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Probing Labdane-Related Diterpenoid Biosynthesis in the Fungal Genus *Aspergillus*

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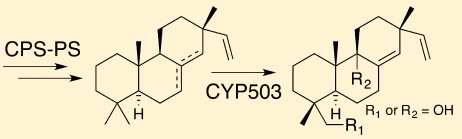
Supporting Information

ABSTRACT: While terpenoid production is generally associated with plants, a variety of fungi contain operons predicted to lead to such biosynthesis. Notably, fungi contain a number of cyclases characteristic of labdane-related diterpenoid metabolism, which have not been much explored. These also are often found near cytochrome P450 (CYP) mono-oxygenases that presumably further decorate the ensuing diterpene, suggesting that these fungi might produce more elaborate diterpenoids. To probe the functional diversity of such biosynthetic capacity, an investigation of the phylogenetically diverse cyclases and associated CYPs from the fungal genus *Aspergillus* was undertaken, revealing their ability to produce isopimaradiene-derived diterpenoids. Intriguingly, labdane-related diterpenoid biosynthetic genes are largely found in plant-associated fungi, hinting that these natural products may play a role in such interactions. Accordingly, it is hypothesized here that isopimarane production may assist the plant-saprophytic lifestyle of *Aspergillus* fungi.

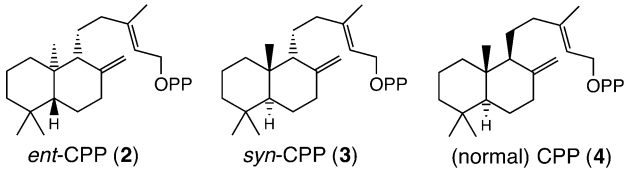


Production of terpenoid natural products is generally associated with plants rather than microbes such as fungi. This is evident for the large superfamily of labdane-related diterpenoids (~7000 members), which is defined by the use of class II diterpene cyclases in their biosynthesis,[1] from a previous genome-mining report. In particular, from the ~1000 sequenced fungal genomes available at NCBI, less than 100 such cyclases were found.[2] Nevertheless, a number of bioactive labdane-related diterpenoid natural products have been identified from fungi,[3] perhaps most prominently pleuromutilin, various derivatives of which are in clinical use and trials.[4]

Class II diterpene cyclases are characterized by a DxDD motif and catalyze protonation-initiated bicyclization of the general diterpene precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP, **1**), forming the eponymous labdadienyl$^+$ diphosphate intermediate.[1] Direct deprotonation of this yields copalyl diphosphate (CPP), which is generated as a distinct stereoisomer (Chart 1).[1] Such cyclases are then termed CPP synthases (CPSs).

CPP is almost invariably subsequently cyclized and/or rearranged by class I diterpene synthases.[1] These enzymes are characterized by a D(D,E)xx(D,E) motif involved in binding the Mg^{2+} cofactor[5] and catalyze ionization of the allylic diphosphate ester to trigger a carbocation cascade reaction that is terminated by deprotonation, either of the carbocation directly, yielding an olefin, or of a water molecule that is first added to the carbocation, yielding a hydroxylated product.[6]

Chart 1. Stereoisomers of Copalyl Diphosphate (CPP)



ent-CPP (**2**) *syn*-CPP (**3**) (normal) CPP (**4**)

In fungi, labdane-related diterpenoid biosynthesis is typically carried out by bifunctional cyclases that represent fusion of a CPS and a class I diterpene synthase. For example, *Phaeosphaeria nodorum* contains a bifunctional cyclase that produces *ent*-kaurene via *ent*-CPP (**2**),[7] and this can be separated into distinct polypeptides with CPS and kaurene synthase (KS) activity,[8] with functionally analogous CPS-KSs reported from other fungi as well.[9−11] In addition, it has been shown that a related bifunctional cyclase from *Phoma betae* is a *syn*-aphidicolan-16*β*-ol synthase (CPS-AoS), produced via the distinct stereoisomer *syn*-CPP (**3**),[12] while *Diaporthe amygdali* contains a bifunctional phyllocladan-16*α*-ol synthase (CPS-PoS), produced via (normal) CPP (**4**).[13]

Phylogenetic analysis of the identified fungal bifunctional diterpene cyclases has shown that the previously characterized CPS-KSs are closely related, and even the functionally distinct CPS-AoS and CPS-PoS are relatively closely related to these as well (i.e., these fall into two neighboring phylogenetic clades).[2] Thus, much of the phylogenetic diversity of these fungal labdane-related diterpenoid biosynthetic enzymes has not yet been explored. The *Aspergillus* genus contains a number of these cyclases, scattered over a significant portion of the remaining, uncharacterized phylogenetic range (Figure 1A). Moreover, the genes encoding these also are often found near those for cytochrome P450 (CYP) mono-oxygenases, which presumably further decorate the hydrocarbon backbone produced by the diterpene synthases, suggesting that these regions represent biosynthetic gene clusters that might produce

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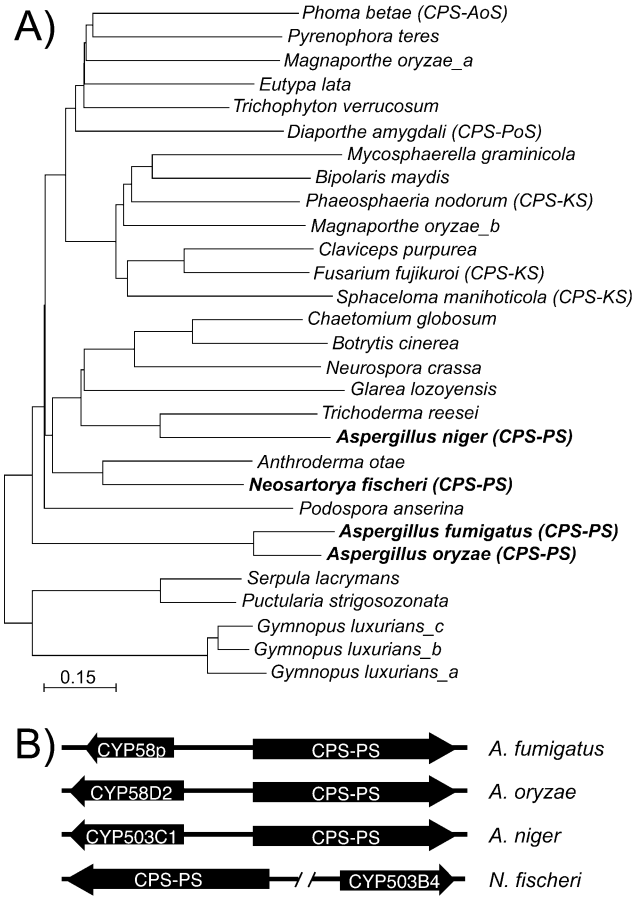

Figure 1. Fungal labdane-related diterpenoid biosynthetic capacity. (A) Representative phylogenetic tree with a selected subset of fungal bifunctional diterpene cyclases, based on a previously reported more complete tree, named by species (letters refer to distinct enzymes found in the same species).[2] Biochemically characterized cyclases are annotated as defined in the text (those identified here are highlighted in bold). (B) Chromosome map for the diterpene cyclases and CYPs investigated here.

more elaborated diterpenoids (e.g., Figure 1B). Here, to both probe fungal labdane-related diterpenoid biosynthesis and further investigate the utility of a previously developed modular metabolic engineering system that relies on functional recombinant expression in *Escherichia coli*[14] for such exploration, biochemical characterization of selected cyclases and neighboring CYPs from the genus *Aspergillus* was carried out.

RESULTS AND DISCUSSION

To begin these studies, the bifunctional diterpene cyclases from *Aspergillus fumigatus* strain AF293, *A. niger* strain CBS 513.88, and *A. oryzae* strain RIB40, as well as the closely related fungi *Neosartorya fischeri* strain NRRL 181, were chosen for analysis. While the cyclases from *A. fumigatus* and *A. oryzae* are closely related, these were selected to explore the hypothesis that their phylogenetic relationship reflects conservation of function. More importantly, together these cyclases cover a significant portion of the remaining uncharacterized phylogenetic range of this fungal enzymatic family (Figure 1A), indicating that biochemical characterization of these, along with the nearby CYPs, may provide insight into the functional diversity of labdane-related biosynthesis in not only the genus *Aspergillus* but fungi more broadly.

The cyclases from *A. fumigatus* and *A. niger* were directly cloned from the relevant fungi, while synthetic genes were obtained for those from *A. oryzae* and *N. fischeri* (these were codon-optimized for expression in the targeted heterologous host *E. coli*). These cyclases were then recombinantly expressed in *E. coli* using a previously reported modular metabolic engineering system, which provides the substrate **1** via coexpression of a GGPP synthase.[15] Analysis by GC-MS of organic solvent extracts from the resulting recombinant cultures and comparison to authentic standards (Figure 2) indicated that the cyclases from *A. fumigatus* and *A. oryzae* produce isopimara-7,15-diene (**5**), while those from *A. niger* and *N. fischeri* produce sandaracopimaradiene (**6**; isopimara-8(14),15-diene). Given that production of these tricycles proceeds via initial cyclization of GGPP to CPP, these bifunctional (iso)pimaradiene synthases were termed CPS-PS (i.e., AfCPS-PS, AoCPS-PS, AnCPS-PS, and NfCPS-PS, respectively).

To investigate the stereochemistry of the CPP intermediate and, hence, that of the derived isopimaradienes, the class I active site of each CPS-PS was inactivated by mutation of the first aspartate (Asp) of the relevant D(D,E)xxE motif to alanine (Ala) (see Table S1 for specific mutations). As expected, each of these mutants no longer exhibits class I activity (i.e., acts as a CPS only). Accordingly, when these were incorporated into the modular metabolic engineering system, only copalol, derived from CPP by endogenous phosphatases, is evident. These PS_{inact} mutants were further coexpressed with class I diterpene synthases specific for either **2** or **4** and found to specifically produce **4** (Figure S1). Thus, these fungal enzymes all produce pimaradienes with normal configuration, as defined by comparison to the analogous A/B rings in cholesterol,[1] as well as C13β-methyl configuration (i.e., isopimaradienes), differing only in placement of the endocyclic double bond that results from alternative sites for deprotonation of a common isopimar-15-en-8-yl$^+$ intermediate (Scheme 1).

To enable investigation of the substrate specificity of the class I active site of these cyclases, the class II active site was inactivated by mutation of Asp from the relevant DxDD motif to Ala. As previously reported for a fungal CPS-KS,[8] mutation of the middle Asp was sufficient to abrogate the CPS activity of NfCPS-PS. However, to block CPS activity, it proved necessary to mutate all three Asp in the other fungal CPS-PS (i.e., only these triple mutants completely lost CPS activity, as the single and double mutants could still react with GGPP to some extent; see Table S1 for final mutants). When these CPS_{inact} mutants were coexpressed in the modular metabolic engineering system with a CPS producing **4**, the expected (iso)-pimaradiene was produced, demonstrating that these retain class I activity. Thus, these are suitable for investigating the substrate specificity of the PS active site.

Accordingly, these CPS_{inact}-PS mutants were coexpressed with CPSs producing either **2** or **3**. Those from *A. fumigatus* and *A. oryzae* did not react with **2**, while those from *A. niger* and *N. fischeri* did and produced the diterpene alcohol *ent*-manool **7**, along with small amounts of the diterpene olefin *ent*-sandaracopimaradiene **8** (Figure S2). On the other hand, those from *A. fumigatus* and *A. oryzae* react with **3**, and both produced an unknown olefin, while those from *A. niger* and *N. fischeri* both produced an unknown alcohol (Figure S3A). To identify these compounds, metabolic flux to terpenoids in the engineered *E. coli* was increased as previously described,[16] and the culture volumes were scaled up, enabling isolation of

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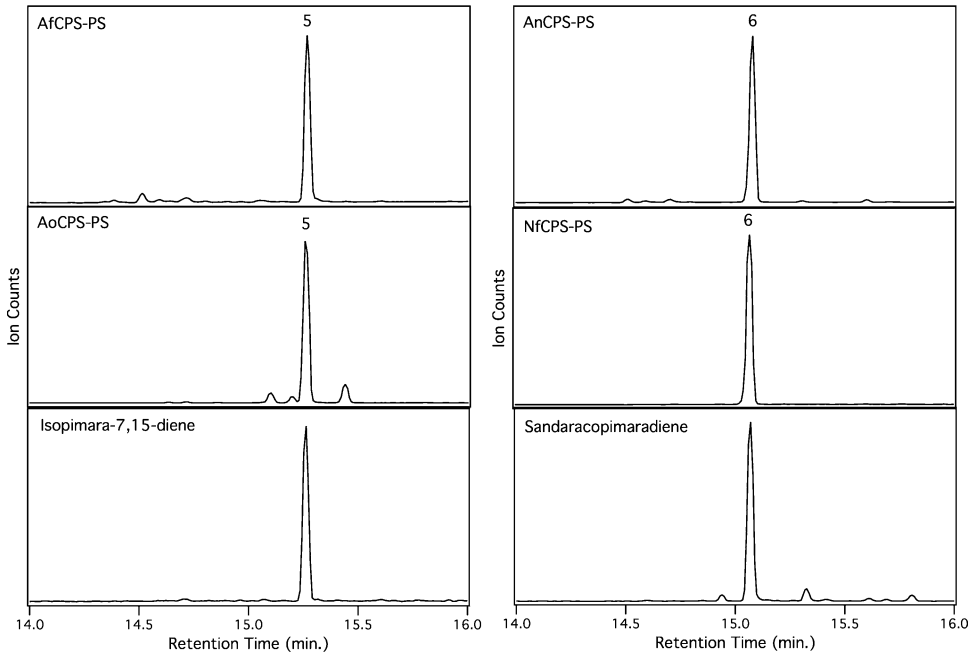
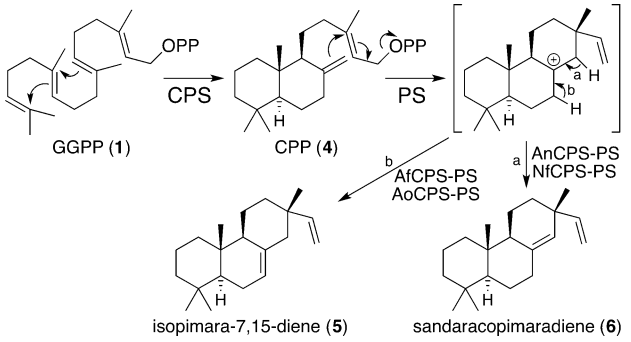


Figure 2. Products of fungal bifunctional diterpene cyclases. GC-MS chromatograms of organic solvent extracts of recombinant *E. coli* expressing the indicated enzyme and engineered to produce **1**, along with those of authentic standards.

Scheme 1. Related Reactions Catalyzed by the Bifunctional Fungal Diterpene Cyclases Characterized Here



sufficient amounts for characterization of each compound by NMR. This analysis indicates that the olefin is *syn*-isopimara-7,15-diene **9** (Table S2 and Figures S4−6), while the alcohol is *syn*-manool **10** (Table S3 and Figures S7−9).

Hence, while the reaction catalyzed by the PS active site with the normal substrate **4** is mechanistically very similar among all four cyclases characterized here (Scheme 1), some differences between them are revealed by their distinct reactivity with these alternative substrates. In particular, the PS active sites from the *A. fumigatus* and *A. oryzae* cyclases are more specific, and their ability to react with **3** and produce *syn*-isopimaradiene suggests that this alternative substrate is oriented in a very similar manner to **4**, enabling cyclization (Figure S3B). By contrast, the greater promiscuity of the PS active sites in the cyclases from *A. niger* and *N. fischeri* leads to the incorporation of water, in an indirect hydrolysis reaction wherein water is added to the

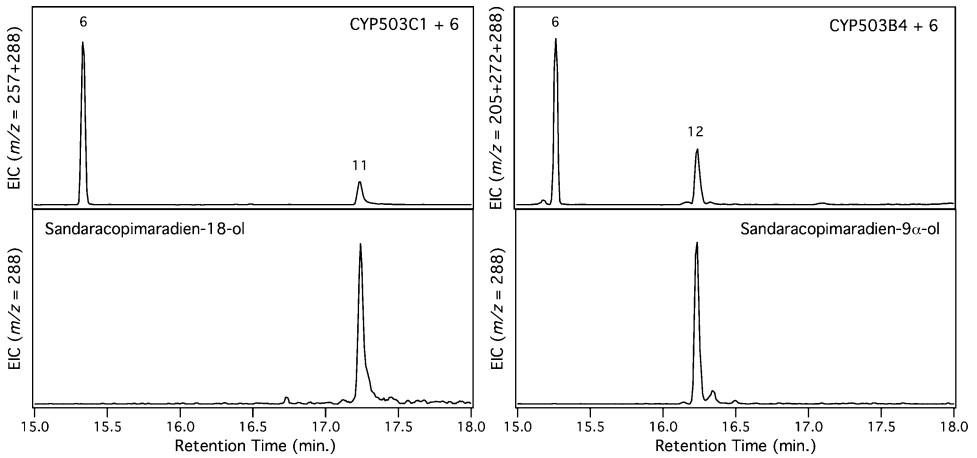


Figure 3. Products from reaction of indicated fungal CYP503 family members with **6**. GC-MS chromatograms of organic solvent extracts of recombinant *E. coli* expressing the indicated CYP, along with the requisite CPR, and also engineered to produce **6**, along with those of authentic standards.

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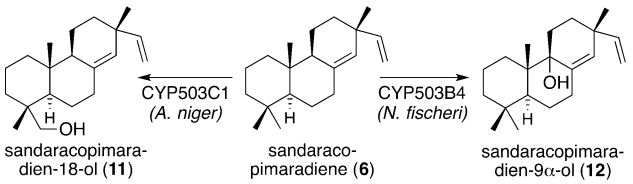
tertiary position of the allylic carbocation generated by initial ionization of the diphosphate ester (Figure S3C). This presumably results from water molecules that are appropriately positioned for such addition in the active site in the presence of the alternative substrates **2** and **3**, but not the native substrate **4**.

Beyond the production of isopimaradienes, the presence of genes encoding CYPs near those encoding the CPS-PS suggests that these fungi may produce more elaborated isopimarane diterpenoids. In *A. fumigatus* and *A. oryzae* these CYPs are members of the CYP58 family, while in *A. niger* and *N. fischeri* these are members of the CYP503 family (Figure 1B). However, the putative CYP58 family member in *A. fumigatus* was predicted to contain a premature stop codon, which was verified here by cloning and sequencing, and this CYP was not further investigated.

The ability of the full-length CYPs to react with the isopimaradiene (**5** or **6**) produced by the neighboring CPS-PSs was investigated with the modular metabolic engineering system. On the basis of previous experience with plant CYPs,[14] synthetic genes codon-optimized for expression in *E. coli* were used in place of the native CYP genes for this purpose. In addition, to provide the requisite electrons, a similarly optimized synthetic gene also was obtained for the CYP reductase from *A. oryzae*. Each CYP was then coexpressed, along with the reductase, as well as the relevant CPS-PS. While no activity was observed with the CYP58D2 from *A. oryzae* (data not shown), with both CYP503 family members a hydroxylated derivative of **6**, the product of their neighboring CPS-PSs, was observed by GC-MS (Figure 3). That produced by the CYP503C1 from *A. niger* was determined to be sandaracopimaradien-18-ol (**11**) by comparison to a previously reported CYP product (i.e., CYP720B4[17]). By contrast, the CYP503B4 from *N. fischeri* was found to instead produce sandaracopimaradien-9α-ol (**12**), again identified by comparison to a previously reported CYP product (i.e., CYP76M8[18]). Thus, despite the common production of **6** by their CPS-PS, the different activity of the nearby CYP503 family members indicates that *A. niger* and *N. fischeri* produce distinct derived isopimarane diterpenoids (Scheme 2).

Scheme 2. Alternative Hydroxylation of Sandaracopimaradiene (6) Catalyzed by CYP503B4 and CYP503C1



Notably, the other previously characterized CYP503 family members both target the same C4α-methyl substituent of a labdane-related diterpene as CYP503C1, albeit in either *ent*-kaurene or *syn*-aphidicolan-16β-ol (i.e., CYP503A1 and CYP503N1, respectively).[19,20] By contrast, CYP503B4 exhibits distinct regiospecificity in targeting C-9 instead. Regardless, while previous work had shown that fungal diterpene cyclases can be functionally expressed in *E. coli*,[9,12,13,21,22] these results further demonstrate that at least some fungal CYPs can be incorporated as well. Although a role for CYP58D2 in elaboration of **5** cannot be ruled out (e.g., this may not be amenable to functional expression in *E. coli*), it seems worth noting that in at least four cases now it has been shown that members of the CYP503 family can carry out initial oxygenation reactions, suggesting a key early role for this CYP family in fungal labdane-related diterpenoid biosynthesis.

It has been previously suggested that a bifunctional diterpene cyclase from *Aspergillus nidulans* produces *ent*-pimara-8(14),15-diene.[23] However, the analytical methods used in that report cannot distinguish between enantiomers. Particularly given that this cyclase is closely related to NfCPS-PS (84% amino acid sequence identity, greater than the 74% identity between the functionally analogous AfCPS-PS and AoCPS-PS characterized here), it seems likely that this *A. nidulans* cyclase also may produce (normal) sandaracopimaradiene (i.e., **6** via **4**). Moreover, the presence of a neighboring CYP503 family member (CYP503B1) similarly closely related to the CYP503B4 from *N. fischeri* further suggests that this fungus also produces a more elaborated derived isopimarane as well.

Intriguingly, almost all of the fungi predicted to produce labdane-related diterpenoids (i.e., contain the relevant diterpene cyclases) are associated with plants as either pathogens or saprophytes,[2] and such biosynthesis (i.e., production of gibberellin phytohormones) by *Fusarium fujikuroi* has been shown to contribute to the virulence of this rice plant pathogen.[24] Thus, it seems at least plausible that biosynthesis of such labdane-related diterpenoids is relevant to the interaction of these fungi with their plant hosts, and the conserved biosynthesis of isopimaranes across the genus *Aspergillus* suggests a role for these diterpenoid natural products in the plant-saprophytic lifestyle associated with these fungi. Indeed, given the ease with which these cyclases can be diverted to alternative reactions by even single amino acid changes,[21] it is striking that these phylogenetically disparate enzymes produce such similar products. Regardless, given the phylogenetic range of the diterpene cyclases characterized here (Figure 1A), these studies provide insight into the labdane-related diterpenoid biosynthetic capacity of not only the fungal genus *Aspergillus* but that of (plant-associated) fungi more generally as well.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were acquired on a Bruker AVIII-700 spectrometer equipped with a 5 mm HCN cryogenic probe, using TopSpin v1.4 software. Analysis was carried out at 25 °C. Chemical shifts were calculated by reference to those known for $CDCl_3$ signals offset from tetramethylsilane (^{13}C 77.23 ppm, ^{1}H 7.24 ppm). All spectra were acquired using standard programs from the TopSpin v1.4 software, with collection of 1D ^{1}H NMR and 2D double-quantum filtered correlation spectroscopy (DQF-COSY), heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), HMQC−COSY, and ROESY (700 MHz), as well as 1D ^{13}C NMR (174 MHz) and distortionless enhancement of polarization transfer (DEPT) spectra. GC-MS analyses were carried out using a 3900 GC with Saturn 2100T ion trap MS (Varian), equipped with an HP-5MS column (Agilent, 0.25 μm, 0.25 i.d., 30 m) with a He flow rate of 1.2 mL/min and the following oven temperature program: 50 °C for 3 min, 15 °C/min to 300 °C, hold 3 min. Samples (1 μL) were injected via splitless injection at 250 °C. Flash chromatography was carried out with a 4 g silica gel column (80−200 mesh) using a Reveleris automated system (Grace, Deerfield, IL, USA) with a 15 mL/min flow rate, 5 mL injections, and UV detection at 200 nm, with the following stepwise gradient: 0%, 5%, 15%, 25% ethyl acetate (in hexane) for 1 min each and a final wash with 100% ethyl acetate for 3 min. HPLC was carried out with an Agilent Poroshell 120 EC-C18 column (4.6 × 150 mm, 4 μm) on an Agilent 1200 series system equipped with fraction collector

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and diode array detector and run at a flow rate of 1 mL/min. The column was pre-equilibrated, and the sample was injected, washed with 50% acetonitrile/dH_2O (0−2 min), and eluted with 50−100% acetonitrile (2−7 min), followed by a 100% acetonitrile wash (7−23 min), with peak-based fraction collection. All reagents were purchased from Thermo-Fisher Scientific unless noted otherwise.

Cloning. To clone their diterpene cyclases (see Table S4 for accession numbers), cultures of *Aspergillus fumigatus* AF293 and *A. niger* CBS 513.88 were obtained from the Fungal Genetics Stock Center (Kansas State University). These fungi were inoculated onto potato dextrose agar plates (39 g/L) and grown at 28 °C for 4 days. These cultures were then harvested by scraping and total RNA isolated using the Concert Plant RNA Reagent (Invitrogen). The genes for the cyclases and CYP58p were amplified by targeted RT-PCR (see Table S5 for primers) and cloned by directional topo-isomerization into pENTR/SD/d-TOPO vectors (Invitrogen). Complete gene sequencing demonstrated that these matched the predicted genes. Thus, the cyclases from *A. oryzae* and *N. fischerii* were simply obtained by gene synthesis. These were amplified by PCR (see Table S5 for primers) and again subcloned into pENTR/SD/d-TOPO vectors. Genes for the targeted CYPs and the CYP reductase from *A. oryzae* (AoCPR) also were obtained by synthesis (see Table S4 for accession numbers). All synthetic genes, including codon optimization for expression in *E. coli*, were purchased from Genscript (the corresponding sequences can be found in the Supporting Information). CPS-PS mutants were generated by whole-plasmid PCR amplification using overlapping mutagenic primers (see Table S5 for primer sequences). All mutants were verified by complete gene sequencing. For CPS-PS expression, wild-type and mutant genes were subcloned by directional recombination into a previously described pGG/DEST vector,[15] which contains a synthase for production of the general diterpene precursor GGPP (**1**). To enable functional CYP expression, AoCPR was first cloned into pET-Duet (Novagen), which is compatible with the pGG/DEST constructs, specifically into the first multiple cloning site, using the NcoI and NotI restriction sites, which were introduced on the 5′ and 3′ ends of AoCPR, respectively, by PCR amplification (see Table S5 for primers). The targeted CYPs were then subsequently cloned into the resulting pET-Duet/AoCPR vector, specifically into the second multiple cloning site, using the NdeI and XhoI restriction sites; again these were introduced on the 5′ and 3′ ends of the CYPs by PCR amplification (see Table S5 for primers). Previous work has suggested that replacement of the N-terminal transmembrane helix sequence found in eukaryotic CYPs with a lysine-rich leader sequence enables functional expression in *E. coli*,[14] which also was attempted here (see Table S5 for relevant primers), but found not to improve activity relative to the full-length constructs. All constructs were verified by complete sequencing of the introduced genes. Additional coexpression of the *ent*-kaurene synthase from *Arabidopsis thaliana* (AtKS), which is specific for *ent*-CPP **2**, or the D404A mutant of the abietadiene synthase from *Abies grandis* (AgAS:D404A; lacking the CPS activity otherwise associated with this bifunctional diterpene cyclase[25]), which is specific for (normal) CPP **4**, utilized previously described pET-Duet-based constructs.[16]

Enzymatic Characterization. The targeted enzymes were characterized using a previously described modular metabolic engineering system in *E. coli*.[15] Accordingly, the relevant constructs were transformed into the C41 OverExpress strain of *E. coli* (Lucigen) and heterologously expressed under the appropriate antibiotic selection. Briefly, the recombinant strains were grown in liquid TB media (12 g casein, 24 g yeast extract, and 8 mL 50% glycerol per L H_2O, with the pH adjusted to 7.0) at 37 °C with shaking at 200 rpm to OD_{600} = 0.6, then transferred to 16 °C, with continued shaking at 200 rpm, for an hour and induced with 1 mM IPTG. At the time of induction, cultures were supplemented with phosphate buffer (pH 7.0) to 100 mM final concentration, as previously described.[16] After 3 days of continued shaking at 200 rpm and 16 °C, enzymatic products were extracted by addition of an equal volume of hexanes and gentle swirling. The organic solvent was separated out and then dried under N_2, with the residue resuspended in fresh hexanes and analyzed by GC-MS. Note that direct analysis of the PS_{inact} mutants described here relied on dephosphorylation of the CPP product by endogenous phosphatases from *E. coli*, which yield the derived primary alcohol (normal) copalol **4′**. The various observed enzymatic products were first tentatively identified by comparison of the associated mass spectra (MS) to the available MS libraries and then direct comparison of retention time and MS to that of authentic standards where available.

Analysis of Unknown Enzymatic Products. To obtain sufficient quantities of the unknown products of $AfCPS_{inact}$-PS and $NfCPS_{inact}$-PS with *syn*-CPP **3** for NMR analysis, 3 L of the relevant bacterial culture was grown as described above. This was extracted twice with equal volumes of hexanes, the phases separated in a separatory funnel, and the pooled hexanes dried by rotary evaporation. The resulting extract was redissolved in 10 mL of fresh hexanes and fractionated via flash chromatography. The resulting fractions were analyzed by GC-MS. Those containing the enzymatic products were dried under N_2 and dissolved in 5 mL of methanol. The compounds were then further purified via HPLC. Again, the fractions containing the enzymatic products were identified by GC-MS analysis. Those containing pure compounds were pooled, dried under N_2, and dissolved in 0.5 mL of $CDCl_3$ (Aldrich) for NMR analysis. Observed HMBC correlations were used to propose the majority of the structures, while COSY correlations between protonated carbons were used to complete the structures, which were further verified by HSQC correlations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00764.

Details of the described structural characterization of enzymatic products, along with other supplemental figures and the sequences of the synthetic genes utilized here (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Peters, R. J. *Nat. Prod. Rep.* **2010**, *27*, 1521−1530.

(2) Fischer, M. J.; Rustenhloz, C.; Leh-Louis, V.; Perriere, G. *BMC Microbiol.* **2015**, *15*, 221.

(3) Quin, M. B.; Flynn, C. M.; Schmidt-Dannert, C. *Nat. Prod. Rep.* **2014**, *31*, 1449−73.

(4) Shang, R.; Wang, J.; Guo, W.; Liang, J. *Curr. Top. Med. Chem.* **2013**, *13*, 3013−25.

(5) Aaron, J. A.; Christianson, D. W. *Pure Appl. Chem.* **2010**, *82*, 1585−1597.

DOI: 10.1021/acs.jnatprod.6b00764
J. Nat. Prod. 2017, 80, 328−333

(6) Zi, J.; Mafu, S.; Peters, R. J. *Annu. Rev. Plant Biol.* **2014**, *65*, 259−286.
(7) Xu, M.; Wilderman, P. R.; Peters, R. J. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 7397−7401.
(8) Kawaide, H.; Sassa, T.; Kamiya, Y. *J. Biol. Chem.* **2000**, *275*, 2276−2280.
(9) Toyomasu, T.; Kawaide, H.; Ishizaki, A.; Shinoda, S.; Otsuka, M.; Mitsuhashi, W.; Sassa, T. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 660−664.
(10) Tudzynski, B.; Kawaide, H.; Kamiya, Y. *Curr. Genet.* **1998**, *34*, 234−240.
(11) Bömke, C.; Rojas, M. C.; Gong, F.; Hedden, P.; Tudzynski, B. *Appl. Environ. Microbiol.* **2008**, *74*, 5325−5339.
(12) Oikawa, H.; Toyomasu, T.; Toshima, H.; Ohashi, S.; Kawaide, H.; Kamiya, Y.; Ohtsuka, M.; Shinoda, S.; Mitsuhashi, W.; Sassa, T. *J. Am. Chem. Soc.* **2001**, *123*, 5154−5155.
(13) Toyomasu, T.; Niida, R.; Kenmoku, H.; Kanno, Y.; Miura, S.; Nakano, C.; Shiono, Y.; Mitsuhashi, W.; Toshima, H.; Oikawa, H.; Hoshino, T.; Dairi, T.; Kato, N.; Sassa, T. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 1038−47.
(14) Kitaoka, N.; Lu, X.; Yang, B.; Peters, R. J. *Mol. Plant* **2015**, *8*, 6−16.
(15) Cyr, A.; Wilderman, P. R.; Determan, M.; Peters, R. J. *J. Am. Chem. Soc.* **2007**, *129*, 6684−6685.
(16) Morrone, D.; Lowry, L.; Determan, M. K.; Hershey, D. M.; Xu, M.; Peters, R. J. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1893−1906.
(17) Hamberger, B.; Ohnishi, T.; Seguin, A.; Bohlmann, J. *Plant Physiol.* **2011**, *157*, 1677−95.
(18) Wang, Q.; Hillwig, M. L.; Okada, K.; Yamazaki, K.; Wu, Y.; Swaminathan, S.; Yamane, H.; Peters, R. J. *J. Biol. Chem.* **2012**, *287*, 6159−6168.
(19) Tudzynski, B.; Hedden, P.; Carrera, E.; Gaskin, P. *Appl. Environ. Microbiol.* **2001**, *67*, 3514−3522.
(20) Fujii, R.; Minami, A.; Tsukagoshi, T.; Sato, N.; Sahara, T.; Ohgiya, S.; Gomi, K.; Oikawa, H. *Biosci., Biotechnol., Biochem.* **2011**, *75*, 1813−7.
(21) Mafu, S.; Potter, K. C.; Hillwig, M. L.; Schulte, S.; Criswell, J.; Peters, R. J. *Chem. Commun. (Cambridge, U. K.)* **2015**, *51*, 13485−7.
(22) Kawaide, H.; Imai, R.; Sassa, T.; Kamiya, Y. *J. Biol. Chem.* **1997**, *272*, 21706−21712.
(23) Bromann, K.; Toivari, M.; Viljanen, K.; Vuoristo, A.; Ruohonen, L.; Nakari-Setala, T. *PLoS One* **2012**, *7*, e35450.
(24) Wiemann, P.; Sieber, C. M.; von Bargen, K. W.; Studt, L.; Niehaus, E. M.; Espino, J. J.; Huss, K.; Michielse, C. B.; Albermann, S.; Wagner, D.; Bergner, S. V.; Connolly, L. R.; Fischer, A.; Reuter, G.; Kleigrewe, K.; Bald, T.; Wingfield, B. D.; Ophir, R.; Freeman, S.; Hippler, M.; Smith, K. M.; Brown, D. W.; Proctor, R. H.; Munsterkotter, M.; Freitag, M.; Humpf, H. U.; Guldener, U.; Tudzynski, B. *PLoS Pathog.* **2013**, *9*, e1003475.
(25) Peters, R. J.; Ravn, M. M.; Coates, R. M.; Croteau, R. B. *J. Am. Chem. Soc.* **2001**, *123*, 8974−8978.

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