J. Antibiot. 59(8): 471–479, 2006

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Geosmin Biosynthesis in *Streptomyces avermitilis*. Molecular Cloning, Expression, and Mechanistic Study of the Germacradienol/Geosmin Synthase

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This paper is submitted in honor of the late Professor Kenneth L. Rinehart, a pioneer in the study of natural products and their biosynthesis.

Received: May 30, 2006 / Accepted: August 3, 2006

Abstract Geosmin (**1**) is responsible for the characteristic odor of moist soil. The Gram-positive soil bacterium *Streptomyces avermitilis* produces geosmin (**1**) as well as its precursor germacradienol (**3**). The *S. avermitilis* gene SAV2163 (*geoA*) is extremely similar to the *S. coelicolor* A3(2) SCO6073 gene that encodes a germacradienol/geosmin synthase. *S. avermitilis* mutants with a deleted *geoA* were unable to produce either germacradienol (**3**) or geosmin (**1**). Biosynthesis of both compounds was restored by introducing an intact *geoA* gene into the mutants. Incubation of recombinant GeoA, encoded by the SAV2163 gene of *S. avermitilis*, with farnesyl diphosphate (**2**) in the presence of Mg^{2+} gave a mixture of (4*S*,7*R*)-germacra-1(10)*E*,5*E*-diene-11-ol (**3**) (66%), (7*S*)-germacrene D (**4**) (24%), geosmin (**1**) (8%), and a hydrocarbon, tentatively assigned the structure of octalin **5** (2%). Incubation of this germacradienol/geosmin synthase with [1,1-$^2H_2$]FPP (**2a**) gave geosmin-d_1 (**1a**), as predicted. When recombinant GeoA from either *S. avermitilis* or *S. coelicolor* A3(2) was incubated with nerolidyl diphosphate (**8**), only the acyclic elimination products β-farnesene (**10**), (*Z*)-α-farnesene (**11**), and (*E*)-α-farnesene (**12**) were formed, thereby ruling out nerolidyl diphosphate as an intermediate in the conversion of farnesyl diphosphate to geosmin, germacradienol, and germacrene D.

Keywords sesquiterpene, biosynthesis, geosmin, *Streptomyces avermitilis*, *Streptomyces coelicolor*

Introduction

Geosmin (**1**) is a well-known, odoriferous metabolite produced by a wide variety of microorganisms, including *Streptomyces*, cyanobacteria, myxobacteria, and various fungi, as well as by higher plants such as liverworts and sugar beets [1~7]. Geosmin, with an especially low detection threshold of 10~100 parts per trillion, is responsible for the characteristic odor of freshly turned earth and is associated with unpleasant off-flavors in water, wine, and fish [8, 9].

Streptomyces avermitilis (*S. avermectinius* is a junior homotypic synonym of *S. avermitilis*), an industrially important Gram-positive bacterium used for the production of the potent anthelminthic macrolide avermectin, also produces geosmin. The 9.03-Mb linear chromosome of *S. avermitilis* harbors genes encoding at least six apparent terpene synthases that are individually implicated in carotene, hopanoid, and sesquiterpene biosynthesis [10, 11]. Among the sesquiterpene synthases, the 2178-bp *geoA* gene (SAV2163) encodes a putative protein of 725 amino acids (aa) with significant similarity to the *S. coelicolor*

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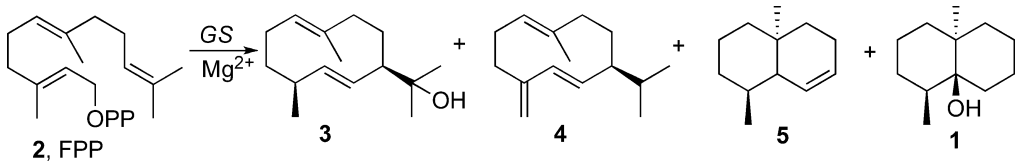
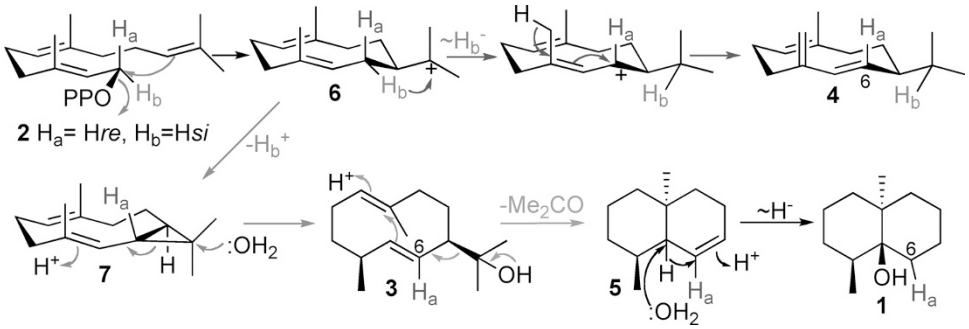


Scheme 1 Conversion of farnesyl diphosphate (FPP, **2**) to geosmin (**1**).



Scheme 2 Mechanism of the cyclization of FPP (**2**) to geosmin (**1**), germacradienol (**3**), germacrene D (**4**), and octalin **5**.

A3(2) SCO6073 gene product [12]. The latter protein has been shown to catalyze the Mg^{2+}-dependent conversion of farnesyl diphosphate (FPP, **2**) to a mixture of (4*S*,7*R*)-germacra-1(10)-diene-11-ol (**3**), germacrene D (**4**), a C_{12} hydrocarbon tentatively identified as octalin **5**, and geosmin (**1**) (Scheme 1) [13～15].

The production of both germacradienol (**3**) and germacrene D (**4**) by the *S. coelicolor* A3(2) germacradienol/geosmin synthase has been shown to involve the partitioning of a common reaction intermediate, proposed to correspond to carbocation **6**, in which loss of the original H-1*si* proton of FPP leads to formation of **3** while a competing 1,3-hydride shift of H-1*si* results in the generation of **4** (Scheme 2) [14]. The H-1*re* proton of FPP is retained at C-6 of both germacradienol and germacrene D, as well as at (most likely) C-6 of geosmin. The relative proportion of geosmin to germacradienol in the product mixtures can be enhanced by increasing either the amount of enzyme or the incubation time, suggesting that germacradienol is transiently released from the cyclase and then rebound before conversion to geosmin [15].

Although the *N*- and *C*-terminal halves of the *S. coelicolor* A3(2) germacradienol/geosmin synthase share a high level of sequence similarity, only the *N*-terminal domain catalyzes the conversion of FPP to germacradienol (**2**), while the *C*-terminal domain has no assigned biochemical function [13]. Molecular genetic experiments with *S. coelicolor* A3(2) deletion mutants have established that the *N*-terminal domain alone can support geosmin formation [14]. We now report the expression and biochemical characterization of the homologous *S. avermitilis* protein GeoA, and the confirmation that the recombinant enzyme is indeed a germacradienol/geosmin synthase. The function of the *geoA* gene product is also supported by the demonstration that a *geoA* deletion mutant does not produce either geosmin or germacradienol. Finally, we describe experiments that rule out the tertiary allylic isomer of FPP, nerolidyl diphosphate, as an intermediate in the enzymatic reaction.

Experimental

General Procedures

General analytical, molecular biological and biochemical methods were as previously described [13, 15, 16]. Recombinant germacradienol/germacrene D synthase, encoded by the SCO6073 gene of *S. coelicolor* A3(2) was expressed from *E. coli* BL21(DE3)pLysS/pRW31, resolubilized from inclusion bodies, purified to homogeneity, and assayed as previously described [13～15]. Farnesyl diphosphate, [1,1-$^2H_2$]farnesyl diphosphate and nerolidyl diphosphate were each prepared as previously described [17, 18].

GC-MS Analysis of Volatile Organic Products of *S. avermitilis*

The spores of wild-type and mutants (see below) of *S. avermitilis* were used to inoculated a 100-ml flask containing 10 ml of vegetative medium (glucose (0.5 g), soy

flour (1.5 g), and yeast extract (0.5 g) per 100 ml, pH 7.2) and the culture was allowed to grow while shaking at 30°C for 2 days. Geosmin production was observed in several media, but a synthetic medium was most suitable for the GC-MS analysis because many peaks were detected in the extract of the complex medium. A 0.1 ml of portion of the culture was used to inoculate a 100-ml flask containing 10 ml of synthetic medium consisting of glucose (60 g), $(NH_4)_2SO_4$ (2 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), K_2HPO_4 (0.5 g), NaCl (2 g), $FeSO_4 \cdot 7H_2O$ (0.05 g), $ZnSO_4 \cdot 7H_2O$ (0.05 g), $MnSO_4 \cdot 4H_2O$ (0.05 g), $CaCO_3$ (5 g), and yeast extract (2 g) per liter, pH 7.0. After incubation while shaking at 28°C for 4 days, the culture was filtered. The supernatant was extracted with 1 ml of *n*-hexane or pentane and the organic layer was dried over Na_2SO_4 and filtered through a 1-cm column of Na_2SO_4 in a Pasteur pipette. A 1 to 5-μl portion of the extract was analyzed by GC-MS (Shimadzu GC-17A, 70 eV, EI, positive ion mode; 30 m×0.25 mm neutral bond-5 capillary column (5% phenylmethylsilicon), using a temperature program of 50～280°C, temperature gradient of 20°C/minute). Geosmin (**1**) and germacradienol (**3**) were identified by comparison with the spectra of the corresponding reference compounds in the NIST/EPA/NIH MS Library (2002 version).

Molecular Cloning and Expression of Recombinant *S. avermitilis* Construction of *geoA*-deletion Mutant

Two segments were amplified by PCR from *S. avermitilis* cosmid CL_228_H03 (http://avermitilis.ls.kitasato-u.ac.jp/). A segment upstream of *geoA* (nt 2,639,510 to 2,637,781) was amplified using the forward (5′-CTCGAG**AAGCTT**TGTCGTTGCCCGCGACCGTCAG-CA-3′) and reverse (5′-CTCGAG**TCTAGA**CATG-GCCGGGCCCTACCCAGGGCC-3′) primers to introduce *Hin*dIII and *Xba*I restriction sites (bold), respectively. The forward (5′-CTCGAG**TCTAGA**TGAGGGGGGGGA-CAGGCGGCTG-3′) and reverse (5′-CTCGAG**AAG-CTT**GCCGCTCAGGCCGAGCAGGTGCAT-3′) primers were used for the amplification of a second segment downstream of *geoA* (nt 2,635,564 to 2,633,807), with introduction of *Xba*I and *Hin*dIII sites (bold), respectively. These two amplified DNA segments were digested with *Hin*dIII and *Xba*I then digested segments were ligated together into *Hin*dIII-cut pKU250 and transformed into *E. coli* DH5α. The resultant plasmid was digested with *Xba*I and the linearized plasmid was ligated with the *Xba*I-fragment of the *aad3″* streptomycin/spectinomycin resistance cassette. The plasmid pKU250::upstream-*aad3″*-downstream construct in *E. coli recA dcm*/pUB307 was introduced into *S. avermitilis* by conjugation. After exoconjugants were selected by vector marker (thiostreptone resistance), *geoA*-deletion mutants, generated by double homologous recombination upstream and downstream of *geoA*, were obtained by selection for thiostreptone-sensitivity and streptomycin/spectinomycin-resistance.

Transfer of an Extra-copy of *geoA* Gene into *S. avermitilis geoA*-deletion Mutants

Cosmid CL_228_H07 was digested with *Kpn*I and the 4,594-bp segment (nt 2,634,026 to 2,638,620) carrying *geoA* was purified by agarose gel electrophoresis and ligated with the *Kpn*I-cut ϕC31-based integrating vector carrying *aphII* as selectable marker. The resulting plasmid was used to transform *E. coli recA dcm*/pUB307::Tn*7* and then transferred into the above-described *S. avermitilis ΔgeoA::aad3″* by conjugation. The *S. avermitilis ΔgeoA::aad3″*/*geoA* exoconjugants, were obtained by selection for neomycin resistance.

Germacradienol/Geosmin Synthase (GeoA, SAV2163p)

The *geoA* gene (SAV2163) was amplified by PCR from template DNA from *S. avermitilis* cosmid CL_228_H03 using the forward (5′-GGTAGG**GAATTC**CCATGAC-GCAGCCGTTCC-3′) and reverse (5′-CTGTCC**CTCGAG**-TCAGCGCGCCACC-3′) primers to introduce *Eco*RI and *Xho*I restriction sites (bold) flanking the normal start and stop codons, respectively. The resulting amplicon and the pET21d(+) vector were digested separately with *Eco*RI and *Xho*I before ligation with T4 DNA ligase (15 : 1 insert:vector, 4°C, 13 hours) and transformation of electrocompetent *E. coli* XL-1 Blue cells. The resultant pXH17 plasmid was purified using a QIAprep Miniprep Kit and the sequence of the insert was confirmed by direct sequencing of both strands. (This construct appends the peptide MASMTGGQQMGRIRIP upstream of the native methionine start codon.) The purified pXH17 plasmid was then used to transform *E. coli* BL21(DE3) which was grown at 37°C in LB media (400 ml, 260 rpm) supplemented with ampicillin (100 mg/liter) and chloramphenicol (34 mg/liter) to an OD_{600}～0.3, then induced by the addition of IPTG (0.4 mM). After a further 5 hours at 30°C, the cells were harvested by centrifugation. The cell pellet was resuspended in 40 ml of lysis buffer (50 mM Tris·Cl, 1 mM EDTA, 20% glycerol (v/v), pH 8.2). Lysozyme (1.5 mg/ml), β-mercaptoethanol (10 mM), benzamidine (0.2 mM) and PMSF (0.2 mM) were added to the cell suspension, which was then incubated at 34°C, with shaking at 100 rpm for 10 minutes. Benzonase (1 μl/liter), $MgCl_2$ (10 mM) and Triton X-100 (0.1%) were added and incubation was continued at 34°C for an additional 7 minutes.

The insoluble fraction containing inclusion bodies was collected by centrifugation (7500 *g*, 10 minutes), washed with 50 ml of lysis buffer, and recentrifuged (7500 *g*, 15 minutes). The harvested inclusion bodies (1.5 g) were resuspended in 16 ml of lysis buffer and added dropwise with stirring at 0°C to 200 ml of 20 mM NaOH containing 5 mM β-mercaptoethanol and 0.02% (v/v) Triton X-100. After centrifugation to remove traces of residual insolubles (15000 *g*, 10 minutes), the basic solution was added at 8 ml/minute with slow stirring at 4°C to 4 liters of refolding buffer (50 mM Tris·Cl, 20% glycerol, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 8.2). The solution was allowed to equilibrate for 6 hours without further stirring, then adsorbed onto Q-Sepharose resin (70 ml, fast-flow) over 3 hours with stirring. The supernatant was decanted from the resin, which was then poured onto a column with a sintered filter, washed with refolding buffer containing 100 mM NaCl (Buffer A) and then eluted with 1 liter of a linear gradient of 100 mM to 500 mM NaCl in lysis buffer (Buffer B). Fractions were monitored by micro-Bradford assay [19] and SDS-PAGE and those containing the purest amounts of the desired protein (M_r ~83000) were combined, diluted 5.3-fold in Buffer A and loaded on a 50-ml Q-Sepharose (fast-flow) at 3 ml/minute. After washing with 150 ml of Buffer A, the column was eluted with 1.5 liters of a linear gradient of Buffer A to Buffer B. The purest fractions were pooled and concentrated by ultrafiltration to 3 ml (4.06 mg/ml, based on uv at 280 nm). The purified protein, which included a vector-derived hexadecapeptide upstream of the native *N*-terminal methionine, exhibited a single band (M_r ~83000) on SDS-PAGE, consistent with the predicted MW of 83402 Da.

Assay of *S. avermitilis* Germacradienol/Geosmin Synthase (GeoA)

The purified recombinant SAV2163 protein was assayed as previously described for the *S. coelicolor* germacradienol/geosmin synthase [13]. To determine the steady-state kinetic parameters, a series of assays was performed in 10-ml glass tubes containing 1 ml of assay buffer (50 mM Tris·Cl, 20% glycerol, 10 mM $MgCl_2$, pH 8.2) and using a range of [1-^{3}H]-FPP concentrations (8, 16, 40, 100, 200, 300, 500, 750, and 1000 nM, 200 mCi/mmol). The reactions, which were carried out in parallel, were initiated by the addition of 2 μl 40-fold diluted GeoA protein solution (200 ng, 2.45 pmol), then immediately overlaid with 1.2 ml of hexane. After 20 minutes incubation at 30°C (less than 10% conversion in all cases), the reaction was quenched by the addition of 75 μl of 0.5 M EDTA (pH 8.1). Control incubations utilized 40 nM and 500 nM [1-^{3}H]FPP in the absence of enzyme. Ether (100 μl) was added and the mixture was vortexed for 30 seconds. The organic layer was passed through a 1-cm SiO_2 column in a Pasteur pipette directly into a scintillation vial containing 7 ml of Opti-Fluor. The aqueous layer was extracted with an additional 1.2 ml of 11 : 1 hexane : ether which was added to the scintillation vial after passage through the SiO_2 column which was then washed with 2×0.75 ml of ether. The steady-state kinetic parameters, k_{cat} and K_m, for the conversion of FPP to combined hydrocarbons and alcohols were calculated by direct fitting of the liquid scintillation data to the Michaelis-Menten equation by non-linear least squares regression using Kaleidagraph V3.6 (Adelbeck Software, Reading, PA, USA).

Metal Ion Dependence

To test divalent cation dependence of GeoA, a series of assays were carried out as above, replacing $MgCl_2$ by 10 mM concentrations of each of the following salts: $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$.

Product Analysis

Preparative scale incubations and GC-MS product analysis were carried out as previously described using 1 mg (12 nmol) of *S. avermitilis* germacradienol/geosmin synthase in 4.5 ml of assay buffer containing 600 ng (270 nM) of FPP and overlaid with 3 ml of HPLC-grade pentane [15]. The incubations were carried out for 20 minutes, 1 hour, and 3.5 hours at 30°C in 18×150 mm glass tubes fitted with a rubber septum. After extraction of the reaction mixture with the pentane layer, the mixtures were extracted with an addition two 3-ml portions of pentane. The combined organic layers were dried over $MgSO_4$, filtered through a 2.5-cm column of $MgSO_4$ in a Pasteur pipette, and concentrated under reduced pressure at 0°C to ~100 μl. A 1-μl portion of the concentrated extract was analyzed by GC–MS (JEOL JMS-600H, 70 eV, EI, positive ion mode; 30 m×0.25 mm HP5MS capillary column, using a temperature program of 60~280°C, temperature gradient of 25°C/minute). The following products were detected for the 3.5-hours incubation: presumed octalin **5** (r.t. 5.18 minutes, $[M]^+$ *m*/*z* 164, base peak *m*/*z* 149), geosmin (**1**) (r.t. 6.20 minutes, $[M]^+$ *m*/*z* 182, base peak *m*/*z* 112), germacrene D (**4**) (r.t. 6.43 minutes, $[M^+]$ *m*/*z* 204, base peak, *m*/*z* 161), and germacradienol (**3**) (r.t. 7.29 minutes, $[M^+]$ *m*/*z* 222, base peak, *m*/*z* 82), as well as a minor sesquiterpene hydrocarbonof asyet unassigned structure (r.t. 6.35 minutes, $[M^+]$ *m*/*z* 204, base peak, *m*/*z* 107). Only germacradienol and germacrene D were detected in the 20 minutes incubation mixture.

Incubation with Nerolidyl Diphosphate

Recombinant *S. coelicolor* germacradienol/geosmin synthase (0.4 mg, 4.8 nmol) was incubated as described above in 4.8 ml of assay buffer containing 600 ng (120 nM) of NPP (**8**) and overlaid with 3 ml of HPLC-grade pentane for 3 hours. A 1-μl portion of the concentrated pentane extract was analyzed by GC-MS (Hewlett-Packard Series 2 GC-MSD, 70 eV, EI, positive ion mode; 30 m×0.25 mm HP5MS capillary column, using a temperature program of 50∼280°C, temperature gradient of 20°C/minute and solvent delay of 4.0 minutes). The following products were detected: β-farnesene (**10**, r.t. 9.58 minutes, $[M]^+$ *m/z* 204, base peak *m/z* 69), (3*Z*)-α-farnesene (**11**, r.t. 9.78 minutes, $[M^+]$ *m/z* 204, base peak, *m/z* 93); and (3*E*)-α-farnesene (**12**, r.t. 9.88 minutes, $[M^+]$ *m/z* 204, base peak, *m/z* 93). Each compound was identified by comparison with the spectra of the corresponding reference compounds in the NIST/EPA/NIH MS Library (2002 version).

Results

Streptomyces avermitilis produces the typical strong odor of moist soil when grown in several different media. GC-MS analysis of the *n*-hexane or pentane extract of a liquid culture revealed two main peaks (Fig. 1B), rt 9.89 minutes $[M^+]$ *m/z* 182 and 11.42 minutes $[M^+ + 1]$ *m/z* 223, that were identical to geosmin (**1**) and germacradienol, (**3**), respectively, by comparison with authentic samples of each compound (Fig. 1).

The *S. avermitilis* open-reading frame, *geoA* (SAV2163), encodes a protein with 77% identity and 87% similarity over 713 aa to the *S. coelicolor* A3(2) germacradienol/gesomin synthase (SCO6073p). Interestingly, the level of identity between the catalytically functional *N*-terminal domains of the *S. avermitilis* and *S. coelicolor* A3(2) proteins is even greater: 89% identity and 98% similarity over 327 aa. Both proteins harbor an aspartate-rich ^{84}**DD**HFL**E** motif and a downstream NSE triad

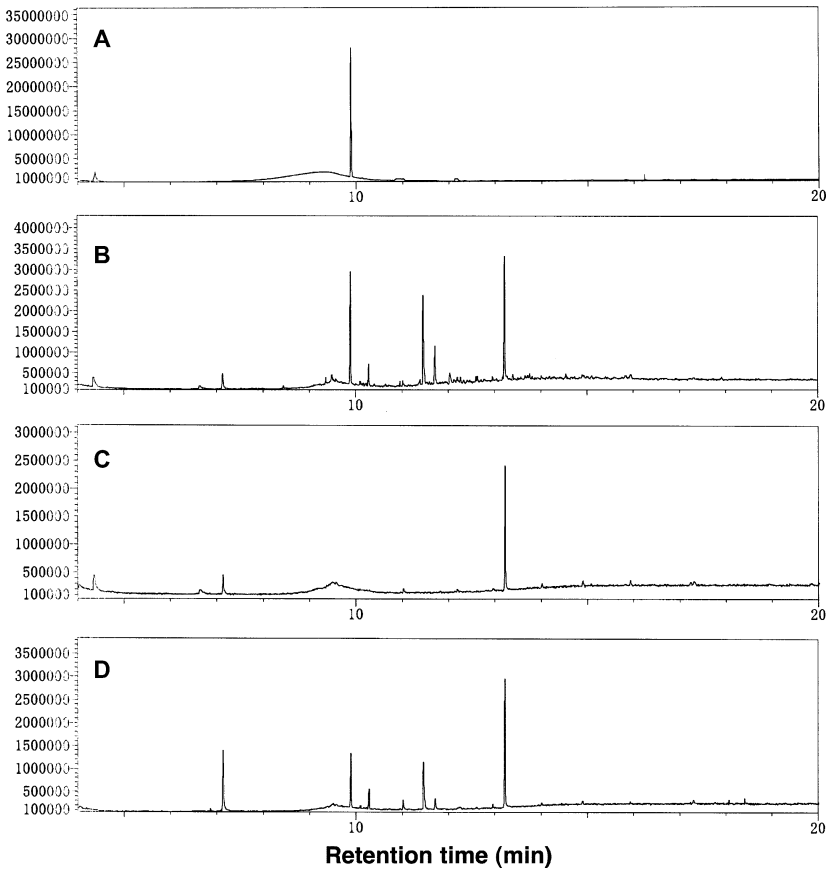


Fig. 1 GC-MS analysis of extracts from cultured broth of *S. avermitilis* and its mutants.

(A) Authentic sample of geosmin (**1**, rt 9.89 minutes, $[M]^+$ *m/z* 182), (B) *n*-hexane extract from wild type strain of *S. avermitilis*. The peak at 11.42 minutes is germacradienol (**3**, $[M^+ + 1]$ *m/z* 223). (C) *n*-hexane extract from *ΔgeoA::aad3″* and (D) *n*-hexane extract from exoconjugant of *geoA*-deletion mutant carrying an extra-copy of *geoA*. (The peak at rt 13.30 minutes is diethyl phthalate ($[M]^+$ *m/z* 222).)

^{224}**N**DLF**S**YQR**E**, the universally conserved Mg^{2+}-binding domains that are found in all known sesquiterpene synthases.

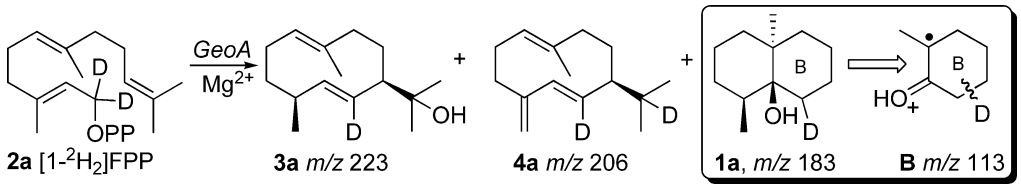
We constructed a *geoA*-deletion mutant by homologous recombination in order to confirm the involvement of the GeoA in geosmin biosynthesis. Two 1.8-kb segments corresponding to both upstream and downstream regions of *geoA* were amplified by PCR and ligated together into an *E. coli*/*Streptomyces* conjugative vector. A resistance gene (*aad3″*) was inserted between these two amplified segments. After introducing the recombinant plasmid into wild-type *S. avermitilis* by selection of the vector marker, thiostreptone-resistance, homologous recombination between the chromosome and the recombinant plasmid gave the desired *geoA*-deletion mutants, which were obtained by selection for the thiostreptone-sensitive and streptomycin/spectinomycin resistance phenotype. The *geoA* deletion mutants failed to produce either geosmin (**1**) or germacradienol (**3**) (Fig. 1C). Introduction of an extra copy of wild-type *geoA* into these deletion mutants restored the production of both geosmin (**1**) and germacradienol (**3**) (Fig. 1D), indicating that GeoA is essential for the formation of both geosmin (**1**) and germacradienol (**3**).

We next used PCR to amplify the 2178-bp coding region of SAV2163, using DNA from *S. avermitilis* cosmid CL_228_H03 as template, while introducing *Eco*RI and *Xho*I restriction sites at the respective 5′- and 3′-termini of the ORF. After ligation of the amplified DNA into the *Eco*RI/*Xho*I cloning sites of the pET21d(+) expression vector, the derived plasmid, pXH17, was purified from *Escherichia coli* XL-1 Blue and used to transform the T7 RNA polymerase-based expression host *E. coli* BL21(DE3). The resultant recombinant protein was obtained as insoluble inclusion bodies that were readily solubilized using 20 mM NaOH and purified to homogeneity, using a protocol previously developed for the homologous *S. coelicolor* A3(2) germacradienol/geosmin synthase [13]. A total of 12 mg of purified recombinant GeoA (SAV2163p) was obtained from 400 ml of culture.

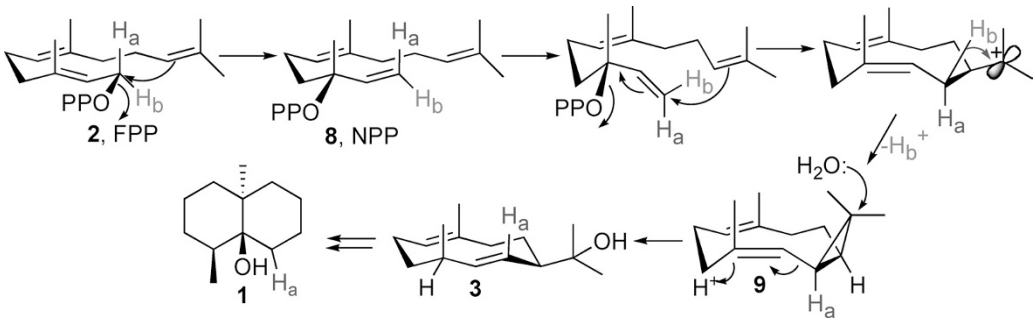
Incubation of 2.5 nM GeoA with 270 nM FPP (**2**) for 3.5 hours at 30°C followed by GC-MS analysis of the pentane-soluble extract revealed the formation of germacradienol (**3**), germacrene D (**4**), octalin **5**, and geosmin (**1**) in a ratio of 66 : 24 : 2 : 8. The individual components were unambiguously identified by direct comparison of GC-retention times and EI-mass spectra with those of authentic **3**, **4**, **5**, and **1** that were generated from FPP in a separate incubation with recombinant *S. coelicolor* A3(2) germacradienol/geosmin synthase [15]. A small amount (3%) of an additional, unidentified sesquiterpene hydrocarbon, *m*/*z* 204, was also detected in the product mixture. As previously observed for the homologous *S. coelicolor* enzyme, no geosmin was detected after only 20 minutes incubation time, while both the absolute yield and the relative proportion of geosmin increased with longer incubation times. Incubation of *S. avermitilis* GeoA with [1,1-$^2H_2$]FPP (**2a**) gave, as expected, germacradienol-d_1 (**3a**) ([M+1] *m*/*z* 223), germacrene D-d_2 (**4a**) ([M+2] *m*/*z* 206, [M+2−C_3H_6D] *m*/*z* 162), and geosmin-d_1 (**1a**) ([M+1] *m*/*z* 183; base peak *m*/*z* 113). As previously observed, the deuterium atom in **1a** could be assigned to ring B, as deduced from the characteristic MS fragment at *m*/*z* 113 ($C_7H_{11}DO$) (Scheme 3) [15, 20].

The activity of recombinant GeoA, which was tested over a pH range from 5.4∼9.5, showed optimal activity between pH 7.6 and 9.0. The reaction showed an absolute requirement for a divalent cation, Mg^{2+} (10 mM) being preferred, with Fe^{2+} and Zn^{2+} exhibiting 50% activity, and Co^{2+}, Cu^{2+}, or Mn^{2+} each showing 10∼20% activity. Neither Ca^{2+} nor Ni^{2+} supported turnover of FPP. The steady-state kinetic parameters for recombinant GeoA were determined using [1-^{3}H]-FPP over a concentration range of 8 nM to 2 μM. The calculated k_{cat} for the formation of combined pentane soluble hydrocarbons and alcohols was $3.10\pm0.09\times10^{-3}\,s^{-1}$, with a K_m for FPP of 75±9 nM, similar to the k_{cat} of $6.2\pm0.5\times10^{-3}\,s^{-1}$ and K_m of 62±8 nM previously established for the recombinant *S. coelicolor* A3(2) enzyme [13].

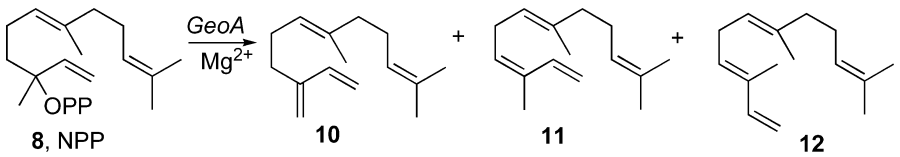
The partitioning of a common cyclization intermediate in the formation of germacradienol (**3**) and germacrene D (**4**), involving a competition between loss of the original H-1*si* of FPP and a 1,3-hydride-shift, has important mechanistic and stereochemical implications. As illustrated in Scheme 2, direct cyclization of (*E*,*E*)-FPP followed by loss of H-1*si* would generate the trans-fused bicyclic intermediate, isolepidozene (**7**), the presumed precursor of



Scheme 3 Cyclization of [1,1-$^2H_2$]FPP (**2a**) to geosmin (**1a**) and MS analysis.



Scheme 4 Hypothetical intermediacy of nerolidyl diphosphate (**8**, NPP) and bicyclohelminthogermacrene (**9**) in geosmin formation.



Scheme 5 Incubation of NPP (**8**) with germacradienol/geosmin synthase.

germacradienol, while prior isomerization to the tertiary allylic isomer, nerolidyl diphosphate (NPP, **8**) would result in the intermediacy of the stereoisomeric, bicyclohelminthogermacrene (**9**) (Scheme 4) [14]. To distinguish between these two possibilities, we carried out separate incubations of NPP with 1 μM recombinant germacradienol/geosmin synthases from both *S. coelicolor* A3(2) and *S. avermitilis* and analyzed the resulting product mixtures by GC-MS. In place of **3**, **4**, **5**, and **1**, the normal products of FPP cyclization, the only compounds generated from NPP were the acyclic elimination products β-farnesene (**10**), (*Z*)-α-farnesene (**11**), and (*E*)-α-farnesene (**12**) (Scheme 5). Nerolidyl diphosphate is therefore firmly excluded as an intermediate in the enzymatic formation of germacradienol, germacrene D, and geosmin.

Discussion

Germacradienol (**3**) was first identified in 1995 as a cometabolite of geosmin in *S. citreus* [4, 21]. Since that time, germacradienol has frequently been observed as a cometabolite of geosmin in streptomycetes, myxobacteria, and liverworts, often accompanied by germacrene D (**4**) [5, 6, 22]. Consistent with the demonstration that *S. coelicolor* A3(2) harbors a germacradienol synthase encoded by the SCO6073 gene, and that this gene is essential for geosmin production, we and others originally proposed a variety of multistep biosynthetic pathways involving both oxidative and reductive transformations to account for the evident conversion of germacradienol to geosmin [4, 13, 20, 23]. Very recently, we have made the unexpected discovery that the protein encoded by SCO6073 can catalyze the entire conversion of FPP to geosmin itself, without requirement for any additional enzymes or redox cofactors [15]. This germacradienol/geosmin synthase is therefore both necessary and sufficient to support geosmin biosynthesis from FPP.

Both germacradienol (**3**) and geosmin (**1**) were found in the culture broth of *S. avermitilis*. The observed ~5 : 6 ratio of germacradienol/geosmin was essentially the same in the several media examined. A *geoA* (SAV2163) deletion mutant constructed by homologous recombination produced neither geosmin nor germacradienol, while production of both compounds could be restored by complementation of the mutation by introducing an extra copy of the *geoA* gene, confirming that GeoA is essential for geosmin biosynthesis.

We have established that the GeoA protein of *S. avermitilis*, encoded by *geoA*, which exhibits a high degree of sequence similarity to the *S. coelicolor* A3(2) enzyme SCO6073p, catalyzes the predicted conversion of FPP to a mixture of germacradienol (**3**), germacrene D (**4**), and geosmin (**1**), as well as the presumed intermediate, octalin **5**. Recombinant *S. avermitilis* GeoA has a product profile, divalent metal ion dependence, pH optimum, and steady-state kinetic parameters that are essentially the same as those previously determined for the *S. coelicolor* A3(2) germacradienol/geosmin synthase [15]. Moreover, incubation of recombinant *S. avermitilis* GeoA with [1,1-

$^2H_2$]-FPP gave deuterated germacradienol (**3a**), germacrene D (**4a**), and geosmin (**1a**) with labeling patterns that were identical to those previously obtained with the *S. coelicolor* A3(2) synthase [15]. Significantly, for both enzymes, the relative proportion of **1** with respect to germacradienol was enhanced by increasing the concentration of synthase or by employing longer incubation times. One would not expect such a result if both geosmin and germacradienol were to be generated by partitioning of a single series of enzyme-bound intermediates. Instead, it appears likely that a substantial fraction of the initially generated germacradienol is released into solution before being subsequently rebound and further converted to geosmin.

The finding that nerolidyl diphosphate is converted only to the acyclic farnesene isomers **10**, **11**, and **12** by both the *S. avermitilis* and the *S. coelicolor* A3(2) germacradienol/geosmin synthase rules out NPP as an intermediate in the conversion of FPP to germacradienol, germacrene D, and geosmin. Since the 2,3*E*-double bond of FPP therefore does not undergo geometric isomerization in the course of the enzyme-catalyzed cyclization, the established loss or migration of H-1*si* leads to the inference that the trans-fused bicyclogermacrene isomer, isolepidozene (**7**), would be the common sesquiterpene precursor of both germacradienol (**3**) and geosmin (**1**). Although we have not yet detected **7** as a product of the germacradienol/geosmin synthase reacton, (−)-isolepidozene (**7**) has been reported as a metabolite of several liverworts, including *Preissia quadrata* [24]. It is also noteworthy that the corresponding alcohol, (4*S**,5*S**,6*R**,7*R**)-1(10)*E*-lepidozen-5-ol (**13**) has been isolated from the liverwort *Dumortiera hirsuta* in which it is a cometabolite of germacradienol (**3**) (Fig. 2) [22]. Both alcohols compounds represent alternative hydration products of isolepidozene. Lepidozenol has also been isolated from the liverwort *Trocholejeunea sandvicensis* [25].

The finding that *S. avermitilis* GeoA converts farnesyl diphosphate to a mixture of germacradienol, germacrene D, and geosmin reinforces the recent discovery that the formation of geosmin from farnesyl diphosphate is mediated by a single enzyme. A similar *geoA* open reading frame that shows 66% identity and 84% similarity over 718 aa to the *S. avermitilis* GeoA has also been found in the recently determined genomic sequence of *S. peucetius* (Prof. J. K. Sohng, personal communication) [26]. The mechanistic details of the remarkable reaction catalyzed by these geosmin synthases are currently under active investigation.

Acknowledgments This work was supported by NIH grant GM30301 to DEC, by Grant of the 21st Century COE Program, Ministry of Education, Culture, Sports, Science and Technology, Japan to H.I. and S.O., Grant-in-Aid for Scientific Research on Priority Areas from Ministry of Education, Culture, Sports, Science and Technology, Japan No. 17019009 to H.I., and by Grant-in-Aid for Scientific Research of the Japan Society for the Promotion of Science No. 17510168 to H.I. We would like to thank Jiaoyang Jiang for helpful advice in the preparation of the manuscript.

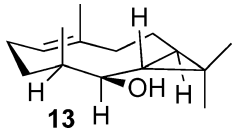


Fig. 2 Lepidozenol (**13**) isolated from the liverworts *D. hirsuta* and *T. sandvicensis*.

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