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A Common Mechanism for Branching, Cyclopropanation, and Cyclobutanation Reactions in the Isoprenoid Biosynthetic Pathway

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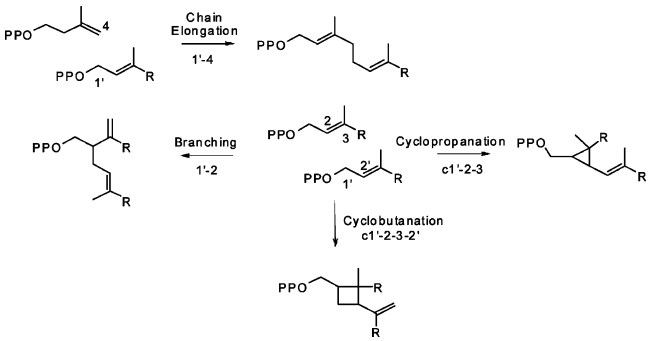
Abstract: Four reactions—chain elongation, cyclopropanation, branching, and cyclobutanation—are used in nature to join isoprenoid units for construction of the carbon skeletons for over 55 000 naturally occurring isoprenoid compounds. Those molecules produced by chain elongation have head-to-tail (regular) carbon skeletons, while those from cyclopropanation, branching, or cyclobutanation have non-head-to-tail (irregular) skeletons. Although wild type enzymes have not been identified for the branching and cyclobutanation reactions, chimeric proteins constructed from farnesyl diphosphate synthase (chain elongation) and chrysanthemyl diphosphate synthase (cyclopropanation) catalyze all four of the known isoprenoid coupling reactions to give a mixture of geranyl diphosphate (chain elongation), chrysanthemyl diphosphate (cyclopropanation), lavandulyl diphosphate (branching), and maconelliyl and planococcyl diphosphate (cyclobutanation). Replacement of the hydrogen atoms at C1 or C2 or hydrogen atoms in the methyl groups of dimethylallyl diphosphate by deuterium alters the distribution of the cyclopropanation, branching, and cyclobutanation products through primary and secondary kinetic isotope effects on the partitioning steps of common carbocationic intermediates. These experiments establish the sequence in which the intermediates are formed and indicate that enzyme-mediated control of the carbocationic rearrangement and elimination steps determines the distribution of products.

The biosynthesis of isoprenoid compounds is nature's most chemically diverse pathway, with over 55 000 known metabolites. Many of these molecules fill key metabolic and structural roles in cells, and the genes encoding isoprenoid enzymes appear in all forms of life except for a few symbiotic bacteria.[1] Better known groups of isoprenoid compounds include sterols (hormones, membrane components), carotenoids (visual pigments, photoprotective agents), prenylated proteins (membrane structure, cell signaling), dolichols (glycoprotein synthesis, bacterial cell wall synthesis), monoterpenes (insect sex pheromones, fragrances), and sesquiterpenes (plant defensive agents). Only the four biosynthetic reactions (see Scheme 1)—chain elongation, cyclopropanation, branching, and cyclobutanation—are used to couple isoprenoid intermediates to construct more complex carbon skeletons.[2] The most common reaction, chain elongation, couples isopentenyl diphosphate (IPP) with an allylic isoprenoid diphosphate to form the 1′−4 linkage characteristic of head-to-tail or "regular" isoprenoids.[3] The enzymes that catalyze 1′−4 coupling are ubiquitous. The other three reactions produce non-head-to-tail or "irregular" carbon skeletons. A c1′−2−3 cyclopropanation reaction is the first pathway-specific step in the biosynthesis of sterols and carotenoids found in all eukaryotes and archaea and in some bacteria.[4] A similar reaction produces the cyclopropane-containing skeleton found in the pyrethrin family of naturally occurring insecticides found in chrysanthemums.[5] Metabolites from 1′−2 branching, which are much less common, are found in a limited number of plants.[6,7] The only documented metabolites from c1′−2−3−2′ cyclobutanation are mealy bug mating pheromones.[8,9]

Scheme 1. Four Fundamental Coupling Reactions



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Farnesyl diphosphate synthase (FPPase) is the most widely distributed of the chain elongation enzymes. It catalyzes two reactions—the sequential addition of the hydrocarbon units of dimethylallyl diphosphate (DMAPP, C_5) and geranyl diphosphate (GPP, C_{10}) to isopentenyl diphosphate (IPP, C_5) to give farnesyl diphosphate (FPP, C_{15}). Because of its central role in isoprenoid biosynthesis, FPP synthase has served as a platform for studying the mechanism of the chain elongation reaction and structure/function relationships for chain elongation enzymes. The isoprenoid units in DMAPP and GPP are joined to IPP by a dissociative electrophilic alkylation of the double bond in IPP by allylic cations generated from the allylic diphosphate substrates.[10] FPPase has a distinctive all α-helical fold, first seen in the X-ray structure of the avian enzyme.[11] In the FPP synthase−substrate complex, the diphosphate moiety of IPP is surrounded by six molecules of water, while the diphosphate group in the allylic substrates interacts directly with three Mg^{2+} atoms and positively charged side chains of arginine and lysine residues.[12] This mode of substrate binding activates the allylic substrate for the electrophilic alkylation while not activating the allylic product formed during each step chain elongation. The iterative addition of a growing allylic chain to IPP requires relocation of the allylic product to the allylic substrate binding site during each step of chain elongation. The ultimate length of the growing isoprenoid chain is determined by the depth of the binding pocket for the allylic substrate and can be altered by site-directed mutagenesis to change the size of the side chains for amino acids in the binding pocket.[13,14]

The α-helical fold originally seen in FPPase is found in a superfamily of enzymes in the isoprenoid biosynthetic pathway whose members also catalyze cyclopropanation in the sterol and carotenoid pathways[15] and the cyclization reactions seen during biosynthesis of mono-, sesqui-, and diterpenes.[16] The fullest expression of structural diversity in the isoprenoid pathway is seen in the constituents of the essential oils of plants. One species, *Artemisia tridentata* ssp. spiciformis (snowfield sagebrush) is unique among all organisms in that its essential oil contains monoterpenes whose structures represent all known irregular isoprenoid skeletons, except for those of the mealy bug mating pheromones and membrane lipid tetraethers in archaea.[6] We recently isolated and cloned the genes for FPPase and CPPase from *A. tridentata* ssp. spiciformis.[17] The encoded proteins have an extraordinarily high level of sequence similarity. Chimeric enzymes constructed by replacing amino acids, beginning at the N-terminus, of one protein with increasing

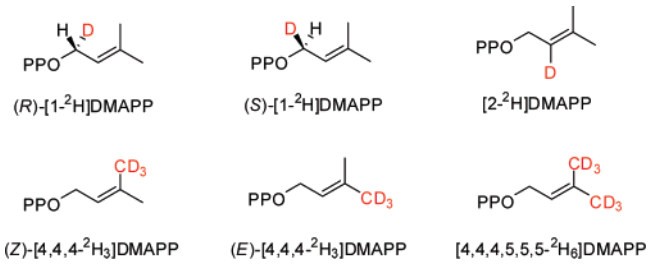

Figure 1. Deuterium labeled substrates.

stretches of sequence from the other show a progression of activities from 1′−4 elongation, through 1′−2 branching and c1′−2′−3′−2 cyclobutanation, to c1′−2−3 cyclopropanation.[2] Three of these proteins catalyzed all four of the coupling reactions, suggesting that chain elongation, branching, cyclopropanation, and cyclobutanation reactions proceed by similar chemical mechanisms. We now describe experiments demonstrating isotopically induced changes in the flux for the cyclopropanation, branching, and cyclobutanation reactions, demonstrating that these metabolites are formed from common carbocationic intermediates through a well-defined sequence of rearrangements and provide insight about how the biosynthetic enzymes can optimize synthesis of an individual structure.

Results

Non-Head-to-Tail Products. DMAPP and the deuterated derivatives shown in Figure 1 were incubated with *A. tridentata* ssp. spiciformis CPP synthase and the c98f chimera, constructed by replacing the first 98 residues in *A. tridentata* ssp. spiciformis FPP synthase with the corresponding sequence from *A. tridentata* CPP synthase.[2] The C_{10} diphosphate-containing products were hydrolyzed with alkaline phosphatase, and the alcohols were extracted with methyl *tert*-butyl ether. The extracts were analyzed by gas chromatography and mass spectrometry. We previously reported that incubation of DMAPP with CPP synthase, followed by alkaline phosphatase, gave a 33:17 mixture of (1*R*,3*R*)-chyrsanthemol ((1*R*,3*R*-COH) and (*R*)-lavandulol ((*R*)-LOH), along with a small amount of (*R*)-maconelliol ((*R*)-MOH) (see Scheme 2). A similar experiment with the c98f chimera gave a 6:5 mixture of (*R*)-LOH and (*R*)-MOH and a small amount of planococcyl/alcohol (POH).

Related structures were formed when chrysanthemyl diphosphate (CPP) synthase and the c98f chimera were incubated with the deuterium labeled DMAPPs (Figure 1). The monoterpene alcohols from incubation of (*R*)- and (*S*)-[1-^{2}H]DMAPP with CPP synthase and mass spectral fragmentation patterns for diagnostic peaks are shown in Scheme 3. COH and LOH from the *R*-enantiomer contained two deuterium atoms as seen by the shift of the molecular ion from *m*/*z* 154 for the unlabeled alcohols to *m*/*z* 156 (Figures S7 and S8). A shift of the *m*/*z* 123 fragment from loss of the hydroxymethyl unit to *m*/*z* 124 in the mass spectra of both alcohols and a shift in the *m*/*z* 69 fragment to *m*/*z* 70 in the mass spectrum of LOH indicate that the labels were not scrambled. In contrast, incubation with (*S*)-[1-^{2}H]-DMAPP gave COH containing a single deuterium due to elimination of deuterium at C3 when the cyclopropane ring was formed, as established by a molecular ion at *m*/*z* 155 and a peak at *m*/*z* 123, showing that the deuterium atom was in the hydroxymethyl unit (Figure S7). Thus, the pro-*S* hydrogen is selectively removed from C1 of the dimethylallyl unit that is

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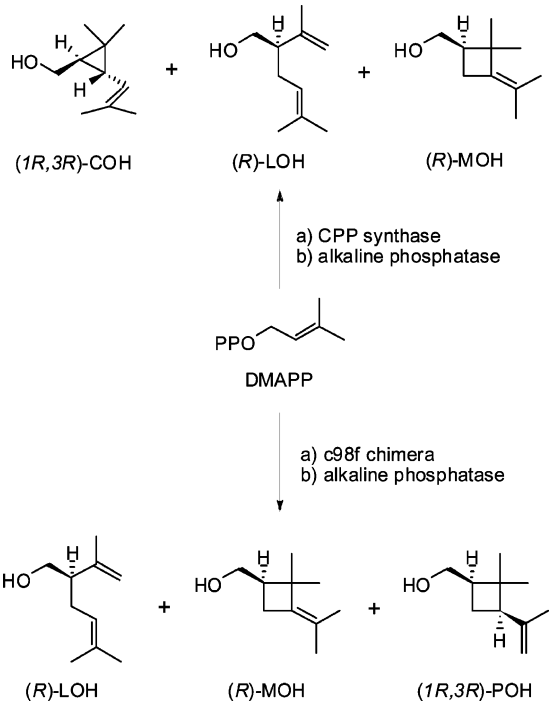
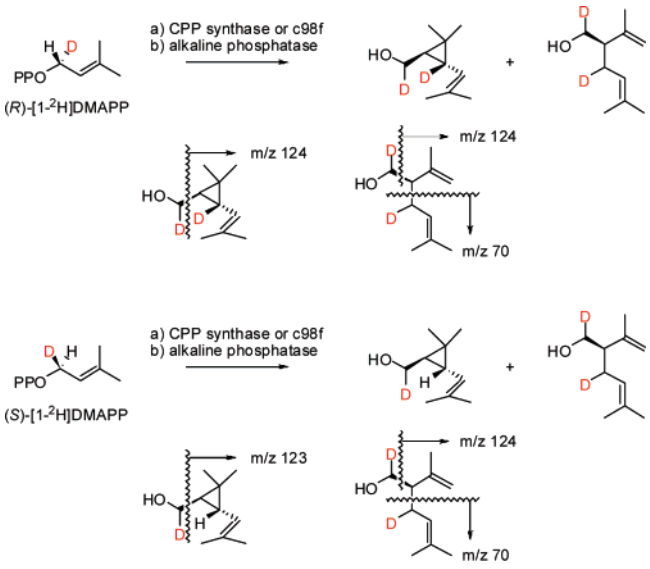
Scheme 2. Non-Head-to-Tail Alcohols after Incubation of DMAPP with CPP Synthase or c98f Followed by Hydrolysis with Alkaline Phosphatase



Scheme 3. Alcohols from Incubation of (*R*)- and (*S*)-[1-^{2}H]DMAPP with CPP Synthase



inserted into the C2−C3 double bond of DMAPP. This is the same stereochemistry that was observed for formation of presqualene diphosphate and prephytoene diphosphate during sterol[18] and carotenoid biosynthesis.[19] The mass spectrum of LOH from (*S*)-[^{1}H]DMAPP was identical to the one obtained for LOH produced from the *R*-enantiomer (Figure S8 and S10).

The products from the three CD_3-labeled DMAPP derivatives and characteristic fragments are shown in Scheme 4. The molecular ions of COH from (*E*)- or (*Z*)-CD_3-DMAPP shift from

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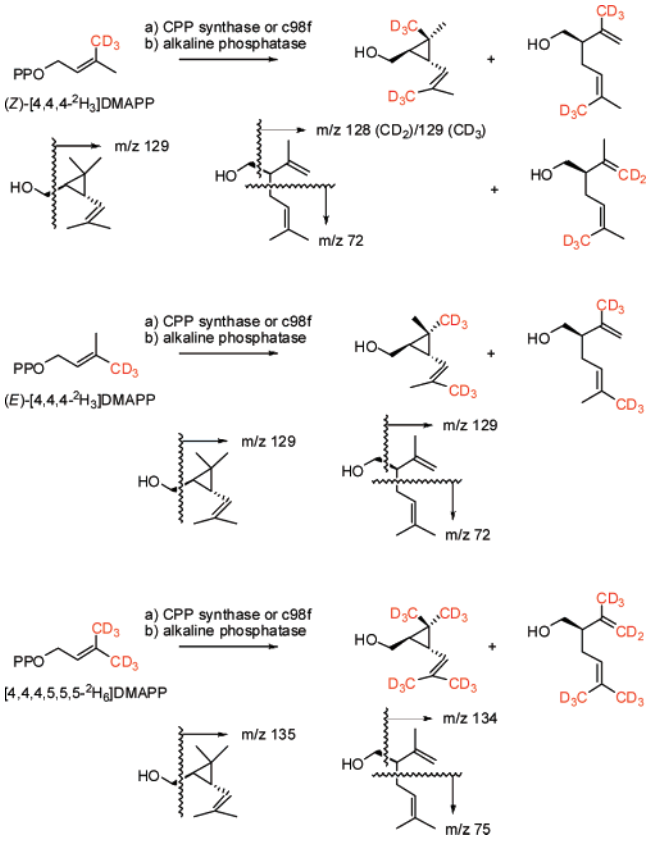
Scheme 4. Alcohols from Incubation of (*E*)- and (*Z*)-[4,4,4-$^2H_3$]DMAPP and [4,4,4,5,5,5-$^2H_6$]DMAPP with CPP Synthase



m/*z* 154 to *m*/*z* 160, and the peak at *m*/*z* 123 from loss of the hydroxymethyl fragment shifts to *m*/*z* 129 (Figures S9 and S12). The molecular ion of COH from $(CD_3)_2$-DMAPP is at *m*/*z* 166, and loss of the hydroxymethyl fragment gives a peak at *m*/*z* 135. The patterns seen for LOH are more complex. LOH from (*E*)- or (*Z*)-CD_3-DMAPP gives a molecular ion at *m*/*z* 159 ($C_{10}H_{13}D_5O$) or 160 ($C_{10}H_{12}D_6O$), depending on whether a hydrogen or deuterium atom is eliminated to give the disubstituted double bond (Figures S12, S13, S18, and S19). The pattern was easier to discern in the more intense peaks at *m*/*z* 128 ($C_9H_{10}D_5$) and *m*/*z* 129 ($C_9H_9D_6$) after loss of the hydroxymethyl fragment. The relative intensities of the *m*/*z* 159/160 and 128/129 peaks varied in scans taken as LOH eluted from the GC column, with the higher mass fragments appearing earlier in the elution. The GC peak for LOH derived from CD_3-DMAPP had a shoulder, indicating that the $C_{10}H_{13}D_5O$ and $C_{10}H_{12}D_6O$ forms of LOH had slightly different retention times with the $C_{10}H_{12}D_6O$ species eluting first (see Figure 2). The relative amount of $C_{10}H_{13}D_5O$ and $C_{10}H_{12}D_6O$ was determined by curve-fitting a plot of peak intensity versus retention time to a model consisting of two overlapping Gaussian peaks in a 71:29 ratio.

Product Distributions. The relative amounts of the non-head-to-tail alcohols from incubations of CPP synthase and the c98f chimera with DMAPP and several deuterated derivatives, followed by hydrolysis with alkaline phosphatase, are shown in Table 1.

Changes in the product ratios are consistent with a combination of primary and secondary deuterium isotope effects on partitioning steps that lead to the products. The largest change

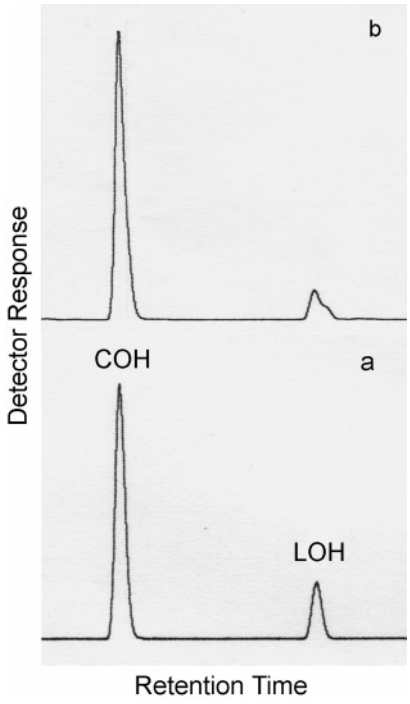


Figure 2. GC trace of C_{10} alcohols from incubation of DMAPP (a) and (Z)-CD_3-DMAPP (b) with *A. tridentata* CPP synthase followed by alkaline phosphatase.

Table 1. Distributions of the Major Products from Incubation of DMAPP with *A. Tridentata* ssp. Spiciformis CPP Synthase (COH/LOH) and the c98f CPP/FPP Synthase Chimera (LOH/MOH and MOH/POH) after Treatment with Alkaline Phosphatase[a]

substrate	COH/LOH	LOH/MOH	MOH/POH
DMAPP	3.9 ± 0.1	1.85 ± 0.03	11.2 ± 0.4
(*R*)-[1-^{2}H]DMAPP	3.7 ± 0.05	-	-
(*S*)-[1-^{2}H]DMAPP	0.81 ± 0.02	-	-
(*Z*)-[4,4,4-$^2H_3$]DMAPP	6.5 ± 0.3	1.83 ± 0.07	10.5 ± 0.9
(*E*)-[4,4,4-$^2H_3$]DMAPP	6.6 ± 0.1	1.80 ± 0.02	10.5 ± 0.5
[4,4,4,5,5,5-$^2H_6$]DMAPP	10.8 ± 0.5	1.27 ± 0.02	13.6 ± 1.3
[2-^{2}H]DMAPP	3.7 ± 0.1	1.86 ± 0.06	9.9 ± 0.9

[a] Each value is the average from three independent incubations.

in the COH/LOH ratio is a 4.9-fold decrease in $[COH/LOH]^H/[COH/LOH]^D$ when DMAPP is replaced by (*S*)-[1-^{2}H]DMAPP. Substitution of one of the methyl groups in DMAPP with a CD_3 moiety results in smaller, but significant, increases in the COH/LOH ratio reflecting isotope effects of $[COH/LOH]^H/[COH/LOH]^D = 1.67$ and 1.69 for the *Z*- and *E*-CD_3 derivatives, respectively. The COH/LOH ratio increased to 10.8 for $(CD_3)_2$-DMAPP, corresponding to $[COH/LOH]^H/[COH/LOH]^D = 2.77$. Deuterium substitution at C2 of DMAPP did not significantly alter the COH/LOH ratio.

A similar study was conducted looking at changes in the LOH/MOH and MOH/POH ratios from incubations of the c98f chimera with [2-^{2}H]DMAPP and the CD_3-DMAPP derivatives. The LOH/MOH ratios seen for DMAPP, [2-^{2}H]DMAPP, and the mono CD_3 DMAPP derivatives were all within experimental error, indicating that there was no discernible isotope effects on the product-forming steps. The ratio decreased from 1.85 to 1.27 for $(CD_3)_2$-DMAPP, a 1.46-fold change in the product forming steps. The MOH/POH ratios for DMAPP and the mono CD_3 DMAPPs were within experimental error. The decrease in the ratio from 11.2 to 9.9 for [2-^{2}H]DMAPP indicates a small ~1.12-fold isotope effect. The MOH/POH ratio increased to 13.6 for $(CD_3)_2$-DMAPP, a 1.21-fold change.

Discussion

The observation that the four reactions used to join isoprenoid units—chain elongation, cyclopropanation, branching, and cyclobutanation—are catalyzed by a single enzyme led us to propose an integrated mechanism for biosynthesis of these structures from shared intermediates based on the dissociative electrophilic alkylation mechanism for chain elongation.[2] The enzymes that catalyze chain elongation and cyclopropanation have similar folds, indicating that they evolved from a common ancestor. Although the enzymes that catalyze branching and cyclobutanation are unknown, the absolute stereochemistries of the chiral centers in naturally occurring COH, LOH, MOH, and POH are identical, as are those in the alcohols obtained from the c98f chimeria of CPP and FPP synthase. These observations are consistent with a scenario where the enzymes that catalyze cyclopropanation, branching, and cyclobutanation evolved from an ancestral chain elongation enzyme through gene duplication/mutagenesis. Thus, the preferred product for a particular enzyme would be determined by the relative efficiencies of the rearrangement and proton elimination steps in the series of reactions initiated by alkylation of DMAPP by the dimethylallyl cation (DMA^+) shown in Scheme 5. The absolute stereochemistries of the products would be dictated by binding interactions within the structurally related chiral environments of the enzyme's active sites. It is, however, possible that cyclopropanation and branching products are synthesized by parallel pathways entered independently during alkylation of DMAPP by DMA^+ to generate the chrysanthemyl cation (C^+) or the lavandulyl cation (L^+). It is also possible that L^+ is the initial alkylation product of DMA^+ and DMAPP, and L^+ subsequently isomerizes to C^+. The maconelliyl cation (M^+) is most likely generated by cyclization of L^+. Computational and experimental studies suggest that the difference in free energy between isomeric protonated cyclopropanes and tertiary carbocations can be sufficiently low to permit rapid equilibration of the two species.[20,21] Thus, any of the three scenarios mentioned above are candidates for the mechanism of the biosynthetic reactions. These issues were resolved by studying the effect of deuterium substitution on the formation of CPP and LPP (lavandulyl diphosphate) by CPP synthase and of LPP and MPP (maconelliyl diphosphate) by the c68f chimera.

During biosynthesis of CPP, the pro-S hydrogen (green) is lost from the C1 position of the electrophilic DMAPP. Incubation of (*S*)-[1-^{2}H]DMAPP with CPP synthase, followed by hydrolytic workup, gives COH with a single deuterium atom in the hydroxymethyl group; whereas, the deuterium atoms from both molecules of DMAPP are incorporated into COH derived from the *R*-enantiomer. Furthermore, the COH/LOH ratio drops ~4.9-fold in incubations with (*S*)-[1-^{2}H]DMAPP relative to the ratio seen for (*R*)-[1-^{2}H]DMAPP or unlabeled DMAPP. These results demonstrate a substantial primary kinetic isotope effect (KIE) on the partitioning of C^+ to CPP (step a) and L^+ (step b) as a result of elimination of deuterium in the CPP product-

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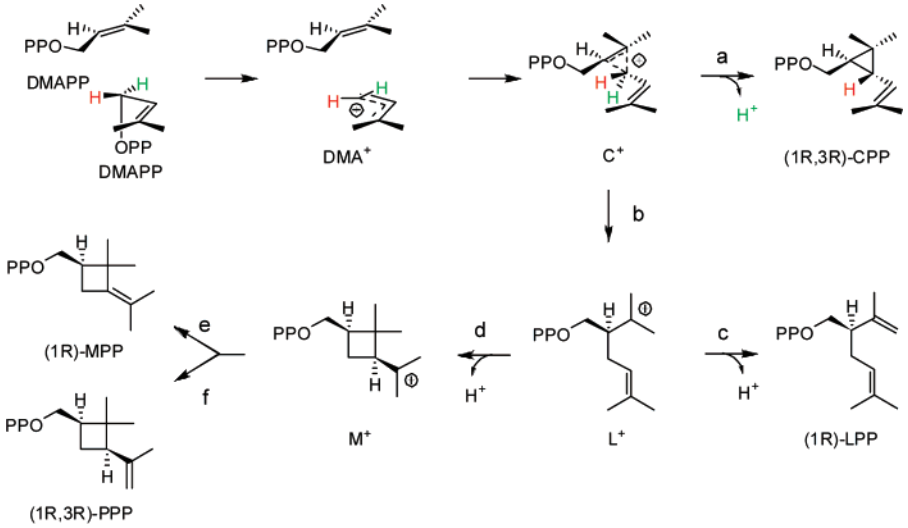
Scheme 5. A Common Mechanism for the Stereoselective Biosynthesis of Irregular Isoprenoid Compounds with c1′−2−3, 1′−2, and c1′−2−3-2′ Skeletons from DMAPP with the C1 Hydrogens Labeled: ProR (Red) and ProS (Green)



forming step.[22] The magnitude of the KIE indicates that C^+ is the direct precursor of L^+.

This hypothesis is confirmed by a KIE on the COH/LOH ratio seen in incubations with (*Z*)- and (*E*)-CD_3-DMAPP and $(CD_3)_2$-DMAPP. The COH/LOH ratios are identical for both stereoisomers and correspond to a positive secondary deuterium KIE of 1.67 for the CD_3 group, which is independent of its stereochemistry on the cyclopropane ring in C^+. The COH/LOH ratio for $(CD_3)_2$-DMAPP corresponds to an observed secondary β-deuterium KIE of 2.77 (or 1.67/CD_3 group). A positive secondary β-deuterium KIE is consistent with rate limiting isomerization of C^+ to L^+ (step b) where the hybridization of the methyl-bearing carbon approaches sp^2 in the transition state.[23]

Mass spectroscopic and gas chromatographic analysis of LOH obtained from (*Z*)-CD_3-DMAPP indicates an ∼2.4-fold preference for elimination of a proton from the CH_3 group rather than a deuterium from the CD_3 in step c. This value reflects a combination of the intrinsic primary KIE and a stereoselective preference imposed by CPP synthase during the elimination step. Values ranging from 2.3 to 5.9 have been reported for related eliminations in reactions catalyzed by monoterpene cyclases.[24]

The LOH/MOH ratio does not change for incubations with DMAPP, [2-^{2}H]DMAPP, and (*Z*)- and (*E*)-CD_3-DMAPP, reflecting no detectible change in the relative rates of steps c and d. However, the ratio decreases ∼1.46-fold when $(CD_3)_2$-DMAPP is the substrate. In this case, mandatory loss of a deuterium in step c results in a modest primary KIE on the partitioning of L^+ to LPP and M^+. Finally, a modest decrease in the MOH/POH ratio when [2-^{2}H]DMAPP is the substrate is consistent with a small primary deuterium KIE on step e, while a small increase in the MOH/POH ratio for $(CD_3)_2$-DMAPP is consistent with a small primary isotope effect of step f.

The mechanism for biosynthesis of the four fundamental building reactions in the isoprenoid pathway presented in Scheme 5 is supported by deuterium isotope effects on key rearrangement and elimination steps. The large isotope effect on the COH/LOH ratio suggests that protonated cyclopropane C^+ is the first intermediate generated upon condensation of DMA^+ with the C2−C3 double bond in DMAPP. The ratio CPP to LPP is dictated by the relative rates for steps a and b. This scenario is consistent with the large primary deuterium KIE seen for step a and the positive β-secondary deuterium isotope effect for step b. Following rearrangement of C^+ to L^+, the tertiary carbocation can lose a proton to give LPP or cyclize to give M^+. Finally, M^+ can lose the cyclobutyl proton to give MPP or a methyl proton to give PPP (planococcyl diphosphate). The rearrangement (b and d) and elimination (a, c, e, and f) steps appear to be irreversible.

Formation of cyclopropanation, branching, and cyclobutanation products by deprotonation of the carbocationic intermediates in the $C^+ \rightarrow L^+ \rightarrow M^+$ cascade of rearrangements provides an opportunity for facile evolution of enzymes for selective biosynthesis of related irregular monoterpenes. The selectivity for synthesis of isoprenoid compounds with a specific carbon skeleton depends on how C^+, L^+, and M^+ partition between the options for elimination and rearrangement. The relative rates of these competitive reactions depend on the ability of an enzyme to stabilize C^+, L^+, and M^+ by a combination of charge−charge and charge−dipole interactions between the carbocations and the enzyme[24] and to promote the regioselective elimination of protons with strategically located bases in the active site.

Materials and Methods

General. Unlabeled DMAPP[25] and deuterium labeled DMAPP[26] were prepared as described in the literature. *Chrysanthemum cinerariaefolium* CPP synthase,[27] *Artemisia tridentata* ssp. spiciformis CPP synthase,[18] and the c98f FPP synthase/CPP synthase *A. tridentata* ssp. spiciformis chimera[2] were purified from *Escherichia coli* hosts as previously reported. Authentic samples and mass spectra of (1*R*,3*R*)-chrysanthemol (COH), (*R*)-lavandulol (LOH), (*R*)-maconelliol (MOH), and (1*R*,3*R*)-planococcol (POH) were available from a previous study.[2]

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Enzymatic Incubations and Product Analysis. Enzyme (400 μg) was added to 35 mM HEPES buffer, pH 7.6, containing 10 mM $MgCl_2$, 5.0 mM β-mercaptoethanol, and 3 mM DMAPP to a final volume of 400 μL. The mixture was incubated for 12 h at 30 °C. The isoprenoid diphosphate products were hydrolyzed to the corresponding alcohols by addition of 80 μL of 500 mM glycine buffer, pH 10.5, containing 5 mM $ZnCl_2$ and 80 units of calf alkaline phosphatase (Sigma) followed by incubation at 37 °C for 2 h. Approximately 0.2 g of NaCl was added, and the aqueous phase was extracted three times with 400 μL of *tert*-butyl methyl ether. The volume of the extract was reduced to ~50 μL with a stream of dry nitrogen. A 1 μL portion of the extract was injected onto a 30 m × 0.32 mm × 0.25 μm 20% β-cyclodextrin capillary GC column (HP-CHIRAL-20B, J & W Scientific) equilibrated at 60 °C followed by a temperature gradient from 60 °C to 120 °C at 2 °C/min, a temperature gradient of 120 °C to 230 °C at 10 °C/min (program 1) or injection on the column equilibrated at 50 °C with 3 min at 50 °C followed by a temperature gradient from 50 °C to 120 °C at 2 °C/min and 120 °C to 230 °C at 10 °C/min (program 2). Helium was used as the carrier gas at a flow rate of 1 mL/min. Analysis by GC/MS was performed under similar conditions using a 30 m × 0.25 mm × 0.25 μm 20% β-cyclodextrin capillary column (HP-CHIRAL-20B, J & W Scientific) at a flow rate of 1 mL of He/min. The structures of the products were verified by co-injection with authentic samples and by mass spectrometry (see Figures S1−S29).[2]

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Supporting Information Available: GC traces for COH, LOH, MOH, and POH. Mass spectra for COH, LOH, MOH, and POH and deuterium-containing derivatives.

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