Taxol biosynthesis: Molecular cloning of a benzoyl-CoA:taxane 2α-*O*-benzoyltransferase cDNA from *Taxus* and functional expression in *Escherichia coli*

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A cDNA clone encoding a taxane 2α-*O*-benzoyltransferase has been isolated from *Taxus cuspidata*. The recombinant enzyme catalyzes the conversion of 2-debenzoyl-7,13-diacetylbaccatin III, a semisynthetic substrate, to 7,13-diacetylbaccatin III, and thus appears to function in a late-stage acylation step of the Taxol biosynthetic pathway. By employing a homology-based PCR cloning strategy for generating acyltransferase oligodeoxynucleotide probes, several gene fragments were amplified and used to screen a cDNA library constructed from mRNA isolated from methyl jasmonate-induced *Taxus* cells, from which several full-length acyltransferases were obtained and individually expressed in *Escherichia coli*. The functionally expressed benzoyltransferase was confirmed by radio-HPLC, ^{1}H-NMR, and combined HPLC-MS verification of the product, 7,13-diacetylbaccatin III, derived from 2-debenzoyl-7,13-diacetylbaccatin III and benzoyl-CoA as cosubstrates in the corresponding cell-free extract. The full-length cDNA has an open reading frame of 1,320 base pairs and encodes a protein of 440 residues with a molecular weight of 50,089. The recombinant benzoyltransferase has a pH optimum of 8.0, K_m values of 0.64 mM and 0.30 mM for the taxoid substrate and benzoyl-CoA, respectively, and is apparently regiospecific for acylation of the 2α-hydroxyl group of the functionalized taxane nucleus. This enzyme may be used to improve the production yields of Taxol and for the semisynthesis of drug analogs bearing modified aroyl groups at the C2 position.

paclitaxel | 2-debenzoyl-7,13-diacetylbaccatin III

The anticancer drug Taxol (generic name paclitaxel), produced by yew (*Taxus*) species, is one of the structurally more complex members of the taxoid family of diterpenoid natural products (1). Of the dozen enzymatic reactions involved in Taxol biosynthesis (2, 3), there are five acyltransferase steps responsible for the addition of five acyl groups present in the final highly functionalized product. The first and third acylation reactions of the Taxol pathway appear to be catalyzed, respectively, by taxadien-5α-ol *O*-acetyltransferase (TAT) (4), which converts the second specific pathway intermediate, taxa-4(20),11(12)-dien-5α-ol, to the acetate ester (5), and by 10-deacetylbaccatin III 10-*O*-acetyltransferase (DAT), which converts 10-deacetylbaccatin III to baccatin III (6) as the last diterpenoid intermediate before Taxol (3) (Fig. 1). cDNAs encoding both of these acetyltransferases have been isolated from an induced *Taxus cuspidata* cell library by a homology-based cloning strategy that yielded a family of related acyltransferase sequences that may contain all of the remaining transferases of Taxol biosynthesis (6, 7).

A survey of the 350 naturally occurring taxoid metabolites characterized to date (1) suggests that the second acylation reaction in the Taxol pathway is the benzoylation of the C2α-hydroxyl group of an advanced taxane intermediate (2, 3) (Fig. 1). Although no advanced, naturally occurring 2-deacyltaxoid metabolites are available to permit assay of 2-*O*-benzoyltransferase activity with benzoyl-CoA as cosubstrate, the semisynthesis of 2-debenzoyl-7,13-diacetylbaccatin III did provide a suitable taxoid substrate for screening the expressed set of *T. cuspidata* transacylase clones for 2α-*O*-benzoyltransferase function. Here, we describe the isolation and analysis of a cDNA encoding the target taxane 2α-*O*-benzoyltransferase (TBT), and we report on the properties of this recombinant enzyme of Taxol biosynthesis.

Materials and Methods

Substrates. Authentic 10-deacetylbaccatin III was obtained from Hauser Chemical Research (Boulder, CO). (2α,5α)-Dihydroxytaxa-4(20),11(12)-diene was a gift from Robert Williams (Colorado State University, Boulder, CO). Benzoyl-CoA as the sodium salt was purchased from Sigma and [7-^{14}C]benzoic acid was from NEN Life Sciences Products. All other reagents were purchased from Aldrich, unless noted otherwise.

Synthesis of 7,13-Diacetylbaccatin III. To a stirred solution of 10-deacetylbaccatin III (160 mg, 294 μmol) in dry CH_2Cl_2 (5 ml) at 25°C under N_2 were added acetic anhydride (20 eq), dimethylaminopyridine (20 eq), and triethylamine (50 μl, 361 μmol). After 16 h, the reaction was diluted with EtOAc (50 ml) and quenched with water (10 ml). The mixture was stirred for 15 min, and the aqueous fraction was separated and extracted with EtOAc (twice with 25 ml). The combined organic fractions were washed with brine, 0.1 M HCl, and water, and dried over anhydrous $MgSO_4$. The organic solvent was evaporated, and the crude product was purified by silica gel flash column chromatography (EtOAc/hexane, 60:40, vol/vol) to yield pure 7,13-diacetylbaccatin III (see Fig. 2) (180 mg, 91% yield, 99% purity by ^{1}H NMR). ^{1}H NMR (300-MHz, $CDCl_3$) δ: 1.10 (s, CH_3), 1.14 (s, CH_3), 1.74 (s, CH_3), 1.77 (ddd, J = 1.8, 10.8, and 14.7 Hz, H-6β), 1.90 (d, J = 1.2 Hz, *allylic*-CH_3) 1.97 (s, C(O)CH_3), 2.12 (s, C(O)CH_3), 2.14 (s, C(O)CH_3), 2.17 (d, J = 8.7 Hz, H-14), 2.29 (s, C(O)CH_3), 2.53 (ddd, J = 7.2, 9.6, and 14.4 Hz, H-6α), 3.89 (d, J = 6.9 Hz, H-3), 4.09 (d, J = 8.4 Hz, H-20α), 4.24 (d, J = 8.4 Hz, H-20β), 4.91 (dd, J = 1.8 and 9.6 Hz, H-5), 5.53 (dd, J = 7.2 and 10.5 Hz, H-7), 5.60 (d, J = 6.9 Hz, H-2), 6.10 (dt, J = 1.2 and 8.7 Hz, H-13), 6.19 (s, H-10), 7.39–8.01 (aromatic

Abbreviations: TBT, taxane 2α-*O*-benzoyltransferase; APCI, atmospheric pressure chemical ionization; Mopso, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF297618).

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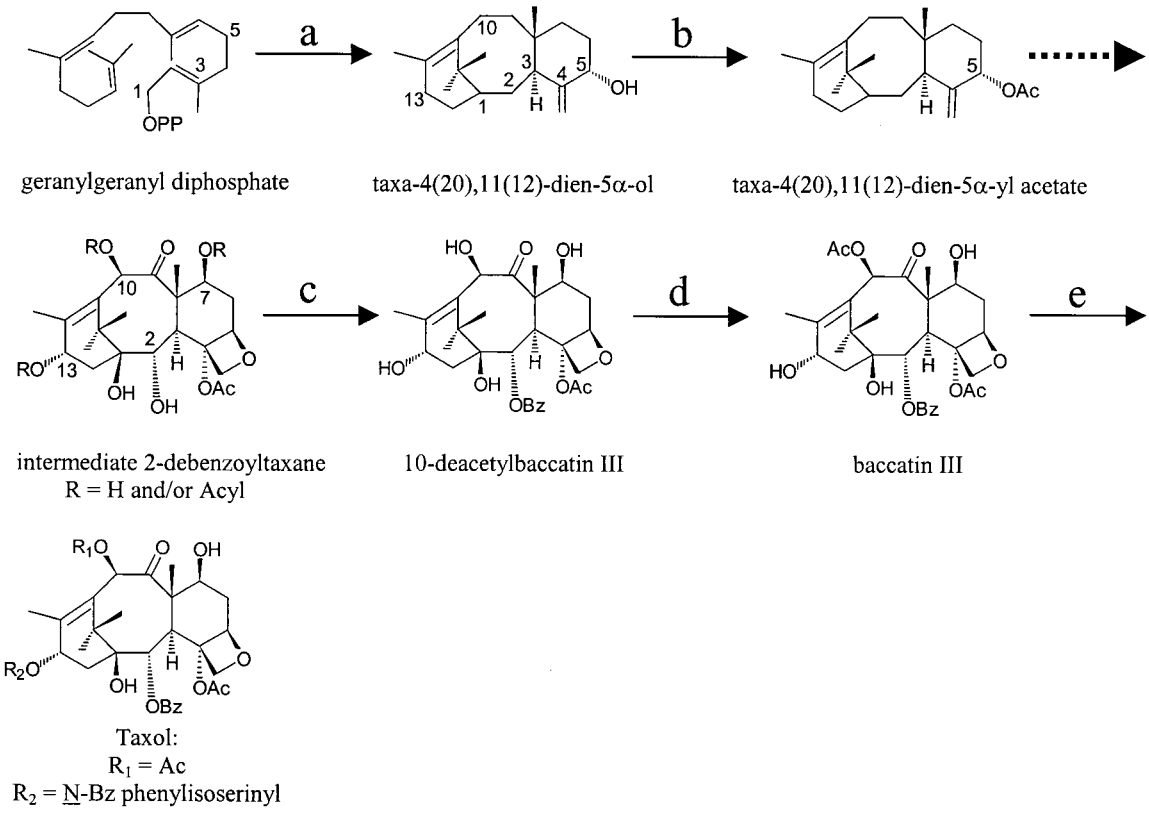


Fig. 1. Outline of the Taxol biosynthetic pathway. The cyclization of geranylgeranyl diphosphate to taxadiene by taxadiene synthase and the hydroxylation to taxadien-5α-ol by taxadiene 5α-hydroxylase (**a**), the acetylation of taxadien-5α-ol by taxa-4(20),11(12)-dien-5α-ol *O*-acetyltransferase (TAT) (**b**), the conversion of a 2-debenzoyl ''taxoid-type'' intermediate to 10-deacetylbaccatin III by a taxane 2α-*O*-benzoyltransferase (TBT) (**c**), the conversion of 10-deacetylbaccatin III to baccatin III by 10-deacetylbaccatin III 10-*O*-acetyltransferase (DBAT) (**d**), and the side-chain attachment to baccatin III to form Taxol (**e**) are illustrated. The broken arrow indicates several as-yet-undefined steps.

protons). Atmospheric pressure chemical ionization (APCI) MS: m/z 671 (PH^+).

Synthesis of 2-Debenzoyl-7,13-diacetylbaccatin III. To a stirred solution of 7,13-diacetylbaccatin III (170 mg, 253 μmol) in dry tetrahydrofuran (2 ml) at 0°C was added bis(2-methoxyethoxy)aluminum hydride (>65 wt % in toluene, 3 eq) dropwise. After stirring for 30 min at 0°C, the reaction was quenched by dropwise addition of saturated NH_4Cl, and the mixture was stirred for 10 min, then warmed to room temperature and diluted with EtOAc (50 ml), followed by addition of water (10 ml). The aqueous phase was separated and extracted again with EtOAc (twice with 25 ml). The combined organic fractions were washed with brine and water, then dried over anhydrous $MgSO_4$. The solvent was evaporated and the crude product was purified by silica gel flash column chromatography (40–60% EtOAc gradient in hexane) to yield 2-debenzoyl-7,13-diacetylbaccatin III (see Fig. 2) (60 mg, 42% yield, 99% purity by ^1H NMR). ^1H NMR (300-MHz, $CDCl_3$) δ: 1.04 (s, CH_3), 1.22 (s, CH_3), 1.77 (s, CH_3), 1.85 (ddd, J = 1.8, 10.8, and 14.4 Hz, H-6β), 1.90 (d, J = 1.2 Hz, *allylic*-CH_3), 2.02 (s, C(O)CH_3), 2.14 (s, C(O)CH_3), 2.15 (s, C(O)CH_3), 2.20 (s, C(O)CH_3), 2.58 (ddd, J = 7.2, 9.6, and 14.4 Hz, H-6α), 2.65 (d, J = 5.1 Hz, OH at C2), 3.58 (d, J = 6.9 Hz, H-3), 3.90 (dd, J = 5.4 and 6.5 Hz, H-2), 4.49 (d, J = 9.6 Hz, H-20α), 4.63 (d, J = 9.6 Hz, H-20β), 4.97 (dd, J = 1.8 and 9.6 Hz, H-5), 5.55 (dd, J = 7.2 and 10.7 Hz, H-7), 6.15 (dt, J = 1.2 and 9.6 Hz, H-13), 6.18 (s, H-10), H-14 proton signal obscured. APCI MS: m/z 567 (PH^+).

Synthesis of [7-^{14}C]Benzoyl-CoA. The method was adapted from a procedure for the synthesis of valproyl-CoA esters (8). To a solution of [7-^{14}C]benzoic acid (3.3 mg, 27 μmol, specific activity 18.5 Ci/mol; 1 Ci = 37 GBq) in CH_2Cl_2/tetrahydrofuran (5:2, vol/vol, 1.4 ml) under N_2 was added 1 M triethylamine in CH_2Cl_2 (3.0 μl, 30 μmol) in one portion, and the mixture was stirred for 10 min at room temperature. Ethyl chloroformate (2.57 μl, 2.9 mg, 27 μmol) was added in one portion and the reaction was stirred for 1 h at room temperature. The solvents were evaporated, and the residue was dissolved in 0.5 ml of *t*-butyl alcohol. CoA as the sodium salt (23 mg, 30 μmol dissolved in 0.5 ml of 0.4 M $NaHCO_3$) was added to the solution and the mixture was stirred for 0.5 h at room temperature, then quenched with 1 M HCl (200 μl) and adjusted to pH 5 with 15 mM NaH_2PO_4 (pH 4.8). The solvents were evaporated under reduced pressure (5 h) at room temperature. The residue was resuspended in 15 mM NaH_2PO_4 (pH 6.9, 7 ml). The crude product was purified by using a C_{18} Sep-Pak cartridge (500 mg C_{18} silica gel, Millipore) that was first washed with methanol and water, then equilibrated with 15 mM NaH_2PO_4 (pH 6.9). The crude sample was loaded onto the column, which was eluted with 5-ml portions of increasing methanol in 15 mM NaH_2PO_4 (pH 6.9) to yield [7-^{14}C]benzoyl-CoA (eluted in 10–15% methanol). The purity (99%) of the CoA thioester was assessed by analytical TLC (silica gel developed with 1-butanol/H_2O/AcOH, 5:3:2, vol/vol/vol) and comparison with authentic cochromatographed benzoyl-CoA (R_f = 0.4). The TLC plate was air dried at 25°C, and the radiochemical purity of the synthetic product was determined to be 99% by liquid scintillation counting of the isolated material.

The [7-^{14}C]benzoyl-CoA was lyophilized, resuspended in water (10 ml) to reconstitute to 15 mM NaH_2PO_4, and adjusted to pH 5 with 1 M HCl, and the solution was extracted with ether to remove trace organic contaminants. The residual ether was evaporated under a stream of N_2 to yield a 1.35 mM solution of [7-^{14}C]benzoyl-CoA.

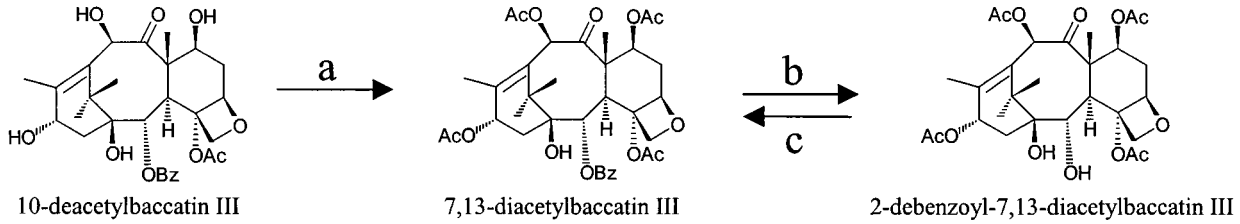
Bacterial Strains, Plasmid Constructs, and Cloning. The isolation of clones encoding transacylases involved in Taxol biosynthesis used a cDNA library derived from *T. cuspidata* cells induced with methyl jasmonate to increase Taxol production (7, 9). Transacylase sequences were amplified by PCR from this enriched library cDNA as template, using the previously described transacylase-specific primers (7). The *Taq* DNA polymerase-catalyzed PCRs were performed at relatively low annealing temperature (PCR conditions: 94°C for 4 min, 32 cycles at 94°C for 45 sec, 45°C for 1.2 min, and 72°C for 2 min, and, finally, 74°C for 5 min; each reaction mixture contained 3 units of *Taq* DNA polymerase and 2 mM $MgCl_2$). Several ≈900-bp fragments (designated pPRB900S) were amplified by this means and were found, by cloning and sequencing, to exhibit significant similarity (64–72%) at the deduced protein level to other acyltransferases of plant origin (6, 7, 10–12), thus suggesting that the pPRB900S mixture represented partial cDNA sequences of related *Taxus* acyltransferases. A mixture of ^{32}P-labeled (13) oligonucleotides derived from pPRB900S was used as a hybridization probe to screen the *T. cuspidata* cDNA library (6.0×10^4 plaques grown in *Escherichia coli* XL1-Blue MRF′ from Stratagene) under low-stringency conditions by standard hybridization and purification procedures (14).

Plaques affording positive signals were purified through two additional rounds of hybridization. Several representatives of each of the seven full-length clones obtained were amplified with sequence-specific mutagenic primers that incorporated *Nde*I and *Xba*I restriction sites at the 5′ and 3′ termini, respectively, for directional ligation into vector pCWori+ (15). Each construct, containing a sequence-verified insert, was used individually to transform *E. coli* JM109 host cells (7).

Bacterial Expression, Benzoyltransferase Assay, and Product Identification. For enzyme preparation, *E. coli* cultures transformed with a pCWori+ vector that harbored a putative transacylase gene were grown overnight at 37°C in 5 ml of Luria–Bertani medium supplemented with 50 μg of ampicillin per ml, and 1 ml of culture was added to 100 ml of Terrific Broth culture medium [6 g of Bacto-tryptone (Difco), 12 g of yeast extract (EM Science, Gibbstown, NJ), and 2 ml of glycerol in 500 ml of water] and grown at 37°C. After 3 h, 1 mM isopropyl β-D-thiogalactopyranoside (for induction) and 50 μg of ampicillin per ml were added, and the cultures were grown at 25°C. After 18 h, the bacteria were harvested by centrifugation, resuspended in 25 ml of assay buffer [25 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (Mopso), pH 7.4] and disrupted by sonication at 0°C, and the resulting homogenate was centrifuged at $15{,}000 \times g$ to pellet debris; the resulting supernatant was centrifuged at $190{,}000 \times g$ to provide the soluble enzyme fraction. A 1-ml aliquot of soluble enzyme preparation was incubated with 2-debenzoyl-7,13-diacetylbaccatin III (100 μM) and [7-^{14}C]benzoyl-CoA (100 μM, 1.9 μCi) for 1.5 h at 31°C. The reaction mixture was then extracted with ether (2 ml), and the organic phases were removed and concentrated under reduced pressure. The resulting crude products were dissolved in acetonitrile (50 μl) and analyzed by radio-HPLC using a Perkin–Elmer HPLC ISS 200 chromatograph coupled to a Packard A100 Radiomatic detector (Canberra, Meriden, CT) (see Fig. 3 for a representative chromatogram). The samples were separated on a Vydac (Hesperia, CA) C_{18} column (5 μm, 4.6 × 250 mm) by elution at 1 ml/min with a linear gradient starting from 30:70 CH_3CN/H_2O to 60:40 CH_3CN/H_2O over 30 min, then to 100% CH_3CN with a linear gradient over 5 min, held at 100% CH_3CN for 5 min, and, finally, returned to the initial conditions. Detection of a biosynthetic product absorbing at 228 nm and possessing a coincidental radioactivity response with the same retention time as authentic 7,13-diacetylbaccatin III directed efforts toward large-scale preparation (4 liters) of the enzyme expressed from a single *E. coli* JM109 transformant bearing the clone designated pKW18/TAX2; this clone was previously amplified with primer set 91NDEF, 5′-GGGAATTCCATATGGGCAGGTTCAATGTAG-3′ and 107XBAR, 5′-CTAGTCTAGATTATAACTTAGAGTTACATA-3′ to introduce *Nde*I and *Xba*I restriction sites, respectively, for directional ligation into expression vector pCWori+. The product generated by large-scale preparation of the putative pKW18/TAX2 benzoyltransferase (≈50 μg) was analyzed by combined liquid chromatography–mass spectrometry (LC-MS) using a Hewlett-Packard Series 1100 MSD in the APCI mode. The sample, dissolved in acetonitrile (200 μl), was loaded (5 μl) onto a Phenomenex (Torrance, CA) Curosil-G column (5 μm, 250 × 4.6 mm) that was eluted at 1 ml/min with 30:70 CH_3CN/H_2O for 5 min, increased linearly to 80:20 CH_3CN/H_2O over 55 min, and then held for 5 min before return to initial conditions (R. E. B. Ketchum and R.C., unpublished work). Additional product (≈500 μg) was purified by silica gel TLC (EtOAc/hexane, 60:40, vol/vol), and the band comigrating with authentic 7,13-diacetylbaccatin III (R_f = 0.33) was isolated, dissolved in 0.5 ml of deuterated chloroform as internal standard, and analyzed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) using a Varian Mercury 300 instrument.

Partial Purification and Characterization of Recombinant *T. cuspidata* Benzoyltransferase. Large-scale (4-liter) cultures of *E. coli* JM109 cells harboring the recombinant *Taxus* benzoyltransferase gene were grown, harvested, and extracted as before. After preparation of the soluble enzyme fraction and demonstration, by SDS/PAGE analysis, that a protein of the appropriate size (≈50 kDa) was expressed in operationally soluble form, this material (100 ml) was applied to a column of *O*-diethylaminoethyl-Sepharose (2.8 × 20 cm; Sigma) that was previously washed with 25 mM Mopso buffer (pH 7.4) containing 3 mM dithiothreitol and 1 M NaCl, and then equilibrated with this buffer (without NaCl). After removal of unbound material, protein was eluted with a linear gradient of from 0 to 200 mM NaCl in equilibration buffer (200 ml total volume, at 3 ml/min). Fractions containing the benzoyltransferase activity (eluting at ≈175 mM NaCl) were combined (≈100 ml) and subjected to ultrafiltration (Amicon YM 10 membrane; Millipore) and repeated dilution to reduce the salt concentration to ≈5 mM. This enzyme preparation was used for kinetic evaluation of the transferase.

For kinetic evaluations, linearity with respect to protein concentration and time was first established, and the concentrations of both cosubstrates (0–1000 μM) were then independently varied while the remaining reactant was maintained at saturation (2 mM). Double-reciprocal plots were constructed for each data set, and the equation of the best-fit line (R^2 = 0.99) was determined (KALEIDAGRAPH, version 3.08, Synergy Software, Reading, PA). To examine the influence of pH on activity, enzyme preparations (1 ml, 90 μg of protein) were concentrated (Nanosep 30 microconcentrator; Gelman) to 150 μl and then diluted, respectively, with 1.35 ml of 2-(*N*-morpholino)ethanesulfonic acid (Mes) (pH 6), Mopso (pH 7), glycine (pH 8), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (Capso) (pH 9), or 3-(cyclohexylamino)-1-propanesulfonic acid (Caps) (pH 10 and 11) buffers, all at 25 mM containing 3 mM dithiothreitol, before the assay.



10-deacetylbaccatin III 7,13-diacetylbaccatin III 2-debenzoyl-7,13-diacetylbaccatin III

Fig. 2. Outline of the synthesis and utilization of 2-debenzoyl-7,13-diacetylbaccatin III. Step **a,** methylene chloride, acetic anhydride, 4-(*N*,*N*′-dimethylamino)pyridine, triethylamine, 25°C, 18 h. Step **b,** tetrahydrofuran, bis(2-methoxyethoxy)aluminum hydride (>65% in toluene), 0°C, 30 min. Step **c** indicates the reaction catalyzed by taxane 2α-*O*-benzoyltransferase in the presence of benzoyl-CoA.

Results and Discussion

Cloning and Heterologous Expression of a Taxane 2α-*O*-Benzoyltransferase from *T. cuspidata*. Biogenetic considerations, based on the relative abundances of naturally occurring taxoids of differing levels of functionalization (1), suggested that benzoylation at the C2α-hydroxyl position of the taxane skeleton represented the second acylation step of the Taxol biosynthetic pathway (2, 3). It also seemed probable that the cDNA encoding the corresponding benzoyltransferase resided among a family of presumptive transacylase clones previously isolated from an induced *Taxus* cell library that had yielded genes for both the first and third acyltransferases of the pathway—i.e., the taxane 5α-*O*-acetyltransferase (7) and the taxane 10β-*O*-acetyltransferase (6). To evaluate this set of clones by functional heterologous expression of the target taxane 2α-*O*-benzoyltransferase, a suitable taxoid substrate was required for use, along with [7-^{14}C]benzoyl-CoA, in developing the cell-free assay. No advanced, naturally occurring 2-deacyltaxoid metabolites were available in sufficient quantities for this purpose. Therefore, 10-deacetylbaccatin III, which is available as a prominent *Taxus* metabolite (16), was acetylated and then selectively debenzoylated to afford 2-debenzoyl-7,13-diacetylbaccatin III (Fig. 2) as a surrogate substrate for surveying the clones by cell-free assay of the corresponding expressed proteins.

For this purpose, the seven full-length cDNA clones obtained by screening the library were transferred into vector pCWori+ and expressed in *E. coli* JM109 host cells for test of function. Semipreparative cultures of each transformed and induced bacteria were generated, and the derived soluble enzyme fraction was assayed under standard conditions (4) with 2-debenzoyl-7,13-diacetylbaccatin III and [7-^{14}C]benzoyl-CoA as cosubstrates. One such enzyme preparation (expressed from the

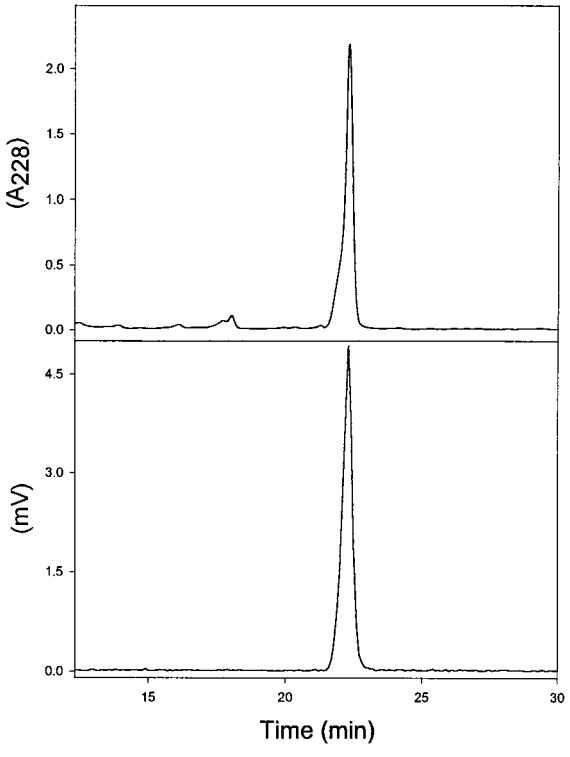


Fig. 3. Radio-HPLC analysis of the biosynthetic product (retention time R_t = 21.9 ± 0.1 min) generated from 2-debenzoyl-7,13-diacetylbaccatin III and [7-^{14}C]benzoyl-CoA by the recombinant benzoyltransferase. The *Upper* trace shows the UV profile (A_{228}) and the *Lower* trace shows the radioactivity profile (in mV), both of which coincide exactly with the retention time of authentic 7,13-diacetylbaccatin III.

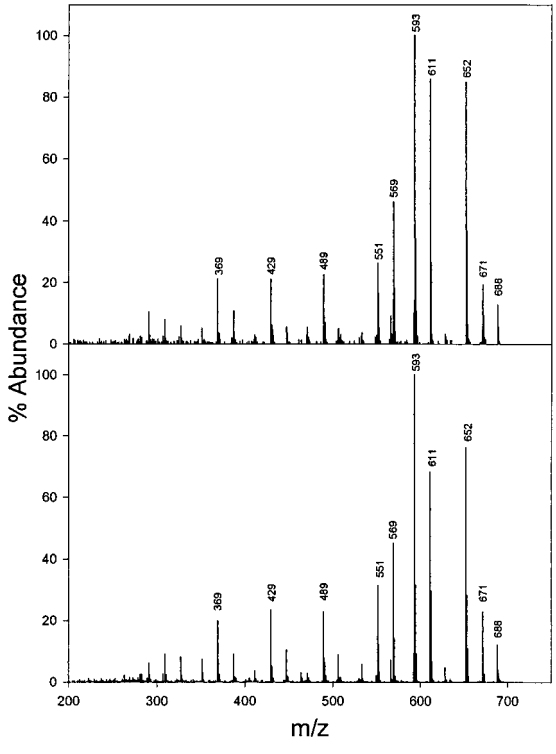


Fig. 4. Coupled reverse-phase HPLC APCI-MS analysis of the biosynthetic product (R_t = 43.6 ± 0.1 min) generated by the recombinant benzoyltransferase with 2-debenzoyl-7,13-diacetylbaccatin III and benzoyl-CoA as cosubstrates (*Upper*) and of authentic 7,13-diacetylbaccatin III (R_t = 43.6 ± 0.1 min) (*Lower*). The diagnostic ions are at *m*/*z* 688 (P + NH_4^+), 671 (PH^+), 652 ($P^{\bullet+}$ − H_2O), 611 (PH^+ − CH_3COOH), 593 (*m*/*z* 652 − CH_3COO), 551 (*m*/*z* 611 − CH_3COOH), and 489 (*m*/*z* 611 − PhCOOH).

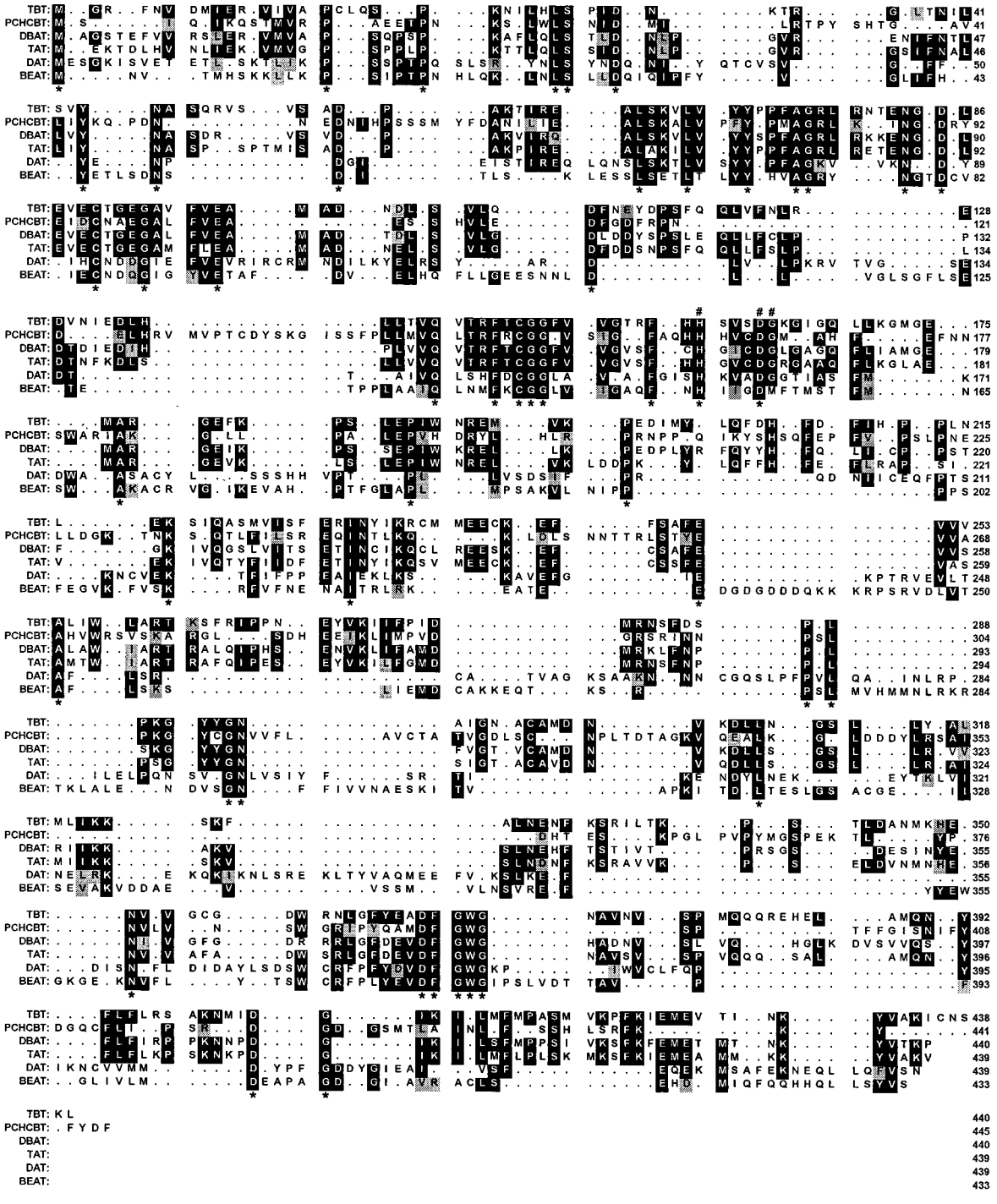


Fig. 5. Deduced amino sequence comparison of TBT (accession no. AF297618) from *T. cuspidata*, anthranilate *N*-hydroxycinnamoyl/benzoyltransferase (PCHCBT, accession no. Z84383) from *Dianthus caryophyllus*, 10-deacetylbaccatin III 10-*O*-acetyltransferase (DBAT, accession no. AF193765) from *T. cuspidata*, taxadien-5α-ol *O*-acetyltransferase (TAT, accession no. AF190130) from *T. cuspidata*, deacetylvindoline 4-*O*-acetyltransferase (DAT, accession no. AF053307) from *Catharanthus roseus*, and benzyl alcohol acetyltransferase (BEAT, accession no. AF043464) from *Clarkia breweri*. Residues boxed in black indicate positional identity for at least three of the compared sequences; similar amino acids are indicated by gray shading. Asterisks (*) indicate conserved residues in all acyltransferase sequences. Pound signs (#) indicate a putative acyl group transfer motif (HXXXDG) present in five of the six sequences. The alignment was created with the PILEUP program (Wisconsin Package Version 10; Genetics Computer Group, Madison, WI).

cDNA designated pKW18/TAX2) yielded a single biosynthetic product that was revealed by reverse-phase radio-HPLC analysis to possess a retention time of 21.9 ± 0.1 min (with coincident radio and UV traces) corresponding exactly to that of authentic 7,13-diacetylbaccatin III (Fig. 3). The expressed protein did not yield product in the absence of either cosubstrate, nor did boiled protein yield product in the presence of both cosubstrates at saturation. Control extracts of *E. coli* host cells transformed with empty vector did not yield detectable product when assayed by identical methods.

After preliminary demonstration that a protein of the appropriate size for an acyltransferase corresponding to the pKW18/TAX2 translation (≈50 kDa, determined by SDS/PAGE) was expressed in operationally soluble form, a large-scale (4-liter) culture of the bacteria carrying the gene was prepared. The soluble enzyme fraction isolated from this preparation was partially purified by anion-exchange chromatography and used to generate sufficient biosynthetic product to identify this material as 7,13-diacetylbaccatin III by combined LC-MS analysis (Fig. 4), a procedure that demonstrated the enzymatic product to possess the identical retention time (43.6 ± 0.1 min) and mass spectrum as the authentic standard. Finally, a purified sample afforded a ^{1}H-NMR spectrum identical to that of authentic 7,13-diacetylbaccatin III, thereby confirming that the *TBT* gene had been isolated by the functional expression strategy.

Characterization of the Recombinant Benzoyltransferase. The enzyme expressed from *TBT* was determined to have a pH optimum of about 8.0, with half-maximal velocities near pH 6.5 and 9.9. This pH optimum is typical for acyltransferases of plant origin (6, 7, 11, 12). The K_m values for 2-debenzoyl-7,13-diacetylbaccatin III and benzoyl-CoA were determined to be 0.64 mM and 0.30 mM, respectively, by Lineweaver–Burk analysis (for both plots, $R^2 = 0.99$). TBT appears to acylate the 2α-hydroxyl group of advanced, well-functionalized, taxoids with a high degree of regioselectivity, since the enzyme does not benzoylate the 1β-, 7β-, 10β-, or 13α-hydroxyl groups of 10-deacetylbaccatin III (see Fig. 2), nor does it benzoylate the 2α- or 5α-hydroxyl groups of taxa-4(20),11(12)-dien-2α,5α-diol (see Fig. 1). The failure of this enzyme to benzoylate the latter simple 2α,5α-diol clearly indicates that a higher level of substitution on the taxane ring is required for productive catalysis and thus validates the use of 2-debenzoyl-7,13-diacetylbaccatin III as a surrogate substrate for the survey of taxane 2α-*O*-benzoyltransferase function.

To evaluate the selectivity of the transferase for the acyl donor, acetyl-CoA was tested as a cosubstrate. Kinetic evaluation indicated a V_{rel} of ≈1.5% compared with benzoyl-CoA, indicating the former to be a substantially less efficient donor than is the aroyl-CoA ester at saturation.

Sequence Analysis. The *TBT* cDNA contains an open reading frame of 1,320 nucleotides (GenBank accession no. AF297618) and encodes a deduced protein of 440 amino acid residues (Fig. 5) with a calculated molecular weight of 50,089, consistent with that of other monomeric plant acyltransferases (≈50 kDa) (6, 7, 10–12). The deduced amino acid sequence bears no N-terminal organellar targeting information, and it resembles that of taxadien-5α-ol acetyltransferase from *T. cuspidata* (7) (68% identity; 74% similarity) and 10-deacetylbaccatin III acetyltransferase from this source (6) (64% identity; 70% similarity), those of other acetyltransferases of plant origin involved in different pathways of secondary metabolism (50–56% identity; 64–65% similarity) (11, 12), and an anthranilate-*N*-cinnamoyl/benzoyltransferase from *Dianthus* (57% identity; 70% similarity) (10). Additionally, TBT possesses the HXXXDG motif (H158, D162, and G163) characteristic of other acyl (aroyl) transferases (6, 7, 10, 12, 17–20) (Fig. 5); this sequence element may function in acyl group transfer from acyl-CoA to the substrate alcohol (12).

Taxol has become a widely used antineoplastic drug in the treatment of ovarian, breast, and lung cancers (21, 22), as well as Kaposi's sarcoma (23). Extensive investigations on the structure–activity relationships of Taxol have shown that the C2 benzoate function is necessary for promoting tubulin stabilization and mitotic arrest, and they have demonstrated that replacement of the C2 benzoxy function by *meta*-azidobenzoates yields taxoid analogs of often greater potency (24, 25). The taxane 2α-*O*-benzoyltransferase described here may facilitate the transfer of such substituted benzoyl groups to the C2 position, and thus find use in the enzymatic semisynthesis of drug analogs.

The isolation of the *TBT* cDNA completes identification of the three transferase genes responsible for acylating the taxane diterpene core, leading to the baccatin III intermediate of Taxol biosynthesis (Fig. 1). In addition to the confirmed taxane 2α-*O*-benzoyltransferase, 10-deacetylbaccatin III 10-*O*-acetyltransferase (6), and taxadienol 5-*O*-acetyltransferase (7), there are two more transacylation steps in the Taxol biosynthetic pathway. These are represented by the baccatin III 13-*O*-phenylisoserinyltransferase and the debenzoyltaxol *N*-benzoyltransferase. The productive cloning strategy described here is likely to soon yield these remaining transacylase genes of Taxol biosynthesis.

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