Prospects for the Bioengineering of Isoprenoid Biosynthesis

D. McCaskill and R. Croteau
Institute of Biological Chemistry, Washington State University, PO Box 646340, Pullman, WA 99164-6340 USA

Over the last decade, our understanding of isoprenoid biosynthesis has progressed to the stage where specific strategies for the bioengineering of essential oil production can be considered. This review provides a current overview of the enzymology and regulation of essential oil isoprenoid biosynthesis. The reaction mechanisms of the synthases which produce many of the basic isoprenoid skeletons are described in detail. Coverage is also provided of the regulation of isoprenoid biosynthesis, including the roles played by tissue and subcellular compartmentation, and by partitioning of intermediates between different branches of isoprenoid metabolism. This provides necessary context for rationally targeting specific enzymes of metabolic pathways for bioengineering essential oil production. Wherever possible, emphasis is placed on research specific to essential oil isoprenoid biosynthesis, although relevant work related to other isoprenoids is also considered when it can provide useful insights. Finally, building upon this understanding of essential oil isoprenoid biosynthesis, several approaches to the bioengineering of isoprenoid metabolism are considered.

Advances in Biochemical Engineering/
Biotechnology, Vol. 55
Managing Editor: T. Scheper

List of Abbreviations

ACAT	acetyl-CoA acetyltransferase
BPP	bornyl diphosphate
DMAPP	dimethylallyl diphosphate
FPP	farnesyl diphosphate
GGPP	geranylgeranyl diphosphate
GPP	geranyl diphosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	HMG-CoA reductase
IPP	isopentenyl diphosphate
LPP	linalyl diphosphate
NPP	nerolidyl diphosphate

1 Introduction

Throughout history aromatic plants and the essential oils derived from them have been highly valued not only for their fragrances but also for their medicinal and culinary uses. Traditionally, essential oil research has focused on the isolation, identification and cataloging of the characteristic aroma constituents present in the oil. Attempts to manipulate the composition and quality of the essential oil produced by the plant relied on conventional plant breeding and agronomic strategies. The composition of essential oils is usually complex and the overall olfactive character of the oil is often determined by a subtle interplay between numerous components. Despite the availability of low cost synthetic aroma chemicals, the use of natural aroma chemicals continues to attract interest for two main reasons [1]. The first derives from the public's understandable, but scientifically unfounded, desire for the use of natural (rather than synthetic) flavorings and fragrances. The second reason is that naturally occurring aroma chemicals often exist in an enantiomerically pure form which would be more expensive to obtain using synthetic routes. It is well known that different enantiomers of the same compound can often have distinctly different aroma characteristics [2, 3].

A family of natural products known collectively as isoprenoids are among the most common constituents found in essential oils. Isoprenoids are the largest single family of compounds found in nature with over 22000 known examples [4], the vast majority of which are produced by plants. The biosynthesis of isoprenoids involves the sequential assembly of branched five carbon isopentanoid units. Classification of the different families of isoprenoids is based on the number of five carbon units present in the skeleton of the compound [5]. The isoprenoids most commonly associated with flavor and/or aroma are the monoterpenes, although sesquiterpenes and to a lesser extent diterpenes also contribute to the overall aromatic character of natural oils. Monoterpenes are the major components of many essential oils of herbs, spices, citrus, and conifer species [6]. The characteristic volatiles of many flowers also contain monoterpenes [7]. Ionones and damascenones, which are common aroma components in tea, tobacco, and many fruits and vegetables, are derived from the degradative breakdown of the tetraterpenoid carotenoids. Turpentine-derived monoterpenes isolated from conifers are used in the manufacture of many low cost flavor and fragrance compounds [8]. In response to concerns about the long-term toxicity and environmental effects of synthetic chemicals, many naturally occurring isoprenoids (including monoterpenes) are also receiving attention in more non-traditional roles including use as agents for the biological control of insect pests and as potential herbicides (see, e.g., [9, 10]).

The following discussion provides an overview of isoprenoid biosynthesis at the biochemical level. Over the last decade, our understanding of the enzymology and regulation of isoprenoid biosynthesis has advanced to a point where specific strategies for the bioengineering of essential oil formation can now be

contemplated. Wherever possible, emphasis will be placed on studies carried out on essential oil biosynthesis. However, much of what is known about isoprenoid biosynthesis at the biochemical level comes from studies unrelated to the production of aromatic isoprenoids. These studies will be described where appropriate in order to provide a broader understanding of isoprenoid biosynthesis and regulation which can then be applied to the specific task of manipulating essential oil formation. Specific strategies for the rational manipulation of individual enzyme activities to influence product formation, the manipulation of pathway fluxes to influence essential oil composition, and approaches to increase yield in the plant without affecting the oil composition will be described. A brief discussion of the use of tissue cultures is also included.

2 Biosynthesis of Isoprenoids

The biosynthesis of isoprenoids from simple, primary metabolites can be conceptually divided into four overall steps: the synthesis of the fundamental precursor isopentenyl diphosphate (IPP) from acetyl-CoA; the formation of the allylic prenyl diphosphates from IPP which serve as the immediate precursors of the different families of isoprenoids; the elaboration of these allylic prenyl diphosphates into the manifold isoprenoid skeletons by specific isoprenoid synthases; and secondary chemical modifications of the parent isoprenoid products of these synthases. Although plants share much of the same enzymology of isoprenoid biosynthesis with animals and microorganisms, significant differences exist. In particular, plants produce a much greater variety of isoprenoids than either animals or microorganisms, and this is reflected in a more complex organization of isoprenoid biosynthesis at the tissue, subcellular and genetic levels. This organizational complexity has greatly hampered studies of isoprenoid biosynthesis in plants. As a result, several fundamental questions about the enzymology and regulation of isoprenoid biosynthesis in plants remain either unanswered or in dispute. A rational approach to the bioengineering of isoprenoid formation in plants requires an appreciation of the contribution of both the pathways and the organization of isoprenoid metabolism.

2.1 Formation of Isopentenyl Diphosphate

Isopentenyl diphosphate (IPP **1**) is the five-carbon building block for the biosynthesis of all isoprenoids. Although the formation of IPP in plants has been the subject of considerable study (reviewed in [11, 12]), many fundamental questions, particularly regarding the regulation of IPP formation, remain unresolved. The basic enzymology of IPP biosynthesis via the mevalonic acid pathway is widely accepted (Fig. 1). The initial steps involve the condensation of

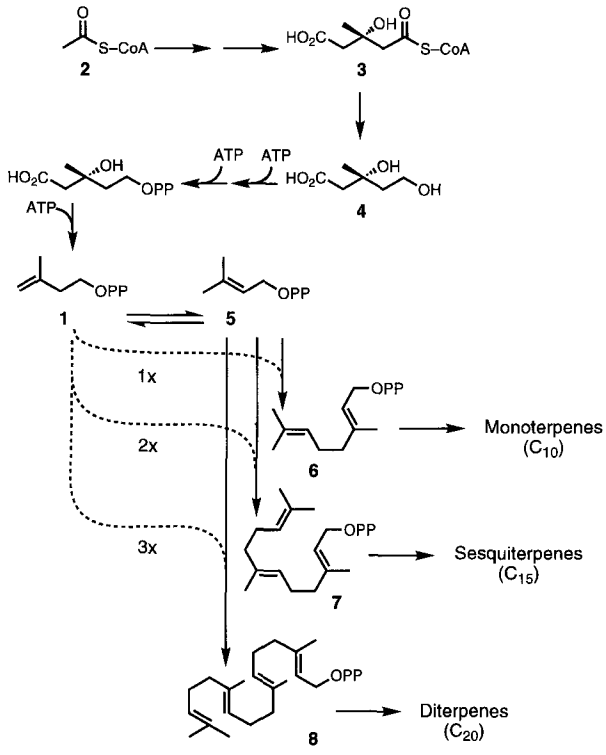

Fig. 1. Formation of the different families of aromatic isoprenoids by the mevalonic acid pathway. The diphosphate moiety is abbreviated as -OPP

three molecules of acetyl-CoA (**2**) to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA **3**), which is subsequently reduced to mevalonic acid (**4**) by HMG-CoA reductase (HMGR). Two sequential phosphorylations of mevalonic acid, followed by a phosphorylation-assisted decarboxylation yields IPP (**1**). In animals and yeast the formation of HMG-CoA from acetyl-CoA is catalyzed by two separate enzymes, acetyl-CoA acetyltransferase (ACAT) and HMG-CoA synthase. In plants, it has been reported that a single enzyme (ACAT/HMG-CoA synthase) may be responsible for both condensations [13], although more recent research is forcing a reevaluation of this earlier work (reviewed in [11]).

HMGR is considered to be one of the most highly regulated enzymes in animals [14] and evidence is also accumulating that HMGR is also highly regulated in plants (reviewed in [15]). In many of the plants examined so far, HMGR is encoded by small gene families which are distinguished from each other by sequence differences in the 3′ untranslated regions of the cDNAs (see, e.g., [16]), These gene families, each of which may contain multiple genes, are commonly designated as *hmg1*, *hmg2*, and *hmg3*. In the case of potato, 12 or more genes for HMGR have been found [17]. Expression of these different gene

families is complex with some members exhibiting tissue-specific expression, constitutive expression, or hormone-inducible expression. Induction of specific HMGR genes by wounding or pathogen infection also occurs. In the case of thale cress (*Arabidopsis thaliana*), the use of alternative promoters which select for different start codons has been shown to result in the tissue-specific expression of two distinct isoforms of HMGR from a single gene [18]. HMGR is also regulated by a protein kinase cascade in which phosphorylation inactivates the enzyme [19, 20]. Other controls involving calcium and calmodulin and proteolytic degradation may also regulate HMGR activity (reviewed in [15]). Despite this wealth of information available on the regulation of HMGR in plants, no unified picture has emerged for the role of the different species of HMGR in regulating the production of the different families of isoprenoids. (period) Indeed, even the subcellular compartmentation of HMGR is still controversial (see Sect. 3.2).

Recently, a new pathway for the biosynthesis of IPP in a wide variety of bacteria has been demonstrated [21]. Based on the labeling patterns observed with different ^{13}C-labeled substrates, it was proposed that IPP is formed in these bacteria by the addition of a two-carbon intermediate to a three-carbon phosphorylated intermediate of glycolysis, resulting in the direct formation of the branched carbon skeleton of IPP in a single step. A similar conclusion has been drawn from the ^{13}C-labeling patterns of the diterpenoid ginkgolides produced in embryos of *Ginkgo biloba* derived from different [^{13}C]glucose substrates, although the labeling patterns of the sterols produced in the same tissue were consistent with the operation of the classical mevalonic acid pathway (discussed in [11, 22]). Although the results of these in vivo studies appear convincing, none of the enzymatic activities involved in this novel pathway to IPP have been demonstrated. Anomalous ^{13}C-labeling patterns of the hemiterpene moiety of prenylated chalcones have also been observed in tissue cultures of mulberry (*Morus alba* L.) [23]. In this case, the labeling pattern was considered to be consistent with the involvement of the mevalonic acid pathway, although it was proposed that the first acetyl-CoA incorporated into IPP was derived from glycolysis, whereas the second and third acetyl-CoAs were derived from the pentose phosphate cycle. These in vivo feeding studies illustrate the pressing need for additional work on the enzymology, regulation and subcellular compartmentation of IPP biosynthesis.

2.2 Prenyltransferases

IPP is utilized in the formation of the allylic diphosphates which serve as the immediate precursors of the different families of isoprenoids. Isomerization of IPP (**1**) by an enzyme known as IPP isomerase produces the allylic isomer dimethylallyl diphosphate (DMAPP **5**). This enzyme has been characterized in a number of plants (reviewed in [5]) and extensively studied in a number of microorganisms (see, e.g., [24] and references therein). DMAPP is a reactive

primer which undergoes elongation by the sequential addition of one, two, or three additional IPP molecules to form either geranyl diphosphate (GPP **6**), farnesyl diphosphate (FPP **7**) or geranylgeranyl diphosphate (GGPP **8**) [25]. GPP and FPP are the ten and fifteen carbon precursors of monoterpenes and sesquiterpenes, respectively. A family of enzymes known collectively as prenyltransferases catalyze this electrophilic elongation sequence. Specific prenyl transferases exist for the formation of GPP, FPP, and GGPP [5] (Fig. 1). The reaction catalyzed by prenyltransferases involves the initial ionization of the allylic diphosphate to generate a delocalized allylic carbocation. This enzyme-bound cation then attacks the double bond of IPP, followed by deprotonation to generate the next allylic diphosphate homolog. In the case of GPP synthase, the first condensation product is released from the enzyme. In the case of FPP and GGPP synthases, the resulting enzyme-bound GPP undergoes further reaction with the addition of another IPP to generate FPP, which is either released in the case of FPP synthase or which undergoes reaction with a third IPP to generate GGPP in the case of GGPP synthase.

GPP synthase has been purified from *Vitis vinifera* [26, 27] and *Lithospermum erythrorhizon* [28] and characterized. However, the most extensively studied prenyltransferase is FPP synthase because of the pivotal role FPP plays in cholesterol biosynthesis in humans. Numerous studies with substrate analogs (see, e.g., [29, 30]) and using site directed mutagenesis [31–34] have been focused on dissecting the role of specific amino acid residues at the active site of the enzyme in substrate binding and catalysis. The crystal structure of recombinant avian FPP synthase has recently been reported at 2.6 Å resolution [35].

2.3 Isoprenoid Synthases

The family of enzymes responsible for the formation of isoprenoids from GPP, FPP, and GGPP are known as monoterpene, sesquiterpene, and diterpene synthases, respectively. These synthases use the acyclic, achiral allylic diphosphates as substrates to form the enormous chemical diversity of carbon skeletons characteristic of isoprenoids. Most isoprenoids are cyclic, and often contain multiple ring systems. The regio- and stereochemistry of the basic ring structure of the isoprenoids are in most cases determined by these highly specific synthases. Isoprenoid synthases that produce cyclic products are also referred to as "cyclases", although examples of synthases producing acyclic products are also known.

The greatest variety of monoterpene synthases that have been studied have been isolated from essential oil-producing plants. By contrast, relatively little is known about the synthases responsible for the production of aromatic sesquiterpenes and diterpenes found in plants. Much of what is known about sesquiterpene and diterpene synthases comes from research carried out on isoprenoids which are not important as aroma chemicals, such as fungal toxins, plant defensive compounds (phytoalexins), hormones, and resin acids. Because of

their central role in producing aromatic essential oils, most of the following discussion will focus on the monoterpene cyclases of plant origin. Consideration will also be given to the sesquiterpene and diterpene cyclases that have been studied, since many of these enzymes produce isoprenoids with the same cyclic carbon skeletons as important aroma compounds, presumably via the same basic reaction mechanisms.

2.3.1 Monoterpene Cyclases

Monoterpene cyclases catalyze the conversion of GPP (**6**) to cyclic monoterpenes with either one or two rings. A unified stereochemical scheme which describes the cyclization of GPP has been proposed (reviewed in [36]); all monoterpene cyclases examined to this point utilize this common strategy (see Fig. 2). Cyclization of GPP requires no cofactors other than a divalent metal ion, typically either Mg^{2+} or Mn^{2+}. The initial step of the cyclization reaction is the ionization of the diphosphate ester bond of enzyme-bound GPP to generate an allylic carbocation-diphosphate anion pair. This ionization is assisted by the divalent cation which helps neutralize the negative charge of the diphosphate, making it a better leaving group. Tight ion pairing of the diphosphate anion with enzyme-bound carbocationic intermediates greatly restricts motion of the diphosphate moiety [37–41] as demonstrated by studies with bornyl diphosphate synthase from sage (*Salvia officinalis*) and tansy (*Tanacetum vulgare*) which produce the phosphorylated products (+)-bornyl diphosphate ((+)-BPP **9**) and (−)-BPP (**10**), respectively [38]. During catalysis, the orientation of the two ends of the diphosphate anion remain fixed relative to the enzyme-bound carbocationic intermediates, and no rotation is permitted along the Pα-O bond axis. Thus, the diphosphate ester oxygen of the BPP product is derived exclusively from the same diphosphate ester oxygen of the GPP substrate.

The initially formed allylic carbocation-anion pair collapses to an enzyme-bound linalyl diphosphate (LPP) intermediate [42, 43] through a *syn*-isomerization process [44] to afford either the 3*R*-(**11**) or 3*S*-(**12**) enantiomer, depending on the helical conformation of the GPP substrate imposed by the binding site of the specific synthase; the chirality of the enzyme-bound LPP determines which antipode of the product is ultimately formed by the cyclase. The formation of enzyme-bound LPP is a prerequisite for cyclization since the *trans*-2,3-double bond of GPP prevents direct C1–C6 ring closure. Rotation about the newly formed C2–C3 single bond of LPP allows the intermediate to adopt a cisoid, anti-endo conformation from which ionization and subsequent cyclization of the resulting tertiary allylic cation yields the corresponding 4*R*- or 4*S*-α-terpinyl carbocation (**13**). The final product released by the synthase is determined by the subsequent steps which the α-terpinyl cation undergoes. These can include additional electrophilic cyclizations, hydride shifts and/or other rearrangements. The reaction is terminated when the carbocation is quenched, either by the addition of a nucleophile such as water (e.g., (−)-*endo*-fenchol (**14**) cyclase in

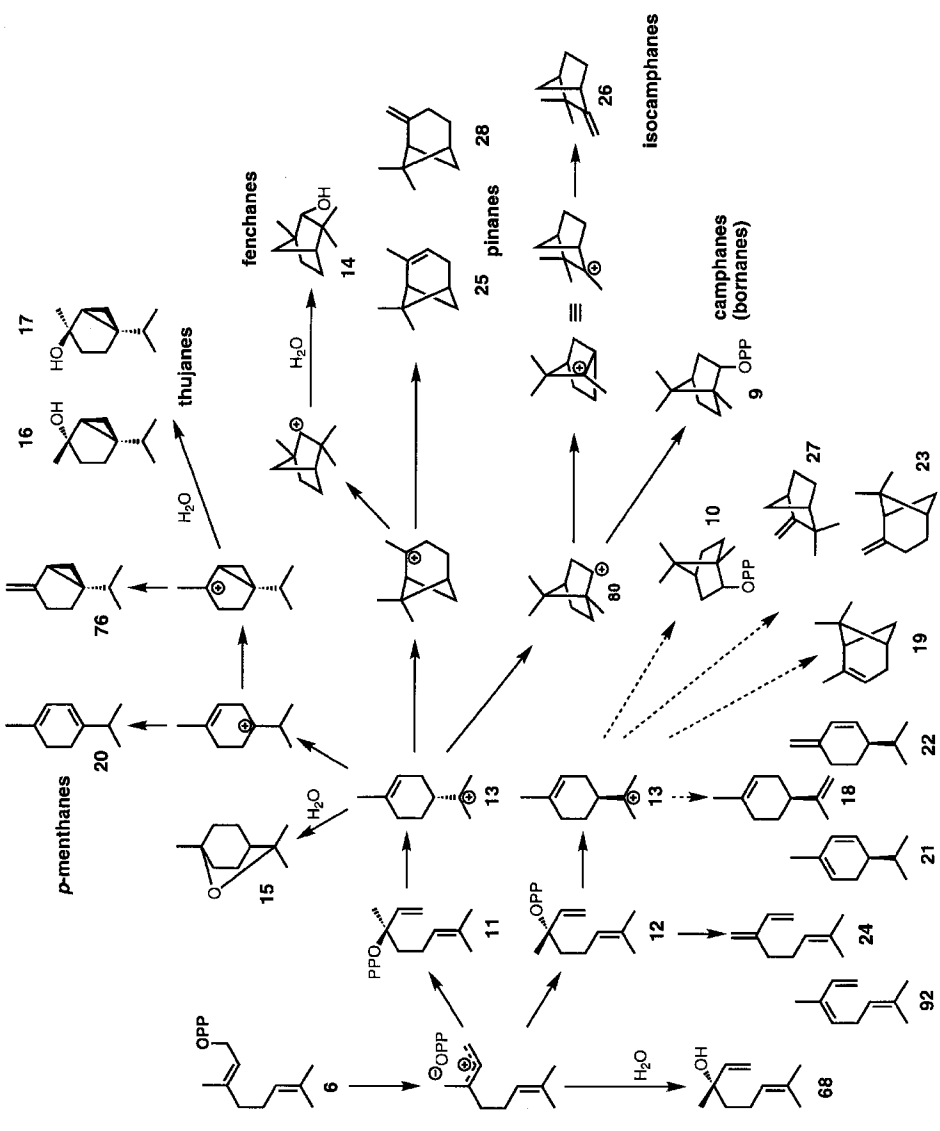

Fig. 2. Cyclization of GPP by monoterpene synthases through either 3*R*-LPP or 3*S*-LPP to produce different monoterpene skeletons

fennel (*Foeniculum vulgare*) [45], 1,8-cineole cyclase (**15**) in common sage (*Salvia officinalis*) [46] and (+)-*trans*-(**16**) and (+)-*cis*-sabinene hydrate (**17**) cyclase from sweet marjoram (*Majorana hortensis*) [47]), or by deprotonation to an olefin (e.g., limonene (**18**) cyclase in peppermint (*Mentha x piperita*) [48]). The terminating step of α-pinene (**19**) synthesis in sage may involve the original diphosphate anion as the base which deprotonates the enzyme-bound pinyl cation [49].

Although the monoterpene cyclases are highly stereospecific in employing only 3*R*- or 3*S*-LPP as the cyclizing intermediate to afford the corresponding 4*S*- or 4*R*-α-terpinyl intermediate and subsequent stereochemically related antipodes, an unusual feature of many of these enzymes is the production of multiple products at the same active site. This is a consequence of the reactive nature of the carbocationic intermediates, the many possible reaction routes, the variety of ways in which the reaction can be quenched, and the permissiveness of the enzymes in allowing multiple catalytic channels. Numerous studies have been carried out to distinguish between the formation of multiple products by enzyme preparations containing more than one synthase species, and the formation of multiple products by a single synthase. Initially, the production of multiple products by a single enzyme was suggested by studies demonstrating copurification of activities, and differential inactivation and inhibition by active site directed reagents [47, 50, 51]. Product analysis of γ-terpinene synthase from thyme (*Thymus vulgaris*) [50] and 4*S*-limonene synthase from peppermint [52] purified to apparent homogeneity demonstrated the formation of primarily γ-terpinene (**20**) and 4*S*-limonene (**18**), respectively, with minor amounts of other products. A more elegant approach has been to use isotopically sensitive branching to probe the partitioning of enzyme-bound carbocationic intermediates between different products [52–55]. This method relies on the kinetic isotope effect produced by deuterium atoms strategically placed in the substrate at positions which normally undergo deprotonation. An induced kinetic isotope effect [56] results in a decrease in the rate of formation of the product arising from deprotonation at the deuterium-bearing carbon with a concomitant increase in the rate of formation of other products, thus indicating that these products arise from a common enzyme-bound intermediate at a branch point rather than from the independent formation of multiple products by separate enzyme species. The formation of multiple products by different monoterpene synthases, as demonstrated by isotopically sensitive branching experiments, is summarized in Table 1. Although isotopically sensitive branching gives strong evidence for the formation of multiple products by a single cyclase species, the ultimate proof of this unusual phenomenon came with the cloning and expression in *Escherichia coli* of a catalytically active recombinant limonene cyclase from spearmint (*Mentha spicata*) which exhibited the same product profile as the native enzyme (see below).

Monoterpene and sesquiterpene cyclases exhibit rather low turnover numbers, typically in the range of 0.01–0.3 sec^{-1} [48, 50, 57–59]. In the case of monoterpene cyclases, the initial isomerization of GPP to LPP is considered to be the rate-limiting step of catalysis. This conclusion is based on the observation

Table 1. The formation of multiple products by monoterpene synthases demonstrated by isotopically sensitive branching

Plant species	Enzyme	Major Products	composition %	Reference
Grand fir (*Abies grandis*)	pinene cyclase	(−)-α-pinene (**19**) (−)-β-pinene (**23**)	35 65	[53]
Lodgepole pine (*Pinus contorta*)	phellandrene cyclase	(−)-α- phellandrene (**21**) (−)-β-phellandrene (**22**)	6 94	[53]
Peppermint (*Mentha* x *piperita*)	limonene cyclase	(−)-4*S*-limonene (**18**) α-pinene (**19**) β-pinene (**23**) mycrene (**24**)	94 2 2 2	[52]
Sage (*Salvia officinalis*)	cyclase I	(+)-α-pinene (**25**) (+)-camphene (**26**)	31 29	[54, 55]
	cyclase II	(−)-α-pinene (**19**) (−)-camphene (**27**) (−) -β-pinene (**23**)	25 31 24	[54, 55]
	cyclase III	(+)-α-pinene (**25**) (+)-β-pinene (**28**)	36 45	[54]

that most cyclases are capable of utilizing the correct antipode of LPP at considerably higher catalytic rates than GPP [38, 41, 44–46, 52, 60–66]. A surprising observation is that some cyclases are also capable of utilizing the incorrect antipode of LPP to form the opposite enantiomeric products from those produced with the natural substrate GPP. In some cases, the enzyme exhibits little catalytic discrimination between the enantiomers of LPP [41, 52, 62, 66], whereas others show a strong preference for the correct enantiomer generated in the natural reaction from GPP [46, 60, 61]. The ability of monoterpene cyclases to utilize either antipode of LPP indicates that formation of the enantiomerically pure end product from the native, achiral substrate GPP is determined by the helical conformation imposed on GPP by binding to the active site; i.e., the cyclase never encounters the incorrect enantiomer of LPP, and only one enantiomeric family of products is ever formed naturally. Utilization of the incorrect antipode of LPP, in addition to the formation of unnatural enantiomeric products, often also results in the formation of either aberrant product ratios or novel products not seen with either GPP or the correct antipode of LPP. This loss of catalytic fidelity in the reaction with the unnatural enantiomer of LPP has been observed with a range of cyclases from angiosperm and gymnosperm species [41, 46, 61, 62, 66].

In addition to sharing a common mechanism for the initial cyclization to the α-terpinyl cation (**13**, Fig. 2), the monoterpene cyclases examined to this point share many common structural features. In general, monoterpene cyclases are operationally soluble, albeit rather hydrophobic, proteins in the range of 40 to 100 kDa [46, 48, 50, 51, 67]. In the few cases where the subunit architecture is

known, the cyclases exist either as a monomer of 50–70 kDa [48, 67] or as an apparent homodimer with subunits of 40–50 kDa [50]. Although monoterpene cyclases from herbaceous angiosperm species have been studied extensively, only recently have the cyclases of conifers (gymnosperms) been examined. Significant differences in the kinetics and active site residues (based on substrate protection against chemical modification reagents) have been found for monoterpene cyclases of angiosperms and gymnosperms. These differences are summarized in Table 2. In general, monoterpene cyclases from gymnosperms exhibit a higher pH optimum and are incapable of utilizing Mg^{2+} as cofactor. Surprisingly, monoterpene cyclases from grand fir (*Abies grandis*) and lodgepole pine also exhibit an absolute requirement for a monovalent cation such as K^+. Angiosperm monoterpene cyclases appear to have one or more catalytically essential histidine and cysteine residues at the active site, whereas this has not been demonstrated for the gymnosperm cyclases examined. In contrast, an active site arginine residue has been implicated in gymnosperm cyclases but not in angiosperm cyclases. The use of polyclonal antibodies has also demonstrated significant structural differences between cyclases. Polyclonal antibodies generated against limonene cyclase from spearmint do not detectably cross-react with any of the cyclases present in either lodgepole pine or grand fir or other angiosperm genera [68]. Similarly, polyclonal antibodies generated against a wound-inducible pinene cyclase in grand fir fail to cross-react with cyclases present in either lodgepole pine, ponderosa pine (*P. ponderosa*), spearmint, peppermint, or sage, although they do cross-react with the other monoterpene cyclases of grand fir [69].

The limonene cyclases catalyze the formation of the corresponding limonene antipodes in a wide variety of plants, including (+)-4*R*-limonene in citrus [64] and (−)-4*S*-limonene (**18**) in *Mentha* species [48]. (−)-4*S*-Limonene synthase has been purified to homogeneity from peppermint and spearmint [48]; the corresponding cDNA has been isolated from spearmint, sequenced and the catalytically active enzyme has been expressed in *E. coli* [75]. The encoded protein contains a putative N-terminal plastidial transit peptide, consistent with

Table 2. Summary of monoterpene cyclase properties from angiosperms and gymnosperms

Property	Angiosperms	Reference	Gymnosperms	Reference
pH optimum	6.0–7.2	[36, 46, 50, 52]	7.5–8.0	[67, 71]
Divalent cation requirement	Mg^{2+}, Mn^{2+}	[36, 46, 50, 52]	Mn^{2+}, Fe^{2+} (Mg^{2+} ineffective)	[67, 71]
Monovalent cation requirement	no	[71]	$K^+ > NH_4^+ \gg Na^+$, Li^+	[71]
Active site residues[a]				
Cys	yes	[46, 47, 50–52, 72–74]	?	[66]
His	yes	[46, 50, 70]	no	[71]
Arg	no	[66]	yes	[66]

[a] Active site residues determined by substrate protection against chemical modification reagents

immunocytochemical localization of the mature enzyme in plastids (Gershenzon and Croteau, unpublished). The precise cleavage site of the transit peptide is not known because the amino terminus of the native limonene cyclase is blocked. Despite the presence of the transit peptide, the recombinant limonene cyclase preprotein is catalytically active, producing precisely the same spectrum of products as the processed native enzyme ($\sim$94% limonene (**18**) with small amounts ($<$ 2%) of α-pinene (**19**), β-pinene (**23**) and myrcene (**24**); see Table 1).

2.3.2 Sesquiterpene Cyclases

Sesquiterpene cyclases catalyze the conversion of FPP (**7**) to over 200 different cyclic skeletons in reactions very similar to those catalyzed by the monoterpene cyclases (reviewed in [76, 77]). However, with 15 carbons and 3 double bonds in the farnesyl substrate, there are considerably more possible reaction routes available compared to GPP as substrate. Few studies have examined the cyclases responsible for the formation of aromatic sesquiterpenes in plants. However, several plant sesquiterpene cyclases involved in the production of defensive compounds (phytoalexins) have been examined. The most extensively studied sesquiterpene cyclases are of fungal origin; these enzymes are involved in the biosynthesis of toxins or antibiotics. The production of these diverse classes of sesquiterpenes involves a relatively limited number of common mechanistic themes and, for this reason, it is instructive to consider the biosynthesis of phytoalexins and fungal toxins as it relates to the origin of aromatic sesquiterpenes.

As with the monoterpene cyclases, the enzymatic cyclization of FPP is initiated by ionization of the diphosphate ester to generate an allylic carbocation. Unlike the case of the geranyl cation with monoterpene cyclases, the farnesyl cation can attack either of two different double bonds. Only a limited number of binding conformations of FPP at the cyclase active site provide the proper orbital alignment necessary for interaction of the double bonds during cyclization (reviewed in [76, 78]). This limits the possible reactions that the farnesyl cation can undergo. The initial cyclization of FPP generates one of four possible carbocations [76, 79]. The formation of the macrocylic *trans*-germacradienyl cation (**29**) and the humulyl cation (**30**) can arise directly from cyclization of the allylic cation generated from FPP (Fig. 3a). However, as with the monoterpene cyclases, the *trans* geometry of the 2,3-double bond of FPP prevents direct cyclization to either the *cis*-germacradienyl cation (**31**) or the bisabolyl cation (**32**) (Fig. 3b). To form these intermediates, the cyclase must first isomerize FPP to the tertiary allylic isomer nerolidyl diphosphate (NPP **33**) by a process similar to the isomerization of GPP to LPP by monoterpene cyclases. The isomerization allows rotation about the newly formed C2–C3 bond of NPP to the cisoid orientation, and reionization of the diphosphate ester then permits cyclization of the resulting cisoid allylic carbocation. As with the monoterpene cyclases, further elaboration of the sesquiterpene cyclase-bound cationic intermediate(s) to the final products may involve additional electrophilic

cyclizations, rearrangements, and hydride shifts before ultimate termination of the reaction by either deprotonation or nucleophile capture.

The *trans*-germacradienyl cation gives rise directly to the widely distributed germacrane family of sesquiterpenes, such as germacrene D (**34**). The germacranes can readily undergo Cope rearrangement to form the elemanes (e.g., δ-elemene (**35**)). A cDNA encoding germacrene C synthase has recently been isolated from tomato (*Lycopersicon esculentum*) and sequenced (Colby et al., unpublished observations). Rearrangement of the *trans*-germacradienyl cation with a concomitant 1,3-hydride shift yields the patchoulane skeleton [80].

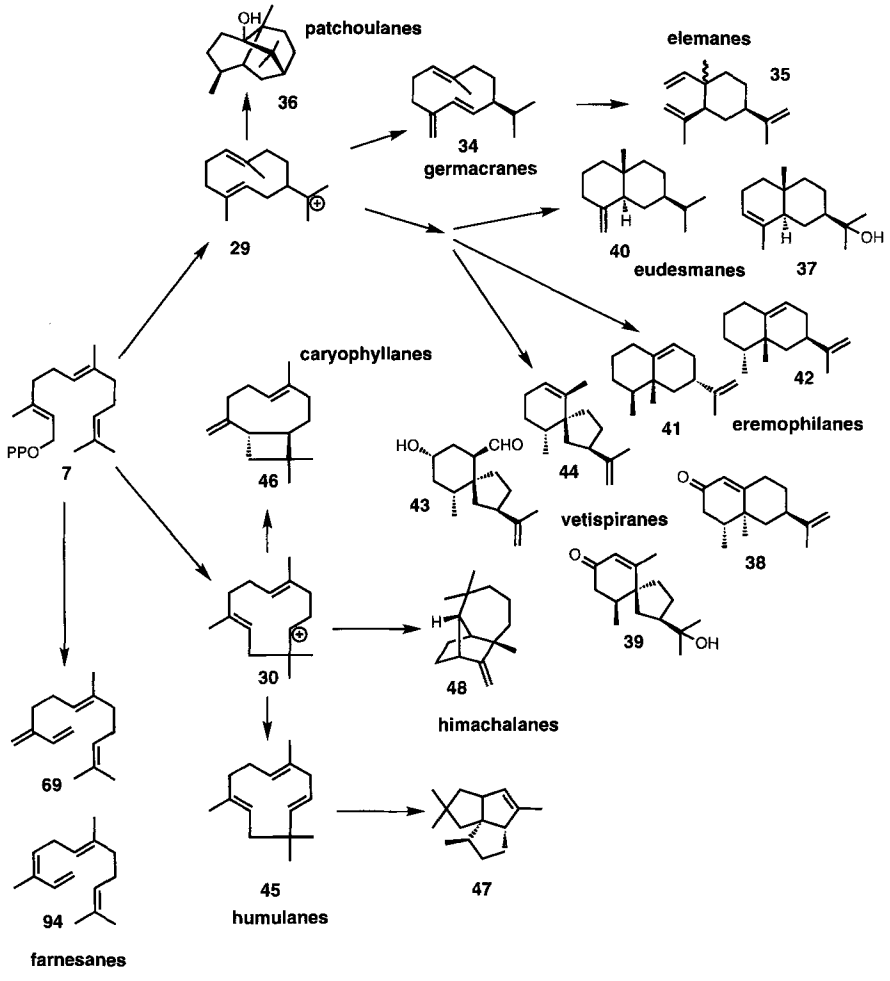

Fig. 3a. Cyclization of FPP by sesquiterpene synthases to produce different sesquiterpene skeletons: **a** direct cyclization of FPP

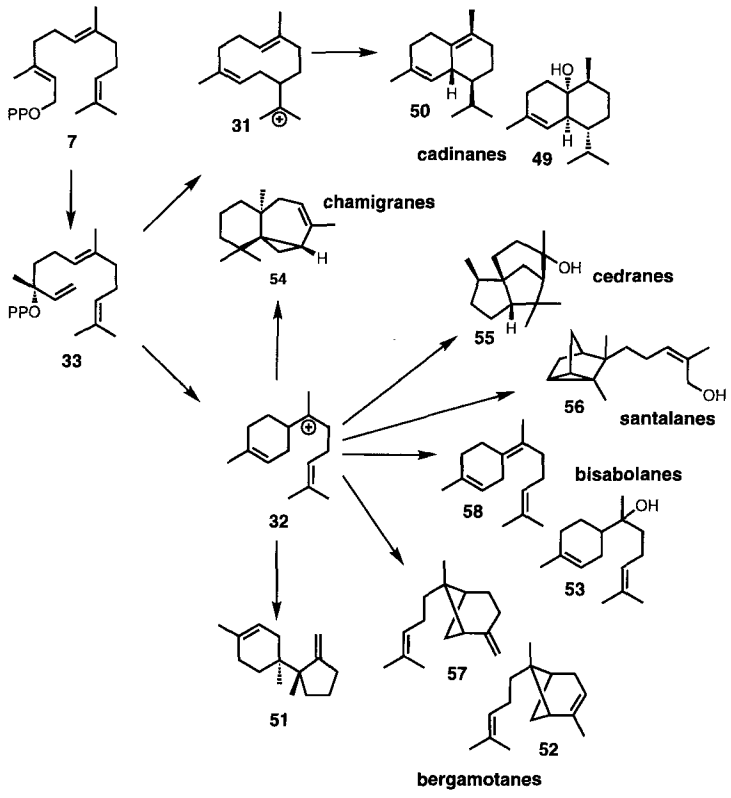

Fig. 3b. cyclization of FPP via an enzyme-bound NPP intermediate

Patchoulol (**36**) synthase has been purified to homogeneity from patchouli (*Pogostemon cablin*) and characterized [57]. Alternate rearrangement of the *trans*-germacradienyl cation leads to the eudesmane, eremophilane, and vetispirane skeletons. Examples of important sesquiterpenes belonging to these families include (+)-α-eudesmol (**37**), (+)-nootkatone (**38**), and β-vetivone (**39**) from eucalyptus, grapefruit, and vetiver, respectively [2]. β-Selinene (**40**) synthase has been partially purified from immature calamondin *Citrofortunella mitis*) fruit and characterized [81]. (+)-Aristolochene (**41**) is found in a variety of fungi while the (−)-enantiomer has been found in the leaf oil of several plants (reviewed in [76]). (+)-Aristolochene synthase from *Penicillium roqueforti* has been purified [82], and the corresponding cDNA overexpressed in *E. coli* [83]. Members of the *Solanaceae* accumulate a wide variety of phytoalexins in response to fungal infection. The formation of the phytoalexin intermediates *epi*-aristolochene (**42**) and lubimin (**43**) (derived from vetispiradiene (**44**)) is induced by fungal infection in tobacco (*Nicotiana tabacum*) [84] and potato (*Solanum tuberosum*) [85], respectively. *epi*-Aristolochene synthase has been purified from induced tobacco suspension cultures [84] and the cDNA has been

cloned and overexpressed in *E. coli* [86]. The formation of vetispirane-derived phytoalexins has been studied in cell-free extracts [85] although no enzyme activities have yet been purified or characterized. A cDNA coding for vetispiradiene synthase has been cloned from *Hyoscyamus muticus* by homology and expressed in *E. coli* [87].

The humulyl cation (**30**) can undergo either deprotonation to humulene (**45**), or an additional ring closure step followed by deprotonation to caryophyllene (**46**). Separate enzymes for the formation of humulene and caryophyllene have been isolated from sage [88] and characterized. Humulene is also believed to be an enzyme-bound intermediate in the formation of the fungal metabolite pentalenene (**47**) [76]. Pentalenene synthase is one of the most extensively studied sesquiterpene cyclases [25, 89–91]; it has been purified and the cDNA cloned and overexpressed at high levels in *E. coli* [58]. Recombinant pentalenene synthase has been crystallized and preliminary X-ray diffraction data have been reported [92]. Although extensive labeling studies have been carried out with the himachalane family of sesquiterpenes (e.g., (+)-longifolene **48**) (reviewed in [93]), none of the responsible enzymes has been characterized.

The cadinane family of sesquiterpenes is widely distributed in nature. Epicubenol synthase has been isolated from *Streptomyces* [79] and shown to catalyze the formation of (+)-epicubenol (**49**) through an initial isomerization of FPP to 3*R*-NPP (**33**), followed by formation of the *cis*-germacradienyl cation (**31**) and subsequent cyclization [94]. (±)-Epicubenol is found in *Juniperus rigida* oil [95] and the (−)-isomer in commercial cubeb oil [96]. δ-Cadinene (**50**) is a component of juniper leaf oil [97] and is also produced by cotton (*Gossypium hirsutum*) as a precursor of phytoalexins formed in response to bacterial pathogens [98]. The cDNA for (+)-δ-cadinene synthase from cotton has recently been cloned and overexpressed in *E. coli* [99].

The most extensively studied sesquiterpene cyclase is trichodiene synthase [100–102]. Trichodiene (**51**) is the parent olefin of the tricothecin family of mycotoxins which are produced by a variety of fungi. Trichodiene synthase has been purified from *Fusarium sprotrichioides* [59] and the gene isolated and overexpressed in *E. coli* [103]. The mechanism of trichodiene synthase is believed to involve the initial formation of enzyme-bound 3*R*-NPP (**33**) followed by cyclization to the bisabolyl cation (**32**) as an intermediate. However, trichodiene synthase exhibits a much lower V_{max} with 3*R*-NPP than with FPP; this has been suggested to result from a conformational change in the enzyme after binding of FPP [104]. Thus, binding of NPP to the enzyme may require a slow conformational change to the form capable of utilizing NPP.

The bisabolyl cation (**32**) also serves as the common intermediate for the formation of the bergamotanes, bisabolanes, santalanes, cedranes, and chamigranes. *trans*-α-Bergamotene (**52**) and α-bisabolol (**53**) are important aroma constituents of bergamot oil [2] and chamomile [105], respectively. Thujopsene (**54**) and (+)-cedrol (**55**), both major constituents of cedarwood oil [2], are typical examples of chamigrane and cedrane sesquiterpenes. α-Santalol (**56**) from sandalwood oil is a typical santalane sesquiterpene [2]. The formation of

trans-β-bergamotene (**57**) and γ-bisabolene (**58**) from FPP has been demonstrated in cell-free extracts of the fungus *Pseudeurotium ovalis* [106, 107] and of tissue cultures of *Andrographis paniculata* [108, 109], respectively. Other than the work with trichodiene synthase, little detailed enzymology has been carried out with sesquiterpene cyclases which utilize the bisabolyl cation intermediate.

2.3.3 Diterpene Cyclases

Because of their higher molecular weight and lower volatility, diterpenes are not commonly considered as aroma chemicals. A notable exception is sclareol (**59**) which is found in clary sage (*Salvia sclarea*) [110]. The aroma profile of clary sage is complex, with ambergris, tobacco-like characteristics. Sclareol isolated from clary sage is used as the starting material for the commercial preparation of the ambergris component Ambrox (**60**) [2]. The enzymatic formation of sclareol from GGPP (**8**) shares similarities with the formation of the labdanoid diterpenes of tobacco trichome exudates and of the abietane diterpene resin acids produced by conifers.

Unlike the monoterpene and sesquiterpene cyclases examined so far, conversion of GGPP to the labdanoid skeleton involves protonation-initiated cyclization from the olefinic terminus of GGPP without the direct involvement (ionization) of the diphosphate ester moiety (Fig. 4). The initial cyclic product formed is (+)-copalyl diphosphate (CPP **61**) which is subsequently converted by ionization-dependent cyclization to either the tobacco exudate components *cis*-abienol (**62**), labdenediol (**63**), or sclareol (**59**), or the resin acid precursor abietadiene (**64**). *cis*-Abienol [111], labdenediol [112], and sclareol [112] cyclase activities have all been localized to tobacco trichomes and partially characterized in cell-free extracts. Abietadiene cyclase has been partially purified from grand fir and lodgepole pine stem extracts and characterized [113], and the corresponding cDNA has recently been cloned and expressed in *E. coli* [114]. The enzymatic formation of these diterpenes shares the common bound intermediate (+)-CPP, and current evidence suggests that a single enzyme is responsible for both the formation of (+)-CPP and the subsequent cyclization of this intermediate to the final product(s). A unified scheme for the formation of several families of diterpenes from (+)-CPP has been proposed [113]. Diterpene cyclization via (+)-CPP contrasts with the formation of the gibberellin family of plant hormones. In this case, initial cyclization of GGPP yields (−)-CPP (**65**) which is subsequently converted to *ent*-kaurene (**66**) by the enzyme *ent*-kaurene synthase. The reaction is carried out by two distinct enzymes constituting *ent*-kaurene synthase A and B activities [115]. The A activity catalyzes the cyclization of GGPP to (−)-CPP; the *ent*-kaurene synthase A gene has recently been isolated from both Arabidopsis [116] and maize [117]. The B activity catalyzes the conversion of (−)-CPP to *ent*-kaurene. *ent*-Kaurene synthase B activity has recently been purified from pumpkin (*Cucurbita maxima*) and characterized [118].

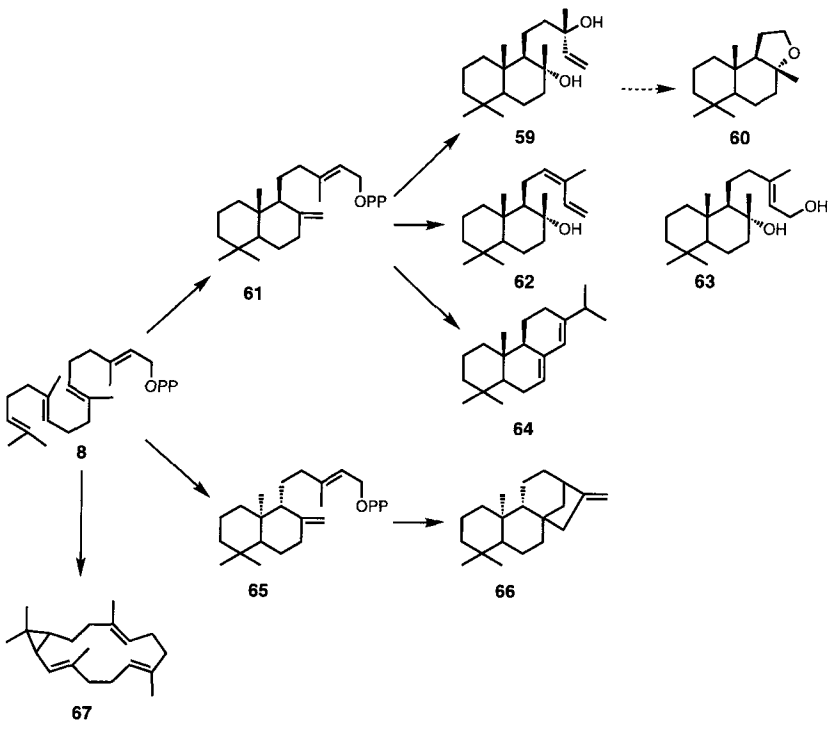

Fig. 4. Cyclization of GGPP by diterpene synthases

Although not involved in the formation of aromatic diterpenes, casbene synthase is another diterpene cyclase which has been extensively studied. Casbene (**67**) is a phytoalexin produced in castor bean (*Ricinus communis*) in response to fungal infection [119]. The cDNA for casbene synthase has been isolated [120] and transcriptional regulation of the gene in response to elicitation has been reported [121]. The reaction catalyzed by casbene synthase is similar to that catalyzed by monoterpene and sesquiterpene cyclases in that it involves the initial ionization of the prenyl diphosphate substrate to an allylic carbocation followed by cyclization and deprotonation to the olefin.

2.3.4 Acyclic Isoprenoid Synthases

Few examples of synthases that catalyze the formation of acyclic isoprenoids have been studied. *S*-Linalool synthase has been purified to homogeneity from the stigma of freshly opened flowers of *Clarkia breweri* [122]. This enzyme produces exclusively *S*-linalool (**68**) from GPP and is an apparent monomer of 76 kDa. The properties of the purified enzyme (pH optimum, divalent cation requirement, K_m for GPP) are similar to those of many of the monoterpene

cyclases. However, unlike most monoterpene cyclases, only a slow rate of conversion to product was observed with 3*S*-LPP as substrate, and the enzyme did not use the 3*R*-antipode. This suggests that formation of linalool involves addition of water directly to the initially generated allylic carbocation without the preliminary formation of enzyme-bound LPP (Fig. 2). This reaction is considerably simpler than that catalyzed by the monoterpene cyclases and may account for the observation that the turnover number of linalool synthase ($31.6\ sec^{-1}$) is much higher than those of the cyclases.

The sesquiterpene olefin *trans*-β-farnesene (**69**) is a common constituent of conifer resin (see references in [123]), is found in hop (*Humulus lupulus*) oil [124], and is also known as an aphid alarm pheromone [10]. *trans*-β-Farnesene synthase has been purified to apparent homogeneity from maritime pine (*Pinus pinaster* Ait.) needles [123]. This soluble enzyme is possibly a homodimer with 45-kDa subunits and its properties (pH optimum, divalent cation requirement, K_m for FPP, sensitivity to thiol-directed reagents) are similar to those of sesquiterpene and monoterpene cyclases.

2.3.5 Structural Similarities among Isoprenoid Synthases

With the availability of cDNA sequences for a number of monoterpene, sesquiterpene, and diterpene cyclases, as well as those for several prenyl transferases, a number of common protein structural features have become apparent. Common to most cyclases [58, 75, 83, 86, 87, 114, 117, 120, 125, 126] and prenyl transferases [127, 128] examined so far is the presence of at least one aspartic acid rich region with a consensus sequence of (I, L, V) XDDXX(D, E). This aspartate rich sequence is believed to be involved in binding of the Mg^{2+} (or Mn^{2+}) salt of the diphosphate substrate [129] and may assist in the initial ionization step of the reaction [130]. Consistent with this supposition is the observation that cyclases, which bind a single diphosphate ester substrate, contain only a single aspartic acid rich region, whereas prenyltransferases, which bind two co-substrates (IPP and an allylic diphosphate), contain two such aspartate rich regions referred to as domains I and II. Site directed mutagenesis of aspartic acid residues in either domain I or II of FPP synthase results in drastically reduced enzymatic activity [32, 34, 130]. The role of these aspartic acid residues in binding the divalent metal ion-complexed substrate is also supported by the X-ray crystal structure of avian FPP synthase [35]. As yet, no directed mutagenesis studies of these putative active site aspartic acid residues have been carried out with any isoprenoid synthase enzyme.

A significant level of homology exists among the plant monoterpene, sesquiterpene, and diterpene synthases. Typically, these enzymes exhibit 30–40% identity and 50–60% similarity at the amino acid level (reviewed in [114]). The organization of introns and exons of the genomic DNA for *epi*-aristolochene synthase [86], vetispiradiene synthase [87], and casbene synthase [120] is also very similar, suggesting that the exons encode functional domains of the enzyme

which have been highly conserved throughout evolution [120]. Other than the highly conserved aspartic acid rich consensus sequence, the plant isoprenoid synthases exhibit very little homology with the fungal sesquiterpene synthases.

Based on sensitivity to amino acid modification reagents, the presence of one or more histidine and cysteine residues at cyclase active sites has also been proposed (see Sect. 2.3.1 and 2.3.2). Supporting this proposal is the observation of three conserved histidine residues and a conserved cysteine residue in the deduced amino acid sequences for 4*S*-limonene synthase [75, 126], *epi*-aristolochene synthase [86], vetispiradiene synthase [87], casbene synthase [120], and abietadiene synthase [114]. When the sequences for the two *ent*-kaurene synthase A activities are taken into account [116, 117], only one of these residues, a histidine, is conserved in all synthases examined to this point. An active site cysteine residue in trichodiene synthase has also been demonstrated by both chemical modification and site directed mutagenesis [131]. The involvement of an active site arginine residue has also been suggested, based on the sensitivity of monoterpene synthases to the arginine-directed reagent phenylglyoxal [66]. This suggestion is also supported by the presence of two strictly conserved and five highly conserved arginine residues in the deduced amino acid sequences of limonene synthase [75, 126] *epi*-aristolochene synthase [86], *ent*-kaurene synthase [116, 117], and casbene synthase [120].

2.4 Secondary Transformations

The isoprenoid synthases previously discussed construct the basic carbon skeletons of the different families of isoprenoids. Once formed, these initial products are often further modified to generate the vast assortment of isoprenoids found in nature. These secondary enzymatic reactions include hydroxylations by P450 cytochromes, redox transformations, isomerizations, and conjugations. Although these secondary transformations are very common in both the sesquiterpene and diterpene series, emphasis will be placed on the secondary transformations of monoterpenes. Because of the great range of modifications possible, only selected, illustrative examples from the monoterpene literature will be presented here.

2.4.1 Mentha

The biosynthesis of the *p*-menthane monoterpenes in *Mentha* (mint) species is well characterized. The essential oils of peppermint and spearmint varieties are distinguished by the position of oxygenation of the *p*-menthane ring, and extensive classical breeding experiments and both in vivo and in vitro studies have firmly established the extended pathways for the formation of (−)-carvone (**70**), (−)-menthone (**71**), and (−)-menthol (**72**) (Fig. 5, reviewed in [132]). Hydroxylation at the C-3 position of the parent olefin limonene leads to the

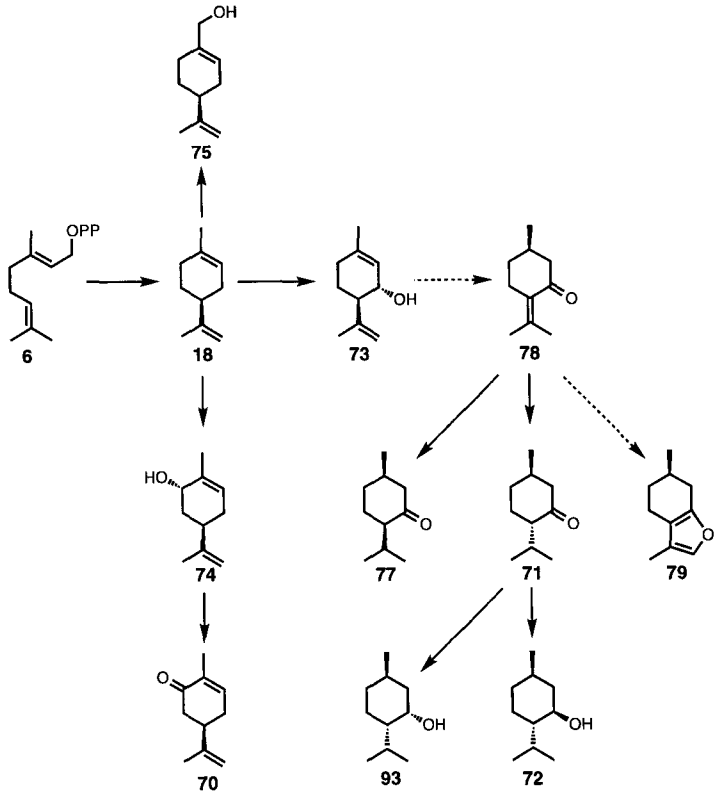

Fig. 5. Secondary transformations of *p*-methane monoterpenes in spearmint, peppermint and perilla. Dashed arrows indicate multiple enzymatic steps

characteristic family of monoterpenes found in peppermint (*M.* x *piperita*) and related species (*M. aquatica*, *M. arvensis*, *M. pulegium*), whereas the monoterpenes of spearmint species (*M. spicata*, *M.* x *gracilis*, *M. crispa*) are characterized by oxygenation at the C6 position of limonene. These reactions are catalyzed by stereospecific cytochrome P-450 oxygenases which hydroxylate (−)-4*S*-limonene at either the C3 position in peppermint varieties to produce (−)-*trans*-isopiperitenol (**73**) or at the C6 position in spearmint varieties to produce (−)-*trans*-carveol (**74**). The two cytochrome P-450s which catalyze these mutually exclusive positional hydroxylations can also be distinguished from each other by differential sensitivity to azole-type inhibitors [133]. Regiospecific, allylic hydroxylation of the parent olefin formed by monoterpene cyclases is a common theme in the biogenesis of many essential oils. Other examples include hydroxylation of (−)-α-pinene (**19**) and (−)-β-pinene (**23**) in hyssop (*Hyssopus officinalis*) [134], hydroxylation of (−)-limonene (**18**) to perillyl alcohol (**75**) in perilla (*Perilla frutescens*) [133], and hydroxylation of sabinene (**76**) to produce the oxygenated thujane monoterpenes (see Sect. 2.4.2).

(−)-*trans*-Carveol (**74**) is subsequently oxidized to (−)-carvone (**70**), the major monoterpene found in spearmint. A similar, but antipodal, pathway has been proposed for the formation of (+)-carvone in caraway seed (*Carum carvi*) [135]. In contrast, the metabolic transformations of (−)-*trans*-isopiperitenol (**73**) in peppermint are much more extensive. A series of redox reactions involving both double bonds and the oxygenated carbon, and a double bond migration, ultimately results in the formation of both (−)-menthone (**71**) and (+)-isomenthone (**77**), and all four stereoisomers of menthol (reviewed in [132]). The relative proportions of the two reductases producing menthone and isomenthone from (+)-pulegone (**78**) varies in commercial mint species from 3:1 to 10:1 [136, 137]. Pulegone is also located at the branch point for the formation of the commercially undesirable oil component (+)-menthofuran (**79**). The biosynthesis of menthofuran from pulegone is believed to involve a cytochrome P-450 catalyzed hydroxylation followed by cyclization and dehydration to provide the furan ring. Although conversion of pulegone to menthofuran by a cytochrome P-450 has been shown in mammalian liver microsomes [138, 139] this activity has not yet been demonstrated in peppermint or related species.

Further insight into the close relationship between the C3 and C6 oxygenated *p*-menthane monterpenes was obtained by examining the essential oil produced by a mutant of Scotch spearmint (referred to as mutant 643) [137]. Instead of producing carvone typical of the wild type spearmint, this mutant produced primarily C3 oxygenated *p*-menthanes, including significant amounts of menthone. Cell-free enzyme assays of both the wild type Scotch spearmint and the mutant 643 demonstrated that both plants contain the same complement of enzymes necessary to produce menthone from *trans*-isopiperitenol, with the only major difference being the specificity of the limonene hydroxylase activity. As expected, the wild type the wild type spearmint contained only the C6 hydroxylase whereas in mutant 643 this activity was entirely replaced by a C3 hydroxylase. The new C3 hydroxylase resembled that found in peppermint in differential sensitivity to azole-type inhibitors. Although spearmint clearly contains most of the same terpene-metabolic machinery as peppermint, these enzymes are normally catalytically silent because carvone is a poor substrate for these subsequent reaction steps.

2.4.2 Salvia

Common sage (*S. officinalis*) has proven to be a particularly rich source of monoterpene cyclases. At least six different major monoterpene cyclases have been isolated from sage oil glands and characterized: pinene synthase I produces mainly (+)-α-pinene (**25**) and (+)-camphene (**26**); pinene synthase II produces mainly (−)-α-pinene (**19**), (−)-β-pinene (**23**), and (−)-camphene (**27**); pinene synthase III produces (+)-α-pinene (**25**) and (+)-β-pinene (**28**) [54]; 1,8-cineole (**15**) synthase [46]; (+)-sabinene (**76**) synthase [140]; and (+)-BPP (**9**) synthase [63]. (−)-BPP (**10**) synthase has been isolated from tansy (*Tanacetum vulgare*)

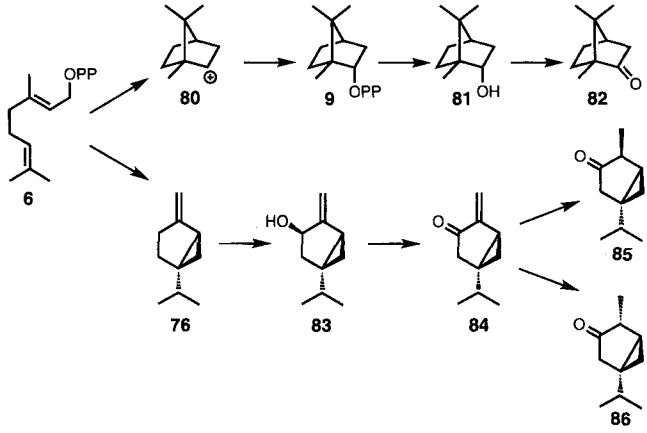

Fig. 6. Secondary transformations of camphane and thujane monoterpenes in sage and tansy

[63]. 1,8-Cineole synthase and BPP synthase illustrate two alternatives to cytochrome P-450 catalyzed hydroxylation for the formation of oxygenated monoterpenes. In the case of 1,8-cineole (**15**) synthase, the cyclic ether oxygen atom is derived by water quenching of the enzyme bound α-terpinyl cation (**13**) (Fig. 2) [46]. BPP synthase catalyzes a second cyclization of the α-terpinyl cation intermediate to produce the bicyclic bornyl (camphyl) cation (**80**), followed by capture of the diphosphate anion to yield the end product [38]. Specific phosphohydrolases cleave BPP (**9**) to borneol (**81**) [73] which is subsequently oxidized to camphor (**82**) [141] by a specific dehydrogenase (Fig. 6).

The thujanes are a widespread family of bicyclic monoterpenes containing an unusual cyclopropane ring (Fig. 6) [142]. (+)-Sabinene (**76**) synthase has been isolated from sage [140]. As with the *p*-menthanes described earlier, allylic hydroxylation of the parent olefin by a microsomal cytochrome P-450 follows to provide (+)-*trans*-sabinol (**83**). A series of redox reactions subsequently yield (+)-sabinone (**84**) and either (+)-3-thujone (**85**) in tansy or (−)-3-isothujone (**86**) in sage [142, 143].

2.4.3 Nepeta

Iridoids are a widely distributed family of monoterpenes that bear the characteristic methylcyclopentanoid-pyran ring structure. Interest in the biosynthesis of iridoids originated because of the role of these monoterpenes in the formation of pharmaceutically important indole alkaloids (reviewed in [144]). Although not known primarily for their aromatic properties, considerable interest in the biosynthesis of the nepetalactone family of iridoids has developed because of the role of these compounds as aphid sex attractants [145]. Nepetalactones are

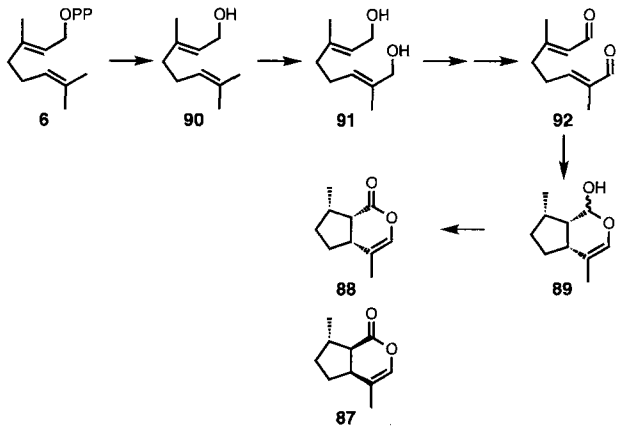

Fig. 7. Proposed biosynthesis of iridoid monoterpenes in catmints

found exclusively in the essential oils of catmints (*Nepeta* spp.) [146, 147], and (4a*S*, 7*S*, 7a*R*)-nepetalactone (**87**) from *Nepeta cataria* is well known as a feline attractant [148, 149]. Biosynthesis of the steroisomeric (4a*R*, 7*S*, 7a*S*)-nepetalactone (**88**) in *N. racemosa* has recently received attention because it possesses the same configuration as the nepetalactol isomer (**89**) employed as a sex pheromone by the damson hop aphid (*Phorodon humuli*) [150].

Unlike the biosynthesis of most cyclic monoterpenes, the formation of iridoids involves the oxidation of geraniol to a highly reactive dialdehyde which is subsequently cyclized to the iridoid skeletons (Fig. 7). After hydrolysis of GPP to geraniol (**90**), a cytochrome P-450 catalyzed hydroxylation yields 10-hydroxygeraniol (**91**). This enzyme has been demonstrated in *N. mussinii* [151] as well as in *Catharanthus roseus* which produces an iridoid glycoside used as an intermediate in indole alkaloid biosynthesis [152, 153]. Efforts to clone this cytochrome P-450 are currently under way [154, 155]. The resulting 10-hydroxygeraniol is then oxidized to the dialdehyde 10-oxogeranial (**92**). An oxidoreductase from *N. racemosa* capable of specifically oxidizing either of the alcohol groups of 10-hydroxygeraniol to the corresponding aldehyde has recently been purified to homogeneity [156]. Cyclization of this reactive dialdehyde affords the iridane skeleton. Although this novel cyclization has been demonstrated in cell-free extracts from tissue cultures of the monoterpene indole alkaloid producing plant *Rauwolfia serpentina* [157], no further work on this enzyme has been reported.

2.4.4 Catabolism

Catabolism normally refers to the degradative loss of an accumulated product through conversion to basic metabolic intermediates which can be recycled by

the plant. Common strategies utilized in catabolism include conjugations, hydroxylations, oxidations, and ring cleavage reactions designed to both increase the water-solubility of the compound and to allow the compound to be broken down into metabolites that can provide both energy and carbon. Non-volatile glycosides of aroma compounds, including many monoterpenes, are widespread in many plant tissues (reviewed in [158]) and are commonly considered as potential aroma precursors [159, 160]; in the plant they likely represent storage or transport derivatives. A consideration of the role of glycosides as precursors of aroma compounds is outside the scope of this paper and they will only be described here in the context of terpene catabolism.

Although the loss of accumulated essential oils during development and senescence has long been recognized (reviewed in [36, 161]) relatively few experiments have examined catabolism of monoterpenes at the biochemical level. Two systems which have been studied in some detail are the loss of essential oil observed in peppermint leaves after floral initiation [162] and a similar loss observed in sage leaves [163]. After floral initiation in peppermint, the composition of the oil changes, reflecting a conversion of menthone to menthol. A significant amount of the menthone is also believed to be converted to neomenthol (**93**), which is subsequently glycosylated to neomenthyl-β-D-glucoside. This glycoside is then transported to the rhizomes where it undergoes oxidative degradation, presumably to provide acetyl-CoA and reducing equivalents for rhizome metabolism (reviewed in [161, 164]). A similar process has recently been shown to occur in sage [165]. In this case flowering results in the loss of approximately 50% of the camphor in leaves [163]. Using both tissue cultures and leaf disks as experimental systems, camphor was shown to undergo hydroxylation to 6-hydroxycamphor followed by a series of oxidative ring cleavage reactions similar to those involved in the microbial degradation of camphor [166]. The cytochrome P-450 responsible for the initial hydroxylation of camphor has been isolated from tissue cultures of sage and characterized [167]. Glycosylation of 6-hydroxycamphor is also observed in both sage leaf disks and tissue cultures [165].

2.5 Regulation

The organization of isoprenoid biosynthesis in plants is complex, involving parallel pathways for the synthesis of IPP in different locales, multiple genes for the expression of different forms of HMGR, and multiple branch points for the partitioning of IPP by prenyltransferases into different families of isoprenoids. The partitioning of intermediates between the formation of isoprenoids essential for cell growth and development (such as sterols, carotenoids, and isoprenoid hormones) and the formation of secondary products such as aromatic oils and phytoalexins represents another crucial bifurcation in isoprenoid metabolism. The composition of the essential oils is determined by the efficiency of competition between multiple monoterpene or sesquiterpene synthases for their

respective precursors and by the relative rates of multiple routes for the secondary transformation of pathway intermediates.

Numerous analytical studies have documented the developmental regulation of essential oil formation at the whole plant level (reviewed in [5, 168]). For example, environmental factors such as photoperiod can affect the formation of specific monoterpenes and sesquiterpenes (see, e.g., [169, 170]). However, little is known about the regulation of essential oil biosynthesis at the biochemical level. HMGR has been suggested to be the primary enzyme that regulates the formation of IPP (reviewed in [11, 15, 171]), although no studies to date have addressed the role of HMGR in regulating essential oil biosynthesis. High levels of glandular trichome-specific expression of the histochemical marker β-glucuronidase fused to an HMGR promoter isolated from *Camptotheca acuminata* and expressed in tobacco has been observed [172] suggesting that specific forms of HMGR may be expressed in these tissues. IPP is a central branch point metabolite between the different families of isoprenoids (see Fig. 1). Several studies have begun to address the role that partitioning of IPP and other prenyl diphosphates plays in regulating isoprenoid biosynthesis. An increase in the levels of chloroplast IPP isomerase during light-stimulated carotenoid biosynthesis in maize has been observed [173]. Carotenoid biosynthesis in ripening bell pepper (*Capsicum annuum*) has also been shown to involve an increase in the levels of GGPP synthase [174]. Competition between monoterpene and sesquiterpene biosynthesis for a common pool of IPP has recently been demonstrated in peppermint oil glands [175] (see also Sect. 3.2). In a somewhat similar fashion, competition for a common pool of GGPP between diterpene and tetraterpene biosynthesis has been suggested by constitutive expression of a phytoene synthase gene in tomato [176]. Collectively, these studies indicate that the regulation of partitioning of either IPP or other prenyl diphosphates between different families of isoprenoids may play a central role in the regulation of isoprenoid biosynthesis.

Production of phytoalexins in tissue cultures in response to fungal elicitation can provide useful insight into the regulation of isoprenoid biosynthesis. Elicitation typically results in the inhibition of sterol biosynthesis and induction of either sesquiterpene [177, 178] or triterpene [179] phytoalexin biosynthesis. This readjustment of FPP partitioning from sterol to phytoalexin formation requires coordinated changes in several enzyme activities. Transient increases in HMGR activity, an increase in the levels of phytoalexin biosynthetic enzymes, and a decrease in sterol biosynthetic capacity all act in concert to redirect FPP metabolism [177–180].

3 Localization of Isoprenoid Biosynthesis

Several levels of organization exist for the compartmentation of isoprenoid biosynthesis and accumulation in plants. Although isoprenoids such as sterols

and carotenoids are found in all types of plant cells, this is not the case with essential oils which are normally synthesized and either stored in, or emitted from, specialized anatomical structures such as glandular trichomes. Complex organization of isoprenoid biosynthesis at the subcellular level also exists and represents a significant challenge to the understanding of how the isoprenoid biosynthetic machinery is integrated and coordinated to provide a diverse array of metabolites including the essential oils. The following sections provide brief coverage of the compartmentation of isoprenoid biosynthesis at both the tissue, cellular, and subcellular levels.

3.1 Tissue Localization

A wide variety of specialized anatomical structures are utilized by plants for the synthesis, and either storage or emission of volatile isoprenoids. These structures include the glandular epidermis of flowers [181, 182], resin blisters and ducts, glandular trichomes, secretory cavities, and secretory idioblasts [182, 183]. Variations in the distribution of structures such as glandular trichomes within a tissue, with attendant variations in the composition and amount of the stored essential oil, are common (reviewed in [183]). Flowers emit a wide variety of fragrant volatiles including fatty acid derivatives, phenylpropanoids, and isoprenoids [7, 184] which are believed to serve as both long- and short-distance attractants for animal pollinators [181]. Many isoprenoids accumulated by the plant in storage structures such as glandular trichomes and resin blisters are believed to serve defensive purposes against herbivores [183]. Storage in specialized structures which segregate the oils from the rest of the plant tissue may also serve to protect the plant from cytotoxic isoprenoids.

Glandular trichomes are perhaps the best studied tissue involved in the biosynthesis of aromatic essential oils. The localization of essential oil formation in such specialized tissues, which often constitute only a small fraction of the plant by mass, has hampered examination of the regulation and enzymology of isoprenoid biosynthesis. Thus, in whole plant extracts, it is often difficult to detect the activity of monoterpene biosynthetic enzymes due to the presence of large amounts of non-specific phosphohydrolases (which degrade substrates such as GPP) and other competing enzymes derived from non-glandular tissue. An early improvement over the use of whole-leaf extracts as a source of monoterpene synthases was the development of methods for preparing surface cell extracts from the leaves of essential oil-producing plants [185]. Subsequently, this method was improved to allow the isolation of intact secretory cells derived from the glandular trichomes of a number of plants [186]. The utilization of isolated secretory cells as a starting material has been instrumental in the purification of several monoterpene biosynthetic enzymes, including limonene synthase from peppermint [48], 1,8-cineole synthase from sage [46], limonene 6-hydroxylase from spearmint [187], and an NADPH-cytochrome P-450 reductase from spearmint [188]. Cell-free extracts prepared from isolated

glandular trichomes of tobacco have also been used as an experimental system to characterize the enzymatic synthesis of the labdane diterpenes *cis*-abienol [111], labdenediol, and sclareol [112].

Intact secretory cells from glandular trichomes of peppermint have also been used to examine the biosynthesis of monoterpenes from basic precursors [189]. This work established that the secretory cells of peppermint glandular trichomes, which are non-photosynthetic, contain all of the enzymatic machinery necessary to produce monoterpenes from an imported carbon source such as sucrose. Similar results have been obtained with photosynthetic glandular trichome cells isolated from tobacco leaves, which were shown to be capable of producing diterpenes from either acetate or carbonate [190, 191]. Several other methods have been reported for the isolation of intact secretory cells from glandular trichomes of sagebrush (*Artemisia tridentata*) [192], rose (*Rosa rugosa*) [193], geranium (*Pelargonium*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), squash (*Cucurbita pepo*), and velvetleaf (*Abutilon theophrasti*) [194], although the use of such cells for examining isoprenoid biosynthesis has been limited. All of these methods rely on abrading the leaf tissue under conditions in which the glandular trichomes can be selectively sheared, followed by separation of the isolated glandular heads from the rest of the leaf tissue. Insights gained from the biochemistry of essential oils in isolated secretory cells can be extended to isoprenoid formation in cells which are not so readily isolated, such as those of the secretory cavities of *Citrus* species and those of resin ducts and blisters in conifers.

Isoprenoids are among the most common volatiles emitted by flowers [184]. Emission of volatiles normally occurs during anthesis, with little aroma emission by flower buds. The most common isoprenoids found by floral head space analysis includes the cyclic monoterpenes limonene (**18**), 1,8-cineole (**15**), α-pinene (**19**), and β-pinene (**23**), and the acyclic monoterpenes myrcene (**24**), ocimene (**94**) and linalool (**68**), although sesquiterpenes such as caryophyllene (**46**) and α-farnesene (**95**) are also commonly encountered [184]. Different compositional patterns of isoprenoids are often emitted from different parts of the flower although few detailed studies of this phenomenon have been reported (reviewed in [181]). Diurnal rhythms in the emission of volatiles by flowers are also well known and are believed to play integral roles in the attraction of day or night pollinators [181]. The basis of this rhythmic emission of volatiles is not known, although the presence of glycosides of monoterpenes [195, 196] and phenylpropanoids [197] in flower tissues has led to the suggestion that release from non-volatile glycosidic pools is involved [196]. Emission of linalool (**68**) from *Clarkia* (Onagraceae) flowers has recently been shown to occur primarily from the petals and to a lesser extent from the pistil and stamens [198]. In contrast, two linalool oxides are emitted almost exclusively by the pistil. Linalool synthase was found in each part of the flower that actively emits either linalool or the linalool oxides. Recently linalool synthase from *Clarkia breweri* has been purified to homogeneity [122] (see Sect. 2.3.4).

3.2 Subcellular Localization

The wide variety of isoprenoids formed by plants, from primary metabolites such as sterols, carotenoids, and isoprenoid-derived hormones, to the natural products (secondary metabolites) such as monoterpenes and sesquiterpenes, has led to the evolution of parallel pathways for isoprenoid biosynthesis at several subcellular sites. This compartmentation can be conceptually divided into the pathways responsible for the formation of IPP and the pathways responsible for the transformation, and thus partitioning, of IPP into the different families of isoprenoids.

The subcellular compartmentation of the mevalonic acid pathway responsible for the formation of IPP in plants (Sect. 2.1) has been the subject of some disagreement [11, 199–201] especially regarding the subcellular localization of HMGR. As indicated earlier, HMGR is encoded by small gene families in plants, with some plants (e.g., *A. thaliana*) containing only 2 genes for HMGR and others (e.g., potato) containing 12 or more [15]. The N-terminal region of the different encoded HMGR proteins (approximately 200 amino acid residues) is highly divergent and does not resemble either a typical endoplasmic reticulum anchoring sequence or a plastidial or mitochondrial targeting peptide sequence [171]. However, the in vitro transcription and translation of both forms of *Arabidopsis* HMGR with subsequent insertion of the translated protein into mammalian microsomes [18, 202] has been used to argue for an exclusively microsomal (endoplasmic reticulum) localization for HMGR. Contradicting these results are studies showing that distinct forms of HMGR reside in plastids and mitochondria, in addition to microsomal fractions (reviewed in [203]). A series of experiments examining the formation of isoprenoids throughout chloroplast development in barley leaves has demonstrated that, early in development, chloroplast isoprenoids are formed from plastid-derived IPP, arguing for the presence of a plastidial mevalonic acid pathway [204, 205]. Purified, immature chloroplasts from barley leaves can synthesize isoprenoids from either pyruvate or acetate, and these organelles contain detectable levels of both mevalonate kinase and mevalonate phosphate kinase [206]. Only one study to date has specifically examined the compartmentation of IPP formation in an essential oil plant. Using secretory cells isolated from glandular trichomes of peppermint as an experimental system, this work demonstrated that the IPP utilized for both monoterpene and sesquiterpene biosynthesis is formed exclusively in the non-photosynthetic plastids (leucoplasts) [175]. The picture that emerges from these studies is that the compartmentation of IPP formation is complex, with parallel pathways existing in the cytoplasm, the plastid and possibly the mitochondria. The relative activities of these parallel pathways can change during development and must play an integral role in partitioning of IPP between the different families of isoprenoids.

In contrast to the situation with the compartmentation of IPP formation, the subcellular compartmentation of IPP utilization is quite well established (reviewed in [5]). FPP synthase, and the subsequent enzymatic steps involved in

sesquiterpene and triterpene biosynthesis from this precursor, are localized in the cytoplasm and endoplasmic reticulum. The formation of monoterpenes and diterpenes from IPP appears to be restricted to the plastid. GPP synthase [207] and GGPP synthase [208] have both been localized to this organelle. A cDNA for GGPP synthase has been isolated and encodes a typical N-terminal plastid targeting sequence [174, 209]. The presence of N-terminal plastid targeting sequences in the preprotein has also been reported for limonene synthase [75], as well as for the diterpene cyclases abietadiene synthase [114], *ent*-kaurene synthase A (involved in gibberellin biosynthesis) [116], and casbene synthase [120]. A cDNA clone for IPP isomerase isolated from the petals and stigma of *Clarkia breweri* flowers has recently been isolated and shown to encode a putative N-terminal plastid targeting sequence [210]. *epi*-Aristolochene synthase and vetispiradiene synthase, the only higher plant sesquiterpene cyclase genes cloned at this time, lack plastidial targeting sequences, consistent with the localization of the corresponding cyclases in the cytoplasm [86, 87].

The subcellular localization of IPP formation and utilization has important implications for the partitioning of IPP between the different families of isoprenoids. Recent studies with isolated secretory cells of peppermint have demonstrated the presence of high levels of the biosynthetic enzymes for both monoterpene and sesquiterpene formation from IPP [175]. However, this potentially high rate of sesquiterpene formation is not reflected in either essential oil composition or the rate of incorporation of basic precursors such as pyruvic acid. This discrepancy results from the formation of IPP exclusively in the leucoplasts of these isolated secretory cells. Thus, the cytoplasmic sesquiterpene pathway competes poorly for the pool of plastidic IPP relative to the plastidic monoterpene pathway. The presence of leucoplasts with well defined morphology has been closely correlated with the formation of monoterpenes in glandular trichomes, resin ducts, secretory cavities, and idioblasts [211]. Based on this observation, it has been suggested that the formation of IPP exclusively in the leucoplasts may be a common feature of these tissues and may play a critical role in the relative ratio of monoterpenes and sesquiterpenes produced [175].

4 Approaches to the Bioengineering of Isoprenoid Biosynthesis

The field of isoprenoid biosynthesis has advanced to the point where several approaches can now be considered for the rational manipulation of essential oil formation. These include the mutagenesis of specific isoprenoid synthases to alter product outcome, the manipulation of a biosynthetic pathway by either altering the activity of an existing enzyme or by introducing novel enzymes, and using genetic approaches to manipulate the formation of the specialized anatomical structures (such as glandular trichomes) involved in the synthesis and storage of

essential oils. An appreciation of the control mechanisms for regulating the flux of intermediates through the pathway, the partitioning of intermediates between branch points, and the accumulation of specific products of the pathway is essential for the rational targeting of specific enzymes for bioengineering. For this reason, metabolic control analysis is briefly described to introduce the concepts necessary for understanding the control of metabolic pathways. The application of tissue cultures for essential oil formation is also briefly considered.

4.1 Manipulation of Specific Enzymes to Alter Product Formation

An appreciation of the detailed kinetic mechanism of a wide variety of monoterpene and sesquiterpene synthases and related enzymes, and an understanding of the role of the active site residues in catalysis along with the availability of primary sequence information sets the stage for engineering product formation. Plant monoterpene and sesquiterpene synthases are particularly well suited for this approach because many of these enzymes already produce multiple products indicating a level of catalytic flexibility.

Two general approaches can be considered for the engineering of product composition by a specific enzyme, directed mutagenesis and domain swapping. Subtle changes in the amino acid residues at the active site of an enzyme can influence substrate binding conformation and the participation of side chain functional groups in catalysis. For example, in the case of monoterpene synthases, one could envision binding alterations to influence the isomerization of GPP to the opposite antipode of LPP, with consequent formation of alternate products. Similarly, modification of specific residues to affect the stability of carbocation intermediates could divert the reaction course along alternate routes to novel products. Although systematic studies of this type have not yet been carried out, two examples are known involving alteration of product outcome by mutation of a single amino acid residue. A yeast mutant which excretes large amounts of geraniol and linalool contains an altered FPP synthase [31] which has a single Lys197 to Glu mutation in a conserved position within a consensus sequence present in all known FPP synthases. The Lys at this position is believed to be involved in substrate binding. Mutation at this position aborts FPP production, although it was not reported if the product released by the mutant enzyme is GPP or geraniol. Recent studies on the site-directed mutagenesis of trichodiene synthase have demonstrated the ability of a fungal sesquiterpene cyclase to form aberrant products following minor modification of the active site [131]. Replacement of Arg304 with Lys results in a drastically decreased rate of trichodiene formation with concomitant formation of several novel sesquiterpene hydrocarbons. Replacement of Tyr305 with Thr results in the formation of approximately 50% of one of these novel products.

The second general approach to engineering product formation involves the construction of chimeric enzymes that combine functional domains from

different synthases. This approach is suggested by the high level of sequence homology observed for plant isoprenoid synthases, and by the nearly identical organization of introns and exons observed with *epi*-aristolochene synthase, vetispiradiene synthase, and casbene synthase (Sect. 2.3.5). The latter observation may indicate evolutionary conservation of functional domains in plant isoprenoid synthases [120], and suggests that chimeric enzymes with different functional domains can be created to synthesize novel products.

4.2 Bioengineering of Pathways

An understanding of the enzymology and regulation of a metabolic pathway (including the roles played by developmental changes, subcellular compartmentation, and the presence of multiple branch points) can permit the manipulation of the flux of intermediates through the pathway, with the goal of increasing the accumulation of a desirable product or decreasing the accumulation of undesirable ones. The bioengineering of the metabolic pathways for isoprenoid formation can involve either the modification of an existing pathway, directed to changing the composition and/or yield of the oil, or the introduction of new or altered enzymes to produce novel products not normally found in the plant.

It is now widely accepted that the control over the flux of intermediates through a metabolic pathway is distributed throughout the enzymes of the pathway, and it is rare that a single enzyme acts to control a truly "rate limiting" step (reviewed in [212]). The biosynthetic pathways used to provide energy and material for cell growth and development have evolved to maintain optimized flux distributions [213]. As a result of this distributed control over flux, metabolic pathways resist attempts to divert significant amounts of biosynthetic precursors from cell growth to the formation of secondary products (such as essential oils). In addition, the presence of branch points in metabolic pathways, and the nature of complex non-linear enzyme kinetics, often result in either minimal or unpredictable changes in pathway flux in response to changes imposed on the activity of a single enzyme (reviewed in [212]). Recent extensions to metabolic control analysis provide experimental strategies for manipulating the output of specific metabolites from a metabolic pathway by modifying two or more enzyme activities without affecting the steady state levels of other metabolites in the pathway (see, e.g., [214, 215]). An appreciation of this distributed flux control provides a sound framework for the successful bioengineering of the output of specific products from a metabolic pathway.

An illustration of the effect that partitioning of metabolites by branch points has on the quality of an essential oil is provided by the *p*-menthane monoterpenes produced in *Mentha* (Fig. 5) in which pulegone (**78**) acts as a branch point between the formation of menthone (**71**) and menthol (**72**), and the commercially undesirable component menthofuran (**79**). The major monoterpenes accumulated in pennyroyal (*M. pulegium*), peppermint (*M.* x *piperita*), and cornmint (*M. arvensis*) are pulegone, menthone and menthol, respectively. After flowering,

the menthone content of peppermint oil decreases with a corresponding increase in the menthol content of the oil. The relative amounts of menthone and menthofuran formed in peppermint oil are controlled by environmental factors such as photoperiod [170]. Thus, pulegone is a branch point between the formation of oils high in menthone and menthol, and those high in menthofuran. The accumulation of high levels of this branch point metabolite is characteristic of the pulegone-rich oil of pennyroyal. The formation of high levels of menthofuran by peppermint under conditions of short photoperiod or high temperature stress is largely beyond the control of the grower. Therefore, the branch point enzymes producing menthofuran and menthone represent promising targets for bioengineering efforts. The simplest approach would involve overexpressing the reductase which converts pulegone to menthone, thereby reducing the flux of pulegone into menthofuran. An alternate approach would involve decreasing conversion of pulegone to menthofuran by either repressing the expression of the putative cytochrome P-450 which converts pulegone to menthofuran, or removing the regulatory element(s) which result in increased expresssion of the P-450 under conditions of short photoperiod, resulting in a low, constitutive level of menthofuran formation.

An alternate approach to bioengineering of a pathway involves the introduction of new or modified enzymes not normally present in the plant. An illustration of the possibilities is provided by the production of C3 hydroxylated monoterpenes by a mutant of Scotch spearmint (see Sect.2.4.1). Replacement of the normal limonene-6-hydroxylase with a C3 hydroxylase activity results in the formation of numerous C3 hydroxylated *p*-menthane monoterpenes not naturally found in spearmint varieties [137]. Many monoterpenes, including limonene, exhibit insecticidal properties (reviewed in [10, 183]). The availability of cDNA clones for limonene synthase [75, 126] raises the possibility of engineering limonene production into crop plants to improve resistance to insect pests. The availability of cDNA clones for other cyclases and related enzymes of monoterpene metabolism provides the opportunity for manipulating composition in essential oil producing plants as well as introducing the formation of bioactive isoprenoids into new crops.

4.3 Trichome Formation

A third goal of bioengineering essential oil production is to increase the yield without affecting organoleptic quality. However, attempts to increase significantly the oil produced may be limited by the fact that these products are synthesized and stored in specialized anatomical structures, such as glandular trichomes, in which the primary metabolic pathways are already optimized to provide the energy and carbon sources necessary for isoprenoid synthesis. For this reason, attempting to increase the supply of IPP available for biosynthesis by increasing the activity of a single enzyme (e.g., HMGR) is unlikely to provide significant increases in total oil production.

Conceptually, the simplest approach to increasing oil production by the plant is to increase the tissue density of the specialized anatomical structures responsible for synthesis and storage. Recent work on the regulation of trichome development in *Arabidopsis* may lead to the development of approaches for increasing the density of glandular trichomes, which could result in a corresponding increase in total oil production. The identification of at least 21 different genetic loci involved in trichome development in *Arabidopsis* has been described [216]. Three of these loci (*GL3*, *TTG*, and *TRY*) appear to be involved in the initiation and surface distribution of trichomes [216–218]. The *GL2* gene has recently been cloned and shown to encode a regulatory transcription factor which is expressed in trichome progenitor cells [219]. Although the studies with *Arabidopsis* involve non-glandular trichomes, the development of glandular trichomes will undoubtedly share many of the same regulatory factors and control elements.

4.4 Tissue Cultures

Plant tissue cultures have long attracted interest as potential sources for the production of high value chemicals, including isoprenoids. However, significant economic and technical hurdles still exist in the utilization of these systems for commercial purposes (see, e.g., [220, 221]). For the most part, production yields of lower isoprenoids, such as monoterpenes and sesquiterpenes, by plant tissue cultures have been disappointing (reviewed in [222, 223]). Callus and suspension cultures established from essential oil-producing plants often produce only very low levels of the isoprenoids characteristic of the intact tissue. The low yield of essential oil isoprenoids is assumed to be due to the presence of undifferentiated cells in tissue culture; without specialized anatomical structures for production and storage, synthesis is suppressed and the activity of catabolic enzymes is enhanced. Studies with tissue cultures of sage have established that, although detectable levels of the enzymes for camphor biosynthesis are present, rapid catabolism of camphor prevents accumulation [224]. The capacity for the catabolism of camphor in tissue cultures of sage is so enhanced compared to intact tissue that cultured cells have been used for characterizing the catabolic pathway [165, 167]. The main value of tissue cultures to this point has been as convenient model systems for studying isoprenoid biosynthesis in response to different environmental stresses such as fungal infection [84, 177–180, 225].

5 Conclusions

This paper has attempted to provide a current overview of essential oil isoprenoid biosynthesis. An understanding of the mechanism of action of monoterpene and sesquiterpene cyclases, along with the availability of an increasing number

of cloned genes encoding isoprenoid synthases and related enzymes, has set the stage for the systematic bioengineering of product composition and yield. In many cases, the secondary transformations of isoprenoids are also understood well enough to rationally target specific enzymes for manipulation, with the goal of modifying essential oil composition. However, significant gaps remain in our understanding of the integration of essential oil biosynthesis with primary metabolism; in particular, the regulation of IPP biosynthesis and the partitioning of IPP remain poorly characterized. Too few studies have addressed the early steps of isoprenoid biosynthesis in essential oil-producing tissues and our understanding of the developmental regulation of essential oil biosynthesis is also fragmentary. These deficiencies need to be addressed before an integrated approach to the bioengineering of essential oil production can be attempted.

Acknowledgements. The work carried out in the authors' laboratory has been supported by grants from the Department of Energy, the National Institutes of Health, the National Science Foundation, and the U.S. Department of Agriculture.

6 References

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