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Stereochemical disposition of the geminal dimethyl groups in the enzymatic cyclization of geranyl diphosphate to (+)-bornyl diphosphate by recombinant (+)-bornyl diphosphate synthase from *Salvia officinalis*

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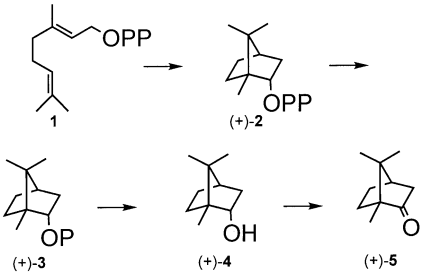
Abstract—Regiospecifically deuterated geranyl diphosphate, in concert with NMR spectrometry, was employed to demonstrate that the *trans*-methyl group (C8) of geranyl diphosphate becomes the C9 carbon of (+)-bornyl diphosphate (geminal methyl *syn* to the diphosphate moiety) and that the *cis*-methyl group (C9) becomes the C8 (geminal methyl *anti* to the diphosphate). The syntheses of the relevant substrates and products, with accompanying spectrometric data are provided.

1. Introduction

The biosynthesis of (+)- and (−)-borneol in plants is unique among monoterpenes in the incorporation of oxygen from the diphosphate during cyclization of the acyclic precursor, geranyl diphosphate (**1**, Scheme 1). Thus, (+)-camphor (**5**) is biosynthesized in culinary sage (*Salvia officinalis*) by cyclization of **1** to (+)-bornyl diphosphate (**2**) followed by sequential hydrolyses to (+)-bornyl monophosphate (**3**), (+)-borneol (**4**), and finally oxidation to the ketone.[1–3]



Scheme 1. Biosynthesis of (+)-camphor (**5**) from geranyl diphosphate (**1**).

Investigations using (+)-bornyl diphosphate synthase (BPPS) from this source led to the recognition that **1** serves as the universal precursor of regular monoterpenes, and subsequent studies with monoterpene syntheses from sage provided evidence for the general mechanistic paradigm illustrated in Scheme 2.[4,5] Thus, the substrate **1** undergoes ionization to a geranyl$^+$/OPP$^-$ ion pair and internal return of the diphosphate anion generates an enzyme-bound linalyl diphosphate (**6A**) intermediate. Rotation about the newly formed C2–C3 single bond to the cisoid conformer (**6B**) followed by re-ionization and S_N' cyclization gives rise to the central α-terpinyl carbocation (**7**) intermediate. Further transformations of this highly reactive species occur by cyclization, hydride shift, and Wagner–Meerwein rearrangements. In the case of BPPS, the bridging cyclization is terminated by a unique anti-Markovnikov capture of the diphosphate anion to form **2**. Oxygen-18 labeling experiments have shown that both diphosphate ionizations and recombinations occur without detectable scrambling of label from the C–O bonds into the other diphosphate oxygens.[6,7] Interestingly, while the sage BPPS produces exclusively (+)-(1*R*, 4*R*)-bornyl diphosphate (**2**), a complementary enzyme from tansy *Tanacetum vulgare* produces the antipodal (−)-(1*S*, 4*S*)-bornyl diphosphate by a mirror-image cyclization reaction sequence.[8]

A fundamental tenet of this general mechanistic model for monoterpene biosynthesis[4] is that the stereochemistry of the α-terpinyl cation intermediate (and hence all subsequent products) is dictated by the initial right- or left-handed helical folding of the substrate that undergoes suprafacial allylic rearrangement of the diphosphate group to generate

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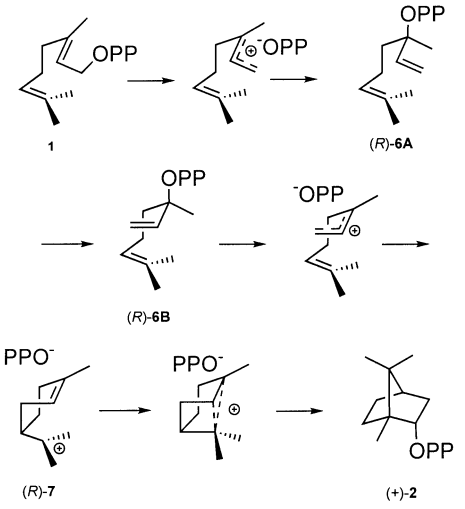


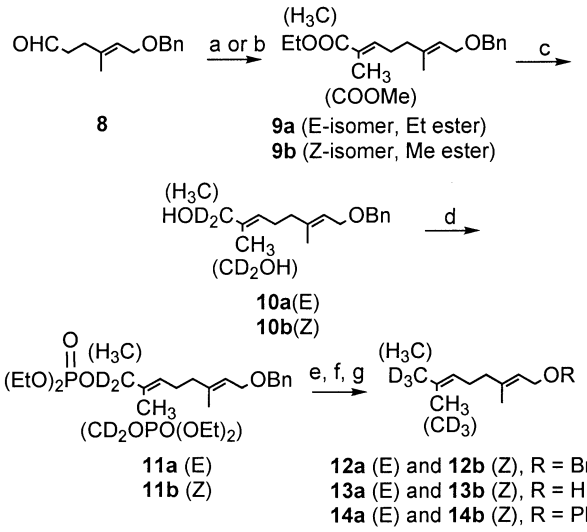
Scheme 2. Enzymatic conversion of geranyl diphosphate to (+)-bornyl diphosphate (**2**) through the intermediacy of (*R*)-linalyl diphosphate (**6**) and the (*R*)-α-terpinyl cation (**7**).

the corresponding (*S*)- or (*R*)-linalyl diphosphate (**6**) and then *anti*,*endo*-S_N' cyclization to the respective (*S*)- and (*R*)-α-terpinyl ions.[9–11] The stereochemical predictions of this model were validated by labeling studies, kinetic measurements, and stereochemical determinations of products.[4,5,12–14] However, the factors that govern the regio- and stereochemical outcome of the later steps remain obscure. Previous studies established that the *E* methyl group of **1** becomes the *exo* methyl of α- and β-pinene,[15] and that proton elimination in limonene biosynthesis occurs either regioselectively at the *Z* methyl or almost randomly at both positions, depending on the particular cyclization enzyme.[16–18] The principal objective of this collaboration was to elucidate the geminal methyl correlation in the cyclization catalyzed by BPPS.

Plans for direct ^{1}H NMR analysis of deuterium labeled **2** required access to relatively large amounts of the cyclase. A cDNA encoding the (+)-BPPS of *S. officinalis* was isolated and the enzyme functionally overexpressed in *E. coli*.[19] The pseudo-mature† form of this recombinant enzyme produces primarily (+)-**2** (75% of product mix) along with a set of stereochemically related olefins (25% of product mix) consisting of (+)-α-pinene (2.9% of total), (+)-camphene (14.5%), limonene (1.9% (+) and 2.4% (−)), and the achiral compound terpinolene (3.3%).‡ The availability of recombinant (+)-BPPS has allowed, for the first time, the assessment of additional stereochemical details of the cyclization reaction. In this paper, we describe the synthesis of (8-$^2H_3$) and (9-$^2H_3$)geranyl diphosphate (**14a** and **13b**), and the use of these labeled substrates, along with NMR based structural evaluation, to define the stereochemical dispositions of the terminal methyl groups in the course of this remarkable enzymatic transformation.

† An N-terminally truncated plasmid construct to remove the putative transit peptide region, thus improving soluble expression of the protein.

‡ Stereochemical analyses of the olefinic products produced by the full-length protein (including transit peptide) previously reported were: (+)-α-pinene [(**16**), 3.4%], camphene [9.5% (+)/0.5% (−)], limonene [3.9% (+)/3.9% (−)], terpinolene (2.1%) and myrcene (1.5%).[19]



Scheme 3. Syntheses of regiospecifically deuterated (8,8,8-$^2H_3$) and (9,9,9-$^2H_3$)geranyl diphosphates (**14a**, **14b**). *Reagents, conditions and yields*: (a) $CH_3C(=PPh_3)CO_2Et$, 90%; (b) $(CF_3CH_2O)_2P(=O)(CH_3)CH$-$CO_2Me$, KHMDS, 18-C-6, THF, −78°C; 93%; (c) AlD_3, ether, ~−10°C; 91, 90%; (d) $(EtO)_2P(=O)Cl$, pyr, CH_2Cl_2: 88, 93%; (e) $LiBEt_3D$, THF, 0°C; 88, 93%; (f) Li, NH_3, THF; 90, 100% (g) (i) MsCl, LiCl, collidine, DMF; (ii) $(Bu_4N)_3HOPP$, CH_3CN; 58, 61%.

2. Syntheses and NMR assignments

The required labeled derivatives of **1** were prepared as shown in Scheme 3 following previously published procedures in most cases.[20,21] Reductive displacements of the (*Z*)-allylic phosphates (**11a** and **11b**) with $LiBEt_3D$ avoided considerable *Z*–*E* isomerization observed in reductions conducted with the corresponding unstable mesylates.[22,23] High purity (≥98%) of the synthetic intermediates **9a**,**b**–**13a**,**b** with regard to the **6**, **7** double bond was ensured by careful chromatographic purifications together with GC and/or NMR analyses at each stage.

One objective of this work was physical characterization of (+)- and (−)-**2** and their unambiguous NMR assignments which were crucial to localizing the deuterium label in the enzymatic cyclization products. Conversion of (+)- and (−)-**4** to the diphosphates was carried out by the Cramer procedure[1,24,25] as modified recently by Danilov.[26] Condensation of the alcohols with tetrabutylammonium hydrogen phosphate (5 equiv.) and CCl_3CN (30 equiv.) in acetonitrile (room temperature, 20 min) afforded a mixture of mono-, di-, and polyphosphates that was separated on a Dowex ion exchange resin by gradient elution with ammonium formate in methanol in a manner similar to literature methods.[27,28] The synthetic (+)- and (−)-**2** were characterized by ^{1}H and ^{31}P NMR spectra that were identical to those of the enzymatic cyclization products. HPLC retention time comparisons between synthetic and enzymatic diphosphates provided confirming evidence.

The ^{1}H spectrum of unlabeled bornyl diphosphate displayed three distinct singlets at δ 0.71, 0.72 and 0.73 ppm, that integrated for a total of nine protons representing the three methyl groups. The lowfield broad triplet at δ 4.33 ppm was assigned to the $H2_X$ proton. Selective irradiation at this frequency produced an NOE to the signals at δ 0.72 and

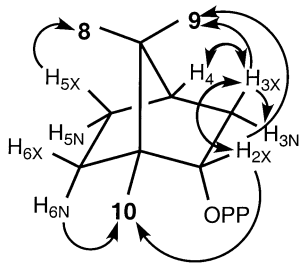


Figure 1. Bornyl diphosphate with selected NOEs depicted by arrows.

0.73 ppm (Fig. 1), indicating that these are the protons on C10 (the bridgehead methyl) and C9 (geminal methyl group *syn* to the phosphate bearing carbon); additionally, an NOE was observed to the signal at δ 2.15 ($H3_X$). Selective irradiation of this signal (δ 2.15) resulted in NOEs of the singlet at δ 0.72, a broad doublet of doublets centered at δ 1.07, a broad triplet at δ 1.51, and the broad triplet at δ 4.33, thereby establishing the resonance at δ 0.72 as the C9 protons, the signal at δ 1.51 as the C4 methine proton, and the doublet of doublets at δ 1.07 as the geminal $H3_N$ (the most intense NOE observed). Irradiation of the signal at δ 1.68 produced an NOE to the signal centered at δ 1.13 and a weak, but discernable, NOE at δ 0.73 (H10), indicating that this resonance represents $H6_N$ with through space dipolar interaction to $H6_X$ and $H5_N$ at δ1.09. An HMQC experiment firmly established that the proton signals at δ 1.68 and 1.13 originate from the same carbon at δ 26.2, whereas the signals at δ 1.57 and 1.09 define protons bound to the carbon at δ 27.7. Selective irradiation at δ 1.57 displayed an NOE to the methyl at δ 0.71, thus establishing this signal as $H5_X$ and the singlet at δ 0.71 as that from the C8 methyl group.

3. Enzymatic cyclizations

Regiospecifically deuterated substrates (8-$^2H_3$)-**14a** and (9-$^2H_3$)-**14b**, as well as unlabeled geranyl diphosphate, were separately incubated with purified bornyl diphosphate synthase for 24–36 h under standard assay conditions[19] resulting in the conversion of >95% of the substrate to products. The olefinic products (predominately α-pinene, camphene and limonene) were removed by extraction into pentane. The olefins generated in this reaction (approximately 25% of the total product) displayed some loss of stereochemical fidelity, as previously reported.[19] Specifically, the composition of the chiral olefins resulting from reaction with unlabeled geranyl diphosphate, as a percent of total product, were camphene [14.5% (+)/trace (−)], α-pinene [(**16**), 2.9% (+)/0% (−)], and limonene [1.9% (+)/2.4% (−)]. The relative proportions of the olefin products resulting from reaction with the two deuterium labeled substrates did not vary significantly from those indicated above. After removing the protein from the reaction solutions by acetone precipitation and centrifugation, the remaining aqueous phase was first vacuum dried then

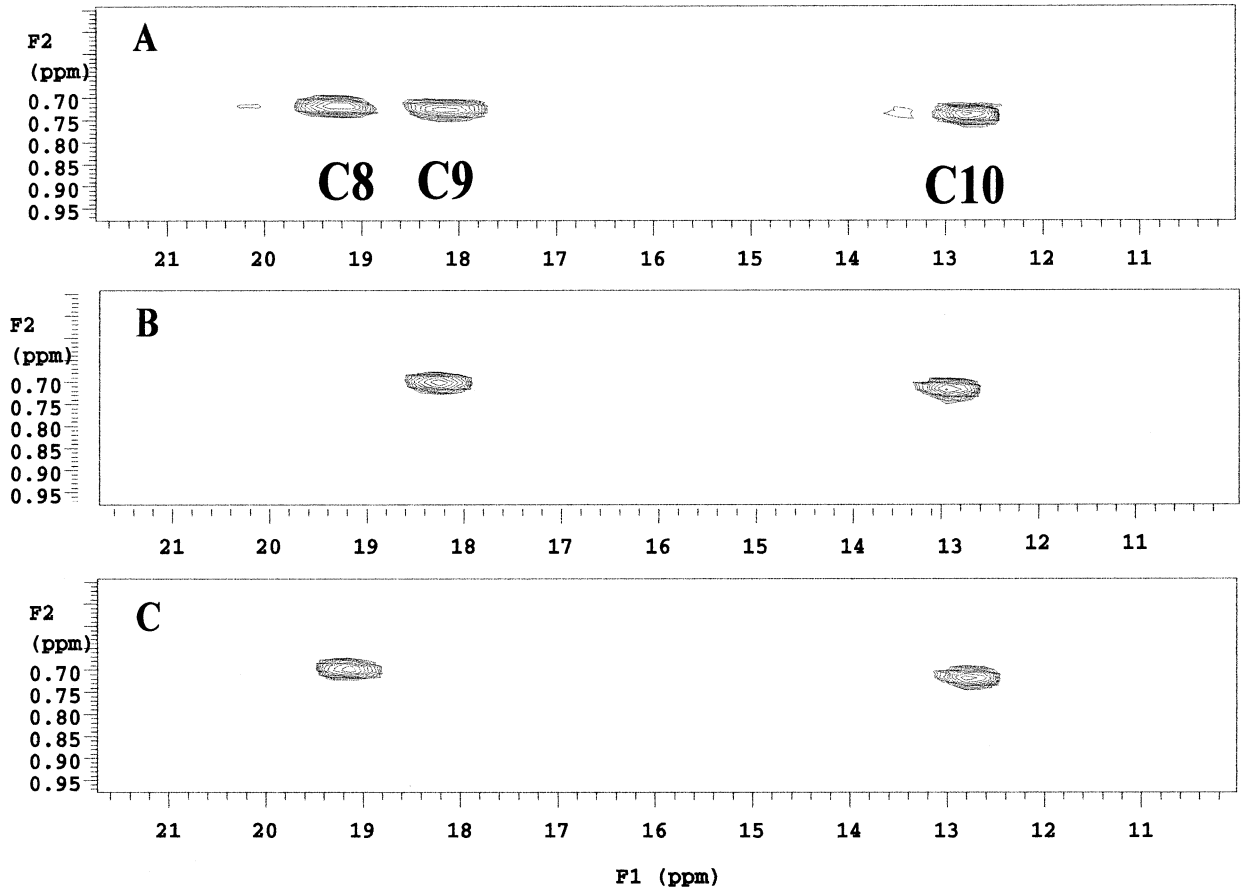
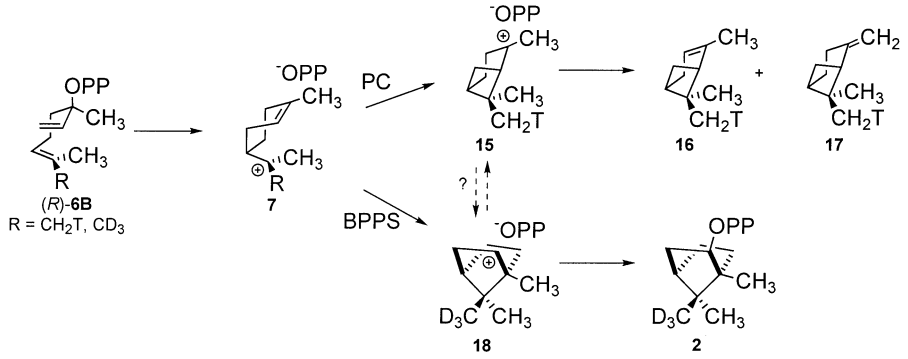


Figure 2. High field region of HMQC spectrum of from bornyl diphosphate derived from: (A) unlabeled geranyl diphosphate; (B) (9-$^2H_3$)-geranyl diphosphate (**14b**) and (C) (8-$^2H_3$)-geranyl diphosphate (**14a**).



Scheme 4. Mechanisms for pinene synthase and BPP synthase cyclizations of (*R*)-linalyl PP bearing tritium and deuterium label in the trans terminal methyl group.

lyophilized before resuspending the resulting product in D_2O for NMR spectrometry. The enantiomeric purity of the bornyl diphosphate (**2**) generated by enzymatic reaction was determined by phosphatase hydrolysis of an aliquot of the unlabeled product to the corresponding alcohol (confirmed by GC/MS), and then chiral phase column capillary GC analysis. The enzymatically generated (+)-bornyl diphosphate was shown to be 100% enantiomerically pure using synthetic (+)- and (−)-bornyl diphosphate as standards for this analytical protocol.

With the assignments of the methyl groups in bornyl diphosphate firmly established, it was possible to interpret an HMQC experiment with the enzymatic reaction products derived from the specifically deuterated substrates. As illustrated in Fig. 2, conversion of (8-$^2H_3$)geranyl diphosphate (**14a**) resulted in loss of the crosspeaks at δ 18.2, F1 (^{13}C) and δ 0.72, F2 (^1H); conversely, conversion of (9-$^2H_3$)geranyl diphosphate (**14b**) resulted in loss of the crosspeaks at δ 19.3, F1 (^{13}C) and δ 0.71, F2 (^1H). These results were further substantiated by NOE experiments, i.e. irradiation of the signal at δ 4.33 in the bornyl diphosphate product generated from (9-$^2H_3$)geranyl diphosphate showed a clear NOE to δ 0.73 and 0.72 (H10 and H9), and irradiation at δ 2.15 produced an NOE to δ 0.72, δ 1.51 and to δ 1.07. The opposite results were seen in NOE experiments with bornyl diphosphate enzymatically produced from **14a**, i.e. NOEs were not observed from irradiation of $H2_X$ or $H3_X$ to the H9 signal, whereas irradiation of the $H5_X$ proton produced a clear NOE to the proton signal at δ 0.71 (H8) and to other protons consistent with those observed in the unlabeled molecule. These data unequivocally demonstrate that enzymatic cyclization of geranyl diphosphate to (+)-bornyl diphosphate converts the substrate *trans* (C8) methyl group to the *syn* (C9) methyl in bornyl diphosphate, and the *cis* (C9) methyl of geranyl diphosphate to the *anti* (C8) methyl group in bornyl diphosphate.

4. Discussion

Previous studies[12–14] employing radiochemical methods and native enzymes from *S. officinalis* established that the cyclizations of **1** to (+)-**2**, (+)-α-pinene (**16**), and (−)-β-pinene (**17**) take place with overall retention of configuration at C1 of **1** (C3 of **2** and C7 of the pinenes), consistent with suprafacial allylic isomerization of the diphosphate in **1** to form (*R*)-**6** and S_N' cyclization to the (*R*)-α-terpinyl ion **7** shown in Scheme 2. Further labeling experiments proved that the *trans* methyl group (C8=CH_2T) of **1** becomes the *exo* methyl of (+)-α-pinene and (−)-β-pinene, consistent with a least motion rotation (30 vs 150°) about the exocyclic C6–C7 bond of **7** (Scheme 4).[15]

A similar radiochemical approach to elucidate the methyl–methyl correlation with (+)-borneol was unattractive because the corresponding hydrogen atoms of the bornane ring are less accessible to unambiguous degradative chemistry. However, the availability of the recombinant, overexpressed (+)-BPPS provided sufficient biosynthetic product to permit use of deuterium-labeled substrates along with NMR-based localization of the relevant hydrogen atoms to reveal the origin of the geminal methyl groups.

It is clear from the NMR data presented above that the *trans* terminal methyl of the substrate is converted to the *syn* geminal methyl of **2**. Thus, cyclization to the bornyl cation occurs on the same face of the original 6,7-double bond to which the S_N' cyclization took place to form the α-terpinyl cation **7**.

As illustrated in Scheme 4, the cyclizations leading to the pinenes and (+)-**2** catalyzed by (+)-pinene cyclase and (+)-BPPS both take place on the *Si* face of a common (*R*)-α-terpinyl intermediate. Whether in the latter case this process occurs by direct anti-Markovnikov addition to the cyclohexenyl double bond to generate the bornyl cation (**7→18**), by indirect formation of the tertiary pinyl cation through a Markovnikov-oriented ring closure and rearrangement (**7→15→18**), or by passage through a bridged, non-classical carbocationic intermediate (see Scheme 2) is not clear. Nevertheless, the lack of oxygen scrambling in the tightly ion-paired diphosphate anion throughout the reaction coordinate[6,7] implies more substantial participation in the cyclization than that of a passive terminating nucleophile. Thus, positioning of the diphosphate over C2 of the cyclohexenyl intermediate **7** could polarize the π-double bond sufficiently to disfavor formation of the tertiary pinyl carbocation **15**, and both promote the anti-Markovnikov addition and internal return to the secondary position to generate **2**. Relevant chemical precedent for stereoselective *endo* capture of bornyl ion pairs exists in the formation of bornyl chloride by rearrangement of pinyl chloride in the hydrochlorination of α-pinene[29] and in the methanolysis of pinyl

p-nitrobenzoates to small amounts of bornyl *p*-nitrobenzoate and methyl ether.[30]

5. Experimental

5.1. Gas chromatographic analysis

GC–MS was performed on a Hewlett–Packard 6890 GC with a quadrupole mass selective detector and Hewlett–Packard Chemstation for data analysis. Cool on-column injection was employed using a temperature program from 40°C (1 min hold) to 200°C at 20°/min, then to 320°C at 40°/min on an HP-5MS column (Hewlett–Packard part # 19091S-433, crosslinked 5% phenyl methyl polysiloxane, 30 m×0.25 mm, 0.25 μm film thickness) with He as the carrier gas at 0.7 mL/min. Chiral phase separations were performed on a Hewlett–Packard 5890 chromatograph by split injection (80:1) on a cyclodex-B capillary column (J and W Scientific #112-2532, 30 m×0.25 mm, 0.25 μm film thickness) using H_2 as the carrier gas at 0.6 mL/min with flame ionization detection. For alcohols, temperature programming was from 70°C (1 min hold) to 180°C at 5°/min, then to 230°C at 30°C/min, and for olefins was from 70°C (10 min hold) to 230°C at 10°/min. The following retention times were determined (min): α-pinene (**16**) 7.45 (−)/7.71 (+), camphene 8.79 (−)/9.07 (+), limonene 12.13 (−)/12.57 (+), borneol (**4**) 15.62 (−)/15.82 (+).

5.2. NMR analysis

NMR data for **2** in D_2O were collected at 125.7 MHz for ^{13}C and at 499.8 MHz for ^1H on a Varian Inova spectrometer. ^{31}P spectra were collected at 121.5 MHz on a Varian Mercury spectrometer. Phase sensitive HMQC data were collected as 2048 complex points in t2 and 128 complex points in t1 using the gradient selected echo-antiecho method for F1 quadrature. The data were linear predicted to 256 points in t1 then zero filled to 1K points and Fourier transformed, yielding a final matrix of 1K×512 real points. Sixty-four transients were collected per t1 increment. One-dimensional NOE data were collected using the Double Pulsed Field Gradient Spin Echo sequence of Stott et al.[31] with a mixing time of 700 ms for transient NOE buildup. Scalar J couplings and exact chemical shifts were determined via single frequency decouplings of each proton in the proton spectrum followed by simulation of the resulting spectrum using the Varian VNMR simulation program.[32] Chemical shifts and *J* values were iteratively adjusted until the simulated spectrum matched the real spectrum as judged by subtraction of the two spectra.

5.2.1. Ethyl (2*E*,6*E*)-8-benzyloxy-2,6-dimethyl-2,6-octadienoate (9a). The *E*,*E*-dienoate was prepared according to a literature procedure[20] using 1.092 g (5.00 mmol) of (4*E*)-6-benzyloxy-4-methyl-4-hexanal (**8**) and 1.824 g (5.03 mmol) of ethyl 2-(triphenylphosphoranylidene)propionate. Purification of the product (*E*/*Z* isomer ratio=96/4 by GC) by flash chromatography with ethyl acetate–hexane (1:10) mixture as eluent afforded 1.365 g (90%) of the product **9a** (~99% *E*,*E*-isomer by GC) as a clear oil. ^1H NMR spectral data are in agreement with the literature values.[20]

5.2.2. Methyl (2*Z*,6*E*)-8-benzyloxy-2,6-dimethyl-2,6-octadienoate (9b). A suspension of 4.13 g (15.6 mmol) of 18-crown-6 and 1.02 g (3.08 mmol) of bis(2,2,2-trifluoroethyl) 1-(methoxycarbonyl)ethylphosphonate[21] in 78 mL of THF was stirred and cooled at −78°C as 623 mg (3.1 mmol) of $KN[Si(CH_3)_3]_2$ in 7 mL of THF was added. After 20 min, 568 mg (2.60 mmol) of aldehyde **8** in 7 mL of THF was added. After 30 min at −78°C, saturated aq. NH_4Cl (50 mL) was added, and the THF was removed at reduced pressure. The remaining aqueous mixture was extracted with hexane (25 mL×3) and the combined extracts were washed with H_2O (50 mL), dried ($MgSO_4$), and concentrated. Purification by chromatography with ethyl acetate–hexane (1:10) mixture as eluent gave 701 mg (93%) of the product **9b** (one isomer by GC) as a clear oil: ^1H NMR (400 MHz, $CDCl_3$) δ 1.65 (s, 3H), 1.89 (d, 3H, *J*=1.2 Hz), 2.14 (t, 2H, *J*=7.6 Hz), 2.61 (q, 2H, *J*=7.5 Hz), 3.73 (s, 3H), 4.03 (d, 2H, *J*=6.8 Hz), 4.50 (s, 2H), 5.43 (td, 1H, *J*=6.7, 1.2 Hz), 5.93 (td, 1H, *J*=7.2, 1.4 Hz), 7.26–7.35 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.3, 20.6, 27.6, 39.0, 51.2, 66.5, 71.9, 121.4, 127.0, 127.5, 127.8, 128.3, 138.5, 139.5, 142.7, 168.3.

5.2.3. (2*E*,6*E*)-(1,1-$^2H_2$)-8-Benzyloxy-2,6-dimethyl-2,6-octadien-1-ol (10a). A solution of 559 mg (13.3 mmol) of $LiAlD_4$ in 15 mL of ether was stirred and cooled in an ice-salt bath as 593 mg (4.45 mmol) of $AlCl_3$ was added.[33,34] After 30 min, 907 mg (3.00 mmol) of **9a** in 7 mL of ether was added to the AlD_3 reagent, and the mixture was stirred for 30 min. The reaction mixture was hydrolyzed at 0°C by adding 0.74 mL of H_2O, 0.75 mL of 15% aq. NaOH, and 2.25 mL of H_2O, successively. After 30 min, the solids were filtered off and the filtrate was concentrated. Purification of the residue by flash chromatography with ethyl acetate–hexane (1:2) mixture as eluent gave 717 mg (91%) of dideutero alcohol **10a**. ^1H NMR (400 MHz, $CDCl_3$) δ 1.49 (br, 1H), 1.65 (s, 3H), 1.66 (d, 3H, *J*=0.7 Hz), 2.07 (t, 2H, *J*=7.6 Hz), 2.18 (q, 2H, *J*=7.3 Hz), 4.02 (d, 2H, *J*=6.6 Hz), 4.51 (s, 2H), 5.39 (m, 2H), 7.27–7.35 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.6, 16.4, 25.7, 39.1, 66.5, 72.1, 121.1, 125.7, 127.5, 127.8, 128.3, 135.0, 138.4, 139.9. ^1H NMR spectral data are in agreement with the literature values[35,36] except the peak for the CD_2OH group (δ 3.86) was absent.

5.2.4. (2*E*,6*E*)-(1,1-$^2H_2$)-8-Benzyloxy-2,6-dimethyl-2,6-octadien-1-yl diethyl phosphate (11a). A solution of 273 mg (1.04 mmol) of **10a** and 145 μL (1.79 mmol) of pyridine in 3 mL of CH_2Cl_2 was stirred and cooled at 0°C as 225 μL (1.49 mmol) of diethyl chlorophosphate was added.[22] The suspension was stirred at 0°C for 4 h, diluted with ether (15 mL), and washed with H_2O (20 mL×2), saturated aq. $NaHCO_3$ (20 mL), and saturated aq. NaCl (20 mL). After drying ($MgSO_4$) and concentration, the residue was purified by flash chromatography with ethyl acetate–hexane (2:3) containing 1% Et_3N as eluent to give 365 mg (88%) of phosphate **11a**. ^1H NMR (400 MHz, $CDCl_3$) δ 1.29 (td, 6H, *J*=7.1, 0.7 Hz), 1.61 (s, 3H), 1.66 (d, 3H, *J*=0.7 Hz), 2.04 (t, 2H, *J*=7.4 Hz), 2.15 (q, 2H, *J*=7.3 Hz), 3.99 (d, 2H, *J*=6.8 Hz), 4.08 (quintet, 4H, *J*=7.3 Hz), 4.46 (s, 2H), 5.37 (td, 1H, *J*=6.7, 1.0 Hz), 5.46 (td, 1H, *J*=6.8, 1.2 Hz), 7.22–7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.4, 16.0 (d, *J*=6.9 Hz), 16.3, 25.8, 38.6, 63.4 (d, *J*=5.4 Hz),

66.3, 71.9, 121.0, 127.4, 127.6, 128.2, 129.3, 130.4 (d, J=6.8 Hz), 138.3, 139.5; ^{31}P NMR (161.9 MHz, $CDCl_3$) δ 2.10.

5.2.5. (2*E*,6*E*)-(8,8,8-$^2H_3$)-1-Benzyloxy-3,7-dimethyl-2,6-octadiene (12a). A solution of 365 mg (0.92 mmol) of **11a** in 5 mL of THF was stirred and cooled at 0°C as 5.5 mL of 1.0 M $LiBEt_3D$ in THF was added. After 1 h, 15 mL of H_2O was slowly added and the product was extracted with hexane (10 mL×3). The combined extracts were washed with 1N HCl (30 mL), saturated aq. $NaHCO_3$ (30 mL), and saturated aq. NaCl (30 mL); dried ($MgSO_4$); and concentrated. Purification of the residue by chromatography with ethyl acetate–hexane (1:30) mixture as eluent provided 199 mg (88%) of **12a**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.61 (s, 3H), 1.65 (s, 3H), 2.06 (t, 2H, J=7.5 Hz), 2.12 (q, 2H, J=7.6 Hz), 4.04 (d, 2H, J=6.5 Hz), 4.51 (s, 2H), 5.11 (td, 1H, J=6.9, 1.3 Hz), 5.41 (td, 1H, J=6.8, 1.3 Hz), 7.27–7.36 (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.4, 17.6, 26.4, 39.6, 66.6, 71.9, 120.9, 124.1, 127.5, 127.8, 128.3, 131.6, 138.7, 140.4. ^{1}H NMR[37–39] and ^{13}C NMR[38,39] spectral data are in agreement with the literature values except the peaks for the CD_3 group (δ_H 1.68, δ_C 25.6) are absent.

5.2.6. (2*E*,6*E*)-(8,8,8-$^2H_3$)-3,7-Dimethyl-2,6-octadien-1-ol (13a). Reduction of benzyl ether **12a** (199 mg, 0.80 mmol) with Li (31.3 mg, 4.51 mmol) in liquid NH_3 (30 mL) and THF (2 mL) was carried out as described previously for geranyl-d_6 benzyl ether.[16] Purification by chromatography with ethyl acetate–hexane (1:5) mixture as eluent followed by Kugelrohr distillation afforded 114 mg (90%) of the (8,8,8-$^2H_3$)geraniol **13a**: ^{1}H NMR (400 MHz, C_6D_6) δ 0.58 (br, 1H), 1.45 (s, 3H), 1.52 (s, 3H), 1.97 (t, 2H, J=7.7 Hz), 2.09 (q, 2H, J=7.4 Hz), 3.96 (d, 2H, J=6.3 Hz), 5.16 (td, 1H, J=7.0, 1.3 Hz), 5.38 (tq, 1H, J=6.7, 1.3 Hz); ^{13}C NMR (100 MHz, C_6D_6) δ 16.1, 17.6, 26.8, 39.9, 59.3, 124.6, 124.9, 131.3, 138.0; ^{2}H NMR (77 MHz, C_6H_6) δ 1.57; Isotope ratio by FI MS, d_3 95.71%, d_2 3.17%, d_1 1.11%.

5.2.7. Diammonium hydrogen (2*E*,6*E*)-(8,8,8-$^2H_3$)-3,7-dimethyl-2,6-octadien-1-yl diphosphate (14a). (8,8,8-$^2H_3$)Geraniol (**13a**, 40.3 mg, 0.256 mmol) was converted to the chloride by Meyers' procedure[40] using LiCl (56.7 mg, 1.338 mmol), 2,4,6-collidine (275 μL, 2.08 mmol), and MsCl (120 μL, 1.55 mmol) in DMF. Conversion of the chloride to (8,8,8-$^2H_3$)geranyl diphosphate was carried out according to Poulter's procedure:[41] yield, 52 mg (58%); ^{1}H NMR (400 MHz, D_2O) δ 1.43 (s, 3H), 1.52 (s, 3H), 1.91 (t, 2H, J=6.8 Hz), 1.96 (q, 2H, J=6.6 Hz), 4.28 (t, 2H, J=6.8 Hz), 5.01 (t, 1H, J=6.6 Hz), 5.25 (t, 1H, J=6.6 Hz); ^{13}C NMR (100 MHz, D_2O) δ 15.7, 17.0, 25.6, 38.9, 62.9 (d, J=5.3 Hz), 119.7 (d, J=8.4 Hz), 124.2, 133.7, 143.0; ^{31}P NMR (400 MHz, D_2O) δ −8.89 (d, J=20.7 Hz), −9.93 (d, J=20.7 Hz).

The *Z*,*E* (or *E*,*Z*) isomers of **10b**–**14b** were prepared and purified as described above for the *E*,*E* isomers **10a**–**14a**. Only the final yields and characterization data are given.

5.2.8. (2*Z*,6*E*)-(1,1-$^2H_2$)-8-Benzyloxy-2,6-dimethyl-2,6-octadien-1-ol (10b). Yield, 529 mg (90%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.65 (s, 3H), 1.79 (d, 3H, J=1.0 Hz), 2.05 (t, 2H, J=7.3 Hz), 2.18 (q, 2H, J=7.3 Hz), 4.00 (d, 2H, J=6.8 Hz), 4.51 (s, 2H), 5.25 (td, 1H, J=7.4, 1.0 Hz), 5.38 (t, 1H, J=6.8 Hz), 7.27–7.36 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.5, 21.1, 25.7, 39.3, 66.2, 72.0, 121.2, 127.1, 127.5, 127.8, 128.3, 134.9, 138.2, 140.0.

5.2.9. (2*Z*,6*E*)-(1,1-$^2H_2$)-8-Benzyloxy-2,6-dimethyl-2,6-octadien-1-yl diethyl phosphate (11b). Yield, 719 mg (91%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33 (td, 6H, J=7.1, 1.0 Hz), 1.64 (s, 3H), 1.78 (d, 3H, J=1.5 Hz), 2.06 (t, 2H, J=7.7 Hz), 2.20 (q, 2H, J=7.6 Hz), 4.02 (d, 2H, J=6.6 Hz), 4.10 (quintet, 4H, J=7.3 Hz), 4.50 (s, 2H), 5.39 (m, 2H), 7.27–7.35 (m, 5H); ^{31}P NMR (161.9 MHz, $CDCl_3$) δ 2.96.

5.2.10. (2*E*,6*Z*)-(8,8,8-$^2H_3$)-1-Benzyloxy-3,7-dimethyl-2,6-octadiene (12b). Yield, 416 mg (93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.66 (s, 3H), 1.70 (d, 3H, J=1.2 Hz), 2.05–2.08 (m, 2H), 2.11 (m, 2H), 4.05 (d, 2H, J=6.8 Hz), 4.52 (s, 2H), 5.13 (td, 1H, J=6.5, 1.0 Hz), 5.43 (td, 1H, J=6.8, 1.2 Hz), 7.27–7.38 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.4, 25.6, 26.3, 39.6, 66.5, 71.9, 120.7, 124.0, 127.5, 127.8, 128.3, 131.5, 138.5, 140.4. ^{1}H NMR[37–39] and ^{13}C NMR[38,39] spectral data are in agreement with the literature values except the peaks for the CD_3 group (δ_H 1.60, δ_C 17.6) are absent.

5.2.11. (2*E*,6*Z*)-(8,8,8-$^2H_3$)-3,7-Dimethyl-2,6-octadien-1-ol (13b). Yield, 276 mg (100%); ^{1}H NMR (400 MHz, C_6D_6) δ 0.69 (br, 1H), 1.46 (s, 3H), 1.65 (d, 3H, J=1.5 Hz), 1.97 (t, 2H, J=7.7 Hz), 2.09 (qd, 2H, J=7.2, 0.9 Hz), 3.96 (d, 2H, J=6.6 Hz), 5.17 (td, 1H, J=7.0, 1.2 Hz), 5.38 (tq, 1H, J=6.7, 1.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.1, 25.7, 26.8, 39.8, 59.3, 124.6, 124.9, 131.3, 138.1; ^{2}H NMR (77 MHz, C_6H_6) δ 1.45; Isotope ratio by FI MS, d_3 93.20%, d_2 3.16%, d_1 2.96%.

5.2.12. Diammonium hydrogen (2*E*,6*Z*)-(8,8,8-$^2H_3$)-3,7-dimethyl-2,6-octadien-1-yl diphosphate (14b). Yield, 64 mg (61%); ^{1}H NMR (400 MHz, D_2O) δ 1.47 (s, 3H), 1.50 (s, 3H), 1.88 (t, 2H, J=6.3 Hz), 1.94 (q, 2H, J=6.5 Hz), 4.25 (t, 2H, J=6.7 Hz), 4.99 (t, 1H, J=6.4 Hz), 5.24 (t, 1H, J=6.7 Hz); ^{13}C NMR (100 MHz, D_2O) δ 15.7, 24.8, 25.6, 38.9, 62.7 (d, J=5.3 Hz), 119.8 (d, J=9.1 Hz), 124.2, 133.7, 142.9; ^{31}P NMR (161.9 MHz, D_2O) δ −4.52 (d, J=22.0 Hz), −7.00 (d, J=20.7 Hz).

5.2.13. (+)- and (−)-Bornyl diphosphates((+)-2 and (−)-ent-2) by chemical synthesis. To a solution of either (+)- or (−)-borneol (**4**) (191 mg, 1.04 mol) in CCl_3CN (3.20 mL) was added $Bu_4NH_2PO_4$ (1.82 g) with constant stirring at room temperature.[26] After 20 min, 2% NH_3 in methanol (10 mL) was added and, after an additional 10 min, the solvent was removed under vacuum. The resulting yellow oil was resuspended in 2% NH_3 in methanol (40 mL) and the mixture was centrifuged to remove precipitated inorganic salts. The supernatant was concentrated under vacuum, and the resulting oil was loaded onto a Dowex® 1×8–400 column (1.0×21 cm^2, formate form) and eluted under gravity with 1.0 L of a 0.05–0.5 M ammonium formate gradient in methanol.[27,28] Fractions containing (+)- or (−)-bornyl monophosphate and (+)- or (−)-**2**), as

Table 1. ^{1}H, ^{31}P and ^{13}C NMR spectral data for bornyl diphosphate in D_2O

Position	^{1}H (ppm)[a]	^{31}P (ppm)[b]	J (Hz)	^{13}C (ppm)[c]
1				49.0
2	4.33		$^{3}J_{3X}$=9.0, $^{4}J_{6X}$=3.0, $^{3}J_{3N}$=2.0	82.4
3*exo*	2.15		$^{2}J_{3N}$=13.6, $^{3}J_{2X}$=9.0, $^{3}J_{4}$=4.5, $^{4}J_{5X}$=3.2	36.8
3*endo*	1.07		$^{2}J_{3X}$=13.6, $^{3}J_{2X}$=2.0	
4	1.51		$^{3}J_{3X}$=4.5, $^{3}J_{5X}$=4.0, $^{4}J_{6X}$=1.0	44.7
5*exo*	1.57		$^{3}J_{6X}$=12.4, $^{2}J_{5N}$=12.0, $^{3}J_{6N}$=4.0, $^{3}J_{4}$=4.0, $^{4}J_{3X}$=3.2	27.7
5*endo*	1.09		$^{2}J_{5X}$=12.0, $^{3}J_{6N}$=9.0, $^{3}J_{6X}$=4.7	
6*exo*	1.13		$^{2}J_{6N}$=12.5, $^{3}J_{5X}$=12.4, $^{3}J_{5N}$=4.7, $^{4}J_{2}$=3.0, $^{4}J_{4}$=1.0	26.2
6*endo*	1.68		$^{2}J_{6X}$=12.5, $^{3}J_{5N}$=9.0, $^{3}J_{5X}$=4.0	
7				47.1
8	0.71			19.4
9	0.72			18.2
10	0.73			12.9
αP		−7.8	$^{3}J_{H2}$=7.6, $^{3}J_{C1}$=5.8, $^{2}J_{C2}$=4.6, $^{3}J_{C3}$=1.0	
βP		−5.1	J_{PP}=16.4	

[a] Referenced to HOD at 4.65 ppm.
[b] Referenced to H_3PO_4 (85%) at 0 ppm.
[c] Referenced to dioxane at 66.8 ppm.

determined by TLC (silica gel; 6:3:1 2-propanol, conc. NH_4OH, H_2O) were pooled separately and repeatedly dried by lyophilization (Scheme 2). NMR: (see Table 1).

5.2.14. Biosynthesis of (+)-bornyl diphosphate. The cDNA encoding bornyl diphosphate synthase from *S. officinalis*[19] was subcloned as a truncated pseudo-mature form into the pSBET plasmid vector.[42] Overexpression of the recombinant protein in *E. coli* provided milligram quantities of purified protein, thereby allowing production of sufficient bornyl diphosphate (approximately 4 mg) for NMR analysis. Details of the subcloning strategy and the expression and purification protocols will be published elsewhere.

To minimize contamination of the water soluble biosynthetic product with buffer components, enzymatic reactions were conducted in 25 mM NH_4HCO_3 (pH 7.2) containing only the required cofactor 10 mM $MgCl_2$. Prior to use, the purified enzyme solution was exchanged into this reaction buffer by repeated concentration and dilution using an Amicon Centriprep 50 concentrator. Approximately 5 mg of geranyl diphosphate was added to 1.0 mg of the purified protein in a total volume of 10 mL buffer divided into five screwcap test tubes, each overlaid with 1.0 mL pentane to trap volatile olefin products. Conversion of the substrate to bornyl diphosphate was monitored by periodically removing 50 μL aliquots of the reaction mixture and enzymatically hydrolyzing the diphosphate components contained therein using bovine intestinal alkaline phosphatase and potato apyrase (in 100 mM Tris (pH 8.0) with 10 mM $MgCl_2$ and 0.1 mM $ZnCl_2$)[43] and extracting the resulting alcohols (borneol and geraniol) for GC/MS analysis. The enzymatic reactions were allowed to proceed at 31°C until less than 5% of the substrate remained (24–48 h). After removing the pentane overlay the protein in the reaction mixture was precipitated with 20% (v/v) acetone. The acetone was removed under vacuum, and precipitated protein was separated by filtration of the concentrated aqueous phase through a glass wool plug into a separate test tube which was brought to dryness under vacuum. The residue was then lyophilized (2–4 h) to reduce residual NH_4HCO_3 salts before transfer of the bornyl diphosphate product in D_2O to an NMR tube.

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