Trichodiene Synthase. Substrate Specificity and Inhibition†

David E. Cane,* Guohan Yang, Qun Xue, and Jae Hoon Shim

Department of Chemistry, Box H, Brown University, Providence, Rhode Island 02912

Received September 1, 1994; Revised Manuscript Received November 18, 1994®

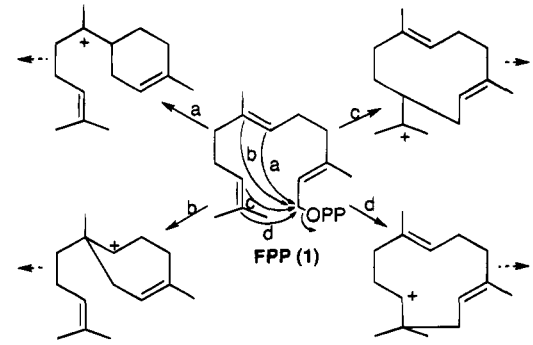
ABSTRACT: The substrate specificity of the sesquiterpene synthase trichodiene synthase was examined by determining the V_{max} and K_m parameters for the natural substrate, *trans,trans*-farnesyl diphosphate (**1**), its stereoisomer, *cis,trans*-farnesyl diphosphate, and the tertiary allylic isomer, (3*R*)-nerolidyl diphosphate (**3**), using both the native fungal and recombinant enzymes. A series of farnesyl diphosphate analogs, **15**, **16**, **20**, **7**, **8**, and **9**, was also tested as inhibitors of trichodiene synthase. 10-Fluorofarnesyl diphosphate (**15**) was the most effective competitive inhibitor, with a K_I of 16 nM compared to the K_m for **1** of 87 nM, while the ether analog of farnesyl diphosphate, **8**, an extremely potent inhibitor of squalene synthase, showed only modest inhibition of trichodiene synthase, with a K_I/K_m of 70.

Sesquiterpene synthases are among Nature's most intriguing and versatile catalysts. Each individual synthase is capable of cyclizing the universal acyclic precursor, farnesyl diphosphate (FPP,[1] **1**), to a single representative of any of 200 known sesquiterpene carbon skeletons (Cane, 1985). Over the last 30 years, extensive biosynthetic investigations, initially at the intact cell level and more recently with cell-free preparations and purified enzymes, have revealed many important details of these cyclization reactions and led to a unified mechanistic and stereochemical model of these complex transformations (Cane, 1985, 1990). According to this picture, farnesyl diphosphate undergoes an ionization to generate the corresponding allylic cation, which can cyclize by electrophilic attack on either the central or distal double bonds (Scheme 1). Further electrophilic cyclizations, coupled with mechanistically well-precedented hydride shifts and carbon skeletal rearrangements, followed by quenching of the positively charged intermediates by deprotonation or the capture of nucleophilic species such as water, can account for the formation of all known carbocyclic skeletons of the sesquiterpene family. According to these schemes, a major determinant of the structure and stereochemistry of the eventually formed product is the precise folding of the FPP substrate at the active site of the cyclase.

In spite of considerable progress in the study of sesquiterpene synthases, several major questions remain unanswered. Among the most fundamental are the following: (1) How does the synthase impose a specific folding on the C_{15} hydrocarbon portion of the substrate? (2) What catalyzes the initial ionization of the pyrophosphate moiety, how is positive charge stabilized in the multiple intermediates, and what determines the precise timing and site of quenching of the positive charge? (3) What amino acid residues are present at the active site of each cyclase, and what is the role of each residue?

By far the most thoroughly studied sesquiterpene synthase is trichodiene synthase. First isolated from the apple mold

Scheme 1: Cyclization of Farnesyl Diphosphate (FPP, **1**)



fungus *Trichothecium roseum* (Evans & Hanson, 1976; Cane et al., 1981), the source of the antibiotic trichothecin, this enzyme was subsequently isolated from *Fusarium sambucinum* (*Gibberella pulicaris*) (Hohn & Beremand, 1989a) and *Fusarium sporotrichioides* (Hohn & VanMiddlesworth, 1986), the producers of the potent mycotoxins diacetoxyscirpenol and T-2 toxin, respectively. The cyclase from the latter organism has been purified to homogeneity and found to be a homodimer of subunit M_r 45 000, as determined by SDS−PAGE. The corresponding structural gene was cloned and shown to encode a protein of M_D 43 999 (Hohn & Beremand, 1989b; Hohn & Plattner, 1989). More recently, we have expressed recombinant trichodiene synthase as 25−30% of soluble protein in *Escherichia coli* (Cane et al., 1993).

Extensive studies using stereospecifically labeled samples of FPP have shed considerable light on the mechanism of formation of trichodiene (**2**) from FPP (Cane, 1990). In order to generate the characteristic cyclohexene ring of the product trichodiene, trichodiene synthase must overcome the geometric barrier to direct cyclization of the *trans,trans*-FPP substrate. In close analogy with the majority of monoterpene synthases, the enzyme accomplishes this task by catalyzing an initial syn isomerization of *trans,trans*-FPP to the corresponding tertiary allylic isomer, (3*R*)-nerolidyl diphosphate (**3**) (NPP) (Scheme 2). 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

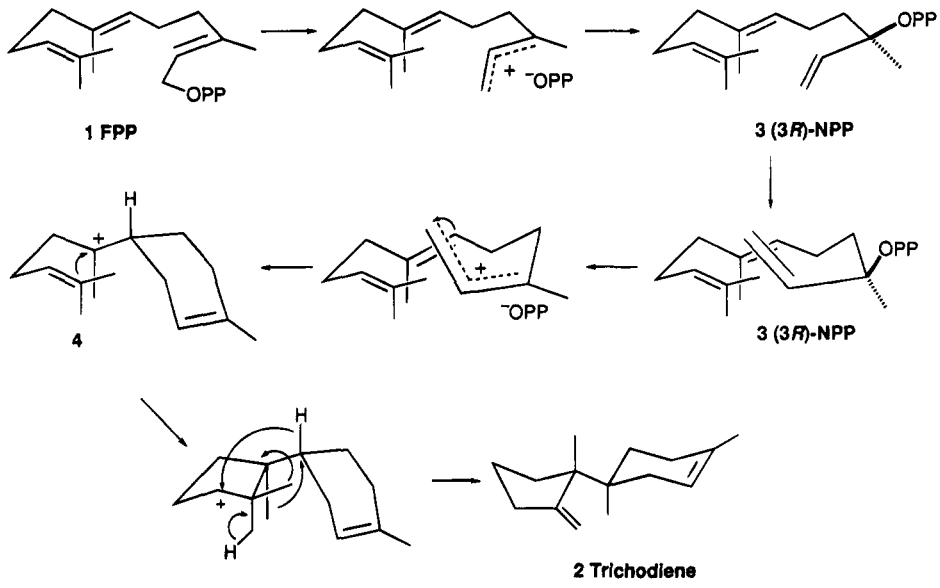
† This research was supported by National Institutes of Health Grant GM-30301 to D.E.C.

® Abstract published in *Advance ACS Abstracts,* February 1, 1995.

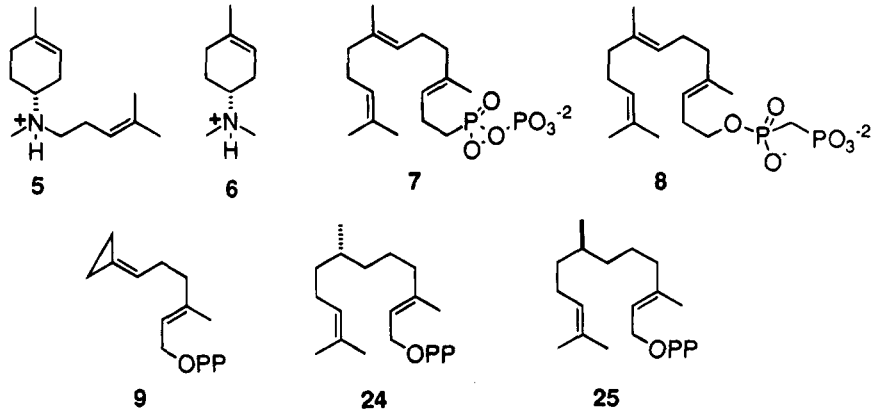
[1] Abbreviations: DTT, dithiothreitol; FPP, farnesyl diphosphate; NPP, nerolidyl diphosphate; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate.

0006-2960/95/0434-2471$09.00/0

Scheme 2: Isomerization–Cyclization of FPP to Trichodiene by Way of (3*R*)-NPP



Scheme 3: Trichodiene Synthase Inhibitors



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.

The intermediacy of (3*R*)-NPP is supported by several lines of evidence, including the observations that cyclization of FPP takes place without the loss of either proton originally attached to C-1 and with the net retention of configuration at this center (Cane et al., 1985), that (3*R*)-NPP can serve as a substrate for trichodiene synthase (Cane & Ha, 1988), and that incubation of 6,7-dihydro-FPP with trichodiene synthase results in the formation of a number of abortive olefinic and alcoholic cyclization products, among which is (3*S*)-6,7-dihydronerolidol (Cane et al., 1990a; Cane & Yang, 1994). On the other hand, competition experiments using labeled samples of both FPP and NPP have established that there is no interconversion of enzyme-free allylic diphosphate substrates and that (3*R*)-NPP is never released from the active site of the enzyme (Cane & Ha, 1988). In the present study, we have determined the steady-state kinetic parameters for *trans,trans*-FPP and (3*R*)-NPP as well as *cis,trans*-FPP for native fungal and wild-type recombinant enzymes.

Further support for the trichodiene synthase mechanism has come from the demonstration that both **5** and **6**, illustrated in Scheme 3, and their respective enantiomers, each of which is an ammonium ion analog of the proposed bisabolyl cation intermediate **4**, show strong synergistic inhibition of trichodiene synthase in the presence of inorganic pyrophosphate (Cane et al., 1992). We describe here the preparation and testing of several FPP analogs as competitive inhibitors of trichodiene synthase in order to probe further the factors affecting substrate recognition by the active site.

MATERIALS AND METHODS

Materials. Trichodiene synthase from *F. sporotrichioides* was a gift from Dr. Thomas M. Hohn of the USDA. Recombinant trichodiene synthase was purified from *E. coli* BL21(DE3)/pZW03 as previously described (Cane et al., 1993; Cane & Yang, 1994). [1-^{3}H]FPP (**1**) (71.7, 204, and 682 mCi/mmol) was synthesized as previously described (Cane & Ha, 1988). (1*Z*)-[1-^{3}H]-(3*R*)-*trans*-Nerolidyl diphosphate ((3*R*)-NPP, **3**) (21.5 mCi/mmol) was synthesized from semisynthetic (3*R*)-nerolidol (Cane et al., 1990b), also as previously described (Cane & Ha, 1988). Farnesylphosphono phosphate (**7**) was synthesized as previously described (Corey & Volante, 1976) and purified as the potassium salt by DEAE-Sephadex ion-exchange chromatography using a linear gradient of potassium carbonate, followed by the removal of inorganic salts using CHP-20P resin (Mitsubishi Chemical Industries) (Biller & Forster, 1990; Biller et al., 1988). Farnesyl (phosphonomethyl)phosphonate tripotas-

sium salt (**8**) was a gift from Dr. Scott Biller of Bristol-Myers Squibb. The methylenecyclopropane analog of geranyl diphosphate (6-cyclopropylidene-3*E*-methylhex-2-en-1-yl diphosphate, **9**) was prepared as previously described (Croteau et al., 1993). Dry tetrahydrofuran and diethyl ether were distilled from sodium, while methylene chloride and acetonitrile were distilled from CaH_2. All other reagents and buffer components used for enzyme assays and protein purification were of the highest quality commercially available.

General Methods. NMR spectra were obtained on Bruker AM 400 or WM 250 spectrometers at 400.134 or 250.133 MHz for ^1H, 100.614 MHz for ^{13}C, and 161.978 MHz for ^{31}P. H_3PO_4 (85%) 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. Capillary gas chromatography was carried out on a Hewlett-Packard 5790 gas chromatograph. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. HPLC was performed on a Waters Model 510 using automated gradient controller Model 680 and Absorbance Detector Model 441. HPLC solvents were purchased from EM Science and were degassed by purging with Ar. High-resolution 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. All analytical TLC plates were visualized by UV light or spraying with 4% *p*-anisaldehyde in 95% ethanol containing 2% H_2SO_4. Steady-state kinetic parameters were initially estimated from Lineweaver–Burk double-reciprocal plots and then calculated by direct fitting of data to the relevant kinetic expression using nonlinear least-squares methods.

Protein concentrations were estimated by the method of Bradford using a commercial reagent (Bio-Rad) (Bradford, 1976). Enzyme incubations were carried out in a GCA/Precision Scientific Thelco 184 constant temperature water bath. Routine assays of trichodiene synthase activity were performed as previously described in buffer T (10 mM Tris Cl, 5 mM $MgCl_2$, 15% glycerol, and 5 mM β-mercaptoethanol, pH 7.8) (Cane et al., 1993). One unit of trichodiene synthase activity corresponded to the conversion of 1 nmol/min of FPP to trichodiene.

Synthesis of Substrates and Inhibitors. (1) [1-^{3}H]-cis, trans-Farnesyl Diphosphate. cis,trans-Farnesal (17.7 mg, 0.08 mmol) was dissolved in isopropyl alcohol and reacted with 25 mCi of [^{3}H]$NaBH_4$ (490 mCi/mmol) at room temperature as previously described for *trans,trans*-farnesal (Cane & Ha, 1988). After 1 h, normal workup gave 13.1 mg of *cis,trans*-farnesol (8.96 mCi, 152.1 mCi/mmol). The product, which was shown by ^1H NMR to contain a minor amount (<10%) *trans,trans*-farnesol, was converted to *cis,trans*-FPP in 27% overall yield using the procedure described for *trans,trans*-FPP (Cane & Ha, 1988); ^1H NMR (D_2O, 400 MHz) δ 1.45 (6H, s), 1.50 (3H, s), 1.57 (3H, s), 1.85 (2H, m), 1.95 (6H, m), 4.27 (2H, t, J = 6.4 Hz), 5.00 (2H, q, m), 5.29 (1H, t, J = 7.1 Hz); ^{31}P NMR (D_2O, 162 MHz) δ −9.61 (d, J = 22 Hz), −5.55 (d, J = 22 Hz).

(2) [1-^{3}H]-trans,trans-10-Fluorofarnesyl Diphosphate (10F-FPP). (a) Triethyl 2-Phosphono-2-fluoroacetate. A mixture of 10.36 g of ethyl bromofluoroacetate (56 mmol) and 13 mL of triethyl phosphite (98%) (76 mmol) was refluxed overnight. The reaction mixture was distilled under vacuum (<1 mmHg), and fractions were collected between 65 and 110 °C to give 8.38 g of triethyl 2-phosphono-2-fluoroacetate (35 mmol, 62%): IR (neat, cm^{-1}) 2983, 2937, 2286, 1762, 1371, 1326, 1240, 1027; ^1H NMR ($CDCl_3$, 250 MHz) δ 1.4 (9H, m), 4.3 (6H, m), 5.25 (1H, dd, J = 12.5, 47 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.9, 16.2, 62.5 (d, J = 6.1 Hz), 64.6 (d, J = 6.5 Hz), 84.9 (dd, J = 162.2, 196.2 Hz), 165.0 (d, J = 20.8 Hz); MS (CI) 243 (M + 1, 100), 215 (38), 187 (22), 159 (28).

(b) Ethyl 2-Fluoro-3-methylbut-2-enoate. Triethyl 2-phosphono-2-fluoroacetate (8.38 g, 35 mmol) in 10 mL of benzene at 0 °C was slowly added to a mixture of 1.67 g of NaH (60%, washed three times with dry pentane) in 15 mL of benzene with vigorous stirring. The mixture was stirred for another 40 min until bubbling stopped. Then 5 mL of acetone (distilled over K_2CO_3 and $KMnO_4$) was added slowly to give a dark orange mixture of solid and liquid, which was stirred for 4.5 h at 0 °C. The reaction was quenched with 5 mL of water and 30 mL of 1 N HCl, and the two layers were stirred for another 15 min at room temperature. The organic layer was separated and the aqueous layer was extracted with ether. The combined organic extracts were washed with aqueous Na_2CO_3 and brine and dried over $MgSO_4$. The filtrate was distilled at atmospheric pressure to remove ether and benzene, and the residue was distilled under aspirator pressure (*ca.* 35 mmHg) to give a fraction boiling between 70 and 82 °C (2.77 g, 19.2 mmol, 55%): IR (neat, cm^{-1}) 2895, 1724, 1671, 1395, 1299, 1220, 1170, 1100, 1050; ^1H NMR ($CDCl_3$, 250 MHz) δ 1.35 (3H, t, J = 7 Hz), 1.88 (3H, d, J = 5 Hz), 2.12 (3H, d, J = 3 Hz), 4.27 (2H, q, J = 7 Hz).

(c) 2-Fluoro-3-methylbut-2-enol. To 1.3 g of ethyl 2-fluoro-3-methylbut-2-enoate (9.0 mmol) in 20 mL of THF at 0 °C was added 400 mg of $LiAlH_4$ with stirring. The reaction was run overnight, quenched with water, extracted with ether, dried over $MgSO_4$, filtered, and concentrated to give 0.84 g of 2-fluoro-3-methylbut-2-enol (8.1 mmol, 90%): IR (neat, cm^{-1}) 3334, 2995, 2925, 1713, 1456, 1394, 1266, 1165, 1128, 1009; ^1H NMR (CD_2Cl_2, 400 MHz) δ 1.65 (3H, d, J = 0.6 Hz), 1.66 (3H, s), 2.38 (1H, br), 4.17 (2H, d, J = 23 Hz); ^{13}C NMR (CD_2Cl_2, 100 MHz) δ 15.7 (d, J = 8.8 Hz), 17.4 (d, J = 5.0 Hz), 57.8 (d, J = 31.4 Hz), 112.5 (d, J = 16.4 Hz), 153.2 (d, J = 241.0 Hz).

(d) 2-Fluoro-3-methylbut-2-enyl Bromide. To 1.20 g of 2-fluoro-3-methylbut-2-enol (11.5 mmol) in 10 mL of ether at 0 °C was added 0.5 mL of PBr_3 dropwise with stirring. After 1 h, normal workup gave an ether solution, which was concentrated at aspirator pressure with the distilling flask immersed in an ice–water bath to give the desired allylic bromide (**10**) (1.3 g, 7.8 mmol, 67%): IR (neat, cm^{-1}) 2961, 2925, 2880, 1645, 1450; ^1H NMR ($CDCl_3$, 250 MHz) δ 1.7 (6H, m), 4.1 (2H, d, J = 23 Hz); MS (EI) 168 (17), 166 (17.3), 87 (100), 67 (23.3), 59 (91). The bromide was used immediately without further purification.

(e) (E)-8-(Phenylsulfonyl)-10-fluorofarnesyl Benzyl Ether (**12**). To 1.39 g of (*E*)-8-(phenylsulfonyl)geranyl benzyl ether (**11**) (3.6 mmol) (Cane et al., 1984) in 10 mL of dry THF at −78 °C was added 2.70 mL of 1.38 M *n*-BuLi, and the mixture was reacted for 1 h. Freshly prepared 2-fluoro-3-methylbut-2-enyl bromide (**10**) (600 mg, 3.6 mmol) in 6 mL of THF was added at −78 °C. The cooling bath was

removed and the reaction mixture was allowed to warm slowly to room temperature. After 2 h, the reaction was complete by TLC. Normal workup gave 1.65 g of crude product, which was chromatographed (hexane/ethyl acetate, 4:1) to provide 1.32 g (2.8 mmol, 78%) of **12**: IR (neat, cm^{-1}) 2996, 2925, 2867, 1720, 1450, 1310, 1160, 1100; ^{1}HNMR ($CDCl_3$, 250 MHz) δ 1.55 (9H, s), 1.65 (3H, s), 1.8 (2H, m), 2.0 (2H, m), 2.9 (2H, br m), 3.78 (1H, dd, J = 5.0, 10 Hz), 4.0 (2H, d, J = 7 Hz), 4.5 (2H, s), 5.12 (1H, t, J = 7 Hz), 5.28 (1H, t, J = 7 Hz), 7.35 (5H, m), 7.51 (2H, m), 7.60 (1H, m), 7.85 (2H, m); ^{13}C NMR ($CDCl_3$,100 MHz) δ 13.7, 15.5 (d, J = 8.9 Hz), 16.3, 17.7 (d, J = 5.5 Hz), 25.4 (d, J = 28.9 Hz), 26.4, 38.3, 66.5, 70.9, 72.1, 110.5 (d, J = 17.4 Hz), 121.2, 126.3, 127.5, 127.7, 128.3 (2C), 128.7 (5C), 133.4, 135.5, 137.9, 138.5, 139.4, 149.3 (d, J = 241.3 Hz); MS (EI) *m/z* (rel intensity): 379 (25), 237 (50), 221 (60), 91 (100).

(f) trans,trans-10-Fluorofarnesol (**13**). *tert*-Butyl alcohol (4 mL) and 1.32 g (2.8 mmol) of (*E*)-8-(phenylsulfonyl)-10-fluorofarnesyl benzyl ether (**12**) dissolved in 20 mL of THF were added through a cannula to a lithium suspension (848 mg of 25% dispersion, washed three times with dry hexanes) with stirring. The reaction was run at room temperature under N_2 for 3 h, after which another portion of Li (880 mg of 25% dispersion, washed with hexanes) was added to the reaction together with 4 mL of *t*-BuOH at 0 °C. The reaction was allowed to run overnight. Normal workup gave 970 mg of a crude mixture of products, which was chromatographed on a $AgNO_3$-impregnated silica gel column (3 × 18 cm, hexanes/ethyl acetate, 2:1) to provide 248 mg (37%) of the desired 10-fluorofarnesol (**13**), as well as 249 mg of the isomer **14**. **13**: IR (neat, cm^{-1}) 3347 (br), 2919, 1715, 1670, 1510, 1450, 1384, 1002; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.52 (3H, s), 1.62 (6H, s), 1.68 (3H, s), 2.1 (6H, m), 2.3 (2H, dt, J = 23, 6.7 Hz), 4.15 (2H, d, J = 7 Hz), 5.15 (1H, t, J = 7.0 Hz), 5.4 (1H, t, J = 7.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.4 (d, J = 9.4 Hz), 15.8, 16.2, 17.6 (d, J = 5.9 Hz), 26.3, 27.5 (J = 29.1 Hz), 36.5, 39.4, 59.4, 107.2 (d, J = 18 Hz), 123.4, 124.5, 134.4, 139.6, 153.9 (d, J = 241.2 Hz); HRMS calcd for $C_{15}H_{25}FO$ 240.1889, found 240.1900; MS (CI) *m/z* (rel intensity) 240 (M^+, 45), 222 (31), 155 (60), 135 (86), 121 (60), 107 (78), 93 (100), 81 (83), 67 (71), 55 (62); capillary GC retention time 4.00 min, isothermal program at 225 °C, (DB-1+, J&W Scientific, 0.25 mm × 30 m). The retention time was the same as that of an authentic sample of **13** provided by Prof. John McCormick of the University of Missouri at Columbia.

(g) [1-^{3}H]-10-Fluorofarnesyl Diphosphate (**15**). To 20 mg (0.09 mmol) of 10-fluorofarnesol (**13**) in 20 mL of dry pentane was added MnO_2 (484 mg, 5.6 mmol), and the mixture was stirred at 0 °C for 1 h. The mixture was filtered and the MnO_2 cake was washed extensively with pentane. The combined filtrate was concentrated and chromatographed on a TLC plate to give 14 mg (0.06 mmol, 71%) of pure *trans,trans*-10-fluorofarnesal: IR (neat, cm^{-1}) 2976, 2922, 2860, 1715, 1674, 1449, 1385, 1194, 1119, 914, 734; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.56 (3H, d, J = 2.6 Hz), 1.59 (3H, d, J = 3.3 Hz), 1.62 (3H, s), 2.2 (11H, m), 5.11 (1H, m), 5.88 (1H, d, J = 8.0 Hz), 9.9 (1H, d, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.3, 15.5 (d, J = 9.4 Hz), 16.0, 17.6 (d, J = 5.1 Hz), 25.7, 27.5 (d, J = 29.3 Hz), 36.5, 40.5, 107.4 (d, J = 17.1 Hz), 123.2, 127.4, 135.7, 153.8 (d, J = 240.1 Hz), 163.6, 191.2; MS (CI) *m/z* (rel intensity) 239 (6.4), 221 (5.8), 201 (6.6), 175 (4.2), 154 (21.8), 135 (37.7), 107 (35.1), 87 (100).

The 14 mg of 10-fluorofarnesal (66 μmol) in 1 mL of isopropyl alcohol was injected into a vial containing [^{3}H]-$NaBH_4$ (25 mCi) at room temperature. The reaction was run overnight and then quenched with H_2O, and the mixture was extracted with ether, dried over $MgSO_4$, concentrated, and purified by preparative TLC to give 6.3 mg of [1-^{3}H]-10-fluorofarnesol (2.94 mCi, 112 mCi/mmol), which was identical to unlabeled 10-fluorofarnesol. A portion of the labeled [1-^{3}H]-10-fluorofarnesol (**13**) (3.1 mg, 12.9 μmol) was diluted with 9.5 mg of unlabeled 10-fluorofarnesol to provide a sample with a specific activity of 27.7 mCi/mmol. Treatment with PBr_3 in THF at 0 °C followed by reaction with inorganic pyrophosphate gave 10-fluoro-FPP (**15**): ^{1}H NMR (D_2O, 250 MHz) δ 1.37 (3H, s), 1.39 (3H, s), 1.42 (3H, s), 1.49 (3H, s), 1.90 (6H, m), 2.19 (2H, dt, J = 6.7, 23.1 Hz), 4.27 (2H, t, J = 6.6 Hz), 5.01 (1H, t, J = 7.7 Hz), 5.25 (1H, t, J = 7.7 Hz); ^{31}P NMR (D_2O, 162 MHz) δ −9.58 (d, J = 22.4 Hz), −5.57 (d, J = 22.4 Hz).

(3) trans,trans-3,7,11-Trimethyl-2,6,11-dodecatrienyl Diphosphate (**16**). *(a) 3-Methyl-3-butenyl Mesylate* (**17**). To 180 mg of 3-methyl-3-buten-1-ol (2.02 mmol) in 5 mL of dry CH_2Cl_2 was added 30 mg of 4-(dimethylamino)pyridine and 300 μL of Et_3N (218 mg, 2.2 mmol). The mixture was cooled in ice−water, 170 μL of MsCl (252 mg, 2.2 mmol) was added dropwise by syringe, and the mixture was stirred overnight while warming to room temperature. Ether was added to the reaction mixture, which was washed successively with dilute HCl, water and aqueous K_2CO_3, and water. The ether layer was dried over $MgSO_4$ and concentrated to provide 220 mg of **17** as a colorless oil: IR (neat, cm^{-1}) 3079, 3029, 2973, 2941, 1652, 1448, 1360, 1168; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.75 (3H, s), 2.44 (2H, t, J = 6.8 Hz), 2.98 (3H, s), 4.31 (2H, t, J = 6.8 Hz), 4.77 (1H, s), 4.85 (1H, s).

(b) (E,E,E)-8-(Phenylsulfonyl)-3,7,11-trimethyl-2,6,11-dodecatrienyl Benzyl Ether (**18**). To 274 mg of (*E*)-8-(phenylsulfonyl)geranyl benzyl ether (**11**) (0.71 mmol) in 3 mL of dry THF was added 440 μL of *n*-BuLi (2.07 M, 0.91 mmol) at −78 °C. After 20 min, a THF solution of freshly prepared 3-methyl-3-butenyl mesylate (**17**) (175 mg, 0.96 mmol, 1.5 mL) was added to the orange anion solution at −78 °C. After 10 min, 3.0 mL of dry DME was added, the cooling bath was removed, and the reaction was allowed to continue for 2 h. The reaction mixture was then diluted with ether, washed with brine and water, and dried over $MgSO_4$. Concentration gave a light yellow oil, which was chromatographed on silica gel (2 × 38 cm, hexane/ethyl acetate, 4:1) to give 209 mg of **18** (0.46 mmol, 65%): IR (film, cm^{-1}) 3066, 3029, 2926, 2857, 1738, 1650, 1449, 1373, 1306; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.57 (3H, s), 1.77 (6H, s), 1.65−2.30 (8H, m), 3.45 (1H, m), 3.98 (2H, d, J = 6.6 Hz), 4.49 (2H, s), 4.63 (1H, s), 4.73 (1H, s), 5.03 (1H, t, J = 6.5 Hz), 5.29 (1H, dt, J = 6.6, 1.3 Hz), 7.35 (5H, m), 7.50 (2H, m), 7.60 (1H, m), 7.80 (2H, m).

(c) trans,trans-3,7,11-Trimethyl-2,6,11-dodecatrien-1-ol (Isofarnesol) (**19**). $EtNH_2$ (3 mL) was distilled into a flask containing 38.5 mg of freshly cut lithium at −78 °C under argon. The mixture was stirred for 10 min, and the cooling bath was removed for 30 min to ensure that the lithium was completely dissolved. The dark blue reaction mixture was cooled to −78 °C, and 100 mg of sulfone **18** (0.22 mmol)

in 2 mL of THF was added slowly by syringe. After 5 min, 0.5 mL of 3-hexyne was added to quench the reaction. The mixture was then partitioned between ether and brine. The organic phase was dried over $MgSO_4$ and concentrated to give a crude product, which was chromatographed on a silica gel column (1 × 30 cm, hexane/ethyl acetate, 4:1) to provide 41.3 mg of **19** (0.18 mmol, 84%): IR (film, cm^{-1}) 3318, 2930, 1668, 1649, 1452, 1381, 1002, 886; ^1H NMR ($CDCl_3$, 250 MHz) δ 1.30 (1H, br), 1.37 (2H, t, J = 8.6 Hz), 1.60 (3H, s), 1.68 (3H, s), 1.72 (3H, s), 2.05 (8H, m), 4.15 (2H, d, J = 6.8 Hz), 4.69 (2H, m), 5.09 (1H, t, J = 5.0 Hz), 5.42 (1H, t, J = 6.8 Hz); HRMS (EI) calcd for $C_{15}H_{26}O$ 222.1983, found 222.1978; MS (EI) 222 (22), 207 (18), 204 (15), 191 (76), 161 (35), 121 (92), 95 (30), 81 (100).

(d) trans,trans-3,7,11-Trimethyl-2,6,11-dodecatrienyl Diphosphate (**16**). The alcohol **19** was converted to its diphosphate ester **16** following the same procedure used for the preparation of FPP. The overall yield was 15%: ^1H NMR (D_2O, 250 MHz) δ 1.34 (2H, t, J = 8.0 Hz) 1.43 (3H, s), 1.52 (6H, s), 1.75−2.05 (8H, m), 4.27 (2H, t, J = 7.1 Hz), 4.28 (2H, m), 5.04 (1H, m), 5.27 (1H, m); ^{31}P NMR (D_2O, 162 MHz) δ −9.62 (d, J = 22.4 Hz), −5.77 (d, J = 22.4 Hz).

(4) trans,trans-3,7,12-Trimethyl-2,6,11-tridecatrienyl Diphosphate (**20**). *(a) 4-Methyl-3-pentenyl Iodide* (**21**). To 204 mg (2.0 mmol) of 4-methyl-3-penten-1-ol in 5.5 mL of CH_2Cl_2 at room temperature was added 31.6 mg of 4-(dimethylamino)pyridine and 300 μL of Et_3N (2.2 mmol). The solution was cooled to 0 °C, and 170 μL of MsCl (252 mg, 2.2 mmol) was added dropwise to give a white cloudy mixture, which was stirred while being warmed up to room temperature over a period of 5 h. The mixture was diluted with ether, washed with K_2CO_3 solution, dilute HCl, and water, dried over $MgSO_4$, and then concentrated to give 144 mg of crude mesylate. This crude sample was dissolved in 10 mL of acetone, to which 1.0 g of LiI was added. The mixture was stirred under gentle reflux. After 30 min, the mixture was cooled, filtered, and concentrated to give a dark orange oil, which was filtered through a silica gel column (1 × 5 cm) and eluted with hexane. The pink hexane eluate was washed with an aqueous K_2CO_3 solution and water, dried over $MgSO_4$, and concentrated to give 230 mg of iodide **21** (53%), which was used immediately for the next step: IR (film, cm^{-1}) 2968, 2927, 2858, 1667, 1448, 1375; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.59 (3H, s), 1.68 (3H, s), 2.56 (2H, m), 3.09 (2H, t, J = 7.4 Hz), 5.07 (1H, t, J = 7.2 Hz).

(b) (E,E,E)-8-(Phenylsulfonyl)-3,7,12-trimethyl-2,6,11-tridecatrienyl Benzyl Ether (**22**). To 76.6 mg (0.20 mmol) of (*E*)-8-(phenylsulfonyl)geranyl benzyl ether (**11**) in 2.0 mL of THF was added 0.11 mL of *n*-BuLi (2.0 M) at −78 °C under N_2 to give a light orange solution. The cooling bath was removed for 5 min, and then the mixture was recooled to −78 °C and 102.5 mg of freshly prepared **21** (0.48 mmol) in 1.0 mL of THF was added. The cooling bath was removed and the reaction was allowed to continue for 3 h. Normal workup gave 111.2 mg of crude oil, which was chromatographed on a silica gel column (1 × 33 cm, hexane/EtOAc, 9:1) to provide 70 mg of **22** (1.5 mmol, 75%): IR (film, cm^{-1}) 3062, 3029, 2924, 2856, 1670, 1337, 1378, 1324, 1145; ^1H NMR (250 MHz) δ 1.52 (3H, s), 1.57 (3H, s), 1.67 (6H, s), 1.4−2.2 (8H, m), 3.48 (1H, dd, J = 3.6, 10.2 Hz), 3.98 (2H, d, J = 6.8 Hz), 4.49 (2H, s), 5.02 (2H, t, J = 7.0 Hz), 5.30 (1H, t, J = 6.8 Hz), 7.3 (5H, m), 7.49 (2H, m), 7.56 (1H, m), 7.78 (2H, m); HRMS (FAB) calcd for $C_{29}H_{39}SO_3$ (M^+ + H) 467.2620, found 467.2626; MS (FAB) *m/z* (rel intensity) 467 (35), 375 (2), 358 (2.5), 217 (20), 147 (18), 91 (95), 69 (100).

(c) trans,trans-3,7,12-Trimethyl-2,6,11-tridecatrien-1-ol (C16 Homofarnesol) (**23**). $EtNH_2$ (2.5 mL) was distilled into a flask containing 40 mg of Li at −78 °C under argon. The mixture was warmed to room temperature and stirred for 30 min until all the lithium had dissolved. The dark blue solution was recooled to −78 °C, and 60 mg (12.8 μmol) of sulfone **22** in 1.0 mL of THF was added through a cannula. After 2 min, the yellow solution was diluted with ether to 35 mL, which was washed with water (3 × 5 mL) and dried over $MgSO_4$. The crude product was chromatographed on a silica gel column (1 × 41 cm, hexane/EtOAc, 3:2) to give 20 mg of C16 homofarnesol (**23**) (85 μmol, 67%): IR (neat, cm^{-1}) 3318, 2965, 2927, 2857, 1669, 1445, 1378, 1003; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.25 (1H, br), 1.45 (2H, m), 1.60 (6H, s), 1.68 (3H, s), 1.69 (3H, s), 2.0 (8H, m), 4.16 (2H, d, J = 6.9 Hz), 5.13 (2H, m), 5.41 (1H, t, J = 6.9 Hz); HRMS (CI) calcd for $C_{16}H_{28}O$ 236.2133, found 236.2127; MS (CI) *m/z* (rel intensity) 237 (2), 236 (22), 219 (10), 218 (6), 175 (17), 151 (20), 149 (58), 137 (25), 135 (27), 123 (45), 121 (35), 109 (62), 107 (62), 95 (100), 93 (60), 69 (73).

(d) trans,trans-3,7,12-Trimethyl-2,6,11-tridecatrienyl Diphosphate (Homo-FPP) (**20**). Homofarnesol **22** was transformed into its diphosphate ester following the procedure for FPP synthesis. The overall yield was 52%: ^{31}P NMR (D_2O, 162 MHz) δ −9.6 (d, J = 22.4 Hz), −5.6 (d, J = 22.4 Hz).

Determination of the Steady-State Kinetic Parameters for (3R)-NPP, cis,trans-FPP, and FPP. Trichodiene synthase from *F. sporotrichioides* was assayed as previously described (Cane et al., 1990a) in buffer H (25 mM Hepes, pH 7.5 (adjusted with NaOH), 3 mM $MgCl_2$, and 1 mM DTT) using varying concentrations of [1-^{3}H]-(3*R*)-NPP (**3**) (22.1 mCi/mmol) and [1-^{3}H]FPP (**1**) (16.7 mCi/mmol). Each series of assays was measured in duplicate using seven different concentrations of NPP, ranging from 22.6 to 681 nM, and seven different concentrations of FPP, from 24.3 to 1217 nM, also in duplicate. A set of seven parallel incubations using the same concentrations of NPP in the absence of enzyme was also run. Each incubation was initiated by the addition of 150 μL (2.2×10^{-2} unit) of trichodiene synthase to give a total volume of 6 mL, and the mixture was overlaid with 2 mL of hexane and incubated for 7 min at 30 °C. The incubation conditions were chosen to give less than 10% conversion to products. The reaction was quenched by the addition of 0.6 mL of 100 mM EDTA sodium salt (pH 7.2 in buffer H), and the mixture was chilled in ice−water. After the mixture was vortex mixed for 20 s and rinsed with 0.5 mL of hexane, the hexane extract was passed through a 0.5 × 3 cm column of silica gel in a Pasteur pipet directly into a scintillation vial containing 5 mL of Optifluor. The aqueous phase was extracted with an additional 2 mL of hexane, which was passed through the same silica gel column. Finally, the column was rinsed with an additional 0.5 mL of hexane. For the greatest precision, all assays were conducted with the same batch of diluted enzyme within the shortest period of time. An analogous set of assays was also carried out in duplicate on both [1-^{3}H]-*cis,trans*-FPP (152 mCi/mmol) (seven concentrations from 6.15 to 369 nM) and [1-^{3}H]FPP (682 mCi/mmol) (seven concentrations from 13.9

Table 1: Comparison of Steady-State Kinetic Parameters of Allylic Diphosphate Substrates of Trichodiene Synthase[a]

substrate	K_m (nM)	V_{max} (rel)	(V_{max}/K_m) (rel)
Native *F. sporotrichioides* Trichodiene Synthase			
t,t-FPP	90 ± 6	100[b]	100
(3*R*)-NPP	89 ± 8	63	63
c,t-FPP	36 ± 3	50	125
Recombinant *F. sporotrichioides* Trichodiene Synthase			
t,t-FPP	91 ± 10	100[c]	100
(3*R*)-NPP	90 ± 5	59	60

[a] Assays were carried out in buffer H as described under Materials and Methods, and the results were fit directly by nonlinear least-squares methods to the Michaelis–Menten equation. [b] Specific activity: 105 units/mg. [c] Specific activity 220 units/mg.

to 697 nM) using 50 μL (1.8×10^{-3} unit) of trichodiene synthase in a total assay volume of 1.5 mL. Incubations were carried out for 10 min at 30 °C before quenching with 0.15 mL of 100 mM EDTA sodium salt (pH 7.2 in buffer H) and extraction and analysis in the usual manner. The assays were also repeated with freshly prepared recombinant trichodiene synthase from *E. coli* BL21(DE3)/pZW03 using [1-^{3}H]FPP (71.7 mCi/mmol) and [1-^{3}H]-(3*R*)-NPP (20.1 mCi/mmol) and following the same procedures.

Inhibition of Trichodiene Synthase. In a typical set of experiments using *F. sporotrichioides* trichodiene synthase, 10-fluoro-FPP (**15**) was tested as an inhibitor using seven concentrations of FPP (682 mCi/mmol) from 20.9 to 628 nM and six concentrations of **15** from 0 to 127 nM. All assays were run in duplicate. Each reaction was initiated by the addition of 10 μL of diluted trichodiene synthase (2.7×10^{-4} unit) to 390 μL of buffer H containing both FPP and 10-fluoro-FPP. The mixture was overlaid with 0.5 mL of hexane and incubated for 10 min at 30 °C before quenching with 150 μL of EtOH, extraction with hexane, and radioactivity analysis.

Analogous procedures were used to test both iso-FPP (**16**) and homo-FPP (**20**) as inhibitors, using six concentrations of FPP (71.7 mCi/mmol) and six concentrations of either **16** or **20**. All assays were carried out in a total volume of 2 mL, and the incubations were quenched with 0.2 mL of 100 mM EDTA sodium salt (pH 7.2 in Buffer H). Farnesylphosphono phosphate (**7**), farnesyl (phosphonomethyl)-phosphonate (**8**), and the cyclopropylidene analog of GPP (**9**) were each tested as inhibitors in an analogous fashion, using recombinant trichodiene synthase in buffer T.

RESULTS

Determination of V_{max} and K_m for FPP, cis,trans-FPP, and (3R)-NPP. To determine the steady-state kinetic parameters for trichodiene synthase, a constant amount of cyclase was assayed in the presence of variable concentrations of substrate ranging from one-sixth to 7–10 times K_m. The data were fit to eq 1 using a nonlinear least-squares regression program to calculate both V_{max} and K_m (Table 1) In the case of (3*R*)-NPP, a blank control was run without enzyme at each NPP concentration to correct for the significant nonenzymatic solvolysis of NPP. To compare precisely the kinetic behavior of FPP and (3*R*)-NPP, as well as that of FPP and *cis,trans*-FPP, the V_{max} and K_m of each pair were determined at the same time using the same enzyme preparation under identical incubation conditions. The values of V_{max} and K_m, which were also determined for FPP and (3*R*)-NPP using overexpressed recombinant trichodiene synthase, were identical within experimental error with those obtained for the native fungal enzyme (Table 1).

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

Inhibitors of Trichodiene Synthase. (1) 10-Fluorofarnesyl Diphosphate (**15**). Reaction of the lithio anion of (*E*)-8-(phenylsulfonyl)geranyl benzyl ether (**11**) with 2-fluoro-3,3-dimethylallyl bromide in THF gave **12**, which was treated with lithium and *tert*-butyl alcohol in THF at room temperature to provide 10-fluorofarnesol (**13**) (37%) along with an equal quantity of 10-fluoro-6,7-dihydro-7,8-dehydrofarnesol (**14**). Oxidation of 10-fluorofarnesol to 10-fluorofarnesal followed by reduction with [^{3}H]$NaBH_4$ gave [1-^{3}H]-10-fluorofarnesol (**14**). After dilution of the tritiated alcohol with unlabeled 10-fluorofarnesol, the product was converted to the allylic bromide by treatment with PBr_3 and thence to 10-fluoro-FPP (**15**, 27.7 mCi/mmol) by reaction with tris-(tetra-*n*-butylammonium) pyrophosphate (Davisson et al., 1986) (Scheme 4).

(2) 3,7,11-Trimethyl-2,6,11-dodecatrienyl Diphosphate (iso-FPP) (**16**) *and 3,7,12-Trimethyl-2,6,11-tridecatrienyl Diphosphate (C_{16} Homo-FPP)* (**20**). Both iso-FPP (**16**) and homo-FPP (**20**) were prepared by routes similar to that used for the synthesis of 10-fluoro-FPP (Scheme 5). Thus, **11** was coupled with either 3-methylbut-3-enyl mesylate (**17**) or 4-methylpent-3-enyl iodide (**21**) to provide sulfone **18** or **22**, respectively. Each sulfone was reduced with Li/$EtNH_2$ to give isofarnesol (**16**) and homofarnesol (**23**), respectively, which were each converted to the corresponding diphosphates **16** and **20** in the usual manner.

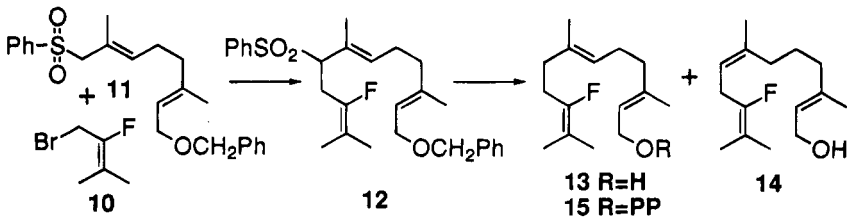
The three FPP analogs, **15**, **16**, and **20**, were each tested as inhibitors of trichodiene synthase. The data were analyzed by double-reciprocal plots, and the K_i for each inhibitor and the K_m for the substrate FPP were obtained by nonlinear least-squares methods by direct fitting to eq 2 for competitive inhibition. A typical set of data is illustrated in Figure 1 for 10-fluoro-FPP (**15**), and the results are summarized in Table 2.

$$v = \frac{V_{max}[S]}{K_m\{1 + 1/K_I\} + [S]} \quad (2)$$

All three substrate analogs proved to be effective competitive inhibitors of trichodiene synthase, with 10-fluoro-FPP being the most potent (K_I = 16 ± 1 nM). Although each analog was turned over to varying degrees, no attempt was made to identify the various olefinic or alcoholic products. Significantly, none of these analogs showed evidence of the time-dependent, irreversible inactivation of trichodiene synthase.

Three additional FPP analogs were also tested as inhibitors using recombinant trichodiene synthase. Farnesylphosphono phosphate (**7**), which is unable to undergo the usual allylic diphosphate ionization, behaved as a mixed noncompetitive inhibitor, as indicated by the double-reciprocal plot (Figure 2), with a K_{I1} for free enzyme of 3.25 ± 1.08 μM and a K_{I2} for the enzyme–substrate complex of 9.10 ± 2.43 μM, compared to the K_m for FPP of 82 ± 16 nM, calculated by fitting to eq 3. A second unreactive FPP analog, the (phosphonomethyl)phosphonate **8**, was a relatively poor

Scheme 4: Synthesis of 10-Fluoro-FPP (**15**)



Scheme 5: Synthesis of Iso-FPP (**16**) and Homo-FPP (**20**)

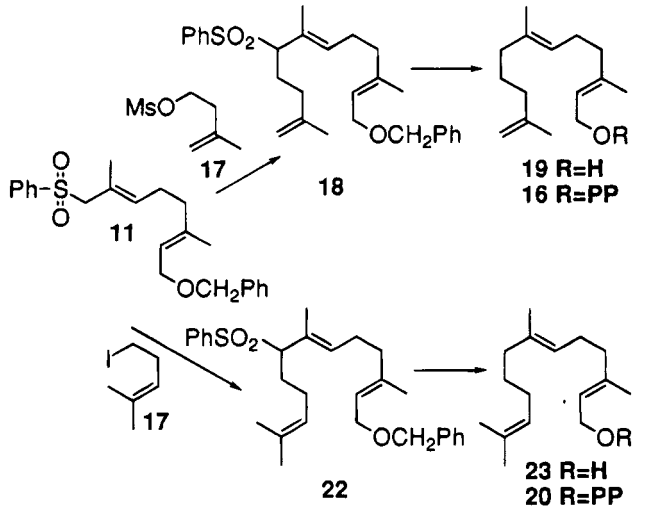


Table 2: Inhibitors of Trichodiene Synthase

inhibitor	inhibition type	K_I (nM)	K_m(FPP) (nM)
15[a]	competitive	16 ± 1	87 ± 5
16[a]	competitive	127 ± 15	109 ± 4[b]
20[a]	competitive	492 ± 126	110 ± 5[b]
7[c]	mixed	K_{I1} = (3.25 ± 1.08) × 10^3	82 ± 16
	noncompetitive	K_{I2} = (9.10 ± 2.43) × 10^3	
8[c]	competitive	(5.25 ± 10.55) × 10^3	70 ● 6
9[c]	competitive	117 ± 19	nd[d]

[a] Assays were carried out using native *F. sporotrichioides* trichodiene synthase in buffer H, as described in Materials and Methods. [b] The slight increase in apparent K_m is probably due to the use of a different preparation of [1-^{3}H]FPP for the assays. [c] Assays were carried out using recombinant trichodiene synthase from *E. coli* BL21(DE3)/pZW03 in buffer T, as described in Materials and Methods. [d] nd, not determined.

competitive inhibitor, with a K_I of 5.3 ± 0.6 μM. Finally, **9**, which is a potent irreversible inhibitor of a variety of monoterpene synthases, was a simple competitive inhibitor of trichodiene synthase with a K_I of 117 nM.

$$v = \frac{V_{max}[S]}{K_m\{1 + 1/K_{I1}\} + [S]\{1 + 1/K_{I2}\}} \quad (3)$$

DISCUSSION

Substrate Specificity. Several lines of evidence have provided strong, albeit indirect, support for the intermediacy of (3*R*)-NPP (**3**) in the conversion of *trans,trans*-FPP to trichodiene (Scheme 2). On the other hand, (3*R*)-NPP has never been directly detected in the incubation, and competition experiments have indicated that (3*R*)-NPP remains enzyme-bound (Cane & Ha, 1988). Comparison of the steady-state kinetic parameters for both (3*R*)-NPP and *cis, trans*-FPP with those of *trans,trans*-FPP has now revealed

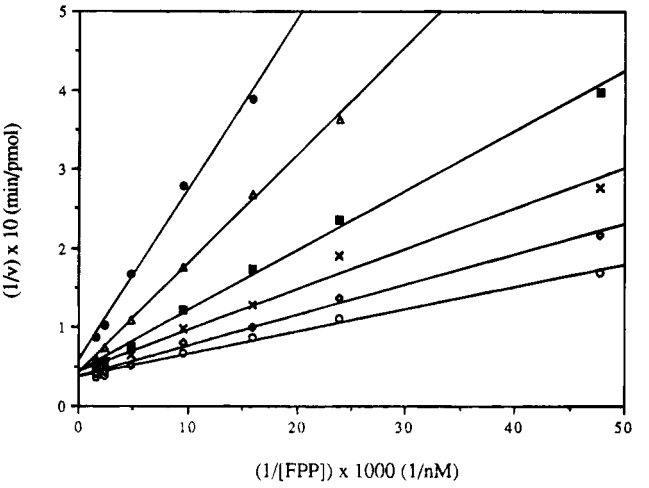


FIGURE 1: Competitive inhibition of trichodiene synthase by 10-fluoro-FPP (I). Activity assays were carried out as described in Materials and Methods. [FPP]: 20.9, 41.9, 62.8, 104.7, 209.4, 418.8, and 628.1 nM. Symbols: ○, [I] = 0 nM; ◇, [I] = 10.6 nM; ×, [I] = 21.2 nM; ■, [I] = 31.8 nM; △, [I] = 63.6 nM; ●, [I] = 127.2 nM.

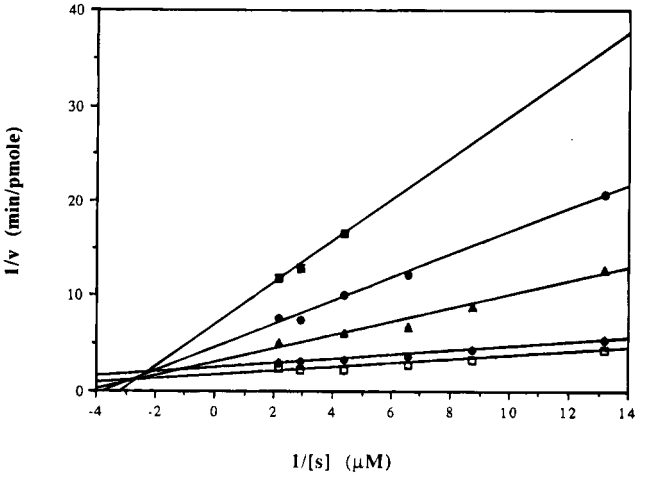
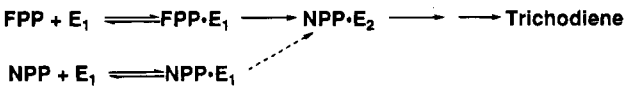


FIGURE 2: Mixed noncompetitive inhibition of trichodiene synthase by farnesyl phosphonate **7**. Activity assays were carried out as described in Materials and Methods. Symbols: □, [I] = 0 μM; ◆, [I] = 2 μM; ▲, [I] = 10 μM; ●, [I] = 15 μM; ■, [I] = 24 μM.

unexpected differences from the well-documented behavior of the analogous allylic diphosphate substrates of monoterpene synthases (Croteau, 1987). Thus, the homologous tertiary allylic esters (3*R*)- or (3S)-linalyl diphosphate, demonstrated intermediates in the formation of a variety of cyclic monoterpenes, typically display values of both V_{max} and V_{max}/K_m that are 2−7 times greater than the corresponding steady-state kinetic parameters for geranyl diphosphate, while the cis isomer, neryl diphosphate, normally has the lowest V_{max} and V_{max}/K_m values of the three substrates (Croteau & Karp, 1979; Croteau et al., 1980a,b, 1985). These results are all consistent with the intermediacy of the

Scheme 6: Postulated Conformational Change in Trichodiene Synthase from Free Enzyme (E_1), Which Can Bind Either FPP or Free NPP, to the Catalytically Active State, Which Cyclizes Enzymatically Generated (3*R*)-NPP (E_2)



tertiary allylic diphosphate ester in the formation of cyclic monoterpenes. By contrast, although the K_m for the natural substrate for trichodiene synthase, *trans,trans*-FPP (**1**), is essentially identical to that for (3*R*)-NPP (**3**), the V_{max} and V_{max}/K_m values for *trans,trans*-FPP are 1.5 times greater than the corresponding values for (3*R*)-NPP.[2] Moreover, although the V_{max} for *cis,trans*-FPP is 50% that of FPP, the K_m is 36% that of the trans,trans substrate, resulting in a slightly higher V_{max}/K_m value for the cis,trans isomer. According to the currently favored mechanism, however, trichodiene synthase does not normally encounter either free (3*R*)-NPP or *cis, trans*-FPP in solution. Thus, the relative V_{max}/K_m values, which reflect the rate of reaction of free enzyme with free substrate, are of uncertain relevance in comparing the relative kinetic competence of the three allylic diphosphate isomers. Instead, the relative values of V_{max}, which depend on the rate at which *bound* substrate is converted to product, may be a better reflection of the inherent kinetic competence of each substrate. On these grounds, *cis,trans*-FPP clearly is the least favored substrate surrogate.

Nonetheless, the apparent V_{max} of the nominal intermediate, (3*R*)-NPP, is at first still surprising since the presumptive intermediate appears to be turned over at two-thirds the rate of its nominal precursor FPP. The source of this apparent anomaly would appear to be that the conformational state (E_2) of trichodiene synthase, which normally binds *enzymatically generated* (3*R*)-NPP, may well be different from the conformation of the free enzyme (E_1), which encounters (3*R*)-NPP in solution (cf. Scheme 6). The measured V_{max} for the tertiary allylic diphosphate **3** therefore may include a slow conformational change of the trichodiene synthase once it has bound (3*R*)-NPP. Such steady-state kinetic anomalies, while rare, are well-precedented and have been described by Cleland in a discussion of the factors that affect the apparent kinetic competence of presumptive enzyme intermediates (Cleland, 1970). Indeed, as shown by Kluger for pyruvate decarboxylase, some intermediates may not be processed at all when encountered free in solution, leading to the notion of closed transition states for some enzyme-catalyzed reactions (Kluger & Smyth, 1981). In the latter respect, it is worth noting that, in catalyzing the isomerization−cyclization of FPP, trichodiene synthase must rigorously shield the intervening carbocationic intermediates from premature quenching by water.

Inhibitors of Trichodiene Synthase. The cyclization of FPP to trichodiene requires not only that the substrate be folded into a specific conformation but also that some degree of control be exercised over the formation of a series of intermediates of varying shape and charge distribution. In order to better understand the role played by the enzyme in this program of conformational and structural changes, we have tested a series of FPP analogs as inhibitors of trichodiene synthase. Simple substitution of a fluorine for the hydrogen atom at C-10 gave an inhibitor, **15**, with a K_I of ca. 20% of the K_m of the natural substrate. Iso-FPP (**16**), in which the 10,11-double bond was replaced by an isomeric 11,12-double bond, was still a reasonably effective inhibitor, with a K_I/K_m of 1.2. By contrast, the additional methylene carbon in homo-FPP (**20**) increased the K_I/K_m to 4.5. The K_I value for **20** can be compared with those of both (7*R*)-6,7-dihydro-FPP (**24**) (K_I = 220 ± 40 nM) and (7*S*)-6,7-dihydro-FPP (**25**) (K_I = 395 ± 40 nM), as well as with that of inorganic pyrophosphate alone (K_I = 495 ± 20 nM) (Cane et al., 1990a).

The phosphono phosphate analog of FPP, **7**, has previously been shown to be an effective competitive inhibitor of squalene synthase, an enzyme that catalyzes the ionization of the allylic diphosphate C−O ester bond of FPP as the first step in the formation of presqualene diphosphate (Corey & Volante, 1976; Biller et al., 1988, 1991). Replacement of the ester oxygen with a methylene gives an unreactive analog that is capable of competing with FPP for the electrophilic active site. When **7** was tested as an inhibitor of trichodiene synthase, it showed mixed noncompetitive inhibition, with the individual inhibition constants each greater than the K_m for FPP by factors of 40−110. The poorer binding of the phosphono phosphate analog is probably due to a combination of the greater steric demand and reduced electronegativity of the methylene group. Similar reductions in binding affinity have been reported for phosphonate analogs of phosphate esters and nucleotides (Blackburn, 1981; Blackburn et al., 1984). The closely related ether derivative **8** is an extremely potent competitive inhibitor of rat liver microsomal squalene synthase, with an I_{50} of 0.05 μM under conditions at which **7** has an I_{50} of only 42 μM (Biller et al., 1988, 1991). Biller has postulated that insertion of the methylene group between the analog of the pyrophosphate moiety and the original allylic ester bridging oxygen atom results in a geometry that may approximate the normal transition-state separation of the farnesyl cation and pyrophosphate anion. He has also speculated that the ether oxygen of **8** might be hydrogen-bonded to a Lewis acid residue, which normally catalyzes substrate ionization. When tested with trichodiene synthase, which catalyzes an analogous ionization of FPP, **8** was only a rather modest competitive inhibitor, with K_I(**8**)/K_m(FPP) = 70. By contrast, the GPP analog **9** was a surprisingly effective competitive inhibitor of trichodiene synthase, with K_I(**9**)/K_m(FPP) = 1.5, in spite of the fact that the prenyl moiety has been shortened by one isoprene unit.

CONCLUSIONS

Sesquiterpene synthases are responsible for the formation of some 200 distinct cyclic sesquiterpenes. In spite of the diversity of individual cyclization products, each of these enzymes uses a common substrate, *trans,trans*-FPP, and variations of a common cyclization mechanism to generate its characteristic product. The means by which each enzyme imposes a specific folding on the initially bound substrate

[2] We have previously reported competition experiments between FPP and racemic NPP, using crude extracts of *T. roseum*, which had led to an estimated relative V_{max}/K_m for NPP that was 1.5−2 times that of FPP. The apparent disagreement with the more precisely determined values of V_{max} and K_m reported here for each substrate with homogeneous *F. sporotrichioides* trichodiene synthases from both native fungal and recombinant sources may reflect either inherent kinetic differences between the *T. roseum* and *F. sporotrichioides* enzymes or, more likely, errors caused by competing phosphatases present in the crude *T. roseum* extracts.

and stabilizes the numerous cyclization intermediates are still obscure. The studies described here have shed new light on the factors affecting substrate and intermediate recognition by trichodiene synthase and revealed a surprising degree of permissiveness in the range of structures that can be bound. The substrates and intermediates described here are expected to be of further utility in the development of mechanism-based inactivators of trichodiene synthase and related enzymes, in the detailed characterization of trichodiene synthase mutants such as those described in the paper that follows (Cane et al., 1995), and in ongoing studies of the structure of the protein itself.

ACKNOWLEDGMENT

We thank Prof. John P. McCormick of the University of Missouri at Columbia for making available samples of 10-fluorofarnesol and for providing unpublished details of the preparation of **10**.

REFERENCES

Biller, S. A., & Forster, C. (1990) *Tetrahedron 46*, 6645−6658.
Biller, S. A., Forster, C., Gordon, E. M., Harrity, T., Scott, W. A., & Ciosek, C. P. (1988) *J. Med. Chem 31*, 1869−1871.
Biller, S. A., Sofia, M. J., Delange, B., Forster, C., Gordon, E. M., Harrity, T., Rich, L. C., & Ciosek, C. P. (1991) *J. Am. Chem. Soc. 113*, 8522−8524.
Blackburn, G. M. (1981) *Chem. Ind. (London)* 134−138.
Blackburn, G. M., Kent, D. E., & Kolkmann, F. (1984) *J. Chem. Soc., Perkin Trans. 1*, 1119−1125.
Bradford, M. (1976) *Anal. Biochem. 72*, 248−254.
Cane, D. E. (1985) *Acc. Chem. Res. 18*, 220−226.
Cane, D. E. (1990) *Chem. Rev. 9*, 1089−1103.
Cane, D. E., & Ha, H. (1988) *J. Am. Chem. Soc. 110*, 6865−6870.
Cane, D. E., & Yang, G. (1994) *J. Org. Chem. 59*, 5794−5798.
Cane, D. E., Swanson, S., & Murthy, P. P. N. (1981) *J. Am. Chem. Soc. 103*, 2136−2138.
Cane, D. E., Abell, C., & Tillman, A. M. (1984) *Bioorg. Chem. 12*, 312−328.
Cane, D. E., Ha, H., Pargellis, C., Waldmeier, F., Swanson, S., & Murthy, P. P. N. (1985) *Bioorg. Chem. 13*, 246−265.
Cane, D. E., Pawlak, J. L., Horak, R. M., & Hohn, T. M. (1990a) *Biochemistry 29*, 5476−5490.
Cane, D. E., McIlwaine, D. B., & Pascoe, K. O. (1990b) *Tetrahedron Lett. 31*, 7553−7554.
Cane, D. E., Yang, G., Coates, R. M., Pyun, H., & Hohn, T. M. (1992) *J. Org. Chem. 57*, 3454−3462.
Cane, D. E., Wu, Z., Oliver, J. S., & Hohn, T. M. (1993) *Arch. Biochem. Biophys. 300*, 416−422.
Cane, D. E., Shim, J. H., Xue, Q., Fitzsimons, B. C., & Hohn, T. M. (1995) *Biochemistry 34*, 2480−2488.
Cleland, W. W. (1970) in *Enzymes* (Boyer, P. D., Ed.) Vol. 2, pp 1−65, Academic, New York.
Corey, E. J., & Volante, R. P. (1976) *J. Am. Chem. Soc. 98*, 1291−1293.
Croteau, R. (1987) *Chem. Rev. 87*, 929−954.
Croteau, R., & Karp, F. (1979) *Arch. Biochem. Biophys. 198*, 512−522.
Croteau, R., Felton, M., & Ronald, R. C. (1980a) *Arch. Biochem. Biophys. 200*, 524−533.
Croteau, R., Felton, M., & Ronald, R. C. (1980b) *Arch. Biochem. Biophys. 200*, 534−546.
Croteau, R., Satterwhite, D. M., Cane, D. E., & Chang, C. C. (1986) *J. Biol. Chem. 261*, 13438−13445.
Croteau, R., Satterwhite, D. M., Cane, D. E., & Chang, C. C. (1988) *J. Biol. Chem. 263*, 10063−10071.
Croteau, R., Alonso, W. R., Koepp, A. E., Shim, J. H., & Cane, D. E. (1993) *Arch. Biochem. Biophys. 307*, 397−404.
Davisson, V. J., Woodside, A. B., Neal, T. R., Stremler, K. E., Muehlbacher, M., & Poulter, C. D. (1986) *J. Org. Chem. 51*, 4768−4779.
Evans, R., & Hanson, J. R. (1976) *J. Chem. Soc., Perkin Trans. 1*, 326−329.
Hohn, T. M., & VanMiddlesworth, F. (1986) *Arch. Biochem. Biophys. 251*, 756−761.
Hohn, T. M., & Beremand, M. N. (1989a) *Appl. Environ. Microbiol. 55*, 1500−1503.
Hohn, T. M., & Beremand, P. D. (1989b) *Gene 79*, 131−138.
Hohn, T. M., & Plattner, R. D. (1989) *Arch. Biochem. Biophys. 275*, 92−97.
Kluger, R., & Smyth, T. (1981) *J. Am. Chem. Soc. 103*, 1214−1216.
Satterwhite, D. M., Wheeler, C. J., & Croteau, R. (1985) *J. Biol. Chem. 260*, 13901−13908.

BI942076B