Biosci. Biotechnol. Biochem., **77** (11), 2278–2282, 2013

Cyclization of All-*E*- and 2*Z*-Geranylfarnesols by a Bacterial Triterpene Synthase: Insight into Sesterterpene Biosynthesis in *Aleuritopteris* Ferns

Junichi SHINOZAKI,[1,†] Masaaki SHIBUYA,[2] Yutaka EBIZUKA,[3] and Kazuo MASUDA[1]

[1]*Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan*
[2]*Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Higashijima, Akiha-ku, Niigata 956-8603, Japan*
[3]*Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

Received July 5, 2013; Accepted August 22, 2013; Online Publication, November 7, 2013
[doi:10.1271/bbb.130543]

***Aleuritopteris* ferns produce triterpenes and sesterterpenes with tricyclic cheilanthane and tetracyclic 18-episcalarane skeletons. The structural and mechanistic similarities between both classes of fern terpene suggest that their biosynthetic enzymes may be closely related. We investigate here whether a triterpene synthase is capable of recognizing geranylfarnesols as a substrate, and is able to convert them to cyclic sesterterpenes. We found that a bacterial triterpene synthase converted all-*E*-geranylfarnesol (1b) into three scalarane sesterterpenes with 18αH stereochemistry (5, 7 and 8), as well as mono- and tricyclic sesterterpenes (6 and 9). In addition, 2*Z*-geranylfarnesol (4) was converted into an 18-episcalarane derivative (10), whose skeleton can be found in sesterterpenes isolated from *Aleuritopteris* ferns. These results provide insight into sesterterpene biosynthesis in *Aleuritopteris* ferns.**

Key words: sesterterpene; triterpene synthase; fern; *Aleuritopteris* spp.

Sesterterpenes derived from all-*E*-geranylfarnesyl diphosphate (**1a**) are a small group of terpenoids, although they exhibit a wide range of structural diversity. They have been isolated from terrestrial fungi, lichens, insects, and marine organisms.[1)] A basis for the structural diversity of fungal and bacterial sesterterpenes has been provided by recent discoveries of sesterterpene synthases. Ophiobolin F synthase from the *Aspergillus clavatus* fungus is a bifunctional enzyme possessing prenyltransferase and terpene cyclase domains.[2)] A tetraprenyl-β-curcumene synthase (TS) homolog from the *Bacillus clausii* (Bcl-TS) bacterium is a sesterterpene/triterpene synthase that converts C25- and C30-head-to-tail substrates to acyclic terpenes.[3)] Although sesterterpenes have also been isolated from limited species of plants, including *Aleuritopteris* (Pteridaceae) ferns,[4–6)] there have been no reports on the enzymes that are required for plant sesterterpene biosynthesis.

An analysis of the chemical structures of sesterterpenes from *Aleuritopteris* ferns predicted that they may have an analogous cyclization mechanism to that found with triterpenes. The fern sesterterpenes isolated to date have possessed either a tricyclic cheilanthane or tetracyclic 18-episcalarane skeleton, respectively represented by cheilanthenediol (**2**) and 18-episcalar-16-ene-6α,19-diol (**3**) (Fig. 1). Like the reaction with squalene, geranylfarnesol (**1b**) or its diphosphate ester (**1a**) is attacked by an electrophile at the terminal double bond to generate a carbocation, leading to a series of ring-forming reactions that yield a carbocation with a 6/6/6- or 6/6/6/6-fused ring system. This subsequently leads to the formation of **2** or **3** by carbocation quenching.

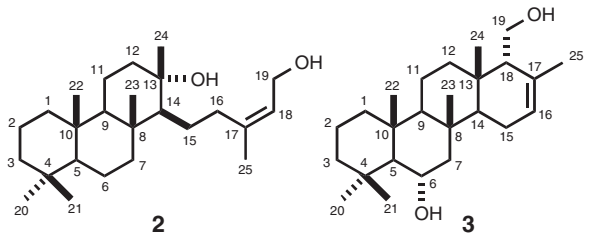

Fig. 1. Sesterterpenes, Cheilanthenediol (**2**) and 18-Episcalar-16-ene-6α,19-diol (**3**), Isolated from *Aleuritopteris* Ferns.

The structural and mechanistic analogies between sesterterpenes and triterpenes derived from ferns led us to hypothesize that sesterterpenes of *Aleuritopteris* ferns would be biosynthesized by triterpene synthases. In fact, early work by Rohmer's group has demonstrated that a squalene-tetrahymanol cyclase from a protozoon *Tetrahymena pyriformis* cyclized all-*E*-geranylfarnesyl methyl ether into a scalarane-type sesterterpene, although stereochemical details are not available.[7)] In addition, sesterterpenes have always been isolated from *Aleuritopteris* ferns together with triterpenes.[4–6)]

We have recently isolated cDNAs encoding triterpene synthases from ferns; these encoded enzymes with homology to bacterial triterpene synthases.[8–10)] Although fern triterpene synthases have been successfully expressed in yeast, the resulting recombinant proteins were not expressed at levels sufficient to perform

† To whom correspondence should be addressed. Fax: +81-42-721-1595; E-mail: sinozaki@ac.shoyaku.ac.jp

Abbreviations: COSY, ^{1}H-^{1}H correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; GC-MS, gas chromatography-mass spectrometry; HMBC, heteronuclear multiple-bond correlation; HPLC, high-performance liquid chromatography; HR-EIMS, high-resolution electron ionization mass spectroscopy; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; PCR, polymerase chain reaction; SHC, squalene-hopene cyclase; TLC, thin-layer chromatography

detailed mechanistic studies. Bacterial triterpene synthases catalyze direct cyclization of squalene to pentacyclic hopanoids. Among these, a squalene-hopene cyclase (SHC) from *Alicyclobacillus acidocaldarius* has been widely used for elucidating the cyclization mechanism of hopanoids, since an overexpression system was successfully established in *Escherichia coli* and produced an enzyme that was stable at ambient temperature.[11–13)]

To test our foregoing hypothesis, we analyzed the products formed by the cyclization of geranylfarnesols by the recombinant SHC enzyme. We found that this enzyme could generate cyclic sesterterpenes with a naturally occurring skeleton, 18-episcalarane, from non-canonical 2*Z*-geranylfarnesol (**4**), but not from all-*E*-geranylfarnesol (**1b**).

Materials and Methods

General experimental procedure. NMR spectra were obtained with an AV 600 spectrometer (Bruker Corporation, Billerica, MA, USA), using tetramethylsilane as an internal standard. Mass spectra were obtained with a JMS-700 MStation spectrometer (Jeol, Tokyo, Japan) in the electronic impact (EI) mode at 70 eV. GC-MS was performed with a 6890N Network GC system (Agilent Technologies, Loveland, CO, USA) and the JMS-700 MStation spectrometer (Jeol) in the EI mode (70 eV), using an HP-5 column (Agilent Technologies; 30 m length, 0.25 mm diameter). Samples were injected in the splitless mode at 50 °C, and after holding for 2 min at 50 °C, the oven temperature was increased at the rate of 20 °C/min to 260 °C, at which it was held for an additional 60 min. MS data from 50 to 500 Da were collected 5 min after injection until the end of the run. An HPLC analysis was performed with an 880-PU pump (Jasco, Tokyo, Japan) and an 830 RI detector (Jasco). Specific rotation was determined with a DIP-140 digital polarimeter (Jasco) in chloroform at 23 °C. Melting point (mp) data were measured by Micro Point apparatus (Yanagimoto, Kyoto, Japan) without correction.

Construction of the SHC expression plasmid. Transformant *E. coli* strain P2[14)] was cultured and propagated in an LB plate and LB medium containing 50 μg/mL of ampicillin. The plasmid harboring a triterpene synthase from *Alicyclobacillus acidocaldarius* (*shc*) (pUC-*shc*) was extracted with an Illustra PlasmidPrep mini spin kit (GE Healthcare, Uppsala, Sweden). To obtain full-length shc by PCR, the following primer set was designed (with the *Nde*I site and the *Bgl*II site underlined in the corresponding primers): forward primer (5′-GAATTCCATATGGCTGAGCAGTTGGTGGAAG-3′) and reverse primer (5′-GAATTCAGATCTTCACCTGCGCTCGATGGCTTG-3′). Synthetic oligonucleotides for the PCR primers were obtained from Nihon Bioservice (Saitama, Japan). Full-length shc was amplified by using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) with 20 ng of pUC-*shc* as a template and the above-mentioned primer set. The PCR conditions were as follows: 94 °C for 2 min, then 30 cycles of 98 °C for 15 s, 60 °C for 30 s, and 68 °C for 2 min. The resulting PCR product was digested with *Nde*I and *Bgl*II, and ligated into pET3a (EMD Millipore, Darmstadt, Germany) to obtain an expression plasmid (pET3-*shc*).

Expression of SHC and enzymatic assay. E. coli BL21(DE3) was transformed with the expression plasmid (pET3-*shc*). The transformant was grown for 20 h at 30 °C in an LB medium containing 50 μg/mL of ampicillin. The harvested cells were disrupted with BugBuster master mix (EMD Chemicals, San Diego, CA, USA). The cell-free lysate was then passed through a PD MidiTrap G-25 column (GE Healthcare) that had been equilibrated with 60 mM sodium citrate (pH 6.0) to yield a cell-free extract.

All-*E*-geranylfarnesol (**1b**) and 2*Z*-geranylfarnesol (**4**) were synthesized according to the literature[15)] with some minor modifications. The resulting cell-free extract as a crude enzyme solution was added to a reaction buffer that consisted of 60 mM sodium citrate (pH 6.0), 0.2% Triton X-100, and 1 mM all-*E*-geranylfarnesol (**1b**) or 2*Z*-geranylfarnesol (**4**). The reactions were incubated for 24 h at 60 °C, and terminated by adding an equal volume of 15% KOH in methanol. After extracting with the same volume of hexane (×2), the extract was concentrated and subjected to a TLC analysis developed with chloroform-methanol (19:1) or hexane-ethyl acetate (17:3), and then to GC-MS.

Purification of enzymatic products 5–10. The hexane extract obtained by the *in vitro* reaction of **1b** (246 mg) was loaded into a Silica Gel 60 column (50 g; Kanto Chemical Co., Tokyo, Japan) with hexane-ethyl acetate as an eluent to obtain two fractions. Fraction A containing **1b** and **5**, and fraction B containing **6–9** were independently combined and evaporated to dryness.

Fraction A (67 mg) was loaded into a silica gel column (25 g) and eluted with hexane-ethyl acetate to yield fraction A-1 containing **5** and fraction A-2 containing **1b**. Fraction A-1 was purified by normal-phase HPLC, using an AQUASIL SS-3251 column (8 mm diameter, 250 mm length; Senshu Scientific Co., Tokyo, Japan) with hexane-ethyl acetate (4:1) to give **5** (13.9 mg, $t_R = 19.6$ min), before recrystallization from acetonitrile.

Fraction B (35.7 mg) was loaded into a silica gel column (20 g) and eluted with hexane-ethyl acetate to yield three fractions, fraction B-1 containing **6** and **7**, fraction B-2 containing **8**, and fraction B-3 containing **9**. Fraction B-1 (7.2 mg) was subjected to Mega Bond Elut Si (5 g; Agilent Technologies), with elution by chloroform-methanol to give **6** and **7**. Product **6** was purified by reverse-phase HPLC in an ODS-3151D column (8 mm diameter, 150 mm length; Senshu Scientific Co.) with acetonitrile to give **6** (1.1 mg, $t_R = 9.5$ min). Product **7** was subjected to BondElut-Si (500 mg; Agilent Technologies), together with elution by chloroform-methanol to give **7** (1.6 mg), before recrystallization from acetone. Fraction B-2 (14.4 mg) was subjected to Mega Bond Elut Si (5 g; Agilent Technologies), with elution by chloroform-methanol to give **8** (13.6 mg), before recrystallization from acetone. Fraction B-3 (8.5 mg) was subjected to BondElut-Si (500 mg; Agilent Technologies), with elution by chloroform-methanol, and then purified by normal-phase HPLC in an AQUASIL SS-3251 column (8 mm diameter, 250 mm length; Senshu Scientific Co.) with chloroform-methanol (9:1) to give **9** (7.5 mg, $t_R = 12.6$ min).

The hexane extract obtained by the *in vitro* reaction of **4** (38 mg) was loaded into a silica gel column (30 g) with hexane-ethyl acetate as the eluent to yield **4** and **10** (0.2 mg).

Spectroscopic and physical data for enzymatic products 5–10. Scalar-17(25)-en-19-ol (**5**). ^{1}H-NMR δ_H (600 MHz, $CDCl_3$): 0.70 (3H, s, H-24), 0.80 (3H, s, H-21), 0.80 (3H, s, H-22), 0.80 (3H, s, H-23), 3.77 (1H, dd, $J = 10.8$, 10.8 Hz, H-19), 3.81 (1H, dd, $J = 3.6$, 10.8 Hz, H-19), 4.62 (1H, d, $J = 1.2$ Hz, H-25), 4.93 (1H, d, $J = 1.2$ Hz, H-25). ^{13}C-NMR δ_C (125 MHz, $CDCl_3$): 16.1 (C-24, q), 16.2 (C-23, q), 17.5 (C-22, q), 17.7 (C-11, t), 18.3 (C-6, t), 18.6 (C-2, t), 21.3 (C-21, q), 22.9 (C-15, t), 33.3 (C-20, q), 33.3 (C-4, s), 37.4 (C-10, s), 37.8 (C-16, t), 38.1 (C-8, s), 39.1 (C-13, s), 39.8 (C-1, t), 40.7 (C-12, t), 41.9 (C-7, t), 42.1 (C-3, t), 56.4 (C-5, d), 58.7 (C-19, t), 59.5 (C-18, d), 60.2 (C-14, d), 61.0 (C-9, d), 105.9 (C-25, t), 147.8 (C-17, s). EIMS *m/z* (%): 358 (M^+, 48), 343 (55), 327 (21), 272 (19), 259 (100), 191 (62), 163 (24), 137 (31), 123 (29), 95 (28). HR-EIMS (M^+) *m/z*: calcd. for $C_{25}H_{42}O$, 358.3236; found, 358.3239. $[\alpha]_D^{23}$ +5.8° (*c* 0.10, $CHCl_3$). Needle-like crystals, mp 167–169 °C.

Preluffariellolide A (**6**). ^{1}H-NMR δ_H (600 MHz, $CDCl_3$): 0.82 (3H, s, H-21), 0.94 (3H, s, H-20), 1.16 (3H, s, H-22), 1.61 (3H, s, H-24), 1.63 (3H, s, H-23), 1.68 (3H, s, H-25), 4.16 (2H, d, $J = 7.2$ Hz, H-19), 5.12 (1H, dd, $J = 6.0$, 6.0 Hz, H-14), 5.13 (1H, dd, $J = 7.2$, 7.2 Hz, H-10), 5.42 (1H, ddd, $J = 7.2$, 7.2, 1.2 Hz, H-18). ^{13}C-NMR δ_C (125 MHz, $CDCl_3$): 16.0 (C-24, q), 16.1 (C-23, q), 16.3 (C-25, q), 20.5 (C-2, t), 21.3 (C-21, q), 23.1 (C-22, q), 24.6 (C-7, t), 26.2 (C-15, t), 26.4 (C-11, t), 32.8 (C-20, q), 35.6 (C-4, s), 39.5 (C-16, t), 39.6 (C-12, t), 41.5 (C-3, t), 42.8 (C-8, t), 43.6 (C-1, t), 56.7 (C-5, d), 59.4 (C-19, t), 74.1 (C-6, s), 123.4 (C-18, d), 123.8 (C-14, d), 124.3 (C-10, d), 135.3 (C-13, s), 136.1 (C-9, s), 139.7 (C-17, s). EIMS *m/z* (%): 358 (M^+-H_2O, 8), 340 (8), 325 (3), 272 (5), 219 (9), 203 (21), 161 (18), 148 (50), 109 (51), 81 (100), 69 (43). HR-EIMS (M^+-H_2O) *m/z*: calcd. for $C_{25}H_{42}O$, 358.3236; found, 358.3239. $[\alpha]_D^{23}$ +6.0° (*c* 0.050, $CHCl_3$), oil.

14βH-Scalarane-17α,19-diol (**7**). ^{1}H-NMR δ_H (600 MHz, $CDCl_3$): 0.79 (3H, s, H-21), 0.81 (6H, s, H-20, H-22), 0.93 (3H, s, H-23), 0.99 (3H, s, H-24), 1.34 (3H, s, H-25), 3.83 (1H, dd, J = 10.8, 2.4 Hz, H-19), 3.92 (1H, dd, J = 10.8, 10.8 Hz, H-19). ^{13}C-NMR δ_C (125 MHz, $CDCl_3$): 16.8 (C-22, q), 18.6 (C-2, C-15, t), 19.6 (C-6 or C-11, t), 20.3 (C-6 or C-11, t), 21.0 (C-21, q), 21.8 (C-24, q), 23.3 (C-23, q), 24.1 (C-25, q), 33.2 (C-20, q), 33.4 (C-4, s), 35.9 (C-7, t), 36.7 (C-13, s), 37.4 (C-8, s), 37.8 (C-10, s), 40.2 (C-1, t), 40.9 (C-12, t), 42.4 (C-3, t), 44.3 (C-16, t), 51.3 (C-9, d), 54.1 (C-14, d), 57.3 (C-5, d), 61.6 (C-19, t), 62.3 (C-18, d), 75.2 (C-17, s). The assignments of C-6 and C-11 are exchangeable. EIMS m/z (%): 358 (M^+-H_2O, 84), 343 (29), 284 (29), 263 (37), 256 (33), 191 (72), 150 (57), 123 (100), 95 (83), 69 (66). HR-EIMS (M^+-H_2O) m/z: calcd. for $C_{25}H_{42}O$, 358.3236; found, 358.3232. $[\alpha]_D^{23}$ -25° (c 0.065, $CHCl_3$), amorphous powder.

Scalarane-17α,19-diol (**8**). ^{1}H-NMR δ_H (600 MHz, $CDCl_3$): 0.79 (3H, s, H-21), 0.84 (3H, s, H-20), 0.85 (3H, s, H-24), 1.15 (3H, s, H-23), 1.19 (3H, s, H-23), 1.36 (3H, s, H-25), 3.91 (1H, m, H-19), 3.93 (1H, m, H-19). ^{13}C-NMR δ_C (125 MHz, $CDCl_3$): 16.3 (C-24, q), 19.1 (C-2, t), 19.4 (C-6, t), 20.1 (C-15, t), 20.6 (C-11, t), 21.9 (C-21, q), 24.6 (C-25, s), 26.4 (C-22, q), 29.5 (C-23, q), 31.1 (C-7, t), 33.4 (C-4, s), 33.5 (C-20, q), 37.0 (C-13, s), 37.9 (C-1, t), 38.5 (C-10, s), 38.8 (C-8, s), 39.6 (C-12, t), 42.5 (C-3, t), 45.1 (C-16, t), 48.4 (C-5, d), 57.2 (C-9, d), 57.7 (C-14, d), 60.7 (C-18, d), 61.0 (C-19, t), 75.1 (C-17, s). EIMS m/z (%): 358 (M^+-H_2O, 100), 343 (29), 325 (13), 263 (37), 259 (25), 191 (63), 150 (44), 123 (62), 95 (38), 81 (25), 69 (18). HR-EIMS (M^+-H_2O) m/z: calcd. for $C_{25}H_{42}O$, 358.3236; found, 358.3232. $[\alpha]_D^{23}$ -32° (c 0.26, $CHCl_3$), plate crystals, mp 261–263 °C.

17E-Cheilanthenediol (**9**). ^{1}H-NMR δ_H (600 MHz, $CDCl_3$): 0.80 (6H, s, H-22, H-23), 0.81 (3H, s, H-21), 0.86 (3H, s, H-20), 1.13 (3H, s, H-24), 1.70 (3H, s, H-25), 4.16 (2H, m, H-19), 5.45 (1H, ddd, J = 7.2, 7.2, 1.2 Hz, H-18). ^{13}C-NMR δ_C (125 MHz, $CDCl_3$): 16.2 (C-22, q), 16.5 (C-25, q), 16.6 (C-23, q), 18.0 (C-6, t), 18.6 (C-2, t), 19.4 (C-11, t), 21.4 (C-21, q), 23.4 (C-15, t), 33.3 (C-20, q), 33.4 (C-4, s), 37.5 (C-10, s), 39.5 (C-8, s), 39.9 (C-1, t), 41.4 (C-7, t), 42.1 (C-3, t), 42.9 (C-16, t), 44.5 (C-12, t), 59.3 (C-19, t), 56.6 (C-5, d), 60.5 (C-9, d), 61.5 (C-14, d), 74.1 (C-13, s), 123.0 (C-18, d), 141.1 (C-17, s). EIMS m/z (%): 376 (M^+, 1), 358 (16), 313 (19), 260 (100), 245 (54), 191 (33), 137 (27), 95 (29), 81 (31), 69 (25). HR-EIMS M^+-H_2O m/z: calcd. for $C_{25}H_{42}O$, 358.3236; found, 358.3233. $[\alpha]_D^{23}$ -11° (c 0.028, $CHCl_3$), oil.

18-Episcalar-17(25)-en-19-ol (**10**). ^{1}H-NMR δ_H (600 MHz, $CDCl_3$): 0.80 (3H, s, H-21), 0.81 (3H, s, H-22), 0.82 (3H, s, H-23), 0.84 (3H, s, H-20), 0.96 (3H, s, H-24), 3.72 (1H, m, H-19), 3.76 (1H, m, H-19), 4.74 (1H, m, H-25), 4.91 (1H, m, H-25). EIMS m/z (%): 358 (M^+, 51), 343 (28), 327 (44), 260 (100), 259 (55), 191 (64), 135 (52), 123 (42). $[\alpha]_D^{23}$ -8° (c 0.02, $CHCl_3$).

Results and Discussion

Incubating all-*E*-geranylfarnesol (**1b**) with a bacterial triterpene synthase provided five cyclic sesterterpenes with tetra-, tri-, and monocyclic-ring systems at a yield of 24%. With the cell-free homogenate from *E. coli* transformants harboring the triterpene synthase gene (*shc*) from *Alicyclobacillus acidocaldarius*,[14] 150 mg of **1b** was incubated for 20 h under the reported conditions (pH 6.0 and 60 °C).[11] A TLC analysis of the hexane extract from the incubated mixture showed four spots in addition to that of unreacted **1b**. Each product was separated by silica gel column chromatography to yield five products (**5**–**9**), two of which (**7** and **8**) showed identical R_f values by TLC. The respective yields of **5**–**9** were 13.9, 1.1, 1.6, 13.6, and 7.5 mg.

The structures of the obtained products (Fig. 2A) were elucidated by NMR (^{1}H- and ^{13}C-NMR, DEPT135, HSQC, HMBC, COSY, and NOESY) and EIMS analyses. The major products were scalar-17(25)-en-19-ol (**5**), scalarane-17α-19-diol (**8**), and 17E-cheilanthenediol (**9**). Products **5** and **8** have a scalarane skeleton with 18αH which was unequivocally determined by NOESY data and biosynthetic reasoning as described later. Tricyclic product **9** was a 17E geometric isomer of naturally occurring **2**. The chemical synthesis of **9** has been reported.[16] The minor products were 14βH-scalarane-17α-19-diol (**7**) and a monocyclic sesterterpenediol (**6**).

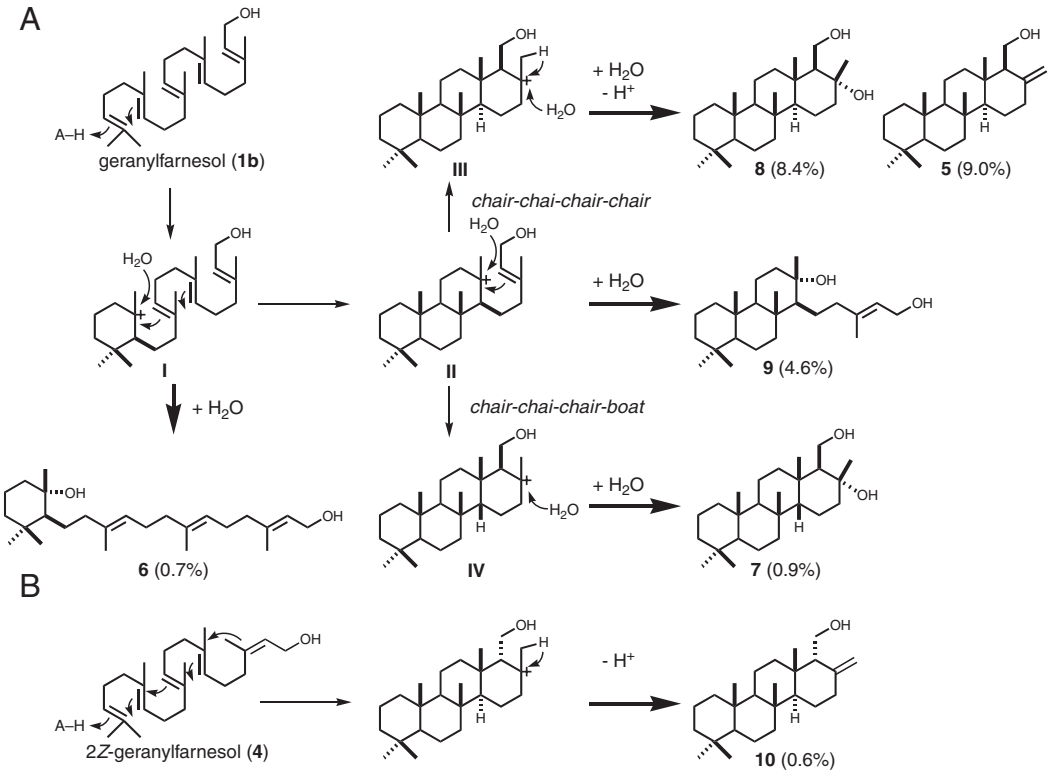

Fig. 2. Proposed Mechanism for the Conversion of (A) All-*E*-geranylfarnesol (**1b**) and (B) 2*Z*-Geranylfarnesol (**4**) to Cyclic Sesterterpenes (**5**–**9**, and **10**).

Product **7** shares a planar structure with product **8**. The relative stereochemistry of **7**, a 14-epimer of **8**, was determined by observing the NOE correlations (Me22/Me23, Me23/H14, H14/Me24, Me24/$CH_2$19, and $CH_2$19/Me25). Novel monocyclic **6** was named preluffariellolide A, because it seems to be a hypothetical intermediate of luffariellolides[17] which has been isolated from a Palauan sponge of *Luffariella* sp.

The reaction mechanisms for the formation of these compounds from **1b** by the bacterial triterpene synthase can be explained as follows (Fig. 2A): The enzyme folds the substrate in an all-pre-chair conformation. An electrophilic residue of the enzyme attacks the terminal double bond to initiate a series of 1,2-additions to double bonds, leading to a tetracyclic all-chair cationic intermediate (**III**) *via* a tricyclic one (**II**). Nucleophilic addition of a water molecule and direct abstraction of a proton respectively yield major products **5** and **8**. Similarly, **9** is produced from **II** by the addition of a water molecule. Folding of the substrate by this enzyme is not robust, allowing some quantity to be folded in a chair-chair-chair-boat conformation to produce sesterterpenediol with a 14βH-scalarane skeleton (**7**) *via* a tetracyclic intermediate with 14βH configuration (**IV**). Quenching of the monocyclic intermediate (**I**) finally gives **6**.

Interestingly, the stereochemistry of the major products (**5** and **8**) was 18αH, which is opposite to that of fern sesterterpene (**3**). The formation of **8** from **1b** requires an all-pre-chair conformation as depicted in Fig. 3. If the bacterial enzyme can also react with 2*Z*-geranylfarnesol (**4**) in the same way, 18-episcalar-16-ene-6α,19-diol (**3**), a naturally occurring sesterterpene, could possibly be produced. We therefore examined this possibility as described next.

Under the same conditions as those just described, incubating 2*Z*-geranylfarnesol (**4**, 28 mg) with the bacterial triterpene synthase afforded the product (**10**) in a 0.6% yield. The hexane extract from the reaction mixture was subjected to silica gel column chromatography, and elution with hexane-ethyl acetate afforded the product (**10**). An MS analysis of **10** showed fragment ions *m*/*z* 259 (derived from the A/B/C ring), *m*/*z* 191 (from the A/B ring), and *m*/*z* 123 and 135 (from the D ring) which are characteristics of a scalarane skeleton.[7,18] These fragment ions were also observed in enzymatic product **5** from **1b**. In addition, the MS data for both products **5** and **10** were almost identical, indicating their structural closeness. Nonetheless, the specific rotation of **10** ($[\alpha]_D^{23}$ -8°) was clearly distinguishable from the value for **5** ($[\alpha]_D^{23}$ $+5.8^\circ$). The ^{1}H-NMR spectrum of **10** revealed the presence of five methyl singlets (δ 0.80, 0.81, 0.82, 0.84, and 0.96), two exomethylene protons (δ 4.74 and 4.91), and two carbinol methylene protons (δ 3.72 and 3.76), suggesting **10** to be a scalarane-type sesterterpene. Four of the five methyl singlets (δ 0.80, 0.81, 0.82, and 0.84) were shifted very close to those of **5** (δ 0.80, 0.80, 0.80, and 0.84), while the remaining one (δ 0.96) was shifted distinctly from Me-24 of **5** (δ 0.72). It is evident that this chemical shift difference of Me-24 reflected the different stereochemistry in an adjacent carbinol side chain. We conclude from these results that the product from **4** was 18-episcalar-17(25)-en-19-ol (**10**, Fig. 2B).

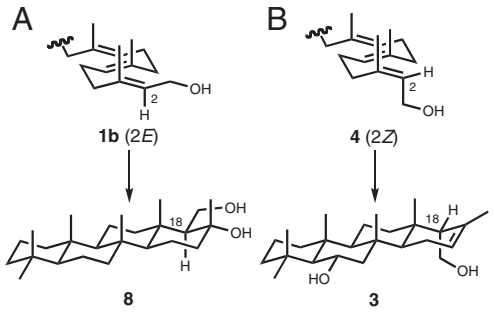

Fig. 3. Relationship between the Geometry of Precursors and the Stereochemistry of the C-18 Side Chain of Tetracyclic Sesterterpenes.

Cyclization of a *Z*-type linear precursor has rarely been observed in nature.[19–25] A few genes involved in the reaction have been reported, such as mono- and sesquiterpene cyclases from flowering plants.[20–22] The present study showed that SHC, a bacterial triterpene synthase, could cyclize 2*Z*-geranylfarnesol (**4**) into **10** with an 18-episcalarane skeleton. The isolation of *Aleuritopteris* cheilanthenediol (**2**), which has a double bond in the side chain with the same *Z*-geometry as 2*Z*-geranylfarnesol (**4**) at the corresponding 2(3) position, strongly suggests the presence of **4** in these ferns. If **4** and SHC-like triterpene synthase(s) are present in *Aleuritopteris* ferns, **2** and **3** can be synthesized without the requirement of additional sesterterpene synthase(s). In fact, there have been several reports of bifunctional terpene synthases that can accept two distinct classes of linear terpene precursors.[3,26–31] It is alternatively possible that sesterterpene synthase(s) are involved in sesterterpene biosynthesis in ferns, as is the case with ophiobolin F synthase from *Aspergillus clavatus*.[2]

This study has shown that SHC, a bacterial triterpene synthase, exhibited plasticity with respect to tolerating C25-polyprenols as a substrate. All of the enzymatic products described here, except for tricyclic sesterterpene (**9**), are novel compounds. It should be noted that the bacterial triterpene synthase catalyzed the cyclization of 2*Z*-geranylfarnesol (**4**) to produce the 18-episcalarane sesterterpene (**10**); these findings provide insight into sesterterpene biosynthesis in *Aleuritopteris* ferns.

Acknowledgment

We thank Professor Karl Poralla of Universität Tübingen for kindly presenting the *E. coli* clone containing *shc* from *A. acidocaldarius* (strain P2). We are also grateful to Dr. Takahisa Nakane of Showa Pharmaceutical University (SPU) for helpful advice on NMR measurements, to Yoichi Takase of SPU for technical advice about obtaining MS data, and to Katsuhiro Matsuzaki and Takefumi Nakamura of SPU for experimental assistance.

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