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Biosynthesis of the Sesquiterpene Antibiotic Albaflavenone in *Streptomyces coelicolor*. Mechanism and Stereochemistry of the Enzymatic Formation of Epi-isozizaene

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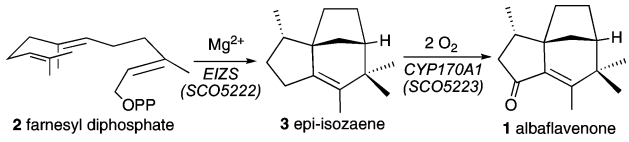
The tricyclic sesquiterpene albaflavenone (**1**), which has been isolated from the gram-positive soil bacteria *Streptomyces coelicolor* and *S. albidoflavus*, has an earthy, camphor-like odor and exhibits antibacterial activity.[1,2] The biosynthesis of albaflavenone in *S. coelicolor* A3(2) is under the control of a two-gene cluster, *sco5222* and *sco5223*.[1a] The 1086-bp *sco5222* gene encodes a protein of 361 amino acids that catalyzes the cyclization of farnesyl diphosphate (**2**, FPP) to the unusual sesquiterpene hydrocarbon (+)-epi-isozizaene (**3**).[1b] The downstream *sco5223* gene, which shares a four-nucleotide ATGA translational overlap with *sco5222*, corresponds to a cytochrome P450 that catalyzes the two-step allylic oxidation of epi-isozizaene to albaflavenone (**1**).[1a]

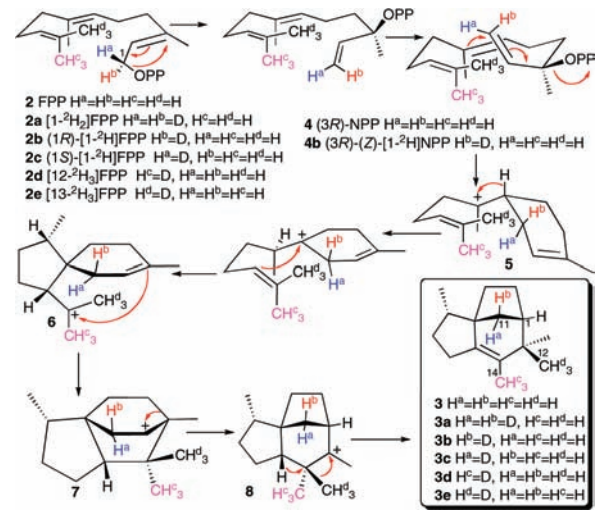
Scheme 1. Biosynthesis of Albaflavenone (**1**) from Farnesyl Diphosphate (**2**) and Epi-isozizaene (**3**)



The cyclization of FPP to epi-isozizaene catalyzed by the SCO5222 protein has been proposed to involve the initial ionization and isomerization of FPP (**2**) to the tertiary allylic isomer, (3*R*)-nerolidyl diphosphate (**4**, NPP) (Scheme 2).[1b] Rotation about the newly generated C-2−C-3 single bond would generate the corresponding *cisoid* NPP conformer that can undergo ionization and cyclization to generate the intermediate bisabolyl cation **5**. Following a 1,2-hydride shift and spirocyclization, the resultant acorenyl cation **6** can undergo further cyclization, ring contraction, 1,2-methyl migration, and final deprotonation to yield (+)-epi-isozizaene (**3**). This proposed mechanism has been supported by incubation of recombinant epi-isozizaene synthase with individual samples of deuterated FPP, [1,1-$^2H_2$]FPP (**2a**), (1*R*)-[1-^{2}H]FPP (**2b**), and (1*S*)-[1-^{2}H]FPP (**2c**) followed by NMR analysis of the resultant labeled **3**.[1b] The combined labeling results are completely consistent with the proposed stereochemical model (Scheme 2), confirming the predicted net retention of configuration in the displacement of the diphosphate group of FPP that reflects the intermediacy of NPP. We now report the direct demonstration of the role of (3*R*)-NPP (**4**) in the formation of epi-isozizaene, including the determination of the detailed stereochemistry of the isomerization−cyclization−rearrangement reaction cascade. We also report the results of site-directed mutagenesis of the universally conserved Mg^{2+}-binding domains and the isolation of abortive cyclization products that reinforce the proposed cyclization mechanism.

Incubation of (3*RS*)-(*Z*)-[1-^{2}H]NPP ((±)-**4b**)[3] with purified recombinant epi-isozizaene synthase resulted in the formation of [11-^{2}H]epi-isozizaene, *m*/*z* 205, as determined by GC−MS analysis.[4] To establish the absolute configuration of the natural NPP intermediate, we carried out competitive incubations between deuterated and undeuterated samples of both (3*S*)- and (3*R*)-(*Z*)-NPP,[3] using selected ion monitoring GC−MS to measure the deuterium content of the resulting epi-isozizaene. Thus incubation of a 1:2 mixture of (3*S*)-(*Z*)-[1-^{2}H]NPP and unlabeled (3*RS*)-NPP gave epi-isozizaene that was devoid of deuterium (>95% d_0). Had only the (3*S*)-NPP been utilized, the observed d_0/d_1 ratio of **3** would have been 1.0, while equal conversion of both enantiomers of NPP would have yielded a d_0/d_1 ratio in the product of 0.5. Complementary incubation of a 1:2 mixture of unlabeled (3*S*)-NPP and (3*RS*)-(*Z*)-[1-^{2}H]NPP with epi-isozizaene synthase gave [^{2}H]epi-isozizaene whose deuterium content indicated a >93:7 preference for (3*R*)-NPP over (3*S*)-NPP. To determine the stereochemistry of the cyclization of the (3*R*)-NPP, we carried out preparative-scale incubations with (3*RS*)-(*Z*)-[1-^{2}H]NPP. The resulting [11_{anti}-^{2}H]epi-isozizaene (**3b**) lacked the characteristic ^{1}H NMR signal at δ 1.47 previously assigned[1b] to H-11_{anti} while displaying a broad singlet for H-11_{syn} that was shifted 0.03 ppm upfield to δ 1.36 due to the isotope effect of the geminal D-11_{anti}. Similarly, the H-11_{anti}/C-11 cross-peak was absent from the HSQC spectrum, while a clear cross-peak for H-11_{syn}/C-11 was evident at δ 1.36 and 36.5 ppm.

Scheme 2. Mechanism and Stereochemistry of the Cyclization of FPP (**2**) to Epi-isozizaene (**3**) via (3*R*)-Nerolidyl Diphosphate (**4**)



These results are completely consistent with the stereochemical model illustrated in Scheme 2, confirming the predicted intermediacy of (3*R*)-NPP (**4**) which is generated by *syn* allylic isomerization of FPP (**2**) and then undergoes *anti*-S_N' cyclization to the bisabolyl cation. This sequence of *syn* isomerization/*anti*-S_N' cyclization to form the bisabolyl cation, with its necessarily *cis* ring double bond, is typical of terpene synthases.[3,5]

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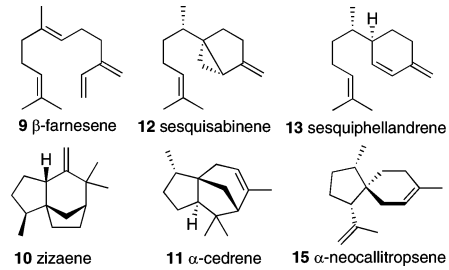
To further test the mechanism of Scheme 2, individual samples of [12,12,12-$^2H_3$]FPP (**2d**)[6] and [13,13,13-$^2H_3$]FPP (**2e**)[6] were each incubated with recombinant epi-isozizaene synthase. The labeling of the resulting *epi*-isozizaene was assigned by ^1H and ^2H NMR. Thus [14,14,14-$^2H_3$]epi-isozizaene (**3d**), derived from [12,12,12-$^2H_3$]FPP (**2d**), lacked the characteristic allylic methyl singlet for H-14 at δ 1.42, while showing the corresponding peak for D-14 in the ^2H NMR spectrum. By contrast, cyclization of [13,13,13-$^2H_3$]FPP (**2e**) gave [12,12,12-$^2H_3$]epi-isozizaene (**3e**) with only a minor singlet at δ 0.99 (<10%), corresponding to geminal methyl H-12, while displaying a singlet at δ 0.99 for D-12 in the ^2H NMR spectrum.[6]

It is therefore established that, in the cyclization of the intermediate acorenyl cation **6**, electrophilic attack engages exclusively the *si* face of the 2-propyl cation side chain to give the postulated tricyclic intermediate cation **7** (Scheme 2).[7] The ^2H-labeling of the geminal methyl groups in **7** and **8** can be inferred from the mandatorily *suprafacial* 1,2-methyl migration. After ring contraction, intermediate **7** undergoes *syn*-1,2-methyl migration and *syn*-deprotonation of **8** to yield [12,12,12-$^2H_3$]epi-isozizaene (**3e**).[8]

Detailed GC−MS analysis of the organic extracts resulting from the incubation of FPP with wild-type epi-isozizaene synthase revealed the formation of at least six additional sesquiterpene hydrocarbons **9**−**14**, *m*/*z* 204, which together comprised ~20% of the total product mixture, with the main components being β-farnesene (**9**, 5%) and zizaene (**10**, 10%) (Chart 1, Table S2). All but compound **14** were identified by comparison of their individual mass spectra and retention indices with those of authentic compounds in the MassFinder 3.0 Database.[9,10] Each aberrant product results from premature quenching or derailment of the normal intermediates of the natural cyclization cascade (Scheme S1).

Chart 1. Additional Sesquiterpenes Produced by Wild-Type and Mutant Epi-isozizaene Synthases



Epi-isozizaene synthase harbors two universally conserved Mg^{2+}-binding domains typical of all sesquiterpene synthases, the apartate-rich ^{99}**DD**RH**D** motif and the characteristic NSE triad ^{240}**N**DLC**S**LPK**E**.[11] Consistent with the importance of each of these domains, the corresponding D99E, D100N, N240D, S244A, and E248D mutants of epi-isozizaene synthase each retained <5% of the FPP cyclase activity of the wild-type enzyme, while the D99N mutant was completely inactive. In common with the behavior of active site mutants of paralogous sesquiterpene synthases,[12] the five epi-isozizaene mutants with residual cyclase activity generated perturbed ratios of the aberrant FPP cyclization products **9**-**14**, as well as an additional sesquiterpene, α-neocallitropsene (**15**) (Table S1).[10] The modified product distributions are thought to reflect compromised binding of the divalent metal ions of the cofactor, with resultant changes in protein conformation and relaxed control over the conformation of the bound substrate FPP and derived intermediates.[12,13]

A BLAST search using as query the *S. coelicolor* epi-isozizaene sequence (SCO5222; UniProt ID Q9K499) reveals likely orthologous terpene synthases in *S. sviceus* (B5HN36, 84% identity), *S. avermitilis* (Q82IV1, 82% identity), and *Streptomyces* sp. SPB74 (B5GD17, 57% identity), each of which has identical conserved Mg^{2+}-binding domains. Moreover, all three epi-isozizaene synthase orthologues have 4-nt ATGA translational overlaps with downstream close homologues of CYP170A1 encoded by *sco5223*, indicating that the albaflavenone biosynthetic pathway may well be widely distributed and highly conserved among Streptomycetes.

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Supporting Information Available: Experimental procedures, GC−MS and NMR data, and supplemental figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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(4) Incubation of (3*RS*)-NPP gave an ~1:1 mixture of epi-isozizaene (**3**) and β-farnesene (**9**). Control experiments showed that >95% of this coproduct **9** was derived from the unnatural enantiomer (3*S*)-NPP, indicating that only (3*R*)-NPP can be cyclized by epi-isozizaene synthase.
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(7) The stereochemistry of the H-4 bridgehead-proton in **6**, **7**, and **8** was deduced based on the assumption of least motion of the 2-propyl cation side chain during the formation of the new C−C bond in **7**.
(8) For analogous sequential 1,2-methyl migration and *syn*-deprotonation, cf. aristolochene synthase (ref 3a) and epi-aristolochene synthase: Schenk, D. J.; Starks, C. M.; Manna, K. R.; Chappell, J.; Noel, J. P.; Coates, R. M. *Arch. Biochem. Biophys.* **2006**, *448*, 31–44.
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