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Geosmin Biosynthesis. *Streptomyces coelicolor* Germacradienol/Germacrene D Synthase Converts Farnesyl Diphosphate to Geosmin

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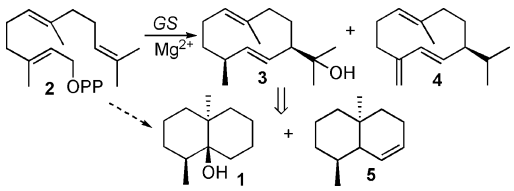
The characteristic odor of moist soil is due to the presence of two terpene derivatives, methyl isoborneol and geosmin (**1**).[1,2] Produced in soil primarily by streptomycetes—ubiquitous Gram-positive, filamentous, saprophytic bacteria—geosmin has also been isolated from a variety of cyanobacteria, fungi, and liverworts.[3] Geosmin has an exceptionally low detection threshold of the order of 10−100 ppt. Besides its pleasant, characteristic earthy aroma, geosmin is also associated with an undesirable musty odor or off flavor in drinking water, as well as in wine, fish, and other food stuffs, making its detection and elimination important in the management of water and food quality.[4]

The structure of geosmin (**1**) was first established as *trans*-1,10-dimethyl-*trans*-9-decalol by Gerber[1c] who detected the volatile oil in 17 different species of *Streptomyces* and a blue green alga following its initial isolation from *S. griseus*.[1] Shortly thereafter, Bentley provided evidence that the C_{12} metabolite geosmin was likely a degraded sesquiterpene, based on the apparent incorporation of both [1-^{14}C]- and [2-^{14}C]acetate into geosmin by strains of *S. antibioticus*.[5]

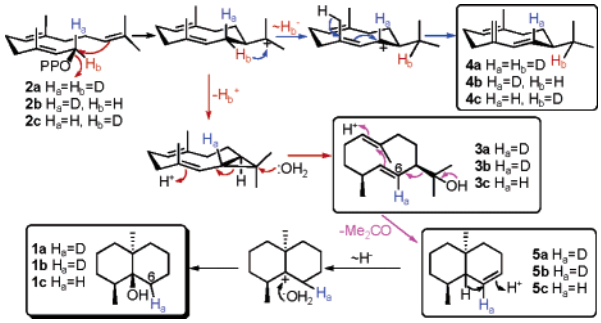
We recently reported expression in *Escherichia coli* of a 2181-bp gene from *S. coelicolor* A3(2) (SCO6073, SC9B1.20) to give a 726 amino acid protein with significant similarity in both the N-terminal and C-terminal halves to the well-characterized sesquiterpene synthase, pentalenene synthase.[6] The full-length recombinant protein was shown to catalyze the Mg^{2+}-dependent conversion of farnesyl diphosphate (FPP, **2**) to a 85:15 mixture of (4*S*,7*R*)-germacra-1(10)*E*,5*E*-diene-11-ol (**3**) and the sesquiterpene hydrocarbon (−)-(7*S*)-germacrene D (**4**), with a k_{cat} of 6×10^{-3} s^{-1} and a K_m for FPP of 60 nM (Scheme 1).[6−8] Both products result from partitioning of a common intermediate, with formation of germacradienol involving loss of the H-1*si* proton of FPP (**2**), H-1*re* being retained at C-6 of **3**, while formation of germacrene D (**4**) involves a competing 1,3-hydride shift of H-1*si* to the isopropyl side chain (Scheme 2).[7] Expression of the N-terminal (366 amino acid) domain of the SCO6073 protein gave a fully functional germacradienol synthase with steady-state catalytic parameters similar to those of the full-length protein.[6] The expressed C-terminal domain of SC06073 had no detectable FPP cyclase activity.[6]

Independently, Chater and co-workers reported that deletion of the entire SCO6073 gene from *S. coelicolor* A3(2) results in the complete loss of geosmin production.[9] In-frame deletion of the N-terminal domain of SCO6073 also abolished geosmin formation, while deletion of only the C-terminal domain had no apparent effect on geosmin biosynthesis. The combined biochemical and molecular genetic evidence thus established conclusively that the SCO6073 gene encodes a germacradienol synthase and that this enzyme catalyzes an essential step in the biosynthesis of geosmin. We originally proposed that formation of geosmin (**1**) from germacradienol (**3**) would involve a multistep biochemical sequence requiring both oxidative and reductive transformations catalyzed by several hypothetical enzymes.[6] Multistep redox pathways have also been

Scheme 1



Scheme 2. Mechanism of Cyclization of FPP (**2**) to Germacradienol (**3**), Germacrene D (**4**), Hydrocarbon **5**, and Geosmin (**1**)



suggested by other investigators.[3] We now report that germacradienol/germacrene D synthase itself generates geosmin (**1**), thereby establishing that the biosynthesis of geosmin from farnesyl diphosphate is *catalyzed by a single enzyme*, without intervention of any additional enzymes and without any requirement for redox cofactors.

GC−MS analysis of the products resulting from incubation of FPP with 2.3 μM recombinant *S. coelicolor* germacradienol synthase (4 h, 30 °C) revealed the presence of a minor (2.5%) but reproducible peak, retention time, 9.79 min, *m*/*z* 182, identical in GC retention time and mass spectrum with (−)-geosmin (**1**), obtained from the volatile extract of *S. coelicolor*, as well as with synthetic (±)-geosmin.[10,11] An additional 1% component of the reaction mixture, retention time, 8.51 min, *m*/*z* 164, was tentatively assigned the octalin structure **5**.[12] Interestingly, increasing the concentration of cyclase from 2.3 to 9.2 μM increased not only the total yield of geosmin (**1**) and hydrocarbon **5** but also the relative *proportion* of both **1** and **5** (13 and 3.5%, respectively), with a concomitant decrease in germacradienol (**3**) from 86 to 74% of the total product mixture. By contrast, the relative proportion of germacrene D (**4**) remained constant at 10−11%, independent of protein concentration. As already established for generation of germacradienol and germacrene D,[6,7] the enzymatic conversion of FPP to geosmin (**1**) showed an absolute requirement for a divalent cation, Mg^{2+} being strongly preferred. Notably, Fe^{2+} or Cu^{2+}, alone or in binary combination with Mg^{2+}, each suppressed the formation of geosmin by a factor of 3 or more, thus ruling out any requirement for either redox-active metal ion. No other organic or inorganic cofactor is required for the formation of geosmin from FPP.

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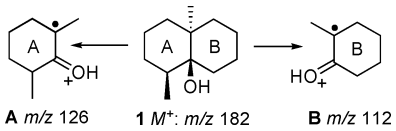
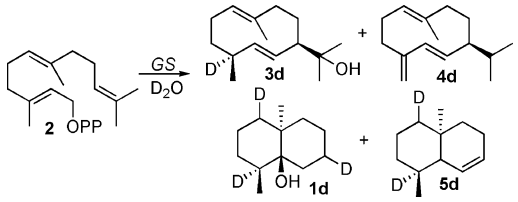


Figure 1. MS fragmentation of geosmin (**1**).

Scheme 3. Cyclization of FPP (**2**) to **1**, **3**, **4**, and **5** in D_2O (predicted labeling)



To explore the mechanism of this unexpected transformation, we carried out incubations of recombinant germacradienol synthase with individual deuterated samples of FPP and analyzed the resulting mixtures of **1**, **3**, **4**, and **5** by GC−MS.[7,11] Cyclization of [1,1-$^2H_2$]FPP (**2a**)[13] or (1*R*)-[1-^{2}H]FPP (**2b**)[13] each gave [6-^{2}H]-germacradienol (**3a** and **3b**) as previously reported,[7] as well as both geosmin-d_1 (**1a** and **1b**; *m*/*z* 183) and octalin-d_1 (**5a** and **5b;** *m*/*z* 165) (Scheme 2). The deuterium atom in both **1a** and **1b**, predicted to be at C-6, could be localized in ring B, on the basis of the observed shift from *m*/*z* 112 to 113 of the characteristic ring B MS fragment ion (Figure 1).[3] The corresponding ring A-derived fragment ion from either **1a** or **1b**, *m*/*z* 126, was devoid of deuterium. As expected, germacradienol (**3c**), geosmin (**1c**), and hydrocarbon **5c** derived from (1*S*)-[1-^{2}H]FPP (**2c**)[13] were all unlabeled, consistent with the previously established loss of H-1*si* of FPP in the formation of germacradienol. The retention and distribution of label in the samples of germacrene D (**4a**, **4b**, **4c**) obtained from each incubation were all as previously reported.[7]

Although the formation of geosmin (**1**) and hydrocarbon **5** from FPP, catalyzed by a single terpene synthase, has no biochemical precedent, the observed labeling results can be fully rationalized by the mechanism illustrated in Scheme 2. Proton-initiated cyclization of germacradienol followed by a retro-Prins-type fragmentation of the resulting eudesmanoid cation will lead to release of the 2-propanol side chain, presumably as acetone, and generation of the trisnorsesquiterpene **5**. Protonation of **5**, followed by a 1,2-hydride shift and capture of the bridgehead cation by water, will generate geosmin (**1**).[14] The observation that the proportion of geosmin is enhanced at increased concentrations of protein suggests that a substantial proportion of the initially generated germacradienol dissociates from the enzyme before being rebound and further converted to **5** and geosmin.

To test further the proposed cyclization mechanism, we carried out a series of incubations with FPP and recombinant germacradienol synthase in D_2O (∼95 atom ^{2}H) using selected ion monitoring (SIM) to quantitate the relevant M, M + 1, M + 2, and M + 3 peaks corresponding to each incubation product (Scheme 3).[11] The resulting germacradienol (**3d**) consisted of 22% d_1-species and 77% d_0, apparently due to a pronounced isotope effect on the first protonation step. Geosmin (**1d**) obtained from each incubation consisted of 13% d_3-, 58% d_2-, 26% d_1, and 3% d_0-species, consistent with the required three protonation steps. Notably, only one of the three deuterium atoms is located in ring B of geosmin (**3d**), as evidenced by the observation of the predicted d_1-fragment at *m*/*z* 113.[14] Hydrocarbon **5d**, resulting from the same D_2O incubations, was a mixture of 6% d_2-, 36% d_1-, and 58% d_0-species. The deuterium isotope effect also resulted in a perturbation of the normal product ratios, with an enhancement of the proportion of germacrene D (**4d**) (24%) and hydrocarbon **5d** (8%), and a decrease in the proportions of germacradienol (**3d**) and geosmin (**1d**) (65 and 5%, respectively.)

The above-described results provide strong experimental evidence for the previously inferred intermediacy of germacradienol (**3**) in geosmin biosynthesis. The demonstration that a single enzyme is both necessary and sufficient to convert farnesyl diphosphate to geosmin solves a long-standing biosynthetic mystery, while expanding our knowledge of the already impressive synthetic virtuosity of terpene synthases. Experiments to establish further details of this remarkable biochemical reaction are in progress.

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Supporting Information Available: Experimental methods, incubation conditions, and GC−MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) Germacradienol (**3**) was first isolated from *S. citreus* as a cometabolite of geosmin (**1**), accompanied by several germacrenoid and eudesmanoid metabolites, including germacrene D (**4**), bicyclogermacrene, and dihydroagarofuran. (a) Gansser, D.; Pollak, F. C.; Berger, R. G. *J. Nat. Prod.* **1995**, *58*, 1790−1793. (b) Pollak, F. C.; Berger, R. G. *Appl. Environ. Microbiol.* **1996**, *62*, 1295−1299. **3**, **4**, and **1** frequently co-occur in *Streptomyces*.
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(11) Earlier incubations (cf. ref 7) had been carried out using 0.5−1.0 μM protein. GC−MS conditions: Hewlett-Packard Series 2 GC−MSD, 70 eV; 30 m × 0.25 mm HP5MS capillary column and temperature program of 50−280 °C, 20 °C/min. Retention time, germacrene D (**4**), 10.24 min; germacradienol (**3**), 11.20 min. See Supporting Information for experimental details.
(12) Although hydrocarbon **5** has not previously been reported, the mass spectrum is very similar, but not identical, to that of the isomeric 4,8a-dimethyl-1,2,3,4,6,7,8,8a-octahydronaphthalene, *m*/*z* 164 (MassFinder 2.3, retention index 1233, scan #479; D. H. Hochmuth, http://www.massfinder.com).
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(14) The deuterium labeling results summarized in Scheme 2 are consistent with the reported conversion of [5,5-$^2H_2$]-1-deoxy-D-xylulose to [$^2H_5$]-geosmin by cultures of *Streptomyces* sp JP95 (ref 3a), as well as the incorporation of [4,4,6,6,6-$^2H_5$]mevalonate into [$^2H_9$]geosmin by both *Myxococcus xanthus* and *Stigmatella aurantiaca* (ref 3b). The formation of germacradienol (**3**) as well as the incorporation of three deuterium atoms from water into geosmin (**1d**) rules out, however, the mechanism of geosmin biosynthesis proposed in ref 3a.

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