J|A|C|S
COMMUNICATIONS

Published on Web 06/28/2002

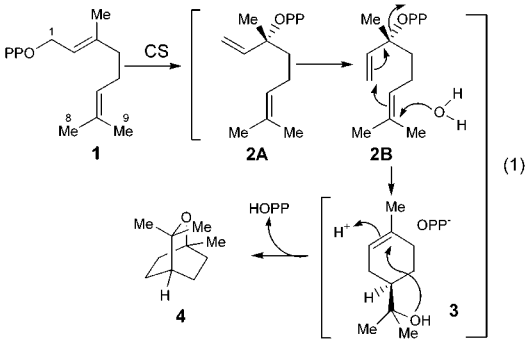
Syn Stereochemistry of Cyclic Ether Formation in 1,8-Cineole Biosynthesis Catalyzed by Recombinant Synthase from *Salvia officinalis*

Mitchell L. Wise,[†,§] Marek Urbansky,[‡] Gregory L. Helms,[||] Robert M. Coates,*[,‡] and Rodney Croteau*[,†]

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164, Department of Chemistry, University of Illinois, Urbana, Illinois 61801, and Department of Chemistry, Washington State University, Pullman, Washington 99164

Received April 17, 2002

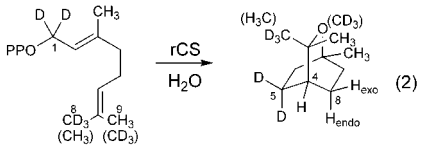
1,8-Cineole (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane, **4**),[1] commonly known as eucalyptol, is a monoterpene biosynthesized by a variety of plants, most notably *Eucalyptus* spp., *Artemisia* spp., and *Salvia* spp. This natural product appears to function in the chemical defense of the producing organism, displaying potent allelopathic effects on competing plant species,[2,3] and the bicyclic ether served as a lead in developing the commercial herbicide cinmethylin.[4] Because 1,8-cineole is both achiral and resistant to chemical degradation, determination of the stereochemical aspects of the enzymatic heterocyclization step has been problematic. We have recently cloned and heterologously overexpressed a cDNA encoding 1,8-cineole synthase (CS) from culinary sage (*Salvia officinalis*).[5] These advances allow conversion of the precursor, geranyl diphosphate (GDP, **1**) to sufficient product for NMR analysis. In this communication we report the results of deuterium-labeling experiments that establish syn stereochemistry in both C−O bond-forming steps of the complex cyclization process catalyzed by the recombinant cyclase.



(1)

The proposed electrophilic mechanism and intermediates in the bicyclization reaction catalyzed by native CS (eq 1) conform to the usual scheme for monoterpene bioynthesis in plants.[6,7] The high enantioselectivity of CS in favoring (*R*)-linaloyl PP (LDP, **2**) over (*S*)-linaloyl PP as an alternative substrate [$(V_{rel}/K_M)_R/(V_{rel}/K_M)_S$ = 54] and the stereoselective incorporation of tritium label from (*R*,*S*)-[1-$^3H_1$]-**1** into the pro-S ethano bridge at C5 [7] are consistent with an anti,endo S_N' cyclization[8,9] of an enzyme-bound (*R*)-LDP intermediate. Although the quantitative incorporation (96 ± 3%) of oxygen-18 from labeled water indicates nucleophilic capture of an α-terpinyl carbocation, the resulting (4-*R*)-α-terpineol (**3**) intermediate must remain tightly bound in the active site since CS is incapable of converting the exogenous monocyclic alcohol to cineole.[7,10] Thus, three discrete reactions take place in the catalytic site(s) of CS: (1) allylic rearrangement of GDP to (*R*)-LDP (**1** → **2A**), (2) S_N' cyclization to α-terpineol (**2B** → **3**), and (3) proton-induced cyclization of the C=C double bond and the tertiary hydroxyl group (**3** → **4**) (eq 1).

We have elucidated the stereochemistry of the water capture in step 2 by double labeling and NMR analysis of the deuterated cineole products, ***syn***- and ***anti*-4-d_5**, with the aid of literature assignments for this monoterpene (eq 2).[11,12] The introduction of deuterium at the C1 position of GDP (C5 in the product) was essential to alter the C_s symmetry of cineole, and thereby allow the determination of the syn or anti configuration of the unlabeled methyl by NOE spectroscopy. (2*E*,6*E*)-[1,1,8,8,8-$^2H_5$]- and (2*E*,6*Z*)-[1,1,9,9,9-$^2H_5$]-GDP ((***E***,***E***)- and (***E***,***Z***)-**1-*d*$_5$**) were synthesized by $LiAlD_4$ reduction of the corresponding methyl geranoate-d_3 isomers[13] followed by conversion to the diphosphates (See Supporting Information). Large-scale incubations of unlabeled and labeled substrates with a purified truncated rCS preparation[14] and subsequent chromatography on silica gel with capillary GC monitoring[13] afforded cineole-d_0 and cineole-d_5 samples that were analyzed by ^1H NMR (500 MHz, $CDCl_3$) and 1D NOE (double pulsed field gradient spin−echo sequence and IBURP-2 pulsing) spectroscopic techniques.[15,16]



(2)

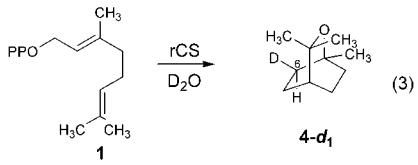
An NOE was observed at the unlabeled geminal methyl (δ_H 1.26 ppm) with cineole-d_5 derived from the E,E-labeled substrate upon irradiation of the $H5_{exo}/H8_{exo}$ protons at δ_H 2.02 ppm, establishing the syn relationship of the CD_2 and CD_3 groups shown in eq 2. This conclusion was confirmed by the complementary NOE measured for the $H8_{exo}$ protons upon irradiation of the C3 CH_3 peak, as well as the absence of the corresponding NOE signals in the otherwise identical NOE spectra of cineole-d_5 produced from the E,Z substrate. Thus, enzymatic cyclizations of (*E*,*E*)- and (*E*,*Z*)-GDP-d_5 gave rise to ***syn***- and ***anti*-4-d_5**, respectively.

The enzymatic conversion of unlabeled GDP was carried out in buffered D_2O to determine the stereochemistry of the proton incorporation at C6 of 1,8-cineole (C2 of GDP, see eq 3). The

* To whom correspondence should be addressed. E-mail: r-coates@uiuc.edu, croteau@mail.wsu.edu.
§ Current address: USDA Cereal Crops Research Unit, 501 Walnut St., Madison, WI 53705.
† Institute of Biological Chemistry, Washington State University.
‡ University of Illinois.
|| Department of Chemistry, Washington State University.

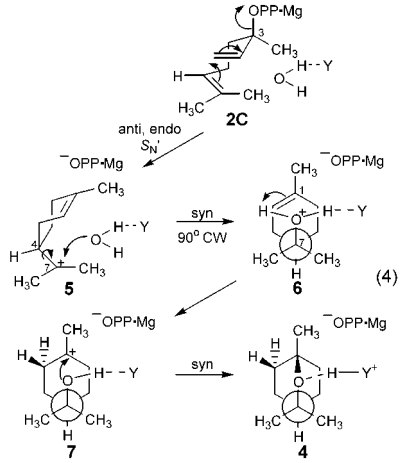
10.1021/ja0265714 CCC: $22.00

cineole-d_1 was isolated as before by elution with $CHCl_3$ for deuterium NMR spectroscopy. The ^2H NMR spectrum (76.73 MHz, $CHCl_3$) showed a single signal at $\delta_D = 1.66$ ppm, thus demonstrating incorporation of a solvent-derived deuterium in the 6 exo position.



(3)

The data presented above, along with prior experimental findings with this[8] and related monoterpene synthases,[7] allow precise stereochemical definition of the multistep cyclization leading to 1,8-cineole (**4**) and a plausible scenario of bond-making and bond-breaking events (eq 4).[10,17] Thus, following ionization and isomerization of GDP to (3*R*)-LDP (**2C**) and anti,endo S_N' cyclization to the (*R*)-α-terpinyl cation (**5**), syn water capture at C7 occurs from the inside face (*re*) of the tertiary carbocation. A 90° clockwise, least-motion rotation about the C4−C7 bond would give rise to the (*R*)-α-terpinyl hydronium ion **6** that protonates itself at the proximal cyclohexene double bond to form a fourth carbocation intermediate **7**. Intramolecular alkylation of the tertiary hydroxyl group in **7**, perhaps ion-paired with the PPi leaving group on the opposite face of the carbocation sp^2 orbital at C1, accompanied by proton transfer to an active site acceptor Y, would complete a second syn addition to form the product.



(4)

These results provide intriguing insight into the catalytic mechanism of this novel enzyme. The syn stereochemistry of ether bond formation contrasts with the common tendency of electrophilic C=C double bond additions to take place predominantly through antarafacial mechanisms,[18] and the outcome conflicts with the usual predictions of the biogenetic isoprene rule.[19] However, cis additions of protic acids to alkenes are well-precedented,[18,20] and examples of syn stereochemistry in terpene synthase reactions are now known.[21] In the present case, the position of the Mg·OPP anion might help to stabilize the intermediates and transition states.

Substrate binding is presumed to occur with the OPP moiety positioned close to the active-site surface and with the geranyl carbon chain positioned deep within a hydrophobic pocket to allow the electrophilic cyclization to occur while protecting early carbocation intermediates from premature capture.[6,7] The water molecule must be precisely held in the active site in close proximity to cationic centers (at C7 as well as delocalized C1−C2−C3) so as to permit covalent bonding only to C7 (i.e., geraniol and linalool are not products of the CS reaction[5,8]).

How the enzyme accomplishes the exact positioning of a water molecule and the highly reactive carbocationic intermediates, and what the identities and specific functions of the surrounding active-site residues are, at present, unclear. However, the availability of this recombinant enzyme may permit crystallographic analysis in the absence and presence of mechanism-based inhibitors to address these critical structural and mechanistic questions.

Acknowledgment. We gratefully acknowledge financial support from the National Institutes of Health to R.M.C. (GM 13956) and to R.B.C. (GM 31354), and the assistance of Reuben J. Peters in the preparation of the cDNA construct.

Supporting Information Available: Details of enzyme incubations, syntheses and spectroscopic data for labeled substrates, additional literature references and figures showing NMR spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) The stereochemical designations and isotope descriptors used in this work are based on the IUPAC conventions. The IUPAC numbering system was used for cineole, whereas the standard positional numbers for acyclic and menthane terpenes were adopted for geraniol and α-terpineol.
(2) Romagni, J. G.; Allen, S. N.; Dayan, F. E. *J. Chem. Ecol.* **2000**, *26*, 303−313.
(3) Romagni, J. G.; Duke, S. O.; Dayan, F. E. *Plant Physiol.* **2000**, *123*, 725−732.
(4) Duke, S. O.; Abbas, H. K. *ACS Symp. Ser.* **1995**, *582*, 348−362.
(5) Wise, M. L.; Savage, T. J.; Katahira, E.; Croteau, R. *J. Biol. Chem.* **1998**, *273*, 14891−14899.
(6) Croteau, R. *Chem. Rev.* **1987**, *87*, 929−954.
(7) Wise, M. L.; Croteau, R. In *Comprehensive Natural Products Chemistry: Isoprenoids Including Steroids and Carotenoids*; Cane, D. E., Ed.; Elsevier Science: Oxford, 1999; Vol. 2, pp 97−153.
(8) Croteau, R.; Alonso, W. R.; Koepp, A. E.; Johnson, M. A. *Arch. Biochem. Biophys.* **1994**, *309*, 184−192.
(9) Godtfredsen, S.; Obrecht, J. P.; Arigoni, D. *Chimia* **1977**, *31*, 62−63.
(10) A possibility that the actual active-site nucleophile is the side chain carboxyl group of an aspartate or glutamate and that the resulting enzyme-bound α-terpinyl ester intermediate undergoes subsequent hydrolysis appears to be excluded by the O-18 labeling result.[7] For precedent see: Yang, G.; Liang P. H.; Dunaway-Mariano D. *Biochemistry* **1994**, *33*, 8527−8531 and references cited therein. We thank W. van der Donk for suggesting this possibility.
(11) Abraham, R. J.; Warne, M. A.; Griffiths, L. *J. Chem. Soc., Perkin. Trans. 2* **1998**, 1751−1757.
(12) The assignments for $H6_{exo}/H7_{exo}$ and $H6_{endo}/H7_{endo}$ were independently confirmed by deuterium labeling and NMR analysis (500 MHz, $CDCl_3$). See Supporting Information.
(13) Wise, M. L.; Pyun, H.-J.; Helms, G.; Assink, B.; Coates, R. M.; Croteau, R. B. *Tetrahedron* **2001**, *57*, 5327−5334.
(14) An N-terminally truncated plasmid construct was prepared to remove the putative transit peptide region and to improve the solubility of the expressed protein. See: Williams, D. C.; McGarvey, D. J.; Katahira, E. J.; Croteau, R. *Biochemistry* **1998**, *37*, 12213−12220.
(15) Stott, K.; Stonehouse, J.; Keeler, J.; Hwang, T. L.; Shaka, A. J. *J. Am. Chem. Soc.* **1995**, *117*, 4199−4200.
(16) Geen, H.; Freeman, R. *J. Magn. Reson.* **1991**, *93*, 93−141.
(17) The possibility that an external general acid of an active-site residue participates directly as proton donor in an antarafacial addition to the 1,2 double bond of the α-terpinyl intermediate is excluded by the syn stereochemical outcome observed. However, a concerted, suprafacial [4 + 2] cycloaddition of a α-terpinyl-$OH_2^+\cdots Y-H$ (Y = O, N, or S) hydrogen-bonded complex is a plausible alternative mechanism.
(18) For reviews and leading references see: (a) Sergeev, G. B.; Smirnov, V. V.; Rostovshchikova, T. N. *Russ. Chem. Rev.* **1983**, *52*, 259−274. (b) Fahey, R. C. *Top. Stereochem.* **1968**, *3*, 237−342. (c) Carey, F. A.; Sundberg, R. J, *Advanced Organic Chemistry, Part B*, 4th ed.; Kluwer-Plenum: New York, 2001; Chapter 6. (d) March, J. *Advanced Organic Chemistry-Reactions, Mechanisms, and Structures*; 4th ed.; Wiley: New York, 1992; Chapter 15.
(19) (a) Eschenmoser, A.; Ruzicka, L.; Jeger, O.; Arigoni, D. *Helv. Chim. Acta* **1955**, *38*, 1890−1904. (b) Ruzicka, L. *Proc. Chem. Soc.*, **1959**, 341−361.
(20) Specific examples are glycals, phenylacetylene, 1,2-dimethylcyclohexene, phenylpropenes, *β-tert*-butylstyrenes, 1-phenyl-4-*tert*-butylcyclohexene, and indene. See ref 18 and Supporting Information.
(21) (a) Pinene cyclase: Coates, R. M.; Denissen, J. F.; Croteau, R. B.; Wheeler, C. J. *J. Am. Chem. Soc.* **1987**, *109*, 4399−4401. (b) Bornyl pp synthase: ref 13. (c) Oxidosqualene cyclase: Corey, E. J.; Virgil, S. C.; Sarshar, S. *J. Am. Chem. Soc.* **1991**, *113*, 8171−8172. (d) Abietadiene synthase: Ravn, M. M.; Coates, R. M.; Flory, J. E.; Peters, R. J.; Croteau, R. *Org. Lett.* **2000**, *2*, 573−576.

JA0265714