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Article

Characterization of a Sesquiterpene Synthase Catalyzing Formation of Cedrol and Two Diastereoisomers of Tricho-Acorenol from *Euphorbia fischeriana*

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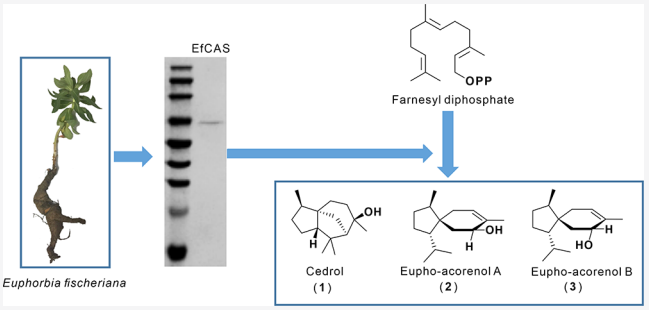
ABSTRACT: A sesquiterpene synthase gene was identified from the transcriptome of *Euphorbia fischeriana* Steud, and the function of its product EfTPS12 was characterized by *in vitro* biochemical experiments and synthetic biology approaches. EfTPS12 catalyzed conversion of farnesyl diphosphate into three products, including cedrol (**1**) and eupho-acorenols A (**2**) and B (**3**) (two diastereoisomers of tricho-acorenol), thereby being named EfCAS herein. The structures of **2** and **3** were determined by spectroscopic methods and comparison of experimental and calculated electronic circular dichroism spectra. EfCAS is the first example of a plant-derived sesquiterpene synthase that is capable of synthesizing acorane-type alcohols. This study also documents that synthetic biology approaches enable large-scale preparation of volatile terpenes and thereby substantially facilitate characterization of corresponding terpene synthases and elucidation of the structures of their products.



Plant volatile oils with sesquiterpenes as their major components have been widely used in the flavor, perfume, cosmetic, and health care industries,[1,2] such as sandalwood oils,[3] patchouli oils,[4] and turmeric oils.[5] Volatile sesquiterpenes also play a variety of critical roles in plant physiology and ecological interactions including plant−plant, plant−herbivore, plant−pathogen, and plant−mutualist interactions.[2,6,7] Sesquiterpenes are synthesized from their common precursor farnesyl diphosphate (FPP) under catalysis of sesquiterpene synthases (sesquiTPSs).[8] Most sesquiTPSs are catalytically promiscuous, thereby producing more than one product with diverse planar skeletons and configurations.[8,9] This makes sesquiterpenes a group of structurally diverse compounds. Due to great complexities of volatile sesquiterpene patterns in plants, the low content of each single sesquiterpene component, and their close chemical properties (e.g., polarity), it is difficult to completely separate these sesquiterpene components and thereby elucidate the complete structures (particularly the absolute configurations). In most cases, volatile sesquiterpenes of plant extracts are simply identified and analyzed by GC-MS-based methods and comparing mass spectra of target peaks with standard spectra.[10] We constructed microbial platforms for the production of plant-derived sesquiterpenes in previous studies,[3,11] which not only laid a foundation for biological production of the corresponding compounds on an industrial scale, but also provided a promising approach to obtain sufficient amounts of products of sesquiTPSs for unambiguous elucidation of their structures.

Euphorbia fischeriana Steud is a plant belonging to the Euphorbiaceae family. Its dry roots, known as "langdu" in traditional Chinese medicine, have been used to treat edema, ascites, and tuberculosis.[12] Although this herb is well known for its diterpene constituents, such as the anti-HIV candidate drug prostratin[13] and the potent cytotoxic agent 17-hydroxyjolkinolide B,[14] its volatile oil (with sesquiterpenes as the major components) has been reported to possess radical scavenging activity.[15] In the present study, we identified the sesquiTPS EfTPS12 from *E. fischeriana* and obtained sufficient amounts of its products for NMR and ECD analysis using an *E. coli*-based synthetic biology approach. The results showed that EfTPS12 catalyzed cyclization of FPP to yield cedrol (**1**) and eupho-acorenols A (**2**) and B (**3**) (two diastereoisomers of tricho-acorenol).

RESULTS AND DISCUSSION

To identify sesquiTPS-encoding genes from *E. fischeriana*, the total RNAs were isolated from a mixture of leaves, stems, and roots and sequenced on an Illumina Novaseq platform. By

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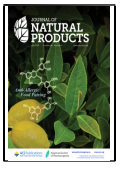

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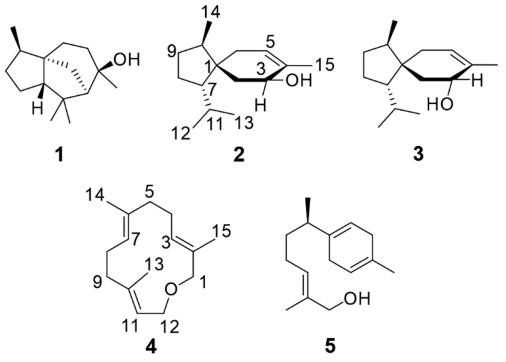

using a Trinity *de novo* assembler,[16] 57 million clean reads including 8.55 G clean bases were assembled after removing reads containing adapters, reads containing poly-N, and low-quality reads, with a Q30 of 92.4%. Subsequently, we used the previously characterized sesquiTPS genes[17,18] (Table S1) as the queries to perform a BLAST search, and 10 candidate genes were identified, including five full-length and five truncated genes. The full-length sequences of five truncated genes were acquired by Rapid Amplification of cDNA ends.

After expressing all of the candidate genes in *E. coli*, soluble expression of EfTPS12 was observed (Figure 1a). EfTPS12 was incubated with FPP, and then the reaction mixture was extracted with *n*-hexane. From the resulting extract, three peaks (**1**−**3**), as well as the peak of farnesol (FOH), were detected by GC-MS, compared with the control sample (the extract of the reaction mixture containing FPP and the inactive EfTPS12, which was heated at 80 °C for 30 min in advance) (Figure 1b−f). The MS spectra of **1**−**3** showed the same molecular ion peaks at *m*/*z* 222 (Figure 1c−e), indicating that they are all sesquiterpene alcohols. Compound **1** was identified to be cedrol, as its MS spectrum is identical with that of the reported one (Figure 1c).[19] This result was consistent with the phylogenetic analysis (Figure S1) in which EfTPS12 was closest to Lc-CedS, a cedrol synthase from *Leucosceptrum canum*.[19]

To unambiguously determine the structures of these three products, we used a synthetic biology approach to obtain sufficient amounts for spectroscopic analysis. EfTPS12 was expressed in an *E. coli* strain in which three lowly expressed genes encoding 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductase (DXR), and isopentenyl diphosphate isomerase (IDI) of the methylerythritol 4-phosphate (MEP) pathway were overexpressed.[20] After fermentation of the resulting *E. coli* strain, the products **1**−**3**, as well as two other compounds, **4** and **5**, were detected in the culture (Figure 2). These five compounds were extracted and isolated, and their structures were elucidated. Compound **1** is indeed cedrol, as its NMR data and specific rotation value ($[\alpha]^{22}_{D}$ +11.6, *c* 0.3, CH_2Cl_2) are identical with the reported data of cedrol.[21,22] Compound **5** was determined

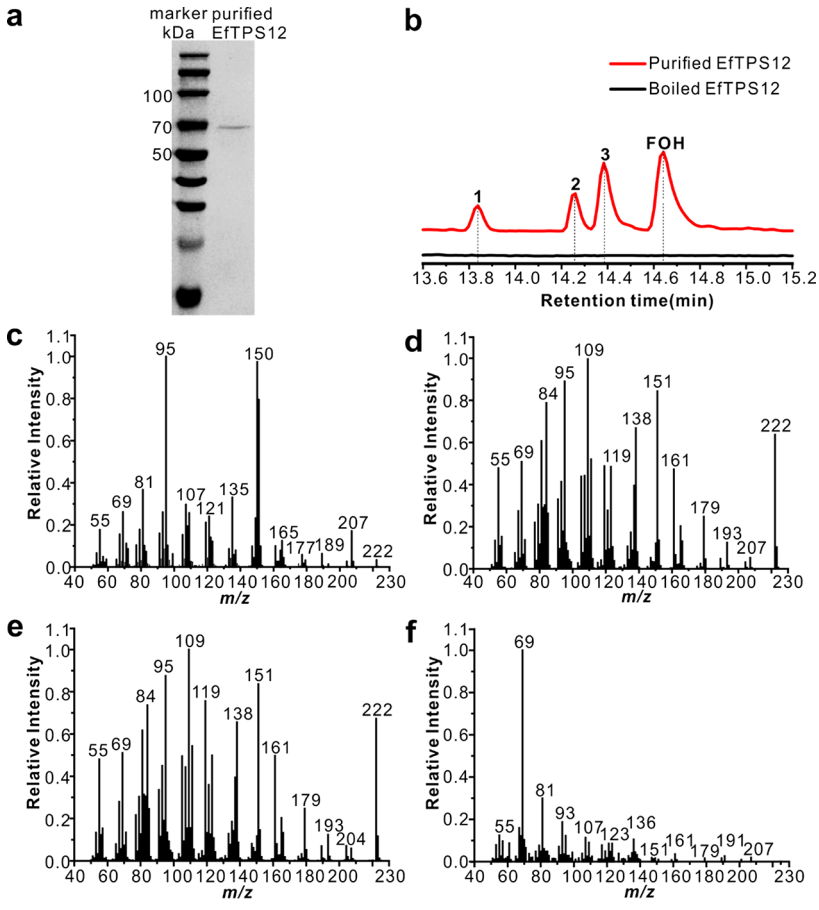

Figure 1. Functional characterization of EfTPS12 by *in vitro* biochemical assays. (a) SDS-page analysis of the recombinant EfTPS12; (b) GC-MS chromatograms of the extracts of the reaction mixtures of FPP with either EfTPS12 (red) or boiled EfTPS12 (black); (c−f) Mass spectra of **1**, **2**, **3**, and farnesol (FOH), respectively.

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J. Nat. Prod. 2021, 84, 1780−1786

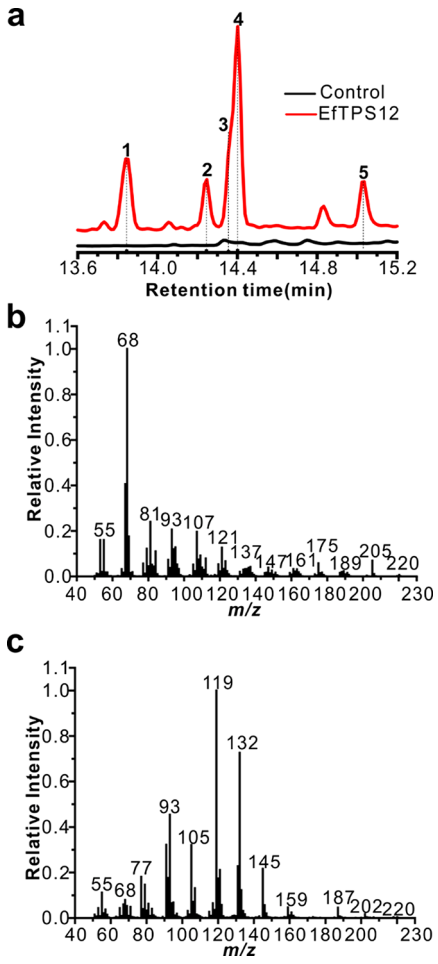

Figure 2. Functional characterization of EfTPS12 by synthetic biology approaches. (a) GC-MS chromatogram of the extracts of the cultures of the *E. coli* C41 strains containing either petDuet-1-ERG20-EfTPS12 (red) or petDuet-1-ERG20 (black); (b and c) mass spectra of **4** and **5**.

as (*E*)-*β*-curcumen-12-ol by comparing its MS (Figure 2c) and NMR data with the literature data,[23] as well.

Compound **2** was obtained as a colorless oil, $[\alpha]^{22}_{D}$ +14.4 (*c* 0.3, CH_2Cl_2). Its HREIMS spectrum showed a molecular ion $[M]^+$ at *m/z* 222.1976, indicating that its molecular formula is $C_{15}H_{26}O$, with three indices of hydrogen deficiency. The ^1H NMR spectrum showed the signals for four methyl groups (δ_H 1.77, 0.89, 0.86, and 0.81) (Table 1). Fifteen carbon signals were preliminarily assigned by the ^{13}C and DEPT NMR spectra, including a trisubstituted double bond (δ_C 134.7 and 125.1), an oxygen-bearing methine group (δ_C 69.3), a quarternary carbon (δ_C 45.7), three nonoxygenated methine groups (δ_C 28.1, 45.1, and 56.2), four methylene groups (δ_C 45.5, 30.8, 25.6, and 22.6), and four methyl groups (δ_C 25.4, 19.3, 19.0, and 15.6), which are consistent with four methyl proton signals in the ^1H NMR spectrum (Table 1). After one-bond linkage relationships between carbons and protons were determined by the HSQC experiment, the planar structure of **2** was constructed by the COSY and HMBC experiments. The correlations of H_2-2/H-3 and H-5/H_2-6 were observed in the COSY spectrum of **2**, and the long-range correlations from H_3-15 to C-3, C-4, and C-5, from H-3 to C-1, C-2, C-4, and C-5, from H-2 to C-3, C-4, and C-6, and from H-6 to C-1 were observed in the HMBC spectrum (Figure 3), indicating the presence of a cyclohexene with a methyl group substituted at C-4 and a hydroxy group at C-3, which is consistent with the hydroxy group signal (3302 cm^{-1}) in the IR spectrum. The HMBC correlations from H_3-14 to C-1, C-9, and C-10, from H_3-12 and H_3-13 to C-7 and C-11, from H-11 to C-1 and C-8, and from H_2-8 to C-9 (Figure 3) suggested the presence of a five-membered ring with an isopropyl group at C-7 and a methyl group at C-10. Thus, the planar structure of **2** was determined and is the same as tricho-acorenol.[24] Because the NMR data of **2** were not completely identical to those of tricho-acorenol[24,25] and (+)-(3*R*)-hydroxy-4-acorene (also called as *ent-epi*-tricho-acorenol),[25,26] **2** is not one of these compounds or their enantiomers.

The relative configuration of **2** was determined by the NOESY correlations between H_2-2 and H_3-14, between H-3 and H-7, between H-5 and H_3-13, between H_2-6 and H_3-12, and between H_2-6 and H_3-13, as shown in Figure 3. The absolute configuration of **2** was subsequently determined by comparing the experimental and calculated ECD spectra (Figure 4).[27] Moreover, cedrol (**1**) is one of the co-products of EfTPS12 and was synthesized from the intermediate **v**, which is also the biosynthetic intermediate of **2** (Scheme 1).[28] This further confirms the absolute configuration of **2**, e.g., 1*R*, 3*S*, 7*S*, 10*R*, a diastereoisomer of tricho-acorenol.[29] Compound **2** was obtained by a chemical synthesis approach before,[30] while this study documents that it is also a natural product. We named it eupho-acorenol A.

Compound **3** was isolated as a colorless oil, $[\alpha]^{22}_{D}$ +4.9 (*c* 0.3, CH_2Cl_2). Its molecular formula, $C_{15}H_{26}O$, was deduced from the molecular ion peak $[M]^+$ at *m/z* 222.1976 in the HREIMS spectrum. The ^1H and ^{13}C NMR data of **2** and **3** were very similar (Table 1). A careful analysis of the HSQC, COSY, and HMBC spectra of **3** indicated that it possesses the same planar structure as **2** (Figure 3). Because the cross-peaks in the NOESY spectrum of **3** acquired in $CDCl_3$ are too crowded to be clearly identified, additional NMR experiments were performed in pyridine-d_5 (Table 1). The NOESY spectrum of **3** acquired in pyridine-d_5 showed the correlations of H-3/H_3-14, H-2a (δ_H 2.23)/H_3-14, and H-2b (δ_H 1.86)/H-7 (Figure 3), suggesting the relative configuration of **3** as shown in Figure 3. Then, its absolute configuration (1*R*, 3*R*, 7*S*, 10*R*) was also determined by comparing its experimental and calculated ECD spectra (Figure 4). The absolute configuration was consistent with the proposed biogenesis (Scheme 1). Like compound **2**, **3** was obtained in the same synthesis effort.[30] We named it eupho-acorenol B.

Compound **4** was obtained as a colorless oil. Its ^1H and ^{13}C NMR data showed the signals assigned to three methyl groups (δ_H 1.54, δ_C 15.5; δ_H 1.58, δ_C 15.8; δ_H 1.63, δ_C 14.0), three trisubstituted double bonds (δ_H 5.27, 5.20, and 4.89; δ_C 135.7, 133.4, 133.2, 125.8, 125.0, and 124.3), and two oxygen-bearing methylene groups (δ_H 4.12 and 3.94; δ_C 77.7 and 69.3) (Table 1). The HSQC experiment was used to assign the one-bond linkage of the protons and carbons (Table 1), and then the structure of **4** was constructed by analysis of its COSY and HMBC spectra. Three structural fragments C-3/C-4/C-5, C-7/C-8/C-9, and C-11/C-12 were obtained by the COSY experiment (Figure S2). In the HMBC spectrum, the correlations from H_3-15 to C-1, C-2, and C-3, from H_3-14 to C-5, C-6, and C-7, from H_3-13 to C-9, C-10, and C-11, from H-1 to C-12, and from H-12 to C-1 constructed a 13-membered ether ring and indicated that three methyl groups (C-15, C-14, and C-13) are located at C-2, C-6, and C-10,

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J. Nat. Prod. 2021, 84, 1780−1786

Table 1. NMR Data for Compounds 2, 3, and 4 (^{1}H 400 MHz and ^{13}C 100 MHz)

	2		3				4	
no.	δ_C,[a] type	δ_H (J in Hz)[a]	δ_C,[a] type	δ_H (J in Hz)[a]	δ_C[b]	δ_H (J in Hz)[b]	δ_C,[a] type	δ_H (J in Hz)[a]
1	45.7, C		46.7, C		46.64		77.7, CH_2	3.94, s
2	45.5, CH_2	1.85[c]	45.9, CH_2	1.88, dd (12.6, 9.9)	46.61	2.23, dd (12.8, 10.1)	133.2, C	
		1.60[c]		1.72[c]		1.86, ddd (12.8, 6.2, 1.7)		
3	69.3, CH	4.29, brt (6.9)	69.6, CH	4.25, m	68.1	4.51, m	125.8, CH	5.20, t (6.6)
4	134.7, C		135.2, C		137.4		24.1, CH_2	2.18[c]
5	125.1, CH	5.52, ddd (6.1, 3.0, 1.4)	125.1, CH	5.52, m	123.4	5.51, m	38.7, CH_2	2.13, m
6	25.6, CH_2	1.84[c]	25.2, CH_2	2.00, dd (17.6, 5.3)	25.2	1.96[c]	133.4, C	
				1.66[c]		1.58[c]		
7	56.2, CH	1.51[c]	58.0, CH	1.28[c]	58.0	1.17[c]	125.0, CH	4.89, t (6.9)
8	22.6, CH_2	1.59[c]	25.8, CH_2	1.29[c]	26.1	1.54[c]	24.7, CH_2	2.18[c]
		1.50[c]				1.18[c]		
9	30.8, CH_2	1.74[c]	31.6, CH_2	1.74[c]	31.6	1.69[c]	38.6, CH_2	2.10, m
		1.18, m		1.27[c]		1.15[c]		
10	45.1, CH	1.68, m	44.6, CH	1.73[c]	44.8	1.70[c]	135.7, C	
11	28.1, CH	1.75[c]	28.3, CH	1.66[c]	28.4	1.53[c]	124.3, CH	5.27, t (6.3)
12	25.4, CH_3	0.89, d (6.9)	24.6, CH_3	0.98, d (6.7)	24.3	0.85, d (6.7)	69.3, CH_2	4.12, d (6.3)
13	19.0, CH_3	0.81, d (6.6)	21.9, CH_3	0.87, d (6.7)	21.9	0.77, d (6.6)	15.5, CH_3	1.54, s
14	15.6, CH_3	0.86, d (6.8)	18.5, CH_3	0.82, d (6.5)	18.5	0.87, d (6.5)	15.8, CH_3	1.58, s
15	19.3, CH_3	1.77, brs	18.9, CH_3	1.74, brs	19.6	1.99, brs	14.0, CH_3	1.63, s

[a]The data were measured in $CDCl_3$. [b]The data were measured in pyridine-d_5. [c]Overlapped by other signals.

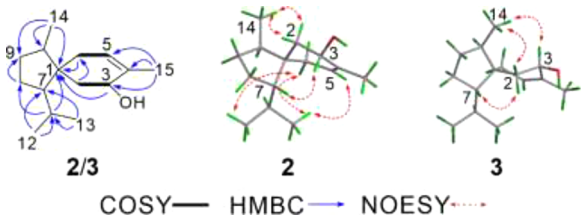

Figure 3. Key COSY, HMBC, and NOESY correlations of **2** and **3**.

respectively (Figure S2). In the NOESY spectrum, the correlation between H_2-1 and H-3 was observed, indicating the configuration of the double bond between C-2 and C-3 is *E*. Thus, the structure of **4** was determined.

It has been reported that *E. fischeriana* volatile oil contains many sesquiterpenes including cedrol (**1**).[15] We detected sesquiterpenes in the roots, stems, and leaves of *E. fischeriana* by GC-MS. **1** and an unknown compound (**6**) were identified from the root extract (Figure S8). The mass spectrum of **6** is very similar to that of acorenone (or *ent*-acorenone) (Figure S8),[25] indicating **6** is one of the stereoisomers of acorenone. The results implied that **2** and **3** could be further modified in *E. fischeriana*, which may explain the reason that **2** and **3** were not detected in the plant tissues (Figure S8).

A cedrol synthase and an *epi*-cedrol synthase have been identified from other plants,[19,31] and the biosynthetic mechanism of **1** has been reported (Scheme 1).[28] In the reaction catalyzed by EfTPS12 (Scheme 1), formation of **1**, **2**, and **3** competes to consume intermediate **v**. In this process, 1,5-hydride shift (path B) may be more favorable than attack of the double bond (path A), which could result in the amounts of **2** and **3** being bigger than that of **1** (Figure 1b). In addition, nucleophilic attack of a water molecule above the plane of the cyclohexene ring could be hindered by CH_3-14 and CH_3-15 (Scheme 1 and Figure 3), due to which more **3** was produced than **2** in the *in vitro* reaction (Figure 1b). In order to clarify the role of EfTPS12 in the formation of **4** and **5**, a handful of experiments were conducted. We synthesized tris(tetrabutylammonium) 12-hydroxyfarnesyl diphosphate and used it as the substrate to perform an *in vitro* reaction, but no product was detected (Figure S5), suggesting EfTPS12 cannot catalyze conversion of 12-hydroxyfarnesyl diphosphate into **4** and **5**. We mutated EfTPS12 by respectively replacing Asp residues of its catalytic motif (DDTYD) with Ala to yield three mutant enzymes, $EfCTS^{D303A}$, $EfCTS^{D304A}$, and $EfCTS^{D307A}$. Coexpression of three mutant enzymes with ERG20 did not result in production of **1**−**5** (Figure S6), indicating that EfTPS12 is indispensable for production of **4**

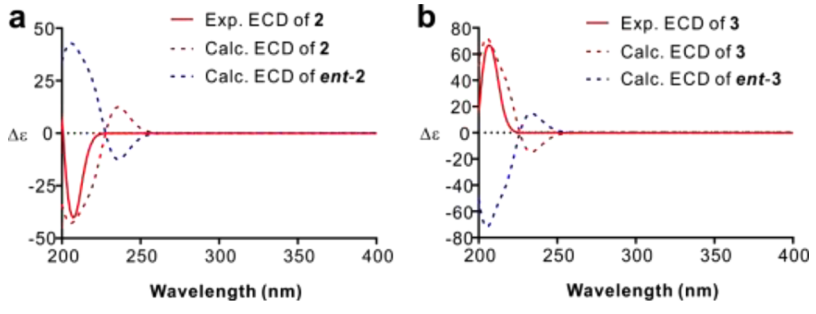

Figure 4. Comparison of the experimental and calculated ECD spectra of **2** and **3**.

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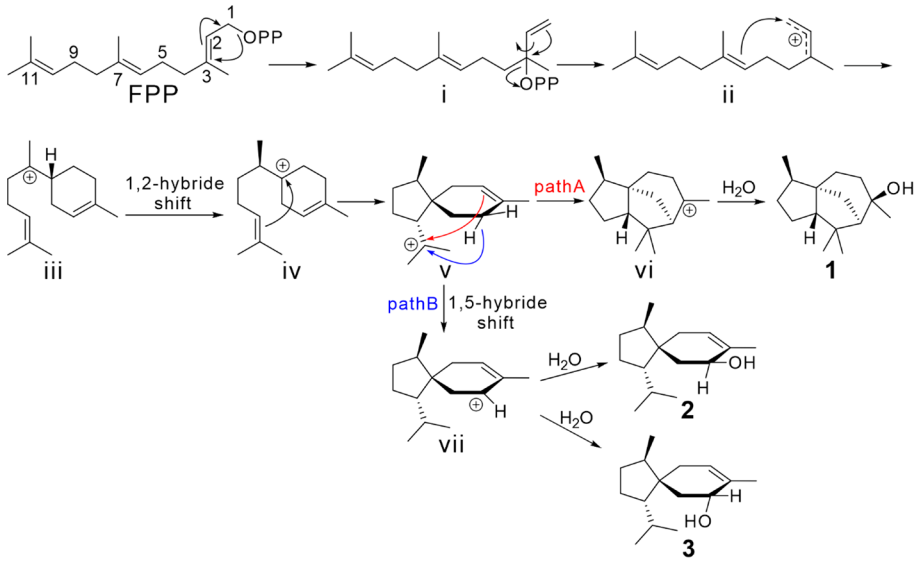
Scheme 1. Plausible Biosynthetic Pathway for 1−3



and **5**. We then coexpressed EfTPS12 with ERG20 in *E. coli* BL21, and production of **1**−**5** was observed (Figure S7), as well. These results indicate that although **4** and **5** do not appear to be the direct products of EfTPS12, they arise by unknown routes that do involve the participation of EfTPS12. Hence, only **1**, **2**, and **3** are the direct products of EfTPS12. Herein, we named it EfCAS.

In the *in vitro* reaction, a large amount of FOH was produced (Figure 1), but no evident FOH peak was detected from the extracts of *E. coli* expressing EfTPS12 and ERG20 (Figure 2). The 6xHis tag may cause a slight change in the structure of the recombinant EfTPS12 used in the *in vitro* reaction, which could result in FOH production.

In conclusion, a new sesquiterpene synthase, EfCAS, was characterized by *in vitro* biochemical experiments and synthetic biological approaches. It can catalyze cyclization of FFP to afford cedrol (**1**) and two tricho-acorenol stereoisomers, eupho-acorenols A (**2**) and B (**3**). Acorenol isomers (*α*-acorenol, *β*-acorenol, *epi*-*α*-acorenol, and *epi*-*β*-acorenol) are the indispensable components of highly valuable plant volatile oils,[32−34] and tricho-acorenol is one of the major volatile metabolites of *Trichoderma* fungi[24,25] which can be used as biocontrol and biostimulant agents.[35,36] Microbial sesquiterpene synthases producing *α*-acorenol and tricho-acorenol were reported,[37−39] while for plant sesquiterpene synthases, TEAS (a tobacco sesquiterpene synthase) was reported to only produce acorane olefins (*α*-acoradiene, 3.9%; 4-*epi*-*α*-acoradiene, 1.3%) in small quantities.[40] EfCAS is the first example of a plant-derived sesquiterpene synthase that is capable of synthesizing acorane-type alcohols. Additionally, this study documents that synthetic biology approaches enable large-scale preparation of volatile terpenoids, thereby substantially facilitating characterization of corresponding terpene synthases and elucidation of the complete structures of their products.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotations were measured on a JASCO P-2000 polarimeter. UV spectra were obtained on a JASCO V-550 spectrophotometer. ECD spectra were measured using a Chirascan spectropolarimeter (Applied Photophysics). NMR spectra were recorded on a Bruker Avance III-400 spectrometer, and TMS was used as the internal standard. Low- and high-resolution GC-MS analysis was respectively performed on an Agilent 7890 GC instrument equipped with a Saturn 2100 ion trap mass spectrometer and an Agilent 7250 GC/Q-TOF system in electron ionization (70 eV) mode, using an HP-5MS column. HPLC separation was performed on an Ultimate 3000 instrument (Thermo Scientific), using a YMC-Pack Pro C18 column (250 × 4.6 mm, 5 μm) or a Phenomenex FMG-AC5-A01-NFC Chiral ND (2) column (250 × 4.6 mm, 5 μm, Guangzhou FLM Scientific Instrument Co. Ltd.). Silica gel (200−300 mesh, Qingdao Marine Chemical Inc.) was used for gravity-driven column chromatography.

Plant Material. The whole herbs of *Euphorbia fischeriana* were collected in Shenyang, Liaoning Province, People's Republic of China, in May 2017, and identified by Prof. Yingni Pan of Shenyang Pharmaceutical University (voucher specimen No. EFIYP-201705). After being washed with tap water and cut into small pieces, the plant tissue pieces were immediately frozen in liquid nitrogen and stored at −80 °C.

Transcriptome Analysis. RNA isolation, sequencing, assembling, annotation, and FPKM calculation were performed according to the previous methods.[11]

RNA Isolation and Gene Cloning. Total RNAs were isolated from a mixture of *E. fischeriana* leaves, stems, and roots and transcribed to cDNAs using RNA Prep Pure Plant kit (TIANGEN) and HiScript II Q Select RT SuperMix kit (Vazyme), respectively. The full-length transcript of EfCAS was amplified using the gene-specific primers by Phanta Max Super-Fidelity DNA polymerase (Vazyme). The amplified fragments were first gel-purified and then introduced into the pEASY-Blunt vector (TransGen Biotech) for sequence analysis.

***In Vitro* Assays.** The coding sequence of EfTPS12 was inserted into the NdeI/XhoI site of the pCold TF vector and transferred into *E. coli* BL21 (DE3) for heterologous expression. The transformants were grown at 37 °C, shaking at 200 rpm. When the OD_{600} of the cultures reached 0.4−0.6, 0.2 mM IPTG was used to induce protein expression overnight at 16 °C. The cells were harvested by centrifugation at 5000*g* for 10 min at 4 °C. Pellets were then resuspended in Tris-HCl buffer (100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM DTT). The cells were disrupted by sonication, and the lysate was centrifuged at 13000*g* for 20 min to obtain the supernatant crude protein, which was subjected to a Ni-NTA (Takara) affinity column successively eluted with buffer A (50 mM Tris-HCl, 5 mM $MgCl_2$, 250 mM NaCl, 10% glycerol, pH 8.4), buffer B (50 mM Tris-HCl, 5 mM $MgCl_2$, 250 mM NaCl, 10% glycerol, 100 mM imidazole, pH 8.4), and buffer C (50 mM Tris-HCl, 5mM $MgCl_2$, 250 mM NaCl, 10% glycerol, 300 mM imidazole, pH 8.4). After removal of imidazole by dialysis, the resulting enzyme solution was concentrated

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J. Nat. Prod. 2021, 84, 1780−1786

by centrifugation. Enzymatic reactions were performed in 500 μL of buffer A containing 5 μM enzyme and 3 mM FPP purchased from Sigma. The reaction mixtures were incubated at 30 °C for 3 h, followed by extraction with *n*-hexane for GC-MS analysis.

Characterization of EfTPS12 by a Synthetic Biology Approach. The EfTPS12 gene was introduced into the NdeI/XhoI site (the first cloning site) of petDuet-1 vector by the cut-paste method to generate the plasmid petDuet-1-EfTPS12. Then, ERG20 (accession no. 97 NC_001142.9) was inserted in the NcoI/NotI site (the second cloning site) of petDuet-1-EfTPS12 to afford petDuet-1-ERG20-EfTPS12. *E. coli* was transformed with petDuet-1-ERG20-EfTPS12 and pIRS,[20] and the resulting constructs were grown in TB media (containing 12 g/L peptone, 24 g/L yeast extracts, 8 mL of 50% glycerol, 9.4 g of K_2HPO_4, 2.2 g of KH_2PO_4) supplied with 50 mg/L ampicillin in 250 mL flasks. At an OD_{600} of 0.4−0.6, expression was induced by adding 1 mM IPTG, and the temperature was set to 16 °C. The cultures were fermented for an additional 72 h at 16 °C and then extracted with 50 mL of *n*-hexanes.

Compound Isolation and Structural Elucidation. A 30 L culture of the recombinant *E. coli* strain containing petDuet-1-ERG20-EfTPS12 and pIRS20 was grown in TB media. After a 72 h growth, the culture was extracted by *n*-hexane. Then, the extracts were dried under vacuum and the residue was subjected to a silica gel column eluted with petroleum ether−EtOAc (50:1 to 10:1) to yield four fractions, Fr1−Fr4. **1** (83 mg) in Fr2 and **2** (72 mg) and **3** (75 mg) in Fr3 were further purified by normal phase HPLC, using a Phenomenex FMG-AC5-A01-NFC chiral-phase column (the mobile phase for **1**: *n*-hexane−isopropanol = 200/1; the mobile phase for **2** and **3**: *n*-hexane−isopropanol = 99/1). Compounds **4** (124 mg) and **5** (70 mg) were respectively purified from Fr1 and Fr4 by reverse phase HPLC, using a YMC-Pack Pro C18 column and methanol−water (80/20) as the mobile phase.

*Eupho-acorenol A (**2**):* colorless oil; $[\alpha]^{22}_{D}$ +14.4 (*c* 0.3, CH_2Cl_2); ECD (4.5 × 10^{-3} M, MeOH) λ_{max} ($\Delta\varepsilon$) 207 (−2.4) nm; IR (KBr) ν_{max} 3302, 2950, 2919, 2867, 1461, 1378, 1274, 1052, 1020, 937, 814 cm^{-1}; ^1H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data, Table 1; HREIMS *m*/*z* 222.1976 $[M]^+$ (calcd for $C_{15}H_{26}O$, 222.1984).

*Eupho-acorenol B (**3**):* colorless oil; $[\alpha]^{22}_{D}$ +4.9 (*c* 0.3, CH_2Cl_2); ECD (4.5 × 10^{-3} M, MeOH) λ_{max} ($\Delta\varepsilon$) 207 (+2.7) nm; IR (KBr) ν_{max} 3318, 2952, 2922, 2868, 1456, 1377, 1052, 1017, 811 cm^{-1}; ^1H (400 MHz, $CDCl_3$ or pyridine-d_5) and ^{13}C (100 MHz, $CDCl_3$ or pyridine-d_5) NMR data, Table 1; HREIMS *m*/*z* 222.1976 $[M]^+$ (calcd for $C_{15}H_{26}O$, 222.1984).

*Compound **4**:* colorless oil; IR (KBr) ν_{max} 2906, 2846, 1438, 1380, 1251, 1087, 1065, 920, 816, 542 cm^{-1}; ^1H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data, Table 1; HREIMS *m*/*z* 220.1820 $[M]^+$ (calcd for $C_{15}H_{24}O$, 220.1827).

Synthesis of Tris(tetrabutyl ammonium) 12-Hydroxyfarnesyl Diphosphate. Following the previous method,[41,42] tris(tetrabutyl ammonium) 12-hydroxyfarnesyl diphosphate was synthesized. Briefly, farnesol reacted with methanesulfonyl chloride to afford farnesyl chloride,[41] which was subsequently oxidized into 12-hydroxyfarnesyl chloride by selenium dioxide and *tert*-butyl hydroperoxide,[42] and 12-hydroxyfarnesyl chloride was then converted into tris-(tetrabutylammonium) 12-hydroxyfarnesyl diphosphate under treatment of tris(tetrabutylammonium) hydrogen pyrophosphate.[42]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.1c00126.

Table of terpene synthases used for performing BLAST analysis against the transcriptome database and those used for construction of phylogenetic tree; figures of phylogenetic analysis of EfTPS12 and the previously characterized terpene synthases from other species; key COSY and HMBC correlations of **4**; details for calculations of ECD spectra of **2** and **3**; GC-MS chromatogram of the extract of the reaction mixture of tris(tetrabutylammonium) 12-hydroxyfarnesyl diphosphate with EfTPS12; GC-MS chromatogram of the extract of the culture of the *E. coli* strain coexpressing ERG20 and each EfTPS12 mutant; GC-MS chromatogram of the extract of the culture of the *E. coli* BL21 strain containing petDuet-1-ERG20-EfTPS12; extraction of *E. fischeriana* tissues; GC-MS chromatograms of the extracts of roots, stems, and leaves of *E. fischeriana*; HREIMS, IR, and NMR spectra of **2**, **3**, and **4**; sequences of the recombinant EfCAS and its encoding gene (PDF)

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Notes

The authors declare no competing financial interest.

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