-stage biosynthesis

The biosynthetic clusters for andrastin A (**71**), austinol (**72**), and terretonin (**73**) have been identified in the genomes of *Penicillium chrysogenum* (the *adr* cluster),[63] *A. nidulans* (the *aus* cluster),[59] and *Aspergillus terreus* (the *trt* cluster),[60] respectively,

and their functions have been investigated by gene deletion and/or heterologous expression approaches. The biosyntheses of **71–73** adopt the same early-stage reactions before the cyclization. At the outset, the NR-PKSs AdrD/AusA/Trt4 produce DMOA (**70**) in a similar manner to that of MpaA in the mycophenolic acid pathway, but use one additional molecule of SAM for the catalysis. The polyketide **70** subsequently undergoes farnesylation by the UbiA-like PTase AdrG/AusN/Trt2 to **66**, methyl-esterification by the methyltransferase AdrK/AusD/Trt5 to **80**, and epoxidation by the FMO AdrH/AusM/Trt8 to **81** (Fig. 8).[60,61] The epoxidation can precede the methylation, but in all of the cases, the methyl ester group is essential for the following cyclization step.[61,63] In the next biosynthetic process, the TPCs AdrI/AusL/Trt1 accept **81** to perform the cyclization to provide the common tetracyclic carbocationic intermediate **82**, which is then transformed into different products according to the position where the deprotonation occurs. Deprotonations from C-11 (H$_a$, AdrI), C-1′ (H$_b$, AusL), and C-9′ (H$_c$, Trt1) yield andrastin E (**83**), protoaustinoid A (**84**), and preterretonin A (**85**), respectively, thus demonstrating that the terpene cyclization diverges the pathway for **71–73** (Fig. 8 and 9).[61,63] It is remarkable that the stereochemistries of the epoxidation and
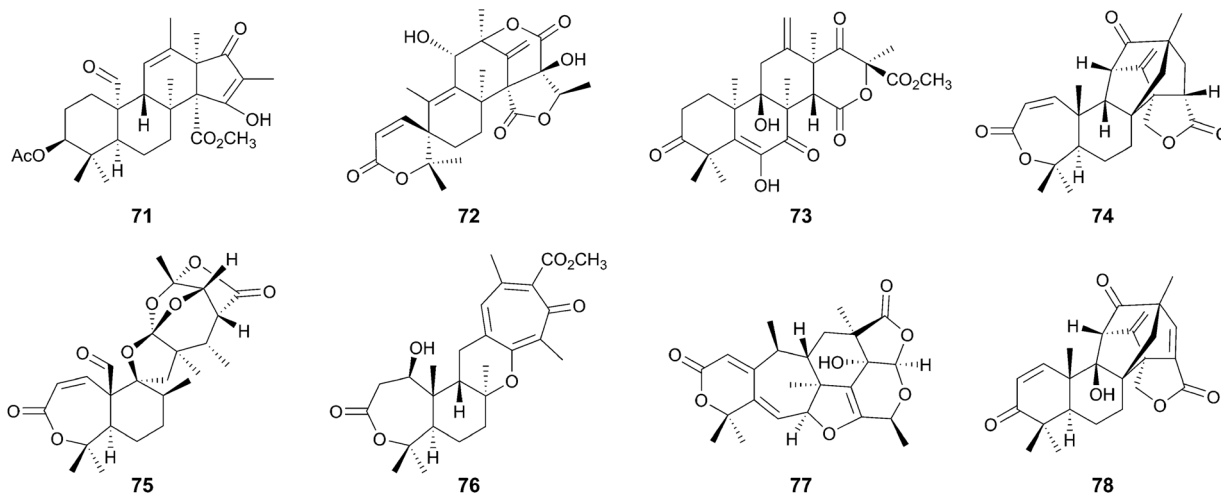

Fig. 7 Representative DMOA-derived meroterpenoids.

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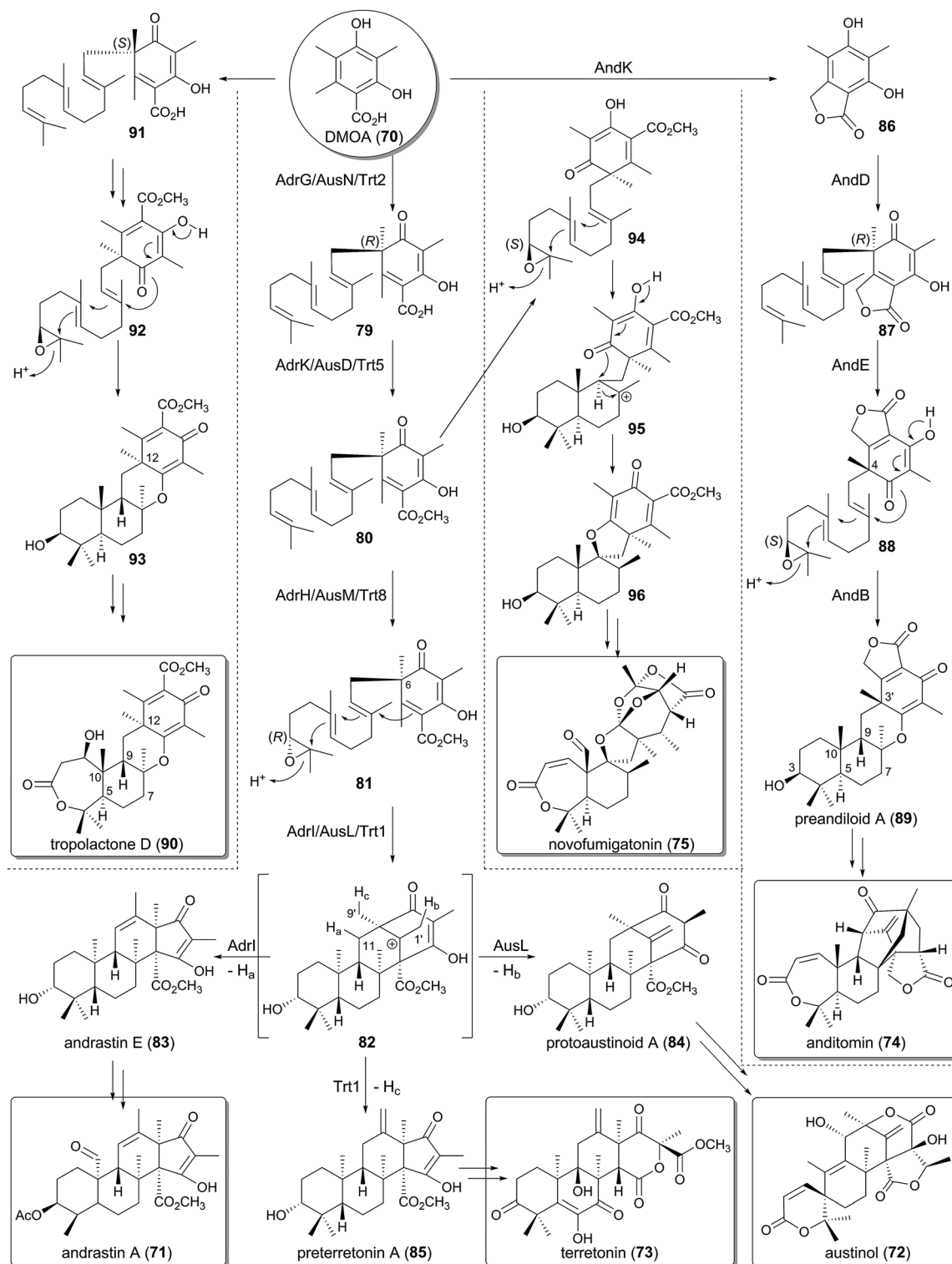This journal is © The Royal Society of Chemistry 2016

Fig. 8 Early-stage biosynthesis of DMOA-derived meroterpenoids.

cyclization reactions are rigidly controlled by the membrane-bound FMOs and the novel family of TPCs, respectively.

The biosynthesis of anditomin (74) involves a different strategy to prepare the substrate for the TPC (Fig. 8), which was revealed by the functional analysis of the *and* cluster discovered

in *Emericella variecolor*.[41] The opening process to produce DMOA (70) by the NR-PKS AndM is identical to that for 71–74, but in the second step, before prenylation occurs, 70 is converted to the phthalide 86 by AndK. AndK is a chimeric protein of P450 monooxygenase and hydrolase and is homologous to

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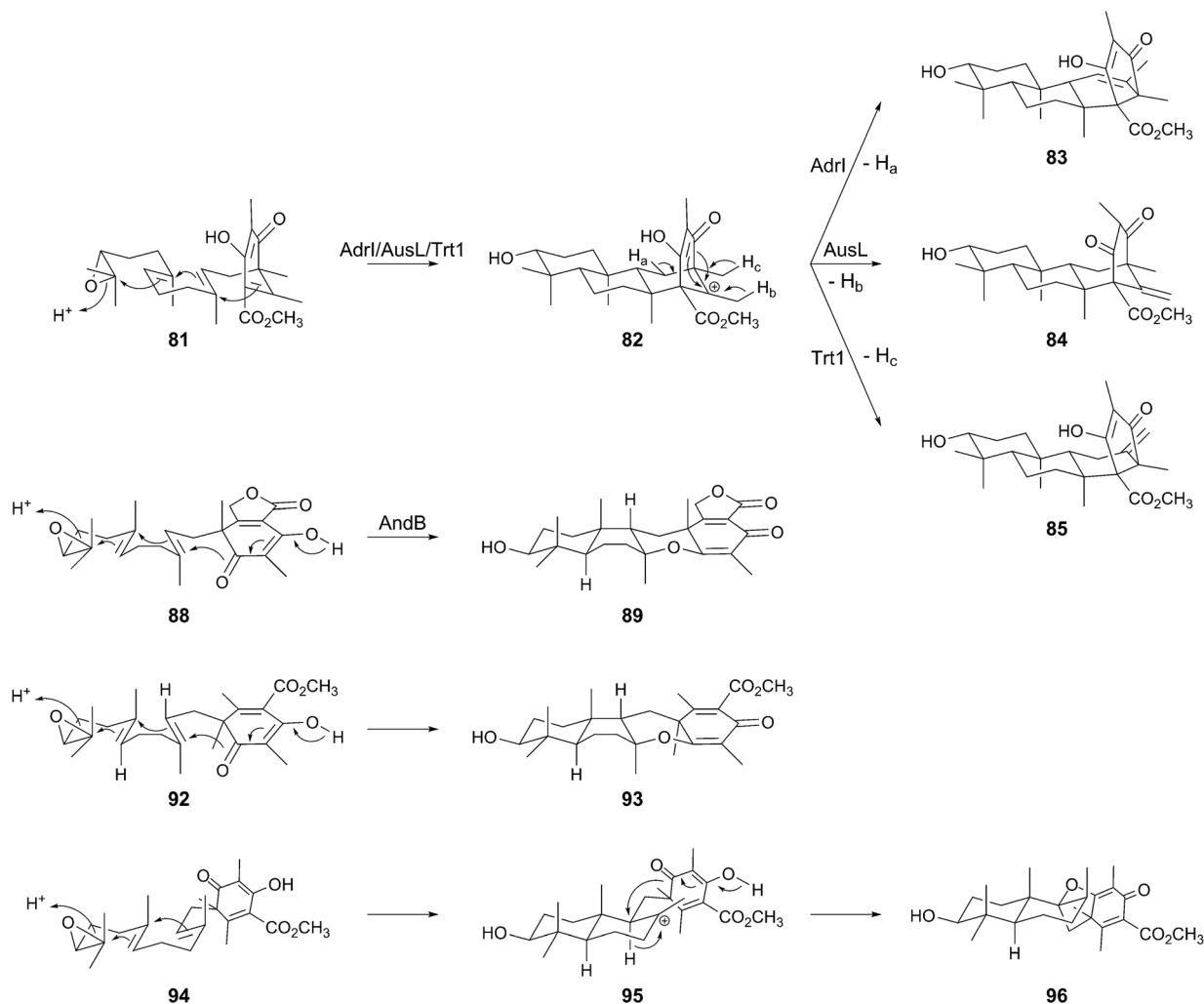Nat. Prod. Rep., 2016, 33, 26–53 | 35

Fig. 9 Comparison of the cyclization reactions in the DMOA-derived meroterpenoids biosynthesis.

MpaDE in the biosynthesis of mycophenolic acid, and thus it catalyzes two-rounds of reactions to provide **86**. The phthalide **86** is then transformed into the pentacyclic intermediate pre-andiloid A (**89**) *via* **87** and **88**, in which the UbiA-like PTase AndD, the FMO AndE, and the TPC AndB are responsible for the conversion (Fig. 8 and 9). Interestingly, the absolute configuration of the terpenoid moiety of **89** is 3S,5R,8S,9S,10S, whereas that for **83–85** it is 3R,5S,8S,9S,10R. These differences could be attributed to the distinct absolute configurations between the precyclization intermediates, **81** and **88**; the absolute configuration of **81** can be deduced to be 6R and 10′R, and that of **88** would be 4R and 10′S. This observation indicates that the anditomin pathway utilizes an epoxidase that forms an epoxide ring with different stereochemistry from that of **71–73**.

Furthermore, similar considerations for the stereochemistry can be applied to the biosynthesis of novofumigatonin (**75**) and tropolactone D (**90**) (Fig. 8 and 9).[54,55] Compound **90** has a similar skeleton to that of preandiloid A (**89**) with 5R,8S,9S,10S configuration, but the stereochemistry of one methyl group at C-12 (C-3′ of **89**) is opposite between the two compounds. This position is the site where the farnesyl group is attached, and

therefore a PTase that generates **91**, an enantiomer of **66**, might be involved in the biosynthesis of **90**. Compound **91** would subsequently be converted to the tetracyclic intermediate **93** *via* **92**, in a similar manner to that observed in the anditomin pathway. Meanwhile, considering its structure, **75** could be synthesized from **96** as the product of a TPC, although no biosynthetic study or proposed biosynthetic pathway has been reported to date. The direct precursor of **96** should be **94**, which would be cyclized to **96** *via* a cationic intermediate **95**. Compound **94** is an epimer of **81** with an (S)-epoxide, suggesting that the novofumigatonin biosynthesis pathway employs an epoxidase with different product selectivity from that of AdrH/ AusM/Trt8.

Collectively, not only the cyclization patterns but also the presence of PTases and epoxidases with different stereo-preferences greatly contribute to the production of the structural diversity of this class of natural products. It should be noted that the prenylation reactions in the DMOA-derived meroterpenoid biosynthesis destroy the aromaticity of the substrate DMOA (**70**), generating a chiral center in the product. This is different from the other PTases described above, in which the

aromaticity of the substrates remains after prenylation, thus contributing to the creation of structural diversity in the DMOA-derived metabolites.

4.2 Biosynthesis of andrastin A and related compounds

Andrastins, including andrastin A (**71**) and andrastins B–D (**94–96**) have been isolated as inhibitors of the protein farnesyltransferase,[48–50,66] and they have relatively simple structures among the DMOA-derived meroterpenoids. Consistent with their simple skeletons, the first tetracyclic intermediate andrastin E (**83**) is converted to the end product **71** with the aid of only four enzymes (Fig. 10).[63] First, **83** is oxidized into the ketone form **96** by the SDR AdrF, which is followed by reduction of the C-3 carbonyl to a β-hydroxyl group to provide andrastin F (**97**), a C-3 epimer of **83**. The reaction is catalyzed by the ketoreductase AdrE, and the discovery of **97** as a product of AdrE ruled out the previous proposal that **97** is directly generated by the terpene cyclization.[67] The β-alcohol of **97** is acetylated by the acetyltransferase AdrJ to yield **95**, and the P450 AdrA finally oxidizes the C-23 methyl group in two successive reactions to produce **94** and **71**.

Although the structure of andrastin A (**71**) is not very complex, **71** appears to be further derivatized into more complicated meroterpenoids in other fungi, which produce citreohybridone J (**98**), other citreohybridones,[67–69] iso-citreohybridones,[67,69–71] and citreohybriddiones.[70,72] Meanwhile, atlantinones A (**99**) and B (**100**) possess the same carbon skeleton as that of **71**, but differ in that they have the 3R configuration.[73,74] This suggests that **83** does not undergo C-3 epimerization in the atlantinones pathway, and the

lactonization of **101** generated by oxidation of the C-23 methyl group of **83** would produce **99**, which is hydroxylated for conversion into **100**.

4.3 Biosynthesis of terretonin and related compounds

The *trt* cluster, responsible for the biosynthesis of terretonin (**73**) and its analogue terretonin C (**102**)[75] lacking the methyl ester group, has been biochemically and molecular biologically analyzed by targeted gene deletion and heterologous expression in *A. oryzae*, leading to the complete determination of the pathway (Fig. 11).[60–62,65] The cyclase Trt1-product, preterretonin A (**85**), is oxidized at the C-3 position to generate preterrenoid (**103**) by the SDR Trt9, which is highly homologous to AdrF involved in the andrastin A pathway. Compound **103** is further hydroxylated at C-16 by the FMO Trt3 to yield terrenoid (**104**), which undergoes multistep oxidations by the P450 Trt6, and subsequent D-ring expansion to produce terretonin H (**106**). The ring-expanded product **106** seems to be derived from **105** by a spontaneous reaction, but the detailed mechanism for this rearrangement has yet to be clarified.

Interestingly, **104** can also be transformed into terretonin D (**107**), which is similar to **106** but contains an additional methyl ester group at the C-16 position, only in the presence of Trt14. An isotope-incorporation experiment with [methyl-¹³C]methionine excluded the possibility that Trt14 is a methyltransferase and indicated the involvement of the intramolecular transfer of the methoxy group.[65] Trt14 shares almost no amino acid sequence identity with any biochemically characterized proteins, but appears to be structurally related to some isomerases as well as epoxide hydrolases.[76–79] The detailed role of
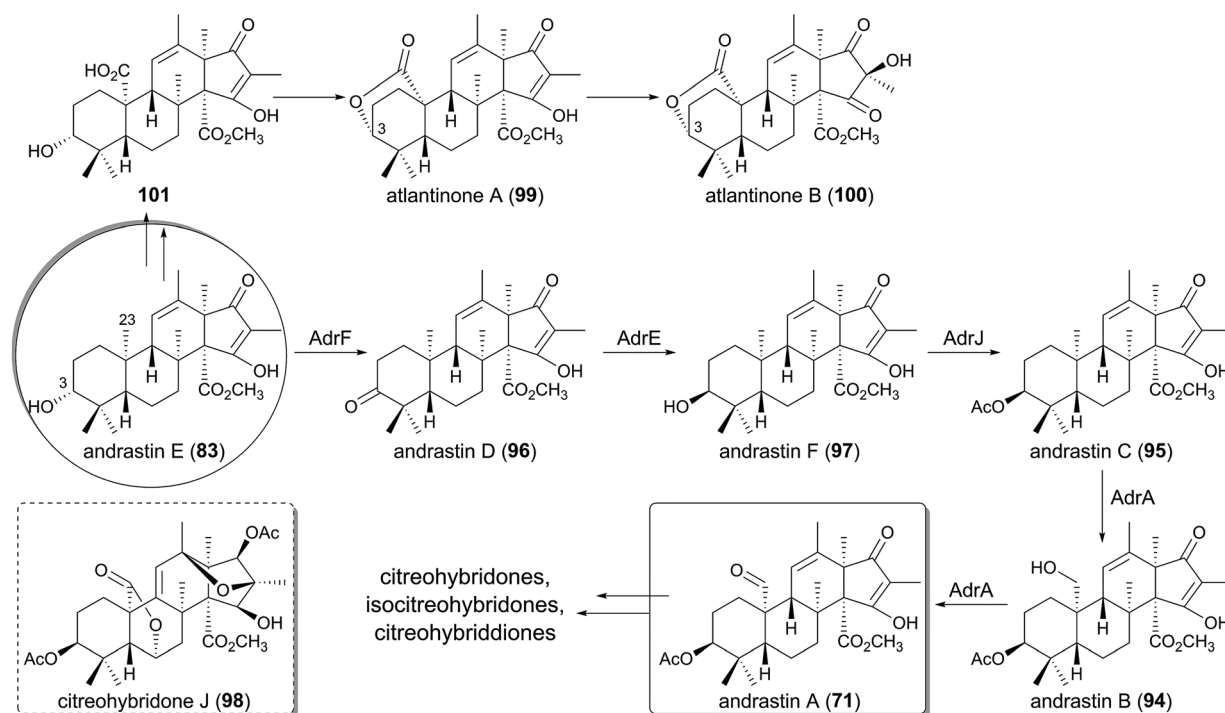

Fig. 10 Biosynthesis of andrastin A and related compounds.

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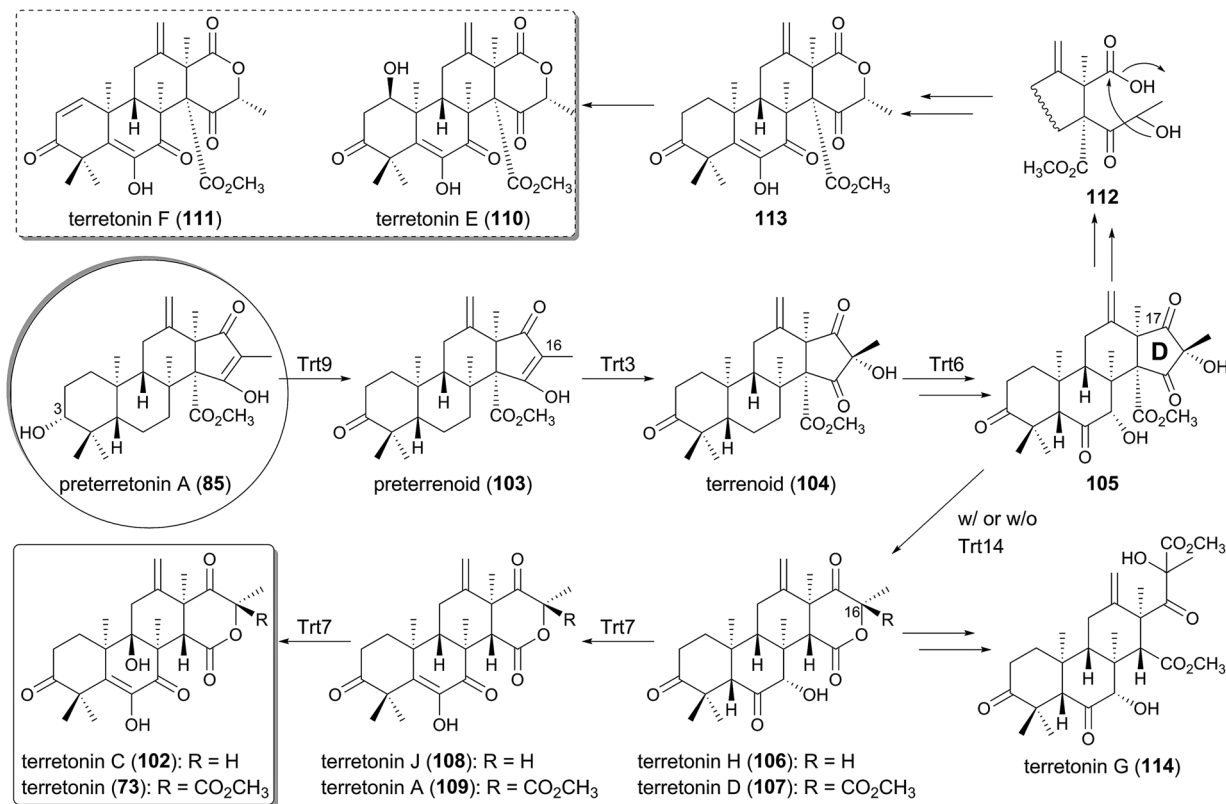*Nat. Prod. Rep.*, 2016, **33**, 26–53 | 37

Fig. 11   Biosynthesis of terretonin and related compounds.

Trt14 also has yet to be elucidated, but Trt14 would somehow be engaged in the intramolecular rearrangement of the methoxy group to afford the terretonin scaffold. Both **106** and **107** are then oxidized in two sequential reactions by the Fe(II)/α-keto-glutarate (α-KG)-dependent dioxygenase Trt7, which is homologous to phytanoyl-CoA 2-hydroxylase (PhyH).[80] In the Trt7-catalyzed reactions, **106** and **107** are first transformed into terretonin J (**108**) and A (**109**), and then further oxidized to terretonin C (**102**) and terretonin (**73**), respectively, to complete the biosynthesis.

Terretonin E (**110**) and F (**111**) have similar carbon skeletons to that of terretonin (**73**), but possess a distinct D-ring system,[81,82] suggesting that they are biosynthesized in a similar but slightly different manner from that for **73**. In the proposed biosyntheses of **110** and **111**, **105** is utilized as the pathway intermediate as well, and is converted to **113** *via* **112** by the nucleophilic attack of water at the C-17 ketone of **105** and the subsequent lactonization (Fig. 11). In contrast, the newest member of this group of meroterpenoids is terretonin G (**114**), from a marine-derived *Aspergillus* sp.,[83] and it is most likely derived from the hydrolysis of the D-ring lactone of **107** and the subsequent formation of a methyl ester (Fig. 11).

4.4   Biosyntheses of austinol/dehydroaustinol and related compounds

The biosyntheses of austinol (**72**) and its oxidized form dehydroaustinol (**115**) have attracted keen interest for biosynthetic

studies because of their intriguing structures, including the unique spiro-lactone system.[84–86] The biosyntheses of **72** and **115** are controlled by the *aus* cluster, which is separately located in two different regions on the chromosomes.[59] Almost the entire austinol pathway was initially elucidated by targeted gene disruption experiments,[58,59] and a subsequent heterologous expression study illuminated the missing link in the biosynthesis, to allow the proposal of the complete pathway for **72** (Fig. 12).[64]

The tetracyclic molecule protoaustinoid A (**84**) is oxidized into berkeleyone A (**116**) by the FMO AusB, which is highly homologous to Trt3. This reaction is followed by C-3 dehydrogenation catalyzed by the PhyH-like Fe(II)/α-KG-dependent dioxygenase AusE to generate preaustinoid A (**117**). In the next process, the FMO AusC inserts one oxygen atom between C-3 and C-4 to yield preaustinoid A1 (**118**), serving as a Baeyer–Villiger monooxygenase (BVMO). Subsequently, AusE again accepts **118** to perform two consecutive oxidations, in which **118** undergoes dehydrogenation to preaustinoid A2 (**119**) and oxidative rearrangement to preaustinoid A3 (**120**). Thus, AusE catalyzes multistep oxidation reactions before and after the Baeyer–Villiger reaction to generate the spiro-lactone ring system.

Meanwhile, in other meroterpenoid pathways, preaustinoid A1 (**118**), with a seven-membered lactone A-ring, is likely to be transformed into berkeleydione (**121**)[87,88] *via* berkeleyone B (**122**),[89] probably in two successive reactions similar to those of the multifunctional dioxygenase AusE. Compound **121** seems to

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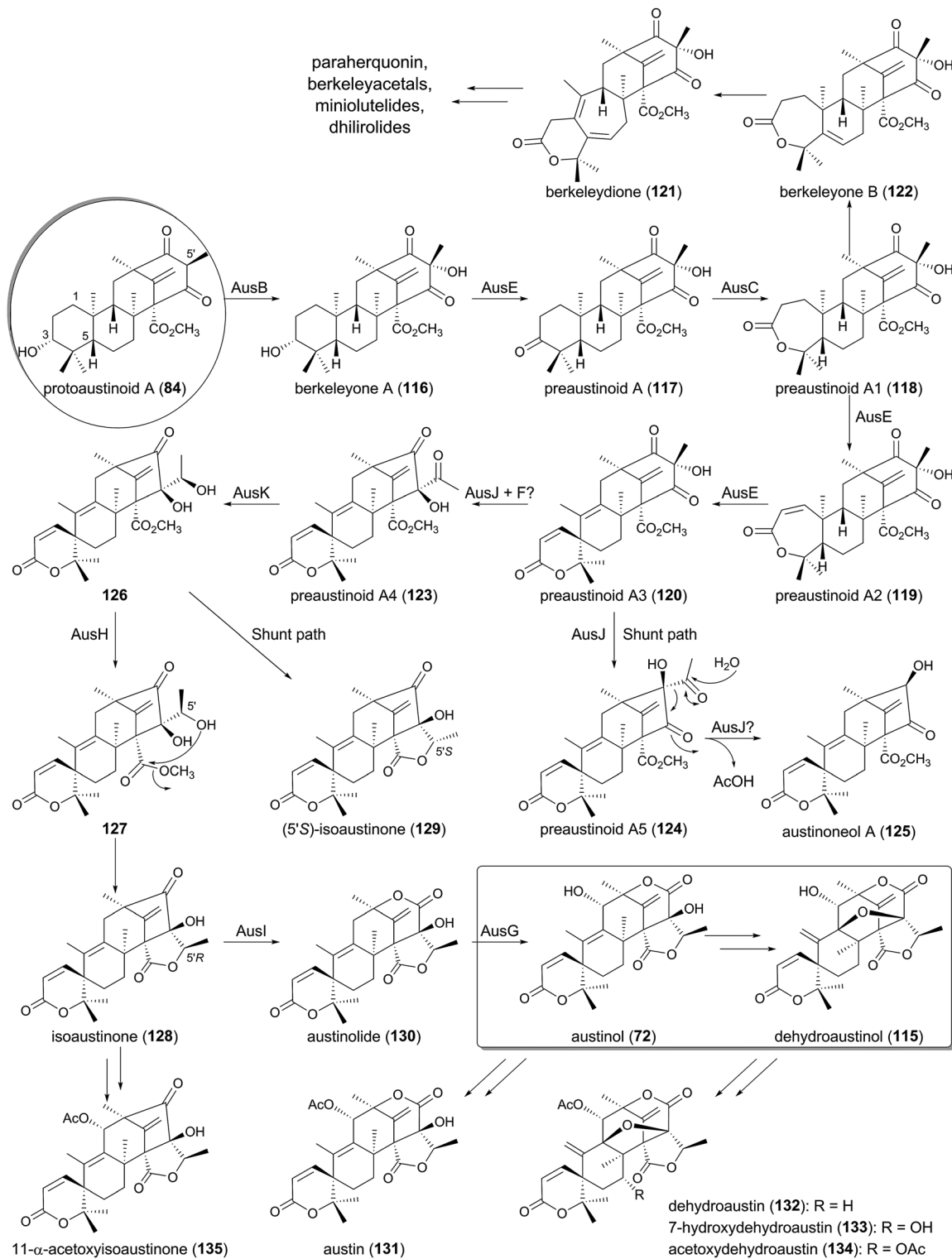This journal is © The Royal Society of Chemistry 2016

Fig. 12 Biosynthesis of austinol/dehydroaustinol and related compounds.

be a common biosynthetic intermediate of many fungal meroterpenoids, including paraherquonin (77),[56] berkeleyacetals,[16] miniolutelides,[90,91] and dhilirolides.[92,93] The possible mechanisms for the spiro-ring formation and the cycloheptadiene-formation of 121 are discussed later in this section.

The next several steps of the pathway are quite interesting, since they apparently involve three novel classes of proteins, AusJ, AusF, and AusH, which are all homologous to Trt14 in the terretonin biosynthesis and share almost no amino acid sequence homology to any characterized enzymes. AusJ

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catalyzes the isomerization of **120** to produce preaustinoid A4 (**123**) with an acetyl group, but the enzyme also seems to produce preaustinoid A5 (**124**), an isomer of **120** and **123**, as a shunt product. Compound **124** is predicted to be further transformed into austinoneol A (**125**), but the reaction mechanism and the enzyme responsible for this conversion have never been proposed. Given the proposed mechanism for Trt14,[65] it is possible that the reaction is also catalyzed by AusJ, in which AusJ performs the deacetylation by retro-Claisen cleavage. Intriguingly, the deletion mutant of *ausF*, encoding a protein homologous to AusJ (54% identity), accumulated **120** and **125** as the major metabolites, although it also produced austinol (**72**) and dehydroaustinol (**115**) in low yields. Thus, AusF was proposed to have a regulatory function in the biosynthesis,[59] but it is also possible that AusJ and AusF work collaboratively to synthesize the pathway intermediate **123**, and that AusJ alone is not sufficient to produce a good yield of **123**.

Preaustinoid A4 (**123**) is then converted into isoaustinone (**128**), with the aid of the ketoreductase AusK and AusH, and, interestingly, **123** is transformed into the shunt metabolite (5′*S*)-isoaustinone (**129**), the C-5′ epimer of **128**, in the absence of AusH. Due to the lack of intermediates between **123** and **128**, the mechanism for the lactonization is still unclear, but it could be proposed as follows. AusK reduces the ketone functionality of the acetyl group to produce **126** with the 5′*R* configuration, which is spontaneously lactonized to yield **129**. Contrary to this, in the presence of AusH, the apparently unstable intermediate **126** is accepted by AusH to undergo C-5′ epimerization to generate **127**, and the following lactonization provides **128**.

Finally, two P450 monooxygenases participate in the formation of austinol (**72**), in which AusI serves as the BVMO to yield austinolide (**130**) and AusG hydroxylates **130** to austinol (**72**). The very last step of the biosynthesis to provide dehydroaustinol (**115**) remains unresolved, since no enzyme for this conversion has been identified. At least, the enzyme(s) does not appear to be encoded in the *aus* cluster. In other fungi, the isoaustinone (**128**) and austinol/dehydroaustinol undergo further decorations, including hydroxylation and acetylation, to produce compounds such as austin (**131**), dehydroaustin (**132**),[94] 7-hydroxydehydroaustin (**133**),[95] acetoxydehydroaustin (**134**),[96] and 11-α-acetoxyisoaustinone (**135**).[95]

One of the most interesting reactions in the austinol pathway is the spiro-ring formation by AusE. Fe(II)/α-KG-dependent dioxygenases are known to initiate their reactions by abstracting a hydrogen atom from the substrate using the active site Fe(IV)-oxo species, which is generated by the oxidative decarboxylation of the cosubstrate α-KG.[97] On the basis of the general mechanism of this class of dioxygenases, several scenarios for the spiro-lactone synthesis can be described (Fig. 13A). In the originally proposed mechanism,[64] the C-5 hydrogen of the substrate preaustinoid A2 (**119**) is abstracted by the enzyme to produce the radical species **136**, which is hydroxylated by the enzyme to generate the hydroxylated intermediate **137** bound to an Fe(II) species. Upon elimination of the C-5 hydroxyl group as a water molecule, the rearrangement and deprotonation from C-9 occur to afford the spiro-lactone preaustinoid A3 (**120**) (Path A). Subsequently, an alternative mechanism has been proposed,

since the above-described mechanism requires a chemically unfavorable *syn*-rearrangement.[98] The other scheme involves the cyclopropane intermediate **138**, which is further rearranged to **139** with the spiro-lactone moiety. The enzymatic reaction is hereby completed by the rebound of the hydroxyl radical to **139** to yield **140**, which undergoes spontaneous dehydration to produce **120** (Path B-1). However, it is still questionable whether the dehydration indeed occurs non-enzymatically, as **120** might be directly generated from **139** by the abstraction of the C-9 hydrogen atom (Path B-2).

Although there is no experimental evidence to distinguish the three proposals described above, the biosynthetic study on andibenin B (**141**) provided the clue to solve this problem. Compound **141** contains a similar spiro-lactone moiety to that of **120**, and is apparently derived from andilesin B (**142**) in a manner resembling that described for AusE. The previous study elucidated that the C-10 hydroxyl group of **141** can be labeled with $^{18}O_2$.[99] If the reaction employs the cationic pathway (similar to Path A), then this position should not be labeled with $^{18}O_2$, as a water molecule would be used for the neutralization. This observation strongly suggests that the spiro-ring formation occurs by the radical-based pathway (similar to Path B), in which **143** exists as a reaction intermediate (Fig. 13B). One major difference regarding the carbaspirocycle-forming reactions in the austinol and andibenin pathways is that the two hydrogen atoms at C-5 and C-9 are both α-oriented in **119**, whereas they have different orientations in **142**. Thus, in the reaction for the synthesis of **141**, the C-9 hydrogen is on the opposite side of the active center, and the enzyme cannot abstract the C-9 hydrogen. This could explain why only the C-10 α-hydroxylated product is obtained in the andibenin pathway. Importantly, there has been no report that **141** undergoes spontaneous dehydration, although its C-9 hydrogen and C-10 hydroxyl group are in more favorable positions for dehydration than those in **140**. Collectively, Path B-2 in Fig. 13A would be the most plausible mechanism for the generation of preaustinoid A3 (**120**).

As mentioned above, berkeleydione (**121**) would also be produced by a similar mechanism from berkeleyone B (**122**) as the substrate (Fig. 13C). The reaction would be initiated by the abstraction of the C-1 hydrogen, yielding the substrate radical **144**. The cyclopropane-forming reaction could then provide **145**, which undergoes the carbon skeletal rearrangement to afford **146**. The hydrogen abstraction and resultant introduction of the double bond would generate the product **121**.

On the basis of the biosynthetic pathway and the enzymes for austinol (**72**) production, likely biosynthetic mechanisms of other related meroterpenoids can now be proposed. Here, the plausible biosyntheses of berkeleyacetal C (**12**) and dhilirolide A (**147**), which are both presumably derived from berkeleydione (**121**), are discussed (Fig. 14). In the berkeleyacetal C pathway, the *exo*-methylene of **121** would initially be oxidized into an aldehyde to generate **148**. Retro-Claisen cleavage would then yield the ring-opened intermediate **149**, and the recyclization of **149** would produce berkeleyacetal A (**150**),[16] which possesses the same scaffold as **12**. The existence of **148** is supported by the isolation of 22-epoxyberkeleydione (**151**) from several fungi.[90,91]

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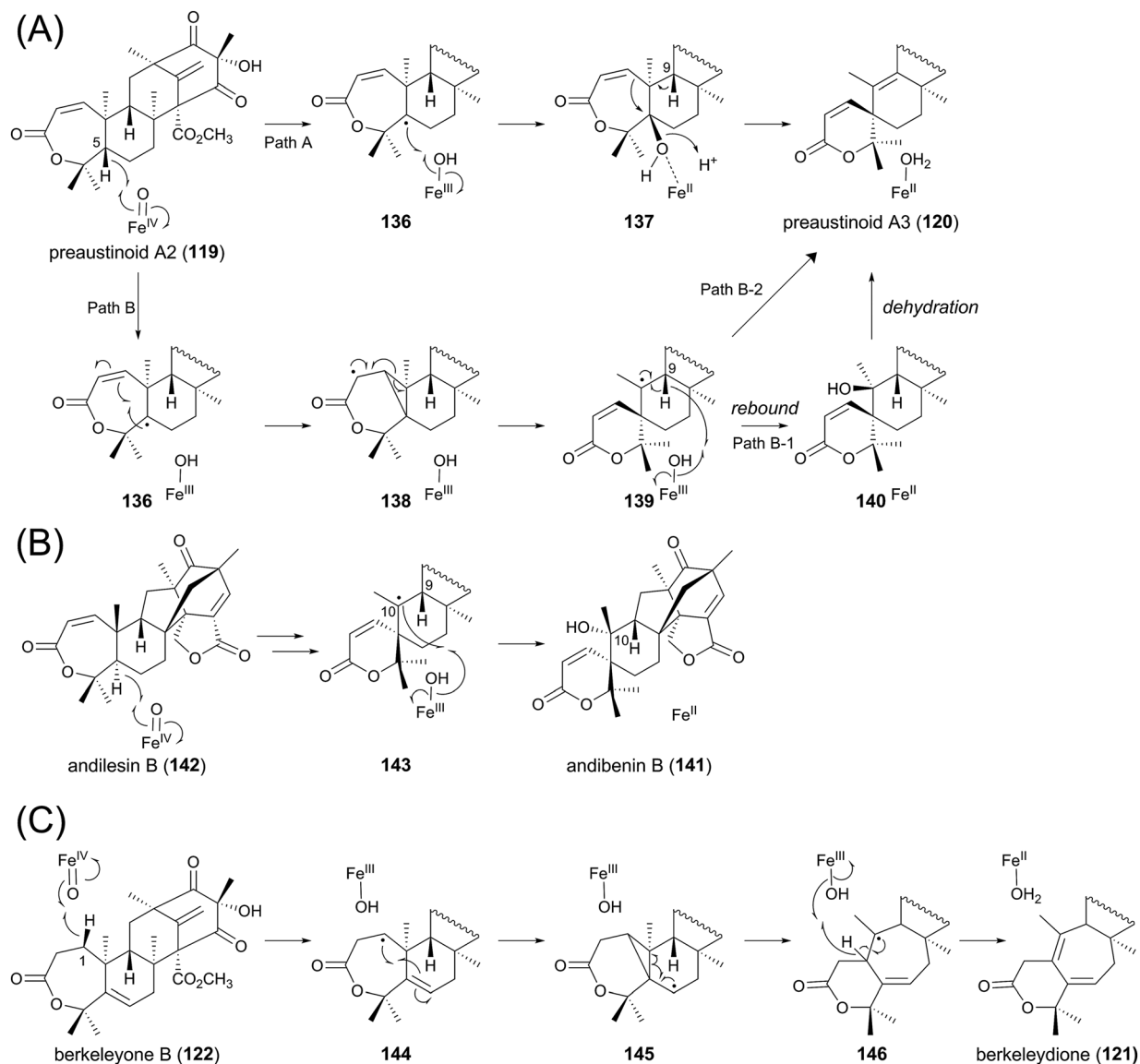This journal is © The Royal Society of Chemistry 2016

Fig. 13 Proposed reaction mechanisms for formation of (A) preaustinoid A3, (B) andibenin B, and (C) berkeleydione.

Indeed, since some P450s can oxidize an *exo*-olefin to both an epoxide and an aldehyde,[100] **151** could be the shunt product generated at the same time as **148**. Double bond isomerization and epoxidation would then transform **150** into berkeleyacetal B (**152**).[16] The pathway from **152** to **12** involves C-7 oxidation and elimination of the methyl ester group, which would proceed *via* the demethylation of the methyl ester followed by the spontaneous decarboxylation of the β-keto acid **153**. Meanwhile, the biosynthesis of **147** has been investigated by isotope-feeding studies, and a possible pathway has been proposed.[93] However, the detailed mechanisms for the biosynthesis and the genuine intermediates have yet to be elucidated. In one rational explanation, **121** would first be converted to a known natural product dhilirolide D (**154**),[92] in which the D- and E-rings should be synthesized in reactions similar to those catalyzed by AusJ and AusK in the austinol biosynthesis, but an AusH-like enzyme is not likely to be involved in the pathway. The next process would

generate the C-9 cation, which induces the ring contraction and the acetyl group formation, to afford **155**. Furthermore, ring-closure reactions could occur on **155** by two successive nucleophilic attacks, leading to the synthesis of dhilirolide J (**156**).[93] Finally, two olefin epoxidations convert **156** to the end product dhilirolide A (**147**).

4.5 Biosyntheses of anditomin and related compounds

The biosyntheses of anditomin (**74**)[53] and its biosynthetic precursors, andilesins A–C (**157**, **142**, **158**),[101,102] have been intensively studied by labeling experiments because of their intriguing intramolecular bridged-ring system,[99,103–106] which was proposed to be synthesized *via* a [4 + 2]-cycloaddition (Diels–Alder reaction) (Fig. 15). The proposed reaction also convinced synthetic chemists to perform biomimetic reactions.[107] The complete biosynthetic pathway of **74** was recently

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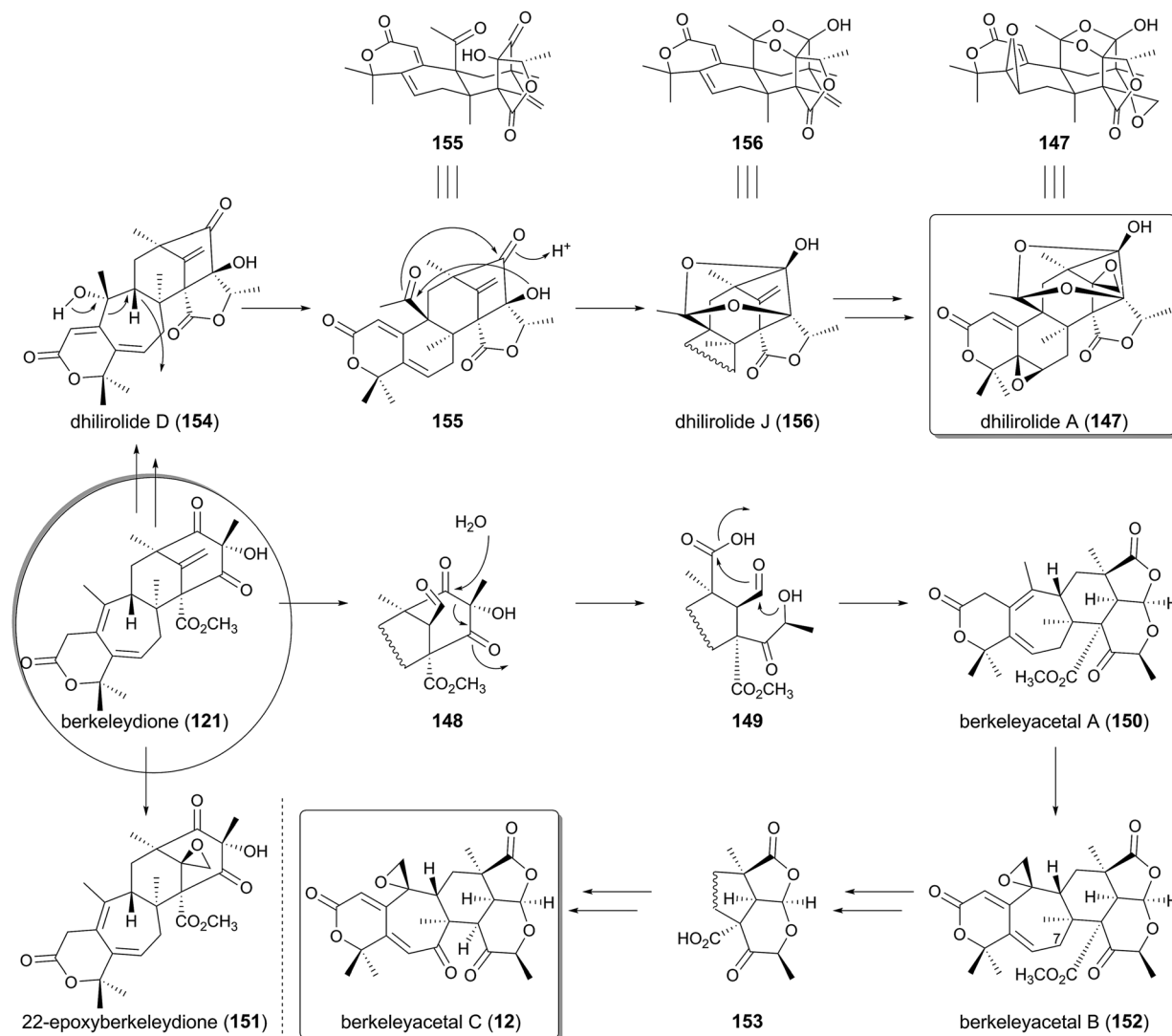*Nat. Prod. Rep.*, 2016, **33**, 26–53 | **41**

Fig. 14 Proposed biosynthesis of berkeleyacetal C and dhilirolide A.

established, mainly by the reconstitution of its biosynthesis in *A. oryzae* (Fig. 16), which confirmed that the Diels–Alder reaction is actually not involved in the biosynthesis.[41]

In the anditomin pathway, the pentacyclic intermediate **89**, generated by the TPC AndB, is oxidized at C-3 by the SDR AndC, which is homologous to AdrF and Trt9, to yield preandiloid B (**159**). AndA, the PhyH-like Fe(II)/α-KG-dependent dioxygenase, subsequently catalyzes two consecutive reactions to convert **159** to andiconin (**161**) *via* preandiloid C (**160**), by constructing the unique bridged framework characteristic of the andilesins.

Fig. 15 Previously proposed mechanism for the bridged-ring system formation in the anditomin/andilesins pathway.

Intermediate **161** then undergoes Baeyer–Villiger oxidation to generate andilesin D (**162**) by the FMO AndJ, which is followed by ketoreduction by the SDR AndI to afford andilesin A (**157**). The acetyltransferase AndG then acetylates **157** to produce **163**, which undergoes spontaneous acetate elimination to yield an enone andilesin B (**142**) due to the generation of a better leaving group. The enoylreduction of **142** by the SDR AndH and the following oxidative rearrangement by another α-KG-dependent dioxygenase AndF eventually produces andilesin C (**158**) and anditomin (**74**), completing the biosynthesis.

Andibenins A–C (**164**, **141**, **165**),[102] analogues of andilesin with a spiro-lactone moiety, should be synthesized by a similar pathway to that of anditomin (**74**); andilesin A (**157**) could be initially oxidized to **164** by the mechanism shown in Fig. 13B, and subsequent dehydration and reduction would produce **141** and **165**. Alternatively, **141** and **165** could be obtained *via* the direct oxidations of andilesin B (**142**) and andilesin C (**158**), respectively.
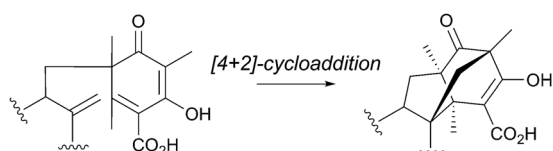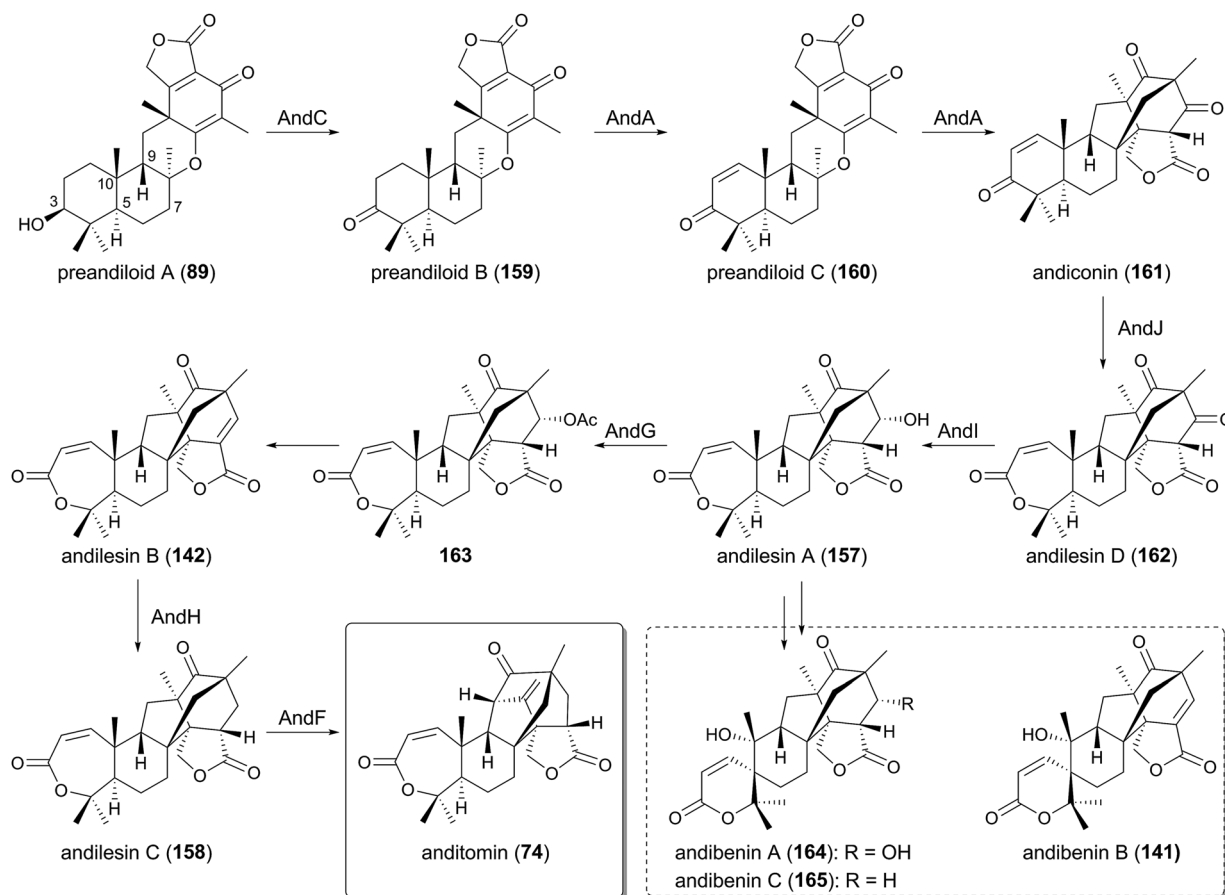
Fig. 16 Biosynthesis of anditomin and related compounds.

One of the most fascinating reactions in the anditomin pathway is the generation of the bicyclo[2.2.2]octane system by the multifunctional Fe(II)/α-KG-dependent dioxygenase AndA. In the proposed mechanism for this rearrangement (Fig. 17), the hydrogen atom of the C-12 methyl group of preandiloid C (**160**) is first abstracted to yield the substrate radical **166**, and the subsequent C–O bond cleavage generates the resonance-stabilized radical species **167**. The electrophilic radical of **167** at C-5′ would then form a C–C bond with the electron-rich olefin at C-12 to generate **168**, which has the nucleophilic radical and the electron-deficient olefin. Thus, C–C bond formation occurs between C-8 and C-2′ to generate the product radical **169**. The reaction by AndA is known to require ascorbate as an essential factor *in vitro*, and therefore the reducing agent should be used at the last step to reduce **169** to the product andiconin (**161**) as well as the ferric ion in the active site. Similarly, FtmF/FtmOx1 from *A. fumigatus*, which is the endoperoxide-generating α-KG-dependent dioxygenase, also reportedly requires a reducing agent for its reaction.[108,109]

The above-discussed mechanism of AndA also provides insight into the biosynthesis of insuetolides A–C (**170–172**), which are analogous to andilesins but possess one additional inserted oxygen atom (Fig. 17).[110] The scaffolds of **170–172** should be generated *via* a similar mechanism to that for AndA: oxygen rebound to **168** would occur, leading to the formation of

173 with a C-8 hydroxyl group. The resultant alcohol could spontaneously attack the electrophilic C-2′ to afford **174**. Further modifications would synthesize **170–172**.

4.6 Biosyntheses of fumigatonin and novofumigatonin

Despite the intriguing structures of fumigatonin (**175**)[111] and novofumigatonin (**75**),[54] isolated from *A. fumigatus* and *Aspergillus novofumigatus*, respectively, with the unusual orthoester moiety, neither biosynthetic studies nor a proposed pathway for these compounds have been reported. The expected intermediate **96** should undergo multistep decorations to afford **75** and **175**. The modification of the A-ring would be performed in a similar manner to that seen in the austinol or anditomin pathways, while the oxidation of the C-13 methyl group resembles the reaction by AdrA in the andrastin A biogenesis. One possible scenario for the orthoester formation can be proposed as follows (Fig. 18). Oxidative cleavage of the C-1′–C-6′ olefin, ketoreduction at C-5′, and reduction of the double bond between C-3′ and C-4′ could provide the intermediates **176** and **177**. In the novofumigatonin pathway, the C-5′ alcohol of **177** initially attacks the C-1′ carbonyl, and the resulting hemiorthoester anion then attacks the C-6′ ketone. Thus, the generated hemiacetal anion again attacks the C-7′ carbonyl to yield the molecular skeleton of novofumigatonin (**75**), along

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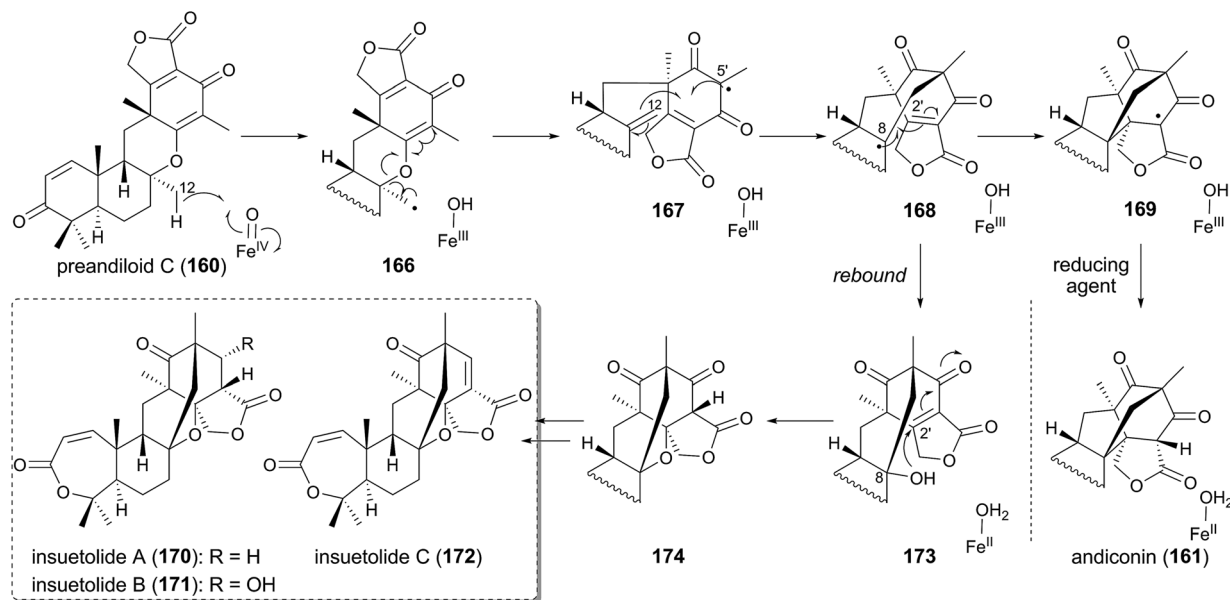*Nat. Prod. Rep.*, 2016, **33**, 26–53 | 43

Fig. 17 Proposed reaction mechanisms for AndA-catalyzed reaction and generation of the insuetolide scaffold.

with the elimination of a methoxide. In contrast, the C-5′ hydroxyl group of the predicted intermediate of **175**, **176**, is acetylated and thus it is unable to initiate the reaction by the nucleophilic attack from the C-5′ alcohol. Alternatively, the C-13 hydroxyl group could be used as the nucleophile to initiate the similar successive nucleophilic attacks, providing the orthoester moiety of fumigatonin (**175**).

5   Indole-diterpenoids

Indole-diterpenoids are a large class of natural products with diverse structures; they are also hybrid molecules consisting of an indole-ring as a non-terpenoid group and a geranylgeranyl pyrophosphate (GGPP)-derived terpenoid moiety. The indole-diterpenoids are further classified into two major groups: pas-paline (**178**)-derived compounds and non-paspaline-derived ones. For example, paxilline (**179**),[112] janthitrem B (**180**),[113] and sulpinine A (**181**)[114] are generated from **178**, whereas emindole SA (**182**),[115] thiersinine A (**183**),[116] radarin A (**184**),[117] anominine (**185**),[118] tubingensin B (**186**),[119] and aflavinine (**187**)[120] are synthesized *via* different pathways (Fig. 19). After the first discovery of the biosynthetic gene cluster for **179** in 2001,[121] the gene clusters for aflatrem (**188**),[122] terpendole K (**189**),[123] loli-trem B (**14**),[124,125] penitrem A (**190**)[126,127] and, shearinine A (**191**)[127] were reported and analyzed. These compounds are all synthesized *via* **178**, and no biosynthetic gene cluster for non-
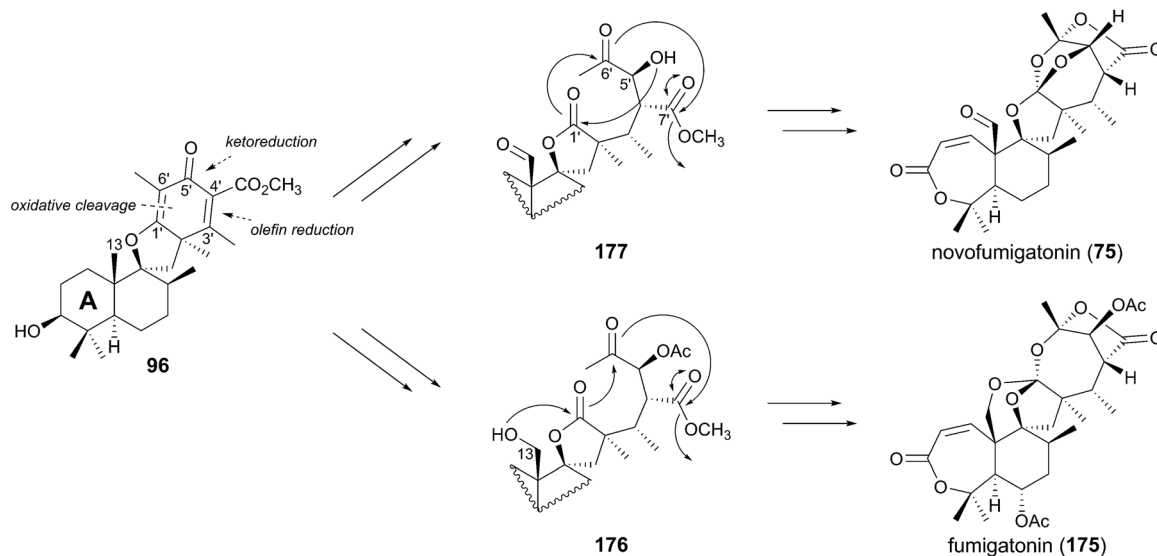

Fig. 18 Proposed reaction mechanisms for orthoester formation in the fumigatonin/novofumigatonin pathways.
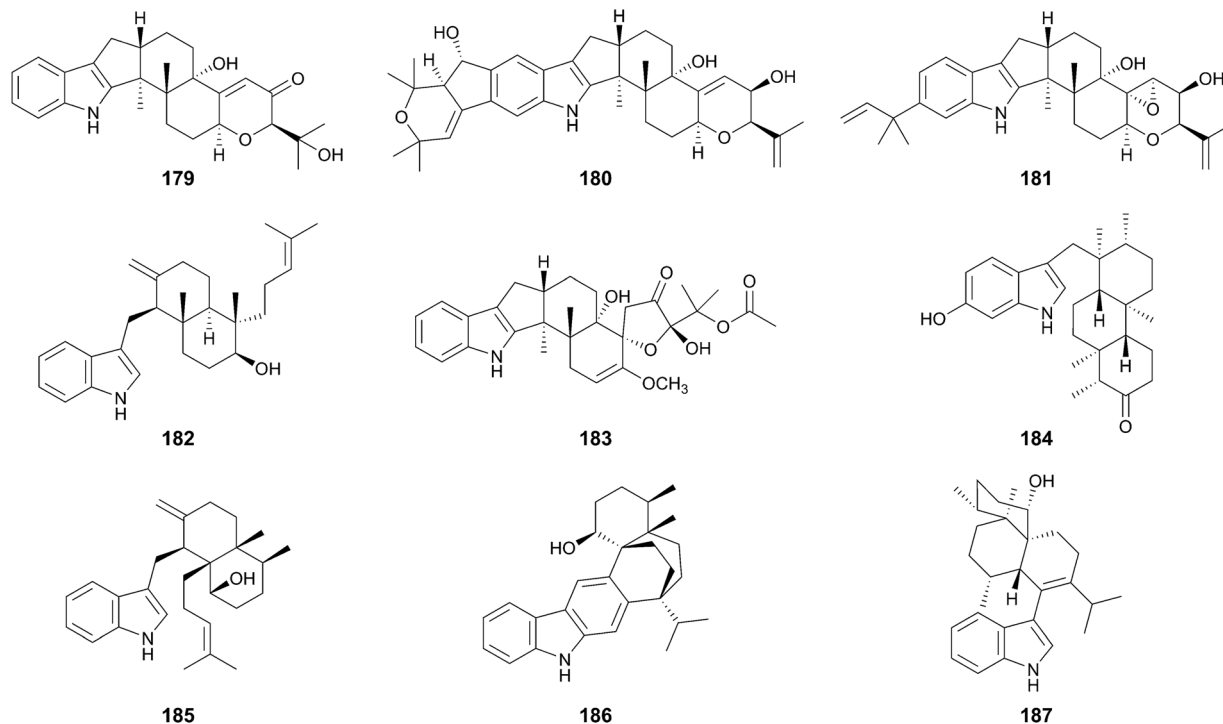
Fig. 19 Representative indole-diterpenoids.

paspaline-derived the indole-diterpenoids has been identified. Although the gene clusters for indole-diterpenoids lack PKS genes, consistent with the fact that they lack a polyketide portion, the biosynthetic schemes are basically similar to those of the other meroterpenoids discussed above. In this section, the biosynthetic studies and pathways for paspaline-derived molecules are summarized (Fig. 20 and 21).

5.1 Biosynthesis of paxilline

The function of the biosynthetic gene cluster for paxilline (**179**) has been investigated by gene deletion, complementation, and heterologous reconstitution experiments, which established the complete biosynthetic route of **179**.[121,128–132] In the paxilline pathway, indole-2-glycerol phosphate (**192**) serves as the indole donor, which is geranylgeranylated by the PTase PaxC to yield 3-geranylgeranylindole (**193**). The prenylation reaction differs from the above-described meroterpenoid pathways in two aspects. First, the prenyl donor geranylgeranyl pyrophosphate (GGPP) is synthesized by the GGPP synthase PaxG encoded by the cluster. Second, PaxC is a soluble protein and shares almost no sequence homology with the transmembrane UbiA-like PTases that are often found in the meroterpenoid biosynthesis.

The 3-geranylgeranylindole (**193**) intermediate then undergoes two rounds of epoxidation–cyclization processes to generate paspaline (**178**). The FMO PaxM initially forms an epoxide ring at C-10 and C-11 to produce **194**, which is accepted by the TPC PaxB for cyclization into emindole SB (**195**). Epoxidation again occurs on **195** to provide **196**, and PaxB-catalyzed cyclization produces **178**. Originally, the bisepoxide **197** was thought to be the biosynthetic intermediate, and it has also

been revealed that PaxB utilizes synthetically prepared **197** as a substrate to afford **178**.[131] However, the heterologous expression of *paxC*, *paxG*, and *paxM* in *A. oryzae* resulted in accumulation of **194**, not **197**, thus indicating that **197** is not used in the natural pathway. The biosynthesis up to **178** seems to be shared by the other biosyntheses for **14** and **188–191** (Fig. 20).

In the late-stage of the biosynthesis, two P450s are responsible for the generation of paxilline (**179**) from paspaline (**178**).[130] PaxP catalyzes multistep oxidative reactions, including demethylation, to yield 13-desoxypaxilline (**198**), which is hydroxylated by PaxQ to afford **179** (Fig. 21).

Interestingly, the *pax* cluster encodes a protein homologous to DMATSs, PaxD, and the function of the PTase has been investigated by *in vitro* reactions using the purified enzyme.[133,134] PaxD attaches a dimethylallyl group at either C-21 or C-22, and also produces diprenylated compounds at both of these positions.[133] These compounds can indeed be detected in a paxilline-producing strain, but the productivity is quite low (~0.5% of **179**),[133] and thus it might not be the intended metabolite in the *pax* pathway. An *in vitro* assay also elucidated that PaxD accepts **178** to perform dimethylallylation at either the C-21 or C-22 position to generate two mono-prenylated products.[134]

Furthermore, the involvement of *paxA* in paxilline biosynthesis is indicated, since the *paxA*-deletion mutant is unable to produce paxilline (**179**) and the deficiency is complemented by the reintroduction of *paxA*.[132] PaxA is predicted to be a six-transmembrane protein and is also present in the other indole-diterpenoid biosynthetic gene clusters. However, reconstitution studies have revealed that *paxA* was not required for the
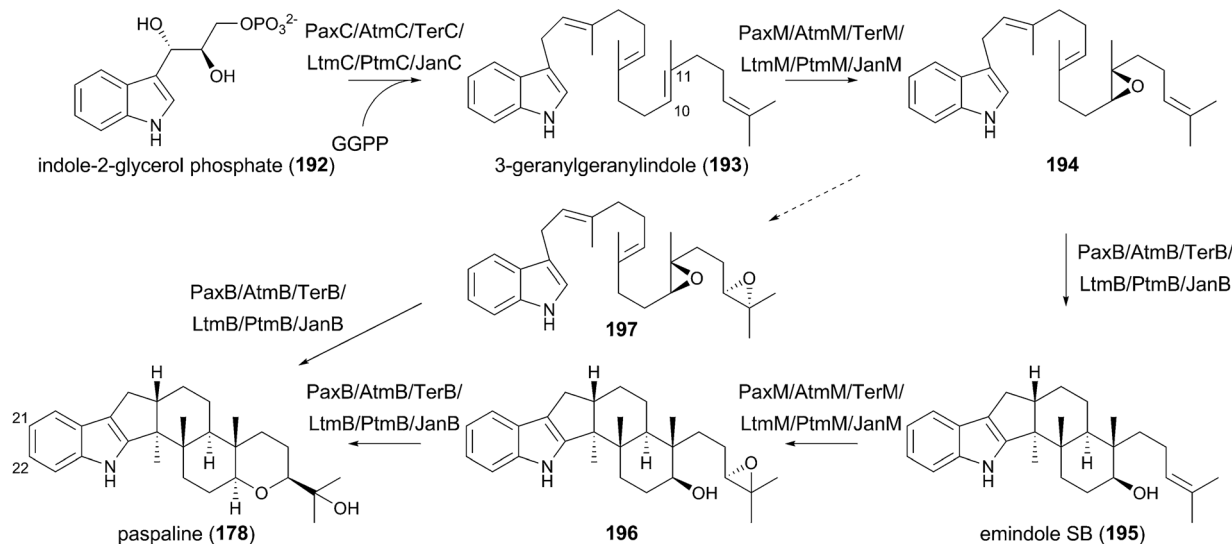
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Fig. 20   Biosynthesis of paspaline.

production of **179**,[131] and therefore the role of *paxA* remains enigmatic.

5.2   Biosyntheses of aflatrem and shearinine A

Aflatrem (**188**) is synthesized *via* a very similar scheme to that for paxilline (**179**) since it utilizes 13-desoxypaxilline (**198**) as the pathway intermediate. The P450 AtmQ is highly homologous to PaxQ; however, it converts **198** into paspalinine (**199**) with an ether linkage.[135,136] The DMATS-type PTase AtmD subsequently catalyzes reverse prenylation at either the C-20 or C-21 position to provide **188** and its isomer β-aflatrem (**200**), respectively (Fig. 21). *In vitro* experiments with purified AtmD illuminated that AtmD can also accept **178** and **179** as substrates to perform prenylation reactions.[134] The entire pathway to produce **188** was reconstructed in *A. oryzae*, verifying the biosynthesis of **188**.[136]

Paspalinine (**199**) is also utilized in the biosynthesis of shearinine A (**191**), which is likely to undergo diprenylation by the DMATS-type PTase JanD to yield shearinine K (**201**).[127] The subsequent oxidation ring closure may be performed by the FMO JanO and/or the P450 PJ-13, leading to the formation of **191** (Fig. 21).

5.3   Biosyntheses of terpendole K and lolitrem B

The biosyntheses of terpendole K (**189**) and lolitrem B (**14**) diverge from the paxilline or aflatrem pathways just after paspaline (**178**) is generated (Fig. 21).[123,125,137] The P450 TerQ/LtmQ oxidizes **178** to generate the C-11 hydroxylated product terpendole E (**13**), which is accepted by the other P450 TerP/LtmP and converted to 13-desoxyterpendole I (**202**). TerQ/LtmQ again hydroxylates **202** to produce terpendole I (**203**). The last several steps in the terpendole K pathway are proposed to involve the PTase TerF and the P450 TerK, in which TerF attaches a dimethylallyl moiety to the C-27 alcohol, and TerK catalyzes the ether-forming ring closure reaction.[123] Finally, an unidentified

dehydrogenase would introduce the double bond between C-6 and C-7 to afford **189**. Meanwhile, the lolitrem B biosynthesis utilizes the homologous proteins of TerF and TerK, LtmF, and LtmK, respectively, which are responsible for the same reactions to construct the eastern part of **14**. Additionally, LtmE and LtmJ, another PTase and P450 set achieve the prenylations at C-20 and C-21 and the oxidative ring closure to afford the western portion of **14**, and complete the biosynthesis.[137]

5.4   Biosynthesis of penitrem A

Among the indole-diterpenoids, one of the most complex molecules, penitrem A (**190**), requires 17 dedicated genes for its biosynthesis.[126] The biosynthetic gene cluster for **190** was initially identified in the genome of *Penicillium simplicissimum* (the *ptm* cluster),[126] and was later discovered in *Penicillium crustosum* as well (the *pen* cluster).[127] Since the complete pathway of **190** has been elucidated using the *ptm* genes, the functions of the Ptm enzymes are focused on below (Fig. 21).

After the formation of paxilline (**179**) in the same manner as that by the Pax enzymes, **179** undergoes ketoreduction at C-10 by PtmH, C-20 dimethylallylation by PtmD, dehydration by PtmI and PtmV, and hydroxylation by PtmO to produce PC-M5 (**204**). Subsequently, the key transformation in the pathway, the construction of the bicyclo[3.2.0]heptane system, is catalyzed by the PTase PtmE to afford PC-M4 (**205**). Finally, four P450s, PtmK, PtmU, PtmL, and PtmJ, as well as the flavin-dependent halogenase PtmN synthesize the end product penitrem A (**190**), in which PtmK is engaged in the ring expansion, and PtmU generates the ether linkage to provide penitrem D (**206**).

6   Miscellaneous meroterpenoids

Although the above-described meroterpenoid biosyntheses generally involve prenylation at the early-stage of the
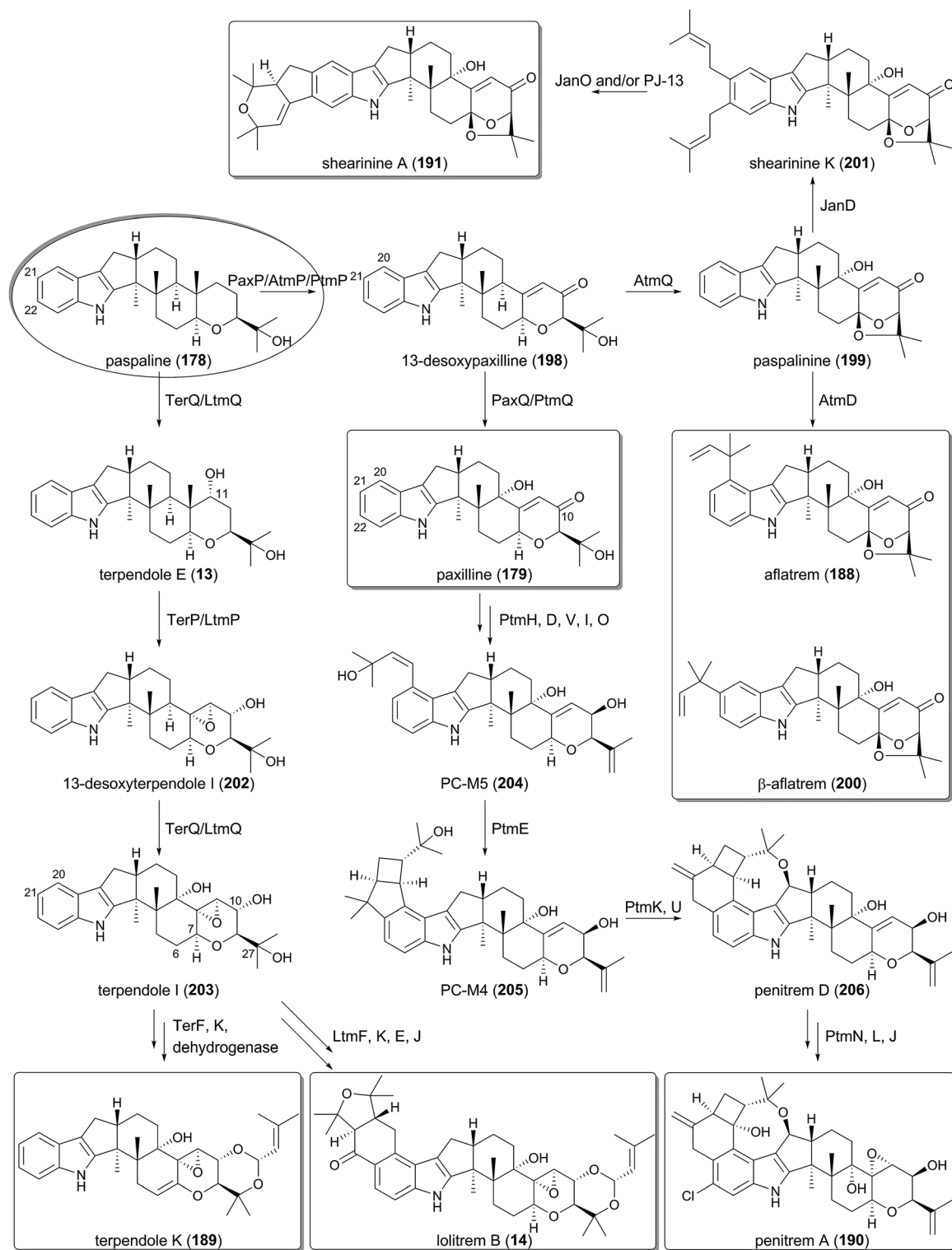
Fig. 21 Late-stage biosynthesis of paxilline-derived indole-diterpenoids.

biosynthesis and cyclization by transmembrane TPCs, meroterpenoids are sometimes generated *via* completely different machineries. This section focuses on the meroterpenoids that require the hybridization of terpenoid and non-terpenoid portions at a later stage and adopt different cyclization strategies for their biosynthesis.

6.1   Biosynthesis of viridicatumtoxin

Viridicatumtoxin (**207**), isolated from *Penicillium aethiopicum*, represents tetracycline-like molecules derived from fungi, and reportedly possesses nephrotoxic or antitumor activities.[138–140] The biosynthesis of **207** has been studied by gene deletion and

in vitro analysis of selected enzymes (Fig. 22).[141–144] The tetracyclic core of **207** is generated collaboratively by the NR-PKS VrtA, the Claisen cyclase VrtG, and the FMO VrtH, from one molecule of malonamoyl-CoA and nine molecules of malonyl-CoA, yielding **208**.[142] The P450 VrtE and the methyltransferase VrtF then convert **208** into **209**, which is further geranylated by the DMATS-type PTase VrtC to generate **210**.[143] The very last step of the biosynthesis involves the cyclization of the geranyl moiety, and interestingly this process is catalyzed by the P450 VrtK and not by the canonical TPCs.[144] Thus, the viridicatumtoxin pathway utilizes neither a transmembrane PTase nor a TPC to afford the cyclized terpenoid moiety.

6.2 Biosynthesis of penigequinolones

Penigequinolones A (**211**) and B (**212**) are insecticidal compounds produced by various fungi, and possess a quinolone alkaloid core structure equipped with a cyclized C_{10} terpenoid moiety.[145] The biosynthetic gene cluster for **211** and **212** (the *pen* cluster)[146] was identified in *Penicillium thymicola* by searching for a similar gene cluster to the *asq* cluster responsible for the biosynthesis of aspoquinolones,[147] which are analogues of **211** and **212**. Indeed, the two pathways appear to share most of their biosynthetic machinery. The early stage of the biosynthesis was investigated with the *asq* cluster, whereas the mid-stage was elucidated by analyzing the *pen* cluster (Fig. 23).

Initially, the non-ribosomal peptide synthase (NRPS) AsqK/PenN condenses anthranilic acid (**213**) and *O*-methyl-L-tyrosine (**214**) to produce the benzodiazepinedione, 4′-methoxycyclopeptin (**215**). Subsequently, the PhyH-like dioxygenase AsqJ/PenM performs oxidative reactions on **215**, and the following spontaneous structural rearrangement provides the quinolone core to generate 4′-methoxyviridicatin (**216**),[147] which is further transformed into **217** by an uncharacterized pathway.

Considering the structures of **211** and **212**, it was thought that the terpenoid motif of these compounds is derived from geranyl pyrophosphate (GPP), but interestingly, a biochemical experiment has revealed that PenI, which is the PTase that accepts **217** as a substrate, only produces the dimethylallylated molecule **218**.[146] In the next step, the FMO PenH catalyzes dehydrogenation to generate the conjugated diene **219**, and the introduced *exo*-methylene hereby serves as a nucleophile required for the second prenylation by the DMATS-like PTase PenG. Thus, PenG catalyzes the prenyl elongation reaction to afford **220** with a C_{10} side chain, in which a water molecule is used to terminate the reaction.

In the following processes, the FMO PenE, which is homologous to PaxM in the paxilline biogenesis, catalyzes epoxidation at the terminal olefin to yield **221**.[146] The epoxide hydrolase PenJ then accelerates the 5-*exo-tet* cyclization to afford yaequinolone C (**222**), which is somehow converted into penigequinolones A (**211**) and B (**212**).

6.3 Biosynthesis of fumagillin

The biosynthesis of fumagillin (**8**) is significantly distinct from all of the other pathways described above, in that the terpenoid moiety is first cyclized and tailored before it is hybridized with the polyketide portion (Fig. 24). A similar synthetic strategy is also employed in other biosyntheses, such as the xenovulene A pathway.[1,148,149] The fumagillin biosynthesis begins with the cyclization of FPP by the TPC Fma-TC to produce β-*trans*-bergamotene (**223**).[150] The Fma-TC-catalyzed reaction is initiated by the elimination of a pyrophosphate group to generate the allylic cation, and therefore it can be classified as a class I TPC. However, Fma-TC is a transmembrane protein homologous to UbiA, and shares no sequence similarity with any known TPCs, although plants can produce several bergamotenes with soluble class I enzymes. At present, no other UbiA-like TPCs have been discovered, but they could also be present in the genomes of different fungal species.

β-*trans*-Bergamotene (**223**) then undergoes multistep oxidations as well as structural rearrangement by the P450 Fma-P450 to yield 5-keto-demethoxyfumagillol (**224**), which is further converted to fumagillol (**225**) with the aid of the PhyH-like dioxygenase Fma-C6H, the methyltransferase Fma-MT, and the ketoreductase, Fma-KR.[151] In contrast, the polyketide
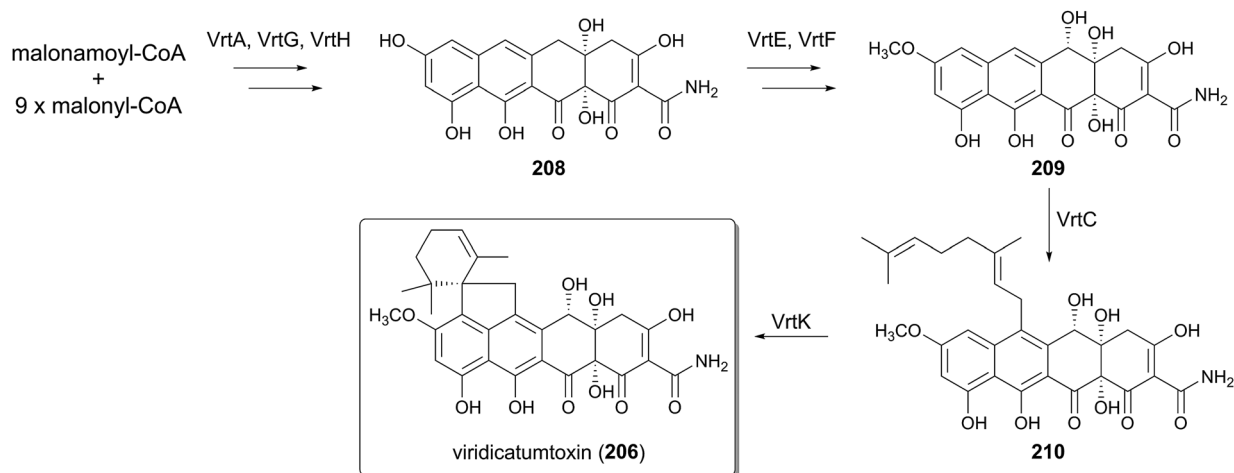

Fig. 22 Biosynthesis of viridicatumtoxin.

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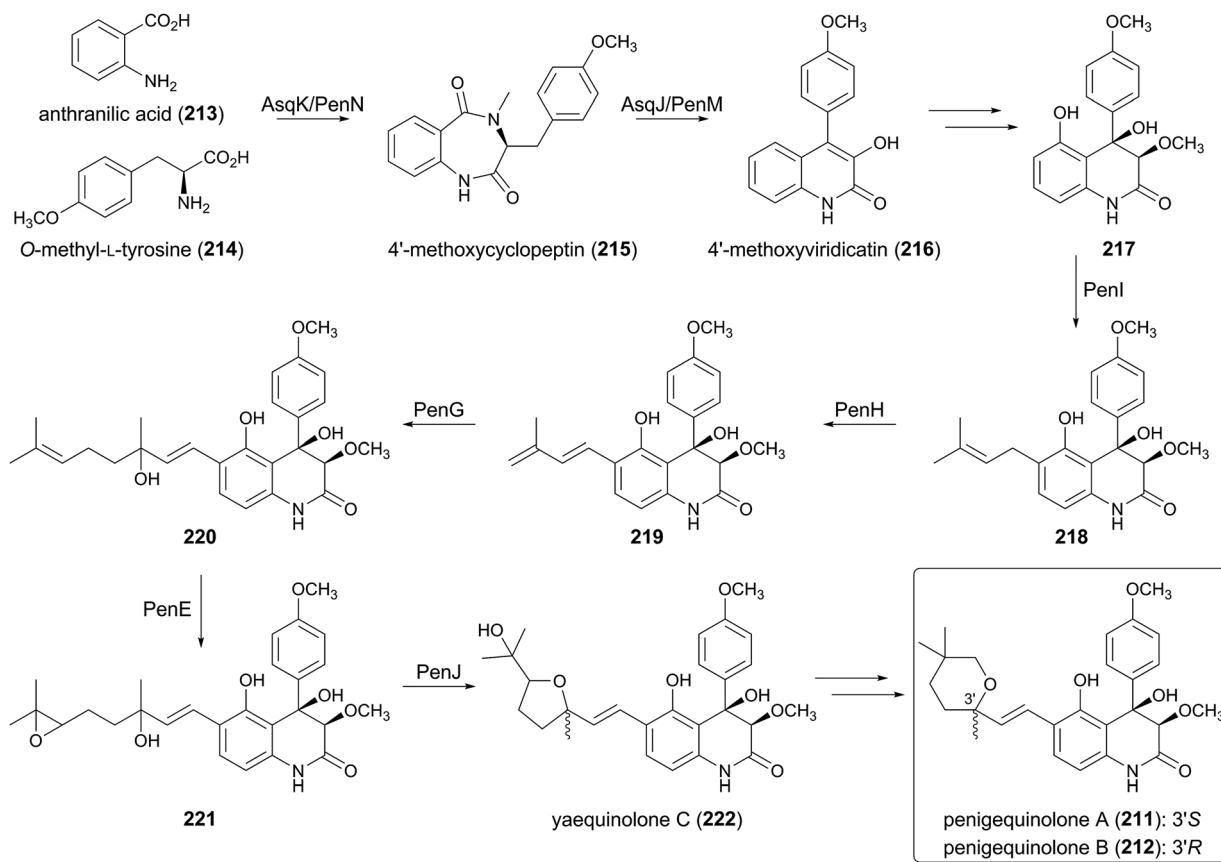This journal is © The Royal Society of Chemistry 2016

Fig. 23   Biosynthesis of penigequinolones.

motif of fumagillin (**8**) is prepared by the highly reducing PKS Fma-PKS, which synthesizes an enzyme-bound dodeca-pentaenoyl group (**226**).[150] The polyketide portion is then *trans*-esterified with **225** by the acyltransferase Fma-AT,

providing prefumagillin (**227**). Finally, Fma-ABM, which is structurally related to the ABM superfamily of monooxygenase, catalyzes the oxidative cleavage of **227** to afford the end product **8**.[151]
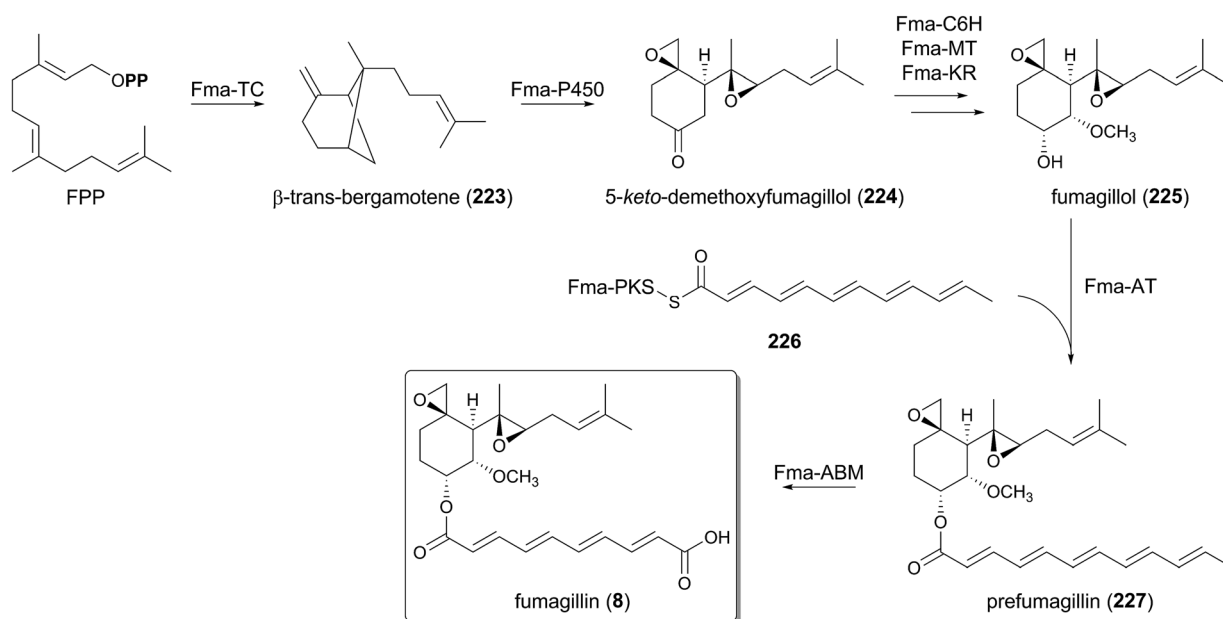

Fig. 24   Biosynthesis of fumagillin.

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7 Conclusions and prospects

This review has summarized recent biosynthetic studies of fungal meroterpenoids at the genetic/enzymatic levels. Although the structures of fungal meroterpenoids are extremely diverse and often highly complex, their biosyntheses employ similar enzymes and mechanisms to synthesize the wide range of natural products. Thus, the fungal meroterpenoid pathways have revealed nature's sophisticated strategy to generate complexity by using simple and achiral starting units.

Biosynthetic studies of unique molecules have indeed led to the discoveries of novel families of enzymes with intriguing activities, such as transmembrane TPCs (both class I and II) and isomerases including Trt14, AusJ, and AusH. However, it should be noted that fascinating reactions are often catalyzed or induced by ordinary enzymes on the basis of their sequences; indeed, oxidative enzymes are especially responsible for amazing transformations in many pathways, and are often multifunctional.[98,152] For instance, PhyH-like Fe(II)/α-KG-dependent dioxygenases are widely utilized in fungal meroterpenoid biosyntheses, and their catalyzing/inducing reactions range from simple hydroxylation (as by Trt7 and Fma-C6H) to drastic structural rearrangement (as by AusE, AndA, and AsqJ).

Finally, due to the characterization of many biosynthetic gene clusters for fungal meroterpenoids and the rapid increase of available fungal genomes, we can now readily discover many putative gene clusters for meroterpenoid biosynthesis with uncharacterized functions. By means of well-established approaches in recent years including heterologous reconstitution techniques, metabolites derived from these gene clusters can be easily obtained, even though the clusters are silent. Furthermore, the genes in both characterized and uncharacterized gene clusters could be used as "genetic building blocks" to construct artificial metabolic pathways to afford unnatural meroterpenoids. Future studies on fungal meroterpenoids should accelerate not only the discovery of enzymes with unique activities but also the accumulation of knowledge to efficiently compile many biosynthetic genes to synthesize desired molecules.

8 Acknowledgements

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