Stereochemistry at C-1 of Geranyl Pyrophosphate and Neryl Pyrophosphate in the Cyclization to (+)- and (−)-Bornyl Pyrophosphate*

Rodney Croteau‡, N. Mark Felton, and Carl J. Wheeler

From the Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340

(1R)-1-3H-labeled and (1S)-1-3H-labeled geranyl pyrophosphate and neryl pyrophosphate were prepared from the corresponding 1-3H-labeled aldehydes by a combination of enzymatic and synthetic procedures. Following admixture with the corresponding 2-14C-labeled internal standard, each substrate was converted to (+)-bornyl pyrophosphate and (−)-bornyl pyrophosphate by cell-free enzyme preparations from sage (Salvia officinalis) and tansy (Tanacetum vulgare), respectively. Each pyrophosphate ester was hydrolyzed, and the resulting borneol was oxidized to camphor. The stereochemistry of labeling at C-3 of the derived ketone was determined by base-catalyzed exchange, taking advantage of the known selective exchange of the exo-α-protons. By comparison of such exchange rates to those of product generated from (1RS)-2-14C,1-3H2-labeled substrate, it was demonstrated that geranyl pyrophosphate was cyclized to bornyl pyrophosphate with net retention of configuration at C-1 of the acyclic precursor, whereas neryl pyrophosphate was cyclized to product with inversion of configuration at C-1. The observed stereochemistry is consistent with a reaction mechanism whereby geranyl pyrophosphate is first stereospecifically isomerized to linalyl pyrophosphate which, following rotation about C-2–C-3 to the cisoid conformer, cyclizes from the anti-endo configuration. Neryl pyrophosphate cyclizes either directly or via the linalyl intermediate without the attendant rotation.

It has long been recognized that geranyl pyrophosphate, the ubiquitous C10 intermediate of isoprenoid metabolism, cannot be converted directly to cyclohexanoid monoterpenes because of the topological constraints imposed by the trans-C-2-C-3 double bond of the acyclic prenyl chain. Many proposals have thus focused on the possible intermediary of neryl pyrophosphate and linalyl pyrophosphate (Fig. 1) in enzymatic cyclization processes and on possible means by which geranyl pyrophosphate might be isomerized to such a sterically more suitable intermediate (1–3). Over the last several years, major advances have been made in understanding the formation of the main classes of monocyclic and bicyclic monoterpenes from their acyclic precursors (3, 4), and from such investigations, as well as studies on related sesquiterpene cyclizations (5), the general features of this reaction type have begun to emerge (6–8).

Investigations of the enzymology of camphor biosynthesis have provided the most detailed information to date regarding a monoterpene cyclization process. The biosynthesis of (+)-camphor in sage (Salvia officinalis) has been shown (9–12) to involve the conversion of geranyl pyrophosphate to (+)-bornyl pyrophosphate which is subsequently hydrolyzed by a distinct pyrophosphatase to (+)-borneol, followed by the NAD-dependent dehydrogenation of the alcohol to the ketone (Fig. 1). (−)-Camphor in tansy (Tanacetum vulgare) and rosemary (Rosmarinus officinalis) is derived by a similar sequence of reactions (9, 15, 14), and sage leaf extracts have also been shown to contain a minor, and readily separable (−)-bornyl pyrophosphate cyclase activity. Studies on the biosynthesis of (+)-bornyl pyrophosphate are of particular significance in establishing for the first time that geranyl pyrophosphate is the preferred substrate for cyclization and that neryl pyrophosphate was not a mandatory intermediate (11). Thus, it was shown with partially purified preparations, free of contaminating phosphohydrolases which compete for the pyrophosphate substrates, that V/Km for geranyl pyrophosphate was 20 times that for the cis-isomer, neryl pyrophosphate. Furthermore, geranyl pyrophosphate and neryl pyrophosphate, as well as the tertiary isomer linalyl pyrophosphate, were converted to the product without detectable interconversion of the three acyclic precursors and without the preliminary formation of any other free intermediate. Additionally, it was demonstrated that [G-14C,1-3H2]geranyl pyrophosphate was converted to (+)-[G-14C,3-3H2]borneyl pyrophosphate (as well as to (−)-[G-14C,3-3H2]borneyl pyrophosphate) without loss of hydrogen from C-1 (14, 15), eliminating earlier proposals for trans-to-cis isomerization involving redox processes (1, 16).

*This investigation was supported in part by Grant GM 31354 from the National Institutes of Health. This is Scientific Paper 5956.

‡To whom correspondence should be addressed.

Fig. 1. Pathway for the conversion of acyclic precursors to camphor. The (+)-(1R,4R)-stereoisomers are illustrated.
Stereochemistry of Cyclization to Bornyl Pyrophosphate

The summary of the evidence clearly indicates that the enzyme is capable not only of the relevant cyclization but also of the required isomerization of geranyl pyrophosphate to a bound intermediate competent to cyclize. The overall conversion is also unusual in that the pyrophosphate moiety of the substrate is retained in the bicyclic product. The bornyl pyrophosphate cyclases have therefore provided a unique opportunity to examine the role of the pyrophosphate moiety in the coupled isomerization-cyclization process (17).

Schemes for the formation of (+)-bornyl pyrophosphate and (-)-bornyl pyrophosphate from geranyl pyrophosphate and neryl pyrophosphate, which are completely consistent with the results of numerous model studies of terpenoid cyclizations (18--23), are illustrated in Fig. 2. The first step is metal ion-assisted ionization with syn-isomerization to the bound tertiary allylic isomer (or its ion-paired equivalent) now free to rotate about the C-2-C-3 bond. Following this rotation, ionization of the tertiary intermediate, with electrophilic attack by C-1 of the cisoid, anti-endo conformer at C-6 of the adjacent double bond, generates the cyclohexanoid ring. Subsequent electrophilic addition to the newly created cyclohexene double bond and capture of the resulting cation by the pyrophosphate anion provide bornyl pyrophosphate with the requisite stereochemistry. One consequence of this scheme is that cyclization should proceed with net retention of configuration at C-1 of geranyl pyrophosphate and with inversion of configuration at C-1 of neryl pyrophosphate (the stereochemical consequences are identical whether neryl pyrophosphate cyclizes directly or via preliminary isomerization to linalyl pyrophosphate).

In this communication, we describe the conversion of both (1R)- and (1S)-1-3H-labeled geranyl pyrophosphate and neryl pyrophosphate to both (+)- and (-)-bornyl pyrophosphate and the stereochemical location of 3H in each product by selective exchange of the exo-α-hydrogen of the derived camphor. The utilization of all four stereospecifically labeled acyclic precursors with the enantiomeric cyclizing enzymes has provided an extremely powerful probe for testing and confirming these stereochemical predictions and, thus, the validity of the coupled isomerization-cyclization scheme.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

The proposed model for the cyclization of geranyl pyrophosphate and neryl pyrophosphate to bornyl pyrophosphate predicts that the configuration at C-1 of the trans-precursor will be retained in the transformation, while that of the cis-precursor will be inverted (Fig. 2). To examine the stereochemical fate of C-1 of these acyclic precursors, the (1R)-1-3H- and (1S)-l-3H-labeled enantiomers of both geranyl pyrophosphate and neryl pyrophosphate were prepared by combination of enzymatic and chemical methods. Each chiral substrate, as well as (1RS)-[1-3H2]geranyl pyrophosphate and (1RS)-[1-3H2]neryl pyrophosphate, was admixed with its 2-14C-labeled counterpart to a 3H/14C ratio of 10, and the positional...
tion of the tritium was verified to be a minimum of 95% of that specified (Table 1). Each substrate was then enzymatically converted to (+)-bornyl pyrophosphate and (−)-bornyl pyrophosphate by partially purified cyclase preparations from S. officinalis (sage) and T. vulgare (tansy), respectively. The purification procedure (Sephadex G-150 chromatography) served to remove some of the competing phosphohydrolases in the crude extracts and to eliminate, from the sage leaf preparation, a low level of (−)-bornyl pyrophosphate cyclase activity (M_{s} \sim 65,000).

Bornyl pyrophosphate generated enzymatically from each substrate was hydrolyzed in situ by the addition of acid phosphatase plus apyrase, and following extraction and pooling of samples from like assays, the borneol was isolated by OsO_{4} treatment to remove unsaturated compounds. The borneol derived from each substrate with the respective cyclase was oxidized (CrO_{3}) to camphor, and this product served to remove some of the competing phosphohydrolases in the purification procedure (Sephadex G-150 chromatography) that specified (Table 1).

These results confirm earlier observations that hydrogen from C-1 of the acyclic precursor is not lost in the conversion to product then diluted to a specific activity of 250 pCi (3H)/mol phosphatase plus apyrase, and following extraction and pooling of samples from like assays, the borneol was isolated by TLC\(^{a}\) after OsO_{4} treatment to remove unsaturated compounds. The borneol derived from each substrate with the respective cyclase was oxidized (CrO_{3}) to camphor, and this product then diluted to a specific activity of 250 pCi (3H)/mol for further studies. The \(^{3}H/^{14}C\) ratios of each borneol sample and its respective derived camphor were identical, within experimental error, to the corresponding prenyl pyrophosphate starting material (Table 1). These results confirm earlier observations that hydrogen from C-1 of the acyclic precursor is not lost in the conversion to (+)- or (−)-camphor (14, 15). Such verification is crucial since the transformation to camphor allows advantage to be taken of the selectivity of exo-hydrogen exchange of this ketone (43) in locating the tritium.

A sufficient number of assays were run with four or five separate cyclase preparations to ensure the production of a minimum of 0.25 μCi (3H) of (+)- and (−)-camphor from each substrate with each preparation. Individual exchange runs with each 0.25 μCi (3H) (1 mmol) sample of camphor were carried out in 0.5 N NaOH/dioxane at 70 °C, and the tritium loss at corresponding time points for like samples was averaged. The averaged exchange curves for (+)-camphor (via (+)-bornyl pyrophosphate cyclase) derived from (1R)-\(^{14}C,1-^{3}H\), (1S)-\(^{14}C,1-^{3}H\), and (1RS)-\(^{14}C,1-^{3}H\) geranyl pyrophosphate are shown in Fig. 3A. The loss of tritium from camphor derived from (1S)-\(^{14}C,1-^{3}H\) geranyl pyrophosphate was far more rapid than that from the product derived from (1R)-\(^{14}C,1-^{3}H\) geranyl pyrophosphate or (1RS)-\(^{14}C,1-^{3}H\) geranyl pyrophosphate, indicating that the 1-proS hydrogen of the acyclic precursor gave rise predominantly to the α-exo-hydrogen of the derived (+)-camphor as predicted from the stereochemical cyclization scheme (Fig. 2).

Comparison of exchange rates from the linear portion of the curves (0-10 min) for the product derived from (1S)- and (1R)-labeled precursors, respectively, gave a ratio (18.5:1) comparable to the known ratio of exchange rates (21:3:1) for the exo- versus endo-α-hydrogens of camphor (43).

The averaged exchange curves for (−)-camphor (via (−)-bornyl pyrophosphate cyclase) derived from (1R)-\(^{14}C,1-^{3}H\), (1S)-\(^{14}C,1-^{3}H\), and (1RS)-\(^{14}C,1-^{3}H\) geranyl pyrophosphate are illustrated in Fig. 3A. In this instance, the rate of tritium loss from camphor derived from (1R)-\(^{14}C,1-^{3}H\) geranyl pyrophosphate was approximately 20 times that of the product derived from (1S)-\(^{14}C,1-^{3}H\) geranyl pyrophosphate and roughly 10 times that from the racemic \(^{14}C,1-^{3}H\)-labeled precursor, when compared at 10 min. Thus, the 1-proR hydrogen of geranyl pyrophosphate gave rise to the α-exo-hydrogen of the derived (−)-camphor, opposite to the location determined for (+)-camphor and in accord with the proposed cyclization scheme. From the summary of these data with the enantiomeric products, it is clear (cf. Fig. 2) that configuration at C-1 of geranyl pyrophosphate is retained in the conversion to bornyl pyrophosphate.

Exchange curves for the (+)-camphor and (−)-camphor derived from the three \(^1H,^{14}C\)-labeled neryl pyrophosphates with the respective (+)- and (−)-bornyl pyrophosphate cyclases are provided in Fig. 3. C and D. Based on arguments similar to those above, the results indicate that the 1-proR hydrogen of neryl pyrophosphate gives rise to the exo-α-hydrogen of (+)-camphor, whereas the 1-proS hydrogen of this precursor gives rise to the exo-α-hydrogen of the enantiomeric (−)-camphor. It is clear, with reference to Fig. 2, that the configuration at C-1 of neryl pyrophosphate is inverted in the cyclization to bornyl pyrophosphate.

The summary of the results with all the \(^1H\)-labeled precursors and both enantiomeric cyclases is entirely consistent with, and thus supports, the cyclization scheme (Fig. 2), which is itself based on numerous model reactions and related biosynthetic studies. Specifically, geranyl derivatives have been shown to cyclize in solution via preliminary conversion to the tertiary allylic, linalyl system (20), this cationic isomerization being favored by the presence of a large counterion such as phosphate (21). The allylic isomerization of geranyl pyrophosphate to linalyl pyrophosphate is considered to occur with overall syn stereochemistry based on an examination of the origin of linalool (46), and the transposition of trans,trans-farnesyl pyrophosphate to nerolidyl pyrophosphate (the C\(_{15}\) analogs of geranyl pyrophosphate and linalyl pyrophosphate,

\[\text{ **Stereochemistry of Cyclization to Bornyl Pyrophosphate**} \]

### Table I

\[^{3}H/^{14}C\] ratios of substrates and derived products

The initially prepared alcohols were analyzed as diphenyletheranes. Aldehydes were prepared by enzymatic oxidation (determined as semicarbazones) and the carboxylic acids by oxidation of the alcohols with MnO\(_{2}\)-Tollens reagent. Phosphatase esters (RCH\(_{2}\)OPP) were examined after enzymatic hydrolysis to the alcohols (as diphenyletheranes). Borneols were obtained by enzymatic hydrolysis of the corresponding pyrophosphates and TLC purification and were determined as the phenylurethanes. Camphors were obtained by CrO\(_{3}\) oxidation of the borneols and TLC purification. Camphors (exchanged) were the products obtained after exhaustive base-catalyzed exchange and TLC purification. Data for borneol (Born) and camphor (Camp) are averages of separate determinations of the product generated from four or five different enzyme preparations. ND indicates not determined.

| Substrate        | RCH\(_{2}\)OH | RCHO | RCO\(_{2}\)H | RCH\(_{2}\)OPP | (+)-Born | (+)-Camp | (+)-Camp (exchanged) | (--)-Born | (--)-Camp | (--)-Camp (exchanged) |
|------------------|---------------|------|-------------|---------------|----------|----------|----------------------|-----------|-----------|----------------------|
| (1RS)-\[^1H\]GPP | 10.1          | ND   | 0.15        | 10.2          | 10.0     | 10.5     | 0.35                 | 10.2      | 10.0      | 0.30                 |
| (1R)-\[^1H\]GPP | 10.4          | 0.42 | 1.19        | 10.2          | 10.2     | 10.4     | 0.36                 | 10.2      | 10.1      | 0.33                 |
| (1S)-\[^1H\]GPP | 10.5          | 10.4 | 0.19        | 10.1          | 9.98     | 10.1     | 0.35                 | 10.3      | 10.0      | 0.31                 |
| (1RS)-\[^1H\]NPP | 9.94          | ND   | 0.14        | 10.0          | 10.1     | 9.87     | 0.34                 | 10.0      | 9.91      | 0.35                 |
| (1R)-\[^1H\]NPP | 10.0          | 0.51 | 0.18        | 9.95          | 9.97     | 9.89     | 0.31                 | 10.2      | 10.3      | 0.30                 |
| (1S)-\[^1H\]NPP | 10.4          | 10.3 | 0.19        | 10.5          | 10.3     | 10.3     | 0.31                 | 10.1      | 9.77      | 0.33                 |

\[^{a}\] The abbreviations used are: TLC, thin-layer chromatography; GLC, gas-liquid chromatography.

Error limits are ±5%. GPP indicates geranyl pyrophosphate. NPP indicates neryl pyrophosphate.
Stereochemistry of Cyclization to Bornyl Pyrophosphate

Results from examination of two distinct and enantiomer-generating enzyme systems provide solid experimental support for the stereochemistry of this crucial monoterpene conversion of trans,trans-farnesyl pyrophosphate to the sesquiterpene trichodiene, a reaction considered to occur by an analogous coupled isomerization-cyclization sequence (8), proceeds with net retention of configuration at C-1 of the trans-acyclic precursor (47), and retention of configuration at C-1 of trans,trans-farnesyl pyrophosphate has been inferred in the cyclization to γ-bisabolene (48). Similar ring-generating allylic displacements in the diterpene series also occur with overall anti-stereochemistry (49–55). Thus, a consistent pattern for terpenoid cyclizations is beginning to emerge.

The absolute configuration of the end products of the bornyl pyrophosphate cyclizations permits prediction of the configuration of the proposed linalyl pyrophosphate intermediates (i.e. (−)-(3R) and (+)-(3S)-linalyl pyrophosphates should give rise, respectively, to (−)- and to (−)-bornyl pyrophosphate, respectively (Fig. 2)); such insight is not possible in the case of symmetrical cyclization products such as γ-terpinene (56) and 1,8-cineole (57). Since the bornyl pyrophosphate cyclases have been shown to utilize (3RS)-(−)-linalyl pyrophosphate as an acyclic precursor, these predictions can be tested directly with the appropriately labeled (3R)- and (3S)-enantiomers. These experiments are now underway.

Acknowledgments—We thank Sali Combelic for raising the plants and Mary Bull for typing the manuscript.

REFERENCES

1. Charlwood, B. V., and Banthorpe, D. V. (1978) in Progress in Phytochemistry (Reinhold, L., Harborne, J. B., and Swain, T., eds) Vol. 5, pp. 65–125, Pergamon Press, New York
2. Cori, O. (1983) Phytochemistry 22, 331–341
3. Croteau, R. (1981) in Biosynthesis of Isoprenoid Compounds (Porter, J. W., and Spurgeon, S. L., eds) Vol. 1, pp. 225–282, John Wiley and Sons, New York
4. Croteau, R. (1984) in Isopentenoids in Plants: Biochemistry and Function (Nes, W. D., Fuller, G., and Tsai, L. S., eds) pp. 31–64, Marcel Dekker, New York
5. Cane, D. E. (1981) in Biosynthesis of Isoprenoid Compounds (Porter, J. W., and Spurgeon, S. L., eds) Vol. 1, pp. 283–374, John Wiley and Sons, New York
6. Cane, D. E. (1978) Tetrahedron 36, 1109–1159
7. Cane, D. E., Iyengar, R., and Shiao, M.-S. (1981) J. Am. Chem. Soc. 103, 914–931
8. Croteau, R., and Cane, D. E. (1985) Methods Enzymol. 110, 352–405
9. Croteau, R., and Karp, F. (1977) Arch. Biochem. Biophys. 184, 77–86
10. Croteau, R., Hooper, C. L., and Felton, M. (1978) Arch. Biochem. Biophys. 188, 182–193
11. Croteau, R., and Karp, F. (1979) Arch. Biochem. Biophys. 198, 512–522
12. Croteau, R., and Karp, F. (1979) Arch. Biochem. Biophys. 198, 523–532
13. Croteau, R., and Felton, M. (1980) Phytochemistry 19, 1343–1347
14. Croteau, R., and Shaskus, J. (1985) Arch. Biochem. Biophys. 236, 545–553
15. Croteau, R., and Felton, M. (1981) Arch. Biochem. Biophys. 207, 460–464
16. Banthorpe, D. V., Modawi, B. M., Poots, I., and Rowan, M. G. (1978) Phytochemistry 17, 1115–1118
17. Cane, D. E., Saito, A., Croteau, R., Shaskus, J., and Felton, M. (1982) J. Am. Chem. Soc. 104, 5891–5893
18. Coates, R. M. (1976) in Progress in the Chemistry of Organic Natural Products (Herz, W., Grisebach, H., and Kirby, G. W., eds) Vol. 33, pp. 73–230, Springer-Verlag, Wien
19. Gottfredsen, S., Obrecht, J. F., and Arigoni, D. (1977) Chimia 31, 62–63
20. Baxter, R. L., Laurie, W. A., and MacHale, D. (1978) Tetrahedron 34, 2195–2199
21. McCormick, J. P., and Barton, D. L. (1978) Tetrahedron 34, 325–330
22. Poulter, C. D., and King, C.-H. R. (1982) J. Am. Chem. Soc. 104, 1420–1424
23. Poulter, C. D., and King, C.-H. R. (1982) J. Am. Chem. Soc. 104, 1422–1424
24. Wadsworth, W. S., Jr., and Emmons, W. D. (1961) J. Am. Chem. Soc. 83, 1733–1738
25. Kosolapoff, G. M. (1960) Organophosphorus Compounds, 1st Ed., Chapter 7, pp. 121–179, John Wiley and Sons, New York
Stereochemistry of Cyclization to Bornyl Pyrophosphate

26. Croteau, R., and Karp, F. (1976) Arch. Biochem. Biophys. 176, 734–746
27. Attenburrow, J., Cameron, A. F. B., Chapman, J. H., Evans, R. M., Hems, B. A., Jansen, A. B. A., and Walker, T. (1952) J. Chem. Soc. 1094–1111
28. Corey, E. J., Gilman, N. W., and Ganem, B. E. (1968) J. Am. Chem. Soc. 90, 5616–5617
29. Caspi, E., and Eck, C. R. (1977) J. Org. Chem. 42, 767–768
30. Mitsunobu, O., and Eguchi, M. (1971) Bull. Chem. Soc. Jpn. 44, 359–390
31. Grynkiewicz, G., and Burzyńska, H. (1976) Tetrahedron 32, 2109–2111
32. Mitsunobu, O. (1981) Synthesis 1, 1–28
33. Barua, R. K., and Barua, A. B. (1964) Biochem. J. 92, 21–22C
34. Donninger, C., and Ryback, G. (1964) Biochem. J. 91, 11p
35. Caspi, E., Shapiro, S., and Piper, J. U. (1981) Tetrahedron 37, 3535–3543
36. Shapiro, S., Piper, J. U., and Caspi, E. (1981) Anal. Biochem. 117, 113–120
37. Cramer, F., and Böhm, W. (1959) Angew. Chem. 71, 775
38. Cornforth, R. H., and Popják, G. (1969) Methods Enzymol. 15, 359–390
39. Dugan, R. E., Rasson, E., and Porter, J. W. (1968) Anal. Biochem. 22, 249–259
40. Eliel, E. L., and Nasipuri, D. (1965) J. Org. Chem. 30, 3809–3814
41. Brown, H. C., and Garg, C. P. (1961) J. Am. Chem. Soc. 83, 2952–2953
42. Thomas, A. F., Schneider, R. A., and Meinwald, J. (1967) J. Am. Chem. Soc. 89, 68–70
43. Tidwell, T. T. (1970) J. Am. Chem. Soc. 92, 1448–1449
44. Abad, G. A., Jindal, S. P., and Tidwell, T. T. (1973) J. Am. Chem. Soc. 95, 6326–6331
45. Guenther, E. (1975) The Essential Oils, Vol. 2 (reprinted), pp. 339–344, 326–336, 429–435, 791–822, Kreiger, Huntington, NY
46. Gottfriedsen, S. E. (1978) Ph.D. dissertation, ETH Zurich, No. 6243 (as cited in Refs. 6 and 7)
47. Cane, D. E. (1984) in Enzyme Chemistry. Impact and Applications (Suckling, C. J., ed) pp. 196–231, Chapman and Hall, London
48. Anastasis, P., Frer, L., Gilmore, C., Mackie, H., Overton, K., Picken, D., and Swanson, S. (1984) Can. J. Chem. 62, 2079–2088
49. Cane, D. E., and Murthy, P. P. N. (1977) J. Am. Chem. Soc. 99, 8327–8329
50. Cane, D. E., Hasler, H., Materna, J., Cagnoli-Bellavita, N., Ceccherelli, P., Madruzza, G. F., and Polonsky, J. (1981) J. Chem. Soc. Chem. Commun. 280–282
51. Coates, R. M., and Cavender, P. L. (1980) J. Am. Chem. Soc. 102, 6358–6359
52. Drengler, K. A., and Coates, R. M. (1980) J. Chem. Soc. Chem. Commun. 856–857
53. Evans, R., Hanson, J. R., and Mulheirn, L. J. (1973) J. Chem. Soc. Perkin Trans. 1, 753–756
54. Hasler, H. (1979) Ph.D. dissertation, ETH Zurich, No. 6359 (as cited in Refs. 6 and 55)
55. Overton, K. H. (1979) Chem. Soc. Rev. 8, 447–473
56. Poulose, A. J., and Croteau, R. (1981) Arch. Biochem. Biophys. 191, 400–411
57. Croteau, R., and Karp, F. (1977) Arch. Biochem. Biophys. 179, 257–265
Supplemental Material:

**Stereochemistry at C1 of Camphor and Camphen Propanediol**

**Preparation of (1S)-1-3H-labeled Alcohol (37)**

1. (1R)-1-3H-labeled alcohol by chemical inversion of (1S). From the preparation of (1S)-1-3H-labeled alcohol by chemical inversion of (1S)-camphor (37). The (1S)-1-3H-labeled alcohol was prepared in an identical manner from (1S)-[2-3H]geraniol (Table 1). Although the (1S)-1-3H-labeled alcohol was dried, derivatized, and subjected to TLC silica gel 0 (with solvent system A). Reactions were performed under nitrogen.

**Abstract of Stereochemistry, Assay, and NMR Spectroscopy**

A mixture of 2.5 mg of authentic carrier followed by preparation of the diphosphonate and crystallization of this derivative to confirm specific activity (8). Alcohols were converted to the acyclic enantiomer to, and chemical inversion of, the (1R)-1-3H-labeled alcohol was not expected to occur in labeling of label (29-32). The specific activity of each alcohol was determined by dilution using appropriate standards. The specific activity and isotope ratio (Table 1). The specific activity of each alcohol was determined by dilution using appropriate standards. In this way the (1S)-1-3H-labeled alcohol was shown to be stereospecifically pure (Table 1), as expected on the basis of the analysis of the (1S)-1-3H-labeled alcohol.

**Preparation of Diphosphonate and Reduction of Sterols**

The reaction was stopped at a suitable time by extraction with ether (2 x 1 ml). Alcohols were derivatized to the corresponding semicarbazones and crystallization of this derivative to confirm specific activity (8). Alcohols were converted to the acyclic enantiomer to, and chemical inversion of, the (1R)-1-3H-labeled alcohol was not expected to occur in labeling of label (29-32). The specific activity of each alcohol was determined by dilution using appropriate standards. In this way the (1S)-1-3H-labeled alcohol was shown to be stereospecifically pure (Table 1), as expected on the basis of the analysis of the (1S)-1-3H-labeled alcohol.

**Preparation of Diphosphonate and Reduction of Sterols**

The reaction was stopped at a suitable time by extraction with ether (2 x 1 ml). Alcohols were derivatized to the corresponding semicarbazones and crystallization of this derivative to confirm specific activity (8). Alcohols were converted to the acyclic enantiomer to, and chemical inversion of, the (1R)-1-3H-labeled alcohol was not expected to occur in labeling of label (29-32). The specific activity of each alcohol was determined by dilution using appropriate standards. In this way the (1S)-1-3H-labeled alcohol was shown to be stereospecifically pure (Table 1), as expected on the basis of the analysis of the (1S)-1-3H-labeled alcohol.

**Preparation of Diphosphonate and Reduction of Sterols**

The reaction was stopped at a suitable time by extraction with ether (2 x 1 ml). Alcohols were derivatized to the corresponding semicarbazones and crystallization of this derivative to confirm specific activity (8). Alcohols were converted to the acyclic enantiomer to, and chemical inversion of, the (1R)-1-3H-labeled alcohol was not expected to occur in labeling of label (29-32). The specific activity of each alcohol was determined by dilution using appropriate standards. In this way the (1S)-1-3H-labeled alcohol was shown to be stereospecifically pure (Table 1), as expected on the basis of the analysis of the (1S)-1-3H-labeled alcohol.

**Preparation of Diphosphonate and Reduction of Sterols**

The reaction was stopped at a suitable time by extraction with ether (2 x 1 ml). Alcohols were derivatized to the corresponding semicarbazones and crystallization of this derivative to confirm specific activity (8). Alcohols were converted to the acyclic enantiomer to, and chemical inversion of, the (1R)-1-3H-labeled alcohol was not expected to occur in labeling of label (29-32). The specific activity of each alcohol was determined by dilution using appropriate standards. In this way the (1S)-1-3H-labeled alcohol was shown to be stereospecifically pure (Table 1), as expected on the basis of the analysis of the (1S)-1-3H-labeled alcohol.

**Preparation of Diphosphonate and Reduction of Sterols**

The reaction was stopped at a suitable time by extraction with ether (2 x 1 ml). Alcohols were derivatized to the corresponding semicarbazones and crystallization of this derivative to confirm specific activity (8). Alcohols were converted to the acyclic enantiomer to, and chemical inversion of, the (1R)-1-3H-labeled alcohol was not expected to occur in labeling of label (29-32). The specific activity of each alcohol was determined by dilution using appropriate standards. In this way the (1S)-1-3H-labeled alcohol was shown to be stereospecifically pure (Table 1), as expected on the basis of the analysis of the (1S)-1-3H-labeled alcohol.
At 10 mi intervals, preference for $\beta$-exchange of 0.15 $\pm$ 0.34 biosynthetically generated $^{14}$C,3Hlcamphor dioxane:0.5 diluted with authentic u-hydrogens of camphor appropriate was either examined directly for $^1$H and $^{14}$C content. The sample was diluted with authentic (+)- or (-)-camphor to a specific activity of 250 $\mu$Ci-3H/mol.

Exchange Procedures—The technique for base-catalyzed exchange of the $\alpha$-hydrogens of camphor was derived from earlier studies (42-44) in which the preference for $\alpha$-exchange was demonstrated ($E_{\alpha/\beta}$ endo = 21.3). A sample of biosynthetically generated $[^1]$C,$^3$Hlcamphor (C$^{25}$ S$^{57}$R, 1 mmol in 1 ml dioxane) was added by syringe to a Teflon-sealed vessel containing 5 ml of dioxane (0.5 N aqueous NaOH, v/v) which was stirred vigorously at 70°C. At 10 min intervals, 0.5 ml aliquots were withdrawn by syringe, added to 1 ml of 0.15 M HNO$_3$ at 0°C, and the mixture extracted with pentane (2 x 2 ml). The pentane extract was concentrated to a small volume, and the camphor reisolated by TLC (silica gel G with solvent system A). The camphor thus obtained was either examined directly for $^1$H and $^{14}$C content, or, following dilution with appropriate carrier, was converted to the oxime which was recrystallized to constant specific activity (subsequent experiments showed the preparation of the oximes to be an unnecessary precaution, and the camphor was thus counted directly). Following the removal of aliquots, the remaining solution (3 ml) was diluted with an additional 3 ml of dioxane:0.5 % NaOH and heated on a steam bath for 40 min. The camphor re-isolated following this exhaustive exchange contained less than 3.15 residual $^3$H in all cases. Tritium lost was determined from the $^{1}$H:$^{14}$C ratio with reference to the starting ketone, and exchange curves for each precursor and (+)- and (-)-product were constructed by averaging comparable data points from 4 or 5 independent runs with separate cyclase preparations.

Preparation of Crystalline Derivatives—The semicarbazones of geranial (mp 183-184°C from methanol) and nerol (mp 172-174°C from methanol), the diphenylurethane of geranial (mp 62-63°C from hexanes), and nerol (mp 52-53°C from hexanes), the phenylurethane of bornyl (mp 138-139°C from hexanes) and the oxime of camphor (mp 127.5-129°C from 78% aqueous ethanol) were prepared by classical techniques described in detail elsewhere (45).

Chromatographic and Determination of Radioactivity—Thin-layer chromatography was done on 0.0 mm layers of silica gel H, silica gel G, or silica gel C containing AgNO$_3$, activated at 110°C for 3 h. Developing solvents are indicated elsewhere in the text. After development, the chromatograms were either scanned with a Berthold TLC Scanner or sprayed with a 0.25 ethanoic solution of 2,7-dichlorofluorescein and viewed under UV light to locate the appropriate component which was eluted from the gel with ether. Radio-GLC was performed on a Packard 550P gas chromatograph (TC detector) attached to a Packard 995 Gas Proportional Counter, and both TC and radio output channels were monitored with a Scintrex Packard 2000 gas chromatograph-UV.

Radioactivity in organic liquid samples and in TLC fractions was assayed with a Packard 2025 LS scintillation spectrometer in a counting solution consisting of 15 ml of 38% ethanol in toluene containing 0.1% (w/v) Omnifluor (New England Nuclear, efficiency for $^3$H = 30%; for $^{14}$C = 72%). All samples were counted to a standard error of less than 1.0.

Stereochemistry of Cyclization to Bornyl Pyrophosphate