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Studies on taxadiene synthase: interception of the cyclization cascade at the verticillene stage and rearrangement to phomactatriene

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Abstract—The cyclization of geranylgeranyl diphosphate (GGDP) to taxadiene catalyzed by taxadiene synthase has been suggested to proceed in stages, involving a transient bicyclic verticillyl carbocation intermediate, which also has been proposed in the biosynthetic pathway leading to phomactatriene by marine fungi of *Phoma* sp. On incubation with des-7-methylGGDP, which would be expected to decrease the stability of a carbocation produced by hydride migration, taxadiene synthase produced phomactatrienes as major products. This indicates that the verticillyl carbocation was indeed formed but underwent further skeletal rearrangements, diverging from the usual pathway taken by GGDP en route to taxadiene. Products were identified using GC–MS, one- and two-dimensional NMR, and X-ray crystallography.

1. Introduction

Isoprenoids are found in virtually all forms of life, and as of 1997, as many as 23,000 isoprenoid compounds are found in the literature.[1] An important member of the diterpenoid family is paclitaxel (**1**, Fig. 1), which has been widely used in the treatment of breast and ovarian cancers since 1992.[2] Paclitaxel is a natural product made by the yew tree (*Taxus*), but extraction of this compound from bark of the tree gave only low yields. Paclitaxel became a leading target for total synthesis in the 1990s,[3–8] but the lengthy sequence of steps and the low overall yields associated with total synthesis served as a drawback for large-scale commercial production. The supply problem has been solved, and paclitaxel is now prepared on a commercial scale either by semisynthesis from 10-desacetyl baccatin III (**2**), which is a renewable advanced intermediate from the needles of *Taxus baccata*,[9] or by fermentation of the Chinese yew *Taxus chinensis*.[10]

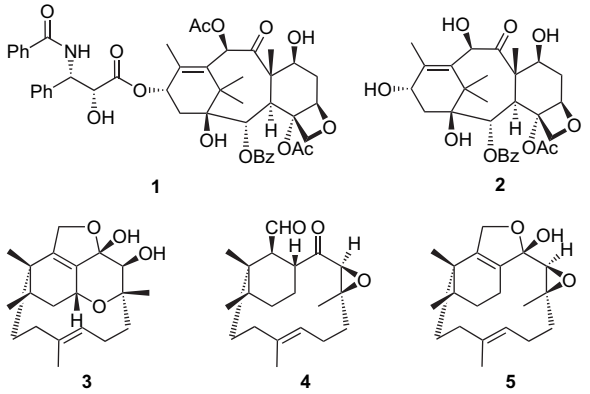

Figure 1. Structures of taxane and phomactin diterpenes.

The phomactins are another important class of terpenoids some of which act as platelet-activating factor (PAF) antagonists.[11] To date, 11 distinct phomactins have been isolated from the marine fungus *Phoma* sp.[11] and are all attractive synthetic targets, with total synthesis already accomplished for phomactins A, D, and G (**3**, **4**, and **5**, Fig. 1).[12]

The biosynthesis of paclitaxel begins with the cyclization of geranylgeranyl diphosphate (GGDP, **6**) to taxa-4(5),11(12)diene (**7**), and followed by a series of monooxygenations on the parent compound and esterification of some of the resulting hydroxyl groups to give paclitaxel. To date, taxadiene synthase, a few monooxygenases, and all five acyl/benzoyl transferases have been found and cloned into common laboratory microorganisms.[13–15] 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.

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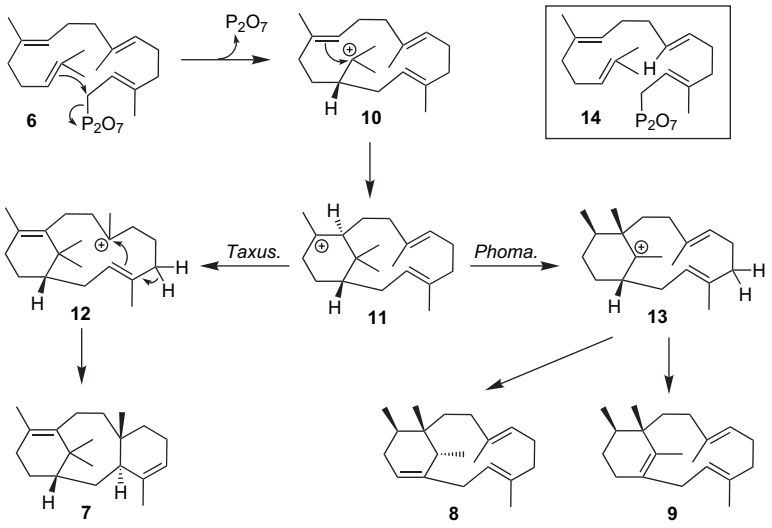


Figure 2. Proposed biosynthetic pathways leading to taxadiene and phomactatrienes.

Similarly, esterifications should occur in the order C5, C2, C10, and C13.[16]

The biosynthesis of the phomactins, like paclitaxel and other isoprenoids in general, should involve an initial cyclization of GGDP to a parent hydrocarbon, followed by oxidative modification of the carbon backbone. Although the biosynthesis of the phomactins has not been demonstrated in vitro, isolation of phomacta-1(14),3(4),7(8)-triene (**8**) and phomacta-1(15),3(4),7(8)-triene (**9**) from *Phoma* sp. supported this mechanism.[17,18]

The biosyntheses of taxadiene **7** and phomactatrienes **8** and **9** are phylogenetically unrelated, with taxadiene following the methyl erythritol phosphate (MEP) pathway,[19a] while the phomactatrienes follow the mevalonate pathway,[19b] but share certain similarities.[17,18] The cyclization of GGDP **6** to taxadiene **3** was proposed to proceed with multiple carbocationic intermediary stages such as **10**–**12**, shown in Figure 2. Formation of intermediate **11** followed by a series of hydride- and methyl migrations would also lead to the phomactatrienes.[17,18]

Under normal circumstances, these carbocation intermediates are buried in the enzyme active site, with short lifetimes that prohibit isolation and direct observation, and a common strategy has been to use substrate analogs that block later enzymatic steps to terminate the process at an intermediate stage.[20] Using this strategy, we have recently demonstrated that cyclization of **6** to **3** could be intercepted at the monocyclic isocembrene stage with suitably-designed GGDP analogs.[21]

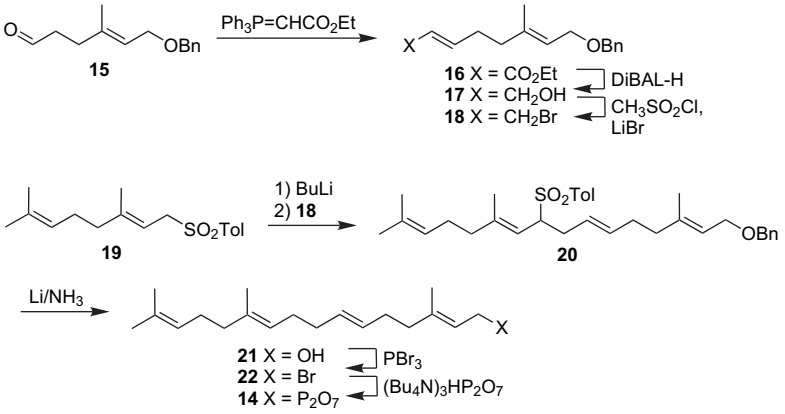
2. Results and discussion

In this paper, we extend the mechanistic study of TS by attempting to intercept the cyclization cascade at the verticillene stage with analog **14** whose synthesis is shown in Scheme 1.

Compared to the natural substrate, this analog lacks the methyl group at C7, which would lead to a less stable carbocation if the trans-annular proton transfer (**11**→**12**) took place. While this work was in progress, Coates et al. published a related work on the taxadiene synthase-catalyzed formation of verticillene-like products.[22]

2.1. Synthesis of substrates

Synthesis of 7-desmethylGGDP **14**, shown in Scheme 1, employed aldehyde **15**, prepared readily from geranyl benzyl ether.[23] A Wittig reaction gave unsaturated ester **16**, having



Scheme 1. Preparation of substrate for biotransformation using taxadiene synthase.

the desired trans geometry at the newly created double bond. Reduction of **16** with DiBAL-H gave allylic alcohol **17** in 75% yield. Conversion of alcohol **17** to bromide **18** was accomplished by treatment with methanesulfonyl chloride followed by lithium bromide. Coupling of bromide **18** with geranyl sulfone **19**, followed by reduction with lithium/ammonia, furnished 7-desmethylGGOH **21** in 28% overall yield from **17**.[24] Alcohol **21** was converted to 7-desmethylGGDP **14** in 24% yield by activation as the bromide **22** (PBr_3, Et_2O, 0 °C), followed by treatment with $(Bu_4N)_3$-HOPP in anhydrous acetonitrile using a modified literature procedure.[25]

2.2. Enzymatic cyclization of 7-desmethylGGDP

Small-scale incubation of analog **14** with recombinant taxadiene synthase[13] gave a mixture of hydrocarbon products, as indicated by GC–MS analysis (Scheme 2). The molecular weight of the all the hydrocarbon products was shown to be 258, consistent with the loss of a proton and the diphosphate group. Large-scale incubation of analog **14** with the enzyme (from 90 L of recombinant *Escherichia coli*) afforded 66 mg of hydrocarbon products, which were further purified, or partially enriched by preparative GC to obtain enough sample of the two major products for rigorous NMR study. The yield of the cyclization reaction of **14** was about 22%, which was comparable to the yield of taxadiene formation. In the control experiment (not shown), incubation of analog **14** with lysate of *E. coli* strain BL21/DE3/plysS (which does not encode taxadiene synthase) failed to give any hydrocarbon products on TLC.

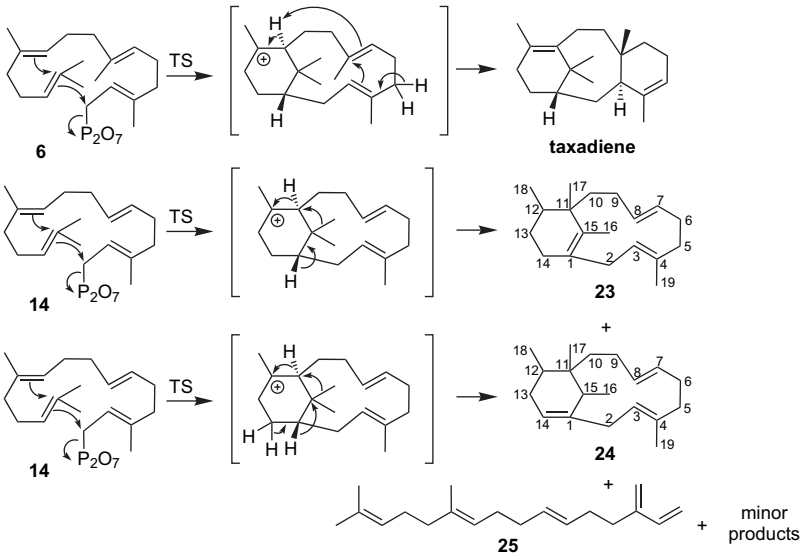
Individual products were separated by preparative thin layer, liquid, and gas chromatographies and analyzed by NMR and mass spectroscopies. The two major components were identified as phomactatriene analogs **23** and **24** by NMR spectroscopy, with details given in Tables 1 and 2 below. In general, ^1H, ^{13}C, and DEPT experiments combined with HSQC data allowed chemical shift measurement and association of individual protons to their attached carbons. HMBC and COSY data were then used to determine connectivity.

Table 1. NMR data for compound **23**

Position	δ_C (mult.)	δ_H (mult., *J*)
1	133.1 (s)	
2	34.1 (t)	3.12 (dd, *J*=12.1, 12.1 Hz), 2.01 (m)
3	125.1 (d)	5.01 (m)
4	130.3 (s)	
5	37.8 (t)	2.00 (m)
6	31.9 (t)	2.13 (m)
7	129.1 (d)	5.04 (m)
8	131.8 (d)	5.16 (m)
9	28.1 (t)	2.09 (m), 1.97 (m)
10	34.0 (t)	1.62 (m), 1.42 (m)
11	40.8 (s)	
12	33.2 (d)	1.78 (m)
13	27.5 (t)	1.45 (m)
14	32.6 (t)	2.32 (m), 1.86 (m)
15	132.7 (s)	
16	21.4 (q)	0.78 (s)
17	15.3 (q)	1.49 (s)
18	16.3 (q)	0.83 (d, *J*=6.8 Hz)
19	16.4 (q)	1.68 (s)

Table 2. NMR data for compound **24**

Position	δ_C	δ_H (mult., *J*)
1	138.6 (s)	
2	35.4 (t)	2.70 (d, *J*=7.6 Hz)
3	127.4 (d)	5.04 (m)
4	134.1 (s)	
5	39.5 (t)	2.13 (m), 2.03 (m)
6	30.5 (t)	2.13 (m)
7	126.9 (d)	5.18 (m)
8	134.3 (d)	5.20 (m)
9	26.1 (t)	2.03 (m), 2.01 (m)
10	36.5 (t)	1.73 (m), 1.21 (m)
11	38.4 (s)	
12	40.6 (d)	1.25 (m)
13	31.2 (t)	2.41 (m), 1.62 (m)
14	121.0 (d)	5.26 (m)
15	37.0 (d)	2.21 (m)
16	14.2 (q)	1.15 (d, *J*=8.0 Hz)
17	22.9 (q)	0.92 (s)
18	17.2 (q)	0.84 (d, *J*=6.8 Hz)
19	15.7 (q)	1.53 (s)



Scheme 2. Products derived from des-7-methylGGDP (**14**) incubation with taxadiene synthase.

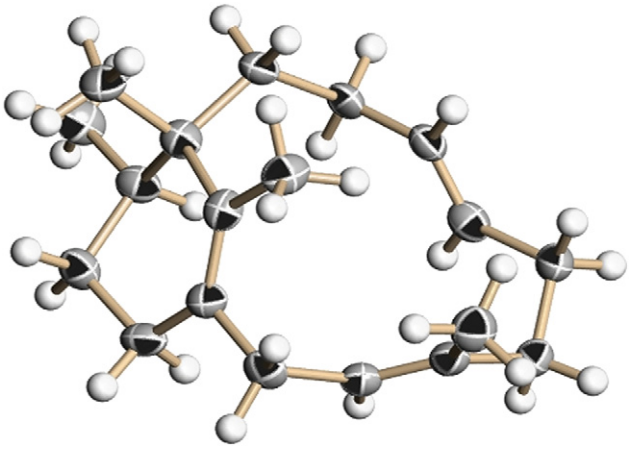


Figure 3. 50% Thermal ellipsoid plot (50% probability) of compound **23**.

Sample **23** crystallized on standing in chloroform and the assigned structure was confirmed by X-ray crystallography, which also was used to confirm stereochemical assignments (Fig. 3).

Acyclic compound **25** found in lower concentration may be an artifact, but none was formed when enzyme was not present, the major product in that case being the alcohol produced on phosphate ester hydrolysis. Smaller amounts of several other compounds were formed, but due to difficulties in separation (and in some cases instability) were not identified.

3. Experimental

3.1. General

NMR spectroscopy was performed on a Bruker ARX-500 instrument using 3 mm H-BB or BB-H probes, $CDCl_3$ solvent at 25 °C. Details of X-ray procedures are available in Supplementary Data. X-ray results have been submitted to the Cambridge Database as CCDC 638433.

3.1.1. 7-Desmethylgeranylgeranyl bromide, 22. To a solution of **21** (1.21 g, 4.4 mmol) in Et_2O (25 mL) at 0 °C, was added PBr_3 (2.2 mmol, 200 μL) using a plastic delivery pipette. The mixture was warmed to room temperature and stirred for 30 min, and additional (200 μL) PBr_3 was added, if TLC showed any unreacted **21**. The reaction was diluted with Et_2O (25 mL), and quenched with satd aq NaCl (50 mL). The organic layer was separated, dried with $MgSO_4$, filtered, and transferred into a cold dry flask, and concentrated in vacuo to give 7-desmethylGGBr **22** (1.46 g, 4.3 mmol, 97%), which was used immediately in the next step.

3.1.2. 7-Desmethylgeranylgeranyl diphosphate, 14. To bromide **22** (1.46 g, 4.3 mmol), was added anhydrous CH_3CN (25 mL), followed by $(Bu_4N)_3HOPP$ (7.76 g, 8.6 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) isopropanol/25 mM aq NH_4HCO_3], loaded onto a column containing 190 mL of Sigma Dowex 50WX8-200 resin (pretreated with concd aq NH_4OH,[26] washed with excess distilled water, then pre-equilibrated with 380 mL of solvent A) and eluted with 380 mL 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, 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) isopropanol/acetonitrile]. The mixture was centrifuged at 11,000*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. As such, cellulose chromatography was not attempted, and the product was purified by silica chromatography instead.[27]

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 isopropanol/concd NH_4OH)] was used for every 3 mL of sample solution. The column was eluted with solvent. 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∼0.5–1.0. When all the impurities have eluted from the column, solvent D [6:3:10 (v/v/v) isopropanol/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 11,000*g* for 10 min. The supernatant was lyophilized to give 7-desmethylGGDP **14** [1.27 g, 1.1 mmol, 24%, $(Bu_4N)_3$ 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) δ 5.4 (m, 3H), 5.10 (t, J=6.8 Hz, 1H), 5.05 (t, J=6.8 Hz, 1H), 4.4 (br, 2H), 3.14 (t-like, J=8.8 Hz, 24H), 2.0 (m, 12H), 1.6 (m, 36H), 1.3 (sextet, J=7.4 Hz, 24H), 0.90 (t, J=7.4 Hz, 36H). ^{13}C NMR (D_2O) δ 143.1, 137.3, 133.7, 133.0, 132.1, 126.7, 126.6, 123.2 (d, $^3J_{P,C}$=9.8 Hz), 64.8 (d, $^2J_{P,C}$=5.4 Hz), 60.6 (Bu_4N), 42.02, 41.99, 35.3, 33.4, 30.5, 29.0, 27.8, 25.7 (Bu_4N), 21.7 (Bu_4N), 19.7, 18.5, 18.2, 15.4 (Bu_4N). ^{31}P NMR (D_2O) δ −8.0 (d-like, 1P), −10.7 (d-like, 1P). ESIHRMS calcd for $C_{19}H_{33}O_7P_2$ 435.1702, found 435.1677.

3.1.3. Incubation of 7-desmethylGGDP with taxadiene synthase. Cell pellets from an 8 L culture of *E. coli* strain BL21(DE3)plysS/pTS79H,[13e] were resuspended and lysed in 200 mL 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 analog **14** (100 mg, 86 μmol) was added, and the mixture was shaken gently for 16–24 h at room temperature. The reaction was extracted with 2×600 mL hexane (HPLC grade); the organic layer was dried with $MgSO_4$, and evaporated. The residue was re-dissolved in 1 mL HPLC hexane, and was loaded onto a 10 mL silica column. The column was eluted with 20 mL HPLC hexane to give 4.9 mg (19 μmol, 22%) crude product after evaporation of solvent. The major products were isolated by preparative GC, see Tables 1 and 2 for ^{1}H and ^{13}C NMR. GC–EIMS: *m*/*z* 258.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2007.03.029.

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26. We initially presumed that used resin (Bu_4N form) would be regenerated to the NH_4 form by treatment 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.
27. 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*, 6145–6160; Keller, R. K.; Thompson, R. *J. Chromatogr.* **1993**, *645*, 161–167.