The biosynthesis of terpene compounds

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Introduction: The essential oils

It has been known for several thousand years that the flowers, fruits, leaves, barks, and roots of many plants contain volatile, odorous substances, collectively called the essential oils. Highly prized by ancient man, the essential oils, in the form of herbs and spices, became one of the earliest recorded items of commerce. It is unlikely that any other commodity has provided such a stimulus to world exploration and to the development of international trade.[1,2] From that ancient trade in raw spices, the isolation and preparation of odorous principles from plants and the later synthetic duplication of the essential oils and their constituents has developed, in modern times, into a large and flourishing industry.

While these oils are very useful to humanity, the classical view has held that essential oils represent inactive waste products of plant metabolism and thus possess no significant biological function.[3] Only recently have we come to realize that the essential oils and their constituents may be of benefit to the plants which produce them; providing a means of chemical communication by which the plant maintains itself against competitors, predators, and pathogens.[4,5]

The ability of essential oils to function as chemical signals appears to stem from their volatility in air (and thus the ability to influence another organism at some distance from the source plant) and from the complexity of composition and structure of the constituents (and thus the ability to transmit a complex and selective biological message). Additionally, recent evidence has shown that essential oil constituents are both rapidly synthesized and catabolized by plants,[6] and thus may play a dynamic though as yet unknown role in metabolism. In this context of a functional role, it is important to note that the synthesis and accumulation of essential oils is generally associated with the presence of specialized glandular structures, most often situated on or near the surface of the plant.[6] (See figure 1.) Such a location is ideal for release of essential oil constituents to the atmosphere, and is consistent with a chemical defense function. The oil glands also provide a means of sequestering essential oil formation from other metabolic processes; yet, the sites are not so isolated as to prevent catabolism and the apparent metabolic recycling of the essential oil constituents.

Monoterpenes and sesquiterpenes: general considerations

Early studies on the chemical composition of the essential oils led to the discovery of a series of related olefins of formula $C_{10}H_{16}$. These compounds became known as terpenes, derived from the German word *terpentine* (turpentine). Related oxygenated compounds with a ten-carbon skeleton were also isolated, and the crystalline ones became known as *camphors* because of their physical resemblance to true camphor. Thus, there was a "thyme camphor" (thymol) and a "peppermint camphor" (menthol). Later, when further structural similarities were noted, the oxygenated compounds were included under the general heading of terpenes, with the term *camphor* now being restricted to a specific compound. The term

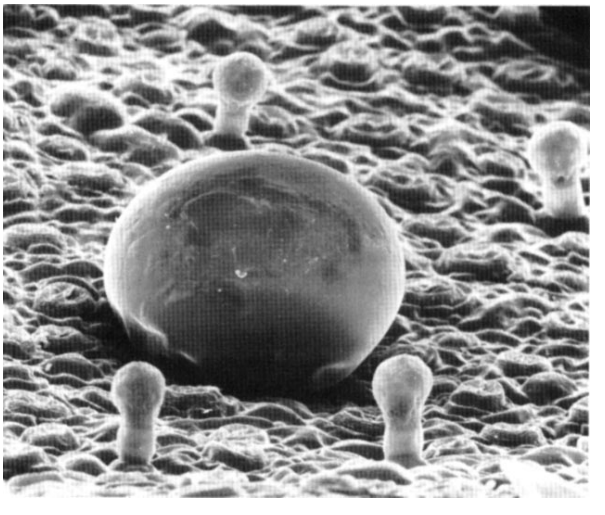

Figure 1. Scanning electron micrograph showing one large 10-celled oil gland surrounded by several smaller 3-celled oil glands on the upper surface of a spearmint leaf. The diameter of the large gland is about 80 μm. (From Croteau (70)).

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terpene is now loosely applied to all compounds of terpenoid (isoprenoid) structure.

The family of structurally related ten-carbon substances, called monoterpenes, were usually constituents of the lower-boiling fractions of essential oils. When attention was later turned to the higher-boiling fractions, the presence of similar fifteen-carbon containing compounds was revealed. These compounds, the sesquiterpenes, have since been found to be fairly widespread in nature, Because of their volatility, they contribute materially to the odor and flavor of essential oils. Higher terpenes such as diterpenes (C_{20}) and triterpenes (C_{30}) are constituents of extracted plant oils, but these substances are generally eliminated on distillation. It is, therefore, the volatile monoterpenes and sesquiterpenes that constitute the characteristic components of many essential oils, and it is these compounds that are the subject of the present review.

A few examples of common monoterpenes and sesquiterpenes are illustrated in figure 2. The apparent unit or building block from which the terpenes are constructed is one of five carbon atoms (that is, the isoprene unit) and this fact was recognized at a quite early stage of chemical investigation. It was, in fact, the finding that monoterpene hydrocarbons were formally derivable by repetitive joining of the C_5-isoprene unit that led Wallach in 1887 to formulate the "isoprene rule."[7] This principle—that most terpenoids could be hypothetically constructed by a head-to-tail joining of isoprene units—was a major advance in terpenoid chemistry. It provided the first unified concept for a common structural relationship among terpenoid natural products. While isoprene itself was once considered to be the actual biological precursor of terpenes in plants, this notion was discarded because isoprene could not then be demonstrated as a natural product (isoprene was shown to be a natural product in 1961[8]). The smallest terpene and, thus, the "terpene unit" was therefore considered to comprise ten-carbon atoms, from which the designations monoterpene (C_{10}), sesquiterpene (C_{15}), diterpene (C_{20}), and so forth originated. Within these broad categories, further divisions are made on the basis of number of carbocyclic rings (such as, acyclic, monocyclic, or bicyclic) and skeletal arrangements (such as, *p*-menthanes, bornanes, or fenchanes).

As the structures of an increasing number of terpenes were determined, it became apparent that not all terpenes conformed to the isoprene rule in its simplest form. Thus, some terpenes were not exact multiples of five, but lacked one or more carbon atoms. Some showed a head-to-head arrangement of isoprene units rather that the regular head-to-tail pattern, while others, because they had undergone rearrangement, did not fit either pattern.

To assemble such a diverse assortment of terpenoids within a common conceptual framework was a formidable task. The task was, however, accomplished by Ruzicka in 1953 when he formulated the "biogenetic isoprene rule."[9] This hypothesis, which ignores the precise character of the biological precursors and assumes only that they are isoprenoid in structure, is based largely on mechanistic considerations. Ruzicka's general scheme for the origin of monoterpenes is reproduced in figure 3. In this hypothesis, the neryl (*cis*) cation undergoes cyclization to the monocyclic α-terpinyl cation, which, by a series of internal additions, hydride shifts, and rearrangements, gives rise to the parent cations of the various structural types. Termination of the ionic reactions is envisioned to proceed by elimination of a proton to give the corresponding olefin, or by addition of a nucleophile (for example, OH^- from water) to give the corresponding alcohol. Similar schemes, based on cationic mechanisms, were formulated for sesquiterpenes and for higher terpenes as well.

While the biogenetic isoprene rule provided a use-

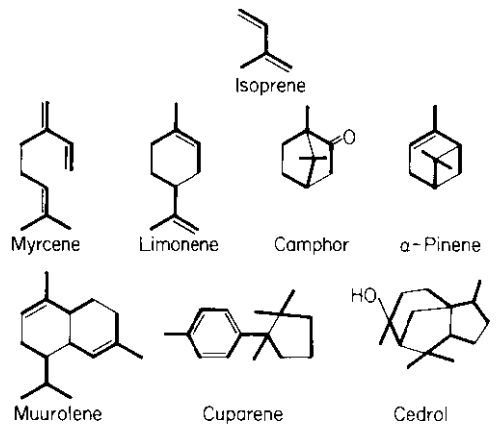

Figure 2. Representative monoterpenes and sesquiterpenes and their division into "isoprene units."

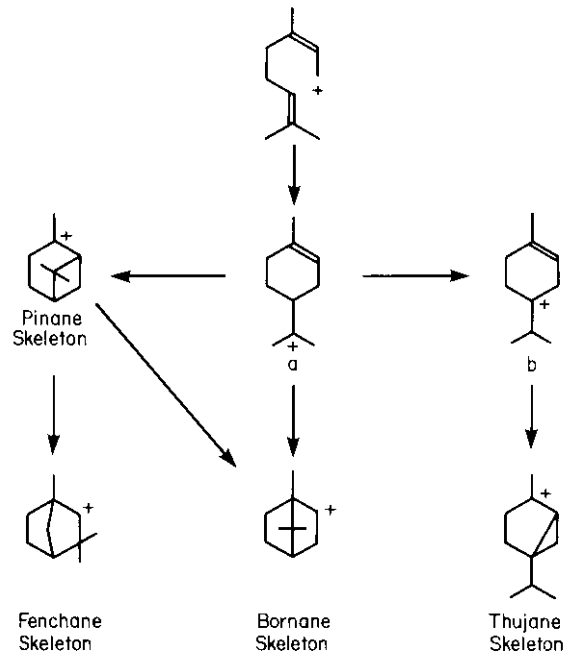

Figure 3. Ionic scheme for the formation of monoterpenes via the α-terpinyl cation (a) and the terpinen-4-yl cation (b) (after Ruzicka (9)).

ful working hypothesis for the biosynthesis of mono- and sesquiterpenes, it was not until the discovery of mevalonic acid and its conversion to isopentenyl pyrophosphate (the biological isoprene unit) that an understanding of terpenoid biosynthesis on a molecular level was possible. The enzymatic conversion of mevalonic acid (the first committed precursor of all terpenoids) to isopentenyl pyrophosphate, and the subsequent conversion of this compound to geranyl pyrophosphate and farnesyl pyrophosphate (the first C_{10} and C_{15} compounds, respectively, to arise by this pathway) are illustrated in figure 4. Detailed discussion of these early steps common to all terpenoid biosynthetic pathways can be found in recent reviews.[10-12]

It is important to note here that the first C_{10} isoprenoid, and the precursor of essentially all higher terpenoids, is geranyl pyrophosphate (that is, the *trans* Δ^2-isomer) (fig. 4), while on stereochemical grounds it was the *cis* Δ^2-isomer that was proposed as the precursor of cyclic monoterpenes (fig. 3). Later authors were to propose neryl pyrophosphate as the probable direct precursor of cyclic monoterpenes,[13,14] and to direct considerable effort toward determining how neryl pyrophosphate might be formed in the plant.[15] However, very recent studies with isolated enzyme systems suggest that the direct precursor of cyclic monoterpenes is, in fact, geranyl pyrophosphate. This work, as well as other selected examples intended to illustrate the application of modern biochemical techniques to the study of terpene biosynthesis at the enzyme level, will be described here. For more comprehensive treatment of the subject, see the recent reviews on monoterpene[15-19] and sesquiterpene biosynthesis.[16, 20-23]

The origin of camphor

The main source of natural camphor is the so-called "camphor tree" *Laurus (Cinnamonum) camphora* of Asia, which produces the *d*-isomer. Numerous other species, however, produce camphor, including many of the common herbs and spices. The *d*-stereoisomer appears to be more widely distributed in nature that the *l*-enantiomer.

Owing to the ease with which it can be isolated and to its reputed medicinal properties, camphor has been known since prehistory, being first mentioned in early far eastern literature.[24] Camphor appears to have been introduced to Europe about the tenth century by Arab traders under the name of *Káfár* or *Kaphur* (from the Sanscrit *Kapūra* meaning "white"). Because of its reported therapeutic properties, camphor was quickly accepted in the West, where much confusion reigned as to the source of this material (it was at one time thought to be a mineral resin). Traders wishing to maintain a monopoly on the supply of

camphor no doubt contributed to this confusion. With the establishment of camphor as a natural plant product, the investigation of the chemical and physical properties of this unique material began. The history of camphor is one of the longest and most involved of any natural product.[25]

One of the first persons to mention camphor in a chemical context was the alchemist Libavius, who in 1595 described the solution of camphor in nitric acid.[26] The next two hundred years witnessed numerous studies on the properties of this compound by some of the most prominent chemists of the time, including Boyle and Lavoisier.[25] The composition and molecular weight of camphor were determined by Dumas in 1833,[27] and the formula for camphor as $C_{10}H_{16}O$ established.

The next sixty years were required to establish the correct structure of camphor, a difficult problem due to the tendency of camphor and its derivatives to undergo intramolecular rearrangements. During this sixty year period over thirty structures were proposed for camphor (some notable examples are illustrated in figure 5), culminating in the correct formulation by Bredt,[28] which was not, however, generally acknowledged until many years later. Once the structure of camphor had been accepted, many of the rearrangements which had misled earlier investigators were explained, and several syntheses of camphor were designed.

The increasing use of camphor as a plasticizer, particularly in the manufacture of celluloid and explosives, soon led to a demand that exceeded the supply of natural material, and from the synthetic studies commercial processes for the production of camphor were developed. Figure 6 illustrates the Bakelite-Xylonite (BXL) process, which can be seen to utilize α-pinene (the primary constituent of turpentine) as the starting material and to proceed through a reaction sequence involving no fewer than three Wagner-Meerwein rearrangements.[29] It was, in part, the rearrangements involved in the synthesis of camphor that formed the basis of studies by Wagner [30] and Meerwein [31] from which this fundamental rearrangement process derived its name. The racemic end-product of the BXL process can be purified to 98% by sublimation or crystallization. World use of this product is probably somewhat in excess of 400 tons yearly.[29] While the industrial significance of camphor has declined over the years, the chemistry of this unique material continues to provide challenge to the modern day researcher.[32-34]

With Wallach's formulation of the isoprene rule[7] and the formulation of the correct structure of camphor,[28] it was immediately realized that camphor was "isoprenoid" or "terpenoid," and thus biogenetically related to other members of this rapidly growing family of natural products. It was Ruzicka, however, who formulated the biogenetic isoprene rule[9] and pro-

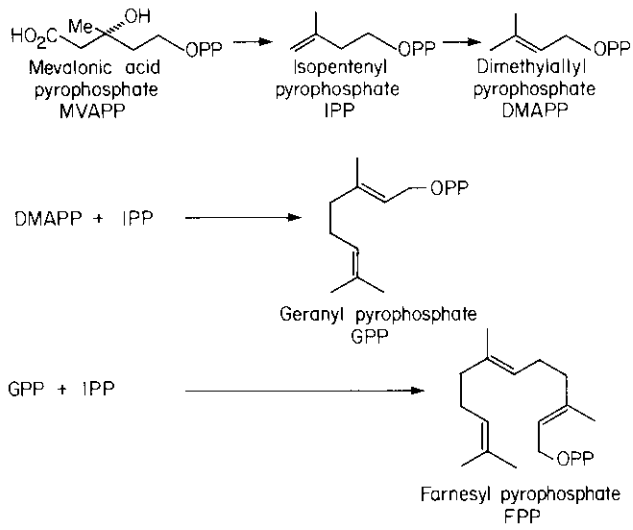

Figure 4. Enzymatic conversion of mevalonic acid pyrophosphate to geranyl pyrophosphate and **trans, trans**-farnesyl pyrophosphate.

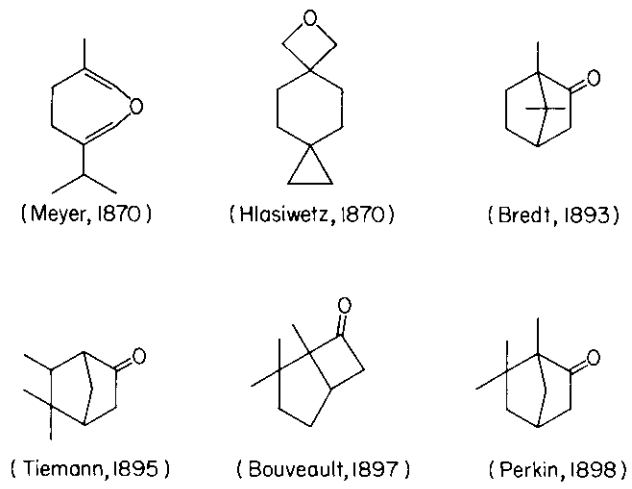

Figure 5. Early proposals for the structure of camphor.

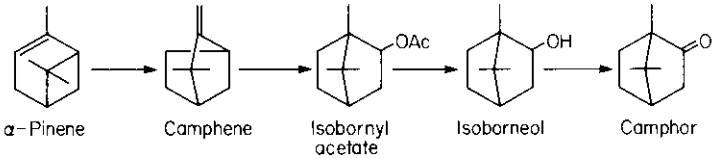

Figure 6. Bakelite-Xylonite process for the synthesis of camphor (from Bean (29)).

vided a theoretical framework for the biosynthetic construction of the bornane (camphane) monoterpenes via the cyclization of the neryl cation (see fig. 3). Later workers were to suggest neryl pyrophosphate as the likely biological precursor,[13,14] and in the early 1970s two groups showed that the labeling pattern of camphor derived from specifically labeled precursors in intact plants was consistent with Ruzicka's cyclization scheme.[35,36]

The first enzymatic synthesis of camphor in a cell-free preparation from leaves of sage (*Salvia officinalis*) was demonstrated in 1976.[37] This soluble enzyme system converted the acyclic precursor [1-^{3}H] neryl pyrophosphate to *d*-[3-^{3}H] borneol in the presence of Mg^{++}, and in the presence of NAD, dehydrogenated the borneol formed to *d*-[3-^{3}H] camphor (fig. 7). The location of the tritium in borneol and camphor indicated a direct conversion of substrate to products, and resolution of the biosynthetic products showed them both to be the d-isomer, the same stereoisomers produced *in vivo*.[38] A specific search of the enzymatic reaction products for the diastereomeric *l*-isoborneol revealed that this alcohol was not formed in detectable yield. Small amounts of the monocyclic alcohol α-terpineol were formed by the crude cell-free preparation; however, subsequent studies showed that α-terpineol was not an intermediate in borneol biosynthesis.[37] This was the first report on the enzymes involved in the biosynthesis of camphor, and the results suggested that camphor was derived by the cyclization of neryl pyrophosphate to borneol followed by dehydrogenation of this bicyclic alcohol (fig. 7).

Attempts to purify the d-borneol synthetase activity by gel permeation chromatography resulted in the apparent loss of catalytic capability; however, subsequent recombination of column fractions demonstrated that two clearly separable enzymatic activities (eluting in the molecular weight regions corresponding to 95,000 and 50,000) were required for the conversion of neryl pyrophosphate to borneol.[38] Several lines of evidence indicated that a water-soluble, dialyzable intermediate (produced by the higher molecular weight component) was involved in this transformation. The intermediate was isolated and conclusively identified as d-bornyl pyrophosphate.[38] Thus, d-borneol appeared to be formed by a pathway involving two discrete enzymatic steps: the cyclization of neryl pyrophosphate to d-bornyl pyrophosphate (by the 95,000 MW protein component) and the hydrolysis of d-bornyl pyrophosphate to d-borneol (by the 50,000 MW protein component) (fig. 8). The intermediate role of *l*-bornyl pyrophosphate in the biosynthesis of *l*-borneol has also been demonstrated in soluble enzyme preparations from rosemary (*Rosmarinus officinalis*), indicating that this pathway for borneol formation is probably widespread in the plant kingdom.[38]

While cyclic pyrophosphates such as copalyl pyrophosphate, presqualene pyrophosphate, and prephytoene pyrophosphate are involved in the biosynthesis of diterpenes, triterpenes, and tetraterpenes, respectively, the discovery of bornyl pyrosphosphate was the first report of a cyclic pyrophosphorylated intermediate in the biosynthesis of monoterpenes. Furthermore, unlike the other cyclic pyrophosphates cited, bornyl pyrophosphate is unique in that the pyrophosphate moiety of the acyclic precursor migrates during the cyclization reaction.

Before discussing bornyl pyrophosphate synthetase, which is the key enzyme of the process, it is necessary to review studies with the other requisite enzymes of *d*-camphor biosynthesis: the bornyl pyrophosphate hydrolase and the borneol dehydrogenase. Detailed examination of the sage leaf enzyme preparation revealed the presence of two bornyl pyrophosphate hydrolase activities, one that eluted in the 50,000 MW range (as mentioned above) and one (much less active) that eluted in the 20,000 MW range. The higher MW activity was further separated

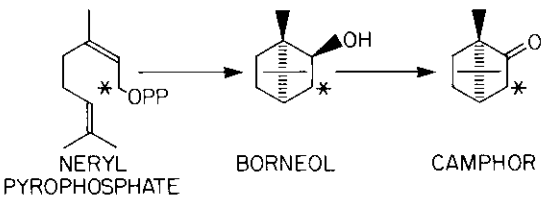

Figure 7. Pathway for the enzymatic conversion of [1-^{3}H]neryl pyrophosphate to **d**-[3-^{3}H]borneol and **d**-[3-^{3}H]camphor. The asterisk indicates the position of tritium in the structures, which are stereochemically correct.

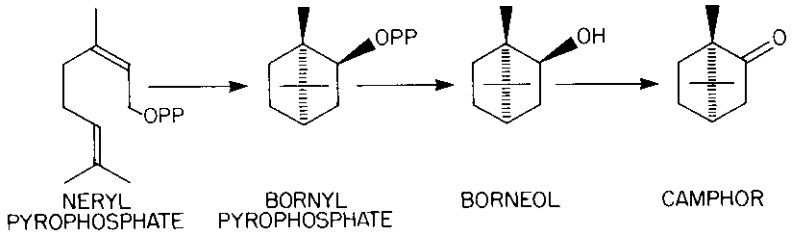

Figure 8. Pathway for the biosynthesis of **d**-borneol and **d**-camphor via **d**-bornyl pyrophosphate.

into a pyrophosphate hydrolase (pyrophosphate to monophosphate) and a monophosphate hydrolase (monophosphate to free alcohol) by chromatography on hydroxylapatite. The other hydrolase (20,000 MW) contained pyrophosphate and monophosphate hydrolase activities, which were inseparable by this or any other chromatographic technique tested. The lower molecular weight phosphohydrolase with both activities was shown to be rather specific for bornyl pyrophosphate, and this observation, along with other data,[40] suggested that this enzyme was responsible for the hydrolysis of bornyl pyrophosphate *in vivo*.

Examination of the other phosphohydrolases indicated that, while bornyl derivatives were hydrolyzed, phosphate and pyrophosphate esters of primary allylic alcohols, such as geraniol and nerol, were the preferred substrates.[39] Of particular significance was the pyrophosphate hydrolase, which was shown to hydrolyze geranyl pyrophosphate to geranyl monophosphate at faster rates than it hydrolyzed neryl pyrophosphate to neryl phosphate. This enzyme, which destroys the substrates for monoterpene biosynthesis, was later proved to be a serious interference in studies on the bornyl pyrophosphate synthetase.

The final step in the biosynthesis of *d*-camphor in sage is the oxidation of *d*-borneol, and the soluble dehydrogenase that catalyzes this conversion was isolated from sage leaf homogenates and resolved from alcohol (ethanol) dehydrogenase.[41] The partially purified enzyme had a molecular weight of about 91,000, exhibited a pH optimum at about 8.0, and preferred NAD over NADP as cofactor. The kinetic properties of the enzyme, as well as the stereochemistry of hydride transfer (B-type), were also examined.[41] In addition to *d*-borneol oxidation, the enzyme readily catalyzed the oxidation of *l*-3-neoisothujanol to *l*-3-isothujone. Several types of evidence indicated that this same enzyme catalyzed the key oxidation step in the biosynthesis of both *d*-camphor and *l*-3-isothujone, which are the major monoterpenes of sage. This last observation prompted us to suggest that monoterpenol oxidations may, in general, be catalyzed by dehydrogenases which are specific for the particular types of monoterpenes produced by the plant,[41] a suggestion supported by subsequent studies with other species.[42]

Preliminary studies with bornyl pyrophosphate synthetase were carried out using neryl pyrophosphate as the substrate because this precursor was usually more efficient than the *trans*-isomer, geranyl pyrophosphate. However, the apparent substrate preference of bornyl pyrophosphate synthetase varied considerably from preparation to preparation. This very puzzling observation was clarified with the discovery of the pyrophosphate hydrolase (described above) that preferentially hydrolyzed geranyl pyrophosphate to the monophosphate, thus preferentially destroying this substrate. It was the variation in the level of this competing enzyme in the crude preparations that produced the variation in substrate preference.

To examine the bornyl pyrophosphate synthetase in any detail, it was therefore necessary to remove the competing phosphatase. This was accomplished by combination of gel filtration on Sephadex G-150,* chromatography on hydroxylapatite, and chromatography on *O*-(diethylaminoethyl)-cellulose.[43] In these fractionation steps, activities for the cyclization of neryl pyrophosphate and geranyl pyrophosphate to bornyl pyrophosphate were coincident, implying that the same enzyme used both substrates.

On the progressive removal of the competing phosphatase, the synthetase showed an increasing preference for geranyl pyrophosphate as substrate. After the complete removal of the phosphatase, the K_m for geranyl pyrophosphate was 1.4 μM with V_{max} of 11.4 nmol/hr-mg, while with neryl pyrophosphate the K_m and V_{max} were 18 μM and 6.9 nmol/hr-mg, respectively.[43] The preference for geranyl pyrophosphate was very clear under conditions where no interconversion of geranyl and neryl pyrophosphates was detected. The partially purified bornyl pyrophosphate synthetase had an apparent molecular weight of 95,000 and required Mg^{++} for catalytic activity. The enzyme exhibited a pH optimum at 6.2, and was strongly inhibited by the thiol-directed reagent *p*-hydroxymercuribenzoate and by the active serine directed reagent diisopropylfluorophosphate.

Bornyl pyrophosphate synthetase was the first monoterpene synthetase to be isolated free from competing phosphatases, and the first to show a clear preference for geranyl pyrophosphate as substrate. Other monoterpene cyclase preparations show an apparent preference for neryl pyrophosphate as substrate,[44-46] while at least an equal number have been shown to utilize both geranyl pyrophosphate and neryl pyrophosphate with roughly equal efficiency in the absence of interconversion of these substrates.[46-48] However, in none of these cases was the removal of all competing phosphohydrolases assured, and in several instances only crude preparations were utilized. Thus, in these cases, it is uncertain whether the specificities observed are those of the monoterpene synthetases, or whether they reflect the presence of competing phosphohydrolases. The findings with bornyl pyrophosphate synthetase, and recent studies with fenchol synthetase from fennel, which also prefers geranyl pyrophosphate,[49] indicate that the traditionally proposed role of neryl pyrophosphate as an obligate precursor of cyclohexanoid monoterpenes (for stereochemical reasons) may need to be reconsidered. Indeed, the present controversy surrounding the possible origins of neryl pyrophosphate as a required precursor of cyclic monoterpenes[15] may be of little significance.

The use of geranyl pyrophosphate as the natural

*Pharmacia Fine Chemicals, Parsippany, NJ

immediate precursor necessitates reconsideration of the mechanistic role of the cyclase enzymes in the biosynthesis of cyclic monoterpenes. As any mechanism for the formation of bornyl pyrophosphate must now accommodate geranyl pyrophosphate as the preferred substrate, a reaction sequence in which the *trans*-isomer must be transformed to an intermediate stereochemically suitable for cyclization is implied. The reaction sequence must also account for the appropriate stereochemistry of the cyclized product and for the fact that the bornane skeleton arises as the pyrophosphate derivative.

With these considerations in mind, a mechanism for the biosynthesis of bornyl pyrophosphate was proposed (fig. 9).[43] To convert geranyl pyrophosphate into a form stereochemically capable of cyclization, it must first react with the enzyme, with concomitant loss of pyrophosphate, to afford a bound linaloyl intermediate (reaction of neryl pyrophosphate with the enzyme in a similar manner accounts for the activity of this *cis*-isomer). An enzyme bound linaloyl structure would permit rotation around the C2-C3 bond, which enables the geranyl pyrophosphate precursor to attain the conformation appropriate for cyclization to bornyl pyrophosphate. While a synchronous cyclization is implied here, this mechanism can be seen to resemble Ruzicka's multistep cationic scheme for the generation of the bornane nucleus (fig. 3). The role of the enzyme is thus to convert geranyl pyrophosphate to a form appropriate for cyclization, to direct the

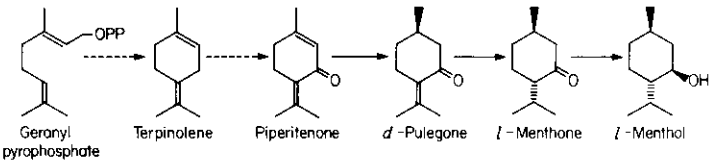

Figure 9. A possible mechanism for the cyclization of geranyl pyrophosphate to **d**-bornyl pyrophosphate.

orientation of entering and leaving groups and thereby achieve the correct stereochemistry, and to provide for the return of the pyrophosphate moiety as the terminating nucleophile. Further details of this hypothetical enzymatic mechanism, including the proposed function of the magnesium cation and the role of a serine residue in binding the pyrophosphate, are described elsewhere.[48]

While the mechanism of figure 9 is consistent with the available data, other feasible mechanisms can be written. Thus, for example, the enzyme bound linaloyl structure could undergo cationic cyclization to an α-terpinyl structure (fig. 10). Subsequent attack of the tertiary ionic center of the α-terpinyl ion on the trisubstituted double bond results in a stabilized pinyl cation. Binding of this pinyl cation could specifically promote Wagner-Meerwein shift to afford a bornyl cation, with the reaction being terminated by pyrophosphate to yield bornyl pyrophosphate. Alternatively, Wagner-Meerwein shift of the methylene group would yield a fenchyl cation, with the reaction being terminated by a hydroxyl ion (from water) to yield *endo*-fenchol. (This mechanism has been proposed to account for the cyclization of geranyl pyrophosphate to *l-endo*-fenchol by an enzyme from fennel.[49]) Finally, loss of either of two protons, with double bond formation, would yield the pinenes. This latter type of termination reaction is probably common to the formation of many monoterpene olefins. This overall mechanism, which assigns a central role to the bound pinyl cation, is highly speculative, but perhaps is a more general model in that it accounts for three different families of cyclic monoterpenes and illustrates three different types of termination steps.

While it seems likely that the conversion of geranyl pyrophosphate to a form stereochemically suitable for cyclization is a common first step in monoterpene cyclizations, the remaining features of the cyclization reactions are open to question. To distinguish between the various possibilities will require far more detailed studies of the monoterpene synthetase enzymes. Although no stereochemistry is indicated in figure 10, this mechanism can be formulated to account for the generation of either naturally occurring enantiomer of the bornane, fenchane, or pinane series. Whether this or another type of mechanism should eventually prove correct, defining how the enzyme actually controls the stereochemistry will certainly prove to be one of the most interesting aspects of monoterpene cyclization reactions.

The origin of l-menthol and its congeners

Menthol was known in Asia over 2,000 years ago, but the first mention of this compound in the Western world is attributed to Gaubius, who in 1771 separated this substance from peppermint oil.[50] As was the case with *d*-camphor, the chemistry of *l*-menthol is of great historical interest, particularly as regards the development of stereochemistry. *l*-Menthol (and its stereoisomers) occurs in nature primarily in the essential oils of *Mentha* species. It is the characteristic component of the oil of peppermint (*Menta piperita* L.) and is responsible for the cooling sensation associated with this product. Menthol and its stereoisomers are found mainly in the free state, but a number of recent reports have described the natural occurrence of glycosides of these monoterpene alcohols.[51-53]

In 1833, Dumas established that menthol had the empirical formula $C_{10}H_{20}O$, but it was another 50

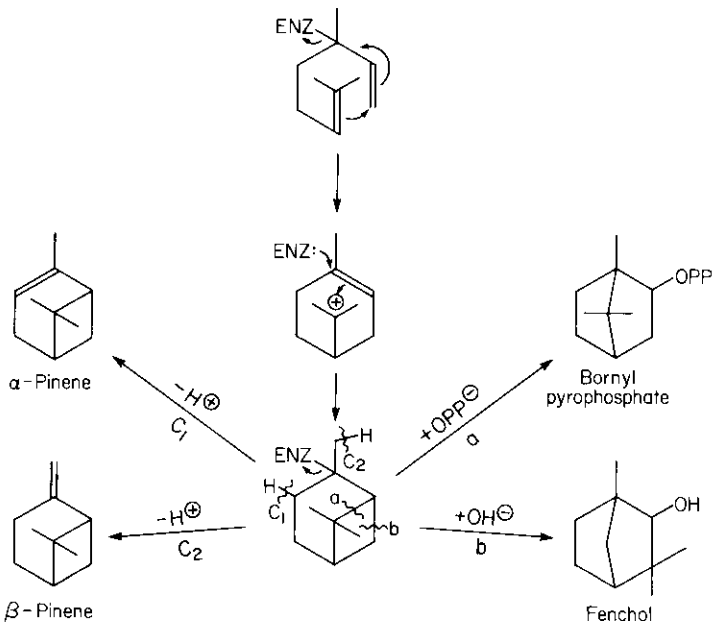

Figure 10. A possible mechanism for the synthesis of bornane, fenchane, and pinane monoterpenes from the same type of intermediate. (Stereochemical considerations are ignored.)

years before it was fully established that menthol was a saturated secondary alcohol.[54,55] Elucidation of the structure of menthol is generally attributed to Beckmann [56] and Semmler, [57] and is based on the observation that pulegone, menthone, and thymol all gave menthol on reduction (fig. 11). That the modern Haarmann and Reimer process for *l*-menthol production is based on the hydrogenation of thymol to menthols [58] attests to the importance of this reaction.

Inspection of menthol indicates the presence of three asymmetric carbons and thus the existence of eight optically active isomers and four corresponding racemates designated as menthols, neomenthols, isomenthols, and neoisomenthols. Menthone, containing two asymmetric centers, therefore exists as four stereoisomers and two racemates designated menthone and isomenthone. The preparation of the pure stereoisomeric menthols and menthones and the elucidation of their configurations was a very difficult problem shared by many workers including Beckmann, Wallach, Pickard, and Read. Accounts of this work, which has contributed in large part to the theoretical development of modern concepts of stereochemistry, are provided by Simonsen[59] and Orloff. [60]

With knowledge of the previously mentioned chemical interconversions of pulegone, menthone, and menthol, and the observation that these and other structurally related monoterpenes were present in peppermint oil, Kremers[61] and Reitsema[62] proposed a biosynthetic sequence for the formation of menthol (fig. 12).

Subsequent investigations with peppermint, primarily by Loomis and coworkers,[63-66] have confirmed this scheme, and the evidence for these reactions comes from several types of experiments. Analysis of the monoterpenes from different leaves of the plant showed a predominance of piperitenone and pulegone in the youngest leaves, with a trend toward menthone and then menthol in the oldest leaves. When $^{14}CO_2$ was administered to the plants, the unsaturated ketones were labeled first, followed by menthone, and later by menthol. Several of the reactions were demonstrated directly by feeding labeled monoterpenes to peppermint leaves. Loomis was also the first to take into account stereochemical considerations in the biosynthetic conversion of piperitenone to *l*-menthol, and this group has recently demonstrated, in cell-free preparations, the stereospecific reductases involved in this sequence of reactions.[67]

By contrast to the biosynthesis of camphor, where asymmetry is introduced in the cyclization step, in the formation of *l*-menthol molecular asymmetry is progressively introduced in three steps from the symmetric precursor piperitenone. The origin of piperitenone is still uncertain, and this proposed series of reactions is illustrated by broken arrows in figure 12. The direct conversion of acyclic precursors to terpinolene has been demonstrated,[68] but virtually nothing is known about the cyclase enzyme involved in this reaction. Similarly, the mechanism of the oxygenation of terpinolene to piperitenone has not yet been determined. While hydroxylation to the corresponding alcohol followed by dehydrogenation to the ketone might be involved, the ready autoxidation of terpinolene to piperitenone also suggests the possible involvement of hydroperoxides in the reaction.[69]

It has long been known to commercial producers of peppermint that the composition of the oil is greatly influenced by environmental factors. Thus, commercially acceptable peppermint oil can be grown only in a few areas, and in these regions the composition of the oil varies from one season to the next. The effects of environmental factors, such as temperature, photoperiod, and light intensity, and agricultural practices, such as irrigation method and fertilizer regime, on the composition and yield of peppermint oil

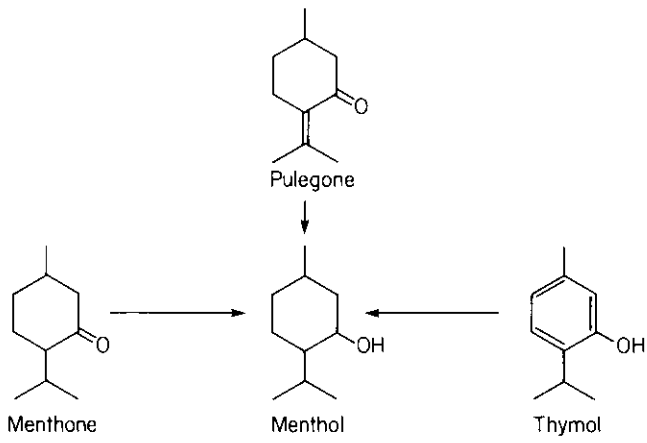

Figure 11. Some chemical reductions yielding menthol.

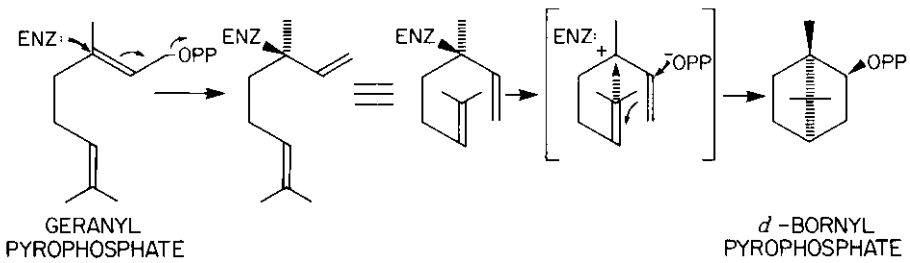

Figure 12. Pathway for the formation of l-menthol in peppermint. Broken arrows indicate postulated reactions.

have been examined, and changes in oil character correlated with the physiological condition of the plants.[64,70,71]

Superimposed on environmental variation and variation imposed by agricultural practices is the metabolic process termed *oil maturation*, whereby menthone is converted to menthol to produce an oil of acceptable quality. This process, which takes place in peppermint grown under a variety of conditions, is especially prominent in mint grown in the Yakima valley of Washington, where maturation occurs at about the time the plants flower (fig. 13). Thus, unlike *de novo* biosynthesis of monoterpenes, maturation takes place relatively late in the growing season and is coincident with an overall loss of oil content (fig. 13).

A notable feature of maturation is the large decrease in menthone content that accompanies this metabolic process. This loss in menthone cannot be accounted for by the increase in menthol or other oil constituents, nor can it be rationalized as due to evaporation. Thus, a catabolic process is implied. When *l*-[G-^{3}H] menthone was administered to leaf discs from flowering peppermint plants and the oil then analyzed, it was found that menthone was converted to *l*-menthol, to a small amount of *l*-menthyl acetate, and to a trace of *d*-neomenthol (all expected products from menthone).[51] However, the bulk of the tritium label from [G-^{3}H] menthone was not present in the essential oil, but rather was located in the water-soluble, nonvolatile fraction, thus giving the first indication that menthone was converted to a metabolite other than an essential oil component.[51]

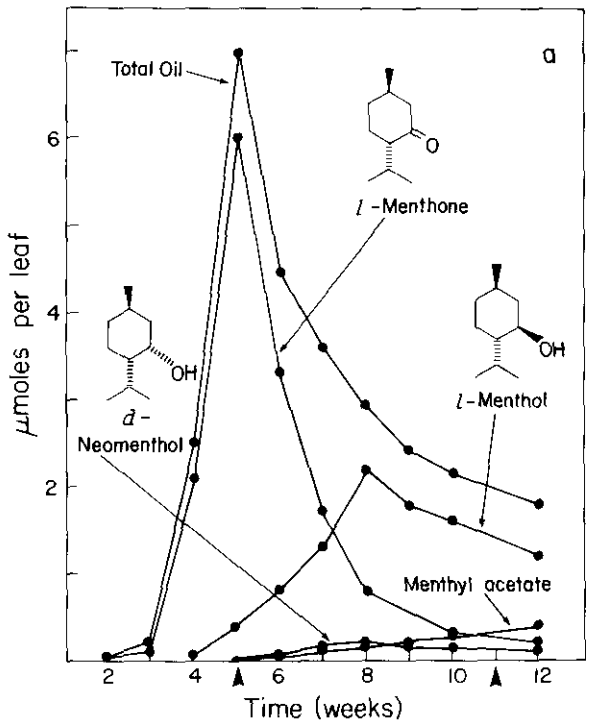

Figure 13. Essential oil composition of midstem peppermint leaves as a function of growth. The first arrow indicates the approximate time of floral initiation; the second arrow indicates the approximate time of full bloom. Menthofuran and pulegone are not plotted, although low levels of these compounds were detected from 3 to 6 weeks (from Croteau and Martinkus (51)).

Analysis of the water-soluble fraction indicated the presence of two labeled components derived from [G-^{3}H] menthone. The minor component was tentatively indentified as a highly oxygenated-menthane monoterpene, while the major metabolite was conclusively identified as *d*-neomenthyl-β-D-glucoside.[51] Menthyl glucoside was recently identified in Japanese mint (*Mentha arvensis*),[52,53] but the amount of menthyl glucoside formed in peppermint (*Mentha piperita*) was negligible compared to the neomenthyl glucoside. This was the first report on the occurrence of a neomenthyl glycoside in nature, and the first evidence implicating glycosylation as an early step in monoterpene catabolism.[51]

Further chemical investigations and studies with $^{14}CO_2$ as a precursor clearly indicated that, during the maturation process, *l*-menthone is reduced to roughly equal amounts of *l*-menthol and *d*-neomenthol.[51] The bulk of the menthol formed appears in the volatile oil (as such, or as the acetate ester) whereas the bulk of the neomenthol formed is converted to the glucoside. The pathways are highly specific in that only menthyl acetate (with little neomethyl acetate) and neomenthyl glucoside (with little menthyl glucoside) are formed (fig. 14). Two possible explanations for this specificity seemed evident: either the transacetylase and the transglucosylase are highly specific for menthol and neomenthol, respectively, or a dehydrogenase specific for menthol production is compartmentalized with a transacetylase, while a dehydrogenase specific for neomenthol formation is accessible only to a glucosylating system.

When *l*-[G-^{3}H]menthol and *d*-[G-^{3}H]neomenthol were separately administered to peppermint leaf discs, equal amounts of *l*-menthyl acetate and *d*-neomenthyl acetate were formed, and equal amounts of *l*-menthyl glucoside and *d*-neomenthyl glucoside were formed.[72] Thus, specificity of product formation (as in fig. 14) was observed when *l*-[G-^{3}H]menthone (or $^{14}CO_2$) was the precursor, but not when the alcohols were administered directly. These results strongly suggested that specificity is determined at the menthone reduction step, that the system is compartmentalized, and that the transacetylase and the transglucosylase are not highly selective enzymes. This latter suggestion was confirmed by isolating the enzymes involved and examining their properties. Thus, the acetyl CoA-dependent transacetylase utilized *l*-menthol and *d*-neomenthol with nearly equal efficiency in the synthesis of the corresponding acetate.[73] Similarly, the UDP-glucose-dependent transglucosylase exhibited nearly identical K_m and V_{max} for *l*-menthol and *d*-neomenthol, synthesizing both β-D-glucosides with equal proficiency.[72]

All of the above results pointed to the enzymatic reduction of *l*-menthone as the key step in the maturation process, and homogenates of leaves from flowering peppermint plants were shown to catalyze

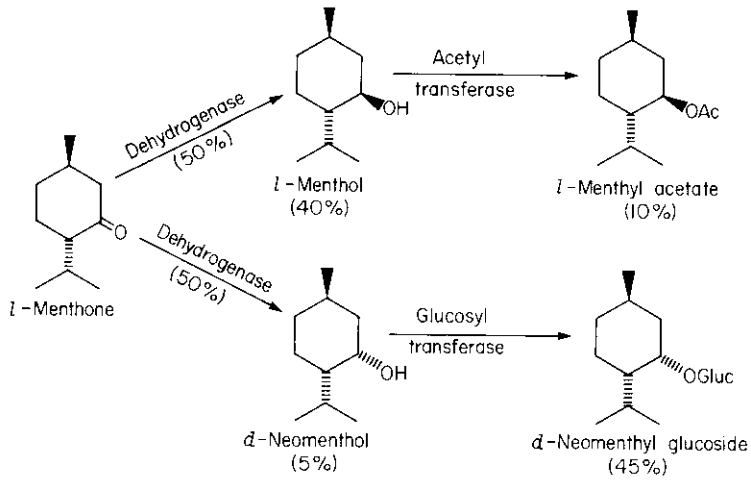

Figure 14. Pathway for the metabolism of l-menthone during maturation in peppermint. The approximate composition of the products derived from menthone is given.

the enzymatic reduction of *l*-menthone to nearly equal quantities of *l*-menthol and *d*-neomenthol, with NADPH as the required cofactor.[73a] The activity was localized in the soluble enzyme fraction and, by combination of gel permeation chromatography and affinity chromatography, two distinct and stereospecific dehydrogenases were isolated (one for reduction of menthone to menthol, the other for reduction to neomenthol). The two enzymes have a number of similar characteristics, but differ in kinetic properties suggesting that their activities may be differentially regulated *in vivo*. While the two dehydrogenases are operationally soluble enzymes, the tissue extraction procedure employed was quite harsh and the integrity of cellular compartments could easily have been destroyed, thereby causing release of their contents to the soluble phase. Thus, while the evidence cited above clearly implicates compartmentation of pathways as an essential feature of the maturation process, the intracellular location of the stereospecific dehydrogenases and their auxiliary enzymes (the transacetylase and transglucosylase) is a key question that remains to be answered.

The role and fate of neomenthyl glucoside are not presently known. However, if during this apparent catabolic phase the monoterpenes are utilized at sites other than the epidermal oil glands, then a means of transporting these lipophilic materials would be required. Monoterpenyl glycosides, many of which have recently been identified,[51] may represent such a transport form. When *l*-[G-^{3}H]menthone is administered to leaves of intact flowering peppermint plants, the neomenthyl glucoside formed in the leaves appears to be specifically transported to the roots, where at least a portion of this material is further metabolized to unidentified products.[51,72]

This observation supports the suggestion that monoterpenyl glycosides are transport derivatives, but much more work will need to be done before gaining a clear understanding of the physiological significance of this observation. Further studies on the properties, regulation, and localization of the enzymes from peppermint, which may be a model for other plants as well, should lead to a better understanding of, and perhaps a rationale for, monoterpene catabolism.

Comparative aspects of monoterpene and sesquiterpene biosynthesis

As the monoterpenes and sesquiterpenes are commonly found together in essential oils, there is a natural tendency to regard these two types of compounds as closely related. Similarities and differences in occurrence, function and sites of synthesis have been described,[6,16] but the comparative biochemistry of these two families of terpenes is still largely a matter of speculation. The sesquiterpenes contain one more isoprene unit than monoterpenes, allowing much greater flexibility in the construction of sesquiterpenoid compounds. Almost two hundred different carbon skeletons containing up to four carbocyclic rings are found in this terpenoid group, and several thousand individual sesquiterpenes have been identified [74,75] (this compares to less than five hundred monoterpenes [75]). The sesquiterpenes are the largest single class of terpenoids, representing a tremendous diversity of structure. The bulk of the sesquiterpenes (like the examples in figure 2) are constructed by the typical head-to-tail fusion of isoprene units, or can be regarded as rearrangement products of more typical head-to-tail structures.

The sesquiterpenes were considered by Ruzicka to originate from the corresponding cation of *trans*-Δ^2-*trans*-Δ^6-farnesol or, where steric considerations appeared to apply, from the *cis*-Δ^2-*trans*-Δ^6-isomer; the *trans,trans*-isomer yielding the ten and eleven membered ring intermediates, and the *cis,trans*-isomer yielding, additionally, six and seven membered ring intermediates (fig. 15).[9]

Within this theoretical framework, transformations

of the monocyclic intermediates could involve further cyclizations by addition of cationic centers to the remaining double bonds, hydride shifts and rearrangements, governed by either electronic or steric considerations.[20,22,76,77] Thus, while the sesquiterpenes are structurally more complex than the monoterpenes, the mechanistic rationale for the formation of both classes of compounds is similar. While Ruzicka's original biogenetic isoprene rule was formulated in terms of the farnesols as precursors, the corresponding *trans,trans*- and *cis,trans*-farnesyl pyrophosphates are now considered to be the more likely direct progenitors of the sesquiterpenes.

Most recently, nerolidyl pyrophosphate has been implicated as an intermediate in the formation of certain sesquiterpenes.[22,23,78] The proliferation of suggested precursors for sesquiterpenes stems in large part from the fact that, while *trans,trans*- farnesyl pyrophosphate is the first C_{15} compound to arise in the normal terpenoid pathway (fig. 4) and is the precursor of most higher terpenes, it is not sterically possible for this *trans,trans*-compound to cyclize directly to all common sesquiterpene structural types. Thus, for example, *cis,trans*-farnesyl pyrophosphate and nerolidyl pyrophosphate have been postulated as precursors of structural types with six or seven membered rings (as in figure 15). This situation is analogous to that in monoterpene biosynthesis, where neryl pyrophosphate has traditionally been regarded as the most likely precursor of cyclohexanoid monoterpenes.

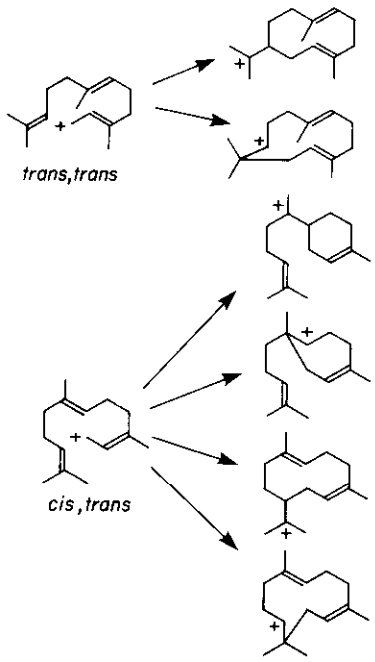

Figure 15. Hypothetical cyclization of the **trans, trans**- and **cis, trans**-farnesyl cations to monocyclic sesquiterpene intermediates.

The recent finding that geranyl pyrophosphate is, in fact, the preferred direct precursor of several cyclohexanoid monoterpenes (see earlier discussion) therefore suggests that the ubiquitous *trans,trans*-farnesyl pyrophosphate could, by analogy, serve as the direct precursor of sesquiterpene types previously postulated to arise from more sterically suitable precursors. Thus, by direct analogy to the proposed cyclization of geranyl pyrophosphate (figs. 9 and 10), preliminary enzymatic attack on the C_{15} precursor could involve the intermediate formation of an enzyme-bound nerolidyl cation (fig. 16), which could cyclize through conformer *a* (equivalent to *trans,trans*-farnesyl pyrophosphate) or conformer *b* (equivalent to *cis,trans*-farnesyl pyrophosphate). The configuration at the enzyme bound carbon, the conformer cyclized, the orientation of the isopropylidene group and the conformation of the remaining chain could explain observed stereochemical preferences, and no other precursor than *trans,trans*-farnesyl pyrophosphate need be invoked. While studies at the enzyme level will be required to examine the above hypothesis, some preliminary *in vivo* studies support the concept,[79] and others can be rationalized on this basis.[22]

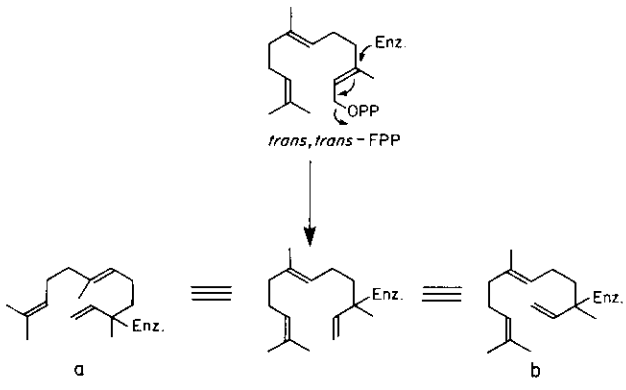

Figure 16. Possible enzyme-bound intermediates in sesquiterpene biosynthesis.

While a large number of sesquiterpenes have no direct monoterpene counterparts, there are many that are, structurally, the C_5-prenylated analogs of common monoterpenes. In fact, the direct sesquiterpene analogs of virtually all common acyclic, monocyclic, and bicyclic monoterpenes are known, and several examples are illustrated in figure 17. Similarly, many sesquiterpenes can be regarded as macrocyclic analogs of common monocyclic monoterpenes. One of the simplest families of macrocyclic sesquiterpenes is that based on the germacrene skeleton, and the hypothetical origin of germacrene A, germacrene B, and hedycaryol from *trans,trans*-farnesyl pyrophosphate (fig. 18) can be seen to resemble similar schemes for the formation of the monoterpenes limonene, terpinolene, and α-terpineol. Hydride shift and proton loss to yield germacrene C and germacrene D resemble similar processes thought to be involved in the biogenesis of the monoterpene dienes α-terpinene, γ-terpinene, and β-phellandrene.

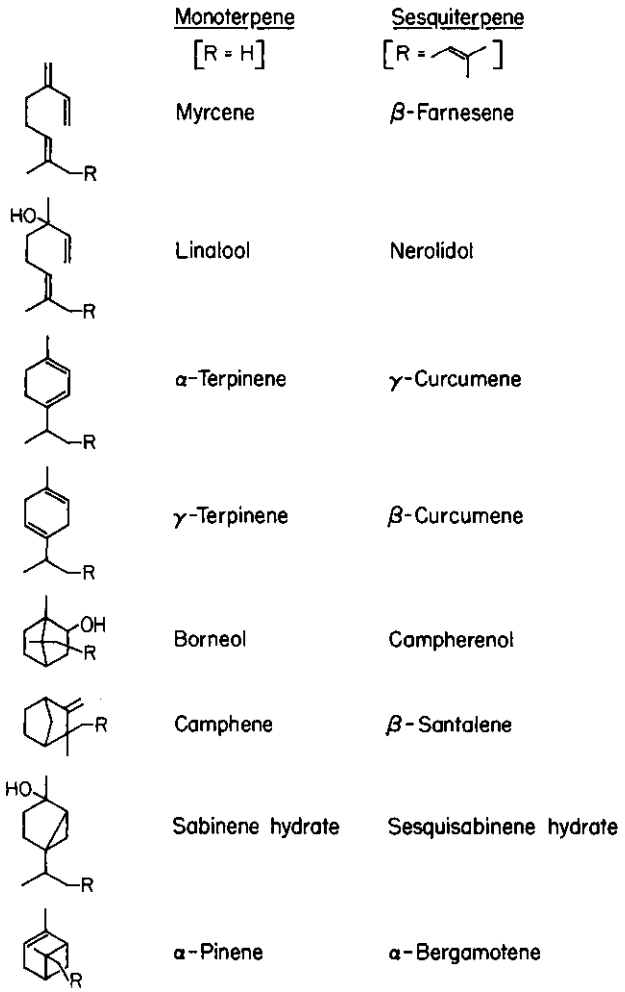

Figure 17. Some monoterpenes and their sesquiterpene analogs.

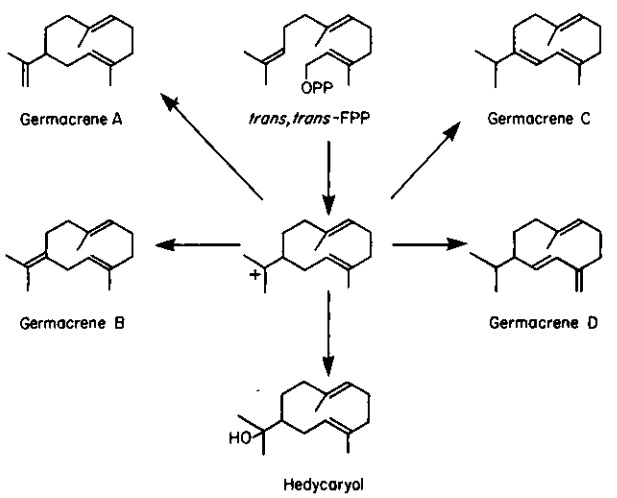

Figure 18. Postualted origin of germacrenes and hedycaryol from **trans, trans**-farnesyl pyrophosphate.

Thus, while there is little evidence to support the suggestion, similar biosynthetic mechanisms (and probably similar enzymes) for the formation of these related monoterpenes and sesquiterpenes are likely. It is even tempting to speculate that the same enzyme might be involved in the synthesis of both C_{10} and C_{15} analogs. If this was the case, one might expect the analogous monoterpenes and sesquiterpenes to co-occur, yet a search of several essential oil compendia suggests such co-occurrence is uncommon.[80-84]

As a number of soluble enzyme preparations capable of synthesizing common monoterpenes from geranyl pyrophosphate are available, the above speculation was tested directly. The ability of these preparations to synthesize a known monoterpene from [1-^{3}H]geranyl pyrophosphate was, thus, compared to the ability of the same preparation to synthesize the analogous sesquiterpene from *trans,trans*-[1-^{3}H] farnesyl pyrophosphate (Table I). *Trans,trans*-[1-^{3}H] farnesyl pyrophosphate was not a very efficient substrate for these monoterpene cyclase preparations, and the appropriate sesquiterpene product was detectable only in the case of monocyclic compounds. Thus, even if *trans,trans*-farnesyl pyrophosphate is available to the enzyme, it seems unlikely that the biosynthesis of sesquiterpenes by monoterpene cyclases has any significance *in vivo*.

Relatively few cell-free systems capable of sesquiterpene biosynthesis are known. Caraway (*Carum carvi*) leaves contain relatively high levels of germacrene D,[85] and a 105,000 *g* supernatant prepared from young leaves was capable of synthesizing germacrene D from *trans,trans*-[1-^{3}H]farnesyl pyrophosphate. When [1-^{3}H]geranyl pyrophosphate was used as the substrate with this relatively crude preparation, several monoterpenes were synthesized (notably limonene), but the monoterpene analog of germacrene D (that is, β-phellandrene, which is a component of caraway oil[86]) was barely detected (Table I). These results provide another indication that monoterpene and sesquiterpene analogs are not synthesized by the same enzymes, and they further indicate that the distinct monoterpene synthetases and sesquiterpene synthetases are rather specific for prenyl pyrophosphate substrates of the appropriate C_{10} or C_{15} chain length. Thus, while it is quite likely that the mechanisms involved in the biosynthesis of analogous monoterpenes and sesquiterpenes are similar, com-

Table I. Comparison of monoterpene and sesquiterpene biosynthesis by cell-free enzyme preparations

Source of enzyme preparation	Monoterpene (% incorp. of GPP)	Sesquiterpene (% incorp. of FPP)
Salvia officinalis	α-pinene (2.3%)	α-bergamotene (< 0.05%)
Salvia officinalis	bornyl pyrophosphate (as borneol) (4.5%)	campherenol (< 0.05%)
Thymus vulgaris	γ-terpinene (3.7%)	β-curcumene (0.13%)
Chenopodium ambrosioides	α-terpinene (5.8%)	γ-curcumene (0.19%)
Carum carvi	β-phellandrene (0.11%)	germacrene D (1.2%)

GPP = (1-^{3}H) geranyl pyrophosphate; FPP = *trans,trans*-(1-^{3}H) farnesyl pyrophosphate

parative studies with monoterpene cyclases and the corresponding sesquiterpene cyclases will be required to explore this point further.

Conclusion

From Wallach's isoprene rule to Ruzicka's biogenetic isoprene rule, the monoterpenes and sesquiterpenes have played a significant role in the development of basic concepts concerning the origin of all terpenoid compounds. Yet, while our understanding of the biosynthesis of higher terpenoids, such as steroids, has flourished, progress in the biochemistry of monoterpenes and sesquiterpenes has been relatively slow—due in large part to the chemical nature of the mono- and sesquiterpenes (volatility, instability, and so forth) and to the experimental difficulties associated with the plants that produce these compounds. Since the last International Symposium,* however, improvements in analytical methods and techniques for plant enzymology have led to significant advances with both the *in vivo* and *in vitro* experimental approach. Progress with cell-free preparations, particularly in the area of monoterpene cyclases and their mechanisms of action, has been very rapid. Enzymes that interconvert the monoterpenes, and thereby increase the complexity of essential oils, are being purified and characterized. Perhaps, for the first time, the door is open to understanding monoterpene catabolism, its regulation, and its physiological rationale. The tools for comparing the origin of monoterpenes and sesquiterpenes on a molecular level are now available. More than any time in the past, recent work has opened up the area of monoterpene and sesquiterpene biosynthesis, and exciting developments are likely to occur in the very near future.

*I International Symposium on Fragrance and Flavor Substances, Bad Pyrmont, Germany, 1974.

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