Cytochrome P450-catalyzed hydroxylation of taxa-4(5),11(12)-diene to taxa-4(20),11(12)-dien-5α-ol: the first oxygenation step in taxol biosynthesis

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Background: The structural complexity of taxol dictates continued reliance on biological production methods, which may be improved by a detailed understanding of taxol biosynthesis, especially the rate-limiting steps. The biosynthesis of taxol involves the cyclization of the common isoprenoid intermediate geranylgeranyl diphosphate to taxa-4(5),11(12)-diene followed by extensive, largely oxidative, modification of this diterpene olefin. We set out to define the first oxygenation step in taxol biosynthesis.

Results: Microsomal enzymes from *Taxus* stem and cultured cells were used to define the first hydroxylation of taxadiene. We confirmed the structure of the reaction product (taxa-4(20),11(12)-dien-5α-ol) by synthesizing this compound. The responsible biological catalyst was characterized as a cytochrome P450 (heme thiolate protein). *In vivo* studies confirmed that taxadienol is a biosynthetic intermediate and indicated that the hydroxylation step that produces this product is slow relative to subsequent metabolic transformations.

Conclusions: The structure of the first oxygenated intermediate on the taxol pathway establishes that the hydroxylation reaction proceeds with an unusual double bond migration that limits the mechanistic possibilities for subsequent elaboration of the oxetane moiety of taxol. The reaction is catalyzed by a cytochrome P450, suggesting that the seven remaining oxygenation steps in taxol biosynthesis may involve similar catalysts. Because the first oxygenation step is slow relative to subsequent metabolic transformations, it may be possible to speed taxol biosynthesis by isolating and manipulating the gene for the taxadiene-5-hydroxylase that catalyzes this reaction.

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Key words: cytochrome P450, taxadiene hydroxylase, taxadienol synthesis, taxol biosynthesis, *Taxus* enzymes

Received: **24 May 1996**
Accepted: **4 Jun 1996**

Chemistry & Biology June 1996, **3**:479–489

Introduction

The complex diterpenoid taxol (paclitaxel) [1] is a potent antimitotic agent with excellent activity against a range of cancers including ovarian and breast cancer [2,3]. The very limited supply of the drug from the original source, the bark of the Pacific yew (*Taxus brevifolia* Nutt.; Taxaceae), prompted the development of alternative means of production [4,5], such as semisynthesis of taxol and its analog taxotere (docetaxel) [6] from advanced taxane diterpenoid (taxoid) metabolites that are more readily available from natural sources [7,8]. Total synthesis of taxol has also been achieved by several elegant routes ([9–12], and references therein), but the yields are too low to be commercially viable. With increasing applications in chemotherapy, both in treatment of additional cancer types and in earlier interventions, the availability and cost of these drugs will remain important issues [13]. It is clear that in the foreseeable future the supply of taxol and its synthetically useful progenitors must continue to rely on biological methods of production, either in *Taxus* species or in cultures of cells derived from these plants [13]. Improving the biological production yields depends upon a detailed understanding of the biosynthetic pathway, the enzymes catalyzing the sequence of reactions, especially the slow steps, and the genes encoding these proteins.

The biosynthesis of taxol (compound **4**, Fig. 1) involves the initial cyclization of geranylgeranyl diphosphate (compound **1**), the universal precursor of diterpenoids [14], to taxa-4(5),11(12)-diene (compound **2**), followed by extensive oxidative modification of the olefin and elaboration of side chains [15] (Fig. 1). The enzyme that catalyzes this reaction, taxadiene synthase, has been purified from yew stem and characterized [16], and the corresponding cDNA has been recently isolated [17]. The cyclization mechanism involves an intramolecular hydrogen transfer and proceeds without free intermediates [18]. It was unexpected to find that the first committed step of the pathway was cyclization to

taxa-4(5),11(12)-diene (compound 2), since the olefinic precursor of taxol was traditionally considered to be the taxa-4(20),11(12)-diene isomer. This theory had been based on the observation that a large number of naturally occurring taxoids bear double bonds in these positions, and that the sequential conversion of the 4(20)-exocyclic methylene group to the oxirane and then to the oxetane ring of taxol could be readily imagined [19–21].

Given the efficient incorporation of taxa-4(5),11(12)-diene into taxol and related compounds, and the elimination of the exocyclic double bond isomer as an intermediate [15,16,18], several additional observations are relevant in considering subsequent metabolic transformations of the precursor olefin. No oxygenated taxoids bearing a 4(5) double bond are known, whereas taxoids with the 4(20)-ene-5-oxy functional grouping are exceedingly common [22]. Furthermore, based on the relative abundances of the various classes of oxygenated taxane metabolites, hydroxylation at C5 of the taxane skeleton is thought to be an early step in the pathway [21,23]. These observations suggest that hydroxylation of taxa-4(5),11(12)-diene at C5, with migration of the double bond, is probably the next step in the taxol biosynthetic sequence. Here, we describe the isolation and characterization of a cytochrome P450 hydroxylase from *Taxus* stem and cultured cells that catalyzes the conversion of taxa-4(5),11(12)-diene (compound 2) to taxa-4(20),11(12)-dien-5α-ol (compound 3) (Fig. 1). We also provide evidence that this taxadienol is the second specific intermediate on the pathway to taxol and related taxoids, and that the hydroxylation step is relatively slow compared to subsequent metabolic transformations in the sequence.

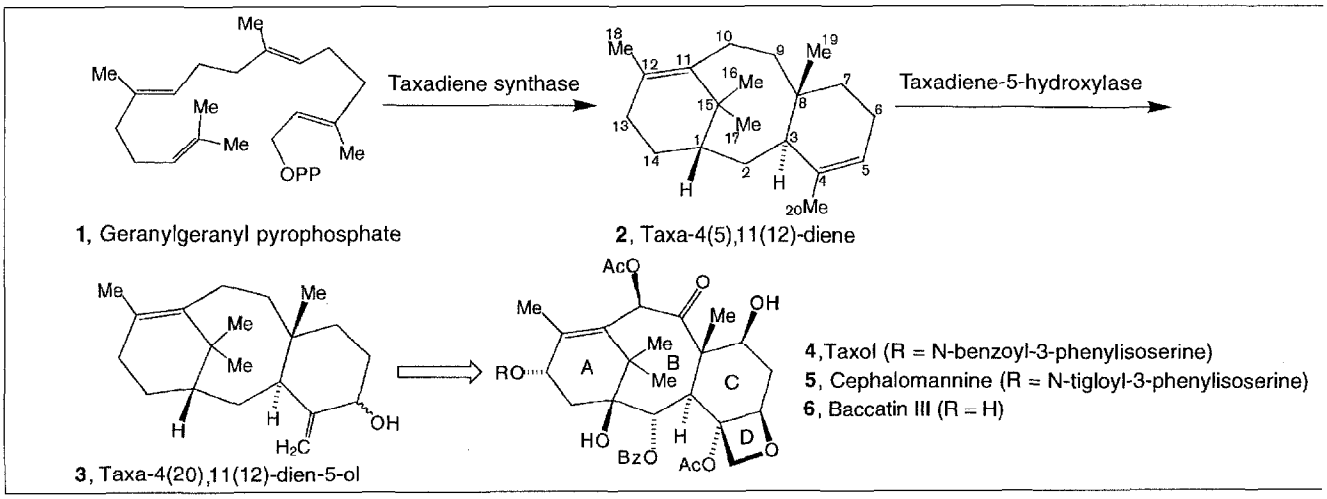
Results and discussion

Demonstration of taxadiene oxygenase activity

Since many terpenoid oxygenation reactions are catalyze by membranous P450 cytochromes [24–26], microsom preparations from extracts of *T. brevifolia* stems or *cuspidata* suspension-cultured cells were examined fc their ability to transform [^{3}H]taxa-4(5),11(12)-diene t more polar products. The microsomal preparations cat lyzed the aerobic, NADPH-dependent conversion of th olefin to a product (in 4–14 % yield depending on th tissue) with chromatographic properties similar to that c the diterpenol standard abieta-7(8),13(14)-dien-18-ol o silica gel thin layer chromatography (TLC) and AgNO argentation TLC. Gas-chromatography mass spectrometi (GC–MS) analysis (selected ion monitoring (SIM) mod of the isolated product indicated that a metabolit (retention time (R_f) 13.39 ± 0.01 min) with ions at *m/z* 28 273, 270 and 255 (ratio ~4:2:1:4) was present, consister with the P^+, P^+-15(CH_3), P^+-18(H_2O), and P^+-15-18 ion respectively, of a diterpene alcohol. Formation of th putative diterpenol from taxadiene was not supported b cumene hydroperoxide or H_2O_2, or by the combination c Fe^{2+}, ascorbate and α-ketoglutarate, eliminating thes alternative modes of olefin hydroxylation.

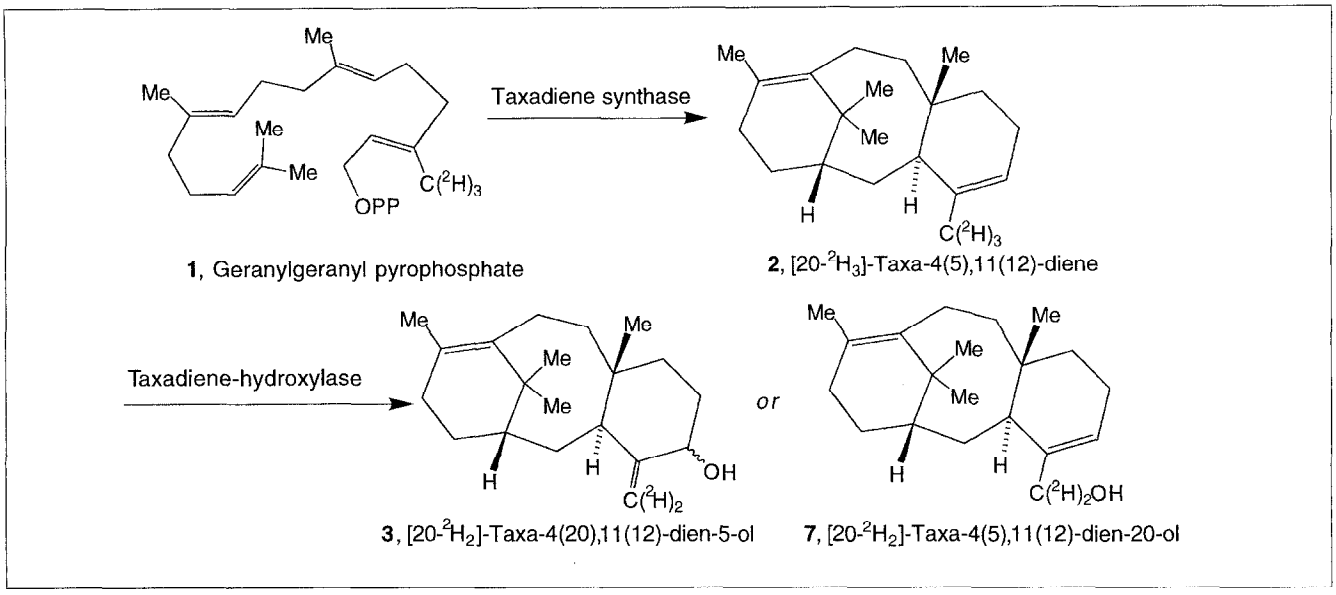
In a preliminary experiment to define the presumptiv diterpenol product, [20-$^2H_3$]taxa-4(5),11(12)-diene w prepared biosynthetically from [20-$^2H_3$]geranylgeran diphosphate using the partially purified taxadiene syn thase from *T. brevifolia* [16,18]. The chromatographicall purified olefin (~80 nmol) was then used as a substrate i the oxygenase reaction and the product was isolated b TLC as before. GC–MS analysis (SIM mode) of th

Figure 1



Cyclization of geranylgeranyl diphosphate (compound **1**) to taxa-4(5),11(12)-diene (compound **2**) and elaboration of the olefin to taxol (compound **4**), cephalomannine (compound **5**), or baccatin III (compound **6**) via a proposed taxa-4(20),11(12)-dien-5-ol intermediat (compound **3**). OPP denotes the diphosphate moiety, Bz the benzoyl group and Ac the acetyl group.

Figure 2



Preparation of [20-$^2H_3$]taxa-4(5),11(12)-diene (compound 20-$^2H_3$-**2**) from [20-$^2H_3$]geranylgeranyl diphosphate (compound 20-$^2H_3$-**1**) using taxadiene synthase, and conversion of the olefin by the microsomal oxygenase to a diterpenol with the loss of one deuterium atom. The most likely products are taxa-4(20),11(12)-dien-5-ol (compound **3**) or taxa-4(5),11(12)-dien-20-ol (compound **7**).

purified material, in this instance, revealed the presence of ions at *m/z* 290 (P^+), 275 (P^+-15), 272 (P^+-18) and 257 (P^+-33). The shift upwards of these diagnostic ions by 2 amu (not 3) indicates the loss of 1 deuterium atom from C20 in the transformation, which can be accounted for by the conversion of taxa-4(5),11(12)-diene (compound **2**) to taxa-4(20),11(12)-dien-5-ol (compound **3**) or to taxa-4(5),11(12)-dien-20-ol (compound **7**) (Fig. 2). Since taxoids bearing a C20 hydroxyl group have not been reported amongst the >100 naturally occurring taxane metabolites now known [22], the result of oxygenation of the deuterated substrate suggested that taxa-4(5),11(12)-diene was hydroxylated at C5 with concomitant allylic migration of the C4,C5 double bond.

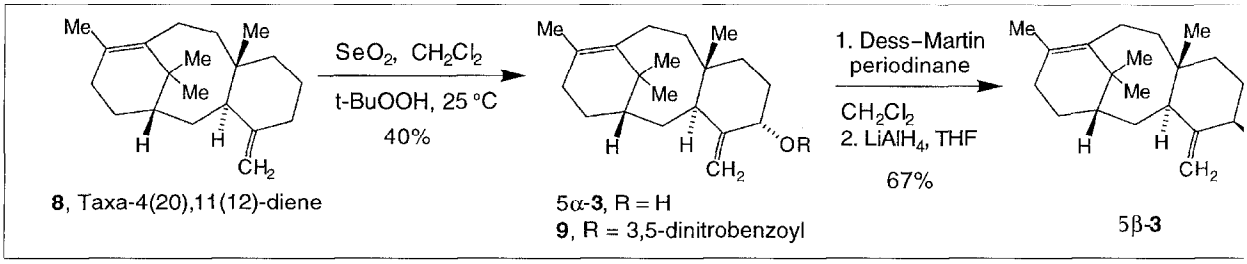
Syntheses of taxa-4(5),11(12)-dien-5α-ol and 5β-ol

To obtain the 5α- and 5β-epimers of taxa-4(5),11(12)-dien-5-ol (compound **3**) for comparative purposes, we used our previously reported total synthesis of taxa-4(5),11(12)-diene (compound **2**) and taxa-4(20),11(12)-diene (compound **8**) [27]. Taxa-4(20),11(12)-diene (compound **8**) was oxidized with SeO_2-*t*BuOOH to give a single hydroxylation product in 40 % yield (compound 5α-**3**, Fig. 3). Extensive NMR-based structural investigations of this substance (^1H NMR, nuclear Overhauser enhancement spectroscopy (NOESY), double quantum filtered correlation spectroscopy (DQF-COSY), heteronuclear multiple quantum coherence (HMQC) spectroscopy and heteronuclear multiple bond correlation (HMBC) spectroscopy, see Supplementary material) revealed that this product was exclusively the 5α-stereoisomer. The corresponding 5β-stereoisomer (compound 5β-**3**) was prepared from compound 5α-**3** by oxidation to taxa-4(20),11(12)-dien-5-one followed by stereoselective reduction with $LiAlH_4$. The corresponding, highly crystalline 3,5-dinitrobenzoate (compound **9**) of compound 5α-**3** was also prepared for co-crystallization studies.

Identification of the biosynthetic product

Chromatographic comparison of the 5α- and 5β-epimers of taxa-4(20),11(12)-dien-5-ol (compound **3**, Fig. 2) to the biosynthetic product prepared from synthetic (±)-[20-^3H]taxadiene with *Taxus* microsomes showed that the labeled diterpenol has identical chromatographic properties to the authentic 5α-alcohol (compound 5α-**3**) on silica gel TLC (retention factor (R_f) 0.5 with pentane:ether, 3:1 v/v) and $AgNO_3$-argentation TLC (R_f 0.5 with pentane:ether, 3:2 v/v). The β-stereoisomer (compound 5β-**3**) differs substantially in chromatographic behavior on both silica gel TLC (R_f 0.3 with pentane:ether, 3:1 v/v) and argentation TLC (R_f 0.4 with pentane:ether, 3:2 v/v), eliminating this diastereomer as the possible biosynthetic product. Preparative incubations of [^3H]taxadiene with *Taxus* microsomes were carried out to accumulate sufficient biosynthetic product (~3 nmol) for full spectrum GC-MS analysis. Comparison of retention time and mass spectrum of the TLC-purified biosynthetic product to those of authentic taxa-4(20),11(12)-dien-5α-ol (compound 5α-**3**) indicated that the two compounds were identical (Fig. 4); the retention time (15.21 min) and mass

Figure 3



8, Taxa-4(20),11(12)-diene

5α-**3**, R = H
9, R = 3,5-dinitrobenzoyl

5β-**3**

Outline of the synthesis of the 5α- and 5β-epimers of taxa-4(20),11(12)-dien-5-ol (compounds 5α- and 5β-**3**) from taxa-4(20),11(12)-die (compound **8**). Compound **9** was prepared for co-crystallization studies.

spectrum of compound 5β-**3** are readily distinguishable from those of compound 5α-**3**. Finally, the purified, tritium-labeled biosynthetic product (42.8 nCi) was converted to the 3,5-dinitrobenzoate ester (90 % yield), diluted with authentic (±)-taxa-4(20),11(12)-dien-5α-yl dinitrobenzoate (compound **9**, 8.0 μmol), and recrystallized to constant specific activity and melting point (4.67 ± 0.09 nCi μmol^{-1}, m.p. 171 °C, decomposing at melting temperature (dec.)). The results of these analyses, including quantitative evaluation of the radiochemical crystallization studies, indicate that a minimum of 87 % of the oxygenation product of taxa-4(5),11(12)-diene (compound **2**) is taxa-4(20),11(12)-dien-5α-ol (compound 5α-**3**).

Radiochemically-guided fractionation of a *T. brevifolia* bark extract (0.25 μCi of (±)-[20-^{3}H]taxa-4(20),11(12)-dien-5α-ol diluted into an extract of 250 kg dry bark), carried out using silica gel, argentation, and reversed-phase chromatography, before and after alkaline hydrolysis of half of the extract, followed by GC–MS analysis of the partially purified product, demonstrated that the alcohol is present in the 5–10 $\mu g\ kg^{-1}$ range and the ester(s) in the 25–50 $\mu g\ kg^{-1}$ range. The low abundance of these oxygenated metabolites in bark tissue is in the range of that previously observed for the olefin substrate of the hydroxylase (5–10 μg taxadiene kg^{-1}). Thus, like the enzymatic cyclization to taxadiene [15,16], the oxygenation of taxadiene appears to be a slow metabolic step relative to subsequent transformations in stem tissue since the reaction product does not appreciably accumulate.

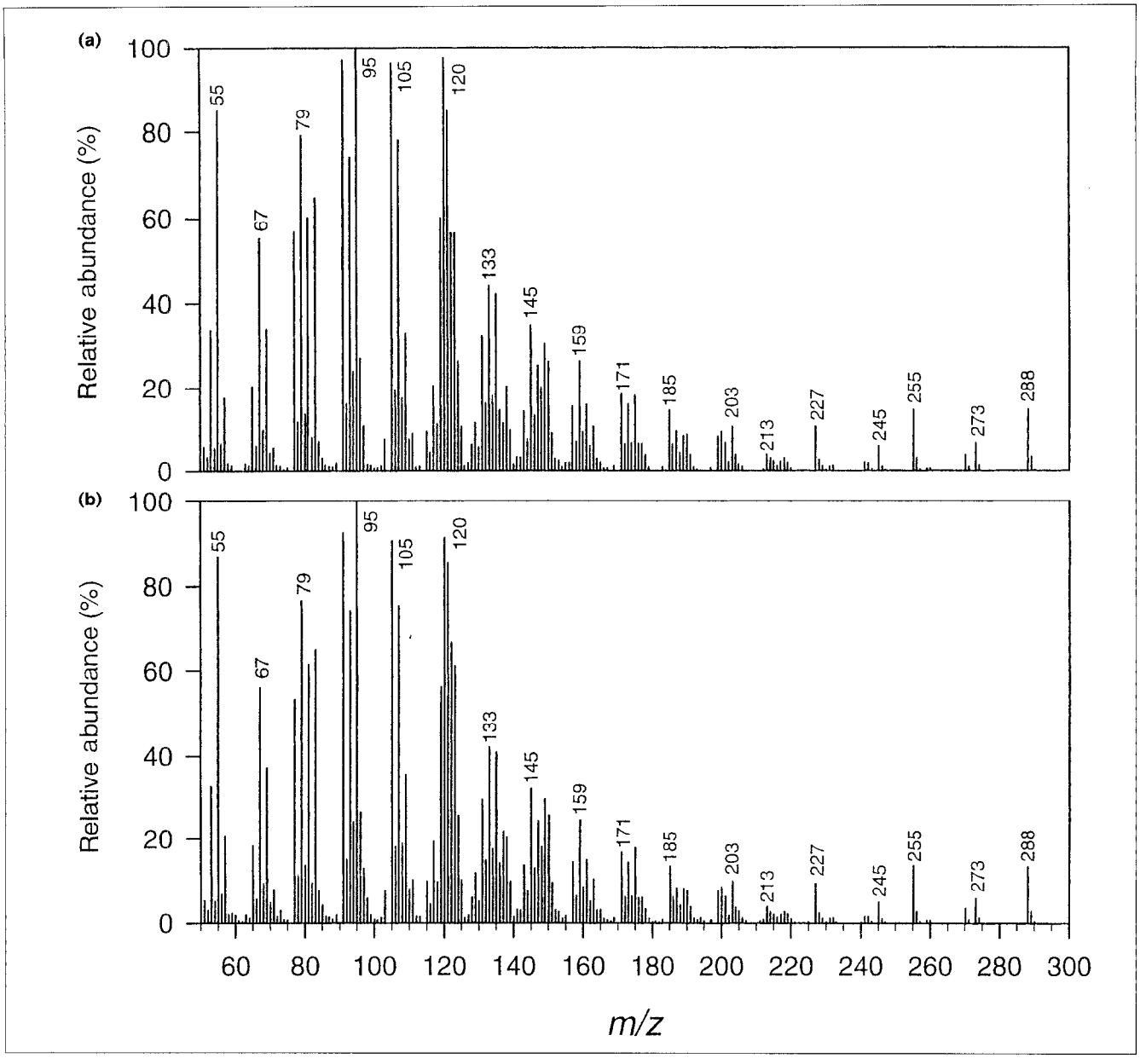
Taxa-4(20),11(12)-dien-5α-ol as a precursor of taxol

To determine if taxa-4(20),11(12)-dien-5α-ol (compound 5α-**3**) could serve as a precursor of taxol (compound **4**) and related taxoids, 4.0 μCi (1 μmol) of the synthetic tritium-labeled alcohol was suspended in 4.5 ml of buffer and equal portions were vacuum-infiltrated into three batches (1.2 g each) of *T. brevifolia* stem discs. Following incubation for two, four and six days, the labeled products were extracted, and the advanced taxoids, 10-deacetylbaccatin III (compound 10-deacetyl-**6**), cephalomannine (cc **5**), and taxol (compound **4**) (see Fig. 1), were iso TLC, diluted with 200 mg of the corresponding u carrier, and crystallized to constant specific acti melting point. The incorporation levels for the re two, four and six day time points were 0.96, 3.07 % for 10-deacetylbaccatin III, 0.16, 0.28 and 0 cephalomannine, and 0.28, 0.58 and 0.77 % for ta incorporation levels into these taxoids, and the mate distribution of label between the three met are comparable to the values obtained previous [^{3}H]taxadiene as an exogenous precursor. These coupled with the detection of very low levels 4(20),11(12)-dien-5α-ol in *Taxus* bark, indicate compound serves as an efficient precursor of t other very closely related taxoids of yew stem.

Characterization of the oxygenase

Centrifugal fractionation of tissue extracts *brevifolia* sapling stems and *T. cuspidata* susp cultured cells indicated that the major portio oxygenase activity (70 %) resides in the light m fraction (190,000g pellet). Approximately 25 % taxadiene-5-hydroxylase activity was located dense membrane fraction (20,000g pellet), pro the result of occlusion of microsomes by dens branes, with the residual activity (~5 %) associa the soluble enzyme fraction (190,000g supe probably due to microsomal membrane solub during tissue homogenization. The inclusion of p lipase inhibitors, 10 mM each of procaine and li in the isolation buffer doubled the microsomal ase activity, presumably by preserving membrar rity. The pH optimum for the reaction (in HEP near 7.2, with a ~50% reduction in activity a pH 6.2 or pH 8.2. The taxadiene-5-hydroxylase was completely dependent on NADPH (1 m inclusion of an NADPH-regenerating system cantly increased activity, further supporting th vation that this reduced pyridine nucleotide co required for activity (Table 1). The inclusion of I FMN (saturation at 2.5 μM) in the reaction mix

Figure 4



GC–MS analysis of the product of the microsomal oxygenase shows that it is identical to synthetic compound 5α-**3**. The mass fragmentation patterns are illustrated for **(a)** authentic (±)-taxa-4(20),11(12)-dien-5α-ol (R_t = 13.38 min) and for **(b)** the diterpenol (R_t = 13.39 min) obtained from taxa-4(5),11(12)-diene when incubated with *Taxus* microsomes in the presence of NADPH and O_2.

improved hydroxylase activity (Table 1), presumably by replacing the flavin prosthetic groups lost during microsome isolation from the NADPH–cytochrome P450 reductase required to couple electron transfer from NADPH to the cytochrome in the course of the reaction [28–30]. Under optimum assay conditions, the K_M value for the substrate (±)-[20-$^3H_3$]taxa-4(5),11(12)-diene was estimated to be ~6.0 μM from Lineweaver–Burk plots of the kinetic data. Because the substrate is racemic and the enzyme system is membranous, this value should be interpreted with caution; K_M values in the micromolar range are typical of terpenoid hydroxylases [25]. Although the substrate prepared biosynthetically from [1-^{3}H]geranylgeranyl diphosphate using the taxadiene synthase ([2-^{3}H]taxa-4(5),11(12)-diene) [15,16] was not available in sufficient amounts to permit kinetic studies, the product of the hydroxylation of this presumably enantiomerically pure olefin was identical (by GC–MS) to the product generated from the synthetic, racemic precursor.

Table 1

Reaction conditions for taxadiene-5-hydroxylase

Conditions	Relative rate
Complete system[a]	100
without flavins	73
without NADPH regenerating system	55
without oxygen	14
without NADPH[b]	0
1:1 $CO:O_2$[c]	
dark	85
9:1 $CO:O_2$[c]	
dark	18
450 nm light	73

[a]Complete system contains 26 μM taxa-4(5),11(12)-diene, 1 mM NADPH, 5 mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 2.5 μM FMN, 2.5 μM FAD and 500 μg microsomal protein per ml.

[b]Relative to boiled control.

[c]Relative to corresponding dark and light incubations (complete system) without CO.

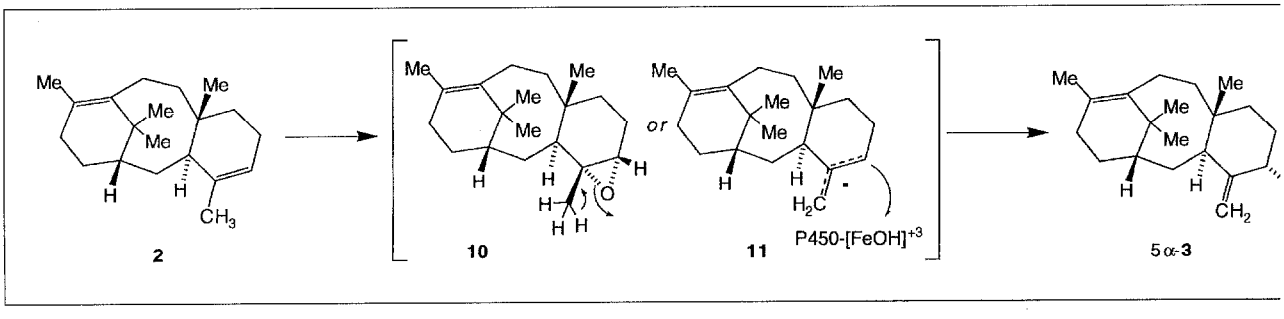
Excluding molecular oxygen from the reaction mixture greatly reduced hydroxylase activity (Table 1); however, the failure to demonstrate an absolute oxygen requirement probably resulted from the inability to remove residual O_2 from the assay buffer, coupled with a relatively high affinity of the cytochrome for this reactant. Hydroxylation of taxadiene was inhibited by CO in the dark, and this inhibition was partially reversed by blue (450 nm) light (Table 1). Finally, the reaction was completely inhibited by clotrimazole and miconazole (at 3 μM) and was partially inhibited by cytochrome *c* (I_{50} ~400 μM). In all defining characteristics (microsomal location, NADPH and oxygen dependence, blue light reversal of CO inhibition, inhibition by substituted imidazoles and cytochrome *c*) the taxadiene hydroxylase resembles a typical cytochrome P450 mixed function oxidase [25,26,31–33].

Mechanistic considerations

Based on precedented modes of cytochrome I mediated oxidations of olefinic substrates ([34–41 references therein), we considered two possible m nisms for the oxidation at C5 of taxadiene (compour which involve a formal allylic transposition (Fig. 5). possibility involves preliminary conversion of the double bond of compound **2** to the corresponding epoxide **10**, followed by ring opening and eliminatio proton from the C20 methyl group to yield allylic al 5α-**3**. However, no evidence for such a free ep intermediate has been obtained by radio-chromatogr or GC–MS analysis of the enzymatic reaction proc Alternatively, cytochrome P450-mediated abstractio hydrogen atom from the C20 methyl group o substrate would yield allylic radical **11**, which could undergo oxidation to compound 5α-**3**.

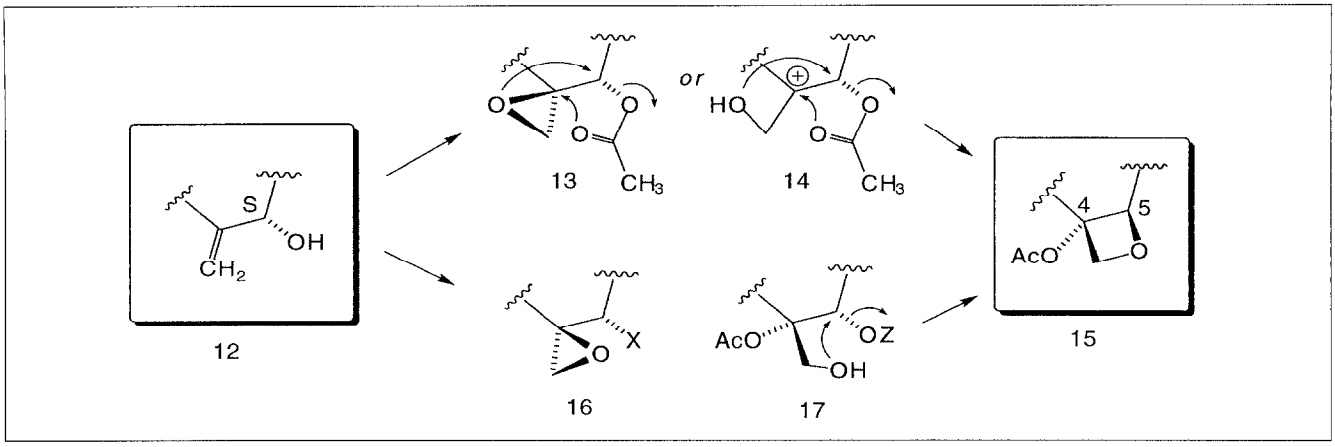
We reasoned that these two mechanistic possibi might be distinguishable using [20-$^2H_3$]taxadiene substrate, since epoxide ring-opening should not inv rate-limiting C20–H bond-breaking step, whereas allylic radical pathway might involve a sufficiently C20–H bond-breaking step to be detectable by the i ence of the primary deuterium kinetic isotope effe the overall reaction rate. To examine this possibilit synthesized [20-$^2H_3$]taxa-4(5),11(12)-diene (see Su mentary material) and tested it as a substrate with microsomes, using parallel incubations with unlal compound **2** as a control. Product quantification by using abietadienol as an internal standard, unexpec revealed that the deuterated substrate yielded sli more taxa-4(20),11(12)-dien-5α-ol than did the unlal substrate. This result indicates that, within the lim experimental error, there was no observable deute kinetic isotope effect on the overall reaction Although this lack of rate suppression might argue in of the epoxidation/ring-opening mechanism, it is possible that the rate of substrate binding or pr

Figure 5



Two possible mechanisms for the taxadiene-5-hydroxylase-catalyzed conversion of taxadiene **2** to taxadienol 5α-**3** based on precedented P450-mediated olefin oxidations involving an allylic transposition. The first mechanism proceeds by preliminary epoxidation of the 4(5) double bond (to form compound **10**) followed by ring opening and deprotonation at C20; the second exploits hydrogen abstraction C20, rearrangement of the resulting allylic radical (compound **11** oxygen rebound at C5.

Figure 6



Possible mechanisms for the construction of the oxetane ring (compound **15**) from the 4(20)-ene-5α-hydroxy functional grouping (compound **12**). Oxidation of the the 4(20) double bond forms an epoxide ring that may be acetylated to form compound **13**. Intramolecular acetate migration and epoxide-ring opening could then furnish the oxetane moiety. Alternatively, the epoxide structure in compound **16** may be subjected to intermolecular acetate opening that could promote oxetane ring closure via compound **17**.

release by the cytochrome, or that of some other step of the reaction cycle, may be slow enough to mask a kinetic isotope effect on the hydrogen abstraction; there is precedent for such masking in cytochrome P450-catalyzed oxidations [38]. We are currently working on preparing the CHD_2-analog of the substrate, to examine the intramolecular isotope effect on deprotonation at C20, and synthesizing epoxide **10** to determine the metabolic relevance of this hypothetical intermediate.

The observation that the taxadiene-5-hydroxylase affords exclusively 5α(*S*)-stereochemistry is consistent with the natural occurrence of a large number of taxoids bearing this configuration [22], and suggests several reasonable mechanisms for the elaboration of the oxetane (D) ring of taxol and related metabolites [20,21] (Fig. 6). Oxidation of the 4(20) double bond of compound **12** to the corresponding epoxide followed by acylation would provide compound **13**. Intramolecular acetate migration concomitant with epoxide ring-opening or a closely related step-wise migration (as in compound **14**) could then furnish the oxetane moiety (compound **15**). Alternatively, conversion of the 4(20) double bond to an activated epoxide (compound **16**) followed by intermolecular acetate opening of the epoxide could promote oxetane ring closure via compound **17**. Efforts are currently under way to exploit double-labeling experiments to examine the timing and mechanism of elaboration of the D ring.

Significance

Chemists and biologists alike have been drawn to the study of taxol because of its spectrum of antineoplastic activities [2,3], its unique mechanism of action in arresting the cell cycle by stabilization of microtubule dynamics [42,43], and the synthetic and biosynthetic challenges posed by the complex and densely functionalized diterpenoid ring system. The limited supply of taxol has hindered the development and use of this natural product as a chemotherapeutic agent since its discovery over 25 years ago in the bark of the Pacific yew [1]. Intensive efforts have thus been made to develop alternative means for the commercial production of this drug. To date, totally synthetic methods have not proven successful as a means of commercial production due to the highly complex nature of the target, which mandates lengthy, expensive synthetic routes with low overall yields [44]. As an interim measure, semi-synthetic approaches have been devised using advanced taxoid metabolites, such as 10-deacetylbaccatin III, which can be obtained from renewable sources, such as needles of the European yew (*T. baccata*) [45]. From the pharmaceutical manufacturing point of view, efficient cell culture production methods would be most desirable. Taxol is produced in *Taxus* cell cultures [46], but production yields are such that this approach is not yet commercially viable.

Improvement of biologically-based processes should be based on a thorough understanding of the target pathway and its rate limiting steps. To this end, we have undertaken a systematic, stepwise approach to deciphering the biosynthesis of taxol and identifying the slow steps in this complex, extended reaction sequence. The first dedicated step of the pathway, at which primary metabolism is diverted to the production of taxol, is the cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene, establishing the taxane skeleton for subsequent oxidative elaboration [15,16]. This taxadiene

synthase-catalyzed step is very slow and is thus an important target for manipulation; the cDNA encoding this synthase has recently been isolated [17].

Here, we have focused on the first oxygenation of taxadiene, a step which studies *in vivo* indicate may also limit pathway flux. The identification of taxa-4(20),11(12)-dien-5α-ol as the second specific intermediate *en route* to taxol has important mechanistic implications for the hydroxylation reaction, which proceeds with a formal allylic rearrangement, and it also constrains the possible means for construction of the oxetane ring of taxol. Identification of the responsible enzyme as a P450 cytochrome, and the development of a specific and sensitive assay, permit a homology-based approach to cloning the corresponding gene. *Agrobacterium tumefaciens*-mediated transformation of *Taxus* species has been described, and the resulting callus cells were shown to produce taxol [47]. It should soon be possible to engineer cells that overexpress the enzymes catalyzing the first two, very slow steps of the pathway to promote improved production yields in these systems.

Materials and methods

Experimental materials

The sources of *T. brevifolia* saplings (2–4 years old) and *T. cuspidata* suspension-cultured cells (14 days post-transfer) have been described [15,48], as have the preparations of [1-^{3}H]geranylgeranyl diphosphate at 90 Ci mol^{-1} [49] and [20-$^2H_3$]geranylgeranyl diphosphate at >95 atom % ^{2}H [18], and the source of abietadienol [50] and of the taxoid standards 10-deacetylbaccatin III, cephalomannine and taxol [15]. The total synthesis of taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene has been described [27]. Modification of this strategy afforded [20-$^3H_3$]-labeled (16 Ci mol^{-1}) and [20-$^2H_3$]-labeled (99 atom % ^{2}H) taxa-4(5),11(12)-diene (see Supplementary material).

Syntheses

*Taxa-4(20),11(12)-dien-5α-ol (compound 5α-**3**)*

To a 0.04 M solution of SeO_2 (7 mg, 0.07 mmol) in CH_2Cl_2 was added *t*BuOOH (26 ml, 0.26 mmol) and the resulting mixture was allowed to stir for 0.5 h. To this solution was added a 0.07 M solution of (+)-taxa-4(20),11(12)-diene (compound **8**) (36 mg, 0.13 mmol) in CH_2Cl_2 and the mixture was allowed to stir for 8 h. The reaction mixture was concentrated and the crude oil was purified by preparative TLC (silica gel, hexanes:ethyl acetate, 4:1 v/v) to give 14 mg (39%) (R_f = 0.75, hexanes:ethyl acetate, 2:1 v/v) of product (compound 5α-**3**) as a colorless oil. IR (neat) 3397, 2925, 1448 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.91 (dd, J = 1.4, 1.4 Hz, 1H), 4.62 (ddd, J = 1.7, 1.1, 0.5 Hz, 1H), 4.23 (dd, J = 2.7, 2.7 Hz, 1H), 3.31–3.28 (m, 1H), 2.82 (dt, J = 13.5, 5.3 Hz, 1H), 2.36–2.28 (m, 1H), 2.24 (ddd, J = 13.1, 13.1, 6.8 Hz, 1H), 2.1–1.93 (m, 3H), 1.87 (ddd, J = 18.6, 10.6, 3.1 Hz, 1H), 1.81 (dd, J = 1.1, 1.1 Hz, 3H), 1.78-1.68 (m, 3H), 1.62 (ddd, J = 15.5, 5.9, 2.4 Hz, 1H), 1.54 (ddd, J = 15.3, 5.3, 2.2 Hz, 1H), 1.32 (s, 3H), 1.28-1.18 (m, 2H), 1.17 (s, OH, 1H), 1.02 (s, 3H), 0.97 (ddd, J = 13.3, 4.3, 2.9 Hz, 1H), 0.59 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 155.9, 136.8, 130.5, 108.7, 74.6, 43.5, 40.0, 39.9, 39.2, 35.4, 32.7, 30.8, 30.16, 30.13, 28.0, 25.4, 24.7, 22.9, 22.1, 21.3. High-resolution mass spectroscopy (HRMS) calc'd for $C_{20}H_{32}O$: 288.2453, found: 288.2448. The synthesis of [20-$^3H_2$]taxa-4(20),11(12)-dien-5α-ol (4.0 Ci mol^{-1}) is described in the Supplementary material.

Taxa-4(20),11(12)-dien-5α-3,5-dinitrobenzoate (c

To a 0.03 M solution of taxa-4(20),11(12)-dien-5-α-c 5α-**3**) (20 mg, 0.07mmol) in CH_2Cl_2 was added pyridin mmol) and the resulting mixture was cooled to 0 °C. T was added 3,5-dinitrobenzoyl chloride (74 mg, 0.32 r mixture was allowed to stir for 4 h as it warmed to 25 °C mixture was quenched with saturated aqueous NaHC extracted with ether (3 x 10 ml). The organic layer was with brine (20 ml), dried over Na_2SO_4, and concentrate which was purified by preparative TLC (silica gel, acetate, 4:1 v/v) to give 26 mg (79 %)(R_f= 0.28, acetate, 4:1 v/v) of product (compound **9**) as a yel analytical sample was purified by recrystallization (m.p. 1 IR(neat) 3098, 2923, 1723, 1626, 1544, 1451, 1344 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.22 (t, J = 2.1 Hz, = 2.1 Hz, 2H), 5.58 (t, J = 3 Hz, 1H), 5.28 (s, 1H), 4.98 (J = 4.2 Hz, 1H), 2.77 (apparent dt, J = 7.2, 14.7 Hz, 1H 7H), 1.85–1.45 (m, 4H), 1.7 (s, 3H), 1.4–1.1 (m, 3H), 1.3 (s, 3H), 0.7 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 1 148.6, 137.7, 134.7, 129.4, 129.1, 122.3, 114.5, 80.8 39.7, 39.1, 37.9, 33.6, 30.6, 30.2, 28.3, 27.9, 25.4, 24. 21.5. HRMS calc'd for $C_{27}H_{34}N_2O_6$: 482.2417, found: 4

For use with the labeled biosynthetic sample, the modified to the 0.5 μmol scale (42.8 nCi of product taxadienol as carrier) with the reagent in ten-fold exc above, gave the product in 90 % isolated yield. The diluted with additional carrier (7.6 μmol of the dinitrob repeatedly crystallized from 30 % aqueous methanol.

5-Keto-taxa-4(20),11(12)-diene

To a 0.01 M solution of taxa-4(20),11(12)-dien-5α-ol (cor (6 mg, 0.02 mmol) in CH_2Cl_2 was added Dess–Martin reagent (29 mg, 0.07 mmol); the resulting mixture was for 2 h. $Na_2S_2O_3$•$5H_2O$ (15 mg, 0.06 mmol) was ad solution of $NaHCO_3$ (4 mg, 0.046 mmol); this solution, ml), was then added to the reaction mixture which was for 30 min. Saturated aqueous $NaHCO_3$ solution (10 m and the mixture was extracted with ether (3 x 10 ml). The was washed twice with saturated aqueous $NaHCO_3$ sol dried over anhydrous Na_2SO_4, and concentrated. The purified by preparative TLC (silica gel, hexanes:ethyl ac to give 4 mg (67 %) (R_f = 0.57; hexanes:ethyl acetate, product ketone as a colorless oil. IR (neat) 2928, 1697, 1 NMR ($CDCl_3$, 300 MHz) δ 5.81 (d, J = 2.5 Hz, 1H), 5. Hz, 1H), 2.87 (sextet, J = 2.5 Hz, 1H), 2.75 (apparent dq, Hz, 1H), 2.51–2.44 (m, 2H), 2.35–1.95 (m, 5H), 1.85–1. (s, 3H), 1.48–1.37 (m, 2H), 1.35 (s, 3H), 1.28–1.15 (m, 3H), 0.7 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 203.9, 129.8, 116.6, 43.2, 40.4, 39.6, 39.3, 37.9, 36.5, 33.9 27.9, 25.5, 24.5, 23.5, 22.4, 21.5. HRMS calc'd f 286.2375, found: 286.2378.

*Taxa-4(20),11(12)-dien-5β-ol (compound 5β-**3**)*

To a 0.01 M solution of 5-keto-taxa-4(20),11(12)-dien described above (4 mg, 0.014 mmol) in tetrahydrofura added $LiAlH_4$ (0.6 mg, 0.014 mmol) and the resulting allowed to stir for 15 min. The reaction mixture was q H_2O (10 ml) and the aqueous layer was extracte (2 x 10 ml). The organic layer was dried over Na_2SO_4 ar to give 4 mg (100%) of the product (compound 5β- colorless oil. An analytical sample was purified by pre (silica gel, hexanes:ethyl acetate, 4.1 v/v; R_f = 0.25). IR 2927, 2858, 1646, 1452 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 M 1H), 4.68 (s, 1H), 3.92–3.8 (m, 1H), 2.83–2.69 (m, 1H 1H), 2.4–2.2 (m, 1H), 2.15–1.82 (m, 5H), 1.82–1.5 (m 3H), 1.5–1.1 (m, 4H), 1.3 (s, 3H), 1.0 (s, 3H), 0.6 (s, 3 ($CDCl_3$, 75 MHz) δ 155.7, 137.7, 129.6, 102.2, 74.2, 43.

39.5, 39.3 35.5, 33.3, 30.8, 30.1, 29.3, 25.4, 24.7, 22.9, 22.7, 21.9. HRMS calc'd for $C_{20}H_{32}O$: 288.2453, found: 288.2449.

Enzyme preparation and assay

Sapling stem sections (60 g) were frozen in liquid N_2, the needles removed, and the bark and cambium pulverized in a hammer mill [49,51]. The resulting frozen powder was added to HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.6, 5 ml g^{-1} fresh tissue wt) containing 1 mM $MgCl_2$, 2 mM EGTA, 1 mM 4'-bromoacetophenone, 10 mM procaine-HCl, 10 mM lidocaine-HCl, 5 mM sodium ascorbate, 5 mM $Na_2S_2O_5$, 5 mM dithiothreitol, 0.1 mM PMSF, 1 μg leupeptin ml^{-1}, 15 % (v/v) glycerol, 1 % (w/v) polyvinylpyrrolidone (relative molecular mass 40,000), 1 % (w/v) polyvinylpolypyrrolidone, and 10 % (w/v) Amberlite XAD-4 polystyrene resin. The mixture was stirred at 4 °C for 30 min then filtered through eight layers of cheesecloth. The filtrate was centrifuged at 3,000g for 20 min to remove cellular debris then again at 20,000g for 20 min to remove dense membranes. This supernatant was then centrifuged at 190,000g for 90 min to obtain the microsomal pellet and the soluble enzyme fraction. The microsomes were resuspended with the aid of a Ten–Broeck homogenizer in 3 ml of assay buffer consisting of 25 mM HEPES (pH 7.2), 1 mM EGTA, 10 mM $MgCl_2$, 25 mM KCl, 5 mM dithiothreitol (not included in cytochrome *c* inhibition experiments), 1 μg leupeptin ml^{-1}, and 20 % (v/v) glycerol. Microsome suspensions were aliquoted, frozen in liquid nitrogen and stored at −80 °C for later use; these preparations were stable for at least two weeks. Microsomes from *T. cuspidata* suspension-cultured cells were prepared as above, except that the filtered, frozen cells were homogenized using a chilled mortar and pestle instead of the hammer mill.

Assays were performed in 10 ml screw-capped culture tubes containing 500 μg of microsomal protein, 2.5 nmol each of FMN and FAD, 1 μmol NADPH, 5 μmol glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase in a total volume of 1 ml of the assay buffer described above. The reactions were initiated by the addition in 5 μl hexane of (±)-[20-$^3H_3$]taxa-4(5),11(12)-diene (26 nmol, saturation) or [2-^{3}H]taxa-4(5),11(12)-diene (typically, 1.8 nmol). The latter substrate was prepared biosynthetically from [1-^{3}H]geranylgeranyl diphosphate using taxadiene synthase [16,18], and was used in preliminary studies before the synthetic substrate was available. Following incubation for 70 min in the dark at 31 °C with gentle shaking, the reaction mixture was chilled on ice, saturated with NaCl, extracted with 3 x 1 ml of pentane:ether (9:1 v/v) and the extract passed over a short column of $MgSO_4$ followed by an additional 3 ml wash. The eluate was evaporated to dryness, and the residue was redissolved in 3 ml of pentane that was passed over a 5 mm x 40 mm column of silica gel (Mallinckrodt SilicAR 60A) and washed with 4 ml of pentane to provide the olefin fraction containing residual substrate. The silica gel column was next eluted with pentane:ether (9:1 v/v) to provide the diterpenol fraction, and was finally eluted with ether (this latter fraction contained low, variable amounts of a label product(s) with chromatographic properties consistent with a diterpene diol). An aliquot of the diterpenol fraction was evaluated by liquid scintillation counting to determine conversion rate and, when appropriate, the product was separated by TLC.

A low, but significant, level of background activity was observed in both boiled controls and in controls lacking NADPH. Controls were included in all experiments and the appropriate background corrections made. All assays were conducted under linear conditions with respect to protein concentration and time. Activity levels measured for taxadiene-5-hydroxylase ranged from 0.14 to 1.7 nmol product per hour per mg protein. Microsomes from stems and cultured cells could be used interchangeably; however, the suspension cultures usually provided the higher activity levels per gram of tissue.

Enzyme characterization

To examine the oxygen requirement for hydroxylase activity, an oxygen scavenging system (consisting of 10 units glucose oxidase, 6 μmol β-D-glucose and 1,300 units catalase [52]) was included in the assay, and the reaction mixture was flushed with Ar through the septum cap for 2 min prior to substrate addition. To test inhibition by substituted imidazoles, these compounds were dissolved in dimethylsulfoxide and added (in 5 μl) to the assays 5 min prior to substrate addition (dimethylsulfoxide alone had no effect on taxadiene hydroxylase activity). To evaluate CO inhibition and blue-light reversal, the reaction mixtures were flushed for 2 min with gas mixtures consisting of 10 % O_2 and 90 % CO, or 10 % O_2, 10 % CO and 80 % N_2, prior to substrate addition. Reactions were incubated in the dark (masked) or were irradiated with blue light (maximum intensity near 450 nm) generated by filtering (0.2 mm filter sheet, Edmund Scientific, Co.) the beam from a 100 W mercury spotlight. All assays were performed in duplicate and typically repeated three times. The data presented are averages with standard error within 10 %.

In vivo *incorporation experiments*

In vivo incorporation experiments with *T. brevifolia* sapling stem discs were carried out exactly as described previously [15], except that the 1.5 ml of incubation buffer for each of the 2, 4 and 6-day time points contained 0.22 mM (0.33 μmol, 1.33 μCi) of (±)-[20-^{3}H]taxa-4(20),11(12)-dien-5α-ol. The labeled taxoids produced by the tissue were recovered as before; the 10-deacetylbaccatin III, cephalomannine and taxol were isolated by preparative TLC, and each was diluted with 200 mg of authentic carrier and crystallized from aqueous methanol to constant specific activity and melting point as previously described [15]. For 2, 4 and 6-day time points, respectively, repeated crystallization gave 10-deacetylbaccatin III (34.7, 59.6 and 111.8 nCi $mmol^{-1}$, m.p. 242–245 °C), cephalomannine (8.8, 15.4 and 19.3 nCi $mmol^{-1}$, m.p. 184–186 °C dec.) and taxol (15.9, 33.0 and 43.8 nCi $mmol^{-1}$, m.p. 198–203 °C dec.). All melting point values (Fisher-Johns) are in agreement with the literature [22].

Analysis of bark extracts

Initial processing of the extract from 750 kg of *T. brevifolia* bark powder has been described, as have the general protocols for radiochemically-guided fractionation of this material [15]. Following isolation of the hydrocarbon fraction by hexane elution through silica gel, two aliquots of the ether eluate (equivalent to 250 kg of starting material) were diluted with 0.25 μCi of [20-$^3H_2$]taxa-4(20),11(12)-dien-5α-ol, one of which was then hydrolyzed with excess 0.1 N KOH to liberate alcohols which were recovered by ether extraction. Subsequent fractionation involved silica gel column chromatography (0–35 % gradient of ether in hexane), argentation column chromatography (10 % $AgNO_3$-silica gel with a 0–50 % gradient of ether in hexane) and reversed-phase column chromatography on Davisil (Alltech C_{18} on silica, 0–20 % CCl_4 in acetonitrile) followed by TLC (silica gel with hexane:ether, 3:1 (v/v)) to provide a fraction ($R_f \approx 0.5$) suitable for GC-MS analysis.

Analytical procedures

GC–MS analyses were performed on a Hewlett–Packard 6890 GC–MSD system using a bonded, FSOT column (0.25 mm inner diameter x 30 m) coated with a 0.2 μm film of AT1000 (Alltech Associates). Cool, on-column injection was used with a He flow rate of 0.7 ml min^{-1} and a temperature program from 50 °C (2 min hold) to 230 °C at 40 °C min^{-1}. Spectra (at 70 eV) were recorded in the total ion or selected ion monitoring (SIM) mode. Procedures for NMR spectrometry are described in the Supplementary material.

Analytical TLC was performed on 0.2 mm silica gel sheets (Chromatogram Kodak) using pentane: ether (3:1, v/v) as developing solvent, with visualization using I_2 vapor. Preparative TLC was performed in 1 mm plates (silica gel G or silica gel G containing 8 % $AgNO_3$) using pentane:ether (3:1, v/v for silica and 3:2, v/v for $AgNO_3$ argentation TLC), or the solvents indicated elsewhere in the text, with visualization using 0.2 % 2,7-dichlorofluorescein in ethanol and viewing under UV light. Liquid scintillation counting was performed in 10 ml of

cocktail consisting of 0.4 % Omnifluor (DuPont/New England Nuclear) in ethanol:toluene (3:7, v/v). Protein concentration was determined by the method of Bradford [53] using bovine serum albumin as a standard and the Bio-Rad protein assay reagent.

Supplementary material available

Structural assignments for taxa-4(20),11(12)-dien-5α-ol (compound 5α-**3**), taxa-4(20),11(12)-dien-5β-ol (compound 5β-**3**) and taxa-4(20),11(12)-diene (compound **8**) using ^{1}H NMR, NOESY, DQF-COSY, HMQC spectroscopy and HMBC spectroscopy. Details of synthesis of [20-$^3H_2$]taxa-4(20),11(12)-dien-5α-ol, [20-$^3H_3$]taxa-4(5),11(12)-diene, [20-$^3H_2$]taxa-4(20),11(12)-diene, [20-$^2H_3$]taxa-4(5),11(12)-diene and [20-$^2H_2$]taxa-4(20),11(12)-diene.

Acknowledgements

This work was supported by grants from the National Institutes of Health (CA-55254 to R.C., CA55138 to D.M.G., and CA43969 to R.M.W.), by McIntire-Stennis Project 0967 from the Agricultural Research Center, Washington State University (to R.C.), and by the Colorado State University Agricultural Experiment Station (USDA SAES Western Project W-122 to R.M.W.). Acknowledgment is made to the donors of the Petroleum Research Fund administered by the American Chemical Society (ACS-PRF No. 288221-AC to R.M.W.). We thank Al Koepp and David Williams for taxoid isolation and MS analyses, Mehri Hezari for conducting the *in vivo* experiment and Chris Rithner (Colorado State University) for NMR analyses.

References

1. Wani, M.C., Taylor, H.L., Wall, M E., Coggon, P. & McPhail, A.T. (1971). Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J. Am. Chem. Soc.* **93**, 2325–2327.
2. Holmes, F.A., Kudelka, A.P., Kavanagh, J.J., Huber, M.H., Ajani, J.A. & Valero, V. (1995). Current status of clinical trials with paclitaxel and docetaxel. In *Taxane Anticancer Agents: Basic Science and Current Status* (Georg, G. I., Chen, T. T., Ojima, I. & Vyas, D. M., eds.), pp. 31–57, American Chemical Society, Washington, DC.
3. Arbuck, S.G. & Blaylock, B.A. (1995). Taxol: clinical results and current issues in development. In *Taxol: Science and Applications* (Suffness, M., ed.), pp. 379–415, CRC Press, Boca Raton, FL.
4. Kingston, D.G.I. (1991). The chemistry of taxol. *Pharmacol. Therapeut.* **52**, 1–34.
5. Cragg, G.M., Schepartz, S.A., Suffness, M. & Grever, M. R. (1993). The taxol supply crisis. New NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents. *J. Nat. Prod.* **56**, 1657–1668.
6. Guénard, D., Guéritte-Voegelein, F. & Potier, P. (1993). Taxol and taxotere: discovery, chemistry and structure–activity relationships. *Accounts Chem. Res.* **26**, 160–167.
7. Holton, R.A., Biediger, R.J. & Boatman, P.D. (1995). Semisynthesis of taxol and taxotere. In *Taxol: Science and Applications* (Suffness, M., ed.), pp. 97–121, CRC Press, Boca Raton, FL.
8. Commerçon, A., Bourzat, J.D., Didier, E. & Lavelle, F. (1995). Practical semisynthesis and antimitotic activity of docetaxel and side-chain analogs. In *Taxane Anticancer Agents: Basic Science and Current Status* (Georg, G. I., Chen, T. T., Ojima, I. & Vyas, D. M., eds.), pp. 233–246, American Chemical Society, Washington, DC.
9. Nicolaou, K.C., *et al.*, & Sorensen, E.J. (1994). Total synthesis of taxol. *Nature* **367**, 630–634.
10. Holton, R.A., *et al.*, & Liu, J.H. (1994). First total synthesis of taxol. 2. Completion of the C and D rings. *J. Am. Chem. Soc.* **116**, 1599–1600.
11. Nicolaou, K.C., *et al.*, & Chadha, R. (1995) Total synthesis of taxol. 4. The final stages and completion of the synthesis. *J. Am.Chem.Soc.* **117**, 653–659.
12. Masters, J.J., Link, J.T., Snyder, L.B., Young, W.B. and Danishefsky, S.J. (1995). A total synthesis of taxol. *Angew. Chem. Int. Ed. Engl.* **34**, 1723–1726.
13. Suffness, M. (1995). Overview of paclitaxel research: progress on many fronts. In *Taxane Anticancer Agents: Basic Science and Current Status* (Georg, G. I., Chen, T. T., Ojima, I. & Vyas, D. M., eds.), pp. 1–17, American Chemical Society, Washington, DC.
14. West, C.A. (1981). Biosynthesis of diterpenes. In *Biosynthesis of Isoprenoid Compounds* (Porter, J.W. & Spurgeon, S.L., eds.), Vol. 1, pp. 375–411, Wiley, New York.
15. Koepp, A.E., *et al.*, & Croteau, R. (1995). Cyclization of ge diphosphate to taxa-4(5),11(12)-diene is the first committe taxol biosynthesis in Pacific yew. *J. Biol. Chem.* **270**, 868
16. Hezari, M., Lewis, N.G. & Croteau, R. (1995). Purification a characterization of taxa-4(5),11(12)-diene synthase from P (*Taxus brevifolia*) that catalyzes the first committed step of biosynthesis. *Arch. Biochem. Biophys.* **322**, 437–444.
17. Wildung, M.R. & Croteau, R. (1996). A cDNA clone for tax synthase, the diterpene cyclase that catalyzes the committ taxol biosynthesis. *J. Biol. Chem.* **271**, 9201–9204.
18. Lin, X., Hezari, M., Koepp, A.E., Floss, H.G. & Croteau, R. (Mechanism of taxadiene synthase, a diterpene cyclase tha the first step of taxol biosynthesis in Pacific yew. *Biochem* 2968–2977.
19. Harrison, J.W., Scrowston, R.M. & Lythgoe, B. (1966). Taxi The constitution of taxine-I. *J. Chem. Soc. C*, 1933–1945.
20. Guéritte-Voegelein, Guénard, D., & Potier, P. (1987). Taxol derivatives. A biogenetic hypothesis. *J. Nat. Prod.* **50**, 9–1
21. Floss, H.G. & Mocek, U. (1995). Biosynthesis of taxol. In *T Science and Applications* (Suffness, M., ed.), pp. 191–20 Press, Boca Raton, FL.
22. Kingston, D.G.I., Molinero, A.A. & Rimoldi, J.M. (1993). The diterpenoids. *Prog. Chem. Org. Nat. Prods.* **61**, 1–206.
23. Croteau, R., Hefner, J., Hezari, M. & Lewis, N.G. (1995). Ta biosynthesis: cyclization and early hydroxylation steps of th *Curr. Top. Plant Physiol.* **15**, 94–104.
24. West, C.A. (1980). Hydroxylases, monooxygenases and cy P-450. In *The Biochemistry of Plants* (Davies, D.D., ed.), V 317–364, Academic Press, New York.
25. Mihaliak, C., Karp, F. & Croteau, R. (1993). Cytochrome P- terpene hydroxylases. *Methods Plant Biochem.* **9**, 261–27
26. Bolwell, G.P., Bozak, K. & Zimmerlin, A. (1994). Plant cyto P450. *Phytochemistry* **37**, 1491–1506.
27. Rubenstein, S.M. & Williams, R.M. (1995). Studies on the of taxol: total synthesis of taxa-4(20),11(12)-diene and tax 4(5),11(12)-diene. The first committed biosynthetic interm *Org. Chem.* **60**, 7215–7223.
28. Hasson, E.P. & West, C.A. (1976). Properties of the systen mixed function oxidation of kaurene and kaurene derivative microsomes of the immature seed of *Marah macrocarpus*. *Physiol.* **58**, 473–478.
29. Aoyama, Y., Yoshida, Y., Kubota, S., Kumaoka, H. & Furumi (1978). NADPH-cytochrome P-450 reductase of yeast mic *Arch. Biochem. Biophys.* **185**, 362–369.
30. Madyastha, K.M. & Coscia, C.J. (1979). Detergent-solubiliz NADPH-cytochrome c (P-450) reductase from the higher *Catharanthus roseus*. *J. Biol. Chem.* **254**, 2419–2427.
31. Testa, B. & Jenner, P. (1981). Inhibitors of cytochrome P-4 their mechanism of action. *Drug Metab. Rev.* **12**, 1–117.
32. O'Keefe, D.P. (1991). Biochemical and biophysical charac plant cytochrome P450s. *Curr. Top. Plant Physiol.* **6**, 204-
33. Correia, M.A. & Ortiz de Montellano, P.R. (1993). Inhibition cytochrome P450 and possibilities for their therapeutic ap In *Frontiers in Biotransformation* (Ruckpaul, K., ed.), pp. 7 Akademie-Verlag, Berlin.
34. Tanaka, K., Kurihara, N. & Nakajima, M. (1979). Oxidative m of tetrachlorocyclohexenes, pentachlorocyclohexenes, and hexachlorocyclohexenes with microsomes from rat liver an abdomen. *Pest. Biochem. Physiol.* **10**, 79–95.
35. Groves, J.T. & Subramanian, D.V. (1984). Hydroxylation by P-450 and metalloporphyrin models: evidence for allylic rearrangement. *J. Am. Chem. Soc.* **106**, 2177–2181.
36. McClanahan, R.H., Huitric, A.C., Pearson, P.G., Desper, J.C S.D. (1988). Evidence for a cytochrome P450 catalyzed al rearrangement with double bond topomerization. *J. Am. C* **110**, 1979–1981.
37. Oliw, E.H., Brodowsky, I.D., Hörnsten, L. & Hamberg, M. (1 allylic hydroxylation of polyunsaturated fatty acids by hepat monooxygenases and its relation to the enzymatic and non formation of conjugated hydroxy fatty acids. *Arch. Biochen* **300**, 434–439.
38. Ortiz de Montellano, P.R. (1995). Oxygen activation and re *Cytochrome P450: Structure, Mechanism, and Biochemis* edn), (Ortiz de Montellano, P.R., ed.), pp. 245–303, Plenur York, NY.
39. Kadkhodayan, S., Coulter, E.D., Maryniak, D.M., Bryson, T.A

Dawson, J.H. (1995). Uncoupling oxygen transfer and electron transfer in the oxygenation of camphor analogues by cytochrome P450-CAM. *J. Biol. Chem.* **270**, 28042–28048,
40. Groves, J.T. (1985). Key elements of the chemistry of cytochrome P-450. *J. Chem. Ed.* **62**, 928–931.
41. Atkins, W.M. & Sligar, S.G. (1987). Metabolic switching in cytochrome $P\text{-}450_{CAM}$: deuterium isotope effects on regiospecificity and the monooxygenase/oxidase ratio. *J. Am. Chem. Soc.* **109**, 3754–3760.
42. Horwitz, S.B. (1992) Mechanism of action of taxol. *Trends Pharmacol. Sci.* **13**, 134–136.
43. Jordon, M.A. & Wilson, L. (1995). Microtubule polymerization dynamics, mitotic block, and cell death by paclitaxel at low concentration. In *Taxane Anticancer Agents: Basic Science and Current Status* (Georg, G.I., Chen, T.T., Ojima, I. & Vyas, D.M., eds.), pp. 138–153, American Chemical Society, Washington, DC.
44. Borman, S. (1994). Total synthesis of anticancer agent taxol achieved by two different routes. *Chem. Eng. News* **72**, 32–34.
45. Nicolaou, K.C., Dai, W.-M. & Guy, R.K. (1994). Chemistry and biology of taxol. *Angew. Chem. Int. Ed. Engl.* **33**, 15–44.
46. Gibson, D.M., Ketchum, R.E.M., Hirasuna, T.J. & Shuler, M.L. (1995). Potential of plant cell culture for taxane production. In *Taxol: Science and Applications* (Suffness, M., ed.), pp. 71–95, CRC Press, Boca Raton, FL.
47. Han, K.-H., *et al.*, & Floss, H.G. (1994). Genetic transformation of mature *Taxus*: an approach to genetically control the *in vitro* production of the anticancer drug, taxol. *Plant Sci.* **95**, 187–196.
48. Ketchum, R.E.B. & Gibson, D.M. (1995). A novel method of isolating taxanes from cell suspension cultures of yew (*Taxus* spp.) *J. Liquid Chromatogr.* **18**, 1093–1111.
49. LaFever, R.E., Stofer-Vogel, B. & Croteau, R. (1994). Diterpenoid resin acid biosynthesis in conifers: enzymatic cyclization of geranylgeranyl pyrophosphate to abietadiene, the precursor of abietic acid. *Arch. Biochem. Biophys.* **313**, 139–149.
50. Funk, C. & Croteau, R. (1994). Diterpenoid resin acid biosynthesis in conifers: characterization of two cytochrome P450-dependent monooxygenases and an aldehyde dehydrogenase involved in abietic acid biosynthesis. *Arch. Biochem. Biophys.* **308**, 258–266.
51. Hefner, J. & Croteau, R. (1996). Microsome preparation from woody plant tissues. *Methods Enzymol* **272**, 243–250.
52. Karp, F., Harris, J.L. & Croteau, R. (1987). Metabolism of monoterpenes: demonstration of the hydroxylation of (+)-sabinene to (+)-*cis*-sabinol by an enzyme preparation from sage (*Salvia officinalis*) leaves. *Arch. Biochem. Biophys.* **256**, 179–193.
53. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.