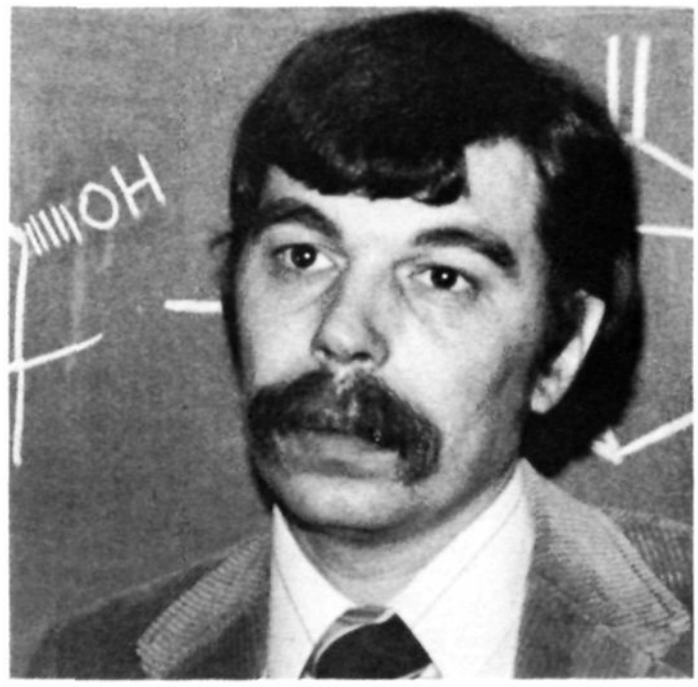
Biosynthesis and Catabolism of Monoterpenoids

RODNEY CROTEAU

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

Received April 20, 1987 (Revised Manuscript Received June 22, 1987)

Contents



Rodney Croteau was born in Springfield, MA, and received his B.S. in 1967 and Ph.D. in 1970 from the University of Massachusetts, Amherst, under the direction of Professors I. S. Fagerson and S. Siggia. Two years as an N.I.H. Postdoctoral Fellow with W. D. Loomis at Oregon State University were followed by additional postdoctoral study with P. E. Kolattukudy at Washington State University where he joined the faculty in 1975. He is now Professor of Biochemistry and Director of the Institute of Biological Chemistry. His research interests have centered on the biochemistry and function of terpenoids.

I. Introduction

The monoterpenes are the C_{10} representatives of the terpenoid family of natural products, and they diverge from higher isoprenoid biosynthesis at the level of geranyl pyrophosphate (see Scheme II), the first C_{10} intermediate to arise in the classical pathway.[1] The vast majority of the several hundred naturally occurring monoterpenes are cyclic (primarily cyclohexanoid), and they represent a relatively small number of skeletal themes multiplied by a very large range of simple derivatives, positional isomers, and stereochemical variants (Figure 1).[2,3] The formation of significant quantities of monoterpenes (>0.1% fresh tissue weight) appears to be confined to some 50 families of higher plants[4–6] in which the monoterpenes are most familiar as components of the essential oils that are synthesized and accumulated in various types of distinct and highly specialized secretory structures (such as glandular trichomes and resin ducts).[7,8] The function of the monoterpenoids in nature is largely still unknown; however, the few documented examples of pollinator attraction,[9] competitive phytotoxicity,[10] and defense against herbivores,[11,12] phytophagous insects, and microbial pathogens[13–15] suggest a general ecological role for this class of compounds.

Research on the biological chemistry of cyclic monoterpenes can be divided into four general areas: the origin of the acyclic precursor, cyclization reactions, secondary transformations of the parent cyclic compounds, and catabolism. It is in this sequence that the present review is organized and through which I will attempt an up-to-date assessment of developments in this field over the last decade, during which the use of cell-free enzyme systems has been brought to bear on the problem permitting direct study of the reactions involved. There is, of course, always the problem of selection. It is impossible to be comprehensive, nor do I wish to be, since even itemizing the nearly two hundred individual compounds and dozen skeletal arrangements presents too great a danger of missing the deeper unifying principles. Fortunately, in this area, the fundamental things, being the simplest in some sense, select themselves, permitting the central themes and underlying unity to be readily discerned. Since I also hope to illustrate the general flow of ideas in this area, I will discuss selected false leads and improvizations that ultimately brought about our current level of understanding. Such inclusion livens the story and provides a warning that our current concepts may too pass into obscurity. Since much of the work described is my own, I fervently hope at least some will survive. The biochemistry of monoterpenoids is periodically reviewed[16,17] and was last broadly dealt with in 1981.[81]

II. Historical Perspective

Such early findings that isoprene (methylbutadiene) was produced by pyrolysis of turpentine (primarily pinenes) and that heating of isoprene produced dipentene (racemic limonene) led Wallach,[19] through a series of detailed structural investigations of nearly a

0009-2665/87/0787-0929$06.50/0

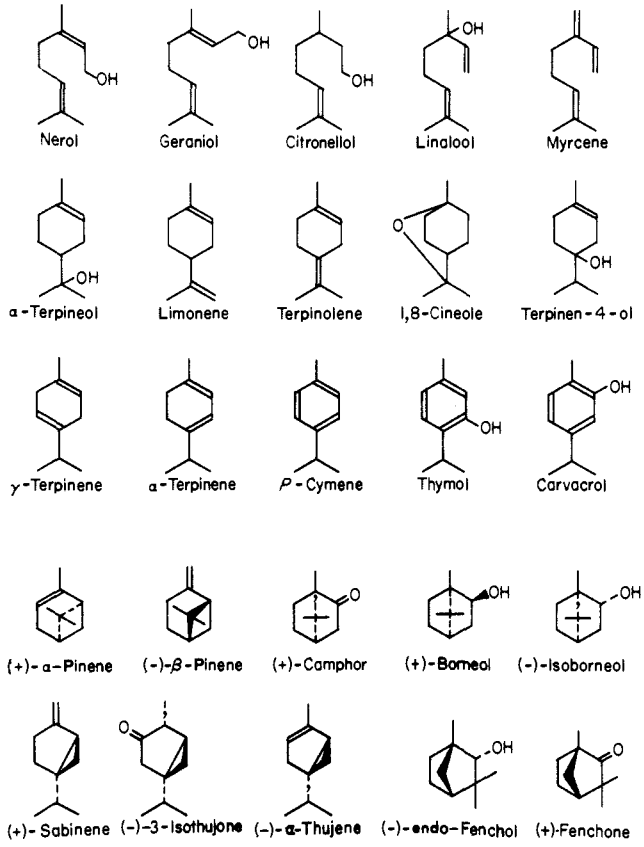
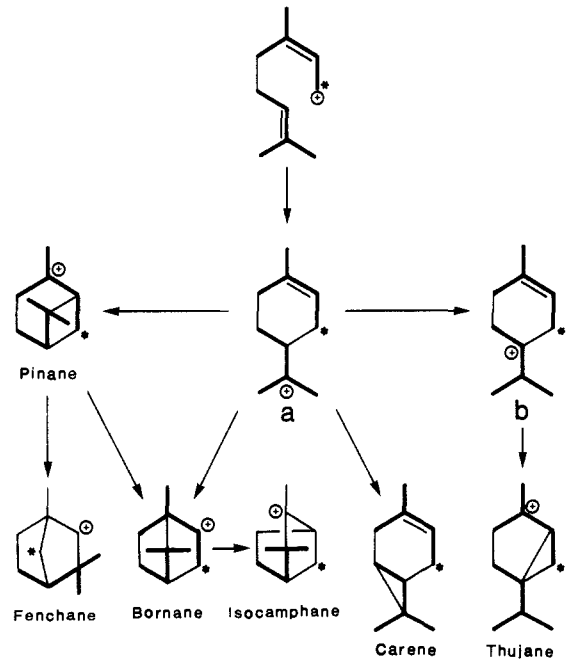


Figure 1. Representative monoterpenes.

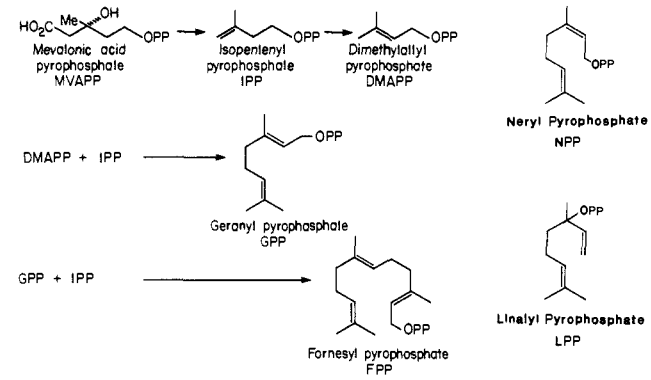
SCHEME I.[a]



[a] Postulated ionic mechanism for the formation of monoterpenes via the α-terpinyl cation (a) and the terpinen-4-yl cation (b). Regular (head-to-tail) structures are divided into isoprene units and the labeling patterns from C1-labeled acyclic precursor are illustrated.

century ago, to formulate the "isoprene rule"—that a certain category of natural products (terpenoids) can be regarded as being constructed of isoprene units, commonly joined in a head-to-tail fashion (Scheme I). A unified conceptual framework for the origin of the various terpenoid types was put forward 3 decades ago

SCHEME II.[a]



[a] The abbreviations indicated are used in subsequent schemes. OPP indicates the pyrophosphate moiety.

by Ruzicka in formulating the "biogenetic isoprene rule".[20] As applied to the monoterpenes, this model (Scheme I) posits intramolecular electrophilic attack of C1 of the neryl cation on the distal double bond to yield a monocyclic (α-terpinyl) intermediate, which by a series of subsequent internal electrophilic additions, hydride shifts, and Wagner–Meerwein rearrangements gives rise to the cationic equivalents of most known skeletal types. The latter species, by deprotonation to the corresponding olefin or capture by a nucleophile, could yield many of the common monoterpenes. Subsequent, often oxidative, modification of these cyclic progenitors could be invoked to explain the generation of most other monoterpenoids, such secondary transformation schemes being based, most often, on chemical precedent consistent with the cooccurrence and genetic inheritance of the various metabolites.[21-23] The early work by Reitsema[24] and Murray[25] among others in "ordering" the metabolites of *Mentha* (mints) is typical. The concept of monoterpene catabolism was late to arrive due in large part to a general reluctance[26-28] to accept the possibility that natural products (as "dead-end" or "waste" metabolites) could be degraded by the organisms that produced them. The question of monoterpene catabolism aside, it was the profound contribution by Ruzicka that set the foundation for nearly all biogenetic investigations to follow; most importantly, in vivo studies using basic precursors, such as [2-^{14}C]-acetate and [2-^{14}C]mevalonate, with which labeling patterns consistent with the biogenetic isoprene rule were demonstrated,[29,30] and time-course studies and direct incorporation of more advanced metabolites, by which precursor–product relationships could be tested.[31,32]

With the growing appreciation of the central role of allylic pyrophosphates in isoprenoid metabolism and, specifically, the finding that geranyl pyrophosphate (see Scheme II) was the first C_{10} intermediate to arise in the general isoprenoid pathway,[1] attempts were made to apply the biogenetic isoprene rule to monoterpene biosynthesis in more explicit mechanistic terms. These attempts brought considerable confusion and some controversy to the field. It had long been known that geraniol and its derivatives cannot cyclize directly (because of the trans double bond at C2), whereas the cis isomer nerol and the tertiary allylic isomer linalool readily cyclize (see Scheme II).[33,34] Thus, to circumvent the topological barrier to the direct cyclization of geranyl pyrophosphate, a number of theories, often con-

flicting and sometimes poorly supported, were proposed for the origin of neryl pyrophosphate and linalyl pyrophosphate (see Scheme II) by direct condensation of C_5 units or by various isomerizations of geranyl pyrophosphate (see ref 18, 35, and 36 for discussion of these now largely historical ideas).

With the availability in the early 1970s of crude cell-free enzyme systems from relevant higher plants, the question of the immediate precursor of the cyclic monoterpenes appeared easily approachable, and early studies with these systems seemingly confirmed neryl pyrophosphate to be a more efficient precursor of various cyclic metabolites than was geranyl pyrophosphate.[37,38] However, partial purification of these crude soluble enzyme systems to remove competing phosphatases and pyrophosphatases (a notorious problem in plant extracts in which substrate hydrolysis may exceed cyclization by a factor of 50[39]) and characterization of these hydrolases revealed the strong preference of such enzymes for geranyl derivatives.[40] Reevaluation of the earlier data lead to the conclusion that relatively little geranyl pyrophosphate present in incubation mixtures would survive intact (being converted largely to the corresponding monophosphate) and that comparison of acyclic precursors under such conditions was inconclusive.[41] Related studies by Suga and associates[42] compared various geranyl, neryl, and linalyl derivatives as acyclic precursors in vivo and in vitro and led to the conclusion that linalyl derivatives were most efficiently cyclized. As in the earlier work, the ultimate fate of each precursor was not examined, and so the comparisons should be regarded with considerable caution; although in this instance the conclusion was correct, linalyl pyrophosphate now being generally considered the key bound intermediate in the cyclization reaction and thus an efficient precursor of cyclic monoterpenoids.

Since these exploratory investigations, several studies[41,43-47] using partially purified preparations nearly freed of competing hydrolases or preparations in which phosphohydrolases were inhibited[48] have made it abundantly clear that geranyl pyrophosphate is efficiently transformed to cyclic monoterpenes without loss of hydrogen from C1 of the trans precursor[49] and without isomerization to *free* neryl or *free* linalyl pyrophosphate or conversion to any other detectable intermediate. Furthermore, geranyl pyrophosphate is, in most cases, cyclized more efficiently that is neryl pyrophosphate and with efficiencies comparable to that of (±)-linalyl pyrophosphate.[44,47] These observations forced the conclusion that monoterpene cyclases (synthases) are capable of catalyzing a multistep process whereby the enzyme carries out an isomerization to a bound intermediate capable of cyclizing, as well as the cyclization reaction itself. The recognition of geranyl pyrophosphate as the universal precursor of cyclohexanoid monoterpenes provided a critical insight that, while not immediately clarifying the detailed mechanism of cyclization, did result in considerable simplification of the problem by eliminating alternatives no longer tenable. It is not necessary at this point to discuss such alternate schemes, since they have been reviewed in detail elsewhere.[18,35,36] Suffice it to say that there is no evidence that has withstood experimental scrutiny for other than geranyl pyrophosphate as the universal precursor of cyclohexanoid monoterpenes. All recent proposals for the biosynthesis of these cyclic compounds have been based on the concept of a tightly coupled isomerization–cyclization reaction[36,43,47,50] and have culminated in the development of a new model, richer in mechanistic detail, that describes the interaction of enzyme and the geranyl pyrophosphate substrate in explicit stereochemical terms.[51]

Our present understanding of monoterpene cyclization has not advanced in a biochemical vacuum and owes much, as will be seen, to the contributions of Poulter and Rilling[52,53] in defining the cationic character of the prenyltransferase reaction and of Cane in the related area of sesquiterpene biosynthesis[54] and in developing general concepts of prenyl pyrophosphate metabolism.[55]

The advent of cell-free enzyme systems[44,47] and the development of associated highly sensitive assay techniques[56] made it possible to examine the myriad secondary transformations of the parent cyclic monoterpenes and, in the few instances examined in detail,[36] forced a reevaluation of earlier proposals based on in vivo investigations. Moreover, studies at the enzyme level allowed broader questions to be addressed concerning general metabolic strategies for these abundant transformations and the specificities of the protein catalysis involved. Efforts in this area have been sporadic thus far, and in no instance have studies progressed to the level achieved, for example, in the metabolism of the diterpenoid kaurene en route to the gibberellin plant hormones.[57,58] Investigations on monoterpene catabolism by the producing organisms have also been slow to develop; yet, in this instance it is only recently that the abundance of evidence supporting catabolic turnover of monoterpenes[18,35,36] has stimulated evaluation of the question at the enzyme level. Progress in some areas has been made, however, particularly in the cases of those structural types for which there is ample precedent in the corresponding microbial degradative pathways.[59]

III. Origin of Geranyl Pyrophosphate

Since the natural substrate for monoterpene cyclization is now considered to be geranyl pyrophosphate, a universal intermediate in the isoprenoid pathway and the initial product of the condensation of dimethylallyl pyrophosphate and isopentenyl pyrophosphate catalyzed by prenyltransferase (Scheme II), there would seem little to add in the context of monoterpene biosynthesis. Indeed, the steps of isoprenoid metabolism from acetyl-CoA through mevalonic acid pyrophosphate, to geranyl pyrophosphate are very well documented,[1,53,60] and the enzymology and mechanism of prenyl transfer have been thoroughly described by Poulter and Rilling.[52,53] However, most prenyltransferases that have been described yield, as major products, higher prenyl pyrophosphates, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate en route to sesqui- and triterpenes and to di- and tetraterpenes, respectively, and they produce geranyl pyrophosphate only as an intermediate in the elongation process. Thus, for example, farnesyl pyrophosphate synthase[53] catalyzes the condensation of dimethylallyl pyrophosphate and isopentenyl pyrophosphate to geranyl pyrophosphate and of geranyl

pyrophosphate and isopentenyl pyrophosphate to farnesyl pyrophosphate (Scheme II), with the latter condensation the more efficient of the two such that geranyl pyrophosphate does not appreciably accumulate.[61]

Two questions thus arise. Does a specific geranyl pyrophosphate synthase exist in monoterpene-producing organisms, and is the enzyme associated with monoterpene biosynthesis? A geranyl pyrophosphate synthase has been isolated from the microorganism *Micrococcus lysodeikticus* where it functions in generating the primer for solanesyl pyrophosphate biosynthesis en route to menaquinone,[62] and crude tissue extracts of monoterpene-producing plants do produce detectable levels of geranyl pyrophosphate from the relevant C_5 precursors; however, farnesyl pyrophosphate is generally the major product in these systems.[63-65] Recently, soluble extracts from leaves of the common sage plant (*Salvia officinalis*; Lamiaceae) were fractionated by combination of gel permeation and hydrophobic interaction chromatography, largely to remove farnesyl pyrophosphate synthase, and shown to possess an electrophoretically distinct prenyltransferase that synthesizes, from dimethylallyl and isopentenyl pyrophosphate, geranyl pyrophosphate as the sole (>95%) product.[66] Moreover, selective extraction of the contents of the epidermal oil glands of sage leaves,[67] the site of monoterpene biosynthesis and accumulation in this species,[68] revealed that essentially all of the geranyl pyrophosphate synthase of the leaf tissue resided in these unique secretory structures. Additionally, the enzyme was absent in extracts of monoterpene-impoverished leaf tissue such as tomato, potato, corn, and wheat. Thus, the two questions raised above can probably be answered in the affirmative. Although the geranyl pyrophosphate synthase of sage leaves has not yet been characterized in detail, in general properties (metal ion requirement, pH optimum, kinetic constants, and molecular weight) it resembles farnesyl pyrophosphate and geranylgeranyl pyrophosphate synthases previously described.[53,69]

IV. Cyclization Reactions

It seems surprising that an understanding of the cyclization mechanism has begun to emerge only in the last decade; however, before studies with cell-free enzyme systems forced a reevaluation of the issue, most biochemists were content with the simple model for the cyclization of neryl pyrophosphate to monoterpenes since there was ample chemical precedent for the reaction, and the transformation of the geranyl system seemed to present an unnecessary stereochemical complication. In describing monoterpene cyclization reactions it is most pertinent to review first the general nature of the cyclase enzymes and the reactions that they catalyze, with illustrative examples and comparison with related proteins. This is conveniently followed by a review of chemical model studies and related investigations upon which the enzymatic cyclization scheme is based. For clarity in the subsequent presentation the general isomerization–cyclization model is then described, followed by the supporting evidence for stereochemical and mechanistic features from which the overall soundness of the scheme, and its predictive value, will become apparent.

A. Enzymology

The crucial cyclization reactions, by which the parent monoterpene carbon skeletons are generated, are catalyzed by enzymes collectively known as cyclases. Many such enzymes have been partially purified (i.e., freed of competing activities such as phosphatase), permitting preliminary characterization. Preparation to homogeneity, permitting detailed physical and chemical study, has been hindered by the somewhat unsavory nature of the rather limited enzyme sources (i.e., terpene-bearing leaves, fruit, and inner bark of certain higher plants) that commonly contain high levels of resins and phenolics, which are detrimental to enzyme activity and stability, as well as competing activities.[47] Plant cells in culture have yet to provide a suitable enzyme source, presumably because differentiation to form an extracellular secretory cavity is a prerequisite for monoterpene production.[70,71] Operational limitations are also imposed by the facts that the cyclases, like most enzymes involved in the biosynthesis of natural products, do not occur in very high intracellular concentrations and the reactions that they catalyze are rather slow (i.e., turnover numbers estimated to be in the 0.01–1.0 s^{-1} range).[47,72] Selective extraction of monoterpene cyclases from leaf epidermal oil glands and affinity chromatography techniques have partially overcome the preparative difficulties[67,73] and, when coupled to sensitive radiochemical assay methods,[44,47] have permitted the study of this class of enzyme.

Multiple cyclases, each producing a different skeletal arrangement from the same acyclic precursor, often occur in higher plants, while single cyclases, which synthesize a limited variety of skeletal types, are also known.[41,44,45,74] Individual cyclases, each generating a simple derivative or positional isomer of the same skeletal type, have been described, as have distinct cyclases catalyzing the synthesis of enantiomeric products.[35,45] The number of monoterpene cyclases in nature is presently uncertain, yet studies on the known examples from a limited number of plant species[18,36] suggest that the total may approach 50. Over the last decade the enzymatic cyclization of geranyl pyrophosphate to some 20 different products has been demonstrated in various preparations from *Salvia*, *Mentha*, *Tanacetum*, *Foeniculum*, *Pinus*, and *Citrus* species, including (−)-limonene, α-terpinene, γ-terpinene, (+)-sabinene, 1,8-cineole, (+)- and (−)-camphene, (+)- and (−)-α-pinene, (+)- and (−)-bornyl pyrophosphate, and (−)-β-pinene. These cyclizations are representative of reactions carried out by the monoterpene cyclases in which a single enzyme catalyzes an extensive series of transformations in which many carbons of the substrate undergo alteration in bonding, hybridization, and configuration. The cyclases, as a class, are thus notable for the apparent complexity and length of the reaction sequence catalyzed from the same acyclic precursor, while maintaining complete regio- and stereochemical control of product formation. In most cases, the relevant enzyme preparations (from diverse plant sources and tissues) have been partially purified and subjected to at least partial characterization.[44,47] On this basis, it seems safe to state that in general properties the monoterpene cyclases (the enzyme type is more properly termed an isomerase–cyclase) resemble not only each other, but also the few sesquiterpene[75,76] and

dieterpene cyclases[77,78] that have been examined as well as the prenyltransferases that catalyze related electrophilic reactions.[52,53]

Cyclases, with apparently rare exception,[79] are operationally soluble enzymes possessing molecular weights in the 50000–100000 range. A singular example (1,8-cineole cyclase of M_r 60000[74]) purified to near homogeneity by traditional chromatographic techniques and examined by SDS–PAGE suggests the absence of subunits.[73] On the basis of a limited number of cases,[45,74,80] the cyclases appear to possess relatively low pI values and to be rather hydrophobic. Several can be assayed in essentially nonaqueous media, a condition under which they are quite stable.[81] The only cofactor required is a divalent metal ion, Mg^{2+} or Mn^{2+} generally being preferred (K_m in the 0.5–5 mM range) but with other metal ions often partially effective.[47,82] In most cases the pH optimum falls within the 6.0–7.0 range and may reflect the ionic state of the pyrophosphate ester–bis(metal ion) complex thought to be the "true" substrate,[83] rather than an inherent property of the cyclase itself. The observed pH curves tend to be sharp, with half-maximum velocity values within a half pH unit of the optimum. Solvolysis (nonenzymatic) of the allylic substrate is appreciable at pH < 6.0.

Most monoterpene cyclases can utilize geranyl pyrophosphate, neryl pyrophosphate, and linalyl pyrophosphate[39,42-48,82,84] as acyclic precursors, without detectable interconversion among these substrates or preliminary conversion to other free intermediates. Michaelis constants for all three substrates with all cyclases studies are in the low μM range. With rare exception, geranyl pyrophosphate is a more efficient substrate than is neryl pyrophosphate based on comparison of respective V/K_m values, and the stereochemically appropriate enantiomer of linalyl pyrophosphate (i.e., the presumptive cyclization intermediate) is more efficient than is geranyl pyrophosphate (see section IVD). Since geranyl pyrophosphate is efficiently cyclized without formation of free intermediates, it is clear that monoterpene cyclases are capable of catalyzing both the required isomerization and cyclization reactions, the overall process being essentially irreversible in all cases. It is assumed, but not yet proven in all cases (by determination of mutually competitive inhibition[44,47,84]), that the isomerization and/or the cyclization (of all the various acyclic precursors) take place at the same active site of the enzyme by the same general mechanism.

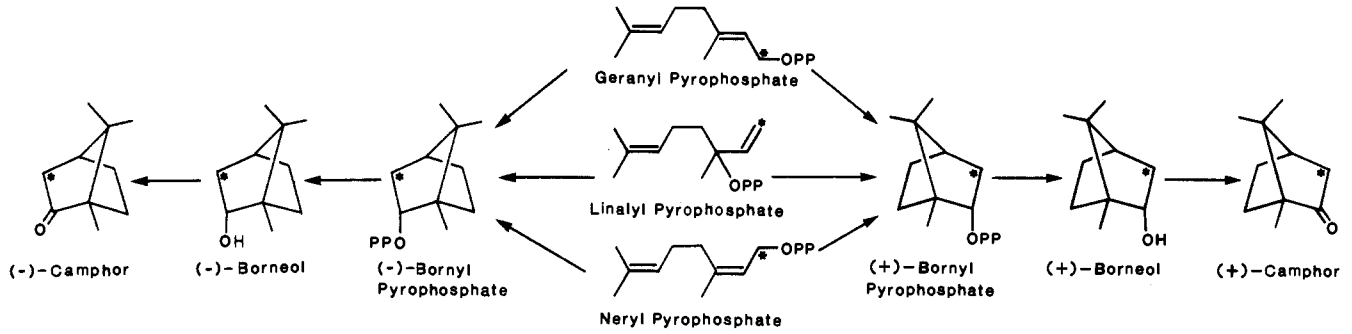
Geranyl phosphate is not a substrate for cyclization, but rather a modest inhibitor of the cyclization of geranyl pyrophosphate.[39,74,84] Inorganic pyrophosphate is inhibitory ($K_i \sim 100$ μM), but the cyclic coproducts of the reaction are not, nor is geraniol itself.[39,47,84] Farnesyl pyrophosphate, the C_{15} homologue of geranyl pyrophosphate (see Scheme II), might be expected to give rise to the sesquiterpene analogues of monoterpene products under the influence of the cyclases. However, such sesquiterpene analogues are not formed, nor are olefins or cyclic products of any type generated in appreciable levels by the monoterpene cyclases examined.[85] A recent study[86] with two cyclases and a series of substrate analogues, all of which were competitive inhibitors and many of which were catalytically competent in partial reactions [isomerization and/or ionization (solvolysis)], allowed deduction of the stereoelectronic features of the substrate required for binding and catalysis. Thus, comparison of the inhibitory properties of geranyl pyrophosphate modified by reduction or epoxidation of the 2,3- or 6,7-double bonds with that of inorganic pyrophosphate showed that the pyrophosphate ester function was the principal determinant of substrate recognition and that the C2–C3 olefin was recognized largely on the basis of geometry, whereas the primary basis of interaction with the C6–C7 olefin was electronic.

Scattered attempts to deduce the presence of specific amino acid residues at the active sites of several cyclases have been made. All cyclases are inhibited by thiol-directed reagents,[18,35,87] and tentative evidence was gained, using selective alkylators to inhibit activity, for the presence of histidine, arginine, serine, and methionine in various cases.[46,73,80,87,88] In too few instances, however, have substrate protection studies been carried out to support the presence of a given residue at or near the active site, and so relatively little can be concluded regarding mechanistic features of the reaction catalyzed. Notable exceptions include an olefin cyclase from *Citrus limonum*, where the Mn^{2+} complex of geranyl pyrophosphate protects the enzyme from benzyl bromide inactivation,[87] and 1,8-cineole cyclase from *S. officinalis*, where inhibition by the thiol-directed reagent methyl methanethiosulfonate could be abolished by preliminary addition of substrate or inorganic pyrophosphate, only in the presence of Mn^{2+}, suggesting a role for a thiol function in binding the diphosphate–metal ion group.[73]

The prime focus of subsequent sections (IVD and IVE) is the cyclization of the universal precursor geranyl pyrophosphate to bicyclic monoterpenes of the bornane, pinane, thujane, and fenchane type. The studies to be described constitute the most detailed biosynthetic investigations in the monoterpene series thus far, in large part because the structural complexity and absolute configuration of the relevant bicyclic products lend themselves readily to stereochemical correlation and allow certain mechanistic deductions to be made with little ambiguity.

Investigations of the enzymology and mechanism of camphor biosynthesis (Scheme III) provided the most revealing information to date regarding a monoterpene cyclization process. The biosynthesis of (+)-(1*R*,4*R*)-camphor in sage was shown to involve the conversion of geranyl pyrophosphate (or other acyclic precursor) to (+)-bornyl pyrophosphate, which is subsequently hydrolyzed by a distinct pyrophosphatase to (+)-borneol, followed by the NAD-dependent dehydrogenation of the alcohol to the ketone.[40,41,88,89] In tansy (*Tanacetum vulgare* L., Asteraceae) and rosemary (*Rosmarinus officinalis* L., Lamiaceae), (−)-(1*S*,4*S*)-camphor is derived by the antipodal sequence of reactions.[88,90,91] The geranyl pyrophosphate:(+)-bornyl pyrophosphate cyclase has been isolated from sage,[41] whereas the enantiomer-producing (−)-bornyl pyrophosphate cyclase has been partially purified from extracts of common tansy.[91] In both cases, C1-labeled acyclic precursor gives C3-labeled cyclic product as predicted (Scheme III). Studies on the biosynthesis of bornyl pyrophosphate were of particular significance in establishing, for the first time, that geranyl pyro-

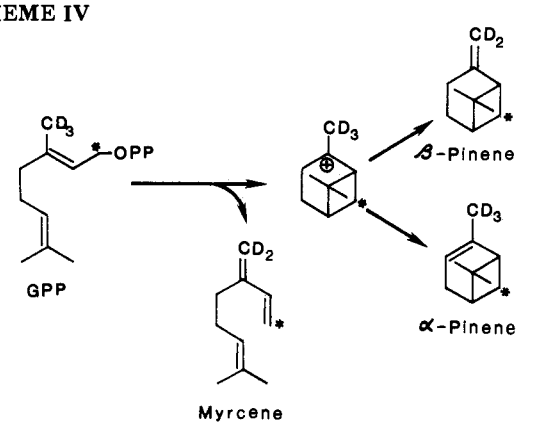
SCHEME III



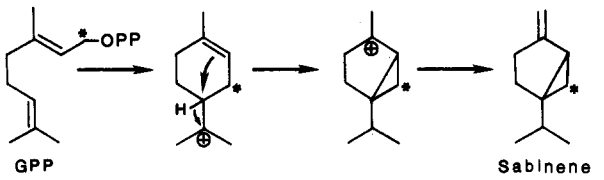
phosphate is the preferred substrate for cyclization, that neryl pyrophosphate (although an alternate substrate for cyclization) is not a mandatory intermediate, and that the pyrophosphate moiety of the substrate is retained in the bicyclic product.[41,91] This reaction type is thus far unique in the monoterpene series and has provided an unusual opportunity to examine the role of the pyrophosphate moiety in the coupled isomerization–cyclization process.

The enzymes catalyzing the synthesis of α-pinene and β-pinene were examined in partially purified preparations from *Citrus* fruit[39,84] and sage leaves.[45,92] Often, α-pinene occurs naturally as a mixture of enantiomers [~70% (+)-isomer in sage], whereas β-pinene occurs almost exclusively as the (−)-isomer. Fractionation of sage leaf extracts by gel filtration produced two regions of pinene cyclase activity (cyclase I of $M_r \sim 96\,000$ and cyclase II of $M_r \sim 55\,000$), which catalyzed cyclizations of opposite enantiomeric specificity.[45] Cyclase I catalyzed the conversion of geranyl pyrophosphate to (+)-α-pinene and to lesser quantities of (+)-limonene, the rearranged monoterpene (+)-camphene, and the acyclic olefin myrcene, whereas cyclase II transformed the acyclic precursor to (−)-α-pinene and (−)-β-pinene, as well as to (−)-camphene, (−)-limonene, and myrcene. Subsequent chromatographic purification and electrophoresis of each enzyme, as well as differential inactivation studies, provided strong evidence that each set of stereochemically related olefins was synthesized by a single cyclase, probably by a mechanism similar to that for bornyl pyrophosphate synthesis, but in this instance involving deprotonation of cationic intermediates to olefins in the termination step.[45] Very recently it was confirmed that (−)-α-pinene and (−)-β-pinene arise from cyclase II by alternate deprotonations of a common pinyl intermediate as determined by comparison of product distributions obtained from 10-$^2H_3$;1-^{3}H-labeled and 1-^{3}H-labeled geranyl pyrophosphate (Scheme IV).[93] Thus, alteration in proportions of the olefinic products generated by cyclase II resulted from the suppression of the formation of (−)-β-pinene (C10 deprotonation) by a primary deuterium isotope effect with a compensating stimulation in the formation of (−)-α-pinene (C4 deprotonation). Both cyalase II and cyclase I also exhibited decreases in the proportion of the acyclic olefin myrcene generated from the deuteriated substrate, accompanied by corresponding increases in the commitment to cyclized products. The observation of isotopically sensitive branching,[94] in conjunction with quantitation of the magnitude of the secondary deuterium isotope effect on the overall rate of product formation by these cyc-

SCHEME IV



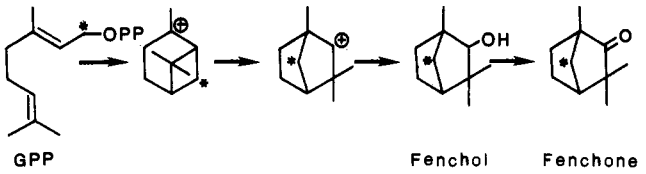
SCHEME V



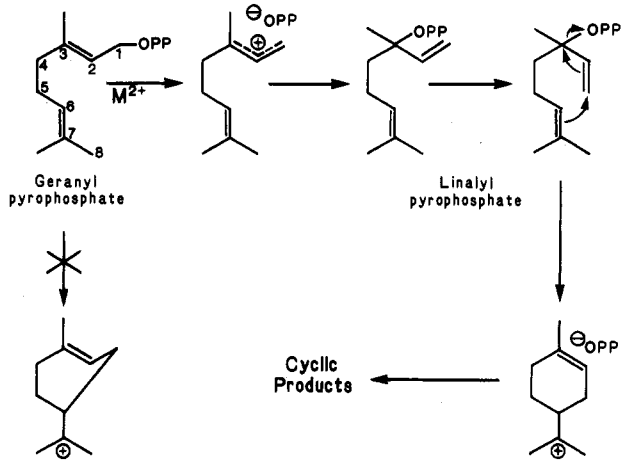
lases, not only confirmed the biosynthetic origin of (−)-α-pinene and (−)-β-pinene by alternative deprotonations of a common enzymatic intermediate, but also supported the origin of the stereochemically related olefin sets by deprotonation of cationic intermediates generated sequentially in the reaction cascade (see Scheme X). The intrinsic isotope effect calculated for the methyl deprotonation (2.4) was quite similar to that determined via natural abundance ^{2}H NMR analysis of commercially available samples of (−)-α-pinene and (−)-β-pinene derived from *Pinus* species (2.1).[95] These results also invalidated an earlier proposal that α-pinene arises by isomerization of β-pinene.[96]

Neryl pyrophosphate and (±)-linalyl pyrophosphate can serve as alternate substrates for olefin synthesis by both cyclases I and II, although in these cases the product distribution differs somewhat from the naturally occurring distribution of olefins found in sage oil, which is produced with geranyl pyrophosphate as the precursor.[45] The aberrant cyclizations of neryl pyrophosphate and the unnatural enantiomer of linalyl pyrophosphate will be described later (section IVD). The availability of the cyclase I and II systems catalyzing formation of enantiomeric products from a common, achiral substrate provided an unusual opportunity to examine the stereochemistry of cyclization. Geranyl pyrophosphate:(+)-sabinene cyclase from sage resembles cyclase II in general properties but is of the opposite stereospecificity (see section IVD) and involves

SCHEME VI



SCHEME VII



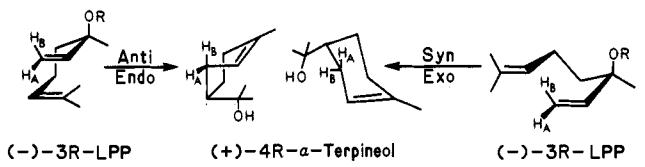
a hydride shift (Scheme V), as demonstrated with the use of [6-^{3}H;1-^{14}C]geranyl pyrophosphate as substrate.[97,98]

The origin of the rearranged bicyclic monoterpene (+)-fenchone derived from the corresponding alcohol (−)-*endo*-fenchol represents another type of cyclization. Partially purified extracts from fennel leaves or fruit (*Foeniculum vulgare* L; Apiaceae) convert [1-$^3H_2$]geranyl pyrophosphate to (−)-*endo*-[7-^{3}H]fenchol, establishing the course of the reaction to involve rearrangement of a pinyl intermediate (Scheme VI).[46] Fenchol cyclase, unlike the aforementioned cyclases, prefers Mn^{2+} to Mg^{2+} as cofactor. Detailed investigation has confirmed that the initial cyclic product is *endo*-fenchol, not the corresponding pyrophosphate ester,[99] indicating that this cyclase employs a different termination step than do the olefin cyclases or bornyl pyrophosphate cyclases. The four cyclases described here involve the different reaction types representative of the bicyclic class, and they are useful, as will be seen, for illustrating stereochemical and mechanistic details.

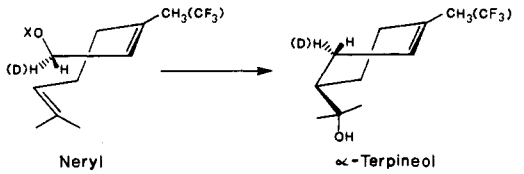
B. Model Reactions

The solvolyses of geranyl, neryl, and linalyl derivatives have provided useful models for monoterpene biosynthesis.[33,34,100-105] Results obtained using diverse systems and conditions have generally shown that neryl and linalyl systems yield monocyclic products (α-terpineol and related olefins) in good yield and at higher rates than do geranyl systems, which afford primarily acyclic products. Geranyl derivatives do give rise to monocyclic products via preliminary conversion to the tertiary, linalyl intermediate,[106-108] and this mode of cyclization (Scheme VII) is favored under conditions where nucleophilic trapping is slow relative to reionization of the tertiary allylic system and where stabilization of intermediate cationic species is favored by ion-pairing (i.e., in solvents of low nucleophilicity in the presence of large, low-charge-density counter ions).[105-111] The importance of ion pairing in both chemical[112,113]

SCHEME VIII



SCHEME IX



and enzymatic[55,114] transformations of allylic derivatives (e.g., pyrophosphate esters) has been repeatedly emphasized. The presence of the 6,7-double bond of the substrate is obviously essential for cyclization to occur, and π participation has been invoked to explain stereochemical features of solvolytic cyclization;[108,115,116] however, kinetic evidence for the influence of this double bond on solvolysis is ambiguous,[101,111,117] and the interpretation is complicated by opposing factors.[105,108,118]

Solvolysis of a number of (3*R*)-linalyl esters leads to (4*R*)-α-terpineol in high enantiomeric excess[115,116,118] (as does linalool itself[33,118,119]), and the cyclization can be formulated as either a syn,exo or anti,endo process (Scheme VIII) (the alternate syn,endo and anti,exo cyclizing conformations are precluded by the absolute configuration of the product, which indicates the face of the 6,7-double bond attacked).[120] Arigoni and coworkers, in a definitive study of the fate of the hydrogens at C1 of 1*E*,2-$^2H_2$-labeled linalyl *p*-nitrobenzoate, deduced that the anti,endo conformation of the linalyl system was preferred during cyclization to α-terpineol,[121] thus disproving a concerted cyclization with π participation by the distal double bond, which earlier had seemed (by analogy to work on the S_N2 reaction[122]) an attractive explanation for the net stereochemistry observed in this allylic displacement.[115,116] Similarly, Poulter and King[123,124] demonstrated that cyclization of *N*-methyl-(*S*)-4-([1′-^{2}H]neryloxy)pyridinium methyl sulfate to the individual enantiomers of α-terpineol proceeds via anti conformation with inversion of configuration at C1, C1–C6 ring closure being faster than reorientation of chiral C1, and that ionization precedes cyclization by analysis of the reaction products generated by solvolysis or a series of fluorinated neryl methanesulfonate analogues (Scheme IX). Thus, cyclization of the allylic system was shown to be both stepwise and stereospecific, providing an elegant demonstration of the preference for ionic over concerted pathways for this reaction type. Recently, biogenetic-type stereoselective cyclization to (+)-limonene was achieved by using chiral neryloxy derivatives.[125]

Several groups examined the influence of pH and divalent cations, such as Mg^{2+} and Mn^{2+}, in catalyzing the solvolysis of allylic pyrophosphates such as geranyl pyrophosphate.[100,126-129] The results strongly suggest that the role of the metal ion in enzymatic transformations of allylic pyrophosphates is to neutralize the negative charge of the pyrophosphate moiety and thus assist in the ionization of the substrate to produce the

allylic cation. The true substrate in the monoterpene cyclase reaction is presumed to be the geranyl pyrophosphate–bis(metal ion) complex;[129] however, there is little to suggest that the bis(metal ion) complex may be formed in meaningful levels at in vivo metal ion concentrations. The departing pyrophosphate anion could serve in shielding the carbocation from premature solvent attack[128] and could possibly function as the base in the terminating deprotonation step of some cyclizations. The assistance of the pyrophosphate moiety in deprotonation was implicated in the prenyltransferase reaction,[52,130] and the process was recently modeled.[131]

Two elements of the cyclization have yet to be addressed: the isomerization of geranyl pyrophosphate to linalyl pyrophosphate (or the equivalent ion pair) and the construction of bicyclic skeleta. Studies on the *biosynthesis* of linalool,[132] and on the analogous nerolidyl system in the sesquiterpene series,[114] showed this allylic transposition to occur by a net suprafacial process, as expected. On the other hand, the *chemical conversion* of acyclic or monocyclic precursors to bicyclic monoterpenes under relevant cationic cyclization conditions has been rarely observed,[109,133-135] and theoretical considerations notwithstanding,[136,137] bicyclizations remain poorly modeled.

Consideration of chemical models, now largely in hindsight, allows broad outlines of an electrophilic cyclization scheme to be delineated. Reaction of geranyl pyrophosphate is initiated by ionization, which is assisted by low pH and divalent metal ion. Conversion of the geranyl to the linalyl system precedes C1–C6 cyclization to the monocyclic intermediate by the established stereochemical course. The overall process occurs stepwise via a series of carbocation–pyrophosphate anion paired intermediates, where topology is maintained between the initial ionization and the termination steps. The function of the enzyme can be crudely described in binding and facilitating the ionization of the substrate, stabilizing and directing reaction intermediates, and promoting the ultimate discharge of the cation in the terminating step. The next sections will attempt to define more precisely the intimate details of cyclase catalysis.

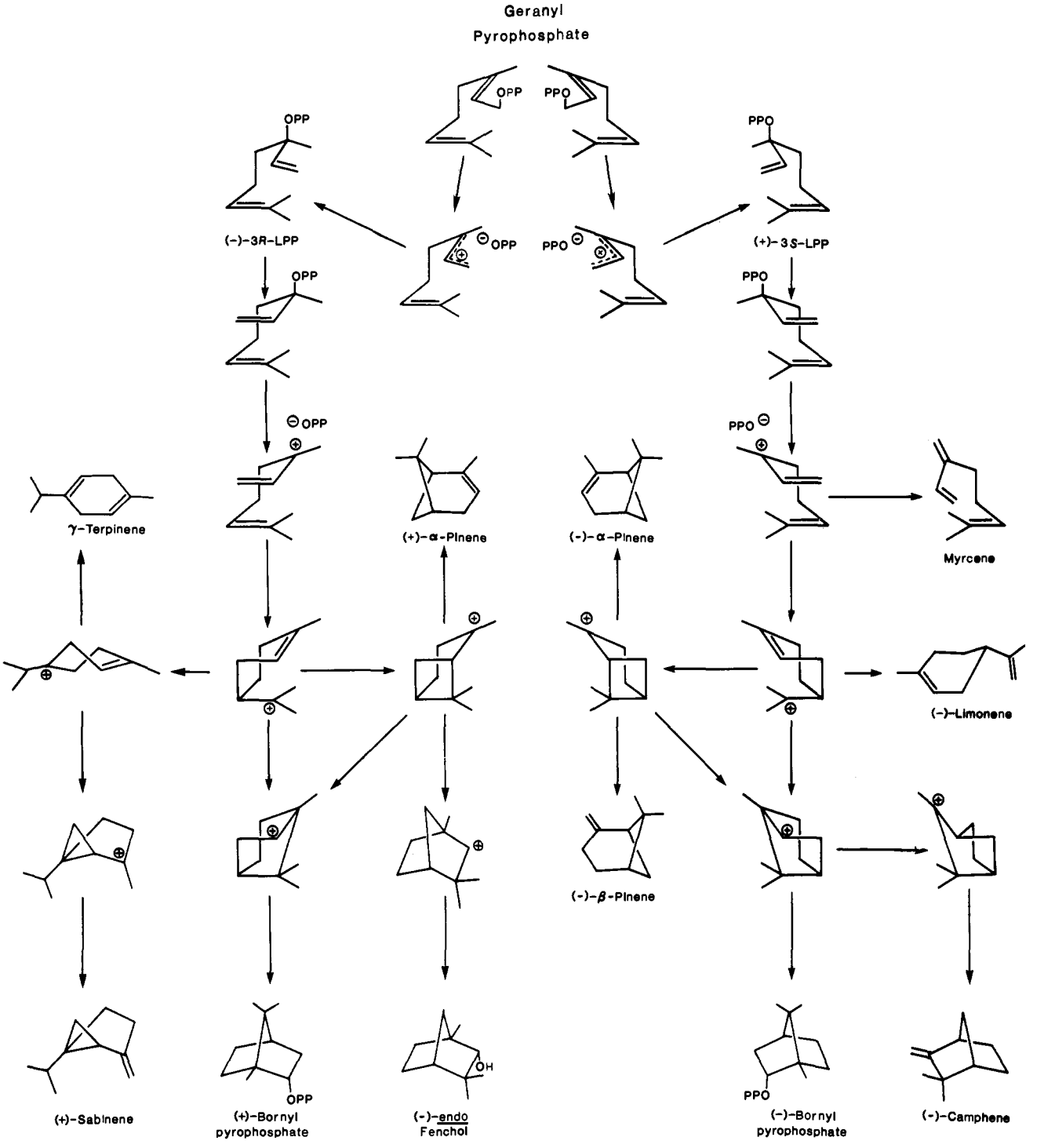
C. Isomerization–Cyclization Scheme

With the preceding reviews of the enzymology of monoterpene cyclization and of model studies relevant to the cyclization process, it is possible to formulate a unified stereochemical scheme for the enzymatic cyclization of geranyl pyrophosphate (Scheme X). The proposal that follows is consistent with the implications of parallel advances in related fields, most notably the contributions of Cane,[50,54,55,72,114] Arigoni,[138] and Coates[139-141] on the stereochemistry of sesquiterpene and diterpene cyclizations, and of Poulter and Rilling[52,53] on the stepwise, ionic mechanism of prenyltransferase, a reaction type of which most monoterpene, sesquiterpene, and diterpene cyclizations are, in a sense, the intramolecular equivalents. Indeed, under certain unusual conditions (with the product farnesyl pyrophosphate alone or with bisubstrate analogues) prenyltransferase can be made to function as a cyclase.[142-144]

It is generally agreed that the cyclase catalyzes the initial ionization of the pyrophosphate moiety to generate an allylic cation–pyrophosphate anion pair, with the assistance of the divalent metal ion and in a manner completely analogous to the action of prenyltransferase. This step is followed by stereospecific syn isomerization to either a 3*R*- or a 3*S*-linalyl intermediate and rotation about the newly formed C2–C3 single bond. Collapse of the initially formed ion pair to enzyme-bound linalyl pyrophosphate is proposed here since the free energy barrier for rotation of an allylic cation is relatively high (i.e., >12 kcal/mol[145]), even when the system is a tertiary-primary resonance hybrid.[146] Subsequent ionization and cyclization of the cisoid, anti,endo conformer of the highly reactive tertiary allylic intermediate affords the corresponding monocyclic 4*R*- or 4*S*-α-terpinyl ion, respectively. Trans orientation of the cationic center and pyrophosphate anion prevents collapse to the relative unreactive α-terpinyl pyrophosphate.[100,126] Following the initial generation of the common α-terpinyl intermediate, the further course of the reaction may involve additional cyclizations via the remaining cyclohexenyl double bond, hydride shifts, and/or rearrangements before termination of the cationic reaction by deprotonation to an olefin or capture by a nucleophile. For example, regiospecific (and probably stereospecific) elimination of a proton from the α-terpinyl cation affords limonene,[147,148] whereas further cyclization to the most highly substituted position of the cyclohexene double bond and capture of the resulting cation by the paired pyrophosphate anion generates the bornyl pyrophosphates. Internal addition to the least substituted position followed by alternate deprotonations yields the pinenes, while Wagner–Meerwein rearrangement of the (+)-pinyl skeleton and capture of the cation by water provides (–)-*endo*-fenchol.[46] A 1,2-hydride shift (C6 → C7) in the original α-terpinyl cation yields the terpinen-4-yl intermediate, which by proton loss produces γ-terpinene and related olefins[80] or by internal electrophilic attack on the cyclohexene double bond generates the cyclopropane ring of (+)-sabinene and related products.[97] The overall process can be viewed as a series of steps: ionization, pyrophosphate migration, bond rotation, ionization, cyclization(s), termination, diverging enantiospecifically in the isomerization sequence and involving numerous regiochemical variants in the generation of the various parent skeleta in which topology is maintained over the course of any given cyclization. The highly reactive electrophilic intermediates are presumed to remain paired with the pyrophosphate anion throughout the multistep reaction sequence, even where charge separation may exceed 3 Å, as in the α-terpinyl cation–pyrophosphate anion pair.

As can be seen, the scheme includes the coupled isomerization component of the multistep reaction necessary to overcome the geometrical impediment to direct cyclization of geranyl pyrophosphate and reformulates earlier concepts of cyclization in explicit stereochemical terms that take into account the conformational constraints[50] imposed by the required 2p orbital alignment for cyclization of an eight-carbon chain containing two trisubstituted π systems and the apparent imperative for allylic displacement of the tertiary pyrophosphate moiety in an anti sense. The scheme accounts for the cyclization of geranyl pyrophosphate, without free intermediates, to all major skeletal types[149]

SCHEME X



and can obviously rationalize the cyclization of neryl and linalyl pyrophosphates as alternate substrates—all routes merging at the cisoid conformer of linalyl pyrophosphate (or the ion-paired equivalent). Although linalyl pyrophosphate is the first explicitly chiral intermediate in the cyclization scheme, it should be emphasized that the eventual configuration is predetermined by the helical conformation of geranyl pyrophosphate achieved on initial binding to the enzyme; the left-handed screw-sense isomer yielding (3*R*)-linalyl pyrophosphate, the right-handed screw-sense isomer affording the 3*S* enantiomer[51,150] (for additional discussion of conformational considerations in mono- and sequiterpene cyclization, see Cane[50]). The scheme applies equally well to monocyclic and bicyclic monoterpenes of either enantiomeric series, but with an obvious degree of uncertainty to symmetrical products, such as 1,8-cineole and γ-terpinene, in which absolute stereochemical inferences based on absolute product configurations are not possible. The following sections (IVD and IVE) present a description of current efforts to test and probe further the implications of this stereochemical model.

D. Stereochemistry

As noted in an earlier section, the labeling patterns of several monoterpenes derived in vivo from basic

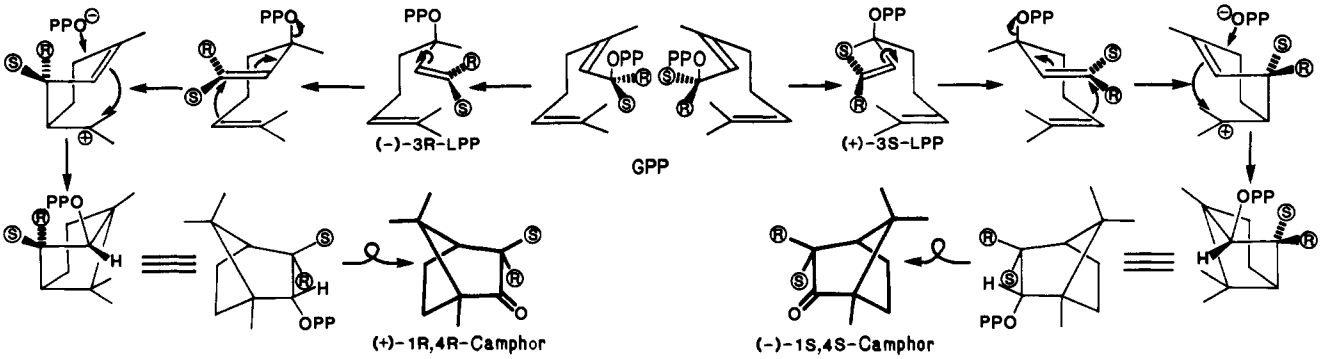
precursors such as [2-^{14}C]mevalonic acid are entirely consistent with the basic cyclization proposal (Scheme X).[29,30] More recently, the labeling patterns of antipodal bornane and pinene monoterpenes from [1-^{3}H]geranyl pyrophosphate were determined [(+)- and (−)-bornyl pyrophosphate are labeled at C3 and (+)- and (−)-pinenes at C7; see Scheme I].[49,88,91,92] These studies, in addition to confirming that cyclization occurs without loss of hydrogen from C1 and thus eliminating redox–isomerization schemes[151,152] and incidently ruling out proposals for the intermediacy of acyclic olefins in the cyclization process,[153] indicated that the antipodes are derived via enantiomeric cyclizations involving antipodal linalyl and α-terpinyl intermediates (Scheme X) rather than by way of a hydride shift or other rearrangement from a common cyclic progenitor. Thus, for these monoterpenes, the corresponding conformation of the presumptive intermediates can be deduced on the basis of the assumption of least motion during the course of the cyclization,[50,150,154] and there exists a direct correspondence between the observed relative and absolute configuration of the terpenoid product and the inferred configuration at C3 and conformation of the cyclizing linalyl intermediate. Stereochemistry of the overall isomerization–cyclization to any given product can be deciphered by examining the alterations at C1 and C3 of the substrate in the course of the reaction sequence.

Although it is not yet fully confirmed whether linalyl pyrophosphate is the true enzyme-bound intermediate of cyclization processes as strongly implied here or simply an efficient substrate analogue as suggested by Cori,[43] it is abundantly clear that cyclases are capable of ionizing and subsequently cyclizing linalyl pyrophosphate, which at minimum must closely mimic the corresponding bound intermediate actually formed at the active site. This feature allows the configuration of the cyclizing intermediate to be determined, in principal, in two ways: either by measuring which enantiomer of a racemic mixture is depleted by the cyclase (via chromatographic resolution of the residual), or by testing directly, and independently, each linalyl pyrophosphate enantiomer as a cyclase substrate. Both approaches were taken in examining the cyclizing intermediate in the formation of (−)-*endo*-fenchol, the major representative of the fenchane family of monoterpenes. Phosphatase-free preparations of the relevant cyclase from *F. vulgare*[99] were incubated with (3*RS*)-[1-^{3}H]linalyl pyrophosphate until roughly 50% of this precursor was converted to the bicyclic monoterpenol endproduct.[155] The residual linalyl pyrophosphate was isolated and enzymatically hydrolyzed (P–O cleavage) to the free alcohol linalool, which was resolved by chiral-phase capillary GLC of the derived threo and erythro mixture of 1,2-epoxides. The predominance of the 3*S* enantiomer in the residual substrate indicated that the 3*R* enantiomer was preferred for the cyclization to (−)-(1*S*)-*endo*-fenchol as predicted (Scheme X).[155] This conclusion was subsequently confirmed by direct testing of (3*R*)-[1*Z*-^{3}H]linalyl pyrophosphate. (3*S*)-[1*Z*-^{3}H]Linalyl pyrophosphate was not a substrate for (−)-*endo*-fenchol biosynthesis but did, by a remarkable anomalous cyclization, give rise to low levels of the enantiomeric (+)-(1*R*)-*endo*-fenchol (resolved by chiral phase GLC of the isopropyl urethane).[155]

The configuration of the tertiary linalyl intermediates in the (+)- and (−)-bornyl pyrophosphate cyclizations was initially examined with [3*RS*-1*E*-^{3}H;3*R*-8,9-^{14}C]-linalyl pyrophosphate (^{3}H:^{14}C = 5.2) as substrate, which was tested with the antipodal cyclases from *S. officinalis* and *T. vulgare* (the cyclic product was converted by combination of enzymatic and chemical means to camphor, which was examined as the crystalline oxime).[156] With the *T. vulgare* derived enzyme this substrate yielded (−)-bornyl pyrophosphate with ^{3}H:^{14}C > 31, indicating specific utilization of (+)-(3*S*)-linalyl pyrophosphate as predicted (Scheme X). With the (+)-bornyl pyrophosphate cyclase from *S. officinalis* the ^{3}H:^{14}C ratio of the product was about 4.2, indicating a preference for the predicted (−)-3*R* enantiomer but the ability also to utilize (+)-(3*S*)-linalyl pyrophosphate. Optically pure (3*R*)- and (3*S*)-[1*Z*-^{3}H]linalyl pyrophosphate were then separately compared with the achiral precursors [1-^{3}H]geranyl pyrophosphate and [1-^{3}H]neryl pyrophosphate as substrates for the cyclizations.[156] All functional precursors afforded optically pure (−)-(1*S*,4*S*)-bornyl pyrophosphate with the cyclase from *T. vulgare* (as determined by chromatographic separation of diastereomeric ketals of the derived ketone camphor). (+)-(3*S*)-Linalyl pyrophosphate was the preferred substrate, and (−)-(3*R*)-linalyl pyrophosphate was inactive. With the (+)-bornyl pyrophosphate cyclase from *S. officinalis*, geranyl, neryl, and (−)-(3*R*)-linalyl pyrophosphates gave the expected (+)-1*R*,4*R* stereoisomer as the sole product, and (−)-(3*R*)-linalyl pyrophosphate was the preferred substrate. However, (3*S*)-linalyl pyrophosphate yielded (−)-(1*S*,4*S*)-bornyl pyrophosphate, albeit at much lower rates, indicating the ability of this enzyme to catalyze the anomalous enantiomeric cyclization. These results established the configurational preferences in the cyclization of the linalyl intermediates to the bornyl systems as predicted and, for both enantiomeric cyclases, indicated that the cyclization of the preferred linalyl pyrophosphate enantiomer was faster than was the coupled isomerization–cyclization of geranyl pyrophosphate.

[3*RS*-^{3}H;3*R*-^{14}C]Linalyl pyrophosphate was also employed as a substrate to investigate the configuration of the cyclizing intermediate in the pinane series.[157] In this case, the (+)-α-pinene produced by the relevant cyclase from *S. Officinalis*, after convertion to the crystalline pinonic acid, bore a ^{3}H:^{14}C ratio of slightly greater than half of the starting material, as expected for the enantioselective conversion of the 3*R* enantiomer to the (+)-pinyl system (Scheme X). The (−)-β-pinene product generated by the antipodal cyclase from the same tissue, following conversion to nopinone oxime, was shown to contain essentially only tritium, consistent with the enantioselective conversion of the 3*S* enantiomer to the (−)-pinyl nucleus. The results with the pinenes indicated a higher degree of enantiomer discrimination by these cyclases than had been observed with the (+)-bornyl pyrophosphate cyclase; yet, even here it was clear that the opposite enantiomer had participated to a detectable degree in the cyclization to each bicyclic product. Configurational preferences in the construction of the antipodal pinenes were confirmed with optically pure (3*R*)- and (3*S*)-[1*Z*-^{3}H]linalyl pyrophosphates [3*R* → (+)-pinenes; 3*S* → (−)-pinenes],

SCHEME XI



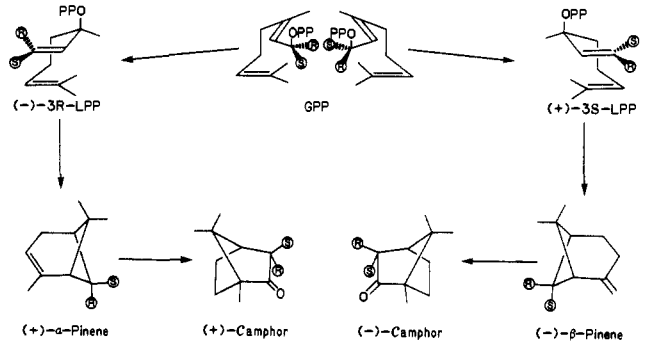
and as before with the fenchyl and bornyl cyclases, the preferred linalyl enantiomer was a more efficient precursor than was geranyl pyrophosphate.

It is curious that certain cyclases, notably (+)-bornyl pyrophosphate cyclase and (−)-*endo*-fenchol cyclase, are capable of cyclizing, at relatively slow rates, the (3*S*)-linalyl pyrophosphate enantiomer to the respective antipodal products (−)-bornyl pyrophosphate and (+)-*endo*-fenchol.[155,156] Since both (+)-bornyl pyrophosphate cyclase and (−)-*endo*-fenchol cyclase produce the designated products in optically pure form from geranyl, neryl, and (3*R*)-linalyl pyrophosphate, the antipodal cyclizations of the 3*S*-linalyl enantiomer are clearly abnormal and indicate the inability to completely discriminate between the similar overall hydrophobic/hydrophilic profiles presented by the linalyl enantiomers in their approach from solution. The spatial similarity of the antipodes is most obvious in the anti,endo conformation, which has been demonstrated to be preferred for cyclization of linalyl derivatives in solution[121] and by these cyclases. That the cisoid, anti,endo conformers of (3*R*)- and (3*S*)-linalyl pyrophosphate are effectively isosteric, while the corresponding helical conformers of geranyl pyrophosphate are not, has been invoked to rationalize the anomalous cyclization of the unnatural enantiomer and to explain the completely stereospecific isomerization–cyclization of the achiral substrate.[156] It need be reemphasized that the anomalous cyclizations of the unnatural linalyl pyrophosphate enantiomer are slow relative to that of the universal achiral precursor geranyl pyrophosphate. However, they are, while completely unexpected, nevertheless stereochemically consistent with the proposed cyclization scheme.

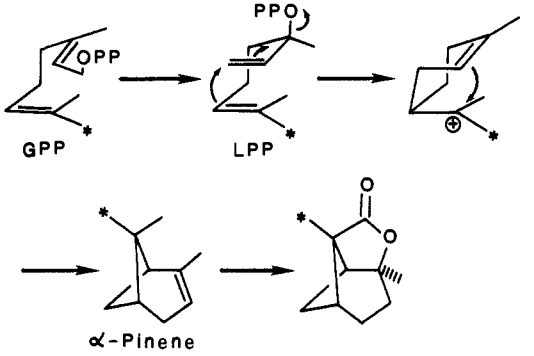
The unusual cyclization of (3*S*)-linalyl pyrophosphate to (+)-*endo*-fenchol by the (−)-*endo*-fenchol cyclase is accompanied by some loss of the normal regiochemical control observed with geranyl or (3*R*)-linalyl pyrophosphates, since aberrant terminations at the acyclic, monocyclic, and bicyclic stages of the cationic cyclization cascade are also observed.[155] The absolute configurations of these abnormal coproducts have yet to be examined. The pinene cyclases too convert the unnatural linalyl enantiomer to abnormal levels of acyclic (e.g., myrcene) and monocyclic (e.g., limonene) terpenes. These aberrant products perhaps arise via ionization of this substrate in the transoid or other partially extended (exo) form,[45,92,157] for which precedent exists in chemical models,[108,118,124] or via alteration in positioning effects of the cationic intermediates with respect to the pyrophosphate counter ion.[157] Such results point out the difficulty in interpreting earlier observations on product formation obtained with the racemic linalyl precursor.[42,43,48] The (+)-pinene cyclase from *S. officinalis* also converts neryl pyrophosphate to abnormally high (relative to geranyl pyrophosphate) levels of limonene.[157] In this instance, the product was resolved chromatographically and shown to be comprised largely of (−)-limonene, instead of the normal (+)-isomer obtained with geranyl pyrophosphate, suggesting that neryl pyrophosphate is cyclized in this instance in the exo conformation.[158] In any event, for all "normal" cyclizations examined thus far, the configuration of the cyclizing linalyl intermediate was confirmed to be that which would be expected on the basis of an anti,endo conformation. Scattered attempts at intercalating the cyclization cascade with chiral analogues of proposed, ion-paired, cyclic intermediates (e.g., α-terpinyl pyrophosphate and 2-pinyl pyrophosphate), not surprisingly, have been completely unsuccessful.[74,92,99]

An important consequence of the anti cyclization, in which the leaving group departs from the allylic system on the side opposite to that which the incoming nucleophilic group of the substrate becomes attached, is that configuration of C1 of the geranyl substrate will be retained. Thus, following the syn-allylic transposition, transoid to cisoid rotation about the newly generated C2–C3 single bond of the linalyl system brings the face of C1 from which the pyrophosphate moiety has departed into juxtaposition with the neighboring *si* face of the C6–C7 double bond from which C1–C6 ring closure occurs (Scheme XI). The cis analogue neryl pyrophosphate, on the other hand, should exhibit inversion of configuration at C1, since the cyclization is either direct or involves isomerization to the linalyl intermediate and cyclization without the attendant C2–C3 rotation. These crucial predictions were confirmed directly with 1*R*-1-^{3}H;2-^{14}C- and 1*S*-1-^{3}H;2-^{14}C-labeled geranyl and neryl pyrophosphates as substrates. Thus, each stereospecifically labeled precursor was separately converted to (+)-bornyl pyrophosphate and (−)-bornyl pyrophosphate by partially purified preparations from *S. officinalis* and *T. vulgare*, respectively.[159] Each pyrophosphate ester was hydrolyzed, and the resulting borneol was oxidized to camphor (Scheme XI). The stereochemistry at C3 of the derived ketone (corresponding to C1 of the acyclic precursor) was determined by taking advantage of the stereoselective base-catalyzed exchange of the exo α-protons.[160,161] By comparison of such exchange rates with those of product generated from the corresponding 1*RS*-1-^{3}H;2-^{14}C-labeled substrate, it was demonstrated that geranyl

SCHEME XII



SCHEME XIII



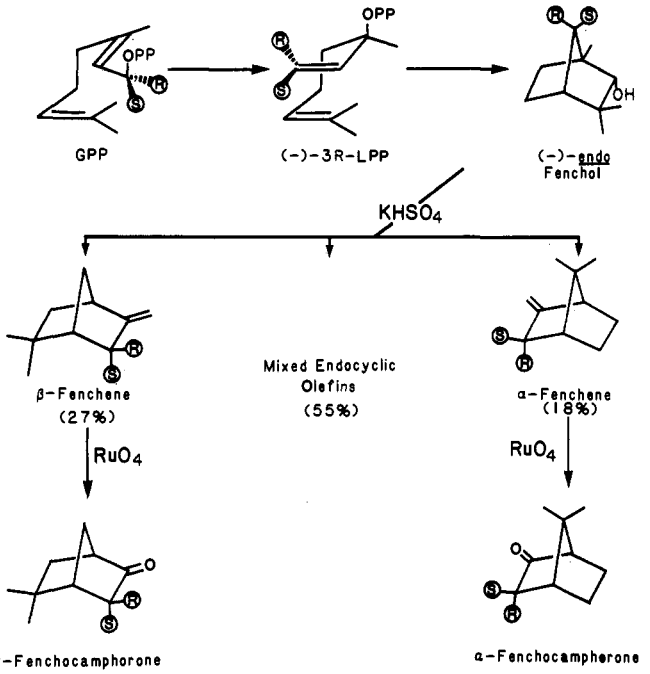
pyrophosphate was cyclized to bornyl pyrophosphate with net retention of configuration at C1, whereas C1 of neryl pyrophosphate was inverted in cyclization to both enantiomers. The observed stereochemistry therefore was entirely compatible with the proposed cyclization scheme. More recently, (+)-bornyl pyrophosphate derived from (3*R*)-[1*Z*-^{3}H]linalyl pyrophosphate and (−)-bornyl pyrophosphate derived from (3*S*)-[1*Z*-^{3}H]linalyl pyrophosphate, by the respective enzymes, were converted to camphor (cf. Scheme XI), and the location of the tritium was determined by similar means.[162] The label was located in the C3 endo position of camphor in both cases, an observation, which taken together with the earlier stereochemical results,[156,159] confirmed the *syn* migration of the pyrophosphate in the overall isomerization and cyclization of geranyl pyrophosphate to these products.

In examining conformational questions in the cyclization of geranyl pyrophosphate to the (+)- and (−)-pinenes by enzymes from *S. officinalis*, the 1*R*- and 1*S*-^{3}H-labeled geranyl precursors were exploited as before.[162] The (+)-α-pinene so obtained was converted to (+)-camphor (^{3}H:^{14}C ratio determined as the crystalline oxime) (Scheme XII). The (−)-β-pinene was similarly converted to (−)-camphor and the ^{3}H:^{14}C ratio determined. Tritium at C3 was then located, as before, by taking advantage of the selective exchange of the exo α-protons and by comparing exchange curves with those of product generated from the racemic 1-^{3}H;^{14}C-labeled substrate. The pinane to bornane skeletal rearrangement proceeds with a degree of racemization (15–20%), placing the tritium at C5 and thus inaccessible to exchange. This minor complication does not alter the results, which indicated that cyclization of geranyl pyrophosphate to the (+)- and (−)-pinenes occurred with retention of configuration at C1 as predicted. Retention of configuration at C1 of geranyl pyrophosphate as a consequence of the C2–C3 rotation in monoterpene cyclization may be contrasted with the inversion of configuration that occurs in the mechanistically related prenyltransferase-mediated condensation of dimethylallyl pyrophosphate with isopentenyl pyrophosphate to give geranyl pyrophosphate itself.[53,163]

The *gem*-dimethyl bridge of the pinenes is a common structural feature of bicyclic monoterpenes, and the stereochemistry of the cyclizations that form the prochiral methyl groups was recently examined using [8*E*-^{3}H]geranyl pyrophosphate as a precursor with the (+)- and (−)-pinene cyclases.[164] The exclusive location of tritium in the exo-methyl group of the (+)- and (−)-[^{3}H]α-pinenes so obtained was established by the complete retention of radioactivity after a degradative sequence involving oxidative conversion of the endomethyl group to the carbonyl carbon of 2-hydroxy-2,6-dimethylbicyclo[3.1.1]heptane-6-carboxylic acid lactone (Scheme XIII). It was therefore clear that in these enantiomeric cyclizations the *E*-methyl of the substrate becomes the exo-methyl group in both (+)- and (−)-α-pinene. Consequently, the initial anti,endo cyclizations of the linalyl antipodes are followed by enantiomeric, least-motion (30 vs. 150° of rotation at C6–C7) cyclizations of the boatlike α-terpinyl intermediates to form the *gem*-dimethyl-bearing cyclobutane rings of these compounds. Since the C6,C7-double bond does not participate in the ionization of the allylic substrate, the observed stereochemical results (i.e., at both C7 and C1) are best accounted for by a preassociation mechanism[165] that minimizes the opportunity for rotational/conformational change during the course of the multistep reaction sequence. Preliminary studies[148] also indicated that with [8*E*-^{3}H]geranyl pyrophosphate as substrate the proportion of limonene is diminished in the olefin mixtures produced by the (+)- and (−)-pinene cyclases. This observation implies that, following cyclization to the respective α-terpinyl intermediates, (+)- and (−)-limonene are formed by regiospecific deprotonation, the *E* methyl of the substrate becoming the methylene of the isopropenyl function. This result, in addition to studies on the enzymatic solvolysis of substrate analogues to be described in section IVE, lend further credence to the concept of a preassociation mechanism whereby topology is maintained between the initial ionization and the termination steps. It is at the same time clear that the cyclase active site cannot rigidly complement the reacting conformation of the geranyl substrate alone, since the enzyme must accommodate the motion attendant to the isomerization and C2–C3 bond rotation events. This concept will be further developed in a later section.

The stereochemical fate at C1 of geranyl pyrophosphate in the isomerization–cyclization to (−)-*endo*-fenchol was also determined in order to address the question of reacting conformations in this transformation.[162] (1*R*)-[1-^{3}H;2-^{14}C]- and (1*S*)-[1-^{3}H;2-^{14}C]-Geranyl pyrophosphates were separately converted to product by preparative incubations with the *F. vulgare* cyclase, and the product of each precursor was stereospecifically dehydrated to α-fenchene and β-fenchene in addition to five other (endocyclic) olefins (Scheme XIV). The mixture was oxidized directly, and the resulting α-fenchocamphorone and β-fenchocamphorone were separated from other more highly oxygenated derivatives and converted to the respective oximes for determination of ^{3}H:^{14}C ratios. Since C1 of

SCHEME XIV



the substrate was now placed adjacent to the carbonyl in each fenchocamphorone, tritium was readily located by taking advantage of the selective exchange of the exo α-hydrogen, much as was done previously in the case of camphor.[159] The exo/endo exchange rate difference for the α-isomer is comparable to that of camphor (~ 20:1), whereas the rate difference for the β-isomer is some 10-fold greater.[161] Comparison of exchange curves for the fenchocamphorones generated from the stereospecifically labeled precursors with those for the corresponding fenchocamphorones obtained from the racemic (1*RS*)-[1-^{3}H;2-^{14}C]geranyl pyrophosphate provided convincing evidence for net retention of configuration of C1 in this cyclization, completely analogous with results from the pinane and bornane series, and additionally confirming the nearly universal preference for anti stereochemistry in mono-, sesqui-, and diterpene cyclizations.[139,166-172]

The summation of the above studies firmly establishes the overall stereochemistry of the isomerization and cyclization of the achiral precursor geranyl pyrophosphate to bornane, pinane, and fenchane monoterpenes. Specifically, the results permitted determination of the reacting helical conformation of geranyl pyrophosphate and the stereochemistry of the initial allylic rearrangement of geranyl to linalyl pyrophosphate (syn), the stereochemistry, following C2–C3 rotation, of the subsequent allylic displacement with C1–C6 cyclization (anti), and the configuration and conformation (anti,endo) of the cyclizing tertiary allylic, linalyl, intermediate and, thus, the face of the C6–C7 π system involved in bonding. Although no attempt has been made here to cover all possible cyclization modes, it is clear that a consistent three-dimensional picture has emerged from the work described, which validates the stereochemical aspects of the proposed syn-isomerization–anti,endo-cyclization model and eliminates other stereochemical alternatives for this enzymatic reaction type (i.e., bicyclization). The isomerizations of geranyl pyrophosphate are stereospecific (the enantiomer formed depending on the initial folding of the precursor), as are the respective cyclizations of the linalyl pyrophosphate enantiomers. With the achiral substrate, stereospecificity is induced at the ionization–isomerization step in that, once chirality of the bound tertiary intermediate is established from the appropriate helical conformer of the geranyl substrate, the stereochemical outcome of the subsequent cyclizations is fixed and thereafter determined relative to the absolute configuration at the C3 tertiary center. A preassociation mechanism is envisioned whereby the initially generated allylic cation–anion pair is first protected from the opposite face by the proximally placed nucleophilic double bond which then, following collapse to linalyl pyrophosphate and C2–C3 rotation, allylically displaces the pyrophosphate moiety in the subsequent ionization–cyclization step.

It should be emphasized that the current paradigm is based on bornane, fenchane, and pinane monoterpenes and that complete stereochemical definition has yet to be applied to monocyclics in general or to anomalous cyclization products, and it is conceivable that such cyclizations involve syn processes and/or extended (exo) conformations. It is not known, for any cyclization, in what way the enzyme controls the required conformation of the reacting substrate yet accommodates the dramatic changes in substrate structure that accompany isomerization–cyclization, what factors determine formation of distinct products arising from apparently identical substrate conformations, or how multiple products in fixed ratio arise or the selection of a single reaction channel is enforced by precise control of substrate conformation and the positioning of the counter ion.

E. Mechanism

The ability of monterpene cyclases to utilize linalyl pyrophosphate as an acyclic precursor permitted determination of configuration of the normally cryptic cyclizing intermediate and additionally allowed separate focus on the cyclization component of the reaction sequence by simply bypassing the normally tightly coupled isomerization step. (It need be emphasized again that there is no evidence in the monoterpene series for a separate isomerase activity; however, a farnesyl to nerolidyl pyrophosphate isomerase was firmly established in the biosynthesis of certain fungal sesquiterpenes.[114]) For bornane, pinane, and fenchane monoterepenes, the K_m values for the appropriate linalyl pyrophosphate enantiomer are lower, in most cases, than those for geranyl pyrophosphate, while the relative velocities for cyclization are substantially higher in all instances, resulting in catalytic efficiencies (V/K_m) up to 10 times higher for the natural linalyl pyrophosphate enantiomer than for the achiral primary allylic isomer.[155-157] These results suggest that the isomerization of geranyl to linalyl pyrophosphate is the slow step of the reaction sequence (compared with the cyclization of the more reactive linalyl intermediate); however, the limiting component of the coupled process is not yet clear in any instance, although it is probably the initial ionization. The difference in enzymatic cyclization velocities observed for geranyl and linalyl pyrophosphates[155-157] is considerably less than that which would be expected from differences in solvolytic reactivity alone,[115,116] indicating that the cyclases are ca-

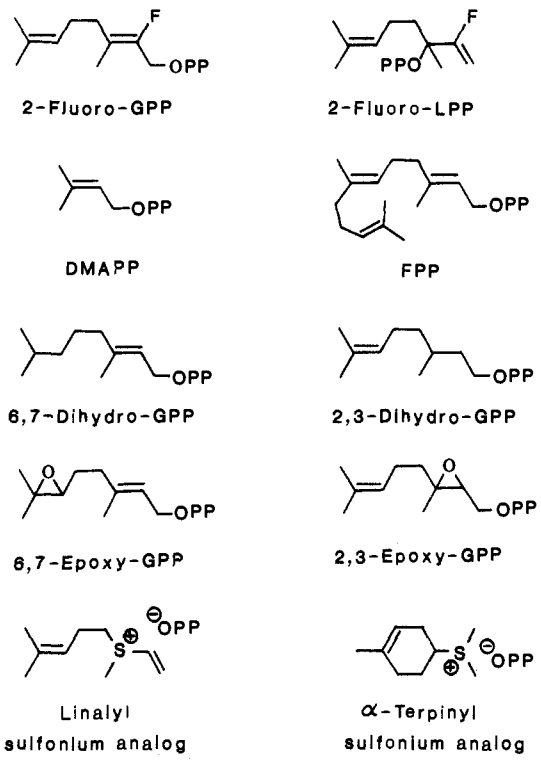


Figure 2. Substrate and reactive intermediate analogues.

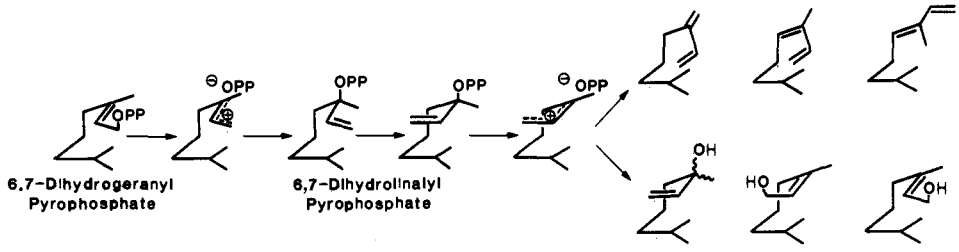
pable of fostering a relative rate acceleration in the case of the primary allylic precursor.

Both the double-bond isomerization and cyclization components of the enzymatic reaction sequence are thought to occur at the same active site and to be initiated by the same event, ionization of the corresponding primary and tertiary allylic pyrophosphates to the ion pair. Evidence for the electrophilic nature of both steps has been obtained with, as alternate substrates, 2-fluorogeranyl pyrophosphate and 2-fluorolinalyl pyrophosphate (Figure 2) in which the electron-withdrawing fluorine substituent would be expected to retard ionization at the respective primary and tertiary centers.[173] A similar approach was exploited earlier by Poulter in deciphering the electrophilic reaction mechanism of prenyltransferase, which involves a single ionization step.[174,175] The rate suppressions observed (2 orders of magnitude at minimum) with these fluorinated C_{10} analogues with several cyclases, by comparison with corresponding rates for solvolysis and nucleophilic displacement,[175] led to the conclusion that this enzyme type functions by ionization of the relevant allylic pyrophosphate in both isomerization (geranyl pyrophosphate) and cyclization (linalyl pyrophosphate) steps and does not involve concerted displacements.[173] The conclusions drawn with fluorinated analogues, which by competitive inhibition studies were shown to closely resemble the substrate in binding behavior, were bolstered by findings with other types of competitive inhibitors bearing the allylic pyrophosphate functionality, but differing markedly in the alkyl substituent.[86] Thus, the C_5 and C_{15} isoprenologues of geranyl pyrophosphate, dimethylallyl and farnesyl pyrophosphate (Figure 2), not only inhibited cyclization to bornyl pyrophosphate and pinene but also were themselves enzymatically solvolyzed at rates approaching those of the normal cyclization of geranyl pyrophosphate. Thus, dimethylallyl pyrophosphate was converted primarily to the corresponding alcohol at 95% the rate of cyclization, whereas farnesyl pyrophosphate was transformed to a mixture of farnesol (trans,trans) and *trans*-nerolidol at 26% the rate of geranyl pyrophosphate cyclization. 6,7-Dihydrogeranyl pyrophosphate, on the other hand, gave rise (at 48% the rate of cyclization) to a mixture of dihydrogeraniol, dihydronerol, and dihydrolinalool (25% of total product) and to a mixture of the corresponding olefins (75% of total product). The relatively high proportion of olefins in this instance indicates ionization of the analogue in a relatively hydrophobic environment in the enzyme active site, where deprotonation of the resulting cation(s) competes favorably with solvent capture. The effect likely results from the relatively tight binding of this analogue and the probability that the isopropyl group can assume the endo position normally occupied by the isopropylidene function of the normal substrate and thus block premature water capture of the developing cation. Cyclase-catalyzed solvolyses of substrate analogues confirmed the electrophilic nature of the isomerization–cyclization reaction, yet such analogues need not be enzymatically reactive to provide useful information.

Of several other analogues tested (2,3-dihydro-, 2,3-epoxy-, and 6,7-epoxygeranyl pyrophosphate; Figure 2) none were catalytically functional; however, an examination of the inhibitor properties, relative to inorganic pyrophosphate, allowed an assessment of substrate binding determinants from which the interrelationships among substrate functional groups within the active site could be approximated and the topology of geranyl pyrophosphate binding to the cyclase formulated.[86] The pyrophosphate ester function is of principal importance in substrate recognition. The C2–C3 olefin is recognized largely on the basis of geometry, whereas the primary basis of interaction with the C6–C7 olefin is electronic. Evaluation of the interactions of the various domains suggested that, on binding, the relatively planar C1–C4 portion of the substrate carbon skeleton is sandwiched by the pyrophosphate moiety on one side and the planar C5–C8 olefinic function on the other side, with direct enzymatic interactions occurring with both the pyrophosphate and C5–C8 regions. In this way, the isopropylidene group assists in promoting the requisite positioning of C1–C4 and the pyrophosphate to optimize orbital alignment for ionization and cyclization and simultaneously excludes water from the active site, thereby precluding abortive quenching of carbocationic reaction intermediates. The formulation of binding topology based on studies with substrate analogues is entirely consistent with, and further refines, the general picture of cyclase–substrate interaction that has emerged from the aforementioned chemical model reactions as well as stereochemical considerations of the enzymatic isomerization–cyclization.

To determine what features of the pyrophosphate moiety itself were critical for enzyme recognition, inorganic pyrophosphate and a series of structurally related analogues were examined as inhibitors of the (+)-bornyl pyrophosphate and (+)-α-pinene cyclases.[176] Analysis of type and magnitude of inhibition revealed that the combination of ionic state (formal charge) at enzyme pH optimum, ability to chelate divalent metal ions, and intramolecular flexibility were required for

SCHEME XV

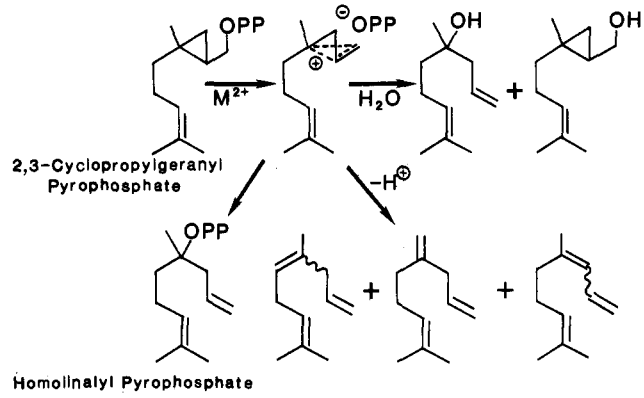


enzyme recognition. For example, methanediphosphonate was nearly as effective an inhibitor of cyclization as was inorganic pyrophosphate. Poulter reported that geranyl methanediphosphonate and geranyl difluoromethanediphosphonate are effective substates for monoterpene cyclases.[177]

Although cyclization of geranyl pyrophosphate is thought to proceed via preliminary isomerization to linalyl pyrophosphate, free linalyl pyrophosphate has never been observed during the conversion of geranyl pyrophosphate to any cyclic product. (3*RS*)-Linalyl pyrophosphate competitively inhibits the conversion of [^{3}H]geranyl pyrophosphate to cyclic product by the various cyclases in a manner predictable from the respective K_m values. Yet, when residual pyrophosphate esters are reisolated from such incubation mixtures and enzymatically hydrolyzed to the respective alcohols, no [^{3}H]linalool can be detected, indicating that the enzyme-generated intermediate is unable to equilibrate with free linalyl pyrophosphate. Unlabeled geranyl pyrophosphate also competitively inhibits the cyclization of (3*RS*)-[^{3}H]linalyl pyrophosphate, and isolation of residual pyrophosphates indicates that [^{3}H]geranyl pyrophosphate is not formed under these conditions. Such results[44,47,84] confirm the utilization of geranyl pyrophosphate and linalyl pyrophosphate at the same site, consistent with a closely coupled isomerization–cyclization mechanism, and indicate, when taken with the K_m values and conversion rates for geranyl and linalyl pyrophosphates,[155-157] that the tertiary intermediate is tightly bound to the cyclase and that cyclization is rapid compared with the rate of dissociation from the enzyme surface or isomerization back to the primary allylic pyrophosphate. Thus, although it has been possible to separately investigate the cyclization step by use of (3*R*)- and (3*S*)-linalyl pyrophosphate and to demonstrate that the cyclization of this precursor is more rapid than the isomerization–cyclization of geranyl pyrophosphate, no ready means has been available to examine the isomerization step in isolation.

In an attempt to dissect the normally cryptic isomerization component from the tightly coupled reaction sequence and directly observe the otherwise transient tertiary intermediate, the noncyclizable substrate analogue 6,7-dihydrogeranyl pyrophosphate was used to probe the cyclizations to (+)-bornyl pyrophosphate and to (+)-α-pinene.[178] Although the C6–C7 double bond of the substrate determines the partitioning to cyclic products, it cannot, for topological reasons, assist in the generation of the initial allylic cation. Thus, saturation of the isopropylidene function prevents cyclization, with minimal effect on binding behavior or on the initial pyrophosphate ionization–migration process. The analogue inhibited the cyclization of both geranyl and linalyl pyrophosphate as noted above and was itself

SCHEME XVI



catalytically active, affording acyclic terpene olefins and alcohols as products (Scheme XV). The enzymatic products generated from 6,7-dihydrogeranyl pyrophosphate qualitatively resembled the solvolysis products of 6,7-*dihydrolinalyl pyrophosphate*,[101] yet they constituted a far higher proportion of olefins, indicating enzymatic product formation in a microenvironment relatively inaccessible to water. 6,7-Dihydrolinalyl pyrophosphate itself was not detected as an enzymatic product in any but trace levels. Since the normal cyclization of geranyl pyrophosphate is considered to proceed via preliminary isomerization to the bound tertiary intermediate (3*R*)-linalyl pyrophosphate, the results suggested that the analogue underwent the normal ionization–migration step, giving rise in this case to (3R)-6,7-dihydrolinalyl pyrophosphate, which was reionized and, because the subsequent cyclizations were precluded, the resulting cation was either deprotonated or captured by water. In divalent metal ion requirement, pH optimum, and other characteristics, the enzymatic "solvolysis" of the analogue resembled the normal monoterpene cyclase reaction.

A far more satisfying result was obtained recently[179] by an alternate attempt to provide direct evidence for the isomerization step as a discrete component of the reaction scheme. This strategy employed a substrate analogue, [1-^{3}H]-2,3-cyclopropylgeranyl pyrophosphate, which in being pseudoallylic was competent to undergo the normal cyclase-catalyzed ionization–isomerization step but which would generate a corresponding homoallylic tertiary pyrophosphate sufficiently unreactive toward the subsequent ionization–cyclization step to allow escape from the active site (Scheme XVI). When incubated with (+)-bornyl pyrophosphate cyclase and (+)-α-pinene cyclase from sage, the racemic analogue gave rise to the products anticipated by discharge of the corresponding cyclopropylcarbinyl cation–pyrophosphate anion pair (31% mixed trienes and 58% mixed alcohols of uncertain stereochemistry), as well as to ~10% of the direct isomerization product, the

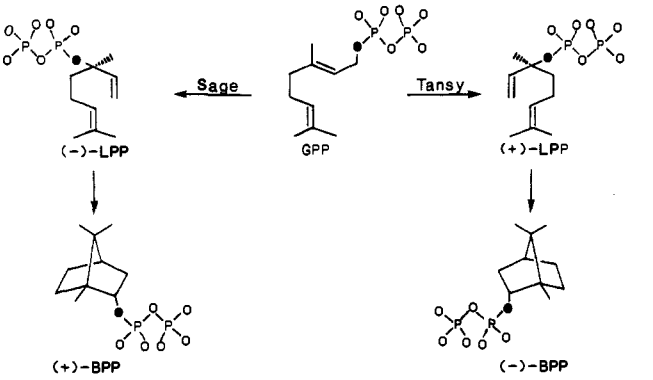
homoallylic pyrophosphate (Scheme XVI). The K_m value for the analogue was over 5 times that of the natural substrate, and the relative rate of enzyme-catalyzed ionization of cyclopropylgeranyl pyrophosphate was approximately 6% of the rate of cyclization observed with geranyl pyrophosphate. In addition to providing the first direct evidence for the cryptic isomerization component of the normally coupled isomerization–cyclization sequence in the conversion of geranyl pyrophosphate to monoterepenes, the cyclase-catalyzed generation of the full spectrum of product types observed from a cyclopropylcarbinyl pyrophosphate now forges a common mechanistic link between three key reactions of isoprenoid metabolism: prenyl transfer and cyclization of allylic pyrophosphates, and ring opening of cyclopropylcarbinyl pyrophosphates such as presqualene and prephytoene pyrophosphates involved in the biosynthesis of sterols[180] and carotenoids,[181] respectively.

The availability of the bornyl pyrophosphate cyclases provided a unique opportunity to directly examine the function of the pyrophosphate moiety in the coupled isomerization–cyclization reaction leading to monoterpenes. Initial studies on the mechanism of the pyrophosphate migration in the conversion of geranyl pyrophosphate to (+)- and (–)-bornyl pyrophosphate established that the two ends of the pyrophosphate moiety of the substrate retained their identities in the cyclization to both products and also indicated that there was no appreciable exchange with exogenous inorganic pyrophosphate in the reaction. Thus, separate incubations of [1-^{3}H;α-^{32}P]- and [1-^{3}H;β-^{32}P]geranyl pyrophosphates with partially purified preparations of each enantiomer-generating cyclase gave [^{3}H;^{32}P]bornyl pyrophosphates (of unchanged isotope ratio) that were selectively hydrolyzed to the corresponding bornyl phosphates. Measurement of ^{3}H:^{32}P ratios of these monophosphate esters indicated that label from only the α-phosphate of the substrate was retained in the derived product.[182]

With the absence of tumbling or end-to-end interchange of the pyrophosphate established for both cyclizations, it became critical to examine the fate of the C–O–P bridge oxygen of the precursor in these transformations. To this end, [8,9-^{14}C;1-^{18}O]geranyl pyrophosphate was prepared and converted to (+)- and (–)-bornyl pyrophosphate by large-scale incubations. Analysis of the products by mass spectrometry of the derived benzoates demonstrated an ^{18}O enrichment identical with that of the original substrate, indicating that the isomerization–cyclization of [1-^{18}O]geranyl pyrophosphate involves essentially no positional oxygen isotope exchange (i.e., the original pyrophosphate ester oxygen of the precursor is the exclusive source of the pyrophosphate ester oxygen of the product).[182]

These results, although implying very tight coupling of the pyrophosphate and terpenoid reaction partners within the enzyme active site, could not distinguish between an initial [1,3]-sigmatropic rearrangement or a tight ion pair in which rotational equilibrium about the Pα–OPβ bond is not achieved, a [3,3]-sigmatropic rearrangement involving the initial attachment of a nonbridge oxygen to C3 of the linalyl system and return of the formerly bridged ^{18}O atom to C2 of the bornyl system, or processes involving bonding of the β-phosphate group at the tertiary center. To examine these various possibilities with respect to the formation and subsequent cyclization of the tertiary intermediate, both α- and β-^{32}P-labeled and 3-^{18}O-labeled linalyl pyrophosphates were prepared. Analysis of products derived from the ^{3}H;^{32}P-labeled substrates, as in the previous experiments, indicated the two ends of the pyrophosphate retained their identities in this cyclization, excluding direct involvement of the β-phosphate of geranyl pyrophosphate in the allylic transposition.[183] Similarly, preparative-scale enzymatic conversions of (±)-[1*E*-^{3}H;3-^{18}O]linalyl pyrophosphate to the enantiomeric bornyl pyrophosphates, followed by mass spectrometric analysis of the derived benzoates, yielded an ^{18}O enrichment of the carbinol oxygen atom of the benzoate esters virtually identical with that of the precursor.[183] The alternative [3,3]-sigmatropic rearrangement was therefore eliminated.

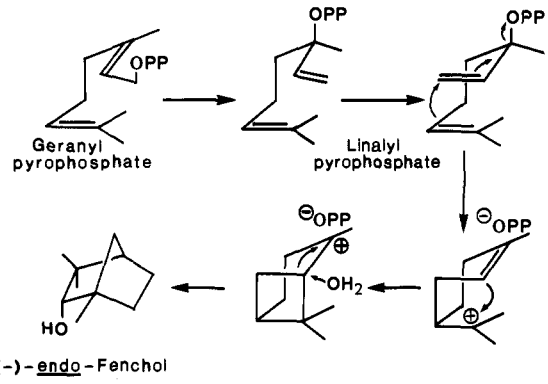
SCHEME XVII.[a]



[a] BPP is bornyl pyrophosphate. The darkened oxygen atom indicates ^{18}O.

The summary of the results clearly indicated that it is solely the pyrophosphate ester oxygen of geranyl pyrophosphate that is involved in all the critical bonding processes in the coupled isomerization–cyclization leading to formation of both (+)- and (–)-bornyl pyrophosphate (Scheme XVII) and, thus, that the pyrophosphate moiety remains closely associated with its terpenyl partner throughout the course of the reaction. These findings strongly support tight ion pairing in the transformation. The observed absence of Pα–Pβ interchange and complete lack of positional ^{18}O-isotope exchange in the case of bornyl pyrophosphate cyclase is particularly notable since the reaction seemingly involves a formal 1,3- followed by a 1,2-migration of the pyrophosphate, with the intervening generation of the transient α-terpinyl cation–pyrophosphate anion pair in which the charge separation is at least 3 Å. It is not yet clear whether the observed lack of positional isotope exchange is due to the strong electrostatic attraction between the inorganic pyrophosphate–metal ion complex and the paired carbocations or whether the restriction in the motion of the pyrophosphate moiety is a more direct result of interaction with the cyclase itself. The results with the bornyl pyrophosphate cyclases may be contrasted with a related study on the role of the pyrophosphate moiety in allylic pyrophosphate isomerization in the sesquiterpene series in which equilibration of the proximal phosphate oxygens was observed in the enzymatic conversion of farnesyl to nerolidyl pyrophosphate.[114] In this instance the oxygen scrambling is presumed to result from reversible con-

SCHEME XVIII



version of the primary and tertiary allylic pyrophosphate isomers at the active site. Conversely, Poulter reported that [1-^{18}O]geranyl pyrophosphate reisolated from incubations with prenyltransferase had not undergone detectable scrambling, in spite of definitive evidence for the generation of allylic cations at the enzyme active site.[130,184]

Results obtained with the (+)- and (−)bornyl pyrophosphate cyclases add to a growing body of evidence supporting the general involvement of ion-pair intermediates in the enzymatic transformation of allylic pyrophosphates[53,55,114,144,184] and imply this common feature for monoterpene cyclases. However, most monoterpene cyclases terminate the reaction by deprotonation of a carbocation to afford an olefin or carbocation capture by water rather than by the pyrophosphate anion. Thus, for example, (−)-*endo*-fenchol derived from [1-^{18}O]geranyl pyrophosphate, in enzyme preparations from *F. vulgare*, bore no detectable ^{18}O label.[185] This result, implying water as the source of oxygen in the cyclic product, is nevertheless fully consistent with the unified cyclization model (Scheme XVIII), in that the stereochemistry of (−)-*endo*-fenchol formation in incompatible with internal return of the pyrophosphate and subsequent P–O bond hydrolysis (i.e., only *exo*-fenchol could possibly be formed via this route).[185]

A rather different approach, employing the sulfonium ion analogues of the linalyl and α-terpinyl cationic intermediates of the cyclization reaction (Figure 2), was also taken to buttress the case for ion pairing.[186] Both analogues were effective inhibitors of the cyclizations of geranyl pyrophosphate to (+)-bornyl pyrophosphate and (+)-α-pinene, with K_i values in the micromolar range. In the presence of inorganic pyrophosphate, however, the K_i values dropped to submicromolar levels (below the range of K_m values for the substrate). Similarly, the K_i values for inorganic pyrophosphate, itself a modest inhibitor, were decreased many fold by the presence of either sulfonium analogue. That the combination of sulfonium analogue and pyrophosphate provided synergistic inhibition of the electrophilic cyclizations suggested that the cyclases bind the paired species more tightly than either partner alone and therefore implicate ion pairing in the transformation of the allylic pyrophosphate substrate. Other anions were not effective in this role, and other diverse trialkylsulfonium salts were ineffective inhibitors of cyclization, thus indicating that inhibition by the terpenoid-like analogues in the presence of pyrophosphate was due to both electronic and structural resemblance to the normal, ion-paired intermediates of the cationic cyclization reactions. The α-terpinyl analogue–pyrophosphate pair was a weaker inhibitor than was the linalyl analogue–pyrophosphate pair.[186] This is not surprising since α-terpinyl pyrophosphate itself is a relatively weak inhibitor of cyclization (and not a substrate), and neither analogue nor α-terpinyl pyrophosphate closely resemble the α-terpinyl cation–pyrophosphate anion pair generated at the cyclase active site where the cationic center and pyrophosphate moiety are trans situated to the cyclohexene ring as a result of the anti,endo cyclization. Synergistic inhibition between an olefinic product and inorganic pyrophosphate was observed in sesquiterpene cyclization,[75] but not thus far with monoterpene cyclases.

The studies outlined above give a strong indication that the pyrophosphate moiety of the substrate is a major contributor to cyclase–substrate interactions, a conclusion consistent with earlier results of studies with both reactive and unreactive substrate analogues. It should also be noted, however, that binding of the sulfonium analogues to the cyclases in the absence of inorganic pyrophosphate is still quite respectable and is presumed to result from the same noncovalent interactions, probably a combination of structural and electronic effects, involved in aligning and stabilizing the substrate as well as the various cationic species generated in the course of the normal catalytic cycle. The topography of these interactions, and thus the relative positioning of the terpenyl partner with respect to the pyrophosphate, almost certainly underlies the inherent regio- and stereochemical features of these cyclization reactions.

It is also possible that the pyrophosphate moiety of the substrate functions as the base in the terminating deprotonation step of some cyclizations. The assistance of the pyrophosphate in deprotonation has been implicated in the prenyltransferase reaction,[52] which the cyclizations clearly resemble in many respects.[144] Should such assistance apply in monoterpene olefin cyclization, a spatial correlation must exist between the position of the pyrophosphate and the proton removed from the proximal face of the corresponding cation. Experimental observation of such a spatial arrangement would provide indirect evidence for this additional function of the pyrophosphate group in olefin synthesis.

Although investigations thus far have been largely confined to a few parent bicyclic skeletal forms, the internal consistency of the many observations describes a coherent scheme for monoterpene cyclization and justifies the assumption that all cyclohexanoid types are generated by variations on the same general mechanism involving only a few possible conformations of the allylic pyrophosphate precursor. A more precise formulation of substrate–cyclase interactions, within the context of this still evolving model, must take into account the substantial changes in charge distribution, hybridization, configuration, and bonding that comprise the isomerization–cyclization process and its component parts. Indeed, half of the carbon atoms of geranyl pyrophosphate undergo such alterations in the conversion to bornyl pyrophosphate. It is not yet understood how the cyclase lowers the activation energy for any of these fundamental bond transformations or enforces the selection of single or multiple reaction channels while

surviving the transient generation of highly reactive electrophilic species at the active site. Covalent interaction between substrate and enzyme now seems extremely improbable and naturally leads to the more difficult ground of determining how, on a molecular level, the enzyme catalyzes the reaction sequence. Such studies can only be accomplished with pure cyclases and will require more suitable means of dissecting the multistep reaction into its component functional parts. Pure enzymes will also permit the study of these catalysts as proteins.

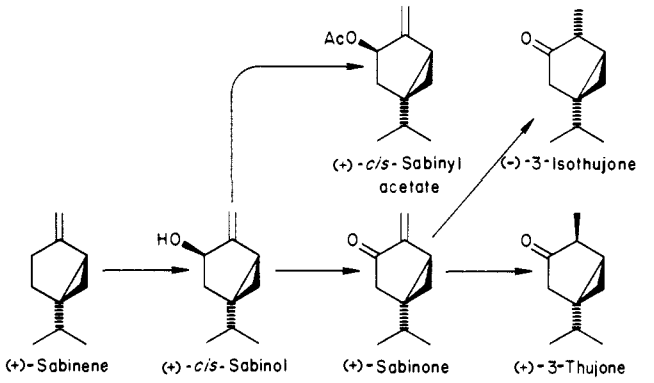
V. Secondary Transformations of Cyclic Parents

A. General Pathways

Whereas relatively few cyclases appear to determine the basic structural character of the monoterpenes produced by a given species, any compendium of monoterpene compounds[2,3] (see also Figure 1) will illustrate the very large number of simple derivatives of each skeletal type found in nature and give some appreciation of the assortment of secondary enzymatic transformations (oxidations, reductions, hydrations, isomerizations, conjugations) presumed to occur among this terpenoid class. Such modifications are generally responsible for imparting the biological functions of many of these compounds. Although the metabolism of the lower terpenes encompasses a diverse range of biochemical transformations, the terpenoids of higher plants, especially those isolated from the essential oils, are commonly olefins or simple oxygenated derivatives. The cooccurrence of such structurally related compounds has led to considerable speculation concerning pathways and precursor–product relationships,[1,23,187] yet few of these proposals have actually been tested via in vivo tracer studies or with cell-free enzyme systems. Reports on the metabolism of monoterpenes by microbial and animal systems are legion,[188–191] and studies on the biotransformation of monoterpenes in plant cell cultures (and derived enzyme systems) obtained from monoterpene-deficient species (e.g., tobacco) have begun to appear.[192,193] The monoterpenes in these cases represent adventitious substrates, and the results are of limited relevance for metabolism in producing species except, of course, as possible models. A number of dehydrogenases and reductases involved in the metabolism of acyclic, monocyclic, and bicyclic monoterpenes have been described[194–196] (for example, the borneol dehydrogenase referred to in a previous section[89]), and these are probably representative of metabolic enzymes of this class. This work has been sporadic and these few isolated examples add little to our overall understanding of terpene metabolism. Rather than compiling a list of such examples or of proposed reaction schemes, most of which have more than ample biochemical precedent, the two central and long-standing questions[197] regarding secondary transformations will be addressed, from which the relevant features of much of the earlier work will become clear. The first question deals with a general strategy for the oxidative modification of monoterpene olefins, while the second question addresses the specificity of the numerous enzymes involved in these transformations.

Surveys of monoterpene cyclization reactions[18,35,44,47] indicate that there are relatively few cases in which

SCHEME XIX



oxygen is introduced in the cyclization step (as, for example, bornyl pyrophosphate, fenchol, and 1,8-cineole) and many instances in which the product of cyclization is an olefin. The formation of oxygenated derivatives of the latter types must necessarily involve subsequent oxidation of the parent olefins, and this process has been one of the most poorly understood aspects of monoterpene metabolism. Recent studies on the origin of two different classes of monoterpenes provided evidence for what is probably a general biosynthetic strategy for the formation of these oxygenated derivatives.

3-Thujone, 3-isothujone, and related thujane-type monoterpenes (Scheme XIX) are widely distributed in the plant kingdom and they often cooccur. An early proposal by Banthorpe and co-workers for the formation of 3-thujone and 3-isothujone via the photooxidation of the olefin sabinene was based on the observations that sabinene rather than the normal product 3-thujone accumulated in tissue cultures of *T. vulgare* as a presumed adaptation to low oxygen tensions[198] and that the photooxidation of this olefin did afford some C3 hydroperoxides that might, by a series of subsequent steps, provide the ketones.[199] The hydration of α-thujene was also suggested as a route to the oxygenated derivatives.[198,200] Later, Banthorpe put forward hypotheses that involved the cyclization of a C_{10} acyclic precursor to various thujanol isomers followed by oxidation to the ketone; these schemes assumed the introduction of oxygen during the cyclization step and thus eliminated the precursor role of the olefins.[30,201] The lack of firm biochemical support for any of these proposals, coupled to the inability to demonstrate the cyclization of acyclic precursors to the thujanols in workable yields,[18] prompted a reexamination of the possibility that oxygenation of the preformed olefin was the correct route to the thujones. *Artemisia absinthium*, a species that produces thujane-type monoterpenes almost exclusively [the volatile oil is comprised of (+)-sabinyl acetate (42%), (+)-3-thujone (32%), (+)-sabinene (12%), and (−)-α-thujene (3%)], was used as a test system to demonstrate that sabinene was the key bicyclic precursor of 3-thujone and other C3-oxygenated compounds of this structural group.[97] Initially, the acyclic precursor [1-^{3}H]geraniol was shown to be incorporated, under aerobic conditions, into the thujane-type monoterpenes in *A. absinthium* leaves in proportion to their natural abundance in this tissue. Light had little effect on the synthesis of these monoterpenes from exogenous geraniol; however, at reduced oxygen levels label from geraniol accumulated in the

olefin sabinene while much less sabinyl acetate and 3-thujone were formed, suggesting a route to the ester and ketone by the allylic, nonphotochemical, oxygenation of sabinene (Scheme XIX). Supporting evidence for the intermediary role of the olefin was provided by isotopic dilution studies in which (+)-sabinene, but not (−)-α-thujene, blocked formation of the oxygenated derivatives from the labeled precursor. (+)-[10-^{3}H]-Sabinene was tested as a substrate in *A. absinthium* leaves and was incorporated directly into both (+)-[10-^{3}H]sabinyl acetate and (+)-3-[10-^{3}H]thujone. [^{3}H]Sabinene was also specifically incorporated into (+)-3-thujone in *T. vulgare* and into the diastereomeric ketone (−)-3-isothujone in *S. officinalis*, confirming the role of this bicyclic olefin as the essential precursor of C3-oxygenated thujane monoterpenes. α-Thujene, α-terpineol, and terpinen-4-ol (Figure 1), previously implicated by Banthorpe as possible intermediates in the biosynthesis of the thujones,[198,201-203] were ruled out of such a role by direct testing of the labeled compounds, while additional isotopic dilution studies provided further evidence to support the pathway illustrated (Scheme XIX).[97] Thus, the various thujyl alcohols and *trans*-sabinol (hydroxyl and cyclopropyl trans) decreased the incorporation of [^{3}H]sabinene into *cis*-sabinyl acetate without trapping counts in the diluent, but had little influence on 3-thujone formation. *cis*-Sabinol, on the other hand, decreased incorporation of [^{3}H]sabinene into *cis*-sabinyl acetate and 3-thujone, and did trap radioactivity. Thus, *cis*-sabinol appeared to be on the pathway to both ester and ketone, whereas the other alcohols tested were not intermediates but did apparently compete as substrates for acetylation. When sabinone was tested in isotopic dilution experiments, the incorporation of [^{3}H]sabinene into 3-thujone was decreased relative to sabinyl acetate, but only traces of labeled sabinone were trapped, perhaps reflecting differing degrees of accessibility to labeled substrate and diluent resulting from the differing subcellular location of various branches of the pathway. From this work it seemed almost certain that the allylic hydroxylation of sabinene to sabinol was the first step in the transformation of the olefin to C3-oxygenated derivatives.[97]

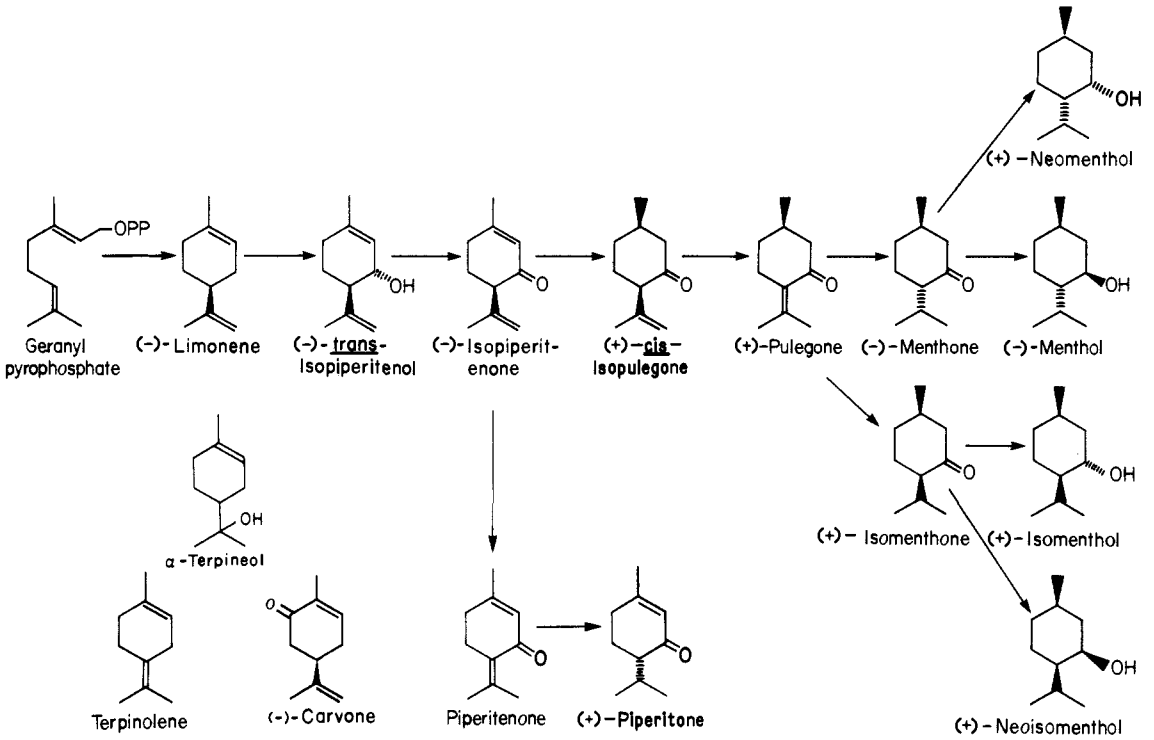
The circuitous route to deducing the pathway to thujane monoterpenes, coupled to the minor content of olefins in the relevant producing species (the production of cyclic products is commonly much slower than is their subsequent metabolism[204]), served to divert early attention from (+)-sabinene as a parent cyclization product, and this compound was undoubtedly overlooked in previous radiochemically-based cyclase assays to which the appropriate carrier was not added to prevent evaporative loss. Adequate cyclase assays[47] of extracts of relevant producing species have since indicated the presence of geranyl pyrophosphate:(+)-sabinene cyclase as a prominent activity, and preliminary examination of the enzyme indicates it to be much like other cyclases in general properties.

Microsomal preparations from the leaf epidermis[67] of *S. officinalis*, *A. absinthium*, and *T. vulgare* were recently shown to catalyze the NADPH and O_2 dependent hydroxylation of (+)-sabinene to (+)-*cis*-sabinol as the sole product.[205] The hydroxylase system was solubilized and characterized with regard to reaction conditions, inhibitors, and activators. Activity was inhibited by rabbit anti-rat cytochrome P-450 and by CO, and the latter inhibition was reversed by 450-nm light. A CO difference spectrum and type I substrate binding spectrum were obtained, and the enzyme met most of the established criteria for a cytochrome P-450 dependent mixed function oxygenase and thus represents one of very few enzyme systems of this type to be isolated from a higher plant. Since background levels of endogenous monoterpenes could be removed from the solubilized preparation, it was possible to carry out substrate specificity studies using unlabeled olefins and very sensitive capillary GLC/MS-based assays. Of the dozen olefins screened, including several such as β-pinene and β-phellandrene that bear an exocyclic methylene, only (+)-sabinene was hydroxylated. A high degree of substrate selectivity distinguishes plant-derived mixed function oxygenases from similar systems of mammalian origin.

The (+)-*cis*-sabinol dehydrogenase was also recently isolated[206] and will be described later. A number of double-bond reductases have been described,[194,196] which are thought to function in the metabolism of monoterpene olefins and enones. The NADPH-dependent stereoselective reduction of (+)-sabinone to either (+)-3-thujone or (−)-3-isothujone was demonstrated in preparations from relevant *Salvia*, *Tanacetum*, and *Artemisia* species, but the enzymes responsible have not yet been characterized.[207] A monoterpenol:acetyl-CoA acetyltransferase operates in the biosynthesis of acetates of menthol and its isomers,[208] and a similar enzyme is probably responsible for the formation of sabinyl acetate in the present case (Scheme XIX). The singular example examined thus far, although highly selective for acetyl-CoA, is not very specific for the monoterpenol cosubstrate, which may serve to explain the in vivo results with *A. absinthium* obtained on adminstering the various thujyl alcohols. The summation of evidence from both in vivo and in vitro studies provides strong support for the biosynthesis of C3-oxygenated thujanes by a pathway that involves the allylic hydroxylation of the parent olefin, oxidation to the corresponding α,β-unsaturated carbonyl compound, and eventual reduction of the conjugated double bond, and it provides an illustrative example of enzymatic exploitation of the inherent reactivity of substrates and intermediates in monoterpene metabolism.

Evidence for the allylic oxidation–conjugate reduction scheme in the metabolism of monoterpene olefins was also obtained from studies on the biosynthesis of oxygenated metabolites in *Mentha* species (Scheme XX), although here again the route to deducing the correct pathway was labyrinthine. Early speculation was that α-terpineol (the presumptive cyclic parent) gave rise to either limonene or terpinolene by dehydration, which were respectively converted to (−)-carvone or (−)-menthone and their congeners.[197] Support for the pathway was seemingly obtained by the cell-free demonstration of the cyclization of geranyl pyrophosphate to α-terpineol,[38,42] but here again failure to appreciate the volatility losses of monoterpene olefins led to an underestimate of their significance as cyclization products. Serious reservation about the proposal arose when it was demonstrated that enzyme preparations capable of converting acyclic precursors to α-terpineol,

SCHEME XX

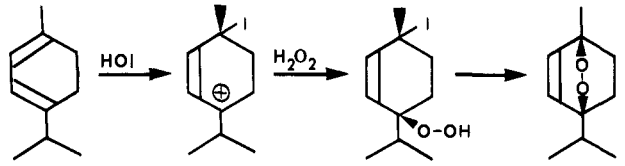


limonene, and terpinolene were incapable of dehydrating α-terpineol to either olefin.[209] With the confirmation that limonene and terpinolene were initial cyclization products (the mechanistically simple cyclization of geranyl pyrophosphate to (-)-limonene predominates in *Mentha*; the cyclase is unremarkable),[147] testing of the labeled olefins as in vivo precursors soon followed, and it was demonstrated that (-)-limonene was the progenitor of both the C3-oxygenated menthone series as well as C2-oxygenated metabolites such as (-)-carvone.[147] Terpinolene was a much less efficient precursor of oxygenated products, supporting the key role of limonene. The subsequent probable steps in the biosynthesis of menthol isomers in peppermint were worked out primarily by Loomis and co-workers[31,197,210-212] using time-course studies and direct feeding experiments with labeled intermediates. Although piperitenone (Scheme XX) was once thought to be a central intermediate of the pathway,[31,197,210] recent studies have shown it to be a dead-end metabolite, giving rise only to piperitone.[213] The bulk of the pulegone and subsequent metabolites are formed by reduction of the endocyclic double bond of (-)-isopiperitenone to (+)-*cis*-isopulegone followed by double-bond isomerization, and not by isomerization of isopiperitenone to piperitenone followed by endocyclic double-bond reduction.

A microsomal cytochrome P-450 mixed function oxidase capable of hydroxylating (-)-limonene to (-)-*trans*-isopiperitenol in the presence of NADPH and O_2 was isolated from the epidermal glands of peppermint (*Mentha piperita*).[207] This system is much like the (+)-sabinene hydroxylase described above, is highly specific for (-)-limonene, and synthesizes (-)-*trans*-isopiperitenol as the singular product; the stereochemistry of this metabolite was confirmed by catalytic hydrogenation to a mixture of (+)-menthol and (+)-isomenthol. The remaining steps of the pathway in peppermint are catalyzed by soluble enzymes that are reasonably active and easily assayed with unlabeled substrates (via GLC/MS) following the removal of endogenous terpenes by treatment of the preparations with beaded polystyrene and partial purification by gel permeation and ion-exchange chromatography.[56,147,213] Notable is the presence of two stereospecific double-bond reductases for the conversion of (+)-pulegone to (-)-menthone and (+)-isomenthone[196] and two stereospecific dehydrogenases responsible for the reduction of (-)-menthone to (-)-menthol and (+)-neomenthol, respectively.[214] The same two enzymes reduce (+)-isomenthone to (+)-neoisomenthol and (+)-isomenthol, respectively. The primary metabolites that accumulate in peppermint are (-)-menthone and (-)-menthol. Thus, allylic oxidation of limonene in this species leads, with an intervening double-bond isomerization, to two conjugate reductions and the eventual reduction of the carbonyl. In spearmint (*Mentha spicata*), hydroxylation of (-)-limonene leads primarily to (-)-*trans*-carveol, which is oxidized to (-)-carvone as the major monoterpene in this species (subsequent reduction products such as dihydrocarvone are minor components). The hydroxylase system of spearmint resembles, in gross properties, that from peppermint but obviously differs in the regiochemistry of the allylic oxidation.[207] The genetic basis for C2 vs. C3 oxygenation of limonene in *Mentha* has been discussed elsewhere.[23,25,215]

The summation of the evidence based on studies of two monoterpene families with several different species provides very strong evidence for the allylic oxidation–conjugate reduction scheme, which can be readily applied to the biosynthesis of many other compounds (e.g., the origin of the myrtenyl and verbenyl series of derivatives from α-pinene). Indeed, a review of monoterpene structures suggests that up to 40% of all naturally occurring oxygenated cyclohexanoids could be accounted for by minor variations on this basic metabolic strategy. A novel mode of monoterpene olefin dioxygenation is the iodoperoxidase-catalyzed conver-

SCHEME XXI



α-Terpinene Ascaridole

sion of α-terpinene to ascaridole (Scheme XXI) demonstrated in cell-free extracts of *Chenopodium ambrosiodes*.[216]

B. Enzyme Specificity

A question often raised with regard to monoterpene interconversions concerns the issue of whether these transformations are carried out by highly specific, or relatively nonspecific, enzymes. Too few examples of monoterpenol dehydrogenases, and double-bond isomerases and reductases, have been studied thus far to allow a definitive answer; however, the enzyme systems that have been examined do exhibit a significant degree of substrate specificity. Thus, the cytochrome P-450 mixed function oxidases are highly selective with regard to substrate; and a (+)-limonene reductase from *Citrus* specifically reduces the exocyclic double bond of this monocyclic diene, and (−)-limonene is not a substrate.[195] Similarly, the aforementioned double-bond reductases involved in monoterpene metabolism in peppermint[196,213] act only on members of the C3-oxygenated series; members of the C2-oxygenated (carvone) series are not substrates. The isomerase and dehydrogenases of mint also use a very limited range of substrates.[56,147,213,214] Several types of evidence indicate that the borneol dehydrogenases isolated from *S. officinalis*,[89] *T. vulgare*, and *F. vulgare*[90] [these enzymes are readily separated from alcohol (ethanol) dehydrogenase] can also use a limited range of thujyl alcohol stereoisomers in the formation of either (+)-3-thujone or (−)-3-isothujone. It, therefore, was once thought that a single dehydrogenase from each species catalyzed the final step of both camphor and thujone or isothujone biosynthesis.[89,90] It is now known that (+)-*cis*-sabinol, not the thujyl alcohols, is the key intermediate in the formation of both thujone and isothujone (Scheme XIX), and the dehydrogenase that oxidizes (+)-*cis*-sabinol to (+)-sabinone, en route to the saturated ketones, is electrophoretically separable from borneol dehydrogenase.[206] The ability of borneol dehydrogenase to utilize thujyl alcohols is of no apparent metabolic significance. By way of contrast to the enzymes involved in oxidative or reductive transformations, the enzymes responsible for the conjugation of monoterpenols, such as acyl- and glucosyltransferases, are rather nonselective with regard to their monoterpenoid cosubstrates.[208,217]

VI. Catabolism

Although the role of monoterpenes in ecological interactions is gaining wide acceptance,[9-15] no function for these components within the plant is obvious, and the sequestration of these natural products within the highly specialized glandular structures that serve as the primary sites of synthesis and accumulation[7] would seem to argue that no physiological or metabolic function is likely.[26-28] The classical perception of monoterpenes as inert waste products permits a simple rationale for the great diversity of monoterpenes produced by plants; i.e., the absence of a specific metabolic or physiological (or other) role allows random structural changes to occur with no disadvantage accruing to the producing species. This simple view is no longer tenable, however, since the aforementioned ecological interactions can impart significant survival value and because considerable evidence has now demonstrated that monoterpenes are by no means inert but are catabolized in a highly specific and ordered fashion. It is the purpose of this section to review the evidence for monoterpene "turnover" and to describe in detail the catabolism of two model cyclic monoterpenes, (−)-menthone and (+)-camphor.

A. Turnover

The apparent loss of monoterpene essential oil constituents during development of several herbaceous species was first recognized at the turn of the century by Charabot,[218] who intuitively reasoned that the process represented the transport and degradation of monoterpenes by the plant. Similar observations of turnover were made numerous times over the intervening years with both herbaceous[197,210] and woody species.[219,220] Although it was once argued that such observations represent only evaporative losses due to lysis of the oil glands,[221] most evidence does not support this view.[197,222,223] Rather, the accumulated evidence is overwhelming that monoterpenes that are stored in, or ultimately exposed to, physiologically active tissues can undergo metabolic turnover. Detailed summations of the supporting arguments are available.[7,18,30,35,36,222]

Two somewhat different types of metabolic turnover phenomena can be distinguished, although at the molecular level they are probably similar if not identical. The first type represents a short-term effect (as, for example, measured by diurnal fluctuation[224-227] or by the time course of radiotracer incorporation[212,227,230]), which is generally thought to involve metabolism of an active intracellular terpene pool and to be highly dependent on the balance between photosynthesis and the utilization of photosynthate.[222] The time course of labeling of monoterpenes in peppermint cuttings after pulse exposure to $^{14}CO_2$ is illustrative.[231] In this instance, nearly 40% of the monoterpenes derived from $^{14}CO_2$ are lost within 10 h of maximal labeling, while overall pool size is increasing since net synthesis exceeds losses.

The second type of turnover occurs during late development and results in a net decrease in monoterpene content. The effect has been observed with numerous species, especially among the Lamiaceae (mints), and monoterpene losses may exceed 50% of the total oil over the course of a few weeks.[232-237] It is clear that under these circumstances the rate of terpene degradation must greatly outpace the rate of terpene synthesis. Unlike the short-term effect, the longer term permanent loss of terpenes necessarily represents the turnover of stored material, most of which is contained in extracellular compartments of the secretory glands. The observed monoterpene turnover is commonly assumed to represent actual catabolism, as the conversion

of exogenous monoterpenoid compounds to primary metabolites, such as amino acids and sugars, was demonstrated in leaf tissues.[29,200] However, most claims for terpene turnover are based on measurements of terpene loss that may also represent conjugation and transport of terpene derivatives out of the tissue being analyzed. Detailed studies on monoterpene turnover were carried out with peppermint (*M. piperita*) as a model system. Turnover of leaf terpenes in this species (30–50% loss) is greatly accelerated late in development[212,217] and is accompanied by collapse of the extracellular oil gland cavities and by extensive ultrastructural modification of both the gland secretory cells and supporting cells.[59]

B. Catabolic Pathways

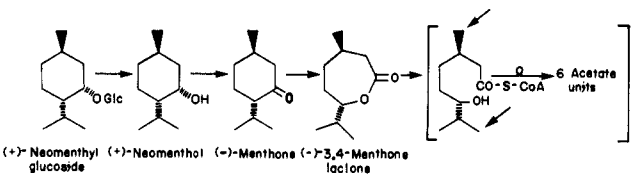
The turnover of monoterpenes in peppermint leaves is coincident with the conversion of (−)-menthone (the major monoterpene constituent) to (−)-menthol and to lesser quantities of (−)-menthyl acetate and (+)-neomenthol (see Scheme XX).[210,212,217] Notably, the significant decrease in menthone content (70–80%) that accompanies the reductive metabolism of this ketone cannot be accounted for by evaporation or by the increase in menthol or other volatile terpenoid constituents. The fate of the missing menthone (30–50%) thus assumes central importance.

Studies on the metabolism of (−)-[G-^{3}H]menthone in leaf discs revealed that menthone loss (not accounted for by volatile metabolites) was a result of the specific conversion of this ketone to a water-soluble metabolite ultimately identified by chemical and enzymatic means as (+)-neomenthyl-β-D-glucoside.[217] Thus, menthone was shown to be reduced to roughly equal amounts of menthol and neomenthol, and while the bulk of the menthol remained in the volatile oil (as such, or as the acetate ester), the bulk of the neomenthol formed was converted to the water-soluble glucoside. These pathways are highly specific in that only menthyl acetate (with little neomenthyl acetate) and only neomenthyl glucoside (with little menthyl glucoside) are formed.

Two possible explanations for such specificity in the metabolic fate of the epimeric alcohols seemed evident; either the enzymes involved exhibited a high degree of selectivity, or the two pathways were compartmentized. Subsequent in vivo studies[238] with labeled menthone, menthol, neomenthol, and CO_2 strongly suggested that specificity was determined at the menthone reduction step, that the systems were compartmentized, and that the transferases were not highly selective enzymes. This latter point was confirmed by isolating the acetyl-CoA-dependent acetyltransferase[208] and the UDPG-dependent glucosyltransferase[238] and examining their specificity for monoterpenols. The two distinct, stereospecific dehydrogenases from *M. piperita* were described earlier.[214]

All of the evidence implicated compartmentation of pathways as an essential feature of monoterpene metabolism in *M. piperita*. By separating the epidermal glands from the remainder of the leaf, it was demonstrated that the menthol dehydrogenase and acetyltransferase were located in the epidermal glands,[239] whereas the neomenthol dehydrogenase and glucosyltransferase were located in the remainder of the leaf that contained the embedded glandular basal cells. Thus, the basis for compartmentation of pathways was intercellular, not intracellular.

SCHEME XXII.[a]



[a] The arrows indicate carboxylation sites. Glc is glucose.

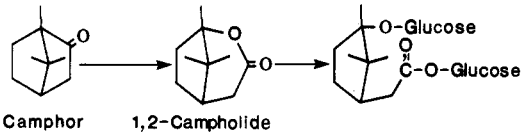
The fact that the glucoside was not further metabolized in peppermint leaf discs.[217] coupled to the water-soluble nature of this material, suggested a possible transport function (which was precluded in leaf disc experiments). By utilizing (−)-[G-^{3}H]menthone as a tracer administered to leaves of intact flowering plants, it was shown that the neomenthyl glucoside generated in the leaf was transported specifically to the rhizome (root) and there converted to other lipidlike metabolites.[217,240] The kinetics of metabolism and transport of exogeneously applied [^{3}H]menthone, if indicative of the in vivo process, appeared sufficient to account for the observed decrease in leaf terpene content.[241]

These results provided the first direct evidence supporting earlier suggestions[242,243] that monoterpenyl glycosides are transport derivatives. They also demonstrated that catabolic transformations can take place at a site quite distant from the glandular site of synthesis. Menthyl-β-D-glucoside was reported to occur in the rhizome of *Mentha arvensis*[244] and is presumed to be the foliar transport derivatives in this species.[245] Over the last several years, many other monoterpenyl glycosides have been found in a variety of species,[238,246] so the basic process outlined for *M. piperita* may be a common phenomenon. The transport of terpenyl glycosides from leaves to flowers was reported in the essential oil rose.[247,248]

Studies on the metabolism of labeled neomenthyl glucoside on reaching the rhizome indicated that the glycosidic derivative is hydrolyzed and the aglycone oxidized back to menthone, which undergoes an unusual oxygenation to 3,4-menthone lactone (Scheme XXII).[241,249] This lactonization reaction accomplishes the crucial ring-opening step that permits a modified β-oxidative degradation of the terpenoid chain.[249] This overall strategy of oxidative ring opening followed by oxidative cleavage of the resulting chain has ample precedent in microorganisms that can utilize monoterpenes as the sole source of carbon and energy.[188–190,250–254]

By employing (−)-[G-^{3}H]3,4-menthone lactone and its progenitors as metabolic tracers with mint rhizomes, the probable pathway for the modified β-oxidation of the derived acyclic terpene skeleton (3,7-dimethyloctanoate) was elucidated.[249] Thus, a series of acidic metabolites was isolated indicating a cleavage sequence to generate acetyl-CoA in a manner analogous to the established microbial pathway, which involves carboxylation of two methyl groups.[250–252] Francis[242] calculated that the complete oxidation of menthone via this route could provide a yield of ATP intermediate between that of glucose and fatty acid. Carbon reutilization, rather than energy generation, appeared to be of primary importance in the present instance, however, since the cata-

SCHEME XXIII



bolism of [^{3}H]-labeled monoterpenes in mint rhizomes was shown to give rise to starch and soluble carbohydrates (via [^{3}H]pyridine nucleotides) as well as to saponifiable acyl lipids (fatty acids from C_{14} to C_{20}) and higher isoprenoid lipids such as squalane and phytosterols (via either [^{3}H]acetyl-CoA or [^{3}H]pyridine nucleotides), whereas little [^{3}H]-water was produced.[249] Following the administration of [^{3}H]menthone lactone to mint rhizomes, the resulting labeled phytosterols and fatty acids (analyzed as methyl esters) could be isolated almost quantitatively from the membranous fraction of a tissue homogenate. The conversion of [^{3}H]geranyl glucoside to waxes, organic acids, and other water-soluble products in rose petals was also described[255] and, while few details of the pathway are available, similarity to the catabolism of menthone lactone in peppermint is anticipated since the carbon skeletons are the same.

The (+)-camphor content of sage (*S. officinalis*) leaves increases as the leaves expand, and the increase is roughly proportional to the number of filled oil glands that appear on the leaf surface during the expansion process.[204] $^{14}CO_2$ is more rapidly incorporated into camphor and its direct progenitors in expanding leaves than in mature leaves, and direct in vitro measurement of the key enzymes involved in the conversion of geranyl pyrophosphate to camphor indicated that these enzymes, including that of the probable rate-limiting cyclization step, are at the highest levels during the period of maximum leaf expansion. Biosynthetic activity declines precipitously as the leaf reaches full size, and the level of camphor ultimately decreases, suggesting the onset of catabolic processes. Since insignificant quantities of volatile metabolites from camphor were produced (e.g., borneol or isoborneol), the conversion of the ketone to a nonvolatile derivative seemed likely.

Examination of the metabolism of (+)-[G-^{3}H]camphor in discs prepared from mature leaves of flowering sage plants revealed that this ketone was converted to a water-soluble metabolite that on chromatographic analysis proved to be considerably more polar than a simple monoterpenyl glycoside. Sufficient labeled product was accumulated to permit mass spectral analysis and degradative studies, which allowed identification of the terpenoid aglycone and indicated that the metabolite contained two glucose residues, one glycosidically linked and the other in ester linkage.[246] All of the evidence was consistent with the initial lactonization of camphor to 1,2-campholide followed by conversion to the β-D-glucoside-6-*O*-glucose ester of the corresponding hydroxy acid (1-(carboxymethyl)-3-hydroxy-2,2,3-trimethylcyclopentane) (Scheme XXIII). The intermediacy of 1,2-campholide was confirmed by direct testing and isotope dilution analysis.

When (+)-[G-^{3}H]camphor was applied to mature leaves of intact sage plants, the glucoside glucose ester of the hydroxy acid was the sole metabolite detected and was, presumably, the transport form in this instance, since tritium was ultimately incorporated into carbohydrates and lipids of the root. The catabolism of camphor in sage was thus reminiscent of menthone catabolism in peppermint, a lactonization step being the key transformation in opening the carbocyclic terpene ring and allowing degradation to simple metabolites that are subsequently reutilized. In the former instance, lactone formation occurs in leaves and precedes transport, whereas in the catabolism of menthone, lactonization occurs following transport to the rhizome; the basic strategy is the same.

Attempts to decipher the pathway by which the bis(glucose) derivative of 1,2-campholide is degraded have been unsuccessful thus far, in large part because catabolism in sage roots is very rapid.[256] Thus, [U-^{14}C]1,2-campholide when incubated with sage root sections gave rise to negligible levels of polar metabolites, the bulk of the incorporated label being located in end products, the acyl lipids and phytosterols of membranes, even after short incubation periods. The lactonization of camphor in sage resembles a similar step in the catabolism of camphor by microorganisms[253,254] and was the first report of this reaction type in higher plants. The subsequent steps in degradation of the lactone may also be analogous to those of the microbial pathway,[253,254] but this point is presently uncertain. The terminal stages of the microbial pathway are still unclear.[257]

The studies reviewed briefly above provide compelling evidence that monoterpenes can be degraded to metabolites that are reutilized in lipid biosynthesis in the developing root or rhizome or can conceivably be further oxidized in energy production. Such evidence should lay to rest the notion that monoterpenes are only inert waste products. The raison d'etre for turnover is not fully established; yet, the conjugation, transport, and catabolism of monoterpenes would seem to suggest a specific and directed process for the salvage of mobile carbon from senescing organs. It seems reasonable to view monoterpene catabolism, in an evolutionary sense, as a means of recovering at least a portion of the initial costs of production of these natural products. An important, and no less obvious, consequence of the above findings is that the accumulation of monoterpenes in plants can no longer be viewed as a passive process dependent only on the rate of biosynthesis. It is now clear that terpene accumulation must be viewed in a regulatory context involving a dynamic balance between biosynthetic and catabolic processes.

A discussion of the regulation of monoterpene metabolism would be an appropriate conclusion to this review. Unfortunately, in spite of much recent progress in this area of biochemistry, little has yet to be put forward regarding regulatory processes at the enzyme, cell, or whole organism level. Numerous development and environmental influences on monoterpene composition and yield are known; however, our understanding of the bases of these effects is rudimentary and likely to remain so until the gap between the gene and enzymatic expression can be bridged and until the means by which these catalysts are controlled at the molecular level can be explained. Monoterpene catabolism appears to be developmentally regulated and is probably associated with senescence. Preliminary studies also suggested developmental regulation of the enzymatic machinery dedicated to camphor biosynthesis,[204] but whether control is exerted by alteration of relevant

enzyme level or activity per se is uncertain, as is the possibility of regulation of the enzymatic steps prior to the committed cyclization.

Acknowledgments. The work from my laboratory described herein was supported in part by grants from the National Science Foundation, the National Institutes of Health, the U.S. Department of Energy and the U.S. Department of Agriculture, and by Research Center Project 0268. It is with pleasure that I acknowledge the collaborative efforts of Professors D. E. Cane of Brown University, A. C. Oehlschlager of Simon Fraser University, and R. M. Coates of the University of Illinois, and the contributions of my dedicated coworkers whose names are cited in the references.

VII. References

(1) Nes, W. R.; McKean, M. L. *Biochemistry of Steroids and Other Isopentenoids*; University Park: Baltimore, 1977.
(2) Devon, T. K.; Scott, A. I. *Handbook of Naturally Occurring Compounds, Terpenes*; Academic: New York, 1972; Vol. II, p 3.
(3) Dev, S.; Narula, A. P. S.; Yadav, J. S. *Handbook of Terpenoids. Monoterpenoids*; CRC: Boca Raton, Vol. I and II, 1982.
(4) Gildemeister, E.; Hoffman, F. *Die Ätherischen Öle*; Akademie Verlag: Berlin, 1955–1960; 8 vol.
(5) Guenther, E. *The Essential Oils*, (reprinted); Krieger: Huntington, 1972–1976; 6 vol.
(6) Gibbs, R. D. *Chemotaxonomy of Flowering Plants*; McGill-Queen's University: Montreal, 1974; Vol. II, p 770.
(7) Croteau, R.; Johnson, M. A. In *Biology and Chemistry of Plant Trichomes*; Rodriguez, E., Healey, P. L., Mehta, I., Eds.; Plenum: New York, 1984; p 133.
(8) Fahn, A. *Secretory Tissues in Plants*; Academic: New York, 1979; p 158.
(9) Blum, M. S.; Crewe, R. M.; Kerr, W. E.; Keith, L. H.; Garrison, A. W. *Science (Washington, D.C.)* **1970**, *164*, 1243.
(10) Muller, C. H.; Chou, C.-H. In *Phytochemical Ecology*; Harborne, J. B., Ed.; Academic: London, 1972; p 201.
(11) Oh, H. K.; Sakai, T.; Jones, M. B.; Longhurst, W. M. *Appl. Microbiol.* **1967**, *15*, 777.
(12) Farentinos, R. C.; Capretta, P. J.; Kepner, R. E.; Littlefield, V. M. *Science (Washington, D.C.)* **1981**, *213*, 1273.
(13) Levin, D. A. *Ann. Rev. Ecol. Syst.* **1976**, *7*, 121.
(14) Monaco, P.; Previtera, L.; Mangoni, L. *Phytochemistry* **1982**, *21*, 2408.
(15) Johnson, M; Croteau, R. In *Ecology and Metabolism of Plant Lipids*; Fuller, G.; Nes, W. D., Eds.; ACS Symposium Series 325; American Chemical Society: Washington, DC, 1987; p 76.
(16) Hanson, J. R. *Nat. Prod. Rep.* **1984**, *1*, 443.
(17) Banthorpe, D. V.; Branch, S. A. *Nat. Prod. Rep.* **1985**, *2*, 513.
(18) Croteau, R. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1981; Vol. 1, p 225.
(19) Wallach, O. *Terpene und Campher*, 2nd ed.; Vit: Leipzig, 1914.
(20) Ruzicka, L.; Eschenmoser, A.; Heusser, H. *Experientia* **1953**, *9*, 357.
(21) Irving, R. S.; Adams, R. P. *Recent Adv. Phytochem.* **1973**, *6*, 187.
(22) Zavarin, E. *Phytochemistry* **1970**, *9*, 1049.
(23) Lawrence, B. M. In *Essential Oils*; Mookherjee, B. D., Mussinan, C. J., Eds.; Allured: Wheaton, 1981; p 1.
(24) Reitsema, R. H. *J. Am. Pharm. Assoc., Sci. Ed.* **1958**, *47*, 26.
(25) Hefendehl, F. W.; Murray, M. J. *Lloydia* **1976**, *39*, 39.
(26) Paech, K. *Biochemie und Physiologie de Sekundären Pflanzenstoffe*; Springer-Verlag: Berlin, 1950.
(27) Sandermann, W. In *Comparative Biochemistry*, Florkin, M., Mason, H. S., Eds.; Academic: New York, 1962; Vol. 3, pp 503, 591.
(28) Mothes, K. *Sitzungsber. Oesterr. Akad. Wiss., Math. Naturwiss. Kl., Abt. I* **1973**, *181*, 1.
(29) Banthorpe, D. V.; Charlwood, B. V.; Francis, M. J. O. *Chem. Rev.* **1972**, *72*, 115.
(30) Charlwood, B. V.; Banthorpe, D. V. *Prog. Phytochem.* **1978**, *5*, 65.
(31) Battaile, J.; Loomis, W. D. *Biochim. Biophys. Acta* **1961**, *51*, 545.
(32) Poulose, A. J.; Croteau, R. *Arch. Biochem. Biophys.* **1978**, *187*, 307.
(33) Stephan, K. *J. Prakt. Chem.* **1898**, *58*, 109.
(34) Zeitschel, O. *Ber. Dtsch. Chem. Ges.* **1906**, *39*, 1780.
(35) Croteau, R. In *Isopentenoids in Plants: Biochemistry and Function*; Nes, W. D., Fuller, G.; Tsai, L. S., Eds.; Marcel Dekker: New York, 1984; p 31.
(36) Croteau, R. In *Herbs, Spices and Medicinal Plants, Recent Advances in Botany, Horticulture and Pharmacology*, Craker, L. E., Simon, J. E., Eds.; Oryx: Phoenix, 1986; Vol. 1, p 81.
(37) George-Nascimento, C.; Cori, O. *Phytochemistry* **1971**, *10*, 1803.
(38) Croteau, R.; Burbott, A. J.; Loomis, W. D. *Biochem. Biophys. Res. Commun.* **1973**, *50*, 1006.
(39) Chayet, L.; Rojas, C.; Cardemil, E.; Jabalquinto, A. M.; Vicuña, R.; Cori, O. *Arch. Biochem. Biophys.* **1977**, *180*, 318.
(40) Croteau, R.; Karp, F. *Arch. Biochem. Biophys.* **1979**, *198*, 523.
(41) Croteau, R.; Karp, F. *Arch. Biochem. Biophys.* **1979**, *198*, 512.
(42) Suga, T.; Hirata, T.; Aoki, T.; Shishibori, T. *Phytochemistry* **1986**, *25*, 2769.
(43) Cori, O. *Phytochemistry* **1983**, *22*, 331.
(44) Cori, O.; Rojas, M. C. *Methods Enzymol.* **1985**, *110A*, 406.
(45) Gambliel, H.; Croteau, R. *J. Biol. Chem.* **1984**, *259*, 740.
(46) Croteau, R.; Felton, M.; Ronald, R. C. *Arch. Biochem. Biophys.* **1980**, *200*, 524.
(47) Croteau, R.; Cane, D. E. *Methods Enzymol.* **1985**, *110A*, 383.
(48) Banthorpe, D. V.; Christou, P. N.; Pink, C. R.; Watson, D. G. *Phytochemistry* **1983**, *22*, 2465.
(49) Croteau, R.; Felton, M. *Arch. Biochem. Biophys.* **1981**, *207*, 460.
(50) Cane, D. E. *Acc. Chem. Res.* **1985**, *18*, 220.
(51) Croteau, R. In *Biogeneration of Aromas*; Parliment, T., Croteau, R., Eds.; ACS Symposium Series 317; American Chemical Society: Washington, DC, 1986; p 134.
(52) Poulter, C. D.; Rilling, H. C. *Acc. Chem. Res.* **1978**, *11*, 307.
(53) Poulter, C. D.; Rilling, H. C. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1981; Vol. 1, p 161.
(54) Cane, D. E. In *Biosynthesis of Isoprenoid Compounds*, Porter, J. W., Spurgeon, S. L., Eds.; Wiley: Vol. 1, New York, 1981; p 283.
(55) Cane, D. E. *Tetrahedron* **1980**, *36*, 1109.
(56) Kjonaas, R. B.; Venkatachalam, K. V.; Croteau, R. *Arch. Biochem. Biophys.* **1985**, *238*, 49.
(57) West, C. A. In *Biosynthesis of Isoprenoid Compounds*, Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1981; Vol. 1, p 375.
(58) MacMillan, J. *Monogr. Br. Plant Growth Regul. Group* **1984**, *11*, 13; *Chem. Abstr.* **1985**, *102*, 41885.
(59) Croteau, R. *Proc. 10th Intern. Cong. Ess. Oils, Flavors Fragrances*; Lawrence, B. M., Mookerjee, B. D., Willis, B., Eds.; Elsevier: Amsterdam, 1987, in press.
(60) Qureshi, N.; Porter, J. W. In *Biosynthesis of Isoprenoid Compounds*, Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1981; Vol. 1, p 47.
(61) Reed, B. C.; Rilling, H. C. *Biochemistry* **1976**, *15*, 3739.
(62) Sagami, H.; Ogura, K.; Seto, S. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 572.
(63) Pauly, G.; Belingheri, L.; Marpeau, A.; Gleizes, M. *Plant Cell Rep.* **1986**, *5*, 19.
(64) De La Fuente, M.; Pérez, L. M.; Hashagen, U.; Chayet, L.; Rojas, C.; Portilla, G.; Cori, O. *Phytochemistry* **1981**, *20*, 1551.
(65) Pérez, L. M.; Lozada, R.; Cori, O. *Phytochemistry* **1983**, *22*, 431.
(66) Purkett, P.; Croteau, R., manuscript in preparation.
(67) Gershenzon, J.; Duffy, M. A.; Karp, F.; Croteau, R. *Anal. Biochem.* **1987**, *163*, 159.
(68) Venkatachalam, K. V.; Kjonaas, R.; Croteau, R. *Plant Physiol.* **1984**, *76*, 148.
(69) Dudley, M. W.; Green, T. R.; West, C. A. *Plant Physiol.* **1986**, *81*, 343.
(70) Charlwood, B. V.; Hegarty, P. K.; Charlwood, K. A. In *Secondary Metabolism in Plant Cell Cultures*; Morris, P., Scragg, A. H., Stafford, A., Fowler, M. W., Eds.; Cambridge University: London, 1986; p 15.
(71) Brown, J. T.; Charlwood, B. V. In *Secondary Metabolism in Plant Cell Cultures*; Morris, P., Scragg, A. H., Stafford, A., Fowler, M. W., Eds.; Cambridge University: London, 1986; p 68.
(72) Cane, D. E. In *Enzyme Chemistry. Impact and Applications*; Suckling, C. J., Ed.; Chapman and Hall: London, 1984; p 196.
(73) Johnson, M. A. Ph.D. Thesis, Washington State University, 1984.
(74) Croteau, R.; Karp, F. *Arch. Biochem. Biophys.* **1977**, *179*, 257.
(75) Cane, D. E.; Pargellis, C. *Arch. Biochem. Biophys.* **1987**, *254*, 421.
(76) Hohn, T. M.; Vanmiddlesworth, F. *Arch. Biochem. Biophys.* **1986**, *251*, 756.
(77) Shen-Miller, J.; West, C. A. *Plant Physiol.* **1984**, *74*, 439.
(78) Moesta, P.; West, C. A. *Arch. Biochem. Biophys.* **1985**, *238*, 325.

(79) Bernard-Dagan, C.; Pauly, C.; Marpeau, D.; Gleizes, M.; Carde, J.; Baradat, P. *Physiol. Veg.* **1982**, *20*, 775.
(80) Poulose, A. J.; Croteau, R. *Arch. Biochem. Biophys.* **1978**, *191*, 400.
(81) Wheeler, C. J.; Croteau, R. *Arch. Biochem. Biophys.* **1986**, *248*, 429.
(82) Rojas, M. C.; Chayet, L.; Portilla, G.; Cori, O. *Arch. Biochem. Biophys.* **1983**, *222*, 389.
(83) Chayet, L.; Rojas, M. C.; Cori, O.; Bunton, C. A.; McKenzie, D. C. *Bioorg. Chem.* **1984**, *12*, 329.
(84) Portilla, G.; Rojas, M. C.; Chayet, L.; Cori, O. *Arch. Biochem. Biophys.* **1982**, *218*, 614.
(85) Croteau, R. In *Fragrance and Flavor Substances*; Croteau, R., Ed.; D&PS Verlag: Pattensen, 1980; p 13.
(86) Wheeler, C. J.; Croteau, R. *J. Biol. Chem.* **1987**, *262*, 8213.
(87) Cori, O.; Chayet, L.; Perez, L. M.; Rojas, M. C.; Portilla, G.; Holuigue, L.; Fernandez, L. A. *Arch. Biol. Med. Exp.* **1981**, *14*, 129.
(88) Croteau, R.; Karp, F. *Arch. Biochem. Biophys.* **1977**, *184*, 77.
(89) Croteau, R.; Hooper, C. L.; Felton, M. *Arch. Biochem. Biophys.* **1978**, *188*, 182.
(90) Croteau, R.; Felton, M. *Phytochemistry* **1980**, *19*, 1343.
(91) Croteau, R.; Shaskus, J. *Arch. Biochem. Biophys.* **1985**, *236*, 535.
(92) Gambliel, H.; Croteau, R. *J. Biol. Chem.* **1982**, *257*, 2335.
(93) Croteau, R. B.; Wheeler, C. J.; Cane, D. E.; Ebert, R.; Ha, H.-J. *Biochemistry*, in press.
(94) Jones, J. P.; Korzekwa, K. R.; Rettie, A. E.; Trager, W. F. *J. Am. Chem. Soc.* **1986**, *108*, 7074.
(95) Pascal, R. A., Jr.; Baum, M. W.; Wagner, C. K.; Rogers, L. R.; Huang, D.-S. *J. Am. Chem. Soc.* **1986**, *108*, 6477.
(96) Banthorpe, D. V.; Ekundayo, O.; Njar, V. C. O. *Phytochemistry* **1984**, *23*, 291.
(97) Karp, F.; Croteau, R. *Arch. Biochem. Biophys.* **1982**, *216*, 616.
(98) Hallahan, T.; Croteau, R., unpublished.
(99) Croteau, R.; Felton, M.; Ronald, R. C. *Arch. Biochem. Biophys.* **1980**, *200*, 534.
(100) Cramer, F.; Rittersdorf, W. *Tetrahedron* **1967**, *23*, 3015.
(101) Rittersdorf, W.; Cramer, F. *Tetrahedron* **1967**, *23*, 3023.
(102) Bunton, C. A.; Hachey, D. L.; Leresche, J.-P. *J. Org. Chem.* **1972**, *37*, 4036.
(103) Stevens, K. L.; Jurd, L.; Manners, G. *Tetrahedron* **1972**, *28*, 1939.
(104) Valenzuela, P.; Cori, O. *Tetrahedron Lett.* **1967**, 3089.
(105) Astin, K. B.; Whiting, M. C. *J. Chem. Soc., Perkin Trans. 2* **1976**, 1160.
(106) Baxter, R. L.; Laurie, W. A.; MacHale, D. *Tetrahedron* **1978**, *34*, 2195.
(107) Haley, R. C.; Miller, J. A.; Wood, H. C. S. *J. Chem. Soc. C* **1969**, 246.
(108) Bunton, C. A.; Cori, O.; Hachey, D.; Leresche, J.-P. *J. Org. Chem.* **1979**, *44*, 3238.
(109) McCormick, J. P.; Barton, D. L. *Tetrahedron* **1978**, *34*, 325.
(110) Kitagawa, Y.; Hashimoto, S.; Iemura, S.; Yamamoto, H.; Nozaki, H. *J. Am. Chem. Soc.* **1976**, *98*, 5030.
(111) Bunton, C. A.; Leresche, J.-P.; Hachey, D. *Tetrahedron Lett.* **1972**, 2431.
(112) Bordwell, F. G. *Acc. Chem. Res.* **1970**, *3*, 281.
(113) Sneen, R. A. *Acc. Chem. Res.* **1973**, *6*, 46.
(114) Cane, D. E.; Iyengar, R.; Shaio, M.-S. *J. Am. Chem. Soc.* **1981**, *103*, 914.
(115) Rittersdorf, W.; Cramer, F. *Tetrahedron* **1968**, *24*, 43.
(116) Winstein, S.; Valkanas, G.; Wilcox, C. F., Jr. *J. Am. Chem. Soc.* **1972**, *94*, 2286.
(117) Brody, E. P.; Gutsche, C. D. *Tetrahedron* **1977**, *33*, 723.
(118) Cori, O.; Chayet, L.; Perez, L. M.; Bunton, C. A.; Hachey, D. *J. Org. Chem.* **1986**, *51*, 1310.
(119) Eastman, R. H.; Noller, C. R. In *Organic Chemistry, An Advanced Treatise*; Gilman, H., Ed.; Wiley: New York, 1953; Vol. 4, p 591.
(120) Endo/exo is used to describe the cyclizing conformers rather than boat/chair.[121] The latter is less descriptive for a cyclization in which four of the six carbons involved in ring formation are trigonal.
(121) Gotfredsen, S.; Obrecht, J. P.; Arigoni, D. *Chimia* **1977**, *31*, 62.
(122) Stork, G.; White, W. N. *J. Am. Chem. Soc.* **1956**, *78*, 4609.
(123) Poulter, C. D.; King, C.-H. R. *J. Am. Chem. Soc.* **1982**, *104*, 1420.
(124) Poulter, C. D.; King, C.-H. R. *J. Am. Chem. Soc.* **1982**, *104*, 1422.
(125) Sakane, S.; Fujiwara, J.; Maruoka, K.; Yamamoto, H. *Tetrahedron* **1986**, *42*, 2193.
(126) Tidd, B. K. *J. Chem. Soc. B* **1971**, 1168.
(127) Brems, D. N.; Rilling, H. C. *J. Am. Chem. Soc.* **1977**, *99*, 8351.
(128) Vial, M. V.; Rojas, C.; Portilla, G.; Chayet, L.; Pérez, L. M.; Cori, O.; Bunton, C. A. *Tetrahedron* **1981**, *37*, 2351.
(129) Chayet, L.; Rojas, M. C.; Cori, O.; Bunton, C. A.; McKenzie, D. C. *Bioorg. Chem.* **1984**, *12*, 329.
(130) Poulter, C. D.; Rilling, H. C. *Biochemistry* **1976**, *15*, 1079.
(131) Jacob, L.; Julia, M.; Pfieffer, B.; Rolando, C. *Tetrahedron Lett.* **1983**, *24*, 4327.
(132) Gotfredsen, S. E. Ph.D. Thesis No. 6243, ETH Zurich, 1978.
(133) Coates, R. M. *Prog. Chem. Org. Nat. Prod.* **1976**, *33*, 73.
(134) Luft, R. *J. Org. Chem.* **1979**, *44*, 523.
(135) Money, T. *Prog. Org. Chem.* **1973**, *8*, 29.
(136) Gascoigne, R. M. *J. Chem. Soc.* **1958**, 876.
(137) Bunton, C. A.; Cori, O. *Interciencia* **1978**, *3*, 291.
(138) Arigoni, D. *Pure Appl. Chem.* **1975**, *41*, 219.
(139) Coates, R. M.; Cavender, P. L. *J. Am. Chem. Soc.* **1980**, *102*, 6358.
(140) Guilford, W. J.; Coates, R. M. *J. Am. Chem. Soc.* **1982**, *104*, 3506.
(141) Coates, R. M.; Koch, S. C.; Hegde, S. *J. Am. Chem. Soc.* **1986**, *108*, 2762.
(142) Saito, A.; Rilling, H. C. *J. Biol. Chem.* **1979**, *254*, 8511.
(143) Saito, A.; Rilling, H. C. *Arch. Biochem. Biophys.* **1981**, *208*, 508.
(144) Davisson, V. J.; Neal, T. R.; Poulter, C. D. *J. Am. Chem. Soc.* **1985**, *107*, 5277.
(145) Deno, N. C.; Haddon, R. C.; Nowak, E. N. *J. Am. Chem. Soc.* **1970**, *92*, 6691.
(146) Allinger, N. L.; Sieffert, J. H. *J. Am. Chem. Soc.* **1975**, *97*, 752.
(147) Kjonaas, R.; Croteau, R. *Arch. Biochem. Biophys.* **1983**, *220*, 79.
(148) Croteau, R.; Coates, R. M., unpublished.
(149) Smith, D. H.; Carhart, R. E. *Tetrahedron* **1976**, *32*, 2513.
(150) Croteau, R. In *Biochemistry of Plant Lipids: Structure and Function*; Stumpf, P. K., Mudd, J. B., Nes, W. D., Eds.; Plenum: New York, 1987; p 11.
(151) Banthorpe, D. V.; Modawi, B. M.; Poots, I.; Rowan, M. G. *Phytochemistry* **1978**, *17*, 1115.
(152) Banthorpe, D. V.; Poots, I. *Phytochemistry* **1979**, *18*, 1297.
(153) Gleizes, M.; Marpeau, A.; Pauly, G.; Bernard-Dagan, C. *Phytochemistry* **1982**, *21*, 2641.
(154) Cane, D. E. *Ann. N.Y. Acad. Sci.* **1986**, *471*, 130.
(155) Satterwhite, D. M.; Wheeler, C. J.; Croteau, R. *J. Biol. Chem.* **1985**, *260*, 13901.
(156) Croteau, R.; Satterwhite, D. M.; Cane, D. E.; Chang, C. C. *J. Biol. Chem.* **1986**, *261*, 13438.
(157) Croteau, R.; Satterwhite, D. M.; Cane, D. E., manuscript in preparation.
(158) Satterwhite, D. M.; Croteau, R., manuscript in preparation.
(159) Croteau, R.; Felton, N. M.; Wheeler, C. J. *J. Biol. Chem.* **1985**, *260*, 5956.
(160) Thomas, A. F.; Schneider, R. A.; Meinwald, J. *J. Am. Chem. Soc.* **1967**, *89*, 68.
(161) Abad, G. A.; Jindal, S. P.; Tidwell, T. T. *J. Am. Chem. Soc.* **1973**, *95*, 6326.
(162) Croteau, R., unpublished.
(163) Donninger, C.; Popják, G. *Proc. R. Soc. London B* **1966**, *163*, 465.
(164) Coates, R. M.; Denissen, J.; Croteau, R. B.; Wheeler, C. J. *J. Am. Chem. Soc.* **1987**, *109*, 4399.
(165) Jencks, W. P. *Acc. Chem. Res.* **1980**, *13*, 161.
(166) Cane, D. E.; Ha, H.-J.; Pargellis, C.; Waldmeier, F.; Swanson, S.; Murthy, P. P. N. *Bioorg. Chem.* **1985**, *13*, 246.
(167) Anastasis, P.; Freer, I.; Gilmore, C.; Mackie, H.; Overton, K.; Picken, D.; Swanson, S. *Can. J. Chem.* **1984**, *62*, 2079.
(168) Drengler, K. A.; Coates, R. M. *J. Chem. Soc., Chem. Commun.* **1980**, 856.
(169) Evans, R.; Hanson, J. R.; Mulheirn, L. J. *J. Chem. Soc., Perkin Trans. 1* **1973**, 753.
(170) Cane, D. E.; Murthy, P. P. N. *J. Am. Chem. Soc.* **1977**, *99*, 8327.
(171) Cane, D. E.; Hasler, H.; Materna, J.; Cagnoli-Bellavita, N.; Ceccherelli, P.; Madruzza, G. F.; Polonsky, J. *J. Chem. Soc., Chem. Commun.* **1981**, 280.
(172) Overton, K. H. *Chem. Soc. Rev.* **1979**, *8*, 447.
(173) Croteau, R.; *Arch. Biochem. Biophys.* **1986**, *251*, 777.
(174) Poulter, C. D.; Argyle, J. C.; Mash, E. A. *J. Biol. Chem.* **1978**, *253*, 7227.
(175) Poulter, C. D.; Wiggins, P. L.; Le, A. T. *J. Am. Chem. Soc.* **1981**, *103*, 3926.
(176) Wheeler, C. J.; Croteau, R., manuscript submitted.
(177) Stremler, K. E.; Poulter, C. D. *Abstracts of Papers*, 192nd National Meeting of the American Chemical Society: Anaheim, CA, 1986; OGRN 57.
(178) Wheeler, C. J.; Croteau, R. *Arch. Biochem. Biophys.* **1986**, *246*, 733.
(179) Wheeler, C. J.; Croteau, R. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4856.
(180) Poulter, C. D.; Rilling, H. C. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1981; Vol. 1, p 413.
(181) Spurgeon, S. L.; Porter, J. W. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1983; Vol. 2, p 1.

(182) Cane, D. E.; Saito, A.; Croteau, R.; Shaskus, J.; Felton, M. *J. Am. Chem. Soc.* **1982**, *104*, 5831.
(183) Croteau, R.; Shaskus, J. J.; Renstrøm, B.; Felton, N. M.; Cane, D. E.; Saito, A.; Chang, C. *Biochemistry* **1985**, *24*, 7077.
(184) Mash, E. A.; Gurria, G. M.; Poulter, C. D. *J. Am. Chem. Soc.* **1981**, *103*, 3927.
(185) Croteau, R.; Shaskus, J.; Cane, D. E.; Saito, A.; Chang, C. *J. Am. Chem. Soc.* **1984**, *106*, 1142.
(186) Croteau, R.; Wheeler, C. J.; Aksela, R.; Oehlschlager, A. C. *J. Biol. Chem.* **1986**, *261*, 7257.
(187) Banthorpe, D. V.; Charlwood, B. V. In *Encyclopedia of Plant Physiology*, New Series; Bell, E. A., Charlwood, B. V., Eds.; Springer-Verlag: Berlin, 1980; Vol. 8, p 185.
(188) Krasnobajew, V. In *Biotechnology: A Comprehensive Treatise*, Rehm, H.-J., Reed, G., Eds.; Verlag-Chemie: Basel, 1984; Vol. 6a, p 97.
(189) Britton, L. N. In *Microbial Degradation of Organic Compounds*; Gibson, D. T., Ed.; Marcel Dekker: New York, 1984; p 89.
(190) Trudgill, P. W. In *Microbial Degradation of Organic Compounds*; Gibson, D. T., Ed.; Marcel Dekker: New York, 1984; p 131.
(191) Santhanakrishnan, T. S. *Tetrahedron* **1984**, *40*, 3597.
(192) Hirata, T.; Hamada, H.; Aoki, T.; Suga, T. *Phytochemistry* **1982**, *21*, 2209.
(193) Ishikura, N.; Nabeta, K.; Sugisawa, H. *Phytochemistry* **1984**, *23*, 2062.
(194) Potty, V. H.; Bruemmer, J. H. *Phytochemistry* **1970**, *9*, 1001.
(195) Potty, V. H.; Bruemmer, J. H. *Phytochemistry* **1970**, *9*, 2319.
(196) Burbott, A. J.; Loomis, W. D. *Plant Physiol.* **1980**, *65*(suppl), 96.
(197) Loomis, W. D. In *Terpenoids in Plants*; Pridham, J. B., Ed.; Academic: New York, 1967; p 59.
(198) Banthorpe, D. V.; Wirz-Justice, A. *J. Chem. Soc., Perkin Trans. 1* **1972**, 1769.
(199) Banthorpe, D. V.; Baxendale, D. *Planta Med.* **1973**, *23*, 239.
(200) Banthorpe, D. V.; Wirz-Justice, A. *J. Chem. Soc. C* **1969**, 541.
(201) Banthorpe, D. V.; Mann, J.; Poots, I. *Phytochemistry* **1977**, *16*, 547.
(202) Banthorpe, D. V.; Doonan, H. J.; Wirz-Justice, A. *J. Chem. Soc., Perkin Trans. 1*, **1972**, 1964.
(203) Banthorpe, D. V.; Bucknall, G. A.; Doonan, H. J.; Doonan, S.; Rowan, M. G. *Phytochemistry* **1976**, *15*, 91.
(204) Croteau, R.; Felton, M.; Karp, F.; Kjonaas, R. *Plant Physiol.* **1981**, *67*, 820.
(205) Karp, F.; Harris, J. L.; Croteau, R. *Arch. Biochem. Biophys.* **1987**, *256*, 179.
(206) Dehal, S. S.; Croteau, R. *Arch. Biochem. Biophys.*, in press.
(207) Karp, F.; Croteau, R., unpublished.
(208) Croteau, R.; Hooper, C. L. *Plant Physiol.* **1978**, *61*, 737.
(209) Croteau, R.; Karp, F. *Arch. Biochem. Biphys.* **1976**, *176*, 734.
(210) Burbott, A. J.; Loomis, W. D. *Plant Physiol.* **1967**, *42*, 20.
(211) Battaile, J.; Burbott, A. J.; Loomis, W. D. *Phytochemistry* **1968**, *7*, 1159.
(212) Burbott, A. J.; Loomis, W. D. *Plant Physiol.* **1969**, *44*, 173.
(213) Croteau, R.; Venkatachalam, K. V. *Arch. Biochem. Biophys.* **1986**, *249*, 306.
(214) Kjonaas, R.; Martinkus-Taylor, C.; Croteau, R. *Plant Physiol.* **1982**, *69*, 1013.
(215) Bugaenko, L. A.; Reznikova, S. A. *Genetika (Moscow)* **1984**, *20*, 2018.
(216) Johnson, M. A.; Croteau, R. *Arch. Biochem. Biophys.* **1984**, *235*, 254.
(217) Croteau, R.; Martinkus, C. *Plant Physiol.* **1979**, *64*, 169.
(218) A review of the Charabot's work is provided by Haagen-Smit, A. J. In *The Essential Oils*; Guenther, E., Ed.; Krieger: Huntington, 1977; Vol. 1, p 15.
(219) Berry, P. A.; Macbeth, A. K.; Swanson, T. B. *J. Chem. Soc.* **1937**, *146*, 1443.
(220) Putievsky, E.; Ravid, U.; Snir, N.; Sanderovich, D. *Isr. J. Bot.* **1984**, *33*, 47.
(221) Schnepf, E. In *Dynamic Aspects of Plant Ultrastructure*; Robards, A. W., Ed.; McGraw-Hill: New York, 1974; p 331.
(222) Loomis, W. D.; Croteau, R. *Recent Adv. Phytochem.* **1973**, *6*, 47.
(223) Tyson, B. J.; Dement, W. A.; Mooney, H. A. *Nature (London)* **1974**, *252*, 119.
(224) Ebermann, R.; Stich, K. *Phytochemistry* **1982**, *21*, 2401.
(225) Weiss, B.; Flück, H. *Pharm. Acta Helv.* **1970**, *45*, 169.
(226) Adams, R. P.; Hagerman, A. *Am. J. Bot.* **1977**, *64*, 278.
(227) Scora, R. W.; Mann, J. D. *Lloydia* **1967**, *30*, 236.
(228) Hefendehl, F. W.; Underhill, E. W.; von Rudloff, E. *Phytochemistry* **1967**, *6*, 823.
(229) Francis, M. J. O.; O'Connell, M. *Phytochemistry* **1969**, *8*, 1705.
(230) Croteau, R.; Loomis, W. D. *Phytochemistry* **1972**, *11*, 1055.
(231) Croteau, R.; Burbott, A. J.; Loomis, W. D. *Phytochemistry* **1972**, *11*, 2459.
(232) Embong, M. B.; Hadziyev, D.; Molnar, S. *Can. Inst. Food Sci. Technol. J.* **1977**, *10*, 201.
(233) Embong, M. B.; Steele, L.; Hadziyev, D.; Molnar, S. *Can. Inst. Food Sci. Technol. J.* **1977**, *10*, 247.
(234) Firmage, D.; Irving, R. *Phytochemistry* **1979**, *18*, 1827.
(235) Hendricks, H.; Van Os, F. H. L. *Phytochemistry* **1976**, *15*, 1127.
(236) Nagasawa, T.; Umemoto, K.; Tsuneya, T.; Shiga, M. *J. Agric. Chem. Soc. Jpn.* **1975**, *49*, 491.
(237) Fugita, S.; Nakana, T.; Fugita, Y. *J. Agric. Chem. Soc. Jpn.* **1977**, *51*, 405.
(238) Martinkus, C.; Croteau, R. *Plant Physiol.* **1981**, *68*, 99.
(239) Croteau, R.; Winters, J. N. *Plant Physiol.* **1982**, *69*, 975.
(240) Kjonaas, R.; Martinkus, C.; Croteau, R. *Plant Physiol.* **1980**, *65*(suppl), 96.
(241) Croteau, R.; Sood, V. K.; Renstrøm, B.; Bhushan, R. *Plant Physiol.* **1984**, *76*, 647.
(242) Francis, M. J. O. In *Aspects of Terpenoid Chemistry and Biochemistry*; Goodwin, T. W., Ed.; Academic: New York, 1971; p 29.
(243) Horster, H. In *Vorkommen und Analytik Ätherischer Öle*; Kubeczka, K. H., Ed.; Georg Thiem Verlag: Stuttgart, 1979; p 34.
(244) Sakata, I.; Koshimizu, K. *Agric. Biol. Chem.* **1978**, *42*, 1959.
(245) Sakata, I.; Koshimizu, K. *J. Agric. Chem. Soc. Jpn.* **1980**, *54*, 1037.
(246) Croteau, R.; El-Bialy, H.; El-Hindawi, S. *Arch. Biochem. Biophys.* **1984**, *228*, 667.
(247) Bugorskii, P. S.; Reznikova, S. A.; Zaprometov, M. N. *Biokhimiya (Moscow)* **1979**, *44*, 1068.
(248) Pogorel'skaya, A. N.; Kholodova, V. P.; Reznikova, S. A. *Fiziol. Rast. (Moscow)* **1980**, *27*, 356.
(249) Croteau, R.; Sood, V. K. *Plant Physiol.* **1985**, *77*, 801.
(250) Seubert, W. *J. Bacteriol.* **1960**, *79*, 426.
(251) Seubert, W.; Fass, E. *Biochem. Z.* **1965**, *341*, 35.
(252) Cantwell, S. G.; Lau, E. P.; Watt, D. S.; Fall, R. R. *J. Bacteriol.* **1978**, *135*, 324.
(253) Chapman, P. J.; Meerman, G.; Gunsalus, I. C. *Biochem. Biophys. Res. Commun.* **1965**, *20*, 104.
(254) Conrad, H. E.; Dubus, R.; Namtvedt, M. J.; Gunsalus, I. C. *J. Biol. Chem.* **1965**, *240*, 495.
(255) Bugorskii, P. S.; Zaprometov, M. N. *Fiziol. Biokhim. Kul't. Rast.* **1983**, *15*, 594.
(256) Croteau, R.; El-Bialy, H.; Dehal, S. S. *Plant Physiol.*, in press.
(257) Ougham, H. J.; Taylor, D. G.; Trudgill, P. W. *J. Bacteriol.* **1983**, *153*, 140.