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Cytochrome P450 Taxadiene 5α-Hydroxylase, a Mechanistically Unusual Monooxygenase Catalyzing the First Oxygenation Step of Taxol Biosynthesis

Stefan Jennewein,[1] Robert M. Long,[1] Robert M. Williams,[2,*] and Rodney Croteau[1,*]
[1]Institute of Biological Chemistry
Washington State University
Pullman, Washington 99164-6340
[2]Department of Chemistry
Colorado State University
Fort Collins, Colorado 80523

Summary

The first oxygenation step in the biosynthesis of the anticancer drug taxol in *Taxus* species is the cytochrome P450-mediated hydroxylation (with double bond migration) of the diterpene olefin precursor taxa-4(5),11(12)-diene to taxa-4(20),11(12)-dien-5α-ol. A homology-based cloning strategy, employing an induced *Taxus* cell library, yielded a cDNA encoding taxadiene 5α-hydroxylase, which was functionally expressed in yeast and insect cells. The recombinant enzyme was characterized and shown to efficiently utilize both taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene (as an adventitious substrate) to synthesize taxa-4(20),11(12)-dien-5α-ol. This hydroxylase resembles, in sequence and properties, other cytochrome P450 oxygenases of taxol biosynthesis. The utilization of both taxadiene isomers in the formation of taxa-4(20),11(12)-dien-5α-ol is novel, suggesting a reaction mechanism involving promiscuous radical abstraction with selective oxygen insertion rather than epoxidation of the C4,C5-alkene of the natural substrate and allylic rearrangement of the resulting taxa-11(12)-en-4,5-epoxide.

Introduction

Taxol [1] (generic name paclitaxel, **1**, Figure 1) is well established as a potent chemotherapeutic agent with excellent activity against a range of cancers [2, 3]. This diterpenoid, derived from yew (*Taxus*) species [4], continues to find wide application both in treatment of additional cancer types and for earlier disease intervention [2, 3, 5]. Several elegant routes have been used to achieve the total syntheses of Taxol (see [6] for recent review), but the yields are too low to be practical. It has been clear for some time [7, 8] that the supply of Taxol and its semisynthetically useful precursors [6] must continue to rely on biological methods of production for the foreseeable future. Improvement of the biosynthetic process in intact yew or derived cell cultures [9–11] should be based upon a full understanding of the pathway of Taxol formation, the enzymes that catalyze this extended sequence of reactions and their mechanisms of action, and the structural genes encoding these enzymes, especially those responsible for the slow steps of the pathway.

*Correspondence: croteau@mail.wsu.edu (R.C.), rmw@chem.colostate.edu (R.M.W.)

Biochemical studies with cell-free enzyme extracts of yew have demonstrated that the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate (**2**) to the committed Taxol precursor taxa-4(5),11(12)-diene (**3**) [12, 13] is followed by cytochrome P450-mediated hydroxylation at C5 of the olefin with double bond rearrangement [14] to yield taxa-4(20),11(12)-dien-5α-ol (**4**) (Figure 1). This unusual hydroxylation reaction at C5 is the first of eight cytochrome P450-catalyzed oxygenations and four CoA-dependent acylations that decorate the taxadiene core en route to Taxol (**1**) [15].

A range of reverse-genetic and homology-based cloning strategies [16, 17] has been employed for isolating cDNAs encoding taxadiene synthase [18], from which the recombinant enzyme [19] was utilized for defining the mechanism of this novel cyclization [20, 21], in addition to a number of regiospecific acyltransferases of the Taxol pathway [16, 17, 22]. In the case of cytochrome P450 oxygenases, differential display of mRNA reverse-transcription PCR served as an alternative to difficult reverse-genetic cloning. Cultured *Taxus* cells induced for Taxol production served as the source [23], and clones were sorted based on sequence relatedness and functional expression [24]. This strategy yielded a family of 16 oxygenases, from which cDNAs encoding the cytochrome P450 taxoid 10β-hydroxylase [24], 13α-hydroxylase [25], and a side-route taxoid 14β-hydroxylase [26] were obtained. Detailed evaluation of this set of heterologously expressed clones failed to yield the taxadiene 5α-hydroxylase. However, because the 5α-hydroxylase is only weakly induced (<2-fold) in methyl jasmonate-treated cell cultures [15], it is possible that the corresponding message was missed by the differential display-based cloning technique [24].

An alternative method for acquiring the taxadiene 5α-hydroxylase gene was a homology-based cloning approach, in which screening probes were generated by PCR with primers directed to regions of very high sequence conservation in cytochrome P450 oxygenases of plant origin. This generic strategy provided suitable probes for screening the induced *Taxus* cell cDNA library, from which the most abundant of the three new clones obtained was expressed in yeast and shown to encode the target 5α-hydroxylase. This cytochrome P450 hydroxylase, which catalyzes the initial oxygenation step of Taxol biosynthesis and resembles other taxoid hydroxylases, was shown to efficiently employ both taxa-4(5),11(12)-diene (with allylic rearrangement) and taxa-4(20),11(12)-diene (directly) in the conversion to taxa-4(20),11(12)-dien-5α-ol. This enzyme's use of the 4(20),11(12)-olefin isomer, although most likely not a physiologically relevant substrate in *Taxus*, has provided mechanistic insight into this unusual cytochrome P450-catalyzed reaction.

Results and Discussion

Homology-Based Cloning of Cytochrome P450 Oxygenases from *Taxus*

For obtaining cytochrome P450 taxoid oxygenase clones that may have been missed by the differential display

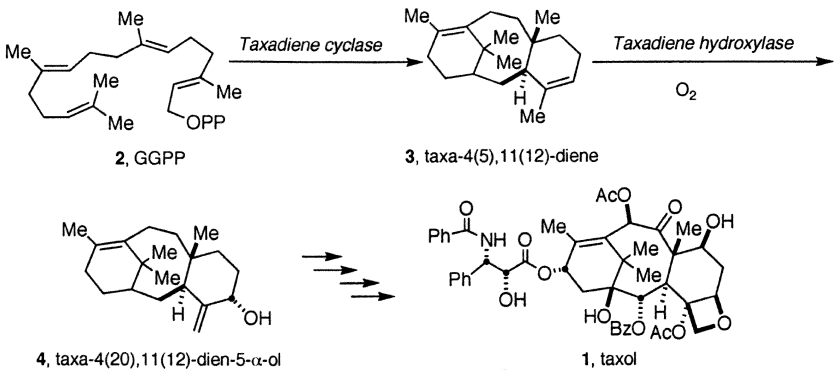

Figure 1. Outline of the Early Steps of Taxol Biosynthesis

Taxol (1) formation involves the cyclization of geranylgeranyl diphosphate (2) to taxa-4(5),11(12)-diene (3) and cytochrome P450-mediated hydroxylation to taxa-4(20),11(12)-dien-5α-ol (4).

method [24] because of their low level of induction [15], a general cloning strategy directed to this gene type was employed based upon the highly conserved PERF motif and the region surrounding the heme binding, invariant cysteine residue of these enzymes [27–29]. PCR amplification, carried out with degenerate primers designed to match these amino acid sequences and the previously described *Taxus* cell cDNA library template, yielded amplicons of the expected length (170 bp), which were then cloned, and sequenced, and shown to resemble other cytochrome P450s. Based on these partial sequences, labeled oligonucleotide probes were synthesized and used to screen the aforementioned *Taxus* cell cDNA library. More than 30 positive plaques were purified by three rounds of hybridization, and after excision, cloning and sequencing led to the identification of several of the cytochrome P450 sequences previously obtained by differential display [24]. However, three new, full length sequences were also acquired, one of which (designated S1) represented the most abundant cytochrome P450 cDNA isolated by this generic homology-based approach.

The 1688 bp S1 clone (GenBank accession number AY289209) contained an apparent 1509 bp ORF encoding a predicted protein of 502 amino acids with a deduced molecular weight of 56,859. This was subsequently shown (see below) to be in agreement with the size of the expressed enzyme observed by SDS-PAGE (~57 kDa). The deduced amino acid sequence of the S1 clone exhibited typical cytochrome P450 characteristics [30], including the oxygen binding domain, an N-terminal membrane anchor, the highly conserved heme binding motif with PFG element (amino acids 437–439), and the absolutely conserved cysteine at position 445. Comparison of the deduced sequence with previously defined cytochrome P450 taxoid hydroxylases, including the taxoid 10β-hydroxylase (AF318211), 13α-hydroxylase (AY056019), and 14β-hydroxylase (AY188177), revealed overall identities in the 61%—63% range and similarities in the 79%—81% range (Figure 2), suggesting that the S1 clone encoded a new taxoid oxygenase.

Functional Expression of Cytochrome P450 Clone S1 in Yeast

To test the function of the encoded enzyme, we exploited the simplicity and reliability of heterologous expression of cDNA clone S1 in *Saccharomyces cerevisiae* strain WAT11, which harbors a galactose-inducible *Arabidopsis thaliana* NADPH-cytochrome P450 reductase that is required for efficient reductive coupling to the cytochrome [31]. This system also permits the testing of catalytic activity via in vivo feeding of taxoid substrates to the transformed yeast [24], thereby eliminating the need for microsome isolation in the preliminary functional screen. For this purpose, the ORF of clone S1 was transferred to the pYES2.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) for expression in the yeast host and for monitoring via immunoblot analysis of the resulting fusion protein created by joining of the the S1 ORF (deleted stop codon) to the vector-encoded simian V5 epitope and C-terminal His_6 tag. The suitability of this C-terminal tagging protocol has been demonstrated by the functional expression of other cytochrome P450 taxoid hydroxylases [26].

Transformed yeast cells, confirmed by immunoblot analysis of membrane protein extracts to express the recombinant S1 cytochrome P450, were tested for catalytic function of the expressed oxygenase via in vivo feeding of exogenous taxane substrates, including taxa-4(20),11(12)-dien-5α-ol, taxa-4(20),11(12)-dien-5α-yl acetate, taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol, taxa-4(20),11(12)-dien-5α,13α-diol, taxusin (the tetraacetate of taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol), and both taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene [32]. As a negative control for the feeding experiments, the yeast host was transformed with the same vector containing a β-glucuronidase insert instead of the cytochrome P450 S1 clone. Extracts of the overnight reaction mixtures were then analyzed by radio-HPLC, which showed that only the two taxadiene isomers were efficiently (almost quantitatively in the case of the 4(5),11(12)-isomer) converted to more polar products. In the case of the taxa-4(5),11(12)-diene substrate, the principal biosynthetic product (>92%) was eluted with a retention time identical to that of taxa-4(20),11(12)-dien-5α-ol [14, 32], and the minor product (<5%) was eluted with a retention time consistent with that of a taxadien-diol. GC-MS analysis (electron impact ionization) confirmed that the major product possessed a retention time and mass spectrum identical to those of authentic taxa-4(20),11(12)-dien-5α-ol [14] with charac-

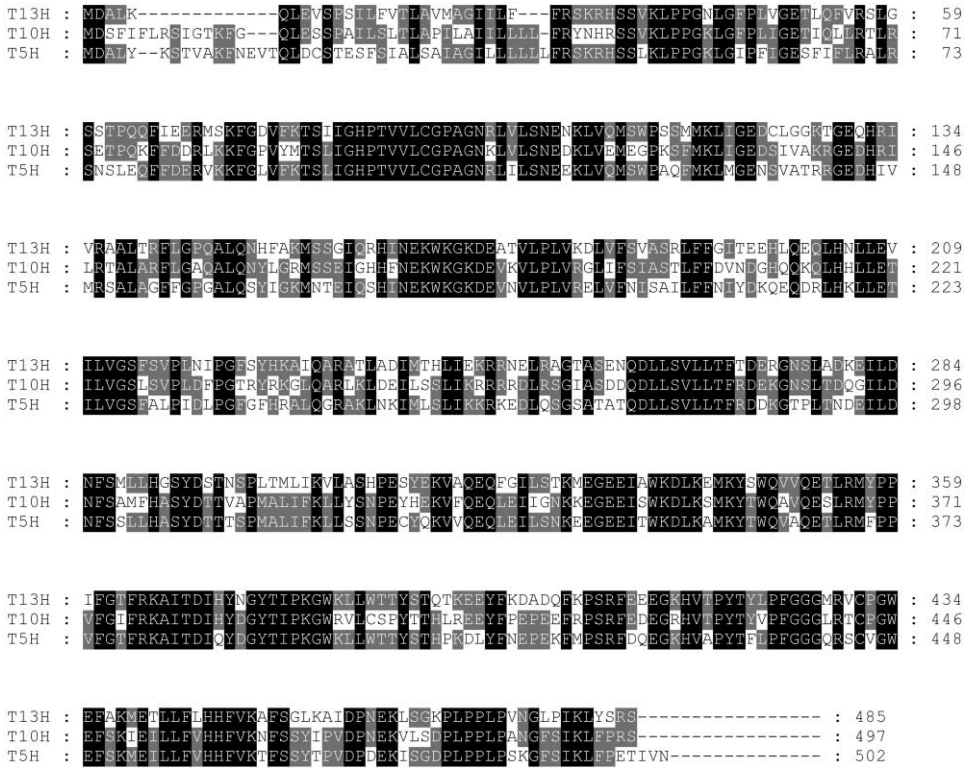

Figure 2. Deduced Amino Acid Sequence Alignment of Taxoid Hydroxylases

The sequences of taxoid 10β-hydroxylase (T10H), taxoid 13α-hydroxylase (T13H), and the clone S1 taxadiene 5α-hydroxylase (T5H) are compared. Black boxes indicate identical residues for the three sequences; gray boxes indicate identical residues for two of the three.

teristic ions at *m/z* 288 (P^+), 273 (P^+-CH_3), 270 (P^+-H_2O), and 255 (P^+-H_2O-CH_3). The minor, more polar product yielded a mass spectrum consistent with that of a taxadien-diol (ions corresponding to the loss of a methyl and two molecules of water from an unobserved parent ion of *m/z* 304); however, neither the retention time nor spectrum matched that of available taxadien-diol standards, and too little of this material was available for structural definition by NMR spectrometry.

In the case of the taxa-4(20),11(12)-diene substrate, the major product (~90%) was again shown, upon radio-HPLC analysis, to possess a retention time identical to that of taxa-4(20),11(12)-dien-5α-ol; this identification was confirmed as before, by GC-MS analysis. The unidentified taxadien-diol side product was also observed (~8%), as were a range of other minor metabolites (at ~2% of the product mix) that were also derived from this substrate in the negative control (yeast that expressed β-glucuronidase). These negative controls did not produce taxa-4(20),11(12)-dien-5α-ol or the unidentified taxadien-diol from either taxadiene isomer. To further support the identity of the biosynthetic taxa-4(20),11(12)-dien-5α-ol, we isolated this radio-labeled product by HPLC and fed the purified material to yeast that functionally expressed the previously characterized taxoid 13α-hydroxylase [25], whereupon this precursor was quantitatively converted to taxa-4(20),11(12)-dien-5α, 13α-diol as expected. These results confirm that cytochrome P450 clone S1 encodes a taxadiene 5α-hydroxylase that catalyzes the first oxygenation step of the Taxol biosynthetic pathway.

Characterization of the Recombinant Hydroxylase

To prepare a sufficient amount of enzyme for comparative analysis of substrate binding and kinetic phenomena, we used a host less prone to interfering activity and artifact formation. Thus, the taxadiene 5α-hydroxylase S1 cDNA clone was transferred to the baculovirus *Spodoptera fugiperda* (Sf9) expression system (which also coexpresses a *Taxus* cytochrome P450 reductase) previously utilized for taxoid hydroxylases [25]. Based on CO-difference spectra [33] of microsomes isolated from Sf9 insect cells expressing the recombinant hydroxylase (versus microsomes from negative control cells expressing the β-glucuronidase gene), this system routinely produced more than 300 pmol of the cytochrome P450 enzyme/mg microsomal protein. These insect cell microsomes enriched in the recombinant hydroxylase were then used for recording binding spectra [34] for both taxadiene isomers (in the absence of NADPH). Evaluation of the substrate binding constant (K_s) over a 100-fold range of substrate concentrations showed K_s to vary somewhat from 3 to 5 μM for taxa-4(20),11(12)-diene and from 5 to 8 μM for taxa-4(5),11(12)-diene (a typical data set at 200 pmol protein concentration is illustrated in Figure 3). These results indicate that both positional isomers of the olefin substrate bind with high affinity to the active site of taxadiene 5α-hydroxylase but that taxa-4(20),11(12)-diene is preferred by a factor of about two.

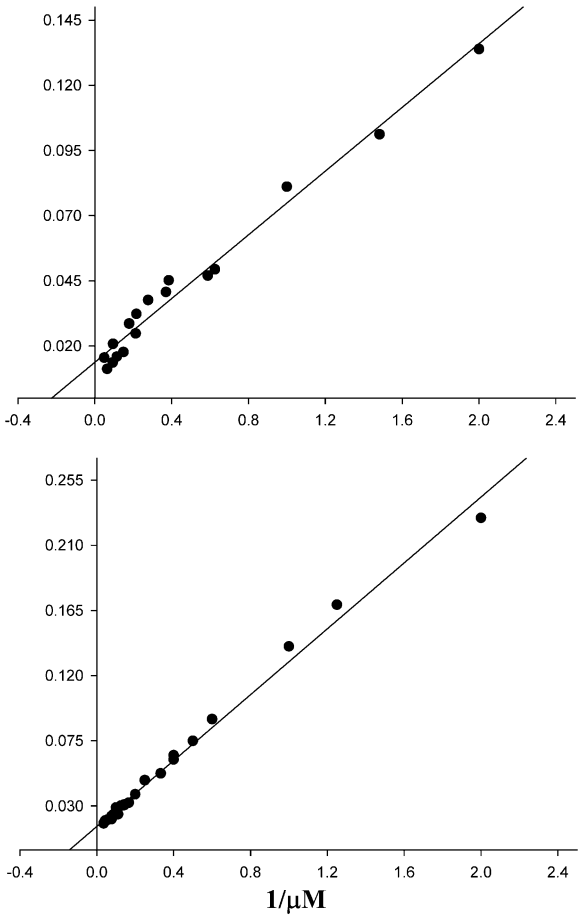

Figure 3. Substrate Binding of Taxadiene Isomers

Microsomes from *S. frugiperda* cells enriched with 200 pmol recombinant taxadiene 5α-hydroxylase (clone S1) were employed. Taxa-4(20),11(12)-diene was assayed over a concentration range from 0.1 to 10 μM, and a binding constant (K_s) of 4 ± 1 μM was determined (A). Taxa-4(5),11(12)-diene was assayed over a concentration range from 0.1 to 20 μM, and a binding constant (K_s) of 6.5 ± 1.5 μM was determined (B).

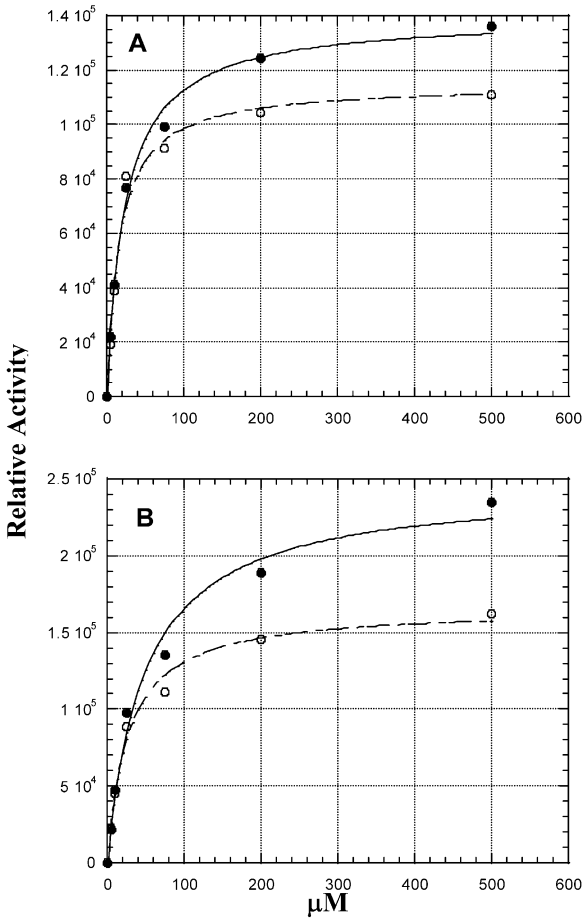

Figure 4. Kinetic Evaluation of Taxadiene Isomers

Taxa-4(20),11(12)-diene (filled circle) and taxa-4(5),11(12)-diene (open circle) were evaluated with microsomes from *S. frugiperda* cells enriched with 50 pmol recombinant taxadiene 5α-hydroxylase (A) and with microsomes from *T. media* suspension cells containing about 50 pmol of total native cytochrome P450 (B) The substrate concentration range varied from 1 to 500 μM in all cases. Taxa-4(20),11(12)-diene yielded an average K_m value of 21.5 μM with V_{rel} of 135, and taxa-4(5),11,12-diene yielded an average K_m value of 36 μM with V_{rel} of 100.

Next, kinetic constants for both isomers were evaluated (at a saturating 200 μM concentration of NADPH plus regenerating system [35]) via the Michaelis-Menten method. Plotting the lines of best fit ($R^2 > 0.99$) provided a K_m value of 16 ± 3.2 μM, with a V_{rel} of 120, for taxa-4(20),11(12)-diene and a K_m value of 24 ± 2.5 μM, with a V_{rel} of 100, for taxa-4(5),11(12)-diene (Figure 4); the latter K_m value compares to a K_m value of ~6 μM determined previously for the 4(5),11(12)-isomer with the native enzyme measured in microsome preparations from yew stem tissue [14]. It is notable that the recombinant hydroxylase expressed from the baculovirus *Spodoptera* system, and assayed in isolated microsomes, produced only the 5α-hydroxy taxadiene from either olefin substrate and that the taxadien-diol produced from these substrates in the intact yeast system was not observed. The diol product was thus attributed to the action of the yeast-host enzyme(s) upon taxadienol; this observation was independently verified by feeding studies with control yeast cells. Comparison of catalytic efficiencies (V_{rel}/K_m) of both substrates with the recombinant enzyme indicated that taxa-4(20),11(12)-diene was preferred to the 4(5),11(12)-isomer by a factor of about two.

Substrate Utilization by the Native Hydroxylase

Because studies with the recombinant 5α-hydroxylase indicated that both taxadiene isomers were functional substrates in the regiospecific formation of taxa-4(20),11(12)-dien-5α-ol, the native enzyme from induced *Taxus* cell microsomes was reevaluated. Previously, this system had never been tested with the 4(20),11(12)-diene isomer [14]. Microsomes from induced cultured cells (this starting material is preferred to stem tissue) were isolated as previously described [14] and, after the confirmation of linear reaction conditions in protein concentration and time, kinetic constants were determined for both [20-^{3}H]taxa-4(5),11(12)-diene and [20-^{3}H]taxa-4(20),11(12)-diene with the optimized assay [14]. The previously described radio-

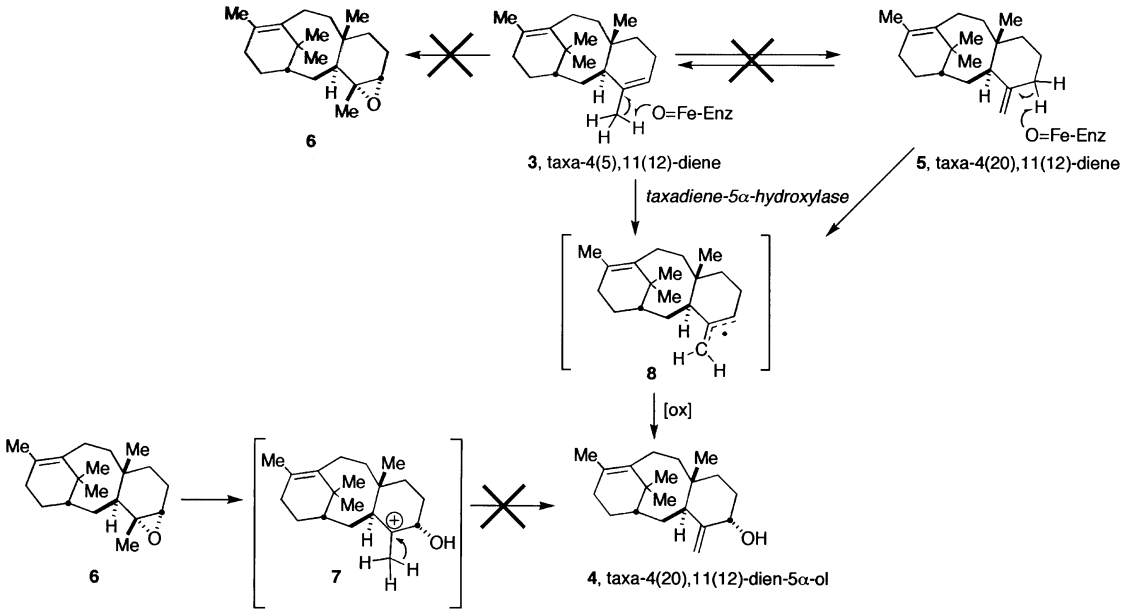

Figure 5. Proposed Mechanism for Cytochrome P450 Taxadiene 5α-Hydroxylase

This cytochrome P450-mediated conversion of taxa-4(5),11(12)-diene (**3**) and taxa-4(20),11(12)-diene (**5**) to taxa-4(20),11(12)-dien-5α-ol (**4**) is proposed to involve hydrogen abstraction from C20 (in **3**) or C5 (in **5**) to provide allylic radical **8**, followed by oxygen insertion at the 5α-face to yield taxadien-5α-ol (**4**). Isomerization of **3** to **5** was not observed, nor does the route via epoxide **6** with rearrangement seem likely.

HPLC-based assay [15] was employed here to permit the summation of taxadien-polyols derived subsequently from the initially formed taxadienol product generated by this microsomal system that contains all of the downstream cytochrome P450 taxoid oxygenases of the pathway [15]. It should be noted that any kinetic isotope effect (KIE) resulting from the C20 deprotonation of [20-^{3}H]taxa-4(5),11(12)-diene was not considered here because previous studies with [20-$^{2}H_3$]taxa-4(5),11(12)-diene (>99 atom % ^{2}H) indicated that hydrogen removal from C20 is apparently not rate limiting in the overall hydroxylation reaction [14]. By this approach, Michaelis-Menten plotting ($R^2 >$ 0.98 for the lines of best fit) indicated a K_m value of 48 μM, and V_{rel} of 100, for taxa-4(5),11(12)-diene, and a K_m value of 27 μM, with V_{rel} of 150, for taxa-4(20),11(12)-diene (Figure 4). Thus, in terms of catalytic efficiency (V_{rel}/K_m), the presumed-to-be-unnatural taxa-4(20),11(12)-diene isomer was again preferred as a substrate in the hydroxylation to taxa-4(20),11(12)-dien-5α-ol by this native enzyme; this is consistent with results obtained with the recombinant form of the enzyme.

Search for a Taxadiene Isomerase and Mechanism of Oxygenase Action

The utilization of taxa-4(20),11(12)-diene as a hydroxylase substrate raised the issue of whether this isomer could be a productive intermediate in vivo, in spite of the fact that the *Taxus* taxadiene synthase (native and recombinant enzyme) produces principally taxa-4(5),11(12)-diene (94%), with very low-level coproduction of taxa-4(20), 11(12)-diene (4.8%) and verticillene (1.2%) and only trace amounts of taxa-3(4),11(12)-diene [19]. Two recently acquired taxadiene synthase isoforms (the expressed enzymes of two less-abundant allelic variants, AY364469 and AY364470) produced (under established assay conditions [19]) a similar distribution of olefins, thus ruling out this route for the generation of significant amounts of taxa-4(20),11(12)-diene in vivo.

No isomerization of taxa-4(5),11(12)-diene to the 4(20),11(12)-diene isomer (or vice versa) was observed in *Taxus* cell microsomes (or *Spodoptera* microsomes enriched in the recombinant 5α-hydroxylase) under assay conditions, in which hydroxylation activity was inhibited [i.e., in the absence of NADPH, or in the absence of O_2 (N_2 atmosphere plus an O_2 scavenging system), or in the presence of CO, 100 μM miconazole, or 100 μM clotrimazole]. Furthermore, no isomerization was observed in boiled controls containing all cofactors and reactants. Similarly, no interconversion of either positional isomer was observed in the presence of magnesium ion, NAD^+, NADH, or $NADP^+$, or flavin cofactors at pH values ranging from 4 to 10. From these studies, we concluded that taxa-4(5),11(12)-diene is not appreciably isomerized to taxa-4(20),11(12)-diene under physiological conditions and that the migration of the double bond from the 4(5) to the 4(20) position in the process of taxadienol formation is an inherent feature of the cytochrome P450 oxygenase reaction with taxa-4(5),11(12)-diene as a substrate. It follows that taxa-4(20),11(12)-diene is but a minor natural *Taxus* product that is formed by taxadiene synthase and is an adventitious, yet efficient, substrate for 5α-hydroxylase.

Previous efforts to evaluate the 5α-hydroxylation reaction by the native enzyme [14], through a search for an epoxide intermediate and through the use of [20-$^{2}H_3$] taxa-4(5),11(2)-diene to examine a KIE on the deprotonation step, failed to distinguish between two reasonable mechanistic possibilities. One of the two mechanistic

manifolds that were considered involved preliminary conversion of the 4(5)-double bond of taxa-4(5),11(12)-diene (**3**) to the corresponding 4(5)-epoxide (**6**); this is followed by ring opening to carbenium ion **7** and elimination of a proton from the C20 methyl group to yield the allylic alcohol product (**4**). The other mechanistic manifold considered was an alternate route involving cytochrome P450-mediated abstraction of hydrogen from the C20 methyl of the substrate (**3**) to yield the allylic radical (**8**), to which adding oxygen at C5 yields **4** (Figure 5). The utilization of the isomeric taxa-4(20),11(12)-diene (**5**) by the hydroxylase, with efficiency comparable to that of the natural substrate, would appear to rule out an intermediate epoxide (**6**) in the reaction cycle and would instead suggest a mechanism involving abstraction of a hydrogen radical from C20 for **3** (or from C5 in the case of the 4(20)-isomer **5**), leading to the common intermediate, allylic radical **8**, followed by selective oxygen insertion from the 5α-face of this radical intermediate to accomplish the net oxidative rearrangement. It also appears that the somewhat tighter binding of the 4(20)-isomer substrate (**5**) may indicate that this isomer more closely mimics the allylic radical intermediate.

It is interesting to note that in 1966 Lythgoe originally proposed taxa-4(20),11(12)-diene (**5**) to be the committed precursor of taxoid biosynthesis [36]. The fact that this diterpene olefin has been shown not to be a significant product of taxadiene synthase but that it is efficiently processed by taxadiene hydroxylase demonstrates the inherent plasticity in substrate specificity that is emerging as a common feature of the enzymes (oxygenases and acyl transferases) of taxoid biosynthesis [16, 17].

Significance

Previous attempts to clone the taxadiene 5α-hydroxylase gene by a method involving differential display of mRNA from induced versus uninduced *Taxus* cells failed because, although the corresponding transcript appears to be fairly abundant, the gene is not highly induced and so was missed in the differential screen. A generic homology-based search for cytochrome P450 cDNA clones yielded the target hydroxylase and thus made the recombinant enzyme available for evaluation of substrate utilization. The efficient conversion of both taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene as substrates allowed us to conclude that the mechanism of this novel oxygenation and rearrangement reaction does not involve initial double bond epoxidation but rather involves hydrogen abstraction from either of two alternate positions of the olefin isomers to form a common allylic radical intermediate, followed by regio- and stereo-specific oxygen insertion at C5α to yield taxadienol. Based on feeding studies and metabolite analyses, we conclude that the 5α-hydroxylation step of Taxol biosynthesis is slow relative to the downstream oxygenations and acylations. Manipulation of the expression of this gene in *Taxus* should increase pathway flux toward Taxol to improve production yields of this drug from its natural, and currently the only commercially viable, source.

Experimental Procedures

Enzymes, Substrates, and Reagents

Enzymes and reagents were obtained from Gibco/BRL (Grand Island, NY), Invitrogen (Carlsbad, CA), New England Biolabs (Beverly, MA), and Stratagene (La Jolla, CA) and were used according to the manufacturers' instructions. Other chemicals described were purchased from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO). The preparations of ($\pm$)-[20-^{3}H]taxa-4(5),11(12)-diene (5.3 Ci/mol), ($\pm$)-[20-^{3}H]taxa-4(20),11(12)-diene (2.6 Ci/mol), and ($\pm$)-taxa-4(20),11(12)-dien-5α-ol (2.0 Ci/mol) have been described [32], as have the preparations of ($\pm$)-taxa-4(20),11(12)-dien-5α-yl acetate (2.0 Ci/mol) [15, 37], ($\pm$)-taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol (2.0 Ci/mol), and ($\pm$)-taxa-4(20),11(12)-dien-5α,13α-diol (2.0 Ci/mol) [25, 26]; the preparation of (+)-[^{3}H-acetyl]taxusin [the tetraacetate of taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol, at 10.0 Ci/mol] will be described elsewhere (M. Chau and R.C., unpublished data).

Homology-Based Cloning of Cytochrome P450 Oxygenases

A generic cloning strategy for cytochrome P450 genes [27–29] was employed based upon two highly conserved regions of these proteins, the commonly occurring PERF sequence and the region surrounding the invariant heme binding cysteine residue [30]. Thus, amino acid sequence alignments of cytochrome P450s of plant origin, available in the public databases, allowed synthesis of degenerate and inosine-containing oligonucleotide primers directed to the PERF motif and its variant forms (i.e., 5′-TTY MGI CCI AGM GIT TYG AR-3′ [forward], 5′-TTY MGI CCI TCI MGI TTY GAR-3′ [forward], 5′-CKI III CCI GCI CCR AAI GG-3′ [forward], 5′-GAR GAR TTY MGN CCN GAR MG-3′ [forward], and 5′-GAR AAR TTY III CCI GAI ARG TTY [forward]) and to the conserved heme binding region (i.e., 5′-GGR CAI III CKI III CCI CCI CCR AAI GG-3′ [reverse], and 5′-CCI GGR CAI ATI MKY YTI CCI GCI CCR AAI GG-3′ [reverse]). Amplification [29] was carried out with a first-strand cDNA template derived from mRNA isolated from *T. cuspidata* cells 16 hr postinduction with methyl jasmonate [23, 24]. This yielded the predicted amplicons of about 200 bp, and these were gel purified, ligated into pGEM-T (Promega, Madison, WI), and transformed into *E. coli* JM109 cells for plasmid preparation and insert sequencing. Based on these sequences, probes 40–50 nt long were synthesized, and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) was used for 5′-labeling with [^{32}P]dCTP (ICN, Irvine, CA). The probes were then used with Rapid-Hyb solution (Amersham Pharmacia, Piscataway, NJ) for screening the previously described induced *T. cuspidata* λ-ZAPII cDNA library [24]. After three rounds of screening, the 32 positives were excised in vivo as pBluescript II SK(−) phagemids by the Stratagene protocol and partially sequenced with T3 and T7 promoter primers so that clones could be sorted into new acquisitions that had not been obtained in the prior differential display screen [24]. These new clones were obtained in full length form (by Marathom 5′-RACE [Clontech, Palo Alto, CA] as necessary) and were fully sequenced.

Cytochrome P450 cDNA Expression in Yeast

For functional expression in *Saccharomyces cerevisiae*, the deduced ORFs of the cytochrome P450 cDNAs were amplified by PCR with a gene-specific forward primer (containing the ATG start codon) and a corresponding reverse primer in which the stop codon was deleted to permit read through when transferred to pYES2.1/V5-HIS-TOPO (Invitrogen). This generated a fusion protein containing the complete cytochrome P450 and the C-terminal-appended simian V5 epitope and histidine (His_6) tag encoded by the vector. This tagging procedure allows detection of the expressed enzyme via immunoblot analysis of the isolated microsomal protein with commercially available antibodies and does not compromise the activity of other recombinant taxoid hydroxylases [26]. Insert size was determined by restriction digests, followed by sequencing with Gal1 (forward) and V5 C-term (reverse) primers (Invitrogen) for orientation confirmation, and then the verified clones were transformed into the yeast host (*S. cerevisiae* strain WAT11 [31]) according to the lithium acetate method [38].

In Situ Screening for Cytochrome P450 Function

Transformed yeast cells were grown to stationary phase in 2 ml of SGIA medium at 30°C with 250 rpm mixing. The cells were then harvested via centrifugation (2000 × g, 10 min), and the cell pellet was suspended in 3 ml YPLA galactose-containing induction medium. Approximately 9 hr after induction, the cells were harvested again via centrifugation. For immunoblotting, the cells were resuspended in lysis buffer (100 mM Tris•HCl [pH 8.5] containing 1 mM DTT and 10% v/v glycerol) and lysed via sonication (VirSonic, microtip probe, medium setting, 3× for 30 s, VirTis, Gardiner, NY) or a Bead Beater (Biospec Products, Bartlesville, OK) and the microsomes were prepared [31]. Protein (50 μg) was then separated by SDS-PAGE (10% denaturing gel), transferred by wet transfer blotting to nitrocellulose, and immobilized by UV crosslinking. The appended C-terminal His_6 tag was then exploited for protein detection, with mouse Penta-His-specific antibody (Qiagen, Valencia, CA) as the primary antibody and alkaline phosphatase-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) as the secondary antibody. The Qiagen protocols were used throughout, with His-size markers as reference, and protein preparations from transformed cells harboring empty vectors as negative controls.

After confirmation of expression by immunoblot analysis, the activities of the recombinant cytochrome P450 enzymes were examined by using a previously developed in vivo feeding procedure [24], which eliminated the uncertainties associated with microsome isolation and in vitro assay. For this purpose, the transformed and induced yeast cells harvested via centrifugation (as above) were resuspended in 3 ml of fresh YPLA medium, to which 30 μM of the labeled test substrate was added. Overnight incubation at 30°C with mixing (250 rpm) followed. The incubation mixture was then treated for 15 min in a sonication bath and extracted twice with 3 ml of hexane:ethyl acetate (4:1 v/v). The organic extract was then dried under N_2, the residue was dissolved in 100 μl of acetonitrile, and an aliquot was separated by reversed-phase radio-HPLC (250 mm × 4.6 mm column of Alltech [Deerfield, IL] Econosil C_{18} [5 μm]; flow rate of 1 ml/min, with radio detection of the effluent [Flow-One-Beta Series A-1000, Radiomatic Corp., Meriden, CT]). The following conditions were employed: solvent A, 97.99% H_2O with 2% CH_3CN and 0.01% H_3PO_4 (v/v); solvent B, 99.99% CH_3CN with 0.01% H_3PO_4 (v/v); gradient, 0–5 min at 100% (A), 5–15 min at 0%–50% (B), 15–55 min at 50%–100% (B), 55–65 min at 100% (B), 65–70 min at 0%–100% (A), and 70–75 min at 100% (A). The HPLC eluant was collected in 1 min fractions and the appropriate fractions containing the radiolabeled product were combined, dried under a stream of N_2, and dissolved in the minimum volume of benzene possible for GC-MS analysis.

GC-MS analyses were performed on a Hewlett-Packard 6890 GC-MSD system with a ZB-5 capillary column (Phenomenex [Torrance, CA]; 30 min length; 0.25 mm inner diameter; coated with a 0.25 μm film of phenyl [5%] polysiloxane). Cool on-column injection was used, with a He flow rate of 0.7 ml/min and a temperature program from 40°C to 320°C at 20°C/min. Spectra were recorded at 70 eV.

Cytochrome P450 cDNA Expression in Insect Cells

The functional expression of cytochrome P450 clones with *Autographa californica* baculovirus and *Spodoptera frugiperda* (Sf9) cells that coexpress the *Taxus* NADPH:cytochrome P450 reductase gene has been described previously [25]. For construction of the recombinant baculovirus harboring cytochrome P450 clone S1, the S1 ORF was amplified by PCR with *Pfu* DNA polymerase and gene-specific primers containing a BamHI site immediately upstream of the start codon and another containing a NotI site downstream of the stop codon. The gel-purified amplicon was first subcloned into the PCR-Blunt vector (Invitrogen), and the BamHI/NotI restriction sites were then used for excising the insert, which was ligated into the similarly digested pFastBac1 vector (Life Technologies, Grand Island, NY). Transforming the *Escherichia coli* strain DH10Bac (Life Technologies) carrying the baculovirus genome allowed this pFastBac1 construct to be used for preparation of recombinbinant Bacmid DNA. As a negative control for this expression system, recombinant baculovirus containing a β-glucuronidase gene, instead of the cytochrome P450 ORF, was used. Baculovirus construction and transfection of Sf9 cells were carried out according to the Life Technologies protocol, and culturing was performed as previously described [25].

Recombinant Enzyme Characterization and Other Assays

For microsome preparation, Sf9 cells were harvested 3 days after transfection, washed twice with 50 mM KH_2PO_4 (pH 7.5) containing 9% (w/v) NaCl, twice with 50 mM HEPES (pH 7.5) containing 0.5 mM EDTA, 0.1 mM DTT, and 10% (v/v) glycerol. For lysing, gentle sonication was used as before in 50 ml of the HEPES buffer system. Centrifugation (10,000 × g, 20 min, 4°C) removed cell debris, and centrifuging the resulting supernatant at 28,000 × g (20 min, 4°C) and then again at 105,000 × g (120 min, 4°C) provided the microsomal membranes, which were resuspended in the same HEPES buffer system without EDTA or in another buffer system, as noted below. Protein content was determined by the Bradford method [39], with bovine serum albumin as the standard.

CO difference spectra [33] and substrate binding spectra [34] were recorded with a Perkin-Elmer Lambda 18 spectrophotometer [40]. The latter were recorded with up to 200 pmol of recombinant microsomal cytochrome P450 enzyme (determination was made with CO difference spectral analysis) per cuvette in 100 mM sodium phosphate buffer (pH 7.5). In preparation for binding studies, the taxadiene isomers were each dissolved in DMSO, and 1 μl additions to the sample were made to achieve a final concentration of 1.5% (v/v). For data analysis, Spectrum for Windows (Perkin-Elmer Corp., Wellesley, MA) and Sigmaplot 7.0 (SPSS, Chicago, IL) were employed, and experiments were run in triplicate.

For catalytic assays, the isolated microsomes were resuspended in 50 mM HEPES (pH 7.5) containing 1 mM DTT and 5% (v/v) glycerol, and the 1 ml reactions (~600 μg protein, 50 μM substrate dissolved in DMSO, and the requisite cofactors) were run as previously described, with the identical protocols for product analysis [25]. There was no influence of DMSO on the reaction. For kinetic evaluation, after the establishment of linear-reaction conditions in protein concentration and time, the response to substrate concentration was plotted via the Michaelis-Menten method (Sigmaplot 7.0) with the calibrated radio-HPLC protocol for product determination. Data from three independent experiments were pooled, and the line of best fit was taken ($R^2 > 0.99$).

Preparation of *Taxus* suspension cell microsomes and assays for the native taxadiene 5α-hydroxylase were carried out as previously described [14, 15], with the following modifications. Unelicited *Taxus media hicksii* cells were harvested 14 days after transfer, separated from the media, frozen in liquid N_2, and ground to a fine powder with a mortar and pestle, with extraction and microsome preparation the same as before [15]. The previously described radio-HPLC-based assay was employed [15] to separate the substrate from taxadien-5α-ol and polyols derived therefrom, which were summed as the "total product" for the purpose of rate determination.

The expression in *E. coli* of the taxadiene synthase allelic variants, and the preparation and assay of the recombinant taxadiene synthase isoforms, were conducted by established methods under capillary GC-MS conditions designed to separate taxadiene positional isomers [19].

The assay for microsomal taxa-4(5),11(12)-diene isomerase activity (and the reverse isomerization) was either carried out under standard cytochrome P450 oxygenase conditions but without NADPH or O_2, or it was carried out in the presence of inhibitory concentrations of CO, miconazole, or clotrimazole (i.e. under conditions described previously for which the rate of 5α-hydroxylation is negligible [14]). A number of additional potential cofactors, including FAD, $FADH_2$, FMN, $FMNH_2$, NAD^+, NADH and $NADP^+$ (all at 2.5 mM), as well as $MgCl_2$ (at 5.0 mM) were also tested. The possibility of pH-dependent isomerization was tested by incubating each isomer (100 μM) in phosphate buffer (pH 4–10) for 12 hr at 31°C, with separation of isomers as previously described [19].

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References

1. Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., and 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. Goldspiel, B.R. (1997). Clinical overview of the taxanes. Pharmacotherapy *17*, 1105–1255.
3. Skeel, R.T. (1999). Antineoplastic drugs and biological response modifiers. Classification, use, and toxicity of clinically useful agents. In Handbook of Chemotherapy, 5th Edition, R.T. Skeel, ed. (Baltimore: Lippincott, Williams & Wilkins), pp. 63–144.
4. Wall, M.E., and Wani, M.C. (1995). Paclitaxel: from discovery to clinic. Am. Chem. Soc. Symp. Ser. *583*, 18–30.
5. Michaud, L.B., Valero, V., and Hortobagyi, G. (2000). Risks and benefits of taxanes in breast and ovarian cancer. Drug Saf. *23*, 401–428.
6. Kingston, D.G.I., Jagtop, P.G., Yuan, H., and Samala, L. (2002). The chemistry of taxol and related taxoids. Prog. Chem. Org. Nat. Prod. *84*, 56–225.
7. Cragg, G.M., Schepartz, S.A., Suffness, M., and 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.
8. Suffness, M. (1995). Overview of paclitaxel research: progress on many fronts. Am. Chem. Soc. Symp. Ser. *583*, 1–17.
9. Ketchum, R.E.B., and Gibson, D.M. (1996). Paclitaxel production in cell suspension cultures of *Taxus*. Plant Cell Tissue Organ Cult. *46*, 9–16.
10. Mirjalili, N., and Linden, J.C. (1996). Methyl jasmonate induced production of taxol in suspension cultures of *Taxus cuspidata*: ethylene interaction and induction models. Biotechnol. Prog. *12*, 110–118.
11. Yukimune, Y., Tabata, H., Higashi, H., and Hara, Y. (1996). Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. Nat. Biotechnol. *14*, 1129–1132.
12. Koepp, A.E., Hezari, M., Zajicek, J., Stofer Vogel, B., LaFever, R.E., Lewis, N.G., and Croteau, R. (1995). Cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene is the committed step of Taxol biosynthesis in Pacific yew. J. Biol. Chem. *270*, 8686–8690.
13. Hezari, M., Lewis, N.G., and Croteau, R. (1995). Purification and characterization of taxa-4(5),11(12)-diene synthase from Pacific yew (*Taxus brevifolia*) that catalyzes the first committed step of Taxol biosynthesis. Arch. Biochem. Biophys. *322*, 437–444.
14. Hefner, J., Rubenstein, S.M., Ketchum, R.E.B., Gibson, D.M., Williams, R.M., and Croteau, R. (1996). 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. Chem. Biol. *3*, 479–489.
15. Lovy Wheeler, A., Long, R.M., Ketchum, R.E.B., Rithner, C.D., Williams, R.M., and Croteau, R. (2001). Taxol biosynthesis: differential transformation of taxadien-5α-ol and its acetate ester by cytochrome P450 hydroxylases from *Taxus* suspension cells. Arch. Biochem. Biophys. *390*, 265–278.
16. Walker, K.D., and Croteau, R. (2001). Taxol biosynthetic genes. Phytochemistry *58*, 1–7.
17. Jennewein, S., and Croteau, R. (2001). Taxol: biosynthesis, molecular genetics and biotechnological applications. Appl. Microbiol. Biotechnol. *57*, 13–19.
18. Wildung, M.R., and Croteau, R. (1996). A cDNA clone for taxadiene synthase, the diterpene cyclase that catalyzes the committed step of Taxol biosynthesis. J. Biol. Chem. *271*, 9201–9204.
19. Williams, D.C., Wildung, M.R., Jin, A.Q., Dalal, D., Oliver, J.S., Coates, R.M., and Croteau, R. (2000). Heterologous expression and characterization of a "pseudomature" form of taxadiene synthase involved in paclitaxel (Taxol) biosynthesis, and evaluation of a potential intermediate and inhibitors of the multistep diterpene cyclization reaction. Arch. Biochem. Biophys. *379*, 137–146.
20. Lin, X., Hezari, M., Koepp, A.E., Floss, H.G., and Croteau, R. (1996). Mechanism of taxadiene synthase, a diterpene cyclase that catalyzes the first step of Taxol biosynthesis in Pacific yew. Biochemistry *35*, 2968–2977.
21. Williams, D.C., Carroll, B.J., Jin, Q., Rithner, C., Lenger, S.R., Floss, H.G., Coates, R.M., Williams, R.M., and Croteau, R. (2000). Intramolecular proton transfer in the cyclization of geranylgeranyl diphosphate to the taxadiene precursor of Taxol catalyzed by recombinant taxadiene synthase. Chem. Biol. *7*, 969–977.
22. Walker, K., Fujisaki, S., Long, R., and Croteau, R. (2002). Molecular cloning and heterologous expression of the C-13 phenylpropanoid side chain-CoA acyltransferase that functions in Taxol biosynthesis. Proc. Natl. Acad. Sci. USA *99*, 12715–12720.
23. Ketchum, R.E.B., Gibson, D.M., Croteau, R.B., and Shuler, M.L. (1999). The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. Biotechnol. Bioengin. *62*, 97–105.
24. Schoendorf, A., Rithner, C.D., Williams, R.M., and Croteau, R. (2001). Taxol biosynthesis: molecular cloning of a cytochrome P450 taxane 10β-hydroxylase cDNA from *Taxus* and functional expression in yeast. Proc. Natl. Acad. Sci. USA *98*, 1501–1506.
25. Jennewein, S., Rithner, C.D., Williams, R.M., and Croteau, R. (2001). Taxol biosynthesis: taxane 13α-hydroxylase is a cytochrome P450-dependent monooxygenase. Proc. Natl. Acad. Sci. USA *98*, 13595–13600.
26. Jennewein, S., Rithner, C.D., Williams, R.M., and Croteau, R. (2003). Taxoid metabolism: taxoid 14β-hydroxylase is a cytochrome P450-dependent monooxygenase. Arch. Biochem. Biophys. *413*, 262–270.
27. Udvardi, M.K., Metzger, J.D., Krishnapillai, V., Peacock, W.J., and Dennis, E.S. (1994). Cloning and sequencing of a full-length cDNA from *Thlaspi arvense* L. that encodes a cytochrome P-450. Plant Physiol. *105*, 755–756.
28. Holton, T.A., and Lester, D.R. (1996). Cloning of novel cytochrome P450 gene sequences via polymerase chain reaction amplification. Methods Enzymol. *272*, 275–283.
29. Pauli, H.H., and Kutchan, T.M. (1998). Molecular cloning and functional heterologous expression of two alleles encoding (S)-N-methylcoclaurine 3′-hydroxylase (CYP80B1), a new methyl jasmonate-inducible cytochrome P-450-dependent mono-oxygenase of benzylisoquinoline alkaloid biosynthesis. Plant J. *13*, 793–801.
30. von Wachenfeldt, C., and Johnson, E.F. (1995). Structures of eukaryotic cytochrome P450 enzymes. In Cytochrome P450: Structure, Mechanism, and Biochemistry, Second Edition, P.R. Ortiz de Montellano, ed. (New York: Plenum), pp. 183–223.
31. Pompon, D., Louerat, B., Bronine, A., and Urban, P. (1996). Yeast expression of animal and plant P450s in optimized redox environments. Methods Enzymol. *272*, 51–64.
32. Rubenstein, S.M., Vazquez, A., Sanz-Cervera, J.F., and Williams, R.M. (2000). Synthesis of stable and radioisotopomers of taxa-4(5),11(12)-diene, taxa-4(20),11(12)-diene and taxa-4(20),11(12)-dien-5α-ol, early intermediates in taxol biosynthesis. J. Label. Compds. Radiopharm. *43*, 481–491.
33. Omura, T., and Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. *239*, 2370–2378.
34. Schenkman, J.B., and Jansson, I. (1998). Spectral analysis of cytochrome P450. Methods Mol. Biol. (Cytochrome P450 Protocols) *107*, 25–33.
35. Shimada, T., and Yamazaki, H. (1998). Cytochrome P450 reconstitution systems. Methods Mol. Biol. (Cytochrome P450 Protocols) *107*, 85–93.
36. Harrison, J.W., Scrowston, R.M., and Lythgoe, B. (1966). Taxine.

Part III. A revised structure for the neutral fragment from *O*-cinnamoyltaxicin-I. J. Chem. Soc. *C*, 1933–1945.
37. Walker, K., Ketchum, R.E.B., Hezari, M., Gatfield, D., Goleniowski, M., Barthol, A., and Croteau, R. (1999). Partial purification and characterization of acetyl coenzyme A: taxa-4(20),11(12)-dien-5α-ol-O-acetyl transferase that catalyzes the first acylation step of taxol biosynthesis. Arch. Biochem. Biophys. *364*, 273–279.
38. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. *153*, 163–168.
39. 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.
40. Haudenschild, C., Schalk, M., Karp, F., and Croteau, R. (2000). Functional expression of regiospecific cytochrome P450 limonene hydroxylases from mint (*Mentha* spp.) in *Escherichia coli* and *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. *379*, 127–136.

Accession Numbers

The complete DNA sequence of taxadiene 5α-hydroxylase has been deposited in the GenBank database with the accession number AY289209.