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A BROADLY SPECIFIC BENZOATE COENZYME A LIGASE IS COUPLED WITH *TAXUS* ACYLTRANSFERASES *IN VITRO* TO BIOSYNTHESIZE PACLITAXEL ANALOGUES

presented by

Sean Austin Sullivan

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Chemistry

Major Professor's Signature

MAY 13, 2010

Date

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By

Sean Austin Sullivan

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Chemistry

2010

ABSTRACT

A BROADLY SPECIFIC BENZOATE COENZYME A LIGASE IS COUPLED WITH *TAXUS* ACYLTRANSFERASES *IN VITRO* TO BIOSYNTHESIZE PACLITAXEL ANALOGUES

By

Sean Austin Sullivan

Herein an optimized *in vitro* coupled assay system that couples the activity of a benzoate coenzyme A ligase and NDTBT is described. Overall product yields are within experimental error (± 5 %) of those found in comparable NDTBT *in vitro* assays with the natural co-substrates. Moreover, it will be demonstrated that the current coupled assay system can be used to biocatalyze several *N*-acyl-*N*-debenzoyl-2′-deoxypaclitaxel analogues, as well as paclitaxel. As important, *in vitro* coupled assays with another *Taxus* 2-*O*-acyltransferase, designated *m*TBT, are also demonstrated, including the effective production of multiple 2-acyl-2-debenzoyl-7,13-diacetylbaccatin III analogues. Paclitaxel analogues with similar functionality to those produced herein have been identified as more efficacious than paclitaxel or docetaxel. The advantages to the coupled assay system include improved substrate availability (compared to *in vitro* assays for the described acyltransferases), coenzyme A recycling capabilities, and counteractivity against benzoyl coenzyme A degradation in solution.

To my mother, Monica Sullivan

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my friends and family for all of their support and understanding throughout this process. I would also like to thank my fellow graduate students in Dr. Walker's lab for experimental support. Danielle Nevarez, Irosha Nawarathne, Behnaz Shafii, and Yemane Mengistu deserve special thanks for their continued assistance while working with the enzymes and substrates researched herein. The undergraduate student Noelle Byrne also deserves thanks for her technical assistance. Finally, I would like to thank Dr. Kevin Walker for his guidance and advice, as well as the other members of my committee, Dr. Dana Spence and Dr. Jetze Tepe.

This research could not have been completed without the *badA* cDNA generously provided by the University of Washington (Seattle, WA). I would also like to thank the Michigan State University Mass Spectrometry Facility and the Genomics Core.

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LIST OF ABBREVIATIONS

Absorption Measurement at 210 nm	A_{210}
Absorption Measurement at 228 nm	A_{228}
Absorption Measurement at 600 nm	A_{600}
Adenosine Triphosphate	ATP
Benzoate Coenzyme A Ligase (specific)	BadA
Benzoate Coenzyme A Ligase (general)	BzCoA Ligase
Benzoate Coenzyme A Ligase Gene Sequence	*badA*
Benzoic Acid	BzOH
Benzoyl Coenzyme A	BzCoA
Bristol-Myers Squibb	BMS
Bovine Serum Albumin	BSA
Centimeter	cm
Collision Induced Dissociation	CID
Coenzyme A	CoASH
Confer	cf.
Electrospray Ionization – Tandem Mass Spectrometry	ESI-MS/MS
Environmental Protection Agency	EPA
Gram	g
Gravity	*g*
High Performance Liquid Chromatography	HPLC
Hour	h

Immobilized-Metal Affinity Chromatography	IMAC
Initial Velocity	v_0
Isopropyl-β-D-thiogalactopyranoside	IPTG
Kilogram	kg
KiloDalton	kDa
Liquid Chromatography – Electrospray Ionization Mass Spectrometry	LC-ESI-MS
Liter	L
Luria-Bertani	LB
Maximum Velocity	V_{max}
Michaelis Constant	K_M
Microgram	μg
Microliter	μL
Micrometer	μm
Micromolar	μM
Milligram	mg
Millimeter	mm
Millimolar	mM
Minute	min
Molar	M
Modified TBT	*m*TBT
Molecular Weight Cutoff	MWCO
Nanometer	nm
Nanomole	nmol

National Cancer Institute	NCI
Normal	N
N-Debenzoyl-(3′*R*)-2′-deoxypaclitaxel	NDB2DT
N-Debenzoyl-2′-deoxypaclitaxel:*N*-benzoyltransferase	NDTBT
Nitriloacetic Acid	NTA
Optical Density at 600 nm	OD_{600}
Plant Cell Culture	PCC
Polymerase Chain Reaction	PCR
Proton Nuclear Magnetic Resonance	^{1}H-NMR
Quadrupole – Time of Flight Mass Spectrometer	Q-ToF
Second	s
Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis	SDS-PAGE
Species	spp.
Thin Layer Chromatography	TLC
Triethanolamine	TEA
Trifluoroacetic Acid	TFA
Tris(hydroxymethyl)aminomethane	Tris
Turnover Number	k_{cat}
Ultraviolet	UV
Volt	V
2′-Deoxypaclitaxel	2DOT
2-*O*-Deacyltaxane:2-*O*-benzoyltransferase	TBT
10-Deacytelbaccatin III	10-DAB

INTRODUCTION

Natural products remain some of the most potent medications used in the pharmaceutical sector to date. Many diseases are treated with natural products or with bioactive compounds derived from natural product precursors.[3] The National Cancer Institute (NCI) conducted a large-scale screen of flora in the Pacific Northwest (USA), out of which paclitaxel (Taxol®) was discovered as an active component in the Pacific yew tree (*Taxus brevifolia*).[4] Paclitaxel was found to be an antimitotic agent that stabilizes the formation of microtubules during cell division, and thus induced cellular apoptosis.[5,6,7] Since its approval for clinical use in 1992, paclitaxel and its synthetically derived analogue docetaxel (Figure 1) have been used to treat multiple cancer types, including ovarian, breast, lung, head, and neck carcinomas.[8] Paclitaxel has also been used in the treatment of coronary artery disease as a preventive measure to reduce the occurrence of in-stent restinosis.[9] In addition, there is some interest in using paclitaxel in the treatment of Alzheimer's disease where it is currently being used as a research tool to counter the effects of the degraded microtubulin cytoskeleton in neuronal cells of patients suffering from this dementia affliction.[10] The broad range of both research and medical uses for paclitaxel (and its analogue docetaxel) has presented a strong demand for this successful antimitotic drug, as well as for modified paclitaxel analogues that could prove more effective in medical applications.

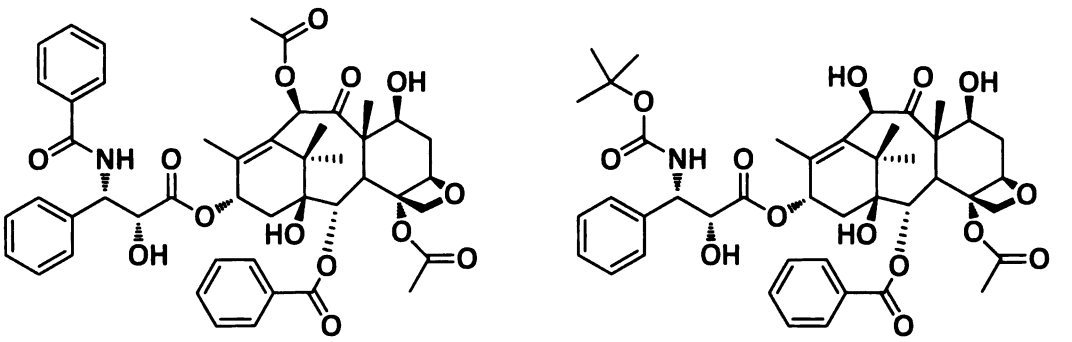

Figure 1. The antineoplastic taxanes paclitaxel (left) and docetaxol (right).

The original source of paclitaxel for preclinical trials was the natural resource *T. brevifolia*, from which only a dearth of paclitaxel could be isolated (1 kg paclitaxel per 6000 kg of *T. brevifolia* bark, i.e. 0.02 % w/w).[11] Harvesting the bark from the plant was a sacrificial process that depredated the plant from old growth forests.[4] The Environmental Protection Agency (EPA) issued an edict that forced the research community to look for alternative methods of production of the pharmaceutical that would be both more efficient and environmentally friendly. The alternative methods for production that have been researched so far include semisynthesis from naturally occurring taxane precursors and plant cell cultures derived from *Taxus* plant species.[12-14] While the total synthesis of paclitaxel was achieved in the 1990's by several different groups,[15,16,17,18,19,20,21,22,23] these syntheses presented elegant examples of multi-step organic chemistry transformations, and were never truly intended to address drug supply issues. Completing the total synthesis of paclitaxel has inspired the synthesis of many other natural products, and has also contributed to some of the methods of production used presently.[24]

The methods used for paclitaxel production today represent the culmination of decades of research; however, these processes do not fully address production efficiency and minimization of environmental impact. The first significant route towards paclitaxel production was a semisynthetic method, which relied on harvesting the leaves of *Taxus* saplings and extracting relatively abundant natural products 10-deacetylbaccatin III (10-DAB) and baccatin III, which are late-occurring metabolites on the paclitaxel biosynthetic pathway.[12] While these precursors can only be isolated in relatively low yields of 0.1 % (w/w) from the leaves, the semisynthetic method utilizes renewable leaves as a source of paclitaxel and its precursors without killing the slow-growing tree.[25] The original contracted producer of Taxol®, Bristol-Myers Squibb (BMS), has been influential in continuing the search for more environmentally-responsible and efficient methods for paclitaxel and its analogues.[4] The current method of synthesis employed by BMS, and other generic companies, is a plant cell culture (PCC) process that is considered a greener method of production compared to the previously used semisynthetic routes.[14] Overall yields of paclitaxel reported for the originally published plant cell culture method range from 0.012 – 0.05 %, which is estimated at 0.3 mg of paclitaxel per gram of dry cell weight per day, which can be maintained for up to 40 days.[13,14] These methods, considered more efficient and green than previous semisynthetic methods, have allowed the supply of paclitaxel to meet the demand of medical and research interests, which is estimated at one metric ton.[26]

While current PCC methods are addressing the supply issue, there remains a sustained demand for paclitaxel in basic research and in the development of taxane

analogues that may aid in the continued fight against cancer and other diseases. Currently, most paclitaxel analogues are only available via the semisynthetic methods due to the lack of investigating the utility of PCCs to manufacture the non-natural paclitaxel compounds *in vivo*.[4] Structure-activity relationships studying the necessity of the different functional groups attached to the taxane core have revealed that some analogues with *N*-acyl-*N*-debenzoyl-substitutions may be equally or more efficacious than the currently used paclitaxel or docetaxel.[27] While interest in such analogues is high,[28] supply issues still plague further studies of such compounds. One possible method for producing such analogues is the engineering of a transgenic host expressing all the necessary genes on the paclitaxel biosynthetic pathway. Current work on such a method has been characterized in yeast (*Saccharomyces cerevisiae)*; however, as of yet, taxadiene is the only diterpenoid that has been successfully isolated from such cultures.[29] Several more enzymatic steps are involved in synthesizing paclitaxel from taxadiene, thus still more research is necessary before such a system can become feasible for industrial scale-up. Two of the enzymes already identified in the final steps of the natural paclitaxel biosynthetic pathway are *N*-debenzoyl-2′-deoxypaclitaxel:*N*-benzoyltransferase (NDTBT) and 2-*O*-deacyltaxane:2-*O*-benzoyltransferase (TBT) have been suggested as possible enzymes for such a transgenic host.[2,30] Both of these *Taxus* acyltransferases exhibit broad substrate specificity that could potentially be used to construct paclitaxel analogues. However, the ability for the biocatalytic production of such analogues relies on the availability of the acyl-coenzyme A (acyl-CoA) co-substrate in the catalyzed reaction.

Currently, the *in vitro* assay procedure described in the analysis of NDTBT and *m*TBT requires incubation with acyl-CoA substrates obtained commercially or through synthetic methods where an acyl acid is coupled to CoASH to form the corresponding thioester.[2,30] The prohibitively high cost of commercially available acyl-CoA substrates is demonstrated in Table I.

Table I. The cost of several acyl-coenzyme A substrates (Sigma-Aldrich).

Acyl-Coenzyme A Substrate	Cost/5 mg
2-Butenoyl Coenzyme A	$ 186.50
Acetyl Coenzyme A	$ 57.07
Arachidonoyl Coenzyme A	$ 180.00
Benzoyl Coenzyme A	$ 184.50
Butyryl Coenzyme A	$ 106.00
Decanoyl Coenzyme A	$ 102.50
Hexanoyl Coenzyme A	$ 119.50

While these acyl-CoA substrates can be synthesized at high reported yields, the general syntheses require several organic solvents and purification steps.[30] One possible alternative to using these costly substrates in assays with the acyl-CoA-dependent acyltransferase is to incubate the enzymes in the presence of a benzoate coenzyme A ligase (BzCoA ligase). Such a coupled assay system would prevent the necessity of completing the difficult mixed-phase syntheses or purchasing the expensive acyl-CoA substrates.

Previous research has been conducted wherein an acyl-CoA synthetase was used to recycle free CoASH in chemical reactions;[31,32,33] however, none of the past research has shown recycling of CoASH with a broadly specific BzCoA ligase. Moreover, there

are no accounts demonstrating such a coupled assay system proposed herein for the production of paclitaxel analogues. The research described in this thesis aims to test the feasibility of a coupled enzyme assay system where a BzCoA ligase from *Rhodopseudomonas palustris* produces the necessary acyl-CoA substrate for the reaction catalyzed via NDTBT or *m*TBT with their respective, diterpene substrates.

CHAPTER 1

Expression, Activity, and Further Studies with Benzoate Coenzyme A Ligase

1.1: Introduction

While plant cell culture methodologies currently dominate the market for production of paclitaxel,[13,14] further studies into heterologous expression hosts could provide a more efficient and environmentally friendly method of production. A heterologous expression host would require multiple enzymes in the paclitaxel biosynthetic route to effectively biocatalyze the complex diterpenoid molecule. Many of the acyltransferases involved in the paclitaxel biosynthetic route in *Taxus* plants have been identified and characterized;[34] however, many of the other important enzymes within the pathway have not yet been elucidated.[34] One of these important enzymes is a putative benzoate coenzyme A ligase (BzCoA ligase), which catalyzes the formation of a thioester bond between benzoic acid (BzOH) and coenzyme A (CoASH), thus yielding benzoyl coenzyme A (BzCoA). BzCoA is an important substrate for the *Taxus* acyltransferase enzymes, yet is not described in many of the feasible hosts for transgenic expression.[35] Since the pertinent BzCoA ligase from *Taxus* plants has not been identified, any heterologous host employed to construct novel paclitaxel or its precursors would require a novel ligase be introduced into the system through a genetic engineering methodology.

The necessity to identify a BzCoA ligase for heterologous expression in a suitable host is clear. More important, the selected BzCoA ligase can offer many added benefits, including a broad substrate specificity range and a high turnover number. The research presented here focuses on the utilization of a BzCoA ligase (BadA), isolated from

Rhodopseudomonas palustris (*R. palustris*), in the production of paclitaxel and baccatin III analogues by coupling BzCoA production to the activity of two acyltransferases involved in the paclitaxel biosynthetic pathway. The reaction catalyzed by the reported BadA is shown in Figure 2. This specific BadA enzyme was chosen because of its previously reported broad substrate specificity, high turnover number, and availability.[1,36]

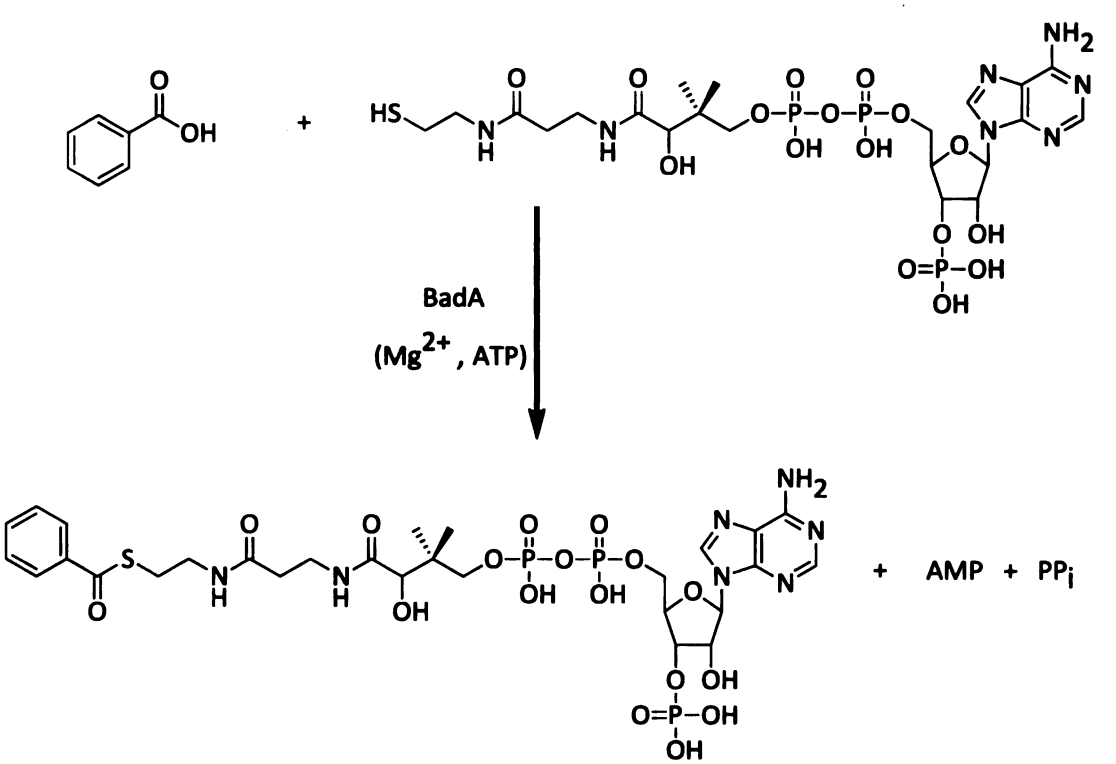

Figure 2. Enzymatic activation of BzOH with CoASH to yield BzCoA proceeds via the activity of BadA, which requires both Mg^{2+} and ATP as cofactors.[1]

1.2: Experimental Section

General. An Agilent 1100 high performance liquid chromatography (HPLC) system (Agilent Technologies, Wilmington, DE) connected to a UV detector was used for separation and analysis of assays. Solvents of reagent grade were obtained from Sigma-Aldrich. Restriction endonucleases were purchased from New England Biolabs (Ipswich, MA). In terms of the performed biochemical work, including microbial and recombinant techniques, most are described in detail by Sambrook.[37]

Substrates. Benzoic acid (BzOH) was purchased from Mallinckrodt (Phillipsburg, NJ). All other acyl-carboxylic acids, as well as BzCoA, adenosine triphosphate (ATP), and $MgCl_2$, were purchased from Sigma-Aldrich. Coenzyme A (CoASH) was obtained from ARC (St. Louis, MO). Initial studies were completed with laboratory stocks of synthesized BzCoA via literature reported syntheses.[30]

Bacterial strains and culture components. All media solutions were prepared in distilled, deionized water. Luria-Bertani (LB) medium contained tryptone (10 g) (Bacto Laboratories Pty Ltd, Australia), yeast extract (5 g) (Bacto) and NaCl (10 g) (Sigma) or 25 g of LB mixture (Neogen, Lansing, MI) per 1 L of distilled, deionized water. Solid medium was prepared by the addition of 1.5% (w/v) agar (Bacto) to the medium with appropriate antibiotics. All media was sterilized via autoclave.

The antibiotic kanamycin (Sigma) was added where appropriate to a concentration of 50 μg/mL. Isopropyl-β-D-thioglucopyranoside (IPTG) (Gold Biotechnology, St. Louis, MO) was added where appropriate to a final concentration of 100 μM. Stock solutions of kanamycin and IPTG were prepared in distilled, deionized

water. Antibiotic and IPTG stock solutions were filter-sterilized through 0.22-μm membrane filters (Millipore, Billerica, MA).

Subcloning *badA* gene from *R. palustris* genome. The Harwood group at the University of Washington (Seattle, WA) generously provided two plasmids pPE202 and pPE204, which contain fragments of the *R. palustris* genome that include the *badA* gene.[36] The *badA* gene was subcloned and directionally inserted into the pET28a vector (Novagen), which incorporated an N-terminal His_6-tag epitope. This was accomplished through amplification of the *badA* gene with the forward primer set (5′-TATGAATGCAGCCGCGGTC-3′; 5′-TGAATGCAGCCGCGGTCAC-3′) and reverse primer set (5′-GTCAGCCCAACACACCC-3′; 5′-TCGAGTCAGCCCAACACACC-3′). The resultant amplicon contained the *badA* gene and incorporated NdeI and XhoI cut sites for directional insertion into the pET28a vector. The recombinant *badA* plasmid, henceforth referred to as pBadA, was transformed into *Escherichia coli* (*E. coli*) cell lines DH5α (for plasmid storage) and BL21(DE3) (for overexpression) following supplier's protocol (Stratagene, La Jolla, CA).

Plasmid sequencing was carried out by the MSU Genomics Core on an ABI PRISM® 3730 Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA), and sequencing comparison was completed using DNASTAR Lasergene software (DNASTAR, Inc., Madison, WI). The pBadA vector containing the *badA* gene purified from the DH5α *E. coli* cell line with the forward primer 5′-CTGCTGACGGCGGACGACTACG-3′ and the reverse primer 5′-TTGGTCAGGCCGTGTTCGTCGG-3′.

BadA overexpression in *E. coli* and purification. The overall procedure for protein overexpression and purification is adapted from previously reported procedures used in the overexpression and purification of NDTBT,[30] with necessary modifications for optimal expression of *badA*. The recombinant *badA* gene was expressed using the BL21(DE3) *E. coli* cell line transformed with the pBadA vector. As described in the company's technical bulletin (Stratagene, La Jolla, CA), the BL21(DE3) cell line contains a T7 polymerase gene inserted into the bacterial chromosome; this gene is induced following the introduction of IPTG. Aliquots (5-mL) of LB medium supplemented with 50 μg/mL kanamycin were inoculated with the described *E. coli* containing the recombinant *badA* gene and incubated overnight at 37 °C. The 5-mL cultures were then used to inoculate 1-L cultures of LB medium, also supplemented with 50 μg/mL kanamycin, and grown at 37 °C to an OD_{600} = 0.7 – 1.0, at which point *badA* gene expression was induced with 100 μM IPTG. Cultures were then grown for 18 h at 18 °C. The cells were harvested by centrifugation at 2000*g* at 4 °C for 20 min; the supernatant was then discarded. The remaining cell pellet was resuspended in 5-mL of Lysis Buffer (50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole at pH 8.0) per gram wet weight. Sonication was used to lyse the resuspended cells with a Misonix XL-2020 sonicator (Misonix Inc., Farmingdale, NY), where the sonicator was set at 50 % power for six consecutive 20 s bursts with 1 min intervals. Clarification of the cell-lysate was accomplished through ultracentrifugation at 149,000*g* for 1.5 h at 4 °C. The clarified cell-lysate will henceforth be referred to as the crude lysate. Cell pellets not immediately used for BadA purification were stored at -20 °C.

Purification of the expressed BadA protein from the crude lysate was carried out using immobilized-metal affinity chromatography (IMAC) in a batch mode. Specifically, the crude lysate (adjusted to pH 8.0) was incubated with Ni - Nitrilotriacetic acid (NTA) Agarose resin (QIAGEN, Valencia, CA) for 10 min at 4 °C with constant shaking. The resin and crude lysate mixture was then transferred to a Poly-Prep® column (BioRad, 0.8 x 4 cm) and the remaining buffer was drained. The resin was then washed with three column volumes of Lysis Buffer to remove any unbound proteins from the column. Bound protein was eluted in a stepwise fashion using one column volume of each of the following buffers: 10 mM, 50 mM, 100 mM, 150 mM, 200 mM, and 250 mM imidazole in Lysis Buffer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify the eluent aliquots containing the highest percentage of purified BadA (described by Sambrook).[37] Acrylamide gels (12 %) cast in a 0.75 mm x 8.3 cm x 7.2 cm mold were used, and electrophoresis was completed in a Bio-Rad apparatus (Mini-PROTEAN Tetra Cell, Bio-Rad) set at 140 V and run until the Coomassie dye reached the bottom of the gel. Protein samples were prepared by diluting 5-μL of sample (adjusted depending on sample volume) to a total volume of 20-μL with sample buffer, boiling the solution at 97 °C for 10 min to ensure protein denaturation, followed by centrifugation to pool the total 20-μL. SDS-PAGE ladder Protein ProSieve® Markers (50547; Lonza, Rockland, ME) was used as a protein standard. Gels were then visualized using Coomassie Blue dye. The aliquots containing purified BadA were then identified and combined, and imidazole was removed using consecutive dilution and concentration, accomplished with 30,000 molecular weight cutoff (MWCO) filters (regenerated

cellulose membrane, Millipore, Billerica, MA). Centrifugation was achieved at 4,000*g* at 4 °C for 20 min. Following concentration, the retentate was washed with Assay Buffer (50 mM sodium phosphate, 5% glycerol, pH 8.0) until imidazole concentration was reduced to 1.5 μM.

Protein concentration was determined via the Bradford method and comparison to bovine serum albumin (BSA) standards. A Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL) was used to prepare all standards and the A_{600} was used for the construction of a Bradford assay calibration curve.

After determining final BadA purity and concentration, the enzyme solution was diluted to 10 mg/mL with Assay Buffer, and 50-μL aliquots, previously flash frozen with liquid nitrogen, were stored at -80 °C.

BadA activity assay. Following purification using Ni-NTA Agarose resin, the activity of the BadA enzyme was tested using the following assay conditions, adapted from previous work by Geissler et al.[1] Briefly, a reaction mixture containing 2.5 mM $MgCl_2$, 1.0 mM ATP, 0.25 mM CoASH, 0.25 mM BzOH and 100 μg/mL BadA in a 20 mM triethanolamine-HCl (TEA) buffer at pH 8.0 was prepared on ice. Following incubation at 31 °C for 10 min, the enzyme activity was quenched with 0.5 N HCl to pH < 1.0.

Because coupled assays with NDTBT were to be completed in the described Assay Buffer (as this buffer has been used in previous studies of NDTBT),[30] the activity of BadA was compared in both the TEA buffer as well as the Assay Buffer. Results from these comparisons showed no statistical difference in activity between the two buffers, hence the Assay Buffer was used in all reported studies on BadA activity.

Following the acid quench, an aliquot from each assay was subjected to HPLC and the eluent was analyzed by ultraviolet (UV) absorbance at 210 nm. Initial assay analyses were completed using the mobile phase gradient as described by Geissler et al.[1] Briefly, separation was achieved via injection with an Agilent 1100 HPLC system (Agilent) onto a C_{18} reverse-phase column (Atlantis™ dC_{18} - 3 μm; Waters, Milford, MA) and elution was completed using a gradient mobile phase composed of solvent A/solvent B [solvent A: 99.9 % H_2O with 0.1 % trifluoroacetic acid (TFA) (v/v); solvent B: 99.9 % acetonitrile with 0.1 % TFA; gradient: 0 – 40 min at 2 – 40 % (B)].[1] However, this method was later altered to allow for more efficient separations without loss of peak resolution. The altered separation technique was achieved using the same instrument and column as before, except the following gradient mobile phase composition was used: [solvent A: 99.9 % H_2O with 0.1 % TFA (v/v); solvent B: 99.9 % acetonitrile with 0.1 % TFA (v/v); gradient: 0 – 5 min at 2 – 17 % (B), 5 – 18 min at 17 – 30 % (B), 18 – 20 min at 30 – 40 % (B), and 20 – 22 min at 40 – 2 % (B)] with a flow rate of 1.0 mL/min and a total run time of 22 min. Detection was completed by monitoring the A_{210} of the effluent. Peak identities in a typical chromatogram were determined through injection of substrate and product standards.

BzCoA product amounts were quantified through comparison to a calibration curve constructed using commercially available BzCoA (see Substrates section above). The calibration curve was constructed using serial dilutions of a 500 μM BzCoA stock solution prepared in distilled, deionized water at pH 5.0 (see Figure 3 on the next page). An acidic solution was used to stabilize the thioester bond in BzCoA, which is prone to

hydrolysis in basic solutions.[38] The reported calibration curve had a linear dynamic range of 0.313 – 10.0 nmol of BzCoA (corresponding to a concentration range of 7.81 – 250 μM BzCoA). At higher concentrations of BzCoA, the analytical signal was no longer linearly dependent on BzCoA amounts.

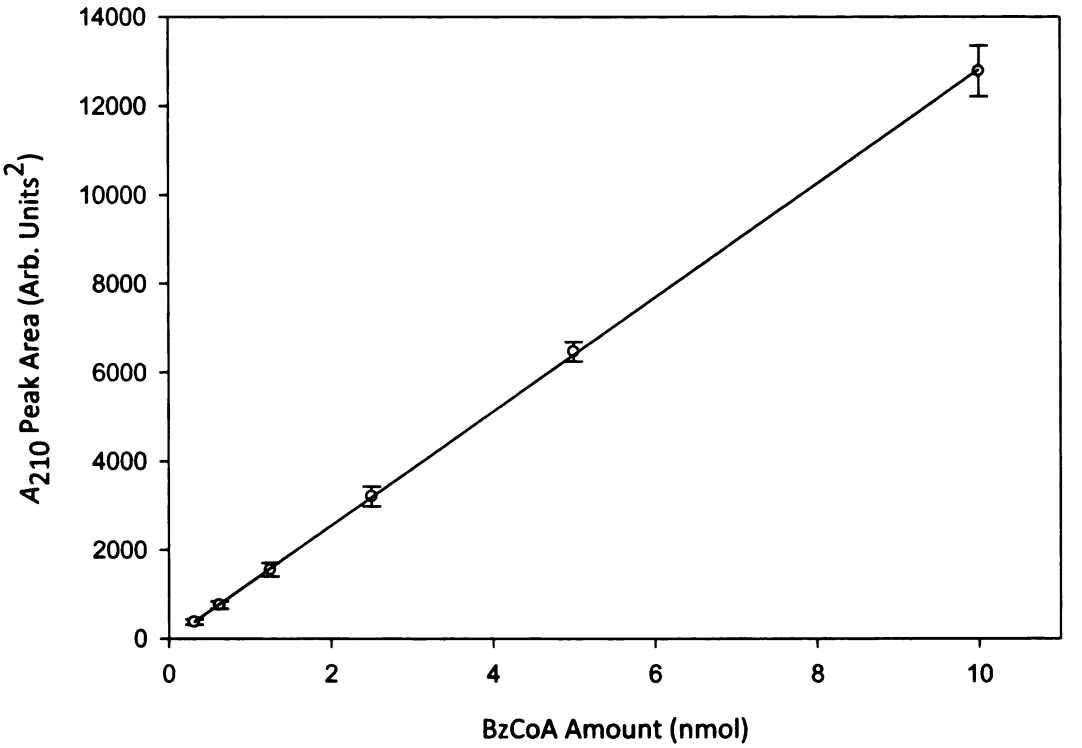

Figure 3. Calibration curve for BadA product quantitation using A_{210} Peak Area as a function of BzCoA Amount, reported in nmol. The 40 μL injection volume allows for conversion of BzCoA amount to concentration. Error bars are reported as the standard deviation of three separate measurements.

While the construction of this calibration curve may seem pedestrian, there were several initial problems when attempting to quantify product amounts from BadA reactions. Several attempts at generating a BzCoA calibration curve were made using a synthesized BzCoA from laboratory stocks. The detailed synthesis of this substrate is

reported fully in literature by Nevarez, et al.,[30] apparent purity was assigned through several methods of analysis, including proton nuclear magnetic resonance (^{1}H-NMR), electrospray ionization – tandem mass spectrometry (ESI-MS/MS) fragmentation, and thin layer chromatography (TLC) preparation. Issues in this thesis research arose when reported product amounts of the BadA reaction exceeded possible theoretical yields by up to 50 %. As the controls run alongside the BadA activity assays showed no residual BzCoA present in any of the other assay components, including the purified enzyme fraction, it was hypothesized that the BzCoA used to construct the calibration curve was not as pure as previously reported(at 95 – 99 %).[30] For this reason, 5 mg of BzCoA was purchased from Sigma to reconstruct a more accurate standard curve. The final calibration curve reported in Figure 3 was therefore constructed using the commercially available BzCoA rather than the synthesized version available from laboratory stocks.

BadA substrate specificity. The two *Taxus* acyltransferases used in later studies exhibit broad substrate specificity with regard to the acyl-CoA substrate. For this reason, the substrate specificity of BadA was tested using four different acyl-carboxylic acid substrates, of which the products correspond to four of the reported acyl-CoA substrates for the *Taxus* acyltransferases.[30,2] The activity of BadA with the four acyl-carboxylic acids 4-methylbenzoic acid, 2-fluorobenzoic acid, 3-furoic acid, and thiophene-2-carboxylic acid was tested using similar assay conditions to those described for BadA activity assays. Briefly, 2.5 mM $MgCl_2$, 1.0 mM ATP, 0.25 mM CoASH, and 0.25 mM carboxylic acid were added to a reaction vessel and diluted to final concentrations with Assay Buffer. The reaction vessel was incubated at 31 °C for 7.5 min, followed by an acid quench with 0.5 N HCl to pH < 1.0. Controls for these substrate specificity studies

were included so that the presence of any *de novo* peaks could be assigned to putative product formation; these controls also allowed for identification of acyl-carboxylic acid peaks in the resultant chromatograms. Separation and analysis was completed using the non-optimized HPLC method described by Geissler et al.[1] Further verification of the production of the expected acyl-CoAs was completed through the coupled assays with both NDTBT and *m*TBT, as discussed in Chapter 2.

1.3: Results and Discussion

Subcloning *badA* gene from *R. palustris* genome. The sequence obtained from the source plasmid pPE204[36] matched the extant recombinant *badA* gene subcloned herein (Figure 4). These results lend support to the conclusion that the BadA used for further studies herein is the same enzyme characterized in previous research.

```
atgaatgcagccgcggtcacgccgccacccgagaagtttaattttgccgagcacctgctgcag
 M  N  A  A  A  V  T  P  P  P  E  K  F  N  F  A  E  H  L  L  Q
accaatcgcgtgcggccggacaagacggcgttcgtcgacgacatctcgtcgctgagcttcgcg
 T  N  R  V  R  P  D  K  T  A  F  V  D  D  I  S  S  L  S  F  A
caactcgaagctcagacgcgtcagctcgccgccgccttacgcgcgatcgggtgaaacgcgaa
 Q  L  E  A  Q  T  R  Q  L  A  A  A  L  R  A  I  G  V  K  R  E
gagcgcgtgctgctgctgatgctcgacggcacggattggccggtggcgtttctcggcgcaatc
 E  R  V  L  L  L  M  L  D  G  T  D  W  P  V  A  F  L  G  A  I
tacgccggcatcgtgccggtcgcggtcaatacgctgctgacggcggacgactacgcctacatg
 Y  A  G  I  V  P  V  A  V  N  T  L  L  T  A  D  D  Y  A  Y  M
ctcgagcattcgcgggctcaggccgtgctggtcagcggcgcgctgcacccggtgctcaaggca
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 A  L  T  K  S  D  H  E  V  Q  R  V  I  V  S  R  P  A  A  P  L
gagccgggcgaggtcgacttcgctgagttcgtcggcgcacatgcgccgcttgagaagcctgcc
 E  P  G  E  V  D  F  A  E  F  V  G  A  H  A  P  L  E  K  P  A
gctacgcaagcggacgatccggcgttctggctgtattcgtcgggttctaccgggcggccgaag
 A  T  Q  A  D  D  P  A  F  W  L  Y  S  S  G  S  T  G  R  P  K
ggcgtggtgcacactcacgccaatccgtactggacctcggagctgtacggccgcaacacgctg
 G  V  V  H  T  H  A  N  P  Y  W  T  S  E  L  Y  G  R  N  T  L
catctgcgcgaagacgacgtctgcttttcggcggccaaactgtttttcgcttacggcctcggc
 H  L  R  E  D  D  V  C  F  S  A  A  K  L  F  F  A  Y  G  L  G
aacgcgctgacgtttccgatgacggtcggcgcgaccacgctgctgatgggcgagcgaccgacg
 N  A  L  T  F  P  M  T  V  G  A  T  T  L  L  M  G  E  R  P  T
ccggacgcggtgttcaagcgctggctcggcggcgtcggcggtgtgaaaccgaccgtgttctac
 P  D  A  V  F  K  R  W  L  G  G  V  G  G  V  K  P  T  V  F  Y
ggcgcgcccaccggctacgccggcatgttggccgcgccgaacctgccgtcgcgcgaccaggtg
 G  A  P  T  G  Y  A  G  M  L  A  A  P  N  L  P  S  R  D  Q  V
gcgttgcggctcgcgtcgtcggcgggcgaagcactgccggcggagattgggcagcgcttccag
 A  L  R  L  A  S  S  A  G  E  A  L  P  A  E  I  G  Q  R  F  Q
cgccatttcggcctcgacatcgtcgatggcatcggctcgaccgagatgctgcacatctttctg
 R  H  F  G  L  D  I  V  D  G  I  G  S  T  E  M  L  H  I  F  L
tcgaacctgccagaccgggtgcgctacggcaccaccggatggccggtgccgggctatcagatc
 S  N  L  P  D  R  V  R  Y  G  T  T  G  W  P  V  P  G  Y  Q  I
gagctgcgcggcgacggcggcggaccggtcgccgacggagagccgggcgatctctacattcac
 E  L  R  G  D  G  G  G  P  V  A  D  G  E  P  G  D  L  Y  I  H
ggcccgtcatcggcgacgatgtattggggcaaccgggccaagagccgcgacaccttccagggc
 G  P  S  S  A  T  M  Y  W  G  N  R  A  K  S  R  D  T  F  Q  G
ggctggaccaagagcggcgacaaatacgtccgcaacgacgacggctcctacacctatgcgggc
 G  W  T  K  S  G  D  K  Y  V  R  N  D  D  G  S  Y  T  Y  A  G
cgcaccgacgacatgctgaaggtcagcggcatctatgtcagcccgttcgagatcgaagcgacg
 R  T  D  D  M  L  K  V  S  G  I  Y  V  S  P  F  E  I  E  A  T
ctggtgcagcatcccggtgtgctcgaagccgcagtggtcgggtcgccgacgaacacggcctg
 L  V  Q  H  P  G  V  L  E  A  A  V  V  G  V  A  D  E  H  G  L
accaaaccgaaggcctatgtggtgccgcggcccggccagaccctgtcggagaccgagctgaag
 T  K  P  K  A  Y  V  V  P  R  P  G  Q  T  L  S  E  T  E  L  K
accttcatcaaggatcgactggcgccgtacaaatatccgcgcagcacggtgttcgtcgccgaa
 T  F  I  K  D  R  L  A  P  Y  K  Y  P  R  S  T  V  F  V  A  E
ttgccgaagacggcgaccggcaagattcagcgcttcaagctgcgcgagggtgtgttgggctga
 L  P  K  T  A  T  G  K  I  Q  R  F  K  L  R  E  G  V  L  G  -
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Figure 4. The nucleotide (5′-3′ top strand) and amino acid (N- to C-terminus translated bottom strand) sequences obtained from the recombinant *badA* gene. Sequencing alignment (not shown) with the cDNA sequence of *badA* in the source vector pPE204[36] showed identical sequences.

BadA overexpression in *E. coli* and purification. SDS-PAGE analysis of the seven separate elutions, each with different imidazole concentrations, was completed with comparison to the aforementioned protein ladder (Lonza). The following image displays a sample SDS-PAGE gel used to determine protein purity and molecular mass.

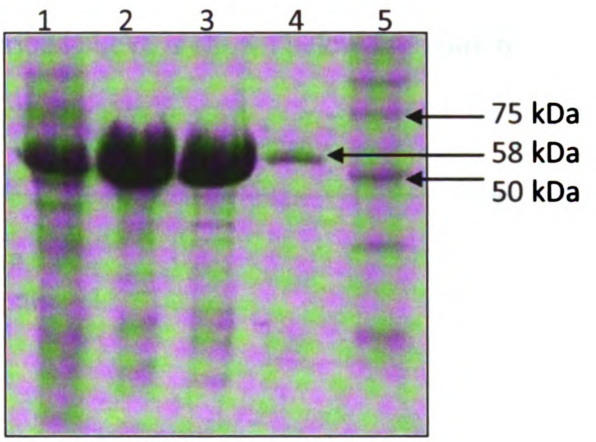


Figure 5. SDS-PAGE analysis of four of the seven separate elutions collected during BadA purification. Lanes: 1, elution at 100 mM imidazole; 2, elution at 150 mM imidazole; 3, elution at 200 mM imidazole; 4, elution at 250 mM imidazole in Assay Buffer; 5, Protein ProSieve® ladder. The two bands in the ladder closest to the putative BadA band are labeled with their respective molecular masses (75 and 50 kDa). The band in Lane 4 was used to estimate BadA mass at ~58 kDa.

In Figure 5, it can be seen that a protein eluting at a molecular mass of ~58 kDa was present in several of the eluents from the purification of BadA. This molecular mass corresponds to that of the previously characterized BadA.[1] The eluents corresponding to Lanes 2, 3, and 4 (150 mM, 200 mM, and 150 mM imidazole in Assay Buffer) were combined with an estimated purity of 95 %, as determined using the SDS-PAGE analysis (see Figure 5). Following buffer exchange and imidazole removal, the total amount of the putative N-terminal His_6-epitope of BadA isolated throughout the prescribed purification process was 6.25 mg BadA per L of harvested culture. While SDS-PAGE

analysis does show that a protein at molecular mass ~58 kDa was successfully purified, it does not conclusively prove the identity of the protein. The following described activity assays were used to lend further support to the identity of the ~58 kDa protein as the hypothesized BadA.

BadA activity assay. A typical chromatogram obtained from the analysis of BadA activity assays as described above is shown below in Figure 6.

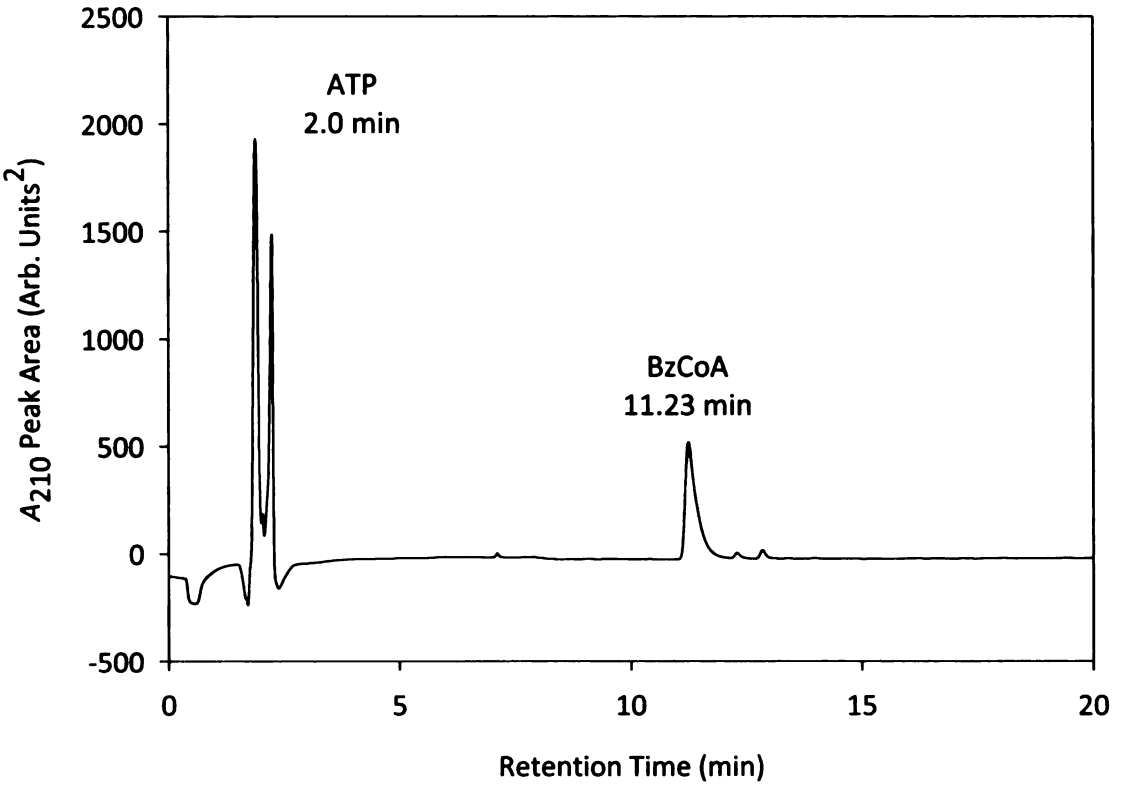


Figure 6. Chromatogram obtained following the analysis of a BadA activity assay. Assay conditions were as follows: 2.5 mM $MgCl_2$, 1.0 mM ATP, 0.25 mM CoASH, 0.25 mM BzOH, 100 μg/mL BadA, incubated at 31 °C for 10 min followed by acid quench.

In the activity assay described above, BzCoA was produced at a 91.5 % yield. This yield was calculated based on comparison of available starting material (0.25 mM BzOH and

CoASH) with the product amount quantified using the calibration curve displayed in Figure 3 of the Experimental Section. In the above chromatogram, a cluster of peaks near 2.0 min was observed in all activity assays. When compared to controls, these peaks were found to have the same retention time as solutions containing ATP; hence, this cluster of peaks is most likely due to ATP as well as AMP, another by-product of the BadA reaction.[1]

For any given set of activity assays completed after purification of the putative BadA, several controls were run to ensure that any observed BzCoA product could be attributed entirely to the activity of the enzyme (Table II).

Table II. Series of assays, including controls, used to determine BadA activity after purification. Y indicates inclusion in the reaction mixture; N indicates absence from the reaction mixture; N/D represents "Not Detectable."

Component Concentration	Activity Assay	BadA Control	$MgCl_2$ Control	ATP Control	$MgCl_2$ and ATP Control
2.5 mM $MgCl_2$	Y	Y	N	Y	N
1.0 mM ATP	Y	Y	Y	N	N
0.25 mM BzOH	Y	Y	Y	Y	Y
0.25 mM CoASH	Y	Y	Y	Y	Y
100 μg/mL BadA	Y	N	Y	Y	Y
Observed BzCoA	0.23 mM	N/D	0.16 mM	N/D	N/D

Not included within the table are controls where BzOH and CoASH were either separately or simultaneously excluded from the overall reaction mixture. All three of

these controls gave no detectable BzCoA product. The results of these assays and controls show that any observed product within a given activity assay is due entirely to the activity of the putative BadA protein. It should be noted that when $MgCl_2$ was not included in the assay ($MgCl_2$ Control), BzCoA was still observed as a product. While this may be the case, the activity of the enzyme is reduced by 31 % or more without the presence of externally introduced $MgCl_2$. One possible explanation for this observed activity is that residual Mg^{2+} could be coordinated with BadA throughout purification procedures. Because previous studies on BadA showed no inhibition of the enzyme with $MgCl_2$ at 2.5 mM,[1] this experimental observation was noted but not deemed a cause for concern. The addition of $MgCl_2$ to the assays improved overall turnover, hence it was routinely used in all subsequent assays of BadA.

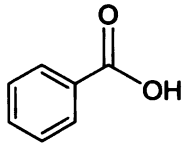
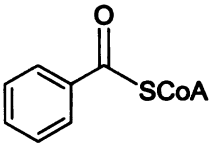
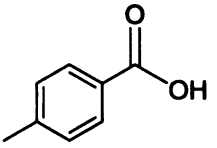
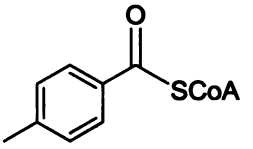
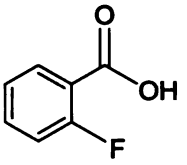
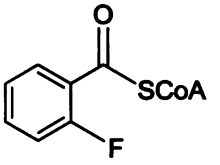
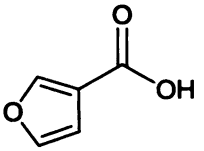
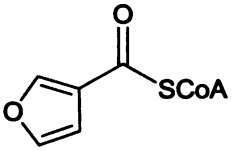
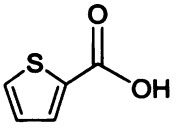
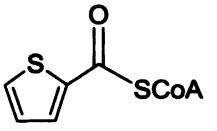
The results of both the SDS-PAGE analysis and the activity assays lend strong support to the argument that the purified protein with molecular mass ~58 kDa does correspond to the previously reported BadA. Additionally, the sequence alignment of the *badA* cDNA in the source plasmid pPE204[36] with the recombinant plasmid pBadA show identical nucleotide sequences, and confirmed the introduction of an N-terminal His_6-tag allowing for Ni – NTA purification.

BadA substrate specificity. The previously published data with regards to the substrate specificity of BadA showed that BadA catalyzes the formation of the thioester bond between several carboxylic acids and CoASH. Included in the previous investigation was the analysis of the relative velocity of BadA with respect to the natural substrate BzOH of both 2-fluorobenzoic acid and 4-methylbenzoic acid.[1] These

productive substrates were utilized in the present investigation, along with 3-furoic acid and thiophene-2-carboxylic acid, which were analyzed for the first time with BadA.

For this reason, the activity of BadA with respect to the four additional substrates discussed was assessed via the assay conditions described in the Experimental section. It should be noted that this data analysis did not allow for quantitation of product amounts as product standards for the acyl-CoA products were not available for the construction of standard curves. Following HPLC separation and UV analysis, the presence of *de novo* peaks was taken as evidence of the production of the putative acyl-CoA product. Further verification of these products was completed through coupled assay activity, as discussed in the next chapter. The observed retention times for the putative acyl-CoA products of the BadA reaction with non-natural carboxylic acid substrate are listed in Table III.

Table III. Retention times for the putative products of the BadA catalyzed reaction when incubated with the reported acyl-carboxylic acid donor.

Acyl-carboxylic Acid Substrate	Putative Acyl-CoA Product	Retention Time
 Benzoic Acid	 Benzoyl-CoA	20.2 min
 4-Methylbenzoic Acid	 4-Methylbenzoyl-CoA	27.8 min
 2-Fluorobenzoic Acid	 2-Fluorobenzoyl-CoA	20.5 min
 3-Furoic Acid	 3-Furanoyl-CoA	15.4 min
 Thiophene-2-carboxylic Acid	 Thiophene-2-carbonyl-CoA	18.3 min

As discussed in the Experimental section above, separation and detection of this finite substrate specificity study was completed using a non-optimized HPLC method. For this reason, the retention time for the natural product, BzCoA, (Table III) does not correspond to the same retention time as the chromatogram displayed in Figure 6 (see page 20), which was obtained via the optimal HPLC method.

The result of this limited substrate specificity study for BadA confirms the previously reported broad substrate specificity of the enzyme, and also lends evidence demonstrating that BadA is active with the two previously untested substrates 3-furoic acid and thiophene-2-carboxylic acid. As a result of these assays, it was hypothesized that coupling the activity of BadA with NDTBT or *m*TBT using non-natural acyl-carboxylic acid donors would result in the production of acyl variants of paclitaxel or baccatin III. The results in Chapter 2 show that BadA must be active with all of the tested non-natural substrates as the expected, substituted paclitaxel and baccatin III analogues were observed.

1.4: Conclusions

The first step in the proposed coupled assays depends on the activity and broad substrate specificity of BadA, thus obtaining a functional and purified BadA was a key step in furthering this research. The successful construction of the recombinant plasmid pBadA was confirmed with sequencing alignment, which offers strong evidence that the BadA expressed via transformed *E. coli* BL21(DE3) cells matches the amino acid sequence of the BadA enzyme previously studied by researchers at the University of Washington.[1,36] The molecular mass of a purified protein obtained through the use of Ni-

affinity chromatography at ~58 kDa matched the molecular mass of the protein BadA. Additionally, activity assays obtained through incubation of the ~58 kDa protein with the natural substrates of the reported BadA enzyme (BzOH and CoASH) led to the observation of BzCoA production as a function of the protein solution. These experimental results lend strong support to the conclusion that the purified, functionally expressed protein has the same amino acid sequence and enzymatic functionality as the previously studied BadA.[1,36] However, the most important aspect of BadA activity, that of production of BzCoA, was confirmed with multiple concentrations of enzyme and substrates. Only when enzyme concentrations were below 1.0 μg/mL in an assay was the observed product yield affected; typical yields of BzCoA were between 90 – 95 % at 5 min. These results led to the initial studies with coupled assay activity.

Another important aspect of the proposed coupled assay system lies in the hypothetical ability to produce substituted paclitaxel or baccatin III products. As the product of the BadA reaction is the acyl-CoA substrate for either of the *Taxus* acyltransferases used in later experiments, the possible substituted products depend on the ability of BadA to produce the necessary acyl-CoA substrate. The limited substrate specificity study performed in this research shows that, when incubated with an acyl-carboxylic acid, BadA catalyzes the production of a *de novo* compound. These novel compounds are most likely the hypothesized acyl-CoA products, and later evidence will show this to be the case.

CHAPTER 2

Coupled Activity of BadA with the *Taxus* Acyltransferases NDTBT and *m*TBT

2.1: Introduction

The production of paclitaxel as a natural product in *Taxus* spp. involves, in part, the activity of five separate acyltransferases, each of which requires a coenzyme A-activated acyl group as a substrate.[34] While these enzymes have been characterized, there have been few studies exploring their utility as biocatalysts for the production of paclitaxel or second generation paclitaxel analogues.[39] As discussed in the Introduction, the implementation of such a proposed biocatalytic system depends greatly on the availability of substrates either *in vitro* or *in vivo*. The research presented in this chapter focuses on coupling the activity of a broadly-specific BzCoA ligase to the activity of two separate *Taxus* acyltransferases *in vitro*.

Displayed on the subsequent pages, Figures 7 and 8 are the proposed coupled reaction schemes for the coupled activity of BadA with NDTBT, and with *m*TBT. In the scheme, there are several important experimental observations listed that helped in the development of the coupled assay system. Firstly, the k_{cat} (turnover) values for both enzymes are reported. Comparison of the turnover number (i.e. product biosynthesized per unit time) showed that BadA operates at a much faster rate than does NDTBT, suggesting that coupled assays conducted herein are limited by the *Taxus* acyltransferase reaction.

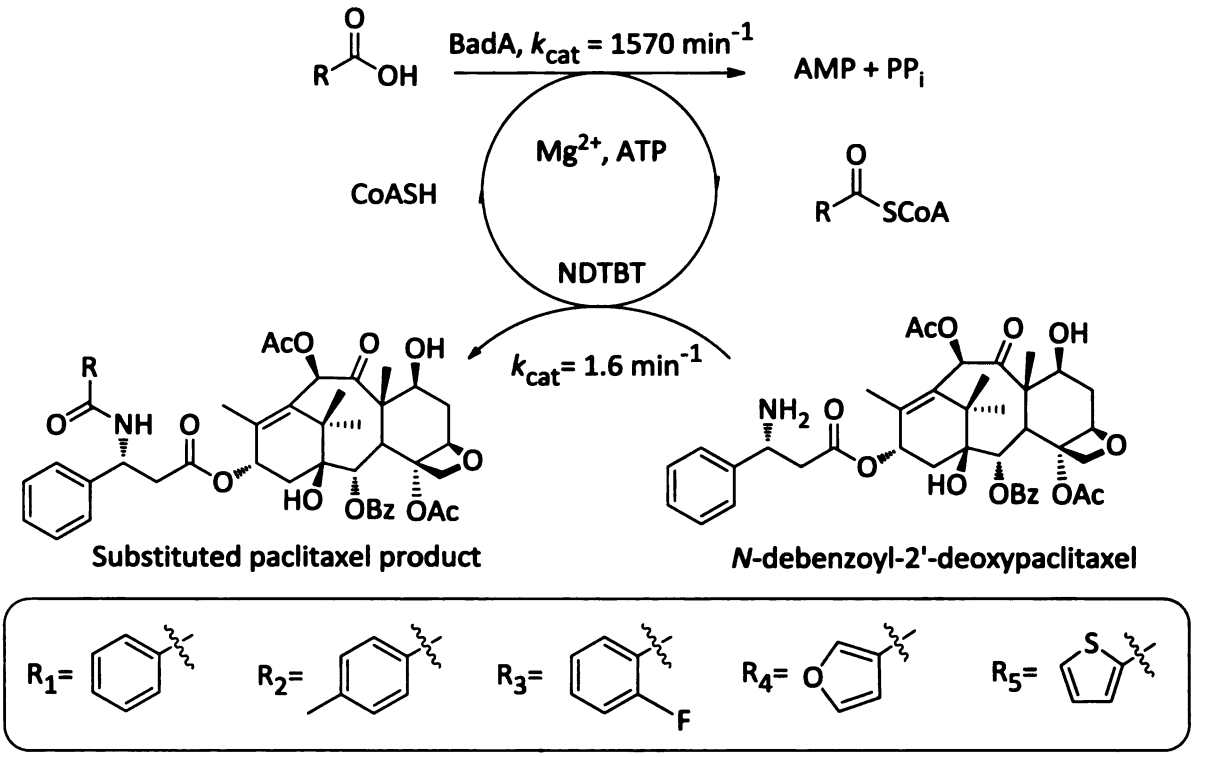


Figure 7. A reaction scheme describing the coupled activity of BadA and NDTBT. The *N*-debenzoyl-2'deoxypaclitaxel is pictured as it is considered the natural substrate for NDTBT. Both the k_{cat} for BadA[1] and NDTBT (reported in this thesis) are listed with their respective natural substrates. The five separate acyl donors displayed describe the substrates that were investigated.

Benzoic acid (likely convergent to the paclitaxel pathway in *Taxus*), along with four surrogate carboxylic acids were tested as substrates in each of the experiments presented in this document. This information provided a primer for assessing possible analogues that could be produced via the coupled reaction scheme.

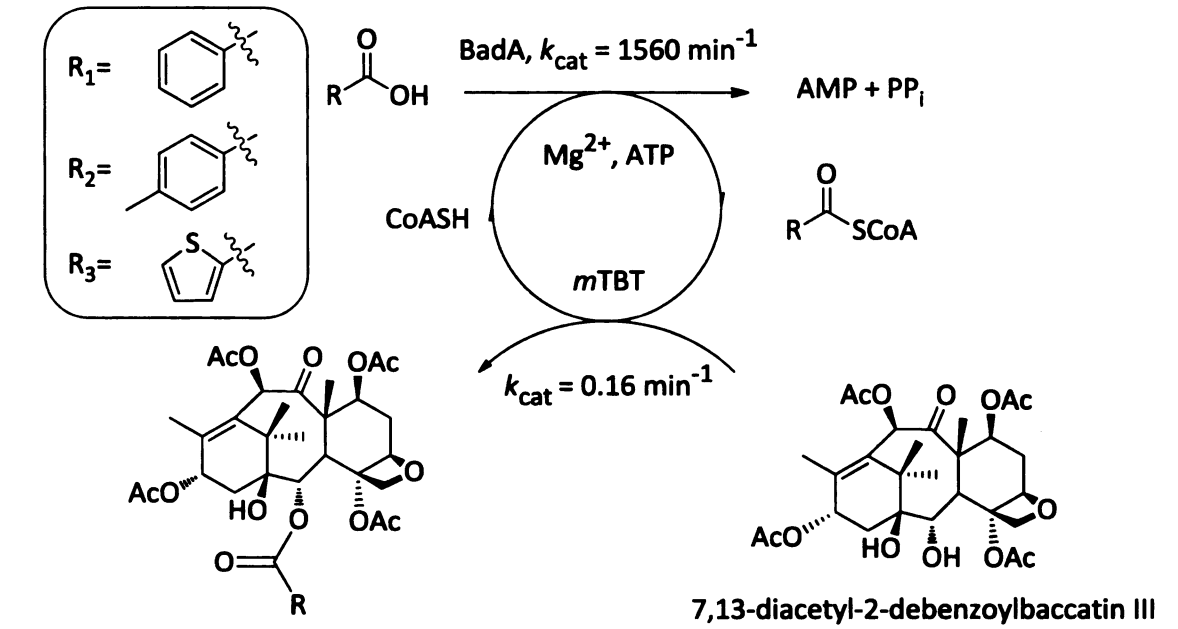


Figure 8. A reaction scheme describing the coupled activity of BadA and *m*TBT. 7,13-diacetyl-2-debenzoylbaccatin III is pictured, and it is considered to be a surrogate substrate for *m*TBT. Both the k_{cat} for BadA[1] and *m*TBT[2] are listed with their respective natural substrates. The three separate acyl donors displayed describe the substrates tested with *m*TBT.

2.2: Experimental

General. Most of the general instruments used in this chapter were identical to those used in Chapter 1. A Q-ToF Ultima Global electrospray ionization tandem mass spectrometer (ESI-MS/MS, Waters, Milford, MA) with a Waters CapLC capillary HPLC was used for mass spectral analysis.

Substrates. The substrates necessary for BadA activity are identical to those reported in the previous chapter. Baccatin III was purchased from Natland (Research Triangle Park, NC). The syntheses of laboratory stocks of *N*-debenzoyl-(3′*R*)-2′-

deoxypaclitaxel (NDB2DT), 2′-deoxypaclitaxel (2DOT), *N*-debenzoylpaclitaxel, and 7,13-diacetyl-2-debenzoylbaccatin III are described in the literature. [2,30] The former two substrates were used in NDTBT and NDTBT/BadA coupled assays, while the latter was used in assays with *m*TBT.

Bacterial strains and culture components. The same LB media, kanamycin, and IPTG solution conditions and concentrations were used for NDTBT expression as those for BadA expression. In addition, the antibiotics ampicillin (Roche) and chloramphenicol (Sigma) were added to media, where appropriate, to final concentrations of 50 μg/mL and 34 μg/mL, respectively. Stock solutions of ampicillin were prepared in water while stock solutions of chloramphenicol were prepared in 100 % ethanol. Antibiotic and IPTG stock solutions were filter-sterilized through a 0.22-μm membrane (Millipore).

2.2.1: NDTBT Experimental

NDTBT subcloning. Ongoing research with the enzyme NDTBT and work towards obtaining a crystal structure for the enzyme has led to the use of a different recombinant vector for expressing *ndtbt* than described previously.[30] The cDNA *ndtbt* was subcloned into the pET28a vector (Novagen) to incorporate a C-terminal His_6-tag, and designated *ndtbt-ct*.

NDTBT overexpression in *E. coli* and purification. Recombinant *ndtbt-ct* was expressed in the described bacterial expression system and harvested according to previous literature, with some modifications.[30] Cultures were grown overnight at 37 °C in 100-mL of LB medium supplemented with 50 μg/mL kanamycin and 34 μg/mL

chloramphenicol. Bacteria transformed with empty vector were processed analogously. To six-850 mL portions of LB medium supplemented with the appropriate antibiotics were added 16-mL of the 100-mL inoculum; the flasks were incubated at 37 °C until the cells reached OD_{600} = 0.5-0.7. Gene expression was induced with 100 μM IPTG, and the culture was incubated at 20 °C for 18 h. The cells were harvested by centrifugation at 4000*g* for 10 min at 4 °C, the supernatant was discarded, and the pellet was stored at -20 °C.

The pellet was resuspended in 3 mL NDTBT Lysis Buffer (50 mM tris(hydroxymethyl)aminomethane (Tris) -HCl, 300 mM NaCl, 10 % glycerol, pH 8.0) per wet weight at 4 °C, and sonicated with a Misonix XL-2020 sonicator (Misonix) set at 60 % power for six consecutive 15 s bursts with 45 s intervals. The cell lysate was clarified by ultracentrifugation at 149,000*g* for 1 h at 4 °C to provide the crude lysate.

The crude lysate was incubated with 1 mL of HIS-Select Nickel Affinity Gel (Sigma) per 8 g of wet pellet in batch mode at 4 °C for 1 h. The mixture was poured into an Econo column (BioRad, 20 cm × 2.5 cm). After the resin settled, the head volume was drained, the resin was washed with five column volumes of NDTBT Wash Buffer (50 mM Tris-HCl, 300 mM NaCl, 10 % glycerol, pH 8.0) to remove any unbound protein from the column, and the bound protein was eluted with 1.5 column volumes of each of the following buffers: 10 mM, 50 mM, 100 mM, 150 mM, 200 mM, and 250 mM imidazole in NDTBT Wash Buffer. SDS-PAGE was used to identify the fractions containing the highest percentage of purified NDTBT.

Samples to be loaded onto the SDS-PAGE gel were prepared by combining 5 μL of enzyme elution with 10 μL of SDS-PAGE sample buffer; samples were subsequently

boiled, centrifuged, and loaded onto the gel. Usually the recombinant protein eluted in the 100 mM and 150 mM imidazole eluent aliquots. The aliquots containing purified NDTBT were then combined, and imidazole was removed using consecutive dilution/concentration cycles with a 30,000 MWCO filter (regenerated cellulose membrane, Millipore, Billerica, MA). Centrifugation was completed at 4,000*g* at 4 °C for 20 min. The concentrated sample was washed with NDTBT Storage Buffer (50 mM Tris-HCl, 10 % glycerol, pH 8.0) until the imidazole concentration was 1.5 μM.

The protein was diluted into 50 mL of NDTBT Storage Buffer before loading onto a column packed with 40 mL of Q-Sepharose Fast Flow (Amersham) that was previously equilibrated with Storage Buffer. Proteins were eluted with a linear NaCl gradient (0-400 mM; 5 mL/min; 33 min), and the bulk of the target enzyme was found in fractions containing 90-150 mM NaCl. These fractions were combined and NaCl was removed by consecutive dilution/concentration cycles as before, using a 30,000 MWCO filter. The protein solution was diluted in NDTBT Gel Buffer (50 mM Tris-HCl, 150 mM NaCl, 10 % glycerol, pH 8.0) to a final concentration of 2 mg/mL.

At least 10 mg of NDTBT protein was loaded onto a self-packed gel filtration column (150 mL of Superdex 200 prep grade in a XK16/70 column (GE Healthcare)) and eluted at 1 mL/min for 2.5 h in Gel Buffer. The enzyme eluted between 75-90 min and was judged to be ~95 % pure by SDS-PAGE and Coomassie blue staining, followed by quantitation on Kodak 1D Image Analysis Software (Version 3.6.3). Fractions containing the protein were combined and NaCl was removed by consecutive dilution/concentration cycles as before, and the protein solution was diluted to 2 mg/mL

in NDTBT Storage Buffer, as determined by the Bradford method. This solution was then separated into 100 μL aliquots, flash frozen in liquid nitrogen, and stored at -80 °C.

NDTBT activity assays and analysis. Functional expression of NDTBT was verified using assays with the following composition: 500 μg/mL purified NDTBT, 200 μM BzCoA, and 200 μM NDB2DT, diluted to a final volume of 200 μL with Assay Buffer. The assays were incubated at 31 °C for 20 min, and the reactions were quenched by the addition of 0.5 N HCl, reducing the pH below 1.0. The acid quench also ensured that the free primary amine of the NDB2DT substrate was protonated to its ammonium ion form so that it would partition into the aqueous fraction during extraction. The assay was then extracted with ethyl acetate (3 x 1 mL), after which the organic fractions were combined and the solvent was evaporated under vacuum. The remaining residue was then resuspended in 100 μL of acetonitrile. The resuspended organic fraction (50 μL) was then injected onto a reverse phase column (Allsphere ODS-25 μm, 250 mm × 4.6 mm, Alltech, Mentor, OH) and eluted by a gradient mobile phase composition with the following solvent A/solvent B ratios [solvent A: 99.9 % H_2O with 0.1 % TFA (v/v); solvent B: 99.9 % CH_3CN with 0.1 % TFA (v/v); gradient: 0 - 5 min at 80 % (A), 5 - 11 min at 80 - 50% (A), 11 - 21 min at 50 – 20 % (A), 21 - 23 min at 20 – 0 % (A), 23 - 25 min at 0 % (A), 25 - 33 min at 0 – 80 % (A)] at a flow rate of 1.5 mL/min and A_{228} monitoring of the effluent on an Agilent 1100 HPLC system (Agilent Technologies, Wilmington, DE) connected to a UV detector.

Products eluting from the HPLC column corresponding to a *de novo* UV-absorbance peak, identified by comparison with assay controls, were collected and further analyzed by liquid chromatography – electrospray ionization mass spectrometry

(LC-ESI-MS), in positive ion mode. A Q-ToF Ultima Global ESI-MS/MS (Waters, Milford, MA) with a Waters CapLC capillary HPLC was used for separation and mass spectral analysis. A C18 reverse phase column (Betasil C18, 5 μm, 150 x 2.1 mm, Thermo Scientific Inc, Waltham, MA) was used during the HPLC separation. Briefly, from the approximate 250 μL collected from the HPLC effluent, 10 μL were injected onto the LC column coupled to the ESI-MS/MS detector. Because the effluent from the Agilent HPLC separation contained trifluoroacetic acid (TFA), a known ionization suppressant in ESI,[40] TFA was separated from the other analytes of interest using a gradient mobile phase composition with the following solvent A/solvent B ratios [solvent A: 99.9 % H_2O with 0.1 % formic acid (v/v); solvent B: 99.9 % CH_3CN with 0.1 % formic acid (v/v); gradient: 0 - 5 min at 98 % (A), 5.00 – 5.01 min at 98 – 50 % (A), 5.01 – 10 min at 50 – 0.0 % (A), 10 – 10.5 min at 0.0 % (A), 10.50 – 10.51 min at 0.0 – 98 % (A), 10.51 – 13 min at 98 % (A),] at a flow rate of 0.3 mL/min. The effluent was diverted for the initial 5.0 min to allow for complete removal of TFA from the sample; from 5.0 – 13 min, the effluent was analyzed using the first-stage mass spectrometer. The mass spectrum obtained from the most abundant peak in the resultant chromatogram had a molecular weight and ionization pattern that were identical to those of authentic 2′-deoxypaclitaxel when analyzed by similar methods.

Kinetic evaluation of NDTBT with substrate *N*-debenzoyl-(3′*R*)-2′-deoxypaclitaxel (NDB2DT). To establish linearity with respect to time and enzyme concentration, varying amounts of NDTBT were incubated with BzCoA (the natural acyl group donor, at 100 μM), and NDB2DT was maintained at saturation (500 μM) in 1.6 mL of Assay Buffer (50 mM sodium phosphate, 5 % glycerol, pH 8.0). Aliquots (200 μL)

from each assay were collected, quenched with 80 μL of 0.5 N HCl at 5, 10, 20, 30, 40, and 60 min time points, and extracted with ethyl acetate (3 × 1 mL). Immediately prior to the acid quench, 4 nmol of baccatin III was added to the reaction mixture as a control for extraction and injection efficiency during further assay processing. Solvent was removed *in vacuo*, and the products in the resultant residue were dissolved in 100 μL of acetonitrile and were analyzed by UV-HPLC with A_{228} monitoring of the effluent, as described for NDTBT activity assays. The peak area corresponding to the biosynthetic 2′-deoxypaclitaxel (2DOT) was converted to concentration by comparison to a calibration curve obtained through serial dilution of authentic 2DOT product standard (linear dynamic range from 0 to 1.25 mM; concentrations greater than 1.25 mM showed loss of linear analytical signal dependence). Kinetic parameters were determined under steady state parameters using 50 μg/mL of protein and a 10 min incubation time. The concentration of BzCoA was independently varied (0-500 μM) in separate assays while NDB2DT was maintained at apparent saturation (500 μM). The initial velocity (v_0) was plotted against substrate concentration for each data set, which exhibited the expected Michaelis-Menten curve. The Hanes-Woolf method of analysis ([BzCoA]/v_0 vs. [BzCoA]) was used, and the equation of the best-fit line (R^2 = 0.97) was determined (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA) to calculate the k_{cat} and K_M parameters.

The effect of CoASH on the NDTBT activity was determined using an inhibition study. Assay compositions were identical to those used to determine the k_{cat} and K_M described above, except for the addition of a single concentration of CoASH, which was

established between 50 μM and 500 μM, in separate assays. The assays were processed by methods identical to those used above, and the Hanes-Woolf method was used to determine the effect of CoASH on NDTBT activity.

Optimizing coupled activity assays for NDTBT and BadA. Initial coupled activity assays were completed using assay concentrations similar to those used for the separate assays of NDTBT and BadA activity. In addition to the proposed coupled assay system, an activity test for NDTBT was run as a control, where the BzCoA concentration was set at 0.25 mM. Briefly, a 500 μL reaction mixture containing 2.5 mM $MgCl_2$, 1.0 mM ATP, 0.25 mM CoASH, 0.25 mM BzOH, 0.50 mM NDB2DT, 500 μg/mL NDTBT, and 200 μg/mL BadA, prepared on ice, was diluted to final concentrations with Assay Buffer. The assays were incubated at 31 °C for 18 h, after which, enzyme activity was quenched by the addition of 0.5 N HCl, reducing the pH below 1.0. Separation and analysis of these initial coupled assays was carried out according to the procedure used to analyze NDTBT activity assays.

Results from the kinetic studies completed on NDTBT activity were used to modify the coupled assay to yield optimal turnover. The following assay conditions were considered optimal after several variations were completed. A reaction mixture with total volume 200 μL containing 2.5 mM $MgCl_2$, 1.0 mM ATP, 0.4 mM BzOH, 0.4 mM CoASH, 0.5 mM NDB2DT, 200 μg/mL NDTBT, and 50 μg/mL BadA, prepared on ice, was diluted to final concentrations with Assay Buffer. The assays were incubated at 31 °C for 2 h, and all remaining steps were completed as before. A series of assays, as discussed in the Results and Discussion section, was used to determine the effects of

BzCoA production through BadA activity on observed 2′-deoxypaclitaxel product amounts.

An analogous set of assays were used to determine whether CoASH could be effectively recycled using the coupled assay system wherein CoASH was added at a concentration 4- to 8-fold led than in previous assays. A reaction mixture with total volume 200 μL containing 0.5 mM NDB2DT, 2.5 mM $MgCl_2$, 1.0 mM ATP, 50 μM CoASH, 500 μg/mL NDTBT, and 200 μg/mL BadA was diluted to final concentrations with Assay Buffer. The assays were incubated for 18 h, followed by an acid quench, as described previously. In one assay, the BzOH concentration was equimolar to the CoASH concentration (50 μM) while in the second assay, the BzOH concentration was twice that of CoASH (100 μM).

Coupled NDTBT and BadA activity with non-natural carboxylic acid and *N*-debenzoylpaclitaxel substrates. Coupled activities with the non-natural carboxylic acid donor substrates for the BadA reaction discussed in the previous chapter were also incubated in the coupled enzyme system. These assays were completed to assess whether the coupled enzyme assay could biosynthesize various *N*-substituted-2′-deoxypaclitaxel analogues. As these assays were completed prior to the optimization of the coupled assay system, the concentrations of the various reaction components were not identical to those described above. A reaction mixture with total volume 250 μL containing 2.5 mM $MgCl_2$, 1.0 mM ATP, 0.25 mM BzOH, 0.25 mM CoASH, 0.25 mM NDB2DT, 250 μg/mL NDTBT, and 250 μg/mL BadA, prepared on ice, was diluted to final concentrations with Assay Buffer. Assays were incubated at 31 °C for 18 h, after which enzyme activity was quenched by the addition of 0.5 N HCl (pH < 1.0). Extraction and

initial separation on the Agilent 1100 HPLC (described previously) was performed as before in the NDTBT activity assays. As before, products eluting from the HPLC column corresponding to a *de novo* UV-absorbance peak, identified via comparison with assay controls, were collected and further by analyzed LC-ESI-MS in positive ion mode. Peak identity was confirmed through the characterization of MS fragmentation, as previously observed for authentic 2′-deoxypaclitaxel product standard. In addition, the mass spectra for the products of this coupled assay system with non-natural carboxylic acid substrates correspond to the previously characterized products of the NDTBT enzymatic reaction when incubated with the corresponding acyl-CoA substrate.[30]

As shown previously, NDTBT is active with several *N*-debenzoyl-taxane variants. The substrate *N*-debenzoylpaclitaxel is important as the hydroxylation at the 2′-position on the C13 side chain of paclitaxel is hypothesized to occur prior to *N*-benzoylation.[41] Coupled assays completed with this taxane substrate were completed with the following compositions: 1.25 mM *N*-debenzoylpaclitaxel, 2.5 mM $MgCl_2$, 1.0 mM ATP, 1.0 mM BzOH, 1.0 mM CoASH, 250 μg/mL NDTBT, and 250 μg/mL BadA. Assays were incubated for 18 h at 31 °C, followed by the same assay processing used for NDTBT activity assays as well as NDTBT and BadA coupled activity assays. However, assays were analyzed by direct injection LC-ESI-MS/MS analysis (as described in the literature[30]) rather than the HPLC separation and UV analysis described in this document. The expected product ion $[M+H]^+$ (m/z = 854) was directed to a fragmentation cell, and the second mass spectrometer was used to scan the array of fragment ions.

2.2.2: *m*TBT Experimental

Bacterial strains and culture components. The same LB media, kanamycin, and IPTG solution conditions and concentrations were used for *m*TBT expression as were used for both NDTBT and BadA expression. *E. coli* BL21(DE3) cells previously transformed with p28PK-TBT were used to overexpress the point-mutant *tbt* as described in the literature.[2] The two point mutations Q19P and N23K introduced in the *tbt* gene isolated from cDNA obtained from *T. cuspidata* allowed increased soluble expression of the modified taxane-2-*O*-benzoyltransferase, and the subsequent protein was renamed modified taxane-2-*O*-benzoyltransferase, or *m*TBT.

Overexpression of *m*TBT in *E. coli* and purification. Laboratory stocks of functional *m*TBT were used throughout the experiments described in this chapter. These stocks were prepared according to the previously described protocols, with some exceptions.[2] In brief, *E. coli* BL21(DE3) transformed with p28K-TBT was used to inoculate a 100-mL culture containing LB medium supplemented with 50 μg/mL kanamycin, which was grown overnight at 37 °C. Aliquots (5-mL) from this 100 mL culture were then separately used to inoculate 1 L of LB media along with the appropriate antibiotic at 37 °C until an OD_{600} = 0.95 – 1.05 was reached. At this point, *mtbt* expression was induced with 50 μM IPTG and the cultures were incubated at 18 °C for 16 h. As before, cultures were centrifuged at 4000*g* for 20 min at 4 °C to harvest the cells. Cell pellets were resuspended in *m*TBT Lysis Buffer (50 mM sodium phosphate, pH 8.0) with 3 mL/g wet cell pellet weight. Sonication at 4 °C was used to lyse the resuspended cells via a Misonix XL-2020 sonicator (Misonix) where the sonicator was set at 50 % power for five consecutive bursts with 1-min intervals followed by sonication

at 70 % power for two consecutive bursts with a 2-min interval. Clarification of the cell lysate was accomplished through ultracentrifugation at 149,000*g* for 1 h at 4 °C; the supernatant from this centrifugation will be referred to as the soluble enzyme fraction.

Purification of *m*TBT from the soluble enzyme fraction was completed in a two-step fashion. First, the soluble enzyme fraction was loaded onto a Whatman DE-52 anion exchange column (2.5 x 6 cm, 15 g resin) to remove any remaining small molecules or cellular debris. The remaining enzyme was eluted from the column using a 200 mM NaCl solution (in *m*TBT Lysis Buffer). The resultant eluted enzyme fraction was then further purified through incubation with His-Select Nickel Affinity Gel (3 g, Sigma-Aldrich) at 4 °C in batch mode. Following an hour of incubation, the mixture was poured into an Econo column (Bio-Rad, 20 x 2.5 cm) and remaining buffer was drained. The resin was then washed with seven column volumes of *m*TBT Wash Buffer (300 mM NaCl, 20 mM imidazole in *m*TBT Lysis Buffer). Bound protein was eluted in a stepwise gradient with 1.5 mL of a solution containing 200 mM imidazole (with 300 mM NaCl in mTBT Lysis Buffer) followed by elution with 1.5 mL of a solution containing 100 mM imidazole (with 300 mM NaCl in *m*TBT Lysis Buffer).

As with both BadA and NDTBT, imidazole was removed through consecutive dilution/concentration cycles via centrifugation (30,000 MWCO, regenerated cellulose membrane, Millipore). Upon imidazole removal, protein was diluted in *m*TBT Assay Buffer (25 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid, 5 % glycerol (v/v), pH 7.4). As stated in literature,[2] SDS-PAGE analysis revealed a 30 % pure protein at molecular mass ~50 kDa, as determined by comparison to molecular mass standards

(Lonza). Total protein concentration was determined by the Bradford method (Pierce, Rockford, IL).

***m*TBT activity assays and analysis.** *m*TBT activity was tested using similar assay conditions to those reported previously with some modifications.[2] Briefly, a 500 μL reaction mixture containing 500 μM 7,13-diacetyl-2-debenzoylbaccatin III, 100 μg/mL total protein, and 500 μM BzCoA, diluted to final concentration with *m*TBT Assay Buffer, was incubated for 15 h at 31 °C. The assay was then moved to ice and 2 x 1 mL of ethyl acetate were used to quench the enzyme activity and extract the product, 7,13-diacetylbaccatin III, from the assay. The organic fractions were then combined and solvent was removed *in vacuo*. Any remaining residue was subsequently resuspended in 100 μL of acetonitrile, and the resultant assay extract was subjected to further analysis.

Assays were separated by reverse phase chromatography and analyzed using ESI-MS. A 10 μL aliquot of the resuspended assay residue was injected onto a C18 column (Betasil C18, 5 μM, 150 x 2.1 mm, Thermo Fisher Scientific Inc, Waltham, MA) eluted with a gradient mobile phase composed of the solvent A/solvent B ratios [solvent A: 99.5 % H_2O with 0.5 % formic acid (v/v); solvent B: 99.5 % CH_3CN with 0.5 % formic acid (v/v); gradient: 0 - 7 min at 70 – 0.0 % (A), 7 - 9 min at 0.0 % (A), 9 – 10.1 min at 0 – 70 % (A), 10.1 - 11 min at 70 % (A)] at a flow rate of 0.3 mL/min on a capillary HPLC system (CapLC capillary HPLC, Waters, Milford, MA). The effluent was monitored via LC-ESI-MS analysis (Q-ToF Ultima Global, Waters, Milford, MA), and the resultant chromatogram was used to identify *de novo* peaks when compared to assay controls.

Coupled activity assays for *m*TBT and BadA. The coupled activity of BadA and *m*TBT was tested in the following manner. A reaction mixture containing 500 μM

7,13-diacetyl-2-debenzoylbaccatin III, 100 μg/mL total protein(containing *m*TBT), 100 μg/mL BadA, 500 μM BzOH, 500 μM CoASH, 2.5 mM $MgCl_2$, and 1.0 mM ATP, diluted to final concentration with *m*TBT Assay Buffer, was incubated for 15 h at 31 °C. Assays were analyzed using the method previously described for analyzing *m*TBT assays.

Coupled activity assays for *m*TBT and BadA with non-natural carboxylic acid substrates. Two of the four non-natural substrates proven to be active in the BadA reaction were incubated in the coupled assays system to determine its possible use in synthesizing *O*-substituted-7,13-diacetyl-2-debenzoylbaccatin III analogues. The assay conditions used were identical to those listed in the procedure testing the coupled activity of *m*TBT and BadA. However, in place of the 500 μM BzOH, the two substrates 4-methylbenzoic acid and thiophene-2-carboxylic acid were incubated at 500 μM. Assay analysis was again identical to the procedure used for assessing *m*TBT activity alone.

2.3: Results and Discussion

2.3.1: NDTBT Results and Discussion

NDTBT activity assays. Analysis of the activity assays discussed in the experimental section yielded chromatograms similar to the following displayed in Figure 9 on the next page.

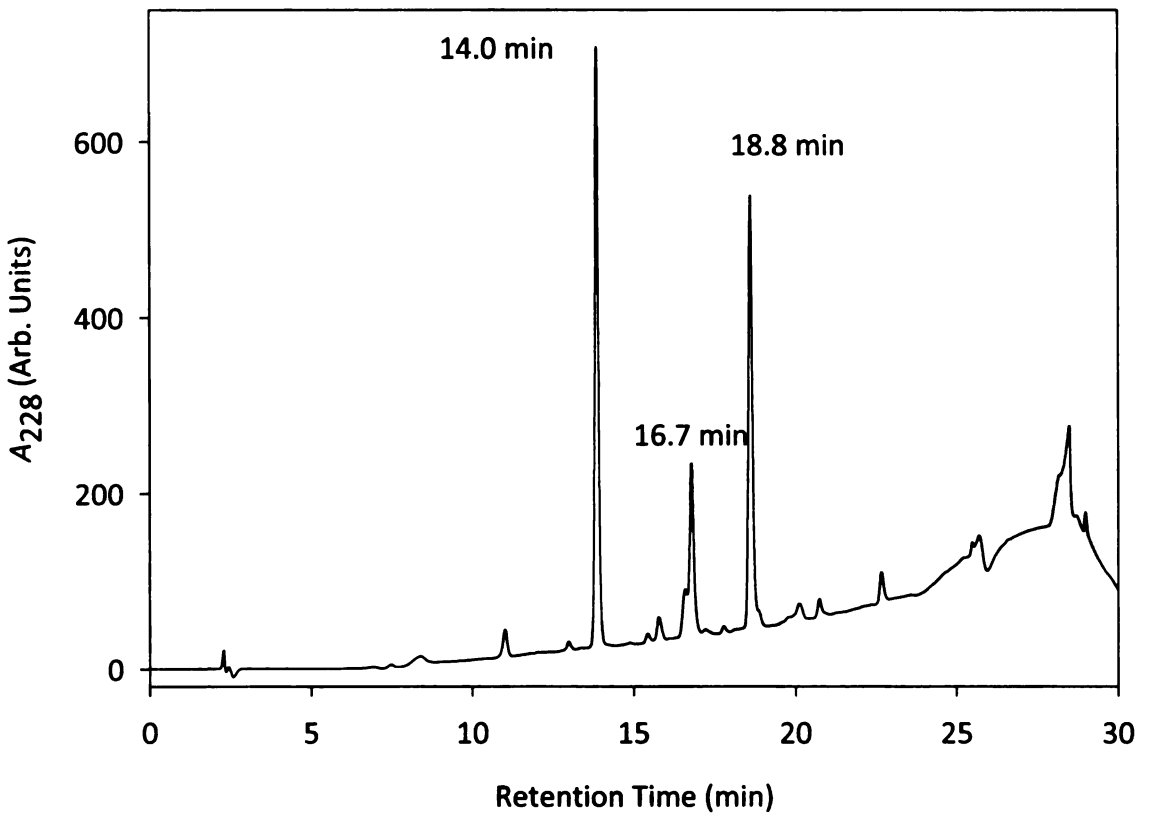


Figure 9. Reverse phase separation followed by UV detection at 228 nm of an activity assay for the enzyme NDTBT. Peak identities: 14.0 min, baccatin III; 16.7 min, *N*-debenzoyl-2′-deoxypaclitaxel; 18.8 min, 2′-deoxypaclitaxel. The baccatin III was included as a standard, as will be discussed in later experiments.

The sample eluting off the HPLC column at 18.8 min was collected and identified as 2′-deoxypaclitaxel by comparison to authentic standard (Figure 9). The peak area was converted to concentration of product using a standard curve constructed using laboratory stocks of 2′-deoxypaclitaxel.

Kinetic Evaluation of NDTBT with *N*-Debenzoyl-(3′*R*)-2′-deoxypaclitaxel (NDB2DT). The proposed coupled assay system requires incubation of NDTBT with the

carboxylic acid and CoASH substrates, along with cofactors, necessary for BadA activity. The affect of these substrates on NDTBT activity have not been previously studied; however, an understanding of these effects could potentially lead to optimization of the coupled assay system. Initial attempts at evaluating these effects were made via comparison of the kinetic parameters k_{cat} and K_M in NDTBT assays incubated with and without one of the newly introduced substrates or cofactors (CoASH, BzOH, $MgCl_2$, and ATP). However, many challenges were faced as the k_{cat} and K_M values for NDTBT alone did not match those previously reported.[30,42] The differences in kinetic evaluation were later rectified on the basis of several observations. First, enzyme used in the original kinetic study of NDTBT was functionally expressed and purified as an N-terminal His_6-tag epitope, whereas the enzyme used in this thesis research was functionally expressed as a C-terminal His_6-tag epitope. Second, the previous studies utilized soluble protein solutions with an estimated purity of roughly 70 % based on SDS-PAGE analysis; the additional purification steps included in the Experimental section above increased protein purity to roughly 95 % based on similar evaluation methodologies. Finally, the BzCoA used in the original kinetic studies was not as pure as originally calculated and contained some residual CoASH salts; as discussed in the previous chapter, actual concentrations of BzCoA were found to be anywhere from 10 – 15 % of the reported concentrations. All of these reasons led to the necessity for the reevaluation of NDTBT kinetics with the purer soluble protein solution and with commercially available BzCoA.

As k_{cat} was reached within the first 5 min of incubation when a protein concentration of 500 μg/mL (used previously)[30] was used, range-finding experiments with varying amounts of NDTBT found that steady state kinetics were observed at an enzyme concentration of 50 μg/mL and a 10 min incubation period. Following incubation of the enzyme with varying benzoyl CoA concentrations (0 – 500 μM) and a constant *N*-debenzoyl-2′-deoxypaclitaxel concentration (500 μM, saturating), the following Hanes-Woolf plot was obtained.

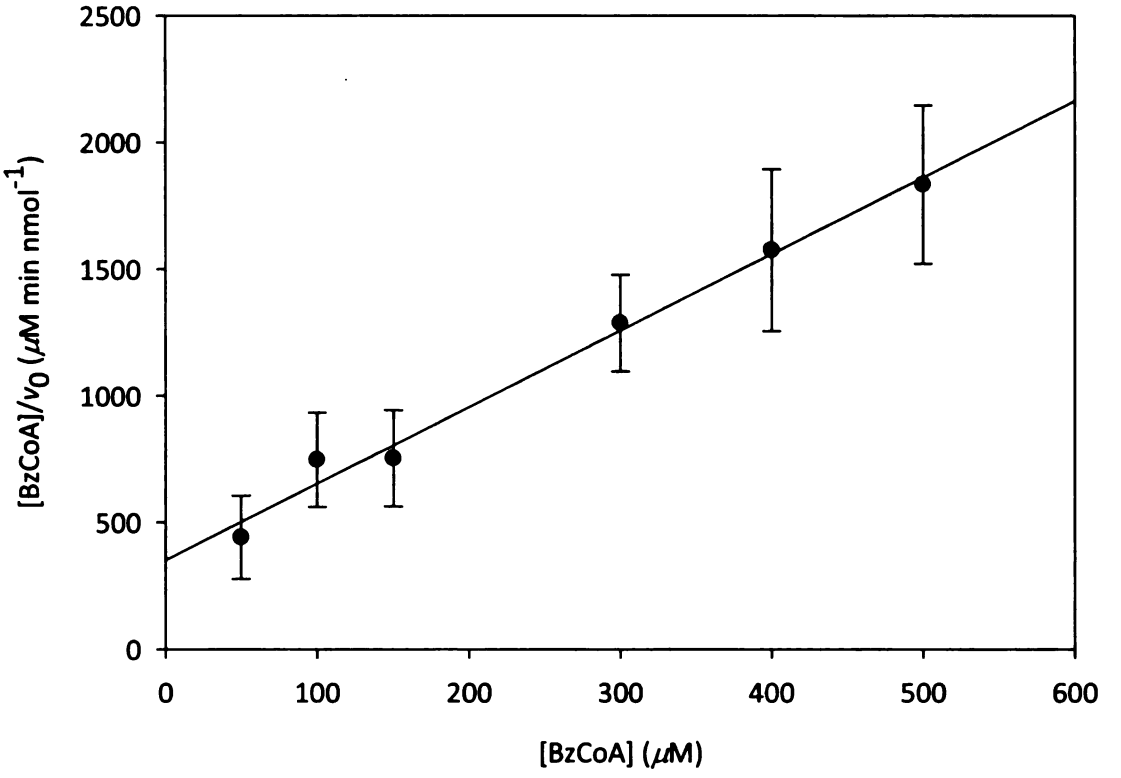


Figure 10. Hanes-Woolf analysis of the data obtained from incubating NDTBT with 500 μM NDB2DT and varying concentrations of BzCoA at a 10 min incubation period. The plot shows the concentration of BzCoA divided by the experimentally determined v_0 (reported as nmols of 2′-deoxypaclitaxel divided by 10 min) vs. BzCoA concentration. Error bars are reported as the standard deviation of three separate measurements.

From this data, it was found that K_M = 116 ± 29 μM benzoyl CoA and V_{max} = 0.33 ± 0.06 nmol 2'-deoxypaclitaxel per minute; taking into consideration the enzyme concentration, it was found that k_{cat} = 1.7 ± 0.3 min^{-1}. When compared to the previously reported values[30,42] evaluated for the same enzyme (K_M = 410 μM, k_{cat} = 1.5 ± 0.3 s^{-1}), it can be seen that the original reported K_M was roughly 3.5 times greater than the newly defined K_M, and that the turnover number k_{cat} was roughly 55 times greater than the newly defined k_{cat}. These significant variations are most likely due to the several reasons listed above.

Following the determination of the newly defined kinetic parameters describing the reaction catalyzed via NDTBT activity, experiments to determine the effect of CoASH on enzyme turnover were completed. Figure 11 displays the results of these experiments, where the same assay conditions were used to determine original kinetic parameters, but CoASH was added at a constant concentration within a given set of v_0 measurements.

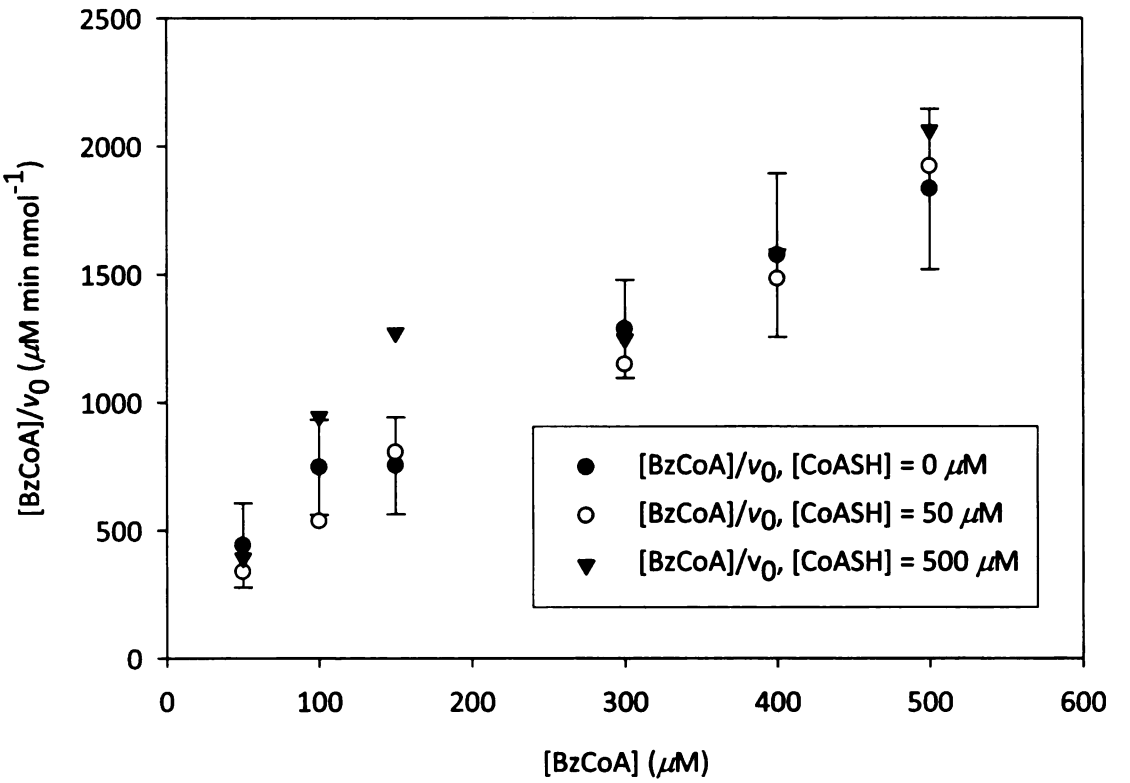


Figure 11. Hanes-Woolf analysis of the data obtained from incubating NDTBT with 500 μM NDB2DT and varying concentrations of BzCoA at a 10 min incubation period upon the addition of CoASH as indicated in the legend. The plot shows the concentration of BzCoA divided by the experimentally determined v_0 (reported as nmol of 2′-deoxypaclitaxel divided by 10 min) vs. BzCoA concentration. Error bars are reported as the standard deviation of three separate measurements.

The results from these experiments show that coenzyme A has no observable effect on NDTBT activity with the given conditions. This conclusion can be drawn from the inhibition data that coincides with the original measurements of NDTBT kinetic parameters, described earlier in this document. Greater concentrations of coenzyme A were not included in this study because coenzyme A concentrations never exceeded 500 μM in the coupled assay system.

Optimizing coupled activity assays for NDTBT and BadA. A set of assays were completed to verify that the coupled enzyme assay system was optimized (Table IV). The evidence provided by these assays is also important in proving several of the other hypotheses made concerning the activity of the coupled assay system. First, by comparing Assays A and B (Figure 12), it can be seen that the 2′-deoxypaclitaxel (2DOT) yield when NDTBT is incubated with $MgCl_2$ and ATP is within experimental error of the 2DOT yield when $MgCl_2$ and ATP are not present. This result confirms the results of several single point inhibition studies completed at various time points with the same enzyme and substrate concentrations, where overall 2DOT production was not affected. Comparing Assays A and C also show that the addition of BadA does not hinder NDTBT turnover. Second, when comparing Assays A and D, product yields are again within experimental error of one another. As Assay A was simply testing NDTBT production, while Assay D was testing production levels in the coupled assay system (with equimolar amounts of BzCoA in Assay A to BzOH and CoASH amounts in Assay D), this result proves that the coupled assay system is just as effective at producing 2DOT as is the simple NDTBT assay system. A comparison of Assays D and E, where the CoASH concentration was halved, again shows product yield within experimental error. This shows that, as hypothesized, CoASH concentrations do not need to be equimolar to the BzOH donor in order to achieve maximal turnover. However, as product levels never exceeded the concentration of CoASH, this series of assays alone does not prove that CoASH can be effectively recycled in the coupled assay system. Assay G, which contained both BzOH and BzCoA at 200 μM, was also designed to test the hypothesis

that CoASH is recycled in the coupled assay system. However, because product levels did not exceed 200 μM, it is not clear whether or not CoASH was recycled.

Interestingly, Assay F shows the lowest production levels in the optimized assay system. One possible explanation for the observed product amounts may be that with CoASH concentrations at 50 μM, the level of BzCoA at any time during the reaction cannot exceed 50 μM. The K_M for NDTBT with respect to BzCoA was previously shown to be 116 ± 29 μM, which implies that at 50 μM BzCoA, the k_{cat} for NDTBT would not be reached. Hence, with BzCoA levels below 116 μM, NDTBT was not operating at optimal velocity, meaning that a two hour time point may not have been long enough to observe the largest product levels for the described assay composition.

Table IV. Assay compositions used in the optimized NDTBT and BadA coupled assay system. Y indicates inclusion in the assay composition.

Final Component Concentration (μM)	Assay Number						
	A	B	C	D	E	F	G
NDB2DT (500)	Y	Y	Y	Y	Y	Y	Y
BzOH (400)				Y	Y	Y	
BzOH (200)							Y
BzCoA (400)	Y	Y	Y				
BzCoA (200)							Y
CoASH (400)				Y			
CoASH (200)					Y		
CoASH (50)						Y	
$MgCl_2$ (2500)		Y	Y	Y	Y	Y	Y
ATP (1000)		Y	Y	Y	Y	Y	Y
BadA (50 μg/mL)			Y	Y	Y	Y	Y
NDTBT (200 μg/mL)	Y	Y	Y	Y	Y	Y	Y

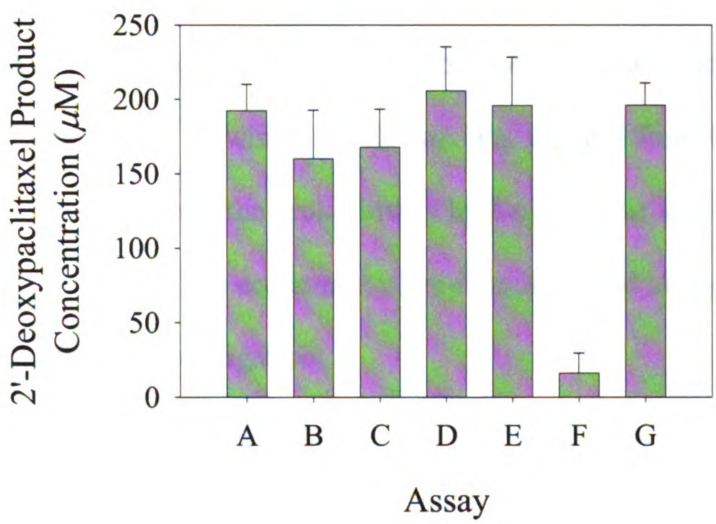


Figure 12. The above chart displays 2′-deoxypaclitaxel concentrations obtained when the assay compositions listed in Table IV were incubated for the prescribed 2 h time point. Error bars are reported as the standard deviation of three independent measurements.

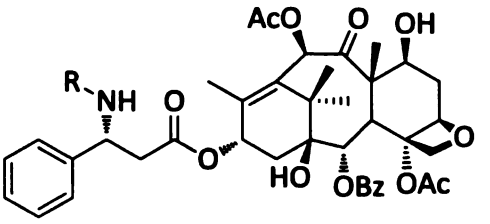
These assay conditions were considered optimal when taking into account several previously completed studies where enzyme and substrate concentrations, as well as time of incubation, were all varied independently of one another. Two hours was chosen as the appropriate stop time because similar assays at 20 min showed less production, while assays at time points up to 48 hours did not show a statistically significant increase in production levels. It should be noted that while similar assays were used to optimize conditions, it was exceedingly difficult to keep specific variables consistent. For example (cf. Chapter 1 Results and Discussion), BzCoA laboratory stocks prepared synthetically via previously described methods[30] were found to be 10 – 15 % pure based on comparison with the BzCoA standard curve constructed with commercially available BzCoA. Many of the preliminary coupled assays were completed using such stocks, and thus prompted all subsequent experiments to be completed with a commercially available stock of BzCoA. Assays conducted with NDTBT alone, with the unknowingly impure stock of BzCoA and the appropriate taxane co-substrate, had production levels that were 50 % less than similar assays in which the BzCoA was derived from BadA activity in a coupled enzyme system. Due to the prohibitively high cost of the acyl-CoA substrate, as discussed in the Introduction, the economics of re-running initial assays were considered too great to warrant further investigation.

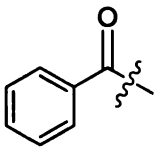
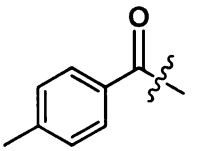
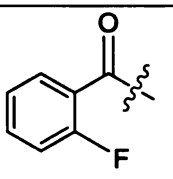
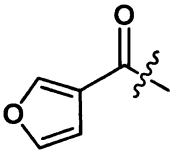
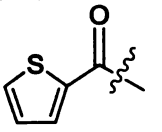
While the assays presented above do not prove that CoASH can be effectively recycled in the coupled assay system, previous preliminary assays did prove this hypothesis. The composition of those assays differed from the optimal set of conditions because these experiments were designed before the optimal conditions were established. In a series of assays, *N*-debenzoyl-2′-deoxypaclitaxel was maintained at 500 μM when

BzOH and CoASH were equimolar at 50 μM. 2′-Deoxypaclitaxel was produced at 85 % yield (7.1 μg, 42.5 μM) in 16 h. However, when BzOH concentration was doubled to 100 μM while CoASH concentration remained constant at 50 μM, 2′-deoxypaclitaxel was produced at 96 % yield (16.5 μg, 98 μM) in 16 h. This shows that CoASH was effectively recycled in the coupled assay system because product yields exceeded CoASH concentration.

Coupled NDTBT and BadA activity with non-natural carboxylic acid and *N*-debenzoylpaclitaxel substrates. The following Table V summarizes the retention times of *de novo* peaks eluting from the column after separation and UV analysis as discussed in the experimental section.

Table V. Retention times (min) for the *N*-substituted-*N*-debenzoyl-2′-deoxypaclitaxel products of the NDTBT and BadA coupled reaction when incubated with the reported acyl-carboxylic acid donor.



R= 				
Benzoic Acid	4-Methylbenzoic Acid	2-Fluorobenzoic Acid	3-Furoic Acid	Thiophene-2-Carboxylic Acid
18.7 min	19.4 min	18.4 min	17.6 min	19.0 min

The identity of each peak was characterized via ESI-MS analysis, as discussed in the experimental section. The following set of Figures (13 - 17) display the mass spectral data obtained as a result of the above peak collection and subsequent analysis. Common fragment ions of the parent ions $[M+H]^+$, as previously resolved in literature,[30] include loss of the C-13 side chain to yield the positively charged taxane core (m/z = 569), the positively charged C-13 side chain (m/z dependent on substrate), and subsequent loss of an acetyl group from the taxane core (m/z = 509). Another commonly observed ion was that of $[M+\text{formic acid}+\text{acetonitrile}+H]^+$, which yields an $[M+88]^+$ ion. This adduct has been reported in previous literature;[43] its presence is not surprising as the mobile phase used to separate the sample before mass spectral analysis contained both acetonitrile and formic acid.

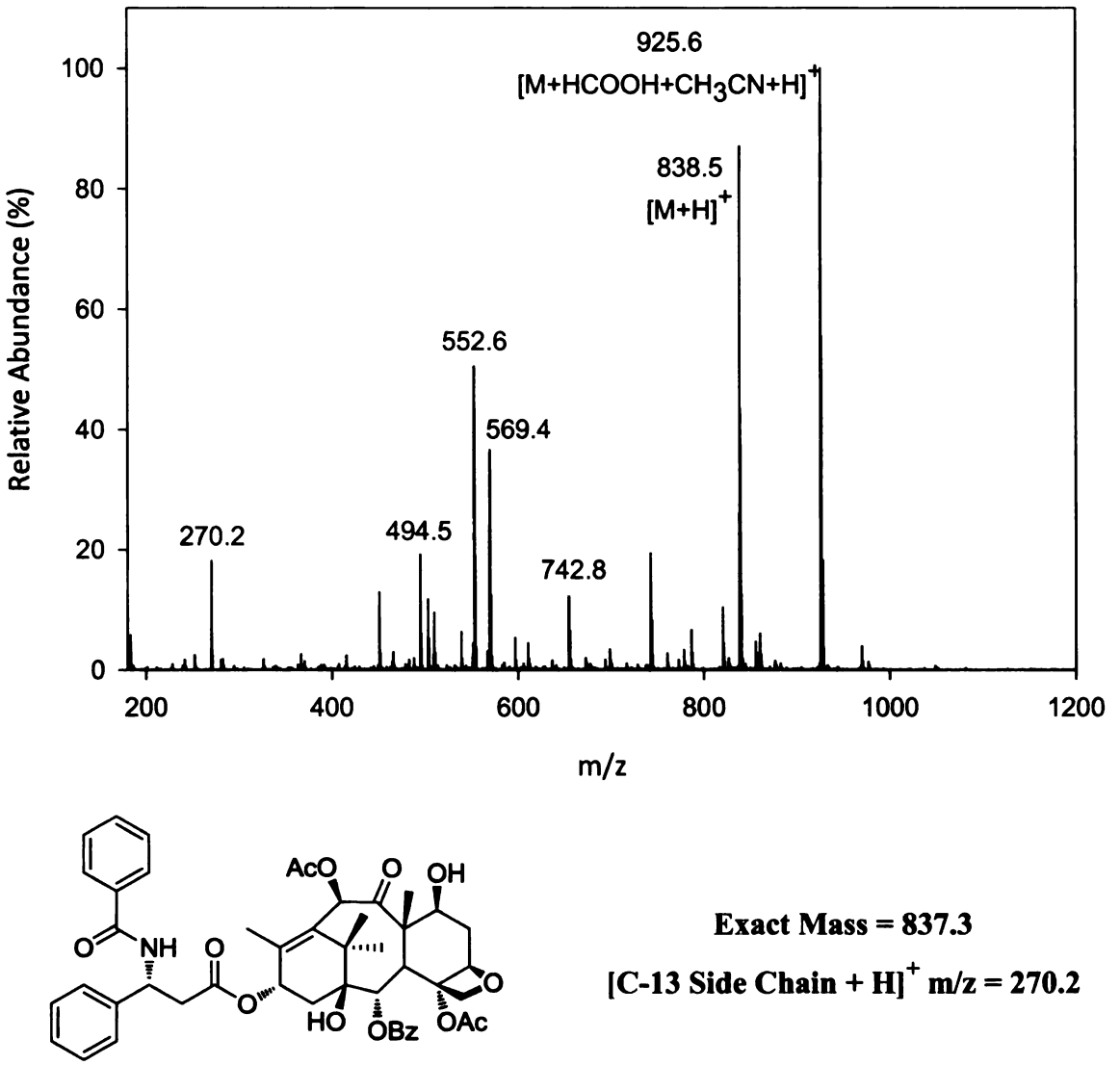
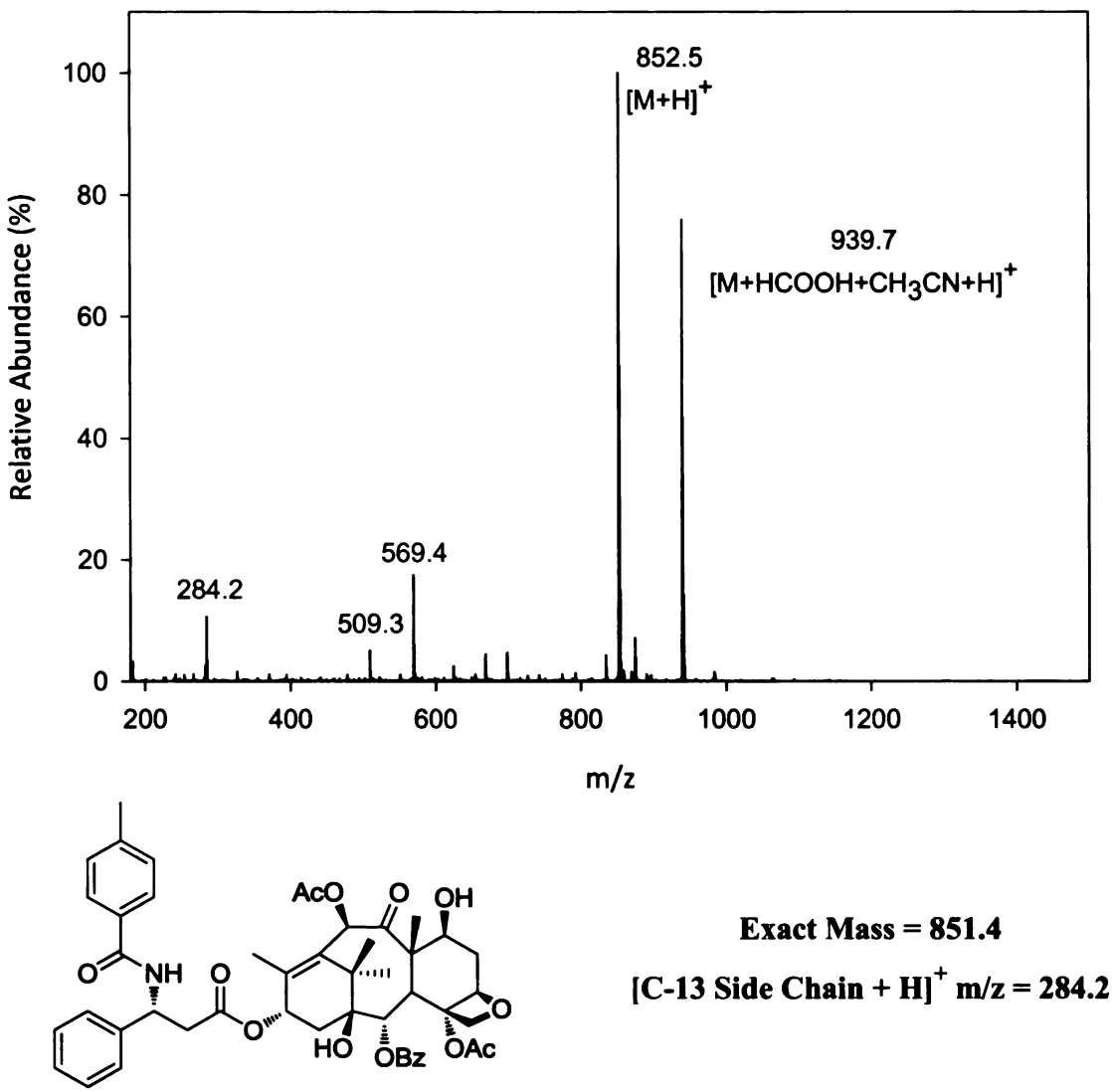


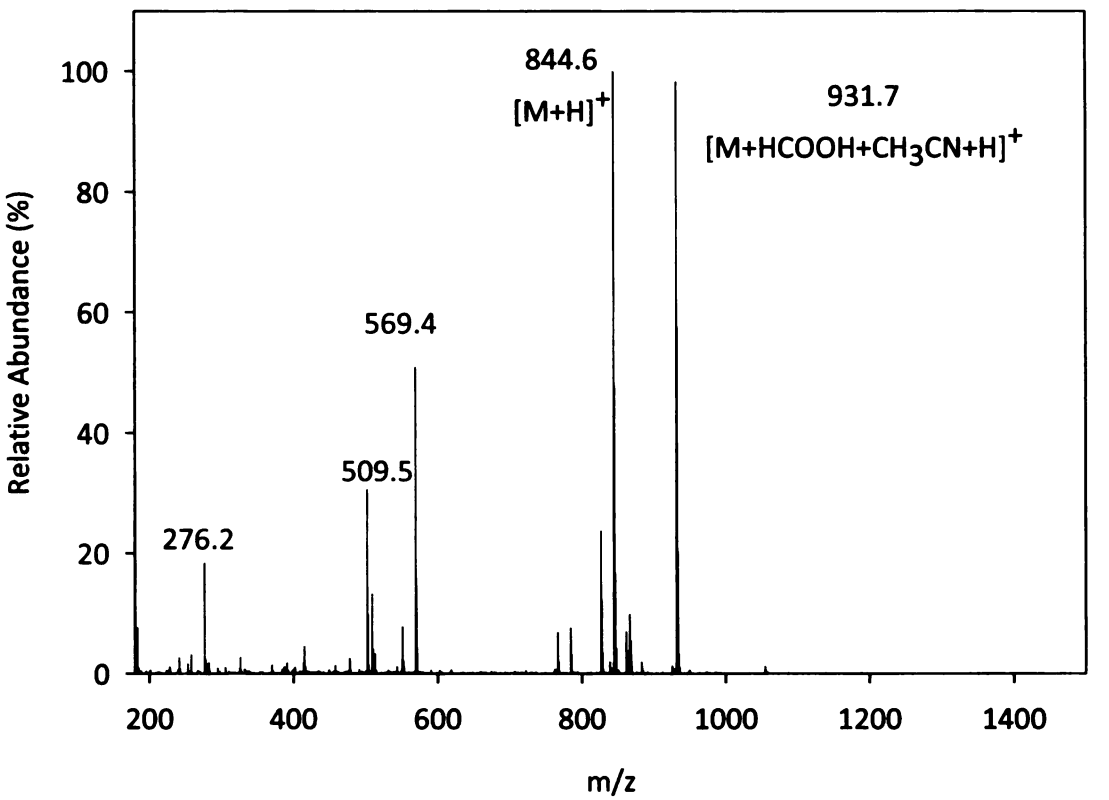
Figure 13. Mass spectrum of the collected *de novo* peak (at a retention time of 18.6 min) from the separation of an NDTBT and BadA coupled reaction incubated with the substrates NDB2DT and BzOH. Presence of the pseudomolecular ion $[M+H]^+$ at 838.5 m/z as well as the regular fragmentation pattern of paclitaxel analogues confirms the identity of the product peak as 2′-deoxypaclitaxel.

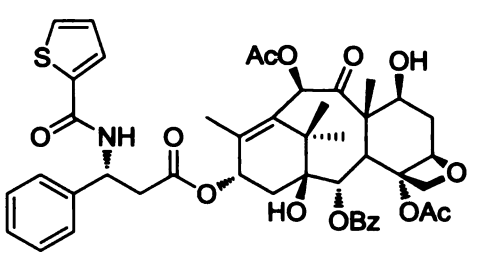


Exact Mass = 851.4

[C-13 Side Chain + H]$^+$ m/z = 284.2

Figure 14. Mass spectrum of the collected *de novo* peak (at a retention time of 19.4 min) from the separation of an NDTBT and BadA coupled reaction incubated with the substrates NDB2DT and 4-methylbenzoic acid. Presence of the pseudomolecular ion [M+H]$^+$ at 852.5 m/z as well as the regular fragmentation pattern of paclitaxel analogues confirms the identity of the product peak as *N*-debenzoyl-*N*-(4-methylbenzoyl)-2′-deoxypaclitaxel.





Exact Mass = 843.3

[C-13 Side Chain + H]$^+$ m/z = 276.2

Figure 15. Mass spectrum of the collected *de novo* peak (at a retention time of 18.4 min) from the separation of an NDTBT and BadA coupled reaction incubated with the substrates NDB2DT and thiophene-2-carboxylic acid. Presence of the pseudomolecular ion [M+H]$^+$ at 844.6 m/z as well as the regular fragmentation pattern of paclitaxel analogues confirms the identity of the product peak as *N*-debenzoyl-*N*-thiophene-2-carbonyl-2′-deoxypaclitaxel.

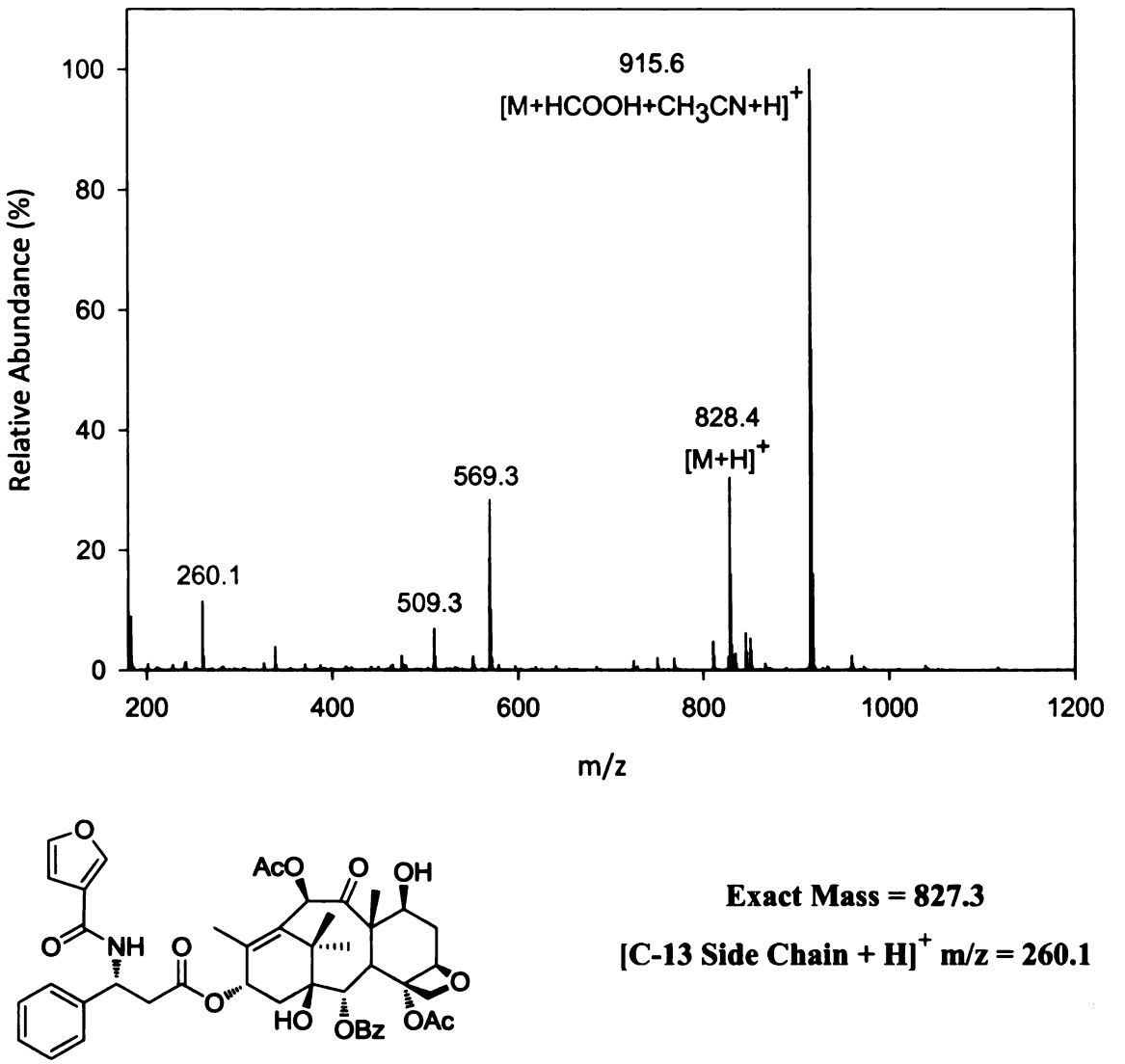
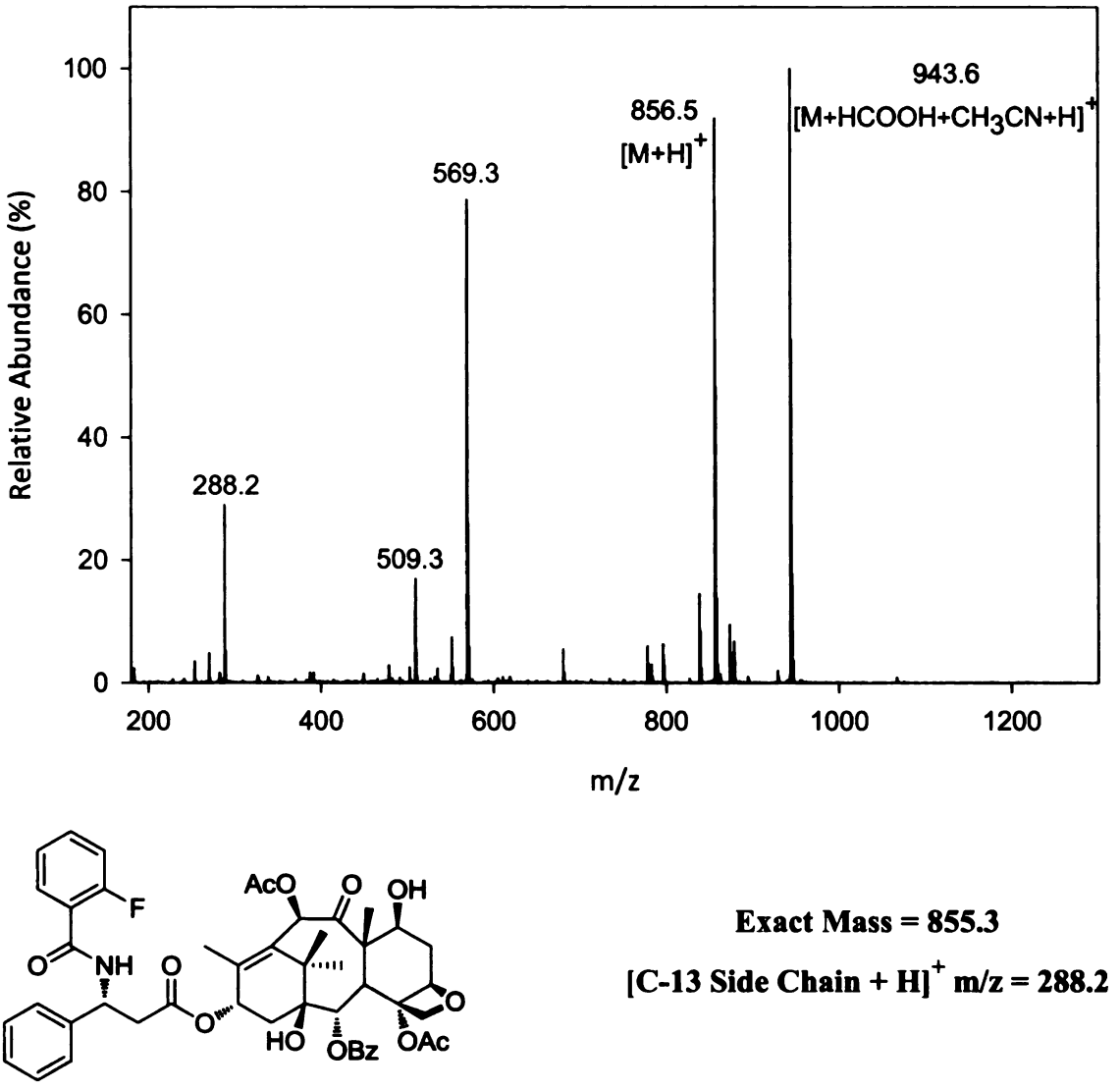


Figure 16. Mass spectrum of the collected *de novo* peak (at a retention time of 17.6 min) from the separation of an NDTBT and BadA coupled reaction incubated with the substrates NDB2DT and 3-furoic acid. Presence of the pseudomolecular ion $[M+H]^+$ at 828.4 m/z as well as the regular fragmentation pattern of paclitaxel analogues confirms the identity of the product peak as *N*-debenzoyl-*N*-(3-furanoyl)-2′-deoxypaclitaxel.



Exact Mass = 855.3

[C-13 Side Chain + H]$^+$ m/z = 288.2

Figure 17. Mass spectrum of the collected *de novo* peak (at a retention time of 19.0 min) from the separation of an NDTBT and BadA coupled reaction incubated with the substrates NDB2DT and 2-fluorobenzoic acid. Presence of the pseudomolecular ion [M+H]$^+$ at 856.5 m/z as well as the regular fragmentation pattern of paclitaxel analogues confirms the identity of the product peak as *N*-debenzoyl-*N*-(2-fluorobenzoyl)-2′-deoxypaclitaxel.

All of the displayed mass spectra indicate that the expected *N*-substituted-*N*-debenzoyl-2′-deoxypaclitaxel product was biosynthesized as a direct result of the coupled assay activity. These are important findings in that they conclusively prove the activity of BadA with the acyl-carboxylic acid substrate to produce the expected acyl-CoA product. This data also proves that the coupled enzyme assay system can be used to produce 2′-deoxypaclitaxel analogues, thus decreasing the reliance on the necessary acyl-CoA substrate in an uncoupled NDTBT assay.

When the coupled assay system was incubated with the taxane substrate *N*-debenzoylpaclitaxel, as discussed in the Experimental section, ESI-MS/MS fragmentation was used for analysis as in previously reported literature.[30] Tandem mass spectrometry was used for analysis because the product yields of the biocatalytic reactions were previously reported as below detection limits using the described UV-HPLC system. The following mass spectrum (Figure 18) was obtained from fragmentation of the parent paclitaxel ion ($[M+H]^+$ = 854 m/z). The fragmentation pattern is similar to that of the 2′-deoxypaclitaxel ions discussed above; however, because fragmentation was induced via collision induced dissociation (CID), the abundances of the fragment ions are much greater than the parent ion peak.

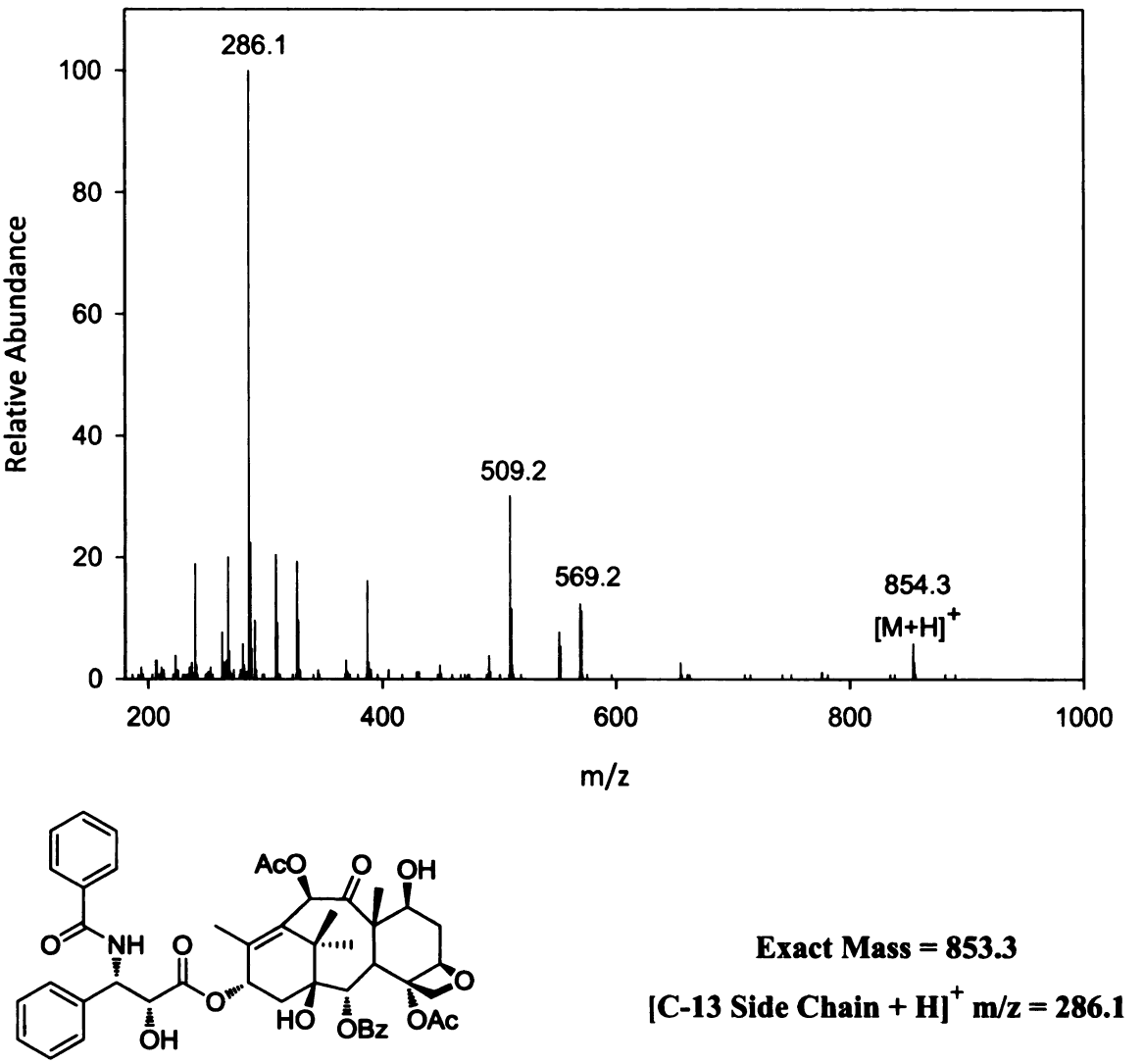


Figure 18. Mass spectrum of the fragmented parent ion 854 m/z from the analysis of an NDTBT and BadA coupled reaction incubated with the substrates *N*-debenzoylpaclitaxel and BzOH. Presence of the pseudomolecular ion $[M+H]^+$ at 854.3 m/z as well as the regular fragmentation pattern of paclitaxel analogues confirms the identity of the product peak as paclitaxel. The base peak 286.1 m/z represents the protonated *N*-benzoylated-phenylisoserinoyl sidechain.

2.3.1: *m*TBT Results and Discussion

Coupled and un-coupled activity assays for *m*TBT and BadA. Activity assays completed for *m*TBT produced results similar to those reported in previous literature.[2] The following Figure 19 displays the mass spectrum obtained by integrating the mass spectra collected during elution of the peak corresponding to the product 7,13-diacetylbaccatin III, as identified by an identical retention time to product standard.

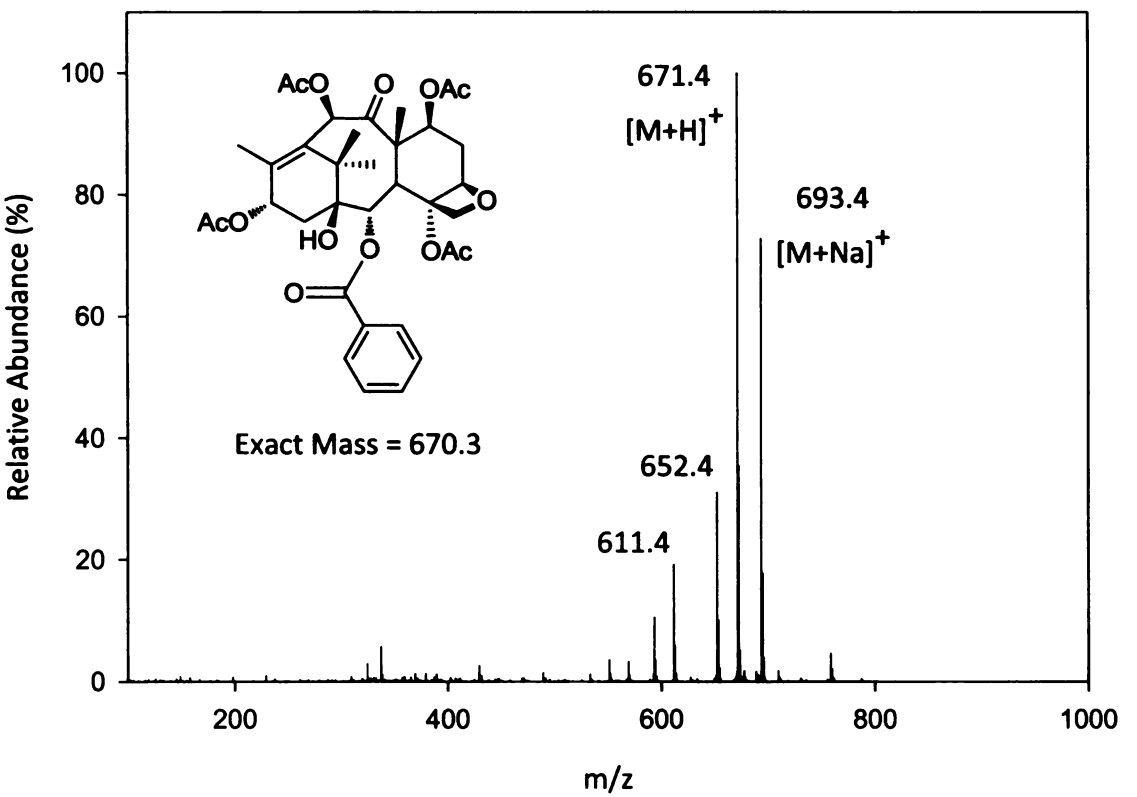


Figure 19. Product mass spectrum for 7,13-diacetylbaccatin III product as a result of an *m*TBT activity assay. The base peak at m/z = 671.4 corresponds to the $[M+H]^+$ pseudomolecular ion.

In Figure 19, the base peak has an m/z corresponding to the $[M+H]^+$ pseudomolecular ion. The next most abundant peak at m/z = 693.4 corresponds to the Na^+ adduct of the

molecular ion. Loss of an acetyl group from both the $[M+H]^+$ ion and the $[M+Na]^+$ ion yield the peaks at m/z = 611.4 and 652.4, respectively.

Following confirmation of *m*TBT activity, the coupled assays (as described in the experimental section) were used to determine whether the activity of BadA could be coupled *in vitro* to the activity of *m*TBT. The resultant mass spectrum matched that of the expected product, as displayed above in Figure 19. This important result shows that the activity of BadA can be coupled to at least two of the *Taxus* acyltransferases involved in the biosynthetic pathway for the production of paclitaxel.

Results of the *m*TBT and BadA coupled assays incubated with the non-natural acyl-carboxylic acid substrates 4-methylbenzoic acid and thiophene-2-carboxylic acid are displayed on subsequent pages in Figures 20 – 21.

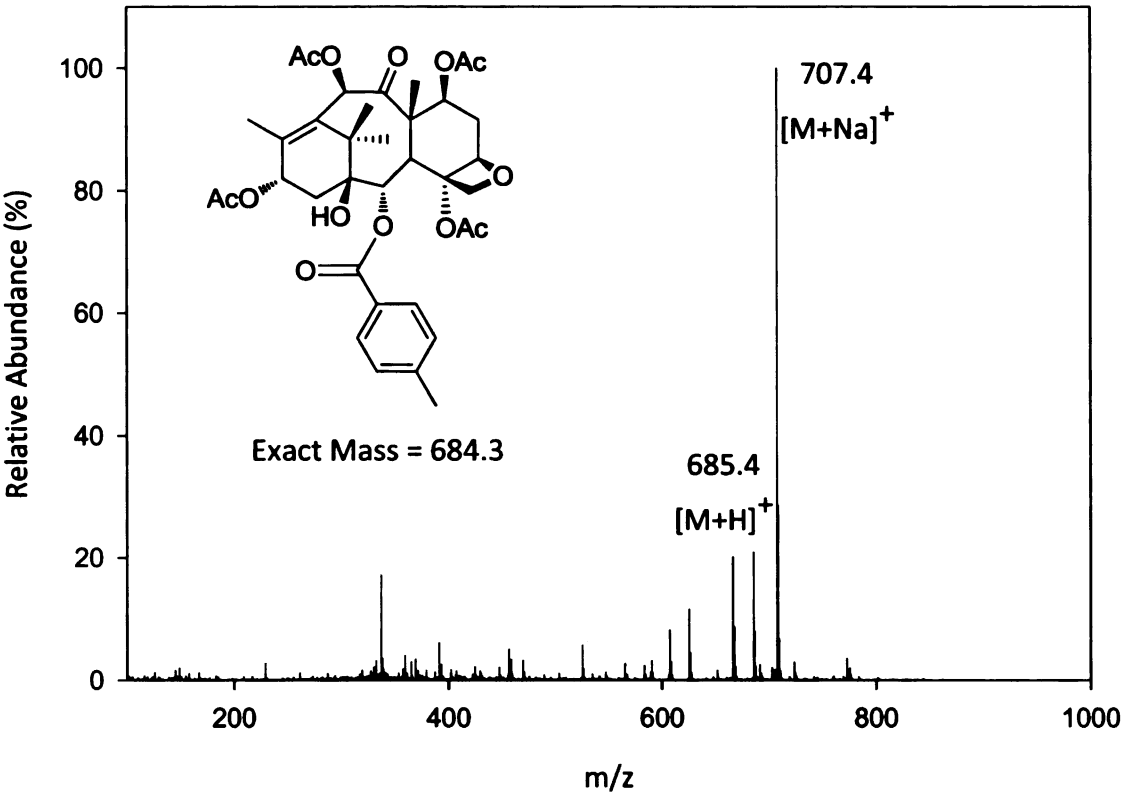


Figure 20. Product mass spectrum for 7,13-diacetyl-2-debenzoyl-2-(4-methylbenzoyl)-baccatin III product as a result of an mTBT and BadA coupled assay. The base peak at m/z = 707.4 corresponds to the $[M+Na]^+$ pseudomolecular ion.

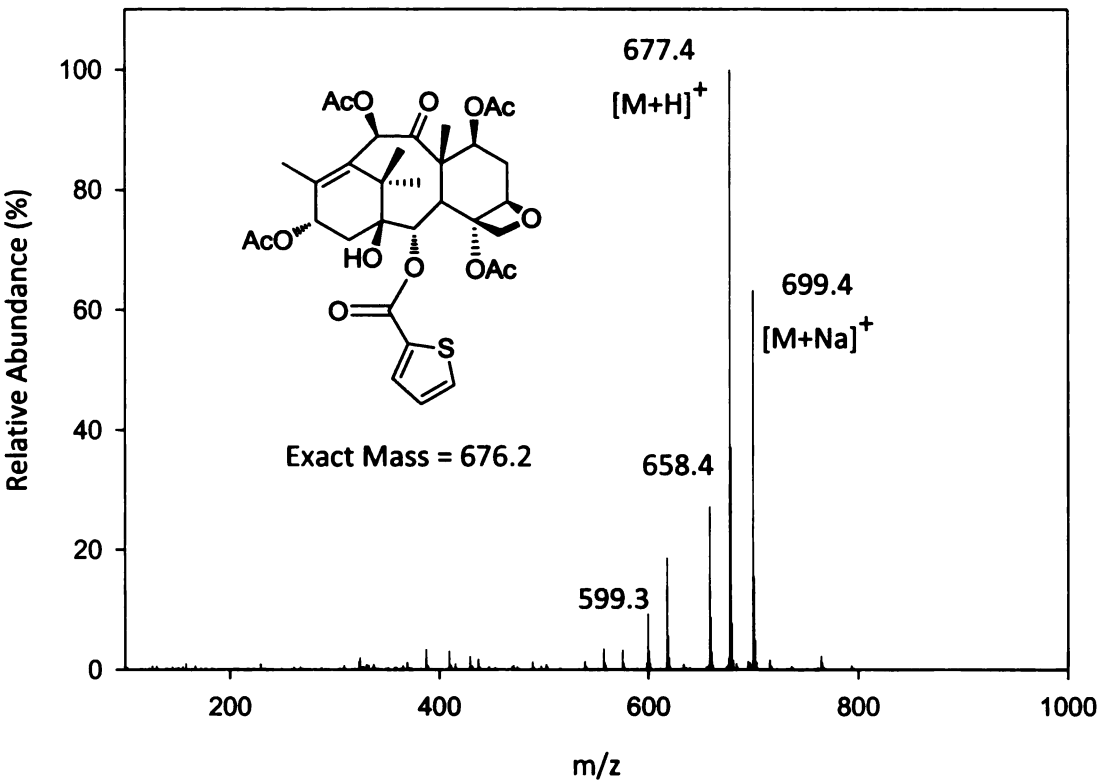


Figure 21. Product mass spectrum for 7,13-diacetyl-2-debenzoyl-2-(thiophene-2-carbonyl)-baccatin III product as a result of an *m*TBT and BadA coupled assay. The base peak at m/z = 677.4 corresponds to the $[M+H]^+$ pseudomolecular ion.

These results indicate that the *m*TBT and BadA coupled activity assays are feasible in the production of 2-*O*-substituted 7,13-diacetyl-2-debenzoylbaccatin III analogues.

2.4: Conclusions

The results of the coupled assay system, where BadA activity is coupled with NDTBT as well as *m*TBT, are promising in many respects. First, when NDTBT is incubated with the natural substrates BzCoA and *N*-debenzoyl-(3′*R*)-2′-deoxypaclitaxel, the amount of observed product 2′-deoxypaclitaxel is statistically identical to that observed when NDTBT is incubated with BadA. These results are important because

they indicate that the activity of NDTBT is unaffected in the coupled assay system. Furthermore, these results prove that the production of 2′-deoxypaclitaxel via the biocatalyst NDTBT can be achieved without the need for synthesizing or purchasing the expensive BzCoA substrate necessary for NDTBT activity. Additionally, the coupled activity with non-natural carboxylic acid substrates further reduces the necessity for synthesized or purchased acyl-CoA substrates. Finally, the results show that a reduced amount of CoASH can be used in the coupled assay system to yield identical product amounts to uncoupled NDTBT assays. This result is especially important because it reduces the need for CoASH, which is an expensive co-substrate (at $ 1,590.00 per g (Sigma)). While CoASH may not be as expensive as the respective acyl-CoA substrates, reducing the necessary amounts within the coupled assay system offers further advantages over the NDTBT assay alone. The studies completed with coupled *m*TBT and BadA activity indicate similar benefits for work with other *Taxus* acyltransferases. While previous work has shown both acyltransferases considered in this research to have broad acyl-CoA substrate specificity, all of the necessary acyl-CoA substrates for such studies had to be synthesized. The use of the coupled assay system circumvents this necessity, allowing for a possibly more efficient method of producing identical substituted paclitaxel analogues without the same high labor and monetary costs.

CHAPTER 3

Future Aims

While the results presented within this thesis research show some promising capabilities of the coupled enzyme assay system discussed, still more research is necessary to further elucidate the complicated nature of such a proposed system. The following brief list outlines some areas of future interest.

1. Optimize the BadA, *m*TBT coupled *in vitro* assay.
2. Compare coupled assay turnover of non-natural carboxylic acid substrates to that of acyltransferase activity with the respective acyl-CoA substrate.
3. Apply coupled assay system with *in vivo* experiments.

The first future aim requires several independent experiments. As with the coupled BadA, NDTBT assay, the first steps would involve elucidating the effects of BadA substrates and cofactors on the reaction catalyzed by *m*TBT. While this may at first consideration seem mundane, it would require the reevaluation of *m*TBT kinetic parameters, as was completed with NDTBT. This would be necessary due to the previous discoveries made concerning the purity of the BzCoA substrates utilized in determining both NDTBT and *m*TBT kinetic parameters.[2,30] With those results in hand, an optimized coupled assay system with BadA and *m*TBT could be devised, leading to the maximal production of baccatin III analogues possible.

As one of the benefits of the coupled assay system discussed throughout this thesis has been the possible production of paclitaxel and baccatin III analogues due to

respective broad substrate specificities, the second future aim is an important one for the viability of such a coupled system. These necessary comparisons would truly demonstrate the possible advantages of the proposed system. However, there are many challenges in completing such research. As an example, recall the drastic changes observed when the NDTBT activity assay, incubated with synthesized BzCoA, showed much less turnover than the analogous coupled assay. These observations were later attributed to the fact that the amount of benzoate donor in the NDTBT activity assay was not equimolar to that of the coupled assay due to impurities. From the discovery of impurities in the synthesized BzCoA, it can be inferred that similar, previously undetected impurities are present in the other acyl-CoA substrates synthesized via the methods presented in previous work.[2] Obviously, if such substrates were used as the acyl donors for NDTBT activity assays, and such assays were compared to coupled assay activity, true comparisons would not be achieved. Thus, the research of aim two is hindered by substrate availability and purity, and would require either newly synthesized or purchased acyl-CoA substrates. This would be an expensive and time consuming endeavor.

While more research is necessary within the *in vitro* coupled assay system, the results of this thesis research have proven that BadA can produce acyl-CoA substrates at a high enough level to be used by two *Taxus* acyltransferases. The third future aim, while perhaps still far off, would be an important one for presenting a complete consideration of the coupled assay system. As one of the arguments in support of this research lies in the possibility of producing non-natural acyl-CoA substrates in a transgenic host cell for use as substrates with *Taxus* acyltransferases, the co-expression of BadA and either

NDTBT or *m*TBT *in vivo* would allow for a complete consideration of such a coupled enzyme system. Incubation of such a transgenic host with the appropriate *N*-debenzoyl-2′-deoxypaclitaxel or 7,13-diacetyl-2-debenzoylbaccatin III substrate, followed by analysis regarding the possible products, would discern the capabilities of such a transgenic host. One main difficulty of such experiments lies in the ability of *E. coli* (or other host cells) to allow the appropriate taxane substrate to permeate the cell membrane. The complex nature of any transgenic host could also prove difficult in determining experimental results. Nevertheless, *in vivo* considerations of the coupled assay system would allow for further implications in the use of transgenic host cells to produce possible second generation paclitaxel analogues.

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