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# Genome Mining in *Streptomyces avermitilis*: Cloning and Characterization of SAV_76, the Synthase for a New Sesquiterpene, Avermitilol

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*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]

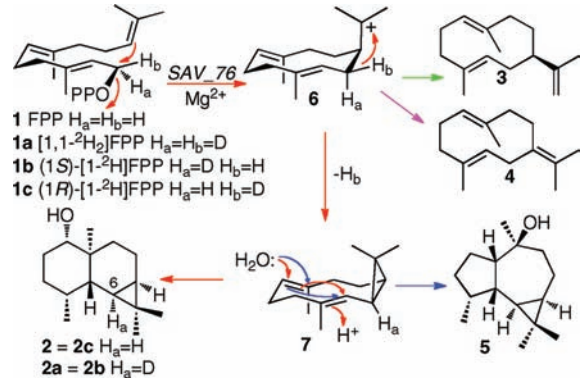
*Streptomyces avermitilis*, a well-studied member of this genus, is used for the industrial production of the important anthelminthic macrolide avermectin.[2,4,5] Sequencing of the *S. avermitilis* genome has revealed four presumptive terpene synthase genes.[4] One of these, *ptlA* (*sav2998*), encodes 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 $Mg^{2+}$-binding motifs, characteristic of essentially all terpene cyclases, are evident in the predicted SAV_76 protein as an aspartate-rich $^{80}$**DD**QF**D** motif and the "NSE" triad motif, $^{239}$**N**DVY**S**LEK**E**.[9] We now report that SAV_76 catalyzes the cyclization of farnesyl diphosphate (**1**, FPP) to a previously unknown tricyclic sequiterpene 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 $His_6$-tag, which was affinity-purified using Ni-NTA chromatography.

Incubation of purified recombinant SAV_76 with FPP in the presence of $MgCl_2$ 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-$^3$H]FPP, were $k_{cat}$ 0.040 ± 0.001 $s^{-1}$ and $K_m$ 1.06 ± 0.11 $\mu$M, 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 $^1$H NMR and HMQC spectra were the two upfield methine proton signals corresponding to H-6 ($\delta$ 0.47; C-6 22.2 ppm) and H-7 ($\delta$ 0.53; C-7 19.3 ppm) attached to a cyclopropane ring, as well as the H-5 ($\delta$ 0.94; C-5 40.5 ppm) and H-$9_{ax}$ ($\delta$ 0.63; C-9 36.8 ppm) protons shielded by the cyclopropyl ring. Four methyl signals were also observed, corresponding to H-12 ($\delta$ 0.99, s; C-12 29.8 ppm), H-13 ($\delta$ 0.91, s; C-13 15.6 ppm), H-14 ($\delta$ 0.87, s; C-14 14.6 ppm), and H-15 ($\delta$ 1.00, d, $J$ = 7.5 Hz; C-15 15.4 ppm), in addition to the alcohol ($\delta$ 1.24) and carbinyl protons, H-1 ($\delta$ 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 $^1$H−$^{13}$C connectivity in **2** was established by a combination of HMQC and HMBC spectroscopy (Figure 1A). Long-range $^1$H−$^{13}$C 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.

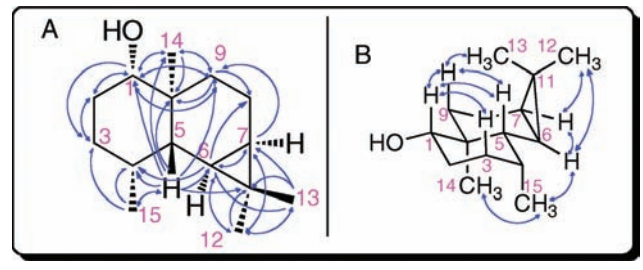



***Figure 1.*** (A) HMBC and (B) NOESY correlations for **2**.

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

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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 $^1$H 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-$^2H_2$]FPP (**1a**), (1*S*)-[1-$^2$H]FPP (**1b**), and (1*R*)-[1-$^2$H]FPP (**1c**) (Scheme 1). Unlabeled **2** resulted from the incubation with (1*R*)-[1-$^2$H]FPP, while [6-$^2$H]-**2a** was formed in incubations with [1,1-$^2H_2$]FPP and with (1*S*)-[1-$^2$H]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 cycloproprane ring of avermitilol (**2**). The position of the deuterium label in [6-$^2$H]-**2a** was established by the absence of the normal H-6 proton signal at $\delta$ 0.47. The concomitant loss of the vicinal couplings between H-6 and both H-5 and H-7 in [6-$^2$H]-**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 $^1$H and $^{13}$C NMR and direct comparison with a reference sample prepared by oxidation of **2** with pyridinium chlorochromate. Cointroduction 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 endogeneous dehydrogenase, since no dehydrogenase gene is evident in the genome of *S. avermitilis* immediately upstream or downstream of the native *sav76* cyclase gene.

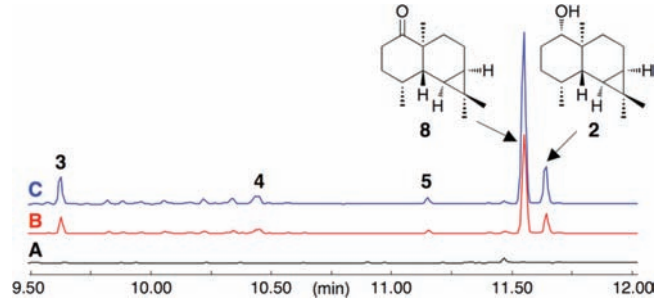


***Figure 2.*** GC-MS analysis of production of avermitilol (**2**) and avermitilone (**8**) along with viridiflorol (**5**), germacrene B (**4**), and germacrene A (**3**) by transformed *S. avermitilis* SUKA17. (A) Control with plasmid pKU460. (B) pKU460-*rpsJp*::*sav76*. C. pKU460-*rpsJp*::*sav76-ptlB*.

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 sequiterpene 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.

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**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.

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