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Enzymatic Formation of Sesquiterpenes

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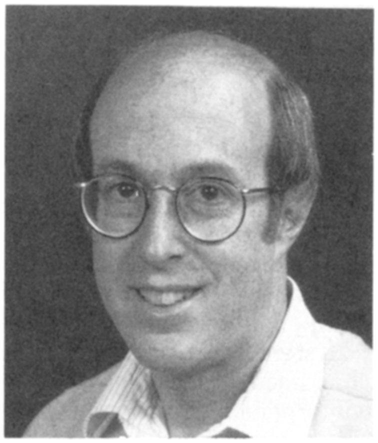
I. Introduction

For more than a century, the study of sesquiterpenes has challenged the ingenuity and technical skill of chemists and biochemists interested in the structure, chemistry, synthesis, and biological origins of this marvelously varied group of compounds. Indeed, the study of sesquiterpene biosynthesis has contributed a particularly rich chapter in the development of modern bioorganic chemistry. To date, representatives of these C_{15} terpenoids comprising more than 200 different carbon skeletons have been isolated from both marine and terrestrial plants as well as fungi and other microorganisms.[1,2] As first pointed out by Ruzicka,[3] all of these substances can be derived by cyclization of a common intermediate, initially proposed to be an activated derivative of the commonly occurring acyclic allylic alcohol farnesol and subsequently shown to be the corresponding diphosphate ester, farnesyl pyrophosphate.[4–6] In the 35 years since the formulation of the biogenetic isoprene rule, numerous studies carried out in a variety of plant and microbial systems have confirmed the essential correctness of Ruzicka's inspired proposal and established many of the details of the formation of cyclic sesquiterpenes. Until recently, the vast majority of such studies had been carried out by traditional incorporation experiments in which isotopically labeled precursors were fed to intact organisms, followed by detailed analysis of the distribution of label in the eventually formed metabolites. An extensive review of such classical precursor-product studies was published in 1981[7] along with comprehensive reviews of the closely related fields of monoterpene[8] and diterpene[9] biosynthesis. A comprehensive review of the metabolism of allylic pyrophosphates has also been published.[10] In subsequent years the biosynthesis of sesquiterpenes has been periodically reviewed.[11–14]



David Cane was born in New York, NY, in 1944. He received his B.A. in 1966 and Ph.D. in 1971 from Harvard University, studying under the direction of Professor E. J. Corey. After 2 years as an NIH Postdoctoral Fellow at the Eidgenossische Technische Hochschule in Zurich with Professor Duilio Arigoni, he joined the faculty of Brown University where he is Professor of Chemistry. He has held fellowships from the Sloan Foundation and the Japan Society for the Promotion of Science as well as a Research Career Development Award and a Fogarty Senior International Fellowship from the NIH. He is currently the holder of a John Simon Guggenheim Memorial Foundation Fellowship and a grant from the Fulbright Commission at the University of Cambridge where he is the Distinguished Visiting Fellow at Christ's College for 1989–1990. He is the recipient of the 1985 Ernest Guenther Award in the Chemistry of Essential Oils and Related Products from the American Chemical Society and the 1990 Simonsen Lectureship of the Royal Society of Chemistry. His research interests include the application of chemical, enzymological, and molecular genetic techniques to the study of the biosynthesis of natural products including macrolides, polyethers, and terpenes.

Although an enormous amount of information has been acquired over the years from precursor–product experiments with intact organisms, it has become increasingly evident that further progress in understanding the formation of sesquiterpenes, or indeed most families of natural products, must come from studies at the cell-free level. Classical biosynthetic incorporation experiments with intact organisms have frequently been hampered by poor uptake of exogenously administered precursors. The use of cell-free systems not only eliminates the natural cellular barriers to substrate utilization, but opens up the possibility of bringing all the powerful tools of modern mechanistic enzymology and protein chemistry to bear on the investigation of biosynthetic transformations.[15,16] Over the last decade, studies of sesquiterpene biosynthesis have entered an entirely new phase, as several individual cyclases catalyzing the conversion of farnesyl pyrophosphate to a variety of sesquiterpenes have been isolated and characterized. The availability of ses-

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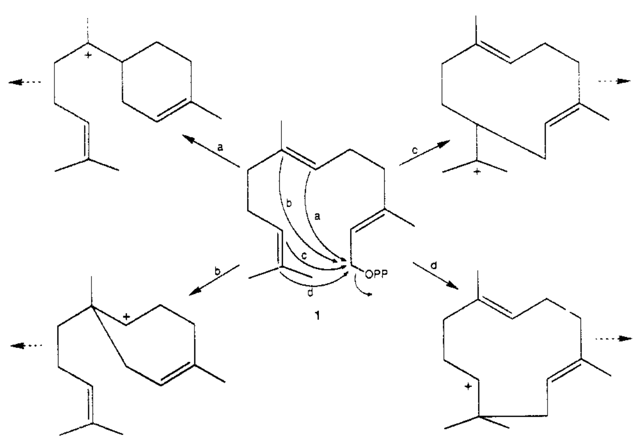

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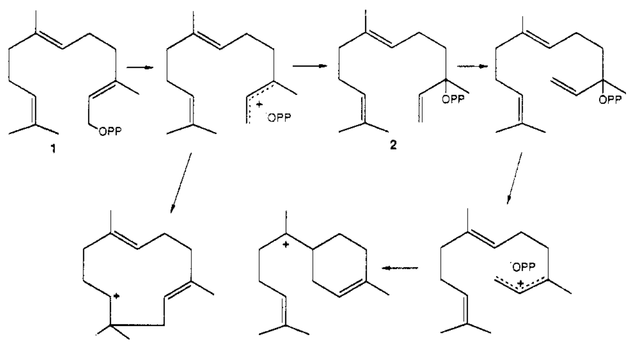


quiterpene synthases, in crude or purified form, has allowed detailed mechanistic analysis of the cyclization reactions themselves which lie at the heart of terpenoid biosynthetic theory. Recent studies of sesquiterpene synthases carried out in our own and other laboratories form the subject of the present review. The isolation and assay of terpenoid cyclases has recently been reviewed[17,18] and a stereochemical theory of sesquiterpene cyclizations has been proposed.[19] A comprehensive review of monoterpene biosynthesis has also appeared recently in this same journal.[20]

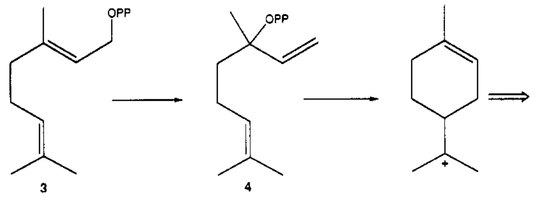
II. Background

According to the currently accepted hypothesis, as originally formulated by Ruzicka and subsequently elaborated by several groups of authors,[21-23] the formation of all cyclic sesquiterpenes can be accounted for by ionization of farnesyl pyrophosphate (FPP, 1) and electrophilic attack of the resultant allylic cation on either the central (paths a and b) or distal (paths c and d) double bond, followed by well-precedented cationic transformations involving further cyclizations and rearrangements, including methyl migrations and hydride shifts, culminated by quenching of the positive charge by loss of a proton or capture of an external nucleophile such as water or the original pyrophosphate anion (Scheme 1). This theory has in general been remarkably successful in rationalizing the results of numerous precursor incorportion experiments and in serving as a paradigm for the design of new experiments. Nevertheless, it was recognized early on that the direct formation of six-membered rings from the trans allylic pyrophosphate precursor is geometrically impossible, as is the analogous formation of ten- and eleven-membered rings containing cis double bonds. While the precise nature of the actual cyclization substrate was at one time a matter of considerable speculation and some controversy,[7,10] it is now generally accepted that the stereochemical barrier to direct cyclization of the *trans,trans*-farnesyl pyrophosphate substrate is overcome by initial isomerization to the corresponding tertiary allylic isomer, nerolidyl pyrophosphate (NPP, 2), which has the appropriate reactivity and conformational flexibility to allow formation of the resulting cyclic products.

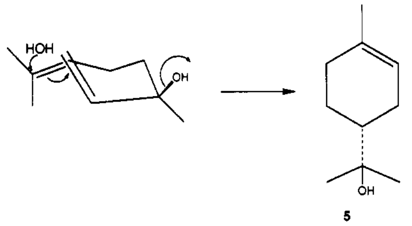
The first step in the enzymatic formation of sesquiterpenes is believed to be the ionization of *trans,trans*-farnesyl pyrophosphate to the corresponding transoid allylic cation–pyrophosphate anion pair (Scheme 2). This ion pair can in principle undergo any of several transformations. Nucleophilic attack on the back face of the allylic cation by the neighboring 10,11 double bond will result in net anti displacement of the pyrophosphate moiety from C-1 and formation of a new C–C bond. On the other hand, the analogous direct capture of the transoid allylic cation by the central double bond is geometrically forbidden. In this case, collapse of the ion pair will either regenerate the primary allylic substrate 1 or yield the transoid conformer of NPP (2) by a net suprafacial allylic rearrangement. The tertiary allylic pyrophosphate ester can undergo simple rotation about the newly generated 2,3 single bond followed by reionization to the corresponding cisoid allylic cation–pyrophosphate anion pair. The latter intermediate can then cyclize by electrophilic attack on either the central or distal double bonds with net anti displacement of the pyrophosphate moiety. According to this model, the two primary transformations involved in terpenoid cyclizations—isomerization and cyclization—although superficially very different in chemical consequences, involve fundamentally the same mechanism—ionization to an allylic cation–pyrophosphate anion pair—with the nature of the eventually formed products a function of the stereochemistry and conformation of the intermediates.

The ion-pair model for the enzymatic cyclization of farnesyl pyrophosphate is supported by related studies of terpene biosynthesis carried out at both the intact cell and enzyme level and is consistent with a substantial body of chemical model reactions. For example, we have demonstrated that the isomerization of FPP to NPP, catalyzed by an enzyme from *Gibberella fujikuroi*, takes place with net syn stereochemistry and have adduced evidence strongly implicating an ion-pair intermediate in this transformation.[24] Studies of the ring-forming reactions involved in the biosynthesis of several diterpenes from allylic pyrophosphates have shown that the allylic displacements by which these metabolites are generated all take place with net anti stereochemistry.[10,25] Most importantly, extensive investigations carried out by Croteau and his collaborators on the enzymic formation of cyclic monoterpenes have established that the first step in these cyclizations is a rate-determining isomerization of geranyl pyrophosphate (GPP) to the corresponding tertiary allylic isomer, linalyl pyrophosphate (LPP), and detailed stereochemical studies have shown that the absolute configuration of the eventually formed products is determined by which optical antipode of LPP is generated in the initial isomerization reaction[20] (Scheme 3). The

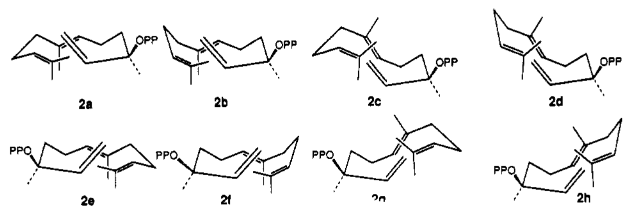
SCHEME 3



SCHEME 4



SCHEME 5



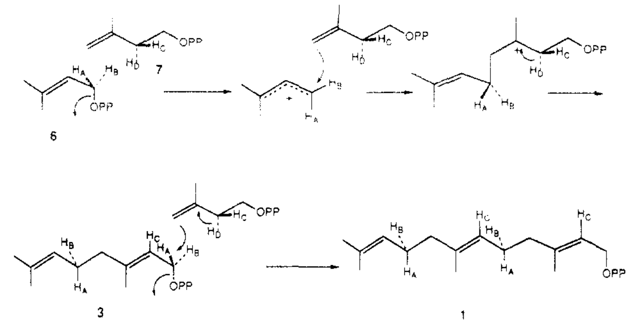
cationic nature of the normal enzymatic reaction intermediates has been further supported by a systematic study of the behavior of a series of substrate and reactive intermediate analogues as competitive inhibitors and anomalous substrates of a variety of monoterpene cyclases,[20,26,27] while an analysis of secondary isotope effects on the rate of formation of several monoterpenes is consistent with the rate-limiting step being ionization of geranyl pyrophosphate.[28] Finally, solvolytic conversion of linalool or its esters to α-terpineol (5), first reported by Stephan in 1898,[29] takes place with high enantiospecificity and by way of the same anti–endo conformation of the substrate which has subsequently been proposed for the enzymatic cyclization of linalyl and nerolidyl pyrophosphate[30] (Scheme 4). Croteau has recently presented a critical discussion of some of the most relevant chemical models of monoterpene cyclizations.[20]

In order for cyclization of either FPP or NPP to occur, it is evident that the π-orbitals of the relevant double bonds must be properly aligned so as to achieve the required geometry for interaction. This condition is met only if the individual double bonds of the allylic substrate are mutually perpendicular to a common plane. A systematic analysis has shown that only a limited number of conformations of the substrate satisfy this condition.[19] The presence of three trisubstituted double bonds in FPP significantly reduces the number of conformational degrees of freedom of the acyclic substrate. Thus C-1–C-4 and the attached methyl group, C-15, are necessarily constrained to a common plane, as are C-5–C-8 and C-14 and C-9–C-13. Similar considerations apply to the two internal double bonds of nerolidyl pyrophosphate. Since only those conformations of FPP or NPP which are competent to cyclize need to be considered, the number of degrees of freedom of the acyclic substrate are further restricted. To satisfy this requirement, C-1 must be brought to within bonding distance of either the central (paths a and b) or distal double bond (paths c and d), with displacement of the pyrophosphate from C-1 of FPP taking place with net anti stereochemistry. For those cases in which cyclization requires the intermediacy of NPP, it is expected that allylic displacement of the pyrophosphate moiety will take place in an anti sense from a cisoid conformation of the tertiary allylic isomer in which the dihedral angle $\theta_{\text{C-1-C-4}}$ is between $-30°$ and $+30°$. Given these constraints, the lowest energy conformations of the cyclizing substrate will naturally be those in which the individual double bonds are each perpendicular to the average plane of cyclization, thereby minimizing transannular interactions. The resultant local energy minima correspond to those conformations which are required for interaction of the relevant π-orbitals. As an illustration, the family of eight possible conformations of NPP which, in principle, are capable of cyclizing by pathways a or b is shown in Scheme 5. For each of these prototype conformations there is a subset whose members may differ in precise dihedral angles, although the relative orientation of the two trisubstituted double bonds and the configuration of the tertiary allylic pyrophosphate remains constant for each limiting conformer. A more detailed analysis of the various cyclizable conformations of farnesyl and nerolidyl pyrophosphate has been given[19] and further illustrations may be found in the examples discussed in the present review.

The stereochemical model for the enzymatic cyclization of farnesyl pyrophosphate has provided a powerful tool for the analysis of sesquiterpene biosynthesis and for the design of mechanistic and stereochemical probes of the cyclization reactions themselves. Underlying this approach is the principle that *the structure and configuration of* a *natural product is not arbitrary but is the natural outcome of its mode of biosynthesis.* It is therefore evident that on the basis of a knowledge of the structure and stereochemistry of a sesquiterpene, as well as an understanding of the underlying mechanistic principles governing its formation, it should be possible to infer the conformation of the cyclizing substrate at the active site of the relevant cyclase. This inferential process leads naturally to testable hypotheses whose experimental verification can lead to further refinements of the general model.

The above discussion has considered terpenoid cyclizations primarily from the point of view of the substrate, intermediates, and products of the cyclization reactions, without ascribing any explicit role to the sesquiterpene synthase itself, the catalyst mediating the transformation. Indeed, only in the last decade has any information become available concerning this synthetically versatile family of enzymes. Although so far only a relative handful out of the some 200 possible sesquiterpene synthases has been isolated and characterized, several generalizations have already begun to emerge. All of the enzymes examined to date, from both plant and microbial sources, are operationally soluble proteins of molecular weight in the range of 40 000–100 000. Several are monomers of M_r 40 000–60 000 while at least two (trichodiene synthase and patchoulol synthase) are homodimers of subunit M_r 40 000–44 000. The enzymes themselves, which are all moderately lipophilic, require no cofactors other than a divalent metal ion, Mg^{2+} usually being preferred. The

SCHEME 6



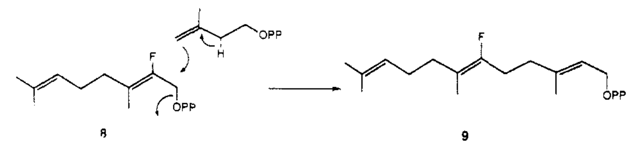
apparent K_m values for the acyclic substrate, farnesyl pyrophosphate, are generally in the 0.5–5 μM range, trichodiene synthase being an exception in having an usually low K_m for FPP of 25 nM. The turnover numbers, where known, are extremely modest, falling in the range 0.02–0.3 s^{-1}. Complicating the isolation and study of these proteins is the fact that many appear to be present at relatively low titres in the host organism. In all these respects the sesquiterpene synthases strongly resemble prenyl transferases, as well as the biogenetically closely related monoterpene and diterpene synthases, which display patterns of molecular weight, lipophilicity, cofactor requirements, substrate binding affinity, and turnover rates. The role of these enzymes would appear to be to bind the substrate in a conformation appropriate for cyclization and to catalyze the ionization of the allylic pyrophosphate ester. The details as to how binding and catalysis are controlled are obscure and essentially nothing is known about the structure of the active sites themselves.

III. Farnesyl Pyrophosphate Synthetase

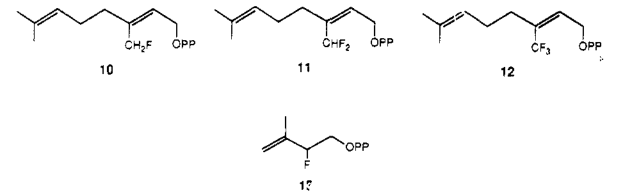
Farnesyl pyrophosphate is formed by the sequential head-to-tail condensation of dimethylallyl pyrophosphate (DMAPP, **6**) with 2 equiv of isopentenyl pyrophosphate (IPP, **7**)[31] (Scheme 6). This well-studied transformation, catalyzed by farnesyl pyrophosphate synthetase (EC 2.5.1.1), has provided both a conceptual and an experimental model for the study of sesquiterpene cyclizations. The reaction has been shown to involve the electrophilic addition of C-1 of DMAPP to C-4 of the cosubstrate, IPP, thereby generating the C_{10} homologue, geranyl pyrophosphate, which itself undergoes condensation with a second equivalent of isopentenyl pyrophosphate to yield farnesyl pyrophosphate. Analogous chain-elongation reactions are responsible for the formation of polyisoprenoids ranging in length from four to several thousand isoprene units. The enzyme-catalyzed cyclization of farnesyl pyrophosphate to generate sesquiterpenes, involving an electrophilic attack on one of the internal double bonds of the acyclic substrate, can be thought of as the intramolecular analogue of the prototypical prenyl transferase reaction.

The full stereochemical details of the farnesyl pyrophosphate synthetase reaction were established by Cornforth and Popjak in the course of their now classical studies of the biosynthesis of cholesterol.[32-35] By using stereospecifically deuterated precursors and analysis by degradation to chirally deuterated succinate, it was shown that the displacement of the pyrophosphate moiety from C-1 of both allylic substrates, DMAPP and GPP, takes place with net inversion of configuration. The latter result was initially interpreted in terms of an S_N2 reaction, implying that breaking of the C–O pyrophosphate ester bond is synchronous with formation of the new C–C bond. In the same series of experiments, electrophilic addition was shown to take place exclusively on the *re* face of the IPP double bond with stereospecific loss of the 2*re* proton, thereby establishing that the formal $S_{E'}$ reaction occurs with net syn stereochemistry. Although the original studies were carried out with the rat liver enzyme, subsequent investigations support the assumption that the same stereochemical course is followed by all prenyl transferases generating *E* double bonds.[31]

SCHEME 7



SCHEME 8



Extensive studies carried out by Poulter and Rilling have shed further light on the detailed mechanism of the prenyl transferase reaction, confirming the cationic nature of the enzymic reaction intermediates and establishing the timing of the various bond-breaking and bond-making steps.[36] By use of a series of fluorinated substrate analogues it was clearly demonstrated that, contrary to the earlier assumption, ionization of the pyrophosphate moiety to generate the corresponding allylic cation precedes formation of the new C–C bond by electrophilic attack on the double bond of IPP. Thus, incubation of 2-fluorogeranyl pyrophosphate (**8**) with farnesyl pyrophosphate synthetase in the presence of IPP was shown to give 6-fluorofarnesyl pyrophosphate (**9**) with a V_{max} nearly 5 orders of magnitude lower than that of the conversion of the natural substrate GPP[37] (Scheme 7). In parallel experiments it was found that the non-enzyme-catalyzed solvolysis of 2-fluorogeranyl mesylate was retarded with respect to the parent geranyl ester by a factor of 4.4 × 10^{-3}, whereas the S_N2 reactivity of the corresponding chloride was actually enhanced by a factor of 2. Further confirmation of the stepwise condensation mechanism came from a systematic structure–activity study by Poulter's laboratory involving a series of GPP analogues, **10–12**, in which the C-3 methyl group was substituted with one, two, and three fluorine atoms, respectively[38] (Scheme 8). In the latter case the relative rates of the farnesyl pyrophosphate synthetase reaction decreased nearly 10 orders of magnitude with increasing fluorine substitution. A Hammett plot comparing the relative rates of the enzyme-catalyzed condensation with the relative rate constant for solvolysis of the corresponding mesylates gave a straight line, indicating that both processes involve similar intermediates. From the observation that the slope of the plot (0.77) was less

SCHEME 9

$$\mathrm{Enz} + \mathrm{GPP\text{-}Mg^{2+}} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{Enz{\cdot}GPP\text{-}Mg^{2+}} \underset{k_4}{\overset{\overset{\mathrm{IPP\text{-}Mg^{2+}}}{k_3}}{\rightleftharpoons}} \mathrm{Enz{\cdot}GPP\text{-}Mg^{2+}{\cdot}IPP\text{-}Mg^{2+}} \xrightarrow{k_5}$$

$$\mathrm{Enz{\cdot}FPP\text{-}Mg^{2+}{\cdot}HPP_i\text{-}Mg^{2+}} \xrightarrow{k_6} \mathrm{Enz} + \mathrm{FPP\text{-}Mg^{2+}} + \mathrm{HPP_i\text{-}Mg^{2+}}$$

than 1, it was further concluded that the enzymatic reaction is in fact more sensitive to the electron-withdrawing groups at C-3, thereby implying a lack of participation of the IPP double bond in the ionization process. Studies with 2-F-IPP (13) have also produced evidence which appears to exclude covalent participation of a nucleophilic X-group in the enzyme active site while indicating that formation of the C–C bond precedes loss of the proton from C-2.[39]

Poulter has used a combination of isotope-partitioning and pulse-chase techniques to demonstrate that the kinetics of the reaction catalyzed by the avian liver enzyme involve an ordered sequential mechanism in which binding of the allylic substrate precedes that of IPP[40] (Scheme 9). By using rapid quench kinetics, it was also demonstrated that at 4 °C the rate-limiting step is release of FPP, the calculated rate of dissociation of the products FPP and inorganic pyrophosphate (k_6), 0.1 s^{-1}, being 50 times slower than the rate constant of the actual condensation step (k_5), 4.7 s^{-1}.

Several groups have described the action of FPP synthetase on a variety of substrate analogues. Of greatest relevance to the present discusion, Rilling has reported that incubation of the enzyme with FPP results in slow solvolysis to a mixture of products containing minor amounts of apparently cyclic sesquiterpenes of as yet undetermined structure.[41] These products presumably result from internal quenching of the allylic cation generated by ionization of the product FPP anomalously bound in the normal allylic pyrophosphate substrate binding site. Poulter has also reported that appropriately designed bisubstrate analogues of GPP and IPP undergo conversion to cyclic products when incubated with FPP synthetase.[42]

In contrast to sesquiterpene synthases, which are confined primarily to plants, fungi, and a limited number of streptomycetes, farnesyl pyrophosphate synthetases appear to occur in all living systems. FPP synthetases that have been isolated and purified from animal, plant, and both eukaryotic and prokaryotic microorganisms are all homodimers of subunit molecular weight 38000–43000 requiring the divalent cations Mg^{2+} or Mn^{2+} for activity. The substrates isopentenyl pyrophosphate, dimethylallyl pyrophosphate, and geranyl pyrophosphate exhibit apparent K_m values of 0.5–2 μM. Partial mapping of the active site of the avian liver enzyme by photoaffinity labeling with (*o*-azidophenyl)ethyl pyrophosphate has led to the identification of a labeled 30 amino acid CNBr peptide fragment shown by automated Edman degradation to have the sequence Leu-Asp-Leu-Ile-Gly-Ala-Pro-Val-Ser-Lys-Val-Asp-Leu-Ser-Thr-Phe-Gln-Glu-Glu-Arg-Tyr-Lys-Ala-Phe-Val-Pro-Tyr-Lys-Ala-Met[43] (Table I). On the basis of the observation that the five amino acids, IGAPV, bore 10% of the total radioactivity, it was suggested that this hydrophobic region might form part of the binding pocket for the hydrocarbon portion of the substrate. A further 30% of the label was located in the sequence ERY, with half the activity being associated with the arginine residue, leading to the inference that this side chain could be involved in binding of the pyrophosphate moiety.

TABLE I. Comparison of Partial Amino Acid Sequences for Farnesyl Pyrophosphate Synthetases from Avian Liver, Rat Liver, Human Fetal Liver, and Yeast

avian liver		**LDLI**G**AP**VS**KVDLS**T**F**Q**E**E**RYK**AF**V**P**YKA**M
rat liver	173	**LDLITAPQG**Q**VDL**GRY**TEKRYKSIVKYKTA**
human fetal liver	166	**LDL**L**TAPQG**N**VDL**V**RFTEKRYKSIVKYKTA**
yeast	170	M**DLITAP**ED**KVDLS**K**F**SL**K**KHSF**IV**T**FKTA**

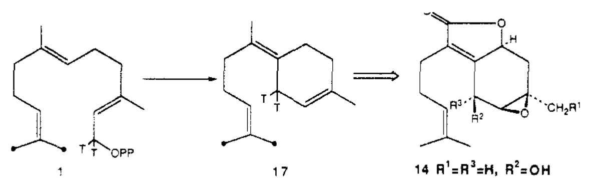
Recently, three groups have independently cloned and sequenced the FPP synthetase gene. Edwards screened a cDNA library with radiolabeled cDNA probes generated from rat liver mRNA obtained from animals with induced levels of cholesterol biosynthesis.[44] The presumptive gene was located by identification of an open reading frame (ORF) encoding a peptide of the appropriate size and amino acid composition, and showing a 57% homology to the previously identified 30 amino acid active site peptide of the avian enzyme (Table I). The function of the ORF was further confirmed by the demonstration that antibodies to a derived fusion protein inhibited rat liver prenyl transferase activity.[45] Subsequently, Sheares et al. screened a human fetal liver cDNA library in λgt11 using rat liver cDNA clones as probes and isolated a partial length cDNA which was shown to code for farnesyl pyrophosphate synthetase.[46] The 1115 bp of cloned cDNA contained an ORF which showed an 85% DNA sequence homology to the rat liver gene and an 88% identity at the deduced amino acid level. Subcloning into the *Eco*RI site of pUC18 allowed expression of a β-galactoside fusion protein which exhibited farnesyl pyrophosphatase synthetase activity and which reacted positively in Western blot analysis with antibodies to chicken liver farnesyl pyrophosphate synthetase. Finally, Poulter has reported the cloning of the analogous enzyme from the yeast *Saccharomyces cerevisiae*.[47] The yeast FPP sythetase gene was located in a 4.5 kb *Eco*RI fragment cloned into pBR322 which hybridized to a radioactively labeled synthetic 33-mer corresponding to the N-terminal sequence of the enzyme and based on the preferred yeast codon usage for a highly expressed gene. Sequencing revealed an ORF coding for a single polypeptide of M_D 40483, consisting of 342 amino acids and showing a significant amino acid homology to the putative liver enzyme as well as to the previously proposed active site fragment of the avian liver enzyme (Table I). Interestingly, while the arginine residue previously implicated in pyrophosphate binding is present in the corresponding rat liver sequence (R-192), the analogous site in the yeast enzyme is occupied by a basic lysine residue (K-189). Although the functional significance of this and other regions remains to be established, the way is now open for analysis of the active site by judiciously chosen site-directed mutagenesis experiments.

IV. Sesquiterpene Cyclases

A. Bisabolene Synthase

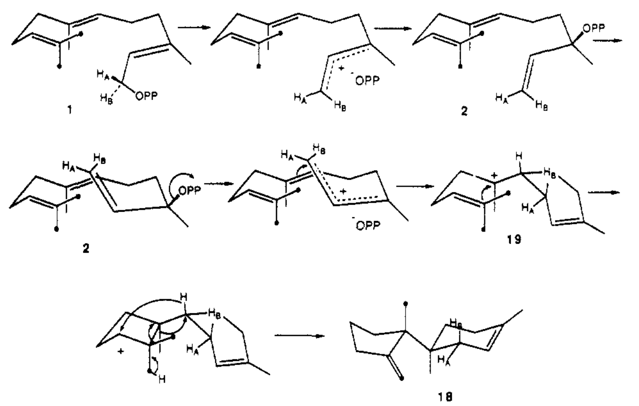
Tissue cultures of *Andrographis paniculata* accumulate a group of bisabolenoid sesquiterpenes, paniculides A–C (14–16)[48] (Scheme 10). Overton reported a number of years ago that a cell-free extract of these

SCHEME 10



14 $R^1=R^3=H$, $R^2=OH$
15 $R^1=R^2=OH$, $R^3=H$
16 $R^1=OH$, $R^2,R^3=O$

SCHEME 11

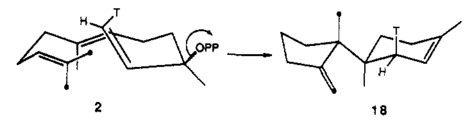


cultures will catalyze the conversion of *trans,trans*-FPP to the parent hydrocarbon (*Z*)-γ-bisabolene (17).[49,50] Since this cyclization is the simplest example of the generation of a six-membered ring by path a of Scheme 1, this work promised to shed light on the then obscure mechanism of isomerization of the trans 2,3 double bond of the substrate. Although early results appeared to support a redox isomerization mechanism,[51-54] subsequent more careful experiments established that the cyclization of FPP to bisabolene takes place without loss of either of the original hydrogen atoms attached to C-1 of the substrate.[55] Thus, incubation of [1-^{3}H,12,13-^{14}C]FPP with the 75000*g* supernatant obtained from *Andrographis* cultures gave rise to bisabolene, which retained all of the original tritium label, on the basis of the absence of change in the observed $^3H/^{14}C$ ratio. These results were further corroborated by cell-free conversion of both (5*R*)- and (5*S*)-[5-^{3}H,2-^{14}C]-mevalonate to bisabolene without change in isotopic ratio. No further work has been reported, however, on the mechanism of the cyclization reaction or on the characterization of the cyclase itself.

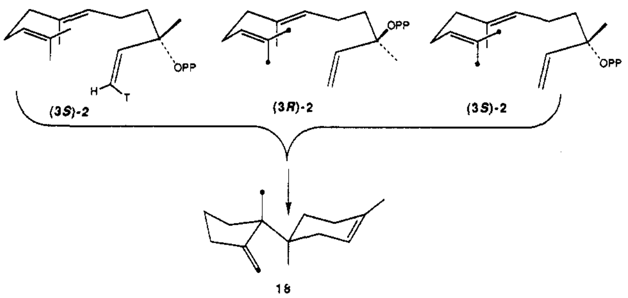
B. Trichodiene Synthase

By far the best studied of the sesquiterpene cyclases is trichodiene synthase, an enzyme isolated from a variety of fungal sources which catalyzes the conversion of *trans,trans*-FPP to trichodiene (18), the parent hydrocarbon of the trichothecane family of antibiotics and mycotoxins.[56,57] The enzyme was first described by Hanson, who prepared crude extracts from the apple mold fungus *Trichothecium roseum*.[58,59] Although it was initially suggested that isomerization of the FPP double bond might involve a redox mechanism, we subsequently demonstrated that conversion of [1-^{3}H,12,13-^{14}C]FPP (1, H_A = H_B = T) to trichodiene takes place without loss of either C-1 hydrogen atom.[60] Degradation of the resulting labeled trichodiene located both equivalents of tritium label at the expected site, C-11. On the basis of this observation, we proposed the cyclization mechanism, illustrated in Scheme 11, in which *trans,trans*-FPP undergoes initial isomerization to NPP (2). By rotation about the newly generated 2,3 single bond, NPP can adopt a conformation capable of cyclizing to the corresponding bisabolyl cation (19). The latter intermediate can then undergo further cyclization followed by a well-documented 1,5-hydride shift and sequential 1,2-methyl migrations to yield, after deprotonation, trichodiene.

SCHEME 12



SCHEME 13



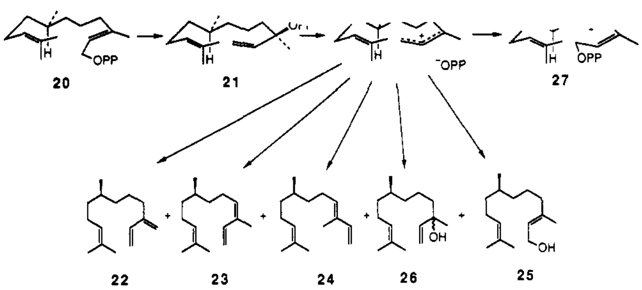
This mechanistic scheme was further tested by determination of the stereochemical course of the cyclization.[61] To this end, (1*S*)- and (1*R*)-[1-^{3}H,12,13-^{14}C]-FPP (1, H_A = T, H_B = H; 1, H_A = H, H_B = T) were separately incubated with trichodiene synthase and the resulting labeled samples of trichodiene were analyzed by the previously developed chemical degradation route to determine the site and stereochemistry of labeling. In this manner it was established that H-1*re* of FPP becomes H-11β of trichodiene, while H-1*si* of FPP ultimately occupies the H-11α position of 18, corresponding to net *retention* of configuration in the displacement of pyrophosphate from C-1 of the allylic substrate. This result, which can be contrasted with the established *inversion* of configuration in the analogous intermolecular condensation reaction catalyzed by farnesyl pyrophosphate synthetase, is the necessary consequence of the required isomerization of the trans-2,3 double bond of FPP which must precede cyclization to the bisabolyl intermediate.

Nerolidyl pyrophosphate was shown to be a viable substrate for the cyclase by incubation of (1*Z*)-[1-^{3}H,12,13-^{14}C]NPP (2) with trichodiene synthase[62,63] (Scheme 12). Analysis of the resulting trichodiene by the usual chemical degradation sequence established that the tritium label was located exclusively at the predicted site, H-11β. The absolute configuration of the enzmatically active enantiomer of NPP was then determined by incubating a mixture of (3*S*,1*Z*)-[1-^{3}H]NPP and (3*RS*)-[12,13-^{14}C]NPP with the cyclase (Scheme 13). The resulting trichodiene was labeled exclusively with ^{14}C, thereby proving that only (3*R*)-NPP is utilized in the cyclization. Finally, we demonstrated that NPP is an enzyme-bound intermediate in the cyclization by carrying out a competitive incubation of [1-^{3}H]FPP and [12,13-^{14}C]NPP. By examining the $^3H/^{14}C$ ratio of the resulting trichodiene as well as of recovered farnesol and nerolidol at short reaction times, it was established that both FPP and NPP can compete for the same active site and that in the conversion of FPP to trichodiene the tertiary allylic pyrophosphate intermediate is not released from the surface of the enzyme. Further analysis of $^3H/^{14}C$ ratios also indicated that the V_{max}/K_m value for (3*R*)-NPP is approximately 1.5–2 times that of FPP. These preliminary values indicate that the rate of cyclization of free NPP is roughly twice that for the isomerization–cyclization of FPP, implying that the rate determining step for the overall transformation may be the initial isomerization step. Direct determination of the actual steady state parameters for NPP is currently in progress. In the meantime, these observations are fully consistent with the results of analogous investigations of monoterpene cyclases which have indicated that the tertiary allylic pyrophosphate, linalyl pyrophosphate, is the preferred substrate for cyclization, based on relative V_{max}/K_m values.[20]

The results of the above-described labeling experiments are fully consistent with the mechanism of Scheme 11. From the fact that cyclization of (3*R*)-NPP takes place on the 1-*re* face of the vinyl double bond, formation of the bisabolyl cation must take place by way of the proposed anti–endo conformation of nerolidyl pyrophosphate. Furthermore the combined knowledge of the stereochemical course of the conversion of both farnesyl and nerolidyl pyrophosphate to trichodiene establishes that the allylic isomerization of FPP to NPP catalyzed by trichodiene synthase takes place by a syn rearrangement, identical with the stereochemical course previously established for the *Gibberella fujikuroi* FPP–NPP isomerase.[24] These stereochemical conclusions are fully consistent with the results of extensive experiments on the cell-free formation of cyclic monoterpenes.[20]

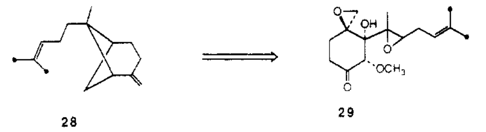
The observation that NPP can serve as a substrate for trichodiene synthase has facilitated the direct study of the cyclase component of the multistep conversion of FPP to trichodiene. Unfortunately, direct observation of the initial isomerization step has not been possible since the intermediate NPP is normally not released from the active site of the cyclase prior to cyclization. To overcome this difficulty, we have examined the action of purified trichodiene synthase from *Fusarium sporotrichioides* on 6,7-dihydrofarnesyl pyrophosphate (**20**). Although this analogue of FPP is in principle capable of undergoing isomerization to 6,7-dihydronerolidyl pyrophosphate (**21**), the latter product cannot be cyclized due to the absence of the 6,7 double bond.[26] In collaboration with Dr. Thomas Hohn, we have found that both (7*S*)- and (7*R*)-*trans*-6,7-dihydrofarnesyl pyrophosphate ((7*S*)-**20** and (7*R*)-**20**) are modest competitive inhibitors of trichodiene synthase.[64] The observed K_I values for (7*S*)-**20** and (7*R*)-**20**, 395 nM and 220 nM, respectively, were 10–15 times the K_m for FPP (25 nM) and roughly half the K_I for inorganic pyrophosphate alone. More significantly, incubation of either enantiomer of the reduced analogue with trichodiene synthase gave rise to a mixture of products shown to consist of 80–85% of the isomeric olefins **22**–**24** and 15–20% of the allylic alcohols **25** and **26** (Scheme 14). The water soluble products resulting from incubation of (7*S*)-**20** also contained 24% of the isomeric *cis*-6,7-dihydrofarnesyl pyrophosphate (**27**). The observed rate of formation of the combined olefinic and alcoholic products was 10% of the V_{max} for the corresponding conversion of FPP to **18**. The observed products presumably result from ionization of the transiently generated 6,7-dihydronerolidyl pyrophosphate at the cyclase active site and alternative quenching of the resultant ion pair by deprotonation, capture of water, or reversion to the isomeric primary allylic pyrophosphate esters. In particular, the cis isomer **27**, which is produced by way of the cisoid conformer of the ion pair, could only be formed through the intermediacy of the tertiary allylic pyrophosphate **21**.

SCHEME 14

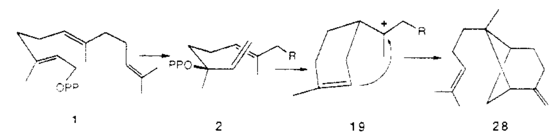


Hohn has purified trichodiene synthase to homogeneity from both *Fusarium sporotrichioides*[65] and *Gibberella pulicaris* (*Fusarium sambucinum*).[66] Both enzymes require Mg^{2+} for activity and are homodimers of M_r 45 000. The *F. sporotrichioides* cyclase exhibits a rate maximum between pH 6.75 and pH 7.75 and a calculated k_{cat} of 0.15 s^{-1}. The similarity of the two enzymes was indicated by the fact that antiserum to the *F. sporotrichioides* cyclase cross-reacts with the *G. pulicaris* enzyme. These antibodies were used to screen a λgt11 library of *F. sporotrichioides* genomic DNA, allowing the isolation of the structural gene for trichodiene synthase.[67] The complete amino acid sequence for a protein of M_r 43 999 has been deduced from the corresponding nucleotide sequence which revealed an 1182 nucleotide (nt) ORF containing an unusual 60 nt, in frame, intron. Excision of the intron and subcloning of the recombinant open reading frame in pDR540 allowed expression of trichodiene synthase activity in *Escherichia coli*.[68] Interestingly, extracts of the *E. coli* transformants were found to contain trichodiene itself, indicating that the recombinant enzyme is able to cyclize endogeneously produced FPP. The recombinant cyclase appeared to be identical with the native fungal enzyme in subunit molecular weight, p*I*, and chromatographic mobility. A truncated copy of the trichodiene synthase gene has also been used in successful gene disruption experiments in which transformants of *F. sporotrichioides* had lost the ability to produce trichothecenes.[66] Very recently, Hohn has also cloned the corresponding trichodiene synthase gene from *G. pulcaris*.[66] Comparison of the nucleotide and deduced amino acid sequences revealed that the two synthases had a 90% homology at the nucleotide level and a 95% homology at the amino acid level, perhaps not surprising in view of the closeness of the two organisms. Experiments are now underway in Hohn's laboratory using site-directed mutagenesis to probe a region of the cyclase enzyme showing apparent homologies to the arginine-containing region of FPP synthetase previously implicated in binding of the pyrophosphate moiety.[66]

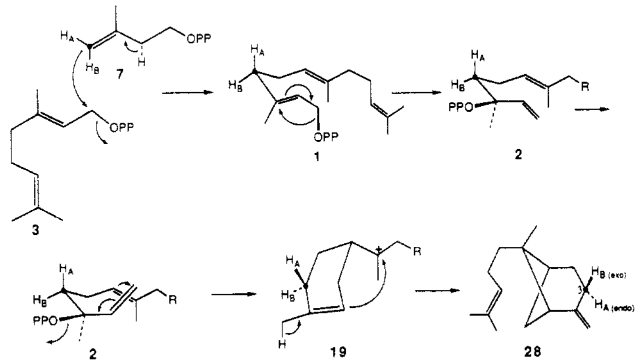
SCHEME 15



SCHEME 16



SCHEME 17

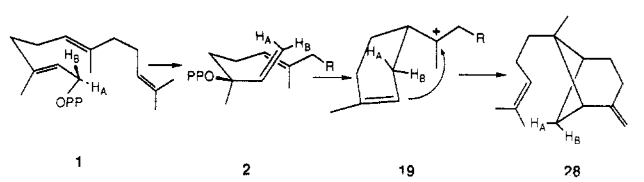


C. Bergamotene Synthase

We have reported the isolation of *β-trans*-bergamotene (**28**) from the mycelia of *Pseudeurotium ovalis*[68] and demonstrated the biosynthetic conversion of labeled **28** to the immunosuppressive antibiotic ovalicin (**29**)[70] (Scheme 15). Incubation of [12,13-^{14}C]FPP (K_m 5 μM) with a cell-free extract of *P. ovalis* gave bergamotene labeled as expected in the terminal methyl groups, as established by oxidative degradation of the side chain.[71] On the basis of analogy to the well-documented conversion of GPP to *β*-pinene, we have proposed that bergamotene is formed by isomerization of FPP to NPP, followed by cyclization of **2** to the bisabolyl cation (**19**) which undergoes further cyclization to the bicyclo[3.1.1]heptane ring system (Scheme 16).

The absolute configuration of (−)-*β-trans*-bergamotene was established to be 1*S*,5*S*,7*R* by a novel combination of enzymatic and spectroscopic methods.[72] Thus avian farnesyl pyrophosphate synthetase was used to prepare (4*S*)- and (4*R*)-[4-^{2}H]FPP (**1**, H_A = D, H_B = H; **1**, H_A = H, H_B = D) from GPP and (4*E*)- and (4*Z*)-[4-^{2}H]IPP, respectively, taking advantage of the known stereochemical course of the prenyl transferase catalyzed reaction[32-35] (Scheme 17). Each of the chirally deuterated FPP samples was separately incubated with crude bergamotene synthase and the resulting labeled bergamotenes were analyzed by high-field (61.4 MHz) ^{2}H NMR. The sample of **28** derived from (4*S*)-[4-^{2}H]FPP displayed a ^{2}H NMR signal at δ 2.53, previously assigned to H-3$_{endo}$ (H-3*si*) of bergamotene. In the complementary experiment the sample of **28** obtained from (4*R*)-[4-^{2}H]FPP gave rise to a ^{2}H NMR signal at 2.24, corresponding to H-3$_{exo}$ (H-3*re*). Since the enzymatic cyclization does not affect the configuration at the chirally deuterated center, the ^{2}H NMR experiment establishes the absolute configuration of the enzymatically generated hydrocarbon.

SCHEME 18



With a knowledge of the absolute configuration of (−)-*β-trans*-bergamotene, it was also possible to determine the stereochemistry of the cyclization of FPP.[71] Incubation of [1,1-$^2H_2$]FPP (**1**, H_A = H_B = D) with the crude cyclase gave bergamotene which displayed the expected ^{2}H NMR signals at δ 1.41 and 2.30, previously assigned to H-6$_{exo}$ and H-6$_{endo}$, respectively (Scheme 18). When the incubation was carried out with (1*S*)-[1-^{2}H]FPP (**1**, H_A = D, H_B = H), the derived bergamotene was labeled only in the H-6$_{exo}$ position, as established by the appearance of the ^{2}H NMR signal at δ 1.41, while cyclization of (1*R*)-[1-^{2}H]FPP (**1**, H_A = H, H_B = D) gave bergamotene enriched exclusively at H-6$_{endo}$. The cyclization therefore occurs with net retention of configuration at C-1 of FPP, as predicted by the proposed intermediacy of NPP.

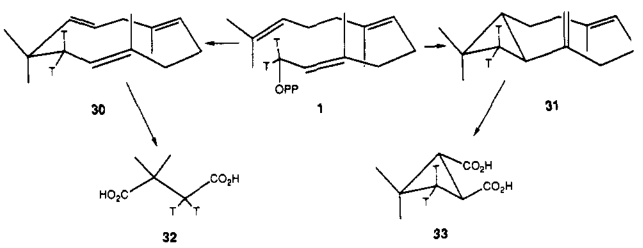
It should also be noted that the use of ^{2}H NMR is particularly convenient for the analysis of enzymatically generated products, avoiding as it does the necessity of elaborate chemical degradations to establish the site and stereochemistry of labeling. We have found that this method is readily applicable when sufficient quantities of sesquiterpene synthase are available to produce >100 nmol of cyclized product.

Based on the absolute configuration of the product *β*-bergamotene, it is expected that the actual cyclization intermediate is (3*S*)-NPP[73] (Scheme 16). Indeed, incubation of (3*S*)-[12,13-^{14}C]NPP with bergamotene synthase did lead to formation of labeled bergamotene. Surprisingly, however, the enzyme was also capable of cyclizing the enantiomeric (3*R*)-[12,13-^{14}C]NPP to bergamotene. This behavior, while unexpected, is reminiscent of the ability of the pinene cyclases of *Salvia officinalis* to cyclize the unnatural enantiomers of their respectively linalyl pyrophosphate intermediates.[20,74,75] Experiments are currently in progress to establish the absolute configuration of the bergamotene products generated from the individual enantiomers of NPP and to determine the relevant steady state kinetic parameters for these substrates. Preliminary experiments have indicated that *β*-bergamotene synthase is also capable of cyclizing the lower homologue GPP to *β*-pinene.

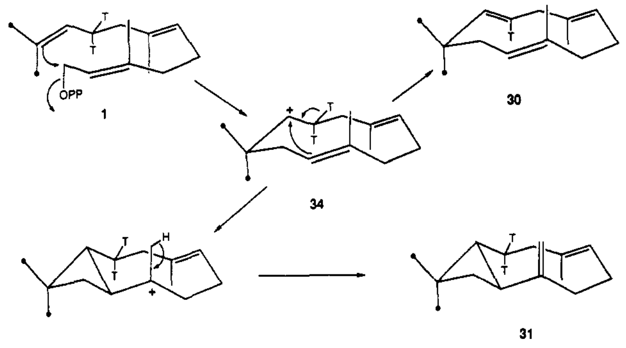
D. Humulene and Caryophyllene Synthases

Humulene (**30**) and *β*-caryophyllene (**31**) are two of the most commonly occurring sesquiterpenes. Croteau has reported that a soluble enzyme preparation obtained from the 105000*g* supernatant of sage (*Salvia officinalis*) leaves will convert [1-^{3}H]FPP to a 2:1 mixture of **30** and **31**.[76] The specificity of labeling was confirmed by oxidative degradation of the enzymatically generated sesquiterpenes to 2,2-dimethylsuccinic acid (**32**) and *trans*-norcaryophyllenic acid (**33**), respectively (Scheme 19). Base-catalyzed exchange of **32** resulted in complete loss of label, thereby locating the tritium at the expected site. The fact that similar base treat-

SCHEME 19



SCHEME 20



ment of **33** failed to remove any label was consistent with the presence of tritium at the methylene carbon of the cyclobutane ring.

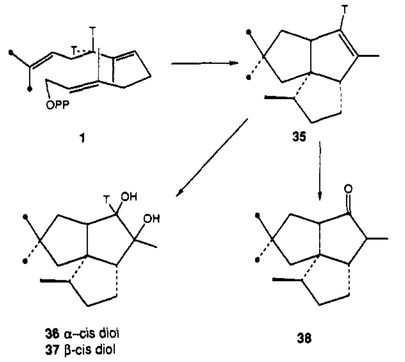
These results can be explained by the cyclization mechanism shown in Scheme 20 in which the humulyl cation **34**, generated by cyclization of FPP by path d (Scheme 1), can either lose a proton from C-9 to form humulene directly, or further cyclize by electrophilic attack on the 2,3 double bond followed by loss of a proton to generate (−)-caryophyllene. Further support for the proposed mechanism came from the observation that cyclization $[9\text{-}^3H,12,13\text{-}^{14}C]$FPP gave caryophyllene of unchanged $^3H/^{14}C$ ratio, whereas the derived humulene had lost half the original tritium.

Partial purification of the extracts obtained from leaf epidermis allowed resolution of the two cyclizing activities and preliminary characterization of each enzyme.[77] Both cyclases had molecular weights M_r 58000 as estimated by their mobility on Sephadex G-100 and depended on a divalent cation, Mg^{2+} being preferred, as usual. The K_m for FPP was also typical, ca. 1.7 μM, and the observed pH optimum, 6.5, was in the usual range. Inorganic pyrophosphate had a slight stimulatory effect on the cyclization rate at low concentrations (50–80 μM), while at higher concentrations it was a modest competitive inhibitor (I_{50} 1 mM). Inhibition by 100 μM *p*-hydroxymercuribenzoate suggested the involvement of an essential thiol residue, while the histidine-directed reagent, diethyl pyrocarbonate, was also an effective inhibitor (I_{50} ca. 500 μM). On the other hand neither arginine- nor serine-directed reagents had any significant effect on the observed cyclase activity.

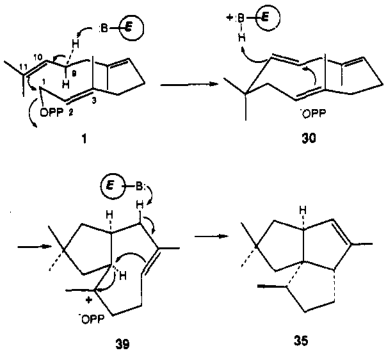
E. Pentalenene Synthase

Pentalenene (**35**),[78] the parent hydrocarbon of the pentalenolactone family of antibiotics, is one representative of a group of several dimethylcyclopentane sesquiterpenes formally derived from humulene.[7] Whereas the majority of these metabolites are formed by fungi, pentalenene is unique in being produced by several species of *Streptomyces*. We have isolated a soluble cyclase from *Streptomyces* UC5319 which catalyzes the cyclization of FPP to pentalenene.[79] In early work, it was shown that incubation of $[8\text{-}^3H,12,13\text{-}^{14}C]$FPP with crude pentalenene synthase gave pentalenene which retained half the original tritium activity, as indicated by the $^3H/^{14}C$ ratio of the derived crystalline diols **36** and **37** (Scheme 21). A simple sequence of hydroboration–oxidation to the ketone **38** served to locate the tritium at C-7 of **35**.

SCHEME 21



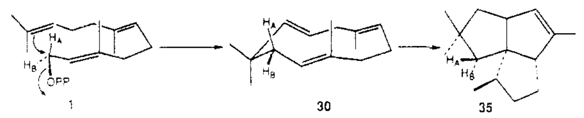
SCHEME 22



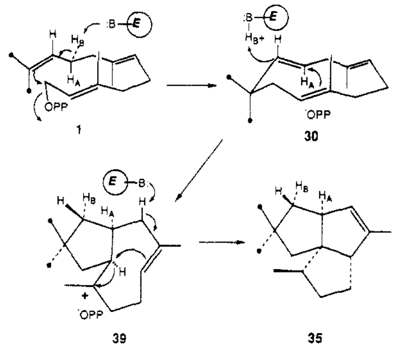
The formation of pentalenene can be readily explained by the cyclization mechanism illustrated in Scheme 22. Initial cyclization of FPP will give humulene (**30**), folded in the *RSR*-CT conformation, as shown.[19,80] Protonation of humulene at C-10 leads to cyclization and formation of the protoilludyl cation **39**, which can undergo a hydride shift followed by further cyclization and deprotonation to generate pentalenene. The proposed conformation of the humulene intermediate can be inferred from the known relative and absolute configuration of the eventually formed pentalenene. Furthermore, the absolute sense of folding of the farnesyl pyrophosphate precursor can be deduced from the results of incorporation experiments, using ^{13}C-labeled precursors and intact cells, which established that C-12 of FPP becomes the β-methyl group (C-12) of pentalenene and derived metabolites.[81,82] This stereochemical model is fully consistent with a battery of incorporation experiments which we have carried out in order to elucidate the mechanistic details of the cyclization.

The stereochemical course of the displacement of the pyrophosphate moiety from C-1 of FPP was established by ^2H NMR analysis of pentalenene derived from chirally deuterated samples of the allylic pyrophosphate substrate.[83,84] Thus incubation of $(1R)\text{-}[1\text{-}^2H]$FPP (**1**, $H_A = H$, $H_B = D$) with pentalenene synthase gave pentalenene carrying deuterium in place of H-3*re*, while cyclization of $(1S)\text{-}[1\text{-}^2H]$FPP (**1**, $H_A = D$, $H_B = H$) gave pentalenene labeled at H-3*si* (Scheme 23). Taken to-

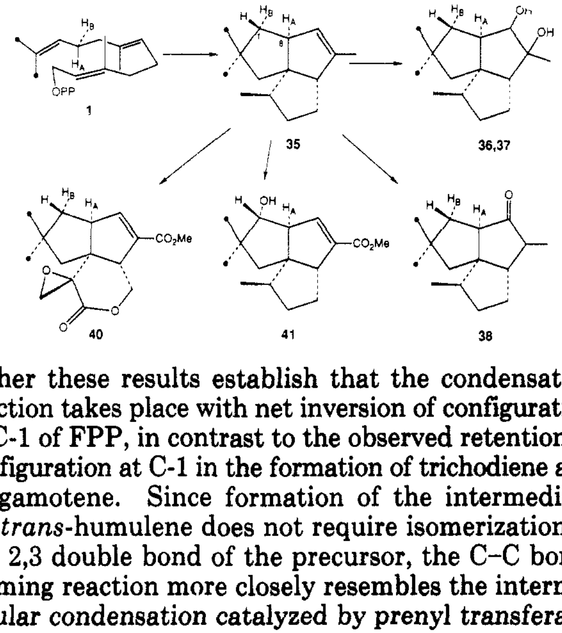
SCHEME 23



SCHEME 24



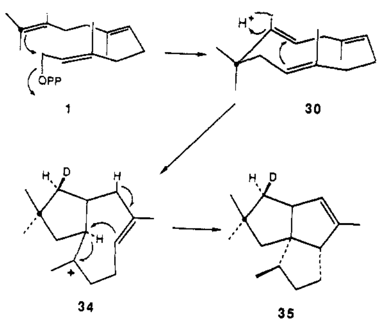
SCHEME 25



gether these results establish that the condensation reaction takes place with net inversion of configuration at C-1 of FPP, in contrast to the observed retention of configuration at C-1 in the formation of trichodiene and bergamotene. Since formation of the intermediate *all-trans*-humulene does not require isomerization of the 2,3 double bond of the precursor, the C–C bond-forming reaction more closely resembles the intermolecular condensation catalyzed by prenyl transferase.

Further insight into the mechanism of the cyclization reaction was gained by incubation of [9-^{3}H,12,13-^{14}C]-FPP (1, H_A = H_B = T) with pentalenene synthase[85] (Scheme 24). The resulting labeled pentalenene required the bulk of the original tritium, as indicated by the measured ^{3}H/^{14}C ratios of the corresponding diols, 36 and 37 (Scheme 25). One equivalent of tritium was located at the bridgehead, C-8, by base-catalyzed exchange of the derived ketone, 38. The remaining equivalent of tritium was located by refeeding labeled pentalenene to cultures of *Streptomyces* UC5319. Comparison of the ^{3}H/^{14}C ratios of the resulting epipentalenolactone F 40 and pentalenic acid 41 established that the tritium had been transferred to C-1. Although the postulated intermediacy of humulene would involve the removal of one of the original C-9 protons of FPP in the initial cyclization step, this same proton could be redonated to humulene were exchange with the external medium slow compared to protonation of the transiently generated humulene intermediate. The efficiency of proton return suggests that the relevant base mediating the transfer must carry no additional hydrogen atoms. Taken together with the conclusions of an earlier stereochemical analysis which indicated that the absolute sense of folding of the farnesyl pyrophosphate is the same as that of the presumed humulene intermediate, the strong implication is that the humulene is generated in the same conformation from which it undergoes further cyclization.

SCHEME 26



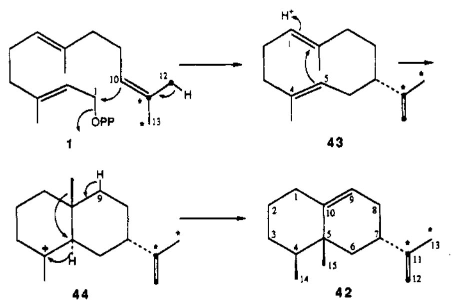
The stereochemistry of the deprotonation step was determined by carrying out separate incubations of (9*R*)- and (9*S*)-[9-^{3}H,4,8-^{14}C]FPP (1, H_A = T, H_B = H; 1, H_A = H, H_B = T) with pentalenene synthase and analysis of the derived samples of pentalenene by the previously developed combination of chemical and microbial degradation sequences[84,86] (Schemes 24 and 25). It was thereby established that H-9*re* of FPP becomes H-8 of pentalenene while H-9*si* of the substrate undergoes intramolecular transfer to C-1 of **35**. Since the cyclization had already been shown to involve electrophilic attack on the *si* face of the 10,11 double bond of FPP, the allylic addition–elimination reaction, a formal S_E' process, must take place with net anti stereochemistry, in contrast to the demonstrated syn stereochemistry for the analogous intermolecular addition–elimination reaction catalyzed by prenyl transferase. The observed stereochemistry of the pentalenene synthase reaction is completely consistent with the postulated *RSR*-CT conformation of the cyclizing FPP substrate which prevents access of any enzymic base to the H-9*re* proton.

The stereochemical course of the subsequent reprotonation at C-10 of humulene was established by incubation of [10-^{2}H,11-^{13}C]FPP with pentalenene synthase. (Scheme 26). Analysis of the resulting pentalenene by a combination of ^{2}H and ^{13}C NMR spectroscopy established that the deuterium atom occupied exclusively the H-1*si*(H-1β) position in pentalenene, consistent with the transfer of the original H-9*si* proton of FPP to the 10*re* face of the 9,10 double bond of humulene, exactly as predicted by the originally proposed stereochemical cyclization model.

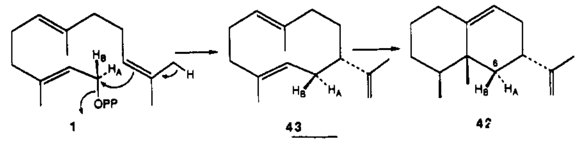
We have previously speculated that the putative base which mediates the original intramolecular proton transfer may also be responsible for the subsequent deprotonation step which results in generation of the ultimate product of the cyclization, pentalenene. It is also conceivable that the inorganic pyrophosphate ion, released in the course of the original condensation reaction, can be used to stabilize positive charge in subsequent intermediates at sites corrsponding to C-1, C-2, and C-3 of the precursor.

To better understand the role of the cyclase itself in controlling the complex sequence of events involved in the cyclization reaction, we have undertaken the purification and characterization of pentalenene syn-

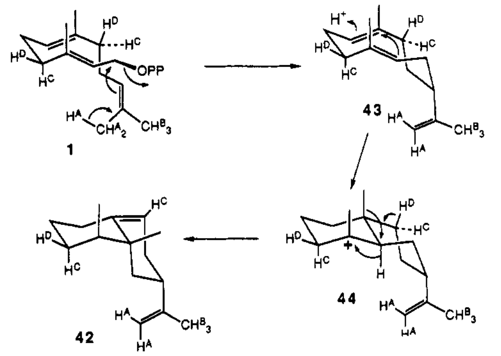
SCHEME 27



SCHEME 28



SCHEME 29



thase.[87,88] The cyclase has an estimated M_r of 48 000 based on FPLC gel filtration on Superose, while sodium decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of apparently homogeneous protein indicated a slightly higher molecular weight of 52 800, suggesting that the native enzyme is a monomer. The K_m for FPP is 3.9 μM and the turnover number was calculated to be 0.2–0.3 s^{-1}. The enzyme, which is moderately lipophilic exhibits the usual requirement for Mg^{2+}; the cyclase is modestly inhibited by inorganic pyrophosphate. Whereas either 10 μM pentalenene or inorganic pyrophosphate alone had little effect on the rate of cyclization, 10 μM concentrations of pentalenene and inorganic pyrophosphate together increased the apparent K_m for FPP by factor of 7, suggesting that both products can bind cooperatively to the active site of pentalenene synthase. Efforts are now underway in our laboratory to isolate the pentalenene synthase gene by screening genomic DNA libraries with homologous probes obtained by using the polymerase chain reaction to amplify specific segments of genomic DNA.[88]

F. Aristolochene Synthase

Eremophilane sesquiterpenes have played a prominent role in the historical development of isoprenoid biogenetic theory. It was pointed out by Robinson in the 1930s that the eremophilane skeleton could be generated by rearrangement of a eudesmane intermediate involving migration of a methyl group from C-10 to C-5, thereby reconciling the apparent deviation of the eremophilanes from the isoprene rule proposed earlier by Ruzicka.[90,91] This suggestion proved to be remarkably accurate and has been amply borne out by a wealth of experiments based on incorporation of labeled precursors using intact organisms.[92–94]

Aristolochene (**42**) is an eremophilane-type sesquiterpene which has been isolated from a variety of species, the (−) enantiomer being found in the plant *Aristolochia indica*[94] and in the leaf oil of *Bixa orellana*,[96] as well as in the defensive secretions of *Syntermes* soldier termites,[97] while the (+) enantiomer has been shown to occur in mycelial extracts of the fungus *Aspergillus terreus*.[98,99] Aristolochene also occurs in *Penicillium roquefortii*,[100] presumably as the (+) enantiomer, where it is the likely precursor of the mycotoxin PR-toxin.[101–103] The corresponding aristolochene synthases have been isolated from both *A. terreus*[103] and *P. roquefortii*.[100]

According to the proposed mechanism, cyclization of farnesyl pyrophosphate by electrophilic attack at C-10 of the distal double bond, followed by loss of a proton from one of the two adjacent methyl groups will generate the known sesquiterpene hydrocarbon germacrene A (**43**) (Scheme 27). The latter intermediate can undergo further cyclization by protonation at C-1 and attack of the resultant cation on the 4,5 double bond to form the bicyclic eudesmane cation **44**, which can rearrange to **42** by sequential 1,2-hydride and methyl migrations, in the manner originally proposed by Robinson. Loss of a proton from C-9 will generate aristolochene. The reaction is catalyzed by a single enzyme, with no evidence for the release of free intermediates. Evidence in support of the proposed mechanism was initially obtained by incubation of [12,13-^{14}C]FPP with crude aristolochene synthase obtained from *A. terreus*.[104] Partial degradation of the resulting labeled aristolochene established that 50% of the ^{14}C activity was located in the C-12 methylene, as expected. These results were further confirmed by cyclization of a mixture of [11,12-$^{13}C_2$]- and [11,13-$^{13}C_2$]FPP. Analysis by ^{13}C NMR revealed the presence of the predicted pair of enhanced and coupled doublets, corresponding to [11,12-$^{13}C_2$]- and [11,13-$^{13}C_2$]aristolochene.

In order to establish the stereochemical course of the initial cyclization reaction, both (1*R*)- and (1*S*)-[1-^{2}H]FPP (**1**, H_A = H, H_B = D; **1**, H_A = D, H_B = H) were separately incubated with the *A. terreus* synthase and the resulting samples of aristolochene were analyzed by ^{2}H NMR (Scheme 28). In this manner it was found that aristolochene derived from (1*R*)-[1-^{2}H]FPP carried deuterium exclusively at H-6_{eq} (H-6*re*), whereas aristolochene obtained from (1*S*)-[1-^{2}H]FPP was labeled at H-6_{ax} (H-6*si*). It was therefore established that cyclization of FPP to aristolochene takes place with the expected inversion of configuration at C-1 of FPP, as demonstrated for the analogous formation of pentalenene involving cyclization at the distal double bond with formation of an 11-membered ring intermediate, and in contrast to the observed retention of configuration associated with cyclization at the central double bond of FPP in the formation of trichodiene and bergamotene.

It was also established that in the formation of the proposed germacrene A intermediate it is the C-12 (cis) methyl group of FPP which undergoes deprotonation[105] (Scheme 29). Thus incubation of [12,12,12-$^2H_3$]FPP (**1**, H_A = D, H_B = H_C = H_D = H) with aristolochene synthase gave rise to **42** which was labeled exclusively with deuterium at the methylene carbon, C-12, as shown by ^{2}H NMR, while [13,13,13-$^{2}H_3$]FPP (1, H_B = D, H_A = H_C = H_D = H) was converted to the corresponding [13,13,13-$^{2}H_3$]aristolochene. These results were consistent with the known stereochemistry of formation of a variety of germacrene A derived sesquiterpenes, including capsidiol,[92,93] 2,3-germacrenediol,[94] lubimin,[94] and hydroxylubimin.[94]

Further insight into the stereochemical course of the cyclization, including the conformation of the FPP at the active site of the cyclase came from enzyme-catalyzed cyclization of (4*R*,8*R*)- and (4*S*,8*S*)-[4,8-$^{2}H_2$]-FPP[105] (1, H_C = D, H_A = H_B = H_D = H; 1, H_D = D, H_A = H_B = H_C = H) (Scheme 29). Analysis by ^{2}H NMR established that it is H-8*si* of FPP which is lost in the formation of the 9,10 double bond of aristolochene. On the basis of the known relative and absolute configuration of (+)-aristolochene, the sequential 1,2-hydride and methyl migrations must take place on opposite faces of the eudesmane intermediate **44**, while the subsequent deprotonation takes place syn to the migrating methyl group. The demonstrated sequence of anti migration, syn deprotonation is consistent with a chair-boat conformation for the substrate FPP and intermediate germacrene A.

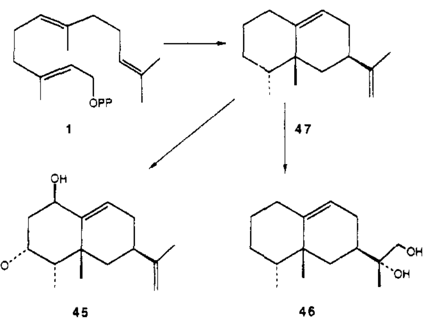
In the meantime, Hohn has purified aristolochene synthase from *P. roquefortii* and established that the cyclase has a subunit molecular weight of M_r 37 000 with the native form probably a monomer.[100] The K_m for FPP was 0.55 μM with a calculated turnover number of 0.04 s^{-1}. The enzyme showed the usual preference for Mg^{2+} and a pH optimum between 6.25 and 7.5. Unlike other known terpenoid cyclizations, however, the reaction was unaffected by the presence of inorganic pyrophosphate, although the protein did show an apparent tendency to aggregate in the presence of pyrophosphate. While the latter phenomenon is as yet unexplained, it was cleverly exploited to simplify the purification of the synthase itself by allowing a pyrophosphate-dependent variation in the behavior of the protein on gel filtration columns.

G. *epi*-Aristolochene Synthase

Many members of the Solanaceae produce sesquiterpenoid phytoalexins in response to cell-wall damage or challenge by various fungal elicitors. One of these defensive substances, capsidiol (**45**), is generated by several species, including *Datura stramonium*,[106] *Capsicum frutescens* (sweet pepper),[107] and *Nicotiana tabacum* (tobacco).[108] Feeding experiments with intact plants using both ^{13}C- and ^{2}H-labeled acetate have established many of the details of the biosynthesis of capsidiol, supporting a pathway involving the rearrangement of a bicyclic eudesmane-type intermediate.[92,93,109]

Recently, two groups have independently observed the induction of a farnesyl pyrophosphate cyclase activity in response to treatment of tobacco cell cultures with either cellulase[109,111] or fungal elicitor prepared from *Phytophtora megasperma* or related species.[112] The resulting cyclization product could be further converted to capsidiol (**45**) and the related metabolite debneyol (**46**) by incubation with a cell-free extract in the presence of NADPH and O_2 or by readministration to elicited tobacco cultures (Scheme 30). Preparative-scale incubation of FPP with the inducible cyclase allowed isolation of a sesquiterpene hydrocarbon whose structure was assigned as 5-*epi*-aristolochene (**47**) by GC/MS and ^{1}H NMR analysis.[113] The latter assignment was subsequently confirmed by unambiguous synthesis of (+)-**47** from capsidiol and direct comparison with the enzymatically generated metabolite.[114]

SCHEME 30

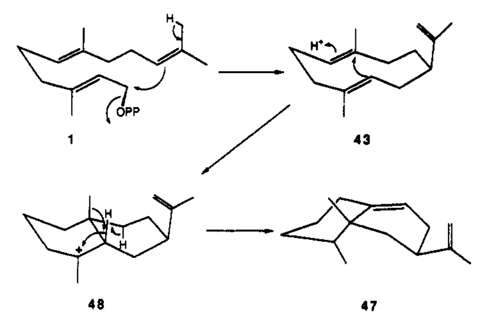


The conversion of farnesyl pyrophosphate to 5-*epi*-aristolochene (**47**) is believed to take place by a mechanism closely related to that implicated in the formation of aristolochene, involving initial formation of a germacrene A intermediate. The trans-4,5-dimethyl stereochemistry of **47** implies that in this case the sequential 1,2-hydride and methyl migrations take place on the same face of the corresponding eudesmane intermediate **48**, as illustrated in Scheme 31. Collaborative experiments between our own group and that in Hull are in progress to elucidate the mechanistic and stereochemical details of the 5-*epi*-aristolochene synthase reaction.

More recently, Chappell has succeeded in purifying 5-*epi*-aristolochene synthase.[115] The purified protein appeared to be a monomer, while SDS-PAGE analysis revealed the preparation to be a mixture consisting primarily of two polypeptides, M_r 61 500 and 63 500, both of which reacted positively in immunoblot analysis with monoclonal antibodies which had been raised to purified cyclase. At the present it is not known whether the two peptides constitute different physiological forms of the cyclase or reflect the partial in vivo degradation of the enzyme. We have observed similar examples of microbial cyclase activities differing in apparent molecular weight and have assumed without further proof that the lower molecular weight material might result from limited proteolysis of the parent synthase. *epi*-Aristolochene synthase displayed a pH optimum of 6.5–6.9 and a p*I* of 4.5–4.8. The K_m for FPP was 2–5 μM and the V_{max} for the most active preparations was 2400–2700 mmol/h mg protein, corresponding to a calculated turnover number of ca. 0.05 s^{-1}, typical of other sesquiterpene cyclases. The enzyme also resembled other cyclases in its absolute requirement for Mg^{2+} and high degree of apparent hydrophobicity. The latter property appeared to result in serious losses during ultrafiltration, as not infrequently observed with several other cyclases.

The demonstration of an inducible cyclase activity has presented a rare opportunity to study the regulation of terpenoid synthase activity at the molecular genetic level. Addition of cycloheximide to tobacco cell cultures along with the normal elicitor completely suppressed the induction of cyclase activity, suggesting that induction involves de novo protein biosynthesis. These conclusions have been further confirmed by Western

SCHEME 31



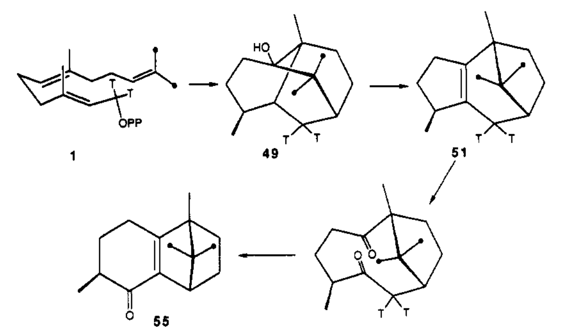
blot analysis which showed a correlation between the titres of immunopositive 61.5- and 63.5-kDa polypeptides and the measured cyclase activity, prior to and subsequent to treatment with cellulase.[116] Pulse labeling with [^{35}S]-methionine of control and elicitor-treated cells, followed by immunoprecipitation of extracts and analysis by SDS-PAGE/autoradiography indicated that exposure to elicitor results in de novo synthesis of the *epi*-aristolochene cyclase. Furthermore the increase in cyclase activity in induced cells could be correlated with increased levels of the corresponding mRNA on the basis of the in vitro translation of mRNA isolated from cellulase-treated cells and analysis of the immunoprecipitatable protein by SDS-PAGE/autoradiography. Cyclase mRNA, which could not be detected in control cultures, reached a maximum 5 h after cellulase induction, then declined rapidly, reaching near basal levels by 20 h, while cyclase activity rose steadily, peaking at ca. 25 h and then remaining constant throughout the time course of the experiment (55 h). Work is currently in progress to isolate the 5-*epi*-aristolochene synthase gene by screening of a cDNA library.

H. Patchoulol Synthase

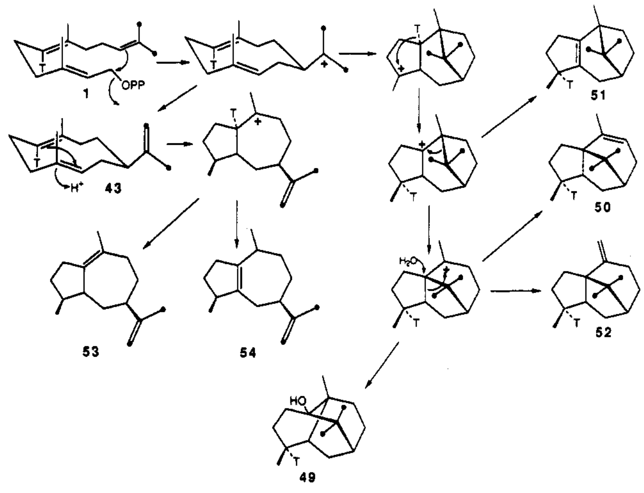
The tricyclic sesquiterpene (−)-patchoulol (patchouli alcohol, (**49**)), an important perfumery raw material, is the major component of the essential oil of patchouli (*Pogestomon cablin*).[117] Cell-free extracts of patchouli leaves have been shown to catalyze the conversion of farnesyl pyrophosphate to patchoulol along with a mixture of cyclic olefins, including α-, β-, and γ-patchoulene (**50–52**) as well as α-bulnesene (**53**) and α-guaiene (**54**).[118] The mechanism of the cyclization has been investigated by Croteau. Incubation of [1-^{3}H,12,13-^{14}C]FPP with the 145000*g* supernatant of *P. cablin* leaf extracts gave patchoulol which was shown to retain tritium at C-1 by a degradative sequence involving acid-catalyzed rearrangement and dehydration of **49** to β-patchoulene (**51**), followed by oxidative cleavage and acid-catalyzed cyclization of the resulting dione to give the cyclohexenone derivative **55**, which was devoid of tritium (Scheme 32).

Further experiments indicated that the formation of patchoulol takes place without the formation of free intermediates and implicated a pathway involving a series of tertiary carbocationic intermediates. Thus isotopic dilution experiments with unlabeled olefins failed to affect the formation of patchoulol from FPP, while a mixture of labeled **50**, **51**, **53**, and **54** was not converted to **49** by patchoulol synthase. Strong evidence for a 1,3-hydride-shift mechanism came from cyclization of [6-^{3}H,12,13-^{14}C]FPP which gave patchoulol of unchanged ^{3}H/^{14}C ratio. A degradative sequence analogous to that described above indicated that the tritium label had been shifted to C-3 in the product. These results have been explained by the cyclization mechanism illustrated in Scheme 33.

SCHEME 32



SCHEME 33



More recently Croteau has purified patchoulol synthase to apparent homogeneity.[119] Gel filtration and SDS-PAGE indicated that the active form of the enzyme has a molecular weight of M_r 80000 and consists of two identical subunits of M_r 40000. The hydrophobicity, isoelectric point (p*I* 5.0), pH optimum of 6.7, and requirement for Mg^{2+} are all typical of sesquiterpene cyclases. The K_m for FPP is 6.5 μM and the calculated turnover number is 0.03 s^{-1}. The cyclase activity could be inactivated by a variety of thiol-directed reagents, including *N*-ethylmaleimide and *S*-methyl methanethiosulfonate, as well as by the histidine-directed reagent diethyl pyrocarbonate. Interestingly, the sesquiterpene olefin cyclase activity present in crude extracts copurified with the patchoulol synthase activity and showed similar pH and metal ion dependence and response to inactivation. It is therefore likely that the various olefinic products are in fact generated by patchoulol synthase by diversion of the various carbocationic intermediates. The formation of product mixtures by a single enzyme, while unusual, is well-precedented in the action of the pinene synthases of sage[28,120,121] and would also appear to be characteristic of the longifolene–sativene biosynthetic enzymes of both plant and fungal origin.[122-124]

V. Unanswered Questions

The dramatic progress made over the last several years in the isolation and characterization of sesquiterpene synthases has provided a wealth of experimental information regarding the detailed mechanism of cyclization of the universal precursor, farnesyl pyrophosphate, to a variety of individual sesquiterpenes, while providing strong support for the fundamental correctness of the general mechanistic and stereochemical scheme for sesquiterpene biosynthesis summarized in section II. The vast majority of the studies to date, however, have focused on the fate of the cyclization substrate, and little is yet known about the structure and active site of the individual cyclases. The fundamental similarities in mechanism of action appear to be reflected in the apparent similarities in overall steady-state parameters, molecular weight, subunit structure, and lipophilicity among the various cyclases. Nonetheless, only one cyclase, trichodiene synthase, has been sequenced at the nucleic acid level. Over the next decade, attention will increasingly shift to investigations of the enzymology and molecular genetics of the cyclases themselves. To achieve a more complete understanding of the formation of cyclic sesquiterpenes the following interrelated questions must be addressed:

1. If the folding of the acyclic substrate, farnesyl pyrophosphate, is a fundamental determinant of the structure and stereochemistry of the eventually formed product, how is this folding controlled? Nothing is known about the factors governing binding and recognition of the lipophilic moiety of FPP, nor is there anything known about the strategies used by the cyclases in enforcing particular conformations on the acyclic substrate and subsequently generated intermediates.

2. How is the ionization which initiates the cyclization catalyzed and how is charge stabilized in the succession of carbocationic intermediates? Similarly, what determines the channeling of intermediates derived from a common folding of the precursor along one of several possible pathways?

3. How is charge neutralized at the end of a given cyclization sequence? What is the nature of the base which catalyzes the final deprotonation in the formation of cyclic olefins and what governs access of internal (pyrophosphate ion) or external (water) nucleophiles? What prevents premature quenching of carbocationic intermediates?

4. How do sesquiterpene synthases avoid self-annihilation which would result from capture of the highly reactive carbocationic intermediates by nucleophilic side chains or peptide bonds within the cyclase itself?

5. What is the structure of the active site of individual cyclases? What amino acid residues are involved, what is their catalytic role, and what are the shape and charge distribution of the catalytic cavity?

6. What is the relationship among the various sesquiterpene cyclases at both the DNA and protein level? Are biogenetically related sesquiterpenes, so-called historically because they would appear to be derived from common reactive intermediates, formed by genetically related cyclases?

7. Can systematic alteration in cyclase active site structure result in a change in cyclization products?

Recent progress in the purification of sesquiterpene synthases and the cloning of the relevant structural genes is certain to allow detailed comparisons of sequences at both the DNA and protein level, while providing sufficient quantities of protein for both kinetic and structural studies, including crystallographic analysis. Further progress along these lines will also require the rational development of substrate-based inhibitors and substrate analogues to assist in the identification of active site residues and to elucidate the intricate details of the cyclization process. The sesquiterpene synthases therefore are certain to provide a particularly fertile ground for the study of fundamental structure–activity relationships. The existence of a naturally occurring family of catalysts, all mediating the cyclization of a common substrate by variations on a common mechanism, suggests that Nature has provided a unique opportunity for the investigation of the effect of DNA and amino acid sequence on protein structure and catalytic action.

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Registry No. Farnesyl pyrophosphate synthetase, 50812-36-7; sesquiterpenoid cyclase, 129215-27-6.

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