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An *N*-Aroyltransferase of the BAHD Superfamily Has Broad Aroyl CoA Specificity *in Vitro* with Analogues of *N*-Dearoylpaclitaxel

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Abstract: The native *N*-debenzoyl-2′-deoxypaclitaxel:*N*-benzoyltransferase (NDTBT), from *Taxus* plants, transfers a benzoyl group from the corresponding CoA thioester to the amino group of the β-phenylalanine side chain of *N*-debenzoyl-2′-deoxypaclitaxel, which is purportedly on the paclitaxel (Taxol) biosynthetic pathway. To elucidate the substrate specificity of NDTBT overexpressed in *Escherichia coli*, the purified enzyme was incubated with semisynthetically derived *N*-debenzoyltaxoid substrates and aroyl CoA donors (benzoyl; *ortho*-, *meta*-, and *para*-substituted benzoyls; various heterole carbonyls; alkanoyls; and butenoyl), which were obtained from commercial sources or synthesized *via* a mixed anhydride method. Several unnatural *N*-aroyl-*N*-debenzoyl-2′-deoxypaclitaxel analogues were biocatalytically assembled with catalytic efficiencies (V_{max}/K_M) ranging between 0.15 and 1.74 nmol·min^{-1}·mM^{-1}. In addition, several *N*-acyl-*N*-debenzoylpaclitaxel variants were biosynthesized when *N*-debenzoylpaclitaxel and *N*-de(*tert*-butoxycarbonyl)docetaxel (i.e., 10-deacetyl-*N*-debenzoylpaclitaxel) were used as substrates. The relative velocity (v_{rel}) for NDTBT with the latter two *N*-debenzoyl taxane substrates ranged between ~1% and 200% for the array of aroyl CoAs compared to benzoyl CoA. Interestingly, NDTBT transferred hexanoyl, acetyl, and butyryl more rapidly than butenoyl or benzoyl from the CoA donor to taxanes with isoserinoyl side chains, whereas *N*-debenzoyl-2′-deoxypaclitaxel was more rapidly converted to its *N*-benzoyl derivative than to its *N*-alkanoyl or *N*-butenoyl congeners. Biocatalytic *N*-acyl transfer of novel acyl groups to the amino functional group of *N*-debenzoylpaclitaxel and its 2′-deoxy precursor reveal the surprisingly indiscriminate specificity of this transferase. This feature of NDTBT potentially provides a tool for alternative biocatalytic *N*-aroylation/alkanoylation to construct next generation taxanes or other novel bioactive diterpene compounds.

Introduction

The antineoplastic agent paclitaxel (Taxol) and docetaxel (Taxotere) (Figure 1) promote and stabilize microtubule assembly and, consequently, disrupt mitosis. This aberration ultimately leads to cell cycle arrest and apoptosis.[1] Application of these drugs remains prominent in the treatment of various cancer types and in the management of heart disease. The pharmaceuticals are also receiving interest in newly developed regimens against Alzheimer's disease and tuberculosis.[2−5] Structure−activity relationship studies have demonstrated that the various acyl group types and their distinct regiochemistry

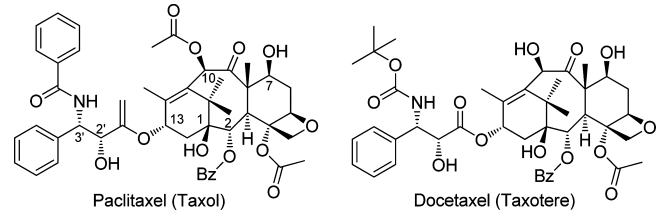

Figure 1. Structures of antineoplastic taxanes: paclitaxel, and docetaxel.

partially define the potency of the active pharmacophore that causes the drugs to bind tubulin and stabilize microtubules.[6] Analysis of the biological activity of paclitaxel analogues derived from various combinatorial chemistry libraries[7−11] has identified several lead compounds with greater efficacy than the parent drug, with regards to better water solubility, tissue-specific targeting, and enhanced blood−brain barrier permeability.[12,13] Several components of the isoserine side chain are required for activity, including the 2′-hydroxyl, 2-aryl, and a 3′-*N*-arylamide.[14] Presently, each derivative is principally

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(1) Schiff, P. B.; Fant, J.; Horwitz, S. B. *Nature (London)* **1979**, *277*, 665–667.
(2) Hayes, D. F.; Thor, A. D.; Dressler, L. G.; Weaver, D.; Edgerton, S.; Cowan, D.; Broadwater, G.; Goldstein, L. J.; Martino, S.; Ingle, J. N.; Henderson, I. C.; Norton, L.; Winer, E. P.; Hudis, C. A.; Ellis, M. J.; Berry, D. A. *N. Engl. J. Med* **2007**, *357*, 1496–1506.
(3) Weissman, N. J.; Ellis, S. G.; Grube, E.; Dawkins, K. D.; Greenberg, J. D.; Mann, T.; Cannon, L. A.; Cambier, P. A.; Fernandez, S.; Mintz, G. S.; Mandinov, L.; Koglin, J.; Stone, G. W. *Eur. Heart J.* **2007**, *28*, 1574–1582.
(4) Seyb, K. I.; Ansar, S.; Bean, J.; Michaelis, M. L. *J. Mol. Neurosci.* **2006**, *28*, 111–124.
(5) Huang, Q.; Kirikae, F.; Kirikae, T.; Pepe, A.; Amin, A.; Respicio, L.; Slayden, R. A.; Tonge, P. J.; Ojima, I. *J. Med. Chem.* **2006**, *49*, 463–466.
(6) Kingston, D. G. I. *J. Nat. Prod.* **2000**, *63*, 726–734.

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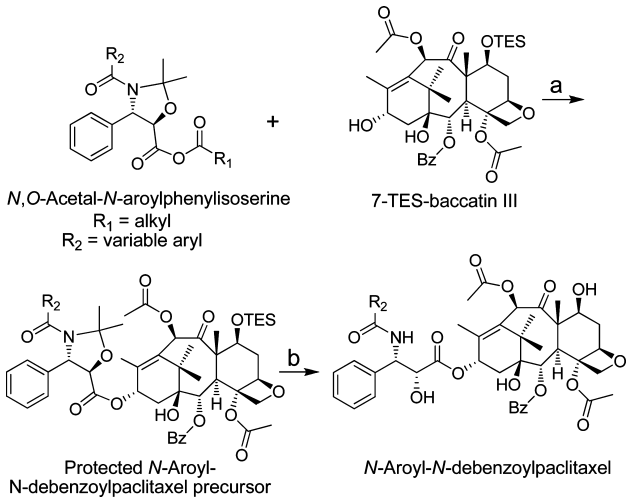
Scheme 1. A Route to *N*-Aroyl-*N*-debenzoylpaclitaxel Analogues[a,b]

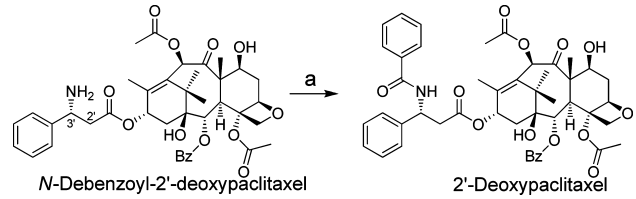


[a] Base, THF. [b] Deprotection.

obtained by semisynthetic methods that require protecting group management, which generally affects overall product yield.[15] At least 10 steps are necessary to install a 3′-(4-*O*-methylbenzamide) in place of the naturally occurring 3′-benzamide in the molecule, five of which include protection/deprotection manipulation of competing reactive centers. Briefly, the semisynthesis of arylamide analogues of paclitaxel includes selective hydroxyl group protection at C7, selective acylation at the C13 hydroxyl with either a *N*,*O*-acetal-*N*-aroylphenylisoserine, activated as a mixed anhydride,[16] or a *N*-aroyllactam precursor of *N*-aroylphenylisoserine,[9] and finally deprotection to construct the target product (Scheme 1).

Conceivably, the application of biocatalytic acylation in the described semisynthetic methods could potentially reduce the number of protection steps in the assembly of these next generation compounds. *Taxus*-derived acyltransferases[17] belong to a superfamily of plant-derived acyltransferases designated BAHD,[18,19] the acronym being derived from the abbreviations of the names (BEAT, AHCT, HCBT, and DAT) of the first

Scheme 2. Enzymatic Benzoylation of *N*-Debenzoyl-2′-deoxypaclitaxel[a]



[a] NDTBT, benzoyl CoA.

four enzymes identified and characterized in this family.[19,20] In general terms, each member of this family requires a coenzyme A-dependent acyl group donor and contains a conserved HXXXD amino acid sequence diad that is presumably involved in the transfer of various acyl groups [alkyl, aroyl, or phenylpropanoyl] to a corresponding hydroxylated and/or aminated substrate.[19] Generally, the specificity of individual members of the BAHD superfamily varies from restricted to broad substrate access *in vitro*[21] and *in vivo*.[22] The paclitaxel biosynthetic pathway in *Taxus* spp. contains five such acyltransferases that transfer acyl/aroyl groups to different taxane structures. The *N*-debenzoyl-2′-deoxypaclitaxel:*N*-benzoyltransferase (designated herein as NDTBT) is unique among the pathway catalysts in terms of recognizing an amine acceptor group of the diterpene cosubstrate; the other *Taxus* acyltransferases convey an alkanoyl or aroyl group to variously positioned hydroxyls. In particular, the monomeric NDTBT catalyzes the formation of a benzamide functional group from cosubstrates benzoyl CoA and *N*-debenzoyl-2′-deoxypaclitaxel (Scheme 2).[23] A previous, limited study revealed that the product derived from NDTBT in assays containing acetyl CoA and the diterpene substrate was below detection limits.[23] In addition, the substrate specificity of NDTBT was recently assessed with the aim of dissecting the function of the catalyst in the biosynthesis of naturally occurring advanced taxanes found in *Taxus* spp.[24] The results from this previous work demonstrated that NDTBT was limited to benzoyl CoA as the acyl donor; whereas, tigloyl, hexanoyl, or butenoyl was not transferred to the *N*-debenzoyl taxanes as assessed by UV-HPLC monitoring of the biosynthetic products.

In the current study, the products isolated from *in vitro* assays with mixtures of NDTBT, *N*-debenzoylpaclitaxel precursors, and acyl CoA thioesters were either analyzed by UV-HPLC in mixed substrate assays to calculate specificity constants or separated by HPLC with the effluent directed toward a tandem mass spectrometer (MS/MS) for selected molecular ion fragmentation analysis. The high sensitivity of this MS/MS method enabled us to reveal the extremely broad substrate specificity of the *N*-benzoyltransferase. Relative kinetic constants are reported for

(7) Ganesh, T.; Norris, A.; Sharma, S.; Bane, S.; Alcaraz, A. A.; Snyder, J. P.; Kingston, D. G. I. *Biorg. Med. Chem.* **2006**, *14*, 3447–3454.
(8) Kingston, D. G. I.; Bane, S.; Snyder, J. P. *Cell Cycle* **2004**, *4*, 279–289.
(9) Ojima, I.; Fumero-Oderda, C. L.; Kuduk, S. D.; Ma, Z.; Kirikae, F.; Kirikae, T. *Biorg. Med. Chem.* **2003**, *11*, 2867–2888.
(10) Baloglu, E.; Hoch, J. M.; Chatterjee, S. K.; Ravindra, R.; Bane, S.; Kingston, D. G. I. *Biorg. Med. Chem.* **2003**, *11*, 1557–1568.
(11) Kingston, D. G. I.; Jagtap, P. G.; Yuan, H.; Samala, L. In *Progress in the Chemistry of Organic Natural Products*; Herz, W., Falk, H., Kirby, G. W., Eds.; Springer-Verlag Wien: New York, 2002; Vol. 84, pp 53−225.
(12) Roh, E. J.; Song, C. E.; Kim, D.; Pae, H. O.; Chung, H. T.; Lee, K. S.; Chai, K.; Lee, C. O.; Un Choi, S. *Biorg. Med. Chem.* **1999**, *7*, 2115–2119.
(13) Xue, M.; Long, B. H.; Fairchild, C.; Johnston, K.; Rose, W. C.; Kadow, J. F.; Vyas, D. M.; Chen, S. H. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1327–31.
(14) Ganesh, T.; Yang, C.; Norris, A.; Glass, T.; Bane, S.; Ravindra, R.; Banerjee, A.; Metaferia, B.; Thomas, S. L.; Giannakakou, P.; Alcaraz, A. A.; Lakdawala, A. S.; Snyder, J. P.; Kingston, D. G. *J. Med. Chem.* **2007**, *50*, 713–25.
(15) Kingston, D. G. I.; Ganesh, T.; Snyder, J. P.; Lakdawala, A. S.; Bane, S. WO2005070414, Virginia Tech Intellectual Properties, Inc., 2005, 78 pp.
(16) McChesney, J. D.; Henri, J. T.; Venkataraman, S. K.; Lamb, R. L.; Foster, J. E.; Sumner, C. M.; Ye, S, Yiannkouros, G. P, Stemphoski, A. M.; Walker, D. G. WO 2007075870, Tapestry Pharmaceuticals, Inc., 2007, 58 pp.
(17) Jennewein, S.; Wildung, M. R.; Chau, M.; Walker, K.; Croteau, R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9149–9154.
(18) D'Auria, J. C. *Curr. Opin. Plant Biol.* **2006**, *9*, 331–340.
(19) St-Pierre, B.; De Luca, V. *Recent Adv. Phytochem.* **2000**, *34*, 285–315.
(20) D'Auria, J. C. *Curr. Opin. Plant Biol.* **2006**, *9*, 331–40.
(21) Souleyre, E. J. F.; Greenwood, D. R.; Friel, E. N.; Karunairetnam, S.; Newcomb, R. D. *FEBS J.* **2005**, *272*, 3132–3144.
(22) Loncaric, C; Ward, A. F; Walker, K. D. *Biotechnol. J.* **2007**, *2*, 266–274.
(23) Walker, K.; Long, R.; Croteau, R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 9166–9171.
(24) Long, R. M.; Lagisetti, C.; Coates, R. M.; Croteau, R. B. *Arch. Biochem. Biophys.* **2008**, *477*, 384–389.

the enzyme with the various combinations of taxane and acyl CoA substrates.

Experimental Section

General. A Varian Inova-300 or a Varian UnityPlus500 instrument was used to acquire proton nuclear magnetic resonance (^{1}H NMR) spectra. A Q-ToF Ultima Global electrospray ionization tandem mass spectrometer (ESI-MS/MS, Waters, Milford, MA) with a Waters CapLC capillary HPLC was used for mass spectral analysis. An Agilent 1100 HPLC system equipped with a UV detector was used for liquid chromatography and absorbance (A_{228}) monitoring of effluent.

Substrates. *N-tert*-Butoxycarbonyl-(3*R*)-*β*-phenylalanine was obtained from Acros Organics (Morris Plains, NJ), docetaxel was obtained from OChem (Des Plaines, IL), coenzyme A was acquired from ARC (St. Louis, MO), and benzoyl CoA and all other reagents were obtained from Sigma-Aldrich and used without further purification, unless indicated otherwise. Baccatin III was purchased from Natland (Research Triangle Park, NC). *N*-Debenzoyl-(3′*R*)-2′-deoxypaclitaxel was synthesized as described,[25,26] except that the enantiomeric *N-tert*-butoxycarbonyl-(3*R*)-*β*-phenylalanine, instead of the racemate, was coupled to 7-TES-baccatin III; deprotection by described methods yielded the desired product.[27]

Preparation of 2′-TBDMS-docetaxel. The following synthesis is based on a similar procedure reported previously.[28] In brief, to a solution of docetaxel (220 mg, 0.27 mmol) dissolved in DMF (2.4 mL) were added imidazole (92 mg, 1.32 mmol, 5 equiv) and *tert*-butyldimethylsilyl chloride (TBDMS-Cl) (204 mg, 1.32 mmol, 5 equiv). The solution was stirred at 23 °C, and then after 18 h, the mixture was diluted with ethyl acetate and extracted with water (2 × 30 mL) and brine (1 × 30 mL). The organic layer was dried ($MgSO_4$), concentrated *in vacuo*, and purified by silica gel column chromatography (1:2 EtOAc/hexane, v/v) to yield 2′-TBDMS-docetaxel as a white powder (246 mg, 0.267 mmol, 99% yield at 99% purity as judged by ^{1}H NMR). ESI-MS (positive ion mode) 944.6 $[M + Na]^+$. ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 8.09 (d, 2H, *J* = 7.5 Hz, *ortho*-ArH of C2 benzoyl), 7.56 (t, 1H, *J* = 7.5, 7.2 Hz, *para*-ArH of C2 benzoyl), 7.46 (t, 2H, *J* = 7.8, 7.2 Hz, *meta*-ArH of C2 benzoyl), 7.36 (m, 4H, ArH of C3′ phenyl), 7.28 (m, 1H, ArH of C3′ phenyl), 6.31 (t, 1H, *J* = 8.7 Hz, H-13), 5.66 (d, 1H, *J* = 7.2 Hz, H-2), 5.43 (br d, 1H, *J* = 9.3 Hz, H-3′), 5.30 (br d, 1H, *J* = 9.0 Hz, NH), 5.18 (s, 1H, H-10), 4.95 (d, 1H, *J* = 8.1 Hz, H-5), 4.5 (d, 1H, *J* = 2.6 Hz, H-2′), 4.32−4.17 (m, 1H, H-7), 4.17 (dd, 2H, *J* = 7.2 Hz, H-20), 3.93 (d, 1H, *J* = 6.6 Hz, H-3), 2.61−2.58 (m, 1H, H-6), 2.53 (s, 3H, C(O)OCH_3 at C4), 2.38−2.39 (m, 3H, H- 6α, H-14), 2.02 (s, 4H, H-18, H- 6*β*) 1.73 (s, 3H, H-19), 1.28 (s, 9H, $OC(CH_3)_3$), 1.28 (s, 3H, H-17), 0.72 (s, 9H, $SiC(CH_3)_3$), −0.13 (s, 3H, $SiCH_3$), −0.34 (s, 3H, $SiCH_3$).

Synthesis of 10-Acetyl-2′-TBDMS-docetaxel. To a solution of 2′-TBDMS-docetaxel (240 mg, 0.26 mmol) and anhydrous cerium chloride (6.3 mg, 0.1 equiv) dissolved in THF (5 mL) was added excess acetic anhydride (10 mL, dropwise). The reaction progress was checked by TLC for the formation of product. After 1 h, the reaction mixture was diluted with EtOAc (100 mL) and washed with saturated aqueous $NaHCO_3$ solution (3 × 40 mL) and then brine (20 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel flash column chromatography (1:1 EtOAc/Hexane, v/v), and the isolated product was concentrated *in vacuo* to give 10-acetyl-2′-TBDMS-docetaxel (231 mg, 0.24 mmol, 93% yield at 95% purity as determined by ^{1}H NMR). ESI-MS (positive ion mode) 986.4 $[M + Na]^+$. ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 8.09 (d, 2H, *J* = 6.9 Hz, *ortho*-ArH of C2 benzoyl), 7.56 (t, 1H, *J* = 7.5 Hz, *para*-ArH of C2 benzoyl), 7.45 (t, 2H, *J* = 7.5 Hz, *meta*-ArH of C2 benzoyl), 7.36 (m, 4H, ArH of C3′ phenyl), 7.28 (m, 1H, ArH of C3′ phenyl), 6.30 (s and br t, 2H, H-10, H-13), 5.66 (d, 1H, *J* = 7.2 Hz, H-2), 5.44 (d, 1H, *J* = 9.6 Hz, H-3′), 5.30 (br d, 1H, *J* = 9.2 Hz, NH), 4.95 (d, 1H, *J* = 7.8 Hz, H-5), 4.5 (d, 1H, *J* = 1.8 Hz, H-2′), 4.42 (dd, *J* = 6.6, 4.5 Hz, 1H, H-7), 4.30 (d, 1H, *J* = 8.4 Hz, H-20α), 4.17 (d, 1H, *J* = 8.1 Hz, H-20*β*), 3.81 (d, 1H, *J* = 7.2 Hz, H-3), 2.53 (s, 3H, C(O)OCH_3 at C-4), 2.41−2.33 (m, 3H, H-6α, H-14), 2.20 (s, 3H, C(O)OCH_3 at C-10), 2.04 (s, 4H, H-18, H-6*β*) 1.83 (s, 3H, H-19), 1.28 (s, 12H, H-16, $OC(CH_3)_3$), 1.13 (s, 3H, H-17), 0.724 (s, 9H, $SiC(CH_3)_3$), −0.14 (s, 3H, $SiCH_3$), −0.32 (s, 3H, $SiCH_3$).

Synthesis of 10-Acetyldocetaxel. To a solution of 10-acetyl-2′-TBDMS-docetaxel (231 mg, 0.24 mmol) dissolved in THF (5 mL) at 0 °C were added pyridine (0.75 mL) and a solution of 70% HF dissolved in pyridine (0.75 mL). The reaction was stirred at 0 °C and then gradually warmed to room temperature. The progress of the reaction was monitored by TLC, showing ∼40% conversion of the starting material. Additional pyridine (0.5 mL) and HF/pyridine solution (0.5 mL) were added at 0 °C, and the mixture was warmed to room temperature and stirred for 10 h to complete the reaction. The solution was diluted with EtOAc (100 mL), and the organic layer was washed successively with a 5% (w/v) solution of $NaHCO_3$ (2 × 10 mL), a 5% (v/v) solution of HCl (2 × 10 mL), water (10 mL), and brine (15 mL). The organic fraction was dried ($MgSO_4$) and concentrated under vacuum, the crude mixture was purified by silica gel column chromatography (1:1 EtOAc/hexane, v/v), and the fractions containing product were concentrated *in vacuo* to provide 10-acetyldocetaxel (193 mg, 0.22 mmol, 89% yield at 95% purity as judged by ^{1}H NMR). ESI-MS (positive ion mode) 872.3 $[M + Na]^+$. ^{1}H NMR ($CDCl_3$, 500 MHz) *δ*: 8.12 (d, 2H, *J* = 7.5 Hz, *ortho*-ArH of C2 benzoyl), 7.62 (t, 1H, *J* = 7.5, 7.0 Hz, *para*-ArH of C2 benzoyl), 7.51 (t, 2H, *J* = 7.5 Hz, *meta*-ArH of C2 benzoyl), 7.40 (m, 4H, ArH of C3′ phenyl), 7.33 (m, 1H, ArH of C3′ phenyl), 6.31 (s, 1H, H-10), 6.25 (t, 1H, *J* = 9.0 Hz, H-13), 5.68 (d, 1H, *J* = 7.0 Hz, H-2), 5.45 (br d, 1H, *J* = 9.3 Hz, H-3′), 5.30 (br d, 1H, *J* = 9.3 Hz, NH), 4.95 (d, 1H, *J* = 8.0 Hz, H-5), 4.64 (d, 1H, *J* = 2.3 Hz, H-2′), 4.42 (dd, *J* = 9.0, 8 Hz, 1H, H-7), 4.30 (d, 1H, *J* = 8.5 Hz, H-20α), 4.17 (d, 1H, *J* = 8.5 Hz, H-20*β*), 3.81 (d, 1H, *J* = 7.5 Hz, H-3), 3.4 (br d, 1H, 2′-OH), 2.61−2.57 (m, 1H, H-6α), 2.38 (s, 3H, C(O)OCH_3 at C-4), 2.33 (m, 3H, H-14), 2.25 (s, 3H, C(O)OCH_3 at C-10), 1.91−1.89 (m, 1H, H-6*β*), 1.86 (s, 3H, H-18), 1.70 (s, 3H, H-19), 1.35 (s, 9H, $OC(CH_3)_3$), 1.27 (s, 3H, H-16), 1.16 (s, 3H, H-17).

Synthesis of *N*-Debenzoylpaclitaxel. To a solution of 10-acetyldocetaxel (170 mg, 0.20 mmol) dissolved in acetonitrile (1 mL) was added (dropwise) 88% formic acid solution (2 mL) at 0 °C, and the reaction was slowly warmed to room temperature. After 4 h, the reaction was judged to be ∼50% complete according to TLC (90:10 chloroform/methanol, v/v, $R_f = 0.3$) and was quenched by partitioning between $NaHCO_3$ solution and chloroform. The aqueous fraction was removed, and the organic layer was extracted with 5% (w/v) $NaHCO_3$ solution (40 mL). The aqueous fractions were combined and back extracted with chloroform (2 × 25 mL). The organic fractions were combined, dried ($MgSO_4$), and concentrated *in vacuo*, and the resultant residue was purified by silica gel column chromatography (8% MeOH in $CHCl_3$) to provide *N*-debenzoylpaclitaxel (105 mg, 0.14 mmol, 70% yield) as a pale yellow solid. ESI-MS (positive ion mode) 750.2 $[M + H]^+$, 768 $[M + Na]^+$. ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 8.07−8.04 (m, 2H, *ortho*-ArH of C2 benzoyl), 7.65 (t, 1H, *J* = 8.3 Hz, *para*-ArH of C2 benzoyl), 7.51 (t, 2H, *J* = 8.1, 6.9 Hz, *meta*-ArH of C2 benzoyl), 7.38 (m, 4H, ArH of C3′ phenyl), 7.28 (m, 1H, ArH of C3′ phenyl), 6.27 (s, 1H, H-10), 6.14 (t, 1H, *J* = 8.0 Hz, H-13), 5.63 (d, 1H, *J* = 7.2 Hz, H-2), 4.93 (d, 1H, *J* = 8.1 Hz, H-5), 4.42−4.34 (m, 1H, H-7), 4.32−4.27 (m, 3H, H-20α,*β* and H-2′), 4.13 (d, 1H, *J* = 8.7

(25) Shiina, I.; Suenaga, Y.; Nakano, M.; Mukaiyama, T. *Bull. Chem. Soc. Jpn.* **2000**, *73*, 2811–2818.
(26) Saitoh, K.; Shiina, I.; Mukaiyama, T. *Chem. Lett.* **1998**, 679–680.
(27) Georg, G. I.; Boge, T. C.; Cheruvallath, Z. S.; Harriman, G. C. B.; Hepperle, M.; Park, H.; Himes, R. H. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 335–8.
(28) van Tilburg, E. W.; Franssen, E. J. F.; van der Hoeven, J. J. M.; van der Meij, M.; Elshove, D.; Lammertsma, A. A.; Windhorst, A. D. *J. Labelled Compd. Radiopharm.* **2004**, *47*, 763–777.

Hz, H-3′), 3.75 (d, 1H, $J = 6.9$ Hz, H-3), 2.56−2.48 (m, 1H, H-6α), 2.24 (s, 3H, C(O)OCH_3 at C10 or C4), 2.23 (s, 3H, C(O)OCH_3 at C10 or C4), 1.98−2.10 (m, 3H, H-14 and H-6β), 1.88 (br s, 3H, H-18), 1.65 (s, 3H, H-19), 1.24 (s, 3H, H-16), 1.12 (s, 3H, H-17).

Synthesis of 10-Deacetyl-*N*-debenzoylpaclitaxel. A solution of docetaxel (100 mg, 0.124 mmol) in concentrated formic acid (25 mL) was stirred for 4 h at room temperature. The mixture was concentrated, and the residual acid was removed as an azeotrope with toluene. The residue was partitioned against 5% (w/v) $NaHCO_3$ solution and ethyl acetate. The aqueous fraction was removed, and the organic layer was again extracted with 5% (w/v) $NaHCO_3$ solution (40 mL). The aqueous fractions were combined and back extracted with ethyl acetate (3 × 25 mL). The organic layer was dried ($MgSO_4$) and concentrated under vacuum. The resulting crude product was purified by silica gel column chromatography (95:5 EtOAc/MeOH, v/v) to obtain 10-deacetyl-*N*-debenzoylpaclitaxel as a white powder (54 mg, 0.075 mmol, 60% yield). ESI-MS (positive ion mode) 708.2 $[M + H]^+$. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.06 (d, 2H, $J = 7.8$ Hz, *ortho*-ArH of C2 benzoyl), 7.62 (t, 1H, $J = 7.5$, 7.2 Hz, *para*-ArH of C2 benzoyl), 7.51 (t, 2H, $J = 7.5$ Hz, *meta*-ArH of C2 benzoyl), 7.39 (m, 4H, ArH of C3′ phenyl), 7.30 (m, 1H, ArH of C3′ phenyl), 6.13 (t, 1H, $J = 9.0$ Hz, 13-H), 5.64 (d, 1H, $J = 7.2$ Hz, H-2), 5.20 (s, 1H, H-10), 4.93 (d, 1H, $J = 7.8$ Hz, H-5), 4.35−4.28 (m, 3H, H-2′, H-3′, H-20α), 4.26 (m, 1H, H-7), 4.15 (d, 1H, $J = 8.4$ Hz, H-20b), 3.88 (d, 1H, $J = 7.2$ Hz, H-3), 2.62−2.52 (m, 1H, H-6α), 2.25 (s, 3H, C(O)OCH_3 at C-4), 2.07−2.02 (m, 1H, 2H, H-14), 1.90 (s, 3H, H-18), 1.82 (m, 1H, H-6β), 1.75 (s, 3H, H-19), 1.21 (s, 3H, H-16), 1.11 (s, 3H, H-17).

Synthesis of CoA Thioesters. Several aroyl CoA donors (heteroaroyls and variously substituted benzoyls) were synthesized *via* a previously described method that proceeds through a mixed ethyl carbonate anhydride.[29] Briefly, triethylamine (3.0 μL, 30 μmol) was added to a solution of the corresponding carboxylic acid (27 μmol) in 5:2 CH_2Cl_2/THF (v/v, 1.4 mL) under N_2 gas. The mixture was stirred for 10 min at 23 °C, ethyl chloroformate (2.9 μL, 30 μmol) was added in one portion, and the reaction was stirred for 1 h at 23 °C. The solvents were evaporated under reduced pressure, and the residue was dissolved in 0.5 mL of *tert*-butyl alcohol. Coenzyme A 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 23 °C, then quenched with dropwise addition of 1 M HCl, and adjusted to pH 3−5. The solvents were evaporated under vacuum, and the residue was dissolved in water (5 mL, pH 5) and loaded onto a C18 silica gel column (10% capped with TMS) that was washed with 100% MeOH (50 mL) and pre-equilibrated with distilled water (100 mL, pH 5). The sample was washed with water (100 mL, pH 5) and then eluted with 15% MeOH in water (50 mL, pH 5). The fractions containing aroyl CoA, as determined by TLC, were combined, and the solvent was evaporated to yield product (95−99% yield at 95−99% purity, assessed by ^{1}H NMR) (see Supporting Information).

Enzyme Expression. Standard microbial and recombinant techniques used throughout this work are described by Sambrook.[30] The full length *ndtbt* cDNA (accession AF466397) was subcloned from its original expression vector pSBET[17] into pET28a (Novagen), which incorporated an N-terminal His_6-tag epitope for immunoblot analysis of the expressed protein and purification by HIS-Select Nickel Affinity Gel (Sigma, St. Louis, MO). To facilitate subcloning, a site-specific mutagenesis protocol was used to remove an internal *Nde*I restriction site with the following oligonucleotide sets [pair 1: T7 promoter primer (5′-TAATACGACTCACTATAGGG-3′) and NdeIKORev primer (5′-GGACATTATCCATTGC**G**TATGCAGTACCAATGGC-3′); pair 2: NdeIKOFor primer (5′-GCCATTGGTACTGCATA**C***G*CAATGGATAATGTCC-3′) and T7 terminator primer (5′-GCTAGTTATTGCTCAGCGG-3′); the nucleotide mutation is bold text and underlined]. Turbo *Pfu* DNA polymerase (Stratagene) was used in the PCR reactions.

The derived blunt-end cDNA amplicon was digested with *Nde*I and *Bam*HI (New England Biolabs, Ipswich, MA) and directionally inserted into the similarly digested pET28a vector by T4 ligase (New England Biolabs, Ipswich, MA) activity. The resultant plasmid containing the *ndtbt* cDNA and the pET28a vector without an insert were used to separately transform *E. coli* BL21-CodonPlus(DE3) following the manufacturer's protocol (Stratagene).

Protein Harvest, Activity Assay, and Purification. Recombinant *ndtbt* was expressed in the described bacterial expression system and harvested according to a previously reported protocol,[23] with slight modifications. Cultures were grown overnight at 37 °C in 5 mL of Luria−Bertani medium supplemented with 50 μg/mL kanamycin and 35 μg/mL chloramphenicol. Bacteria transformed with empty vector were processed analogously. The 5-mL inoculum was added and grown at 37 °C to $OD_{600} = 0.5-0.7$ in 1 L of Luria−Bertani medium supplemented with the appropriate antibiotics, and then gene expression was induced with 100 μM isopropyl-β-D-1-thiogalactopyranoside, and the culture was incubated at 20 °C. After 18 h, the cells were harvested by centrifugation at 2000*g* for 20 min at 4 °C, the supernatant was discarded, and the pellet was resuspended in Lysis Buffer (50 mM sodium phosphate, 300 mM sodium chloride, and 10 mM imidazole, pH 8.0 at 3 mL/g cells) at 4 °C. The cells were lysed at 4 °C by sonication (6 × 20 s bursts at 60% power at 1 min intervals) with a Misonix XL-2020 sonicator (Misonix Inc., Farmingdale, NY), and the cell-lysate was clarified by ultracentrifugation at 46 000*g* for 1 h at 4 °C.

To verify functional expression of the NDTBT, 5 mg of total protein from the clarified lysate were withdrawn from the supernatant of lysed bacteria that expressed *ndtbt* and from the supernatant of similarly processed cells that were transformed with empty vector. Protein concentrations were assessed by the Bradford method (Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL)) and compared against a series of bovine serum albumin protein standards of varying concentrations. Each soluble enzyme fraction (1 mL) was separately assayed with benzoyl CoA and *N*-debenzoyl-2′-deoxypaclitaxel (each at 1 mM) at 31 °C. After 2 h, the assays were quenched with 0.1 N HCl until pH 4−5 to partition the amine substrate to the aqueous fraction. The assays were then separately extracted with ethyl acetate (2 × 1 mL), the respective organic fractions were combined, the solvent was evaporated, and the residue was redissolved in 50 μL of acetonitrile. A 25-μL aliquot was loaded onto a reversed-phase column (Allsphere ODS-25 μm, 250 mm × 4.6 mm, Alltech, Mentor, OH) and eluted isocratically with 50:50 (v/v) solvent A/solvent B [solvent A: 97.99% H_2O with 2% CH_3CN and 0.01% H_3PO_4 (v/v); solvent B: 99.99% CH_3CN with 0.01% H_3PO_4 (v/v); flow rate of 1 mL/min; A_{228} monitoring of the effluent for 20 min] on an Agilent 1100 HPLC system (Agilent Technologies, Wilmington, DE) connected to a UV detector. UV-absorbance profiles of the biosynthetic product isolated from the assay containing crude enzyme extract of *ndtbt*-expressing cells were compared to absorbance profiles of the products identically isolated from control assays containing extracts of cells transformed with empty vector. Products eluting from the HPLC column corresponding to a *de novo* UV-absorbance peak were collected and further analyzed by direct injection-electrospray ionization mass spectrometry, in positive ion mode. The isolated product had a molecular weight and ionization pattern (data not shown) that were identical to those of authentic 2′-deoxypaclitaxel when analyzed by similar methods.

After identifying that NDTBT was functionally expressed, the supernatant of the lysed bacterial cells was incubated with HIS-Select Nickel Affinity Gel in batch mode at 4 °C. After 2 h, the mixture was poured into an Econo column (BioRad, 20 cm × 2.5 cm), the Lysis Buffer was drained. The resin was washed with five column volumes of Wash Buffer (50 mM sodium phosphate and 300 mM sodium chloride, pH 8.0) containing 20 mM imidazole,

(29) Loncaric, C.; Merriweather, E.; Walker, K. D. *Chem. Biol.* **2006**, *13*, 1–9.
(30) Sambrook, J.; Russell, D. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: 2001.

and the bound protein was eluted with one column volume of Wash Buffer containing 250 mM imidazole. The imidazole was removed from the eluent by consecutive concentration by ultracentrifugation (30 000 MWCO, YM30 membrane, Millipore, Billerica, MA) and dilution in Assay Buffer (50 mM sodium phosphate, 5% glycerol, pH 8.0) until the imidazole was 1.5 μM. The recombinant protein migrated to an R_f consistent with the calculated molecular weight of NDTBT (~49 kDa) on SDS-PAGE, which was stained with Coomassie Blue dye. The protein was recognized in a Western blot analysis according to a method based on the 1-Step TMB-Blotting Kit (Pierce, Rockford, IL) with the following antibodies: Monoclonal Anti-polyHistidine and Anti-Mouse IgG Peroxidase Conjugate (Sigma, St. Louis, MO). KODAK 1D Image Analysis Software (Version 3.6.3) was used to integrate the relative intensity of the enzyme band against BSA standards ranging from 2 to 10 mg/mL, and overexpression was verified by immunoblot analysis. By comparison, NDTBT was not detectable in soluble extracts isolated from bacteria transformed with empty vector.

Kinetic Evaluation of NDTBT with *N*-Debenzoyl-(3′*R*)-2′-deoxypaclitaxel and Benzoyl CoA. To establish linearity with respect to time and enzyme concentration, varying amounts of NDTBT were incubated with benzoyl CoA (the natural acyl group donor, at 50 μM), and *N*-debenzoyl-(3′*R*)-2′-deoxypaclitaxel was maintained at saturation (1 mM) in 2 mL of Assay Buffer. Aliquots (200 μL) from each assay were collected, quenched with 0.1 N HCl at 30 min intervals over 2.5 h, and extracted with ethyl acetate. The organic solvent was removed, and the products in the resultant residue were dissolved in 50 μL of acetonitrile and were analyzed by UV-HPLC with A_{228} monitoring of the effluent, as before. The peak area corresponding to the biosynthetic 2′-deoxypaclitaxel was converted to concentration by comparison to the peak area obtained with authentic 2′-deoxypaclitaxel (0 to 30 μM at 5 μM intervals). Kinetic parameters were determined under steady state parameters using 0.5 mg/mL of protein and a 20-min incubation time. The concentration of benzoyl CoA was independently varied (0−1000 μM) in separate assays while *N*-debenzoyl-(3′*R*)-2′-deoxypaclitaxel was maintained at apparent saturation (1 mM). The initial velocity (v_o) was plotted against substrate concentration for each data set, and the equation of the best-fit line ($R^2 = 0.96$) was determined (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA) to calculate the V_{max} and K_M parameters.

Kinetic Evaluation of NDTBT with *N*-Debenzoyl-2′-deoxypaclitaxel and Competing CoA Substrates. The general NDTBT assay to identify productive aroyl CoA substrates contained purified enzyme (~100 μg) in 500 μL of Assay Buffer, *N*-debenzoyl-2′-deoxypaclitaxel, and an aroyl CoA, each at 500 μM. The reaction was incubated for 3 h at 31 °C, quenched by the addition of 0.1 N HCl, and extracted with ethyl acetate (1 × 2 mL). The solvent was concentrated *in vacuo*, and the remaining residue was dissolved in 50 μL of acetonitrile and analyzed by HPLC, as described above. UV-Absorbance profiles of isolated crude product derived from these assays were compared to absorbance profiles of a sample identically isolated from control assays, in which one cosubstrate and/or enzyme was omitted. Eluent from the HPLC column that correlated with a *de novo* UV-absorbance peak was further analyzed by ESI-MS (positive ion mode) to confirm product identity.

After productive CoA substrates were identified, the procedure to calculate the relative kinetic constants of NDTBT for multiple competing CoA substrates was adapted from a method used to study the kinetics of a *Taxus* acyltransferase with multiple substrates.[29] This relative kinetics evaluation method was advantageous toward conserving the dearth of semisynthetically derived *N*-debenzoyl-2′-deoxypaclitaxel and acyl CoA cosubstrates by reducing the number of replicate assays needed to construct plots to evaluate the kinetics of the transferase with each acyl CoA substrate. The substrate specificity of NDTBT for the productive acyl CoA's, identified above, was assessed by incubating each CoA thioester (500 μM) separately with 100 μM benzoyl CoA, 100 μg of NDTBT enzyme, and 500 μM *N*-debenzoylpaclitaxel, in a 200 μL assay. After 20 min, the samples were acidified, and the biosynthetic products were isolated by extraction with ethyl acetate as described previously. The products were loaded onto the reversed-phase HPLC column and eluted by a gradient of solvent A/solvent B [solvent A: 99.99% H_2O with 0.1% TFA (v/v); solvent B: 99.99% CH_3CN with 0.1% TFA (v/v); gradient: 0−5 min at 80% (A), 5−11 min at 80−50% (A), 11−21 min at 50−20% (A), 21−23 min at 20−0% (A), 23−25 min at 0% (A), 25−33 min at 0−80% (A)] at a flow rate of 1.5 mL/min and A_{228} monitoring of the effluent.

The following calculations were used to correct the variance in the molar absorptivity of the different aromatic chromophores of the various biosynthetically *N*-aroylated products. The area under the absorbance peak corresponding to 2 nmol of each aroyl free acid (A_{acid}) was independently calculated. The A_{acid} values were each added to the peak area for the absorbance of the substrate *N*-debenzoyl-2′-deoxypaclitaxel (A_{taxane}) monitored at the same wavelength and concentration as the free acids to approximate the total absorbance peak area ($A_{total} = A_{acid} + A_{taxane}$). The quotient derived by dividing A_{total} for the benzoic acid/taxane pair by A_{total} for each free acid/taxane pair was used to calculate the relative molar absorptivity. This ratio was multiplied by the area under the UV absorbance (A_{228}) peak for each biosynthetic *N*-aroyl product, eluting from the HPLC; this normalized peak area was directly compared to the absorbance peak corresponding to the 2′-deoxypaclitaxel (made biosynthetically in the same mixed-substrate assay). The acquired absorbance ratios were each multiplied by the specificity constant (V_{max}/K_M) of NDTBT calculated for benzoyl CoA and *N*-debenzoyl-2′-deoxypaclitaxel as substrates, and the resultant values are reported as specificity constants relative to that of NDTBT for each surrogate acyl CoA substrate.

Relative Velocities of NDTBT with *N*-Debenzoylpaclitaxel Analogues. In general, the conversion rate of each *N*-debenzoyl isoserinoyl taxane substrate to its *N*-benzoyl isoserine derivative by NDTBT catalysis was estimated to be 10−20-fold less compared to the conversion rate for the *N*-debenzoyl-2′-*deoxy*paclitaxel substrate. Therefore, to optimize the amount of biosynthetic product for detection by LC-ESI/MS/MS analysis, *N*-debenzoylpaclitaxel or 10-deacetyl-*N*-debenzoylpaclitaxel (derived from docetaxel) and several different acyl CoA thioesters, all substrates at apparent saturation (1 mM), were incubated for 2 h with NDTBT (100 μg in 200 μL of assay buffer, as described previously) to calculate the relative velocity (v_{rel}) of each set of diterpene and acyl CoA substrate. The isoserinoyl taxanes and CoA thioester substrates at 1 mM were judged to be at steady state (<10% conversion of substrate) at the termination of the reactions. Each assay was extracted with ethyl acetate (2 × 1 mL), the solvent was removed *in vacuo*, the remaining residue was dissolved in 50 μL of acetonitrile, and a 20-μL aliquot of the sample was analyzed by LC-ESI tandem mass spectrometry. The abundance of the selected molecular ion (MH^+), derived in the first-stage MS, of the authentic standard was linearly proportional to a concentration below 25 μM, and the ion abundance profiles in the MS/MS spectra of authentic compounds were comparable to those observed for each biosynthetic product that was structurally similar (see Supporting Information). Based on these correlations, the relative amounts of *N*-aroylated biosynthetic product (paclitaxel, its *N*-acyl-, or 10-deacetyl-*N*-acyl derivatives) made by the NDTBT was assessed by comparing the area under the corresponding ion abundance peak for each of the selected molecular ions (MH^+).

Results

Bacterial Expression and Activity Assay of the *N*-Benzoyltransferase. To obtain a sufficient quantity of functional catalyst needed for this investigation, where several activity screens and kinetic assays were conducted, methods used previously to express *ndtbt* in bacteria were modified to scale up the production of enzyme.[23] Briefly, the *ndtbt* cDNA was subcloned into expression vector pET28a to incorporate

an N-terminal His_6-tag epitope for immunoblot analysis and purification by nickel-affinity resin chromatography. To optimize the enzyme expression level, the resulting plasmid was transferred into *E. coli* BL21-CodonPlus (DE3)-RIPL cells, to express tRNA (rare in wild-type *E. coli*) that recognize codons present in the recombinant *ndtbt* cDNA derived from *Taxus* plants. The NDTBT-His_6 fusion (1 mg) was used at 70% purity.

The function of the benzoyltransferase was assessed similarly to a previously described method.[23] To produce sufficient *de novo* biosynthetic product for characterization, larger amounts of NDTBT (0.5 mg/mL) were used in 200 μL assays in the present investigation compared to the 0.5 μg/mL used in a previous study.[23] The cosubstrate concentrations of the donor and acceptor substrates (0.40 and 0.42 mM, respectively) were approximately 2.5 times their reported K_m values.[23] After reacting under these conditions, the biosynthetic product was isolated as described previously[23] and was characterized by ^{1}H NMR and direct injection LC-ESI/MS to confirm its identity as 2′-deoxypaclitaxel, thus verifying that the isolated enzyme was functional.

Relative Substrate Specificity of NDTBT with Various Aroyl CoA Thioesters and *N*-Debenzoyl-2′-deoxypaclitaxel. *N*-Debenzoyl-2′-deoxypaclitaxel at 1 mM and the benzoyltransferase were assayed separately with different aroyl CoA substrates that were synthesized by a previously described process, which involved coupling a mixed anhydride carboxylic acid with coenzyme A under mildly basic aqueous conditions.[29] Acidifying the aqueous assay solutions to pH 4, to terminate the assays prior to extraction of the assay buffer with organic solvent, ensured that the amine substrate persisted as the ammonium ion and thus partitioned to the water fraction, while the *N*-aroylated products partitioned to the organic phase. The extracted products were further authenticated as *N*-acyl analogues by mass spectrometry. Analogous assays conducted with enzyme isolated from *E. coli* harboring the empty vector did not show a detectable product.

All of the aroyl CoA's examined, except for 2-methylbenzoyl CoA, in this study were productive with NDTBT and *N*-debenzoyl-2′-deoxypaclitaxel. The specificity constant (V_{max}/K_M) of NDTBT for each aroyl CoA was estimated from the amount of *N*-aroylated taxane made from the corresponding thioester in a competitive substrate reaction under typical assay conditions. The resulting product mixture was analyzed by reversed-phase HPLC with UV monitoring (A_{228}) of the effluent. To assess the relative molar absorptivity for quantifying each biosynthetic *N*-aroyl product would have required the synthesis of several different *N*-aroylated authentic standards *via* the *N*-debenzoyl substrate. This route would have quickly exhausted our supply of this costly diterpene substrate. Therefore, instead of using a linear regression correlation of concentration and absorbance to quantify the biosynthetic products in the competitive assays, the absorbance (A_{228}) of each *N*-acyl-*N*-debenzoyl-2′-deoxypaclitaxel derivative was approximated by first individually determining the A_{228} of each free acid corresponding to the *N*-acyl group of a biosynthetic product. This absorbance value was then added to that of the *N*-debenzoyl-2′-deoxypaclitaxel substrate at the same concentration and wavelength used with the free acids to calculate the molar absorptivity. From these values, the relative specificity constants (Table 1) were calculated based on that of NDTBT for benzoyl coenzyme A (V_{max}/K_M = 1.6 nmol·min^{-1}·mM^{-1}) (Entry 1A); the catalytic efficiency for 4-methylbenzoyl CoA (1.7 nmol·min^{-1}·mM^{-1}, Entry 4A) was nearly equal to that of the natural substrate. The efficiencies of NDTBT for the other coenzyme A thioesters of substituted-aroyls or heterole carbonyls ranged from 0.15 to 1.1 nmol·min^{-1}·mM^{-1}. The *para*-substituted aroyls displayed the highest efficiency compared to their *meta*- and/or *ortho*-regioisomers (Table 1).

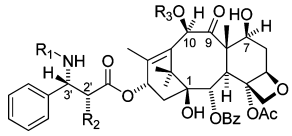
Relative Velocity of NDTBT with *N*-Debenzoylisoserinoyl Taxane Analogues and Various Aroyl CoA's. In a previous survey,[31] cytochrome P450 hydoxylase activity in microsome preparations derived from *Taxus* cell cultures was found to hydroxylate at C2′ of *N*-debenzoyl-2′-deoxypaclitaxel to produce *N*-debenzoylpaclitaxel. This data suggested that *N*-benzoylation occurs as the last step in the paclitaxel biosynthetic pathway and hinted that the benzoyltransferase could transfer an acyl group to the substrate amino group located vicinal to a hydroxyl group (Scheme 3). In contrast, a nontaxane acyltransferase, anthranilate *N*-benzoyltransferase, has narrow substrate specificity for the amine acceptor substrate; intriguingly, 3- or 4-hydroxyanthranilate substrates were not productive substrates of the anthranilate benzoyltransferase.[32] These results prompted us to examine the effect of the hydroxyl group vicinal to the amine acceptor group of isoserinoyl taxanes on NDTBT catalysis with a broad range of non-natural aroyl CoA substrates. *N*-Debenzoylpaclitaxel and *N*-de(*tert*-butoxycarbonyl)docetaxel (i.e., 10-deacetyl-*N*-debenzoylpaclitaxel) substrates required for this investigation were both prepared from docetaxel (Scheme 4).

Because of the prohibitive expense of docetaxel to semisynthesize adequate *N*-debenzoylpaclitaxel analogues, the use of these analogues needed to be rationed, in the present study. Therefore, to conserve the supply of these derivatives, the substrates were incubated at a single concentration (1 mM) with each of the 16 aroyl CoA's (at 1 mM) for 2 h, in duplicate runs. Each sample was analyzed by ESI-MS/MS to verify *N*-aroylated product identity and to quantify the relative rate at which the biosynthesized products were formed. The first-stage mass spectrometer was set to select for the MH^+ ion of the product, which was directed into a fragmentation chamber, and the resulting fragment ions were analyzed by the second-stage mass spectrometer set to scan mode. Typical diagnostic fragment ions were *m*/*z* 509 (for the 10-acetyl and 10-deacetyl taxane substrates), 569 (for the 10-acetyl taxane substrates), and the distinct side chain fragment ion for product characterization (Figure 2 and see Supporting Information). In these assays, the kinetic parameters of NDTBT were unknown, regarding whether the non-natural aroyl CoA's were first-order or at saturation at 1 mM; however, to provide a rough approximation of the relative velocities (v_{rel}) of NDTBT (100 μg) for each CoA thioester (Table 1), the rates were estimated to be at steady state and first-order.

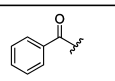
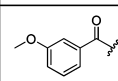
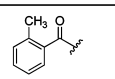
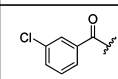
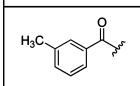
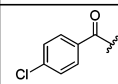
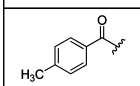
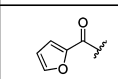
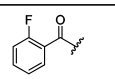
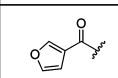
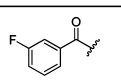
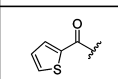
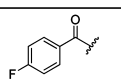
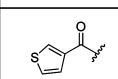
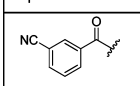
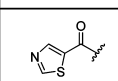
The rate (~10 pmol/h) at which benzoyl CoA and *N*-debenzoylpaclitaxel was converted to paclitaxel by NDTBT was set at 100% and compared to the rates of NDTBT with each of various aroyl CoA's and the same taxane substrate (cf. Table 1 for the following entries). Surprisingly, the v_{rel} of 2-furanoyl CoA was 200% (Entry 12B) relative to benzoyl CoA, whereas 3-furanoyl CoA was 19% (Entry 13B). The v_{rel} values for the other 2- and 3-heterole carbonyl CoA's displayed similar kinetics to that of the 3-furanoyl thioester, ranging between 19% and 22% (Entries 14−16B). NDTBT was productive with

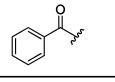
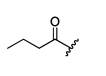
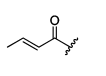
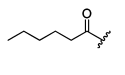
(31) Long, R. M.; Croteau, R. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 410–417.
(32) Yang, Q.; Reinhard, K.; Schiltz, E.; Matern, U. *Plant Mol. Biol.* **1997**, *35*, 777–789.

Table 1. Relative Kinetics of NDTBT after Incubation of Aroyl- (*Top Panel*) and Short Hydrocarbon Chain- (*Bottom Panel*) CoA's with *N*-Debenzoylpaclitaxel Analogues[a]



R_1=H, R_2=H, R_3=Ac: *N*-debenzoyl-2'-deoxypaclitaxel
R_1=H, R_2=OH, R_3=Ac: *N*-debenzoylpaclitaxel
R_1=H, R_2=OH, R_3=H: 10-*O*-deacetyl-*N*-debenzoylpaclitaxel

R_1 Derived from CoA		***A*** (V_{max}/K_M) (nmol•min^{-1}•mM^{-1}) R_2=H, R_3=Ac	***B*** v_{rel} R_2=OH, R_3=Ac	***C*** v_{rel} R_2=OH, R_3=H	R_1 Derived from CoA		***A*** (V_{max}/K_M) (nmol•min^{-1}•mM^{-1}) R_2=H, R_3=Ac	***B*** v_{rel} R_2=OH, R_3=Ac	***C*** v_{rel} R_2=OH, R_3=H
	**1**	1.6	100%	100%		**9**	0.38	85%	11%
	**2**	Not detectable	Not detectable	3%		**10**	0.37	<1%	8%
	**3**	0.27	27%	33%		**11**	1.1	<1%	40%
	**4**	1.7	33%	Not Detectable		**12**	0.31	200%	36%
	**5**	0.85	8%	38%		**13**	0.15	19%	43%
	**6**	0.34	11%	19%		**14**	0.39	19%	5%
	**7**	0.97	11%	68%		**15**	0.37	22%	3%
	**8**	0.15	9%	17%		**16**	0.16	22%	29%

R_1 Derived from CoA		***D*** v_{rel} R_2=H, R_3=Ac	***E*** v_{rel} R_2=OH, R_3=Ac	***F*** v_{rel} R_2=OH, R_3=H
	**17**	100%	13%	12%
	**18**	47%	85%	35%
	**19**	3%	100%	92%
	**20**	2%	2%	3%
	**21**	36%	68%	100%

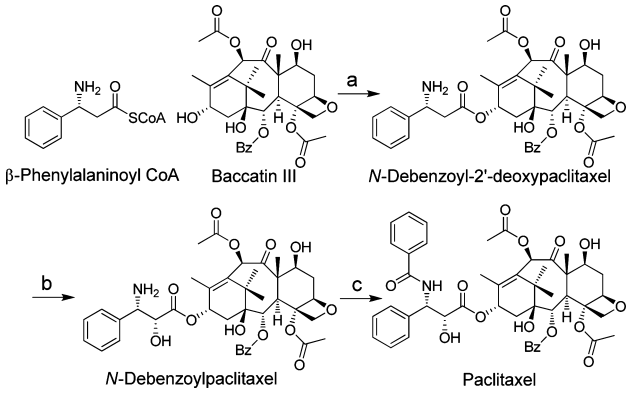
[a] V_{max}/K_M values are listed as nmol•min^{-1}•mM^{-1} (SE ≈ ±0.05); v_{rel} values are listed as percent (SE ≈ ±5%).

3-methoxybenzoyl CoA, which was utilized at 85% (Entry 9B) of the rate for benzoyl CoA. The relative rates of the catalyst for the methyl-, fluoro-, and cyano-substituted benzoyl CoAs were modest (Entries 3−8B), with the exception of 2- and 3-chlorobenzoyl CoA (Entries 10−11B); the products derived from the latter were only marginally detectable, at v_{rel} < 1% (data not shown).

In a parallel study, we assessed whether the absence of the acetyl group at C10 of the *N*-debenzoylpaclitaxel affected the specificity of the NDTBT with the array of aroyl CoA's. The rate (~20 pmol/h) at which benzoyl CoA and 10-deacetyl-*N*-debenzoylpaclitaxel was converted to 10-deacetylpaclitaxel (Entry 1C) by NDTBT was set at 100% and compared to the rates at which the enzyme converted each of various aroyl CoA's and the taxane substrate to a respective product. 4-Fluorobenzoyl CoA (v_{rel} = 68%, Entry 7C) and 3-furanoyl CoA (v_{rel} = 43%, Entry 13C) were used at a rate most comparable to that at which NDTBT used benzoyl CoA. Interestingly, all of the other aroyl CoA's tested were productive (with relative conversion rates between 3% and 38%), except for 4-methylbenzoyl CoA (Entry 4C), which did not produce detectable product.

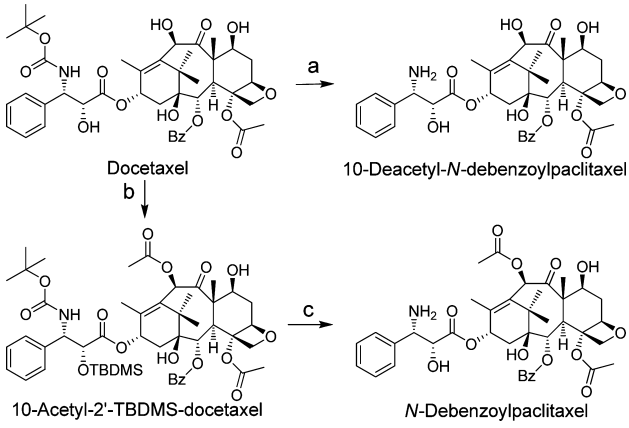
Relative Velocity of NDTBT with *N*-Debenzoyl Taxane Analogues and Various Alkanoyl/Alkenoyl CoA's. Four non-aromatic CoA thioesters (acetyl CoA, butyryl CoA, butenoyl CoA, and hexanoyl CoA) were assayed with the three *N*-debenzoyl taxoids, described herein. Each of the isolated

Scheme 3. Late-Occurring Steps on the Paclitaxel Biosynthetic Pathway[a,b,c]



[a] Phenylpropanoyl CoA transferase. [b] C2′ P450 hydroxylase. [c] NDTBT, benzoyl, COA.

Scheme 4. Synthesis of *N*-Debenzoylisoserinoyltaxanes[a,b,c]



[a] *i*. Formic acid, *ii*. $NaHCO_3$, 60% yield. [b] *i*. TBDMS-Cl, imid., 99% yield, *ii*. Ac_2O, $CeCl_3$, 93% yield. [c] *i*. HF/pyridine, *ii*. Formic acid, *iii*. $NaHCO_3$, 62% yield.

biosynthetic products was not detectable by HPLC with UV-monitoring (A_{228}), likely due to low abundance and/or coelution of the biosynthetic products from the HPLC column with other unknown compounds extracted from the assay mixture. As an alternative, the samples were analyzed by the more selective LC-ESI-MS/MS procedure utilized earlier in this study. As before, typical diagnostic fragment ions were *m/z* 509, 569, and the side chain fragment ion (ordinarily the base peak) that confirmed the identity of the *N*-alkanoyl/alkenoyl taxane (cf. Figure 2 and see Supporting Information). The relative rates of product formation were calculated from the mass spectrometry data by a method analogous to that used for the *N*-aroylation products.

N-Debenzoyl-2′-deoxypaclitaxel was incubated with NDTBT and benzoyl CoA, and the relative rate (~200 nmol/h) of the benzoyl group transfer to the *N*-debenzoyl taxane was compared to the rate of acyclic carbonyls. The v_{rel} for the transfer of acetyl (shortest chain) and hexanoyl (longest chain) groups were 47% and 36%, respectively (Entries 18D and 21D), relative to the transfer rate for the benzoyl group. The v_{rel} for the C_4 chains, butenoyl and butanoyl, were only at 2% and 3%, respectively. For *N*-de(*tert*-butoxycarbonyl)docetaxel and *N*-debenzoylpaclitaxel (i.e., 2′-hydroxylated substrates), the relative velocity of the most productive acyclic CoA substrate was set to 100%. In contrast to *N*-debenzoyl-2′-deoxypaclitaxel, the alkanoyl CoA's (Entries 18E/F, 19E/F, and 21E/F) gave superior turnover with

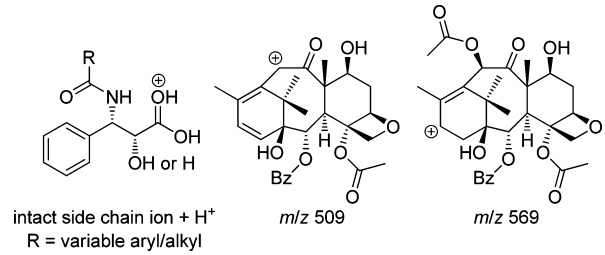


Figure 2. Typical fragment ions observed in the MS/MS profiles of isoserinoyl taxanes.

the 2′-hydroxylated taxoids, compared to benzoyl CoA (~10 and ~20 pmol/h, Entries 17E/F, respectively). The 2-butenoyl group was transferred the slowest by NDTBT to all of the taxoid cosubstrates (Entries 20D/E/F), which may be a consequence of the extended, fixed geometry of the *trans*-double bond.

Discussion

Broad substrate specificity of enzymes on secondary metabolic pathways has been suggested as important for the evolution of metabolite diversity.[33] The results of this study demonstrated the extraordinarily broad substrate specificity of the recombinantly expressed NDTBT enzyme in purified form when incubated with several acyl-CoA donor substrates and three *N*-debenzoylpaclitaxel derivatives, *N*-debenzoyl-2′-deoxypaclitaxel, *N*-debenzoylpaclitaxel, or 10-deacetyl-*N*-debenzoylpaclitaxel. Other BAHD family acyltransferases, benzoyl CoA:benzyl alcohol benzoyl transferase (*Clarkia breweri* plant),[34] and alcohol acyltransferases from wild strawberry (*Fragaria vesca*), cultivated strawberry (*Fragaria* × *ananassa*), and banana (*Musa sapientum*)[35] also have broad substrate specificity with varying acyl CoA donors and a range of alcohol acceptor substrates. As structural information becomes available for these BAHD acyltransferases, including NDTBT, the rationale for their broad substrate specificity can likely be understood, and the mechanism defining the variable substrate access can be systematically compared.

A prior seminal description of NDTBT[23] primarily focused within the context of paclitaxel biosynthesis and thus screened the function of the catalyst with benzoyl CoA and a single diterpene substrate, *N*-debenzoyl-2′-deoxypaclitaxel. Substrate specificity analysis was limited to acetyl CoA and phenylacetyl CoA, neither of which was found to be productive in this earlier study.[23] In a more recent biosynthesis investigation,[24] the assembly of the isoserinoyl side chain of paclitaxel was investigated. The superior catalytic transfer of benzoyl to *N*-debenzoylpaclitaxel compared to *N*-debenzoyl-2′-deoxypaclitaxel (cf. Scheme 3) supported an earlier claim that 2′-hydroxylation precedes *N*-benzoylation.[31] This prior study also concluded that NDTBT did not transfer other naturally occurring short chain alkanoyl/alkenoyl groups to the amino group of the *N*-debenzoylpaclitaxel substrate as assessed by UV-HPLC analysis alone.[24]

In contrast, the current investigation was formulated by consideration of the several described next-generation paclitaxel molecules derived by synthetic replacement of the *N*-benzoyl

(33) Firn, R. D.; Jones, C. G. *Nat. Prod. Rep.* **2003**, *20*, 382–391.
(34) D'Auria, J. C.; Chen, F.; Pichersky, E. *Plant Physiol.* **2002**, *130*, 466–476.
(35) Beekwilder, J.; Alvarez-Huerta, M.; Neef, E.; Verstappen, F. W. A.; Bouwmeester, H. J.; Aharoni, A. *Plant Physiol.* **2004**, *135*, 1865–1878.

of the natural product with several different kinds of non-natural *N*-aroyl groups.[10,15] The hypothesis developed herein was that NDTBT could feasibly transfer non-natural aroyl moieties in addition to transferring a benzoyl to the amino functional group of various derivatives of *N*-debenzoylpaclitaxel. To evaluate this theory, the paclitaxel pathway *N*-benzoyltransferase was examined for its utility to *N*-aroylate analogues of advanced taxane metabolites. NDTBT was shown to indiscriminately transfer aroyl groups, including heteroles, 2-, 3-, and 4-substituted benzoyls, alkanoyl, and alkenoyl (cf. Tables 1) to either of the *N*-debenzoylated taxanes used as a cosubstrate. More importantly, these results indicate that the *N*-benzoyltransferase is not limited to *N*-debenzoyl-2′-deoxypaclitaxel as a substrate that contains the β-phenylalanine side chain. The diverse specificity for *N*-aroylation was largely unaffected by the presence of the vicinal hydroxyl group at C2′ of the phenylisoserinoyl diterpenes and thus supports the observation described in a previous study.[24] In addition, the lack of a C10-acetyl group on the taxane substrate did not affect the function of the enzyme, and therefore, conceivably, C10-acetylation could occur as a last step in the biosynthesis of paclitaxel. Furthermore, *ortho*-, *para*-, or *meta*-substitution on the benzoyl group transferred from CoA generally did not affect NDTBT activity, although the *para*-regioisomers within a homologous series were typically transferred faster.

It was very apparent that the scale of the assays used in this investigation produced a paucity of isoserinoyl taxane products on the order of pmol/h and thus precluded their detection by UV-HPLC. The application of a selective and more sensitive HPLC electrospray ionization tandem mass spectrometric analysis enabled the detection of the array of biosynthetic products made by NDTBT catalysis, described herein; moreover, this mode of analysis enabled categorical identification of the fragment ion of the intact side chain for each of the biosynthetically acquired *N*-acyl derivatives.

In conclusion, we demonstrated that the wild-type *Taxus* *N*-benzoyltransferase functions as a general *acyl*transferase. The broad substrate specificity of NDTBT for a variety of acyl CoA thioesters provides momentum for the eventual application of this biocatalyst toward the production of modified paclitaxel compounds and likely accounts for the variety of *N*-acyl derivatives of paclitaxel analogues found in *Taxus* plants and derived cell cultures.[36] In addition, the described substrate specificity will expand the current knowledge of the selectivity of enzymes that belong to the BAHD acyltransferase superfamily. It is still premature nonetheless, particularly without enzyme structural data, to speculate why NDTBT displays the unique kinetic parameters in terms of substituent regiochemistry in the aroyl CoA substrate, with regards to sterics and inductive effects. Furthermore, it was anticipated that NDTBT, a benzoyltransferase, would primarily aroylate the *N*-debenzoyl substrates; thus, it was intriguing to see alkanoyl and butenoyl groups transferred by the catalyst to the acceptor substrate. Moreover, the presence of a 2′-hydroxy group on the phenylpropanoyl side chain of the taxane substrate increased the rate of *N*-alkanoylation/alkenoylation over *N*-benzoylation. At the early stages of defining the scope of NDTBT specificity, the underlying effect of the 2′-hydroxyl on preferential alkanoyl CoA binding remains a mystery. Conceivably, when structural data become available for NDTBT, valuable insight into the mechanism of substrate specificity can be dissected, and directed mutational analysis can be employed to potentially produce new catalyst derivatives that are able to transfer a greater or refined scope of novel acyl groups to the taxane core or other diterpene scaffolds. The production of efficacious paclitaxel analogues through biocatalytic means *in vitro* or *in vivo* in a suitable host system will facilitate semibiosynthetic methods that interface synthetic chemistry and molecular biology.

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Supporting Information Available: ^{1}H NMR data for each of the synthesized aroyl CoA analogues; tandem mass spectrometry data for each of the biosynthesized *N*-benzoyl paclitaxel analogues derived by effective catalysis of NDTBT. This material is available free of charge via the Internet at http://pubs.acs.org.

JA900545M

(36) Kingston, D. G. I. In *Taxol: Science and Applications*; Suffness, M., Ed.; CRC-Press: Boca Raton, FL, 1995; pp 287−315.