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Characterization of a silent sesquiterpenoid biosynthetic pathway in *Streptomyces avermitilis* controlling *epi*-isozizaene albaflavenone biosynthesis and isolation of a new oxidized *epi*-isozizaene metabolite

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Summary

The genome-sequenced, Gram-positive bacterium *Streptomyces avermitilis* harbours an orthologue (SAV_3032) of the previously identified *epi*-isozizaene synthase (SCO5222) in *Streptomyces coelicolor* A3(2). The *sav3032* is translationally coupled with the downstream *sav3031* gene encoding the cytochrome P450 CYP170A2 analogous to SCO5223 (CYP170A1) of *S. coelicolor* A3(2), which exhibits a similar translation coupling. *Streptomyces avermitilis* did not produce *epi*-isozizaene or any of its oxidized derivatives, albaflavenols and albaflavenone, under in any culture conditions examined. Nonetheless, recombinant SAV_3032 protein expressed in *Escherichia coli* catalysed the Mg^{2+}-dependent cyclization of farnesyl diphosphate to *epi*-isozizaene. To effect the production of *epi*-isozizaene in *S. avermitilis*, the *sav3032* gene was cloned and placed under control of a copy of the native *S. avermitilis* promoter *rpsJp* (*sav4925*). The derived expression construct was introduced by transformation into a large-deletion mutant of *S. avermitilis* SUKA16 and the resulting transformants accumulated *epi*-isozizaene. The previously characterized oxidized *epi*-isozizaene metabolites (*4R*)- and (*4S*)-albaflavenols and albaflavenone, as well as a previously undescribed doubly oxidized *epi*-isozizaene derivative were isolated from cultures of *S. avermitilis* SUKA16 transformants in which *sav3032* was coexpressed with the P450-encoding *sav3031*. This new metabolite was identified as 4β,5β-epoxy-2-*epi*-zizaan-6β-ol which is most likely formed by oxidation of (*4S*)-albaflavenol.

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Introduction

Terpenoid compounds are used as antibiotics, hormones, flavour and/or odour constituents, and pigments. Some of these compounds also possess other physiologically or commercially important properties (Glasby, 1982; Sacchettini and Poulter, 1997) and many have been shown to play an important role in intra-species and inter-species interactions. Terpenoid metabolites are produced by a wide variety of terrestrial and marine plants and by fungi, while only a relatively minor fraction have been isolated from bacterial species. Sesquiterpene synthases catalyse the cyclization of farnesyl diphosphate to any of 300 sesquiterpene hydrocarbons or alcohol. These enzymes are widely distributed among plants and fungi. Only relatively recently bacterial sesquiterpene synthases, primarily from *Streptomyces*, have been identified. The first to be characterized was pentalenene synthase from *Streptomyces exfoliatus* (Cane *et al.*, 1994), which generates the parent hydrocarbon of the antibiotic pentalenolactone, a metabolite that has been isolated from numerous species of *Streptomyces*. Analysis of the genome sequences of *Streptomyces coelicolor* A3(2) and *Streptomyces avermitilis* has revealed additional terpene synthases encoding the biosynthesis of germacradienol and geosmin, and of *epi*-isozizaene and the derived antibiotic albaflavenone. Recent genomics-based investigations have revealed that bacterial terpenoid metabolism may be more widespread than previously assumed. Following the discovery of the gene encoding formation of the bacterial monoterpene (2-methylisoborneol synthase) in a variety of species of *Streptomyces*, a protein-family search using a hidden Markov model revealed that bacterial terpene synthases, classified into three groups – monoterpene, sesquiterpene and diterpene synthases – are in fact widely distributed in many bacteria, especially

Actinomytales microorganisms, all of which are therefore predicted to have the ability to produce wide variety of terpenoid metabolites (Komatsu *et al*., 2008).

The genome sequence of the industrially important Gram-positive bacterium *S. avermitilis*, which is used for the production of the potent anthelminthic macrolide avermectin (Omura *et al*., 2001; Ikeda *et al*., 2003; Nett *et al*., 2009), revealed the presence of at least four genes encoding sesquiterpene synthases. One of these genes, *sav2998*, was found to encode pentalenene synthase (Tetzlaff *et al*., 2006) while *sav2163* was shown to be a germacradienol/geosmin synthase (Cane *et al*., 2006). Recently *sav76* has been explained to encode a new sesquiterpene (avermitilol) synthase (Chou *et al*., 2010). The remaining gene, *sav3032*, shows a high level of similarity at the predicted amino acid level to *epi*-isozizaene (**1**) synthase, a sesquiterpene synthase recently identified in *S. coelicolor* A3(2) (Lin *et al*., 2006). *Epi*-isozizaene synthase has itself been shown to catalyse the committed step in the biosynthesis of the antibiotic albaflavenone (**3**), an α, β-unsaturated sesquiterpene ketone with an earthy, camphor-like odour that was originally isolated from the highly odorous *Streptomyces* species *S. albidoflavus* (Gürtler *et al*., 1994). In *S. coelicolor* A3(2) the albaflavenone biosynthetic gene cluster consists of two adjacent ORFs, *sco5222* and *sco5223*. The 1086 bp *sco5222* gene encodes a protein of 361 amino acids that catalyses the cyclization of farnesyl diphosphate (**2**) to *epi*-isozizaene (**1**) (Lin *et al*., 2006; Lin and Cane, 2009; Aaron *et al*., 2010). The *sco5223* gene, which shares a four-nucleotide ATGA transcriptional overlap with *sco5222* at its 5′-end, has been demonstrated to catalyse the two-step allylic oxidation of *epi*-isozizaene (**1**) to albaflavenone (**3**) through the intermediacy of an epimeric mixture of albaflavenols (**4a** and **4b**) (Zhao *et al*., 2008; 2009).

Although *S. avermitilis* also harbours a two-gene cluster (*sav3032–sav3031*, nt 3 788 761–3 791 219) homologous to the *sco5222–sco5223* pair in *S. coelicolor* A3(2), neither albaflavenone nor related compounds have been isolated from *S. avermitilis*. We now report the experimental demonstration of the biochemical function of the *S. avermitilis sav3032–sav3031* cluster, which are shown to encode the predicted *epi*-isozozaene synthase and the cytochrome P450, *epi*-isozizaene mono-oxygenase, resulting in the biosynthesis of albaflavenone and a previously uncharacterized sequiterpene metabolite, 4β,5β-epoxy-2-*epi*-zizaan-6β-ol (**5**).

Results

Orthologue of epi-*isozizaene synthase in* S. avermitilis

The gene *sav3032*, encoding an orthologue of the recently characterized *S. coelicolor* A3(2) *epi*-isozizaene synthase gene, is located at 3 790 128–3 791 219 nt of the *S. avermitilis* genome. The predicted 363 aa gene product of *sav3032* shows 82% identity and 87% positive matches to *S. coelicolor* A3(2) *epi*-isozizaene synthase (361 aa; SCO5222). Notably, *sav3032* is also translationally coupled by a four-nucleotide ATGA overlap to the downstream *sav3031* gene that encodes an apparent cytochrome P450 of 456 aa with 80% identity and 89% positive matches to CYP170A1 of *S. coelicolor* A3(2) (461 aa; SCO5223) (Lamb *et al*., 2003). It is therefore very likely that *S. avermitilis* also is competent to produce albaflavenone, and that the biochemical pathway is identical to that which has been established in *S. coelicolor* A3(2) (Lin *et al*., 2006; Zhao *et al*., 2008; 2009; Lin and Cane, 2009; Aaron *et al*., 2010).

Both terpene synthases, SAV_3032 and SCO5222, harbour the two universally conserved Mg^{2+}-binding domains, the aspartate-rich motif -**DD**RH**D**-, beginning at Asp101 in SAV_3032, and the characteristic triad **N**DLC**S**-LPK**E**, at Asn242 of SAV_3032 (Komatsu *et al*., 2008). The universally conserved cysteine residue found in the C-terminal region of all cytochrome P450s and which binds the proximal face of the protohaem co-factor is found in SAV_3031 (-RK**C**PS-) at Cys405 and in SCO5223 (-RK**C**PS-) at Cys410 respectively. Both of these cytochrome P450s also harbour an unusual aspartate-rich motif (-AK**DD**NG**D**PI-), which has not been found in bacterial cytochrome P450s, and which has recently been implicated in the conversion of farnesyl diphosphate to a mixture of acyclic farnesene isomers by recombinant SCO5223 (Zhao *et al*., 2009).

In vitro *Mg^{2+}-dependent cyclization of farnesyl diphosphate to* epi-*isozizaene catalysed by recombinant SAV_3032 protein*

To confirm the predicted biochemical function of the SAV_3032 protein, the recombinant protein was expressed using a standard *Escherichia coli* T7-RNA polymerase-based expression host–vector system. Although the C-terminal His_6-tagged SAV_3032 was initially obtained as inclusion bodies, the insoluble protein was readily resolubilized and refolded, then purified to homogeneity by ion-exchange chromatography to yield 3 mg of SAV_3032 protein from 1 l of culture. Incubation of the purified soluble recombinant SAV_3032 protein with farnesyl diphosphate (**2**) in the presence of Mg^{2+} ions and analysis of the resulting products by GC-MS revealed the formation of a major sesquiterpene hydrocarbon product, $C_{15}H_{24}$, *m/z* 204 (Fig. 1, retention time 10.13 min), identical in both retention time and mass spectral fragmentation pattern by direct comparison with authentic *epi*-isozizaene, generated by SCO5222 *epi*-isozizaene synthase (Lin *et al*., 2006). This result unambiguously

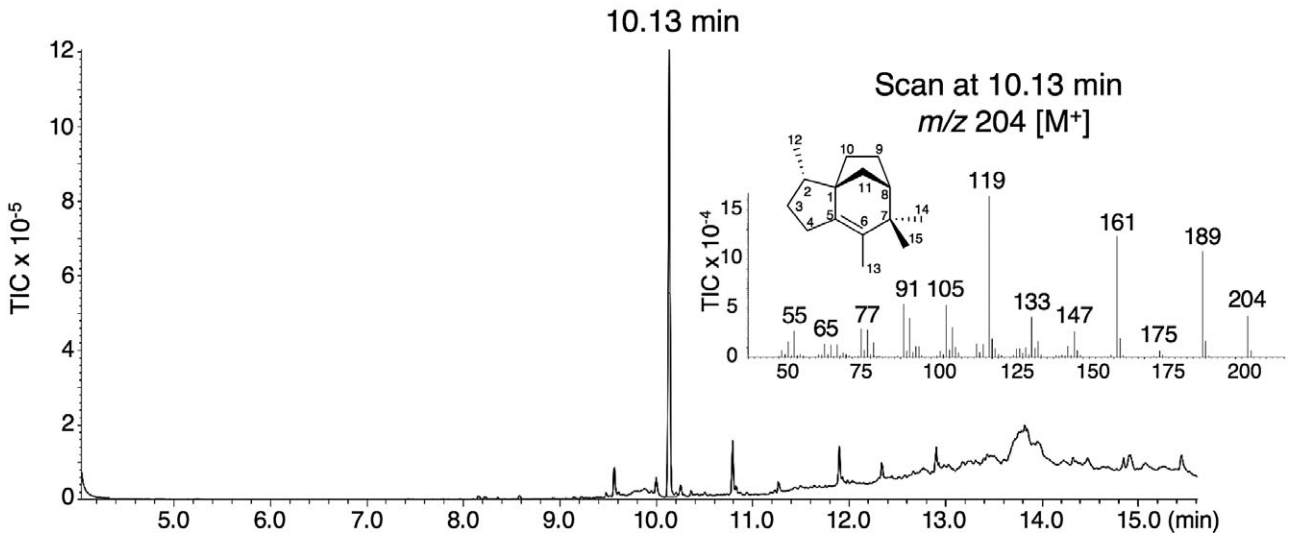

Fig. 1. GC-MS analysis of *n*-pentane extracts from incubation of farnesyl diphosphate and C-terminal His_6-tagged SAV_3032 protein in the presence of Mg^{2+}.

demonstrates that SAV_3032 catalyses the cyclization of farnesyl diphosphate (**2**) to *epi*-isozizaene (**1**).

The pH optimum for the SAV_3032-catalysed cyclization was found to be pH 7.0. The steady-state kinetic parameters were determined by carrying out a series of 60 min incubations at 30°C in pH 7.0 PIPES using a range of concentrations of farnesyl diphosphate, giving a k_{cat} of $(2.58 \pm 0.06) \times 10^{-5}$ s^{-1} and a K_m (farnesyl diphosphate) of 125 ± 11 nM. Substitution of 10 mM Mn^{2+} for Mg^{2+} gave relative rates of formation of **1** of 32%. Other divalent cations were significantly less effective: Zn^{2+} (5%), Fe^{2+} (1%), Fe^{3+} (5%), Co^{2+} (1%), Cu^{2+} (2%) and Ni^{2+} (1%).

Production of epi-*isozizaene and related compounds from* S. avermitilis

Although several attempts were made to produce *epi*-isozizaene (**1**) and its oxidized derivatives from *S. avermitilis*, none of the metabolites could be detected in either the *S. avermitilis* wild-type or its derivates under any culture conditions examined. Since both DNA microarray and real-time PCR analyses indicated that *sav3032* was not expressed under any of these conditions, we placed *sav3032* under the control of an alternative promoter, *rpsJp*, which is a constitutive promoter of the gene encoding ribosomal protein S10 of *S. avermitilis* and we had previously shown can efficiently control the expression of genes encoding monoterpene synthase, 2-methylisoborneol synthase, in *S. avermitilis* SUKA16 (Komatsu *et al*., 2008). Transformants of *S. avermitilis* SUKA16 (pKU460::*rpsJp-sav3032*) indeed produced *epi*-isozizaene (**1**) as a major component, as established by GC-MS comparison with authentic *epi*-isozizaene (Fig. 2A). To detect further oxidized derivatives of *epi*-isozizaene, *sav3032* was coexpressed with *sav3031* which encodes the cytochrome P450 CYP170A2. While the measured titres of *epi*-isozizaene in the resulting transformants were significantly reduced, several additional GC-MS peaks sesquiterpenoid components were observed. As shown in Fig. 2B, the peaks at 11.38 min and 11.44 min corresponded to the allylic oxidation products (*4S*)-albaflavenol (**4a**) and (*4R*)-albaflavenol (**4b**), respectively, by comparison with authentic albaflavenols prepared by allylic oxidation of *epi*-isozizaene with SeO_2 (Lin *et al*., 2006). A compound eluting at 11.90 min was also identical to the further oxidation product, albaflavenone (**3**), by direct comparison with an authentic sample. In addition to these three previously identified derivatives of *epi*-isozizaene, a fourth metabolite (**5**) that eluted at 11.83 min with parent M^+ *m/z* 236 did not match the EI-MS fragmentation pattern of any compounds in public MS databases.

*Structure elucidation of the new compound (**5**)*

Fermentation products were extracted with methanol from cultured mycelia and the organic extract was purified by silica gel column chromatography. Ultimately 2.6 mg of the new compound (**5**) was obtained from 5 l of culture as a colourless oil with a mild odour. The molecular formula $C_{15}H_{24}O_2$ of **5** was obtained from HRMS (FAB), indicating that the structure results from the addition two oxygen atoms to *epi*-isozizaene (**1**). Comparative analysis of EI-MS fragments between albaflavenols (**4a**, **4b**) and **5** indicated that the new compound (**5**) was not a simple hydroxylation product of albaflavenols (**4a**, **4b**), based on

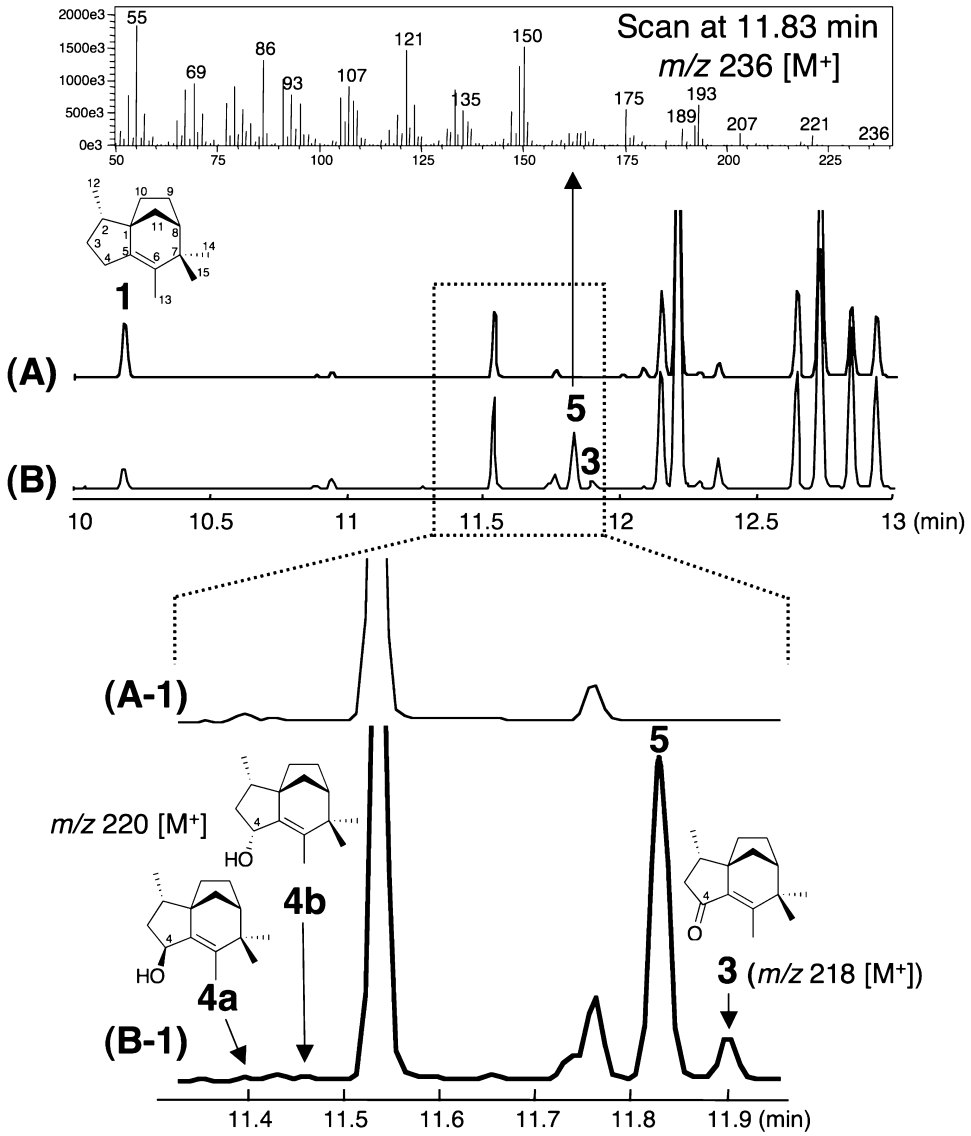

Fig. 2. GC-MS analysis of *n*-hexane extracts from 120 h cultures of *S. avermitilis* SUKA16 carrying pKU460::*rpsJp–sav3032* (A) or pKU460::*rpsJp–sav3032–sav3031* (B), with EI-MS of component at 11.83 min corresponding to compound **5**. In traces A-1 and B-1, the region from 11.30 to 11.95 min is expanded for chromatograms A and B. The peaks eluted from 11.54 to 11.77 min (A-1 and B-1) and from 12.0 to 13.0 (A and B) were identical to short- and medium-chain fatty acids.

the absence of a characteristic dehydration fragmentation peak at *m/z* 218. In other respects, the new compound (**5**) showed an EI-MS pattern similar to but distinct from all other known *epi*-isozizaene derivatives.

The ^{1}H- and ^{13}C NMR spectra of **5** (described in Materials and Methods in *Supporting information*) showed signals for a secondary methyl signal (δ_H 0.99, δ_C 21.53) and for three methyl signals (δ_H 0.887, δ_C 20.44; δ_H 1.04, δ_C 21.77; δ_H 0.893, δ_C 27.69), suggesting that the structure of **5** corresponds to typical tricyclic sesquiterpene (Gürtler *et al.*, 1994). In the ^{1}H-^{1}H COSY spectrum of **5**, the connection between the methyl signal at δ 0.99 (*d*, *J* = 7.3 Hz, H-12) and the methine at δ 3.49 (1H, broad singlet, H-4) through the H-2 methine (δ 1.74) and H-3 methylene (δ 1.98, δ 1.68), and the methylene signals at H-10 (δ 1.93, δ 1.50) and H-11 (δ 1.77, δ 1.20) through H-9 (δ 2.27, δ 1.61) and H-8 (δ 1.74) could be traced. These results indicated that the partial structure of **5** is similar to that of albaflavenols (**4a**, 4**b**) (Zhao *et al.*, 2008). In the ^{13}C NMR and HMBC spectra of **5**, typical geminal methyl signals at C15 (δ 21.77) and C14 (δ 27.69) connected to C7 (δ 40.52) were similar to those of albaflavenols (**4a**, 4**b**), except for quaternary carbon signals at C5 (δ 76.50) and C6 (δ 74.98) compared with those of the quaternary olefinic carbon at C5 (δ 147.5) and C6 (δ 135.5) of **4a**. In consideration of the four degrees of unsaturation and the ^{13}C NMR chemi-

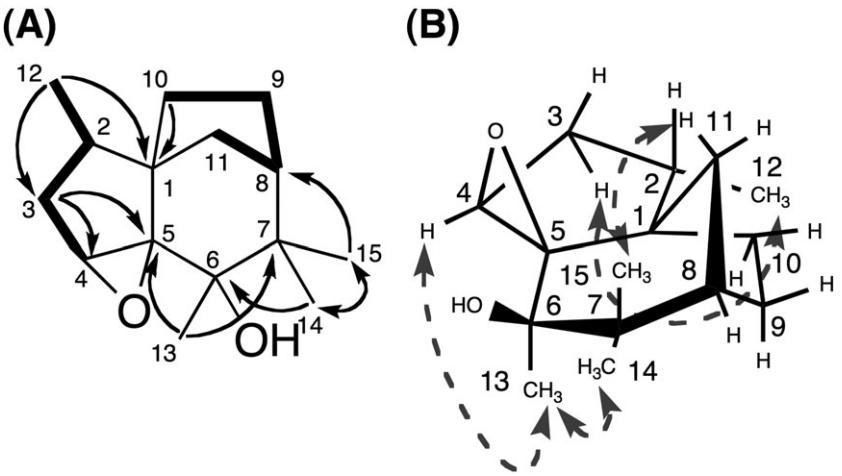

Fig. 3. COSY, HMBC (A) and NOESY (B) analysis of **5**. Bold lines in (A) represent ^{1}H-^{1}H correlations. Arrows in (A) shows key HMBC correlations (^{1}H <-> ^{13}C), while dashed arrows in (B) represent NOESY correlations.

cal shifts of **5**, the carbon chemical shifts at δ 65.61 (C4) and δ 76.50 (C5) were assigned to epoxy carbons, instead of the characteristic secondary hydroxyl carbon signal at δ 70.8 (C4) and quaternary carbon at δ 147.5 (C5) of **4a**. Among the four methyl carbons, a remaining tertiary methyl (δ_H 0.887, δ_C at 20.44 C13) and hydroxyl group were clearly connected to C6 (δ 74.98) in the HMBC spectrum of **5**. To combine MS and NMR spectral data, structure **5** was determined as 4,5-epoxy-2-*epi*-zizaan-6-ol shown in Fig. 3A. The complete stereochemistry was assigned from the NOESY spectrum of **5**, which displayed correlation peaks between the 12α-methyl (δ 0.99) and H-3α (δ 1.68), H-4α (δ 3.49) and 13α-methyl (δ 0.887), and 13α-methyl (δ 0.887) and 15α-methyl (δ 1.04), thereby establishing the relative configuration of the 12α-methy (2-*epi*), 4β, 5β-epoxy and 6β-hydroxy substituents, respectively, in **5** (Fig. 3B). The absolute configuration could then be assigned with confidence based on the derivation from *epi*-isozizaene, giving the assignment of **5** as 4β,5β-epoxy-2-*epi*-zizaan-6β-ol.

Distribution of epi-*isozizaene synthase orthologues in other bacterial genomes*

Since two verified *epi*-isozizaene synthases exhibiting a high level of mutual amino acid sequence identity and similarity had thus been demonstrated from *S. coelicolor* A3(2) and *S. avermitilis*, we created a hidden Markov model for *epi*-isozizaene synthase using the two amino acid sequences as well as those of predicted *epi*-isozizaene synthases that were identified by screening public databases of bacterial proteins using this model. At least nine additional predicted *epi*-isozizaene synthases were found as follows: *Streptomyces albus* J1074, *S. lividans* TK24, *S. ghanaensis* ATCC 14672, *S. griseoflavus* Tu4000, *S. sviceus* ATCC 29083, *S. viridochromogenes* DSM 40736, *Streptomyces* sp. SPB74 and SPB78, and *Burkholderia pseudomallei* 668 (Table S1). The albaflavenone biosynthetic gene clusters in both *S. avermitilis* and *S. coelicolor* A3(2) consist of two adjacent ORFs, *epi*-isozizaene synthase and a downstream, translationally coupled gene encoding a cytochrome P450 belonging to the CYP170A family of mixed function monooxygenases. Indeed CYP170-family cytochrome P450 genes were located immediately downstream of the predicted *epi*-isozizaene synthase genes in all cases that were examined except that of *B. pseudomallei* (Table S1). These predicted *epi*-isozizaene synthases (Fig. S1) all contained the canonical aspartate-rich motif -WFF[AV]W**DD**[RQ]H**D[**RE]**D**- and the characteristic triad motif -A[WL]Y**N**DLC**S**LPK**E**-, respectively, separated by some 140 ± 5 amino acids. The three aromatic amino acid residues, F95, F96 and F198 in SCO5222, that appeared to be oriented to stabilize carbocation intermediates in the cyclization cascade through cation-π interactions (Aaron *et al*., 2010) were universally conserved in all predicted terpene synthases. Furthermore, the characteristic aromatic amino acid-rich motif -V**YWF**HHES[GD]R**Y**- was found in the C-terminus regions of each of these predicted terpene synthases (Fig. S1).

To confirm the production of *epi*-isozizaene or its oxidized derivatives in five *Streptomyces* strains, *S. albus*, *S. ghanaensis*, *S. lividans*, *S. sviceus* and *S. viridochromogenes*, each microorganism was cultivated and their *n*-hexane extracts of mycelia were subjected to GC-MS analysis using authentic samples (Table S2). The production of *epi*-isozizaene was detected from mycelia in *S. ghanaensis* and *S. lividans*. *Streptomyces albus* produced not only *epi*-isozizaene but also albaflavenols and albaflavenone in the mycelium.

Discussion

The *S. avermitilis epi*-isozizaene synthase (SAV_3032) displays an unusually low k_{cat} of 2.58 ± 0.06 × 10^{-5} s^{-1}

compared with that of the SCO5222 protein ($0.049 \pm 0.001\ s^{-1}$), and indeed all other previously characterized terpene synthases. This discrepancy probably results from the necessity of refolding the SAV_3032 protein from insoluble inclusion bodies, resulting in only a small fraction of the refolded SAV_3032 protein that is catalytically competent. The fact that the K_m for farnesyl diphosphate (125 ± 11 nM) is very close to that of the *S. coelicolor* A3(2) synthase (147 ± 14 nM) suggests that the active protein is properly folded, as distinguished from the formation of a large proportion of partially active, misfolded recombinant protein. In any case, it is absolutely clear that the SAV_3032 protein catalyses the cyclization of farnesyl diphosphate (**2**) to *epi*-isozizaene (**1**).

We have recently shown that mutants of *S. avermitilis* such as SUKA16, harbouring deletions of more than 1.4 Mb, including the gene clusters for the majority of the natural products produced by wild-type *S. avermitilis*, are exceptionally useful for the production of a variety of both homologous and heterologous natural products, including terpenoids, based on the re-introduction of the relevant biosynthetic gene clusters under control of strong, tunable promoters (Komatsu *et al*., 2008; 2010). Thus although the *sav3032* gene encoding *epi*-isozizaene synthase was found to be silent in *S. avermitilis* under the culture conditions examined, placing *sav3032* under the control of the alternative promoter, *rpsJp*, resulted in efficient expression of *sav3032* and consequent generation of *epi*-isozizaene. Coexpression of *sav3032* with the *sav3031* gene encoding the CYP170A-family oxygenase (SAV_3031; CYP170A2) led to the production of the expected allylic oxidation products, the epimeric albaflavanols (**4a**, **4b**) and albaflavenone (**3**), as well as a previously unknown compound, 4β,5β-epoxy-2-*epi*-zizaan-6β-ol (**5**). The results confirming that the two-gene, translationally coupled *S. avermitilis* operon, *sav3032–sav3031*, is responsible for albaflavenone biosynthesis. The production of derived metabolites such as albaflanols, albaflavenone, and the newly discovered 4β,5β-epoxy-2-*epi*-zizaan-6β-ol (**5**) requires the coexpression of *sav3031* encoding CYP170A2 since the SUKA16 transformant carrying pKU460::*rpsJp–sav3032* produced only *epi*-isozizaene. Formation of compound **5** from *epi*-isozizaene (**1**) requires two successive oxidation reactions. In principle, compound **5** might be generated from (*4S*)-albaflavenol by successive epoxidation and rearrangement, with ring opening of the initially formed epoxide involving net S_N1-like retention of configuration (Fig. 4), but this has yet to be demonstrated directly. Whatever the detailed mechanism, it is also likely that the second reaction is also catalysed by CYP170A2, although we cannot yet rule out the action of one of the 20 kinds other cytochrome P450s (Lamb *et al*., 2003) or the putative epoxidase (*sav1745*) that are evident in the genome of *S. avermitilis*.

A bioinformatics-based search for *epi*-isozizaene synthases in the bacterial protein databases has revealed that nine additional bacterial strains harbour orthologous gene encoding *epi*-isozizaene synthase, all but one of which is translationally coupled to a downstream member of the CYP170A family of oxygenases. This deduction is supported by the demonstrated production of *epi*-isozizaene by three of these *Streptomyces* strains, *S. albus*, *S. ghanaensis* and *S. lividans*. The formation of albalflavenols and albaflavenone by *S. albus* also indicates that the coupled P450 is active in this strain under general culture conditions.

Experimental procedures

Bacterial strains, vector plasmid and bioinformatics

The large-deletion mutant *S. avermitilis*, SUKA16 (Komatsu *et al*., 2008; 2010), was used for production of sesquiterpenoid compounds. *Streptomyces ghanaensis* ATCC 14672, *S. sviceus* ATCC 29083, *S. viridochromogenes* DSM 40736 were obtained from the culture collection of the RIKEN Bioresource Center, Japan. *Streptomyces albus* J1074 and *S. lividans* TK24 were from the John Innes Centre, UK. The integrating vector containing *attP* and *int* from actinophage φC31 used for the expression of the gene encoding the terpene synthase was described previously (Komatsu *et al*., 2010). The *S. avermitilis* cosmid clones used are listed at http://avermitilis.ls.kitasato-u.ac.jp. Culture condition and recombinant DNA procedures were described in Materials and Methods in *Supporting information*. The procedures for bioinformatics were described previously (Komatsu *et al*., 2008).

Detection of sesquiterpenoid compounds from transformants of S. avermitilis *SUKA16*

The spores of *S. avermitilis* transformants were transferred into vegetative medium (Cane *et al*., 2006) and the culture was grown with shaking at 30°C. A 0.1 ml portion of the culture was inoculated into 10 ml of production medium (Cane *et al*., 2006) in a 125 ml flask. After growth at 28°C on a rotary shaker at 200 r.p.m., the mycelia were harvested by centrifugation and the mycelial products were extracted with 4 ml of methanol for 15 min at room temperature. Mycelia were removed by the centrifugation and the supernatant was extracted with 1 ml of *n*-hexane. After separation of phases, the upper *n*-hexane layer was collected and a portion of the *n*-hexane extract was directly subjected to GC-MS as described previously (Komatsu *et al*., 2008).

*Isolation and purification of 4β,5β-epoxy-2-*epi*-zizaan-6β-ol (**5**)*

Spores of *S. avermitilis* SUKA16 carrying pKU460::*rpsJp–sav3032–sav3031–sav3129–sav5765* (*rpsJp* indicates relatively strong and constitutively controlled promoter in *S. avermitilis*. *sav3129* and *sav5765* are genes encoding ferredoxin and ferredoxin reductase respectively. The con-

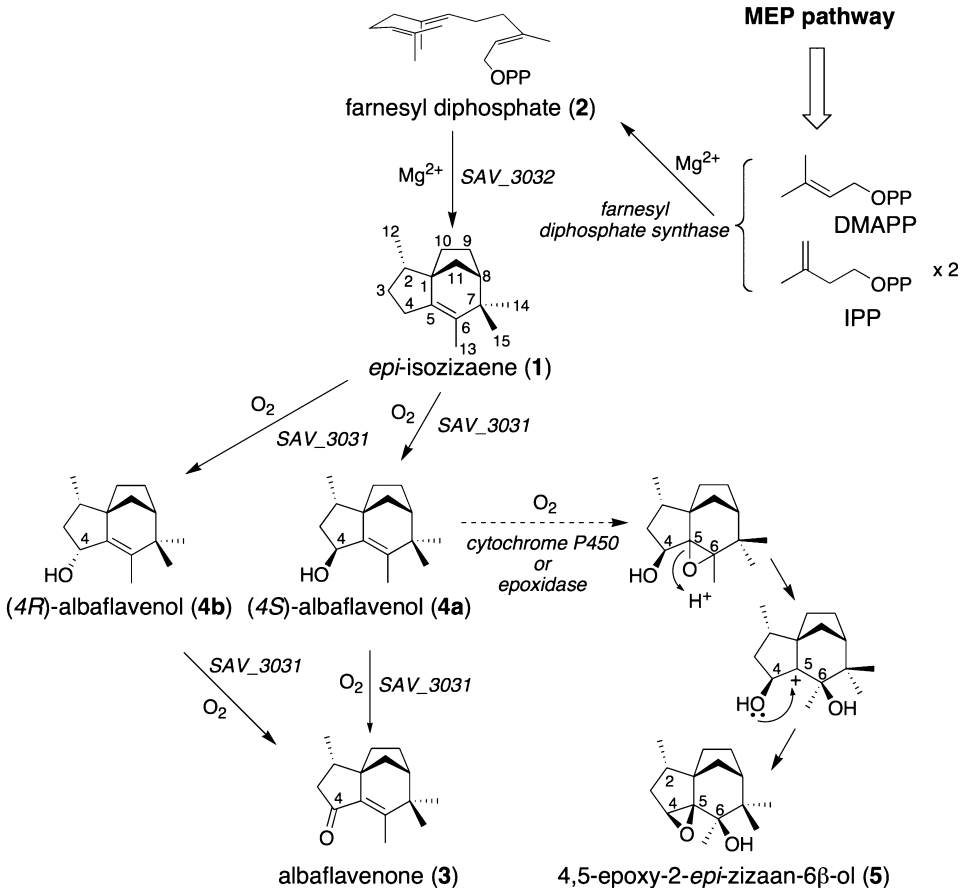

Fig. 4. Biosynthetic pathway in *S. avermitilis* SUKA16 carrying pKU460::*rpsJp–sav3032–sav3031* from farnesyl diphosphate to albaflavenone (**3**) and proposed mechanism of formation of 4β,5β-epoxy-2-*epi*-zizaan-6β-ol (**5**). Abbreviations are as follows: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; MEP, 2-*C*-methylerythritol-4-phosphate.

struction of the plasmid was described in Materials and Methods in *Supporting information*) were cultured under the same conditions as described above. After cultivation for 5 days, the 5 l culture was filtered to harvest the mycelia which were washed with deionized water. The mycelial products were extracted with 500 ml of methanol and then the mycelia were removed by filtration. The methanol extract was extracted three times with 300 ml of *n*-hexane. After separation of the phases, the *n*-hexane layers were combined and concentrated under the reduced pressure to obtain 296 mg of a brownish oil. The crude concentrated extract was dissolved in a small volume of *n*-hexane and subjected to silica gel (70–230 mesh) column chromatography (10 mm in diameter × 85 mm). The previously unknown compound (**5**) was eluted with *n*-hexane-EtOAc (10:1) and confirmed by GC-MS analysis. Pure compound (**5**) (2.6 mg) was obtained using a second silica gel column (230–400 mesh) chromatography (10 mm in diameter × 60 mm) eluted with *n*-hexane-EtOAc (100:1).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alignment of amino acid sequences of known and predicted *epi*-isozizaene synthases (A). Shadow boxes indicate identical amino acids. The four segments illustrate the conserved motifs characteristic of *epi*-isozizaene synthases. Reversed characters indicate aromatic amino acids of which aromatic rings were oriented to stabilize carbocation intermediates in the cyclization cascade through cation-π interactions in *S. coelicolor* A3(2) *epi*-isozizaene synthase. Underlined characters were aromatic amino acids in the fourth conserved motifs. Abbreviations are as follows: SAV, *S. avermitilis*; SCO, *S. coelicolor* A3(2); SAL, *S. albus* J1074; SGH, *S. ghanaensis* ATCC 14672; SGRIS, *S. griseoflavus* Tu4000; SLI, *S. lividans* TK24; SSV, *S. sviceus* ATCC 29083; SVI, *S. viridochromogenes* DSM 40736; SPB74, *Streptomyces* sp. SPB74; SPB78, *Streptomyces* sp. SPB78; BPS668, *Burkholderia pseudomallei* 668. Maps of gene clusters encoding *epi*-isozizaene synthase and cytochrome P450 CYP170 family in *Streptomyces* strains (B). Closed arrows indicate *epi*-isozizaene synthase and greyed arrows are cytochrome P450. Characters above the junction of the two arrows are overlapping nucleotide sequences between the translationally coupled *epi*-isozizaene synthase and cytochrome P450 genes.
Table S1. Summary of predicted *epi*-isozizaene synthase and CYP170A-family cytochrome P450 in bacterial database.
Table S2. Production of sesquiterpenoid compounds from *Streptomyces* strains.

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