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Preliminary assessment of the C13-side chain 2′-hydroxylase involved in Taxol biosynthesis

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Abstract

The biosynthesis of the anticancer drug Taxol in yew (*Taxus*) species is thought to involve the preliminary formation of the advanced taxane diterpenoid intermediate baccatin III upon which the functionally important *N*-benzoyl phenylisoserinoyl side chain is subsequently assembled at the C13-*O*-position. In vivo feeding studies with *Taxus* tissues and characterization of the two transferases responsible for C13-side chain construction have suggested a sequential process in which an aminomutase converts α-phenylalanine to β-phenylalanine which is then activated to the corresponding CoA ester and transferred to baccatin III to yield β-phenylalanoyl baccatin III (i.e., *N*-debenzoyl-2′-deoxytaxol) that undergoes subsequent 2′-hydroxylation and N-benzoylation to afford Taxol. However, because the side chain transferase can utilize both β-phenylalanoyl CoA and phenylisoserinoyl CoA in the C13-O-esterification of baccatin III, ambiguity remained as to whether the 2′-hydroxylation step occurs before or after transfer of the amino phenylpropanoyl moiety. Using cell-free enzyme systems from *Taxus* suspension cells, no evidence was found for the direct hydroxylation of β-phenylalanine to phenylisoserine; however, microsomal preparations from this tissue appeared capable of the cytochrome P450-mediated hydroxylation of β-phenylalanoyl baccatin III to phenylisoserinoyl baccatin III (i.e., *N*-debenzoyltaxol) as the penultimate step in the formation of Taxol and related N-substituted taxoids. These preliminary results, which are consistent with the proposed side chain assembly process, have clarified an important step of Taxol biosynthesis and set the foundation for cloning the responsible cytochrome P450 hydroxylase gene.

Keywords: Taxol; Paclitaxel; Biosynthesis; Side chain assembly; Cytochrome P450 oxygenase; β-Phenylalanoyl baccatin III-2′-hydroxylase; *N*-Debenzoyltaxol; *Taxus*; Yew

Taxol [1] (generic name paclitaxel, Fig. 1) is well established as a potent antineoplastic drug with excellent activity against a range of cancers [2]. This taxane diterpenoid (taxoid), derived from yew (*Taxus*) species [3], continues to find wide application in the treatment of additional cancer types, for earlier use in disease intervention, and in combination with other chemotherapeutic agents [4]. Total syntheses of Taxol have been achieved by several elegant routes [5] but the approach is commercially impractical [6]. The increased demand for the drug therefore shifted destructive isolation from the original, low-yielding source (the bark of the Pacific yew, *Taxus brevifolia*) to semisynthetic approaches [7]. These methods involve synthetic side chain attachment to the C13-*O*-position of more readily available baccatin III derivatives (Fig. 1) that can be isolated in higher yield from the needles of various *Taxus* species as a renewable resource. For the foreseeable future, the supply of Taxol and its precursors will continue to rely on yew species [8] or cell cultures derived therefrom [9], and drug sourcing and patient treatment costs will remain critical issues [10]. For these reasons, it is important to understand the biosynthesis of Taxol and its underlying molecular genetics, with the view to improving drug yields by transgenic means. This approach holds most promise for *Taxus* cell culture production methods [11].

The Taxol biosynthetic pathway is considered to require 19 enzymatic steps from the universal diterpenoid precursor geranylgeranyl diphosphate which is cyclized, in the committed step, to taxa-4(5),11(12)-diene [12]. This

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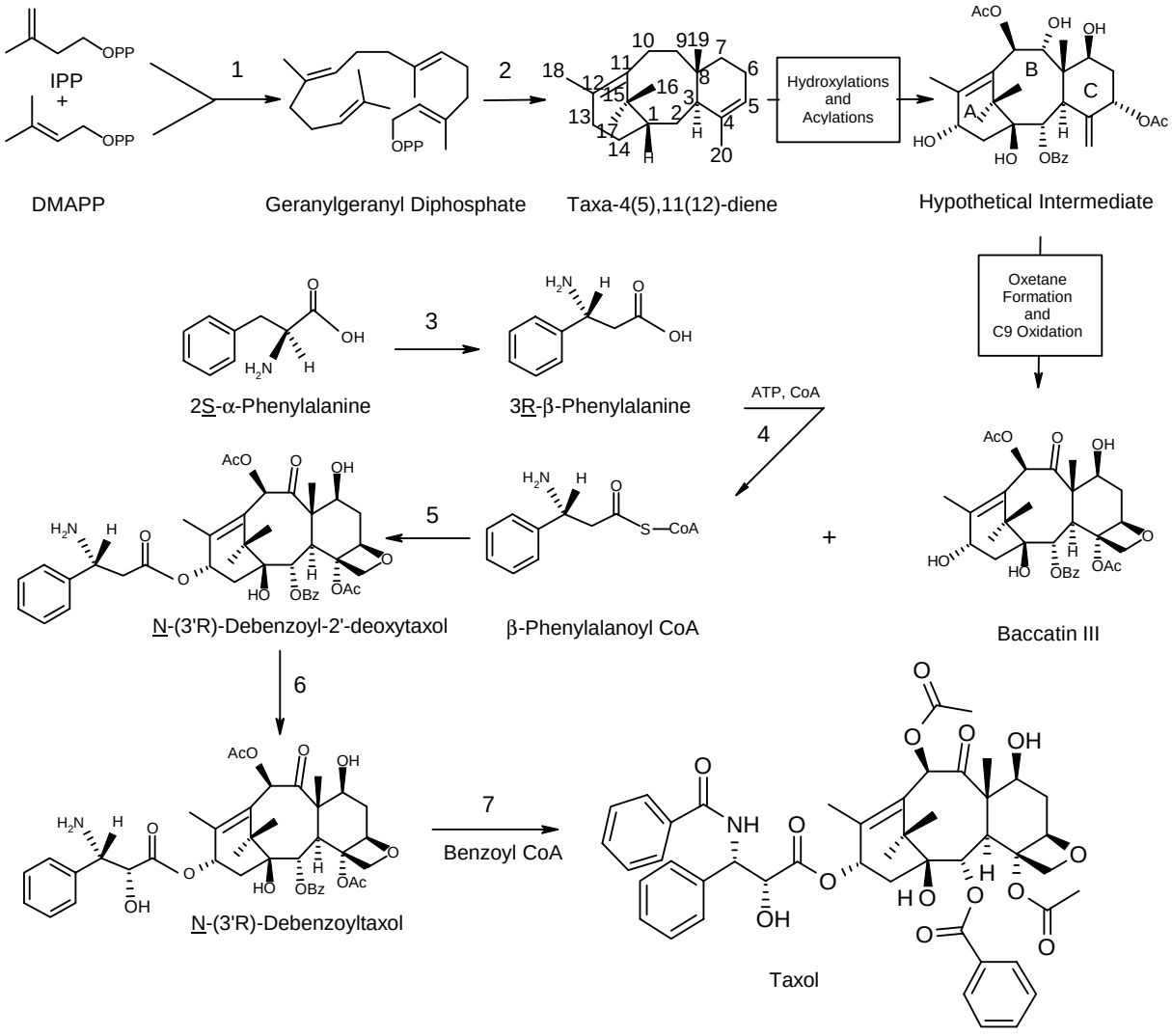

Fig. 1. Outline of Taxol biosynthesis involving the construction of baccatin III and the final assembly of the C13-side chain. The enzymatic steps indicated are: 1, geranylgeranyl diphosphate synthase; 2, taxadiene synthase; 3, phenylalanine aminomutase; 4, amino phenylpropanoyl CoA ligase; 5, amino phenylpropanoyl-13-*O*-transferase; 6, side chain-2′-hydroxylase; and 7, benzoyl-*N*-transferase. Ac is the acetyl group, Bz is the benzoyl group, and OPP is the diphosphate moiety.

parental olefin (Fig. 1) is then functionalized by a series of eight cytochrome P450-mediated oxygenations and three CoA-dependent acylations, and undergoes oxidation at C9 and a ring expansion step to form the oxetane group en route to the late stage intermediate baccatin III. Considerable information is now available about the enzymes and genes involved in the construction of baccatin III [13], to which the functionally important side chain at C13 [14] is then appended (in five additional steps) to complete the pathway to Taxol.

Previous in vivo studies with *Taxus*, in which labeled amino acids were used to elucidate the mode of assembly of the C13-side chain, showed that the intact side chain (*N*-benzoyl phenylisoserine) was not incorporated into Taxol, whereas β-phenylalanine and, to a lesser extent, α-phenylalanine and phenylisoserine were utilized as precursors [15]. These results, along with earlier work on the origin of β-phenylalanine from α-phenylalanine [16,17], suggest that β-phenylalanine (derived by mutation of α-phenylalanine [18]), as the free amine, is attached to baccatin III, and that this side chain is hydroxylated and N-benzoylated to complete the route to Taxol (Fig. 1).

These biosynthetic results prompted a search for the enzymes and genes involved in C13-side chain assembly, which has recently yielded the phenylalanine aminomutase [19] and the two responsible transferases [20,21]. In the case of the transfer reactions, functional screening of a family (58–69% sequence identity within the group) of previously acquired *Taxus* acyl/aroyl transferase cDNAs [12,22] afforded the benzoyl CoA-dependent, regio- and stereo-selective transferase responsible for the final benzamidation step of the Taxol pathway [20], and the C13 phenylpropanoid side chain-CoA acyl transferase that mediates the critical 13-O-esterification of baccatin III [21]. The kinetic properties and specificity of this latter recombinant transferase, which catalyzes the attachment of the biologically important

Taxol side chain precursor, indicate that, at saturation with the baccatin III and amino phenylpropanoyl CoA co-substrates, the enzyme prefers β-phenylalanoyl CoA ($V_{rel} = 1.0$) over 3-phenylisoserinoyl CoA ($V_{rel} = 0.4$); α-phenylalanoyl CoA and *N*-benzoyl phenylisoserinoyl CoA are not productive acyl donors ($V_{rel} < 0.01$).

The evidence thus far suggests that the biogenesis of the side chain begins with the isomerization of α-phenylalanine to β-phenylalanine by the aminomutase [19], which is converted by an as yet undefined ligase to the reactive CoA ester and transferred to the C13-hydroxyl of baccatin III to yield 13-*O*-β-phenylalanoyl baccatin III (i.e., *N*-debenzoyl-2′-deoxytaxol), as a true intermediate on the pathway to Taxol, which subsequently undergoes C2′-hydroxylation and N-benzoylation as final side chain modifications [20,21]. However, the lack of absolute selectivity of the C13-side chain transferase leaves open the possibility that 2-hydroxylation of β-phenylalanine precedes transfer (i.e., of phenylisoserine) to the baccatin III core rather than transfer of β-phenylalanine and subsequent 2′-hydroxylation of the 13-O-substituted baccatin III. This ambiguity in timing has stimulated a more direct evaluation of the side chain hydroxylation step using cell-free extracts of *Taxus* suspension cell cultures that produce Taxol [23]. The failure thus far to demonstrate direct hydroxylation of β-phenylalanine to phenylisoserine in these cell-free enzyme systems under reaction conditions described for other amino acid hydroxylases [24], and the fact that all other oxygenations of the taxoid core are seemingly mediated by cytochrome P450 enzymes [13] led to an evaluation of *Taxus* cell microsomes for the target activity. The results described herein are entirely consistent with the cytochrome P450-catalyzed 2′-hydroxylation of β-phenylalanoyl baccatin III (*N*-debenzoyl-2′-deoxytaxol) to phenylisoserinoyl baccatin III (*N*-debenzoyltaxol), and they support the proposed assembly sequence in which side chain transfer precedes 2′-hydroxylation.

Materials and methods

Plant cell cultures. The source and preparation of (uninduced) suspension cell cultures of *Taxus* × *media* cv Hicksii (cell line Mh00D) have been described, as have the methods for taxoid isolation and LC–MS analysis [23,25].

Substrates and reagents. [13-^{3}H]*N*-Debenzoyl-(3′*R*)-2′-deoxytaxol (19.5 Ci/mol) was synthesized at the 100 mg scale by literature methods [26,27], except that *N*-Boc-(3*R*)-β-phenylalanine (Peptech, Burlington, MA) was coupled with [13-^{3}H]7-triethylsilyl-baccatin III with deprotection as described [28]. The free-amine product was partially purified via preparative, normal-phase TLC by development twice with CH_2Cl_2/MeOH (95:5, v/v), and a diffuse, low R_f band was collected and the silica gel was eluted with MeOH. The eluate was brought to pH 9 by dropwise addition of conc. NH_4OH, then extracted with CH_2Cl_2 (3×), and the extract was concentrated for separation by tapered-layer silica gel TLC (Analtech, Newark, DE) by development twice with CH_2Cl_2/MeOH as before. The product ($R_f = 0.3$) was eluted from the gel with MeOH, and the identity as *N*-debenzoyl-2′-deoxytaxol (β-phenylalanoyl baccatin III) was confirmed by LC–MS.

The conversion of [13-^{3}H]*N*-debenzoyl-(3′*R*)-2′-deoxytaxol to [13-^{3}H](3′*R*)-2′-deoxytaxol (of the same spec. act.) was carried out by the Schotten–Baumann N-benzoylation procedure as described [28]. [13-^{3}H]Baccatin III (72 Ci/mol) was synthesized as described [29] but included an additional deprotection step of the 7-triethylsilyl derivative using HF [28]. [7-^{14}C]Benzoyl CoA (0.019 Ci/mol) was prepared by literature procedure [28], and other acyl CoA thioester salts were purchased from Sigma Chemical (St. Louis, MO). The synthesis of [1-^{14}C]3*R*-β-phenylalanine (3-amino-3-phenylpropanoic acid; 0.14 Ci/mol) was carried out as described [30] starting from [^{14}C]NaCN (Sigma). Ferredoxin and ferredoxin reductase, as well as all of the inhibitors and biochemicals mentioned below, were obtained from Sigma.

Taxus cell feeding studies. To compare in vivo conversions and relative incorporation rates of putative taxoid intermediates and side chain precursors, [^{3}H]*N*-debenzoyl-(3′*R*)-2′-deoxytaxol (10^7 dpm at 19.5 Ci/mol), [^{3}H](3′*R*)-2′-deoxytaxol (10^7 dpm at 19.5 Ci/mol), [^{3}H]baccatin III (10^7 dpm at 19.5 Ci/mol), and [^{14}C]3*R*-β-phenylalanine (10^7 dpm at 0.14 Ci/mol) were each dissolved in 0.5 mL MeOH and added to separate wells of a sterile six-well plate (Cellstar, Copell, TX). After evaporation of the solvent, 10 mL of 8-day-old *T.* × *media* (cell line Mh00D) cell suspension was added to each well, and the plate was shaken at 120 rpm at room temperature for 10 days in the dark. The media were then removed by vacuum filtration and extracted with an equal volume of CH_2Cl_2, and the filtered cells were also extracted CH_2Cl_2 assisted by sonication. The organic extracts were dried under vacuum, and the residues were redissolved in CH_3CN, filtered through 0.2 μm PTFE syringe filters, and analyzed by radio-HPLC as described below.

Enzyme isolation and assay. The soluble enzyme fraction from 14-day-old *T.* × *media* suspension cell cultures (line Mh00D) was prepared by methods previously described to isolate operationally soluble taxoid acyl transferases from this tissue [22] except that, for the isolation of putative oxygenases, the initial centrifugation step was carried out at 3000*g* for 20 min, and the buffer was 100 mM Hepes (pH 7.2) containing 15% (v/v) glycerol, 1 mM $MgCl_2$, 2 mM EGTA, 5 mM dithiothreitol, 5 mM $Na_2S_2O_5$, 10 mM glycerol-1-phosphate, 10 mM lidocaine, 10 mM procaine, and 1 mM benzamidine.

The microsomal enzyme fraction from 14-day-old *T.* × *media* suspension cell cultures (line Mh00D) was prepared by methods employed previously to examine cytochrome P450 oxygenases of taxoid metabolism [31,32] with the following modifications: frozen, pulverized cells, after transfer to extraction buffer, were further homogenized using a glass Ten-Broek tissue grinder; the intermediate centrifugation stop at 20,000*g* was omitted; and the resulting microsomal pellet was resuspended in 25 mM Hepes buffer (pH 7.2) containing 5% (v/v) glycerol, 1 mM EGTA, 10 mM $MgCl_2$, 1 mM KCl, 1 mM dithiothreitol, and 25 μg leupeptin/mL.

Aliquots of the soluble enzyme or microsomal fractions (500 μg protein) were assayed with either [^{14}C]β-phenylalanine (400 μM, 28.6 nCi), [^{3}H]*N*-debenzoyl-(3′*R*)-2′-deoxytaxol (130 μM, 2.5 μCi), or [^{3}H](3*R*′)-2′-deoxytaxol (130 μM, 2.5 μCi) as substrate, in the presence of all possible combinations of ferredoxin (1.5 mM), ferredoxin reductase (5 μM), Fe^{2+} (5 mM), α-ketoglutarate (50 or 100 μM), sodium ascorbate (5 mM), FMN (2.5 μM), FAD (2.5 μM), NADPH (1 mM), and a regenerating system (for NADPH where appropriate) consisting of glucose-6-phosphate (2 mM) and glucose-6-phosphate dehydrogenase (0.2 U), with incubation for 1 h at 27 °C in air, in the dark.

The target product [^{14}C]3-phenyl-(3*R*)-isoserine (from [^{14}C]3*R*-β-phenylalanine) was isolated by adjusting the assay mixture to pH 9 (with sat. $NaHCO_3$) to neutralize the free amine, followed by extraction with three volumes of ethyl acetate, and concentration of solvent. To facilitate the analysis by reversed-phase radio-HPLC, the target product was derivatized by N-benzoylation and methylation (with diazomethane) to the 2-*N*-benzoyl-3-phenylisoserine methyl ester by methods previously described [28]. The target product [^{3}H]*N*-debenzoyltaxol (from [^{3}H]*N*-debenzoyl-(3′*R*)-2′-deoxytaxol) was extracted from the assay mixture with ethyl acetate after adjustment to pH 9, as before. For the analysis by radio-HPLC, the product was converted to Taxol via N-benzoylation [28].

The N-acylated derivatives were analyzed by reversed-phase radio-HPLC using an Agilent 1100 Series HPLC coupled to a Packard A-100

flow-through radio-detector using 3a70b scintillation cocktail (RPI, Mt. Prospect, IL). The primary analytical method employed a Phenomenex Curosil 5 μPFP column (250 × 4.6 mm) developed (at 1 mL/min) with CH_3CN/H_2O starting at 20% CH_3CN to 80% CH_3CN (linear gradient over 60 min), then to 100% CH_3CN (10 min hold), followed by re-equilibration to starting conditions. Product identification was determined by matching retention times of radio-peaks to those of authentic standards. Additional verification was provided by substituting [ring-$^2H_5$]benzoyl chloride in the N-benzoylation derivatization reaction to permit analysis of any stable isotope-labeled product by LC–MS methods previously described [25]. Analysis of the free amine forms (of substrates and products) was also performed directly by radio-HPLC using the same instrumentation and column as above but with development (at 1 mL/min) using CH_3CN: 10 mM ammonium formate (pH 3) starting at 30% CH_3CN to 100% CH_3CN (linear gradient over 40 min with 10 min hold), followed by 5% CH_3CN wash (10 min) and re-equilibration to starting conditions.

Enzyme characterization. In addition to the aforementioned substrates, enzyme preparations were also evaluated with [^{3}H]baccatin III (200 μM) and [^{3}H]benzoyl CoA (50 μM) as substrates to determine the presence of endogenous side chain precursors and the relevant side chain transferase [21] and *N*-benzoyl transferase activities [20]. Cytochrome P450 CO-difference spectra were obtained by methods previously described for *Taxus* cell microsomes [32]. The influence of CO saturation on enzyme activity was also determined by sparging the reaction mixture with CO for 4 min prior to initiating the assay by addition of NADPH and incubation in air. Miconazole and clotrimazole were tested as inhibitors by separate addition (at 100 μM) to the assay mixtures prior to initiating the reaction by addition of NADPH. NADPH and flavin (FAD and FMN) dependence were tested by omission of the appropriate cofactor from the reaction mixture. Positive control reactions with microsomal preparations were carried out for the cytochrome P450 taxoid 5α-hydroxylase, 10β-hydroxylase, and 13α-hydroxylase as previously described [33–35].

Results

Preliminary studies

In an initial attempt to define the sequence of the assembly steps of C13-*O*-side chain construction in Taxol biosynthesis, labeled β-phenylalanine, baccatin III, *N*-debenzoyl-2′-deoxytaxol, and 2′-deoxytaxol (see Fig. 1) were compared as precursors by in vivo feeding studies with *T.* × *media* cells. Incubation under standard conditions, followed by isolation and radio-HPLC analysis of the derived metabolites, revealed incorporation of radiolabel from baccatin III, β-phenylalanine, and *N*-debenzoyl-2′-deoxytaxol into Taxol, taxol B, (cephalomannine) and taxol C (Fig. 2) in a roughly equal proportion, with the highest conversion efficiency for baccatin III; [^{3}H]2′-deoxytaxol was not detectably incorporated into Taxol (or either

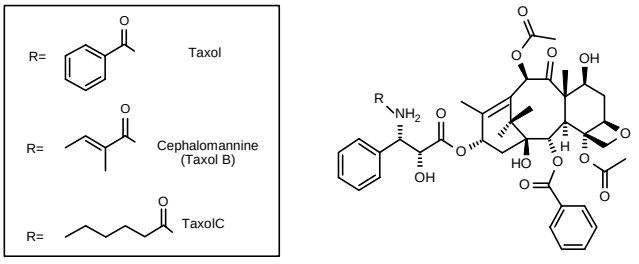

Fig. 2. The structures of advanced taxoids.

of the two N-acylated taxol homologues). These results, particularly the conversion of *N*-debenzoyl-2′-deoxytaxol coupled to the apparent failure of 2′-deoxytaxol to undergo hydroxylation, are consistent with previous feeding studies with intact yew tissue [15] and with the selectivities of the recombinant transferases involved in side chain construction [20,21], and they support the proposed pathway to Taxol (Fig. 1) involving transfer of β-phenylalanine (via the CoA ester) to baccatin III, followed by 2′-hydroxylation and final N-benzoylation.

However, the apparently more efficient incorporation of baccatin III than the presumed advanced intermediate *N*-debenzoyl-2′-deoxytaxol, as well as the failure of baccatin III to detectably label the supposed intermediates *N*-debenzoyl-2′-deoxytaxol and *N*-debenzoyltaxol on route to Taxol and its congeners, were unanticipated; the seemingly less efficient labeling from β-phenylalanine can be rationalized as resulting from dilution with endogenous material [19]. It was not feasible to determine possible differences in uptake efficiency between baccatin III and the two side chain derivatives (especially the free amine), nor to account for potential complications arising from endogenous esterases that might hydrolyze the side chain derivatives back to baccatin III with reincorporation of the latter. Both of these uncertainties could easily confound the interpretation. Nevertheless, the results (particularly the incorporation of *N*-debenzoyl-2′-deoxytaxol) are highly suggestive, but do not prove, that 2′-hydroxylation follows the transfer of β-phenylalanine to baccatin III.

Cell-free enzyme systems

To examine in greater detail the presumptive hydroxylation step of the C13-*O*-side chain assembly process, the candidate taxoid substrates *N*-debenzoyl-2′-deoxytaxol (β-phenylalanoyl baccatin III) and 2′-deoxytaxol (3-phenylisoserinoyl baccatin III), and the potential amino acid substrate β-phenylalanine were evaluated under a broad range of oxygenase reaction conditions using cell-free enzyme preparations from *T.* × *media* cells. Assay of the soluble enzyme fraction with the three potential substrates failed to yield the target products, phenylisoserine from β-phenylalanine or 2′-hydroxylated derivatives of the taxoids, or any other recognizable oxygenated metabolites, although control incubations with [^{3}H]benzoyl CoA to affect the terminal transfer step to *N*-debenzoyltaxol [20] readily demonstrated the presence of the functional transferase in these preparations.

With the microsomal fraction, β-phenylalanine was not detectably converted to 3-phenylisoserine, nor was 2′-deoxytaxol converted to Taxol, under any reaction conditions tested. However, *N*-debenzoyl-2′-deoxytaxol was converted to *N*-debenzoyltaxol (as evidenced by the radio-HPLC determination of labeled Taxol following synthetic N-benzoylation of the reaction products) in the presence of NADPH/O_2 (Fig. 3). Sporadic conversion was also observed in the presence of Fe^{2+}/α-ketoglutarate/ascorbate

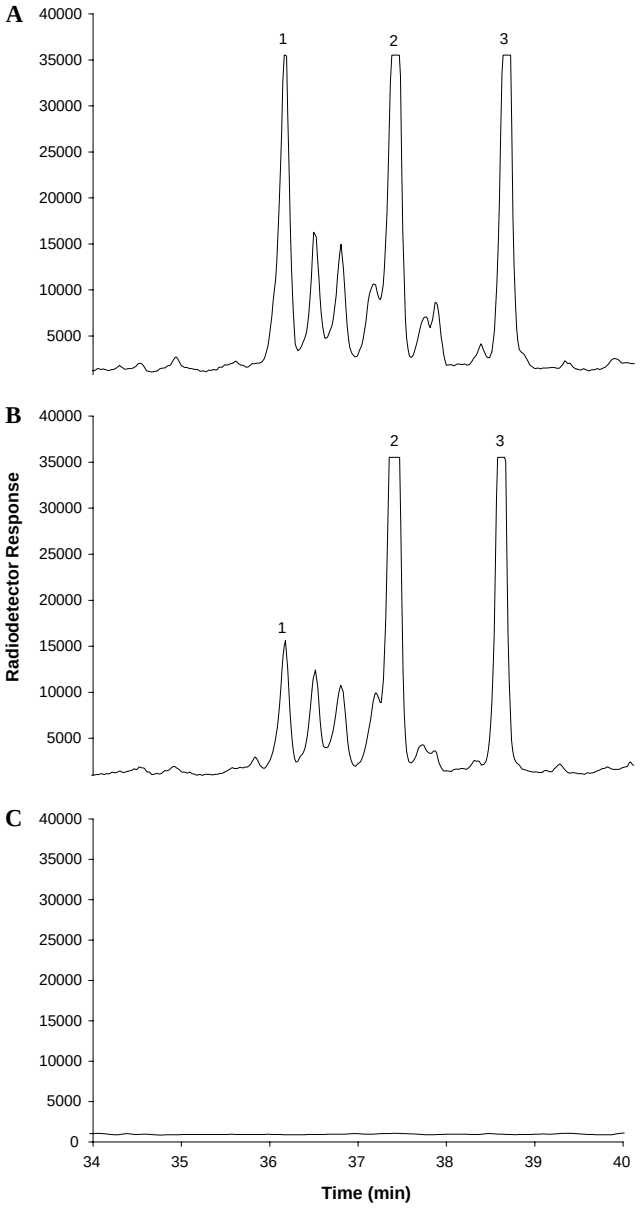

Fig. 3. Radio-HPLC analysis of (A) the biosynthetic products cephalomannine (1), Taxol (2), and taxol C (3) generated from *N*-debenzoyl-2′-deoxytaxol by *T.* × *media* cell microsomal preparations in the presence of NADPH and O_2; (B) the biosynthetic products from the identical experiment following synthetic N-benzoylation of the reaction mixture; and (C) the biosynthetic products from the identical experiment conducted in the presence of 100 μM clotrimazole. Peaks were identified by retention time coincidence of each radiolabeled peak with that of the authentic standard determined by the absorbance profile (A_{254}). The absence of the minor unidentified products in (C) suggests that these metabolites are also 2′-hydroxylated taxoids but which have undergone O-deacylation by a small amount of contaminating esterase activity in the preparation. This phenomenon has been observed previously with acylated taxoid substrates using *Taxus* microsomes [32,39].

but only when NADPH and the cytochrome P450 inhibitors clotrimazole or miconazole were also present in the reaction mixture.

Interestingly, labeled taxol B (cephalomannine) and taxol C (Fig. 2) were also observed as reaction products (Fig. 3) from [^{3}H]*N*-debenzoyl-2′-deoxytaxol (along with labeled Taxol after synthetic N-benzoylation), suggesting that, following the 2′-hydroxylation step, N-acylation had also occurred in these microsomal preparations. To test this possibility, the experiment was repeated, but without the additional N-benzoylation step, with the result that Taxol, cephalomannine, and taxol C were observed in the same proportions as before and no *N*-debenzoyltaxol was detected (Fig. 3). Final confirmation of this phenomenon was provided by conducting the N-benzoylation reaction with [$^2H_5$]benzoyl chloride, a procedure that failed to yield deuterium-labeled Taxol. Thus, it was clear that, following the microsomal hydroxylation of *N*-debenzoyl-2′-deoxytaxol to *N*-debenzoyltaxol, sufficient transferase activity and acyl/aroyl CoA precursors were present in these membranous preparations to affect essentially quantitative conversion (albeit at very low radiotracer scale) to the N-substituted derivatives Taxol, cephalomannine, and taxol C (Fig. 2).

Several observations can be gleaned from these results. The conversion of *N*-debenzoyl-2′-deoxytaxol (β-phenylalanoyl baccatin III), coupled to the failure of β-phenylalanine to undergo hydroxylation, suggests that hydroxylation does not occur at the level of the amino acid but rather that β-phenylalanine is activated and transferred to baccatin III prior to the hydroxylation step. The failure of 2′-deoxytaxol to undergo 2′-hydroxylation, however, indicates that the 2′-hydroxylase does not operate on N-acylated taxoid derivatives but rather on the free amine (i.e., *N*-debenzoyl-2′-deoxytaxol). This result is consistent with prior evidence suggesting that N-benzoylation is the final step of Taxol biosynthesis [20] (N-acylation in the case of cephalomannine and taxol C formation). Finally, it is notable that the 2′-hydroxylation product of *N*-debenzoyl-2′-deoxytaxol (i.e., *N*-debenzoyltaxol) was not directly observed because this metabolite was efficiently transformed to the N-substituted terminal taxoids Taxol, cephalomannine, and taxol C in these microsomal preparations. This surprising observation indicates that both transferase activity and acyl/aroyl CoA cosubstrates are present in these membranes at levels sufficient to conduct at least radiochemical scale conversions, in spite of all prior evidence indicating that these side chain transferase systems are operationally soluble [20,21]. It is of additional significance that no 2′-deoxytaxol was detected as a product of *N*-debenzoyl-2′-deoxytaxol with these "transferase competent" microsomes, nor was 2′-deoxycephalomannine or 2′-deoxytaxol C apparently formed (based on retention time estimates, since these two 2′-deoxytaxoid standards were not available). Given that *N*-debenzoyl-2′-deoxytaxol is a competent substrate for the *N*-benzoyl transferase [20], the absence of 2′-deoxytaxoids in these reactions indicates that the 2′-hydroxylation and N-acylation are closely coupled in sequence. The physiological significance of this apparent channeling, observed at these relatively low rates of in vitro conversion, is not known and warrants further study.

Enzyme characterization

The 2′-hydroxylase activity was restricted to the microsomal fraction prepared from *T.* × *media* suspension culture cells, and so was further characterized under conditions of pH, buffer composition, and cofactor composition that had been optimized for other microsomal cytochrome P450 taxoid hydroxylases from this tissue [31,32]. Under these conditions, the specific activity for 2′-side chain hydroxylation (based on total protein or cytochrome P450 content determined by CO-difference spectrometry) was nearly 5-fold less than that for the positive controls measured for the previously characterized cytochrome P450 taxoid 5α-hydroxylase, 10β-hydroxylase, and 13α-hydroxylase [33–35] (e.g., 1 nmol/h/mg microsomal protein compared to 5 nmol/h/mg microsomal protein for taxadiene-5α-hydroxylase). The 2′-hydroxylation activity was absolutely dependent on O_2 and the cofactor set of NADPH, FAD, and FMN, and was completely inhibited by pre-treatment with CO or the inhibitors miconazole or clotrimazole at 100 μM added prior to NADPH (Fig. 3); under these conditions, activity was negligible (<0.1 nmol/h/mg microsomal protein). The 2′-hydroxylation reaction was not supported by the combination of Fe^{2+}, α-ketoglutarate, and ascorbate. However, this cofactor set, in the presence of NADPH, FAD, FMN, and the azole inhibitors (at sufficient levels to eliminate the NADPH-dependent reaction), did sustain detectable 2′-hydroxylation activity, a curious observation for which we have no explanation. These results suggest that the 2′-hydroxylation reaction is mediated by a cytochrome P450 oxygenase, as are other taxoid hydroxylation reactions, only in this case operating on the C13-side chain rather than the taxane core.

The 2′-hydroxylation reaction is presumed to give rise to the naturally occurring 2′*R*-configured biosynthetic products based upon the radiochromatographic coincidence with authentic Taxol, cephalomannine, and taxol C. Authentic 2′*S*-configured taxoid standards are not available for comparison but these diastereoisomers would almost certainly be chromatographically distinguishable from the 2′*R*-configured natural products.

Discussion

Previous in vivo feeding studies with *Taxus* tissues [15] and in vitro characterization of the responsible transferases [20,21] provided broad outlines of the C13-side chain assembly process but could not resolve the issue of 2′-hydroxylation timing [13]. Thus, transfer of β-phenylalanine to baccatin III to afford *N*-debenzoyl-2′-deoxytaxol, as well as 2-hydroxylation of β-phenylalanine to 3-phenylisoserine followed by transfer of the phenylisoserinoyl moiety to baccatin III to yield *N*-debenzoyltaxol, appeared possible based on the extant data and the feeding studies with *Taxus* cells described herein. Although the phenylpropanoyl CoA ligase involved in side chain assembly has not yet been characterized, and β-phenylalanoyl CoA has not yet been tested as a hydroxylation substrate, the present failure to affect the direct conversion of β-phenylalanine to 3-phenylisoserine coupled to the preliminary demonstration of cytochrome P450-mediated conversion of β-phenylalanoyl baccatin III (*N*-debenzoyl-2′-deoxytaxol) to 3-phenylisoserinoyl baccatin III (*N*-debenzoyltaxol) indicates that transfer of the amino phenylpropanoid side chain (as β-phenylalanoyl CoA) precedes the 2′-hydroxylation step.

By clarifying the sequence of side chain assembly reactions, the present results have implicated both 13-*O*-β-phenylalanoyl baccatin III (*N*-debenzoyl-2′-deoxytaxol) and 13-*O*-3-phenylisoserinoyl baccatin III (*N*-debenzoyltaxol) as true intermediates on the pathway to Taxol, although neither metabolite was detectably labeled in *Taxus* cell feeding studies in which [^{3}H]baccatin III was readily incorporated into Taxol and its congeners. Both of these 13-O-substituted baccatins have been previously reported as minor metabolites of *Taxus* [36,37], and *N*-debenzoyltaxol (but not *N*-debenzoyl-2′-deoxytaxol, 2′-deoxytaxol or phenylisoserine) was detected in extracts of *T.* × *media* cells (unpublished data). These observations, along with the relative abundances of β-phenylalanine and baccatin III measured in vivo [38], suggest that the rate-limiting step in side chain assembly resides beyond the aminomutase [19] (i.e., at the aroyl CoA ligase reaction) but that, in the presence of both β-phenylalanoyl CoA and baccatin III co-substrates, the subsequent steps of the process are relatively fast and efficiently coupled because neither downstream intermediate on route to Taxol appears to appreciably accumulate.

The finding that the biosynthetic product of the 2′-hydroxylation reaction, *N*-debenzoyltaxol, was subsequently converted to Taxol (by *N*-benzoyl transfer), cephalomannine (by *N*-tigloyl transfer), and taxol C (by *N*-hexanoyl transfer) (Fig. 3) by these microsomal preparations, in rough proportion to their natural occurrence in *Taxus* cells [25], was surprising in indicating that sufficient transferase activities (and the relevant acyl/aroyl CoA co-substrates) were present in these membranous preparations to conduct the observed reactions, at least at the radiochemical level. This finding, which may suggest the operation of a metabolome for the kinetically efficient process of side chain assembly without accumulation of intermediates, bears further investigation, and, along with the above considerations regarding the seeming limitation in ligase-mediated β-phenylalanoyl CoA supply, may have important implications for the transgenic manipulation of *Taxus* cell cultures to improve Taxol production yields.

The finding that the side chain 2′-hydroxylation step is apparently catalyzed by a cytochrome P450 oxygenase is also of significance, in that all of the other known hydroxylases of taxoid metabolism (that hydroxylate at the 2α, 5α, 7β, 10β, 13α, and 14β positions of the taxane core), and for which the corresponding genes have been defined, are also cytochrome P450 oxygenases [12,13]. Although the 2′-hydroxylase operates on the C13-side chain, rather than on the taxane core as do the other pathway hydroxylases (see Fig. 1), the observations that the reaction is likely cyto-

chrome P450-mediated and that the substrate is typically "taxoid" in structure suggest that the corresponding gene resides within the previously isolated family of *Taxus* cytochrome P450 cDNAs [12,13] and provide impetus for the cloning effort. Functional screening is underway, and it is anticipated that the gene will soon be available for biotechnological evaluation, and that the recombinant hydroxylase will permit determination of specificity and kinetics unencumbered by the difficulties encountered with the native, microsomal enzyme.

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