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THE SYNTHESIS OF TAXANE SUBSTRATES AND PHENYLPROPANOID COENZYME A THIOESTERS TO PROBE THE MECHANISM OF TAXUS ACYLTRANSFERASES

presented by

YEMANE A. MENGISTU

has been accepted towards fulfillment
of the requirements for the

Master of Science degree in Chemistry

Major Professor's Signature

12/18/2009

Date

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BY

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Chemistry

2009

ABSTRACT

THE SYNTHESIS OF TAXANE SUBSTRATES AND PHENYLPROPANOID COENZYME A THIOESTERS TO PROBE THE MECHANISM OF *TAXUS* ACYLTRANSFERASES

By

Yemane A. Mengistu

The use of *Taxus* acyltransferase enzyme could minimize the several protection and deprotection steps involved in the semisynthesis of next generation paclitaxel analogues. *N*-Debenzoyl-2'-deoxypaclitaxel:*N*-benzoyltransferase (NDTBT) transfers a benzoyl group to the free amine of the phenylpropanoid side chain of *N*-debenzoyl-2'-deoxypaclitaxel. To investigate the substrate specificity of NDTBT; various congeners of *N*-debenzoylpaclitaxel were made and incubated with several acyl CoA thioesters. It was found that NDTBT transferred several different acyl groups to the amino group of these substrates.

The paclitaxel pathway enzyme baccatin III:3-amino-3-phenylpropanoyltransferase (BAPT) shown to transfer β-phenylalanine analogues to the baccatin III core contains a glycine in place of a conserved histidine residue in the active site; thus, the substrate is hypothesized to participate in the catalytic mechanism. Thioesters with modifications at the β-carbon were synthesized and site-directed mutagenesis of BAPT was conducted.

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LIST OF ABBREVIATIONS

THF	Tetrahydrofuran
DCM	Dichloromethane
br	Broad
s	Singlet
d	Doublet
t	Triplet
Boc	*t*- Butyloxycarbonyl
ESI-MS	Electrospray Ionization Mass Spectrometry.
CoA	Coenzyme A Salt
NMR	Nuclear Magnetic Resonance
Ac_2O	Acetic Anhydride
TBDMSCl	*t*-butyldimethylsilyl chloride
NDTBT	*N*-debenzoylpaclitaxel benzoyltransferase
BAPT	Baccatin III:3-aminophenylpropanoyltransferase
DBAT	10-Deacetylbaccatin III-10-*O*-acetyltransferase

CHAPTER ONE

BROAD SUBSTRATE SPECIFICITY OF *N*-DEBENZOYLPACLITAXEL ACYLTRANSFERASE (NDBAT)

1. Introduction

1.1 Paclitaxel Background and Development

The natural product paclitaxel (Figure 1) is a diterpene pharmaceutical that is widely used as an antimitotic agent[1] The drug was first identified in and isolated from the Pacific yew (*Taxus brevifolia*) in 1963.[2] The mechanism of action involves promoting polymerization of β-tubulin subunits, and specifically by inhibiting microtubule depolymerization during cell division causing cell arrest and apoptosis.[3, 4] Paclitaxel (Taxol®) and docetaxel (Taxotere®) (Figure 2) are important anticancer agents in the treatment of a broad range of malignancies including carcinoma of the ovary, lung, head, neck, bladder, and esophagus. They have also demonstrated effectiveness in the initial therapy against earlier stages of cancer, a setting in which any new therapy is likely to make its greatest impact.[5, 6] Currently, a combinations of docetaxel and other anticancer drugs have shown good activity in advanced stage pretreated non-small cell lung cancer pretreated with other cancer agents[7]and node positive breast cancer.[8] These pharmaceuticals are also receiving great attention for the treatment of Alzheimer's and coronary restenosis.[9, 10]

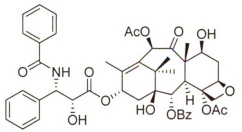


Figure 1: Paclitaxel (Taxol®)

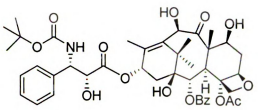


Figure 2: Docetaxel (Taxotere®)

Paclitaxel stabilizes microtubules against depolymerization, which is its primary mechanism that inhibits cell proliferation. Moreover pleiotropic effects exhibited by this drug may be responsible for its success where other chemotherapeutic agents fail.[11, 12] This could be because paclitaxel also modulates the expression of genes that encode enzymes for membrane assembly and those that participate in cell proliferation and apoptosis--processes with mechanisms independent of microtubule stabilization.[11, 13] In addition, anti-angiogenic activity, also independent of microtubule assembly, has been shown at 1-10 nmol/L of paclitaxel.[14, 15] Although paclitaxel has led to improvement in the duration and quality of life for some cancer patients, the majority will develop progressive disease after initially responding favorably to paclitaxel treatment.[16] Despite

the success of paclitaxel and docetaxel in chemotherapy, there are demands to maximize the potential benefit of paclitaxel and its analogues in order to minimize the resistance developed against paclitaxel and docetaxel.[17]

Various approaches have been undertaken to obtain better analogues of paclitaxel with increased water solubility, specificity, and improved activity against multi-drug resistant (MDR) tumors.[18] The targets include the synthesis of next generation analogues and prodrugs with better and enhanced blood-brain barrier permeability, bioavailability, and cancer-target specificity. Preparations of new formulations with improved physical properties, and cocktail therapy along with other drugs based on the combinatorial chemistry libraries of several paclitaxel analogues are also being evaluated. Investigations of the biological activity of these analogues revealed lead compounds with better efficacy.[19-25]

The structure-activity relationships of paclitaxel have been studied for several years.[26] and some of the results are summarized in Figure 3. These drug congeners have provided a foundation for the design of new generation paclitaxel analogues. Each functional group of the C-13 isoserinyl side chain of paclitaxel is essential for the bioactivity of paclitaxel.[27, 28] However, substitutions of the phenyl group of the naturally occurring phenylisoserine with other alkyl groups improve the activity of paclitaxel. [29] As shown in Figure 4, combinations of acetyl group substitutions at C-10 and benzoyl at C-2 result in more efficacious taxoids compared to the parent drug.[30, 31]

Various modifications of the 3'-*N*- acyl group of paclitaxel demonstrates better activity. Ojima and coworkers have synthesized several analogues by replacing the phenyl group of 3'-*N*-benzoyl with other groups such as cyclopentyl, tertbutyloxy, cyclohexyl and other groups which resulted in equipotent or more active analogues compared to the parent drug.[1, 24]. For example, cyclohexyl in place of phenyl (Figure 4) creates a paclitaxel analog that is 20 times more potent than paclitaxel in non small cell carcinoma.[24]. The ester group at C-2 and C-4, along with the oxetane and the rigid taxane are essential for activity,[26] however substituting the C-2 benzoyl with meta substituted benzoyl groups produce a more cytotoxic drug.[32]

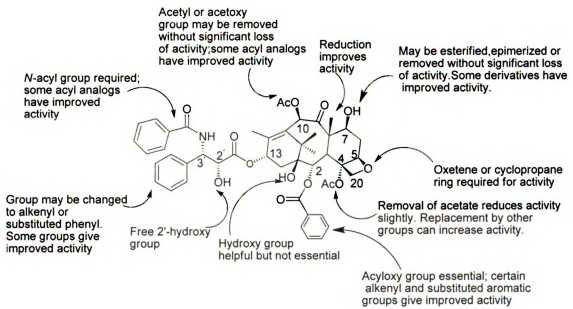


Figure 3: Structure activity relationship of paclitaxel

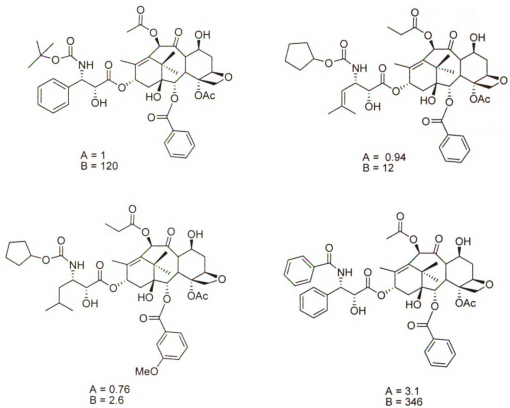


Figure 4: The IC_{50} (nm/L) of various modified paclitaxel analogs compared with paclitaxel

A = Human breast cancer cell line; B = multidrug resistant human breast cancer cell line

Replacement of the natural acyl groups with surrogate groups at the 3'-*N*-, 2-*O* and/or the 10-*O*-positions (Figure 4) by synthetic means has provided paclitaxel analogues that are more efficacious than the parent drug, regarding physical, chemical and biological properties.[24, 25] This demonstrated that these sites were central to the development of next generation paclitaxel compounds[22] (Figure 3).

1.2 Application of Biocatalysis in the Construction of Paclitaxel Analogues

1.2.1 Introduction

The tremendous demand for paclitaxel in basic research and cancer chemotherapy forced researchers to secure better ways of resourcing paclitaxel. The total synthesis of paclitaxel was a major achievement that involved greater than 90 steps, and thus was never considered to address commercial supply.[26, 33-35] A significant source of paclitaxel is from *Taxus* cells, which are grown in a suitable media and later extracted to acquire the pharmaceutical.[36] This biological production circumvents the several steps of purification needed to fraction plant chlorophylls, lignins and other non-essential plant products from the desired taxane products when using the natural resources, the *Taxus* brevifolia tree; [37] Bristol-meyers Squibb adopted and optimize this method for the current supply of Taxol®. [38, 39]

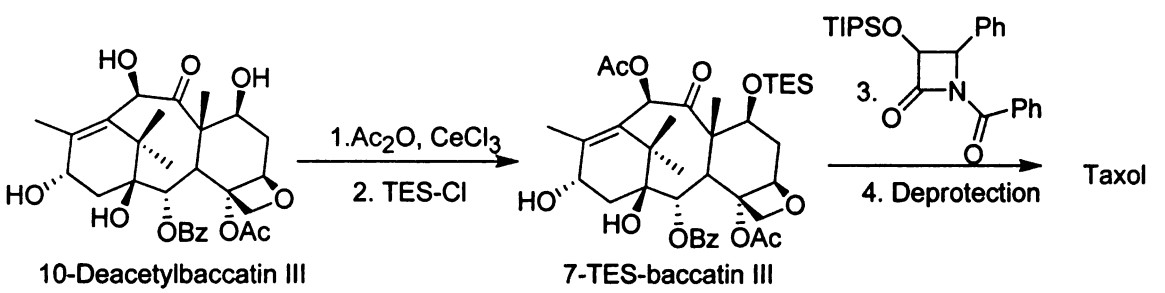


Figure 5: Ojima-Holton coupling for the synthesis of Paclitaxel

An alternative method of semisynthesizing paclitaxel and its analogues[40] was developed wherein the *N*-benzoylisoserinyl side chain was coupled to baccatin III that is derived synthetically from an abundant naturally occurring precursor of paclitaxel, 10-deacetylbaccatin III, found in the needles of *Taxus baccata*.[41] This semisynthetic method, however, involves silyl protection at the C7-hydroxyl, acetylation of 10-deacetylbaccatin III at C10 to afford 7-*O*-protected baccatin III, synthetic attachment of the *N*-Bz phenylisoserine side chain at the C-13 hydroxyl, and, finally, deprotection to yield paclitaxel (see Figure. 5); implementing biosynthetic (i.e. enzymatic) methods to construct paclitaxel and its analogues would circumvent these protecting group manipulations. The demand for enantiopure pharmaceuticals and fine chemicals and corporate awareness of the impact of their environmental footprint has prompted initiatives to employ environmentally friendly synthesis as an alternative to chemical synthesis laden with petroleum based solvents. Biocatalysis is being implemented into methods that facilitate the production of rare pharmaceuticals and precursor compounds, particularly for those that require many synthetic steps.[42]

1.2.2. Acyltransferases on Paclitaxel Pathway

Acyltransferases are a key class of enzymes in the biosynthesis of important natural products, and catalyze regioselective acylation reactions during the assembly of compounds such as paclitaxel, daunorubicin, lovastatin and penicillin.[43]

The gene encoding the 10-deacetylbaccatin III-10-*O*-acetyltransferase (DBAT), taxane-2α-benzoyltransferase (TBT), taxadiene-5α-O-acetyltransferase (TAT), *N*-debenzoyl-2-deoxypaclitaxel:*N*-benzoyltransferase(NDTBT), and baccatin III:3-amino-3phenylpropanoyltransferase(BAPT) have been isolated, expressed, and the corresponding enzymes characterized.[44]

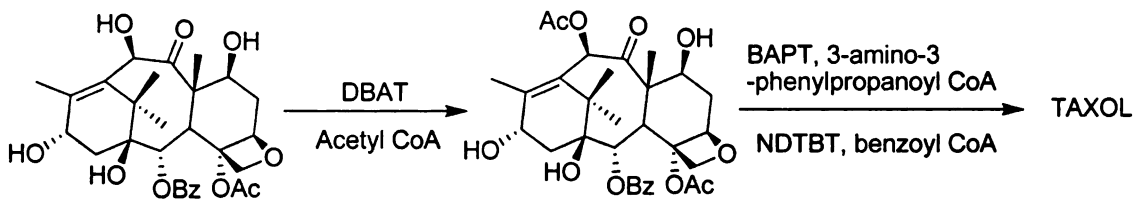
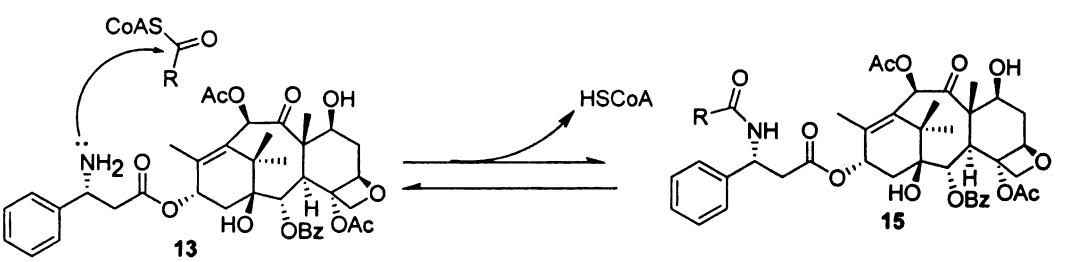


Figure 6: Biocatalytical route for the synthesis of paclitaxel

Even though paclitaxel and its precursors like baccatin III and 10-deacetylbaccatin III are important taxoids,[45] there are many naturally occurring taxoids such as cephalomanine, taxol C, taxuspinanane J, taxuspine N, 10-deacetylcephalomanine, and other compounds that have different acylation patterns and a variety of acyl and aroyl combinations. The functional groups include esters of acetate, propionate, butyrate, butyrate, (including hydroxylated and branched derivatives), tigloate, benzoate and phenylpropionate , as well as occasional glycosyl groups.[46, 47] The variety of different acyl group types at a specific regiocenter acyltransferase suggests that the acyltransferases catalyzing the acyl group attachment could have broad substrate specificity that generates the taxane variants at a particular hydroxyl or amino group.

1.2.3 Synthesis of Taxane Substrates

The paclitaxel biosynthetic pathway in *Taxus* spp. contains five acyltransferases that transfer acyl/aroyl groups to different taxane structures. The *N*-debenzoyl-2'-deoxypaclitaxel: *N*-benzoyltransferase (NDTBT) catalyzes the last step of paclitaxel biosynthesis by transferring the benzoyl group to the free amine position to form a benzamide functional group.(Scheme 1).[48] NDTBT belongs to a large superfamily of acyltransferases, designated BAHD,[49, 50] acyltransferase which was named according to the first letter of each of the first four enzymes first characterized in this family (benzylalcohol O-acetyltransferase (BEAT),anthocyanin *O*-hydroxycinnam-oyltransferase, (AHCT) *N*-hydroxycinnamoyl/benzoyltransferase (HCBT), and deacetylvindoline 4-*O*-acetyltransferase (DAT)).[51]



Scheme 1: Proposed mechanism of NDTBT

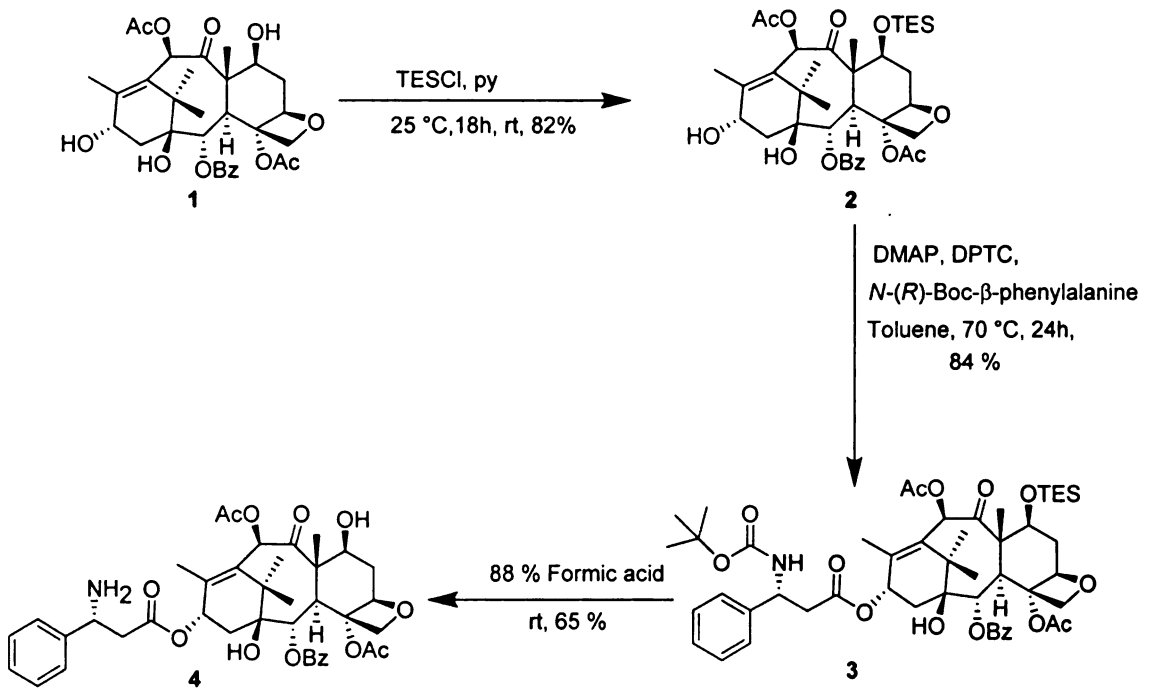
With the aim of biocatalytically synthesizing several *N*-acyl-*N*-debenzoylpaclitaxel variants and to test the broad specificity of NDTBT, it is imperative to synthesize various *N*-debenzoyl taxane substrates.

Thus, *N*-debenzoylpaclitaxel, and *N*-debenzoyl-2'-deoxypaclitaxel were synthesized to assess enzyme selectivity for the diterpene co-substrate. In particular, we wanted to evaluate the dependence of 2'-hydroxylation on the *N*-acylation and to determine whether *N*-acylation precedes hydroxylation. The other taxane 10-deacetyl-*N*-debenzoylpaclitaxel, would aid in dissecting whether the acetyl group at C-10 would affect *N*-acylation by NDTBT catalysis. The relevant substrates were synthesized from baccatin III and docetaxel, which are commercially available. The synthesis of these substrates discussed in the next section.

1.3 Results

1.3.1 Synthesis of *N*-Debenzoyl-2'-deoxypaclitaxel

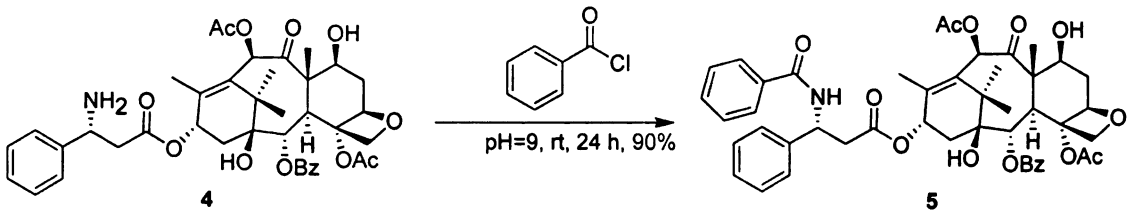
N-Debenzoyl(3'*R*)-2'-deoxypaclitaxel was synthesized first by selectively protecting the 7-hydroxyl of baccatin III (**1**), which is the most reactive hydroxyl group of baccatin-III,[52] to give **2** (Scheme 2). The protected baccatin III was coupled to *N*-Boc-*R*-β-phenylalanine using *O*,*O*-di(2-pyridyl) thiocarbonate (DPTC) in the presence of a catalytic amount of 4-(*N*,*N*-dimethylamino)pyridine (DMAP).[53,54] to form ester **3** (*N*-Boc-*N*-debenzoyl-2'-deoxypaclitaxel), followed by deprotection to give **4** (3'*R*-*N*-debenzoyl-2'-deoxypaclitaxel[55]) as a single diasteromer.



Scheme 2: Synthesis of 3'*R*-*N*-debenzoyl-2'-deoxypaclitaxel

1.3.2. Synthesis of 2'-deoxypaclitaxel

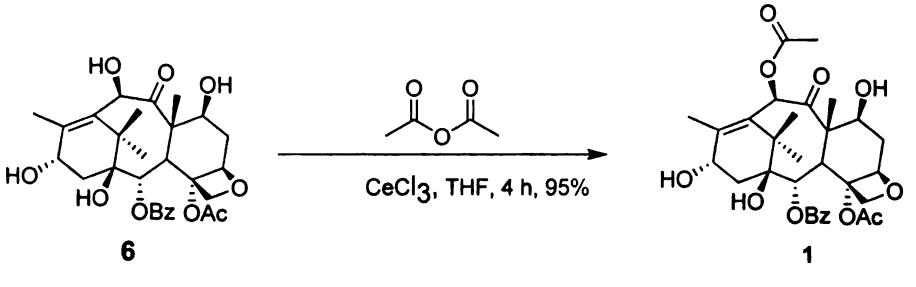
The 2'-deoxypaclitaxel was synthesized by the Schotten–Baumann method[56] where 3'*R*-*N*-debenzoyl-2'-deoxypaclitaxel (**4**) was acylated by treatment with benzoyl chloride under basic conditions (Scheme 3). The final product was used as a product standard for comparison against the biosynthetically derived product **5** (2'-deoxpaclitaxel).



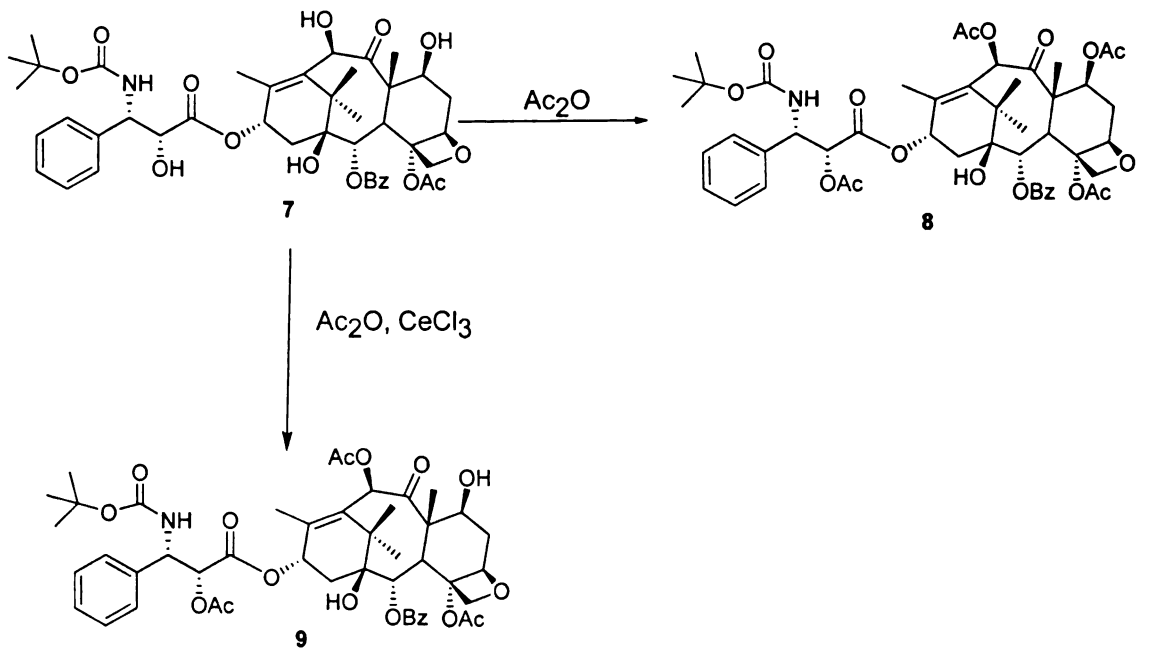
Scheme 3 The synthesis of 2'-deoxypaclitaxel

1.3.3 Synthesis of *N*-Debenzoylpaclitaxel Substrate

Previous studies on the acetylation of 10-deacetylbaccatin III (**6**) using acetic anhydride and pyridine resulted in a mixture of 7-acetylbaccatin III,[57]10-deacetyl-7-acetylbaccatin III, and baccatin III (**1**) (i.e.,C10 acetylation exclusively).[52] Therefore, we initially attempted to acetylate the C10 hydroxyl of docetaxel (Scheme 5) using acetic anhydride, hoping to acquire a mixture of acetylated regioisomers, from which the desired product would be isolated. Regrettably, 7, 10, 2'-triacetylated docetaxel (**8**) was formed exclusively.

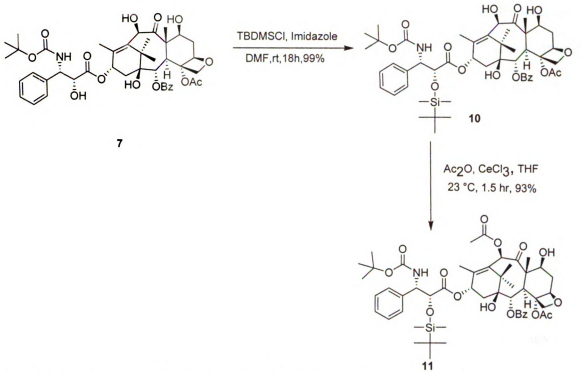


Scheme 4: Selective acylation of 10-deacetylbaccatin III

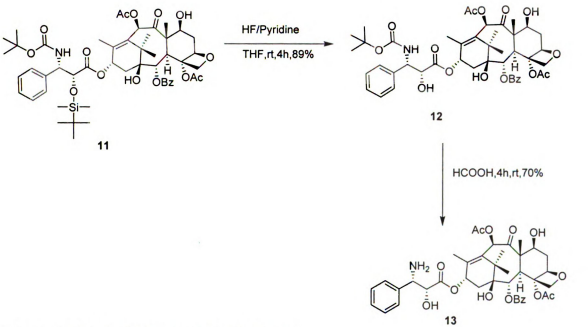


Scheme 5: Acetylation of docetaxel with and without Lewis acid.

As an alternative, the synthesis of *N*-debenzoylpaclitaxel started by chemoselectively protecting the 2'-hydroxyl position of commercially available docetaxel(**7**)[58] using *tert*-butyldimethylsilyl chloride, (TBDMS-Cl) (Scheme 6) followed by regioselective acetylation using cerium chloride as a Lewis acid to give 10-acetyl-2'-*O*-(*tert*-butyldimethylsilyl) docetaxel (**11**) at more than 95% yield.[59] The method was adopted from selective acetylation of 10-deacetylbaccatin III (10-DAB) to produce baccatin III[59](Scheme 4).



Scheme 6: Preparation of 10-acetyl-2'-*O*-(*tert*-butyldimethylsilyl) docetaxel

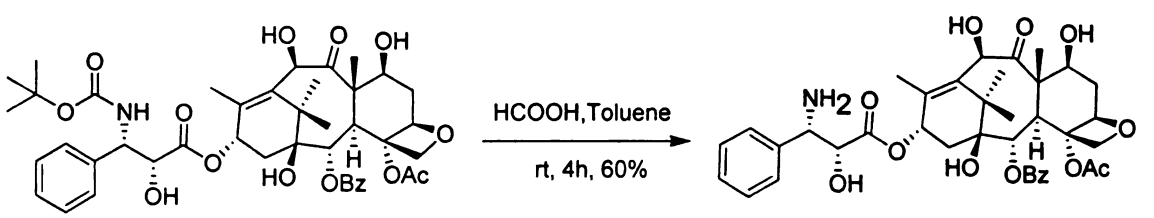


Scheme 7: Synthesis of *N*-debenzoylpaclitaxel

The resulting product (**11**) was deprotected with HF/pyridine to give 10-acetylated docetaxel (**12**). The 10-acetyl-docetaxel was further deprotected by formic acid treatment to give *N*-debenzoylpaclitaxel (**13**) (Scheme 7), which was to be used as a substrate for NDTBT enzyme.[60]

1.3.4 Synthesis of 10-Deacetyl-*N*-debenzoylpaclitaxel

Various analogues of paclitaxel with the 3'*N*-benzoyl of paclitaxel replaced with other acyl groups[61] and the acyl group at C10 replaced with various alkyl group are largely provided by synthetic means.[62] These synthetic methods require several protecting group manipulation steps that ultimately affect the optimal yield of the final target compound. Alternatively the use of chemo-and regioselective acyltransferases could reduce the number of protecting group steps. 10-Deacetyl-*N*-debenzoylpaclitaxel (**14**) is a model substrate to test whether NDTBT can bioscatalytically *N*-acylate. paclitaxels either acylated or deacylated at C-10. To obtain **14**, docetaxel (**7**) was deprotected with formic acid as shown below (Scheme 8).



7 **14**

Scheme 8: Synthesis of 10-deacetyl-*N*-debenzoyl paclitaxel

1.3.5 Protein Harvest and Activity Assay of the NDTBT

With the taxanes substrates in-hand, the NDTBT enzyme needed to be overexpressed in *E. coli*, isolated, and tested for activity. Recombinant *ndtbt* cDNA was expressed and harvested following standard procedures.[48] Briefly, the *ndtbt* cDNA was subcloned into expression vector pET28a, and the resulting plasmid transformed into *E.coli* cells. The bacteria was grown to log phase, then induced with 0.1 mM IPTG at 16°C for 16 h. The cells were harvested by centrifugation and the cell pellet was resuspended in lysis buffer. The soluble protein fraction was purified and the resulting enzyme was assayed by the following procedure.

The presumed natural substrate *N*-debenzoyl-2'-deoxytaxol and other *N*-debenzoylpaclitaxel substrates (10-deacetyl-*N*-debenzoylpaclitaxel and *N*-debenzoyl-paclitaxel) were incubated with NDTBT in the presence of various CoA donors including benzoyl; *ortho*-, *meta*-, and *para*-substituted benzoyl; various heteroyls, alkanoyls; and butenoyl CoAs. The choice of aroyl, alkanoyl and heteroyl CoA donors was based on the biological activity of various non-natural aroyl, and alkanoyl *N*-acylated taxanes made synthetically by replacing the benzoyl group with these unnatural groups.[22, 63]

To assess whether a *de novo* product was formed, the assays were extracted, and the product mixture was analyzed by either UV-HPLC in mixed substrate assays to calculate the relative specificity constants, or the mixture was separated by HPLC with the effluent directed toward a tandem mass spectrometer (MS/MS) for selected molecular ion fragmentation analysis. The UV and mass spectrometric data revealed that several

different types of *N*-acylated paclitaxel analogues were biosynthesized by NDTBT catalysis.

1.3.6 Relative Substrate Specificity of NDTBT with Various Aroyl CoAs.

The relative substrate specificity of NDTBT for various aroyl CoA and *N*-debenzoyl-2'-deoxypaclitaxel was calculated by competitive assay method where benzoyl CoA was incubated in pair with each aroyl CoA used herein. 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 relative specificity constants were calculated based on that of NDTBT for benzoyl CoA (V_{max}/K_M = 1.6 nmol•min^{-1}•mM^{-1}) (Table 1; Entry 1A).

The relative velocity, instead of the specificity constants, of NDTBT with *N*-debenzoylpaclitaxel and 10-deacetyl-*N*-debenzoylpaclitaxel substrates was calculated in order to conserve the dearth amount of these substrates. Thus, accordingly, each was incubated separately at a single concentration done (1 mM) with each of the 16 aroyl CoAs (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. In these assays, the kinetic parameters of NDTBT were unknown, regarding whether the non-natural aroyl CoAs 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 and 2, entries B and C), the rates were estimated to be at steady state and first-order.

The non-aromatic CoA thioesters (acetyl CoA, butyryl CoA, butenoyl CoA, and hexanoyl CoA) were assayed with the three *N*-debenzoyl taxoids. The relative velocity was determined by using LC/MS/MS to increase detection and the velocity was compared with the benzoyl CoA.

As shown in table (1 and 2), NDTBT displayed a range of catalytic efficiencies and relative velocities with various CoAs with each of the *N*-debenzoylpaclitaxel substrates.

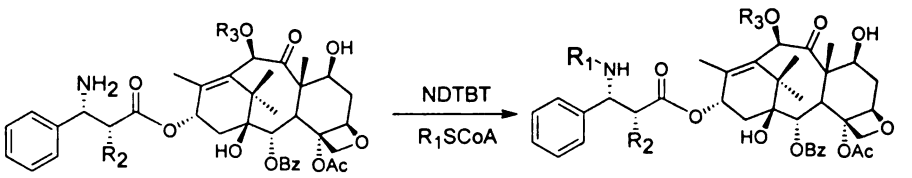


Table 1: Relative kinetics of the benzoyl, substituted aroyl and heteroaroyl groups

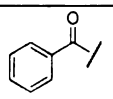
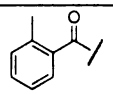
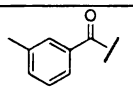
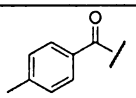
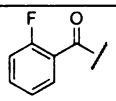
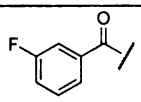
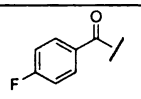
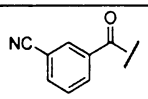
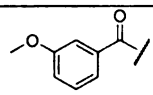
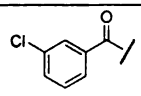
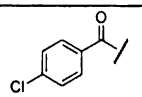
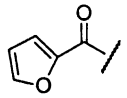
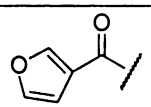
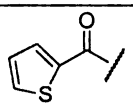
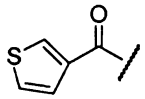
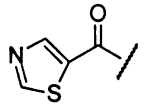
R_1 Derived from CoA		**A** (V_{max}/K_M) (nmol·min^{-1}·mM^{-1}) R_2 = H, R_3 = Ac	**B** V_{rel} R_2 = OH, R_3 = Ac	**C** V_{rel} R_2 = OH, R_3 = H
	1	1.6	100	100
	2	Not detectable	Not detectable	13%
	3	0.27	27%	33%
	4	1.7	33%	Not detectable
	5	0.85	8%	38%
	6	0.34	11%	19%
	7	0.97	<11%	68%
	8	0.15	< 9%	17%
	9	0.38	85%	11%
	10	0.37	< 1%	8%

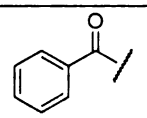
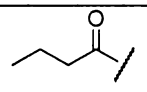
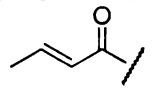
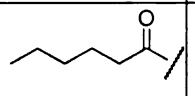
Table 1: Continued Relative kinetics of NDTBT for heteroaroyl CoA groups

R_1 Derived from CoA		A (V_{max}/K_M) R_2= H, R_3 = Ac	B V_{rel} R_2 = OH, R_3 = Ac	C V_{rel} R_2 = OH, R_3 = H
	11	1.1	< 1%	40%
	12	0.31	200%	36%
	13	0.15	19%	43%
	14	0.39	19%	5%
	15	0.37	22%	3%
	16	0.16	22%	29%

V_{max}/K_M values are listed as nmol·min^{-1}·mM^{-1}

A = *N*-debenzoyl-2'-deoxypaclitaxel, B = *N*-debenzoylpaclitaxel, C= 10-deacetyl-*N*-debenzoylpaclitaxel

Table 2: Relative kinetics of NDTBT for alkanoyls CoA with taxane substrates

R_1		V_{rel} R_2 = H, R_3 = Ac	V_{rel} R_2 = OH, R_3=Ac	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%

1.4 Discussion

Broad substrate specificity of enzymes on secondary metabolic pathways has been suggested as important for the evolution of metabolite diversity.[64] 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, and 10-deacetyl-*N*-debenzoylpaclitaxel.

1.4.1 Substrate Specificity

Previous work with the intent to dissect the paclitaxel biosynthetic pathway has shown that only benzoyl CoA was transferred when NDTBT was incubated with a single taxane substrate, *N*-debenzoyl-2'-deoxypaclitaxel, while acetyl CoA and phenylacetyl CoA were not productive.[48] In a more recent biosynthetic investigation,[65]the assembly of the isoserinoyl side chain of paclitaxel was investigated. The result showed that benzoyl CoA was transferred superior on to *N*-debenzoylpaclitaxel compared to *N*-debenzoyl-2'-deoxypaclitaxel supporting an earlier claim that 2'-hydroxylation precedes *N*-benzoylation.[66] 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.[65]

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- and 3-substituted benzoyls, alkanoyl, and alkenoyl (cf. Tables 1) to either of the *N*-debenzoylated taxanes used as a co-substrate.

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 biosynthetic study.[65] 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.

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.

1.4.2. Kinetics Analyses

The catalytic efficiency V_{max}/K_M for benzoyl CoA was calculated with respect to the *N*-debenzoyl-2'-deoxytaxol whereas 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 the same assay conditions.

p-Methylbenzoyl CoA is best transferred by the NDTBT (1.7 $nmol \cdot min^{-1} \cdot mM^{-1}$) compared to the natural substrate when using the presumed taxane substrate (*N*-debenzoyl-2'-deoxypaclitaxel) whereas the 2-methylbenzoyl CoA was not productive. In the case of halogen-substituted benzoyl CoA, p-chlorobenzoyl CoA with catalytic specificity of (1.1 $nmol \cdot min^{-1} \cdot mM^{-1}$, entry 11A) is the best among halogen substituted benzoyl CoA.

The relative velocity (V_{rel}) for various aroyl and non-aroyl CoAs were calculated by setting the rate for benzoyl CoA at 100% relative to the rates of NDTBT with various aroyl CoAs and the two taxane substrates, *N*-debenzoylpaclitaxel and 10-deacetyl *N*-debenzoylpaclitaxel (Table 1, Columns B and C). The data shows that the velocities are distinctly variable with respect to the *N*-debenzoylpaclitaxel substrates.

For the halogen substituted such as 2- and 3-chloro benzoyl CoA had relative velocities of less than 1%. (See Table 1, Entries 10 B and 11 B), while the rest of acyl CoA substrates had relative velocities between 19 to 85% of the velocity for benzoyl CoA.

The catalytic efficiencies for heteroaroyl CoAs (Table 1, entries 12A, 14A, 15A) is only around one fourth of that of benzoyl CoA (Table 1, entry 1A). Intriguingly the relative velocity for 2-furoyl CoA was shown to be surprisingly doubled (200%) compared to benzoyl CoA whereas, the rate for 3-furoyl was only 19% when these CoAs were incubated with *N*-debenzoylpaclitaxel.

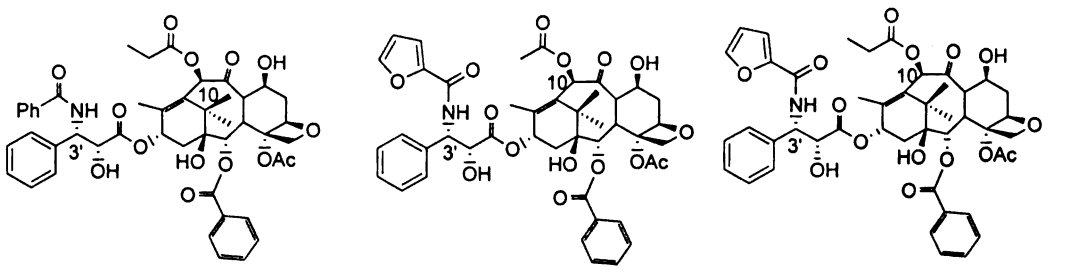
For the alkanoyl CoAs (Table1, Entries 18, 19, 20, 21) each incubated separately with the three taxanes and NDTBT; the relative velocities were compared with of benzoyl CoA. In this study, acetyl CoA was converted with greater rate than other alkanoyl CoA with *N*-debenzoyl-2'-deoxpaclitaxel (column A, Table 2) as the substrate. In contrast, butenoyl CoA showed the maximum relative velocity (V_{rel} =100%) with NDTBT and *N*-debenzoylpaclitaxel (column B, Table 2) while the natural substrate benzoyl CoA was lower (V_{rel} = 13%). For 10-deacetyl-*N*-debenzoyltaxol (column C, Table 2), hexanoyl CoA showed the maximum conversion set at V_{rel} = 100% while benzoyl CoA was shown to have a V_{rel} = 12 %.

1.4.3. Biocatalytic Applications

These results demonstrate that NDTBT can catalyze the conversion of several *N*-debenzoylpaclitaxel analogues to various acylated derivatives of paclitaxel regardless of the hydroxyl group at the 2'-position and acetyl group at the C10-hydroxyl. Most interestingly, some of the biocatalytically derived compounds are the next generation paclitaxel compounds that have been derived by synthetic means, such as 3'-*N*-furanoyl-*N*-debenzoyl taxane derivative.

1.4.3.1 Paclitaxels Modified at 3'N and C10

Figure 5 shows promising paclitaxel analogues (**16**, **17**, and **18**) that have greater cytotoxicity against cancer cells and microtubule assembly compared to paclitaxel. It has been reported that aliphatic and hetero-aromatic *N*-acyl analogues (**18**) with a propanoyl for acetyl replacement at C10 was more cytotoxic and have better microtubule assembly that paclitaxel.[52, 67] Notably, when the *N*-benzoyl was replaced with *N*-(2-furanoyl), the efficacy of the analogue was decreased relative to that of paclitaxel; however, when the *N*-(2-furanoyl) replacement was coupled with a propanoyl for acetyl replacement at C10, the cytotoxicity of the analog for human ovarian cancer increased 20-fold while the microtubule assembly action increased slightly (Figure 6).[22]



17

A = <0.001 [0.02]
B = 0.71 [0.44]

16

A = 14.6 [0.02]
B = 2.0 [0.44]

18

A =0.004[0.02]
B =0.49 [0.44]

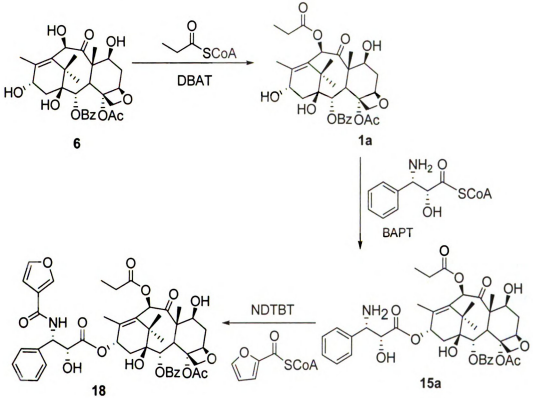
Figure 6: Promising paclitaxel analogues derived from a combinatorial chemistry library.

A is the IC_{50} (μg/mL) cytotoxicity for human ovarian cancer and B is the I_{50} (μM) for microtubule assembly. Paclitaxel values in brackets.

1.4.3.2 Semisynthetic Approaches to Modified Taxanes

The semisynthesis of arylamide analogs of paclitaxel includes selective hydroxyl group protection at C7, selective acylation at the C13 hydroxyl with either an *N*, *O*-acetal-*N*-aroylphenylisoserine, activated as a mixed anhydride,[68] or an *N*-aroyllactam precursor of *N*-aroylphenylisoserine,[21] and finally deprotection to construct the target product.[63] A typical example is the synthesis of the unnatural taxane containing a 3′-*N*-(*p*-flurobenzoyl)-3'-debenzoylpaclitaxel which would require a number of steps to install in place of the naturally occurring 3′-benzamide in the molecule.[69] Currently, analog (**18**) is made by Holton-Ojima β-lactam synthon method using modified baccatin III analogue and needs more than 13 steps for semisynthesis of **18.**[59, 70] The application of biocatalytic

acylation in the described semi-synthetic methods could potentially reduce the number of protection steps in the assembly of these second generation compounds.[71] Conceivably, *Taxus* acyltransferases NDTBT and the moderately promiscuous DBAT (10β-*O*-acetyltransferase) can facilitate the assembly of analogues such as **18.** As noted, NDTBT can transfer 2-furanoyl to the 3'-amino group of the paclitaxel side chain, while DBAT catalyze the transfer of propionyl and butyryl from the corresponding acyl CoA to 10-deacetylbaccatin III to form unnatural analogues of baccatin III.[72] Therefore, analog **18** is a reachable target via biocatalytic production as proposed in Scheme 9.



Scheme 9: Use of acyltransferase enzymes to biocatalytically synthesizes potent analogues of paclitaxel.

DBAT=10-deacetylbaccatinIII:10-*O*-acetyltransferase, NDTBT=*N*-debenzoylpaclitaxel-*N*-benzoyltransferase BAPT=Baccatin III:3-amino-3-phenylpropanoyltransferase

1.5 Conclusion

The data described herein shows that the wild type *taxus* *N*-benzoyltransferase(NDTBT) functions as a general acyltransferase. 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.

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'-hydroxyl 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 alkanoyl CoA binding remains a curiosity. Conceivably, when structural data becomes 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 semi-biosynthetic methods that interface synthetic chemistry and molecular biology.

CHAPTER 2

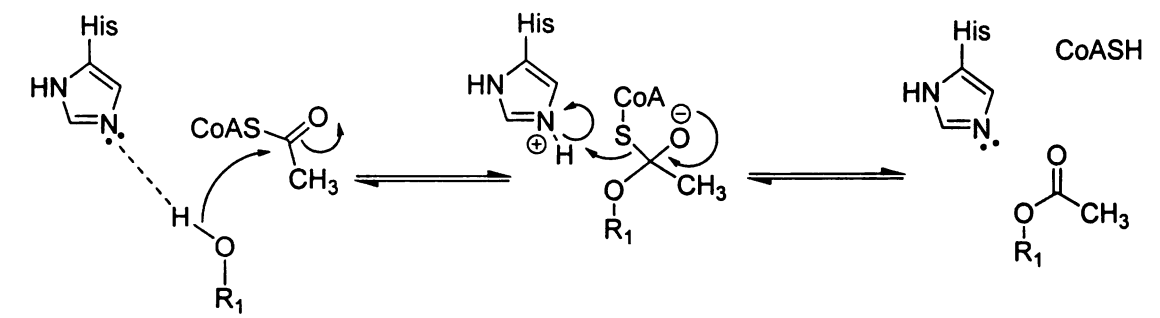
THE STUDY OF A PROPOSED SUBSTRATE-ASSISTED-CATALYSIS MECHANISM FOR THE PHENYLPROPANOYLTRANSFERASE (BAPT)

2.1 Introduction

The acyltransferases involved in paclitaxel biosynthesis all belong to a superfamily designated BAHD. Enzymes in this family have played a principal role in the evolution of secondary metabolism in several plant species.[73, 74] The five acyltransferases involved in paclitaxel biosynthesis are taxadien-5-ol-*O*-acetyltransfease (TAT), taxane-2-*O*-benzoyltransferase (TBT), 10-deacetylbaccatinIII:10-*O*-acetyltransferase (DBAT), *N*-debenzoylpaclitaxel-*N*-benzoyltransferase (NDTBT), and baccatin III:3-amino-3-phenylpropanoyltransferase (BAPT).[49] The latter enzyme transfers a β-phenylalanoyl group to a hydroxytaxane acceptor, and the derived product is an intermediate on paclitaxel biosynthetic of paclitaxel and its analogues.[55] Dissecting the mechanism of the acyl group transfer catalyzed by BAPT will be central topic of this chapter.

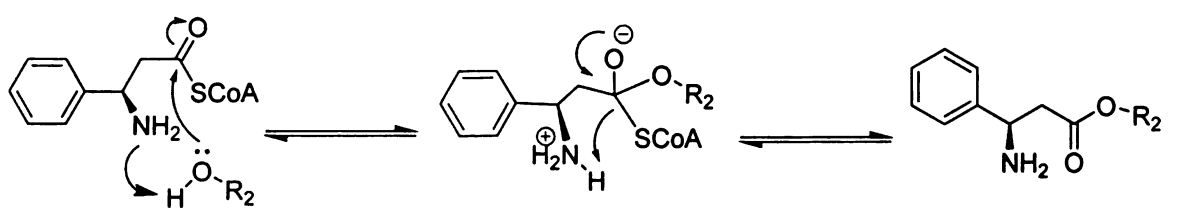
The amino acid sequences of plant-derived acyltransferases in the BAHD family, including the acyltransferases on the paclitaxel biosynthetic pathway, reveals an HXXXD motif that is highly conserved in this family of catalysts and is presumed to reside in the active site.[49] The amino acid identity among the *taxus* acyltransferase is approximately 56% (with functionally equivalent residues at about 73% similarity). However, the BAPT sequence is unique in that the proposed catalytic motif contains a natural G for H mutation (i.e., GXXXD). Previously, a study was carried out with the BAHD

acetyltransferase, vinorine synthase, wherein a site-directed mutation changed the catalytic His_{160} to a catalytically inert alanine. The operationally expressed mutant was rendered functionally inactive.[76] This suggested that His_{160} was indispensable for acetyltransferase activity.[77, 78] His_{160} is proposed to serve solely as a general base in the mechanism of the vinorine synthase catalysis (Scheme 10), whereas the Asp_{164} in the HXXXD motif is considered to serve a role in maintaining enzyme structure but not necessary for enzyme function.



R_1OH = 17-deacetylvinorine

Scheme 10: Proposed catalytic mechanism of vinorine synthase involves His^{160} as a general base.



R_2OH = baccatin III

Scheme 11: Proposed substrate-assisted mechanism for BAPT catalysis.

The mutagenesis study with vinorine synthase revealed the importance of the His_{160} in the HXXXD motif in vinorine synthase catalysis.[77,78] Imaginably, this conserved histidine

residue can be considered essential among members of the BAHD family. However, the *Taxus* derived BAPT is functional despite having a H→G mutation in this motif. It is therefore proposed that the mechanism of BAPT utilizes the free amine of the β-amino-3-phenylpropanoyl CoA thioester.[55] To test this hypothesis, several different phenylpropanoid CoA thioesters bearing various replacement groups at the C3 of the phenylpropanoid in place of the amine were synthesized.[55, 79] The CoA thioesters of phenylpropionate, 3-keto-phenylpropionate, 3-hydroxy-3-phenylpropionate, and β-phenylalanyl were separately incubated with radiolabeled baccatin III.

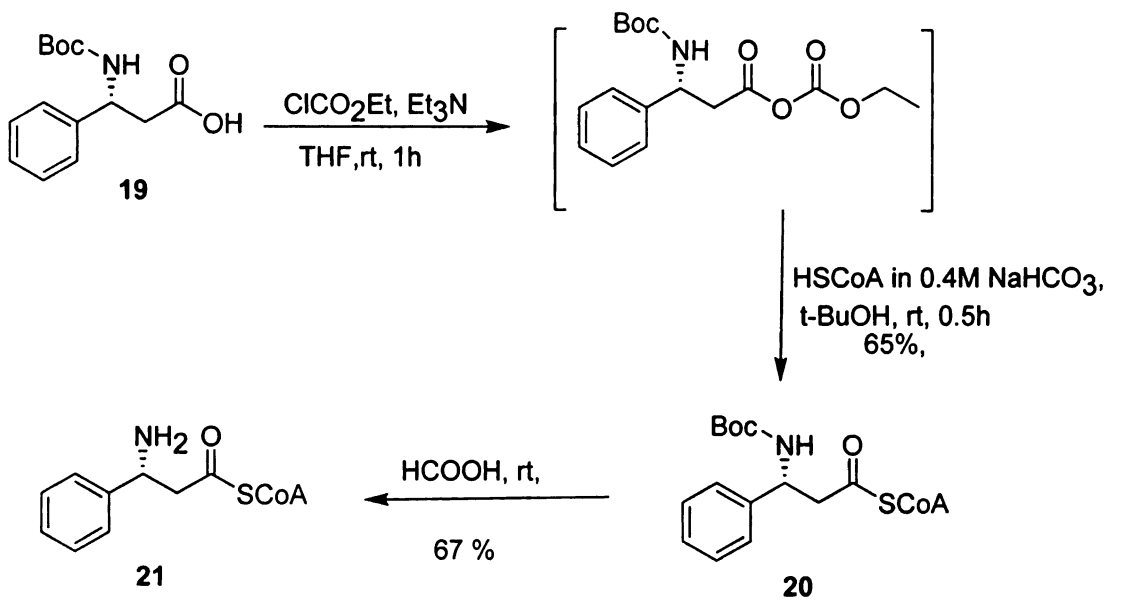
2.2 Results

2.2.1 The synthesis of R-β-phenylalanoyl CoA thioester

The conventional method of synthesizing CoA thioesters typically proceeds *via* a mixed anhydride, but the presence of functional groups (hydroxyl, amine, or β-keto) required protecting group manipulations to manage the reactivity of the competing functional group. Consequently, different approaches were accessed to synthesize the proposed CoA thioesters as follows.

To synthesize (*R*)-β-phenylalanyl CoA, commercially available *N*-Boc-3-amino-phenylpropanate acid was mixed with ethyl chloroformate to form the mixed anhydride, and the mixed anhydride which was subsequently coupled to CoASH under basic conditions. After quenching the reaction with HCl, the product was isolated at 65 % which was significantly lower than the typical>90% product yield obtained when

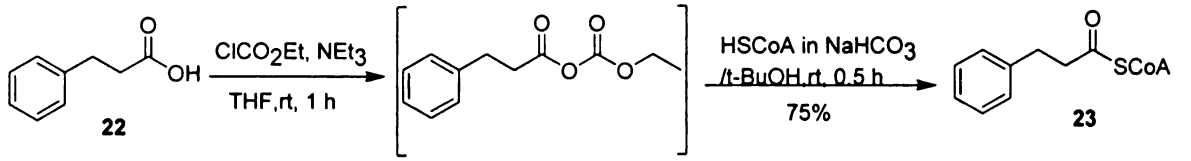
coupling aroyl or aliphatic carbonyl anhydrides with the CoASH under similar conditions.[60] The Boc deprotection step with 88% formic acid was slow, and after 17 h, 65% of the starting material was converted to product (21) (Scheme 12).



Scheme 12: synthesis of 3*R*-β-phenylalanoyl CoA thioester

2.2.2 The Synthesis of 3-Phenylpropanoid CoA

The 3-phenylpropanoid CoA was prepared to test whether the absence of the amine functional group at C3 (cf. Scheme 13) of the substrate would affect the substrate selectivity of BAPT, and provide preliminary evidence supporting a substrate-assisted catalysis (SAC)-based mechanism. The mixed anhydride procedure described previously[55] was used to make (**22**). The yield was 75%, which was comparable to other phenylpropanoids containing functional group at C3.



Scheme 13: The synthesis of phenylpropanoyl CoA

2.2.3 The synthesis of 3*R*-hydroxy-3-phenylpropanoyl CoA

In the process of synthesizing 3*R*-hydroxy-3-phenylpropanoyl CoA using the mixed anhydride method, as explained previously, no product was formed, but LC-MS analysis showed the formation of polymerized anhydride products that were consistent with the putative structures.

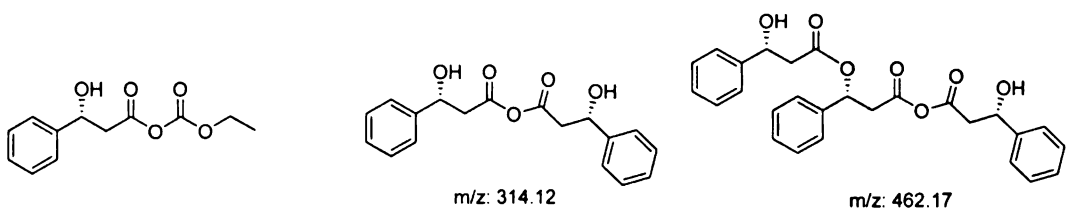
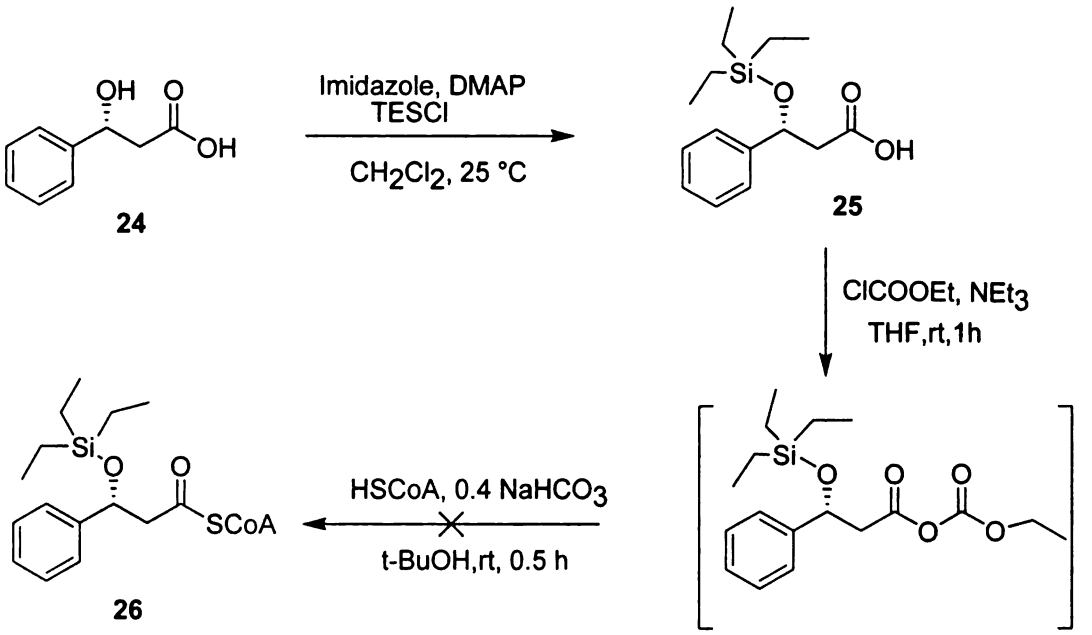


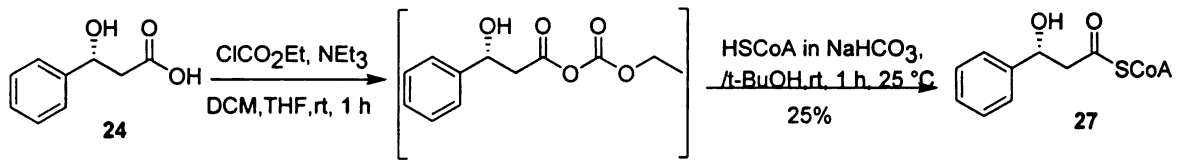
Figure 7: Products detected in an attempt to synthesize 3-hydroxy-3-phenylpropanoyl CoAs.

To eliminate the formation of polymerization product, the C3-hydroxyl group was protected with triethylsilyl (TES), and the thioesterification with CoASH was attempted by methods previously described (Scheme 14). Still, no thioester product was detected by LC-MS analysis of the reaction mixture.



Scheme 14: Attempted Synthesis of 3-hydroxy-3-phenylpropanoyl CoA using *O*-triethylsilyl protection chemistry

The presence of the TES group, because of its steric effect may prevent the coupling of the CoA to the phenylpropanoate. Therefore, we re-explored the direct conversion of the 3-hydroxyphenylpropanoid to its thioester without a protecting group. The solvent system was changed from 100% THF to the combination THF:DCM (5:2 v/v) and the reactions were diluted 3-fold compared to previous attempt described earlier; the latter dilution was made to reduce the effect of polymerization. In this instance, the reaction mixture contained predominantly the desired mixed anhydride (Scheme 15), as assessed by mass spectrometry and ^{1}H-NMR analysis. The thioesterification of the mixed anhydride of **24** resulted in product **27** at 25% yield (Scheme 15). The yield was judged suitable to commence with our preliminary enzyme assays.

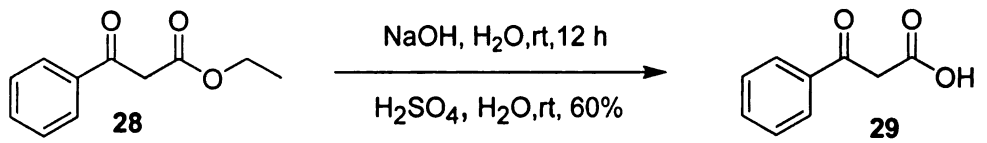


Scheme 15: Synthesis of 3*R*-hydroxyphenylpropanoyl CoA

2.2.4 Synthesis of 3-Keto-3-phenylpropanoyl CoASH thioester

2.2.4.1 Synthesis via a Mixed Anhydride Approach

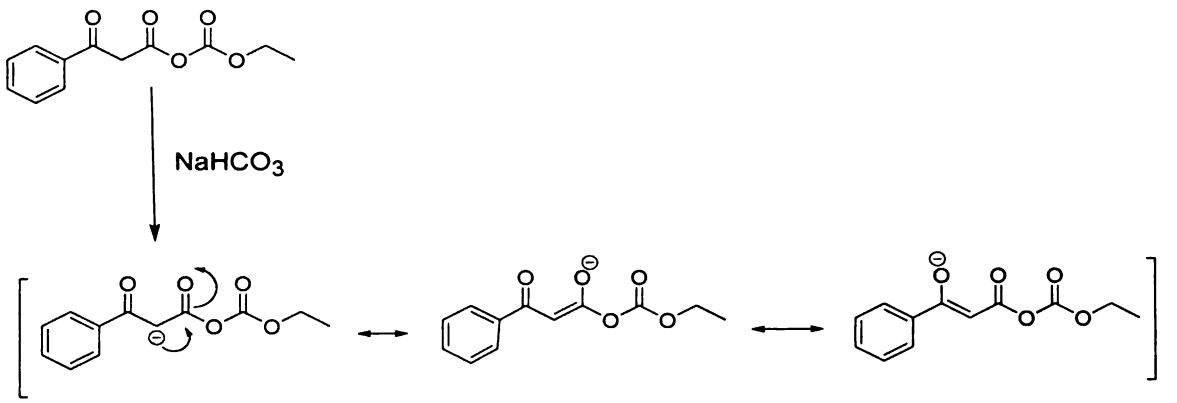
The synthesis of 3-keto-3-phenylpropionyl CoA was started with the synthesis of 3-oxo-3-phenylpropionic acid by hydrolyzing the ethyl ester of 3-oxo-3-phenyl propionate to obtain 3-oxo-3-phenylpropionic acid[80](Scheme 16).



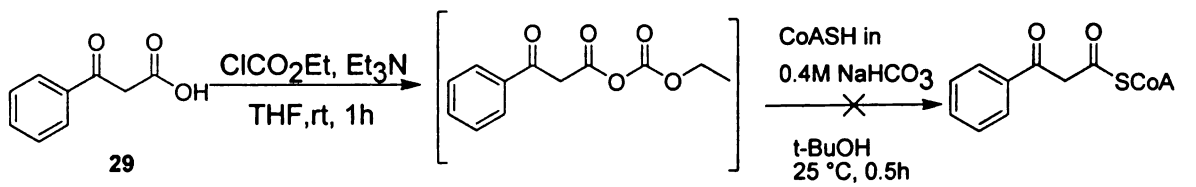
Scheme 16: Synthesis of 3-oxo-3-phenylpropanoic acid

The preparation of the mixed anhydride in the case of β-keto acids was designed to eliminate or minimize the condensation reaction that could partially occur as a result of the acidic α-proton of the starting material.[81] The β-keto acid (29) was dissolved in THF and added dropwise to a mixture of ethyl chloroformate and 1 equivalent of triethylamine. The resulting keto-enol mixture of the anhydride was verified by ^{1}H NMR.

Unfortunately, displacement of the ethyl carbonate by CoASH under basic conditions did not yield the desired acyl CoA. One of the reasons for the failure of the reaction was probably due to the facile formation of the keto-enol in the presence of base or acid. The resonance effect of the tautomer likely stabilizes the α-carbon (Scheme 17) and thus reduces the strength of the nucleophilic attack by CoASH (Scheme 18).



Scheme 17: The effect of base on tautomerization of (ethyl carbonic)-3-oxo-phenyl propanoic anhydride

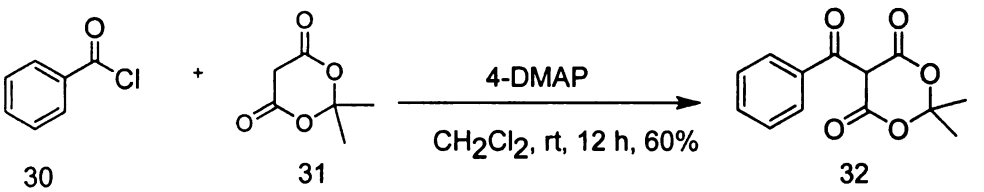


Scheme 18: An attempt to synthesize 3-Oxo-3-phenylpropanoyl CoA

2.2.4.2 Synthesis through Meldrum's acid

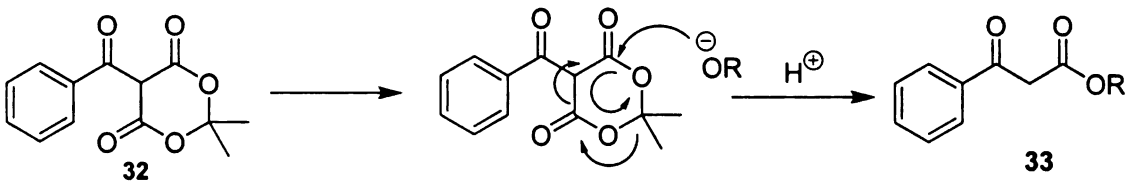
The failure to synthesize β-keto thioesters using the proven mixed anhydride method promoted an alternative method. In this next approach, Meldrum's acid was used to activate the carbonyl group of the carboxylate for nucleophilic attack by CoASH. Meldrum's acid derivatives have been used successfully in previous investigations for the synthesis of β-ketoamides and β-thioesters.[82] As an example the synthesis of β-keto-β-phenylpropanoamides, proceeded from benzoyl Meldrum's acids wherein the derivative was refluxed with an alcohol, amine, or thiol nucleophile to give the corresponding ester.[82] Herein, typical reaction conditions for coupling an acyl Meldrum's acid with a nucleophile were modified as follows.

The benzoyl Meldrum's acid was prepared according to a literature procedure [82](Scheme 19), and the resulting derivative was dissolved in THF, and the CoASH was added slowly as a solution in $NaHCO_3$. The reaction was heated at 40 °C overnight instead reflux temperature (66 °C for THF) to minimize possible decomposition of the CoA reactant and/or the resultant CoA thioester The progress of the reaction was checked by ESI-MS (negative mode) but no product formation was observed.



30 31 32

Scheme 19: Synthesis of benzoyl Meldrum's acid



Scheme 20: Method of synthesis of β-keto esters using benzoyl Meldrum's acid

After promoting no reaction at 40 °C, the stability of CoASH and benzoyl Meldrum's acid was empirically evaluated in refluxing THF. After 3h the benzoyl Meldrum's acid was opened up (MW = 145) but not totally degraded as determined by ESI-MS while the CoASH remained intact (Figure 8). But no thioesterification product, β-keto thioester, was detected. It was presumed that the thioester might be unstable at that high temperature or not formed at all.

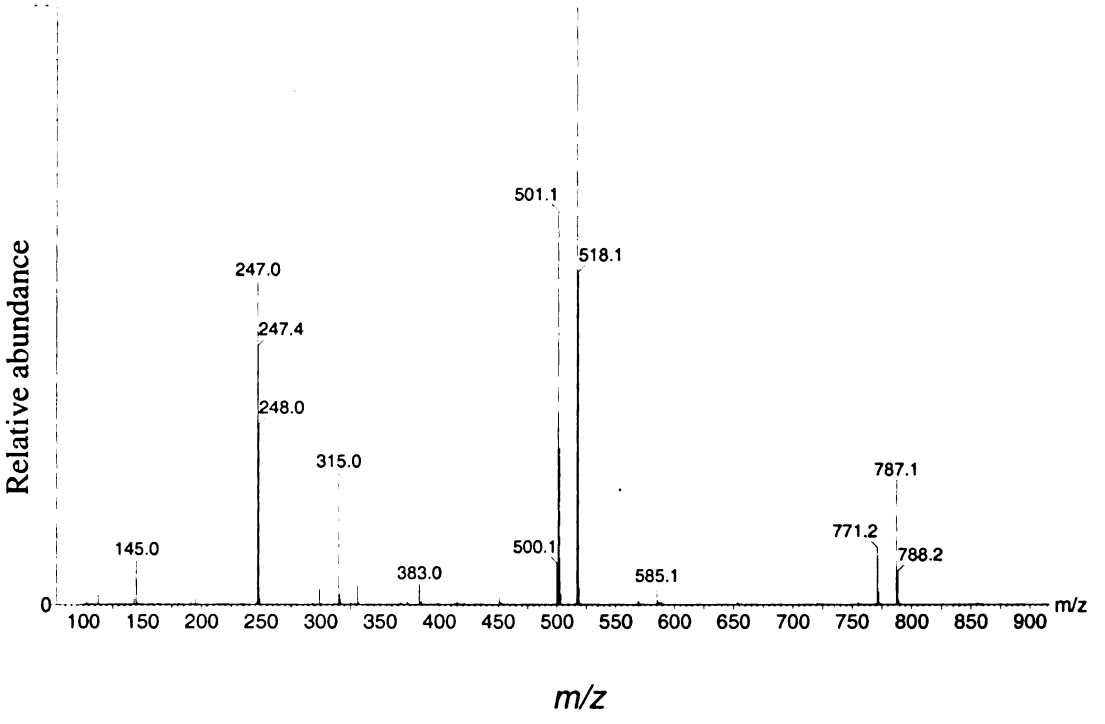


Figure 8: ESI-MS of the reaction product for the synthesis of β-keto thioesters using Meldrum's acid

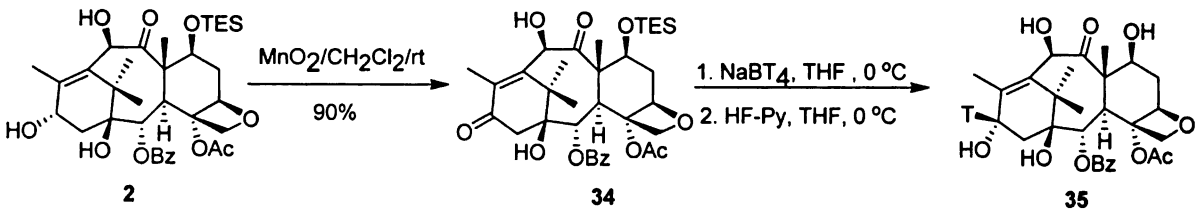
Shown unreacted CoA salt = $[M + Na - H]^{-1}$= 787.1, benzoyl Meldrum's acid, $[M-H]^{-1}$ = 247.4, $[2M + Na - H]^{-1}$ = 517

To avoid long reflux time and thus minimize decomposition, a microwave synthesizer was used to keep the temperature at 40 °C for five minute while keeping all other reagents/reactants the same as in previous attempts. The reaction mixture was analyzed by ESI-MS (negative mode) and benzoyl Meldrum's acid, 3-oxo-3-phenylpropanoic acid β-ketoacid, and a significant amount of CoASH were detected. Increasing the reaction time for an additional 5 min showed an increase in the hydrolysis of the benzoyl Meldrum's acid and the decomposition of CoASH. The challenge to provide sufficient energy for the coupling reaction employing the benzoyl Meldrum's acid and CoASH

resulted in overshooting a fine balance to overcome the energy burner for nucleophilic attack of the CoASH on the acid and to prevent CoASH from decomposing. The obligatory high temperature generally needed to activate by ring opening Meldrum's acids [82]made the synthesis of the β-keto thioester challenging.

2.2.5 Synthesis of Radioactive Baccatin III

To increase the sensitivity of the detection of biosynthetic products derived from baccatin III, a tritium labeled baccatin III substrate was synthesized from 7-TES-baccatin III (**2**) according to a described procedure. Briefly, **2** was oxidized followed by reduction with tritium labeled sodium borohydride and deprotection (Scheme 21) provided the [13-^{3}H]baccatin III which had the same R_f (retention factor) as authentic baccatin III on silica gel thin layer chromatography and the same retention time as authentic baccatin III on radio-HPLC.



Scheme 21: **Synthesis of [13-^{3}H]baccatin III**

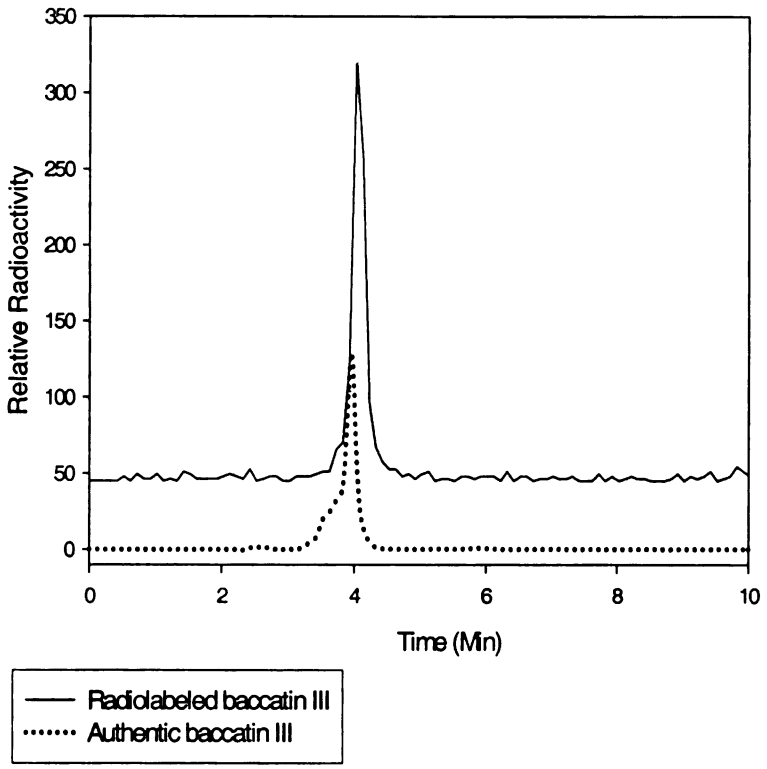


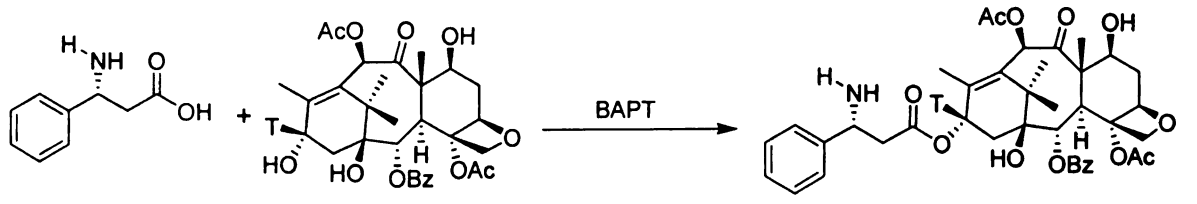
Figure 9: Confirmation of [13-^{3}H] baccatin III with HPLC

2.3 Enzyme expression and Activity assay of BAPT

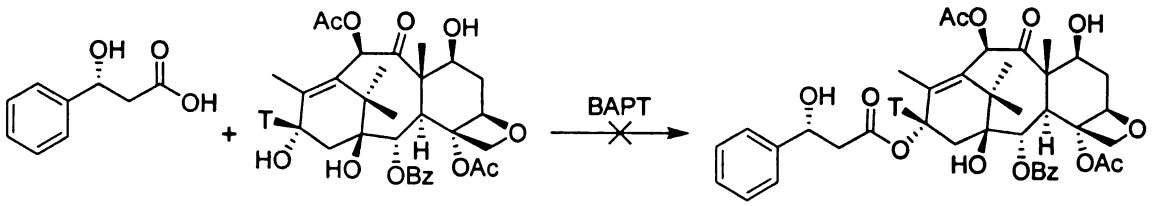
Standard microbial and recombinant techniques used throughout this work are described by Sambrook.[83] The full length *bapt* cDNA (accession AY082804) was subcloned from

its original expression vector pSBET[55] 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). The resultant plasmid containing the *bapt* cDNA in pET28a was transferred into an expression vector; *E. coli* BL21-CodonPlus® (DE3), the bacteria was grown, the recombinant protein was over expressed by S-isopropyl β-D-1-thiogalactopyranoside, the protein was harvested and purified.

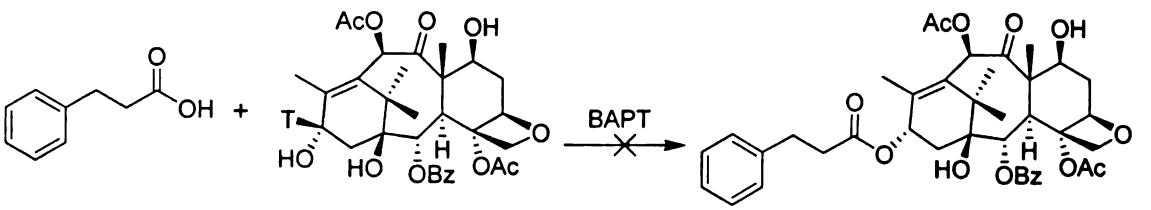
The operationally soluble BAPT was verified as functional by incubating the enzyme with *R*-β-phenylalanine CoA and [13-^{3}H]baccatin III. After 2 h, the assay mixture was extracted with ethyl acetate, the solvent was evaporated, and the remaining residue was analyzed by radio HPLC and ESI-MS. UV-absorbance profiles of the biosynthetic product isolated from the assay containing crude enzyme extracts from cells transformed with empty vector showed no *de novo* radioactive peak corresponding to the biosynthetic product.



Scheme 22: Assay of the natural substrate phenylalanoyl CoA and baccatin III with BAPT



Scheme 23 : Assay of unnatural 3-hydroxy-3-phenylpropanoid CoA



Scheme 24: Assay of unnatural 3-phenylpropanoyl CoA

The products were purified by HPLC, and the corresponding *de novo* UV-absorbance peak were collected and further analyzed by a direct injection-electrospray ionization mass spectrometry, in positive ion mode. Further diagnostics by LC-ESI-MS/MS of selected ion *m/z* 734$[M+H]^+$ revealed (Figure 10) typical diagnostic fragment ions of *m/z* 509, 569, and the β-phenylalanyl side chain fragment ion (*m/z* 166, ordinarily the base peak) that confirmed the identity of the product as *N*-debenzoyl-2'-deoxypaclitaxel when compare to authentic sample analyzed by similar methods (figure 13). The only productive acyl CoA substrate was β-phenylalanyl CoA, and UV-absorbance profiles of the biosynthetic product isolated from the assay containing crude enzyme extracts from cells transformed with empty vector showed no *de novo* radioactive peak corresponding to the biosynthetic product.

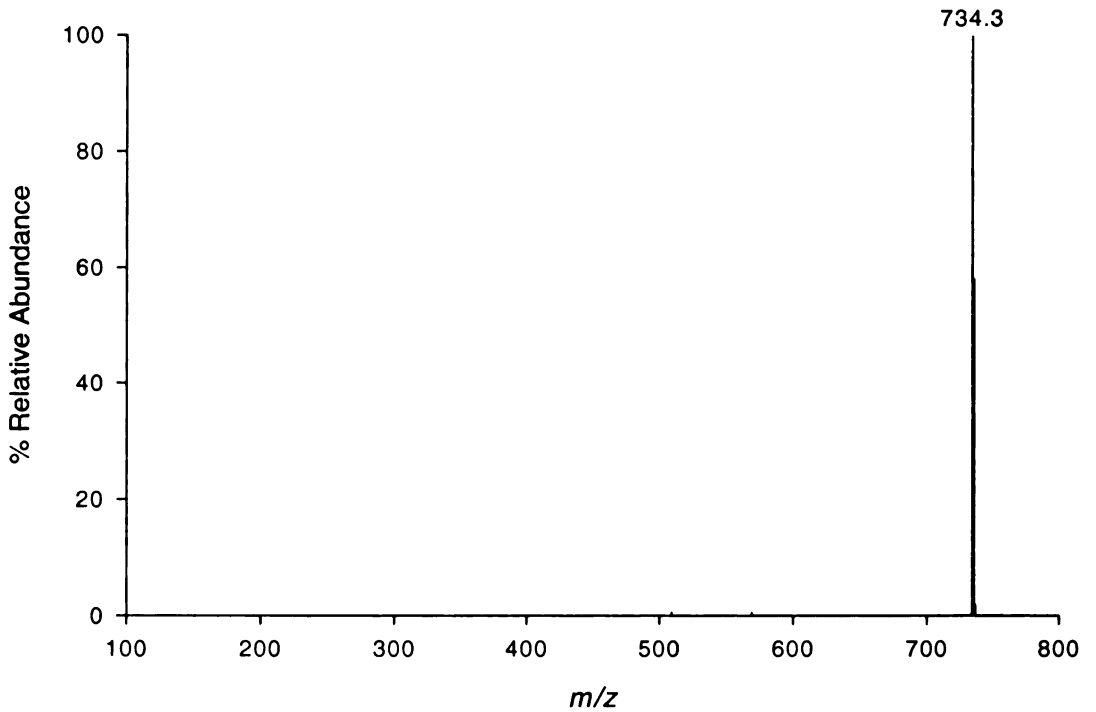


Figure 10: mass spectrum of product formation

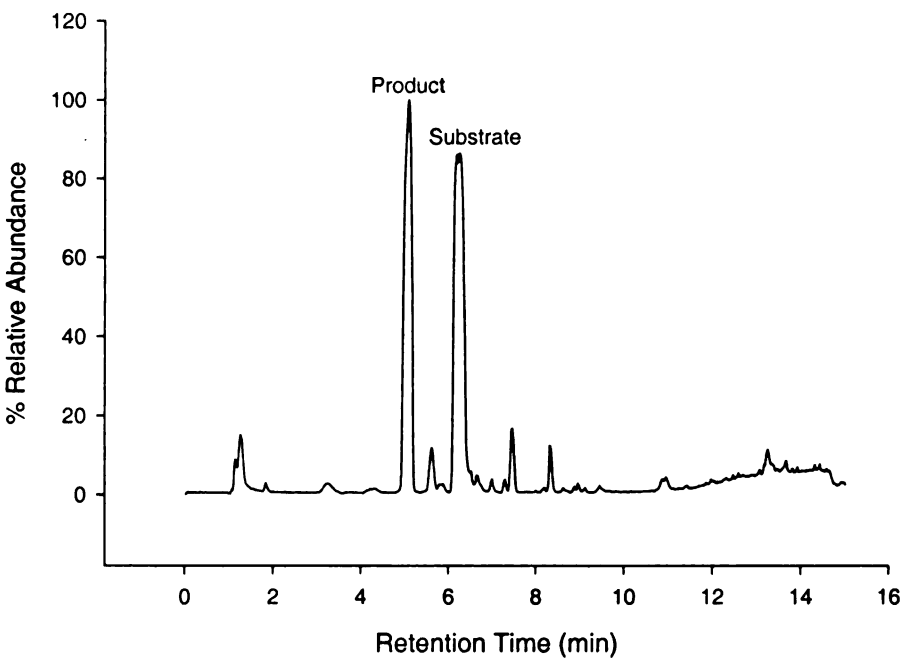


Figure 11: Liquid chromatography of the extracted product

The results show the enzyme is very productive based on the approximately 50% conversion of substrate to product, after 1h incubation with BAPT (100 μg). Several β-amino acid CoA thioesters will be synthesized to assess the scope of the substrate specificity of BAPT.

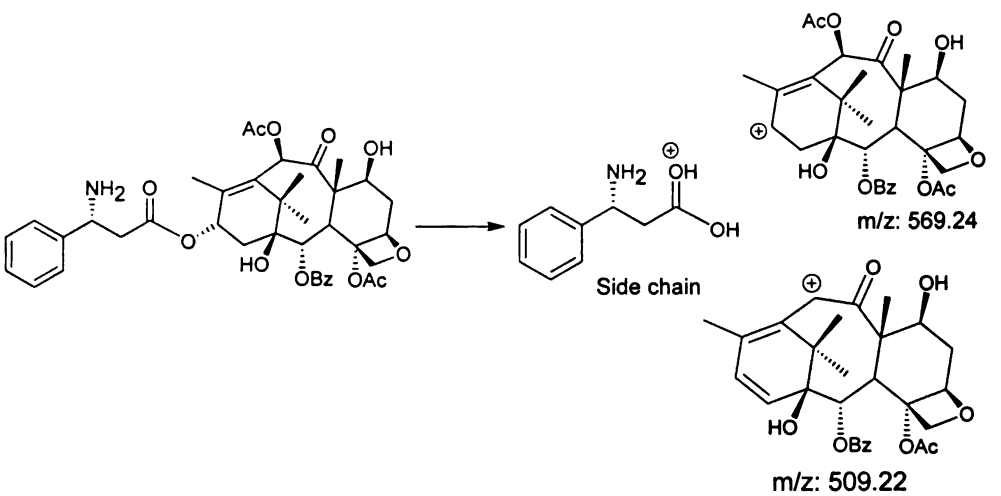


Figure 12: Fragmentation pattern of *N*-debenzoyl-2'-deoxypaclitaxel

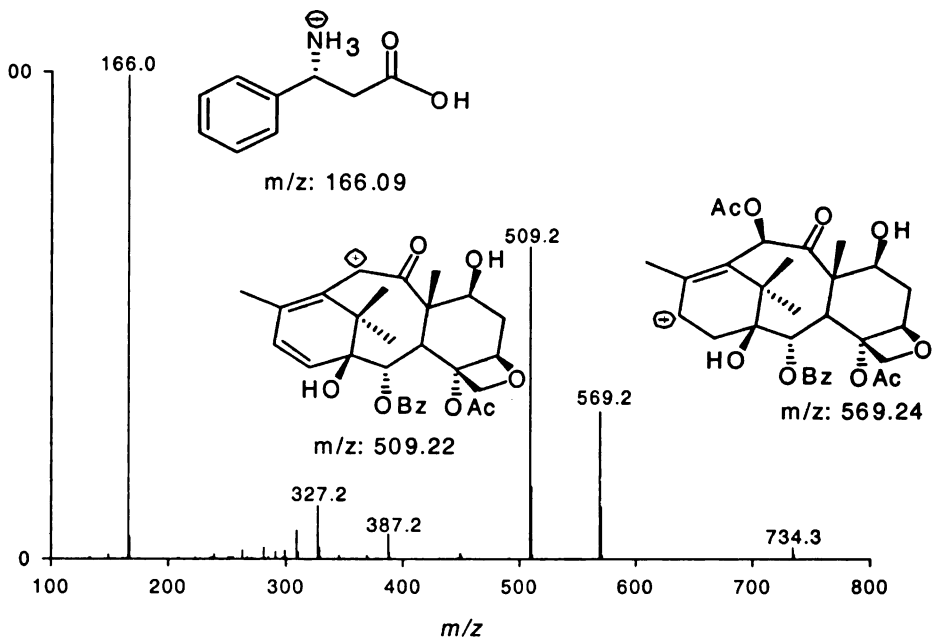


Figure 13: MS/MS of the product identified by side chain fragment

2.4 Discussion

The result of the assay showed that optimization of the expression of BAPT enzyme by introducing N-terminal His_6- tag epitope and the use of high expression vector enable us to get enough quantities of active enzyme activity of enzyme so that the biosynthetic products could be easily detected with HPLC even without the use of radioactive baccatin III. In this experiment three phenylpropanoid CoA cosubstrates were synthesized to probe the role of free amine in proposed substrate assisted catalysis mechanism. In a previous study, a diasteromeric mixture of (*R,S*)-β-phenylalanoyl CoA was utilized in the seminal assays with BAPT, while in this experiment enantiopure *R*-β-phenylalanoyl CoA was used. The use of enantiopure CoA could increase the possibility of product formation as there is no competitive binding from the racemic mixture towards the binding site. In

addition new phenylpropanoid CoA thioesters (3*R*-hydroxy-3-phenylpropanoyl CoA, and 3-phenylpropanoyl CoA) were used and these thioester CoAs were incubated individually with the other co-substrate baccatin III in the presence of BAPT. Among these the only productive substrate was the β-phenylalanoyl CoA and the product was identified by LC-MS and LC-MS/MS. The other phenylpropanoid thioester CoAs have similar carbon skeletons, except they have different functional groups at C3-i.e., OH or H instead of free amine. The synthesis of 3-oxo-phenylpropanoid CoA as a substrate failed and thus will be a future goal of the project. Conclusively, these results support the role of the free amine to potentially aid BAPT catalysis, particularly considering that the BAPT enzyme naturally lacks the lone conserved histidine residue purportedly required for catalysis of the superfamily in which BAPT belongs. Further investigation to examine the substrate specificity of BAPT for other derivatives of β-aminopropanoid CoA thioester is imminent.

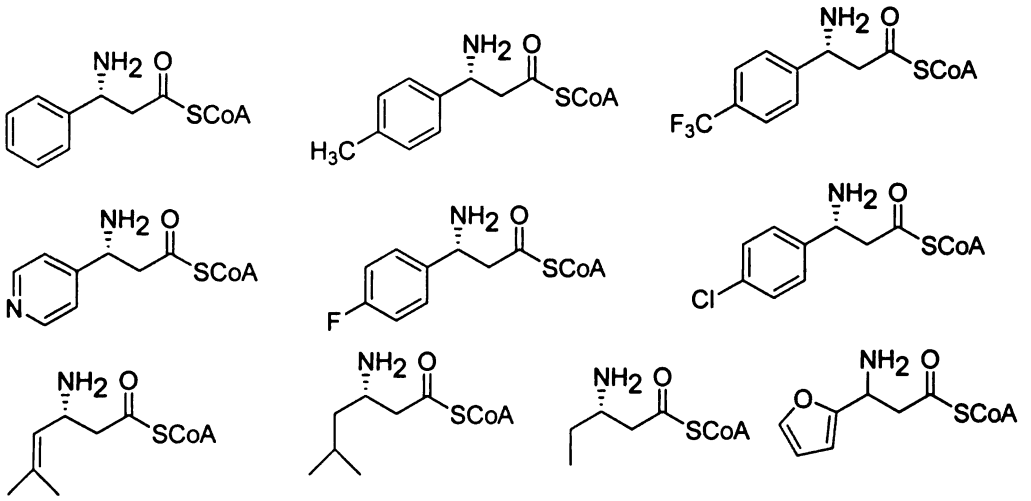


Figure 14: Future targets of β-amino acid CoA thioester

2.5 CONCLUSION

The BAPT acyltransferase enzyme couples the phenylpropanoid to the baccatin III core. This work has shown preliminary data that likely suggests that the BAPT enzyme involves substrate assisted catalysis mechanism to transfer the phenylpropanoid CoA to the Baccatin III. The presence of an amine group at the β-position of the substrate is seemingly crucial. As the scope of the substrate specificity examination is expanded we might reveal that BAPT also promotes substrate assisted catalysis of unnatural β-aminopropanoids. Conceivably BAPT could be utilized in biocatalytic process that reduce the number of steps to synthesize these paclitaxel analogs.

3. EXPERIMENTAL SECTION

3.1 General Methods:

CoASH as a trilithium salt was purchased from ARC (St. Louis, MO). (*R,S*)-3-(*tert*-butoxycarbonylamino)-3-phenylpropanoic acid (*R*)-3-(*tert*-butoxycarbonylamino)-3-phenylpropanoic acid, (*R*)-3-hydroxy-3-phenylpropanoic acid were purchased from Alfa Aesar (Ward Hill, MA). *N-tert*-Butoxycarbonyl-(3*R*)-phenylalanine was obtained from Acros Organics (Morris Plains, NJ), docetaxel was obtained from OChem (Des Plaines, IL), baccatin III was purchased from Natland (Research Triangle Park, NC). C-18 (carbon 11%) reversed phase silica gel was purchased from Silicycle, (QC, Canada). Tetrahydrofuran and dichloromethane were obtained from dry still packed with activated alumina that was pressurized with nitrogen gas. Silica gel (230-400 Mesh) and aluminium backed silica gel 60 Thin Layer Chromatography (TLC) plates, embedded with A_{254} chromophores were purchased from EMD™ chemicals Inc (Gibbstown, NJ).Ethyl chloroformate, ethyl benzoylacetate, and all other reagents were purchased from Sigma-Aldrich and used without purification unless noted otherwise.

All reported yields are for isolated materials. ^{1}H and ^{13}C NMR spectra were acquired on a Varian Inova-300 or a Varian Unity plus 500 spectrometer and were referenced to residual solvent signals either at 7.24 ppm or at 4.67 ppm for $CDCl_3$ and D_2O respectively. All apparent coupling constants (J values) were measured at the indicated field strengths and expressed in hertz (Hz).

A Q-ToF Ultima Global electrospray ionization tandem mass spectrometer (ESI-MS, ESI-MS/MS, Waters, Milford, MA) with a waters CapLC capillary HPLC was used for mass spectral analysis at the Mass Spectrometry Facility Research Training and Support Facility, Michigan State University. An Agilent 1100 HPLC system equipped with a UV detector was used for liquid chromatography and absorbance (A_{228}) monitoring of effluent.

Synthesis of 7-TES-baccatin III (2):

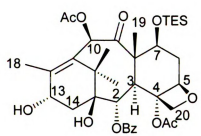


Figure 15: 7-TES-baccatin III

To a solution of baccatin III (165 mg, 0.28 mmol) in pyridine (10 mL) was added chlorotriethylsilane (1.42 mL, 8.45 mmol) dropwise. The solution was stirred at 25 °C for

24 h. After dilution with ethyl acetate (100 ml), the solution was washed with aqueous $CuSO_4$ (3 x 20 mL) and brine (20 mL). The organic extract was dried ($MgSO_4$), concentrated, and purified by flash chromatography using silica gel as stationary phase and 35% ethyl acetate in hexane as a mobile phase to give 7-TES-baccatin III(160 mg, 82.0%). R_f = 0.50 (35:65 ethyl acetate in hexane), ESI-MS (positive ion mode) 701.3$[M+H]^+$, 723.28 $[M+Na]^+$. ^1H NMR ($CDCl_3$, 300 MHz) δ: 8.07 (dd, *O*-Ph,), 7.60 (t, J = 1.4, J = 7.4, *p*-Ph), 7.49 (m, Ph) , 6.59 (s, H-10), 5.69 (d, *J* = 6.9 Hz, H-2), 4.92 (d, *J* = 8.2 Hz, H-5), 4.8 (t, H-13) 4.48 (dd, *J* = 6.9 Hz, H-7), 4.33 (d, *J* = 8.0 Hz, H-20a), 4.12(d, *J* = 8.0 Hz, H-20b), 3.91(d, *J* = 6.9 Hz, H-3), 2.96 (d, *J* = 20 Hz, H-14a), 2.65 (d, *J* = 20. Hz, H-20b), 2.50 (m, H-6α), 2.23 (s, $C(O)OCH_3$ at C4), 2.19 (s, C-10 $C(O)OCH_3$ and H-18), 1.67, 1.28, 1.19 (s, H-16, H-17 and H-19), 0.90 (dd. 9H, Si-CH_2 CH_3), 0.5(m, Si-CH_2 CH_3)

Synthesis of (*3R*)-*N*-Boc-7-TES-2'-deoxytaxol (3):

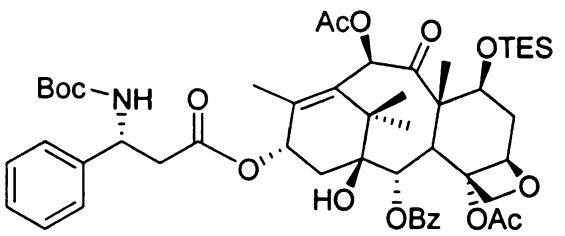


Figure 16: (*3R*)-*N*-Boc-7-TES-2'-deoxytaxol

Dipyridyl thiocarbonate (0.305 g, 1.30 mmol) was added to a solution of 3-(*R*)-*N*-Boc-β-phenylalanine (0.344 g, 1.30 mmol), *N,N*-dimethylaminopyridine (0.167 g, 1.3 mmol) and 7-TES-baccatin III (0.150 g, 0.22 mmol) in toluene (24 mL). The reaction was stirred at 70 °C for 24 h. The solvent was evaporated under reduced pressure and the crude residue was purified by flash column chromatography using silica gel and 35% ethyl acetate in hexane as the mobile phase. Fractions containing the product were combined and solvent evaporated under reduced pressure to obtain the desired product as pale yellowish solid (175 mg, 85%). R_f = 0.80 (35: 65 ethyl acetate in hexane), ESI-MS (positive ion mode): 970.4 $[M+Na]^+$. ^{1}H-NMR (500 MHz , $CDCl_3$) δ: 8.07 (dd, *O*-Ph), 7.60 (t, J = 1.4, J = 7.4, *p*-Ph), 7.49 (m, Ph) , 6.59(s, H-10), 5.69(d, *J* = 6.9 Hz, H-2), 5.2 (s, H-3'), 4.92 (d, *J* = 8.2 Hz, H-5), 4.48 (dd, *J* = 6.9 Hz, H-7), 4.33 (d, *J* = 8.0 Hz, H-20a), 4.12 (d, *J* = 8.0 Hz, H-20b), 3.91 (d, *J* = 6.9 Hz, H-3), 3.1 (dd, H-2') ,2.96 (d, *J* = 20 Hz, H-14a), 2.65 (d, *J* = 20. Hz, H-20b), 2.50 (m, H-6α), 2.23 (s, $C(O)CH_3$ at C-4),

2.19 (s, $C(O)CH_3$ at C-10 and H-18), 1.67, 1.28, 1.19 (s, H-16, H-17 and H-19), 1.5 (s,, $C(CH_3)_3$), 0.9 (dd, $SiCH_2CH_3$), 0.6 (m, $SiCH_2CH_3$)

Synthesis of *N*-debenzoyl-2'-deoxypaclitaxel (4)

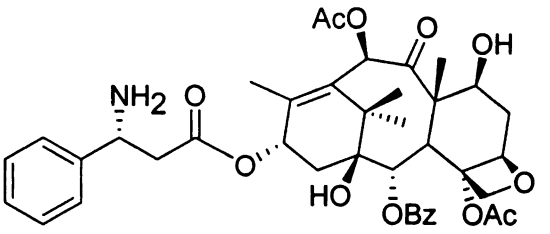


Figure 17: *N*-debenzoyl-2'-deoxypaclitaxel

Formic acid (0.5 ml, 88% aqueous solution) was added to a solution of *N*-Boc-7-TES-2'-deoxypaclitaxel (100 mg, 106 μmol) in 2 mL of dichloromethane and stirred at room temp. The completion of the reaction was monitored by TLC and the residual formic acid was removed under reduced pressure. The reaction mixture was then diluted with ethyl acetate and the solution was washed with 5% $NaHCO_3$ (2 x 10 mL), water (2 x 10 mL), brine (2 x 10 mL) successively. The organic extract was dried over Na_2SO_4 and concentrated. The residue was purified by PTLC to get *N*-debenzoyl-2'-deoxypaclitaxel (52 mg, 65%). R_f = 0.375 (10: 90 methanol: ethyl acetate), ESI-MS (positive ion mode): 734.2 $[M+H]^+$. ^{1}H-NMR (500 MHz , $CDCl_3$) δ: 8.07 (dd, *O*-Ph), 7.60 (t, J = 1.4, J = 7.4, *p*-Ph), 7.49 (m, Ph) , 6.40 (s, H-10), 6.00 (t, *J* = 7.2 Hz, H-13), 5.75 (br,NH) 5.69 (d, J =

6.9 Hz, H-2) 5.2 (s, H-3'), 4.92 (d, J = 8.2 Hz, H-5), 4.48 (dd, J = 6.9 Hz, H-7), 4.33 (d, J = 8.0 Hz, H-20a), 4.12(d, J = 8.0 Hz, H-20b), 3.91 (d, J = 6.9 Hz, H-3), 3.1 (dd, H-2') 2.96 (d, J = 20 Hz, H-14a), 2.65 (d, J = 20. Hz, H-20b), 2.50 (m, H-6α), 2.23 (s, $C(O)CH_3$ at C-4), 2.19 (s, $C(O)CH_3$ at C-10 and H-18), 1.87-1.8 (m,H-6β) 1.67, 1.28, 1.19 (s, H-16, H-17 and H-19)

Synthesis of 2'-deoxypaclitaxel (5):

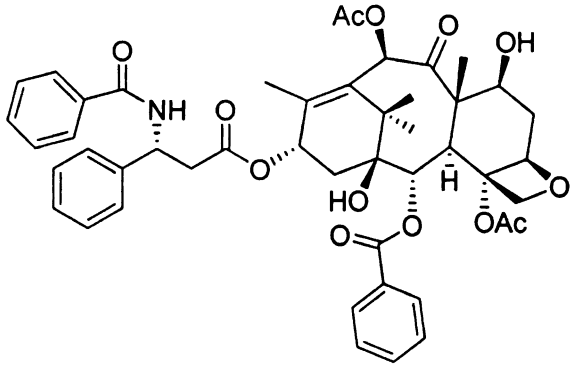


Figure 18: 2'-deoxypaclitaxel

In a 10-mL tube *N*-debenzoyl-2'-deoxypaclitaxel (8.3 mg, 0.01 mmol) was dissolved in ethyl acetate and saturated with $NaHCO_3$ (1 mL) to maintain the pH at 9.00. Benzoyl chloride (40 μL, 0.01 mmol) was added and the mixture was vigorously vortexed for 30 minute and the reaction proceeds at 25 °C. The reaction was diluted with ethyl acetate after 24 h, washed with water and brine successively, dried over $MgSO_4$ and concentrated and purified using PTLC using (methanol: ethyl acetate) to get a pale white

solid 2'-deoxypaclitaxel (8.51 mg, 90%). R_f = 0.875 (10:90, methanol: ethyl acetate); ESI-MS (positive ion mode): 861 $[M+Na]^+$ ^{1}H-NMR, (300 MHz , $CDCl_3$) δ: 8.02 (,d, *J* = 7, *O*-Ph), 7.60-7.26 (m, , phenyl), 6.36 (s, H-10), 6.1 (d, *J* = 6.3, 0.9 Hz, H-13), 5.66 (b, NH) 5.62 (d, *J* = 6.9 Hz, H-2), 5.18 (b, H-3') , 4.90 (dd, *J* = 5.4, 2.1, H-5), 4.4 (dd, *J* = 6.9 Hz, H-7), 4.24 (d, *J* = 8.0 Hz, H-20a), 4.12 (d, *J* = 8.0 Hz, H-20b), 3.72 (d, *J* = 6.6, 1H, H-3), 3.05 (dd, *J* = 10.5, 9.9 Hz, H-2'), 2.96 (d, *J* =20 Hz, H-14a), 2.65 (d, *J* = 20 Hz, H-20b), 2.50 (m, H-6α), 2.23 (s, $C(O)CH_3$ at C-4), 2.19 (s, $C(O)CH_3$ at C-10 and H-18) 1.67, 1.28, 1.19 (s, H-16, H-17 and H-19).

Preparation of 2'-TBDMS-docetaxel (7)

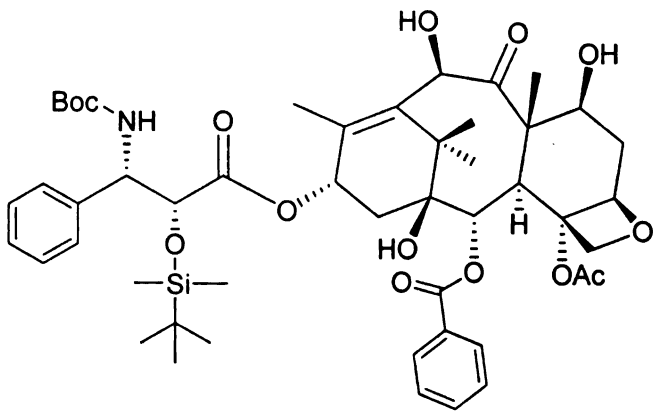


Figure 19: 2'-TBDMS-docetaxel

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 after 18 h, the mixture was diluted with ethyl acetate and washed 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 (v/v), EtOAc/hexane to yield 2′-TBDMS-docetaxel as a white powder (246 mg, 0.267 mmol, 99%) yield at 99% purity as judged by TLC and UV absorbance visualization. ESI-MS (positive ion mode): 944.6 $[M+Na]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.09 (d, J = 7.5 Hz, *o*-ph at C2 benzoyl), 7.56 (t, J= 7.5, 7.2 Hz, *p*-ArH of C2- benzoyl), 7.46 (t, J = 7.8, 7.2 Hz, *m*-ph of C2- benzoyl), 7.36 (m, C3′ phenyl), 7.28 (m, ArH of C3′ phenyl), 6.31 (t, J = 8.7 Hz, H-13), 5.66 (d, J = 7.2 Hz, H-2), 5.43 (br d, J = 9.3 Hz, H-3′), 5.30 (br, d, J = 9.0 Hz, NH), 5.18 (s, H-10), 4.95 (d, J = 8.1 Hz, H-5), 4.5 (d, J = 2.6 Hz, H-2′), 4.32-4.17 (m, H-7), 4.17 (dd, J = 7.2 Hz, H-20), 3.93 (d, J = 6.6 Hz, H-3), 2.61-2.58 (m, H-6), 2.53 (s, $C(O)OCH_3$ at C4), 2.38-2.39 (m, H- 6α, H-14), 2.02 (s, H-18, H-6) 1.73 (s, H-19), 1.28 (s, *N*-Boc), 1.28 (s, H-17), 0.72 (s, $SiC(CH_3)_3$), -0.13 (s, $SiCH_3$), -0.34 (s, $SiCH_3$).

Synthesis of 10-acetyl-2′-TBDMS-docetaxel. (8)

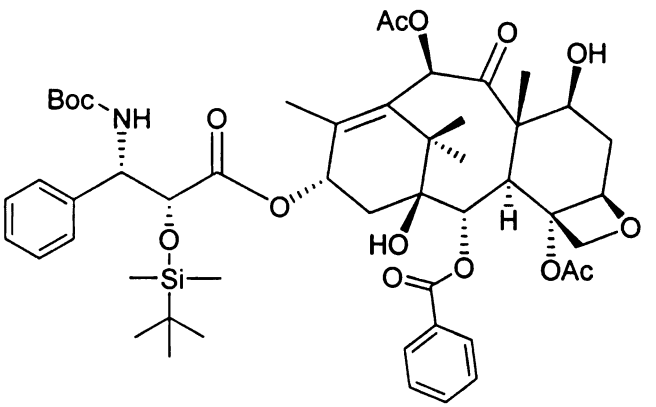


Figure 20: 10-acetyl-2′-TBDMS-docetaxel.

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 monitored 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 v*acuo* 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 (300 MHz, $CDCl_3$) δ: 8.09 (d, J = 7.5 Hz, *o*-ph at C2 benzoyl), 7.56 (t, J= 7.5, 7.2 Hz, *p*-ArH of C2- benzoyl), 7.46 (t, 2H, J = 7.8, 7.2 Hz, *m*-ph of C2- benzoyl), 7.36 (m, C3′ phenyl), 7.28 (m, 1H, ArH of C3′

phenyl), 6.30 (s and br, t, H-10, H-13), 5.66 (d, J = 7.2 Hz, H-2), 5.44 (br d, J = 9.6 Hz, H-3′), 5.30 (br, d, J = 9.0 Hz, NH), 4.95 (d, J = 7.8 Hz, H-5), 4.5 (d, J = 1.8 Hz, H-2′), 4.42 (dd, J = 6.6 , H-7), 4.30 (dd, J = 8.4 Hz, H-20b), 4.17 (d, J= 8.1 Hz, H-20a) 3.81 (d, J = 7.2 Hz, H-3), 2.53 (s, $C(O)OCH_3$ at C4), 2.41-2.33 (m, H- 6α, H-14), 2.20 (s, $C(O)OCH_3$ at C10), 2.04 (s, H-18, H-6), 1.83 (s, H-19), 1.28 (s, H-16, *N*-Boc), 1.13 (s, H-17), 0.72 (s, $SiC(CH_3)_3$), -0.14 (s, $SiCH_3$), -0.32 (s, $SiCH_3$).

Synthesis of 10-acetyldocetaxel (10)

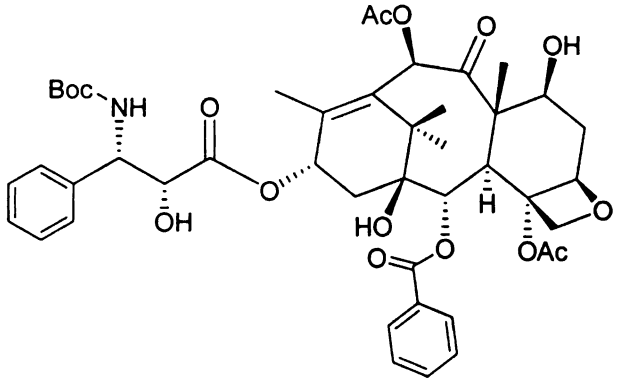


Figure 21: 10-acetyldocetaxel

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% hydrogen fluoride 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 (0.5mL) 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(v/v) EtOAc/ hexane), 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 1H NMR). ESI-MS (positive ion mode): 872.3 $[M+Na]^+$ ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 8.12 (d, J = 7.5 Hz, *o*-ph of C2 benzoyl), 7.62 (t, J = 7.5, 7.0 Hz, *p*-ArH of C2 -benzoyl), 7.51 (t, J = 7.5 Hz, *m*- ArH of C2- benzoyl), 7.40 (m, ArH of C3′ phenyl), 7.33 (m, ArH of C3′ phenyl), 6.31 (s, H-10), 6.25 (t, J = 9.0 Hz, H-13), 5.68 (d, J = 7.0 Hz, H-2), 5.45 (br d, J = 9.3 Hz, H-3′), 5.30 (br d, J= 9.3 Hz, NH), 4.95 (d, J= 8.0 Hz, H-5), 4.64 (d, J= 2.3 Hz, H-2′), 4.42 (dd, J = 9.0, 8 Hz, H-7), 4.30 (d, J = 8.5 Hz, H-20a), 4.17 (d, J= 8.5 Hz, H-20b), 3.81 (d, J= 7.5 Hz, H-3), 3.4 (br d, 2′-OH), 2.61-2.57 (m, H-6α), 2.38 (s, 3H, C(O)OCH3 at C-4), 2.33 (m, H-14), 2.25 (s, $C(O)OCH_3$ at C-10), 1.91-1.89 (m, H-6), 1.86 (s, H-18), 1.70 (s, H-19), 1.35 (s, $OC(CH_3)_3$), 1.27 (s, H-16), 1.16 (s, H-17).

Synthesis of *N*-Debenzoylpaclitaxel. (11)

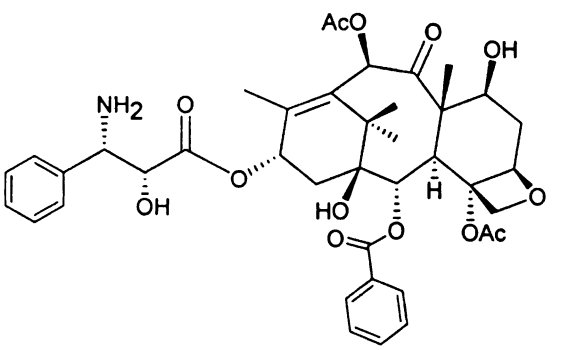


Figure 22: *N*-debenzoylpaclitaxel

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 by TLC (90:10 (v/v) chloroform/methanol, 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 (**11**). (70% yield, 105 mg, 0.14 mmol,) as a pale yellow solid. ESI-MS (positive ion mode): 750.2 $[M + H]^+$, 768 $[M + Na]^+$ ^{1}H-NMR (CDCl3, 300 MHz) δ: 8.07-8.04 (m, *o*-ArH of C2 -benzoyl), 7.65 (t, J = 8.3 Hz, *p*- ArH

of C2- benzoyl), 7.51 (t, 2H, J = 8.1, 6.9 Hz, *meta*-ArH of C2 benzoyl), 7.38 (m, ArH of C3′ phenyl), 7.28 (m, ArH of C3′ phenyl), 6.27 (s, H-10), 6.14 (t, J = 8.0 Hz, H-13), 5.63 (d, J = 7.2 Hz, H-2), 4.93 (d, J = 8.1 Hz, H-5), 4.42-4.34 (m, H-7), 4.32-4.27 (m, H-20a, and H-2′), 4.13 (d, J = 8.7Hz, H-32), 3.75 (d, J = 6.9 Hz, H-3), 2.56-2.48 (m, H-6a), 2.24 (s, $C(O)OCH_3$ at C10 or C4), 2.23 (s, $C(O)OCH_3$ at C10 or C4), 1.98-2.10 (m, H-14 and H-6b), 1.88 (br s, H-18), 1.65 (s, H-19), 1.24 (s, H-16), 1.12 (s, H-17).

Synthesis of 10-Deacetyl-*N*-debenzoylpaclitaxel (12)

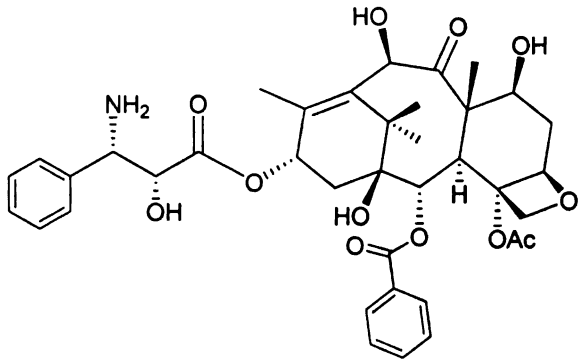


Figure 23: 10-Deacetyl-*N*-debenzoylpaclitaxel

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 (v/v) EtOAc/MeOH) 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 (300 MHz, $CDCl_3$) δ: 8.06 (d, J = 7.8 Hz, *o*-ArH of C2 benzoyl), 7.62 (t, J = 7.5, 7.2 Hz, *p*-ArH of C2- benzoyl), 7.51 (t, J =7.5 Hz, *m*-ArH of C2 benzoyl), 7.39 (m, ArH of C3′phenyl), 7.30 (m, ArH of C3′phenyl), 6.13 (t, J = 9.0 Hz, 13-H), 5.64 (d, J = 7.2 Hz, H-2), 5.20 (s, H-10), 4.93 (d, J = 7.8 Hz, H-5), 4.35-4.28 (m, H-2′, H-3′, H-20a), 4.26 (m, H-7), 4.15 (d, J = 8.4 Hz, H-20b), 3.88 (d, J = 7.2 Hz,H-3), 2.62-2.52 (m, H-6a), 2.25 (s, $C(O)OCH_3$ at C-4), 2.07-2.02 (m, H-14), 1.90 (s, H-18), 1.82 (m, H-6), 1.75 (s, H-19), 1.21 (s, H-16), 1.11 (s, H-17).

Synthesis of -13-Oxo-7-TES-baccatin III (27)

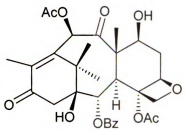


Figure 24: 13-Oxo-7-TES-baccatin III

7-TES-baccatin III (100 mg, 0.14 mmol) was dissolved in dichloromethane (6 mL), three batches of MnO_2 (2 g) were added with constant stirring. The reaction was followed up using TLC, and after most of the reaction converted into product, the reaction suspension was filtered and diluted with ethyl acetate. The organic solvent concentrated and purified by silica gel chromatography (Ethyl acetate: Hexane 35:65, as mobile phase) to get a white powder of 13-oxo-7-TES-baccatin III (85 mg, 86.9%).ESI- MS (positive mode): 700.3 $[M+ Na]^+$ ^{1}H-NMR (300 MHz , $CDCl_3$) δ: 8.03 (d, J = 7.2 Hz,o-ArH of C2 benzoyl), 7.62 (t, J = 7.5 Hz, Bz), 7.45 (t, J = 7.5 Hz, Bz), 6.55 (s, 10-H), 5.67 (d, J = 6.0 *Hz*, 2-H), 4.88 (d, J = 7.5 Hz, 5-H), 4.44 (dd, J = 10.3, 6.9 *Hz*, 7-H), 4.28 (d, J = 8.7 Hz, 20-H_a), 4.10 (d, J = 8.4 Hz, 20H_b), 3.87 (d, J = 6.6 Hz, 3-H), 2.92 (d, J = 20.1 Hz, 14-H_a), 2.61(d, J = 20.7 Hz, 14- H_b), 2.50 (m, 6-H), 2.21 (s, 3H, OAc), 2.17 (s, 3 H, OAc), 2.06 (s, 18-CH_3), 1.82 (m, 6-H), 1.65 (s, 19-CH_3), 1.25 (s, 16-CH_3), 1.17 (s, 17-CH_3), 0.884 (t, J = 8.4 Hz, Si($CH_2CH_3)_3$, 0.58-0.51 (band, 6H, Si(CH_2 $CH_3)_3$).

Synthesis of [13-H^3] baccatin III.(28)

13-Oxo-7-TES-baccatin III (20 mg, 0.029 mmol) was dissolved in THF (250 μL) and added to the original vial containing [^{3}H]$NaBH_4$ (50 mCi, specific activity, 150 mCi/mmol, ARC Inc.) and stirred at 0 °C in ice bath for 30 minutes and the temperature was kept at room temperature for 2 h after which 0.1 ml of 0.01 N sodium hydroxide solution was added and the reaction was followed by TLC and kept for additional 2 h and the reaction quenched with water (0.5 ml). Finally, the reaction was extracted with ethyl acetate (3 x 5ml), dried over sodium sulfate, and the solvent was evaporated under nitrogen. The resulting residue was purified by PTLC to get [13-^{3}H]-7-TES baccatin III (yield = 15 mg), and verified with authentic 7-TES-baccatin III by comparison against authentic sample on TLC. [13-H^3]-7-TES baccatin III (15mg, 0.021mmol) was dissolved in 1 mL of THF, and 0.3 mL of pyridine was added and stirred at 0 °C and HF/pyridine (0.3 mL) added dropwise over 0.5 h, and the reaction was warmed to room temperature and stirred for 2 h. The reaction progress was monitored by TLC, and the reaction was diluted with EtOAc (10 mL) and washed with sat. $NaHCO_3$ (2 x 2 mL), 10% NaOH solution (2 mL), and finally with brine. The organic layer was separated from the aqueous layer, and, the organic fraction was dried over sodium sulfate. The solvent was removed under a stream nitrogen gas, and the crude product was purified by PTLC using (ethyl acetate: hexane, 50:50) and 8 mg, (0.013mmol, 65%). The specific activity of baccatin III (1.01 x10^8 DPM, 0.0455 mCi, and specific activity 3.46 mCi/mmol) was determined using liquid scintillation counting on a 14/4C Wallace Instruments counter (Copley, OH)

3.2 General Procedure for the synthesis of thioesters CoASH

Triethylamine (1.1 equiv.) was added to a solution of the corresponding carboxylic acid (1.0 equiv.) in 5:2 (v/v)CH_2Cl_2 / THF (1.4 mL) under N_2 gas. The mixture was stirred for 10 min at 23 °C, ethyl chloroformate (1.2 eq.) was added in one portion, and the reaction was stirred for 1 h. The solvents were evaporated under reduced pressure, and the residue was dissolved in 0.5 mL of *tert*-butyl alcohol. CoASH as the sodium salt (1.1 equiv.) in 0.4 M $NaHCO_3$ was added to the solution, and the mixture was stirred for 0.5 h at room temperature (23 °C), then quenched by dropwise addition of 1M HCl and adjusted to pH 3-5. The solvents were evaporated under vacuum, and the residue was dissolved in water (5 ml, pH 3-5) and loaded onto a C-18 silica gel column (10 % capped with trimethyl silyl (TMS)) that had been 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 3-5) and then eluted with 15% MeOH in water (50 mL, pH 3-5). The fractions containing Coenzyme A thioesters, as determined by TLC, were combined and the solvent was evaporated, and the derived solution containing the product was lyophilized. The purity of the product was assessed by analytical TLC, and the identities of the compounds were then confirmed by ESI-MS and ^{1}H-NMR spectroscopy.

Phenylpropanoyl CoASH (17)

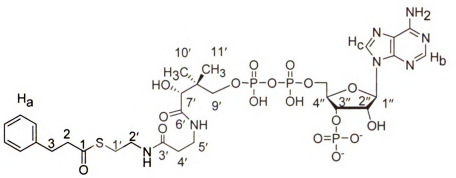


Figure 25: phenylpropanoyl CoA

Following the general procedure, 3-phenylpropanoic acid (9.8 mg, 65 μmol) was dissolved in CH_2Cl_2 /THF (5:2 (v/v), 3.0 mL) and 1 M triethylamine in DCM (8 μL, 78 μmol) was added and mixed for 10 min. ethyl chloroformate (11 μL, 97.5 μmol) was added to one portion under nitrogen. The reaction stirred for 1 h at room temperature and the reaction progress was monitored by TLC. The resulting acid anhydride was dried overnight under vacuum to remove traces of ethyl chlorformate. The resulting residue was dissolved in 2.5 ml of *t*-BuOH. The CoA was dissolved (as the trilithium salt, 50 mg, 60 μmol) in $NaHCO_3$ (0.4 M, 2.5 mL) and added to the solution. The reaction was stirred for 1 h at room temperature, quenched with 1M HCl, adjusted to pH 3-5 and the solvents were evaporated under reduced pressure at room temperature. The residue was purified by reverse-phase flash chromatography on C_{18} silica gel chromatography (using water and methanol as a mobile phase). The fractions containing the product were combined

together, and the solvent was evaporated in *vacuo,* and the remaining water was removed by lyophilization to provide the phenylpropanoyl CoASH as a white solid (69 mg, 51%). The purity (98%) was assessed by analytical TLC, R_f = 0.46 (n-butanol/H_2O/AcOH, 5:3:2 v/v/v) and visualization under UV on the TLC plate. The compound was confirmed by ESI-MS and ^{1}H-NMR. ESI-MS: calculated 899, experimental $[M-H]^-$ 898, $[M-2H]^{-2}$ 448.5. ^{1}H-NMR (500 MHz, D_2O) see figure for numbering δ (in ppm): 0.67 (s, H-10'), 0.81 (s, H-11'), 2.26 (t, J = 6, H-4'), 2.44 (dd, J = 5, H-2), 2.84 (1H, m, H-1'), 3.16 (dd, J = 5.5, H-2'), 3.25 (m, H-3), 3.31 (dd, J = 6.5, H-5'), 3.48 (m, H_a-5"), 3.75 (m, H_b-5"), 3.94 (s, H-7'), 4.14 (s, H-9', 2H), 4.5 (br,H-4"), 4.68 (D_2O, H-2", H-3"), 6.05 (d, J=5, H-1"), 7.08-7.26 (5H, m, phenyl proton), 8.12 (s, CH-adenine), 8.45(s, CH-adenine)

Synthesis of (*RS*)-Phenylalanoyl CoA (20)

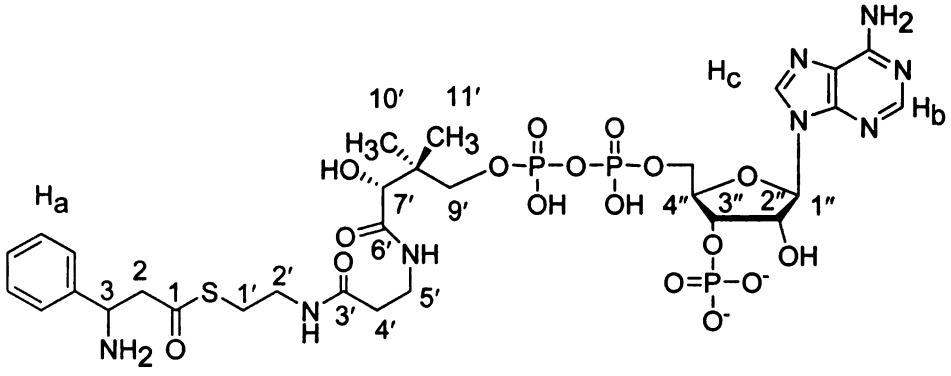


Figure 26: (*RS*)-Phenylalanoyl CoA

(*R,S)-N*-Boc-β-3-Phenylalanine CoA was prepared similarly to the procedures described previously except (*R,S)-N*-boc-β-3-Phenylalanine (17 mg, 65 μmol) was dissolved in 5:2 (v/v) CH_2Cl_2 / THF (1.4 mL) and 1 M triethylamine in DCM (8 μL, 78 μmol) was added and mixed for 10 min. Ethyl chloroformate (11 μL, 97.5 μmol) was then added on to the reaction solution under nitrogen. The reaction was stirred for 1 h at room temperature, and the progress of the reaction was monitored by analytical TLC. The resulting acid anhydride was dried over night under vacuum to remove traces of ethyl chlorformate. To the resulting residue was added in 2.5 ml of *t*-BuOH. CoA (as the trilithium salt, 50 mg, 60 μmol) was dissolved $NaHCO_3$ (0.4 M, 2.5ml) and added to the solution containing the mixed anhydride. The mixture was stirred for 2 h at room temperature, the reaction was quenched with 1M HCl, adjusted to pH 3-5 and the solvents were evaporated under reduced pressure at room temperature. The residue was purified by reverse phase flash chromatography on C_{18} silica gel chromatography, using water and methanol as a gradient mobile phase. The product comes with about 10% methanol in water and the solvent of these fractions were combined removed under high vaccum. The residual water was lyophilization to yield (*RS)-N*-Boc-β-phenylalnine CoA as a white solid (46 mg, 45 μmol, 69%).The purity (98%) was assessed by analytical TLC (R_f = 0.47 (n-butanol: acetic acid: water, (5:2:3)as mobile phase).The derived product was confirmed by ESI-MS and ^{1}H-NMR. ESI-MS: analysis calculated mw 1013. The Boc protected product was dissolved in 1 mL of water and the Boc group was removed by 88% formic acid solution. The reaction progress was followed up by TLC. At completing the reaction

was diluted with water and concentrated under vacuum. The dilution/concentration process was repeated two more times, and the sample was lyophilized to obtain *RS*-phenylalanyl CoA (22 mg, 24 μmol, 53%) ESI-MS (negative ion mode) 913 $[M-H]^{-1}$

^{1}H-NMR (500 MHz, D_2O) δ: 0.66 (s, H-10'), 0.78 (s, H-11'), 2.16 (2H, t, H-1') 2.8 (2H, m, H-1'), 3.1 (t, H-2'), 3.26-3.28 (m, H-2 & H-5'), 3.44 (dd, J= 4.8 and 9.6 Hz, H_a-5″), 3.66 (dd, J = 4.8 and 9.6 Hz, H_b-5″), 3.86 (s, H-7') , 4.06 (s, H-9'), 4.43 (ddd, J = 2.7 and 5.3 Hz, H-4″), 4.56- 4.66 (m, H-2″ and H-3″), 6.0- 6.1 (two doublets; one set from each stereoisomer, J = 6.9 Hz for both, H-1″), 7.21-7.27 (m, H_A phenyl protons), 8.26 (s, H_C adenine-CH), 8.33 (s, H_B adenine-CH)

Synthesis of 3(*R*)-β-phenylalanoyl CoASH (15)

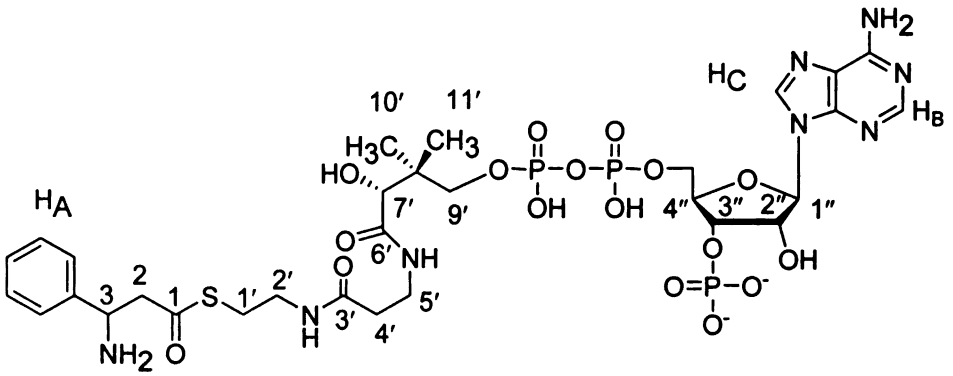


Figure 27: 3*R*-β-phenylalanoyl CoA

Following the same procedure as preparation of (*RS*)-β-phenylalanoyl CoASH, but we use the *R* enantiomer of *N*-Boc-*R*-β-phenylalnine, to get (20 mg, 21μmol, 50%) *R*-β-

phenylalanine CoA, verified by LC-MS. Compound then confirmed by ESI-MS(negative mode) 913[M-H]$^-$, ^{1}H-NMR (500 MHz, D_2O) , δ: 0.66 (s, H-10′) , 0.78 (s, H-11′), 2.16 (t, H-1′) 2.8 (m, H-1′), 3.1 (t, H-2′), 3.26-3.28 (m, H-2 & H-5′), 3.44 (dd, J= 4.8 and 9.6 Hz, H_a-5″), 3.66 (dd, J= 4.8 and 9.6 Hz, H_b-5″), 3.86 (s, H-7′), 4.06 (s, H-9′), 4.43 (ddd, J = 2.7 and 5.3 Hz, H-4″), 4.56- 4.66 (m, H-2″ and H-3″), 6.0- 6.1 (dd, *J* = 6.9 Hz , H-1″), 7.21-7.27 (m, H_A phenyl protons), 8.26 (s, H_C adenine-CH), 8.33 (s, H_B adenine-CH).

Synthesis of (*R*)-3-hydroxy-3-phenylpropanoyl CoA (20)

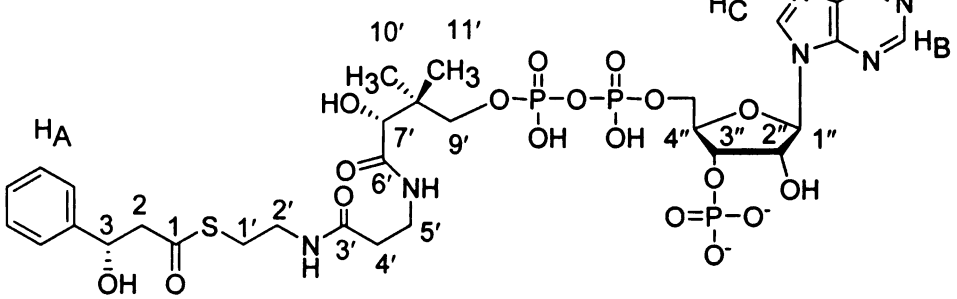


Figure 28: (*R*)-3-hydroxy-3-phenylpropanoyl CoA

Following the general procedure, *R*-3-hydroxy-3-phenylpropanoyl CoA was similarly prepared as the previous except *R*-3-hydroxy-3-phenylpropanoic acid (10mg, 65 μmol) was used with slight variation in the composition of solvent in a 5:1 CH_2Cl_2: THF (v/v, 1.8 mL) to yield (*R*)-3-hydroxy-3-phenylpropanoyl CoA (15 mg, 16 μmol, 25%) ESI-MS: calculated 915.17, experimental [M-H]$^-$ 914.09. ^{1}H-NMR (500 MHz, D_2O) , δ 0.66 (s, H-10') , 0.78 (s, H-11'), 2.39 (t, H-4'), 2.8 (m, H-1'), 3.20 (t, H-2'), 3.26-3.28 (m, H-2

& H-5'), 3.46 (dd, J = 4.8 and 9.6 Hz, H_a-5″), 3.80 (dd, J = 4.8 and 9.6 Hz, H_b-5″), 3.96 (s, H-7′) , 4.06 (s, H-9′), 4.46 (dd, J = 2.7 and 5.3 Hz, H-4″), 4.60 - 4.82 (m, H-2″ and H-3″), 5.80 (m, H-3), 6.40 (dd, J=6.9 Hz , H-1″), 7.21-7.27 (5H, m, H_A phenyl protons), 8.20 (1H,s, H_C adenine-CH), 8.43 (1H,s, H_B adenine-CH)

Synthesis of benzoyl-2, 2-dimethyl-1, 3-dioxane-4, 6-dione

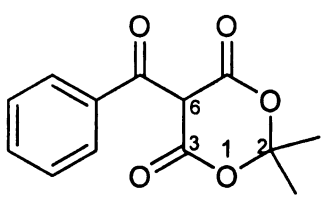


Figure 29: benzoyl-2, 2-dimethyl-1, 3-dioxane-4, 6-Dione

A solution of 2,2–dimethyl-1,3-dioxane-4,6-dione (1.2 g, 8.32 mmol) and 4 (dimethylamino)pyridine (DMAP, 2.4g, 10.9 mmol) in DCM (25 ml) was cooled in an ice-salt bath to -10 °C, and a solution of benzoyl chloride (1.62 g, 9.25 mmol) in dichloromethane (10 mL) was added dropwise over 2 h. The resulting yellow solution was kept in the cooling bath for 4 h and allowed to stir at room temperature overnight. The Orange-yellow solution was then cooled in an ice-water bath and washed with 3 x 5 mL of a 1 M aqueous HCl solution and water. The organic layer was dried over $MgSO_4$ and filtered, and the filtrate was evaporated to dryness. The residue was triturated with hexanes (10 ml), and dried under vacuum to give the benzoyl-2,2–dimethyl-1,3-dioxane-

4,6-dione as a yellow powder. A suspension of 1g of the (4 mmol) was made in 40 ml of toluene, was stirred at room temperature for 45 min and then filtered to remove insoluble material. The toluene solution was then treated with neat isopropyl amine (300 μL, 0.14 g, and 3.5 mmol) added drop wise through a syringe. The resulting precipitate was isolated by filtered and washed with toluene (3 x 5 ml) and hexanes (2 x 5 ml) to afford a salt as a yellow solid. The salt was then suspended in dichloromethane and washed with 1 M aqueous HCl solution. The organic layer was separated, dried ($MgSO_4$), filtered, and concentrated to give a yellow solid.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 15.5 (br, s, 1H, H-5), 7.7 (d, J = 8.1, 2H, *o*-Ph), 7.64 (dd, J = 7.5, 1H, *p*-Ph), 7.05 (dd, J = 7.2, 2H, *m*-Ph), 1.88 (s, 6H, CH_3).

3.3 Enzyme expression, Protein Harvest, Protein Purification.

3.3.1 Enzyme Expression.

Standard microbial and recombinant techniques used throughout this work are described by Sambrook. The full length *bapt* cDNA (accession AY082804) was subcloned from its original expression vector pSBET[55] 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 The resultant plasmid containing the *bapt* cDNA in pET28a was transformed into expression vector, *E. coli* BL21-CodonPlus® (DE3).

3.3.2. Protein Harvest

Recombinant *bapt* was expressed in the described bacterial expression system and harvested according to a previously reported protocol[48] with slight modifications. Cultures were grown overnight at 37°C in 5 mL Luria-Bertani medium supplemented with 50 μg/mL kanamycin. Bacteria transformed with empty vector were processed analogously. The 5-mL inoculum was added to and grown at 37°C to OD_{600} = 0.7 in 1 L Luria-Bertani medium supplemented with the kanamycin (50 mg/L) antibiotic, and then gene expression was induced with 100 μM isopropyl-β-D-1-thiogalactopyranoside, and the culture was incubated at 18°C. After 18 h, the cells were harvested by centrifugation at 2000*g* for 20 min at 4°C, the supernatant discarded, and the pellet was resuspended in

Lysis Buffer (25 mM 2-(*N*-morpholino) ethanesulfonic acid, pH 7.0 at 5 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 45000*g* for 1 h at 4°C.

3.3.3 Protein Purification

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, 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 (30000 MWCO, YM30 membrane, Millipore, Billerica, MA) and dilution in Assay Buffer (50 mM sodium phosphate, 5% glycerol, pH 8.0)

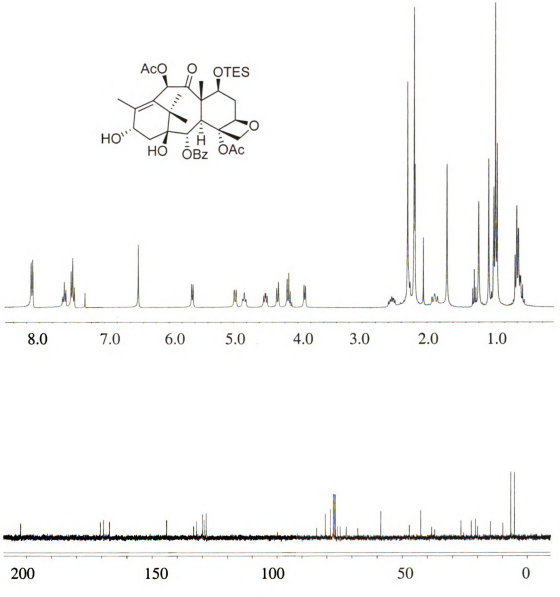
3.3.4. Activity and Assay of BAPT

To verify functional expression of the BAPT, 100 μg of the purified soluble protein was withdrawn and assayed with three acyl CoAs 100 μmol of *R*-β-phenylalanine CoA and [13-^{3}H]baccatin III (70 μmol, 0.5μC$_i$) at 31°C. The assay mixture was quenched with saturated sodium bicarbonate and extracted with ethyl acetate (2 × 1 mL), the organic fractions were combined, the solvent was evaporated, and the residue was redissolved in

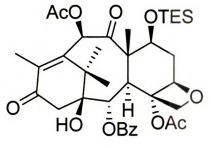
50 μL acetonitrile. A 25-μL aliquot was loaded onto a reverse-phase column (Allsphere ODS2-5μm, 250 × 4.6 mm, Alltech, Mentor, OH) and eluted isocratically with 50:50 (v/v) solvent A:solvent B [solvent A: 97.99% H_2O with 2% CH_3CN and 0.01% H_3PO_4 (v/v); solvent B: 99.99% CH_3CN with 0.01% H_3PO_4 (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 *bapt*-expressing cells were compared to absorbance profiles of the products identically isolated from control assays containing extracts of cells transformed with empty vector

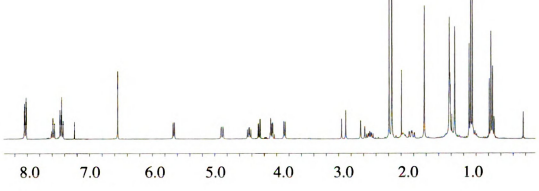
Appendix

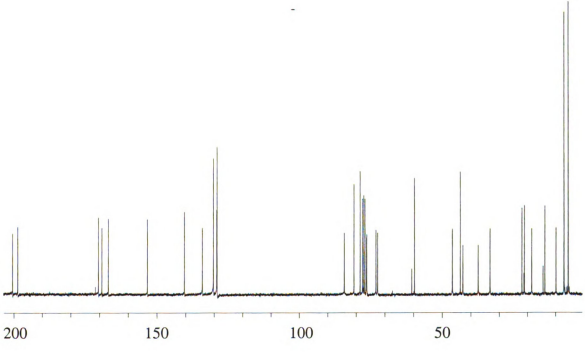
Spectra 1: ^{1}H and ^{13}C NMR for 7-TES-Baccatin III



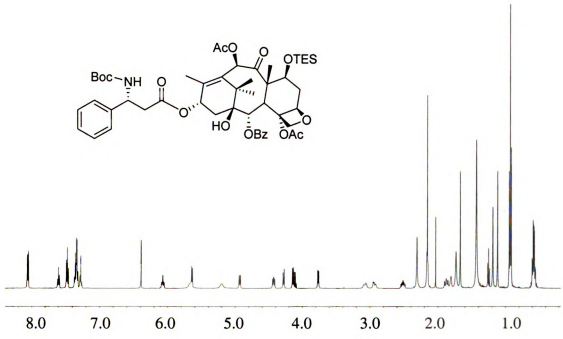
Spectra 2: ^{1}H and ^{13}C NMR for 13-Oxo-7-TES-Baccatin III

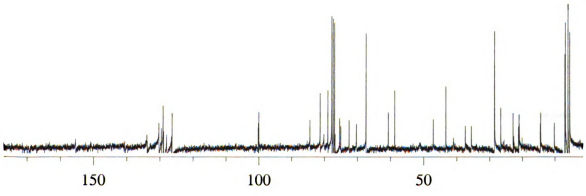




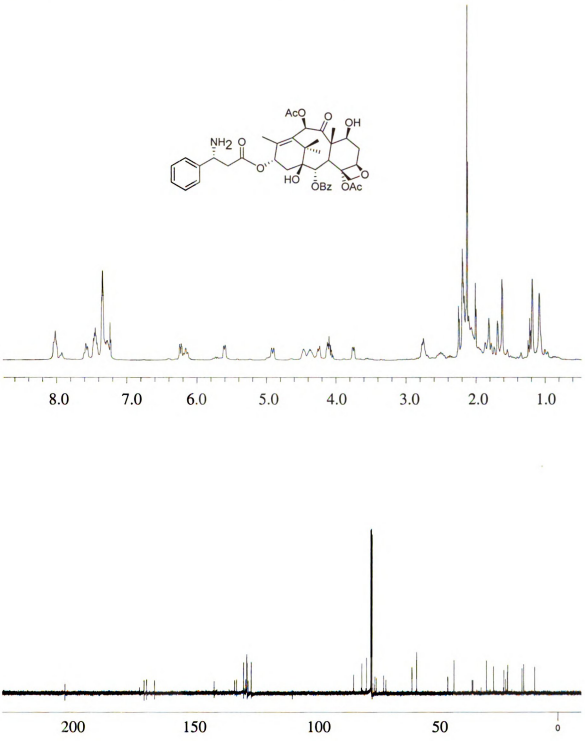


Spectra 3: ^{1}H and ^{13}C NMR for N-boc-N-debenzoyl-7-TES -2'-deoxytaxol

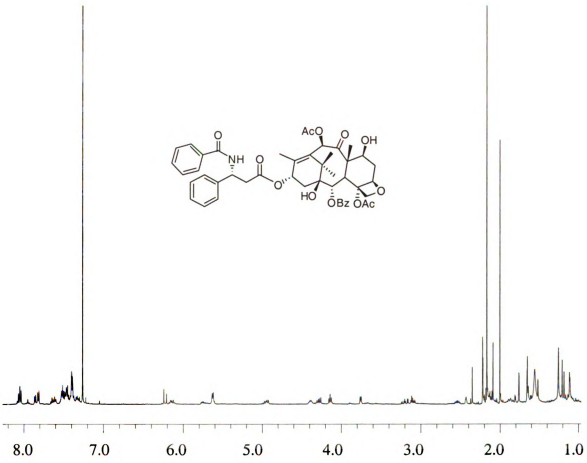




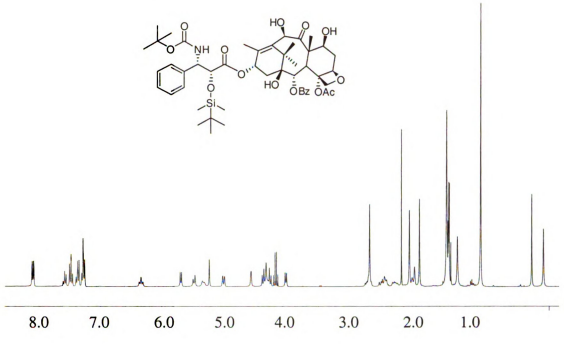
Spectra 4: ^{1}H and ^{13}C NMR for N-debenzoyl-2'-deoxytaxol



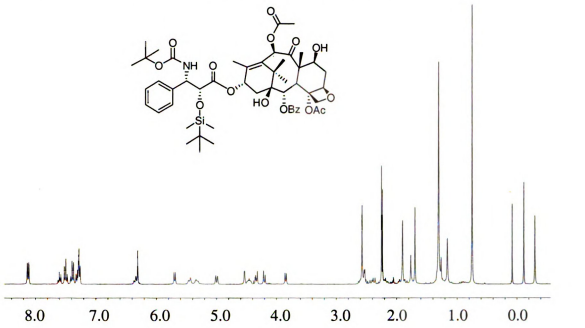
Spectra 5: ^{1}H NMR for 2'-Deoxypaclitaxel

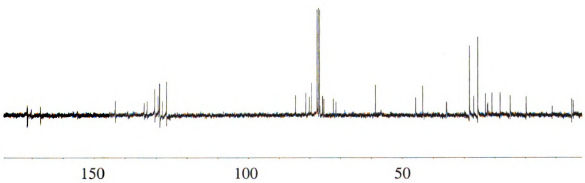


Spectra 6: ^{1}H and ^{13}C NMR for 2-TBDMS-docetaxel

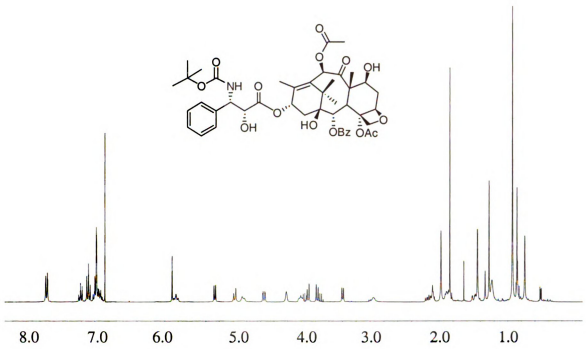


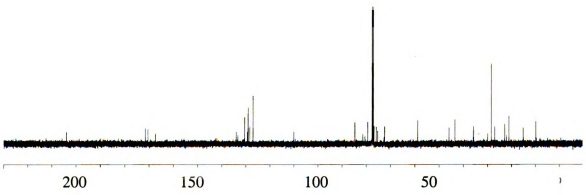
Spectra 7: ^{1}H and ^{13}C NMR for 10-acetyl-2-TBDMS-docetaxel



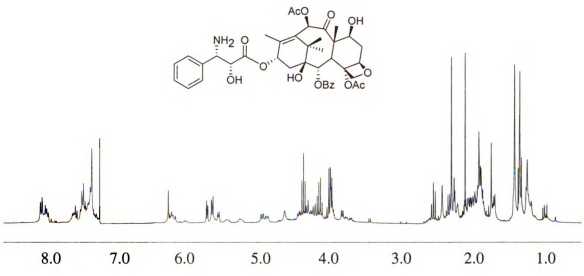


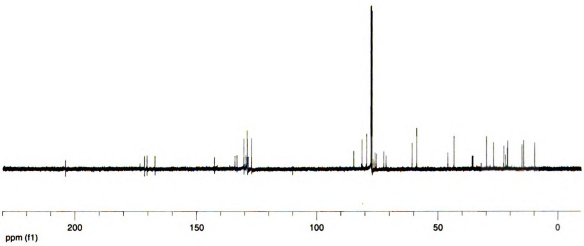
Spectra 8: ^{1}H and ^{13}C NMR for 10-acetyl-docetaxel



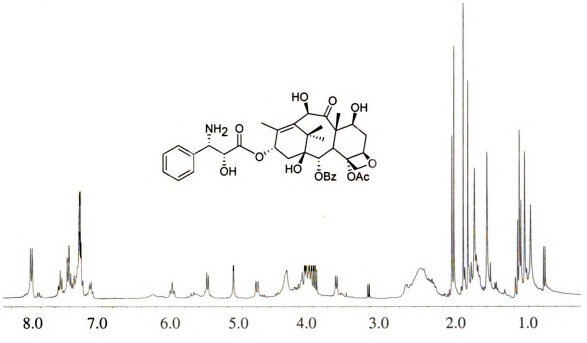


Spectra 9: ^{1}H and ^{13}C NMR for N-debenzoylpaclitaxel

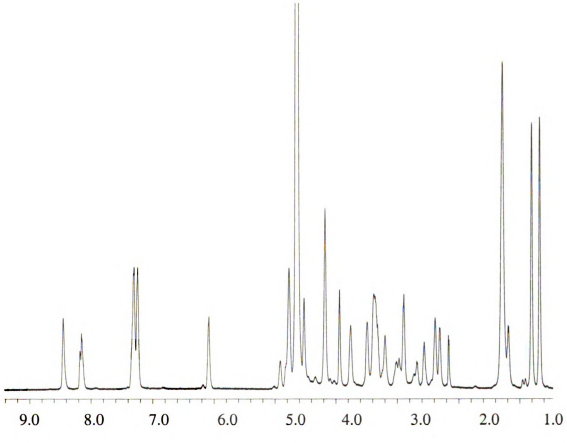




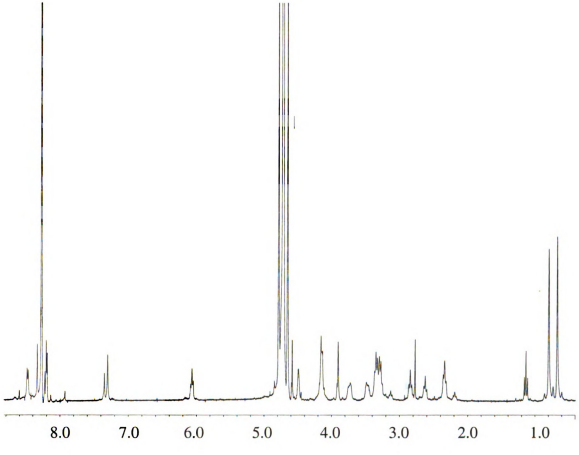
Spectra 10: ^{1}H *NMR for* 10-deacetyl-*N*-debenzoyltaxol



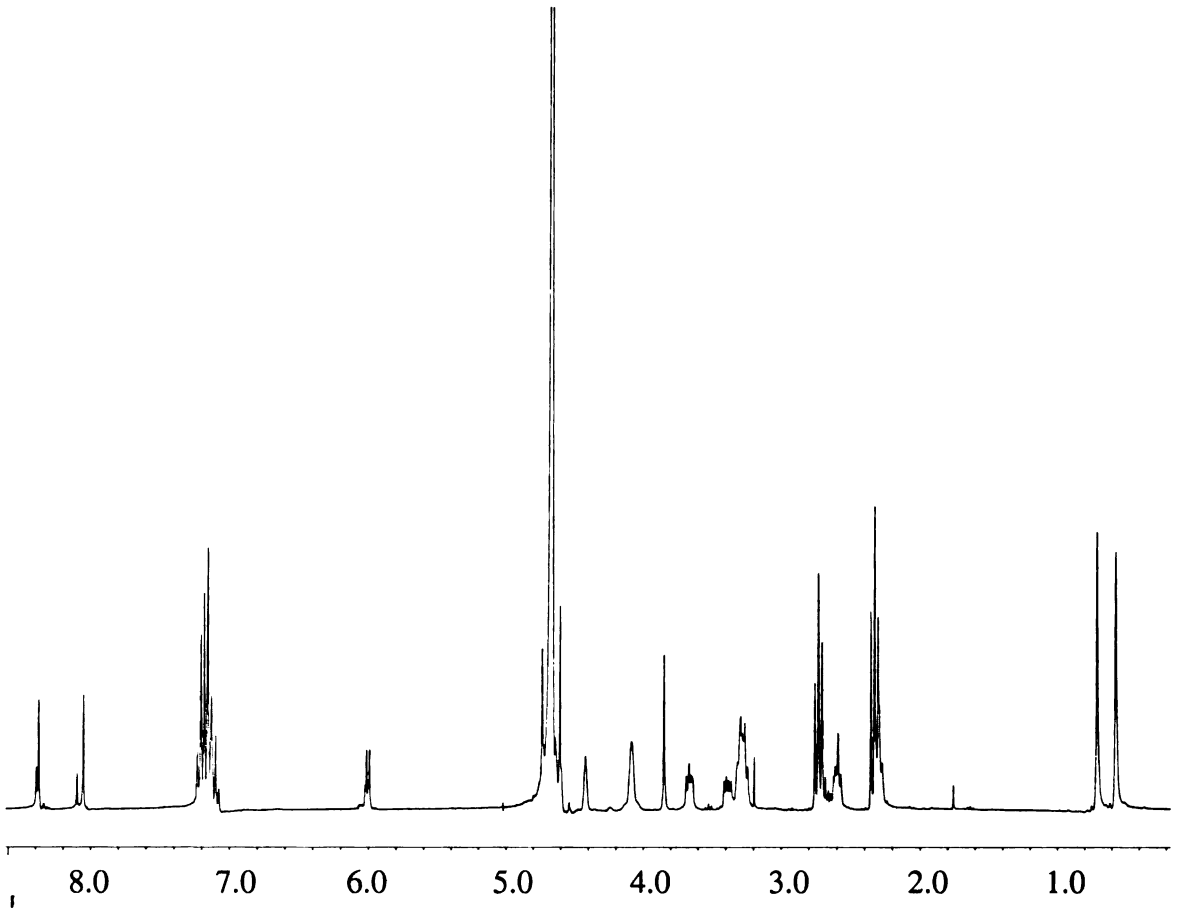
*Spectra 11: ^{1}H NMR for N-*Boc β-phenylpropanoyl CoA



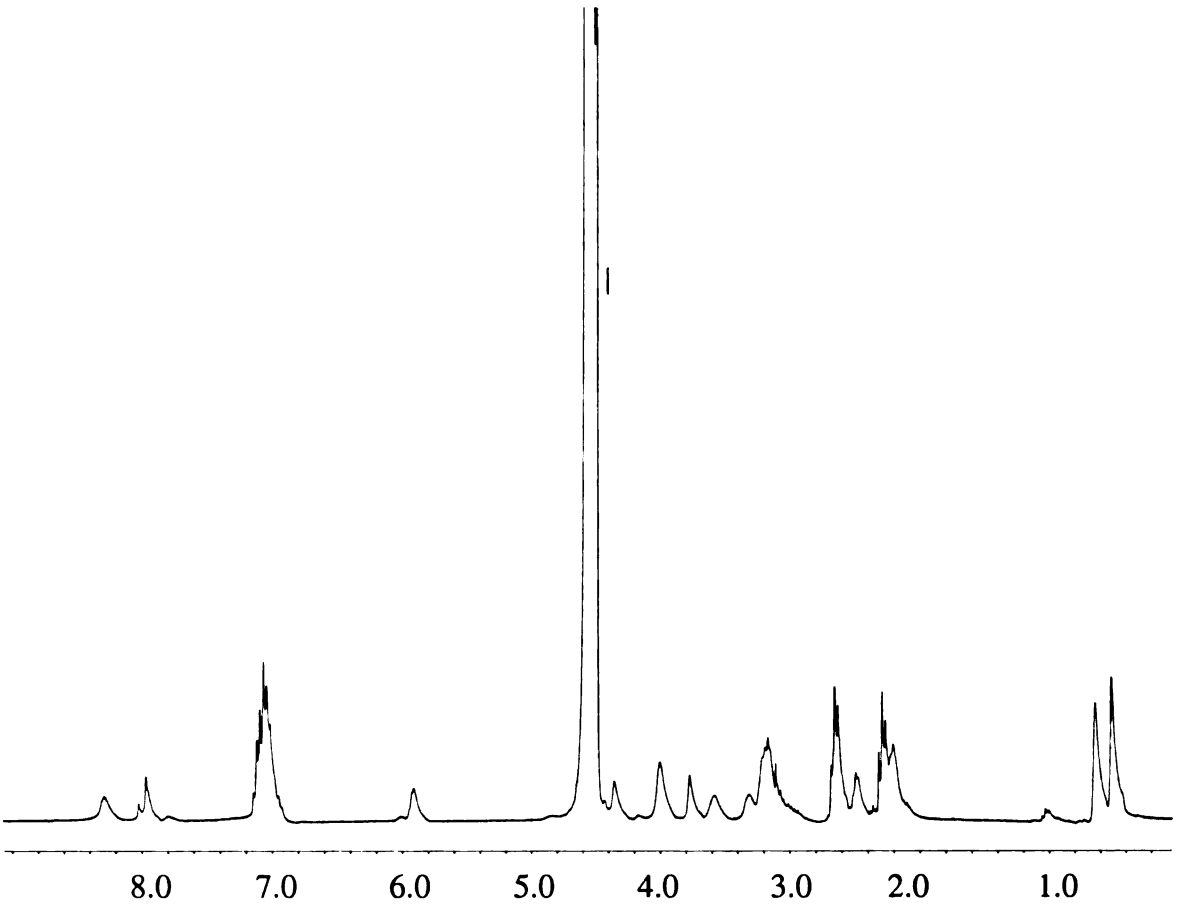
Spectra 12: ^{1}H NMR for N- β-phenylpropanoyl CoA



Spectra 13: ^{1}H NMR for 3-Phenylpropanoyl CoA

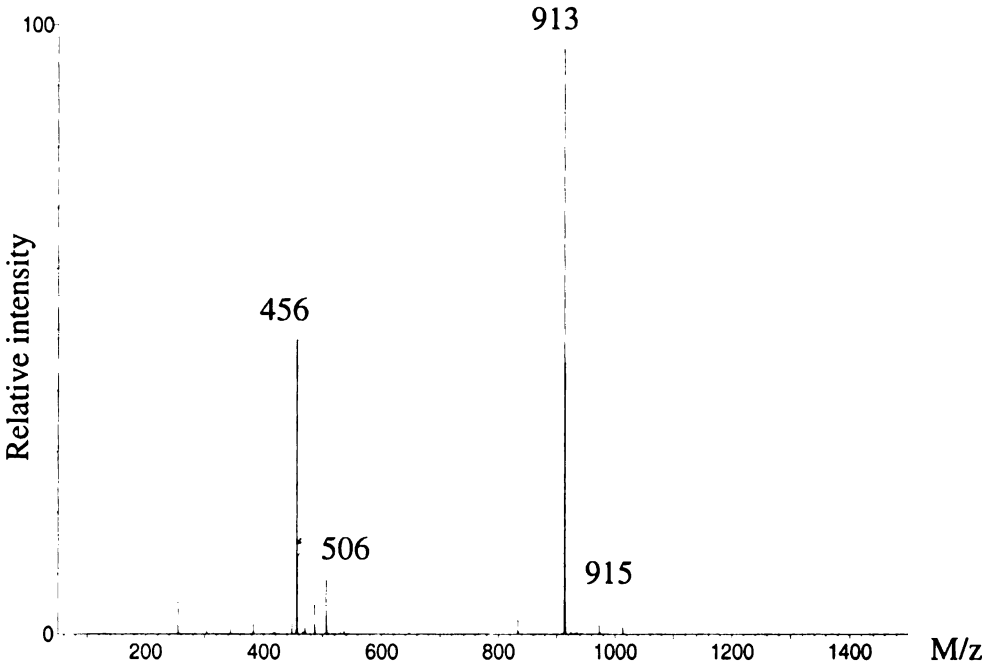


Spectra 14: ^{1}H *NMR for* 3-Hydroxy-3-phenylpropanoyl CoA



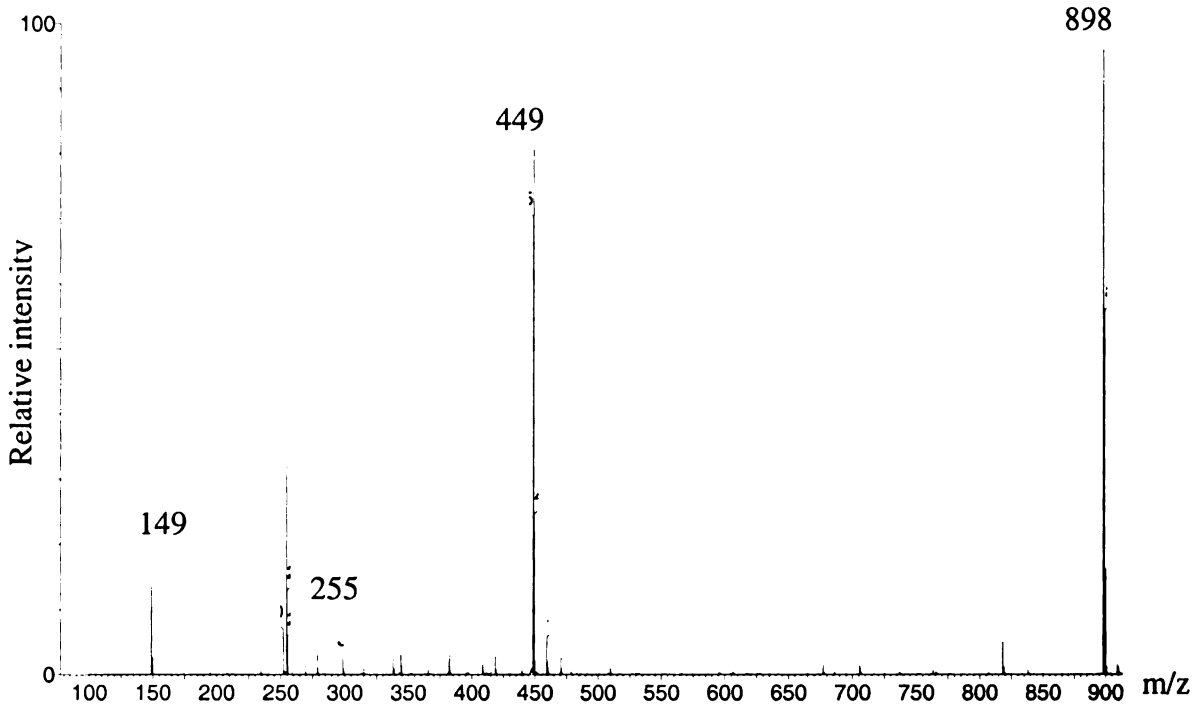
Electro spray Mass spectrum for CoA thioesters

Spectra 15: ESI-MS (-) for β-phenylalanoyl CoA



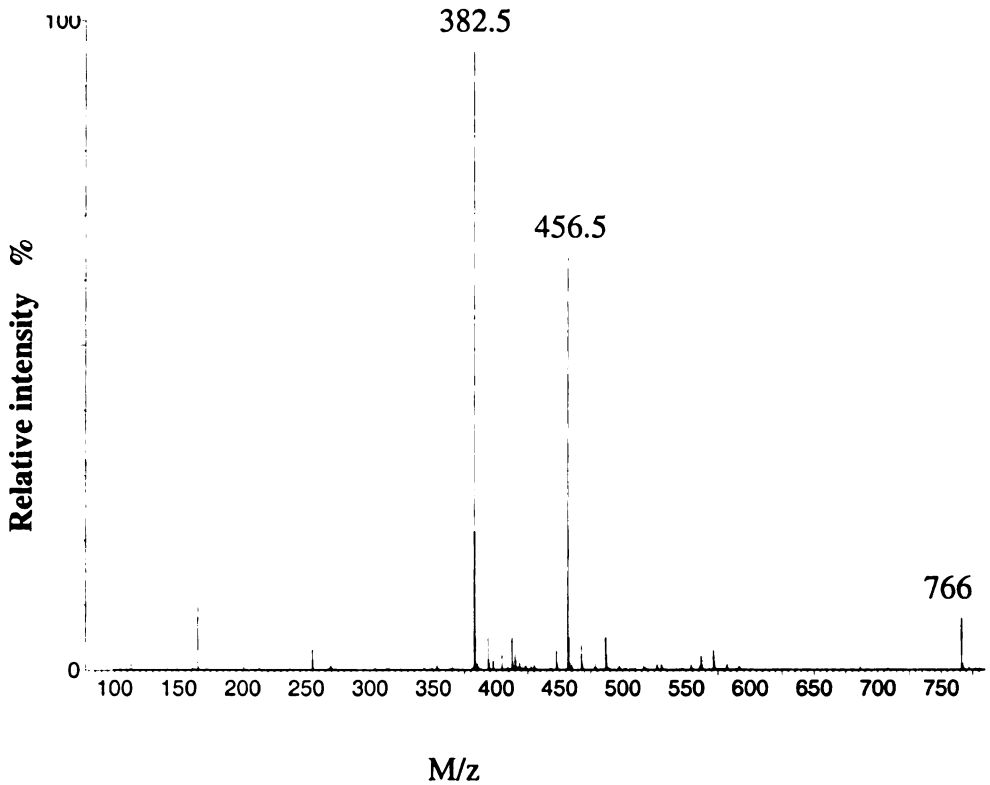
ESI-MS of *R*-β-phenylalanoyl CoA $[M-H]^{-} = 913$, $[M-2H]^{-2} = 456.0$

Spectra 16: ESI-MS (-ve) for Phenylpropanoid CoA



ESI-MS (-) 3-phenylpropanoyl CoA $[M-2H]^{-2} = 448.5$ $[M-H]^{-} = 898$,

Spectra 17: ESI-MS (-ve) for 3-hydroxy-3-phenylpropnoyl CoA



ESI-spectra of 3R-hydroxy -3-phenyl propanoyl CoA, the result is dominated by the CoA salt, 456.5 $[M-2H]^{-2}$. 382.5 [HSCoA-2H]

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