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Biochemistry and Molecular Genetics of the Biosynthesis of the Earthy Odorant Methylisoborneol in *Streptomyces coelicolor*

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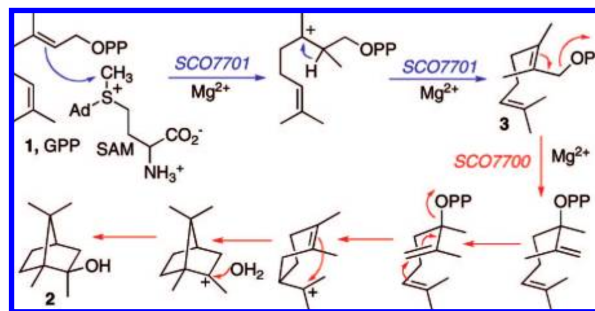
Streptomyces are ubiquitous, gram-positive soil bacteria that are known to produce an enormously wide range of pharmaceutically useful compounds.[1] The 8.7-Mb linear chromosome of *S. coelicolor* A3(2), the most thoroughly genetically characterized representative of the genus, harbors 7825 predicted genes[1] that include open-reading frames (ORFs) for at least three known or presumptive terpene synthases: *sco6073* encoding germacradienol/geosmin synthase;[2a] *sco5222*, encoding epi-isozizaene synthase;[3] and a third gene of unassigned function, *sco7700*. The predicted SCO7700 protein contains a **DD**CYC**ED** motif, an unusual variant of the universally conserved Mg^{2+}-binding, aspartate-rich DDXXD characteristic of terpene synthases,[4] as well as a second conserved Mg^{2+}-binding "NSE" triad,[4] **N**DLY**S**YTK**E**, located 148 amino acids downstream of the aspartate-rich motif. We now report the biochemical characterization of the *S. coelicolor* terpene synthase, SCO7700, and the demonstration that the encoded protein works in combination with the *C*-methyl transferase SCO7701 to catalyze the two-step conversion of geranyl diphosphate (GPP) (**1**) to 2-methyl-isoborneol (**2**) by way of 2-methylgeranyl diphosphate (**3**) (Scheme 1).

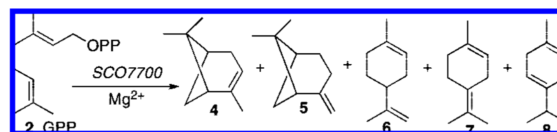
The 1.3-kb *sco7700* gene, encoding a protein of 440 amino acids was amplified by polymerase chain reaction (PCR) from *S. coelicolor* A3(2) genomic DNA and ligated into the pET28a expression vector. The derived construct, pET28a-*sco7700*, was transformed into *Escherichia coli* BL21(DE3). After induction with IPTG, the expressed soluble SCO7700 protein, carrying an N-terminal His_6-tag, was purified to homogeneity (>95% pure) by Ni-NTA chromatography. MALDI-TOF analysis gave a M_D of 49937 ± 25 (calcd, 49955). Incubation of recombinant SCO7700 with farnesyl diphosphate or geranylgeranyl diphosphate did not result in the formation of any detectable sesquiterpene or diterpene products. By contrast, GC−MS analysis of the products resulting from the incubation of 60 μM GPP (**2**) with 25 μM recombinant SCO7700 revealed the formation of a complex mixture of cyclic monoterpenes, consisting of α-pinene (**4**) (6%), β-pinene (**5**) (23%), limonene (**6**) (32%), γ-terpinene (**7**) (29%), and δ-terpinene (**8**) (10%) accompanied by traces of the monoterpene alcohols β-terpineol, 4-terpineol, and α-terpineol (Scheme 2). Although similar monoterpene mixtures have been reported for several plant monoterpene synthases and the K_m value of 2.8 ± 0.5 μM for cyclization of GPP by SCO7700 was well within the range typical for such synthases,[5] the unusually low k_{cat} of $3.0 \pm 0.2 \times 10^{-5}$ s^{-1} suggested that GPP might possibly be a surrogate substrate rather than the natural substrate for the SCO7700 protein.

Although few microorganisms are reported to produce monoterpenes,[6] 2-methylisoborneol (**2**) is a common volatile organic metabolite of many actinomycetes, as well as cyanobacteria and myxobacteria, with an extremely low threshold for detection (parts per trillion).[7a] Together with geosmin, methylisoborneol is responsible for the characteristic odor of moist soil and can also impart an unpleasant musty or muddy off-taste to drinking water and food

Scheme 1. Biosynthesis of Methylisoborneol



Scheme 2. Cyclization of GPP by SCO7700



products.[7b] Little was known about the biosynthesis of 2-methylisoborneol, other than the 1981 report that feeding of either labeled acetate or methionine to cultures of *S. antibioticus* gave labeled methylisoborneol, indicating that methylisoborneol is probably a methylated monoterpene.[8] We therefore were intrigued that the 3′-end of the *S. coelicolor sco7700* gene is only16 nucleotides upstream of the *sco7701* ORF, which is annotated only as a putative C-methyltransferase. We thus speculated that *sco7700* and *sco7701* might together comprise a two-gene operon for the biosynthesis of 2-methylisoborneol from geranyl diphosphate.

To test this hypothesis, we used PCR to amplify the 879-bp *sco7701* gene from *S. coelicolor* genomic DNA, ligated the resulting DNA into pET28a, and used the derived pET28a-*sco7701* to transform *E. coli* BL21(DE3). After induction with IPTG, the expressed soluble N-terminal His_6-tagged SCO7701 protein was purified (>95%) by Ni-NTA affinity chromatography. The protein had a M_D by MALDI-TOF of 34993 ± 17 (calcd, 34987). Incubation of recombinant SCO7701 (25 μM) with 60 μM GPP and 120 μM *S*-adenosyl-L-methionine (SAM) in 4 mL of assay buffer (50 mM PIPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.0; 18 h, 37 °C) was followed by the addition of 25 μM of purified recombinant SCO7700 and further incubation (18 h, 30 °C). After extraction with pentane/Et_2O, the combined, concentrated organic extracts were analyzed by capillary GC−MS, revealing formation of 2-methylisoborneol (**2**, $C_{11}H_{20}O$, *m/z* 168) as the major product, identical by GC retention time and mass spectrum with an authentic sample of **2**, accompanied by a trace amount of 2-methylbornene ($C_{11}H_{18}$, *m/z* 150).

We next examined the SCO7701-catalyzed reaction directly. Following incubation of SCO7701 (25 μM) with GPP (60 μM) and SAM (25 μM) (18 h, 37 °C), the reaction mixture was treated

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with a mixture of apyrase and acid phosphatase (2 h, 30 °C) to hydrolyze the diphosphate esters. GC−MS analysis of the ether-extractable products confirmed the formation of (*E*)-2-methylgeraniol (**9**) ($C_{11}H_{20}O$, *m/z* 168), identical in retention time and mass spectrum to an authentic sample of **9**.[9] To further characterize the reaction products, a preparative-scale incubation (100 mL, 18 h, 37 °C) was carried out with 6 μmol of GPP, 12 μmol of SAM, and a total of 0.2 μmol of SCO7700 protein, added in equal batches at 0, 6, and 12 h. After washing with Et_2O, the water-soluble products were conveniently purified by absorption onto a CHP-20P column which was then washed with water followed by 5% aq acetonitrile to elute 2-methylgeranyl diphosphate (**3**). The ^{1}H-decoupled ^{31}P NMR spectrum of **3** showed a pair of doublets ($J = 21$ Hz) corresponding to the presence of the expected diphosphate monoester. Hydrolysis of **3** with apyrase/phosphatase then yielded 2-methylgeraniol whose ^{1}H NMR spectrum was identical with that of synthetic **9**. These results firmly demonstrate that the SCO7701 protein catalyzes the SAM-dependent electrophilic methylation of GPP to yield (*E*)-2-methyl-GPP (**3**) (Scheme 1). The steady-state kinetic parameters for this reaction were determined using *S*-[*methyl*-^{3}H]SAM and a standard acid-lability assay to monitor the formation of 2-methyl-GPP, giving K_m (GPP) 13.1 ± 1.4 μM, K_m (SAM) 4.3 ± 0.3 μM, and k_{cat} $7.5 \pm 2.5 \times 10^{-3}$ s^{-1}.

Incubation of synthetic (*E*)-2-methyl-GPP (**3**) (60 μM) with SCO7700 (25 μM) in 4 mL of assay buffer (18 h, 30 °C) and analysis of the ether−pentane extract by GC−MS confirmed the expected formation of 2-methylisoborneol (Scheme 1) along with a trace amount of 2-methylbornene. Using [1-^{3}H]-**3**, the reaction exhibited typical Michaelis−Menten kinetics, with a K_m (2-MeGPP) of 26 ± 12 μM and k_{cat} of $3.9 \pm 0.9 \times 10^{-2}$ s^{-1}.

While these studies were nearing completion, the specific incorporation of labeled mevalonolactone and [*methyl*-^{13}C]-Met into methylisoborneol by cultures of the myxobacterium *Nannocystis exedens* was reported, as well as the isolation of (*E*)-2-methylgeraniol (**9**), which was also labeled by [*methyl*-^{13}C]-Met.[10] It is thus firmly established that the electrophilic methyl of SAM is introduced into methylisoborneol by the initial *C*-methylation of GPP followed by the cyclization of the resulting 2-methyl-GPP (**3**) (Scheme 1). The latter cyclization most likely occurs by a mechanism closely resembling that established for the conversion of GPP to bornyl diphosphate,[5] except that the penultimate bornyl cation is quenched on the *exo* face by water, analogous to the formation of fenchol,[11] rather than by internal return of the pyrophosphate to the *endo* face.

Close homologues of the *sco7700/sco7701* two-gene operon can be found in several streptomycetes, including *S. griseus*, *S. ambofaciens*, and *S. scabies*, as well as *Saccharopolyspora erythraea*. Interestingly, the predicted methylisoborneol synthase proteins all consist of ∼435 amino acids, about 100 amino acids larger than typical microbial terpene synthases. Each of these proteins carries a ∼100-amino acid peptide leader sequence of unknown function with an unusually high (25−30%) proline content. The biosynthesis of the volatile metabolite methylisoborneol appears to be highly conserved among actinomycetes carrying this pathway.[12]

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Supporting Information Available: Sequence comparisons, experimental methods, GC−MS data, and full citation for ref 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Shortly after submission of the original version of this manuscript, Komatsu et al published the results of an independent study of methyl isoborneol synthesis which used bioinformatic analysis to identify the two candidate genes constituting the methylisoborneol synthase operon in seven *Streptomyces* species, including *S. coelicolor*, and the confirmation of this assignment by PCR amplification and heterologous expression in *S. avermitilis* of the operons from *S. ambofaciens*, *S. lasaliensis*, and *Sac. erythraea.* They also report the in vitro formation of methylisoborneol by incubation of GPP and SAM with recombinant *S. lasaliensis* methyl transferase and methylisoborneol synthase, as well as the conversion of GPP to 2-methylGPP by the *S. lasaliensis* methyl transferase. Komatsu, M.; Tsuda, M.; Omura, S.; Oikawa, H.; Ikeda, H. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 7422–7427.

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