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Identification of a Key Prenyltransferase Involved in Biosynthesis of the Most Abundant Fungal Meroterpenoids Derived from 3,5-Dimethylorsellinic Acid.

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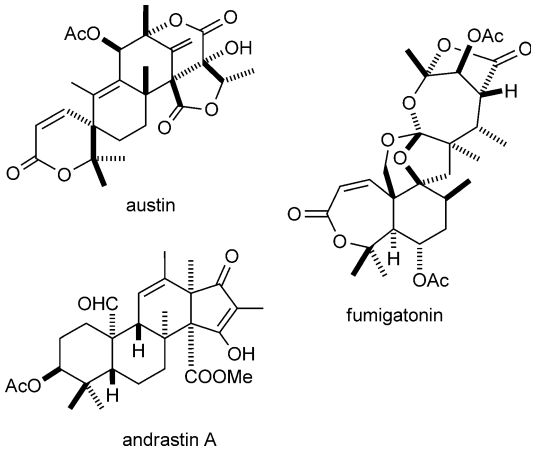
Meroterpenoids, found widely in plants and microbes, are a unique class of natural products that have structures synthesized by both polyketide and terpenoid pathways. The fact that many of them possess potent biological activity points to the effectiveness of nature's ability to create such hybrid molecules. Fungi are known to produce numerous meroterpenoids with extremely broad structural diversity, which can be classified according to the structure of the incorporated polyketide unit.[1] These units include triketide pyrone and various tetraketide orsellinic acid derivatives. Among meroterpenoids, those derived from 3,5-dimethylorsellinic acid (DMOA, **1**) are the most widespread in terms of the number of compounds reported.[1] These meroterpenoids often undergo extensive structural modifications such that the original aromatic ring is no longer present in the final compounds.[1] Examples include andibenin, austin, andrastin, citreohybridone, tropolactone, fumigatonin, and terretonin (**2**) (Scheme 1).[1] Here, we describe the first identification of the biosynthetic gene cluster for meroterpenoids of this series (presumably that for **2**) from *Aspergillus terreus*, and detection of the long-hypothesized common key intermediate, farnesyl-DMOA (**3**) for the first time, which was produced by the prenyltransferase (PT) found in the cluster. This PT catalyzes an unusual electrophilic "aromatic addition" reaction of a prenyl group to **1** that sacrifices the aromaticity of the phenyl ring.

Since we have recently succeeded in identifying the first fungal meroterpenoid biosynthetic gene cluster for pyripyropene (the *pyr* cluster) from *Aspergillus fumigatus*,[2] we searched the genomic DNA database of *A. terreus* NIH2624 (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html) to find similar biosynthetic gene clusters for closely related meroterpenoids.[3] Such a cluster should



Scheme 1. Representative meroterpenoids.

contain the characteristic set of genes for meroterpenoid biosynthesis: a fungal type I polyketide synthase (PKS), a prenyltransferase (PT), and a novel terpene cyclase (CYC) that is predicted to consist entirely of transmembrane helices.[2] This led us to find the 36 kb *trt* cluster of 13 genes, including genes predicted to code for PKS, PT, CYC (similar to *pyr4* from the pyripyropene cluster), FAD-dependent monooxygenase (FMO), and other genes (Table 1). A high degree of sequence similarity was observed between PT, CYC, and FMO genes and the corresponding genes from the *pyr* cluster. However, the PKS exhibited significant difference from Pyr2 in the predicted domain architecture (ketosynthase (KS), acyltransferase (AT), acyl carrier protein (ACP), methyltransferase (MeT), and thioesterase (TE) domains; Figure S1 in the Supporting Information). The presence of both MeT and TE domains downstream of ACP is a unique structural feature among fungal type I PKSs. This PKS exhibited high sequence identity (50%) to PksCT (citrinin biosynthesis from the fungus *Monascus purpureus*), with a similar domain architecture (Figure S2).[4] Therefore, we postulated that this cluster might be involved in the biosynthesis of **2** (Scheme 2). Terretonin, a mycotoxin produced by *A. terreus*, has a unique tetracyclic structure that resembles that of steroids.[5] The meroterpenoid nature of **2** has been demonstrated by tracer experiments, and the polyketide portion was found to be derived from **1**.[6]

In order to test whether the gene for PKS (Trt4) of this cluster produces **1**, the gene was expressed in a heterologous fungal expression system (*Aspergillus oryzae* M-2-3).[7] The full-length gene for Trt4 (ATEG_10080) was amplified by PCR from genomic DNA of *A. terreus* ATCC 46038 (a strain that produces

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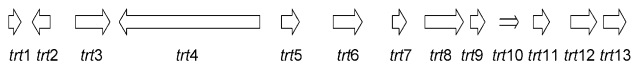
Table 1. Proposed functions of each open reading frame (*trt* cluster) in *A. terreus*, and their amino acid similarity/identity with other known proteins.



Gene	Protein homologue, origin	Identity [%]	Similarity [%]
*trt*1	terpene cyclase, Pyr4 (*Aspergillus fumigatus*)	33	57
*trt*2	UbiA-like prenyltransferase (*Neurospora crassa*)	36	56
*trt*3	FAD-dependent mono-oxygenase (*Mixococcus xanthus*)	36	54
*trt*4	citrinin polyketide synthase (*Monascus purpureus*)	32	50
*trt*5	methyltransferase (*Nitrosococcus oceani*)	26	42
*trt*6	P450 mono-oxygenase, CypX (*Gibberella fujikuroi*)	43	61
*trt*7	Phytanoyl-CoA dioxygenase, PhyH (*Raistonia eutropha*)	34	56
*trt*8	FAD-dependent mono-oxygenase, PaxM (*Penicillium paxilli*)	47	61
*trt*9	short-chain dehydrogenase (*Herpetosiphon aurantiacus*)	40	55
*trt*10	unknown		
*trt*11	isochorismatase family hydrolase (*Pseudomonas putida*)	50	72
*trt*12	tyrosinase or mono-oxygenase (*Gibberella zeae*)	72	83
*trt*13	peptidoglycan binding domain (*Oceanicaulis alexandrii*)	36	47

2) and ligated into the fungal expression plasmid pTAex3R under the control of the α-amylase promoter (*amyB*). Upon comparison with other fungal PKS sequences, we noticed that the database sequence for ATEG_10080 has misassigned the start codon, and we deduced the true start site to be 16 bp upstream of the annotated ATG. This results in a different N-terminal amino acid sequence, because of the frameshift relative to the database sequence (Figure S3). The resulting plasmid was transformed into *A. oryzae* M-2-3 to give *A. oryzae* M-2-3/pTA-trt4 for expression of the PKS gene. After three days induction of the culture in Czapek–Dox medium, the cells were harvested, extracted, and analyzed by LC-MS. The result showed the production of a compound that was not seen in the control experiment (without the PKS gene; Figure 1). The molecular formula of $C_{10}H_{12}O_4$ (*m/z* calcd for $C_{10}H_{12}O_4$ $[M+H]^+$: 197.0808; found: 197.0809) was deduced by HRMS (Figure S4). From a large-scale culture (200 mL), 13 mg of the compound was extracted, purified, and subjected to extensive NMR analysis. Both ^1H and ^{13}C NMR spectra were consistent with the presence of six aromatic carbons, a carboxylic acid, and three methyl groups attached to an aromatic ring (Figure S5). Finally, HMBC and HMQC spectra confirmed the structure to be that of **1**. Therefore, Trt4 was established to be a PKS that produces the polyketide precursor of **2**. Two methyl transfer reactions take place during the first two rounds of condensation steps. Very recently, a PKS that produces the same polyketide **1** was

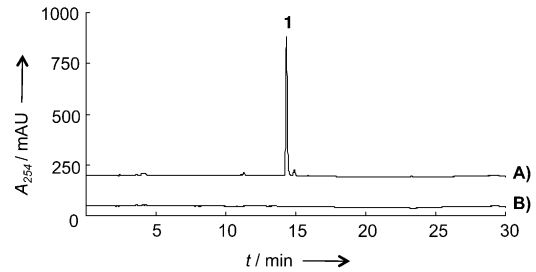
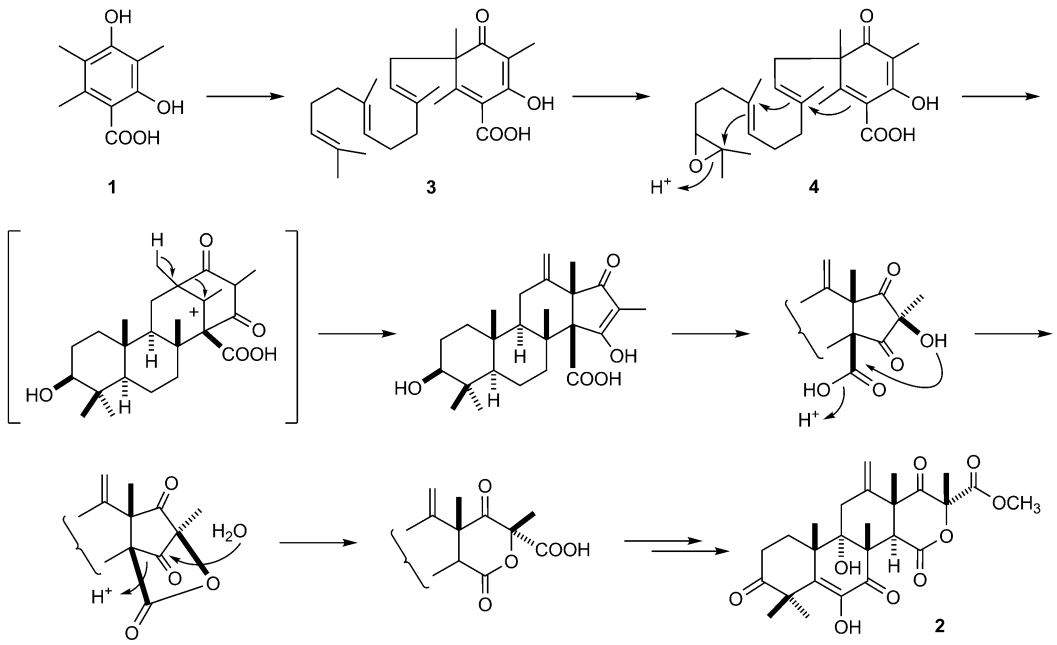


Figure 1. HPLC profiles of extracts of a culture media from *A. oryzae* transformant harboring A) pTA-trt4, and B) empty vector.



Scheme 2. Proposed biosynthetic pathway to terretonin **2**.

identified from *A. nidulans* (AN8383); it exhibits 67% sequence identity to Trt4, and has the same domain architecture.[8]

Next, to examine the function of the next enzyme in the pathway, the PT gene for Trt2 (ATEG_10078) was cloned and coexpressed with the PKS gene. Similarly to *pyr6* (the PT from the *pyr* cluster), *trt2* exhibited sequence similarity to *ubiA* (ubiquinone biosynthesis).[2] The full-length *trt2* gene was introduced under control of an *amyB* promoter into a second plasmid, pPTR I, which harbors the pyrithiamine resistant marker, and the resulting construct was transformed into *A. oryzae* M-2-3/pTA-trt4 to give transformant *A. oryzae* M-2-3/pTA-trt4,pPTR-trt2 that expresses the two genes simultaneously. After induction for three days, the cells were harvested and extracted with acetone. The extract was analyzed by LC-MS and showed a peak that was not seen in the control (Figure 2). The

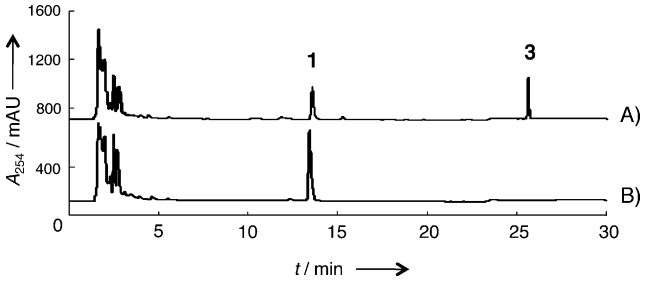
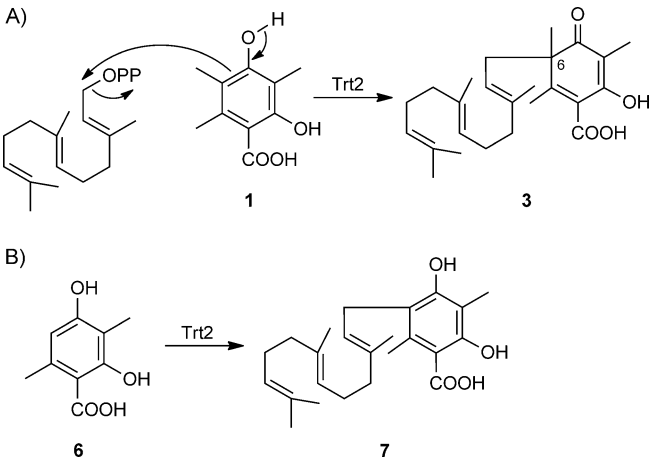


Figure 2. HPLC profiles of extracts of cells from *A. oryzae* transformant harboring A) pTA-trt4 and pPTR-trt2, and B) pTA-trt4 only.

molecular formula of $C_{25}H_{36}O_4$ (*m/z* calcd for $C_{25}H_{37}O_4$ $[M+H]^+$: 401.2686; found: 401.2681) was deduced by HRMS, and a fragment ion peak for **1** $[196+H]^+$ (*m/z* 197.0866) was consistent with the attachment of a farnesyl group to **1** (Figure S6). Samples (6 mg) from a large-scale culture (2 L) were prepared and subjected to extensive NMR analysis. Both ^1H and ^{13}C NMR spectra showed the presence of 25 carbons (one signal overlapped with a solvent peak; Figure S7, S8). The presence of a tertiary aliphatic methyl group (^1H: $\delta = 1.41$, ^{13}C: $\delta = 24.2$) and six vinylic or aromatic methyl groups (^1H: $\delta = 1.53$, 1.58, 1.59, 1.65, 1.78, 2.17) was consistent with the structure of farnesyl-DMOA (**3**). Two ^{13}C signals ($\delta = 179.7$, 184.7), which were assigned to C-3 and C-5 carbons, exhibited significant peak broadening, thus indicating that the two carbonyls are in keto–enol tautomerization (Figure S9). A similar effect was observed in the spectra of andrastin A.[9] Finally, the structure was confirmed by HMBC and HMQC spectra. Therefore, it was established that Trt2 catalyzes the addition of a farnesyl group to **1** to produce the key intermediate **3** en route to the presumed terretonin. While **3** has been proposed to serve as a key intermediate in the biosynthesis of various related meroterpenoids,[1] this is the first report of the detection of **3** and its spectroscopic characterization. The reaction catalyzed by this PT is remarkable as it represents an electrophilic aromatic addition reaction, rather than the usual aromatic substitution reaction (Scheme 3A). As **1** lacks any hydrogen atom in the aromatic ring, inevitably the aromaticity is destroyed upon accommodation of the incoming prenyl group. Clearly, the driving force for the reaction is the electron donation from the adjacent phenolic hydroxyl moiety that ends up as a ketone. This is the first report on a PT that catalyzes such an aromatic addition reaction onto a phenyl ring. Considering that Trt2 is homologous to UbiA, the ability to catalyze such a reaction in this class of PT underscores the catalytic versatility. We also studied whether Trt2 could catalyze a normal aromatic substitution reaction, by employing an analogue of **1**, 3-methylorsellinic acid (MOA, **6**), that lacks the critical methyl group at C-5. Thus, **6** was incubated with the *A. oryzae* M-2-3/pTA-trt2 transformant that expresses only the PT gene. Remarkably, production of a prenylated compound was observed, and its structure was confirmed as farnesyl-MOA (**7**; Figures S10–S13). Therefore, Trt2 was shown to possess the ability to catalyze a normal type of aromatic prenylation reaction (Scheme 3B). We also demonstrated that Trt2 even catalyzes prenylation of an unrelated pyrone intermediate, 4-hydroxy-6-(3-pyridinyl)-2*H*-pyran-2-one (HPPO) from pyripyropene biosynthesis (Figures S14–S15).[2] Further study is needed to fully understand the substrate specificity of this PT. Some preliminary in vitro studies on metal ion dependency were also carried out; these showed that Mg^{2+} is the most effective ion (Figure S16).



Scheme 3. Reactions catalyzed by Trt2. A) Plausible mechanism of aromatic addition reaction of farnesyl diphosphate onto **1**. C-6 represents an undetermined chiral center. B) Trt2-catalyzed aromatic substitution reaction of **6** to produce **7**.

We noticed some decomposition of **3** back to **1** upon long exposure to solvents. This clearly indicates that **3** is thermodynamically less stable than **1**. Therefore, nature employs an unstable intermediate at this stage to destroy the aromaticity, and thereby trigger the modification of an otherwise stable aromatic unit and incorporate it into the backbone of the tetracyclic meroterpenoid skeleton of the molecule. What remains to be studied is the absolute configuration of the quaternary chiral center—a challenging task, given the structure of this molecule—and this might require X-ray crystallographic structure determination. The instability of **3**, however, might hamper such an attempt. Among the meroterpenoids derived from **1**, two have been reported with opposite absolute configurations: terretonin and andrastin (the *ent* series).[9] It is the

PT step that determines the critical chiral center and thus the absolute configuration of the final compounds.

We further studied the next expected step on the pathway, an FMO gene (Trt8; ATEG_10085). The full-length gene was cloned and introduced into pPTR I (under control of the *amyB* promoter) together with *trt2*, to give a construct harboring two genes similar to that in our previous pyripyropene studies.[2] This was transformed into *A. oryzae* M-2-3/pTA-trt4 to give a transformant *A. oryzae* M-2-3/pTA-trt4,pPTR-trt2 + trt8 that expressed the three genes simultaneously. As expected, a product specific to the introduced *trt8* gene was found in inducted culture, and this was identified as epoxyfarnesyl-DMOA (**4**; Figures S17–S20). In addition, we also observed production of dihydroxyfarnesyl-DMOA (**5**), which was apparently derived from **4** through hydrolysis of the epoxide (Figures S21–S23). Therefore, Trt8 was established as an epoxidase that catalyzes the oxygenation of the terminal double bond of the farnesyl moiety in **3** to produce the direct precursor for the following cyclization reaction.

The enzymatic preparation of **3** has now opened a new opportunity to study the biosynthesis of this important class of meroterpenoids in detail. The functional characterization of the remaining genes in the cluster is now underway. Our works will provide rich chemical diversity by engineering meroterpenoid biosynthetic genes and pathways.

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Keywords: biosynthesis · meroterpenoids · polyketides · prenyltransferases · terpenes · terretonin

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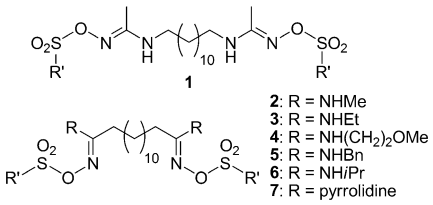

Destroying aromaticity: A novel prenyltransferase (Trt2) involved in fungal meroterpenoid biosynthesis was shown to catalyze an unusual aromatic addition reaction onto a fully substituted aromatic ring. The prenylated product serves as a key intermediate in the biosynthesis of the most abundant series of meroterpenoids in fungi.

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