# Taxol biosynthesis: Taxane 13$\alpha$-hydroxylase is a cytochrome P450-dependent monooxygenase

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**A central feature in the biosynthesis of Taxol is oxygenation at multiple positions of the taxane core structure, reactions that are considered to be mediated by cytochrome P450-dependent monooxygenases. A PCR-based differential display-cloning approach, using *Taxus* (yew) cells induced for Taxol production, yielded a family of related cytochrome P450 genes, one of which was assigned as a taxane 10$\beta$-hydroxylase by functional expression in yeast. The acquired clones that did not function in yeast were heterologously expressed by using the *Spodoptera fugiperda*-baculovirus-based system and were screened for catalytic capability by using taxa-4(20),11(12)-dien-5$\alpha$-ol and its acetate ester as test substrates. This approach allowed identification of one of the cytochrome P450 clones (which bore 63% deduced sequence identity to the aforementioned taxane 10$\beta$-hydroxylase) as a taxane 13$\alpha$-hydroxylase by chromatographic and spectrometric characterization of the corresponding recombinant enzyme product. The demonstration of a second relevant hydroxylase from the induced family of cytochrome P450 genes validates this strategy for elucidating the oxygenation steps of taxane diterpenoid (taxoid) metabolism. Additionally, substrate specificity studies with the available cytochrome P450 hydroxylases now indicate that there is likely more than one biosynthetic route to Taxol in yew species.**

paclitaxel | taxa-4(20),11(12)-dien-5$\alpha$,13$\alpha$-diol | *Taxus cuspidata* | yew

Taxol (generic name paclitaxel; Fig. 1) is a highly effective anticancer drug used widely in the treatment of various carcinomas, melanomas, and sarcomas (1). This structurally complex taxane diterpenoid (taxoid) was first isolated from the bark of the Pacific yew (*Taxus brevifolia* Nutt.) (2). Despite great difficulties in obtaining sufficient quantities of Taxol for clinical trials from this source (3, 4), the unique mode of action of this drug, as well as its outstanding potency against several tumor cell lines, ultimately led to the development of Taxol as one of the most efficacious anticancer agents in current use (5, 6).

Taxol is presently derived largely by semisynthesis from the advanced taxoid 10-deacetylbaccatin III, which can be obtained from needles of the European yew, *Taxus baccata* (7–9). However, approval of Taxol for the treatment of additional types of cancer, as well as the use of the drug much earlier in the course of intervention, is very likely to increase the demand for Taxol and its precursors for semisynthesis. Additionally, extensive studies on the naturally occurring taxoids, now numbering over 350 (10), have led to the discovery of novel structures and to the preparation of new chemical derivatives with improved biological activity (11–14). These "second generation" taxoid drugs, like Taxol itself, still depend on the isolation of natural products from the yew tree. Several elegant total synthetic routes to Taxol and related taxoids have been developed (15–20); however, low yields and high costs preclude them from commercial production (21) and, for the foreseeable future, sourcing must continue to rely on *Taxus* species or, potentially, cell cultures derived therefrom (6). Improved biological production methods depend on an understanding of the biosynthetic pathways leading to Taxol and related taxoids and definition of the responsible enzymes and genes.

Taxol biosynthesis involves about 20 enzymatic steps from primary plant metabolism, and several of these steps have now been defined (22–24). The committed step of the pathway is the initial cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate to taxa-4(5),11(12)-diene to establish the taxane core structure (refs. 25 and 26; Fig. 1). This parent olefin is next hydroxylated at the C5 position by a cytochrome P450 enzyme (27), representing the first of eight oxygenation steps (of the taxane core) on route to Taxol. The resulting intermediate, taxa-4(20),11(12)-dien-5$\alpha$-ol, can be acetylated (Fig. 1) by a well defined acetyltransferase (28), for which the corresponding gene has been identified (29). Recent studies, using microsomal preparations from *Taxus* cells induced for Taxol production (30), have shown that taxa-4(20),11(12)-dien-5$\alpha$-yl acetate is converted, by cytochrome P450-dependent reactions, to polyoxygenated taxoids by way of taxa-4(20),11(12)-dien-5$\alpha$,10$\beta$-diol 5-acetate (31). This same set of experiments also demonstrated the microsomal cytochrome P450-dependent conversion of taxa-4(20),11(12)-dien-5$\alpha$-ol itself to polyoxygenated derivatives by means of taxa-4(20),11(12)-dien-5$\alpha$,13$\alpha$-diol (Fig. 1), thereby allowing some uncertainty as to the precise order of the early pathway hydroxylation and acylation steps diverging from the confirmed intermediate taxadienol (31).

The central importance of cytochrome P450-mediated oxygenation reactions in Taxol biosynthesis, and the uncertainties surrounding the precise order of hydroxylation of the taxane core, prompted the development of an approach based on differential display of mRNA-reverse transcription-PCR (using induced and uninduced *Taxus* cells) to isolate a family of related cytochrome P450 clones that could be screened by expression in yeast (32). This strategy led to the isolation of a cDNA encoding the above-indicated taxane 10$\beta$-hydroxylase (32). However, not all of the acquired cytochrome P450 clones could be functionally expressed in yeast, thus forcing the development of an alternate means of expression based on the *Spodoptera fugiperda*-baculovirus system (33, 34). This alternative approach has allowed definition of a second important clone encoding the taxane 13$\alpha$-hydroxylase, thereby confirming the utility of the original cloning strategy and allowing substrate specificity studies with the recombinant enzymes to examine the order of early hydroxylation steps and the possible routes to Taxol.

## Materials and Methods

**Enzymes, Substrates, and Reagents.** Enzymes and reagents were obtained from the indicated sources and were used according to the manufacturers' instructions. The preparation of [20-$^3$H]taxa-4(5),11(12)-diene, [20-$^3$H]taxa-4(20),11(12)-dien-5$\alpha$-ol, and [20-$^3$H]taxa-4(20),11(12)-dien-5$\alpha$-yl acetate (all at 2 Ci/mol) have been described (27, 31, 35). The [20-$^3$H]taxa-4(20),11(12)-dien-

Abbreviation: Sf9, *S. fugiperda* cells.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY056019).

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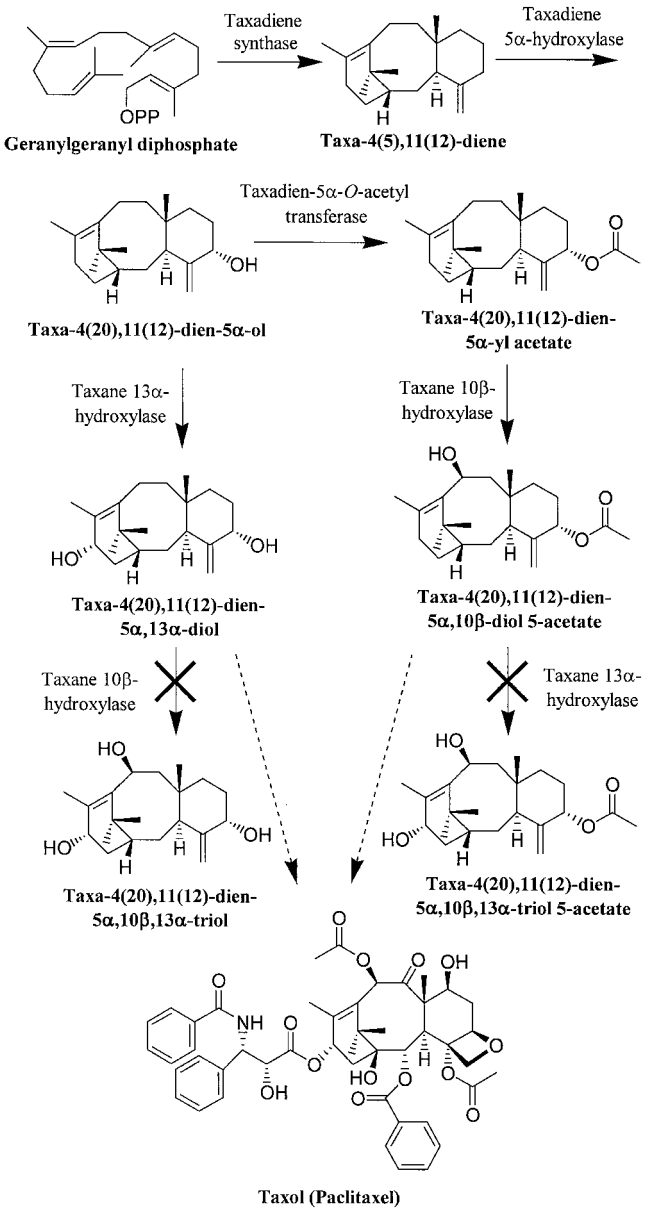


**Fig. 1.** Outline of the Taxol biosynthetic pathway illustrating the cyclization of geranylgeranyl diphosphate to taxadiene, the hydroxylation to taxadien-5α-ol, acetylation to the ester, and subsequent 10β-hydroxylation and 13α-hydroxylation of the appropriate intermediates. The large X indicates that the illustrated reaction did not occur with the noted enzyme. The broken arrows signify undefined steps.

5α,10β-diol 5-acetate was obtained from [20-$^3$H]taxa-4(20),11(12)-dien-5α-yl acetate via biotransformation, using microsomal membranes obtained from *S. fugiperda* (Sf9) cells coinfected with two recombinant baculoviruses containing, respectively, the taxane 10β-hydroxylase cDNA (32) and the *Taxus* NADPH:cytochrome P450 reductase gene. For details of the procedure, including product isolation by HPLC, see below.

**Heterologous Expression of Cytochrome P450 Clones in Insect Cells.** For the functional identification of the previously acquired *Taxus cuspidata* cytochrome P450 clones that did not express efficiently in yeast (32), an alternative approach based on the Sf9-baculovirus (*Autographa californica*) expression system (33, 34) was used. For the construction of recombinant baculovirus, the cytochrome P450 ORFs were amplified by PCR using *Pfu* DNA polymerase and gene-specific primers containing appropriate restriction sites immediately upstream of the starting ATG and downstream of the stop codon. Additionally, a reverse primer was designed with six additional codons for histidine residues immediately upstream of the stop codon to permit installation of a C-terminal $His_6$-tag in the expressed protein. The amplicons, so obtained by standard PCR procedures, were gel-purified, subcloned first into pCR-Blunt vectors (Invitrogen), and then the ORFs were excised, using the added restriction sites, and ligated into similarly restricted pFastBac1 vectors (Life Technologies, Grand Island, NY). The sequence and the correct insertion of the cytochrome P450 clones were confirmed, and the pFastBac1/cytoP450ORF constructs were then used for the preparation of the corresponding recombinant Bacmid DNA by transformation of *Escherichia coli* strain DH10Bac (Life Technologies). This protocol as well as the transfection of Sf9 cells were carried out accordingly to the manufacturer's protocol.

Sf9 cells were propagated either as adherent monolayer cultures in Grace insect cell medium supplemented with 10% FCS (both from Life Technologies) or as suspension cultures in Grace medium containing 10% FCS/0.1% Pluronic F-68 (Sigma). The adherent cell cultures were maintained in a chamber at 28°C, and the suspension cultures were incubated in a rotary shaker at 28°C and 150 rpm.

For functional characterization of the cytochrome P450 clones, recombinant baculovirus carrying the cytochrome P450 ORF was coexpressed with recombinant baculovirus carrying the *T. cuspidata* NADPH:cytochrome P450 reductase (S.J. and R.C., unpublished data). To the insect cell suspension (1.5–2.0 $\times$ $10^6$ log-phase cells per ml), the two recombinant baculoviruses were each added at a multiplicity of infection of 1 to 5 [viral titers were determined by the end-point dilution method (36)]. For infection, the insect cells of a 750-ml cell culture (1 liter conical flask) were collected by centrifugation (700 $\times$ *g* for 10 min at room temperature) resuspended in 75 ml of the original cell culture volume, and incubated for 1 h at 28°C and 80 rpm in the presence of added baculovirus. The culture volume was then brought to 750 ml with standard medium, and hemin (Sigma) was added to a final concentration of 2 $\mu$g/ml to compensate for the low heme synthetic capability of the insect cells. The infected cells were then incubated for 48 h in a gyratory shaker at 28°C and 140 rpm, harvested by centrifugation (700 $\times$ *g* for 10 min at room temperature), and washed and harvested twice with 50 mM $KH_2PO_4$ (pH 7.5) containing 0.9% NaCl. The washed cell pellet was then resuspended in 5 ml of 50 mM Hepes buffer (pH 7.5) containing 0.5 mM EDTA/0.1 mM DTT/10% (vol/vol) glycerol, and the cells were lysed by sonication, using the microtip probe (VirSonic, VirTis). Cell debris was removed by centrifugation (10,000 $\times$ *g* for 10 min at 4°C), and the resulting supernatant was then centrifuged at 105,000 $\times$ *g* for 1 h at 4°C to provide the microsomal enzyme preparation, which was either assayed immediately or frozen for later use in liquid $N_2$ and stored at −80°C. The protein content of these microsomal preparations was determined by the Bradford method (37) with BSA as standard. Control experiments were identically conducted, except that a recombinant baculovirus containing a β-glucuronidase gene was substituted for that containing the cytochrome P450 gene.

**Assay for Cytochrome P450 Oxygenase Activity.** The standard assay was conducted in a total volume of 1 ml of 0.25 M Tris·HCl buffer (pH 7.5) containing 200 $\mu$M NADPH/2.5 $\mu$M FMN/2.5 $\mu$M FAD/2 mM glucose-6-phosphate/2 units glucose-6-phosphate dehydrogenase/≈600 $\mu$g microsomal protein/50 $\mu$M substrate {[20-$^3$H]taxa-4(5),11(12)-diene, [20-$^3$H]taxadien-5α-ol, [20-$^3$H]taxadien-5α-yl acetate, or [20-$^3$H]taxadien-5α,10β-diol 5-acetate}. Samples were incubated at 32°C for 1 h after which 1 ml of saturated aqueous NaCl was added, and the reaction mixture was extracted twice with 2 ml of hexane:ethyl acetate

(4:1 vol/vol). The combined organic extracts were then evaporated under $N_2$ and either dissolved in hexane and subjected to GC-MS analysis as described (27, 31) or in $CH_3CN$ for separation by RP radio-HPLC [250 mm × 4.6 mm column of Alltech Associates Econosil $C_{18}$ (5 μm)] [solvent A: 97.99% $H_2O$ with 2% $CH_3CN$ and 0.01% $H_3PO_4$ (vol/vol); solvent B: 99.99% $CH_3CN$ with 0.01% $H_3PO_4$ (vol/vol); 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), 70–75 min at 100% (A); flow rate of 1 ml/min with radio-detection of effluent (Flow-One-Beta Series A-1000; Radiomatic Flo-One, Meriden, CT)]. This same procedure was used to isolate biosynthetically prepared [20-$^3$H]taxa-4(20)11(12)-dien-5α,10β-diol 5-acetate described previously.

For preparative scale conversions, to obtain product for NMR analysis, a substrate concentration of 250 μM was used, and the incubation was extended overnight. The product was isolated as before and subjected to an additional normal-phase TLC purification step [20 cm × 20 cm × 0.5 mm silica gel 60 F254 (Merck, Darmstadt, Germany); developing solvent toluene:acetone (3:1 vol/vol); detection by radio-monitoring], after which the material ($R_f$ ≈0.5) was eluted from the plate with $CH_3CN$, dried under $N_2$, and then repeatedly dissolved in and taken to dryness from perdeuterobenzene to remove traces of the original solvents. The putative taxadiendiol (≈600 μg by scintillation counting and >99% pure by GC) was finally dissolved in deuterobenzene to a final concentration of 2 mM for NMR analysis, using a Varian Unity-500 spectrometer, equipped with a very sensitive proton indirect detection probe, under described conditions (27, 31).

**Enzyme Characterization.** CO-difference spectra (38) were recorded by using a Perkin–Elmer Lambda 18 spectrophotometer under conditions described elsewhere (31). To monitor heterologous expression and subcellular localization of the gene product, immunoblot analysis was performed by taking advantage of the appended C-terminal $His_6$-tag, using, as primary Ab, the Penta-His-specific Ab (Qiagen, Valencia, CA) and, as secondary Ab, alkaline phosphatase-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch). For this purpose, 50 μg of soluble or microsomal protein per lane was separated by SDS/PAGE (10% denaturing gel), transferred by blotting to nitrocellulose, and immobilized by UV-crosslinking. Membrane preparation, Ab hybridization, and colorimetric detection were carried out according to the Qiagen protocols.

The pH optimum of the microsomal taxane 13α-hydroxylase was determined in 0.25 M Tris·HCl (pH 7–9) and phosphate (pH 5.5–8.5) buffers over intervals of 0.5 pH unit. $K_m$ and $V_{rel}$ values were determined under standard assay conditions with substrate concentrations ranging from 2 to 100 μM. The SPSS (Chicago) SIGMAPLOT ENZYME KINETICS 1.10 software package was used, with the Michaelis–Menten method, and the data reported are the means ± SD of triplicate analyses.

## Results and Discussion

**Expression of *Taxus* Cytochrome P450 Monooxygenases in Insect Cells.** The initial steps in the biosynthesis of Taxol involve the cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene (25, 26) followed by cytochrome P450-mediated hydroxylation at C5 of the olefin, with allylic rearrangement, to yield taxa-4(20),11(12)-dien-5α-ol (ref. 27; Fig. 1). Further transformations on the pathway to Taxol are considered to involve multiple, additional cytochrome P450-dependent oxygenations of the taxane core (39) and several CoA-dependent acylations (29, 40, 41) of which acetylation at C5 may be the first (28); however, the precise order of these reactions diverging from taxadienol is not known (22–24). A recent study, using induced *Taxus* cell microsomal preparations with taxa-4(20),11(12)-dien-5α-ol and taxa-4(20),11(12)-dien-5α-yl acetate as substrates, demonstrated the cytochrome P450-mediated conversion of the acetate ester to polyoxygenated monoacetate products via taxadien-5α,10β-diol 5-acetate and the P450-dependent conversion of the 5α-alcohol to polyoxygenated products via taxadien-5α,13α-diol (ref. 31; Fig. 1). Both pathways seemed to be kinetically competent *in vitro*, suggesting multiple, perhaps intersecting, early routes for taxoid metabolism. However, the plethora of oxygenated products formed by *Taxus* microsomes (31) greatly complicated the interpretation of these results and suggested that further definition of the pathway would require cloning and characterization of the individual cytochrome P450 species involved.

Protein purification of cytochrome P450 enzymes from *Taxus* microsomes as the basis for cDNA cloning was eliminated as not feasible, because the number of P450 species involved and their likely similarity in physical properties would almost certainly preclude bringing the individual proteins to homogeneity for amino acid microsequencing. As an alternative approach to cytochrome P450 cloning by reverse genetics, a strategy (32) was used that was based on a differential display of mRNA-reverse transcription-PCR method for the isolation of transcriptionally active cytochrome P450s in *Taxus* cells induced (with methyl jasmonate) for Taxol production (30). This strategy led to the acquisition of a family of unique but closely related cytochrome P450 sequences that were expressed in yeast (*Saccharomyces cerevisiae*) and yielded the taxane 10β-hydroxylase as a 1497-bp cDNA encoding a 498-residue cytochrome capable of transforming taxadienyl acetate to the 10β-hydroxy derivative. However, only about half of the full-length clones afforded a characteristic CO-difference spectrum with absorbance maximum near 450 nm (38), indicating the presence of functional heme-containing and properly folded recombinant cytochrome P450 in the yeast host (32). To further develop and validate this general approach to defining the oxygenation steps of the Taxol biosynthetic pathway, a more reliable functional expression system was required.

As an alternative means for functional expression of the *Taxus* cytochrome P450 clones, the *Spodoptera fugiperda*-baculovirus system (33, 34) was used. In preliminary control experiments, Sf9 insect cells were infected with recombinant *A. californica* baculovirus containing either the cytochrome P450 reductase or a β-glucuronidase gene. Neither the crude insect cell lysate nor the derived microsomal fraction from uninfected or control Sf9 insect cells was capable of detectably metabolizing the test substrates (taxadiene, taxadien-5α-ol, taxadien-5α-yl acetate, and taxadien-5α,10β-diol 5-acetate) under cytochrome P450 assay conditions, thereby confirming that these cells were devoid of endogenous activity. Next, the previously acquired taxane 10β-hydroxylase (GenBank accession no. AF318211), as the C-terminal $His_6$-tagged version, was similarly expressed, and the derived microsomes were shown to possess respectable 10β-hydroxylase activity for the conversion of taxadien-5α-yl acetate to taxadien-5α,10β-diol 5-acetate. The CO-difference spectrum indicated the presence of ≈240 pmol of cytochrome P450 per mg of microsomal protein. These results demonstrated that the His-tagged version of a *bona fide* taxane hydroxylase could be functionally expressed with the *Spodoptera*-baculovirus system.

Although functional expression of the test clone was achieved, CO-difference spectra (38) of microsomes (or of solubilized preparations) harboring other recombinant cytochrome P450s sometimes showed only moderate increases in absorption at 450 nm compared with the controls (see Fig. 2 for example), and in a few cases, the dithionite-reduced CO-bound species was highly unstable. Thus, for the cytochrome P450 clone designated F16, the absorption maximum at 450 nm of the difference spectrum illustrated (Fig. 2) vanished within 3–5 min. Nevertheless, immunoblot analysis of the recombinant preparations (exploiting the His-tag epitope) confirmed expression of the cytochrome P450 transgenes (and the exclusive localization of the recombi-

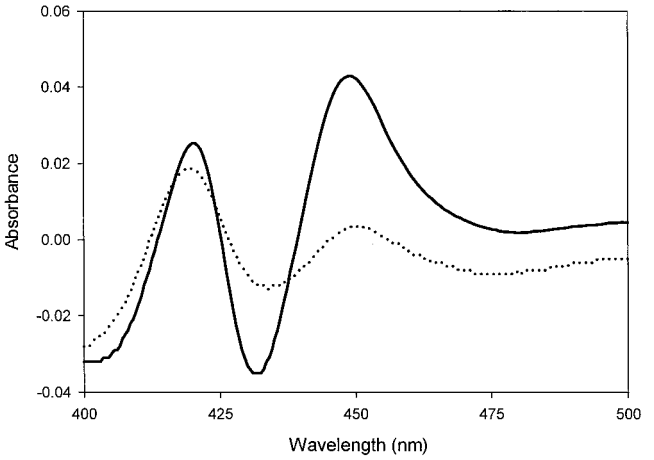


**Fig. 2.** CO-difference spectra recorded with solubilized microsomal protein from *S. fugiperda* cells infected with recombinant *A. californica* baculovirus harboring either cytochrome P450 clone F16 (solid line) or the $\beta$-glucuronidase gene (negative control, dotted line). For solubilization with Emulgen 911 (Kao Chemical, Tokyo), 6 mg of microsomal protein was used and a solubilization efficiency of about 50% was achieved.

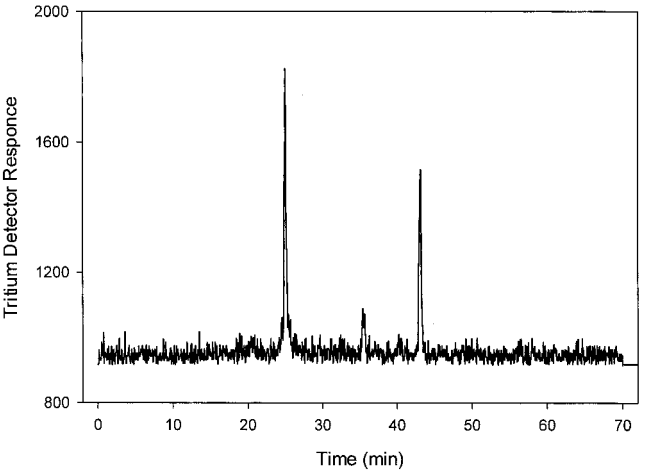


**Fig. 3.** Radio-HPLC analysis of the biosynthetic product ($R_t$ = 25.6 min) generated from taxadien-5$\alpha$-ol ($R_t$ = 43.4 min) as substrate by the recombinant cytochrome P450 hydroxylase clone F16 coexpressed with the *Taxus* cytochrome P450 reductase.

nant gene products to the microsomal fraction; data not shown) and suggested that these cytochrome P450 proteins were often present in excess of that deduced from the CO-difference spectra of the chemically reduced enzymes.

For functional characterization of the *Taxus* cytochrome P450 cDNAs, the insect cells were coinfected with a recombinant baculovirus harboring the cytochrome P450 gene under consideration and with a recombinant baculovirus containing the *Taxus* NADPH:cytochrome P450 reductase gene. The rationale for this coinfection protocol is based on the low level of endogenous cytochrome P450 reductase in *S. fugiperda* cells ($\approx$12 milliunits/mg of microsomal protein) and on the need to maximize compatibility in electron transfer between the reductase and the cytochrome P450 enzymes (42). This expression strategy increased cytochrome P450 reductase activity of the insect cell microsomes to over 240 milliunits/mg of protein.

With the utility of this coexpression system thus demonstrated, Sf9 cells coinfected with the reductase and with each of the *Taxus* cytochrome P450 clones (32) were cultured, the corresponding light membranes were prepared and evaluated by CO-difference spectra and immunoblotting, and each was assayed with the test substrates (taxadiene, taxadien-5$\alpha$-ol, and taxadien-5$\alpha$-yl acetate), after which the organic extracts were analyzed by radio-HPLC. By using this HPLC-based screen, the gene product of one of the clones, designated F16, was shown to convert taxa-4(20),11(12)-dien-5$\alpha$-ol to a more polar product (Fig. 3). GC-MS analysis of the isolated product revealed the presence of a taxadiendiol with parent ion ($P^+$) of $m/z$ 304 [taxadienol molecular weight = 288, plus *O* (mass 16)] (Fig. 4). Analysis of the full spectrum, including diagnostic ions at $m/z$ 286 ($P^+ - H_2O$), 271 ($P^+ - H_2O - CH_3$) and 253 ($P^+ - H_2O - CH_3 - H_2O$), suggested a tentative identification of the enzymatic product as taxa-4(20),11(12)-dien-5$\alpha$,13$\alpha$-diol, which was observed previously by *in vitro* assay of *Taxus* microsomes (31). Large-scale microsomal preparation allowed the generation and TLC-based isolation of about 600 $\mu$g of the putative taxadiendiol (>99% purity by GC) for NMR analysis. Because all of the $^1H$ resonances of taxa-4(20),11(12)-dien-5$\alpha$,13$\alpha$-diol had been previously assigned, the proton spectrum (Fig. 5) and its correlation spectra were readily interpreted along the lines originally described (31). Confirmation of the identity of the biosynthetic product as taxadien-5$\alpha$,13$\alpha$-diol indicated that a cDNA (clone F16) encoding the cytochrome P450 taxane 13$\alpha$-hydroxylase had been isolated.

**Sequence Analysis.** The translated nucleotide sequence of clone F16 (GenBank accession no. AY056019; ORF 1,458 bp) encodes a 485-aa protein for which a molecular weight of 54,652 was calculated, in agreement with the size of the enzyme observed by SDS/PAGE. Analysis of the deduced amino acid sequence revealed several characteristics typical of cytochrome P450 enzymes (43), including an N-terminal membrane anchor, the oxygen-binding domain, a highly conserved heme-binding motif with PFG element (amino acids 435–438), and an absolutely conserved cysteine at position 431. Alignment of the *T. cuspidata* taxane 13$\alpha$-hydroxylase with the previously acquired taxane 10$\beta$-hydroxylase (ref. 32; Fig. 6) demonstrated an overall sequence identity of 63% with a sequence similarity of 72%. Both hydroxylases resemble most closely the sequences of cytochrome P450 oxygenases involved in the metabolism of gibberellins and brassinosteroids (44–46), two other classes of complex polycyclic terpenoids of plant origin.

**Enzyme Characterization.** For characterization of the taxane 13$\alpha$-hydroxylase, microsomal preparations from *S. fugiperda* cells that coexpressed the F16 clone and the *Taxus* NADPH:cyto-

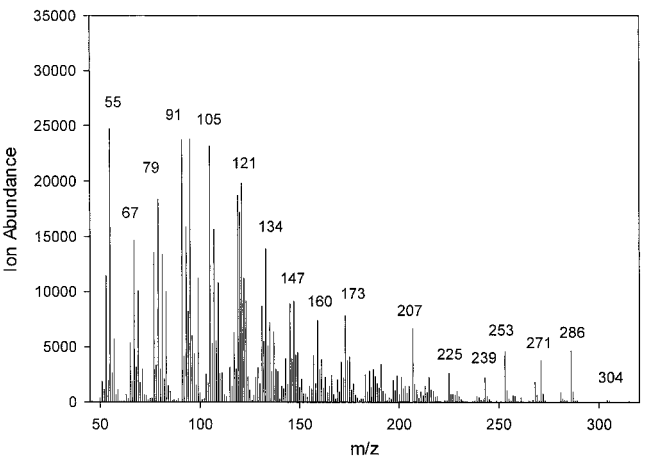


**Fig. 4.** GC-MS analysis of the biosynthetic product derived from taxadien-5$\alpha$-ol by the recombinant cytochrome P450 hydroxylase clone F16 coexpressed with *Taxus* cytochrome P450 reductase.

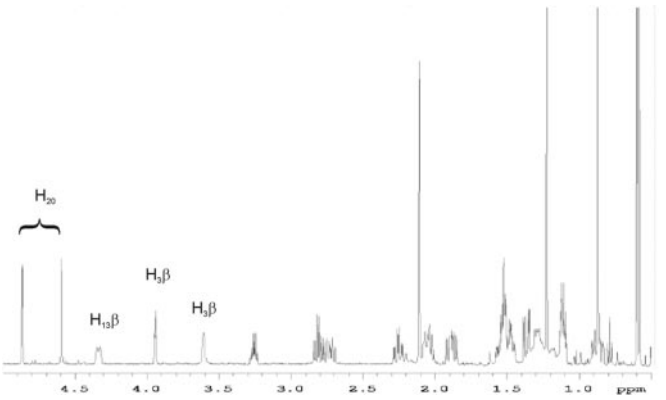


**Fig. 5.** $^{1}$H-NMR analysis (in deuterobenzene) of the biosynthetic product derived from taxadien-5$\alpha$-ol by the recombinant cytochrome P450 hydroxylase clone F16 coexpressed with *Taxus* cytochrome P450 reductase.

chrome P450 reductase gene were used. The pH optimum for this microsomal hydroxylase was determined to be near pH 7.5 (with broad activity profile), and the highest activity was observed in Tris·HCl buffer. Kinetic evaluation of the 13$\alpha$-hydroxylase was carried out with both taxadienol and taxadienyl acetate as substrates. The $K_m$ values for the alcohol and acetate ester were determined to be 24 $\pm$ 9 $\mu$M and 14 $\pm$ 4 $\mu$M, respectively. These values are essentially identical to those measured with native enzyme in *Taxus* microsomes (31). The rate of conversion of the acetate ester to the 13$\alpha$-hydroxylated derivative was only about 6% of that for the conversion of taxadien-5$\alpha$-ol to the 5$\alpha$,13$\alpha$-diol.

To examine the possibility that the 13$\alpha$-hydroxylase acted on the product of the previously defined 10$\beta$-hydroxylase and *vice versa*, the required substrates were prepared biosynthetically by using the corresponding *Spodoptera*-baculovirus expression system. Thus, [20-$^{3}$H]taxa-4(20),11(12)-dien-5$\alpha$,10$\beta$-diol 5-acetate was prepared from taxadien-5$\alpha$-yl acetate, using the recombinant 10$\beta$-hydroxylase, and [20-$^{3}$H]taxa-4(20),11(12)-dien-5$\alpha$,13$\alpha$-diol was prepared from taxadien-5$\alpha$-ol, using the recombinant 13$\alpha$-hydroxylase. Incubation of taxadien-5$\alpha$,10$\beta$-diol 5-acetate, under standard cytochrome P450 assay conditions, with the recombinant 13$\alpha$-hydroxylase did not yield a product, nor did taxadien-5$\alpha$,13$\alpha$-diol yield a detectable polar metabolite when incubated with the recombinant 10$\beta$-hydroxylase under standard assay conditions. These results, demonstrating apparently rather strict substrate selectivity for both recombinant hydroxylases, indicate that the trihydroxylated intermediate of Taxol biosynthesis is not likely taxa-4(20),11(12)-dien-5$\alpha$,10$\beta$,13$\alpha$-triol or the corresponding 5$\alpha$-monoacetate ester (see Fig. 1).

## Conclusions

The development of the *Spodoptera*-baculovirus system based on coexpression with the *Taxus* cytochrome P450 reductase has allowed functional screening of additional *Taxus* cytochrome P450 clones and has led to the acquisition of a taxane 13$\alpha$-hydroxylase, which is similar in sequence and properties to the taxane 10$\beta$-hydroxylase (32). These results validate the original strategy for acquisition of taxane-metabolizing cytochrome P450s and suggest that the target cytochrome P450 genes responsible for the eight oxygenation steps (of the taxane core) on route to Taxol reside within the family of related clones now in hand (32).

The order of the regiospecific oxygenation steps of the Taxol pathway [beyond the hydroxylation at the C5$\alpha$-position (27)] is not known; however, consideration of the relative abundances of known taxoids bearing oxygen functions at different positions of the

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f14 : MDSFIFLRSIGTKFGQLESSPAILSLTLAPILAIILLLLPRYNHRSSVKLPPGKLGFPLIGETIQLLRTTRSETP :  75
f16 : ----------MDALKQLEVSPSILFVTLAVMAGTILF--FRSKRHSSVKLPPGNLGFPLVGETLQFVRSLGSSTP :  63

f14 : QKFFDDRLKKFGPVYMTSLIGHPTVVLCGPAGNKLVLSNEDKLVEMEGPKSFMKLIGEDSIVAKRGEDHRILRTA : 150
f16 : QQFIEEFMSKFGDVFKTSIIGHPTVVLCGPAGNRLVLSNENKLVQMSWPSSMMKLIGEDCLGGNTGEQHRIVRAA : 138

f14 : LAPFLCAQALQNYLGRMSSETGHHFNEKWKGKDEVKVLPLVRGLIFSIASTLFFDVNDGHQQKQLFHLLETI--G : 223
f16 : LTRFLGPQALQNHFAKMSSGIQRHINEKWKGKDEATVLPLVKDLVFSVASRLFFGITEEHLQEQLFNLLEVILVG : 213

f14 : GLSVPLDFPGTRMRKGLQARLKLDETLSSLIKRRRRDLRSGIASDDQDLLSVLLTFRDEKGNSLTDQGLLDNFSA : 298
f16 : SFSVPLNIPGFSMHKAIQARATLADIMTHLIEKRRNELRAGTASENQDLLSVLLTFTDERGNSLADKELLDNFSM : 288

f14 : MFHASYDTLVAPMALIFKLLYSNPEYHEKVFQEQLEIIGNKKEGEEISWKDLKSMKYTWQAVQESLRMYPPVFGI : 373
f16 : LLHGSYDSLNSPLTMLIKVLASHPEVSYEKVAQEQFGILSTKMEGEEIAWKDLKEMKYSWQVVQETLRMYPPIFGT : 363

f14 : FRKAITDITYDGYTIP-GWRVLCSPYTTHLREEYFPEPEEFRPSRFEDEGRHVTPYTYVPFGGGLRTCPGWEFSK : 447
f16 : FRKAITDTTYNGYTIPKGWKLLWTTYSTQTKEEYTKDADQFKPSRFEEGKHVTPYTYLPFGGGMRVCPGWEFAK : 438

f14 : IEILLFVHHFVKNFSSYIPVDPNEKVLSDPLPPLPANGFSIKLFPRS---------- : 494
f16 : METLLFLHHFVKAFSGLKAIDPNEKLSGKPLPPLPVNGLPIKLYSRS---------- : 485
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**Fig. 6.** Deduced sequence alignment of the taxane 10$\beta$-hydroxylase (clone F14) and the taxane 13$\alpha$-hydroxylase (clone F16). Shaded residues indicate identical amino acids.

taxane core suggests that the sequence of oxygenations is at C5 then C10, followed by oxygenation at C2 and C9, then at C13 (47). Oxygenations at C7 and C1 of the taxane nucleus are considered to be very late introductions. It is important to note that this proposal for the sequence of oxygenation steps is based solely on tabulation of the several hundred known taxane structures (10), most of which cannot reside on the Taxol pathway and must represent metabolic diversions and dead-ends, and therefore some caution in the interpretation is advised. The finding of a taxane 13$\alpha$-hydroxylase that operates on a taxadien-monool substrate, in combination with the previous *in vitro* studies with *Taxus* microsomes (31), now suggest that hydroxylation at the C13 position may occur earlier in the *Taxol* pathway than previously thought.

Substrate selectivity studies with the available recombinant taxane hydroxylases have now shown that the 10$\beta$-hydroxylase and 13$\alpha$-hydroxylase pathway routes do not likely intersect at the level of a taxadien-5$\alpha$,10$\beta$,13$\alpha$-triol (Fig. 1), thus leaving several possible interpretations. It is possible that one of the hydroxylases is being viewed out of sequence and that the taxadienol or taxadienyl acetate is simply an adventitious substrate with very competent kinetics for the corresponding enzyme. Alternatively, at least two pathways to Taxol, one via taxadien-5$\alpha$,10$\beta$-diol 5-acetate and the other via taxadien-5$\alpha$,13$\alpha$-diol, may exist and may possibly intersect at a later polyoxygenated intermediate. To resolve these issues, it is necessary to search for additional taxane hydroxylases, particularly those responsible for the subsequent metabolism of the 5$\alpha$,13$\alpha$-diol and the 5$\alpha$,10$\beta$-diol 5-monoacetate.

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