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Specificity of the N-benzoyl transferase responsible for the last step of Taxol biosynthesis[☆]

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Abstract

The last few steps in the biosynthesis of the anticancer drug Taxol in yew (*Taxus*) species are thought to involve the attachment of β -phenylalanine to the C13-Q-position of the advanced taxane diterpenoid intermediate baccatin III to yield N-debenzoyl-2'-deoxytaxol, followed by hydroxylation on the side chain at the C2'-position to afford N-debenzoyltaxol, and finally N-benzoylation to complete the pathway. A cDNA encoding the N-benzoyl transferase that catalyzes the terminal step of the reaction sequence was previously isolated from a family of transferase clones (derived from an induced *Taxus* cell cDNA library) by functional characterization of the corresponding recombinant enzyme using the available surrogate substrate N-debenzoyl-2'-deoxytaxol [Walker et al., Proc. Natl. Acad. Sci. USA 99(2002) 9166–9171]. Semi-synthetic N-debenzoyltaxol was prepared by coupling of 7-triethylsilylbaccatin III and (2R,3S)- β -phenylisoserine protected as the N-Boc N,Q-isopropylidene derivative by means of carbodiimide activation and formic acid deprotections. The selectivity of the recombinant N-transferase for N-debenzoyltaxol was evaluated, and the enzyme was shown to prefer, by a catalytic efficiency factor of two, N-debenzoyltaxol over N-debenzoyl-2'-deoxytaxol as the taxoid co-substrate in the benzoyl transfer reaction, consistent with the assembly sequence involving 2'-hydroxylation prior to N-benzoylation. Selectivity for the acyl/aroyl-CoA co-substrate was also examined, and the enzyme was shown to prefer benzoyl CoA. Transfer from tigloyl-CoA to N-debenzoyltaxol to afford cephalomannine (taxol B) was not observed, nor was transfer observed from hexanoyl-CoA or butanoyl-CoA to yield taxol C or taxol D, respectively. These results support the proposed sequence of reactions for C13-Q-side chain assembly in Taxol biosynthesis, and suggest that other N-transferases are responsible for the formation of related, late pathway, N-acylated taxoids.

Keywords

Taxol; Paclitaxel; Biosynthesis; Side chain assembly; N-Benzoyl transferase; N-Debenzoyltaxol; Cephalomannine; Taxol A, B, C and D; *Taxus*; Yew

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²**Abbreviations used:** DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Mopso, 3-(N-morpholino)-2-hydroxypropanesulfonic acid; Ac, acetyl; Ph, phenyl; Bz, benzoyl; Et, ethyl; Me, methyl; Boc, *t*-butoxycarbonyl; DCC, N,N'-dicyclohexylcarbodiimide; DMAP, 4-(N,N-dimethylamino) pyridine; pyr, pyridine; TsOH, *p*-toluenesulfonic acid.

Taxol [1] (generic name paclitaxel, **7**, Fig. 1) is a taxane diterpenoid (taxoid) derived from yew (*Taxus*) species [2] that is now well established as a potent antineoplastic drug of wide application in cancer intervention [3,4]. Elegant total syntheses of Taxol have been devised [5] but none are commercially viable [6], and the destructive isolation of the drug from the original, low-yielding source (the bark of the Pacific yew, *Taxus brevifolia*) could not be sustained [7]. The increased demand for Taxol has thus led to semisynthetic methods of production [8] involving the attachment of the functionally important *N*-benzoyl phenylisoserine side chain to the C13-*O*-position of more readily available derivatives of baccatin III (**4**, Fig. 1) that can be isolated in higher yield from the renewable needles of various *Taxus* species. It is clear that, for the foreseeable future, the supply of Taxol and its precursors for semisyntheses must continue to rely on yew species [9] or derived cell cultures [10], and that drug sourcing and cost will remain significant issues [11]. It is important, therefore, to understand the biosynthesis of Taxol and its underlying molecular genetics, with the aim of improving drug yields by transgenic means, particularly by *Taxus* cell culture production methods [12].

The biosynthesis of Taxol requires nearly 20 steps beginning with the prenyl transferase-mediated construction of the universal diterpenoid precursor geranylgeranyl diphosphate which is cyclized, in the committed step, to taxa-4(5),11(12)-diene [13]. This parental olefin is then functionalized by a series of oxygenations, acylations, and other reactions to yield the late stage intermediate baccatin III (**4**, Fig. 1) [14], upon which the functionally significant *N*-benzoyl phenylisoserine side chain [15] is then assembled at the C13-*O*-position to complete the pathway to Taxol (**7**).

Feeding studies with *Taxus* tissues, using both amino acid precursors and advanced taxoid intermediates, metabolite profiling of *Taxus* cells in culture, and demonstration of relevant activities in cell free enzyme systems (see [14,16,17] for reviews of this work) have indicated that side chain construction requires five steps (Fig. 1) involving aminomutase-catalyzed conversion of (*S*)- α -phenylalanine (**1**) to (*R*)- β -phenylalanine (**2**), ligation to the corresponding CoA ester (**3**), transfer of the aminoacyl group to the C13-hydroxyl of baccatin III (**4**) to yield *N*-debenzoyl-2'-deoxytaxol (i.e., 13-*O*- β -phenylalanoyl baccatin III, **5**), hydroxylation at the 2'-position in the side chain to afford *N*-debenzoyltaxol (i.e., 13-*O*-phenylisoserinoyl baccatin III, **6**), and *N*-benzoylation as the final side chain modification.

Genes encoding the phenylalanine aminomutase [18] and the two responsible side chain transferases [19,20] have been isolated. In the case of the transfer reactions, functional screening of a family of previously acquired *Taxus* acyl/aroacyl transferase cDNAs [13] yielded the C13 phenylpropanoid side chain-CoA acyl transferase that mediates the 13-*O*-esterification of baccatin III [20], and the benzoyl CoA-dependent transferase responsible for the final benzamidation step of the Taxol pathway [19]. The lack of absolute substrate specificity by the recombinant transferases led to some ambiguities with regard to the precise timing of hydroxylation and acyl transfer steps in side chain assembly [14] that were only recently resolved by demonstration that the cytochrome P450 2'-hydroxylase operates only on *N*-debenzoyl-2'-deoxytaxol (**5**), thus implicating the construction sequence of *O*-esterification, followed by 2'-hydroxylation, followed by *N*-benzamidation [17]. In this context, it is important to note that the recombinant terminal *N*-transferase was characterized using the more readily available *N*-debenzoyl-2'-deoxytaxol (**5**) as a surrogate taxoid co-substrate [19]. With the recent preparation of *N*-debenzoyltaxol (**6**) described herein, the selectivity of this transferase for both acyl/aroacyl-CoA and taxoid co-substrates was revisited. The implications of these findings for the production of Taxol (**7**), and for the origin of the closely related taxoids cephalomannine (taxol B, **8**), taxol C (**9**) and taxcultine (taxol D, **10**) (Fig. 1), are discussed.

Materials and methods

Substrates and reagents

Acetyl-CoA, butanoyl-CoA and hexanoyl-CoA, all as the sodium salts, were purchased from Sigma Chemical Co. (St. Louis, MO). Benzoyl-CoA, and [7-¹⁴C]benzoyl-CoA (1.1 mCi/mol) employed for preliminary kinetic assay development, were prepared as previously described, as were tigloyl-CoA (from tiglic acid) and *N*-debenzoyl-2'-deoxytaxol (**5**) [17,19].

N-Debenzoyltaxol (**6**) was prepared from the commercially available 10-deacetylbaecatin III (**11**) (Natland International, Research Triangle Park, NC) by a combination of various literature procedures with considerable modification as outlined in Fig. 2. Regioselective silylation of the C7 hydroxyl group [21,22] followed by purification and acetylation [22] afforded the 7-triethylsilyl-10-acetyl derivative **13**. In our experience, silylations without purification of **12** [21] were inconsistent and usually gave mixtures from which **13** was difficult to isolate in pure form. The side chain on the 13 α -hydroxyl group was then introduced by carbodiimide activation of excess enantio-pure *N*-*t*-butoxycarbonyl-protected oxazoline carboxylic acid **14** (2.5 equiv.) [23]. Simultaneous hydrolysis of the *N*-*t*-butoxycarbonyl, acetonide, and triethylsilyl protecting groups with formic acid for 30 min at room temp [24] gave *N*-debenzoyltaxol (**6**, 7 mg, overall yield 28% from **1**), the identity and purity of which were established by TLC, 500 MHz ¹H NMR spectra, and FAB MS data. The NMR data for **6** and its *N*-*t*-butoxycarbonyl derivative (**16**) corresponded well with the literature values for these known compounds [25,26].

Variations in the specific conditions for the formic acid-catalyzed cleavage of the protecting groups showed that the 30-min reaction time and removal of the formic acid by co-evaporation with toluene under reduced pressure with a bath temp below 30°C were critical in obtaining the optimal 60% yield. The order of the three deprotection steps is unknown. The reliability of the preceding methods for side chain attachment is indicated by the successful preparation of *N*-debenzoyl 2'-deoxytaxol in deuterium-labeled forms by similar acylations of **13** and **13-d₁** with the *N*-Boc derivative (**14**) of (*R*)-(+)- β -phenylalanine (2.7 equiv, 1 equiv DMAP, 2.7 equiv DCC, room temp, overnight, 70–71%) followed by formic acid hydrolyses (excess HCO₂H, room temp, 30 min, 54–60%).

The *N*-*t*-butoxycarbonyl oxazoline side chain precursor **14** was obtained from (*S*)-(+)-phenylglycine (**17**) in 7 steps and 12% overall yield according to known methods [27–29] as summarized in Fig. 3. For further details of the above synthetic procedures, analytical methods, characterization data, and NMR spectra, see Supplementary Material.

Taxol (taxol A, **7**), cephalomannine (taxol B, **8**), taxol C (**9**) and taxcultine (taxol D, **10**) were previously identified as metabolites produced in *Taxus* cell culture [30–32], and these standards were prepared from this source. 2'-Deoxytaxol was synthesized by Schotten-Baumann *N*-benzoylation of *N*-debenzoyl-2'-deoxytaxol (**5**) using a published procedure [33]. We noted in the course of this study that the classical Schotten-Baumann chemistry, when performed on *N*-debenzoyltaxol (**6**) at the micro-scale, can result in significant 2'-deoxygenation to yield 2'-deoxytaxol. To minimize this unusual side reaction, chemical *N*-benzoylation of 2'-hydroxy derivatives was carried out by dissolving the taxoid in a minimum volume of ethyl acetate followed by the addition of a slight excess of benzoyl chloride diluted 10-fold in dry tetrahydrofuran. The mixture was gently mixed for 30 seconds and the solvents were immediately removed under an argon stream. The resulting residue was dissolved in acetonitrile for subsequent purification (>95% yield). Substitution of ethyl acetate in the reaction by acetonitrile, methylene chloride, acetone, methanol or water resulted in reduced yield and/or 2'-deoxygenation.

Enzyme preparation and assay

A pSBET plasmid construct harboring the previously identified *N*-benzoyl transferase (designated TAX10) was transformed into *Escherichia coli* BL21 (DE3) for expression of the recombinant enzyme as before [19]. A single transformed bacterial colony was used to inoculate 1 liter of Luria-Bertani medium supplemented with kanamycin (50 µg/mL) and grown at 37°C to an $A_{600} = 0.8$, whereupon expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.4 mM. The temperature was then reduced to 18°C, and the culture was shaken at 230 rpm for 16 h. Bacteria were harvested by centrifugation (2,500g, 15 min), resuspended in 40 mL of extraction buffer (50 mM Mopso, pH 7.5, with 5% (v/v) glycerol, 1 mM EDTA and 0.5 mM DTT), and then disrupted by sonication for 2 × 30 s at 0°C with a Virsonic 475 (Virtis, Gardiner, NY) using a 1.5-cm sonication probe at maximum power. The resulting homogenate was clarified by centrifugation (40,000g, 1 h) to provide the soluble enzyme fraction.

After the addition of MgCl₂ to a final concentration of 5 mM, the soluble protein extract (30 mL, 150 mg total protein) was loaded onto a diethylaminoethyl cellulose (Whatman International, Clifton, NJ) column (2.5 × 20 cm, 35 mL bed volume) that had been equilibrated with buffer A (50 mM Mopso, pH 7.5, with 10% (v/v) glycerol, 15 mM MgCl₂, 5 mM EDTA, 1 mM leupeptin and 0.5 mM DTT). Unbound protein was removed by washing the column at 3 mL/min with three column volumes of the same buffer, and bound protein was then eluted with a linear NaCl gradient (0–250 mM, 250 mL, 3 mL/min) in the same buffer. The target protein eluted between 120–200 mM NaCl [19], and these fractions were collected, pooled and loaded onto a 40-µm type I ceramic hydroxyapatite (Bio-Rad Laboratories, Hercules, CA) column (2.5 × 20 cm, 35-mL bed volume) that had been equilibrated with buffer A containing 160 mM NaCl. The column was washed with three column volumes of this buffer to remove unbound protein, and the column was then eluted with a linear gradient of 0–100 mM potassium phosphate (pH 7.2, 100 mL, 3 mL/min). The target protein eluted between 25–45 mM phosphate, and these fractions were collected, pooled and concentrated (to ~2 mL at 10 mg protein/mL) using a 10 kD cutoff centrifugal concentrator (Amicon Centriprep 10, Millipore Corp., Bedford, MA), and then exchanged into assay buffer (50 mM Mopso, pH 7.5, with 5% (v/v) glycerol and 5 mM MgCl₂) using an Econopac 10DG 30 × 10 mL disposable desalting column (Bio-Rad Laboratories, Hercules, CA).

The partially purified transferase was flash frozen (liquid N₂) in 100-µL aliquots and stored at –80°C. SDS-PAGE with Coomassie Blue staining of this partially purified material indicated that the target enzyme was ~75% pure, yielding a stock solution of 3.5 mg TAX10 protein/mL. The corresponding protein fraction from an empty vector control incubation was identically prepared.

The standard assay protocol for the *N*-benzoyl transferase [19] was modified slightly in preliminary studies to optimize reaction conditions and establish linearity in kinetic assays. A 100-µL aliquot of protein solution (up to 100 µM TAX10 protein) in the above noted buffer was combined with up to 500 µM of either *N*-debenzoyl-2'-deoxytaxol (**5**) or *N*-debenzoyltaxol (**6**) and up to 500 µM [7-¹⁴C]benzoyl-CoA in the same buffer, and incubated at 31°C for up to 1 h. Assay mixtures were then extracted with two 0.1-mL portions of CH₂Cl₂, and the organic extracts were pooled and reduced to dryness under a stream of purified N₂. The residue was dissolved in 50 µL of CH₃CN, filtered through a 0.2-µm nylon syringe filter (Millex-GN, Millipore, Bedford, MA) into a sample vial, and analyzed by HPLC with UV- and radio-monitoring (Agilent 1100 Series with G1311A quaternary pump, and coupled to a G1314A UV-detector and Packard Series A-100 radio-detector). Separation was achieved using a Phenomenex (Torrance, CA) reversed-phase Curosil column (5 µm, 4.6 × 250 mm) by eluting at 1 mL/min with 25:75 (v/v) CH₃CN:H₂O for 5 min, followed by a linear gradient to 65:35 (v/v) CH₃CN:H₂O over 40 min, then washing at 85:15 (v/v) CH₃CN:H₂O for 10 min before

return to starting conditions. Products were analyzed by calibrated radio-monitoring and/or UV absorbance (A_{228}) using the authentic standards. Additionally, background profiles were generated via assays performed with protein prepared from empty vector (pSBET) control cultures; these control assays showed no detectable transferase activity with any combination of co-substrates.

Kinetic evaluation

Kinetic assays were performed under linear reaction conditions of protein concentration and time (10 μ M protein, 10 min), at pH 7.5, by varying the concentration of N-debenzoyltaxol (**6**) or N-debenzoyl-2'-deoxytaxol (**5**) (0–500 μ M) with benzoyl-CoA held at saturation (1 mM). The reverse condition was also evaluated by varying the concentration of benzoyl-CoA (0–500 μ M) with each taxoid co-substrate held at saturation (1 mM). The resulting data sets were evaluated as double-reciprocal plots via the program Enzyme Kinetics Pro (ChemSW, Fairfield, CA), using direct linear plots for comparison, and the lines of best fit were determined. All data are reported as the averages of triplicate, independent assay sets, with an SE of less than $\pm 12\%$ of the mean. For evaluating the use of acetyl-CoA, butanoyl-CoA, hexanoyl-CoA and tigloyl-CoA by the N-transferase, the linear assay was conducted with both the CoA ester and taxoid co-substrate at a concentration of 500 μ M.

Results

Selectivity for the taxoid co-substrate

Considerable evidence now indicates that the final stage of Taxol biosynthesis involves assembly of the N-benzoyl phenylisoserinoyl side chain at the C13-O-position of the advanced intermediate baccatin III (**4**, Fig. 1), with the terminal step of the reaction sequence being the N-benzoylation of 3'-N-debenzoyltaxol (**6**) to complete the pathway to Taxol (**7**) [14,16,17]. A cDNA encoding the presumptive N-benzoyl transferase was previously isolated by functional screening of a family of *Taxus* acyl/aroyl transferase clones [13], and the recombinant enzyme was characterized using the surrogate substrate 3'-N-debenzoyl-2'-deoxytaxol (**5**), not 3'-Ndebenzoyltaxol (**6**), because the former was more readily available [19]. With the recent synthetic preparation of N-debenzoyltaxol, described herein, it became possible to compare the presumed natural substrate of the reaction with the previously employed surrogate substrate, and to determine whether selectivity of this enzyme for the taxoid co-substrate is consistent with the proposed side chain assembly sequence (Fig. 1) in which 2'-hydroxylation precedes N-benzoylation, and not the reverse in which N-benzoylation (to 2'-deoxytaxol) precedes 2'-hydroxylation.

The recombinant N-benzoyl transferase (the TAX10 clone, previously designated DBTNBT for 3'-N-debenzoyl-2'-deoxytaxol N-benzoyl transferase) was prepared using protocols similar to those described previously [19] which involve expression of the corresponding cDNA in *E. coli* and purification of the resulting soluble enzyme (to 75% purity by SDS-PAGE) by chromatographic means. Assay conditions were optimized, and reaction linearity with respect to protein concentration and time was established, in preliminary experiments conducted by a method involving enzymatic transfer from [7- 14 C]benzoyl-CoA to the taxoid co-substrate followed by separation of the derived product, and a purification and quantification by radio-HPLC. Enzyme assays were also carried out with protein identically prepared from empty vector controls, and these preparations evidenced no detectable transferase activity.

Replicate kinetic assays, with three independent enzyme preparations, were then carried out at saturating levels of unlabeled benzoyl-CoA (1 mM) with varying concentrations of the taxoid co-substrate (0–500 μ M); in this instance, product purification and quantification were

conducted by HPLC-UV detection (A_{228}). Rate data were plotted, and the resulting kinetic constants are reported as averages $\pm$ SE.

With N-debenzoyl-2'-deoxytaxol (**5**) as variable co-substrate (benzoyl CoA at 1 mM), a K_m value of $346 \pm 70 \mu\text{M}$ and V_{max} of $688 \pm 59 \text{ nmol/min}$ ($V/K_m = 1.99$) were determined (Fig. 4). With benzoyl CoA as variable co-substrate (taxoid at 1 mM), a K_m value of $529 \pm 59 \mu\text{M}$ and V_{max} of $517 \pm 29 \text{ nmol/min}$ ($V/K_m = 0.98$) were determined. These values are very close to those previously reported for this recombinant enzyme using benzoyl CoA and the surrogate substrate [19]. In the case of N-debenzoyltaxol (**6**) as the variable co-substrate (benzoyl CoA again at 1 mM), a K_m value of $78 \pm 11 \mu\text{M}$ and V_{max} of $349 \pm 15 \text{ nmol/min}$ ($V/K_m = 4.47$) were determined (Fig. 4). With benzoyl CoA as variable co-substrate (taxoid at 1 mM), a K_m value of $375 \pm 67 \mu\text{M}$ and V_{max} of $439 \pm 33 \text{ nmol/min}$ ($V/K_m = 1.17$) were similarly determined. The difference in kinetic efficiencies for benzoyl transfer between N-debenzoyltaxol (**6**) and N-debenzoyl-2'-deoxytaxol (**5**), while small (about two-fold, reflected primarily in difference in K_m values), appears significant given the very small difference in size and polarity between these two taxoids, and is consistent with the proposal for side chain assembly in which the N-transferase would be expected to operate preferentially with N-debenzoyltaxol as substrate for the terminal pathway reaction.

Selectivity for the CoA ester co-substrate

In addition to Taxol (**7**), *Taxus* species produce several closely related N-acylated taxoids [34,35], including cephalomannine (taxol B, N-tigloyl, **8**), taxol C (N-hexanoyl, **9**) and taxcultine (taxol D, N-butanoyl, **10**), presumed to originate (see Fig. 1) by similar N-transfer from the corresponding CoA ester to N-debenzoyltaxol (**6**) [14]. In *Taxus* cell cultures, the experimental system for much of our work on Taxol biosynthesis [14], Taxol (taxol A), cephalomannine (taxol B), taxol C and taxcultine (taxol D) are produced in roughly equal proportion [32]. Therefore, it was of interest to determine whether one or more N-transferases are responsible for the biosynthesis of these related taxoids as previous work with the N-benzoyl transferase (TAX10) had eliminated only acetyl CoA as a possible acyl donor with this enzyme (using N-debenzoyl-2'-deoxytaxol (**5**) as co-substrate) [19].

To test the possibility that the TAX10 N-transferase might catalyze N-acyl transfer in the formation of these other advanced taxoids, we incubated tigloyl-CoA, hexanoyl-CoA, and butanoyl-CoA individually with the transferase using N-debenzoyltaxol (**6**) as co-substrate under optimal, and saturating, conditions determined for the benzamidation reaction.

Under these conditions, the recombinant enzyme did not detectably N-transfer from tigloyl-CoA to N-debenzoyltaxol to produce cephalomannine (taxol B, **8**). An unknown compound was produced, at rates roughly 15% of the corresponding benzamidation reaction, that possessed an HPLC retention time close to that of cephalomannine but which was not coincident with any taxoid previously observed by metabolite profiling [30–32]. This compound might arise by the less efficient C1-, C7-, or C2'-O-acylation of N-debenzoyltaxol (**6**); HPLC-MS analysis of this material was suggestive of such a taxoid, but insufficient material was available to permit identification.

Similarly, the recombinant enzyme did not detectably N-transfer from hexanoyl-CoA to N-debenzoyltaxol to form taxol C (**9**). Again, in this case, an unknown compound was produced, at rates roughly 5% of the benzamidation reaction, that bore an HPLC retention time close to that of taxol C but which was not coincident with any known taxoid. As before, this apparently aberrant compound might be the product of inefficient O-acylation of N-debenzoyltaxol by the recombinant transferase, a reaction seemingly not observed in vivo.

Finally, the N-transferase showed no detectable activity in the presence of N-debenzoyltaxol (**6**) and butanoyl-CoA to effect the formation of taxcultine (taxol D, **10**), nor were any products formed via transfer from acetyl-CoA to N-debenzoyltaxol, consistent with the earlier observation [19] that the TAX10 transferase was inactive in the presence of acetyl-CoA and N-debenzoyl-2'-deoxytaxol (**5**). If it is assumed that these in vitro results reflect the in vivo specificity of this enzyme, then these data indicate that the TAX10 N-transferase catalyzing the last step of Taxol biosynthesis is specific for benzoyl-CoA, of the five CoA esters tested, and clearly cannot be responsible for the biosynthesis of the related N-acylated taxoids, taxol B, C and D (Fig. 1).

Discussion

Because the N-benzoyl transferase catalyzes the final step in side chain construction, it was anticipated that this enzyme would show a kinetic preference for the last intermediate of the Taxol biosynthetic pathway N-debenzoyltaxol (**6**) [14]. As demonstrated in the present work, however, the selectivity of the N-benzoyl transferase for N-debenzoyltaxol over N-debenzoyl-2'-deoxytaxol (**5**) as substrate is quite modest (i.e., a two-fold difference in kinetic efficiency based on V/K_m); nevertheless, it appears to be sufficient, in combination with the specificity of the 2'-hydroxylase for N-debenzoyl-2'-deoxytaxol (**5**) as substrate [17], to direct the pathway sequence as proposed by 2'-hydroxylation in the side chain followed by N-benzoylation. The reverse sequence, involving N-benzoylation of N-debenzoyl-2'-deoxytaxol prior to 2'-hydroxylation would yield 2'-deoxytaxol, and this product would be expected to accumulate because it is not an effective substrate for the 2'-hydroxylase to afford Taxol [17]. 2'-Deoxytaxol, however, is not observed in metabolite profiling of *Taxus* cell cultures [30–32], although both N-debenzoyltaxol and N-debenzoyl-2'-deoxytaxol have been noted at low levels.

The related N-acylated derivatives taxol B, C and D (**8 – 10**) are, like Taxol (**7**), prominent metabolites produced in *Taxus* cell cultures [32]. The second, significant observation to derive from the present work is that the N-benzoyl transferase responsible for Taxol formation does not effectively catalyze N-acylation of N-debenzoyltaxol (**6**) using tigloyl-, hexanoyl-, or butanoyl-CoA as co-substrate, and so cannot be involved in the biosynthesis of these related metabolites. Another transferase, or several transferases, must be responsible for converting N-debenzoyltaxol to these N-acylated taxoids, and the corresponding cDNA(s) likely reside within the available family of transferase clones [13] now being expressed and functionally screened for these alternative transferase activities.

The observed selectivity of the N-benzoyl transferase has biotechnological implications for the production of Taxol in *Taxus* cell cultures, for which a stable transformation system has recently been developed [36]. Overexpression of the TAX10 N-benzoyl transferase in culture might be expected to increase the biosynthesis of Taxol at the expense of taxols B, C and D, with the potential for improving production yield nearly three-fold, if the N-benzoyl transferase fully outcompetes N-acyl transferases in the utilization of N-debenzoyltaxol as co-substrate. Transgenic experiments to test this possibility, and to determine the influence of gene specific silencing, are underway.

Supplementary Material

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Fig. 1. Outline of the final steps in the biosynthesis of Taxol (7) and related taxoids from baccatin III (4) and (S)-phenylalanine (1).

Fig. 2.
Outline of the partial synthesis of *N*-debenzoyletaxol (**6**) from 10-deacetylbaccatin III (**11**) and the *N*-*t*-butoxycarbonyl oxazoline side chain precursor **14**.

Fig. 3. Outline of the synthesis of the enantio-pure C13-O-side chain precursor (**14**) from (*S*)-(+)-phenylglycine (**17**).

Fig. 4. Kinetic evaluation of the N-benzoyl transferase by Lineweaver-Burk analysis using N-debenzoyltaxol (**6**) and N-debenzoyl-2'-deoxytaxol (**5**) as substrates (0–500 μ M) with benzoyl CoA as saturating co-substrate (1 mM). Incubation conditions and assay procedures are described in Materials and methods.