Trichodiene Synthase. Stereochemical Studies of the Cryptic Allylic Diphosphate Isomerase Activity Using an Anomalous Substrate

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Incubation of (7*S*)-6,7-dihydrofarnesyl diphosphate with trichodiene synthase gave, as previously described, a mixture consisting of 80% dihydrofarnesene isomers **6**–**8** accompanied by 6% *trans*-6,7-dihydrofarnesol (**10**) and 13% 6,7-dihydronerolidol (**9**). The latter product has now been shown to be (3*S*,7*S*)-6,7-dihydronerolidol [(3*S*,7*S*)-**9**] by direct comparison with synthetic (3*S*,7*S*)-6,7-dihydronerolidol and (3*RS*,7*S*)-6,7-dihydronerolidol using chiral capillary gas chromatography. The observed formation of (3*S*,7*S*)-**9** can be accounted for by backside attack of water on an allylic cation–pyrophosphate anion pair generated by syn allylic rearrangement of dihydrofarnesyl diphosphate (**5**) and ionization of the resulting (3*R*,7*S*)-6,7-dihydronerolidyl diphosphate (**14**). These results provide further support for the proposed isomerization of farnesyl diphosphate (**1**) to (3*R*)-nerolidyl diphosphate (**3**) in the enzymatic formation of trichodiene (**2**).

Trichodiene synthase catalyzes the cyclization of farnesyl diphosphate (**1**) (FPP) to the sesquiterpene trichodiene (**2**), the parent hydrocarbon of the trichothecane family of fungal antibiotics and mycotoxins.[1] The enzyme was first isolated from the apple mold fungus *Trichothecium roseum*,[1,2] the producer of the antibiotic trichothecin, and subsequently from *Fusarium sambucinum* (*Gibberella pulicaris*)[3] and *Fusarium sporotrichioides*,[4] the producers of the potent mycotoxins diacetoxyscirpenol and T-2 toxin, respectively. The cyclase from the latter organism has been purified to homogeneity and shown to be a homodimer of subunit M_r 45000.[4] The corresponding structural gene was cloned and shown to encode a protein of M_D 43999.[5] More recently, we have expressed recombinant trichodiene synthase as 25–30% of soluble protein in *Escherichia coli*.[6] In the meantime, homologous trichodiene synthase genes have been characterized from the trichothecane producers *G. pulicaris*,[7] *Myrrothecium roridum*,[8] and *Gibberella zeae*.[8]

Extensive studies of both the native and recombinant enzymes have established many of the mechanistic and stereochemical details of the cyclization of *trans,trans*-FPP to trichodiene[9] (Scheme 1). According to the proposed scheme, the substrate *trans,trans*-FPP (**1**) undergoes initial syn isomerization to the tertiary allylic isomer, nerolidyl diphosphate (**3**) (NPP). Rotation about the 2,3-single bond, allows NPP to adopt a conformation that, upon ionization of the pyrophosphate moiety and backside capture of the resultant cisoid allylic cation–pyrophosphate anion pair by the neighboring 6,7-double bond, undergoes cyclization to the corresponding bisabolyl cation (**4**). The latter intermediate then undergoes conversion to trichodiene by a well-documented sequence involving cyclization, hydride shift, consecutive methyl migrations, and deprotonation. In support of this mechanistic proposal is the observation that (3*R*)-NPP can act as a kinetically competent substrate for trichodiene synthase.[9b] On the other hand, competition experiments have established that cyclization of FPP to trichodiene takes place without any release of detectable free intermediates,[9b] including NPP, an observation consistent with the behavior of all other known sesquiterpene[10] and monoterpene synthases.[11]

Since (3*R*)-NPP is not released from the enzyme active site prior to the cyclization, direct observation of the inherent isomerase activity of trichodiene synthase has not been possible. One approach that we have used to address this problem has been the use of (7*S*)- and (7*R*)-6,7-dihydrofarnesyl diphosphate as inhibitors and anomalous substrates of trichodiene synthase.[9d] In principle, the enzyme should be able to isomerize each of these substrate analogs to the corresponding tertiary allylic diphosphate, 6,7-dihydronerolidyl diphosphate, but further cyclization would be blocked due to the absence of normal 6,7-double bond. Indeed, analysis of the abortive products has provided details of this otherwise cryptic process.

In previously reported studies, incubation of both (7*S*)- and (7*R*)-*trans*-6,7-dihydrofarnesyl diphosphate ((7*S*)-DHFPP (**5**) and (7*R*)-DHFPP) with phosphatase-free, homogeneous, native trichodiene synthase from *F. sporotrichioides* was shown to give a mixture of products consisting of 80–85% isomeric olefins (**6**–**8**) and 15–20% of the allylic alcohols **9** (10–14%) and **10** (4–5%)[9d] (Scheme 2). The configuration of the tertiary allylic alcohol **9** was not determined. No *cis*-dihydrofarnesol (**11**) was detected in either incubation. The water-soluble

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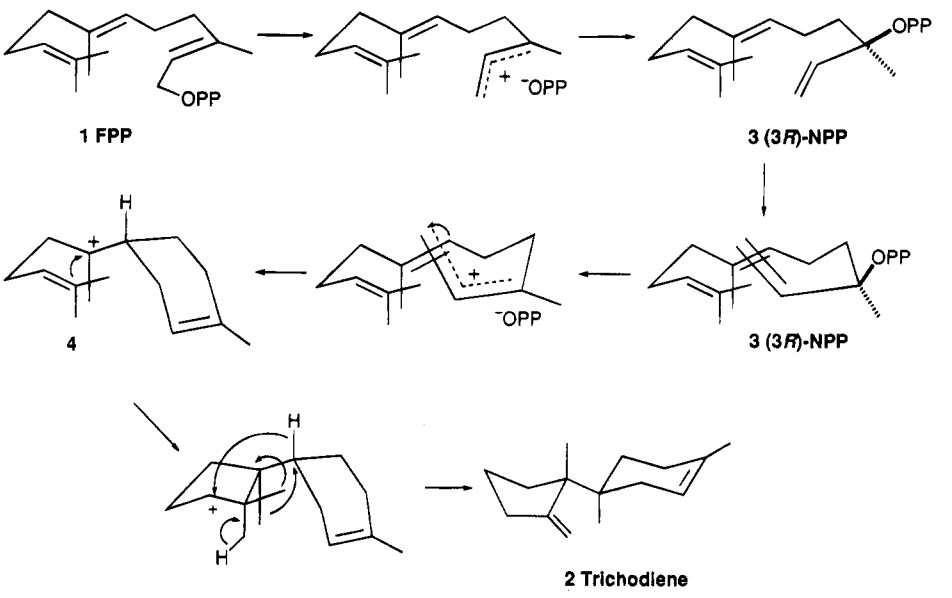
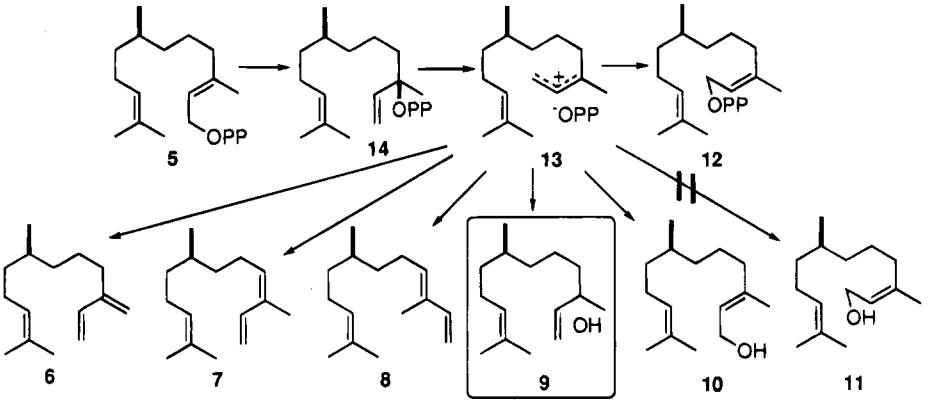
Scheme 1



Scheme 2



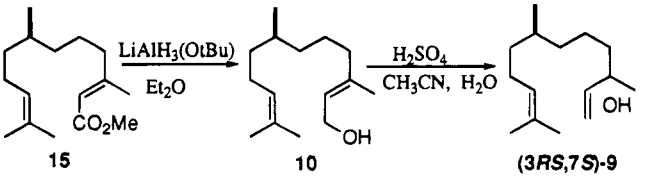
products resulting from the incubation of (7*S*)-DHFPP (**5**) contained 24% of the isomeric (7*S*)-*cis*-6,7-dihydrofarnesyl diphosphate (**12**) which could be formed through capture of the pyrophosphate moiety at C-1 by the cisoid ion-pair **13**. By contrast, incubation of (7*R*)-DHFPP with trichodiene synthase did not give any *cis*-6,7-dihydrofarnesyl diphosphate, perhaps due to the configuration at C-7 and the precise geometry of binding. Notably, neither (7*R*)- nor (7*S*)-DHFPP gave detectable 6,7-dihydronerolidyl diphosphate (**14**), the presumed intermediate in the formation of the various olefinic, alcoholic, and water-soluble products.

Although **14** could not be isolated, the corresponding alcohol, 7*S*-6,7-dihydronerolidol (**9**), was generated by trichodiene synthase as 10–15% of the total product mixture. In the original study, the diastereomeric purity and configuration at C-3 of **9** were not determined. Since this information might shed light on the formation of dihydronerolidyl diphosphate and the manner in which it is quenched by water, we set out to establish unambiguously the complete stereochemistry of **9**.

Results

In order to establish the stereochemical course of the enzymatic isomerization of (7*S*)-6,7-dihydroFPP (**5**) to 6,7-dihydronerolidol by trichodiene synthase, we required reference samples of both (3*RS*,7*S*)- and (3*S*,7*S*)-6,7-dihydronerolidol ((3*RS*,7*S*)-**9** and (3*S*,7*S*)-**9**, respectively). The requisite (3*RS*,7*S*)-**9** was prepared from (−)-methyl *trans*-(7*S*)-6,7-dihydrofarnesoate (**15**) as previously described[9d] (Scheme 3). Thus reduction of **15** with lithium *tert*-butoxyaluminum hydride gave the alcohol **10** which was exposed to sulfuric acid in aqueous acetonitrile at room temperature to give (3*RS*,7*S*)-**9** as a mixture of two diastereomers which were readily resolved by capillary GC on a chiral Cyclodex B column.

Scheme 3



Enantiomerically pure (3*S*,7*S*)-6,7-dihydronerolidol ((3*S*,7*S*)-**9**) was prepared from (3*R*)-isoprene oxide (**16**) by a route based on the synthesis of (*S*)-(+)-linalool developed by Eliel.[12,13] Thus (3*R*)-isoprene oxide (**16**), prepared in nine steps from (+)-pulegone, was reacted with the lithio anion of (3*R*)-(+)-β-citronellyl phenyl sulfone (**17**) that had been synthesized from 3*R*-(+)-β-

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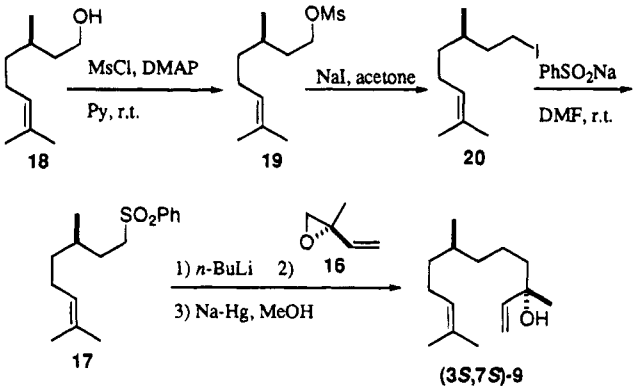
Scheme 4



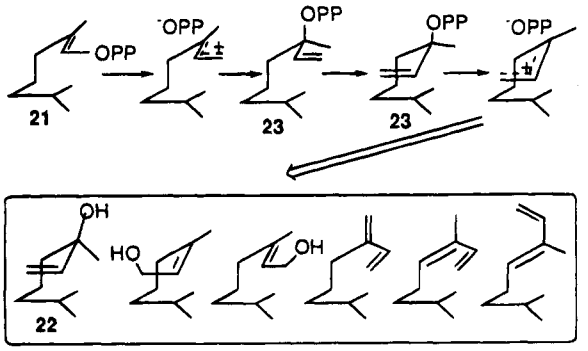
citronellol (**18**) as previously described[14] (Scheme 4). Reductive cleavage of the phenylsulfonyl moiety was achieved by treatment of the product with sodium amalgam in methanol[15] to provide (3*S*,7*S*)-6,7-dihydronerolidol ((3*S*,7*S*)-**9**) which was purified by silica gel chromatography. Analysis of (3*S*,7*S*)-**9** by capillary GC on Cyclodex B confirmed that the sample consisted of a single enantiomer.

Preparative scale incubation of (7*S*)-6,7-dihydroFPP (**5**), synthesized as previously described,[9d] with phosphatase-free, homogeneous, recombinant trichodiene synthase for 70 min at 30 °C gave a mixture of hydrocarbons and alcohols (100% yield, 1.02 μmol). Analysis by capillary GC (175 °C, DB-1+, 0.25 mm × 30 m) revealed the following product composition: **6** (68.8%), **7** (5.1%), **8** (6.9%), **9** (12.9%), **10** (6.2%). These results were essentially the same as those previously obtained with native trichodiene synthase from *F. sporotrichioides*.[9d] When the enzymatic incubation products were analyzed on a chiral Cyclodex B column, the dihydronerolidol component **9** eluted as a single sharp peak (retention time of 80.95 min). Under the same conditions, authentic (3*S*,7*S*)-6,7-dihydronerolidol ((3*S*,7*S*)-**9**) had a retention time of 80.93 min, whereas synthetic (3*RS*,7*S*)-6,7-dihydronerolidol ((3*RS*,7*S*)-**9**) emerged as two peaks with retention times of 78.86 and 80.36 min. Coinjection of the enzymatically generated dihydronerolidol and synthetic (3*S*,7*S*)-6,7-dihydronerolidol gave a single peak while coinjection with the synthetic mixture of dihydronerolidol diastereomers gave two peaks in which the component with the longer retention time was enhanced. These results establish unambiguously that the 6,7-dihydronerolidol which is generated by abortive cyclization of (7*S*)-6,7-dihydroFPP (**5**) has the (3*S*)-configuration.

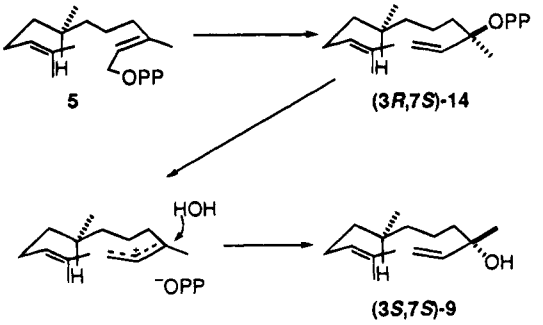
Discussion

In studies of monoterpene biosynthesis, Croteau has employed the substrate analog 6,7-dihydrogeranyl diphosphate (**21**) to probe the cryptic isomerization step in the conversion of geranyl diphosphate to (+)-bornyl diphosphate and (+)-α-pinene by way of the tertiary allylic ester linalyl diphosphate[16] (Scheme 5). The dihydro-analog **21**, which inhibited the cyclization of both geranyl and linalyl diphosphate, was itself converted by each cyclase to a

Scheme 5



Scheme 6



mixture of acyclic olefins and alcohols, including 6,7-dihydrolinalool (**22**) of undetermined configuration at C-3. The predominance of olefins in the mixture of abortive cyclization products suggested that the microenvironment of the enzyme active site was relatively inaccessible to water. In contrast, nonenzymic acid-catalyzed solvolysis in water gave mainly the alcohol products.[17] It was suggested that the reduced substrate analog had undergone the normal ionization–isomerization step to give dihydrolinalyl diphosphate (**23**), which in turn reionized to the corresponding cisoid cation. The latter intermediate, which is incapable of cyclization, would then serve as the proximal precursor of the observed mixture of olefins that result from deprotonation or capture of water.

According to the proposed mechanism of trichodiene biosynthesis (Scheme 1), (3*R*)-NPP (**3**) is generated by isomerization of FPP (**1**). Although (3*R*)-NPP itself can serve as a viable substrate for trichodiene synthase, the intermediacy of (3*R*)-NPP cannot be directly observed since the enzyme-generated (3*R*)-NPP is not released from the active site. The results from the incubation of the anomalous substrate 6,7-dihydrofarnesyl diphosphate are consistent with the proposed cationic nature of the normal allylic isomerization–cyclization process (Scheme 6). The observed (3*S*)-configuration of the dihydronerolidol (**9**) which results from abortive isomerization–cyclization would result from stereospecific attack on the back face of the allylic cation–pyrophosphate anion pair by adventitious water, which normally would be excluded from the active site. Thus isomerization of **5** to (3*R*)-**14** could take place by syn migration of the pyrophosphate moiety via a transoid allylic cation–pyrophosphate anion pair. Reionization of (3*R*)-6,7-dihydronerolidyl diphosphate (**14**) from either the transoid or cisoid conformer would regenerate the corresponding ion pair which would be subject to nucleophilic capture by water only from the

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opposite face, thereby generating the observed (3*S*,7*S*)-6,7-dihydronerolidol. The formation of a single enantiomer of the product rules out nonenzyme-catalyzed solvolysis of either **5** or **14**, while the observed (3*S*)-configuration of the dihydronerolidol excludes phosphatase-catalyzed hydrolysis (3*R*,7*S*)-6,7-dihydronerolidyl diphosphate (**14**). The observed formation of the primary allylic isomer, (7*S*)-*trans*-6,7-dihydrofarnesol (**10**), would result from alternative nucleophilic attack of water at C-1 of the transoid cation–pyrophosphate anion pair. On the other hand, the absence of the *cis* isomer **11** might indicate that water could not penetrate the hydrophobic active site deep enough to reach C-1 of the cisoid allylic cation. Attempts to directly trap the nerolidyl diphosphate intermediate of the normal cyclization are in progress.

Experimental Section

Instrumentation. NMR spectra were obtained on Bruker AM 400 or WM 250 spectrometers at 400.134 or 250.133 MHz for ^{1}H, 100.614 MHz for ^{13}C, and 161.978 MHz for ^{31}P. 85% H_3PO_4 was used as external reference for ^{31}P-NMR. Abbreviations used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. IR spectra were obtained on a Perkin-Elmer 1600 series FTIR infrared spectrometer. Mass spectra were obtained on a Kratos MS80 instrument. Radioactivity measurements were obtained on a Beckman LS 5801 liquid scintillation counter with 5 mL of Optifluor cocktail (Packard) and were automatically quench corrected. Capillary gas chromatography was carried out on a Hewlett-Packard 5790 gas chromatograph with flame ionization detection. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter.

Materials and Methods. Dry tetrahydrofuran and diethyl ether were distilled from sodium, while methylene chloride and acetonitrile were distilled from CaH_2. Silver nitrate-impregnated silica gel for column chromatography was made by mixing EM Science silica gel 60 with 10% $AgNO_3$ (w/v) and drying overnight under vacuum. All analytical TLC plates were visualized by UV light or spraying with 4% *p*-anisaldehyde in 95% ethanol containing 2% H_2SO_4. DEAE A-25 and bulk matrix Q-Sepharose were obtained from Sigma (St. Louis, MO). Bio-Gel P-6DG was purchased from Bio-Rad (Richmond, CA). Methods of trichodiene synthase assay, protein determination, and SDS-PAGE were as previously described.[6] All reagents and buffer components were analytical grade or higher. All buffers were made using doubly deionized water from a Barnstead Nanopure II Ion Exchange system.

(3*RS*,7*S*)-6,7-Dihydronerolidol ((3*RS*,7*S*)-9). To 31.3 mg of $LiAlH_4$ (0.82 mmol) in 0.8 mL of dry ether at 0 °C was added 60.2 mg of *t*-BuOH (0.79 mmol) in 1 mL of ether dropwise via syringe. After 5 min, 97.4 mg of methyl (7*S*)-6,7-dihydrofarnesoate[9d] (**15**) (0.387 mmol) in 1 mL of ether was added dropwise and stirring was continued for 3 h. After normal workup, the crude product was chromatographed to give 72 mg of pure (7*S*)-*trans*,*trans*-6,7-dihydrofarnesol (**10**): IR (neat, cm^{-1}) 3314, 2920, 1670, 1459, 1377, 1002; ^{1}H-NMR ($CDCl_3$, 250 MHz) δ 0.86 (3H, d, 7.6 Hz), 1.25 (7H, m, br), 1.60 (3H, s), 1.65 (6H, s), 1.98 (4H, m), 4.15 (2H, t, 5.7 Hz), 5.09 (1H, t, 7 Hz), 5.40 (1H, t, 5.7 Hz).

The alcohol **10** (40 mg, 0.18 mmol) was dissolved in 5 mL of CH_3CN and 1 mL of water. Concentrated H_2SO_4 (5 drops, 36 N) was added and the mixture was stirred at room temperature overnight. The resulting crude product was chromatographed to provide 17.8 mg of pure (3*RS*,7*S*)-*trans*-6,7-dihydronerolidol ((3*RS*,7*S*)-**9**): IR (neat, cm^{-1}) 3386, 2925, 1646, 1456, 1376, 1119, 995, 920; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.85 (3H, d, 7.6 Hz), 1.10 (2H, m), 1.30 (3H, s), 1.3–1.5 (7H, m), 1.60 (3H, s), 1.69 (3H, s), 1.95 (2H, m), 5.05 (1H, dd, 1.03 Hz, 1.7 Hz), 5.10 (1H, m), 5.20 (1H, dd, 16.7 Hz, 1.7 Hz), 5.92 (1H, dd, 16.7 Hz, 10.3 Hz).

(3*S*,7*S*)-6,7-Dihydronerolidol ((3*S*,7*S*)-9). To a solution of 56.0 mg of (*R*)-(+)-2-methyl-2-hydroxy-3-buten-1-yl *p*-toluenesulfonate ($[\alpha]^{25}_D$ +13.49 (*c*, 2.98, $CHCl_3$)), prepared as described,[12] in 1 mL of THF was added 96 mg of ground KOH at room temperature. The mixture was cooled to 0 °C and stirred for 1 h and then filtered and diluted with 2 mL of THF. The THF solution was distilled at atmospheric pressure and the resultant solution of isoprene oxide **16** in 1.5 mL of THF was used immediately for reaction with the lithio anion of citronellyl phenyl sulfone (**17**).

To (3*R*)-(+)-β-citronellol (**18**) (1.0 g, 6.4 mmol) in 10 mL of CH_2Cl_2 containing 0.12 g of 4-(dimethylamino)pyridine and 1 mL of Et_3N (7.2 mmol) in 10 mL of CH_2Cl_2 at 0 °C was added 0.56 mL of MsCl (7.1 mmol) dropwise with stirring. The mixture was stirred at 0 °C for 3.5 h and filtered. The filtrate was washed successively with 10% HCl, aqueous $KHCO_3$ and water, dried over $MgSO_4$, and concentrated to give 1.47 g of crude mesylate **19**. The crude product was dissolved in 25 mL of acetone, 1.9 g of NaI was added, and the mixture was brought to reflux with stirring for 3 h. After cooling to room temperature, the reaction mixture was filtered and concentrated to give a reddish oil which was applied to a short silica gel column (2 × 4 cm) and eluted with 30 mL pentane, 50 mL pentane:ethyl acetate (100:4), and 50 mL pentane:ethyl acetate:ether (100:4:4). The collected fractions were concentrated to give 1.5 g of citronellyl iodide (**20**). The crude iodide was mixed with 2.0 g of the sodium salt of benzenesulfinic acid in 10 mL of DMF and the mixture was stirred overnight at room temperature. Normal workup gave 1.53 g of crude oil which was chromatographed on a silica gel column (4.5 cm × 23 cm, hexane–ethyl acetate, 9:1) to provide 1.26 g of pure sulfone **17** (4.5 mmol, 70%): $[\alpha]^{25}_D$ −11.4 (*c* 6.31 g/100 mL, $CHCl_3$); IR (neat, cm^{-1}) 3064, 2917, 1713, 1673, 1586, 1447, 1381, 1306, 1144, 1087; HRMS (M^+ + H) calcd for $C_{16}H_{24}SO_2$ 281.1575, found 281.1570; MS (CI), *m/z* (rel intensity) 281 (M^+ + 1, 9%), 169 (5%), 143 (75%), 139 (100%), 138 (68%), 123 (27%), 109 (16%), 95 (45%), 83 (62%), 69 (48%); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.81 (3H, d, 6.5 Hz), 1.15 (2H, m), 1.45 (2H, m), 1.50 (3H, s), 1.62 3H, s), 1.70 (1H, m), 1.87 (2H, m), 3.06 (2H, m), 4.98 (1H, t, 5.7 Hz), 7.53 (2H, m), 7.62 (1H, m), 7.88 (2H, m); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 17.5, 19.0, 25.1, 25.6, 29.0, 31.4, 36.3, 54.3, 124.0, 128.0 (2 × C), 129.2 (2 × C), 131.6, 133.5, 139.2.

To 231 mg (0.82 mmol) of phenyl (*R*)-citronellyl sulfone (**17**) in 0.4 mL of THF was added 0.8 mL of *n*-BuLi (1.37 M, 1.1 mmol) at −78 °C. After 45 min, 1 mL of THF was added to the resultant slurry and the distilled (3*R*)-isoprene oxide (**16**) was added to the yellow solution at −78 °C. The cooling bath was removed and after 3 h the reaction mixture was subjected to normal workup. The resulting oil was dissolved in 2 mL of MeOH, and 2.6 g of ground Na–Hg (5%) was added at room temperature. The mixture was stirred overnight, and after normal workup 140 mg of crude product was obtained which was chromatographed on silica gel (1 cm × 46 cm, hexane–ethyl acetate, 10:1). The desired fractions were located by GC and combined to give a GC-pure product (10 mg, 14% from (*R*)-(+)-2-methyl-2-hydroxy-3-buten-1-yl *p*-toluenesulfonate). The IR and ^{1}H-NMR spectra of (3*S*,7*S*)-**9** were identical to those of (3*RS*,7*S*)-6,7-dihydronerolidol ((3*RS*,7*S*)-**9**). $[\alpha]^{25}_D$ +5.7 (*c* 0.49, hexane); HRMS (M^+ − H_2O) calcd for $C_{15}H_{24}$ 206.2035, found 206.2038; MS (EI), *m/z* (rel intensity) 206 (65%), 191 (45%), 177 (25%), 163 (73%), 149 (68%), 109 (58%), 95 (45%), 81 (60%), 71 (100%), 55 (75%), 41 (80%); ^{13}C-NMR ($CDCl_3$,100 MHz) δ 17.6, 19.5, 21.3, 25.6, 25.7, 27.7, 32.4, 37.1, 37.3, 42.7, 73.3, 111.5, 125.0, 131.0, 145.3.

(7*S*)-*trans*-6,7-Dihydrofarnesyl Diphosphate. Oxidation of 35 mg (0.16 mmol) of (7*S*)-*trans*-6,7-dihydrofarnesol (**10**) with MnO_2 in hexane at 0 °C to give 30.7 mg (0.14 mmol) of the corresponding aldehyde. The aldehyde was reduced with 25 mCi [^{3}H]$NaBH_4$ in 2-propanol at 0 °C and the product was chromatographed to give 11.2 mg of [1-^{3}H]-(7*S*)-*trans*-6,7-dihydrofarnesol (9.81 mCi, 197 mCi/mmol). A portion of this sample (137 μCi) was mixed with 35.2 mg of unlabeled alcohol **10** (final specific activity, 0.88 mCi/mmol) and converted to the corresponding diphosphate ester following the usual procedure of conversion to the allylic bromide with CBr_4 and PPh_3, and diphosphorylation with $(n\text{-}Bu_4N)_3HP_2O_7$ in CH_3-

CN.[9b,18] The resulting diphosphate ester was purified on a DEAE A-25 anion-exchange column by elution with a gradient of 0.05 to 1.0 M $Et_3NH_2CO_3$ (pH 8) followed by chromatography on C-18 silica gel eluted with a water to methanol gradient. The overall yield of **5** from the alcohol **10** was 24%: ^{1}H-NMR (D_2O, 400 MHz) δ 0.66 (3H, d, 6.6 Hz), 0.9–1.3 (7H, m), 1.42 (3H, s), 1.49 (3H, s), 1.51 (3H, s), 1.84 (4H, m), 4.27 (1H, t, 6.5 Hz), 5.03 (1H, t, 7.2 Hz), 5.25 (1H, t, 7.1 Hz); ^{31}P-NMR (D_2O, 162 MHz) δ −9.85 (d, 22 Hz), −5.90 (d, 22 Hz).

Preparation of Trichodiene Synthase.[6] A single colony of *E. coli* BL21(DE3)/pZW03 was inoculated into 200 mL of LB media (DIFCO bacto-tryptone 10 mg/mL, DIFCO bacto-yeast extract 5 mg/mL, and NaCl 10 mg/mL, pH 7.0), and incubated at 37 °C and 250 rpm for 11 h to reach an A_{600} of 0.23. Then six portions of 20 mL each were inoculated into six 2.5-L flasks containing 800 mL each of LB medium, and the cultures were incubated at 37 °C to an A_{600} of 2.1. IPTG (1 M, 0.8 mL) was added to each flask to induce the production of trichodiene synthase, and the cultures were incubated at 30 °C for 2 h. The cells were harvested by centrifugation at 8000*g* (7,000 rpm) for 10 min. The cells were disrupted by grinding with glass beads (0.1–0.15 mm) in a Bead Beater in buffer T (10 mM Tris·HCl, 5 mM $MgCl_2$, 15% v/v glycerol, 5 mM $HSCH_2CH_2OH$, pH 7.8) containing 1 mM PMSF at 4 °C. The mixture was centrifuged to remove the glass beads, and the milky supernatant was collected and subjected to ultracentrifugation at 150000*g* at 4 °C for 65 min. The supernatant (170 mL) was diluted with Buffer T to 1.2 l, to which 571 g of ammonium sulfate was added slowly at 4 °C. The milky protein solution was centrifuged at 18000*g* for 35 min and the protein precipitate was redissolved in 20 mL of Buffer T and desalted on a Bio-gel P6-DG column (2.5 cm × 85 cm) using buffer T. The protein was applied to a Q-Sepharose column (2.5 cm × 13 cm) which was eluted with 150 mL of Buffer T followed by a gradient of 125 mM to 300 mM KCl in buffer T (total 200 mL). The desired fractions were located by activity assay with FPP,[6,9] combined, and then concentrated to 10 mL by ultrafiltration through a DIAFLO ultrafiltration membrane. The concentrated enzyme preparation was stored at −80 °C. The results of the purification are summarized in Table 1.

Preparative-Scale Incubation of [1-^{3}H]-(7*S*)-6,7-Dihydrofarnesyl Diphosphate (DHFPP) with Trichodiene Synthase. [1-^{3}H]-(7*S*)-6,7-DHFPP (**5**) (1.02 μmol, 0.875 mCi/mmol) in 200 μL of 0.01 N NH_4OH was diluted with 4.4 mL of Buffer H (25 mM HEPES, 3 mM $MgCl_2$, 1 mM DTT, pH 7.2), and 1 mL Q-Sepharose-purified trichodiene synthase (1.76 mg, 390 nmol/min) was added to give a final concentration of DHFPP 182 μM. The mixture was then incubated at 30 °C for 70 min. The incubation was carried out in duplicate, along with a pair of blank controls without trichodiene synthase.

Each incubation mixture was vortexed twice with freshly distilled ether (2 × 3.0 mL) for 60 s. The two phases were separated by centrifugation and the ether extract was withdrawn and passed through a silica gel column (0.5 × 2 cm). The eluent was concentrated on a rotavapor using an ice–water bath to cool the distillation flask. The residue was taken up in 100 μL of spectroscopic grade hexane. Counting of a 1 μL aliquot by liquid scintillation showed an almost 100% turnover of substrate while the blank control gave 0.3% ether-extractable radioactivity based on **5**.

The hexane solution obtained above was then subjected to GC analysis on a Cyclodex B column (0.25 mm × 30 m, J &W Scientific) along with authentic synthetic (3*RS*,7*S*)-6,7-dihydronerolidol ((3*RS*,7*S*)-**9**) and (3*S*,7*S*)-6,7-dihydronerolidol ((3*S*,7*S*)-**9**) (column pressure 18 psi, split ratio of 3:100, 120 °C, isothermal program). Under these conditions the pure diastereomer (3*S*,7*S*)-**9** showed a retention time of 80.93 min while the mixture of (3*RS*,7*S*)-**9** diastereomers eluted at 78.86 and 80.36 min. The enzymatically generated product mixture was injected alone and in separate injections in admixture with either (3*RS*,7*S*)-**9** and authentic (3*S*,7*S*)-**9**.

Table 1. Purification of Trichodiene Synthase from *E. coli* BL21(DE3)/pZW03

step	total protein, mg	total activity, nmol/min	spec activity, nmol/min mg^{-1}	recovery, %
15000*g* supernatant	1200	66300	55	100
$(NH_4)_2SO_4$ precipitation	192	17850	93	27
Q-Sepharose	17.5	3900	222	5.9

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Supplementary Material Available: Copies of ^{1}H and ^{13}C NMR spectra of **17** (1 page). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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