Chemistry & Biology 13, 309–317, March 2006 DOI 10.1016/j.chembiol.2006.01.006

Profiling a Taxol Pathway 10β-Acetyltransferase: Assessment of the Specificity and the Production of Baccatin III by In Vivo Acetylation in *E. coli*

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Summary

The 10β-acetyltransferase on the biosynthetic pathway of the antineoplastic drug Taxol catalyzes the regiospecific transfer of the acetyl group of acetyl-coenzyme A (CoA) to 10-deacetylbaccatin III. We demonstrate that in addition to acetyl group transfer, the overexpressed enzyme also catalyzes the exchange of propionyl and *n*-butyryl from the corresponding CoA thioester to the hydroxyl group at C10 of the co-substrate. Also, in vivo studies revealed that *E. coli*, producing endogenous acetyl-CoA and overexpressing the recombinant acetyltransferase, can convert exogenously supplied 10-deacetylbaccatin III to baccatin III. Potentially, this heterologous in vivo production method in bacteria could be optimized to couple various unnatural acyl-CoA analogs to myriad amino and/or hydroxyl acceptors by acyltransferase catalysis; conceivably, this process could facilitate the preparation of second-generation Taxols.

Introduction

The biosynthetic pathway to the diterpenoid Taxol (paclitaxel), a potent mitotic drug used to treat several types of cancer, includes five acyltransferase-catalyzed steps. (Paclitaxel is the generic name for Taxol, which is a registered trademark of Bristol-Myers Squibb. Because of the greater familiarity with the word "Taxol," this will be used throughout the manuscript.) Each operationally soluble transacylase delivers an acyl group from a corresponding acyl-coenzyme A (acyl-CoA) thioester to a Taxol pathway intermediate [1–3]. Knowledge of the structure-activity relationships of this drug reveals that most of the acyl groups play a principal role in drug efficacy. Notably, the *N*-benzoylphenylisoserine side chain at C-13, the benzoyl at C-2, and the acetyl at C-4 are important pharmacophoric descriptors (Figure 1A) required for activity, whereas modification in the "northern hemisphere" of the molecule at either the C-7, C-9, or C-10 hydroxyl has negligible effect [4–6]. However, recent investigations have demonstrated that when acyl group substitution (propionyl or butyryl for acetyl) at the C-10 hydroxyl is coupled with simultaneous aroyl exchange (2-furanoyl for benzoyl) at the C3′ amide of Taxol (Figure 1B), a synergistic effect with regard to enhanced drug potency is observed for the modified Taxol compared to the parent compound [7]. The current general method used to synthesize analogs with various combinations of acyl group substitutions at, for example, C-3′N/C-10, starts with synthetic acylation of the C-10 hydroxyl of natural product 10-deacetylbaccatin III by treatment with an acyl anhydride and cerium chloride catalyst (Figure 2), followed by silyl group protection of the C-7 hydroxyl, addition of the C-13 side chain via a synthetically derived *N*-substituted β-lactam precursor, and final deprotection [7] (Figure 2).

The favorable application of the described modified Taxols possessing increased efficacy in both cytotoxicity and microtubule assembly assays has prompted a survey to assess whether the acyltransferases on the Taxol biosynthetic pathway can regiospecifically transfer novel acyl groups to the hydroxyls of advanced metabolites on the Taxol pathway. The incorporation of such biocatalysts into synthetic routes could potentially eliminate the required protecting group manipulation chemistry and circumvent the multistep aspect of synthetic approaches toward the production of Taxol analogs. Additionally, these enzyme catalysts can potentially be used in mixed reactions pools to produce an array of related molecules that have enhanced bioactive properties.

Furthermore, it is postulated that the metabolic flux of the Taxol biosynthetic pathway can be increased or decreased by altering the primary pathway or competitive routes, respectively, through genetic modification of *Taxus*-derived cell cultures [8, 9], and/or by engineering a suitable host, e.g., *Arabidopsis*, to manufacture the drug [10]. However, the complex matrix of taxoid metabolites in the *Taxus* plant, which includes Taxol, likely contains pathway catalysts possessing broad substrate specificity, particularly, since these enzymes have essentially evolved in the presence of very closely related compounds [11]. This consideration adds to the complexity of genetically engineering the native organism, or another suitable host, with a goal of directing pathway flux to a *single* target molecule. It is conceivable, however, that each Taxol pathway enzyme could be altered for desired catalytic properties through application of random mutagenesis techniques, which often introduce fortuitous mutations into a particular target gene [12–16]. In general, these enzyme "breeding" methods have produced catalysts with desired, targeted function, such as improved small-molecule enantioselectivity [12, 13] and modified substrate specificity [14–16]. Conceptually, such modification of Taxol pathway genes could yield expressed enzymes with enhanced catalytic efficiency for the natural substrates, and, hypothetically, these genes could be engineered into cell cultures of *Taxus* plants to increase pathway flux.

Although before the specificity of the Taxol pathway acyltransferases can be altered through mutagenic methods for application in transgenic biotechnology, relevant physiological substrates that compete with the desired natural substrate for enzyme active site occupancy must first be identified. For example, variously substituted naturally occurring taxoids bear an acetyl or benzoyl group at C10 [11], and, feasibly, the *Taxus* 10β-acetyltransferase could catalyze transfer of both

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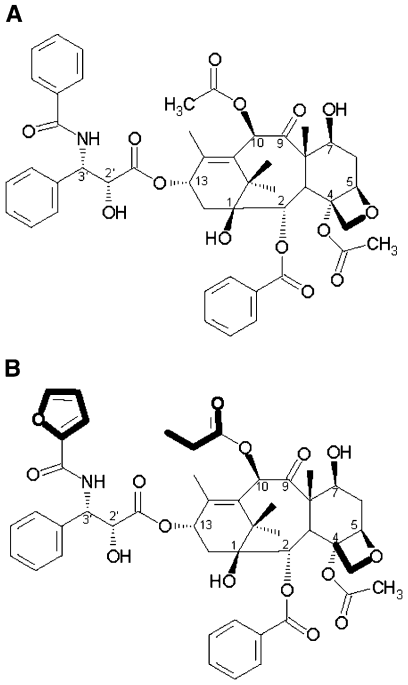

Figure 1. Taxol and an Example of a Second-Generation Taxol Analog

(A and B) (A) Taxol in its native form; (B) a promising Taxol analog derived from a combinatorial chemistry library possessing a propanoyl for acetyl substitution at C10 and a furanoyl for benzoyl replacement at the β-amine of the isoserinoyl side chain at C13.

groups. An assessment of the range of substrates utilized by the acetyltransferase will determine substrate binding and selectivity [17] and will provide a basis for directing random or rational tailoring of the residues to broaden or narrow specificity as desired.

Herein, we report on the regio- and substrate specificity of the 10-deacetylbaccatin III 10β-*O*-acetyltransferase (DBAT), which was characterized initially by its ability to catalyze the transfer of an acetyl group from acetyl-CoA to 10-deacetylbaccatin III to form baccatin III [18] (Figure 2). A collection of acyl-CoA thioester substrates was obtained from commercial sources or via synthetic methods, and each thioester was incubated in vitro with DBAT and 10-deacetylbaccatin III to profile the selectivity and relative steady-state kinetics of the enzyme for each CoA substrate. We also describe an in vivo method utilizing *E. coli*, expressing the DBAT enzyme, to produce baccatin III, a direct precursor of Taxol, from exogenously fed 10-deacetylbaccatin III and endogenous acetyl-CoA made by the bacteria.

Results and Discussion

The initial survey to identify *Taxus* acyltransferases (among 16 members) that operate on the Taxol pathway was based on the assembly of a sizable assay matrix that included screening each transacylase against several taxane variants and CoA thioesters that bore the acyl groups of putative pathway intermediates. Therefore, an assessment of extended substrate specificity of DBAT was not conducted originally. However, now that each of the five acyl/aroyltransferases involved in Taxol biosynthesis has been isolated and characterized, a more manageable assay matrix is evident, and, thus, an evaluation of the substrate selectivity for novel acyl-CoA thioesters of each functionally characterized acyltransferases has been initiated. The remaining 11 *Taxus* acyltransferase clones of unknown function will ultimately be incorporated into this investigation to characterize their mode of action.

The selectivity of DBAT was examined with radiolabeled natural substrate [13-^{3}H]-10-deacetylbaccatin III and several acyl-CoA compounds under standard assay conditions [18], and bacteria expressing empty vector or vector containing an acyltransferase that does not acylate at C10 were examined as control systems, as described elsewhere [18]. Assays with the natural substrate acetyl-CoA yielded a single peak eluting at 5.53 min (with coincident radioactivity and UV traces) from

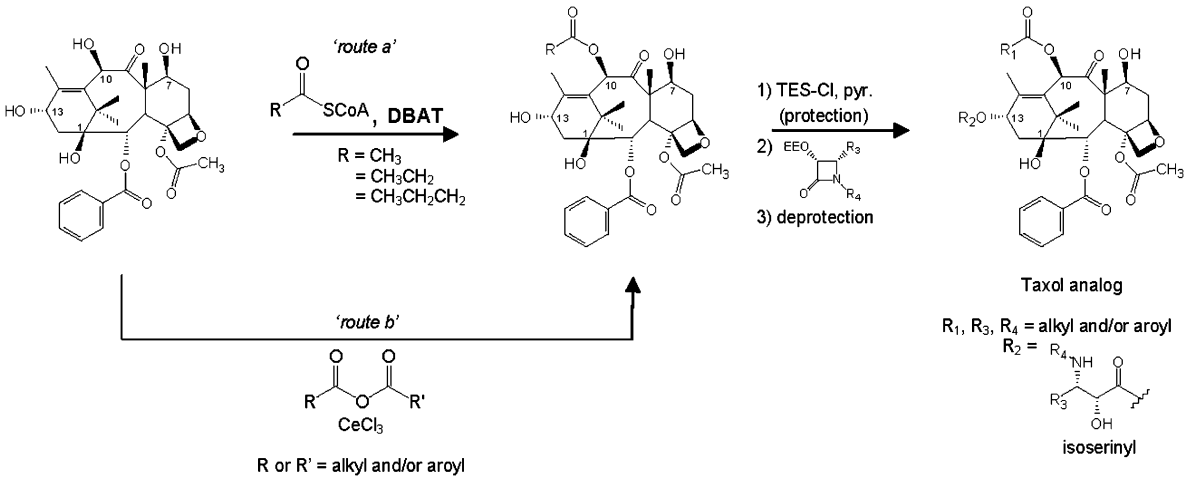

Figure 2. Biosynthetic and Synthetic Routes to Taxol Analog Precursors

Outline of the biosynthetic reaction catalyzed by the 10-deacetylbaccatin III 10β-*O*-acetyltransferase (DBAT) in the presence of acetyl-CoA (R = CH_3), propionyl-CoA (R = CH_3CH_2), and butyryl-CoA (R = $CH_3CH_2CH_2$) (*route a*). Outline of the semisynthesis of 10-acyl analogs of baccatin III from 10-deacetylbaccatin III, acyl anhydrides, and cerium chloride ($CeCl_3$) (*route b*). Abbreviations: TES-Cl, triethylsilyl chloride; pyr., pyridine.

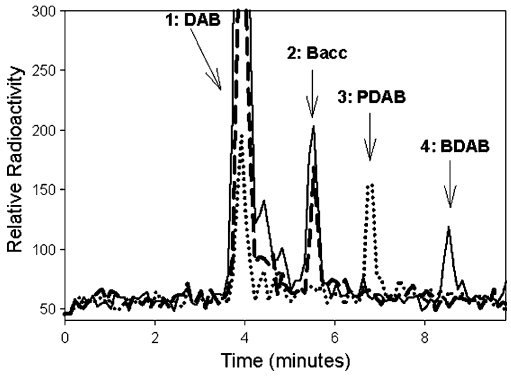

Figure 3. Radio-HPLC Analysis of Products Catalyzed by the 10-β Acetyltransferase

Radio-HPLC analysis of the biosynthetic products (retention time R_t = 5.53, 6.73, and 8.53 ± 0.01 min, respectively) generated from [^{3}H]-10-deacetylbaccatin III and acetyl-, propionyl-, and butyryl-CoA by the recombinant 10-deacetylbaccatin III acetyltransferase. Peak 1, [^{3}H]-10-deacetylbaccatin III (DAB); peak 2, [^{3}H]-baccatin III (Bacc); peak 3, [^{3}H]-10-propionyl-10-deacetylbaccatin III (PDAB); peak 4, [^{3}H]-10-butyryl-10-deacetylbaccatin III (BDAB). HPLC conditions: 40:60 acetonitrile/water.

the reverse-phase radio-HPLC (Figure 3). The retention time of the biosynthetic product was identical to that of authentic baccatin III. Identical radio-HPLC analysis of enzyme-catalyzed products extracted from similar assays incubated with the surrogate substrates propionyl-CoA and butyryl-CoA also revealed single peaks eluting at 6.73 min and 8.63 min, respectively (Figure 3); each identified peak possessed a coincident UV peak. A taxane 2α-benzoyltransferase (TBT), from *Taxus*, was expressed and isolated from JM109 cells as described [19]. Assays with TBT, [13-^{3}H]-10-deacetylbaccatin III, and either acetyl-CoA, propionyl-CoA, or butyryl-CoA showed no detectable product when analyzed by radio-HPLC as described.

A preparative-scale (6 L) culture of the JM109 bacteria harboring the *dbat* gene was grown for expression of the operationally soluble DBAT enzyme, which was subsequently isolated and partially purified as described previously [18]. Portions of this concentrated enzyme fraction were incubated with 10-deacetylbaccatin III and acetyl-CoA, propionyl-CoA, or butyryl-CoA in an attempt to generate enough biosynthetic product to elucidate the structure and determine the regiochemistry of the isolated material as the 10-acetylated material, baccatin III, 10-propionyl-10-deacetylbaccatin III, or 10-butyryl-10-deacetylbaccatin III, respectively, by ^{1}H-NMR analysis. Only baccatin III and the putative 10-propionyl-10-deacetylbaccatin III analog were made in sufficient quantity (≥1 mg, ~1.6 μmol) to confirm by ^{1}H-NMR that the product was monoacylated by DBAT at only the C10 hydroxyl, and not at the C1, C7, or C13 hydroxyl of the diterpene (cf. Figure 2 for numbering), thus validating the regiospecificity of the enzyme. The ^{1}H-NMR data for the biosynthetically derived baccatin III were identical to the data reported for this compound [18]. The diagnostic singlet observed at δ 6.29 corresponds to the signal for H10 that is attached to a carbon bearing the C10 acetoxy functional group. Protons H7 and H13 of methine carbons (CH), each bearing a hydroxyl group, were observed comparatively as upfield signals at δ 4.43 and δ 4.83, respectively (Table 1). Likewise, the ^{1}H-NMR spectrum of the 10-propionyl-10-deacetylbaccatin III analog contains a diagnostic singlet for the H10 signal at δ 6.32 (Table 1) that is consistent with the chemical shift of hydrogen attached to a methine bonded to an acyloxy functional group. The H7 and H13 signals are upfield at δ 4.44 and δ 4.88, respectively, as observed for baccatin III. The relative deshielding effect of the acyloxy at C10 on the chemical shift of H10 is apparent if the upfield H10 signal (at δ 5.24) of 10-deacetylbaccatin III, possessing only a hydroxyl at C10 (Table 1), is compared. The identity of the propionyl group is further confirmed by upfield chemical shifts for

Table 1. A Listing of the ^{1}H-NMR Data of 10-Deacetylbaccatin III and Its Various Biosynthetically Derived 10-Acetylated Analogs

H	10-DAB	Baccatin III	10-PDAB
2	5.62, d, 7	5.59, d, 7	5.60, d, 7
3	4.27, m	3.84, d, 7	3.87, d, 7
5	4.96, d, 8	4.98, bd, 8	4.97, d, 8
6a	2.58, m	2.52, m	2.54, m
6b	1.86, m	1.83, m	1.85, m
7	4.29, m	4.43, m	4.44, m
10	**5.24, s**	**6.29, s**	**6.32, s**
14	2.32, d, 6.0	2.26, d, 6.3	2.28, d, 5.4
13	4.86, bt	4.83, bt	4.88, m
16 + 17	1.08, s	1.07, s	1.09, s
18	2.12, s	2.02, s	2.04, s
19	1.81, s	1.64, s	1.65, s
20a	4.15, d, 8	4.27, d, 8	4.29, d, 8.5
20b	3.99, d, 7	4.12, d, 8	4.13, d, 8.5
Ar	8.03, d; 7.62, t; 7.51, t	8.07, d; 7.58, t; 7.45, t	8.1, d; 7.59, t; 7.46, t
4-OAc	2.36, s	2.25, s	2.27, s
10-OAcyl	**not applicable**	**2.21, s**	**2.52, m; 1.22, t, 4**

300 MHz ^{1}H-NMR data for authentic 10-deacetylbaccatin III (10-DAB), and for baccatin III and 10-propionyl-10-deacetylbaccatin III (10-PDAB) derived biosynthetically from assays incubated with acetyl-CoA and propionyl-CoA, respectively, 10-DAB, and DBAT. The bold text highlights the distinct chemical shift differences between the 10-deacyl- and 10-acylbaccatin III molecules at H10 and the side chain protons of the 10-acyl group. Samples were dissolved in $CDCl_3$ and were analyzed at 300 K: δ in ppm, multiplicity, *J* (coupling) in Hz. Abbreviations: s, singlet; d, doublet; t, triplet; bt, broad triplet; m, multiplet.

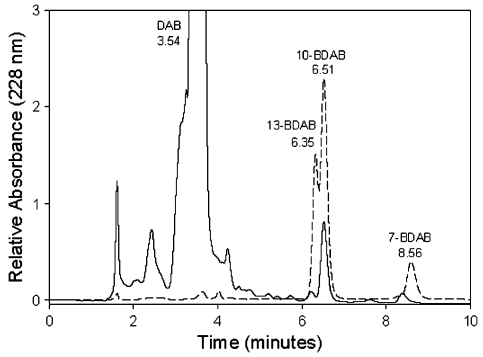

Figure 4. Comparison of a Biosynthetically Derived Butyryl-10-Deacetylbaccatin III Regioisomer to Authentic Standards

The solid line trace shows the HPLC-UV profile (A_{228}) of the compounds isolated from the enzyme-catalyzed reaction of 10-deacetylbaccatin III and butyryl-CoA incubated with recombinant DBAT enzyme. The de novo product elutes at R_t = 6.51 ± 0.01 min. The large peak at 3.54 min is unconsumed 10-deacetylbaccatin III (DAB) substrate. The superimposed dashed line trace shows the HPLC-UV profile (A_{228}) of a mixture of authentic 13-butyryl-10-deacetylbaccatin III (R_t = 6.35 min, 13-BDAB), 10-butyryl-10-deacetylbaccatin III (R_t = 6.51 min, 10-BDAB), and 7-butyryl-10-deacetylbaccatin III (R_t = 8.56 min, 7-BDAB). The peak corresponding to the biosynthetically derived product coincides with that for the authentic 10-butyryl analog and thus verifies that DBAT transfers the butyryl group of the corresponding coenzyme thioester specifically to the C10, and not to the C7 or C13, hydroxyl of 10-deacetylbaccatin III. HPLC conditions: 60:40 acetonitrile/water.

the C*H*$_3$ and C*H*$_2$ protons at δ 1.22 and δ 2.52, respectively, of the alkanoyl side chain.

The comparatively poorer catalytic efficiency of DBAT in assays with butyryl-CoA and 10-deacetylbaccatin III resulted in low production (~0.16 μmol) of the butyryl baccatin analog. Consequently, the acquisition of ^{1}H-NMR data for this biosynthetic product was precluded, despite efforts to process assays on a preparative-scale slightly greater than that used in assays with propionyl-CoA (described above). Therefore, the regiospecificity of the butyryl group transfer to the taxane core had to be confirmed by comparison of HPLC-UV profiles of authentic standards to that of the product catalyzed by DBAT in the presence of 10-deacetylbaccatin III and butyryl-CoA. The retention time (R_t = 6.51 min) of authentic 10-butyryl-10-deacetylbaccatin III was identical to that of the biosynthetic butyryl product, while authentic 7- and 13-butyryl-10-deacetylbaccatin III eluted at 8.56 min and 6.35 min, respectively (Figure 4), thus indicating that DBAT transfers the butyryl group from the corresponding CoA thioester to the C10 hydroxyl of the diterpene cosubstrate.

Further product analysis included combined LC-electrospray ionization mass spectrometry or fast atom bombardment ionization to confirm that products of the correct molecular weight were obtained for monoacylated product (MH^+ at *m/z* 587 for baccatin III, at *m/z* 601 for the proprionyl derivative, and at *m/z* 615 for the butyryl derivative). The MH^+ molecular ions of each biosynthetically derived product fragmented into diagnostic ions at *m/z* 527 (M^+ − alkanoic acid at C10) and *m/z* 509 (*m/z* 527 − H_2O).

The relative specificity constant (V_{max}/K_m) of each processed CoA was calculated from the amount of 10-acylated product made from the corresponding thioester in a competitive substrate reaction under typical assay conditions, and the resulting product mixture was analyzed by reverse-phase HPLC with UV monitoring (A_{228}) of the effluent. The relative absorbance maxima of each baccatin derivative were compared directly (the extinction coefficient of each product was assumed to be virtually identical for each alkanoyl baccatin analog since no additional strongly UV-absorbing chromophore was added to the substrate during catalysis). Relative to the specificity constant of DBAT for the native acetyl-coenzyme substrate (V_{max}/K_m = 3.0 s^{-1}•nM^{-1}), propionyl-CoA was at 1.6 s^{-1}•nM^{-1} and butyryl was lower at 0.16 s^{-1}•nM^{-1}. CoA thioesters of branched chain alkanoyls (*iso*-butyryl and *iso*-valeryl, also known as 2-methylpropionyl and 3-methylbutyryl, respectively), *n*-alkanoyls greater than C_4 (*n*-pentanoyl and *n*-hexanoyl), cyclohexanecarboxyl, benzoyl, and, intriguingly, the C_4 *trans*-butenoyl were all unproductive substrates; i.e., transesterification by DBAT with these substrates was below detectable limits. These data suggest that the length of the alkyl side chain plays an important role in the steric interaction between substrate and enzyme. Additionally, in light of the productive transfer by DBAT of the C_4 *n*-butyryl group to the hydroxytaxane, the presence of the π bond in the *trans*-butenoyl-CoA suggests that the active site of DBAT is conformational exclusive of the locked C_4-transoid configuration and/or that the π electrons are electronically restrictive.

Second-generation Taxol analogs have been synthesized with propionyl or butyryl substitutions at C10 of the taxoid core, and such compounds have demonstrated improved cytotoxicity and tubulin binding properties relative to the parent drug [7]. Therefore, DBAT biocatalysis holds promise as an alternative method for the production of unnatural baccatin III compounds that can serve as intermediates in biosynthetic or semisynthetic routes to manufacture modified Taxols.

Potential Application of DBAT in the Semisynthesis of Taxol and Its Analogs

Naturally occurring Taxol is found in several subspecies of the Yew (*Taxus*) at relatively low concentrations compared to other taxanes, including the closely related advanced biogenetic precursor 10-deacetylbaccatin III, which is extractable at ~5- to 10-fold higher concentrations from renewable portions of the Yew [11]. A current practical semisynthesis of Taxol derives from coupling a phenylisoserinyl precursor to baccatin III, which is synthesized by selective acetylation of 10-deacetylbaccatin III at C10 [20]. This latter acylation has posed a synthetic challenge due to the order of increasing reactivity of the hydroxyl groups (at C1 < C13 < C10 < C7, the most reactive) of 10-deacetylbaccatin III (see Figure 1 for numbering) that can be acylated [21–23]. Consequently, methods have been developed to regioselectively acetylate the hydroxyl at C10, and not at the more reactive C7. These procedures include the addition of a removable silyl protecting group at C7 followed by acetylation at C10, and direct acetylation of 10-deacetylbaccatin III (without the use of protecting groups) is achieved by reaction with methyliminium salts [24] and acetic anhydride in the presence of a Lewis acid ($CeCl_3$, LiCl, $ZnCl_2$, or $YbCl_3$) (Figure 2) [25].

However, regardless of whether the protecting group is added before or after acetylation of the hydroxyl at C10 of 10-deacetylbaccatin III, the C7 hydroxyl ultimately still needs to be protected prior to coupling the synthetically derived phenylisoserine side chain precursor at C13 of baccatin III enroute to Taxol [26–29]. Alternatively, semisynthetic approaches that do not rely on protecting group manipulation chemistry to construct Taxol and its analogs will foreseeably involve enzyme catalysts. In this context, DBAT has been shown to regiospecifically transfer acetyl (natural donor) as well as propionyl and *n*-butyryl from the corresponding CoA thioesters to the C10 hydroxyl of 10-deacetylbaccatin III by using "green chemistry" methodology without heavy metal Lewis acid catalysts, organic solvents, or silyl and 2,2,2-trichloroethyl chloroformate protecting groups at other reactive hydroxyls.

Furthermore, in vivo biosynthetic production of metabolites occurring early in the Taxol biosynthetic pathway has been successful in bioengineered *Arabidopsis* [10] and *E. coli* [30]. Engineering additional downstream enzymatic steps on the pathway to Taxol and related compounds into a bacterial system may be limited by the need to target and express in active form at least 19 total genes that include a family of membrane-targeted cytochrome P450 monooxygenases. Therefore, *Taxus* plant genes will likely be more suitably expressed in the native host or in another plant system such as *Arabidopsis* or suitable crop plant [10] that has the requisite plant organelles for enzyme and metabolite targeting and trafficking. However, we have demonstrated that *E. coli*, expressing the *dbat* gene, can be used as a pliant host to synthesize an obligatory intermediate on the Taxol pathway via an abundant natural product precursor. Bacterial culture (100 ml) expressing *dbat* converts ~20% (50 nmol) of exogenously fed 10-deacetylbaccatin III (at 250 μM) to baccatin III in 16 hr (without optimization of expression or growth conditions), and the in vivo DBAT enzyme uses endogenous acetyl-CoA from *E. coli* as a cosubstrate. Separate HPLC analyses with UV monitoring (A_{228}) of organic extracts derived from the medium, and the pelleted cells of JM109 *E. coli* used in the feeding study, showed that approximately 90% of the baccatin III produced (eluting at 7.69 ± 0.01 min) (Figure 5) was found in the medium, while the remaining 10% of de novo product was associated with the cells. This observation suggests that the *E. coli* cells export a bulk of baccatin III to the growth broth, thus making the purification of baccatin III markedly easier than acquiring this metabolite from its natural resource, *Taxus* leaves. For a negative control experiment, JM109 *E. coli* cells possessing a plasmid expressing TAX02 (a related *Taxus*-derived 2α-*O*-*benzoyl*transferase) were processed identically to bacteria expressing DBAT; these cells did not produce baccatin III above the detection limits of the analysis.

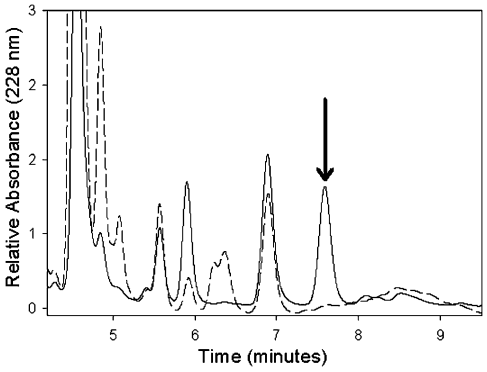

Figure 5. HPLC Analysis of the In Vivo Production of Baccatin III from 10-Deacetylbaccatin III

HPLC analysis of the biosynthetic product (retention time R_t = 7.69 ± 0.01 min, arrow) generated in vivo from 10-deacetylbaccatin III fed to *E. coli*. The solid line trace shows the UV (A_{228}) profile of products isolated from *E. coli* cells expressing DBAT enzyme (TAX06); the dashed line trace shows the UV profile of products isolated from *E. coli* expressing TAX02, which regiospecifically acylates the C2 hydroxyl of taxoids. HPLC conditions: 30:70 acetonitrile/water. See Experimental Procedures for further details.

Significance

Recombinantly expressed DBAT has modest substrate flexibility for alkanoyl-CoA cosubstrates and catalyzes the regiospecific transfer of the alkanoyl to the C10 hydroxyl of 10-deacetylbaccatin III. This preliminary screen of DBAT provides a baseline for guiding enzyme "breeding" procedures that will supply mutants of the acyltransferase; the so-derived mutant enzymes will be screened for novel acyltransferase function. Ideally, mutant enzymes that possess enhanced turnover for the natural substrate or expanded selectivity for unnatural substrates can be acquired by these random mutagenesis methods. In addition to being tractable for the discovery of novel catalytic function, these investigations will, in general, provide a priori insight into the structural/functional domains and conserved motifs of the family of *Taxus* acyltransferases, which belong to a larger superfamily, designated BAHD [31]. Currently, the regio- and substrate specificity of the other acyltransferases on the Taxol pathway are being investigated through in vitro assays, and, as with DBAT, mutants of these enzymes will be developed and screened for novel function.

Furthermore, the in vivo production of baccatin III is attainable in *E. coli* transformed with DBAT, and thus precludes the need to employ genetic engineering techniques on *Taxus* plant cell cultures to express the acetyltransferase for the production of elevated levels of the metabolite. Optimization of the semibiosynthetic method in bacteria potentially provides a practical means of developing commercial-scale production of baccatin III, which serves as a key intermediate to Taxol in semisynthesis and biosynthesis. Moreover, application of this in vivo method with the other acyltransferases in the Taxol biosynthetic pathway imaginably could spawn alternative methods for the semibiosynthetic production of a library of second-generation pharmaceuticals with modified acyl groups. A practical model of the application of the heterologous expression of plant genes in bacteria is the large-scale, in vivo production, in *E. coli*, of pharmaceutically important analogs of the plant-derived atemisinin, a secondary natural product of *Artemisia* used to treat malaria [32].

Experimental Procedures

General

A Varian Inova-300 or a Varian UnityPlus500 instrument was used to acquire proton nuclear magnetic resonance spectra. A Finnigan LC-ESI LCQ Deca XP tandem mass spectrometer (Thermo Electron Corp.) or a JEOL AX-505 Fast Atom Bombardment ionization double focusing mass spectrometer was used for mass spectral analysis. An Agilent 1100 HPLC system equipped with a Packard Radiomatic Flow-One Beta 150TR radioactivity detector (PerkinElmer, Shelton, CT) was used for tandem liquid chromatography with UV detector and radioactivity monitoring of effluent mixed with 3a70B Complete Counting Cocktail (Research Products International, Mount Prospect, IL).

Substrates and Reagents

[13-^{3}H]-10-Deacetylbaccatin III (1.2 Ci/mol) was synthesized as described elsewhere [33]. [^{3}H]Sodium borohydride was purchased from American Radiolabeled, Inc. (St. Louis, MO). 10-Deacetylbaccatin III was purchased from Natland (Research Triangle Park, NC). CoA, as the lithium salt, and acetyl, propionyl, *n*-butyryl and *iso*-butyryl, 2,3-butenoyl (crotonyl), *iso*-valeryl-, *n*-hexanoyl-, and benzoyl-CoA thioesters were purchased from Sigma. All other reagents were purchased from Sigma-Aldrich and were used without further purification unless indicated otherwise. Valeryl- and cyclohexanecarboxylic acid-CoA thioesters were synthesized by established procedures [19, 34]. Briefly, to a solution of pentanoic acid (Aldrich) or cyclohexanecarboxylic acid (Aldrich) (55 μmol) in CH_2Cl_2/tetrahydrofuran (5:2, vol/vol, 10.5 ml) under N_2 was added triethylamine (60 μmol, 8.5 μl) in one portion, and the mixture was stirred for 10 min at room temperature. Ethyl chloroformate (55 μmol, 5.3 μl, 1 equivalent) was added in one portion, and the reaction was stirred for 1 hr at room temperature. The solvents were evaporated, and the residue was dissolved in 0.5 ml *t*-butyl alcohol. CoA as the sodium salt (47.6 mg, 61 μmol dissolved in 1.0 ml of 0.4 M $NaHCO_3$) was added to the solution, and the mixture was stirred for 0.5 hr at room temperature, then quenched with 1 M HCl (4 ml, 1 equivalent relative to $NaHCO_3$) and adjusted to pH 5 with 15 mM NaH_2PO_4 (pH 4.8). The solvents were evaporated under reduced pressure (5 hr) at room temperature. The residue was resuspended in 15 mM NaH_2PO_4 (pH 6.9, 7 ml). The crude product was purified by flash chromatography on a C_{18} silica gel (VWR) column that was first washed with methanol and water, then equilibrated with 15 mM NaH_2PO_4 (pH 6.9). The crude sample was loaded onto the column, which was eluted with 5 ml portions of increasing methanol in 15 mM NaH_2PO_4 (pH 6.9) to yield valeryl- or cyclohexanoyl-CoA (eluted in 10%–15% methanol). The purity (estimated at 96%) of each CoA thioester was assessed by analytical thin layer chromatography (TLC) (silica gel developed with 1-butanol/H_2O/AcOH, 5:3:2, vol/vol/vol) and ^{1}H-NMR analysis. The ^{1}H-NMR spectra compared favorably with those of other, closely related and commercially available alkanoyl-CoA thioesters. The valeryl-CoA or cyclohexanoyl-CoA esters were lyophilized, weighed, and resuspended in water to ~500 μM.

Synthesis of 7-Butyryl-10-Deacetylbaccatin III

To a solution of 10-deacetylbaccatin III (50 mg, 92 μmol) in 2 ml pyridine was added butyryl chloride (95 μl, 0.92 mmol), and the reaction mixture was stirred overnight at room temperature. The mixture was partitioned between water (20 ml) and ethyl acetate (20 ml) and was stirred for 30 min. The aqueous layer was isolated and further extracted with ethyl acetate (3 × 20 ml); the organic fractions were combined and extracted with aqueous saturated copper sulfate solution (4 × 20 ml) to remove pyridine. The ethyl acetate was dried over magnesium sulfate, filtered, and evaporated in vacuo. The 7-butyryl-10-deacetylbaccatin III (R_f = 0.44) was purified from the reaction mixture by preparative TLC (40:60 hexane/ethyl acetate). The yield of the target product was 10 mg (18% yield [without optimization of the synthesis], 98% pure by ^{1}H-NMR). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.90 (t, *J* = 7.5 Hz, C*H*$_3$CH$_2$CH$_2$C=O), 1.04 (s, H16), 1.06 (s, H17), 1.57 (tq, *J* = 7.5 Hz, *J* = 7.5 Hz, CH$_3$C*H*$_2$CH$_2$C=O), 1.82 (s, H19), 1.90 (m, H6$_b$), 2.10 (bs, H18), 2.20 (t, *J* = 7.5 Hz, CH$_3$CH$_2$C*H*$_2$C=O), 2.23 (d, *J* = 5.1 Hz, H14), 2.28 (s, C*H*$_3$C=O at C4), 2.52 (m, H6$_a$), 4.06 (d, *J* = 6.9 Hz, H3), 4.13 (d, *J* = 8.1 Hz, H20$_b$), 4.32 (d, *J* = 8.1 Hz, H20$_a$), 4.86 (bt, *J* = 8.1, H13), 4.94 (d, *J* = 9.6 Hz, H5), 5.33 (s, H10), 5.51 (dd, *J* = 10.8 Hz, *J* = 7.2 Hz, H7), 5.61 (d, *J* = 6.9 Hz, H2), 7.48–8.10 (aromatic-H).

Synthesis of 10-Butyryl-10-Deacetylbaccatin III

The following procedure is adapted from established methods [35]. To a solution of 7-triethylsilyl-10-deacetylbaccatin III (32 mg, 50 μmol) [36] in 1.5 ml dry THF at −40ºC was added lithium bis(trimethylsilyl)amide (65 μl, 66 μmol). The solution was stirred for 5 min, and butyryl chloride (7 μl, 66 μmol) was added dropwise. The reaction was warmed to 0ºC, quenched by the addition of saturated aqueous ammonium chloride solution (20 ml), and then extracted with ethyl acetate (3 × 10 ml). The organic fractions were combined and washed with brine and were dried over anhydrous magnesium sulfate. The solvent was decanted then removed in vacuo, and the remaining crude residue containing the acylated product was dissolved in 1 ml dry THF and cooled to 0ºC. A solution of 30% HF in pyridine was added dropwise (over 1 hr) to the material to remove the silyl protecting group. The reaction was quenched by the addition of sodium bicarbonate to neutralize the reaction and partitioned against ethyl acetate. The organic layer was isolated and saved, and the aqueous fraction was extracted with ethyl acetate (2 × 5 ml). The organic fractions were combined and extracted in series with brine and water; the organic solvent was dried over anhydrous magnesium sulfate, decanted, and removed in vacuo. The remaining crude residue was loaded onto a preparative silica gel TLC plate and eluted with 40:60 hexane/ethyl acetate. The band of silica gel corresponding to the product (R_f = 0.37) was isolated and extracted with ethyl acetate, and the organic layer was decanted and evaporated to yield pure 10-butyryl-10-deacetylbaccatin III (10 mg, ~30% yield [without optimization of the synthesis], 98% purity by ^{1}H-NMR). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.01 (t, *J* = 7.5 Hz, C*H*$_3$CH$_2$CH$_2$C=O), 1.08 (s, H16 and H17), 1.65 (s, H19), 1.73 (tq, *J* = 7.5 Hz, *J* = 7.5 Hz, CH$_3$C*H*$_2$CH$_2$C=O), 1.84 (m, H6$_b$), 2.03 (s, H18), 2.26 (s, C*H*$_3$C=O at C4), 2.27 (d, *J* = 7.7 Hz, H14), 2.46 (m, CH$_3$CH$_2$C*H*$_2$C=O), 2.54 (m, H6$_a$), 3.87 (d, *J* = 7 Hz, H3), 4.13 (d, *J* = 8.4 Hz, H20$_b$), 4.29 (d, *J* = 8.4 Hz, H20$_a$), 4.45 (dd, *J* = 10.8 Hz, *J* = 6.9 Hz, H7), 4.87 (bt, *J* = 8, H13), 4.97 (dd, *J* = 9.6 Hz, *J* = 1.8 Hz, H5), 5.60 (d, *J* = 7 Hz, H2), 6.30 (s, H10), 7.46–8.08 (aromatic-H).

Synthesis of 13-Butyryl-10-Deacetylbaccatin III

Briefly, to a solution of 7-triethylsilyl-10-deacetylbaccatin III [37] (100 mg, 152 μmol) in 3 ml dry THF, cooled to −40ºC, was added 1.0 M lithium bis(trimethylsilyl)amide (167 μl, 167 μmol). The solution was stirred for 5 min, and triethylsilyl chloride (28 μl, 167 μmol) was added dropwise. The reaction was warmed to 0ºC, quenched by the addition of saturated aqueous ammonium chloride solution (20 ml), and extracted with ethyl acetate (3 × 10 ml). The organic fractions were combined and dried over anhydrous magnesium sulfate; the solvent was decanted and removed in vacuo. The crude bis(silyl)-protected product was purified by preparative TLC (50:50 hexane/ethyl acetate), the sample at R_f = 0.8 was isolated from the silica gel by extraction with ethyl acetate, and the solvent was evaporated. To the remaining residue, 7,10-bis(triethylsilyl)-10-deacetylbaccatin III (~30 mg, 39 μmol), dissolved in 3 ml dry THF at −40ºC was added lithium bis(trimethylsilyl)amide (42 μl, 43 μmol). The solution was stirred for 5 min, and butyryl chloride (4.6 μl, 43 μmol) was added dropwise. The reaction was stirred for 30 min, warmed to 0ºC, quenched by the addition of saturated aqueous ammonium chloride solution (20 ml), and then extracted with ethyl acetate (3 × 10 ml). The organic fractions were combined and dried over anhydrous magnesium sulfate. The solvent was decanted then removed in vacuo, and the remaining crude residue containing the 13-*O*-butyryl analog was dissolved in 1 ml dry THF and was cooled to 0ºC. The remaining deprotection step with HF/pyridine is identical to the procedure described above for the synthesis of the 10-butyryl isomer. The product (R_f = 0.33) was purified by preparative TLC (40:60 hexane/ethyl acetate) to yield pure 13-butyryl-10-deacetylbaccatin III (5 mg, 21% [without optimization of the synthesis; based on the 7,10-bis(triethylsilyl)-10-deacetylbaccatin III], 95% purity by ^{1}H-NMR). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.99 (t, *J* = 7.5 Hz, C*H*$_3$CH$_2$CH$_2$C=O), 1.10 (s, H16), 1.20 (s, H17), 1.25 (tq, *J* = 7.5 Hz, *J* = 7.5 Hz, CH$_3$C*H*$_2$CH$_2$C=O), 1.73 (s, H19), 1.84 (m, H6$_b$), 2.02 (s, H18), 2.20 (d, *J* = 9.0 Hz, H14), 2.30 (s, C*H*$_3$C=O at C4), 2.40 (m, CH$_3$CH$_2$C*H*$_2$C=O),

2.58 (m, $H6_a$), 3.93 (d, J = 7.2 Hz, H3), 4.16 (d, J = 8.2 Hz, $H20_b$), 4.20 (dd, J = 10.8 Hz, J = 6.9 Hz, H7), 4.30 (d, J = 8.4 Hz, $H20_a$), 4.95 (dd, J = 9.6, J = 1.8 Hz, H5), 5.20 (s, H10), 5.65 (d, J = 6.9 Hz, H2), 6.17 (t, J = 8.7, H13), 7.46–8.08 (aromatic-H).

Expression, Activity Assay, and Partial Purification of DBAT

The large-scale (6L) procedure for the expression of the recombinant DBAT from pCWori$^+$ in *E. coli* JM109 cells, activity assay, and partial purification of the enzyme by anion exchange chromatography has been described previously [18], with the following modifications of the assay and purification schemes. After eluting the target protein from a column of *O*-diethylaminoethylcellulose (2.5 × 14 cm, Whatman DE-52) at ≈100 mM NaCl in 25 mM MOPSO (3-(*N*-morpholino)-2-hydroxypropanesulfonic acid) buffer containing 3 mM dithiothreitol, fractions containing DBAT activity were combined (50 ml) and concentrated (Amicon Diaflo YM 30 membrane, Millipore, Billerica, MA). The DBAT concentration was estimated to be at ~140 μg/ml, as determined by SDS-PAGE and Coomassie blue staining.

Screening for Productive CoA Substrates and Analysis of Products

The general DBAT assay contained a 1 ml aliquot of the dialyzed protein (~140 μg), which was incubated with [13-^{3}H]-10-deacetylbaccatin III (75 μM, 90 nCi, specific activity at 1.2 Ci/mol) and an acyl-CoA (500 μM) for 1 hr at 31ºC. The reaction was then quenched by extraction with ethyl acetate, and the solvent was concentrated in vacuo. The residue was dissolved in 50 μl acetonitrile and analyzed by radio-HPLC. The sample was loaded onto an Econosphere C_{18} column (5 μm; 250 × 4.6 mm) (Alltech, Mentor, OH), eluted isocratically with 40:60 (vol/vol) acetonitrile/water at 1 ml/min. Isolated crude product derived from DBAT assays incubated with productive CoA esters (acetyl-, propionyl-, and butyryl-CoA thioesters) each showed a single, major radioactivity peak with a unique chromatographic retention time upon elution from the HPLC column at 5.53, 6.73, and 8.63 min, respectively (cf. Figure 3).

To confirm the identity of each novel 10-acylbaccatin III product made by DBAT, each productive acyl-coenzyme cosubstrate (500 μM) was incubated separately in 20 ml assays for 3 hr with DBAT (1.1 mg) and unlabeled 10-deacetylbaccatin III (500 μM) buffered with 25 mM MOPSO buffer containing 3 mM dithiothreitol and 100 mM sodium chloride. The putative target product was purified by preparative TLC (silica gel developed with a mobile phase of 40:60 hexane/ethyl acetate); the TLC retention factor (R_f) of both products isolated from DBAT assays containing the productive substrates propionyl-CoA and butyryl-CoA were virtually identical at ~0.40. The TLC-purified material was further purified by reverse-phase HPLC eluted isocratically with 50:50 (vol/vol) acetonitrile/water at 1 ml/min with UV monitoring (A_{228}) of the effluent. The products eluting from the column with identical retention times as those for the radioactive products derived from the corresponding analytical radiochemical assays (described earlier) were isolated. The solvent from each fraction containing purified product was evaporated, and the residue was dissolved in deuterated chloroform as internal standard for analysis by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). Purified product (~1 mg, 1.6 μmol) isolated from the preparative-scale DBAT assay incubated with propionyl-CoA was sufficient for ^{1}H-NMR analysis, while the low concentration of the sample (at ≤ 0.1 mg, 0.16 μmol) isolated from a similar assay incubated instead with butyryl-CoA was below the detection limits of ^{1}H-NMR.

Comparison of the retention time on C_{18} reverse-phase HPLC of the biosynthetically derived butyryl baccatin isomer to those of authentic 7-, 10-, and 13-butanoyl-10-deacetylbaccatin III isomers was conducted to assess the regiochemistry of the biosynthetic product. Briefly, a preparative-scale (20 ml) assay containing butyryl-coenzyme (500 μM) was incubated for 3 hr with DBAT (1.1 mg) and unlabeled 10-deacetylbaccatin III (500 μM) buffered with 25 mM MOPSO buffer containing 3 mM dithiothreitol and 100 mM sodium chloride. The assay was extracted with ethyl acetate (3 × 20 ml), and the organic solvent was dried over magnesium sulfate, decanted, and evaporated in vacuo. The remaining residue of crude product was dissolved in acetonitrile (50 μl), a fraction (10 μl) was injected onto a reverse-phase HPLC column that was eluted isocratically with 60:40 acetonitrile/water, and the effluent was monitored at 228 nm. Each synthetic authentic butyryl-10-deacetylbaccatin III standard (see above in Experimental Procedures) was independently assessed by separately dissolving ~1 mg of each in acetonitrile (300 μl), a fraction of each (2 μl) was injected onto the HPLC reverse-phase column eluted with 60:40 acetonitrile/water, and the effluent was monitored at 228 nm. After the retention times of authentic 7-, 10-, and 13-butyryl-10-deacetylbaccatin III were established at 8.56 min, 6.51 min, and 6.35 min, respectively, an equimolar mixture of the butyryl isomers was prepared in acetonitrile (100 μl), and a fraction (2 μl) was injected onto the HPLC reverse-phase column eluted with 60:40 acetonitrile/water and UV monitoring at 228 nm (cf. Figure 4). The HPLC-UV profile of the mixture of authentic standards was compared to that of the de novo biosynthetic product.

Additionally, a fraction of each isolated acylbaccatin was further analyzed by combined liquid chromatography-mass spectrometry (LC-ESI/MS) or fast atom bombardment mass spectrometry (FAB-MS). Diagnostic ions with ion abundance are as follows. FAB ionization (positive ion mode) of the putative baccatin III: *m/z* 587 (MH^+, 43%), 527 ($M^+ - C(O)CH_3$, 43%), and 509 (*m/z* 527 − H_2O, 14%), and the putative 10-propionyl-10-deactylbaccatin III: *m/z* 601 (MH^+, 55%), 527 ($M^+ - C(O)CH_2CH_3$, 40%), and 509 (*m/z* 527 − H_2O, 10%); LC-electrospray ionization (positive ion mode) of putative 10-butyryl-10-deacetylbaccatin III: *m/z* 615 (MH^+, 10%), 527 ($M^+ - C(O)CH_2CH_2CH_3$, 65%), and 509 (*m/z* 527 − H_2O, 24%); LC-electrospray ionization (positive ion mode) of authentic 10-deacetylbaccatin III: *m/z* 545 (MH^+, 12%), 527 ($M^+ - H_2O$, 32%) and 509 (*m/z* 527 − H_2O, 10%).

Kinetic Evaluation of Competing Substrates

The procedure used to calculate the relative kinetic constants of DBAT for multiple CoA substrates was adapted from a method used to calculated the specificity constants for a NodH sulfotransferase [38]; this relative kinetics evaluation method was advantageous toward conserving the radiolabeled 10-deacetylbaccatin III cosubstrate by reducing the duplicate assays required to construct parallel kinetic plots for each acyl-CoA substrate. In brief, evaluations of the linearity of product formation with respect to time and protein concentration were first established with the natural substrates, and then the concentration of acetyl-CoA (5–100 μM) was independently varied while the [13-^{3}H]-10-deacetylbaccatin III (400 μM, 0.49 μCi at 1.2 Ci/mol) was maintained at saturation. Assay conditions and analysis were performed as described previously [18]. A double reciprocal plot was constructed for the data set, and the equation of the best-fit line (R^2 = 0.98) was determined (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA) to calculate the V_{max} and K_m.

The substrate specificity of the catalytically productive CoA thioesters was assessed by mixing each thioester (each at 50 μM) in a single assay tube, along with ~30 μg DBAT enzyme and unlabeled 10-deacetylbaccatin III (500 μM), and incubating for 1 hr. The product ratio of each individual novel acylbaccatin III and baccatin III (NAB_n/Bacc, where *n* = derived acylbaccatin III product) formed in the mixed reaction was used along with the specificity constant (V_{max}/K_m) of DBAT for acetyl-CoA to calculate the relative specificity constant of each surrogate acyl-CoA substrate: relative $(V_{max}/K_m)_n$ = ($[NAB_n/Bacc] \times [V_{max}/K_m]$).

In Vivo Conversion of 10-Deacetylbaccatin III to Baccatin III by Recombinant DBAT in *E. coli*

10-Deacetylbaccatin III (100 μM) was fed to a 100 ml culture of transformed JM109 *E. coli* expressing the DBAT protein from pCWori$^+$. The bacteria were grown to A_{600} = 1.0 at 37ºC with ampicillin selection, then expression was induced with 1 mM isopropyl-D-thiogalactoside, and the transformed bacteria were grown at 31ºC for 18 hr. The approximate acetyl-CoA concentration in these transformed JM109 *E. coli* cells was assessed to be at saturation (i.e., >100 μM) for DBAT (at ~30 μg/ml), as determined by assaying an aliquot of supernatant, without purification, from a cell-free extract of bacterial lysate as follows. A 1 ml aliquot of cell-free extract of the bacteria was incubated for 1.5 hr with radiolabeled substrate [13-^{3}H]-10-deacetylbaccatin III at saturation (250 μM and 500 μM), with no exogenous acetyl-CoA added. Extraction of the assay supernatant with

ethyl acetate (2 × 1 ml), evaporation of solvent, and radio-HPLC analysis of the residue dissolved in 50 μl acetonitrile revealed that baccatin III was produced at maximum velocity. Analogously, when the cofactor was removed from *E. coli* extracts by partial purification of DBAT by anion exchange chromatography prior to the addition of substrate, standard assays containing [13-^{3}H]-10-deacetylbaccatin III (at saturation) and exogenous acetyl-CoA added at 250 μM revealed zero order production of baccatin III (i.e., DBAT was at V_{max}), as determined by radio-HPLC analysis of the effluent and comparison to a kinetic curve for these substrates. This observation suggested that the acetyl-CoA present in the cell-free extract (100 ml) of JM109 cells at $A_{600} \approx 1.0$ is $\geq$ 250 μM, which is consistent with typical endogenous acetyl-CoA concentrations ranging between 20 and 600 μM in *E. coli* [39].

The bacteria cells from the in vivo feeding experiment described above were harvested by centrifugation at 5000 × *g* (10 min), and the supernatant was isolated and saved for later. The cells were resuspended in 10 ml brine and lysed by brief sonication (five 20 s bursts at 50% power [Misonix sonicator, Farmingdale, NY]). Lastly, ethyl acetate (3 × 5 ml) was added, and the colloidal suspension was extracted carefully to avoid emulsion.

To the supernatant, saved previously, was added NaCl (~30 g) to minimize emulsion, and the resulting brine solution was extracted with ethyl acetate (3 × 50 ml). The organic fractions from the extraction of the cellular debris and supernatant were processed separately, dried with sodium sulfate, and filtered. The solvent of the filtrates was evaporated, and the samples were dissolved in 200 μl acetonitrile and analyzed by reverse-phase HPLC with UV monitoring (A_{228}) of the effluent (isocratic elution with 30:70 acetonitrile/water) (cf. Figure 5). For a negative control experiment, JM109 cells possessing a plasmid expressing TAX02 (a related *Taxus*-derived 2α-*O*-benzoyltransferase) were processed identically to bacteria expressing DBAT. The level of baccatin III produced by these cells was below the detection limits of the analysis.

Acknowledgments

The authors thank Dr. Rodney Croteau (Washington State University) and the Washington State University Research Foundation (WSURF) for the Taxus 10β-acetyltransferase clone. We also acknowledge Kimberly Jerkins, Susan Achberger, Christopher Johnson, and Allyson Boers for their technical assistance.

Received: October 26, 2005
Revised: December 22, 2005
Accepted: January 18, 2006
Published online: March 24, 2006

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