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Letter

Identification and Characterization of a Cryptic Bifunctional Type I Diterpene Synthase Involved in Talaronoid Biosynthesis from a Marine-Derived Fungus

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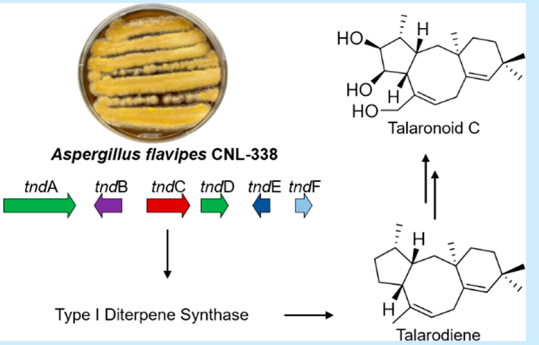
ABSTRACT: We report the identification of the *tnd* biosynthetic cluster from the marine-derived fungus *Aspergillus flavipes* and the in vivo characterization of a cryptic type I diterpene synthase. The heterologous expression of the bifunctional terpene synthase led to the discovery of a diterpene backbone, talarodiene, harboring a benzo[*a*]cyclopenta[*d*]-cyclooctane tricyclic fused ring system. The conversion of geranylgeranyl diphosphate to talarodiene was investigated using ^{13}C-labeling studies, and stable isotope tracer experiments showed the biotransformation of talarodiene into talaronoid C.



Terpenes are the largest class of natural products and are produced by all kingdoms of life. These compounds possess enormous structural diversity and exhibit various biological activities ranging from anticancer and antimalarial activity to being carcinogens and mycotoxins.[1] Despite their structural complexity, all terpenes are derived from the universal C_5 hemiterpene precursors dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). Coupling of these C_5 precursors, facilitated by prenyltransferases (PTs), generates linear, achiral polyprenyl diphosphates that can be transformed by terpene cyclases (TCs) into complex scaffolds containing multiple fused rings and stereogenic centers.[2−6] The structural diversity associated with terpenes often originates from the cyclization step, and TCs catalyze some of the most complex reactions in natural product chemistry.

In fungi, although condensation and cyclization reactions mostly occur independently, bifunctional terpene synthases have been characterized where the C-terminal half is responsible for producing the polyprenyl diphosphate and the N-terminal half catalyzes the cyclization reaction. Depending on the cyclization reaction for initial carbocation formation, TCs are generally categorized into two distinct classes (type I and type II). An alkene−cation cyclization mechanism is initiated in type I reactions following the heterolytic cleavage of the diphosphate, whereas the protonation of an alkene triggers cyclization in type II TCs.[4,5] The first fungal type I diterpene (C_{20}) synthase, PaFS, was characterized in 2007 from *Phomopsis amygdali* and shown to produce fusicoccadiene (**1**).[7] The first type I sesterterpene (C_{25}) synthase, AcOS, was characterized in 2013 from *Aspergillus clavatus* and shown to be responsible for the biosynthesis of ophiobolin F (**2**).[8] Because of their potential to synthesize diverse hydrocarbon skeletons, subsequent genome mining efforts focused on identifying additional cryptic type I bifunctional terpene synthases. As a result, a number of fungal type I sesterterpene synthases were characterized.[9−17] However, since the discovery of PaFS, only a limited number of type I diterpene synthases have been identified, including those responsible for the production of variediene (**3**),[18] phomopsene (**4**),[19] brassicicene (**5**),[20] a precursor to the cyclopiane-type diterpenes (**6**),[21] and dolasta-1(15),8-diene (**7**)[22] (Figure 1). Given our limited knowledge of type I diterpene synthases, the discovery and biochemical characterization of new enzymes would bring to light cryptic natural products, unveil novel cyclization reactions, and allow for more informed bioinformatic predictions. In this work, we describe the discovery and in vivo characterization of a cryptic bifunctional type I diterpene synthase from a marine-derived fungus that synthesizes a tricyclic 5−8−6 hydrocarbon skeleton. The use of stable tracer isotope experiments also allowed us to show the biotransformation of the diterpene

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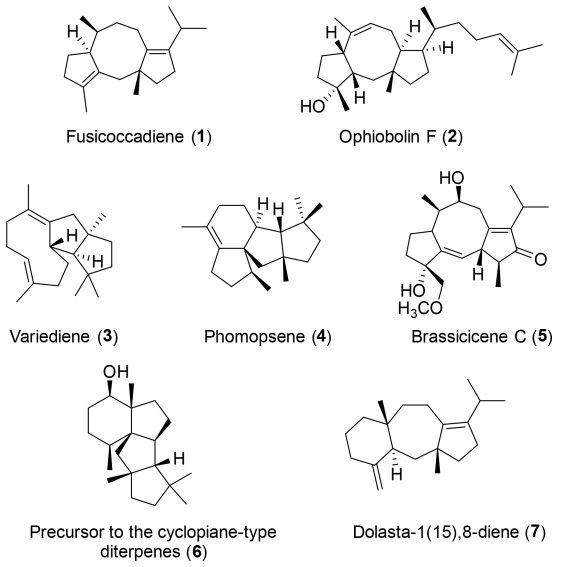

Figure 1. Structures of selected fungal diterpenes and sesterterpenes produced by type I bifunctional terpene synthases.

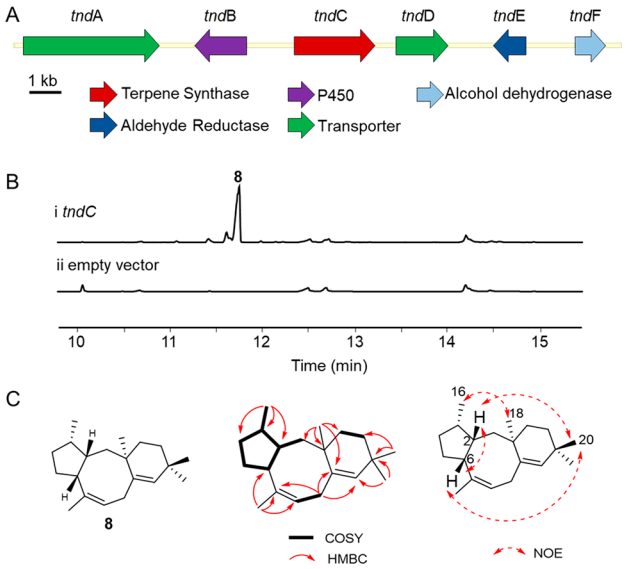

Figure 2. Characterization of the type I diterpene synthase *tndC* from *A. flavipes* CNL-338. (A) Organization of the *tnd* biosynthetic gene cluster in *A. flavipes* CNL-338. (B) GC-MS analysis (TIC) of extracts from *S. cerevisiae* ZXM144 transformed with (i) a plasmid-borne *tndC* or (ii) an empty vector. (C) Structure identification of compound **8** and key 2D NMR correlations.

backbone into the talaronoid class of natural products and ultimately characterize a cryptic biosynthetic cluster.

It is known that marine organisms are prolific producers of bioactive natural products and often produce molecules not observed in their terrestrial counterparts.[23] The previously characterized type I bifunctional terpene synthases were identified exclusively from terrestrial fungi; given the tremendous promise that marine organisms hold for characterizing novel biosynthetic enzymes, we turned to marine-derived fungi as an underexplored resource to identify and characterize type I terpene synthases. Recently, our group sequenced the genome of the marine-derived fungus *Aspergillus flavipes* CNL-338[24] and, using the PaFS and AcOS sequences as probes, scanned the genome for bifunctional terpene synthases. A 21-kb biosynthetic cluster harboring a cryptic chimeric synthase, *tnd*C, was identified (Figure 2A), and the bioinformatic analysis of TndC revealed that the 764 amino acid-containing protein possessed both PT and TC domains. A multiple sequence alignment also showed that TndC contained the conserved aspartate-rich DDxxD motif for Mg^{2+} binding in both the PT and TC domains in addition to a second NSE Mg^{2+}-binding motif in the TC domain indicative of type I cyclases (Figure S2). The phylogenetic comparison of the cryptic chimeric synthase with known fungal-derived diterpene and sesterterpene synthases showed that TndC clades between PaFS and the astellifadiene sesterterpene synthase EvAS[25] and stellata-2,6,19-triene sesterterpene synthase EvSS[26] (Figure S1), suggesting that TndC could produce a new terpene skeleton; however, it was not clear if the product was a diterpene or sesterterpene.

Initial efforts at expressing recombinant TndC from *Escherichia coli* and *Saccharomyces cerevisiae* failed to generate any soluble protein. Thus, to elucidate the product of TndC, we heterologously expressed intron-free *tnd*C in *Saccharomyces cerevisiae* ZXM144.[27] Compared to an empty vector control, the GC-MS analysis of crude extracts of *S. cerevisiae* ZXM144 transformed with *tnd*C revealed the presence of a new major product, **8**, with *m*/*z* 272 $[M]^+$ (Figures 2B and S7), supporting the production of a diterpene instead of a sesterterpene. HRESIMS (Figure S7) coupled with 1D and 2D NMR experiments (Figures S11−S15 and Table S2) identified that the planar structure of **8**, which was named talarodiene, contained a benzo[*a*]cyclopenta[*d*]cyclooctane tricyclic hydrocarbon backbone (Figure 2C). NOESY correlations were used to assign the relative configuration of **8** (Figures S16−S22), and ECD calculations (Figure S8) were used to determine the absolute configuration as (2*S*,3*S*,6*R*,11*R*).

With the isolation of **8**, the cyclization mechanism that converts geranylgeranyl diphosphate (GGPP) into the 5−8−6 tricyclic hydrocarbon skeleton was investigated using ^{13}C-labeling studies. [1-^{13}C]Acetate, [2-^{13}C]acetate, and [1,2-$^{13}C_2$]acetate were administered independently to *tnd*C-transformed *S. cerevisiae* ZXM144, and the corresponding labeling patterns of ^{13}C-enriched **8** were analyzed by NMR spectroscopy (Figures S23−S25 and Table S3). From the [1,2-$^{13}C_2$]acetate labeling patterns and given the similarity of TndC to EvAS and EvSS, a cyclization mechanism similar to the first steps in the biosynthesis of astellifadiene and stellata-2,6,19-triene is proposed in Figure 3. Cleavage of diphosphate followed by 1,11- and 10,14-cyclization reactions converts GGPP to the bicyclic tertiary cation intermediate $\mathbf{9}^+$. Ring expansion of $\mathbf{9}^+$ from a 1,2-alkyl shift forms the cation intermediate $\mathbf{10}^+$, which is transformed into the tertiary cation intermediate $\mathbf{11}^+$ following a transannular proton transfer. A 1,2-hydride shift and 2,6-cyclization form intermediate $\mathbf{12}^+$, and deprotonation at C-8 ultimately yields **8**.

After the heterologous expression of the cryptic *tnd*C gene led to the isolation of **8**, we turned back to the original host and evaluated *A. flavipes* CNL-338 for its production of this new tricyclic diterpene (Figure 4). Unfortunately, we were unable to detect the presence of **8** in crude extracts using GC-MS and LC-MS analyses, suggesting that **8** is not the final natural product and is instead an intermediate that is modified by tailoring enzymes encoded in the *tnd* gene cluster. A closer inspection of the regions upstream and downstream of *tnd*C

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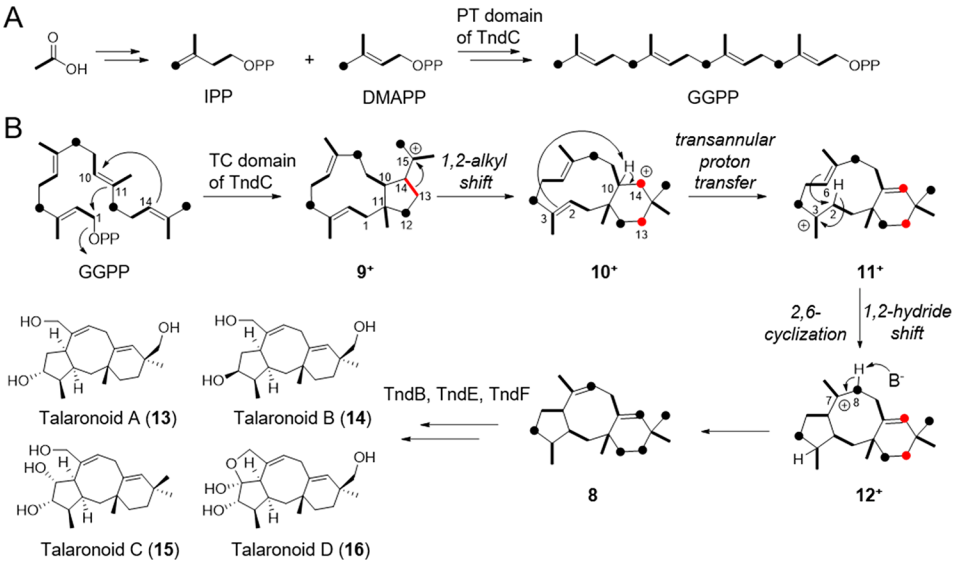

Figure 3. Proposed biosynthesis of the talarodiene backbone. (A) Biosynthesis of the acyclic precursor geranylgeranyl diphosphate (GGPP) using the C-terminal prenyltransferase (PT) domain of TndC. (B) Formation of the 5−8−6 tricyclic talarodiene backbone **8** via the N-terminal cyclization (TC) domain of TndC. [1,2-$^{13}C_2$]Acetate labeling patterns are shown as black bold lines and dots to signify double and single enrichments, respectively. Red dots indicate C−C bond breakage of an intact acetate unit.

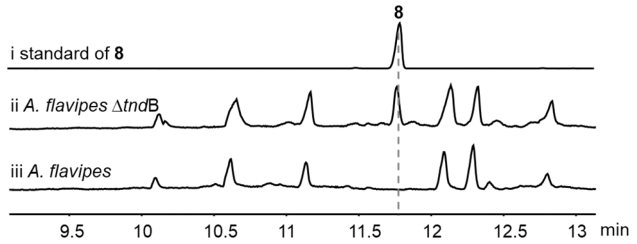

Figure 4. GC-MS chromatograms (TIC) of (i) a standard of compound **8**, (ii) a crude extract of the Δ*tndB* strain, and (iii) a crude extract from wild-type *A. flavipes* CNL-338.

revealed that the *tnd* cluster encodes several oxidative enzymes in addition to the diterpene synthase, including a cytochrome P450 enzyme (*tnd*B), an aldehyde reductase (*tnd*E), and an alcohol dehydrogenase (*tnd*F) (Table S4). Given the type of tailoring enzymes present, we speculated that the cytochrome P450 TndB would be the next enzyme in the biosynthetic pathway. Indeed, GC-MS analysis of the Δ*tnd*B mutant showed the accumulation of **8** (Figures 4 and S10).

While the gene inactivation experiments unequivocally linked the *tnd* biosynthetic cluster to **8** in *A. flavipes* CNL-338, the final natural products produced by the pathway were unknown. Recently, a group of diterpenoids, namely, talaronoids A (**13**), B (**14**), C (**15**), and D (**16**), containing a 5−8−6 fused ring system were isolated from the terrestrial fungus *Talaromyces stipitatus* (Figure 3).[28] Using the amino acid sequence of TndC as a biosynthetic hook, we scanned the genome of *T. stipitatus* and identified a 24-kb cluster that harbored an assortment of genes similar to those in the *tnd* biosynthetic cluster from *A. flavipes* CNL-338. When aligned, the two *tnd* clusters were organized similarly, with both clusters containing genes coding for the cytochrome P450 enzyme (*tnd*B), the bifunctional type I terpene cyclase (*tnd*C), the MFS multidrug transporter (*tnd*D), and the aldehyde reductase (*tnd*E) (Figures S3−S5). Further annotation upstream and downstream of the four *tnd* genes in *T. stipitatus* revealed a number of transposable elements suggestive of putative boundaries for the biosynthetic cluster, whereas *A. flavipes* contained genes coding for a putative drug-resistant protein (*tnd*A), an alcohol dehydrogenase (*tnd*F), and a putative short-chain dehydrogenase (orf-1) (Figure S3 and Table S4).Without independently knocking out each *tnd* gene, we cannot unequivocally define the *tnd* cluster boundaries. However, given the variability between the two organisms upstream and downstream of *tnd*B and *Tnd*E, respectively, we can predict that the minimal *tnd* cluster consists of *tnd*B, *tnd*C, *tnd*D, and *tnd*E. Although both organisms share the same four core *tnd* genes, when we scanned crude extracts of *A. flavipes* CNL-338 for the presence of **13**−**16**, the compounds were not detected. It is worth noting that only limited quantities of the talaronoids were originally reported from a large-scale solid-phase fermentation of *T. stipitatus*.[28] We thus assumed that much like the terrestrial strain, the talaronoids were also produced in trace amounts in the marine-derived fungus *A. flavipes* CNL-338.

To determine if **8** was indeed an intermediate in talaronoid biosynthesis, we biosynthetically prepared ^{13}C-enriched **8** in *S. cerevisiae* using [1-^{13}C]acetate. Labeled material was administered to *A. flavipes* CNL-338, and HRESIMS inspection of the crude extract showed the production of a new compound not observed in the DMSO control. The isotopic fragmentation pattern of the new compound also indicated it was derived from the labeled material (Figure 5A). Closer inspection of the new compound showed that its retention time and *m*/*z* matched those of an authentic standard, talaronoid C (**15**) (Figure 5B), thereby confirming that **8** had been biotransformed into **15**. Thus, the stable tracer isotope experiment confirmed **8** as an intermediate in the talaronoid biosynthetic pathway.

In summary, we identified and characterized the *tnd* biosynthetic cluster responsible for the production of talaronoid C from the marine-derived fungus *A. flavipes* CNL-338. The heterologous expression of a cryptic type I bifunctional terpene synthase led to the discovery of a diterpene possessing a benzo[*a*]cyclopenta[*d*]cyclooctane

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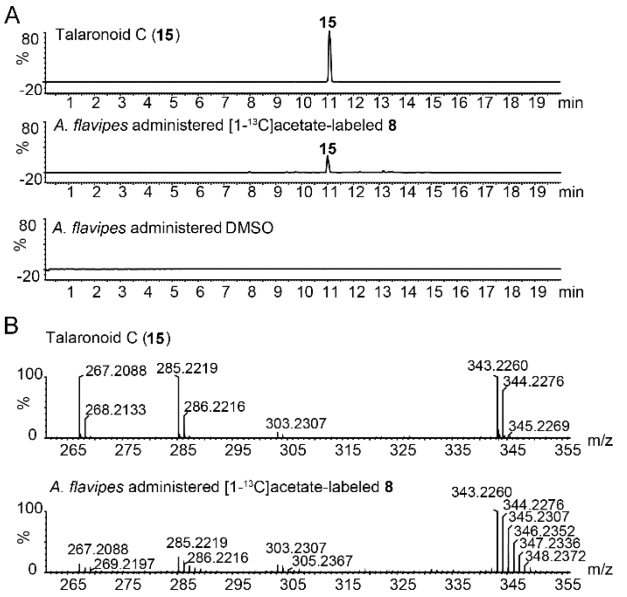

Figure 5. In vivo conversion of the talarodiene backbone **8** in *A. flavipes* CNL-338. (A) High-resolution LC-MS analysis (EIC = 343 *m*/*z*) of the $[M + Na]^+$ adduct of the talaronoid C standard **15** compared to *A. flavipes* CNL-338-administered [1-^{13}C]acetate-labeled **8** or a DMSO control. All traces are shown on the same scale. (B) HRESIMS fragmentation pattern of the talaronoid C standard compared to the product observed after [1-^{13}C]acetate-labeled **8** was administered to *A. flavipes* CNL-338.

ring system and demonstrated that a single enzyme was responsible for the synthesis of this complex hydrocarbon scaffold. ^{13}C-Labeling studies helped elucidate a possible cyclization mechanism that would convert geranylgeranyl diphosphate to the 5−8−6 tricyclic hydrocarbon skeleton, and stable tracer isotope experiments validated **8** as an intermediate in talaronoid biosynthesis. Our work thus brought to light the product of a cryptic terpene biosynthetic cluster, and information gleaned from the characterization of TndC can assist with future genome mining predictions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.2c02904.

Experimental details and NMR spectroscopic data (PDF)

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Author Contributions

P.Z. and G.W. contributed equally. P.Z, G. W., and J.M.W. designed the project. All authors carried out experiments, analyzed data, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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