

# Intramolecular proton transfer in the cyclization of geranylgeranyl diphosphate to the taxadiene precursor of taxol catalyzed by recombinant taxadiene synthase

David C Williams<sup>1</sup>, Brian J Carroll<sup>2</sup>, Qingwu Jin<sup>3</sup>, Christopher D Rithner<sup>4</sup>, Steven R Lenger<sup>4</sup>, Heinz G Floss<sup>2</sup>, Robert M Coates<sup>3</sup>, Robert M Williams<sup>4</sup> and Rodney Croteau<sup>1</sup>

**Background:** The committed step in the biosynthesis of the anticancer drug taxol in yew (*Taxus*) species is the cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene. The enzyme taxadiene synthase catalyzes this complex olefin cation cyclization cascade involving the formation of three rings and three stereogenic centers.

**Results:** Recombinant taxadiene synthase was incubated with specifically deuterated substrates, and the mechanism of cyclization was probed using MS and NMR analyses of the products to define the crucial hydrogen migration and terminating deprotonation steps. The electrophilic cyclization involves the ionization of the diphosphate with closure of the A-ring, followed by a unique intramolecular transfer of the C11 proton to the *re*-face of C7 to promote closure of the B/C-ring juncture, and cascade termination by proton elimination from the  $\beta$ -face of C5.

**Conclusions:** These findings provide insight into the molecular architecture of the first dedicated step of taxol biosynthesis that creates the taxane carbon skeleton, and they have broad implications for the general mechanistic capability of the large family of terpenoid cyclization enzymes.

## Introduction

The diterpenoid taxol [1] (paclitaxel, **1**, Figure 1) is now well-established as a potent chemotherapeutic agent, showing excellent activity against a range of cancers and is currently approved for treating refractory ovarian, metastatic breast, and non-small-cell lung cancers, as well as Kaposi's sarcoma [2]. (Paclitaxel is the generic name for Taxol, a registered trademark of Bristol-Myers Squibb. Because of its greater familiarity, 'taxol' is used throughout.) The limited supply of the drug from the original source, the bark of the Pacific yew (*Taxus brevifolia* Nutt.; Taxaceae), prompted intensive efforts to devise alternative means of production [3,4]. These efforts have yielded a commercially viable semisynthesis of taxol and its analogs from advanced taxane diterpenoid (taxoid) metabolites that are more readily available from yew [5,6]. However, with increasing applications in chemotherapy, both in treatment of additional cancer types and for earlier disease interven-

tion, the supply and cost of these drugs will remain important issues [7]. Total syntheses of taxol have been achieved by several elegant routes [8–13] but the yields are too low to be practical, and it is clear that in the foreseeable future the supply of taxol and its synthetically useful progenitors must rely on biological methods of production [7]. The development of improved biological processes must be based upon a detailed understanding of the pathway for taxol biosynthesis, the enzymes which catalyze the sequence of reactions and their mechanisms of action, and the genes encoding these enzymes, especially those responsible for slow steps of the pathway.

Taxadiene synthase from *Taxus* species catalyzes the first committed step in the biosynthesis of taxol and related taxoids by the cyclization of the universal diterpenoid precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP, **2**) to the parent olefin (**6**), which undergoes an extended series

<sup>1</sup>Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA

<sup>2</sup>Department of Chemistry, University of Washington, Seattle, WA 98195-1700, USA

<sup>3</sup>Department of Chemistry, University of Illinois, Urbana, IL 61801, USA

<sup>4</sup>Department of Chemistry, Colorado State University, Fort Collins, CO 80523, USA

Correspondence: Robert M Williams  
E-mail: rmw@chem.colostate.edu

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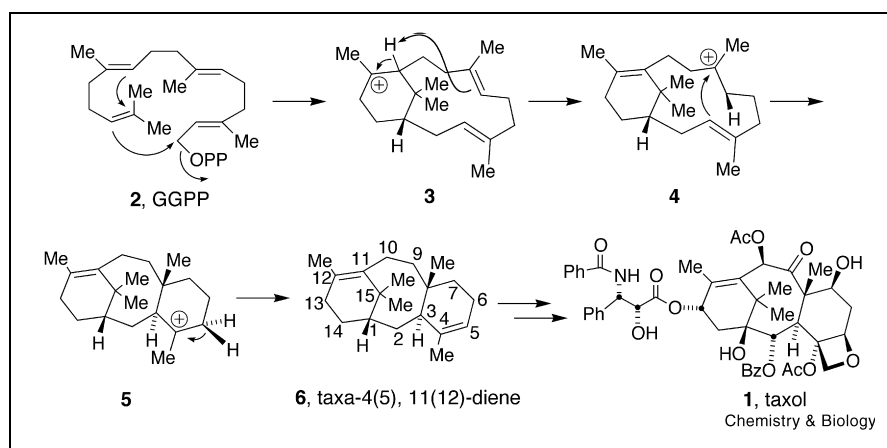
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**Figure 1.** Cyclization of geranylgeranyl diphosphate to taxol.

of oxygenation and acylation reactions (Figure 1) [14,15]. This enzyme, which has been isolated from both yew saplings [16] and cell cultures [17], catalyzes a slow, but apparently not rate limiting, step in the taxol biosynthetic pathway [17]. Initial assessment of the mechanism of taxadiene synthase [18] indicates that the reaction involves the ionization and cyclization of GGPP to a transient verticillyl intermediate (3), proposed to have the 11R configuration to allow intramolecular transfer of the C11 proton to C7 (4) to initiate transannular B/C-ring closure to the taxenyl cation (5), followed by deprotonation at C5 to yield taxa-4(5),11(12)-diene (6). No other details of the stereochemical mechanism of this novel enzyme are known.

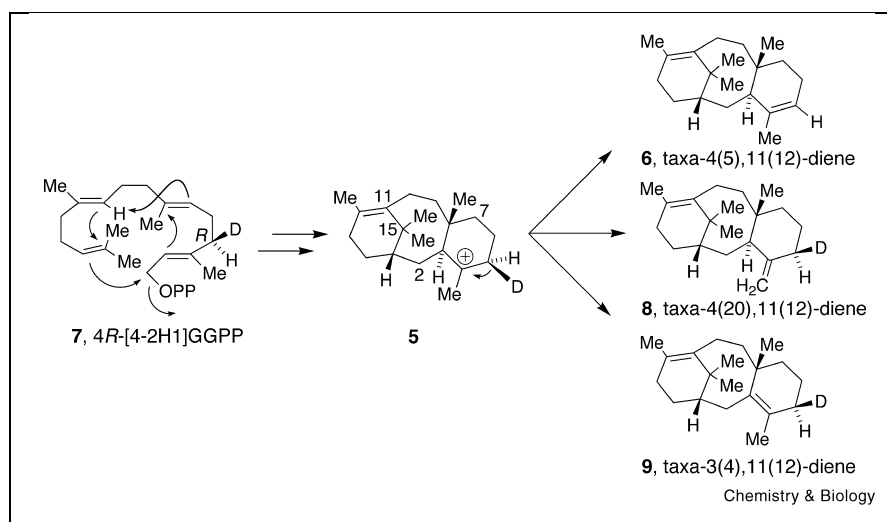
A cDNA encoding the taxadiene synthase from *T. brevifolia* has been obtained by a homology-based PCR cloning method [19]. A truncated version of the enzyme, in which

the plastidial targeting peptide of the preprotein has been deleted, has been functionally overexpressed in *Escherichia coli* and shown to resemble the native enzyme in kinetic properties [20]. This advance has made available sufficient amounts of the enzyme, and thus the enzyme product, to permit a more detailed study of the electrophilic cyclization reaction cascade.

## Results and discussion

### Chain termination: C5-deprotonation

To examine the stereochemistry of the C5-deprotonation of the terminal taxenyl C4-carbocationic intermediate of the reaction sequence (5, Figure 1), the appropriate deuterium labeled substrate, (4*R*)-[4-<sup>2</sup>H<sub>1</sub>]GGPP (7, Figure 2) (84 mol% <sup>2</sup>H), was prepared and incubated at preparative scale with the purified recombinant taxadiene synthase. The olefinic products of the reaction were isolated and subjected to GC-MS analysis for comparison to the prod-



**Figure 2.** Cyclization of deuterium labeled GGPP.

ucts identically obtained from the recombinant enzyme using unlabeled substrate. The taxa-4(5),11(12)-diene (**6**) enzymatically synthesized from the deuterium labeled acyclic precursor bore no detectable deuterium compared to the product prepared from the unlabeled precursor, thereby indicating that the C4  $\beta$ -hydrogen is selectively and entirely lost in the terminating deprotonation step (see Figure 2). Interestingly, the use of the alternate substrate also permitted observation of the phenomenon of isotopically sensitive branching [21,22], in which the deuterium-dependent slowing of the C5-deprotonation of the common taxenyl cation intermediate promoted alternative deprotonations for terminating the reaction cycle. With the unlabeled substrate, taxadiene synthase yields principally taxa-4(5),11(12)-diene (**6**, 94%), with lesser amounts ( $\sim 5\%$ ) of the 4(20),11(12)-isomer (**8**) resulting from deprotonation from the C20 methyl group, and negligible proton loss from the bridgehead methine (C3) to give the 3(4),11(12)-isomer (**9**) [20]. With the deuterated substrate, the proportion of taxa-4(5),11(12)-diene (**6**) in the enzymatic cyclization products decreased to 77.6%, with a concomitant increase in the proportions of the 4(20),11(12)-isomer (**8**) to 11.1% and of the 3(4),11(12)-isomer (**9**) to 9.2%; the latter two isomers fully retained the deuterium at C5.

Although there was relatively little change in the absolute rate of formation of the 4(20)-isomer, partitioning of the taxenyl carbocation to the 3(4)-isomer was substantially enhanced in response to the primary deuterium kinetic isotope effect on the C5 methylene deprotonation. An overall rate suppression resulting from the deuterium substitution on total olefin formation of about 50% was determined from the total ion chromatogram (compared to control experiments run in parallel with unlabeled substrate), indicating a kinetic isotope effect for the C5-deprotonation of  $k_H/k_D \sim 2.1$ . This latter result is of note, since earlier studies based on apparent secondary kinetic isotope effects had suggested that the initial ionization of the diphosphate ester substrate was rate limiting [18] and poten-

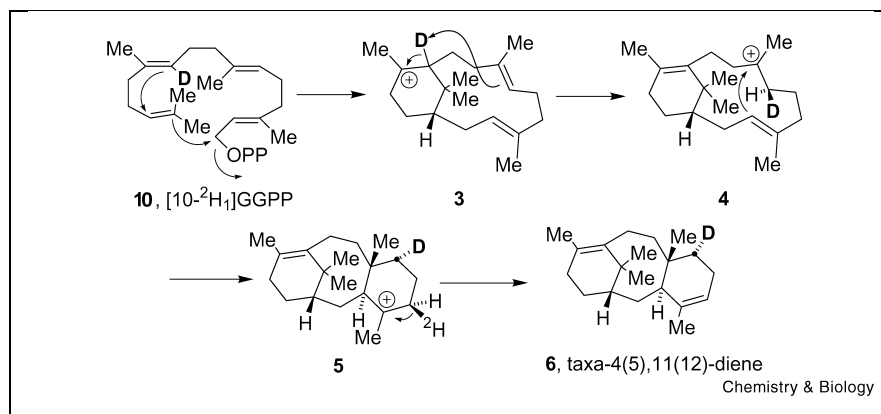
tially capable of masking downstream isotopically sensitive steps.

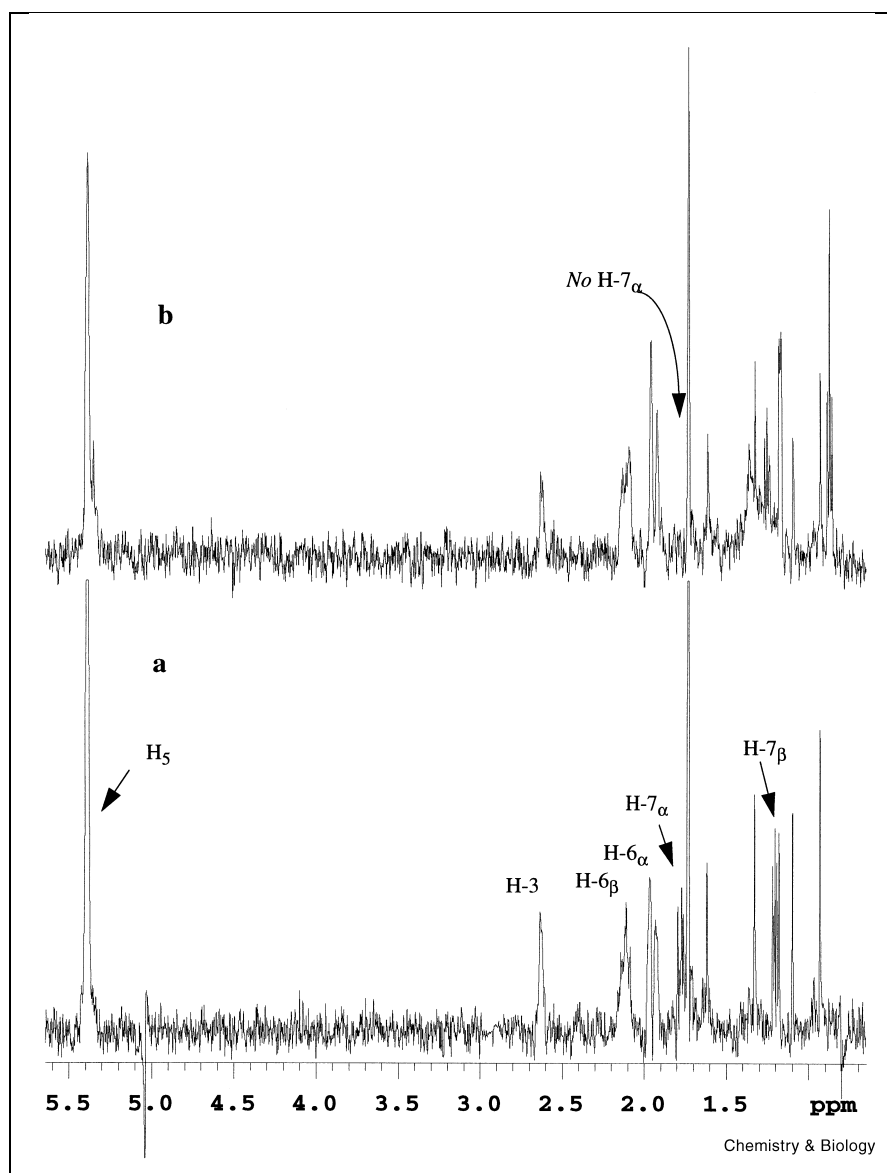
### A-ring to C-ring cascade

To examine the stereochemistry of the intramolecular hydrogen migration from C11 of the verticillyl intermediate (**3**) to C7 in the second ring closure step,  $[10\text{-}^2\text{H}_1]\text{GGPP}$  (**10**,  $>99\%$  mol%  $^2\text{H}$ , Figure 3) was prepared as previously described [18]. Incubation at preparative scale with the purified recombinant enzyme at saturating levels of the deuterated substrate yielded  $\sim 100\ \mu\text{g}$  of taxa-4(5),11(12)-diene (**6**) by calibrated GC-MS analysis of the reaction products (an overall rate suppression of  $\sim 50\%$  was also observed due to C10 deuterium substitution, yielding  $k_H/k_D = 2.0$  similar to that previously noted for the C5-deprotonation). Evaluation of the mass spectrum revealed that  $>99\%$  of the deuterium originally present at C10 of GGPP (C11 of the intermediate) had migrated to C7 of the taxane skeleton as assessed by shift of the C-ring fragment ion at  $m/z$  122 to  $m/z$  123 (and of the parent ion from  $m/z$  272 to  $m/z$  273), thereby demonstrating the complete fidelity of the intramolecular proton transfer.

The olefin fraction of the enzymatic reaction products was purified by a combination of open column silica gel chromatography (with pentane) and reversed phase ( $\text{C}_{18}$ ) HPLC (with acetonitrile) to afford  $\sim 10\ \mu\text{g}$  of pure C7-deuterated taxa-4(5),11(12)-diene (**6**) following transfer to deuterobenzene and removal of residual pentane used to partition the product from the HPLC solvent. The complete assignment of the  $^1\text{H}$  NMR spectrum of taxa-4(5),11(12)-diene in  $\text{CDCl}_3$  has been described previously [14] and these assignments have been reconfirmed in  $\text{C}_6\text{D}_6$  for the present work. Since earlier work had indicated that deuterium from  $[10\text{-}^2\text{H}_1]\text{GGPP}$  should reside at C7 of taxadiene following transfer from C11 of the verticillyl intermediate [18], the assignments most relevant to defining the regiochemistry of the product include those for the C-ring H5, H6 ( $\alpha$  and  $\beta$ ), H7 ( $\alpha$  and  $\beta$ ), and the H19 methyl protons.

**Figure 3.** Stereochemistry of intramolecular hydrogen migration.





**Figure 4.** Double pulsed field gradient spin echo-selective TOCSY of **6** ( $C_6D_6$ ) produced from **10(b)** and of unlabeled **6(a)**. The H5 signal at  $\delta 5.38$  was selectively excited in each case followed by a 65 ms mixing period. The predominant signals following the mixing period are those of the C-ring. Note the presence of the  $7_\alpha$  proton signal in **a** at  $\delta 1.76$  but its absence in **b** following deuterium atom transfer. Other features to note are the collapse of the  $7_\beta$ ,  $6_\alpha$ , and  $6_\beta$  proton fine structure in **b** as compared with **a**.

The relatively small amount of biosynthetic [ $^2H$ ]taxadiene available ( $\sim 10 \mu g$ ) precluded the use of two-dimensional NMR techniques. Consequently, 1D DPGSE-TOCSY [23] experiments were done to explore ways of discriminating among the various spin systems in the olefin, with the hope that an unambiguous excitation of the H7 protons would result. The presence of H5 at  $\delta 5.38$  ppm, alone in this region of the spectrum, provided an entry point to define the C-ring system. Figure 4 illustrates the results of the 1D DPGSE-TOCSY experiment, following selective excitation of H5 and a 65 ms mixing period. The most notable features of this spectrum are the doublet of doublets at  $\delta 1.19$  ppm (assigned to  $H_{7\beta}$ ), the multiplet at  $\delta 1.76$  ( $H_{7\alpha}$ ), the singlet at  $\delta 1.73$  (C20 methyl), and the broad triplet and doublet at  $\delta 2.11$  and  $\delta 1.94$  ( $H_{6\beta}$  and  $H_{6\alpha}$ , re-

spectively). Since the interpretation of the result of deuterium incorporation into taxadiene rests upon the correct stereochemical assignments for the C7 protons, it was imperative to confirm these assignments.

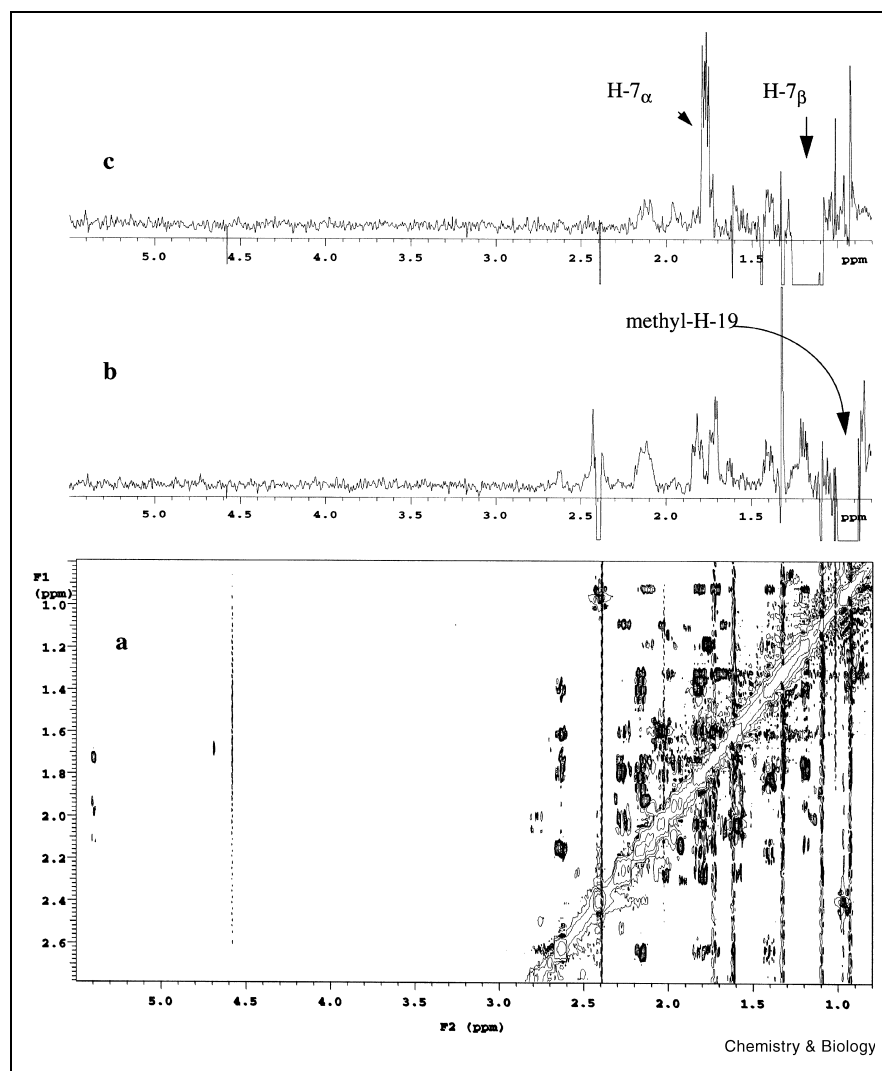
A two-dimensional NOESY-NMR experiment (with a 400 ms mixing time) was conducted for this purpose (Figure 5). Through-space correlations among protons on the  $\beta$ - (top) face of the molecule were examined. Cross-peaks were observed correlating the diagonal signal at 0.93 ppm (C19 methyl) with signals at  $\delta 1.19$  (C17 methyl), at  $\delta 1.39$  and  $\delta 1.81$  ( $H_{9\alpha,\beta}$ ),  $\delta 2.11$  ( $H_{6\beta}$ ),  $\delta 1.72$  ( $H_{2\beta}$ ), and  $\delta 1.19$  ( $H_{7\beta}$ ). No cross-peak was observed between the C19 signal and the signal at  $\delta 1.76$  ppm, which is consistent with the latter assignment as the  $H_{7\alpha}$  proton. Correlations with the diag-

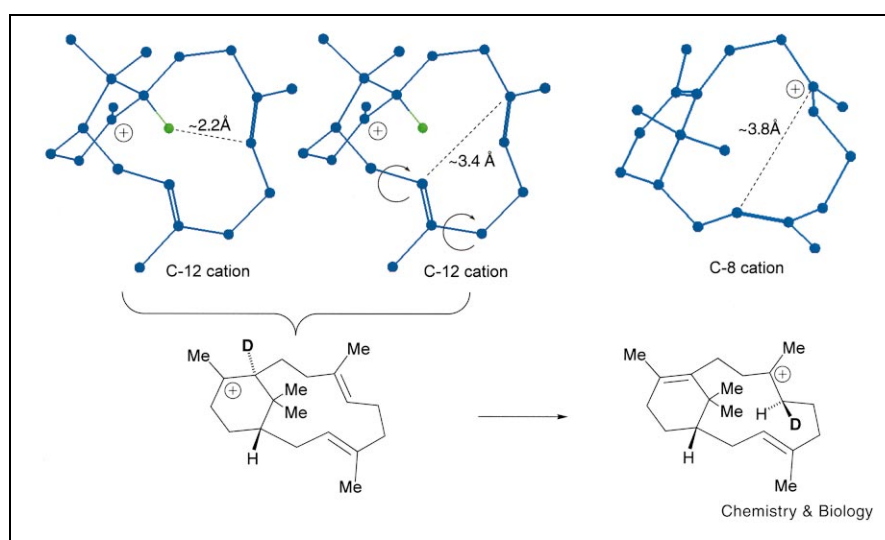
onal signal for the H7<sub>β</sub> proton (δ1.19 ppm) were carefully examined. As expected, there was a strong cross-peak found at δ1.76 ppm assigned to the H7<sub>α</sub> proton. There were also other cross-peaks at δ0.93 (C19 methyl), δ1.39 (H9<sub>α</sub>), δ1.94 and δ2.11 (H6<sub>α</sub> and H6<sub>β</sub>, respectively). In contrast, the signal assigned to the H7<sub>α</sub> proton (δ1.76 ppm) had fewer correlations, including those for H3 (δ2.62) and the C18 methyl (δ1.60), which is consistent with the stereochemical assignment to the α- (bottom) face of the olefin.

With completion of the regio- and stereochemical NMR assignments, the enzymatically derived, deuterium labeled taxadiene sample was examined. Residual pentane (from the final solvent partitioning step) was removed from the sample by transfer to and repeated evaporation from C<sub>6</sub>D<sub>6</sub>; otherwise, pentane signals completely dominate the 1D <sup>1</sup>H NMR spectrum in the crucial δ0.5–δ1.5 ppm region (Fig-

ure 4). By this means, the pentane signal intensities were reduced sufficiently to permit repetition of the 1D DPGSE-TOCSY experiment, this pulsed field gradient technique being especially efficient at de-phasing those signals not correlated with the selected proton resonance. The 1D DPGSE-TOCSY spectrum of the deuterium labeled olefin, with selective excitation of the H5 proton at δ5.38 ppm, is clear of artifacts including residual pentane signals (Figure 4b) and appears remarkably similar to that of the unlabeled standard (Figure 4a) with a few important exceptions. The signal for the H7<sub>α</sub> proton (1.76 ppm), clearly present in the standard, has disappeared in the labeled olefin. The signal at δ1.19 ppm, assigned to the H7<sub>β</sub> proton, has collapsed to an apparent doublet ( $J = 6$  Hz) consistent with the loss of its geminal H7<sub>α</sub> partner. Finally, the NMR patterns for both of the H6 protons have been altered. These results are entirely consistent with transfer of the deuterium atom from C10 of the acyclic

**Figure 5.** NOESY NMR spectrum of **6** (C<sub>6</sub>D<sub>6</sub>). Inset **a** is a 500 MHz two-dimensional NOESY spectrum. Insets **b** and **c** are slices of the 2D spectrum taken through the methyl H19 and H7<sub>β</sub> positions, respectively.





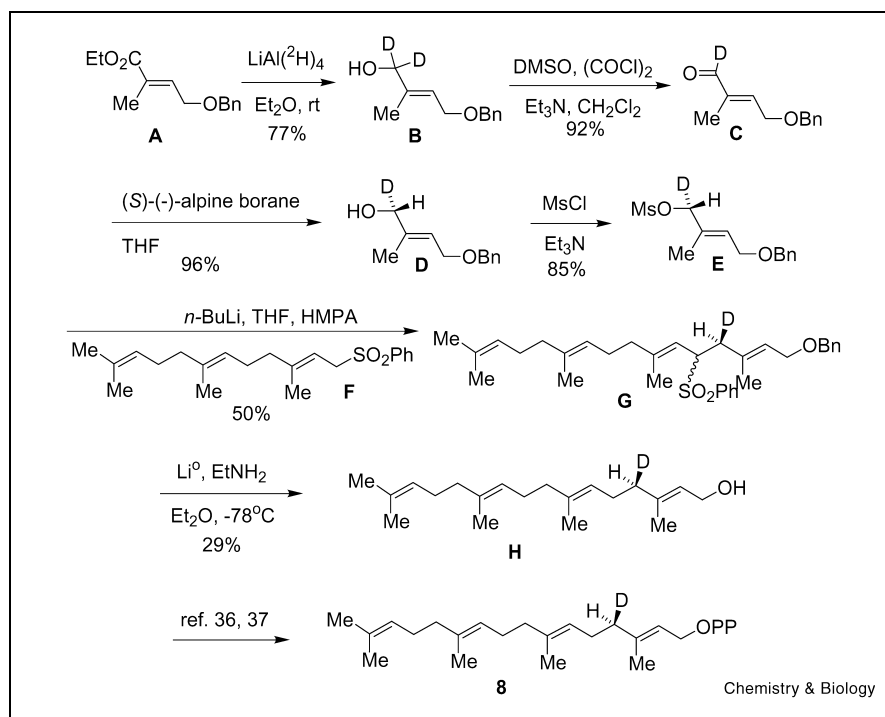
**Figure 6.** Molecular modeling of the C11 to C7 hydrogen migration converting the verticillen-12-yl carbocation to its 8-yl isomer prior to cyclization of the C-ring.

precursor exclusively to the H7 $_{\alpha}$  position of the taxadiene product.

#### Transition state modeling

Modeling of the intermediate C12 carbocation resulting from the C11–C15 closure in the initially formed macrocycle (Figure 6), with minimization of this structure using MacSpartan (AM1 basis set, overall molecular charge of +1, trivalent carbon at C12), reveals that the C11 proton is within  $\sim 2.2$  Å of the C7 carbon and seemingly perfectly

poised for the transannular migration to the *re*-face at C7. Furthermore, inspection of the conformation of the C12 carbocation strongly suggests that it is unlikely that an amino acid at the enzyme active site could gain access to the C11 proton to mediate the C11–C7 transfer, since this proton is buried deep within the concave face of the 12-membered macrocycle. Based on both the experimental evidence and the conformational analysis, it appears that an unassisted intramolecular transfer is the most plausible mechanism for the initiation of the final ring closure step



**Figure 7.** Synthesis of all *trans*-(4*R*)-[4-<sup>2</sup>H<sub>1</sub>]GGPP (**8**).

mediated by taxadiene synthase. Thus, the C7–C8 olefin serves as the Brønsted base that quenches the incipient carbocation at C12 and no active site enzyme base needs to be invoked for this process.

Of further interest in this enzymatic reaction is the facial bias of the final C8–C3 olefin cation cyclization. The established stereochemistry of the taxane B/C-ring junction dictates attack by the C3/C4  $\pi$ -electrons on the C8 cation (formed immediately following the C11 to C7 proton migration) from *the same face* to which the newly installed proton at C7 has migrated (overall *syn* addition of H<sup>+</sup> and C3 to the 7,8 double bond). Modeling of this process (Figure 6), which formally involves a criss-cross of bond formation across the C7/C8 double bond, proved to be insightful. The molecular model of the C8 carbocation suggests that, upon pyramidalization at C7 and fashioning of the C11/C12 olefin, the conformation of the 12-membered ring twists, relative to the C12-cationic center, thereby rocking the C3/C4  $\pi$ -electrons into the correct facial orientation for capture of the C8 cation and establishing the *trans*-fused B/C-ring juncture.

There is precedent in enzymatic terpenoid cyclization reactions for apparently similar deprotonation–reprotonation steps involving transient formation of olefinic intermediates, with reprotonation of a distal double bond to initiate subsequent rearrangements or ring closures. For example, in the diterpene series, an intramolecular proton transfer in a pimarenyl intermediate promotes a *syn* addition/methyl migration to yield the abietane skeleton catalyzed by recombinant abietadiene synthase [24]. Related internal proton transfers also occur in cyclization reactions catalyzed by sesquiterpene synthases [25,26]; however, in the best defined example (5-*epi*-aristolochene synthase), the proton transfer step is clearly mediated by an active site base [27].

### A remarkable olefin cation cascade

The overall stereochemical mechanism for the conversion of GGPP to taxa-4(5),11(12)-diene (Figure 3) is shown to involve the following five processes: (1) Walden inversion at the diphosphate-bearing carbon (C1) coupled with anti-periplanar C1–C14 and C11–C15 bond formation initiated by the ionization of the diphosphate group and leading to the verticillen-12-yl carbocation (**3**) (the stereochemical outcome at C1 and C15 was elucidated by separate enzymatic cyclizations of (*R*)-[1-<sup>2</sup>H<sub>1</sub>]- and (*S*)-[1-<sup>2</sup>H<sub>1</sub>]-, and (*E*)-[16,16,16-<sup>2</sup>H<sub>3</sub>]GGPP and 600 MHz <sup>1</sup>H NMR analyses of the resulting [2-<sup>2</sup>H<sub>1</sub>]- and [16,16,16-<sup>2</sup>H<sub>3</sub>]taxadienes; unpublished results, D.C.W., Q.J., R.M.C., R.C.); (2) conformational inversion of the A-ring accompanied by rotations of the macrocyclic loop; (3) a unique intramolecular transfer of the C11 proton to the *re*-face of C7 to generate the isomeric verticillen-8-yl carbocation; (4) bond rotations and closure of the B/C-ring junction via capture of the C8 carbocation by the transannular C3/C4  $\pi$ -electrons to

form the penultimate taxen-4-yl intermediate; and (5) elimination of the 5- $\beta$  proton from a twist boat conformer to form taxadiene.

### Significance

Taxadiene synthase is a remarkable terpene cyclase that appears to function by binding and ionization its substrate GGPP to mediate an enantio- and face-selective polyolefin cation cascade that involves the formation of three carbon–carbon bonds, three stereogenic centers, and the loss of hydrogen in ‘a single step’. The seemingly unassisted intramolecular proton transfer mechanism of taxadiene synthase is thus strikingly unusual in this regard, and suggests that this enzyme type is capable of mediating complex olefin cation cyclizations, *with absolute stereochemical fidelity, by conformational control alone*. These findings provide insight into the molecular architecture of the first dedicated step of taxol biosynthesis that creates the taxane carbon skeleton, and these observations have broad implications for the general mechanistic capability of the large family of terpenoid cyclization enzymes that catalyze similar electrophilic reaction cascades.

### Materials and methods

#### Substrate preparation

All *trans*-GGPP (**2**, 98%) and [10-<sup>2</sup>H<sub>1</sub>]GGPP (**10**, >90%; >99 mol% <sup>2</sup>H) were prepared and purified as previously described [18]. All *trans*-(*R*)-[4-<sup>2</sup>H<sub>1</sub>]GGPP (**8**, 93%; 84 mol% <sup>2</sup>H) was prepared from previously described [28] ester **A** (25:1 *E:Z* ratio by <sup>1</sup>H NMR) and the known [29–31] sulfone **F** (Figure 7). Ester **A** was reduced with LiAlH<sub>4</sub> to dideuterated alcohol **B**, oxidized to monodeuterated aldehyde **C** by the Swern method, and reduced asymmetrically to **D** with (*S*)-alpine borane. Camphanate derivatization and <sup>1</sup>H NMR analysis showed a 95:5 enantiomeric ratio for **D** [32]. Following conversion to the mesylate **E** and coupling [33] to sulfone **F**, the product **G** was obtained in 50% yield after purification. The sulfone group and benzyl ether protecting group were removed by reduction with Li in EtNH<sub>2</sub> at –78°C to give (*R*)-[4-<sup>2</sup>H<sub>1</sub>]geranylgeraniol **H** in 29% yield following AgNO<sub>3</sub> silica gel argentation chromatography to remove a contaminating double bond isomer. To determine the enantiomeric purity of **H**, the asymmetric epoxidation [34] of the 2,3 double bond was carried out to differentiate the two protons adjacent to the epoxide ring by <sup>2</sup>H NMR analysis. Integration of the relevant peaks at 1.51 and 1.35 ppm showed a ratio of 1:8 which, by assuming the same enantioselectivity as in the epoxidation of geraniol [34], gave an estimated *R:S* enantiomer ratio of 91:9. Alcohol **H** was converted to the corresponding diphosphate ester (30% yield) by established procedures [35,36]. A single component was observed upon cellulose TLC and no impurities were observed in the <sup>1</sup>H and <sup>31</sup>P NMR spectra; the latter (D<sub>2</sub>O, 162 MHz) displayed two doublets at –9.54 and –5.72 ppm (*J*=20.8 Hz).

#### Enzymatic conversions

The truncated (M60) version of recombinant taxadiene synthase, in which the plastidial transit peptide has been deleted, was overexpressed in *E. coli* and purified (>96%) as previously described [20]. Incubations were carried out in 3 ml of the standard assay buffer [20] containing 1 mg of enzyme and a 2 mM concentration of the appropriate substrate. After immediate extraction with pentane to remove any organic soluble contaminants, the reaction mixture was maintained at 31°C until in excess of 100  $\mu$ g of olefin product had been generated. Following incubation, the pentane-soluble reaction products were extracted for capillary GC-MS analysis [20] to establish overall conversion, the distri-

bution of taxadiene isomers, and the deuterium content of these olefinic products. In preparation for the NMR analysis of the product derived from [ $10\text{-}^2\text{H}_1$ ]GGPP (**10**), the olefin mixture was purified by silica gel column chromatography with hexane, followed by reversed phase ( $C_{18}$ ) HPLC with acetonitrile. The concentrated acetonitrile eluent was partitioned between pentane and water, and the pentane phase was transferred to deuterobenzene (99.96 mol%) and repeatedly concentrated under  $N_2$  (without drying which results in decomposition [14]) to remove residual solvent, ultimately yielding  $\sim 10\ \mu\text{g}$  of pure taxa-4(5),11(12)-diene (**6**) in a 500  $\mu\text{l}$  sample volume.

#### MS and NMR spectrometry

Protocols for capillary GC-MS analysis of the taxadiene isomers have been described in detail elsewhere [14,18,20]. All NMR spectra were recorded on a Varian Unity-500 spectrometer at  $25^\circ\text{C}$  using a very sensitive  $^1\text{H}$  indirect detection probe. 1D double pulsed field gradient spin echo TOCSY (DPFGSE-TOCSY) spectra were obtained with a mixing time of 65 ms. 2D NOESY spectra were acquired using mixing times of 400 ms. 2D spectra were collected as  $256\ (t_1) \times 2048\ (t_2)$  complex points with a sweep width of 7 kHz in each dimension. Data were processed using Varian, Inc. VNMR software. The final data size, after linear prediction in  $t_1$  and zero-filling in both dimensions, was  $1024\ (f_1) \times 4096\ (f_2)$  complex points.

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