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Published on Web 02/14/2004

Mechanism and Stereochemistry of the Germacradienol/Germacrene D Synthase of *Streptomyces coelicolor* A3(2)

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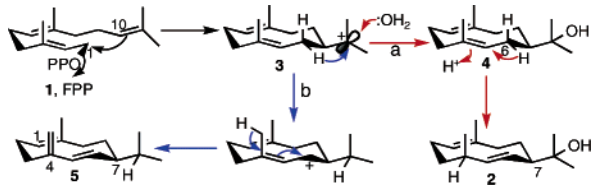
We recently reported the biochemical characterization of a new sesquiterpene cyclase,[1] germacradienol synthase, encoded by the SC9B1.20 (SCO6073) gene of *Streptomyces coelicolor* A3(2),[2] which catalyzes the cyclization of farnesyl diphosphate (**1**, FPP) to (4*S*,7*R*)-germacra-1(10)*E*,5*E*-diene-11-ol (**2**).[2,3] Parallel studies by Chater and his collaborators[4] independently established that germacradienol synthase mediates a key step in the biosynthesis of geosmin, a widely occurring metabolite of numerous streptomycetes, bacteria, and fungi with a characteristic earthy odor.[5] The mechanism for the formation of germacradienol (**2**) (Scheme 1, path a) was proposed to involve ionization of FPP and cyclization to generate the 10-membered ring geracradienyl cation intermediate (**3**), which would react with water to give the (7*R*)-germacra-1(10),4-diene-11-ol (**4**). Protonation of **4** at C-4 followed by deprotonation at C-6 (corresponding to C-1 of FPP) would result in migration of the double bond and formation of germacradienol (**2**).

Analysis by GC-MS of the pentane-extractable products of the enzymatic cyclization of FPP revealed formation of 15% of a sesquiterpene hydrocarbon coproduct with a characteristic *m*/*z* 204, corresponding to $C_{15}H_{24}$, and a fragmentation pattern similar to that for germacrene D (**5**) in the NIST 98 Mass Spectral Database.[6] The structure assignment was rigorously confirmed by extensive 1D and 2D NMR analysis of 1.6 mg of **5** obtained by a preparative scale incubation[7] and comparison with reported data for germacrene D.[8] The 400 MHz ^{1}H NMR displayed the expected signals for three olefinic protons (δ 5.79, d, J = 15.9 Hz, 1H; 5.26, dd, J = 15.8, 9.9 Hz, 1H; 5.14, m, 1H), plus two olefinic exomethylene protons (δ 4.80, d, J = 1.6 Hz, 1H, and δ 4.75, d, J = 2.3 Hz, 1H), as well as an allylic methyl group (δ 1.52, br s, 3H) and a pair of geminal isopropyl methyls (δ 0.87, d, J = 6.8 Hz, 3H, and δ 0.82, d, J = 6.7 Hz, 3H). Both the ^{13}C NMR and the DEPT spectra also matched the published data for germacrene D (**5**), and the assignment was further supported by both ^{1}H−^{1}H COSY and HMQC spectra (see Supporting Information). The formation of **5** could be readily explained by diversion of the germacradienyl cation **3** by a 1,3-hydride shift, followed by deprotonation of the C-4 methyl group (Scheme 1, path b).

The absolute configuration of the enzymatically generated germacrene D was established by direct comparison by chiral capillary GC-MS with an authentic sample of (±)-germacrene D from *Solidago canadensis*.[9] The enantiomeric (+)- and (−)-germacrene D eluted with retention times of 19.09 and 19.28 min, respectively.[9] Under the same conditions, germacrene D produced by germacradienol/germacrene D synthase from *S. coelicolor* A3(2) had a retention time of 19.29 min. This result was confirmed by co-injection with (±)-**5**, thereby establishing the absolute configuration of the coproduct of the enzymatic cyclization as (−)-(7*S*)-germacrene D (**5**), consistent with formation of both **2** and **5** by a common folding of the substrate FPP.

Scheme 1. Mechanism Originally Proposed for Formation of Germacradienol (**2**) and (−)-Germacrene D (**5**) from FPP (**1**)



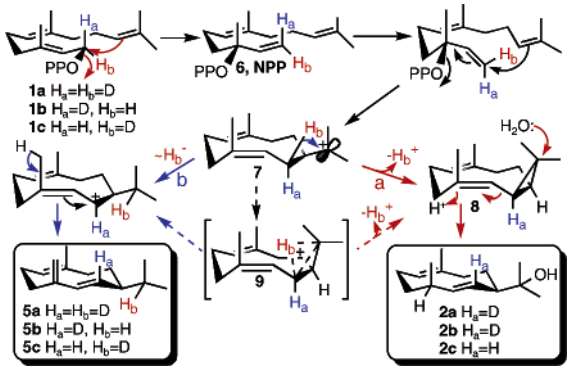
To establish the stereochemistry of formation of both germacradienol (**2**) and germacrene D (**5**), we carried out a series of incubations with stereospecifically deuterated samples of FPP (**1**). The requisite samples of [1,1-^{2}H$_2$]FPP (**1a**), (1*R*)-[1-^{2}H]FPP (**1b**), and (1*S*)-[1-^{2}H]FPP (**1c**) were synthesized as previously described.[10] Incubation of [1,1-^{2}H$_2$]FPP (**1a**) with germacradienol synthase gave germacradienol (**2a**) that retained only one of the two original deuterium atoms, as was evident from the intensities of the parent peak, *m*/*z* 223 [M + 1]$^+$, and a peak at *m*/*z* 83 [M + 1 − $C_9H_{16}O$]$^+$ in the mass spectrum of **2a**. Incubation of (1*R*)-[1-^{2}H]FPP (**1b**) gave germacradienol (**2b**) that displayed a mass spectrum identical to that of **2a**. By contrast, incubation of (1*S*)-[1-^{2}H]FPP (**1c**) generated germacradienol **(2c**) lacking deuterium, with a mass spectrum identical to **2** derived from unlabeled FPP (**1**), including the parent peak at *m*/*z* 222 and a peak at *m*/*z* 82 [M − $C_9H_{16}O$]$^+$. The site of deuterium retention was established by a combination of ^{1}H, ^{2}H, and ^{13}C NMR. Thus, the ^{1}H NMR spectrum of [6-^{2}H]-**2a** derived from incubation of [1,1-^{2}H$_2$]FPP (**1a**) lacked the characteristic signal for H-6 (δ 4.99, dd), while the signal for H-5 (δ 5.68) now appeared as a broad singlet. Consistent with this assignment, the ^{2}H NMR spectrum of [6-^{2}H]-**2a** displayed a signal at δ 4.98 corresponding to D-6, while the corresponding signal for C-6 at 124.1 ppm in the ^{13}C NMR spectrum of [6-^{2}H]-**2a** was strongly attenuated. Taken together, these results established that H-1*si* of FPP is lost in the formation of (4*S*,7*R*)-germacra-1(10)*E*,5*E*-diene-11-ol (**2**), with H-1*re* of FPP becoming H-6 of **2** (Scheme 2).

EI-MS analysis of the (−)-germacrene D (**5**) formed in the same series of incubations established that formation of **5** involves a stereospecific 1,3-hydride shift of H-1*si* of FPP. Incubation of [1,1-^{2}H$_2$]FPP (**1a**) gave [6,11-^{2}H$_2$]-**5a** that displayed a parent peak at *m*/*z* 206 [M + 2]$^+$ and a d_1-base peak at *m*/*z* 162, [M + 2 − $CDMe_2$]$^+$, formed by loss of the deuterated isopropyl side chain. The migration of H-1*si* was established by parallel incubations of (1*R*)-[1-^{2}H]FPP (**1b**) and (1*S*)-[1-^{2}H]FPP (**1c**) with germacradienol/germacrene D synthase to give [6-^{2}H]-**2b** and [11-^{2}H]-**2c**, which both had d_1-parent peaks at *m*/*z* 205 but base peaks at *m*/*z* 162 [M + 1 − $CHMe_2$]$^+$ and 161 [M + 1 − $CDMe_2$]$^+$, respectively, corresponding to fragmentation of the relevant protiated and deuterated side chains.

Incubation of (1*S*)-[1-^{2}H]FPP (**1c**) with germacradienol/germacrene D synthase resulted in an unexpected increase in the relative proportion of the minor product (−)-germacrene D (**5**), with a 35:

10.1021/ja039929k CCC: $27.50

Scheme 2. Mechanism and Stereochemistry of the Cyclization of FPP (**1**) to Germacracradienol (**2**) and (−)-Germacrene D (**5**)



65 ratio of **5**/**2**, as compared to the 15:85 ratio observed for unlabeled FPP (**1**). Cyclization of [1,1-$^2H_2$]FPP (**1a**) gave an intermediate product ratio of 25:75 **5**/**2**, while (1*R*)-[1-^{2}H]FPP (**1b**) gave the same proportion of products as unlabeled FPP. The perturbation in the product ratios as a consequence of isotopic substitution requires that both products be formed from a common intermediate, with the partitioning between the two pathways being subject to a deuterium isotope effect. It is therefore evident that the originally proposed mechanistic pathway illustrated in Scheme 1 cannot be correct. Thus, loss of the H-1*si* proton of FPP from H-6 of germacradienol **4** (Scheme 1, path a) would occur too late to have any effect on the partitioning of the common cationic intermediate **3**. An alternative mechanism for the cyclization of FPP (**1**) that fully accounts for the observed isotopically sensitive branching as well the stereochemistry of formation of both germacradienol and germacrene D involves rearrangement of FPP (**1**) to the tertiary allylic isomer, (3*R*)-nerolidyl diphosphate (**6**, NPP).[1,11] Rotation about the 2,3-bond followed by ionization and electrophilic attack of the newly generated cisoid allylic cation on C-10 will give the helminthogermacradienyl cation **7**.[11] The latter then serves as the common intermediate that partitions to either **2** or **5**. Thus, cyclization of carbocation **7** with loss of the original H-1*si* of FPP (Scheme 2, path a) would give isobicyclogermacrene (**8**).[12] Protonation of the vinyl cyclopropane moiety of **8** with ring opening of the resultant cyclopropyl carbinyl cation and capture by water would give germacradienol **2**. The deprotonation of **7** competes with the 1,3-hydride shift of the same H-1*si*-derived hydrogen to generate (−)-germacrene D (**5**) (path b). In principle, the competing deprotonation and hydride shift pathways may involve partitioning of an edge-protonated cyclopropane corresponding to the common intermediate or transition state **9**.[13] The isotope effect on the loss of H_b-6 of **7** (^Dk_a) is apparently greater than that for the competing hydride migration of H_b-6 (^Dk_b) (or alternatively, ring-opening of **9** with net migration of H_b), resulting in an increase in partitioning along path b, with a concomitant increase in the proportion of (−)-germacrene D (**5**) as compared to germacradienol (**2**).[14] This net isotope effect on partitioning ($^Dk_a/^Dk_b$) is apparently attenuated by an α-secondary deuterium isotope effect in the cyclization of [1,1-$^2H_2$]FPP (**1a**).

The stereospecificity of the hydride shift in the formation of (−)-germacrene D by the germacradienol/germacrene D synthase of *S. coelicolor* A3(2) is opposite to that previously observed for the (−)-germacrene D synthase of *S. canadensis*.[9a] In the latter case, H-1*re* of FPP undergoes the 1,3-hydride shift, in contrast to the migration of H-1*si* mediated by the *S. coelicolor* (−)-germacrene D synthase. On the other hand, due to the differences in geometry of the germacradienyl cation intermediates **3** and **7**, the stereoelectronics of hydride migration are the same for both cyclizations. Notably, (+)-germacrene D synthase of *S. canadensis* catalyzes the formation of the enantiomeric sesquiterpene by a pair of consecutive 1,2-hydride shifts. Thus, not only are the enantiomers of germacrene D formed from FPP by distinct mechanisms, but formation of (−)-germacrene D can also take place with different mechanisms, but similar stereoelectronics, depending on the source of the synthase.

Acknowledgment. (±)-Germacrene D from *Solidago canadensis* provided by Dr. H. J. Boowmeester of the Research Institute for Agrobiology and Soil Fertility, Wageningen, The Netherlands, was a generous gift of Prof. W. A. König of the Universität Hamburg, Germany. We thank Prof. Duilio Arigoni of the ETH, Zürich for critical comments leading to the formulation of the mechanism of Scheme 2. This work was supported by NIH Merit Award Grant GM30301 to D.E.C.

Supporting Information Available: NMR spectra and GC/MS data for **2** and **5** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) Germacradienol synthase (5.0 nmol) and FPP (**1**) (600 nmol) in 10 mL of assay buffer (50 mM Tris−Cl, 20% (v/v) glycerol, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, pH 8.2) overlaid with 2.0 mL of pentane (HPLC grade 99+%) was incubated at 30 °C for 3 h. The dried pentane extracts were passed through SiO_2, concentrated, and analyzed on an Agilent 6890 GC/JEOL JMS-600H mass spectrometer, using a 30 m × 0.25 mm HP5MS capillary column in EI (positive) mode using a temperature program of 60−280 °C, with a gradient of 20 °C/min and a solvent delay of 3.5 min.

(7) In preparative scale incubations, FPP (50 mg, 100 μmol) was incubated with germacradienol synthase (1.5 μmol, added in four equal portions every ∼6 h) in 1.4 L of modified assay buffer containing 0.2 mM β-mercaptoethanol overlaid with 300 mL of pentane (HPLC grade) at 30 °C for 24 h. The dried pentane extracts were purified by flash SiO_2 chromatography (pentane) to give **5** (1.6 mg) as a colorless oil. Chiral GC-MS analysis was carried out using a 25-m fused silica capillary column of heptakis(6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin (Macherey-Nagel).[9]

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(14) This result would be consistent with the greater degree of C−H bond conservation in the transition state for cyclopropane ring opening of **9** as compared to the competing deprotonation that gives **8**. Cf. More-O'Ferrall, R. A. *J. Chem. Soc. B* **1970**, 785−790.

JA039929K