Communications to the Editor

Aristolochene Biosynthesis. Stereochemistry of the Deprotonation Steps in the Enzymatic Cyclization of Farnesyl Pyrophosphate

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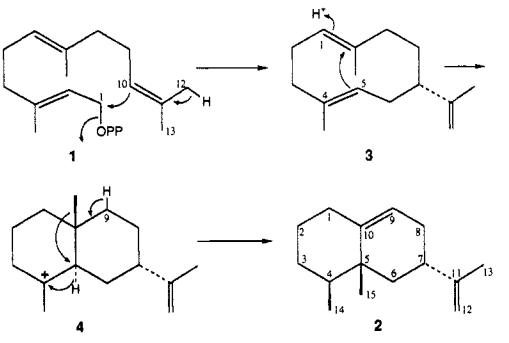
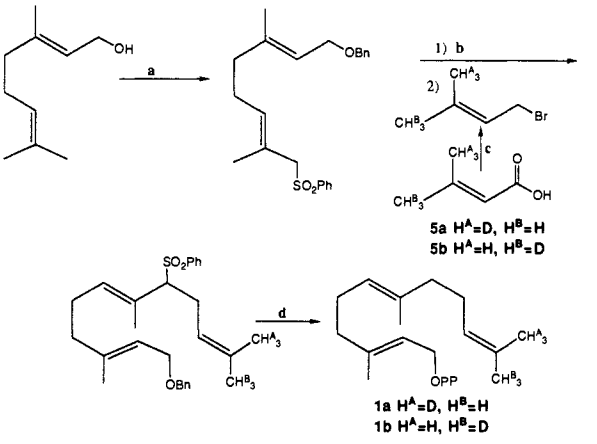
Aristolochene synthase, which has been isolated from cell-free extracts of the fungi *Aspergillus terreus*[1] and *Penicillium roquefortii*,[2] catalyzes the cyclization of *trans,trans*-farnesyl pyrophosphate (**1**) (FPP) to the eremophilane sesquiterpene (+)-aristolochene (**2**)[3] (Scheme I). According to the proposed mechanism, ionization of the allylic pyrophosphate and electrophilic attack of the resulting cation at C-10 of the distal double bond, followed by loss of a proton from one of the two adjacent methyl groups, will generate the monocyclic intermediate germacrene A (**3**). Protonation of **3** at C-1 is thought to initiate further cyclization by intramolecular electrophilic attack on the 4,5-double bond to form the bicyclic eudesmane cation **4**. The latter intermediate can in turn rearrange to **2** by sequential 1,2-hydride and methyl migrations followed by loss of a proton from C-9.[4] We have recently reported the results of labeling studies that support this proposal and that establish that the cyclization of FPP to aristolochene takes place with net inversion of configuration at C-1 of the allylic pyrophosphate.[1] The purification of aristolochene synthase from *P. roquefortii* has shown that the entire sequence of reactions is catalyzed by a single polypeptide of M_r 37 000,[2] with no evidence for release of any free intermediates. We now report experiments that establish the stereochemical course of the deprotonation steps involved in the formation of aristolochene and that shed light on the conformation of the substrate FPP as it is folded at the active site of the cyclase.

In order to determine which methyl group of FPP undergoes deprotonation, we carried out incubations of [12,12,12-$^2H_3$]FPP (**1a**) and [13,13,13-$^2H_3$]FPP (**1b**) with crude aristolochene synthase isolated from *A. terreus* as previously described.[1,5] The requisite substrates were prepared from (*Z*)-[4,4,4-$^2H_3$]-3-methyl- and (*E*)-[4,4,4-$^2H_3$]-3-methylcrotonic acids (**5a** and **5b**),[6] respectively, by the procedure developed earlier[7] and summarized in Scheme II. Analysis by 61.42-MHz ^2H NMR spectroscopy of the sample of aristolochene (**2a**) (700 nmol) obtained from **1a** revealed a single olefinic peak at δ 4.71, corresponding to deuterium at C-12, whereas the sample of **2b** (1.35 μmol) derived from the *E* isomer, **1b**, gave rise to a resonance at δ 1.69 (D-13).[8]

Scheme I

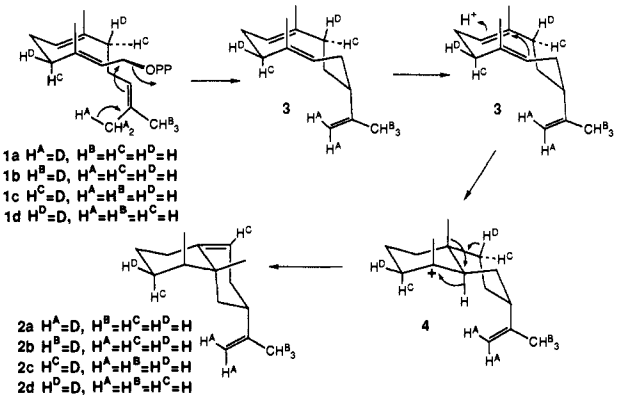


Scheme II[a]



[a] Reagents: (a) (i) NaH, benzyl bromide; (ii) SeO_2, EtOH; (iii) PBr_3; (iv) $PhSO_2Na$. (b) *n*-BuLi, HMPA, THF. (c) (i) $LiAlH_4$; (ii) PBr_3. (d) (i) Li, $EtNH_2$; (ii) CCl_4, PPh_3; (iii) $(n\text{-}Bu_4N)_3HP_2O_7$, CH_3CN.

Scheme III



Thus it is the original C-12 (cis) methyl group of FPP that undergoes deprotonation in the formation of the presumed intermediate germacrene A.[9]

(1) 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.
(2) Hohn, T. M.; Plattner, R. D. *Arch. Biochem. Biophys.* **1989**, *272*, 137.
(3) Cane, D. E.; Rawlings, B. J.; Yang, C.-C. *J. Antibiot.* **1987**, *40*, 1331. Cane, D. E.; Salaski, E. J.; Prabhakaran, P. C. *Tetrahedron Lett.*, in press.
(4) The methyl migration by which the eremophilane sesquiterpenes are generated from eudesmane intermediates was first proposed by Robert Robinson. Cf.: Penfold, A. R.; Simonsen, J. L. *J. Chem. Soc.* **1939**, *87*. Robinson, R. *The Structural Relations of Natural Products*; Clarendon: Oxford, 1955; p 12.
(5) Preparative-scale incubations of labeled samples of FPP (3–13 μM) and the isolation and purification of the derived aristolochene were carried out as previously described[1] by using the cell-free extract from 500 mL of *A. terreus* culture. Each FPP sample contained [12,13-^{14}C]FPP as internal standard to allow determination of substrate turnover.
(6) Aberhart, D. J. *J. Org. Chem.* **1980**, *45*, 5218 and references cited therein. The *E* isomers **5b** and the derived **1b** each contained 10% of the minor corresponding *Z* isomer.
(7) Cane, D. E.; Abell, C.; Tillman, A. M. *Bioorg. Chem.* **1984**, *12*, 312.
(8) The spectrum of **2b** also displayed a minor signal (ca. 10%) at δ 4.92 due to the presence of the *Z*-deuterated isomer in the farnesyl pyrophosphate substrate.
(9) Incorporations of [1,2-$^{13}C_2$]acetate have established a similar origin for the isopropenyl substituents of the plant phytoalexins capsidiol,[10] 2,3-germacrenediol,[11] lubimin,[11] and hydroxylubimin.[11] Each of these substances is derived from FPP, presumably by way of germacrene A. Capsidiol has also been shown to be biosynthesized by oxidation of the aristolochene diastereomer 5-epiaristolochene.[12]

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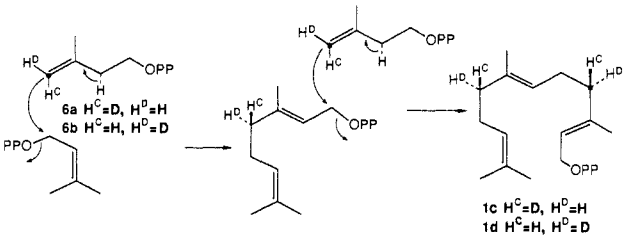
Scheme IV



The proton that is lost from C-9 of **4** originates at C-8 of farnesyl pyrophosphate. To establish the stereochemical course of this deprotonation step, we prepared both (4*R*,8*R*)- and (4*S*,8*S*)-[4,8-$^2H_2$]FPP (**1c** and **1d**) by enzyme-catalyzed condensation of (4*Z*)- and (4*E*)-[4-^{2}H]isopentenyl pyrophosphate (**6a** and **6b**),[13,14] respectively, with dimethylallyl pyrophosphate in the presence of avian prenyl transferase[14,15] (Scheme IV). After incubation of each of the stereospecifically deuterated FPPs with aristolochene synthase, the resulting samples of aristolochene were analyzed by ^{2}H NMR. As summarized in Scheme III, aristolochene **2c** (730 nmol) retained both deuterium atoms, as evidenced by the presence of signals at δ 1.44 and 5.35, corresponding to D-3a and D-9, while **2d** (500 nmol) showed a single peak at δ 1.36, corresponding to D-3b. These results demonstrate conclusively that it is H-8$_{si}$ that is lost in the formation of the 9,10-double bond of aristolochene.

On the basis of the known relative and absolute configuration of (+)-aristolochene,[3] it can be inferred that the sequential 1,2-hydride and methyl migrations take place on opposite faces of the bicyclic intermediate. Loss of H-8$_{si}$, which becomes H-9β (H-9$_{si}$) in **4**, establishes that the proton that is lost must be syn to the migrating methyl group. The sequence of anti migration, syn deprotonation is readily explained by invoking a chair–boat conformation for the cyclizing FPP and intermediate germacrene A. Further experiments to test the proposed cyclization mechanism and to characterize the cyclase itself are in progress.

Acknowledgment. This work was supported by a grant from the National Institutes of Health, GM30301. We thank Dr. D. John Aberhart of the Worcester Foundation for Experimental Biology, Shrewsbury, MA, for a generous gift of stereospecifically deuterated 3-methylcrotonic acids, **5a** and **5b**. We also thank Steven W. Weiner for the preparation of [1^{3}H]dimethylallyl pyrophosphate.

(10) Baker, F. C.; Brooks, C. J. W. *Phytochemistry* **1976**, *15*, 689. Baker, F. C.; Brooks, C. J. W.; Hutchinson, S. A. *J. Chem. Soc., Chem. Commun.* **1975**, 293.

(11) Birnbaum, G. I.; Huber, C. P.; Post, M. L.; Stothers, J. B.; Robinson, J. R.; Stoessl, A.; Ward, E. W. B. *J. Chem. Soc., Chem. Commun.* **1976**, 330.

(12) Threlfall, D. R.; Whitehead, I. M. *Phytochemistry* **1988**, *27*, 2567. Whitehead, I. M.; Threlfall, D. R.; Ewing, D. F. *Phytochemistry* **1989**, *28*, 775. Vogeli, U.; Chappel, J. *Plant Physiol.* **1988**, *88*, 1291. Whitehead, I. M.; Prabhakaran, P. C.; Ewing, D. F.; Cane, D. E.; Threllfall, D. R. *Phytochemistry* **1990**, *29*, 479.

(13) Ogura, K.; Koyama, T.; Kobayashi, M.; Ito, M. *Biochemistry* **1987**, *26*, 4745.

(14) Cane, D. E.; McIlwaine, D. B.; Oliver, J. S. *J. Am. Chem. Soc.* **1990**, *112*, 1285.

(15) The ionization–condensation–elimination reaction has been shown to take place on the *re* face of the isopentyl pyrophosphate double bond.[16] A typical incubation involved 20 μmol of (4*Z*)-[4-^{2}H]isopentenyl pyrophosphate, 0.46 μCi of [4-^{14}C]isopentenyl pyrophosphate, and 20 μmol of [1-^{3}H]dimethylallyl pyrophosphate in 6.8 mL of 20 mM Tris buffer, pH 7.7, containing 10 mM $MgCl_2$, 7 mM DTE, and 508 units of avian prenyl transferase which had been purified from chicken livers to the hydroxy apatite step [specific activity 14 nmol min^{-1} (mg of protein)$^{-1}$] (Reed, B. C.; Rilling, H. C. *Biochemistry* **1975**, *14*, 50). After 12 h at 30 °C, the resulting **1c** was purified by a sequence of Sephadex G-25 gel filtration, C_{18} reverse-phase ion pairing, and DEAE-Sephadex ion-exchange chromatography, to yield 1.3 μmol of (4*R*,8*R*)-[4,8-$^2H_2$]FPP.[14,17]

(16) Cornforth, J. W.; Cornforth, R. H.; Popjak, G.; Yengoyan, L. *J. Biol. Chem.* **1966**, *241*, 3970. Donninger, C.; Popjak, G. *Proc. R. Soc. London, B* **1966**, *163*, 465. Cornforth, J. W.; Cornforth, R. H.; Donninger, C.; Popjak, G. *Proc. R. Soc. London, B* **1966**, *163*, 492. Popjak, G.; Cornforth, J. W. *Biochem. J.* **1966**, *101*, 553.

(17) Cane, D. E.; Oliver, J. S.; Harrison, P. H. M.; Abell, C.; Hubbard, B. R.; Kane, C. T.; Lattman, R. *J. Am. Chem. Soc.*, in press.

Tetrahedral Copper(II) Complexes Supported by a Hindered Pyrazolylborate. Formation of the Thiolato Complex, Which Closely Mimics the Spectroscopic Characteristics of Blue Copper Proteins

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Synthetic examples of tetrahedral copper(II) complexes are rare, despite current interest in their unusual electronic structure from both physicochemical and biological points of view.[1] We now report that a hindered tripod nitrogen ligand,[2] HB(3,5-iPr$_2$pz)$_3^-$, stabilizes the tetrahedral coordination geometry with reasonable durability in the solid state or in noncoordinating solvents.

The reaction of KHB(3,5-iPr$_2$pz)$_3$ with 1 equiv of $CuCl_2 \cdot 2H_2O$ in dry acetone gives Cu(Cl)(HB(3,5-iPr$_2$pz)$_2$) (**1**) as a deep brown microcrystalline solid in 60–70% yield.[3] The same reaction with KHBpz$_3$ and KHB(3,5-Me$_2$pz)$_3$ yields only the disproportionation products, Cu(HBpz$_3$)$_2$[4] and Cu(HB(3,5-Me$_2$pz)$_3$)$_2$,[5] respectively. The crystal structure of **1** is shown in Figure 1.[6] The copper, chlorine, boron, and one pyrazole ring lie on a crystallographically imposed mirror plane. The mean bond lengths of Cu–Cl and Cu–N are close to one another, 2.13–1.98 Å, with the dihedral angles approximately 90°. Hence, the coordination geometry of **1** is described as a tetrahedron that is slightly elongated toward the chlorine atom. Although several examples[7] of tetrahedral copper(II) complexes have been reported, their dihedral angles are not comparable to 90°, but lie in the range 50–70°, owing to significant flattening or elongation.

The absorption spectrum of a solution of **1** in a noncoordinating solvent (CH_2Cl_2, toluene, or pentane) is essentially identical with the reflectance spectrum of the solid sample of **1**, implying that the tetrahedral structure is preserved in these solvents. However, addition of a slight amount of a coordinating solvent such as DMSO and DMF into a CH_2Cl_2 solution of **1** causes the immediate formation of the solvent adduct. The DMF adduct Cu(Cl)(DMF)(HB(3,5-iPr$_2$pz)$_3$) (**3**) was isolated, and the structure was established by X-ray crystallography.[8] As shown in Figure 2, the adduct is a pentacoordinated complex of square-pyramidal geometry with one pyrazole nitrogen as an apical ligand. Owing to the formation of the adduct, the d–d band of

(1) (a) Yokoi, H.; Addison, A. W. *Inorg. Chem.* **1977**, *6*, 1341. (b) Gewirth, A. A.; Cohen, S. L.; Schugar, H. J.; Solomon, E. I. *Ibid.* **1987**, *26*, 1133. (c) Gewirth, A. A.; Solomon, E. I. *J. Am. Chem. Soc.* **1988**, *110*, 3811.

(2) Kitajima, N.; Fujisawa, K.; Fujimoto, C.; Moro-oka, Y. *Chem. Lett.* **1989**, 421.

(3) Anal. Calcd for $C_{27}H_{46}N_6BCuCl$: C, 57.45; H, 8.21; N, 14.89; Cl, 6.28. Found for crystals of **1**: C, 57.03; H, 8.06; N, 14.82; Cl, 6.12. IR (KBr, cm^{-1}): 2545 (BH). UV-vis [λ_{max}, nm (ϵ, cm^{-1} M^{-1})]: (in CH_2Cl_2) 996 (150), 510 (310), 362 (1900), 262 (1600); (in DMF) 758 (100), 530 (80), 346 (1100), 269 (4400). The reaction with $CuBr_2$ gave Cu(Br)(HB(3,5-iPr$_2$pz)$_3$) (**2**). Calcd for $C_{27}H_{46}N_6BCuBr$: C, 53.25; H, 7.61; N, 13.80; Br, 13.12. Found for **2**: C, 53.36; H, 7.72; N, 13.74; Br, 13.09.

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(5) Kitajima, N.; Moro-oka, Y.; Uchida, A.; Sasada, Y.; Ohashi, Y. *Acta Crystallogr.* **1988**, *C44*, 1876.

(6) **1** crystallizes in space group $P2_1/m$ with a = 10.039 (1) Å, b = 16.519 (3) Å, c = 9.868 (2) Å, β = 102.60 (1)°, V = 1597.1 (4) Å^3, and D_{calcd} = 1.18 g cm^{-1} for Z = 2. Full-matrix least-squares refinement of the model based on 1093 reflections ($F_o > 3\sigma(F_o)$) converged to a final R = 8.03% and R_w = 7.93%. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms except for the ones on the methyl groups were calculated and fixed in the refinement (d(C–H) = 1.0 Å).

(7) Knapp, S.; Keenan, T. P.; Zhang, X.; Fikar, R.; Potenza, J. A.; Schugar, H. J. *J. Am. Chem. Soc.* **1987**, *109*, 1882 and references cited therein.

(8) Anal. Calcd for $C_{30}H_{53}N_7BOCuCl$: C, 56.51; H, 8.38; N, 15.38; Cl, 5.56. Found for crystals of **3**: C, 56.50; H, 8.70; N, 15.16; Cl, 5.82. IR (KBr, cm^{-1}): 2543 (BH) 1640 (CO). **3** crystallizes in space group $P2_1/a$ with a = 27.252 (4) Å, b = 14.103 (2) Å, c = 9.469 (1) Å, β = 96.99 (1)°, V = 3612.4 (9) Å^3, and D_{calcd} = 1.20 g cm^{-1} for Z = 4. For 5283 independent reflections with $F_o > 3\sigma(F_o)$, the final R = 7.98% and R_w = 6.63%.

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