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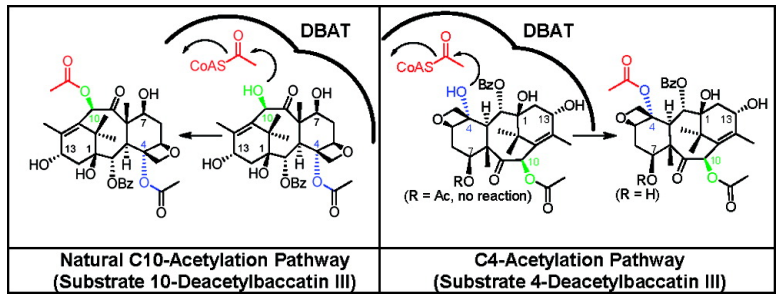

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The Taxol Pathway 10-*O*-Acetyltransferase Shows Regioselective Promiscuity with the Oxetane Hydroxyl of 4-Deacetyltaxanes

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Abstract: The 10-deacetylbaccatin III:10β-*O*-acetyltransferase isolated from *Taxus cuspidata* regiospecifically transfers short-chain alkanoyl groups from their corresponding CoA thioesters to the C10 hydroxyl of 10-deacetylbaccatin III. This 10-*O*-acetyltransferase along with five other *Taxus* acyltransferases on the paclitaxel (Taxol) biosynthetic pathway and one additional *Taxus*-derived acyltransferases of unknown function were screened for 4-*O*-acetyltransferase activity against 4-deacetylbaccatin III, 7-acetyl-, 13-acetyl-, and 7,13-diacetyl-4-deacetylbaccatin III. These 4-deacyl derivatives were semisynthesized from the natural product baccatin III via silyl protecting group manipulation, regioselective reductive ester cleavage with sodium bis(2-methoxyethoxy)aluminum hydride, and regioselective acetylation with acetic anhydride. Assays with the 4-deacetylated diterpene substrates and acetyl CoA revealed the taxane 10β-*O*-acetyltransferase was able to catalyze the 4-*O*-acetylation of 4-deacetylbaccatin III to baccatin III and 13-acetyl-4-deacetylbacatin III to 13-acetylbaccatin III, although each was converted at lesser efficiency than with the natural substrate. In contrast, this enzyme was unable to acetylate 7-acetyl-4-deacetylbaccatin III and 7,13-diacetyl-4-deacetylbaccatin III substrates at C4, suggesting that the C7 hydroxyl of baccatin III must remain deacylated for enzyme function. The biocatalytic transfer of an acyl group to the tertiary hydroxyl on the oxetane moiety at C4 of the taxane ring demonstrates that the regiochemistry of the 10β-acetyltransferase is mutable.

Introduction

Paclitaxel (Taxol) (Figure 1) continues to be used in its well-known regimen against cancer.[1] The clinical application of the pharmaceutical now also includes treatment against restenosis that commonly occurs in the management of heart disease,[2] and variants of paclitaxel are being developed for potential inclusion in Alzheimer's disease therapy to stabilize the tubulin scaffold in neuronal cells.[3] The biosynthetic pathway to this potent mitotic drug in *Taxus* plants and derived cell cultures includes five acyltransferase-catalyzed steps. Each operationally soluble transacylase delivers an acyl group from a corresponding acyl coenzyme A (acyl CoA) thioester to a pathway intermediate.[4−6] Two 5α-*O*-acetyltransferases (designated TAX01 and TAX19) acetylate hydroxyl groups positioned at C5 and the 10-*O*-acetyltransferase acetylates at C10 of the taxane ring, early and late along the pathway, respectively (Figure 2). In the presence

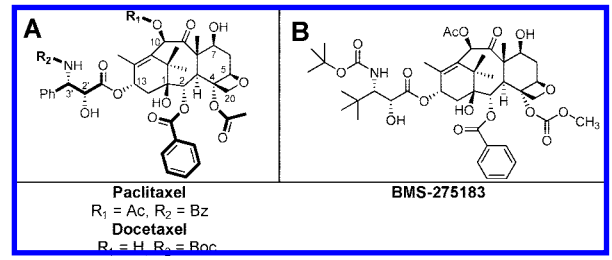

Figure 1. (A) Anticancer pharmaceuticals paclitaxel (Taxol), docetaxel (Taxotere), and (B) a new lead compound from Bristol-Myers Squibb BMS-275183.

of acetyl CoA, TAX01 and TAX19 were shown to acetylate an early stage taxadiene-5α-monool, which is a likely candidate for the first hydroxylated intermediate in the biosynthesis of paclitaxel. The ene-acetoxy functional group of the resultant taxa-4(20),11(12)-dienyl-5α-acetate is considered to transform biosynthetically to a 5-acetoxy-4(20)-epoxy grouping. This epoxide is proposed to isomerize to a 4-acetoxy-4(20),5-oxetane group via intramolecular transfer of the acetoxy group from C5 to C4 with concomitant ring expansion of the epoxy of advanced taxanes in planta (Figure 2, inset).

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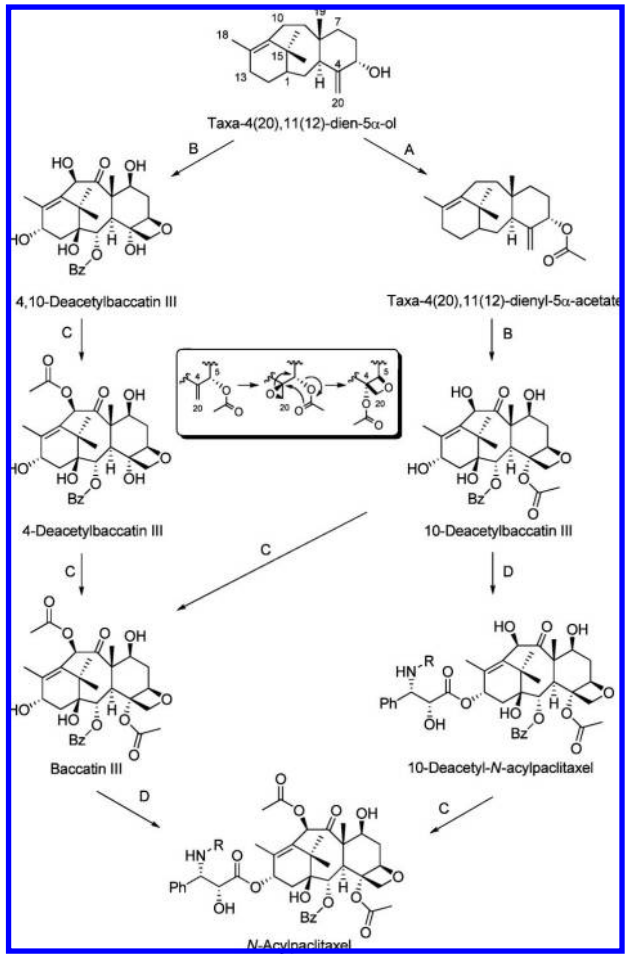

Figure 2. Proposed biosynthetic matrix to paclitaxel, showing parallel routes with alternative acetylation sequences. Step A: 5-*O*-Acetylation by TAX01 or TAX19. Step B: Several oxidation and acylation reactions. Step C: 10-Deacetylbaccatin III acetyltransferase-catalyzed acetylation. Step D: 13-*O*-Acylation by the *Taxus* phenylpropionyl transferase and side chain elaboration. R = *tert*-butoxycarbonyl (synthetically derived) or benzoyl. (Inset) Proposed biogenesis of the oxetane ring of advanced taxanes in *Taxus* spp.

While TAX01 and TAX19 were both characterized as 5α-*O*-acetyltransferases in earlier investigations,[7,8] it later became apparent that the regioselectivity of members of the *Taxus* acyltransferase family depends on the level and regiochemistry of hydroxylation on the taxane substrate. In a previous study, TAX01 and TAX19 showed distinct regiochemical acylation when each catalyst was separately incubated with a surrogate substrate, taxadiene-5,9,10,13-tetraol, and acetyl CoA[7] (Figure 3). TAX01 preferentially acetylated the tetraol substrate primarily at the C9 and/or C10 hydroxyls; no C5- or C13-acetylated product was detected, while TAX19 was more selective for the hydroxyls at C13 and/or C5 in the east−west pole positions (Figure 3). In summary, the data reported in the prior study demonstrated the divergent regiospecificity of TAX01 and TAX19 with variously hydroxylated taxane substrates and implied that the other *Taxus* acyltransferases might likely demonstrate new regiochemistry when incubated with deacylated analogs of their natural pathway taxoid substrates.

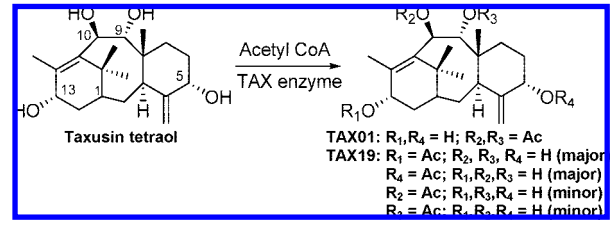

Figure 3. Major (and minor) differentially acetylated products obtained when heterologously expressed *Taxus* acyltransferases, designated TAX01 and TAX19, were incubated with taxusin tetraol and acetyl CoA. Ac = acetyl.

In the study described herein, advanced taxanes deacetylated at C4 were used as substrates and revealed that recombinantly expressed *Taxus* 10-DAB 10β-*O*-acetyltransferase (DBAT)[9] catalyzes novel regioselective acetylation at the tertiary C4 on the oxetane ring of 4-DAB compounds. This observation suggested that an alternative, parallel pathway must be considered along with the isomerization/ring expansion route, described above, for the formation of the 4-acetoxy-4(20),5-oxetane ring functional grouping. The Michaelis constants are reported for the acetyltransferase with its native cosubstrates and compared to the kinetic parameters calculated for surrogate 4-DAB substrates by mixed substrate analysis.

Materials and Methods

General Procedures, Substrates, and Reagents. A Varian Inova-300 or a Varian UnityPlus500 instrument was used to acquire proton nuclear magnetic resonance (^{1}H NMR) spectra with $CDCl_3$ as an internal reference. A Q-ToF Ultima Global electrospray ionization tandem mass spectrometer (Waters, Milford, MA) was used for mass spectral analysis. An Agilent 1100 HPLC system (Agilent Technologies, Wilmington, DE) was employed for chromatographic analyses and connected in series with a UV detector and a Packard Radiomatic Flow-One Beta 150TR radioactivity detector (Perkin Elmer, Shelton, CT), which mixed the effluent with 3a70B Complete Counting Cocktail (Research Products International, Mount Prospect, IL). Reaction products were purified by Kieselgel-60 F254 fluorescent preparative thin-layer chromatography (PTLC) and visualized by absorbance of UV light at 254 nm. Baccatin III and 10-DAB were purchased from Natland Corp. (Research Triangle Park, NC). Unlabeled and tritium-labeled acetyl coenzyme A thioesters were obtained from Sigma-Aldrich as were all other reagents, which were used without purification unless otherwise indicated. Docetaxel (Taxotere) was acquired from OChem Inc. (Des Plaines, IL).

Synthesis of 4-DAB Analogs. 4-Deacetylbaccatin III. 4-DAB was synthesized via an established procedure[10] as follows. To a solution of baccatin III (338 mg, 0.41 mmol) in dry *N,N*-dimethylformamide (6 mL) was added imidazole (330 mg, 4.9 mmol), and the solution was stirred for 5 min, after which chlorotriethylsilane (1.36 mL, 8.2 mmol) was added dropwise at 23 °C. The reaction was heated at 50 °C for 6 h, cooled to room temperature, quenched by adding brine (10 mL), and then diluted with ethyl acetate (EtOAc) (10 mL). The organic layer was decanted, the remaining aqueous layer was extracted with EtOAc (3 × 20 mL), the organic fractions were combined, dried (Na_2SO_4), and the solvent was removed in vacuo. The crude product was crystallized from EtOAc with addition of hexanes to obtain the 7,13-bis(triethylsilyl)baccatin III at >95% yield and judged to be 99% pure by ^{1}H NMR analysis.

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To 7,13-bis(triethylsilyl)baccatin III (287 mg, 0.35 mmol) dissolved in dry *N,N*-dimethylformamide (2 mL) at 0 °C was added imidazole (72 mg, 1.1 mmol), and the solution was stirred for 2 min. Chlorodimethylsilane (0.11 mL, 1.1 mmol) was added dropwise; the reaction was stirred at 0 °C for 45 min, diluted with EtOAc (30 mL), and washed with water (4 × 20 mL). The organic phase was dried (Na_2SO_4) and concentrated under vacuum. The product was purified from the crude mixture by PTLC (15:85 (v/v) EtOAc:hexanes) to give 1-dimethylsilyl-7,13-bis(triethylsilyl) baccatin III product (95% yield, 99% purity by ^{1}H NMR analysis).

Sodium bis(2-methoxyethoxy)aluminum hydride (110 μL) was added dropwise to a solution of 1-dimethylsilyl-7,13-bis(triethylsilyl)baccatin III (120 mg, 0.14 mmol) in dry tetrahydrofuran (THF) (3 mL) at 0 °C. The reaction was stirred for 40 min and then quenched with 1 mL of saturated sodium tartarate solution for 10 min. The solution containing crude product was diluted with EtOAc (40 mL), washed with an equal amount of water, and dried (Na_2SO_4). The organic layer was removed under vacuum, and the crude product was purified by PTLC (20:80 (v/v) ethyl acetate: hexane) to give 1-dimethylsilyl-4-deacetyl-7,13-bis(triethylsilyl) baccatin III (70% yield, 99% purity by ^{1}H NMR).

To a solution of 1-dimethylsilyl-4-deacetyl-7,13-bis(triethylsilyl)baccatin III (56.6 mg, 0.068 mmol) in THF (2 mL) was added pyridine (400 μL). The solution was stirred and cooled to 0 °C, and 60% HF/pyridine solution (400 μL) was added dropwise over 20 min to remove the silyl protecting groups. The reaction was warmed to room temperature and, after 6 h, diluted with EtOAc (20 mL). The aqueous phase was removed, and the remaining organic fraction was washed with sodium bicarbonate (0.4 M), water, and brine (2 × 20 mL each), dried over sodium sulfate, filtered, and finally concentrated under vacuum. The product was purified by PTLC (80:20 (v/v) EtOAc/hexanes, $R_f = 0.24$) to give the final product, 4-DAB (60% yield, 99% purity by ^{1}H NMR analysis). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.10 (s, CH_3-16), 1.15 (s, CH_3-17), 1.61 (s, CH_3-19), 2.14 (s, CH_3-18), 2.29 (s, $OC(O)CH_3$ at 10β), 2.50 (m, 6α, β), 2.58 (m, 14α, β), 3.50 (bs, 4α-OH), 3.63 (d, $J = 6$ Hz, 3α), 4.09 (dd, $J = 6$ Hz, $J = 6$ Hz, 7α), 4.18 (d, $J = 8$ Hz, 20α), 4.43 (d, $J = 8$ Hz, 20β), 4.67 (bt, 13β), 4.85 (dd, $J = 4$ Hz, $J = 4$ Hz, 5α), 5.64 (d, $J = 6$ Hz, 2β), 6.34 (s, 10α), 7.50 (m), 7.64 (m), 8.09 (m) [*m*-H, *p*-H, *o*-H of OBz, respectively].

Synthesis of 7-Acetyl-4-DAB. To a solution of 4-DAB (20 mg, 0.037 mmol) in *N,N*-dimethylformamide (2 mL) were added imidazole (25 mg, 0.37 mmol) and triethylsilyl chloride (12.4 μL, 0.074 mmol), and the reaction was stirred at 45 °C for 3 h under nitrogen to obtain a 1:1 mixture of starting material and 13-triethylsilyl-4-DAB, which was purified by PTLC (80:20 (v/v) EtOAc/hexanes). To a solution of this silylated product (6 mg, 0.01 mmol) in tetrahydrofuran (2 mL) was added dimethylaminopyridine (5.6 mg, 0.05 mmol), triethylamine (1.3 μL, 0.01 mmol), and acetic anhydride (172 μL, 0.2 mmol). The solution was stirred for 3 h at 23 °C and concentrated in vacuo; the sample was then loaded onto a PTLC plate and eluted with 80:20 (v/v) EtOAc:hexanes to give 7-acetyl-13-triethylsilyl-4-DAB (99% yield). To the latter product (7 mg, 0.01 mmol) in THF (2 mL) and pyridine (60 μL) at 0 °C under nitrogen was added (dropwise) a 60% solution of HF in pyridine (60 μL) over 5 min, and the reaction was warmed to room temperature. After 2 h, the solution was diluted with EtOAc (10 mL), quenched with water (5 mL), and extracted with EtOAc (2 × 5 mL). The organic extracts were dried (Na_2SO_4) and evaporated in vacuo. The 7-acetyl-4-DAB was purified by PTLC (80:20 (v/v) EtOAc/hexanes, $R_f = 0.59$) and isolated in 95% yield, 94% purity by ^{1}H NMR analysis. ESI-MS (positive ion mode), *m/z*: 587 (MH^+), 604 ($M + NH_4^+$), 609 ($M+Na^+$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.02 (s, CH_3-16), 1.13 (s, CH_3-17), 1.66 (s, CH_3-19), 2.04 (s, $OC(O)CH_3$ at 7β), 2.12 (s, CH_3-18), 2.18 (s, $OC(O)CH_3$ at 10β), 2.44 (m, 6α), 2.49 (m, 14α, β), 2.64 (m, 6β), 3.68 (d, $J = 6$ Hz, 3α), 4.2 (d, $J = 8$ Hz, 20β), 4.30 (d, $J = 8$ Hz, 20α), 4.62 (bd, 13β), 4.84 (dd, $J = 3$ Hz, $J = 4$ Hz, 5α), 5.21 (dd, $J = 7$ Hz, $J = 7$ Hz, 7α), 5.57 (d, $J = 5$ Hz, 2β), 6.22 (s, 10α), 7.46 (m), 7.57 (m), 8.06 (m) [*m*-H, *p*-H, *o*-H of OBz, respectively].

Synthesis of 13-Acetyl-4-DAB. To a solution of 4-DAB (20 mg, 0.04 mmol) in THF (2 mL) were added dimethylaminopyridine (23 mg, 0.18 mmol), triethylamine (5 μL, 0.04 mmol), and acetic anhydride (4 μL, 0.04 mmol). The solution was stirred for 15 min at 0 °C, the reaction was concentrated in vacuo, and the 13-acetyl-4-DAB was purified by PTLC (80:20 (v/v) EtOAc/hexanes, $R_f = 0.41$) (65% yield, at >98% purity by ^{1}H NMR analysis). ESI-MS (positive ion mode), *m/z*: 587 (MH^+), 604 ($M + NH_4^+$), 609 ($M + Na^+$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.15 (s, CH_3-16), 1.18 (s, CH_3-17), 1.38 (s, CH_3-19), 1.94 (s, CH_3-18), 2.19 (s, $OC(O)CH_3$ at 10β), 2.23 (s, $OC(O)CH_3$ at 13α), 2.39 (m, 6α), 2.49 (m, 14α, β), 2.59 (m, 6β), 2.69 (bs, 4α-OH), 3.28 (d, $J = 6$ Hz, 3α), 3.98 (dd, $J = 6$ Hz, $J = 6$ Hz, 7α), 4.08 (d, $J = 9$ Hz, 20β), 4.32 (d, $J = 9$ Hz, 20α), 4.81 (dd, $J = 4$ Hz, $J = 4$ Hz, 5α), 5.62 (d, $J = 6$ Hz, 2β), 5.94 (m, 13β), 6.29 (s, 10α), 7.46 (t, $J = 6$ Hz, $J = 6$ Hz), 7.57 (m), 7.80 (m) [*m*-H, *p*-H, *o*-H of OBz, respectively].

Synthesis of 7,13-Diacetyl-4-DAB. Analogous to the procedures described above for the synthesis of 13-acetyl-4-DAB, to a solution of 4-DAB (18 mg, 0.033 mmol) dissolved in THF (3 mL) were added dimethylaminopyridine (19 mg, 0.16 mmol), triethylamine (8.8 μL, 0.06 mmol), and acetic anhydride (9 μL, 0.09 mmol). The reaction was stirred at 23 °C for 20 min, diluted with 10 mL of EtOAc, and washed with water (pH 1.0). The organic layer was concentrated, and the 7,13-diacetyl-4-DAB was purified by PTLC (80:20 (v/v) ethyl acetate:hexanes, $R_f = 0.74$), isolated in 75% yield, and judged to be >99% pure by NMR. ESI-MS (positive ion mode), *m/z*: 630 (MH^+), 647 ($M + NH_4^+$), 652 ($M + Na^+$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.12 (s, CH_3-16), 1.15 (s, CH_3-17), 1.7 (s, CH_3-19), 2.00 (s, CH_3-18), 2.04 (s, $OC(O)CH_3$ at 10β), 2.17 (s, $OC(O)CH_3$ at 7β), 2.21 (s, $OC(O)CH_3$ at 13α), 2.45 (m, 6α, β), 2.48 (m, 14α, β), 2.68 (bs, 4α-OH), 3.42 (d, $J = 7$ Hz, 3α), 4.14 (d, $J = 8$ Hz, 20β), 4.29 (d, $J = 8$ Hz, 20α), 4.82 (dd, $J = 3$ Hz, $J = 3$ Hz, 5α), 5.19 (dd, $J = 7$ Hz, $J = 7$ Hz, 7α), 5.62 (d, $J = 6$ Hz, 2β), 5.92 (m, 13β), 6.20 (s, 10α), 7.46 (t, $J = 9$ Hz), 7.59 (m), δ 8.00 (m) [*m*-H, *p*-H, *o*-H of OBz, respectively].

Synthesis of 13-Acetylbaccatin III. 13-Acetylbaccatin III was synthesized similarly to a procedure described for the synthesis of 13-butyrylbaccatin III,[11] except acetic anhydride was used in place of butyryl chloride as the acyl donor. ESI-MS (positive ion mode), *m/z*: 629 (MH^+), 646 ($M + NH_4^+$), 651 ($M + Na^+$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.13 (s, CH_3-16), 1.21 (s, CH_3-17), 1.65 (s, CH_3-19), 1.89 (d, $J = 2$ Hz, CH_3-18), 2.19 (s, $OC(O)CH_3$ at 10β), 2.22 (s, $OC(O)CH_3$ at 4α), 2.23 (m, 6α, β), 2.31 (s, $OC(O)CH_3$ at 13α), 2.54 (m, 14α, β), 3.81 (d, $J = 7$ Hz, 3α), 4.14 (d, $J = 9$ Hz, 20α), 4.29(d, $J = 9$ Hz, 20β), 4.42 (dd, $J = 7$ Hz, $J = 7$ Hz, 7α), 4.95 (dd, $J = 2$ Hz, $J = 2$ Hz, 7α), 5.64 (d, $J = 7$ Hz, 2β), 6.16 (m, 13β), 6.28 (s, 10α), 7.46 (m), 7.59 (m), 8.05 (m) [*o*-H, *p*-H, *m*-H of OBz, respectively].

Expression of Acyltransferases and Screening Enzyme Activity with 4-DAB Compounds. The *Taxus cuspidata* cDNA clones of the five acyltransferases on the paclitaxel pathway were used in their original recombinant expression system as described in previous studies[12] or subcloned into a new expression vector and transferred to a suitable bacterial host. For directional ligation of a full-length acyltransferase into a new expression vector cut with restriction enzymes PCR primers were used to install appropriate 5′- and 3′-overhangs into the DNA amplicon by a cohesive-end ligation method described previously.[13] The 2α-*O*-benzoyltransferase was expressed from pCWori$^+$ in *E. coli* JM109;[14] the 5α-*O*-acetyltransferase[8] was expressed from pSBET

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in JM109 as described. DBAT was subcloned from pCWori$^+$ into pET28,[15] the 13-*O*-phenylpropanoyltransferase[5] was subcloned from pSBET and transferred to pET14, and the 3′*N*-benzoyltransferase[12] was subcloned from pSBET to pET28; each new vector was separately expressed in *E. coli* BL21(DE3). A *Taxus* acyltransferase cDNA clone, designated TAX19,[7] which acetylates a taxadiene-monool and -tetraol, was expressed from pSBET in *E. coli* JM109, and a *Taxus* acyltransferase clone, designated TAX05, of unknown function was expressed from pSBET in *E. coli* BL21(DE3).[12] Each of the *E. coli* cells transformed with a respective acyltransferase were grown overnight at 37 °C in 5 mL of Luria−Bertani medium supplemented with the appropriate antibiotic at 50 μg/mL. The 5-mL inoculum was added to and grown in 1 L Luria−Bertani medium supplemented with antibiotic (50 μg/mL) at 37 °C to $OD_{600} = 1.0$. The cultures were induced with isopropyl-β-D-thiogalactopyranoside (1 mM final concentration), and the cultures grown at 16−18 °C for 18 h. The subsequent steps were performed at 4 °C, unless otherwise indicated. The cultures were harvested and centrifuged at 6000*g* for 20 min, and the pellet was resuspended in MOPSO buffer (pH 7.2), which was supplemented with 3 mM dithiothreitol and glycerol (5% v/v). The cells were lysed at 4 °C by sonication (3 × 30 s bursts at 50% power (Misonix sonicator, Farmingdale, NY) with 2 min intervals between each burst), and the cell-lysate was centrifuged at 10 000*g* for 20 min to remove cell debris and clarified by ultracentrifugation at 100 000*g* for 2 h. Empty vectors of each type described above were expressed in the corresponding *E. coli* in control assays, and the cell free extracts were obtained by procedures identical to those described above.

To verify functional expression of the enzymes, except for the TAX05 enzyme of unknown function, a 1 mL aliquot of each crude extract was assayed with its natural cosubstrates,[12] each at 1 mM. A 1 mL aliquot of each extract containing active paclitaxel pathway acyltransferases, the TAX05 enzyme, and enzymes isolated from hosts engineered for empty vector expression was incubated with surrogate substrate 4-DAB (1 mM) and [^{3}H]acetyl coenzyme A (0.1 μmol, 1 μCi) under similar assay conditions at 30 °C. After 3 h, ethyl acetate (2 mL) was added to the assays to stop the reactions. The organic solvent was removed, and the assays were extracted twice more with EtOAc (2 mL). The organic fractions were combined, the solvent was evaporated, the residue was redissolved in 50 μL of acetonitrile, and a 25 μL aliquot was analyzed. The sample was loaded onto a reverse-phase column (Zorbax 5 μm XDB-C18, 4.6 × 250 mm, Hewlett-Packard) eluted at 1 mL/min with 20:80 (v/v) for 5 min, then with a linear gradient of CH_3CN in H_2O over 20 min to 100% CH_3CN, and finally returned to initial conditions with a 5 min hold and continuous UV-absorbance detection and radioactivity monitoring of the effluent mixed with counting cocktail.

Mass Spectrometry and ^{1}H NMR Analysis of Biocatalyzed Product. For the assay in which 4-DAB was incubated with [^{3}H]acetyl CoA and recombinantly expressed DBAT, radio-HPLC analysis of the biosynthetic product isolated from these assays revealed a de novo compound displaying a new radioactivity signal that was of identical retention time to that of authentic baccatin III. To verify the identity of this unknown compound, large-scale (6 L) *E. coli* cultures expressing the *dbat* clone by IPTG induction were grown for 18 h at 20 °C, the cells were lysed by sonication, and the cell debris was pelleted by centrifugation. The soluble enzymes (80 mL) were decanted and loaded onto a Whatman DE-52 anion-exchange column (2.5 × 6 cm, 20 g resin) to remove small molecules and cell debris. The protein fractions containing active acyltransferases were eluted between 100 and 120 mM NaCl, pooled (150 mL), and concentrated 20-fold by ultrafiltration (10 000 kDa MWCO, Billerica, MA); this enzyme preparation was judged to be >50% pure and deemed sufficient for this preparative assay. The overexpressed protein was of the correct molecular weight (~50 kDa), as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining. The quantity (100 μg/mL) and purity of concentrated enzyme was assessed using Kodak 1D image analysis software (version 3.6.3) to integrate the relative intensities of the scanned protein bands compared to a standardized concentration series of bovine serum albumin.

4-DAB (1 mM, 0.001 mmol) was incubated with acetyl coenzyme A (1 mM, 0.01 mmol) in separate enzyme assays containing 5 mL of the concentrated enzyme, described above. Each reaction was quenched after 3 h by mixing with EtOAc to denature the protein. The organic fraction was decanted, the aqueous fraction was extracted twice more with 5 mL of ethyl acetate, the organic fractions were combined, and the solvent was evaporated under vacuum. The resulting residue was redissolved in acetonitrile (100 μL), and the total volume was loaded onto a reverse-phase HPLC column in 20 μL aliquots per load. The sample elution parameters were the same as those described in the previous section. The fraction eluting with identical retention time (14.94 min) as the biosynthetic radioactive compound identified in the preliminary screening assays, discussed previously, was collected, and the effluent was evaporated. A small fraction of the resulting residue was dissolved in methanol (10 μL) and analyzed by electrospray ionization mass spectrometry (Q-ToF Ultima Global, Milford, MA) in the positive ion mode. A solution of the remaining sample in $CDCl_3$ (100 μL) in a solvent-matched Shigemi tube was analyzed by ^{1}H NMR.

Kinetic Evaluations. For kinetic studies, DBAT was harvested as before except the enzyme was further subjected to Nickel affinity gel chromatography to obtain protein at >80% purity (3 mg /mL). Linearity of the DBAT rate with respect to protein concentration and time was first established with the natural substrate 10-DAB at 50 μM, while acetyl CoA was maintained at saturation (500 μM). Aliquots (1 mL) were collected and quenched at 30 min and 1, 2, 3, and 5 h. Protein at 5 μg/mL was used for kinetic evaluations in assays run for 20 min in order to maintain steady-state conditions. For nonlinear regression analysis of the reaction rate, the concentration of the diterpene was then independently varied (0−1000 μM) in separate assays, while acetyl CoA was maintained at saturation (500 μM). The initial velocity (v_o) was plotted against substrate concentration, and the equation of the best-fit line ($R^2 > 0.98$) was determined (Microsoft Excel 2003, Microsoft Corp., Redmond, WA).

Kinetic Evaluation of DBAT with Competing 4-DAB Substrates. The procedure used to calculate the relative kinetic constants of DBAT for multiple diterpene substrates was adapted from methods used to calculate the specificity constants for DBAT[15] and a NodH sulfotransferase[16] in separate, previous studies. This relative kinetics evaluation method was advantageous toward conserving the costly radiolabeled acetyl CoA substrate and the dearth of synthetically derived 4-deacetylbaccatin III cosubstrate by reducing the duplicate assays required to construct parallel kinetic plots for each taxane substrate. In brief, the kinetic parameters (k_{cat} and K_M) obtained from the Lineweaver−Burk plot constructed for DBAT and its commercially available natural substrates (acetyl CoA and 10-DAB) were used to assess the relative catalytic efficiencies of competing 4-DAB substrates in mixed substrate assays with either docetaxel or 10-DAB.

The relative substrate specificity constant of the catalytically productive 4-DAB substrate was assessed indirectly by comparison against the constant $(k_{cat}/K_M)_{Bacc1}$ calculated for the natural substrate with DBAT. In summary, 10-DAB (35 μM) and docetaxel (35 μM) [a productive 10-deacetyltaxoid substrate of DBAT] were mixed along with the DBAT enzyme (~5 μg) and [^{3}H]acetyl CoA (1 μCi, 0.1 μmol) in a single assay tube and incubated for 20 min. The molar ratio ([$Bacc_1$]/[10AcD]) of baccatin III (from 10-DAB) and

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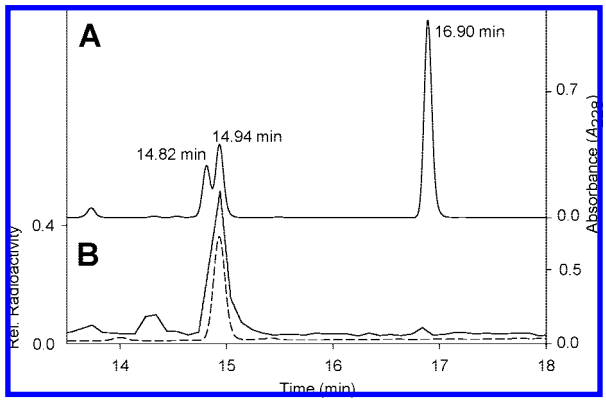

Figure 4. (A) Partial reverse-phase HPLC profile of a mixture of authentic 13-acetyl-4-deacetylbaccatin III (R_t = 14.82), baccatin III (R_t = 14.94), and 7-acetyl-4-deactylbacccatin III (R_t = 16.90) with A_{228} monitoring of the effluent. (B) Partial HPLC profile showing A_{228} (dotted line) and radioactivity (solid line) of a mixture of authentic unlabeled baccatin III with the [^{3}H]-labeled biosynthetic product (R_t = 14.94 min) derived by incubation of DBAT with acetyl CoA and 4-deacetylbaccatin III.

10-acetyldocetaxel formed in the mixed reaction was used to convert the specificity constant $(k_{cat}/K_M)_{Bacc1}$ of DBAT to a relative specificity constant for the docetaxel substrate with the following equation: $(k_{cat}/K_M)_{Bacc1}([10AcD]/[Bacc_1]) = (k_{cat}/K_M)_{10AcD}$. In a separate mixed substrate assay docetaxel (35 μM) was mixed with 4-DAB (35 μM) and DBAT (~5 μg), and the molar ratio ([10AcD]/[$Bacc_2$]) of 10-acetyldocetaxel and baccatin III (the latter derived from 4-DAB) formed was used to calculate the relative specificity constant of DBAT with 4-DAB (i.e., $(k_{cat}/K_M)_{4\text{-}DAB}$) from the specificity constant of DBAT with docetaxel with the following equation: $(k_{cat}/K_M)_{Bacc1}([Bacc_1]/[10AcD])([10AcD]/[Bacc_2]) = (k_{cat}/K_M)_{4\text{-}DAB}$. Analogous competitive assays were run to obtain the relative kinetic parameters of DBAT with the productive substrate 13-acetyl-4-DAB.

Results

Expression and Screening Acyltransferase Function with 4-DAB. Seven full-length *Taxus* acyltransferase cDNA clones were recombinantly expressed in bacteria to screen for novel enzyme activity that acylates the C4 hydroxyl of 4-DAB via acyl group transfer from [^{3}H]acetyl CoA. Six of the described clones encoded enzymes on the paclitaxel pathway, and one encoded an enzyme of unknown function. Despite the characterization of the pathway acyltransferases with their natural substrates, no extended investigation of the substrate specificity has been systematically conducted to dissect the range of acyl groups these catalysts can transfer to their natural regiocenter. Particularly, the specificity of each catalyst has never been explored for acetyl CoA and the unique 4-deacetylbaccatin III substrates used in this study. Therefore, all of the extant acyltransferases (despite the classification of some as non-*acetyl*transferases) were included in the assay screens since it was difficult to predict their behavior with the given set of cosubstrates. Semipreparative cultures of each transformed and induced bacteria were generated, and the derived soluble enzyme fraction was partially purified and assayed under standard conditions using synthetically derived 4-DAB as substrate along with tritium-label acetyl CoA. One such enzyme preparation (expressed from the *dbat* cDNA) yielded a single radioactive biosynthetic product with a retention time of 14.94 ± 0.01 min on reverse-phase radio-HPLC that corresponded exactly to that of authentic baccatin III (Figure 4). Expressed protein that was either boiled (and incubated with both cosubstrates at saturation) or assayed in the absence of either cosubstrate yielded no detectable products nor did enzyme isolated from *E. coli* host cells transformed with empty vector when assayed by identical methods.

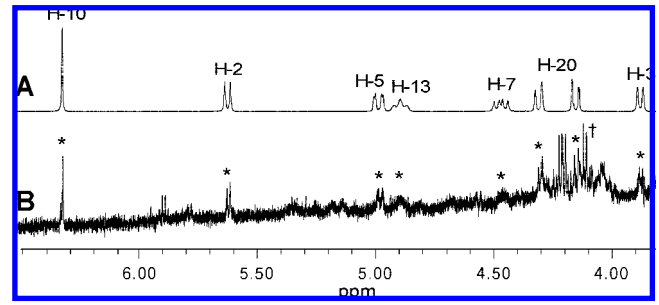

Figure 5. (A) Partial ^{1}H NMR profile of diagnostic signals of authentic baccatin III. (B) Partial ^{1}H NMR profile of the biosynthetic product derived by incubating DBAT with acetyl CoA and 4-deacetylbaccatin III and purified by reverse-phase HPLC. Peaks highlighted with an asterisk (*) are proton signals of the biosynthetically derived sample that are consistent with those of authentic baccatin III. The other minor signals in the spectrum are consistent with those of the starting material 4-deacetylbaccatin III. The dagger (†) indicates peaks from residual ethyl acetate (solvent).

After demonstrating that a protein was expressed in operationally soluble form of appropriate size for DBAT (~50 kDa, determined by SDS-PAGE),[12] a large-scale (6 L) culture of the bacteria carrying the gene was prepared. The soluble enzyme fraction isolated from this preparation was purified by anion-exchange chromatography to obtain protein at >50% purity. This enzyme preparation was satisfactory to generate sufficient biosynthetic product, derived by incubation of unlabeled acetyl CoA and 4-DAB with DBAT. The product was purified by reverse-phase HPLC and then shown by direct injection ESI-MS analysis to have the same molecular weight (m/z = 587, MH^+) as baccatin III. An aliquot of the purified biosynthetic sample (~0.1 μg) was analyzed by ^{1}H NMR and due to limited sample displayed a low signal-to-noise spectrum that was however in most respects identical to that of authentic baccatin III (Figure 5). Particularly diagnostic was the proton signal (δ = 3.86) of H-3 of the biosynthetic sample that matched the resonance of the H-3 proton of baccatin III (Figure 5), which possesses an acetate group at C4. In contrast, the ^{1}H NMR signal for H-3 of 4-DAB is further upfield at δ = 3.58 when the functional group at C4 is a hydroxyl (data not shown). In addition, the biosynthetic product has diagnostic proton resonances at δ 4.89 and 4.49, which, respectively, match the H-13 and H-7 ^{1}H NMR signals of authentic baccatin III (Figure 5).

Taken together, the mass spectrometric and ^{1}H NMR data provided preliminary evidence that DBAT was able to convert 4-DAB to baccatin III. However, these data alone were not sufficient to validate the regiochemistry of the acyltransferase reaction, and sample limitations made the less sensitive ^{13}C NMR-dependent techniques impractical. Therefore, the regiochemistry of the DBAT reaction with 4-DAB and acetyl CoA was further verified by comparing the biosynthetic product against synthetically derived 7-acetyl-4-DAB and 13-acetyl-4-DAB regioisomers by relative retention time on UV-HPLC. The retention time (14.94 ± 0.01 min) of authentic baccatin III was identical to that of the biosynthetic product, while authentic 7-acetyl-4-DAB and 13-acetyl-4-DAB eluted at 16.90 ± 0.01 and 14.82 ± 0.01 min, respectively (Figure 4). These data strongly support that DBAT transfers the acetyl group from the corresponding CoA thioester to the hydroxyl at C4 on the oxetane ring of the diterpene cosubstrate.

C4 Acetylation of 13-Acetyl-4-DAB. The occurrence of several naturally occurring taxoids acylated at C13, including paclitaxel (cf. Figure 1), prompted the use of synthetically derived 13-acetyl-4-DAB as a substrate along with [^{3}H]acetyl CoA to assess whether enzymatic C4-hydroxyl acylation by DBAT catalysis was viable when the C13-hydroxyl is capped. The soluble DBAT enzyme used for the acetylation of 4-DAB was incubated with the 13-acetyl analog under standard assay conditions, and reverse-phase radio-HPLC analysis of the sample mixture revealed a de novo biosynthetic product with a retention time of 17.05 ± 0.01 min. This product had identical retention time on HPLC as authentic 13-acetylbaccatin III. Enzyme-catalyzed acetylation at the C7 rather than at the C4 hydroxyl was ruled out due to the difference in the retention time (Δt = +1.23 min) on reverse-phase HPLC of the biosynthetic product and that of authentic 7,13-diacetyl-4-DAB (data not shown).

Kinetic Parameters of the Recombinant 10β-Acetyltransferase with 4-DAB Compounds. The specificity constant (k_{cat}/K_M) of DBAT with its natural diterpene substrate 10-DAB was calculated to be 10^4 $s^{-1}\cdot M^{-1}$(k_{cat} = 0.58 s^{-1}, K_M= 57.6 μM), indicating the proficient turnover of this enzyme. The DBAT kinetic constants with its natural substrate were used to calculate the relative catalytic constants of DBAT for 4-DAB or 13-acetyl-4-DAB with acetyl CoA in mixed substrate assays.[15,16] The common product, baccatin III, obtained from both 10-DAB and 4-DAB in a mixed reaction with DBAT and acetyl CoA precluded direct comparison of the relative steady-state rate (v_o) for conversion of each substrate to product in a single assay. Therefore, docetaxel (a 10-deacetyltaxane) was employed to facilitate the calculations of the specificity constants. Docetaxel (50 μM) was demonstrated to be a productive substrate after incubating it for 1 h with DBAT (5 μg) and [^{3}H]acetyl CoA (0.1 mM, 1 μCi). The identity of the product was verified as 10-acetyldocetaxel by identical retention time on reverse-phase HPLC and mass spectrometry fragmentation as those of authentic standard (data not shown). For the kinetic assays, 4-DAB (35 μM) was incubated with docetaxel (35 μM) and [^{3}H]acetyl CoA (0.1 mM, 1 μCi) to obtain the relative rates of acetylated product formed by DBAT (5 μg) catalysis from each substrate. Similarly, docetaxel (35 μM), 10-DAB (35 μM), and [^{3}H]acetyl CoA (0.1 mM, 1 μCi) were incubated together with DBAT (5 μg), and the ratio of the acetylated products from their respective substrates was assessed. A similar sequence of assays was conducted between docetaxel and 13-acetyl-4-DAB as competitive substrates. The relative catalytic efficiency values were calculated to be 190 and 15 $s^{-1}\cdot M^{-1}$ for 4-DAB and 13-acetyl-4-DAB, respectively.

Discussion

Implications on Paclitaxel Biosynthesis. The ability of the 10-acetyltransferase to acetylate the oxetane ring of advanced taxanes at the C4-hydroxyl has profound implications on the proposed order of the paclitaxel biosynthetic pathway in *Taxus* plants and derived cell cultures. The current dogma is that the oxetane ring (cf. Figure 1) of taxanes is formed by conversion of 4,20-ene-5-ol to a 4,20-oxirane-5-acetoxy functional group by an acetylation and epoxidation sequence (cf. Figure 2) in *Taxus* plants that make paclitaxel.[17] The latter functional group is proposed to isomerize and require the concomitant intramolecular migration of the acetate group from C4 to C5. The present investigation suggests that a potential alternative biosynthetic route may be operable wherein the oxetane ring is formed by an acyl-independent mechanism prior to acetylation of the C4 hydroxyl. However, since DBAT is ~50-fold less catalytically efficient with 4-DAB than with 10-DAB and since no naturally occurring 4-deacetyl taxanes have yet been detected,[18] the regioselective C4 acylation by DBAT is cautiously considered an operational step on the pathway. It is notable to mention that the lower efficiency of DBAT with the 4-deacetyl substrates was anticipated, particularly since the 4-DAB analogs were deemed surrogate substrates and possessed markedly different positioning of the hydroxyl group ("southeast" orientation) compared to that in the natural diterpene substrate ("northwest" orientation).

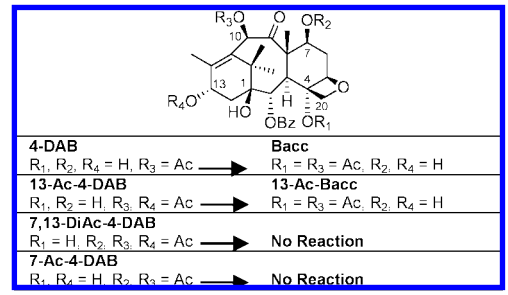

Figure 6. 4-Deacetylbaccatin III analogs utilized in the reaction catalyzed by the 10-*O*-acetyltransferase in the presence of acetyl CoA. 13-Ac-Bacc: 13-acetylbaccatin III. 7-Ac-4-DAB: 7-acetyl-4-deacetylbaccatin III. 13-Ac-4-DAB: 13-acetyl-4-deacetylbaccatin III. 7,13-DiAc-4-DAB: 7,13-diacetyl-4-deacetylbaccatin III. Bacc: baccatin III. 4-DAB: 4-deacetylbaccatin III.

It is evident, however, that the regiospecificity of DBAT is linked to the acylation regiochemistry present in the substrate. Theoretical models suggest that enzyme specificity is governed, in part, by the spatial orientation of the substrate in the active site. In this context, the natural cosubstrate acetyl CoA likely docks in the DBAT active site in its native conformation, while the deacetylated baccatin III is oriented so that the free hydroxyl at either C10 (in the natural substrate 10-DAB) or C4 (of the surrogate 4-DAB analogs) is directed toward the proposed catalytic histidine of the acyltransferases in the BAHD family, including DBAT and the other functional *Taxus* acyltransferases.[19] The catalytic competence of DBAT with these surrogate substrates thus categorizes the described biocatalyst as multifunctional, with the ability to acetylate at secondary- and tertiary-hydroxyl regiocenters on the taxane skeleton at C10 and C4, respectively. Likely, the other functionally defined *Taxus* acyltransferase will show altered selectivity with modified taxane substrates or possibly nontaxane hydroxyterpenes; investigations to test the specificity of this family of acyltransferases are ongoing.

Interestingly, 4-DAB analogs acetylated at C7 and/or both C7 and C13 were not productive substrates of DBAT, suggesting that acylation at C7 of advanced taxanes precludes C4 acetylation catalysis (Figure 6). In contrast, DBAT was able to catalyze the acetylation of 13-acetyl-4-DAB (in which the C7 hydroxyl is free) (Figure 6) in the presence of acetyl CoA but with >10-fold lesser efficiency (15 $s^{-1}\cdot M^{-1}$) compared with 4-DAB (190 $s^{-1}\cdot M^{-1}$). Incidentally, no naturally occurring

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taxanes have been isolated that bear an acyl group at the C7 hydroxyl of advance taxoids; thus, a C4-hydroxy/oxetane taxoid conceivably could be acetylated by DBAT in *Taxus* plants. Consequently, a putative parallel pathway on which C4-acetylation could proceed after formation of the oxetane ring must be considered in the paclitaxel biosynthetic pathway (see Figure 2). Additionally, 10-*O*-acetylation of docetaxel (cf. Figure 1) by DBAT (at its natural C10 hydroxyl position) further supported the notion that a side chain at C13, even larger than an acetyl group, does not affect substrate recognition by DBAT. This finding not only enabled docetaxel to be used as an intermediary substrate for calculating the relative kinetic constants of DBAT with 4-DAB analogs in this study, but also suggested that acetylation at the C10 hydroxyl can occur after attachment of the phenylpropanoyl side chain to 10-deacetylbaccatin III on the biosynthetic pathway (Figure 2). Baccatin III, which is acetylated at C10, is widely accepted as the last diterpene intermediate on the paclitaxel pathway, but apparently, parallel routes with alternative placement of the C10 acetylation step must be taken into account (Figure 2), according to the current findings.

Altered Synthetic Acetylation Regiochemistry. Intriguingly, synthesis of the 13-acetyl-4-DAB substrate used during this investigation proceeded directly from 4-DAB via regioselective acetylation without a protecting group series. This selectivity was unexpected considering that the hydroxyl at C7 of baccatin III is significantly more reactive than the sterically hindered C13 hydroxyl; consequently, the C7 hydroxyl ordinarily needs to be protected prior to conducting synthetic acylation chemistry at C13. When 4-DAB was acetylated at C13 by synthetic means, no protecting group chemistry was required due to an apparent inversion in the rates of reactivity of the hydroxyls at C7 and C13 when the functional group at C4 of baccatin III is hydroxyl, rather than acetoxy. This exchange in hydroxyl group reactivity potentially provides a synthetic route for directly attaching unnatural paclitaxel side chains at C13 without protecting group chemistry to make new generation paclitaxel compounds.

Potential Application of DBAT in Semisynthesis. Several structure−activity relationship analyses have guided the semisynthesis of several analogs of paclitaxel[20,21] in order to optimize drug pharmacodynamics. Presently, each paclitaxel analog is principally obtained by semisynthetic methods that require protecting group steps, which generally affect production yields. To partially address this synthesis bottleneck, Bristol-Myers Squibb recently reported a biocatalytic method where 10-deacetylbaccatin III (10-DAB) is regiospecifically deacetylated at the C4 hydroxyl by the function of esterase activity found in whole cells and cell-free extracts of *Rhodococcus* sp. or *Stenotrophomonas maltophilia*.[22] The so-derived 4-deacetyl intermediate was subjected to protecting group manipulations, followed by synthetic methyl carbonation, and then synthetically elaborated to an efficacious 4-deacetyl-4-(methyl carbonate)paclitaxel (BMS-275183) (Figure 1).[23] Potentially DBAT could be engineered for enhanced catalytic efficiency and regiospecificity for optimal C4 acylation to complement the C4-esterase process developed by BMS. The coupled esterase/transferase production process would eliminate the remaining silyl protection/deprotection steps involved in production of 4-acyl-4-DAB precursors.[18,22]

Consideration of the described next-generation paclitaxel molecule derived by synthetic replacement of the acetyl group at C4, in part, encouraged this investigation in which the *Taxus* acyltransferases were screened for 4-*O*-acetylation activity against 4-deacylated analogs of advanced taxane metabolites. Currently, C4-hydroxyl acylation or methyl carbonation of next generation taxanes incorporates a protecting group series prior to modifying the C4-hydroxyl of the taxane. This requirement inflates the number of reaction steps and consequently reduces product yield.[22,24] The present exploratory investigation demonstrated that the wild-type *Taxus* 10-*O*-acetyltransferase also functions as a 4-*O*-acetyltransferase (Figure 6), and thus provides preliminary evidence that formulates a basis for the potential development and implementation of DBAT into a biocatalytic process. It is apparent that the catalytic efficiency of DBAT with 4-DAB analogs and acetyl CoA is 2 orders of magnitude lower than with the natural diterpene substrate. Thus, the wild-type DBAT is understandably not suitable for immediate biocatalytic application. However, foreseeably this Taxol pathway enzyme could be altered for desired catalytic properties through precedent application of random mutagenesis techniques, which often introduce fortuitous mutations into a particular target gene.[25−29] In general, these enzyme "breeding" methods have produced catalysts with desired targeted function, such as improved efficiency,[30] enhanced small-molecule enantioselectivity,[25,26] and modified substrate specificity.[27−30] Enhancing the 10-*O*-acetyltransferase activity toward modifying the C4-hydroxyl with general acyl groups potentially provides an essential bridge to construct a linear biocatalytic route to construct intermediates such as 4-carbonate-4-DAB for subsequent synthesis of new generation taxanes.

Potential in Vivo Assembly of Modified Taxanes. A previous in vivo *Taxus* acyltransferase study was conducted to assess whether novel baccatin III analogs could be assembled from 10-DAB substrate fed to *E. coli*, which was engineered to express DBAT and a butyric acid CoA ligase in the presence of the endogenously expressed short-chain acid CoA ligase. Accordingly, acetate, propionate, and butyrate fed to the cells were converted to their CoA thioesters for use as substrates by DBAT, which converted 10-DAB to baccatin III analogs variously acylated at C10.[11] By analogy, 4-DAB could replace 10-DAB in this in vivo system for the biocatalytic production of novel 4-acyl-4-DAB compounds. This biochemical acylation could greatly impact production of (but not limited to) C4 methyl carbonate paclitaxel analogs, e.g.,

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BMS-275183 (currently in clinical trials),[23] and other C4-acyl analogs of baccatin III. The substrate specificity of the wild-type DBAT for longer chain (C_3 and C_4) acylation and carbonation at C4 is currently being investigated.

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