

Genome Mining in *Streptomyces avermitilis*: Cloning and Characterization of SAV_76, the Synthase for a New Sesquiterpene, AvermitilolWayne K. W. Chou,[†] Immacolata Fanizza,[†] Takuma Uchiyama,[‡] Mamoru Komatsu,[‡] Haruo Ikeda,[‡] and David E. Cane^{*,†}

Department of Chemistry, Box H, Brown University, Providence, Rhode Island 02912-9108 and Laboratory of Microbial Engineering, Kitasato Institute for Life Sciences, Kitasato University, 1-15-1 Kitasato, Sagami-hara, Kanagawa 228-8555, Japan

Received April 12, 2010; E-mail: David_Cane@brown.edu

Streptomyces are gram-positive bacteria known for their production of an enormous variety of biologically active secondary metabolites.¹ The growing number of completed *Streptomyces* genome sequences has revealed that only a fraction of the biosynthetic potential of these versatile bacteria has been uncovered.^{1–3} Genome mining has thus provided a powerful new tool for the discovery of both known and previously unknown natural products and the elucidation of new biochemical transformations and biosynthetic pathways.³

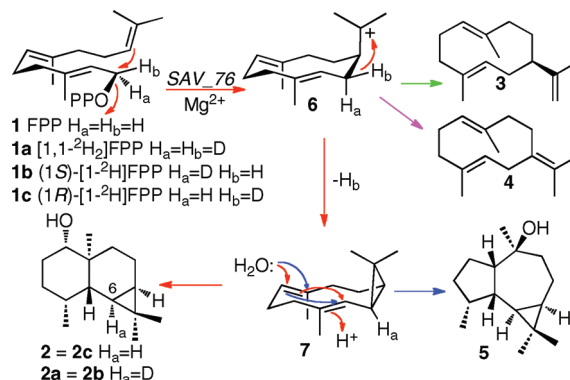
Streptomyces avermitilis, a well-studied member of this genus, is used for the industrial production of the important anthelmintic macrolide avermectin.^{2,4,5} Sequencing of the *S. avermitilis* genome has revealed four presumptive terpene synthase genes.⁴ One of these, *ptIA* (*sav2998*), encodes a pentalenene synthase,⁶ a second, *geoA* (*sco6073*), is a germacradienol/geosmin synthase,⁷ and a third, *sav3032*, encodes an epi-isozizaene synthase.⁸ The function of the remaining putative terpene synthase gene, *sav76*, has not previously been established. Two highly conserved Mg²⁺-binding motifs, characteristic of essentially all terpene cyclases, are evident in the predicted SAV_76 protein as an aspartate-rich ⁸⁰DDQFD motif and the “NSE” triad motif, ²³⁹NDVYSLEKE.⁹ We now report that SAV_76 catalyzes the cyclization of farnesyl diphosphate (**1**, FPP) to a previously unknown tricyclic sesquiterpene alcohol, which we have named avermitilol (**2**) (Scheme 1).

A synthetic *sav76* gene, with codons optimized for expression in *E. coli*, was cloned into the pET28a(+) expression vector. The resultant plasmid was transformed into *E. coli* BL21(DE3) and used for high-level expression of recombinant SAV_76 protein carrying an N-terminal His₆-tag, which was affinity-purified using Ni-NTA chromatography.

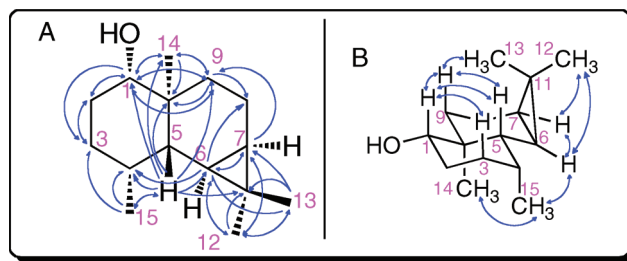
Incubation of purified recombinant SAV_76 with FPP in the presence of MgCl₂ gave a sesquiterpene alcohol (**2**) (*m/z* 222) as the major enzymatic reaction product (85%), accompanied by germacrene A (**3**) (1%), germacrene B (**4**) (5%), and the known tricyclic sesquiterpene alcohol, viridiflorol (**5**) (3%), as well as several unidentified minor sesquiterpene products, as determined by capillary GC-MS analysis. The steady-state kinetic parameters for the SAV_76-catalyzed reaction, measured by monitoring the formation of **2** using [1-³H]FPP, were *k*_{cat} 0.040 ± 0.001 s^{−1} and *K*_m 1.06 ± 0.11 μM, comparable to those for typical terpene synthases.^{6–9}

The structure of sesquiterpene alcohol **2** was assigned by a combination of 1-D and 2-D NMR spectroscopy. Key resonances observed in the ¹H NMR and HMQC spectra were the two upfield methine proton signals corresponding to H-6 (δ 0.47; C-6 22.2 ppm) and H-7 (δ 0.53; C-7 19.3 ppm) attached to a cyclopropane ring, as well as the H-5 (δ 0.94; C-5 40.5 ppm) and H-9_{ax} (δ 0.63; C-9

36.8 ppm) protons shielded by the cyclopropyl ring. Four methyl signals were also observed, corresponding to H-12 (δ 0.99, s; C-12 29.8 ppm), H-13 (δ 0.91, s; C-13 15.6 ppm), H-14 (δ 0.87, s; C-14 14.6 ppm), and H-15 (δ 1.00, d, *J* = 7.5 Hz; C-15 15.4 ppm), in addition to the alcohol (δ 1.24) and carbinyl protons, H-1 (δ 3.13; C-1 79.7 ppm). The absence of any olefinic protons or allylic methyl groups indicated that **2** was a tricyclic, cyclopropane-containing, secondary alcohol.

Scheme 1. Cyclization of FPP (**1**) to Avermitilol (**2**)

The ¹H–¹³C connectivity in **2** was established by a combination of HMQC and HMBC spectroscopy (Figure 1A). Long-range ¹H–¹³C correlations defining the cyclopropane ring were observed between the methyl H-13 protons and C-6, C-7, and C-11 of the cyclopropane ring, in addition to the reciprocal crosspeaks between the protons and carbons of the geminal methyl pair. Pairwise 2- and 3-bond correlations between H-5 and C-1, C-4, C-6, C-9, C-10, C-11, C-14, and C-15 established the central position of the bridgehead H-5 proton. Additional crosspeaks between H-1 and C-2, C-3, C-9, C-10, and C-14 as well as between H-9_{ax} and C-1, C-5, C-8, C-10, and C-14 established the remaining connectivity.

Figure 1. (A) HMQC and (B) NOESY correlations for **2**.

The relative stereochemistry of sesquiterpene **2** was readily deduced from the NOESY NMR spectrum. Major NOESY

[†] Brown University.
[‡] Kitasato University.

correlations established the presence of a *trans*-decalin ring system with a *cis*-fused dimethylcyclopropane ring as deduced from NOESY crosspeaks among the H-6 and H-7 methine protons and the H-12 methyl signals (Figure 1B). The H-6 proton also displayed a cross peak to the H-15 secondary methyl, which itself had a crosspeak to the H-14 methyl at the ring junction. Additional NOESY correlations were also observed between H-1 and each of its 1,3-diaxial partners H-3_{ax}, H-5, and H-9_{ax}. This H-9_{ax} proton signal also displayed crosspeaks with both H-5 and the H-13 methyl.

The absolute configuration of **2** was assigned by ¹H NMR analysis of the derived (*R*)- and (*S*)-Mosher esters of the C-1 secondary alcohol,¹⁰ leading to the assignment of the absolute configuration of **2** as 1*S*, 4*R*, 5*S*, 6*R*, 7*R*, 10*S*. Avermitilol (**2**) is a new tricyclic sesquiterpene alcohol whose isolation has not been previously reported.¹¹

To probe the stereochemical course of the SAV_76-catalyzed reaction, recombinant SAV_76 was incubated in separate experiments with [1,1-²H₂]FPP (**1a**), (1*S*)-[1-²H]FPP (**1b**), and (1*R*)-[1-²H]FPP (**1c**) (Scheme 1). Unlabeled **2** resulted from the incubation with (1*R*)-[1-²H]FPP, while [6-²H]-**2a** was formed in incubations with [1,1-²H₂]FPP and with (1*S*)-[1-²H]FPP. The GC-mass spectra of **2a** and **2b** had parent peaks *m/z* 223, corresponding to [M+1]⁺, indicating that the H-1_{si} proton of FPP is retained while the H-1_{re} of FPP is lost during formation of the cyclopropane ring of avermitilol (**2**). The position of the deuterium label in [6-²H]-**2a** was established by the absence of the normal H-6 proton signal at δ 0.47. The concomitant loss of the vicinal couplings between H-6 and both H-5 and H-7 in [6-²H]-**2a** also supported the assigned position of the deuterium label.

These labeling experiments are consistent with a mechanism for the formation of avermitilol (**2**) in which FPP undergoes initial ionization with electrophilic attack on the *si*-face of the distal double bond to form a germacradienyl cation (**6**) (Scheme 1). Insertion of the 2-propyl cation into the C–H bond with loss of the original H-1_{re} proton of FPP would result in formation of the enzyme-bound bicyclogermacrene (**7**). Proton-initiated anti-Markovnikov cyclization of **7** and quenching of the tricyclic secondary carbocation by water would yield avermitilol (**2**). Consistent with this proposed mechanism is the observed formation of the minor products germacrene A (**3**) and B (**4**) by alternative deprotonation of the germacradienyl cation. The coproduction of the isomeric viridiflorol (**5**) can be explained by competing proton-initiated Markovnikov cyclization of bicyclogermacrene to form the *cis*-fused 5,7-ring system followed by capture of water.

Although, avermitilol (**2**) was not detected in extracts of wild-type *S. avermitilis*, the *in vivo* activity of the *sav76* gene could be directly demonstrated using a genome-minimized mutant, *S. avermitilis* SUKA17, from which >1-Mb of DNA had been deleted, including the genes for the major endogenous secondary metabolites produced by the parent strain.¹² GC-MS analysis of hexane extracts of cultures of *S. avermitilis* SUKA17 harboring *sav76* under control of the native *S. avermitilis* promoter *rpsJp* (*sav4925*) showed the presence of avermitilol (**2**, 15%), accompanied by small quantities of germacrene A (**3**, 10%), germacrene B (**4**, 5%), and viridiflorol (**5**, 2%) (Figure 2). The major component of the mixture was ketone avermitilone (**8**, 67%, *m/z* 220), whose structure was confirmed by ¹H and ¹³C NMR and direct comparison with a reference sample prepared by oxidation of **2** with pyridinium chlorochromate. Coinroduction of the *ptlB* gene (*sav2997*), encoding the native *S.*

avermitilis FPP synthase, along with *sav76* increased the titers of both **2** and **8**. The formation of avermitilone (**8**) may result from adventitious oxidation of **2** by an endogenous dehydrogenase, since no dehydrogenase gene is evident in the genome of *S. avermitilis* immediately upstream or downstream of the native *sav76* cyclase gene.

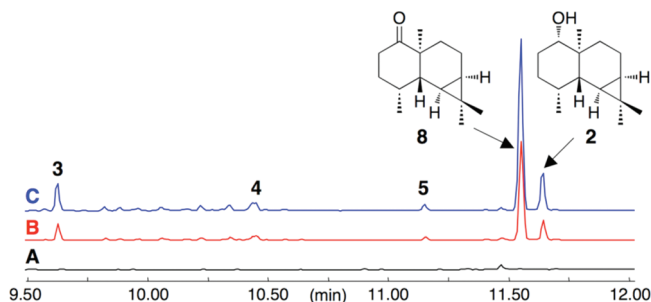


Figure 2. GC-MS analysis of production of avermitilol (**2**) and avermitilone (**8**) along with viridiflorol (**5**), germacrene B (**4**), and germacrene A (**3**) by transformed *S. avermitilis* SUKA17. (A) Control with plasmid pKU460. (B) pKU460-*rpsJp::sav76*. C. pKU460-*rpsJp::sav76-ptlB*.

We have now assigned the biochemical functions of all four terpene synthases originally revealed by the sequencing of the *S. avermitilis* genome.^{6–8} Avermitilol (**2**) is a new sesquiterpene whose isolation has not previously been reported. The *sav76* gene product has one close orthologue, SSAG_00457 (Uniprot ID B4UXV1) which is found in *Streptomyces* sp. Mg1, with 78% identity and 85% positive matches over 334 amino acids.

Acknowledgment. This work was supported by NIH grant GM30301 (D.E.C.), and by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) and from the Japan Society for the Promotion of Science (JSPS) 20310122 (H.I.). We thank Tun-Li Shen for assistance with mass spectrometry and Russell Hopson for assistance with NMR.

Supporting Information Available: Sequence comparisons, experimental methods, and NMR and GC-MS data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Bentley, S. D.; et al. *Nature* **2002**, *417*, 141–147.
- (2) Omura, S. et al. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12215–12220.
- (3) (a) Walsh, C. T.; Fischbach, M. A. *J. Am. Chem. Soc.* **2010**, *132*, 2469–2493. (b) Corre, C.; Challis, G. L. *Nat. Prod. Rep.* **2009**, *26*, 977–986.
- (4) Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Shinose, M.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M.; Omura, S. *Nat. Biotechnol.* **2003**, *21*, 526–531.
- (5) Lamb, D. C.; Ikeda, H.; Nelson, D. R.; Ishikawa, J.; Skaug, T.; Jackson, C.; Omura, S.; Waterman, M. R.; Kelly, S. L. *Biochem. Biophys. Res. Commun.* **2003**, *307*, 610–619.
- (6) Tetzlaff, C. N.; You, Z.; Cane, D. E.; Takamatsu, S.; Omura, S.; Ikeda, H. *Biochemistry* **2006**, *45*, 6179–6186.
- (7) Cane, D. E.; He, X.; Kobayashi, S.; Omura, S.; Ikeda, H. *J. Antibiot. (Tokyo)* **2006**, *59*, 471–479.
- (8) Takamatsu, S.; Lin, X.; Nara, A.; Komatsu, M.; Cane, D. E.; Ikeda, H. *Microb. Biotech.*, submitted.
- (9) Christianson, D. W. *Chem. Rev.* **2006**, *106*, 3412–3442.
- (10) Hoye, T. R.; Jeffrey, C. S.; Shao, F. *Nat. Protoc.* **2007**, *2*, 2451–2458.
- (11) A commercially available stereoisomer of **2**, of unspecified stereochemistry and origin, is listed in CAS (Registry No 1008931-42-7) but with no literature references. We have determined the full relative stereochemistry of this stereoisomer (Supporting Information) and shown that it is distinct from **2** by direct NMR and GC-MS comparison.
- (12) Komatsu, M.; Uchiyama, T.; Omura, S.; Cane, D. E.; Ikeda, H. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 2646–2651.

JA103087W