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Identification of Ophiobolin F Synthase by a Genome Mining Approach: A Sesterterpene Synthase from *Aspergillus clavatus*

Ryota Chiba,[†] Atsushi Minami,[†] Katsuya Gomi,[‡] and Hideaki Oikawa*,[†]

Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan, and Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan

hoik@sci.chem.hokudai.ac.jp

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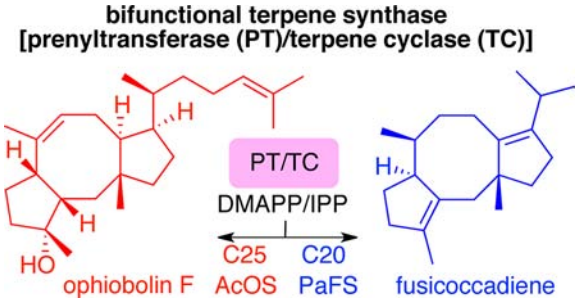
ABSTRACT



During a screening of putative diterpene synthase genes found in public databases using the *Aspergillus oryzae* expression system, it was found that a single transformant with the ACLA_76850 gene from *A. clavatus* produced a sesterterpene alcohol, ophiobolin F, and three minor sesterterpene hydrocarbons. The sesterterpene synthase has two catalytically independent domains (prenyltransferase/terpene cyclase) which are homologous to those of diterpene synthase, fusicoccadiene synthase. Coevolution of both domains and reaction mechanisms of these terpene synthases are discussed.

Fungal genomic analysis showed the presence of numerous gene clusters for biosynthesis of secondary metabolites, which are known as useful drug lead compounds.[1] The analysis of eight known *Aspergillus* genomes revealed the presence of 30−40 gene clusters in individual species, most of which are not expressed.[2,3] To date, various approaches, including promoter exchange, transcriptional regulator overexpression, and chemical epigenetics, have been applied to identify novel products of these cryptic gene clusters.[2] Alternatively, the Fujii−Ebizuka group introduced a powerful method, heterologous expression of backbone synthetic genes with the *Aspergillus oryzae* expression system.[4] This reliable system enabled them to produce a number of polyketides using plasmids harboring genes from genomic DNA.[5] This approach was further extended to the partial or total reconstitution of the biosynthetic machinery of natural products, including meroterpenoids (pyripyropene,[6] teretonin[7]), the PKS-NRPS-derived metabolite (tenellin[8]), and diterpene (aphidicolin[9]).

[†] Hokkaido University.
[‡] Tohoku University.

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Recent genomic analysis suggested that the number of biosynthetic genes for fungal metabolites is frequently less than that for metabolites from actinomycetes. Therefore, a reconstitution approach may be applicable to the genome mining of fungal metabolites.

In 2007, Sassa and co-workers reported fusicoccadiene synthase (PaFS),[10] which is an unusual bifunctional class-I

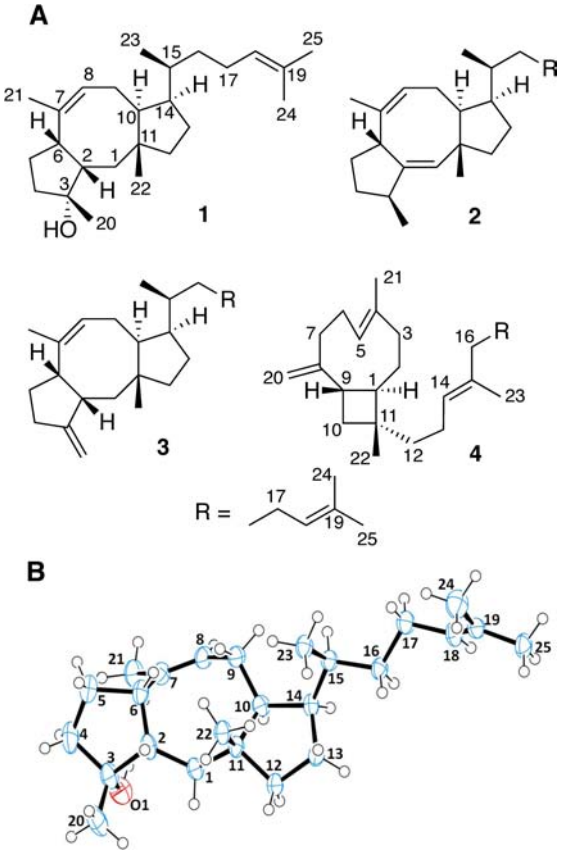

Figure 1. (A) Structures of sesterterpenes **1**–**4** isolated in this study; (B) ORTEP drawing of ophiobolin F (**1**).

diterpene synthase[11] possessing prenyltransferase (PT) and terpene cyclase (TC) domains. As the PaFS-like diterpene synthases have a characteristic sequence conservation for PT/TC domains, they can be easily identified in public databases using bioinformatics analysis. Recently, we successfully applied the *A. oryzae* expression system for functional analysis of diterpene synthase and for total biosynthesis of diterpene aphidicolin (yield 130 mg/L equivalent).[9] For extension of this system, we performed genome mining for novel diterpenes. Here, we used a genome mining approach, identified the first sesterterpene synthase, *A. clavatus ophiobolin F synthase (AcOS)*, and discuss the evolutionary relationships between prenyltransferase and terpene cyclase domains between PaFS and AcOS.

To find candidates for the genome mining of diterpenes, we carried out a Blast search to screen for geranylgeranyltransferase-diterpene cyclase hybrid genes using the PaFS gene as a query sequence. After preparing expression plasmids for putative bifunctional class-I diterpene synthase genes using the pTAex3 vector, the resultant plasmids were transformed into the auxotrophic mutant *A. oryzae* NSAR-1[12] as in the case of diterpene aphidicolin. During our screening of the transformants, we found that a single transformant with the ACLA_76850 gene from *Aspergillus clavatus* NRRL 1 produced novel terpenes.

The ACLA_76850 transformant was initially cultured in CD medium supplemented with maltose to induce target gene expression.[9] GC-MS analysis of the partially purified fraction from the mycelial extracts showed new peaks that were not found in a control culture of the wild-type strain (Figures S1, S2). Surprisingly, molecular ion peaks of all products in the MS spectra were not observed at either m/z 272 or 290, characteristic of diterpenes, but at m/z 340 and 358, corresponding to those of sesterterpene hydrocarbons and alcohols. To determine their structures, the mycelial acetone extract of the *A. oryzae* NSAR1 transformant obtained from large-scale incubation using solid medium was partitioned with hexane–acetonitrile. The resultant hexane extract was purified by silica gel column chromatography and by reversed-phase HPLC to give the major product **1** (124 mg/kg medium) and minor products **2**–**4** (less than 0.3 mg/kg medium).

HR-EIMS of the isolated alcohol **1** indicated a molecular formula of $C_{25}H_{42}O$ (unsaturation: 5). NMR spectral data indicated the presence of two double bonds (Table S1), and the degree of unsaturation indicated that this alcohol had a tricyclic system. A literature search on the natural sesterterpene alcohols reported allowed us to identify ophiobolin F, which has the 5–8–5-membered tricyclic fused structure. The ^{1}H NMR and MS spectral data, optical rotation, and melting point of **1** were identical to those reported for ophiobolin F.[13] Further confirmation of the structure of **1** was obtained by X-ray crystallographic analysis (Figure 1).

Repeated chromatography led to the isolation of three minor C25 hydrocarbons, **2**, **3**, and **4**. Among these compounds, ^{1}H and ^{13}C NMR spectral data of **2** and **3** were closely related to those of **1** (Table S1). In place of the methyl and tertiary alcohol in **1**, **2** shows a new doublet methyl signal (1.14 ppm) while **3** has signals (4.83, 4.87 ppm) indicating an exomethylene. 2D NMR analyses allowed us to determine the planar structures and relative configurations of two minor ophiobolanes, as shown in Figure 1. The structure of **3** was further confirmed by dehydration of **1** with the Burgess reagent[14] to give **3** (*exo*)

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and its regioisomer (*endo*). Isolation of sesterterpene **1** as a predominant product indicated that the ACLA_76850 gene in the *A. clavatus* genome encodes ophiobolin F synthase (AcOS).

The third component, **4**, named clavaphyllene, was structurally distinct from the ophiobolanes **1**−**3**. The HR-EIMS of the isolated hydrocarbon had a molecular formula of $C_{25}H_{40}$ (unsaturation: 6). Extensive NMR data analysis, including COSY, HMQC, and HMBC, showed that **4** had a geranyl chain, and the remaining sesquiterpene bicyclic core with two double bonds and its ^{1}H and ^{13}C NMR spectra were very similar to those of β-caryophyllene[15] (Table S2). All NMR data and the apparent NOEs supported this planar structure and revealed the relative configuration of **4** (Figures 1and S3). The structurally related diterpene alcohol xeniaphyllenol was isolated from a soft coral.[16]

Using ophiobolin F synthase cDNA obtained from the *A. oryzae* transformant, *AcOS* was cloned into the pCold I vector and expressed as an *N*-terminal His_6-tagged fusion protein in *Escherichia coli*. Recombinant AcOS was purified by Ni-NTA column chromatography (Figure S4). As the bifunctional diterpene synthase PaFS, AcOS contains two putative domains, i.e., a terpene cyclase domain at the N-terminus and a geranylfarnesyl diphosphate (GFPP) synthase domain at the C-terminus (Figure S5). To examine the catalytic activity of AcOS, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) with AcOS were incubated in the presence of Mg^{2+}. Subsequent GC-MS analysis revealed a single peak of **1**. As expected, this enzyme accepted geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) as starter units as in the case of PaFS.

The AcOS reaction strongly suggested that the PT domain produced GFPP as the predominant product. We were interested in the catalytic activity of the TC domain and whether this domain accepts GGPP to afford diterpene product because the *A. oryzae* transformant produced a small amount of diterpene hydrocarbons (Figure S2). GC-MS analysis of the products obtained by incubation of GGPP as a single substrate with AcOS gave a trace amount of the diterpene, which was identical to those from the *A. oryzae* transformant. Its extremely low abundance hampered structure determination of the diterpene. When we carried out incubation of GFPP with PaFS, no product was obtained. These experiments suggested that the TC domains of the bifunctional enzymes AcOS and PaFS are rather specific in substrate recognition and product formation. We speculated on the coevolution of bifunctional PaFS-type terpene synthases. The identification of AcOS as an ortholog of the diterpene synthase PaFS implies that the PT domain of sesterterpene synthase AcOS probably originated from a bifunctional diterpene synthase by initial mutation changing the product chain length from GGPP to GFPP and that the broad substrate specificity of the ancestral TC domain may accept GFPP to give the C25 cyclization product. A second mutation at the TC domain chose only GFPP to avoid producing a mixture of diterpene and sesterterpene. Although identification of the signature sequence in the PT domain by changing the product chain length may be useful to distinguish between diterpene and sesterterpene synthases, our preliminary efforts using site-directed mutagenesis of the proposed residues[17] were unsuccessful.

As the first sesterterpene synthase is available and both AcOS and PaFS produce similar molecular skeletons (5−8−5 fused ring system), comparison of the cyclization mechanism may be useful. The degree of sequence identity between AcOS and PaFS (41%) is similar to that of two fungal gibberellin KS/CPS synthases (45%) from different fungal species,[18,19] suggesting that the active sites may adopt a similar carbocationic intermediate. Based on the results of feeding experiments and enzymatic reactions with specific isotope-labeled precursors, the detailed cyclization mechanisms for ophiobolin synthase reactions[20]and fusicoccadiene synthase reactions[21] have been proposed, as shown in Scheme 1.

As in the case of the other terpene synthases,[22,23] the active sites of both enzymes accept the linear chain substrate (GGPP or GFPP) as a similar preorganized conformation from which the initial C1−C11 cyclization followed by the second C10−C14 cyclization may proceed to give the dolabelladien-15-yl cation ($\mathbf{5}^+$) via 12-geranylhumulen-9-yl cation $\mathbf{6}^+$. The subsequent hydride shift from $\mathbf{5}^+$ determines the two cyclization pathways; AcOS chooses the 1,5-H shift (C8−C15), while PaFS chooses the 1,2-H shift (C14−C15), which requires counterclockwise rotation of the C14−C15 bond for smooth interaction between the C14−H σ bond and the C15 carbocation p-orbital derived from C10−C14 cyclization (Scheme 1, Figure S6). For some reason (possibly steric hindrance of the extra C5 unit), AcOS prefers the 1,5-H shift from the conformation allowing interaction between the p-orbital of the C15 carbocation of $\mathbf{5}^+$ and Hα-8, while the active site of PaFS may allow the corresponding rotation for the 1,2-H shift (Figure S6). In AcOS cyclization, formation of **4**[24] via $\mathbf{6}^+$ and configurations of the ophiobolane A−B ring

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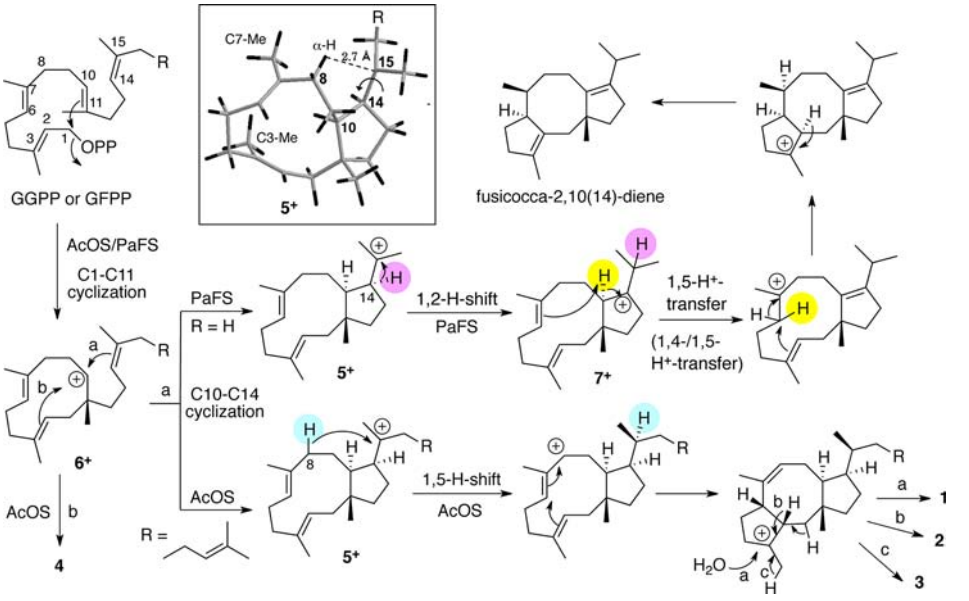
Scheme 1. Proposed Mechanism of Bifunctional Class-I Diterpene Synthases AcOS and PaFS[a]



[a] The conformation of carbocation intermediate $\mathbf{5}^+$ for the 1,5-H shift from C8 to C15 is shown within the square. The distance between proximate 8H and C15 in the carbocation is shown.

juncture (C2 and C6) provided information on the pre-organized conformation (C3-Me down, C7-Me down) of $\mathbf{5}^+$ (Scheme 1). On the other hand, an alternative conformation (C3-Me up, C7-Me up) for dolabelladien-14-yl cation $\mathbf{7}^+$ was proposed in the PaFS cyclization mechanism.[21] Therefore, product switching between AcOS and PaFS reactions is likely to be achieved by stabilizing the distinct conformation and controlling initial hydride shift modes with or without the sterically demanding additional C5-unit.

In conclusion, we described the identification and functional analysis of the first sesterterpene synthase, AcOS, by a genome mining approach with the *A. oryzae* expression system to produce sesterterpene ophiobolin F while screening for "diterpene synthases." This unexpected observation indicated that compulsory expression of bifunctional class-I PaFS-like terpene synthase genes results in identification of both di- and sesterterpene synthases, which may be distinguished by characteristic motifs for size determination (C20/C25) in the corresponding PT domain. The recent success in the reconstitution[6–9] of the biosynthetic machinery of natural products using genomic data provides further options for this genome mining approach to supply bioactive metabolites. We are currently engaged in studies for the total biosynthesis of more elaborate ophiobolins using genes adjacent to *AcOS*.

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Supporting Information Available. Experimental procedures, GC-MS data, CIF file for ophiobolin F, and ^{1}H and ^{13}C NMR spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.