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Genome Mining for Sesterterpenes Using Bifunctional Terpene Synthases Reveals a Unified Intermediate of Di/Sesterterpenes

Ying Ye,[†] Atsushi Minami,[†] Attila Mandi,[‡] Chengwei Liu,[†] Tohru Taniguchi,[‡] Tomohisa Kuzuyama,[§] Kenji Monde,[‡] Katsuya Gomi,[∥] and Hideaki Oikawa*[,†]

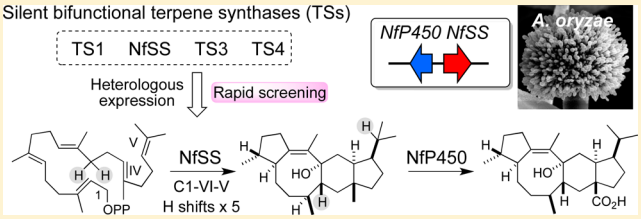
[†]Division of Chemistry, Graduate School of Science, and [‡]Faculty of Advanced Life Science, Frontier Research Center for Post-Genome Science and Technology, Hokkaido University, Sapporo 060-0810, Japan

[§]Biotechnology Research Center, The University of Tokyo, Tokyo 113-8657, Japan

[∥]Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan

Supporting Information

ABSTRACT: Genome mining is a promising method to discover novel secondary metabolites in the postgenomic era. We applied the *Aspergillus oryzae* heterologous expression system to functionally characterize cryptic bifunctional terpene synthase genes found in fungal genomes and identified the sesterfisherol synthase gene (*NfSS*) from *Neosartorya fischeri*. Sesterfisherol contains a characteristic 5−6−8−5 tetracyclic ring system and is modified by cytochrome P450 monooxygenase (NfP450) to sesterfisheric acid. The cyclization mechanism was proposed on the basis of the analysis of in vivo and in vitro enzymatic reactions with isotopically labeled precursors. The mechanism involves C1 cation−olefin IV−olefin V cyclization followed by five hydride shifts, allowing us to propose a unified biogenesis for sesterterpenes branching from bicyclic (5−15), tricyclic (5−12−5), and tetracyclic (5−6−8−5) cation intermediates. Furthermore, the mechanism is distinct from that of a separate class of di/sesterterpenes including fusicoccins and ophiobolins. The difference between mechanisms is consistent with phylogenetic analysis of bifunctional terpene synthases, suggesting that the amino acid sequence reflects the initial cyclization mode, which is most likely related to the initial conformation of a linear prenyl diphosphate.



INTRODUCTION

Advances in genome sequencing technology and development of bioinformatics tools such as SMURF,[1] antiSMASH,[2] and 2ndFind[3] revealed a large number of biosynthetic gene clusters of natural products in fungi. For instance, 40−80 such clusters were found in each of the eight known species of *Aspergillus*,[4,5] most of which are not expressed in standard fermentation conditions. Expression of these silent genes enhances opportunities to find novel natural products. Indeed, various approaches such as promoter exchange,[6] epigenetic regulation,[7] and activation of cluster-specific transcription factors[8] have been used to artificially control expression in the source organism. A reconstitution strategy utilizing *Aspergillus oryzae* as a host strain also emerges as a method for the biosynthetic study of fungal natural products. This strategy has been applied to elucidate the biosynthetic machinery of polyketides,[9,10] terpenes,[11] indolediterpenes,[12,13] and other meroterpenoids.[14,15] Reconstitution of the entire penitrem biosynthetic pathway involving 17 genes shows the reliability of the *A. oryzae* expression system for studying the biosynthesis of fungal natural products.[16] Most notably, the heterologous expression system does not require isolation and characterization of the source organism, thus enabling a sequence-oriented search of natural products. Rapid progress of DNA synthetic technologies would strongly support this approach. Additionally, we need no special knowledge of the complicated signal transduction. Therefore, the *A. oryzae* expression system with reliable promoters such as P_{amyB}[17] is a powerful method for genome mining in the postgenomic era.

Among various terpene cyclases, bifunctional terpene synthases, including fusicoccadiene synthase (PaFS)[18] and phomopsene synthase (PaPS),[19] have a distinct domain organization composed of a C-terminal prenyltransferase domain and an N-terminal class I terpene cyclase domain[20] that catalyzes cyclization via a cation intermediate derived from cleavage of pyrophosphate, allowing us to find a number of homologues in the fungal genome deposited in a public database. The putative terpene synthases are potential candidates involved in the biosynthesis of novel terpenoids. Recently, we applied the *A. oryzae* expression system to characterize those terpene synthases and identified the first sesterterpene synthase (AcOS), which mediates geranylfarnesyl diphosphate (GFPP) synthesis followed by its cyclization to afford ophiobolin F with a characteristic 5−8−5 tricyclic ring system (Scheme S1).[21] The successful identification prompted us to continue this genome mining approach for novel di/sesterterpenes.

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Here, we describe the identification and characterization of bifunctional sesterterpene synthase (*NfSS*) and cytochrome P450 monooxygenase (*NfP450*), which participate in the biosynthesis of sesterfisherol (**1**) and sesterfisheric acid (**2**), respectively (Figure 1). We elucidated the cyclization

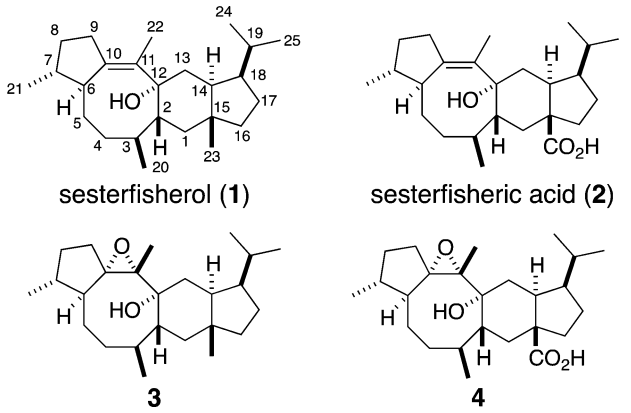

Figure 1. Chemical structures of sesterfisherol (**1**) and sesterfisheric acid (**2**) and their derivatives (**3**, **4**).

mechanism for the characteristic 5−6−8−5 ring system by in vivo and in vitro functional analysis of NfSS using isotopically labeled substrates. On the basis of this mechanism, we propose a unified biosynthetic pathway of structurally related sesterterpenes in fungi and plants.

RESULTS AND DISCUSSION

Genome Mining for Terpene Synthases. A phylogenetic analysis of N-terminal terpene cyclase domains in 15 bifunctional terpene synthases found in five fungal genomes suggested that these are separated into five clades (clades A−E, Figure S1). Intriguingly, four functionally characterized terpene synthases (PaFS,[18] AbFS,[22] PaPS,[19] and AcOS[21]) fall into clade B. Assuming that the amino acid sequence reflects the cyclization mechanism, terpene synthases in all other clades may potentially synthesize di- or sesterterpenes with new carbon skeletons. Additionally, to our knowledge, no terpenoids have been isolated from *Neosartorya fischeri* and *A. oryzae*, suggesting that terpene synthase genes in these species are silent in standard fermentation conditions. Accordingly, the terpene synthase genes {NFIA_41470 (NfTS1), NFIA_55500 (NfTS2), and NFIA_62390 (NfTS3) from *N. fischeri*; AOR_1_1216074 (AoTS1) from *A. oryzae*} were selected as targets for heterologous expression in *A. oryzae*.

For rapid functional analysis of these terpene synthases, we constructed an *A. oryzae* transformant expressing all four genes by the tandem transformation method[14] using two plasmids, pUARA2-NfTS1/NfTS3 and pUARA2-NfTS2/AoTS1. GC−MS analysis of the extract from the resultant quadruple transformant showed production of a new metabolite with a molecular ion peak (M^+, m/z 358) corresponding to the sesterterpene alcohol **1** (Figure 2, Figure S2A). The MS fragmentation pattern was different from that of ophiobolin F. A double transformant (pUARA2-NfTS2/AoTS1) also produced **1** at 52.5 mg/kg of rice medium, while a single transformant (pUARA2-AoTS1) did not (Figure 2). Taken together, we speculated that NFIA_55500 (NfTS2) mediates construction of **1**.

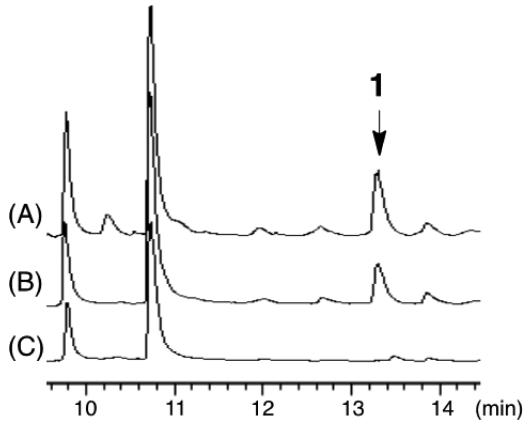

Figure 2. GC−MS profiles of metabolites produced by (A) *A. oryzae* expressing NfTS2 and AoTS1, (B) *A. oryzae* expressing NfTS1, NfTS2, NfTS3, and AoTS1, and (C) *A. oryzae* NSAR1.

HR-MS analysis of **1** indicated a molecular formula of $C_{25}H_{42}O$ (unsaturation 5). ^1H and ^{13}C NMR spectra showed the presence of one double bond and one oxygenated quaternary carbon, and the degree of unsaturation indicated that this alcohol contains a tetracyclic ring system. However, broadening of ^{13}C NMR signals owing to slow conformational changes around the eight-membered ring prevented complete signal assignment. A similar observation was reported in NMR analysis of ciguatoxin, which harbors a fused ring system that includes a medium-sized ring.[23] Subsequently, the highly strained nature of **1** was soon realized by the fact that **1** in hexane solution was slowly converted into the corresponding epoxide **3** at room temperature for several days. The same epoxide **3** was obtained as a single diastereomer by treatment of **1** with *m*-chloroperoxybenzoic acid for <1 min at 0 °C.

Fortunately, **3** showed sharp signals in the NMR spectra, enabling us to determine the planar structure (Table S1). Detailed analysis of the NOESY spectrum revealed the relative stereochemistry of **3** except at C7 (Figure S3). The stereochemistry at C7 was determined by NOESY analysis of **4**, which is the oxidation product of **3** described later. This analysis indicates that the C21 methyl group and H6 lie on the same face of the molecule (Figure S3). The absolute stereochemistry of **3** was determined by the vibrational circular dichroism (VCD) technique in conjunction with density functional theory (DFT) calculations,[24] which revealed five low-energy conformers (Figure S4). The Boltzmann-averaged VCD spectrum has a good agreement with the experimental spectrum (Figure 3), indicating that the absolute stereochemistry of **3** was 2*R*, 3*S*, 6*S*, 7*R*, 10*S*, 11*R*, 12*R*, 14*S*, 15*R*, and 18*R*. The elucidated structure of **1**, named as sesterfisherol, is closely related to that of nitidasin[25] except for the oxidation state and the C7 stereochemistry. In addition to the successful formation of **1** in the reaction with the recombinant terpene synthase from the NFIA_55500 gene described later, all experimental results showed that this gene encodes a sesterfisherol synthase, which was thus named *N. fischeri* sesterfisherol synthase (NfSS).

Identification of a Sesterfisheric Acid Biosynthetic Gene Cluster. Database analysis shows that *NfSS* is encoded downstream of a cytochrome P450 monooxygenase (NFIA_55490) and no other modification enzymes can be found around the *NfSS* gene. Thus, the NFIA_55490 gene product appears to be a sole candidate to modify **1**. Since a

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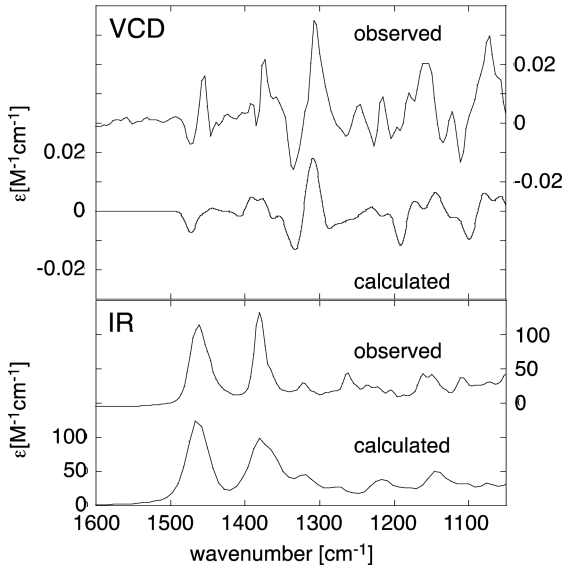

Figure 3. Vibrational circular dichroism and infrared spectra of **3** measured in $CDCl_3$ (c = 0.2 M, l = 100 mm) and calculated by B3LYP/TZVP with the polarized continuum model (PCM) for $CHCl_3$.

bioinformatics search of the deposited sequence on the TMHMM server[26] showed that the gene product lacks an N-terminal membrane-binding region, which is essential for localization to the endoplasmic reticulum membrane,[27] we carefully examined the upstream region of NFIA_55490 and found a new start codon 154 bp upstream (Figure S5).

To characterize NfP450, a double transformant with the *NfSS* and *NfP450* genes was prepared. LC−MS analysis showed that the transformant produced **2** at 20 mg/kg with m/z 387 ($[M - H]^-$) (Figure 4, Figure S2B). The molecular

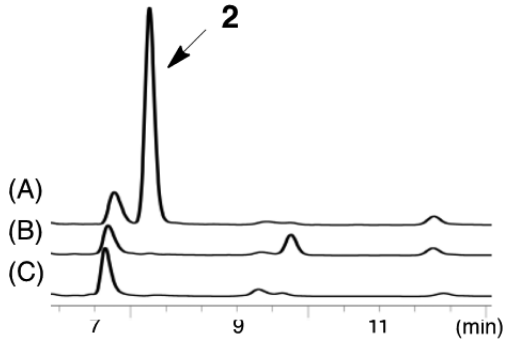

Figure 4. HPLC profiles of metabolites produced by the transformants: (A) *AO*-NfSS/NfP450, (B) *AO*-NfSS, and (C) *A. oryzae* NSAR1.

formula of **2** was determined by HR-MS analysis to be $C_{25}H_{40}O_3$. As in the case of **1**, several missing signals of **2** became visible in the corresponding epoxide **4** (Table S2), which enabled determination of its structure. The results indicated that NfP450 catalyzes oxidative modifications of **1** to **2**, which is named as sesterfisheric acid.

Diterpenes and sesterterpenes such as **2**, which have rather simple functionalities such as hydroxyl, carbonyl, and carboxylic acid, are frequently found in lichen, ascomycete, and basidiomycete fungi as shown in Scheme S2. The physiological roles of these metabolites are currently unknown, but may include switching of life cycles, as in the case of conidiogenone, a potent inducer of conidiogenesis in *Penicillium cyclopium*.[28]

Cyclization Mechanism Catalyzed by NfSS. The C1−C19 carbon framework of **1** is essentially the same as that in GFPP, including the positions of the branching methyl groups. This implies that cyclization of GFPP into **1** involves no backbone rearrangements but does a series of hydride shifts. As exemplified in the biosynthetic study of structurally complex steroids, labeling with [1-^{13}C,$^{2}H_3$]acetate provided important evidence on the hydride shifts.[29] Therefore, in vivo isotopic labeling experiments with [1-^{13}C,$^{2}H_3$]acetate were then conducted. The ^{13}C NMR spectrum of labeled **3**, prepared from labeled **1**, showed that the ^{13}C-labeling pattern is identical to that of GFPP biosynthesized via the mevalonate pathway, indicating no backbone rearrangements during cyclization. Of ten ^{13}C signals labeled, seven carbon signals accompanied by upfield-shifted signals were observed at C3 (number of upfield-shifted signals 3), C7 (3), C11 (3), C13 (1), C15 (3), C17 (1), and C19 (3) (Table S3). In contrast, no upfield-shifted signals at C1, C5, and C9 were observed (Table S3), suggesting that deuterium labels at C2, C6, and C10 (H2, H6, and H10) were lost by hydride shifts. In addition, a 1,5-hydride shift at C12 may be involved early in cyclization (Scheme 1), as observed in the cyclization of ophioblin F[30] (Scheme S1).

To examine the hydride transfers at C12, we then prepared a recombinant NfSS protein fused to the maltose binding protein in *Escherichia coli* for in vitro enzymatic reaction (Figure S6). When NfSS was incubated with geranylgeranyl diphosphate (GGPP) and isopentenyl diphosphate (IPP), a single peak corresponding to **1** was observed by GC−MS analysis (Figure S7), confirming that recombinant NfSS mediates construction of **1** via GFPP. In addition, the prenyltransferase domain in NfSS was also capable of using dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), and farnesyl diphosphate (FPP) as starter units (Figure S7). With the active NfSS recombinant protein in hand, we then carried out a large-scale enzymatic reaction with (8,8-$^{2}H_2$)GGPP. In the ^{1}H NMR spectrum of the corresponding epoxide **3**, the characteristic doublet signals due to H24 and H25 methyl groups in nonlabeled **3** were changed to a pair of singlets in labeled **3**, suggesting a 1,5-H shift from C12 to C19. This was further supported by the disappearance of the correlation peaks of H24 and H25 in TOCSY analysis (Figure 5 A). Similarly, the correlation peak between H1-a and H3 disappeared (Figure 5B), suggesting a 1,2-H shift from C12 to C2.

Considering the experimental results shown above and related AcOS[21,30] and CotB2[31] catalyzing cyclizations involving a similar 1,5-H shift, a cyclization mechanism for NfSS is proposed as shown in Scheme 1. Initiated by the elimination of a pyrophosphate group of GFPP, two successive cyclizations at C1−C15 and C14−C18 give the bicyclic cation **5** with a 5−15 fused ring system. A subsequent 1,5-H shift from C12 to C19 affords fusaproliferan-12-yl cation **6**. A second C6−C10 cyclization yields 6-*epi*-variculane-7-yl cation **7a** with a tricyclic system. Sequential 1,2-H shifts, followed by a third C2−C12 cyclization, give the tetracyclic cation **8**, which undergoes another round of sequential 1,2-H shifts (C2 to C3 and C12 to C2) followed by quenching of sesterfisheran-12-yl cation **9** with water to furnish **1** (Scheme 1 B). Thus, five hydride shifts, including unusual shifts of both C12-methylene hydrogens, are involved in the NfSS-catalyzed cyclization to construct the elaborated fused ring system in **1**.

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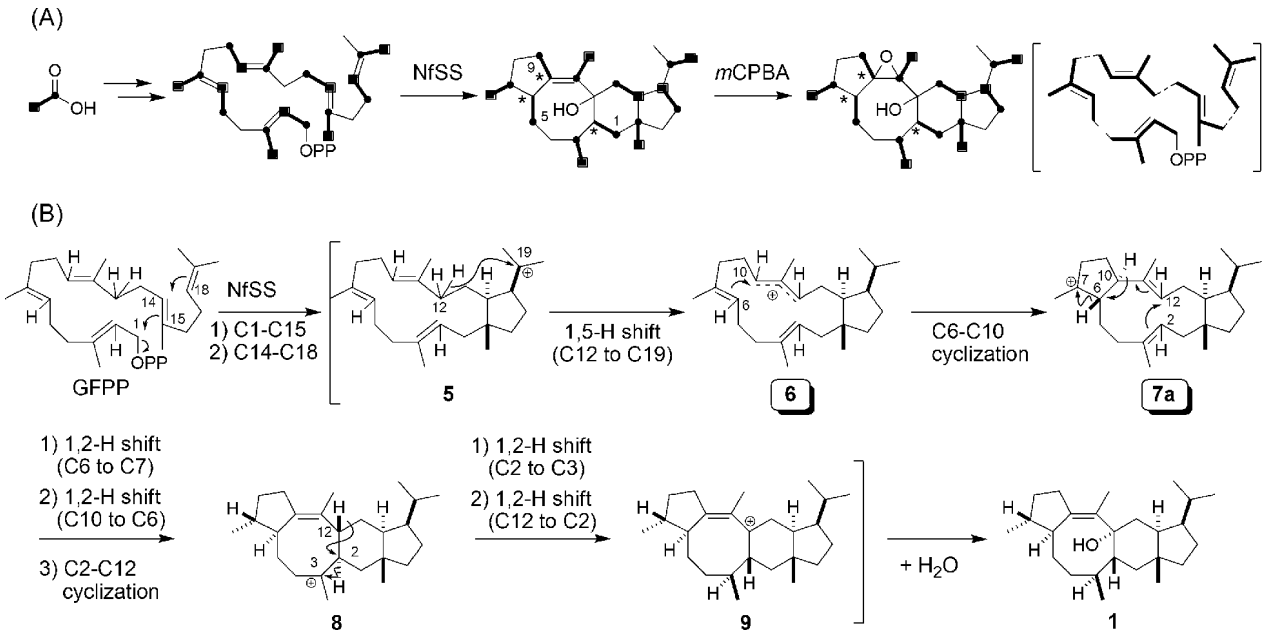
Scheme 1. (A) Summary of Feeding Experiments with [1-^{13}C,$^{2}H_3$]Acetate[a] and (B) Proposed Cyclization Mechanism Catalyzed by NfSS



[a]Bold lines indicate acetate units. Black circles and white squares show carbon-13 and deuterium atoms, respectively. Asterisks mark carbons from which deuterium labels were lost. The structure in parentheses shows isoprene units derived from mevalonate. Due to ^{2}H washing out during interconversion of acetyl-CoA and malonyl-CoA, the shifted signals derived from the methyl group of GFPP varied among 1−3.

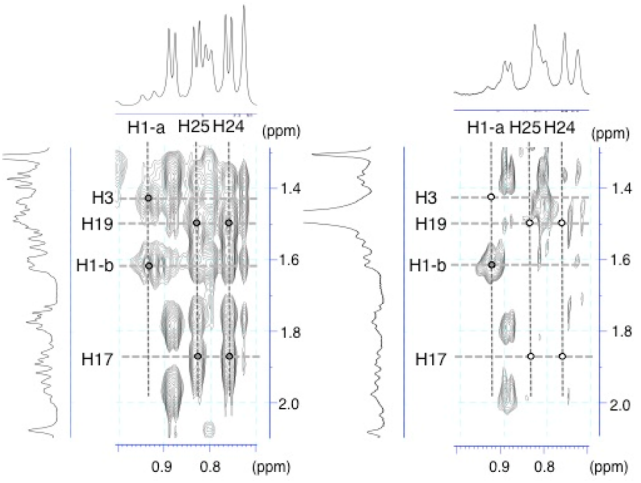


Figure 5. TOCSY spectra of (A, left) unlabeled and (B, right) deuterium-labeled **3**.

Unified Biogenesis of Sesterterpenes. Biosynthetic studies on fusicoccin A[18] (C20) and ophiobolin A[21]/mangicol A[32]/variecolin[33] (C25) suggested that these metabolites are biosynthesized via dolabelladien-15-yl cation (C20) or the corresponding C25 cation intermediate. These cationic intermediates are commonly constructed by the cyclization with C1 cation−olefin III−olefin IV of the corresponding polyprenyl diphosphates (GGPP/GFPP), which is defined as type B cyclization. Including structurally related methyl phomopsenate (C20),[19] we classified these di/sesterterpenes as group B (Scheme 2, Scheme S1). On the other hand, the cyclization mechanism of **1** suggested that an alternative cyclization with C1 cation−olefin IV−olefin V of GFPP, termed as type A cyclization, is involved in the biosynthesis of structurally related sesterterpenes such as bicyclic fusaproliferin[34] (**10**), tricyclic variculanol[35] (**11**), tetracyclic astellatol[36] (**12**) and aleurodiscal[37] (**14**), pentacyclic retigeranic acid[38] (**13**), and others (alborosin,[39] nitidasin,[25,40] nitiol,[41] YW3548,[42] and YW3699[43]), which are classified into group A sesterterpenes (Schemes 2 and 3, Scheme S2).

On the basis of the type A cyclization mechanism elucidated in the NfSS reaction, we now propose a unified biosynthetic pathway for all sesterterpenes in group A (Scheme 3, Scheme S2). As described, initial cyclization of GFPP gives fusaproliferan-12-yl cation **6**, quenching of which with water provides **10**. Alternatively, C6−C10 cyclization gives variculan-7-yl cation **7b**, which is deprotonated to **11**. The tricyclic ring system **7b** and the enantiomeric form of **7a**/**7b** may also be converted into various products such as **12**, alborosin, YW3548/YW3699, or nitidasin/nitiol (Scheme 3, Scheme S2). Furthermore, the structurally similar **7c** may be used to generate the tetra- and pentacyclic products **13** and **14**, respectively (Scheme 3). On the basis of the involvement of key cationic intermediates **6**, **7**, and related cations for biogenesis of group A sesterterpenes, NfSS can be regarded as a model terpene synthase.

Of particular interest is that cyclization-based classification of di/sesterterpenes closely reflects the phylogenetic relationships among bifunctional terpene synthases. NfSS is a clade A enzyme, while the type B di/sesterterpene synthases, PaFS, AbFS, PaPS, and AcOS, belong to clade B. The correlation suggests that the amino acid sequence of the terpene cyclase domain may reflect the first cyclization mode (C1−IV−V vs C1−III−IV), which most likely depends on the initial conformation of the linear prenyl diphosphates GGPP and GFPP. Although this hypothesis requires experimental support, the data imply that phylogenetic analysis is a valuable roadmap to characterize new di/sesterterpene synthases by genome mining.

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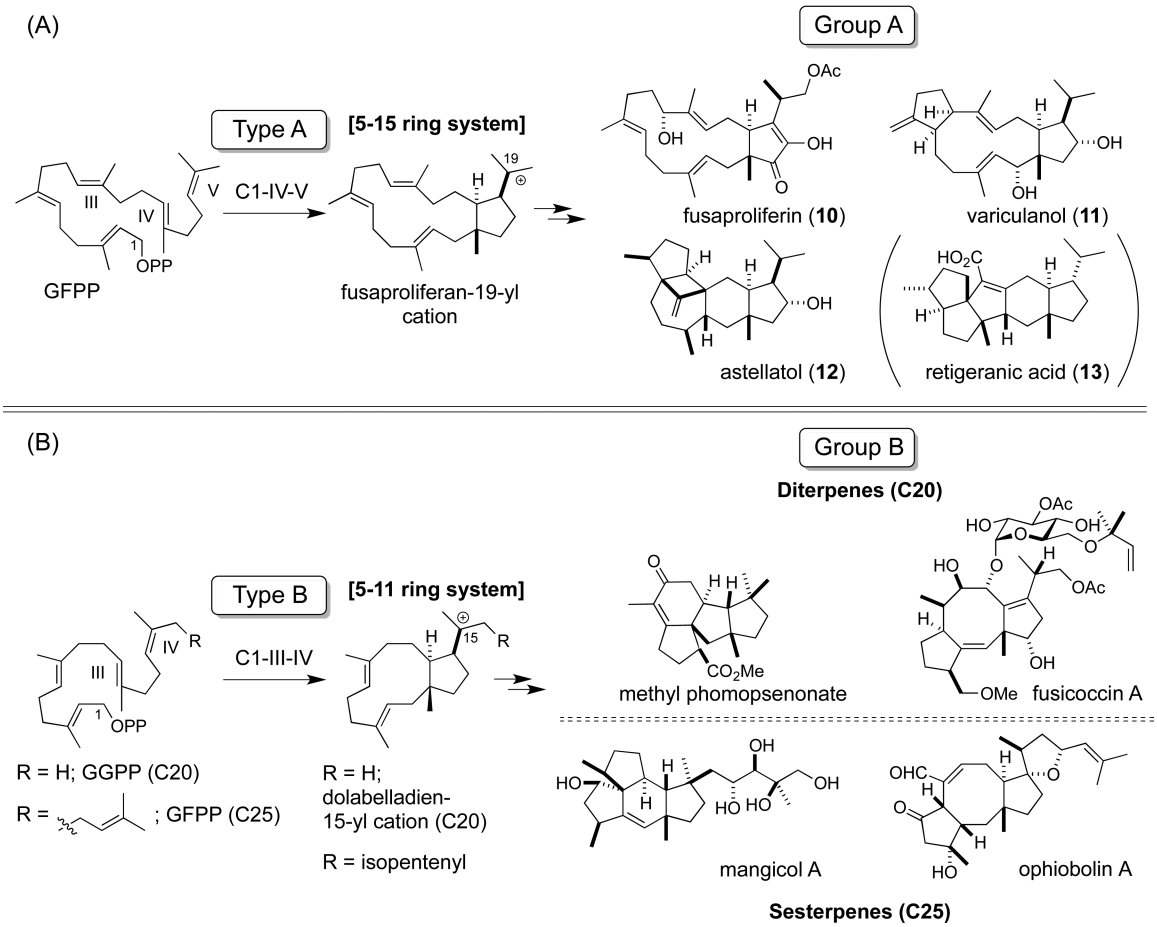
Scheme 2. Classification of Di/Sesterterpenes Based on Initial Cyclization: (A) Group A and (B) Group B Di/Sesterterpenes



CONCLUSIONS

Genome mining is a promising method to discover novel natural products. Among several strategies for genome mining, heterologous expression can be a powerful method that allows a sequence-oriented search of novel natural products. In this study, we successfully developed a rapid screening system for cryptic macrocyclic di/sesterterpene synthases using the reliable *A. oryzae* expression system. This system enabled us to isolate sesterfisherol and sesterfisheric acid by expressing a small gene cluster consisting of a terpene synthase (*NfSS*) and a cytochrome P450 monooxygenase (*NfP450*). In vivo and in vitro analyses of the bifunctional sesterterpene synthase NfSS with isotopically labeled precursors establish an intriguing cyclization mechanism, in which five hydride shifts are required to form a 5−6−8−5 tetracyclic system without any C−C bond rearrangement. On the basis of this mechanism, we proposed a unified biogenesis for group A sesterterpenes from bicyclic (5−15), tricyclic (5−12−5), and tetracyclic (5−6−8−5) cation intermediates.

EXPERIMENTAL PROCEDURES

Strain and Culture Conditions. *E.* HST08 and *E. coli* DH5α were used for cloning, following standard recombinant DNA techniques. *E. coli* BL21-Gold(DE3) was used for protein expression. A fungal host strain used in this study was *A. oryzae* NSAR1, a quadruple auxotrophic mutant (*niaD*$^{-}$, *sC*$^{-}$, Δ*argB*, *adeA*$^{-}$) for fungal expression.

Preparation of Expression Plasmids. The NFIA_041470, NfP450, NFIA_055490, NfSS, NFIA_062390, and AOR_1_1216074 were amplified from genomic DNA with a primer set as shown in Table S3. Polymerase chain reactions (PCRs) were performed with the KOD-Plus-Neo (TOYOBO). Each PCR product was inserted into the appropriate restriction site (site 1 and/or site 2) of pUARA2 or pUSA2 using the In-Fusion Advantage PCR cloning kit (Clontech Laboratories) to construct expression plasmids.

Transformation of *A. oryzae*. Transformation of *A. oryzae* NSAR1 or the transformant (1.0×10^8 cells) was performed by the protoplast−polyethylene glycol method reported previously.[11] To construct transformants harboring terpene synthase genes, three plasmids, pUARA2-*NfTS1*/*NfTS3*, pUARA2-*NfTS2*/*AoTS1*, and pUARA2-*AoTS1*, were used for the transformation. The transformant with *NfSS* was further transformed with pUSA2-*NfP450* to construct *AO*-NfSS/NfP450.

Extraction of Metabolites. Mycelia of *A. oryzae* transformants were inoculated into a solid medium containing polished rice (100 g) and adenine (10 mg) in 500 mL Erlenmeyer flasks. Each culture was incubated at 30 °C for 2 weeks. After extraction with ethyl acetate, the extract was then concentrated in vacuo and the residues were extracted with ethyl acetate (100 mL × 2). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo to afford crude extracts.

Analysis of the Metabolites. After partial purification of the crude extracts, the metabolites were analyzed by GC−MS with a DB-1 MS capillary column (0.32 mm × 30 m, 0.25 mm film thickness; J&W Scientific) or LC−MS with a ZORBAX XDB-C18 column (50 mm × 2.1 mm) at the following conditions.

GC conditions to analyze sesterfisherol (**1**): Each sample was injected into the column at 100 °C in the splitless mode. After a 3 min isothermal hold at 100 °C, the column temperature was increased at

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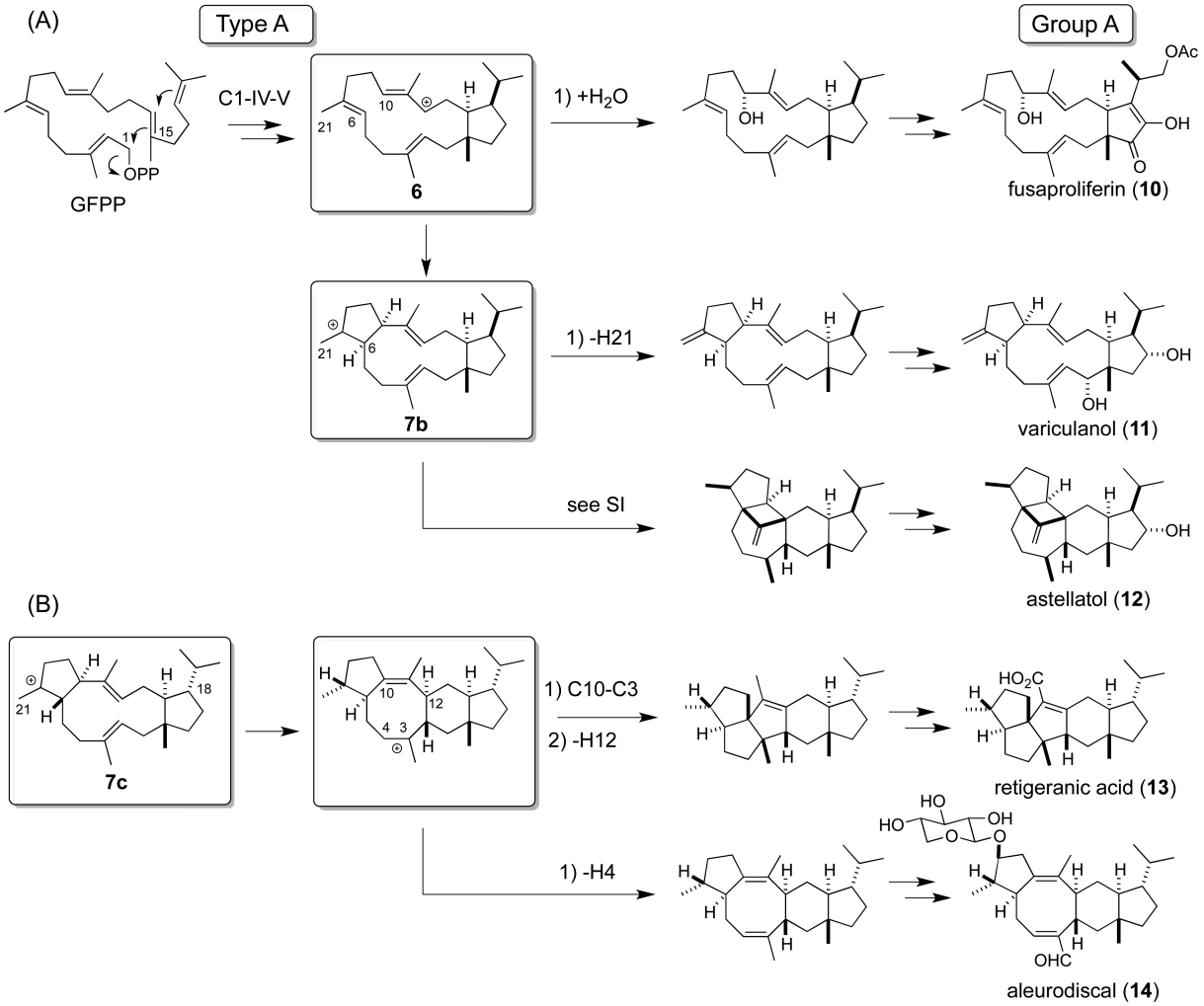
Scheme 3. Proposed Cyclization of Group I Sesterterpenes: (A) Cyclization Mechanism of 10−12 via Bicyclic Cation 6 and (B) Cyclization Mechanism of 13 and 14 via 7c



14 °C min^{-1} to 268 °C, with a 4 min isothermal hold at 268 °C. The flow rate of helium carrier gas was 0.66 mL min^{-1}.

LC conditions to analyze sesterfisheric acid (**2**): 95% acetonitrile for 15 min at a flow rate of 0.2 mL/min with 210 nm detection.

Sesterfisherol (1). Isolation yield: 52.5 mg from 1 kg of rice medium. $[\alpha]_D^{24}$ −48.3 (*c* 1.5, $CHCl_3$). HR-EIMS analysis (positive): *m*/*z* calcd for $C_{25}H_{42}O$ $[M]^+$ 358.3230, found 358.3243. ^{1}H NMR and ^{13}C NMR data are summarized in Table S1.

Sesterfisheric Acid (2). Isolation yield: 20.0 mg from 1 kg of rice medium. $[\alpha]_D^{25}$ −11.1 (*c* 0.3, C_6H_6). HR-ESIMS analysis (negative): *m*/*z* calcd for $C_{25}H_{39}O_3$ $[M - H]^-$ 387.2905, found 387.2919. ^{1}H NMR and ^{13}C NMR data are summarized in Table S2.

Epoxidation of Sesterterpene. To a solution of **1** (5.0 mg, 14.0 mmol) in CH_2Cl_2 (1.0 mL) was added *m*CPBA (4.9 mg, 28.4 mmol for **1** or 4.0 mg, 23.2 mmol for **2**) at 0 °C, and the mixture was stirred for 10 min. After addition of a satd Na_2SO_3 solution, the reaction mixture was extracted with EtOAc. The combined organic extracts were washed with satd $NaHCO_3$, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The 10,11-epoxysesterfisherol (**3**) (4.8 mg from **1**) was directly used for NMR analysis. The same procedure was applied for the epoxidation of **2** (2.4 mg, 6.2 mmol) to afford 10,11-epoxysesterfisheric acid (**4**) (2.6 mg from **2**).

10,11-Epoxysesterfisherol (3). $[\alpha]_D^{24}$ 1.3 (*c* 0.4, $CHCl_3$). HR-ESIMS analysis (positive): *m*/*z* calcd for $C_{25}H_{43}O_2$ $[M + H]^+$ 375.3258, found 375.3302. ^{1}H NMR and ^{13}C NMR data are summarized in Table S1.

10,11-Epoxysesterfisheric Acid (4). $[\alpha]_D^{26}$ 0.2 (*c* 0.4, $CHCl_3$). HR-ESIMS analysis (negative): *m*/*z* calcd for $C_{25}H_{39}O_4$ $[M - H]^-$ 403.2854, found 403.2902. ^{1}H NMR and ^{13}C NMR data are summarized in Table S2.

Spectral Measurements. IR and VCD spectra were measured on a BioTools Chiral*ir*-2X at a resolution of ca. 8 cm^{-1} under ambient temperature for 32 and 13504 scans, respectively. The sample was dissolved in $CDCl_3$ at a concentration of 0.2 M, and the solution was placed in a 100 mm CaF_2 cell. All spectral data were corrected by a solvent spectrum obtained under identical experimental conditions.

Feeding Experiments with Isotopically Labeled Acetate. Mycelia of the *A. oryzae* transformant were inoculated into MPY medium (maltose−polypeptone−yeast extract; 3% maltose, 1% polypeptone, 0.5% yeast extract; final volume 100 mL) containing 8 mg of [1-^{13}C,^{2}H$_3$]acetate in 500 mL Erlenmeyer flasks (two flasks) for 4 days at 200 rpm. After extraction with acetone, the extract was then concentrated in vacuo and the residues were extracted with ethyl acetate (100 mL × 2). The combined organic layers were washed with water, concentrated in vacuo, and partitioned between *n*-hexane and $CHCl_3$. The *n*-hexane layer was purified by silica gel chromatography (cyclohexane/$CHCl_3$ = 10/1) to give labeled **1** (3.2 mg). Epoxidation of **1** gave **3** (3.0 mg).

NfSS Cloning and Expression in *E. coli*. To generate the overexpression construct for *NfSS*, *NfSS* was amplified using the codon-optimized synthetic DNA of the *A. oryzae* transformant using the following primer sets: NfSS-F (5′-**GATGACGATGACAAG**-ATGGAAGTTTGGGAACATAG-3′; bold characters indicate the complementary sequence for the In-Fusion reaction), NfSS-R (5′-**AGGATCCGAATTCCG**TTAGTTTTCTTTAACGCTCAG-3′; bold

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characters indicate the complementary sequence for the In-Fusion reaction). The PCR products were directly inserted into the *Kpn*I-digested pMALc4E to generate pMALc4E-*NfSS*. pMalc4E-*NfSS* was separately introduced into *E. coli* BL21-Gold(DE3) for overexpression. The transformant was grown at 37 °C at an OD_{600} of ∼0.6 in a 500 mL flask. After the culture was cooled at 4 °C, isopropyl β-D-thiogalactopyranoside (0.1 mM) was added to it. After incubation at 20 °C for 18 h, the cells were harvested by centrifugation at 4000 rpm. The harvested cells were resuspended in disruption buffer (50 mM Tris−HCl (pH 7.5), 200 mM NaCl) and disrupted by sonication. After centrifugation, the supernatant was applied to an amylose column to purify the expressed protein.

NfSS Assay. Typical conditions are as follows: A reaction mixture (50 μL of Tris−HCl buffer (pH 7.5)) containing GGPP (220 μM), IPP (660 μM), $MgCl_2$ (5 mM), DTT (2 mM), and NfSS (10 μM) was incubated at 30 °C for 3 h. The reaction was quenched by the addition of EtOAc (50 μL), and the resultant mixture was vortexed and centrifuged at 12000 rpm. The upper EtOAc layer was collected and analyzed by GC−MS with the same GC conditions used to analyze **1**.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b08319.

Experimental procedures, HR-MS data, NMR data, LC−MS data, and DFT calculation data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*hoik@sci.hokudai.ac.jp

Notes

The authors declare no competing financial interest.

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