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Stereochemistry of the Macrocyclization and Elimination Steps in Taxadiene Biosynthesis through Deuterium Labeling

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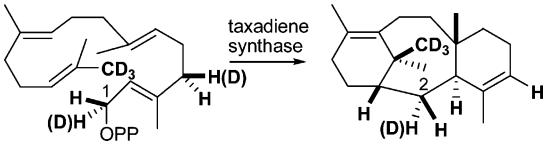

Taxadiene synthase catalyzes the cyclization of (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP) to taxa-4(5),11(12)-diene (Scheme 1, **5** → **2**) as the first committed step of Taxol biosynthesis. Deuterated GGPPs labeled stereospecifically at C-1, C-4, and C-16 were synthesized and incubated with recombinant taxadiene synthase from *Taxus brevifolia* to elucidate the stereochemistry of the cyclization reaction at these positions. The deuterium-labeled taxadienes obtained from (*R*)-[1-$^2H_1$]-, (*S*)-[1-$^2H_1$]-, and [16,16,16-$^2H_3$]GGPPs (**9**, **10**, and **23b**) were established to have deuterium in the 2α and 2*β* CH_2 and 16CH_3 positions, respectively, by high-field ^1H NMR spectroscopy (eqs 1−3). Incubation of (*R*)-[4-$^2H_1$]GGPP (**17**) with the recombinant enzyme gave a 10:10:80 mixture of [5*β*-$^2H_1$]taxa-3(4),11(12)-diene, [5*β*-$^2H_1$]taxa-4(20),11(12)-diene, and unlabeled taxa-4(5),11(12)-diene according to GC/MS analyses of the products (eq 4). It follows that C-1 of GGPP underwent inversion of configuration, that the A ring cyclization occurs on the *si* face of C15, and that the terminating proton abstraction removes H5*β* from the final taxenyl carbocation intermediate. Thus, the C1−C14 and C15−C10 bonds are formed on the opposite faces of the 14,15 double bond of the substrate, i.e., overall anti electrophilic addition. The implications of these findings for the mechanism of the cyclization and rearrangement are discussed.

Introduction

Taxol (**1**) is an established anticancer drug with annual sales exceeding one billion US dollars. The use of this diterpene alkaloid for treatment of ovarian and breast cancer was approved by the FDA in the early 1990s.[1] Despite considerable effort in the synthetic community and completion of total syntheses by several laboratories,[2−5] the commercial production by purely chemical means is not viable owing to the length and low overall yields of these schemes.[6] Semisynthetic production developed by Bristol-Myers-Squibb involves harvesting 10-deacetylbaccatin III and attaching the side chain chemically.[7a] Since 2002, production by BMS has switched

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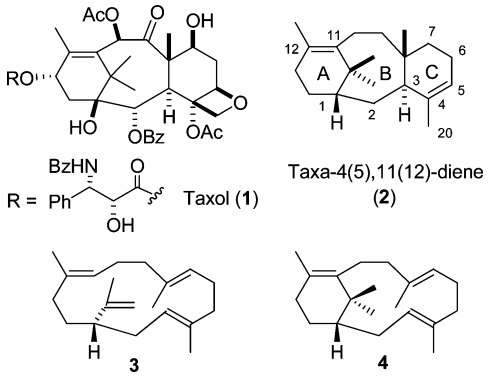
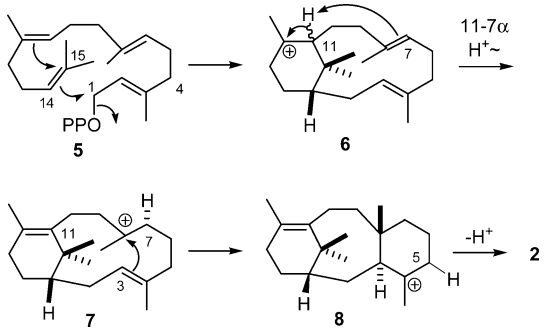
to a plant cell culture-based method, but most generic Taxol suppliers still rely on semisynthetic methods.[7b]



Considerable progress has been made in characterizing the initial and final stages of Taxol biosynthesis. The first and possibly rate-limiting step is the cyclization of (*E,E,E*)-geranylgeranyl diphosphate (GGPP, **5**) to the parent tricyclic diterpene, taxa-4(5),11(12)-diene (**2**, Scheme 1).[8] This complex reaction is catalyzed by the

SCHEME 1



enzyme taxadiene synthase, the mechanism of which will be discussed in this and a closely related paper submitted separately.[9] The structure of the cyclization product was confirmed by total synthesis of (±)-taxadiene.[10] Microsomal preparations from *Taxus brevifolia* catalyze the conversion of taxadiene to taxa-4(20),11(12)-dien-5α-ol with migration of the C-ring double bond into the exocyclic position, a structural characteristic of many taxane diterpenes.[11]

Taxadiene synthase isolated from *T. brevifolia*[12,13] has a molecular weight of 79 000, an optimal pH of 8.5, and a dependence upon a divalent metal ion for activity. The gene for this novel cyclase has been cloned and overexpressed in *E. coli*.[14,15] Taxadiene synthase shares more gene sequence homology with abietadiene synthase from grand fir[16,17] than it does with casbene synthase from castor beans,[18] despite the very different polyene cyclization effected by the former and the similar macrocyclization catalyzed by the latter, indicating that phylogeny is of greater significance than mechanistic similarity.[19]

The mechanism proposed for the cyclization is shown in Scheme 1.[16,20,21] GC/MS analysis of [$^2H_1$]taxadiene arising from incubation of [10-$^2H_1$]GGPP with taxadiene synthase established that the deuterium is transferred from C11 to the C ring of the product.[8] NMR data demonstrated that this deuterium is located at C-7 on the α-face, and a mechanism involving an intramolecular 11α→7α proton transfer was advanced.[21,22] Incubations of the related macrocyclic diterpenes, cembrene A (**3**) and verticillene (**4**), with taxadiene synthase did not produce taxa-4(5),11(12)-diene.[8] However, it remains a possibility that partially cyclized intermediates are tightly bound in the active site and have no opportunity to exchange with exogenous additives during the catalytic cycle. An alternative mechanism in which the C-ring is formed first was ruled out by the lack of incorporation of deuterium label from 2,7-*cyclo*-GGPP-d_2.[15,23] Recent labeling results support a similar macrocyclization via the same verticillen-12-yl intermediate (**6**) in the biosynthesis of the diterpene precursor to phomactin.[24]

Several stereochemical issues are presented by the mechanism shown in Scheme 1, i.e., inversion or retention of configuration at C1 of GGPP, *re* vs *si* face attack at C15 of GGPP, H11α vs H11*β* stereochemistry of the verticillen-12-yl ion intermediate **6**, and Hα vs H*β* elimination in the formation of the 4,5 double bond. Both inversion and retention have been observed at the diphosphate-bearing carbon in the cyclizations catalyzed by monoterpene and sesquiterpene synthases.[25] Retentive stereochemistry is attributed to prior allylic rearrangement to transient tertiary diphosphate intermediates that undergo anti S_N' cyclizations. This paper reports the elucidation of three of these stereochemical unknowns by means of deuterium labeled substrates. A separate

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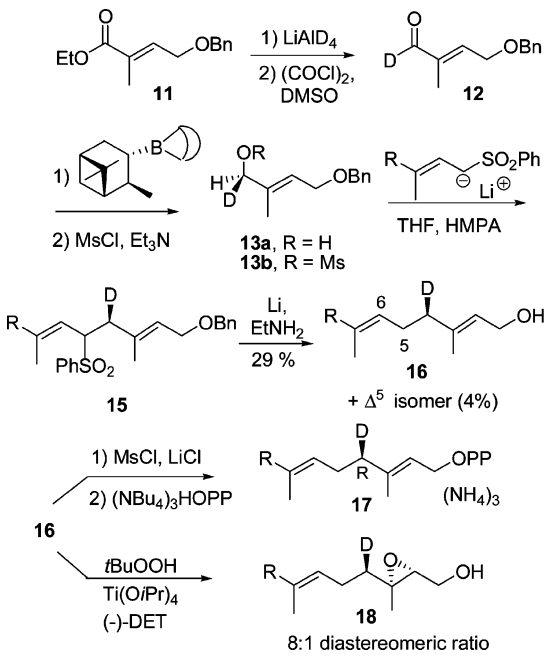
SCHEME 2



publication deals with the C11 stereochemistry of the verticillenyl intermediate in the cyclization.[9]

Synthesis of Deuterium-Labeled GGPPs. Substrates labeled with deuterium in the $1H_R/1H_S$, $4H_R/4H_S$, and 16(*E*-CH_3)/17(*Z*-CH_3) positions were required to elucidate the cryptic stereochemistry occurring at C1, C4, and C15 in the cyclization mechanism. Both (*R*)- and (*S*)-[1-$^2H_1$]GGPPs (**9** and **10**, see eqs 1 and 2) were prepared by reduction of the 1-deuterio aldehyde precursor, (*E*,*E*,*E*)-geranylgeranial, with (*R*)- and (*S*)-alpine boranes, conversion to diethyl phosphates, and S_N2 displacements with tris(tetrabutylammonium) diphosphate.[26] The enantiopurities were assumed to be the same as those of (*R*)- and (*S*)-[1-$^2H_1$]geranyl PP (98−99%) prepared in exactly the same way from deuterium-labeled geraniols. Thus, enzymatic hydrolysis of (*R*)-[1-$^2H_1$]geranyl PP with bacterial alkaline phosphatase, derivatization of (*R*)-[1-$^2H_1$]-geraniol with (1*S*)-camphanoyl chloride, and ^1H NMR analysis indicated the diasteromeric purity to be 99:1.[26, 27]

(*R*)-[4-$^2H_1$]Geranylgeraniol (**16**) was synthesized by means of a C_{15} + C_5 Biellmann coupling approach outlined in Scheme 2.[11,28] Reduction of ethyl 4-benzyloxy-2-methyl-2*E*-butenoate (**11**)[29] with $LiAlD_4$ (ether, 0 °C, 77%) followed by Swern oxidation (DMSO, $(COCl)_2$, Et_3N, CH_2Cl_2, −78 °C)[30] afforded the corresponding deuterio aldehyde (**12**, 92%). Enantioselective reduction with (*S*)-alpine borane (THF, 25 °C) gave the chiral alcohol (1*R*)-[1-$^2H_1$]-4-benzyloxy-2-methyl-2*E*-buten-1-ol (**13a**, 96%). The enantiomeric purity was determined to be 95*R*/5*S* by means of ^1H NMR analysis of the camphanate ester derivative.[27,31] Reaction of the labeled alcohol with methanesulfonyl chloride (Et_3N, CH_2Cl_2, 0 °C) provided mesylate **13b** (97%) for the alkylation reaction.

The key coupling reaction was carried out by lithiation of (*E*,*E*)-farnesyl phenyl sulfone (**14**)[32] (1 equiv of ^{n}BuLi, 3:1 THF−HMPA, −78 and −23 °C, 10 and 60 min) followed by alkylation of the sulfonyl-stabilized carbanion with mesylate **13b** (1 equiv, −78 and −23 °C, 1 and 2 h). The resulting sulfone ether (**15**, 50%) was reduced with Li/$EtNH_2$ (4:3 ether−$EtNH_2$, −78 °C) to cleave both the allylic C−S bond and the benzyl ether. (*R*)-[4-$^2H_1$]-Geranylgeraniol (**16**, 29%) was purified by chromatography on 15% $AgNO_3$−silica gel to remove a small amount of the 5,6 double-bond isomer (∼7:1 6,7−5,6 isomer ratio) formed in the reaction. The enantiomeric purity of (*R*)-[4-$^2H_1$]geranylgeraniol was determined by asymmetric epoxidation of the 2,3-double bond ($Ti(OiPr)_4$, D-(−)-diethyl tartrate, tBuOOH, CH_2Cl_2, −20 °C).[33] ^2H NMR analysis (77 MHz, C_6H_6) of the resulting (2*R*,3*R*,4*R*)-[1-$^2H_1$] epoxide (**18**) showed an 8:1 ratio for the two diastereomers (δ_D 1.35 major and 1.51 minor) which corresponds to 91:9 ratio of 4*R* and 4*S* enantiomers after allowance for the expected 95:5 enantioselectivity of the asymmetric epoxidation. The labeled alcohol **16** was converted to (*R*)-[4-$^2H_1$]GGPP (**17**) by activation as the chloride (MsCl, LiCl, collidine, DMF, 0 °C)[34] and allylic displacement (Bu_4NHOPP, CH_3CN, 25 °C, 30%) followed by ion exchange to the NH_4 salt and cellulose chromatography.[35]

GGPP labeled with deuterium in the trans terminal methyl group (**23b**) was accessed by regioselective cleavage of the 14,15 double bond of (*E*,*E*,*E*)-geranylgeranyl benzyl ether[36,37] and *E*-selective Wittig olefination. The polyene ether was converted to the trisnoraldehyde (**19**) in four steps (38% overall yield): (a) terminal hypobromination[38] with NBS in aqueous THF (4:7 H_2O/THF, 0 °C, 2.5 h, 62%), (b) epoxide formation (KOH pellets, ether, 25 °C, 78%),[39] (c) hydrolysis to the 14,15 diol ($HClO_4$, 2:1 THF/H_2O, 25 °C, 82%), and (d) periodate cleavage ($NaIO_4$, 1:1 THF/H_2O, 25 °C, 96%).[38] Wittig reaction of the aldehyde with ethyl 2-(triphenylphosphoranylidene)-propionate (1.5 equiv, CH_2Cl_2, 25 °C, 98%) gave the α,β-unsaturated ester **20** with an *E*/*Z* selectivity of 97:3

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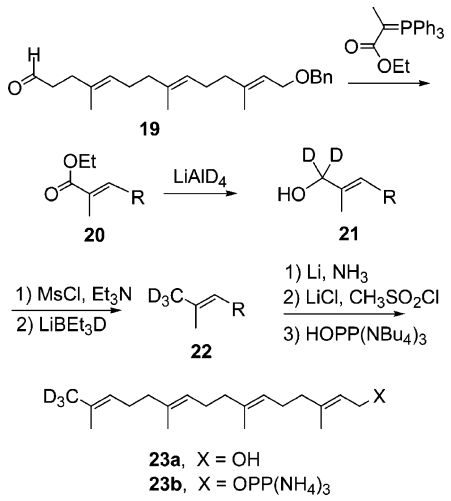
SCHEME 3



(Scheme 3). The identification of the trans and cis isomers was based on the ^{1}H NMR chemical shifts for the C14 vinyl protons (δ_H 6.74 *E* isomer, 5.90 *Z* isomer) and comparisons with literature data for similar compounds.[36]

Reduction of ester **20** with $LiAlD_4$ (Et_2O, 25 °C, 2 h) afforded the dideuterated alcohol (**21**, 82%). The trans CD_2OH group was converted to CD_3 by the mesylation ($MeSO_2Cl$, Et_3N, CH_2Cl_2, −20 °C, 30 min), and reductive displacement with $LiBEt_3D$ (1.0 M in THF, 20 equiv, −20 °C, 45 min, 82%) in the same solution afforded labeled ether **22**.[40] Reductive cleavage of the benzyl protecting group by lithium in NH_3−THF (−78 °C, 30 min) gave [16,16,16-$^2H_3$]geranylgeraniol (**23a**, 96%).[36] The localization of the deuterium predominantly in the trans terminal methyl group was verified by the absence of the corresponding signals in the ^{1}H and ^{13}C NMR spectra (δ_H 1.72 and δ_C 25.6 absent). The allylic alcohol was converted to [16,16,16-$^2H_3$]GGPP (**23b**) following known procedures.[34,35]

^{1}H NMR Assignments for Taxadiene in C_6D_6 ^{1}H and ^{13}C NMR assignments for taxadiene in $CDCl_3$ were previously reported for the product formed enzymatically[41] and for synthetic (±)-taxadiene.[10] The proton resonances arising from the C-ring hydrogens, specifically H5, H6α and H6*β*, H7α and H7*β*, and the H19 methyl protons, were assigned by Floss and Rithner using 1D DPFGSC-TOCSY and NOESY NMR techniques.[21] Halsall reported the ^{13}C NMR spectra for a series of 4(20),11-taxadiene derivatives.[42]

In the present work, a typical amount of taxadiene obtained from an enzyme incubation was 50−100 *μ*g. At this scale, the presence of a small amount of HCl from air oxidation of $CDCl_3$ might jeopardize the integrity of the sample and the quality of the spectra. Accordingly, we decided to obtain independent assignments in C_6D_6, in which solvent the microgram samples of taxadiene were expected to be more stable. ^{1}H and ^{13}C NMR spectra were obtained with synthetic (±)-taxa-4(5),11(12)-diene kindly provided by R. M. Williams.[10] The data and assignments in C_6D_6 (Table 1) correspond well with those previously reported[41] in $CDCl_3$, the maximum deviations being 0.15 ppm for ^{1}H NMR and 0.7 ppm for ^{13}C NMR.

HMBC, HMQC, TOCSY, and NOE spectral correlations were critical in securing unambiguous NMR assignments for taxadiene. The HMBC long-range correlation grid shows cross-peaks between both methyl resonances at δ_H1.09 and 1.32 ppm and the vinylic carbon at δ_C137.5 ppm. This indicates that the ^{13}C peak at δ_C 137.5 can be assigned to C11. These methyl resonances were also correlated with peaks at δ_C 38.9 and 44.2 ppm on the HMBC plot. According to the DEPT spectra, the signal at 44.2 ppm is a methine and the peak at 38.9 ppm is a quaternary carbon. Thus, these two peaks in the ^{13}C NMR spectrum were assigned to C1 and C15, respectively, and the signals at δ_H 1.09 and 1.32 belong to the geminal methyl groups (C16 and C17).

The NOE data in Table 2 allowed stereochemical assignments to be made. The CH_3 group at δ_H 0.93 (C19) showed a small positive NOE (0.7%) upon irradiation at 1.32 ppm. This allows assignment of the 1.32 and 1.09 ppm peaks to the C17 and C16 geminal methyl groups, respectively. These assignments are supported by NOEs between the C16 CH_3 (δ_H 1.09) and both H13*β* (δ_H 2.24, 2.1%) and H14*β* (δ_H 2.02, 1.2%) on the A ring. The C-5 vinyl proton clearly belongs to the peak at δ_H 5.38 ppm, and by HMQC correlation, its ^{13}C resonance was assigned to 121.3 ppm. The C5 carbon resonance is also correlated by HMBC to the C-20 methyl group at δ_H 1.73 ppm. The peaks at 137.5 and 129.3 ppm were related to the C-18 vinyl methyl peak at 1.60 ppm. In the COSY spectrum, the vicinal protons at δ_H 1.94 and 2.11 ppm for C-6 showed cross-peaks with the vinyl proton at 5.38 ppm. The C5 proton is correlated on the HMBC map with the C7 carbon peak at 38.5 ppm, which in turn is matched with vicinal proton peaks at δ_H 1.19 and 1.74 ppm in the HMQC plot. Irradiation of the C19 methyl peak resulted in a positive NOE (1.1%) at 1.19 ppm, identifying the 7*β* proton.

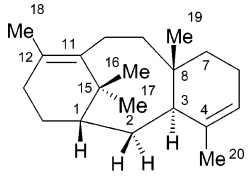
Rigorous assignments for H2α and H2*β* were complicated by the proximity of their signals (δ_H 1.62 and 1.72) to each other and to that for H1 (δ_H 1.66) in the proton spectrum and the extensive overlap in this region. However, NOEs observed at δ_H 1.72 (1.8 and 2.1%) after irradiation at the C17 and C19 methyl signals enabled attribution of the higher field resonance to H2*β*.

An examination of Dreiding models shows the proximity of the C16 and C19 methyl groups, and the resulting steric repulsion is a serious limiting factor for the conformations available to the molecule. According to the coupling constants between the H1, H2α, H2*β*, and H3 protons (Table 1), the following dihedral angles can be deduced:[43] 1/2α, 36°; 1/2*β*, 58°; 3/2α, 112°; 3/2*β*, 132°. The 7α proton shows two additional couplings of 5.9 Hz besides the geminal coupling, and the 7*β* proton shows one coupling of 6.2 Hz. The dihedral angels between these protons and the C6 protons can be deduced from the coupling constants:[43] 6α/7*β*, 90°; 6*β*/7*β*, 28°; 6α/7α, 32°; 6*β*/7α, 143°. The C ring needs to adopt a pseudo-chair conformation to fulfill these dihedral angles.

The 13*β*, 14α, and 14*β* protons show vicinal coupling constants greater than 10 Hz. Thus, the conformation of

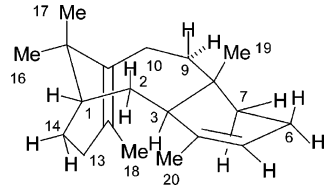
(40) Andres, H.; Morimoto, H.; Williams, P. G. *J. Chem. Soc., Chem. Commun*. **1990**, 627.
(41) Koepp, A. E.; Hezari, M.; Zajicek, J.; Vogel, B. S.; LaFever, R. E.; Lewis, N. G.; Croteau, R. *J. Biol. Chem.* **1995**, *270*, 8686.
(42) Halsall, T. G. *Org. Magn. Reson.* **1983**, *21*, 524.
(43) Karplus, M. *J. Chem. Phys*. **1959**, *30*, 11.

TABLE 1. 600 MHz ^{1}H and 150 MHz ^{13}C NMR Data and Assignments for (±)-Taxa-4(5),11(12)-diene (2) in C_6D_6



C no.	δ_C	δ_H	m	*J* (Hz)	C no.	δ_C	δ_H	m	*J* (Hz)
1	44.2	1.66	dd	7.4, 6.8	11	137.5			
2α		1.62	ddd	15.7, 5.2, 2.1	12	129.3			
	28.4								
2*β*		1.72	ddd	15.5, 6.4, 1.9	13α		1.79		
						29.6			
3	39.8	2.62			13*β*		2.24	br t	14.7
4	138.1				14α		1.58	ddd	14.7, 10.6, 4.3
						23.3			
5	121.3	5.38	m		14*β*		2.02	dddd	14.9, 12.1, 8.8, 3.1
6α		1.94	br d	17.4	15	38.9			
	22.7								
6*β*		2.11	m		16	30.6	1.09	s	
7α		1.76			17	26.1	1.32	s	
	38.5								
7*β*		1.19			18	21.2	1.60	s	
8	37.3				19	21.5	0.93	s	
9α		1.39	dt	14.9, 5.9	20	23.9	1.73	s	
	41.4								
9*β*		1.81	ddd	15.0, 10.0, 5.1					
10α		2.15							
	24.5								
10*β*		2.63	ddd	15.0, 10.4, 5.4					

TABLE 2. NOE Enhancements in the 600 MHz ^{1}H NMR Spectrum of (±)-Taxa-4(5),11(12)-diene (2) in C_6D_6 after Irradiations at 0.93, 1.09, and 1.32 ppm



irradiation at 16 CH_3 (1.09 ppm)			irradiation at 17 CH_3 (1.32 ppm)			irradiation at 19 CH_3 (0.93 ppm)		
proton	δ_H	NOE (%)	proton	δ_H	NOE (%)	proton	δ_H	NOE (%)
17CH_3	1.32	1.6	19CH_3	0.93	0.7	7*β*	1.19	1.1
1CH	1.66	1.6	16CH_3	1.09	1.7	17CH_3	1.32	0.7
14*β*	2.02	1.2	1CH	1.66	1.1	9α	1.39	0.7
13*β*	2.24	2.1	2*β*	1.72	1.8	2*β*	1.72	2.1
3 CH	2.62	0.5	9*β*	1.81	1.9	9*β*	1.81	1.2
			10α	2.15	1.5	6*β*	2.11	2.2
			3CH	2.62	0.1	3CH	2.62	0.5

the A ring is likely a twisted pseudo-boat form to match these coupling constants. These conclusions about the conformation of taxa-4(5),11(12)-diene are in agreement with the X-ray crystal structure and data published by Williams and Rubenstein for the related synthesis of 20-nor-4,5-dihydrotaxen-20-one.[10]

Incubations of Labeled GGPPs with Taxadiene Synthase. Because the full-length taxadiene synthase cDNA is translated as a catalytically impaired preprotein,[14] a series of N-terminally truncated enzymes was generated by expression in *E. coli* of the corresponding 5′-truncated cDNAs from a suitable vector.[15] A pseudomature recombinant form of taxadiene synthase having 60 amino acids deleted from the preprotein was found to be most efficient, with kinetics comparable to those of the mature native enzyme.[15] Incubations were performed under established assay conditions in the presence of 1 mM $MgCl_2$, the labeled GGPPs, and a small amount of [1-^{3}H]GGPP. The resulting nonpolar products were extracted into pentane, and the solvent was removed with a nitrogen stream before GC and GC−MS analyses. Typical yields were ∼6%. Enzymatic cyclization with unlabeled subtrate afforded a 5:95 mixture of exocyclic and endocyclic taxadiene isomers according to GC analyses.

The location and configuration of the deuterium in the [2-$^2H_1$]taxadiene epimers formed from (*R*)- and (*S*)-[1-$^2H_1$]-GGPPs (**9** and **10**) were elucidated by 1D TOCSY ^{1}H NMR spectra (Figure 1 and the Supporting Information). The presence of one deuterium in both [2-$^2H_1$]taxadiene samples is apparent from their nearly identical GC−MS spectra (Supporting Information) that show M^+ at *m/z* 273 and the most intense fragment ion at *m/z* 123, both

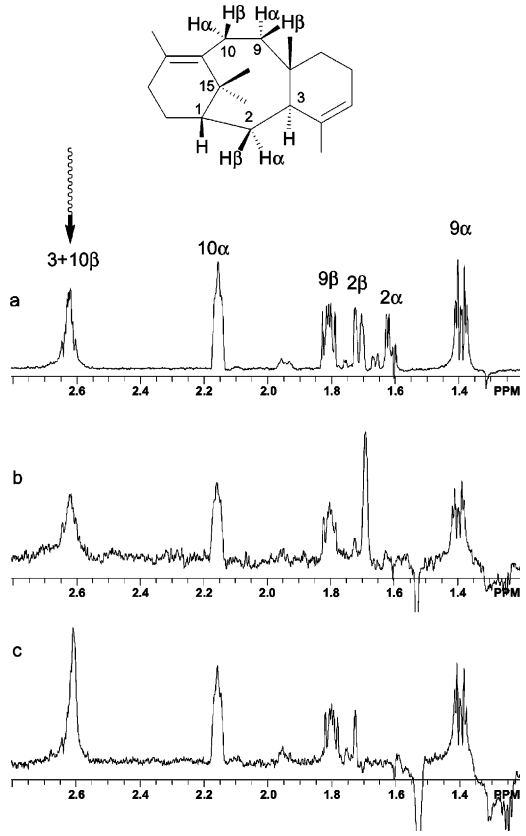
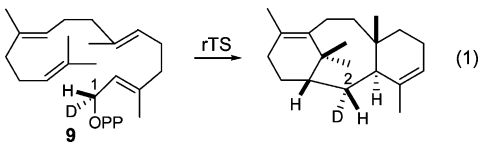


FIGURE 1. 1D TOCSY NMR spectra (750 MHz, C_6D_6, 15 °C) of taxadienes generated by irradiations at 2.62 ppm: (a) unlabeled taxadiene; (b) [2-$^2H_1$]taxadiene from (*R*)-[1-$^2H_1$]-GGPP (**9**); (c) [2-$^2H_1$]taxadiene from (*S*)-[1-$^2H_1$]GGPP (**10**).

increased by 1 amu compared to those in the GC−MS of unlabeled **2**. The TOCSY pulse sequence was employed to eliminate peak overlap in the region of interest, i.e., 1.62 (H2α) and 1.72 (H2*β*), which coincided with, or overlapped, peaks for H1 (1.66), H20 CH_3 (1.73), H7α (1.76), and H9*β* (1.81). The TOCSY spectrum of unlabeled taxadiene following irradiation at 2.62 (H3 and H10*β*) led to five peaks at 1.39 (H9α), 1.62 (H2α), 1.72 (H2*β*), 1.81 (H9*β*), and 2.15 (H10α) (Figure 1a). The TOCSY spectrum (2.62 irradiation) of the labeled product from (*R*)-[1-$^2H_1$]GGPP (**9**) displays a strong peak at 1.69 and no detectable peak in the vicinity of 1.62 (Figure 1b). The small upfield displacement (Δ*δ* −0.03 ppm) is typical for a geminal deuterium isotope shift.[44] This spectrum clearly locates the deuterium in the 2α position (eq 1).



In contrast, the TOCSY spectrum of the [2-$^2H_1$]-taxadiene from (*S*)-[1-$^2H_1$]GGPP (**10**) generated by the same method showed no peaks in the same region. The absence of a TOCSY signal for H2α can be explained by the assumption that the spin propagation from H3 to H2α

(44) Bovey, F. A. *Nuclear Magnetic Resonance Spectroscopy*, 2nd ed.; Academic Press: San Diego, 1988; p 141.

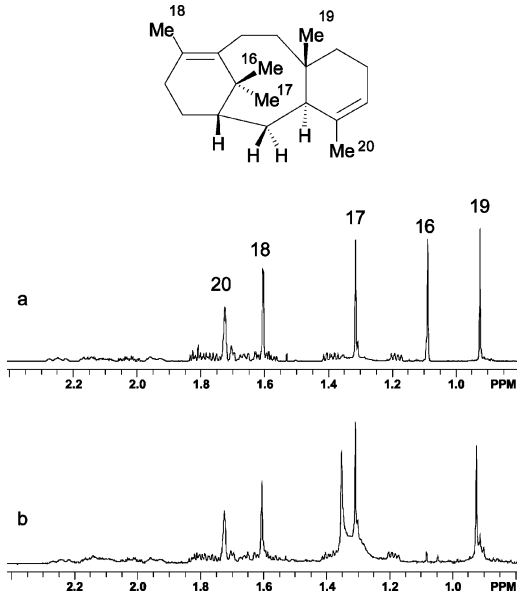
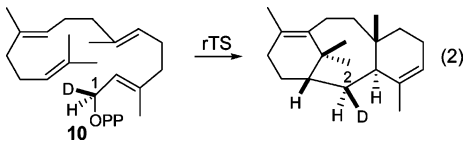
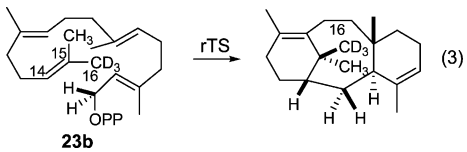


FIGURE 2. ^1H NMR spectra (600 MHz, C_6D_6, 15°C) of unlabeled (±)-taxadiene (**2**) (a) and [$^2H_3$]taxadiene biosynthesized from [16,16,16-$^2H_3$]GGPP (**23b**) (b). The positional numbers are shown above the five methyl signals in panel a. The signal at 1.09 (16 CH_3) is absent in panel b. The extra peak at 1.36 in the lower spectrum is from an impurity.

occurs mainly by means of the geminal H2*β*-H2α coupling interaction, owing to the small magnitude of the direct vicinal coupling interaction, i.e., $J_{3-2\alpha.}$ = 2.1 Hz. The presence of deuterium at H2*β* would block the H2*β*−H2α spin transmission path. The presence of hydrogen in the 2α position of the sample was confirmed by the observation of an NOE at 1.59 upon irradiation at 1.73 (C20 CH_3). The puckered conformation of the B ring in taxadiene brings the C-ring vinyl methyl (C20) into close proximity with H2α. We conclude that enzyme-catalyzed cyclization of (*S*)-[1-$^2H_1$]GGPP gave rise to [2*β*-$^2H_1$]-taxadiene (eq 2).

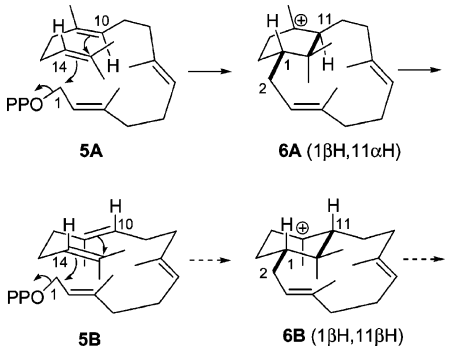


The ^1H NMR spectrum of the taxadiene product from incubation of [16,16,16-$^2H_3$]GGPP (**23b**) showed a negligible peak at 1.09 ppm and a normal sized peak at 1.32 ppm (Figure 2). This clear-cut result proves that the C16 methyl group of the [$^2H_3$]taxadiene product is deuterated (eq 3).

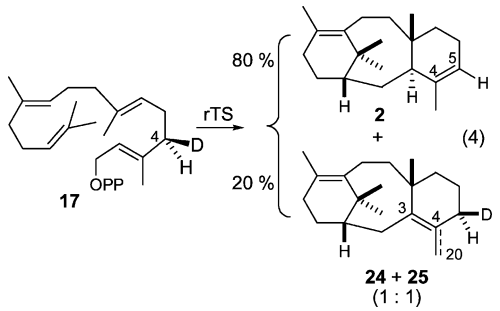


GC−MS analysis of the incubation products from (*R*)-[4-$^2H_1$]GGPP (**17**) showed three peaks in a 10:10:80 area ratio with retention times of 10.29, 10.49, and 10.70 min (MS, M^+ 273, 273, and 272, respectively). The second and third peaks in retention time order were identi-

SCHEME 4



fied as [5-$^2H_1$]taxa-4(20),11(12)-diene (**25**) and unlabeled 4(5),11(12)-taxadiene (**2**) by GC and MS comparisons with synthetic samples (eq 4).[10] The first component eluted was assigned tentatively as [5-$^2H_1$]taxa-3(4),11(12)-diene (**24**), from its molecular ion peak in the MS and the absence of the usual C-ring fragment ion.[41] This product was barely detectable (~1%) in the mixture from unlabeled substrate while the proportion of the exocyclic isomer **25** was typically 4−5%. Presumably, the primary kinetic isotope associated with D5β elimination from the taxen-4-yl ion to form the 4,5 double-bond isomer magnifies the extent of competition of the minor pathways (H3α and H20 eliminations) that gives rise to the minor taxadiene isomers.[15] These results confirm that proton abstraction occurs predominantly on the β-face of C5 of taxen-4-yl ion.[15,21]

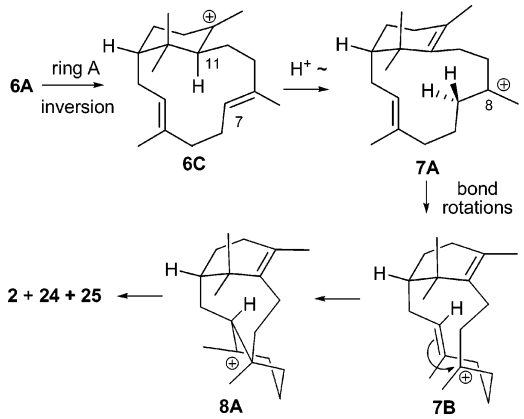


Discussion

The known 1βH stereochemistry of taxadiene and the new finding that the trans terminal CH_3 of GGPP becomes the C16 methyl in the tricyclic product (eq 3) require that the C1−C14 and C15−C10 bonds are formed on the opposite faces of the 14,15 double bond (14 *re*, 15 *si*) of GGPP, corresponding to an anti addition. The observed inversion of configuration at CH_2OPP (eqs 1 and 2) indicates the likelihood of a stereoelectronically favorable antiperiplanar macrocyclization and renders unlikely the possibility of allylic rearrangement followed by S_N' cyclization. A boat or chair folding of the terminal diene of GGPP would allow antiperiplanar alignment of C1 with the 14,15 and 10,11 double bonds (Scheme 4, **5A** vs **5B**). Addition to the C11−C12 double bond might occur on the *re-re* or *si-si* face to generate trans (H1β−H11α) or cis (H1β−H11β) bridged verticillen-12-yl ion intermediates (**6A** vs **6B**).

All known natural products belonging to the biogenetically related verticillane group of diterpenes[45,46] and retaining both bridgehead protons are shown in the

SCHEME 5



literature with trans (1H−11H) hydrogens on the six-membered A ring. In the case of verticillol,[45] the trans bridging was firmly established by means of an X-ray crystal structure determination.[45c,47] The same holds for phomactatriene, the rearranged verticillane diterpene derived from the 1β,11α-*trans*-verticillen-12-yl ion (**6A**) by vicinal 11αH → 12αH and 15βMe → 11βMe rearrangements.[24] Recent results from incorporation of labeled acetate into phomacatriene point to an initiating macrocyclization similar to that shown in Scheme 4. The parallel finding in this laboratory that taxadiene synthase-catalyzed cyclization of 6-fluoroGGPP to predominantly 7-fluoroverticilla-3,7,12(18)-triene with trans H1β/H11α bridgehead configurations affords compelling evidence in favor of the trans-bridged verticillen-12-yl$^+$ ion intermediate **6A**.[9]

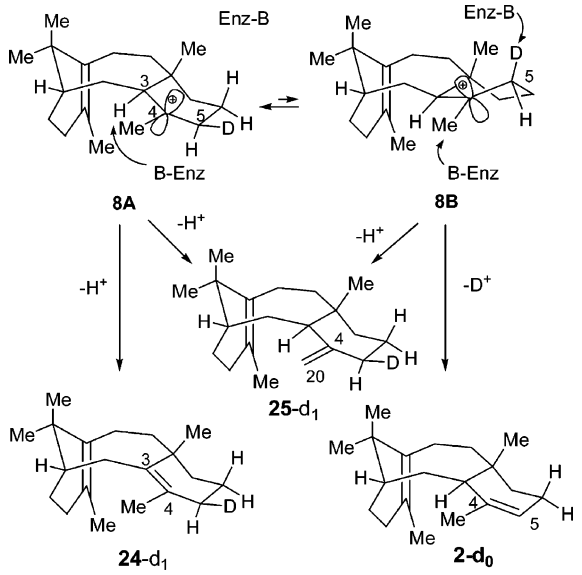
The six-membered A ring of the initially formed trans-bridged verticillen-12-yl positive ion **6A** must undergo conformational inversion to enable formation of the C ring (Scheme 5). The intramolecular transfer of the C11 bridgehead proton to C7 shown has been verified by deuterium labeling.[8,21] Molecular modeling indicates that a direct intramolecular 11α → 7α proton transfer is sterically and energetically favorable.[21] Alternatively an indirect process mediated by an active-site residue would appear feasible. Similar nonexchanging proton translocations take place in cyclization mechanisms producing the sesquiterpene pentalenene[22] and the diterpene abietadiene.[17] In the case of pentalenene biosynthesis, it was postulated that a histidine residue was responsible for carrying out a vicinal deprotonation−reprotonation.[48] However, site-directed mutagenesis showed that this enzyme base is not essential for catalytic activity.[49]

(45) (a) Kaneko, C.; Hayashi, S.; Ishhikawa, M. *Chem. Pharm. Bull.* **1964**, *12*, 1510. (b) Erdtman, H.; Norin, T.; Sumimoto, M.; Morrison, A. *Tetrahedron Lett.* **1964**, *51*, 3879. (c) Karlsson, B.; Pilotti, A.-M.; Söderholm, A.-C.; Norin, T.; Sundin, S.; Sumimoto, M. *Tetrahedron* **1978**, *34*, 2349.

(46) (a) Harrison, L. J.; Tori, M.; Taira, Z.; Asakawa, Y. *The 28th Symposium on the Chemistry of Terpenes, Essential Oils, and Aromatics*; Kanasawa, Japan, 1984; p 285. (b) Nagashima, F.; Toyota, M.; Asakawa, Y. *Phytochemistry* **1990**, *29*, 2169. (c) Asakawa, Y. In *Progress in the Chemistry of Natural Products*; Herz, W., Kirby, G. W., Moore, R. E., Steglich, W., Tamm, Ch., Eds); Springer: Vienna, 1995; Vol. 65, p 1. (d) Nagashima, F.; Tamada, A.; Fujii, N.; Asakawa, Y. *Phytochemistry* **1997**, *46*, 1203. (e) Basar, S.; Koch, A.; Konig, W. A. *Flavour Frag. J.* **2001**, *16*, 315. (f) Duh, C.-Y.; El-Gamal, Ali Ali H.; Wang, S.-K.; Dai, C.-F. *J. Nat. Prod.* **2002**, *65*, 1429.

(47) The absolute configuration of (+)-verticillol has been revised as H1β/H11α (1*S*,11*S*) through anomalous dispersion analysis in the X-ray crystallographic determination of its *p*-iodobenzoate derivative.[9]

SCHEME 6



Inversion of the cyclohexene ring to a half-boat form and rotations of the nonenyl chain (**7A** → **7B**) are required in order to bring the carbocation site into position for cyclization onto the *re-re* face of the 3(4) double bond to generate the taxen-4-yl carbocation, **7B** → **8A**.

The loss of deuterium from (*R*)-[4-$^2H_1$]GGPP in the final step of the mechanism indicates that proton elimination forming taxa-4(5),11(12)-diene occurs exclusively on the β-face (Scheme 6). The C ring of the tax-11-en-4-yl$^+$ ion presumably adopts a twist-boat conformation (**8A**→ **8B**) in order for a stereoelectronically favorable elimination of a ψ-axial 5β proton to occur. It is not clear why the ostensibly higher energy elimination of the 5β proton predominates, other than enforced proximity to an active site base. The elimination of H3α in the formation of the tetrasubstituted isomer **24**-d_1 indicates access of a proton acceptor on the opposite face. The increased proportions of the 3(4) and 4(20) double bond isomers (**24** and **25**) of taxadiene reflect the operation of a substantial primary kinetic isotope effect.[15, 21]

Conclusions

The labeling results reported herein indicate that the initiating macrocyclization step in taxadiene biosynthesis occurs by a stereoelectronically favorable alignment of CH_2OPP with the 14,15 and 10,11 double bonds of the GGPP substrate. The potentially concerted alkylation/π-cyclization proceeds directly to a verticillen-12-yl carbocation intermediate having either a trans (1β,11α) or cis (1β,11β) stereochemical relationship of the bridgehead hydrogens (**6A** vs **6B**). The taxadiene synthase-catalyzed conversion of 6-fluoroGGPP to 7-fluoroverticilla-3,7,12-(18)-triene with trans bridgehead stereochemistry and other evidence strongly favor 1,11-trans verticillenyl ion **6A**.[9] Inversion of the A ring followed by direct or indirect 11 → 7α H$^+$ transfer, cyclization onto the 3,4 double bond, and terminating elimination of the 5β hydrogen from a twist-boat conformation of a taxa-11-en-4-yl ion intermediate complete the complex tricyclization mechanism.

Experimental Section

Representative preparative procedures and characterization data for (*R*)-[4-$^2H_1$]GGPP (**17**) and [16,16,16-$^2H_3$]GGPP (**23b**), and for the enzymatic incubation experiments, are given below. General experimental aspects, procedures, and characterization data for the other compounds and NMR spectra and GC−MS traces of taxadiene and deuterium-labeled taxadienes are available in the Supporting Information.

(*4S,E,E,E*)-[4-$^2H_1$]5-Benzenesulfonyl-1-benzyloxy-3,7,-11,15-tetramethyl-2,6,10,14-hexadecatetraene (15). The procedure of Yee and Coates for a different compound was followed.[50] A patent by Kuraray Co. mentioned the preparation of the same compound in unlabeled form in their synthesis of *all-trans*-polyprenols.[51] A solution of sulfone **14** (412 mg, 1.19 mmol) in HMPA (2 mL) and THF (6 mL) was stirred and cooled at −78 °C as *n*-BuLi in hexane (0.76 mL, 1.56 M, 1.19 mmol) was added dropwise. After 10 min at − 78 °C and 1 h at − 23 °C, the solution was cooled back to −78 °C. A solution of mesylate **13b** (322 mg, 1.19 mmol) in THF (3 mL) was added dropwise, stirring was continued for 1 h at −78 °C and 2 h at −23 °C, and MeOH (10 drops) was added. The yellow solution was allowed to warm to 25 °C, and H_2O (30 mL) and 1 M HCl (2 mL) were added. The product was extracted with hexane (4 × 30 mL), and the combined organic extracts were washed with H_2O (3 × 50 mL), dried (Na_2SO_4), and evaporated to give a yellow oil (760 mg). Purification by flash chromatography (15% EtOAc in hexane) provided 312 mg (50%) of sulfone **15**: R_f 0.44 (30% EtOAc in hexane); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.55 (s, 3 H, C*H*$_3$), 1.56 (d, 3 H, J = 1.0 Hz, C*H*$_3$), 1.57 (s, 3 H, C*H*$_3$), 1.59 (s, 3 H, C*H*$_3$), 1.67 (d, 3 H, J = 1.0 Hz, C*H*$_3$), 1.89−2.09 (m, 9 H, C*H*$_2$), 3.91 (m, 1 H, C*H*), 3.96 (t, 2 H, J = 6.5 Hz, C*H*$_2$), 4.43 (s, 2 H, C*H*$_2$), 4.93 (d, 1 H, J = 10.5 Hz, vinyl *H*), 5.02 (m, 1 H, vinyl *H*), 5.07 (t of quintets, 1 H, J = 6.8, 1.5 Hz, vinyl *H*), 5.39 (t, 1 H, J = 6.7 Hz, vinyl *H*), 7.25−7.88 (m, 10 H, Ar*H*); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 15.8, 16.3, 17.6, 25.6, 26.2, 26.6, 39.56, 39.60, 63.03, 63.05, 66.0, 71.6, 116.81, 116.84, 123.2, 124.1, 124.66, 124.70, 127.43, 127.56, 128.2, 128.6, 129.1, 133.3, 135.5, 137.6, 138.2, 145.4.

(*4R,E,E,E*)-[4-$^2H_1$]-3,7,11,15-Tetramethyl-2,6,10,14-hexadecatetraen-1-ol (16). The procedure of Yee and Coates for a different compound was followed.[50] To a solution of sulfone **15** (266 mg, 0.51 mmol) in Et_2O (6 mL) at −78 °C was added $EtNH_2$ (8 mL) through Tygon tubing by evaporation from the metal container (40 °C) and condensation in the reaction flask (−78 °C). Lithium pieces (141 mg, 20.4 mmol) were added, and the suspension was stirred at −78 °C until the blue color persisted. 1-Hexyne (1 mL) was added, the Li pieces were removed, and the yellow color was quenched by addition of MeOH (1 mL). The solution was allowed to warm to 25 °C, and the solvents were evaporated. The residue was partitioned between H_2O (20 mL) and ether (20 mL), and the aqueous layer was extracted with ether (3 × 25 mL). The combined organic extracts were washed with 1% HCl (1 × 40 mL), satd NaCl (1 × 40 mL), satd $NaHCO_3$ (1 × 40 mL), and satd NaCl (1 × 40 mL). The ethereal solution was dried (Na_2SO_4) and evaporated to give a yellow oil (106 mg). Purification by flash chromatography (15% $AgNO_3$ in SiO_2, 10% EtOAc in hexane to 50% EtOAc in hexane) gave (4*R*,*E*,*E*,*E*)-[4-$^2H_1$]GGOH (**16**, 43 mg, 29%). (The $AgNO_3$-impregnated silica gel was prepared as follows: To 85 g of SiO_2 and 15 g of $AgNO_3$ was added 50 mL of CH_3CN. After the mixture was stirred for 30 min at 25 °C, the solvent was removed under reduced pressure until the

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weight was constant.) The ^{1}H NMR spectrum of **16** was identical with that of unlabeled GGOH except that the CH_2 peaks at 1.94−2.13 ppm showed one less proton upon integration: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.13 (s, 1 H, O*H*), 1.60 (s, 9 H, C*H*$_3$), 1.68 (s, 6 H, C*H*$_3$), 1.94−2.13 (m, 11 H, C*H*$_2$), 4.15 (dd, 2 H, J = 5.9, 3.2 Hz, C*H*$_2$), 5.11 (m, 3 H, vinyl *H*), 5.42 (t of quintets, 1 H, J = 6.8, 1.2 Hz, vinyl *H*).

(4*R*,*E*,*E*,*E*)-[4-^{2}H$_1$]-3,7,11,15-Tetramethyl-2,6,10,14-hexadecatetraen-1-yl Diphosphate, Triammonium Salt (17). The diphosphorylation was carried out according to the procedure reported by Poulter.[35] To a solution of LiCl (22 mg, 0.52 mmol), collidine (112 mg, 0.93 mmol), and alcohol **16** (30 mg, 0.10 mmol) in DMF (4 mL) at 0 °C was added CH_3SO_2Cl (71 mg, 0.62 mmol). After 1 h at 0 °C, ice−water (20 mL) was added, and the product was extracted with cold pentane (3 × 20 mL). The combined organic extracts were washed with satd $Cu(NO_3)_2$ (5 × 20 mL), satd NaCl (2 × 20 mL), and satd $NaHCO_3$ (1 × 20 mL). Drying (Na_2SO_4) and evaporation gave the chloride as a yellow oil (32 mg, quantitative). The chloride (32 mg, 0.10 mmol) was dissolved in CH_3CN (1 mL), and powdered 3 Å molecular sieves (200 mg) and HOPP $(NBu_4)_3$ (136 mg, 0.14 mmol) were added. After 16 h at 25 °C, CH_3CN (30 mL) was added, and the solids were filtered. The filtrate was washed with hexane (4 × 10 mL), and the acetonitrile layer was evaporated to give a brown oil (186 mg). The oil was dissolved in ion exchange buffer (2 mL) and loaded onto an ion-exchange column (6 mL ion-exchange resin). Elution with ion-exchange buffer (12 mL) and lyophilization gave a yellow solid, which was purified by cellulose chromatography (10 mL cellulose, cellulose buffer) to give 15.3 mg (30%) of the labeled diphosphate **17** as a white solid: R_f 0.35 (cellulose TLC, 2:1:1 2-propanol/acetonitrile/0.1 M NH_4HCO_3). The ^{1}H NMR spectrum was the same as the unlabeled GGPP except that integration for the CH_2 peaks at 1.76−1.98 ppm changed from 12 to 11 H: ^{1}H NMR (D_2O, 500 MHz) δ 1.41 (s, 6 H, C*H*$_3$), 1.43 (s, 3 H, C*H*$_3$), 1.48 (s, 3 H, C*H*$_3$), 1.53 (s, 3 H, C*H*$_3$), 1.76−1.98 (m, 11 H, C*H*$_2$), 4.28 (t, 2 H, J = 6.2 Hz, C*H*$_2$), 4.97 (m, 3 H, vinyl *H*), 5.26 (t, 1 H, J = 6.7 Hz, vinyl *H*); ^{31}P NMR (D_2O, 162 MHz) δ −9.54 (d, J = 20.8 Hz), −5.72 (d, J = 20.8 Hz).

(*E*,*E*,*E*,*E*)-[16,16,16-^{2}H$_3$]-1-Benzyloxy-3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraene (22). The mesylation followed Woggon's procedure,[52] and the reduction followed Williams' procedure.[40] To a solution of alcohol **21** (104 mg, 0.26 mmol) and Et_3N (106 mg, 1.04 mmol) in CH_2Cl_2 (6 mL) at − 20 °C was added CH_3SO_2Cl (39 mg, 0.34 mmol). After 30 min at − 20 °C, $LiBEt_3D$ (5.2 mL, 1.0 M in THF, 5.2 mmol) was added dropwise. After 45 min at − 20 °C, the reaction was quenched by slow addition of H_2O (20 mL). The aqueous layer was extracted with hexane (3 × 20 mL), and the combined organic extracts were washed with 1% HCl (2 × 30 mL), satd $NaHCO_3$ (1 × 30 mL), and satd NaCl (1 × 30 mL). Drying (Na_2SO_4) and evaporation gave a colorless oil (160 mg), which was purified by flash chromatography (30% CH_2Cl_2 in pentane) to give **22** (82 mg, 82%) as a pale yellow oil. The ^{1}H and ^{13}C NMR spectra were identical to the unlabeled compound except that the peak at 1.72 ppm (d, 3 H, J = 1.0 Hz, C*H*$_3$) was absent in the ^{1}H NMR spectrum and the 25.6 ppm peak was absent in the ^{13}C NMR spectrum. Data for **22**: R_f 0.40 (35% CH_2Cl_2 in pentane); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.61 (s, 9 H, C*H*$_3$), 1.66 (s, 3 H, C*H*$_3$), 1.95−2.17 (m, 12 H, C*H*$_2$), 4.04 (d, 2 H, J = 6.6 Hz, C*H*$_2$), 4.51 (s, 2 H, C*H*$_2$Ph), 5.12 (m, 3 H, vinyl *H*), 5.42 (t of sextets, 1 H, J = 6.6, 1.2 Hz, vinyl H), 7.26−7.38 (m, 5 H, aryl *H*); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 15.87, 15.90, 16.4, 17.5, 26.2, 26.5, 26.6, 39.50, 39.57, 39.59, 66.4, 71.8, 120.7, 123.7, 124.1, 124.3, 127.4, 127.7, 128.2, 131.0, 134.8, 135.2, 138.5, 140.3.

(*E*,*E*,*E*,*E*)-[16,16,16-^{2}H$_3$]-3,7,11,15-Tetramethyl-2,6,10,14-hexadecatetraen-1-ol (23a). The deprotection was carried out according to Coates' procedure.[50] To a solution of **22** (82 mg, 0.21 mmol) in THF (6 mL) and NH_3 (9 mL) at − 78 °C was added Li (30 mg, 0.43 mmol). After 30 min at − 78 °C, 3-hexyne (1 mL) was added, and after 5 min, satd NH_4Cl (5 mL) was added slowly. The white suspension was allowed to warm to rt, and satd NH_4Cl (15 mL) was added. The aqueous layer was extracted with hexane (4 × 20 mL), and the combined organic extracts were washed with satd NaCl (1 × 25 mL). Drying (Na_2SO_4) and evaporation gave a light yellow oil (67 mg), which was purified by flash chromatography (20% EtOAc in hexane) to give (*E*,*E*,*E*,*E*)-[16,16,16-^{2}H$_3$]-geranylgeraniol (60 mg, 96%) as a yellow oil. The ^{1}H NMR spectrum was identical to that of unlabeled GGOH except that the integration of the singlet at 1.68 ppm changed from 6 to 3 H: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.60 (s, 9 H, C*H*$_3$), 1.68 (s, 3 H, C*H*$_3$), 1.95−2.14 (m, 12 H, C*H*$_2$), 4.15 (d, 2 H, J = 7.0 Hz, C*H*$_2$), 5.11 (m, 3 H, vinyl *H*), 5.42 (td, 1 H, J = 7.0, 1.1 Hz, vinyl *H*).

(*E*,*E*,*E*,*E*)-[16,16,16-^{2}H$_3$]-3,7,11,15-Tetramethyl-2,6,10,14-hexadecatetraen-1-yl Diphosphate, Triammonium Salt (23b). Diphosphorylation was carried out as described above for **17**. Alcohol **23a** (26 mg, 0.088 mmol) gave 8.7 mg (20%) of **23b**, R_f 0.34 (cellulose TLC, cellulose buffer). The ^{1}H NMR spectrum was identical to that of unlabeled GGPP except that the CH_3 resonance at 1.48 ppm was absent: ^{1}H NMR (D_2O, 400 MHz) δ 1.39 (s, 3 H, C*H*$_3$), 1.42 (s, 3 H, C*H*$_3$), 1.52 (s, 3 H, C*H*$_3$), 1.75−1.95 (m, 12 H, C*H*$_2$), 4.26 (t, 2 H, J = 6.1 Hz, C*H*$_2$O), 4.89−5.00 (m, 3 H, vinyl *H*), 5.25 (t, 1 H, J = 6.8 Hz, vinyl *H*); ^{31}P NMR (D_2O, 162 MHz) δ −9.5 (d, J = 20.8 Hz), −5.7 (d, J = 20.8 Hz).

Preparative Incubations with Recombinant Taxadiene Synthase. The truncated (M60) version of recombinant taxadiene synthase, from which the plastidial transit peptide had been deleted, was overexpressed in *E. coli* and purified (>96%) as previously described.[15] Preparative incubations were carried out in 3 mL of the standard assay buffer (25 mM Hepes, pH 8.0, containing 10% (v/v) glycerol) in the presence of 1 mM $MgCl_2$ and saturating levels of deuterated GGPP plus a trace amount of [1-^{3}H]GGPP (10^6 dpm) in order to monitor the conversion easily. Up to 1 mg of protein was employed in each incubation for 8−12 h at 31 °C, which afforded pentane-soluble product yields in the 5−10% range.

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Supporting Information Available: General experimental aspects, preparative procedures, and chracterization data for compounds not given in the Experimental Section and other NMR figures and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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