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Taxol Biosynthesis: Molecular Cloning and Characterization of a Cytochrome P450 Taxoid 7β-Hydroxylase

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Summary

Biosynthesis of the anticancer drug Taxol in yew species involves eight cytochrome P450-mediated oxygenations and four coenzyme A-dependent acylations of the diterpenoid core. A family of cytochrome P450 genes, obtained from a yew cell cDNA library, were functionally expressed and screened with taxusin (taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol tetraacetate) as a surrogate substrate. One clone converted this substrate to an oxygenated derivative that was identified as 7β-hydroxytaxusin. The structure and properties of this 7β-hydroxylase are similar to those of other taxoid hydroxylases. Kinetic and binding assays indicated selectivity of the 7β-hydroxylase for polyoxygenated and acylated taxoid substrates, an observation consistent with the operation of this enzyme in the central portion of the Taxol biosynthetic pathway. Although the 7β-hydroxyl of Taxol is not essential for antimitotic activity, this functional group provides a convenient means for preparing taxoid derivatives.

Introduction

The highly functionalized diterpenoid Taxol (generic name paclitaxel, Figure 1), derived from yew (*Taxus*) species [1], is a potent mitotic inhibitor used successfully in the treatment of a wide variety of cancers [2, 3]. The limited supply of this drug from its natural source has prompted the development of alternative means of production [4]. Total syntheses of Taxol have been achieved by several elegant routes (see [5] for recent review) but the yields are too low to be practical, and the principal source of the drug is via semisynthesis [6] from advanced taxane diterpenoid (taxoid) precursors (e.g., 10-deacetylbaccatin III, Figure 1) isolated from needles of various *Taxus* species. Thus, for the foreseeable future, the supply of Taxol and its semisynthetically useful precursors [5] must continue to rely upon biological methods of production [4, 7]. Improvement of the biosynthetic process in intact yew or derived cell cultures [8, 9] should be based upon a full understanding of the pathway for Taxol formation, the enzymes which catalyze this extended sequence of reactions and their mechanisms of action, and the structural genes encoding these enzymes, especially those responsible for slow steps of the pathway.

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The Taxol biosynthetic pathway consists of approximately 20 enzymatic steps from the primary intermediate geranylgeranyl diphosphate [10] which is cyclized, in the first committed step of the pathway, to taxa-4(5),11(12)-diene [11] to establish the core skeleton [12] (Figure 1). This parental taxane then undergoes a series of eight cytochrome P450-mediated oxygenations and several acylations, including C13 side-chain assembly and attachment, en route to Taxol [13, 14]. The predicted order of hydroxylation, based on the relative abundances of the several hundred defined taxoids bearing oxygen functions at the various positions, begins with C5 and C10, then C2, C9, and C13, and later C7 and C1 [15]. Biochemical studies are consistent with the proposed order in the early part of the pathway, C5, C10, and C13 hydroxylations have been demonstrated [16, 17]. All of the core acylations have also been described, as have those involved in C13 side-chain assembly and transfer [18]; the latter appears to occur after construction of the oxetane ring (see Figure 1). The intermediate steps of the Taxol biosynthetic pathway remain largely undefined.

Because natural product biosynthetic genes of plant origin are rarely clustered [19], a range of reverse genetic and homology-based cloning strategies, as well as EST mining [13, 14], have been employed to isolate cDNAs encoding geranylgeranyl diphosphate synthase [10], taxadiene synthase [20], and all of the CoA-dependent acyl and aroyl transferases of the Taxol pathway [18, 21–24]. In the case of the cytochrome P450 oxygenases, an additional approach was taken based on differential display of mRNA-reverse transcription-PCR, using cultured *Taxus* cells induced for Taxol production as the transcript source [25], and with sorting of clones by sequence relatedness and functional expression [26]. These combined strategies yielded a family of about two dozen related oxygenases from which cDNAs encoding the cytochrome P450 taxoid 5α-hydroxylase [27], 10β-hydroxylase [26], 13α-hydroxylase [28], and a side-route taxoid 14β-hydroxylase [29] were obtained to confirm the early part of the Taxol biosynthetic pathway.

Intermediate oxygenation steps of the pathway (i.e., from the level of a taxadien triol onward) have been much more difficult to approach. This midsection of the pathway is not defined in reaction order and the intermediates are not known or, if predicted, are not readily available for testing. This serious difficulty necessitates the preparation and evaluation of "surrogate" substrates to explore these "central" hydroxylations. In the present instance, taxusin (the tetraacetate of taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol) was employed as a surrogate substrate to functionally evaluate the extant cytochrome P450 clones for taxoid C1, C2, and C7 hydroxylase activities. Taxusin is a prominent metabolite of yew heartwood [30, 31] in which it is considered a dead-end metabolite, not an intermediate in Taxol formation [15]; nevertheless, the natural occurrence in *Taxus* of a broad range of taxusin-like metabolites bearing additional oxygen functional groups at C1, C2, or

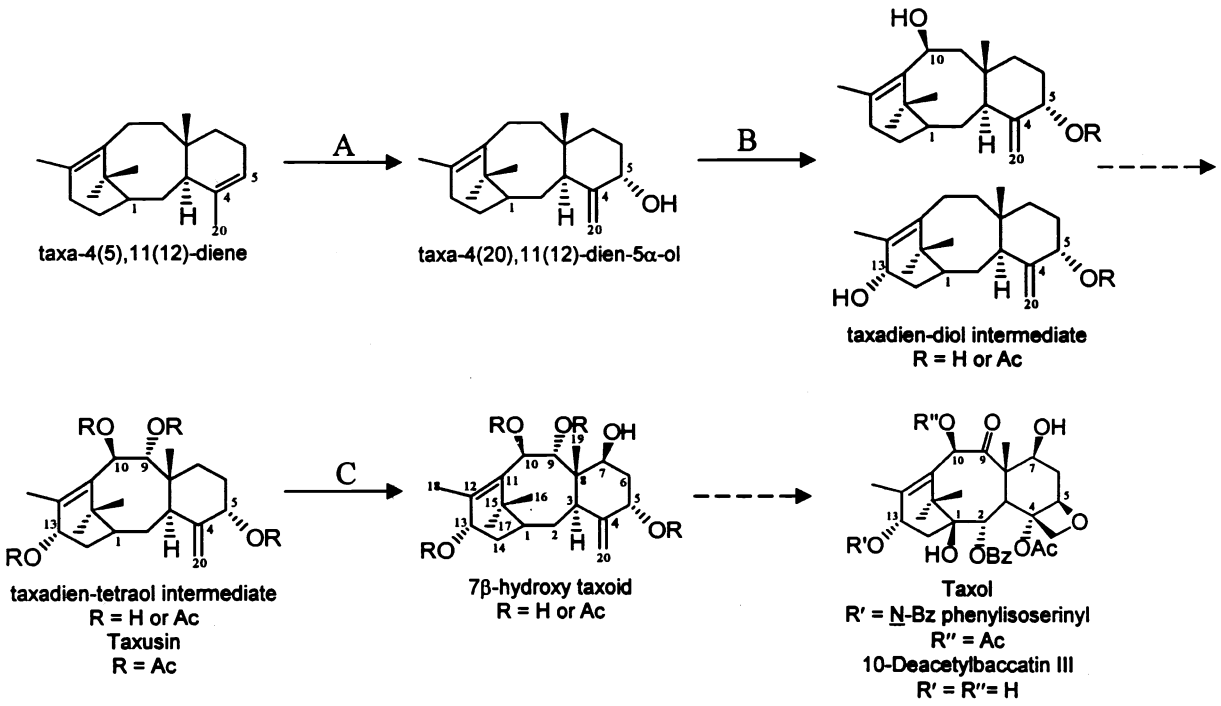

Figure 1. Outline of the Taxol Biosynthetic Pathway

Hydroxylation of the committed precursor taxa-4(5),11(12)-diene to the monool by the 5α-hydroxylase (A), hydroxylation to the level of a diol by the 10β-hydroxylase or 13α-hydroxylase (B), and hydroxylation of a tetraol derivative by the 7β-hydroxylase (C) are illustrated. Broken arrows indicate multiple steps, several of which remain undefined.

C7 [31–33] encouraged the use of this material as an alternate substrate for the present purpose. This approach led to the acquisition and characterization of a seemingly regioselective taxoid 7β-hydroxylase presumed to operate in the middle section of the multistep Taxol biosynthetic pathway.

Results and Discussion

Preliminary Studies

To determine whether taxusin might be a suitable surrogate substrate for identifying oxygenases relevant to taxoid metabolism, microsomes prepared from cultured *Taxus* cells were incubated with [^{3}H-acetyl]taxusin under standardized cytochrome P450 assay conditions [16, 17]. The resulting reaction products were extracted and separated by radio-HPLC to reveal several metabolites more polar than taxusin (i.e., presumptive hydroxylated derivatives), thereby suggesting that *Taxus* microsomes do contain cytochrome P450 enzyme(s) capable of oxygenating taxusin.

Functional Screening of Cytochrome P450 Clones

To test the ability of the encoded enzymes of the previously acquired family of cytochrome P450 clones [26] to oxygenate taxusin, we exploited the simplicity and reliability of heterologous expression of these cDNAs in *Saccharomyces cerevisiae* strain WAT11 which harbors a galactose-inducible NADPH-cytochrome P450 reductase from *Arabidopsis thaliana* that is required for efficient reductive coupling to the cytochrome [34]. This system also permits test of catalytic activity by in vivo feeding of taxoid substrates to the transformed yeast [26–29], thereby eliminating the need for microsome isolation in the preliminary functional screen.

Transformed yeast cells, confirmed to express the recombinant cytochrome P450s by CO-difference spectra [35] as previously described [26], were tested for the ability to oxygenate taxusin by overnight incubation with [^{3}H-acetyl]taxusin. Extracts of the overnight reaction mixture of the 19 clones evaluated were then analyzed by radio-HPLC, and it was shown that one clone (designated F31) was capable of the nearly quantitative conversion of taxusin to more polar products (i.e., one large radio peak flanked by two smaller radio peaks [data not shown]). LC-MS analysis of this material revealed a mass spectrum of the principal product that was consistent with that of a hydroxytaxusin. Although the parent ion (P^+) at *m/z* 521 (taxusin MW of 504 plus *O* [mass 16] + H^+) was not observed, diagnostic fragment ions were observed at *m/z* 461 (P^+-CH_3COOH), 401 (P^+-$2CH_3COOH$), 359 (P^+-$2CH_3COOH$-CH_3CO), 341 (P^+-$3CH_3COOH$), 299 (P^+-$3CH_3COOH$-CH_3CO), and 281 (P^+-$4CH_3COOH$). MS analysis of the two minor products yielded parent ions and diagnostic fragment ions suggestive of hydrolysis products of both the taxusin substrate and the putative hydroxytaxusin product, presumably arising via hydrolytic metabolism in the yeast host. For this reason, the products generated from taxusin by cytochrome P450 clone F31, as well as those generated from taxusin by *Taxus* microsomes (see above), were peracetylated prior to re-analysis by LC-MS and GC-MS. These analy-

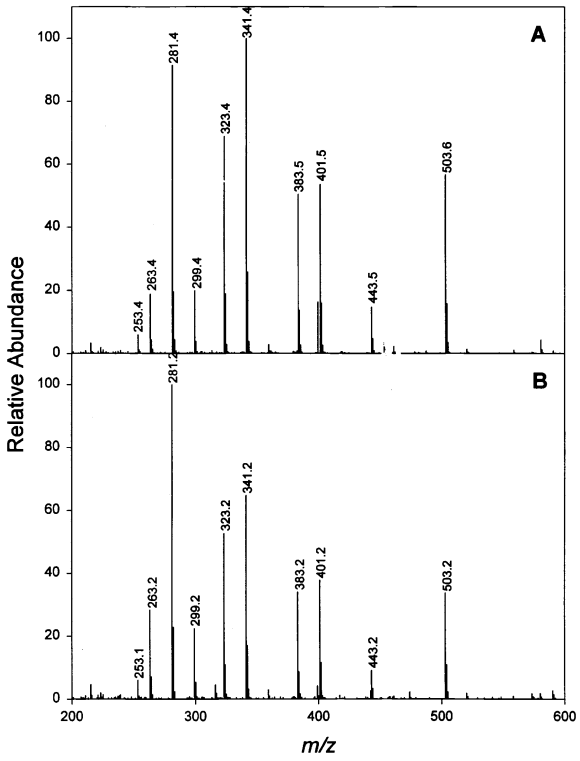

Figure 2. LC-MS of the Biosynthetic Product Derived from (+)-Taxusin

The mass spectra (APCI) of the presumptive hydroxytaxusin (following peracetylation) derived from taxusin by *Taxus* cell microsomes (A) and by recombinant cytochrome P450 clone F31 expressed in yeast (B) are illustrated; the LC retention times of both products (A and B) were also identical (33.6 min).

ses of the derivatized material revealed that the minor hydrolytic products had disappeared (by collapsing into either tetraacetylated substrate or pentaacetylated product), and that the major product of taxusin metabolism by clone F31 and by *Taxus* microsomes had the identical retention time by HPLC (33.6 min) and by GC (17.66 min) and the identical mass spectrum by APCI and electron impact ionization (Figure 2). By APCI MS, the presumptive acetylated hydroxytaxusin product did not yield a detectable parent ion at *m/z* 563 (acetoxytaxusin [MW = 562] + H^+); however, diagnostic fragment ions were observed at *m/z* 503 (P^+-CH_3COOH), 443 (P^+-$2CH_3COOH$), 401 (P^+-$2CH_3COOH$-CH_3CO), 383 (P^+-$3CH_3COOH$), 341 (P^+-$3CH_3COOH$-CH_3CO), 323 (P^+-$4CH_3COOH$), 299 (P^+-$3CH_3COOH$-$2CH_3CO$), 281 (P^+-$4CH_3COOH$-CH_3CO), and 263 (P^+-$5CH_3COOH$). These data indicated that taxusin was converted to the same hydroxylated product by yeast harboring clone F31 and by the cytochrome P450(s) of *Taxus* cell microsomes.

Product Identification

To confirm the identity of the product generated from taxusin by cytochrome P450 clone F31, and to determine the position and stereochemistry of the newly appended hydroxyl group, the transformed yeast incubations were scaled up 25-fold, and the resulting product was isolated and chromatographically purified to yield about 1 mg of material (>95% purity by HPLC, free of hydrolysis products and underivatized) for NMR analysis (Table 1). The previously determined ^{13}C-NMR shift assignments for taxusin [36] aided in assigning the chemical shifts for the putative hydroxytaxusin. The ^{1}H-NMR spectrum of the putative hydroxytaxusin was also quite similar to that of taxusin, as expected; however, a unique doublet of doublets in the sample was observed at 4.34 ppm, consistent with the chemical shift of a proton attached to a carbon bearing a hydroxyl group. The only protons that could exhibit this type of coupling are either at C2 or C7. 2D-NOESY and 2D-TOCSY experiments revealed the regiochemistry of the hydroxyl substituent and the complete ^{1}H assignments. The NOESY experiment showed the doublet of doublets (δ 4.34) to have correlations between H6α and H6β. These 2D-NMR data confirm the hydroxyl to be on the C-ring at C7. The stereochemistry of the C7 proton was determined by nuclear Overhauser effect difference (NOE-DIF) spectroscopy, which revealed strong correlations with the α-oriented protons H3 (δ 2.83) and H10 (δ 6.23), and those of the C9α-acetate methyl (δ 2.07), and moderate coupling with H6α (δ 2.09). The strong correlations with H3 and H10 (both α face) would be exhibited only if H7 was α oriented, since a β-oriented hydrogen could not be so coupled. The strong correlation with H6α also indicated H7 to be of the gauche conformation; consistent with α stereochemistry (models show H6β and H7α to be nearly orthogonal). These correlations confirm that H7α is attached to a carbon bearing a β-hydroxyl

Table 1. Complete ^{1}H and ^{13}C NMR Assignments for the Biosynthetic Product Derived from Taxusin by the Recombinant Cytochrome P450 Expressed from Clone F31

Position No.	α-Proton δ (ppm)	β-Proton δ (ppm)	Carbon δ (ppm)
1		1.85 (*m*)	35.66
2	1.72 (*m*)	1.77 (*m*)	31.42
3	2.83 (*br d*)		40.21
4			146.89
5		5.39 (*br t*)	75.15
6	2.09 (*m*)	1.63 (*m*)	36.49
7	4.34 (*dd*)		70.65
8			46.72
9		6.05 (*d*)	79.36
10	6.23 (*d*)		71.34
11			134.57
12			137.63
13		5.92 (*t*)	70.37
14	1.05 (*dd*)	2.66 (*dt*)	27.37
15			39.50
16	1.14 (*s*, exo)		27.37
17	1.63 (*s*, endo)		31.22
18	2.1 (*s*)		12.27
19		0.80 (*s*)	15.05
20	4.93 (*d*, exo)		115.38
	5.27 (*d*, endo)		
OAc	2.1 (*s*)		20.81
	2.07 (*s*)		21.01
	2.02 (*s*)		21.46
	2.16 (*s*)		21.77
			168.90
			169.68
			169.77
			170.41

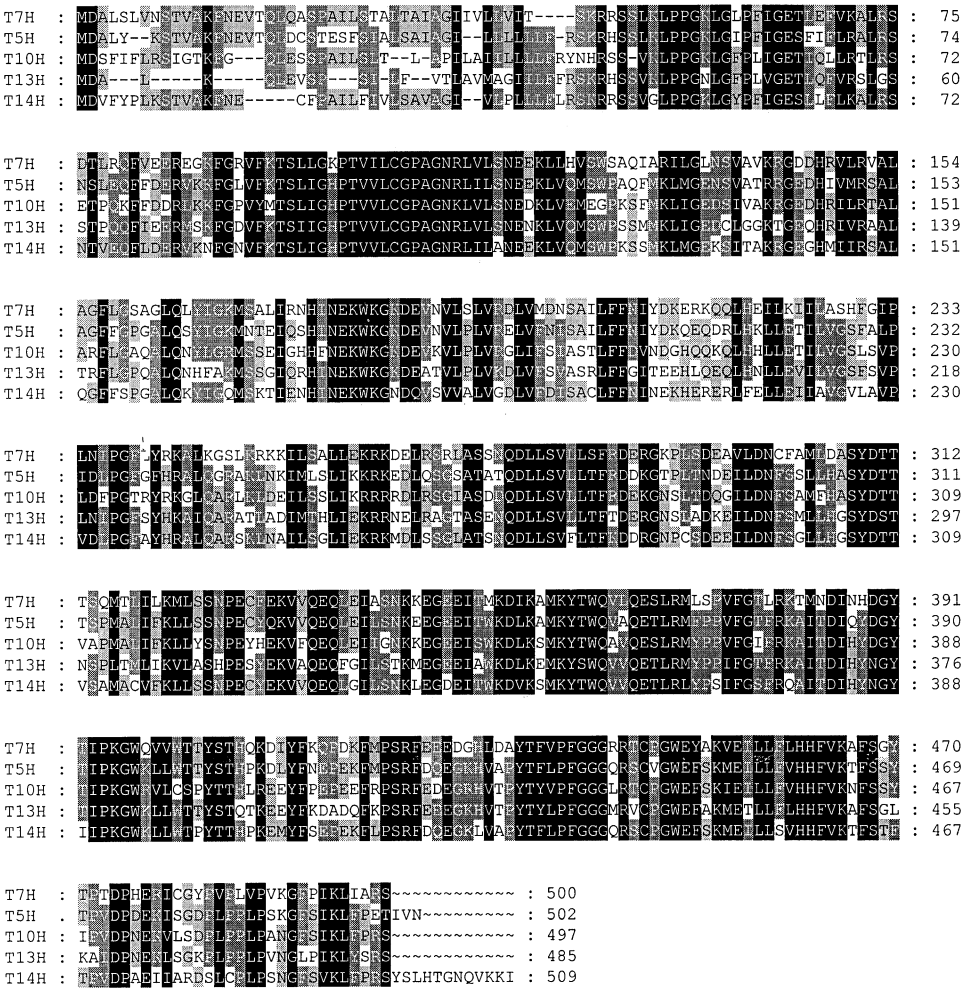

Figure 3. Deduced Sequence Alignment of Taxoid Hydroxylases

Alignments of cytochrome P450 taxoid 7β-hydroxylase (T7H) with taxoid 5α-hydroxylase (T5H), 10β-hydroxylase (T10H), 13α-hydroxylase (T13H), and 14β-hydroxylase (T14H) are illustrated. Black boxes indicate identical residues; gray boxes indicate identical residues for at least three of the sequences.

group and, thus, that the product of clone F31 is 7β-hydroxytaxusin.

The proton spectrum also revealed the presence of what appeared to be a significant contaminant in the sample that was not observed at the HPLC purification step (UV monitoring), suggesting that the sample had undergone modification after isolation. The presence of this contaminant complicated the ^{13}C-NMR and ^{1}H-NMR analyses of the biosynthetic product because the spectra of the contaminant resembled those of 7β-hydroxytaxusin in many aspects. However, the two-dimensional NMR experiments clearly indicated the presence in the contaminant of a free hydroxyl at C9, and that the characteristic doublet of doublets had shifted downfield to δ 5.48, consistent with the chemical shift of a proton attached to a carbon (C7) now bearing an acetoxy group. These results were highly suggestive of intramolecular migration of the acetyl group at C9 of the 7-hydroxytaxusin product to C7. Nonenzymatic shifts of this type have been observed previously in similarly functionalized taxoids [37]; the in vivo significance of such reactions is uncertain.

Sequence Analysis

The translated nucleotide sequence of clone F31 (GenBank accession number AY307951, orf 1503 bp) encodes a 501 residue protein with a calculated molecular weight of 56,323. Analysis of the deduced sequence revealed several features that are characteristic of cytochrome P450 enzymes, such as an N-terminal membrane anchor, the oxygen binding domain, the reductase binding domain, the conserved PERF motif (aa 421-424), the highly conserved heme binding motif with PFG element (aa 438-440), and the essential cysteine at position 446 [38]. Sequence alignment (Figure 3) of the taxoid 7β-hydroxylase from *T. cuspidata* with the previously acquired taxoid hydroxylases revealed significant identity and high similarity scores; for the 7β-hydroxylase versus the 5α-hydroxylase [27] (63% I, 82% S), the 10β-hydroxylase [26] (55% I, 75% S), the 13α-hydroxylase [28] (53% I, 72% S), and the 14β-hydroxylase [29] (53% I, 72% S). The 7β-hydroxylase is most similar in sequence to the 5α-hydroxylase which also hydroxylates in the taxane C ring. However, the taxoid 5α-hydroxylase also bears high sequence identity to the

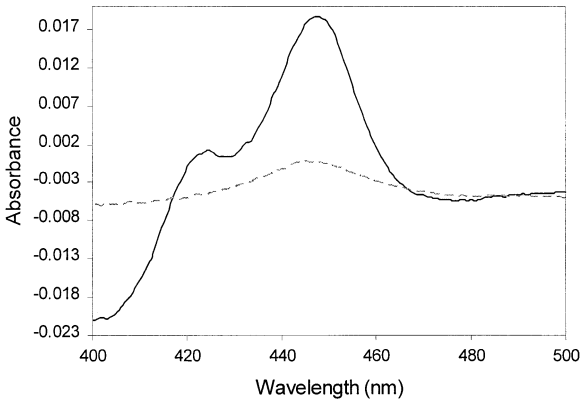

Figure 4. Reduced CO-Difference Spectrum of the Recombinant Hydroxylase

The spectrum was recorded using a microsomal preparation from Sf9 cells which express recombinant cytochrome P450 clone F31 (solid line); the dotted line is the negative control spectrum identically obtained using microsomal preparations from Sf9 cells which express recombinant β-glucuronidase.

taxoid 14β-hydroxylase which functionalizes the A ring [29]. Thus, the correlation of sequence similarity with hydroxylation regiochemistry is inexact. In an EST library derived from *Taxus* cells induced for taxoid production with methyl jasmonate [25], clone F31 is the third most abundant cytochrome P450 transcript.

Characterization of the 7β-Hydroxylase

Because previous studies with taxoid hydroxylases have shown the *Spodoptera fugiperda*-baculovirus-based expression system to be superior to yeast for the characterization of *Taxus* cytochrome P450 enzymes (this highly efficient system coexpresses the *Taxus* NADPH cytochrome P450 reductase, permits ready isolation of catalytically functional microsomes, and is often less prone to interfering activities from the host), the 7β-hydroxylase clone F31 was transferred to the appropriate vector for this purpose [28]. Expression in these insect cells and microsome isolation by established protocols [27, 28] confirmed the efficient production of the recombinant cytochrome (~1 nmol/mL of resuspended microsomes) by CO-difference spectrometry [35] (Figure 4) and by standardized assay [27, 28] for the direct conversion of labeled taxusin to the 7β-hydroxy derivative (see Figure 5).

Using this microsomal system, the pH optimum for the 7β-hydroxylase was determined to be about pH 7.5, similar to other taxoid hydroxylases [28, 29]. Overall hydroxylase activity was slightly higher in HEPES buffer than in Tris-HCl; however, hydrolytic activity (enzymatic deacylation of both substrate and product) was also more prominent in HEPES. Therefore, Tris-HCl was selected as the buffer for kinetic assays, but, even with this, it was still necessary to peracetylate the mixture prior to analysis to permit accurate quantification (Figure 5). By using this assay and Eadee-Hofstee plotting (R^2 = 0.93 for the line of best fit), the K_m value for (+)-taxusin with the recombinant microsomal 7β-hydroxylase was

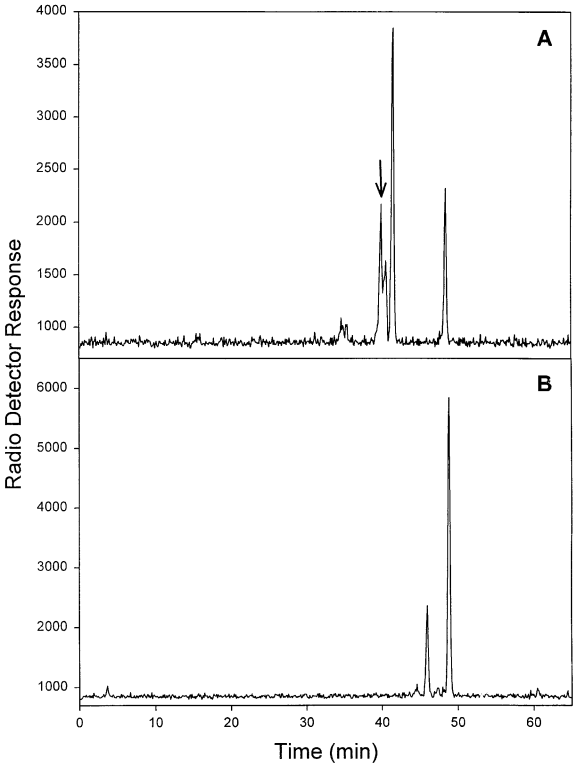

Figure 5. Radio-HPLC Analysis of the Products Derived from [^{3}H]Taxusin

(A) illustrates the mixture of products generated from [^{3}H-acetyl]taxusin by the microsomal cytochrome P450 hydroxylase expressed from clone F31 in Sf9 cells, with the substrate at 49 min, the hydroxytaxusin product at 40 min (arrow), and a mixture of partial hydrolysis products with retentions between 40 and 41 min. (B) illustrates the separation of the same mixture following chemical peracetylation, with the substrate (tetraacetate) eluting at 49 min and the derived pentaacetate product eluting at 46 min.

determined to be 7.6 ± 0.1 μM; this value is somewhat lower than those of other taxoid hydroxylases for their presumed natural substrates [27–29].

The occurrence in *Taxus* species of many 7β-oxygenated taxoids that vary in the oxygenation pattern and acyl substitutions on the taxane core [33], and the apparent abundance of the F31 clone, suggest that the cytochrome P450 7β-hydroxylase could be a prominent catalyst in vivo that is promiscuous in substrate utilization and thus responsible for generating a range of 7β-hydroxy taxoids. To evaluate the specificity of the 7β-hydroxylase, a number of tritium-labeled, simpler taxa-4(20),11(12)-diene derivatives (taxadiene, taxadien-5α-ol, taxadien-5α-yl acetate, taxadien-5α,13α-diol, taxadien-5α-acetoxy-10β-ol, and taxadien-5α-acetoxy-9α,10β,13α-triol [Figure 6E]) were tested for catalytic conversion by feeding to the transformed yeast harboring clone F31, with [^{3}H-acetyl]taxusin as the positive control. None of these test substrates was detectably oxygenated in overnight assays under conditions where (+)-taxusin was nearly quantitatively converted to 7β-hydroxy taxusin. These results indicated that the 7β-hydroxylase is rather specific in being unable to uti-

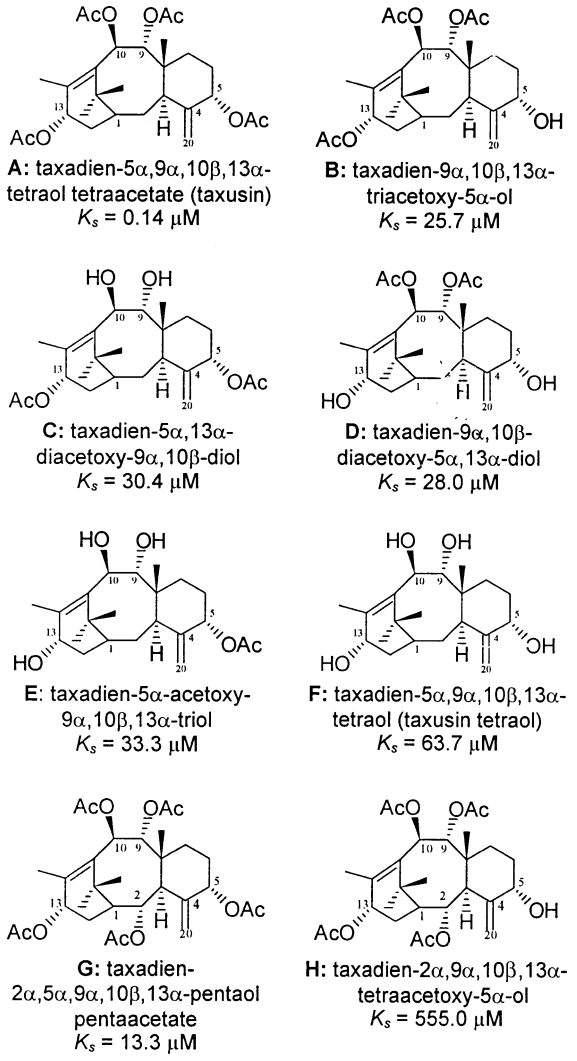

Figure 6. Taxoid Substrates Used in Binding Studies with the 7β-Hydroxylase

The binding constant (K_s) is shown below each polyfunctionalized taxoid.

lize simple (relatively unsubstituted) taxoids as substrates, and they implied that the actual substrate for this enzyme is more highly substituted (hydroxylated and acylated), which is consistent with an intermediate of Taxol biosynthesis that resides somewhere in the middle of this extended pathway.

To assess this possibility, and obtain more information on the substrate selectivity of this enzyme, a range of more highly substituted taxoids was evaluated (see Figure 6). These compounds were not available in radiolabeled form, thereby eliminating the use of the simple in vivo catalytic assay in transformed yeast and necessitating the use of binding competence [39] (as an indirect measure of substrate selectivity) with *Spodoptera* microsomal preparations harboring the recombinant hydroxylase, again with (+)-taxusin as the positive control. The evaluation of these taxadien tetraol and pentaol acetates (Figure 6) showed taxusin (A) to exhibit the tightest binding (i.e., the lowest K_s value), followed in order by the pentaol pentaacetate (G), the tetraol triacetate (B) and the two tetraol diacetates (C and D), the tetraol monoacetate (E), the tetraol corresponding to taxusin (F), and finally the pentaol tetraacetate (H) with the weakest binding affinity. Control microsomes (devoid of the clone F31 hydroxylase) did not appreciably bind these taxoid substrates.

Comparison of binding affinity between taxusin (A) and the corresponding tetraol triacetate (B) (180-fold difference), and between pentaol pentaacetate (G) and pentaol tetraacetate (H) (40-fold difference) indicated that the C5-acetate group is a very important binding determinant. Comparison of the K_s value of the tetraol monoacetate (E) with that of the corresponding tetraol (F) also supports the importance of the C5-acetate (i.e., 2-fold difference in binding). Interestingly, comparison of monoacetate (E) with taxusin (A) reveals a 230-fold difference in binding, indicating that the C5-acetate alone is insufficient for tight binding and catalysis and suggesting that acylations elsewhere on the taxane core contribute to binding. This suggestion is also supported by comparing binding kinetics between the tetraol (F) and its various diacetylated (C and D) and triacetylated (B) derivatives (roughly 2-fold difference). Additionally, comparison of the taxoids with either a free hydroxyl or acetate at C13 (cf. B and D; C and E) shows only minor differences in the K_s values, suggesting that acetylation at C13 is of little significance in binding. The addition of a C2-acetoxy group (cf. A and G) decreases binding by nearly 100-fold (perhaps due to steric interactions), although binding of the pentaacetate (G) is still more favorable (by a factor of two) than any of the partially acylated tetraols (cf. G to B, C, D, and E). Although productive binding is not necessarily indicative of functional oxygenation of the test substrate, the data do strongly suggest that the actual substrate for the 7β-hydroxylase is a well functionalized taxoid, possibly an acylated taxadien-tetraol bearing a 5α-acetoxy group, and that (+)-taxusin was a fortuitous choice as a surrogate for this mid-pathway intermediate. These data relating to the specificity of the cytochrome P450 taxoid 7β-hydroxylase (derived from both the catalytic and binding assays) also suggest that this enzyme is relatively selective, which is not so unusual for a biosynthetic cytochrome P450 of this type. This conclusion implies that the large number of highly functionalized, naturally occurring 7β-hydroxy taxoid derivatives [33] do not arise via promiscuous substrate utilization by the 7β-hydroxylase but rather by the action of more promiscuous oxygenases and acyltransferases that lie downstream of the selective, and seemingly regiospecific, 7β-hydroxylation step.

Significance

A functional screen of *Taxus* cytochrome P450 clones using taxusin as a surrogate substrate yielded a taxoid 7β-hydroxylase that is similar in structure and properties to other taxoid hydroxylases, and that likely operates in the middle section of the Taxol biosynthetic pathway. Consistent with its placement on the pathway, selectivity studies suggest that the true substrate

for this enzyme is a polyoxygenated and acylated taxoid bearing a 5α-acetoxy group. The 7β-hydroxy taxoids, and their C7 acetyl, benzoyl, and xylosyl derivatives, are common metabolites of *Taxus* species [33]. Although the 7β-hydroxyl of Taxol is not essential for antimitotic activity [40], this functional group is the most reactive and accessible hydroxyl of the taxane core [5], and it has been exploited in the synthesis of biologically active photoaffinity probes [41–43], cleavable water soluble derivatives [44, 45], and the C7-methylthiomethyl analog as a highly effective "second generation" taxoid drug [46]. This cytochrome P450 7β-hydroxylase could prove useful in the improved production of Taxol in yew and for the preparation of other 7β-hydroxy taxoids as starting materials for subsequent modification at this position.

Experimental Procedures

Plant Material, Reagents, and Substrates

The *Taxus* cell suspension cultures have been previously described [25]. The preparations of the racemic, simple taxoids taxa-4(5),11(12)-diene, taxa-4(20),11(12)-dien-5α-ol and taxa-4(20),11(12)-dien-5α-yl acetate, taxa-4(20),11(12)-dien-5α,13α-diol, and (+)-taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol (all [20-^{3}H]-labeled at 2 Ci/mol) have been described [16, 17, 28, 47]. (+)-Taxusin was isolated from the heartwood of *T. brevifolia* [11], and (+)-taxa-4(20),11(12)-dien-2α,9α,10β, 13α-tetraacetoxy-5α-ol was a gift from Dr. Tohru Horiguchi (Colorado State University, Fort Collins, CO).

[^{3}H-acetyl]Taxusin

(+)-Taxusin (the tetraacetate of taxa-4(20),11(12)-dien-5α,9α, 10β,13α-tetraol, 75 μmol) was hydrolyzed to the tetraol in excess magnesium methoxide-methanol (8 mL) under reflux for 16 hr, followed by dilution with brine and extraction into CH_2Cl_2. Solvent evaporation provided the tetraol (60% isolated yield), the identity of which was confirmed by comparison to the authentic standard. The tetraol (50 μmol) was reacetylated in CH_2Cl_2 (1 mL) containing dimethylaminopyridine (100 μmol), triethylamine (1 mmol) and [^{3}H]acetic anhydride (5 mCi, 100 μmol, NEN Life Sciences Products) by stirring for 16 hr at room temperature [48], followed by the addition of dimethylaminopyridine (50 μmol), triethylamine (200 μmol), and acetic anhydride (200 μmol) and stirring for another 20 hr to complete the reaction. The reaction was quenched with saturated $NaHCO_3$, extracted with ethyl acetate (3 × 2 mL), and the pooled organic fraction was washed with brine and evaporated. The crude product was purified by preparative TLC (silica gel developed twice with hexane:acetone [3:1, v/v]) to give (+)-[^{3}H-acetyl]taxusin (4.4 μmol, 146 Ci/mol, >95% purity by radio-HPLC).

Taxadien-5α-Acetoxy-9α,10β,13α-Triol

Because of the differing reactivities of the taxadien-tetraol hydroxyls (the 5α-hydroxyl is the most difficult to acylate, followed by 13α, 9α, and 10β), protection was required to direct regiochemistry. The vicinal diol function was protected as the acetonide by stirring a solution of the tetraol (90 μmol) in 0.5 ml dimethylformamide containing *p*-toluene sulfonic acid (1.3 μmol) and 2,2-dimethoxy propane (2 mL) under reflux. The resulting acetonide was purified by silica gel flash column chromatography (hexane:acetone [3:1, v/v]) to give an isolated yield of 95% at purity >95% as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.84 (*s*, CH_3), 0.97 (*s*, CH_3), 1.21 (*dd*, H14α, *J* = 3.9 Hz), 1.43 (*d*, CH_3 in acetonide, *J* = 0.6 Hz), 1.46 (*d*, CH_3 in acetonide, *J* = 0.3), 1.48 (*s*, CH_3), 1.67 (*m*, H2), 1.68 (*m*, H1), 1.74 (*m*, H7), 1.76 (*m*, H6), 2.08 (*d*, *allylic*-CH_3, *J* = 1.2 Hz), 2.85 (*dt*, H14β, *J* = 4.2, 9.8, 15.5 Hz), 3.16 (*br t*, H3, *J* = 2.1 Hz), 4.13 (*d*, H9, *J* = 9.3 Hz), 4.33 (*t*, H5, *J* = 2.5 Hz), 4.37 (*br d*, H13, *J* = 9.3 Hz), 4.70 (*d*, H20, *J* = 1.2 Hz), 4.90 (*d*, H10, *J* = 9.6 Hz), and 5.07 (*d*, H20, *J* = 1.2 Hz).

To protect the 13α-hydroxyl, the TES ether was prepared from the acetonide (77 μmol) in 0.5 ml CH_2Cl_2 containing chlorotriethylsilane (13 μL), dimethylaminopyridine (50 μmol) and triethylamine (50 μL) by stirring at room temp for only 30 min to prevent silylation at the 5α-position. The reaction was quenched and extracted, and the product was purified by silica gel flash column chromatography as before to give an isolated yield of 34% at purity >95% as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.23 (*q*, TES methyl, *J* = 8.0 Hz), 0.63 (*t*, TES methylene, *J* = 8.0 Hz), 0.68 (*s*, CH_3), 0.94 (*dd*, H14α, *J* = 2.0 Hz), 1.16 (*s*, CH_3 in acetonide), 1.20 (*s*, CH_3 in acetonide), 1.29 (*s*, CH_3), 1.48 (*m*, H2), 1.50 (*m*, H1, 1.60 (*m*, H7), 1.64 (*m*, H6), 1.85 (*d*, *allylic*-CH_3, *J* = 1.2), 2.56 (*dt*, H14β, *J* = 3.0, 9.2, 15.3 Hz), 3.47 (*br t*, H3, *J* = 4.8 Hz), 4.33 (*d*, H9, *J* = 9.6 Hz), 4.37 (*t*, H5, *J* = 2.6 Hz), 4.49 (*br d*, H13, *J* = 9.6 Hz), 4.66 (*d*, H20, *J* = 1.2 Hz), 5.04 (*d*, H20, *J* = 1.2 Hz), and 5.23 (*d*, H10, *J* = 9.3 Hz). The C13-TES ether, 9,10-acetonide of the tetraol was then acetylated at C5 as above, and the product was purified by silica gel flash column chromatography (hexane:acetone [19:1, v/v]) to give an isolated yield of 36% at purity >95% as determined by ^{1}H-NMR. (300 MHz, $CDCl_3$) δ: 0.48 (*q*, TES methyl, *J* = 8.0), 0.88 (*t*, TES methylene, *J* = 8.0 Hz), 0.88 (*s*, CH_3), 1.13 (*s*, CH_3), 1.10 (*dd*, H14α, *J* = 8.0 Hz), 1.46 (*s*, CH_3 in acetonide), 1.50 (*s*, CH_3 in acetonide), 1.60 (*s*, CH_3), 1.70 (*m*, H2), 1.72 (*m*, H1), 1.76 (*m*, H7), 1.78 (*m*, H6), 2.02 (*s*, $COCH_3$), 2.02 (*d*, *allylic*-CH_3, *J* = 1.2 Hz), 2.40 (*dt*, H14β, *J* = 4.8, 9.6, 14.1 Hz), 2.88 (*br t*, H3), 4.32 (*d*, H9, *J* = 9.6 Hz), 4.62 (*d*, H20, *J* = 0.9 Hz), 4.77 (*t*, H13, *J* = 8.6 Hz), 5.00 (*d*, H10, *J* = 9.3 Hz), 5.07 (*d*, H20, *J*, = 0.9 Hz), and 5.46 (*t*, H5, *J* = 2.7 Hz). The TES and acetonide groups were then removed in 0.5 N HCl by a published method [49], and the product was purified by silica gel TLC as above to give an isolated yield of 40% at purity >90% as determined by ^{1}H-NMR. (300 MHz, $CDCl_3$), δ: 0.90 (*s*, CH_3), 1.01 (*s*, CH_3), 1.25 (*dd*, H14α), 1.47 (*s*, CH_3), 1.68 (*m*, H2), 1.70 (*m*, H1), 1.72 (*m*, H7), 1.89 (*m*, H6), 2.06 (*d*, *allylic*-CH_3, *J* = 1.5 Hz), 2.10 (*s*, $COCH_3$), 2.86 (*dt*, H14β, *J* = 3.3, 9.9, 15.3 Hz), 3.01 (*br t*, H3, *J* = 3.8 Hz), 4.06 (*d*, H9, *J* = 9.3 Hz), 4.44 (*dd*, H13, *J* = 2.5 Hz), 4.82 (*d*, H10, *J* = 9.9 Hz), 4.86 (*d*, H20, *J* = 1.2 Hz), 5.19 (*d*, H20, *J* = 1.2 Hz), and 5.35 (*t*, H5, *J* = 3 Hz).

This procedure was repeated using [^{3}H]acetic anhydride to generate the radiolabeled form at 53 Ci/mol.

Taxadien-2α,5α,9α,10β,13α-Pentaol Pentaacetate

The pentaacetate (see Figure 6H) was prepared from the corresponding tetraacetate (see Figure 6G), and purified by TLC as above, followed by HPLC (Metachem 5 μ Taxsil column [250 × 4.6 mm] eluted with CH_3CN:H_2O [1:4, v/v; 5 min hold] followed by a linear gradient [1 mL/min] to CH_3CN:H_2O [4:1, v/v] with UV monitoring) to give an isolated yield of 80% at purity >95% as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.89 (*s*, CH_3), 1.14 (*s*, CH_3), 1.5 (*dd*, H14α, *J* = 7.2 Hz), 1.76 (*s*, CH_3), 1.73 (*m*, H7), 1.88 (*m*, H6), 1.94 (*br d*, H1, *J* = 9.6 Hz), 2.14 (*d*, *allylic*-CH_3, *J* = 1.2 Hz), 2.01 (*s*, $COCH_3$), 2.02 (*s*, $COCH_3$), 2.5 (*s*, $COCH_3$), 2.1 (*s*, $COCH_3$), 2.22 (*s*, $COCH_3$), 2.56 (*dt*, H14β, *J* = 4.2, 9.9, 15 Hz), 3.28 (*d*, H3, *J* = 6.6 Hz), 4.77 (*d*, H20), 5.29 (*t*, H5, *J* = 1.8 Hz), 5.34 (*d*, H20), 5.47 (*dd*, H2, *J* = 2.6 Hz), 5.91 (*br t*, H13, *J* = 8.9 Hz), 5.93 (*d*, H9, *J* = 10.5 Hz), and 6.03 (*d*, H10, *J* = 10.8 Hz).

Taxadien-5α,13α-Diacetoxy-9α,10β-Diol

The 9,10-acetonide of taxusin tetraol prepared previously was acetylated at C5 and C13, deprotected, and purified as described above to give the diacetoxy diol at a purity of >95% as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.91 (*s*, CH_3), 1.06 (*dd*, H14α, *J* = 8 Hz), 1.17 (*s*, CH_3), 1.52 (*s*, CH_3), 1.66 (*m*, H2), 1.72 (*m*, H7), 1.78 (*m*, H1), 1.82 (*m*, H6), 1.95 (*d*, *allylic*-CH_3, *J* = 1.8 Hz), 2.07 (*s*, $COCH_3$), 2.14 (*s*, $COCH_3$), 2.67 (*dt*, H14β, *J* = 4.5, 9.5, 14.8 Hz), 2.94 (*br t*, H3, *J* = 4.8 Hz), 4.14 (*d*, H9, *J* = 9.6 Hz), 4.80 (*d*, H10, *J* = 9.6 Hz), 4.82 (*d*, H20, *J* = 1.5 Hz), 5.19 (*d*, H20, *J* = 1.5 Hz), 5.35 (*t*, H5, *J* = 3 Hz), and 5.54 (*br t*, H13, *J* = 8.7 Hz).

Taxadien-9α,10β,13α-Triacetoxy-5α-ol

Taxusin tetraol was partially acetylated as before but without protecting groups, and the reaction was monitored by analytical TLC and quenched after the completion of triacetate formation (1 hr). The triacetoxy monool product was purified by flash chromatography as above to give an isolated yield of 60% at purity >90% as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.71 (*s*, CH_3), 0.99 (*s*, CH_3), 1.13 (*dd*, H14α, *J* = 4.9 Hz), 1.57 (*s*, CH_3), 1.72 (*m*, H7), 1.75 (*m*, H1), 1.78 (*m*,

H2), 1.82 (*m*, H6), 2.10 (*d*, *allylic*-CH_3, J = 1.5 Hz), 2.01 (*s*, $COCH_3$), 2.05 (*s*, $COCH_3$), 2.07 (*s*, $COCH_3$), 2.79 (*dt*, H14β, J = 4.8, 10.1, 15.3 Hz), 3.29 (*d*, H3, J = 3.9 Hz), 4.29 (*t*, H5, J = 3 Hz), 4.75 (*d*, H20, J = 1.5 Hz), 5.12 (*d*, H20, J = 1.5 Hz), 5.71 (*dd*, H13, J = 3.9 Hz), 5.76 (*d*, H9, J = 10.5 Hz), and 6.09 (*d*, H10, J = 10.5 Hz).

Taxadien-9α,10β-Diacetoxy-5α,13α-Diol

Taxusin tetraol was regioselectively acetylated with recombinant taxoid 10-*O*-acetyltransferase [22] which is capable of acylating hydroxyls at both C9 and C10 positions. The isolated product was purified by HPLC as above to give the diacetoxy diol at >95% purity as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.70 (*s*, CH_3), 0.90 (*s*, CH_3), 1.20 (*dd*, H14α, J = 3.9 Hz), 1.50 (*s*, CH_3), 1.70 (*m*, H1), 1.70 (*m*, H6), 1.74 (*m*, H2), 1.92 (*m*, H7), 2.22 (*d*, *allylic*-CH_3, J = 1.2 Hz), 2.00 (*s*, $COCH_3$), 2.04 (*s*, $COCH_3$), 2.81 (*dt*, H14β, J = 3.3, 9.1, 15.6 Hz), 3.26 (*d*, H3, J = 4.5 Hz), 4.31 (*t*, H5, J = 2.7 Hz), 4.33 (*dd*, H13, J = 3 Hz), 4.71 (*d*, H20, J = 1.2 Hz), 5.07 (*d*, H20, J = 1.2 Hz), 5.71 (*d*, H9, J = 10.5 Hz), and 6.07 (*d*, H10, J = 10.2 Hz).

Taxus Cell Microsome Preparation and Assay

Taxus cuspidata suspension cell cultures (cell line P93AF) were induced with methyl jasmonate, the frozen cells (40 g) were extracted (1 mM benzamidine was substituted for phenylmethylsulfonyl fluoride in the buffer), and microsomes were prepared by established protocols [16, 17]. [H^3-acetyl]Taxusin (10^6 dpm, 10 Ci/mol) was added to a 10 ml screw-capped tube and the assay was preformed as previously described [17]. The diethyl ether extract was evaporated, and the residue was dissolved in 2 ml of pentane:ether (1:1, v/v) and loaded onto a small column of silica gel (Mallinckrodt SilicAR 60 Å) which was washed with 6 ml of the same solvent to remove residual substrate, followed by elution with 8 ml diethyl ether to obtain the more polar products. The latter fraction was evaporated, and the residue was dissolved in CH_3CN and separated by radio-HPLC (Metachem Taxsil column developed with CH_3CN:H_2O [1:4, v/v; 5 min hold] followed by a linear gradient [1 mL/min] from 20% to 80% CH_3CN with monitoring using a Packard A-100 flow-through radio-detector).

Cytochrome P450 Expression and In Vivo Assay in Yeast

Procedures for the functional expression of *Taxus* cytochrome P450 cDNA clones in *S. cerevisiae* WAT11 cells [34] and the in vivo assay have been previously described [26, 29]. For the purpose of product identification, a 250 ml expression culture (A_{600} ~0.8) was divided into 10 ml aliquots from which the transformed cells were harvested by centrifugation and each resuspended in 3 ml of induction medium to which 100 μM (+)-taxusin (in DMSO) was added. Following incubation for 16 hr at 30°C with shaking, each tube was treated in a sonication bath (10 min), saturated with NaCl, and extracted with hexane (3 × 2 mL). The pooled extracts were evaporated, and the residue was dissolved in pentane:ether (1:1, v/v) and loaded onto and eluted from a silica gel column with the same solvent to remove residual substrate. The presumptive hydroxyl taxusin product was then eluted from the column with ether, the solvent concentrated, and the material purified by TLC (silica gel developed once with hexane:acetone [19:1, v/v] and twice with hexane:acetone [3:1, v/v]), eluted from the gel with acetone, concentrated and dissolved in CH_3CN, and further purified by HPLC (Alltech Adsorbosphere HS C18, 5 μ column [250 × 4.6 mm]) with an isocratic gradient of 40% CH_3CN in H_2O. Fractions containing the product were combined, concentrated, and extracted with pentane to provide pure material for spectrometric analysis.

Spectrometric Analyses

To simplify MS analysis of the putative hydroxyl taxusin (that contained partially deacetylated material from host-derived esterases), the mixture was peracetylated and purified by reverse-phase HPLC as described above. LC-MS (atmospheric pressure chemical ionization, APCI) analysis was performed on a Hewlett-Packard (Agilent) series 1100 HPLC with model 1946A mass detector. The product was resolved on a Supelcosil Discovery HS-F5 column (250 × 4.6 mm) with a Metachem Metaguard HS-H5 guard column. The column was developed with CH_3CN:H_2O (1:19) at 5% to 100% CH_3CN over a 50 min linear gradient (1 ml/min). The MS method has been previously described [32].

Coupled capillary GC-MS analyses were performed on a Hewlett-Packard 6890 MSD system (electron impact at 70 eV ionizing voltage) using a Phenomenex ZB-5 column (30 min × 0.25 mm, 5% phenyl polysiloxane) and cool on-column injection, with a previously described method [17].

For NMR analysis of the purified product, the material was repeatedly dissolved in and evaporated to dryness from $CDCl_3$ and then dissolved in 0.5 ml $CDCl_3$. ^{1}H-NMR was performed on a 300 MHz Varian Mercury NMR spectrometer, and ^{13}C-NMR was performed on a 600 MHz Bruker NMR spectrometer. The nuclear Overhauser effect difference (NOE-DIF) experiment, two-dimensional NOE spectroscopy (NOESY), and two-dimensional homonuclear total correlation spectroscopy (TOCSY) were performed on the Varian 300 MHz instrument.

Cytochrome P450 Expression in Sf9 Cells

Heterologous expression of *Taxus* cytochrome P450 cDNA clones in *Spodoptera furgiperda* (Sf9) cells that coexpress the *Taxus* NADPH:cytochrome P450 reductase was carried out as previously described [28], except that the baculovirus infection period was extended to 45 min and harvest was extended to 60 hr post-infection. Microsome preparation was also as before [28] but, in this case, the lysis buffer contained 20 mM HEPES (pH 7.5), 5 mM DTT, 10 mM lidocaine, 10 mM procaine, and 1 mM benzamidine, and the final resuspension buffer was 25 mM Tris (pH 7.5) containing 20% (v/v) glycerol. The concentration of microsomal cytochrome P450 was determined by CO-difference spectroscopy [35], and the assay for oxygenation of taxusin (and other taxoids) was as before (with 100 pmol of cytochrome P450 in a 0.5 ml assay volume) but the NADPH concentration was reduced to 0.5 mM and a regenerating system (2 mM glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase) was added. Incubation was for 16 hr at 31°C in the dark, with product extraction and purification as before. Control experiments were identically carried out but with a β-glucuronidase gene replacing the cytochrome P450 insert.

Characterization of the 7β-Hydroxylase

The pH optimum of the recombinant taxoid 7β-hydroxylase (cytochrome P450 clone F31 expressed in *Spodoptera*) was determined in MOPSO (pH 6.5–7.5), HEPES (pH 7.0–8.0), Tris (pH 6.5–8.0), and phosphate (pH 5.0–9.5) buffers over intervals of 0.5 pH units. The K_m value for (+)-taxusin (substrate concentration range of 5 to 300 μM) was determined by Eadie-Hofstee plotting using Microsoft Excel software. To simplify the radio-HPLC assay described above, the products were peracetylated prior to analysis. Conversion was determined by integration of the pentaacetate product radio-peak relative to that of the substrate, and the reported values are the means ± SD of three independent replicates.

Substrate binding assays [39] were performed with a Perkin-Elmer Lambda 18 spectrophotometer using established protocols [17] and *Spodoptera* microsomes containing the 7β-hydroxylase. Double-reciprocal plotting of the difference between the maximum (390 nm) and minimum (420 nm) absorbance for each substrate concentration was used to determine the binding constant (K_s).

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