Aristolochene Synthase. Elucidation of the Cryptic Germacrene A Synthase Activity Using the Anomalous Substrate Dihydrofarnesyl Diphosphate

David E. Cane* and Youla S. Tsantrizos[1]

Contribution from the Department of Chemistry, Box H, Brown University, Providence, Rhode Island 02912

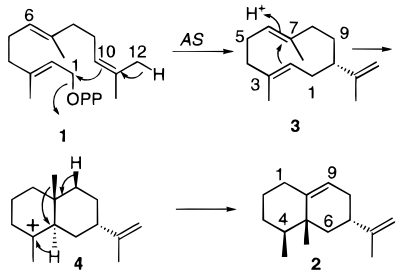
Received June 12, 1996[⊗]

Abstract: Germacrene A (**3**) has been proposed to be an intermediate in the cyclization of farnesyl diphosphate (**1**) to the sesquiterpene hydrocarbon (+)-aristolochene (**2**). Under normal circumstances, however, neither **3** nor any other intermediate is released from the cyclase active site. When the anomalous substrate (7*R*)-6,7-dihydrofarnesyl diphosphate (**5**) is incubated with aristolochene synthase from *Aspergillus terreus*, the resultant dihydrogermacrene (**6**) cannot be further cyclized and is released from the active site. The structure of the abortive cyclization product, *m*/*z* 206, was confirmed as dihydrogermacrene (**6**) by direct GC−MS comparison with an authentic synthetic sample prepared from (−)-(3*S*)-*β*-citronellol.

Aristolochene synthase catalyzes the cyclization of farnesyl diphosphate (**1**, FPP) to (+)-aristolochene (**2**), the parent hydrocarbon of a large group of fungal toxins.[2] The enzyme from *Penicillium roqueforti*,[3a] a monomer of M_r 38 000, has been cloned[3b] and overexpressed[3c] in *Escherichia coli*. The *Aspergillus terreus* enzyme,[4] of apparently the same molecular weight, has also recently been purified to homogeneity.[5] Investigations with stereospecifically labeled substrates[4] and with a mechanism-based inhibitor[6] have supported a cyclization mechanism in which FPP undergoes an initial cyclization to germacrene A (**3**) by electrophilic attack of an allylic cation on C-10 of the distal double bond, followed by loss of a proton from the attached (*Z*)-methyl group (Scheme 1). Reprotonation of germacrene A at C-6 and further cyclization would give the bicyclic eudesmane cation **4**. Consecutive methyl migrations and deprotonation can then generate aristolochene (**2**).

Under normal circumstances, germacrene A is never released from the cyclase active site and therefore cannot be directly detected. One effective strategy for the investigation of terpenoid synthases catalyzing multistep transformations in which the intermediates are sequestered by the enzyme has been to use substrate analogs suitably designed so as to prevent completion of the normal catalytic cycle, either by stabilization of an otherwise reactive intermediate or by the absence of a critical reactive center.[7,8] The abortive cyclization products thus generated are released from the enzyme and are diagnostic of the structure and stereochemistry of the normal enzyme-bound intermediates. For example, we have previously used both (7*S*)- and (7*R*)-6,7-dihydrofarnesyl diphosphate (**5**) to study the cryptic isomerization of farnesyl to nerolidyl diphosphate catalyzed by trichodiene synthase.[8] Incubation of either substrate analog with trichodiene synthase led to formation of dihydronerolidyl diphosphate which, due to the absence of the central double bond, was incapable of undergoing cyclization to the normal bisabolyl cation intermediate. Instead, ionization of dihydronerolidyl diphosphate and subsequent deprotonation or capture by water resulted in the formation of a mixture of acyclic hydrocarbons and alcohols (Scheme 2).

Scheme 1. Intermediacy of Germacrene A (**3**) in the Cyclization of Farnesyl Diphosphate (**1**) to (+)-Aristolochene (**2**)



In order to investigate the formation of germacrene A by aristolochene synthase, we envisaged that cyclization of the same substrate analog, (7*R*)-6,7-dihydrofarnesyl diphosphate (**5**), would result in formation of 6,7-dihydrogermacrene A (**6**) (Scheme 3) The absence of a 6,7-double bond in **6**[9] would not only block the protonation that normally takes place at C-6 but

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1996.

(1) Current address: Department of Chemistry & Biochemistry, Concordia University, 1455, de Maisonneuve Blvd. W., Montreal, Québec H3G 1M8 Canada.

(2) *Encyclopedia of Terpenoids*; Glasby, J. S., Ed.; Wiley: Chichester, England, 1982.

(3) (a) Hohn, T. M.; Plattner, R. D. *Arch. Biochem. Biophys.* **1989**, *272*, 137−143. (b) Proctor, R. H.; Hohn, T. M. *J. Biol. Chem.* **1993**, *268*, 4543−4548. (c) Cane, D. E.; Wu, Z.; Proctor, R. H.; Hohn, T. M. *Arch. Biochem. Biophys.* **1993**, *304*, 415−419.

(4) Cane, D. E.; Rawlings, B. J.; Yang, C. *J. Antibiot.* **1987**, *40*, 1331−1334. Cane, D. E.; Prabhakaran, P. C.; Salaski, E. J.; Harrison, P. H. M.; Noguchi, H.; Rawlings, B. J. *J. Am. Chem. Soc.* **1989**, *111*, 8914−8916. Cane, D. E.; Prabhakaran, P. C.; Oliver, J. S.; McIlwaine, D. B. *J. Am. Chem. Soc.* **1990**, *112*, 3209−3210.

(5) Cane, D. E.; Kang, I., unpublished results.

(6) Cane, D. E.; Bryant, C. *J. Am. Chem. Soc.* **1994**, *116*, 12063−12064.

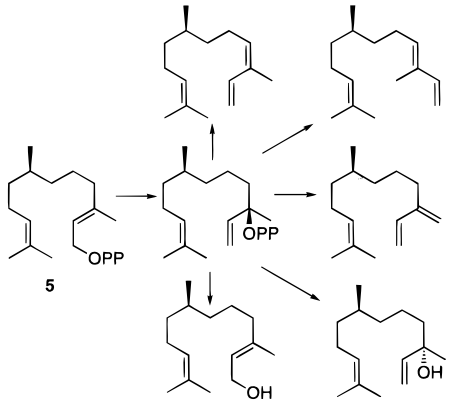
(7) (a) Monoterpene biosynthesis: Wheeler, C. J.; Croteau, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4856−4859. Wheeler, C. J.; Croteau, R. *Arch. Biochem. Biophys.* **1986**, *246*, 733−742. (b) Steroid biosynthesis: Corey, E. J.; Virgil, S. C. *J. Am. Chem. Soc.* **1991**, *113*, 4025−4026. Corey, E. J.; Virgil, S. C.; Liu, D. R.; Sarshar, S. *J. Am. Chem. Soc.* **1992**, *114*, 1524−1525. Corey, E. J.; Virgil, S. C.; Sarshar, S. *J. Am. Chem. Soc.* **1991**, *113*, 8171−8172. Corey, E. J.; Virgil, S. C.; Cheng, H. M.; Baker, C. H.; Matsuda, S. P. T.; Singh, V.; Sarshar, S. *J. Am. Chem. Soc.* **1995**, *117*, 11819−11820.

(8) Cane, D. E.; Pawlak, J. L.; Horak, R. M.; Hohn, T. M. *Biochemistry* **1990**, *29*, 5476−5490. Cane, D. E.; Yang, G. *J. Org. Chem.* **1994**, *59*, 5794−5798.

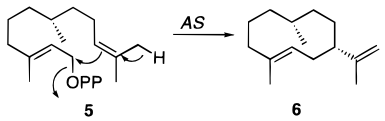
(9) For clarity, numbering in dihydrogermacrene A (**6**) has been based on numbering of the corresponding carbon atoms in the precursor dihydrofarnesyl diphosphate (**5**).

S0002-7863(96)01981-6 CCC: $12.00

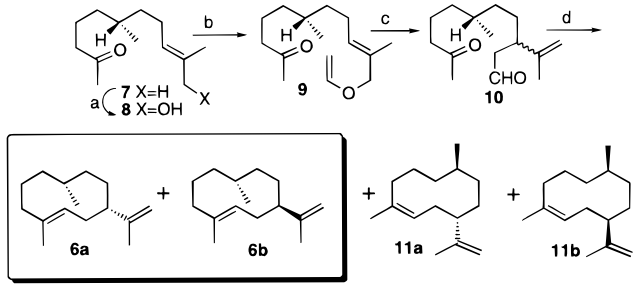
Scheme 2. Isomerization of (7*S*)-6,7-Dihydrofarnesyl Diphosphate (**5**) to Dihydronerolidyl Diphosphate and Conversion to Acyclic Hydrocarbons and Alcohols by Trichodiene Synthase



Scheme 3. Cyclization of (7*R*)-6,7-Dihydrofarnesyl Diphosphate (**5**) to Dihydrogermacrene (**6**) by Aristolochene Synthase



Scheme 4. Synthesis of Dihydrogermacrene (**6**)[a]



[a] (a) SeO_2, *t*-BuOOH, CH_2Cl_2, 0 °C, 3.5 h; (b) $Hg(OTf)_2$, $EtOCH{=}CH_2$, reflux, 36 h; (c) sealed tube, 200 °C, 6 h; (d) (i) 10 equation of $TiCl_3(DME)_{1.5}$, 35 equiv of Zn−Cu couple, DME, reflux 5 h under Ar; (ii) add **9** over 30 h by syringe pump, reflux, 5 h.

the abortive cyclization product that accumulated would not be subject to the facile Cope rearrangement characteristic of germacrene A.[10]

Results

A reference sample of **6** was prepared as a mixture of two stereoisomers by a simple variation of McMurry's efficient synthesis of germacrene A and helminthogermacrene.[11] To this end, (6*R*)-5,6-dihydrogeranylacetone (**7**), prepared as previously described from (−)-(3*S*)-β-citronellol,[8] was converted to the corresponding allylic alcohol **8** followed by treatment with ethyl vinyl ether in the presence of $Hg(OTf)_2$ to give the allylic vinyl ether **9** in overall 45−55% purified yield (Scheme 4) Claisen rearrangement of **9** proceeded smoothly to give the corresponding aldehyde **10** (65%) as a mixture of diastereomers. Reductive coupling with the McMurry reagent[12] gave a 45% yield of a mixture of the *trans* and *cis* isomers, 6,7-dihydrogermacrene A (**6**) and 6,7-dihydrohelminthogermacrene (**11**), respectively, each of which was present as a pair of diastereomers, as evidenced by ^{1}H NMR data and capillary GC/CI-MS analysis, in an overall ratio of 1.0:1.1:1.6:2.0 for **6a**, **6b**, **11a**, and **11b**. The *cis* and *trans* isomers, **6** and **11**, which were separated by argentation SiO_2 column chromatography, were readily distinguished by difference NOE experiments, which displayed the expected positive NOE between the olefinic H-2 proton and the *(Z)*-methyl in both **11a** and **11b**, but not in **6a** and **6b**.

(7*R*)-6,7-Dihydrofarnesyl diphosphate (**5**), prepared as previously described,[8] acted as a very efficient competitive inhibitor of aristolochene synthase, with an observed K_I of 0.18 μM, compared to a K_m of 3.28 μM for FPP, indicating that the substrate analog was capable of binding tightly to the cyclase active site. Preparative-scale incubation of **5** with *A. terreus* aristolochene synthase[13] produced a single hydrocarbon product, *m/z* 206, which upon capillary GC−MS analysis was identical in retention time and mass spectrum with the minor, faster eluting, diastereomer **6a** of synthetic *trans*-dihydrogermacrene. The configuration of the 2-propenyl side chain in **6a** has not yet been directly determined but has been provisionally assigned as *S*, based on the known configuration of the corresponding center in (+)-aristolochene.[14,15] The observed rate of formation of **6a** (1 nmol h^{-1} (mg of protein)$^{-1}$ was a factor of 75 slower than turnover of the natural substrate, FPP (75 nmol h^{-1} (mg of protein)$^{-1}$), in comparable incubations with partially purified aristolochene synthase.

Conclusions

The observed formation of dihydrogermacrene A from 6,7-dihydrofarnesyl diphosphate provides strong evidence for the intermediacy of germacrene A in the conversion of FPP to aristolochene. Although the configuration of the 2-propenyl side chain in the abortive cyclization product has not yet been definitively assigned, there is now no doubt that the first step in the cyclization sequence is the generation of the 10-membered ring, germacrene A intermediate. The generality of these results is enhanced by the fact that germacrene A has been proposed as an intermediate in the conversion of farnesyl diphosphate to numerous eudesmanoid, eremophilane,[16] and related sesquiterpenes including valencene, eremophilene, epi-aristolochene,[17] and vetaspiradiene[18] and their derived metabolites (Scheme 5).

Experimental Section

Instrumentation and General Methods. General instrumentation and methods were as previously described.[8] [1-^{3}H]Farnesyl diphosphate (58 mCi/mmol) was prepared as previously described.[8] Unlabeled and [1-^{3}H]-(7*R*)-6,7-dihydrofarnesyl diphosphate (**5**; 59.8 mCi/mmol) were each prepared from enantiomerically pure (−)-(3*S*)-β-citronellol (Aldrich) as previously described.[8]

***trans*-(7*R*)-Dihydrogermacrenes (6a,6b) and *cis*-(7*R*)-dihydrogermacrenes (11a,11b). (a) (9*E*,6*R*)-6,10-dimethyl-2-oxo-9-undecen-**

(10) Weinheimer, A. J.; Youngblood, W. W.; Washecheck, P. H.; Karns, T. K. B.; Ciereszko, L. S. *Tetrahedron Lett.* **1970**, 497−500.

(11) McMurry, J. E.; Kocovsky, P. *Tetrahedron Lett.* **1985**, *26*, 2171−2172.

(12) McMurry, J. E.; Lectka, T.; Rico, J. G. *J. Org. Chem.* **1989**, *54*, 3748−3749.

(13) In preliminary incubations, (7*R*)-6,7-dihydrofarnesyl diphosphate (**5**) was turned over by aristolochene synthase about twice as efficiently as was the corresponding (7*S*)-6,7-dihydrofarnesyl diphosphate. For this reason we decided to concentrate our efforts on the 7*R* enantiomer.

(14) Cane, D. E.; Salaski, E. J.; Prabhakaran, P. C. *Tetrahedron Lett.* **1990**, *31*, 1943−1944.

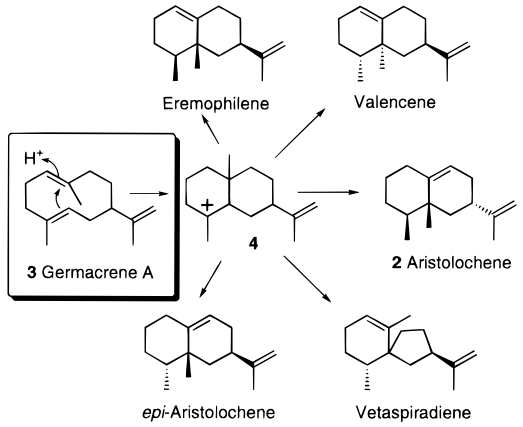
(15) We cannot as yet rule out aberrant binding of dihydrofarnesyl diphosphate so as to generate dihydrogermacrene A with the 2-propenyl group in the unnatural configuration. Several derivatives of synthetic **6b** have been prepared, but to date none have been suitable for X-ray crystallographic analysis (D. E. Cane and S. Tsubotani, unpublished).

(16) Penfold, A. R.; Simonsen, J. L. *J. Chem. Soc.* **1939**, 87−89. *The Structural Relations of Natural Products;*; Robinson, R., Clarendon: Oxford, England, 1955; p 12.

(17) Vögeli, U.; Freeman, J. W.; Chappell, J. *Plant Physiol.* **1990**, *93*, 182−187. Facchini, P. J.; Chappell, J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11088−11092.

(18) Back, K.; Chappell, J. *J. Biol. Chem.* **1995**, *270*, 7375−7381.

Scheme 5. Intermediacy of Germacrene A in Sesquiterpene Biosynthesis



11-ol (8). SeO_2 (520 mg, 4.7 mmol) was added to dry CH_2Cl_2 (2 mL), and the mixture was cooled in an ice bath under N_2. A solution of anhydrous *t*-BuOOH in 2,2,4-trimethylpentane (3 M , 10 mL, 30 mmol) was added dropwise via a syringe followed by the addition of (6*R*)-5,6-dihydrogeranyl acetone (1.53 g, 7.8 mmol), prepared as previously described from (−)-(3*S*)-*β*-citronellol,[8] dissolved in 3 mL of CH_2Cl_2. The reaction was monitored by TLC and it was stopped as soon as trace amounts of allylic aldehyde were detected (3.5 h). The reaction mixture was then diluted with 150 mL of EtOAc, extracted with H_2O (100 mL) and saturated aqueous $NaHCO_3$ (100 mL), dried over $MgSO_4$, and evaporated to dryness. Flash column chromatography using a linear solvent gradient from 10 to 25% EtOAc in hexane led to the isolation of 930 mg of **8** (88% yield, based on recovered starting material), accompanied by 100 mg of the corresponding unsaturated aldehyde (9%). **8**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (CH_3, d, J = 6.5 Hz), 1.12−1.25 (2H, m), 1.27−1.45 (3H, m), 1.48−1.59 (2H, m), 1.65 (CH_3, s), 1.95−2.10 (3H, m), 2.14 (CH_3, s), 2.41 (2H, t, J = 7.5 Hz), 3.99 (2H, d, J = 4.7 Hz), 5.38 (1H, t, J = 7.2 Hz); ^{13}C NMR (400 MHz, $CDCl_3$) δ 13.34, 19.16, 21.06, 24.81, 29.55, 31.99, 36.05, 36.31, 43.74, 68.40 (C-11), 125.88 (C-9), 134.35 (C-10), 209.28 (C-2).

(b) Allyl Vinyl Ether 9. Allylic alcohol **8** (580 mg, 2.7 mmol) and $Hg(CF_3COO)_2$ (700 mg, 1.6 mmol) were dissolved in ethyl vinyl ether (40 mL) and refluxed for 1.5 days under anhydrous conditions. The reaction mixture was then diluted with CH_2Cl_2 (100 mL), extracted with 5% KOH (3 × 50 mL), dried over $MgSO_4$, and evaporated to dryness. The allyl vinyl ether **9** was found to be very unstable to chromatography on silica gel or active alumina. The product was partially purified on deactivated alumina (prepared by the addition of H_2O (1:10 w/w) to activated neutral alumina (Brockmann I, Aldrich)) using a gradient of 5−10% EtOAc in hexane. **9**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (CH_3, d, J = 6.5 Hz), 1.05−1.22 (2H, m), 1.22−1.31 (1H, m), 1.31−1.45 (2H, m), 1.47−1.64 (2H, m), 1.67 (CH_3, s), 1.96−2.10 (2H, m), 2.14 (CH_3, s), 2.40 (2H, t, J = 7.5 Hz), 3.99 (1H, dd, J_1 = 6.8 Hz, J_2 = 1.8 Hz), 4.08 (2H, s), 4.22 (1H, dd, J_1 = 14.2 Hz, J_2 = 1.8 Hz), 5.44 (1H, t, J = 7.2 Hz), 6.45 (1H, dd, J_1 = 14.2 Hz, J_2 = 6.8 Hz); ^{13}C NMR (400 MHz, $CDCl_3$) δ 13.78, 19.37, 21.32, 25.16, 29.85, 32.30, 36.35, 36.39, 44.03, 74.59, 86.92, 129.34, 130.80, 151.60, 209.18.

(c) Claisen Rearrangement of 9 to 10. Vinyl ether **9** (740 mg, 3.1 mmol) was heated in a sealed glass tube to 200 °C for 5−6 h. Purified **10** was isolated as a mixture of diastereomers in 65% yield (475 mg) after flash column chromatography using a linear gradient of 5−10% EtOAc in hexane. **10**: HRMS (EI, high resolution) *m*/*z* 238.1908 (calcd for $C_{15}H_{26}O_2$ 238.1926); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86 (CH_3, dd, J_1 = 6.6 Hz, J_2 = 1.9 Hz), 1.00−1.18 (2H, m), 1.18−1.32 (2H, m), 1.32−1.45 (3H, m), 1.45−1.65 (2H, m), 1.65 (CH_3, m), 2.13 (CH_3, d, J = 0.4 Hz), 2.39−2.44 (4H, m), 2.58−2.68 (1H, m), 4.77 (2H, dm, J = 12.2 Hz), 9.66 (1H, t, J = 2.4 Hz); ^{13}C NMR (400 MHz, $CDCl_3$) δ 18.31 and 18.50, 19.06 and 19.27, 20.93 and 29.97, 2 × 29.51, 30.07 and 30.18, 32.16 and 32.35, 33.74, and 33.84, 35.83 and 36.20, 41.41 and 41.50, 43.58 and 43.59, 47.19 and 47.34, 111.91 and 112.05, 145.63 and 145.78, 201.88 and 201.91, 2 × 208.59.

(d) *trans*-(7*R*)-Dihydrogermacrene (6a, 6b) and *cis*-(7*R*)-dihydrogermacrenes (11a, 11b). $TiCl_3(DME)_{1.5}$ and Zn−Cu couple were prepared according to McMurry's procedure[12] except that commercially available anhydrous dimethoxyethane was used after distillation over Na metal using benzophenone as the indicator. All transfers were carried out in a glovebag under an atmosphere of dry argon, and samples were weighed in sealed vials filled with argon. $TiCl_3(DME)_{1.5}$ (2.82 g, 9.7 mmol) and Zn−Cu couple (2.11 g, 33 mmol) were transferred into an oven-dried flask (200 mL) to which DME was added (100 mL) with stirring. The flask was sealed, removed from the glovebag, and connected to a reflux condenser under a strong flow of argon. The black suspension was then stirred for 5 h at reflux. A solution of **10** (225 mg, 0.94 mmol) in dry DME (25 mL) was added to the activated Ti metal, at a rate of 0.7−1 mL/h using a syringe pump over a period of 30 h, while the temperature was maintained at reflux. Following addition of **10**, reflux was continued for an additional 5 h. The reaction mixture was then cooled in an ice bath, pentane was added (50 mL), and the mixture was filtered through a pad of florisil to remove metal salts. Most of the solvent was evaporated *very slowly* on the rotary evaporator; the receiving flask was immersed in a dry ice/2-propanol bath, and the evaporation flask was kept at about room temperature. When a very small amount of solvent was left, the crude dihydrogermacrene solution was transferred to a small flask (using only pentane or CH_2Cl_2) and the remainder of the solvent was removed with a flow of N_2 while the flask was immersed in an ice bath: GC/MS, Chrompack CP-Sil 5 CB, 25 m × 0.32 mm × 0.12 μm, 100 °C, 2 min, 10 °C/min to 250 °C; retention times, *trans*-(7*R*)-dihydrogermacrenes **6a**, R_t 5.45 min, and **6b**, R_t 5.63 min, *cis*-(7*R*)-dihydrogermacrenes **11a**, R_t 5.93 min, and **11b**, R_t 6.18 min; ratio 1.0:1.1:1.6:2.0. In order to optimize resolution of components, the GC conditions were slightly changed for the GC/MS experiments with the enzymatic dihydrogermacrene products (see below for details). Under the latter conditions, the retention times of the two *trans* products were **6a** 4.75 min and **6b** 5.07 min. Flash column chromatography of the crude mixture of dihydrogermacrenes on Ag^+-impregnated silica gel, using a linear gradient from 10% CH_2Cl_2 in pentane to 100% CH_2Cl_2, led to the separation of the two *cis* compounds (**11a**, **11b**) from the two *trans* isomers (**6a**, **6b**). The major *trans* compound was isolated almost completely free of the minor component; further separation of each compound was not attempted, although the two *trans* diastereomers are clearly separated on TLC. MS (CI, NH_3) for both **6a** and **6b** were identical, *m*/*z* (% rel intensity) 206 (12), 150 (20), 135 (32), 121 (40), 109 (66), 95 (94), 81 (100), 68 (99), 55 (37). Flash column chromatography on $AgNO_3$−SiO_2 (linear gradient 10% CH_2Cl_2/pentane to 100% CH_2Cl_2) gave pure **6b** and a mixture of **6a** and **6b**. **6b:** ($AgNO_3$−SiO_2; CH_2Cl_2/EtOAc 4:1, R_f 0.45) ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.79 (d, J = 6.5 Hz, 3H, C*H*$_3$CH), 0.96−1.04 (m, 1H), 1.15−1.38 (m, 5H), 1.45−1.65 (m, 3H), 1.71 (br s, 6H, C*H*$_3$C═C), 1.78−1.88 (m, 1H), 2.1−2.25 (m, 4H), 4.61 (br s, 1H, methylene C*H*), 4.67 (br s, 1H, methylene C*H*), 5.40 (t, J = 7.4 Hz, 1H, C*H*═C); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 18.9, 20.5, 22.3, 23.9, 32.5, 33.8, 34.5, 35.2, 35.4, 38.5, 49.3, 107.94, 124.6, 134.9, 151.9. Partial spectra **6a**: ($AgNO_3$−SiO_2; CH_2Cl_2/EtOAc 4:1, R_f 0.35) ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.81 (d, J = 6.5 Hz, 3H, C*H*$_3$CH), 1.71 (br s, C*H*$_3$C═C), 4.61 (br s, 1H, methylene C*H*), 4.67 (br s, 1H, methylene C*H*), 5.40 (t, J = 7.4 Hz, 1H, C*H*═C); ^{13}C NMR (100.6 MHz, $CDCl_3$) 107.92, 126.3, 139.8, 151.5. (7*R*)-*cis*-dihydrogermacrene (**11a**, **11b**): Ag^+-SiO_2 TLC (CH_2Cl_2/EtOAc 4:1) R_f 0.85; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.81 (0.5 CH_3, d, J = 6.7 Hz, minor diastereomer), 0.87 (0.5 CH_3, d, J = 6.8 Hz, major diastereomer), 0.95−1.75 (12H, m), 1.67 (CH_3, m), 1.76 (CH_3, dm, J = 3.3 Hz), 2.0−2.4 (2H, m), 4.65 (0.5H, m, minor diastereomer), 4.75 (0.5H, m, minor diastereomer), 4.69 (0.5H, s, major diastereomer) and 4.80 (0.5H, s, major diastereomer); ^{13}C NMR (400 MHz, $CDCl_3$) δ 14.04, 21.58, 22.13 (br), 22.35, 22.71, 22.74, 23.03, 23.16, 24.56, 27.69, 28.38, 28.88 (br), 29.58, 29.73, 30.48 (br), 31.52 (br), 43.46, 43.62, 108.74, 109.13, 123.53, 125.13 (br), 135.40 (br), 136.20, 148.61, 149.90. (7*R*)-*trans*-dihydrogermacrene (**11a**, **11b**): Ag^+-SiO_2 TLC (CH_2Cl_2/EtOAc 4:1) R_f 0.4−0.5, 0.3−0.4; ^{1}H NMR (400 MHz, $CDCl_3$) (major diastereomer) δ 0.79 (CH_3, d, J = 6.5 Hz), [the equivalent signal of the minor diastereomer appears at δ 0.81 (CH_3, d, J = 6.5 Hz)], 0.96−1.04 (1H, m), 1.15−1.38 (5H, m), 1.45−1.65 (3H, m), 1.71 [2CH_3, m (sharp)], 1.78−1.88 (1H m), 2.1−2.25 (4H,

m), 4.61 [1H, m(sharp)], 4.67 [1H, m(sharp)], 5.40 (1H, t, J = 7.4 Hz); ^{13}C NMR (400 MHz, $CDCl_3$) of major diastereomer (minor diastereomer) δ 18.9, 20.5, 22.3, 23.9, 32.5, 33.8, 34.5, 35.2, 35.4, 38.5, 49.3, 107.94 (107.92), 124.6 (126.3), 134.9 (139.8), 151.9 (151.5).

Isolation of Aristolochene Synthase from *A. terreus*. Cultures of *A. terreus* NRRL 11,156 were grown by a modification of the previously described method.[4] Each 100 mL of liquid seed culture (consisting of 49 mg of $MgSO_4$, 4 g of α-D-glucose, 300 mg of Pharmamedia, 131 mg of K_2HPO_4, 100 mg of soybean meal, 1 g of $CaCO_3$, and 50 mg of NaCl in a total volume of 100 mL of deionized H_2O, pH 7.0−7.1) in a 500-mL bottom-baffled flask was inoculated with the spores produced on a single potato dextrose agar plate of *A. terreus.* The culture was incubated in an incubator shaker at 28 °C at 275 rpm for 72 h. After that period, the seed culture was stored at 4 °C for 10−12 h. Large-scale cultures (5 × 500 mL of media consisting of 245 mg of $MgSO_4$, 20 g of α-D-glucose, 1.5 g of Pharmamedia, 660 mg of K_2HPO_4, 500 mg of soybean meal, 5 g of $CaCO_3$, and 250 mg of NaCl in a total volume of 500 mL of deionized H_2O, pH 7.0−7.1 in 3-L bottom-baffled flasks) were inoculated with 15−18 mL of seed culture and they were incubated under the same conditions for a period of 45−48 h. The mycelium from one or two 500-mL cultures was collected by filtration, washed with cold deionized H_2O (50 mL) and 50 mL of cold Tris buffer [10 mM Tris, 5 mM $MgCl_2 \cdot 6H_2O$, 1 mM DTT (DL-dithiothreitol), 15% glycerol, and 0.1 mM PMSF (phenylmethanesulfonyl fluoride) in degassed H_2O, pH adjusted to 7.8 with 2 M HCl], and then suspended in 200 mL of Tris buffer in a Bead Beater containing 100 mL of 0.5-mm glass beads (prewashed with deionized H_2O and Tris buffer). The outer jacket was cooled with ice/water, and the cells were lysed with 10 × 30-s pulses with a 30-s interval between each pulse. The crude enzyme homogenate was decanted, centrifuged at 20 000 rpm for 30 min, and immediately either frozen at −80 °C or loaded on to a DE-52 ion exchange column. The crude enzyme is very unstable at 4 °C, losing almost all of its activity within 12 h. The enzymatic activity of the crude homogenate was always found to be between 3 and 5 nmol of FPP turned over per 1 mg of total crude protein per hour.

Aristolochene Synthase Assay. Aliquots of 100 μL of enzyme solution were typically incubated with 10 μL of [1-^{3}H]FPP (1 nmol) and 390 μL of HEPES buffer (10 mM HEPES, 5 mM $MgCl_2 \cdot 6H_2O$, 1 mM DTT , 15% glycerol, and 0.1 mM PMSF in degassed H_2O, pH adjusted to 7.5 with 2 N NaOH) at 30−31 °C for 10 min. (It was found to be important to adjust the protein concentration in each assay to less than 0.04 mg/mL in order to maintain a linear dependence of enzyme activity on protein concentration.) When the assay tubes were removed from the incubation bath, they were immediately immersed into an ice/salt water bath (0−2 °C). Each sample was extracted with hexane (3 × 1 mL), and the hexane extracts were filtered through a Pasteur pipet column containing 4−5 cm of silica gel and 1−1.5 cm of anhydrous $MgSO_4$ before analysis by liquid scintillation counting.

Partial Purification of Aristolochene Synthase. (a) DE-52 Anion Exchange. The crude cell-free extract isolated from four or five growth flasks (500 mL of liquid culture/flask, 270−350 mL of crude enzyme solution, and 130−160 mg of total protein) was loaded at a flow rate of 60−70 mL/h onto a 2.5 × 42-cm column of (diethylamino)ethyl-cellulose column (DE-52 Whatman) that had been previously equilibrated with 10 mM Tris buffer. The column was eluted overnight with a linear gradient of 0−350 mM KCl in 10 mM Tris buffer (total volume 540 mL) at a flow rate of 24 mL/h. All fractions (83 fractions of 6.5 mL or 250 drops) were checked for both protein content by the BioRad protein assay and aristolochene synthase activity. Aristolochene synthase eluted from the column at concentrations of 280−310 mM KCl. A 4−5-fold increase in the specific activity of the enzyme was observed in the combined active fractions (66−74), which were immediately frozen at −80 °C. The specific activity could further be increased (13−16 fold from the crude) by rechromatographing the desalted enzyme solution on a new DE-52 column, using a linear gradient of 150−250 mM KCl in 10 mM Tris buffer.

(b) Ethyl Agarose. A 90-mL KCl solution of aristolochene synthase (25 mg of protein in 500 mM KCl, 10 mM Tris buffer) isolated from 8 × 500-mL growth cultures of *A. terreus* and partly purified by DE-52 ion exchange chromatography was loaded onto an ethyl agarose column (2.5 × 42 cm) that had been preequilibrated with a 500 mM KCl, 10 mM Tris buffer. The column was eluted with a decreasing linear gradient from 500 to 250 mM KCl, 10 mM Tris buffer (total volume 600 mL) at a flow rate of 25 mL/h at 4 °C. All fractions collected (100 fractions of 3.5 mL or 150 drops each) were assayed for enzyme activity and protein content. Although maximum enzyme activity was observed in the fractions that eluted at 450−400 mM KCl (40−60), the amount of specific activity increase obtained was almost negligible.

(c) Sephacryl S-200. The most active fractions obtained from the ethyl-agarose column (fractions 41−47) were combined and concentrated to a volume of 1-mL ultrafiltration in a Centricon microconcentrator (Amicon) using a YM30 membrane. This solution was then applied to a pre-equilibrated Sephacryl S-200 column and eluted with Tris buffer at a flow rate of 25 mL/h, collecting 1.5 mL fractions. The most active fractions were assayed for total protein content. An average 300-fold increase in the specific activity of the enzyme was observed as compared to the original crude extract.

Steady-State Kinetic Analysis. **Competitive Inhibition of Aristolochene Synthase by (7*R*)-Dihydrofarnesyl Diphosphate.** Aristolochene synthase activity was assayed at five different FPP concentrations ranging from 0.46 to 2.3 μM in the presence of varying amounts of (7*R*)-dihydrofarnesyl diphosphate. The data were initially analyzed by Lineweaver−Burk double-reciprocal plots, which were consistent with competitive inhibition and that were used to obtain initial estimates of the relevant steady-state kinetic parameters. The values of V_{max}, K_m, and K_I were then obtained by direct fitting of data by nonlinear least-squares methods to the form of the Michaelis−Menten equation for competitive inhibition.

Preparative Incubation of [1-^{3}H]-(7*R*)-6,7-Dihydrofarnesyl Diphosphate with Aristolochene Synthase. Partially purified *A. terreus* aristolochene synthase (15−20-fold after the DE-52 step, 15 mg, specific activity 50−65 nmol of FPP h^{-1} mg^{-1} of protein) was incubated with 20 nmol of [1-^{3}H]-(7*R*)-6,7-dihydrofarnesyl diphosphate (**5**) in 70 mL of buffer (10 mM pH 7.5 HEPES, 5 mM $MgCl_2$, 1 mM DTT, 15% glycerol, and 9.1 mM PMSF) for 2 h at 30 °C. The incubation mixture was extracted with 4 × 50 mL of HPLC-grade pentane which had been pretreated with concentrated H_2SO_4 to remove olefinic impurities and predistilled from CaH_2 before use. The pentane extracts were dried ($MgSO_4$) and passed through a SiO_2 column before concentration and analysis by capillary GC/CI-MS.

Acknowledgment. This work was supported by a grant from the NIH, GM30301.

JA961981B