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Studies of the Cryptic Allylic Pyrophosphate Isomerase Activity of Trichodiene Synthase Using the Anomalous Substrate 6,7-Dihydrofarnesyl Pyrophosphate[†]

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ABSTRACT: Two enantiomeric analogues of farnesyl pyrophosphate (**1**) were tested as inhibitors and anomalous substrates of trichodiene synthase, which catalyzes the cyclization of *trans,trans*-farnesyl pyrophosphate (**1**) to the sesquiterpene hydrocarbon trichodiene (**2**). The reaction has been shown to involve preliminary isomerization of **1** to the tertiary allylic isomer nerolidyl pyrophosphate (**3**) which is cyclized without detectable release of the intermediate from the active site of the cyclase. Both (7*S*)-*trans*-6,7-dihydrofarnesyl pyrophosphate (**7a**) and (7*R*)-*trans*-6,7-dihydrofarnesyl pyrophosphate (**7b**), prepared from (3*R*)- and (3*S*)-citronellol (**9a** and **9b**), respectively, proved to be modest competitive inhibitors of trichodiene synthase. The values of K_i(**7a**), 395 nM, and K_i(**7b**), 220 nM, were 10-15 times the observed K_m for **1** and half the K_i of inorganic pyrophosphate alone. Incubation of either **7a** or **7b** with trichodiene synthase resulted in formation of a mixture of products which by radio/gas-liquid chromatographic and GC/selected ion mass spectrometric analysis was shown to be composed of 80-85% isomeric trienes **19-21** and 15-20% allylic alcohols **12** and **18**. Examination of the water-soluble products resulting from incubation of **7a** also revealed the generation of 24% of the isomeric *cis*-6,7-dihydrofarnesyl pyrophosphate (**26**). The combined rate of formation of anomalous alcoholic and olefinic products was 10% the V_{max} determined for the conversion of **1** to **2**. The results can be explained by initial enzyme-catalyzed isomerization of dihydrofarnesyl pyrophosphate (**7**) to the corresponding tertiary allylic isomer dihydronerolidyl pyrophosphate (**8**). Since the latter intermediate is unable to cyclize due to the absence of the 6,7-double bond, ionization of **8** and quenching of the resulting ion pair by deprotonation, capture of water, or collapse to the isomeric primary pyrophosphate esters will generate the observed spectrum of anomalous products.

Trichodiene synthase catalyzes the cyclization of *trans,trans*-farnesyl pyrophosphate (**1**) (FPP)[1] to trichodiene (**2**), the parent hydrocarbon of the trichothecane family of antibiotics and mycotoxins (Cane et al., 1981a, 1985). The enzyme has been isolated from a variety of sources, including *Fusarium sambucinum* (*Gibberella pulicaris*) (Hohn & Beremand, 1989a) and *Fusarium sporotrichioides* (Hohn & VanMiddlesworth, 1986), producers of the potent mycotoxins diacetoxyscirpenol and T-2 toxin, respectively, as well as the apple mold fungus *Trichothecium roseum* (Cane et al., 1981a, 1985; Evans et al., 1973), the source of the antibiotic trichothecin. The *F. sporotrichioides* cyclase has been purified to

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[1] Abbreviations: FFAP, free fatty acid phase; FPP, farnesyl pyrophosphate; GC/MS, capillary gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate; HPLC, high-pressure liquid chromatography; HRMS, high-resolution mass spectrometry; MPLC, medium-pressure liquid chromatography; NMR, nuclear magnetic resonance; PP$_i$, inorganic pyrophosphate; THF, tetrahydrofuran.

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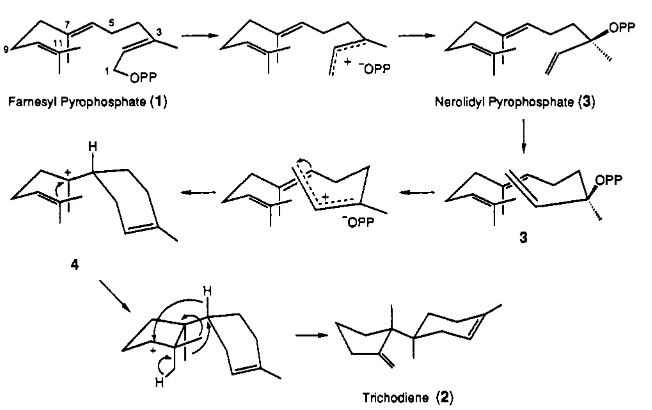
Scheme I

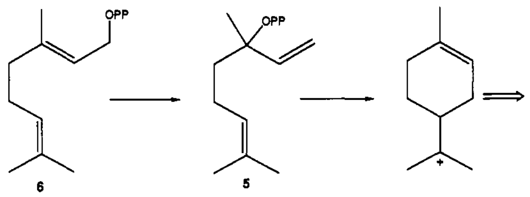


homogeneity and shown to be a homodimer of subunit M_r 45 000 (Hohn & VanMiddlesworth, 1986). In common with all other known terpenoid cyclases (Croteau & Cane, 1985), the only required cofactor is a divalent metal cation, Mg^{2+} being strongly preferred over Mn^{2+} which is inhibitory at higher concentrations (Hohn & VanMiddlesworth, 1986). The trichodiene synthase gene from *F. sporotrichoides* has been cloned and its nucleotide sequence determined (Hohn & Beremand, 1989b). It was shown to contain a 60-nucleotide intron and to code for a protein of M_r 43 999. Specific deletion of the intron sequence and subcloning of the modified open reading frame into pDR540 led to expression of trichodiene synthase activity in *Escherichia coli* (Hohn et al., 1989). Preliminary investigations have shown the recombinant cyclase to be identical with the native fungal enzyme in subunit molecular weight, p*I*, and chromatographic mobility.

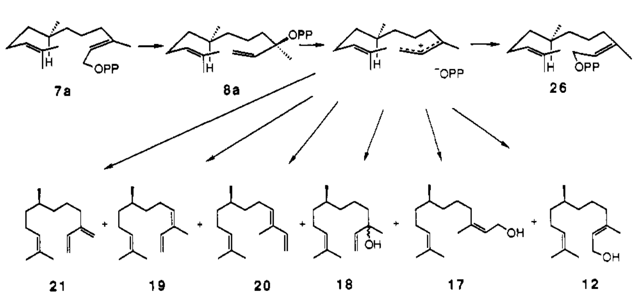
In parallel with the above studies, extensive investigations have established many of the mechanistic and stereochemical details of the conversion of *trans,trans*-farnesyl pyrophosphate to trichodiene. Thus cyclization of FPP has been shown to take place without loss of either of the original H-1 hydrogen atoms and with net retention of configuration at C-1 of the allylic pyrophosphate substrate (Cane et al., 1985). These results have been explained by the cyclization mechanism illustrated in Scheme I, in which *trans,trans*-farnesyl pyrophosphate undergoes initial syn isomerization to the tertiary allylic isomer, nerolidyl pyrophosphate (**3**). By rotation about the newly generated 2,3-single bond, nerolidyl pyrophosphate can adopt a conformation suitable for cyclization to the corresponding bisabolyl cation (**4**) which can be converted in turn to trichodiene by a well-established sequence of hydride shifts and methyl migrations. In further support of this mechanistic picture, (3*R*)-nerolidyl pyrophosphate has been shown to be a substrate for trichodiene synthase with a V_{max}/K_m at least 1.5–2 times that of FPP (Cane & Ha, 1986, 1988). Furthermore, competition experiments have established that, whereas under in vitro conditions both allylic substrates can compete for the same active site in trichodiene synthase, cyclization of the primary allylic pyrophosphate **1** normally takes place without detectable release of free intermediates.

The proposed scheme for the cyclization of FPP to trichodiene is consistent with the results of extensive studies of monoterpene cyclases which have established the intermediacy of the tertiary allylic pyrophosphate, linalyl pyrophosphate (**5**), in the conversion of geranyl pyrophosphate (**6**) to all the major classes of cyclic monoterpenes (Croteau, 1987) (Scheme II). Trichodiene synthase is therefore believed to be an *isomerase–cyclase* in which both the initial isomerization of farnesyl to nerolidyl pyrophosphate and the subsequent cyclization of the tertiary allylic pyrophosphate take place at the same active site by variations of a common mechanism involving the generation of allylic cation–pyrophosphate anion pairs (Cane, 1980, 1985). Whereas the transoid allylic cation–pyrophosphate anion pair resulting from the ionization of *trans,-trans*-FPP is geometrically incapable of cyclization and can therefore only collapse to the tertiary allylic isomer **3**, the cisoid allylic cation–pyrophosphate anion pair derived from the corresponding *anti-endo* conformer of the intermediate nerolidyl pyrophosphate can readily cyclize by electrophilic attack on the neighboring 6,7-double bond. Kinetic studies of several terpenoid synthases have indicated that the intial isomerization step is rate-limiting (Croteau, 1987). The finding that exogenous nerolidyl pyrophosphate can serve as a substrate for trichodiene synthase has allowed the direct study of the *cyclase* component of this complex reaction sequence. Direct observation of the inherent *isomerase* activity of trichodiene synthase is complicated by the fact that the tertiary allylic pyrophosphate intermediate is not released from the active site prior to cyclization.

Scheme II



Scheme III



In order to further elucidate the details of the cyclization mechanism, we have examined the role of 6,7-dihydrofarnesyl pyrophosphate (**7**) as an inhibitor and anomalous substrate of trichodiene synthase, using the homogeneous enzyme from *F. sporotrichioides*. In principle, trichodiene synthase should be capable of isomerizing either enantiomer of **7** to the corresponding 6,7-dihydronerolidyl pyrophosphate (**8**), but the resultant product cannot undergo cyclization due to the absence of the 6,7-double bond found in the normal substrate (Scheme III). We report below the results of studies that give further insight into the cryptic isomerase activity of trichodiene synthase and that lend further support to the mechanism summarized in Scheme I.

EXPERIMENTAL PROCEDURES

Instrumentation. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR spectra were obtained on a Perkin-Elmer 681 infrared spectrometer. NMR spectra were obtained on a Bruker AM 400 or WM 250 spectrometer at 400.134 or 250.133 MHz (^1H), 100.614 or 62.856 MHz (^{13}C), and 161.978 MHz (^{31}P) in $CDCl_3$, unless otherwise indicated. Chemical shifts are reported as parts per million downfield of Me_4Si (^1H and ^{13}C) or H_3PO_4 (^{31}P). Abbreviations used are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Electron impact mass spectra were obtained on a Kratos MS80 instrument operating at 70 eV. The samples were introduced either by direct inlet or in GC/MS mode. Unless otherwise noted, radioactivity measurements were obtained on a Beckman LS 5801 liquid scintillation counter using 5-mL solutions in Optifluor cocktail (Packard) and were automatically quench corrected. Capillary gas chromatography was performed on a Hewlett-Packard 5790 gas chromatograph using Carbowax 20M (0.2 mm × 25 m, film thickness 0.2 μm) or cross-linked methyl silicone (0.253 mm × 30 m, film thickness 0.25 μm) as the stationary phase. Radio/gas chromatography was performed on a Gow-Mac 560P thermal conductivity gas chromatograph connected to a Radiomatic Flo-One\Beta Model GCR radioactive detector using a 10% FFAP (8 ft × $^1/_8$ in., stainless steel) on Chromosorb WHP column (Croteau & Cane, 1985).

Materials and Methods. All buffers were prepared with deionized water obtained from a Sybron Barnstead Nanopure II Ion Exchange system or a Milli-Q (Millipore) system. Alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa was obtained from Sigma (P 7640). Inorganic pyrophosphatase (EC 3.6.1.1) from bakers' yeast was purchased from Boehringer-Mannheim. Trichodiene synthase was isolated from *F. sporotrichioides* and purified as previously described (Hohn & VanMiddlesworth, 1986). The homogeneous enzyme was stored frozen, at either −75 °C or liquid N_2 temperature, in 10 mM Hepes buffer (pH 7.5) that contained 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol. The enzyme preparation consisted primarily of the M_r 45 000 polypeptide (≥90%) and had a concentration of 0.2 mg of protein/mL. The enzyme was thawed in an ice bath and used undiluted for preparative incubations or diluted an appropriate amount with the same buffer for kinetic studies. Tetrahydrofuran and diethyl ether were distilled from sodium, while methylene chloride and acetonitrile were distilled from CaH_2. Triethylamine was distilled before use in buffers or distilled from KOH and stored over KOH for anhydrous reactions. Mesyl chloride was vacuum distilled prior to use. [1-^{3}H]-Farnesyl pyrophosphate (725 mCi/mmol) was prepared as previously described (Cane et al., 1981b; Davisson et al., 1986b). Sodium borotritide (319 mCi/mmol) was purchased from New England Nuclear. $(n\text{-}Bu_4N)_3HP_2O_7$ was prepared by the method of Poulter (Davisson et al., 1986a,b). Glycerol, hexane (HPLC grade), and magnesium chloride were purchased from Fisher Scientific. Dithiothreitol, ammonium bicarbonate, and tetrasodium pyrophosphate used in the kinetic studies were purchased from Sigma. Hepes was obtained from Research Organics or Sigma. All other reagents were obtained from Aldrich and were used without additional purification. Alkyllithium reagents were titrated with 2,5-dimethoxybenzyl alcohol (Winkle et al., 1980) under an N_2 atmosphere. Flash chromatography (Still et al., 1978) was performed on EM Science silica gel 60 (230–400 mesh). Silver nitrate impregnated silica gel for column chromatography was prepared by mixing EM Science silica gel 60 (100 g) with $AgNO_3$ (15 g) in $EtOH/CH_3CN$ (1:1, 150 mL). The solvent was removed by rotary evaporation followed by drying overnight under high vacuum at 70 °C. Silver nitrate impregnated analytical TLC plates were produced by dipping the plate in 10% $AgNO_3$ in EtOH, followed by drying overnight in darkness at 100 °C. Compounds on analytical TLC plates were visualized by UV light or charring after dipping in *p*-anisaldehyde reagent. Pyrophosphate esters were chromatographed on DEAE–Sephadex (A-25-120, bead size: 40–120 μm, Sigma) at 4 °C with a linear gradient (0.05–1.0 M) of pH 8.0 $Et_3NH(HCO_3)$ buffer. The buffer was prepared by adjusting the pH of a mixture of nanopure water and Et_3N to 8.0 with gaseous CO_2. Preparative-scale enzyme incubations were done in Wheaton-type reaction vials sealed with Teflon-lined, screw-cap closures. In general, all nonaqueous reactions were done under N_2 in oven-dried glassware, mixtures were magnetically stirred, and the progress was monitored by analytical TLC. All organic extracts were dried over $MgSO_4$, filtered by gravity, and evaporated under reduced pressure (water aspirator). Any remaining solvent was removed under high vacuum. A Sage Model 341B syringe pump was used to add sensitive reagents slowly by syringe.

Kinetic Studies of the Inhibition of Trichodiene Synthase by **7a**, **7b**, *and* PP_i. The reaction mixture contained 25 mM Hepes, pH 7.5 (adjusted with NaOH), 3 mM $MgCl_2$, and 1 mM dithiotheitol in a final volume of 0.25 mL. Dilutions of FPP and 6,7-dihydro-FPP were made in 15 mM NH_4CO_3 and the resulting concentrations determined by liquid scintillation counting. Additions of substrate and inhibitors were in 10-μL volumes. The final concentration of NH_4CO_3 in all reactions was 1.2 mM. The stock solution of trichodiene synthase was diluted between 1/100 and 1/400 in a buffer containing 10 mM Hepes, pH 7.5, 5 mM $MgCl_2$, and 10% glycerol just prior to the start of the assay. The reaction was started by the addition of 10 μL of enzyme, and incubation was for 10 min at 30 °C. All reactions were performed in 1.5-mL microcentrifuge tubes preincubated for 2 min at 30 °C. The reaction was stopped by the addition of 0.25 mL of ethanol. The product trichodiene was extracted through the addition of 0.7 mL of hexane, followed by vortexing for 15 s and centrifugation in a microcentrifuge for 15 s.[2] The hexane layer was removed and counted in minivials after the addition of the scintillation cocktail OCS (Amersham). Vials were counted in a Beckman LS3081 liquid scintillation counter for 10 min. Less than 10% of substrate was converted to trichodiene over the time course of the reaction. All data points were measured in duplicate over a range of substrate and inhibitor concentrations, and each set of experiments was carried out two to three times. Preliminary evaluations of inhibition patterns and estimates of steady-state kinetic parameters were obtained from double-reciprocal (Lineweaver–Burk) plots. The appropriate kinetic parameters for competitive inhibition were obtained by computer fitting data to the following equation using a nonlinear least-squares regression program KINFIT, written by Professor Vernon Anderson and based on the algorithm of Marquardt (1963):

$$v = VA/[A + K_m(1 + I/K_i)]$$

where $V = V_{max}$, A = concentration of FPP, and I = concentration of inhibitor.

Determination of the Enantiomeric Purity of Citronellol. To (+)-(3*R*)-β-citronellol (**9a**) (11 mg, 70 μmol) prepared as described from (*R*)-(+)-pulegone (Plesek, 1957; Valentine et al., 1976) in CH_2Cl_2 (2 mL) under N_2 were added several crystals of 4-(*N*,*N*-dimethylamino)pyridine (DMAP), Et_3N (2 drops), and finally (+)-MTPA-Cl (30 μL, 160 μmol, 2.3 equiv) (Dale et al., 1969), and the mixture was stirred overnight at room temperature. After 24 h, the mixture was passed through a pipet column of silica gel [EtOAc/hexanes (1:10), 5 mL; 0.5 × 2 cm column] to give the desired ester in the first 5-mL fraction. This was concentrated to give a quantitative yield of the Mosher ester of citronellol (**10a**): ^{1}H NMR δ 7.68 (2 H, dd, J = 7.8, 0.6 Hz), 7.08 (3 H, m), 5.09 (1 H, tm, J

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[2] Preparative reactions should not be run in plastic microcentrifuge tubes since the hexane will extract material from the tube itself. These impurities do not affect the results of kinetic determinations based solely on measurement of extractable radioactivity.

= 7.3 Hz), 4.08 (2 H, m), 3.41 (3 H, d, J = 1.1 Hz), 1.85 (2 H, m), 1.66 (3 H, d, J = 1.0 Hz), 1.52 (3 H, s), 1.43 (1 H, m), 1.31 (1 H, m), 1.16 (2 H, m), 1.00 (1 H, m), 0.67 (3 H, d, J = 6.6 Hz). The enantiomeric purity was determined by sequential additional of aliquots of Eu(fod)$_3$ in benzene-d_6, followed by ^{1}H NMR analysis. Base-line separation of all three diastereomeric methyl resonances could be achieved, and the percent enantiomeric excess (% ee) was calculated from the integration of these peaks. If none of the minor diastereomer was observed, the sample was spiked with the Mosher ester of racemic citronellol as a control. The sample (+)-(3*R*)-β-citronellol (**9a**), prepared from pulegone, was found to have a % ee of ≥98%.

Mosher esters of (+)- and (−)-β-citronellol (Aldrich catalog nos. C8,320-1 and 24,733-2, respectively) were produced as described above. The % ee of (−)-β-citronellol (**9b**) was 62–64%, while the % ee of "(+)-β-citronellol" (**9**) was found to be 0–1%. The latter sample was therefore used as the source of racemic citronellol.

(−)-(*7R*)-*Citronellyl Iodide* [(−)-**11a**]. To (+)-(3*R*)-β-citronellol (**9a**) (5.143 g, 32.9 mmol) in CH_2Cl_2 (50 mL) were added 4-(*N*,*N*-dimethylamino)pyridine (0.595 g, 4.9 mmol, 0.15 equiv) and Et_3N (5.0 mL, 36 mmol, 1.1 equiv), and the system was flushed with N_2 and then cooled in an ice/H_2O bath. Mesyl chloride (2.8 mL, 36 mmol, 1.1 equiv) was added over several minutes by syringe to give a copious precipitate. The mixture was warmed to room temperature and stirred overnight. After 20 h, the reaction mixture was filtered by suction; the filter cake was rinsed with several small portions of CH_2Cl_2 (10 mL total). The combined filtrates were washed with 5% aqueous HCl (25 mL, 3 × 10 mL CH_2Cl_2 back-extractions) and 5% aqueous $NaHCO_3$ (25 mL, 3 × 10 mL CH_2Cl_2 back-extractions). The combined extracts were dried, filtered, and concentrated to give 7.42 g of yellow liquid (96% crude yield). This material was used without further purification in the next reaction.

To the crude mesylate (7.42 g, 32 mmol) in acetone (150 mL) was added NaI (9.5 g, 63 mmol, 2.0 equiv), and the mixture was heated overnight at a gentle reflux. After 22 h, the mixture was cooled to room temperature and filtered by suction. The filter cake was rinsed with acetone (50 mL), and the filtrate was concentrated to a reddish liquid. The liquid was dissolved in CH_2Cl_2 (50 mL) and washed with $NaHSO_3$ (50 mL, 25 mL). The aqueous portions were back-extracted with CH_2Cl_2 (20 mL). The combined organic portions were dried, filtered, and concentrated to give 7.47 g of yellow liquid. The crude iodide was purified by elution through flash silica gel (4 × 2 cm column) with pentane (600 mL). The iodide eluted in the first 200 mL; the solvent was removed to give 6.90 g (79%) of (−)-**11a** as a colorless liquid: $[\alpha]^{28}{}_D$ −12.3° (*c* 15.11, $CHCl_3$); IR (neat) 2960, 2920, 1450, 1375, 1180 cm^{-1}; ^{1}H NMR δ 5.09 (1 H, tm, J = 7.1 Hz), 3.20 (2 H, br m), 1.95 (3 H, m), 1.68 (3 H, d, J = 0.9 Hz), 1.65 (1 H, m), 1.61 (3 H, s), 1.56 (1 H, m), 1.34 (1 H, m), 1.18 (1 H, m), 0.89 (3 H, d, J = 6.6 Hz); ^{13}C NMR δ 131.2 (C), 124.4 (CH), 40.9 (CH), 36.2 (CH or CH_3), 33.5 (CH_2), 25.7 (CH_2), 25.3 (CH or CH_3), 18.6 (CH_2), 17.6 (CH_2), 4.82 (CH or CH_3); mass spectrum, *m*/*z* (rel intensity) 266 (M^+, 50), 197 (8), 155 (22), 127 (24), 97 (37), 83 (93), 67 (55), 41 (100), 39 (80), 27 (78); HRMS, calcd for $C_{10}H_{19}I$ 266.0531, found 266.0524.

(−)-*Methyl* (*7S*)-*7,11-Dimethyl-3-oxo-10-dodecenoate* [(−)-**13a**]. A 50% oil dispersion of NaH (2.40 g, 50 mmol, 2.1 equiv) in a flame-dried flask was suspended in dry THF (100 mL). The mixture was cooled to 0 °C in an ice/H_2O bath; the methyl acetoacetate (5.2 mL, 48 mmol, 2.0 equiv) was added over 10 min by syringe pump. After evolution of H_2 ceased, a solution of *n*-BuLi (1.30 M, 35.7 mL, 46 mmol, 1.95 equiv) in hexanes was added over 20 min by syringe pump to give a milky white solution. After stirring at 0 °C for 3.5 h, a solution of iodide (−)-**11a** (6.333 g, 23.8 mmol) in dry THF (10 mL) was added via cannula; additional THF (2 × 2 mL) was used to ensure complete transfer. After 10 min, the resulting orange mixture was warmed to room temperature and stirred for 24 h. TLC analysis indicated incomplete reaction, so an additional equivalent of the dianion of methyl acetoacetate was prepared as described above from 50% NaH (1.2 g, 25 mmol, 1.05 equiv), methyl acetoacetate (2.6 mL, 24 mmol, 1.0 equiv), and *n*-BuLi (1.30 M, 17.9 mL, 23 mmol, 0.98 equiv) in dry THF (25 mL). This was added by cannula to the bright orange reaction mixture at 0 °C, and the resulting solution was warmed to room temperature and stirred overnight. After a total of 41 h, the reaction was quenched by pouring the mixture into saturated aqueous KH_2PO_4 (30 mL). The mixture was concentrated by rotary evaporation to ~10 mL volume and then filtered by suction. The filter cake was rinsed with EtOAc (2 × 20 mL). The layers of the two-phase filtrate were separated, and the aqueous layer was diluted with saturated aqueous KH_2PO_4 (30 mL) and then extracted with EtOAc (3 × 30 mL). The combined extracts were washed with saturated aqueous KH_2PO_4 (30 mL) that was also saturated with NaCl. The combined aqueous portions were extracted with additional EtOAc (2 × 30 mL). The combined organic extracts were dried, filtered, and concentrated to give 11.33 g (>100%) of an orange-red liquid. The crude mixture was purified by flash chromatography on silica gel [EtOAc/hexanes (1:15), 3 L; 400-mL forefraction; 20-mL fractions; 6.5 × 25 cm column] to give the desired product in fractions 39–80. Concentration gave 3.329 g (55%) of (−)-**13a** as a liquid: $[\alpha]^{28}{}_D$ −2.0° (*c* 10.47, $CHCl_3$); IR (neat) 2960, 2920, 1755, 1720, 1450, 1440, 1240 cm^{-1}; ^{1}H NMR δ 11.98 (s, OH of enol form), 5.03 (1 H, tm, J = 7 Hz), 4.94 (s, C*H*=OH of enol form), 3.69 (3 H, s), 3.67 (s, OCH_3 of enol form), 3.40 (2 H, s), 2.47 (2 H, t, J = 7 Hz), 1.90 (2 H, m), 1.66 (3 H, s), 1.55 (3 H, s), 1.00–1.75 (7 H, m), 0.82 (3 H, d, J = 6 Hz); ^{13}C NMR δ 202.6, 167.5, 130.9, 124.6, 52.1, 48.8, 43.1, 36.7, 36.1, 32.1, 25.5, 25.3, 20.8, 19.2, 17.4; HRMS, calcd for $C_{15}H_{26}O_3$ 254.1882, found 254.1884.

(−)-*Methyl trans*-(*7S*)-*3*-[(*Diethoxyphosphoryl*)*oxy*]-*7,11-dimethyl-2,10-dodecadienoate* [(−)-**14a**]. To a suspension of 50% NaH (0.762 g, 15.9 mmol, 1.2 equiv, oil dispersion) in dry Et_2O (8 mL) under N_2 at 0 °C was added a solution of (−)-**13a** (3.366 g, 13.23 mmol) in dry Et_2O (6 mL) via cannula over 15 min. Additional Et_2O (2 × 2 mL) was used to complete the transfer. After 15 min, diethyl chlorophosphate (2.70 mL, 18.7 mmol, 1.4 equiv) was added slowly by syringe, and the resulting milky mixture was warmed to room temperature and stirred overnight. After 12 h, the mixture was recooled to 0 °C and additional 50% NaH (0.127 g, 2.6 mmol, 0.2 equiv, oil dispersion) and diethyl chlorophosphate (0.48 mL, 3.3 mmol, 0.25 equiv) were added. After a total of 24 h, the reaction was quenched by the addition of half-saturated aqueous NH_4Cl (30 mL). The two-phase mixture was diluted with Et_2O (30 mL), and the layers were shaken well in a separatory funnel. The aqueous phase was extracted with Et_2O (2 × 20 mL). The combined etheral portions were washed with 5% aqueous $NaHCO_3$ (20 mL), which was back-extracted with additional Et_2O (3 × 20 mL). The combined Et_2O layers were dried, filtered, and concentrated to give 6.115 g (>100% crude) of a yellow liquid. ^{1}H NMR analysis indicated ~97% conversion to a 9:1 trans:cis mixture of isomers. The crude material was purified by flash chromatography on silica gel [EtOAc/hexanes (1:3), 800 mL; (1:1), 600 mL; 400-mL forefraction; 20-mL fractions; 6.5 × 21 cm column] to give the desired product in fractions 48–130. Concentration gave 3.952 g (76%) of (−)-**14a** as a slightly yellow liquid: $[\alpha]^{27}_{D}$ −2.1° (*c* 8.12, $CHCl_3$); IR (neat) 2960, 2920, 1730, 1665, 1435, 1285, 1210, 1150, 1035, 985 cm^{-1}; ^{1}H NMR δ 5.37 (1 H, s), 5.08 (1 H, tm, $J = 7.1$ Hz), 4.28 (2 H, q, $J = 7.1$ Hz), 4.26 (2 H, q, $J = 7.1$ Hz), 3.69 (3 H, s), 2.41 (2 H, m), 1.95 (2 H, m), 1.68 (3 H, d, $J = 1.0$ Hz), 1.60 (3 H, s), 1.59 (2 H, m), 1.38 (3 H, m), 1.37 (6 H, t, $J = 7.1$ Hz), 1.16 (2 H, m), 0.88 (3 H, d, $J = 6.5$ Hz); ^{13}C NMR δ 163.9 (C), 161.8 (C), 130.7 (C), 124.5 (CH), 104.4 (CH), 64.4 (CH_2), 64.4 (CH_2), 50.7 (CH_3), 36.7 (CH_2), 35.8 (CH_2), 35.2 (CH_2), 31.9 (CH), 25.4 (CH_3), 25.2 (CH_2), 23.6 (CH_2), 19.1 (CH_3), 17.3 (CH_3), 15.8 (CH_3), 15.7 (CH_3); mass spectrum, *m/z* (rel intensity) 390 (M^+, 11), 359 (6.5), 345 (4.3), 247 (4.8), 220 (8.2), 204 (14), 189 (14), 176 (10), 162 (12), 161 (22), 155 (100), 127 (58), 99 (79), 81 (56), 69 (85), 55 (55), 41 (75); HRMS, calcd for $C_{19}H_{35}O_6P$ 390.2171, found 390.2181.

(−)-Methyl trans-(7S)-6,7-Dihydrofarnesoate [(−)-**15a**]. A two-necked flame-dried flask under N_2 was charged with CuI (3.78 g, 19.9 mmol, 2.0 equiv) and dry Et_2O (25 mL), and then the gray suspension was cooled to −78 °C (dry ice/2-propanol). To the cold mixture was added a solution of CH_3Li (1.46 M, 25.8 mL, 37.7 mmol, 3.8 equiv) in Et_2O over 25 min by syringe pump. The slurry became bright yellow and then white as the addition proceeded. After complete addition, the mixture was warmed to 0 °C for 5 min, at first turning clear and colorless and then slightly yellow with some solid visible, and finally was recooled to −78 °C. A solution of phosphonate (−)-**14a** (3.869 g, 9.91 mmol) in dry Et_2O (5 mL) was added over 15 min by cannula to give an orange, cloudy solution. Additional Et_2O (2 × 2 mL) was used to ensure complete transfer. After 2 h at −78 °C, the reaction mixture was warmed to −25 °C (dry ice/2-propanol/H_2O) to give a red solution. After 1 h, the reaction was quenched by the addition of half-saturated NH_4Cl (30 mL). The mixture was warmed to room temperature and, after the evolution of gas ceased, was filtered by suction through analytical Celite. The filter cake was rinsed with several portions of EtOAc (30 mL total), and the two-phase filtrate was transferred to a separatory funnel and shaken well. The deep blue aqueous layer was further extracted with EtOAc (5 × 20 mL). The combined extracts were dried, filtered, and concentrated to give 2.451 g (98% crude) of a slightly yellow liquid. ^{1}H NMR analysis indicated ~96% conversion to a 94:6 mixture of trans:cis isomers. The crude material was partially purified by flash chromatography on silica gel [EtOAc/hexanes (1:20), 700 mL; 75-mL forefraction; 20-mL fractions; 6.5 × 10 cm column] to give a mixture of cis and trans isomers in fractions 8–26. Concentration of these fractions gave 2.269 g of liquid, which was further purified by MPLC on silica gel [EtOAc/hexanes (1:50), 3 L; 300-mL forefraction; 20-mL fractions; flow rate 15 mL/min; Lobar LiChroprep Si 60, size C (3.7 × 44 cm)] to give pure trans isomer in fractions 29–45. Earlier fractions were recycled once by MPLC to effect complete separation of cis and trans isomers. A total of 0.089 g (3%) of the cis isomer was isolated (analytical data reported below), while a total of 2.096 g (84%) of pure (−)-**15a** was obtained: $[\alpha]^{26}_{D}$ −4.6° (*c* 9.487, $CHCl_3$); IR (neat) 2930, 1720, 1650, 1435, 1380, 1360, 1225, 1150 cm^{-1}; ^{1}H NMR δ 5.66 (1 H, s), 5.10 (1 H, tm, $J = 7$ Hz), 3.68 (3 H, s), 2.16 (3 H, s), 2.11 (2 H, t, $J = 8$ Hz), 1.96 (2 H, m), 1.68 (3 H, s), 1.60 (3 H, s), 1.20–1.55 (5 H, m), 1.13 (2 H, m), 0.87 (3 H, d, $J = 6$ Hz); ^{13}C NMR δ 167.2 (C), 160.4 (C), 131.0 (C), 125.0 (CH), 115.2 (CH), 50.6 (CH_3), 41.2 (CH_2), 37.1 (CH_2), 36.5 (CH_2), 32.3 (CH), 25.6 (CH_3), 25.5 (CH_2), 24.9 (CH_2), 19.5 (CH_3), 18.7 (CH_3), 17.6 (CH_3); mass spectrum, *m/z* (rel intensity) 252 (M^+, 2.3), 221 (21), 178 (16), 141 (38), 128 (57), 123 (45), 109 (82), 95 (82), 86 (70), 84 (84), 83 (64), 82 (78), 81 (67), 67 (72), 55 (79), 43 (96), 41 (100); HRMS, calcd for $C_{16}H_{28}O_2$ 252.2089, found 252.2075.

(−)-trans-(7S)-6,7-Dihydrofarnesol [(−)-**12a**]. To a flame-dried flask charged with $LiAlH_4$ (119 mg, 3.14 mmol, 2.0 equiv) in dry Et_2O (5 mL) under N_2 and cooled to 0 °C was added absolute EtOH (190 μL, 3.24 mmol, 2.1 equiv) by syringe over 5 min. The mixture was stirred for another 10 min, and then a solution of (−)-**18** (394 mg, 1.56 mmol) in dry Et_2O (3 mL) was added over 5 min by cannula. Additional Et_2O (2 × 1 mL) was used to ensure complete transfer. The resulting gray suspension was stirred for 20 h at 0 °C, and then for another 7 h at room temperature. The mixture was recooled to 0 °C, and H_2O (120 μL), 15% NaOH (120 μL), and H_2O (360 μL) were cautiously added at 10-min intervals to quench the reaction. The white, flocculent precipitate was removed by suction filtration through analytical Celite. The filter cake was rinsed with additional Et_2O (15 mL total). The filtrate was dried, filtered by suction, and concentrated to give 341 mg (97% crude yield) of a 94:6 mixture of desired and overreduced product. Initial purification was accomplished by flash chromatography on silica gel [EtOAc/hexanes (1:100), 200 mL; (1:10), 700 mL; 200-mL forefraction; 15-mL fractions; 3.5 × 7 cm column]. Fractions containing (−)-**12a** (10–38) were combined and concentrated to a liquid. The residue was further purified by flash chromatography on $AgNO_3$-impregnated silica gel [EtOAc/hexanes (1:50), 400 mL; (1:20), 200 mL; (1:10), 1000 mL; (1:5), 100 mL; 650-mL forefraction; 20-mL fractions; 3.5 × 17 cm column]. Fractions 20–41 were concentrated to give 222 mg (63%) of pure (−)-**12a**: $[\alpha]^{27}_{D}$ −2.7° (*c* 5.16, $CHCl_3$); IR (neat) 3600–3100, 3120, 1665, 1450, 1375, 1000 cm^{-1}; ^{1}H NMR δ 5.41 (1 H, tm, $J = 7$ Hz), 5.10 (1 H, tm, $J = 7$ Hz), 4.15 (2 H, d, $J = 7$ Hz), 1.97 (4 H, m), 1.69 (3 H, d, $J = 1$ Hz), 1.67 (3 H, s), 1.59 (3 H, s), 1.20–1.50 (6 H, m), 1.18 (2 H, m), 0.87 (3 H, d, $J = 6$ Hz); ^{13}C NMR δ 140.1 (C), 130.9 (C), 125.1 (CH), 123.3 (CH), 59.4 (CH_2), 39.9 (CH_2), 37.1 (CH_2), 36.7 (CH_2), 32.4 (CH), 25.7 (CH_3), 25.6 (CH_2), 25.2 (CH_2), 19.6 (CH_3), 17.6 (CH_3), 16.2 (CH_3); mass spectrum, *m/z* (rel intensity) 224 (M^+, 6.9), 206 (6.1), 191 (3.4), 177 (2.9), 163 (5.5), 149 (11), 137 (8.5), 135 (12), 123 (24), 121 (29), 109 (84), 107 (22), 95 (75), 82 (68), 81 (88), 71 (86), 68 (64), 67 (69), 55 (100), 43 (66), 41 (92); HRMS, calcd for $C_{15}H_{28}O$ 224.2140, found 224.2151.

(−)-trans-(7S)-6,7-Dihydrofarnesal. To a solution of alcohol (−)-**12a** (120.5 mg, 0.537 mmol) in CH_2Cl_2 (5 mL) under N_2 was added the Dess–Martin periodinane **16** (251 mg, 0.59 mmol, 1.1 equiv). The resulting white, heterogeneous mixture was stirred overnight at room temperature. After 22 h, the reaction was diluted with Et_2O (25 mL) and then poured into a mixture of 5% aqueous $NaHCO_3$ (10 mL) and $Na_2S_2O_3$ (680 mg, 4.30 mmol, 8.0 equiv). The cloudy two-phase mixture was stirred until both phases were clear and colorless (20 min). The layers were separated, and the organic phase was washed with 5% aqueous $NaHCO_3$ (10 mL) and H_2O (10 mL). The combined aqueous layers were extracted with Et_2O (2 × 10 mL). The organic layers were dried, filtered, and concentrated to give 120 mg (100% crude yield) of a slightly yellow liquid. The crude material was purified by flash chromatography on silica gel [EtOAc/hexanes (0:1), 50 mL;

(1:15), 200 mL; 7-mL fractions; 2 × 12 cm column] to give the desired aldehyde in fractions 7–10. Concentration gave 95.3 mg (80%) of pure (−)-dihydrofarnesal: $[\alpha]^{25}_{D}$ −4.7° (*c* 8.26, $CHCl_3$); IR (neat) 2920, 2850, 1675, 1450, 1380, 1195, 1125 cm^{-1}; ^{1}H NMR δ 9.99 (1 H, d, *J* = 8.0 Hz), 5.88 (1 H, d, *J* = 8.0 Hz), 5.09 (1 H, tm, *J* = 7.2 Hz), 2.19 (2 H, t, *J* = 7.4 Hz), 2.16 (3 H, d, *J* = 1.1 Hz), 1.96 (2 H, m), 1.68 (3 H, s), 1.60 (3 H, s), 1.48 (3 H, m), 1.31 (2 H, m), 1.14 (2 H, m), 0.87 (3 H, d, *J* = 6.6 Hz); ^{13}C NMR δ 191.2 (CH), 164.2 (C), 131.1 (C), 127.2 (CH), 124.7 (CH), 40.8 (CH_2), 36.9 (CH_2), 36.4 (CH_2), 32.2 (CH), 25.6 (CH_3), 25.4 (CH_2), 24.5 (CH_2), 19.4 (CH_3), 17.6 (CH_3), 17.4 (CH_3); mass spectrum, *m/z* (rel intensity) 222 (M^+, 23), 207 (3.6), 189 (2.6), 179 (3.8), 161 (4.8), 149 (6.2), 137 (27), 69 (100), 97 (78), 95 (74), 84 (85), 67 (68), 55 (91), 43 (64), 39 (75), 29 (66); HRMS, calcd for $C_{15}H_{26}O$ 222.1984, found 222.1985.

Ammonium trans-[1-^{3}H]-(7S)-6,7-Dihydrofarnesyl Pyrophosphate (**7a**). To $NaBH_3T$ (3.0 mg, 78 μmol, 25 mCi, 0.82 atom equiv) under argon, cooled in an ice/H_2O bath, was added an ice-cold solution of (−)-*tran*-(7*S*)-6,7-dihydrofarnesal (84.7 mg, 381 μmol) in dry EtOH (1.5 mL) by syringe over 10 min. The resulting clear, colorless solution was stirred at 0 °C for 3 h, and then the reaction was chased by the slow addition of ice-cold unlabeled $NaBH_4$ (15 mg, 400 μmol, 4.2 atom equiv) in EtOH (1 mL) by syringe. The resulting solution was warmed to room temperature and stirred overnight. After a total of 22 h, the reaction was quenched by carefully pouring the mixture into ice-cold half-saturated aqueous NH_4Cl (5 mL). The mixture was gently swirled and then shaken to completely mix the layers. The aqueous layer was further extracted with Et_2O (5 × 5 mL). The combined extracts were dried, filtered, and concentrated to give a quantitative crude yield of colorless liquid. The residue was purified by flash chromatography on $AgNO_3$-impregnated silica gel [EtOAc/hexanes (1:20), 200 mL; (1:10), 800 mL; 10-mL fractions; 2 × 16 cm column]. Concentration of fractions 29–50 gave 64 mg (75% chemical yield) of [1-^{3}H]-**12a** as a colorless liquid. A portion of this material was counted by liquid scintillation, and the total activity for the entire sample was determined to be 20.6 mCi (82% radiochemical yield). The specific activity was determined by serial dilution of a known amount of the alcohol, followed by scintillation counting of an aliquot, and was found to be 72.1 mCi/mmol.

To [1-^{3}H]-**12a** (18.1 mg, 80.7 μmol, 5.82 mCi, specific activity 72.1 mCi/mmol) in CCl_4 (0.5 mL) under argon was added triphenylphosphine (44.5 mg, 170 μmol, 2.1 equiv), and the mixture was heated at reflux. After 8 h, the mixture was cooled, additional triphenylphosphine (9.9 mg, 37.7 μmol, 0.5 equiv) was added, and the solution was again gently refluxed for another 10 h. The mixture was cooled, diluted with pentane (3 mL), and filtered through a pipet column of analytical Celite (0.5 × 2 cm). The residue was rinsed with additional pentane (10 mL). The filtrate was concentrated, and the residue was triturated with fresh pentane (5 mL) and again filtered through analytical Celite as described before. Concentration of the pentane eluant provided the chloride which was used in the subsequent reaction without further purification.

To the chloride under argon was added a solution of $(n\text{-}Bu_4N)_3HP_2O_7$ (146 mg, 162 μmol, 2.0 equiv) in dry CH_3CN (0.3 mL) by syringe. After 8 h, additional $(n\text{-}Bu_4N)_3HP_2O_7$ (70 mg, 78 μmol, 1.0 equiv) was added and the reaction was continued overnight. After a total of 21 h, the solvent was removed under a gentle stream of N_2 to give a viscous, light brown liquid. The crude mixture was dissolved in 100 mM Tris-HCl (pH 7.4, 1.0 mL), inorganic pyrophosphatase (200 units) and 20 μL of 100 mM $MgCl_2$ (2.0 μmol, 0.02 equiv) were added, and the mixture was incubated at 30 °C for 20 min. The reaction mixture was cooled to 4 °C, diluted with 0.05 M $Et_3NH(HCO_3)$ (pH 8.0, 5 mL), and applied to a DEAE-Sephadex column previously equilibrated with 0.05 M $Et_3NH(HCO_3)$ at 4 °C. The column was eluted with a linear gradient of pH 8.0 0.05 M $Et_3NH(HCO_3)$ (200 mL) to 1.0 M $Et_3NH(HCO_3)$ (200 mL) at a flow rate of 1.0 mL/min. Fractions (5 mL) were collected automatically and analyzed by scintillation counting. Fractions 41–49 were combined and lyophilized repeatedly from 10 mM NH_4OH to remove volatile buffer salts. Analysis by ^{31}P NMR spectroscopy showed the presence of desired allylic pyrophosphate **7a** (26% yield), which was contaminated with PP_i in a ratio of 1:2.5.[3] Fractions 50–59 were combined and lyophilized repeatedly from 10 mM NH_4OH. Analysis by ^{31}P NMR spectroscopy showed the material to be pure allylic pyrophosphate **7a** (44% yield). This material was dissolved in 10 mM NH_4OH (5 mL) and stored frozen at −20 °C: ^{1}H NMR (D_2O) δ 5.25 (1 H, tm, *J* = 7.0 Hz), 5.03 (1 H, tm, *J* = 7.0 Hz), 4.27 (2 H, t, *J* = 6.6 Hz), 1.83 (4 H, m), 1.51 (3 H, s), 1.49 (3 H, s), 1.42 (3 H, s), 1.23 (3 H, m), 1.10 (2 H, m), 0.94 (2 H, m), 0.65 (3 H, d, *J* = 6.6 Hz); ^{31}P NMR (10 mM NH_4OH + 3 drops of D_2O) δ −5.68 (P_β, d, *J* = 22 Hz), −9.54 (P_α, d, *J* = 22 Hz).

A sample of **7a** (specific activity 0.65 mCi/mmol) was produced in the same manner from a sample of [1-^{3}H]-**12a** which had been diluted with unlabeled (−)-**12a**. A sample of **7a** with a specific activity of 40.5 mCi/mmol was produced by mixing known quantities of the 72.1 mCi/mmol material with the 0.65 mCi/mmol material.

(−)-trans-(7S)-[1,1-$^{2}H_2$]-6,7-Dihydrofarnesol [(−)-**12a**]. To a flame-dried flask charged with $LiAlD_4$ (36.5 mg, 0.859 mmol, 2.0 equiv) in dry Et_2O (2 mL) under N_2 was added absolute EtOD (54 μL, 0.919 mmol, 2.1 equiv) by syringe over 2 min. The mixture was cooled to 0 °C, and then a solution of (−)-**15a** (109 mg, 0.432 mmol) in dry Et_2O (1 mL) was added over 2 min by cannula. Additional Et_2O (2 × 0.5 mL) was used to ensure complete transfer. The resulting gray suspension was stirred for 12 h at 0 °C, and then for another 6 h at room temperature. The mixture was recooled to 0 °C, and H_2O (120 μL), 15% NaOH (120 μL), and H_2O (360 μL) were cautiously added at 5-min intervals to quench the reaction. The white, flocculent precipitate was removed by suction filtration through analytical Celite. The filter cake was rinsed with additional Et_2O (10 mL total). The filtrate was dried, filtered by suction, and concentrated to give 91.6 mg (94% crude yield). Initial purification was accomplished by flash chromatography on silica gel [EtOAc/hexanes (1:100), 250 mL; (1:10), 500 mL; 250-mL forefraction; 15-mL fractions; 2 × 4 cm column]. Fractions containing (−)-[1-$^{2}H_2$]-**12a** (0–8) were combined and concentrated. The residue was further purified by MPLC on silica gel [EtOAc/hexanes (1:100), 250 mL; (1:10), 600 mL; 100-mL forefraction; 10-mL fractions; flow rate 8 mL/min; Lobar LiChroprep Si 60, size B (2.5 × 31 cm)] to give the desired alcohol in fractions 46–57. Evaporation of the solvent gave 68.2 mg (70%) of (−)-[1,1-$^{2}H_2$]-**12a** as a colorless liquid. Capillary GC analysis indicated

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[3] In order for the pyrophosphatase reaction to proceed at a reasonable rate, it is generally advisable for the concentration of $MgCl_2$ to exceed that of inorganic pyrophosphate and pyrophosphate esters combined. In the present example, the deficiency in $MgCl_2$ concentration resulted in incomplete hydrolysis of inorganic pyrophosphate under the conditions described.

that this material was ≥99% pure: $[\alpha]^{27}{}_D$ −2.6° (*c* 2.16, $CHCl_3$); IR (neat) 3600–3100, 2920, 2180, 2090, 1660, 1450, 1375, 1080 cm^{-1}; ^1H NMR δ 5.39 (1 H, s), 5.10 (1 H, tm, J = 7.1 Hz), 1.98 (2 H, t, J = 7.9 Hz), 1.97 (2 H, m), 1.68 (3 H, d, J = 1.0 Hz), 1.66 (3 H, d, J = 1.3 Hz), 1.60 (3 H, s), 1.23–1.50 (6 H, m), 1.11 (2 H, m), 0.86 (3 H, d, J = 6.6 Hz); ^{13}C NMR δ 140.1 (C), 130.9 (C), 124.9 (CH), 123.3 (CH), 58.6 (CD_2), 39.8 (CH_2), 37.0 (CH_2), 36.5 (CH_2), 32.2 (CH), 25.6 (CH_3), 25.5 (CH_2), 25.0 (CH_2), 19.5 (CH_3), 17.5 (CH_3), 16.1 (CH_3); mass spectrum, m/z (rel intensity) 226 (M^+, 15), 208 (12), 193 (4.0), 179 (2.4), 177 (2.6), 165 (9.6), 149 (6.0), 138 (8.3), 137 (13), 123 (39), 121 (8.9), 109 (100), 95 (62), 84 (81), 83 (54), 82 (66), 81 (51), 73 (46), 70 (40), 69 (97), 55 (45), 49 (51), 41 (36); HRMS, calcd for $C_{15}D_2H_{26}O$ 226.2264, found 226.2259.

Ammonium trans-(7S)-[1,1-$^2H_2$]-6,7-Dihydrofarnesyl Pyrophosphate (*[1,1-$^2H_2$]*-**7a**). A sample of low specific radioactivity alcohol was prepared by mixing (−)-[1,1-$^2H_2$]-**12a** (11.2 mg, 49.5 μmol) with [1-^3H]-**12a** (2.40 × 10^7 dpm, 10.8 μCi, 0.15 μmol, specific activity 72.1 mCi/mmol) to give material that was >99% deuterated with a specific activity of 0.22 mCi/mmol. This material was converted to the pyrophosphate ester as described for the synthesis of **7a**. This gave a 2:1 mixture of [1,1-$^2H_2$]-**7a** (23.1 μmol, 5.08 μCi, 47%) and inorganic pyrophosphate. The allylic pyrophosphate was purified by incubation with inorganic pyrophosphatase (200 units) in 50 mM Tris-HCl (pH 7.4) that contained 6 mM $MgCl_2$ (36 μmol, 1.6 equiv with respect to allylic pyrophosphate) in a total volume of 6.0 mL. The mixture was incubated for 30 min at 30 °C, cooled to 4 °C, and then diluted with 50 mM $Et_3NH(HCO_3)$ (pH 8.0, 5 mL), 100 mM NH_4OH (2 mL), and 25 mM EDTA (pH 7.5, 2.5 mL, 62.5 μmol). The clear, colorless solution was purified by chromatography on DEAE-Sephadex as was described for the purification of **7a**. Repeated lyophilization from 10 mM NH_4OH removed the volatile buffer salts and gave [1,1-$^2H_2$]-**7a** (20.5 μmol, 4.51 μCi, 41%) which was free of PP_i as judged by ^{31}P NMR spectroscopy: ^1H NMR (D_2O) δ 5.25 (1 H, s), 5.03 (1 H, tm, J = 7.1 Hz), 1.83 (4 H, m), 1.51 (3 H, s), 1.49 (3 H, s), 1.42 (3 H, s), 1.23 (3 H, m), 1.10 (2 H, m), 0.94 (2 H, m), 0.65 (3 H, d, J = 6.6 Hz); ^{31}P NMR (10 mM NH_4OH + 3 drops of D_2O) δ −5.69 (P_β, d, J = 23 Hz), −9.53 (P_α, d, J = 23 Hz).

Purification of Racemic Ammonium trans-6,7-Dihydrofarnesyl Pyrophosphate (**7**). Racemic **7** (953 μCi, 14.4 μmol, specific activity 66.1 mCi/mmol), which had been shown by ^{31}P NMR to be contaminated with PP_i (23.5 μmol), was incubated at 30 °C with inorganic pyrophosphatase (100 units) in 50 mM Tris-HCl (pH 7.4) that contained 4.1 mM $MgCl_2$ (25 μmol, 1.7 equiv with respect to allylic pyrophosphate) in a total volume of 6.1 mL. After 30 min, the heterogeneous mixture was cooled to 4 °C and diluted with 50 mM $Et_3NH(HCO_3)$ (pH 8.0, 5 mL), 100 mM NH_4OH (10 mL), concentrated NH_4OH (0.5 mL), and 25 mM EDTA (pH 7.5, 1.0 mL, 25 μmol) to give a clear, colorless solution. The cold solution was purified by chromatography on DEAE-Sephadex as described for the purification of **7a**. The fractions that contained the desired allylic pyrophosphate were lyophilized repeatedly from 10 mM NH_4OH; the final volume was adjusted to 1.0 mL. In order to remove all protein, the sample was passed through an Amicon Centricon 10 microconcentrator by centrifugation at 4 °C for 2 h at 5000*g*. Additional 10 mM NH_4OH (2 × 1 mL) was passed through the microconcentrator under the same conditions to ensure complete elution of the pyrophosphate. The eluant was again purified on DEAE-Sephadex, and buffer salts were removed by repeated lyophilization from 10 mM NH_4OH. The purity of the allylic pyrophosphate **7** that was obtained (756 μCi, 11.4 μmol, 79% recovery) was confirmed by ^1H and ^{31}P NMR spectroscopy.

Purification of Ammonium trans-(7R)-6,7-Dihydrofarnesyl Pyrophosphate (**7b**). Enantiomerically enriched (7*R*)-**7b** (64% ee) (1.24 mCi, 27 μmol, specific activity 45.9 mCi/mmol), which had been shown by ^{31}P NMR to be contaminated with PP_i (153 μmol), was purified by incubation with inorganic pyrophosphatase as described above, yielding 232 μCi (5.06 μmol, 19% recovery) of (7*R*)-**7b** that was pure by both ^1H and ^{31}P NMR spectroscopy.

3-Carbethoxy-6,10-dimethyl-2-oxo-9-undecene (**22**). To a solution of NaOEt (39.8 mmol, 1.05 equiv) in a flame-dried flask under N_2 was added ethyl acetoacetate (5.3 mL, 41.6 mmol, 1.1 equiv) by syringe. After 5 min, this solution was added by cannula to citronellyl iodide (**11**) (10.086 g, 37.9 mmol) over 5 min. The clear, slightly orange mixture was heated at a gentle reflux (oil bath temperature 90 °C) for 8 h, then cooled to room temperature, and concentrated to a final volume of 5 mL by rotary evaporation. The concentrate was taken up in EtOAc (100 mL) and washed with 5% aqueous HCl (30 mL, 2 × 15 mL; 2 × 15 mL EtOAc back-extractions) and 5% aqueous $NaHSO_3$ (2 × 30 mL; 2 × 15 mL EtOAc back-extractions). The combined extracts were dried, filtered, and concentrated to give a quantitative crude yield of a slightly yellow liquid. The crude material was purified by flash chromatography on silica gel [EtOAc/hexanes (1:50), 500 mL; (1:25), 500 mL; 1:10, 1000 mL; 1000-mL forefraction; 20-mL fractions; 6 × 20 cm column]. Concentration of fractions 16–38 gave 7.605 g (75%) of **22** as a colorless liquid (Azerad & Cyrot, 1965): IR (neat) 2960, 2920, 1740, 1715, 1450, 1365 cm^{-1}; ^1H NMR δ 5.08 (1 H, t, J = 7.1 Hz), 4.20 (2 H, q, J = 7.0 Hz), 3.36 (1 H, t, J = 7.7 Hz), 2.22 (3 H, s), 1.90 (5 H, m), 1.68 (3 H, d, J = 0.9 Hz), 1.60 (3 H, s), 1.42 (1 H, m), 1.30 (1 H, m), 1.27 (3 H, t, J = 7.0 Hz), 1.13 (2 H, m), 0.88 (3 H, d, J = 6.6 Hz); ^{13}C NMR δ 203.1 (C), 169.8 (C), 131.0 (C), 124.6 (CH), 61.1 (CH_2), 60.0 (CH), 36.6 (CH_2), 34.3 (CH_2), 32.1 (CH), 28.5 (CH_3), 25.6 (CH_2), 25.5 (CH_3), 25.3 (CH_2), 19.2 (CH_3), 17.5 (CH_3), 14.0 (CH_3); mass spectrum, m/z (rel intensity) 268 (M^+, 15), 250 (7.7), 223 (5.3), 222 (5.0), 207 (8.1), 183 (12), 179 (17), 161 (19), 143 (42), 123 (34), 109 (74), 97 (45), 95 (70), 82 (63), 67 (49), 55 (100), 43 (94), 41 (81), 29 (56); HRMS, calcd for $C_{16}H_{28}O_3$ 268.2038, found 268.2031.

6,10-Dimethyl-2-oxo-9-undecene (**23**). To ester **22** (7.603 g, 28.3 mmol) in THF (100 mL)/H_2O (50 mL) cooled to 0 °C was added 1.0 M NaOH (57 mL, 57 mmol, 2.0 equiv) over several minutes. The cloudy mixture was warmed to room temperature and stirred well. After 44 h, the mixture was concentrated by rotary evaporation to remove most of the THF, and EtOAc (75 mL) was added. The two-phase mixture was vigorously stirred and carefully acidified with concentrated HCl (~4 mL). After the evolution of CO_2 subsided, the mixture was heated on a H_2O bath at 80 °C for 1 h and then cooled to room temperature. The layers were separated and the aqueous layer was saturated with solid NaCl and extracted with EtOAc (2 × 50 mL). The combined extracts were washed with 5% aqueous $NaHCO_3$ (2 × 50 mL) and saturated $NaHCO_3$ (50 mL). The combined washes were back-extracted with additional EtOAc (2 × 25 mL). The organic layers were dried, filtered, and concentrated to give 5.42 g (98% crude) of a slightly yellow liquid. The liquid was purified by flash chromatography on silica gel [EtOAc/hexanes (1:25), 2 L; (1:15), 0.5 L; (1:10), 0.5 L; 0.5-L forefraction; 20-mL fractions; 6.5 × 19 cm column]. Concentration of fractions 18–40 gave 4.776 g (86%) of **23** as a colorless liquid: IR (neat) 2950, 2920, 1715, 1450, 1375, 1360, 1165 cm^{-1}; ^{1}H NMR δ 5.08 (1 H, t, *J* = 7.1 Hz), 2.40 (2 H, t, *J* = 7.4 Hz), 2.13 (3 H, s), 1.97 (2 H, m), 1.67 (3 H, d, *J* = 1.0 Hz), 1.60 (3 H, s), 1.57 (2 H, m), 1.40 (1 H, m), 1.30 (2 H, m), 1.13 (2 H, m), 0.88 (3 H, d, *J* = 6.6 Hz); ^{13}C NMR δ 208.7 (C), 130.8 (C), 124.7 (CH), 43.8 (CH$_2$), 36.8 (CH$_2$), 36.2 (CH$_2$), 32.1 (CH), 29.6 (CH$_3$), 25.5 (CH$_3$), 25.3 (CH$_2$), 21.2 (CH$_2$), 19.2 (CH$_3$), 17.4 (CH$_3$); mass spectrum, *m/z* (rel intensity) 196 (M$^+$, 10), 163 (2.1), 153 (1.6), 138 (3.8), 123 (8.2), 109 (15), 95 (14), 82 (15), 71 (17), 67 (13), 55 (28), 43 (100), 41 (40); HRMS, calcd for $C_{13}H_{24}O$ 196.1827, found 196.1829.

Methyl cis-6,7-Dihydrofarnesoate (**24**). To a mixture of ketone **23** (805 mg, 4.10 mmol) and trimethyl phosphonoacetate (2.0 mL, 12.4 mmol, 3.0 equiv) under N_2 was added a 1.0 M solution of $NaOCH_3$ (10.3 mL, 10.3 mmol, 2.5 equiv) in CH_3OH. The slighly yellow solution was stirred overnight at room temperature. After 24 h, the mixture was poured into half-saturated NH_4Cl (20 mL) and extracted with EtOAc (3 × 20 mL). The aqueous layer was concentrated by rotary evaporation to remove CH_3OH and then extracted with additional EtOAc (2 × 20 mL). The combined organic layers were dried, filtered, and concentrated to give a two-phase residue. The residue was partitioned between H_2O (10 mL) and hexanes (20 mL). The hexanes layer was washed with more H_2O (10 mL), and the combined aqueous layers were extracted with hexanes (4 × 10 mL). The extracts were dried, filtered, and concentrated to give 891 mg (86% crude) of a slightly yellow liquid. Capillary GC analysis showed this to consist of a 2:1 mixture of trans:cis isomers (**15**:**24**). The mixture was partially purified by flash chromatography on silica gel [EtOAc/hexanes (1:10), 250 mL; 50-mL fractions; 3 × 3 cm column]. Concentration of the first fraction gave a mixture of prepurified trans:cis isomers which were separated by MPLC on silica gel [Et_2O/hexanes (1:50); 300-mL forefraction; 20-mL fractions; flow rate 15 mL/min; Lobar LiChroprep Si 60, size C (3.7 × 44 cm)]. Concentration of fractions 38–50 gave 365 mg (35%) of pure trans isomer **15**, which was identical with **15** synthesized as described above. Concentration of fractions 17–34 gave 204 mg (20%) of cis isomer **24**: IR (neat) 2920, 1725, 1650, 1440, 1380, 1230, 1190, 1150 cm^{-1}; ^{1}H NMR δ 5.65 (1 H, s), 5.10 (1 H, tm, *J* = 7.2 Hz), 3.67 (3 H, s), 2.60 (2 H, tm, *J* = 5.7 Hz), 1.96 (2 H, m), 1.88 (3 H, s), 1.68 (3 H, s), 1.60 (3 H, s), 1.26–1.56 (5 H, m), 1.15 (2 H, m), 0.88 (3 H, d, *J* = 6.5 Hz); ^{13}C NMR δ 166.7 (C), 161.1 (C), 130.9 (C), 125.0 (CH), 115.5 (CH), 50.7 (CH$_3$), 37.1 (CH$_2$), 36.9 (CH$_2$), 33.6 (CH$_2$), 32.3 (CH), 25.7 (CH$_3$), 25.6 (CH$_2$), 25.5 (CH$_2$, 25.1 (CH$_3$), 19.4 (CH$_3$, 17.6 (CH$_3$); mass spectrum (CI, isobutane), *m/z* (rel intensity) 253 (M$^+$ + 1, 10), 221 (3.6), 193 (7.0), 128 (4.4), 109 (6.8), 69 (9.8), 58 (49), 57 (100), 56 (59), 55 (41), 44 (40), 40 (47); HRMS (CI, isobutane), calcd for $C_{16}H_{29}O_2$ (M$^+$ + 1) 253.2167, found 253.2173.

cis-6,7-Dihydrofarnesol (**17**). To a flame-dried flask charged with $LiAlH_4$ (69 mg, 1.81 mmol, 2.0 equiv) in dry Et_2O (3 mL) under N_2 and cooled to 0 °C was added absolute EtOH (112 μL, 1.91 mmol, 2.1 equiv) by syringe over 5 min. The mixture was stirred for another 10 min, and then a solution of **24** (229 mg, 0.91 mmol) in dry Et_2O (2 mL) was added over 5 min by cannula. Additional Et_2O (2 × 0.5 mL) was used to ensure complete transfer. The resulting gray suspension was stirred for 18 h at 0 °C, and then for another 30 h at room temperature. The mixture was recooled to 0 °C, and H_2O (69 μL), 15% NaOH (69 μL), and H_2O (209 μL) were cautiously added at 5-min intervals to quench the reaction. The mixture was diluted with Et_2O (10 mL), and the white, flocculent precipitate was removed by suction filtration through analytical Celite. The filter cake was rinsed with additional Et_2O (10 mL total). The filtrate was dried, filtered by suction, and concentrated to give 203 mg (100% crude yield) of a 90:10 mixture of desired and overreduced product. The crude liquid was purified by flash chromatography on silica gel [EtOAc/hexanes (1:100), 150 mL; (1:20), 200 mL; (1:10), 550 mL; (1:5), 200 mL; 200-mL forefraction; 20-mL fractions; 3.5 × 9 cm column]. Concentration of fractions 19–20 gave 61 mg (30%) of **17** that was contaminated with ~7% of 2,3,6,7-tetrahydrofarnesol. This material was suitable for use as a carrier compound without any additional purification. Concentration of fractions 17–18 and 21–42 gave 114 mg (56%) of the cis alcohol which was contaminated with 2,3,6,7-tetrahydrofarnesol (11%) and some **12** (2%). Additional purification gave an analytically pure sample of **17** for characterization: IR (neat) 3600–3100, 2920, 1670, 1450, 1375, 1000 cm^{-1}; ^{1}H NMR δ 5.41 (1 H, tm, *J* = 7.0 Hz), 5.10 (1 H, tm, *J* = 7.1 Hz), 4.11 (2 H, d, *J* = 7.0 Hz), 2.04 (2 H, t, *J* = 7.8 Hz), 1.95 (2 H, m), 1.73 (3 H, s), 1.68 (3 H, s), 1.60 (3 H, s), 1.22–1.50 (6 H, m), 1.12 (2 H, m), 0.86 (3 H, d, *J* = 6.6 Hz); ^{13}C NMR δ 140.3 (C), 131.0 (C), 124.9 (CH), 124.0 (CH), 59.0 (CH$_2$), 37.0 (CH$_2$), 36.7 (CH$_2$), 32.2 (CH), 32.1 (CH$_2$), 25.6 (CH$_3$), 25.6 (CH$_2$), 25.5 (CH$_2$), 23.4 (CH$_3$), 19.5 (CH$_3$), 17.5 (CH$_3$); mass spectrum, *m/z* (rel intensity) 224 (M$^+$, 0.3), 206 (1.8), 191 (1.8), 177 (0.8), 163 (0.9), 149 (2.5), 135 (4.0), 123 (8.8), 121 (11), 109 (37), 95 (27), 82 (26), 81 (40), 71 (31), 68 (23), 67 (26), 55 (50), 43 (19), 41 (100); HRMS, calcd for $C_{15}H_{28}O$ 224.2140, found 224.2144.

6,7-Dihydronerolidol (**18**). To ketone **23** (0.465 g, 2.37 mmol) in dry THF (10 mL) under N_2 at 0 °C was added a 1.0 M solution of vinylmagnesium bromide (2.90 mL, 2.90 mmol, 1.2 eq) in THF over 10 min by syringe. The brown solution was stirred at 0 °C for 10 h and then warmed to room temperature. After a total of 22 h, the reaction was quenched by addition of saturated aqueous NH_4Cl (15 mL), followed by extraction with EtOAc (30 mL, 4 × 15 mL). The combined organic layers were dried, filtered, and concentrated to a yellow liquid. The crude liquid was purified by flash chromatography on silica gel [EtOAc/hexanes (1:25), 250 mL; (1:15), 200 mL; (1:10), 400 mL; 20-mL fractions; 3 × 17 cm column]. Concentration of fractions 17–23 gave 311 mg (58%) of **18** that was contaminated with a small amount of a dimeric side product. Additional purification was accomplished by flash chromatography on $AgNO_3$-impregnated silica gel [EtOAc/hexanes (1:10), 300 mL; 100-mL forefraction; 20-mL fractions; 3 × 12 cm column]. Concentration of the forefraction gave 207 mg (40%) of fairly pure **18** which was used for the formation of olefins without further purification. Concentration of fractions 1–6 gave 68.4 mg (13%) of analytically pure **18**: IR (neat) 3600–3300, 2930, 2870, 1460, 1380, 995, 920 cm^{-1}; ^{1}H NMR δ 5.91 (1 H, dd, *J* = 17.3, 10.8 Hz), 5.20 (1 H, dd, *J* = 17.3, 1.3 Hz), 5.09 (1 H, t, *J* = 7.0 Hz), 5.04 (1 H, dd, *J* = 10.8, 1.3 Hz), 1.96 (3 H, m), 1.68 (3 H, d, *J* = 1.0 Hz), 1.50 (2 H, s), 1.40 (8 H, m), 1.28 (3 H, s), 1.12 (2 H, m), 0.86 (3 H, d, *J* = 6.6 Hz); ^{13}C NMR δ 145.3 (CH), 131.0 (C), 125.0 (CH), 111.5 (CH$_2$), 73.3 (C), 42.7 (CH$_2$), 37.3 (CH$_2$), 37.1 (CH$_2$), 32.4 (CH), 27.7 (CH$_3$), 25.7 (CH$_3$), 25.5 (CH$_2$), 21.3 (CH$_2$), 19.5 (CH$_3$), 17.6 (CH$_3$); mass spectrum, *m/z* (rel intensity) 206 (M$^+$ − H_2O, 3.6), 191 (2.3), 177 (1.5), 163 (1.2), 149 (2.3), 135 (3.3), 123 (7.2), 121 (8.8), 109 (27), 95 (17), 81 (27), 71 (100), 55 (33), 41

(49); HRMS, calcd for $C_{15}H_{26}$ ($M^+ - H_2O$) 206.2034, found 206.2031.

trans-6,7-Dihydrofarnesol, THP Ether (**25**). To alcohol **14** (30.5 mg, 136 μmol) in dry CH_2Cl_2 (1 mL) under N_2 were added dihydropyran (31 μL, 340 μmol, 2.5 equiv) and pyridinium *p*-toluenesulfonate (7 mg, 28 μmol, 0.2 equiv) (Miyashita et al., 1977). The clear, colorless solution was stirred at room temperature overnight. After 25 h, the mixture was diluted with Et_2O (20 mL) and washed with half-saturated NaCl (10 mL). The aqueous layer was extracted with Et_2O (2 × 10 mL), and the combined extracts were dried, filtered, and concentrated to give a colorless oil. The oil was purified by flash chromatography on silica gel [EtOAc/hexanes (0:1), 20 mL; (1:40), 200 mL; 5-mL fractions; 1.5 × 12 cm column]. Concentration of fractions 9–12 gave 34.5 mg (82%) of **25**: IR (neat) 2930, 2860, 1450, 1375, 1115, 1025 cm^{-1}; ^1H NMR δ 5.35 (1 H, tm, J = 6.9 Hz), 5.10 (1 H, tm, J = 7.2 Hz), 4.63 (1 H, dd, J = 4.2, 2.8 Hz), 4.23 (1 H, dd, J = 11.9, 6.4 Hz), 4.03 (1 H, dd, J = 11.9, 7.4 Hz), 3.90 (1 H, m), 3.51 (1 H, m), 2.00 (2 H, t, J = 7.4 Hz), 1.95 (2 H, m), 1.84 (1 H, m), 1.73 (1 H, m), 1.68 (3 H, d, J = 1.0 Hz), 1.66 (3 H, s), 1.60 (3 H, s), 1.22–1.62 (9 H, m), 1.11 (2 H, m), 0.86 (3 H, d, J = 6.6 Hz); ^{13}C NMR δ 140.6 (C), 131.0 (C), 125.0 (CH), 120.4 (CH), 97.8 (CH), 63.6 (CH_2), 62.3 (CH_2), 39.9 (CH_2), 37.1 (CH_2), 36.6 (CH_2), 32.3 (CH), 30.7 (CH_2), 25.7 (CH_3), 25.6 (CH_2), 25.5 (CH_2), 25.0 (CH_2), 19.6 (CH_2), 19.5 (CH_3), 17.6 (CH_3), 16.3 (CH_3); mass spectrum, m/z (rel intensity) 308 (M^+, 11), 290 (9.7), 225 (43), 224 (40), 206 (54), 191 (45), 177 (43), 163 (41), 149 (48), 137 (56), 123 (54), 109 (58), 95 (67), 85 (93), 67 (82), 53 (100), 29 (77); HRMS, calcd for $C_{20}H_{36}O_2$ 308.2715, found 308.2700.

6,7-Dihydro-β-farnesene (**21**). To a solution of ether **25** (32 mg, 104 μmol) in dry THF (1.0 mL) under N_2 were added 18-crown-6 (53 mg, 201 μmol, 1.9 equiv) and potassium *tert*-butoxide (116 mg, 1.03 mmol, 10 equiv) (Otera et al., 1986). The heterogeneous mixture was refluxed gently for 1.5 h, cooled to room temperature, and then partitioned between benzene (10 mL) and H_2O (5 mL). The benzene layer was washed with additional H_2O (10 mL), and the combined aqueous layers were extracted with pentane (2 × 5 mL). The organic layers were dried, filtered, and concentrated by rotary evaporation at 0 °C to give 19.3 mg of yellow liquid. The residue was purified by chromatography on a $AgNO_3$-impregnated silica gel plate [EtOAc/hexanes (1:20); 20 × 20 cm × 250 μm]. The bands were visualized by UV, and the silica gel was scraped off and then eluted with EtOAc/hexanes (1:20). The eluant from the lower band (R_f 0.49) was concentrated to give 8.1 mg (38%) of pure olefin **21**. The eluant from the middle band (R_f 0.54) was concentrated to give an additional 6.5 mg of **21** that was ~90% pure. This material was rechromatographed as described before to give 4.0 mg (19%) of **21**: IR ($CHCl_3$) 3080, 2920, 2860, 1590, 1455, 1375, 990, 895 cm^{-1}; ^1H NMR δ 6.37 (1 H, dd, J = 17.4, 10.8 Hz), 5.23 (1 H, dd, J = 17.4, 0.6 Hz), 5.10 (1 H, tm, J = 7.2 Hz), 5.05 (1 H, d, J = 10.8 Hz), 5.00 (1 H, s), 4.98 (1 H, s), 2.18 (2 H, t, J = 7.8 Hz), 1.98 (2 H, m), 1.68 (3 H, d, J = 1.0 Hz), 1.56 (3 H, s), 1.20–1.60 (5 H, m), 1.15 (2 H, m), 0.87 (3 H, d, J = 6.5 Hz); ^{13}C NMR δ 146.6 (C), 139.0 (CH), 131.0 (C), 125.0 (CH), 115.4 (CH_2), 113.0 (CH_2), 37.2 (CH_2), 36.9 (CH_2), 32.3 (CH), 31.7 (CH_2), 25.7 (CH_3), 25.6 (CH_2), 25.6 (CH_2), 19.6 (CH_3), 17.6 (CH_3); mass spectrum, m/z (rel intensity) 206 (M^+, 16), 191 (6.6), 177 (10), 163 (21), 149 (13), 135 (20), 123 (27), 121 (35), 109 (81), 95 (69), 82 (49), 81 (73), 67 (59), 55 (65), 41 (100), 14 (65); HRMS, calcd for $C_{15}H_{26}$ 206.2031, found 206.2029.

α-cis- and α-trans-6,7-Dihydrofarnesene (**19** *and* **20**). To a solution of **18** (54.5 mg, 243 μmol) in pyridine (120 μL, 1.48 mmol, 6.1 equiv) at 70 °C under N_2 in a flask sealed with a rubber septum was added $POCl_3$ (45 μL, 483 μmol, 2.0 equiv) by syringe (Anet, 1970). After 5 min, the cloudy, viscous mixture was cooled in an ice/H_2O bath and then partitioned between H_2O (10 mL) and pentane (10 mL). A small amount of NaCl was added to aid in the separation of the layers. The pentane layer was washed with additional H_2O (2 × 10 mL), and the combined aqueous portions were extracted with pentane (10 mL). The pentane extracts were dried, filtered, and concentrated by rotary evaporation at 0 °C. The residue was purified by flash chromatography on 10% $AgNO_3$-impregnated silica gel (pentane, 150 mL; 7-mL fractions; 1.5 × 6 cm column). Concentration of fractions 2–17 gave 13.9 mg (28%) of a 34:21:45 mixture of **21**:**19**:**20**, which was characterized without separation of the isomers: IR ($CHCl_3$) 3080, 2960, 2920, 2850, 1640, 1605, 1590, 1450, 1375, 1075, 985, 895 cm^{-1}; (*α-cis*-**19**) ^1H NMR δ 6.78 (1 H, ddd, J = 17.2, 10.8, 0.8 Hz), 5.38 (1 H, tm, J = 7.4 Hz), 5.18 (1 H, d, J = 17.2 Hz) 5.10 (1 H, m), 5.08 (1 H, m), 2.16 (2 H, m), 1.97 (2 H, m), 1.81 (3 H, d, J = 1.1 Hz), 1.68 (3 H, s), 1.60 (3 H, s), 1.28–1.52 (3 H, m), 1.16 (2 H, m), 0.88 (3 H, d, J = 6.5 Hz); GC/MS, m/z (rel intensity) 206 (m^+, 21), 191 (12), 177 (6.1), 163 (16), 149 (9.2), 135 (15), 123 (25), 121 (44), 109 (97), 95 (63), 93 (53), 82 (43), 81 (100), 79 (50), 67 (49), 55 (50), 41 (78), 14 (32); GC/HRMS, calcd for $C_{15}H_{26}$ 206.2031, found 206.2039; (*α-trans*-**11**) ^1H NMR δ 6.37 (1 H, dd, J = 17.4, 10.8, Hz), 5.48 (1 H, tm, J = 7.4 Hz), 5.10 (1 H, m), 5.07 (1 H, d, J = 17.4 Hz), 4.92 (1 H, d, J = 10.8 Hz), 2.16 (2 H, m), 1.97 (2 H, m), 1.74 (3 H, d, J = 0.6 Hz), 1.68 (3 H, s), 1.60 (3 H, s), 1.28–1.52 (3 H, m), 1.16 (2 H, m), 0.89 (3 H, d, J = 6.5 Hz); GC/MS, m/z (rel intensity) 206 (M^+, 19), 191 (7.3), 177 (3.5), 163 (11), 149 (7.2), 135 (11), 123 (10), 121 (41), 109 (95), 95 (52), 93 (51), 82 (42), 81 (100), 79 (49), 69 (79), 67 (36), 55 (42), 41 (73); GC/HRMS, calcd for $C_{15}H_{26}$ 206.2031, found 206.2039.

Preparative Incubation of Ammonium trans-(7S)-[1-^3H]-6,7-Dihydrofarnesyl Pyrophosphate (**7a**) *with Trichodiene Synthase*. To a solution of **7a** (2.35 × 10^6 dpm, 14.7 nmol, specific activity 72.1 μCi/μmol, final concentration 9.8 μM; done in duplicate) in 25 mM Hepes buffer (pH 7.5) that also contained 3 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) was added trichodiene synthase (25 μL, ~0.1 μg/μL). This gave a final volume of 1.5 mL. The mixture was gently swirled, carefully overlaid with hexanes (1 mL), and then incubated at 30 °C in a Teflon-lined, screw-capped vial. A blank (no trichodiene synthase) was run as a control. After 2 h, the vials were cooled in an ice/H_2O bath, and the layers were vigorously mixed on a vortex mixer for 30 s. The extract was removed by pipet and passed through a silica gel pipet column (0.5 × 2 cm). The aqueous layer was further extracted with Et_2O (2 × 1 mL), and the extracts were passed through the pipet column. The pipet column was eluted with additional Et_2O (1 mL). An aliquot of the combined extracts was counted to determine the percent conversion to both olefinic and alcoholic products, while the entire extract of the blank was counted to determine the level of background solvolysis. Authentic olefins and alcohols (~0.5–1 mg of each component) were added, the solution was dried by passage through a small plug of $MgSO_4$, and the eluant was concentrated by rotary evaporation. The residue was taken up in hexanes (~100 μL) for analysis by radio-GLC (Table II and Figure 1).

In order to determine the product distribution of the water-soluble pyrophosphate esters, any remaining extraction solvent was removed from the aqueous layer, for both the trichodiene synthase treated solution and the blank, under a stream of N_2 while the solution was cooled to 0 °C (10–15 min). The cold solution was diluted with fresh reaction mix buffer (25 mM Hepes, 3 mM $MgCl_2$, 1 mM DTT; 0.35 mL), a solution of alkaline phosphatase (0.2 mL, 100 units) in reaction mix buffer was added, and the mixture was incubated at 30 °C. After 30 min, the mixture was cooled to 0 °C, additional alkaline phosphatase (0.2 mL, 100 units) in reaction mix buffer was added, and the mixture was again incubated at 30 °C. After a total of 2 h, the mixture was again cooled to 0 °C and then extracted with hexanes (1 mL) and Et_2O (2 × 1 mL). Separation of the layers was greatly facilitated by centrifugation at 8000*g* for 30–60 min after each extraction. The extracts were passed through the pipet column used earlier, and an aliquot of the eluant was counted to determine the amount of products produced. After the addition of authentic compounds (~0.5–1 mg of each component), the solution was dried by passage through a small plug of $MgSO_4$, and the eluant was concentrated by rotary evaporation. The residue was taken up in hexanes (~100 μL) for analysis by radio-GLC (Table II).

The product distribution from background solvolysis was determined after incubation of **7a** under the same conditions described above, except that the total volume was 15 mL (done in duplicate). After 2 h at 30 °C, the solution was worked up as before, except that the volumes of the extraction solvents were doubled. After addition of authentic carrier compounds (~0.5–1 mg of each component), the extracts were dried, filtered, and concentrated. The residue was dissolved in hexanes (~100 μL) for radio-GLC analysis (Table II).

Preparative Incubation of Ammonium trans-(7R)-[1-^3H]-6,7-Dihydrofarnesyl Pyrophosphate (**7b**) *with Trichodiene Synthase.* The incubation of enantiomerically enriched (7*R*)-**7b** (1.50 × 10^6 dpm, 14.7 nmol, specific activity 45.9 μCi/μmol) with trichodiene synthase was carried out by the same procedure used for the incubation of **7a**. The product distribution of the water-soluble pyrophosphate esters for both the trichodiene synthase treated solution and the blank (no trichodiene synthase) was determined by radio-GLC analysis after hydrolysis with alkaline phosphatase (Table II).

Preparative Incubation of Ammonium trans-(7S)-[1,1-$^2H_2$]-6,7-Dihydrofarnesyl Pyrophosphate ([*1,1-$^2H_2$*]-**7a**) *with Trichodiene Synthase.* The incubation of [1,1-$^2H_2$]-**7a** (specific activity 0.22 μCi/μmol) with trichodiene synthase was carried out in the manner described above, with the following modifications. HPLC-grade pentane (2 × 1 mL) was substituted for hexanes for extraction of the incubation solutions. The pipet column was eluted with additional pentane (1 mL). An aliquot of the pentane eluant was counted to determine the percent conversion to olefinic products, an appropriate amount of unlabeled carrier olefins (2–4 μg per component) was added, and the mixture was very carefully concentrated at 0 °C by rotary evaporation. The residue was taken up in hexanes (~25 μL) for analysis by GC/MS. The incubation solution was then extracted with HPLC-grade Et_2O (2 × 1 mL, washed with 5% $NaHSO_3$ to remove peroxides), and the extracts were passed through the pipet column. An aliquot was counted to determine the percent conversion to alcoholic products, an appropriate amount of unlabeled carrier alcohols (2–3 μg per component) was added, and the mixture was very carefully concentrated at 0 °C by rotary evaporation. The residue was taken up in hexanes (~25 μL) for analysis by GC/MS. After removal of excess extraction solvent, the remaining pyrophosphates were hydrolyzed with alkaline phosphatase as was described before. After incubation for 2 h, the solution was extracted with pentane (1 mL) and Et_2O (2 × 1 mL). The extracts were passed through the pipet column, which was eluted with additional Et_2O (1 mL). An aliquot was counted to determine the percent conversion to alcoholic products, appropriate amounts of unlabeled carrier alcohols (6–12 μg per component) were added, and the mixture was very carefully concentrated at 0 °C by rotary evaporation. The residue was taken up in hexanes (~25 μL) for analysis by GC/MS (Table III).

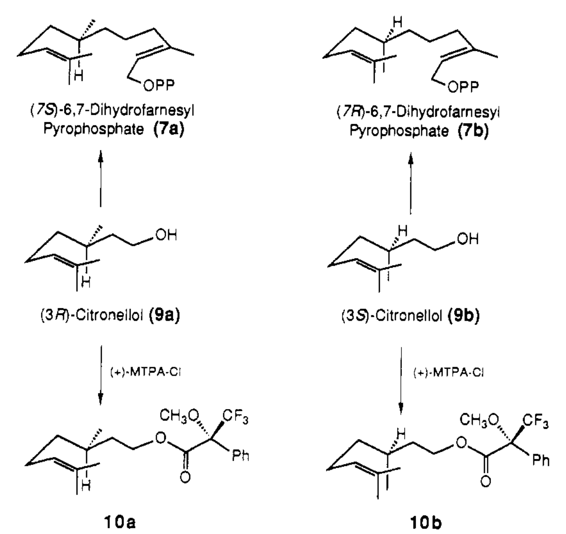
Kinetic Studies of the Conversion of **7a** *and* **7b** *to Anomalous Products by Trichodiene Synthase.* Steady-state kinetic parameters were determined for the formation of anomalous products from **7a** and **7b** by incubation of each substrate with trichodiene synthase in 25 mM Hepes, 3 mM $MgCl_2$, and 1 mM DTT in a total volume of 250 μL. The enzyme was diluted with enough ice-cold storage buffer to give 1–10% conversion of substrate during the 15-min assay (typically 1/4 dilution of stock enzyme solution) and was used within 15 min after dilution. A parallel incubation was also carried out with FPP as substrate and trichodiene synthase diluted by another factor of 50. The assays were initiated after preincubation for 5 min by addition of trichodiene synthase. After incubation for 15 min at 30 °C, the reaction was quenched by addition of EtOH (250 μL) followed by cooling to 0 °C. The products resulting from incubation of **7a** or **7b** were extracted with Et_2O (2 × 1 mL), while the trichodiene produced from FPP was extracted with hexanes (2 × 1 mL). Each extract was passed through a silica gel pipet column (0.5 × 2 cm) directly into a scintillation vial, and the pipet columns were eluted with additional extraction solvent (1 mL). Liquid scintillation counting gave the amount of products formed over the 15-min incubation time. All points were measured in duplicate, and the data were analyzed as described by standard graphical methods and by computer fitting to the hyperbolic rate expression using the KINFIT program. The instability of the enzyme under the assay conditions lead to some scatter in the data for low substrate concentrations (Table IV).

RESULTS

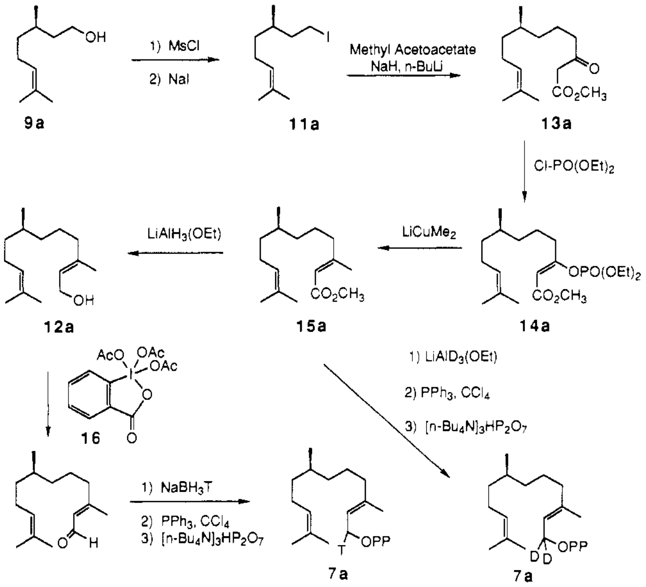
Preparation of Anomalous Substrates and Products. Although Washburn (Washburn & Kow, 1977) had previously described a preparation of racemic *trans*-6,7-dihydro-FPP (**7**), we required samples of the individual enantiomers of **7**. We therefore devised a new synthesis of (7*S*)-**7a** and (7*R*)-**7b**, using as starting materials (+)-(3*R*)- and (−)-(3*S*)-citronellol (**9a**) and (**9b**), respectively. The enantiomeric purity of commercially available samples of citronellol was first established by formation of the corresponding Mosher's ester **10**, followed by ^1H NMR analysis in the presence of the shift reagent $Eu(fod)_3$ (Dale et al., 1969) (Scheme IV). By integration of the methyl resonances of the individual diastereomers, (−)-(3*S*)-citronellol (**9b**) was found to consist of a mixture of 82% (3*S*)- and 18% (3*R*)-**9** (64% ee), whereas "(+)-(3*R*)-citronellol" was shown to be essentially racemic (<1% ee). The required (+)-(3*R*)-citronellol (**9a**) (≥98% ee) was therefore prepared from (*R*)-pulegone according to the literature procedure (Plesek, 1957).

Treatment of **9a** with mesyl chloride followed by displacement with sodium iodide gave (−)-citronellyl iodide (**11a**) in 79% yield (Scheme V). The latter intermediate was efficiently converted to (−)-*trans*-6,7-dihydrofarnesol (**12a**) by using the homologation sequence developed by Weiler (Sum & Weiler, 1979). Thus reaction of (−)-citronellyl iodide (**11a**) with the dianion of methyl acetoacetate gave ester (−)-**13a**, which was reacted with diethyl chlorophosphate to give the enol phos-

Scheme IV



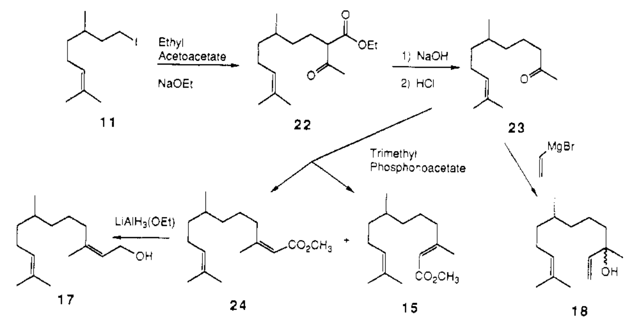
Scheme V



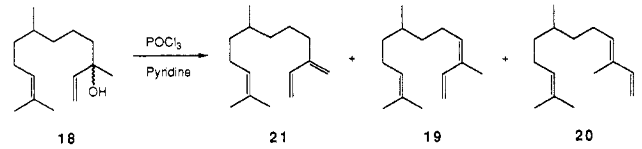
phonate (−)-**14a** in 42% yield for the two steps. Conjugate addition of lithium dimethyl cuprate gave (−)-methyl *trans*-6,7-dihydrofarnesoate (**15a**) which was reduced with LiAlH$_3$(OEt) to give (−)-**12a** in 53% overall yield. The synthesis of (+)-**12b** as well as racemic (±)-**12** was carried out in essentially the same manner.

The allylic alcohols were each labeled at C-1 with tritium by oxidation to the aldehyde and reduction with NaBH$_3$T. Thus treatment of (−)-**12a** with the Dess–Martin periodinane reagent **16** (Dess & Martin, 1983) followed by reaction with [^{3}H]NaBH$_4$ gave (−)-[1-^{3}H]-*trans*-6,7-dihydrofarnesol (**12a**). The resulting labeled alcohol was then converted to the pyrophosphate ester by a modification of the method of Poulter (Davisson et al., 1986a,b). Accordingly, reaction of [1-^{3}H]-**12a** with triphenylphosphine in refluxing CCl$_4$ gave the corresponding allylic chloride which was displaced with tris(tetrabutylammonium) pyrophosphate to give the desired pyrophosphate ester [1-^{3}H]-**7a**. The crude reaction product was treated with yeast inorganic pyrophosphatase in order to remove contaminating inorganic pyrophosphate, and the resulting mixture was chromatographed on DEAE-Sephadex to give **7a** in 26% overall yield from (−)-**12a**. The corresponding samples of (7*R*)-[1-^{3}H]-*trans*-6,7-dihydro-FPP (**7b**) and (7*RS*)-[1-^{3}H]-*trans*-6,7-dihydro-FPP (**7**) were prepared in like manner, the inorganic pyrophosphatase being removed by ultrafiltration. To prepare [1-^{2}H$_2$]-*trans*-6,7-dihydro-FPP ([1-^{2}H$_2$]-**7a**), (−)-**15a** was reduced with LiAlD$_3$(OEt) to give [1-^{2}H$_2$]-**12a**, which was pyrophosphorylated in the usual manner. The chemical and stereochemical purity of each of the resulting allylic pyrophosphate esters was confirmed by ^{1}H and ^{31}P NMR analysis.

Scheme VI



Scheme VII



Although trichodiene synthase is in principle capable of catalyzing the allylic isomerization of 6,7-dihydro-FPP, enzymatic solvolysis of the resulting 6,7-dihydronerolidyl pyrophosphate would be expected to generate a variety of abortive products resulting from deprotonation of the intermediate ion pair or quenching with water. Preliminary incubations of **7a** with trichodiene synthase indicated that the anomalous substrate was indeed converted to a mixture of olefins and alcohols. In order to confirm the structure of each of the reaction products, we required authentic samples of the isomeric alcohols **17** and **18**, as well as the α- and β-dihydrofarnesenes **19**–**21**.

Treatment of racemic citronellyl iodide (**11**) with the monoanion of ethyl acetoacetate gave the β-keto ester **22** (Scheme VI). Alkaline hydrolysis of **22** followed by acidification effected smooth decarboxylation to yield 2,3-dihydrogeranyl acetone (**23**) in 65% overall yield from **11** (Azerad & Cyrot, 1965). Wittig reaction of **23** with trimethyl phosphonoacetate gave a mixture of esters which were separated by chromatography to give the previously prepared **15** in 35% yield and the corresponding cis isomer **24** in 20% yield. Reduction of **24** with LiAlH$_3$(OEt) gave *cis*-6,7-dihydrofarnesol (**17**) in 86% yield. To prepare 6,7-dihydronerolidol (**18**), 2,3-dihydrogeranyl acetone (**23**) was reacted with vinylmagnesium bromide to give the desired tertiary allylic alcohol in 53% yield as an inseparable mixture of diastereomers.

The 6,7-dihydronerolidol was also used to prepare the required conjugated dienes **19**–**21**. Treatment of **18** with POCl$_3$ in pyridine produced a 3.5:2.0:4.5 mixture of the three hydrocarbons **19**–**21** in 27% yield (Scheme VII). The stereochemical assignments for the individual α-isomers were based on analysis of the high-field ^{1}H NMR spectrum and comparison with the chemical shifts and coupling constants of the previously described α-farnesenes (Anet, 1970). Thus the H-2 proton in the cis isomer, (3*Z*)-dihydro-α-farnesene (**19**), appeared as a doublet of doublet of doublets at δ 6.78 (J = 17.0, 10.8, and 0.8 Hz) compared to chemical shifts of δ 6.74 and 6.77 reported for the corresponding protons in (3*Z*,6*E*)- and (3*Z*,6*Z*)-α-farnesene, respectively. By contrast, the H-2 proton

Scheme VIII

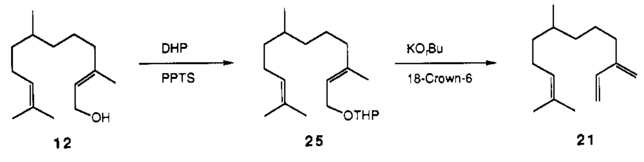


Table I: Inhibition of Trichodiene Synthase

inhibitor	K_i (nM)	inhibitor	K_i (nM)
(7*S*)-**7a**	395 ± 40	(7*RS*)-**7**	265 ± 30
(7*R*)-**7b**[a]	235 ± 35	PP_i	495 ± 20
(7*R*)-**7b**[b]	220 ± 40[b]		

[a] (7*R*)-**7b**, 64% ee. [b] (7*R*)-**7b**, 100% ee, calculated value.

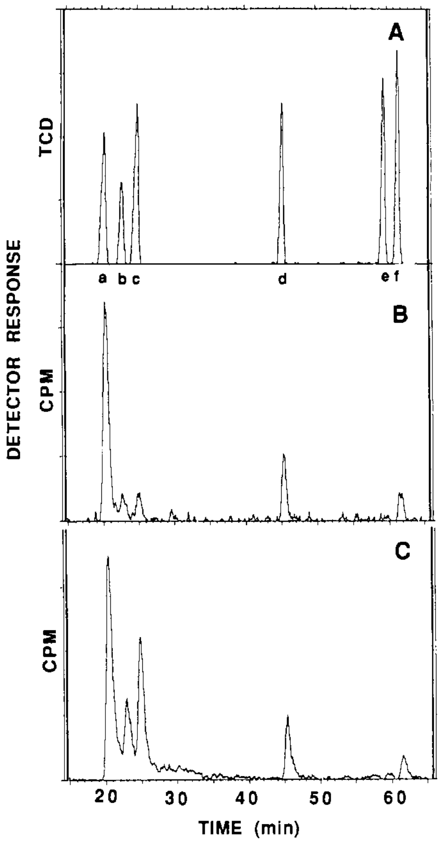


FIGURE 1: Radio-GLC analysis of products derived from incubation of dihydro-FPP enantiomers with trichodiene synthase. (A) Thermal conductivity profile of authentic standards: (a) dihydro-β-farnesene (**21**); (b) *cis*-dihydro-α-farnesene (**19**); (c) *trans*-dihydro-α-farnesene (**20**); (d) dihydronerolidol (**18**); (e) *cis*-dihydrofarnesol (**17**); (f) *trans*-dihydrofarnesol (**12**). (B) Radiodetector profile of products generated by incubation of (7*S*)-dihydro-FPP (**7a**). (C) Radiodetector profile of products generated by incubation of (7*R*)-dihydro-FPP (**7b**). See Experimental Procedures for description of incubation conditions and radio-GLC analysis.

in the trans isomer, (3*E*)-dihydro-α-farnesene (**20**), appeared as a doublet of doublets at δ 6.37 (J = 17.0 and 10.8 Hz) consistent with the chemical shifts of δ 6.30 and 6.35 corresponding to H-2 in (3*E*,6*E*)- and (3*E*,6*Z*)-α-farnesene. For most purposes the olefin mixture could be used directly, although the individual isomers could be readily separated by capillary GLC and characterized by GC/MS analysis. Alternatively, 6,7-dihydro-β-farnesene (**21**) could be obtained in 57% yield by conversion of *trans*-6,7-dihydrofarnesol to the corresponding tetrahydropyranyl ether **25**, which underwent elimination upon exposure to potassium *tert*-butoxide and 18-crown-6 in refluxing THF (Otera et al., 1986) (Scheme VIII).

Kinetic Studies. With samples of both enantiomers of *trans*-6,7-dihydro-FPP (**7**) in hand, we first examined the ability of these anomalous substrates to bind to the active site of trichodiene synthase, as measured by their inhibitory effect on the normal cyclization reaction. Increasing concentrations of each substrate analogue were therefore incubated with trichodiene synthase over a range of FPP concentrations. Analysis of the resulting data by double-reciprocal (Lineweaver–Burk) plots indicated that both **7a** and **7b** were modest competitive inhibitors of the cyclase. The actual steady-state kinetic parameters for each analogue were obtained by directly fitting the data to the relevant expression for competitive inhibition (see Experimental Procedures), giving a K_i of 395 ± 40 nM for (7*S*)-*trans*-6,7-dihydro-FPP (**7a**) and a K_i of 235 ± 35 nM for (7*R*)-**7b** (Table I). On the basis of the the known enantiomeric composition of **7b** (64% ee 7*R*), the intrinsic K_i of (7*R*)-*trans*-6,7-dihydro-FPP was calculated to be 220 ± 40 nM.[4] As expected, racemic (±)-**7** showed an intermediate K_i = 265 ± 30 nM, consistent with a predicted K_i of 285 ± 35 nM calculated from the inhibition constants of the individual enantiomers. By contrast, the K_i determined for inorganic pyrophosphate alone was 495 ± 20 nM compared to K_m of 25 ± 2 nM for the natural substrate, FPP (**1**).

Product Studies. Preliminary experiments indicated that trichodiene synthase would convert either enantiomer of 6,7-dihydro-FPP to a mixture of hydrocarbons and alcohols, as judged by chromatographic mobility on silica gel. We therefore carried out a preparative-scale incubation in which 14.7 nmol (2.35 × 10^6 dpm, 72.1 μCi/μmol) of (7*S*)-6,7-dihydro-FPP (**7a**) was incubated with 2.5 mg of trichodiene synthase for 2 h at 30 °C. The incubation mixture was extracted with successive portions of hexane and ether, following which 0.5–1-mg samples of synthetic olefins **19**–**21** and alcohols **12**, **17**, and **18** were added to the organic extract as internal standards. Analysis of the resulting mixture by radio-GLC showed the products to consist of more than 80% acyclic olefins, among which the major component was identified as dihydro-β-farnesene (**21**) (65%), along with the corresponding *cis*- and *trans*-6,7-dihydro-α-farnesenes (**19** and **20**) (8% each) (Table II and Figure 1). The alcoholic constituents consisted of 6,7-dihydronerolidol (**18**) (14%) as well as *trans*-6,7-dihydrofarnesol (**12**) (5%). On the other hand, none of the isomeric *cis*-6,7-dihydrofarnesol (**17**) could be detected among the abortive reaction products. The water-soluble products of the incubation were determined by treatment of the ether-extracted aqueous phase with alkaline phosphatase and detection of the liberated alcohols by radio-GLC analysis of the corresponding ether extract after addition of authentic allylic alcohols as internal standards. The derived allylic alcohols consisted of a 76:24 mixture of *trans*-6,7-dihydrofarnesol (**12**) and *cis*-6,7-dihydrofarnesol (**17**), indicating that considerable enzyme-catalyzed isomerization of the double bond had occurred under the conditions of the incubation. Interestingly, none of the tertiary allylic alcohol **18** which would have been derived from 6,7-dihydronerolidyl pyrophosphate (**8**) was detected. A control experiment, in which the anomalous substrate **7a** was incubated with alkaline phosphatase, confirmed the stereochemical integrity of the starting material (99% trans). We also carried out blank incubations in the absence of synthase which in-

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[4] For a mixture of enantiomers, the observed inhibition constant K_i(obsd) is given by the equation: $1/K_i(\text{obsd}) = x/K_i(R) + y/K_i(S)$, where x is the mole fraction of the *R*-enantiomer, y is the mole fraction of the *S*-enantiomer, $K_i(R)$ is the inherent inhibition constant of the *R*-enantiomer, and $K_i(S)$ is the inherent inhibition constant of the *S*-enantiomer.

Table II: Distribution of Products from Incubation of Anomalous Substrates with Trichodiene Synthase[a]

		product distribution (%)[b]						
substrate	conditions[c]	**21**	**19**	**20**	**18**	**17**	**12**	total conversion (%)
(7*S*)-**7a** (9.8 μM)	TS	65	8	8	14	<1	5	18
	TS + APase	<1	<1	<1	<1	24	76	75
	APase	<1	<1	<1	<1	1	99	89
	control	4	1	2	66	5	22	0.5
(7*S*)-**7a** (9.8 μM)	TS	44	14	28	10	<1	4	19
	TS + APase	<1	<1	<1	<1	10	90	59
	APase	<1	<1	<1	<1	8	92	77

[a] Incubation for 2 h at 30 °C as described under Experimental Procedures. [b] Determined by radio-GLC analysis. [c] Abbreviations: TS, trichodiene synthase; TS + APase, trichodiene synthase followed by alkaline phosphatase treatment of water-soluble products; APase, alkaline phosphatase hydrolysis of substrate; control, incubation in the absence of enzyme.

Table III: Incubation of **7a** with Trichodiene Synthase and Analysis of Products by GC/Selected Ion Mass Spectrometry

		P/(*P* + 2) intensity ratios					
conditions	*P*/(*P* + 2)	**21**	**19**	**20**	**18**	**17**	**12**
TS[a]	224/226	–[b]	–	–	–	ND[c]	13
	206/208	15	(78)	(108)	7	ND	11
	191/193	12	(73)	(162)	8	ND	3
TS + APase	224/226	ND	ND	ND	ND	11	2
	206/208	ND	ND	ND	ND	7	2
	191/193	ND	ND	ND	ND	4	2

[a] Olefinic and alcholic products analyzed separately, as described under Experimental Procedures. Abbreviations: TS, trichodiene synthase; TS + APase, trichodiene synthase followed by alkaline phosphatase treatment of water-soluble products. [b] No peak detected. [c] ND, not determined.

Table IV: Comparison of FPP (**1**), **7a**, and **7b** as Substrates for Trichodiene Synthase

substrate	V_{rel}	K_m (nM)
FPP (**1**)	100	14 ± 2[a]
(7*S*)-**7a**	10	440 ± 250
(7*R*)-**7b**	11	770 ± 125

[a] This value is based on only five data points. The K_m for FPP was shown to be 25 ± 2 nM in a separate experiment involving a larger number of data points.

dicated that competing, non-enzyme-catalyzed solvolysis was negligible (3%). The products of an analogous nonenzymatic solvolysis reaction carried out on 10 times the normal scale were analyzed by radio-GC and shown to consist predominantly (93%) of a mixture of allylic alcohols, in which dihydronerolidol and *trans*-dihydrofarnesol were present in a 3:1 ratio, accompanied by small quantities of the corresponding olefinic hydrocarbons.

Preparative-scale incubation of (7*R*)-6,7-dihydro-FPP (**7b**) (64% ee) with trichodiene synthase gave the results summarized in Table II and illustrated in Figure 1. The distribution of products was qualitatively very similar to that obtained upon incubation of the pure enantiomer **7a**. Once again the major products of the enzyme-catalyzed reaction were olefinic, with alcoholic products comprising only 14% of the mixture. By contrast, in the hydrocarbon fraction the proportion of dihydro-β-farnesene (**21**) had decreased (44%), while that of *trans*-α-farnesene **20** had risen to 28%. No *cis*-6,7-dihydrofarnesol (**17**) could be detected. Control experiments confirmed that background solvolysis could account for less than 3% of the products observed. Although analysis of the water-soluble products once again indicated the presence of *cis*-6,7-dihydro-FPP (**26**) (10%), this result must be viewed with caution since alkaline phosphatase treatment of **7b** at the time of the incubation indicated contamination with as much as 8% of the cis isomer. (The latter isomer had been shown not to be a contaminant of the sample of **7b** used in the determination of competitive inhibition parameters described above.)

The identities of the enzymatically generated solvolysis products were confirmed by the use of GC/selected ion MS. For this purpose, (7*S*)-6,7-dihydro-FPP, doubly labeled with deuterium at C-1 ([1-$^2H_2$]-**12a**), was incubated with trichodiene synthase in the usual manner. At the end of the 2-h incubation period, authentic samples of the olefins **19–21**, and allylic alcohols **12**, **17**, and **18**, were added to the separated hydrocarbon and alcoholic fractions, respectively. In similar fashion, the allylic alcohol standards were also added to the extracts obtained after alkaline phosphatase catalyzed hydrolysis of the aqueous products of the trichodiene synthase incubation mixture. The amounts of added carrier were estimated so as to correspond to molar ratios of carrier to anticipated product between 10:1 and 100:1. The *P*/(*P* + 2) ratio was measured for selected ions of each GC component. Thus for the olefins **19–21**, the molecular ions M^+ (206 and 208) and ($M^+ - CH_3$) (191 and 193) were measured, while for the alcohols **12**, **17**, and **18** the parent ion M^+ (224 and 226), as well as the fragments ($M^+ - H_2O$) (206/208) and ($M^+ - H_2O - CH_3$) (191/193), was analyzed. (No parent ion was detectable for the tertiary allylic isomer **18**.) As summarized in Table III, the isotopic content of the observed parent and fragment ions, as measured by the *P*/(*P* + 2) ratios, were in reasonable agreement for the three allylic alcohols as well as for dihydro-β-farnesene (**21**). Unfortunately, the relatively small proportion of the presumed *cis*- and *trans*-dihydro-α-farnesene components (**19** and **20**) produced in this experiment made precise measurement of the intensities of the (*P* + 2) peaks difficult,[5] and the identification of these latter minor components must rest primarily on the radio-GLC results, consistent with literature precedent (Wheeler & Croteau, 1986; Rittersdorf & Cramer, 1967).

Kinetics of Anomalous Product Formation. The steady-state kinetic parameters for the conversion of **7a** and **7b** to anomalous products were determined by incubation of varying amounts of each substrate with an amount of trichodiene synthase sufficient to give 1–10% turnover during the 15-min assay period. As a control, parallel incubations were also

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[5] The observed *P*/(*P* + 2) ratios are uncorrected for the contribution due to isotopic natural abundance for which the expected values of *P*/(*P* + 2) are 67 (*m*/*z* 124/226), 78 (*m*/*z* 204/206), and 90 (*m*/*z* 191/193) (Beynon & Williams, 1963).

carried out with the natural substrate, FPP, using $^1/_{50}$ the concentration of trichodiene synthase. The V_{rel} for both (7*S*)- and (7*R*)-dihydro-FPP (**7a** and **7b**) were essentially equal, corresponding to roughly 10% the V_{max} measured for FPP (Table IV). The calculated K_m values for **7a** and **7b** were considerably less precise, but were within a factor of 1–3 times the corresponding K_i values determined for each enantiomer.

DISCUSSION

Trichodiene synthase is one of a large class of terpenoid cyclases which catalyze the conversion of the acyclic precursors farnesyl pyrophosphate (**1**) and geranyl pyrophosphate (**6**) to six-membered ring-containing sesquiterpenes and monoterpenes, respectively (Cane, 1981, 1985; Croteau, 1981, 1987). Extensive mechanistic and stereochemical evidence now supports the proposal that the first step in these cyclizations is the isomerization of the primary trans-allylic pyrophosphate substrate to the corresponding tertiary allylic isomer, nerolidyl or linalyl pyrophosphate (**3** and **5**). There is also good evidence that both the isomerization and cyclization events take place at the same active site and by closely related mechanisms involving the corresponding transoid or cisoid allylic cation–pyrophosphate ion pairs. Unfortunately, direct observation of the isomerization step has not been possible since the tertiary allylic pyrophosphate intermediate is never released from the active site of the cyclase.

To address this problem, we chose to study the action of trichodiene synthase on the substrate analogue 6,7-dihydrofarnesyl pyrophosphate (**7**). It was expected that although one or both enantiomers of **7** could in principle undergo enzyme-catalyzed isomerization to the corresponding tertiary allylic pyrophosphate ester **8**, cyclization by the normal pathway would be prevented by the absence of the 6,7-double bond normally present in the natural substrate FPP. Croteau has reported similar experiments using the geranyl pyrophosphate analogue 6,7-dihydrogeranyl pyrophosphate (Wheeler & Croteau, 1986). In the presence of a mixture of (+)-α-pinene synthase and (+)-bornyl pyrophosphate synthase obtained from extracts of sage (*Salvia officinalis*), dihydrogeranyl pyrophosphate was converted to a 55:45 mixture of acyclic dienes (6,7-dihydromyrcene, -*cis*-ocimene, and -*trans*-ocimene) and allylic alcohols (6,7-dihydrogeraniol and -linalool, as well as small amounts of the cis isomer, 6,7-dihydronerol).[6] The observed product mixtures were in fact very similar to those produced when either enantiomer of **7** was incubated with trichodiene synthase, with the exception that in neither of the latter cases was any of the corresponding cis isomer, (2*Z*)-6,7-dihydrofarnesol (**17**), detected. Examination of the polar products resulting from incubation of (7*S*)-6,7-dihydro-FPP (**7a**) with trichodiene synthase did reveal, however, that significant amounts of *cis*-6,7-dihydro-FPP (**26**) had been generated. Although **26** was also detected among the products of the incubation of the enantiomeric analogue (7*R*)-6,7-dihydro-FPP (**7b**), the excess over the blank was too small to interpret with any confidence. Unfortunately, in neither case was any of the tertiary allylic pyrophosphate, 6,7-dihydronerolidyl pyrophosphate (**8**), directly observed. Similar observations have been reported by Croteau, who was unable to detect any dihydrolinalyl pyrophosphate among the products resulting from the enzymatic isomerization of dihydrogeranyl pyrophosphate.

The binding of dihydro-FPP to the active site of trichodiene synthase was supported by the fact that both enantiomers, **7a** and **7b**, acted as modest competitive inhibitors of the cyclization of FPP to trichodiene. The 7*R*-isomer, **7b**, was only slightly more tightly bound than was **7a**, with the observed K_i values a factor of 8–16 higher than the corresponding K_m for FPP itself. These values were comparable in magnitude with the corresponding K_m values determined for the trichodiene synthase catalyzed solvolysis of either **7a** or **7b**. A substantial portion of the binding of the anomalous substrate must in fact be due to the polar pyrophosphate moiety, since the measured K_i for inorganic pyrophosphate alone was ca. 500 nM, that is 20 times the K_m for FPP.[7] The intermediate values determined for the dihydro-FPP analogues indicate that saturation of the central double bond of the substrate has a negative effect on the overall binding affinity in comparison with FPP. Analogous results have been reported by Croteau, who found that the K_i/K_m ratio of dihydrogeranyl to geranyl pyrophosphate for pinene and bornyl pyrophosphate synthases ranged from 35:1 to 55:1, with the apparent K_m for turnover of the anomalous substrate somewhat lower than the measured K_i.

These results are readily understood in terms of the isomerization–cyclization model for the conversion of FPP to trichodiene. Binding and isomerization of either **7a** or **7b** is expected to generate the tertiary allylic isomer **8**. Kinetic studies of both monoterpene and sesquiterpene cyclizations are consistent with this step being rate-limiting in the processing of the normal substrates geranyl pyrophosphate or FPP. Rotation about the newly generated 2,3-single bond in **8** followed by ionization to the corresponding cisoid allylic cation–pyrophosphate ion pair cannot lead to cyclized product, however, due to the absence of the central double bond. Instead, the intermediate cation must undergo either deprotonation or capture by a nucleophile. The predominant formation of olefinic products is consistent with the expected exclusion of water from the cyclase active site which under normal circumstances must be protected against premature quenching of the cationic intermediates. By contrast, background solvolysis, which accounts for no more than 3% of the observed products, leads almost exclusively to the generation of a mixture of allylic alcohols consisting primarily of dihydronerolidol (**18**) and *trans*-dihydrofarnesol (**12**) in a 3:1 ratio.[8] Similarly, Rittersdorf and Cramer (1967) have reported that acid-catalyzed solvolysis of 6,7-dihydrogeranyl pyrophosphate results in formation of a 4:1 mixture of dihydrolinalool and dihydrogeraniol, with less than 2% total olefinic products, among which were dihydromycrene and the dihydroocimene isomers. The allylic alcohol products resulting from the incubation of **7a** or **7b** with trichodiene synthase also consisted of a (2–3):1 mixture of tertiary to primary isomers, **18** and **12**. These cannot have arisen from simple enzyme-catalyzed hydrolysis of the corresponding pyrophosphate esters since the homogeneous preparation of trichodiene synthase was free of contaminating phosphohydrolase activity. It is evident therefore that both the primary and tertiary allylic alcohol products, **12** and **18**, must have been formed by capture of the abortively generated allylic cation by adventitious water at the active site of the cyclase. At the moment, the configuration

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[6] Croteau has also found that incubation of 6,7-dihydrogeranyl pyrophosphate with sabinene hydrate synthase from sweet marjoram (*Majorana hortensis*) leads exclusively to the formation of 6,7-dihydromyrcene (Hallahan & Croteau, 1989).

[7] Since tris(tetra-*n*-butylammonium) pyrophosphate was used in the preparation of **7a** and **7b**, treatment with inorganic pyrophosphatase was introduced into the purification procedure in order to avoid contamination of each anomalous substrate/inhibitor with inorganic pyrophosphate.

[8] The observed background solvolysis is most likely catalyzed by Mg^{2+}, which has been shown to catalyze the solvolysis of allylic pyrophosphates (Vial et al., 1981).

at C-3 of the enzymatically generated **18** is unknown. The alternative fate of the allylic cation–pyrophosphate ion pair would be to collapse to the corresponding pyrophosphate esters which might then be released into the medium. Indeed, incubation of **7a** with trichodiene synthase did lead to formation of significant quantities of *cis*-6,7-dihydro-FPP (**26**). In fact, the rate of formation of **26** was approximately equal to the rate of production of total olefinic and alcoholic products, on the basis of the product distribution data summarized in Table II. Moreover, **26** could only have been produced by collapse of the cisoid conformer of the ion pair, which itself would be formed *only through the intermediacy of the tertiary allylic pyrophosphate* **8**. The absence of **26** in quantities meaningfully above the blank after incubation of the 7*R*-isomer **7b** may result from differences in the precise geometry of binding of the two enantiomers or may simply reflect variations in the precise incubation conditions used for the two experiments. The absence of *cis*-dihydrofarnesol (**17**) in the products of either incubation may indicate either that **26**, once formed, is never rebound by the enzyme or that the cisoid conformer of the allylic cation is completely shielded from attack by exogenous water.

The use of appropriately designed substrate analogues can serve as a powerful tool for the elucidation of the details of multistep enzyme reactions. By selectively stabilizing key intermediates or preventing otherwise facile reactions, it may be possible to divert the normal enzyme-catalyzed process so as to generate abortive reaction products whose structures reflect the existence of otherwise undetectable intermediates. Extension of these ideas to the investigation of later stages of terpenoid cyclizations, as well as the eventual development of active-site-directed inhibitors, is under continuing study.

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