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THE SYNTHESIS OF ARYL PROPANOID COENZYME A THIOESTERS AND TAXOL ANALOGS FOR BIOCHEMICAL INVESTIGATION OF A TAXOL PATHWAY ACYLTRANSFERASE

presented by

SANJIT SANYAL

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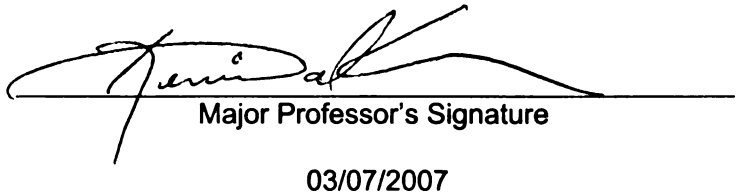
of the requirements for the

MASTER OF SCIENCE degree in CHEMISTRY



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Date

THE SYNTHESIS OF ARYL PROPANOID COENZYME A THIOESTERS AND TAXOL ANALOGS FOR BIOCHEMICAL INVESTIGATION OF A TAXOL PATHWAY ACYLTRANSFERASE

By

Sanjit Sanyal

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ABSTRACT

THE SYNTHESIS OF ARYL PROPANOID COENZYME A THIOESTERS AND TAXOL ANALOGS FOR BIOCHEMICAL INVESTIGATION OF A TAXOL PATHWAY ACYLTRANSFERASE

By

Sanjit Sanyal

Coenzyme A thioesters have attracted considerable attention since they represent an important class of activated intermediates in various biological pathways. It is estimated that approximately 4% of all known enzymes require CoA or CoA thioesters as substrates. The present investigation describes the optimization of the synthesis of surrogate coenzyme A thioesters that will be used to dissect the proposed mechanism of an acyltransferase. Modifications of the previously described method by Walker et al. (Walker, K., Fujisaki, S., Long, R. & Croteau, R. *Proc. Natl. Acad. Sci., U.S.A.* **2002**, *99*, 12715) resulted in improvement of yields from submiligram quantity (~30%) to near quantitative conversion (65-70%) with identical reaction conditions; this was most apparent upon re-synthesis of two productive 3-amino-3-phenylpropanoid CoAs synthesized in the previous study. Modification of an existing method for the coupling of the 3'-amino-3'-phenylpropanoid sidechain of Taxol® onto C13 of baccatin III is described that will give access to a library of Taxol® derivatives including second-generation taxanes in a short synthetic route. Furthermore, synthesis of radiolabeled baccatin III, an advanced taxol biosynthetic pathway intermediate, is also described.

To my parents and wife for their support and love

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Last but not least, I would like to express my appreciation and love to my wife Sulagna, and to my father, mother and sister for their love, encouragement and emotional support.

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LIST OF ABBREVIATION

THF	Tetrahydrofuran
DCM	Dichloromethane
GC/MS	Gas Chromatography - Mass Spectrometry
br	Broad
s	Singlet
d	Doublet
t	Triplet
q	Quartet
Boc	*tertiary*Butyloxy Carbonyl
ESI-MS	Electrospray Ionization Mass Spectrometry
CoA	Coenzyme A
TFA	Trifluoroacetic acid
NMR	Nuclear Magnetic Resonance Spectrum
TES	Triethylsilyl
DMAP	N, N-Dimethylaminopyridine
DPTC	Dipyridyldithiocarbonate

CHAPTER 1

SYNTHESIS OF ARYL PROPANOID COENZYME A THIOESTERS

1. 1 INTRODUCTION:

Natural products with interesting structural or biological function within the host organism or that possess unique bioactivity against disease such as antimalarials[1-2] or anticancer,[3-5] usually are initially isolated and structurally characterized by a variety of analytical methods. Advances in molecular genetic techniques (e.g., EST library construction)[6-8] have facilitated the isolation of biosynthetic genes on the pathway of corresponding natural products. Mutagenic methods to alter a single gene on the pathway coupled with a metabolomic survey of the resulting pathway intermediates, particularly after feeding labeled precursors to the host organisms, can often unequivocally establish the function of the encoded enzyme.[9] However, in systems where *in vivo* feeding of substrates to auxotroph, or fully-functional organisms, is precluded by impermeability to cell membranes or by complications in the culturability of the host organisms,[10] individual genes are alternatively expressed in a heterologous host, and the function of the corresponding enzyme is validated by *in vitro* assay with rationally selected substrate(s).

Accordingly, *in vitro* assay procedures have been used to determine the function of coenzyme A (CoA)-dependent acyltransferases that impart diverse biological processes throughout all kingdoms of organisms.[11] Acyl CoA thioesters serve as acyl

group donors along the metabolic pathways of amino acids,[12,13] lipids,[14-17] secondary metabolites,[18,19] and carbohydrates[20]. The acyltransferases on the corresponding pathways utilize the relatively labile thioester bond to convey acyl groups to an acceptor molecule. Examples are demonstrated in the formation of carbon-carbon bonds during iterative Claisen condensation steps in fatty acid[21] and polyketide biosyntheses,[22] of carbon-oxygen bonds during the acylation of hydroxyl groups in biosynthesis of vinorine[23] and paclitaxel,[8] or of carbon-nitrogen bonds in amino group acylation of penicillin G[24] and *N*-acetylsphingosine[25] biosyntheses.

Coenzyme A contains a reactive nucleophilic thiol functional group that interacts with an activated carboxylic acid to produce acyl coenzyme A thioesters. One of the most common synthetic methods to activate carboxylic acid involves treatment of the acid with an alkyl/aryl chloroformate under basic condition to form a mixed anhydride (Figure 1)

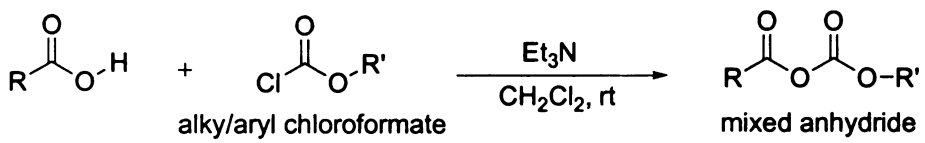


Figure 1: Activation of carboxylic acids for the production of esters and thioesters

that will make the carbonyl group of the carboxylic acid more reactive towards an incoming nucleophile, thiol function in this case leading to the formation of coenzyme A thioesters (Figure 2).

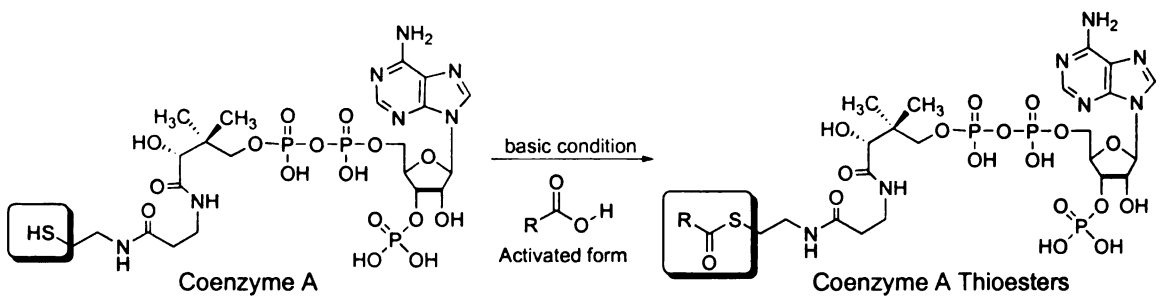


Figure 2: Synthesis of coenzyme A thioesters

Commercially available CoA thioesters for biochemical studies are largely limited to alkanoyl/alkenoyl/fatty acid-substitution, with the exception of benzoyl-CoA (Figure 3). Additionally the high cost of commercially available coenzyme As is due in large part to production cost from natural resources.[26] Consequently, only a limited selection of the acyl thioesters is available.

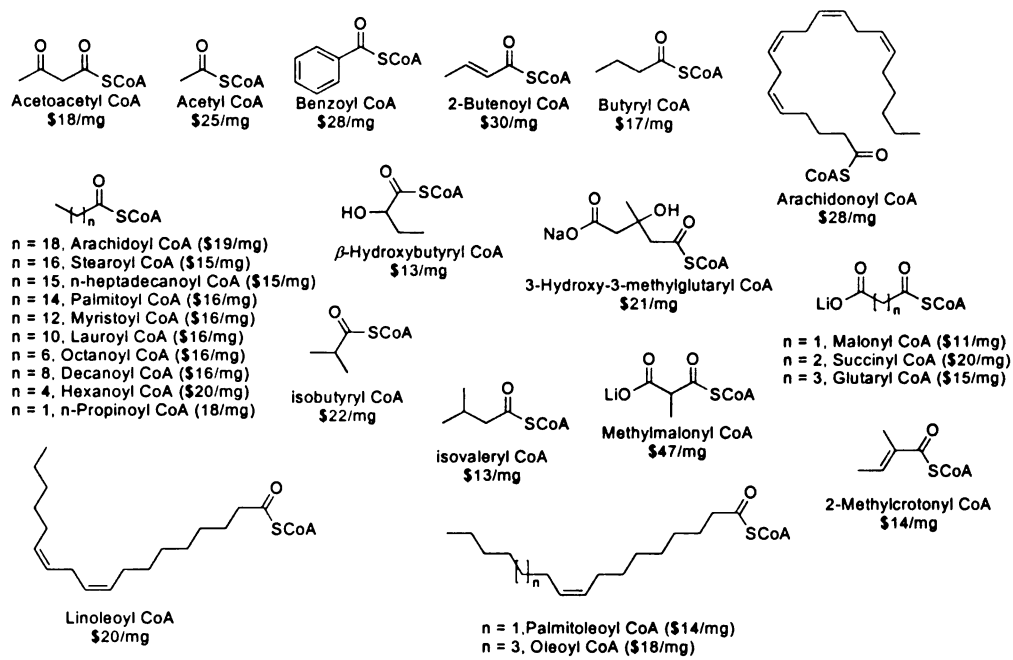


Figure 3: Commercially available coenzyme A (from Sigma Catalog, 2006)

The present acyl coenzyme A library lacks acyl groups that possess reactive functional groups, and acyl-CoAs of this type remain a challenge to the synthetic community. The synthesis and purification of various acyl CoAs in microgram scale was reported earlier using CoA ligases[27] but the substrate specificity of these enzymes restricts access to a broad series of products. Recent methods incorporating catalytic RNA in the synthesis of CoA thioesters[28] emerge as an interesting approach, although the limited substrate specificity of these RNA-based catalysts remains a major drawback toward practical application of these methods. Synthetic procedures for acyl-CoA have also been

reported,[29-33] but most processes outline the activation of an alicyclic acyl module that is void of functional groups with competing reactivity.

In 1991, Walt and his coworkers demonstrated that various acyl-CoAs can be synthesized using S-acylthiocholine iodide as an acylating agent,[34] but the CoAs they synthesized are all commercially available and the acyl groups are mainly unfunctionalized hydrocarbons (Figure 4).

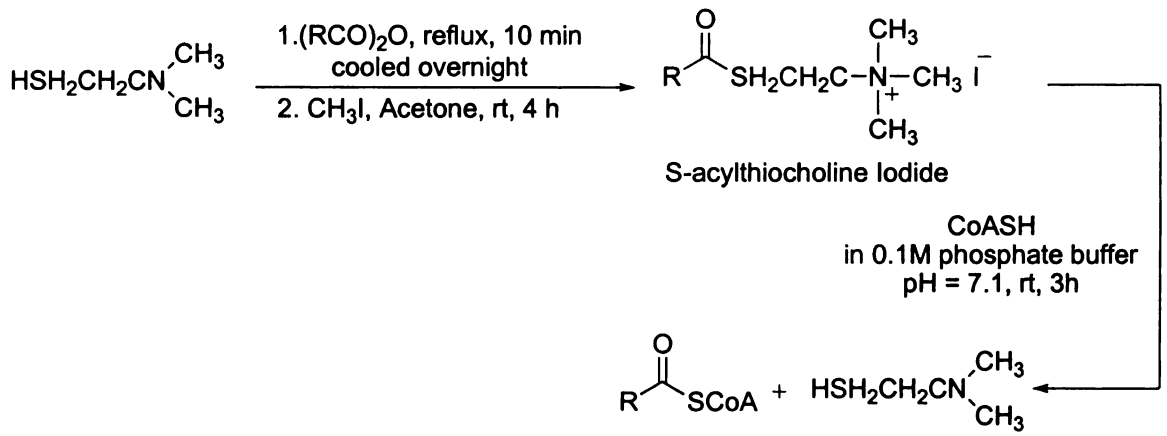


Figure 4: Synthetic route developed by Walt et al.

In 1995, Strohl and his coworkers reported a synthetic route[35] using 1,1'-carbodiimidazole as an activating agent (Figure 5) for synthesizing acetyl CoA and propionyl CoA, which are relatively simple in complexity and, moreover, are commercially available.

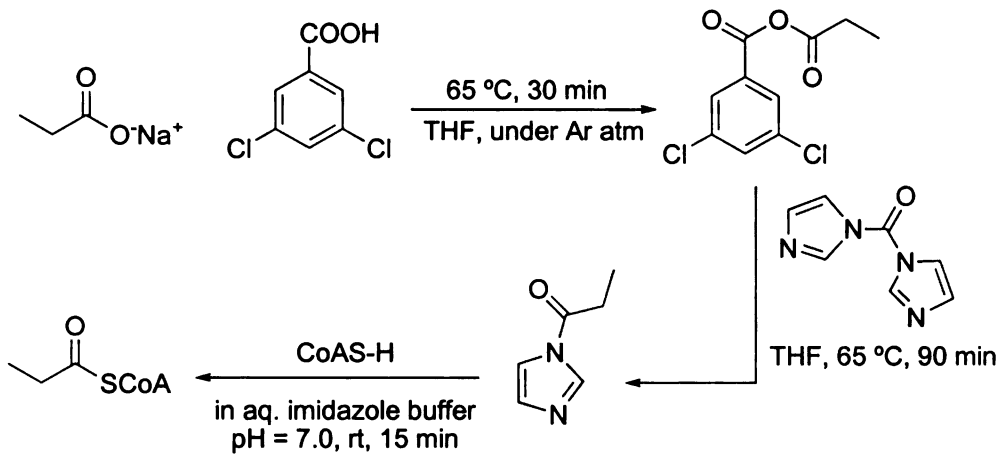


Figure 5: Synthesis of propionyl CoA by Strohl et al.

The first example of the synthesis of a substituted phenylpropanoyl CoA came from Noel and his coworkers in 2000.[36] They have described a two-step synthesis of coumaryl-CoA involving the generation of an N-hydroxysuccinimide ester of 4-coumaric acid followed by a thioester exchange with CoA (Figure 6). Regrettably, experimental details, such as yield and spectroscopic characterizations are missing in this report.

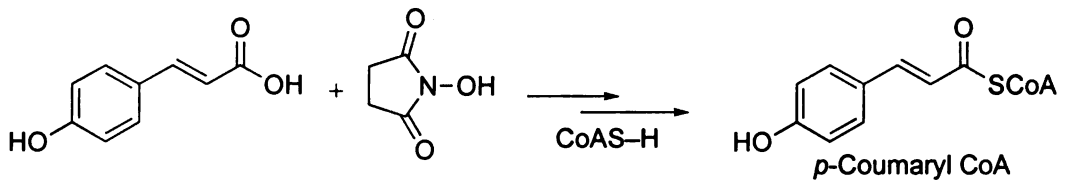


Figure 6: Synthesis of *p*-coumaryl CoA by Noel et al.

The aforementioned syntheses describe the production of target CoA thioesters used to investigate aspects of primary metabolism in various organisms. However, in 2002, the first synthetic procedure for making milligram quantities of amino- and aminohydroxy phenylpropanoyl CoA was reported.[37] In order to assess the biosynthetic origin of the phenylpropanoid side chain of paclitaxel, several amino acid-CoA thioesters were employed in a previous investigation by Walker and his coworkers.[37] *β*-

Phenylalanoyl- and phenylisoserinoyl-CoA (Figure 7) were synthesized and identified as productive substrates of the expressed taxane 13β-*O*-phenylpropanoyltransferase.[37]

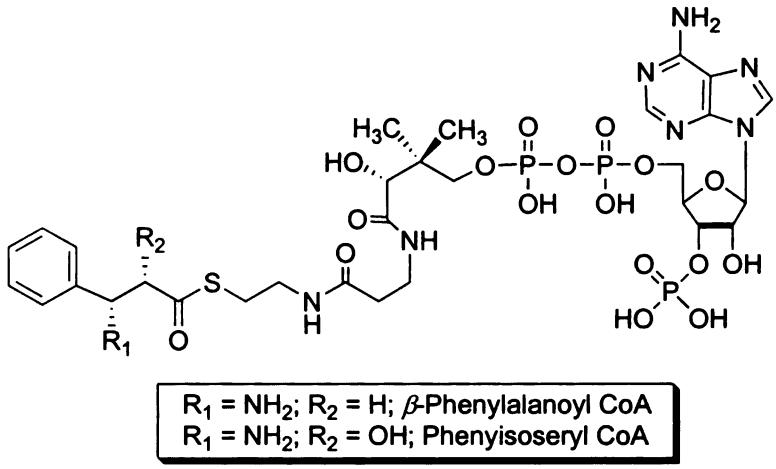


Figure 7: β-phenylalanoyl- and phenylisoserinoyl-CoA

The apparent void in this area of research encouraged efforts to develop a direct process for synthesizing quantitative amounts of novel acyl CoA thioesters. With an emerging focus on biocatalysis, it becomes imperative to have access to a variety of surrogate substrates in order to survey the substrate specificity of a particular enzyme. Therefore to probe the specificity of the aminophenylpropanoyl transfer reaction in the Taxol® biosynthetic pathway an expanded library of CoA thioesters bearing a variety functional groups on the acyl moiety are explored and are described herein. The primary goal was to optimize the reaction conditions of previously developed methods to make the process more practical, economical, and general for the synthesis of a series of unnatural CoA thioesters.

Herein is described a general procedure for synthesizing milligram quantities of various aryl substituted propanoid coenzyme A thioesters. Also described is a modified method to remove butoxycarbonyl from reactive amines by mild deprotection with

formic acid, which replaces the less selective trifluoroacetic acid-based deprotection that partially degrades the CoA thioester.[37] In general, the described methods, demonstrate the incorporation of a mixed anhydride intermediate of a reactive carboxylic acid to convert variously substituted aryl propanoic acids to their corresponding CoA thioester for investigations on a phenylpropanoyltransferase enzyme on the paclitaxel biosynthetic pathway. Several of the thioesters produced in this study can also be applied toward investigation on phenylpropanoid metabolism that includes lignin and lignan biosyntheses.[38-40]

1. 2 RESULTS AND DISCUSSION:

The target coenzyme A thioesters were designed in such a way that can produce a library of CoA thioesters of various functionalities with competing reactivity in them (Figure 8).

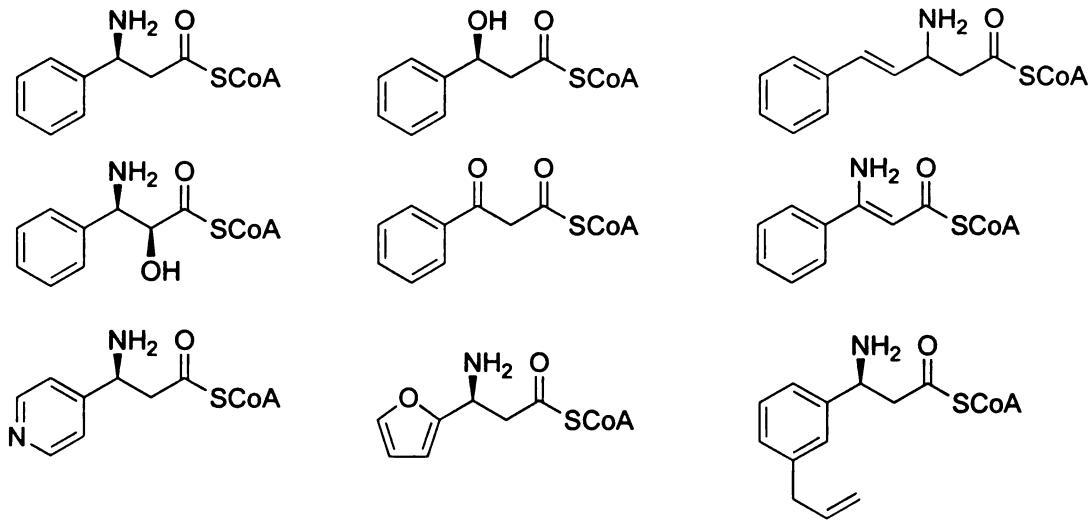


Figure 8: Target coenzyme A thioesters

In order to investigate the substrate specificity of the 3-amino-3-phenylpropanoyltransferase, the amounts of natural and surrogate CoA thioester substrates derived synthetically need to consistently fall in milligram scale. These relatively large gravimetric quantities are required to compare relative kinetic constants of the enzyme for each substrate. Furthermore, this also ensures that large-scale biosynthetic assays can be conducted to produce enough product for characterization by sample-limited techniques, such as ^{1}H- and ^{13}C-NMR.

The synthesis of β-phenylalanoyl- and phenylisoserinoyl CoAs in a previous study were not optimized for maximal yield.[37] Instead, the focus of this earlier investigation was framed in the context of characterizing the function of enzymes in a library of acyltransferases expressed from genes derived from *Taxus* plants. The assays

were developed to incorporate natural substrates, which are likely very catalytically efficient. To assess the efficiency of surrogate substrates significant substrate may be required to achieve apparent saturation of the enzyme for maximal turnover.

Following the literature procedure that describes the synthesis of *β*-phenylalanoyl CoA a trial synthesis was conducted to gain insight of the steps and processes of the procedure that could compromise the yield. Various aspects of the synthesis were modified to increase the yield from ~30%[37] to ~66%. The commercially available *β*-phenylalanine was first converted to its corresponding *N*-Boc protected amino methyl ester (Figure 9).

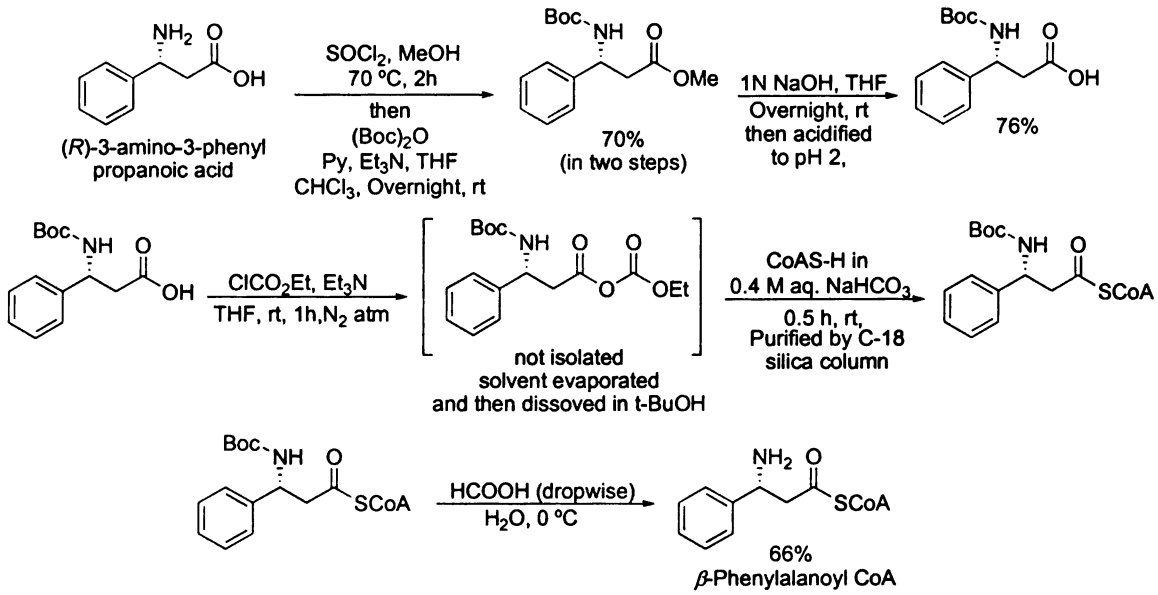
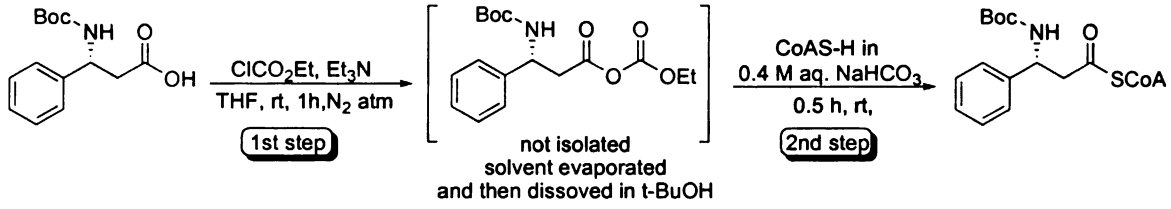


Figure 9: Protocol for synthesis of *β*-phenylalanoyl CoA

Then CoA as trilithium salt dissolved in a 0.4 N aqueous solution of sodium bicarbonate was added into the reaction. Stirring the reaction for half hour and then quenching with hydrochloric acid produced the *N*-Boc protected desired thioester. The *N*-Boc protected acyl CoA was isolated by evaporating the solvent under reduced pressure and then purified by anion exchange column using C-18 silica gel chromatography. To deprotect

the *N*-Boc group 88% aqueous formic acid was employed in place of trifluoroacetic acid that was used in previous work.[37] By virtue of formic acid being a weaker acid, the deprotection step was slower than when more reactive trifluoroacetic acid used in the previous method, however, degradation of the product was minimized upon deprotection with the milder reagent.

Thus far described have been procedures to optimize the first two steps and the last steps of the CoA thioester synthesis described in the previous investigation.[37] Yet, the formation of the mixed anhydride and the ensuing coupling reaction with CoA still needed to be optimized. Increasing the CoA concentration from one to two equivalents did not significantly improve the yield, which remained approximately at 61%. This overall yield was sensitive to the reaction time of the first step where the carboxylate substrate is converted to the anhydride (Table 1).



Entry	time for 1st step (min)	time for 2nd step (min)	% Yield
1	60	30	61
2	30	30	38
3	30	60	40
4	90	30	60
5	90	60	61

Table 1: Optimization of reaction time for synthesis of coenzyme A thioesters

From the results, it is apparent that the reaction time for anhydride formation is an important factor to obtain a better yield. Since increasing the time for the trans-

thioesterification reaction did not significantly change the yield, it can be assumed that the first step is rate-limiting, and is therefore guiding the overall product formation, i.e., the yield of the mixed anhydride is directly proportional to observed mass 913.27 in this sequential reaction. The identity of the coenzyme thioester was verified by ^{1}H-NMR and confirmed with ESI-MS analysis (negative ion mode) (Figure 10).

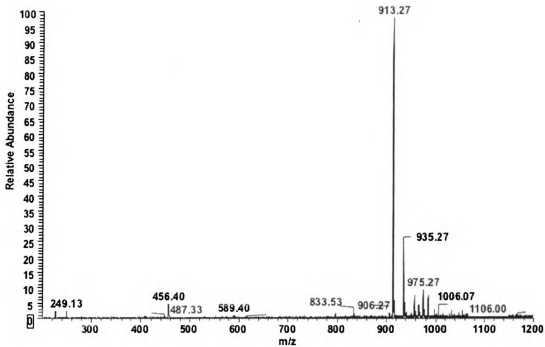


Figure 10: ESI-MS of β-phenylalanoyl CoA

The next coenzyme thioester target had a hydroxy group instead of amino group at the C3 of the β-phenylpropanoyl CoA. The general procedure described previously was performed except that the last deprotection step was unnecessary since no Boc-protection was needed for the starting material. The hydroxy group on the phenylpropanoid sidechain was anticipated to not be as nucleophilic as the CoA thiol in the polar protic solvent (*t*-BuOH) toward the anhydride. One equivalent of ethyl chloroformate was used to convert the 3-hydroxylphenylpropanoic acid to the

corresponding CoA thioester outlined in Figure 11. Notably, while the thioesterification step remained the same as in the synthesis of other CoA thioesters, the solvent system of the initial step was modified from THF to a mixture of dichloromethane and THF to solvate the starting material.

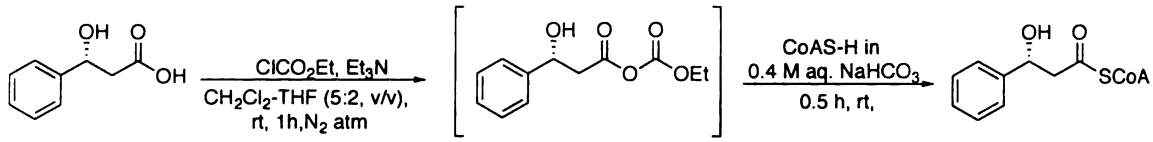


Figure 11: Synthesis 3-hydroxy-3-phenylpropanoyl CoA

However, ESI-MS analysis revealed the presence of an analyte (at m/z 838.27) with an ion abundance similar to that of the target thioester product (at m/z 914.20) (Figure 12).

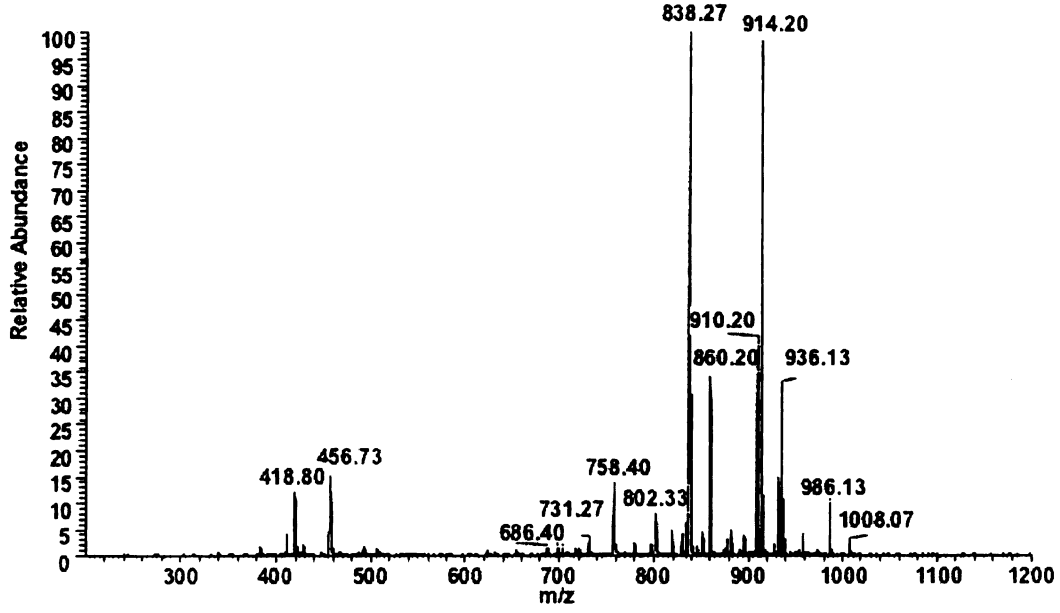


Figure 12: ESI-MS showing desired 3-hydroxy-3-phenylpropanoyl CoA (m/z 914.20) and an impurity (m/z 838.27)

Further analysis suggested that the compound is also a CoA thioester and was putatively identified as a coupling product of ethyl chloroformate and the CoA salt (Figure 13).

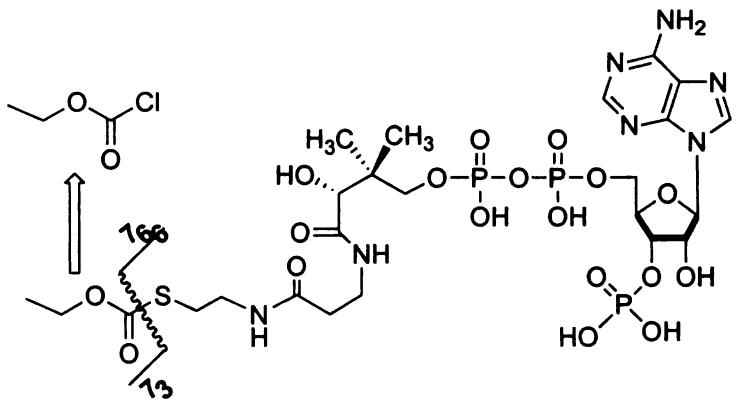


Figure 13: Structure elucidation of the by-product generating molecular ion m/z 839

There are two possibilities for how this compound was generated. Either the CoA-SH can attack the carbonate carbonyl instead of the acyl-carbonyl of the intermediate anhydride or it can displace chloride from unreacted ethyl chloroformate to form a thiocarbonate ethyl ester as shown in Figure 14.

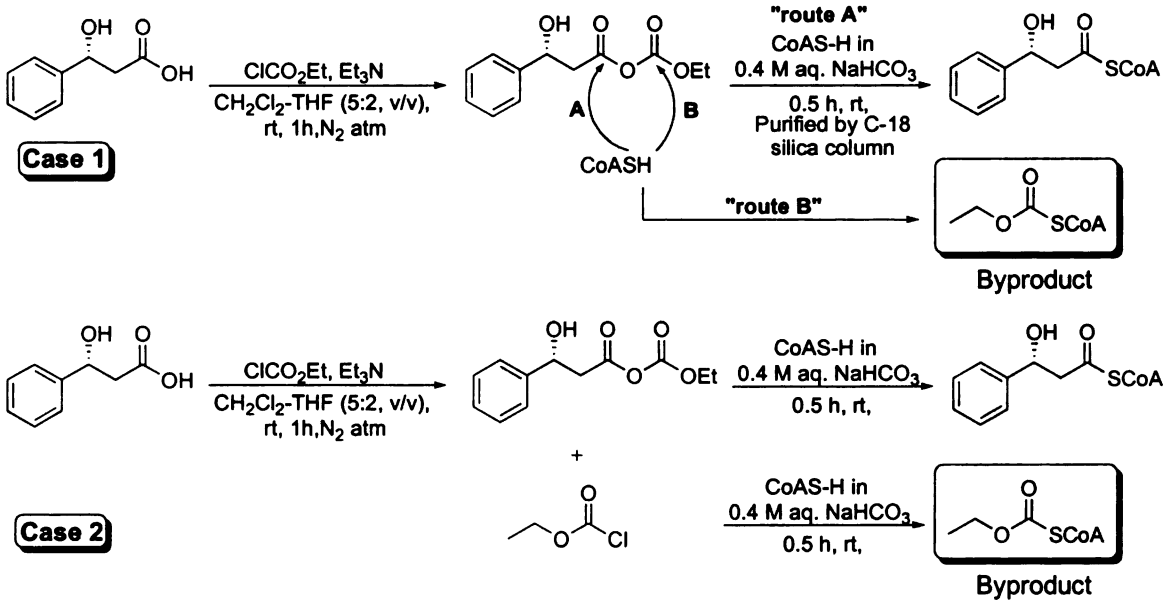


Figure 14: Two possible ways of forming the by-product with molecular ion m/z 839

It is worth noting that in principle the acyl-carbonyl should be a more reactive electrophilic center than the carbonate carbonyl. Contributing resonance structures demonstrate that the lone pairs of electrons on the oxygen atoms flanking the carbonate

carbonyl contribute electron density to this reactive carbon center, thus minimizing the electrophilicity at this site (Figure 15). In contrast, the acyl carbonyl has only one oxygen atom that donates electrons to reactive carbonyl carbon, and therefore the acyl carbonyl is considered less deactivated than the carbonate carbonyl.

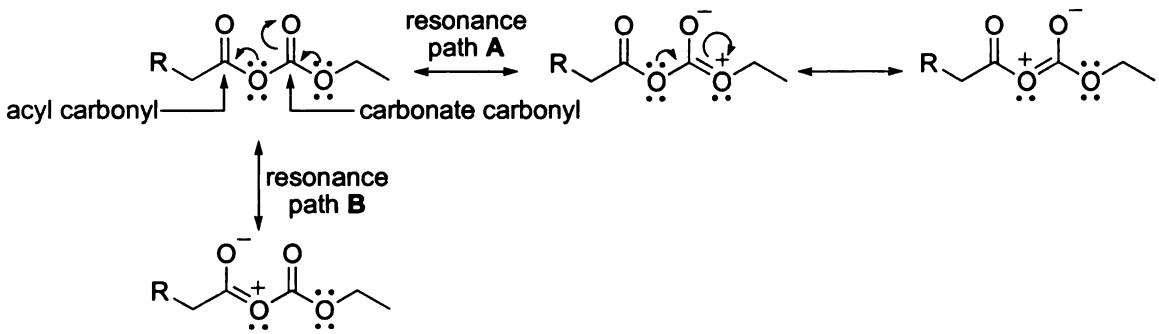


Figure 15: Origin of differential reactivity of carbonate carbonyl and acyl carbonyl

If indeed CoASH is not regioselective for attack on a carbonyl in the asymmetric anhydride, several options were considered to bias nucleophilic attack toward the acyl carbonyl e.g. use of *t*-butyl chloroformate with a bulky alkyl group as the acid-activating agent and *t*-butyl would likely diminish the CoASH attack towards the carbonate carbonyl due to the steric hindrance of the *t*-butyl group.

Alternatively, less ethyl chloroformate (0.5 equivalent) was employed to minimize the potential reaction of CoASH with excess chlorocarbonate reagent. ESI-MS analysis of the product from the modified coupling reaction revealed that a major portion of CoASH was unreacted ([M-H], m/z 766.27) and that the desired thioester ([M-H], m/z 914.20) was in relatively lower abundance (Figure 16).

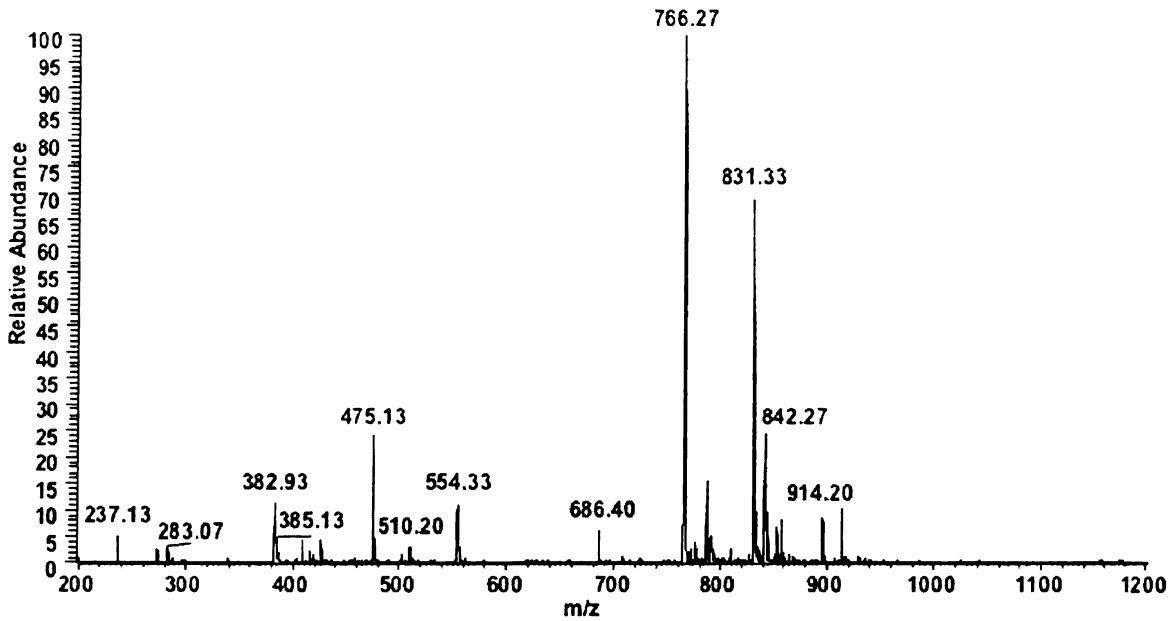
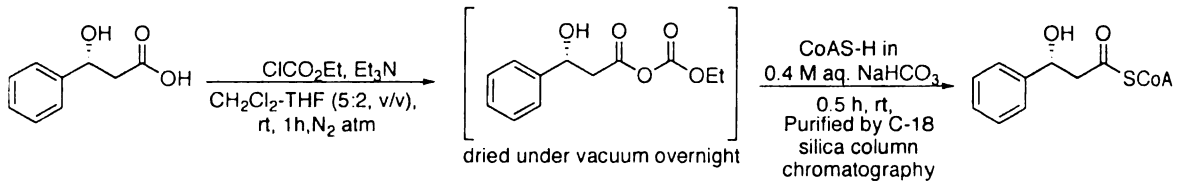


Figure 16: ESI-MS showing unreacted CoA as a major component at m/z 766.27

A third option was attempted prior to converting the acid to a formate ester bearing a bulky *t*-butyl group. Instead of carrying the mixed anhydride to the next step in the reaction series, after only removing the methylene chloride/THF solvents, the product residue was dried under high vacuum for 24 h. This simple variation was considered to potentially remove excess unreacted chloroformate (b. p. 95 °C), which is relatively volatile. The resulting residue was isolated as a white solid compared to previous cases where the residue containing the anhydride was obtained as a pale-white paste. Addition of the CoASH under standard reaction conditions to the anhydride (as a white solid) in the second step of reaction yielded the desired product as a single product, which was verified by the abundant ion ([M-H], m/z 914.08) in the ESI-MS spectrum (Figure 17).



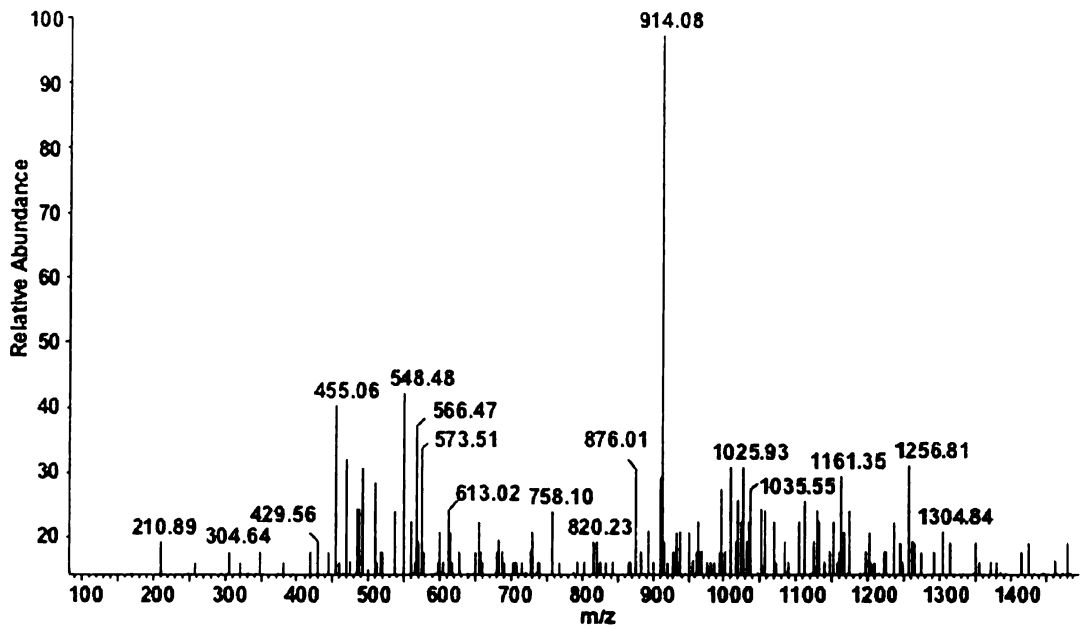
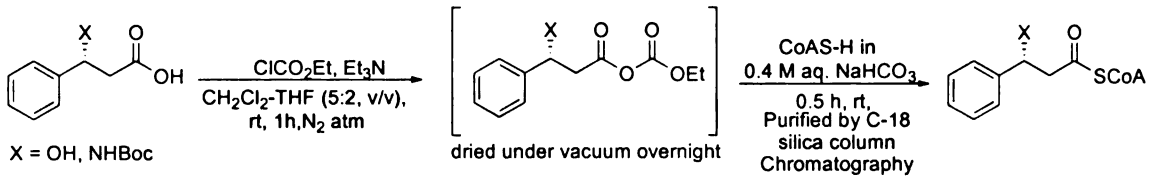


Figure 17: ESI-MS showing the desired thioester (m/z 914.08) as major product

Therefore, it is concluded that the production of the ethyl formate thioester of CoA by-product in previous attempts at this reaction sequence with 3-hydroxy-3-phenylpropanoic acid resulted from the reaction between unreacted ethyl chloroformate and CoASH (Figure 14). Drying the reaction overnight under vacuum removed all of the unreacted ethyl chloroformate; thus the CoASH nucleophile attacked only the activated acid and not the competing chloroformate reagent.

Now that a convenient method for purifying the mixed anhydride was developed, optimization of the ethyl chloroformate used in the reaction was pursued. Ethyl chloroformate at 1.5 and 2.0 equivalents gave maximal yield at 68% (Table 2) indicating that 1.5 equivalent of the activating agent is ideal for these reactions. These modified steps were applied to synthesis of other phenylpropanoyl CoA thioesters.



X	Entry	Equiv. of Chloroformate	% Yield
OH	1	1.0	31
OH	2	1.5	68
OH	3	2.0	68
NHBoc	1	1.0	61
NHBoc	2	1.5	66

Table 2: Optimization of amount of ethyl chloroformate

The synthesis of *β*-phenylalanoyl CoA, described in an earlier report[31], was reinvestigated, wherein the amount of ethyl chloroformate was optimized and the mixed anhydride was dried overnight under vacuum, as described above to remove unreacted chloroformate prior to conducting the next reaction step. However, the addition of excess ethyl chloroformate (1.5 equivalents) showed no significant effect on the yield (Table 2). Thus treatment was followed by the general procedure for CoASH coupling, and ESI-MS analysis of the ensuing target product confirmed that the by-product ([M-H], m/z 838.27) (cf. Figure 12), derived by the coupling of ethyl chloroformate with CoASH, was absent; a single abundant peak corresponding to *N*-Boc-*β*-phenylalanoyl CoA was observed ([M-H], m/z 1013.40) (Figure 18).

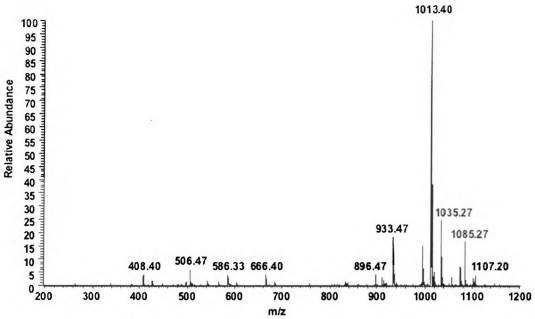


Figure 18: Absence of the byproduct in synthesis of β-phenylalanoyl CoA

Intriguingly, even when excess chloroformate was not removed under vacuum prior to initiating the final thioesterification step, the CoA nucleophile preferentially attacked the β-phenylalanoyl mixed anhydride species. Relatively, none of the ethyl carbonate thioester at [M-H], m/z 838.27 (cf. Figure 12) was observed by ESI-MS analysis (Figure 18). This observation is in stark contrast to that for the similar reaction with β-hydroxyphenylpropanoic acid where a significant amount of carbonate thioester was produced. Conceivably, when the free OH-group of the β-hydroxyphenylpropanoid is not protected a non-bonded interaction (like H-bonding) might low the activity of the acid towards the mixed anhydride formation (Figure 19).

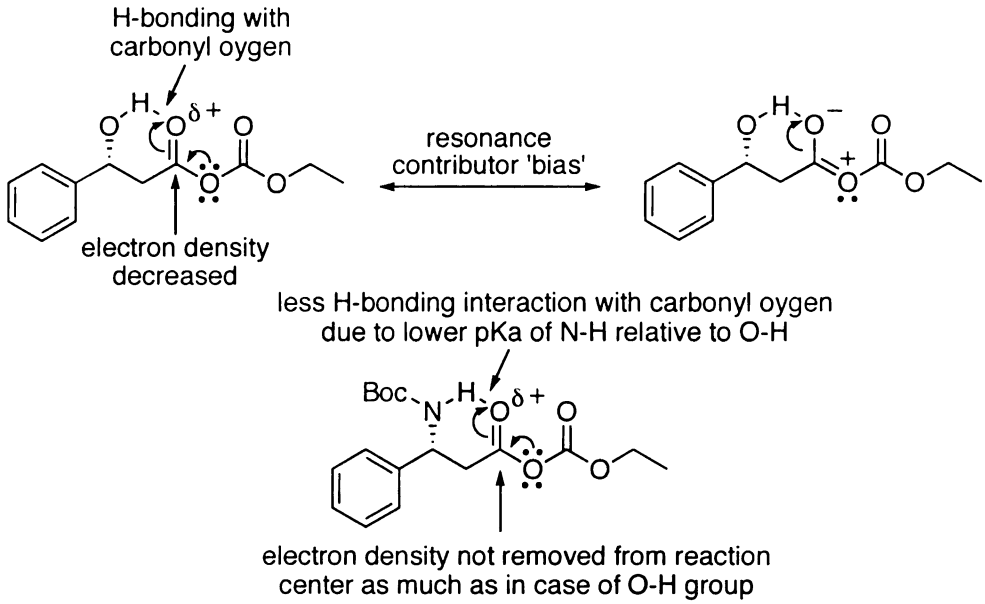


Figure 19: Origin of differential reactivity of acyl carbonyl in *β*-hydroxy- and *N*-Boc-*β*-aminophenylpropanoyl anhydride

Notably, the O-H bond is more labile than an N-H bond and, therefore, the O-H group is more apt to engage in hydrogen bonding. Consequently, an intramolecular H-bonding interaction with the carbonyl oxygen should increase the electrophilic character of the carbonyl carbon and thus should accelerate the reaction rate. Ironically, this is not observed. The reason for this differential reactivity of the CoA nucleophile is unclear, and experiments are currently being investigated to explore the phenomenon.

The *β*-styrylalanoyl CoA was made from *β*-styrylalanine that was produced during the preparation of the product standard in the phenylaminomutase (PAM) project.[41] Since the *β*-amino acid was not commercially available, the first challenge was to synthesize this amino acid derivative via modification of a literature procedure.[42] (*S*)-3-Amino-5-phenylpentanoic acid was converted first to its *N*-Boc methyl ester derivative. Photochemical benzylic bromination of the *N*-Boc protected amino ester was proceeded with *N*-bromosuccinimide treatment followed by a dehydrobromination to produce the *N*-

Boc styryl-β-alanine methyl ester. Saponification of the ester and thioesterification of the carboxylate with CoA followed the general procedure described earlier. Final N-Boc deprotection with formic acid yielded styryl-β-alanoyl CoA (Figure 20). The identity of the desired CoA thioester was confirmed by ESI-MS analysis ([M-H], m/z 939.33) (Figure 21).

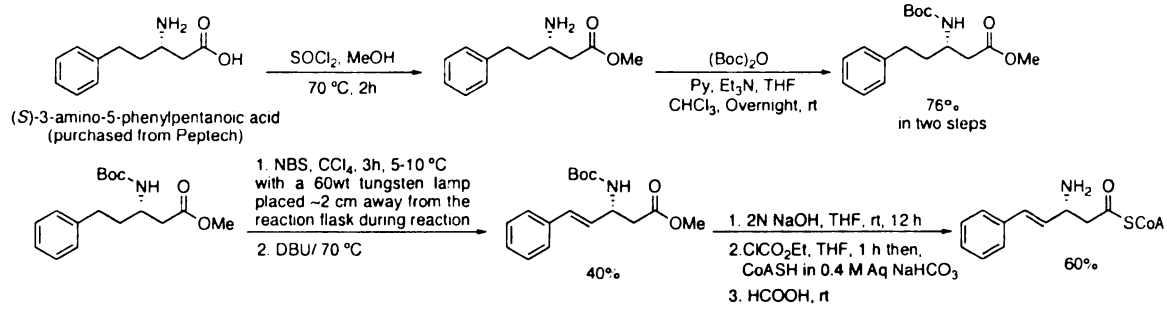


Figure 20: Synthesis of styryl-β-alanoyl CoA

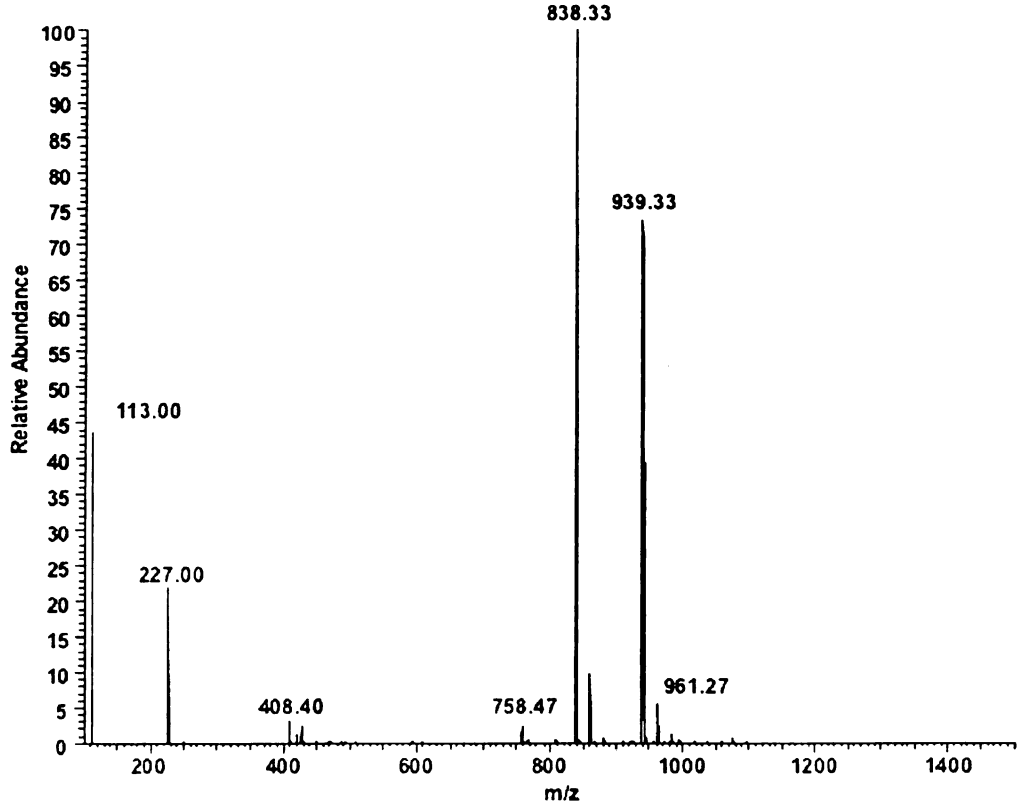


Figure 21: ESI-MS spectrum of styryl-β-alanoyl CoA ($[M-H]^{-1}$, m/z 939.33)

The procedure for the synthesis phenylisoserinoyl CoA is reported in the literature.[37] However, repeating this synthetic process revealed that trifluoroacetic acid-mediated deprotection was harsh and difficult to control, resulting in compromised yields. Formic acid, a milder deprotecting agent was used instead of TFA to remove the Boc-group (Figure 22).

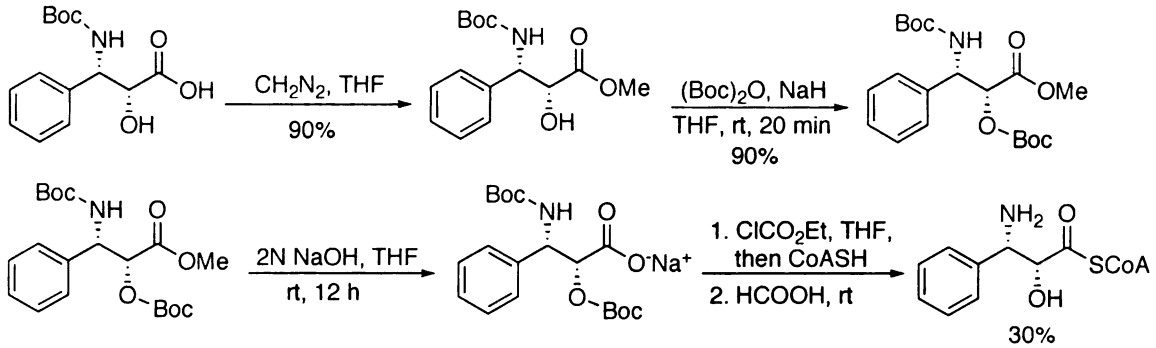


Figure 22: Synthesis of phenylisoserinoyl CoA

N-Boc-phenylisoserine was first converted to its methyl ester by diazomethane treatment then C2 hydroxyl group was protected as *O*-Boc group with di-*tert*-butoxydicarbonate and sodium hydride treatment. The *N*- and *O*- protected intermediate was saponified and converted to the thioester following the procedure described earlier. Final deprotection with formic acid produced the desired CoA thioester, as verified by ESI-MS analysis ([M-H], m/z 928.8) (Figure 23).

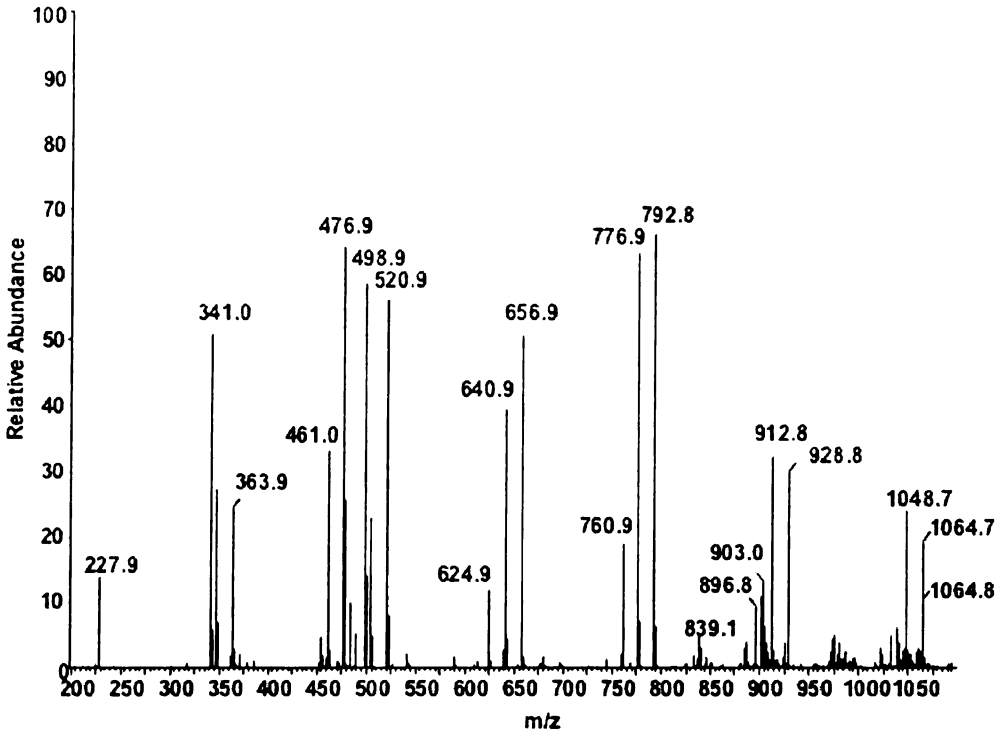


Figure 23: ESI-MS spectrum of phenylisoserinoyl CoA ([M-H]$^{-1}$, m/z 928.8)

Encouraged by the synthesis of 3-hydroxy-3-phenylpropanoyl CoA without hydroxy group protection has prompted a reinvestigation of the synthesis of phenylisoserinoyl CoA without *O*-Boc protection (Figure 24).

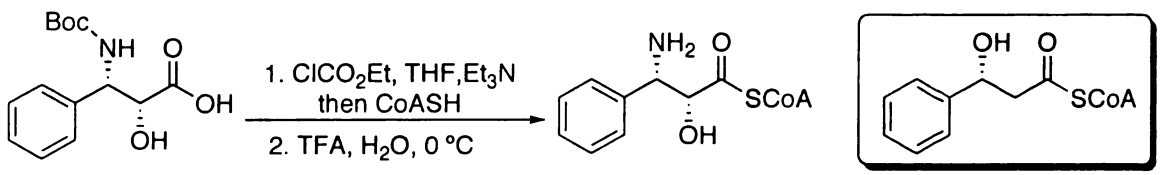


Figure 24: Future synthesis of phenylisoserinoyl CoA thioester

Currently, experiments are ongoing in order to synthesize a series of coenzyme A thioesters in which the acyl moiety possess a variety of structural features and functional groups that have competing nucleophilic reactivities. The main challenge is gaining access to the corresponding β-amino acids. For example, 3-pyridyl-3-aminopropionic acid is not available commercially, and, consequently, the synthesis of this pyridyl amino

acid was attempted by following a literature procedure.[43] The first step of the reaction involved the formation of an enamine with excess ammonium acetate dissolved in ethanol under reflux. The water, by-product of this reaction, hydrolyzed the enamine back to the starting material, ultimately, even when attempts were made to remove the water with Dean-Stark trap and by adsorption with 4Å molecular sieves. The challenge was overcome by using tetraethoxy orthosilicate that serves as a very powerful dehydrating agent during the reaction (Figure 25). The product will be converted to its corresponding CoA thioester.

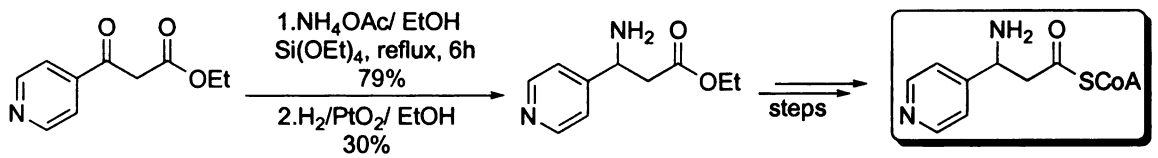


Figure 25: Synthesis of 3-pyridyl-3-aminopropionic acid

3-Furyl-3-aminopropionic acid was purchased and was converted to its N-Boc methyl ester derivative (Figure 26).

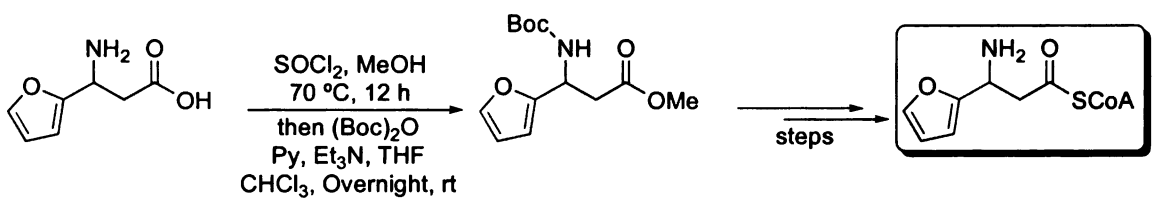


Figure 26: Synthesis of methyl N-Boc-3-furyl-3-aminopropionic acid

Ethyl benzoylacetate was used to synthesize benzoylacetic acid following a literature procedure.[44] It was also used to prepare a unique enamino acid (Figure 27) following a literature procedure.[43] All these β-amino acids will be converted into corresponding CoA thioesters in near future.

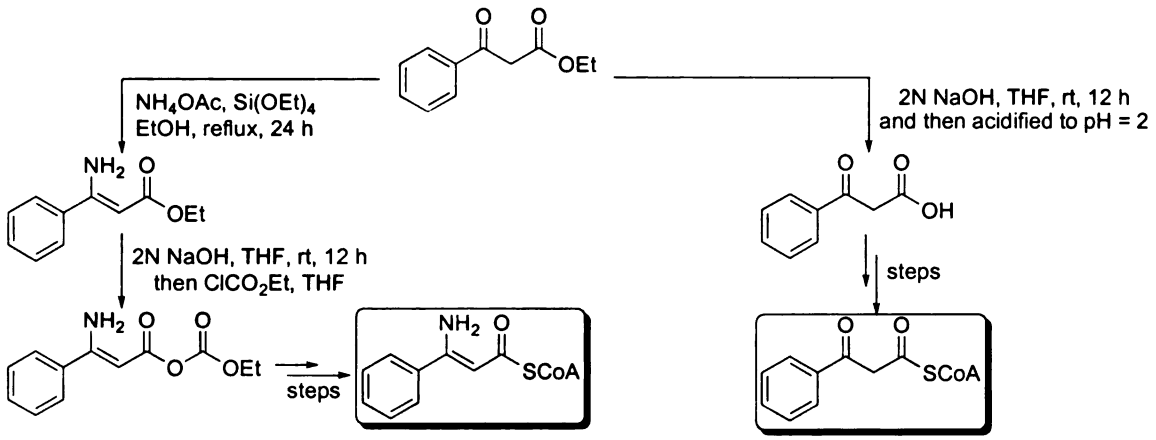


Figure 27: Synthesis using ethyl benzoylacetate as starting material

Conclusion:

The routes toward the production of milligram quantities of various aryl substituted propanoid compounds have been described. Also reported is a modified method that removes *t*-butoxycarbonyl from reactive amines by mild deprotection with formic acid in place of the less selective trifluoroacetic acid that partially degrades the CoA thioester, used in a previous procedure.[37] Presently efforts are ongoing to synthesize a series of unnatural CoA thioesters possessing different functional groups in the acyl side chain. Foreseeably, application of these CoA thioesters toward elucidating the function and mechanism of various secondary metabolite pathway enzymes is feasible.

CHAPTER 2

SYNTHESIS OF A TAXOL® ANALOG AND 13-^{3}H-BACCATIN III TO ELUCIDATE THE MECHANISM OF A TAXOL® PATHWAY BAHD ACYLTRANSFERASE

2. 1 INTRODUCTION:

Taxol® (Figure 28), a taxane diterpenoid (generically known as paclitaxel), isolated from various yew plant species (*Taxus*), is a potent pharmaceutical agent[45-46] that has current and potential application in the treatment of 3 of the top 4 deadliest diseases in the world, including heart disease[47], cancer[48] and Alzheimer's disease[49], and thus has became one of the best selling drugs in history, (netting $3 billion in sales in 2000).[45-46]

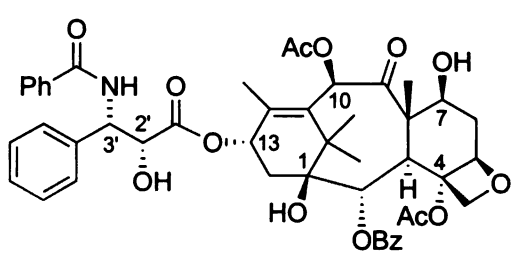


Figure 28: Taxol®

Despite approval by FDA in 1992 for clinical trials the poor solubility of Taxol® in blood plasma affected drug delivery and efficacy. Improvement in infusion adjuvant brought Taxol® to the forefront as a chemotherapeutic against ovarian and breast cancers. Recently, however, improvements in Taxol® delivery in clinical use have been hugely

bolstered by the production of Abraxane™ (Abraxis Oncology, Bridgewater, NJ). This drug surrogate is derived by a formulation process that suspends the drug in a protein albumin-based emulsion that has fewer side effects than direct delivery of the drug with toxic chemical solvents, such as Cremophor® EL.[50] Still, the production of Taxol® was suffering from the isolation of the drug from the bark of the limited natural resource, the pacific yew.[46] That isolation procedure was not only inefficient (yield of Taxol® from the bark extract was 0.014%)[46] but was also a destructive isolation technique that killed the tree. The projected increase in the use of Taxol® for basic research, cancer chemotherapy, arterial stent treatment, and potential Alzheimer's disease application warrants effort to improve existing production processes. The total synthesis of the drug,[51] while a significant accomplishment, is costly and low yielding, and thus provides an unrealistic alternative for commercial supply. Semisynthesis,[52-53] currently the major route to Taxol®, involves a limited number of synthetic steps to convert more abundant, advanced Taxol® pathway intermediates (for example, 10-deacetylbacatin III isolated from various *Taxus* needles) (Figure 29) to the target drug.

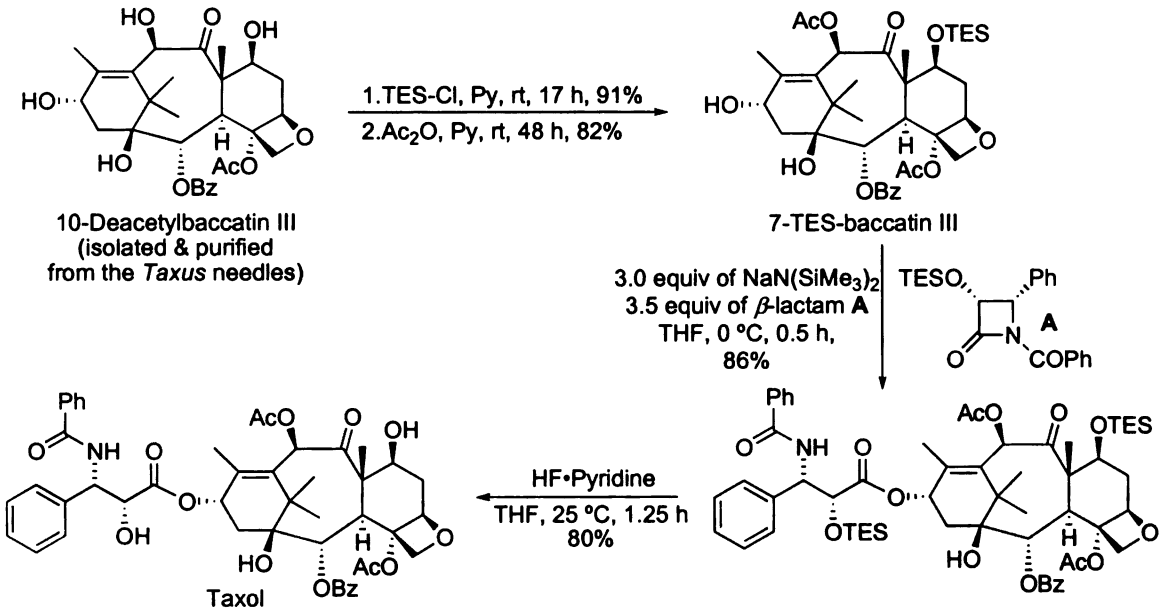


Figure 29: Semisynthesis of Taxol®- the process begins with the isolation of 10-deacetylbacatin III from the needles of *Taxus*.

However, while the semisynthetic process addresses the supply issue, several challenges[54] remain obvious in terms of technical difficulties like the isolation and separation of 10-deacetylbacatin III from other metabolites and cellular organelles. Also consistency in crop production remains a concern with regards to the product yield of the necessary starting material.

Since the production of Taxol® will, for now, depend on biological means of production, it remains imperative to understand the biosynthetic pathway of Taxol® *in planta*. Advances in molecular genetic engineering techniques and biotechnology, coupled with the recent isolation and characterization of sixteen Taxus-derived acyltransferases, six of which are functionally characterized, include the C10-acyltransferase, the C2-hydroxyl and C3'-amino benzoyl transferases, and C13-*O*-phenylpropanoyl transferase (Figure 30).[54]

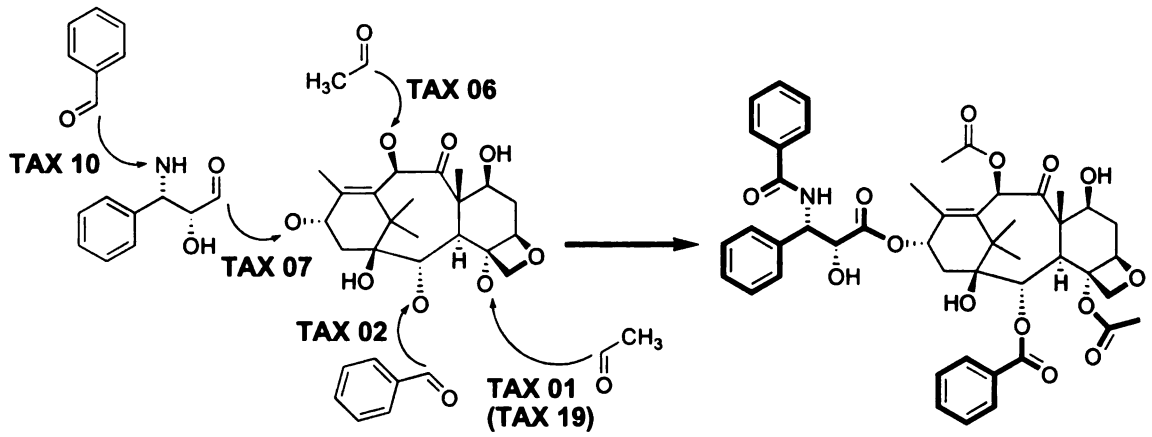


Figure 30: Biosynthetic conversion of 10-deacetylbaccatin III to Taxol® – the last few steps involve BAHD superfamily acyltransferases including C10-acetyltransferase, the C2-hydroxyl and C3'-amino benzoyltransferases, and C13-*O*-phenylpropanoyl transferase

The acyltransferases involved in Taxol® biosynthesis all belong to the Benzyl alcohol acetyl-, Anthocyanine-O-hydroxy-cinnamoyl-, anthranilate-N-Hydroxy-cinnamoyl/benzoyl-, Deacetylvindoline acetyltransferase (BAHD) enzyme superfamily[55] that have played a principal role in the evolution of secondary metabolism in several plant species. BAHD acyltransferases are involved in the biosynthesis of diverse natural plant products including volatile flower scent in *Clarkia*, capsaicin biosynthesis in *Capsicum*, and the biosynthesis of morphine and the anti-cancer drug vincristine.[56-59]

Despite the importance of BAHD acyltransferases and the products they create, little is known about their catalytic mechanism, subtrate specificity, and evolution. Several reports have highlighted that sequence similarity does not correlate with enzyme function. For example, even when two acyltransferases share greater than 90% similarity, the reaction they catalyze can be completely different.[60] These enzymes require an acyl coenzyme A cosubstrate as the acyl donor, and have a signature conserved sequence motif (HXXXD) in the putative active site (Table 3).[61]

Region	A B	Acyl Group Transferred
Consensus	...C.....HXXXDG	———
TAX01	...C.....HGVCDG	5-*O*-Acetyl
TAX02	...C.....HSVSDG	2-*O*-Benzoyl
TAX06	...C.....HGICDG	10-*O*-Acetyl
TAX10	...C.....HSVCDG	3'-*N*-Benzoyl
TAX07	...C.....GSACDA	13-*O*-(3-Amino-3-Phenylpropanoyl)

Table 3: Conserved sequence motif in Taxol® pathway acyltransferases

Three residues (C, H, D) in this motif are postulated to constitute a catalytic triad that is directly involved in acyl group transfer from the CoA donor to an amino, hydroxyl, or thio group of the acceptor (Figure 31).[61]

However, TAX07 (3-amino-3-phenylpropanoyltransferase) contains a natural Gly→His and Ala→Gly substitutions in this motif (Table 3); this latter exchange (A→G) should be relatively benign. Although based on the obligatory function of the histidine residue in proposed mechanism (Figure 31 A), the Gly substitution for His would be expected to dramatically abrogate or at least compromise acyl-group transfer catalysis for this indeed-functional transcyclase. Therefore, an alternative mechanism can be suggested for TAX07 that incorporates the NH_2-nitrogen of the TAX07 cosubstrate 3-amino-3-phenylpropanoyl CoA as crucial acid-base catalyst in place of imidazole of the missing His residue (Figure 31 B).

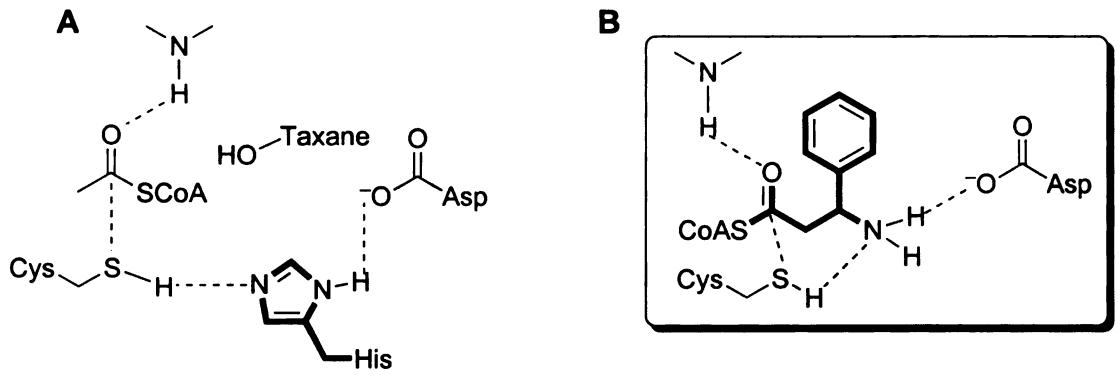


Figure 31: A) Schematic of proposed mechanism of the active site motif C....HXXXD motif during the acyl group transfer from CoA to the hydroxytaxane; B) Complementary role of the amino phenylpropanoid acyl group of the CoA thioester in the catalytic triad

The first crystal structure of a BAHD acyltransferase, vinorine synthase (VS; EC 2.3.1.160), was reported recently[62], and thus serves as a structural model for the *Taxus* transacylases. Interestingly, while the putative catalytic residues, HXXXD, are located within or proximate to the active site, another highly conserved region DFGWGKP is distally located, thus raises an interesting question regarding the function of this motif during catalysis. Analysis of the 3D-structural data of the acetyl coenzyme A-dependent acetyltransferase, vinorine synthase, from *Rauwolfia* plant, reveals that the enzyme is composed of two structural domains (Figure 32), but shares only moderate amino acid sequence relatedness (~45% similarity; ~24% identity) with the *Taxus* acyltransferases. However, like the *Taxus* transferases, vinorine synthase and all of the catalytic residues are found in domain I, including the signature HXXXD motif. This structural conservation suggests that substrate variability might be encoded in the mutable domain II.

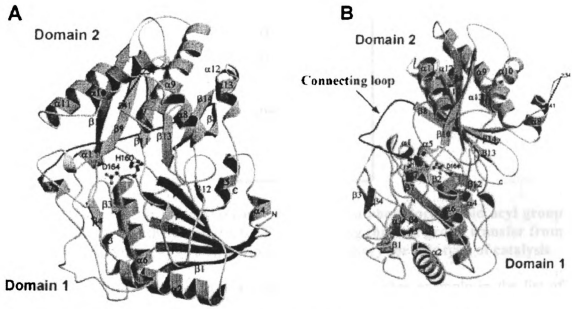


Figure 32: Structure of vinorine synthsase. A and B represent orthogonal views of the VS structure as depicted in *ribbon representation*. *N* and *C* denote the termini of VS. The secondary structure elements are labeled (a1–a13 and b1–b14), and domains 1 and 2 are indicated. The large crossover loop (amino acids 201-213) connects both domains. The conserved and catalytic residues His^{160} and Asp^{164} are shown in *ball-and-stick representation*.

Furthermore, reports on the VS mechanism suggest that the His residue is postulated to be the only catalytic residue, whereas the Asp residue is likely for maintaining the enzyme geometry; the Cys residue is not considered in the proposed mechanism for VS. This latter postulate deviates from the previously proposed catalytic mechanism involving an acid/base triad for hydrogen bonding (cf. Figure 31).[62] Since the 3-amino-3-phenylpropanoyl transferase, designated as TAX07 lacks this purported His residue, a new mechanism can be proposed wherein the amino nitrogen of the co-substrate (3-amino-3phenylpropanoyl coenzyme A) serves as the sole general base involved in catalysis (Figure 33).

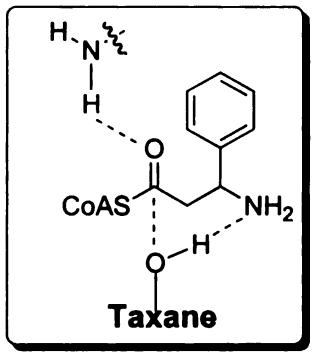


Figure 33: Proposed complementary role of the amino phenylpropanoid acyl group of the CoA thioester in the catalytsis by TAX07 during the acyl group transfer from CoA to the hydroxytaxane; Cys and Asp are not necessary for this type of catalysis

Validation of the proposed mechanism will add another example in the list of substrate-assisted catalysis (SAC),[63-65] which marks a unique mode of catalysis that will be explored.

A series of unnatural coenzyme A thioesters will be used to screen TAX07 in order to test whether the hypothesized SAC mechanism is viable (Figure 34).

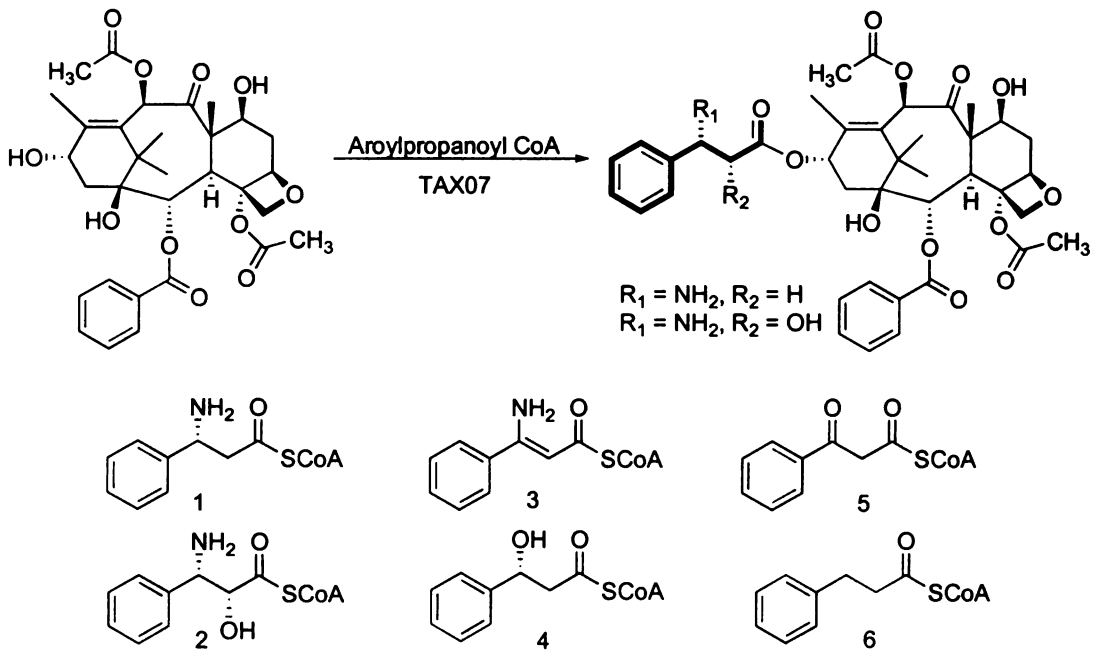


Figure 34: Future plans to validate the SAC mechanism. According to that mechanism (cf. Figure 33) CoA thioesters with 1, 2, 3 will be probably productive substrates but not the 4, 5, 6 CoA thioesters

2. 2 RESULTS AND DISCUSSION:

The initial challenge to investigate the mechanism of the TAX07 acyltransferase was to synthesize radiolabeled baccatin III substrate (Figure 35) at ~10 Ci/mmol specific activity in order to gain μmol-scale sensitivity of the product formed in the assays and analyzed by high performance liquid chromatography and radioactivity detection. Another objective was to synthesize product standards [2'-deoxyTaxol, 13-*O*-(3'-hydroxy-3'-phenylpropanoyl)baccatin III, 13-*O*-(3'-keto-3'-phenylpropanoyl)baccatin III, 13-*O*-(3'-phenylpropanoyl)baccatin III, and 13-*O*-(3'-imino-3'-phenylpropanoyl)baccatin III] that will confirm the formation of a putative biosynthetic product derived from C13 acylation of baccatin III by TAX07 catalysis with variously substituted phenylpropanoids.

Synthesis of 13-^{3}H-Baccatin III:

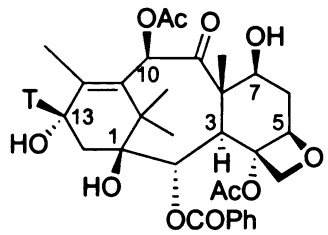


Figure 35: 13-^{3}H-Baccatin III

Since the discovery in 1971 of the diterpenoid Taxol®, with its anticancer activity and unusual ability to stabilize the assembly of microtubules, there has been a need for radiolabeled Taxol® and related compounds to facilitate pharmacological studies.[52] Recent developments in Taxol® chemistry have provided methods for regiospecifically tritium-labeling of the phenylpropanoyl sidechain or the baccatin III ring system.

In 1993, Kepler and his coworkers demonstrated the syntheses of [3"-^{3}H] Taxol® and [13-^{3}H] Taxol®.[66] They found that adaptations of the methods by Holton[67-68] and Ojima[69] were the most effective for the small-scale required for synthesis of radiolabeled Taxol® at high specific activity. These methods were applied in the present synthesis of 13-^{3}H-baccatin III. The C7 hydroxy group of commercially available 10-deacetylbaccatin III (10-DAB) (Natland Inc., Research Triangle Park, NC) was first protected with the triethylsilyl ether using triethylsilylchloride and pyridine. The resulting 7-TES-baccatin III was acetylated at C10 using acetyl chloride and pyridine (Figure 36).

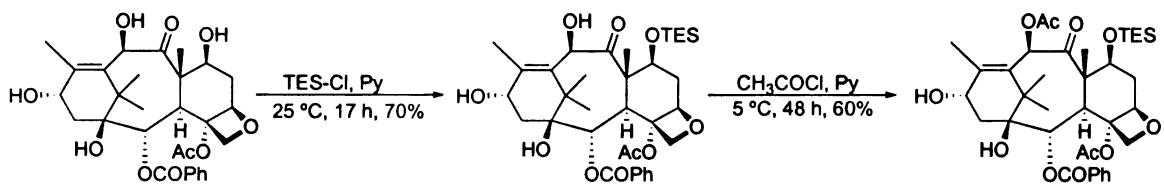


Figure 36: Synthesis of 7-TES-baccatin III from commercially available 10-DAB

In the next steps, 7-TES-baccatin III was oxidized to its 13-keto analogue using neutral, activated manganese dioxide as per literature procedure.[66] Various methods are described for reducing the keto group to the hydroxyl and each were considered. Treatment of that ketone with sodium borohydride in ethanol reported[66] to give 35% yield of the silylbaccatin III with considerable side reactions resulting from cleavage of the ester groups on the baccatin III core. Borane-tetrahydrofuran complex is reported[70] to selectively reduce the α, β-unsaturated ketone of progesterone. Based on this latter report, the 13-oxobaccatin III was treated with sodium borotritide (ARC, Inc., St. Louis, MO) in tetrahydrofuran and afforded quantitative conversion yielding 13-^{3}H-7-TES-baccatin III that was finally converted to the desired 13-^{3}H-baccatin III by removing the triethylsilyl group with HF•pyridine (Figure 37).

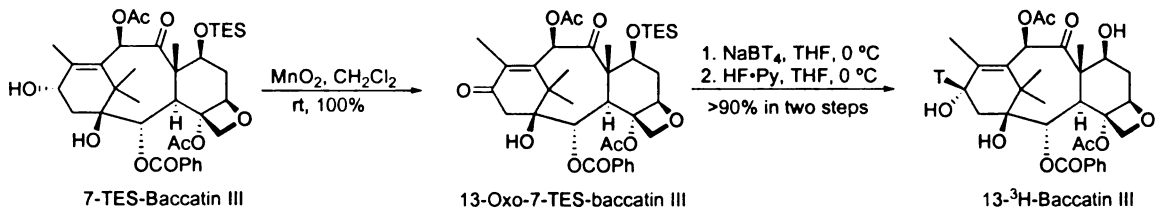


Figure 37: Synthesis of 13-^{3}H-Baccatin III using sodium borotritide in THF

Synthesis of 2'-DeoxyTaxol®:

N-Debenzoyl-2'-deoxytaxol and 2'-deoxytaxol were synthesized by modifying a previous method.[71] The former metabolite will serve as an intermediate to investigate the substrate specificity of the *Taxus*-derived N-acyl transferase[72], and likely serve as a productive substrate to screen for a putative cytochrome P450-dependent 2'-hydroxylase. Also, the *N*-Debenzoyl-2'-deoxytaxol can serve as a synthethic precursor toward the development of modified second-generation taxanes.

The second-generation taxanes mostly have modifications at 3'-amino and 13-hydroxyl functions. For example, non-aromatic 3'-N-acyl analogs **1** are more cytotoxic than Taxol®[73], and thiocarbamates **2** and **3** have greater cytotoxicity and tubulin polymerization effectiveness than the unmodified drug[74] (Figure 38). Perhaps the most promising propanoyl sidechain analog is the 3'-*tert*-butyl-3'-N-thienoyl **4**, which in addition to possessing 2-fold better tubulin assembly properties and cytotoxicity against B16 melanoma cells relative to Taxol®, is 25 times more water soluble than the parent drug.[75]

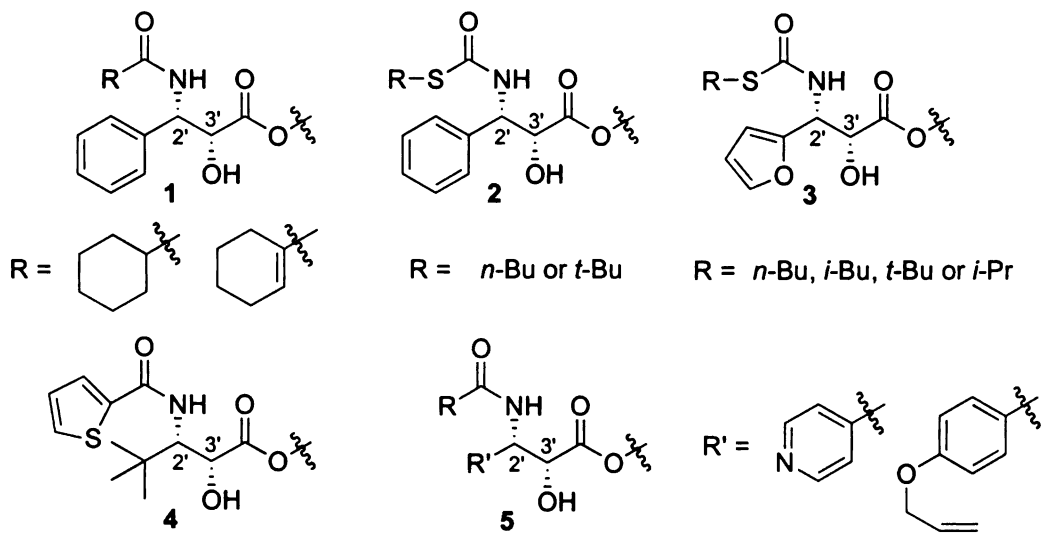


Figure 38: Second generation Taxol® derivatives

Although most of efforts have been directed at modifications of the ring system, there are very few alternate ways of coupling the side chain to baccatin III. It has been reported[76] that the C-13 hydroxyl group of baccatin III is very resistant to acylation, in part because of its hindered location at the concave face the molecule. Thus the acylating agents need to have compact structure. Though Ojima's β-Lactam protocol[77] is the most extensively used method for the sidechain attachment to baccatin III, oxazoline[78] and dioxo-oxaisothiazole[79] ring systems have also been utilized for this purpose. Less obviously, it is also possible to prepare modified Taxol® analogues by direct modification of an existing sidechain. This approach was demonstrated few years ago by developing a conversion of cephalomannine to Taxol®[80], but unfortunately Taxol® itself proved resistant to such manipulation. Another approach, that involves a dehydration condensation step, utilizes *O,O'*-di(2-pyridyl) thiocarbonate (DPTC) and DMAP to add the side chain on baccatin III (Figure 39).[81]

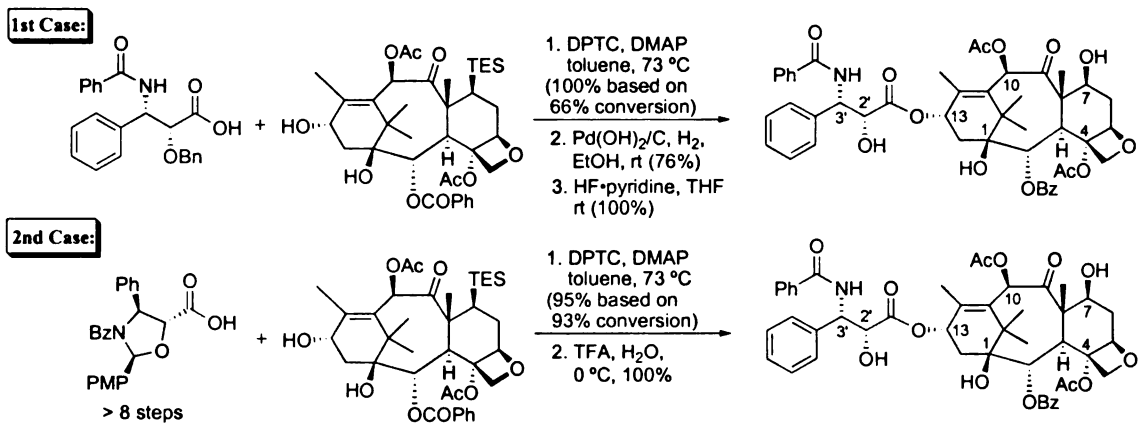


Figure 39: Mukaiyama's dehydration-condensation protocol between 7-TES-baccatin III and protected *N*-benzoylphenylisoserines

Analysis of Mukaiyama's protocol has indicated that direct attachment of the protected sidechain (1st case in Figure 39) is difficult and very low yielding (100% based on 66% conversion after 4 times operation, therefore one individual step yields less than 20% of product)[81]. It involves two deprotection steps (7-*O*–TES & 2'-*O*–Bn) leading to Taxol® whereas any further manipulation at 3'-N, that will be necessary to generate second generation taxanes, needs an extra deprotection steps of the 3'-N-benzoyl group on the sidechain. Secondly, the sidechain protected as *N,O*-cyclic acetal (2nd case in Figure 39) seemed to be more reactive, because of their less hindered structure, and very efficient (the reaction yielded 95% based on 93% conversion and involves only one deprotection steps[81]. But the major limitation of this option lies into the fact the synthesis of that *N,O*-cyclic acetal involves more than eight steps and again the 3'-N-benzoyl group needs to be deprotected for any further manipulation towards other Taxol® analogs.

By adapting and modifying the above dehydation-condensation protocol our goal was to synthesize 2'-deoxyTaxol® that can be utilized both as biosynthetic as well as synthetic precursor of modified Taxol® analogues. Whereas it can be used as a product standard of TAX07 and TAX10 acyltransferases, its N-debenzoyl analog (3'-N-

debenzoyl-2'-deoxyTaxol®) can also be used as a substrate for 2'-hydroxylase[82] and TAX10[83] (Figure 40). Moreover, after 2'-hydroxylation, the free 3'-amino group can be further manipulated, synthetically via a known acylation method[84], or biochemically using TAX10, to synthesize several second-generation taxanes.

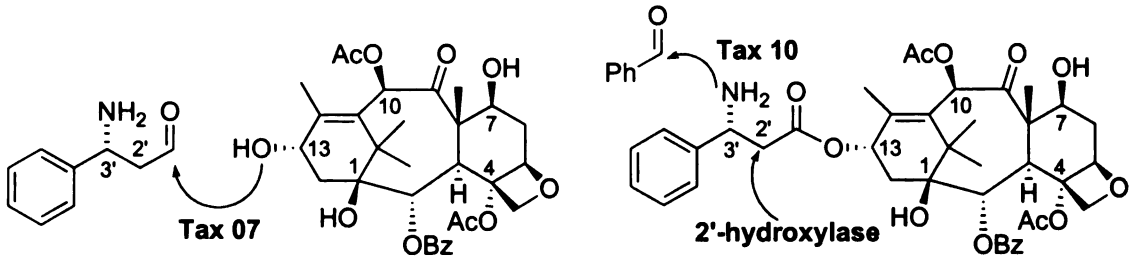


Figure 40: Taxol® biosynthetic pathway

First step was to synthesis of 7-TES-baccatin III that was done by the method mentioned before (Figure 41). Attempted coupling of 7-TES-baccatin III with N-benzoyl-*β*-phenylalanine using DPTC and DMAP failed to produce the Taxol® analog indicating towards the fact that 2'-oxygen might be a condition for this coupling reaction.

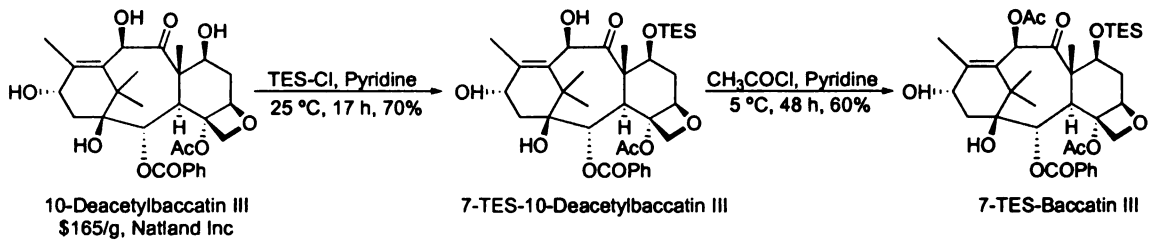
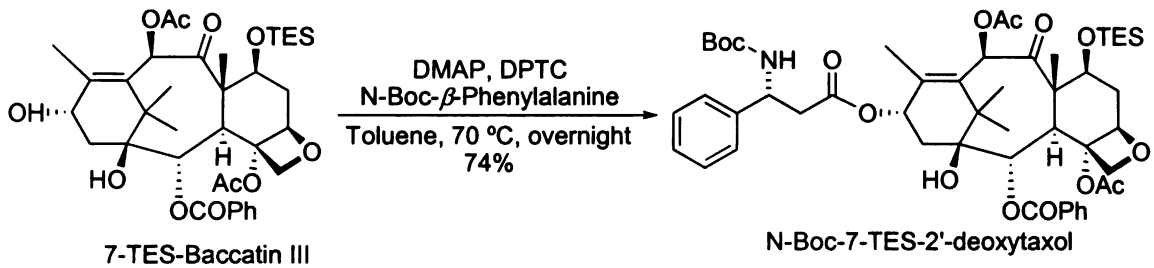


Figure 41: Synthesis of 7-TES-baccatin III

However, after several unsuccessful attempts with N-benzoyl-*β*-phenylalanine at varying reaction conditions, it was observed that changing the 3'-amino protecting group from benzoyl to *t*-butyloxycarbonyl (Boc) allow the reaction to proceeds successfully with good yield (Figure 42). It is important to note that excess (6.0 equivalent) DPTC and N-Boc-*β*-phenylalanine was used instead of 2.0 equivalents of each as reported earlier.[85]

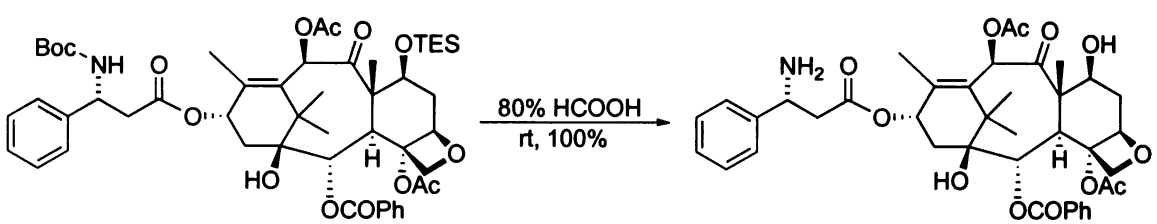


7-TES-Baccatin III

N-Boc-7-TES-2'-deoxytaxol

Figure 42: *N*-Boc-7-TES-2'-deoxyTaxol®

HF•pyridine was introduced to deprotect the TES group at C7. After complete deprotection of 7-hydroxy function deprotection of 3'-N-Boc group was attempted. Trifluoroacetic acid was tried for this purpose. Surprisingly complete decomposition of the substrate under TFA condition was observed. Reaction at low temperature (0 °C) was also attempted which resulted in no change of substrate. Altering the deprotection sequence (3'-*N*-Boc followed by 7-TES) caused decomposition at the very beginning (during deprotection of 3'-*N*-Boc). It was apparent that deprotection with TFA was decomposing the substrate perhaps due to its very high acidity. To avoid use of this strong acid, 80% aqueous formic acid was employed and it was observed that both 7-TES and 3'-*N*-Boc group were deprotected with formic acid (Figure 43). Use of formic acid thus not only afforded desired product (3'-*N*-debenzoyl-2'-deoxyTaxol®) but also shortened the synthetic path by eliminating one extra deprotection step.



N-Boc-7-TES-2'-deoxytaxol

N-Debenzoyl-2'-deoxytaxol

Figure 43: Synthesis of *N*-debenzoyl-2'-deoxyTaxol®

Whereas the intermediate 3'-*N*-debenzoyl-2'-deoxyTaxol® can be used as a substrate for TAX10, the benzoylation was done chemically also to have a substrate for

2'-hydroxylase and product standard for TAX07 and TAX10 Taxol® pathway acyltransferases. The benzoylation of the 3'-amino group was done by a literature method[84] to obtained quantitative conversion of the substrate to 2'-deoxyTaxol®, the target compound (Figure 44).

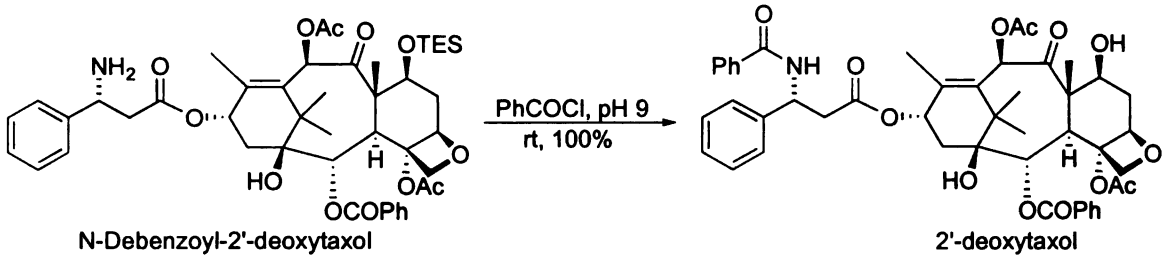


Figure 44: Synthesis of 2'-deoxyTaxol®

Conclusion:

Modification of an existing method for the coupling of the 3'-amino-3'-phenylpropanoid sidechain of Taxol® onto C13 of baccatin III is described. Although many methods for the attachment of C13 sidechain to baccatin III for the synthesis of Taxol® and related compounds were developed in the last few years by several groups, the described method, will give access to a library of Taxol® derivatives including second-generation taxanes in a short synthetic route. Furthermore, synthesis of radiolabeled baccatin III, an advanced taxol biosynthetic pathway intermediate, is also described. Foreseeably, application of these Taxol® analogues toward elucidating the putative SAC mechanism of TAX07 acyltransferase is feasible.

CHAPTER 3

EXPERIMENTAL METHODS

General Methods:

Coenzyme A as trilithium salt was purchased from Sigma. (*R,S*)-3-(*tert*-butoxycarbonylamino)-3-phenylpropanoic acid and (*R*)-3-hydroxy-3-phenylpropanoic acid was purchased from Alfa Aesar. Benzoylacetic acid was prepared per literature procedure.[86] (*Z*)-ethyl-3-(tert-butoxycarbonylamino)-3-phenylacrylic acid was prepared as per literature procedure[87] from ethyl isonicotinoylacetate (purchased from AK Scientific, Inc, Mountain View, CA). (*R,E*)-3-(tert-butoxycarbonylamino)-5-phenylpent-4-enoic acid was prepared from (*S*)-3-amino-5-phenylpentanoic acid (purchased from Chem-Impex International, Inc. Wood Dale, IL) as per literature procedure.[88] *N*-Boc-(*2R,3S*)-3-phenylisoserine was purchased from Chem-Impex International, Inc. (Wood Dale, IL). Ethyl chloroformate, ethyl benzoylacetate and all other reagents were purchased from Aldrich unless notified otherwise. C18 (carbon 11%) reversed phase silica gel was purchased from Silicycle, (QC, Canada).

All substrates were used without further purification. Tetrahydrofuran and dichloromethane were obtained from dry still packed with activated alumina that was pressurized with nitrogen gas. Silica gel (230-400 Mesh) and aluminium backed silica gel

60 TLC plates, embedded with A_{254} chromophores, were purchased from EMD™ Chemicals Inc (Gibbstown, NJ).

All reported yields are for isolated materials. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova-300 (300.11 and 75.47 MHz respectively), Varian VXR-500 or Varian Unity-500-Plus spectrometer (499.74 and 125.67 MHz respectively) and were referenced to residual solvent signals either at 7.24 ppm or at 4.67 ppm for $CDCl_3$ and D_2O respectively. All apparent coupling constants (J values) were measured at the indicated field strengths.

Tandem gas chromatography/mass spectrometry analysis was conducted by loading 1 µl of sample onto an HP 5HS GC column (0.25-mm inner diameter x 30 m, 0.25-µm film thickness) (Agilent, Palo Alto, CA) coupled to mass selective detector (model 5973 inert®, Agilent) in ion scan mode from 50-300 atomic mass units. Electospray Ionization Mass Spectrometry (ESI-MS) analysis was conducted by loading 0.1 mM solution of thioester samples in water (pH = 5) onto a Q-Tof Ultima™API (Micromass, Beverly, MA) mass spectrometer coupled with a LC-system (model 2795, Waters, Milford, MA) at the Mass Spectrometry Facility, Michigan State University.

General Procedure:

The carboxylic acid (1 equivalent) was suspended in THF was added ethyl chloroformate (1.5 equivalents) and 1M Et_3N in THF (1.2 equivalents) under N_2 to form the mixed anhydride. The reaction was stirred for one hour at room temperature with monitoring by analytical TLC. After completion of the reaction, the solvents were evaporated under reduced pressure followed by vacuum and the residue was dissolved in

t-BuOH. CoA (as trilithium salt, 1.2 equivalents) in 0.4 M $NaHCO_3$ was added to the solution and the mixture was stirred for half hour at room temperature. Then quenched with 1M HCl and adjusted to pH 5. The solvents were evaporated under reduced pressure at room temperature. The residue was purified by flash column chromatography using C_{18}-silica gel that was first loaded and washed with methanol and then with water (pH=5). The residue, after loading on the column, was first eluted with water (10-20 ml) and then with increasing concentrations of methanol (5-100%) in water (pH=5). The product comes with about 10-20% methanol. The derived product was then lyophilized. To remove the N-Boc protection, the residue was dissolved in 1 ml water, cooled to 0 °C, and 1 ml of trifluoroacetic acid was added dropwise with stirring for 1h to deprotect the amino group. The mixture was then warmed to room temperature and stirred an additional 1 h. The progress of decarbonylation of the N-Boc compound was monitored by silica gel analytical TLC (1-butanol/H_2O/AcOH, 5:3:2, vol/vol/vol) with detection by UV absorbance. After complete deprotection, the reaction was diluted with 50 ml of water and concentrated to 0.5 ml under vacuum; this dilution and evaporation process was repeated three times to remove residual trifluoroacetic acid. Finally the sample was concentrated to dryness and resuspended in 5 ml of water. The product was purified by C_{18} silica gel flash column chromatography as described above. The CoA thioesters were eluted with 15-20% methanol, which was removed in *vacuo* and finally lyophilized to obtain a white solid. Purity of the product was assessed by analytical TLC. Compound was then confirmed by ESI-MS and ^1H NMR spectroscopy.

Experimental Details and Spectroscopic Data:

(3*RS*)-*β*-Phenylalanoyl coenzyme A.

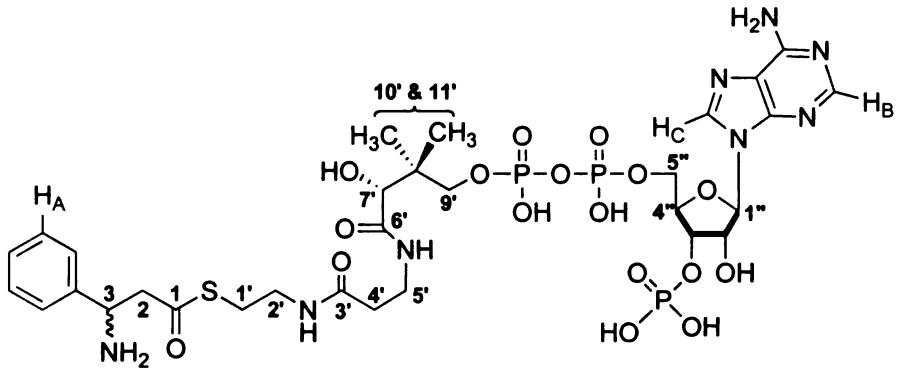


Figure 45: (3*RS*)-*β*-Phenylalanoyl coenzyme A.

Following the general procedure, *N*-Boc-*β*-phenylalanine (26 mg, 98 μmol) suspended in THF (1.4 ml) was added ethyl chloroformate (18 μl, 15.9 mg, 147 μmol) and 1M Et_3N in THF (13 μl, 118 μmol) under N_2 to form the mixed anhydride. The reaction was stirred for one hour at room temperature with monitoring by analytical TLC. After completion, the solvents were evaporated under reduced pressure followed by vacuum and the residue was dissolved in 2 ml *t*-BuOH. CoA (as trilithium salt, 83.7 mg, 108 μmol) in 2 ml 0.4 M $NaHCO_3$ was added to the solution and the mixture was stirred for another half hour at room temperature. Then quenched with 1M HCl and adjusted to pH 5. The solvents were evaporated under reduced pressure at room temperature. The residue was purified by the described method using C_{18} silica gel chromatography. The product comes with about 10-15% methanol. The derived *N*-Boc-*β*-phenylalanoyl Coenzyme A was then lyophilized, and the Boc group was deprotected by above described method and the residual trifluoroacetic acid was removed. Finally the sample was concentrated to dryness and resuspended in 5 ml of water. The product was purified

by C_{18} silica gel flash column chromatography as described above. The CoA thioester was eluted with 20% methanol, which was removed in *vacuo* and finally lyophilized to obtain a white solid. The purity was assessed by analytical TLC which was found to be 98-100%. Compound was then confirmed by ESI-MS and ^{1}HNMR spectroscopy. Yield = 66% based on CoA. ESI-MS: Calculated 914.18, Experimental [M–H] 913.27, [M+H] was not possible. ^{1}H-NMR (500 MHz, D_2O) (see Figure 45 for numbering) δ (in ppm) : 0.66 (3H, s, H-10'), 0.78 (3H, s, H-11'), 2.16 (2H, t, H-4'), 2.8 (2H, m, H-1'), 3.1 (2H, t, H-2'), 3.26-3.28 (4H, m, H-2 & H-5'), 3.44 (1H, dd, J = 4.8 and 9.6 Hz, H_a-5"), 3.66 (1H, dd, J = 4.8 and 9.6 Hz, H_b-5"), 3.86 (1H, s, H-7'), 4.06 (2H, s, H-9'), 4.43 (1H, ddd, J = 2.7 and 5.3 Hz, H-4"), 4.56-4.66 (2H, m, H-2" and H-3"), 6.0-6.1 (two doublets; one set from each stereoisomer, J = 6.9 Hz for both, H-1"), 7.21-7.27 (5H, m, H_A phenyl protons), 8.26 (1H, s, H_C adenine-CH), 8.33 (1H, s, H_B adenine-CH).

***(R)*-3-Hydroxy-3-phenylpropanoyl coenzyme A.**

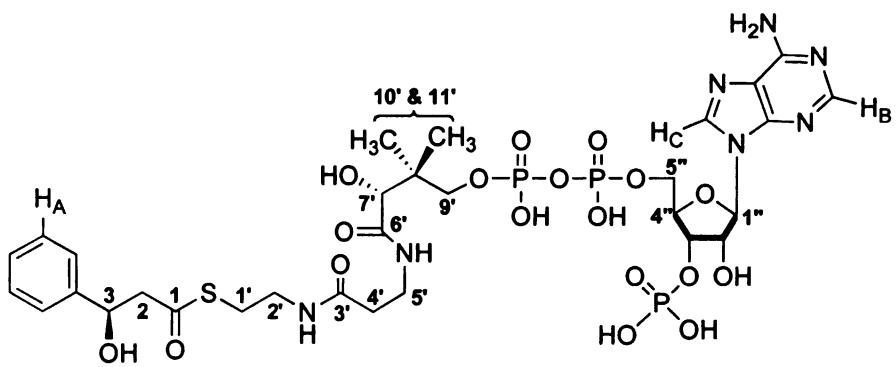


Figure 46: *(R)*-3-Hydroxy-3-phenylpropanoyl coenzyme A.

Following the general procedure, *(R)*-3-hydroxy-3-phenylpropanoic acid (21 mg, 125 μmol) suspended in THF (1.8 ml) was added ethyl chloroformate (23 μl, 20.3 mg, 188 μmol) and 1M Et_3N in THF (17 μl, 150 μmol) under N_2 to form the mixed anhydride. The reaction was stirred for one hour at room temperature with monitoring by

analytical TLC. After completion, the solvents were evaporated under reduced pressure followed by vacuum and the residue was dissolved in 2.5 ml *t*-BuOH. CoA (as trilithium salt, 107 mg, 138 μmol) in 2.5 ml 0.4 M $NaHCO_3$ was added to the solution and the mixture was stirred for another half hour at room temperature. Then quenched with 1M HCl and adjusted to pH 5. The solvents were evaporated under reduced pressure at room temperature. The residue was purified by the described method using C_{18} silica gel chromatography. The product comes with about 15% methanol which was evaporated under *vacuo* and the residue was finally lyophilized to white solid. The purity was assessed by analytical TLC which was found to be 98-100%. Compound was then confirmed by ESI-MS and ^{1}HNMR spectroscopy. Yield = 68% based on CoA. ESI-MS: Calculated 915.17, Experimental [M–H] 914.09, [M+H] was not possible. ^{1}H-NMR (500 MHz, D_2O) (see Figure 46 for numbering) δ (in ppm) : 0.66 (3H, s, H-10'), 0.78 (3H, s, H-11'), 2.39 (2H, t, H-4'), 2.80 (2H, m, H-1'), 3.20 (2H, t, H-2'), 3.36-3.38 (4H, m, H-2 & H-5'), 3.46 (1H, dd, J = 4.8 and 9.6 Hz, H_a-5"), 3.80 (1H, dd, J = 4.8 and 9.6 Hz, H_b-5"), 3.96 (1H, s, H-7'), 4.06 (2H, s, H-9'), 4.46 (1H, ddd, J = 2.7 and 5.3 Hz, H-4"), 4.60-4.82 (2H, m, H-2" and H-3"), 5.80 (1H, m, H-3), 6.40 (1H, d, J = 6.9 Hz H-1"), 7.21-7.27 (5H, m, H_A phenyl protons), 8.20 (1H, s, H_C adenine-CH), 8.43 (1H, s, H_B adenine-CH).

(*3R*)- Styryl-*β*–alanoyl coenzyme A.

Synthesis of Sodium (3R, 4E)-N-Boc-3-amino-5-phenylpent-4-enoate:

Thionyl chloride (0.1 ml, 1.5 mmol) at 0°C was added to the reaction mixture dissolved in methanol at a rate to keep the stirred suspension of (*S*)-3-amino-5-phenylpentanoic acid (193 mg, 1 mmol) at reflux. After the initial refluxing ceased, the mixture was heated at 70 °C for an additional 2 h, then the reaction was cooled, and the

methanol was removed under reduced pressure to give a crude methyl ester hydrochloride salt of (*S*)-3-amino-5-phenylpentanoate, which was used in next reaction step without further purification. For reference, a small amount of the amine (R_f = 0.2) was evaluated on analytical silica gel TLC (95:5 CH_2Cl_2/methanol, v/v).

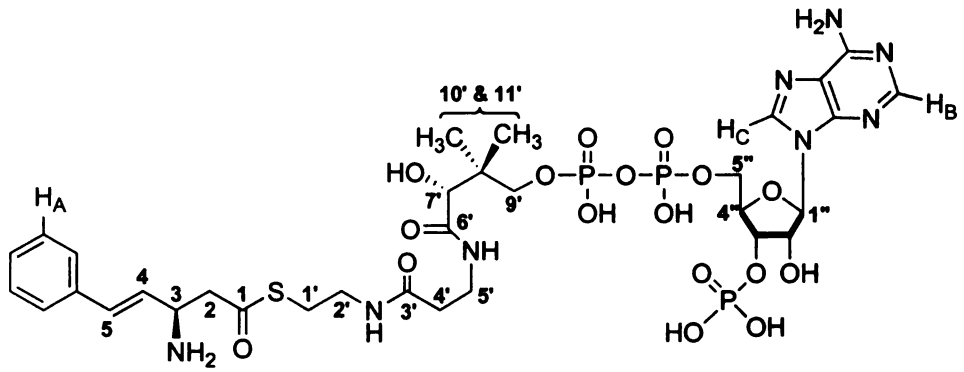


Figure 47: (3*R*) Styryl-*β*-alanoyl coenzyme A.

To the crude mixture of the *β*-amino methyl ester intermediate (193 mg, 0.8 mmol), triethylamine (0. 12 ml, 0.84 mmol), pyridine (0.06 ml, 0.84 mmol) dissolved in THF (0.6 ml) and $CHCl_3$ (0.6 ml) at 0 °C was added solid di-*tert*-butyldicarbonate (185 mg, 0.84 mmol). The solution was stirred at 0 °C for half hour, and then overnight at room temperature. The solution was washed with 15% phosphoric acid, aqueous $NaHCO_3$ solution and saturated NaCl solution. The organic fraction was dried over Na_2SO_4, filtered and the solvent evaporated to dryness under reduced pressure. The crude product was purified by silica gel flash column chromatography (90: 10 hexane/ethyl acetate, v/v); the fractions containing methyl (*S*)-*N*-Boc-3-amino-5-phenylpentanoate were combined, and the solvent was evaporated to give the product as a colorless oil which solidified upon overnight drying *in vacuo* (76% yield, R_f = 0.35 in 90:10 hexane/ethyl acetate, v/v on silica gel TLC). ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H ppm 1.42 [9H, s, $C(CH_3)_3$], 1.77-1.91 (2H, m, $PhCH_2CH_2$), 2.50-2.85 (4H, m, $PhCH_2CH_2$ and

CH$_2$CO), 3.68 (3H, s, OCH_3), 3.90-4.01 (1H, m, NH*CH*), 4.97 (1H, d, J = 9.9 Hz, NH) and 7.13-7.31 (5H, m, aromatic protons).

The *N*-Boc protected amine, described above, was converted to the unsaturated methyl (3*R*, 4*E*)-*N*-Boc-3-amino-5-phenylpent-4-enoate according to a literature procedure that describes a sequential and selective benzylic bromination, by *N*-bromosuccinimide treatment followed by a dehydrobromination.[88] Under an inert atmosphere of nitrogen, a solution of *N*-Boc ester (0.5 mmol, 0.153 g) in dry CCl_4 (2 ml) was treated with *N*-bromosuccinimide (0.56 mmol, 0.097 g). The reaction mixture was illuminated for 3 hour under a 60 W tungsten lamp held ~ 2 cm away from the flask while maintaining the temperature at less than 10 °C (in cold room). The product mixture was purified by silica gel flash column chromatography (90:10 hexane/ethyl acetate, v/v); the fractions containing the alkene intermediate were combined, and the solvent was evaporated to give the product as a white solid (40 % yield, R_f = 0.52 in 70:30 hexane/ethyl acetate, v/v on silica gel TLC). ^{1}H-NMR (300 MHz, $CDCl_3$) δ_H ppm 1.46 [9 H, s, $C(CH_3)_3$], 2.67-2.74 (2 H, m, CH_2CO), 3.70 (3 H, s, OCH_3), 4.62-4.78 (1 H, m, NH*CH*), 5.23-5.37 (1 H, m, NH), 6.19 (1 H, dd, J = 6.2 and 15.7, PhCH=*CH*), 6.55 (1 H, d, J = 15.7, Ph*CH*) and 7.20-7.38 (5H, m, aromatic protons).

The *N*-Boc methyl ester, methyl (3*R*, 4*E*)-*N*-Boc-3-amino-5-phenylpent-4-enoate (20 mg, 65 μmol), described above, in THF (1.2 ml) was hydrolyzed for 12 h with NaOH (65 μmol, 32.5 μl of a 2M aqueous solution) to the yield the sodium salt of (3*R*, 4*E*)-*N*-Boc-3-amino-5-phenylpent-4-enoic acid.

Synthesis of (3R)-Styryl-β–alanoyl Coenzyme A:

This carboxylate sodium salt was suspended in THF (1.4 ml) to which was added ethyl chloroformate (7.0 µl, 7.8 mg, 72 µmol) under nitrogen to form the mixed anhydride. The mixture was stirred vigorously at room temperature for 1 h. The transesterification of the mixed anhydride with CoA (as trilithium salt, 60 mg, 78 µmol in 1.4 ml 0.4 M $NaHCO_3$), the purification of *N*-Boc protected CoA ester, and the *N*-deprotection with trifluoroacetic acid were all performed as described in general procedure. The final product (3R)-styryl-*β*–alanoyl coenzyme A, was eluted from the C_{18} silica gel column with 15-20% methanol which was evaporated and the residue was lyophilized to give the product as white solid. The purity was assessed by analytical TLC which was found to be 98-100%. Compound was then confirmed by ESI-MS and ^{1}HNMR spectroscopy. Yield = 60% based on CoA. ESI-MS: Calculated 940.20, Experimental [M–H] 939.33, [M+H] was not possible. ^{1}H NMR (500 MHz, D_2O) (see Figure 47 for numbering) δ (in ppm) : 0.68 (3H, s, H-10'), 0.76 (3H, s, H-11'), 2.16 (2H, t, H-4'), 2.82 (2H, m, H-1'), 3.11 (2H, t, H-2'), 3.26-3.28 (4H, m, H-2 & H-5'), 3.44 (1H, dd, J = 4.8 and 9.6 Hz, H_a-5"), 3.66 (1H, dd, J = 4.8 and 9.6 Hz, H_b-5"), 3.86 (1H, s, H-7'), 4.06 (2H, s, H-9'), 4.43 (1H, ddd, J = 2.7 and 5.3 Hz, H-4"), 4.56-4.66 (2H, m, H-2" and H-3"), 4.67-4.78 (1H, m, H-3) 6.0-6.1 (1H, d, J = 6.9 Hz, H-1"), 6.19 (1H, dd, J = 6.2 and 15.7, H-4), 6.55 (1H, d, J = 15.7, H-5), 7.21-7.27 (5H, m, H_A phenyl protons), 8.26 (1H, s, H_C adenine-CH), 8.33 (1H, s, H_B adenine-CH).

(2*R*, 3*S*)-3-phenylisoserinoyl coenzyme A.

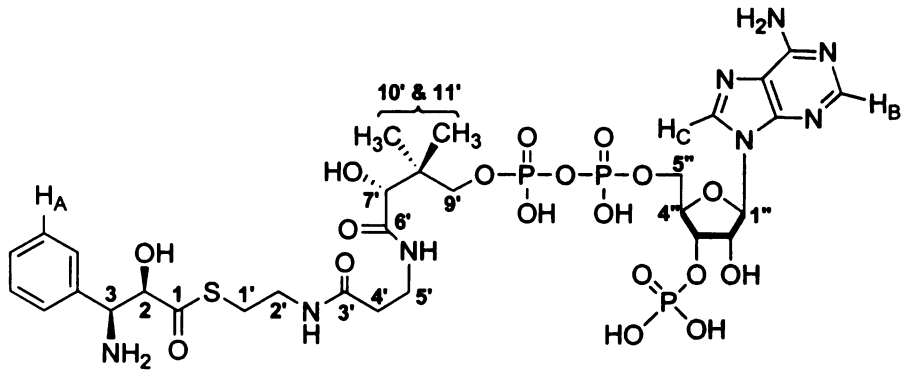


Figure 48: (2*R*, 3*S*)-3-phenylisoserinoyl coenzyme A

N-Boc-(2*R*,3*S*)-3-phenylisoserine (62 mg, 0.2 mmol) in 2.5 ml of THF was esterified by diazomethane treatment. The crude ester was subjected to silica gel flash column chromatography (50:50 hexane/ethyl acetate, v/v) to yield the pure ester derivative (0.18 mmol, 90% yield). This methyl ester (55 mg, 0.18 mmol) was dissolved in THF (3.7 ml) and added to a stirred suspension of sodium hydride (1.1 mmol) in THF (2.5 ml) under nitrogen. To the mixture was added di-*t*-butyl dicarbonate (46 mg, 0.2 mmol) in THF (5 ml), and the suspension was stirred at room temperature for 20 minute. The mixture was then chilled on ice, quenched with 1 ml of water, and finally filtered. The filtrate was collected and the solvent evaporated. The product was purified by silica gel flash column chromatography (65:35 hexane/ethyl acetate, v/v) to yield pure *N,O*-di-Boc-(2*R*,3*S*)-3-phenylisoserine methyl ester (0.16 mmol, 90% yield). This methyl ester (26 mg, 65 μmol), described above, in THF (1.2 ml) was hydrolyzed for 12 h with NaOH (65 μmol, 32.5 μl of a 2M aqueous solution) to the yield the sodium salt of *N,O*-di-Boc-(2*R*,3*S*)-3-phenylisoserine. This carboxylate sodium salt was suspended in THF (1.4 ml) to which was added ethyl chloroformate (7.0 μl, 7.8 mg, 72 μmol) under nitrogen to form the mixed anhydride. The mixture was stirred vigorously at room temperature for 1 h.

The transesterification of the mixed anhydride with CoASH (as trilithium salt, 60 mg, 78 μmol in 1.4 ml 0.4 M $NaHCO_3$), the purification of *N,O*-di-Boc protected CoA ester, and the *N,O*-deprotection with trifluoroacetic acid were all performed as described in general procedure. The final product (2*R*,3*S*)-3-phenylisoserinoyl coenzyme A, was eluted from the C_{18} silica gel column with 15-20% methanol which was evaporated and the residue was lyophilized to give the product as white solid. The purity was assessed by analytical TLC which was found to be 98-100%. Compound was then confirmed by ESI-MS and ^{1}HNMR spectroscopy. Yield = 67% based on CoA. ESI-MS: Calculated 930.1, Experimental [M–H] 928.8, [M+H] was not possible. ^{1}H NMR (300 MHz, CD_3OD) (see Figure 48 for numbering) δ (in ppm) : 0.83 (3H, s, H-10'), 1.05 (3H, s, H-11'), 2.44 (2H, t, H-4'), 2.79 (2H, m, H-1'), 3.45 (2H, m, H-2'), 3.47 (2H, dd, J = 6.6 Hz, H-5'), 3.57 (1H, dd, J = 6.6 and 10.5 Hz, H_a-5"), 3.98 (1H, dd, J = 5.4 and 9.9 Hz, H_b-5"), 4.06 (1H, s, H-7'), 4.25 (2H, s, H-9'), 4.49 (1H, br ddd, H-4"), 4.69-4.90 (4H, m, H-2, H-3, H-2" and H-3"), 6.13 (d, J = 6.0 Hz, H-1"), 7.25-7.41 (5H, m, H_A phenyl protons), 8.18 (1H, s, H_C adenine-CH), 8.57 (1H, s, H_B adenine-CH).

2'-Deoxytaxol.

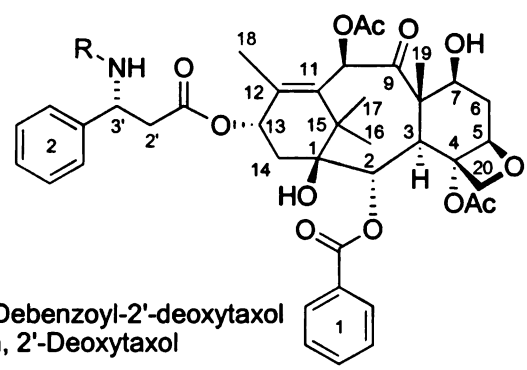


Figure 49: *N*-Debenzoyl-2'-deoxytaxol and 2'-Deoxytaxol.

Preparation of 7-TES-10-deacetylbaccatin III: To a solution of commercially available 10-deacetylbaccatin III (0.45 g, 0.8 mmol) in pyridine (20 mL) was added TES-Cl (2.5 mL, 16 mmol, 20 equivalents) dropwise. The solution was stirred at 25 °C for 17 h. After dilution with ether (100 ml), the solution was washed with aqueous $CuSO_4$ solution (3 x 30 mL) and brine (3 x 20 mL). The organic layer was dried over $MgSO_4$, concentrated and purified by flash column chromatography (65:35 hexanes/ethyl acetate, v/v) to produce 7-TES-10-deacetylbaccatin III as white solid (yield = 70%).

Preparation of 7-TES-baccatin III: To a solution of 7-TES-10-deacetylbaccatin III (0.3 g, 0.46 mmol) in pyridine (10 mL) at 0 °C was added acetyl chloride (0.17 mL, 2.3 mmol, 5 equivalents) dropwise. The solution was stirred at 5-10 °C (in cold room) for 48 h. After dilution with ether (100 ml), the solution was washed with aqueous $CuSO_4$ solution (3 x 30 mL) and brine (3 x 20 mL). The organic layer was dried over $MgSO_4$, concentrated and purified by flash column chromatography (65:35 hexanes/ethyl acetate, v/v) to produce 7-TES-baccatin III as white solid (yield = 60%).

Preparation of N-Boc-7-TES-2'-deoxytaxol: Di-pyridylthiourea (0.3 g, 1.3 mmol) was added to a solution of 3-(*R,S*)-*N*-Boc-*β*-phenylalanine (Alfa Aesar, Ward Hill, MA) (0.3 g, 1.3 mmol), *N,N*-dimethylaminopyridine (0.167 g, 1.3 mmol) and 7-TES-baccatin III (0.15 g, 0.22 mol) in toluene (24 mL). The reaction was stirred at 70 °C for overnight. The solvent was evaporated under reduced pressure and the crude residue was purified by flash column chromatography using 65:35 hexanes/ethyl acetate, v/v. Fractions containing the product were combined and solvent was evaporated under reduced pressure to obtain the desired product as pale white solid (yield = 74%).

Preparation of N-debenzoyl-2'-deoxytaxol: 0.15 mL of formic acid (88% aqueous solution) was added to a solution of *N*-Boc-7-TES-2'-deoxytaxol (50 mg, 53 μmol) in 1 mL dichloromethane and stirred at room temperature with monitoring by TLC. Residual formic acid was removed on a vacuum pump upon completion of the reaction indicated by TLC (no starting material was observed). The reaction mixture was then diluted with ethyl acetate and the solution was washed with 5% $NaHCO_3$ (2 x 10 mL), water (2 x 10 mL), brine (2 x 10 mL) and then dried over sodium sulfate. The solution was then filtered and solvent was removed under reduced pressure. The residue was purified by PTLC (90: 10 ethyl acetate/methanol, v/v) to obtain the desired product as yellow solid (yield = 100%). ^{1}H NMR (500 MHz, $CDCl_3$) (see Figure 49 for numbering) δ (in ppm): 1.14 (3H, s, H-16), 1.24 (3H, s, H-17), 1.68 (3H, s, H-19), 1.79 (3H, d, J = 1.5 Hz, H-18), 1.88 (1H, ddd, J = 2.3 Hz, 11.0 Hz, 14.7 Hz, H_b-6), 2.23 (3H, s, 10-OAc), 2.28 (2H, s H-14), 2.38 (3H, s, 4-OAc), 2.54 (1H, ddd, J = 6.7 Hz, 9.7 Hz, 14.8 Hz, H_a-6), 2.98 (1H, d, J = 2.7, H-2'), 3.24 (1H, br d, H-3') 3.79 (1H, dd, J = 1.0 Hz, 7.0 Hz, H-3), 4.19 (1H, dd, J = 1.0 Hz, 8.5 Hz, H_b-20), 4.30 (1H, dd, J = 1.0 Hz, 8.4 Hz, H_a-20), 4.40 (1H, dd, J = 6.7 Hz, 10.9 Hz, H-7), 4.94 (1H, d, J = 0.8, H-5), 5.67 (1H, d, J = 7.1 Hz, H-2), 6.37 (1H, s, H-10), 7.35 (1H, t, t, *p*-Ph-2), 7.42 (2H, m, *m*-Ph-2), 7.48 (2H, m, *o*-Ph-2), 7.51 (2H, m, *m*-Ph-1), 7.61 (1H, t, t, *p*-Ph-1), 8.13 (2H, two doublets, *o*-Ph-1).

Preparation of 2'-deoxytaxol:

In a screw-cap tube 8.3 mg (10 μmol) of *N*-debenzoyl-2'-deoxytaxol was taken and dissolved in 0.1 N aqueous NaOH solution to maintain the pH at 9.0. Benzoyl chloride (50 μL) was then added to it. The mixture was stirred vigorously on a vortex for 30 minute. The aqueous layer was then extracted with ethyl acetate (5 mL x 2). The organic

fraction was then washed with brine (5 mL x 2), dried over sodium sulfate, filtered. Solvent was evaporated under reduce pressure and the residue was purified by PTLC (65:35, hexanes/ethyl acetate, v/v) to obtain the desired product as a pale white solid (yield = 90%). ^{1}H NMR (500 MHz, $CDCl_3$) (see Figure 49 for numbering) δ (in ppm): 1.14 (3H, s, H-16), 1.24 (3H, s, H-17), 1.68 (3H, s, H-19), 1.79 (3H, d, J = 1.5 Hz, H-18), 1.88 (1H, ddd, J = 2.3 Hz, 11.0 Hz, 14.7 Hz, H_b-6), 2.23 (3H, s, 10-OAc), 2.28 (2H, s H-14), 2.38 (3H, s, 4-OAc), 2.54 (1H, ddd, J = 6.7 Hz, 9.7 Hz, 14.8 Hz, H_a-6), 2.98 (1H, d, J = 2.7, H-2'), 3.79 (1H, dd, J = 1.0 Hz, 7.0 Hz, H-3), 4.19 (1H, dd, J = 1.0 Hz, 8.5 Hz, H_b-20), 4.30 (1H, dd, J = 1.0 Hz, 8.4 Hz, H_a-20), 4.40 (1H, dd, J = 6.7 Hz, 10.9 Hz, H-7), 4.94 (1H, d, J = 0.8, H-5), 5.24 (1H, br d, H-3'), 5.67 (1H, d, J = 7.1 Hz, H-2), 6.37 (1H, s, H-10), 7.35 (1H, t, t, *p*-Ph-2), 7.40 (2H, m, *m*-Ph-N), 7.42 (2H, m, *m*-Ph-2), 7.48 (2H, m, *o*-Ph-2), 7.49 (1H, m, *p*-Ph-N), 7.51 (2H, m, *m*-Ph-1), 7.61 (1H, t, t, *p*-Ph-1), 7.74 (2H, two doublets, *o*-Ph-N), 8.13 (2H, two doublets, *o*-Ph-1).

13-^{3}H-Baccatin III.

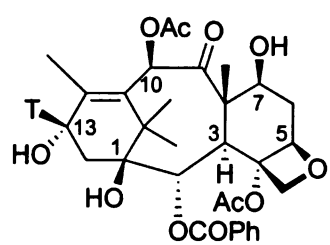


Figure 50: 13-^{3}H-Baccatin III

Preparation of 13-Oxo-7-TES-baccatin III: 7-TES-baccatin (0.1 g, 0.14 mmol), prepared by the method described above, was dissolved in dichloromethane (10 mL) and manganese (IV) oxide powder was added and stirred slowly with monitoring the reaction

by TLC. Upon completion of the reaction, (i.e. when TLC showed no starting material present), the reaction mixture was filtered and solvent was evaporated under reduced pressure. The residue was then purified by flash column chromatography using 65:35, hexanes/ethyl acetate, v/v. Fraction containing the desired product were then combined and solvent was evaporated under reduced pressure to obtain the product as white solid (yield = 100%).

Preparation of 13-^{3}H-baccatin III:

In a vial 13-Oxo-7-TES-baccatin III (10 mg, 0.014 mmol) in THF (0.5 mL) was taken and stirred at 0 °C. In another vial excess ^{3}H-$NaBH_4$ (50 mCi, specific activity 100-500 mCi/mmol), solid, ARC Inc.) was dissolved in minimum amount of 0.01N sodium hydroxide solution which was then slowly added by a syringe to the vial containing the substrate. The reaction was then stirred at 0 °C for 10 minute and then at room temperature with occasional monitoring by TLC. The reaction was quenched by adding the starting material (10 mg) and was stirred for another 2 h. Finally water (0.5 ml) was added to the reaction. The reaction mixture was then extracted with ethyl acetate (5 mL x 2) and the solvent was evaporated under flow of nitrogen gas. Without further purification (to avoid loss of radioactivity), the residue was taken to the next deprotection step and the 7-TES-group was deprotected in the same way as described above. The residue obtained after solvent evaporation was then purified by PTLC (60:40, hexanes/ethyl acetate, v/v) to get the product as white solid (8 mg, 0.014 mmol, 100% chemical yield). The radioactivity of the product was then determined by a liquid scintillation counter (model 14/4C Wallace Instruments, Copley, OH) (0.493 x 10^{9} DPM, 0.22 mCi, specific activity 16 mCi/mmol). The identity of the product was confirmed by

eluting it in HPLC, which, under same condition, indicated same retention time with commercially available authentic baccatin III sample.

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