1.16 Sesquiterpenes

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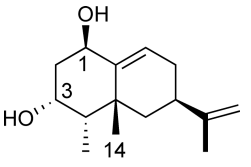
1.16.1 Introduction

Sesquiterpenes, like so many other classes of terpenes and other natural products, have attracted significant interest because of the roles they play in biological systems and their utility for human uses. However, one of the features that serves to distinguish sesquiterpenes from many of the other families of natural products is the thousands of different sesquiterpene compounds that have been identified. The source of this diversity arises in two ways. First is the structural diversity that arises in the assembly of the 15-carbon skeletons making up the backbone of all sesquiterpenes. The second source of diversity is due to the layering of functional groups and substituents upon the structural scaffolds in distinct regio- and stereospecific manners. Hence, while some recent reviews of sesquiterpenes have focused on the number of distinct and new sesquiterpenes identified,[1] the current chapter will initially discuss theoretical considerations for how the sesquiterpene scaffolds arise, then attempt to rationalize this information with what is now known about the enzymes mediating these key biosynthetic reactions in a manner similar to the mechanistic approaches of Cane,[2,3] Hohn,[4] and Christianson.[5] We will then turn our attention to emerging details about the biochemical mechanisms responsible for elaborating the sesquiterpene scaffolds with substituent and functional groups. Lastly, we will raise the question of how well do we understand sesquiterpene biosynthesis. That is, do we understand the enzyme mechanisms and the physiological context of how these compounds are made in cells to actually engineer the biosynthesis of novel or unique sesquiterpene compounds?

The structural complexity of sesquiterpenes is readily illustrated by what initially appears to be a simple structure. Capsidiol (**1**), an eremophilane-type sesquiterpenoid first isolated from pepper,[6] *Capsicum annum*, contains two hydroxyl substituents. Hence, the trivial name capsidiol. However, more important to note is the complexity inherent in this structure. For a 15-carbon or C_{15} compound, capsidiol contains five chiral centers and three different substituents (hydroxyl, methyl and isopropenyl groups) arrayed precisely (regio- and stereospecifically) around a bicyclic scaffold. What we intend to suggest in this chapter is that if we can truly understand the mechanisms responsible for the biosynthesis of molecules like capsidiol, then we should be able to modify these enzymes so they, for example, yield the inverted stereochemistry of the C14 methyl substituent, or alter the regio- or stereochemical positioning of the hydroxyl at C1, thus creating novel molecules, which may have benefits to the organisms producing them as well as man-made applications.



Capsidiol (**1**)

1.16.2 Classification of Sesquiterpenes

1.16.2.1 Carotenoid Cleavage Products

The issue of how to classify sesquiterpenes is not as straightforward as one might imagine. While the majority of sesquiterpenes arise directly from farnesyl diphosphate (FPP), a key C_{15} diphosphorylated intermediate of the mevalonate (MVA) biosynthetic pathway, a number of biologically important sesquiterpenes arise as breakdown products from terpenes synthesized by the nonmevalonate or methylerythritol phosphate (MEP) pathway, which in plants operates simultaneously and in parallel to the MVA pathway except in the plastid compartment (**Figure 1**). Perhaps the most familiar of these carotenoid breakdown products is abscisic acid (ABA, **4**, **Scheme 1**), a well-known plant growth regulator.[7] An important discovery in elucidating the biosynthetic pathway for ABA was the functional mapping of the viviparous mutants of maize. The *vp* mutants and especially *vp*14 were distinguished by having corn kernels that sprouted prematurely during corn cob development.[8] The premature sprouting was subsequently correlated with the developing seeds having abnormally low levels of ABA, which typically arrests premature seed germination.

Functional characterization of the wild-type VP14 locus demonstrated that it encoded for a carotenoid cleavage dioxygenase (CCD) that cleaves 9-*cis*-neoxanthoxin or 9-*cis*-violaxanthoxin (**2**) at the C11═C12 double bond, introducing oxygens from molecular O_2 into both cleavage products and liberating xanothoxin (**3**), which undergoes successive oxidations and an epoxide ring opening to yield ABA (**4**, **Scheme 1**).[9] The CCD genes are now well recognized as part of a much larger and diverse gene family, widely distributed in bacteria, plants, and animals, and characterized for their specificity of cleavage of various carotenoids substrates at the 7,8; 9,10; 11,12; and 15,15′ double bonds distributed across the conjugated polyene chains. The CCD

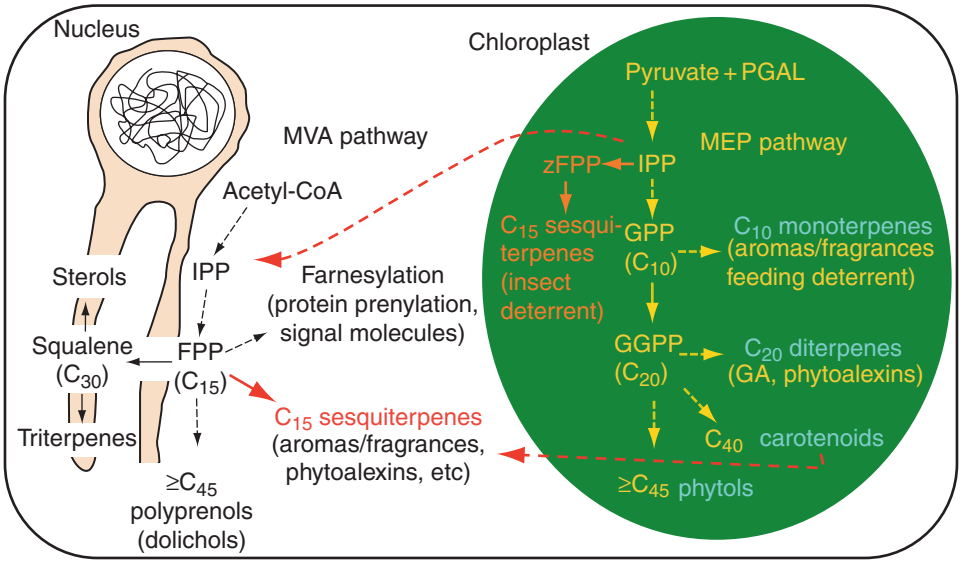
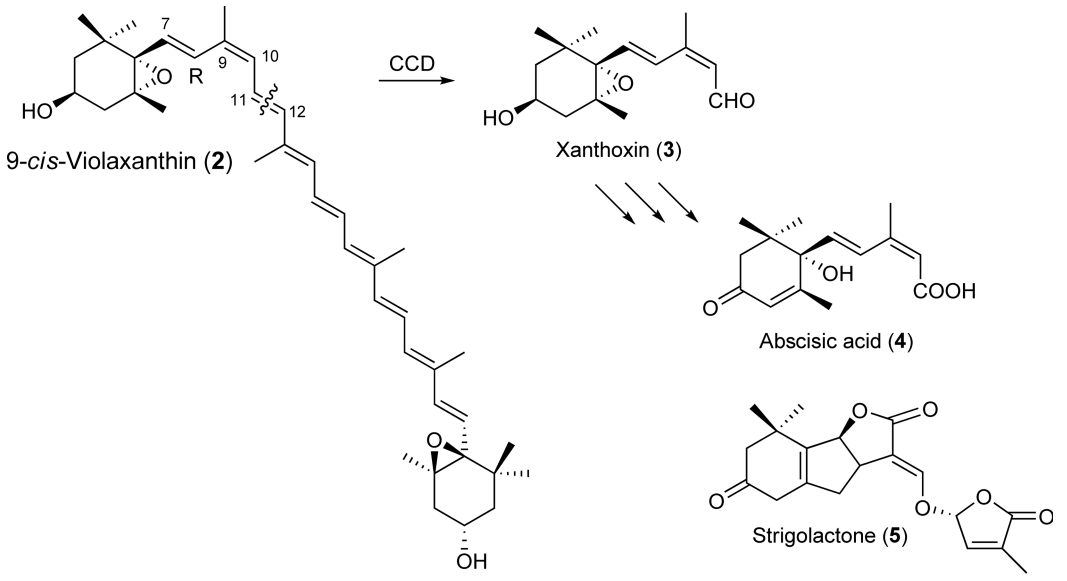


Figure 1 Cartoon depiction of terpene metabolism in a typical plant cell with an emphasis on the different carbon sources contributing to sesquiterpene biosynthesis and showing their biological functions.



Scheme 1

homologs found in animals have been associated with the conversion of dietary carotenoids into essential retinal pigments important for visual perception.[10] In plants, several of the CCDs have been found associated with the biosynthesis of strigolactones, (e.g., **5**) secreted sesquiterpenoids that serve as signal molecules for the establishment of mycorrhizal relationships[11] as well as their colonization by parasitic plants like *Striga* and *Orobanche* species.[12,13]

More recently, new members of the CCD family of enzymes have been associated with the biosynthesis of a new class of hormones controlling axillary bud growth in plants,[13,14] essentially regulating the above-ground architecture, biomass accumulation, and agricultural yields of fruit-bearing branches. Genetic mutants exhibiting constitutive and unregulated outgrowth of axillary buds were mapped to genetic loci coding for enzymes possessing amino acid signature domains for CCDs. Interestingly, the newly identified growth regulatory molecule was identified as strigolactone (**5**), the same sesquiterpenoid previously shown to mediate plant associations with mycorrhizal fungi and parasitic plants,[12] yet derived by independent carotenoid cleavage and modification reactions occurring in the roots rather than the aerial portions of the plant.

1.16.2.2 Farnesanes

The farnesane family of sesquiterpenes (**Figure 2**) is derived from FPP (**14**) in one of the two ways. Farnesol (**6**) represents the simplest of the acyclic sesquiterpenes and it can arise from the action of phosphatases cleaving the terminal phosphate substituents.

Biologically, FPP and farnesol are important molecules monitored intracellularly, which are thought to provide for homeostatic control of carbon flux into the MVA pathway.[15,16] Although there are suggestions in the literature of possible FPP-specific phosphatases, none have been confirmed to date. Instead, phosphatases associated with lipid metabolism as well as other nonspecific phosphatases have been shown to use FPP as a substrate.[17] In contrast, successive phosphorylation of farnesol by a CTP-dependent kinase(s) has been described,[18] thus at least in theory providing for the recycling of farnesol back into the active pool of carbon feeding the MVA pathway.

The family of acyclic sesquiterpenes is much larger in terms of family members, and most are initially derived by the action of specific terpene synthases (**Scheme 2**). In common with most terpene synthases, nerolidol[19,20] and farnesene[21] synthases appear to initiate catalysis by cleavage of the diphosphate substituent leaving a delocalized carbocation with positive charge distributed between C1 and C3. Capture of a hydroxyl

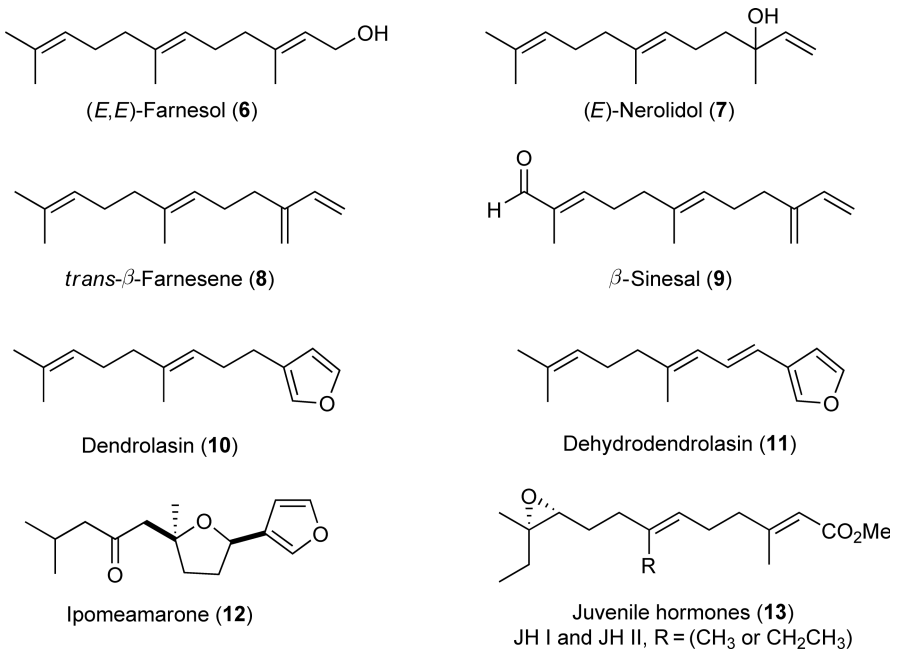
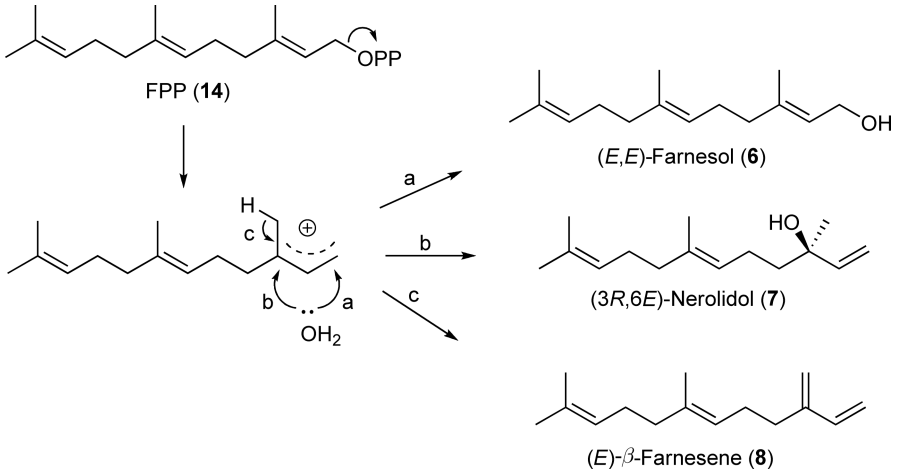


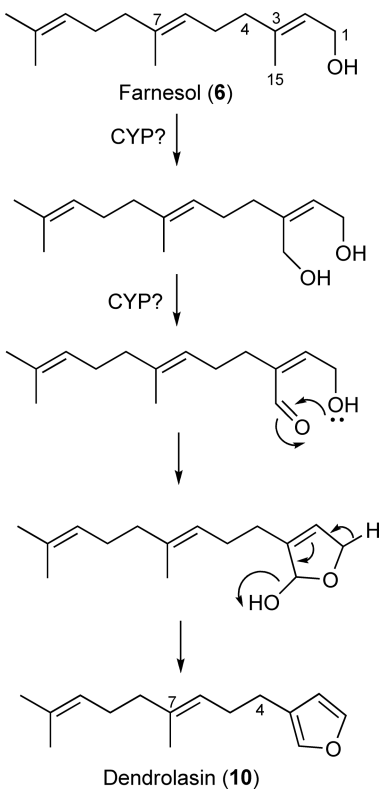
Figure 2 Structures and names of selected farnesane sesquiterpenes.



Scheme 2

group from the bulk solvent at C1 or C3 yields farnesol (**6**) and nerolidol (**7**), respectively. Nerolidol is a fragrance emitted by snapdragon flowers, which is thought to attract pollinators.[22] Alternatively, proton abstraction from the C15 methyl group yields β-farnesene (**8**), an alarmone first described as a volatile emitted from herbivore-damaged plants serving to agitate and disperse aphids.[23] β-Farnesene has been associated with many other activities including its action as an attractant for predator insects to herbivore-damaged plants.[24]

The acyclic sesquiterpenes are subject to further modifications including hydroxylation/oxidations that generate compounds like β-sinesal[25] (**9**), a flavor component of citrus. Reductions of C═O and C═C double bonds, dehydrogenations, formation of furan rings, epoxidations, and acylations serve to diversify further the straight-chain sesquiterpene backbone. Dendrolasin (**10**) and dehydrodendrolasin (**11**) are examples of furan-containing sesquiterpenes characterized as allomones.[26] In marine organisms, these compounds are found in various sponge species where they deter feeding behaviors of fish and other invertebrates,[27] and may play a



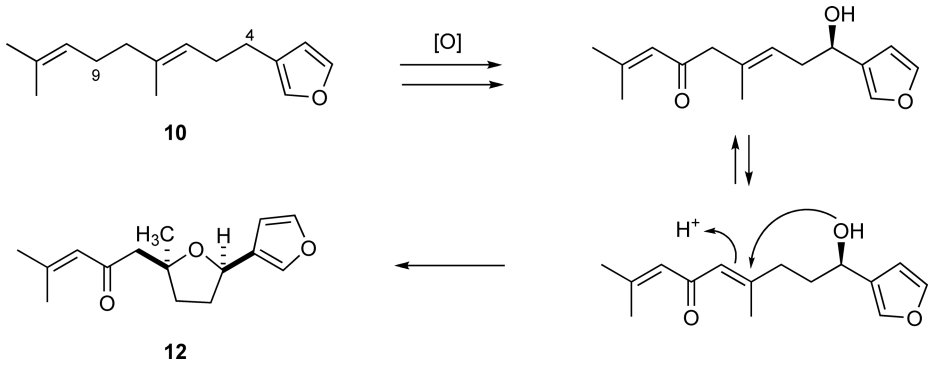
Scheme 3

similar role when emitted from angiosperm trees in response to insect predation.[28] Ipomeamarone (**12**), an antimicrobial sesquiterpene produced by sweet potato in response to pathogen and elicitor challenge,[29] appears to be an interesting derivative of dendrolasin characterized by an internal tetrahydrofuran ring.

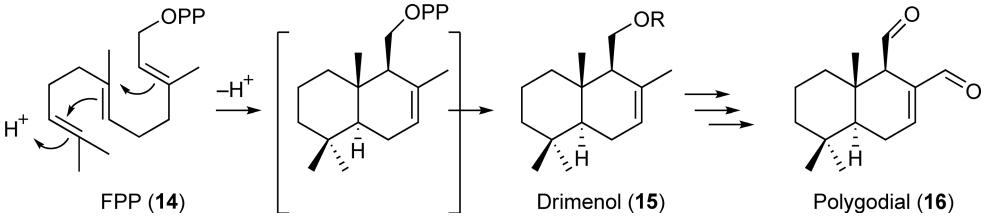
Although furan rings commonly occur within terpene structures, the biochemical mechanism responsible for the introduction of such heterocycles is still not understood. Gaikwad and Madyastha[30] provided evidence for an enzyme-mediated reaction. More specifically, these authors suggested that oxidized substituents could provide specificity for binding in the active site of a cytochrome P-450 enzyme, orienting the substrate such that a methyl group syn to the hydroxyl/carbonyl becomes oxidized. Proximity to, and correct spatial orientation of, the hydroxylmethyl group to the carbonyl group would then allow for spontaneous cyclization to the hemiacetal, which would readily undergo dehydration to yield the furan final product.[30] **Scheme 3** illustrates one possible reaction sequence in which farnesol is successively oxidized at the C15 methyl group to the aldehyde oxidation state, followed by cyclization and dehydration to form the furan ring of dendrolasin (**10**). Introduction of the internal tetrahydrofuran ring of ipomeamarone (**12**) could plausibly occur by the reaction sequence illustrated in **Scheme 4**. A succession of oxidations at C4 and C9, isomerization of the 6,7 double bond into the conjugated 7,8 position, and stereospecific conjugate addition of the C4 hydroxyl onto C7 of the resulting α,β-enone would afford ipomeamarone. The juvenile hormones JH 1(**13**, R = CH_2CH_3) and JH II (**13**, R = CH_3), isolated from male Cecropia silk worms, are homosesquiterpenes involved in maintaining the juvenile stage of development in the life cycle of insects and Crustacea (see also **Figure 10**).

1.16.2.3 Drimanes

Another rather unique group of decalin derivatives comprises the drimane class of sesquiterpenes.[31] These natural products have been found in association with many marine sponges[31] and with primitive and evolutionarily lower plants like liverworts,[32] but not exclusively. Polygodial (**16**) is a common drimane sesquiterpene occurring in various plant species and documented for its rather strong anti-insecticidal activities.[33] The biosynthetic route to the drimanes is not yet fully resolved (**Scheme 5**) and no genes coding for the



Scheme 4

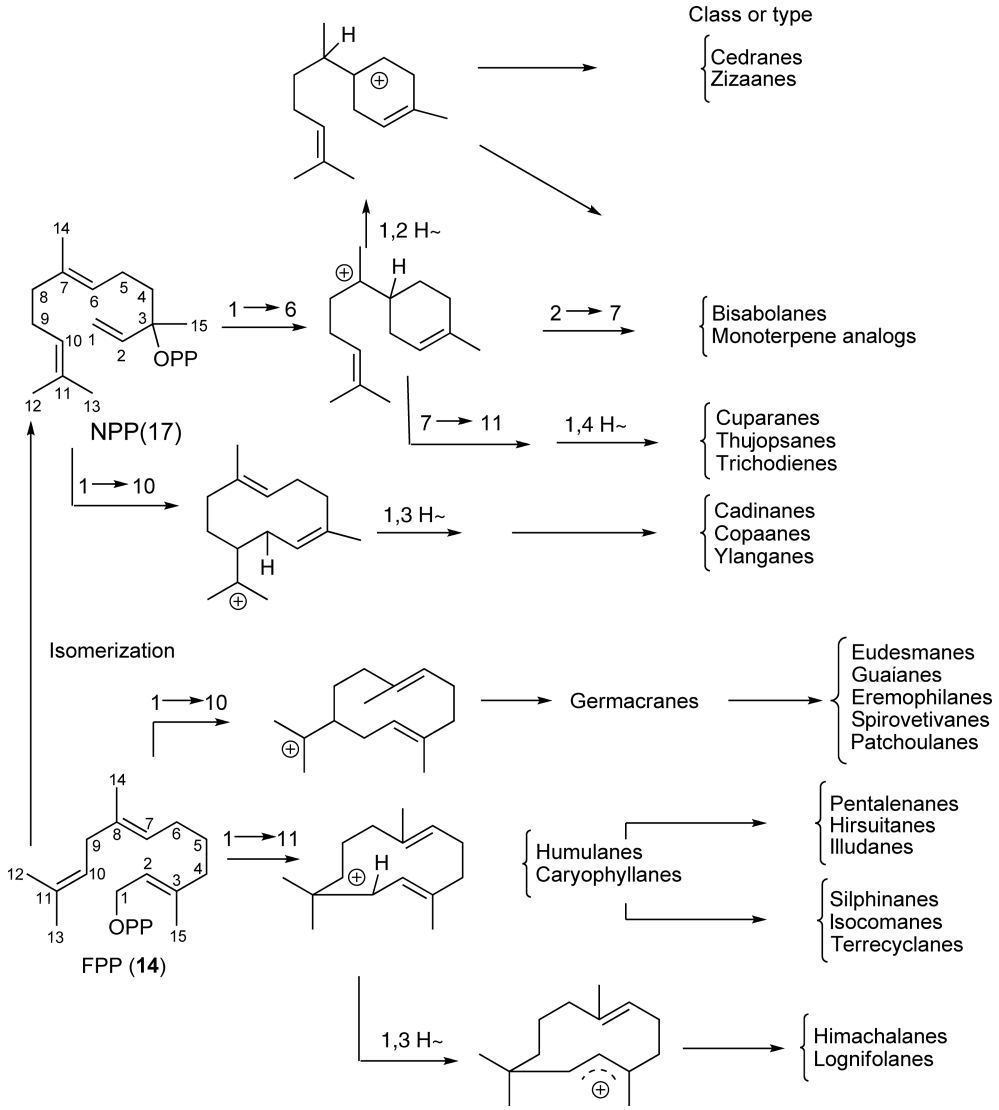


Scheme 5

corresponding synthases and oxidases have yet been reported in the literature. Nonetheless, the mechanism assumed to be involved in the biosynthesis of drimanes has precedent in the labdane diterpene synthases,[34] as well as triterpene cyclases like cycloartenol and β-amyrin synthases.[35] Interestingly, the labdane diterpenes arise from two-step cyclization cascades associated with a single enzyme in fungi[36,37] and two successive enzymes in plants.[34,38] The first step (A activity) consists of a proton-initiated cyclization of geranylgeranyl diphosphate (GGPP) to form copalyl diphosphate (CPP), a diphosphorylated, decalin A,B ring intermediate (or its enantiomer, *ent*-copalyl diphosphate). The second step is initiated by the ionization of the diphosphate substituent, followed by carbocation-mediated cascades to give various tricyclic and tetracyclic diterpenes.[39,40] In contrast, triterpene cyclases initiate catalysis by proton addition to squalene and squalene 2,3-epoxide, nonphosphorylated intermediates, which then undergo multiple cyclizations to form a variety of polycyclic structures with the imposition of strict conformational and stereochemical specificities.[41,42] Drimenol biosynthesis has been demonstrated with a partially purified enzyme preparation from *Polygonum hydropiper*[43] that utilized FPP as the substrate. However, a phosphorylated intermediate was not observed in the case of drimenol (**15**) biosynthesis. The existence of proton-initiated sesquiterpene synthases has speculative significance because this crucial, high-energy step has been suggested by some to represent an evolutionary link between the more primitive ionization-induced mechanism of cyclization and the evolutionarily advanced proton addition mechanism.[31]

1.16.2.4 Cyclic Sesquiterpenes

The vast majority of sesquiterpenes isolated from microbial and plant sources have cyclized structures and, at first glance, a classification scheme based on overall structure seems reasonable. That is, gathering what initially appears to be a disparate array of compounds into classes like monocyclic, bicyclic, tricyclic, and macrocyclic compounds classifies these compounds into groups resembling one another. However, a more cogent classification scheme based on chemical rationalizations for the biogenetic pathways leading to particular classes of compounds is possible, as illustrated in **Scheme 6**. The value of this classification as we hope to illustrate below is that it lends itself to experimental exploration into the origins for this chemical diversity. To avoid confusion, we consistently refer to carbon position numbers for the farnesyl chain marked on structure **14** (**Scheme 6**).



Scheme 6

The same positional numbers will be used in the following schemes to designate individual carbon atoms in intermediate carbocations and the final cyclic sesquiterpene products.

The bewildering diversity of cyclic structures associated with naturally occurring sesquiterpenes can be rationalized by different combinations of relatively few individual reactions of carbocation intermediates. It seems worthwhile at the outset to offer some generalizations about the relative stabilities of carbocations, the basic types of carbocation reactions, and the thermodynamic driving forces in the transformations. Carbocation formation is most commonly initiated by heterolysis of the C–O bond of FPP to generate the allylic farnesyl$^+$/OPP$^-$ ion pair. Less common initiating steps in sesquiterpene biosynthesis are proton transfers to C═C double bonds or epoxides, the former previously presented in the context of drimane biosynthesis. The polycyclic structures are then elaborated by permutations and combinations of the following individual propagating steps: (1) carbocation + C═C double bond cyclizations; (2) 1,2-, 1,3-, and 1,4-hydride shifts; and/or (3) Wagner–Meerwein rearrangements. The cascade of propagating steps is then terminated by 1,2- or 1,3-proton eliminations forming C═C double bonds or cyclopropane rings, or by capture of a water molecule to produce alcohols.

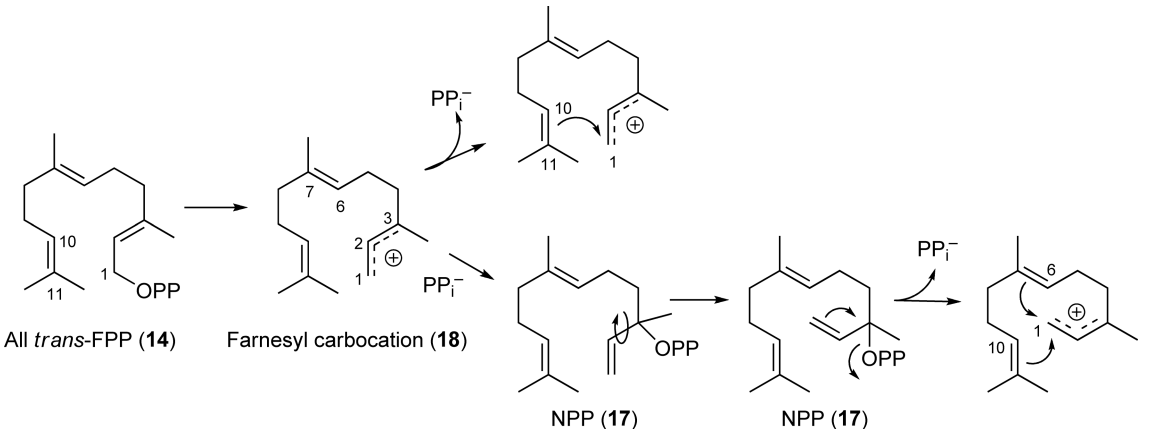
In the special case of the FPP– nerolidol diphosphate (NPP) interconversion to be discussed later, the farnesyl carbocation in the farnesyl^{+}/OPP^{-} ion pair is captured by the pyrophosphate counterion at C3.

The stabilities of carbocations vary greatly depending upon the substituents attached to the electron-deficient site. Tertiary carbocations are more stable than similar secondary ions by 15–20 kcal mol^{-1}. Allylic carbocations like the farnesyl ion are greatly stabilized by delocalization of the positive charge between the C1 and C3 termini. Cyclizations of carbocations with C═C double bonds are typically exothermic by 20 kcal mol^{-1} that arises from the conversion of the C═C double bond into two C–C single bonds, if the stabilities of the reactant and product carbocations are otherwise similar. The strain energies associated with carbocyclic rings are likely to be significant factors in different types of cyclizations; approximate strain energies of cyclopropane, cyclobutane, cyclopentane, and cyclohexane are approximately 25, 25, 7, and 0 kcal mol^{-1}, respectively.

In general, the multistep schemes in sesquiterpene biosynthesis will proceed to generate more stable carbocations from less stable ones. Relatively high-energy secondary carbocations, usually regarded as key intermediates, may be generated by exothermic carbocation + C═C cyclizations such as the case of the secondary ion intermediate (**24**) formed by anti-Markovnikov cyclization of the bisabolyl ion in trichodiene biosynthesis (see **Scheme 9**). The enzyme-bound carbocation intermediates may be stabilized by specific interactions with peptide residues surrounding the active sites of sesquiterpene synthases, e.g., cation-pi interaction, with aromatic rings of phenylalanine, tyrosine, and tryptophan; dipole–dipole interactions with amide linkages; and ion pairing with carboxylate groups in the side chains of aspartate and glutamate residues. Carbocations are exceptionally strong acids similar in proton-donating ability to concentrated sulfuric acid. Consequently it follows that the common terminating steps of 1,2-proton elimination and nucleophilic capture of water are both highly exothermic transformations. The increased solvation of the liberated pyrophosphate ion and its associated magnesium ion ligands contributes added driving force to the overall cyclization processes. It should be evident that biosyntheses of most sesquiterpenes from FPP are highly exothermic chemical transformations.

The usual starting substrate for all these different sesquiterpene classes is (*E,E*)-FPP (**14**), which undergoes an initial cleavage of the diphosphate substituent leaving a reactive farnesyl carbocation. It is the subsequent cascade of events of this carbocation intermediate that leads to the final reaction products, the hydrocarbon skeleton characterizing a particular class of sesquiterpenes. And, these cascades are dictated by the configurations and conformations of the various reactive intermediates, positioning the reactive carbocation centers into proximity and alignment with other atoms and bonds within the C_{15} scaffold, which then allows for particular steps to proceed while limiting or preventing others. Later in this chapter, we illustrate how the terpene synthases might provide geometric landscapes that essentially create the mold to direct an ionized farnesyl intermediate down a specific catalytic cascade.

As shown in **Scheme 7**, the initial ionization leads to a 2,3-transoid farnesyl carbocation ion pair (**18**) that can either lose the ionized diphosphate counterion, thus permitting bond formation between C1 and C10 or C11 and leading to a series of sesquiterpenes derived from these macrocyclic intermediates, or the ions may

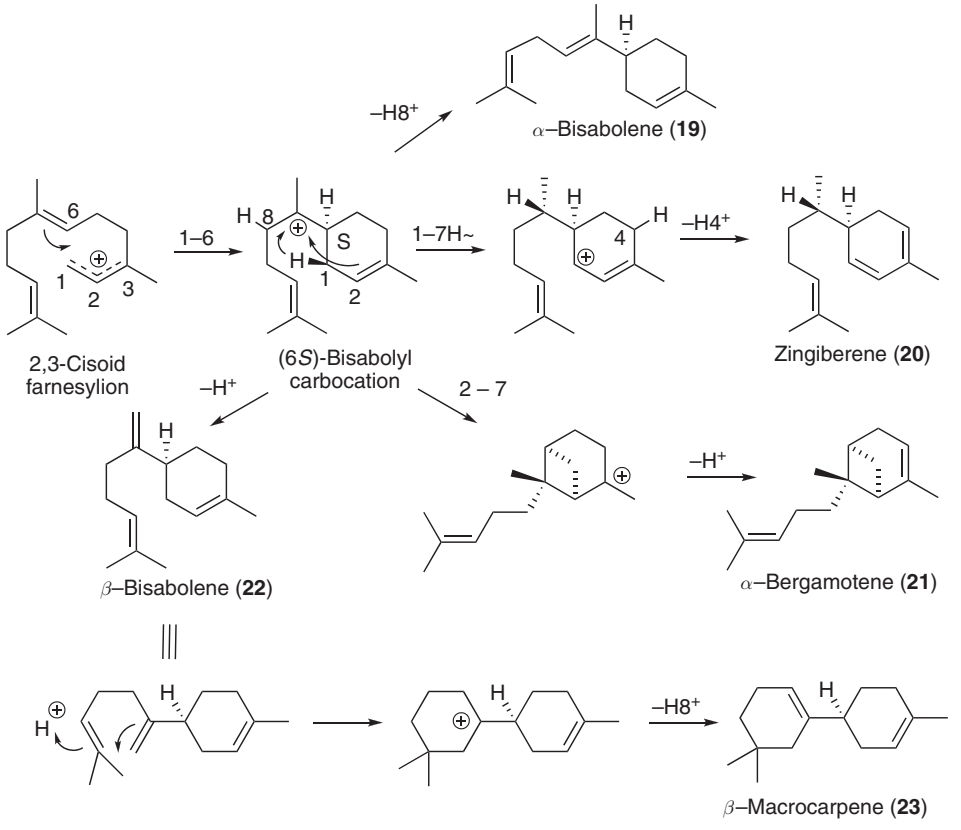


Scheme 7

recombine to form a new intermediate, NPP (**17**).[2] NPP is an important intermediate because it allows for rotation around the newly formed single bond between C2 and C3. A second ionization of the 2,3-cisoid NPP thus creates a new, stereochemically distinct intermediate, positioning the carbocation for ring formation between C1 and C6 or C10, and opening the way to several more classes of sesquiterpenes distinguished by a *cis* configuration, rather than *trans* configuration, about the C2═C3 double bond.

1.16.2.4.1 The bisabolane series

The bisabolane group includes sesquiterpenes found in the fragrances from familiar plants like lavender,[44] as well as many coniferous species.[45,46] This class may also appear to be among the simplest of sesquiterpenes because of their structures, consisting of many, but not exclusively, monocyclic members (**Scheme 8**). They are however representative of the same level of complexity found in all the cyclic sesquiterpenes. Several of the early cDNAs isolated encoding for terpene synthases were for enzymes catalyzing the conversion of (*E*,*E*)-FPP to (*E*)-α-bisabolene (**19**) from gymnosperms,[45] and these have been useful in illustrating the putative reaction mechanism. As noted in **Scheme 7**, formation of the 2,3-cisoid rotomeric form of NPP (**17**) is followed by an ionization step generating a carbocation with charge distributed between C1 and C3, and, if oriented correctly, allowing for ring formation between C1 and C6 in a stereospecific manner. With the carbocation site now centered at C7, proton abstraction at C8 terminates the reaction cascade yielding α-bisabolene (**19**), known sequiterpenes as both the 7E and 7Z isomers. Zingiberene (**20**) biosynthesis proceeds up to the same bisabolyl carbocation intermediate, but is then presumably followed by a 1,3-hydride shift and subsequent proton abstraction to yield the final 1,3-cyclohexadiene product.[47] The biosynthesis of α-bergamotene (**21**) illustrates yet another degree of freedom within these synthase reactions with the formation of a bicyclo[3.1.1]heptane ring system arising from the formation



Scheme 8

of a new C2–C7 bond and recentering of the carbocation at C3. Final proton abstraction from C4, similar to that for zingiberene biosynthesis, yields a new double bond between C3 and C4 and formation of α-bergamotene.[44,48]

The biosynthesis of β-macrocarpene (**23**) illustrates a fourth permutation on the fate of the bisabolyl carbocation intermediate. Abstraction of a proton from C14 yields β-bisabolene (**22**), which is commonly found as a final product in many plant species,[49] but in the case of two highly similar maize enzymes the triene actually undergoes yet another round of catalytic transformations initiated by a proton addition.[50] In this case, the addition of a proton, either from a water molecule bound in the active site or the proton abstracted in an earlier step, to the C10═C11 double bond positions a favorable tertiary carbocation at C11. This carbocation, if brought into close enough proximity to C14 and with the proper stereochemical orientation to the pi orbital of the C7═C14 double bond, allows for a new C–C bond to form, creating a second six-membered ring with gem dimethyl substituents and shifts the carbocation to C7. A second proton abstraction from C8 thus yields β-macrocarpene (**23**).

Support for the chemical rationalization of β-macrocarpene biosynthesis by the maize TPS6/11 enzymes comes from several experimental observations.[50] First, GC-MS profiling of the reaction products identified the predominate product as β-macrocarpene (**23**) with β-bisabolene (**22**) as a significant minor product. Although time course experiments did not demonstrate a precursor–product type relationship between β-bisabolene and β-macrocarpene, the presence of β-bisabolene demonstrates that these two enzymes are capable of biosynthesizing this compound. Second, reinitiation of the second round of reactions via a proton donation was demonstrated by rehydrating lyophilized TPS6 enzyme in D_2O, then observing the incorporation of one extra mass unit into the appropriate MS fragments of the β-macrocarpene reaction product. Third, using a strategy employed by Rising *et al.*[51] for mapping active site residues involved in proton addition and reinitiation of catalysis for 5-epi-aristolochene synthase (EAS), another sesquiterpene synthase discussed below, Köllner *et al.*[50] observed that mutation of a putative active site tyrosine (position 522) to phenylalanine abolished β-macrocarpene biosynthesis without any alteration in the proportion of β-bisabolene accumulating. One interpretation of this result, as concluded by the authors, is that tyrosine 522 is responsible for the reinitiation of catalysis, thus enhancing substrate flux through the enzyme and accumulation of the macrocarpene product. But this conclusion, unlike that of Rising *et al.*,[51] is not consistent with standard biochemical expectations. Knocking out the second series of reactions in these maize enzymes should eliminate β-macrocarpene accumulation, but then β-bisabolene accumulation should be enhanced to a level equal to β-macrocarpene plus β-bisabolene accumulation by the wild-type enzyme. But this was not observed, suggesting that the mutation compromised the enzyme in at least two ways – knocking out the second series of reactions and reducing the flux of substrate through the enzyme, possibly by hindering product release.[52,53]

The bisabolane family of sesquiterpenes is notable because of the different ways in which stereocenters are introduced and regioisomers are created. Hydride shifts and ring closures can lead to the creation of stereoisomers, while the generation of configurational isomers can arise from regiospecific proton abstractions, as illustrated in **Scheme 8**. For example, the maize TPS6 enzyme creates the 6S stereochemistry in the initial 1–6 cyclization, and directs elimination of H8 to yield **23**, rather than an isomer if H14 were abstracted generating a 7,14 double bond.

Köllner *et al.*[54] have also provided compelling evidence that another maize terpene synthase, TPS4, possesses two active site pockets rather than one as observed in the available three-dimensional structures solved for sesquiterpene synthases.[5,55–59] TPS4 catalyzes the biosynthesis of 14 bisabolane type sesquiterpenes, many containing only a single 1,6 ring, and a second family of bicyclic compounds derived after a second 2,6 ring closure, like that in bergamotene biosynthesis. Using the structural coordinates of EAS to build molecular models of TPS4, these investigators noted the possibility of two distinct pockets within the TPS4 enzyme, both sufficiently large to accommodate binding of various proposed reaction intermediates (**Figure 3**). To test if one pocket might direct conversion of FPP to the initial bisabolene intermediate, and the second functions to introduce 2,6 ring closures, they created site-directed mutants in each pocket that were evaluated for the reaction products generated upon incubation with *trans*,*trans*-FPP or *cis*,*trans*-FPP, a possible isomeric intermediate proposed as essential for the 1,6 ring closure. Overall, Köllner *et al.* reported that mutations in pocket I residues prevented formation of reaction products from (*E*,*E*)-FPP, but not (*Z*,*E*)-FPP, and that mutants in pocket II residues lost their capacity for the biosynthesis of bicyclic compounds, but maintained their ability to generate acyclic and monocyclic bisabolane-type compounds. Hence, the results are consistent with reactions

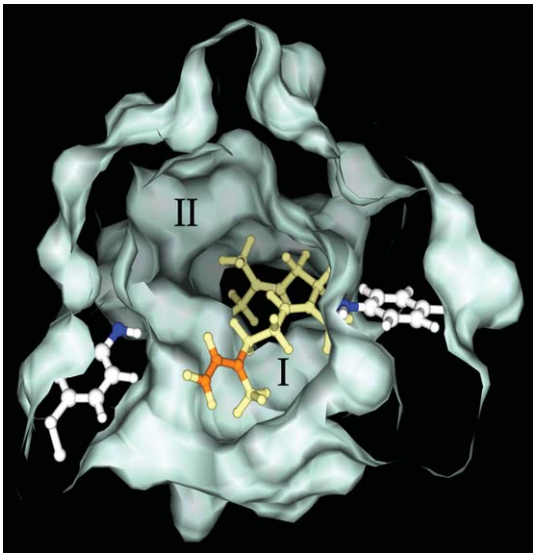


Figure 3 A molecular model of the maize TPS4 enzyme, highlighting the two putative active site pockets, I & II. Reproduced from T. G. Köllner; P. E. O'Maille; N. Gatto; W. Boland; J. Gershenzon; J. Degenhardt, *Arch. Biochem. Biophys.* **2006**, *448* (1–2), 83–92.

leading up to the first ring closure occurring in pocket I, and the diversification of these bisabolene intermediates, including a second ring closure, occurring in pocket II.

More recently, several groups investigating the terpene biosynthetic potential of trichomes (surface appendages that secrete rich arrays of plant secondary compounds) using microarray and expressed sequence tags (EST) DNA sequencing approaches have uncovered new synthases that catalyze the biosynthesis of sesquiterpenes in the bisabolane family.[47,60] The report by Sallaud *et al.*[60] is particularly intriguing; they found that the Sst2 locus in wild tomato, *Solanum habrochaites*, was responsible for the biosynthesis and accumulation of several bisabolene-derived sesquiterpenes, including bergamotene and santalene isomers, and their acidic forms that provide insect resistance to the wild tomato species. Molecular sequencing of the Sst2 locus identified two closely linked terpene synthases, one coding for a prenyltranferase, FPP synthase (FPS), and the other for santalene and bergamotene synthase (SBS), a multifunctional synthase catalyzing the biosynthesis of several isomers each for santalene and bergamotene from FPP. However, what makes this observation especially unique is that both the FPS and the SBS contain plastid-targeting signal sequences at their amino termini, which were shown to mediate vectorial transport of these enzymes into the plastid compartment. Along with the work from Besser *et al.*,[61] a unique pathway for sesquiterpene biosynthesis has been now been localized to the plastid compartment (see **Figure 1**).

Sallaud *et al.*[60] reported two additional extraordinary findings. First, the *S. habrochaites* FPS utilized IPP and DMAPP to synthesize (*cis*,*cis*)-FPP rather than the universally observed all-*trans* FPP, and second, not only did the SBS enzyme readily utilize the (*cis*,*cis*)-FPP to generate stereochemically unique forms of santalene and bergamotene, but based on informatic comparisons, the synthase utilizes a proton addition mechanism to initiate catalysis similar to that found with di- and tri-terpene synthases.[34,35] These observations clearly indicate that a very unique form of coevolution must have been occurring leading up to this novel sesquiterpene biosynthetic capacity in plastids. The evolution of the genes producing (*cis*,*cis*)-FFP synthase and the SBS cyclase that utilizes the geometrically isomeric substrate was explained[60] as a means to circumvent the strong regulatory constraints associated with the biosynthesis of cytosolic (*trans*,*trans*)-FPP.

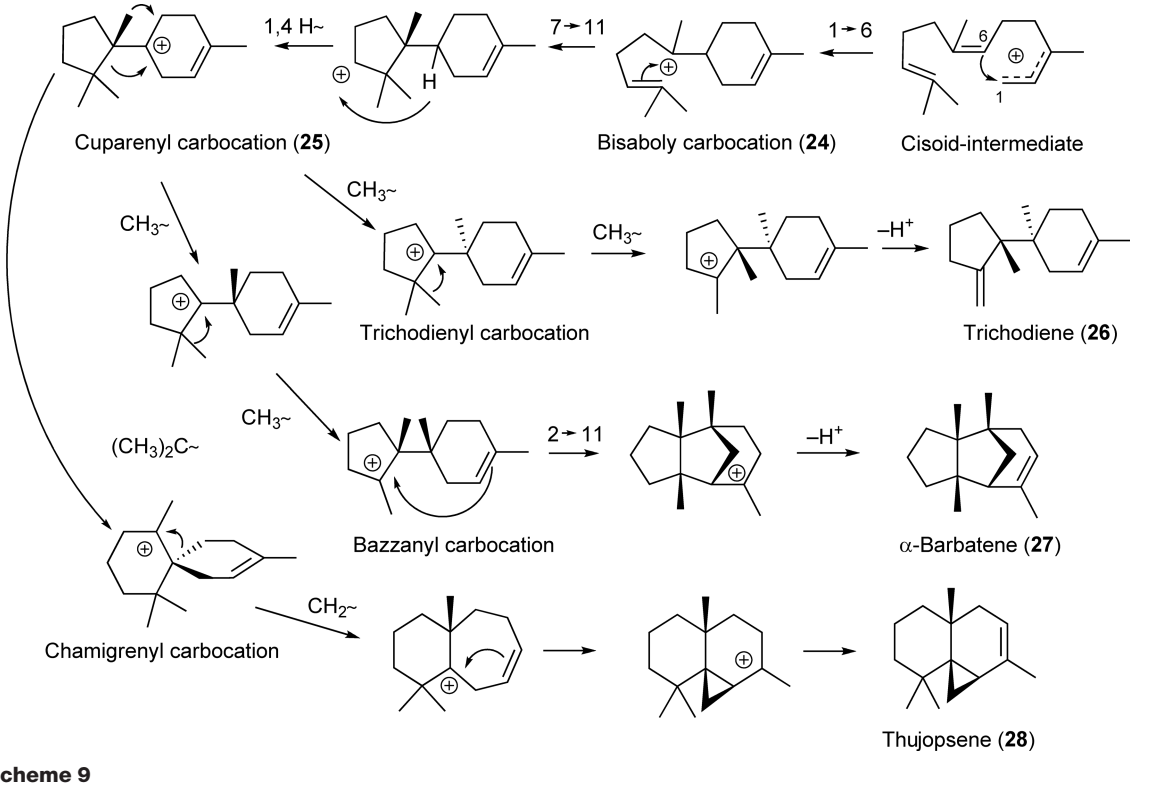
1.16.2.4.2 The cuparane series

The cuparane series of sesquiterpenes is one of the best understood classes of compounds from the perspective of their biological importance and their biosynthesis. Their chemical appreciation arises because some of the most potent and regulated mycotoxins, the trichothecanes, are members of this class.[62] The trichothecanes are derived from the tricyclic hydrocarbon trichodiene (**26**) produced by pathogenic fungi that colonize cereals like

oat, corn, and wheat, and consist of a family of derivatives containing oxidized and epoxide functionalities associated with the biological activity of these mycotoxins.[63] The effects of these compounds on humans and livestock have been studied extensively because of the severity of symptoms caused upon ingestion, which include severe digestive disorders like vomiting and diarrhea, hemolysis, disruption of cognitive function, and death.[64] The permissible level of these mycotoxins in foods is strictly regulated by federal agencies and, while levels below 2 ppm are considered tolerable, there has been movement to lower these allowable levels substantially in the recent past.[65] Investigations have identified protein translation as the primary mode of toxicity[66] for the trichothecanes, and in particular have identified interactions of trichothecanes with the L3 protein of the large ribosomal subunit.[67]

Many of the genes for trichodiene and trichothecane biosynthesis have been isolated and functionally characterized.[62,63] Interestingly, given their fungal origin, the respective genes appear to be clustered, somewhat analogous to the clustering of terpene biosynthetic genes observed in prokaryotic organisms like *Streptomyces*.[68] Clustering is thought to provide some advantages for coordinate expression of the resident genes.

The biosynthesis of trichodiene, the immediate precursor to the entire trichothecane family, has been studied intensively and the results largely support the chemical rationale illustrated in **Scheme 9**. Trichodiene biosynthesis proceeds from a bisabolyl carbocation intermediate (**24**) as discussed above by a ring closure between C7 and C11, followed by a 1,4-hydride shift to yield the cuparenyl intermediate (**25**). Several features of this intermediate distinguish it from other sesquiterpene classes and position it uniquely for the following transformation pathways. First, it consists of a bicyclic ring system, one five-membered and the other six-membered, that are not fused, but rather coupled by simple carbon–carbon linkage. Second, formation of the five-membered ring can result in the methyl substituent at C7 being in one of two stereo configurations. Trichodiene synthases appear to produce exclusively the 6*S*-enantiomer of the trichodienyl carbocation,[3] while the recently characterized α-barbatene synthase from a plant generates the 6*R*-enantiomer shown in **Scheme 9**.[69] The possibility that the (6*R*)-trichodienyl carbocation precursor to α-barbatene actually arises from the 7S stereoisomer of the cuparenyl carbocation (**25**) cannot be excluded at this time. Lastly, generation of the key cuparenyl carbocation intermediate (**25**) requires a unique 1,4-hydride shift, which cannot proceed



Scheme 9

by successive 1,2 shifts, because of the quaternary carbons at C7 and C11. Instead, the 1,4-hydride shift must occur directly between carbons 6 and 10 without the involvement of any intervening carbons, and because C6 and C10 are positioned in close enough proximity to one another, in the proper stereoorientation (the so-called synperi-planar orientation), to allow for the shift to occur. It should also be noted that the 1,4-hydride shift converts a high-energy secondary carbocation to the much more stable tertiary isomer (**25**). Alternatively, quantum mechanical calculations[70] indicate that this overall transformation might occur in two steps by a lower-energy mechanism involving proton transfer from C6 to C10 of (**24**) to form an isomeric bisabolyl carbocation followed by 7–11 cyclization to cuparenyl ion (**25**). Two 1,2 methyl migrations and a regiospecific proton abstraction of the rearranged cuparenyl intermediate thus yields trichodiene (**26**).

Experimental evidence for trichodiene chemical rationalization comes for diverse approaches including inhibitors designed to mimic proposed intermediates,[71–74] site-directed mutagenesis,[75] structural elucidation of the trichodiene synthase with and without ligand binding,[57] and combinations of these efforts.[71,76,77] The three-dimensional structure determined by Rynkiewicz *et al.*,[57] for the *Fusarium sporotrichioides* trichodiene synthase pointed out the importance of three metal cofactors binding near the entrance to the active site, the binding coordination between these metal cofactors and the diphosphate substituent of FPP, and this work identified a significant conformational change that occurred at the entrance into the active site cavity upon ligand binding. These authors were also able to model various intermediates into the enzyme's active site and suggest ways that FPP binding might induce conformation changes within the active site protecting carbocation intermediates, yet positioning active site residues, particularly hydrophobic ones, to coax intermediates down a particular cyclization cascade. Development of substrate analogs to inhibit particular steps in the trichodiene synthase-catalyzed reaction has been described and demonstrated to induce conformation changes in the enzyme much like substrate binding.[71]

However, given the large number of mutants created and critically evaluated, few if any of these mutants have terminated the reaction cascade at a particular step and resulted in the predominate production and release of one intermediate. Instead, mutants like N225D or Y295F exhibited a small increase in the amount of cuparenene (2.4% increased to 10.4%) or bisabolene (0.9–17.5%) released, respectively, while still accumulating the lion's share of product as trichodiene.[76] This type of observation is not particularly surprising once one considers that it is the contour of the active site and not particular active site residue interactions with reaction intermediates that direct catalysis.

The cuparenyl carbocation (**25**, **Scheme 9**) is the likely progenitor of many other important families of sesquiterpenes, including those in the bazzanane, gymnomitrane, and chamigrane families, illustrated by α-barbatene (**27**) (a gymnomitrane member) and thujopsene (**28**) (a chamigrane member). These sesquiterpene families appear to be highly represented in the lower plants, especially liverworts,[78] and until recently were not considered common in higher plants. Two independent efforts recently identified an *Arabidopsis* α-barbatene synthase, one using a reverse genetics approach[79] and the other a novel mechanism for functionally characterizing genes identified in genome sequencing projects.[69] Interestingly, the plant enzyme generates three major products in roughly equal amounts, along with eight or more minor products. This is distinctly different from the *Fusarium* trichodiene synthase that biosynthesizes predominately one reaction product, trichodiene, in greater than 82% yield. However, given the notion from Cane's and Christianson's[5] work that active site geometry dictates reaction product specificity for this synthase, a significant question is whether a single enzyme species can yield multiple products or if there are distinct enzyme conformers responsible for each type of reaction product. The former explanation would suggest that the active site cavity is large enough and flexible enough to accommodate a large geometric variation in the intermediates, but that some dynamic in the protein–ligand structure imparts a particular geometric tunnel (catalytic cascade) to a substrate once it is bound. The latter explanation would suggest that the folding algorithms for a particular amino sequence result in slightly but significantly different protein conformers, and that the relative abundance of a particularly folded conformer would correspond to the relative synthesis rate for a particular reaction product. The current information is insufficient to distinguish between these possibilities. However, given the preponderance of these families of sesquiterpenes in liverworts, it will be very interesting to examine the corresponding synthases for conservation of amino acid sequences and functional conservation in terms of their catalytic and reaction product specificities.

1.16.2.4.3 The cadinane series

Gossypol (**29**) and artemisinin (**30**) (see **Figure 4**) are two of the most prominent members of the cadinane family of sesquiterpenes. Gossypol consists of two highly oxygenated forms of cadinene (hemi-gossypol) joined together by a binaphthyl linkage to form a potent toxin that affords an unparalleled level of insect resistance to cotton plants, *Gossypium barbadense*, and related species.[80] Gossypol accumulates in lysigenous glands found in stems, leaves, and the cotyledons of seeds and knockout mutants of cadinene synthase, the key enzyme dedicating FPP to gossypol biosynthesis, does result in cotton plants much more susceptible to microbe and insect predation, yet the seed meal resulting after oil extraction is now suitable for animal consumption.[81,82] Desoxyhemigossypol and hemigossypol also accumulate in wild-type cotton plants and cells in response to fungal pathogen or elicitor challenge, serving as phytoalexins or defense compounds to microbial infection.[83]

Artemisinin (**30**) is a potent antimalaria drug now being developed from *Artemisia annua*.[84] *A. annua* has been used for hundreds of years in traditional Chinese medicines and teas to treat a variety of ailments including malaria, and artemesinin, a cadinane-type sesquiterpene containing a unique peroxide bridge, was subsequently identified as the active ingredient. Artemisinin appears to be synthesized in leaf/floral trichomes, 4–8 cell surface structures that accumulate and secrete compounds mediating plant–environment interactions. Extensive efforts are currently underway to build high-yielding production platforms for artemisinin using transgenic plants[85] and fermentable organisms;[86] hence considerable information about the genes responsible for the artemisinin biosynthetic pathway and its metabolic engineering is now available.[87]

The biosynthesis of gossypol proceeds from FPP via a δ-cadinene intermediate (**32**), while that for artemisinin utilizes amorph-4,11-diene (**33**, **Scheme 10**). And while these two intermediates share overall structural similarities common to all cadinanes, these sesquiterpene scaffolds now appear to be formed by distinctly different mechanisms. In the case of δ-cadinene, the cisoid farnesyl carbocation derived from (*E,E*)-FPP (**Scheme 7**) undergoes an initial C1–C10 ring closure to generate the macrocyclic germacradienyl intermediate (**31**) having the proper stereochemistry found in the final cadinene compound. A 1,3-hydride shift repositions the carbocation to facilitate a second ring closure, a C1–C6 cyclization, to yield a putative cadinenyl carbocation. A final proton abstraction from C6 releases the final δ-cadinene product (**32**).

The rationale for the *cis*-germacradienyl pathway has been challenged by the possibility of a bisabolyl pathway (**Scheme 10**).[88] In this scenario, the first ring closure is a C1–C6 cyclization, followed by a 1,3-hydride shift and the second C1–C10 ring closure. However, a conceptual difficulty with this pathway is the generation of the carbocation centered at C11 and the necessity for the charge to migrate to C7 in order to allow for the final proton abstraction at C6. Although 1,5-hydride shifts are known to occur readily in long-lived carbocations in solution, the 1,5-hydride shift illustrated in **Scheme 10** would require that the disubstituted cyclohexane ring adopt a high-energy boat conformation with the methyl and isopropyl on the prow positions. Nonetheless, this option must be considered because of additional experimental observations. Although incubation of δ-cadinene synthase with FPP yielded 98% δ-cadinene as the sole reaction product, incubation of this enzyme with NPP (**17**) yielded 62.1% δ-cadinene, 8.4% β-bisabolene, 15.8% α-bisabolene, and 9.8% farnesene.[88] Hence, either this enzyme utilizes a bisabolyl cyclization cascade and incubation with NPP supports the premature release of reaction intermediates, or the enzyme must possess the capacity to catalyze distinct cyclization cascades independently.

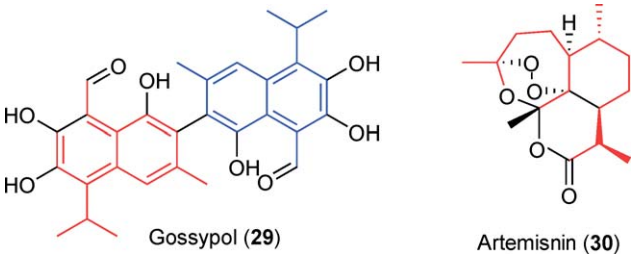
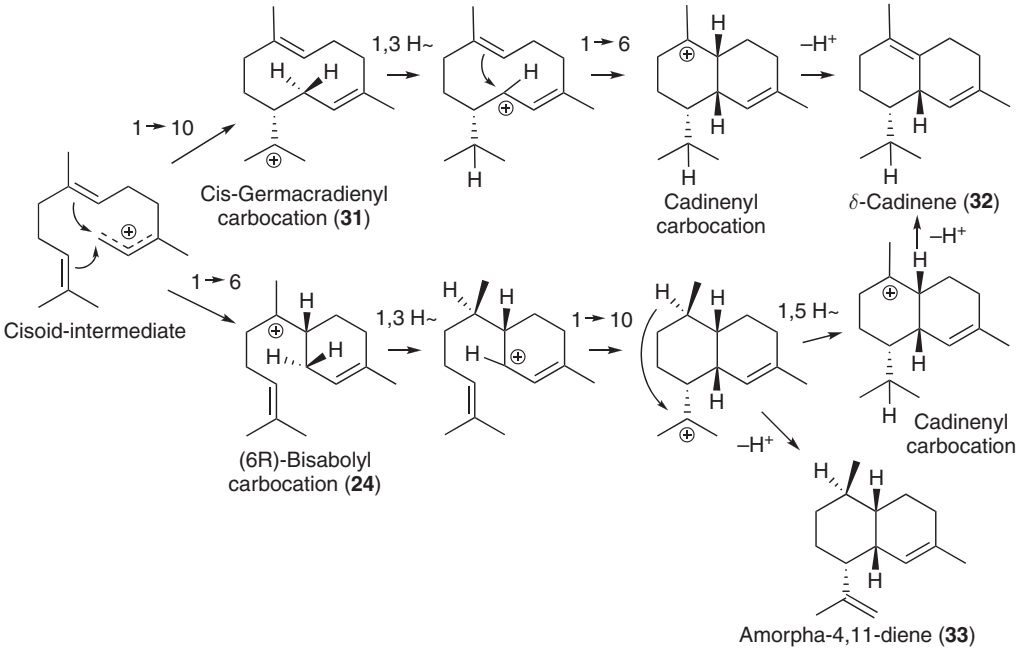


Figure 4 Structures of gossypol and artemisinin.



Scheme 10

In contrast, Yoshikuni *et al.*[89] have provided compelling evidence for the germacradienyl pathway leading to δ-cadinene using a molecular mutagenesis approach. These investigators sought to evoke new catalytic potential from the δ-cadinene synthase by subjecting the gene to a random mutagenesis protocol, then screening the mutant enzymes for their catalytic specificities. Importantly, these investigators observed the development of a germacrene D-4-ol synthase activity within independently derived, mutant cadinene synthase enzymes, but never observed the evolution of a mutant capable of synthesizing bisabolene-type sesquiterpenes. One interpretation of these results is that the authors only generated mutants that terminated catalysis after the initial C1–C10 ring closure and, because of a fortuitously positioned water molecule in the active site, the reaction was terminated upon water capture.

Conversely, the bisabolene pathway seems likely to be the prevailing mechanism operating within the amorphadiene synthase (ADS), and the germacradienyl pathway does not appear to be present in this enzyme. Picaud *et al.*[90] reasoned that because standard incubations of ADS with FPP reliably yielded small amounts of bisabolene-type reaction products, these might be the result of a premature quenching of the reaction cascade via a bisabolene pathway to amorphadiene. In contrast to the observation of Yoshikuni *et al.*[89] with cadinene synthase, Picaud *et al.* also noted the lack of any germacrane-type reaction products in any of their enzyme incubations. These investigators also noted an interesting parallel that incubation of ADS with GPP resulted in several cyclic monoterpene products while EAS, another sesquiterpene synthase previously demonstrated to utilize a germacrenyl intermediate for sesquiterpene biosynthesis,[51] only generated acyclic monoterpenes, consistent with the utilization of an isomerization step by ADS. Following up on these observations, Picaud *et al.* then prepared specific deuterium-labeled FPP and followed the incorporation of the deuterium into the amorphadiene product by NMR analyses. The labeling patterns were fully consistent with the bisabolene cyclization cascade.

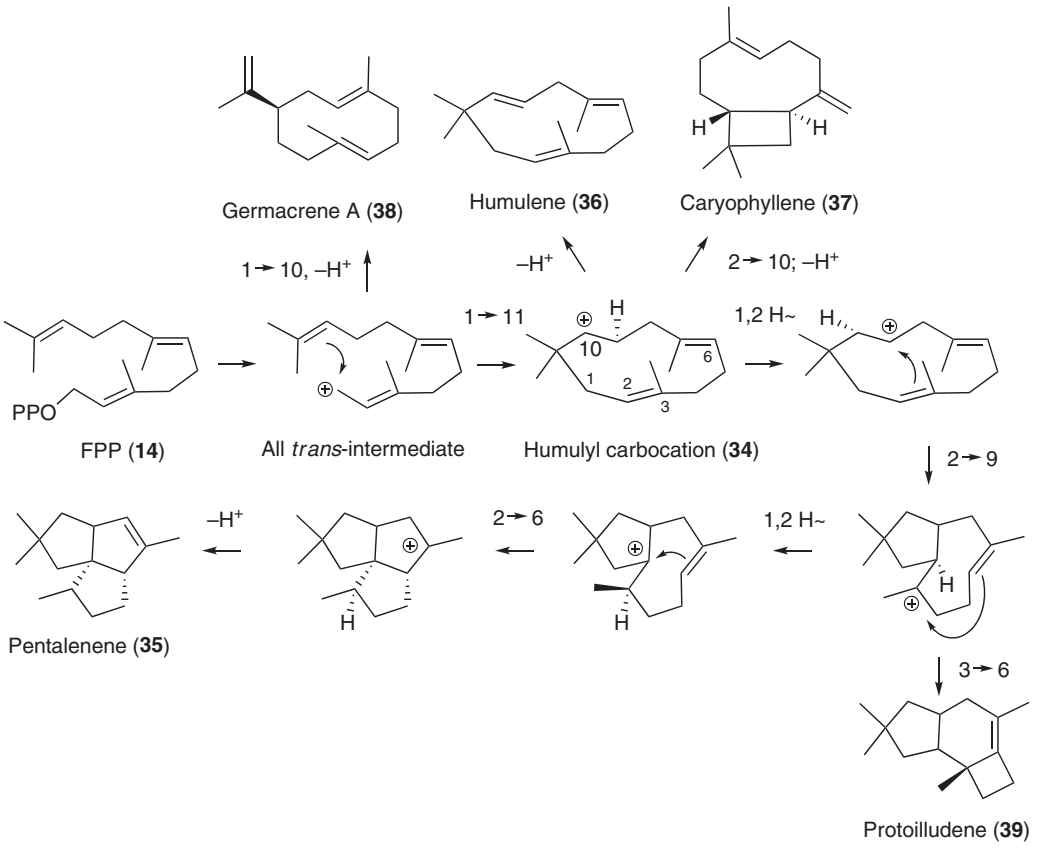
The observation that the cadinane family of sesquiterpenes arises from synthases using different reaction mechanisms shakes our intuitive notions about the origins of this family of compounds. Most researchers in the field naturally assumed that the key synthase enzymes for this class of compounds would rely on a common biosynthetic mechanism, and that somehow this mechanism must have been fixed and selected for over evolutionary time. This is clearly not the case, at least as it pertains to the cadinane family of sesquiterpenes, and may indicate independent evolution of these capacities in plants. Cotton, the species

representing cadinene synthase, is in Malvaceae, which split from Asteraceae, of which *A. annua* is a member, some 113 million years ago.[91] Whether the terpene synthase enzymes found in these extant species arose from a common ancestor or from independent ancestors does not seem to be the key issue here, but rather more fascinating is the development of enzymes producing sesquiterpenes within a common chemical family via distinctly different reaction mechanisms.

1.16.2.4.4 The humulane series

Humulene and caryophyllene are representative of the sesquiterpenes derived from an initial macrocyclic intermediate formed by a C1–C11 ring closure. These sesquiterpenes, like the germacrene series discussed below, are distinguished by being directly derived from the all-*trans* FPP (**14**) substrate and not requiring isomerization to the cisoid conformation or nerolidyl diphosphate intermediate. And in this regard the humulane sesquiterpenes might be considered the simplest of sesquiterpenes. However, their biosynthesis utilizes what appears to the same conserved mechanisms as imposed by all terpene synthases as illustrated by the in-depth studies of pentalenene synthase.

The chemical rationalization put forward by Cane[2] for pentalenene (**35**) formation is depicted in **Scheme 11**. FPP is initially ionized, generating the carbocation positioned in proximity to the pi orbital between C10 and C11, thus allowing for an anti-Markovnikov ring closure between C1 and C11. Proton abstraction from the humulyl carbocation intermediate (**34**) results in formation of the macrocyclic triene humulene (**36**). A second ring closure between C2 and the carbocation centered at C10 prior to a final proton abstraction is also possible to yield the unique sesquiterpene caryophyllene (**37**), having a *trans*-fused bicyclo[7.2.0]undecane nucleus. If the humulyl carbocation is however constrained to a third possible conformation,



Scheme 11

a 1,2-hydride shift is the preferred next step leading to a ring closure between C2 and C9. A second hydride shift, followed by a third ring closure within the cyclooctene ring, and the final proton abstraction yields the pentalenene final product.

Although investigated extensively using very sophisticated approaches of structural biology[59] and site-directed mutagenesis,[92] the catalytic cascade for pentalenene biosynthesis is largely an inferred pathway. For instance, a humulyl intermediate has not been observed. Instead, mutagenesis informed by the three-dimensional structure for pentalenene synthase[92] and parallel studies with other terpene synthases lead to mutant enzymes that yielded significant levels of germacrene A (**38**), sometimes in combination with protoilludene (**39**), another possible premature termination product, with a direct and proportional decline in pentalenene biosynthesis. The biosynthesis of germacrene A by the mutant enzymes is significant because it suggests a flexibility within the active site allowing for the initial ring closure between C1 and either C10 or C11. This flexibility may result as a consequence of flexibility in the spatial orientation of amino acids making up the surface contour of the active site. A similar argument can be made for the biosynthesis of protoilludene by some of the mutants, although in this case a greater conformational constraint would have to be imposed upon the putative bicyclic intermediate by the mutant enzyme in order for the reaction to proceed to the protoilludene product.

Tantillo and coworkers[70,93–95] have attempted to provide additional insight into several terpene synthase reactions, and in particular for the pentalenene synthase reaction, by applying a quantum chemical approach. Suggested intermediates were first energy-minimized, transition states between intermediates were sought using various density functional algorithms, and then predicted catalytic cascades were assembled. Overall, a net exothermic reaction of 41–52 kcal mol^{-1} for converting the humulyl carbocation intermediate (**34**) to pentalenene was calculated with relatively small energetic barriers between transition states. One consequence of the algorithms used in these calculations however was the suggestion of two catalytic paths, neither of which is identical to that shown in **Scheme 11**. One alternative positions a protoilludyl carbocation intermediate into the reaction cascade prior to a final reorganization of the fused six- and four-membered rings into two five-membered rings. The second proposal inserts a hyperconjugated intermediate, a secoilludyl carbocation, which rearranges to another high-energy intermediate positioning a proton between two C═C double bonds, which then leads to the penultimate pentalenyl carbocation. Gutta and Tantillo[95] recognize the speculative nature of these alternative pathways and considered the original pathway as suggested by Seemann *et al.*[92] more likely based on experimental observations with genetic mutants. Regardless of the catalytic cascade, the calculations by Gutta and Tantillo point to the penultimate step as that having the greatest energetic barrier once the diphosphate substituent has been ionized from FPP, but for the most part, transition barriers to be rather modest and readily overcome. These observations, the authors suggest, indicate that the pentalenene synthase active site architecture not only serves to promote and stabilize specific intermediates and transition states, but it must also impose destabilizing forces to disfavor other intermediates/transition states. The possibility that the proposed hydride shifts might occur by equivalent proton transfers suggests a more direct role of the enzymes in catalysis.[70] In the present case (**Scheme 11**), the 1,2-hydride shift might take place by successive proton transfers to and from the synthase active site with participation of an enzyme-bound humulene intermediate.

These notions have been experimentally tested. Previously, Steele *et al.*[96] made the surprising observation that several terpene synthases from grand fir (*Abies grandis*) catalyzed the generation of over 30 reaction products when incubated with FPP. Because of the multitude of reaction products, these enzymes were described as promiscuous, which Yoshikuni *et al.*[97] interpreted as synthases possessing active sites allowing for a wide range of catalytic cascades. Focusing on the *A. grandis* α-humulene synthase as a template, these investigators first identified 19 putative active sites within the α-humulene synthase on the basis of molecular modeling against the EAS crystal structure.[55] They then introduced mutations into all of these sites, not one mutation at a time, but in a combinatorial fashion and evaluated the reaction products of each by GC-MS. Interestingly, while the wild-type enzyme generated approximately 28% α-humulene, 15% sibirene, 11.8% longifolene, and lesser amounts of many other sesquiterpenes like 3.9% for β-bisabolene, single and combinatorial mutants catalyzed reactions dominated by single reaction products (**Figure 5**). Like Gutta and Tantillo,[95] the work of Yoshikuni *et al.* suggests that the geometry of the active site plays a role in defining not only what reactions are allowed, but what reactions are not allowed.

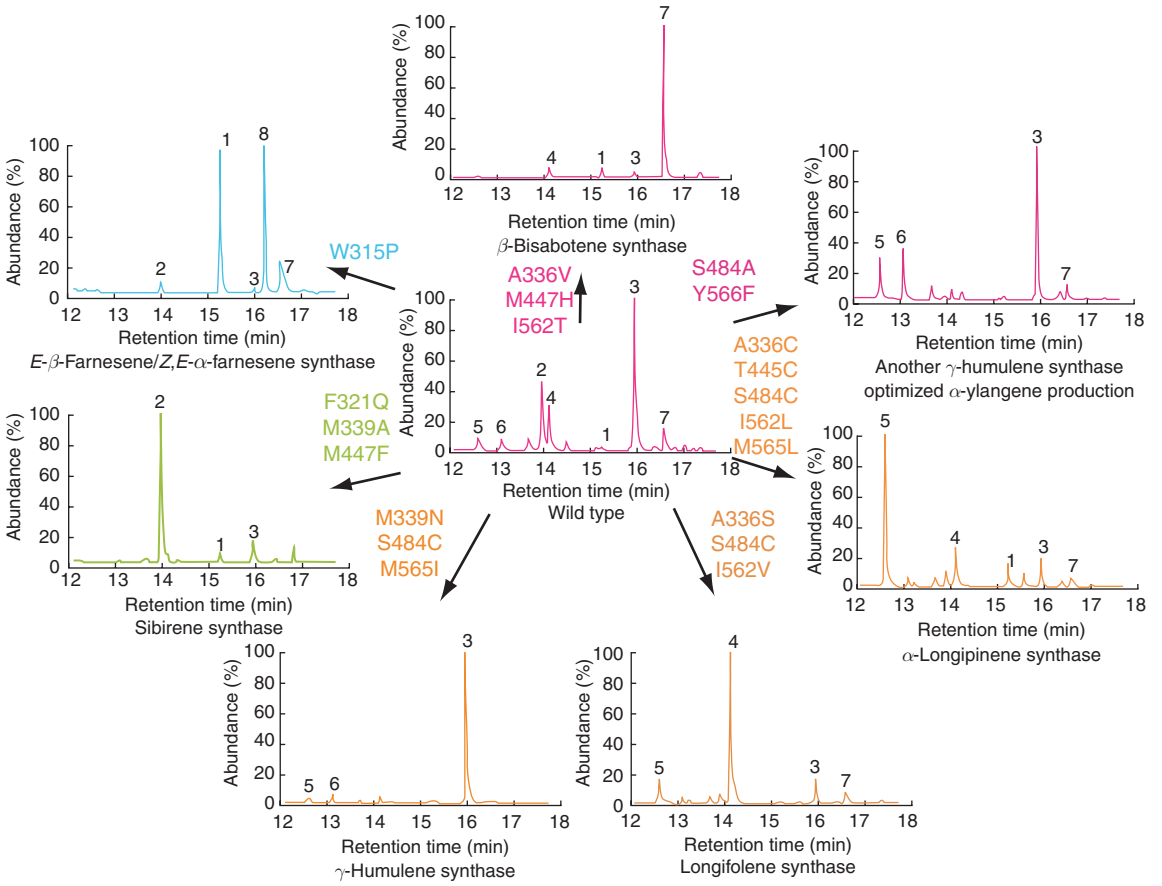
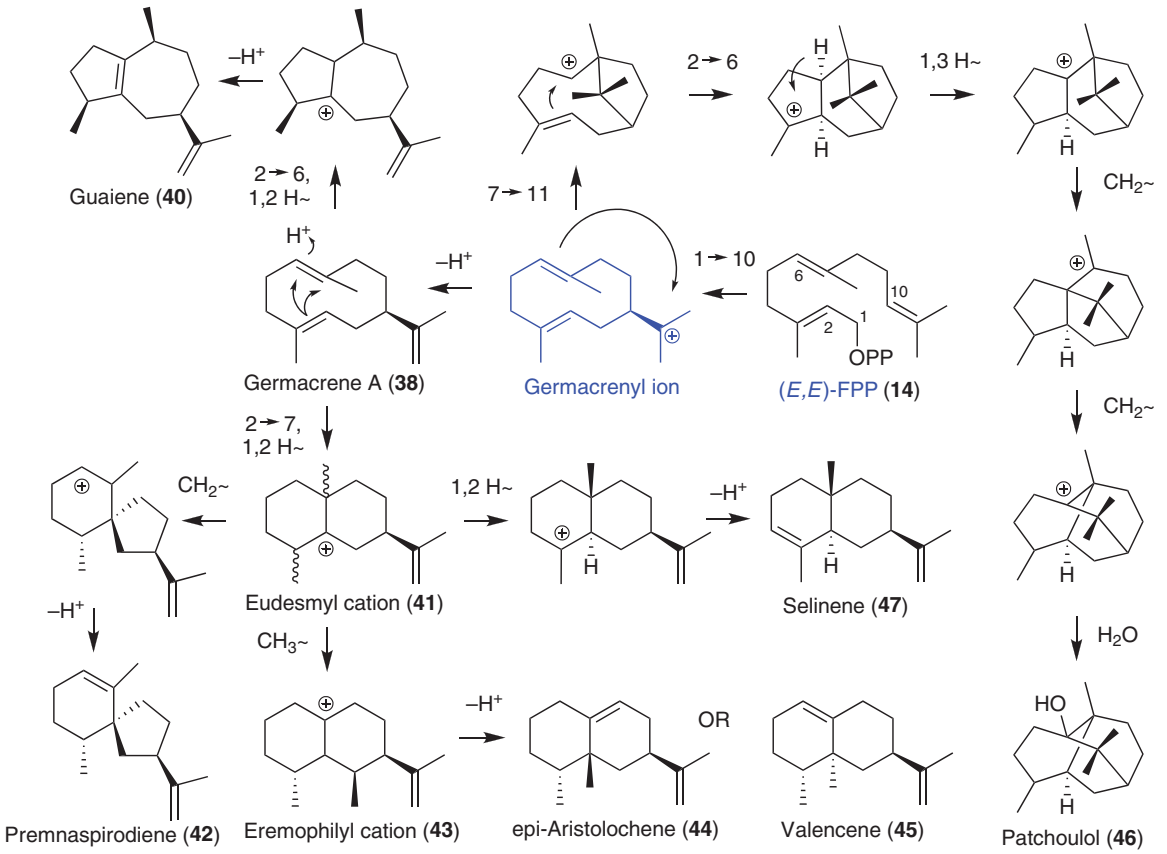


Figure 5 Narrowing down reaction product specificity in mutated forms of α-humulene synthase. Reproduced from Y. Yoshikuni; T. E. Ferrin; J. D. Keasling, *Nature* **2006**, *440* (7087), 1078–1082.

1.16.2.4.5 The germacrane series

Although one may not recognize it, the germacrane-derived sesquiterpenes are some of the most common compounds we interact with in our environment on a daily basis. Patchoulol (**46**) is the key fragrance in many perfumes, colognes, soaps, and all kinds of cleaning products. Nootkatone, the C2 oxidation product of valencene (**45**), is the key citrus flavor in many of our processed foods and soft drinks. Less common, but no less appreciated, are those derivatives like rishitin and capsidiol (**1**), sesquiterpene phytoalexins derived from epi-aristolochene (**44**) by solonaceous plants in response to pathogen challenge and possessing antimicrobial activities.[98] The germacrane sesquiterpenes are also produced by fungi and bacteria. For instance, geosmin (see **Scheme 14**), produced by *Streptomyces coelicolor*, is a major contributor to the odor of soil.[99,100] Aristolochene (**48**, see **Scheme 13**) produced by *Penicillium roqueforti* and *Aspergillus terreus* serves as the precursor to highly oxygenated mycotoxins, like the PR-toxin.[101]

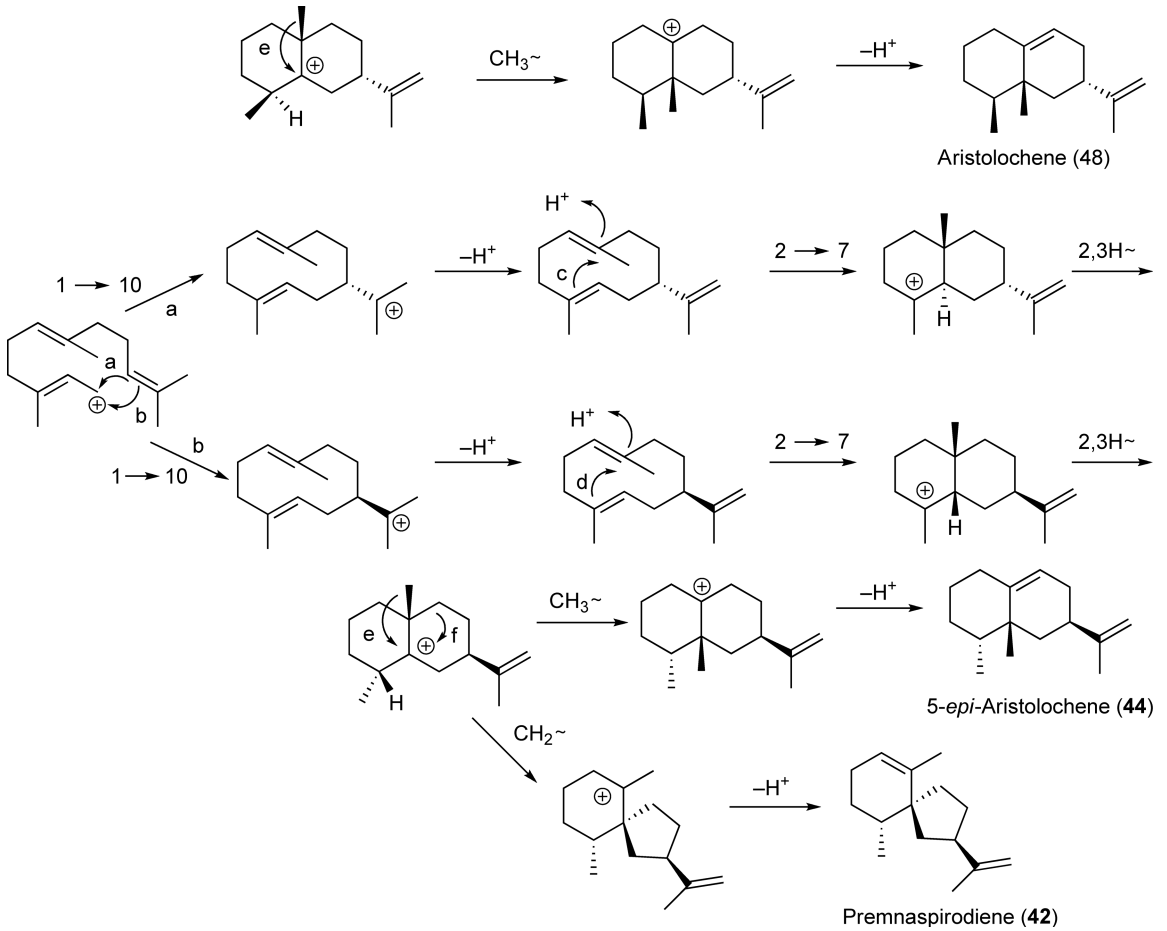
The biosynthesis of the germacrane family of sesquiterpenes has been extensively investigated, accelerated in large part by successes in obtaining and heterologously expressing a wide range of plant and microbial genes coding for the corresponding terpene synthase enzymes.[102–104] These advances have enabled an amazing array of experimental approaches, from presteady-state kinetics[52,53] to solving and rationalizing three-dimensional structures with function.[55,58]. The key chemical entry point into this class of sesquiterpenes is initiated by a C1–C10 ring closure of the all-*trans* farnesyl carbocation. This is an important distinction with other classes of sesquiterpenes, especially those that rely on an isomerization about the C2═C3 double bond prior to the first ring closure reaction. As illustrated in **Scheme 12**, this first ring closure yields an (*E*,*E*,*R*)-germacrenyl carbocation (**39**) that then undergoes a multitude of catalytic pathways to a very diverse range of molecules.



Scheme 12

These include macrocyclic derivatives (i.e., germacranes), bicyclic compounds with fused, bridged, and spiro five-, six-, and seven-membered ring systems (compare guaiene (**40**) and premnaspirodiene (**42**)), bicyclic forms differentiated by how methyl substituents are regio- and stereochemically arrayed about the ring systems (illustrated by the dashed bonds for the eudesmanes and eremophilanes), and compounds consisting of complex bridged ring systems like patchoulol (**46**). The generation of this diversity arises from partial steps or reactions that have already been discussed with regard to other sesquiterpene classes: C–C bond formation mediated by electron flow from double bonds to carbocations; hydride, methyl, and methylene migrations; proton donations and abstractions; and water capture.

The dissection of the reactions leading to aristolochene (**48**), 5-epi-aristolochene (**44**), and premnaspirodiene (**42**) are illustrative of our understanding of the biochemical processes contributing to the germacrane family of sesquiterpenes (**Scheme 13**). Interestingly, some of the first terpene synthases genes to be cloned were those for a fungal aristolochene synthase (AS)[103] and 5-epi-aristolochene synthase (EAS)[105] from a higher plant. Although the two enzymes share very little similarity in primary sequence alignments (<40% similarity), both proteins adopt what has become known as the characteristic 'terpene synthase fold'.[55,56,58] From a chemical perspective, the initial C1–C10 cyclization of the farnesyl carbocation occurs to establish the first chiral center at C10 with the isopropenyl substituent taking on opposite orientations, followed by proton abstractions from the *cis* terminal methyl groups (C13).[105,106] Given the geometric differences between these two reaction intermediates, the enzyme active site surfaces must be very different from one another in order to stabilize one configuration of the germacradienyl intermediate (**39**) versus the other. A similar inference is also suggested to account for the selective stereochemistries created for the methyl substituents at C3 and C7 as a result of the second ring closure reaction.



Scheme 13

Evidence for a germacrenyl intermediate in the initial step of catalysis comes from two different approaches. First, incubation of the fungal AS with 6,7-dihydroFPP, an FPP analog with the C6═C7 double bond saturated, yields 6,7-dihydrogermacrene as the sole reaction product.[107] Similar results were observed using fluorofarnesyl diphosphate analogs with either the plant[108] or fungal[109] enzymes. In both of these cases, a fluorogermacrene A derivative was the only reaction product, an outcome attributed to a premature termination product of catalysis. The second approach relied on molecular modeling according to the crystal structure of EAS, which lead to site-directed mutagenesis of an active site residue positioned to serve as the proton donor initiating the second ring closure steps. When tyrosine 520 was mutated to phenylalanine in EAS, the only reaction product was germacrene A (**38**).[51] An analogous mutation in the *P. roqueforti* AS enzyme, tyrosine 92 to phenylalanine, resulted in germacrene A biosynthesis as well.[110] However, unlike the EAS mutant, the Y92F AS mutant accumulated substantial amounts of aristolochene, prompting further investigations concluding that Y92 was probably not responsible for donating a proton to the germacrene A intermediate and initiating the second series of carbocation-mediated reactions.[111] Instead, acidic residues of the *Pencillium* AS enzyme involved in coordinating and binding the divalent metal cofactors and thought to facilitate binding of the diphosphate substituent of FPP and possibly aligning the allylic tail into the active site, were shown to be crucial for the conversion of germacrene A to aristolochene.[111] Other AS mutants yielded germacrene A in addition to several other unexpected derivatives, providing further evidence for how active site residues might serve to stabilize and promote particular transformations.[112,113]

Abstraction of a proton from the *cis* C13 CH_3 in generating the germacrene A intermediate and the possible recycling of this proton to initiate a second round of catalysis has also been examined. Schenk *et al.*[114] prepared

FPP bearing deuterium and tritium at C13, then, after incubation with EAS or the *Hyoscyamus muticus* premnaspirodiene synthase (HPS), examined the retention of label in epi-aristolochene and premnaspirodiene and its possible redistribution to other positions in the final reaction products by NMR analyses. No recycling for the labels was observed, but a clear secondary kinetic isotope effect on the reaction rate was noted, fully consistent with a concerted cyclization and proton elimination mechanism leading directly from FPP to germacrene A.[114]

The stereoselective incorporation of one deuterium atom into the 6β position of epi-aristolochene following incubation of FPP with EAS in fully deuterated buffer established a *trans* antiperiplanar alignment in the protonation–cyclization of the 6,7 and 2,3 double bonds of the germacrene A intermediate in the conversion to the following *cis*-eudesmyl carbocation intermediate.[114] This work also demonstrated the predominant loss of deuterium after incubation of (*S*)-[8-$^{2}H_{1}$]FPP with EAS, and complementary label retention in the incubation of the enantiomeric labeled FPP, proving that the pseudo-axial allylic hydrogen at C8 is eliminated in forming the ring double bond of epi-aristolochene.

One of the implicit goals driving much of the molecular dissection of terpene biosynthetic enzymes has been that such knowledge might lead to the rational design of enzymes that generate new desired products. While we are still far from such a goal, progress in defining the roles of domains and residues in the catalytic process have been significant.[89,97,115–117] In this regard, the molecular comparisons of EAS, the plant enzyme responsible for epi-aristolochene biosynthesis, to HPS, the enzyme from *H. muticus* responsible for premnaspirodiene production have been particularly useful. As illustrated in **Scheme 13**, the four steps leading up to the *cis*-eudesmyl carbocation intermediate are identical for both these enzymes. They then differ with either a methyl (EAS) or a methylene (HPS) migration, followed by a proton abstraction at C8 (EAS) or C6 (HPS). The EAS and HPS enzymes share greater than 70% primary sequence identity, and very interestingly, separate regions of each protein were shown to mediate the methyl versus methylene migrations in earlier domain-swapping experiments.[115] Based on the crystal structure for the EAS enzyme and molecular modeling of the HPS enzyme upon that scaffold, the active sites cavities for both EAS and HPS were predicted to be identical. This surprising result argued that catalytic specificity must lie outside the active site and most likely with residues that impinge upon the active site residues to alter the active site geometry and architecture.

To discern which residues might be playing a key role in directing methyl versus methylene migrations, Greenhagen *et al.*[116] mapped the active site residues within a van der Waals radius (3.4 Å) of each carbon of a farnesyl analog also resolved within the EAS crystal structure (**Figure 6**). Then the residues outside the active site residues, the so-called second-tier residues, those that were within a van der Waals radius of the first-tier (active site) residues were mapped. Continuation of this contact mapping exercise was extended to a third tier of interacting residues, and this information was then compiled into a comparative algorithm. As shown in a left-hand column of the upper panel of **Figure 6**, the second-tier residues of EAS interacting with the active site residues are arrayed and the specific interactions are denoted by a corresponding solid sphere in the body of the table. The second vertical column in **Figure 6** lists only the second-tier residue differences in HPS generated by molecular model comparisons of HPS and EAS. These differences, highlighted in red, are tracked forward to identify which carbons within FPP are affected. These residues were then targeted for mutagenesis. That is, the identified residues within EAS were mutated to those found in HPS at the corresponding positions, and the converse mutations were effected as well.

When nine of the second- and third-tier residue differences in HPS were introduced into EAS, the M9-EAS mutant enzyme synthesized a reaction profile dominated by premnaspirodiene with very little epi-aristolochene (**Figure 7**). Substituting eight of the same positions within HPS for the corresponding amino acid found in EAS was sufficient to convert the M8-HPS reaction profile to that of EAS (**Figure 7**). Both the M9-EAS and M8-HPS enzymes were comparable to the wild-type enzymes in terms of their catalytic properties, that is, K_m and k_{cat} values.

The notion that specific residues in specific positions within the protein regulate discrete reaction coordinates is of course appealing because it suggests that diversification of the synthase reactions is simply a matter of what amino acids are in these key positions. Unfortunately, this notion is too simplistic. First, what has been reported is that mutations in these particular positions are sufficient to interconvert the reaction specific to the respective enzymes. That does not exclude the possibility that other combinations might be equally or more effective. In fact, O'Maille *et al.*[118] recently tested this possibility by preparing a combinatorial library of tobacco epi-aristolochene synthase (TEAS) mutants, with various combinations of mutations at these nine positions, and demonstrated that more than one combination of mutants was able to interconvert the reaction specificities between TEAS and HPS.

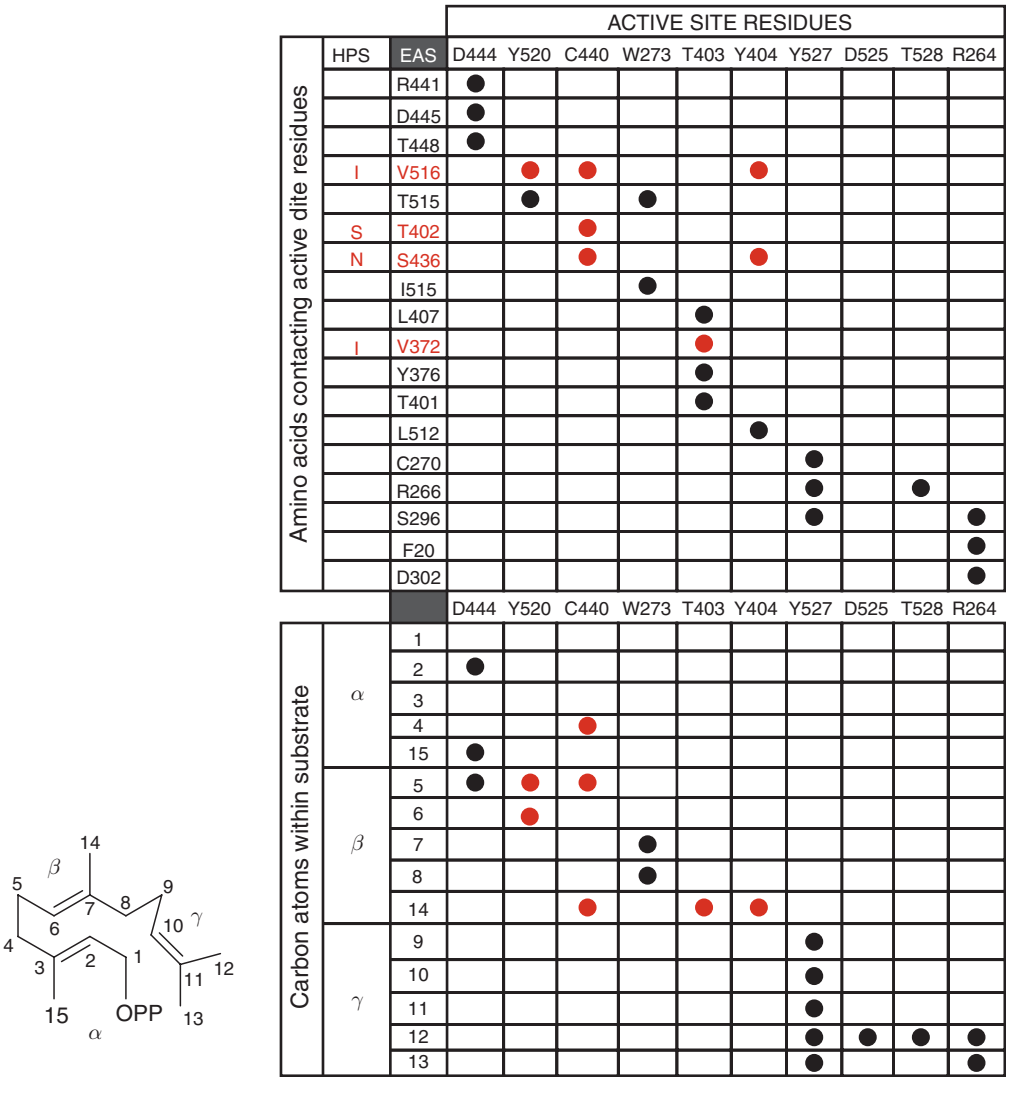
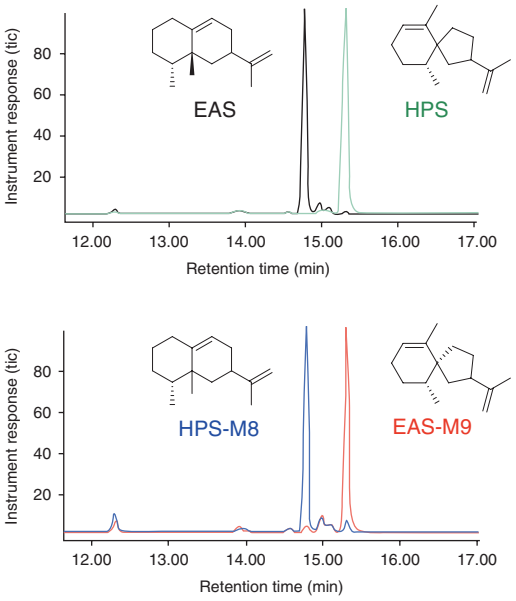


Figure 6 An algorithm developed by Greenhagen *et al.*[116] to map how active site residues of EAS impinge upon specific carbons of FPP (left) bound in the reaction pocket, and then how residues outside the active site, second-tier residues, impinge upon the active site residues.

Equally important, none of the 518 mutants in this collection produced a novel reaction product. That is, the mutants produced products evident in the parental types, but varied in their relative amounts and ratios relative to one another. This would imply that what Greenhagen *et al.*[116] identified were positions within particular synthases important for two particular types of chemical transformations, methyl versus methylene migrations. Whether these positions could play a similar role in synthases catalyzing the biosynthesis of other class of sesquiterpenes, or even how these positions might influence catalysis with substrate analogs modified by regio- and stereochemical variations, remain unknown.

1.16.3 Decorating the Sesquiterpene Scaffolds

Many sesquiterpene hydrocarbons and especially some of those that are synthesized in reactions that terminate the catalytic cascade by water capture forming C_{15} alcohols are potent fragrances. Patchoulol (**46**, **Scheme 12**), already mentioned above, is an extremely popular fragrance in colognes, perfumes, and a wide assortment of



% of the reaction products formed

Synthase/ mutant	epi-Aristolochene	epi-Eremophilene	Premnaspirodiene
EAS	96	<4	<2
EAS-M2	28	70	<2
EAS-M4	16	33	18
EAS-M5	23	24	27
EAS-M7	11	19	43
EAS-M9	3	8	71
HPS	0	<1	98

Figure 7 Identification of the residues controlling reaction product specificity within EAS and HPS. Greenhagen *et al*.[116] identified first-, second-, and third-tier residues serving to create the geometric surface of the active site of two sesquiterpene synthases, EAS and HPS, and then created reciprocal mutants in each. That is, these investigators substituted those contact residues of HPS into EAS and vice versa, creating up to nine mutations (M9), and evaluated each mutant enzyme for its reaction product specificity by GC-MS. Adapted from B. T. Greenhagen; P. E. O'Maille; J. P. Noel; J. Chappell, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (26), 9826–9831.

cleaning reagents. However, in terms of biological activities, some of the most active sesquiterpene scaffolds are further modified, often by at least some form of oxygenation, but also by halogenation,[119] acetylation,[120] and glycosylation.[121] Two notable examples illustrate this point nicely. First, artemisinin (**30**, **Figure 3**) is a highly oxygenated sesquiterpene containing a unique peroxide bridge, derived initially from the cadinene scaffold amorpha-4,11-diene (**33**, **Scheme 10**).[84] Artemisinin is a potent antimalaria drug the use of which is currently limited by its availability. Second, solanaceous plants defend themselves against microbial challenges by producing oxygenated germacrene-derived sesquiterpenes like capsidiol, solavetivone, rishitin, and lubimin.[98] Although progress in defining the biochemical mechanisms responsible for these secondary modifications (decorations) has lagged behind that for the synthases, recent efforts have focused on the identification and characterization the oxygenation reactions. The rationale for this focus is in part because the hydroxyl substituents provide the handles for subsequent modifications, like O-glycosylations and acetylations.

Figure 8 illustrates examples of recently reported oxidation reactions for bacteria, fungi, and plants. Elucidation of the pentalenolactone antibiotic biosynthetic pathway has benefited from detailed bioinformatic inspection of the *Streptomyces avermitilis* genome,[122] and especially the identification of a gene cluster responsible for pentalenolactone biosynthesis. You *et al*.[68] subsequently demonstrated that the ptlH gene within this cluster encodes for a nonheme iron dioxygenase that inserts a hydroxyl function at C11 of only the putative deoxypentalenic acid intermediate, but not pentalenene or a C13 hydroxylated form. Oxidation of sesquiterpenes however is more commonly mediated by cytochrome P-450 oxidases. The albaflavenone antibiotic produced by *S. coelicolor* is initiated by successive hydroxylations of epi-isozizaene at the C4 position to yield the cisoid α,β-unsaturated ketone albaflavenone.[123] The reaction is catalyzed by a typical prokaryotic P-450 enzyme designated CYP170A1, which was found clustered with the corresponding sesquiterpene synthase gene. In contrast to the specificity found in plant P-450 hydroxylases (see 1.16.3), CYP170A1 did not exhibit much specificity for the stereochemistry of the first hydroxylation.

Multifunctional hydroxylases are also found in fungal species. Trichothecene mycotoxins in *Fusarium* species originate from a bisabolene sesquiterpene scaffold, trichodiene (**26**, **Scheme 9** and **Figure 8**) and include hydroxyl groups, oxygen heterocycles, and several O-acetylated substituents.[62,63] The DNA sequence

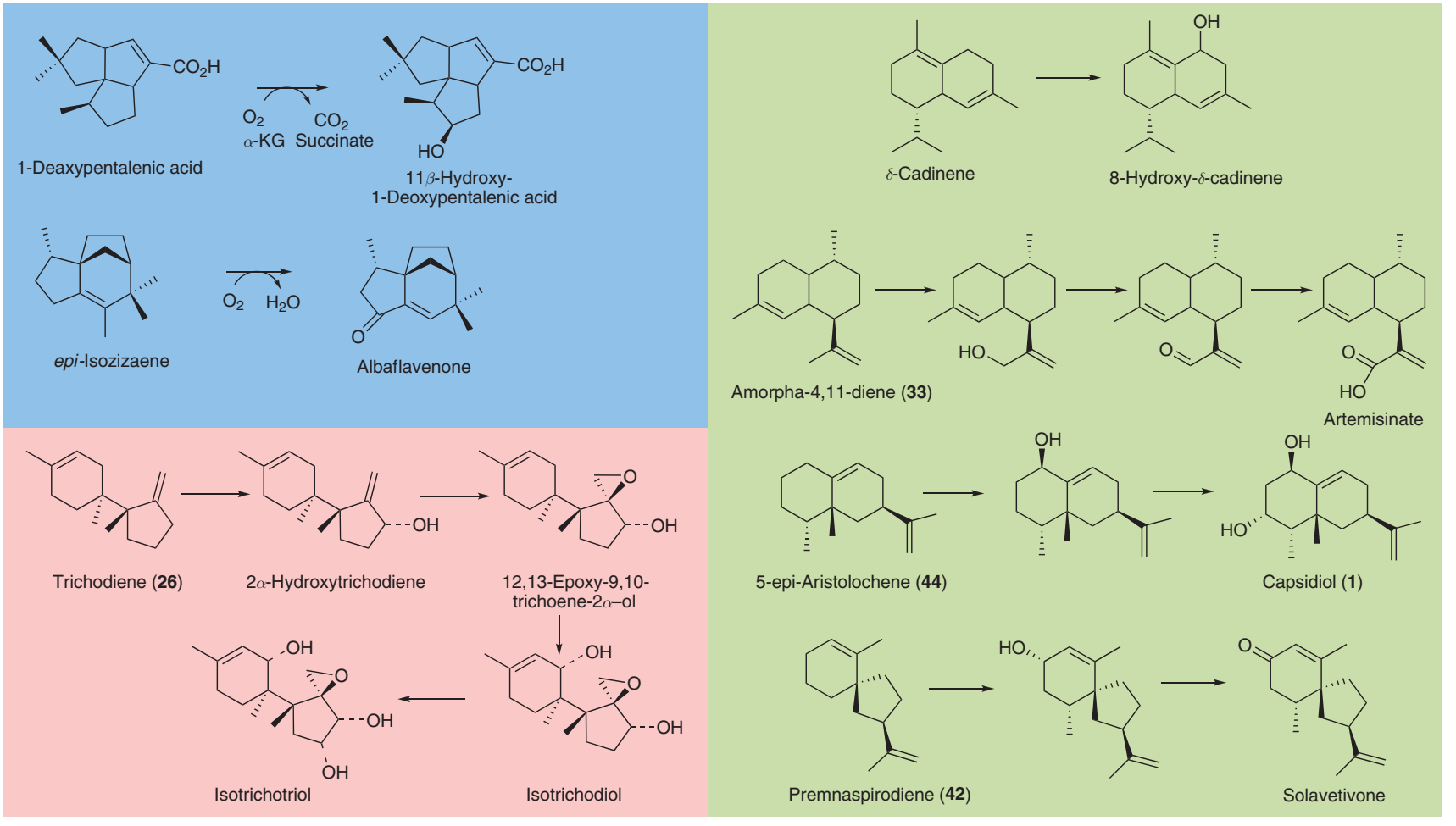


Figure 8 Examples of sesquiterpene oxidation reactions occurring in bacteria (blue panel), fungi (red panel), and plants (green panel). α-KG = α-keto-glutarate.

of the *Fusarium graminearum* genome has been determined, and similar to the sesquiterpene biosynthetic genes in *Streptomyces*, the trichothecene biosynthetic genes also appear to be clustered.[62,63] Interestingly, the Tri4 gene was shown by genetic and biochemical means to catalyze four successive hydroxylations of trichodiene[124,125] (red panel, **Figure 8**). While fungal and plant multifunctional P-450 terpene hydroxylases were recognized earlier,[40,126,127] the Tri4 enzyme is distinctive in catalyzing the introduction of oxygen at very distal positions within the trichodiene molecule and the unusual insertion of oxygen across a double bond to create a spiro-epoxide.

Mono- and multifunctional P-450s acting on sesquiterpene scaffolds are common in plants as well (**Figure 8**, green panel). The hydroxylation of δ-cadinene (**32**) at the C8 position has been reported as possibly the first of several independently regulated biochemical hydroxylations.[128] In comparison, CYP71av1 catalyzes the introduction of a carboxylate group into a terminal methyl of the isopropenyl substituent of amorphadiene (**33**), another cadinene sesquiterpene and intermediate in artemisinin biosynthesis.[86,129]

Hydroxylases/oxygenases responsible for the generation of antimicrobial sesquiterpenes in plants have also been functionally identified. Epi-aristolochene hydroxylase (EAH) has been shown to be responsible for the highly specific successive hydroxylation of 5-epi-aristolochene.[130] In contrast to the multifunction hydroxylase of *S. coelicolor*, the tobacco CYP71D20 enzyme first catalyzes the introduction of a hydroxyl at C1 in the β orientation, followed by stereospecific insertion of a second hydroxyl in the α orientation at C3. In a parallel study, these investigators also demonstrated that premnaspirodiene (**42**), another germacrene-derived scaffold, was successively hydroxylated at C2 by a highly homologous P-450 (to EAH) to yield the spiro α,β-unsaturated ketone, solavetivone (**Figure 8**).[131] Unlike the *Streptomcyes* P-450 responsible for introducing a ketone into epi-zizaene,[123] the *H. muticus* enzyme, hyoscyamus premnaspirodiene oxidase (HPO), exhibited selectivity by introducing the first hydroxyl at C2 in the α orientation.

Using an analogous approach to that of Greenhagen *et al.*[116] for terpene synthases, Takahashi *et al.*[131] began a similar effort for assessing those features of the respective P-450 that might mediate their catalytic specificities. In their initial efforts, molecular models built upon three-dimensional structures for mammalian enzymes were used to direct mutagenesis of putative active site/substrate interacting residues (**Figure 9**). While residues controlling substrate selectivity or the regiospecificity of the hydroxylations were not found, residues dictating kinetic control were. For instance, mutant V482I, A484I improved the k_{cat} of the HPO enzyme for several substrates 10-fold. While these attempts were not successful in mapping residues that control regio- and stereospecificity, they did improve the overall catalytic efficiency of these enzymes, providing new approaches to efforts for improving biological production platforms for modified sesquiterpene products.[132]

Sesquiterpenes also undergo interesting structural modifications by C–C bond cleavage reactions that give rise to norsesquiterpenes, as illustrated by the two examples in **Scheme 14**. Many plants respond to herbivore attack by releasing a mixture of volatile organic compounds from their leaves, which contain high proportions

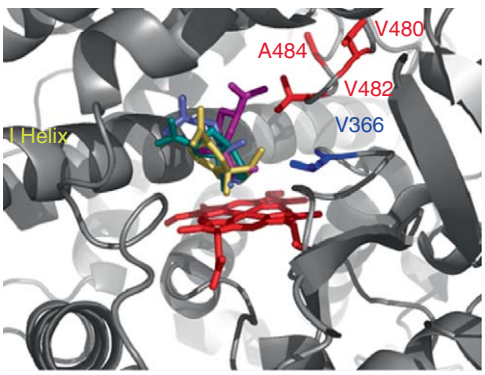
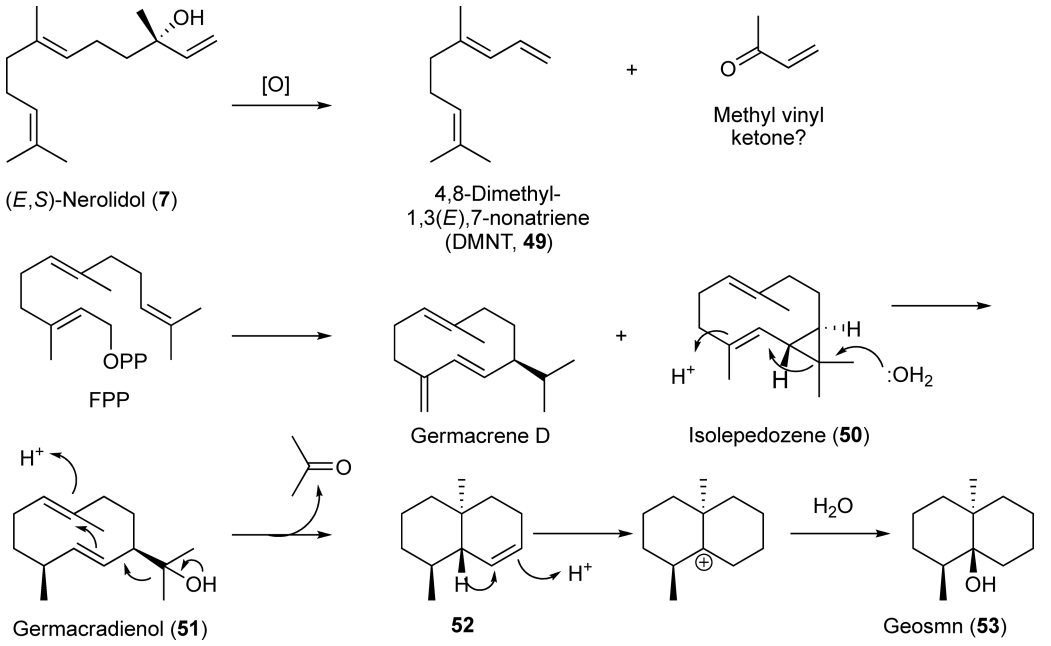


Figure 9 A molecular model of HPO, premnaspirodiene oxidase from *H. muticus*, a cytochrome P-450 enzyme. HPO is responsible for a series of successive hydroxylations at C2 of premnaspirodiene and various orientations of the substrate (cyan, blue, gold, and magenta stick figures) are rendered into the active site, along with the highlighting of residues shown to influence catalysis. Reproduced from S. Takahashi; Y. S. Yeo; Y. X. Zhao; P. E. O'Maille; B. T. Greenhagen; J. P. Noel; R. M. Coates; J. Chappell, *J. Biol. Chem.* **2007**, *282* (43), 31744–31754.



Scheme 14

of the norsesquiterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT, **49**) and/or its norditerpene relative (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).[133] These polyenes, often referred to inappropriately as 'homoterpenes,' are defensive phytochemicals that attract predators of the herbivore insects. DMNT and TMTT are biosynthesized in plant leaves by stereoselective oxidative cleavage of (*E,S*)-nerolidol (**7**),[134] and presumably the same enantiomer of the diterpene precursor, geranyllinalool. Deuterium-labeling experiments established a high stereoselectivity for removal of the pro S allylic hydrogen at C5 of (*E,S*)-nerolidol and a syn-periplanar alignment of the C–H and C–C bonds cleaved in this fragmentation reaction.[135,136] The expected by-product methyl vinyl ketone has apparently never been detected in the volatile emissions, prompting the suggestion of alternative pathways proceeding through geranylacetone and farnesylacetone intermediates, which then undergo oxidative cleavage to DMNT and TMTT with acetate as the by-product.[135] Although there seems to be no information available about the nature of the novel oxidases that catalyze the C–C cleavage reactions, nerolidol synthases associated with production of DMNT in cucumber (*Cucumis sativus* L.) and lima bean (*Phaseolus lunatus* L.) have been identified,[137] and a gene coding for geranyllinalool synthase in *Arabidopsis thaliana* has been characterized.[138] These enzymes are upregulated in response to herbivore attack and catalyze stereospecific hydrolysis of (*E,E*)-FPP and (*E,E,E*)-GGPP to the respective tertiary alcohols as substrates for the cleavage enzymes.

Geosmin is a rather simple dimethyldecalol (**53**) responsible for the characteristic strong odor of moist soil and associated with unpleasant off-flavors of water, wine, and fish. This trisnorsesquiterpene alcohol is biosynthesized by many microbes, including most *Streptomyces* species. Jiang and Cane[99] recently characterized a single 726-amino acid protein from *S. coelicolor*, which catalyzes the remarkable multistep conversion of (*E,E*)-FPP to geosmin by way of the *trans*-fused bicyclogermacrene, isolepidozene (**50**), (*E,E*)-germacradienol (**51**), and dimethyloctalin (**52**) intermediates (**Scheme 14**). The nonoxidative Prins-type fragmentation of the germacradienol intermediate is triggered by proton-induced cyclization of the (*E,E*)-1,6-cyclodecadiene moiety generating the dimethyloctalin (**52**) and a molecule of acetone. The N-terminal domain of the bifunctional germacradienol-geosmin synthase catalyzes the initial cyclizations of FPP while the C-terminal domain effects the Prins fragmentation step and the proton-induced conversion of **52** to geosmin.

A number of C16 and C17 homosesquiterpenes bearing one or two extra methyl groups that act as hormones or pheromones have been identified (**Figure 10**). The epoxy homofarnesoate esters are designated juvenile hormones I and II (JH I and II) since they, and the corresponding epoxy farnesoate parent (JH III), function by

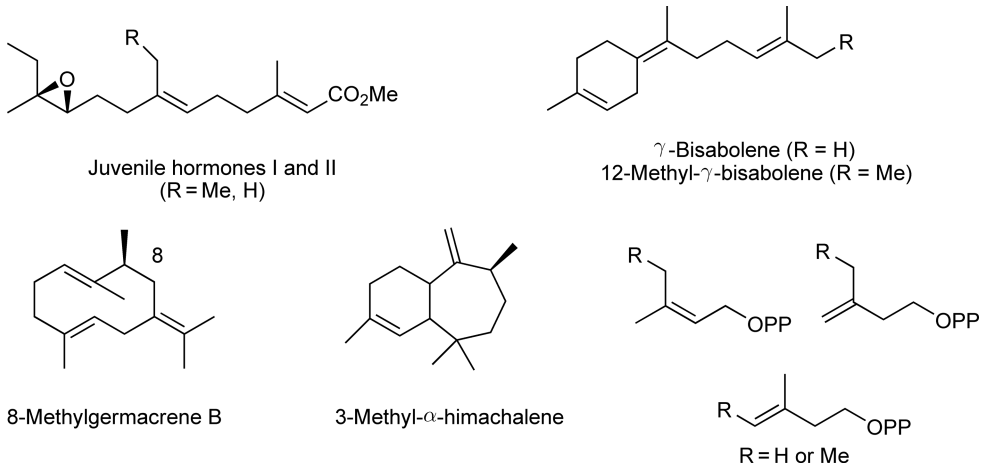


Figure 10 Structures of homosesquiterpenes and homo analogs of dimethylallyl and isopentenyl diphosphates.

maintaining the larval stage of the Cecropia silkworm, *Manduca sexta*.[139] 12-Methyl-γ-bisabolene[140] is a major component of the male sex pheromone for the cereal pest *Eurygaster integriceps Puton*, and the methyl-substituted homologs of germacrene B[141] and α-himachelene[142] are sex pheromones for the Brazilian sandfly *Lutzomyia longipalpis*. JH I and II, and presumably the other known homosesquiterpenes, are biosynthesized by the actions of oxidases and cyclases on the appropriate homologs of FPP, which in turn are produced by prenyl synthases from various combinations of methyl-substituted DMAPPs and IPPs derived from homomevalonate.[139]

1.16.4 Future Challenges

Much has been learned about the chemistry and biochemistry of sesquiterpenes in microbes and plants. The ability to clone and express sesquiterpene biosynthetic genes in heterologous hosts has been immensely helpful, especially for putting the chemical rationalizations together with biochemical functions of these enzymes. Nonetheless, two features of the enzymological conversion of FPP to such a rich diversity of scaffolds standout as unresolved.

First, there are two major divisions within sesquiterpenes that are differentiated by either initial cyclizations of the 2,3-transoid farnesyl carbocation intermediate or initial cyclizations of the 2,3-cisoid farnesyl isomer derived via nerolidyl PP. While many sesquiterpene synthases employ this isomerization activity, many do not. Perhaps adding more confusion to this matter, some synthases appear to utilize both mechanisms. Defining the enzymological features, the amino acids and their respective position within the active site pocket, responsible for the isomerization reaction remain major challenges to the field of sesquiterpene biosynthesis, and this has equally important ramifications for the entire field of isoprenoid biochemistry. This is because sesquiterpenes and isoprenoids in general are known to possess biological activities and these activities are due to the rich stereo- and regiochemistry of these molecules. Elucidating the biochemical mechanisms that impose these features will provide us with tools to better understand, for example, how evolutionary changes came about to give microbes and plants the abilities to occupy ecological niches dependent on their communications with their environment via chemical signals and cues.

A second major challenge is to develop better informatic tools, tools that can identify genes with their true biochemical functions. As is, terpene synthases are readily identified by consensus elements found in the primary sequence of each. The DDXXD motif, a domain associated with metal cofactor binding and coordinating the binding of allylic diphosphate substrates via the negatively charged diphosphate substituent. Sesquiterpene synthases are often differentiated from mono- and di-terpene synthases because they lack an amino terminal domain responsible for targeting the enzymes to the chloroplast compartment. The work of Sallaud *et al.*[60] clearly demonstrates how such a simplistic criterion might fail, because they have documented a

sesquiterpene synthase having just such a targeting signal sequence. Beyond the distinction of mono- versus sesqui- versus di-terpene synthase, what is needed is a tool to predict the geometry of the active site that can then be used to predict specific biochemical transformations. Perhaps coupling physical renderings with quantum chemical calculations, like those of the Tantillo group,[93–95] could be used to identify preferred catalytic cascades within newly discovered sesquiterpene synthase gene sequences.

The remarkable discoveries of Sallaud *et al.*[60] about sesquiterpene biosynthesis in the wild tomato *S. habrochaites* discussed in Section 1.16.2.4.1 contradict many current assumptions, that is, that sesquiterpenes are formed only in the cytosolic compartment by the mevalonate pathway, and that the universal substrate for the cyclases is (*E*,*E*)-FPP. The existence of a (*Z*,*Z*)-FPP synthase raises the question whether (*Z*,*Z*)-, (*E*,*Z*)-, and (*Z*,*E*)-FPP might be involved is sesquiterpene biosynthesis in other plants. Might (*Z*)-specific prenyl transferases and cyclases participate in diterpene biosynthesis? Do the (*Z*)-specific prenyl PPs arise from the mevalonate or MEP pathways? What are the stereochemistry and mechanisms of the coupling reactions catalyzed by (*Z*)-specific prenyl transferases and the cyclizations brought about by (*Z*)-specific cyclases? How do the three-dimensional structures and active site residues of (*E*)- and (*Z*)-specific prenyl transferases and cyclases compare? Do (*E*)- and (*Z*)-specific sesquiterpene synthases coexist in the same plants? How can the evolution of the (*Z*)-specific enzymes be rationalized?

Given the value of sesquiterpenes for agricultural, medical, and industrial applications, there have been numerous efforts to develop robust and sustainable production platforms for these chemicals in microbial and plant systems. For instance, Martin *et al.*[143] described how engineering of the MVA pathway into *Escherichia coli*, which natively has only the MEP pathway, and Wu *et al.*[85] described how engineering novel sesquiterpene branch pathways into the plastids of transgenic plants resulted in high-sesquiterpene-producing platforms. While highly significant, these efforts have not yet fully captured an appreciation for the normal regulatory mechanism imposed upon these pathways. The apparent diurnal (circadian?) rhythms of sesquiterpene metabolism[144,145] and the differential contribution of the MVA and MEP pathways to sesquiterpene biosynthesis by various tissues over development time[144,146] are examples of sesquiterpene physiology not well understood, but which must provide advantages to the organisms reliant upon them. Future investigations are likely to provide insights into the importance of these mechanisms for normal growth and developmental processes, and provide new alternatives for the generation of sesquiterpenes for mankind's use.

Glossary

anti-Markovnikov orientation Reactions of C═C double bonds with electrophiles (e.g., carbocations R^+ or H^+) that generate the less stable carbocation.

carbocation intermediates Reactive trivalent carbon species bearing a positive charge that are intermediates in sesquiterpene biosynthetic mechanisms.

cDNA Copy or complimentary DNA prepared from mRNA as the starting template.

configuration A general term referring to the stereochemistry of molecules.

conformations Refers to the spatial arrangement of the carbon atoms and substituents of a molecules differing by rotations about C–C and/or C–X single bonds.

diterpenes Organic natural products having 20-carbon skeletons formed by combination of four isoprene units.

elicitors Compounds released or produced by microbes that are detected by plants and lead to production of phytoalexins.

enantiomer One of two nonsuperimposable mirror image forms, usually of carbon compounds bearing one or more chiral centers.

first-tier residues The amino acid residues lining the three-dimensional active site of enzymes, which could be in contact with the substrate in the enzyme–substrate complex.

GC–MS An analytical instrument consisting of a gas chromatograph coupled to a mass spectrometer.

heterologous expression A term referring to the expression of a gene taken from one organism and introduced into a completely different host cell.

homosesquiterpenes Sesquiterepenes having a carbon skeleton composed of more than 15 carbon atoms.

knockout mutants Mutations in a specific genetic locus or gene, introduced by either chemical means or the insertion/deletion of DNA fragments into the locus.
lysigenous glands Epidermal and subepidermal reticulated cavities found in select plant species that serve as storage chambers for the accumulation of natural products.
methylerythritol phosphate pathway The biosynthetic pathway operating in the plastids that converts pyruvate and glyceraldehyde-3-phosphate into dimethylallyl and isopentenyl phosphates by way of deoxyxylulose-5-phosphate and 4-*C*-methylerythritol-1-phosphate intermediates.
mevalonate pathway The biosynthetic pathway operating in the cytosolic compartment in plants, which converts three molecules of acetyl-CoA into dimethylallyl and isopentenyl diphosphates by way of mevalonic acid.
monoterpenes Organic natural products having 10-carbon skeletons formed by combination of two isoprene units.
mycotoxins Toxic natural products produced by fungal species, often in association with their colonization of plant material.
norsesquiterpenes Sesquiterepenes having a carbon skeleton composed of less than 15 carbon atoms.
phosphatases Hydrolytic enzymes that can hydrolyze the terminal phosphate substituents from phosphorylated intermediates.
phytoalexins Antimicrobial compounds produced by plants in response to microbial attack.
prenyl transferases Synthases that produce acyclic polyprenyl phosphates by condensation dimethylallyl and isopentenyl phosphates, for example, farnesyl phosphate synthase.
Prins fragmentation Reactions that involve heterolytic cleavage of a C–C bond of an alcohol between the α and β positions with respect to a carbocation or a carbon bearing a leaving group.
second- and third-tier residues The amino acid residues forming concentric layers of residues impinging upon those residues making up the active site of an enzyme.
sesquiterpenes Fifteen-carbon compounds formed by combinations of three isoprene units, which are produced by the isoprenoid biosynthetic pathway.
site-directed mutagenesis A technical means for introducing fragments of nonsense DNA into a specific genetic site.
stereochemistry A term usually referring to the three-dimensional structures of molecules.
terpene hydroxylases Enzymes catalyzing the introduction of oxygen substituents into the hydrocarbon scaffolds of linear and cyclized mono-, sesqui-, and di-terpenes.
terpene synthase fold The α-helical fold that characterizes the three-dimensional structures of terpene synthases in the solid state.
terpene synthases Enzymes catalyzing the initial ionization/cyclization reactions using prenyl diphosphate intermediates. These enzymes are also called ‘‘cyclases’’.
transgenic plants Plants that have been genetically engineered with novel genes.
trichomes Epidermal appendages on plant leaves, stems, petals, and other above-ground plant tissues that synthesize unique arrays of natural products, some of which provide benefit for plant–environment interactions.
triterpenes Organic natural products having 30-carbon skeletons formed by combination of six isoprene units.
Wagner–Meerwein rearrangements Skeletal reorganizations of carbocations occurring by 1,2-migrations of a carbon substituent adjacent to the cationic site, usually generating a more stable carbocation from a less stable one.

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Biographical Sketches

Joseph Chappell has been on the faculty at the University of Kentucky since April 1985, where he has developed an internationally recognized research program pioneering the molecular genetics and biochemistry of natural products in plants. Dr. Chappell earned his B.A. degree in biology from the University of California, San Diego in 1977, his Ph.D. in biology in 1981 from the University of California, Santa Cruz, and pursued postdoctoral studies at the University of Freiburg, Germany and the Max Planck Institute, Cologne,

Germany, where he worked on the isolation and characterization of the first genes cloned for plant biosynthetic pathways. At the University of Kentucky, Dr. Chappell's research has focused on the mechanisms plants use to defend themselves against microbial pathogens and especially the biosynthesis of antimicrobial terpene-type compounds.

Robert M. Coates has been emeritus professor of chemistry at the University of Illinois at Urbana-Champaign (UIUC) since his retirement in 2008. He received his BS in chemistry from Yale University in 1960 and his Ph.D. from the University of California, Berkeley in 1964. After his postdoctoral research at Stanford University, he joined the faculty at the UIUC in 1965. His major research interests lie in synthetic and mechanistic organic chemistry, natural products, and biosynthesis, especially the chemistry and biochemistry of isoprenoid compounds. His research publications are primarily concerned with the synthesis and investigation of novel carbocyclic structures, carbocation rearrangements, isotope labeling for elucidation of stereochemistry, and design and synthesis of inhibitors of key enzymes on the isoprenoid biosynthesis pathway. He has been a member of the editorial board for the journal *Steroids* since 2001, and served as an editor for the *Encyclopedia of Reagents for Organic Synthesis* during 1992–98. He has been on the board of directors of Organic Syntheses, Inc. since 1984 and president since 2002.