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Metabolic Engineering-Based Rapid Characterization of a Sesquiterpene Cyclase and the Skeletons of Fusariumdiene and Fusagramineol from *Fusarium graminearum*

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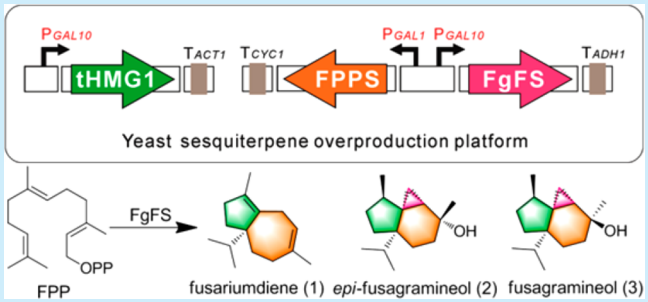
Supporting Information

ABSTRACT: The potential power of sesquiterpene synthase FgJ03939 from *Fusarium graminearum* was fully exploited in a farnesyl diphosphate-overexpressing *Saccharomyces cerevisiae* chassis to produce the novel sesquiterpenes fusariumdiene (**1**), *epi*-fusagramineol (**2**), and fusagramineol (**3**) with 5/7 bicyclic and 5/6/3 tricyclic ring systems, respectively, as well as five known sesquiterpenes (**4**−**8**). The structure of the unusual skeletons was characterized, and an absolute configuration was proposed. A mechanism for the biosynthesis of **1**−**8** was also proposed.



Terpenoids represent the most diverse group of natural products; their structural diversity endows them with a wide range of applications.[1] Nevertheless, with the development of genome sequencing and annotation, a growing number of functionally unknown terpene synthases have been revealed, implying that our understanding of the diversity of terpenoids is still lacking. Advances in metabolic engineering have made it possible for researchers to establish efficient universal C5 precursor isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) based systems for the overexpression of bioactive terpenoids and the functional characterization of terpene synthases and to accelerate the discovery of new terpenoids.[2] Sesquiterpenes, which are produced by the cyclization of the universal precursor farnesyl diphosphate (FPP), represent a wide range of natural products with highly diverse structures and bioactivity. Currently, there are at least 121 different sesquiterpene carbon skeletons whose structures have been characterized; those with 5/7 bicyclic (4%) and 5/6/3 tricyclic (7%) ring systems are considered rare skeletons.[3] Only one 5/6/3 tricyclic ring system (laurene) has been formed by the 1,6-cyclization of FPP upon isomerization to nerolidyl pyrophosphate (NPP) (Figure S1).[4]

Filamentous fungi are producers of structurally diverse terpenoid compounds that can produce a large number of advanced terpenoids, such as the fusicocca-2,10(14)-diene,[5] variediene and (2*E*)-α-cericerene,[6] and GJ1012 A−E and mangicdiene.[2b] However, only specific sesquiterpenes have been characterized from a limited number of fungi (e.g., trefolane A and sterhirsutins).[7] *Fusarium* is a class of phytopathogenic fungi found in cereal crops that is known to produce a large number of terpenoids.[8] Phylogenetic analysis showed that 27 class I terpene synthases of genus *Fusarium* (anamorph; *Gibberella* in teleomorph) with an identity lower than 70% were widely distributed (clade II−V) across the phylogenetic tree; those from clade I were not found (Figure S2). Recently, we characterized the promiscuous terpene cyclases FgMS and FgGS, which are responsible for the production of two new sesterterpenes mangicdiene and variecoltetraene, as well as five new diterpenes, GJ1012 A−E, with three new skeletons, from *F. graminearum*.[2b] Bioinformatics analysis showed that *F. graminearum* can produce nine class I terpene synthases (Figure S3, named FgJ0xxxx and marked in red), which indicates its powerful ability for terpenoid production. Herein, we report the metabolic engineering-based rapid characterization of a sesquiterpene cyclase (FgJ03939) from *F. graminearum* that was capable of producing novel 5/7 bicyclic and 5/6/3 tricyclic new skeletons, as well as five known sesquiterpenes (Figure 1), in *Saccharomyces cerevisiae*.

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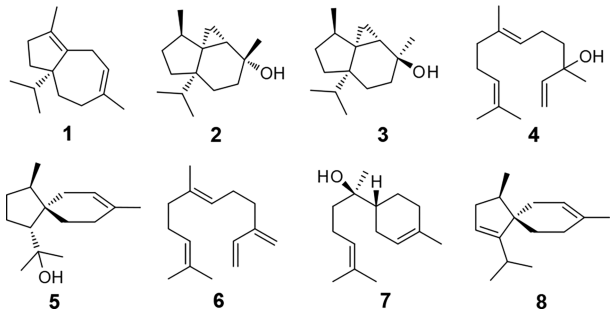

Figure 1. Structures of sesquiterpenes **1**−**8** produced by FgFS.

Phylogenetic data showed that the *FgJ03939*, together with Hyp3 and FfAAS, belonged to clade IV of class I terpene synthases and formed a new sub-branch. Multiple sequence alignments with fungal terpene synthases with known functions showed that the protein sequence of FgJ03939 shared 66% identity with FfAAS, which produces α-acorenol.[9] Sequence alignment showed that the highly conserved aspartate-rich motif ^{144}**DD**V**I**E, the NSE/DTE triad ^{266}**N**D**L**Y**S**Y**D**K**E**,[10] the pyrophosphate sensor ^{222}**R**, and the ^{358}**RY** dimer[11] (Figure S4) were present in FgJ03939. The *FgJ03939* gene was amplified by reverse transcriptase PCR (RT-PCR) and cloned to pET28a. The protein was then expressed, purified (Figure S5), and incubated with geranyl diphosphate (GPP), FPP, and geranylgeranyl diphosphate (GGPP). Finally, the products were detected and analyzed by gas chromatography/mass spectrometry (GC/MS). Different from the STC3 and STC5 in *Fusarium fujikuroi*,[8a] our *in vitro* assay showed that FgJ03939 can use GPP, FPP, and GGPP as substrates to produce monoterpene, sesquiterpene, and diterpene, respectively (Figure S6). Catalytic efficiency calculations (k_{cat}/K_m) showed that FPP is the most efficient substrate for FgJ03939 (Table S3).

To analyze the function of this putative sesquiterpene synthase, metabolic engineering was employed to create an FPP overproduction platform in *S. cerevisiae* to express enough products for structural characterization. Using the information provided by our in vitro data,[2a] the mutant *S. cerevisiae* YZL141 was constructed by overexpressing the key enzyme of mevalonate (MVA) pathway, tHMG1, to produce sufficient IPP and DMAPP for the production of terpenoids. Thereafter, the downstream sesquiterpene-production pathway was engineered by overexpressing *ERG20* (FPP synthase) and *FgJ03939* in *S. cerevisiae* YZL141. After 95 h fed-batch fermentation, similar to the *in vitro* assay, eight sesquiterpenes (with compound **2** and **3** as the main products; Figure 2 and Figure S7) in the hexane-extracted layer were detected by GC/MS with a titer of 712 mg/L. Through this metabolic engineering approach, the potential power of FgJ03939 was released by a robust FPP supply platform; we then isolated and characterized the eight compounds produced by this enzyme.

The structures of the three new compounds were established using one- and two-dimensional NMR spectroscopy. Compound **1**, $[\alpha]^{22}_D$ −13.8 (*c* 0.4, $CHCl_3$), was obtained as a colorless oil. Its molecular formula was determined as $C_{15}H_{24}$ through HR-ESI-MS at *m*/*z* 203.1794 $[M - H]^-$ (calcd 203.1792). Through ^{1}H NMR spectroscopy, we found that compound **1** exhibited two singlet methyls, two doublet methyls, and one olefinic methane [δ_H 5.30 (m, 1H, H-2), Figure S8a] through ^{13}C NMR and HSQC spectroscopy (Figure S8b and S8c), it was confirmed that compound **1** has

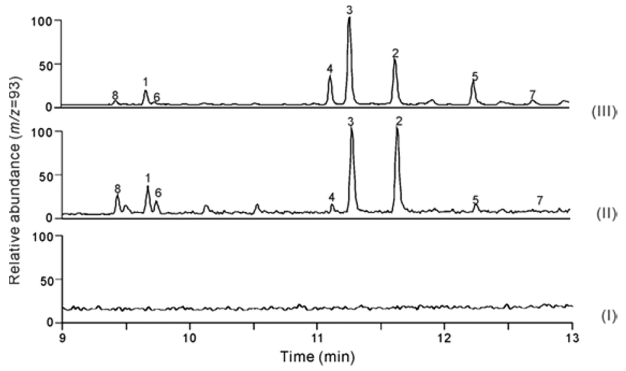

Figure 2. GC/MS chromatogram of sesquiterpenes produced by (I) boiled FgFS with FPP; (II) FgFS with FPP; and (III) *S. cerevisiae* YZL141 mutant containing FgFS.

one sp^3 quaternary carbon (C-10), three sp^2 quaternary carbons [δ_C 138.3 (C-3), 137.9 (C-6), 130.7 (C-7)], one olefinic methine [δ_C 121.9 (C-2)], and one aliphatic methine, five aliphatic methylenes, and four methyls; the ^{1}H NMR signals for the directly coupled hydrogens were assigned from the HSQC spectrum (Table S4). These data indicated that compound **1** was a bicyclic sesquiterpene. Additionally, the ^{1}H−^{1}H-COSY spectroscopy showed four connected spin systems (C-1−2, C-4−5, C-8−9, and C-14−11−15, Figure 3A, Figure S8d). The

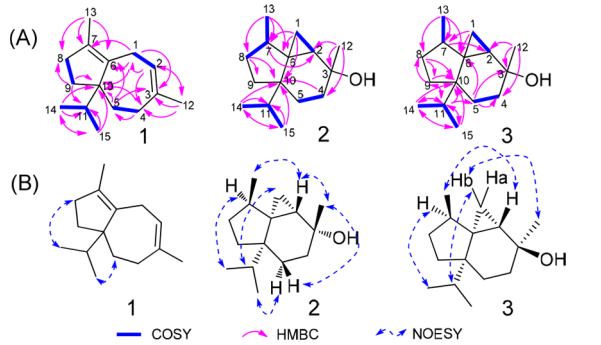

Figure 3. (A) ^{1}H−^{1}H COSY and key HMBC correlations in compounds **1**−**3**. (B) Key NOESY correlations in compounds **1**−**3**.

HMBC (Figure 3A) from H_3-14 and H_3-15 to C-10 indicated a C-10−11 bond, while peaks from H_3-13 to C-6, C-7, and C-8, and from H-1 to C-6, C-7, and C-10 revealed a C-8−7(13)-6(10)-1 connection. Correlations from H-9 to C-10 and C-11 revealed that a C-9−10 connection, forming a five-membered ring. Furthermore, the correlations from H_3-12 to C-2, C-3, and C-4, and from H-4 and H-5 to C-10 indicated a C-2−3(12)-4 and C-5−10 connection (Figure S8e). Thus, the planar structure of compound **1** was identified as a 5/7-membered bicyclic sesquiterpene. The theoretical optical rotation of **1a**, and its enantiomer **1b** (the two configurations of **1**), were then calculated by time-dependent density function theory (TD-DFT) at the b3lyp/aug-cc-pvdz level. Lastly, the self-consistent reaction field (SCRF) method was employed to perform optical rotation calculations of compound **1** in methanol at the b3lyp/aug-cc-pvdz level. The specific rotation value of 1 ($[\alpha]^D_{22}$ −13.8) matched the computated value ($[\alpha]^D_{22}$ −38.0). Thus, the absolute configurations at C-10 of **1** were determined as *S*.

Compound **2**, $[\alpha]^{22}_D$ −45.6 (*c* 0.26, $CHCl_3$), was obtained as a white solid. Its molecular formula was determined as $C_{15}H_{26}O$ through HR-ESI-MS at *m*/*z* 205.1951 $[M-OH]^+$ (calcd

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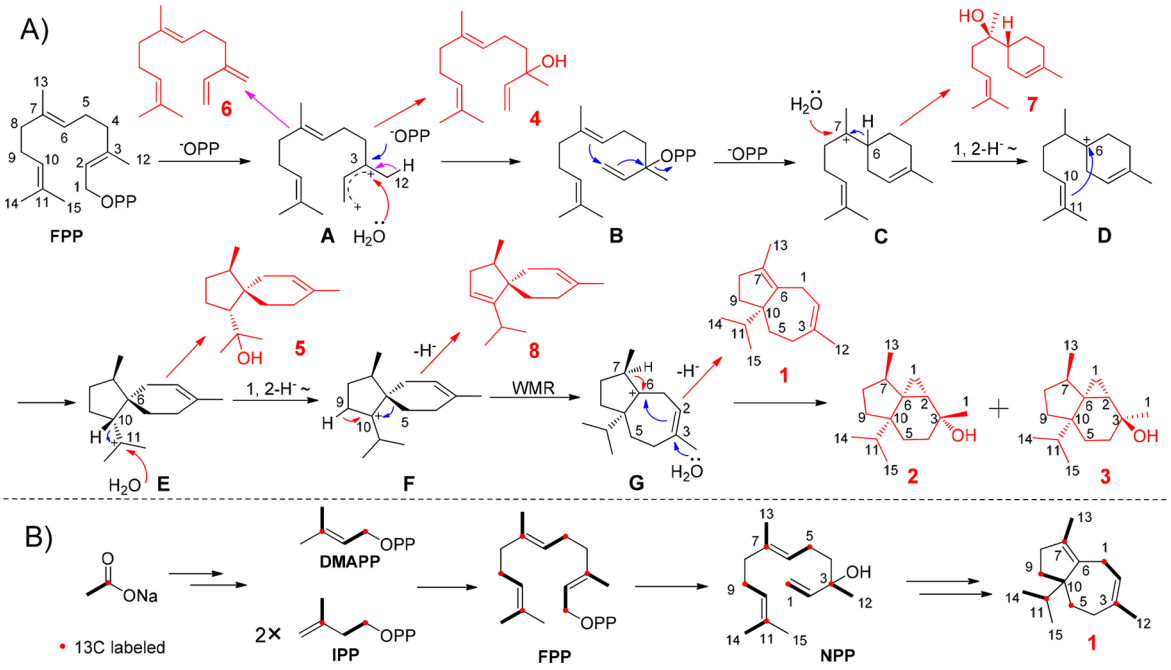

Figure 4. (A) Proposed mechanisms for the enzymatic cyclization of FPP to compounds **1**–**8**. (B) Summary of feeding experiments with [1-^{13}C, $^{2}H_3$] sodium acetate.

205.1950). The ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) spectra revealed that compound **2** contained 15 carbons (three sp^3 quaternary carbons, three aliphatic methines, five aliphatic methylenes, and four methyls, Figure S9b,c). One of the quaternary carbons [δ_C 70.39 (C-3)] was bound to oxygen, indicating a tricyclic system. The directly linked hydrogens were identified by HSQC (Table S5 and Figure S9d). Through ^{1}H–^{1}H-COSY and DQF-COSY, we identified four connected spin systems (C-1–2, C-4–5, C-8–7–13, and C-14–11–15, Figure 3A and Figure S9e,f); further connections were established using the HMBC spectra (Figure 3A and Figure S9g). Correlations from H_3-14 and H_3-15 to C-10 and from H_3-12 to C-2, C-3, and C-4 indicated C-10–11, C-2–3(12)-4 connections. Furthermore, the peaks from H_3-13 to C-6, from H-8 to C-9 and C-10, and from H-7 to C-2 revealed C-2–6–7, and C-8–9–10 connections. The correlations from H-4 to C-10 and from H-1 to C-6, C-7, and C-10 indicated a C-1–6–10–5 connection, forming a 5/6/3-membered tricyclic structure. The relative configuration was assigned as 2*S**,3*R**,6*S**,7*R**,10*R** through analysis of the key NOESY spectrum: H-1 and H-11, H-7 and H_3-14, H_3-12 and H-2/H-5α, and H_3-15 and H-5β (Figure 3B and Figure S9h).

Compound **3**, $[\alpha]^{22}_{D}$ −72.3 (*c* 0.25, $CHCl_3$), was obtained as a white solid. The molecular formula was determined as $C_{15}H_{26}O$ by HR-ESI-MS at *m*/*z* 205.1951 $[M - OH]^+$ (calcd 205.1951). The ^{1}H and ^{13}C NMR spectra of compound **3** were similar to those of compound **2**; combined with HSQC, it was confirmed that compound **3** also contained 15 carbons (one oxygenated carbon [δ_C 69.26 (C-3)], two sp^3 quaternary carbons, three aliphatic methines, five aliphatic methylenes, and four methyls, Figure S10a–c), and the directly linked hydrogens were also identified (Table S6). Through ^{1}H–^{1}H-COSY, we identified four spin systems (C-1–2, C-4–5, C-7–13, and C-14–11–15, Figure 3A and Figure S10d). HMBC (Figure 3A and Figure S10e) from H_3-14 and H_3-15 to C-10; from H-11 to C-5, C-9, and C-10; and from H-8 to C-9 and C-10 revealed a C-5–10(11)-9–8 connection. Peaks from H_3-13 to C-6 and C-8, from H-7 to C-2, and from H_3-12 to C-2, C-3, and C-4 indicated a C-8–7(13)-6–2–3–4 connection, forming a nine-membered ring. The C-1–6–10 connection was inferred from correlations from H-5 to C-6 and from H-1 to C-6 and C-7, resulting in a 5/6/3-membered tricyclic structure, as in compound **2**. The relative configuration was assigned as 2*S**,3*S**,6*S**,7*R**,10*R** through the analysis of the key NOESY correlations: H-1b and H-11/H_3-12/H_3-14; H-7 and H_3-14; and H_3-13 and H-2 (Figure 3B, Figure S10f). It was found that only the C-3 configuration of compound **3** was reversed in compound **2**. Additionally, the absolute configurations of compound **2** and **3** could be inferred from the intermediates **5** and **8**, as the C-7 of compound **5** and **8** was *R*; therefore, the absolute configurations of compound **2** and **3** were designed as 2*S*,3*R*,6*S*,7*R*,10*R*, and 2*S*,3*S*,6*S*,7*R*,10*R*, respectively.

Compounds **4**–**8** were purified and analyzed by NMR spectroscopy (Figure S11–15), showing structures of nerolidol (**4**), (−)-α-acorenol (**5**), (*E*)-β-farnesene (**6**), (+)-α-bisabolol (**7**), and (−)-acoradiene (**8**).[2b,9,12] Compounds **1**–**3** were named fusariumdiene, *epi*-fusagramineol, and fusagramineol, respectively, and the sesquiterpene synthase was designated FgFS (*F. graminearum* fusariumdiene and fusagramineol synthase).

Since compounds **1**–**3** contained two unusual skeletons, it was necessary to clarify the cyclization mechanisms of these skeletons. Based on the information provided by the well-defined cyclization mechanisms of several known sesquiterpenes, such as α-acorenol (**5**),[13] our proposed cyclization mechanism for the enzymatic conversion of FPP into sesquiterpenes by FgFS is outlined in Figure 4A. The mechanism starts with the departure of the pyrophosphate group of **FPP**; next, the formed farnesyl cation **A** is reattacked by a pyrophosphate group to form **NPP B**. From this process, two side-products nerolidol **4** and farnesene **6**, could be isolated. **B** undergoes C-1–6 ring closure to form the bisabolyl cation **C**; traces of the product (+)-α-bisabolol **7** is also formed.

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Then, cation **C** undergoes a 1,2-H^- shift and C-10−6 ring closure to form acorenyl cation **E**, which is attacked by water to form (−)-α-acorenol **5**. Intermediate **E** then undergoes a 1,2-H^- shift to form cation **F**; this deprotonation forms the side product (−)-acoradiene **8**. Cation **F** undergoes a Wagner−Meerwein rearrangement to form the fusariumdisyl cation **G**. **G** is attacked by water at C-3 to form *epi*-fusagramineol (**2**) or fusagramineol (**3**) or processes the deprotonation at C-7 to form fusariumdiene (**1**).

To obtain further insight into the FgFS-catalyzed cyclization, an isotope-labeling experiment was carried out using [1-^{13}C,$^{2}H_3$] sodium acetate as the carbon source. Compound **1** was labeled at C-1, C-3, C-5, C-7, C-9, and C-11, and upfield β-shifted resonances were observed for C-1 (one signal), C-3 (broad signal), C-7 (broad signal), and C-11 (three signals); these indicated that C-1−2, C-3−12, C-7−13, and C-11−14 contained intact acetate units (Figure 4B, Figures S16 and S17, and Table S8). Compound **8** was also labeled at C-1, C-3, C-5, C-7, C-9, and C-11, and upfield β-shifted resonances were observed for C-1 (one signal), C-3 (broad signal), C-7 (two signals), and C-11 (two signals); these indicated that C-1−2, C-3−12, C-7−13, and C-11−14 contained intact acetate units (Figures S18 and S19 and Table S9). This labeling pattern was consistent with the proposed mechanism (Figure 4A).

In summary, with the aid of metabolic engineering, we have characterized the function of FgFS, a novel sesquiterpene synthase from *F. graminearum*. Fusagramineol, its isomer *epi*-fusagramineol, and the byproduct fusariumdiene contained novel skeletons. Using the information from the known compounds **4**−**8**, we proposed that compounds **1**−**3** were generated by the 1,6-cyclization of FPP upon isomerization to NPP, which is a new mechanism for the formation of 5/7 bicyclic sesquiterpene, and a rare mechanism for the formation of 5/6/3 tricyclic sesquiterpenes. Furthermore, through isotopic labeling experiments, we confirmed these proposed cyclization mechanisms of FgFS, which provided a reasonable mechanism for the construction of these sesquiterpenes. Different from our previously metabolic engineering work in *E. coli*,[2b] our study provides a more efficient and systematic approach for the functional and mechanistic characterization of terpene cyclases using FPP overproduction platform in *S. cerevisiae*; this approach can be further extended to sesquiterpene synthases in various origins. This may accelerate the process of discovering novel terpene skeletons and provide bioactive or high value-added compounds for drug discovery and industrial applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b00366.

Experimental procedures, phylogenetic analysis, NMR data, GC/MS and LC/MS data, and quantum chemical calculation data

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Author Contributions

‖G.B. and A.H. contributed equally to this work.

Notes

The authors declare the following competing financial interest(s): A patent application based on the results in this study has been filed by J1 Biotech Co., Ltd.

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