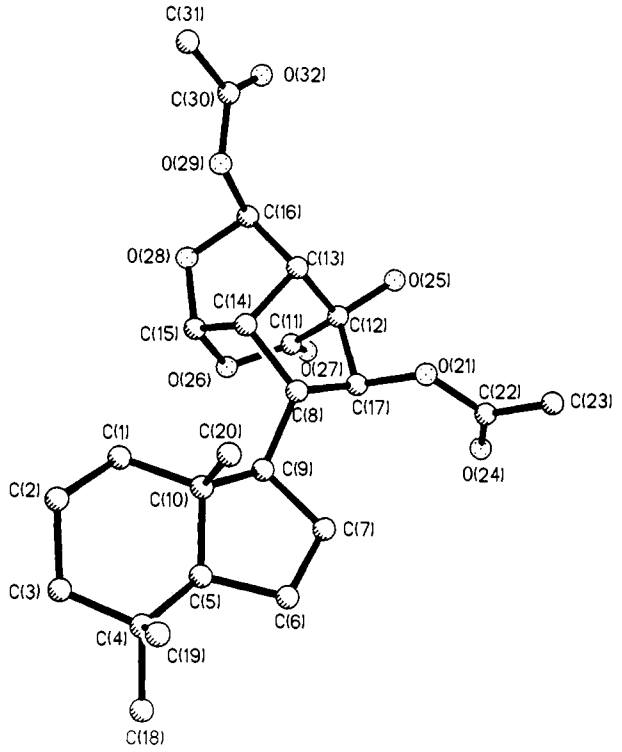

Figure 1. A computer-generated perspective drawing of the final X-ray model of chromodorolide A (**1**). Hydrogens are omitted for clarity, and no absolute configuration is implied.

cyclic in order to satisfy its unsaturation number. ^{1}H COSY,[6] ^{1}H double resonance, NOE, and one bond HETCOR[6] NMR experiments routinely elaborated the spin system associated with the network of methine protons in the heterocyclic portion of **1** (H8 to H16, Table I). It was not possible to accommodate all of the identified fragments on any known diterpene carbon skeleton so the structure of chromodorolide A (**1**) was solved by single-crystal X-ray diffraction analysis.[7] A computer-generated perspective drawing of the final X-ray model of chromodorolide A is given in Figure 1.

Chromodorolide A (**1**) is a rearranged spongian diterpene with a new carbon skeleton for which we suggest the name chromodorane. Biogenesis of the chromodorane skeleton may proceed via formation of a new carbon-carbon bond (C12-C17) subsequent to the degradation and rearrangement steps that generate the norrisane skeleton.[2a] Chromodorolide A (**1**) displays both cytotoxic and antimicrobial activities.[8]

Acknowledgment. Financial support at UBC was provided by NSERC and the National Cancer Institute of Canada and at Cornell by NIH CA24487 and the New York Sea Grant. We thank Sandra Millen for identifying the nudibranch.

Supplementary Material Available: Tables of atomic positions, thermal parameters, interatomic distances, interatomic angles, and torsional angles for chromodorolide A (**1**) (6 pages). Ordering information is given on any current masthead page.

(6) Bax, A. *Two-Dimensional Nuclear Magnetic Resonance in Liquids*; Reidel: Boston, 1982; Chapter 2.

(7) Crystals of chromodorolide A belonged to space group $P2_12_12_1$ with a = 8.653 (2) Å, b = 9.662 (3) Å, c = 30.743 (9) Å and one molecule of composition $C_{24}H_{34}O_8 \cdot CH_3OH$ forming the asymmetric unit. All unique reflections with $2\Theta \leq 112°$ were collected with 2Θ:Θ scans and CuKα radiation (1.54178 Å). A total of 1498 (77%) had $|F_o| \geq 5\sigma(F_o)$ and were used in subsequent calculations. The structure was phased with direct methods and refined by full-matrix least-squares techniques to a conventional discrepancy index of 0.043 for the observed data. Additional crystallographic information is available and described in the paragraph entitled Supplementary Material at the end of this manuscript.

(8) L1210 ED_{50} 20 μg/mL; P388 T/C 125% 4 mg/Kg; *Bacillus subtilis*: MIC 60 μg/disc; *Rhizoctonia solani* MIC 60 μg/disc.

Stereochemical Studies of Botryococcene Biosynthesis: Analogies between 1′-1 and 1′-3 Condensations in the Isoprenoid Pathway

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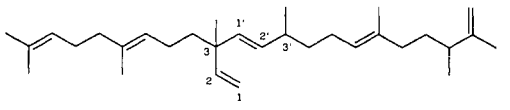
Received September 29, 1988

Botryococcenes are triterpenes with an unusual 1′-3 fusion between two farnesyl residues.[1] Biosynthetic feeding experiments suggest that a parental C_{30} botryococcene (**5**, R = $C_{11}H_{19}$) is constructed from two molecules of farnesyl diphosphate (**1**, R = $C_{11}H_{19}$) and that higher members of the family are generated by successive methylations with *S*-adenosyl methionine.[2-4] Although only a few 1′-3 fused isoprenoids are known in nature,[5] model studies[6,7] for biosynthesis of squalene (**6**, R = $C_{11}H_{19}$), the precursor of all steroids, suggest the common mechanism for biosynthesis of **5** and **6** from presqualene diphosphate (**2**, R = $C_{11}H_{19}$) shown in Scheme I.[8] Recent experiments established the quaternary centers at C3 in a C_{32},[1] and a C_{34}[9] botryococcene had the same absolute stereochemistry as the corresponding center in **2** (R = $C_{11}H_{19}$), consistent with the product-precursor relationship indicated in Scheme I. We now present stereochemical studies which further strengthen the mechanistic link between 1′-1 and 1′-3 condensations.

Upon incubation, [5-^{3}H, 1-^{14}C]farnesol ([5-^{3}H, 1-^{14}C]**7**) was efficiently incorporated (ca. 2%) into the C_{30}-C_{34} hydrocarbons of *Botryococcus braunii* var. *showa* without prior degradation.[1,2] In subsequent feeding experiments with [1-^{2}H]-, [2-^{2}H]-, (*S*)-[1-^{2}H]-, (*R*)-[1-^{2}H]-, or (*S*)-[1-^{2}H,1-^{13}C]**7**, the botryococcene fraction was isolated,[1] and a mixture of two noncyclic C_{31} isomers (**8** and **9**) was obtained by HPLC.[10] Labeling patterns were



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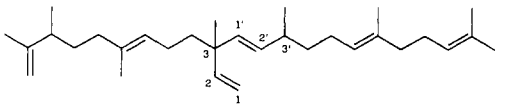

9

determined by ^{2}H NMR without separation of the isomers.[12] The

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(2) Wolf, F. R.; Nemethy, E. K.; Blanding, J. H.; Bassham, J. A. *Phytochem.* **1985**, *24*, 733-737.

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(8) Only atoms C-1 to C-3 and C-1′ to C-3′ of the original farnesyl residues are designated with numbers. This designation is maintained in all subsequent structures derived from **1**.

(9) White, J. D.; Somers, T. C.; Reddy, G. N. *J. Am. Chem. Soc.* **1986**, *108*, 5352-5353.

(10) The structure of **8** was first reported by P. Metzger et al.[11] Isomer **9** gave similar ^{1}H and ^{13}C spectra. The site of methylation was determined from ^{1}H, ^{13}C, and COSY spectra as previously described for the cyclic C_{32} botryococcenoid braunicene.[1] A complete characterization of **9** will be published elsewhere.

(11) Metzger, P.; Casadevall, E.; Pouet, M. J.; Pouet, Y. *Phytochem.* **1985**, *24*, 2995-3002.

(12) ^{1}H and ^{13}C chemical shifts for atoms in the center of **8** and **9** were identical. The compounds were not separated in order to provide sufficient quantities for the 2D NMR measurements.

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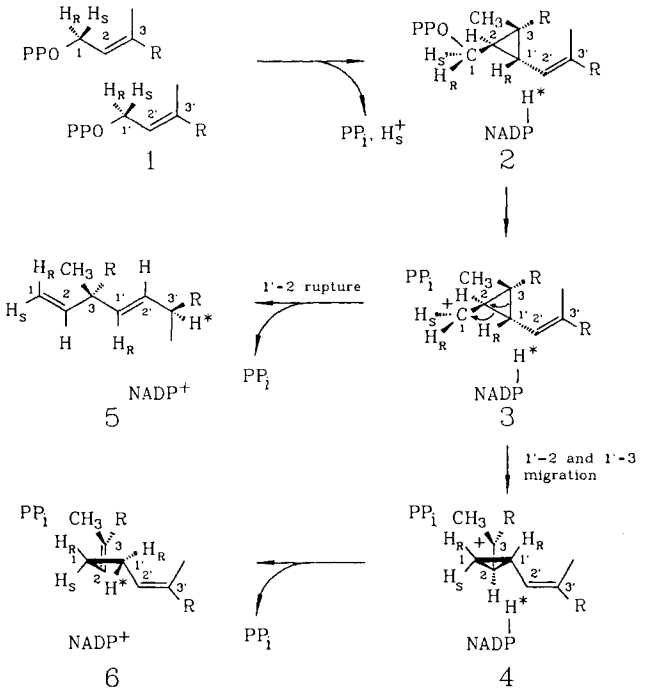
Scheme I. A Mechanism for Biosynthesis of Botryococcene (**5**, R = $C_{11}H_{19}$) and Squalene (**6**, R = $C_{11}H_{19}$) from Presqualene Diphosphate (**2**, R = $C_{11}H_{19}$)



^{2}H spectrum of material from racemic [1-^{2}H]**7** had intense peaks at 4.91 (^{2}H at C-1) and 5.39 ppm (^{2}H at C-1′) with a relative intensity of 2:1, while the ^{2}H spectra from incubation with [2-^{2}H]**7** had intense peaks at 5.24 (^{2}H at C-2′) and 5.78 ppm (^{2}H at C-2) in a 1:1 ratio. These results demonstrate that the farnesyl residue in the 1′-branch of the 1′–3 linkage lost a single deuterium from C-1′, and the residue in the 3-branch was incorporated without loss of deuterium. Furthermore, **8** and **9** obtained by incorporation of (*R*)-[1-^{2}H]**7** gave a ^{2}H NMR spectrum with intense peaks (1:1) at 4.91 (^{2}H at C-1) and 5.39 ppm (^{2}H at C-1′), while incorporation of (*S*)-[1-^{2}H]**7** gave a single intense resonance at 4.91 ppm (^{2}H at C-1). Thus, the pro-*S* proton was lost from C-1 of the farnesyl residue in the 1′-branch.

Resonances for the *E* and *Z* deuterons were not sufficiently well-resolved at 61.6 MHz to deduce the stereochemistry at C-1 in the 3-branch from the ^{2}H spectra of **8** and **9** derived from (*R*)- or (*S*)-[1-^{2}H]**7**. The problem was solved by a ^{1}H–^{13}C HETCOR experiment.[13,14] The region near 4.90/111.9 ppm in the 2D map (see Figure 1) of **8** and **9** obtained from an incubation with (*S*)-[1-^{2}H,1-^{13}C]**7** had two intense peaks at 4.888/111.870 and 4.934/111.870 ppm for the respective *E* and *Z* protons at C-1 attached to naturally abundant ^{13}C.[15] In addition, a triplet ($J_{^2H-^{13}C}$ = 24 Hz) was centered at 4.925/111.607 ppm. The magnitude of the coupling constant exactly matched the predicted value based on the observed one-bond coupling constant ($J_{^1H-^{13}C}$ = 155 Hz) and the gyromagnetic ratios for protons and deuterons ($\gamma_{^2H}/\gamma_{^1H}$ = 0.154) for a C^{1}H^{2}H methylene unit at C-1. The triplet is slightly upfield of the single peak assigned to the (*Z*)-^{1}H-^{13}C unit containing naturally abundant ^{13}C in both dimensions ($\Delta\delta^{^1H}$ = 0.009 ppm, $\Delta\delta^{^{13}C}$ = 0.263 ppm). Since deuterium is known to generate small *upfield* shifts at hydrogen[16] and carbon[17] in methylene units, the triplet was assigned to the (*Z*)-^{1}H-^{13}C unit at C-1 in **8** and **9** derived from (*S*)-[1-^{2}H,1-^{13}C]**7**. The absence of an upfield triplet to accompany the resonance at 4.888/111.870 ppm established that deuterium from (*S*)-labeled farnesol was stereoselectivity incorporated into the *E* position at C-1.

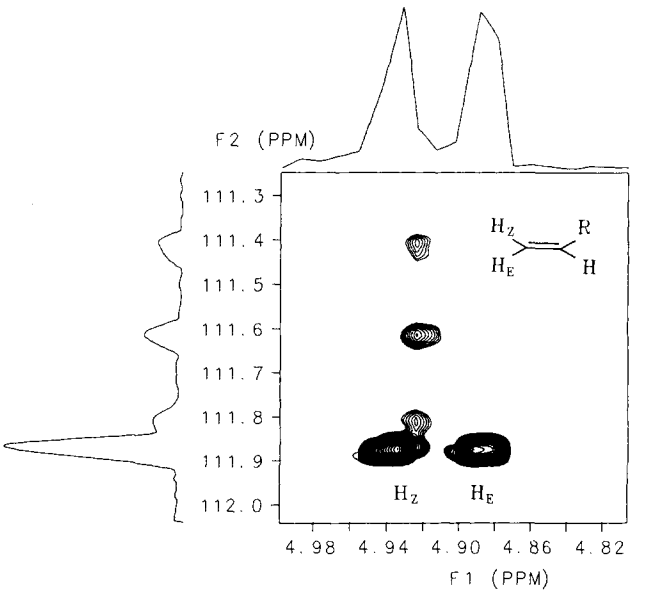
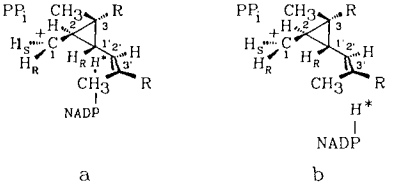


Figure 1. A ^{1}H–^{13}C HETCOR spectrum of the botryococcene mixture from (*S*)-[1-^{2}H,1-^{13}C]**7**. The spectrum was recorded on a Varian VXR-500 spectrometer in a 1:9 mixture of cyclohexane-d_{12} and C_6F_6 at 26 °C by using the protocol of Bax and Morris[14] with a sweep width of 1900 Hz in the carbon dimension and 335 Hz in the proton dimension. Only one-bond correlations were detected.

Chart I. Structures of Primary Cation **3**[a]



[a] With NADPH positioned near C-1 in an orientation (a) that precludes attack and near C-3′ in an orientation and (b) that produces an *R* stereocenter at C-3′ and an *E* C-1′–C-2′ double bond.

Our stereochemical studies support the contention that squalene (**6**) and botryococcene (**5**) are synthesized from presqualene diphosphate (**2**). First, the quaternary centers (C-3) in **2** and **5** have the same absolute stereochemistry.[1,9] Furthermore, the pro-*S* hydrogen is lost from C-1 of the 1′-farnesyl residue in both **5** and **6**.[18] During squalene biosynthesis, this loss occurs during formation of the cyclopropane ring in **2**.[18,19] A similar result is expected if **5** is also derived from (*R*,*R*,*R*)-**2**. Finally, rearrangement of **2** to squalene occurs with inversion of configuration at C-1 and C-1′.[18] For stereoelectronic reasons[20,21] inversion at C-1 requires that the cyclopropylcarbinyl substrate adopt a conformation in the active site of squalene synthetase in which the C-1-oxygen bond is syn to the C-2–C-3 cyclopropane bond as shown in Scheme I. If one assumes that (*R*,*R*,*R*)-**2** is the immediate presursor of **5**, stereoselective formation of (*E*)-[^{2}H]**5** from (*S*)-[1-^{2}H]**1** requires that the conformation of (*R*,*R*,*R*)-**2**, when bound to botryococcene synthetase, be similar to that in squalene synthetase.

We[20,21] proposed that the regioselective carbocationic rear-

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(15) Chemical shifts were assigned on the basis of vicinal coupling constants between the proton at C-2 and the C-1 methylene protons at 4.934 (J_{trans} = 17.4 Hz) and 4.888 ppm (J = 10.7 Hz) and an NOE from the proton at C-2 to the E proton at C-1.
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rangements of **2** to **6** are governed by electrostatic interactions between cyclopropylcarbinyl cations and PP_i, with PP_i acting as a template for the rearrangement. We also suggested premature capture of **3** is thwarted by binding NADPH near C-1′ in an orientation where a stereoelectronic barrier prevents attack at that position prior to rearrangement to **4**. If the cofactor is moved to a position where hydrogen transfer to C-3′ is possible, the stereoelectronic barrier to nucleophilic attack is removed, and reduction of **3** can occur with concomitant rupture of the C-1′–C-2 bond. Since the absolute stereochemistry at C-3′ in **5** is R, a relative minor displacement of the cofactor by approximately 2.5 Å along the bottom face of the substrate as shown in Chart I suffices to explain the regioselective formation of either product.

Acknowledgment. We thank Dr. Fred Wolf of Amoco Corporation for his generous assistance in establishing cultures of *B. braunii* in our laboratory. This project was supported by NIH Grant GM 21328.

Molecular Recognition in Cytochrome P-450: Alteration of Regioselective Alkane Hydroxylation via Protein Engineering

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Received November 17, 1988

The utility of synthetic catalysts in the oxidation of unactivated alkanes and alkenes has been the focus of intense interest.[1-5] In particular, the oxidative capability of the cytochrome P-450 monooxygenases has been modelled through the synthesis of shape-selective, regiospecific metalloporphyrins for use as potential synthetic tools.[6-10] Protein engineering now offers a viable alternative for the design of oxidative catalysts with the predictable regio- and stereospecificity inherent in biological systems. Here we report the manipulation of the regiospecificity of hydroxylation of several bicyclic substrates by the site-directed mutagenesis of cytochrome $P\text{-}450_{cam}$.[11]

Characterization of the gene encoding the soluble, bacterial cytochrome $P\text{-}450_{cam}$[12] and the X-ray crystal structures of several forms of the protein have been published.[13-15,31] The high resolution crystal structures of $P\text{-}450_{cam}$ indicate the existence of several specific active site residues which may differentially affect the energetically favorable binding orientations of various organic substrates. Collectively, these protein–substrate interactions serve to juxtapose the 5-position of the camphor skeleton directly above the heme iron,[17] for efficient hydrogen abstraction followed by stereospecific oxygen rebound[18,19] to afford the 5-*exo*-hydroxycamphor. Three active site residues, Val-295, Val-247, and Tyr-96, have been mutated and the present study undertaken to determine the resulting regiospecificity of hydroxylation for a series of structurally related bicyclic alkanes when these active site residues of cytochrome $P\text{-}450_{cam}$ are altered. Camphor (**1**), 1-CH_3-norcamphor (**2**), and norcamphor (**3**), were examined in order to assess the recognition of substrate methyl groups by the enzyme. An active site hydrogen bond between the substrate camphor and Tyr-96 is clearly indicated in the X-ray structure of $P\text{-}450_{cam}$ and was found to be important in the control of substrate affinity and

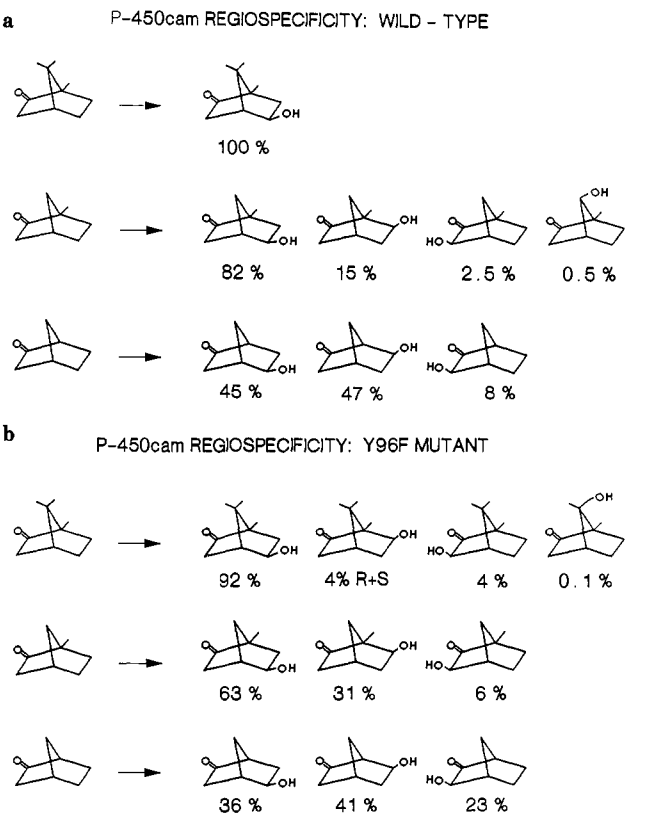

Figure 1. Comparison of regiospecificity of hydroxylation of bicyclic substrates. When the hydrogen bond which normally orients these substrates is removed by site-directed mutagenesis (Y96F), a new metabolite profile is obtained with increased specificity for the 3-position of the substrate. Enzymatic reactions were performed with 2–3 μM $P\text{-}450_{cam}$, 10 μM putidaredoxin, 2 μM putidaredoxin reductase, 200 mM KCl, 500 μM substrate, and 500 μM NADH in Tris buffer, pH 7.4. After reactions had reached completion, the mixtures were diluted to 1 mL with water and extracted twice with equal volumes of $CHCl_3$. The concentrated organic extract was analyzed by gas chromatographic mass spectrometry and compared to authentic standards, with a Hewlett Packard 3700 gas chromatograph equipped with a 30 m capillary DB-5 column in line with a Hewlett Packard 7070E mass spectrometer. Spectra were obtained in the electron impact mode, with an ionizing potential of 70 eV. Relative product yields were obtained by integration of peak areas of the GC traces. GC conditions varied according to the substrate used. Typically, a temperature ramp from 70 °C to 200 °C at 3 °C/min after an isothermal period of 3–5 min.

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