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Recombinant Squalene Synthase. Synthesis of Non-Head-to-Tail Isoprenoids in the Absence of NADPH

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Abstract: Squalene synthase (SQase) catalyzes two consecutive reactions in sterol biosynthesis. The first is the condensation of two molecules of farnesyl diphosphate (**FPP**) to form a cyclopropylcarbinyl intermediate, presqualene diphosphate (**PSPP**). The subsequent conversion of **PSPP** to squalene (**SQ**) involves an extensive rearrangement of the carbon skeleton and a NADPH-dependent reduction. Incubation of a truncated soluble form of recombinant yeast SQase with **FPP** in buffer lacking NADPH gave (1*R*,2*R*,3*R*)-**PSPP**. As the incubation continued, SQase catalyzed the subsequent conversion of **PSPP** to a mixture of triterpenes. Two of the major products, (*Z*)-dehydrosqualene (**DSQ**) and (*R*)-12-hydroxysqualene (**HSQ**), have the same 1′−1 linkage between the farnesyl units from **FPP** that is found in squalene. The other major product, (10*S*,13*S*)-10-hydroxybotryococcene (**HBO**), has a 1′−3 linkage between the farnesyl units. Small quantities of (*S*)-**HSQ** and (10*R*,13*S*)-**HBO** were also formed. Three additional triterpenes, the allylic isomers of **HSQ** and **HBO**, and an unidentified alcohol were produced in minor amounts. A methyl ether corresponding to **HSQ** was detected when methanol was present in the incubation buffer. These compounds are the expected "solvolysis" products from **PSPP**. They provide strong support for mechanisms that propose cyclopropylcarbinyl cations as intermediates in the SQase-catalyzed rearrangement of **PSPP** to **SQ** and unambiguously demonstrate that the catalytic machinery of SQase is capable of synthesizing a variety of irregular isoprenoids.

Over 33 000 isoprenoid compounds are known. In most cases their carbon skeletons consist of five-carbon isoprene units connected in a "regular" 1′−4 or head-to-tail pattern.[1] However, there are many examples where some of the five-carbon units in a complex structure are joined in an irregular or "non-head-to-tail" manner. Two of the most familiar non-head-to-tail terpenes are squalene, with a 1′−1 linkage between two farnesyl residues, and phytoene, with a 1′−1 linkage between two geranylgeranyl moieties.[2] These compounds are important intermediates in the sterol and carotenoid biosynthetic pathways, respectively, and representative examples are widely distributed in nature. The greatest diversity of irregular structures is found in components of the essential oils of plants in the *Asteraceae* family, where monoterpenes with 1′−1,[3] 1′−2,[3,4] 1′−3,[3,4] 2−1′−3,[3,4,5] c1′−2−3,[3,4,6,7] and c1′−1−2 [3,5,8] structures (see Figure 1) have been reported. The essential oil of *Artemisia tridentata speciformis*, a subspecies of sage brush common to the Great Basin of the western United States, contains compounds representing all of the structures illustrated in Figure 1.[3−8]

The discovery by Rilling[10,11] that presqualene diphosphate (**PSPP**),[9] a triterpene with a c1′−2−3 fusion between two farnesyl moieties, is an intermediate between farnesyl diphosphate (**FPP**) and squalene (**SQ**) in the cholesterol biosynthetic pathway (see Scheme 1) stimulated considerable speculation about the biogenesis of isoprenoids with 1′−1 linkages. Shortly afterward, Altman and co-workers[12] reported that prephytoene diphosphate (**PPPP**), a C_{40} analogue of **PSPP**, is an intermediate between geranylgeranyl diphosphate (**GGPP**) and the 1′−1 hydrocarbon phytoene (**PH**) in the carotenoid biosynthetic pathway. During this period, several groups proposed a variety

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(9) Abbreviations: BITIP, binaphthol; BO, botryococcene; DMAP, *N*,*N*-(dimethylamino)pyridine; DSQ, dehydrosqualene; er, enantiomeric ratio; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; HBO, hydroxybotryococcene; HSQ, hydroxylsqualene; IPP, isopentenyl diphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MTBE, methyl *tert*-butyl ether; MTP, methoxytrifluoromethylphenylacetyl; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PPPP, prephytoene diphosphate; PSPP, presqualene diphosphate PH, phytoene; PHase, phytoene synthase; SQ, squalene; SQase, squalene synthase.
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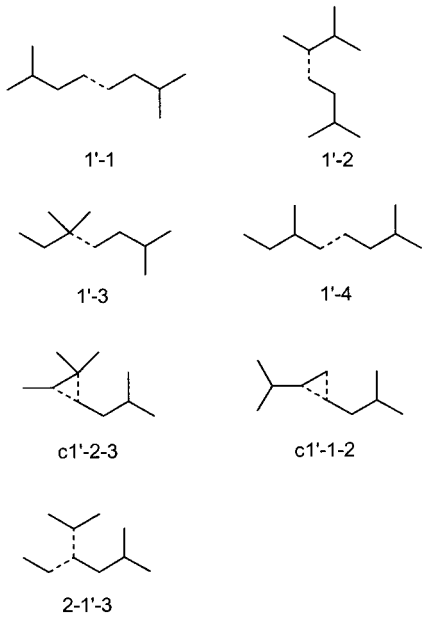
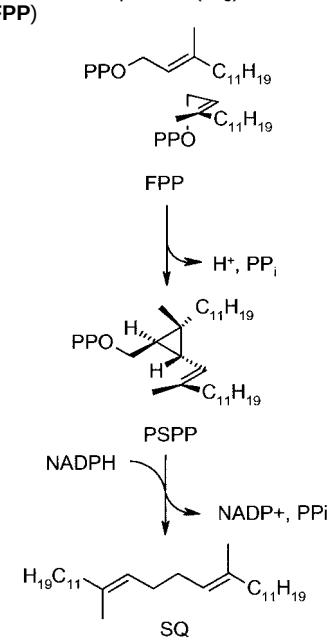
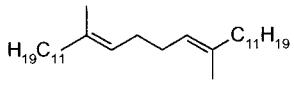


Figure 1. Regular and irregular attachments of isoprenoid units.

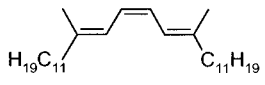
Scheme 1. Synthesis of Squalene (**SQ**) from Farnesyl Diphosphate (**FPP**)



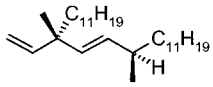
of mechanisms based on rearrangements of cyclopropylcarbinyl cations to account for the conversion of **PSPP** to **SQ**.[12−16] In addition to the 1′−1 structures, it was recognized that cationic rearrangements of c1′−2−3 cyclopropylcarbinyl cations could explain the formation of several other irregular isoprenoid skeletons found in nature, including those with 1′−3 and 2−1′−3 structures.[4,16] Subsequent labeling experiments with stable isotopes provided strong evidence that **PSPP** is an intermediate in the biosynthesis of the 1′−3 triterpene botryococcene (**BO**), a major constituent of the oil produced by *Botryococcus braunii*, a photosynthetic algae commonly found in freshwater ponds.[17] Model studies with derivatives of chrysanthemol, a naturally occurring monoterpene with a c1′−2−3 structure, were consistent with both regiochemical and stereochemical features in the proposals by Epstein and Poulter for biogenesis of irregular isoprenoids.[1,18−25]



Squalene (SQ)



Phytoene (PT)



Botryococcene (BO)

Squalene synthase (SQase) catalyzes two distinct chemical transformations. The first is a condensation between two molecules of **FPP** to give **PSPP**. The second involves the rearrangement and NADPH-dependent reduction of **PSPP** to generate **SQ** as outlined in Scheme 1.[26] These reactions are the first pathway-specific steps in cholesterol metabolism, and SQase has attracted attention as a logical target for development of cholesterol-lowering drugs.[27] A similar set of reactions is catalyzed by phytoene synthase (PHase) during synthesis of **PH** from **GGPP**.[28] Rilling discovered that **PSPP** accumulated when **FPP** was incubated with yeast microsomes in buffer that did not contain NADPH.[10] However, the diphosphate ester was quickly hydrolyzed by phosphatases in the microsomal preparation, and presqualene alcohol was typically isolated from preparative-scale incubations.[29]

SQase is extraordinarily difficult to purify from yeast microsomes.[30] When the gene for the yeast enzyme was characterized, Jennings et al.[31] identified a 26-amino acid consensus sequence for a membrane-spanning α-helix at the C-terminus of the protein. Zhang et al.[32] subsequently constructed an *E. coli* clone that produced a truncated version of recombinant yeast SQase without the putative membrane-spanning helix. The recombinant protein was soluble, directed the synthesis of **SQ** when incubated with **FPP** and NADPH, and was easily purified.[32] Like the wild-type microsomal enzyme, recombinant SQase rapidly converted **FPP** to **PSPP** in the absence of NADPH. Upon further incubation, the purified

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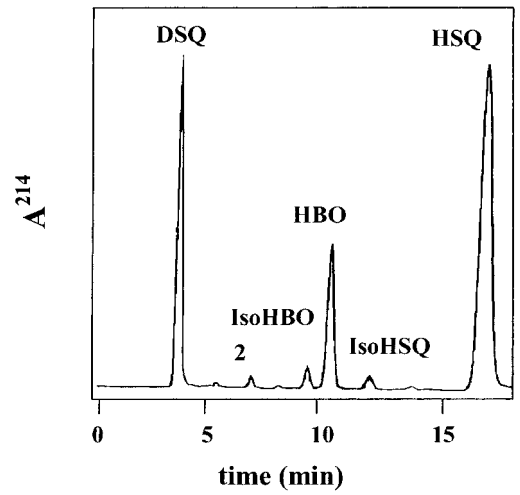

Figure 2. HPLC trace of the SQase-catalyzed solvolysis of **PSPP**. Analysis on a Rainin microsorb MV silica gel column with isocratic elution using methyl *tert*-butyl ether/heaxane (1:19 v/v).

recombinant enzyme subsequently converted **PSPP** to a mixture of triterpenes with 1′−1 and 1′−3 skeletons[33] similar to those obtained in the earlier model studies.[1,18−24] We now describe an in-depth study of the SQase-catalyzed "solvolysis" of **PSPP** and its implications for the biogenesis of irregular isoprenoid compounds.

Results

Product Studies. (a) Aqueous Buffer. FPP was rapidly converted to **PSPP** when incubated with recombinant SQase, as determined by reversed-phase TLC analysis of the *n*-butanol-soluble diphosphates (data not shown). As the incubation continued, **PSPP** was, in turn, converted into a mixture of compounds soluble in methyl *tert*-butyl ether (MTBE). A normal-phase HPLC trace of the mixture is shown in Figure 2. The components were purified by HPLC and analyzed by mass spectrometry. The first compound to elute gave a molecular ion at *m*/*z* 408.3749, consistent with an unsaturated $C_{30}H_{48}$ triterpene hydrocarbon, and compounds representing five of the other peaks gave molecular ions at *m*/*z* 426 with exact masses characteristic of isomeric $C_{30}H_{50}O$ triterpene alcohols. The mass spectra of three other trace components were inconsistent with structures derived from **PSPP** and were probably impurities unrelated to the enzyme-catalyzed reaction. When SQase was heat inactivated at 95 °C for 30 min before an incubation, the protein did not catalyze the synthesis of **PSPP** from **FPP** or the conversion of **PSPP** to MTBE-soluble materials.

The triterpene hydrocarbon eluting at approximately 4 min was identified as (*Z*)-12,13-dehydrosqualene (**DSQ**) (see Figure 3). A ^{1}H NMR spectrum of the hydrocarbon was consistent with a symmetrical structure containing four different methyl groups, 16 methylene protons, and three sets of olefinic protons in a ratio of 2:2:4. The UV spectrum of **DSQ** had a λ_{max} at 286 nm with shoulders at 275 and 297 nm in a ratio of 1.14:1.40:1.00. (*E*,*Z*,*E*)-Phytoene (**PH**), the C_{40} analogue of **DSQ**, and (*E*,*E*,*E*)-**PH** have characteristic UV spectra that are distinctive for the stereochemistry of the central double bond in the triene chromophore.[34,35] The (*E*,*Z*,*E*) isomer has a λ_{max} at 286 with

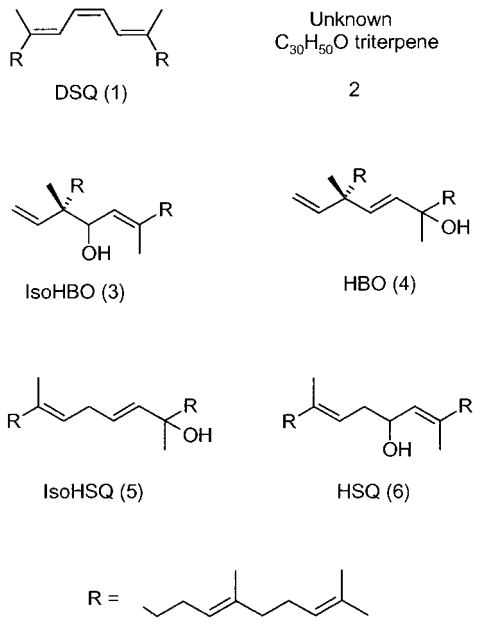

Figure 3. Products of the SQase-catalyzed solvolysis of **PSPP**.

shoulders at 276 and 297 nm. The relative absorbances at 276, 286, and 297 nm are 1.13:1.34:1.00, respectively. This pattern is similar to the UV spectrum of **DSQ**. In contrast, the UV spectrum of (*E*,*E*,*E*)-**PH** has distinct maxima at 275, 286, and 298 nm with relative absorbances of 0.94:1.39:1.00. It is clear from comparisons of the UV spectrum of **DSQ** with those of (*E*,*Z*,*E*)- and (*E*,*E*,*E*)-**PH** that **DSQ** has a *Z* central double bond. The stereochemistries of the C(10)−C(11) and C(14)−C(15) double bonds are less certain. However, the symmetry of the ^{1}H NMR spectrum indicates that stereochemistries of the double bonds in each of the farnesyl units in **DSQ** are identical. In addition, **HSQ** is the (*E*)-10,11, (*E*)-14,15 stereoisomer (see below). We assume that **DSQ**, formed in the same reaction, has the same stereochemistry for the C(10)−C(11) and C(14)−C(15) double bonds. Finally, the (*E*)-6,7 and (*E*)-18,19 double bonds in **DSQ** are derived directly from the (*E*)-6,7 double bond in **FPP**. These assignments are consistent with the structure of **DSQ** produced by incubation of **FPP** with a microsomal preparation of SQase in the presence on Mn^{2+}.[36,37]

The structures of the two major triterpene alcohols, 10-hydroxybotryococcene (**HBO**) and 12-hydroxysqualene (**HSQ**), were deduced from their ^{1}H NMR spectra. **HBO**, which eluted at 10.5 min, had a characteristic three-proton pattern for a vinyl group with resonances at 4.94, 4.97, and 5.79 ppm and an AB quartet (J = 16 Hz) for the (*E*)-11,12 double bond. **HSQ**, which eluted at 17 min, gave a broad doublet for the olefinic proton at C(11) and a doublet of triplets for the proton at C(12). Both of these structures were confirmed by independent synthesis as illustrated in Scheme 2. **HBO** was synthesized by solvolysis of presqualene dinitrobenzoate (**7**) under conditions known to convert chrysanthemyl dinitrobenzoate to yomogi alcohol, a 1′−3 monoterpene alcohol.[1] **HSQ** was synthesized by treating farnesal with the barium reagent derived from farnesyl chlo-

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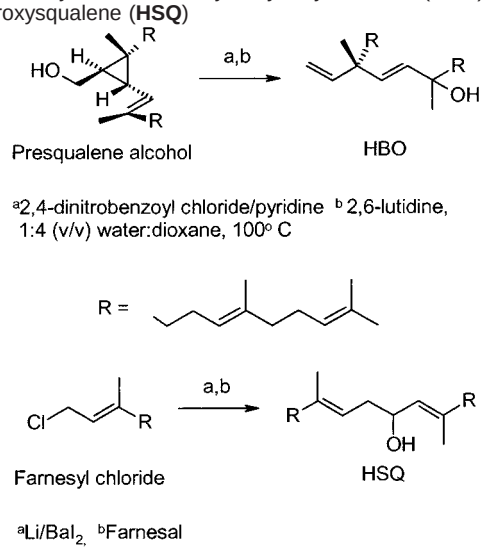
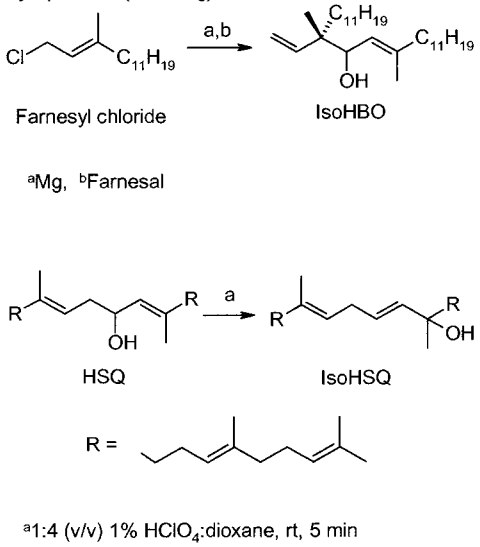
Scheme 2. Synthesis of 10-Hydroxybotryococcene (**HBO**) and 12-Hydroxysqualene (**HSQ**)



Scheme 3. Synthesis of 12-Hydroxybotryococcene (**isoHBO**) and 10-Hydroxysqualene (**isoHSQ**)



ride.[38] The mass spectra and ^{1}H NMR spectra for **HBO** and **HSQ** isolated from the SQase-catalyzed solvolysis of **PSPP** were identical to those of the synthetic alcohols.

As mentioned above, three of the minor products gave mass spectra with peaks at *m*/*z* 426 (M), 408 (M − H_2O), and 69 (C_5H_9, the ω-isoprene unit[39]) characteristic of a triterpene alcohol. On the basis of previous model studies,[1] we surmised that two of the minor alcohols might be the allylic isomers of **HBO** and **HSQ**. Iso**HBO** and iso**HSQ** were synthesized as outlined in Scheme 3. Addition of farnesylmagnesium bromide to farnesal gave iso**HBO** as a mixture of inseparable diastereomers. Iso**HSQ** was obtained by briefly treating **HSQ** with a mixture of 1% perchloric acid in dioxane. The samples of synthetic iso**HBO** and iso**HSQ** coeluted with their respective enzymatic products on normal-phase HPLC and gave identical mass spectra. The structure of triterpene alcohol **2** has not been assigned.

Table 1. Effect of Divalent Metal Ions on Product Distribution

	%[a]	
compound	Mg^{2+}	Mn^{2+}
DSQ	20	32
2	1	<0.5
IsoHBO	2	<0.5
HBO	17	3
IsoHSQ	1	<0.5
HSQ	59	65

[a] Incubations in 50 mM MOPS buffer, pH 7.2 containing 10 mM of the divalent metal ion.

(b) Buffer Containing Methanol. When FPP was incubated with SQase in buffer containing 20% methanol, a new product was formed in addition to **DSQ** and the five triterpenes alcohols. A mass spectrum of the material had a molecular ion at *m*/*z* 440, consistent with a triterpene methyl ether derived from **PSPP**. This was confirmed by the presence of major peaks at *m*/*z* 408 (M − CH_3OH), 235 (a $C_{16}H_{27}O$ fragment from cleavage of the C(12)−C(13) bond), and 69 (C_6H_9, ω-isoprene unit). The fragmentation pattern suggests that the new product is the methyl ether corresponding to **HSQ**. Interestingly, the methyl ether corresponding to **HBO** was not detected among the products.

(c) Influence of Mg^{2+} and Mn^{2+}. Squalene and phytoene synthases require a divalent metal for catalysis. Although Mg^{2+} is thought to be the biologically relevant metal for SQase, the enzyme is active in buffers containing Mn^{2+}.[32] We found a substantial change in the product distribution for recombinant SQSase when Mn^{2+} was substituted for Mg^{2+}. Most noticeable was an increase in the regioselectivity for synthesis of 1′−1 products from 80% to 97% of the total (see Table 1). In addition, the ratio of **DSQ** to **HSQ** increased from approximately 1:3 to 1:2. Under some conditions, a higher specific activity is seen for phytoene synthase with Mn^{2+}.[40] However, it is not known which metal is physiologically relevant.

Stereochemical Studies. (a) Stereochemistry of HBO. HBO has chiral centers at C(10) and C(13). Since no bonds are formed or broken at C(13) during the course of the enzyme-catalyzed transformation of **PSPP**, we assumed that the absolute stereochemistry at the quaternary cyclopropyl carbon was retained in **HBO**. Thus, C(13) should have an *S* configuration. The absolute stereochemistry at C(10) was determined by correlation with the chiral center in linalool (**8**) as shown in Scheme 4. **HBO** was treated with ozone, and the resulting mixture of ozonides was reduced with sodium borohydride to give a mixture of alcohols that included 2-methyl-1,2,5-pentanetriol (**9**) containing C(7)−C(11) from the original hydrocarbon chain. Upon chromatography of the mixture, triol **9** copurified with 2-methyl-2-hydroxymethyl-1,4-butanetriol (**10**). The triols were treated with (*S*)-Mosher's chloride to give the triester of **9** (**9-OMTP**) and the triester of **10** (**10-OMTP**). The esters were separated by column chromatography. (+)-(*S*)-Linalool was synthesized from geraniol by an asymmetric Sharpless epoxidation[41] followed by conversion of the optically active epoxide to the corresponding mesylate, followed by dissolving metal reduction.[42] ^{1}H and ^{19}F NMR spectra of the (*S*)-Mosher's ester of

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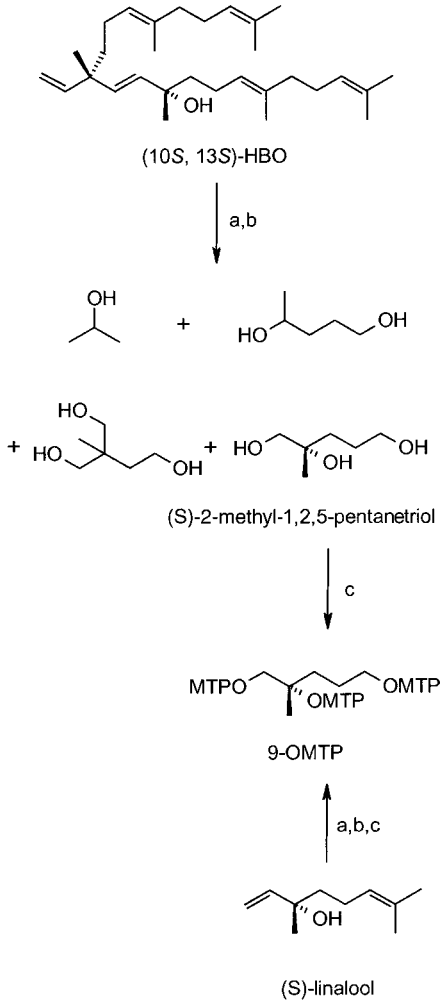
Scheme 4. Correlation of C(10) in 10-Hydroxybotryococcene (**HBO**) with the Chiral Center in Linalool



the alcohol indicated that the *S* enantiomer was formed preferentially with an enantiomeric ratio (er) of 11.5. The optically active alcohol was subjected to the ozonolysis/reduction procedure described for **HBO** to give triol **9**, which was then converted to **9-OMTP**. These experiments were repeated with **HBO** synthesized from presqualene dinitrobenzoate and racemic linalool.

Although attempts to resolve the diastereomers of **9-OMTP** by HPLC on several different analytical columns were unsuccessful, we were able to achieve an excellent separation on a Chiracel OD column. Two peaks of equal area were seen for **9-OMTP** obtained from racemic linalool. The slower eluting diastereomer was identified as the 2*S* isomer by co-injection with **9-OMTP** derived from racemic and (*S*)-linalool. The relative proportion of the **9-OMTP** diastereomers obtained from (*S*)-linalool after ozonolysis and reduction, as determined by HPLC, agreed with a ^{1}H NMR analysis of the enantiomeric purity of the sample of (*S*)-linalool used in the reactions. Thus, the ozonolysis/reduction sequence did not racemize the optically active center. The same HPLC conditions were used to analyze the mixture of **9-OMTP** diastereomers from degradation of **HBO** obtained from the enzyme-catalyzed reaction and from solvolysis of presqualene dinitrobenzoate. As seen in Figure 4,

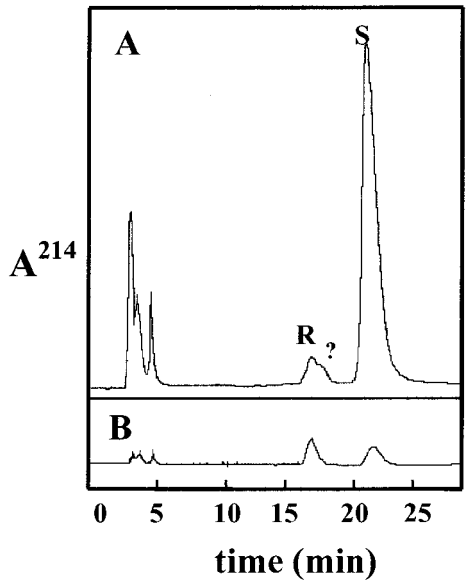


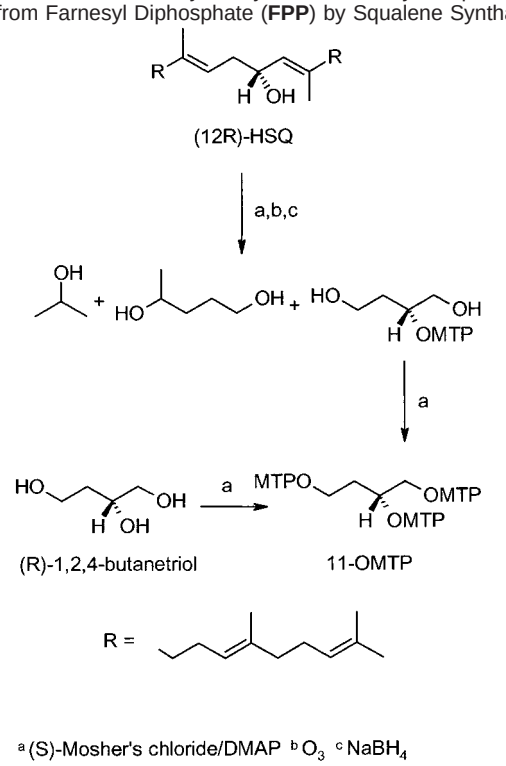
Figure 4. HPLC trace of **9-OMTP** from ozonolysis/reduction of **HBO**. Analysis on a Chriacel OD column with isocratic elution using 2-propanol/hexane (1:99) at a flow rate of 1 mL/min. (A) **9-OMTP** from **HBO** isolated from an incubation of **FPP** with SQase. (B) **9-OMTP** from racemic **HBO**.

the HPLC trace for **9-OMTP** obtained from **HBO** produced from SQase was substantially enriched in the *S* diastereomer, while the triester from the synthetic sample gave equal amounts of the *S* and *R* diastereomers. MS analysis confirmed that the peaks were diastereomers of **9-OMTP**. A mass spectrum of the major diastereomer gave major peaks at *m*/*z* 549 for M − $C_9H_8OF_3$ and *m*/*z* 189 for $C_9H_8OF_3$. A spectrum of the minor diastereomer also gave signals at *m*/*z* 549 and 189, along with peaks at *m*/*z* 447, 315, 103, and 99 from a contaminant that appeared as a shoulder on the peak for the minor diastereomer. On the basis of the HPLC trace from enzymatic **HBO**, the 10*S* stereoisomer constituted at least 92% of the *R*/*S* mixture of enantiomers.

(b) Stereochemistry of HSQ. The stereochemistry at C(12) in **HSQ** isolated from the SQSase-catalyzed solvolysis of **PSPP** was determined by comparing with a Mosher's ester derivative of 1,2,4-butanetriol derived from the triterpene alcohol with Mosher's esters of samples of (*R*)- and (*S*)-1,2,4-butanetriol as outlined in Scheme 5. During exploratory experiments with racemic **HSQ** to optimize the ozonolysis/reduction protocols, we obtained higher recoveries from the Mosher's ester (**6-OMTP**) than from the alcohol. When chromatographed on a Chiracel OD column, **6-OMTP** gave peaks of equal intensity at 18 and 21 min whose mass spectra were identical. Racemic **6-OMTP** was treated with ozone, and the ozonide was reduced with $NaBH_4$, The resulting diol was purified by flash chromatography and then converted to **11-OMTP**. Chromatography of the tris-Mosher's ester on a normal-phase column gave two peaks of equal area (see Figure 5b). The mass spectra and retention times were identical to those of authentic samples of (*R*)- and (*S*)-**11-OMTP**.

In a similar sequence of reactions, **HSQ** produced by SQase was converted to **6-OMTP**. Chromatography of **6-OMTP** on a Chiracel OD column again gave peaks at 18 and 21 min (data not shown); however, the relative peak areas were now 2:23. When **6-OMTP** was converted to **11-OMTP** and the mixture of tris-Mosher's ester diastereomers was chromatographed, the

Scheme 5. Stereochemistry for Synthesis of Dehydrosqualene (**DSQ**) from Farnesyl Diphosphate (**FPP**) by Squalene Synthase



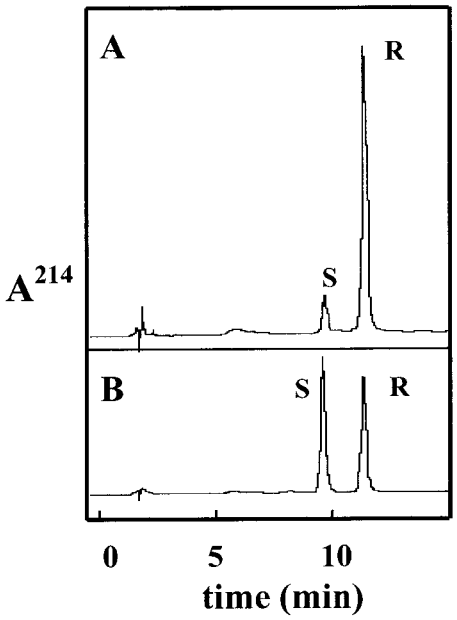


Figure 5. HPLC trace of **11-OMTP** from ozonolysis/reduction of **HSQ**. Analysis on a Chriacel OD column with isocratic elution using 2-propanol/hexane (1:99) at a flow rate of 1 mL/min. (A) **11-OMTP** from **HSQ** isolated from an incubation of **FPP** with SQase. (B) **11-OMTP** from racemic **HSQ**.

peaks for (*S*)- and (*R*)-**11-OMTP** were obtained in a ratio of 2:23 (see Figure 5a). Identical results were obtained when this analysis was repeated with **HSQ** produced from a second incubation of **FPP** with SQase. The compounds giving the peaks in Figure 5a were confirmed as the 2*S* and 2*R* diastereomers of **11-OMTP** by co-injection with authentic standards and by mass spectrometry. The absolute configuration of the major enantiomer of **HSQ** is consistent with a previous determination by exciton coupled circular dichroism[43] and by a previous correlation to 1,2,4-butanetriol.[36] The absolute stereochemistry for addition of nucleophilic water to C(12) of **HSQ** during the SQSase-catalyzed solvolysis of PSPP is identical to that for addition of the hydrogen atom from NADPH to the equivalent carbon atom in squalene.[44,45]

(c) Stereochemistry of the Conversion of FPP to DSQ. When two molecules of **GGPP** are condensed to give (*Z*)-**PH**, the *si* hydrogen atoms at C(1) are lost,[46] one during the cyclopropanation reaction to give **PPPP** and the second during the rearrangement/elimination step that generates **PH**. The stereochemistries of the related reactions were established for the conversion of **FPP** to **DSQ** catalyzed by SQase. (*R*)-[1-^{2}H]-Farnesol ((*R*)-[1-^{2}H]**12**) was prepared by the Bu_3SnD reduction of farnesal using a catalyst prepared from titanium tetraisopropoxide and (*R*)-binaphthol ((*R*)-BITIP) as described by Keck and Krishnamurthy.[47] ^{1}H NMR and MS spectra of the alcohol established that the deuterium content was >98%, and the ratio of *R*/*S* enantiomers was >99, as determined from the ^{1}H NMR spectrum of the Mosher's ester of the labeled alcohol. Initially, (*R*)-[1-^{2}H]**FPP** was prepared by the method of Davisson et al.[48] by converting (*R*)-[1-^{2}H]**13** to the chloride followed by treatment with tris(tetra-*n*-butylammonium) hydrogen diphosphate. However, this series of transformations lowered the *R*/*S* enantiomeric ratio of the diphosphate to approximately 9:1.

Since optical activity was presumably lost because of multiple displacements during the chlorination step, we used the Danilov modification[49] of the Cramer reaction, a reaction that does not break the carbon−oxygen bond in the alcohol, to phosphorylate the deuterium-labeled farnesol. Thus, (*R*)-[1-^{2}H]**13** was converted to (*R*)-[1-^{2}H]**FPP** with an *R*/*S* enantiomeric ratio of >99, as determined by treatment with alkaline phosphate and conversion of the alcohol to the corresponding Mosher's ester. (*S*)-[1-^{2}H]**13** was produced in 65% yield (>98% incorporation) by reduction of farnesal using (*S*)-BITIP and converted to corresponding diphosphate (er > 99) by the Danilov procedure.

DSQ isolated from an incubation of (*R*)-[1-^{2}H]**FPP** and SQSase gave a mass spectrum with a molecular ion at *m*/*z* 410 (see Figure 6a), whereas a mass spectrum of **DSQ** synthesized from unlabeled **FPP** had a molecular ion at *m*/*z* 408 (Figure 6b). Thus, **DSQ** synthesized from two molecules of (*R*)-[1-^{2}H]-**FPP** retained both of the deuterium atoms, while both were lost during the synthesis of **DSQ** from (*S*)-[1-^{2}H]**FPP**. The mass spectrum of **DSQ** synthesized from unlabeled **FPP** was identical to that obtained for **DSQ** from (*S*)-[1-^{2}H]**FPP** (data not shown). Thus, the stereochemistries for the cyclopropanation, rearrangement, and elimination steps for formation of **DSQ** from **FPP** by SQSase and (*Z*)-**PH** from **GGPP** by PHase (see Scheme 6) are identical.[46]

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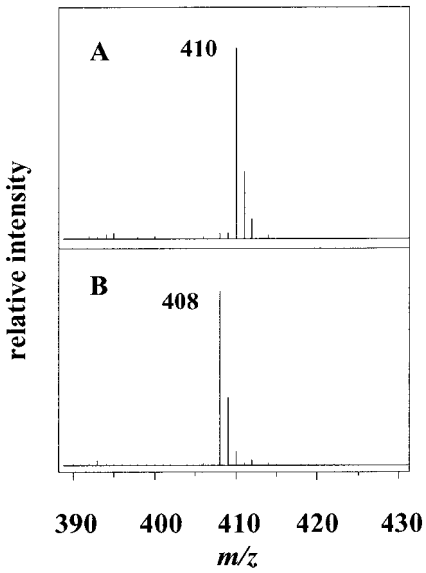
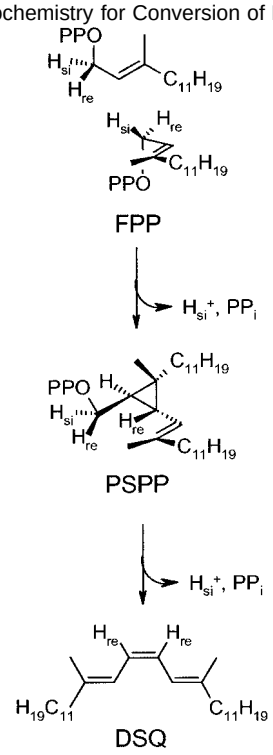


Figure 6. Molecular ion cluster for **DSQ** synthesized from (A) **(*R*)-[1-^{2}H]FPP** and (B) from **(*S*)-[1-^{2}H]FPP**.

Scheme 6. Stereochemistry for Conversion of **FPP** to **DSQ**



Discussion

The 1′−4, or "regular", linkage between isoprene units is a consequence of the fundamental chain elongation reaction in the pathway. During each step, the hydrocarbon chain in the allylic diphosphate substrate is extended by adding its hydrocarbon moiety to the double bond in isopentenyl diphosphate (**IPP**) to generate an allylic product containing an additional isoprene unit. The mechanism for chain elongation is an electrophilic alkylation of the double bond in **IPP** by the carbocation derived from the allylic substrate.[50] The enzymes that catalyze chain elongation are selective for the length of the hydrocarbon chain and the stereochemistry of the newly formed double bond. They fall into one of two distinct families based on the stereochemistry of the double bond in the allylic product. Those enzymes that catalyze formation of *E* double bonds are all α-helical proteins[50,51] and are related to several isoprenoid cyclases,[52,53] while those that synthesize *Z* double bonds are structurally unrelated.[51] Amino acid sequences for SQase from a wide variety of organisms do not show substantial similarity to other enzymes in the isoprenoid pathway. However, a recent X-ray structure for human SQase[54] shows that the protein has a fold similar to the all-α-helix "isoprenoid fold" first reported for farnesyl diphosphate synthase.[50−53]

"Irregular" isoprenoids are formed by joining two molecules of an allylic diphosphate and, except for the irregular monoterpenes derived from dimethylallyl diphosphate (**DMAPP**), also contain 1′−4 linkages generated during chain elongation. The most prominent examples are the triterpenes derived from squalene, which has a 1′−1 linkage between two farnesyl residues, and the carotenoids derived from phytoene, which has a 1′−1 linkage between two geranylgeranyl units. The 1′−1 bond is formed in two distinct reactions. In the first, C(1) of one of the allylic diphosphates is embedded in the C(2)−C(3) double bond of the other allylic substrate to generate a cyclopropylcarbinyl diphosphate with a c1′−2−3 structure. In this manner, **FPP** yields (1*R*,2*R*,3*R*)-**PSPP**, and **GGPP** gives (1*R*,2*R*,3*R*)-**PPPP**.[2] In a second reaction, the cyclopropylcarbinyl diphosphates rearrange to 1′−1 structures. An alternative rearrangement of **PSPP** produces **BO** with a 1′−3 linkage between two C_{15} residues.[2,17]

Mechanisms proposed for the conversion of **PSPP** to squalene have centered on the rearrangement of c1′−2−3$^+$ to c1′−1−2$^+$ (see Scheme 7).[13−16] These suggestions were subsequently expanded to include stereochemical features related to the biosynthesis of squalene and phytoene as well as the formation of other irregular isoprenoid linkages.[4] Key aspects of these proposals were verified in studies using the C_{10} chrysanthemyl system as a model for the presqualene and prephytoene rearrangements.[1,18−25] It is now possible to firmly link the model studies with the behavior of SQase. In a normal incubation of the enzyme with **FPP** and NADPH, squalene is the sole product. However, when NADPH is *not* present, both the regioselectivity and stereoselectivity normally associated with the reaction catalyzed by SQase are compromised, and a mixture of products is obtained. The major components of the mixture are the 1′−1 triterpenes **DSQ**, a C_{30} analogue of (*Z*)-**PH**, and **HSQ**, a hydroxy analogue of **SQ**. **HBO**, a hydroxy analogue of **BO**, which has a 1′−3 linkage between the farnesyl residues, is also formed along with minor amounts of the allylic isomers of **HSQ** and **HBO**. The major triterpene alcohols formed during the reaction were (12*S*)-**HSQ** and (10*S*,13*R*)-**HBO**. However, approximately 8% of the 12*S* and 10*S*,13*R* stereoisomers were seen as well. In the absence of NADPH, SQase appears to catalyze the "solvolysis" of **PSPP** to give rearranged products similar to those seen during the model reactions. The loss of stereocontrol

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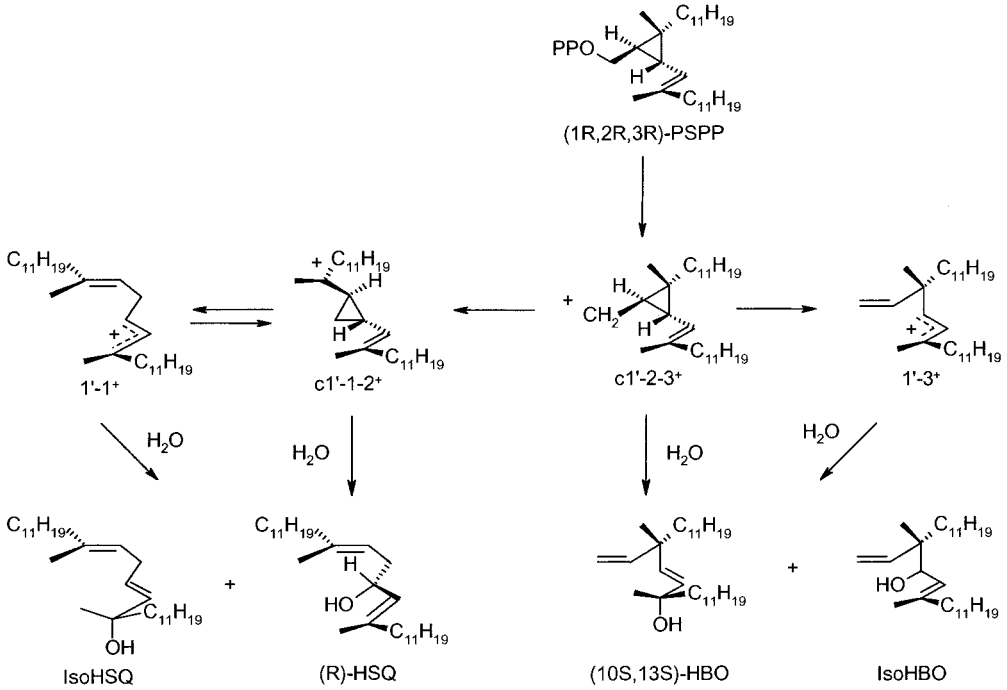
Scheme 7. A Mechanism for Formation of 1′−1 and 1′−3 Triterpenes from Presqualene Diphosphate



and the formation of allylic isomers of **HSQ** and **HBO** most likely arise by isomerization of c1′−2−3$^+$ and c1′−1−2$^+$ to 1′−3$^+$ and 1′−1$^+$, respectively, in analogy to what was observed in the model studies.[2] However, when one compares the product distributions of the model and enzyme catalyzed reactions, it is clear that the enzyme clearly exerts a substantial influence on both the stereochemistry and regiochemistry of the rearrangements. In addition, a methyl ether corresponding to **HSQ** was detected among the products when the incubation buffer contained methanol. This result provides additional support for the mechanism outlined in Scheme 7 and suggests that the active site of SQase contains bound molecules of methanol when the alcohol is present in the buffer.

The reactions catalyzed by SQase are apparently sensitive to subtle structural and electronic effects. As seen in Table 1, the percentage of 1′−3 products decreased substantially from 18% to only 3% with concomitant increase in the amount of 1′−1 products from 80% to 97% when Mg^{2+} was replaced by Mn^{2+}. Divalent metals are typically required for catalysis by prenyltransferases,[55] and in the crystal structure of farnesyl diphosphate synthase they form a bridge between the diphosphate group in the allylic substrate and active-site carboxylate residues. The X-ray structure of human SQase suggests that the diphosphate moieties are also bound through metal bridges to active site carboxylates. It is interesting to note that there is no change in the relative proportions of primary and tertiary substitution products when FPP and GPP are solvolyzed in aqueous buffers containing Mn^{2+} or Mg^{2+}.[56,57] Thus, we conclude that the perturbations due to metal ions are specific to the SQSase·carbocation·PP_i·metal complex.

In conclusion, the products formed when SQase is incubated with **FPP** in the absence of NADPH demonstrate that the catalytic machinery of the enzyme can synthesize 1′−1 compounds typical of those seen in the sterol and carotenoid biosynthetic pathways, as well as the 1′−3 structures found in botryococcene. The cyclopropylcarbinyl cationic rearrangements seen in model studies that previously formed the basis for an integrated mechanism for the biosynthesis of irregular isoprenoids have now been detected in enzyme-catalyzed transformations as well.

Experimental Section

Methods. ^{1}H NMR spectra were recorded at 300 MHz and referenced to $CDCl_3$, C_6D_6, CD_3OD, or DDS. ^{13}C NMR spectra were proton decoupled, recorded at 75 MHz, and referenced to $CDCl_3$, CD_3OD, or DDS. ^{31}P NMR spectra were proton decoupled and referenced to external 10% H_3PO_4. Purification by chromatography refers to flash chromatography on silica gel (grade 60, 230−400 mesh) unless otherwise stated. Concentrations for optical rotations are reported in grams per 100 mL. Thin-layer chromatography (TLC) was performed with silica gel 60 plates using the indicated solvent system, visualized by UV light, and developed with phosphomolybdic acid or *p*-anisaldehyde. All synthetic reactions were performed under a nitrogen atmosphere in oven-dried glassware. Organic extracts of reactions were dried over anhydrous Na_2SO_4, filtrated, and concentrated by rotary evaporation unless otherwise indicated. Normal-phase HPLC analysis was performed on a Rainin 4.6 × 250 mm microsorb MV 5 μm silica gel column or a Phenomenex 4.6 × 250 mm Luna silica gel column. Reversed-phase HPLC analysis was performed on a Phenomenex 4.6 × 250 mm Prodigy ODS column. Protein concentrations were determined by the method of Bradford.[58]

Materials. Solvents were dried and purified according to published procedures.[59] Reagents for organic transformations were ACS reagent grade and used without further purification unless otherwise noted. Isoprenoid alcohols and halides were distilled prior to use and stored under nitrogen. Mosher's chloride was prepared using the method of Gao et al.[41] Pure truncated recombinant yeast SQSase was obtained as

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described by Zhang et al.[32] except that the Phenyl Superose column was replaced by a Phenyl Source (Pharmacia). 3-(*N*-Morpholino)-propanesulfonic acid (MOPS) was from Sigma. **FPP** was prepared by the method of Davisson et al.,[48] and presqualene alcohol was a provided by Eugene Yi.

Analysis of Products from Incubations of FPP with SQase. (a) Analytical Scale. Typically, 130 μg (0.3 μmol) of **FPP** was incubated with 1 mg (23 nmol) of recombinant yeast SQase in 1 mL of 50 mM MOPS buffer, pH 7.2, containing 10 mM $MgCl_2$ for 1.5 h at 30 °C. The buffer was degassed to minimize inactivation of SQase by oxidation. After the incubation, the reaction mixture was saturated with solid NaCl. Individual reaction mixtures were extracted with 3 × 1 mL of methyl *tert*-butyl ether (MTBE) to obtain hydrocarbons, alcohols, or methyl ethers or with 3 × 1 mL of *n*-butanol to obtain **FPP** and **PSPP**. Emulsions that formed during the extractions were clarified by centrifugation at 3000*g*. The MTBE extracts were concentrated under a stream of nitrogen and dissolved in hexanes. The products were analyzed by normal-phase HPLC with isocratic elution with MTBE/hexanes (1:19 v/v). Diphosphates were detected by concentrating the *n*-butanol extract under a stream of nitrogen and dissolving the white residue in 25 mM NH_4HCO_3. The solution was analyzed by reversed-phase HPLC with a 5 min elution with 25 mM NH_4HCO_3, followed by a 0% to 100% linear gradient of CH_3CN at 0.7 mL/min over 25 min. Under these conditions, **FPP** eluted at 15-min and **PSPP** eluted at 30 min.

The products were detected and their relative abundances were determined from their absorbance at 214 nm. The extinction coefficients for **HSQ** ($\epsilon_{214} = 11\,450\ M^{-1}\ cm^{-1}$), iso**HSQ** ($\epsilon_{214} = 11\,500\ M^{-1}\ cm^{-1}$), **HBO** ($\epsilon_{214} = 11\,650\ M^{-1}\ cm^{-1}$), and iso**HBO** ($\epsilon_{214} = 11\,700\ M^{-1}\ cm^{-1}$) were measured for synthetic samples in hexane. The extinction coefficient for **DSQ** ($\epsilon_{214} = 11\,110\ M^{-1}\ cm^{-1}$) was estimated from the extinction coefficient reported at 285 nm ($\epsilon_{285} = 42\,200\ M^{-1}\ cm^{-1}$)[60,61] and the ratio of the absorbances for **DSQ** at 285 and 214 nm ($A_{285}/A_{214} = 3.85$). An extinction coefficient ($\epsilon_{214} = 11\,600\ M^{-1}\ cm^{-1}$), based on the average extinction coefficients of the other C_{30} alcohols, was arbitrarily assigned to the unidentified triterpene alcohol (**2**).

(b) Preparative Scale. Typically, 14 mg (32 μmol) of **FPP** and 30 mg (0.68 μmol) of SQSase were incubated in 30 mL of 50 mM MOPS buffer, pH 7.2, containing 10 mM $MgCl_2$ at 30 °C. To minimize the precipitation of the magnesium salts of **FPP** and **PSPP**, **FPP** was added in four equal portions at 15-min intervals, and SQSase was added in three equal portions at 20-min intervals. After 2 h, the reaction mixture was saturated with solid NaCl and extracted with MTBE (3 × 30 mL). The combined extracts were dried, concentrated, and purified by normal-phase HPLC on a Rainin microsorb MV silica gel column (1:19 (v/v) MTBE/hexanes) to afford the following products in order of elution.

Dehydrosqualene (DSQ, 1) (3.0 min): UV (hexanes), λ_{max} 286 nm; ^{1}H NMR ($CDCl_3$) δ 6.3 (2H, m), 6.1 (2H, m), 5.14−5.04 (4H, br m), 2.2−1.9 (16H, br m), 1.8−1.5 (24H); MS (EI, 70 eV) *m*/*z* 408 (M, 18), 69 (100); HRMS for $C_{30}H_{48}$ calcd 408.3756, found 408.3749.

Unidentified Triterpene Alcohol (2) (7 min): MS (EI 70 eV) *m*/*z* 426 (M, 2), 408 (M − H_2O, 25), 81 (40), 69 (100).

12-Hydroxybotryococcene (IsoHBO, 3) (9 min): MS (EI 70 eV) *m*/*z* 426 (M, 10), 408 (M − H_2O, 20) 339 (20), 271 (5), 258 (20), 205 (10), 203 (20), 137 (80), 121 (60), 95 (60), 81 (80) 69 (100); HRMS (EI 70 eV) for $C_{30}H_{50}O$ calcd 426.3861, found 426.3865.

10-Hydroxybotryococcene (HBO, 4) (10.5 min): ^{1}H NMR ($CDCl_3$) δ 5.79 (1H, dd, J = 17.5, 10.8 Hz), 5.60 (1H, d, J = 16.0 Hz), 5.43 (1H, d, J = 16.0 Hz), 5.15−5.05 (4H, m), 4.97 (1H, dd, J = 10.8, 1.4 Hz), 4.94 (1H, dd, J = 17.5, 1.4 Hz), 2.1−1.85 (14H, br m), 1.65 (6H, s), 1.56 (9H, s), 1.52 (3H, s), 1.42−1.3 (2H, m), 1.26 (3H, s), 1.1 (3 H, s); MS (EI, 70 eV) *m*/*z* 426 (M, 2), 408 (M − H_2O, 27), 203 (19) 69 (100); HRMS for $C_{30}H_{50}O$ calcd 426.3861, found 426.3848.

10-Hydroxysqualene (IsoHSQ, 5) (12 min, 1%): MS (EI, 70 eV) *m*/*z* 426 (M^+, 1), 408 (M − H_2O, 10), 271 (40) 229 (2), 81 (40), 69 (100); HRMS (EI, 70 eV) $C_{30}H_{50}O$ calcd 426.3861, found 426.3848.

12-Hydroxysqualene (HSQ, 6) (16 min): ^{1}H NMR ($CDCl_3$) δ 5.18 (1H, d, J = 7.4 Hz), 5.07 (5H, br m), 4.33 (1H, dt, J = 7.4, 6.9 Hz), 2.28 (1H, m), 2.17 (1H, m), 2.15−1.90 (16H, br m) 1.65−1.55 (24H); MS (EI, 70 eV) *m*/*z* 426 (M, 1), 408 (M − H_2O, 20), 271 (5), 221 (37), 206 (100), 69 (70); HRMS for $C_{30}H_{50}O$ calcd 426.3861, found 426.3890.

Synthesis of 12-Hydroxybotryococcene (isoHBO, 3). Farnesyl chloride (482 mg, 2 mmol) was added over the course of 1 h to a stirred suspension of 100 mg (4.1 mmol) of magnesium turnings in 5 mL of THF. Stirring was continued for 1 h, the solution was brought to reflux, and 200 mg (0.9 mmol) of farnesal (**7**)[62] in 1 mL of THF was added over 10 min. After 30 min, the mixture was allowed to cool to room temperature, and 2 mL of HCl (1 M) was added, followed by 5 mL of ether. The organic layer was separated and washed with 10 mL of a saturated NH_4HCO_3, followed by 10 mL of brine. The organic layer was dried, concentrated, and purified by chromatography (1:19 (v/v) EtOAc/hexanes) to give 80 mg (20%) of a colorless oil: TLC R_f = 0.43 (3:7 (v/v) EtOAc/hexanes); IR (neat, cm^{-1}) 3455 (br); ^{1}H NMR ($CDCl_3$, 3:2 mixture of diastereomers) δ 5.83, major, 5.82, minor (1H, dd, J = 15, 10.5 Hz), 5.25−5.15 (2H, m), 5.11−5.02 (5H, m), 4.09 (1H, d, J = 9 Hz), 2.2−1.85 (16H, m), 1.71−1.69 (9H, m), 1.61 (9H, s), 1.59 (3H, s), 1.38 (1H, t, J = 8 Hz), 0.98, major, 1.07, minor (3H, s); ^{13}C NMR ($CDCl_3$, 3:2 mixture of diastereomers) δ 144.1, 143.2, 140.3, 139.9, 135.4, 134.9, 134.9, 131.5, 125.0, 124.7, 124.5, 124.5, 124.3, 124.0, 123.9, 115.4, 114.9, 74.7, 73.9, 45.8, 45.3, 40.2, 40.1, 40.0, 39.9, 37.5, 36.6, 27.0, 26.9, 26.6, 25.9, 22.9, 22.8, 19.1, 17.9, 17.3, 16.7, 16.3, 16.2, 16.0; MS (EI 70 eV) *m*/*z* 426 (M, 10), 408 (M − H_2O, 20) 339 (20), 271 (5), 258 (20), 205 (10), 203 (20), 137 (80), 121 (60), 95 (60), 81 (80) 69 (100); HRMS (EI 70 eV) for $C_{30}H_{50}O$ calcd 426.3861, found 426.3876.

Synthesis of 10-Hydroxybotryococcene (HBO, 4). Presqualene 2,4-Dinitrobenzoate (24). Presqualene alcohol (300 mg, 0.7 mmol) was dissolved in 4 mL of pyridine. 2,4-Dinitrobenzoyl chloride (185, 0.8 mmol) was added, and the mixture was stirred for 5 h at 50 °C. Ether (30 mL) was added, and the mixture was washed with 2 × 20 mL of saturated NH_4HCO_3. The ether layer was dried and concentrated. The yellow residue was purified by chromatography (1:5 (v/v) EtOAc/hexanes) to give 250 mg (59%) of a yellow oil: TLC R_f = 0.53 (1:19 (v/v) EtOAc/hexanes); ^{1}H NMR ($CDCl_3$) δ 9.2 (1H, s), 9.1 (2H, s), 5.2−5.05 (4H, m), 4.98 (1H, d, 6 Hz), 4.5 (1H, dd, J = 12, 8 Hz), 4.4 (1H, dd, 12 Hz, 8 Hz), 2.2−1.9 (16H, m), 1.75 (3H, s), 1.68 (6H, s), 1.6 (12H, s), 1.45 (1H, m), 1.3, (1H, m), 1.1 (3H, s); ^{13}C NMR ($CDCl_3$) δ 162.5, 148.6, 140.0, 135.0, 134.2, 131.3, 129.3, 124.2, 123.8, 121.1, 117.8, 65.8, 42.7, 39.7, 39.7, 26.8, 26.7, 26.6, 26.4, 25.8, 25.7, 25.1, 25.1, 17.7, 16.9, 16.1, 16.0, 13.1.

10-Hydroxybotryococcene (HBO, 4). Presqualene dinitrobenzoate (**7**) (110 mg, 0.18 mmol) was dissolved in 5 mL of 80% dioxane/water containing 80 μL (0.71 mmol) of 2,6-lutidine. The solution was heated at 100 °C for 24 h, allowed to cool to room temperature, diluted with 10 mL of ether, and washed with 2 × 10 mL of saturated NH_4HCO_3 followed by 10 mL of brine. The ether layer was dried, concentrated, and purified by normal-phase HPLC (isocratic elution with hexanes for 5 min, followed by a 40 min linear gradient to 3:7 (v/v) TBME/hexanes at 1 mL/min) to give 10 mg (13%) of a clear oil: ^{1}H NMR ($CDCl_3$) δ 5.79 (1H, dd, J = 17.5, 10.8 Hz), 5.6 (1H, d, J = 16.0 Hz), 5.43 (1H, d, J = 6.0 Hz), 5.15−5.05 (4H, m), 4.97 (1H, dd, J = 10.8, 1.4 Hz), 4.94 (1H, dd, J = 17.5, 1.4 Hz), 2.1−1.85 (14H, br m), 1.65 (6H, s), 1.56 (9H, s), 1.52 (3H, s), 1.42−1.3 (2H, m), 1.26 (3H, s), 1.1 (3 H, s); ^{13}C NMR ($CDCl_3$) δ 146.0, 135.4, 135.0, 134.3, 131.3, 124.6, 124.2, 111.6, 73.2, 42.7, 41.9, 41.2, 39.7, 28.5, 28.5, 26.8, 26.7, 25.7, 23.5, 23.4, 23.1, 22.9, 17.7, 16.0, 15.9; MS (EI, 70 eV) *m*/*z* 426 (M,

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(61) Suzue, G.; Tsukada, K.; Tanaka, S. *Biochim. Biophys. Acta* **1968**, *164*, 88−93.
(62) Griffith, W. P.; Ley, S. V. *Aldrichimica Acta* **1990**, *23*, 13−19.

2), 408 (M − H_2O, 27), 203 (19), 69 (100); HRMS for $C_{30}H_{50}O$ calcd 426.3861, found 426.3848.

Synthesis of 10-Hydroxysqualene (isoHSQ, 5). A sample of 30 mg (0.07 mmol) of **HSQ** was treated with 5 mL of 4:1 (v/v) dioxane/1% aqueous perchloric acid. This mixture was stirred 10 min, transferred to a separatory funnel, diluted with 5 mL ether, and washed with 5 mL of saturated NH_4HCO_3 followed by 5 mL of brine. The organic layer was dried and concentrated. The residue was purified by chromatography (1:19 (v/v) EtOAc/hexanes) to give **HSQ** (15 mg, 50%) as a colorless oil and **IsoHSQ** (15 mg, 50%) as a pale yellow oil. **IsoHSQ** was further purified by normal-phase HPLC (1:19 (v/v) MTBE/hexanes) to give 5 mg (17%) of a pale yellow oil: TLC R_f = 0.38 (1:4 (v/v) EtOAc/hexanes); IR(neat, cm^{-1}) 3460 (br); ^1H NMR ($CDCl_3$) δ 5.57 (1H, dd, J = 15, 6 Hz), 5.48 (1H, d, J = 15 Hz), 5.16−5.03 (5H, m, H), 2.72 (2H, t, J = 6 Hz), 2.08−1.92 (16H, m), 1.66 (3H, s), 1.58 (15H, s), 1.53 (3H, s), 1.24 (3H, s); ^{13}C NMR ($CDCl_3$) δ 136.9, 136.5, 135.7, 135.6, 135.3, 131.5, 126.9, 126.8, 124.6, 124.6, 124.5, 124.4, 124.3, 122.1, 105.9, 73.3, 42.7, 40.1, 39.9, 31.9, 31.0, 28.4,26.9, 26.8, 25.9, 23.1, 17.9, 16.3, 14.4; MS (EI, 70 eV) *m*/*z* 426 (M,5), 408 (20, M−H_2O), 365 (5), 339 (20), 271 (70) 229 (20), 215 (25), 203 (10), 81 (40), 69 (100); HRMS (EI, 70 eV) $C_{30}H_{50}O$ calcd 426.3861, found 426.3860.

Synthesis of 12-Hydroxysqualene (HSQ, 6). HSQ was prepared according to the procedure of Yanagisawa et al.[38] using 82 mg (11.6 mmol) of lithium, 1.8 g (11.5 mmol) of biphenyl, 3 g (8.0 mmol) of vacuum-dried $BaI_2 \cdot 2H_2O$, 1.2 g (5 mmol) of farnesyl chloride, and 0.6 g (2.7 mmol) of **7**. Chromatography (1:19 (v/v) EtOAc/hexanes) afforded 0.62 g (67%) of a clear oil: ^1H NMR ($CDCl_3$) δ 5.18 (1H, d, J = 7.4 Hz), 5.07 (5H, br m), 4.33 (1H, dt, J = 7.4, 6.9 Hz), 2.28 (1H, m), 2.17 (1H, m), 2.15−1.90 (16 H, br m), 1.65−1.55 (24H, br m); ^{13}C NMR ($CDCl_3$) δ 138.8, 138.7, 135.2, 131.3, 131.2, 127.2, 124.3, 124.0, 123.8, 119.6, 68.4, 40.0, 39.7, 39.6, 36.4, 26.8, 26.6, 26.4, 25.7, 17.7, 16.7, 16.4, 16.1, 16.0; MS (EI, 70 eV) *m*/*z* 426, 408 (20), 221 (37), 271 (5), 206 (100), 69 (70); HRMS for $C_{30}H_{50}O$ calcd 426.3861, found 426.3860.

Stereochemical Analysis of HBO. 2-Methyl-1,2,5-pentanetriol (8) (General Ozonolysis Procedure). A solution of 50 mg (0.33 mmol) of racemic linalool in 10 mL of 1:1 (v/v) CH_3OH/CH_2Cl_2 was cooled to −78 °C. Ozone was bubbled through the stirred solution until a blue color persisted. Stirring was continued for 5 min before a gentle stream of nitrogen was used to disperse the excess ozone. $NaBH_4$ (30 mg, 0.8 mmol) was added to the stirred solution in small portions. The reaction was allowed to warm to 0 °C, and stirring was continued overnight before 0.2 mL of saturated NH_4Cl was added. After an additional 1 h of stirring, the mixture was filtered through Celite, followed by repeated washing of the Celite with 1:9 (v/v) methanol/CH_2Cl_2. The eluent was concentrated and purified by chromatography (1:9 (v/v) methanol/CH_2Cl_2) to yield 33 mg (80%) of a clear oil: TLC R_f = 0.15 (1:9 (v/v) methanol/CH_2Cl_2); IR (neat, cm^{-1}) 3400 (br); ^1H NMR (CD_3OD) δ 3.55 (2H, t, J = 6.3 Hz), 3.35 (2H, s), 1.66−1.56 (2H, m), 1.54−1.48 (2H, m), 1.13 (3H, s); ^{13}C NMR (CD_3OD) δ 74.6, 71.6, 64.8, 37.0, 29.0, 25.0; MS (CI) *m*/*z* 135 (M + 1, 1), 117 (40, M − H_2O), 99 (100), 81 (75), 57 (40); HRMS (CI) calcd for $C_6H_{15}O_3$ (M + 1) 135.1021, found 135.1007.

Bis-Mosher's Ester of 2-Methyl-1,2,5-pentanetriol (8-OMTP) (General Procedure for Preparing Mosher's Esters).[41,63] A solution of 5 mg (0.05 mmol) of triol **8**, 30 μL (0.16 mmol) of (*S*)-Mosher's chloride, and 30 mg (0.25 mmol) of DMAP in 500 μL of CH_2Cl_2 was stirred for 5 min at room temperature. The reaction was terminated by the addition of 1 mL of hexanes, followed by filtration of the suspension through silica. Solvent was removed with a stream of nitrogen, and the residue was purified by chromatography (1:19 (v/v) EtOAc/hexanes). HPLC analysis on a Baker Chiralcel OD column (1:99 (v/v) 2-propanol/hexanes) gave two peaks of equal intensity with retention times at 18 min [MS (EI) *m*/*z* 549 (20), 189 (100); HRMS (EI) calcd for $C_{26}H_{27}O_6F_6$ (M^+ − $C_9H_8OF_3$) 549.1712 found 549.1730] and 21 min [MS (EI) *m*/*z* 549 (20), 189 (100); HRMS (EI) calcd for $C_{26}H_{27}O_6F_6$ (M^+ − $C_9H_8OF_3$) 549.1712, found 549.1700].

(*S*)-Linalool ((*S*)-10). The Sharpless[41] asymmetric epoxidation was used to convert geraniol (5 g, 36 mmol) to 4 g (72%) of the 2,3-epoxide (83% ee by analysis of the ^{19}F NMR spectrum of the ester from (*S*)-Mosher's chloride): $[\alpha]^{25}_D = -5.1$ (*c* 4.0, $CHCl_3$) (lit.[37] $[\alpha]^{25}_D = -5.89$ ($CHCl_3$)); ^1H NMR ($CDCl_3$) δ 5.05 (3 H, t, J = 7.2 Hz), 3.83−3.72 (1 H, m), 3.68−3.57 (1 H, m), 2.9 (1 H, dd, J = 6.9, 4.2 Hz), 2.5, (1 H, br s), 2.04 (2 H, q, J = 7.5 Hz), 1.64 (3H, s), 1.56 (3H, s), 1.49−1.36 (1 H, m), 1.25 (3H, s); ^{13}C NMR ($CDCl_3$) δ 132.1, 123.3, 63.0, 61.4, 61.1, 38.4, 25.6, 23.6, 17.6, 16.7.

Following the procedure of Yasuda et al.,[42] the epoxide (1 g, 6.5 mmol) was converted to (*S*)-linalool to give 720 mg (80%, 83% ee as determined by analysis of the ^{19}F NMR spectrum of the ester derived from (*S*)-Mosher's chloride): $[\alpha]^{25}_D = +16.0$ (*c* 6.95, $CHCl_3$); ^1H NMR (C_6D_6) δ 5.89 (1H, dd, J = 17.4, 10.8 Hz), 5.19 (1H, dd, J = 17.4, 1.5 Hz), 5.10 (1H, m), 5.02 (1H, dd, J = 10.8, 1.5 Hz), 2.1−1.9 (2H, m), 1.66 (3H, s), 1.58 (3H, s), 1.58−1.50 (3H, m), 1.26 (3H, s); ^{13}C NMR (C_6D_6) δ 145.9, 131.9, 124.2, 11.6, 73.5, 42.1, 27.9, 25.7, 22.8, 17.7.

(*S*)-2-Methyl-1,2,5-pentanetriol ((*S*)-8). (*S*)-Linalool (400 mg, 2.6 mmol) was treated with ozone and 250 mg (6.6 mmol) of $NaBH_4$ according to the procedure described for racemic **8** to give 266 mg (77%) of a clear oil: TLC R_f = 0.15 (1:9 (v/v) methanol/CH_2Cl_2); IR (neat, cm^{-1}) 3400 (br); $[\alpha]^{25}_D = -1.54$ (*c* 13.3, 1:1 MeOH/CH_2Cl_2); ^1H NMR (CD_3OD) δ 3.55 (2H, t, J = 6.3 Hz), 3.35 (2H, s), 1.66−1.56 (2H, m), 1.54−1.48 (2H, m), 1.13 (3H, s); ^{13}C NMR (CD_3OD) δ 74.6, 71.6, 64.8, 37.0, 29.0, 25.0; MS (CI) *m*/*z* 135 (M + 1), 117 (40, M − H_2O), 99 (100), 81 (75), 57 (40); HRMS (CI) calcd for $C_6H_{15}O_3$ (M + 1) 135.1021, found 135.1001.

Bis-Mosher's Ester of (*S*)-2-Methyl-1,2,5-pentanetriol ((*S*)-8-OMTP). (*S*)-2-Methyl-1,2,5-pentanetriol (5 mg, 0.05 mmol) was treated with 30 μL (0.16 mmol) of (*S*)-Mosher's chloride and 30 mg (0.25 mmol) of DMAP according to the procedure described for **8-OMTP**. HPLC analysis on a Baker Chiracel OD column (1:99 (v/v) 2-propanol/hexanes) gave peaks at 18 min (8%) [MS (EI, 70 eV) *m*/*z* 549 (M, 20), 189 (100); HRMS (EI) calcd for $C_{26}H_{27}O_6F_6$ (M − $C_9H_8OF_3$) 549.1712, found 549.1720] and 21 min (92%) [MS (EI, 70 eV) *m*/*z* 549 (M, 20), 189 (100); HRMS (EI) calcd for $C_{26}H_{27}O_6F_6$ (M − $C_9H_8OF_3$) 549.1712, found 549.1718].

Racemic HBO. The general ozonolysis procedure was followed for 1 mg (2 μmol) of an equal mixture of racemic **HBO** diastereomers dissolved in 4 mL of 1:1 (v/v) MeOH/CH_2Cl_2. The ozonide was treated with 5 mg (0.13 mmol) of $NaBH_4$, and the products were purified by TLC (1:9 (v/v) MeOH/CH_2Cl_2). Alcohols **8** and **9** copurified. The mixture was dissolved in 1 mL of 1:9 (v/v) MeOH/CH_2Cl_2 and transferred to a vial, where the solvent was removed with a stream of nitrogen followed by a vacuum. The residue was treated according to the procedure described for **8-OMTP** using 5 μL (30 μmol) of Mosher's chloride and 5 mg (41 μmol) of DMAP in 250 μL of CH_2Cl_2. The residue was purified by chromatography to yield a mixture of **8-OMTP** and the tri-ester of meso alcohol **9**. Bis-Mosher's ester **8-OMTP** was further purified by HPLC on a Baker Chiracel OD column (1:19 (v/v) 2-propanol/hexanes). Analysis of the purified diastereomers of **8-OMTP** by HPLC on a Baker Chiracel OD column (1:99 (v/v) 2-propanol/hexanes) gave peaks of equal intensity at 18 min [MS (EI, 70 eV) *m*/*z* 549 (M, 20), 189 (100); HRMS (EI) calcd for $C_{26}H_{27}O_6F_6$ (M − $C_9H_8OF_3$) 549.1712, found 549.1730] and 21 min [MS (EI, 70 eV) *m*/*z* 549 (M, 20), 189 (100); HRMS (EI) calcd for $C_{26}H_{27}O_6F_6$ (M − $C_9H_8OF_3$) 549.1712, found 549.1750]. The tris-ester of **9** was analyzed by HRMS (EI) calcd for $C_{27}H_{29}O_6F_6$ (M − $C_9H_8OF_3$) 563.1868, found 563.1876.

HBO from Incubation of FPP and SQase. Following the procedure for racemic **HBO**, 200 μg (1 μmol) of **HBO** from an incubation of **FPP** with SQSase was ozonized and reduced with 5 mg (100 μmol) of

(63) Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543−2549.

$NaBH_4$ in 4 mL of 1:1 (v/v) MeOH/CH_2Cl_2. The residue was purified by TLC (1:9 (v/v) CH_3OH/CH_2Cl_2), and the Mosher's esters were prepared according to the procedure described for **8-OMTP** using 5 μL (30 μmol) of Mosher's chloride and 5 mg (41 μmol) of DMAP in 250 μL of CH_2Cl_2. Analysis of the purified diastereomers of **8-OMTP** from enzymatic **HBO** by HPLC on a Baker Chiracel OD column (1:99 (v/v) 2-propanol/hexanes) gave peaks at 18 min (7%) [MS (EI, 70 eV) *m*/*z* 549 (M − $C_9H_8OF_3$, 20), 447 (40), 315 (20), 189 (100), 103 (15), 99 (85); HRMS (EI) calcd for $C_{26}H_{27}O_6F_6$ (M − $C_9H_8OF_3$) 549.1712, found 549.1725] and 21 min (93%) [MS (EI, 70 eV) *m*/*z* 549 (M − $C_9H_8OF_3$, 20), 189 (100); HRMS (EI) calcd for $C_{26}H_{27}O_6F_6$ (M − $C_9H_8OF_3$) 549.1712, found 549.1697].

Stereochemical Analysis of HSQ. Tris-Mosher's Ester of 1,2,4-Butanetriol (11-OMTP). 1,2,4-Butanetriol (11 mg, 0.10 mmol) was treated with 110 μL (0.56 mmol) of (*S*)-Mosher's chloride and 70 mg (0.57 mmol) of DMAP in 3 mL of CH_2Cl_2 according to the procedure described for **8-OMTP**. The residue was purified by chromatography (1:4 (v/v) EtOAc/hexanes) to afford 70 mg (93%) of a clear oil: TLC R_f = 0.22 (1:19 (v/v) EtOAc/hexanes); HPLC (3:17 (v/v) MTBE/hexanes, 1.5 mL/min) peaks of equal intensity at 9.5 and 11 min; ^{1}H NMR (mixture of diastereomers, $CDCl_3$) δ 7.5−7.3 (30 H, m), 5.35−5.25 (2H, m), 4.61−4.52 (2H, m), 4.40−4.30 (2H, m), 3.55 (6H, m), 3.48 (3H, m), 3.40 (9H, m), 2.2−1.9 (4H, m); MS (EI, 70 eV) *m*/*z* 754 (M), 521 (25), 189 (100), 105 (45), 77 (25); HRMS (EI) for $C_{34}H_{31}O_9F_9$ calcd 754.1824, found 754.1817.

The procedure was repeated with commercial samples of (*S*)-1,2,4-butanetriol to give **(*S*)-11-OMTP**), TLC R_f = 0.21 (1:4 (v/v) EtOAc/hexanes); HPLC (3:17 (v/v) MTBE/hexanes, 1.5 mL/min) a single peak at 9.5 min; MS (EI, 70 eV) *m*/*z* 754 (M), 521 (25), 189 (100), 105 (45), 77 (25); HRMS (EI) for $C_{34}H_{31}O_9F_9$ calcd 754.1824, found 754.1840; and with (*R*)-1,2,4-butanetriol to give **(*R*)-11-OMTP**), TLC R_f = 0.23 (1:4 (v/v) EtOAc/hexanes); HPLC (3:17 (v/v) MTBE/hexanes, 1.5 mL/min) peaks at 9.5 min (2%) and 11 min (98%); MS (EI, 70 eV, mixture of diastereomers) *m*/*z* 754 (M), 521 (25), 189 (100), 105 (45), 77 (25); HRMS (EI) for $C_{34}H_{31}O_9F_9$ calcd 754.1824, found 754.1835.

Racemic HSQ. The procedure described for **8-OMTP** was followed using 1 mg (2.3 μmol) of **HSQ**, 10 μL (0.053 mmol) of (*S*)-Mosher's chloride, and 10 mg (0.082 mmol) of DMAP in 500 μL of CH_2Cl_2. The residue was purified by chromatography (1:99 (v/v) EtOAc/hexanes) to yield 1 mg (68%) of a clear oil: TLC R_f = 0.66 (1:9 (v/v) EtOAc/hexanes); HPLC (Chiracel OD, 100% hexanes) peaks of equal intensity at 17 and 21 min; MS (EI, 70 eV) *m*/*z* 642 (M, 1), 408 (50), 271 (50), 203 (70), 189 (100), 136 (60), 81 (80), 69 (100).

The sample was treated with ozone, followed by 10 mg (0.27 mmol) of $NaBH_4$ according to the procedure described for **8** with the following modifications. Ten minutes after addition of $NaBH_4$, 1 mL of saturated NH_4Cl was added, and the mixture was stirred vigorously for 30 min. The organic layer was washed with 2 mL of saturated $NaHCO_3$ and 1 mL of brine. The organic layer was dried over Na_2SO_4 and filtered through glass wool. After solvent was removed with a stream of nitrogen, 500 μL of CH_2Cl_2, 5 mg (0.041 mmol) of DMAP, and 5 μL (0.03 mmol) of (*S*)-Mosher's chloride were added. After the solution was stirred for 5 min, 1 mL of hexane was added, and the resulting cloudy mixture was filtered through silica. HPLC (3:17 (v/v) MTBE/hexanes, 1.5 mL/min) gave equal peaks at 9 min [MS (EI, 70 eV) *m*/*z* 754 (M, 2), 189 (100), 105 (45)] and 11 min [MS (EI) *m*/*z* 754 (M, 2), 521 (25), 189 (100), 105 (20), 77 (10)].

HSQ from Incubation of FPP and SQase. The procedure was repeated with 500 μg (1.6 μmol) of **HSQ** isolated from an incubation of **FPP** and SQase, 5 mg (0.041 mmol) of DMAP, and 5 μL (0.03 mmol) of (*S*)-Mosher's chloride. HPLC (Chiracel OD, 100% hexanes) gave peaks at 17 min (8%) [MS (EI, 70 eV) *m*/*z* 642 (M, 1), 408 (50), 271 (50), 203 (70), 189 (100), 136 (60), 81 (80), 69 (100)] and 21 min (92%) [MS (EI, 70 eV) *m*/*z* 642 (M, 1), 408 (50), 271 (50), 203 (70), 189 (100), 136 (60), 81 (80), 69 (100)].

The material was treated with ozone, $NaBH_4$, and (*S*)-Mosher's chloride as described above. HPLC (3:17 (v/v) MTBE/hexanes, 1.5 mL/min) gave peaks at 9 min (8%) [MS (EI, 70 eV) *m*/*z* 754 (M, 2), 189 (100), 105 (45), 77 (25)] and 11 min (92%) [MS (EI) *m*/*z* 754 (M, 2), 521 (25), 189 (100), 105 (20), 77 (10)].

Stereochemical Analysis of DSQ. (*S*)-[1-^{2}H](*E*,*E*)-3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol ((*S*)-[1-^{2}H]Farnesol, (*S*)-[^{2}H]13). A mixture of 58 mg (0.2 mmol) of (*S*)-(−)-BINOL, 100 μL (0.1 mmol, 1 M in CH_2Cl_2) of Ti(O-*i*-Pr)$_4$, 6 μL (0.5 M in CH_2Cl_2) of CF_3CO_2H, and 400 mg of oven-dried 4 Å molecular sieves in 4 mL of CH_2Cl_2 was heated at reflux for 1 h.[45] The mixture was allowed to cool to room temperature, and 220 mg (1 mmol) of farnesal was added. The mixture was stirred for 5 min and then was cooled to −78 °C before 206 mg (1.2 mmol) of Bu_3SnD was added. The mixture was stirred for 10 min and allowed to stand without stirring for 24 h at 20 °C. Saturated $NaHCO_3$ (2 mL) was added. The resulting mixture was stirred for 1 h and then filtered through Celite. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried over $NaSO_4$ and concentrated. The crude material was purified by flash chromatography (1:20 (v/v) acetone/hexane) to give 145 mg (65%) of a clear oil: ^{1}H NMR ($CDCl_3$) δ 5.40 (1H, d, J = 6.6 Hz), 5.15−5.04 (2H, m), 4.15 (1H, d, J = 6.6 Hz), 2.12−1.90 (8H, m), 1.80, (1H, br s), 1.69 (6H, br s), 1.59 (6H, br s); ^{13}C NMR ($CDCl_3$) δ 140.1, 135.6, 131.6, 124.5, 124, 123.5, 59.2 (t, J_{C-D} = 22 Hz), 39.9, 39.7, 26.9, 26.5, 25.9, 17.9, 16.5; MS (EI, 80 eV) *m*/*z* 223.1 (M, 30), 206 (M − OH, 100), 191 (M − CH_3OH, 20), 137 (80), 123 (40), 81 (70), 69 (C_5H_9, 70); HRMS (EI, 80 eV) calcd for $C_{15}H_{25}O^2H$ 223.2046, found 223.2039.

(*R*)-[1-^{2}H](*E*,*E*)-3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol ((*R*)-[1-^{2}H]Farnesol, (*R*)-[^{2}H]13). Farnesal (220 mg, 1 mmol) was treated with catalyst prepared from 58 mg (0.2 mmol) of (*R*)-(−)-BINOL, 100 μL (0.1 mmol, 1 M in CH_2Cl_2) of Ti(O-*i*-Pr)$_4$, 6 μL (0.5 M in CH_2Cl_2) of CF_3CO_2H, and 400 mg of 4 Å oven-dried molecular sieves in 4 mL of CH_2Cl_2 and 206 mg (1.2 mmol) of Bu_3SnD as described for synthesis of **(*S*)-[^{2}H]13**. The crude material was purified by flash chromatography (1:20 (v/v) acetone/hexane) to give 134 mg (60%) of a clear oil: ^{1}H NMR ($CDCl_3$) δ 5.40 (1H, d, J = 6.6 Hz), 5.15−5.04 (2H, m), 4.15 (1H, d, J = 6.6 Hz), 2.12−1.90 (8H, m), 1.80, (1H, br s), 1.69 (6H, br s), 1.59 (6H, br s); ^{13}C NMR ($CDCl_3$) δ 140.1, 135.6, 131.6, 124.5, 124.0, 123.5, 59.2 (t, J_{C-D} = 22 Hz), 39.9, 39.7, 26.9, 26.5, 25.9, 17.9, 16.5, 16.2; MS (EI, 80 eV) *m*/*z* 223.1 (M, 20), 205 (M − H_2O, 10), 191 (M − CH_3OH), 136 (50), 123 (25), 95 (20), 81 (60), 69 (100, C_5H_9); HRMS (EI, 80 eV) calcd for $C_{15}H_{25}O^2H$ 223.2046, found 223.2051.

(*S*)-[1-^{2}H]Farnesyl Diphosphate ((*S*)-[1-^{2}H]FPP, (*S*)-[1-^{2}H]14). Trichloroacetonitrile (0.28 mL, 1.31 mmol) was added to a stirred solution of (*S*)-[^{2}H]**13** (60 mg, 0.27 mmol) and tetra-*n*-butylammonium dihydrogen phosphate (370 mg, 1.08 mmol) in 1 mL of CH_3CN.[49] After 1 h, the solution was concentrated under vacuum. The residue was dissolved in 3 mL of 1:49 (v/v) 2-propanol/25 mM NH_4HCO_3, loaded on a 2 × 10 cm column of DOWEX AG 50W-X8 cation-exchange resin (NH_4^+ form), and eluted with 60 mL of 1:49 (v/v) 2-propanol/25 mM NH_4HCO_3. After lyophilization, the resulting powder was purified by flash chromatography on a 3 × 6 cm cellulose column (8.5:1.5 (v/v) tetrahydofuran/0.1 M NH_4HCO_3) to give two fractions, one enriched in monophosphate and the other in diphosphate. The material enriched in diphosphate was further purified by reversed-phase HPLC using a 5-min isocratic elution with 25 mM NH_4HCO_3 followed by a 25-min linear gradient to 100% CH_3CN at a rate of 0.7 mL/min to give 12 mg (10%) of the ammonium salt of (*S*)-[1-^{2}H]**FPP** [^{1}H NMR (D_2O) δ 5.45 (1H, d, J = 6.5 Hz), 5.20 (2H, m), 4.41 (1H, dd, J = 6.5 Hz, $J_{H,P}$ = 6.5 Hz, H at C1), 2.05−1.95 (8H, m), 1.71 (3H, s), 1.67 (3H, s), 1.60 (6H, s); ^{31}P NMR (D_2O) δ −11.46 (1P, d, J = 20 Hz), −10.50 (1P, d, J = 20 Hz); (−) FAB MS 382 (M − 1)] and 25 mg (27%) of the ammonium salt of (*S*)-[1-^{2}H]farnesyl phosphate [^{1}H NMR (D_2O) δ 5.37 (1 H, d, J = 7.0 Hz) 5.12 (2H, m), 4.25 (1H, dd, J = 7, 6.5 Hz),

2.05−1.95 (8H, m), 1.66 (3H, s), 1.63 (3H, s), 1.57 (3H, s), 1.56 (3H, s); ^{31}P NMR (D_2O) δ 3.54 (s)].

(*R*)-[1-^{2}-H]Farnesyl Diphosphate ((*R*)-[1-^{2}H]FPP, (*R*)-[1-^{2}H]14). The procedure described for (*S*)-[1-^{2}H]**FPP** was followed using trichloroacetonitrile (0.25 mL, 1.16 mmol), (*R*)-[^{2}H]**13** (50 mg, 0.22 mmol), and tetra-*n*-butylammonium dihydrogen phosphate (350 mg, 1.02 mmol) to give 4 mg (4%) of (*R*)-[1-^{2}H]**FPP** [^{1}H NMR (D_2O) δ 5.45 (1H, d, J = 6.5 Hz), 5.20 (2H, m), 4.41 (1H, dd, J = 6.5 Hz, $J_{H,P}$ = 6.5 Hz), 2.05−1.95 (8H, m), 1.71 (3H, s), 1.67 (3H, s), 1.60 (6H, s); ^{31}P NMR (D_2O) δ −11.52 (1P, d, J = 20 Hz), −9.9 (1P, d, J = 20 Hz); (−) FAB MS 382 (M − 1)**]** and 30 mg (40%) of (*R*)-[1-^{2}H]farnesyl phosphate [^{1}H NMR (D_2O) δ 5.37 (d, J = 7.0 Hz, 1 H) 5.12 (m, 2H) 4.25 (dd, J = 7, 6.5 Hz, 1H), 2.05−1.95 (m, 8H), 1.66 (s, 3H), 1.63 (s, 3H), 1.57 (s, 3H), 1.56 (s, 3H); ^{31}P NMR (D_2O) δ 3.54 (s)].

Incubation of (*S*)-[1-^{2}H]FPP with SQase. Following the general protocol for analytical scale reactions, (*S*)-[1-^{2}H]**FPP** (130 μg, 0.3 μmol) and 1 mg (23 nmol) of SQase were incubated in 1 mL of 50 mM MOPS buffer, pH 7.2, containing 10 mM $MgCl_2$. The products were purified by normal-phase HPLC, and **DSQ** was analyzed by profile scanning EI MS (80 eV) (10 scans): 408 (100), 409 (29.96), 410 (6.97).

Incubation of (*R*)-[1-^{2}H]FPP with SQSase. (*R*)-[1-^{2}H]FPP (130 μg, 0.3 μmol) and 1 mg (23 nmol) of SQSase were incubated as described for the *S* enantiomer. **DSQ** was purified from the reaction mixture and analyzed by profile scanning EI MS (80 eV) (10 scans): 408 (1.21), 409 (1.41), 410 (100), 411 (29.26), and 412 (5.25).

Incubation of FPP with SQSase in Buffer Containing Mn^{2+}. The general procedure for incubation protocol was followed using **FPP** (130 μg, 300 μmol) and 1 mg (23 nmol) of SQSase in 1 mL of 50 mM MOPS buffer, pH 7.2, containing 10 mM $MnCl_2$. Products were extracted into MTBE, analyzed by normal-phase HPLC (1:19 (v/v) MTBE/hexane), and identified by co-injection with authentic materials. When the incubation was conducted in the presence of 10 mM $MgCl_2$ and 1 mM $MnCl_2$, the product distribution was the same as for Mn^{2+}.

Incubation of FPP with SQSase in Buffer Containing Methanol. The general incubation protocol was followed using **FPP** (130 μg, 300 μmol) and 1 mg (23 nmol) of SQSase in 1 mL of 50 mM MOPS buffer, pH 7.2, containing 1:4 (v/v) methanol/water and 10 mM $MgCl_2$. Reaction buffer was extracted with MTBE. Analysis of the extract by normal-phase HPLC (1:19 (v/v) MTBE/hexanes) gave **DSQ** (3.00 min, 18%), **2** (7.00 min, 1%), **IsoHBO** (9.00 min, 2%), **HBO** (10.5 min, 16%), **IsoHSQ** (15 min, 2%), and **HSQ** (16.0 min, 43%). In addition, a new peak was seen at 4.00 min (18%). The material was collected, MTBE was removed under vacuum, and the residue was analyzed by mass spectrometry to give MS (EI, 70 eV) *m*/*z* 440 (1, M), 408 (40, M − CH_3OH), 235 (100, $C_{16}H_{27}O$), 69 (90).

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