Isoprenoid Biosynthesis. Stereochemistry of the Cyclization of Allylic Pyrophosphates

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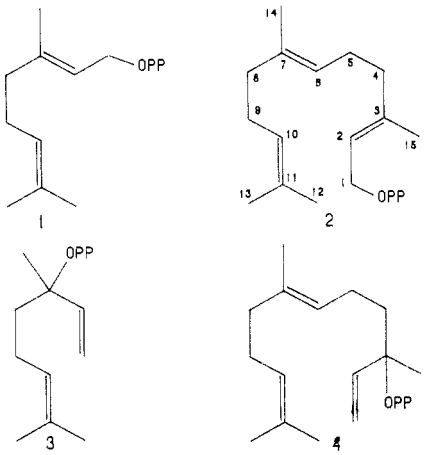
For more than 30 years, the biogenetic isoprene rule has served as a central and unifying hypothesis for the rationalization of terpenoid biogenesis and the design and interpretation of biosynthetic experiments.[1] As initially formulated by Ruzicka and elaborated subsequently by several groups of authors,[2] intramolecular electrophilic cyclization of the simple acyclic substrates geranyl pyrophosphate (1) and farnesyl pyrophosphate (2) followed by appropriate carbocation transformations, including rearrangements, deprotonations, and further cyclizations, can account for the formation of the enormous variety of cyclic monoterpenes and sesquiterpenes, respectively.

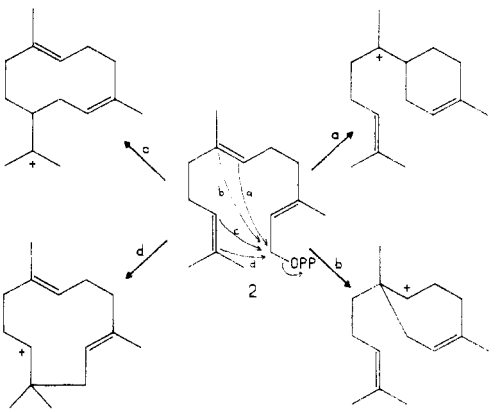
The biogenetic isoprene rule was itself a *mechanistic* reformulation of a purely structural hypothesis, the isoprene rule, first proposed by Ruzicka himself in 1923 and based on the perceived structural similarity between farnesol and the known classes of cyclic sesquiterpenes.[3] The evolution from structural to mechanistic formulation has many parallels in the development of biogenetic theory. Thus, although the structural resemblance between cholesterol and the acyclic hydrocarbon squalene was recognized by Robinson in the 1930s,[4] it was the specific cyclization scheme proposed by Bloch and Woodward[5] some 20 years later which not only accounted for the results of detailed biosynthetic investigations incompatible with the earlier hypothesis but also provided a convincing *mechanistic* framework for the understanding of squalene cyclization. Within a remarkably short time, moreover, this new hypothesis was reformulated in explicit *stereochemical* terms which provided a dramatically simple rationalization of the biogenesis of tetracyclic and pentacyclic triterpenes based on a small group of folding patterns for the activated squalene precursor.[6]

Surprisingly, there has as yet been no comparable development of a general stereochemical model for monoterpene and sesquiterpene biogenesis.[7] The need for such a model becomes evident if one considers the limitations of the conventional mechanistic representation of the cyclization of farnesyl pyrophosphate. As illustrated in Scheme II, the initial step in the formation of cyclic sesquiterpenes is envisaged as an intramolecular attack by the carbon bearing the pyrophosphate ester on either the central (paths a and b) or distal (path c and d) double bond of the farnesyl substrate. An analogous scheme is applicable to the cyclization of the

Scheme I
Monoterpene and Sesquiterpene Precursors: Geranyl Pyrophosphate (1), FPP (2), Linalyl Pyrophosphate (3), and Nerolidyl Pyrophosphate (4)



Scheme II
Cyclization of Farnesyl Pyrophosphate



David E. Cane received his B. A. from Harvard College in 1966 and his Ph. D. in 1971 from Harvard University under the direction of Professor E. J. Corey. After 2 years as an N.I.H. Postdoctoral Fellow in the laboratories of Professor Duilio Arigoni at the Eidgenossische Technische Hochschule in Zurich, Switzerland, he joined the faculty of Brown University where he is now Professor and Chairman of the Department of Chemistry. He has held fellowships from the Sloan Foundation and the Japan Society for the Promotion of Science as well as a Research Career Development Award from the N. I.H. Dr. Cane is the recipient of the 1985 Ernest Guenther Award in the Chemistry of Essential Oils and Related Products. His research interests have centered on the biosynthesis of natural products, including terpenoids, macrolides, and polyether ionophores, as well as application of stable isotope NMR methods and cell-free enzyme systems to the study of these problems.

(1) Ruzicka, L.; Eschenmoser, A.; Heusser, H. *Experientia* **1953**, *9*, 357. Ruzicka, L. *Pure Appl. Chem.* **1963**, *6*, 493. Ruzicka, L. *Proc. Chem. Soc.* **1959**, 341.

(2) (a) Hendrickson, J. *Tetrahedron* **1959**, *7*, 82. (b) Richards, J. H.; Hendrickson, J. B. "The Biosynthesis of Steroids, Terpenes, and Acetogenins"; W. A. Benjamin: New York, 1964; pp 225–239. (c) Parker, W.; Roberts, J. S.; Ramage, R. *Q. Rev., Chem. Soc.* **1967**, *21*, 331. (d) Devon, T. K.; Scott, A. I. "Handbook of Naturally Occurring Compounds"; Academic Press: New York, 1972; Vol. 2, pp 56–57.

(3) Ruzicka, L. *Helv. Chim. Acta* **1922**, *5*, 923.

(4) Robinson, R. *Chem. Ind. (London)* **1934**, *53*, 1062.

(5) (a) Woodward, R. B.; Bloch, K. *J. Am. Chem. Soc.* **1953**, *75*, 2023. (b) Dauben, W. G.; Abraham, S.; Hotta, S.; Charkoff, I. L.; Bradlow, H. L.; Soloway, A. H. *J. Am. Chem. Soc.* **1953**, *75*, 3038.

(6) Eschenmoser, A.; Ruzicka, L.; Jeger, O.; Arigoni, D. *Helv. Chim. Acta* **1955**, *38*, 1890.

(7) For recent comprehensive reviews of monoterpene and sesquiterpene biosynthesis see: (a) Croteau, R. In "Biosynthesis of Isoprenoid Compounds", Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1981; pp 225–282. (b) Cane, D. E. *Ibid.*, pp 283–374. (c) Croteau, R.; Cane, D. E. In "Methods in Enzymology (Steroids and Isoprenoids)"; Law, J. H., Rilling, H. C., Eds.; Academic Press: New York, 1985; pp 383–405. (d) Cane, D. E. *Tetrahedron* **1980**, *36*, 1109.

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lower isoprenologue, geranyl pyrophosphate, for which only paths a and b are relevant.

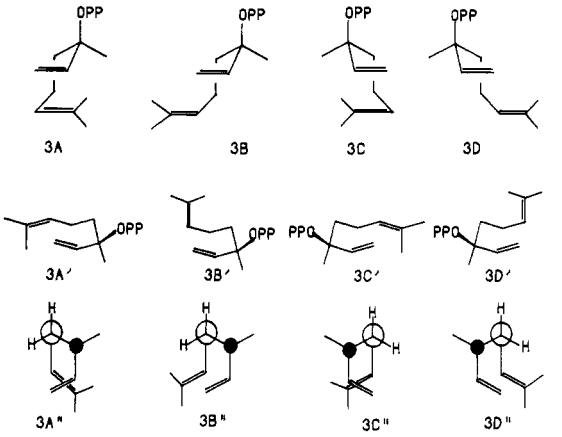
This mechanistic scheme has two serious stereochemical deficiencies, one purely geometric and the second conformational. (1) The direct formation of six-membered rings from the trans allylic pyrophosphate precursor is geometrically forbidden since the resultant cyclohexene derivative must contain a cis double bond. A similar constraint applies to the attack on the distal double bond of farnesyl pyrophosphate leading to formation of 10- and 11-membered ring products with cis double bonds. On the basis of the evidence obtained in several laboratories,[7] it is now generally accepted that such cyclization processes involve the intermediacy of the corresponding tertiary allylic pyrophosphate ester, nerolidyl pyrophosphate or its lower isoprenologue linalyl pyrophosphate, which can each undergo rotation about the 2,3-single bond prior to ionization and cyclization. (2) The conventional planar representation of farnesyl pyrophosphate clearly does not correspond to the actual cyclizing species. In order for cyclization to occur, the π-orbitals of the farnesyl pyrophosphate double bonds must be properly aligned. The requisite geometry for interaction can be achieved only if the individual double bonds of the substrate are mutually perpendicular to a common plane. In fact, there are only a relatively small number of conformations of farnesyl pyrophosphate which meet this condition.

Conformations of Allylic Pyrophosphate Substrates

In considering the conformations which are available to cyclizing allylic pyrophosphate substrates, it should be apparent that the presence of two or three trisubstituted double bonds in geranyl pyrophosphate or farnesyl pyrophosphate significantly reduces the number of degrees freedom of the respective C_{10} or C_{15} hydrocarbon moiety. Thus C-1–C-4 and C-15 of farnesyl pyrophosphate must be coplanar, as are C-5–C-8 and C-14, as well as C-9–C-13. Conformations which are competent for cyclization may be generated by imposing a number of additional constraints on the allylic pyrophosphate substrate. (1) The planes of the two (three) double-bond systems of geranyl pyrophosphate (farnesyl pyrophosphate) must be perpendicular to a common plane. (2) C-1 must be brought to within bonding distance of either the C-6,7 double bond (paths a and b) or C-10,11 (paths c and d), with displacement of the pyrophosphate moiety from C-1 taking place in an anti sense. (3) For those cases in which a cis double bond is generated as a consequence of cyclization, the cyclization substrate will be either linalyl pyrophosphate (**3**) or nerolidyl pyrophosphate (**4**). Allylic displacement of the pyrophosphate moiety takes place in an anti sense from a cisoid conformation of the tertiary allylic pyrophosphate in which the dihedral angle $\theta_{C\text{-}1\text{-}C\text{-}4}$ has a value between −30° and +30°.

If the above constraints are imposed, it will be seen that for linalyl pyrophosphate cyclizing by path a or b there are only four possible conformations, which can be grouped into two diastereomeric sets of enantiomeric pairs of conformations (Scheme III). For nerolidyl pyrophosphate cyclizing by path a or b there are eight possible conformations, comprised of four diastereomeric sets of enantiomeric conformational pairs

Scheme III
Conformations of Linalyl Pyrophosphate for Cyclization by Pathways a or b

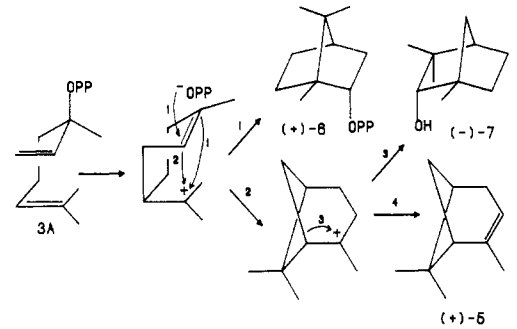


(Scheme VIII), while for nerolidyl pyrophosphate cyclizing by path c or d there are a comparable 8 conformations (Scheme XVI). Finally, for farnesyl pyrophosphate cyclizing by path c or d there are yet another eight conformations available (Scheme XI). Each of these cases is analyzed in more detail below.

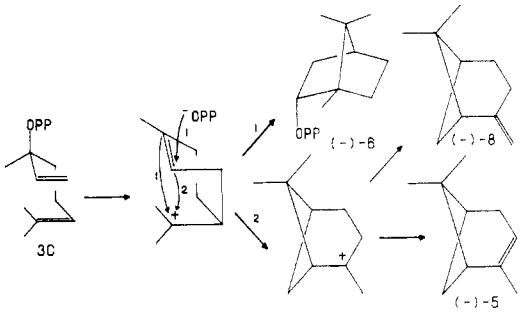
Monoterpene Cyclizations. In Scheme III are illustrated the four prototype conformations for cyclization of linalyl pyrophosphate **3A**, **3B**, **3C**, and **3D**. Alternative projections, **3A′**–**3D′** and **3A″**–**3D″**, useful for visualizing the reactive conformations, are also shown. The pair **3A**/**3C** represent enantiomeric conformations as do the corresponding diastereomeric pair **3B**/**3D**. **3A** and **3B** have the same configuration (3*R*) at the single chiral center of linalyl pyrophosphate but differ as to the face of the 6,7-double bond, *si* or *re*, respectively, which undergoes electrophilic attack during cyclization. Conformation **3A** is usually referred to as *anti-boat*, but the designation *anti-chair* is less useful for **3B**. A more appropriate description would be based on the configuration of the tertiary allylic pyrophosphate and the crossed or parallel[8] arrangement of the 1,2- and 6,7-double bonds. For most cyclic monoterpenes, the corresponding conformation of the linalyl pyrophosphate precursor can usually be deduced readily on the basis of the assumption of least motion during the course of the cyclization. There is thus a one-to-one correspondence between the observed relative and absolute *configuration* of the terpenoid product and the inferred *conformation* of the precursor. For example, bicyclic monoterpenes, formed by cyclization path a, are derived only from conformer **3A** or **3C**. On the basis of these arguments, it is evident that the bicyclic monoterpenes (+)-α-pinene (**5**), (+)-bornyl pyrophosphate (**6**), and (−)-*endo*-fenchol (**7**) are formed from conformer **3A** of (3*R*)-linalyl pyrophosphate (Scheme IV). The antipodal series of monoterpenes, including (−)-α-pinene (**5**), (−)-β-pinene (**8**), and (−)-bornyl pyrophosphate (**6**), is likewise derivable from (3*S*)-linalyl pyrophosphate by way of conformer **3C** (Scheme V). The inferred configurations of the linalyl pyrophosphate precursor have in fact been confirmed by incorporation experiments using cell-free preparations of the appropriate cyclases.[9]

(8) Cf. Sutherland, J. K. *Tetrahedron* **1974**, *30*, 1651.
(9) Cane, D. E.; Chang, C.; Croteau, R.; Satterwhite, M., unpublished results.

Scheme IV
Derivation of (+)-Bornyl Pyrophosphate (6), (−)-Fenchol (7), and (+)-α-Pinene (5) from (3*R*)-Linalyl Pyrophosphate Folded in Conformer 3A



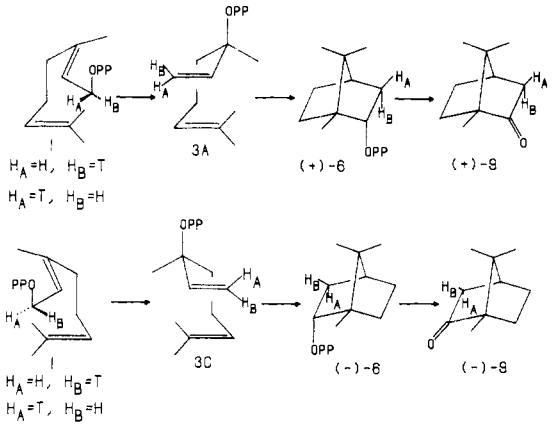
Scheme V
Derivation of (−)-Bornyl Pyrophosphate (6), (−)-α-Piene (5), and (−)-β-Pinene from (3*S*)-Linalyl Pyrophosphate Folded in Conformer 3C



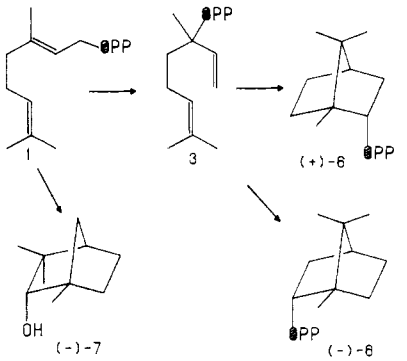
A useful model must not only explain existing data; it must also serve as the inspiration for new experiments. Consideration of monoterpene cyclizations in explicit stereochemical terms has generated a series of especially informative investigations. Two examples will suffice here. Since all cyclases investigated to date utilize geranyl pyrophosphate as substrate, formation of cyclic products requires initial isomerization to the tertiary allylic ester, linalyl pyrophosphate, which is capable of free rotation about the newly generated 2,3-single bond. This isomerization is believed to take place at the same active site at which the subsequent cyclization occurs, by essentially the same general mechanism: ionization to form an allylic cation–pyrophosphate anion pair. The fate of this ion pair depends on its conformation, with the transoid conformer undergoing simple recombination at the allylic site (allylic rearrangement) while the cisoid ion pair can suffer backside nucleophilic attack by the neighboring double bond (cyclization). The monoterpene cyclases are therefore isomerase—cyclases for which the initial isomerization activity is normally cryptic. It has, however, been possible to study this isomerase activity directly in one case, using a fungal enzyme which catalyzes the conversion of farnesyl to nerolidyl pyrophosphate.[10] With the latter enzyme, labeling studies have demonstrated that the allylic rearrangement takes place in a suprafacial manner with respect to the allylic system. At the active site of a cyclase, rotation about the 2,3-bond of linalyl pyrophosphate brings the face of C-1 from which the pyrophosphate moiety has departed into juxtaposition with the neighboring 6,7-double bond. As a consequence, it is expected that

(10) Cane, D. E.; Iyengar, R.; Shiao, M.-S. *J. Am. Chem. Soc.* **1981**, *103*, 914.

Scheme VI
Cyclization of (1*R*)-[1-^{3}H]- and (1*S*)-[1-^{3}H]Geranyl Pyrophosphate by Both (+)- and (−)-Bornyl Pyrophosphate Synthetases with Retention of Configuration at C-1



Scheme VII
Cyclization of [1-^{18}O]Geranyl Pyrophosphate and [3-^{18}O]Linalyl Pyrophosphate to (+)- and (−)-Bornyl Pyrophosphate, with Complete Retention of ^{18}O in the Ester Oxygen and Derivation of the Hydroxyl Oxygen Atom of (−)-Fenchol (7) from Water

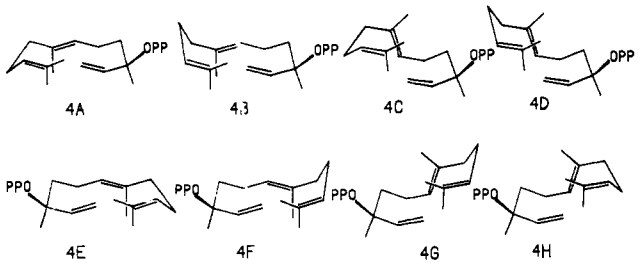


cyclization of geranyl pyrophosphate by path a (or b) will result in net *retention* of configuration at C-1 of the precursor.

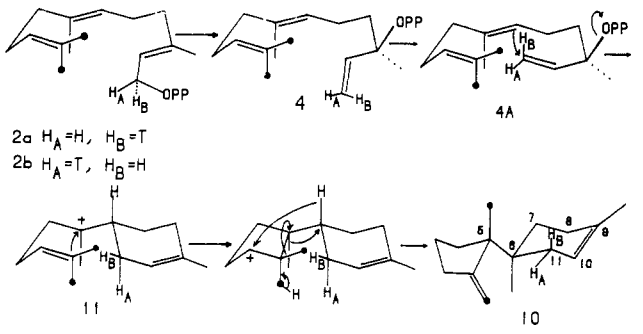
This prediction has in fact been confirmed directly by Croteau who has carried out separate incubations of (1*R*)-[1-^{3}H]- and (1*S*)-[1-^{3}H]geranyl pyrophosphate with both (+)- and (−)-bornyl pyrophosphate synthetases followed by stereoselective exchange of the resulting derived samples of tritiated camphor (**9**)[11] (Scheme VI). The same stereochemical model has also been invoked to account for the fact that fenchyl pyrophosphate does not appear to be an intermediate in the formation of (−)-*endo*-fenchol (**7**), an assertion which is supported by the demonstration that the alcoholic oxygen atom of fenchol is not derived from the pyrophosphate moiety of geranyl pyrophosphate,[12] whereas conversion of both [1-^{18}O]geranyl pyrophosphate[13] and [3-^{18}O]linalyl pyrophosphate[14] to either (+)- or (−)-bornyl pyrophosphate results in complete retention of the ester oxygen atom in the bornyl pyrophosphate product (Scheme VII).

(11) Croteau, R.; Felton, N. M.; Wheeler, C. J. *J. Biol. Chem.* **1985**, *260*, 5956.
(12) Croteau, R.; Shaskus, J.; Cane, D. E.; Saito, S.; Chang, C. *J. Am. Chem. Soc.* **1984**, *106*, 1142.
(13) Cane, D. E.; Saito, A.; Croteau, R.; Shaskas, J.; Felton, M. *J. Am. Chem. Soc.* **1982**, *104*, 5831.
(14) Croteau, R.; Shaskus, J. J.; Renstrom, B.; Felton, N. M.; Cane, D. E.; Saito, A.; Chang, C. *Biochemistry*, in press.

Scheme VIII
Conformations of Nerolidyl Pyrophosphate for Cyclization by Pathways a or b



Scheme IX
Cyclization of (1*R*)-[1-^{3}H,12,13-^{14}C]Farnesyl Pyrophosphate (2a) and (1*S*)-[1-^{3}H,12,13-^{14}C]Farnesyl Pyrophosphate (2b) to Trichodiene (10) by Way of Conformer 4A of Nerolidyl Pyrophosphate and the Bisabolyl Cation 11 with Retention of Configuration at C-1



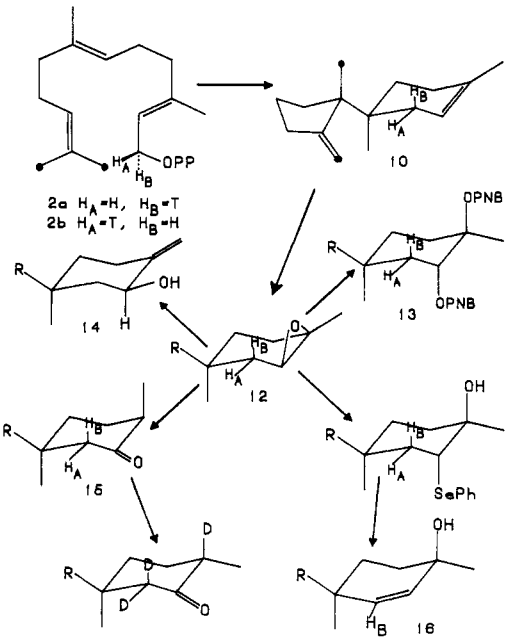
Sesquiterpene Cyclizations. Paths a and b. Cyclization of farnesyl pyrophosphate by paths a or b requires initial isomerization to nerolidyl pyrophosphate (**4**). Whereas only four conformations of linalyl pyrophosphate are competent to cyclize, the presence of an additional double bond in nerolidyl pyrophosphate increases the number of reactive conformers to eight, illustrated in Scheme VIII as the enantiomeric pairs **4A/4E**, **4B/4F**, **4C/4G**, and **4D/4H**. The conformation of the nerolidyl pyrophosphate intermediate can once again be inferred from the observed relative and absolute configuration of the cyclized product. Of course, for those cases in which only the central double bond undergoes reaction the conformation of the distal double bond will remain cryptic.

Conformer **4A** of nerolidyl pyrophosphate can be inferred as the precursor of the fungal sesquiterpene trichodiene (**10**)[15] (Scheme IX). Extensive experimental studies of the biosynthesis of the trichothecane family of antibiotics are in accord with this model, including the demonstrated 1,4-hydride shift and the proof that the C-5 methyl group is derived from C-2 of mevalonic acid.[7b,16] We have been studying the enzymatic cyclization of farnesyl pyrophosphate to trichodiene itself. Initially we reported that a preparation of trichodiene synthetase from the fungus *Trichothecium roseum* would convert [1-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate to trichodiene without loss of tritium label,[15a] thereby refuting earlier claims that this conversion involves a redox mechanism requiring loss of isotope from C-1.[17] The distribution of tritium label

(15) (a) Cane, D. E.; Swanson, S.; Murthy, P. P. N. *J. Am. Chem. Soc.* **1981**, *103*, 2136. (b) Cane, D. E.; Ha, H.-J.; Pargellis, C.; Waldmeier, F.; Swanson, S.; Murthy, P. P. N. *Bioorg. Chem.* **1985**, *13*, 246.

(16) Tamm, Ch.; Breitenstein, W. In "The Biosynthesis of Mycotoxins"; Steyn, P. S., Ed.; Academic Press: New York, 1980; pp 69–104.

Scheme X
Degradation of Trichodiene Derived from (1*R*)-[1-^{3}H,12,13-^{14}C]Farnesyl Pyrophosphate and (1*S*)-[1-^{3}H,12,13-^{14}C]Farnesyl Pyrophosphate To Establish the Stereochemistry of Labeling at C-11

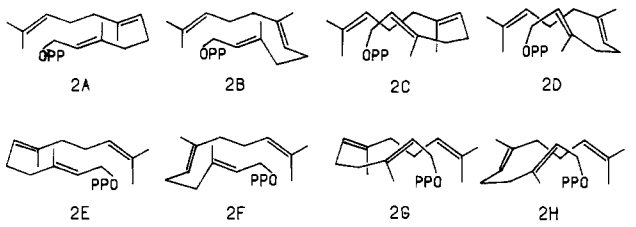


was confirmed by degradation of the labeled trichodiene. We have now shown that formation of trichodiene takes place with net retention of configuration at C-1 of the farnesyl pyrophosphate precursor, as required by the intermediacy of nerolidyl pyrophosphate and in accordance with the stereochemical model represented by **4A**.[15b] Thus separate incubations of (1*R*)-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate and (1*S*)-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate with trichodiene synthetase gave labeled samples of trichodiene which were degraded by the route summarized in Scheme X. Treatment of trichodiene with *m*-chloroperbenzoic acid gave the epoxide **12**, a portion of which was converted to the crystalline bis(*p*-nitrobenzoate) ester **13** by hydrolysis with aqueous $HClO_4$ and esterification of the resulting diol. The configurational assignment of the epoxide was confirmed by conversion of an unlabeled sample to the corresponding allylic alcohol **14** and analysis by 270-MHz ^{1}H NMR. The presence of tritium at C-11 of each trichodiene sample was demonstrated by rearrangement of **12** to the ketone **15** by treatment with $LiClO_4$ and exchange with NaOD in D_2O/dioxane.

Finally the stereochemistry of tritium labeling was established by conversion of the epoxide to the hydroxyphenyl selenide followed by treatment with $NaIO_4$ and cis elimination to give the allylic alcohol **16**. Whereas the sample of **16** derived from (1*R*)-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate retained all the tritium label, as judged by the unchanged $^3H/^{14}C$ ratio, the corresponding sample of **16** originating from (1*S*)-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate was devoid of tritium. These results are completely in accord with those obtained independently by Croteau[11] for the formation of (+)- and (−)-bornyl pyrophosphate (**6**) and support the intermediacy of nerolidyl pyrophosphate. It should be noted, however, that to date there has been

(17) Evans, R.; Hanson, J. R. *J. Chem. Soc., Perkin Trans. 1* **1976**, 326.

Scheme XI
Conformations of Farnesyl Pyrophosphate Cyclizing by Pathways c or d



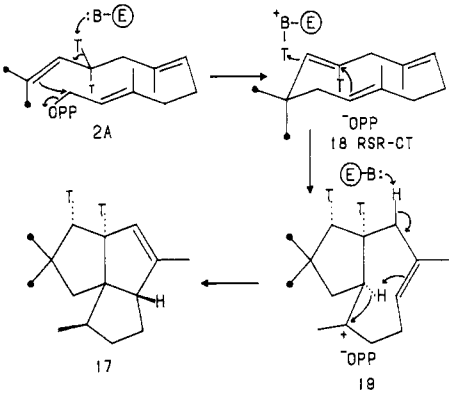
no direct demonstration of the role of nerolidyl pyrophosphate in the formation of sesquiterpenes. Experiments are in progress in this laboratory to address this question and to probe further the implications of the stereochemical cyclization model.

Sesquiterpene Cyclizations. Paths c and d. A large and varied group of sesquiterpenes are derivable, at least formally, from germacradienyl or humulyl ring systems generated by cyclization of farnesyl pyrophosphate at the distal double bond according to paths c or d, respectively. For products in which the trans 2,3-double bond of the precursor is preserved, cyclization of farnesyl pyrophosphate by paths c and d is possible from eight prototype conformations **2A–2D** and their respective enantiomers **2E–2H** (Scheme XI). We have previously discussed the biosynthesis of a group of biogenetically related dimethylcyclopentane sesquiterpenes including the microbial metabolites quadrone, fommanosin, pentalenolactone, marasmic acid, botrydial, hirsutic acid, the coriolins, and the illudins.[7b] Each of these substances is formally derivable from farnesyl pyrophosphate by way of conformers **2A**, **2B**, or **2F**.

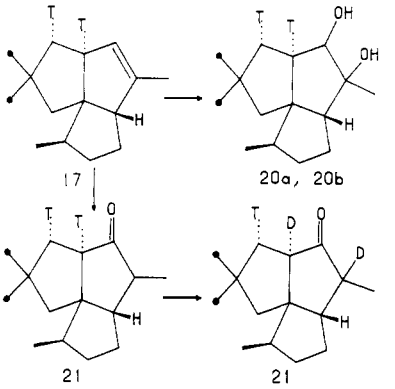
One pathway which we have investigated in considerable detail is the enzymatic conversion of farnesyl pyrophosphate to the tricyclic sesquiterpene pentalenene (17), the parent hydrocarbon of the pentalenolactone family of antibiotics.[18] Initial experiments utilizing intact cells of *Streptomyces* UC5319 and ^{13}C-labeled precursors, combined with analysis by ^{13}C NMR, had suggested a pathway for pentalenene biosynthesis involving initial cyclization of farnesyl pyrophosphate to humulene (18) followed by cyclization of 18 to pentalenene, as illustrated in Scheme XII.[19] Furthermore, the labeling data had indicated that the absolute sense of folding of the farnesyl pyrophosphate precursor was the same as that for the humulyl intermediate, a correlation to which we had previously drawn attention for the biosynthesis of related dimethylcyclopentane sesquiterpenes formally derivable from an intermediate humulene.[20]

One likely explanation for this striking correlation is that in each case the humulene is being generated and further cyclized at the same active site. One way of directly testing this theoretical prediction is to examine the fate of the proton removed from C-9 of farnesyl pyrophosphate in the formation of humulene. Cyclization of the resultant humulene at a distinct active site would be expected to result in complete loss of this

Scheme XII
Conversion of Farnesyl Pyrophosphate, Folded in Conformer 2A, to Pentalenene (17), via Humulene (18) and the Bicyclic Cation 19 with Retention of the Proton Derived from C-9 of Farnesyl Pyrophosphate



Scheme XIII
Degradation of Pentalenene Derived from [9-$^3H_2$,12,13-^{14}C]Farnesyl Pyrophosphate and Location of Half the Tritium Label at C-8

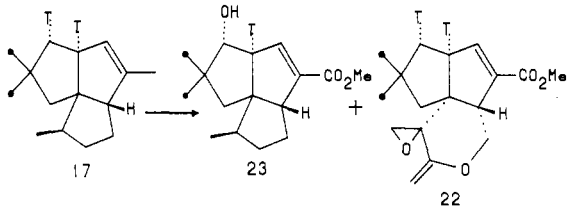


proton, whereas formation of pentalenene at the same active site might involve reprotonation at C-10 of humulene by the original C-9 proton, at least some fraction of the time. The extent of net proton return would depend on the partitioning of the transiently generated conjugate acid of the enzyme base between exchange with the medium and reprotonation of the substrate. Alternatively, a simple hydride shift might interconvert the 10- and 9-humulyl cations, thereby resulting in complete retention of the proton in question.

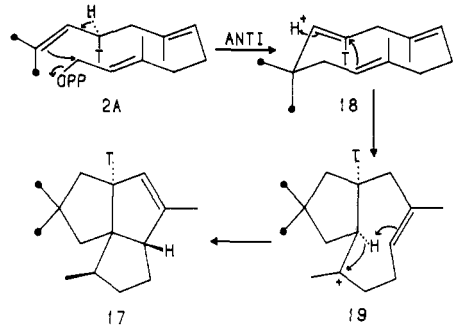
Incubation of [9-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate ($^3H/^{14}C$ atom ratio 2:2) with a partially purified preparation of pentalenene synthetase gave labeled pentalenene which was subjected to a combined chemical and microbial degradation sequence to locate the sites of tritium labeling. A portion of the pentalenene was converted to the mixture of diastereomeric cis diols **20a** and **20b** by treatment with OsO_4 (Scheme XIII). Recrystallization of the individual diol isomers to constant activity and isotope ratio indicated that the bulk of the tritium activity had been retained ($^3H/^{14}C$ atom ratio 1.7:2). Half of the tritium label was located at the bridgehead, C-8, by hydroboration–oxidation to the ketone **21** which after basic exchange exhibited a $^3H/^{14}C$ atom ratio of 0.9:2. The remainder of the tritium was shown to be at C-1 by reincubation of the enzymatically generated sample of pentalenene with an actively fermenting culture of *Streptomyces* UC5319 and isolation of the resulting oxidized metabolites (Scheme XIV). Whereas the derived pentalenolactone F methyl ester

(18) Cane, D. E.; Abell, C.; Tillman, A. M. *Bioorg. Chem.* **1984**, *12*, 312; Cane, D. E.; Tillman, A. M. *J. Am. Chem. Soc.* **1983**, *105*, 122.
(19) Cane, D. E.; Rossi, T.; Tillman, A. M.; Pachlatko, J. P. *J. Am. Chem. Soc.* **1981**, *103*, 1838.
(20) Cane, D. E.; Nachbar, R. B. *J. Am. Chem. Soc.* **1978**, *100*, 3208 (correction **1979**, *101*, 1908).

Scheme XIV
Microbial Degradation of Pentalenene (17) Derived from [9-$^3H_2$,12,13-^{14}C]Farnesyl Pyrophosphate and Location of Half the Tritium Label at C-1



Scheme XV
Conversion of (9*R*)-[9-^3H,12,13-^{14}C]Farnesyl Pyrophosphate to Pentalenene

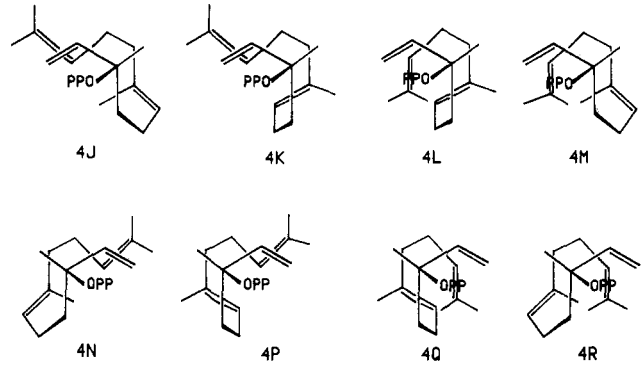


(22) retained all the original tritium of the pentalenene precursor ($^3H/^{14}C$ atom ratio 1.7:2), the corresponding sample of pentalenic acid methyl ester (**23**) had lost half the tritium label ($^3H/^{14}C$ atom ratio 0.9:1). Assuming for the moment that introduction of the hydroxyl oxygen atom of pentalenic acid has occurred with the usual retention of configuration, the observed loss of half the tritium suggests that the tritium in pentalenene has the H-1$_\alpha$ configuration in accord with the result predicted from the stereochemical model based on conformer **2A** of farnesyl pyrophosphate and the intermediacy of humulene folded in the RSR-CT[8,18] conformation.

Recently we have further extended our studies of pentalenene biosynthesis to a determination of the stereochemical course of the proton (hydride) transfer reaction.[21] Electrophilic attack of C-1 of the farnesyl pyrophosphate precursor at C-11 of the distal double bond, coupled with transient loss of the C-9 proton, constitutes a formal S_E' process analogous to the addition–elimination reaction catalyzed by prenyl transferase by which first dimethylallyl pyrophosphate and then geranyl pyrophosphate are condensed with the double bond of isopentenyl pyrophosphate in the biosynthesis of farnesyl pyrophosphate itself. Extensive studies by Cornforth and Popjak have established the net syn stereochemistry of the latter process.[22] Cyclization of farnesyl pyrophosphate is in a sense the intramolecular equivalent of the prenyl transferase reaction. Consideration of the geometric requirements of conformer **2A**, however, leads to the prediction that the initial intramolecular S_E' reaction must take place with net anti stereochemistry. We have now confirmed this prediction by incubation of (9*R*)-[9-^3H,12,13-^{14}C]-farnesyl pyrophosphate with pentalenene synthetase (Scheme XV). The bulk of the tritium from this

(21) Cane, D. E.; Lattman, R., unpublished results.
(22) Cornforth, J. W.; Cornforth, R. H.; Donninger, C.; Popjak, G. *Proc. R. Soc. London, Ser. B.* **1966**, *163*, 492. Cornforth, J. W.; Cornforth, R. H.; Popjak, G.; Yengoyan, L. *J. Biol. Chem.* **1966**, *241*, 3970.

Scheme XVI
Conformers of Nerolidyl Pyrophosphate Cyclizing by Pathways c or d To Give Products with a Cis Double Bond



precursor was shown by a degradative sequence analogous to that illustrated in Schemes XIII and XIV to reside at C-8, in accord with the proposed stereochemical model. Complementary experiments with (9*S*)-[9-^3H,12,13-^{14}C]farnesyl pyrophosphate are now in progress. It should also be noted that the C-4,5 bond of pentalenene is also formed by an intramolecular S_E' reaction; the stereochemical details of this latter transformation are also under study.

Among the sesquiterpenes formally derivable from a germacradienyl or humulyl intermediate, a substantial number involve initial generation of a cis double bond corresponding to C-2,3 of the *trans,trans*-farnesyl pyrophosphate precursor. The requisite double bond isomerization is most likely achieved by initial conversion to nerolidyl pyrophosphate, followed by rotation about the newly generated 2,3-single bond. The intermediate nerolidyl pyrophosphate, containing one chiral center and two trisubstituted double bonds, has available to it eight conformers capable of cyclizing by paths c or d: **4J/4N**, **4K/4P**, **4L/4Q**, and **4M/4R** (Scheme XVI). Strong stereochemical evidence for the intermediacy of nerolidyl pyrophosphate in the biosynthesis of longifolene and related cadalene-derived sesquiterpenes has been obtained by Arigoni.[23] Detailed and elegant studies by the Zürich group led to an ingenious stereochemical model based on cyclization of nerolidyl pyrophosphate in conformer **4K** which could account not only for the absolute and relative configuration of (−)-sativene and (−)-longifolene but could readily explain the observed stereochemical course of the 1,3-hydride transfers leading to 10- or 11-membered ring intermediates.[24]

Discussion

Many of the ideas elaborated here have been developed to some extent in previously published work.[2] In addition to the above-referenced studies of Arigoni,[23] Andersen has pointed out the relationship between the configuration of nerolidyl pyrophosphate and the absolute configuration of derived sesquiterpenoid metabolites and has illustrated these notions by an impressive series of biomimetic cyclization reactions.[25] It should be noted, however, that although nerolidyl pyro-

(23) Arigoni, D. *Pure Appl. Chem.* **1975**, *41*, 219.
(24) Dorn, F. Dissertation, ETH Zurich, 1975, No. 5554. Dorn, F.; Bernasconi, P.; Arigoni, D. *Chimia* **1975**, *29*, 24.
(25) Andersen, N. H.; Ohta, Y.; Syrdal, D. D. In "Bio-Organic Chemistry"; van Tamelen, E. E., Ed.; Academic Press: New York, 1977; Vol. 2, pp 1–37. Andersen, N. H.; Syrdal, D. D. *Tetrahedron Lett.* **1972**, 2455.

phosphate and linalyl pyrophosphate are the first explicitly chiral intermediates in the biosynthesis of cyclized terpenes, their eventual configuration is already implicit in the chiral conformations of the acyclic, achiral primary allylic pyrophosphates farnesyl and geranyl pyrophosphate. Shirahama and Matsumoto have analyzed the conformational properties of humulene and discussed the chemical and enzymatic cyclizations of this substrate.[26] We have simply extended these ideas to a consideration of the conformation of the farnesyl pyrophosphate precursor.

Although no attempt has been made in the foregoing discussion to provide an exhaustive analysis of each hypothetical cyclization mode or to rationalize the formation of the more than 200 known monoterpene and sesquiterpene carbon skeleta, several generalizations have begun to emerge. The first is that only a small fraction of the total possible precursor conformations appear to be utilized by monoterpene and sesquiterpene cyclases. Furthermore, whereas trans addition across double bonds is accepted to be the rule for cyclization of squalene and related polyolefins,[6] monoterpene and sesquiterpene cyclizations appear to involve net cis addition to the 6,7- and, where applicable, 10,11-double bonds of farnesyl and geranyl pyrophosphate. (For those cases, however, in which positive charge is quenched by an *external* nucleophile, such as water or the pyrophosphate moiety, net addition remains trans.) The stereochemical course of enzyme-catalyzed electrophilic addition to double bonds may therefore be a function of conformational constraints on the substrate rather than an expression of an inherent stereochemical imperative for polar addition reactions.

It is also evident that the representations used here for the various prototype cyclization conformers are essentially cartoons, distorted to a great extent for convenience of two-dimensional projection. More extensive analysis using the tools of molecular mechanics will be necessary to achieve more realistic representations of the prototype conformers and to establish their relative conformational stabilities. Although it is assumed that the precise conformation of the substrate will be dictated to a large extent by binding to the cyclase active site, no information is as yet available to relate the prototype cyclization conformers to either global or local minima of the substrate. Taken literally, for example, the representation of conformer **4A** for nerolidyl pyrophosphate involves an unacceptable overlap of C-1 and C-12. Obviously both C-1 and C-11 cannot simultaneously be within bonding distance of the central double bond.

The recognition that several groups of distinct metabolites are derivable from a single parent conformation of the allylic pyrophosphate precursor leaves open the question as to the role of the cyclase itself in controlling product specificity. Any model of substrate–active site interaction must take into account the extensive changes in substrate geometry which accompany a typical terpenoid cyclization. In the formation of trichodiene (**10**), for example, 10 of the original 15 carbon atoms of *trans,trans*-farnesyl pyrophosphate have undergone substantial changes in hybridization, bonding, or configuration during the course of the cyclization, as have 9 of the 15 carbons of pentalenene (**17**) or 5 of 10 carbon atoms in bornyl pyrophosphate (**6**). These dramatic changes in substrate structure cannot be accommodated by an active site model rigidly complementary to the initial conformation of the allylic pyrophosphate substrate.

To assure fidelity in the formation of its characteristic products from among many available kinetic channels, the cyclase must somehow exercise strict conformational and electronic control over the entire cyclization process. On the other hand, recent experiments which we have carried out in collaboration with Professor Croteau of Washington State University have led to the remarkable observation that (+)- and (−)-bornyl pyrophosphate synthetases can each cyclize either enantiomer of linalyl pyrophosphate with only modest enantiomeric discrimination,[27] in spite of the demonstrated ability of each cyclase to synthesize enantiomerically pure products from the achiral precursor geranyl pyrophosphate.[28] Similar results have been obtained by Croteau for the formation of (−)- and (+)-fenchol by (−)-fenchol synthetase.[29] It should also be pointed out that the role of the cyclase in generating, steering, and eventually quenching positive charge is essentially completely unexplored.

Consideration of terpenoid cyclizations in explicit stereochemical terms has revealed that a relatively small number of substrate conformations are responsible for the vast majority of cyclized monoterpene and sesquiterpene products. Most significantly, stereochemical analysis has provided a coherent framework for the posing of new questions about the cyclization process itself. With the increasing availability of cell-free cyclase preparations, the next several years should witness substantial advances in our understanding of the mechanisms by which nature synthesizes this fascinating arry of natural products.

The work from the laboratory of DEC described here was supported by the NIH, GM 22172 and GM 30301. The work on cell-free studies of monoterpene biosynthesis was carried out in close collaboration with Professor Rodney Croteau of Washington State University. It is also a pleasure to acknowledge the contributions of a group of skilled and dedicated co-workers whose names are cited in the references.

(26) Shirahama, H.; Osawa, E.; Matsumoto, T. *J. Am. Chem. Soc.* **1980**, *102*, 3208.

(27) Cane, D. E.; Chang, C.; Croteau, R.; Satterwhite, D. M., unpublished results.

(28) Croteau, R.; Karp, F. *Arch. Biochem. Biophys.* **1979**, *198*, 512; **1977**, *184*, 77. Croteau, R.; Shaskus, J. *Arch. Biochem. Biophys.* **1985**, *236*, 535.

(29) Satterwhite, D. M.; Wheeler, C. J.; Croteau, R. *J. Biol. Chem.*, in press.