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Studies on Taxadiene Synthase: Interception of the Cyclization Cascade at the Isocembrene Stage with GGPP Analogues

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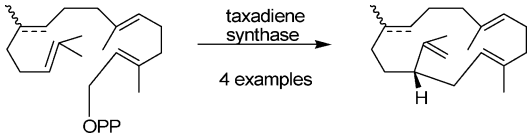

The cyclization of GGPP to taxadiene, catalyzed by taxadiene synthase, has been suggested to proceed through a series of monocyclic isocembrenyl- and bicyclic verticillyl-carbocationic intermediary stages. A set of GGPP analogues with abolished or perturbed π-nucleophilicity at the Δ^{10} double bond (GGPP numbering) was synthesized and incubated with taxadiene synthase to intercept the cyclization cascade at the monocyclic stage. Each analogue was transformed by taxadiene synthase in vitro to hydrocarbon products in varying yields, and the structures of the major product in each reaction were solved by GCEIMS and one- and two-dimensional (^{1}H and ^{13}C) NMR and found to be 14-membered monocyclic isocembrenyl diterpenes, indicating that the first C−C bond formation catalyzed by taxadiene synthase could be uncoupled from the other subsequent bond formation events by using suitably designed substrate analogues. The formation and isolation of these isocembrenyl diterpene products using taxadiene synthase supports proposals that the isocembrenyl cation is an intermediate in the cyclization of GGPP to taxadiene.

Introduction

Paclitaxel (**1**, Figure 1) is an important natural product belonging to the isoprenoid family.[1] Originally isolated from the bark of the yew tree *Taxus brevifolia* by Wani and Wall in 1963, paclitaxel was approved as a chemotherapy agent for breast and ovarian cancer in late 1992.[2] The clinical effectiveness and the scarcity of paclitaxel makes this drug very precious, and to date, paclitaxel has achieved $9 billion in sales worldwide.[3] Because paclitaxel can be isolated only in low yields from the bark of the yew tree, at one time it was thought that continuous production would eventually cause a supply shortage of the drug. As such, paclitaxel became a leading target for total synthesis, but commercial-scale production has not been possible.[4−9] The supply problem was solved in 1995, and paclitaxel is now prepared in approximately 80% yield from a related compound, 10-desacetyl baccatin III (**2**),[3] isolated from the renewable needles of *Taxus baccata*. Since 2002, Bristol-Myers-Squibb replaced the semisynthetic route with an even more environmentally friendly method using cell fermentation. In this process, plant cells from *Taxus chinensis* are initially grown on a petri dish and later transferred to a large-scale fermentor. The development of this method was honored with a presidential award and is expected to provide a steady supply of paclitaxel.[10]

The biosynthesis of paclitaxel begins with the formation of taxa-4(5),11(12)diene (**3**), which is then trans-

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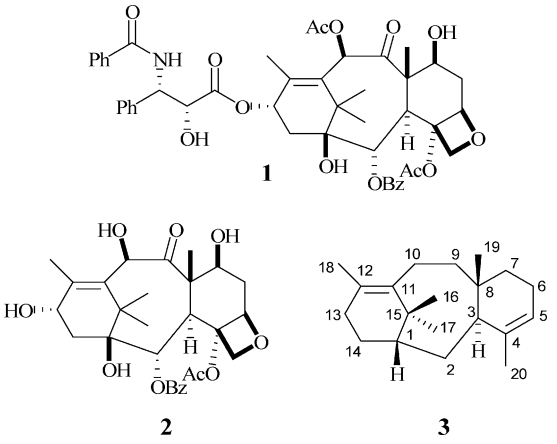
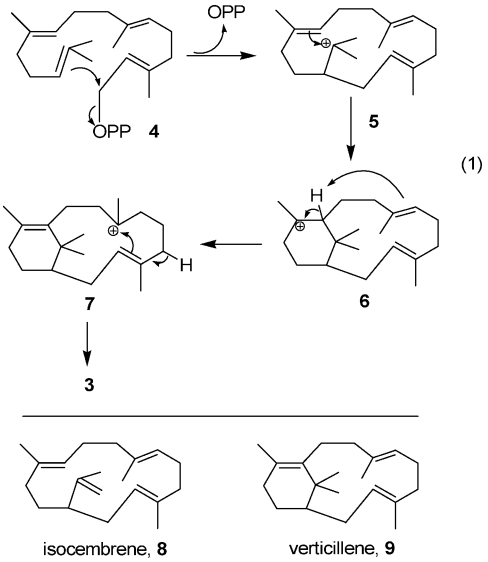


FIGURE 1. Structures of taxane diterpenes.

formed to 10-desacetylbaccatin III (**2**) in several steps and eventually to paclitaxel. The many steps that link taxadiene to 10-desacetylbaccatin III (**2**) and to paclitaxel obviously involve monooxygenations of the taxane skeleton and the esterification of some of the resulting hydroxyl groups.[11] To date, taxadiene synthase, a few monooxygenases, and all five acyl/benzoyl transferases have been found and cloned into common laboratory microorganisms.[12−14] Although the exact sequence of the transformations is not known, a statistical survey of the oxygenation pattern of the entire taxoid family suggests that oxygenation of taxadiene should occur in the order C5, C10, C2, C9, C1, and C13 en route to paclitaxel. Similarly, esterifications should occur in the order C5, C2, C10, and C13.[15]

The cyclization of geranylgeranyl diphosphate (GGPP, **4**) to taxadiene **3** is a remarkable reaction that involves the formation of three C−C σ bonds, three carbocyclic rings, and three new stereocenters from the achiral, acyclic substrate. This reaction represents the committed step in the biosynthetic pathway to all taxanes. The chemical structure of taxadiene has been verified independently by total synthesis,[16] and conversion of radiolabeled taxadiene to radiolabeled paclitaxel with yew stem sections proved that taxadiene is the progenitor of paclitaxel.[12a] Like other terpene-cyclase-catalyzed reactions, this cyclization reaction has been traditionally formulated to proceed with multiple carbocationic intermediary stages, as shown in eq 1.



The intermediate carbocations **5**−**7** are structurally related to diterpenes isocembrene (**8**) and verticillene (**9**), and both compounds have been proposed as possible intermediates in the cyclization of GGPP to taxadiene.[17] However, early experiments designed to test the intermediacy of these putative monocyclic or bicyclic compounds were unsuccessful. Treatment of cembrene (not isocembrene) or verticillene with acid in biomimetic reactions did not convert these materials to products having the desired tricyclic taxane skeleton.[18] Furthermore, incubation of yew extracts with verticillene in inhibition, trapping, and direct conversion experiments showed that verticillene is not a freely exchangeable intermediate in the enzyme-catalyzed reaction.[19] Nonetheless, the existence of carbocations **5**−**7** as tightly bound intermediates in the active site of the enzyme remains plausible. Under normal circumstances, these intermediates are reactive, with short lifetimes which

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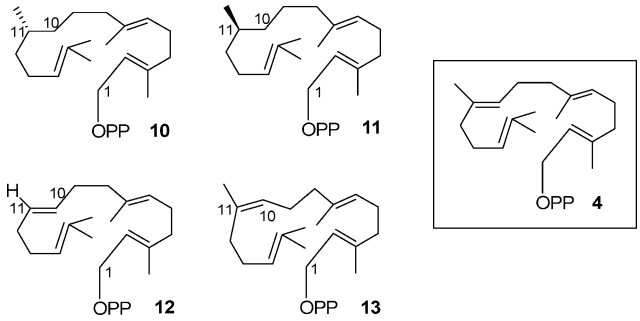

FIGURE 2. Analogues **10**−**13** used in this study, which are dissimilar to GGPP **4** at the Δ^{10} double bond.

may prohibit isolation and direct observation. To observe the intermediates in such situations, a common strategy has been to use substrate analogues that are unable to undergo the complete enzymatic cascade, thereby terminating at the intermediate stage.

The availability of taxadiene synthase, overexpressed in recombinant *E. coli,* allowed the mechanistic investigation of the cyclization of GGPP to taxadiene in vitro.[20] In this paper, we report the synthesis of four GGPP analogues **10**−**13** (Figure 2) that are dissimilar to GGPP **4** at the Δ^{10} double bond and incubation of these compounds with taxadiene synthase. By design, the termini of these analogues remain unchanged with respect to the natural substrate in order to maintain the ability to form the C1−C14 bond (GGPP numbering). For analogues **10** and **11**, the monocyclic **5**-like intermediates, if formed, would obviously not be able to undergo the subsequent C−C σ bond formation step because the requisite Δ^{10} double bond is absent. Although analogues **12** and **13** both contain the Δ^{10} double bond, formation of the bicyclic **6**-like intermediate would be be more difficult because the Δ^{10} double bond of **12** is only disubstituted and hence less nucleophilic, while in **13**, the unnatural Z-geometry of the Δ^{10} double bond may not present the proper approach vector to effect C−C bond formation. If not completely unreactive, these analogues were expected to halt the cyclization cascade at the monocyclic isocembrene level. A similar strategy had been used successfully by others to intercept, for example, the conversion of oxidosqualene to lanosterol and the conversion of farnesyl diphosphate to aristolochene.[21,22] While this work was in progress, Coates et al. published a related work on the taxadiene synthase-catalyzed formation of verticillene-like products.[20d]

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Results

Synthesis of Substrates. The synthesis of 10,11*R*-dihydroGGPP **10** was accomplished in six steps from the commercially available *S*-citronellyl bromide **14**, as shown in Scheme 1. Treatment of bromide **14** with sodium iodide in acetone, followed by sodium *p*-toluenesulfinate in DMF at 0 °C, gave sulfone **16** in 89% yield over two steps. Sulfone **16** was subjected to a Beillmann coupling at −78 °C with the known allylic bromide **17**,[23] and subsequent reduction gave the desired known[24] alcohol **19** in 32% yield over two steps. Alcohol **19** was converted to 10,-11*S*-dihydroGGPP **10** in 35% by activation as the bromide **20** (PBr_3, Et_2O, 0 °C), followed by treatment with $(Bu_4N)_3HOPP$ in anhydrous acetonitrile using a modified literature procedure.[25] Using the identical procedure, commercially available *R*-citronellyl bromide **21** was used to synthesize 10,11S-dihydroGGPP (**11**).

Synthesis of 11-desmethylGGPP **12** and 2*E*,6*E*,10*Z*-GGPP **13**, shown in Scheme 2, employed the common starting aldehyde **23**, prepared readily from farnesyl benzyl ether **22** in 28% yield.[26] A Wittig reaction gave unsaturated ester **24** in 91% yield, having the desired *trans* geometry at the newly created double bond. Reduction of **24** with DiBAL-H gave alcohol **25** in 93% yield. Conversion of alcohol **25** to bromide **26** was accomplished in one pot by treatment with methanesulfonyl chloride followed by lithium bromide. Beillmann coupling of bromide **26** with sulfone **27**, followed by reduction with lithium/ammonia, furnished 11-desmethyl GGOH **29** in 45% overall yield from **25**.[23c] Alcohol **29** was converted to 11-desmethylGGPP **12** in 46% yield as described above for analogue **10**.

For the preparation of 2*E*,6*E*,10*Z*-GGPP **13**, aldehyde **23** was subjected to a Still−Gennari olefination to furnish unsaturated ester **31**, with a *Z*/*E* ratio of 98:2 (separable by column chromatography to 100:0 as determined by ^{1}H NMR).[27] A similar set of transformations, as described for **24** → **12**, provided 2*E*,6*E*,10*Z*-GGPP **13** in similar yields.

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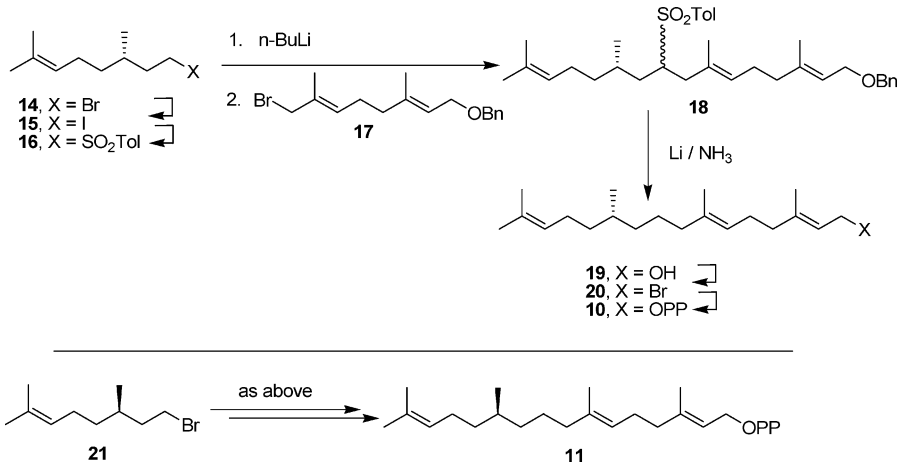
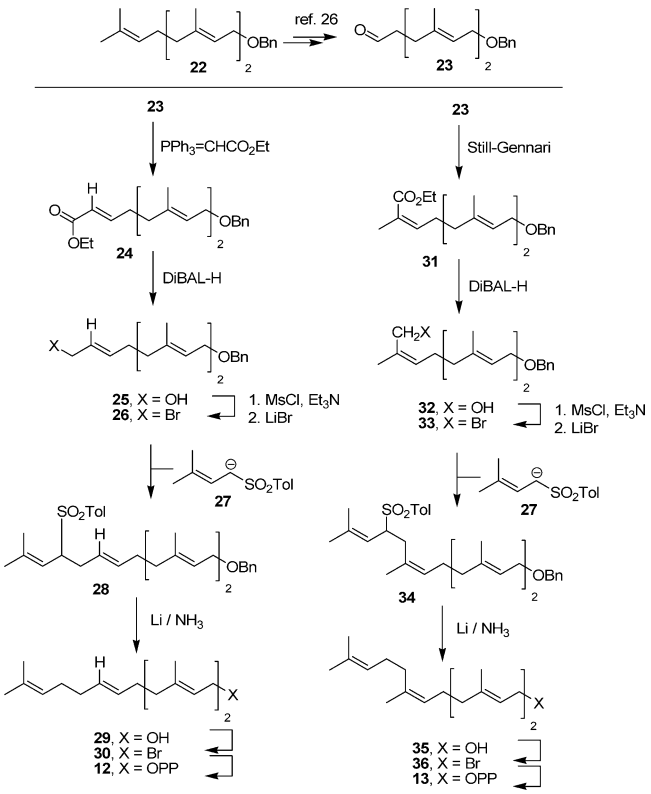
SCHEME 1



SCHEME 2



Enzymatic Cyclization of GGPP Analogues to Isocembrenoid Diterpenes. *E. coli* strain BL21(DE3)-plysS/pTS79H, described previously,[12e] was used to overexpress taxadiene synthase. After optimization, the crude enzyme derived from 8 L of cell culture could effect conversion of 89 μmol of all-trans GGPP to 5.7 mg (21 μmol) of the hydrocarbon product, corresponding to 23% isolated yield.[28] TLC of products from small-scale incubations of analogues **10**−**13** with taxadiene synthase, along with GCMS analyses (see Figure 3), indicated that each analogue gave predominantly one major product comprising approximately 50−60% of the overall hydrocarbon mixture. The molecular weight of the major products from **10** to **13** were shown to be 274, 274, 258, and 272, respectively, consistent with the loss of a proton and the diphosphate group. Large-scale incubations were conducted to obtain more product, and the results are summarized in Table 1.

NMR Analyses of the Incubation Products. After purification of the crude hydrocarbon products by preparative GC, we obtained sufficient amounts (~1 mg) of the major products for rigorous structural elucidation by NMR (^{1}H, ^{13}C, DEPT, COSY, NOESY, HSQC, HMBC, and TOCSY). NMR analysis in all cases used the following procedures. The ^{1}H spectrum was first used to count

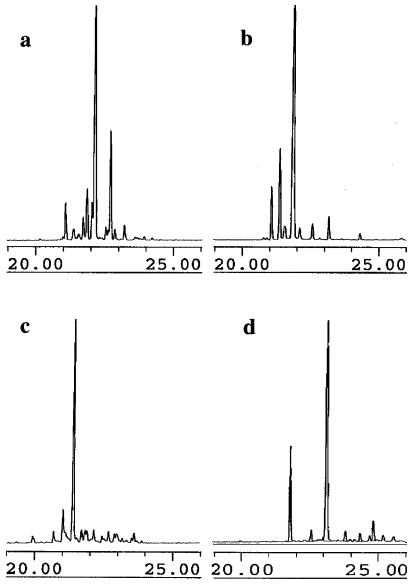


FIGURE 3. GCMS analyses, displaying the total ion current of the hydrocarbon products from the incubation of taxadiene synthase with analogues **10** (panel a), **11** (panel b), **12** (panel c), and **13** (panel d). GCMS analyses were performed using a 30 M, 0.25 mm i.d., 0.25 μM DB-5MS column, programmed 120 °C for 2.00 min, to 280 °C at 5 °C/min, final time 15 min. In control experiments, incubation of analogues **10**−**13** with BL21/DE3/plysS (which does not encode taxadiene synthase) failed to give any hydrocarbon products on TLC.

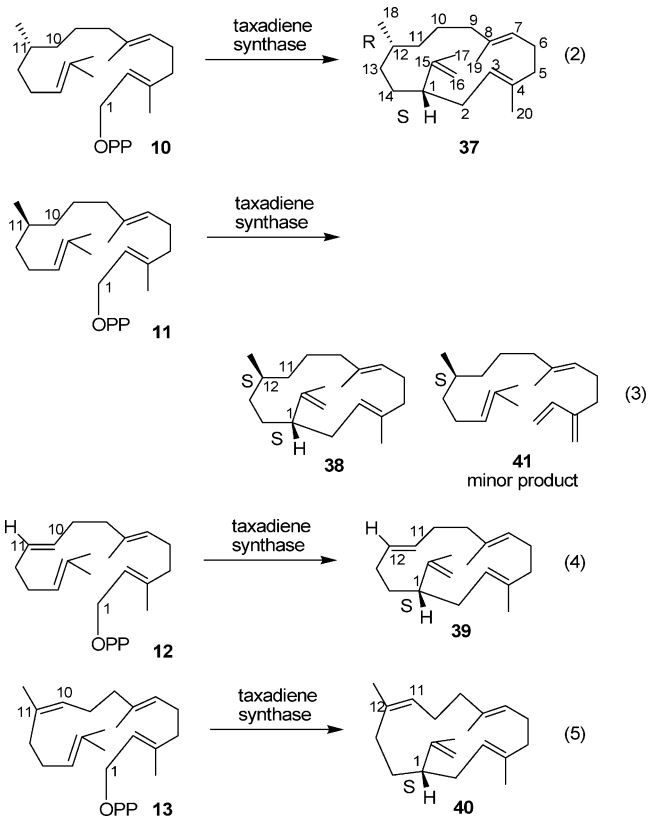
(28) We opted to use soluble crude lysate instead of purified enzyme because we felt that the crude lysate could contain pyrophosphatase activity, which would hydrolyze inorganic phosphate from the reaction, thereby eliminating product inhibition by diphosphate. A previous similar experience with the enzyme SAM-synthetase showed that the soluble crude lysate functioned better than the purified enzyme, probably for the same reason. See: Park, J.; Tai, J.; Roessner, C. A.; Scott, A. I. *Bioorg. Med. Chem.* **1996**, *4* (12), 2179−2185.

TABLE 1. Large-Scale Incubation of Taxadiene Synthase with Analogues 10−13

substrate		enzyme		hydrocarbon products	
analogue	amount (μmol)	cell (L)	lysate (mL)	mass (mg)	yield (%)
10[a]	377	30	750	5.9[c]	6
11[a]	300	32	800	15.7[c]	19
12[b]	770	39	975	∼1−2[d]	<1
13[b]	n.d.	8	200	n.d.[e]	∼3
GGPP[b]	89	8	200	5.7[c]	23

[a] [$(Bu_4N)_2NH_4$ salt], see ref 25c. [b] [$(Bu_4N)_3$ salt], see ref 25c. [c] Isolated yield. [d] Estimated yield. [e] In some runs, substrate **13**, contaminated with ∼10% all-trans GGPP, gave ∼1:1 monocyclic products/taxadiene. Given that the yield of GGPP → taxadiene was 23%, the yield of products from **13** was estimated at ∼3%. See ref 27b for more details.

vinyl protons and methyl groups and determine methyl group multiplicities. ^{13}C and DEPT experiments then allowed double bond and attached hydrogen numbers to be determined. HSQC associated ^1H chemical shifts to appropriate carbons. In HMBC analysis, correlations between each methyl ^1H peak and ^{13}C of nearby carbons revealed in all cases the directly attached carbon and at least two others, usually saturated methylene and two vinyl carbons. Vinyl ^1H correlations to adjacent methylenes completed individual isoprene unit assignments. TOCSY spectra then supplied connectivity between groups. The major products from the respective reactions were assigned structures corresponding to 1*S*,12*R*-11,12-dihydroisocembrene **37**, 1*S*,12*S*-11,12-dihydroisocembrene **38**, 1*S*-12-desmethylisocembrene **39**, and 1*S*-11,12-*Z*-isocembrene **40** as shown in eqs 2−5 on the basis of these NMR experiments.[29] In every case, the stereochemistry at C1 was assumed to be the same as in C1 of taxadiene, i.e., an "*S*" configuration. The ^{13}C and ^1H chemical shifts of **37**−**40** are summarized in Tables 2 and 3, and additional NMR data are shown in the Supporting Information. Due to limited amounts of material, we were unable to characterize the minor products in each reaction, except for one case. For the incubation of **11**, one of the minor products was shown by GCMS and ^1H NMR analysis to be 10,11-dihydrogeranylmyrcene **41**.[30m]



Discussion

The cyclization of GGPP to taxadiene had been a subject of continued interest since 1966.[17] The availability of taxadiene synthase, overexpressed in recombinant *E. coli,* allowed the mechanistic investigation of the cyclization of GGPP to taxadiene in vitro.[19,20] For example, by the use of appropriately deuterated substrates, the

TABLE 2. ^1H (500 MHz) and ^{13}C NMR (125 MHz) Data for Compounds 37 and 38 in $CDCl_3$

	37		**38**	
position[a]	δ_C	δ_H (mult, *J*, int)	δ_C	δ_H (mult, *J*, int)
1	49.2	1.91 (m, 1H)	45.9	1.98 (m, 1H)
2	33.4	1.96, 2.04[b] (m, 2H total)	33.9	1.97 (m, 2H)
3	125.0	5.17 (dd 9.3, 5.4 Hz, 1H)	124.7	5.18 (br t, 7.2 Hz, 1H)
4	135.3	-	135.1	-
5	39.4	2.14, 2.23[b] (m, 2H total)	39.4	2.17 (m, 2H)
6	24.6	2.24 (m, 2H)	24.9	2.25 (m, 2H)
7	123.4	5.07 (t, 6.4 Hz, 1H)	124.6	5.02 (t, 6.5 Hz, 1H)
8	134.7	-	134.1	-
9	37.5	1.89, 2.06[b] (m, 2H total)	38.3	1.93, 2.04[b] (m, 2H)
10	21.6	1.350, 1.44[b] (m, 2H total)	21.9	1.411 (m, 2H)
11	32.1	1.16, 1.31[b] (m, 2H total)	31.0	0.92, 1.412[b] (m, 2H)
12	32.6	1.349 (m, 1H)	29.0	1.49 (m, 1H)
13	34.2	0.76, 1.29[b] (m, 2H total)	32.1	1.05, 1.27[b] (m, 2H)
14	28.0	1.09, 1.53[b] (m, 2H total)	25.3	1.14, 1.51[b] (m, 2H)
15	149.4	-	149.0	-
16	109.7	4.72, 4.73 (m, 2H total)	110.1	4.71, 4.73 (each br s, 1H)
17	19.3	1.69 (s, 3H)	18.8	1.67 (s, 3H)
18	21.0	0.86 (d, 6.7 Hz, 3H)	20.6	0.83 (d, 6.7 Hz, 3H)
19	17.5	1.63 (s, 3H)	16.6	1.61 (s, 3H)
20	15.1	1.59 (s, 3H)	15.4	1.60 (s, 3H)

[a] See eq 2 for the representative numbering system. [b] The assignments of diastereotopic protons as the α or β configurations are not possible because of the flexibility of the skeleton.

TABLE 3. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Data for Compounds 39 and 40 in $CDCl_3$

	39		40	
position[a]	δ_C	δ_H (mult, *J*, int)	δ_C	δ_H (mult, *J*, int)
1	47.1	2.052 (m, 1H)	48.4	2.01 (m, 1H)
2	33.0	1.97, 2.049[b] (m, 2H)	33.1	1.99, 2.079[b] (m, 2H)
3	124.9	5.10 (br t, 7.9 Hz, 1H)	124.8	5.09 (m, 1H)
4	135.0	-	135.3	-
5	39.3	2.08, 2.19[b] (m, 2H)	39.6	2.081 (m, 2H)
6	24.9	2.09, 2.28[b] (m, 2H)	24.8	2.071, 2.28[b] (m, 2H)
7	124.3	5.03 (t, 7.0 Hz, 1H)	124.6	5.04 (t, 7.14 Hz, 1H)
8	135.5	-	136.6	-
9	39.0	2.055, 2.16[b] (m, 2H total)	39.7	1.934, 2.21[b] (m, 2H)
10	26.8	2.18, 2.22[b] (m, 2H total)	29.3	2.12 (m, 2H)
11	131.7	5.35 (m, 1H)	126.0	5.10 (m, 1H)
12	128.8	5.51 (m, 1H)	135.5	-
13	24.9	1.81, 1.91[b] (m, 2H total)	30.6	1.65, 1.93[b] (m, 2H)
14	31.3	1.30, 1.45[b] (m, 2H total)	29.8	1.29, 1.44[b] (m, 2H)
15	149.0	-	148.8	-
16	110.0	4.74, 4.75 (m, 2H total)	110.1	4.75, 4.76 (each br. s, 1H)
17	19.2	1.70 (s, 3H)	19.3	1.71 (s, 3H)
18	-	-	24.3	1.68 (s, 3H)
19	17.5	1.62 (s, 3H)	17.6	1.60 (s, 3H)
20	15.6	1.59 (s, 3H)	15.5	1.58 (s, 3H)

[a] See eq 2 for the representative numbering system. [b] The assignments of diastereotopic protons as the α or β configurations are not possible because of the flexibility of the skeleton.

stereochemical course of the cyclization had been elucidated in detail.[19,20a,e] Also, alkylation of the enzyme by potential suicide inhibitors had been attempted, unfortunately, with little success.[20b] Using a fluorinated analogue, Coates et al. recently observed verticillene synthase activity by taxadiene synthase directly,[20d] which supports the early biogenetic postulates.[17] In this publication, we refine the cyclization mechanism of taxadiene synthase by demonstrating isocembrene synthase activity by taxadiene synthase through the isolation of isocembrenoid diterpene analogues **37**−**40**.

In the active site of taxadiene synthase, the terminal double bond of GGPP is brought into close contact with C1 to initiate the cyclization reaction. It appears that a similar conformation can be adopted by analogues **10**, **11**, and **13** despite the increased rotational freedom or altered geometry relative to *all-trans* GGPP at the Δ^{10} double bond, indicating that the enzyme allows a certain amount of steric flexibility for binding of these analogues. The total hydrocarbon yield from **10** (6%) was significantly lower than the yield from **11** (19%), suggesting that the chirality of the binding pocket fits **11** better than **10**.

In contrast, 11-desmethyl GGPP analogue **12**, which is able to fit into the binding pocket as readily as the natural substrate, gave a very low (<1%) yield. There are a few possibilities that would explain the low hydrocarbon yield: (a) the analogue remains dormant in the active site without reacting, (b) the analogue is hydrolyzed back to the corresponding alcohol **29**, either by taxadiene synthase or by other hydrolases, (c) cyclization does take place, but the resulting desmethyl isocembrenyl cation is quenched by capture of water to give a desmethyl cembrenol, or (d) the substrate is irreversibly attacked by a nucleophilic site in the enzyme, giving rise to inhibition. Possibility (a), that analogue **12** is unreactive, seems unlikely due to the significantly higher yields seen with the other analogues. Possibilities (b), (c), and (d) have precedents in other terpene-cyclase systems, but remain unexplored for this reaction.[31] In our reactions, because the incubations were performed using soluble crude lysate containing many polar endogenous metabolites, we did not attempt to locate any potentially unreacted substrate or alcoholic products from the incubation of **12**.[28] We are currently reinvestigating this reaction to explore these possibilities.

In this work, the analogues **10**−**13** were designed without any modification to the Δ^{14} double bond in order to maintain the ability to form the C1−C14 bond (GGPP numbering). It appears that the Δ^{14} double bond is the preferred site of reaction despite the presence of Δ^6 (and Δ^{10} for **12** and **13**) double bond in all these analogues to potentially compete for ring closure, and the observed regioselectivity is undoubtedly imposed by the enzyme. When farnesyl diphosphate (FPP) was incubated with taxadiene synthase, the absence of the Δ^{14} double bond allowed the otherwise disfavored formation of six-membered bisabolyl sesquiterpenes.[20c] Finally, we note that the major products from each analogue are terminal olefins that resulted from deprotonation at C16, rather than at C1, which would lead to the more stable Zaitsev-like product, suggesting that an enzymatic base could be responsible for regioselective deprotonation.

Conclusion

The results presented in this publication indicate that the first C−C bond formation catalyzed by taxadiene synthase can be uncoupled from the other subsequent bond formation events by using suitably designed substrate analogues. The formation and isolation of isocembrenyl diterpene products using taxadiene synthase

(29) Compounds **37**−**39** are new compounds. Compound **40** had been previously synthesized using a nonenzymatic method. The ^{1}H NMR of **40** showed good agreement with the literature data. See: Kato, T.; Suzuki, M.; Kobayashi, T.; Moore, B. P. *J. Org. Chem*. **1980**, *45*, 1126−1130.

(30) The olefinic region in the ^{1}H NMR of our product was similar to that of geranylmyrcene, less a triplet. See: Baeckstrom, P.; Li, L. *Tetrahedron* **1991**, *47*, 6533−6538. See also the Supporting Information for more details.

support the proposals that the isocembrenyl cation is possibly an intermediate in the cyclization of GGPP to taxadiene.

Experimental Section

10,11*R*-Dihydrogeranylgeranyl Bromide (20). To a solution of **19** (0.62 g, 2.1 mmol) in Et_2O (21 mL) at 0 °C was added PBr_3 (0.7 mmol, 68 μL) using a plastic delivery pipet. The mixture was warmed to room temperature and stirred for 30 min, and additional (68 μL) PBr_3 was added if TLC showed any **19** that remained unreacted. The reaction was diluted with Et_2O (21 mL) and quenched with saturated aqueous NaCl (42 mL). The organic layer was separated, dried with $MgSO_4$, filtered, transferred into a cold dry flask, and concentrated in vacuo to give 10,11*R*-dihydroGGBr **20** (0.63 g, 1.8 mmol, 85%), which was used immediately in the next step.

10,11R-Dihydrogeranylgeranyl Diphosphate, 10. To bromide **20** (0.64 g, 1.8 mmol) was added anhydrous CH_3CN (10 mL), followed by $(Bu_4N)_3HOPP$ (3.25 g, 3.6 mmol). The reaction was stirred at room temperature for 2 h and concentrated using a rotary evaporator (bath temperature ≤ 40 °C). The clear syrup obtained was dissolved in minimal amount of solvent A [1:49 (v/v) 2-propanol/25 mM aq NH_4HCO_3], loaded onto a column containing 80 mL of Sigma Dowex 50WX8-200 resin (pretreated with concd aq NH_4OH,[32] washed with excess distilled water, and then preequilibrated with 60 mL of solvent A) and eluted with 160 mL of solvent A. Typically, the product would elute immediately, and fractions containing the product were cloudy and/or yellow colored, as followed by a silica gel TLC with anisaldehyde visualization. The desired fractions were pooled and concentrated in vacuo using a rotary evaporator (≤40 °C), followed by lyophilization to dryness to give either a gum or a thick liquid.

Solid−liquid extraction of the crude product was performed by following the literature procedure.[25b] The lyophilized product was dissolved in a minimal amount of 0.1 M aq NH_4HCO_3 and treated with solvent B [1:1 (v/v) 2-propanol/acetonitrile]. The mixture was centrifuged at 11000*g* for 10 min, and the clear supernatant was collected and saved. After two identical treatments, the supernatants were combined and concentrated in vacuo using a rotary evaporator (bath temperature ≤ 40 °C) to give a thick yellow liquid, which was either stored frozen at −20 °C or used directly in the next chromatography step. ^1H and ^{13}C NMR in D_2O at this stage showed significant presence of the Bu_4N group, indicating that the initial cation-exchange step was not completely successful.[32] As such, cellulose chromatography was not attempted, and the product was purified by silica chromatography instead.[33]

The thick yellow liquid from the solid−liquid extraction was dissolved in a minimal amount of 0.1 M NH_4HCO_3 and loaded onto the silica column, saving a small amount for TLC. Typically, approximately 250 mL of silica gel [equilibrated with solvent C (2:1 v/v 2-propanol/concd NH_4OH)] was used for every 3 mL of sample solution. The column was eluted with solvent C, and the eluents were collected in fractions. A TLC analysis (in solvent C, anisaldehyde stain) of the crude mixture showed the desired product at the baseline R_f 0.0−0.1, along with impurities with $R_f \sim 0.5-1.0$. When all the impurities had eluted from the column, solvent D [6:3:11 (v/v/v) 2-propanol/concd NH_4OH/doubly distilled H_2O (ddH_2O)] was used to elute the desired product from the column. The fractions containing the product were pooled and concentrated in vacuo using a rotary evaporator (bath temperature ≤ 40 °C) to remove bulk solvent. Rotary evaporation was discontinued when the product solution started to bubble, and the cloudy product solution was lyophilized to give a colorless gum. The product was taken up in ddH_2O and centrifuged at 11000*g* for 10 min. The supernatant was collected, transferred to a tared flask, and lyophilized to give 10,11*R*-dihydroGGPP **10** [0.71 g, 0.74 mmol, 35%, $(Bu_4N)_2NH_4$ salt by ^1H NMR]. The product was kept as a frozen aqueous solution (25 mg/mL) at −20 °C in plastic centrifuge tubes: solvent suppressed ^1H NMR (D_2O, 500 MHz) 5.39 (br t, J = 6.5 Hz, 1H), 5.13 (br t, J = 6.8 Hz, 1H), 5.08 (br t, J = 6.8 Hz, 1H), 4.45 (br, 2H), 3.17 (m, 16H), 2.14−1.87 (8H), 1.76−1.51 (m, 28H), 1.47−1.24 (br, 4H and sextet, J = 7.4 Hz, 16H) 1.19−1.00 (m, 3H), 0.94 (t, J = 7.5 Hz, 24H), 0.87 (t, J = 6.35 Hz, 3H); ^{13}C NMR (D_2O, 125 MHz) δ 143.1, 137.3, 132.9, 127.5, 127.0, 123.3 (d, J = 8.5 Hz), 65.0 (d, J = 5.5 Hz), 60.8 (Bu_4N), 42.7, 42.4, 39.9, 39.4, 34.9, 29.3, 28.20, 28.17, 28.10, 25.9 (Bu_4N), 22.1, 21.8 (Bu_4N), 20.0, 18.7, 18.3, 15.6 (Bu_4N); ^{31}P NMR (D_2O, 202 MHz) δ −9.1 (d, J = 20.0 Hz), −10.8 (d, J = 18.7 Hz); ESIHRMS calcd for $C_{20}H_{37}O_7P_2$ 451.2015, found 451.2031.

Incubation of 10,11*R*-DihydroGGPP with Taxadiene Synthase. Cell pellets from an 8 L culture of *E. coli* strain BL21(DE3)plysS/pTS79H[12e] were resuspended and lysed in 200 mL of buffer consisting of 30 mM NaHEPES, 5 mM sodium metabisulfite, 2.5 mM L-ascorbic acid, 10 mM KF, 2 mM dithiothreitol, 2 mM β-cyclodextrin hydrate, and 1 mM $MgCl_2$ at pH 8.4. Substrate analogue **10** (96 mg) was added, and the mixture was shaken gently for 16−24 h at room temperature. The reaction was extracted with 2 × 600 mL of hexane (HPLC grade); the organic layer was dried with $MgSO_4$ and evaporated. The residue was redissolved in 1 mL of HPLC hexane and was loaded onto a 10 mL silica column. The column was eluted with 20 mL of HPLC hexane to give 1.6 mg (5.7 μmol, 6%) of crude product after evaporation of solvent. The major product **37** was isolated by preparative GC; see Table 2 for ^1H and ^{13}C NMR: GCEIMS *m/z* 274.

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Supporting Information Available: Complete reference information, synthesis procedures, and characterization data for compounds not shown in the Experimental Section. This material is available free of charge via the Internet at http://pubs.acs.org.

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(32) We initially presumed that used resin (Bu_4N form) would be regenerated to the NH_4 form by treatement with concd NH_4OH. A closer inspection of the literature (ref 25b) suggested that the resin (Bu_4N form) should first be converted to the H^+ form before conversion to the NH_4 form.

(33) Purification of isoprenoid diphosphates by silica chromatography has been previously used by others; see: Ohnuma, S.; Ito, M.; Koyama, T.; Ogura, K. *Tetrahedron* **1989**, *45*, 6145−60. Keller, R. K.; Thompson, R. *J. Chromatogr.* **1993**, *645*, 161−167.