Mechanism of Taxadiene Synthase, a Diterpene Cyclase That Catalyzes the First Step of Taxol Biosynthesis in Pacific Yew†

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ABSTRACT: The first committed step in the formation of taxol has been shown to involve the cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene. The formation of this endocyclic diterpene olefin isomer as the precursor of taxol was unexpected, since the exocyclic isomer, taxa-4(20),11(12)-diene, had been predicted as the initial product of the taxol pathway on the basis of metabolite co-occurrence. [1-$^2H_2$,20-$^2H_3$] and [20-$^2H_3$]geranylgeranyl diphosphates were employed as substrates with the partially purified taxadiene synthase from Pacific yew (*Taxus brevifolia*) stems to examine the possibility of a preliminary cyclization to taxa-4(20),11(12)-diene followed by isomerization to the more stable endocyclic double bond isomer. GLC-MS analysis of the derived taxa-4(5),11(12)-diene, via selected ion monitoring of the parent ion and the P-15 and C-ring fragment ions, compared to those of unlabeled standard, showed the olefin product to possess a deuterium enrichment essentially identical to that of the acyclic precursor, thus ruling out the putative isomerization step. With [4-$^2H_2$]geranylgeranyl diphosphate as substrate, similar product analysis established the enzymatically derived taxa-4(5),11(12)-diene to contain only one deuterium atom, consistent with direct formation from a taxenyl cation by deprotonation at C5. (±)-Casbene, (±)-verticillene, and (±)-taxa-4(20),11(12)-diene were tested as possible olefinic intermediates in taxa-4(5),11(12)-diene formation by a series of inhibition, trapping, and direct conversion experiments; no evidence was obtained that these exogenous olefins could serve as intermediates of the cyclization reaction. However, GLC-MS analysis of the taxadiene product derived by enzymatic cyclization of [1-^{3}H]-geranylgeranyl diphosphate in ^2H_2O indicated little incorporation of deuterium from the medium and suggested a rapid internal proton transfer in a tightly bound olefinic intermediate. Analysis of the enzymatic product generated from [10-$^2H_1$]geranylgeranyl diphosphate confirmed the intramolecular hydrogen transfer from C11 of a verticillyl intermediate to the C-ring of taxa-4(5),11(12)-diene. From these results, a stereochemical mechanism is proposed for the taxadiene synthase reaction involving the initial cyclization of geranylgeranyl diphosphate to a transient verticillyl cation intermediate, with transfer of the C11 α-proton to C7 to initiate transannular B/C-ring closure to the taxenyl cation, followed by deprotonation at C5 to yield the taxa-4(5),11(12)-diene product directly.

The novel diterpenoid taxol (**8**) (paclitaxel)[1] (Wani et al., 1971) is now well-established as a potent chemotherapeutic agent, showing excellent activity against a range of cancers including ovarian and breast cancer (Holmes et al., 1995; Arbuck & Blaylock, 1995). The limited supply of the drug from the original source, the bark of the Pacific yew (*Taxus brevifolia* Nutt.; Taxaceae), prompted intensive efforts to develop alternate means of production (Kingston, 1991; Cragg et al., 1993). Semisyntheses of taxol and its analog taxotere (docetaxel)[1] (Guénard et al., 1993), from advanced taxane diterpenoid (taxoid) metabolites that are more readily available from natural sources, have been developed as an interim measure (Holton et al., 1995; Commerçon, 1995). However, with increasing applications in chemotherapy, both in treatment of additional cancer types and in use much earlier in the course of intervention, the supply and cost of these drugs will remain important issues (Suffness, 1995). Total synthesis of taxol has been achieved by several elegant routes (Nicolaou et al., 1994; Holton et al., 1994a,b), but the yields are too low to be commercially viable (Borman, 1994) and it is clear that in the foreseeable future the supply of taxol and its synthetically useful progenitors must rely on biological methods of production, either in *Taxus* species or in cell cultures derived therefrom (Suffness, 1995). The development of improved biological processes should be based upon a detailed understanding of the pathway for taxol biosynthesis, the enzymes catalyzing the sequence of reactions, especially the slow steps, and the genes encoding these proteins.

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[1] Paclitaxel is the generic name for taxol, which is now a registered trademark of Bristol-Myers Squibb. Because of the far greater familiarity with the word taxol, we use it in this paper in lieu of paclitaxel. Docetaxel is the generic name for taxotere which is a registered trademark of Rhone-Poulenc. The structural differences between taxol (Scheme 1) and taxotere are a *tert*-butoxycarbonyl group instead of a benzoyl group on the nitrogen atom at C3′ of the C13 side chain and a hydroxyl function instead of an acetate at the C10 position of the taxane B-ring.

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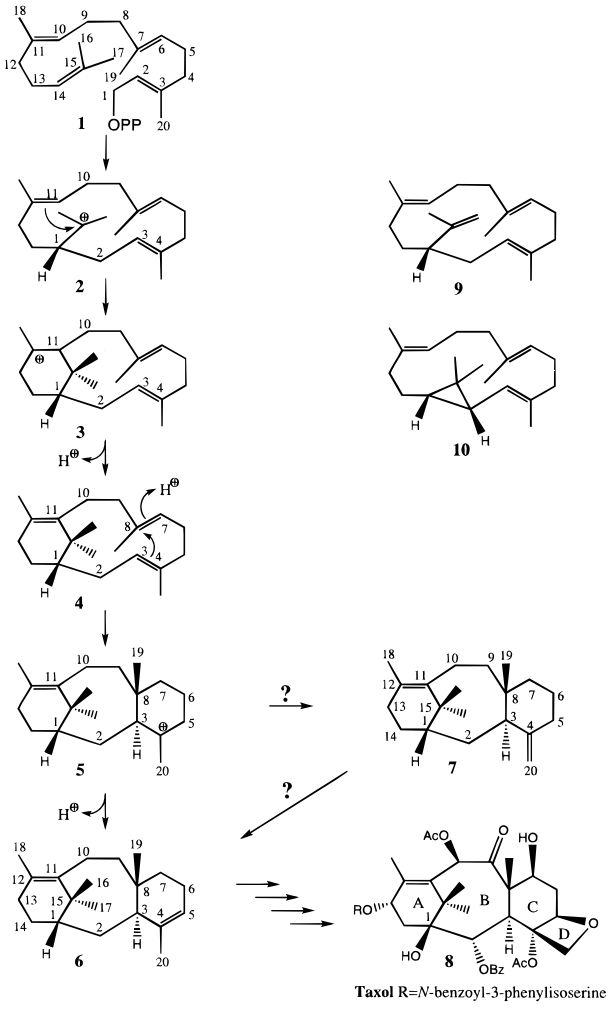
Scheme 1. Proposed Scheme for Cyclization of Geranylgeranyl Diphosphate to Taxadiene Showing Possible Olefinic Intermediates of the Reaction



Taxol R=*N*-benzoyl-3-phenylisoserine

The biosynthesis of taxol is considered to involve the initial cyclization of geranylgeranyl diphosphate (**1**), the universal precursor of diterpenoids (West, 1981), to an olefin with the taxane skeleton, followed by extensive oxidative modification and elaboration of the side chains (Guéritte-Voegelein et al., 1987; Floss & Mocek, 1995). The initial cyclization reaction traditionally has been formulated (Scheme 1) to proceed by conversion of geranylgeranyl diphosphate (**1**) to a verticillene (**4**) intermediate followed by protonation at C7, closure of the B- and C-rings, and deprotonation to taxa-4(20),11(12)-diene (**7**) (Harrison et al., 1966; Guéritte-Voegelein et al., 1987). Like verticillene (**4**), casbene (**10**) or a cembrene-type olefin (**9**) (Scheme 1) could conceivably serve as a possible intermediate in the conversion of geranylgeranyl diphosphate to taxadiene; however, chemical model studies have thus far yielded no evidence to support the proposed intermediacy of such olefins in taxane formation (Dauben et al., 1979; Begley et al., 1990).

Postulation of taxa-4(20),11(12)-diene (**7**) as the initial cyclic product with the taxane skeleton and the olefinic progenitor of taxol (**8**) was largely based upon the now well-established observation (Kingston et al., 1993) that a large number of naturally occurring taxoids bear double bonds in these positions, and that the sequential conversion of the 4(20)-exocyclic methylene group to the oxirane and, thence, to the oxetane ring of taxol could be readily envisioned (Guéritte-Voegelein et al., 1987; Floss & Mocek, 1995). However, it was recently demonstrated that a cell-free preparation from Pacific yew stems catalyzed the cyclization of [1-^{3}H]geranylgeranyl diphosphate (**1**) to [^{3}H]taxa-4(5),-11(12)-diene (**6**) as the only detectable product and that this endocyclic olefin isomer was converted in good yield to taxol (**8**) and closely related taxoids *in vivo* (Koepp et al., 1995). The responsible enzyme, taxadiene synthase, has been partially purified from extracts of yew stem and characterized and shown to be a fairly typical terpenoid cyclase (Hezari et al., 1995). Thus, it appears that the first dedicated step in taxol biosynthesis is the conversion of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene (**6**), rather than to the 4(20),11(12)-diene isomer (**7**) previously suggested on the basis of metabolite abundance. However, it is possible that taxa-4(20),11(12)-diene (**7**) could be formed first followed by isomerization to the more stable 4(5),11(12)-isomer (**6**) (Scheme 1), either as a natural consequence of the enzymatic cyclization reaction or, potentially, as an artifact. A series of experiments were carried out to test this possibility and to examine the involvement of verticillene (**4**) and other potential olefinic intermediates in the reaction cycle.

EXPERIMENTAL PROCEDURES

Materials and General Synthetic Procedures. [1-^{3}H]-Geranylgeranyl diphosphate (90 Ci/mol) and (−)-[18-^{3}H]-abieta-7(8),13(14)-diene (117 Ci/mol) were prepared as previously described (LaFever et al., 1994; Funk & Croteau, 1994). (±)-Casbene was a gift from Gerald Pattenden, University of Nottingham, (−)-sandaracopimaradiene was a gift from Robert M. Coates, University of Illinois, and (±)-[20-^{13}C]taxa-4(20),11(12)-diene, recently prepared by total synthesis (Rubenstein & Williams, 1995), was a gift from Robert M. Williams, Colorado State University. ^2H_2O (100.0 atom % ^{2}H) was from Aldrich.

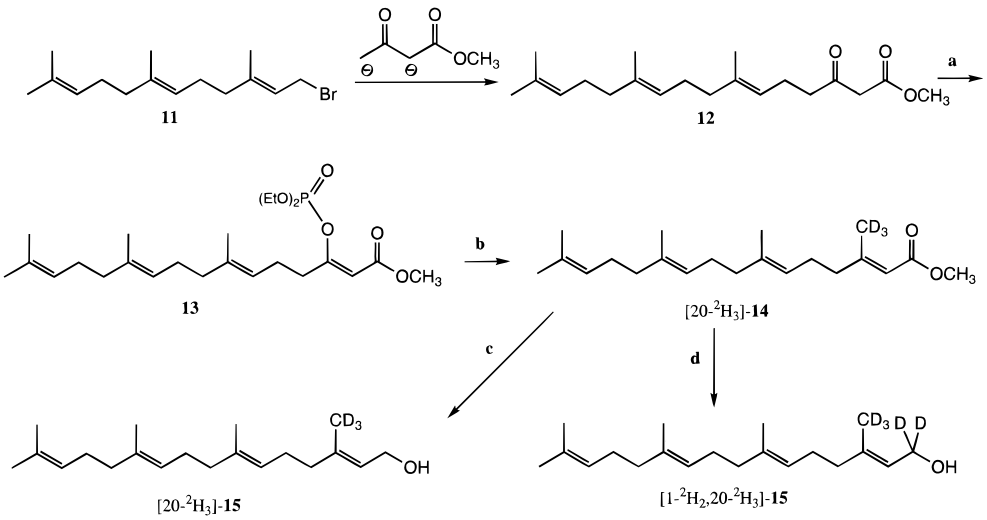
Proton and carbon NMR[2] spectra were recorded in deuterochloroform at the indicated frequencies using a Bruker AF300 Spectrometer with a deuterium lock unless otherwise noted. Chemical shifts (δ, ppm) are referenced to TMS (δ 0) or $CHCl_3$ (δ 7.24) for ^{1}H and to $CHCl_3$ (δ 77.00) for ^{13}C. Deuterium NMR spectra were recorded in benzene, unless otherwise noted, at the indicated frequency using a Bruker AC 200 spectrometer, with chemical shifts referenced to benzene (δ 7.15). Mass spectra were obtained on a Hewlett-Packard 5985 spectrometer.

Flash chromatography was performed on silica gel 60 using the indicated mixture of ethyl acetate in hexane as eluant unless otherwise noted. Analytical TLC was carried out on commercially prepared plates (0.25 mm Merck Kieselgel GF_{254}) with visualization by fluorescence quenching (254 nm UV light), or by staining with I_2, aqueous $KMnO_4$, or 4% phosphomolybdic acid hydrate in ethanol. Ion exchange chromatography was carried out on Dowex AG 50W-X8 (100−200 mesh) using the indicated eluant.

Reagents and solvents were purified and dried by standard protocols (Perrin et al., 1980). Geranylgeraniol was converted, via the corresponding chloride (Corey et al., 1972), to the diphosphate ester (40−65% yield) by a literature

[2] Abbreviations: EI, electron impact; GLC, gas−liquid chromatography; LSC, liquid scintillation counting; MS, mass spectrometry, NMR, nuclear magnetic resonance (spectrometry); THF, tetrahydrofuran; TLC, thin layer chromatography; TMS, tetramethylsilane; UV, ultraviolet.

Scheme 2. Synthesis of [20-$^2H_3$] and [1-$^2H_2$, 20-$^2H_3$]Geranylgeranyl Diphosphate



procedure (Davisson et al., 1986), and the product was isolated by anion exchange chromatography with isopropanol/25 mM NH_4HCO_3 (1:49, v/v) while monitoring the purification via analytical TLC [R_f geranylgeranyl diphosphate = 0.17 with iPrOH/NH_4OH/H_2O (6:3:1, v/v/v)]. Following purification and removal of solvent, geranyl geranyl diphosphate was dissolved in 10 mM aqueous NH_4HCO_3 at a working concentration of 1.5 mM.

Synthesis of [20-$^2H_3$] and [1-$^2H_2$,20-$^2H_3$]Geranylgeranyl Diphosphate. Commercially available farnesyl bromide (**11**) (95%, Aldrich) served as the starting material for these syntheses (Scheme 2). The dianion generated from methyl acetoacetate by deprotonating with sodium hydride and then lithium diisopropyl amide in dry THF (Huckin & Weiler, 1974) was reacted with farnesyl bromide (**11**) in THF at 0 °C to afford β-keto ester **12** in 83% yield following extractive work up and column chromatography (Huckin & Weiler, 1974; Sum & Weiler, 1981). ^{1}H NMR: 1.56 (s, 2×CH_3), 1.58 (s, CH_3), 1.65 (s, CH_3), 1.97 (m, 8H), 2.25 (m, 2H), 2.54 (m, 2H), 3.42 (s, 2H), 3.71 (s, CH_3), 5.05 (m, 3H); ^{13}C NMR: 178.5, 167.6, 136.7, 135.0 131.2, 124.0, 123.0, 122.0, 52.2, 51.0, 49.0, 43.2, 39.7, 26.7, 26.5, 25.6, 22.1, 17.6, 15.9.

Deprotonation of β-keto ester **12** with sodium hydride in dry diethyl ether at 0 °C under Ar, followed by addition of diethyl chlorophosphate-trapped the enol as phosphate ester **13** in quantitative yield following quenching of the reaction with NH_4Cl and filtration through Celite (Sum & Weiler, 1979). ^{1}H NMR: 1.34 (t, CH_3), 1.38 (t, CH_3), 1.56 (s, 2×CH_3), 1.60 (s, CH_3), 1.64 (s, CH_3). 1.90−2.11 (m, 8H), 2.25 (t, CH_3), 2.42 (t, CH_3), 3.63 (s, CH_3), 4.18−4.30 (m, 4H), 5.02−5.61 (m, 3H), 5.31 (s, 1H); ^{13}C NMR: 164.1, 161.5, 137.0, 135.0, 131.1, 124.2, 123.9, 121.6, 104.9, 104.8, 65.7, 64.8, 64.6, 63.7, 51.0, 39.6, 35.1, 26.6, 26.5, 25.6, 24.8, 17.6, 16.0, 15.9, 15.5.

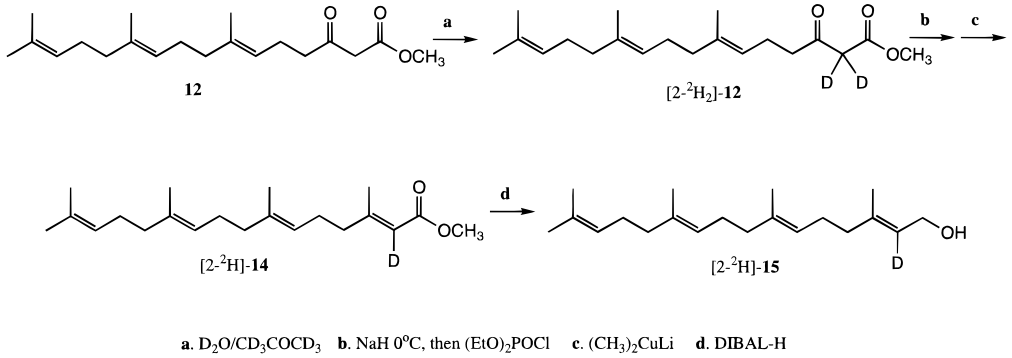
Addition of the enol phosphate (**13**) to $(C^2H_3)_2CuLi$, generated from C^2H_3Li (>99 atom % ^{2}H, Aldrich) and cuprous iodide in dry diethyl ether under Ar at −5 °C, gave, after stirring overnight while warming to room temperature, methyl [20-$^2H_3$]geranylgeranoate ([20-$^2H_3$]-**14**) in 87.5% yield following quenching of the reaction mixture with aqueous NH_4Cl, extraction with diethyl ether, and purification by column chromatography (Corey et al., 1980). ^{1}H NMR: 1.56 (s, 3×CH_3), 1.64 (s, CH_3), 1.98 (m, 8H), 2.12 (m, 4H), 3.65 (s, CH_3), 5.05 (m, 3H), 5.64 (s, 1H); ^{13}C NMR: 167.2, 160.0, 136.2, 135.0, 131.2, 124.4, 124.1, 122.8, 115.3, 50.7, 40.9, 39.7, 39.6, 26.7, 26.6, 25.9, 25.7, 17.6, 16.0.

Reduction of the methyl ester **14** with excess diisobutyl aluminum hydride in dry THF at −70 °C, followed by quenching with methanol, partitioning with diethyl ether/saturated $NaHCO_3$, and chromatographic purification, gave trideuteroalcohol [20-$^2H_3$]-**15** in 90.4% yield. ^{1}H NMR: 1.58 (s, 3×CH_3), 1.66 (s, CH_3), 1.92−2.11 (m, 12H), 4.13 (d, 2H), 5.09 (m, 3H), 5.40 (t, 1H); ^{2}H NMR: 1.63 (s); ^{13}C NMR: 139.7, 135.3, 134.9, 131.2, 124.4, 124.1, 123.8, 123.3, 59.4, 39.7, 39.5, 39.3, 26.7, 26.6, 26.3, 25.7, 17.6, 16.0. Alcohol [20-$^2H_3$]-**15** was pyrophosphorylated (Davisson et al., 1986) to provide [20-$^2H_3$]geranylgeranyl diphosphate (>95 atom % ^{2}H).

To prepare the pentadeuterated substrate, [20-$^2H_3$]-geranylgeranoate (**14**) was reduced with excess $LiAl^2H_4$ in dry THF to afford the corresponding alcohol ([1-$^2H_2$, 20-$^2H_3$]-**15**) in 74.2% yield following work-up and chromatographic purification. ^{1}H NMR: 1.60 (s, CH_3), 1.69 (s, CH_3), 1.94−2.14 (m, 12H), 5.09 (m, 3H), 5.42 (s, 1H); ^{2}H NMR: 1.62 (s), 4.13 (s); ^{13}C NMR: 139.9, 135.5, 134.9, 131.2, 124.3, 124.1, 123.4, 120.2, 39.7, 39.5, 39.3, 26.7, 26.1, 25.6, 17.6, 16.0. Pyrophosphorylation, as before, afforded [1-$^2H_2$,-20-$^2H_3$]geranylgeranyl diphosphate (>95 atom % ^{2}H).

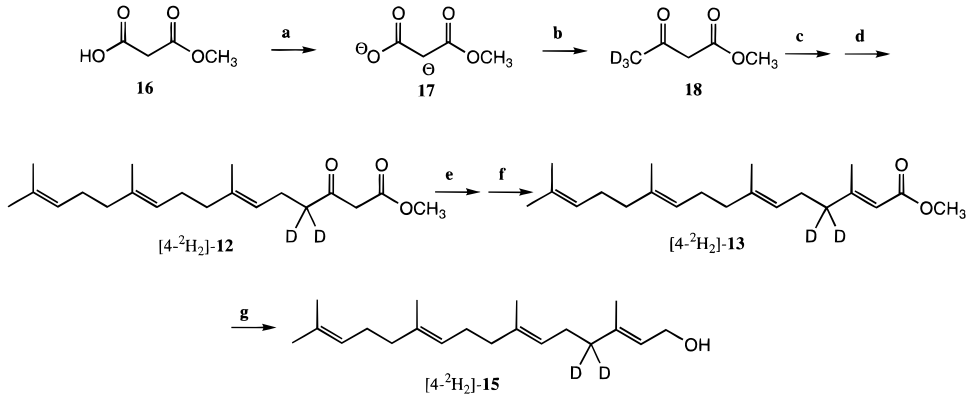
Synthesis of [2-^{2}H]- and [4-$^2H_2$]Geranylgeranyl Diphosphate. For the preparation of the C2-deuterated substrate, β-keto ester **12** was first exchange labeled in ^2H_2O/perdeuteroacetone to provide [2-$^2H_2$]-**12** (Scheme 3). The latter was deprotonated with NaH and trapped as an enol phosphate ester with diethyl chlorophosphate, and the phosphate ester was reacted with lithium dimethylcuprate as before (Scheme 2) to provide methyl [2-^{2}H]geranylgeranoate ([2-^{2}H]-**14**). Reduction of the methyl ester (**14**) with diisobutyl aluminum hydride in dry THF afforded the corresponding alcohol [2-^{2}H]-**15** in 60.4% yield, which was transformed to [2-^{2}H]-geranylgeranyl diphosphate ([2-^{2}H]-**1**) as before (84 atom % ^{2}H by MS analysis of alcohol [2-^{2}H]-**15**).

Scheme 3. Synthesis of [2-^{2}H]Geranylgeranyl Diphosphate



a. D_2O/CD_3COCD_3 **b**. NaH 0°C, then $(EtO)_2POCl$ **c**. $(CH_3)_2CuLi$ **d**. DIBAL-H

Scheme 4. Synthesis of [4-$^2H_2$]Geranylgeranyl Diphosphate



a. 2 eq LDA **b**. CD_3COCl **c**. NaH 0°C, then BuLi -10°C **d**. farnesyl bromide
e. NaH 0°C, then $(EtO)_2POCl$ **f**. $(CH_3)_2CuLi$ **g**. DIBAL-H

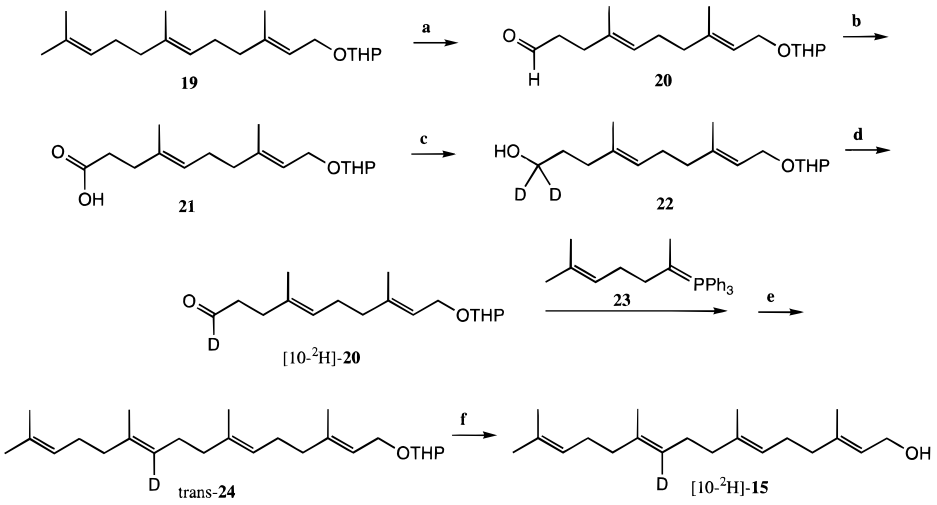
The synthesis of the 4-$^2H_2$-labeled substrate proceeded along the same lines as the preparation of the 20-$^2H_3$-labeled material (Scheme 2) but, in this instance, starting with methyl [4-$^2H_3$]acetoacetate (**18**) (Scheme 4). Addition of two equivalents of *n*-butyllithium to monomethyl malonate (**16**) (Strube, 1963) yielded the dianion (**17**) which was reacted with trideuteroacetyl chloride to produce the desired reagent (**18**) in 59% yield (Wierenga & Skulnick, 1979). Subsequent transformations, as before, provided the alcohol [4-$^2H_2$]-**15** [^{1}H NMR: 1.58 (s, 3×CH_3), 1.66 (s, CH_3), 1.92−2.11 (m, 10H), 4.13 (d, 2H), 5.09 (m, 3H), 5.40 (t, 1H); ^{2}H NMR: 1.94 (s); ^{13}C NMR: 139.7, 135.4, 135.0, 131.3, 124.4, 124.2, 123.8, 123.4, 59.4, 39.7, 26.8, 26.2, 25.7, 17.7, 16.2, 16.0], which was converted to [4-$^2H_2$]geranylgeranyl diphosphate (91 atom % ^{2}H).

Synthesis of [10-^{2}H]Geranylgeranyl Diphosphate. The protected farnesol (**19**) (Greene & Wuts, 1991) was ozonized in CH_2Cl_2/pyridine (50:1, v/v) at −78 °C, followed by decomposition of the ozonides with dimethyl sulfide (Cane & Tandon, 1994; Corey & Yamamoto, 1970; Arigoni et al., 1973) and chromatographic purification to afford the C_7 aldehyde (25% yield) and the desired C_{12} aldehyde (**20**, 16% yield) (Scheme 5). Oxidation of trisnoraldehyde **20** with pyridinium dichromate (Corey at al., 1968) gave the corresponding acid (**21**) which was reduced with $LiAl^2H_4$ in diethyl ether at −30 °C to furnish the deuterated alcohol (**22**) that was finally reoxidized with pyridinium chlorochromate (Maucuso et al., 1978) to produce the deuterated C_{12} trisnoraldehyde ([10-^{2}H]-**20**) in 66% overall yield. ^{1}H NMR: 1.48−1.58 (m, 3H), 1.62 (s, CH_3), 1.66 (s, CH_3), 1.68−1.88 (m, 3H), 2.04 (m, 2H), 2.12 (m, 2H), 2.30 (t, 2H), 2.49 (t, 2H), 3.50 (dt, 1H), 3.86 (dt, 1H), 3.99 (dd, 1H), 4.22 (dd, 1H), 4.60 (t, 1H), 5.11 (t, 1H), 5.33 (t, 1H); ^{13}C NMR: 139.8, 133.2, 125.0, 120.8, 97.8, 63.6, 62.3, 41.9, 39.3, 31.8, 30.7, 26.1, 25.5, 19.6, 16.3, 16.1.

6-Methyl-5-hepten-2-yl triphenylphosphonium iodide (**23**) was prepared by conversion of 6-methyl-5-hepten-2-ol (99%, Aldrich) to the iodide via the tosylate (Carson & Correia, 1961) followed by reflux with triphenylphosphine in toluene and recrystallization of the product from acetonitrile in about 50% yield (Wittig & Schollkopf, 1954; Wittig & Haag, 1955; Vedejs et al., 1988; Vedejs & Marth, 1988). Addition of *n*-butyl lithium to a solution of **23** in dry THF generated the ylide which was condensed with deuterated aldehyde **20** *in situ* (Vedejs et al., 1988; Vedejs & Marth, 1988) to provide a mixture of cis and trans protected alcohols (**24**) that was separated by argentation TLC [10% $AgNO_3$-silica gel with hexane/ethyl acetate (9:1, v/v); R_f (trans) ∼0.17; R_f (cis) ∼0.20]. Deprotection of each in methanolic HCl gave [10-^{2}H]nerylgeraniol and [10-^{2}H]geranylgeraniol ([10-^{2}H]-**15**) [^{1}H NMR: 1.57 (s, 3×CH_3), 1.65 (s, 2×CH_3), 1.94−2.12 (m, 12H), 4.12 (d, 2H), 5.08 (m, 2H), 5.39 (t, 1H); ^{13}C NMR: 139.8, 135.4, 134.9, 131.2, 124.4, 123.8, 123.3, 59.4, 39.7, 39.6, 39.5, 26.7, 26.6, 26.3, 25.7, 17.7, 16.3, 16.0; ^{2}H NMR: 5.09 (s, 1^{2}H)] which were pyrophosphorylated as before (>95 atom % ^{2}H).

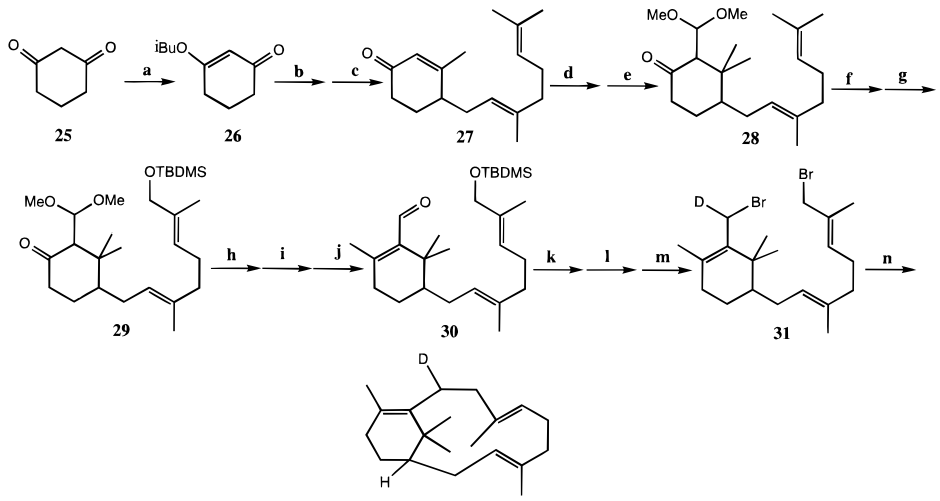
Synthesis of [10-^{2}H]Verticillene. The labeled olefin was synthesized by extensive modification of an earlier protocol (Begley et al., 1990; Hitchcock & Pattenden, 1992) (Scheme 6). Refluxing 1,3-cyclohexanedione (**25**) with isobutanol in toluene in the presence of *p*-toluenesulfonic acid gave 3-isobutoxycyclohex-2-enone (**26**) in nearly quantitative yield

Scheme 5. Synthesis of [10-^{2}H]Geranylgeranyl Diphosphate



a. O_3, then $(CH_3)_2S$ **b**. PDC **c**. $LiAlD_4$ **d**. PCC **e**. separation by argentation TLC **f**. HCl/CH_3OH

Scheme 6. Synthesis of (±)-[10-^{2}H]Verticillene



a. 2-butanol, p-TsOH/$PhCH_3$ **b**. LDA/THF, then geranyl bromide **c**. CH_3Li/THF, then H^+ **d**. $(CH_3)_2CuLi$, then TMSCl, Et_3N **e**. $(CH_3O)_3CH$, $TiCl_4/CH_2Cl_2$ **f**. SeO_2, t-BuOOH **g**. TBDMSCl, imidazole **h**. CH_3MgI **i**. PPTS/CH_3COCH_3 **j**. MsCl, DMAP **k**. $NaBD_4/CeCl_3$ **l**. NH_4F/THF **m**. PBr_3/Et_2O **n**. $Ni(CO)_4$

(Stork & Danheiser, 1973). Alkylation of the enolate of **26**, generated with lithium diisopropylamide in dry THF, with geranyl bromide (97%, Aldrich), followed by methylation at the carbonyl group with methyl lithium and hydrolysis with dilute acid provided the disubstituted cyclohexenone (**27**) in 78% yield. The cyclohexenone, by coupling with lithium dimethylcuprate, was transformed into an enolate which was trapped with chlorotrimethylsilane as a silyl enol ether (Rasmussen, 1977; Patterson & Fried, 1974; Mukaiyama & Hayashi, 1974). The enolate, regenerated with titanium tetrachloride, was then reacted with trimethyl orthoformate to give the *cis*-keto acetal (**28**) in 51% yield.

Selective oxidation of the ketoacetal with SeO_2 in CH_2Cl_2 in the presence of *tert*-butyl hydroperoxide (Bhalerao & Rapoport, 1971; Umbreit & Sharpless, 1977) provided the allylic alcohol that was protected as the *tert*-butyldimethylsilyl ether (**29**). Addition of methylmagnesium bromide to the protected ketoacetal (**29**) provided the tertiary alcohol-acetal which upon mild hydrolysis gave the corresponding tertiary alcohol-aldehyde that was dehydrated upon mesylation to yield the α,β-unsaturated aldehyde (**30**) in an overall yield of 66% for these three steps. Reduction of the α,β-unsaturated aldehyde (**30**) with NaB^2H_4 in the presence of $CeCl_3$ provided the allylic alcohol which was deprotected with NH_4F to yield the diallylic alcohol that was converted to the diallylic bromide (**31**) with PBr_3. Slow addition of a solution of the diallylic bromide (**31**) in *N*-methylpyrrolidone to a solution of $Ni(CO)_4$ in *N*-methylpyrrolidone over 48 h at 50 °C (Corey & Semmelhack, 1966; Corey & Hamanaka, 1967) provided (±)-[10-^{2}H]verticillene ([10-^{2}H]-**4**, 94 atom % ^{2}H) in 15% yield following purification. ^{1}H NMR: 0.81 (s, CH_3), 0.89 (s, CH_3), 1.20−1.34 (m, 3H), 1.42 (s, CH_3), 1.58 (s, 2×CH_3), 1.70−2.20 (m, 10H), 2.28−2.34 (m, 1H), 4.90 (m, 1H), 4.92 (m, 1H); ^{2}H NMR: 1.97 (s, 0.5 ^{2}H), 2.37 (s, 0.5 ^{2}H).

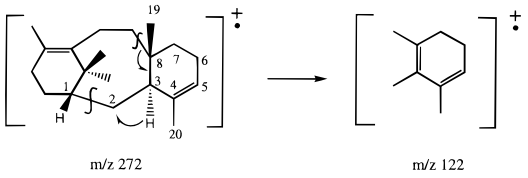
Enzyme Isolation and Assay. Two-year-old Pacific yew saplings in active growth were used as the enzyme source, and the taxadiene synthase was partially purified from stem extracts as previously described (Hezari et al., 1995). All assays were conducted under optimum, linear conditions at

saturating concentrations of geranylgeranyl diphosphate (15 μM) and divalent metal ion cofactor ($MgCl_2$ at 1.0 mM) (Hezari et al., 1995). Trapping experiments, in an attempt to interpose intermediates in the conversion of [^{3}H]geranylgeranyl diphosphate to taxadiene, were carried out by the addition (in hexane followed by evaporation of solvent) of the presumptive diterpene olefin intermediates (at a level of 15 μM) to the above described reaction mixtures. To test direct conversion of presumptive olefinic intermediates to taxa-4(5),11(12)-diene (**6**), a level of 5 μM of the olefin replaced the geranylgeranyl diphosphate substrate (**1**) in the standard assay. Based on the water solubility of *n*-eicosane and comparison to the solubilities of a range of other *n*-alkanes and their corresponding cycloalkanes and cycloalkenes (Robotham and Gill, 1989), it was estimated that the solubility of polycyclic diterpene olefins in the glycerol-containing assay buffer was in the 5−10 μM range. The addition of these olefins to the enzyme incubation mixtures at 5−15 μM produced no detectable turbidity by measurement of change in absorbance at 600 nm. For preparative purposes, the 0.5 mL reaction mixture (10−20 μg of protein/mL) was directly scaled up by 6-fold. In all cases, the assay mixtures were gently washed with an equal volume of hexane to remove trace levels of nonpolar contaminants from the tissue extracts and buffer components before initiation of the reaction by addition of geranylgeranyl diphosphate and/or the presumptive olefin intermediate. This treatment with hexane had no adverse effect on taxadiene synthase activity. To examine incorporation of deuterium from ^2H_2O into the olefin product, reaction mixtures containing all buffer components and other additives, with the exception of the substrate, were quick frozen and lyophilized. The lyophilized powder was then hydrated in ^2H_2O, followed by the addition of substrate and assay. Identically prepared control samples were hydrated in ^1H_2O in order to determine the rate suppression in ^2H_2O.

Product Analysis. Following incubation, 1 mL of pentane was added to the reaction mixture, and the contents were vigorously vortex mixed. Following centrifugation to separate phases, the pentane layer was removed and passed through a glasswool-plugged column of silica gel [2−3 g of SilicAR 60A (Mallinckrodt) in a glass Pasteur pipette] surmounted by a layer of anhydrous $MgSO_4$ to dry the sample. The reaction mixture was extracted twice more with 1 mL of pentane that was also passed through the silica gel column, followed by a final 1 mL pentane wash, to afford the hydrocarbon fraction, free of oxygenated metabolites. Elution of the column with diethyl ether gave a measure of [^{3}H]geranylgeraniol liberated from the ^{3}H-labeled substrate by competing phosphatases. In the case of the ^{3}H-labeled substrate, an aliquot of the hydrocarbon fraction was analyzed by LSC in 10 mL of a cocktail consisting of 0.4% (w/v) Omnifluor (DuPont/New England Nuclear) dissolved in 30% ethanol in toluene (^{3}H efficiency ∼42%) to verify the activity of the preparation; the identity and purity of the taxadiene product were confirmed by radio-GLC as before (Koepp et al., 1995). Boiled controls evidenced negligible activity with [1-^{3}H]geranylgeranyl diphosphate as substrate.

With ^{2}H- and ^{13}C-labeled substrates and putative intermediates, the stable isotope content of the enzymatically derived taxadiene was determined by combined capillary GLC-MS analysis of an aliquot of the hydrocarbon fraction prepared as above using a Hewlett-Packard 5840A/5985B system equipped with a 30 m × 0.25 mm diameter fused silica column with a 0.25 μm film of AT 1000 (Alltech) operated at 10 psi He and programmed from 35 °C (cool on-column injection) at 30 °C/min to 50 °C (5 min hold) and then at 10 °C/min to 220 °C. EI spectra were recorded at 70 eV with an electron multiplier voltage of 2800 V. In each case, the following ion clusters in the EI spectrum were evaluated by selected ion monitoring (SIM) with reference to authentic taxa-4(5),11(12)-diene: *m/z* 272 [P^+]; 257 [P^+-15(CH_3)]; and 122 [the base peak, C-ring double cleavage fragment (C_9H_{14})]. This fragment ion (equivalent to trimethylcyclohexadiene) could theoretically arise from the taxadiene A- or C-ring but was shown to arise exclusively from the C-ring, and thus to be comprised of taxadiene carbons 2, 3, 4, 5, 6, 7, 8, 19, and 20, by labeling of this fragment ion of the olefin derived from geranylgeranyl diphosphate that carried deuterium at carbons 1, 2, 4, and 20. The origin of this relatively stable radical cation can be rationalized by the double cleavage and hydrogen migration illustrated in Scheme 7. An estimate of the conversion of stable isotope-labeled substrates was obtained by summing the relevant ion intensities, following background correction, relative to those of taxadiene obtained from [^{3}H]geranylgeranyl pyrophosphate in parallel experiments; isotopic abundance was calculated from the molecular ion(s) following background correction.

Scheme 7. Proposed Origin of the C-Ring Fragment Ion from Taxadiene

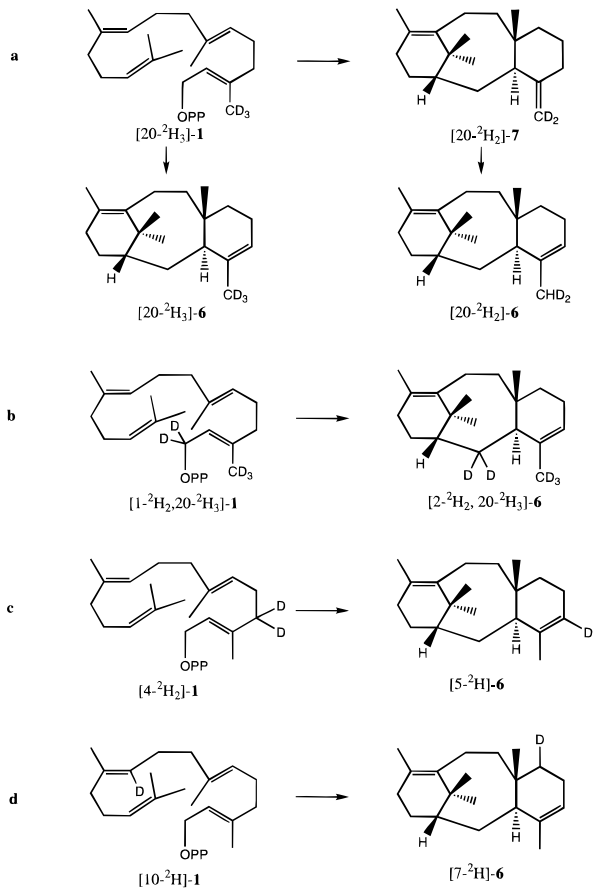


In the case of the diterpene olefin trapping experiments, the hydrocarbon fraction from a duplicate incubation was diluted with 250 μg of the authentic olefin and subjected to repeated argentation TLC [8% $AgNO_3$-silica gel G with hexane/diethyl ether (97:3, v/v)] with location by methanolic 2,7-dichlorofluorescein spray and UV light [casbene R_f ∼0.46; verticillene R_f ∼0.51; taxa-4(20),11(12)-diene R_f ∼0.56; taxa-4(5),11(12)-diene R_f ∼0.62; sandaracopimaradiene R_f ∼0.64]. Following each round of purification of the material (eluted in diethyl ether), approximated 50 μg of the isolated olefin was evaluated for tritium content by LSC as above. Calibration of this purification sequence with [^{3}H]abieta-7(8),13(14)-diene (LaFever et al., 1994; Funk & Croteau, 1994) indicated a total recovery of diterpene olefin in excess of 85%.

RESULTS

A partially purified preparation of taxadiene synthase from Pacific yew stems (Hezari et al., 1995) was used in all experiments. This preparation was essentially free of phosphatase activity (measured by the liberation of geranylgeraniol from [^{3}H]geranylgeranyl diphosphate) and, when assayed by LSC and capillary GLC-MS under optimum condition (at 15 μM geranylgeranyl diphosphate and 1 mM $MgCl_2$), was capable of transforming the substrate to taxa-4(5),11(12)-diene in 4−5% yield (∼1 nmol) without detectable formation of cembrene, casbene, verticillene, or taxa-4(20),11(12)-diene (<0.05% conversion of substrate, <7

Scheme 8. Possible Labeling Patterns of Taxadiene Derived from Deuterium Labeled Geranylgeranyl Diphosphate (D = ^{2}H)



pmol/h). The latter observation argues against any of these four olefins as free intermediates of the reaction sequence but does not address the possibility that such intermediates are enzyme-bound.

Conversion of Deuterium-Labeled Geranylgeranyl Diphosphate to Taxadiene. To test the possibility that the cyclization of geranylgeranyl diphosphate (**1**) proceeds through the initial formation of taxa-4(20),11(12)-diene (**7**) followed by isomerization to and release of the endocyclic double bond isomer (**6**), the enzyme catalyzed transformations of [1-^{2}H$_2$,20-^{2}H$_3$]geranylgeranyl diphosphate and [20-^{2}H$_3$]-geranylgeranyl diphosphate to taxadiene were examined (Scheme 8). GLC-MS analysis of the derived taxa-4(5),11(12)-diene indicated conversion efficiencies for the pentadeuterium- and trideuterium-labeled substrates of 3.6% and 4.0%, respectively, compared to 4.2% for the tritium-labeled substrate as a control assayed under identical conditions. The observed rate suppressions probably reflect, at least in part, the operation of secondary deuterium isotope effects on the ionization step of the reaction catalyzed by taxadiene synthase. Similar kinetic isotope effects on terpenoid cyclizations have been observed previously (Croteau et al., 1987; Wagschal et al., 1991, 1994) and suggest that ionization of the allylic diphosphate substrate may be the rate-limiting step in these reactions.

Inspection of the parent ion clusters of the taxa-4(5),11-(12)-diene derived from the deuterium labeled substrates (Scheme 8a,b), and comparison with the corresponding peak intensities of the molecular ion cluster of an unlabeled reference sample of taxadiene recorded under identical

Table 1: GLC-MS (Selected Ion Monitoring) Analysis of Taxa-4(5),11(12)-diene Derived from Labeled Geranylgeranyl Diphosphate (GGPP)

		fragment ions (*m*/*z*) [atom % ^{2}H][a]	
substrate (atom % ^{2}H)[a]	P	P-15(CH_3)	C-ring (C_9H_{14})
[1-^{3}H$_1$]GGPP	272	257	122
[1-^{2}H$_2$,20-^{2}H$_3$] GGPP (>95)	277 [>95]	262	127
[20-^{2}H$_3$] GGPP (>95)	275 [>95]	260	125
[4-^{2}H$_2$] GGPP (91)	273 [90]	258	123
[2-^{2}H$_1$] GGPP (84)	273 [82]	258	123
[1-^{3}H$_1$] GGPP/^{2}H$_2$O (100)	273 [8 ± 2]		
[10-^{2}H] GGPP (>95)	273 [85 ± 5]	258	123

[a] Atom % deuterium corrected for background contribution. No entry indicates not reliably determined.

conditions, indicated a quantitative shift in the parent ion from *m*/*z* 272 to *m*/*z* 277 for the pentadeuterated substrate and to *m*/*z* 275 for the trideuterated substrate. Identical results were obtained by measurement of the P-15 (CH_3) and C-ring fragment ions (Table 1), confirming the derived taxa-4(5),11(12)-dienes to possess deuterium enrichments essentially identical to those of the corresponding acyclic precursors. These results revealed that the cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene is direct and does not involve preliminary formation of taxa-4(20),11(12)-diene followed by isomerization to the endocyclic isomer since, had this indirect pathway operated, one deuterium atom would have been lost at the C20-deprotonation step (Scheme 8a,b). By similar reasoning, the conversion of [1-^{2}H$_2$,20-^{2}H$_3$]geranylgeranyl diphosphate to taxa-4(5),11(12)-diene without loss of deuterium (Scheme 8b) would appear to eliminate casbene as an intermediate of the reaction sequence since one hydrogen atom would have been lost from C1 of the acyclic precursor in the deprotonation leading to the cyclopropane function of this olefin (Scheme 1).

To examine the presumptive C5-deprotonation step in the cyclization to taxa-4(5),11(12)-diene, [4-^{2}H$_2$]geranylgeranyl diphosphate was next examined as a substrate (Scheme 8c). In this instance, the conversion efficiency assessed by GLC-MS was slightly less than that of the tritium-labeled substrate (4.1 vs 4.3% for the control run in parallel), and inspection of P, P-15 and C-ring fragment ions indicated the loss of one deuterium atom in the derived taxadiene (i.e., a shift of *m*/*z* 272 to 273, *m*/*z* 257 to 258, and *m*/*z* 122 to 123) (Table 1). The loss of one deuterium atom in the transformation is entirely consistent with direct cyclization of [4-^{2}H$_2$]-geranylgeranyl pyrophosphate to the taxenyl cation **5** with C5-deprotonation in the C-ring to the taxadiene product.

Potential Olefinic Intermediates of the Reaction Sequence. Several tests were made in an attempt to evaluate the role of potential olefinic intermediates in the reaction cascade leading to taxa-4(5),11(12)-diene: (1) by interception of an intermediate and dilution of the product generated from [^{3}H]-geranylgeranyl diphosphate (measured by apparent inhibition of [^{3}H]taxadiene formation); (2) by trapping of exchangeable, labeled intermediate in the conversion of [^{3}H]geranylgeranyl diphosphate to taxadiene in the presence of a large excess of olefin (determined by reisolation and purification of the "trapped" intermediate); and (3) by direct testing of the olefin as a precursor of taxa-4(5),11(12)-diene (by GLC-MS analysis of product).

Interception was tested by separate incubation of [1-^{3}H]-geranylgeranyl diphosphate (15 μM plus 1 mM $MgCl_2$) in the presence of 15 μM of each putative olefin intermediate [(±)-[10-^{2}H]verticillene (**4**), (±)-[20-^{13}C]taxa-4(20),11(12)-diene (**7**), and (±)-casbene (**10**) as well as (−)-sandaracopimaradiene as a control that could not possibly serve as an intermediate in taxadiene biosynthesis]. Apparent inhibition of [^{3}H]taxa-4(5),11(12)-diene formation on the order of 9−14% was observed in all cases, including sandaracopimaradiene, indicating that the effect was nonspecific, and that little, if any, intervention of the olefins in the reaction cascade had occurred. GLC-MS analysis of the taxa-4(5),-11(12)-diene (P^+, P-15 and the C-ring fragment ions) that had been generated from [^{3}H]geranylgeranyl diphosphate in the presence of [10-^{2}H]verticillene or [20-^{13}C]taxa-4(20),-11(12)-diene revealed negligible stable isotope incorporation into the product [below the limit of detection by this method (<0.05% conversion), under conditions where the 5% conversion of [^{3}H]geranylgeranyl diphosphate to the taxadiene product was easily observed].

The diterpene olefins, (±)-casbene (**10**), (±)-verticillene (**4**), and (±)-taxa-4(20),11(12)-diene (**7**) (each at 15 μM), that were separately employed as potential traps of radioactivity from [1-^{3}H]geranylgeranyl diphosphate (15 μM) en route to taxa-4(5),11(12)-diene, and the control diterpene olefin, sandaracopimaradiene similarly tested, were recovered from the enzymatic reaction mixtures by pentane extraction. An aliquot of the extract, representing [^{3}H]taxadiene and any trapped olefin, was counted, and the remainder was then diluted with 250 μg of the corresponding authentic carrier and subjected to three rounds of purification by argentation TLC. The casbene, taxa-4(20),11(12)-diene and sandaracopimaradiene so recovered (in ∼85% yield) contained negligible radioactivity (background), and the verticilline contained less than 3% of the label [6 dpm/μg vs 210 dpm/μg for taxa-4(5),11(12)-diene]. Thus, none of the presumptive olefin intermediates tested trapped appreciable label from [^{3}H]geranylgeranyl diphosphate in the conversion of this substrate to taxa-4(5),11(12)-diene.

In a final test, the conversion of (±)-casbene, (±)-verticillene, and (±)-taxa-4(20),11(12)-diene to taxa-4(5),11(12)-diene was examined directly and independently in preparations previously verified to catalyze the efficient conversion of geranylgeranyl diphosphate to this product (4−5% yield; 600−750 pmol/h). These potential olefin precursors were examined at the 5 μM level to avoid the minor, nonspecific inhibition previously observed at 15 μM concentrations. GLC-MS analyses of the hydrocarbon fractions isolated from the incubation mixtures revealed no detectable taxa-4(5),11(12)-diene (<0.1% conversion; <5 pmol/h).

Intramolecular Hydrogen Transfer in a Verticillene Intermediate. In considering the overall efficiency of the cyclization reaction, the preliminary closure to a macrocyclic cembrene-type intermediate (**9**) or to the bicyclic olefin casbene (**10**) (Scheme 1) seems an unnecessary step, since reprotonation of the same carbon of the isopropenyl or cyclopropane group is required to regenerate the tertiary carbocationic center involved in closure to the trimethyl cyclohexene A-ring of the product. The involvement of a 1*S*-verticillene intermediate in the reaction, however, seems inescapable, since deprotonation to generate the A-ring bridgehead double bond is almost certainly required before protonation at the remote C7 position needed to close the B/C-ring junction. Although this postulated intermediacy of verticillene would necessitate the removal of the original C10 proton of geranylgeranyl diphosphate in the initial cyclization step, this same proton could conceivably be redonated to C7 of a transiently generated verticillene intermediate, if the transfer was sufficiently fast compared to competing exchange processes. Inspection of models (Floss & Mocek, 1995) indicates that if a verticillyl cation is formed by C−C bond formation on the C11 *re*-face, the 11α-proton is sufficiently close to C7 that a single active site base in the enzyme could mediate the intramolecular proton transfer from C11 to C7, with the resulting cation at C8 initiating the transannular cyclization to taxadiene as before (Scheme 1).

To examine the possibility of such an intramolecular proton transfer, the enzymatic conversion of [1-^{3}H]geranylgeranyl diphosphate to taxadiene in ^2H_2O was examined; under these conditions, the reaction rate was attenuated by roughly 60% compared to that in ^1H_2O. The taxadiene so generated, when analyzed by GC-MS (Table 1), was shown to be largely devoid of deuterium (8 ± 2% $^2H_1$), a result consistent with intramolecular hydrogen transfer since little external protonation at C7 was observed.

In a final experiment, the intramolecular hydrogen transfer was examined directly using [10-$^2H_1$]geranylgeranyl diphosphate as substrate (Scheme 8d). The bulk of the enzymatically produced taxadiene bore one deuterium atom (85 ± 5% $^2H_1$), and the shift of the fragment ion at *m*/*z* 122 to *m*/*z* 123 indicated that the deuterium atom now resided on the taxane C-ring (Table 1). This result is entirely consistent with the migration of the hydrogen at C10 of the geranylgeranyl substrate (the 11α-hydrogen of the verticillyl cation) to C7 of the taxane skeleton in the course of the enzymatic transformation to taxadiene. Another olefinic product was detected in this reaction mixture at roughly 10% the level of taxadiene. This product bore no deuterium ($P^+ = 272$) and had a GC retention time identical to that of verticillene, but the partial spectrum (the SIM-MS parameters were optimized for taxadiene analysis) was insufficient to permit identification. The *cis*-isomer of the geranylgeranyl substrate, [10-$^2H_1$]nerylgeranyl diphosphate (a coproduct of the synthesis in Scheme 5), was an ineffective substrate for taxadiene synthase, yielding no detectable cyclic product when incubated with the enzyme at a concentration of 15 μM (saturation with the *trans*-isomer).

DISCUSSION

Studies with [1-$^2H_2$,20-$^2H_3$], [20-$^2H_3$], and [4-$^2H_2$]geranylgeranyl diphosphate have demonstrated the direct conversion of the acyclic precursor to taxa-4(5),11(12)-diene, without the intervention of taxa-4(20),11(12)-diene followed by isomerization, since all hydrogens at the C3-methyl group of the acyclic precursor (C20 of geranylgeranyl diphosphate; C20 of taxadiene) are retained in the cyclization whereas one hydrogen atom from the C4-methylene group of geranylgeranyl diphosphate (C5 of taxadiene) is lost. This result cannot be readily explained by other than formation of the taxenyl cation **5** and deprotonation at the adjacent C5-methylene (taxane numbering) to yield taxa-4(5),11(12)-diene directly.

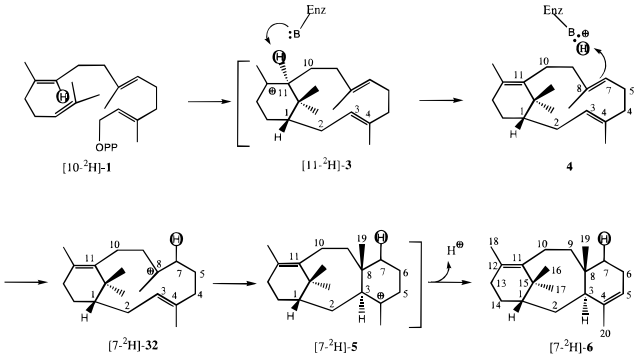
Negative results regarding the intervention, trapping, and conversion of potential olefinic intermediates of the reaction,

including the failure to demonstrate the direct, independent isomerization of the exocyclic isomer under these otherwise functional catalytic conditions for geranylgeranyl diphosphate as substrate, as well as the conversion of [1-$^2H_2$,20-$^2H_3$]-geranylgeranyl diphosphate to product without loss of deuterium, argue strongly against the involvement of taxa-4(20),11(12)-diene as well as casbene in the reaction cycle. However, the negative results do not eliminate the possible involvement of tightly bound verticillene as an intermediate that is not exchangeable with exogenous material in the time frame of the reaction, e.g., because it is generated at a site on the enzyme (and/or at a time in the reaction cycle) not accessible to exogenous verticillene. Thus, on binding the prenyl diphosphate substrate, the taxadiene synthase may undergo conformational alteration as a means of shielding from water the carbocations generated in ionization and subsequent cyclization steps. Such conformational change would as a consequence also prevent the egress of any stable intermediate from the active site or the approach from solution of an exogenous olefin. Similar stable, but tightly bound, intermediates are thought to participate in related cyclizations in the sesquiterpene series, notably in the initial cyclization of farnesyl diphosphate to the macrocyclic olefins humulene and germacrene A en route to pentalenene and aristolochene, respectively (Cane, 1990).

The failure of the olefin product to acquire significant label from ^2H_2O in the enzymatic cyclization suggested an intramolecular proton transfer in the reaction. This supposition was confirmed by the results of incorporation of label from [10-^{2}H]geranylgeranyl diphosphate into taxa-4(5),11-(12)-diene which clearly established that significant transfer of deuterium from C10 of the substrate (C11 of the proposed verticillyl intermediate) to the C-ring of taxadiene (most likely to C7) does occur. Thus, the same proton removed from the substrate in the initial cyclization step to form the A-ring is redeployed in the protonation of the transiently generated verticillene intermediate to complete the cyclization sequence in the closure of the B/C-ring juncture leading to the taxenyl cation **5**. The efficiency of proton return with the geranylgeranyl substrate (85%) indicates that exchange with the external medium must be slow relative to the intramolecular transfer step and furthermore suggests that the enzyme base mediating the transfer is monoprotic; were the base polyprotic, the operation of a primary deuterium kinetic isotope effect should have been evident in significantly decreasing deuterium incorporation unless the base does not exchange protons with solvent between turnovers. The evidence implicates rapid intramolecular hydrogen transfer and a very short-lived, tightly bound 1*S*-verticillene intermediate in the catalytic cycle. Similar intramolecular hydrogen transfers have been previously demonstrated in the sesquiterpene series in the cyclization of farnesyl diphosphate to humulene and its subsequent conversion to pentalenene (Cane et al., 1991).

Taken together with the earlier results, a cyclization scheme for the transformation of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene can now be formulated with a minimum number of steps and a single, enzyme-bound, olefinic intermediate (Scheme 9). Thus, ionization of the geranylgeranyl diphosphate ester (**1**) promotes C−C bond formation between C1 and C14 of the substrate, followed by closure of the A ring via *re*-face attack at C10; deprotonation of the resulting 11α-verticillyl cation (**3**) by

Scheme 9. Stereochemical Mechanism for Cyclization of Geranylgeranyl Diphosphate to Taxa-4(5),11(12)-diene Involving 1*S*-Verticillene as a Transient Intermediate



removal of the 11α-hydrogen then affords 1*S*-verticillene (**4**). This bound intermediate is rapidly reprotonated at C7, via the same enzyme base responsible for the earlier deprotonation step, to initiate transannular cyclization to generate the taxenyl cation (**5**), which upon deprotonation at C5 yields the endocyclic double bond of the taxadiene product (**6**).

These studies on taxadiene synthase constitute the first mechanistic description of any enzyme of taxol biosynthesis, and they confirm that the first committed product of the pathway is taxa-4(5),11(12)-diene, not taxa-4(20),11(12)-diene, thus correcting earlier biogenetic proposals based on metabolite co-occurrence (Harrison et al., 1966; Guéritte-Voegelein et al., 1987). The metabolic step catalyzed by this diterpene cyclase, which delineates the taxane skeleton, is slow and perhaps rate-limiting in this extended biosynthetic sequence (Koepp et al., 1995). The cyclization reaction, therefore, has clear implications for improving taxol yield, with the taxadiene synthase as an obvious target for gene isolation.

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