

Probing the stereospecificity and chemospecificity of polyketide thioesterases

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Abstract

Macrocyclization is a synthetically challenging step in the total synthesis of natural products. The success of chemical approaches such as the Corey-Nicolaou, Yamaguchi and Keck macrolactonization is heavily based on the conformation and stereochemistry of the substrate. While there have been some advances in computational modeling, it has been difficult to predict whether the above-mentioned reactions will work. We have begun characterizing polyketide thioesterase catalytic activity and substrate tolerance to find more efficient and dependable routes towards macrolactonization and macrolactamization.

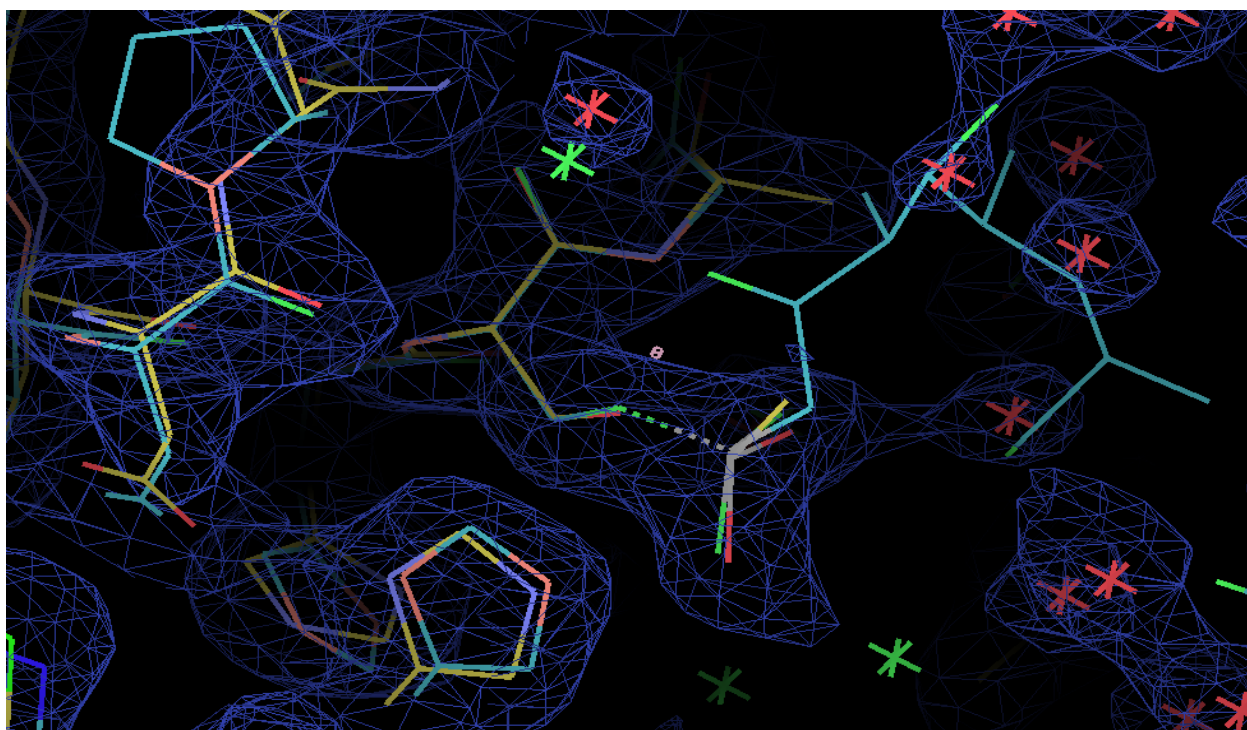


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Appendix A: Publications

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List of Abbreviations

6-dEB	6-Deoxyerythronolide B
ACP	Acyl carrier protein
AT	Acyltransferase
CoA	Coenzyme A
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	N,N'-dicyclohexylcarbodiimide
DCM	Dichloromethane
DEAD	Diethyl Azodicarboxylate
DEBS	6-Deoxyerythronolide B synthase
DH	Dehydratase
DIAD	Diisopropyl Azodicarboxylate
DIEA	Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
dTNB	5,5'-Dithiobis-2-nitrobenzoic acid
EDC	N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide
ER	Enoylreductase
FAS	Fatty Acid Synthase
HKR	Hydrolytic Kinetic Resolution
HPLC	High performance liquid chromatography
HWE	Horner-Wadsworth-Emmons reaction
Ipc ₂ BAllyl	B-Allyldiisopinocampheylborane
JohnPhos	(2-Biphenyl)di-tert-butylphosphine

KR	Ketoreductase
KS	Ketosynthase
LC-MS	Liquid chromatography/Mass spectrometry
LDA	Lithium Di-tert-butyl Amine
MALDI	Matrix-assisted laser desorption/ionization
mCPBA	meta-Chloroperoxybenzoic acid
MT	Methyltransferase
NAC	N-acyl cysteamine
NMR	Nuclear magnetic resonance
NRPS	Non-ribosomal peptide synthetase
PIKS	Pikromycin synthase
PKS	Polyketide synthase
Rad	Radical
RCM	Ring closing metathesis
SAM	S-adenosylmethionine
SNAC	N-acetylcysteamine thioesters
TBAF	Tetrabutylammonium fluoride
TBAI	Tetrabutylammonium iodide
TBS	tert-Butyldimethylsilyl
TBTU	O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
TE	Thioesterase
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Zea	Zearelenone

Chapter 1. Synthesis and biosynthesis of polyketides: an overview on how nature and synthetic chemists synthesize macrolactones

"This is an important point: neither biology nor chemistry would be served best by a development in which all organic chemists would simply become biological such that, as a consequence, research at the core of organic chemistry and, therefore, progress in understanding the reactivity of organic molecules, would dry out. Progress at its core in understanding and reasoning is not only essential for organic chemistry itself, but for life science as a whole. Life science needs an Organic Chemistry that remains strong." - Albert Eschenmoser

1.1 Introduction

Many pharmaceutical drugs have been discovered by extracting biologically active materials from nature. Whether it is derived from a fungus or the sap of a tree, these medicinal molecules are essential to the health of the world. With an ever growing population, demand for these drugs increases. Unfortunately, many of the methods used to acquire these natural products either require an exorbitant amount of crude material to generate a single dose or are expensive to produce. Techniques are constantly being invented and improved to lower the costs of drug manufacturing. Synthetic chemists have begun addressing the production of medicinal natural products by understanding how nature produces these essential molecules and designing syntheses based on their respective biosyntheses.

1.1.1 Enzyme catalysis could be the next stage in drug development

Paclitaxel, first isolated by Wall and Wani in 1971, is an example of an effective anti-tumor treatment that is difficult to obtain in large quantities.¹ For initial biological analysis, Paclitaxel required the 1,200 kg of bark to produce 28 kg of crude extract which in turn yielded on 10 g of pure material.² As the therapeutic went through Phase I and Phase II clinical trials, over 33,000 kg of bark was needed and concerns began to rise as to the ecological impact. Due to its biological activity and overharvesting of its natural source, the drug became a highly contested target for synthetic chemists. The anticancer agent was simultaneously synthesized by Holton³ and Nicolaou⁴ in 1994 but, as is the case in a total synthesis, only milligram quantities were produced. While advances have been made by the Baran group and other research groups,

the core of paclitaxel is still difficult to synthesize from commercially available chemicals.⁶ Today, Taxol[®] is produced by Bristol-Myers Squibb⁵ and is generated chemically from 10-deacetylbaccatin III extracted from the needles of European yew trees. While this process is not inefficient, a biochemical approach could reduce the consumption of plant material.

To date, there have been few uses of enzymes in mass pharmaceutical production. Sitagliptin, a new potential treatment for type II diabetes, is currently being manufactured by Merck with the use of an engineered transaminase. (Figure 1.1.1) Their first generation process was a 9-step chemical synthesis which included a wasteful EDC coupling-Mitsunobu sequence that provided the product at a 52 % overall yield.⁷ A few years later, they improved their synthesis to a 3-step chemical synthesis including an impressive one pot transformation from the starting beta-keto ester to an enamine.⁸ Unfortunately, both syntheses were plagued by the problematic enantioselective reduction of the enamine. While they found conditions for quantitative yields and high enantiopurity using a rhodium-based catalyst⁸, the reaction required specialized high pressure hydrogenation equipment and produced heavy metal waste. Transaminases have the biochemical machinery to perform the desired chemistry but, like many enzymes, are quite substrate-specific. Through active site mutation and directed evolution, Merck Research Laboratories were able to produce an enzyme that would biocatalytically synthesize sitagliptin from a diketo-version of the substrate.⁹ By switching from a chemical synthesis to a biochemical transformation, they were able to decrease total waste including heavy metals, avoid the need for costly hydrogenation equipment and increase overall yield and productivity.

Drug development and synthesis is a lengthy and costly process; research and development alone often extends for over a decade. According to a 2002 poll, from research and development to clinical trials to marketing, the average cost for developing a new drug was estimated at US\$ 802 million.¹⁰ As businesses try to decrease cost and increase efficiency, pharmaceutical companies search the literature and do research in-house to improve their methodologies. Research into the biosynthesis of natural products has been a source of inspiration and innovation with regards to synthetic routes. Unfortunately, many biosynthetic pathways are yet to be discovered and the process required to discover the pathways is lengthy

and low producing. If enzymes were tailored specifically for difficult chemical transformations, production of pharmaceutical drugs would surely improve.

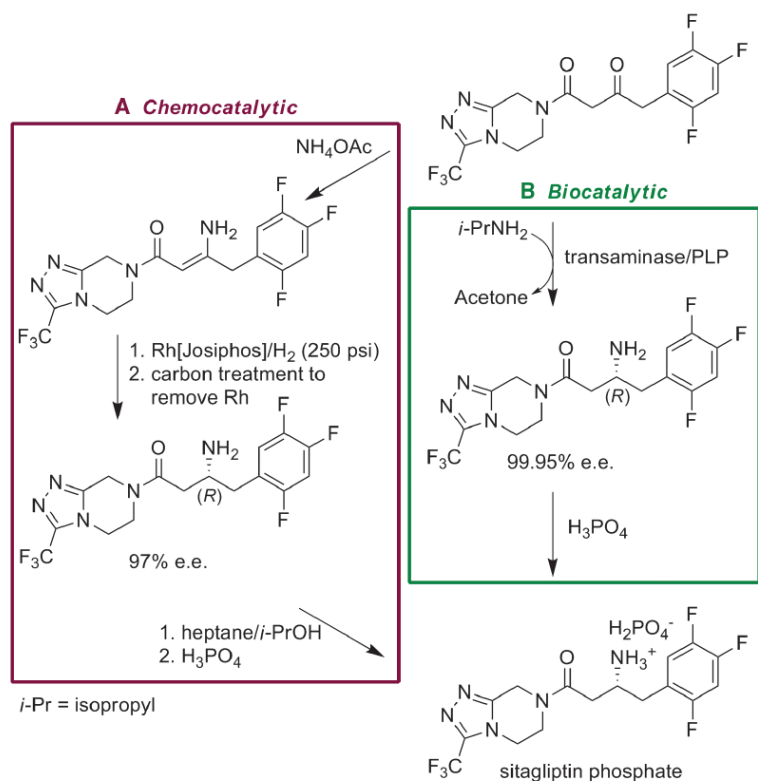


Figure 1.1.1 Biocatalytic hydrogenation is a more efficient route towards large scale preparation of sitagliptin

1.1.2 Polyketide natural products

Polyketide natural products are among the most structurally diverse and biologically active group of natural products.¹¹ (Figure 1.1.2) They comprise 20 % of the top-selling drugs worldwide and combine for revenues in excess of \$10 billion every year.¹² Most polyketides can be separated into three different types based on their respective biosyntheses: Type I, Type II and Type III polyketide synthases (PKSs). While they are structurally diverse, each biosynthesis will undergo a similar sequence: initiation, elongation and off-loading.

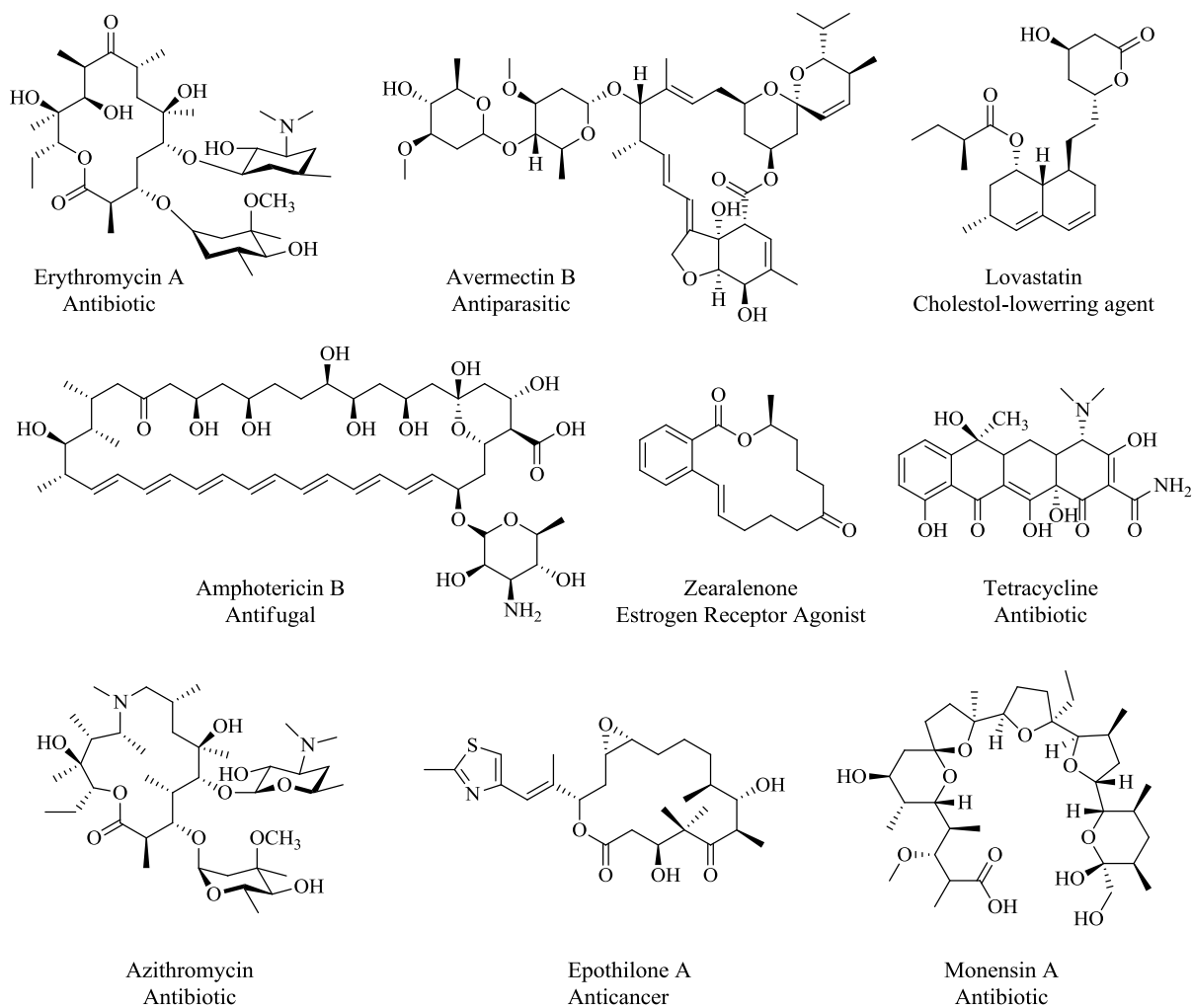


Figure 1.1.2 A selection of polyketide natural products with a range of biological and structural diversity.

While PKSs utilize the same biochemical machinery, their biosyntheses differ in which domains are present and their modular separation. Type I PKSs separate each transformation of the CoA-starter units into separate modules encoded on a single polypeptide chain.¹³ These multi-domain proteins typically produce fungal polyketides such as lovastatin and bacterial polyketide like erythromycin and epothilone. Type II PKSs can be subdivided into two groups, Iterative and Modular Type II PKSs, which produce bacterial aromatic natural products and secondary metabolites respectively. Iterative synthases allow for several unreacted carbonyls to be adjacent to one another. These repeating moieties can, unlike other pathways that include aromatic moieties from starter units, produce aromatic portions to molecules through simple dehydration. Modular Type II PKSs contain distinct modules that involve multiple copies of the

active site. Each starter unit is transformed before being loaded onto the next module and this continues until the linear substrate is fully formed. Examples of Type II PKS products include the anticancer agent doxorubicin and the antibacterial tetracyclines. Finally, Type III PKSs are homodimers that catalyze the extension of substrates through iterative decarboxylative Claisen condensation reactions.¹⁴ Unlike the other two types, Type III synthases do not contain an acyl carrier protein to transfer one substrate to the next module. These PKSs load the starter unit, transform the substrate and continue to grow either attached to the KS or bound to a CoA.

1.1.3 Polyketide biosynthesis

The biosynthesis of polyketide natural products utilizes simple acyl-coenzyme A (acyl-CoA) monomers to build remarkably complex molecules.¹⁵ The biochemical machinery used to synthesize these metabolites can be split into three steps: chain elongation, processing and off-loading. The simplest example of this machinery at work can be seen in the closely related biosynthetic pathways of fatty acid synthases (FAS).¹⁶

Chain elongation occurs through Claisen-type condensation reactions. The incorporation of several acetyl-CoA units is through the nucleophilic attack of an enzyme-bound thioester.¹⁷ The first step is catalyzed by the acyl transferase (AT) which transfers an acetyl group from the CoA to the acyl carrier protein (ACP). Once the monomer is bound to the ACP, it contains an alpha carboxylated center that allows for nucleophilic attack of substrates bound to the ketosynthase (KS). The repetition of these steps forms all of the C-C bonds in the core of the molecule and beta-carbonyl functionality can be modified in the processing step.

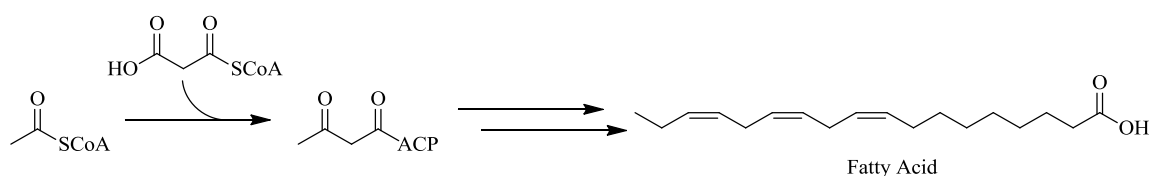


Figure 1.1.3 An example of chain elongation by the addition of acetyl-CoA to form fatty acids

Through stereospecific reductions, methylations and, regiospecific dehydration, the ACP bound substrate will be transformed to give the desired product. Figure 1.1.4 shows complete the biosynthesis of 6-deoxyerythronolide B (6-dEB), the precursor to the antibiotic erythromycin.¹⁸ As seen in module 1, the newly formed β -ketone is selectively reduced by the ketoreductase

(KR) to give the R-stereoisomer. A second stereospecific reduction by a KR occurs on Module 2 producing the opposite stereochemistry. Introduction of methyl groups in PKSs can be done in two ways: introduction from a malonyl starter unit (seen in module 3) or through a methyltransferase (MT). While not common in Type II PKSs such as deoxyerythronolide B (DEBS) synthase, MTs can be found in Type III PKS pathways using S-adenosyl methionine (SAM) as a methyl donor.¹⁹ Module 4 shows the tandem use of dehydratase (DH) and enoyl reductase (ER) to give a fully saturated carbon center. The use of one and both domains can be seen in the biosynthesis of the omega-3 fatty acid alpha-linolenic acid, the product in Figure 1.1.3.

The final step of the polyketide biosynthesis is chain termination. While there is a wide range of termination mechanisms, enzymatic macrolactonization are most commonly catalyzed by the use of a thioesterase (TE).¹¹ This process has been shown to be regio- and stereoselective process.²⁰ Many efforts have been made to understand the cyclization of linear molecules into large macrolactones²¹ but few have characterized TEs in an attempt to catalyze macrolactonization in natural product total synthesis. Following cyclization, post tailoring steps, such as glycosylation, oxidation and reduction, will further modify the unbound polyketide to complete the biosynthesis.²¹

able to elucidate a crystal structure with a substrate in the hydrophobic channel and gain important insight into the macrolactonization of picromycin.³⁰ (Figure 1.1.6)

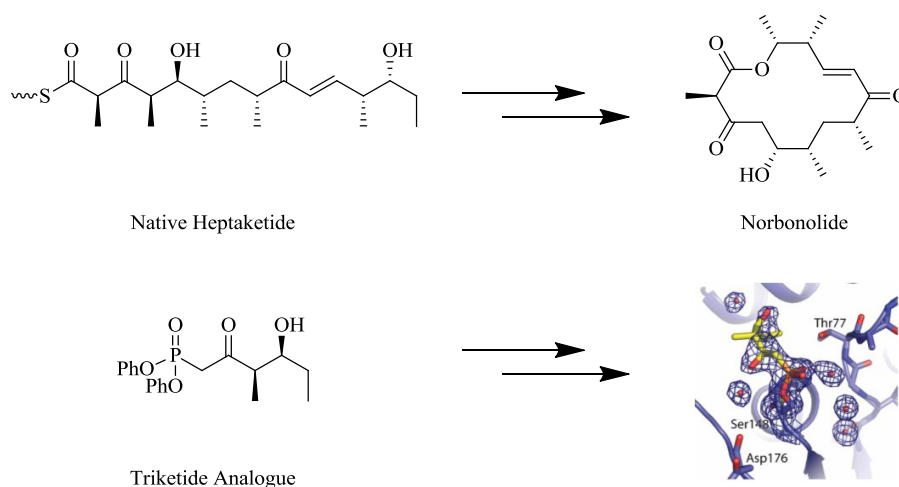


Figure 1.1.6 Crystal structures were elucidated with the treatment a thioesterase with a triketide analogue of the native substrate containing a phosphonate moiety

The mechanism and stereospecificity for the macrolactonization of erythromycin's core, 6-deoxyerythronolide B, is yet to be discovered. When Khosla *et al.* first elucidated the crystal structure of the thioesterase responsible, they commented that docking studies showed that only one stereochemical orientation can be accommodated in the active site.²² They believed that the mechanism selecting macrolactonization over hydrolysis was due to extensive hydrogen-bonding interactions. Through site-directed mutations of the binding cavity of DEBS TE, the Boddy lab showed that hydrogen bonding between substrate and enzyme does not mediate substrate specificity but rather non-polar interactions dominate substrate recognition.²³ A study similar to that done for pikromycin would surely give insight into the mechanism and stereospecificity of DEBS TE.

1.2 The inherent difficulties and chemical approaches of macrolactonization

The biologically and structurally diverse selection of polyketide natural products seen in Figure 1.1.2 has one thing in common; they are all macrolactones. Biosynthetically, they are all made by the attack of a hydroxyl group to an enzyme-bound substrate. Chemically, however, the cyclization of these molecules has been through a wide range of methods. While utilization of RCM³², intramolecular cross-coupling³³ and HWE³⁴ methods have been used to synthesize

polyketides, the lactone formation has been the synthetic approach for most of these targets. There are two schools of thought when forming a macrolactone: carboxylic acid activation or alcohol activation. Campagne *et al.* have a thorough review²¹ updated in 2013 on both types of activation. Polyketide biosynthesis involves activation of the acid portion of the molecule and will be the theme of this thesis.

1.2.1 *Macrolactonization is sterically and energetically unfavored*

Whether it is peptide synthesis or total synthesis of natural products, developing efficient techniques to couple a carboxylic acid to another fragment are essential. There are a wide range of reagents that activate a carboxylic acid by forming a new ester or thioester bond. By forming a more electrophilic center, the activation energy required to form the carbon-nucleophile bond decreases and nucleophilic attack is more energetically favored. Often, these reactions are employed in the later stages of a total synthesis due to the complexity of the natural product target and have to be both dependable and protecting group tolerant.

Macrolactonization is based on activation energies and steric hindrance. Beginning with the simplest case, Mandolini and Illuminati synthesized omega-bromoalkane carboxylic acids with varying lengths and analyzed the kinetics involved in lactone formation.³⁵ Their work confirmed that there is a relation between ring size and activation energy but, after 12-membered lactones, the ring formation energy does not play a large role. There is also a competition between inter- and intramolecular reactivity. Similar to RCM³⁶, formation of undesired dimers occurs but high dilution techniques and solid phase syntheses have addressed this issue.³⁷ The major obstacle for macrolactonization is the substituents surrounding the carboxylic acid and alcohol. In rare cases, the geometry of the starting material can lead to lactone formation with heat as can be seen in the total synthesis of Callipeltoside A.³⁸ Configuration and substitution can also disfavor macrocyclization as was discovered by Woodward in his total synthesis of erythromycin A.³⁹⁻⁴¹ Seventeen stereoisomers, differing only in the configuration of the reactive alcohol, were protected with various linear and acetal protecting groups and subjected to the Corey-Nicolaou conditions. (Figure 1.1.7) Only the 9S substrate with two cyclic acetal protecting group proceeded with respectable yield. Successful macrolactonization occurred only when a combination of the stereochemistry of the reactive alcohol and the geometry of the linear molecule were aligned.

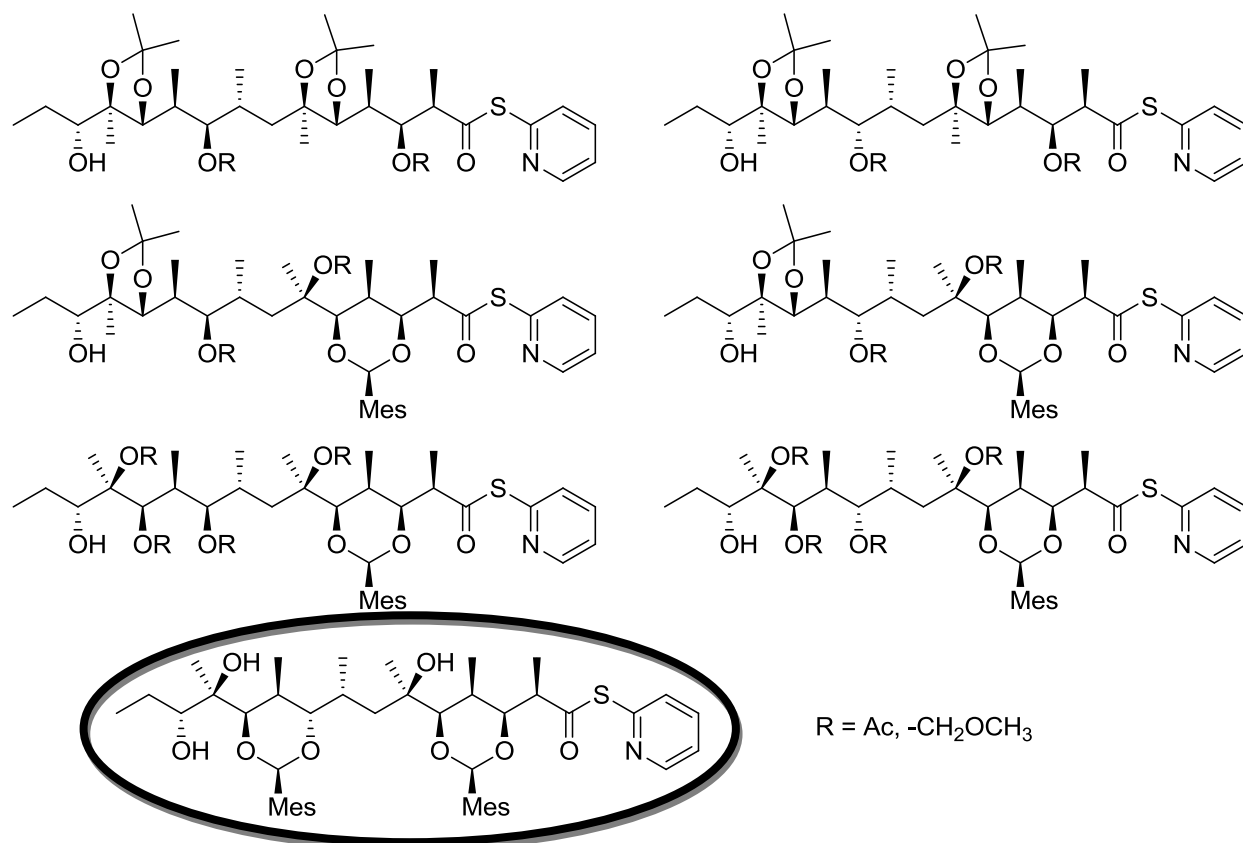


Figure 1.2.1 Woodward synthesized 17 different molecules, varying in configuration and protecting groups, to find the correct configuration to form the core of erythromycin A

1.2.2 Common acid activation reactions

Activation of a carboxylic acid requires the selective reaction or displacement of the $-OH$ group with a moiety that can be easily displaced. Formation of mixed anhydrides, activated esters, thioesters, O-acyl ureas and *in situ* acid chlorides has been used towards the synthesis of polyketide natural products. As synthetic organic chemistry evolves, three reactions have become staples in macrolactone formation: Corey-Nicolaou, Yamaguchi and Keck. (Figure 1.1.8)

1.2.3 Corey-Nicolaou macrolactonization

Finding mild conditions for the lactonization of complex polyketides was a challenge for many synthetic chemists in the 1970s including E.J. Corey and K.C. Nicolaou. Ideally, they desired a method that has low reaction temperatures and activation of both the carboxyl and the hydroxyl group. Using 2-pyridinethiol esters as their test substrates, they theorized that these

substrates would cyclize due to a favorable proton transfer from the hydroxyl to the carbonyl.⁴² After confirming their hypothesis, they were able to synthesize the anti-fungal zearalenone by treating the corresponding acid with 2,2'-dipyridyl disulfide at room temperature followed by refluxing until completion. While they did comment on the difficulty of formation of nine-membered rings, the Corey-Nicolaou cyclization conditions would prove to be useful for the formation of 6- to 16-membered lactones.

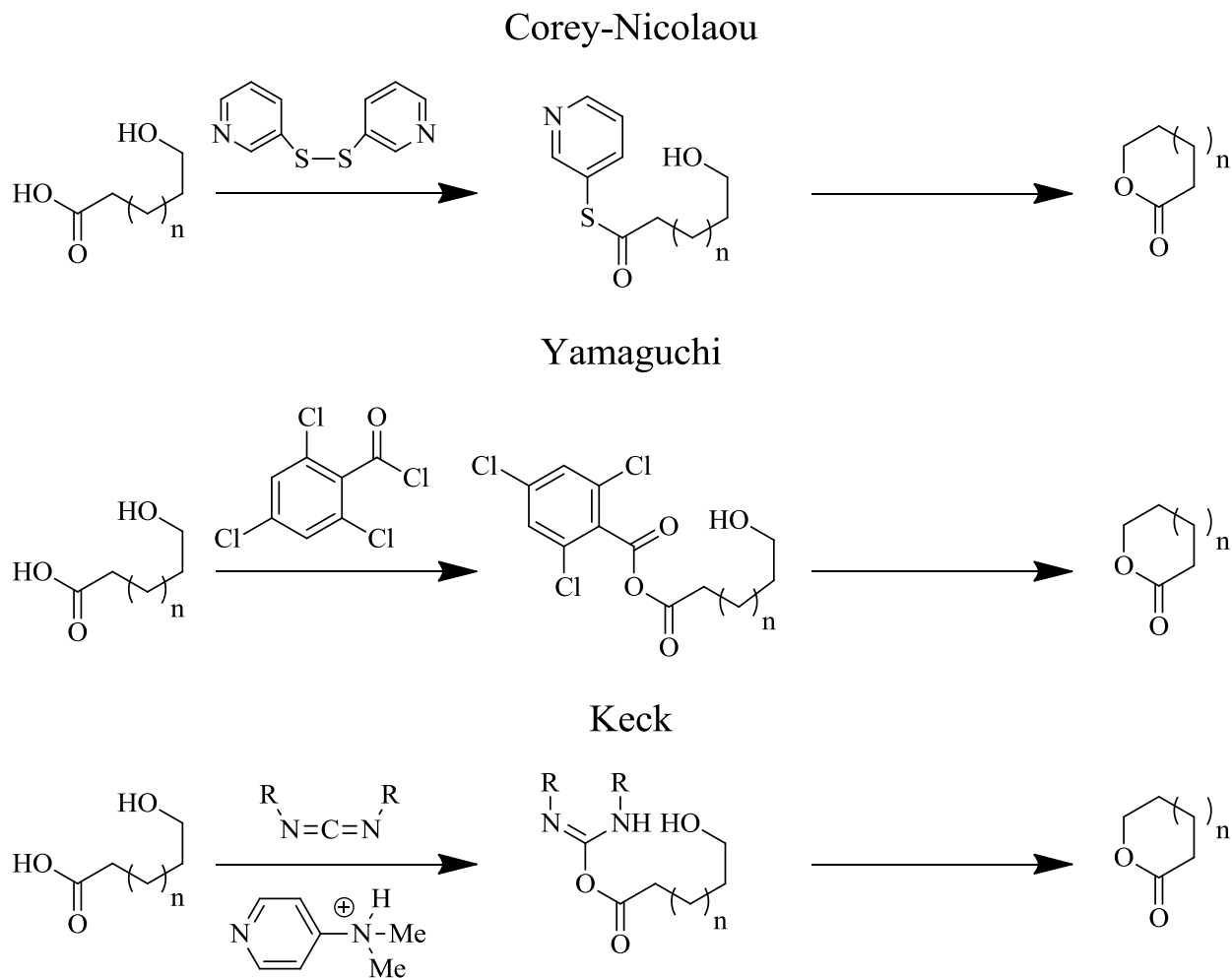


Figure 1.2.2 The three most common carboxylic acid activations reactions to form macrolactones

Over the years, modifications of the original method were introduced to eliminate triphenylphosphine oxide by-products and further activate the thiopyridone. The Corey-Clark modification used the thionylchloroformate foregoing the PPh_3 activation of the acid.⁴³ Gerlach *et al.* introduced a metal (usually Ag, Cu or Hg) that would activate the carboxylic acid by

complexing the carbonyl and the pyridine.^{44,45} This complex would allow for milder conditions and has been utilized in the synthesis of many natural products such as pamamycin.⁴⁶ (Figure 1.1.9) While this method has produced 7- to 48-membered lactones, it has recently been a less popular choice for synthetic chemists possibly due to its long reaction time and the high concentration of cuprate or silver salts.⁴⁷

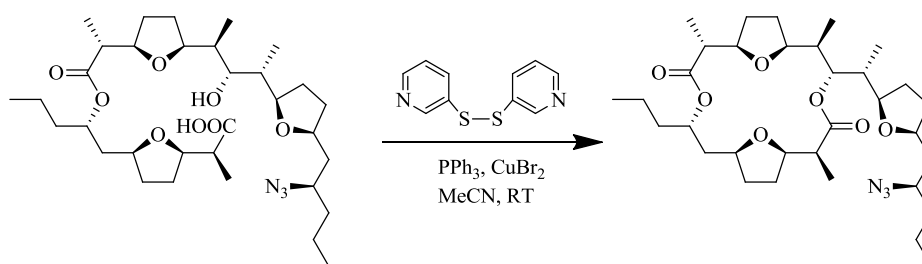


Figure 1.2.3 Use of the Gerlach-modified Corey-Nicolaou macrolactonization conditions towards the total synthesis of pamamycin

1.2.4 Yamaguchi macrolactonization

In 1976, Masaru Yamaguchi sought after mild conditions for the rapid conversion of Ω -hydroxycarboxylic acids to a macrolactone.⁴⁸ Having seen the effectiveness of DMAP in acyl transfer reactions, a reagent was needed to further activate the carboxylic acid to overcome the unfavourable entropic factors. Through methodological studies, it was found that 2,4,6-trichlorobenzoyl chloride was the best candidate to form the more reactive mixed anhydride. These conditions allowed Yamaguchi to synthesize the 12-membered macrolactone of a polyketide from an acid-sensitive seco-acid with no formation of unwanted furan derivatives.⁴⁸

To date, the Yamaguchi protocol has allowed for the synthesis of over 340 molecules containing 8- to 72-membered macrolactones.²¹ An exemplary of this methodology can be seen in the total synthesis of dictyostatin. (Figure 1.1.10)⁴⁹ Patterson *et al.* set out to synthesize the potent cytotoxic macrolide by a convergent synthesis utilizing Still-Gennari-type olefination, Stille cross coupling and HWE coupling. Having installed 10 of the 11 stereogenic centres, they were able to cyclize the seco acid cleanly with a 77 % yield. As the synthesis of natural products evolves, efficient reactions dominate and the Yamaguchi conditions are now considered by some researchers as standard protocol for macrolactonization.⁵⁰

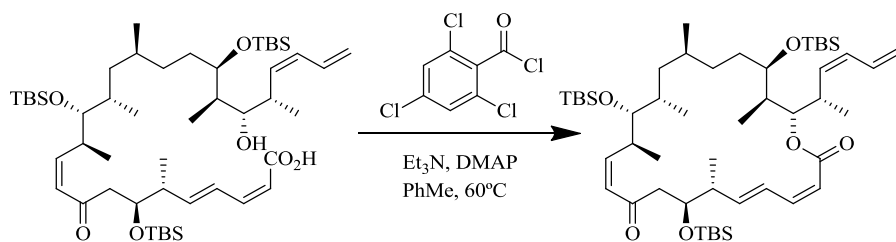


Figure 1.2.4 Use of Yamaguchi macrolactonization conditions towards the total synthesis of dictyostatin

1.2.5 Keck macrolactonization

Amidst the total synthesis of the macrocyclic bis-lactone colletodiol⁵¹, Keck *et al.* had difficulty with their macrolactonization. After an extensive literature examination, they wanted to not only successfully finish their synthesis but address the current issues for generating an activated ester: *in situ* as opposed to discrete activation, destruction by adventitious moisture and issues complicating conventional chromatography.⁵² As the bimolecular Steglich esterification⁵³ is high yielding and purification of urea by product was chromatographically simple, these researchers attempted to use the same conditions to form macrolactones. After addressing issues with the key proton transfer step, they found that a combination of DCC, DMAP and DMAP•HCl allowed for a high yielding reaction.

The Molinski lab at the UC San Diego also had troubles with lactone formation in their total synthesis of enigmazole A.⁵⁰ Having tried the Yamaguchi macrolactonization conditions and its variants with no success, the researchers were able to form a macrolactone with the Keck conditions. Unfortunately for them, the ring formed was the 16-membered ring, not the 18-membered ring found in the natural product. By switching the order of the previous hydrogenation step and the macrolactonization, they were able to form the desired 18-membered lactone intermediate.(Figure 1.1.11)

Struggles with ring formation are not unique to the above-mentioned syntheses. The confirmation of the starting material and the conditions used play a large role in the success of lactonization. Research into computational methods⁵⁴ and other reaction conditions continue to keep researchers interested in finding efficient, substrate tolerant and regio-selective methods to form macrolactones.

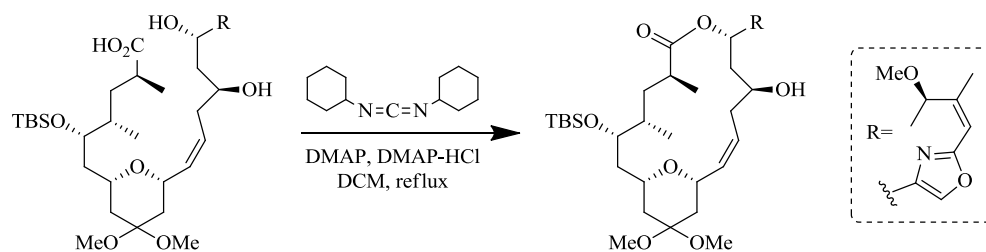


Figure 1.2.5 Use of Keck Conditions towards the total synthesis of enigmazole A

1.3 Conclusion

Many pharmaceutical drugs are polyketide natural products with macrolactone cores.⁵⁵ While synthetic chemists attempt to improve their syntheses, macrolactonization is still a challenge. Thioesterases have been shown as possible candidates for macrolactonization²⁵ and enzymes can adopt conformations to overcome high entropic barrier.⁵⁶ While the Corey-Nicolaou, Yamaguchi and Keck conditions are effective in the formation of macrolactones, there may be a biosynthetic approach to this problem. By analyzing the regio- and stereospecificity of thioesterases and attaining crystal structures of the enzyme to understand their mechanisms of action, we may be able find a biosynthetic solution like that used in the synthesis of sitagliptin⁹ to solve this synthetic problem.

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Chapter 2. Characterization of thioesterases: Formation of an acyl-enzyme intermediate to elucidate a crystal structure of DEBS TE in its active confirmation

"Few scientists acquainted with the chemistry of biological systems at the molecular level can avoid being inspired. Evolution has produced chemical compounds exquisitely organized to accomplish the most complicated and delicate of tasks. Many organic chemists viewing crystal structures of enzyme systems or nucleic acids and knowing the marvels of specificity of the immune systems must dream of designing and synthesizing simpler organic compounds that imitate working features of these naturally occurring compounds." - Donald J. Cram

2.1 Introduction

In nature, thioesterases (TEs) are responsible for the cyclization of many successful polyketide pharmaceuticals.^{1,2} The capability of DEBS TE to cyclize erythromycin³ as well as various ring sizes⁴ stereospecifically⁵ from various complex linear molecules makes it a great target for synthetic purposes. While researchers have successfully been able to elucidate the structure of the active site⁶, understanding how macrocyclization versus hydrolysis is controlled is difficult due to quick off-loading of the acyl-enzyme intermediate.⁷ Formation of a non-hydrolysable acyl-enzyme intermediate similar to studies^{8,9} done for Pik TE will allow for the elucidation of the crystal structure of DEBS TE. The Boddy laboratory has synthesized a mimic of the linear form of erythromycin that has been shown to cyclize when treated *in vitro* with isolated DEBS TE.⁵ By synthesizing a linear mimic that has a phosphonate moiety than can covalently modify the active site, we may be able to gain insight into the mechanism and stereochemical requirements for cyclizing non-native substrates.

2.1.1 Using biochemical machinery to synthesize polyketide natural product analogues

Interest into the biosynthesis of polyketide natural products has increased since Birch discovered the incorporation of acetate units into 6-methylsalicylic acid.² For many macrolide polyketides, the whole or partial genome sequences for the bacteria that encode them have been discovered enabling identification of the genes responsible for polyketide biosynthesis. As DEBS is the most extensively characterized polyketide synthase¹⁰, researchers have begun mutating portions of the sequence biosynthetic genes⁴, introducing non-native starting units¹¹ and linking modules in a particular order to produce non-native molecules.¹² Since small changes in

overall chemical structure have been shown in many pharmaceutical drugs to increase their potency,¹³ many researchers have used these rationally mutated biosynthetic pathways to produce a variety of new complex substrates whose biological activity in some cases supersedes that of their parent molecule.

Precursor-directed biosynthesis involves introducing a non-native molecule to a synthase in hopes of forming the corresponding unnatural substrate.¹⁴ It has been shown that inactivation of selected residues of a module deactivates the entire module¹⁵ and N-acetylcysteamines (NAC) thioesters mimic the phosphopantetheine arm of ACPs.¹⁶ From this, it can be hypothesized that inactivation of the KS in module 1 would allow for introduction of an unnatural diketide starting unit at module 2 thus using the other biosynthetic machinery to produce 6-dEB homologues.¹⁷ (Figure 2.1.1)

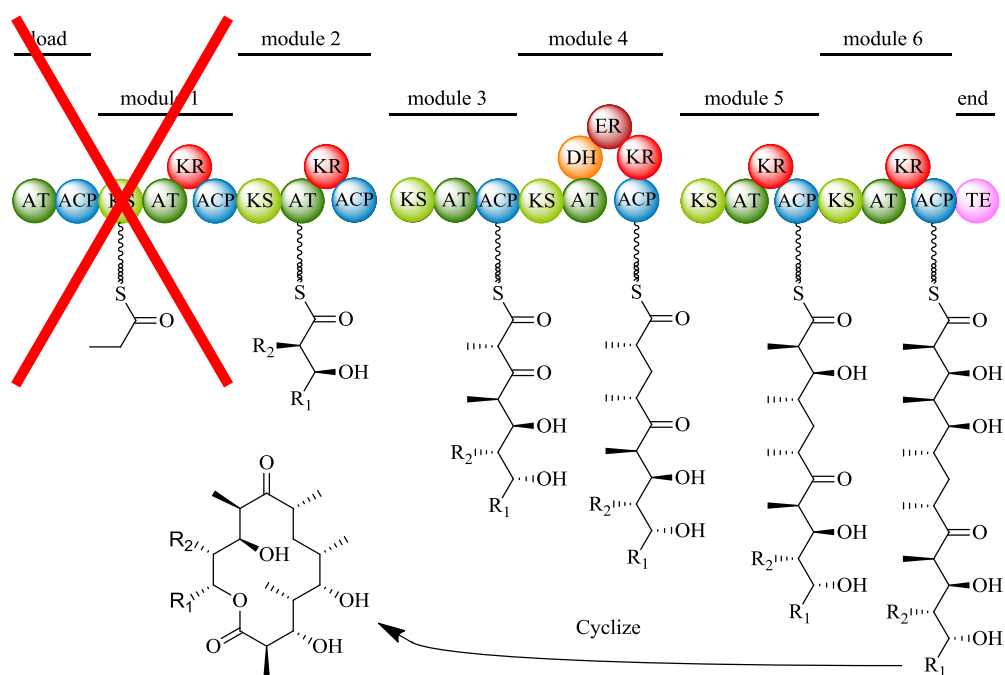


Figure 2.1.1 Knock-out of module 1 in DEBS leads to precursor-directed biosynthesis of 6-deoxyerythronolide analogues

Starting with the simplest case, researchers were able to biosynthesize a 13-ethyl erythronolide analogue.¹⁷ They were able to effectively knock-out module 1 by specific introduction of null mutations at KS1 and incorporate an unnatural ethyl-malonyl NAC thioester into the pathway. Chaitan Khosla and David E. Cane among others continued to explore the

scope of this methodology, changing the R groups at position 12 and 13 as well as forming 16-membered rings.^{10,17,18} (Figure 2.1.2) While the biological significance is not yet known, these advancements opened the door for considering DEBS as a synthetic tool.

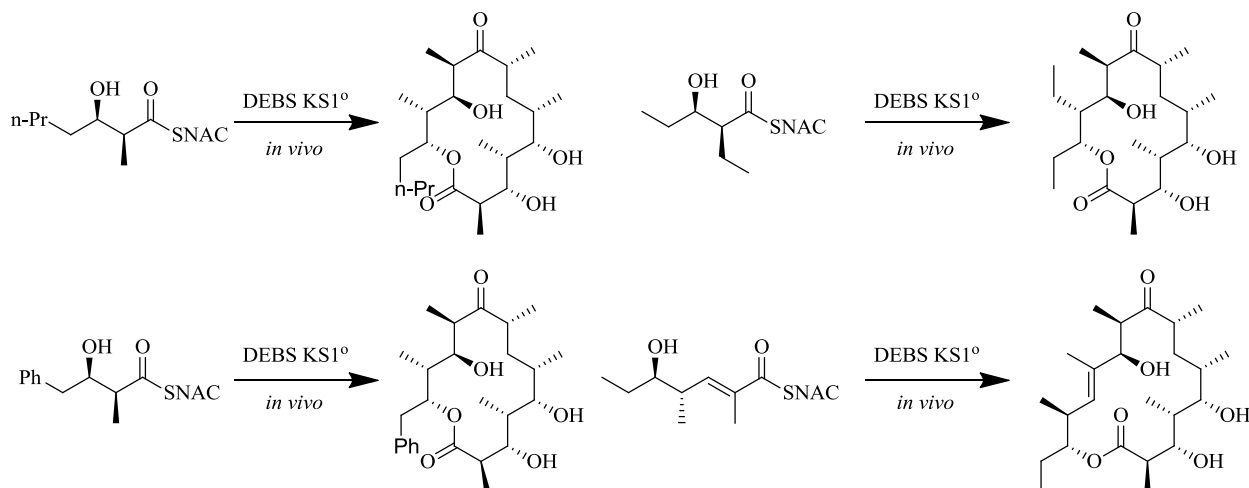


Figure 2.1.2 A few examples of 6-deoxyerythronolide analogues formed by precursor-directed biosynthesis

While exploring for evidence of two independent active sites within a modular PKS, researchers began engineering synthase mutants with their KS or ACP inactivated and stumbled upon another possible synthetic tool.¹⁹ Pairing modules 1 and 2 together and selectively inactivating KS1, they discovered the formation of a 6-membered lactone. Further investigation and further permutations of fused module pairs allowed for the production of 6-, 8-, 12-, 14- and 16-membered rings.^{4,20,21} (Figure 2.1.3) Through ¹³C labeled substrates, they were also able to prove the incorporation of chemically synthesized diketide thioesters into the production of 6-membered rings²². Santi *et al.* took this methodology further and cloned each pair of modules individually. Through the use of combinatorial biosynthesis and selective deactivations, they produced a large library of modified polyketide macrolactones.²³

As knowledge accrues on the mechanisms involved in the biosynthesis of 6-dEB, experiments continue to show the synthetic potential of these synthases. Currently, work is being done to utilize precursor-directed biosynthesis and directed mutagenesis but these methodologies do not utilize the macrolactonization power of DEBS TE. The mechanism of thioesterases are not yet fully understood, partially due to the quick off-loading of the substrate.⁷

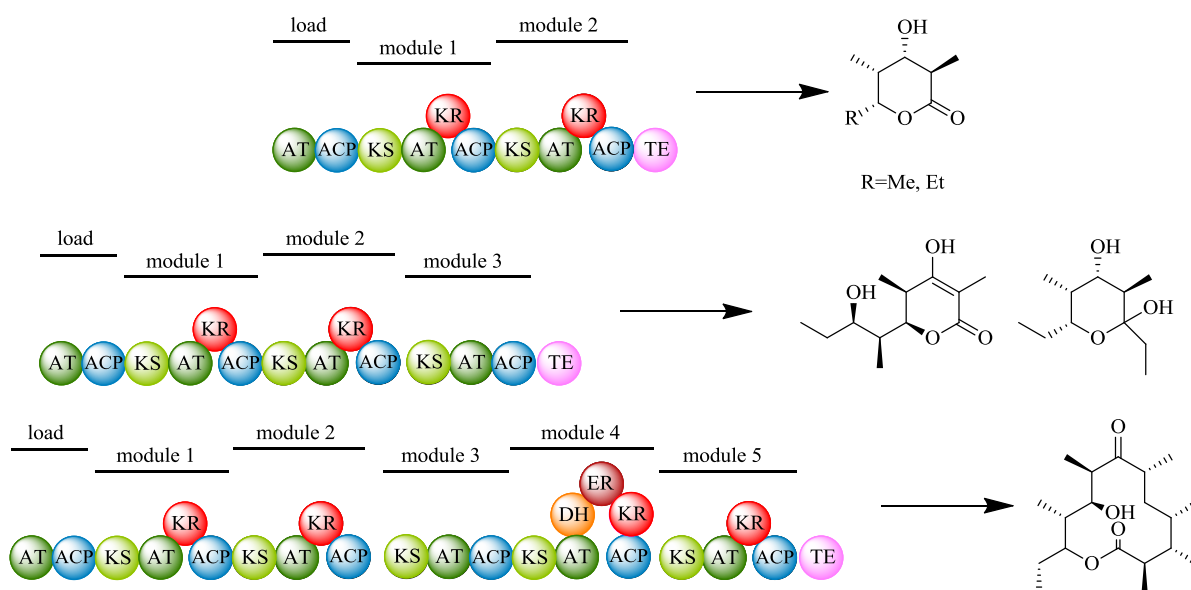


Figure 2.1.3 Directed mutagenesis of DEBS leads to the formation of various ring sizes

2.1.2 Factors affecting substrate off-loading of thioesterases

The TE domain embedded at the end of PKS pathways is responsible for the off-loading of the substrate. Once loaded onto the TE, there are two possible outcomes: attack by an external nucleophile (usually water) or attack from an internal nucleophile.²⁴ The outcome will be based on the conformation of the linear substrate in the substrate channel.²⁵ Once it was shown that an excised TE domain could be cloned and catalyze cyclization *in vitro*^{26,27}, the mystery of why one substrate would either hydrolyze or macrolactonize began. The active sites of thioesterases were analyzed and speculations were based on computational docking of macrocycles into the active site from crystal structures.⁷ Ground breaking studies on the TE responsible for the biosynthesis of nabonolide (the core of pikromycin)^{8,9} allowed for the formation of a non-hydrolysable intermediate in the active site. This substrate-soaked enzyme was by X-ray crystallography and shed the first light on solving the regio-, chemo- and stereospecificity²² of thioesterases.

While chemists and biochemists have been interested in polyketide synthases since the early 1950s²⁸, utilizing the pathways for chemoenzymatic synthesis began within the last couple decades. It began with isolation²⁹ of the TE domain of DEBS through over expression in *E. coli* and further analysis into the use of NAC thioesters as phosphopantetheine arm mimics³⁰. Once

isolated, TE domains were tested with non-native substrates in hopes of seeing some macrocyclization³¹. Unfortunately, hydrolysis was the only catalytic activity observed until a study on the enzyme responsible for the macrolactonization of epothilone²⁷. By hydrolyzing the ester linkage and activating the carboxylic acid through a NAC thioester, the Khosla lab was able to test epothilone-TE for macrolactonization. They were able to show through HPLC analysis that the excised epothilone TE domain can catalyze the formation of the macrocycle. Cane *et al.* explored the reactivity of another thioesterase, Pik TE.²⁶ While the unmodified seco-10-deoxymethynolide substrate underwent cyclization when treated with the enzyme, the substrate with the hydroxyl at 7-position produced the hydrolysis product. (Figure 2.1.4) Small changes to the molecular structure were shown to greatly affect enzymatic activity and thus encouraged investigations into the residues and sterics of the active site.

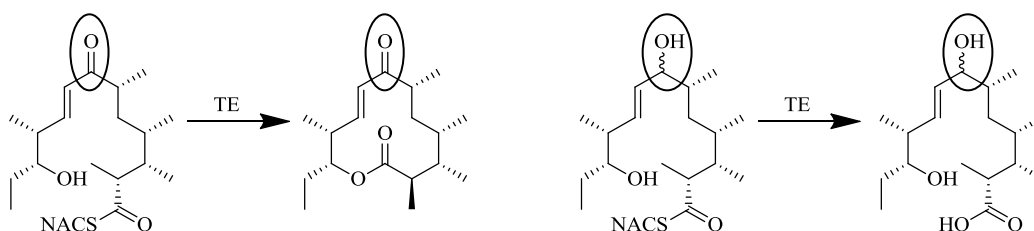


Figure 2.1.4 Oxidation state of distant oxygen affects TE-catalyzed macrocyclization

Many factors may contribute to macrolactonization. Researchers studying the closely related NRPS thioesterase domain of surfactin³² and the FAS TE domain of Orlistat³³ believe aromatic and hydrophobic residues in the active site contribute to stability and binding. Docking simulations of DEBS TE suggested that the presence of hydrogen bonds to nonreactive functional groups may influence substrate specificity.⁶ More recently, single site-directed mutants for DEBS TE were constructed that exchanged residues thought to be responsible for hydrogen bonding.⁷ Data collected from these mutants disagreed with the hydrogen bonding hypothesis and the researchers propose that hydrophobic interactions between the binding cavity and substrate likely drive substrate specificity. The hydrophobic interaction hypothesis is in agreement with data collected a few years earlier from the first structural data found for a non-hydrolysable acyl-enzyme intermediate docked into the active site of pikromycin TE.

2.1.3 Challenges of characterizing thioesterase catalysis and a mechanistic study of Pik TE

To recognize why the Pik TE work was ground breaking, it helps to understand the two-step mechanism involved in TE-mediated macrocyclization.²⁴ As seen in Figure 2.1.5 for the biosynthesis of 6-dEB, the first step involves loading the linear substrate onto the serine residue of the active site. It has been shown that this acylation is rate limiting for related TEs *in vitro*³⁴⁻³⁶ and pre-steady state burst kinetics for PKS TEs have not been detected.⁷ It follows that, once the substrate is loaded onto the thioesterase, it is rapidly off-loaded quickly either through hydrolysis or cyclization. Since the intermediate is not readily isolatable, the approach taken by the Fecik, Smith and Sherman laboratories was to form a non-hydrolysable acyl-enzyme intermediate with a molecular mimic of their polyketide, which is stable and isolatable, and characterize this material instead.

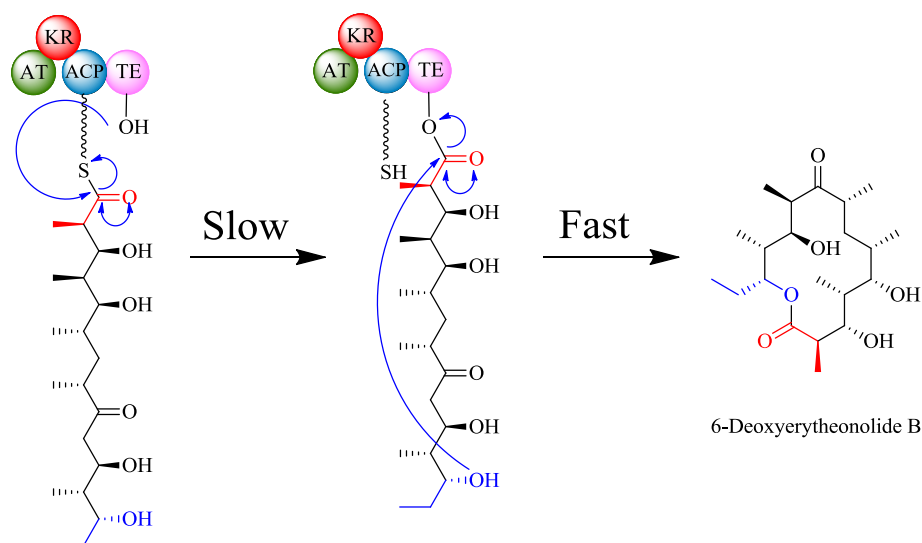


Figure 2.1.5 TE-catalyzed macrolactonization occurs in two steps and substrate loading is rate-determining

The goal was to develop a substrate-based affinity label that would have low chemical reactivity but would be able to covalently modify the active site serine⁸. Thioesterases are part of the serine hydrolase family and peptidyl α -aminoalkylphosphonate diphenyl esters had been used to inhibit the activity of the closely related serine proteases³⁷. Crystal structures of Cbz-(4-AmPh-Gly)P(OPh)₂ bound to bovine trypsin³⁸ confirmed the inhibition was due to phosphorylation of the active site serine. The Pik TE researchers' next challenge was to synthesize a molecular mimic of their polyketide with this new phosphonate moiety.

The pikromycin synthase is responsible for formation of narbonolide and 10-dexoymethynolide, the macrocyclic cores to pikromycin and methymycin respectively.⁹ With the recent work on thioesterase-catalyzed macrocyclization of 10-dexoymethynolide²⁶, they designed three triketides that mimicked C1-C6 portion of the heptaketide responsible for the formation of the closely related narbonolide. (Figure 2.1.6) Synthetically, installation of the methyl group at C2 would be difficult and as such, these reagents lack this functionality but the authors noted that they did not expect it to contribute substantially to the binding.⁹

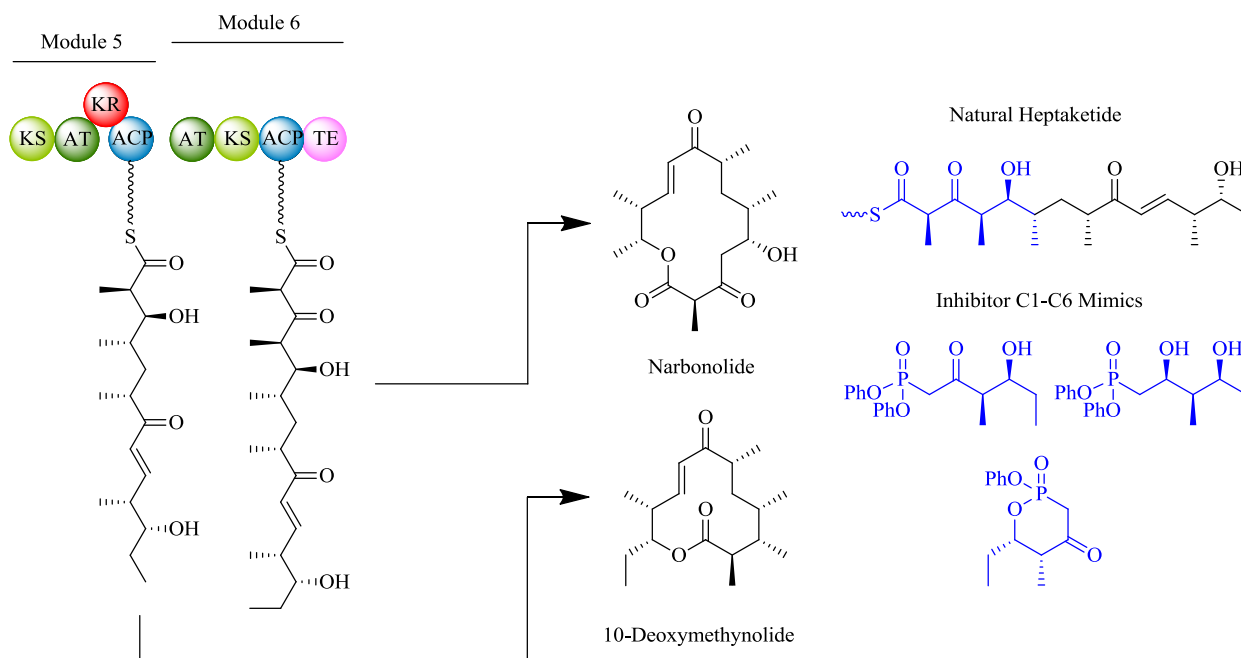


Figure 2.1.6 Pikromycin synthase can biosynthesize both narbonolide and 10-deoxymethynolide making it a good candidate for characterization

With the triketide mimics in hand, the biochemical analysis and crystallography began.⁸ After the TE was incubated with the triketides for 8 hours, the rates of enzymatic hydrolysis of p-nitrophenylpropionate showed substantial inhibition. LC-MS analysis of proteolytic digests confirmed that Pik TE was covalently modified and thus the crystals were soaked to prepare for X-ray crystallography. With the high resolution crystal structures such as those seen in Figure 2.1.7, the researchers discovered that a) this methodology works for thioesterases, b) substrate are not necessary held into the active site with H-bonding and c) the oxyanion of the tetrahedral intermediate is stabilized by only one hydrogen bond from the protein.

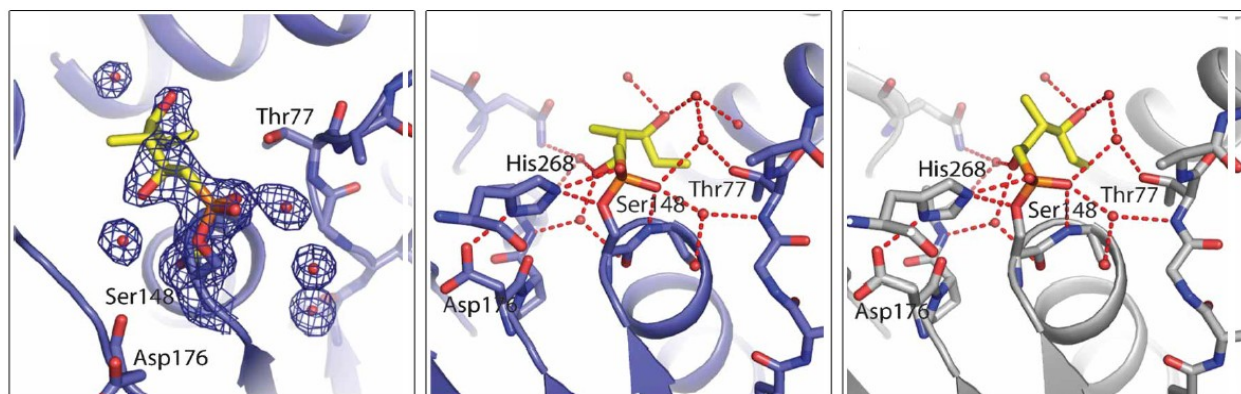


Figure 2.1.7: Crystal structures of Pik TE showing the substrates entered the active site and that the TE can be crystallized (O, red; P, orange; N, blue; water, red spheres)

Unfortunately, the triketide was not long enough to allow for the complete mechanistic analysis of Pik TE macrolactonization. The full heptaketide with the phosphonate moiety would be the ideal substrate but the synthesis of that molecule would be quite difficult. These studies were, however, the first steps into characterizing the mechanism and a good roadmap of steps involved in characterizing thioesterase activity. First, find a synthetic substrate that is known to cyclize *in vitro* with an excised thioesterase and synthesize a mimic containing the phosphonate moiety. Initial kinetic analysis will determine if inhibition occurs and LC-MS or spectroscopic methods will determine if the inhibition is due to covalent modification of the active site. Finally, the crystals will be soaked with the phosphonate mimic, the crystal structure will be solved and the mechanism elucidated.

2.1.4 Unusual stereochemical requirements of DEBS TE catalyzed macrolactonization

Work in our lab recently showed the ability of DEBS TE to hydrolyze short peptidyl thioester-containing substrates³⁹ and this work led to the synthesis of full 6-dEB analogs.⁵ (Figure 2.1.8) Unlike the native substrate, this peptidyl analogue was designed to be quickly prepared by simple chemical transformations. The design included a phenyl backbone as a chromophore to simplify the analysis of UV traces obtained by LC-MS and the native substrate's 1,3 diol to provide insight into the regioselectivity of 12- and 14-membered lactone formation.

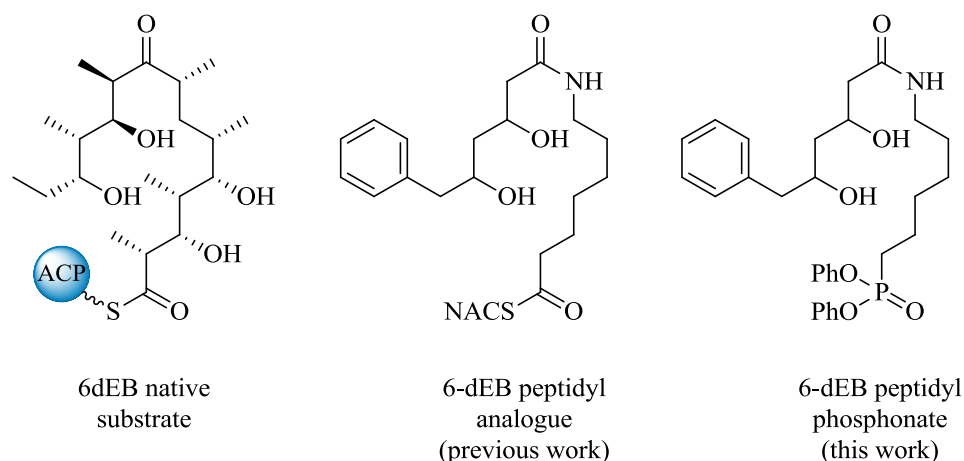


Figure 2.1.8: Rational design for synthesis of 6-dEB analogues for analysis of DEBS TE activity

Pinto *et al.* were successful in synthesizing the four enantioenriched 6-dEB analogues. Their LC-MS analysis was the first example of TE mediated cyclization of a non-native substrate.⁵ An overview of their results can be seen in Figure 2.1.9. The community had agreed that the confirmation of the alcohols plays a major role in macrocyclization;^{26,40} the stereochemistry of the alcohol on position 14 had to be R for cyclization to occur. This was not untrue in the case of this study but the surprising result was that the confirmation of the alcohol at position 12 also played a part. The 1,3 diol present in 6-dEB has the two alcohols with a relative *anti* confirmation. The substrate with the native confirmation should undergo cyclization but surprisingly, it did not. Instead, only the more sterically hindered *syn*-1,3 diol cyclised. It was concluded that understanding the interactions that control thioesterase catalyzed reaction would require high-resolution, structural data.

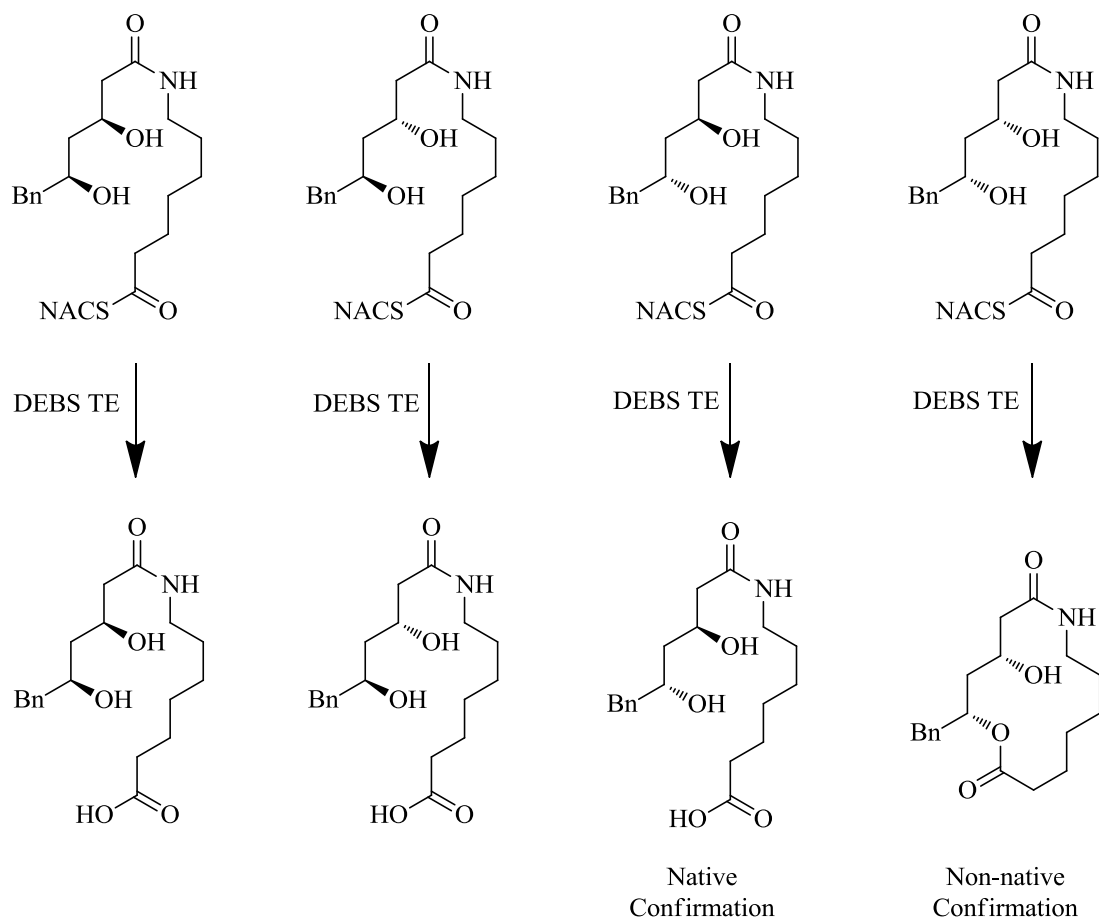


Figure 2.1.9: Results from the 6-dEB NAC-thioester study shows only one of four substrates cyclizes

This work highlights the need of a reagent for the formation of a non-hydrolysable acyl-enzyme intermediate in the active site of DEBS TE. Furthermore, this substrate will be used to elucidate a high-resolution crystal structure of the enzyme-substrate complex. Herein, we present: (1) our study on the ability of alkyl-phosphonates to form non-hydrolysable acyl-enzyme intermediates, (2) synthesis of the phosphonate 6-dEB substrates (3) results of the crystallographic analysis and (4) a brief synopsis of our future efforts towards this project.

2.2 Results and Discussion

2.2.1 Synthesis and analysis of alkyl phosphonates as targets to characterize DEBS TE

While Fecik *et al.* showed that phosphonate diphenyl esters were able to covalently modify the active site of Pik TE⁹, we wanted to do ensure that DEBS TE ad a comparable activity towards phosphonate esters embarking on the synthesis of more complex substrates.

Furthermore, unlike Fecik *et al.*'s substrate, the carbon-phosphorus bond that we required was not adjacent to a carbonyl. While the Arbuzov reaction would oxidize the trialkyl phosphate or phosphonous acid ester up to the dialkyl phosphonate⁴¹, it is a reaction that requires high temperature and we required a less harsh reaction that would be more functional group tolerant. Our first attempt was to react diphenyl phosphoryl chloride with commercially available alkyl Grignard and alkyl lithium reagents to form test substrates⁴². This methodology produced alkyl phosphonate **2.2-2.6** with low to moderate yield. (Figure 2.2.1) The α -aminohexylphosphonate diphenyl esters **2.6** was prepared by formation of an alkyl lithium⁴³ from the corresponding ω -bromo-amino alkane **2.12**.

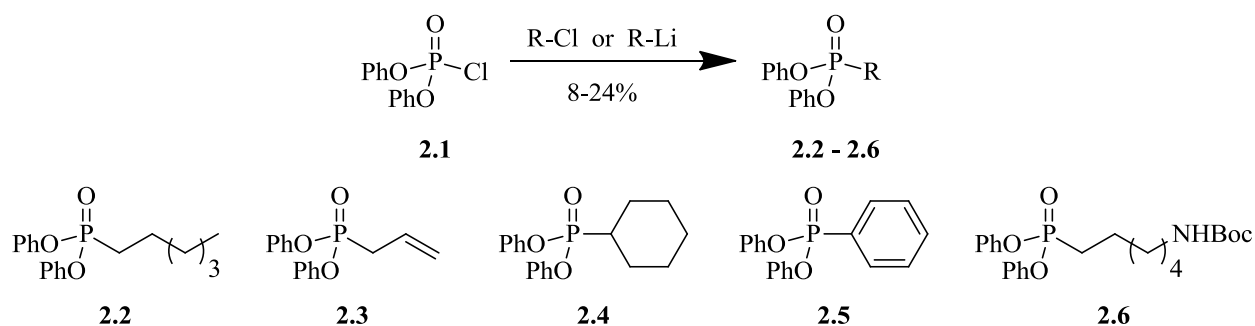


Figure 2.2.1: Synthesis of alkyl phosphonates **2.2-2.6**

Adapting the procedures for DEBS TE hydrolytic assay developed by Khosla *et al.*,²⁹ the freshly prepared DEBS TE was treated with the phosphonates and incubated for 18 hours at room temperature. As it was shown previously by the Boddy lab, DEBS TE will catalyze the hydrolysis of NAC thioesters and this reaction releases the NAC thiol.³⁹ 5-5'-dithiobis-(2-nitrobenzoic acid) or dTNB reacts with free thiols to produce nitrophenyl thiolates that can be quantified spectrophotometrically at 412 nm.⁴⁴ This reagent, known as Ellman's reagent, was included in the assay to quantify thioester hydrolysis and to evaluate the ability of our phosphonates to inhibit DEBS TE-mediated hydrolysis. An overview of the experiment can be seen in Figure 2.2.2.

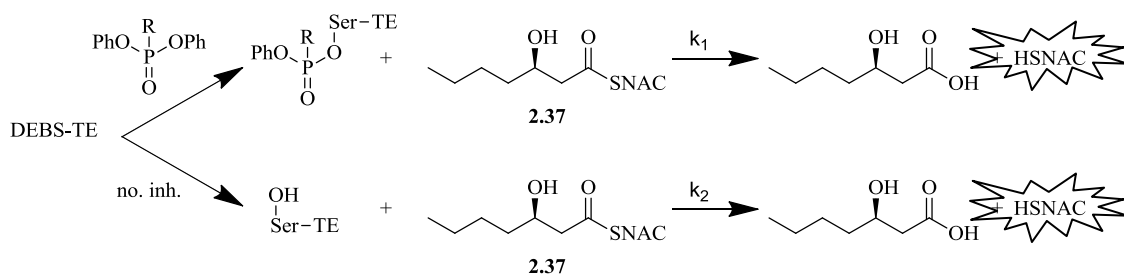
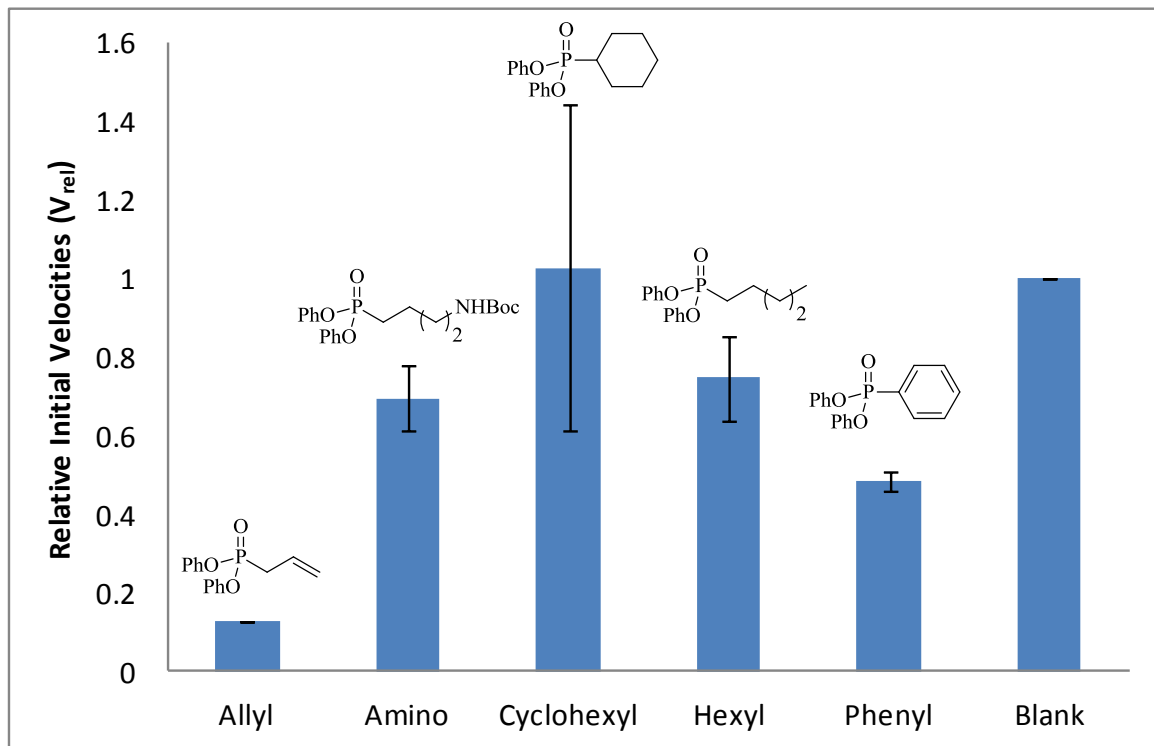


Figure 2.2.2: Kinetic assay of DEBS TE to see if phosphonates can inhibit thioesterase activity

To quantify the phosphonate inhibition of TE-catalyzed hydrolysis, we set up six simultaneous assays, one with each phosphonate and a blank. The wild type DEBS TE (243 μM) employed in our assays was expressed and purified as described.⁴⁵ Our enzymatic reactions were prepared by generating a solution containing 5 mM substrate **2.37**³⁹, 50 mM phosphate buffer, 4 % *v/v* dTNB from a saturated aqueous solution and 10 % *v/v* DMSO as a solubility additive. The incubated, inhibited DEBS TE was added to the 200- μL reaction and monitored for 60 minutes by UV-Vis. The relative initial velocities can be seen in Figure 2.2.3. Going forward, this data confirmed that inhibition can occur with the addition of phosphonate esters, especially the allyl and phenyl phosphonates. We, however, wanted to confirm that this inhibition was due to the covalent modification of the active site and not other factors involved in introducing another molecule to the assay.



Figure

2.2.3: Relative initial velocities for the inhibited and uninhibited DEBS TE hydrolysis of an SNAC substrate

Matrix-assisted laser desorption/ionization (MALDI) has been used to confirm the molecular weight of many biomolecules and organic macromolecules including some FAS TEs⁴⁶. For our purposes, we wanted to utilize this soft ionization technique to confirm that DEBS TE was covalently modified by the phosphonate inhibitor. The spectra in Figure 2.2.4 are the results from MALDI-TOF analysis. We hypothesized that from the crystal structure analysis of the phosphonate modified Pik TE, the first phenoxy group would be displaced by the serine residue and the second phenoxy group would be hydrolyzed.⁹ As such, we expected to observe an increase in molecular weight corresponding to the mono- or di-hydrolyzed version of the phosphonate if covalent modification of the enzyme was occurring. From the gene sequence, the mass of DEBS TE is approximately 33 kDa⁶ and the experimental values fall between the mass increase of a mono- and di- hydrolyzed phosphonate with a mass increase corresponding to the differing alkyl groups. While the data is somewhat ambiguous with respect to whether one or both phenoxy groups are hydrolyzed, our goal of determining if the active site was covalently modified was confirmed.

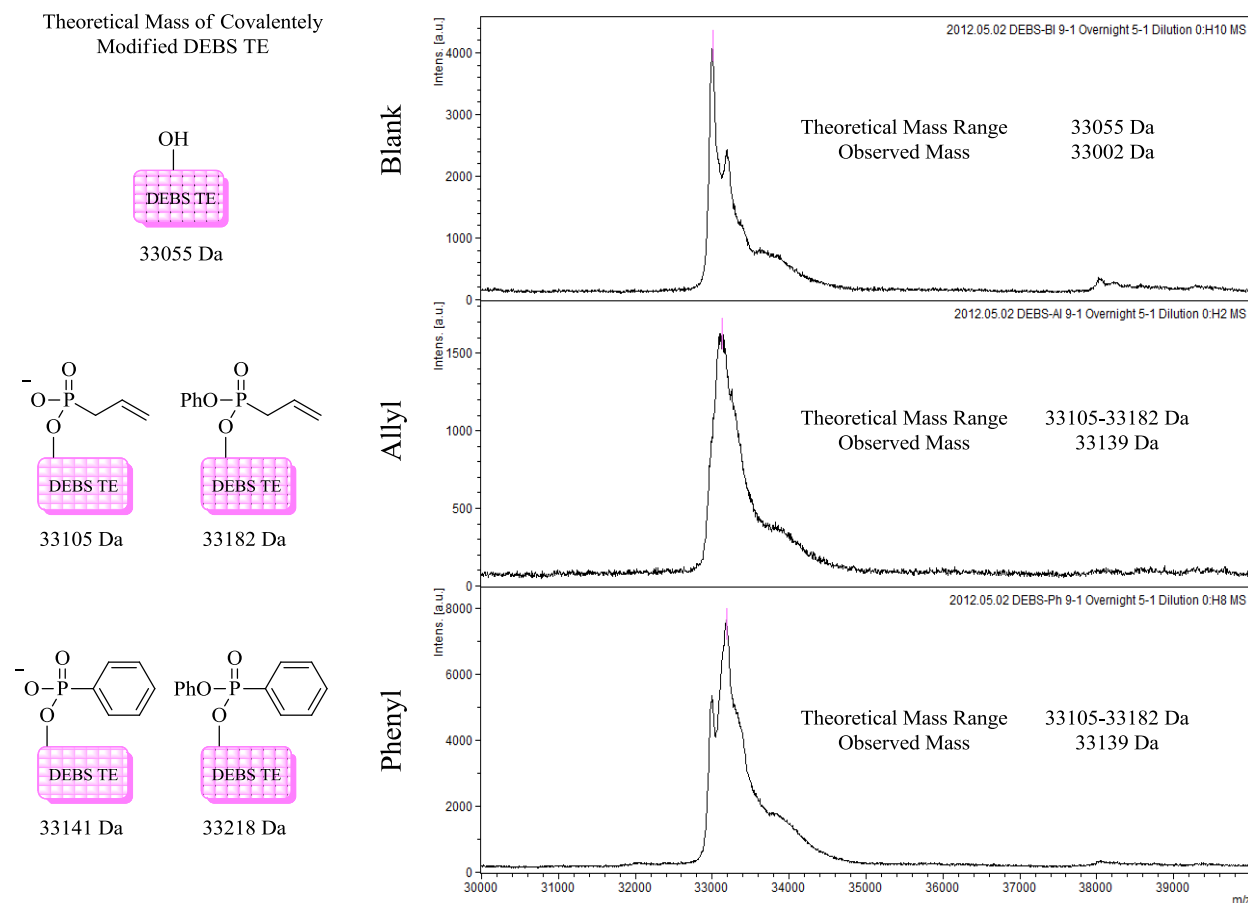


Figure 2.2.4: MALDI-TOF analysis showing possible modification of the active site of DEBS TE by an allyl phosphonate

Test substrates were synthesized and tested as viable targets for TE inhibition. Their relative velocities showed that inhibition did occur and MALDI-TOF analysis showed that there is an increase in molecular weight when the enzymes are incubated with the phosphonates. We, however, wanted to unambiguously confirm that reagents were modifying the active site serine prior embarking upon the synthesis of the full linear polyketide mimic. We therefore needed to obtain a crystal structure with a phosphonate bound to the enzyme. This work was carried out in collaboration with crystallographers Dr. Martin Schmeing and his post-doctoral fellow Dr. Fabien Bergeret at the McGill University.

The Schmeing lab cloned, expressed and purified an optimized DEBS TE sequence and was able to obtain outstanding high-resolution structural data. In the original published structure of DEBS TE⁶, the N and C termini were not resolved and were thought to decrease resolution.

By removing these extremities and cloning this newly optimized construct, their protein crystallized readily. The uninhibited DEBS-TE modified by the Schmeing lab produced crystals that diffracted to an incredible 1.8 Å and they were able to solve the structure using molecular replacement. Ultimately, the structure of the enzyme containing the allyl-phosphonate was also solved to 2.1 Å. This structure showed a distance between the phosphorus of the phosphonate and the oxygen of the active site serine to be 1.59 Å and the oxygen bound to the phosphonate was 2.85 Å from the histidine, results consistent with the work by Fecik *et al.* of the phosphonate in their active site. (Figure 2.2.5) They also did an overlay to compare their result with the crystallographic data found for Pik TE⁹. (Figure 2.2.6) These results were a proof of concept that the active site of DEBS TE can be covalently modified by a phosphonate-based molecule and allowed us to continue on to the synthesis of the phosphonate mimic of 6-dEB.

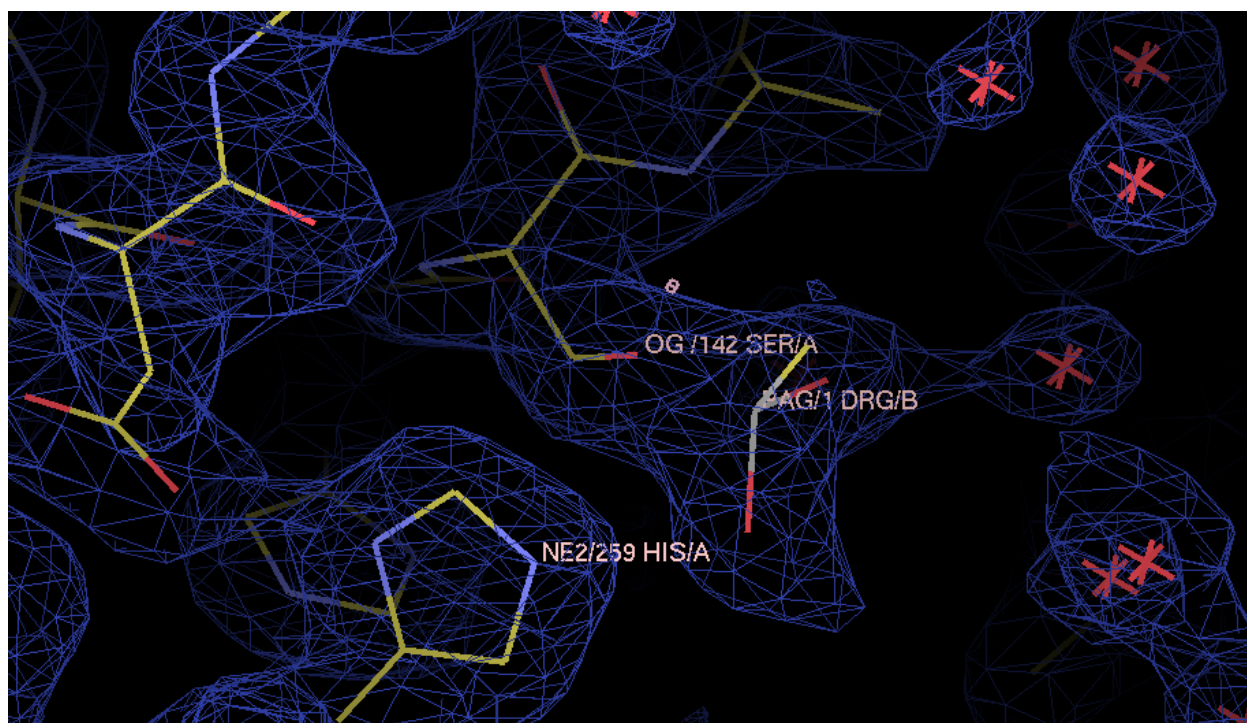


Figure 2.2.5: Crystal structure of allyl-phosphonate entering and modifying the active site DEBS TE (O, red; P, grey; N, blue; carbon, yellow)

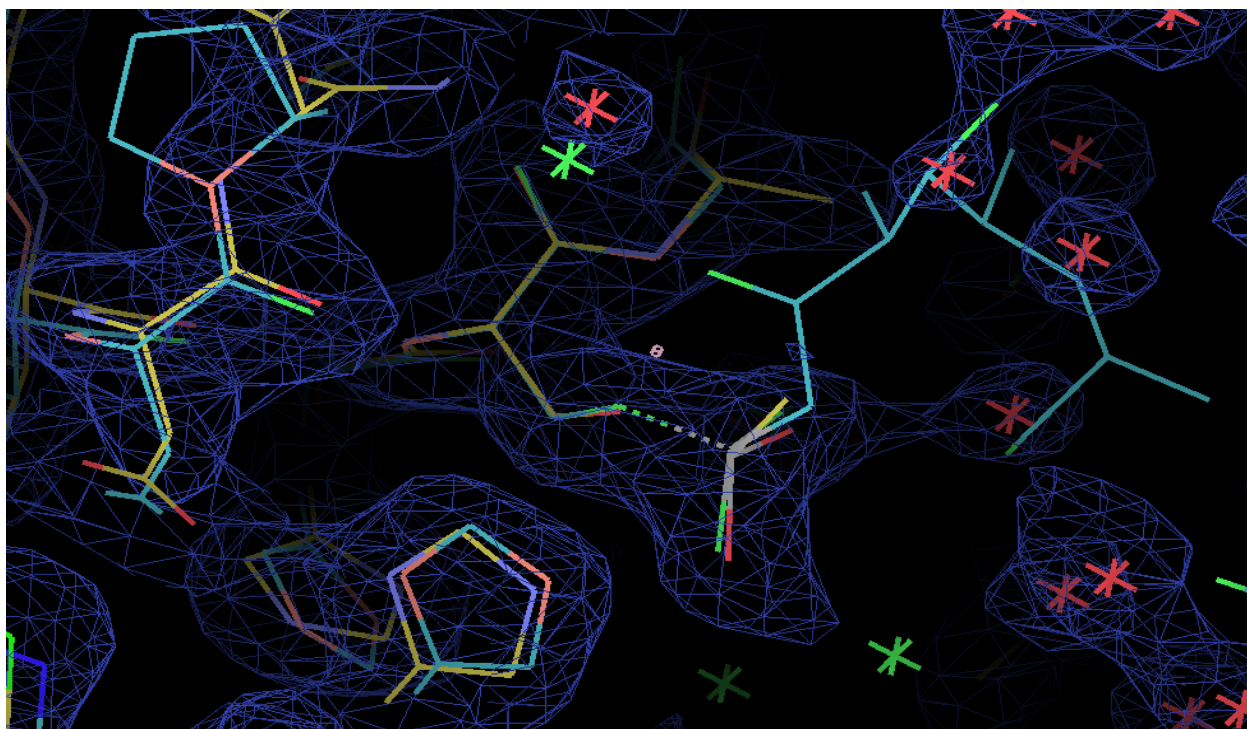


Figure 2.2.6: Overlaid crystal structures of Pik TE and DEBS TE mechanism studies (O, red; P, grey; N, blue; carbon, yellow)

2.2.2 Retrosynthesis of phosphonate 6-dEB mimics 2.35 and 2.36

The four enantioenriched substrates synthesized and characterized by Pinto *et al.* provided the first example of a non-native substrate that was cyclized by DEBS TE. This study required access to all four stereoisomers to address its hypothesis.⁵ Using this synthetic route as a starting point, we optimized the synthesis. In particular, our route differs in a few steps that proved to be low yielding and the coupling partner is different. For this study, we synthesized two diastereometrically and enantiomerically pure substrates: the native (*S,S*) configuration and the cyclizing (*R,S*) configuration.

To access stereoisomers, there are two options: synthesize each isomer selectively or perform a resolution of stereoisomers from a mixture. Having to access all four stereoisomers, Pinto *et al.* elected to do the latter. From the TBS protected alcohol **2.25**, they formed the epoxide, which underwent the Jacobsen hydrolytic kinetic resolution⁴⁸ (HKR) and opened their enantiopure epoxides through cuprate addition⁴⁹ of a vinyl Grignard. This sequence proved to be

cumbersome as the kinetic resolution also formed a triol with the undesired stereochemical configuration. (Figure 2.2.7) Having developed a route to recycle the triol, the cuprate addition also proved challenging. The yields were low and quite varied for the addition of the vinyl group and as such, we decided to address these issues by investigating an alternate route. Having mastered the Brown allylation from the first sequence of the reaction, we elected to use sequential Brown allylations to set the stereochemistry. While the Brown allylation is notorious for being moderately yielding, it is consistent and avoided unwanted side products. From **2.25**, the vinyl group would be dihydroxylated⁵⁰ and oxidized to the aldehyde and the asymmetric allylation performed to get the desired **2.27**⁵¹.

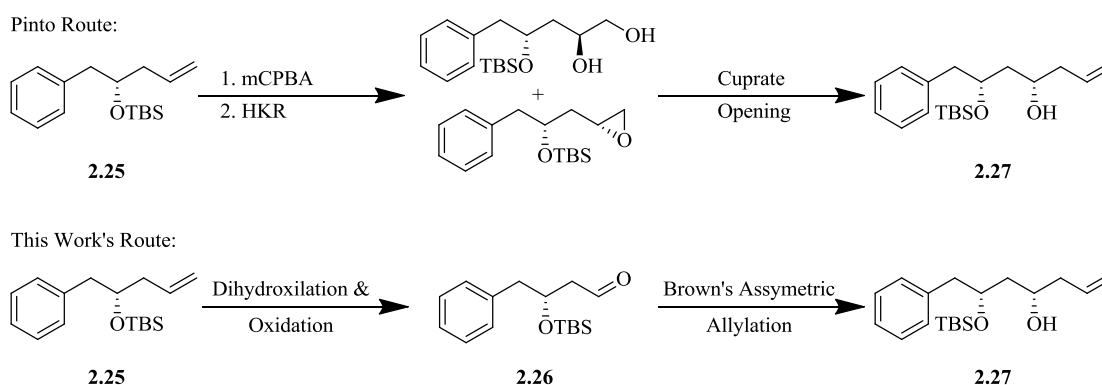


Figure 2.2.7: Diol **2.27** is accessed through asymmetric allylation instead of Jacobsen's HKR

Our syntheses also differ in the formation of the carboxylic acid. Pinto *et al.* chose to do a one pot permanganate cleavage and oxidation of the vinyl diol and couple the acid and amine portions by a carbodiimide cross-coupling reaction. (Figure 2.2.8) As was the case with the cuprate addition, the yield of the one pot cleavage-oxidation-coupling varied. Carbodiimide cross-coupling reactions are generally reliable³⁹ so we hypothesized that the oxidation was deemed to be the issue. Recovery and purification of the product from periodate-permanganate oxidation⁵² is often difficult so we decided to first form the aldehyde and use the high yielding Pinnick oxidation⁵³ to acquire the desired carboxylic acid. We also elected to use TBTU as a coupling reagent having seen the success it has in solid-phase peptide synthesis.

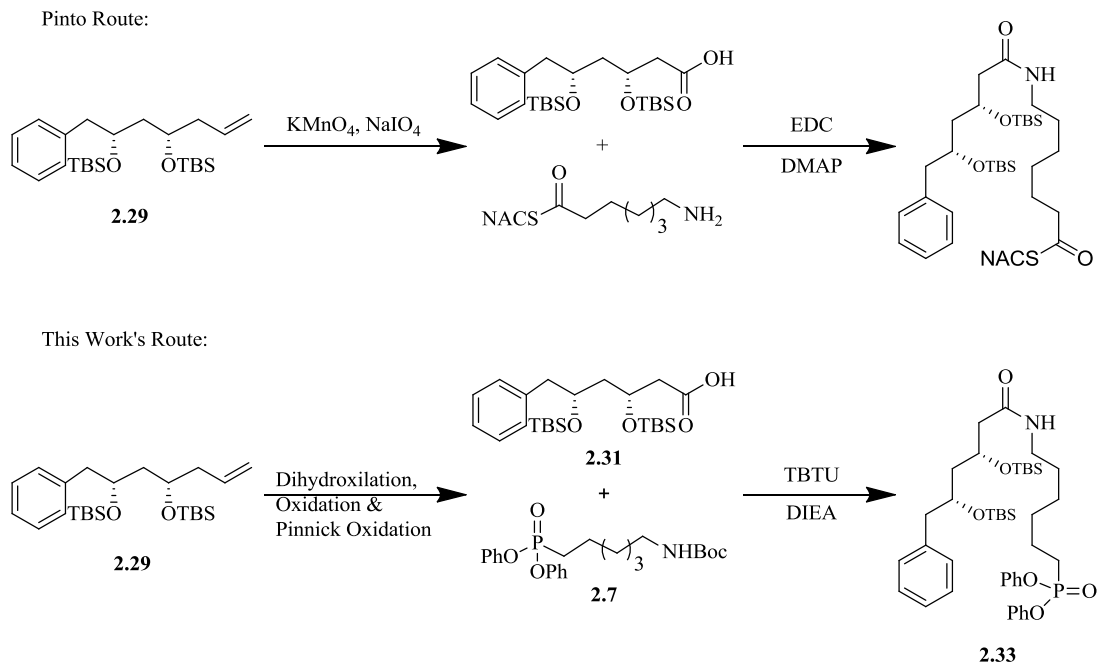


Figure 2.2.8: Different oxidation and coupling conditions pursued in hopes of improving the synthesis of **2.33**

Having identified the challenges with the Pinto enantioselective syntheses, we our second generation approach. (Figure 2.2.9) Disconnection of amide **2.35** reveals carboxylic acid **2.31** and free amine **2.18**. While methodology for the formation of C-P bonds is scarce, the mild success with Grignard additions leads us to believe that we could form the protected **2.7** from the bromo-amine **2.12** and either the diphenyl chloro-phosphonate **2.1** or the phosphate **2.17**. Carboxylic acid **2.31** could arise from oxidative cleavage of the terminal alkene of **2.29**. Formation of the bond shown in protected diol **2.29** could be formed from an asymmetric allylation of aldehyde **2.26**. Unlike the first generation approach, the two diastereomers would be synthesized separately through opposite Brown allylations of **2.26**. This aldehyde could be accessed through the oxidative cleavage of **2.25** which itself could be formed from another Brown allylation of commercially available phenylacetaldehyde **2.23**.

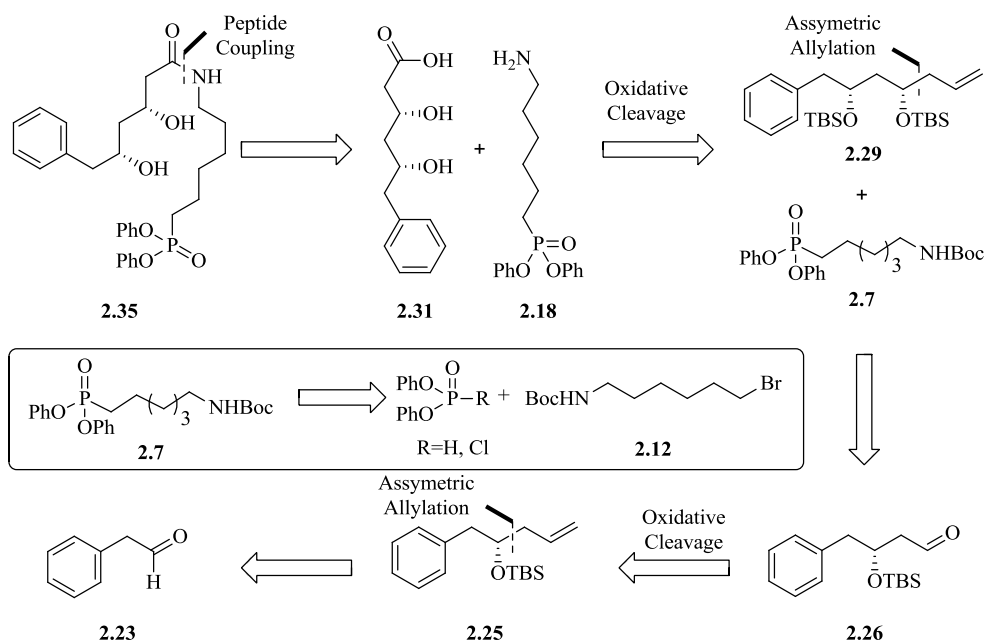


Figure 2.2.9: Retrosynthesis of the (*S,S*) 6-dEB phosphonate substrate

2.2.3 Synthesis of the amino-phosphonate 2.7

Alkyl phosphonates **2.2-2.5** were prepared from commercially available alkyl Grignard and alkyl lithium reagents and the synthesis was both low yielding and difficult to purify. The first attempt to synthesize amino-phosphonate **2.6** was by the same methodology, with the Grignard reagent formed *in situ*. Unfortunately, formation of a Grignard reagent was unsuccessful and we conceded to synthesize it by a halogen-metal exchange approach.⁴³ This procedure proved even lower yielding than the Grignard addition, led to a reaction mixture difficult to purify and had the added challenge of requiring highly reactive reagent KH. In an attempt to find a procedure that would be more general, higher yielding and safer, we embarked on direct formation of C-P bonds. (Figure 2.2.10)

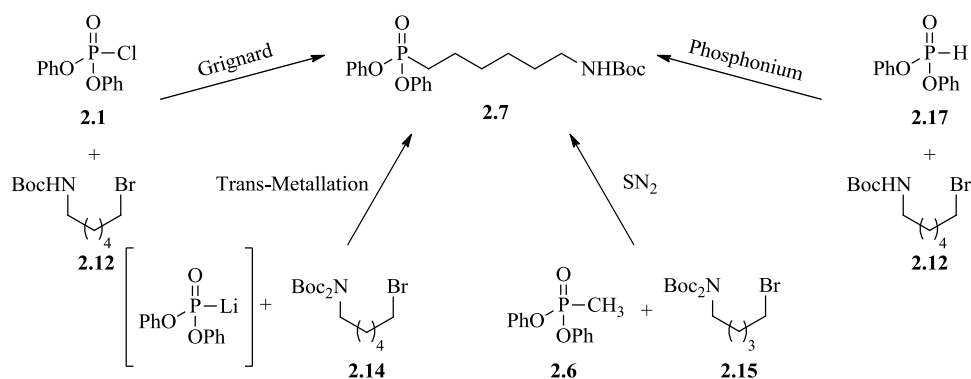
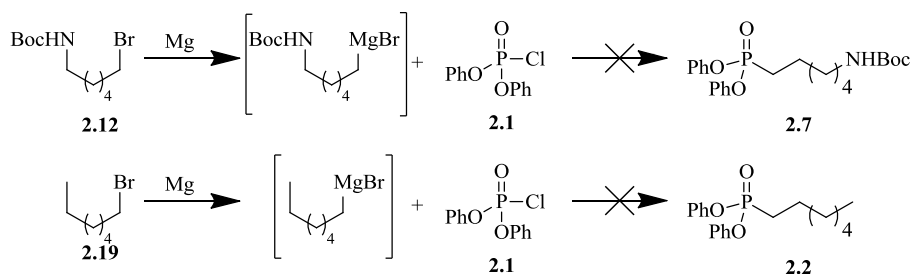


Figure 2.2.10: Various synthetic approaches to the synthesis of **2.7**

Formation of a Grignard from the corresponding alkyl bromide or chloride did not proceed. As seen in Scheme 2.2.1, the formation of the Grignard from **2.12** and slow addition of the chloro-phosphonate **2.1** under N₂ atmosphere gave no reaction. Addition of an iodine crystal did not encourage formation of the Grignard⁵⁴ reagent either, so the problem was believed to be the steric bulk of the phenoxy groups. To confirm this as an unviable option for synthesis, we tried the bromo-hexane since it had been the least hindered substituent of the previously synthesized phosphonate. This too gave no formation of **2.2**.

Scheme 2.2.1: Failed attempts of forming **2.7** through a Grignard reaction

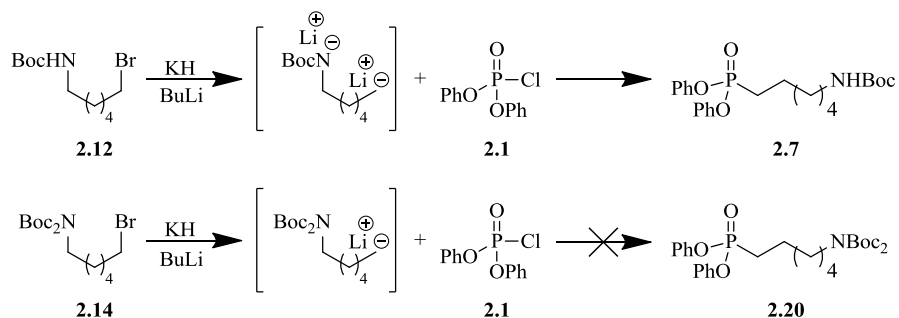


Reactions and Conditions: Mg, alkyl bromide, diphenyl phosphoryl chloride, THF, reflux, 1h

The amino-phosphonate **2.7** had been previously synthesized by transmetallation⁴³ but the yields were quite low. The next attempt was to use the methodology that worked and to improve upon it by trying to ensure that there was nothing quenching the reagent. (Scheme 2.2.2) To ensure that the bromine was the nucleophilic portion of the molecule and that neither the KH nor the butyllithium were quenched, the nitrogen was diprotected. It was hypothesized

that while mono-protection⁵⁵ does decrease the nucleophilicity, if it were di-protected⁵⁶, there would not only be very little electron density left but also steric bulk that would encourage formation of the primary carbanion. Unfortunately, this reaction also afforded no product.

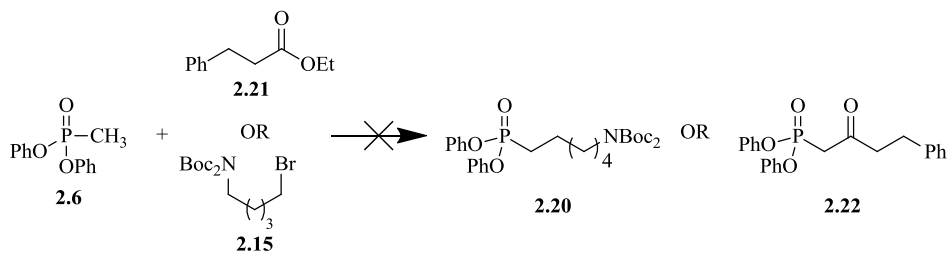
Scheme 2.2.2: Failed attempts of forming **2.7** through trans-metallation



Reactions and Conditions: KH, THF, 0 °C, 0.5h; then t-BuLi, diphenyl phosphoryl chloride, -78 °C to rt, overnight

It occurred to us that maybe that phosphonate **2.1** is a poor electrophile due to its steric bulk. While we were successful in forming alkyl phosphonates, perhaps the low yield is due to the electrophile and not the commercially available reagents. Our next attempt would reverse the roles of electrophile and nucleophile. (Scheme 2.2.3) Methylphosphonate **2.6** was easily prepared from **2.1** with MeMgBr and **2.15** was prepared by the same methodology as **2.14**; its chain being one carbon less. To ensure there were again no complications with nucleophilic nitrogen, it was di-protected. This attempt utilized the non-nucleophilic base LDA to deprotonate the phosphonate. Our hopes were dashed once again as it did not generate **2.20** and further testing with the more electrophilic ethyl 3-phenylethanoate did not produce **2.22**.

Scheme 2.2.3: Failed Attempts of Forming **2.7** through deprotonation of a methyl phosphonate



Reactions and Conditions: LDA, THF, -78 °C, 2h

Discouraged by the lack of reactivity of the electrophilic chloro-phosphonate and the nucleophilic methyl phosphonate, we investigated alternative approaches to phosphonate construction. A report in *Tetrahedron Letters* showed the synthesizing phosphonates from the corresponding phosphite. The Salvatore lab had done a methodological study⁵⁷ showing the use of metal carbonates as bases to form alkyl phosphonates. (Figure 2.2.11) From a wide range of linear and sterically hindered alkanes, their yields ranged from 47-77 % for reaction times of between 2 and 6 days. They also showed that the use of TBAI as an iodine donor increased the yields three fold. So these were the next conditions to try to form **2.7** and gratifyingly, this did produce the amino-phosphonate with an isolated yield of 16 % after 2 days.

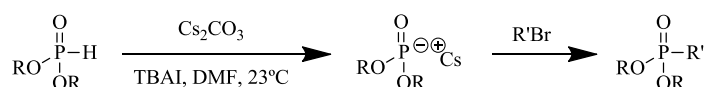
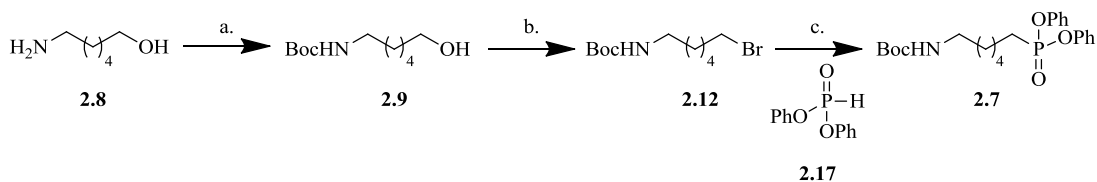


Figure 2.2.11: Salvatore *et al.* proposed mechanism for formation of alkyl phosphonates from phosphites

Having found reaction conditions that reliably produced **2.7**, our attentions were directed now towards increasing the yield and decreasing the reaction time. Deeper search into the use of various bases to deprotonate phosphites yielded a paper⁵⁸ by Montchamp *et al.*. Their study used DBU to promote alkylation of phosphonates with shorter reaction times and without the use of TBAI. Furthermore, they tested their methodology in various polar aprotic solvents at various temperatures. They found the best conditions for their reactions were using CH₃CN as a solvent and reaction temperatures of 0 °C to room temperature. Reaction of **2.8** and the phosphite beginning at 0 °C and warming to room temperature overnight produced a gratifying 28 % yield. Assuming the TBAI was used for trans-halogenation in the CsCO₃ conditions, we also synthesized the iodo-alkyl amine **2.16** and incorporated it into our new conditions but the yield increase was marginal. With a set of conditions for moderately yielding C-P bond formation, Scheme 2.2.4 below details the total synthesis of the amino-phosphonate **2.7**. Increased yields could be accomplished through the use of different bases and further decreasing the temperature as the Montchamp study only looked at temperatures above -20 °C. Furthermore, we discover, through NMR analysis, that some of the materials was being consumed by a competing O-alkylation. While this could be pursued in the future, we found that further optimization would be unnecessary for our purposes.

Scheme 2.2.4: Synthesis of amino-phosphonate **2.7**



Reactions and Conditions: (a) Boc_2O , K_2CO_3 , 1:1 Dioxane: H_2O , rt., overnight, 93 %; (b) CBr_4 , PPh_3 , DCM, 0 °C, 1h, 90 %; (c) DBU, diphenyl phosphite, MeCN, 0 °C to rt., overnight, 26 %

2.2.4 Synthesis of the carboxylic acids **2.31** and **2.32**

Having amino-phosphonate **2.7** synthesized, it was now time to concentrate on the synthesis of the diastereometrically pure carboxylic acids **2.31** and **2.32**. This second generation synthesis (seen in Figure 2.2.12) includes some improvements along with some difficulties. The first challenge of the synthesis was producing **2.25** on a large scale and with high enantiomeric excess. The preparation of (+)- $\text{Ipc}_2\text{BAllyl}$ borane *in situ* generated a reagent that produced lower selectivities, so both diastereomers were ultimately not synthesized with the same procedure. Finally, a few approaches were attempted for the final oxidative cleavage to form the carboxylic acids.

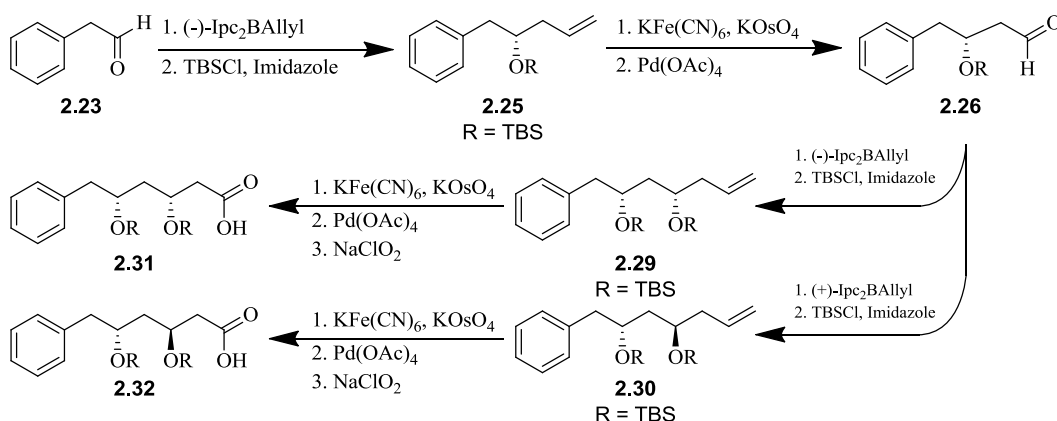
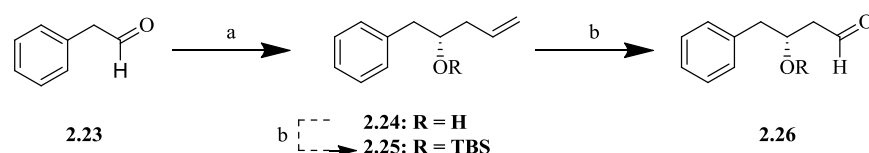


Figure 2.2.12: Overview of the synthesis of the carboxylic acids **2.31** and **2.32**

The set of reactions seen in Scheme 2.2.6 begins with the first of two asymmetric allylations using allyldiisopinocampheylborane as outlined by Brown *et al.*⁵¹ As the enantiomeric excess is heavily dependent on maintaining the low temperature⁵⁹, scale up of this reaction can be problematic. The increase in reaction temperature through addition of the

nucleophile has enough of an effect to lower *ee* substantially. Small quantities of $\text{Ipc}_2\text{B}(\text{allyl})\text{borane}$ dissolved in pentanes are commercially available but a recent paper from the Roush lab⁶⁰ outlined a large scale *in situ* preparation. They showed the use of $\text{Ipc}_2\text{B}(\text{allyl})\text{borane}$ to produce 7 times more product than the methodology used by Pinto *et al.* and maintained 80-95 % *ee*. The *in situ* preparation of (-)- $\text{Ipc}_2\text{B}(\text{allyl})\text{borane}$ was used to synthesize (*S*)-1-Phenylpent-4-en-2-ol (**2.24**) with a 3-fold increase of product formed. The yield of formation was comparable to that of the commercially available borane and the *ee* for the alcohol was 85 % *ee* (determined by chiral HPLC). TBS protection of the alcohol produced **2.25** with no trouble. Sharpless dihydroxylation⁶¹ and lead tetraacetate oxidative cleavage provided the aldehyde **2.26** with a 54 % yield over the two steps.

Scheme 2.2.5: Synthesis of aldehyde **2.26**



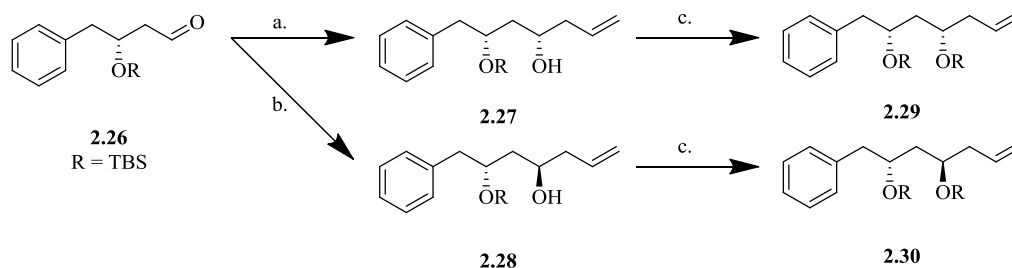
Reactions and Conditions: (a) (-)- Ipc_2BOMe , allylMgBr , Et_2O , 0 °C, 1h; then phenylacetaldehyde, Et_2O , -100 °C, 2h, 60 %; (b) imidazole, TBSCl, DMF, rt., overnight, 92 %; (c) K_2CO_3 , $\text{K}_3\text{Fe}(\text{CN})_6$, $\text{K}_2\text{OsO}_7 \cdot (\text{H}_2\text{O})_2$, 1:1 t-BuOH:H₂O, rt, overnight; then $\text{Pb}(\text{OAc})_4$, DCM, 0.5h, 54 %

The aldehyde was then split into two reactions to produce the two diastereomers through a second asymmetric allylation. (Scheme 2.2.6) While treatment of **2.23** with *in situ* generated (-)- $\text{Ipc}_2\text{B}(\text{allyl})\text{borane}$ gave **2.24** in high *ee*, treatment of **2.23** with *in situ* (+)- $\text{Ipc}_2\text{B}(\text{allyl})\text{borane}$ did not generate sufficient enantiopurity. Chiral HPLC analysis showed that the *ee* for the enantiomer of **2.24** was at best 40 %. Having observed that the *in situ* conditions were not favorable for both enantiomers and having seen that the commercially available (+)- $\text{Ipc}_2\text{B}(\text{allyl})\text{borane}$ did produce high enantiopurity, **2.27** was synthesized with the *in situ* conditions and **2.28** with the commercially available (+)- $\text{Ipc}_2\text{B}(\text{allyl})\text{borane}$.

While isopinocampheylboranes are great chiral auxiliaries for stereospecific reactions, they produce isopinocampheol, a by-product that is very hard to separate from the mixture. To address this problem, we attempted to TBS protect crude alcohol **2.24** and purify after the protection. We tested this theory on less progressed alcohol **2.24** and the reaction proceeded

with negligible yield of **2.25**. It was presumed that the presence of isopinocampheol and the steric hindrance of putting a second TBS group decrease the overall yield of **2.29** and **2.30**.

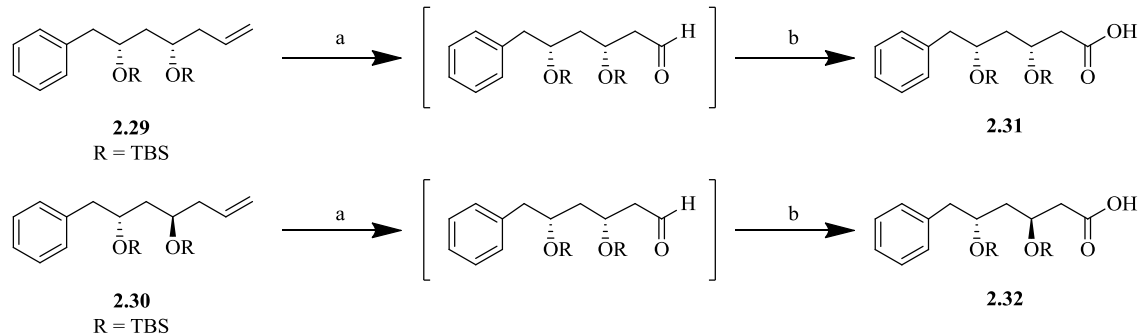
Scheme 2.2.6: Synthesis of the protected diol **2.29** and **2.30**



Reactions and Conditions: (a) (-)-Ipc₂BOME, allylMgBr, Et₂O, 0 °C, 1h; then **2.16**, Et₂O, -100 °C, 2h, 62 %; (b) (+)-Ipc₂B(allyl)borane, Et₂O, -100 °C, 2h, 35 %; (c) imidazole, TBSCl, DMF, rt., overnight, 65 % (**2.29**), 51 % (**2.30**)

While there are many options for oxidative cleavage of a double bond, many of the common ones are either low yielding (KMnO₄), require special equipment (ozonolysis) or are expensive and toxic (OsO₄). Since it was deep into the synthesis, we wanted to ensure we could find a set of conditions that was dependable and protecting group tolerant. Dr. von Rudloff and the famous Canadian chemist Dr. Lemieux discovered a set of conditions⁵² utilizing varying concentrations of NaIO₄ to cleave an alkene into two carboxylic acids. While it is not a common method for cleavage likely due to the use of CCl₄, we wanted explore it as a possible route. Using **3.15** as a test substrate, the alkene was successfully oxidized however the terminal TBS group was lost under the reaction conditions. As we didn't want to have to reprotect the alcohols before the peptide coupling reaction, we opted to synthesize **2.31** and **2.32** by a three step oxidation. (Scheme 2.2.8) The dependable Pinnick oxidation⁶² was employed after the same dihydroxylation/oxidative cleavage procedures previously used to generate the two acids in a comparable yield for this reaction.

Scheme 2.2.7: Oxidation of protected diols **2.21** and **2.22**



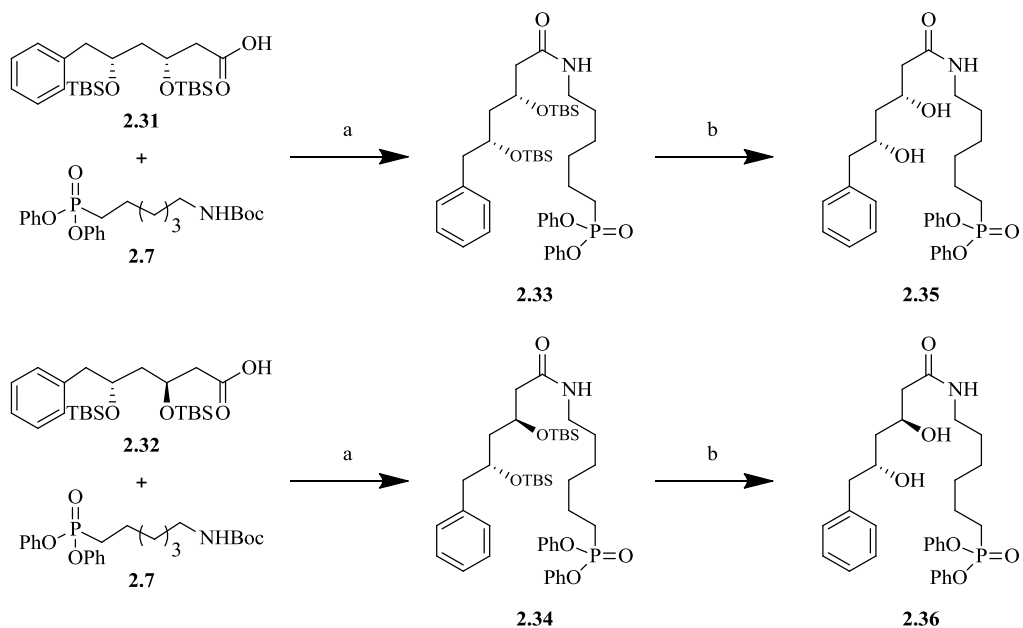
Reactions and Conditions: (a) K_2CO_3 , $\text{K}_3\text{Fe}(\text{CN})_6$, $\text{K}_2\text{OsO}_7 \cdot (\text{H}_2\text{O})_2$, 1:1 t-BuOH:H₂O, rt, 12h; then $\text{Pb}(\text{OAc})_4$, DCM, 0.5h; (b) NaClO_2 , NaH_2PO_4 , H₂O, 2-methyl-2-butene, t-BuOH, rt., overnight, 63 % (**2.31**), 44 % (**2.32**)

2.2.5 Completing the synthesis of 6-dEB phosphonate substrates **2.35** and **2.36**

The final steps of the synthesis were a peptide coupling and deprotection. (Scheme 2.2.9) While there are many options⁶³ for amide bond formation, our lab began experimenting with the use of TBTU as a peptide coupling reagent. It proved to be a clean, effective reaction proceeding with a moderate yield. After performing the deprotection conditions⁵ used by Pinto *et al.*, we were pleasantly surprised to see the phosphonate had survived the acidic conditions and gave us our final product. The next step of the study was to send the substrates to the crystallographers and see if we could form the non-hydrolysable acyl-enzyme intermediate.

We also had an interesting result when using different NMR solvents. Deuterated chloroform was used for virtually every NMR analysis and we used it initially as our solvent to analyze purified **2.36**. The spectrum of **2.36** dissolved in CDCl_3 contained the two expected protons adjacent to the freshly deprotected alcohols but there were also many unexpected peaks in alkyl region. At first, we assumed these alkyl peaks were impurities but before embarking on a second purification, we elected to try another deuterated solvents. We were very pleased when deuterated methanol provided a spectrum with a different story. (Both spectra can be seen in Figure 2.2.13) Unlike the CDCl_3 spectrum, the CD_3OD spectrum had integrations that matched with the amount of hydrogen atoms present and better resolved the aromatic region of the molecule. Unfortunately, this was at the cost of peak resolution. Overall, this reinforced the notion that solvation of the analyte can have a drastic effect on the number and type of conformers present in your sample dramatically affecting the spectrum.

Scheme 2.2.8: Coupling and deprotection to completing the synthesis of amides **2.25** and **2.26**



Reactions and Conditions: (a) **2.5**, HCl•Dioxane, rt., 1h; then **2.21** or **2.22**, TBTU, DIEA, DMF, rt., overnight, 43 % (**2.33**), 45 % (**2.34**) (b) HF, pyridine, MeCN, rt., 5h, 49 % (**2.35**), 33 % (**2.36**)

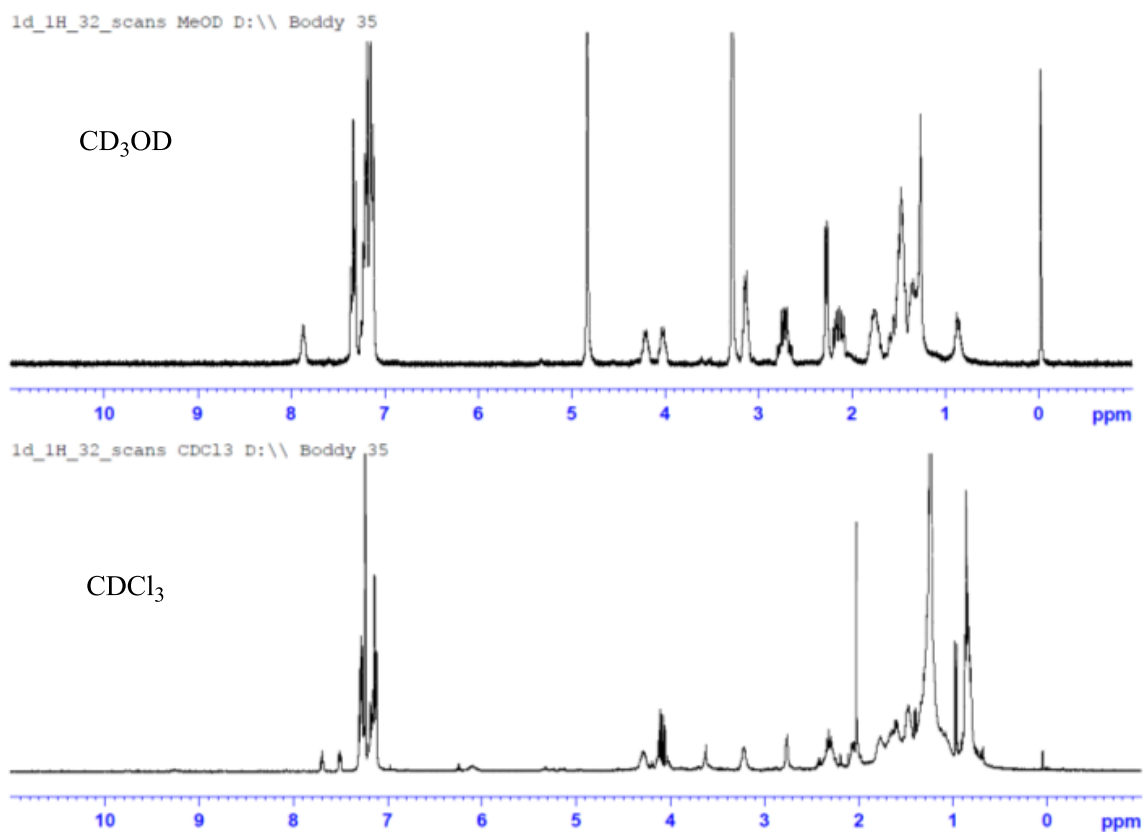


Figure 2.2.13 Different NMR solvents effects resolution in two spectra of **2.36**

2.2.6 X-ray crystallography results of 2.35 and 2.36 in the active site of DEBS TE

The freshly synthesized phosphonate substrates were sent to the Schmeing lab to once again elucidate a crystal structure. The first attempt at soaking the crystals was successful but the electron density maps showed that it was not the phosphonate but rather a molecule of polyethylene glycol (PEG) used as a crystallization agent that occupied the active site. With this result, they approached this problem anew knowing now that the substrate was in competition with PEG. By decreasing the concentration of inhibitor and increasing the incubation time to 8-10 days, they were able to increase the formation of the acyl-enzyme intermediate to almost 100%. We were delighted when the Schmeing lab was able to collect two electron density maps from the two substrates. The calculated crystal structures with 2.1 Å resolution can be seen in Figures 2.2.13 and 2.2.14. The electron densities show that full modification of DEBS TE occurs with both substrates but there is not enough continuous density to build in the substrate. The Schmeing lab work is ongoing as they continue to try different techniques to acquire enough electron density to confirm that the entire substrate is in the active site.

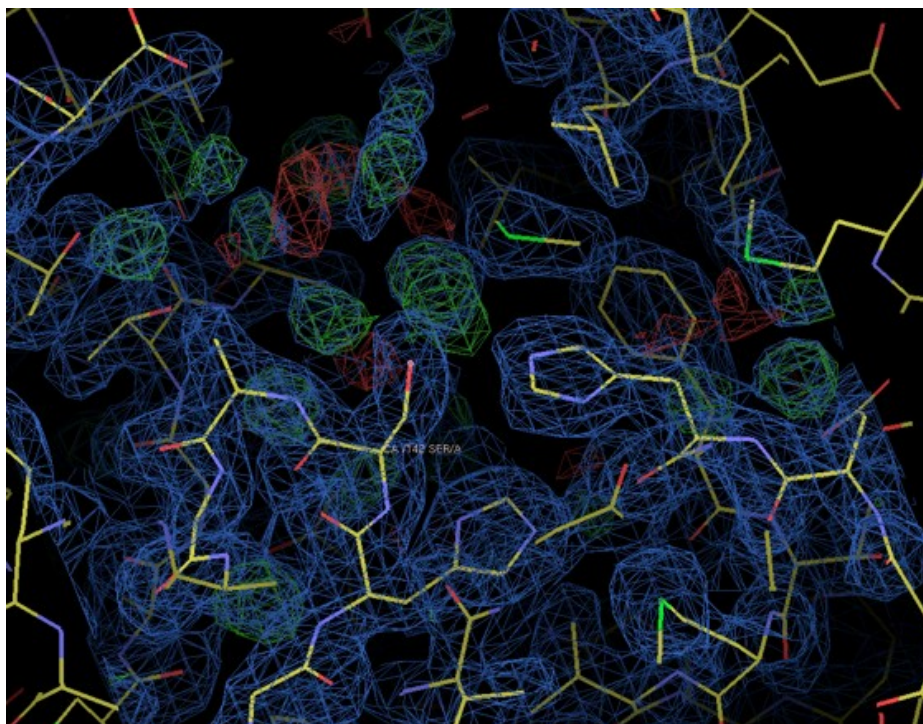


Figure 2.2.13 Electron density map of a crystallized DEBS TE with phosphonate **2.35** in the active site (O, red; P, grey; N, blue; carbon, yellow)

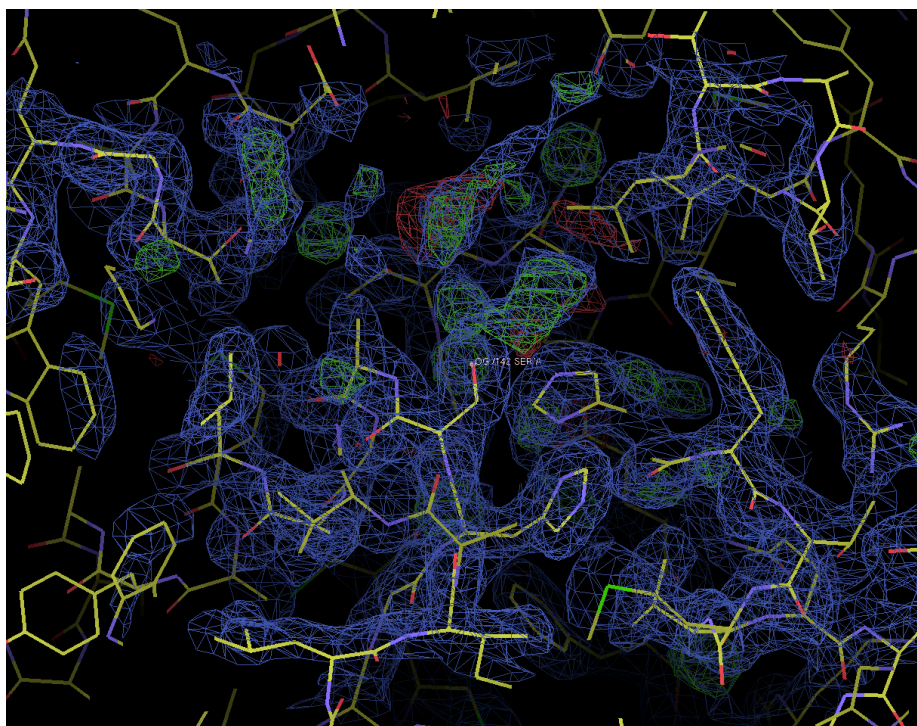


Figure 2.2.14 Electron density map of a crystallized DEBS TE with phosphonate **2.36** in the active site (O, red; P, grey; N, blue; carbon, yellow)

2.3 Conclusion and future work

There have been many studies pertaining to the catalytic activity of the 6-dEB synthase. Precursor-directed biosynthesis¹⁸ and directed mutagenesis⁴ have given insight into the products that can be produced by this PKS but there had not yet been a study on how this PKS cyclised non-native substrates until Pinto *et al.* synthesized their model system⁵. From this model, they discovered substrates that would cyclize *in vitro* with an excised DEBS TE but their result did not follow the accepted hypothesis for stereoselective macrolactonization. To understand the mechanism involved in the cyclization of DEBS TE, they would have to synthesize a substrate, similar to the work⁹ done by Fecik *et al.*, to form a non-hydrolysable acyl-enzyme intermediate that could be crystallized in the active site. The work above is the synthesis of phosphonate substrates that combines the structure of Pinto *et al.* with the phosphonate portion of the molecule responsible for forming the intermediate.

Before embarking on a total synthesis, we wanted to first confirm that a phosphonate-based substrate could modify the active site of DEBS TE. Through the use of some simple alkyl

phosphonates, we were able to show that enzyme activity decreases with the addition of these substrates and that the inhibition was due to covalent modification of the active site through MALDI-TOF and x-ray crystal analysis. The synthesis of the phosphonate substrates was a second generation approach to the synthetic route put forward by Pinto et al differing in approach to the carboxylic acid portion of the molecule and with a different amine nucleophile. Whilst discovering methods to form C-P bonds directly and consistent conditions for asymmetric allylation, two diastereometrically pure phosphonate substrates were sent to our crystallographer collaborators. The crystal structures confirmed that full modification of the active site occurs but work is still ongoing to confirm the position of the substituents in the substrate.

Future work depends on the results from the crystallographers. If they are unable to form crystals to fully characterize DEBS TE, we could synthesize phosphonates that are more easily hydrolyzable⁶⁴ or synthesizing a triketide phosphonate similar to the study⁸ done by Fecik *et al.*. Another interesting study, while difficult to synthesize, would be to form a cyclic phosphonate and see if that would enter the active site. Also, while Fecik *et al.* noted that the methyl group adjacent to the phosphonate was negligible, synthesizing that mimic would confirm whether these methyl groups assist in holding the alkyl chain in the active site. Hopefully, this approach can be used for analysis of thioesterases in the future and a general understanding of the stereochemical requirements for molecular recognition can be discovered so that synthetic chemists have stereospecific chemoenzymatic methodology to form macrolactones.

2.4 References

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2.5 Experimental Section

2.5.1 General Methods

Reactions were carried out under argon or nitrogen atmosphere with dry solvents and dried glassware under anhydrous conditions unless specified otherwise. All reactions in dry THF, Et₂O and DCM as solvent were dried through activated alumina columns. Reagents were purchased at the highest commercial quality and without further purification. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as a visualization and/or ceric ammonium molybdate (CAM), p-anisaldehyde (PA) and potassium permanganate (KMnO₄) as staining solutions. Flash chromatography was performed with E. Merck silica gel (60, particle size 0.040-0.063 mm) and preparative thin-layer chromatography (PTLC) separations were carried out on 0.25 mm E. Merck silica gel plates (60F-254).

¹H NMR, ¹³C NMR, ³¹P NMR spectra were recorded on the Bruker DPX-300 or AMX-300, AMX-300II and AMX-400 spectrometers and calibrated using residual undeuterated solvents as internal references. Data is reported as follows: chemical shifts in ppm (δ); multiplicities are indicated by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, m = multiplet, br = broad; coupling constants in Hz (J). Infrared spectra (IR) were collected on a Shimadzu FTIR-8400S in solution and reported in wavenumbers (cm⁻¹). HRMS were obtained on a Kratos Analytical Concept instrument (University of Ottawa Mass Spectrum Center).

LCMS data for enantiopurity were collected with an Agilent 1100 series HPLC with a DAD detector and Chiralpak IB column. The conditions for the separation were 98:2 Hexane:isopropyl alcohol at a rate of 0.8 mL/min and detection at 220 nm by Roxanne Clément in the CCRI at the University of Ottawa. MALDI-TOF traces were collected with a Bruker microflex LT with sinapinic acid sandwich. UV-Vis traces done on a Thermo Scientific Evolution 300 UV-Vis.

Crystal structures were collected with a Rigaku MicroMax-007HF X-Ray generator, Rigaku SATURN 994 camera and a Rigaku VariMax-HF monochromator by Dr. Fabien Bergeret at McGill University. Initial phosphonates **2.2-2.7** were soaked with the TE overnight

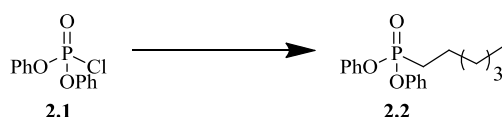
with the phosphonates then collected crystals were analyzed. Full phosphonate substrates **2.35** and **2.36** were soaked with the TE for 8-10 days then collected crystals were analyzed.

2.5.2 Experimental Procedures

General procedure for formation of alkyl phosphonates 2.2-2.6

To diphenyl phosphoryl chloride **2.1** (1 equiv.) dissolved in ether (0.2 M) was added an alkyl Grignard (2.5 equiv.) at -78 °C. The reaction was stirred for 3 hours and allowed to warm to room temperature. The reaction mixture was quenched with saturated ammonium chloride (15 mL) and diluted with water. The aqueous layer was then extracted with EtOAc (3 x 10 mL) and the resulting organic layer washed with brine (2 x 10 mL), dried with MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography.

Diphenyl hexylphosphonate (**2.2**)



The procedure outlined above was employed in the conversion of **2.1** (0.5 mL, 2.41 mmol, 1 equiv.) with hexylmagnesium bromide (3.0 mL, 6.03 mmol, 2.5 equiv) yielding 0.143 g (0.46 mmol, 19 %) of diphenyl hexylphosphonate **2.2**.

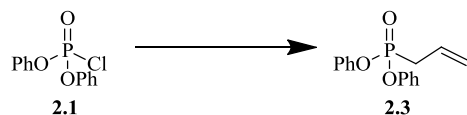
R_f: 0.35, 15 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.33-7.27 (m, 4H), 7.16-7.12 (m, 6H), 2.11-1.98 (m, 2H), 1.83-1.72 (m, 2H), 1.50-1.18 (m, 9H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 150.2, 130.0, 124.8, 121.1, 36.0, 29.2, 26.8, 26.0, 25.8, 25.7, 25.6 ppm.

NMR Spectra are consistent with those previously reported.¹

Diphenyl allylphosphonate (2.3)



The procedure outlined above was employed in the conversion of **2.1** (0.5 mL, 2.41 mmol, 1 equiv.) with allylmagnesium bromide (6.0 mL, 6.03 mmol, 2.5 equiv) yielding 0.106 g (0.38 mmol, 16 %) of diphenyl allylphosphonate **2.3**.

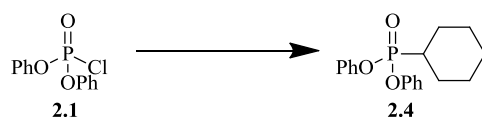
R_f: 0.24, 20 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.32-7.28 (m, 4H), 7.16-7.12 (m, 6H), 6.02-5.94 (m, 1H), 5.34-5.42-5.33 (m, 2H), 3.01 (dt, *J* = 7.4, 1.4 Hz) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 129.8, 126.3, 125.2, 125.2, 121.4, 120.6, 32.4, 31.0 ppm.

NMR Spectra are consistent with those previously reported.²

Diphenyl cyclohexylphosphonate (2.4)



The procedure outlined above was employed in the conversion of **2.1** (0.5 mL, 2.41 mmol, 1 equiv.) with cyclohexylmagnesium bromide (3.0 mL, 6.03 mmol, 2.5 equiv) yielding 0.136 g (0.42 mmol, 15 %) of diphenyl cyclohexylphosphonate **2.4**.

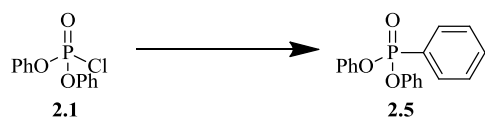
R_f: 0.36, 15 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.31-7.26 (m, 4H), 7.16-7.12 (m, 6H), 2.21-2.09 (m, 1H), 1.92-1.84 (m, 4H), 1.50-1.32 (m, 6H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 150.4, 129.6, 124.7, 120.6, 36.0, 29.2, 25.8, 25.7, 25.6 ppm.

NMR Spectra are consistent with those previously reported.³

Diphenyl phenylphosphonate (2.5)



The procedure outlined above was employed in the conversion of **2.1** (0.5 mL, 2.41 mmol, 1 equiv.) with phenyl lithium (2.4 mL, 6.03 mmol, 2.5 equiv) yielding 0.105 g (0.34 mmol, 14 %) of diphenyl phenylphosphonate **2.5**.

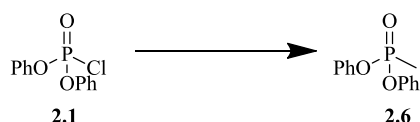
R_f: 0.25, 20 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.32-7.27 (m, 4H), 7.16-7.12 (m, 6H), ppm.

¹³CNMR (100 MHz, CDCl₃): δ 150.4, 150.4, 133.2, 132.3, 132.2, 129.7, 128.7, 128.6, 125.1, 120.7, 120.6 ppm.

NMR Spectra are consistent with those previously reported.⁴

Diphenyl methylphosphonate (2.6)



The procedure outlined above was employed in the conversion of **2.1** (0.5 mL, 2.41 mmol, 1 equiv.) with hexylmagnesium bromide (3.0 mL, 6.03 mmol, 2.5 equiv) yielding 0.191 g (0.77 mmol, 32 %) of diphenyl methylphosphonate **2.6**.

R_f: 0.35, 15 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.32-7.27 (m, 4H), 7.16-7.12 (m, 6H), 1.79 (d, *J* = 18.2Hz) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 150.4, 130.1, 124.9, 121.0, 36.2, 35.8 ppm.

NMR Spectra are consistent with those previously reported.¹

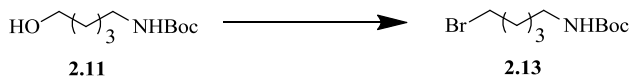
R_f: 0.57, 20 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 4.48 (br s), 3.66 (t, *J* = 6.8Hz, 2H), 3.09 (t, *J* = 6.5Hz, 2H), 1.88-1.82 (m, 2H), 1.47 (s, 9H), 1.54-1.18 (m, 6H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 156.1, 62.9, 51.1, 32.8, 28.5, 26.4, 25.4 ppm.

NMR Spectra are consistent with those previously reported.⁶

5-(tert-butoxycarbonyl-amino) 1-bromo pentane (2.13)



The procedure outlined for the production of **2.12** was employed here with **2.11** (1.136 g, 5.53 mmol, 1 equiv.), CBr₄ (1.83 g, 5.53 mmol, 1 equiv.) and PPh₃ (1.450 g, 5.53 mmol, 1 equiv.). The reaction yield was 1.207 g (4.53 mmol, 82 %)

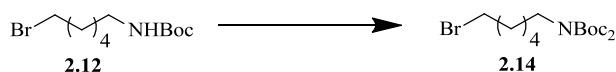
R_f: 0.58, 20 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 4.52 (s, 1H), 3.64 (t, *J* = 6.8Hz, 2H), 3.12 (t, *J* = 6.5Hz, 2H), 1.89-1.84 (m, 2H), 1.47 (s, 9H), 1.54-1.18 (m, 4H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 156.0, 62.7, 50.9, 32.6, 26.4, 25.3 ppm.

NMR Spectra are consistent with those previously reported.⁶

6-(di-tert-butoxycarbonyl-amino) 1-bromo hexane (2.14)



To **2.12** (0.430 g, 1.54 mmol, 1 equiv.) in dry THF (3 mL) was added DMAP (0.094 g, 0.77 mmol, 0.5 equiv.) and allowed to stir for 15 minutes at room temperature. Di-tert-butyl – dicarbonate (0.7 mL, 3.08 mmol, 2 equiv.) was added to the reaction and allowed to stir overnight. The mixture was quenched with H₂O (3 mL), extracted with EtOAc (3 x 5 mL) and washed with 10 % HCl (2 x 5 mL) and brine (2 x 5 mL). The resulting organic layer was dried over MgSO₄, concentrated *in vacuo* and purified by column chromatography to yield 0.440 g of **2.14** (1.20 mmol, 78 %).

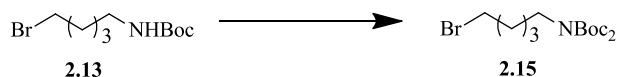
R_f: 0.52, 60 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 3.55 (t, *J* = 7.1Hz, 2H), 3.38 (t, *J* = 6.8Hz, 2H), 1.86-1.82 (m, 2H), 1.46 (s, 9H), 1.54-1.18 (m, 6H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 154.2, 44.8, 33.4, 30.2, 29.9, 28.4, 25.7 ppm.

NMR Spectra are consistent with those previously reported.⁷

5-(di-tert-butoxycarbonyl-amino) 1-bromo pentane (2.15)



The procedure outlined for the production of **2.14** was employed here with **2.13** (0.770 g, 2.90 mmol, 1 equiv.), DMAP (0.177 g, 1.45 mmol, 0.5 equiv.), Di-tert-butyl-dicarbonate (1.4 mL, 5.80 mmol, 2 equiv.). The reaction yield was 0.714 g (2.03 mmol, 70 %)

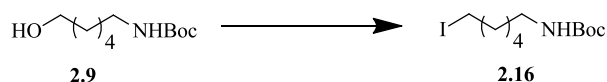
R_f: 0.48, 60 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 3.50 (t, *J* = 7.1Hz, 2H), 3.40 (t, *J* = 6.8Hz, 2H), 1.88-1.81 (m, 2H), 1.46 (s, 9H), 1.52-1.20 (m, 4H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 154.0, 46.8, 34.3, 30.1, 30.0, 28.6, 26.7 ppm.

NMR Spectra are consistent with those previously reported.⁷

6-(tert-butoxycarbonyl-amino) 1-iodo hexane (2.16)



To **2.9** (0.400 g, 2.76 mmol, 1 equiv.) in dry dichloromethane (10 mL) was added imidazole (0.398 g, 5.85 mmol, 3 equiv.) and PPh₃ (0.766 g, 2.92 mmol, 1.5 equiv.) at room temperature. After complete dissolution, the reaction was then put to 0 °C, protected from light and iodine (0.741 g, 2.92 mmol, 1.5 equiv.) was added. The resulting mixture was allowed to warm to room temperature over 2 hours. The reaction was quenched with NaHCO₃. The layer was washed with brine (2 x 10 mL), dried over MgSO₄ and concentrated *in vacuo*. The product was purified by column chromatography to yield 0.708 g of **2.16** (2.26 mmol, 82 %).

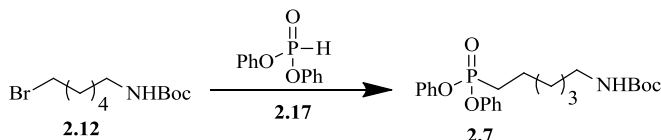
R_f: 0.35, 20 % EtOAc/Hex

¹H NMR (400 MHz, CDCl₃): δ 4.49 (s, 1H), 3.15 (t, *J* = 6.8 Hz, 2H), 3.09 (t, *J* = 7.0 Hz, 2H), 1.84-1.74 (m, 2H), 1.46 (s, 9H), 1.54-1.18 (m, 6H) ppm.

¹³C NMR (100 MHz, CDCl₃): δ 156.0, 40.5, 33.4, 30.2, 29.9, 28.4, 25.7, 7.0 ppm.

NMR Spectra are consistent with those previously reported.⁸

6-(tert-butoxycarbonyl-amino)-1-diphenoxyphoryl hexane (**2.7**)



To diphenyl phosphite **2.17** (0.2 mL, 1.04 mmol, 1 equiv.) in acetonitrile (4 mL) is added DBU (0.18 mL, 1.15 mmol, 1.1 equiv.) at 0 °C. This solution is mixed for 10 minutes and **2.12** (0.329 g, 1.15 mmol, 1.1 equiv.) is added slowly over 10 minutes. The resulting mixture is allowed to warm to room temperature and stirs overnight. The reaction was diluted with H₂O (3 mL), extracted with EtOAc (3 x 5 mL) and washed with 10 % HCl (2 x 5 mL) and brine (2 x 5 mL). The resulting organic layer was dried over MgSO₄, concentrated *in vacuo* and purified by column chromatography to yield 0.126 g of **2.7** (0.29 mmol, 28 %).

R_f: 0.50, 60 % EtOAc/Hex

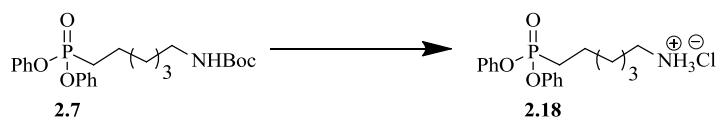
¹H NMR (400 MHz, CDCl₃): δ 7.32-7.28 (m, 4H), 7.16-7.13 (m, 6H), 4.48 (s, 1H, -NH), 3.09 (t, *J* = 6.0 Hz, 2H), 2.11-2.00 (m, 2H), 1.83-1.73 (m, 2H), 1.57-1.42 (m, 3H), 1.36 (s, 9H), 1.35-1.33 (m, 3H) ppm.

¹³C NMR (100 MHz, CDCl₃): δ 156.0, 150.4, 129.8, 125.1, 120.6, 120.5, 77.23, 30.2, 30.0, 26.5, 26.2, 25.2, 22.3, 22.3 ppm.

³¹P NMR (100 MHz, CDCl₃): δ 25.80 (tt, *J* = 17.4, 12.5 Hz) ppm.

HRMS (EI) *m/z* calcd for C₂₃H₃₂NO₅P [(M-H)⁺] 434.2096, found 434.1998

6-amino-1-diphenoxyphoryl hexane (2.18)



To **2.7** (0.166 g, 0.383 mmol, 1 equiv.) was added HCl/Dioxane (1.0 mL, 2.9 mmol, 10 equiv.) at room temperature. The reaction was stirred for an hour and concentrated *in vacuo*. The free amine was isolated as a thick yellow liquid. The reaction yield was 0.125 g (0.375 mmol, 98 %) and the product was taken onto the next step without further purification.

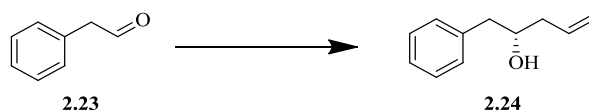
¹HNMR (400 MHz, D₂O): δ 7.28-7.24 (m, 4H), 7.15-7.13 (m, 2H), 7.11-7.00 (m, 4H), 3.58 (tt, $J = 36.2\text{Hz}, 5.7\text{Hz}, 2\text{H}$), 2.79 (t, $J = 7.3\text{Hz}, 2\text{H}$), 2.12-2.05 (m, 2H), 1.66-1.62 (m, 2H), 1.60-1.57 (m, 2H), 1.55-1.48 (m, 2H), 1.46-1.29 (m, 2H) ppm.

¹³CNMR (100 MHz, D₂O): δ 149.4, 149.3, 130.2, 126.0, 120.6, 120.5, 39.3, 28.9, 28.7, 26.4, 24.9, 23.5, 21.1 ppm.

³¹PNMR (100 MHz, D₂O): δ 31.00 (p, $J = 15.5\text{Hz}$) ppm.

HRMS (EI) m/z calcd for C₁₈H₂₅NO₃P [(M-H)⁺] 334.1572, found 334.1538

(R)-1-Phenylpent-4-en-2-ol (2.24)



To (-)-B-methoxydisopinocampheylborane (5.0 g, 15.81 mmol, 1.25 equiv.) dissolved in ether (15.2 mL) was added allylmagnesium bromide (15.8 mL, 15.18 mmol, 1.20 equiv.) and stirred vigorously at 0 °C for 1 hour. A large amount of white solid (presumably MgBrOMe) precipitates upon addition of the allylmagnesium bromide. The reaction was allowed to warm to room temperature over 2 hours. A premixed solution of phenylacetaldehyde **2.23** (1.5 mL, 12.65 mmol, 1 equiv.) in ether (9.7 mL) and the reaction are put to -100 °C. The phenylacetaldehyde solution is added drop wise to the borane solution ensuring that the temperature remains at -100 °C and the drops go along the side of the flask. The ether/liquid N₂ bath was maintained for 1 hour and then allowed to warm to room temperature. A solution of 15 % NaOH (6 mL) and 30 % H₂O₂ (9 mL) was premixed and added to the reaction drop wise at 0 °C to ensure the vigorous reaction does not overflow. This mixture was equipped with a water

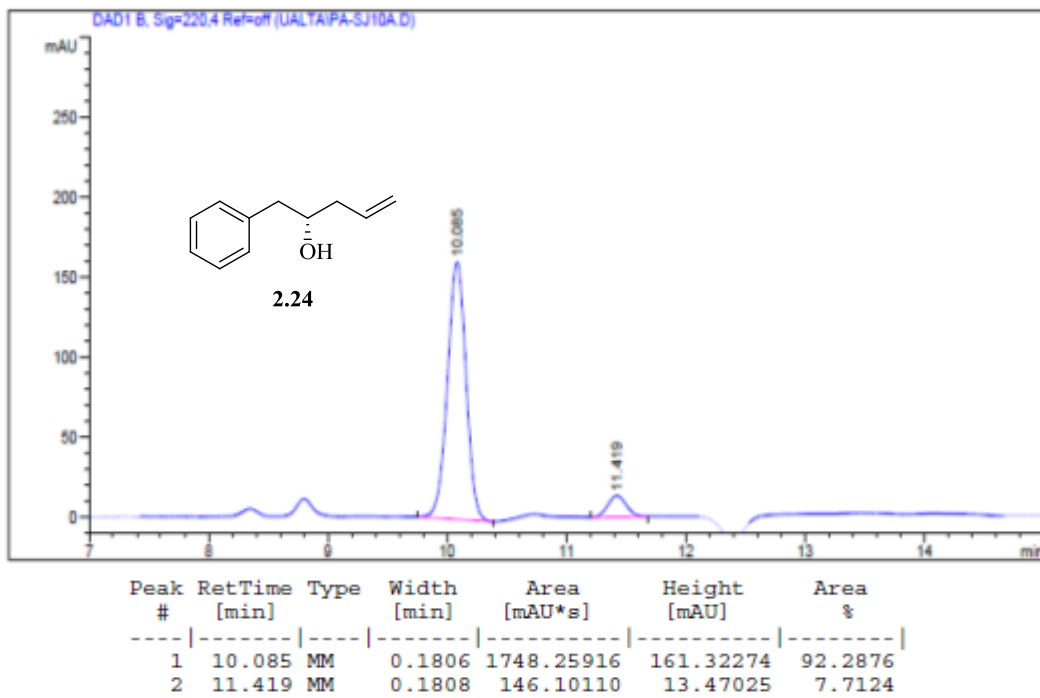
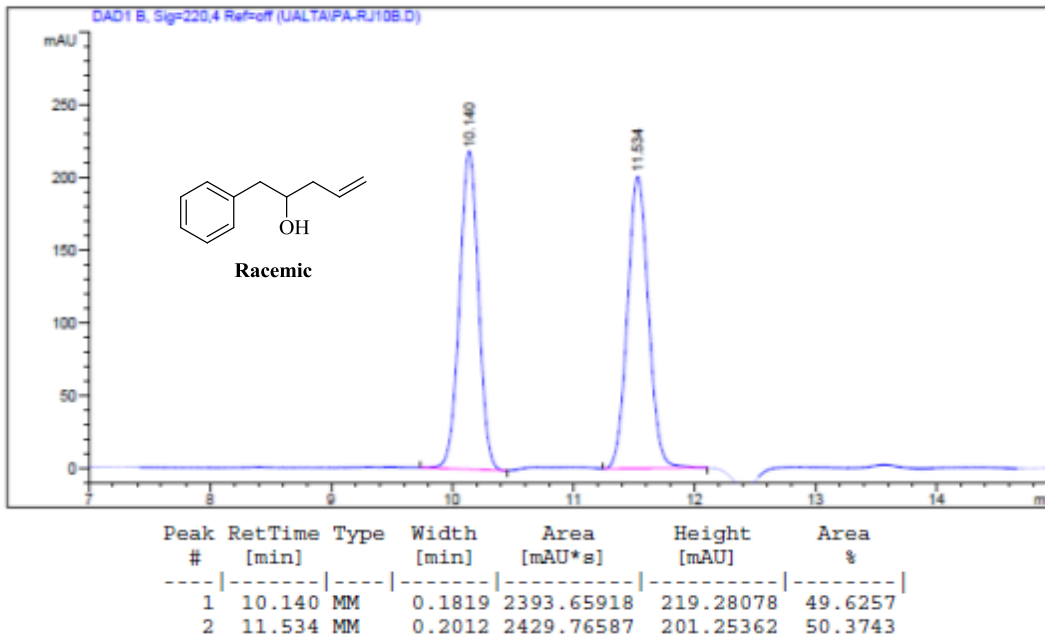
condenser and refluxed for 45 minutes. The reaction was diluted with EtOAc (12 mL) and the aqueous layer extracted with EtOAc (2x20 mL) The organic layer was washed with NaCl (2x15 mL), dried over MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (2 % EtOAc/Hexanes) to yield 1.26 g of **2.24** (7.5 mmol, 60 %, 85 % *ee*).

R_f: 0.38, 10 % EtOAc/Hex

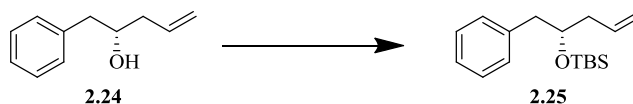
¹H NMR (400 MHz, CDCl₃): δ 7.33-7.19 (m, 5H), 5.92-5.79 (m, 1H), 5.18-5.14 (m, 1H), 5.12 (t, *J* = 1.2Hz, 1H), 3.92-3.83 (m, 1H), 2.84-2.68 (m, 2H), 2.37-2.29 (m, 1H), 2.25-2.16 (m, 1H), 1.62 (br s, 1H). ppm.

¹³C NMR (100 MHz, CDCl₃): δ 138.4, 134.7, 129.4, 128.6, 126.5, 118.2, 71.7, 43.3, 41.2 ppm.

NMR Spectra are consistent with those previously reported.⁹



tert-Butyldimethyl-(R)-1-Phenylpent-4-en-2-silane (2.25)



To **2.24** (2.13 g, 13.13 mmol, 1 equiv.) dissolved in anhydrous DMF (13.1 mL) was added imidiazole (1.97 g, 28.89 mmol, 2.2 equiv.) and TBSCl (2.97 g, 19.70 mmol, 1.5 equiv.) at room temperature and mixed overnight. After checking by TLC for completion, the reaction mixture was quenched with saturated ammonium chloride (15 mL) and diluted with EtOAc. The aqueous layer was then extracted with EtOAc and the resulting organic layer washed with water whilst increasing the concentration of brine to 100 %. After one final brine wash, the organic layer was dried with MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (2 % EtOAc/Hexanes) to yield 3.34 g of **2.25** (12.08 mmol, 92 %)

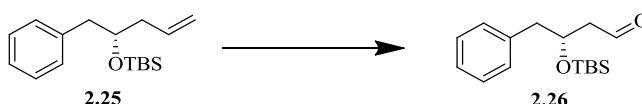
R_f: 0.22, 10 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.27-7.14 (m, 5H), 5.88-5.80 (m, 1H), 5.07-5.01 (m, 2H), 3.88-3.84 (m, 1H), 2.78-2.63 (m, 2H), 2.34-2.18 (m, 2H), 0.82 (s,9H) -0.08 (s,3H) -0.25 (s,3H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 139.3, 135.1, 129.9, 128.1, 126.1, 117.1, 73.5, 43.6, 41.7, 41.7, 26.0, 18.1, -4.8, -5.1 ppm.

HRMS (EI) *m/z* calcd for C₁₆H₂₆O₂Si [(M-H)⁺] 219.1200, found 219.1192

(S)-3-tert-Butyldimethylsilyl-1-Phenylpentanal (2.26)



To **2.25** (1.26 g, 4.56 mmol, 1 equiv.) dissolved in anhydrous 1:1 ^tBuOH:H₂O (46 mL) was added K₂CO₃ (1.89 g, 13.68 mmol, 3 equiv.) and K₃Fe(CN)₆ (4.50 g, 13.68 mmol, 3 equiv.) at room temperature. The reaction was stirred at room temperature until most of the solid is dissolved followed by the addition of K₂O₈ (0.020 g, 0.05 mmol, 0.01 equiv.) in 15 % NaOH (1.5 mL) which was mixed overnight. After checking by TLC for completion, the reaction mixture was diluted with H₂O and extracted with EtOAc (3 x 30 mL). The pooled organic layer was washed with brine, dried with MgSO₄ and concentrated *in vacuo*. The product was then dissolved in DCM (18 mL) and Pd(OAc)₄ (2.43 g, 5.47 mmol, 1.2 equiv.) and stirred for 1 hour. The reaction is then concentrated *in vacuo*, redissolved in EtOAc and silica was added to be

loaded onto a flash column for purification. (5 % EtOAc/Hexanes) to yield 0.686 g of **2.26** (2.46 mmol, 54 %)

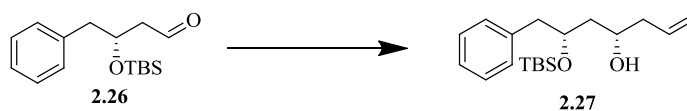
R_f: 0.45, 10 % EtOAc/Hex)

¹HNMR (400 MHz, CDCl₃): δ 9.76 (t, *J* = 2.6Hz, 1H), 7.29-7.13 (m, 5H), 4.36 (p, *J* = 6.2Hz, 1H), 2.88-2.74 (m, 2H), 2.49 (dd, *J* = 5.7, 2.5Hz, 2H), 0.84 (s, 9H) -0.01 (s, 3H) -0.12 (s, 3H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 202.0, 137.8, 129.7, 128.4, 69.6, 50.6, 44.6, 25.8, 18.0, -4.8, -4.9 ppm.

HRMS (EI) *m/z* calcd for C₁₆H₂₆O₂Si [(M-H)⁺] 277.1701, found 277.1610

(4*S*,6*S*)-6-(tert-Butyldimethylsilyloxy)-7-phenylhept-1-en-4-ol (**2.27**)



To (-)-B-methoxydisopinocampheylborane (2.33 g, 7.36 mmol, 1.25 equiv.) dissolved in ether (7.4 mL) was added allylmagnesium bromide (7.0 mL g, 7.07 mmol, 1.20 equiv.) and stirred vigorously at 0 °C for 1 hour. A large amount of white solid (presumably MgBrOMe) precipitates upon addition of the allylmagnesium bromide. The reaction was allowed to warm to room temperature over 2 hours. A premixed solution of **2.26** (1.64 g, 5.89 mmol, 1 equiv.) in ether (4.5 mL) and the reaction are put to -100 °C. The aldehyde solution was added drop wise to the borane solution ensuring that the temperature remains at -100 °C and the drops go along the side of the flask. The ether/liquid N₂ bath was maintained for 1 hour and then allowed to warm to room temperature. A solution of 15 % NaOH (6 mL) and 30 % H₂O₂ (9 mL) was premixed and added to the reaction drop wise at 0 °C to ensure the vigorous reaction does not overflow. This mixture was equipped with a water condenser and refluxed for 45 minutes. The reaction was diluted with EtOAc (12 mL) and the aqueous layer extracted with EtOAc (2x20 mL) The organic layer was washed with NaCl (2x15 mL), dried over MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (2 % EtOAc/Hexanes) to yield 1.170 g of **2.27** (3.65 mmol, 62 %)

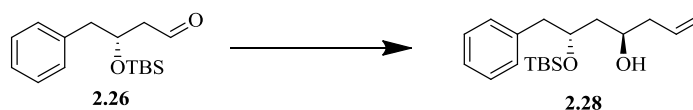
R_f: 0.38, 10 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.46-7.07 (m, 5H), 5.89-5.66 (m, 1H), 5.17-4.96 (m, 2H), 4.15-4.02 (m, 1H), 3.82-3.71 (m, 1H), 2.91-2.65 (m, 2H), 2.19-2.08 (m, 2H), 1.64-2.06 (m, 2H) 0.88 (s,9H) 0.08 (s,3H) -0.00 (s,3H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 138.6, 134.9, 129.7, 128.8, 126.5, 117.8, 74.0, 70.1, 44.9, 43.2, 42.8, 41.9, 26.0, -3.9, -4.7 ppm.

NMR Spectra are consistent with those previously reported.⁹

(4S,6R)-6-(tert-Butyldimethylsilyloxy)-7-phenylhept-1-en-4-ol (**2.28**)



To **2.26** (1.20 g, 4.31 mmol, 1 equiv.) dissolved in anhydrous ether (14 mL) was added (+)-Ipc₂BAllyl (5.0 mL, 5.00 mmol, 1.2 equiv.) at -100 °C. The ether/liquid N₂ bath was maintained for 1 hour and then allowed to warm to room temperature. A solution of 15 % NaOH (3 mL) and 30 % H₂O₂ (4.5 mL) was premixed and added to the reaction drop wise at 0 °C to ensure the vigorous reaction does not overflow. This mixture was equipped with a water condenser and refluxed for 45 minutes. The reaction was diluted with EtOAc (12 mL) and the aqueous layer extracted with EtOAc. (2x20 mL) The organic layer was washed with NaCl (2x15 mL), dried over MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (2 % EtOAc/Hexanes) to yield 0.484 g of **2.28** (1.51 mmol, 35 %)

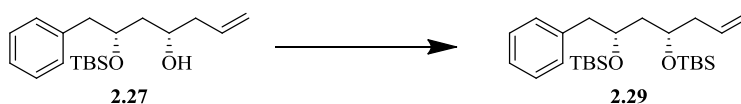
R_f: 0.34, 10 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.29-7.11 (m, 5H), 5.87-5.70 (m, 1H), 5.11-5.00 (m, 2H), 4.22-4.15 (m, 1H), 4.12-4.03 (m, 1H), 3.37-3.30 (d, *J* = 1.9Hz, 1H) 2.95-2.75 (m, 2H), 2.26-2.07 (m, 2H), 1.57-1.52 (m, 2H) 0.86 (s,9H) 0.03 (s,3H) -0.15 (s,3H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 138.8, 134.9, 129.7, 128.6, 126.5, 117.6, 73.1, 67.9, 43.2, 42.3, 40.8, 25.6, 18.2, -4.8 ppm.

NMR Spectra are consistent with those previously reported.⁹

(4S,6S)-4,6-bis(tert-Butyldimethylsilyloxy)-7-phenylhept-1-ene (2.29)



To **2.27** (1.09 g, 3.56 mmol, 1 equiv.) dissolved in anhydrous DMF (3.6 mL) was added imidazole (0.53 g, 7.82 mmol, 2.2 equiv.) and TBSCl (0.65 g, 4.27 mmol, 1.2 equiv.) at room temperature and mixed overnight. After checking by TLC for completion, the reaction mixture was quenched with saturated ammonium chloride (15 mL) and diluted with EtOAc (3 mL). The aqueous layer was then extracted with EtOAc (2 x 5 mL) and the resulting organic layer washed with water whilst increasing the concentration of brine to 100 %. After one final brine wash, the organic layer was dried with MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (2 % EtOAc/Hexanes) to yield 1.01 g of **2.29** (2.31 mmol, 65 %)

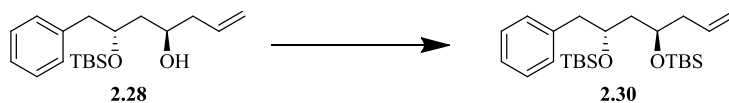
R_f: 0.63, 5 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.26-7.13 (m, 5H), 5.73-5.70 (m, 1H), 4.99-4.93 (m, 2H), 3.94-3.91 (m, 1H), 3.86-3.83 (m, 1H), 2.78-2.63 (m, 2H), 2.22-2.20 (m, 2H), 0.87 (s, 9H), 0.82 (s, 9H), 0.06 (s, 3H), 0.04 (s, 3H), -0.07 (s, 3H), -0.20 (s, 3H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 139.1, 134.8, 129.9, 128.1, 126.0, 117.0, 70.9, 69.1, 44.5, 44.2, 42.0, 25.9, 18.1, -4.2, -4.4, -4.7, -4.8 ppm.

HRMS (EI) *m/z* calcd for C₂₀H₃₅O₄Si₂ [(M-H)⁺] 377.2327, found 377.2310

(4S,6R)-4,6-bis(tert-Butyldimethylsilyloxy)-7-phenylhept-1-ene (2.30)



To **2.28** (0.460 g, 3.56 mmol, 1 equiv.) dissolved in anhydrous DMF (1.5 mL) was added imidazole (0.22 g, 3.23 mmol, 2.2 equiv.) and TBSCl (0.26 g, 1.75 mmol, 1.2 equiv.) at room temperature and mixed overnight. After checking by TLC for completion, the reaction mixture was quenched with saturated ammonium chloride (15 mL) and diluted with EtOAc (3 mL). The aqueous layer was then extracted with EtOAc (2 x 5 mL) and the resulting organic layer washed with water whilst increasing the concentration of brine to 100 %. After one final brine wash, the organic layer was dried with MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (2 % EtOAc/Hexanes) to yield 0.789 g of **2.30** (1.82 mmol, 51 %)

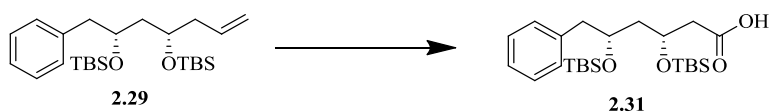
R_f: 0.63, 5 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.27-7.12 (m, 5H), 5.82-5.72 (m, 1H), 5.05-4.97 (m, 2H), 3.95-3.91 (m, 1H), 3.85-3.77 (m, 1H), 2.73-2.71 (m, 2H), 2.23-2.19 (m, 2H), 1.63-1.54 (m, 2H), 0.82 (s,9H), 0.82 (s,9H), -0.01 (s,3H), -0.02 (s,3H), -0.04 (s,3H), -0.17 (s,3H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 139.1, 135.2, 130.0, 128.2, 126.1, 117.0, 71.6, 70.0, 45.0, 44.7, 42.5, 26.0, 18.1, -4.0, -4.3, -4.4 ppm.

HRMS (EI) *m/z* calcd for C₂₀H₃₅O₄Si₂ [(M-H)⁺] 377.2327, found 377.2560

(4S,6S)-4,6-bis(tert-Butyldimethylsilyloxy)-7-phenylheptanoic acid (2.31)



To **2.29** (0.343 g, 0.66 mmol, 1 equiv.) dissolved in anhydrous 1:1 ^tBuOH:H₂O (8 mL) was added K₂CO₃ (0.332 g, 1.98 mmol, 3 equiv.) and K₃Fe(CN)₆ (0.782 g, 1.98 mmol, 3 equiv.) at room temperature. The reaction was stirred at room temperature until most of the solid is dissolved followed by the addition of K₂OsO₇ (0.006 g, 0.015 mmol, 0.02 equiv.) in 15 % NaOH (1.5 mL) which was mixed overnight. After checking by TLC for completion, the reaction mixture was diluted with H₂O and extracted with EtOAc (3 x 30 mL). The pooled organic layer was washed with brine, dried with MgSO₄ and concentrated *in vacuo*. The product was then dissolved in DCM (3 mL) and Pd(OAc)₄ (0.409 g, 0.91 mmol, 1.2 equiv.) and stirred for 1 hour. The reaction is then concentrated *in vacuo* and the crude product was checked by NMR for the aldehyde peak.

To this aldehyde (0.302 g, 0.65 mmol, 1 equiv.) dissolved in ^tBuOH (9 mL) was added NaClO₂ (0.182 g, 1.99 mmol, 3 equiv.) and NaH₂PO₄ (0.483 g, 3.98 mmol, 6 equiv.) dissolved in water (2 mL) drop wise over 5 minutes. 2-methyl-2-butene (3.3 mL, 6.61 mmol, 10 equiv.) was added to reaction and allowed to mix at room temperature for 24 hours. The reaction mixture was then washed with 10 % HCl (2x2 mL) and diluted with DCM (4 mL). The aqueous layer was then extracted with DCM (3x4 mL) and the resulting organic layers were pooled and washed with brine (3x2 mL). The organic layer was dried with MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (5 % EtOAc/Hexanes) to yield 0.656 g of **2.31** (1.45 mmol, 63 %)

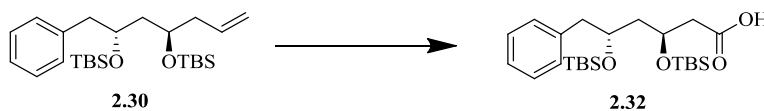
R_f: 0.12, 10 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.27-7.14 (m, 5H), 4.32-4.26 (m, 1H), 3.96-3.90 (m, 1H), 2.62-2.54 (m, 2H), 2.54-2.38 (m, 2H), 1.74-1.60 (m, 2H), 0.86 (s,9H), 0.85 (s,9H), 0.04 (s,6H), -0.01 (s,3H), -0.14 (s,3H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 177.2, 138.5, 129.7, 128.3, 126.3, 70.6, 66.7, 44.4, 44.2, 42.3, 25.9, 25.8, -4.4, -4.7, -4.8 ppm.

HRMS (EI) *m/z* calcd for C₂₀H₃₅O₄Si₂ [(M-H)⁺] 395.2068, found 395.2050

(4S,6R)-4,6-bis(tert-Butyldimethylsilyloxy)-7-phenylheptanoic acid (2.32)



To **2.30** (0.33 g, 0.75 mmol, 1 equiv.) dissolved in anhydrous 1:1 ^tBuOH:H₂O (8 mL) was added K₂CO₃ (0.31 g, 2.26 mmol, 3 equiv.) and K₃Fe(CN)₆ (0.75 g, 2.26 mmol, 3 equiv.) at room temperature. The reaction was stirred at room temperature until most of the solid is dissolved followed by the addition of K₂O₈ (0.005 g, 0.015 mmol, 0.02 equiv.) in 15 % NaOH (1.5 mL) which was mixed overnight. After checking by TLC for completion, the reaction mixture was diluted with H₂O and extracted with EtOAc (3 x 30 mL). The pooled organic layer was washed with brine (3 x 15 mL), dried with MgSO₄ and concentrated *in vacuo*. The product was then dissolved in DCM (3 mL) and Pd(OAc)₄ (0.40 g, 0.91 mmol, 1.2 equiv.) and stirred for 1 hour. The reaction is then concentrated *in vacuo* and the crude product was checked by NMR for the aldehyde peak.

To this aldehyde (0.33 g, 0.72 mmol, 1 equiv.) dissolved in ^tBuOH (9 mL) was added NaClO₂ (0.02 g, 2.17 mmol, 3 equiv.) and NaH₂PO₄ (0.05 g, 4.34 mmol, 6 equiv.) dissolved in water (2.2 mL) drop wise over 5 minutes. 2-methyl-2-butene (3.6 mL, 7.24 mmol, 10 equiv.) was added to reaction and allowed to mix at room temperature for 24 hours. The reaction mixture was then washed with 10 % HCl (2x2 mL) and diluted with DCM (4 mL). The aqueous layer was then extracted with DCM (3x4 mL) and the resulting organic layers were pooled and washed with brine (3x2 mL). The organic layer was dried with MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (5 % EtOAc/Hexanes) to yield 0.149 g of **2.32** (0.33 mmol, 44 %)

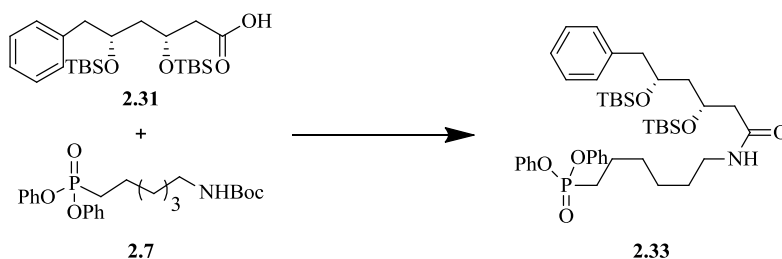
R_f: 0.12, 10 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.26-7.13 (m, 5H), 4.18-4.12 (m, 1H), 3.94-3.88 (m, 1H), 2.82-2.60 (m, 2H), 2.58-2.44 (m, 2H), 1.76-1.60 (m, 2H), 0.85 (s,9H), 0.79 (s,9H), 0.00 (s,3H), -0.03 (s,3H), -0.04 (s,3H), -0.11 (s, 3H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 176.1, 138.5, 129.9, 128.3, 126.4, 71.7, 67.8, 44.9, 44.6, 42.9, 25.9, 25.7, -4.4, -4.5, -4.7 ppm.

HRMS (EI) *m/z* calcd for C₂₀H₃₅O₄Si₂ [(M-H)⁺] 395.2068, found 395.2061

Diphenoxy-7-((3S,5S)-3,5-bis(tert-butylidimethylsilyloxy)-6-phenylhexanamido)phosphonate
(2.33)



2.7 (0.05 g, 0.13 mmol, 1.3 equiv) was dissolved and stirred in HCl/Dioxane (0.3 mL, 1.95 mmol, 13 equiv.) at room temperature under argon. After 1 hour, the solvent was evaporated *in vacuo* and the free amine **2.18** was used neat in the next step of the reaction.

2.31 (0.04 g, 0.15 mmol, 1 equiv.) was dissolved in DMF (1.0 mL) and added drop wise to the freshly deprotected amine. TBTU (0.04 g, 0.18 mmol, 1.2 equiv.) and DIEA (50 μL, 0.45 mmol, 3 equiv.) were added to this solution and the reaction was mixed under argon for 24 hours at room temperature. After checking by TLC for completion, the reaction mixture was poured over brine (10 mL) and extracted with EtOAc (3 x 10 mL). The organic layer was dried with MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (20 % Acetone/Hexanes) to yield 0.043 g of **2.33** (0.056 mmol, 43 %)

R_f: 0.45, 30 % Acetone/Hex

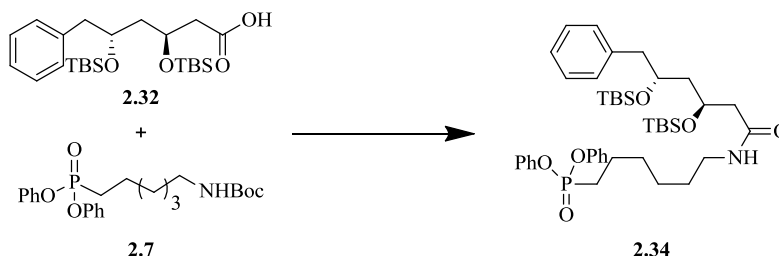
¹HNMR (400 MHz, CDCl₃): δ 7.31-7.11 (m, 15H), 6.31 (br t, *J* = 5.1 Hz, 1H), 4.19-4.15 (m, 1H), 3.95-3.92 (m, 1H), 3.21-3.07 (m, 2H), 2.80-2.71 (m, 2H), 2.47-2.20 (m, 2H), 2.06-1.99 (m, 2H), 1.79-1.73 (m, 4H), 1.44-1.34 (m, 8H), 0.85 (s,9H), 0.84 (s,9H), 0.05 (s,3H), 0.04 (s, 3H), -0.00 (s,3H), -0.13 (s, 3H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 170.7, 150.4, 138.4, 129.8, 129.7, 128.2, 126.2, 125.1, 120.6, 120.5, 70.5, 67.2, 44.4, 43.6, 43.4, 39.1, 30.2, 30.0, 29.3, 26.6, 26.5, 25.9, 25.8, 22.3, 22.3, 18.0, 17.9, -4.6, -4.8 ppm.

³¹PNMR (100 MHz, CDCl₃): δ 25.70 (tt, *J* = 17.4, 12.5Hz) ppm.

HRMS (ESI) *m/z* calcd for C₃₆H₅₂NO₆PSi [(M-H)⁺] 653.3302, found 653.9926

Diphenoxy-7-((3S,5R)-3,5-bis(tert-butylidimethylsilyloxy)-6-phenylhexanamido)phosphonate
(2.34)



6-(tert-butoxycarbonyl-amino) 1-bromo hexane (0.09 g, 0.20 mmol, 1.3 equiv) was dissolved and stirred in HCl/Dioxane (0.5 mL, 1.95 mmol, 13 equiv.) at room temperature under argon. After 1 hour, the solvent was evaporated *in vacuo* and the free amine **2.18** was used neat in the next step of the reaction.

2.32 (0.066 g, 0.15 mmol, 1 equiv.) was dissolved in DMF (1.5 mL) and added drop wise to the freshly deprotected amine. TBTU (0.058 g, 0.18 mmol, 1.2 equiv.) and DIEA (0.1 mL, 0.45 mmol, 3 equiv.) were added to this solution and the reaction was mixed under argon for 24 hours at room temperature. After checking by TLC for completion, the reaction mixture was poured over brine (10 mL) and extracted with EtOAc (3 x 10 mL). The organic layer was dried with MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (20 % Acetone/Hexanes) to yield 0.052 g of **2.34** (0.068 mmol, 45 %)

R_f: 0.45, 30 % Acetone/Hex

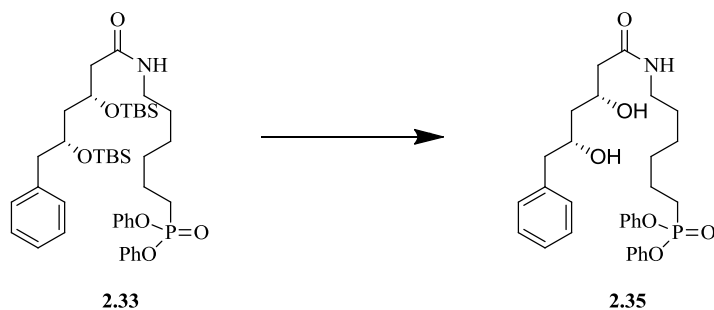
¹HNMR (400 MHz, CDCl₃): δ 7.31-7.12 (m, 15H), 6.33 (br t, *J* = 5.1 Hz, 1H), 4.08-4.02 (m, 1H), 3.90-3.88 (m, 1H), 3.28-3.04 (m, 2H), 2.47-2.28 (m, 2H), 2.06-1.00 (m, 2H), 1.78-1.65 (m, 4H), 1.45-1.28 (m, 8H), 0.82 (s, 9H), 0.81 (s, 9H), 0.00 (s, 6H), -0.03 (s, 3H), -0.18 (s, 3H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 170.7, 150.4, 138.6, 129.8, 128.2, 126.3, 125.1, 120.5, 71.3, 67.7, 44.4, 44.2, 44.1, 39.1, 30.2, 30.0, 29.3, 26.6, 26.5, 25.9, 25.8, 22.3, 22.3, 18.0, 17.9, -4.5, -4.5, -4.6, -4.7 ppm.

³¹PNMR (100 MHz, CDCl₃): δ 25.68 (tt, *J* = 17.4, 12.5 Hz) ppm.

HRMS (ESI) *m/z* calcd for C₄₂H₆₆NO₆PSi₂ [(M-Na)⁺] 790.4064, found 790.4021

Diphenoxy-7-(3*S*,5*S*)-3,5-dihydroxy-6-phenylhexanamido Phosphonate (**2.35**)



To **2.33** (0.026 g, 0.034 mmol, 1 equiv.) was placed in an Eppendorf tube and dissolved in MeCN (1.7 mL) at room temperature. Pyridine (55 μL, 0.677 Mmol, 10 equiv.) followed by 48 % aqueous HF (285 μL, 6.77 Mmol, 100 equiv.) were added to the reaction and the mixture was vortexed for 10 seconds. The reaction proceeded with no stirring for 5 hours. The reaction was then diluted with water (1 mL) extracted with EtOAc (3 x 1 mL) and dried with MgSO₄. The organic layers were pooled, concentrated *in vacuo* and purified by flash chromatography (2 % MeOH-DCM) yielding 0.012 g of **2.35** (0.022 mmol, 65 %).

R_f: 0.51, 5 % Methanol/DCM

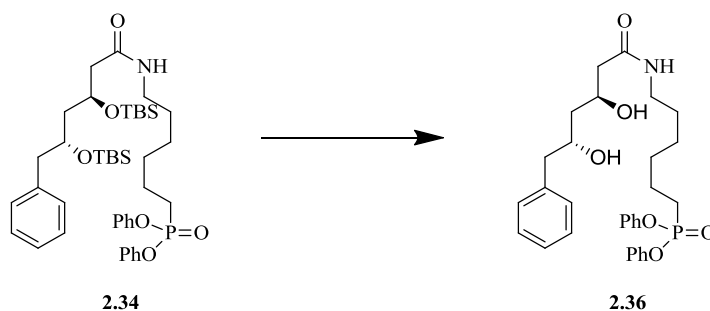
¹HNMR (400 MHz, CD₃OD): δ 7.88 (t, *J* = 4.8Hz, 1H) 7.44-7.06 (m, 15H), 4.27-4.15 (m, 1H), 4.09-3.96 (m, 1H), 3.19-3.08 (m, 2H), 2.72 (qd, *J* = 13.4, 6.9Hz, 2H), 2.27 (d, *J* = 6.6 Hz, 2H), 2.22-2.05 (m, 2H), 1.85-1.67 (m, 2H), 1.50-1.29 (m, 10H) ppm.

¹³CNMR (100 MHz, CD₃OD): δ 172.3, 150.2, 138.5, 131.0, 129.6, 127.8, 125.8, 125.1, 120.2, 70.8, 67.3, 43.6, 43.2, 42.5, 38.8, 29.4, 28.6, 27.6, 26.0, 25.8, 23.9, 22.3, 21.8 ppm.

³¹PNMR (100 MHz, CD₃OD): δ 27.03 (tt, *J* = 17.4, 12.5Hz) ppm.

HRMS (ESI) *m/z* calcd for C₃₀H₃₈NO₆P [(M-H)⁺] 540.2438, found 540.2515

Diphenoxy-7-(3*S*,5*R*)-3,5-dihydroxy-6-phenylhexanamido Phosphonate (**2.36**)



To **2.34** (0.052 g, 0.068 Mmol, 1 equiv.) was placed in an Eppendorf tube and dissolved in MeCN (1.7 mL) at room temperature. Pyridine (55 μL, 0.677 Mmol, 10 equiv.) followed by 48 % aqueous HF (285 μL, 6.77 Mmol, 100 equiv.) were added to the reaction and the mixture was vortexed for 10 seconds. The reaction proceeded with no stirring for 5 hours. The reaction was then diluted with water (1 mL) extracted with EtOAc (3 x 1 mL) and dried with MgSO₄. The organic layers were pooled, concentrated *in vacuo* and purified by flash chromatography (2 % MeOH-DCM) to yield 0.022 g of **2.36** (0.041 mmol, 60 %)

R_f: 0.52, 5 % Methanol/DCM

¹H NMR (400 MHz, CD₃OD): δ 7.88 (br t, *J* = 4.8 Hz, 1H) 7.37-7.13 (m, 15H), 4.25-4.17 (m, 1H), 4.06-3.98 (m, 1H), 3.17-3.11 (m, 2H), 2.70 (qd, *J* = 13.4, 6.9 Hz, 2H), 2.27 (d, *J* = 6.6 Hz, 2H), 2.22-2.05 (m, 2H), 1.85-1.48 (m, 2H), 1.50-1.29 (m, 10H) ppm.

¹³C NMR (100 MHz, CD₃OD): δ 172.5, 150.2, 138.8, 129.6, 129.1, 127.8, 125.7, 125.1, 120.3, 120.2, 68.8, 65.3, 44.2, 43.8, 43.0, 38.7, 29.7, 29.4, 28.6, 25.8, 21.8 ppm.

³¹P NMR (100 MHz, CD₃OD): δ 25.87 (tt, *J* = 17.4, 12.5 Hz) ppm.

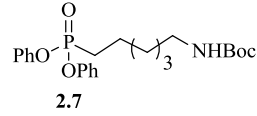
HRMS (ESI) *m/z* calcd for C₃₀H₃₈NO₆P [(M-Na)⁺] 62.2337, found 562.2341

2.5.3 Kinetic analysis of hydrolysis by DEBS TE

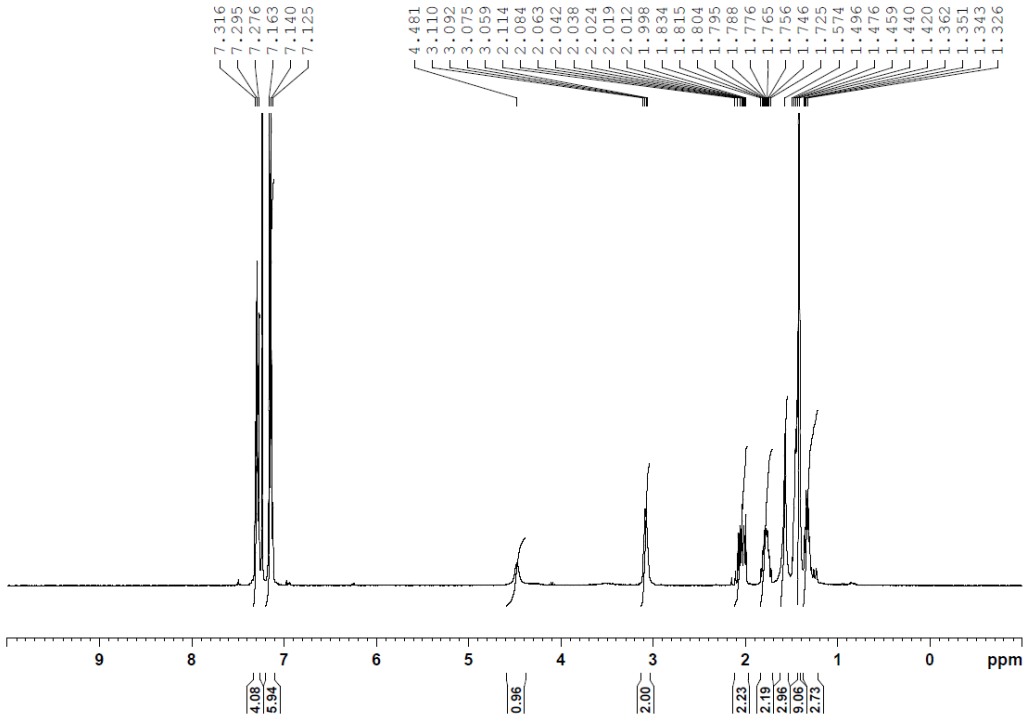
TE-catalyzed hydrolysis of thioester substrates were monitored by the observation of the formation of 5-thio-2-nitrobenzoate by the reaction released N-acetylcysteamine with 5,5'-dithiobis-2-nitrobenzoic acid (dTNB). A typical kinetic assay mixture consisted of 2.5 μM inhibited DEBS TE, 50 mM phosphate buffer (pH 7.38), 4 % (v/v) saturated dTNB in water, 5 mM substrate (100 mM stock solution in DMSO), and 10 % (v/v) DMSO in a total volume reaction of 200 μL.

The formation of the free thiol was quantified by measuring the absorption at 412 nm using a Thermo Scientific Evolution 300 UV-Vis. The reactions were monitored over 60 minutes at room temperature and data points were collected at 5, 10, 15, 20, 25, 30, 45 and 60 minute intervals. Initial velocities were determined by linear regression analysis and compared to the reaction with an uninhibited sample of DEBS TE.

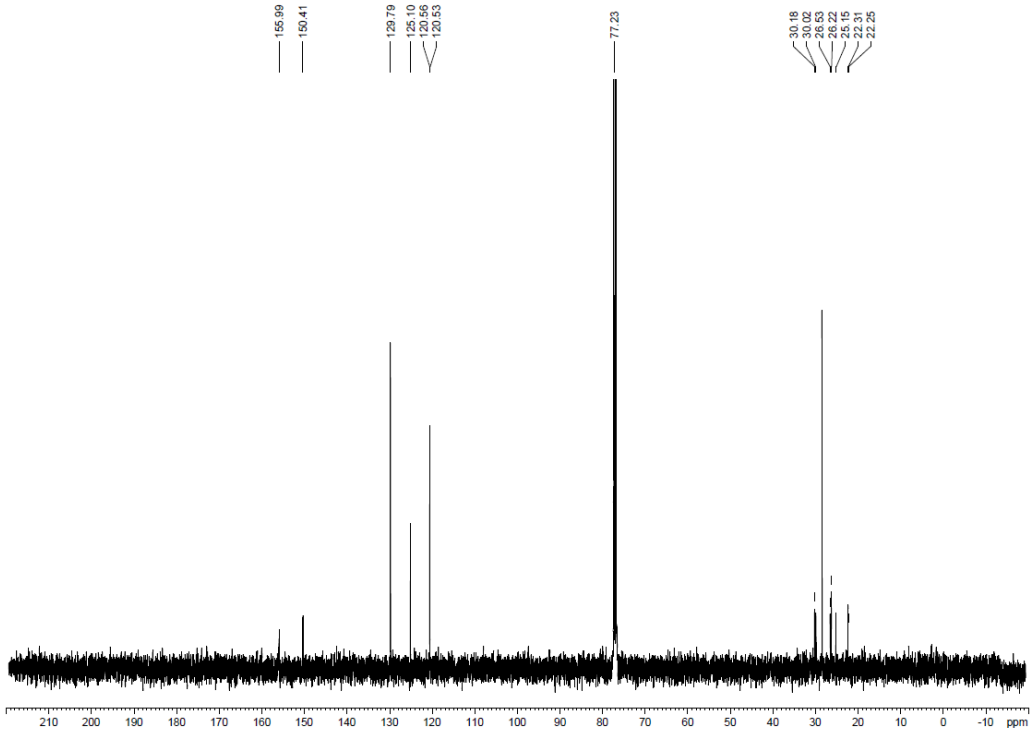
2.5.4 Selected 1D NMR spectra



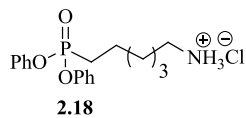
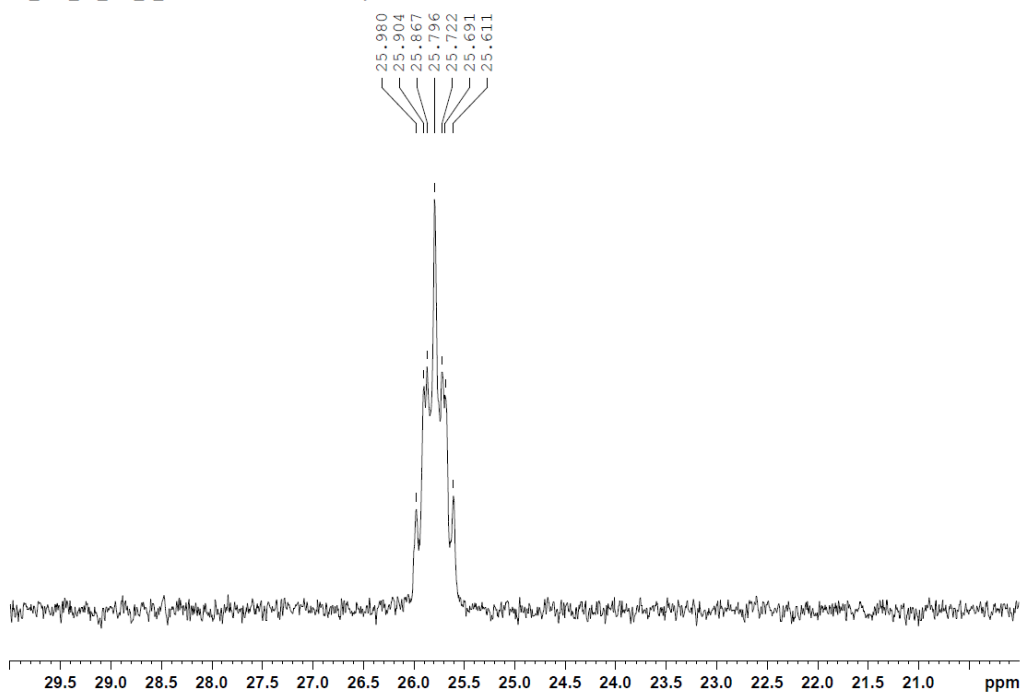
1d_1H_32_scans CDCl3 D:\Boddy 37



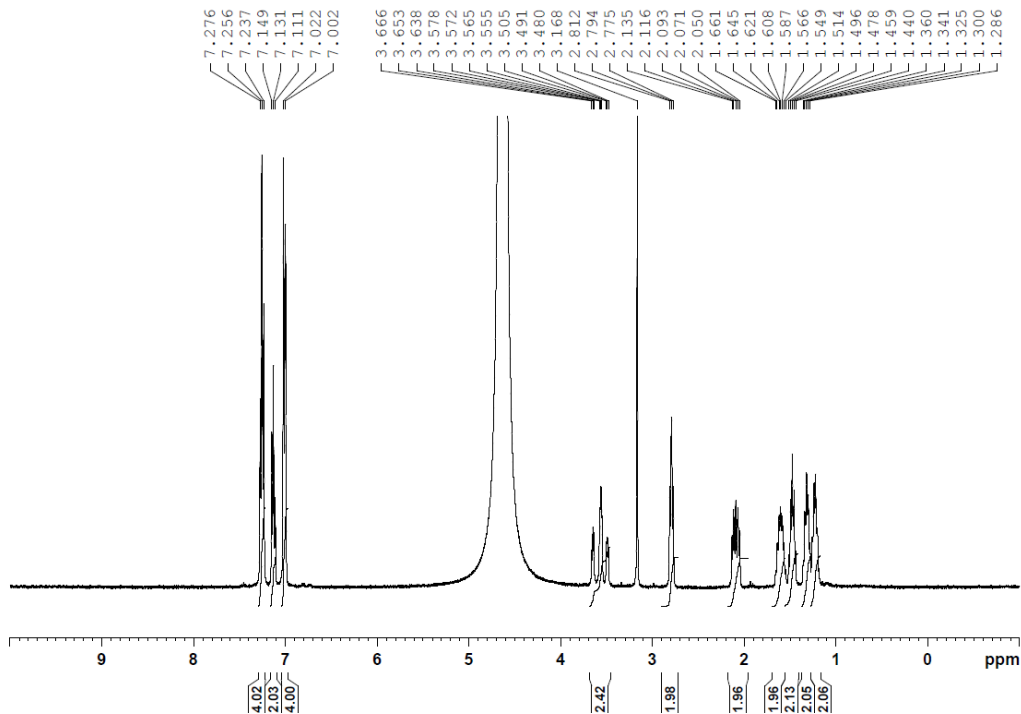
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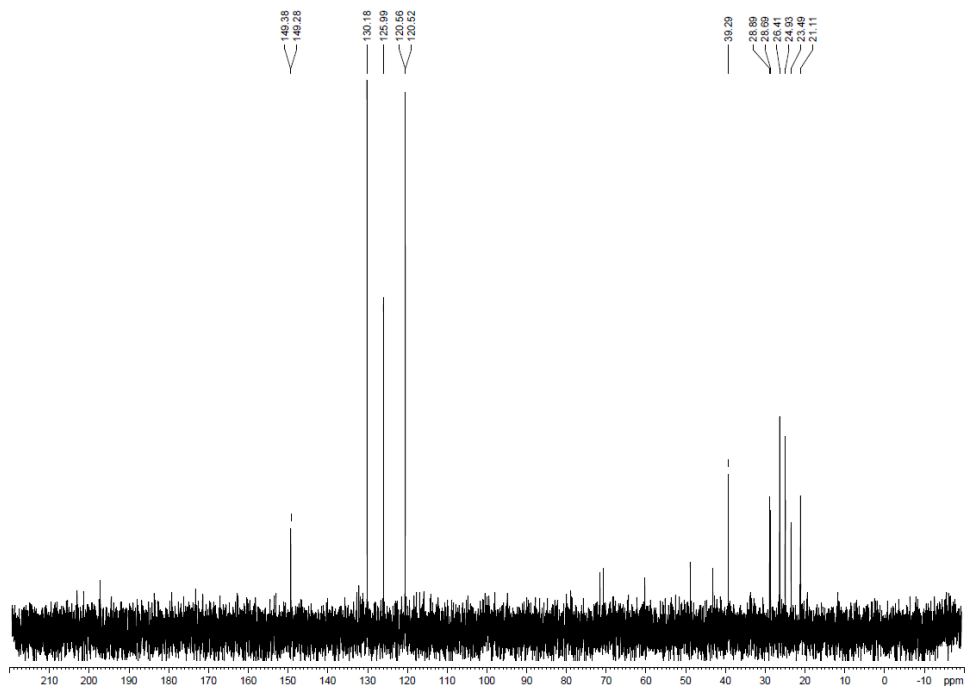
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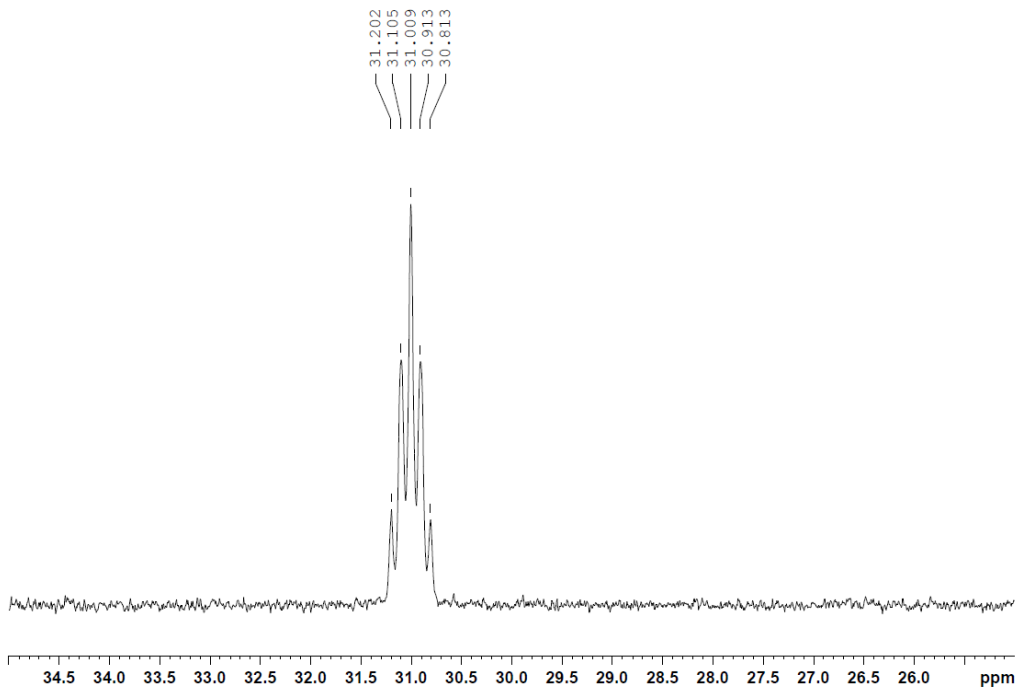
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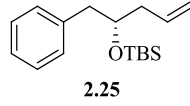


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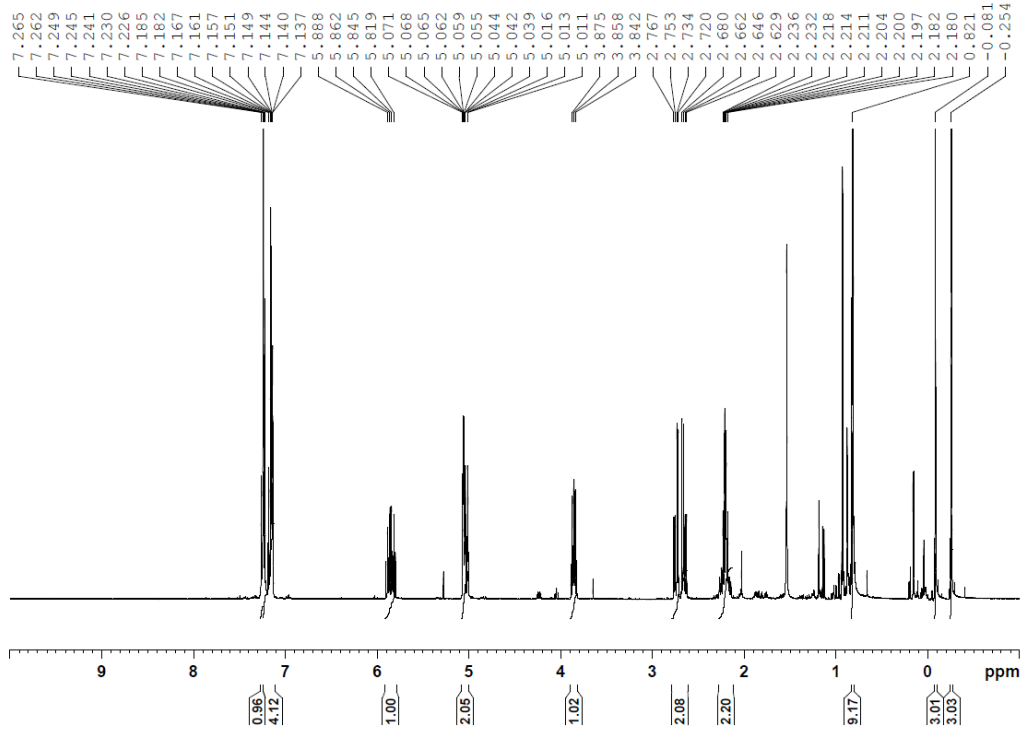


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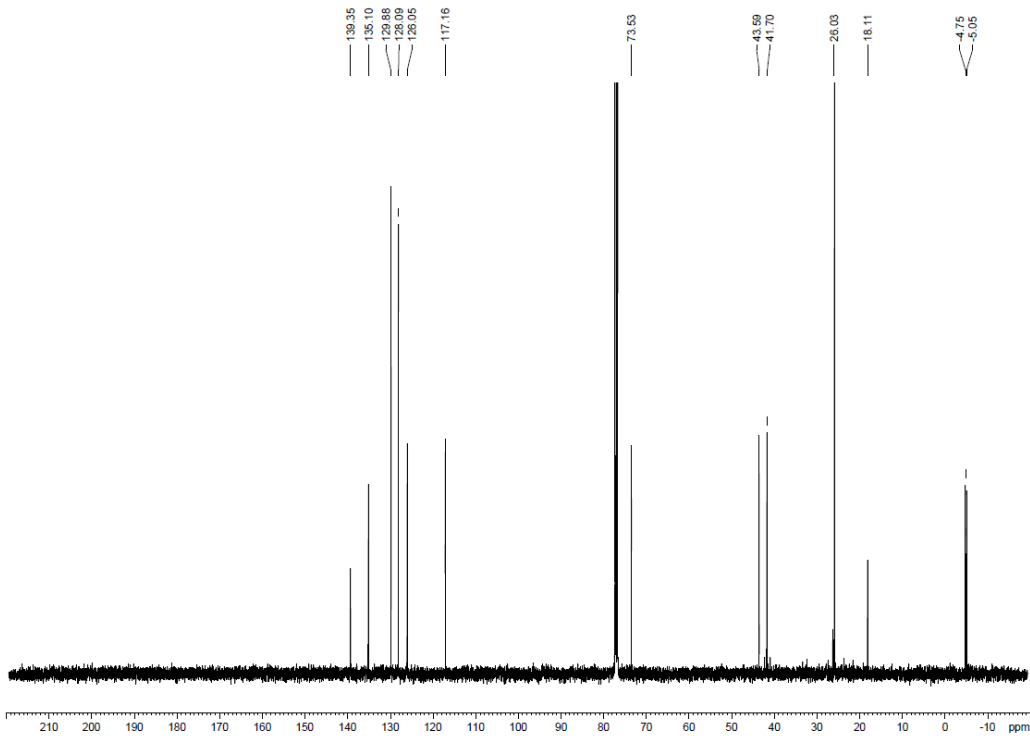


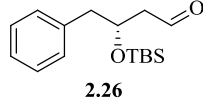


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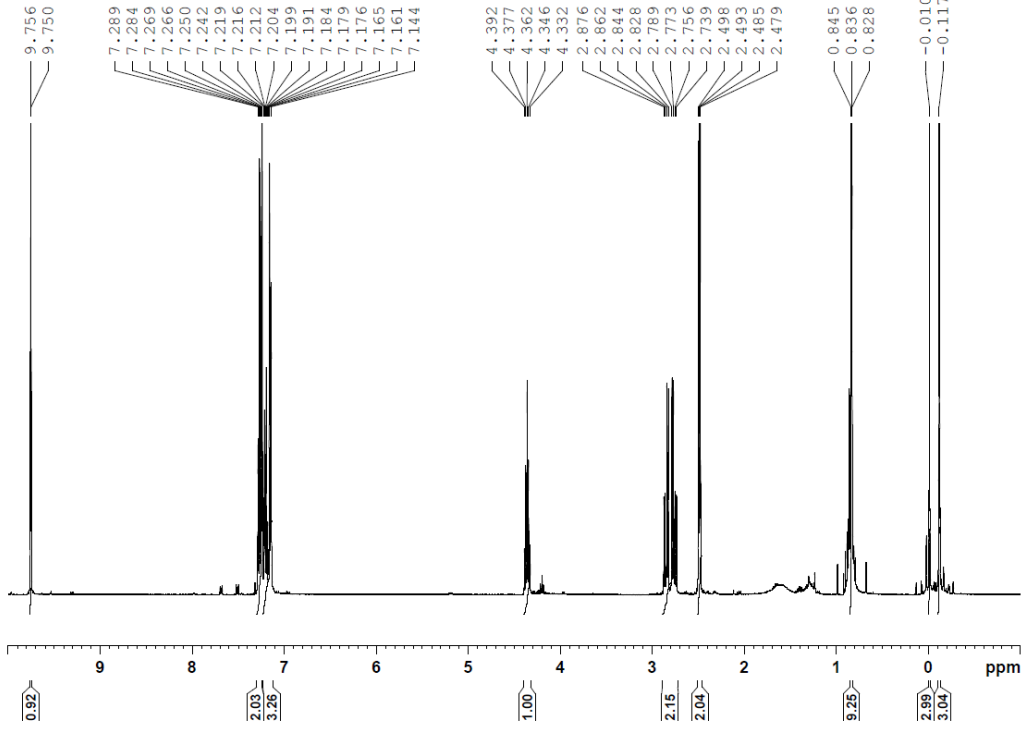


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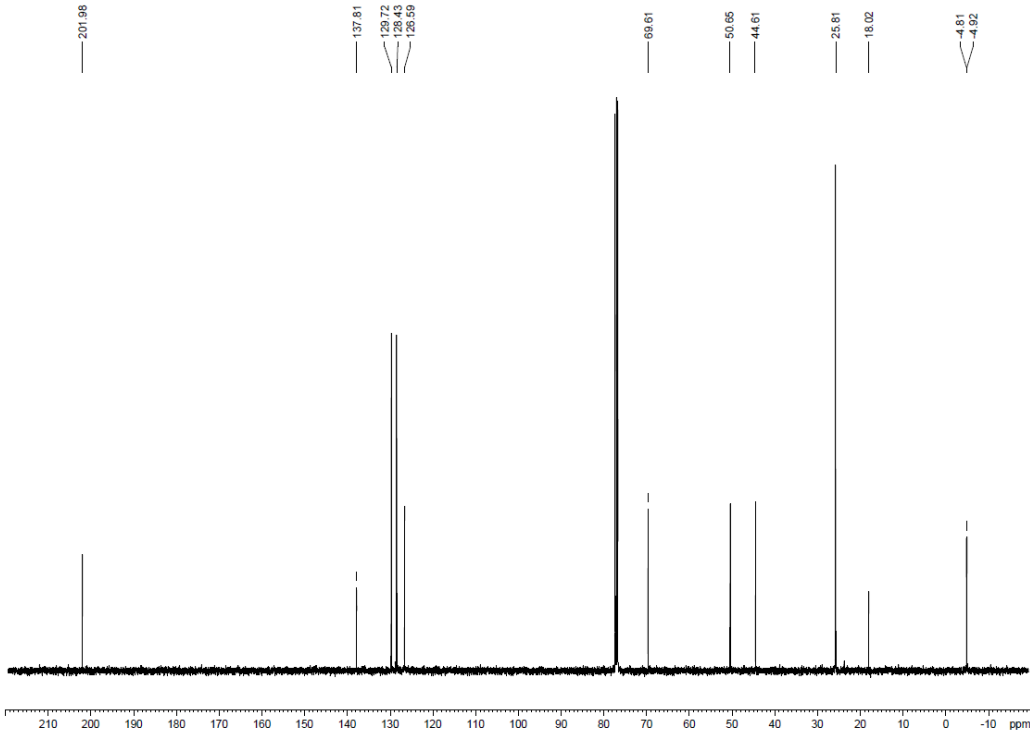


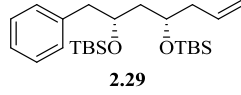


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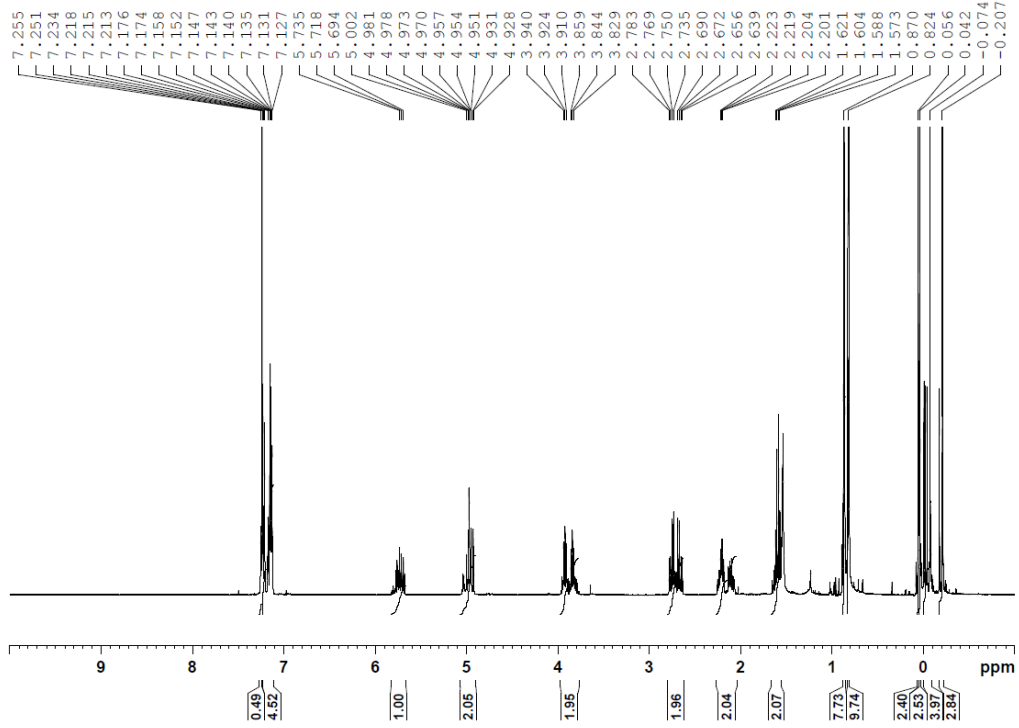


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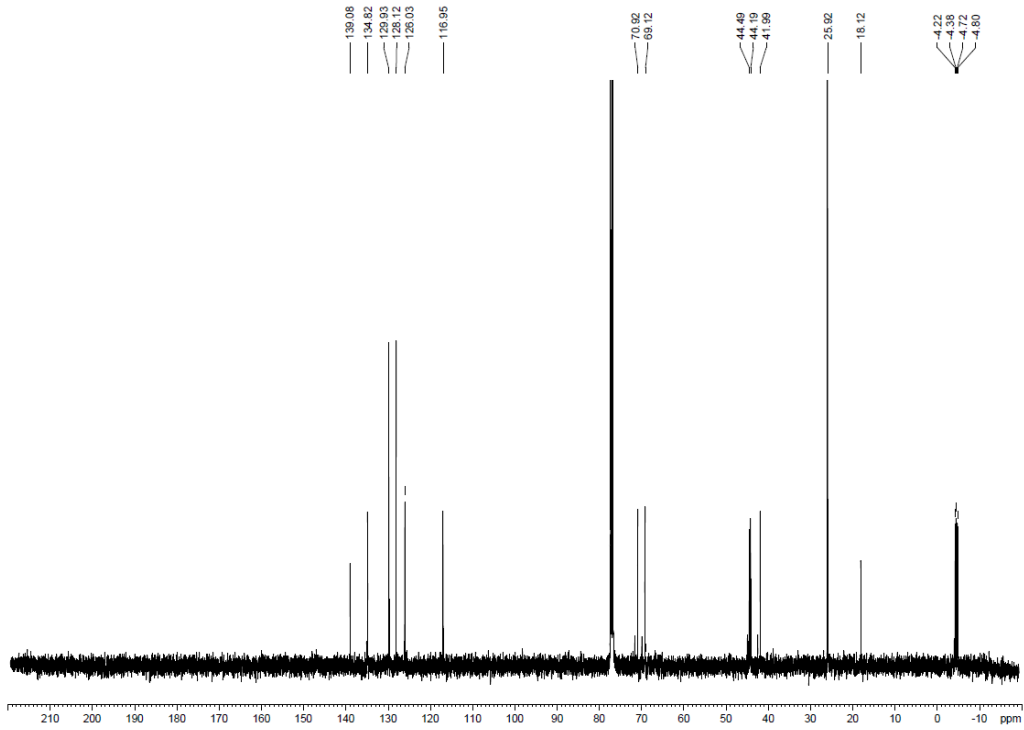


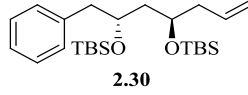


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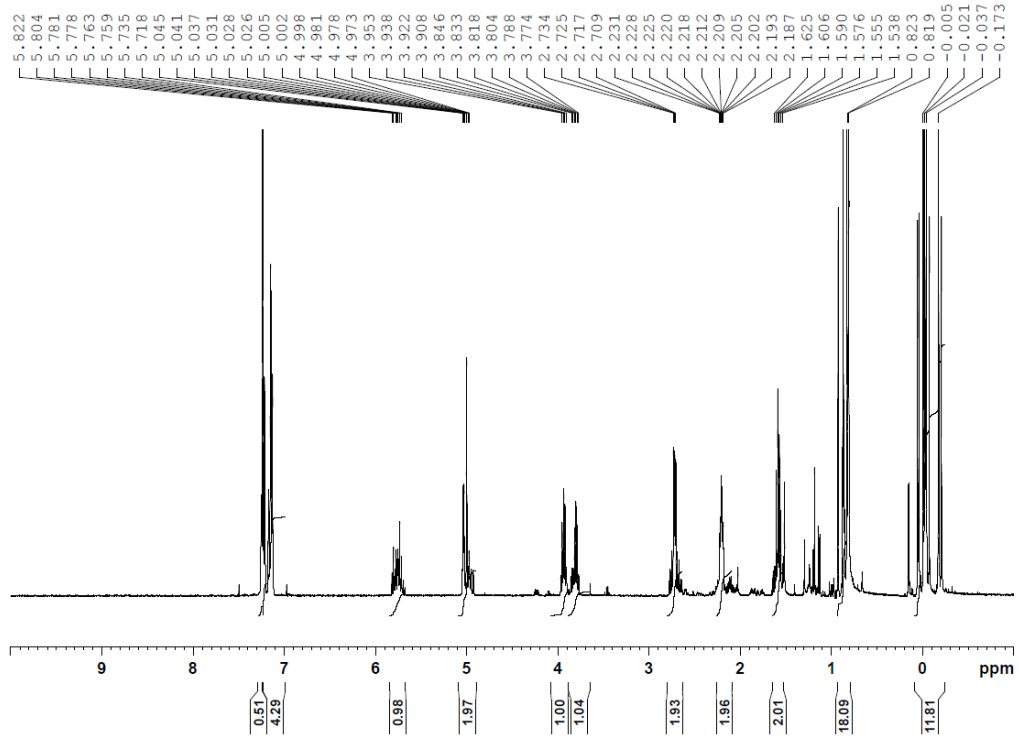


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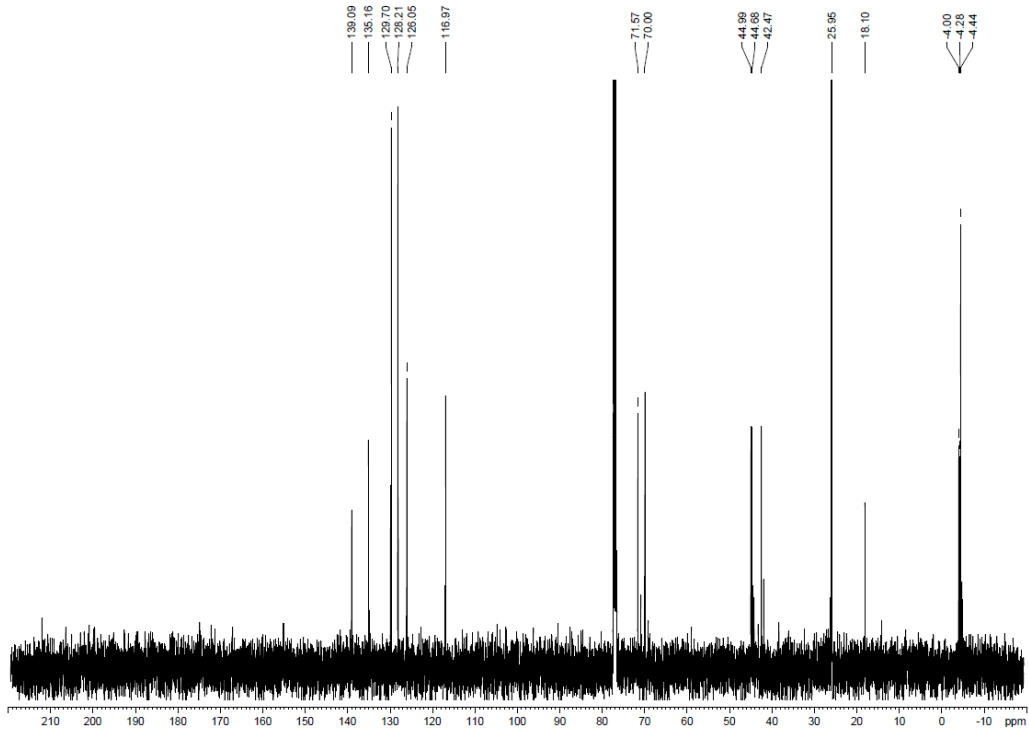


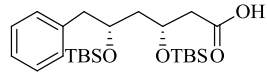


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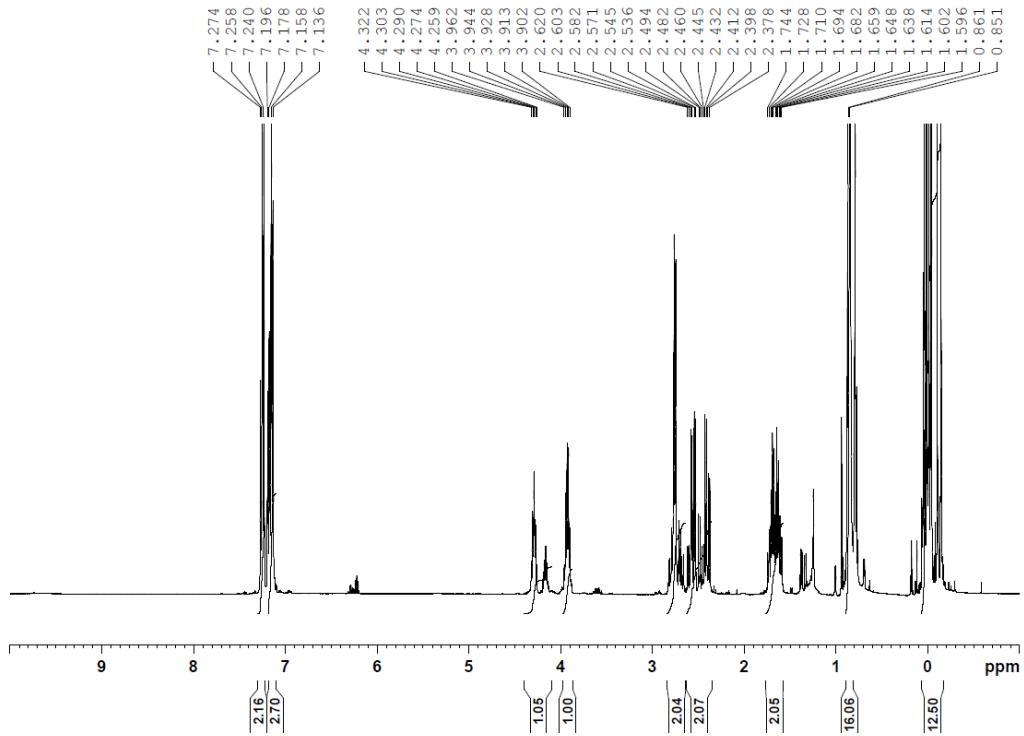
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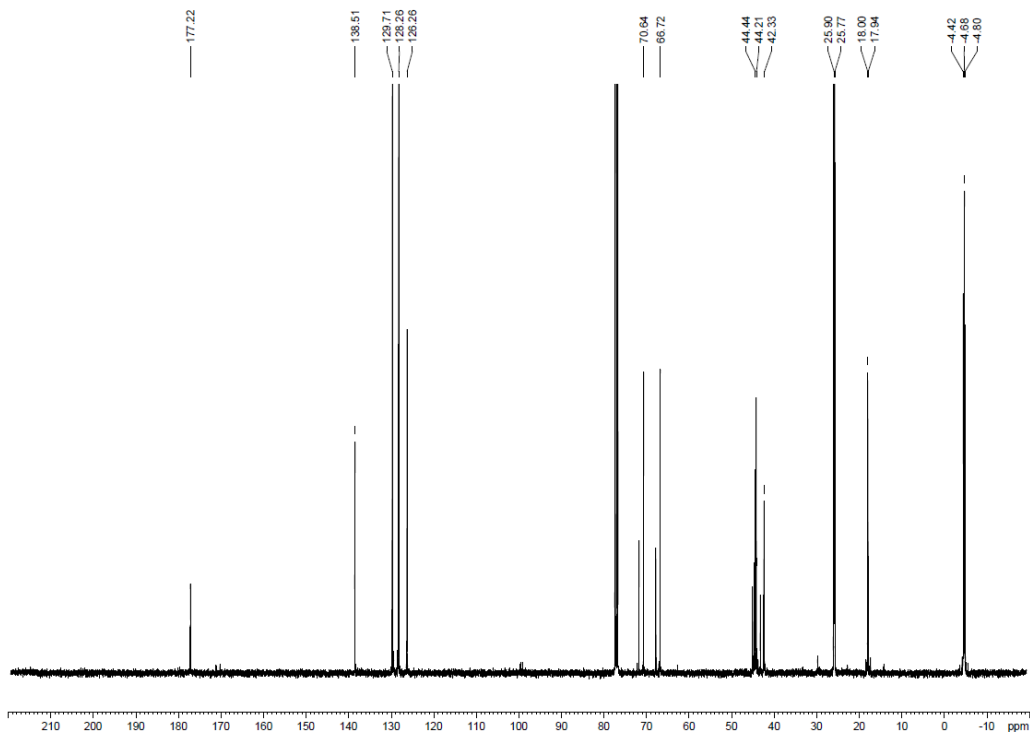


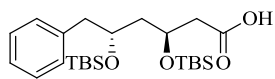
2.31

1d_1H_32_scans CDC13 D:\Boddy 9



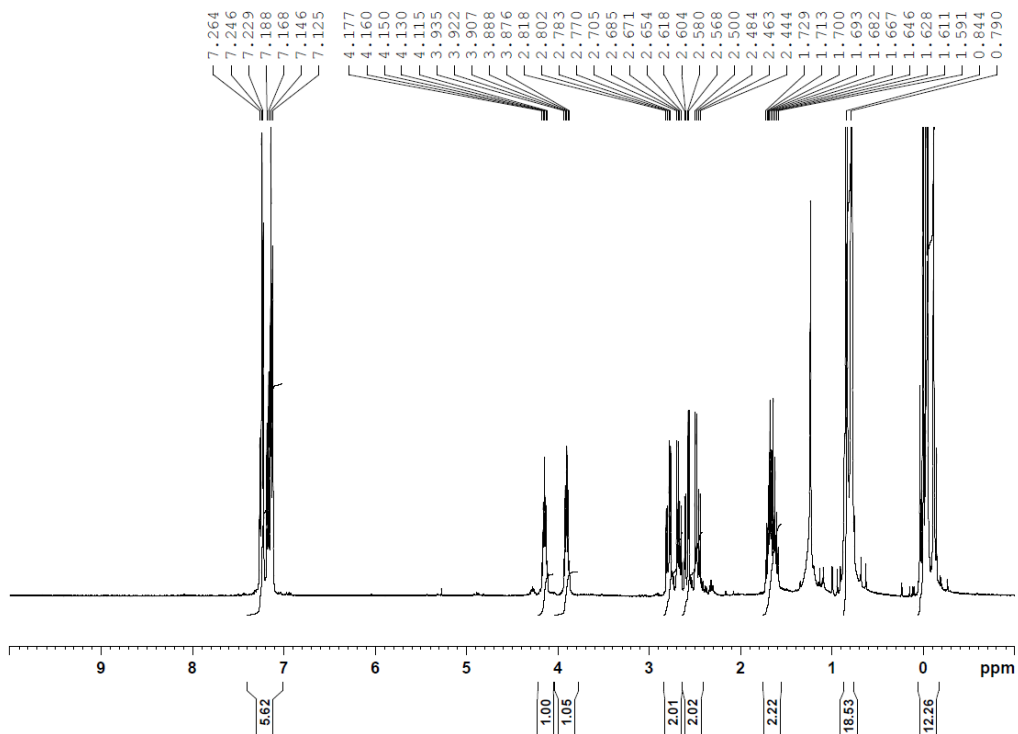
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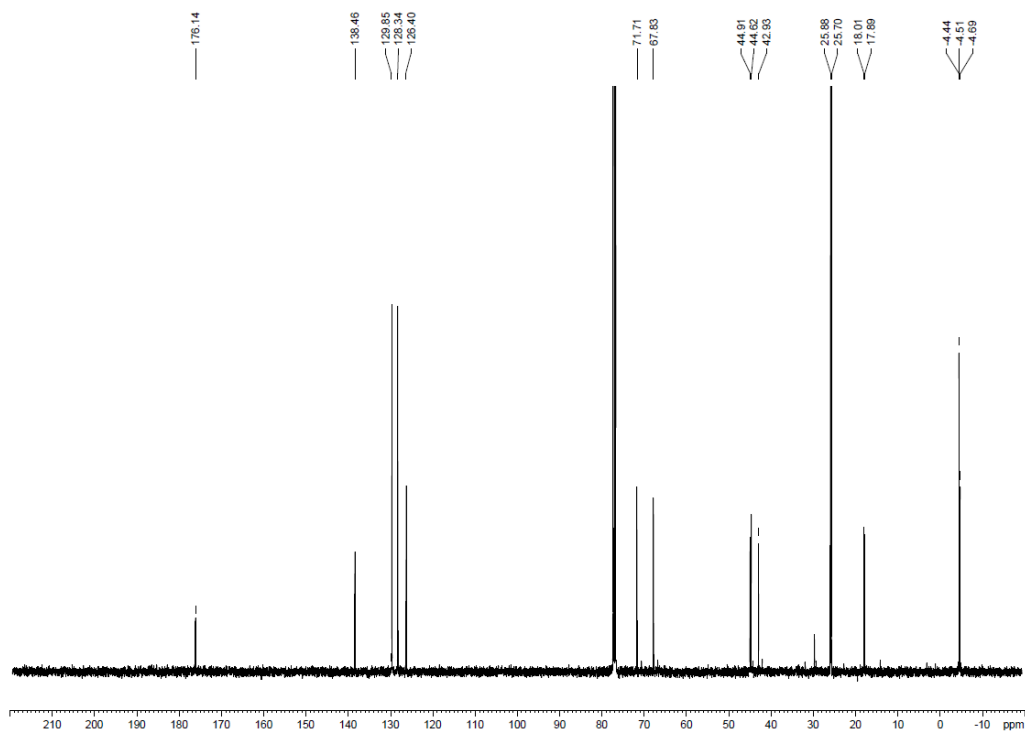


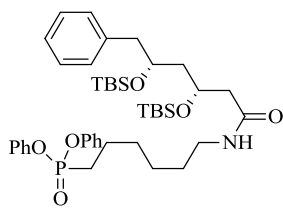
2.32

1d_1H_32_scans CDCl3 D:\Boddy 23



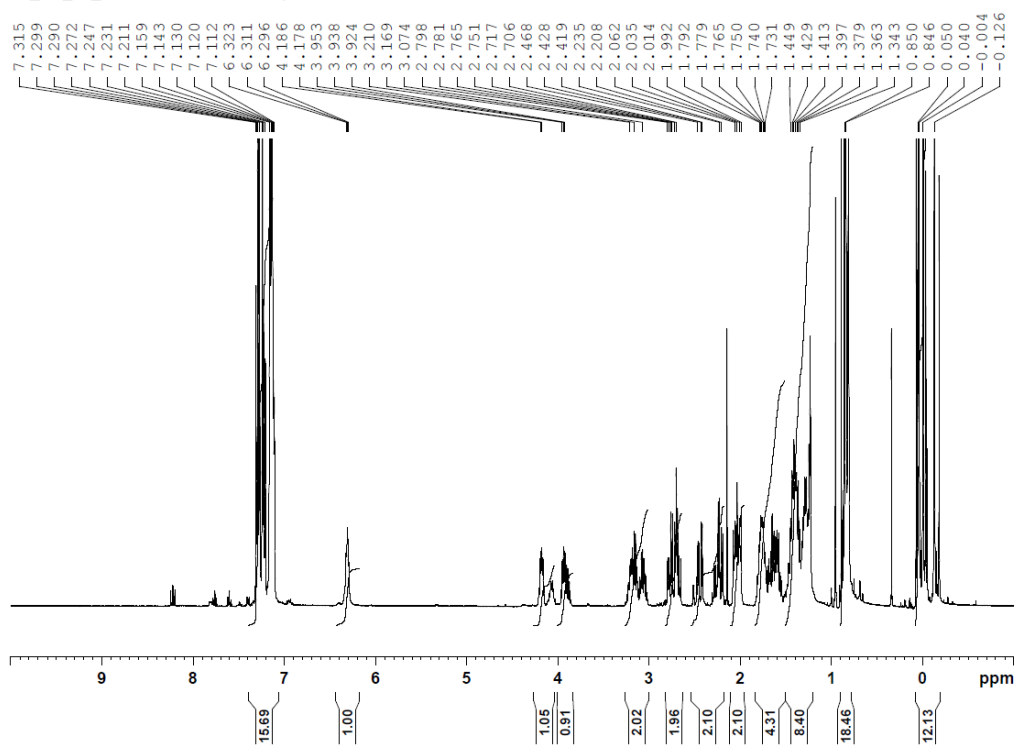
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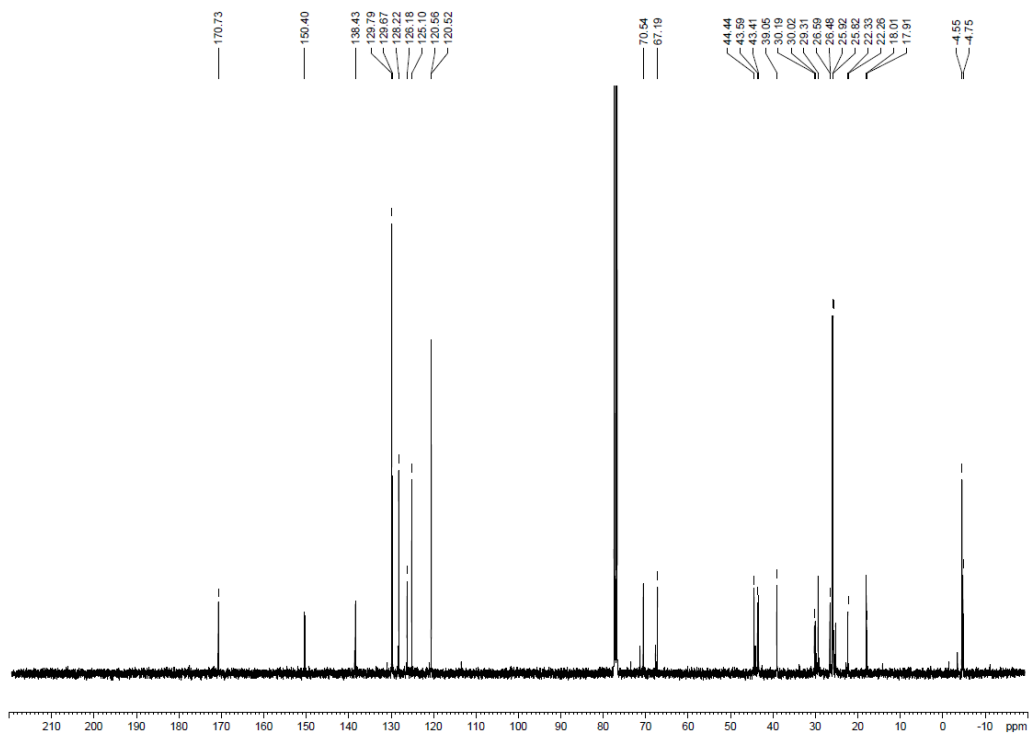


2.33

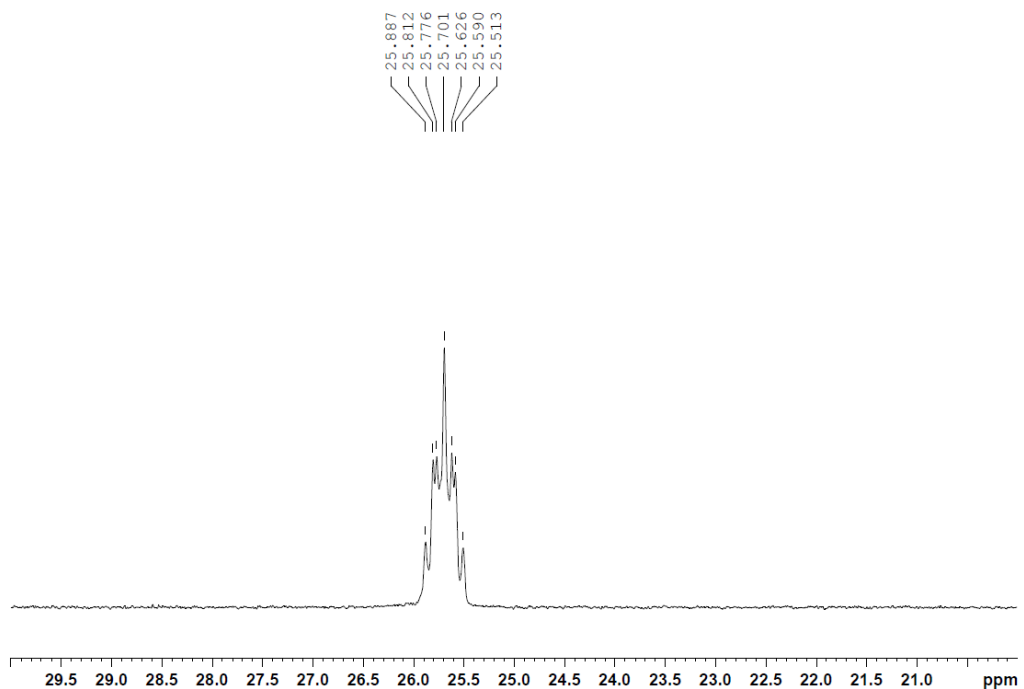
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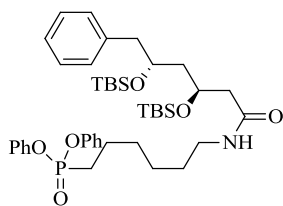


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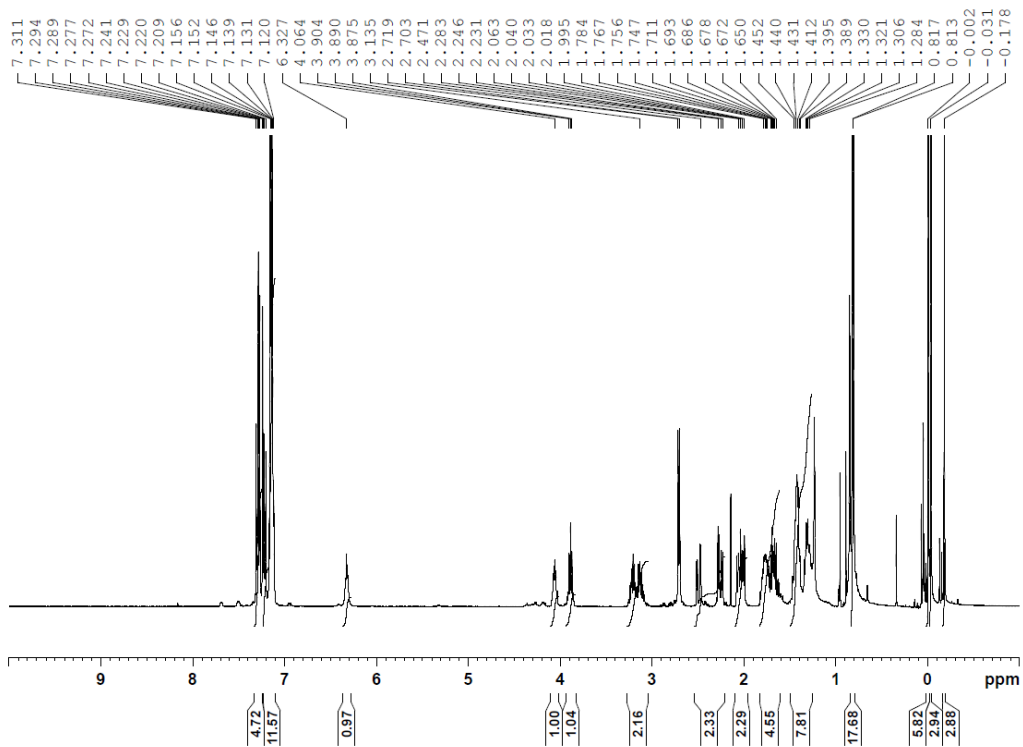
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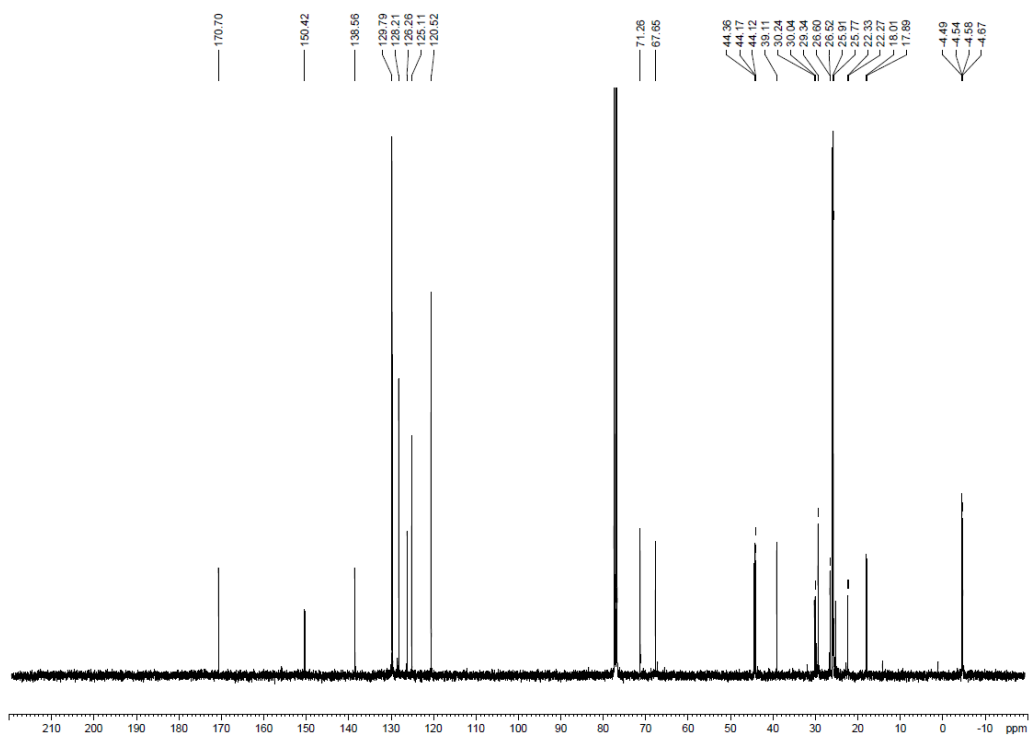


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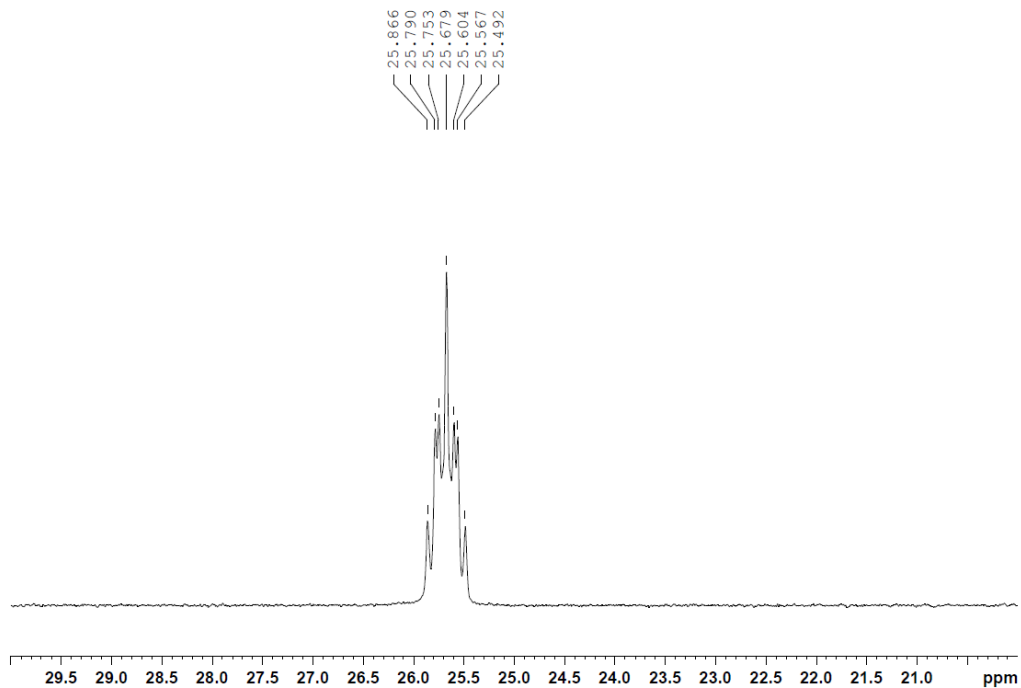
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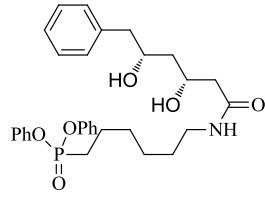


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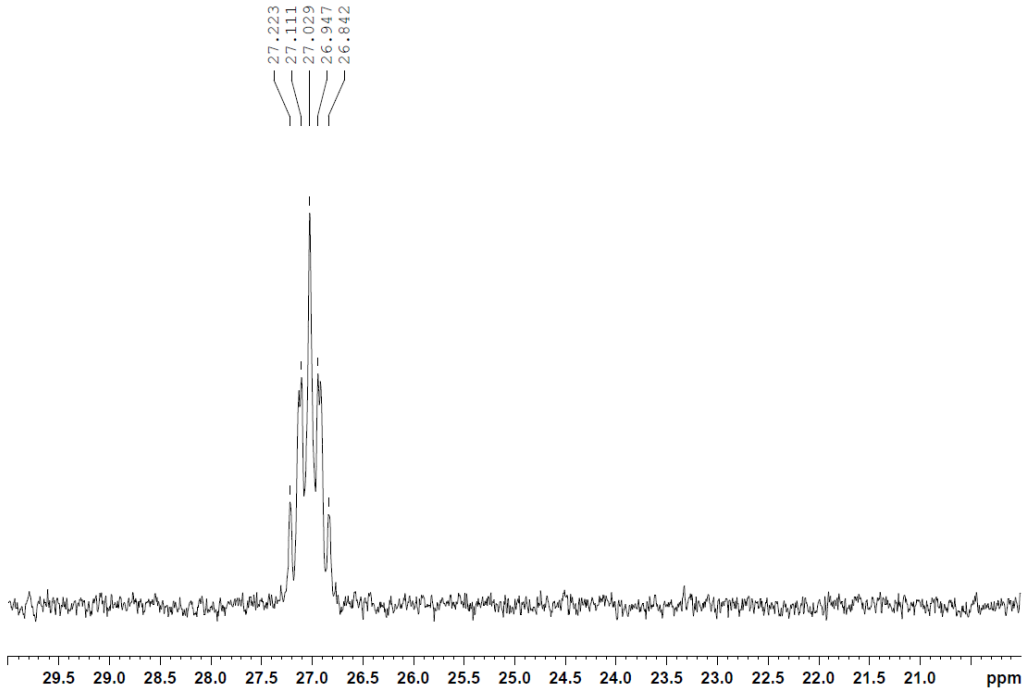
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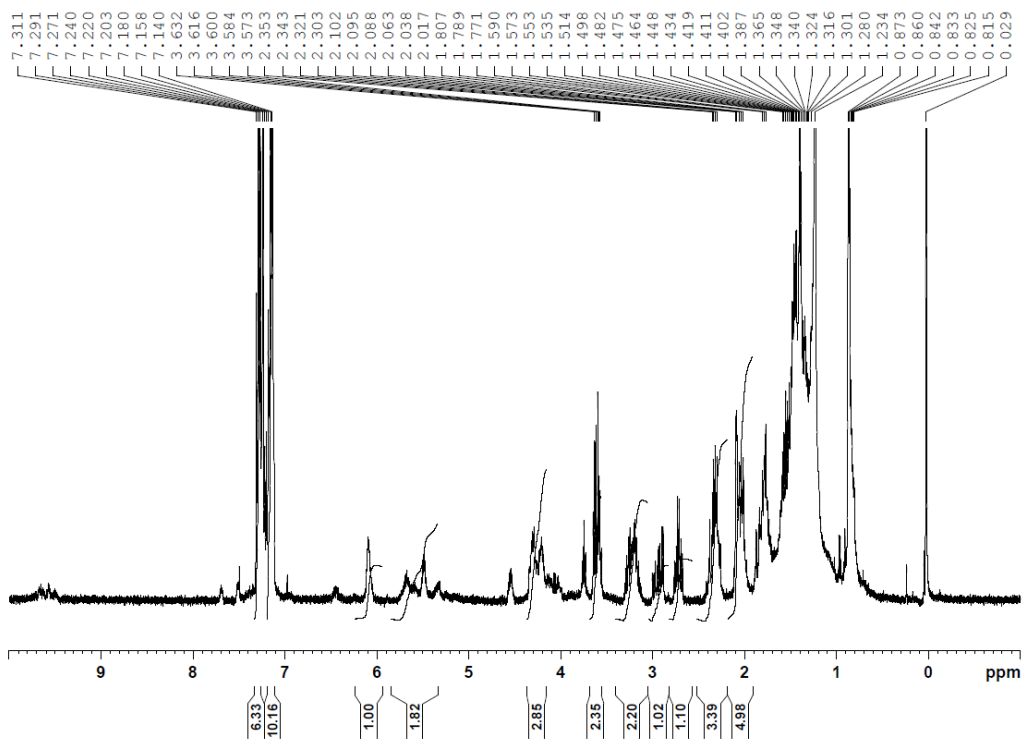


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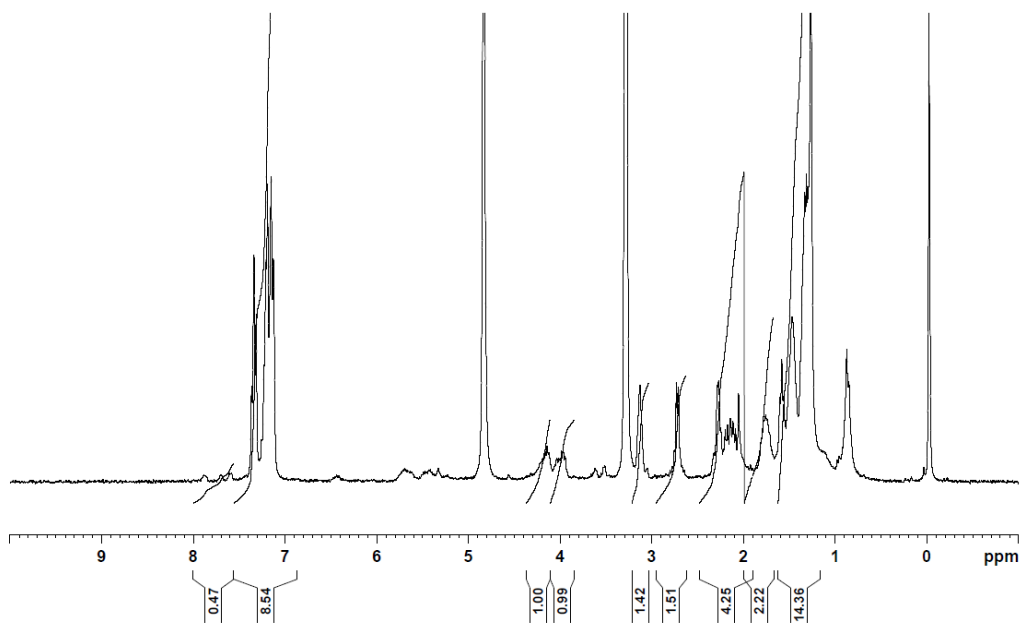
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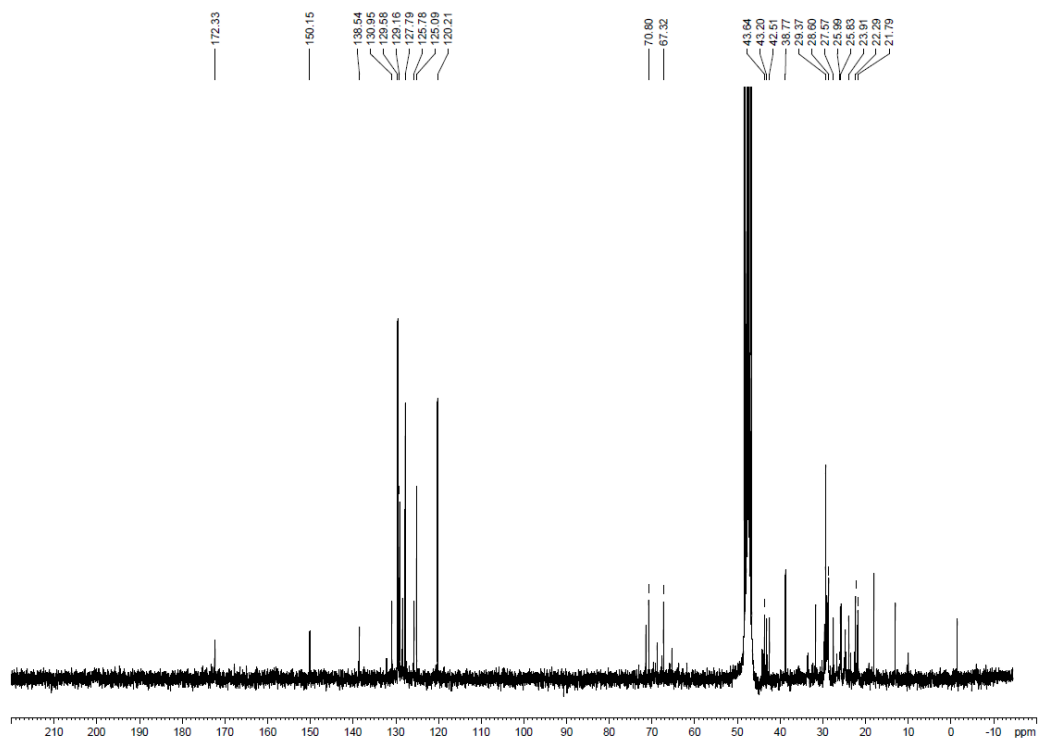
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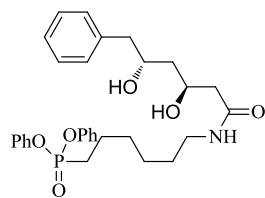


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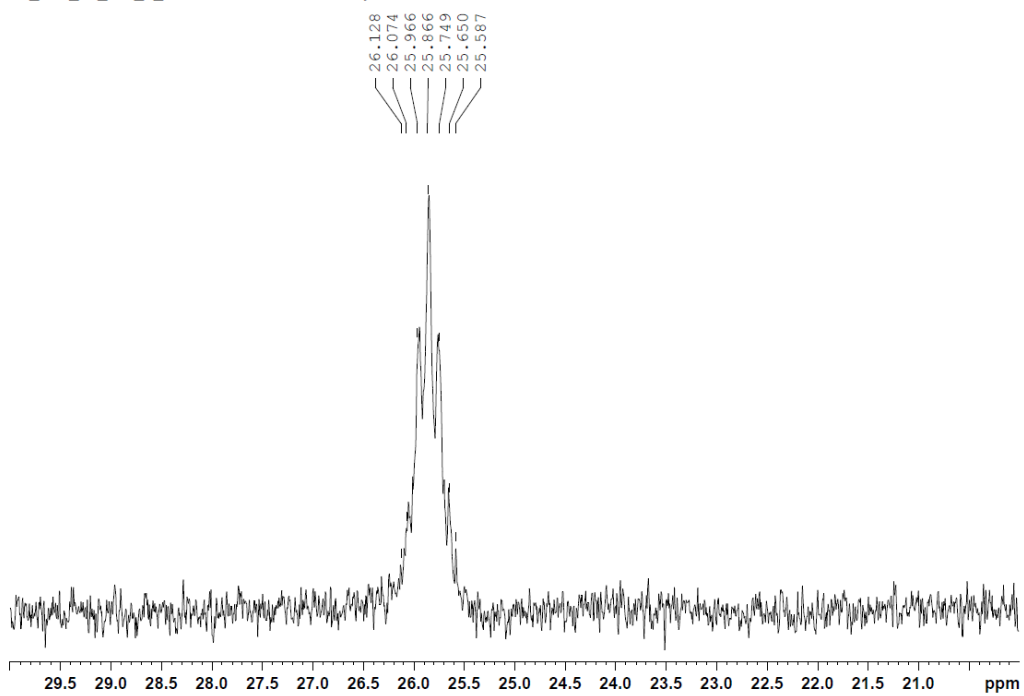
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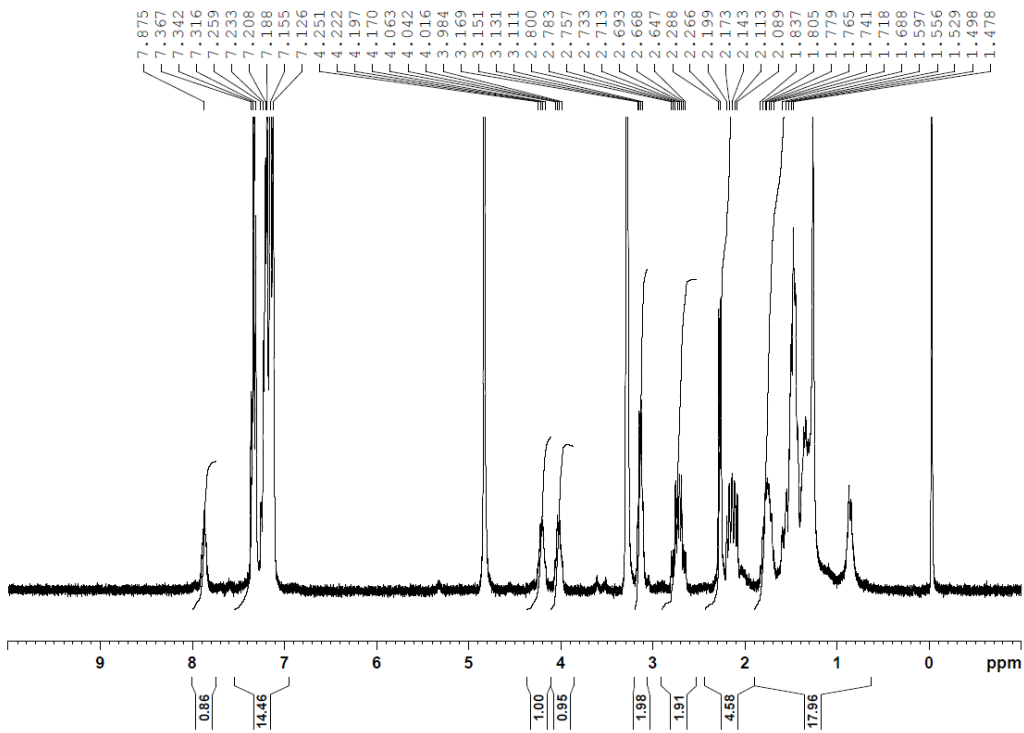


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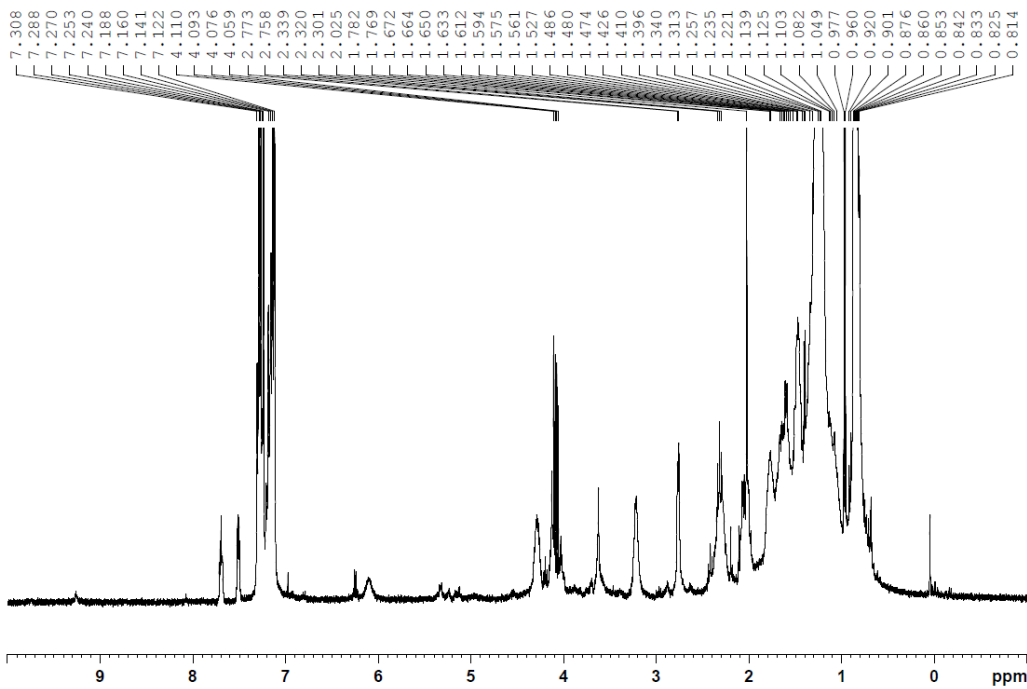
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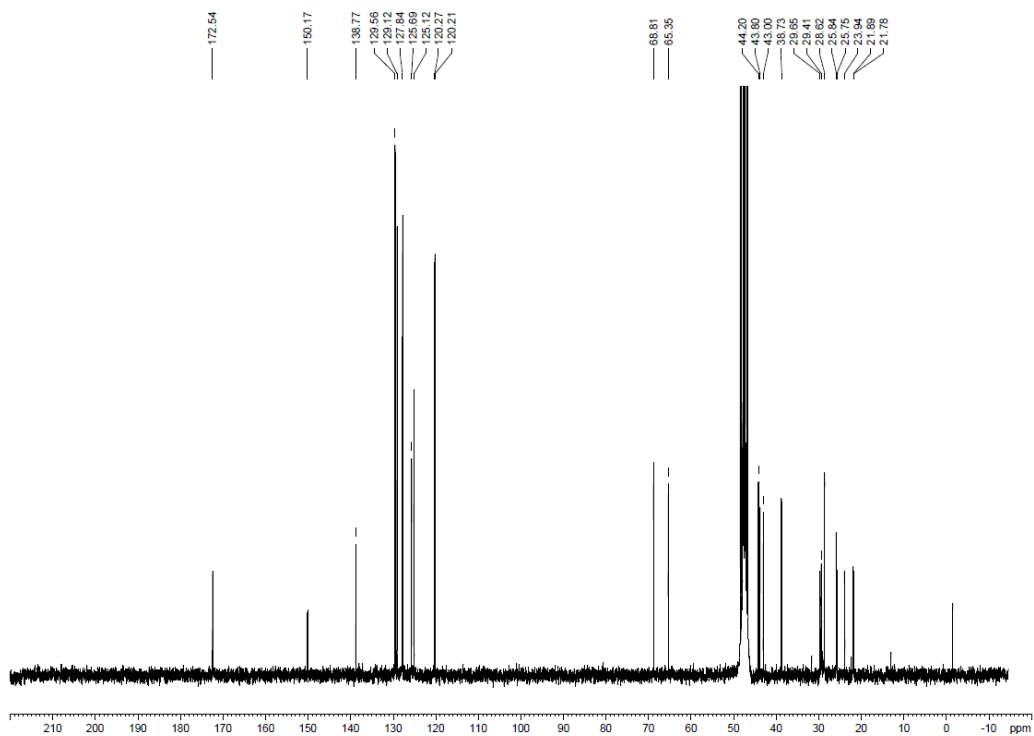
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1d_1H_32_scans CDCl3 D:\Boddy 35



¹³C NMR with proton decoupling



Chapter 3. Utilizing the biochemical machinery of thioesterases in the formation of macrolactams.

"One reason which has led the organic chemist to avert his mind from the problems of biochemistry is the obsession that the really significant happenings in the animal body are concerned in the main substrates of such high molecular weight and consequent vagueness of molecular structure as to make their reactions impossible of study by his available and accurate methods. " – Sir Frederick Gowland Hopkins

3.1 Introduction

Bristol-Myers Squibb made several analogs¹ of the antitumor agent epothilone B in hopes of improving in vivo activity. They found that exchanging the lactone oxygen for a nitrogen, generating a lactam, the molecules increased stability and activity². The cyclization of the polyketide epothilone B is catalyzed by a thioesterase domain and there are macrolactam polyketides³. It follows that if macrolactams can be formed from TEs, we could utilize this biochemical machinery to create a chemoenzymatic approach to lactam-derivatives of natural products. While their bacterial origins, termination pathways and chemosynthetic approaches may vary, there is precedent that makes this a viable synthetic tool. The closely related NRPS-TEs cyclize macrolactams⁴ and PKS TEs have been shown to cross couple thioester-substrates and amines⁵. Unpublished work⁶ by Monica Wirz in the Boddy lab has also shown *in vitro* that some thioesterases have a broad substrate tolerance for lactone formation. Synthesis of an amino version of Wirz's substrates would give insight into the possibility of using those TEs to catalyze the formation of lactams.

3.1.1 Reasons for switching nitrogen atoms for oxygen atoms in pharmaceuticals

When the antitumor activity of taxanes was discovered, microtubule stabilizing agents became a new and effective treatment of cancer. Unfortunately, resistance builds up with these cytotoxic drugs and the search must begin again for new drugs that will stabilize the microtubules by a different mechanism. The epothilone family of natural products showed promise in this regard but poor metabolic stability and unfavorable pharmacokinetics forced researchers to find another solution². Bristol-Myers Squibb rationally designed a set of

semisynthetic analogues hoping to address the stability issue and noticed that hydrolysis of the ester linkage may be an issue.

They addressed this issue by synthesizing ixabepilone, a semi synthetic version of epothilone B that replaced the lactone oxygen atom with a nitrogen atom. (Figure 3.1.1) To their satisfaction, this macrolactam was not susceptible to hydrolysis by esterases, had improved water solubility and required less harmful solubilising agents. Through *in vivo* and *in vitro* evaluation, Bristol-Meyers Squibb had found among other things, their synthetic analogue had a higher antitumor efficacy and lower cytotoxicity than the natural analogue epothilone B. Unlike many pharmaceutical drugs, ixabepilone survived all three phase of clinical trial⁷ and is currently being sold under the trade name Ixempra for the treatment of breast cancer when other chemotherapy medicines are ineffective⁸.

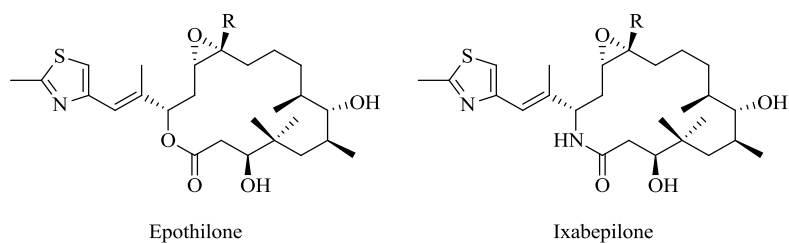


Figure 3.1.1 Epothilone and the chemically derived ixabepilone

The semi-synthetic approach⁹ used by Bristol-Meyers Squibb is impressive; noticing that the lactone of epothilone was actually an allylic ester, they formed a π -allylpalladium complex and trapped it with an azide. (Figure 3.1.2) Hydrolysis of the azide through a modified Staudinger reaction and subsequent recyclization with a coupling reagent provided ixabepilone. This paper also assessed the practicality of a total synthesis of ixabepilone. They did attempt the synthesis and their preliminary results showed that it was unlikely this route would produce sufficient quantities of material. The key step, a ring closing metathesis, either reversed the alkene stereochemistry or closed a more highly substituted olefin leading to the low yield. Their use of an RCM is a common way of synthesizing polyketide natural products¹⁰ but what if there were another approach? There are polyketides with lactam¹¹ cores, *in vitro* cyclization studies on thioesterases^{12,13} and TEs that form macrolactams;^{14,15} could we use a thioesterase to form this amide bond?

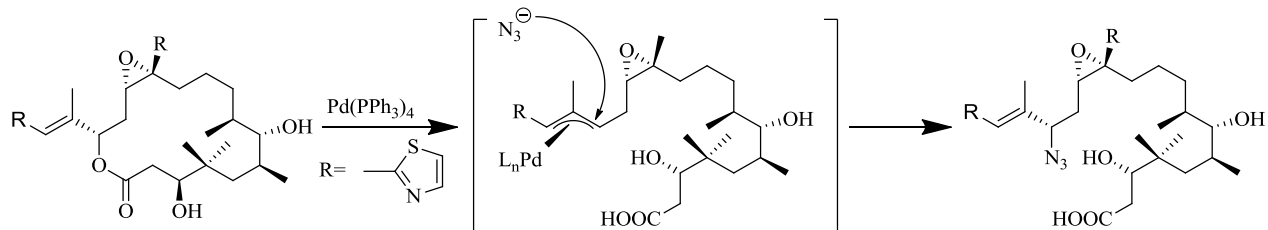


Figure 3.1.2 Semi-synthesis of ixabepilone by Pd-catalyzed nucleophilic substitution

3.1.2 Macrolactamization compared to macrolactonization

While macrolactam polyketides all have an amide bond in their rings, their only other similarity is that they were all extracted from bacterial sources. Some of the gene sequences contain thioesterases and others are cyclize through an ACP. Their syntheses vary from forming the ring through peptide coupling and through similar techniques used in macrolactone syntheses. Regardless of their differences, biochemical macrolactonization and macrolactamization fundamentally arise from the attack of a terminal nucleophile which is catalyzed by a folding enzyme.

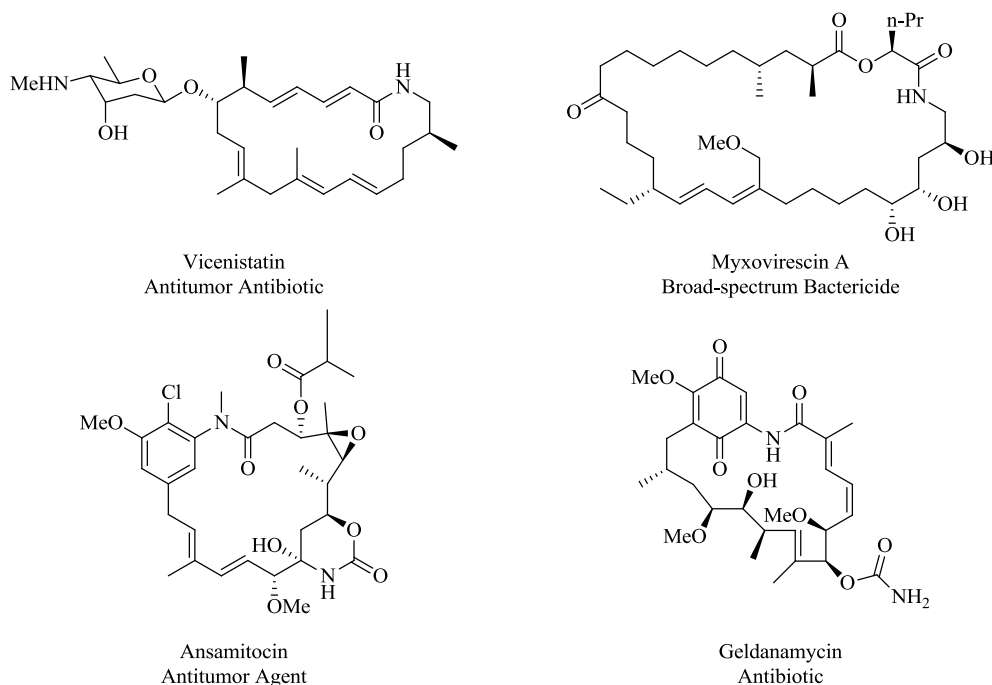


Figure 3.1.3 A few examples of macrolactam polyketides natural products

The four natural products seen in Figure 3.1.2 are representative of polyketide macrolactams. They all contain multiple ketide units and their structures are consistent with type I polyketides biosynthesis. The genera of producing organisms vary substantially. While they are all isolated from bacterial sources, vicenistatin and ansamitocin are derived from *Streptomyces*¹⁶ and *Nocardia*¹⁷ respectively and myxovirescin is isolated from the distantly related delta-proteobacteria *myxococcus virens*¹⁵. In trying to find a thread to determine a general biosynthetic approach towards macrolactam formation, it is safe to say it is not dependent on the genera of the bacteria.

Synthetic chemists usually try and find handles in which to make disconnections in their retrosyntheses. While there have been total syntheses of vicenistatin¹⁹ and myxovirescin A²⁰, their approaches are represented in the total syntheses of dechloroansamitocin²¹ and geldanamycin²². Unlike macrolactones, activation of carboxylic acids for peptide coupling reactions²³ is a well explored field and this is how Kirschning *et al.* synthesized the core of geldanamycin. (Figure 3.1.4) The more common approach (and the first unsuccessful approach for Kirschning) is through ring closing metathesis. The Andrus lab's synthesis of dechloroansamitocin (Figure 3.1.5) utilizes the Grubbs I catalyst to form their ring. As is the case with RCM²⁴, there was a 3:1 mixture of stereoisomers and it was difficult to separate. While there are different approaches to lactam synthesis, if there was chemoenzymatic methodology, it could be utilized to decrease unwanted by-products.

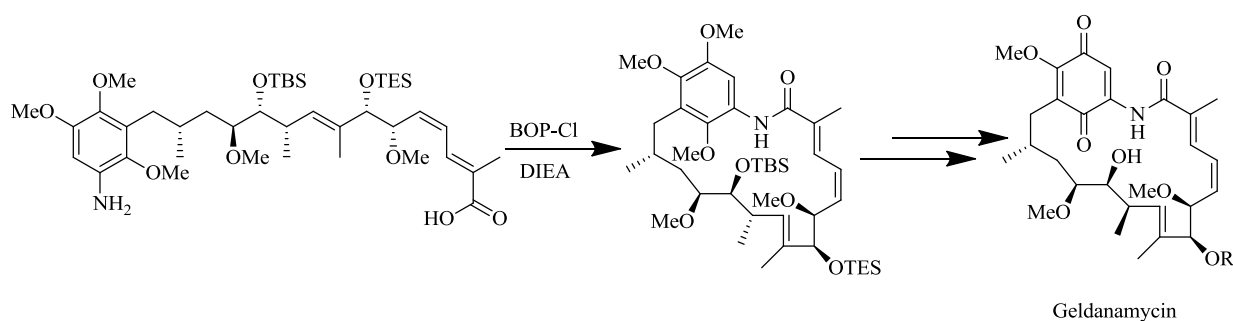


Figure 3.1.4 Carboxylic activation through BOP-Cl towards the total synthesis of geldanamycin

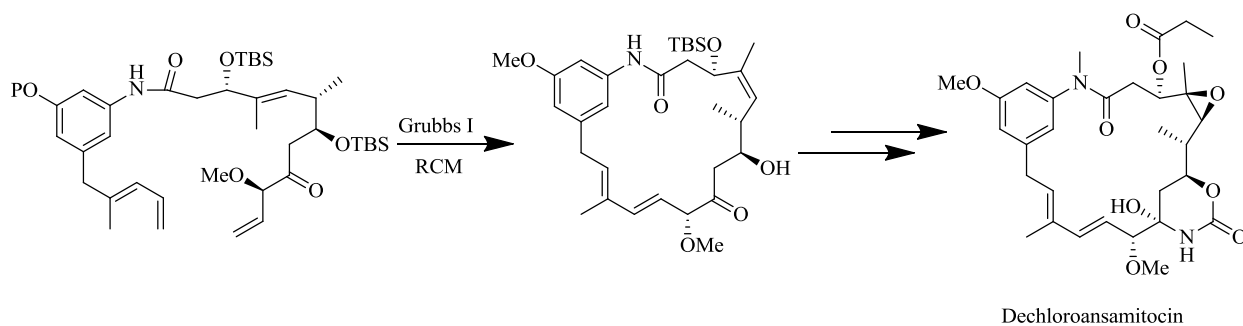


Figure 3.1.5 Ring-closing metathesis towards the total synthesis of dechloroansamitocin

The four above-mentioned lactam polyketides also have major differences with respect to the biosynthesis of their cyclic cores. Cyclization is typically catalyzed by a thioesterase domain and this is the case with the non-benzene containing polyketides vicenistatin¹⁴ and myxovirescin A²⁵. However, the biosynthesis of vicenistatin¹⁴ and geldanamycin²⁶ are loaded onto the final ACP and released by a separate “downloading” enzyme²⁷. While macrolactam formation in Type I PKSs are inconsistent, the closely related NRPSs for their C-N bonds almost exclusively with TEs. Furthermore, studies done in the Boddy lab have showed that Zea TE is substrate tolerant⁶ and can incorporate an exogenous nitrogen-based nucleophile through cross coupling.⁵ The studies discussed below will show that there is good reason to believe that PKS TEs can be reliably used to macrolactamize polyketide substrates.

3.1.3 Zea TE and related thioesterases are good candidates for macrolactamization

Resorcylic acid lactones (RALs), like zearalenone and radicicol, are a group of type I polyketide natural products produced by a variety of different fungal strains²⁸. (Figure 3.1.6) Their biosyntheses includes a thioesterase domain, Zea TE and Rad-TE for zearalenone and radicicol respectively, that has been studied for their substrate tolerance in forming 10-18 membered lactones.⁶ Investigation into the ring-closing ability of RAL TEs stemmed from the discovery that Zea TE not only shows *in vitro* macrocyclization activity for a non-native substrate but can also incorporate other nucleophiles. Our goal is determine if this broad substrate tolerance and chemoselective reactivity will allow for the use of an internal amino nucleophile for form a macrolactam.

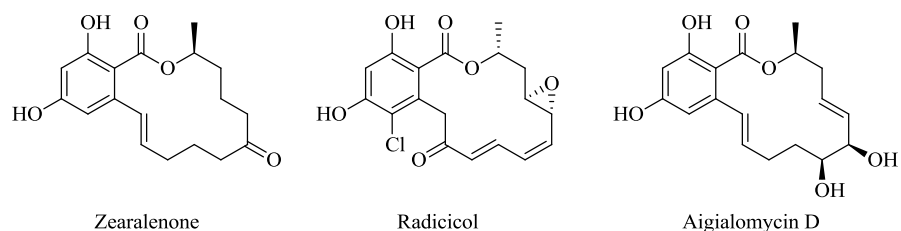


Figure 3.1.6 A few examples of resorcylic acid lactones (RALs)

Wirz *et al.* designed and synthesized simplified SNAC thioesters modeled from Zearalenone as well as substrates with varied the chain lengths and subjected them to enzymatic cyclization conditions. (Scheme 3.1.7) This *in vitro* chemoenzymatic assay showed that the native 14-membered ring and other ring sizes were cyclised by both Zea TE and Rad TE. Furthermore, unlike TEs from bacterial sources such as DEBS TE¹³, the substituents surrounding the macrolactonization are unnecessary for substrate recognition. This means that the substrates fed to the fungal TEs have fewer structural requirements to enter the active site. Unfortunately, this substrate tolerance was shown for hydroxyl groups only but Zea TE was shown to be able to incorporate nitrogen as a nucleophile as well⁵.

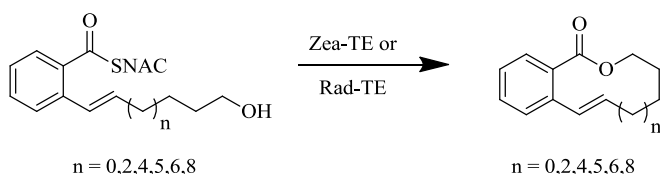


Figure 3.1.7 Work by Wirz *et al.* showing the substrate tolerance of Zea TE and Rad-TE catalyzed macrolactonization

Wang *et al.* studied⁵ the activity of Zea TE exclusively and their results include kinetic characterization and cross-coupling activity. Kinetic analysis indicated that the specificity constant for the catalysis of macrocyclization by Zea TE was 1000-fold greater than those seen for thioesterases of epothilone and 6-dEB. The more interesting result came from the cross coupling experiment. *In vivo* characterization of the module containing Zea TE has been shown to form ethanol and butanol esters instead of the hydrolysis product in the presence of their corresponding alcohols.²⁹ Wang *et al.* showed that this transesterification was catalyzed by the thioesterase. (Scheme 3.1.8) He incubated the NAC thioester with various oxygen and nitrogen based nucleophiles and found their nucleophilic attack were faster than water. Unlike most characterized thioesterases, Zea TE underwent cross coupling in an aqueous buffer with a low

concentration of an exogenous nucleophile and incorporated both oxygen and nitrogen atoms.

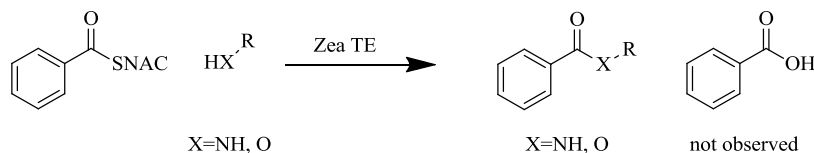


Figure 3.1.8 Work by Meng *et al.* showing Zea TE can catalyze cross-coupling of oxygen and nitrogen based nucleophiles

3.1.4 Conclusion

As was shown by ixabepilone, the exchange of a nitrogen atom for an oxygen atom can substantially impact activity. Synthesizing these analogues through a total synthesis route is difficult to scale-up and a semi-synthetic requires the natural product. While chemosynthesis of macrolactams is easier than macrolactones, the sterics and entropy still factor into formation of the ring system. The biochemical machinery also varies in the formation of macrolactams of polyketide natural products. Fungal TEs could provide a chemoenzymatic approach to this synthetic need because they are substrate tolerant and can use nitrogen-based substrates as nucleophiles.

This work addresses the need for chemoenzymatic methodology to synthesize macrolactams. By synthesizing a simplified SNAC thioester modeled from Zearalenone with a terminal nitrogen, treatment of this substrate with Zea TE will show if *in vivo* macrolactamization by a non-native substrate is possible. Herein, we present: (1) the three routes towards this substrate (2) a likely successful synthetic route towards the product and (3) a brief synopsis of our future efforts towards this project.

3.2 Synthesis of macrolactam 3.8

Wirz *et al.* showed⁶ that 14-membered lactones can be formed catalytically through an *in vitro* reaction with either Zea TE or Rad-TE. To have a study comparable to theirs, we wanted to synthesize a similar substrate with a nitrogen nucleophile. (Figure 3.2.1) Their thioester substrate was actually synthesized by the ring opening of the macrolactone. This has two advantages: they are able to have an authentic standard for LC-MS and NMR analysis and it's likely the highest yielding approach to the synthesis. For our route, we wanted to synthesize the

macrolactam so that we had a standard but wanted to see if we could decrease the number of steps required to get to the target. Herein, we present three routes towards the macrolactam.

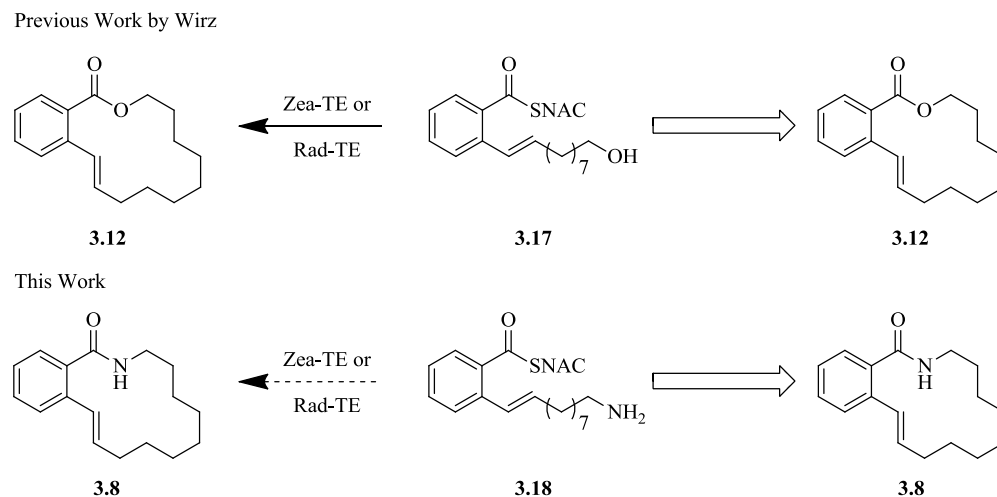


Figure 3.2.1 Comparing the previous enzymatic macrolactonization substrate to the macrolactamization substrate in this work

3.2.1 Retrosynthetic Analysis of Macrolactam 3.8

We envisioned formation of the macrolactam **3.8** to happen from one of two possible disconnections: through a ring-closing metathesis at the alkene position or a peptide coupling in amide bond formation. (Figure 3.2.2) The first approach was direct formation of the macrolactam from the amide **3.7**. Route II, the shortest route, would be cross metathesis from the vinyl ester **3.2** and protected amine **3.8**. Finally, a variation on the route taken by Wirz et al., Route III forms macrolactone (**3.12**) followed by a methoxide opening to afford **3.13** and conversion of the alcohol to an amine for peptide coupling forms the target molecule.

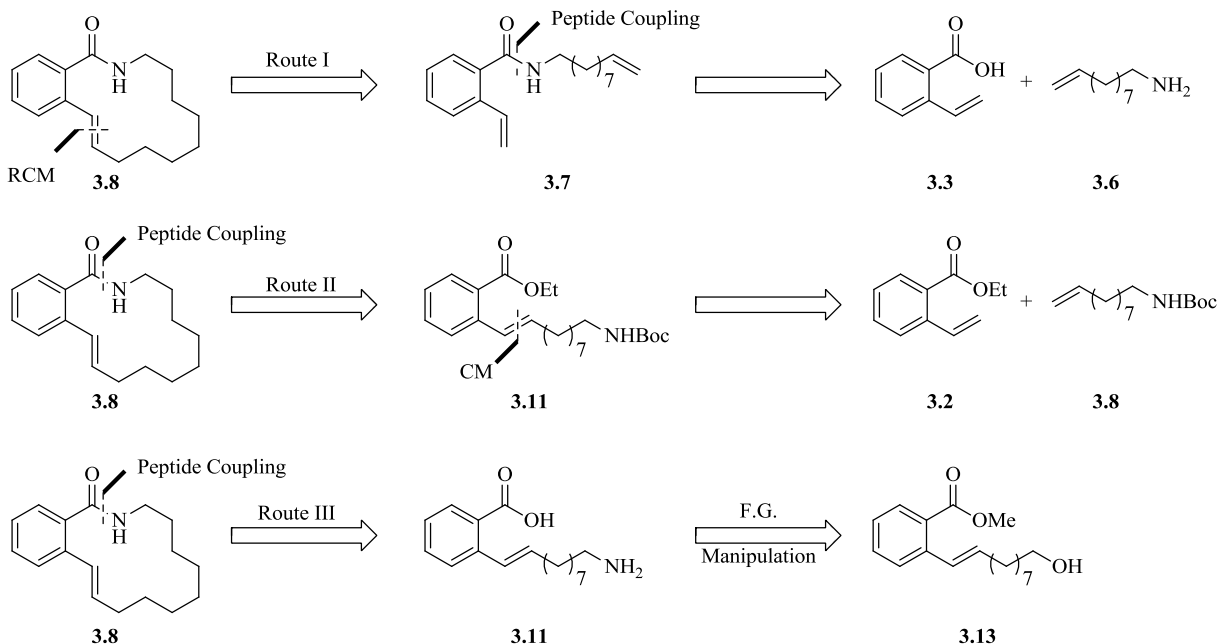
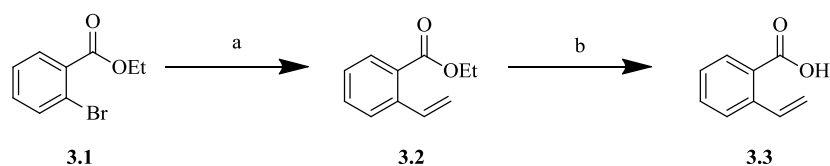


Figure 3.2.2 The three routes towards the synthesis of macrolactam **3.8**

3.2.2 Route I: Synthesis through Ring Closing Metathesis

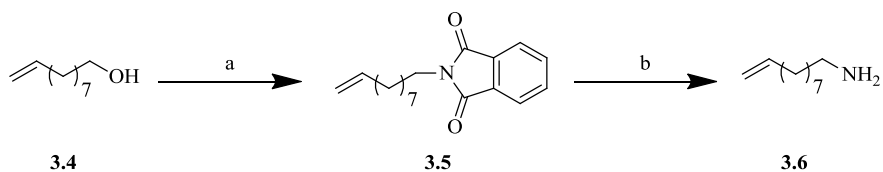
Vinyl benzoic acid **3.3** and decenamine **3.6** are not commercially available so the first point of order was to synthesize them. (Scheme 3.2.1 and Scheme 3.2.2) Denmark *et al.* developed high yielding palladium cross coupling conditions³⁰ that use an inexpensive, non-toxic siloxane as opposed to the highly toxic stannanes used in Stille coupling. This converts the bromo ethyl benzoate **3.1** to the vinyl ester **3.2** in good yield and hydrolysis forms our first peptide coupling partner **3.3**. Through a Mitsunobu reaction³¹ with phthalamide, decenol **3.4** was converted to **3.5** and subsequent reflux with hydrazine hydrate formed decenamine **3.6**, the other coupling partner.

Scheme 3.2.1 Synthesis of vinyl benzoic acid **3.3**



Reactions and Conditions: (a) JohnPhos, PdBr₂, 2,4,6,8-tetramethyl-2,4,6,8-tetravinylcyclotetrasiloxane, TBAF, THF, 50 °C, 6h, 92 % (b) LiOH, H₂O, MeOH, rt., overnight, 96 %;

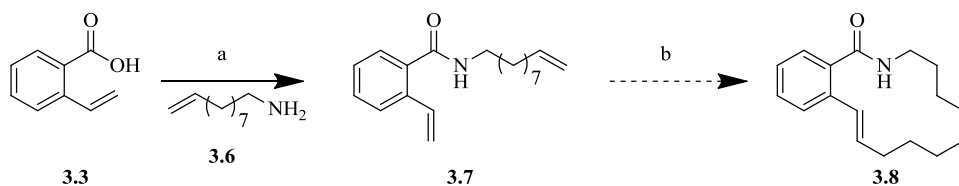
Scheme 3.2.2 Synthesis of decenamine **3.6**



Reactions and Conditions: (a) phthalimide, PPh₃, DIAD, Toluene, THF, 0 °C to rt., overnight, 89 %; (b) H₂N-NH₂·H₂O, EtOH, 85 °C, 3h, 76 %;

Coupling of the vinylbenzoic acid **3.3** and decenamine **3.6** could have been accomplished by many ways but EDC was used as peptide coupling reagent in the lactone substrate so it was a good place to begin. Having formed amide **3.7** with the EDC conditions in low to moderate yield, we found that using HOBt proceeded with more success. With **3.7** in hand, ring closing metathesis was the next step. (Scheme 3.2.3) While free amines have been known to cause trouble in RCM,³² lactams have been successfully synthesized from the corresponding amides. A study³³ by Marcaurrelle *et al.* showed the formation of 13- to 18-membered macrolactams via ring-closing metathesis is possible. For 14-membered rings, the conversion is equally effective with whichever generation catalyst used so we decided to go with the more stable Grubbs II catalyst.

Scheme 3.2.3 Route I: Attempted synthesis of macrolactam **3.8** by RCM of **3.7**

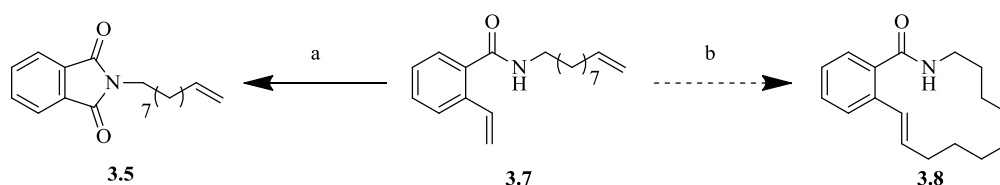


Reactions and Conditions: (a) **3.6**, TBTU, DIEA, DMF, rt., overnight, 94 %; (b) Grubbs II, toluene, 85 °C, overnight, 0 %;

We attempted the synthesis in the fume hood using the conditions outlined by Marcaurrelle and no macrolactam was formed but we were able to isolate a by-product. (Scheme 3.2.4) Reaction in toluene at 65 °C for 6 hours actually produced **3.5** through the incorporation of a water molecule and oxidation. While ruthenium oxidation is not uncommon³⁴, this result did take us by surprise and confirmation of the product was easy considering we had previously synthesized **3.5**. Assuming the water incorporation was due to the solvent and less air-sensitive

techniques, we attempted the synthesis in a glove box. Ring-closing metathesis reactions are typically done in either toluene or DCM³⁵ so we attempted the synthesis with DCM as a solvent. With the change in solvent, we also had to change the temperature and duration of reaction. Our final conditions employed DCM, the same catalyst loading and reaction for 36 hours at 45 °C in a glove box. By NMR, a doublet corresponding to the alkene proton adjacent to the benzene ring appeared so we were hopeful that the reaction had occurred but the yield was quite low. Although **3.8** may have formed, we were unable to isolate macrolactam from this route. Our hypotheses regarding the failure of product formation were either the reaction was happening too slowly or the alkene directly attached to the benzene ring was difficult to complex with and therefore difficult to form a ring.

Scheme 3.2.4 Using toluene for RCM produces surprising side product

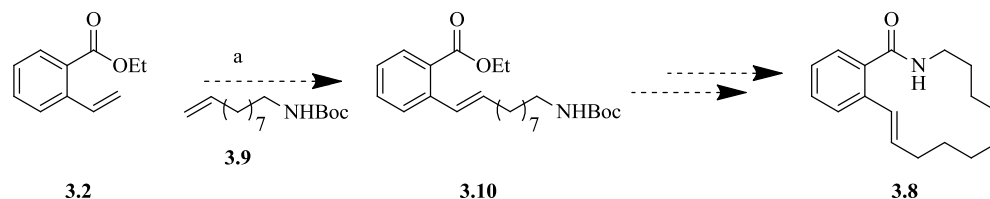


Reactions and Conditions: (a) Grubbs II, toluene, 85 °C, overnight, 15%; (b) GII, DCM, 45°C, 36h, 0%;

3.2.3 Route II: Synthesis through peptide coupling from cross metathesis

Having seen that ring closing metathesis was difficult with **3.7**, we anticipated that cross metathesis would likely have similar issues. That being said, we had the reagents at hand and this was a quick route to an intermediate that could form the macrolactam. (Scheme 3.2.5) Since free amines have been known to complex with the ruthenium of Grubbs catalysts³², the amine was Boc-protected. Using the same DCM conditions above for the RCM, we attempted the cross-metathesis reaction however no product was generated.

Scheme 3.2.5: Route II: Synthesis of **3.10** from cross metathesis

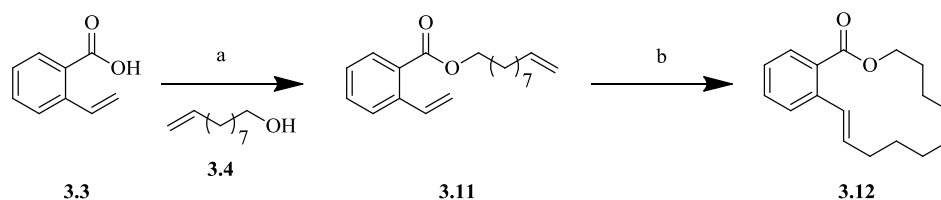


Reactions and Conditions: (a) Grubbs II, DCM, 45 °C, 36h, 0 %

3.2.4 Route III: Synthesis through peptide coupling from functional group transformation

Having failed with the two direct routes towards lactam **3.8**, the next approach began the same as the approach taken by Wirz *et al.*: form the macrolactone then open it with a nucleophile. Her sequence begins with an EDC coupling of decenol **3.4** to vinyl benzoic acid **3.3** to form **3.11**. (Scheme 3.2.6) Unlike the unsuccessful RCM with the macrolactam, **3.11** did form the macrolactone **3.12** but it should be noted that the yields were variable and typically low.

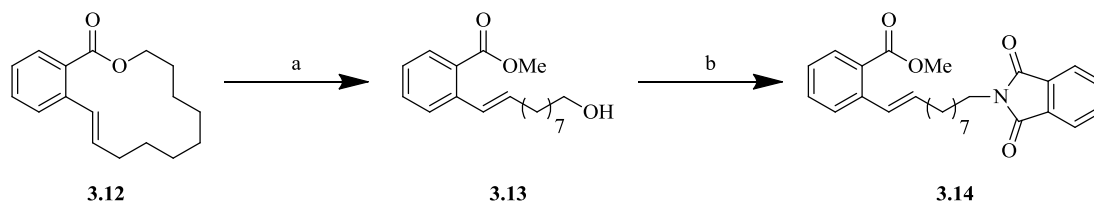
Scheme 3.2.6 Route III: Synthesis of macrolactone **3.12**



Reactions and Conditions: (a) 3.4, EDC·HCl, DMAP, Et₃N, DCM, rt., 24h, 72 %; (b) Grubbs II, toluene, 85 °C, overnight, 63 %;

Macrolactone **3.12** was opened with a methoxide to give the ring opened form **3.13**. (Scheme 3.2.7) Having successfully used a phthalimide to convert decenol **3.4** to decenamine **3.6**, there was precedent for using this methodology to convert the terminal alcohol of **3.13** into an amine. Unfortunately, the use of refluxing hydrazine used to uncover the amine would destroy the ester moiety of **3.13** so we sought different conditions. Work by Ganem *et al.* had successfully converted phthalimides to primary amines through a two stage NaBH₄/acetic acid³⁶ procedure and this seemed like an approach worth pursuing. We used the same Mitsunobu conditions to form the phthalimide **3.14** and we were now ready to remove the protecting group.

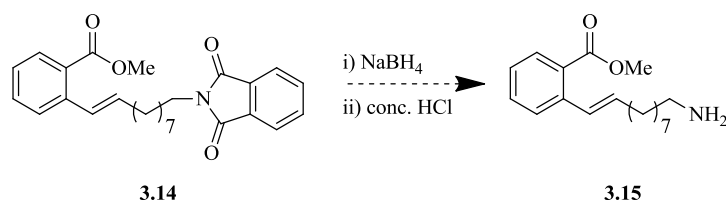
Scheme 3.2.7 Route III: Synthesis of ring opened **3.14**



Reactions and Conditions: (a) NaOMe, MeOH, rt., overnight, 72 %; (b) phthalimide, PPh₃, DIAD, Toluene, THF, 0 °C to rt., overnight, 71 %;

Unfortunately, the conditions never produced free amine **3.14**. (Scheme 3.2.8) As we followed by proton NMR and TLC, the NaBH₄ would never fully deprotect the phthalimide but instead partially reduced it. We tried various conditions to try removing the reduced phthalimide but our efforts were fruitless. There were also conditions³⁷ shown by Sparatore *et al.* showing that they had removed a phthalimide through refluxing HCl but that also did not produce **3.15**. Having consumed all of reserves of **3.14** trying to resolve this issue, we had decided that we had to pursue another functional group transformation route.

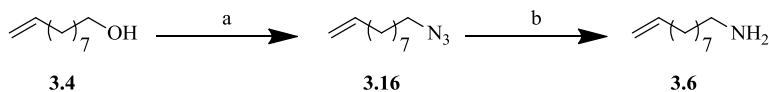
Scheme 3.2.8 Route III: Unsuccessful removal of phthalimide moiety of **3.14**



Reactions and Conditions: (i) NaBH₄, AcOH, 6:1 isopropanol:H₂O, rt., 24h, 0 %; (ii) 6N HCl, 1:1 EtOH:H₂O, reflux, 24h, 0 %;

It was suggested by a fellow researcher to form the primary amine **3.15** through a Staudinger reaction from the corresponding azide. Scheme 3.2.9 shows the conditions on a test substrate. A Mitsunobu reaction was employed using DPPA as a nucleophile to form **3.16**. To our content, the Staudinger reaction successfully unmasked the azide to form **3.6**.

Scheme 3.2.9 Synthesis of **3.6** through azide formation then the Staudinger reaction



Reactions and Conditions: (a) DPPA, PPh₃, DEAD, THF, 0 °C to rt., overnight, 89 %; (b) PPh₃, H₂O, rt., overnight, 68 %;

3.3 Conclusion and future work

Exchanging an oxygen atom for a nitrogen atom in macrocycles improves the stability of the substrate and can lead to increased biological activity.² There exists lactam polyketide natural products and some of these amide bonds are formed by a thioesterase. While zearelenone is a macrolactone, the substrate tolerance⁶ and ability of Zea TE to catalyze nitrogen-based nucleophilic attack⁵ leads us to believe that this thioesterase could also catalyze the chemoenzymatic synthesis of a macrolactam.

Researchers Wirz *et al.* synthesized a zearelenone mimic that was successfully macrocyclized *in vitro* by an excised Zea TE⁶. This work aimed at synthesizing a substrate similar to that used in the zearelenone study that would use a primary amine as its nucleophile. Three routes were unsuccessful at synthesizing the substrate through RCM, cross metathesis and conversion of a phthalimide to a primary amine. It was, however, successful in determining a hopeful route towards the intended target.

Future work would be to synthesize the substrate as seen in Figure 3.2.3 and successfully show the substrate tolerance of Zea TE. A study showing this enzyme catalyzing macrolactamization and macrothiolation on various ring sizes would also follow.

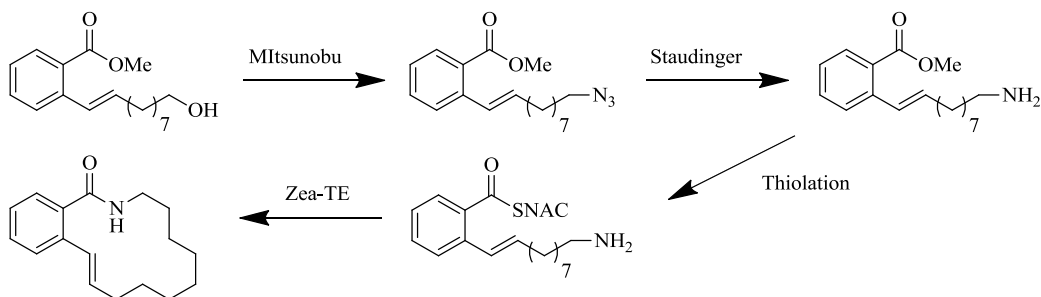


Figure 3.3.1 Future work in synthesizing a substrate that can undergo TE-catalyzed macrolactamization

3.4 References

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3.5 Experimental Section

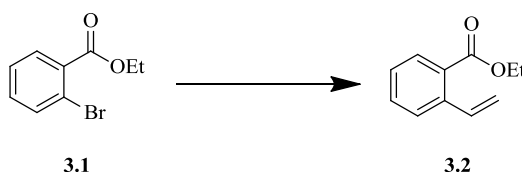
3.5.1 General Methods

Reactions were carried out under argon or nitrogen atmosphere with dry solvents and dried glassware under anhydrous conditions unless specified otherwise. All reactions in dry THF, Et₂O and DCM as solvent were dried through activated alumina columns. Reagents were purchased at the highest commercial quality and without further purification. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as a visualization and/or ceric ammonium molybdate (CAM), p-anisaldehyde (PA) and potassium permanganate (KMnO₄) as staining solutions. Flash chromatography was performed with E. Merck silica gel (60, particle size 0.040-0.063 mm) and preparative thin-layer chromatography (PTLC) separations were carried out on 0.25 mm E. Merck silica gel plates (60F-254).

¹H NMR, ¹³C NMR, ³¹P NMR spectra were recorded on the Bruker DPX-300 or AMX-300, AMX-300II and AMX-400 spectrometers and calibrated using residual undeuterated solvents as internal references. Data is reported as follows: chemical shifts in ppm (δ); multiplicities are indicated by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, m = multiplet, br = broad; coupling constants in Hz (J). Infrared spectra (IR) were collected on a Shimadzu FTIR-8400S in solution and reported in wavenumbers (cm⁻¹). HRMS were obtained on a Kratos Analytical Concept instrument (University of Ottawa Mass Spectrum Center).

3.5.2 Experimental Procedures

Ethyl 2-vinylbenzoate (3.2)



To a dry 50 mL flask, (2-biphenyl)di-tert-butylphosphine (0.094 g, 0.32 mmol, 0.1 equiv.) and palladium (II) bromide (0.100 g, 0.38 mmol, 0.1 equiv.) were added. After flushing

the flask with argon, 2,4,6,8-tetramethyl-2,4,6,8-tetravinylcyclotetrasiloxane (0.54 mL, 1.57 mmol, 0.5 equiv.), tetrabutylammonium fluoride (6.4 mL, 6.30 mmol, 2.0 equiv.) and THF (0.2 mL) were added. The mixture was stirred for 10 minutes and ethyl-2-bromobenzoate (0.5 mL, 3.15 mmol, 1 equiv.) was added. The reaction was warmed to 50 °C for 6 hours then taken off the heat and diluted with ether (20 mL). The reaction mixture was concentrated to near dryness and put through a silica plug. The reaction yield was 0.550 g (0.31 mmol, 92 %) and the product was taken onto the next step without further purification.

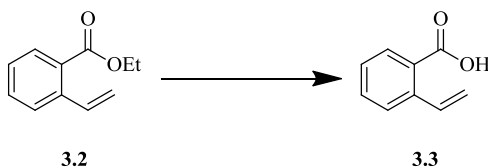
Rf: 0.35, 10 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.87 (dd, 1H, 9.26 Hz, 1.32 Hz), 7.63-7.48 (m 3H), 7.35 (td, *J* = 7.7 Hz, 1.4 Hz, 1H), 5.65 (dd, 1H, 17.5 Hz 1.4 Hz), 5.37 (dd, 1H, 11.0 Hz, 1.34 Hz), 4.38 (q, 2H, 7.0 Hz), 1.41 (t, 3H, 7.1 Hz) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 167.4, 139.4, 135.5, 132.1, 130.0, 129.0, 127.4, 127.2, 166.6, 61.0, 14.4 ppm.

NMR Spectra are consistent with those previously reported.¹

2-Vinylbenzoic acid (3.3)



To **3.2** (0.700 g, 3.97 mmol, 1 equiv.) dissolved in methanol (40 mL) at room temperature was added lithium hydroxide monohydrate (1.581 g, 37.74 mmol, 9.8 equiv.) in H₂O (16 mL). The reaction mixture was stirred overnight. The reaction was quenched with 10 % HCl until a pH of 2 and extracted with ether (3 x 20 mL). The organic layer was washed with brine (2 x 20 mL), dried over MgSO₄ and concentrated *in vacuo*. The compound was purified by column chromatography (silica gel, 20 % EtOAc/Hexane) to yield 0.565 g of **3.3** (3.81 mmol, 96 %).

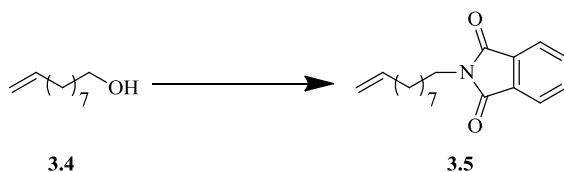
Rf: 0.18, 50 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 8.02 (dd, 1H, 9.26 Hz, 1.32 Hz), 7.63-7.49 (m 3H), 7.35 (td, *J* = 7.7 Hz, 1.4 Hz, 1H), 5.65 (dd, 1H, 17.5 Hz 1.4 Hz), 5.37 (dd, 1H, 11.0 Hz, 1.34 Hz) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 173.2, 140.4, 136.2, 133.2, 131.4, 127.7, 127.6, 127.2, 116.7 ppm.

NMR Spectra are consistent with those previously reported.¹

2-(dec-9-enyl)isoindoline-1,3-dione (3.5)



To 9-decene-1-ol (1.0 mL, 5.61 mmol, 1.0 equiv.) dissolved in THF (21 mL) was added phthalimide (0.825 g, 8.41 mmol, 1.5 equiv.) and triphenyl phosphine (2.206 g, 5.61 mmol, 1.0 equiv.). The solution is cooled to 0 °C. Diisopropyl azodicarboxylate (1.7 mL, 8.41 mmol, 1.5 equiv.) was dissolved in toluene (4.2 mL) and added to the previous solution at 0 °C. The resulting mixture was slowly put to room temperature and stirred overnight. The mixture was concentrated *in vacuo* and purified by column chromatography to afford yielding 1.425 g of yellow oil **3.5** (5.00 mmol, 89 %)

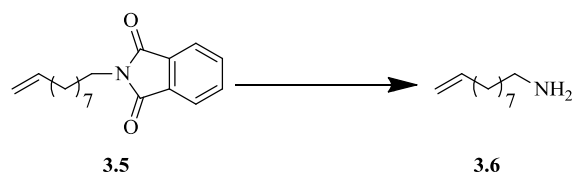
Rf: 0.31, 10 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.89-7.68 (m, 4H), 5.79 (tq, 1H, 6.9 Hz, 9.4 Hz), 5.02-4.88 (m, 2H), 3.66 (t, *J* = 6.6 Hz, 2H), 2.06-1.94 (m, 2H), 1.71-1.57 (m, 2H), 1.44-1.21 (m, 10H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 168.0, 139.0, 133.7, 132.0, 122.9, 113.9, 37.7, 33.6, 29.0, 29.0, 28.8, 28.7, 28.4, 26.6 ppm.

NMR Spectra are consistent with those previously reported.²

Dec-9-enylamine (3.6)



To 2-(dec-9-enyl)isoindoline-1,3-dione (0.950 g, 3.32 mmol, 1 equiv.) dissolved in ethanol (17 mL) was added hydrazine hydrate (0.4 mL, 6.64 mmol, 2 equiv.). The solution was refluxed at 85°C for 3 hours. The resulting foamy solution was taken out of the oil bath and placed in an ice bath (0 °C). The solid was filtered out and concentrated *in vacuo*. The solution was dissolved in 0.1 M HCl and extracted with EtO₂ (3 x 8 mL). The ether layers were combined, washed with brine (2 x 5 mL) and dried over MgSO₄. The solution was filtered, concentrated to dryness *in vacuo*. The reaction yield was 0.392 g (2.52 mmol, 76 %) and the product was taken onto the next step without further purification.

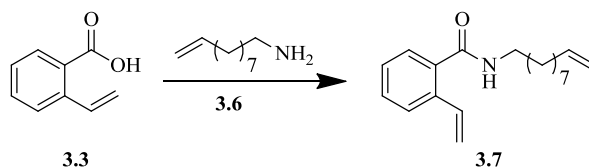
Rf: 0.50, 50 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 6.87 (br s, 1H), 5.79 (tq, 1H, 6.9 Hz, 9.4 Hz), 5.00-4.87 (m, 2H), 2.66 (br t, 2H, 7.2 Hz), 2.06-1.94 (m, 2H), 1.71-1.57 (m, 2H), 1.44-1.21 (m, 12H) ppm

¹³CNMR (100 MHz, CDCl₃): δ 139.5, 116.2, 40.9, 34.4, 29.2, 29.2, 29.1, 29.0, 29.0, 27.0 ppm

NMR Spectra are consistent with those previously reported.³

2-Vinyl benzoic acid dec-9-enyl amide (3.7)



Vinyl benzoic acid **3.3** (0.100 g, 0.67 mmol, 1 equiv.) was dissolved in DMF (7 mL) and added drop wise to the free amine **3.6** (0.126 g, 0.81 mmol, 1.2 equiv.). TBTU (0.260 g, 0.81 mmol, 1.2 equiv.) and DIEA (0.35 mL, 2.01 mmol, 3 equiv.) were added to this solution and the reaction was mixed under argon for 24 hours at room temperature. After checking by TLC for completion, the reaction mixture was poured over brine (10 mL) and extracted with EtOAc (3 x 10 mL). The organic layer was dried with MgSO₄ and concentrated *in vacuo*. The product was

purified by flash column chromatography (20 % Acetone/Hexanes) to yield 0.181 g of **3.7** (0.63 mmol, 94 %)

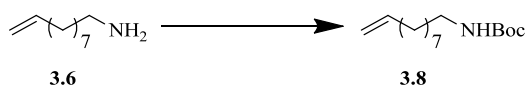
Rf: 0.31, 20 % Acetone/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.54 (d, 1H, 8.36 Hz), 7.40 (dt, 2H, 8.1Hz, 15.6Hz), 7.28 (t, 1H, 7.5Hz), 7.01 (dd, 1H, 17.6Hz, 14.4Hz), 5.85-5.68 (m, 3H), 5.33 (d, 1H, 17.6Hz), 5.00-4.90 (m, 2H), 3.41 (q, 2H, 6.4Hz), 2.02 (q, 2H, 7.2Hz), 1.610-1.22 (m, 12H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 193.3, 139.2, 135.7, 134.6, 130.1, 127.8, 127.4, 126.23, 116.7, 114.2, 50.0, 33.8, 29.6, 29.4, 29.2, 29.0, 28.9, 27.0 ppm.

HRMS (EI) *m/z* calcd for C₁₉H₂₇NO [(M-H)⁺] 285.2093, found 285.2078

1-(tert-butoxycarbonyl-amino) 9-hexene (**3.8**)



To Dec-9-enylamine (0.600 g, 3.86 mmol, 1 equiv.) dissolved in a 1:1 dioxane/water mixture (16 mL) was added K₂CO₃ (2.140 g, 15.46 mmol, 4 equiv.) and mixed at room temperature for 10 minutes. Di-tert-butyl-dicarbonate (1.1 mL, 4.64 mmol, 1.2 equiv.) was added to the mixture and allowed to mix overnight. The reaction was diluted with EtOAc (12 mL) and the organic layer separated. The layer was washed with 10 % HCl (2 x 10 mL), dried over MgSO₄ and concentrated *in vacuo*. The reaction yield was 0.897 g (3.51 mmol, 91 %) and the product was taken onto the next step without further purification.

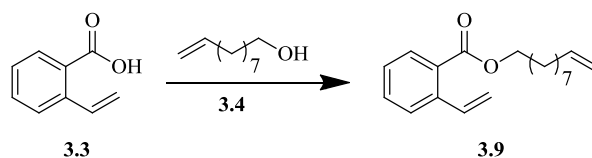
Rf: 0.31, 20 % Acetone/Hex

¹HNMR (400 MHz, CDCl₃): δ 5.79 (tq, 1H, 6.9 Hz, 9.4 Hz), 5.00-4.87 (m, 2H), 4.47 (br s, 1H), 3.08 (t, 2H, 7.2 Hz) 2.06-1.94 (m, 2H), 1.71-1.57 (m, 2H), 1.42 (s, 9H), 1.44-1.21 (m, 6H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 122.6, 113.7, 37.7, 33.4, 29.2, 29.0, 28.9, 28.7, 28.5, 26.6, 25.4 ppm.

NMR Spectra are consistent with those previously reported.⁴

2-Vinyl benzoic acid dec-9-enyl ester (3.9)



To 2-Vinylbenzoic acid (0.620 g, 4.18 mmol, 1 equiv.) in dichloromethane (21 mL) was added EDC•HCl (0.975 g, 6.28 mmol, 1.5 equiv.), DMAP (0.051 g, 0.42 mmol, 0.1 equiv.) and **3.4** (1.2 mL, 6.28 mmol, 1.5 equiv.). The mixture was stirred for 20 minutes and then Et₃N (0.90 mL, 6.28 mmol, 1.5 equiv.) was added. The solution was mixed for 24 hours at room temperature. Reaction was quenched with NH₄Cl (5 mL), extracted with EtOAc (2 x 5 mL), washed with NaCl (2 x 5 mL) and dried with MgSO₄. The resulting organic layer was concentrated and purified by column chromatography to yield 0.861 g of **3.9** (3.01 mmol, 72 %)

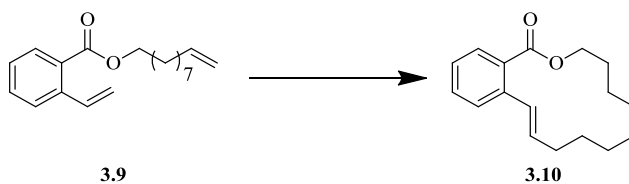
Rf: 0.75, 10 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.89 (dd, *J* = 7.9, 1.3 Hz, 1 H), 7.63-7.30 (m, 3 H), 5.82 (m, 1 H), 5.66 (dd, *J* = 17.4, 1.3 Hz, 1 H), 5.36 (dd, *J* = 10.9, 1.3 Hz, 1 H), 5.03 (m, 2 H), 4.32 (t, *J* = 6.8 Hz, 2 H), 2.11-2.01 (m, 2 H), 1.84-1.66 (m, 2 H), 1.50-1.20 (m, 10 H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 167.4, 139.5, 139.1, 136.0, 132.2, 130.1, 129.1, 127.6, 127.2, 116.4, 114.2, 65.3, 33.9, 29.4, 29.2, 28.9, 28.8, 26.1 ppm.

NMR Spectra are consistent with those previously reported.¹

7,8,9,10,11,12,13,14-octahydro-6-oxa-benzocyclotetradecen-5-one (3.10)



To **3.9** (0.420 g, 1.47 mmol, 1 equiv.) dissolved in toluene (294 mL) was added Grubbs II (0.062 g, 0.007 mmol, 0.05 equiv.) and refluxed at 85°C overnight. The reaction was filtered through a silica plug and concentrated *in vacuo*. The reaction yield was 0.240 g (0.92 mmol, 63 %) and the product was taken onto the next step without further purification.

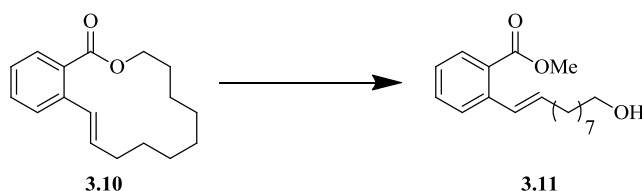
Rf: 0.76, 10 % EtOAc/Hex

¹H NMR (400 MHz, CDCl₃): δ 7.89 (dd, *J* = 7.9, 1.3 Hz, 1 H), 7.63-7.30 (m, 3 H), 6.91 (d, *J* = 15.8 Hz, 1 H), 5.93 (dt, *J* = 15.7, 7.2 Hz, 1 H), 4.34 (t, *J* = 5.2 Hz, 2 H), 2.33-2.24 (m, 2 H), 1.80-1.65 (m, 2 H), 1.50-1.20 (m, 10 H) ppm.

¹³C NMR (100 MHz, CDCl₃): δ 169.0, 138.2, 133.2, 131.8, 130.5, 130.0, 29.4, 127.3, 126.8, 65.6, 30.7, 27.2, 26.7, 26.6, 24.2, 23.9, 23.3 ppm.

NMR Spectra are consistent with those previously reported.¹

2-(10-Hydroxy-dec-1-enyl) methyl benzoate (3.11)



To **3.10** (0.522 g, 2.02 mmol, 1 equiv.) dissolved in methanol (10 mL) was added NaOMe (0.164 g, 3.03 mmol, 1.5 equiv.) and allowed to stir overnight. The reaction was quenched with 10 % HCl and subsequent HCl was added to a pH of 2. This aqueous layer was extracted with EtOAc (2 x 10 mL), the organic layers were pooled, washed with brine (2 x 5 mL) and dried over MgSO₄. The resulting organic layer was concentrated and purified by column chromatography to yield 0.861 g of **3.9** (3.01 mmol, 72 %)

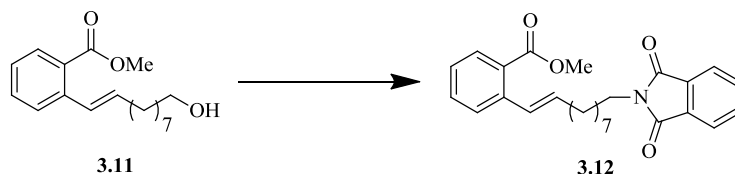
Rf: 0.21, 20 % EtOAc/Hex

¹H NMR (400 MHz, CDCl₃): δ 7.89 (dd, *J* = 7.9, 1.3 Hz, 1 H), 7.63-7.30 (m, 3 H), 6.91 (d, *J* = 15.8 Hz, 1 H), 5.93 (dt, *J* = 15.7, 7.2 Hz, 1 H), 3.88 (s, 3 H), 3.67 (t, *J* = 6.2 Hz), 2.33-2.23 (m, 2 H), 1.79-1.67 (m, 2 H), 1.50-1.20 (m, 10 H) ppm.

¹³C NMR (100 MHz, CDCl₃): δ 168.1, 139.7, 134.1, 131.8, 130.4, 128.4, 128.1, 127.1, 126.6, 63.1, 52.1, 33.2, 32.8, 29.4, 29.3, 29.2, 29.2, 25.7 ppm.

NMR Spectra are consistent with those previously reported.¹

2-(10-(isoindoline-1,3-dione)-dec-1-enyl) methyl benzoate (3.12)



To **3.11** (0.050 g, 0.17 mmol, 1.0 equiv.) dissolved in THF (0.7 mL) was added phthalimide (0.038 g, 0.26 mmol, 1.5 equiv.) and triphenyl phosphine (0.045 g, 0.17 mmol, 1.0 equiv.). The solution is cooled to 0 °C. Diisopropyl azodicarboxylate (50 μ L, 0.26 mmol, 1.5 equiv.) was dissolved in toluene (0.1 mL) and added to the previous solution at 0 °C. The resulting mixture was slowly put to room temperature and stirred overnight. The mixture was concentrated *in vacuo* and purified by column chromatography to afford yellow oil yielding 0.035 g of **3.5** (0.12 mmol, 71 %)

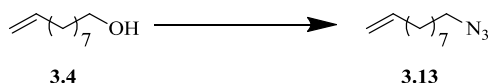
Rf: 0.20, 10 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 7.85-7.65 (m, 4H), 7.79 (d, $J = 7.9$, 1.4 Hz, 1 H), 7.51 (dd, $J = 8.0$, 1.0 Hz, 1 H), 7.41 (td, $J = 7.1$, 1.0 Hz, 1 H), 7.20 (dd, $J = 7.5$, 1.3 Hz, 1 H), 6.91 (d, $J = 15.8$ Hz, 1 H), 5.93 (dt, $J = 15.7$, 7.2 Hz, 1 H), 3.88 (s, 3 H), 3.65 (t, $J = 7.3$ Hz, 2 H), 2.26-2.15 (m, 2 H), 1.72-1.65 (m, 2 H), 1.52-1.15 (m, 10 H) ppm.

¹³CNMR (100 MHz, CDCl₃): δ 168.4, 168.1, 139.6, 134.0, 133.8, 132.2, 131.9, 130.2, 128.3, 128.1, 127.1, 126.4, 123.1, 68.9, 52.0, 38.0, 33.2, 29.3, 29.2, 29.1 ppm.

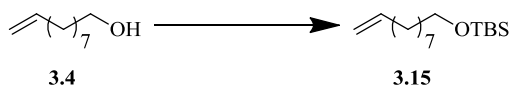
NMR Spectra are consistent with those previously reported.¹

1-Azido-Dec-9-ene (3.13)



To 9-decene-1-ol (1.0 mL, 5.61 mmol, 1.0 equiv.) dissolved in THF (56 mL) was added diphenylphosphoryl azide (1.3 mL, 8.40 mmol, 1.5 equiv.) and triphenyl phosphine (2.203 g, 8.40 mmol, 1.5 equiv.). The solution is cooled to 0 °C. Diethyl azodicarboxylate (1.3 mL, 8.40 mmol, 1.5 equiv.) was added to the previous solution at 0 °C. The resulting mixture was slowly put to room temperature and stirred overnight. The mixture was concentrated *in vacuo* and

(9-decen-1-yloxy)-(1,1-dimethylethyl)dimethyl-silane (3.15)



To **3.4** (0.6 mL, 3.36 mmol, 1 equiv.) dissolved in anhydrous DMF (3.5 mL) was added imidazole (0.230 g, 7.39 mmol, 2.2 equiv.) and TBSCl (0.612 g, 4.03 mmol, 1.2 equiv.) at room temperature and mixed overnight. After checking by TLC for completion, the reaction mixture was quenched with saturated ammonium chloride (15 mL) and diluted with EtOAc. The aqueous layer was then extracted with EtOAc and the resulting organic layer washed with water whilst increasing the concentration of brine to 100 %. After one final brine wash, the organic layer was dried with MgSO₄ and concentrated *in vacuo*. The product was purified by flash column chromatography (2 % EtOAc/Hexanes) to yield 0.802 g of **3.15** (2.99 mmol, 89 %)

Rf: 0.18, 10 % EtOAc/Hex

¹HNMR (400 MHz, CDCl₃): δ 5.79 (tq, 1H, 6.9 Hz, 9.4 Hz), 5.00-4.87 (m, 2H), 3.75 (br t, 2H, 7.0 Hz), 2.06-1.94 (m, 2H), 1.44-1.21 (m, 12H), 0.81 (s, 9H), 0.00 (s, 3H) -0.08 (s, 3H) ppm.

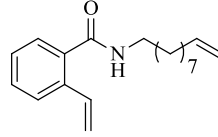
¹³CNMR (100 MHz, CDCl₃): δ 140.1, 114.6, 63.1, 33.3, 32.2, 28.4, 25.3, 25.0, 17.6, -5.0 ppm

NMR Spectra are consistent with those previously reported.⁶

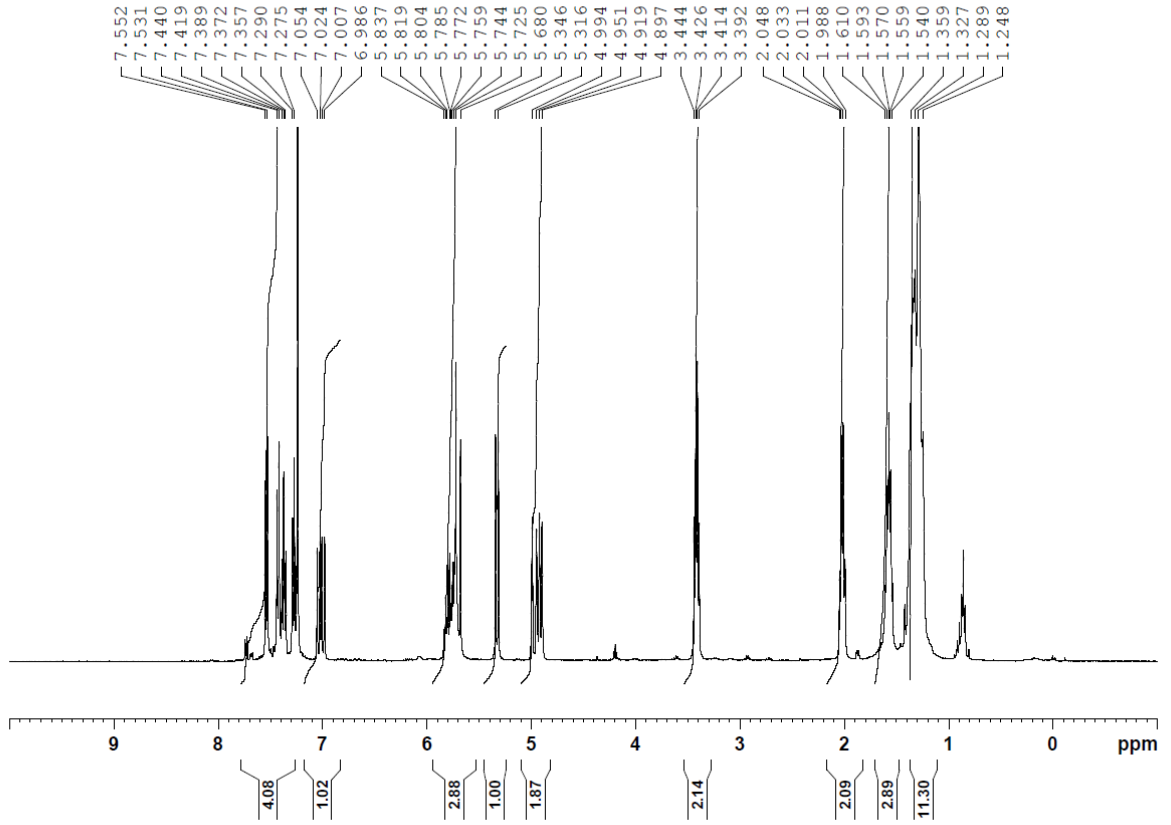
3.5.2. References

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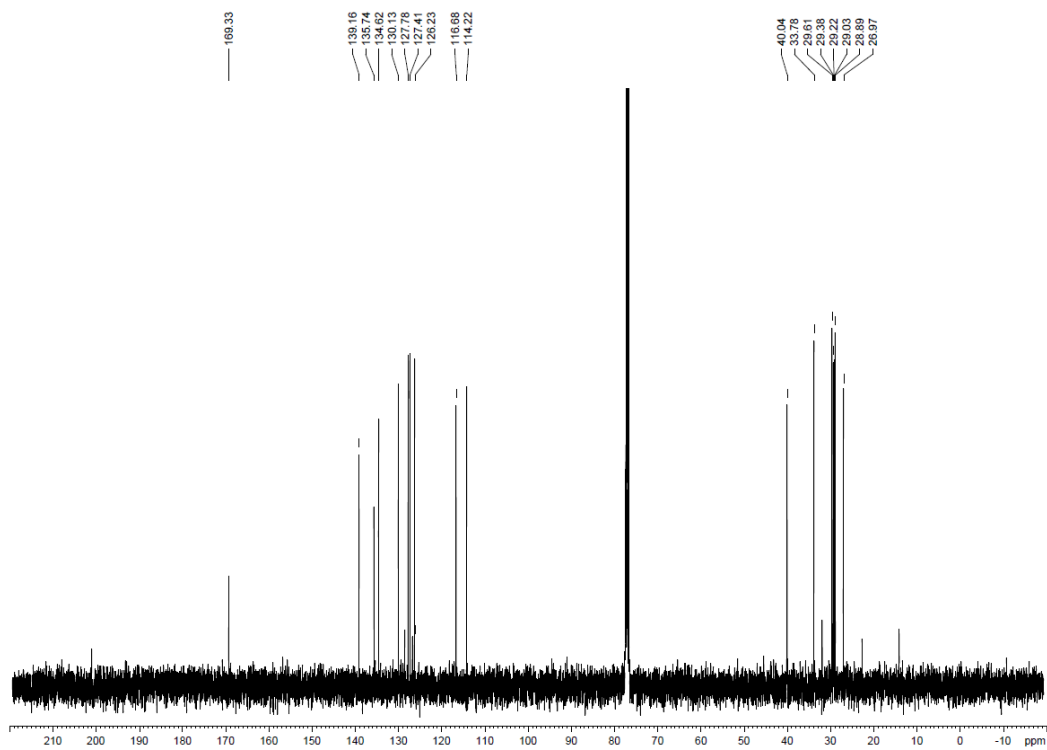
3.5.3 Selected NMR spectra



1d_1H_32_scans CDCl3 D:\ Boddy 50



1d_13C_1_hour CDCl3 D:\Boddy 50



(...) Every substance, every single object, is made up of those atoms, the possible combinations of which are infinite in an infinity of ways. The objects exist as long as the atoms constituting them remain together; they cease to exist when their atoms move away from one another. The endless changes of reality are due to the continual aggregation and disaggregation of atoms.

-- Democritus

The organic chemist is more than a logician and a strategist; he is an explorer strongly influenced to speculate, to imagine, and even to create

-- E. J. Corey

When we have been faced with a problem of effecting a chemical synthesis, we have sought known methods. We have not paused to think why we do not invent a new method every time. If we adopt this philosophy, we are going to be extremely busy till the end of the century (a) trying to equal the enzymes, and (b) thinking of new ways of synthesis.

-- Sir Derek H. R. Barton

Carbon is not a man, not salt nor water nor calcium. He is all of these, he is much more, much more; and the land is so much more than its analysis.

-- John Steinbeck

As soon as we touch the complex processes that go on in a living thing, be it plant or animal, we are at once forced to use the methods of chemistry. No longer will the microscope, the kymograph, the scalpel avail for the complete solution of the problem. For further analysis of these phenomena which are in flux and flow, the investigator must associate himself with those who have labored in fields where molecules and atoms, rather than multicellular tissues or even unicellular organisms, are the unit of study

-- John Jacob Abel

ORIGINAL ARTICLE

Biosynthesis of ebelactone A: isotopic tracer, advanced precursor and genetic studies reveal a thioesterase-independent cyclization to give a polyketide β -lactone

Morgan A Wyatt^{1,2,5}, Yasodha Ahilan^{1,5}, Panos Argyropoulos³, Christopher N Boddy³, Nathan A Magarvey^{1,2,4} and Paul HM Harrison¹

Macrocyclization of polyketides generates arrays of molecular architectures that are directly linked to biological activities. The four-membered ring in oxetanones (β -lactones) is found in a variety of bioactive polyketides (for example, lipstatin, hymegluslin and ebelactone), yet details of its molecular assembly have not been extensively elucidated. Using ebelactone as a model system, and its producer *Streptomyces aburaviensis* ATCC 31860, labeling with sodium [$1-^{13}\text{C},^{18}\text{O}_2$]propionate afforded ebelactone A that contains ^{18}O at all oxygen sites. The pattern of $^{13}\text{C}-^{18}\text{O}$ bond retention defines the steps for ebelactone biosynthesis, and demonstrates that β -lactone ring formation occurs by attack of a β -hydroxy group onto the carbonyl moiety of an acyclic precursor. Reaction of ebelactone A with *N*-acetylcysteamine (NAC) gives the β -hydroxyacyl thioester, which cyclizes quantitatively to give ebelactone A in aqueous ethanol. The putative gene cluster encoding the polyketide synthase (PKS) for biosynthesis of **1** was also identified; notably the ebelactone PKS lacks a terminal thioesterase (TE) domain and no stand alone TE was found. Thus the formation of ebelactone is not TE dependent, supporting the hypothesis that cyclization occurs on the PKS surface in a process that is modeled by the chemical cyclization of the NAC thioester.

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Keywords: biosynthesis; ebelactone; gene cluster; isotope shift; polyketide; *Streptomyces*

INTRODUCTION

A number of naturally occurring β -lactones (oxetan-2-ones) have been identified (Figure 1). Thus, natural β -lactones derived from terpenes (for example, varanisatin), amino acids (for example, SQ 26 517), fatty acids and polyketides are all well known.¹ Representative examples of the polyketide group include ebelactones A (**1**) and B (**2**) and hymegluslin (F-244, **3**), while lipstatin (**4**) is formed by degradation of fatty acids.^{2–4} β -Lactones formed *via* mixed metabolic pathways include the cinnabaramides (**5**) and salinosporamides (**6**).^{5,6} Many β -lactones are potent irreversible enzyme inhibitors; for example, **4** inhibits pancreatic lipase and the tetrahydro-derivative is used clinically as Orlistat in treatment of type 2 diabetes; **3** is an inhibitor of HMG-CoA synthase, an important enzyme in the mevalonate pathway to sterols; and salinosporamide A is a proteasome inhibitor that is in clinical trials.^{6–8}

The ebelactones are produced by the actinomycete, *S. aburaviensis* ATCC 31860.⁹ They are known inhibitors of esterases, lipases,

cutinases, homoserine transacetylase and cathepsin A, as well as modulators of the mTOR pathway.^{10–14} This wide range of biological activities has triggered interest in the synthesis of these compounds.¹⁵

Prior studies of ebelactone biosynthesis by Uotani and co-workers with ^{13}C -labeled sodium acetate, propionate and butyrate showed that **1** is derived from a 'starter' acetate unit and six 'extender' units of propionate condensed in the typical 'head-to-tail' polyketide arrangement.¹⁶ Likewise, **2** derives from 1 unit of acetate, 5 units of propionate and 1 terminal unit of butyrate. However, the origin of the oxygen atoms in **1** and **2** was not determined, and with it the biosynthesis of the most intriguing component of these molecules, the β -lactone ring, was left unresolved.

Several possible pathways for the biosynthesis of β -lactones such as **1** are feasible based on biosynthetic and/or chemical precedents (Scheme 1). In mechanisms *a–e* within Scheme 1, a polyketide intermediate (for example, hexaketide **7** in the case of **1**) is extended on the presumed ebelactone polyketide synthase (PKS), and the

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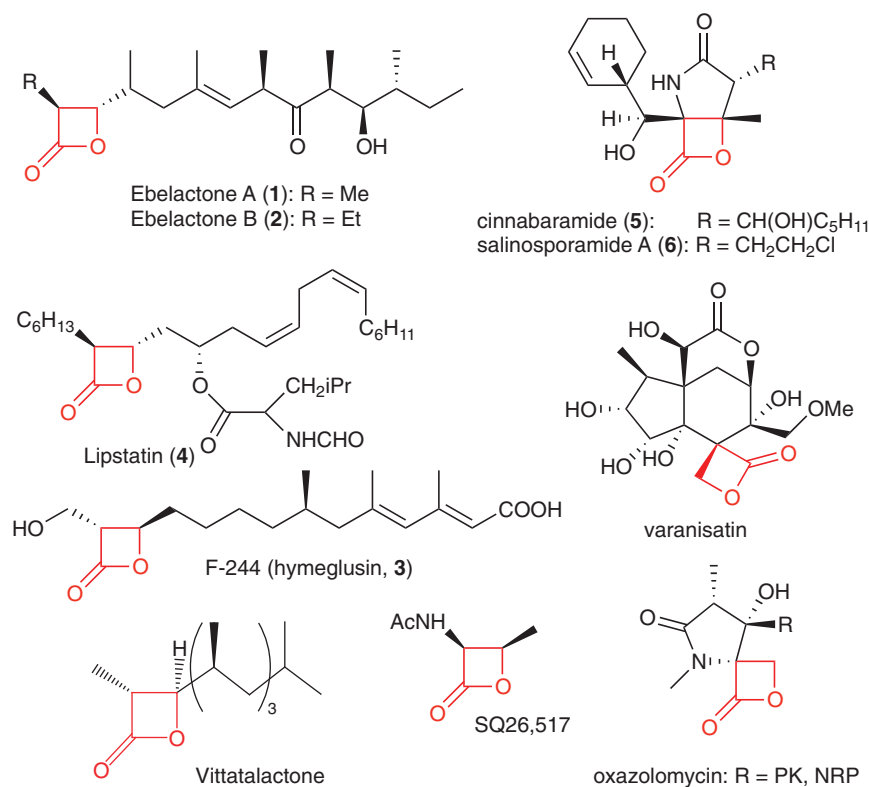
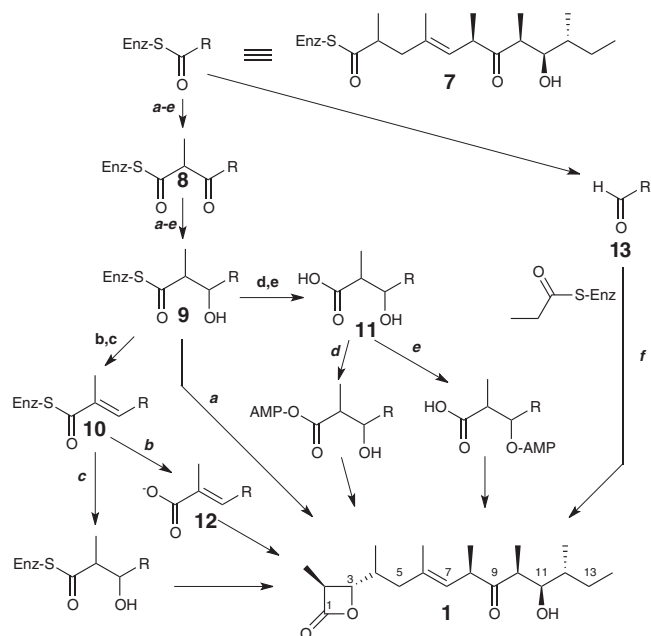


Figure 1 Examples of naturally occurring secondary metabolites that contain a β -lactone ring, including ebelactones A and B (1, 2), F-244 (3) and lipstatin (4).



Scheme 1 Possible biosynthetic routes for lactone ring formation in β -lactones such as 1.

resulting β -ketoacyl moiety (for example, 8, formed from 7 and methylmalonyl-CoA for the ebelactone A case) is reduced to give the β -hydroxyacyl-enzyme (for example, 9 *en route* to 1).

In mechanism *a*, intermediate 9 cyclizes directly by attack of the C- β hydroxy group onto the carboxylic acid derivative, whether with

or without the intervention of a PKS thioesterase (TE)-dependent reaction. If a TE is present, then this mechanism gives the small ring lactone in a route that is analogous to formation of macrocyclic lactones such as erythromycin *in vivo*, and indeed TEs are found in the salinosporamide and cinnabaramide biosynthetic gene clusters, although their function remains unproven.^{17–19} This route also resembles the chemical lactonization of β -hydroxy-thioesters.^{20,21} In mechanism *b*, the β -hydroxyacyl-enzyme is dehydrated (for example, 9–10), and hydrolyzed from the PKS. The resulting α,β -unsaturated acid (for example, 12) could cyclize via protonation at C- α and nucleophilic attack by the carboxylate at C- β . This process is reminiscent of the chemical bromo-lactonization of β,γ -unsaturated acids.^{20,22}

In the biosynthesis of lipstatin, a variation on pathway *b* has been proposed based on deuterium labeling experiments. A Claisen-like condensation between two esters derived from fatty acid degradation is followed by reduction of the resulting ketone. The ensuing alcohol (cf 9, Scheme 1) then undergoes dehydration–rehydration to effect epimerization at C- β before cyclization.²³ An analogous process for ebelactone biosynthesis would correspond to mechanism *c*.

In mechanisms *d* and *e*, the β -hydroxyacyl-enzyme is hydrolyzed to give the β -hydroxy acid (for example, 9–11), which is then activated by, for example, adenylation by ATP, either at the carboxylate (path *d*) or at the β -hydroxy group (path *e*). This activation facilitates cyclization by mechanisms analogous to the chemical synthesis of β -lactones by activation of β -hydroxyacids at carboxylate and alcohol groups, respectively.^{20,24}

Lastly, by contrast, in mechanism *f*, the polyketide precursor is cleaved reductively from the enzyme to furnish an aldehyde (for example, hexaketide 7–13).²⁵ This aldehyde can then undergo aldol-type condensation with an acyl donor (for example, propionyl or

methylmalonyl-CoA add to **13**), and the resulting aldol adduct can then cyclize directly in a process analogous to chemical tandem aldol-lactonization reactions.^{20,21} Indeed, a tandem aldol-lactonization-like mechanism has been suggested in the biosynthesis of the salinosporamides.¹⁸

Mechanisms **b** and **e** require that the lactone ring oxygen atom is derived from the same biosynthetic unit as the C-1 carbonyl oxygen, and involve cleavage of the C-β-O bond during biosynthesis. In contrast, in mechanisms **a**, **d** and **f** the C-O bond at C-β remains intact from the hexaketide precursor **7**. Finally, the oxygen atom at C-β is predicted to be lost from precursor **7** in route **c**.

In order to probe the mechanism of lactone ring formation, we used the ¹⁸O-induced isotopic shift method to study the origin of the oxygen atoms in F-244 (**3**) in *Fusarium* sp. ATCC 20788.²⁶ The results showed that the β-lactone ring is derived from an intact bond between the β-carbon and the ring oxygen atom, while the carbonyl oxygen atom is derived from an intact C=O bond in the adjacent acetate unit. Thus pathways **b**, **c** and **e** are excluded as possible mechanisms for lactone formation in this fungal metabolite.

However, polyketide biosynthesis in fungi is different from that in bacteria, and we thus wished to establish whether these results still hold true in the *Streptomyces* genus, and to further discriminate between the remaining mechanisms. Here, we report the results of incorporation experiments of [1-¹³C,¹⁸O₂]propionate into **1**, the conversion of an acyclic precursor into **1**, and the identification of the putative biosynthetic gene cluster encoding formation of **1**.

RESULTS AND DISCUSSION

Isotope labeling experiments

Addition of sodium [1-¹³C,¹⁸O₂]propionate (mixed with sodium [1-¹³C,¹⁶O₂]- and unlabeled propionate to avoid excessive incorporation rates) to cultures of *S. aburaviensis* ATCC 31860 followed by purification gave labeled **1** (**1a**).²⁷ The ESI mass spectrum of this material showed a distribution of peaks corresponding to molecular ions of unlabeled **1** (MH⁺, *m/z* 339) and labeled **1a** (MH⁺, *m/z* 340–345) (Figure 2), along with peaks at *m/z* 356–362, due to the corresponding [M + NH₄]⁺ ions. Thus, unusually high levels of isotopic enrichment were observed in this metabolite.

The ¹³C NMR spectrum of this sample showed high levels of enrichment at all positions derived from C-1 of propionate, similar to the results reported by Uotani *et al.*¹⁶ The high-resolution ¹³C NMR spectrum exhibited isotopically shifted signals induced by the presence of ¹⁸O, which were observed at all four oxygen-bearing

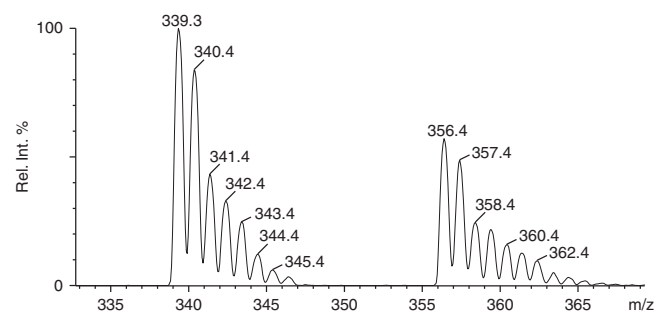


Figure 2 ESI mass spectrum of **1a** revealing the distribution of isotopomers for unlabeled and labeled ebelactone A. *m/z* 339 corresponds to MH⁺ for unlabeled **1** and 340–346 to MH⁺ of ¹³C- and ¹⁶O- or ¹⁸O-labeled ebelactone A (**1a**). The peaks at *m/z* 356–362 are due to the corresponding [M + NH₄]⁺ ions.

positions (C-1, C-3, C-9 and C-11), indicating that these carbon atoms were incorporated from propionate with the relevant C-O bonds remaining intact (Figure 3 and Table 1). Isotopic shifts of 0.053 p.p.m. and 0.039 p.p.m. were observed at C-9 and C-1, respectively (Table 1), consistent with values expected for C=O double bonds. Isotopic shifts of 0.029 p.p.m. and 0.024 p.p.m. were observed for C-3 and C-11, respectively: these fall in the range (0.010–0.035 p.p.m.) expected for C-O single bonds.^{28,29}

These results demonstrate that four carbon–oxygen bonds in ebelactone A remain intact during biosynthesis from propionate (Scheme 2), showing that the ring formation takes place by attack of the hydroxyl group at C-3 of a polyketide precursor onto the terminal carbonyl group, and not by the attack of a carboxylate onto the C-3 carbon of the polyketide. Thus pathways **b** and **e** (Scheme 1) can be excluded as possible mechanisms for the formation of the β-lactone moiety. Likewise, oxygen exchange as proposed for lipstatin in

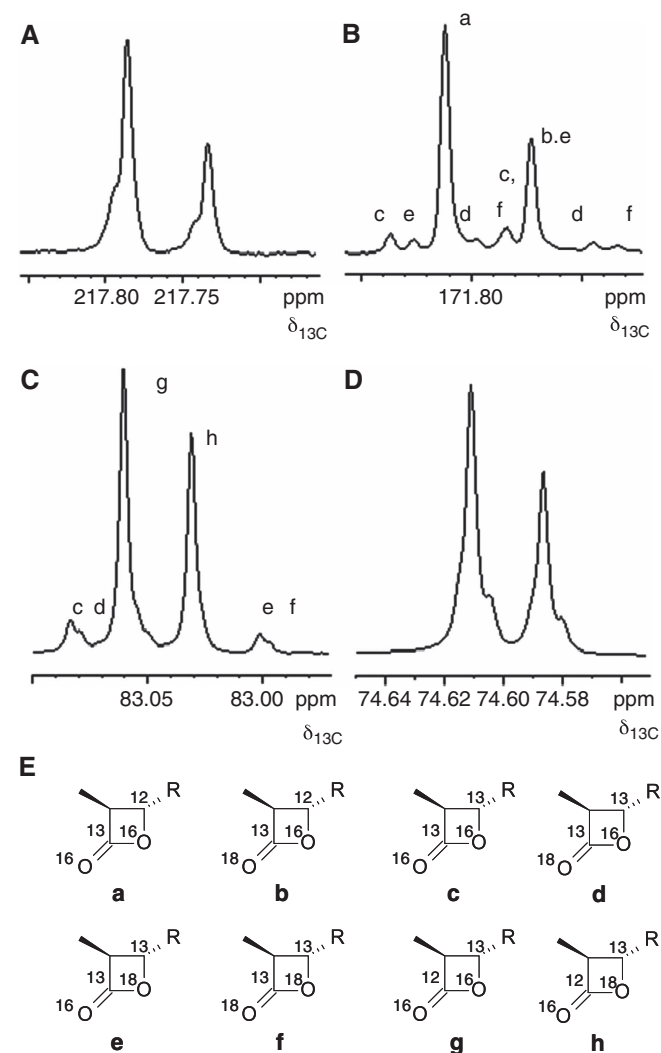
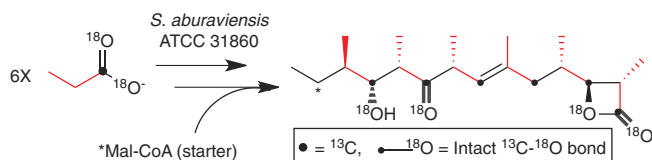


Figure 3 ¹³C NMR signals for **1** from cultures of *S. aburaviensis* enriched with sodium [1-¹³C, ¹⁸O₂]propionate: (A) Signal for C-9; (B) Signal for C-1: a: natural abundance and ¹³C-¹⁶O-labeled molecules; b: ¹³C-¹⁸O labeled; c, d, e, f: signals with ¹³C-¹³C couplings due to isotopomers c, d, e, f (E). (C) Signal for C-3 (see E for isotopomer assignments); (D) Signal for C-11; (E) isotope labeling patterns of the β-lactone ring in **1**. Isotopomers a–h give rise to peaks marked a–h, respectively, in the ¹³C NMR spectrum in B, C. For R, see Scheme 1.

Table 1 Isotope shifts and ^{18}O enrichments observed for **1a**

$\delta^{13}\text{C}^a$	C# ^b	$\Delta\delta^c$	$^{18}\text{O}/^{16}\text{O}^d$
217.79	9	0.053	0.56
171.81	1	0.039	0.51
83.06	3	0.029	0.69
74.61	11	0.024	0.69

^aCarbon-13 chemical shift, measured in CDCl_3 at 175 MHz.^bSee Scheme 1 for numbering.^cIsotope shift due to oxygen-18.^dMeasured by peak height ratios.**Scheme 2** Ebelactone A (**1**) showing the labeling pattern due to intact incorporation of ^{13}C and ^{18}O from propionate units.

mechanism **c** does not occur in biosynthesis of **1**.²³ This result is in agreement with our previous findings in the biosynthesis of F-244, suggesting that these fungal and bacterial β -lactone rings are made by similar mechanisms.²⁶

The remaining mechanisms (**a**, **d** and **f**) are all consistent with the qualitative results obtained, as each atom has the same biosynthetic origin in all three cases.

However, an essentially equal level of oxygen-18 enrichment was observed at C-1, when compared with C-3, C-9 and C-11 (Table 1). This result indicates that a free carboxylic acid (for example, **11**) cannot be involved in the biosynthetic pathway, because hydrolysis of an intermediate acyl-enzyme species (for example, **9**) would require incorporation of unlabeled water. Cyclization of the ensuing acid would then result in loss of one-half of the labeled oxygen atoms. Hence, pathway **d** can also be excluded.

Therefore, it can be concluded that the lactone ring of **1** is either formed by direct displacement of the acyl intermediate while still attached to the PKS, thereby releasing the cyclized product (mechanism **a**) (Scheme 1); or through a route similar to the tandem aldol lactonization (**f**) proposed in the biosynthesis of salinosporamides.¹⁸ In the former case, such a reaction might be catalyzed by a thioesterase domain that is tailored for formation of the strained four-membered ring. Mechanism **f** by contrast would require a novel enzymatic system that must still account for the equal levels of labeling in the units of the hexaketide precursor **7** and the final group that forms the ring, despite the different roles of these moieties; nonetheless, this hypothesis would account for the formation of both ebelactone A and B, as propionate and butyrate precursors are unselectively incorporated into the terminal ketide unit.

Further, the presence of oxygen-18 in both the ketone and hydroxy moieties (C-9 and C-11) of **1a** shows that ebelactone is synthesized in a manner in which the oxidation state is controlled by the PKS, and not by introduction of these oxygen atoms into a more highly reduced polyketide. These results allow a detailed prediction of the nature and sequence of the presumably modular PKS that forms ebelactone.

In addition to the large peaks shown in Figure 3, lower intensity peaks were observed at many of the ^{13}C -labeled positions in **1a**. These peaks were consistent with incorporation of multiple labeled

propionate moieties into adjacent units within the same molecule, due to the high incorporation rate. Thus, long-range two-bond ^{13}C - ^{13}C couplings between C-11 and C-9, C-9 and C-7, C-7 and C-5, and C-5 and C-3 account for all the observed minor peaks at these sites. In addition, a two-bond ^{13}C - ^{13}C coupling of 9.3 Hz was observed between the C-1 and C-3 positions (Figures 3B and C). This unusually large coupling constant is fully consistent with literature values for $^2J_{^{13}\text{C}-^{13}\text{C}}$ in cyclobutanone, and can be explained by the two separate two-bond coupling paths across the lactone ring.³⁰ Figure 3E shows all the possible isotope labeling patterns for the lactone ring of **1a**.

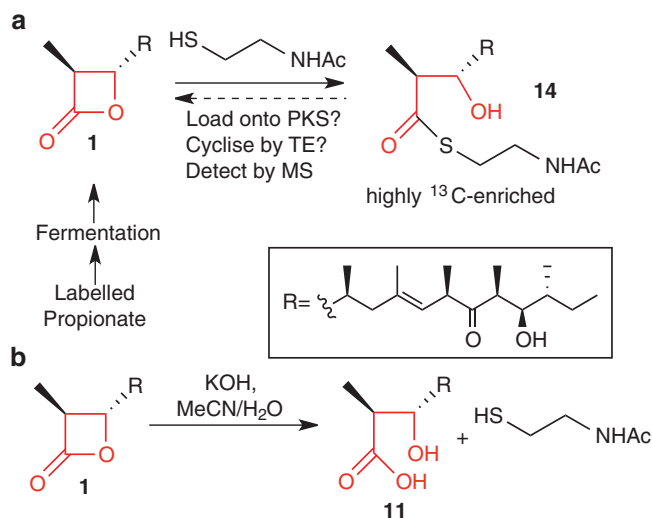
As mechanisms **a** and **f** are not readily distinguishable through isotope labeling in precursors, it was clear that further understanding of the assembly of the β -lactone ring requires identification of advanced biosynthetic intermediates.

Advanced precursor studies

The formation of the β -lactone ring by cyclization through mechanism **a** implies that a β -hydroxy-acyl carrier protein (ACP)-bound thioester would be formed before cyclization. As *N*-acetylcysteamine (NAC) derivatives of a variety of biosynthetic intermediates have been successfully incorporated into polyketide and other natural products, we next envisioned that feedings may provide insight into the route of cyclization if the appropriate labeled *N*-acetylcysteamine derivative (that is, **14**) would load onto the relevant cyclizing domain of the PKS, and undergo cyclization to give **1** (Scheme 3a).^{31,32} Rather than prepare **14** through total synthesis, we recognized that this *N*-acetylcysteamine derivative could be prepared from ebelactone A, using the unique reactivity of the lactone to effect nucleophilic attack with *N*-acetylcysteamine; and further that the high level of isotopic labeling observed in incorporation of ^{13}C - and/or ^{18}O -labeled propionate into **1** could be used to introduce an adequate level of isotope to act as a tracer in this biosynthetic experiment.

Therefore, the isotopically labeled **1a** from above was treated with *N*-acetylcysteamine and TEA in DCM, and the product isolated and rigorously purified to ensure removal of unreacted **1**. Analysis of the product revealed that the lactone ring had opened, and careful examination of the NMR spectra established that ring opening had occurred by nucleophilic attack on the carbonyl carbon, and not by attack at C- β . Direct evidence for this addition, and support for the proposed structure (**14**) came from the three-bond HMBC correlations between the methylene protons adjacent to sulfur and the thioester carbonyl carbon. Mass spectral analysis revealed that this sample of **14** had the same distribution of molecular ions and hence the same isotopic signature as described above for the ebelactone **1a**.

This sample was next used in a biosynthetic incorporation experiment in which **14** was added to growing cultures of *S. aburaviensis*. After growth as for the experiments above, the cultures were extracted with ethyl acetate and the organic extracts were analyzed by HPLC-MS (Figure 4). Organic extracts obtained from *S. aburaviensis* fed with exogenous precursors were compared directly with those not receiving precursor feedings, and with precursor added to sterile, uninoculated medium, using LC-MS. The unfed controls led to identification of ebelactone A at the expected retention time and with an isotopomer distribution corresponding to natural abundance and identical to authentic standard ebelactone (Figure 4). Culture extract derived from the incorporation of **14** gave ebelactone comprised of natural abundance isotopomers (as above), superimposed with isotopomers of higher mass that derive from the precursor (Figure 4). Therefore, **14** is cyclized to **1** under these conditions. However, to our surprise, the samples in which labeled **14** was added to uninoculated culture medium also resulted in formation



Scheme 3 (a) Formation of labeled ebelactone from labeled propionate, followed by reaction with *N*-acetylcysteamine, gives the thioester **14**, which acts as a putative advanced precursor for formation of **1**. For details of the cyclization, see text. Thioester synthesis: Et₃N, CH₂Cl₂, RT, 4 h, 60%. (b) Hydrolysis of **1** yields **11**.

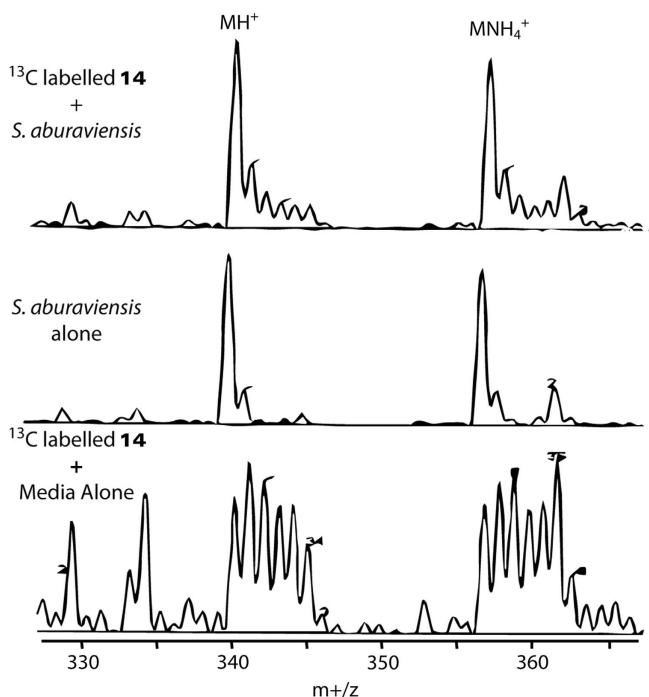


Figure 4 Cyclization of **14** by *S. aburaviensis* and media alone result in ebelactone A formation. Cyclization of ¹³C-labeled **14** to give labeled **1** was detected by HPLC-MS, in the presence and absence of *S. aburaviensis*. Mass spectra at the retention time of ebelactone A are shown.

of the β-lactone **1**: these samples showed no unlabeled **1**, but rather ebelactone A with the same isotopomer distribution as it had before formation of the *N*-acetylcysteamine derivative (Figure 4). The level of production was, within experimental error, the same as that detected in the biosynthetic incorporation sample. Clearly, this experiment demonstrates that the *N*-acetylcysteamine derivative **14** undergoes chemical cyclization to the β-lactone without enzymatic catalysis.

This cyclization in aqueous solution is almost unprecedented: although the formation of β-lactones *via* cyclization of β-hydroxy-thioesters is used in organic synthesis, these reactions are normally performed under anhydrous conditions in organic solvents, and generally require the use of Lewis acidic cations to assist departure of the thiolate leaving group (for example, thiophenyl with mercury).^{20,33} Literature precedent for this aqueous cyclization is rare, and only one example could be found where a β-lactone is formed in aqueous milieu, and this is as a transient intermediate, which is rapidly cleaved by hydrolysis in a subsequent step. Thus, hydrolysis of lactocystin proceeds through the intermediate *clasto*-lactocystin β-lactone, but only a maximum of 10% of the theoretical yield of lactone is present during this reaction.³⁴ Therefore, we wondered whether the *N*-acetylcysteamine thioester **14** was undergoing hydrolysis through a similar mechanism. Hence, we prepared the β-hydroxy acid **11** through hydrolysis of ebelactone A (NaOH/H₂O, tetrahydrofuran, RT, 8 h, 67%) and used this material as a standard for analysis of the extracts from above (Scheme 3b). In no case was there any evidence for formation of **11**, and thus the ebelactone is being formed without hydrolysis of either **14** or **1**.

The formation of ebelactone from **14** under a variety of conditions was confirmed by other results from HPLC-MS, and it was eventually found that while **14** was stable in aprotic organic solvents such as chloroform without any sign of cyclization, simply diluting into 50:50 EtOH:H₂O effected slow cyclization over the course of 24 h. While a concentrated solution failed to react to completion, dilute samples (*ca.* 50 mg l⁻¹) cyclized efficiently and essentially completely to furnish **1**. These observations, therefore, show that the system reaches an equilibrium position that moves toward the lactone as the rate of lactone opening falls with decreasing concentration of both lactone and *N*-acetylcysteamine.

While these results supported the TE-independent formation of the lactone, we also considered whether an alternative cyclization was occurring: if the C-11 hydroxy group attacked the thioester, then the product would be a 12-membered macrocycle, which would have the same mass as ebelactone, and could conceivably co-elute in the HPLC-MS analysis. To eliminate this possibility we performed a preparative experiment in which **14** was dissolved in EtOH:H₂O to a final concentration of 50 mg l⁻¹. The reaction was allowed to proceed at room temperature for 24 h, and then the mixture was quickly evaporated and separated by chromatography. The eluted **1** was identical to standard in all respects, including proton NMR.

Taken together, these results establish that the β-hydroxyacyl thioester **14** undergoes spontaneous cyclization to ebelactone A, and thus raise the possibility that an analogous process during the biosynthesis of **1** could occur to cleave the chain from the PKS. Thus a dedicated thioesterase would not necessarily be required during the formation of **1**.

To determine whether a conventional thioesterase could form a β-lactone, or if it would catalyze cyclization from the C-11 hydroxy group to form the macrocycle mentioned above, we, therefore, incubated **14** with deoxyerythronolide B (DEBS) synthase-TE, the TE responsible for formation of the 14-membered macrocyclic ring in erythromycin.³⁵ The reaction mixture was analyzed by HPLC-MS, along with authentic standards of **1**, **14**, and acid **11**. The results show that comparable levels of **1** are formed when **14** is incubated with or without enzyme. However, in the enzymatic sample only, extensive formation of acid **11** occurred (Figure 5a). There was no evidence for formation of the putative macrocycle. Further, kinetic characterization of DEBS-TE-mediated hydrolysis showed saturation, clearly indicating that this is an enzymatic process (Figure 5b). The kinetic parameters

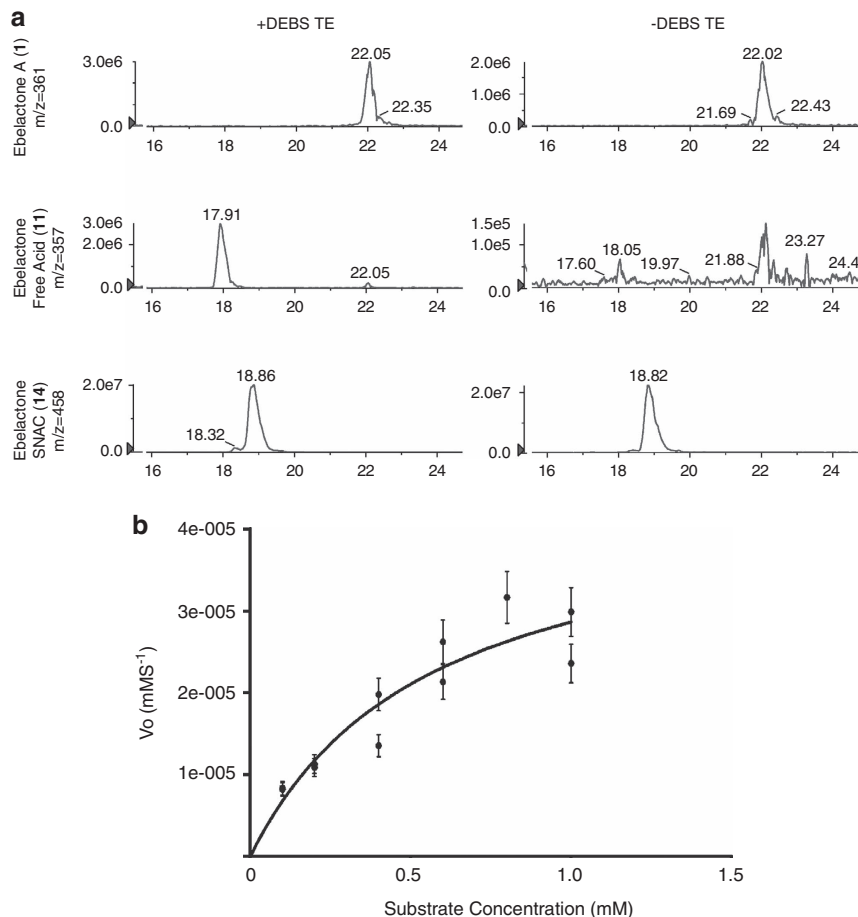


Figure 5 Hydrolysis of **14** with DEBS-TE results in hydroxy acid **11**. (a) Extracted ion chromatograms from HPLC-MS analyses of incubations of **14** with and without DEBS-TE. Chromatograms are, from top to bottom, for $[1 + Na]^+$ ($m/z=361$), $[11 + H]^+$ ($m/z=357$), and $[14 + H]^+$ ($m/z=458$) ion extractions. Note that the ion extraction chromatogram for $[11 + H]^+$ also produces a peak at the retention time for **1** (22.0 min). This is presumably due to hydration of the ketone in **1** during ionization. (b) Plot of the initial rate of hydrolysis (V_0) of **14** by DEBS-TE versus substrate concentration. The data is directly fit to the Michaelis-Menten model. Conditions: $5 \mu M$ DEBS-TE, pH 7.4. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

are in accord with those expected for hydrolysis of other *N*-acetylcysteamine derivatives with DEBS-TE ($k_{cat} = 0.53 \pm 0.1 \text{ min}^{-1}$; $K_M = 0.5 \pm 0.2 \text{ mM}$; $k_{cat}/K_M = 16 \pm 7 \text{ M}^{-1} \text{ s}^{-1}$).^{36–38} Test incubations of DEBS-TE with ebelactone A failed to catalyze any hydrolysis reaction.

Putative ebelactone gene cluster

The biosynthesis of a number of macrocyclic polyketide lactones has been extensively studied and many PKS gene clusters identified. These lactones include 5-, 6-, 10-, 12-, 14- and 16-membered rings, as well as larger ring sizes. Of the gene clusters identified from which a four-membered, β -lactone ring is formed (oxazolomycin, cinnabaramide and salinosporamide), their biogenesis is not directed by canonical polyketide biosynthetic machinery.^{18,19,39} Moreover, in each of these instances the exact mechanistic details remain ambiguous and as yet unproven. The cluster for oxazolomycin biosynthesis has two non-ribosomal peptide synthase-type Condensation (C) domains, one of which is suggested to cyclize the C-terminal serine residue to afford the β -lactone.³⁹ In the cluster for salinosporamide there is a thioesterase (*SalF*), but this has been suggested to perform an editing role and a tandem aldol-lactonization sequence has been proposed to form the β -lactone ring.¹⁹ In the closely related cinnabaramide, a thioesterase (*CinE*) was identified, which has

sequence similarity to *SalF*, but which was suggested to make the lactone from a β -hydroxyacyl-enzyme.¹⁸ However, this role has not been proven. Therefore, we wished to identify the PKS that effects biosynthesis of **1** and in particular to further examine the process of β -lactone ring formation.

The genome of *S. aburaviensis* was sequenced, resulting in a number of putative biosynthetic gene clusters. The cluster that encodes ebelactone biosynthesis was found on a single contig and is readily recognized through its characteristic PKS domains, that define the predicted sequence of biosynthetic reactions for ebelactone. The cluster is characterized by seven genes (*ebeA-G*) (Table 2), which are expected to encode seven proteins (EbeA-G) that assemble to make a functional PKS (Figure 6). There are six modules, each containing a ketosynthase (KS) domain to effect chain elongation. Also present in some modules are ketoreductase, dehydratase and enoylreductase domains that are largely correctly placed to correspond to the necessary processing of the growing chain to install β -hydroxy, alkene and methylene groups. The loading module contains a characteristic KS^Q domain, which loads malonyl-CoA and decarboxylates the malonyl-enzyme to generate the starter acetate unit.⁴⁰ Specificity of the first acyltransferase domain is consistent with the loading of malonyl-CoA, whereas the remaining acyltransferase domains all have specificity for methylmalonyl-CoA, consistent with the final structure

Table 2 Ebelactone gene cluster analysis

Gene	Size, bp	Deduced role	Protein homolog	Accession number	Protein identity/similarity %/%
<i>ebeA</i>	3192	PKS	NP_821594 (<i>Streptomyces avermitilis</i> MA-4680)	NP_821594.1	58/68
<i>ebeB</i>	6615	PKS	BAH02268 (<i>Streptomyces platensis</i>)	BAH02268.1	57/67
<i>ebeC</i>	4761	PKS	ZP_05000638 (<i>Streptomyces</i> sp. MG1)	ZP_05000638.1	57/67
<i>ebeD</i>	2154	PKS	YP_004815656 (<i>Streptomyces violaceusniger</i> Tu 4113)	YP_004815656	63/75
<i>ebeE</i>	2130	PKS	YP_004959634 (<i>Streptomyces bingchenggensis</i> BCW-1)	YP_004959634.1	48/61
<i>ebeF</i>	7410	PKS	CAQ64687 (<i>Streptomyces lasaliensis</i>)	CAQ64687.1	55/65
<i>ebeG</i>	6537	PKS	CBA11583 (<i>Streptomyces lydicus</i>)	CBA11583.1	57/68
<i>orf1</i>	1698	Adenylation domain	ZP_10549985 (<i>Streptomyces auratus</i> AGR0001)	ZP_10549985.1	65/76
<i>orf2</i>	876	Reductase	YP_003341831 (<i>Streptosporangium roseum</i> DSM43021)	YP_003341831.1	81/89
<i>orf3</i>	807	Hypothetical protein	YP_004902238 (<i>Kitasatospora setae</i> KM-6054)	YP_004902238.1	62/71

Results generated by BLASTx analysis.

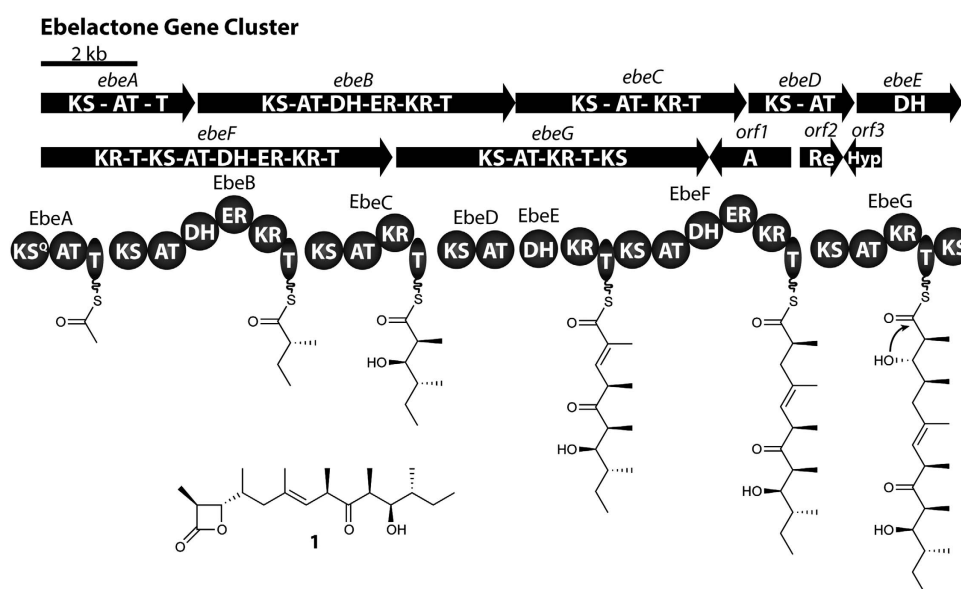


Figure 6 Ebelactone gene cluster of *S. aburaviensis* ATCC 31860. Shown are the putative genes for ebelactone biosynthesis (*ebeA-G*) and the corresponding protein assembly-line (EbeA-G). The assembly-line contains the following polyketide synthase domains: KS, acyltransferase (AT), thiolation (T), dehydrogenase (DH), enoylreductase (ER), ketoreductase (KR). Downstream genes of possible biosynthetic relevance are also shown although their putative functions are not required in the final maturation of ebelactone (**1**). These include an adenylation domain (A), a reductase (Re), and a hypothetical protein (Hyp)(*orf1-3*). Tethered polyketide intermediates are shown on the ebelactone assembly-line. The ebelactone (**1**) release mechanism through β -hydroxy attack is shown.

of **1**. Extension on the next module using methylmalonate by the KS is followed by processing by ketoreductase, dehydratase and enoylreductase domains to generate the fully reduced diketide 2-methylbutyrate, while module three contains only KS and ketoreductase and thus produces a hydroxylated triketide. Module 4 is more complex, and may be used twice: in the first iteration, only the KS is used, leaving the required ketone intact, while in the second round we speculate that the KS is used again, but now the growing chain is processed by EbeE (dehydratase protein) and the ketoreductase domain of EbeF to generate the pentaketide intermediate shown. Normal modular chain extension continues for two further rounds to generate the acyclic, enzyme-bound precursor to ebelactone, attached to the final thiolation domain.

The cluster clearly does not contain a thioesterase to effect β -lactone ring formation. This can be understood in terms of the results described above: ebelactone can form by direct cyclization of the

β -hydroxyacyl moiety on the thiolation domain in a process that is preceded by the chemical cyclization of **14**. Whether this process is accelerated by a component of the PKS is unclear, and is the subject of further investigation. Intriguingly, the PKS does contain a KS domain beyond the presumed terminal T domain. This could perform a catalytic role in cyclization, or alternatively it is possible that the 'normal' natural product of this cluster is that from further chain extension on the PKS, and the formation of ebelactone is the result of derailment through accidental cyclization on the terminal thiolation domain. Investigations to further clarify these issues are on-going.

CONCLUSION

The oxygen-18 isotopic distribution studies and the organization of the putative gene cluster establish that the biosynthetic pathway to ebelactone involves a starter malonate, which is decarboxylated by the

first KS^Q domain. Six methylmalonate units are added in the head-to-tail fashion of polyketide biosynthesis, introducing hydroxy and ketone groups with intact C–O or C=O bonds to generate a β -hydroxyacyl intermediate. This is cyclized with retention of the C(β)-O bond, consistent with nucleophilic attack of the hydroxy group onto the acyl carbon.

The putative intermediacy of a β -hydroxyacyl intermediate was then tested by synthesis of the *N*-acetylcysteamine derivative **14**, which in this unique situation was readily prepared by reaction of *N*-acetylcysteamine with ebelactone A, thereby furnishing this complex intermediate in one step without requiring extensive synthetic work. Remarkably, **14** was found to cyclize spontaneously to afford ebelactone A in virtually quantitative yield. We are not aware of any reports of corresponding cyclization of β -hydroxyacyl *N*-acetylcysteamine compounds, despite their widespread application in biosynthetic studies. We are currently continuing to investigate this process to determine whether the nature of the polyketide chain and/or the *N*-acetylcysteamine leaving group determine whether such substrates are capable of cyclization without the need for either chemical or enzymatic catalysis. Further, we were surprised that DEBS-TE, a thioesterase that might well have cyclized the β -hydroxyacyl *N*-acetylcysteamine thioester, resulted in enzymatic hydrolysis. Taken together, then, the results point away from a TE-catalyzed generation of the β -lactone ring, and suggest that cyclization might occur spontaneously on a β -hydroxyacyl moiety tethered to the PKS.

Lastly, we identified a putative gene cluster in the producing organism with the correct domain architecture for ebelactone formation. Consistent with the results from oxygen-18 labeling and advanced precursor studies, the cluster does not possess any identifiable thioesterase genes or domains, neither at the end of the ebelactone PKS nor as a distinct stand-alone TE. Taken together, this leads us to the conclusion that the β -lactone ring formation occurs on a different PKS component. This could be the ACP at the end of module 7, in a process that is simulated by the chemical cyclization of **14**. Alternatively, it is intriguing to speculate that the cluster is designed to make a different natural product, using the domain(s) found beyond the presumed terminal ACP; in this case the β -lactone could be the result of an accidental cyclization as the growing polyketide chain passes along the assembly-line, that is, premature release. Which situation occurs will demand *in vitro* experiments that install the ebelactone chain onto the terminal ACP and reveal the rates of β -lactone formation.

METHODS

General

ISP2 agar (Difco 277010, Becton Dickinson and Co., Sparks, MD, USA), fish meal (Sigma, Oakville, ON, Canada or Rothsay, Guelph, ON, Canada), CaCO₃ (Aldrich, Oakville, ON, Canada), glycerol (Caledon Laboratories, Georgetown, ON, Canada) and pure water were used as supplied to prepare all media. Silica gel 60 (Merck, Montreal, QC, Canada) was used for normal phase flash chromatography columns and a prepacked Merck Lobar RP-18 column (240 × 10 mm) for reverse phase chromatography. Normal phase TLC was performed on Machery Nagel alugram sil G/UV₂₅₄ or Merck plates, while reverse phase TLC used Merck silica gel RP-18 F₂₅₄ plates. HPLC was performed on a C18 column (100 mm × 2.1 mm, 3 μ) with a 0.2 ml min⁻¹ flow rate running a gradient from 0% B to 100% B over 30 min. A = 5% MeCN, 0.05% formic acid in H₂O; B = 5% H₂O, 0.05% formic acid in MeCN. Proton and ¹³C NMR spectra were recorded on Bruker AV-600 (proton at 600 MHz) and AV-700 (proton at 700 MHz) spectrometers.

Growth of *S. aburaviensis* ATCC 31860 (*sp.* MG7-G1)

Cultures of *S. aburaviensis* from American Type Culture Collection (ATCC 31860) were grown in Petri dishes at 28 °C for 5 days on ISP medium 2 agar

plates (36.6 g in 1 l pure water). The resulting spores were suspended in sterile water and transferred to liquid growth medium containing 3% glycerol, 2% fish meal and 0.2% CaCO₃ in 100 ml pure water in a 500-ml Erlenmeyer flask, which had been autoclaved at 121 °C for 20 min. The flask was incubated in a rotary shaker-incubator at 28 °C and 180 r.p.m. for 2 days. Aliquots of the resulting culture (2 ml) were inoculated into twenty-two 500 ml Erlenmeyer flasks containing the same sterile liquid medium as described above. The flasks were incubated in the rotary shaker-incubator at 28 °C and 180 r.p.m. for 2 days.

Extraction and isolation of ebelactones A and B

Ethyl acetate (100 ml) was added into each culture flask and the mixture was shaken on the shaker-incubator at 180 r.p.m. for 1 h. The resulting liquid was centrifuged at 4000 r.p.m. at 4 °C for 10 min. The ethyl acetate layer was removed, and the remaining aqueous layer was then extracted twice with equal volumes of ethyl acetate (4.4 l of ethyl acetate in total). The combined organic layers were concentrated to give ~3 g of crude extract. Flash chromatography with 5:5:1 hexanes:chloroform:ethyl acetate gave fractions containing **1**, which were combined and concentrated. The residue was then filtered using a RP-18 SPE cartridge (E. Merck, adsorbex 400 mg) with 50:50 methanol-water as eluent. The filtrate was concentrated by azeotropic distillation with ethanol, and then subjected to reverse phase chromatography with a step gradient elution from 50:50 to 70:30 methanol-water to give fractions containing pure ebelactone A (**1**). Yield: 4 mg of white solid. Both one-dimensional and two-dimensional spectra were obtained by NMR; the data agreed with literature assignments made during the initial structure determination.²

Addition of labeled sodium [1-¹³C, ¹⁸O₂]propionate

A mixture of unlabeled sodium propionate (Fisher Laboratories, Ottawa, ON, Canada, 250 mg), sodium [1-¹³C, ¹⁸O₂] propionate (150 mg) (76% ¹⁸O/site) and sodium [1-¹³C]propionate (100 mg) dissolved in sterile water (8.8 ml) was added to 22 flasks prepared as described above, at 21 and at 45 h after inoculation (200 μ l per flask per feeding). Ebelactone A was isolated as above. ¹³C NMR (CDCl₃, 150 MHz): as previously reported, except for: 171.1 (enhanced C-1, $\Delta\delta$ 0.039 p.p.m.), 83.0 (enhanced C-3, $\Delta\delta$ 0.029 p.p.m.), 43.4 (enhanced C-5), 126.9 (enhanced C-7), 217.1 (enhanced C-9, $\Delta\delta$ 0.053 p.p.m.), 74.6 (enhanced C-11, $\Delta\delta$ 0.029 p.p.m.); MS (ESI +ve) *m/z* 339.3 (100%), 340.4 (85%), 341.4 (45%), 342.4 (35%), 343.4 (28%), 344.4 (12%), 346.0 (3%).

Preparation of *N*-acetylcysteamine adduct **14**

A mixture of ebelactone A (**1**) (12 mg, 30 μ mol), *N*-acetylcysteamine (9.5 mg, 80 μ mol), triethylamine (7.5 mg) and dichloromethane (0.2 ml) was stirred at room temperature for 4 h. The mixture was purified by chromatography on silica (2–4% MeOH:CHCl₃) to yield **14** (9.5 mg, 20 μ mol, 63%) as a colorless liquid. ¹H NMR (CDCl₃, 600 MHz) δ 5.78 (1H, br s, NH), 5.00 (1H, d, *J* = 10 Hz, H-7), 3.58 (1H, dq, 8, 7 Hz, H-8), 3.49 (1H, m, H-11), 3.45 (2H, t, 6 Hz, CH₂-NH), 3.42 (1H, dd, *J* = 8, 4 Hz, H-3), 3.15 (1H, br s, OH), 2.95 (2H, t, 6 Hz, CH₂-S), 2.93 (1H, dq, 4, 7 Hz, H-2), 2.86 (1H, dq, 2, 7 Hz, H-10), 2.54 (1H, d, 7 Hz, H-4), 2.38 (1H, dd, 13, 4 Hz, H-5), 1.98 (3H, s, MeC=O), 1.79 (1H, dd, 13, 10 Hz, H-5), 1.76 (2H, dq, 8, 3 Hz, H-13), 1.72 (3H, s, 6-Me), 1.45 (1H, m, H-12), 1.28 (3H, d, 7 Hz, 2-Me), 1.19 (1H, m, H-13), 1.12 (3H, d, 7 Hz, 8-Me), 1.09 (3H, d, 7 Hz, 10-Me), 0.89 (3H, t, 7 Hz, 14-Me), 0.87 (3H, d, 7 Hz, 4-Me), 0.77 (3H, d, 7 Hz, 12-Me); ¹³C NMR (CDCl₃, 150 MHz) δ 217.4 (C-9), 204.2 (C-1), 169.9 (MeC=O), 136.5 (C-6), 125.2 (C-7), 78.1 (C-3), 74.0 (C-11), 50.3 (C-2), 45.1 (C-8), 45.0 (C-10), 41.9 (C-5), 40.8 (C-4), 36.1 (CH₂-N), 34.0 (C-12), 28.8 (CH₂-S), 24.5 (C-13), 22.7 (CH₃-C=O), 16.1 (6-Me), 15.8 (8-Me), 15.5 (12-Me), 14.3 (2-Me), 13.6 (C-14), 10.4 (4-Me), 8.9 (10-Me); IR (CHCl₃, cm⁻¹) 3323, 1690, 1660; MS (ESI +ve) *m/z* 480.5 ([M + Na]⁺, 475.5 ([M + NH₄]⁺), 458.5 ([M + H]⁺). High-resolution mass data was obtained on a Waters Q-TOF Global Ultima giving an *m/z* = 458.2930 (ESI +ve, [M + H]⁺) with a calculated error of 2.2 p.p.m.

Preparation of hydroxy acid **11**

To a solution of ebelactone A (3.1 mg, 9.2 μ mol) in tetrahydrofuran (0.5 ml) was added aqueous NaOH (0.071 M, 141 μ l, 10 μ mol) and the mixture was stirred at room temperature for 8 h. The mixture was neutralized with HCl (1

equiv.), the THF was evaporated and MeOH (0.5 ml) was added. The mixture was loaded onto an RP-18 cartridge filter column, which was progressively eluted with MeOH:H₂O (50:50, 60:40, 70:30 and 100:0). Fractions containing **11** were combined and concentrated, then further purified on silica gel (CHCl₃:hexane: EtOAc:AcOH 33:33:33:1) to afford **11** (2.2 mg, 6.2 μmol, 67%). ¹H NMR (CDCl₃, 600 MHz) δ 5.01 (1H, d, *J* = 10 Hz, H-7), 3.55 (1H, dq, 10, 7 Hz, H-8), 3.49 (1H, dd, *J* = 9, 2 Hz, H-11), 3.41 (1H, t, *J* = 5 Hz, H-3), 2.85 (1H, dq, 7, 2.5 Hz, H-10), 2.75 (1H, dq, 5, 7 Hz, H-2), 2.33 (1H, q, 9 Hz, H-5), 1.81 (2H, m, H-5), 1.73 (1H, dq, 14, 7 Hz, H-13), 1.68 (3H, br s, 6-Me), 1.42 (1H, dq, 3, 7 Hz, H-12), 1.30 (3H, d, 7 Hz, 2-Me), 1.12 (3H, d, 7 Hz, 8-Me), 1.12 (1H, m, H-13), 1.07 (3H, d, 7 Hz, 10-Me), 0.86 (3H, t, 7 Hz, 14-Me), 0.85 (3H, d, 7 Hz, 4-Me), 0.77 (3H, d, 7 Hz, 12-Me); ¹³C NMR (CDCl₃, 150 MHz) δ 217.6 (C-9), 178.0 (C-1), 137.3 (C-6), 125.7 (C-7), 78.0 (C-3), 74.5 (C-11), 45.4 (C-8), 44.9 (C-10), 42.5 (C-5), 41.7 (C-2), 36.6 (C-12), 34.7 (C-4), 24.9 (C-13), 16.5 (6-Me), 16.4 (8-Me), 16.3 (4-Me), 15.2 (2-Me), 14.8 (12-Me), 10.8 (14-Me), 9.3 (10-Me); MS (ESI +ve) *m/z* 374.5 ([M + NH₄]⁺), 379.4 ([M + Na]⁺). High-resolution mass data was obtained on a Waters Q-TOF Global Ultima giving an *m/z* = 379.2460 (ESI +ve, [M + Na]⁺) with a calculated error of 0.9 p.p.m.

Cyclization of 14

A solution of **14** (0.95 mg, 2.8 μmol) in EtOH (10 ml) and water (10 ml) was stirred at room temperature for 18 h. The solvent was evaporated and the residue purified on silica gel (CHCl₃:hexane:EtOAc 5:5:1) to afford **1** (1 mg, ~100%), which was identical to authentic standard by proton NMR.

Enzymatic hydrolysis of 14

14 was incubated with or without recombinant purified DEBS-TE (5 μM). Each assay contained 1.2 mM substrate in 50 mM phosphate buffer (pH 7.4) at 20 °C and dimethyl sulfoxide (10% *v/v*). After 18 h, the assay was diluted fourfold with 1:1:0.0005 water, acetonitrile and formic acid, centrifuged to remove insoluble material and analyzed by ESI-LCMS.

ESI-positive LCMS was performed using a reverse phase BDS (base-deactivated silica) Hypersil C18 100 × 2.1 mm, particle size 3 μm (Thermo Scientific) column with mobile phase A (0.05% formic acid 5% acetonitrile in water) and mobile phase B (0.05% formic acid, 5% water in acetonitrile). A gradient from 0% B to 100% B over 30 min with a flow rate of 0.2 ml min⁻¹ was used for the chemical separation. An ionization voltage of 5500V was used in the ESI TurboSpray probe interfaced with an API2000 triple quadrupole mass spectrometer.

Kinetic analysis of DEBS-TE-mediated hydrolysis

The TE-catalyzed consumption of **14** was monitored by observation of the formation of 5-thio-2-nitrobenzoate by reaction of released *N*-acetylcysteamine with 5,5'-dithio-2-nitrobenzoic acid. Kinetic assay mixtures consisted of 5 μM DEBS-TE, 50 mM phosphate buffer (pH 7.43), 4% (*v/v*) saturated solution of dithio-2-nitrobenzoic acid in 50 mM phosphate buffer (pH 7.43), substrate (at 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) and 10% (*v/v*) dimethyl sulfoxide. The formation of free thiol was quantified by measuring the absorption at 412 nm using a ScanDrop 250 Nano Volume Spectrophotometer. The reactions were performed at room temperature and data points were collected every 30 s for 1.5 h. Initial velocities were determined by linear regression analysis of the data. All steady state kinetic assays were performed in duplicate. The kinetic parameters *k*_{cat} and *K*_M were calculated from the direct fit of the slope of *v* versus [S] to the Michaelis-Menten equation.

Genome sequencing and ebelactone cluster identification

A single colony of *S. aburaviensis* ATCC 31860 was grown 3 days at 28 °C, 250 r.p.m. in DSMZ Media 65 (Deutsche Sammlung von Mikroorganismen und Zellkulturen). Genomic DNA was harvested using a GenElute Bacterial Genomic DNA Kit (Sigma). Genomic DNA was sent for library preparation and Illumina sequencing at the Farncombe Metagenomics Facility at McMaster University, using an Illumina MiSeq DNA sequencer. Contigs were assembled using the ABySS genome assembly program and with Geneious bioinformatic software. The ebelactone gene cluster was identified using BLAST with PksJ from the bacillaene biosynthetic assembly-line of *Bacillus subtilis* str. 168 as the

query. The entire ebelactone cluster was found on a single contig. Gene functions and domain architectures were determined using AntiSMASH and BLAST analysis against the NCBI database.⁴¹ The Genbank accession number for the ebelactone gene cluster is KC894072.

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PANOS ARGYROPOULOS

SKILLS AND PROFICIENCIES

- Proficient at communicating science to scientific and non-scientific audiences
- Effective French and English communicator with a working knowledge of Greek
- Exposed to and interested in different aspects of chemistry, biochemistry and materials
- Experienced in training undergraduate students for chemical synthesis and monitoring students in an educational environment
- Integral part of team building in both laboratory and sport settings
- Recognized as an important contributor to problem solving in a laboratory setting and within the chemistry and university graduate student societies

EDUCATION

- MASTER OF SCIENCE – University of Ottawa (Ottawa, Ontario) 2014
- Research interests with Dr. Chris Boddy include:
 - Characterization of enzyme-substrate interaction in polyketide biosynthesis
 - The use of thioesterases in polyketide natural product synthesis
 - Courses taken at the University of Ottawa
 - Synthesis and Properties of Natural and Designed Organic Structures
 - Organic Structure Determination using NMR Spectroscopy
 - Characterization and Applications of Soft Materials
 - Physical Organic Chemistry
 - Community Outreach and Media Relations in the Sciences
- FROM IDEAS TO LAUNCH – TRANSFORMING BUSINESS IDEAS TO REALITY – McGill University (Montreal, Quebec) 2012
- Completed a program aimed at providing practical tools for planning and launching a successful business
 - Addressed topics such as discovery and development of business ideas, writing and execution of a business plan and practical financing
- SECOND LANGUAGE CERTIFICATION COURSE– University of Ottawa (Ottawa, Ontario) 2012
- Completed FLS3500, a course designed to acknowledge proficiency in French
- BACHELOR OF SCIENCE – Simon Fraser University (Burnaby, B.C.) 2010
- Graduated in chemistry with interests in French, Mathematics and Philosophy
 - Research interests included Natural Product Synthesis under Dr. Robert Briton and Liquid Crystal Characterization with Dr. Vance Williams
- UNIVERSITY FORMAL EXCHANGE PROGRAM –University of Strathclyde (Glasgow, Scotland) 2009
- Exploration into Forensic Chemistry and Teaching Science to Primary School Children
- UNIVERSITY TRANSFER PROGRAM – Capilano University (North Vancouver, B.C.) 2007
- Graduated in chemistry with interests in French and Philosophy
- HIGH SCHOOL DIPLOMA– Handsworth Secondary School (North Vancouver, B.C.) 2005
- Graduated with a French Immersion diploma

EMPLOYMENT

- UNIVERSITY OF OTTAWA (Ottawa, Ontario) 2011 - 2013
Teaching Assistant, Department of Chemistry
- Weekly discussion and review sessions of organic chemistry for 20-400 students
 - Responsible for training and monitoring undergraduate students in a laboratory setting
- PD PLUS (Vancouver, B.C.) 2011
Tutor
- Tutoring High School Students in Science, Mathematics and French
- BRITISH COLUMBIA LIQUOR DISTRIBUTION BRANCH (Vancouver, B.C.) 2008 - 2011
Store Clerk
- Providing customer service and stocking the shelves

PUBLICATIONS AND CONFERENCES

3 MINUTE THESIS COMPETITION

- Placed 1st at the University of Ottawa and competed in the Ontario-wide competition of oratory explanation of research to a non-scientific audience

95TH CANADIAN CHEMISTRY CONFERENCE AND EXHIBITION

- Presented a poster entitled, "Synthesis of Phosphonate to Characterize Enzyme-Substrate Interactions in Macrolactonization"

96TH CANADIAN CHEMISTRY CONFERENCE AND EXHIBITION

- Presented a poster entitled, "Probing the Stereospecificity of DEBS-TE by Formation of a Non-Hydrolyzable Acyl-Enzyme Intermediate"

"Biosynthesis of Ebelactone A: Isotopic tracer, advanced precursor and genetic studies reveal a thioesterase-independent cyclization to give a polyketide β -lactone" Wyatt, Ahilan, [Argyropoulos](#), Boddy, Magarvey, Harrison – J Antibiot 2013 Jul; 66(7):421-30.

EXTRACURRICULAR INVOLVEMENT

- ASSISTANT COACH FOR THE GIRLS U15 OTTAWA INTERNATIONALS SOCCER CLUB 2013 - 2014
- Certified by the Ontario Soccer Association to coach up to U18 and assists in running soccer practices and games for the Ottawa Internationals
- COMPETE AND VOLUNTEER IN BOTH MAJOR POETRY SLAMS IN OTTAWA 2013 - 2014
- Compete semi-regularly in the 3-minute memorized performance of original poetry and contribute to the poetry community regularly by scorekeeping the competition
- GSAED REPRESENTATIVE FOR THE DEPARTMENT OF CHEMISTRY 2011 - 2013
- Contribute to discussion pertaining to the graduate student budget and social activities

INSTRUMENTATION AND COMPUTER SKILLS

- Experienced in the use of Bruker and Avance-based NMR spectrometers, GC, HPLC, IR, MALDI-TOF and Electrophoresis systems
- Proficient in Microsoft Office, ChemBioOffice, Bruker TOPSPIN among other NMR analysis software, Scifinder, Reaxsys, Adobe Photoshop, eMolecules