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THE STEREOCHEMISTRY OF ALLYLIC PYROPHOSPHATE METABOLISM

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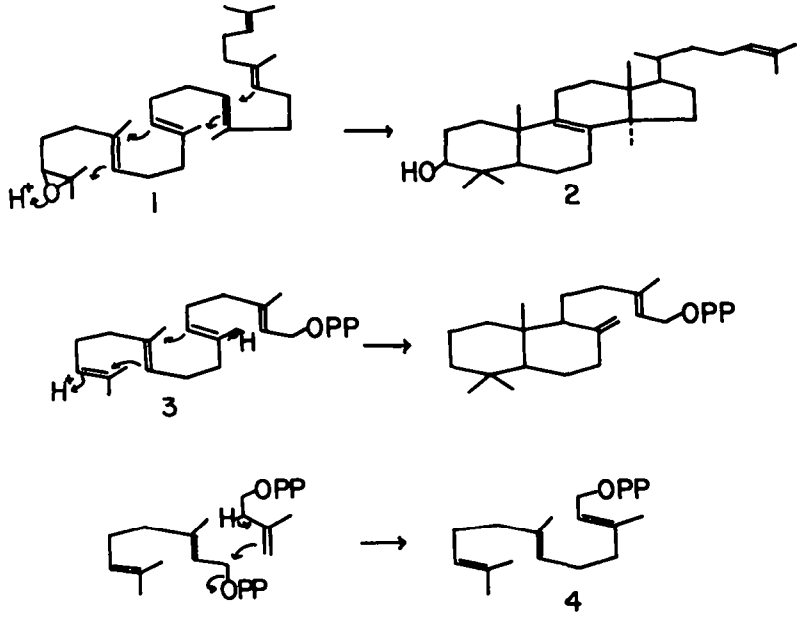
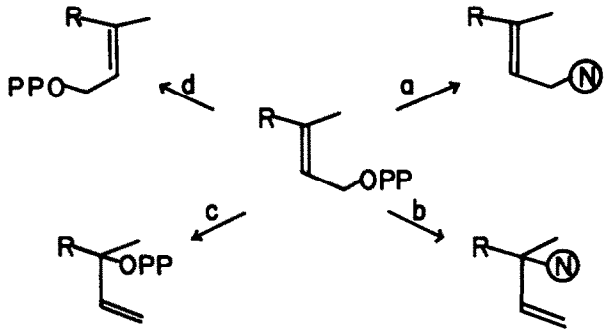
I. INTRODUCTION

In considering the biological synthesis of organic molecules, one is struck by the relatively small number of reaction types which Nature has selected for the formation of C–C bonds. The pathways of central metabolism as well as the biosynthesis of sugars, fatty acids, and polyketides, are dominated by the chemistry of the CO function and reactions such as Claisen, aldol, and acyloin condensations as well as related carboxylations appear repeatedly. By contrast, in the biosynthesis of terpenoids the predominant strategy for the generation of C–C bonds is electrophilic attack of a cationic center or its equivalent on one or more C–C double bonds. For example in the formation of lanosterol, a key intermediate in the biosynthesis of cholesterol, four new C–C single bonds and eight contiguous asymmetric centers are generated in a single enzyme catalyzed step by protonation and consequent cyclization of squalene oxide. The recognition that the origin and stereochemistry of all triterpenes by cyclization of all-*trans*-squalene can be explained by invoking well-known carbonium ion reactions and straightforward stereochemical principals[1] not only remains one of the great triumphs of modern biogenetic theory but has stimulated the efforts of a generation of synthetic chemists in the area of biogenetic polyolefin cyclizations.[2] In most enzymatic processes electrophilic reactions are initiated in one of two ways: (1) By addition of a proton to an epoxide, as in the cyclization of squalene oxide (1) to lanosterol (2), or to a double bond, as in the formation of bicyclic diterpenes from the acyclic precursor geranylgeranyl-pyrophosphate (3) or (2) by ionization of an allylic pyrophosphate, as for example, in the biosynthesis of farnesyl pyrophosphate (4), an allylic pyrophosphate itself derived by attack of allylic pyrophosphate



Scheme 1.

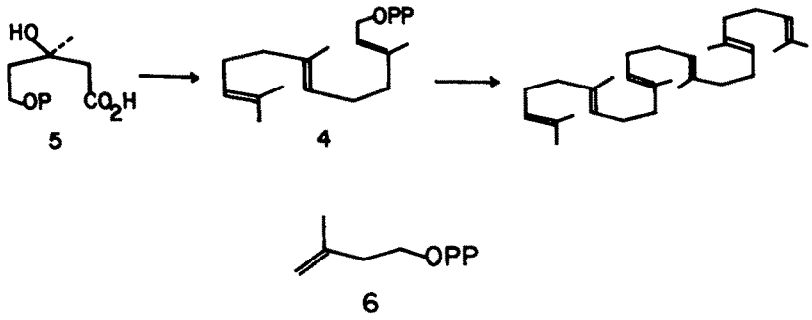
precursors on successive units of isopentenyl pyrophosphate. The central role played by allylic pyrophosphates in the biosynthesis of isoprenoid metabolites is the subject of this review. After a brief introduction four major reaction types involved in a variety of transformations will be considered: (Scheme 2) (1) direct displacements (pathway a); (2) allylic displacements (pathway b); (3) allylic transpositions (pathway c); and (4) double bond isomerizations (pathway d). The emphasis will be on the stereochemical details of each of the reactions considered and, where applicable, the relevance of this information to an understanding of the chemical and catalytic mechanisms by which these transformations occur.



Scheme 2.

Discovery

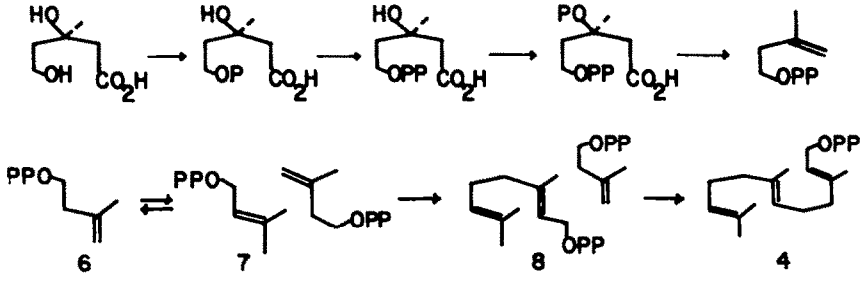
The existence of allylic pyrophosphates was first recognized by Lynen in early investigations of the conversion of mevalonic acid to cholesterol.[3] Experiments with a crude yeast extract indicated that when 5-phosphomevalonate (5) was incubated with Mg^{2+} and ATP, in the absence of NADPH, a water soluble, collidine extractable material was formed which was unambiguously identified as farnesyl pyrophosphate by comparison with authentic material and by chemical degradation. Furthermore, reincubation of this farnesyl pyrophosphate with yeast enzymes in the presence of NADPH gave squalene. While farnesol itself had been known for many years as a constituent of the essential oils of higher plants, Lynen's finding was the first direct evidence for the existence of an activated farnesol derivative first suggested by Ruzicka some 30 years earlier.[4] Lynen also found that if the yeast extracts were first treated with iodoacetamide, no farnesyl pyrophosphate was formed from 5-phosphomevalonate and ATP, but in its place a new substance, 3-methyl-3-butenyl-1-pyrophosphate, dubbed isopentenyl pyrophosphate (6), was generated. This latter substance could itself be converted to farnesyl pyrophosphate and thence to squalene and thus constituted the long sought "Biological Isoprene Unit."[4,5]



Scheme 3.

Subsequent work in a number of laboratories established the details of the formation of farnesyl pyrophosphate from mevalonate (Scheme 4).[6] Thus mevalonic acid is converted by the action of mevalonate kinase and ATP to 5-phosphomevalonate which in turn undergoes phosphorylation mediated by a second kinase to 5-pyrophosphomevalonate. Decarboxylation and dehydration, presumably by way of 3-phospho-5-pyrophosphomevalonate, yields isopentenyl pyrophosphate, which serves as the fundamental building block for all terpenoid substances. Enzymatic isomerization of isopentenyl pyrophosphate gives the corresponding allylic isomer, dimethylallyl pyrophosphate (7). Condensation of a unit of dimethylallyl pyrophosphate with a molecule of isopentenyl pyrophosphate generates a new C–10

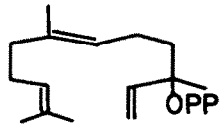
allylic pyrophosphate, geranyl pyrophosphate (8), which itself may undergo further condensation with another equivalent of isopentenyl pyrophosphate to yield the C-15 homologue farnesyl pyrophosphate (4). This sequence is believed to be universal for all forms of life, from bacteria (which produce no sterols but do synthesize long chain bactoprenols), through fungi and higher plants, to mammals. The stereochemistry of each of the above steps has been worked out in great detail and the story of these investigations constitutes one of the great epics in the history of chemical and biochemical inquiry. Furthermore the above pathways may branch at several points with, for example, geranyl pyrophosphate serving as the precursor of the monoterpenes and farnesyl pyrophosphate being the universal precursor of the sesquiterpenes.



Scheme 4.

Chemical properties

Although a detailed discussion of the chemical properties of allylic pyrophosphates is beyond the scope of this review, it is worthwhile to mention a few of the most salient chemical and physical properties.[7] Allylic pyrophosphates are water soluble substances which can be extracted from aqueous solution with butanol or collidine. Treatment of the pyrophosphate esters with alkaline phosphatase yields the corresponding allylic alcohols by P–O bond cleavage and with retention of configuration at the carbinyl carbon.[8] Alkaline solutions of the allylic pyrophosphates are reasonably stable at room temperature or lower. For example, heating solutions of farnesyl pyrophosphate in 0.1 N KOH for 1 hr at 100°, 80° or 60° results in 15%, 10% or 8% hydrolysis respectively. On the other hand acidic solutions of allylic pyrophosphates are markedly unstable. At pH 3.19 and 22° farnesyl pyrophosphate is about 15% hydrolyzed within 30 min and 50% hydrolyzed after 9 hr. The rate of hydrolysis is strongly pH dependent, the reaction being essentially complete within 6 hr at pH 1, 75% complete at pH 2.5, and 25% complete at pH 3.5, while above pH 5 the substrate remains unchanged. The behavior, which in detail resembles a titration curve, has been discussed by Popják and interpreted as evidence that it is the undissociated acid which is the unstable species, assuming the pKa of the allylic monoester to be essentially that of pyrophosphoric acid, pKa 1.9.[7] In a later study Tidd has compared the hydrolytic behavior of allylic and nonallylic phosphates and pyrophosphates.[9] Whereas nonallylic phosphoesters undergo predominant P–O bond cleavage, the allylic esters hydrolyze with C–O bond cleavage by an apparent acid-catalyzed S_N1 mechanism, the driving force being the enhanced stability of the intermediate allyl cation. Dimethylallyl phosphate is reported to hydrolyze at some 350,000 times the rate of ethyl phosphate. Below pH 6, dimethylallyl pyrophosphate hydrolyzes at approximately twice the rate of the corresponding phosphate ester. The pH behavior for pyrophosphate hydrolysis has been interpreted by Tidd as reflecting hydrolysis of the monoanion in the pH range 2–6, with the neutral species playing a more important role under even more acidic conditions. The products of the acid hydrolysis are both rearranged and unrearranged allylic alcohols formed in varying proportions, and inorganic pyrophosphate. Tertiary allylic pyrophosphates, such as nerolidyl pyrophosphate (9), are considerably more labile toward acid than their primary isomers. At 25° and pH 2.9 nerolidyl pyrophosphate is completely hydrolyzed within 30 min, under which conditions farnesyl pyrophosphate will be only 55% hydrolyzed. Similarly, at pH 3.9, nerolidyl pyrophosphate is 30% hydrolyzed after 60 min, while the farnesyl isomer



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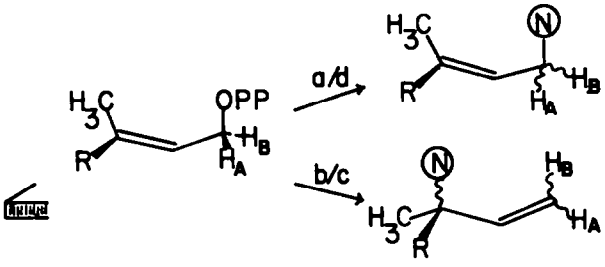
remains essentially unchanged. Recently Rilling[10] has re-examined in some detail Cori's[11] original observation that divalent cations such as Mg^{2+} and Mn^{2+} catalyze the hydrolysis of the allylic pyrophosphates even at neutral pH. For example at pH 7.6, 1 M Mg^{2+} causes an approximate 10-fold increase in the rate of hydrolysis of geranyl pyrophosphate compared to the corresponding rate measured at the same ionic strength in the absence of divalent cation. Based on the dissociation constants of Mg-geranyl-PP, Rilling has proposed that the actual reacting species responsible for the enhanced solvolysis must be Mg_2-geranyl-PP. Since Rilling has previously shown that two metal ions are bound per catalytic site of the enzyme prenyltransferase when substrate is present,[12] he has postulated that the role of the two metals is to ionize the substrate, giving rise to an allylic cation, which then reacts with the 3,4-double bond of isopentenyl pyrophosphate.

Rationale

It is interesting to note that few of the intermediates of central metabolism are ever converted to more than one product by different enzymes which employ only small variations of a single catalytic mechanism. For example, although glucose-6-phosphate can give rise, under the influence of the appropriate enzymes, to four distinct products, glucose, glucose-1-phosphate, 6-phosphogluconate, or fructose-6-phosphate, the details of each of these conversions are completely unrelated. On the other hand a substance such as farnesyl pryophosphate is believed to lead nearly 200 different sesquiterpene carbon skeletons by variations on essentially a single mechanism: ionization of the allylic pyrophosphate followed by electrophillic attack on the central or distal double bond followed by appropriate deprotonations, Wagner–Meerwein rearrangements, or hydride shifts. It is assumed that a distinct cyclase has evolved to mediate the formation of each sesquiterpene. Whereas stereoelectronic factors may play one role among many in the rate-determining (rate enhancing) steps of glucose-6-phosphate metabolism, our notions of both the *rate* and *product*-determining factors in the catalysis of farnesyl pyrophosphate cyclizations and isoprenoid biosynthesis in general are still almost purely stereoelectronic, with orientation of the substrate at the active site of the enzyme presumed to exert a controlling influence on the conversion of substrate to product. It is only natural therefore that stereochemical investigations, which provide detailed 3-dimensional descriptions of the chemical events taking place at the enzyme active site, have played such an important role in the study of isoprenoid biosynthesis. Indeed, the availibility of an enormous amount of data on the stereochemistry of numerous enzyme-catalyzed reactions has led in recent years to speculation on the origin and evolution of the mechanisms of enzyme catalysis itself.[13]

While stereochemical information is extremely useful in understanding the details of catalysis, it is subject to a number of important limitations. In classical organic chemistry stereochemical probes have proved invaluable in distinguishing synchronous and non-synchronous processes during nucleophilic substitions, elimination reactions, and pericyclic processes. In studying enzyme mediated reactions, however, the conclusions which may justifiably be drawn from stereochemical data are more limited. Keeping in mind that few if any fundamental ionic chemical processes are subject to a strict stereochemical inperative (for example, both *cis* and *trans* E2 eliminations have been observed[14] and S_N2' displacements appear to take place with either *syn* or *anti* stereochemistry[15]), it is unclear to what extent such stereoelectronic requirements play a role in the development of catalytic mechanisms which can result in as much as 10^6-fold or greater rate enhancements. Furthermore, even for a direct displacement such as the S_N2 reaction which invariably occurs with inversion, the observation of inversion during enzyme catalyzed displacement cannot be used by itself to establish the synchrony of bond-making and bond breaking events, since the chiral environment provided by the enzyme will almost always impose rigid stereospecificity on a given reaction.

With these caveats in mind, it is nonetheless clear that stereochemical investigations can be an extremely versatile and powerful probe of biological reactions. As should be evident, a consideration of the role of allylic pyrophosphates is central to an understanding of mechanisms of terpenoid biosynthesis and of the general problem of enzyme catalyzed formation of C–C bonds. The studies reviewed below concern four fundamental classes of allylic pyrophosphate transformations. While the individual systems studied and the approaches used vary considerably, certain similarities exist in the analytical techniques employed. In each case depicted in Schemes 2 and 5 a prochiral center at C(1) of the allylic pyrophosphate is transformed into either a prochiral sp^3 (pathways a and d) or sp^2 (pathways b and c) center in the product. Determination of the stereochemical course of the transformation at C(1) requires a synthetic or biosynthetic method for replacing one or the other of the attached stereoheterotopic



Scheme 5.

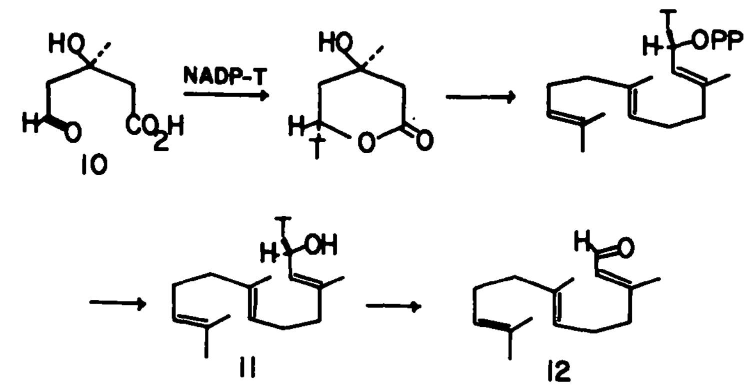
hydrogens in the starting material with deuterium or tritium and a means of analysing by spectroscopic, chemical, or enzymatic methods the stereochemistry of labeling at the corresponding center in the product. In recent years analysis of an sp^3 center which is chiral by virtue of isotopic substitution has become, in principle, rather straightforward, requiring in most cases only that the substrate itself or a suitable degradation product be submitted to an enzymatic or chemical process of known stereochemical consequence. Until recently, however, no general methods have been available for determining the stereochemistry of tritium substitution at the terminus of a vinyl group. As described in Sections III and IV, analysis of such prochiral sp^2 centers has required the development of powerful and often subtle new techniques including the use of chiral methyl analysis and deuterium magnetic resonance. Pathways b and c also involve a change in bonding at C(3) in which a prochiral center is converted to a new chiral (or occasionally prochiral) center by external or internal substitution respectively. For determining the stereochemistry of substitution at C(3) it is necessary only to know the absolute configuration of the resulting chiral center and the stereochemistry of the original double bond.

II. DIRECT DISPLACEMENTS

Of all the classes of allylic pyrophosphate reactions, the direct displacements have been the most intensively studied and are probably the best understood. While the majority of known examples of direct displacement involve the π-electrons of an olefinic or aromatic double bond, O- and even N-allylated substances are not uncommon. The chain elongation reactions catalyzed by prenyl-transferase, which were the first pyrophosphate displacements to be recognized, have been particularly thoroughly studied and it is now possible to evaluate a number of competing mechanistic proposals as a result of more recent kinetic investigations employing substrate analogs.

Prenyltransferase and double bond allylation

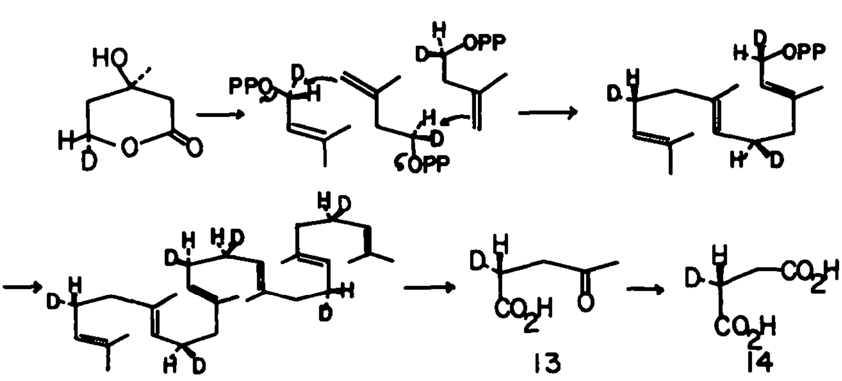
The stereochemistry of the prenyltransferase reaction was first determined by Cornforth and Popják, in advance of the isolation and purfication of the enzyme itself.[16] These stereochemical investigations grew out of an exhaustive study of the biosynthesis of cholesterol and took advantage of the ability of suitably prepared rat liver enzyme extracts to synthesize squalene from mevalonate by way of farnesyl pyrophosphate, according to the pathway already outlined. Thus in the biosynthesis of farnesyl pyrophosphate, two molecules of isopentenyl pyrophosphate are added, under control of prenyltransferase, first to DMAPP and then to geranyl pyrophosphate with displacement of inorganic pyrophosphate and generation of two new C–C bonds (Scheme 4). The question arose whether these displacements occur with retention or inversion at the carbinyl carbon of the allylic pyrophosphate. The solution of the problem took advantage of the fact that the C(5) hydrogens of mevalonate become the C(1) hydrogens of the allylic pyrophosphate and therefore a sample of mevalonate, stereospecifically labeled in a known manner at C(5) with either deuterium or tritium, was required. To prepare the requisite mevalonate, mevaldic acid (**10**) was reduced with either ^{2}H- or ^{3}H-NADH using the enzyme mevaldate reductase from rat liver (Scheme 6). (The latter enzyme, which at one time was thought to be involved in the normal biosynthesis of mevalonate, reduces both the 3*R* and 3*S*-enantiomers of mevaldate, in contrast to hydroxymethyl glutaryl CoA reductase which accepts only the 3*S*-form of HMGCoA and is the enzyme involved in the *in vivo* pathway.[17] In fact it is now believed that the mevaldate reductase, while a convenient biochemical tool, is simply a rather non-specific dehydrogenase and plays no essential role in normal mevalonate metabolism.[18]) The reduction yielded samples of the desired [5-^{2}H]- and [5-^{3}H]-mevalonates whose absolute configuration had to be determined. The stereochemistry at C(5) was established by converting the [5-^{3}H]-mevalonate sample, mixed with [4-^{14}C]-mevalonate as internal standard, to farnesyl pyrophosphate using a soluble enzyme preparation



Scheme 6.

from rat liver. The farnesyl pyrophosphate was in turn cleaved by alkaline phosphatase to free farnesol which was labeled at C(1), C(5) and C(9) with ^{3}H. Since none of the enzymatic reactions employed disturbed the bonding to C(1) of the farnesol,[8] the stereochemistry at this center would be identical to that of the mevalonate from which it was derived. Treatment of the [^{3}H, ^{14}C]-farnesol with liver alcohol dehydrogenase and an excess of NAD$^+$ resulted in the formation of farnesal (**12**) from which one third of the tritium had been lost. Assuming the absolute specificity of alcohol dehydrogenase to be the same for farnesol as had already been demonstrated unambiguously for ethanol,[19] the tritium which was removed must have occupied the 1-proR position of farnesol, implying that the mevalonate precursor was 5R-[5-^{3}H]-mevalonate. This conclusion has been supported by a direct correlation of [1-^{3}H]-geraniol, obtained by reduction of gerianal with ^{3}H-NADH in the presence of LADH, with 1R-[1-^{3}H]-ethanol.[20] More recently, Popják has reconfirmed these conclusions by direct NMR analysis of 5R-[5-^{2}H]-mevalonate.[18] (These latter experiments incidentally also provided interlocking evidence for the retention of configuration at the carbinyl carbon during alkaline phosphatase hydrolysis of farnesyl pyrophosphate.[8])

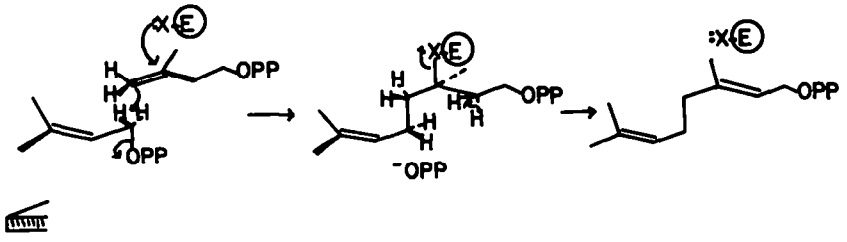
With the stereochemistry of the mevaldate reductase-catalysed reaction established, a sample of 5R-[5-^{2}H]-mevalonate was converted to squalene by a system containing microsomes and soluble enzymes from rat liver (Scheme 7). The stereochemistry at carbons 5 and 9 of each of the intermediate farnesyl pyrophosphates is established during the prenyltransferase mediated steps and is unaffected by the subsequent conversion to squalene. In order to determine the stereochemistry of deuteration at the corresponding centers in squalene, the latter substance was subjected to ozonolysis followed by performate oxidation to yield four equivalents of levulinic acid (**13**) in which C(2) is ultimately derived from C(5) of mevalonate. Sodium hypoiodite oxidation gave succinate (**14**) which was laevorotatory and displayed a negative ORD curve corresponding to that of a known 2R-[2-^{2}H]-succinate standard prepared by an unambiguous route starting with fumarase catalyzed hydration of fumarate in D_2O to 2S, 3R-[3-^{2}H]-malate and followed by substitution of the hydroxyl function by hydrogen.[21] Determination of the absolute configuration of the succinate therefore established the stereochemistry of the corresponding positions in squalene and farnesyl pyrophosphate and demonstrated conclusively that in the addition of an allylic pyrophosphate to isopentenyl pyrophosphate the new C–C bond is formed with *inversion of configuration* at the center from which the pyrophosphate is displaced.



Scheme 7.

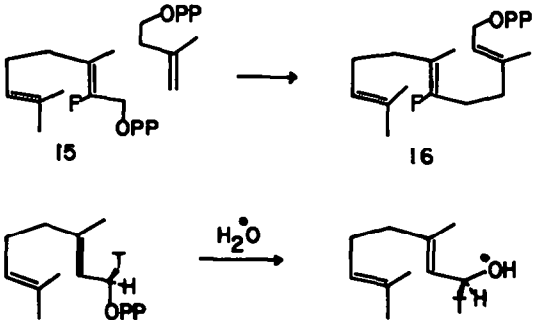
The studies of Conforth and Popják are particularly important, not only because they represent the first such stereochemical experiments, but because the results obtained and methods employed have served as a standard for all subsequent investigations. Prenyl transferases from numerous organisms with a variety of chain length specificities are now known. An implicit assumption has been that all these enzymes, while differing slightly in substrate compatibility, catalyze displacement of pyrophosphate with inversion of configuration. In fact it has been commonly accepted that the biosynthesis of prenyl pyrophosphates in all organisms involves the same stereochemical pathway as that which has been conclusively established as part of the biosynthesis of cholesterol in yeast and mammalian liver. These assumptions, while not often explicitly examined, have nonetheless been supported in those relatively few cases where they have been experimentally tested. A number of studies of terpenoid biosynthesis which independently confirm inversion of configuration during prenyl transfer will be cited in Sections III and V.

Based on the observed inversion of configuration at C(1) of each allylic pyrophosphate intermediate, Cornforth and Popják proposed that the displacement of the pyrophosphate probably resembles an S_N2 process in which formation of the new C–C bond is synchronous with departure of inorganic pyrophosphate from C(1) (Scheme 8). Ionization preceding bond formation, it was argued, would lead to scrambling of configuration, by analogy to the well-known stereochemistry of the S_N1 reaction. Taking into account the fact that prenyl transfer to isopentenyl pyrophosphate occurs by an overall suprafacial process in which C(1) of the allylpyrophosphate becomes attached to the *re* face of the C(3)–C(4) double



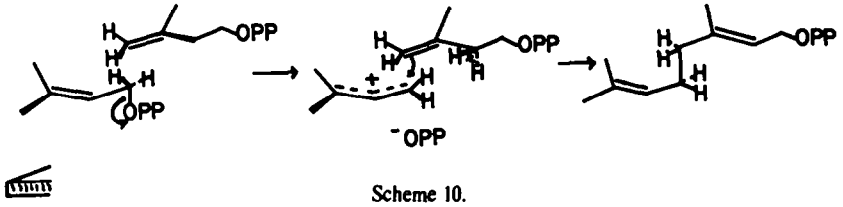
Scheme 8.

bond of isopentenyl pyrophosphate with concomitant loss of 2-H_{re} and generation of a new *trans* double bond,[16,22] a condensation-elimination mechanism was proposed in which an unspecified nucleophilic X-group on the enzyme stabilizes the developing positive charge at C(3) by ion-pairing or covalent bond formation and is then eliminated in a second step. The proposed mechanism, it was felt, would not only account for the observed stereochemistry but involved well-precedented *anti* addition and elimination. More recent work by Poulter and Rilling has indicated, however, that the actual timing of the bond-making and bond-breaking processes may be somewhat different.[23] Briefly summarized, these workers found that when 2-fluorogeranyl pyrophosphate (**15**) was incubated with IPP and hog liver prenyl transferase, a new product, 6-fluoro farnesyl pyrophosphate (**16**), was formed at a rate 8.4×10^{-4} that of the normal prenyl transfer reaction (Scheme 9). The effect was clearly due to a change in k_{cat} since the fluorinated substrate showed a Michaelis constant ($K_M = 1.1 \pm 0.2\ \mu M$) close to that of geranyl pyrophosphate ($K_m = 0.8 \pm 0.2\ \mu M$) and could act as a competitive inhibitor of the latter substrate ($K_i = 2.4 \pm 0.5\ \mu M$). Whereas bimolecular substitution of 2-fluorogeranyl chloride with cyanide took place at twice the rate of the corresponding reaction with geranyl chloride, first order solvolysis of fluorinated



Scheme 9.

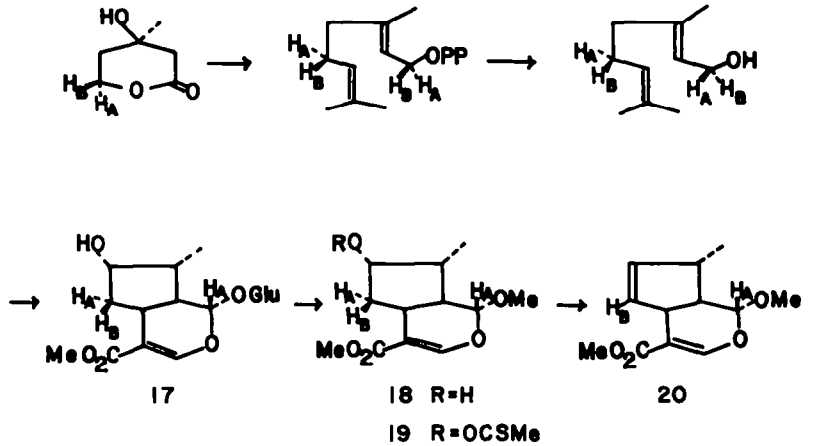
geranyl methanesulfonate occurred 4.4×10^{-3} times slower than the normal solvolysis rate. The similar retardation of both the model S_N1 reaction and the enzymatic reaction implies that the rate determining step in enzymatic prenyl transfer is largely the ionization of the allylic substrate, suggesting an overall sequence of ionization-condensation-elimination. Loss of stereochemical integrity at C(1) of the ionized allylic pyrophosphate is prevented by the barrier to rotation about the C(1)–C(2) bond of the allylic cation, estimated to be some 28 kcal,[23,24] and inversion is imposed by the enzyme itself which holds the normal cosubstrate IPP on the face opposite to that from which the pyrophosphate ion departs (Scheme 10).



Scheme 10.

Additional stereochemical data has been advanced in support of the above hypothesis. When geranyl pyrophosphate was incubated with prenyl transferase in the absence of IPP, the allylic substrate underwent hydrolysis at about 2% the rate of the normal prenyltransferase catalyzed reaction (Scheme 9). This hydrolysis was autocatalytic and required inorganic pyrophosphate for maximum activity. By using 1S-[1-^{3}H]-geranyl pyrophosphate and incubating the derived geraniol with liver alcohol dehydrogenase it was demonstrated that the hydrolysis took place with inversion of configuration at C(1). Furthermore, experiments conducted in $H_2{}^{18}O$ resulted in formation of [1-^{18}O]-geraniol, consistent with C–O bond cleavage of the pyrophosphate ester. As suggested by Poulter and Rilling, these observations indicate that cleavage of the allylic substrate does not require participation of the double bond of IPP. Metal ion promoted ionization, referred to earlier, can generate the necessary allylic cation which, in the absence of IPP, is trapped by water. The hypothesis that water is occupying the normal IPP binding site, as implied by the observed inversion of configuration accompanying hydrolysis, is supported by the requirement for inorganic pyrophosphate and the finding that 2-fluoroisopentenyl pyrophosphate, a competitive inhibitor for prenyl transferase, also inhibits the anomalous hydrolysis.

Only one other example of formation of a prenol with C–O bond cleavage has been cited in the literature. Arigoni and Escher,[25] in experiments on the biosynthesis of loganin and derived indole alkaloids by *Catharanthus roseus* found that stereospecifically tritiated samples of geraniol were incorporated into loganin (17) with complete loss of the 1-H_{re} hydrogen but retention of 1-H_{si} (Scheme 11). Surprisingly, parallel incorporations of 5R- and 5S-[5-^{3}H]-mevalonates gave the opposite result: the H atom originally 5-H_{re} of mevalonate and therefore 1-H_{re} of geranyl pyrophosphate was retained in loganin, while that from 5-H_{si} was lost. The inescapable conclusion is that the intermediate geranyl pyrophosphate must have been hydrolyzed to geraniol with inversion of configuration, prior to incorporation into loganin. The latter series of experiments also established the stereochemistry of the *C. roseus* prenyltransferase reaction. Conversion of loganin derived from 5S-[5-^{3}H]-mevalonate to 1-0-



Scheme 11.

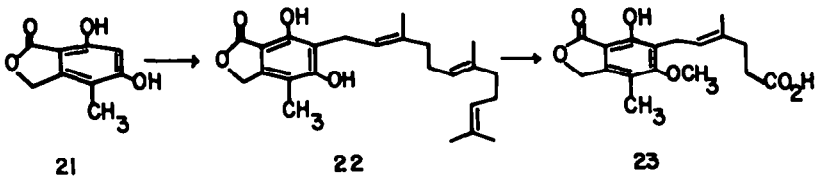
methyl-loganin aglucone (**18**) followed by pyrolytic *cis* elimination of the derived xanthate **19** gave the olefin **20** which retained one equivalent of tritium at C(6), consistent with inversion of configuration during prenyl transfer.

Aromatic prenylation

Prenylated aromatic metabolites are extremely common. Among the many examples are several hundred prenylated coumarins, flavonoids, rotenoids, phenols and quinones.[26] Only a few of these have been subjected to biosynthetic study and the results of two stereochemical investigations are summarized below.

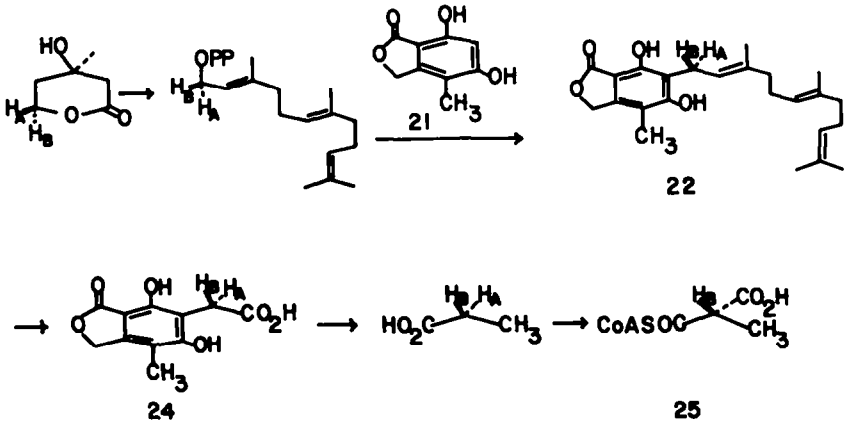
Mycophenolic acid

The biosynthesis of mycophenolic acid (**23**) by *Penicillium brevicompactum* has been shown to involve degradation of an intermediate, 6-farnesyl-5, 7-dihydroxy-4-methylphthalide (**22**)[27] (Scheme 12).



Scheme 12.

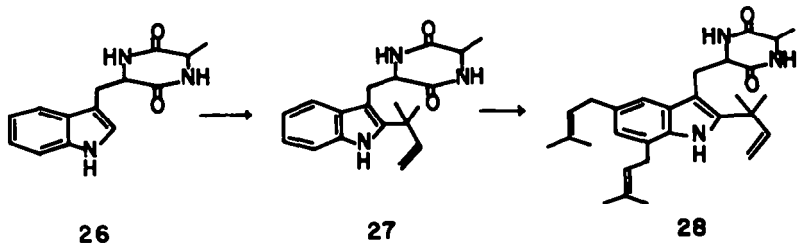
Phillips has described a cell-free system from *P. brevicompactum* which will support biosynthesis of **22** from 5,7-dihydroxy-4-methylphthalide (**21**) and either mevalonate or farnesyl pyrophosphate[28] (Scheme 13). Incubation of 5*R*-[5-^{3}H, 5-^{14}C]- and 5*S*-[5-^{3}H, 5-^{14}C]-mevalonate in separate experiments with this cell free preparation gave labeled samples of **22** which were ozonized to the carboxylic acid **24**. Reduction of the carboxylate to a Me group by way of the mesylate followed by chromic acid oxidation gave [2-^{3}H, 2-^{14}C]-propionic acid. The chirality of each propionate sample was determined by formation of the corresponding propionyl CoA ester and treatment with propionyl CoA carboxylase to give 2*S*-methylmalonyl CoA (**25**), a reaction known to involve removal of the 2-proR hydrogen of the proprionate substrate.[29] The sample of [2-^{3}H, 2-^{14}C]-propionate derived from 5*R*-[5-^{3}H]-mevalonate gave methylmalonyl CoA containing 120% of the tritium present in propionyl CoA, while the corresponding propionate from incorporation of 5*S*-[5-^{3}H]-mevalonate retained only 20% of the tritium upon conversion to methylmalonyl CoA. Since the configuration at C(1) of farnesyl pyrophosphate is identical with that at C(5) of the precursor mevalonate, the results obtained imply that C(1) of the allylic pyrophosphate undergoes inversion during the aromatic prenylation. The slight deviation from theoretical of the actual values for tritium retention may reflect some scrambling of tritium into the methyl of propionate during the reduction of the intermediate mesylate.



Scheme 13.

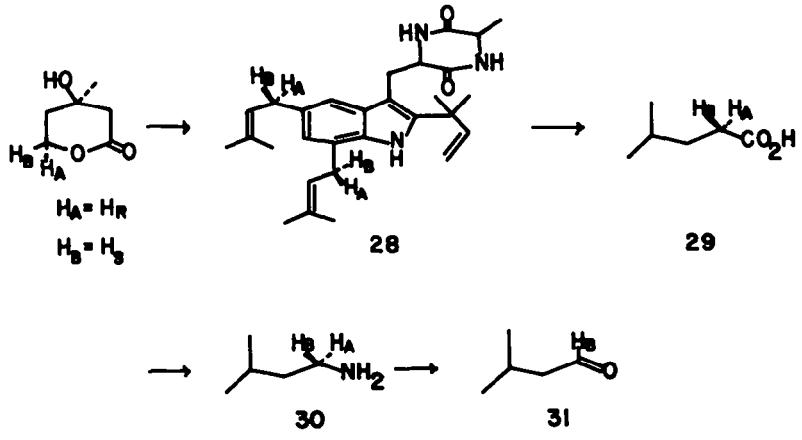
Echinulin

The fungal metabolite echinulin (**28**) is formed by prenylation of the di-ketopiperazine **26**.[30] A cell free extract of *Aspergillus amstelodami* which adds dimethylallyl pyrophosphate to **26** to give **27** by an apparent S_N2' reaction has been described (Scheme 14).[31] Incorporation of [1,2-$^{13}C_2$]-acetate into



Scheme 14.

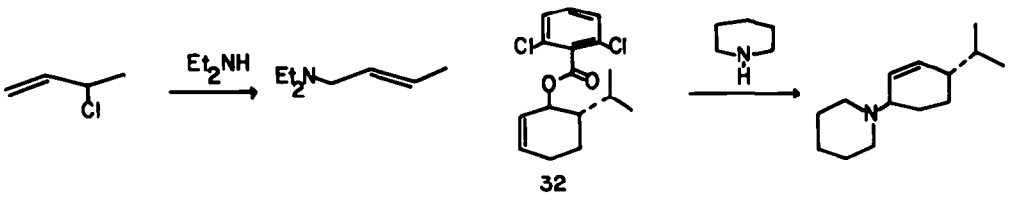
echinulin using whole cells of *A. amstelodami* established that the dimethylallyl substituents possessed the normal isoprenoid labeling, with the *E*-Me of each substituent being derived from C(2) of mevalonate.[32] In order to determine the stereochemistry of the aromatic prenylation reactions, Barrow fed both 5*R*- and 5*S*-[5-^{3}H]-mevalonate to intact cells of *A. amstelodami*[32] (Scheme 15). The derived echinulins were reduced with diimide and subjected to vigorous oxidation with fuming nitric acid to give isocaproic acid (**29**). Each isocaproate sample was degraded to the corresponding C(1)-labeled isopentylamine (**30**), which was oxidized to the isopentenal (**31**) by pea seedling diamine oxidase. It could be shown using authentic samples of 1*R*- and 1*S*-[1-^{3}H]-isopentylamine that the oxidase catalyzes removal of the 1-H_{si} atom, in accord with independent findings of Battersby on the oxidation of [1-^{3}H]-benzylamines.[33] In the oxidation of the biosynthetically derived samples of isopentyl amine, the amine derived from 5*R*-[5-^{3}H]-mevalonate lost >90% of its tritium while that from 5*S*-[5-^{3}H]-mevalonate lost <10% of the tritium. These results demonstrated once again, that prenyl transfer had occurred with inversion of configuration at the allylic center.



Scheme 15.

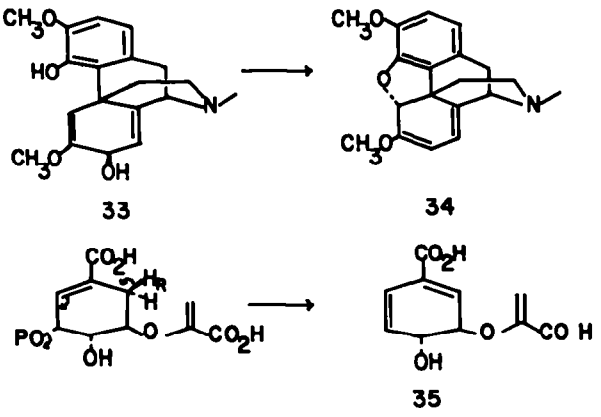
III. ALYLIC DISPLACEMENTS

Since the first suggestion by Hughes in 1938 that nucleophilic substitutions can occur at a site allylic to a leaving group,[34] the S_N2' reaction has been a source of stimulation and considerable controversy among organic chemists.[35] The first examples of S_N2' reactions were announced in 1949 by Winstein who found that α- and γ-methylallyl chlorides reacted with sodium malonate esters or diethylamine to give rearranged products by a second order kinetic process.[36] In 1953 Stork and White reported that the 2,6-dichlorobenzoate ester of *trans*-6-isopropyl-2-cyclohexen-1-ol (**32**) underwent displacement by both piperidine and malonate ion with exclusive *syn* stereochemistry[37] (Scheme 16). This paper was enormously influential and led to the nearly universal belief over the next twenty years that the *syn* mode was an inherent property of the S_N2' reaction. This influence can be seen, for example in contemporary



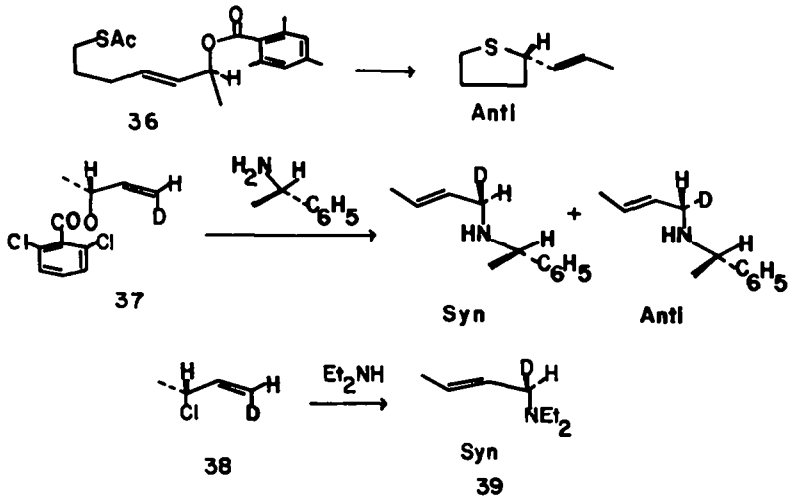
Scheme 16.

interpretations put forward to account for the stereochemistry of two biosynthetic processes (Scheme 17). In the course of investigating the biosynthesis of morphine, Barton established that thebaine (34) was formed by cyclization of salutaridinol I (33) which he determined to be the *R*-alcohol.[38] In order to explain this apparent *anti* allylic displacement it was felt necessary to postulate either prior allylic rearrangement of the substrate followed by S_N2 displacement or participation of an enzyme X-group by S_N2 reaction at C(7), followed by *syn* S_N2' displacement. Similarly the finding that 1,4 elimination of



Scheme 17.

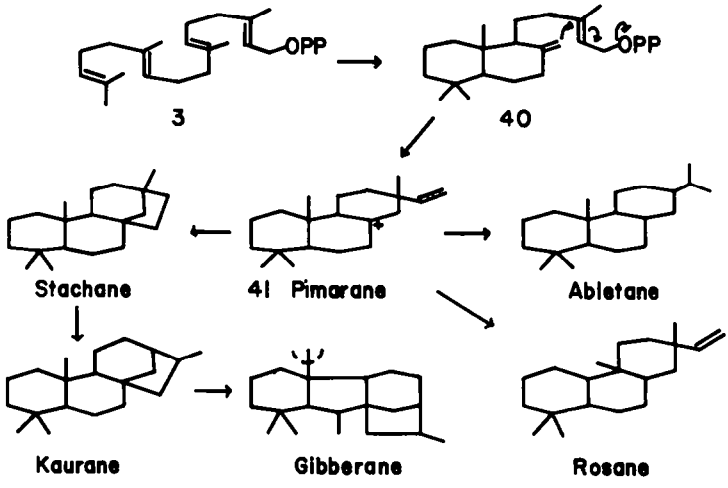
phosphate by chorismate (35) synthetase took place with *anti* stereochemistry[39] was rationalized by hypothesizing initial *syn* S_N2' displacement of phosphate, again by an enzyme X-group, followed by anti 1,2-elimination. Within the last three years new information has become available concerning the stereochemistry of the S_N2' reaction. Using modern analytical techniques Stork has reconfirmed his original finding of *syn* stereochemistry in the reaction of cyclohexenyl benzoates with piperidine.[40] On the other hand sulfide nucleophiles gave varying proportions of *syn* and *anti* products and the use of an acyclic substrate 36 resulted in predominant *anti* stereochemistry (Scheme 18). Whereas Overton found that reaction of (*R*) or (*S*)-α-methyl[γ-^{2}H]allyl or α-n-pentyl[γ-^{2}H] allyl 2,6-dichlorobenzoates (37) with (*R*) or (*S*)-phenethylamine gave S_N2' products in which the ratio of *syn* to *anti* displacement was only



Scheme 18.

1.4–1.8,[41] Magid found that reaction of the analogous deuterated methylallyl chloride (38) with diethylamine gave exclusively *syn* S_N2' product 39.[42] Against this background are a number of theoretical arguments which purport to rationalize, if not predict, the *syn* or *anti* course of the S_N2' reaction.[43] The concept of the S_N2' reaction itself has been strongly critized by Bordwell who has argued that both the stereochemical and kinetic properties of these reactions are satisfactorily accounted for by intimate ion pair mechanisms.[44]

Formal S_N2' or S_N1' reactions have been proposed to account for the biosynthesis of a significant number of natural products. The currently accepted hypothesis for the biogenesis of most tricyclic and tetracyclic diterpenes involves electrophilic cyclization of geranylgeranyl pyrophosphate (3) to give the bicyclic labda-8(17), 13-dien-15-yl pyrophosphate (40)[45] (Scheme 19). A second cyclization by allylic displacement of the terminal pyrophosphate generates ring C and the key pimarenyl cation 41. Subsequent hydride shifts, methyl migrations, olefin alkylations, and deprotonations can account for the formation of the majority of diterpene skeletons. Strong experimental support for this hypothesis has been obtained in a number of cases, and some of these studies are referred to in the discussion which follows. Only in the last 2 years has the stereochemistry of the allylic pyrophosphate displacements been determined.

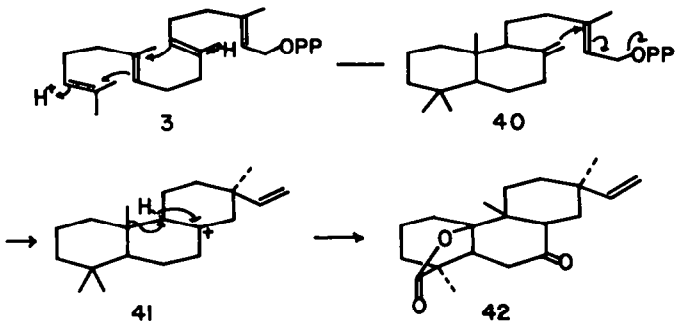


Scheme 19.

Biosynthesis of rosenonolactone

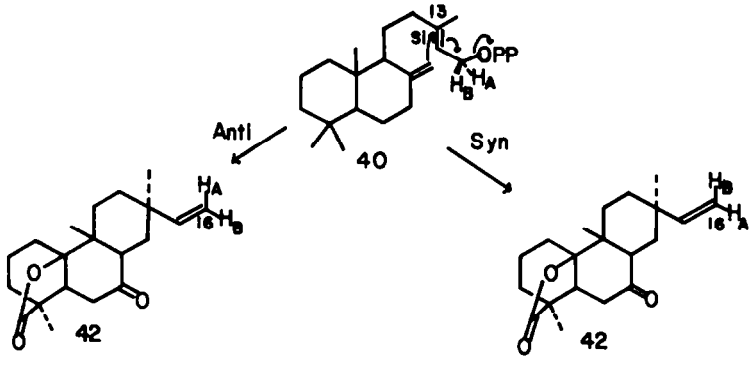
The isolation of rosenonolactone (42), the major mycelial constituent of the apple mold *Trichothecium roseum*, was first reported in 1949.[46] Some 10 years later two groups reported the structure based on extensive chemical degradation.[47] The absolute stereochemistry was defined shortly thereafter by ORD and CD methods[48] and by a complete X-ray crystal analysis of dibromorosenonolactone.[49] In 1969 Overton reported a bigenetically modelled synthesis of rosenonolactone beginning with isocupresic acid.[50]

Rosenonolactone was one of the first non-steroidal terpenoid metabolites whose biosynthesis was studied in detail. Studies by Arigoni[47] and Birch[48] and later by Hanson[51] have led to a rather complete picture of the rearrangements leading to the rosane skeleton, and the hydride and methyl migrations which accompany formation of ring C have been documented by careful labeling studies (Scheme 20).



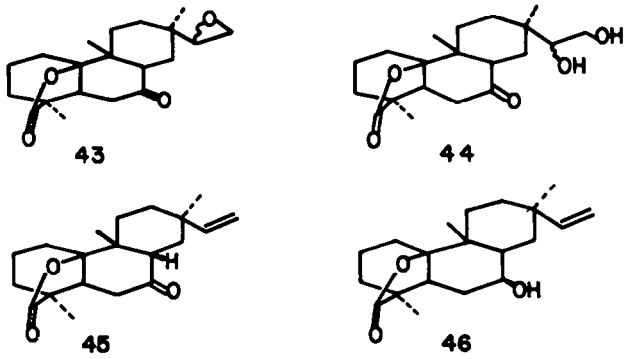
Scheme 20.

From the known absolute configuration of rosenonolactone, it follows that during the allylic displacement by which ring C is formed, cyclization occurs on the *si* face of the 13,14 double bond of **40**. In order to determine in which sense, *syn* or *anti*, the pyrophosphate departs, we had to determine which of the prochiral hydrogens at C(16) of **40** becomes cis(H-16Z) and which becomes *trans*(H-16E) to the C–C bond in the terminal vinyl group of rosenonolactone[52] (Scheme 21). The solution of this problem was achieved using a powerful new spectroscopic method, ^{2}H NMR.[53] This technique has a number of attractive features. (1) The natural abundance of deuterium is only 0.015%. An enrichment with deuterium of only 1% therefore leads to a sixty-fold enhancement of the natural abundance signal. (2) Shielding of deuterium is the same as for protons in a given compound. Shift assignments may therefore be made from the ^{1}H NMR spectrum. (3) In proton-decoupled ^{2}H NMR there is no NOE due to the dominant intraatomic quadrupole relaxation mechanism. For the same reason relaxation times T_1 are relatively short (usually 0.1–1 s), minimizing the possibility of partial saturation. Both these factors make integration of deuterium spectra meaningful. (4) The direct determination of the positions of labeling makes laborious degradative sequences unnecessary. On the other hand the somewhat broad signals (4–8 Hz at half-height for deuterons in substrates MW 200–500) and the reduced field width (1/6.5 that of the corresponding proton spectrum at a given magnetic field), require high-field spectrometers for sensitive applications.



Scheme 21.

Analysis of the 270-MHz ^{1}H NMR of rosenonolactone allowed the assignment of the vinyl protons which appeared as an ABX pattern: H-16Z = 4.97 ppm, H-16E = 4.90 ppm, H-15 = 5.80 ppm ($J_{H\text{-}16Z\text{-}H\text{-}16E}$ = 1.0 Hz, $J_{H\text{-}16Z\text{-}H\text{-}15}$ = 17.5 Hz, $J_{H\text{-}16E\text{-}H\text{-}15}$ = 10.6 Hz). In an attempt to improve the separation of the protons at C(16), rosenonolactone was converted to a mixture of diastereometric epoxides (**43**), whose NMR spectrum in the region of interest was unfortunately no longer first order. Furthermore, while the protons of the corresponding diol **44** were well resolved, the signals could not be unambiguously assigned. Alternatively, formation of isorosenonolactone (**45**) led to no improvement in the vinyl region of the spectrum, and a europium shifted spectrum of rosenololactone (**46**) gave no better resolution. ^{2}H NMR analysis was therefore confined to the parent rosenonolactone.



In the biosynthetic experiment, a series of specifically deuterated substrates, sodium[5-$^2H_2$]-mevalonate, 5*R*-[5-^{2}H]-mevalonate, and 5*S*-[5-^{2}H]-mevalonate, was fed to cultures of *T. roseum* and the resulting labeled rosenonolactone isolated and purified (Scheme 22). Parallel incorporation of a [2-^{14}C]-mevalonate internal standard indicated enrichments of 3–6% deuterium at each labeled site in the

diterpene, and a control in which incorporation of [2-^{14}C, 5-$^{3}H_2$]-mevalonate (^{3}H/^{14}C, 3.15) gave rosenonolactone (^{3}H/^{14}C, 2.98) indicated no loss of label from C(5) of mevalonate. Each of the biosynthetically deuterated samples of rosenonolactone was analyzed by 41.44-MHz ^{2}H NMR (Fig. 1). The rosenonolactone (**42A**) derived from feeding [5-$^{2}H_2$]-mevalonate showed a signal at 4.97 ppm with an upfield shoulder at ~4.90 ppm, identical with the pattern of authentic specimens of [16-$^{2}H_2$]-or a mixture of *cis* and *trans*-[16-^{2}H]-rosenonolactone (**42D**). The terminal methylene signals which were separated by only 0.07 ppm (3 Hz) were therefore not clearly resolved. Rosenonolactone (**42B**), derived from 5*R*-[5-^{2}H]-mevalonate exhibited a signal at 5.01 ppm ($\nu_{1/2} = 3.5$ Hz) while **42C** (from 5*S*-[5-^{2}H]-mevalonate) gave rise to a signal at 4.92 ppm ($\nu_{1/2} = 7.5$ Hz). The positions of the observed signals in each spectrum were confirmed by doping each sample with ~1/3 part of the *cis*, *trans*-[16-^{2}H]-rosenonolactone. The resulting spectra from **42B** showed an upfield tail, while that from **42C** had maxima at 4.98 and 4.93. It could also be shown that the broad signal observed for **42A** corresponded to the superposition of a

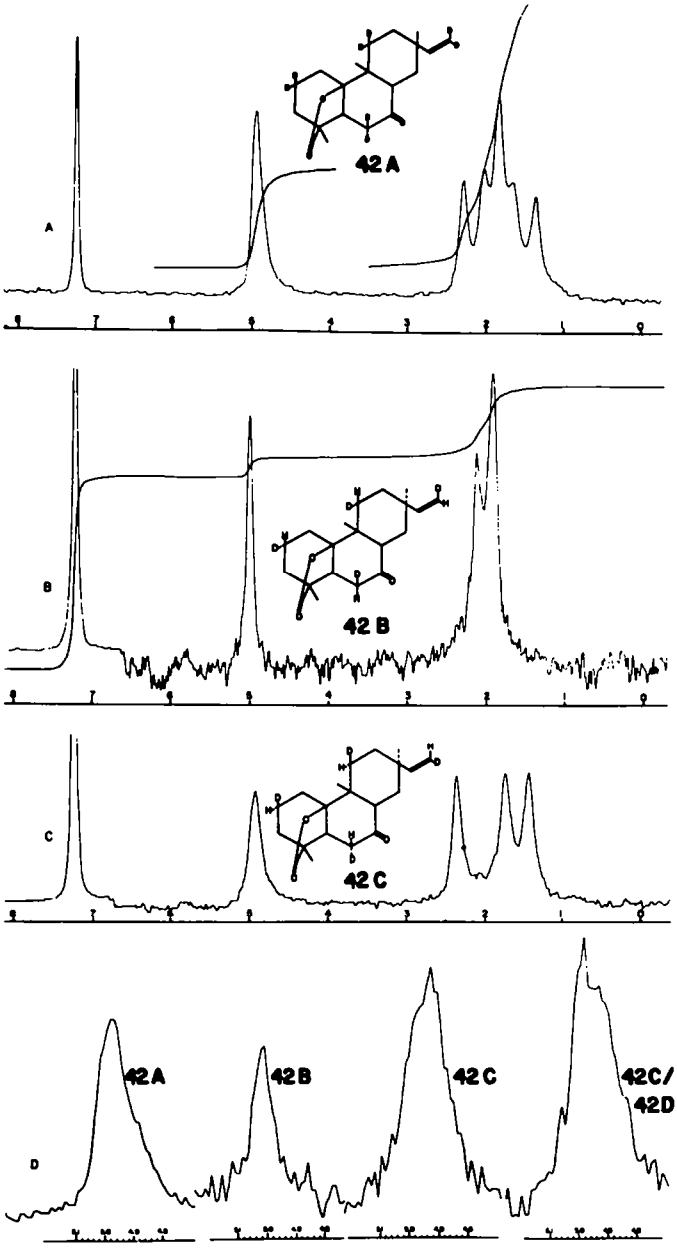
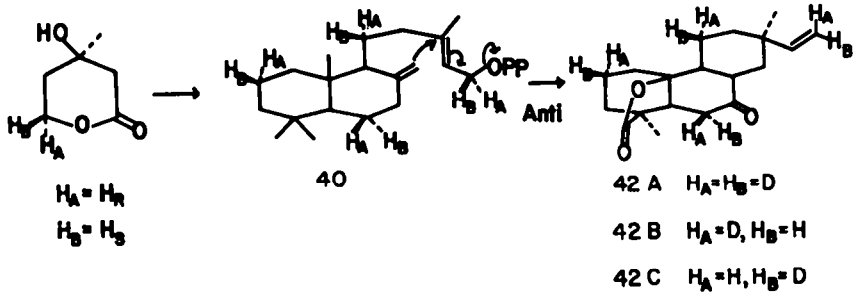


Fig. 1. Proton decoupled ^{2}H NMR spectra of labeled **42**: A, 0.13 mmol of **42A** (from feeding of [5-$^{2}H_2$]-mevalonate), 7030 transients, line broadening (LB) = 0.5 Hz; B, 0.032 mmol of **42B** (from feeding of (5R)-[^{2}H]-mevalonate), 27970 transients, LB = 0.5 Hz; C, 0.085 mmol of **42C** (from feeding of (5*S*)-[^{2}H]-mevalonate), 6708 transients, LB = 0.5 Hz; D, expanded spectra of olefinic regions of deuterated rosenonolactone samples, including mixture of **42C** and ~30% **42D**.



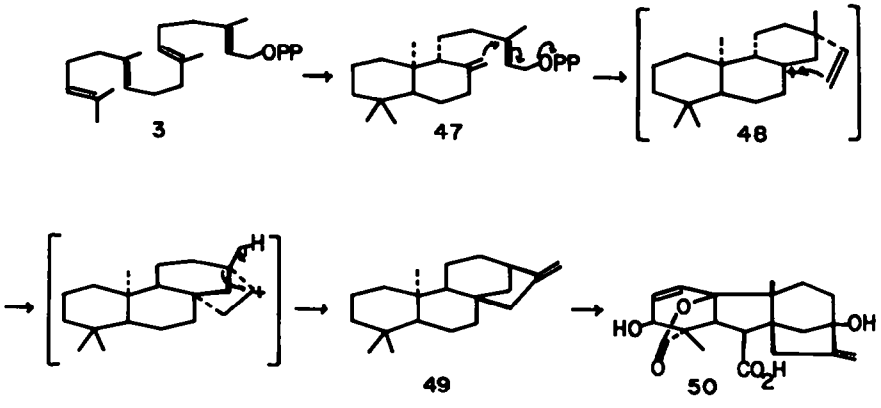
Scheme 22.

narrow downfield signal and a broader upfield signal, consistent with these assignments. These observations therefore established that 5-H_{re} of mevalonate became the 16Z H atom of rosenonolactone. Conversely the 16E hydrogen of **42** was derived from 5-H_{si} of mevalonate. These results, taken together with the known direction of attack on the 13,14 double bond of **40**, established that the allylic displacement which generates ring C of rosenonolactone takes place with overall *anti* stereochemistry. This ^2H NMR study was, in fact, the first explicit determination of the stereochemistry of an enzyme catalyzed allylic displacement of a pyrophosphate.

Analysis of the ^2H NMR spectra of the various biosynthetic rosenonolactone samples also provided additional information on the stereochemistry of the prenyltransferase reactions by which the precursor geranylgeranyl pyrophosphate is generated. The hydrogens at C(6) of rosenonolactone are also derived from C(5) of mevalonate by way of C(1) of geranyl pyrophosphate. The spectrum of **42B**, derived from 5*R*-[^2H]-mevalonate, displayed a signal at 2.13 ppm, previously assigned in the proton spectrum to H-6β, while the spectrum of **42C** had a corresponding signal at 2.37 ppm (H-6α). The labeling pattern in each case is consistent with inversion of configuration in the prenyltransfer reaction. Similarly, if one makes the reasonable, if unproven, assumption that of the signals corresponding to deuterium at C(2) and C(11) it is the higher field peak within each pair which corresponds to the axial position,[54] then 5*R*-[^2H]-mevalonate is seen to label the C(2α) and C(11α) positions and 5*S*-[^2H]-mevalonate the C(2β) and C(11β) positions, again consistent with the usual finding of inversion of configuration.

Kaurene and the gibberellins

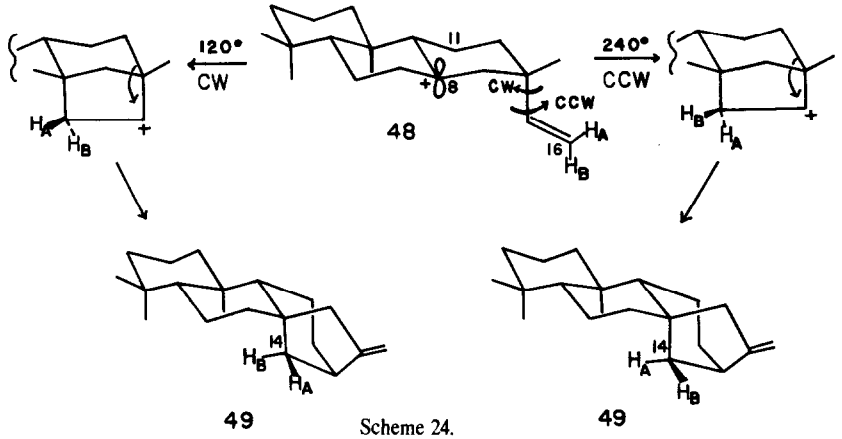
Since the isolation and structure of elucidation of gibberellic acid (**50**) from the rice pathogen *Gibberella fujikuroi*, more than fifty members of this important class of plant growth hormones have been identified from both fungal and higher plant sources.[55] As a result of extensive investigations in several laboratories, the biosynthetic pathway for these diterpenes is now known in intimate detail (Scheme 23). Cyclization of geranylgeranyl pyrophosphate (**3**) gives a bicyclic intermediate, copalyl pyrophosphate (**47**), which is enantiomeric to the corresponding labdadiene intermediate **40** of rosenonolactone biosynthesis. Allylic displacement of pyrophosphate generates a cation at C(8) which adds to the newly formed vinyl double bond. Wagner–Meerwein rearrangement and deprotonation produce the tetracyclic hydrocarbon *ent*-kaurene (**49**). Subsequent ring contraction, demethylation, and oxidations then produce the several gibberellins by a sequence which is now reasonably well understood.



Scheme 23.

West has isolated and purified 170-fold the kaurene synthetase from *G. fujikuroi* which catalyzes the two step conversion of geranylgeranyl pyrophosphate to *ent*-kaurene.[56] While it was not possible to separate the geranylgeranyl pyrophosphate cyclizing (A) activity, from the *ent*-kaurene synthesizing (B) activity, inhibition and pH optimum experiments suggested a distinct active site for each activity. Kaurene synthetase has also been obtained from immature seeds or seedlings of several higher plants, including *Ricinis communis* (castor bean),[57] *Cucurbita maxima* (pumpkin),[58] and *Marah macrocarpus* (formerly *Echinocystis macrocarpa*, wild cucumber).[59]

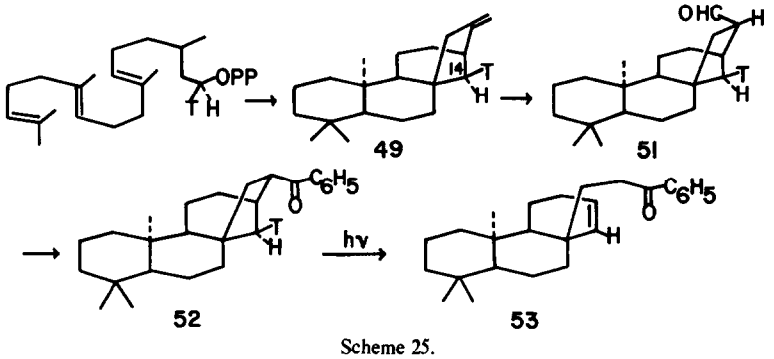
Determination of the steric course of allylic displacement associated with kaurene formation suffers from an inherent ambiguity since the transiently generated vinyl group, which would carry any stereochemical information, is further transformed by attachment to C(8) of the pimarenyl cation **48** (Scheme 24). In order to relate the stereochemistry of the newly formed sp^3 center at C(14) of



Scheme 24.

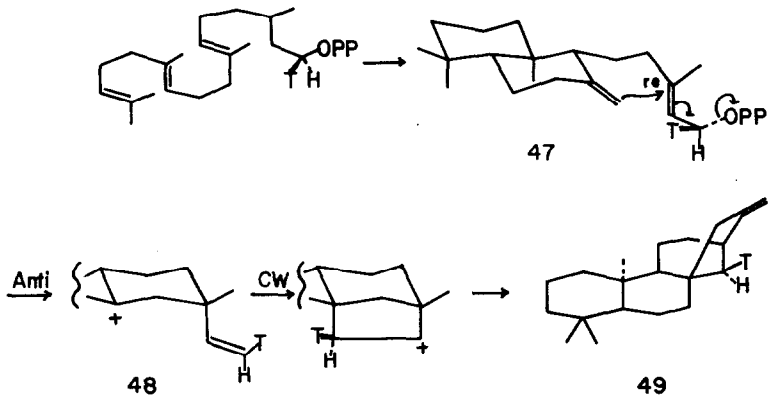
ent-kaurene to the corresponding sp^2 C(16) carbon of the transient pimarenyl cation, one must first know which face, *re* or *si*, of the vinyl group is involved in formation of the new bond. Conversely, one can only obtain the latter information if the stereochemistry of labeling at C(16) of the pimarenyl cation is known, which in turn requires a knowledge of the *syn* or *anti* course of the allylic displacement by which this intermediate is itself generated. It is possible, however, to make a provisional interpretation of the net stereochemistry of the copalyl pyrophosphate to *ent*-kaurene transformation. Inspection of the pimarenyl intermediate indicates that in order for further ring closure to occur, the vinyl group must rotate through 120° in a clockwise sense or 240° in a counterclockwise sense in order to approach within bonding distance of the C(8) cation. The two modes are distinguished by which face of the terminal vinyl carbon becomes attached to C(8). Although models indicate either bonding geometry might be feasible, it can provisionally be assumed that the 120° rotation which involves the *least motion* at the active site is favored. This clockwise motion also avoids unfavorable steric interaction with H(11α). By applying these assumptions one can then interpret the results obtained by two research groups who have independently studied the enzymatic formation of *ent*-kaurene.

Coates *et al.*[60] have prepared 1*S*-[1-^{3}H]-geranylgeraniol by enzymic reduction of [1-^{3}H]-geranylgeranial with liver alcohol dehydrogenase and an NADH recycling system consisting of NAD^+ and excess ethanol.[61] The corresponding pyrophosphate was incubated with a cell-free preparation of *M. macrocarpa* enzymes[59] to give tritiated *ent*-kaurene (Scheme 25) which was diluted with inactive carrier and



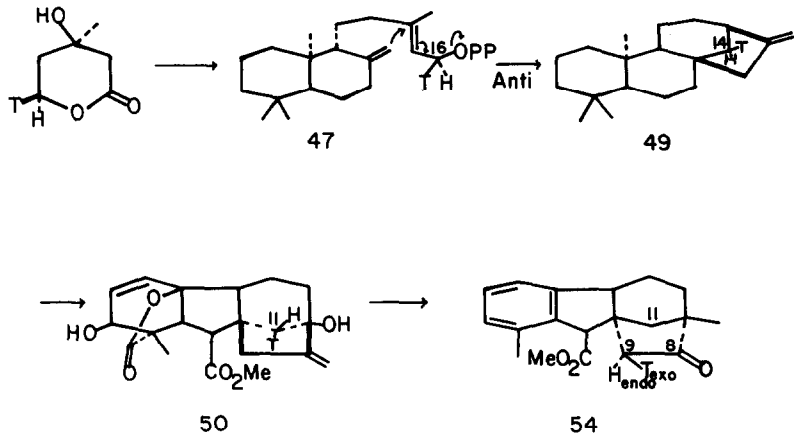
Scheme 25.

recrystallized to constant specific activity. A parallel experiment was carried out with 1*R*, *S*-[1-^{3}H]-geranylgeranyl pyrophosphate. In order to establish the stereochemistry of tritium labeling at C(14), each of the labeled kaurene samples was treated with perbenzoic acid and the resulting epoxide exposed to boron trifluoride etherate to give the corresponding *endo*-carboxaldehyde **51**. Treatment with phenyl Grignard reagent followed by Collins oxidation and base-catalyzed epimerization gave the *exo*-phenyl ketone **52** which was recrystallized to constant specific activity. Upon photolysis in t-butanol the phenyl ketone underwent a Norrish type II cleavage with abstraction of the 14-H syn to the ethano bridge and formation of the tricyclic phenyl ketone **53** in 13% yield. Whereas starting with *ent*-kaurene derived from 1*R*, *S*-[1-^{3}H]-geranylgeranyl pyrophosphate, 55% of the tritium activity remained in the recrystallized semicarbazone of **53**, the same sequence carried out on the *ent*-kaurene from 1*S*-[1-^{3}H]-geranylgeranyl pyrophosphate led to recovery of only 0.8% of the tritium specific activity. The presence of all tritium derived from 1*S*-[1-^{3}H]-geranylgeranyl pyrophosphate in the 14β position of *ent*-kaurene corresponds to a net inversion of configuration at the original pyrophosphate bearing carbon (Scheme 26). If one considers the known absolute configuration of *ent*-kaurene and applies the above discussed assumption of least motion of the transiently generated vinyl group, the results are consistent with an *anti* allylic displacement of pyrophosphate. Of course the opposite conclusion would apply to counterclockwise vinyl rotation.



Scheme 26.

A similar result was obtained by Hanson *et al.* who examined the biosynthesis of gibberellic acid from 5*S*-[5-^{3}H]-mevalonate[62] (Scheme 27). Using a cell-free cell system (−)-*ent*-kaurene (**49**) was prepared from 5*S*-[5-^{3}H, 2-^{14}C]- mevalonate. In order to determine the labeling stereochemistry at C(14), the kaurene was fed to intact cells of *G. fujikuroi* by which it was converted to gibberellic acid (**50**). Treatment of the methyl ester of this gibberellic acid (^{3}H/^{14}C 3.79) with refluxing 2N HCl gave the rearrangement product methyl gibberate (**54**) (^{3}H/^{14}C 2.78) which had lost one tritium. It could be shown that tritium loss was due to acid-catalyzed exchange at C(9) which accompanies formation of **54**. Thus when a sample of [11-^{3}H$_2$]-gibberellic acid, biosynthesized from [16-^{3}H$_2$]-copalyl pyrophosphate, was

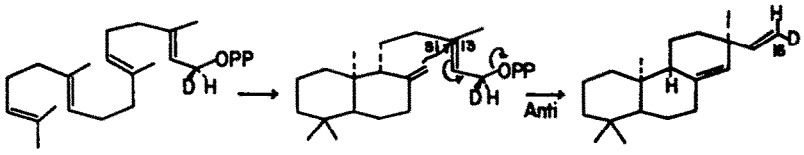


Scheme 27.

subjected to a similar sequence of reactions, the derived methyl gibberate contained only 46% of the initial tritium activity The proton which was lost during this exchange must therefore originate at the 5-H_{si} position of mevalonate. The stereochemistry of the exchange was determined in the following manner. The two proton signal from the H-9 H atoms in the NMR spectrum of **54** appeared at δ2.66 and 2.46 as an AB quartet. The upfield signal also showed an extra 3.0 Hz splitting which was ascribed to a long range *W*-coupling, presumably to the corresponding proton at C(11). The upfield resonance was therefore assigned to the 9-*endo* H atom, by analogy with the presence of similar couplings in simpler bicyclo [2.2.1] heptanes. Additional confirmation of the shift assignments was obtained from the $Eu(fod)_3$ shifted spectrum of the derived C(8) *endo* alcohol. It was then found that both base and acid catalyzed exchange in deuterated media to loss of the signal for the low field proton of **54** and appearance of a broad singlet corresponding to the *endo*-H atom. It is therefore the *exo*-proton which undergoes preferential exchange, as is also observed for both camphor and isofenchone. Since the exchangeable C(9) *exo*-proton of **54** corresponds to H-11β of gibberellic acid and H-14β of *ent*-kaurene, these protons must ultimately be derived from 5-H_{si} of mevalonate. Hanson's results are identical, therefore, to those of Coates, obtained several years later by the entirely different approach described above. Although not explicitly stated at the time, the labeling stereochemistry is consistent with an *anti* allylic displacement if the least motion assumption discussed earlier is applied.

Sandarocopimaradiene

Coates and Drengler have also studied the formation of a second copalyl pyrophosphate-derived diterpene, sandarocopimaradiene (**55**), using a soluble enzyme extract from castor bean seedlings (*Ricinus communis*).[60a] (Scheme 27A) The stereochemistry of the angular vinyl group of **55** indicates that the allylic displacement which generates ring C has taken place on the *si* face of the 13,14 double bond of **47**, in contrast to the *re* attack at this site involved in the formation of kaurene. By incubating 1*S*-[1-^{2}H]-geranylgeranyl pyrophosphate and analyzing the resulting sandarocopimaradiene by 220 MHz ^{1}H NMR, it was found that the signal corresponding to H-16E was no longer evident at 4.98 ppm, while the signal for H-16Z appeared as a doublet (J = 17.3 Hz) at 5.04 ppm coupled to the H-15 doublet at 5.87 ppm. These results are consistent only with *anti* stereochemistry for the allylic displacement. The fact that formation of the rosane, kaurane, and pimarane skeletons all involve *anti* allylic displacements, independent of the configuration of either the precursor or the products, suggests that the observed *anti* stereochemistry is an inherent property of the enzyme-catalyzed reaction.

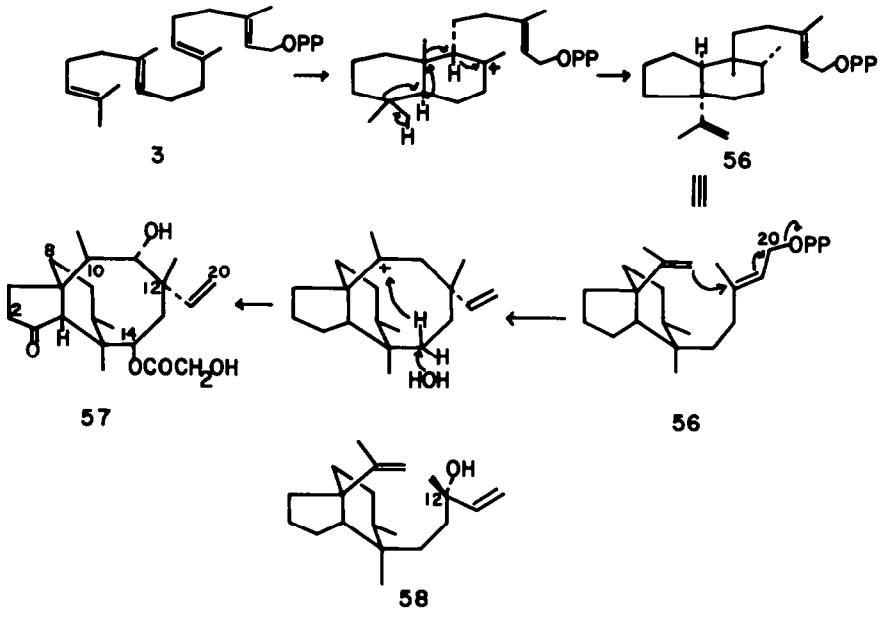


47 55

Scheme 27A.

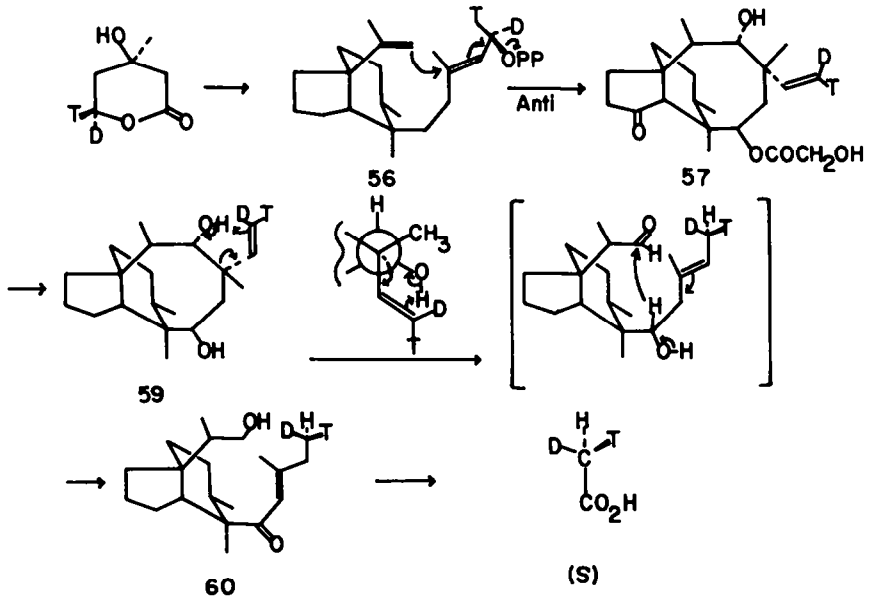
Pleuromutilin

The stereochemistry of one additional allylic displacement has been established, and although again for a diterpene, in this case one whose structure and detailed biogenesis differs considerably from the diterpenes just discussed. As a result of the work of Arigoni *et al.*, our knowledge of the biosynthesis of the *Pleurotus mutilus* metabolite pleuromutilin (**57**) rests on especially firm ground[63] (Scheme 28). Thus geranylgeranyl pyrophosphate undergoes proton initiated cyclization to produce a labdenyl cation diastereomeric to both the corresponding intermediates of rosane and kaurane biosynthesis. Sucessive migration of a hydride, methyl, and hydride, followed by ring contraction and deprotonation generates the bicyclic intermediate **56**. Formal S_N2' displacement of the allylic pyrophosphate by the double bond of the isopropenyl side chain then closes the 8-membered ring with formation of the angular vinyl group and generation of a cation which is quenched by a transanular hydride shift and addition of water. Subsequent oxidation and formation of the glycolate ester complete the biosynthesis. The various hydride shifts and carbon skeletal migrations have all been thoroughly documented by elegant labeling and degradative studies. Recently direct confirmation of the second cyclization step has been obtained by incorporation of [20-$^3H_2$]-**56**, chemically synthesized from a sample of the tertiary allylic alcohol **58** which could be isolated from culture extracts of *P. mutilus*.[64]



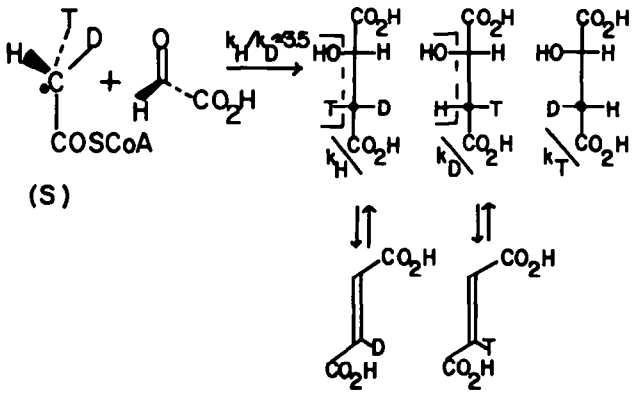
Scheme 28.

The stereochemistry of the allylic pyrophosphate displacement has now been established by Arigoni and Hasler using two independent methods.[64] In the first approach a sample of 5*S*[5-^{2}H, ^{3}H]-mevalonate was fed to a culture of *P. mutilus* (Scheme 29). The stereochemistry of labeling in the vinyl group of the resultant pleuromutilin of course depends on the *syn* or *anti* geometry of the key cyclization step.



Scheme 29.

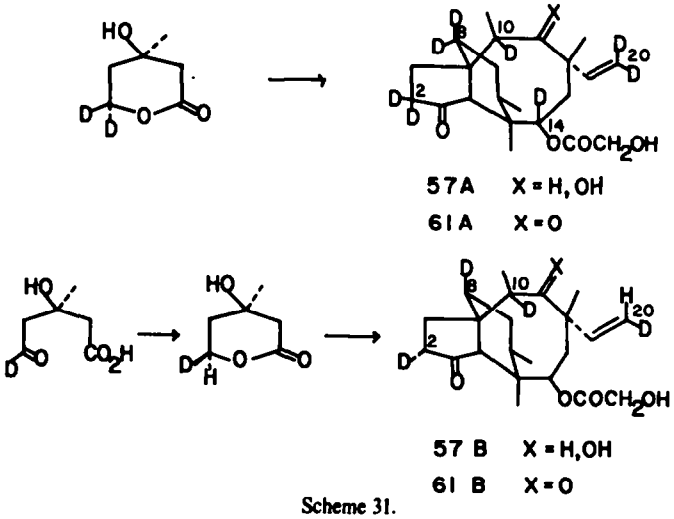
Hydrolysis of this labeled pleuromutilin sample followed by a novel electrochemical reduction gave the diol **59** which was subjected to pyrolysis at 225° to produce the hydroxyenone **60** in good yield. This latter transformation involved an initial retro *ene* cleavage of the homoallylic alcohol, followed by redox hydride transfer and movement of the new double bond into conjugation with the ketone. Examination of models indicates that in the fragmentation reaction hydrogen transfer can only take place from a quasi-chair conformation and to only one face of the vinyl double bond. Kuhn–Roth degradation of the hydroxyenone **60** served to carve out the labeled Me group as acetic acid in which the Me carbon is chiral by virtue of substitution with tritium, deuterium, and protium. Elegant procedures for determining the sense of chirality of any given acetate sample have previously been developed by both Cornforth and Arigoni[65] (Scheme 30). In this methood of analysis, the chiral acetate is first converted to 2*S*-malate



Scheme 30.

by condensation with glyoxylate under the action of malate synthetase. The resulting malate is of course a mixture of three isotopically labeled species generated by the respective loss of protium, deuterium, or tritium. The proportions of these three species depend on both the stereochemistry of the condensation with glyoxylate, which occurs with inversion, and the intramolecular primary isotope effect, $k_H/k_D \simeq 3.5$. Subsequent incubation with fumarase washes out 3-H_{re} of each malate sample. Beginning with authentic *R* and *S* acetates, fumarase incubation has been shown to result in retention of 78% and 22% of the tritium, respectively, in the reisolated malate samples. When the acetate obtained from the above described degradation of pleuromutilin was subjected to the standard analysis, a value of 34% retention of tritium after fumarase treatment was obtained, corresponding to 2*S*-[2-^{2}H, ^{3}H]-acetate. The substantial optical purity of the derived acetate confirmed the anticipated stereospecificity of the retro-*ene* reaction, and since the Kuhn–Roth degradation did not affect the chirality of the acetate Me, it can be inferred that the labeled pleuromutilin bore its tritium and deuterium respectively *trans* and *cis* to C(12). The established labeling is therefore consistent only with an *anti* allylic displacement of pyrophosphate. Interestingly these conclusions are independent of a knowledge of the absolute configuration of pleuromutilin. In principle there exist two enantiomeric *anti* transition states, each corresponding to a given antipode of pleuromutilin, and diastereomeric to the corresponding *syn* transition states. Although *ent*-pleuromutilin from 5*S*-[5-^{2}H, ^{3}H]-mevalonate would carry tritium and deuterium respectively *cis* and *trans* on its vinyl group, the corresponding retro-*ene* reaction would necessarily involve proton transfer to the opposite face of the vinyl double bond, resulting in exclusive formation of S-acetate.

The second experimental approach used by Arigoni and Hasler was based on ^{2}H NMR (Scheme 31). Feeding of [5-$^2H_2$]-mevalonate gave deuterated pleuromutilin (**57A**) which was oxidized to the corresponding 11-ketone **61A** for which the 61.4 MHz ^{2}H NMR was recorded (Fig. 2). The resulting spectrum displayed well resolved signals at 5.39 and 5.08 ppm, previously assigned to the 20-*trans* and 20-*cis* H atoms. Also present as expected, were signals at 6.02 ppm (D(14)) and 3.28 ppm (D(10)). Prior in-



Scheme 31.

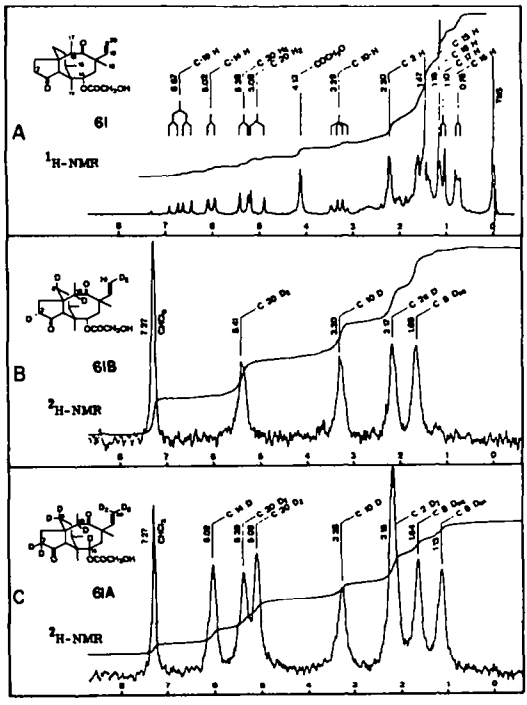
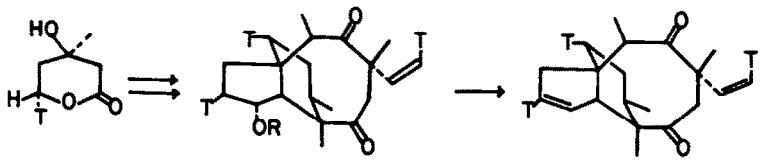


Fig. 2. Proton and deuterium NMR spectra of **61**: A, ^{1}H NMR (60 MHz) of **61**; B, ^{2}H NMR (61.4 MHz) of **61B** (from feeding [5-^{2}H]-mevaldate); C, ^{2}H NMR (61.4 MHz) of **61A** (from feeding [5-$^2H_2$]-mevalonate); spectra reproduced from ref. 64 by permission of Dr. H. Hasler.

incorporation experiments with tritiated mevalonates,[63] referred to earlier, had firmly established that the isotope at C(10) originates at the 5-H_{si}-position of mevalonate, while that at C(14) corresponds to the 5-H_{re}. The ^{2}H NMR spectrum also contained a two-deuterium signal at 2.16 ppm (2D(2)) and singlets at 1.64 and 1.13 ppm, provisionally assigned as $D(8)_{eq}$ and $D(8)_{ax}$, respectively. The requisite stereospecifically deuterated mevalonate was then obtained by a particularly ingenious device (Scheme 31). Feeding of a sample of [5-^{2}H]-mevaldic acid gave pleuromutilin (**57B**) which was enriched at the unusually high level of 7% deuterium per labeled site, as determined by measurement of internal standard ^{14}C. Furthermore conversion of the pleuromutilin as before to the 14-ketone (**61B**) and analysis of the resulting ^{2}H NMR spectrum revealed that the signal corresponding to D(10) had been enhanced while that derived from D(14) was completely absent, thereby establishing that the *in situ* microbial reduction of mevaldate had yielded exclusively 5*S*-[5-^{2}H]-mevalonate. This result is all the more impressive when one realizes that the exogeneously administered mevaldate was potentially a substrate for two enzymes of opposite stereospecificity, *P. mutilus* mevaldate reductase (which apparently is responsible for the observed product) and HMG-CoA reductase (which would have produced the opposite configurational isomer). The ^{2}H NMR also displayed a signal at 5.41 ppm, derived from D(20)-*trans*, while no deuterium was apparent at 5.08 ppm. If one takes into account the absolute configuration of pleuromutilin, previously assigned by chiroptical methods,[66] then the finding that 5*S*-[5-^{2}H]-mevalonate exclusively labels D(20)-*trans* establishes that the intervening allylic displacement of pyrophosphate takes place with *anti* stereochemistry . On the other hand, if one considers the independent assignment of *anti* stereochemistry deduced from chiral methyl analysis, it is amusing to note that the ^{2}H NMR experiment actually unambiguously establishes the absolute configuration of pleuromutilin!

One further piece of information could be extracted from analysis of the ^{2}H NMR spectrum of the 5*S*-[5-^{2}H]-mevalonate-derived pleuromutilin. The presence of a signal at 1.65 ppm, assumed to correspond to $D(8)_{eq}$, is consistent with inversion of configuration at the corresponding carbon in the formation of the precursor geranylgeranyl pyrophosphate. A similar conclusion had already been drawn for carbon (2) as a result of earlier, radioisotopic experiments[63] (Scheme 32). In this latter investigation pleuromutilin, obtained after incorporation of 5*R*-[5-^{3}H]-mevalonate, was converted to the diketo-alcohol **62**. Benzoylation and pyrolytic *cis* elimination gave the dehydration product **64** which retained all the tritium activity of the precursor. The tritium from 5*R*-[5-^{3}H]-mevalonate must therefore occupy



62 R = H
63 R = C_6H_5CO

64

Scheme 32.

the H(2β) position, and this is indeed the stereochemistry expected on the basis of an inversion of configuration at this center during isoprenoid chain extension.

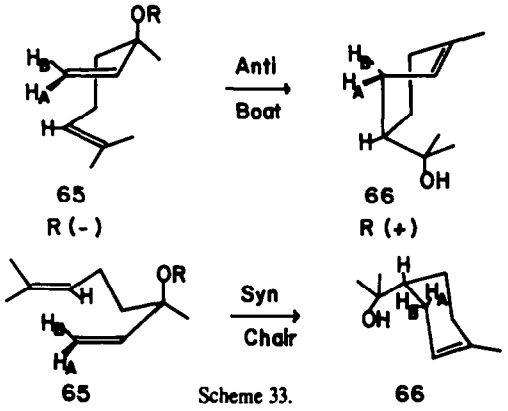
From the observed preference for *anti* stereochemistry in the four biological allylic displacements already discussed, it is now possible to draw a number of preliminary conclusions. The possibility that these reactions are occurring by a two step process involving *syn* allylic rearrangement (see Section IV) followed by direct S_N2 displacement with inversion, while not explicitly excluded, is unlikely. No such intermediates have been detected for the kaurene synthetase catalyzed cyclizations although it should be rather straightforward to prepare and test the appropriate tertiary allylic pyrophosphate. The previously mentioned tertiary allylic alcohol **58** found in *P. mutilus* extracts is interesting in this regard. Preliminary data based on comparison of molecular rotations of a series of tertiary allylic alcohols indicate that this alcohol **58** may have the 12*S* configuration. This finding suggests that **58** is formed by displacement of the allylic pyrophosphate from the same face of the double bond as that usually engaged by the action of the cyclase. In this view alcohol **58** can be regarded as an anomalous hydrolysis product, formed by a process analogous to the formation of geraniol by prenyltransferase. Were **58** to have been formed by *syn* allylic rearrangement and subsequent *phosphatase* hydrolysis, the alcohol would be expected to have the epimeric 12*R* configuration.

From the results of the previously cited chemical model experiments, the concerted S_N2' reaction may have only a relatively small, if any, preference for *syn* stereochemistry. For example a 10:1 preference, if exclusively the result of stereoelectronic factors, would correspond to at most a 1.4 kcal difference in transition state free energy. For an enzyme, which may have to achieve catalytic factors of say 10^6, corresponding to a net 8.4 kcal lowering of the transition state, 1.4 kcal may be an acceptable price to pay if offset by suitable advantages. Whether allylic displacement at the active site occurs by a stepwise or concerted process (the stereochemical data cannot supply this information), it would appear advantageous to have the leaving group depart from the face of the allylic system opposite that to which the nucleophilic group of the substrate becomes attached. This not only allows the path of the pyrophosphate to be unencumbered by the incoming nucleophilic double bond and attached carbon atoms, but separates the complementary enzymatic machinery for initiating or assisting ionization of the pyrophosphate from that which aligns the remainder of the molecule with the developing positive charge. According to this hypothesis, enzymatic allylic displacement would not be significantly different mechanistically from direct enzymatic displacement, the only difference being the ultimate site of attachment of the nucleophile. Looking ahead to Section IV, the finding that allylic rearrangement also involves a sequence of ionization—internal recombination is completely consistent with the developing picture of biological allylic displacements. In fact, if all these reactions require an initial ionization step, the experimental distinction between prior allylic rearrangement followed by displacement at the same active site or simple allylic displacement becomes experimentally meaningless.[67]

Chemical model reactions

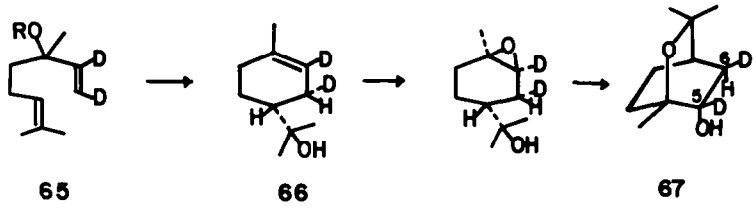
In addition to the previously mentioned studies of chemical allylic displacement processes, a recent report from Arigoni's group is particularly relevant to the present discussion.[69] Since the recognition by Stephan in 1898 that solvolysis of (−)-*R*-linalool (**65**, R = H) affords optically active α-terpineol (**66**),[70] this reaction and its analogs have been subjected to careful scrunity by several groups of investigators.[71] Andersen has taken advantage of this type of asymmetric induction in a study of the solvolytic conversion of (*S*)-(+)-nerolidyl derivatives to (−)-β-bisabolene in up to 37% optical yield and applied this procedure to a biomimetic synthesis of cedrene.[72] These solvolysis reactions are therefore of interest not only as examples of allylic displacements, but as potential models of the terpenoid cyclizations to be discussed in Section V. A consideration of the known absolute configuration of both reactants and products indicates that the solvolysis of the *p*-nitrobenzoate of (−)-linalool (**65**, R = PNB) may proceed in

principle through a *syn-chair* or *anti-boat* transition state, the former process having been favored for many years by analogy to Stork's earlier work on the S_N2' reaction[71] (Scheme 33). As illustrated, an



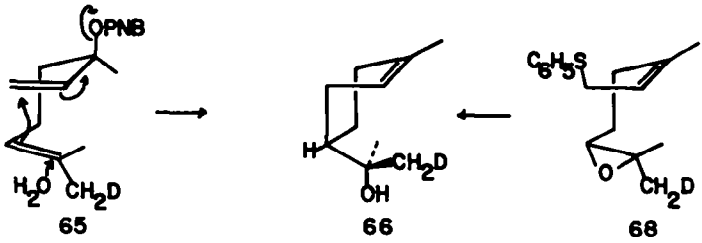
Scheme 33.

experimental distinction of the two processes becomes possible if one follows the hydrogens H_A and H_B through cyclization. Reduction of dehydrolinalool with deuterium gas and a soluble tris(triphenylphosphine)rhodium chloride catalyst gave *rac*-[1,2-$^2H_2$]-linalool which was converted to its corresponding *p*-nitro-benzoate and solvolyzed for 6 days at 50° in 70% aqueous acetone (Scheme 34). From the resulting complex mixture of products, the labeled α-terpineol was isolated and converted to hydroxycineol (**67**) by treatment with *m*-chlorperbenzoic acid. The derived [5,6-$^2H_2$]-hydroxycineol was a convenient substrate for ^1H NMR analysis since previous decoupling experiments on unlabeled material had convincingly established that in the presence of 29.56 mol% $Eu(dpm)_3$, the signal corresponding to H_{6exo} appeared at 6.90 ppm downfield of TMS, whereas the H_{6endo} proton was more strongly shifted to 8.19 ppm. Furthermore H_{6exo} displayed a 2 Hz W-coupling to H_{2exo} whereas H_{6endo} was unsplit. Analysis of the dideuterated hydroxycineol revealed that the signal corresponding to H_5 was diminished in intensity to 0.1 proton while that for H_{6exo} integrated as 0.1 proton. After correction for a small amount of proton scrambling, it was calculated that 90% of the deuterium at C(1) of α-terpineol was located *cis* to the isopropyl side chain. Reference to the previous analysis (Scheme 33, $H_A = D$, $H_B = H$) indicated that between 80 and 90% of the reaction occurred via the *anti-boat* transition state.



Scheme 34.

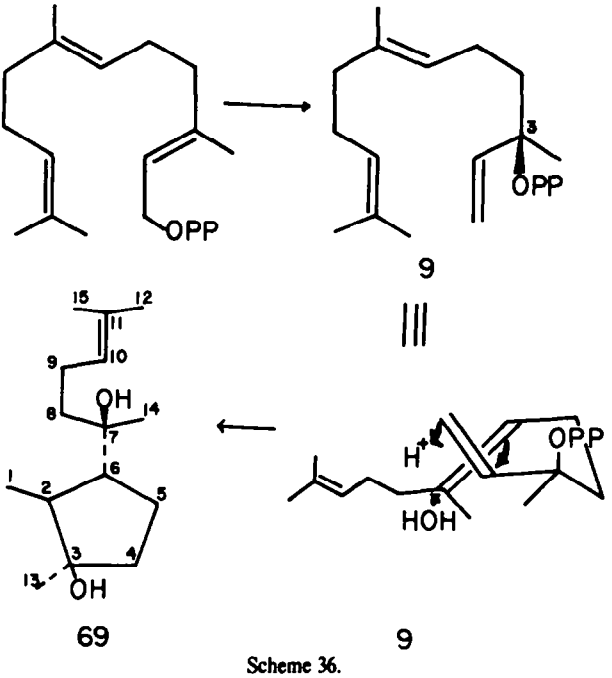
Further insight into the stereochemistry of the solvolytic displacement was gained using [8-^{2}H]-linalool (Scheme 35). When this latter substrate was solvolyzed as its *p*-nitrobenzoate, ^{13}C NMR analysis indicated that one of the two diastereotopic methyls of the derived α-terpineol was preferentially labeled (*ca* 2:1). Furthermore comparison with a sample of deuterated α-terpineol synthesized by an unambiguous route involving cyclization of the epoxide **68**, established the sense of preferential labeling to be as shown. Attack by external nucleophile on the 6,7-double bond therefore occurs with preferential *anti* stereochemistry coupled with the *anti*-attack on the solvolyzed allylic ester.



Scheme 35.

IV. ALLYLIC REARRANGEMENTS

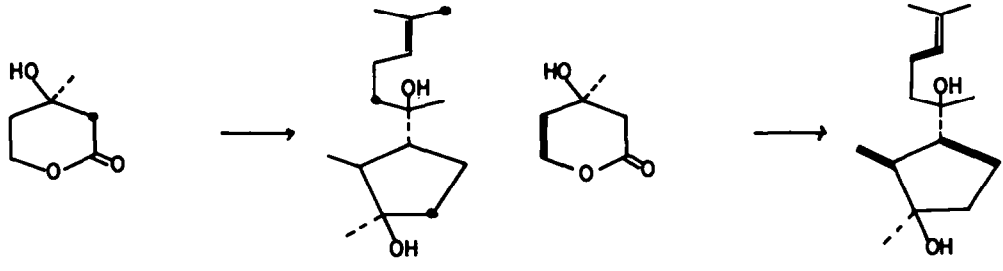
Chemical model studies of the sort just described illustrate the potential importance of tertiary allylic pyrophosphates and emphasize the need for understanding the biochemical mechanisms by which these substrates are formed from primary allylic precursors. Although present in the essential oils of numerous higher plants[73] and recognized by Ruzicka more than 70 years ago as a potential precursor of cyclic sesquiterpenes,[4] nerolidol and its biologically activated ester **9** have remained a biosynthetic enigma until only recently. Early claims for the intermediacy of nerolidyl pyrophosphate in squalene biosynthesis[7,74] have been refuted by Rilling[8] and, until 1978, there was still no direct experimental evidence for the biochemical conversion of nerolidyl pyrophosphate to cyclized material. It was therefore with some interest that we noted the isolation of the sesquiterpene cyclonerodiol (**69**) from the culture broths of a number of fungi.[75] Consideration of the unusual structure of this cyclopentanoid metabolite suggested that cyclonerodiol might be formed by addition of the elements of water across the vinyl and central double bonds of nerolidol or its pyrophosphate ester (Scheme 36). It was immediately apparent that this process, if substantiated, might then serve as a useful vehicle to study the formation of nerolidyl pyrophosphate itself from farnesyl pyrophosphate.



Scheme 36.

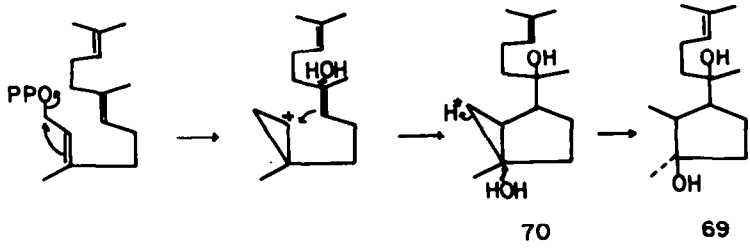
Cyclonerodiol biosynthesis and the enzymatic conversion of farnesyl to nerolidyl pyrophosphate

Our early experiments with cyclonerodiol focussed on placing our biosynthetic hypothesis on a firm experimental footing.[76] Incorporation of [2-^{14}C]-mevalonate, using growing cultures of *Gibberella fujikuroi*, gave cyclonerodiol which was subjected to extensive chemical degradation in order to establish the positions of labeling (Scheme 37). The finding that carbons (4) and (8) each bore exactly one third of the ^{14}C-activity with the remainder confined to the isopropylidene carbons was in keeping with the presumed pathway. During the course of this early work, support for the biogenetic scheme was provided by Hanson *et al.* who reported a cleverly conceived incorporation of [4,5-$^{13}C_2$]-mevalonate into cyclonerodiol from *T. roseum* resulting in a ^{13}C NMR spectrum in which the pairs of signals



Scheme 37.

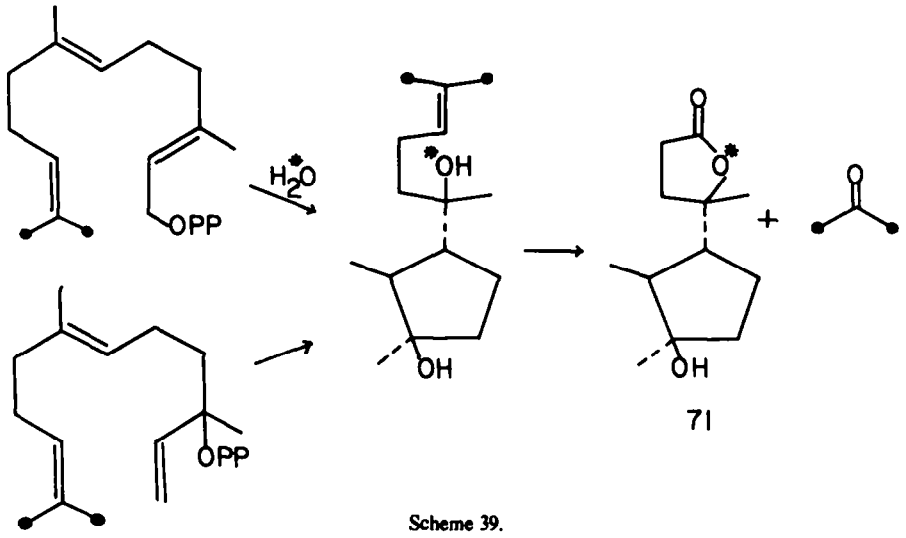
corresponding to C(1) and C(2), C(5) and C(6), and C(9) and C(10) were enhanced and coupled.[77] The Sussex group also found that feeding of [^{3}H,^{14}C]-farnesyl pyrophosphate gave rise to radioactive cyclonerodiol of unchanged ^{3}H/^{14}C ratio, although no attempt was made to locate the label. Attempted incorporation of nerolidol, however, was unsuccessful, prompting the suggestion that either nerolidol, might be present as an enzyme bound intermediate, or that cyclonerodiol could arise by hydration of a bicyclo [3.1.0]-hexane **70** formed by cyclization of a cyclopropyl cation generated directly from farnesyl pyrophosphate (Scheme 38).



70 69

Scheme 38.

Experiments in our own laboratory using intact cells and either nerolidol or nerolidyl pyrophosphate were equally unsuccessful. We therefore turned to the development of a suitable cell-free system from *G. fujikuroi*, which after some experimentation proved to be reasonably easy to prepare and manipulate.[78] Indeed incubation of [12,13-^{14}C]-farnesyl pyrophosphate with an early version of this enzyme preparation gave a 1% yield of cyclonerodiol which was diluted with carrier and purified (Scheme 39).

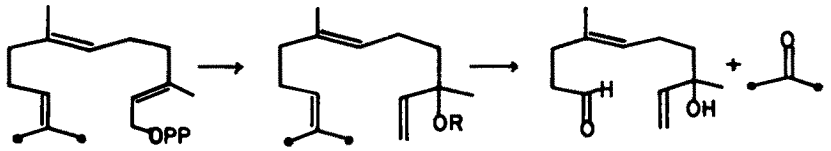


71

Scheme 39.

Using improved versions of this enzyme preparation conversions of up to 9% from farnesyl pyrophosphate have recently been obtained. Treatment of the labeled cyclonerodiol with osmium tetroxide-sodium periodate gave acetone, isolated as the semicarbazone and bearing >99% of the ^{14}C-activity, as well as a cyclic hemiacetal which was converted to the inactive crystalline trisnorlactone **71**. This degradation sequence in fact proved to be quite versatile and has been utilized in a number of analytical sequences. The cell-free system, when supplemented with ATP, was also capable of converting mevalonate to cyclonerodiol, albeit in lower overall conversion (0.07%). Incubation of [12,13-^{14}C]-nerolidyl pyrophosphate gave a 0.8% yield of cyclonerodiol which was degraded as before to establish that the label resided exclusively in the isopropylidene carbons. Interestingly a similar incubation with free [12,13-^{14}C]-nerolidol failed to give labeled product, suggesting that in the biosynthetic sequence cyclization precedes pyrophosphate ester hydrolysis. While this latter hydrolysis could be assumed to occur with P–O bond cleavage, by analogy to the known mode of action of alkaline phosphatase, direct support for this hypothesis was obtained by carrying out the conversion of farnesyl pyrophosphate to cyclonerodiol in $H_2{}^{18}O$.[79] Mass spectrometric analysis of the derived trisnorlactone established that only the side chain OH at C(7) of cyclonerodiol is derived from water. The parent (M^+), as well as M^+-CH_3, M^+-H_2O, and M^+-CH_3-H_2O fragments of labeled **71** showed the same ^{18}O enrichment. The absence of any M+4 peak and the fact that the isotopic enrichment was unchanged in the fragments corresponding

to loss of water from the ring both establish that pyrophosphate ester hydrolysis takes place with exclusive P–O cleavage and support the proposed hydration of the nerolidyl double bonds while conclusively ruling out Hanson's cyclopropane intermediate. Direct evidence for the farnesyl–nerolidyl interconversion was provided by quenching a short term (15 min) incubation of [12,13-^{14}C]-farnesyl pyrophosphate and, after initial extraction of the neutral products and addition of carrier nerolidyl pyrophosphate, extracting the prenyl pyrophosphates with butanol (Scheme 40). Purification of the recovered

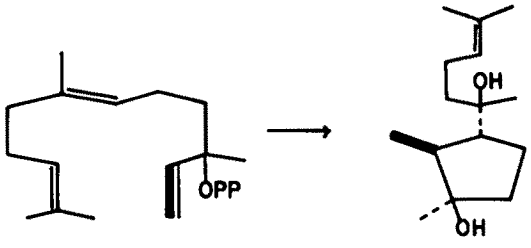


9 R = PP 72

R = H

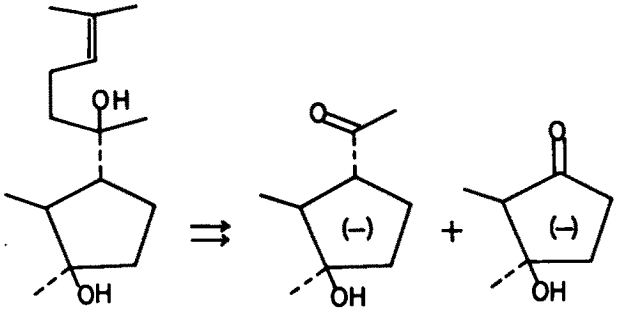
Scheme 40.

nerolydyl pyrophosphate by ion-exchange chromatography followed by hydrolysis with alkaline phosphatase gave radioactive nerolidol. Dilution with inactive carrier and straightforward oxidative degradation gave inactive trisnoraldehyde 72 and acetone, whose semicarbazone carried all the ^{14}C activity of the nerolidol precursor. Further insight into the biosynthetic sequence was gained by incubating a sample of [1,2-$^{13}C_2$]-nerolidyl pyrophosphate with the cell-free preparation in an NMR tube (Scheme 41). The enzymatic reaction was intermittently interrupted by cooling to 0–3° and the sample analyzed by ^{13}C NMR. Spectra recorded after 5, 10, and 20 min total reaction at 25° revealed the presence of a pair of 38.4 Hz doublets centered at 44.54 and 14.67 ppm, assigned to C(2) and C(1) of cyclonerodiol, respectively, and which linearly increased in intensity with time. No evidence was found for the accumulation of any farnesyl pyrophosphate under these conditions. Needless to say none of the above described enzymatic conversations could be observed using boiled controls.



Scheme 41.

Having established beyond doubt the role of nerolidyl pyrophosphate, the stage was now set for more detailed stereochemical investigations.[80] The stereochemistry of folding of the nerolidyl pyrophosphate, as well as the direction of attachment of the water oxygen at the side chain is apparent from the absolute configuration of cyclonerodiol, previously established by correlation with 73 and 74, two known conversion products of (-)-*R*-linalool[81,82] (Scheme 42). Since hydrolysis of the pyrophosphate

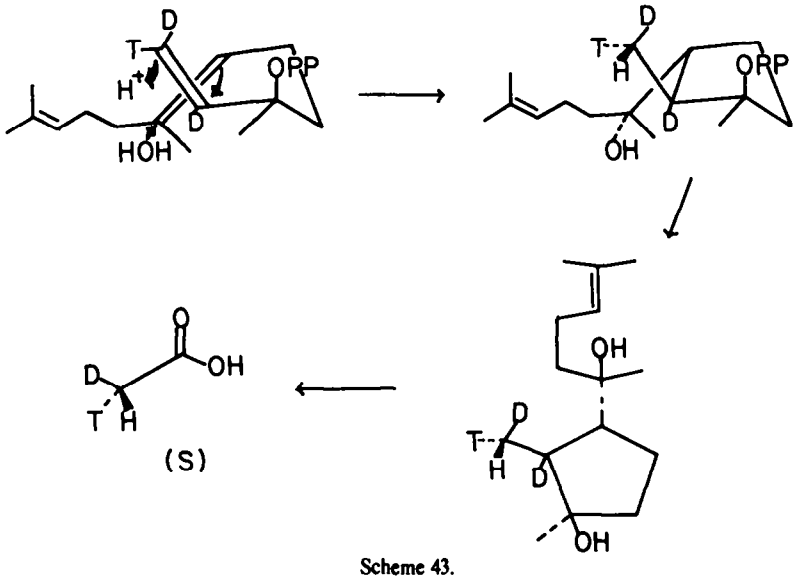


73 74

Scheme 42.

ester occurs with P–O bond cleavage, the intermediate nerolidyl pyrophosphate would then have the 3*R* configuration. This latter point was confirmed by incubating a mixture of 3*S*-[12,13-^{14}C]- and 3*R*,*S*-[1-^{3}H]-nerolidyl pyrophosphate with the *G. fujikuroi* enzyme extract.[79] The resultant tritiated cyclonerodiol was devoid of ^{14}C activity, indicating that only the tritiated 3*R*-enantiomer serves as a precursor.

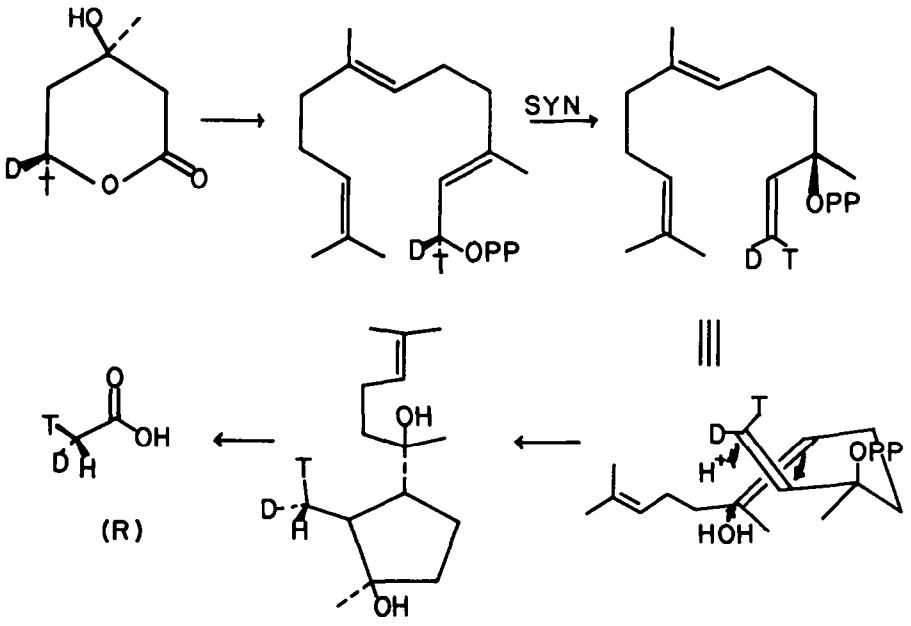
During the cyclization step, protonation of the terminal vinyl carbon of nerolidyl pyrophosphate generates the C(1) Me group of cyclonerodiol (Scheme 43). In order to determine the stereochemistry of this latter protonation we required a sample of **9** stereospecifically labeled with both deuterium and



Scheme 43.

tritium at C(1).[80] Protonation at C(1) would then generate a Me group with a chirality dependent on the stereochemistry of proton addition. The requisite sample of [1,2-^{2}H$_2$,1-^{3}H]-(*E*)-nerolidol, obtained by lithium aluminum deuteride–sodium methoxide reduction of [1-^{3}H]-dehydronerolidol followed by deuterated water quench, was converted to the pyrophosphate and incubated with the usual cell-free system. The resultant labeled cyclonerodiol was subjected to Kuhn–Roth oxidation and the acetate obtained, which consisted of chirally labeled acetate from the C(1) and C(2) of cyclonerodiol, diluted with unlabeled acetate originating from the C(13), C(14), and either the C(12) or C(15) methyls of **69**, was analyzed by the standard malate synthetase–fumarase procedure. The finding that upon fumarase treatment the derived malate retained only 29.6% tritium established the chirality of the acetate as 2*S*-[2-^{2}H,^{3}H]-acetate. Since Kuhn–Roth oxidation did not affect the observed chirality, the C(1) Me of cyclonerodiol derived from [1,2-^{2}H,1-^{3}H]-(*E*)-nerolidyl pyrophosphate must also have been of *S* chirality, implying that protonation at C(1) of nerolidyl pyrophosphate must have occurred on the *re* face. The overall stereochemistry of the addition of water across the vinyl and central double bonds is thus completely *trans*.

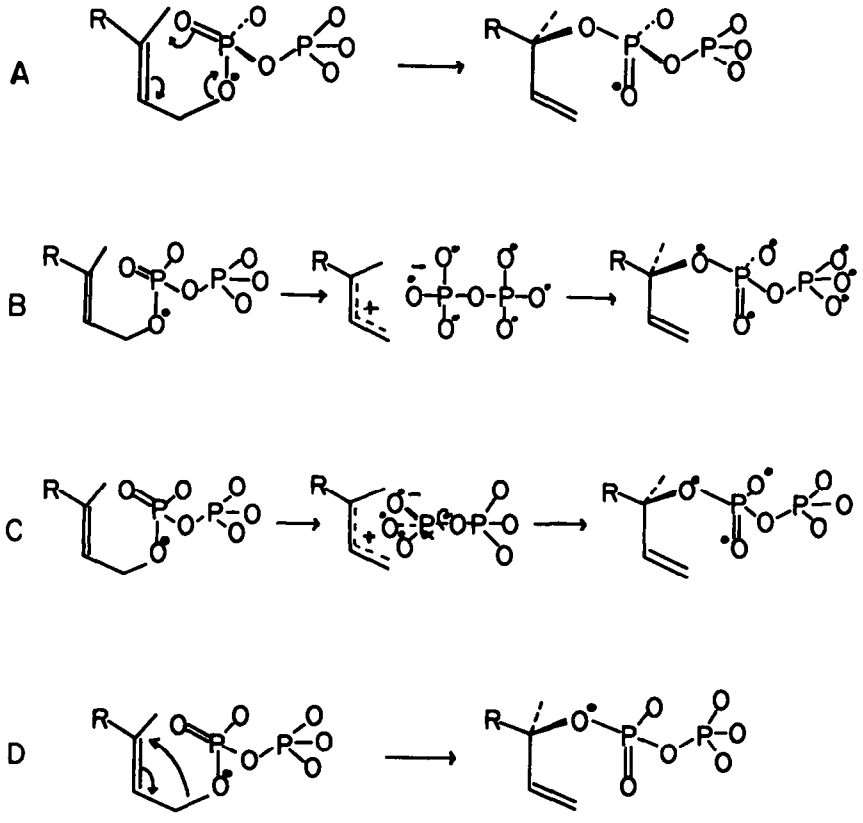
With the above determination of the stereochemistry of the enzymatic conversion of nerolidyl pyrophosphate to cyclonerodiol, a means was at hand for analyzing samples of labeled nerolidyl pyrophosphate of unknown stereochemistry at C(1) (Scheme 44). For example, isomerization of 1*R*-[1-^{2}H,^{3}H]-farnesyl pyrophosphate would give either [1-^{2}H,^{3}H]-(*E*)-or-(*Z*)-nerolidyl pyrophosphate whose stereochemistry could be determined by conversion to cyclonerodiol and determination of the chirality of the resultant C(1) methyl. In actual practice, it was found most convenient to feed 5*R*- and 5*S*-[5-^{2}H,^{3}H]-mevalonates separately to intact cultures of *G. fujikuroi* and to allow the microorganism to carry out all the biosynthetic steps. Isolation of the derived cyclonerodiol and Kuhn–Roth oxidation gave rise to a pair of chiral acetate samples which were each analyzed by the malate synthetase–fumarase procedure. It was thus established that incorporation of 5*R*-[5-^{2}H,^{3}H]-mevalonate gave rise to 2*R*-[2-^{2}H,^{3}H]-acetate (68.9% tritium retention) whereas 5*S*-[5-^{2}H,^{3}H]-mevalonate resulted in 2*S*-[2-^{2}H,^{3}H]-acetate (30.8% tritium retention). These results imply that the allylic rearrangement of farnesyl pyrophosphate to nerolidyl pyrophosphate is a *syn* process. Thus 5*R*-[5-^{2}H,^{3}H]-mevalonate gives rise to 1*R*-[1-^{2}H,^{3}H]-farnesyl pyrophosphate, which is then isomerized to 3*R*-nerolidyl pyrophosphate. *Syn* isomerization gives the [1-^{2}H,^{3}H]-(1*Z*)-nerolidyl stereoisomer which, upon cyclization and pyrophosphate ester hydrolysis, yields 1*R*-[1-^{2}H,^{3}H]-cyclonerodiol and therefore the corresponding 2*R*-[2-^{2}H,^{3}H]-



Scheme 44.

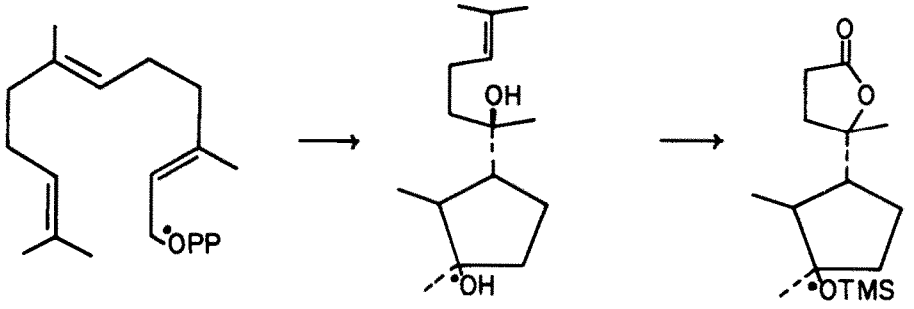
acetate upon oxidation. The results with 5*S*-[5-^{2}H,^{3}H]-mevalonate are entirely complementary and fully support the above argument.

Further insight into the allylic rearrangement process was then gained by examining the role of the pyrophosphate moiety during the established *syn* isomerization.[79] Four mechanisms, illustrated in Scheme 45 might be advanced, *a priori*, to account for this allylic rearrangement: (A) a concerted



Scheme 45.

phospho-Claisen or ionic stepover mechanism. In this process it is important to note that the oxygens attached to P_α remain stereochemically distinct in both the forward and reverse reactions and therefore would not be expected to become scrambled by any enzyme-catalyzed process; (B) a stabilized allylic cation in which free inorganic pyrophosphate is formed, allowing complete scrambling of all six non-bridge oxygens; (C) an ion-pair intermediate in which there is sufficient time for rotation about the P_α–OP_β bond; and (D) a 1,3-sigmatropic rearrangement or tight ion-pair in which P_α–OP_β rotation is restricted. Each of these mechanisms could be distinguished experimentally by determining the relationship of the ester oxygen of the primary allylic pyrophosphate to the corresponding ester oxygen of the resulting tertiary allylic pyrophosphate. Furthermore, the previously established finding that the OH oxygen at C(3) of cyclonerodiol corresponds to the C(3) pyrophosphate ester oxygen of the intermediate nerolidyl pyrophosphate allows the analysis to be carried out on a suitable derivative of cyclonerodiol itself (Scheme 46). Thus incubation of [1-^{18}O]-farnesyl pyrophosphate, containing a [12,13-^{14}C]-farnesyl



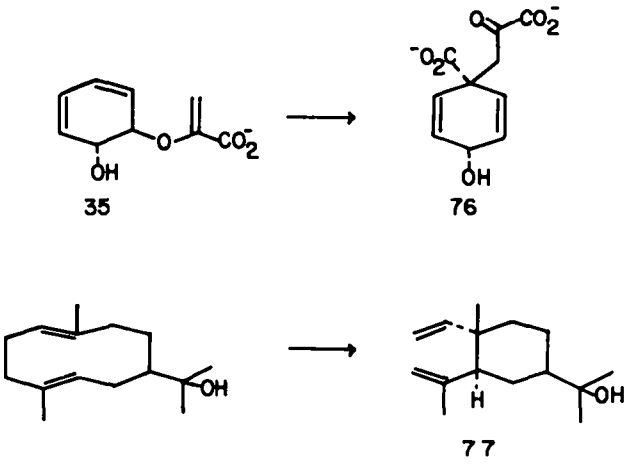
75

Scheme 46.

pyrophosphate internal standard, gave cyclonerodiol which was shown to carry almost exactly one third of the ^{18}O of the precursor at C(3), as established by mass spectrometric analysis of the derived trimethylsilylether (**75**) of the trisnorlactone. Measurement of the specific activity of the cyclonerodiol established that no dilution with endogenous substrates had occurred. Finally, as a result of the phosphatase activity which was a persistent contaminant of the usual cell-free preparation,[83] it was also possible to reisolate farnesol from the incubation mixture. The mass spectrum of the derived farnesyl acetate indicated the ^{18}O-enrichment of the recovered farnesol to be unchanged from that of the precursor, indicating that no scrambling of starting material had occurred and incidentally establishing that the *G. fujikuroi* phosphatase also acts by P–O bond cleavage. These results conclusively rule out mechanisms A, B and D for the allylic pyrophosphate rearrangement and strongly support the ion-pair of pathway C. The experiment, of course, measures only net scrambling of nerolidyl pyrophosphate released from the isomerase. In the absence of purified enzyme it is not possible to determine the scrambling for a single turnover, nor the number of times nerolidyl and farnesyl pyrophosphate are interconverted at the active site prior to product release. From results of analysis of the previously described ^{13}C NMR and nerolidyl pyrophosphate trapping experiments, however, it is possible to make a crude estimate that the cyclase will convert nerolidyl pyrophosphate to cyclonerodiol between 2 and 10 times as fast as rebinding of the free tertiary allylic pyrophosphate to the isomerase, making scrambling by such a mechanism unlikely.

The last experiments also have obvious implications for the *timing* of the rearrangement process since bond breaking must clearly precede bond making. The implication that the oxygens attached to P_α are free to rotate in spite of a presumably strong interaction with Mg^{2+} is particularly striking and is reminiscent of the results of both Rose[84] and Lowe[85] who have independently observed enzyme mediated scrambling of the terminal oxygens of transiently generated ADP during investigation of a number of ATP-dependent processes. Recalling the conclusion of Rilling and Poulter that in prenyltransferase catalysis ionization precedes bond-making, a coherent picture of allylic pyrophosphate biochemistry begins to emerge.

The preference for an ionic over a concerted rearrangement pathway also bears further comment. Few examples of electrocyclic concerted processes are to be found among known biochemical reactions. A possible exception to this rule is of course the chorismate mutase reaction involving apparent Claisen rearrangement of a phosphenol pyruvate ester to prephenate (**76**),[86] yet no data is available to exclude the alternative ion pair pathway (Scheme 47). The ready formation of elemanes such as **77** from germacradiene precursors[87] has led to the belief that this process may occur *in vivo*, but neither this



Scheme 47.

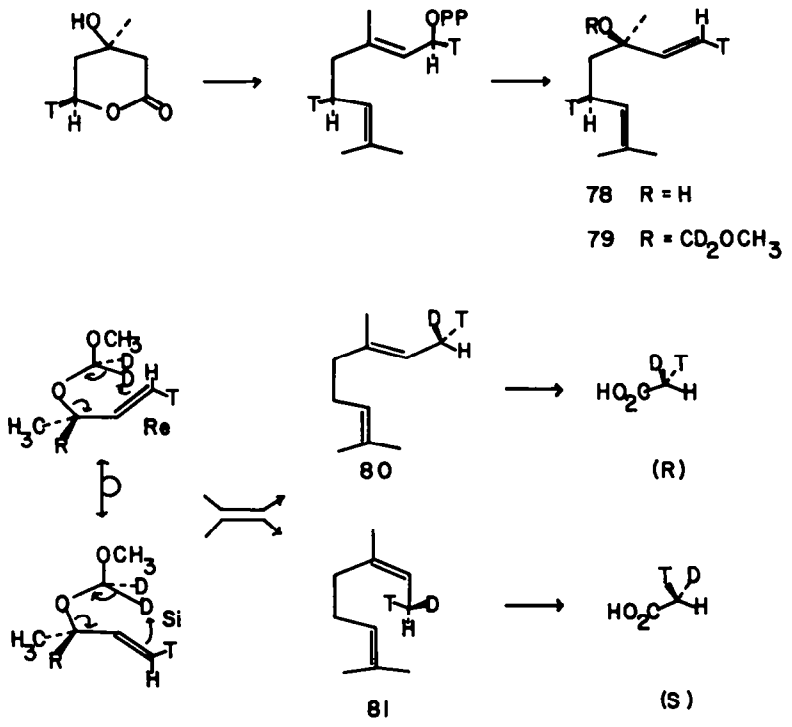
reaction nor the well known photochemical conversion of ergosterol to Vitamin D_2[88] have been shown to be enzyme mediated. The currently favored theory for the interconversion of the three great classes of indole alkaloids posits intramolecular Diels–Alder reaction of a dihydropyridine with a vinyl acrylate ester.[89] These latter reactions can as readily be interpreted as two step Michael addition—aldol condensations, especially since many of the usual criteria for concertedness—stereospecificity, insensivity to solvent polarity, secondary isotope effects—are largely moot for an enzymatic reaction. In many other cases where electrocyclic processes have been suggested as part of biosynthetic pathways the substrates themselves have turned out not to be intermediates. For example the proposition that sulfenic acids might intervene in the formation of penam and cepham systems was based on the well-known chemistry of penicillin sulfoxides.[90] Labeling studies, however, have conclusively ruled out such intermediates for the *in vivo* pathway.

The conspicuous scarcity of concerted electrocyclic biochemical reactions is perhaps not too surprising when one recalls the insensitivity of this class of reactions to most forms of catalysis. Only recently has it been found possible to accelerate these reactions significantly by using ionized substituents or transition metals. The evolution of enzyme catalysis would, of course, involve not only rate enhancement but *control*, over both rate and products. Such speculation, however, comes largely with hindsight. Electrocyclic reactions, being inherently stereospecific, might have been expected to be ready candidates for evolutionary selection. Moreover, a significant element of control might be possible for the enzyme-catalyzed process since precise binding at an active site would provide the highly ordered transition state required to overcome the large negative entropy of activation for pericyclic reactions.

Biosynthesis of linalool

The finding of *syn* stereochemistry for the farnesyl–nerolidyl interconversion has been matched by a similar conclusion for the biosynthesis of the monoterpene analog linalool (**78**).[91] Investigations by Suga[92] had previously established that (−)-*R*-linalool is biosynthesized from [2-^{14}C]-mevalonate by the tree *Cinnamomum camphora* and degradations had established that the label was roughly equally distributed between the two isoprene units, in contrast to the frequently observed preferential labeling of the isopentenylpyrophosphate fragment associated with bicyclic monoterpenes. The respectable incorporation levels of 0.01–0.05% provided a convenient opportunity to determine the stereochemistry of linalool biosynthesis from suitably labeled precursors. This study was undertaken in collaboration with Arigoni *et al.*[91] Initial feeding of [5-$^3H_2$]-mevalonate indicated incorporation of mevalonate without loss of tritium activity. A sample of more than 2 mCi of 3*R*,5*R*-[5-^{3}H]-mevalonate was then administered to branches of *C. camphora* and the resultant (−)-*R*-linalool isolated and purified (Scheme 48).

The labeled linalool was degraded in Arigoni's laboratory by a procedure similar to that developed previously by the ETH group for the synthesis of chiral acetates.[93] Thus pyrolysis of the derived dideuteromethylene methoxymethyl ether **79** of linalool at 250°C for 5 h gave two isomeric alkenes **80** and **81** which were separated by preparative gas chromatography. Consideration of the transition state for this reaction clearly indicates that the *cis*-isomer **81** is formed by approach of the migrating deuterium atom to the 1-*si*-face of the vinyl double bond whereas transfer to the 1-*re*-face produces the



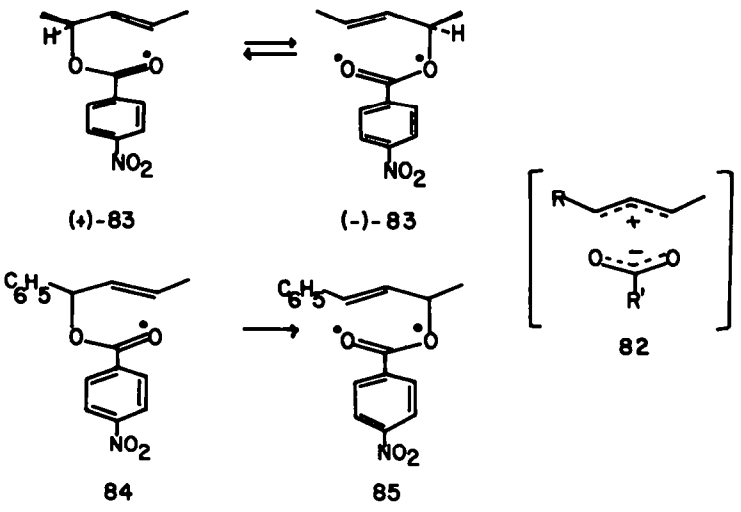
Scheme 48.

corresponding *trans*-isomer **80**. Since the terminal vinyl carbon is expected to carry tritium, the pyrolysis generates in each case a chiral methyl whose chirality is dependent on the stereochemistry of tritium substitution and the direction of deuterium transfer. Analysis of each chiral acetate sample, obtained by ozonolysis of the corresponding alkene, established that the acetate derived from the *trans*-alkene had the *R*-configuration (fumarase value = 70.4) whereas the acetate from the *cis*-alkene was of the complementary *S*-configuration (fumarase value = 32.9). These results established that the linalool biosynthesized from 5*R*-[5-^{3}H]-mevalonate was labeled with tritium *cis* to the sidechain. Applying the apparently secure, if as yet unproven, assumption that hydrolysis of the corresponding linalyl pyrophosphate takes place in this plant with P–O bond cleavage, the above experiments once again demonstrate that the allylic rearrangement of geranyl to linalyl pyrophosphate involves *syn* stereochemistry.

Chemical models

As a result of the extensive work of Goering *et al.*, a particularly well-documented picture of the rearrangements of allylic carboxylate esters has emerged.[94] Strong evidence has been advanced to show that in polar solvents these processes occur by tight ion-pair intermediates such as **82** and thorough labeling and kinetic experiments have established a preference for *syn* stereochemistry and concurrent scrambling of the carboxylate ester oxygens to an extent determined by the relative stability of the derived allylic cation. Briefly summarized it was found that rearrangement of optically active *trans*-α-,γ-dimethylallyl *p*-nitrobenzoate (**83**) in 90% aqueous acetone resulted in interconversion of the enantiomers by way of a tight ion pair in which the rate of scrambling of the ester oxygens is 1/2.9 times that of racemization (Scheme 49). The analogous transformation of *trans*-α-phenyl-γ-methyl *p*-nitrobenzenoate (**84**) to *trans*-α-methyl-γ-phenylallyl *p*-nitrobenzoate (**85**) differed in that it involved a more stable intermediate cation and was essentially irreversible. Kinetic and labeling studies revealed that the rearrangement involved almost complete randomization of the carboxyl oxygens accompanied by partial loss of optical purity, consistent with a longer-lived or looser ion-pair.

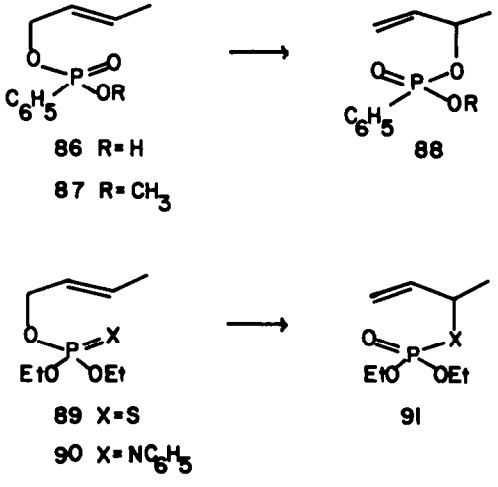
Few rearrangements of allylic phosphate or pyrophosphate esters have been subjected to such careful analysis. Most mechanistic interpretations for these latter reactions have been based on observed product distributions.[71,95] The one exception has been an NMR examination by Herriot of the rearrangement of crotyl phenylphosphonate (**86**) to α-methylallyl phenylphosphonate (**88**) which suggested an



Scheme 49.

ionic rather than a concerted mechanism[96] (Scheme 50). The reaction was accelerated by addition of trifluoroacetic acid and was strongly inhibited by added bases such as pyridine. A modest rate enhancement of 2.5 was achieved by using nitrobenzene in place of chlorobenzene as solvent. The reaction could not be observed in hydroxylic solvents which simply intercepted the allyl cation. Finally, isomerization of the corresponding methyl ester **87** proceeded at a rate 1/20 that of **86** at the same temperature and concentration.

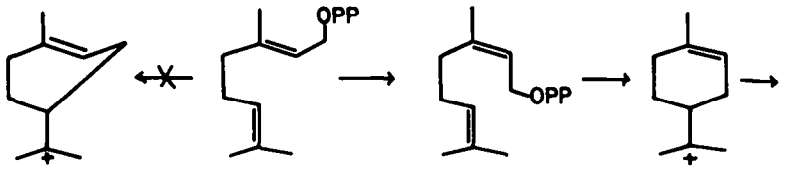
When the phosphate ester contains a heteroatom in place of the ester oxygen a [3.3]-sigmatropic shift is reported to result. In the rearrangement of the thio and iminophosphates **89** and **90**, the driving force presumably comes from formation of the more stable P=O bond.[97]



Scheme 50.

V. DOUBLE BOND ISOMERIZATION

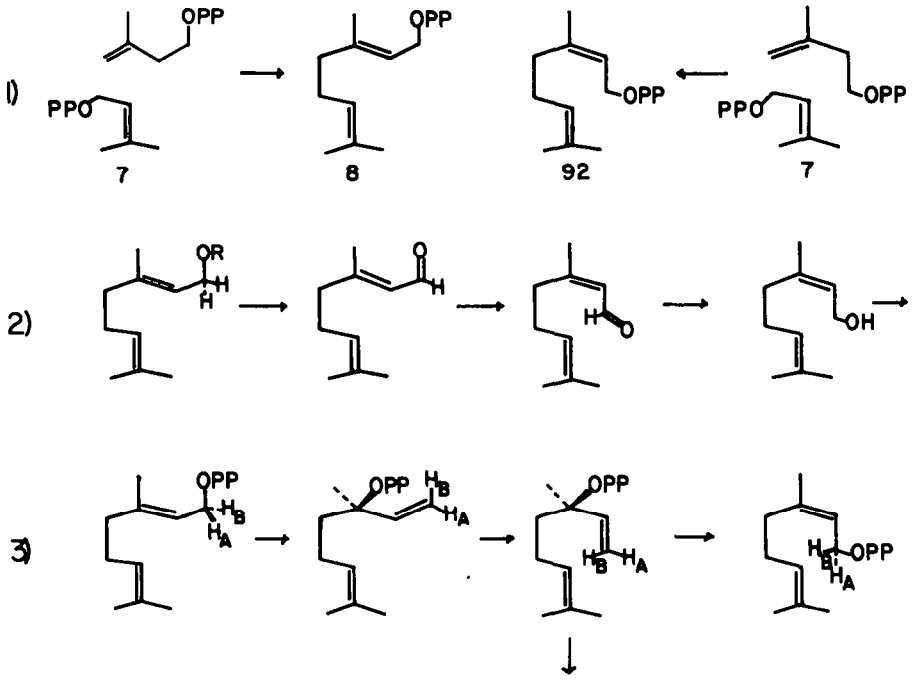
Although the recognition of geranyl and farnesyl pyrophosphate as the universal precursors of cyclized mono-and sesquiterpenes has provided a powerful theoretical tool for the understanding of terpenoid biogenesis, the intimate details of the cyclization process are still poorly understood in most cases. A major difficulty stems from the realization that the formation of 6-membered rings from allylic pyrophosphates requires a prior isomerization of the *trans*-double bond in order to avoid formation of *trans*-cyclohexane (Scheme 51). Most reviews of biogenetic theory have merely skirted this inherent problem by simply classifying sesquiterpenes, for example, as derivatives of *trans,trans*- or *cis,trans*-farnesyl pyrophosphate.[98] Unfortunately a coherent theory of the cyclization process has yet to emerge. Studies of the biosynthesis of monoterpenes in higher plants have been hampered by low incorporations, compartmentation effects, non-uniform labeling, and poor reproducibility.[99] Significant progress has



Scheme 51.

recently been made by the development of suitable cell-free systems but, with a few notable exceptions, much of this work is still characterized by an unfortunate reliance on purely chromatographic methods for characterization and analysis of radioactive products and a regrettable failure to satisfactorily locate the positions of isotopic labeling. The result has been a near morass of half-supported theories and conflicting data, some of dubious validity or significance. Investigations of sesquiterpene biosynthesis have generally been a good deal more thorough if less often at the enzyme level. Many of the studies have involved oxygenated fungal metabolites and one difficulty has been that the intermediate sesquiterpene hydrocarbons are often present in only inconveniently small quantities. In some otherwise suitable systems useful labeling information is obliterated by subsequent metabolic steps.

Attempts to identify the mechanism of the *trans-cis* isomerization, or to prove that it occurs at all, have generated at least three conflicting theories. These hypotheses are distinguished by the fate of the hydrogens directly attached to C(1) of the allylic pyrophosphate (Scheme 52).



Scheme 52.

(1) Direct formation of neryl (92) or *cis,trans*-farnesyl pyrophosphate by addition of isopentenyl pyrophosphate to the respective precursors, dimethylallyl (7) and geranyl pyrophosphate, avoids the isomerization problem altogether. In principle, the hydrogens at C(1) of the newly formed pyrophosphate would be unaffected by such a process.

(2) Various redox schemes for the *trans-cis* isomerization have been put forward. These processes all involve removal of one of the C(1) hydrogens and generation of intermediates in which may occur about a C(2)–C(3) single bond.

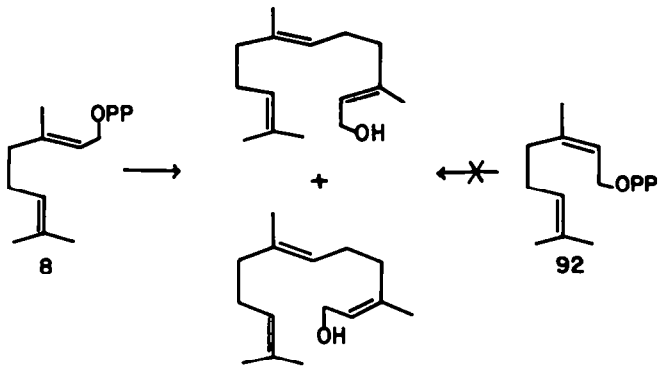
(3) *trans-cis* Isomerization via an intermediate tertiary allylic pyrophosphate such as linalyl or nerolidyl pyrophosphate has been suggested by several schools. Again the transiently generated C(2)–C(3) single bond allows the necessary rotation. Assuming the now established *syn* stereochemistry for allylic rearrangement, the combination of primary–tertiary and tertiary–primary isomerizations and intermediate bond rotation will lead to net inversion at C(1) as a consequence of double bond isomerization but with no loss of C(1) hydrogens. A variant of this hypothesis, encouraged by the known

course of linalyl and nerolidyl solvolyses reviewed above, posits direct cyclization of the tertiary allylic pyrophosphate intermediate.

For convenience the discussion which follows has been divided somewhat arbitrarily according to the fate of the hydrogens at C(1) of the allylic pyrophosphate.

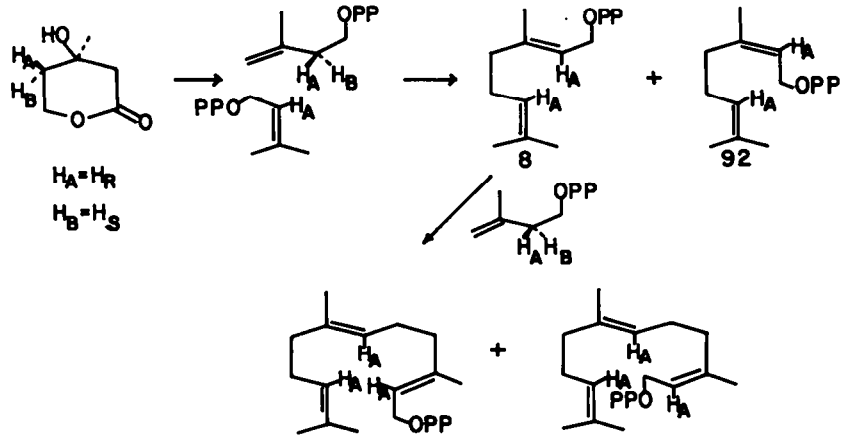
Direct formation of cis*-allylic pyrophosphate or isomerization during cyclization*

The major experimental support for this hypothesis has come from the work of Cori *et al.* These workers have descrived a cell-free system from *Citrus sinensis* (orange) flavedo which catalyzed the conversion of mevalonic acid into isopentenyl pyrophosphate, dimethylallyl pyrophosphate, farnesyl pyrophosphate, and hydrocarbons.[100] Incubation with isopentenyl pyrophosphate led to formation of dimethylallyl, geranyl, and neryl pyrophosphates. Although the system would convert either neryl or geranyl pyrophosphate to limonene in 1–3% yield, no interconversion of these isomeric acyclic substrates could be detected. For example incubation of geranyl pyrophosphate gave no nerol, nor did neryl pyrophosphate yield geraniol. Furthermore only the *trans* isomer, geranyl pyrophosphate (**8**), was converted to *trans,trans*- and *cis,trans*-farnesol (Scheme 53). Evidence for direct formation of the *trans* or *cis* isomers came from examining the fate of the C(4) hydrogens of the precursor mevalonate. It has



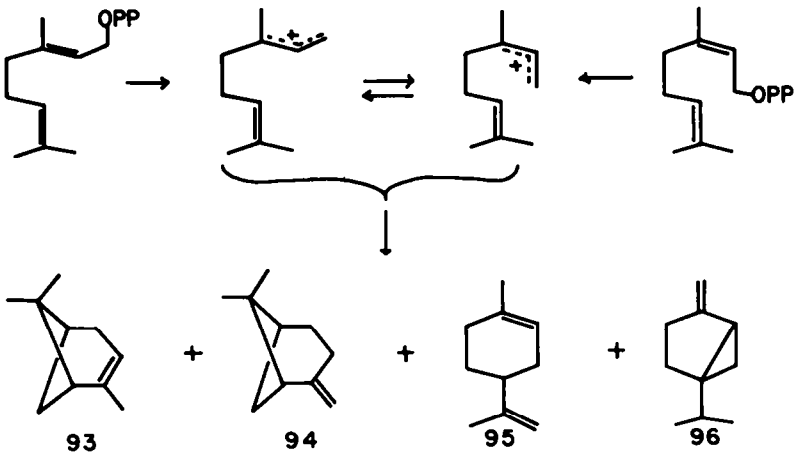
Scheme 53.

come to be accepted that formation of biogenetically *trans* double bonds involves retention of 4-H_{re} and loss of 4-H_{si} of mevalonate, whereas *cis* double bonds require loss of corresponding 4-H_{re} and retention of the 4-H_{si} atoms.[16,22,101] Incubation of 4*R*- and 4*S*-[4-^{3}H-mevalonates with the orange flavedo extract led to retention of the tritium from the 4*R*-^{3}H precursor and loss from the 4*S*-^{3}H-mevalonate in not only geranyl and *trans,trans*-farnesyl pyrophosphate, but in *cis,trans*-farnesyl pyrophosphate as well[100] (Scheme 54). A similar experiment with cell-free enzymes from *Pinus radiata* and stereospecifically tritiated isopentenyl pyrophosphate once again indicated retention of 4-H_{re} from mevalonate in all four products, geranyl, neryl, *trans,trans*- and *cis,trans*-farnesyl pyrophosphate. In the absence of demonstrated interconversion of the unbound substrates, direct formation of either double bond isomer by different prenyl transferases or a single



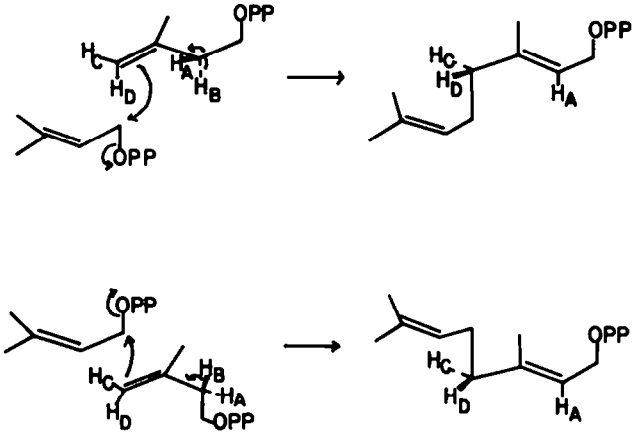
Scheme 54.

transferase with different active site geometries was suggested. The significance of these isomerizations to the *in vivo* cyclization process is uncertain, especially since either double bond isomer acted as a precursor of limonene. Indeed the finding that an extract of *Citrus limonum* (lime) would convert either geranyl or neryl pyrophosphate to α- and β-pinenes (**93** and **94**), limonene (**95**), and sabinene (**96**), but not into each other (Scheme 55), has led Cori to modify his previously suggested theory to cyclization of either isomer via a common enzyme bound intermediate.[102] A number of stereochemical experiments



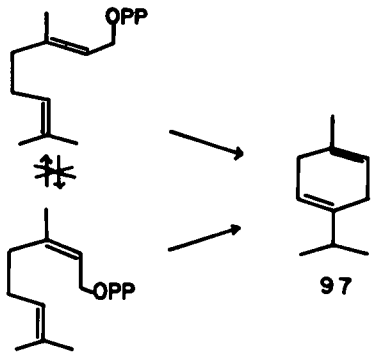
Scheme 55.

which might help sort-out some of the conflicting data have not yet been done. According to one view, the prenyl transferase(s) can preserve the *syn* stereochemistry associated with formation of *trans* double bonds and still remove 4-H_{si} of mevalonate while forming a *cis* double bond if the *si* rather than the *re* face of the isopentenyl pyrophosphate 3,4-double bond is attacked (Scheme 56). Determination of the resulting stereochemistry in the derived geraniol, nerol, *and* cyclized material would be very useful. In addition, as pointed out before, direct formation of *cis* double bonds should not affect the allylic hydrogens at C(1) derived from C(5) of mevalonate and this point might easily be checked.



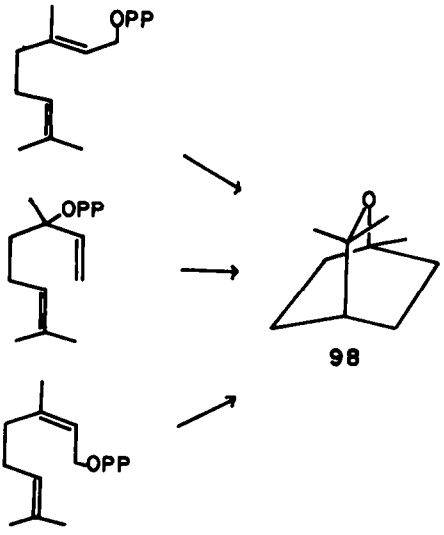
Scheme 56.

In a series of carefully executed experiments Croteau has isolated and partially purified a number of monoterpene cyclases from *Salvia officinalis* (sage) and *Thymus vulgaris* (thyme).[68,103] Enzymes from the latter system transformed both [1-^{3}H]-geranyl and neryl pyrophosphates to γ-terpinene (**97**) with similar K_m and V_{max} while failing to interconvert the double bond isomers (Scheme 57). No attempt was made to determine the extent of tritium retention from geranyl precursor by the use of double labeling and once again cyclization via a common enzyme bound intermediate was suggested. On the other hand 1,8-cineole (**98**) synthetase from sage showed a marked preference for neryl pyrophosphate, geranyl and linalyl pyrophosphates being converted at only 9% and 15% the rate, respectively, of the neryl precursor



Scheme 57.

(Scheme 58). The lowered conversions could, of course, be due to loss of any separate isomerase activity during enzyme extraction and purification and does not necessarily imply direct formation of the *cis*-isomer. It would also be useful to know whether these substrates are competing for the same active site.



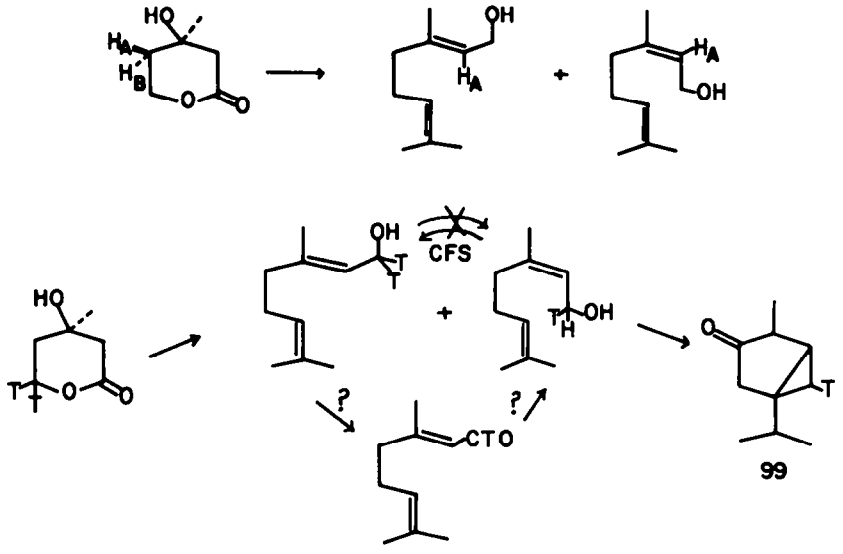
Scheme 58.

Studies by Banthorpe have also demonstrated that the loss of 4-H_{si} of mevalonate is not incompatible with the formation of terpenoids containing a *cis* double bond.[104] Both geranyl and neryl β-D-glucosides were formed in petals of *Rosa dilecta* with retention of the 4-proR hydrogen. Similarly (+)-α-pinene biosynthesized by *Pinus attenuata* retained tritium from only 4*R*-[-^{3}H]-mevalonate, although the positions of labeling were only inferred by analogy to ^{14}C degradation studies.

Banthorpe's group has also examined the biosynthesis of geraniol and nerol by cell-free extracts of *Tanacetum vulgare*.[105] Under conditions where no interconversion of geraniol and nerol or their respective phosphate or pyrophosphate esters could be observed, incubation of [^{14}C]-isopentenyl pyrophosphate gave nerol and geraniol in proportions ranging from 7:1 to 0.25:1 and in yields of 0.2–3.1%, depending on the time of preparation of the extracts. The variability in product ratios was used to argue in favor of direct formation of the two double bond isomers by distinct prenyl transferases. Further investigation of the concomitant formation of cyclized product isothujone (**99**) by these extracts has produced a good deal more complex picture and required some modification of these views, as described in the next section.

Isomerization with removal of a C(1) *hydrogen*

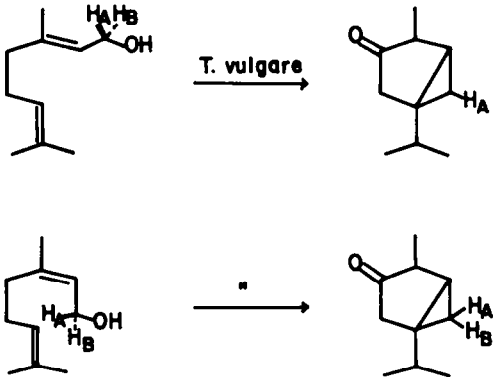
When the *T. vulgare* cell-free extract was incubated in separate experiments with 4*R* and 4*S*-[4-^{3}H,2-^{14}C]-mevalonate, the derived geraniol and nerol each carried tritium from only the 4*R*-^{3}H precursor, as indicated by the ^{3}H/^{14}C ratio[105] (Scheme 59). This result was consistent with either the previously



Scheme 59.

hypothesized direct formation of the double bond isomers or an initial formation of geraniol followed by isomerization. To test this latter notion [5-$^3H_2$,2-^{14}C]-mevalonate was used as substrate. Previous degradations had indicated a preferential labeling of the isopentenyl derived portion of geraniol and nerol even by cell-free extracts and evidence had been advanced for an enzyme-bound pool of dimethylallyl pyrophosphate.[106] Indeed both geraniol and nerol resulting from feeding of [5-$^3H_2$]-mevalonate lost all tritium activity upon oxidation to their corresponding acids. The two substances differed significantly, however, in their retention of tritium from the precursor, the $^3H/^{14}C$ ratio in geraniol being unchanged while that for nerol corresponded to 0.47 that of mevalonate. The latter results are inconsistent with simple direct formation of each isomer and suggest an initial formation of geraniol or a derivative, followed by redox isomerization with consequent loss of one C(1) hydrogen. Parallel experiments with whole leaves of *T. vulgare* supported this notion since the isothujone (**99**) which was isolated after administration of [5-$^3H_2$,2-^{14}C]-mevalonate had lost one half the tritium, although the residual tritium was not explicitly located. Also consistent with previous observations was the finding that only 4-H_{re} of mevalonate is incorporated into isothujone.

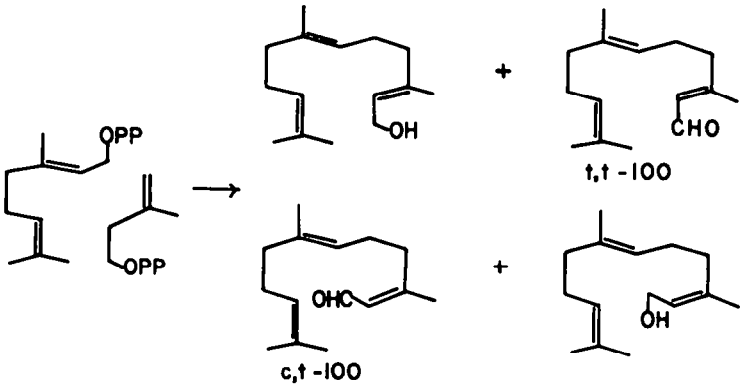
Further attempts to demonstrate the redox interconversion of geraniol and nerol or their pyrophosphate esters were unsuccessful using the cell-free extracts, which nonetheless were capable of converting mevalonic acid to not only geraniol and nerol but apparently to isothujone. Curiously it was found that with foliage of *T. vulgare*, the expected geraniol–nerol interconversion did take place (Scheme 60). Feeding of both 1*R*- and 1*S*-[1-^3H]-geraniol and nerol gave isothujone which retained both tritium atoms from nerol but had lost the 1*S*-^3H from geraniol, as indicated by the observed isotope ratios. The rather unlikely suggestion was made that the failure to observe interconverison in the



Scheme 60.

cell-free system was due to compartmentation or enzyme-bound intermediates *not encountered with the whole plant*! The whole plant results in fact cast doubt on the revalance of the cell-free experiments to the *in vivo* pathway and it may even be possible that several sets of enzymes exist within the plant for different purposes. At present there is data available to support or contradict almost any theory and further work is clearly needed before any solid conclusions can be drawn.

Further documentation of the redox pathway has come from Cori's laboratory.[100] Working again with enzymes from *C. sinensis* flavedo these investigators found that [1-^{3}H]-geranyl pyrophosphate in the presence of isopentenyl pyrophosphate was converted to an isomeric mixture of farnesols (Scheme 61).



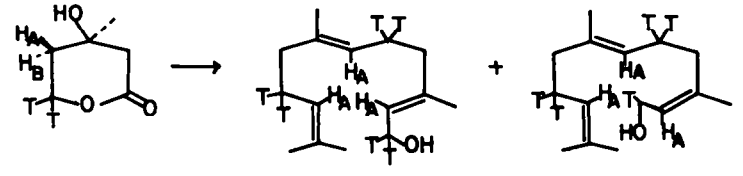
Scheme 61.

Neryl pyrophosphate was inactive as a precursor, arguing against interconversion of pyrophosphate esters. Similar observations had been made with a *P. radiata* extract.[107] With the *Citrus* enzymes, however, it was possible to detect formation of the corresponding *trans,trans*- and *cis,trans*-farnesals (**100**), although the identification of these substances rested exclusively on the chromatographic behavior of the aldehydes and their dinitrophenylhydrazone derivatives. Addition of NAD$^+$ to the extract favored formation of the *cis*-isomer. The significance of the suggested isomerization sequence to the *in vivo* cyclization pathway is unclear.

A number of *cis-trans* isomerizations of farnesols which are clearly artefacts have been reported. Jommi *et al.* have investigated the liver alcohol dehydrogenase catalyzed interconversion of *trans,trans*- and *cis,trans*-farnesol via the corresponding aldehydes.[108] The isomerization, which was shown to depend directly on the dehydrogenase itself and most likely involve an active site–SH group, proceded at a rate *ca.* 10^{-3} that of the normal oxidation.

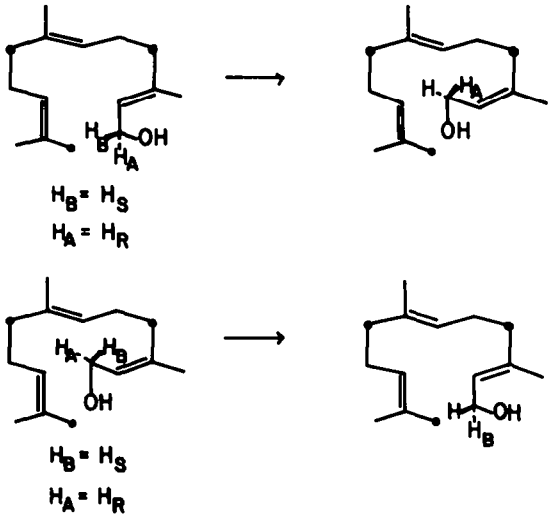
Similarly Suzuki has observed interconversion of *trans,trans* and *cis,trans*-farnesol as well as *trans,trans*- and *cis,trans*-10,11-epoxyfarnesol brought about by cell suspensions of *Helminthosporium sativum* and reported the isolation of the corresponding aldehyde intermediates.[109] During these conversions 1-H_{re} of farnesol was lost.

The most thorough documentation of the isomerization of farnesol and its derivatives has come from the work of Overton with a cell-free system derived from suspension tissue cultures of *Andrographis paniculata*.[110] These enzymes are capable of converting mevalonic acid to both farnesol isomers as well as bisabolene. Experiments with 4*R*- and 4*S*-[4-^{3}H]-mevalonates indicated stereospecific removal of 4-H_{si} and retention of 4-H_{re} at all three olefinic positions of both *trans,trans*- and *cis,trans*-farnesol (Scheme 62). Evidence for an isomerization came from the fact that incubation of [5-$^3H_2$,2-^{14}C]-mevalonate gave



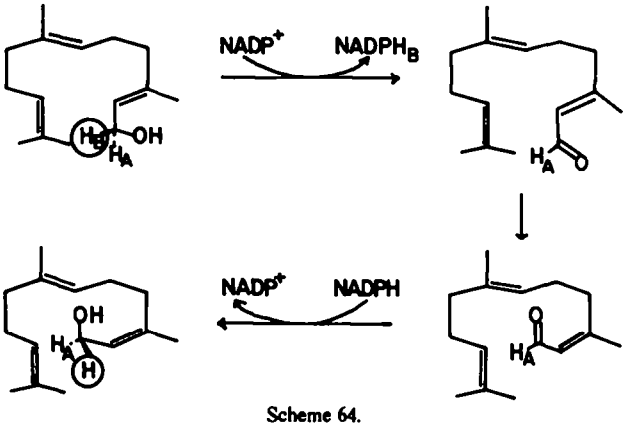
Scheme 62.

trans,trans-farnesol of unchanged ^{3}H/^{14}C ratio but *cis,trans*-farnesol which had lost one-sixth of the total tritium. This isomerization process was examined more directly by using [1-$^3H_2$,4,8,12-$^{14}C_3$]-farnesols (Scheme 63). The *trans,trans*-isomer lost 50% of its tritium label upon isomerization to



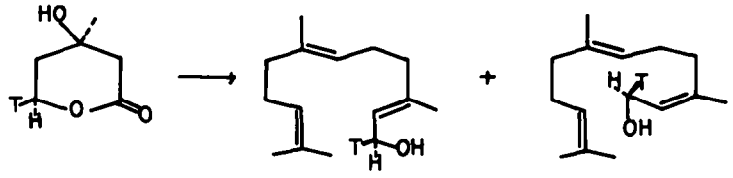
Scheme 63.

cis,trans-farnesol, while the reverse *cis,trans*-isomerization led to a similar 50% loss. The stereochemistry of this process was then investigated in detail. When 1*S*-[1-^{3}H]-*trans,trans*- and 1*R*-[1-^{3}H]-*cis,trans*-farnesols were used as substrates, isomerization led to loss of isotope, whereas incubation of the corresponding 1*R*-[1-^{3}H]-*trans,trans*- and 1*S*-[1-^{3}H]-*cis,trans*-farnesols resulted in retention of label in the isomerized material. These results established that conversion of *trans* to *cis* involves removal of 1-H_{si} but exchange of 1-H_{re} in the reverse reaction. These results were confirmed by the observation that the *cis,trans*-farnesol resulting from isomerization of 1*R*-[1-^{3}H]-*trans,trans*-farnesol (with retention of tritium) retained its label upon subsequent incubation with liver alcohol dehydrogenase, known to be specific for H_{re}. These results were explained by a scheme involving formation of an intermediate aldehyde **100** or its equivalent, 2,3-bond rotation, and reduction from the stereochemically opposite but geometrically equivalent face, as illustrated (Scheme 64).



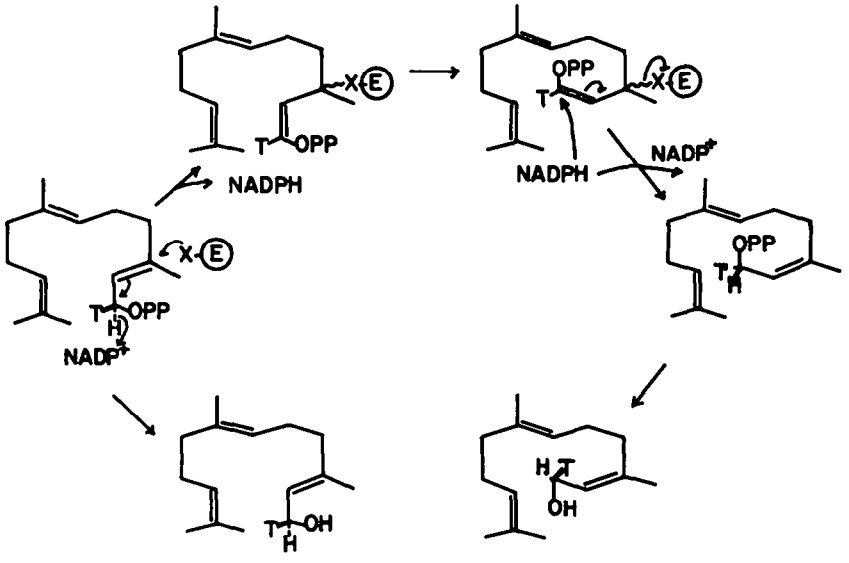
Scheme 64.

Experiments conducted with mevalonate stereospecifically tritiated at C(5) provided some surprises. Incubation of 5*S*-[5-^{3}H,2-^{14}C]-mevalonate was expected, in accord with the known formation of 1*S*-[1-^{3}H]-farnesyl pyrophosphate and the above isomerization results with free farnesol, to give *trans,trans* and *cis,trans*-farnesol both devoid of tritium at C(1). In fact, both products completely retained tritium label at C(1) and in the *si* position as established by exchange with an alcohol dehydrogenase–diaphorase system (Scheme 65). When experiments by Popjak,[18] referred to in Section II, reconfirmed the authenticity of the assigned mevalonate stereochemistry at C(5), it was thought that hydrolysis of farnesyl pyrophosphate by *A. paniculata* enzymes might have occurred with C–O bond cleavage and inversion at C(1). Controls involving *Andrographis* phosphatase, alkaline phosphatase, and lithium aluminium hydride cleavage of authentic 1*R*- and 1*S*-[1-^{3}H]-*trans,trans*-farnesyl pyrophosphates all indicated retention of configuration associated with regeneration of the free alcohols. These



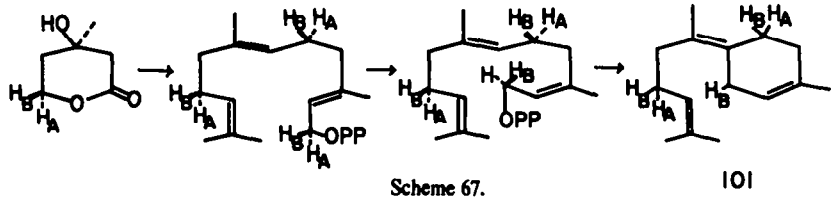
Scheme 65.

experiments were complicated by a curious prior racemization of the stereospecifically labeled pyrophosphates which, while apparently real, has not been observed in several other laboratories. Hydrolysis conducted in $H_2{}^{18}O$ using *A. paniculata* extracts also resulted in no observable incorporation of ^{18}O into farnesol, again consistent only with P–O bond cleavage. The only remaining explanation for the difference between the results of incubation of 5*S*-[5-^{3}H]-mevalonate and those for free farnesols is that different enzymes, with different specificities, isomerize farnesol and its pyrophosphate (Scheme 66).



Scheme 66.

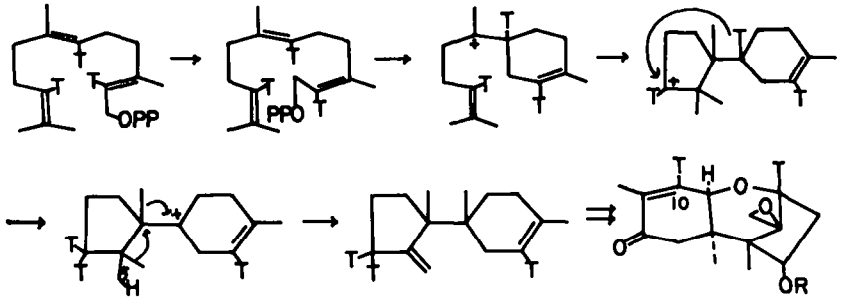
Further insight was gained by examining the formation of cyclized material, the sesquiterpene γ-bisabolene (**101**), from 5-tritiated mevalonates (Scheme 67). When [5-$^3H_2$,2-^{14}C]-mevalonate was incubated with the cell-free system the derived bisabolene had retained five-sixths of the original tritium while there was no loss of tritium when 5*S*-[5-^{3}H,2-^{14}C]-mevalonate was used as a precursor. The latter result is consistent with the presumed isomerization of *trans,trans*- to *cis,trans*-farnesyl pyrophosphate with retention of 1-H_{si}. Interestingly the system also incorporated *cis,trans*-farnesol, apparently without need for prior enzymatic pyrophosphorylation. Unfortunately no further stereochemical results of this interesting work have appeared.



Scheme 67. **101**

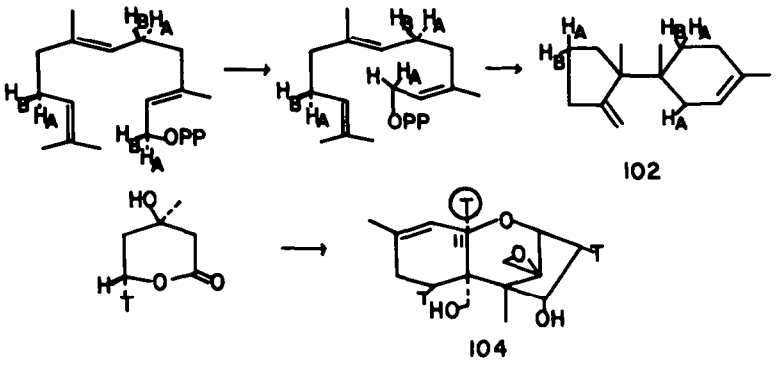
Working with cell-free enzymes of *Trichothecium roseum*, Hanson has provided further evidence for a redox isomerization of farnesyl pyrophosphates.[111] These extracts were capable of converting *trans,trans*-farnesyl pyrophosphate to the sesquiterpene hydrocarbon trichodiene (**102**), the demonstrated precursor of the trichothecane family of antibiotics.[112] The incorporation of the *trans,trans*-isomer was in keeping with the previous observation, made with whole cells, that 4-H_{re} of mevalonate labeled trichothecin (**103**) at C(10), the position derived from the proximal double bond of farnesyl

pyrophosphate.[113] Indeed incubation with the cell-free system of [2,6,10-$^3H_3$]-farnesyl pyrophosphate, formed enzymically from 4*R*-[4-^{3}H,2-^{14}C]-mevalonate, resulted in trichodiene which retained all three tritium atoms (one of which had undergone a previously demonstrated hydride shift)[114] (Scheme 68).



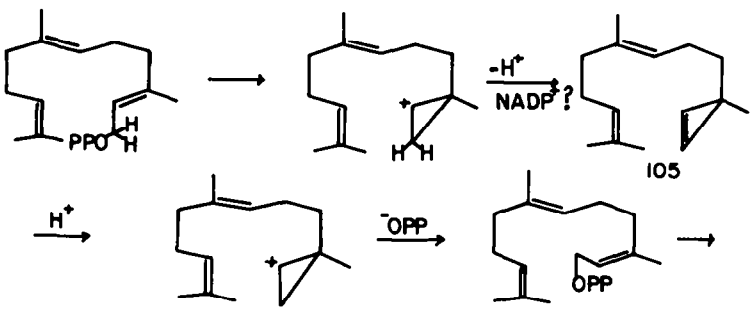
Scheme 68.

When [1,5,9-$^3H_6$, 4,8,12-$^{14}C_3$]-*trans,trans*-farnesyl pyrophosphate was employed, the resulting trichodiene carried only five-sixths of the tritium, as indicated by the change in $^3H/^{14}C$ ratio. Moreover, of the reisolated farnesols, the *trans,trans*-isomer had lost no tritium while the *cis,trans*-farnesol bore five-sixths of the original hydrogen isotope. Use of 1*R*-[1-^{3}H]-*trans,trans*-farnesol pyrophosphate gave trichodiene which retained all the tritium label, establishing that in the isomerization 1-H_{si} is lost, in accord with parallel results (Scheme 69) from Tamm's laboratory which showed that 5*R*-[5-^{3}H]-



Scheme 69.

mevalonate labeled the corresponding C(11) hydrogen of the trichothecane skeleton when fed to cultures of *Myrothecium verrucaria*.[115] The demonstrated stereospecificity is opposite to that observed by Overton for the *Andrographis* enzymes. In the *Trichothecium* system, however, free farnesol was not a substrate.[111] Further support for a redox process came from the observation that use of 4*S*-[4-^{3}H]-NADPH gave tritiated trichodiene, although once again neither the position nor the stereochemistry of the transferred tritium was determined. Hanson's work is marred by the implausible suggestion that isomerization occur by way of a cyclopropene intermediate **105** requiring that nicotinamide coenzyme act as a *proton* donor and acceptor! (Scheme 70). Unfortunately this suggestion has



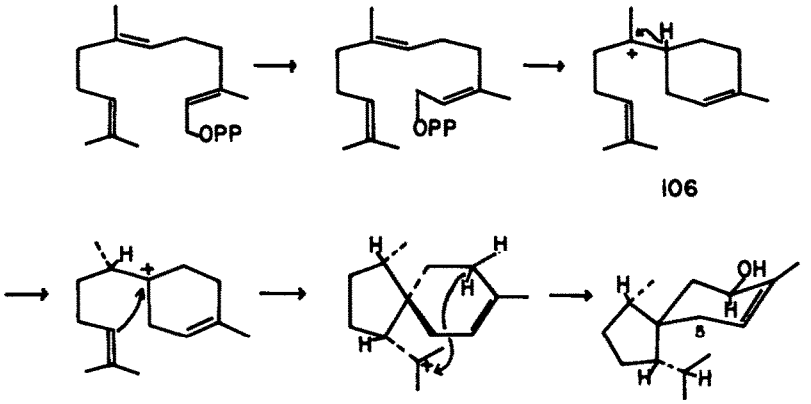
Scheme 70.

actually been cited at least once in the literature to account for double bond isomerization, if only to be rejected as unprecedented in favor of an alternative hypothesis.[105]

Isomerization without loss of C(1) *hydrogens*

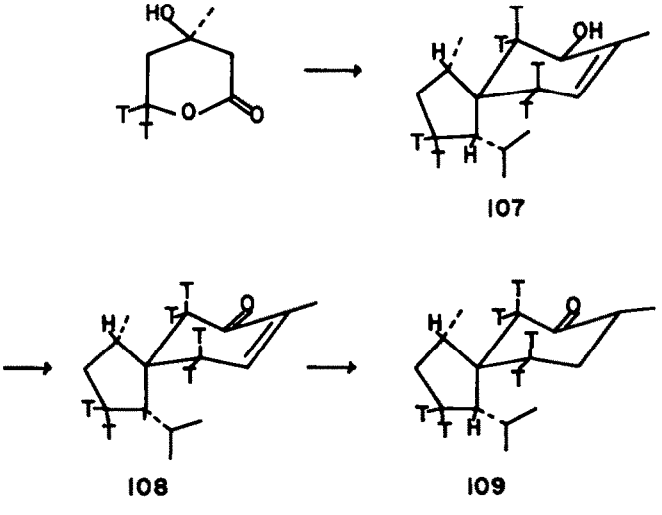
Although various redox schemes for double bond isomerization have received experimental support from a variety of sources, several examples of isomerization without compulsory loss of hydrogen in the biosynthesis of sesquiterpenes have been reported by Arigoni and his collaborators.

The sesquiterpene coccinol (**107**) is a metabolite of the fungus *Fusidium coccineum*.[116] Incorporations of [2-^{14}C]-mevalonate, [1-^{13}C]-acetate and [1,2-$^{13}C_2$]-acetate have supported a scheme for coccinol biosynthesis in which initial cyclization of a farnesyl pyrophosphate precursor gives a bisabolyl cation **106**, analogous to that implicated in the previously described work of Overton and Hanson (Scheme 71).



Scheme 71. 107

The bisabolyl cation in this case would undergo a hydride shift and ring closure, followed by a second 1,5-hydride shift and quenching of the positive charge by addition of water. Coccinol is particularly well suited for the study of farnesyl pyrophosphate isomerization since the presence of the 6-membered ring precludes direct formation from *trans,trans*-farnesyl pyrophosphate, while C(5), which is derived from C(1) of the farnesyl precursor, still carries two H atoms. In order to examine whether or not conversion of *trans,trans*-farnesyl pyropnosphate to coccinol involves loss of one of the C(1) hydrogens, a sample of [5-$^{3}H_2$,2-^{14}C]-mevalonate (^{3}H/^{14}C 13.34) was administered to *F. coccineum* (Scheme 72). The resulting coccinol, recrystallized as its phenylurethane showed an essentially unchanged ^{3}H/^{14}C ratio (12.52), corresponding to at most loss of 0.36 tritium equivalents. When the



Scheme 72.

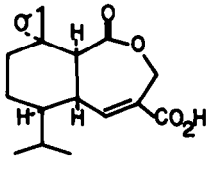
coccinol was converted to the ketone **108** and the latter exchanged with base under conditions known to exchange both C(1) and C(5), the $^3H/^{14}C$ ratio fell to 4.32, indicating exchange of two-thirds of the tritium. When the ketone was first reduced to the saturated derivative **109** and then subjected to exchange, only one-third of the tritium could be washed out, resulting in a $^3H/^{14}C$ of 8.81. These results clearly illustrate that loss of a full equivalent of hydrogen from C(1) of the *trans,trans*-farnesyl pyrophosphate precursor is not compulsory. The retention of hydrogen may be rationalized in one of three ways:

(1) Isomerization of *trans,trans*- to *cis,trans*-farnesyl pyrophosphate via nerolidyl pyrophosphate or by direct cyclization of the intermediate nerolidyl pyrophosphate. A related mechanism would involve isomerization and cyclization at the same active site via a set of stereoisomeric allylic cation—pyrophosphate anion pairs.

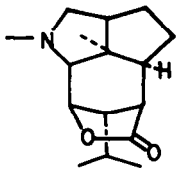
(2) Intervention of a redox process would be consistent with the observed retention of hydrogen only if the transiently generated NADPH failed to exchange its hydride with endogeneous oxidized coenzyme before reducing the intermediate aldehyde or its equivalent.

(3) Direct formation of *cis,trans*-farnesyl pyrophosphate, while conceivable, appears to be unlikely.

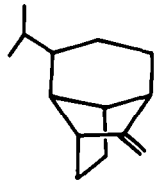
Further examples of the retention of C(1)-hydrogen during farnesyl pyrophosphate isomerization have come from a particularly thorough series of investigations of the biosynthesis of a group of cadalane and humulane-derived sesquiterpenes conducted by Arigoni *et al.*[117–119] Detailed incorporation experiments backed up by exhaustive chemical degradations have established that in the biosynthesis of avocettin (**110**), dendrobine (**111**), (−)-sativene (**112**), and (+)- and (−)-longifolene (**113**), the C(1)-



110



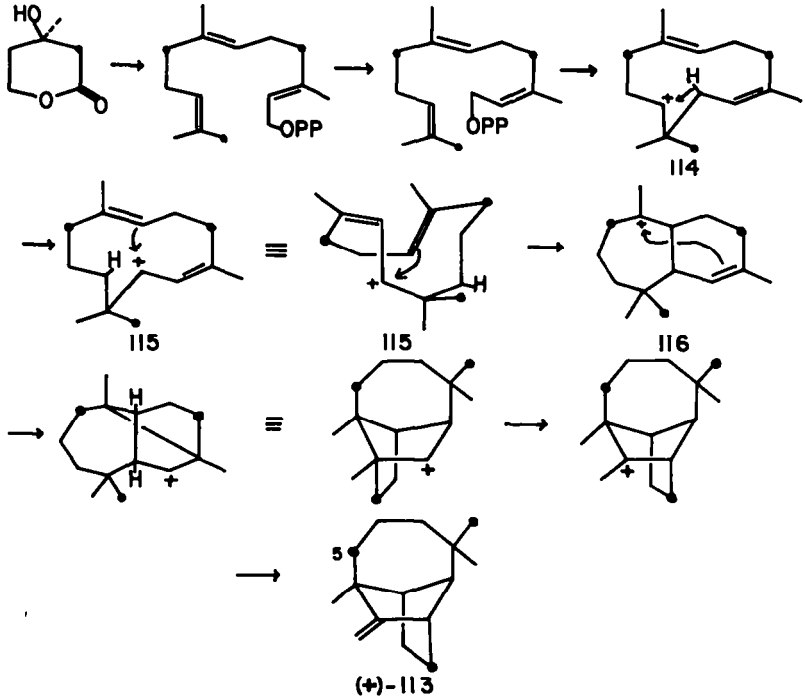
111



112

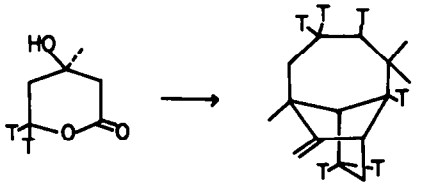
hydrogens of the farnesyl precursor are completely retained. Only the work with the antipodes of longifolene will be considered in any detail here.

(+)-Longifolene (**113**) is a constituent of the essential oils of several species of pine. Evidence for the biosynthetic pathway summarized in Scheme 73 was initially gained when administration of [2-^{14}C]-mevalonate to fresh cuttings of *Pinus ponderosa* gave radioactive longifolene (0.1% incorporation)



Scheme 73.

which was shown by degradation to contain one-third of the label at C(5). When [5-$^3H_2$,2-^{14}C]-mevalonate was incorporated the resulting longifolene showed no loss of tritium, as implied by the unchanged $^3H/^{14}C$ ratio, and subsequent degradations unambiguously located all six tritium atoms (Scheme 74).

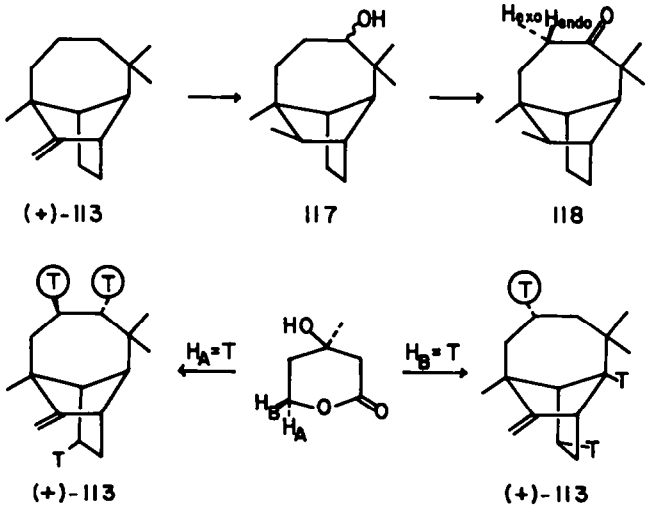


(+)-113

Scheme 74.

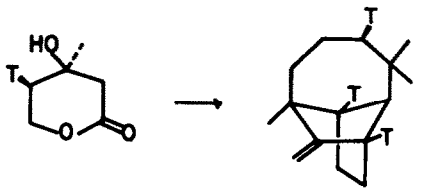
Besides providing further support for the general features of the proposed biosynthetic pathway (Scheme 73), the above feeding and degradation experiments established two important points: (1) cyclization of farnesyl pyrophosphate, by whatever mechanism, does not involve any loss of hydrogen from C(1) and (2) transfer of charge in a first-formed cyclization product **114** takes place by a 1,3-hydride shift. This shift generates a *cis*-humulyl cation **115** which further cyclizes to a himachalane cation **116** in which the necessarily *cis*-double bond of the 6-membered ring is derived from the 2,3-double bond of the farnesyl pyrophosphate. The proximal acyclic precursor must therefore be *cis,trans*-farnesyl pyrophosphate. That this *cis,trans*-farnesol derivative is formed by isomerization of an initially generated *trans,trans*-farnesyl pyrophosphate is supported by the finding that incorporation of 4*R*-[4-^{3}H]-mevalonate gave longifolene which contained all three possible tritium atoms.

Feeding of 5*R*-[5-^{3}H,2-^{14}C]- and 5*S*-[5-^{3}H,2-^{14}C]-mevalonates established that in the 1,3-hydride shift it is 5-H_{re} (H_A) which migrates and 5-H_{si} of mevalonate (H_B) which remains at C(1). (Scheme 75, circles



Scheme 75.

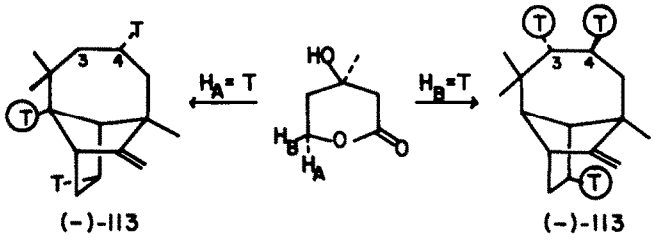
indicate those labels located by degradation.) Hydroboration of longifolene followed by autooxidation gave, in addition to longifolol, longifolan-3-ol (**117**) formed by intramolecular radical abstraction of the C(3)-*endo* hydrogen and capture of the C(3) radical with oxygen. Jones oxidation gave the corresponding longifolan-3-one (**118**). ORD and NMR measurements established that it was H(4)*exo* which underwent rapid exchange upon treatment of **118** with sodium hydroxide whereas removal of the H(4) *endo* proton required a more drastic reaction with methoxide in refluxing methanol for three days. Application of this sequence to (+)-longifolene (**113**) derived from stereospecifically 5-tritiated mevalonates established that the migrating hydrogen from 5-H_{re} becomes H(3)*exo*. This stereochemistry is consistent only with cyclization onto the *si*-face of the distal double bond. The labeling established at C(4) of longifolene by the ketone exchange experiments is of course that predicted from the usual inversion of configuration associated with prenyltransferase action. Similar degradations of longifolene derived from the above-mentioned feeding of 4*R*-[4-^{3}H]-mevalonate established that the corresponding tritium occupied the H(3)*endo* position again consistent with *si*-attack on the distal double bond of farnesyl pyrophosphate (Scheme 76).



(+)-113

Scheme 76.

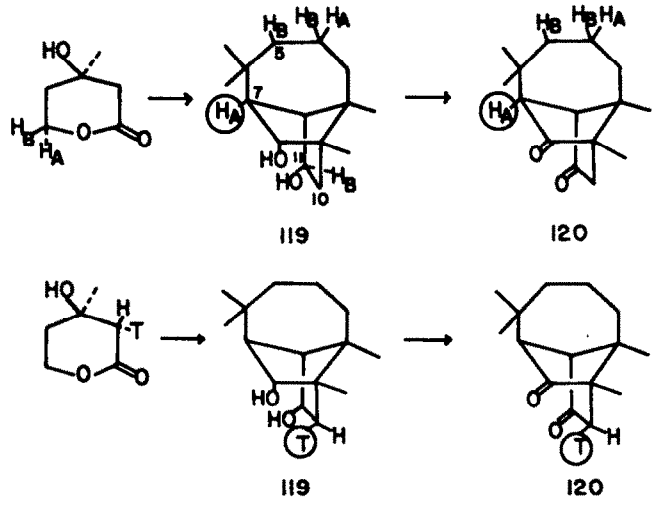
An unusual opportunity was provided by the discovery that cultures of *Helminthosporium sativum* produce the enantiomeric (−)-longifolene, albeit in small amounts, along with larger quantities of (−)-sativene.[117,119] Feedings of specifically tritiated mevalonates and degradations of the sort already described established the interesting facts that 1,3-hydride transfer involved migration of 5-H_{si} of mevalonate (H_B), while the presence of this newly transferred tritium at H(3)*exo* is in this case consistent only with attack on the *re* face of the distal double bond of farnesyl pyrophosphate (Scheme 77). Once again the stereochemistry of tritium labeling at C(4) and C(10) reconfirmed prenyltransferase catalyzed inversion of configuration.



(−)-113 (−)-113

Scheme 77.

A similar result has been obtained by Hanson in an investigation of the biosynthesis of culmorin (**119**) by *Fusarium culmorum*.[120] Culmorin belongs to the same configurational series as (−)-longifolene. Incorporation of [5-$^3H_2$,2-^{14}C]-mevalonate gave culmorin bearing all five possible tritium atoms, the sixth having been replaced by the OH at C(11) (Scheme 78). Feeding of 5*R*-5-^{3}H,2-^{14}C]-mevalonate and base-catalyzed exchange of the corresponding diketone **120** derived from culmorin established the presence of 5-H_{re} of mevalonate at C(7), implying that it was 5-H_{si} which migrated, presumably to C(5). It could also be shown by selective base exchange of the diketone **120** derived from feeding of 2*S*-[2-^{3}H,2-^{14}C]-mevalonate that 2-H_{si} of mevalonate became H(10)*endo*. This latter result established that introduction of the third isopentenyl pyrophosphate unit in the formation of farnesyl pyrophosphate took place on the usual *re* face, in spite of the subsequent intermediacy of *cis,trans*-farnesyl pyrophosphate.

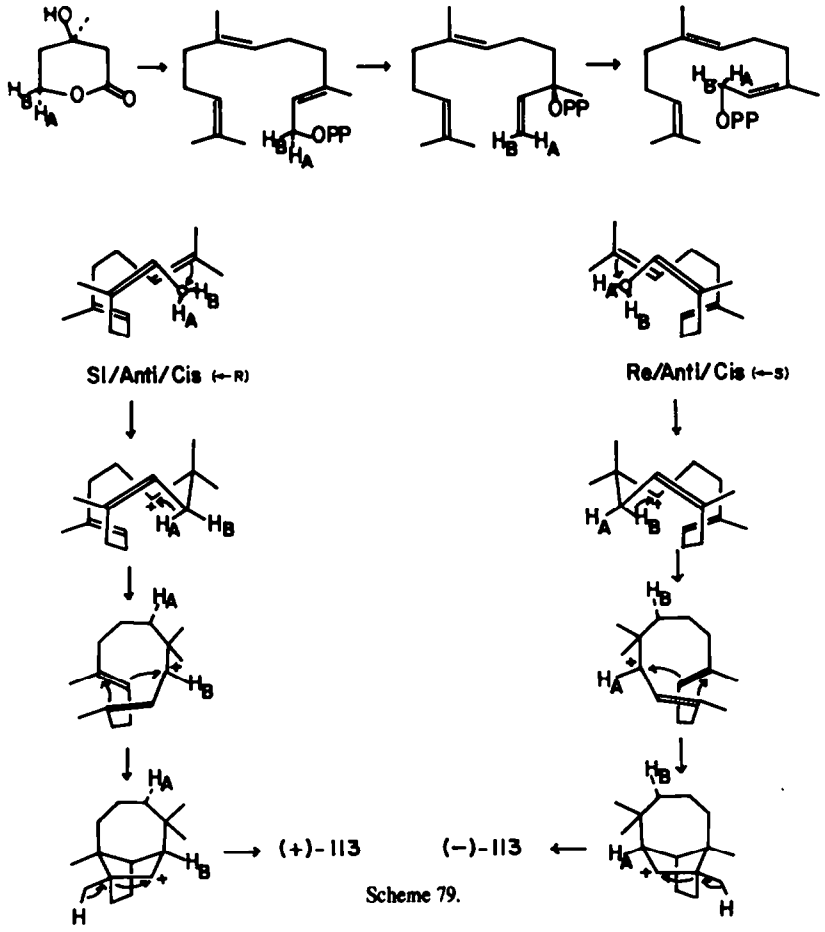


119 120

119 120

Scheme 78.

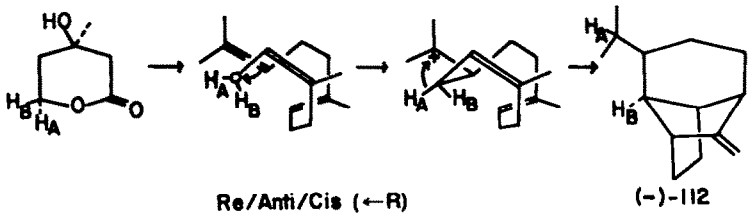
To explain these various results Arigoni has advanced an elegant stereochemical theory which relates the folding and stereochemistry of the farnesyl pyrophosphate precursor to both the hydrogen transferred in 1,3-migration and the resultant absolute configuration of the product (Scheme 79). In the formation



Scheme 79.

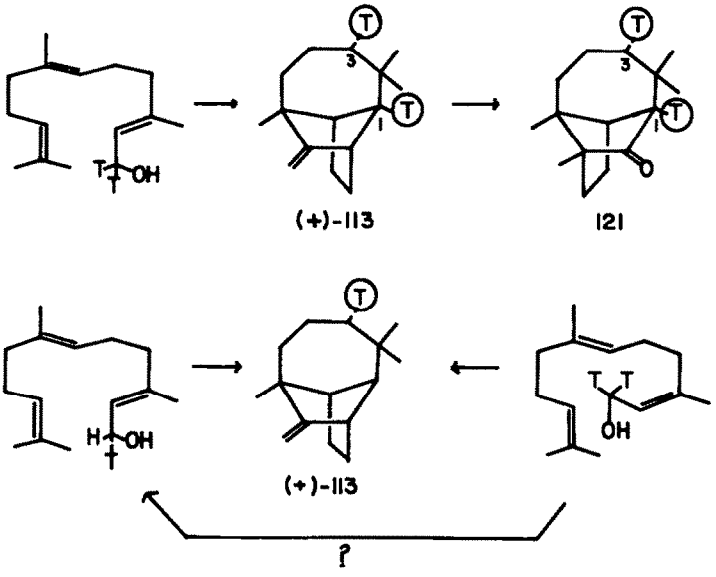
of (+)-longifolene initial cyclization of *cis,trans*-farnesyl pyrophosphate takes place on the *si* face of the distal double bond. In order for the observed transfer of H_A (derived from the 5-H_{re} of mevalonate) to take place, the *cis*-farnesyl precursor must fold in a conformation such that C(1) is *anti* to the olefinic hydrogen at C(10). The sequence is symbolized as *si, anti, cis* (←*R*). Examination of models indicates that the alternative *si, syn, trans* mode is sterically inaccessible, besides leading to an all-*trans*-humulyl cation which would be unable to undergo the necessary cyclization to the himachalane ring system as described above. For (−)-longifolene, cyclization of *cis*-farnesyl pyrophosphate takes place to the *re*-face of the distal double bond, in an anti relationship to the corresponding olefinic hydrogen, necessarily leading to complementary transfer of H_B (*re, anti, cis* (←*S*)). This theory requires that isomerization of *trans,trans*- to *cis,trans*-farnesyl pyrophosphate take place without loss of hydrogen from C(1) but with concomitant inversion at this center. As already pointed out, isomerization via nerolidyl pyrophosphate will necessarily account for the described result. It is also worthwhile noting that the observed retention of hydrogen associated with double bond migration takes place in two species, *Pinus* and *Helminthosporium*, previously implicated in redox isomerization processes leading to loss of hydrogen, as described above.

In further keeping with this theory are the results for (−)-sativene (**112**), also biosynthesized by *H. sativum* along with (−)-longifolene[119] (Scheme 80). Incorporation experiments, summarized elsewhere,[117] established that attack occurred on the *re* face of the distal double bond of farnesyl pyrophosphate with formation of a cadalane cation and 1,3-hydride transfer of H_A (5-H_{re}). The sequence of events is symbolized as *re, anti, cis* (←*R*). In each case the hydrogen which migrates is that which is best able to overlap with the initially formed cation. Similar conclusions have been drawn for the biosyntheses of avocettin (**110**) and dendrobine (**111**), although in these latter two cases the alternative direct cyclization of a *trans,trans*-farnesyl pyrophosphate precursor could not be ruled out.



Scheme 80.

Returning to a consideration of (+)-longifolene further insight was gained by feeding of specifically labeled farnesols[118] (Scheme 81). Administration of 1*RS*-[1-^{3}H]-*trans,trans*-farnesol to shoots of *P. ponderosa* supplemented with ATP gave longifolene with a respectable 0.3% incorporation. Tritium was

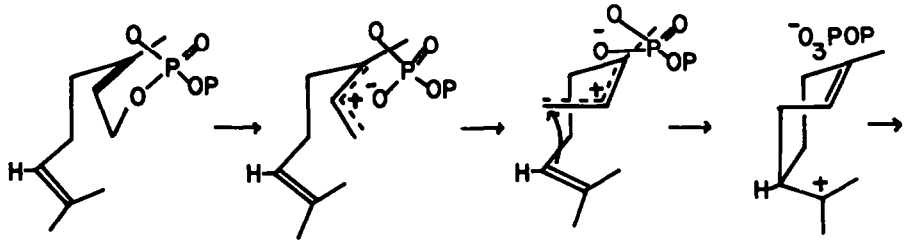


Scheme 81.

located at C(1) by acid catalyzed conversion to racemic longiborneol which was oxidized to (±)-longicamphor (121). Vigorous exchange with potassium t-butoxide served to remove one tritium from the bridgehead C(1) position. When 1*R*-[1-^{3}H]-farnesol was incorporated, degradation by the usual sequence established the presence of tritium at the expected H(3)*exo* position, consistent with the earlier feedings of [5-^{3}H]-mevalonate. Surprisingly, however, feeding of 1*RS*-[1-^{3}H]-*cis,trans*-farnesol resulted in loss of one tritium, the remainder being located once again at H(3)*exo*. This unexpected loss was ascribed to redox isomerization of the free alcohol, as observed for other *Pinus* species, and pyrophosphorylation of only the *trans,trans*-isomer. The possibility therefore exists that, as also observed for *A. paniculata*, different mechanisms may operate within a given organism for isomerization of free prenols and their pyrophosphate esters.

From the above discussion it should be evident that a clear and universally accepted picture of allylic pyrophosphate isomerizations has yet to emerge. Furthermore no easily identifiable trends are apparent and there is no facile correlation of the results according to whether they involve higher plants or fungi, whole cells or enzymes, mono- or sesquiterpenes, cyclized or acyclic substrates. In those cases where hydrogen is retained during isomerization, further work will be needed to determine whether this is definitive evidence in favor of a nerolidyl pyrophosphate intermediate or simply an artefact of the failure of transiently generated NADPH to undergo rapid equilibration with exogeneous oxidants. A variation of the tertiary allylic pyrophosphate isomerization mechanism is also worth considering. As already discussed, there is good evidence that ion-pair intermediates can account for direct displacements of allylic pyrophosphates as well as for the rearrangement of primary allylic pyrophosphates to tertiary allylic pyrophosphates. There is little reason to believe that the reconversion of the tertiary to the primary isomer would not also involve an ion pair. Furthermore the possibility has also been raised that such an ionic mechanism may be involved in allylic displacements, while the *in vitro* linalool solvolyses

provide an enticing model for terpenoid cyclization. Considering now the conversion of a *trans*-allylic pyrophosphate to cyclized product, the first step should be generation of an ion pair. Collapse of this ion pair at the tertiary center would generate the tertiary allylic pyrophosphate in a transoid conformation which is readily converted by 2,3-bond rotation to its cisoid conformational isomer. Reionization generates a second pair which can be attacked at C(1) by the neighboring double bond. Should the cisoid ion-pair collapse to the primary *cis*-allylic pyrophosphate, further reaction at C(1) would still require reionization and generation of the same ion-pair. According to this line of reasoning, formation of the primary *cis*-allylic isomer occurs by a non-productive equilibrium side reaction from the cisoid ion pair. The question then arises whether the tertiary allylic pyrophosphate is itself a mandatory intermediate. The answer hinges on the ability of the initially generated transoid ion pair to undergo isomerization to the corresponding cisoid structure (Scheme 82). The (gas phase) free energy barrier to rotation about the



Scheme 82.

2–3 bond of a tertiary allylic cation has been calculated to be 13.1 kcal,[24] considerably less than that estimated for the corresponding 1–2 bond rotation (28 kcal). Although a 13.1 kcal rotational barrier still corresponds to a modest maximum rate of *ca.* 3000 sec^{-1}, the barrier should actually be lower for the ion pair, which may well have an unsymmetrical structure in which a greater degree of positive charge is at the tertiary center. (Presumably the tighter the ion pair, the lower the rotational barrier. In the limit, obviously, there would be a covalently bonded tertiary allylic pyrophosphate! The question is whether this limit need be reached for 2,3-bond rotation to occur at a catalytically competent rate.) In solution, capture of the initially generated *trans*-allylic cation by solvent nucleophile competes favorably with any isomerization.[71,95] At the active site of the cyclase, however, the cation would be protected on one face by the paired pyrophosphate ion and on the other by the nucleophilic double bond of the substrate. Bond rotation and cyclization might therefore occur without formation of a covalent tertiary allylic pyrophosphate ester. Similar proposals have indeed been put forward at various times by Cori, Banthorpe and Croteau. The cyclase might be able to act on either double bond isomer as well as the tertiary allylic ester since any of these substrates can eventually lead to the same intermediate ion-pair. It is therefore interesting to recall that some of the terpenoid cyclases isolated from Sage and described by Croteau[103] are capable of utilizing geranyl and/or linalyl pyrophosphates in place of neryl pyrophosphate with no apparent requirement for nicotinamide coenzyme. It is not yet known whether the latter preparations contain a separate isomerase and it would therefore be extremely important to know whether isomerization and cyclization take place at the same active site. Careful studies of relative rates and the effects of inhibitors should soon be feasible and there is a possibility of some real progress in this confused area. Unfortunately, one of the drawbacks of a theory which postulates double bond isomerization catalyzed by the cyclase itself is the absence of a stable, characterizable intermediate. In fact to date much of the argument in favor of such a theory is necessarily negative, being based on evidence contradicting alternative hypotheses. Of these alternative mechanisms the various redox processes which have been observed must still be shown to be a *compulsory* part of the isomerization process if these theories are to be accepted. Arigoni's finding that in *Pinus ponderosa* different mechanisms may operate for farnesol and farnesyl pyrophosphate equilibration as well as Overton's similar conclusion for *A. paniculata* are particularly significant in this regard. The failure to identify a single mechanism for isomerization is particularly disturbing in light of the almost universal stereochemical course of essentially all other features of isoprenoid metabolism, many of which have been referred to repeatedly in this review. The details of the isomerization process, in fact, remain one of the last areas of allylic pyrophosphate metabolism about which there is still substantial disagreement. It is hoped that the next several years will yield the necessary experimental findings to resolve these issues.

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