

Resorcylic Acid Lactone Thioesterases as Potential Biocatalysts

By

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Abstract

A key missing tool in the chemist's toolbox is an effective biocatalyst for macrocyclization. Macrocycles limit the conformational flexibility of small molecules, often improving their ability to bind selectively and with high affinity to a target, making them a privileged structure in drug discovery. Resorcylic acid lactones (RALs) are a class of fungal macrocyclic polyketides that exhibit anti-cancer and anti-malarial activity among others. The thioesterases (TEs) found in the biosynthetic pathways for the zearalenone (*Zea*) and radicicol (*Rdc*) resorcylic acid lactones are responsible for macrocyclization and show promising traits as biocatalysts. These RAL TEs show the highest substrate tolerance of any polyketide thioesterase to date. These TEs can efficiently cyclize 12- 18-membered rings, 14-membered macrolactams, and amino acid containing substrates. Their robustness is evident in their ability to retain activity after lyophilization/re-suspension and in high DMSO concentrations. Furthermore, the ability of *Zea* and *Rdc* TEs to macrocyclize depsipeptide substrates illustrates the first time a polyketide synthase TE has efficiently processed a peptide-containing substrate. The unique substrate tolerance of this class of TEs shows great potential as a viable biocatalyst. Herein we describe the synthesis and enzymatic results of diverse group of substrates, with the TEs from the radicicol and zearalenone biosynthetic pathways, as well initial results on the chemoenzymatic synthesis of asperterrestide A.

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List of Abbreviations

9-BBN	9-borabicyclo[3.3.1]nonane
A	Adenylation domain
ABA	<i>o</i> -aminobenzoic acid
ACN	Acetonitrile
ACP	Acyl-carrier protein
ATA	<i>Arthrobacter</i> transaminase
Boc	Tert-butoxy carbonyl
BuOAc	Butyl acetate
C	Condensation domain
CDCl ₃	Deuterated chloroform
CD ₃ OD	Deuterated methanol
CoA	Co-enzyme A
Crp TE	Cryptophycin thioesterase
d	Doublet
DCM	Dichloromethane
DEAD	Diethyl azodicarboxylate
DEBS TE	6-deoxyerythronolide B thioesterase
DH	Dehydratase
DIPEA	Diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DMSO-d ₆	Deuterated dimethylsulfoxide
Dpt TE	Daptomycin thioesterase
DTNB	5,5'-Dithio-bis-(2-nitrobenzoic acid)
<i>ee</i>	Enantiomeric excess
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
Epo TE	Epothilone thioesterase

ER	Enoyl reductase
ESI	Electrospray ionization
Et ₂ O	Diethyl ether
EtOAc	Ethyl acetate
FA(S)	Fatty acid synthase
GDH	Glucose dehydrogenase
h	Hour
HATU	Hexafluorophosphate azabenzotriazole tetramethyl uronium
Hex	Hexane
HF	Hydrofluoric acid
HHDH	Halohydrin dehalogenase
HMG	3-Hydroxy-3-methylglutaryl
HOBt	Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HR-PKS	Highly reducing polyketide synthase
Hsp90	Heat shock protein 90
HSV	Herpes simplex virus
HWE	Horner-Wadsworth-Emmons
<i>i</i> -PrOH	isopropanol
K _i	Inhibition constant
KR	Ketoreductase
KS	Ketosynthase
KRED	Ketoreductase
m	Multiplet
M	Molar
M ⁺	Molecular ion
MAO	Monoamine oxidase
MAP kinase	Mitogen activated protein kinase
MAT	Malonyl acyl-transferase
Me	Methyl

MeOH	Methanol
mg	milligram
MgSO ₄	Magnesium sulfate
MHz	Megahertz
MeI	Methyl iodide
min(s)	Minute(s)
mm	Millimeter
mM	millimolar
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NEt ₃	Triethyl amine
NH ₄ OAc	Ammonium acetate
nm	nanometers
NMR	Nuclear magnetic resonance
NR-PKS	Non-reducing polyketide synthase
NRP(S)	Non-ribosomal peptide (synthetase)
O ₂	Oxygen gas
OsO ₄	Osmium tetroxide
PCP	Peptidyl-carrier protein
Pd/C	Palladium on carbon
pH	Power of hydrogen
Phe	Phenylalanine
Pim TE	Pimaricin thioesterase
PK	Polyketide
PK/PD	Pharmacokinetic/pharmacodynamic
PKS	Polyketide synthase
PLP	Pyridoxal phosphate
PPh ₃	Triphenyl phosphine
psi	Pounds per square inch
PT	Product template domain
PyBOP	Benzotriazole-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate

q	Quartet
Q-TOF	Quadripole time of flight
RAL	Resorcylic acid lactone
Rdc TE	Radical thioesterase
R _f	Retention factor
RT	Room temperature
s	Singlet
SAT	Starter-unit acyl-transferase
S _N 2	2-Step nucleophilic substitution
SNAC	N-acetyl cysteamine
t	Triplet
TAK-1	Transforming growth factor β-activated kinase
TBS(Cl)	Tert-butyl dimethyl silyl (chloride)
TE	Thioesterase domain
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
Tyc TE	Tyrosidine thioesterase
UV	Ultra-violet
Vlm TE	Valinomycin thioesterase
v/v	Volume by volume
V _{rel}	Relative velocity
Zea TE	Zearalenone thioesterase
μL	Microliter
μM	micromolar

Chapter 1 introduction

1.1 Biocatalysis

1.1.1 What is Biocatalysis?

Biocatalysis is the use of a biological system or part of, to catalyze chemical reactions.¹ The field of biocatalysis uses enzymes found in a variety of different organisms, from prokaryotes like bacteria, to eukaryotes such as plants and fungi.^{2,3} Scientific focus in this field is to harness the power of these enzymes to increase the productivity (overall yields, stereochemical purity, waste reduction etc.) of chemical reactions. This can range from undergraduate students in a research lab working on a 10 mg scale, all the way to big pharmaceutical companies, like Pfizer, making kilograms of Lipitor a week to send across the world.⁴ Biocatalysis benefits from highly functionalized enzymes that can catalyze extremely selective, high-yielding transformations efficiently and under mild conditions. With the technology we have today the field of biocatalysis is growing, with many examples of biocatalysis being used not only for research but in large industrial processing.^{2,4-6}

1.1.2 Biocatalysis in Industry.

Over the past 15 years there has been a spike in the number of biocatalysts being implemented into industrial processing. With advances in protein engineering, generating viable biocatalysts is becoming more and more practical. A breakthrough in 2010 by a group at Codexis, established an improved chemoenzymatic synthesis of the anti-diabetes drug, Sitagliptin (Januvia).⁵ Until 2010, the synthesis of sitagliptin revolved around an asymmetric reductive amination, via a rhodium-catalyzed hydrogenation (Fig. 1.1).

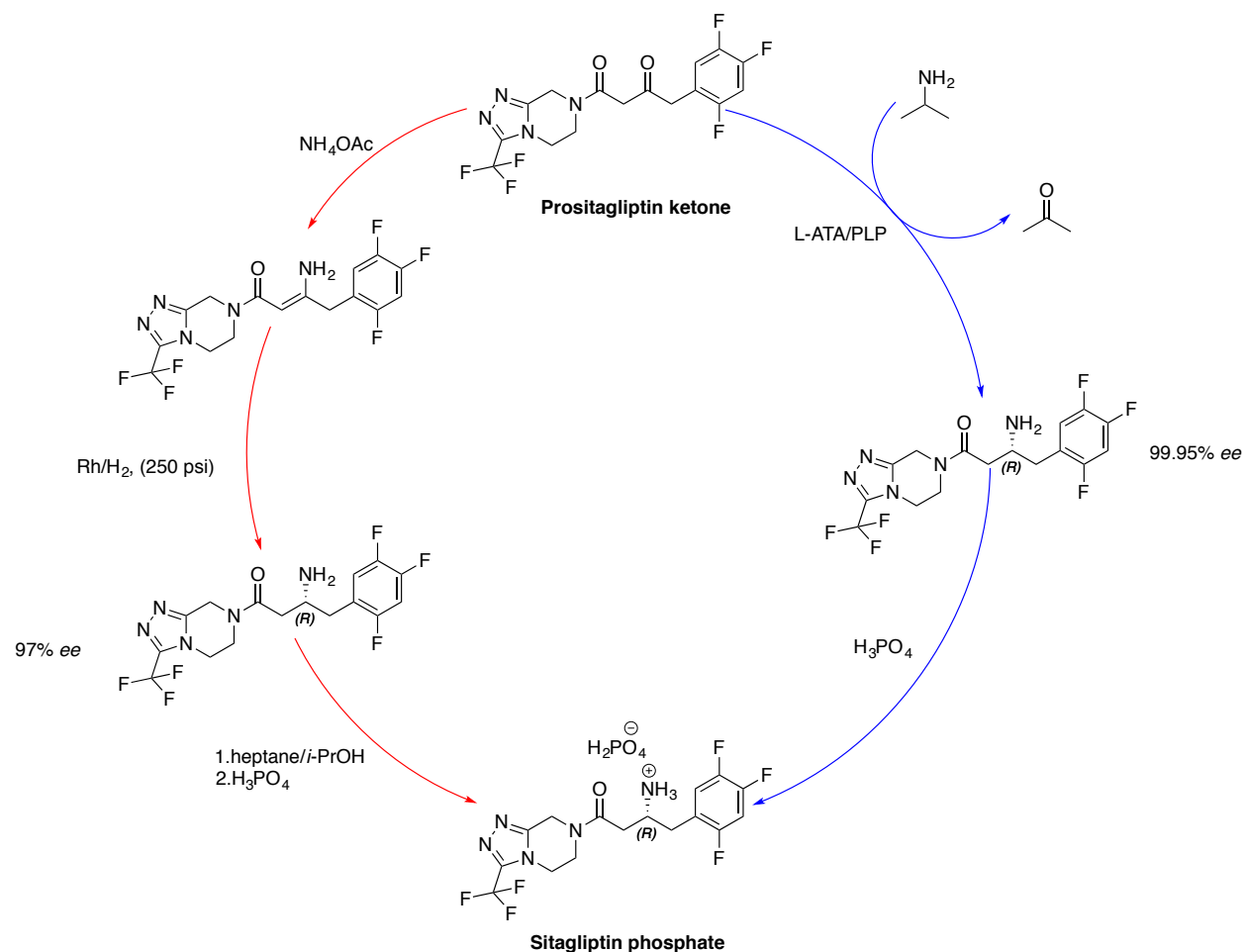


Figure 1. 1 Improved chemoenzymatic transamination of prositagliptin ketone to sitagliptin phosphate.⁵

The original method yielded sitagliptin with a 97% *ee* and required additional purification steps to remove toxic and expensive rhodium catalysts. Along with additional purifications to remove the rhodium catalyst, the hydrogenation had to be run under H_2 at 250 psi, requiring additional expensive equipment.⁵ Savile *et al.*, using an engineered (*R*)-selective transaminase homolog (ATA-117), were able to increase the stereoselectivity of this transformation (from 97% to 99.95% *ee*) while removing the need for high pressure reaction conditions and toxic catalysts. Overall they increased the yield by 13%, their productivity by 53%, and decreased their waste by 19%.⁵

Another example of the benefits of industrial biocatalysis is in the production of atorvastatin (Lipitor) (Fig. 1.2).

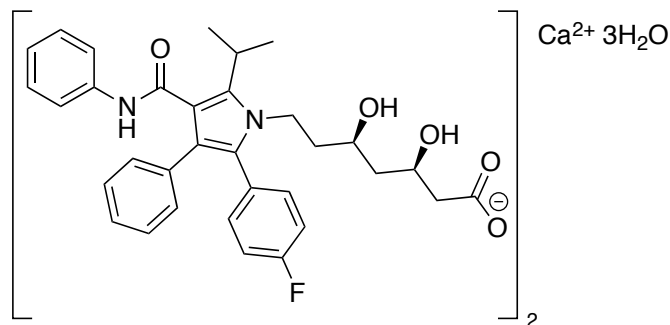


Figure 1. 2 Structure of atorvastatin calcium (Lipitor).

Lipitor is the first commercial drug to exceed \$10 billion dollars in annual sales. It acts as an HMG-CoA reductase inhibitor, suppressing the synthesis of cholesterol in the liver.⁴ One of the key intermediates in its synthesis is hydroxynitrile **1.4** (Fig. 1.3). In 2009, Codexis engineered a 2-step, 3 enzyme process to produce **1.4** from the cheap, commercially available chloro ketone **1.1**.

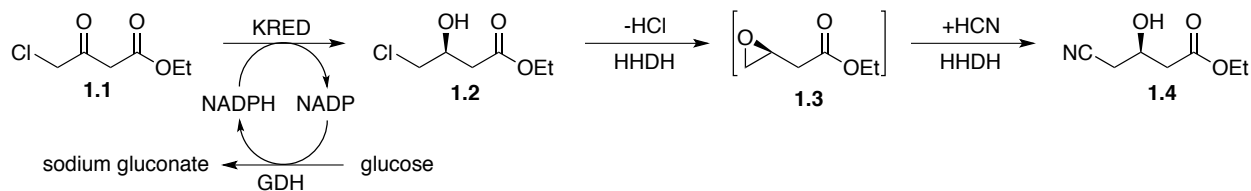


Figure 1. 3 2-step, 3 enzyme synthesis of hydroxynitrile **1.4**. KRED-ketoreductase, HHDH-halohydrin dehydrogenase, GDH-glucose dehydrogenase.⁴

Codexis showcased the first example of a multi-step system using multiple enzymes. A ketoreductase was used to selectively reduce the ketone in compound **1.1** to the alcohol using an NADPH cofactor as the hydride donor. One issue with biocatalysis is the use of expensive cofactors, in this example Codexis was able to overcome this issue by adding a glucose dehydrogenase to re-generate the NADPH through the oxidation of glucose. The second step involved the use of a halohydrin dehydrogenase to form the epoxide intermediate **1.3**, that is subsequently re-opened, with high stereoselectivity, to form **1.4**. Due to the mass amounts of this intermediate required to reach the demand of Lipitor, this green-chemistry approach to compound **1.4** has greatly reduced waste.⁴ Overall this method had a yield increase from 85% to >90%, with an *ee* of >99.9%. Since the reaction was performed in biodegradable, aqueous buffer, the only waste came from extractions using BuOAc, which was recycled at a rate of 85%. This green-by

design process is a good example where this field is heading, the use of biocatalysts for multi-step reactions.²⁻⁴

The former two examples (sitagliptin & hydroxynitrile **1.4**) use reductive enzymes to form highly pure stereocentres (KRED, ATA-117, HHDH).^{2,4,5} This is where a majority of biocatalysis research lies, but there are also examples where oxidative enzymes are employed to set stereocentres. Boceprevir, marketed under the trade name Victrelis, is a first in class treatment for chronic hepatitis C (Fig. 1.4).⁶

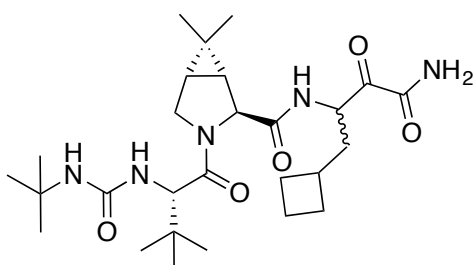


Figure 1. 4 Structure of boceprevir (Victrelis).

Looking at the structure from a chemist's point of view, the obvious bottleneck for boceprevir synthesis is the formation of the bicyclic proline moiety, containing 3 stereocentres. A collaboration between Codexis and Merck in 2012 developed a biocatalytic method, using a monoamine oxidase (MAO), to set these 3 stereocentres.⁶

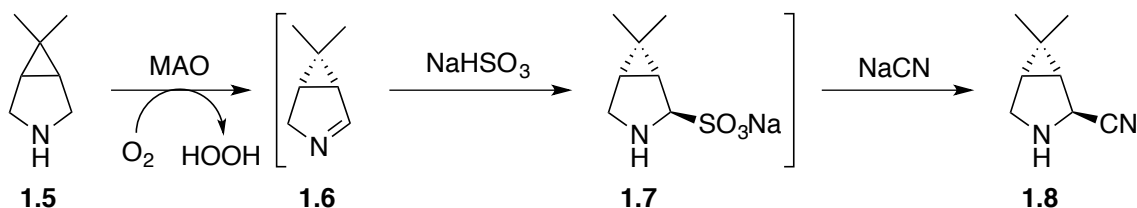


Figure 1. 5 Biocatalytic synthesis of bicyclic intermediate **1.8**.⁶ MAO – monoamine oxidase.

The starting compound, **1.5**, undergoes an oxidative desymmetrization by a MAO using oxygen gas to selectively produce **1.6**, which is then sulfonated to make **1.7** (Fig. 1.5). The addition of sodium cyanide forms the bicyclic proline nitrile **1.8**. This enzymatic desymmetrization resulted in an *ee* of 99% for compound **1.8**, a 60% reduction in raw materials need, and a 60.7% reduction

in waste.⁶ The monoamine oxidase used in boceprevir synthesis illustrates that the scope of potential biocatalysts is expanding to include oxidative enzymes.^{2,6}

1.1.3 Limitations of Biocatalysis.

There are an increasing number of examples showcasing the favourable usage of biocatalysts over traditional synthetic organic methods.² Increased efficiencies, reduced reaction times, high stereoselectivity, and massive waste reductions highlight a few of the benefits of employing biocatalytic methods in small molecule production. Unfortunately, there are still many limitations impeding the viability and development of biocatalysts.

The stability and reactivity of these enzymes are issues when it comes to optimizing biocatalytic assays. Proteins often require aqueous, pH specific and temperature specific conditions for optimal results.⁷ When working with hydrophobic compounds, co-solvents are required for solubility, and these co-solvents can disrupt protein structure and potentially abolish catalytic activity. Often in synthetic chemistry, substrate solubility can be increased by pH manipulation or a temperature increase, in a biocatalytic assay this can lead to the proteins being denatured thereby lowering yields. Another fundamental concern is that the reactions catalyzed by these enzymes are often reversible, and it can sometimes be difficult to drive the equilibrium to the desired product.^{8,9} In the case of sitagliptin phosphate, the transaminase works in a reversible fashion from the ketone to the amine. To drive the equilibrium forward the enzymatic reaction had to be run at a reduced pressure to cause the acetone to evaporate once it had formed (Fig. 1.1).⁵ Although enzyme stability and reaction equilibrium are an issue, the most significant bottle-neck in biocatalysis optimization stems from the tolerance of the enzymes to accept the desired substrate.^{2,3}

Over millions of years, enzymes have evolved to work selectively and efficiently.¹ The amazing efficiency of these enzymes is the main drive to use them biocatalytically, and the selectivity is the barrier that must be overcome. In a biological system, enzymes need to be very selective on what substrates they catalyze reactions for. Without this selectivity many undesirable reactions can occur *in vivo* that may disrupt or kill the organism. For biocatalysis the lack of

substrate tolerance greatly limits catalytic efficiency.^{7,9} As seen in sitagliptin, substrate range studies suggested that the native enzyme would only accept substrates that contained a methyl group substituent to the ketone.⁵ The native enzyme likely would have shown no activity with pro-sitagliptin (Fig. 1.1). These selective enzymes require significant mutagenesis to subtly modify the protein fold to enable catalysis of the desired reaction. In the case of sitagliptin, over 27 mutations had to be made to the native protein to accept pro-sitagliptin as a substrate.⁵ Thus protein engineering plays an essential role in biocatalyst development.

1.1.4 Protein Engineering.

Protein engineering is the process of making useful proteins by modifying known proteins, or making them *de novo*.¹⁰ Common methods for protein engineering include directed evolution, random mutagenesis, *de novo* protein synthesis etc..^{5,10,11} Random mutagenesis consists of rounds of mutated proteins being screened with the desired substrate. As enzyme efficiency increases with respect to the desired substrate, key mutations can be confirmed and further manipulated. This often requires multiple rounds of mutagenesis and can be a long process.¹² Computational modelling based on protein crystal structures can be a useful tool in deciding what mutations may be beneficial or non-deleterious, and can aid in *de novo* protein design.¹³ The advances in protein engineering using these tools has increased the practicality of biocatalyst optimization, but more research is needed to make biocatalysis an everyday strategy for making small molecules.^{7,9,11}

1.2 Macrocyclic Natural products

1.2.1 Macrocyclic Natural Products in Medicine.

Macrocyclic natural products are secondary metabolite small molecules consisting of a ring(s) larger than 8 atoms. They are found in most living organisms and possess a large variety of bioactivities, from antimicrobials, to anticancer chemotherapeutics etc. (Fig. 1.6).¹⁴

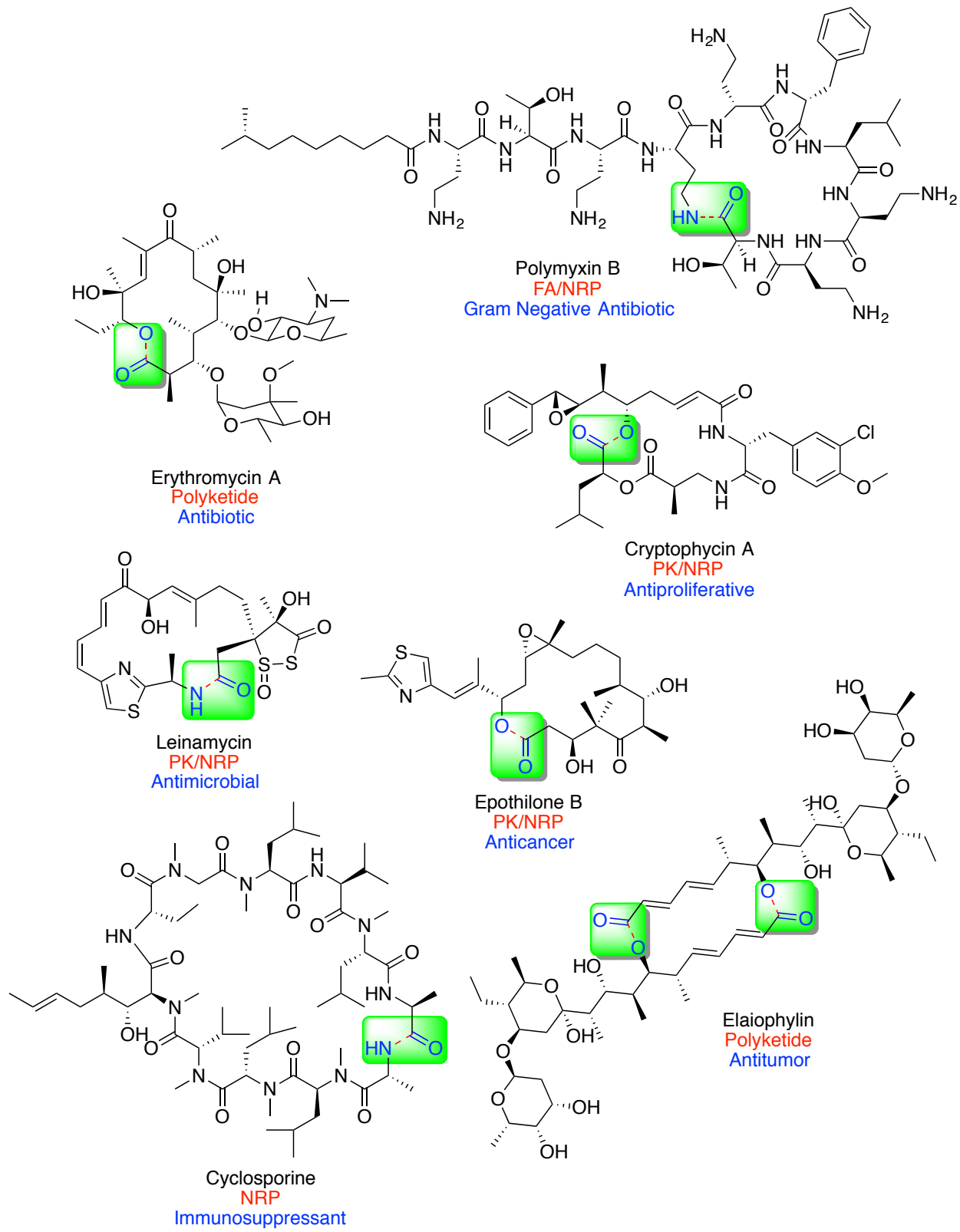
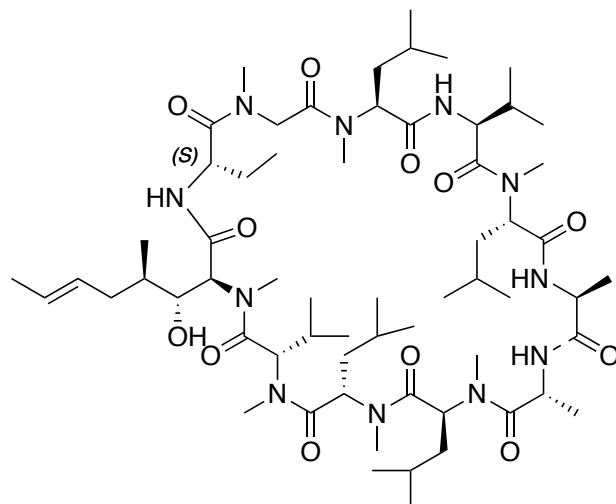


Figure 1. 6 Bioactive, macrocyclic natural products. Highlighted site of cyclization.

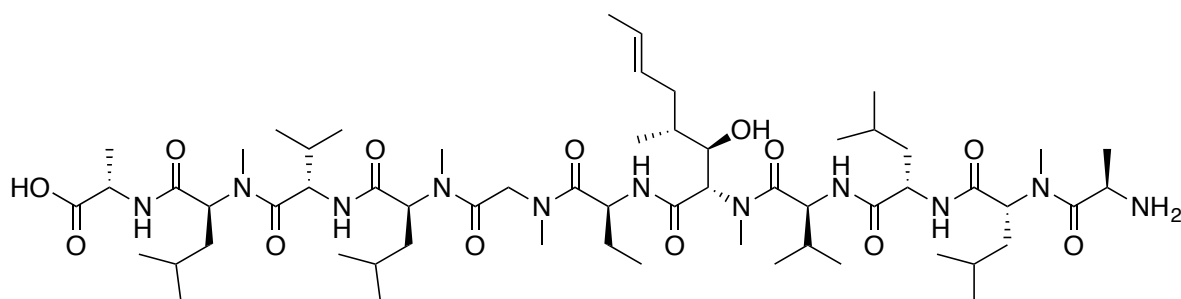
Many of these compounds have found their way into commercial production as pharmaceuticals.¹⁵ Compounds such as erythromycin A (antibiotic), and cyclosporine (immunosuppressant) can be found at your local pharmacy.^{16,17}

1.2.2 Structural Characteristics.

It is the unique structural features of macrocycles that enable them to be highly bioactive. The macrocyclic scaffold adds a conformational restriction to these compounds making them ideal for selective binding, as well as other favourable drug-like properties such as increased solubility, cell permeability, and PK/PD properties.¹⁵ By retaining a cyclic structure, the flexibility of these compounds is limited. This limited movement can increase effective binding, lowering K_i values of these compounds, improving their potency.¹⁸ Macrocycles allow for an increase in compound functionality while still maintaining membrane permeability. Cyclosporine, an immunosuppressant that inhibits the activation of T-cells through binding cytosolic cyclophilin (immunophilin), relies on cell penetration to be effective.¹⁹ The linear cyclosporine undecapeptide analog drug containing 11 peptides would likely be too polar for membrane permeation, however once cyclized, the decrease in rotatable bonds, and increased hydrophobicity, would allow for membrane penetration (Fig. 1.7).



Cyclosporine



Linear Cyclosporine

Figure 1. 7 Cyclosporine and its hydrolyzed linear precursor.

These desirable characteristics make macrocyclic natural products highly successful in new drug development. A critical issue with macrocycles however, is the difficulty in their chemical synthesis. In fact most macrocyclic natural products produced in industrially and used clinically, are made biologically, using a host organism.²⁰

1.2.3 Synthetic Macrolactonization Techniques

Research on macrocyclization reactions has been vigorous over the past 70 years. Because macrocycles are so structurally important to small molecules and medicinally beneficial, a general synthetic method would be ideal.^{15,21,22} There have been many breakthroughs in tackling this issue with reactions such as the Yamaguchi, Corey-Nicolaou, and Mitsunobu reactions along with many

others.²² Two fundamental issues with macrocyclization are overcoming the large entropic and enthalpic barriers, as well as maintaining chemo-, regio-, and stereo-selectivity. After decades of research one of the more common methods to form macrocycles is via lactone/lactam formation. This typically involves a nucleophilic attack from either an alcohol or amine, to an activated carbonyl.²³ Due to the enthalpic and especially entropic barrier for intramolecular reactivity, transesterification via an inter-molecular reaction typically competes. To overcome this unwanted reactivity, macrocyclizations typically need to be carried out at low concentrations.²³

The Yamaguchi reaction is the gold standard when it comes to forming macrolactones. Developed in 1978 at the Kyushu University in Japan, this technique uses a *seco*-acid (hydroxy acid) substrate and involves the formation of a mixed anhydride followed by intra-molecular nucleophilic attack forming a lactone (Fig. 1.8).²⁴

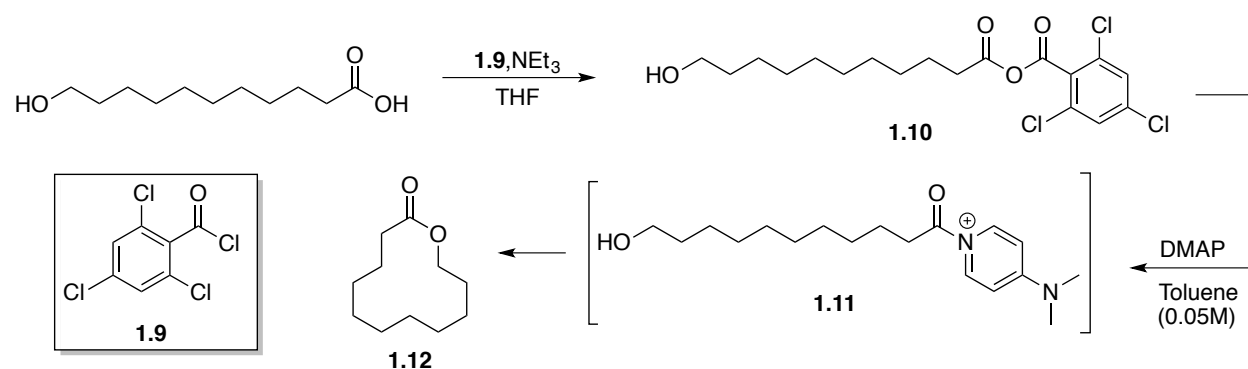


Figure 1. 8 Yamaguchi macrocyclization reaction sequence.

The Yamaguchi reagent, 2,4,6,-trichloro benzoyl chloride **1.9** activates the free carboxylic acid of the linear substrate to form the anhydride intermediate **1.10**. To further activate the anhydride, DMAP is used to form compound **1.11**. Upon formation of **1.11** it will rapidly react with any nearby nucleophiles; therefore, the concentration must be low to favour the intra-molecular reaction. This is often controlled by slowly injecting the solution of compound **1.10** into a solution of DMAP, minimizing the concentration of **1.11** at any given time. In a dilute solution **1.11** favours an intra-molecular macrocyclization to form **1.12**.²⁴ This method has been used in the total synthesis of luffalactone²⁵, macrolactin A²⁶, oleanolide²⁷, as well as many other natural products.

The Corey-Nicolaou reaction uses a pyridinyl disulfide compound **1.13** to activate both the nucleophilic ω -hydroxyl group and the free carboxylic acid (Fig. 1.9).²³

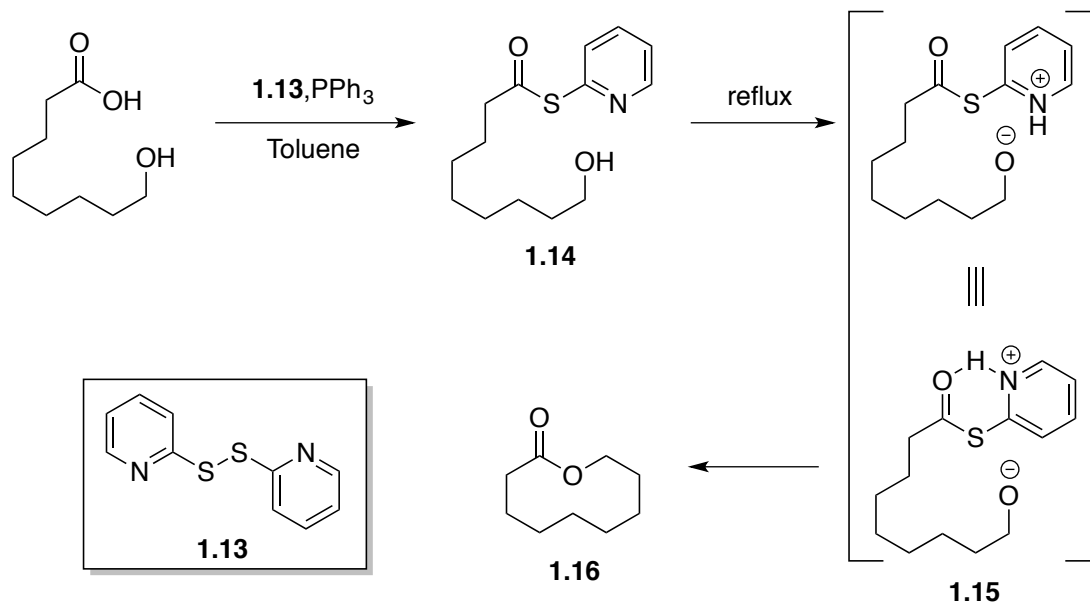


Figure 1.9 Corey-Nicolaou macrolactonization.²³

The activated thioester **1.14** is formed upon the addition of **1.13** and triphenyl phosphine. Thioester **1.14** is then further activated under reflux conditions with the pyridinyl nitrogen acting as a base deprotonating the ω -alcohol to form **1.15**. At low concentrations this alkoxide attacks the activated thioester forming the macrolactone product **1.16**. The Corey-Nicolaou reaction is quite useful because it uses mild reaction conditions.²⁸ Several total syntheses have employed this method including the total synthesis of zearalenone.²⁹

The Mitsunobu reaction has been a popular choice in the ester formation, because it provides an inversion of the ω -hydroxyl configuration, which can be quite useful.³⁰ Its utility to form macrolactones has also been studied and shown to work quite well. The Mitsunobu differs from the Yamaguchi and Corey-Nicolaou reactions in the sense that the ω -hydroxy group is acting as the electrophile and the carboxylate becomes the nucleophile (Fig. 1.10).

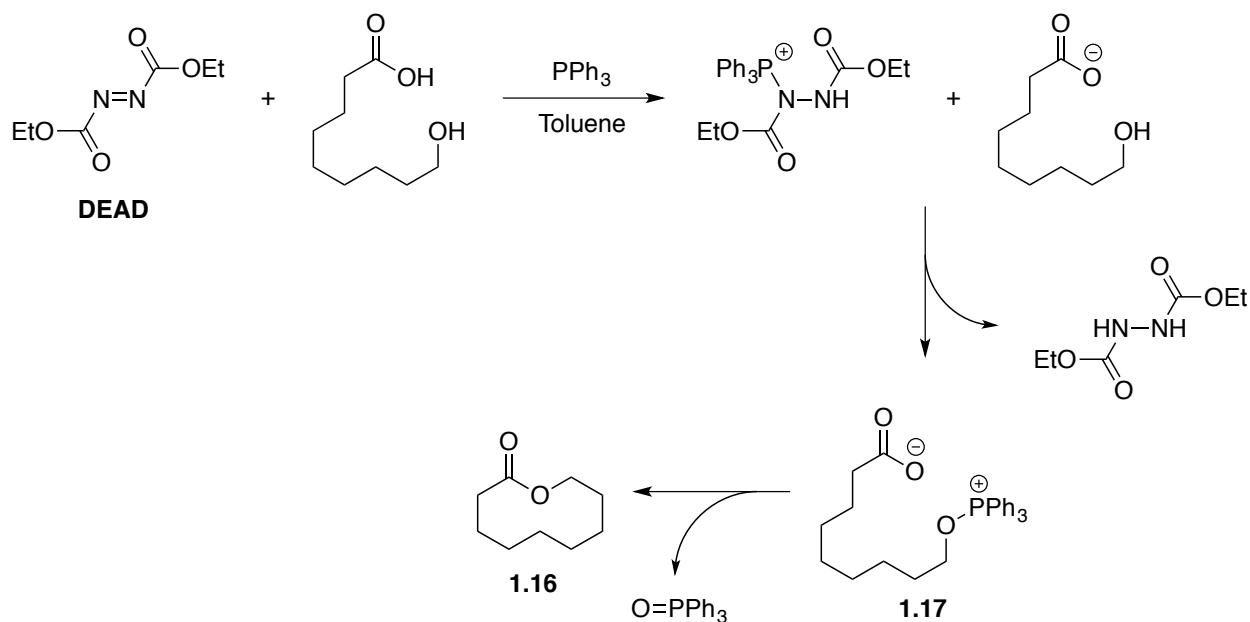


Figure 1. 10 Mitsunobu macrolactonization reaction.

Triphenyl phosphine and diethyl azodicarboxylate (**DEAD**) are used to form the oxy-phosphonium intermediate **1.17**. Compound **1.17** then undergoes an $\text{S}_{\text{N}}2$ reaction where the carboxylate is the nucleophile and the oxy-phosphonium ion is the leaving group, yielding compound **1.16**.²³ The stereochemical control of the Mitsunobu macrolactonization has made it favourable for many total syntheses such as amphidinolide K³¹, cyclothialidine³², and hypothemycin³³.

All these macrolactonization techniques, although useful, suffer from the same setbacks in terms of chemo-, regio-, and stereoselectivity. Lack of chemo-selectivity means, any other reactive groups on these linear precursors, require protecting groups, adding multiple steps to these syntheses. Macrolactonization is increasingly difficult with more complex molecules and leads to lack of regioselectivity. Lastly, the lack of stereoselectivity can greatly reduce overall yields of macrolactonization reactions. The total synthesis of erythromycin exhibits how difficult it can be to sufficiently produce a macrocycle with correct configuration, and a practical yield.¹⁶ Over 200 reaction conditions were tested, and all hetero-atoms required protecting groups to achieve macrolactonization. Another universal problem for all these techniques, is the need for dilute conditions. For small-scale research reactions this is not a serious concern, but at industrial scale,

it becomes prohibitive. In overcoming these issues, biocatalytic macrocyclization enzymes, specifically thioesterases, provide a useful alternative.

1.3 Thioesterases For Enzymatic Macrocyclization

1.3.1 What Are Thioesterases?

All macrocyclic natural products undergo a cyclization reaction, usually near the end of their biosynthesis. These can be spontaneous (rare) or enzyme catalyzed reactions. Thioesterases (TEs) are a group of enzymes responsible for macrocyclization in polyketide (PK) and non-ribosomal peptide (NRP) synthesis.³⁴ Depending on the biosynthetic pathway, TEs can also catalyze hydrolysis³⁵ and in some cases transesterification.³⁶ Figure 1.11 shows a typical thioesterase macrocyclization mechanism. The linear substrate is loaded on the thioesterase from the acyl carrier protein (ACP) via a nucleophilic attack from the active-site serine (in some cases an active site cysteine). A second nucleophilic attack from an internal amine or alcohol is catalyzed by the TE, to release the macrocyclic product.³⁴ The thioesterase is then free to catalyze another macrocyclization. Thioesterase hydrolysis follows the same mechanism except an external water molecule acts as the nucleophile opposed to an internal hetero-atom. Advances in molecular biology over the last 40 years have allowed for a more in depth look at these chemoenzymatic powerhouses.³⁷

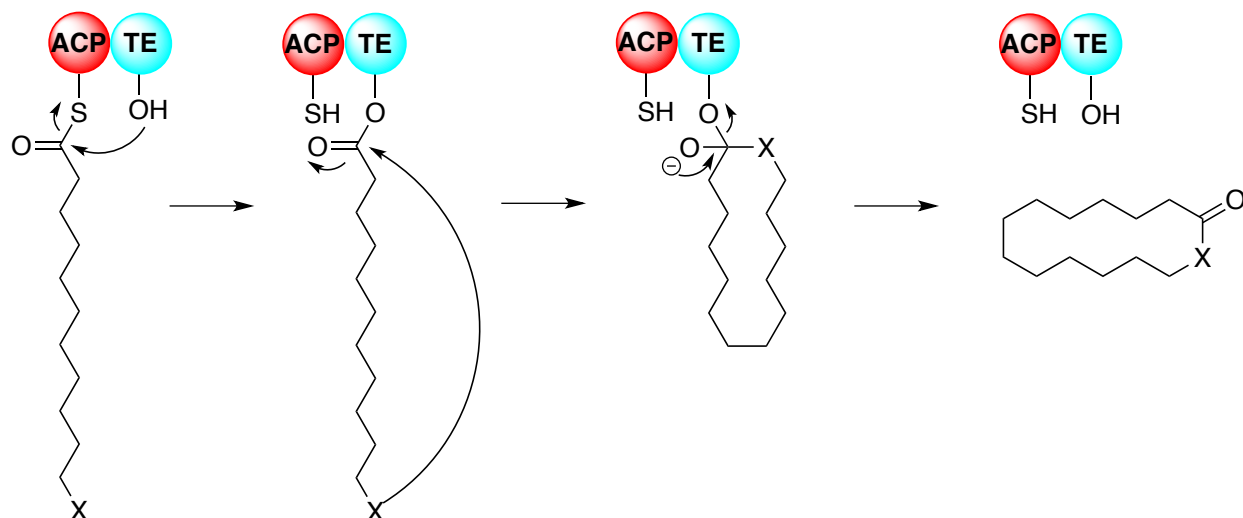


Figure 1. 11 Thioesterase macrocyclization mechanism. X = OH of NH₂.

1.3.2 Thioesterases In Non-ribosomal Peptide Biosynthesis.

Non-ribosomal peptides represent a large group of natural products including macrocyclic peptides. Many of these macrocyclic peptides have shown excellent bioactivities and are being used in modern medicine.^{19,38} The linear intermediates of these compounds are cyclized during biosynthesis using peptidyl thioesterases. NRP biosynthesis consists of a large assembly-line protein called an NRPS (non-ribosomal peptide synthetase) that terminates with a thioesterase domain (Fig. 1.12).

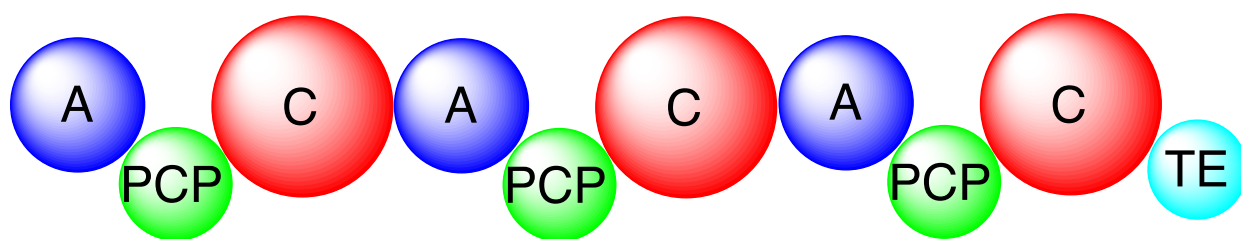


Figure 1. 12 NRPS protein with terminal thioesterase. A = adenylation domain, C = condensation domain, PCP = peptidyl carrier protein domain, TE = thioesterase domain.

For linear peptides the TE will hydrolyze the substrate once it has been properly elongated³⁹, and for macrocyclic peptides it will either macrolactamize, or macrolactonize the substrate.³⁸

Daptomycin, a potent last chance antibiotic, contains a 10-amino acid macrocycle that is formed using the Dpt TE. Cyclization using the Dpt TE is a great example in understanding just how efficient the chemo-, and regioselectivity of these thioesterases can be.³⁸

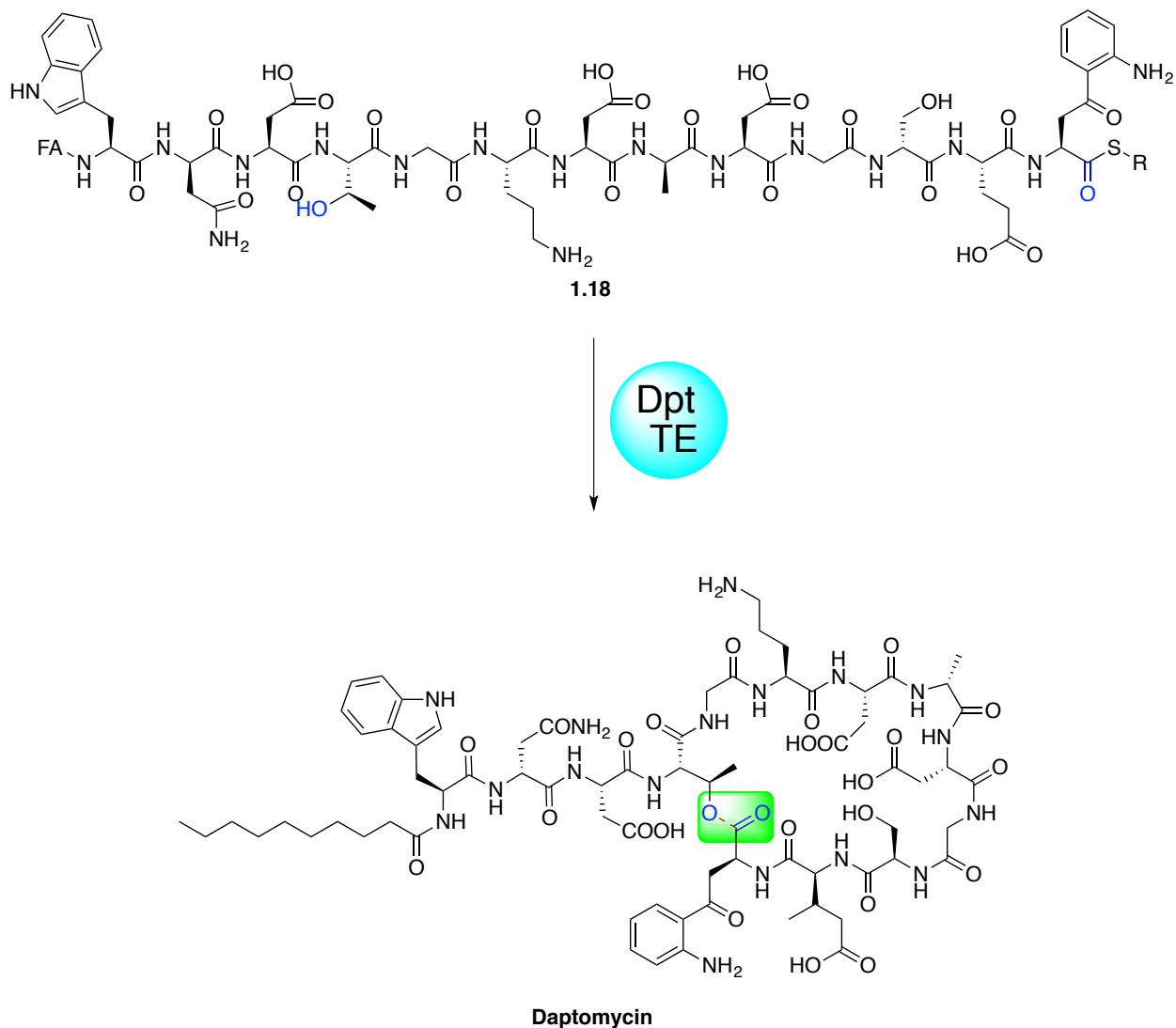
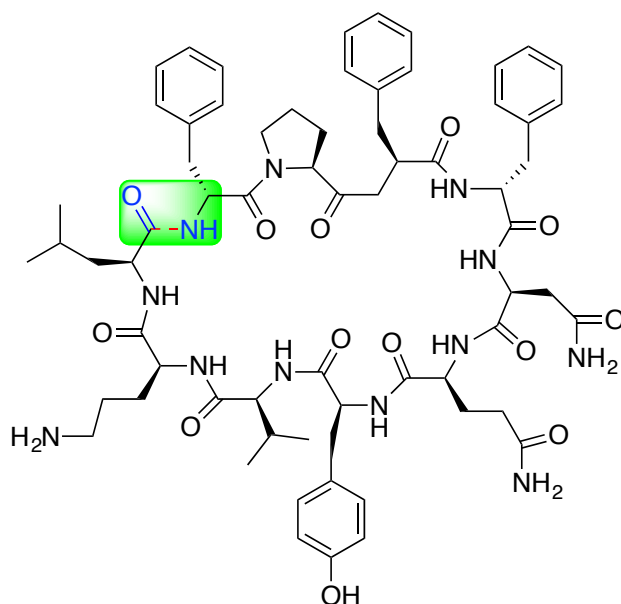


Figure 1. 13 Selective macrolactonization of daptomycin by Dpt TE.

Catalysis using the Dpt TE selectively forms a lactone between the threonine residue and the α -carboxylate of the terminal kynurenine, 10-amino acids down the linear chain, even though there are several other nucleophilic residues capable of forming a lactone or lactam (Fig. 1.13). Several examples of daptomycin total syntheses require protecting groups on the nucleophilic hetero-atoms before macrocyclization is performed, and even then the macrocyclization only produced moderate yields.^{40,41} The Dpt TE has been recombinantly expressed and purified, in attempts to use it to produce daptomycin analogs.⁴² Chemoenzymatic assays run on native-like precursors and analogs with the Dpt TE have been effective when minor changes to the native amino acids have been made. Further deviations away from the native substrate does show a drop in efficiency as well as

product distribution.⁴² The work done with the Dpt TE illustrates that the recombinant thioesterase is active as its own sub-domain and can catalyze macrolactonization of native like substrates. The selectivity of this TE does show sensitivity with manipulations of the amino acid sequence, but shows promise as a biocatalyst for daptomycin analog synthesis.⁴²

Tyrocidine (Fig. 1.14) is another cyclic NRP natural product, and an extensive amount of research has been done on its thioesterase.



Tyrocidine

Figure 1. 14 Tyrocidine, showing lactam formation at D-Phe-L-Leu.

The tyrocidine thioesterase (Tyc TE) forms a macrolactam, whereas the Dpt TE forms a macrolactone. Since the Tyc TE favours the macrolactam, the possibilities for peptide manipulation are increased. Many groups that have worked with this TE have proven its broader substrate tolerance compared to the Dpt TE.^{43,44} The Dpt TE relies on a lactone formation, warranting an alcohol containing amino acid, greatly limiting what amino acids can be swapped in and out of the linear precursor. Since Tyc TE employs a simple N-terminal macrolactam formation with the C-terminal thioester, the possibilities are much greater. Tyc TE has been shown to catalyze macrocyclization of over 300 amino acid containing analogs, making it an ideal biocatalyst in the formation of cyclic peptides.⁴⁴

Valinomycin is another macrocyclic peptide that employs a unique thioesterase that catalyzes two transesterification reactions prior to macrolactonization (Fig. 1.15).

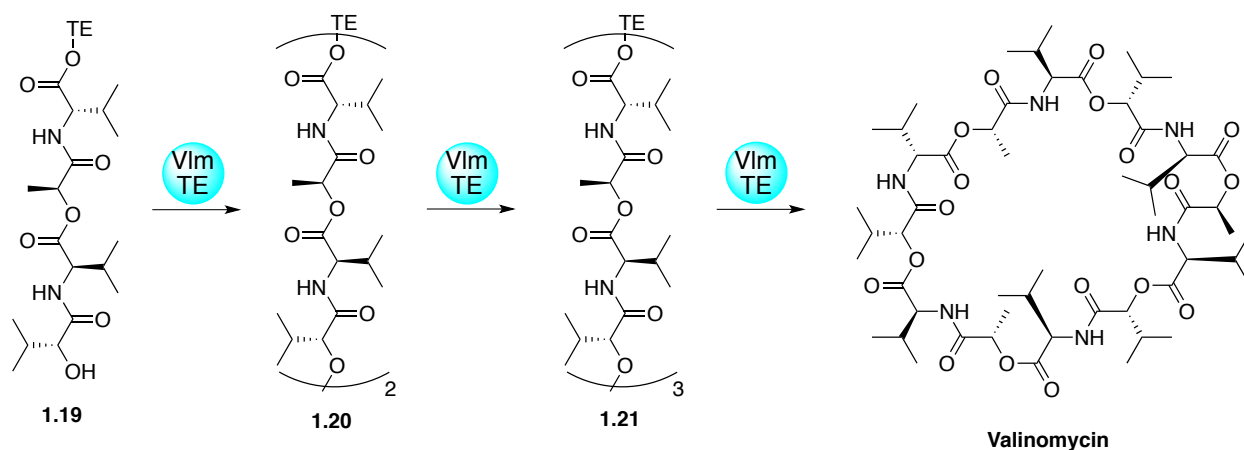


Figure 1. 15 Oligomerization and macrolactonization by Vlm TE to form valinomycin.

The valinomycin thioesterase (Vlm TE), starting from tetradepsipeptide **1.19**, catalyzes two oligomerization reactions forming octadepsipeptide **1.20**, and then dodecadeepsipeptide **1.21**. When **1.21** is formed, the Vlm TE, which the linear substrate is bound to, will catalyze macrolactonization to form valinomycin. The Vlm TE will not initiate macrocyclization, on a quantitatively relevant scale, until the dodecapeptide has formed.⁴⁵ This TE shows the high selectivity some NRP TEs have, but also the polyfunctionality that is possible with thioesterases.

NRP biosynthetic pathways represent a hot spot for biocatalytic macrocyclization tools. TEs found in these pathways show vast diversity with respect to catalytic transformations (lactone, lactam, hydrolysis, and transesterification reactions)^{42,43,45}, as well as substrate tolerance,⁴³ though the substrate selectivity seen with NRP TEs still relies on the peptidyl nature of the substrate.

1.3.3 Polyketide Biosynthesis.

Polyketides (PK) represent a large group of natural products, found in both prokaryotes and eukaryotes. They are constructed from acyl-CoA units to form linear carbon chains, which are then released through hydrolysis or cyclization.⁴⁶

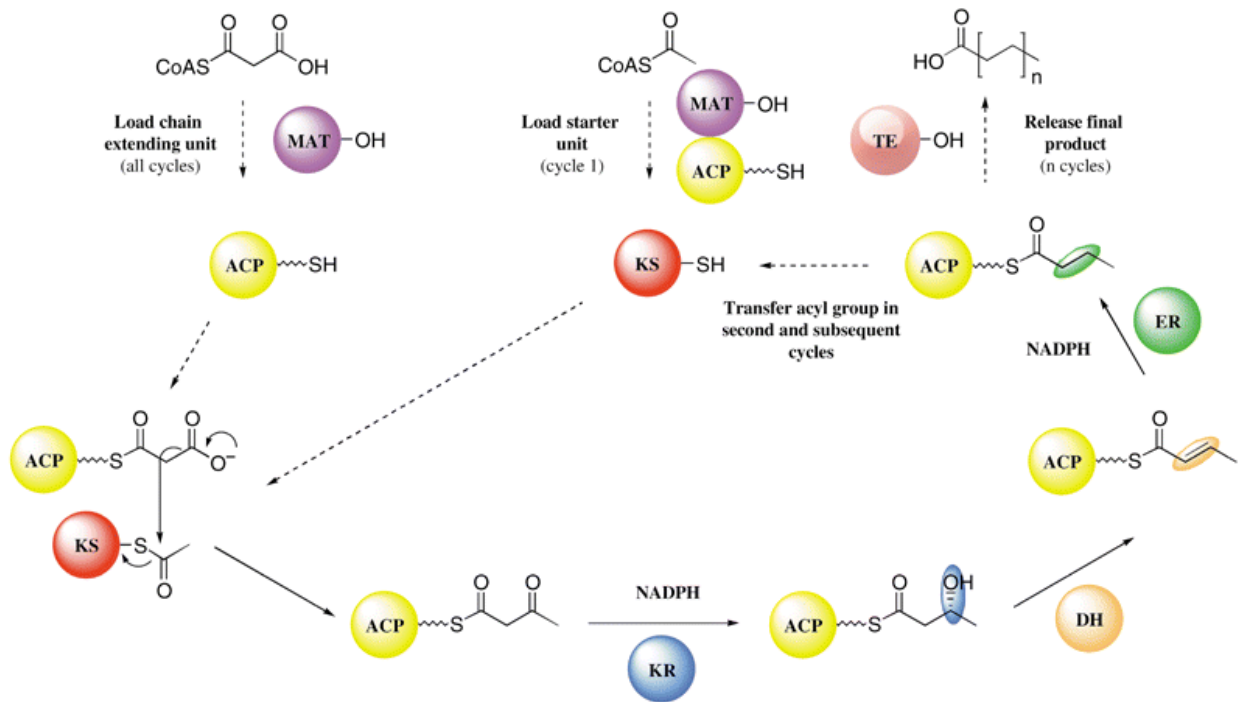


Figure 1. 16 Chain elongation in polyketide biosynthesis.⁴⁶

The biosynthesis of these compounds is analogous to fatty-acid synthesis except varying degrees of reduction on the carbon chain are possible (Fig. 1.16). PK biosynthesis uses mega-enzymes containing multiple catalytic domains. The acyl-carrier protein (ACP) domain is what the substrate is bound to during the elongation process. An acyl-transferase (AT) domain initiates elongation by transferring the first acyl-CoA unit to the ACP. The keto-synthase (KS) then adds an additional acyl-CoA unit onto the ACP. This ketone intermediate can then be sequentially reduced all the way to a saturated carbon chain using keto-reductase (KR), dehydratase (DH), and enoyl-reductase (ER) domains. Polyketide synthesis is terminated by the thioesterase domain, either hydrolyzing the linear intermediate to the free acid, or forming a macrocycle.⁴⁶ Thioesterases found in PK biosynthesis have been extensively studied and their catalytic ability to form macrocycles has shown promise as potential biocatalysts.⁴⁷

1.3.4 Thioesterases in Polyketide Biosynthesis.

Similar to the TEs in NRP biosynthesis, polyketide TEs have shown efficient catalytic activity through *in vitro* testing of recombinantly purified TE domains.⁴⁷⁻⁵¹ Lactone and lactam

formation, hydrolysis, and transesterification has been observed with many of these TE's, showing a similar diversity to NRP TEs.

The DEBS (6-deoxyerythromycin B) TE, found in the erythromycin pathway, catalyzes the formation of 6-deoxyerythronolide B via macrolactonization. The chemo- and regio-selectivity seen in this process is quite amazing (Fig. 1.17).

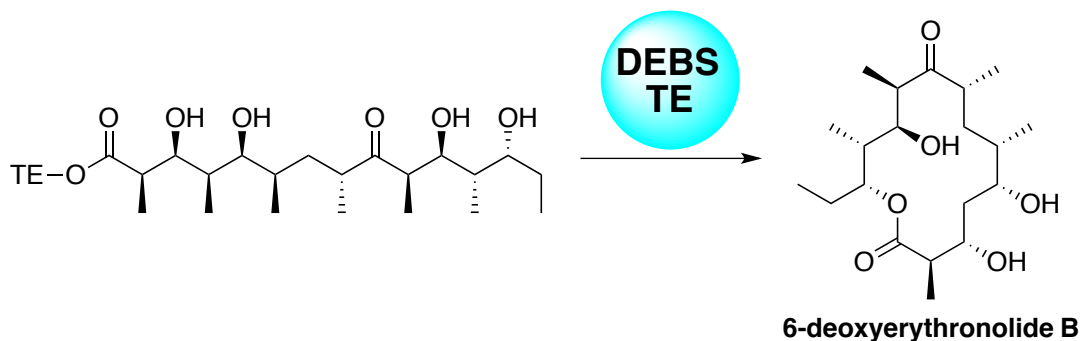


Figure 1. 17 Formation of 6-deoxyerythronolide B using the DEBS TE.

With 4 free hydroxyl groups, the ability to selectively form the 14-membered ring shows how superior this biocatalytic macrolactonization is to top of the line synthetic techniques.⁴⁹ Work done by Pinto *et al.*, tested to see how this TE works when modifications are made to the native linear precursor. They showed that by changing the configuration at the C-11 and C-13 hydroxyl groups, macrocyclization ability varies greatly (Fig. 1.18). Substrates **1.22-1.25** were tested and the only diastereomer that cyclized was compound **1.22**. Interestingly compound **1.23** is the analogous diastereomer to the native compound, meaning the variations in the substrates tested also impact macrocyclization ability.⁵²

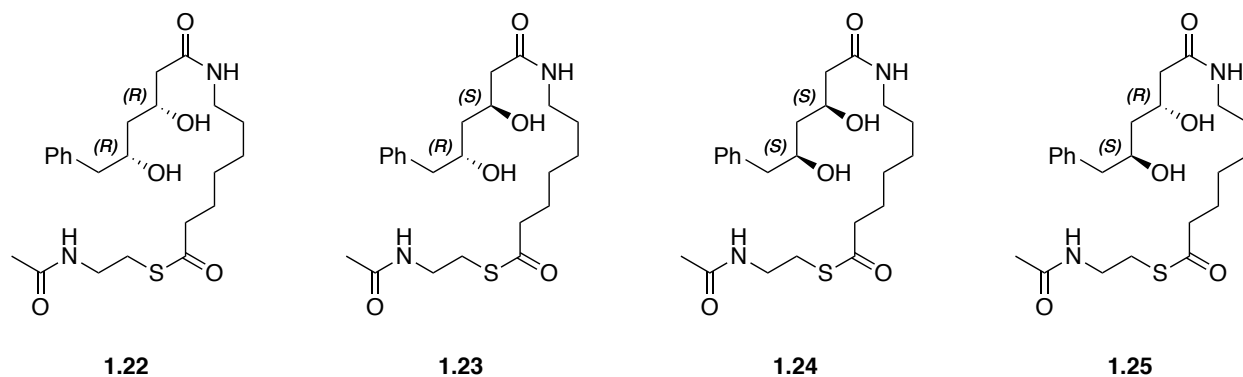


Figure 1. 18 Substrates tested with the DEBS TE by Pinto et al.⁵²

The DEBS TE is a great example of how selective PK TEs can be, which is ideal for its biosynthesis but remains an issue in terms of biocatalysis utility.

A majority of thioesterases found in PK biosynthesis, contrary to NRP biosynthesis, catalyze the formation of a macrolactone. Epothilone C, a 17-membered anti-cancer drug⁴⁸, amphotericin B, a C₆₀ antifungal agent⁵³, and pikromycin, a 14-membered antibiotic⁵³, all contain macrolactones. Macrolactonization is preferred in these compounds because PK biosynthesis favours the use of alcohols over other nucleophiles. There are a few cases where macrolactamization is seen in polyketide biosynthesis. Rifamycin B, a bacterio-static antibiotic⁵⁴, and geldanamycin, a gram-negative antibiotic⁵⁵, are both polyketides that form a lactam upon macrocyclization. Interestingly, in both cases the cyclization is performed by a lactam synthase (post-PKS enzyme catalyzing amide formation) rather than a thioesterase (Fig. 1.19).^{54,55}

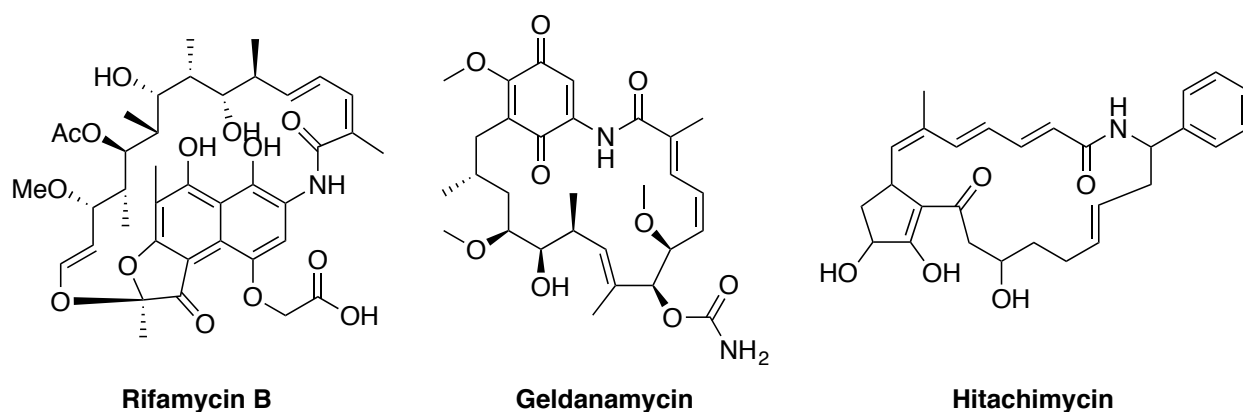


Figure 1. 19 Structure of rifamycin B, geldanamycin, and hitachimycin

The only example to date where a PKS TE is used to form a macrolactam is with hitachimycin (Fig. 1.19) and β -amino fatty acid containing analogs.⁵⁶ Macrolactam formation *in vitro*, using recombinant protein has also been seldom seen, the only example of a PKS TE able to catalyze both lactam and lactone formation was with the resorcylic acid lactone thioesterase (RAL TEs) zearalenone and radicicol.⁵¹

There has been a large increase over the past 20 years looking at the biocatalytic potential of thioesterases. NRPS TEs show potential for making cyclic peptide libraries (Tyc TE), and

PKS TEs show promise for macrocyclization of non-peptidyl compounds. The search for a universal thioesterase that shows efficient catalysis with, chemo-, regio-, and stereo-selectivity, while at the same time being tolerant enough for non-native substrate remains a challenge.

1.3.5 Thesis Objectives

The main objective of this thesis is to explore the substrate tolerance of the radicicol and zearalenone thioesterase enzymes. Chapter 2 showcases results with three non-phenyl containing compounds. Chapter 3 elicits the catalytic results of 6 amino acid containing substrates with both TEs. Chapter 4 describes the initial attempts to use the zearalenone and radicicol TEs to chemoenzymatically synthesize asperterrestide A.

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Chapter 2: Substrate Tolerance of Two Resorcylic Acid Lactone Thioesterases

2.1 Introduction:

2.1.1 Fungal Resorcylic Acid Lactones

A majority of polyketide discovery has revolved around bacteria, due to genetic simplicity (no introns) and accessible culturing techniques. Due to this focus on bacterial natural products, fungal polyketides haven't been explored to the same extent. One group of macrocyclic polyketides that illustrates the powerful enzymes found in fungal polyketide biosynthesis, are resorcylic acid lactones (RALs).¹ Their biological functions cover a vast range of utility, from antibiotics, to kinase inhibitors, even antimalarial properties (Fig. 2.1).¹

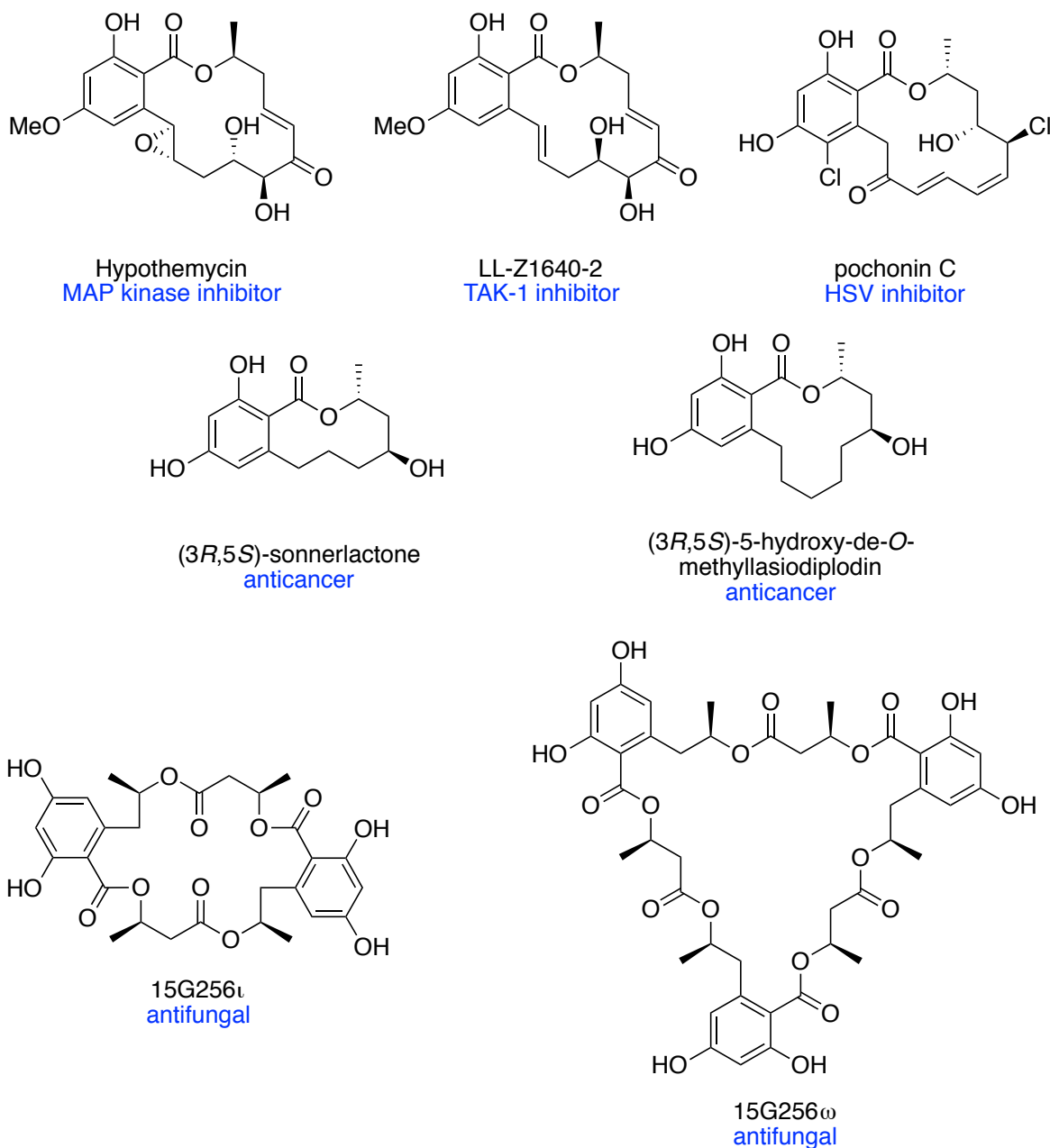


Figure 2. 1 10-30 membered resorcylic acid lactones and their bioactivities.

These macrolides can range from 10-16 membered rings although a few higher order compounds have been isolated. The most abundantly found group consists of a 14-membered macrocycle, containing a resorcylate moiety and an α -methyl group adjacent to the lactone ester (Fig. 2.2).^{1,2}

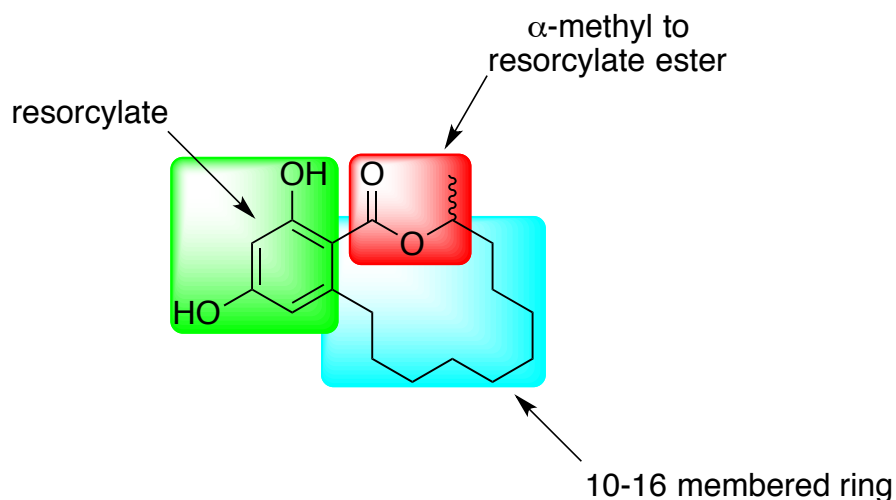


Figure 2. 2 Resorcylic acid lactone base scaffold.

The diverse bioactivities, as well as unique structure of these polyketides have warranted an increasing amount of research over the past 30 years.^{1,3-6} Upon original discovery in the 60s it was thought that these compounds were, for the most part, inactive or undesirable to the medical community. But increasing recent research has sparked the interest of these compounds as possible pharmaceuticals, with a few that have reached *in vivo* testing (radicol & hypothemycin).¹

2.1.2 Resorcylic Acid Lactone Biosynthesis

Resorcylic Acid Lactones rely on a type I iterative PKS system for their biosynthesis. Two PKS mega-proteins are employed to synthesize the base scaffold of these compounds (Fig. 2.3). The iterative nature of these proteins allows for multiple rounds of ketide units being condensed onto the ACP substrate.⁷

The first PKS protein is a highly-reducing polyketide synthase (HR-PKS). This protein employs 6 catalytic domains (MAT, ACP, KS, KR, DH, ER) and is responsible for the chain-elongation of the natural product. This consists of adding 4-7 ketide units, with varying degrees of reduction, onto the ACP. Unique to fungal type I iterative PKS systems is the ability for domains to turn “on and off” depending on the length of the ACP bound substrate.⁸ Once the linear chain

is complete, the intermediate is transferred to a second, non-reducing polyketide synthase (NR-PKS).^{4,5,7-10}

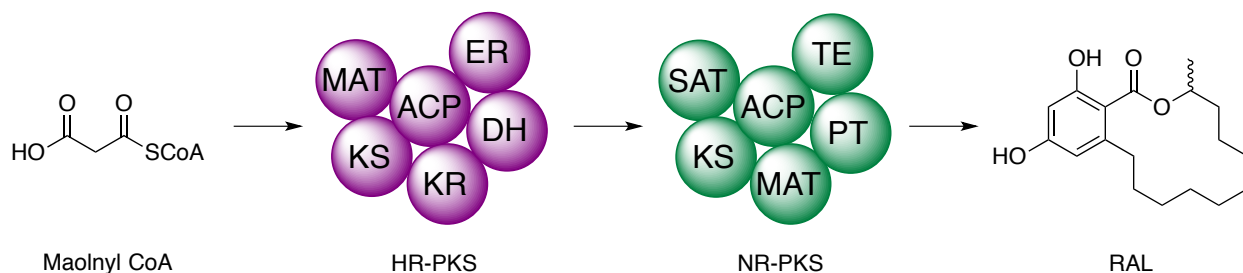
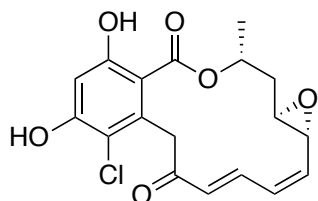


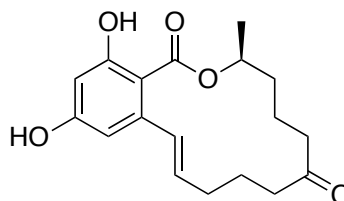
Figure 2. 3 RAL Biosynthesis.

The NR-PKS protein is responsible for the formation of the resorcylate moiety as well as the macrocyclization step. Unlike the HR-PKS, this protein does not contain a KR, DH, or ER domain, instead it employs a product template domain (PT) as well as a thioesterase domain (TE). Upon the addition of three, fully oxidized, ketide units, the product template domain initiates formation of the resorcylate functionality via an aldol condensation. Once the resorcylate has formed, the TE domain macrocyclizes the linear substrate, which is sequentially released as the macrocycle product.⁷ Similar to the TE found in the valinomycin pathway, the RAL TE does not initiate cyclization until a specific linear intermediate is formed.⁸ This unique property of RAL TEs has led to research being done on the radicicol and zearalenone thioesterases (Rdc TE & Zea TE).^{6,11}

2.1.3 Radicicol & Zearalenone



Radicicol
Hsp90 inhibitor



Zearalenone
estrogen receptor agonist

Figure 2. 4 Radicicol & zearalenone structure and biological activity.

Radicicol and Zearalenone (Fig. 2.4) are 14-membered, fungal resorcylic acid lactones. Radicicol was first isolated in 1953 from *Monosporum Bonorden* and shows Hsp90 inhibition at nanomolar concentrations. The two PKS proteins (Rdc5, and Rdc1) synthesize the macrocyclize precursor monocillin II, which is culminated by macrocyclization using the Rdc TE. Upon release monocillin II undergoes oxidative tailoring to form radicicol (Fig. 2.5).⁵

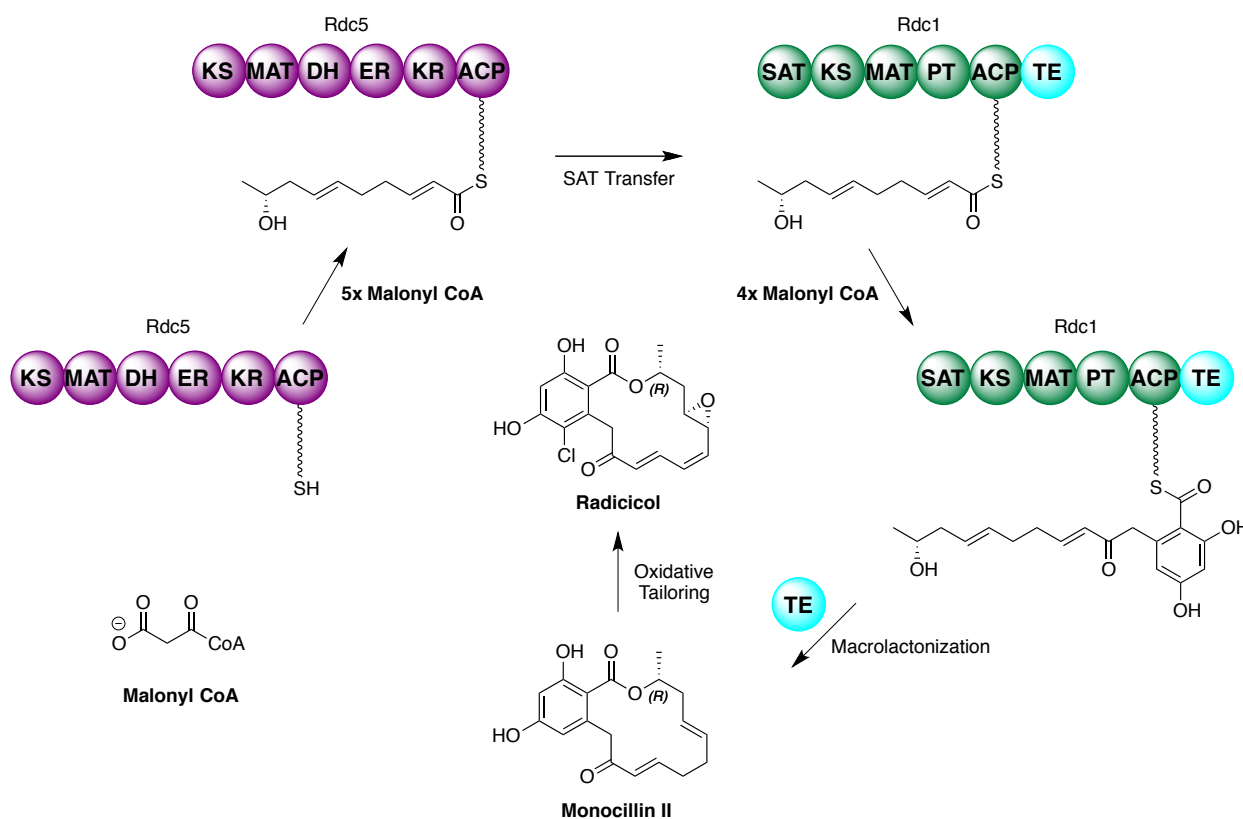


Figure 2. 5 Radicicol Biosynthesis.⁵

Zearalenone is a mycotoxin first isolated from *Fusarium graminearum*. It acts as an estrogen receptor agonist, and is a large cause of infertility in swine.¹ Zearalenone biosynthesis (Fig. 2.6) uses PKS-13 (HR-PKS) and PKS-4 (NR-PKS) to form β -zearalenol, followed by an oxidation to make zearalenone. The oxidation states of the linear intermediate differ from radicicol

as well as the number of ketide units added by each PKS, but both undergo similar macrolactonizations once the resorcyate is formed.

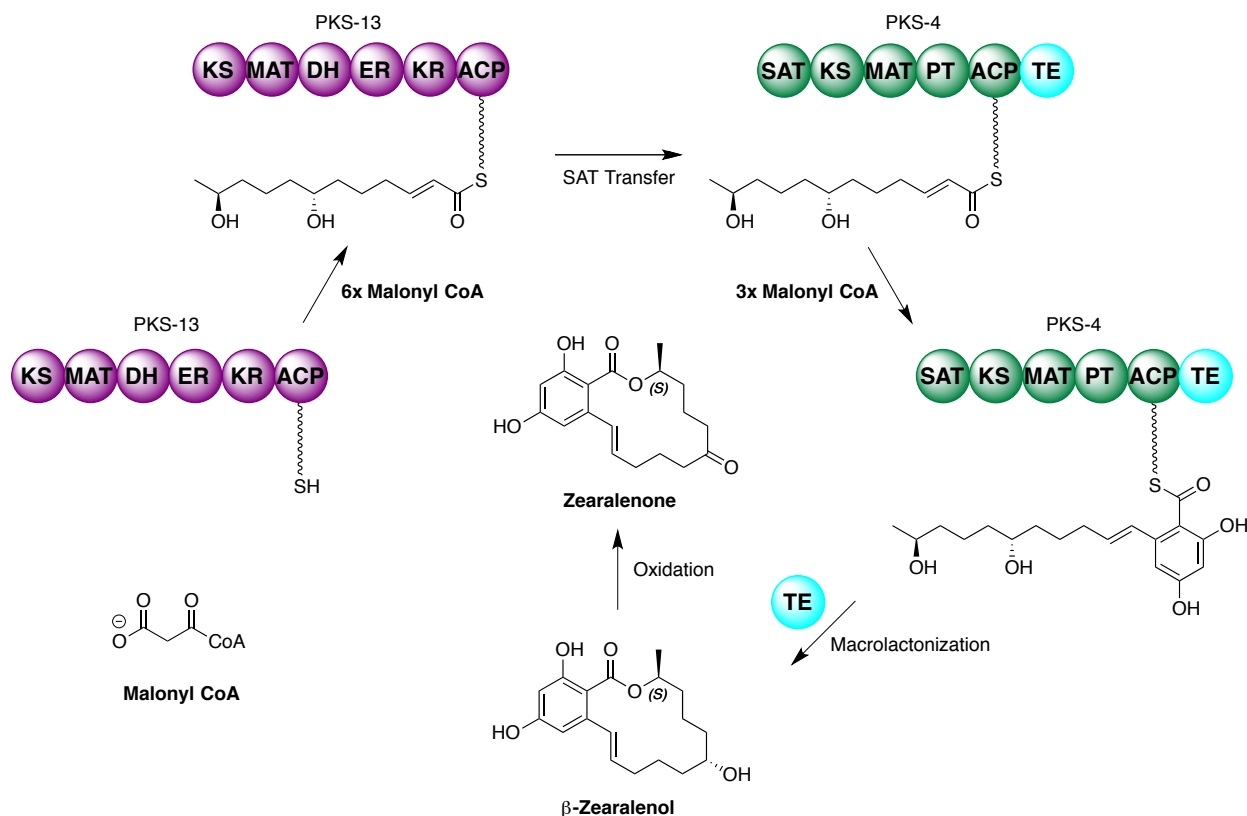


Figure 2. 6 Zearalenone Biosynthesis.⁴

2.2 Previous RAL Work

2.2.1 Zearalenone and Radicol thioesterases show catalytic stereoselectivity, ring-size tolerance, as well as macrolactamization ability.

Prior work with these two thioesterases was done by the Boddy group.^{3,6,11} A group of N-acetyl cysteamine substrates (SNAC), were synthesized. SNAC mimics the linkage between the substrate and the ACP domain. SNAC derivatized substrates have been used extensively to study the activity of thioesterases.^{3,6,8,10}

The Boddy group was able to show that substrates with ring sizes from 12- to 18-members can be efficiently macrocyclized with initial velocities similar to the native precursor. They also demonstrated that both (*R*) and (*S*) configured secondary hydroxyl nucleophiles are tolerated by both TEs and macrolactamization is also achievable (Fig. 2.7). Lastly, they demonstrated that these thioesterases are active with up to 40% DMSO (v/v) in enzymatic assays (Fig 2.8) and they can be lyophilized, stored for months and will retain activity upon re-suspension.^{3,6}

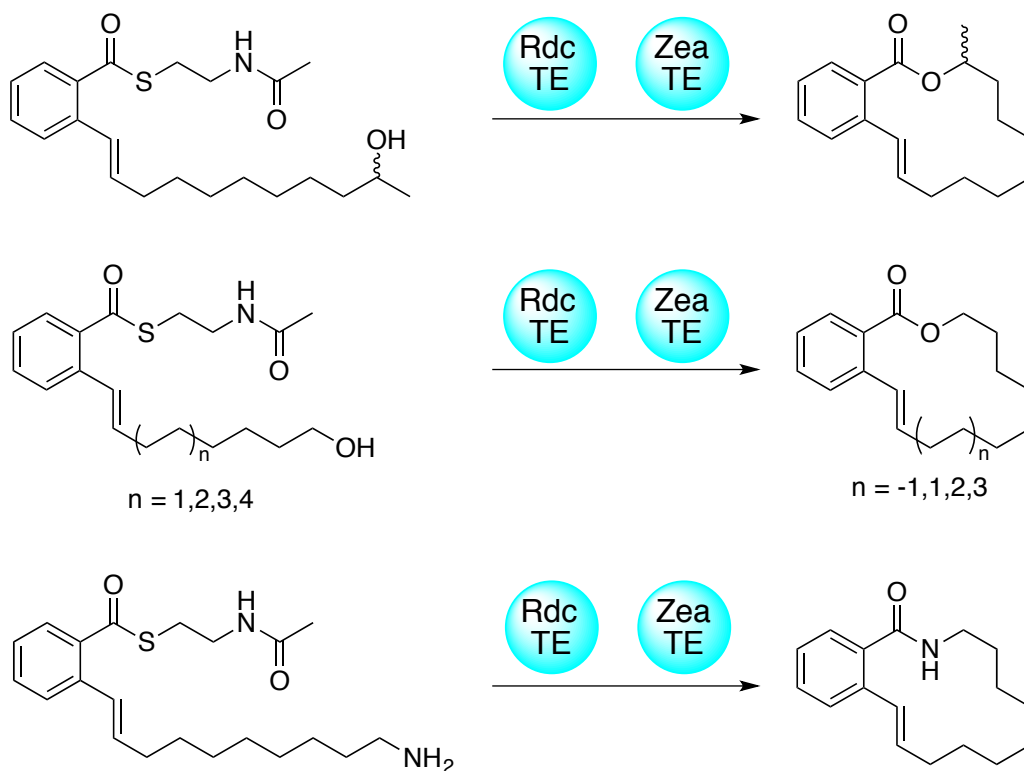


Figure 2. 7 Previously macrocyclized substrates with Zea and Rdc TEs.

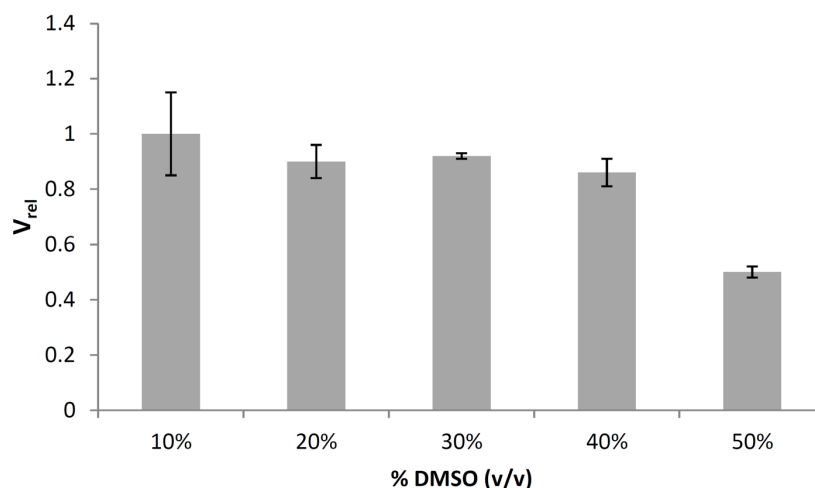


Figure 2. 8 Radicol enzymatic efficiency with varying DMSO concentrations.

Relative velocities for the ring size and aza compounds relative to the 14-membered native-like substrate **2.1** were recorded to provide initial kinetic information about these substrates (Fig 2.9).

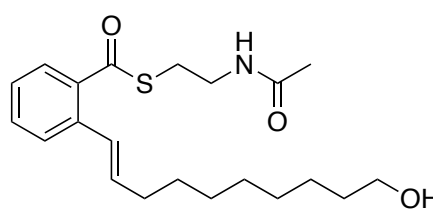
Structure	Ring size	v_{rel} Rdc TE	v_{rel} Zea TE
	14	1.00 ± 0.03	1.00 ± 0.03
	10	$\sim 10^{-4} \pm 10^{-4}$	$\sim 10^{-4} \pm 10^{-4}$
	12	0.19 ± 0.06	0.88 ± 0.01
	14	0.66 ± 0.02	1.00 ± 0.03
	16	3.13 ± 0.01	1.89 ± 0.05
	18	0.22 ± 0.05	0.55 ± 0.17
	Aza-14	0.32 ± 0.06	0.11 ± 0.01

Figure 2. 9 Relative velocities of ring size/aza compounds with Zea and Rdc TEs compared to substrate **2.1**.

The kinetic data shows that 12-18 membered rings and macrolactam substrates are catalyzed with comparable initial velocities to **2.1**. The ability to maintain efficient catalysis with these non-native substrates is reassuring that these TEs have potential as biocatalysts.³ To further investigate the substrate scope of these TEs additional work reported in this thesis commenced in August 2017.

2.3 Results and Discussion

The prior work done with these TEs highlighted the flexibility of these proteins with respect to substrate tolerance. One area that was not investigated, was the relevance of the phenyl ring in the linear substrate. In RAL biosynthesis, the thioesterase does not act on the linear intermediate until the resorcyate phenyl ring has been formed. The speculation being that the linear substrate needs the resorcyate functionality adjacent to the ACP thioester in order for the thioesterase to catalyze macrocyclization.⁸ The following research was performed to test this hypothesis. Three substrates were designed, synthesized, and tested to understand how the α - β site adjacent to the SNAC thioester impacts macrolactonization when the phenyl ring is removed and/or changed.

2.3.1 Synthesis of saturated substrate 2.2 and macrocyclic standard 2.4

The first substrate was synthesized to establish whether the RAL TEs can macrocyclize without an adjacent phenyl ring to the SNAC thioester. Compound **2.1** was previously used by the Boddy lab, as the native-like substrate to the intermediates found in the zearalenone and radicicol biosynthetic pathways. Compound **2.2** is a simplified version lacking a phenyl ring and γ,δ -unsaturation as seen in substrate **2.1**.

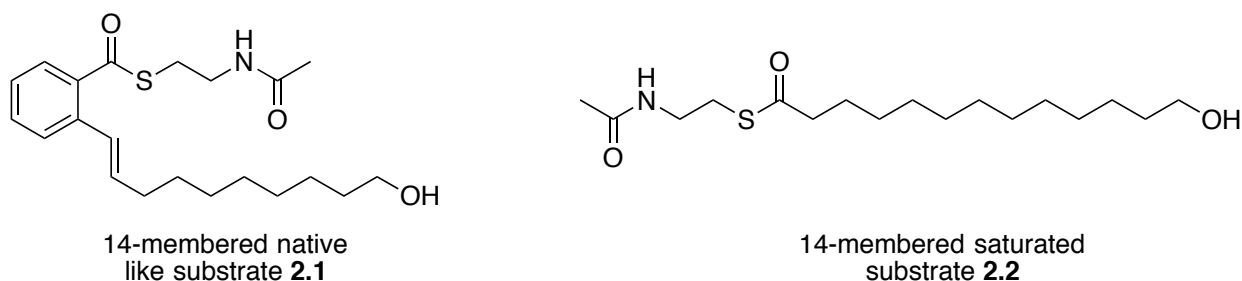
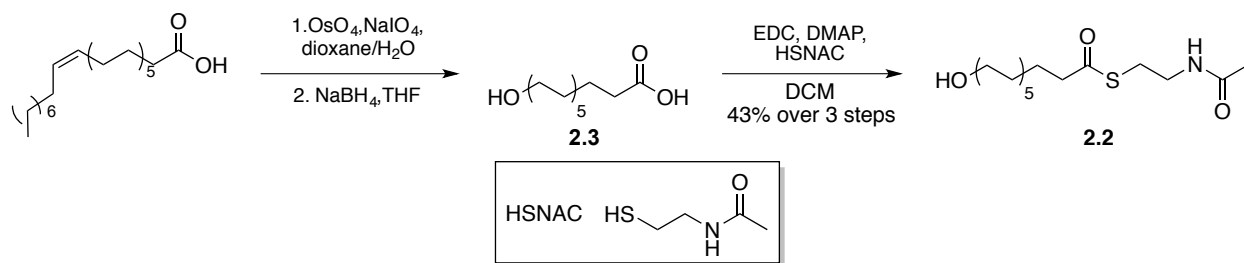


Figure 2. 10 Structures of compound 2.1 and 2.2.

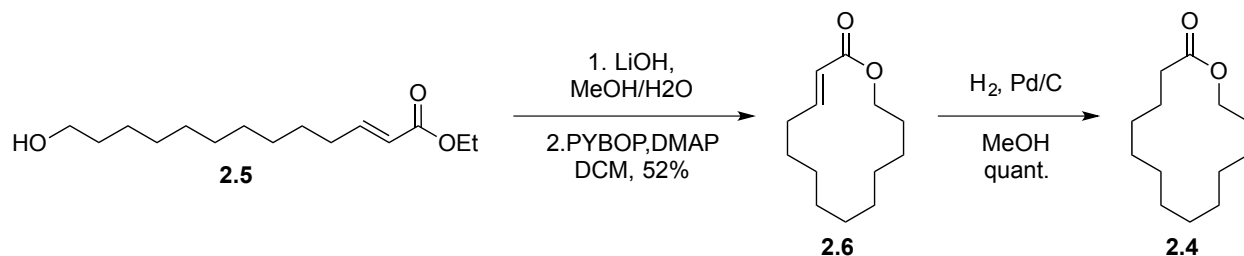
2.2 was synthesized in 3 steps starting from erucic acid (Scheme 2.1). A Lemieux-Johnson oxidation¹² was used to cleave the ω -9 alkene of erucic acid to the aldehyde, followed by a

reduction of the aldehyde to the alcohol using NaBH₄. The 14-carbon *seco*-acid intermediate **2.3**, was coupled with SNAC using EDC and DMAP to afford compound **2.2**.



Scheme 2.1 Synthetic scheme for compound **2.2**.

To provide a standard of the proposed macrocyclic product, compound **2.4** was synthesized in a two-step process.



Scheme 2.2 Synthesis of macrocycle standard **2.4**.

Hydroxy ester **2.5** was hydrolyzed using LiOH and macrocyclized via a PyBOP/DMAP coupling to form compound **2.6**. **2.6** was hydrogenated using H₂ with Pd/C to yield the 14-membered saturated macrocycle standard **2.4**.

2.3.2 Zea & Rdc TE enzymatic assays with compound **2.2**.

Biocatalytic assays were performed using recombinant purified zearalenone thioesterase (pMW29) and radicicol thioesterase (pMW14).¹¹ Assays were run analogous to the prior work done by the Boddy lab.³ Assays were run at a volume of 50 μ L containing 50 mM phosphate buffer (pH 7.4), 1.0 mM substrate, 5.0 μ M enzyme, and 10% DMSO (v/v) for 24 h, at RT.

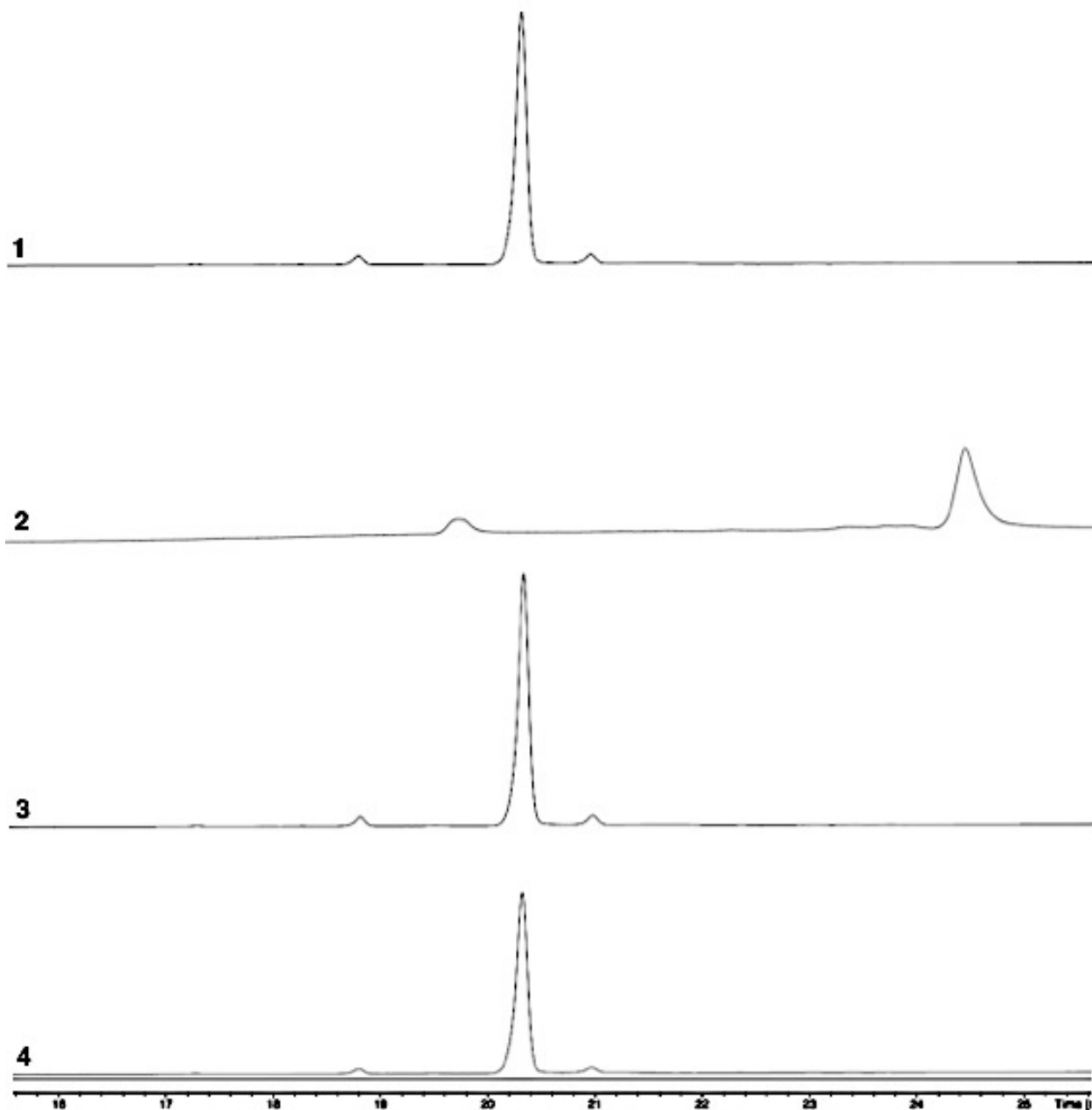


Figure 2. 11 HPLC traces of **2.2** enzymatic assays with *Zea* and *Rdc* TEs.

1) Substrate **2.2** standard 2) Macrocycle **2.4** standard(24.5 min) 3) *Rdc* TE + **2.2** 4) *Zea* TE + **2.2**

The HPLC trace in figure 2.11 shows that after a 24 h incubation of substrate with both thioesterases, there is no significant product formed, or starting material lost. It was hypothesized that due to the lack of a phenyl ring at the α,β position, the thioesterase will not catalyze macrocyclization. This is demonstrated in the assays run with substrate **2.2**. We can now confidently say that the resorcyate moiety found in resorcylic acid lactones plays a significant role

in the thioesterase's ability to macrocyclize the linear precursor. To investigate whether conjugation of the resorcyate with the ACP thioester plays a significant role in RAL TE activity, substrate **2.7** was synthesized and tested (Fig. 2.12).

2.3.3 Synthesis of α,β -unsaturated substrate **2.7** and macrocyclic standard **2.6**

The second substrate synthesized to probe phenyl ring relevance was designed after the 14-membered native like substrate **2.1**. Substrate **2.7** contains a 14-membered carbon chain with an α,β -unsaturation adjacent to the SNAC thioester. **2.7** was designed to probe whether the conjugated system of the native precursor confers enough affinity to initiate macrocyclization.

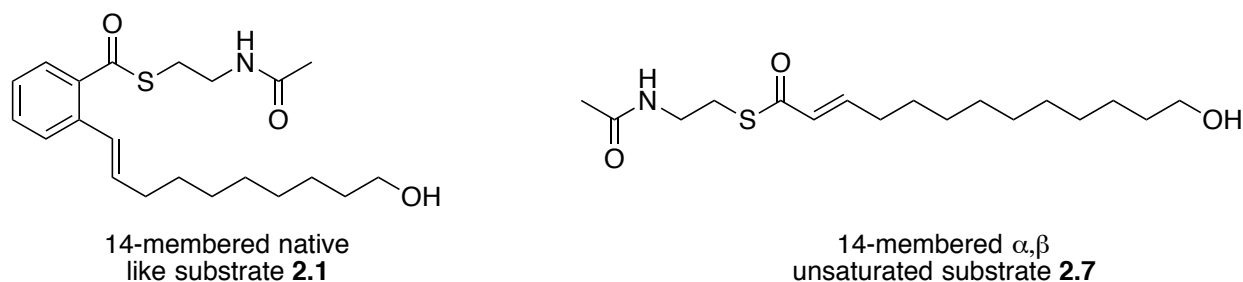
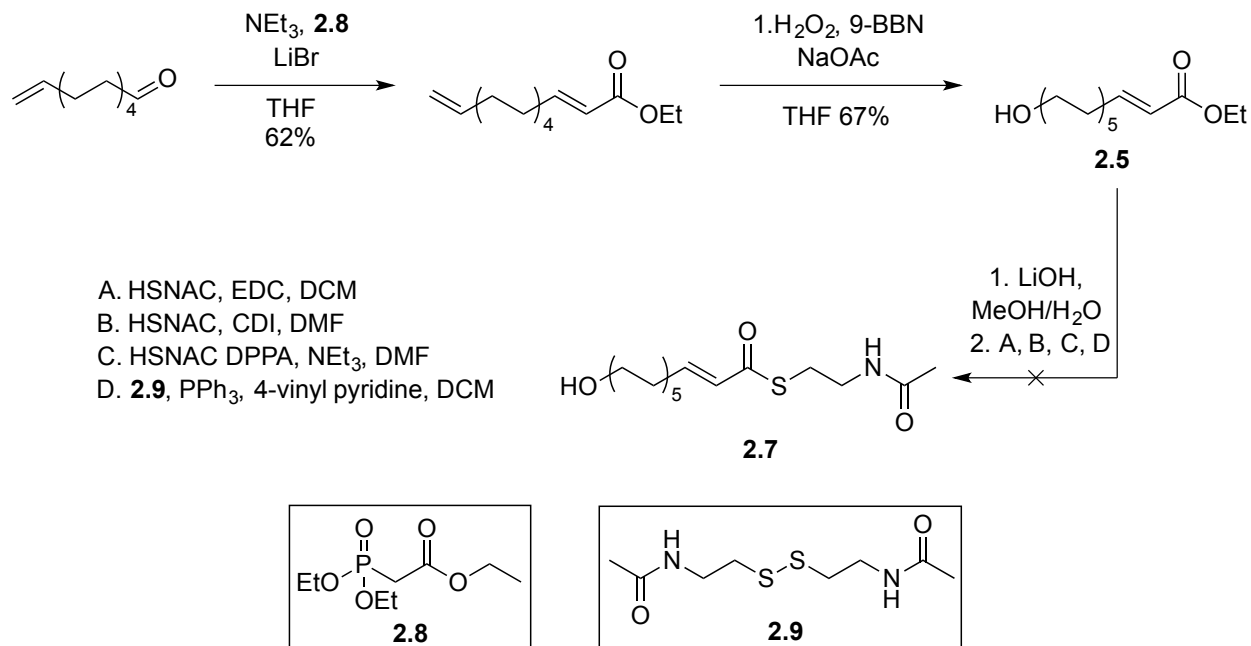


Figure 2. 12 Structures of compound **2.1** and **2.7**

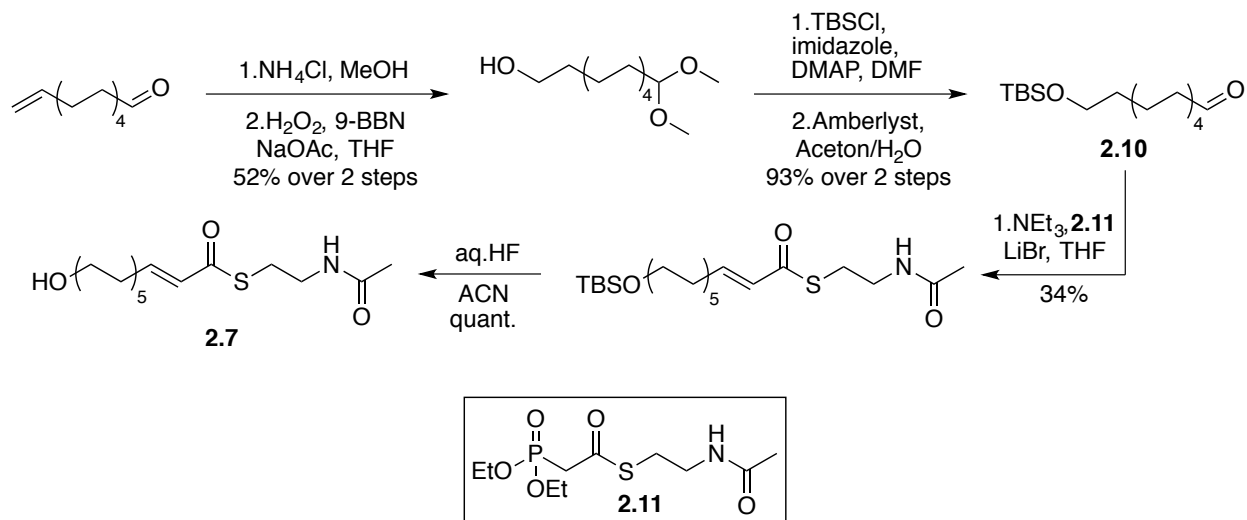
Initial attempts at synthesizing compound **2.7** started with formation of compound **2.5**, followed by subsequent hydrolysis and thioester coupling with N-acetyl cysteamine.



Scheme 2.3 Initial synthetic route to compound 2.7

Formation of the **2.5** began with a Horner-Wadsworth-Emmons¹³ (HWE) condensation between **2.8** and 10-undecenal. A selective oxidation of the terminal alkene using 9-BBN and H₂O₂ was used to afford the 14-carbon hydroxyl ester **2.5**. Following ester hydrolysis, several different coupling agents were tested to form the SNAC thioester, but the soft nature of the nucleophilic thiol caused a 1,4 Michael addition¹⁴ to occur in each case. The final attempt consisted of a Corey-Nicolaou¹⁵ type thioesterification. This consisted of first reducing the N-acetyl cystamine disulfide **2.9** with triphenyl phosphine, followed by the acid activation to the phosphonium intermediate and subsequent nucleophilic attack of the free thiolate anion. Unfortunately, this attempt also resulted in a 1,4 addition.

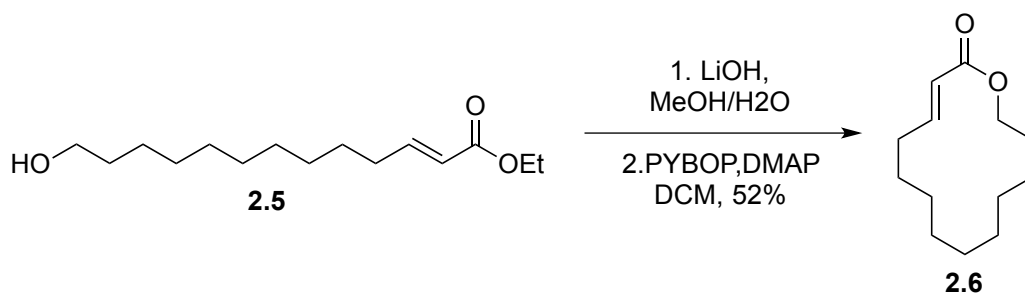
The next synthetic strategy also consisted of an HWE reaction to form the α,β -unsaturation, but the HWE analog was modified to have the N-acetyl cysteamine thioester pre-installed (compound **2.11**).



Scheme 2.4 Modified synthetic route to compound **2.3**.

Due to the base-labile nature of the thioester, oxidation of the terminal alkene and subsequent hydroxyl protection had to be done prior to the HWE condensation. Once **2.10** was synthesized the HWE reaction with **2.11** followed by TBS deprotection yielded compound **2.7**.

To confirm whether any macrocycle product was formed in the thioesterase assays, a synthetic standard had to be made (Scheme 2.5). This was done by hydrolyzing compound **2.5** followed by a PYBOP/DMAP macrocyclization method to form **2.6**.



Scheme 2.5 Synthesis of macrocycle standard **2.6**.

Upon completion of linear substrate **2.7** and macrocyclic standard **2.6**, enzymatic macrocyclization assays were performed with both Zea and Rdc TEs.

2.3.4 Zea & Rdc TE enzymatic assays with compound 2.7.

Enzymatic assays were performed with analogous conditions to section **2.3.3**. Assays were run at a volume of 50 μL containing 50 mM phosphate buffer (pH 7.4), 1.0 mM substrate, 5.0 μM enzyme, and 10% DMSO (v/v) for 24 h, at RT.

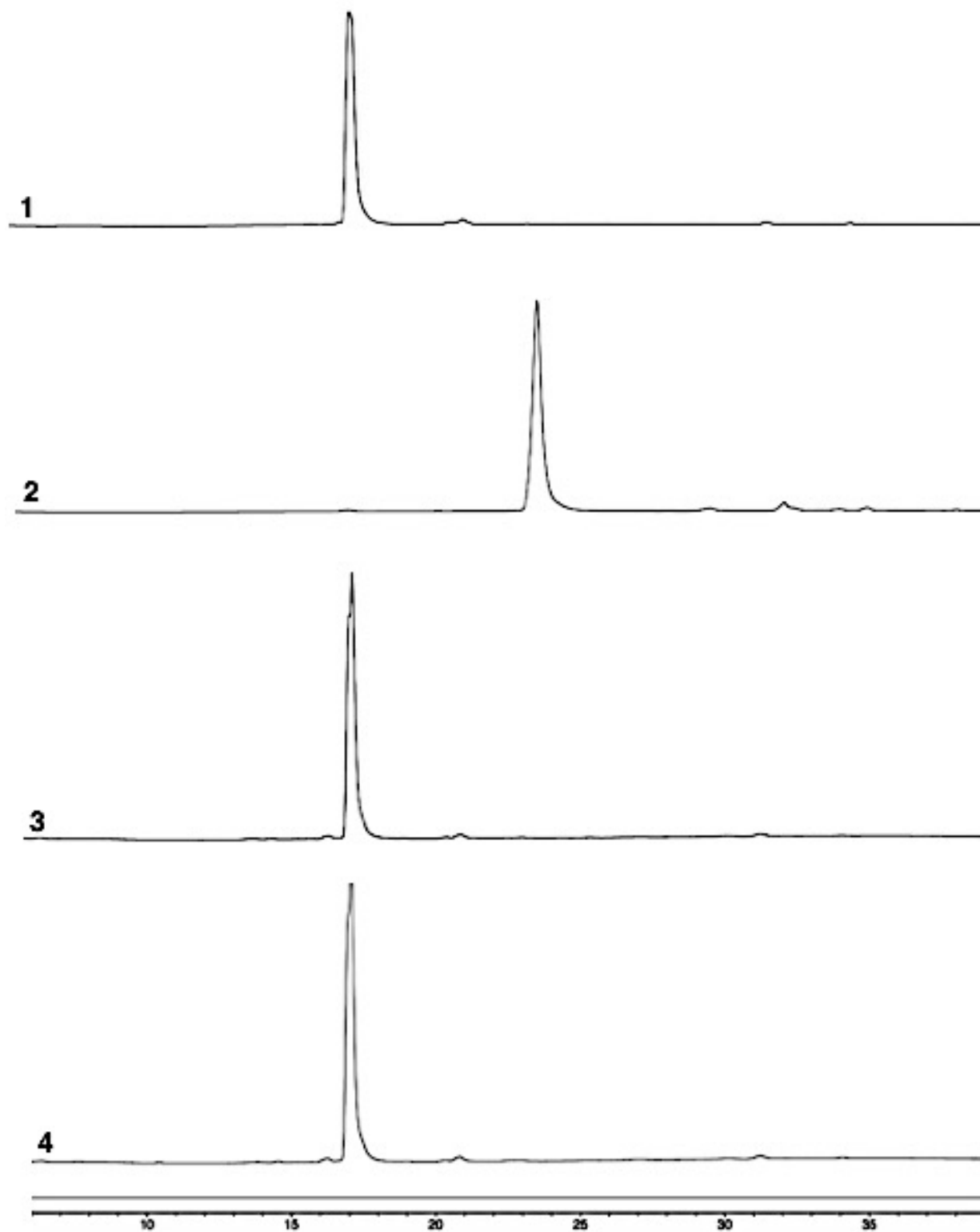


Figure 2. 13 HPLC traces of 2.3 enzymatic assays with Zea and Rdc TEs.

1) Substrate **2.7** standard. 2) Macrocycle **2.6** standard. 3) Rdc TE + **2.7**. 4) Zea TE + **2.7**.

Looking at the HPLC traces for the thioesterase assays (Fig. 2.13), both Zea and Rdc TEs show no enzymatic activity. After the 24 h incubation period the only peak seen is the starting material. Not only is there no macrocycle, there are also no other products present such as hydrolysis, glycerolysis, or oligomerization. This suggests that **2.7** does not load onto the TEs. Assay results confirm that the conjugated system of the ACP thioester is not the driving force for thioesterase recognition, hinting that the resorcyate phenyl ring is mandatory for enzymatic activity.

2.3.5 Synthesis of glycine containing substrate **2.12** and macrocyclic standard **2.15**.

The third substrate, compound **2.12**, was designed to change the functionality of the phenyl ring, to an amide. Since complete removal of the phenyl group abolishes activity, we wanted to probe, if a different functional group replacing the phenyl ring can be cyclized, by these thioesterases.

2.12 still possessed the same 14-membered N-acetyl cysteamine structure, but with a glycine inserted adjacent to the thioester.

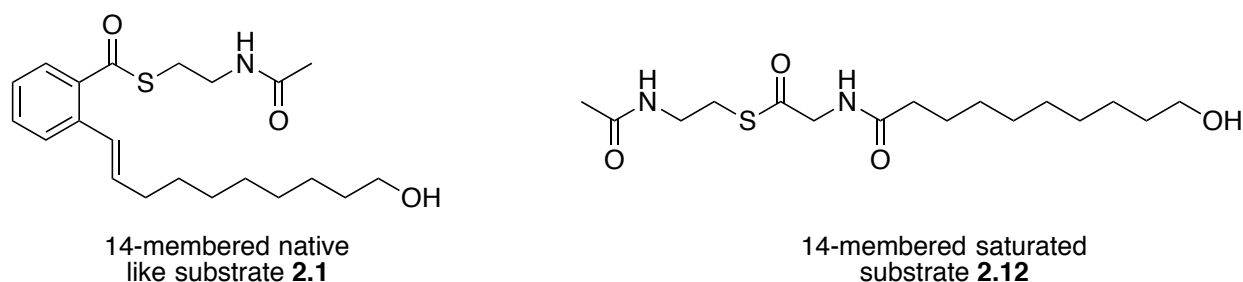
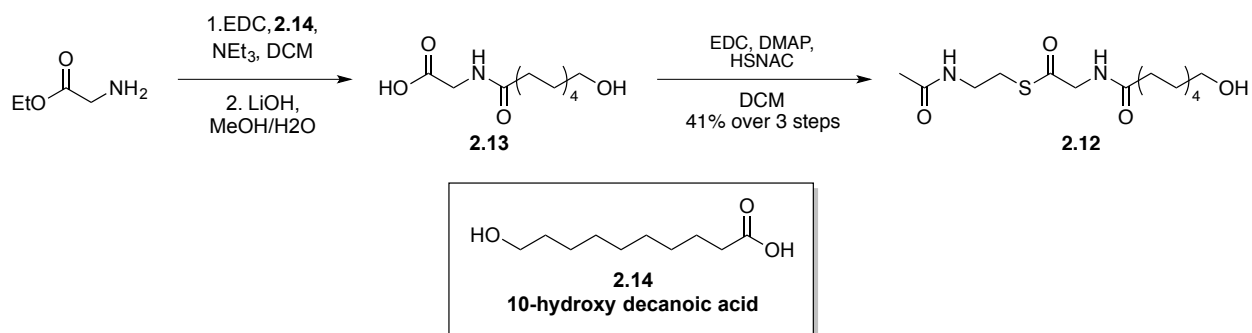


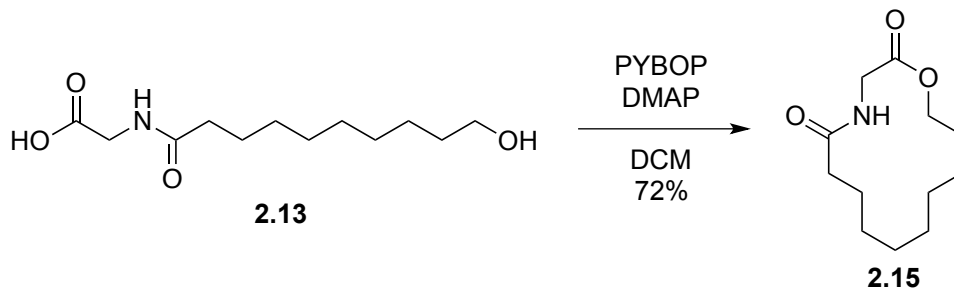
Figure 2. 14 Structures of compound **2.1** and **2.12**.

2.12 was synthesized in 3 steps starting with an amide coupling between glycine ethyl ester and 10-hydroxy decanoic acid, which was then hydrolyzed to give *seco* acid **2.13**. The last step was a thioesterification between **2.13** and SNAC, using EDC and DMAP (Scheme 2.6).



Scheme 2. 6 Synthesis of compound 2.12.

The macrocyclic standard for **2.12** was synthesized in one step starting with intermediate *seco* acid **2.13**, using a PYBOP/DMAP macrocyclization to yield compound **2.15**.



Scheme 2. 7 Synthesis of macrocycle standard 2.15.

2.3.6 Zea & Rdc TE enzymatic assays with compound **2.12**.

Substrate **2.12** was assayed with both Zea and Rdc TEs analogously to **2.2** and **2.7**. After a 24 h incubation, the following HPLC data was recorded.

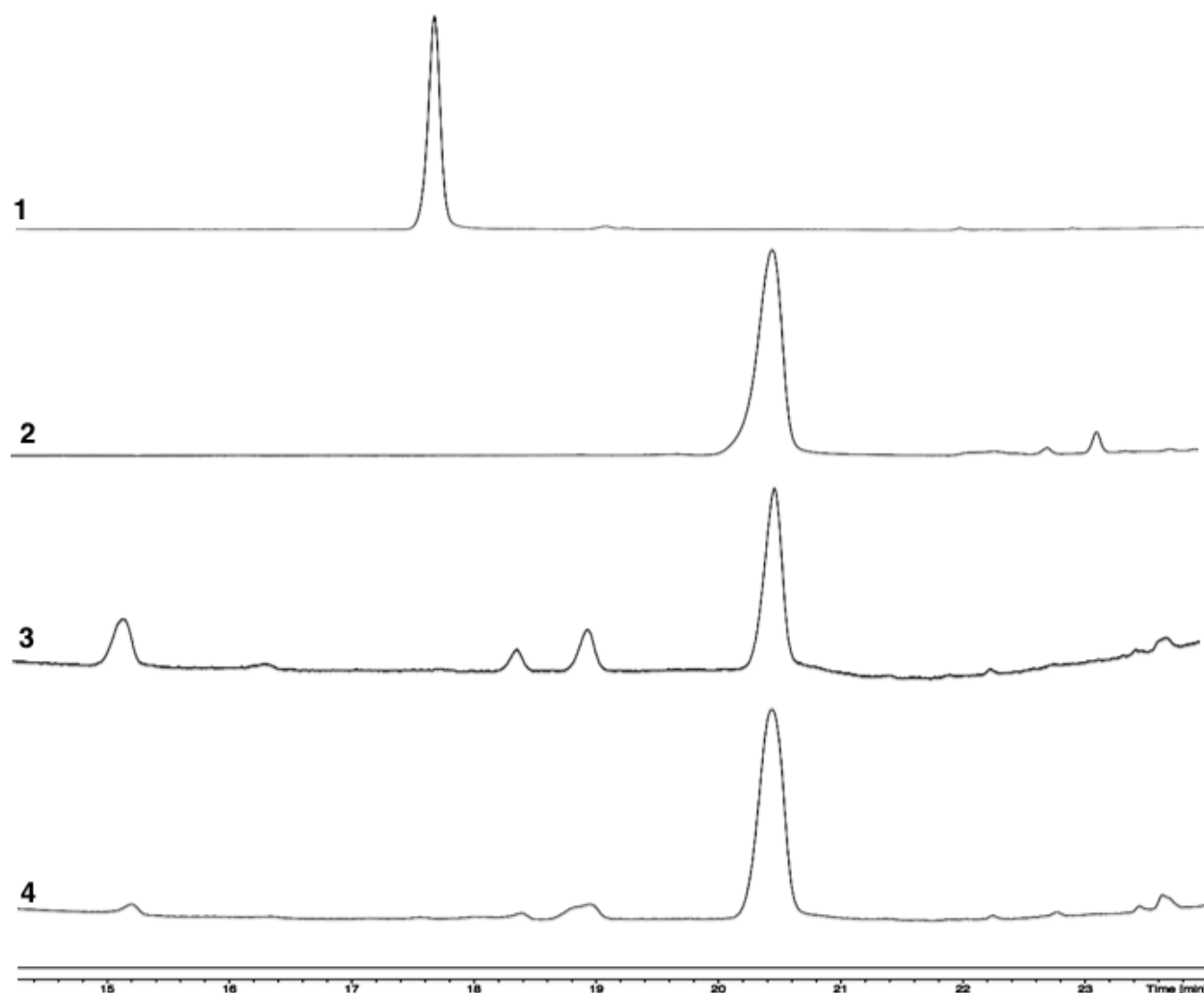


Figure 2.15 HPLC traces of **2.4** enzymatic assays with Zea and Rdc TEs.

1) Substrate **2.12** standard. 2) Macrocycle **2.15** standard. 3) Rdc TE + **2.12**. 4) Zea TE + **2.12**.

The HPLC data shows that after a 24-hour incubation, we see no remaining starting material. Product distribution for the Zea TE is almost 100% macrocycle with a small amount of hydrolysis, and the Rdc TE assay shows about 70/30 macrocycle to hydrolysis. The high catalytic turnover observed with substrate **2.12** was quite interesting. The ability of these two TEs to efficiently catalyze this non-native, glycine containing substrate is quite remarkable. These TEs are derived from polyketide biosynthesis, whose evolutionary basis would never have been challenged with an amino acid containing substrate. To see this extent of catalytic activity really speaks to the substrate tolerability of these thioesterases.

To further explore this catalytic macrocyclization rate of compound **2.12**, relative velocities compared to the 14-member native like substrate **2.1** were recorded with both thioesterases. Relative velocities were determined using a continuous Ellman's assay. Ellman's reagent reacts with free thiols, producing an absorbance at 412 nm. The initial loading step of these thioesterases releases free SNAC, can be readily quantified from reaction with Ellman's reagent.

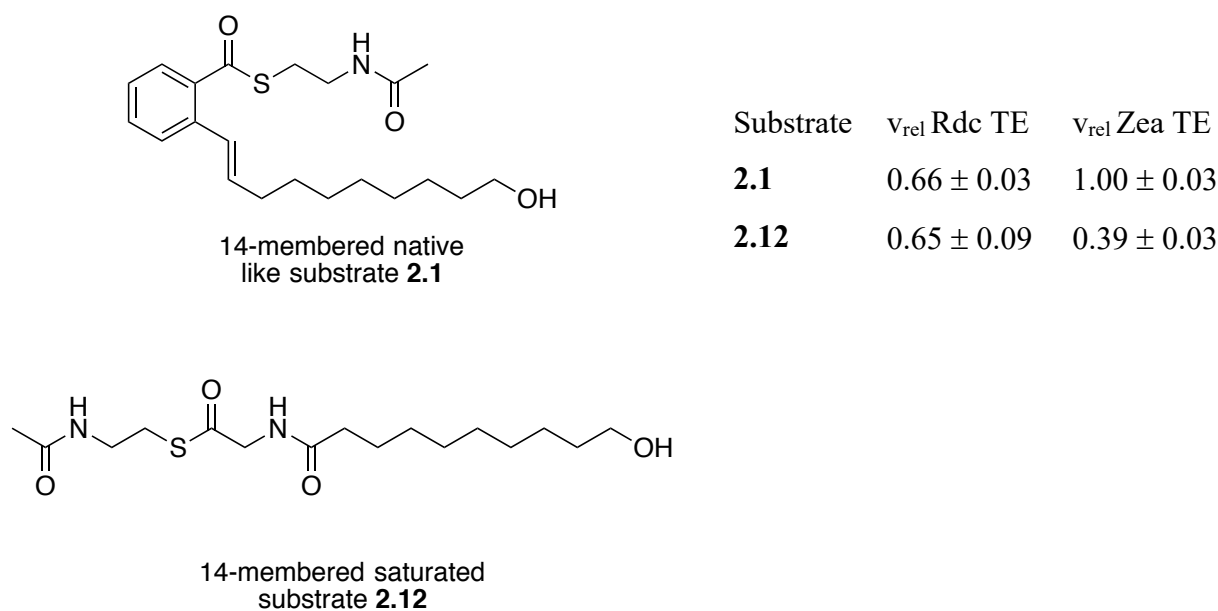


Figure 2. 16 Relative velocities of 2.12 with Zea and Rdc TEs compared to 2.1

The relative velocities for the glycine inserted substrate **2.12** are quite comparable to **2.1**, meaning with respect to efficiency, by introducing this completely foreign glycine moiety, both TEs can still work at a catalytically relevant rate. These results indicate, that non-native functional groups can be added to these linear substrates and still bind to the RAL TEs. This also indicates that the α,β -phenyl ring is not mandatory for enzymatic activity.

2.4 Conclusions

In conclusion the substrate scope for Rdc and Zea TEs has been further explored. Adding to the ring size and lactam accessibility, these thioesterases can also efficiently macrocyclize a 14-member SNAC substrate, containing a completely non-native glycine insertion. Enzymatic assays

with compounds **2.2** and **2.7** have shown that these substrates do require some functionality adjacent to the ACP bound thioester. The data from substrate **2.12** confirms that a phenyl ring is not mandatory and that an amide group in its place can still produce favorable biocatalytic efficiencies. This could be due to the cis conformation **2.12** provides, or a pi-stacking interaction with the amide. The formation of macrocycle **2.15** dictates the first example of a native PKS TE catalyzing the formation of an amino acid containing depsipeptide macrolactone.³ This data has paved the way for a more in depth look into the substrate tolerability of Zea and Rdc TEs in the terms of depsipeptides. These RAL TEs are by far the most substrate tolerant PKS thioesterases characterized to date and show promise as potential biocatalysts.

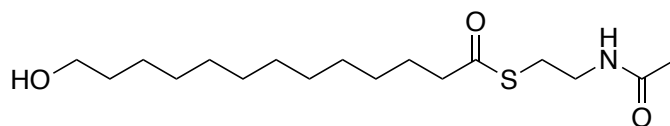
2.5 Experimental

2.5.1 General Synthetic Methods

All reagents were purchased from Sigma-Aldrich or Oakwood chemicals at the highest available purity and used without further purification. All solvents were purchased from Fisher Scientific. All reactions were conducted using dry solvents under an argon atmosphere unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) and carried out on 0.25 mm E. Merck silica gel plates using UV light (254 nm) as a visualizing agent and phosphomolybdic acid stain and heat as developing agent. NMR spectroscopy was performed with a Bruker Avance II, operating 400 MHz for ¹H spectra, and 100 MHz for ¹³C spectra. All chemical shifts are reported in parts per million (δ), integration and coupling constants in Hz and corrected using the solvent residual peak as internal standard. High-resolution mass spectroscopy (HRMS) was conducted on a Micromass Q-TOF I for ESI measurements (John L. Holmes Mass Spectroscopy).

2.5.2 Synthetic Protocols

S-(2-acetamidoethyl) 13-hydroxytridecanethioate (2.2)



40 mg **2.3** (0.174 mmol) was dissolved in dry DCM under argon and cooled to 0°C. 50 mg EDC (0.26 mmol 1.5 eq.) and 2.6 mg DMAP (0.017 mmol, 0.1 eq.) was added and the solution stirred at 0°C for 10 minutes. 25 mg of N-acetyl cysteamine (0.21 mmol, 1.2 eq.) was added and the solution was stirred at room temperature for 20 h. The reaction was quenched with NH₄Cl, extracted with DCM, washed with Brine, dried over MgSO₄, and concentrated. The crude product was purified by column chromatography (3:97 MeOH/DCM) to yield 25 mg of pure **2.2** (44%) as a grey solid.

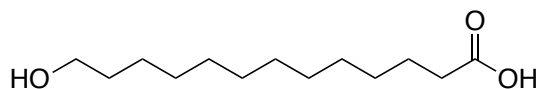
R_f = 0.21 (3:97 MeOH/DCM)

¹H NMR (400 MHz, CDCl₃) δ 5.86 – 5.71 (m, 1H), 3.67 – 3.57 (m, 2H), 3.41 (q, *J* = 6.4 Hz, 2H), 3.00 (t, *J* = 6.4 Hz, 2H), 2.59 – 2.50 (m, 2H), 1.94 (s, 3H), 1.67 – 1.60 (m, 2H), 1.54 (m, 2H), 1.24 (m, 16H).

¹³C NMR (100 MHz, CD₃OD) δ 199.21, 172.03, 61.61, 43.40, 38.78, 32.27, 29.33, 29.28, 29.26, 29.19, 29.11, 28.94, 28.54, 27.68, 25.54, 25.30, 21.08.

HRMS (ESI⁺) calc. for C₁₇H₃₃NO₃Na (M+Na) 354.2079; obsd. 354.2058

13-hydroxytridecanoic acid (**2.3**)

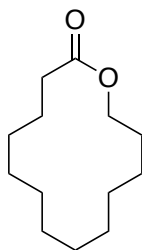


To a solution of 2 g of erucic acid (5.9 mmol) in dioxane/water 3:1, was added 1.4 ml 2,6-lutidine (11.8 mmol, 2 eq.), 53 mg potassium osmate dihydrate (0.59 mmol, 0.1 eq.), and 5 g sodium periodate (23.6 mmol, 4 eq.). The solution was stirred at room temperature for 16 h. The solution was diluted with water, extracted with EtOAc, washed with NaHCO₃, brine, dried over MgSO₄ and concentrated. The crude product was dissolved in dry THF and cooled to 0°C. 70 mg of NaBH₄ (1.10 mmol, 0.5 eq.) was added slowly over 15 minutes under argon. The solution was stirred for 3 h at room temperature, at completion 1.0 M HCl was added and the solution was extracted with EtOAc, dried over MgSO₄, and concentrated. The crude product was purified by column chromatography (40:60 EtOAc/hexanes) to yield 1.1 g of pure **2.3** (81%) as a grey solid. Characterization is consistent with previously reported data.¹⁶

$R_f = 0.19$ (40:60 EtOAc/hexanes)

¹H NMR (400 MHz, CDCl₃) δ 3.63 (t, $J = 6.6$ Hz, 2H), 2.33 (t, $J = 7.4$ Hz, 2H), 1.55 (m, 4H), 1.25 (m, 16H).

oxacyclotetradecan-2-one (2.4)

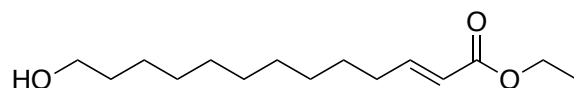


12 mg **2.6** (0.06 mmol) was dissolved in a mixture of THF/MeOH (1:1), containing 5 mg Pd/C catalyst. The flask was stirred for 24 h under 1 atm of H₂. The solution was diluted with THF, filtered through celite and concentrated. The crude product was purified by column chromatography (3:97 EtOAc/hexanes) to yield 9 mg **2.4** (75%) as a colorless oil. Characterization is consistent with previously reported data.¹⁷

$R_f = 0.1$ (3:97 EtOAc/hexanes)

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ 4.17 – 4.09 (m, 2H), 2.39 – 2.30 (m, 2H), 1.62 (dd, $J = 11.7, 6.2$ Hz, 4H), 1.42 – 1.21 (m, 16H).

ethyl (E)-13-hydroxytridec-2-enoate (2.5)

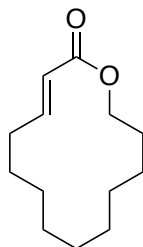


To a solution of 3.04 g ethyl (*E*)-trideca-2,12-dienoate (10.7 mmol) in dry THF was added 20 ml of 9-BBN THF solution (0.5 M, 10.7 mmol, 1.1 eq.). The solution was stirred for 1 h at room temperature followed by the addition of 15 ml NaOAc solution (3 M, 32.1 mmol, 3 eq.) and 2.5 ml of water. The solution was cooled to 0°C and 7.5 ml of 30% H_2O_2 in water (42.8 mmol, 4 eq.) was slowly added over 30 minutes and stirred for 16 h. The solution was extracted with Et_2O , dried over MgSO_4 and concentrated. The crude product was purified by column chromatography (50:50 EtOAc/hexanes) to yield 2.0 g **2.5** (61%) as a colorless oil. Characterization is consistent with previously reported data.¹⁸

$R_f = 0.45$ (50:50 EtOAc/hexanes)

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ 6.94 (dt, $J = 15.6, 7.0$ Hz, 1H), 5.78 (d, $J = 15.6$ Hz, 1H), 4.20 – 4.13 (m, 2H), 3.62 (t, $J = 6.6$ Hz, 2H), 2.16 (q, $J = 7.2$ Hz, 2H), 1.53 (m, 2H), 1.42 (m, 2H), 1.30 – 1.24 (m, 15H).

(E)-oxacyclotetradec-3-en-2-one (2.6)

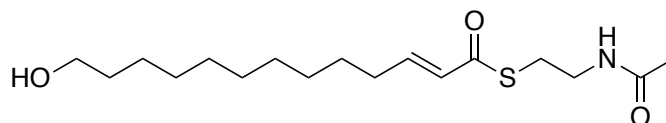


63 mg of **2.5** (0.424 mmol) was dissolved in a MeOH/THF/H₂O (4:3:1) mix, followed by 80 mg LiOH (4.2 mmol). The solution was heated to 50°C and stirred for 12 hours. Upon completion the solution was acidified to pH = 2, extracted with EtOAc, dried over MgSO₄ and concentrated. The seco acid was dissolved in dry DCM and added to a solution of 705 g DMAP (5.8 mmol, 30 eq.) and 407 mg PyBOP (0.8 mmol, 4 eq.), over a 10-hour period at room temperature to final concentration 0.004 M. The solution was stirred an additional 2 h, quenched with NH₄Cl, extracted with EtOAc, dried over MgSO₄ and concentrated. The crude product was purified by column chromatography (3:97 EtOAc/hexanes) to yield 24 mg **2.6** (52%) as a colorless oil. Characterization is consistent with previously reported data.¹⁷

R_f = 0.3 (5:95 EtOAc/hexanes)

¹H NMR (400 MHz, CDCl₃) δ 7.05 – 6.92 (dt, *J* = 15.6, 7.0 Hz, 1H), 5.79 (d, *J* = 15.7 Hz, 1H), 4.29 – 4.18 (m, 2H), 2.24 (m, 2H), 1.66 (m, 2H), 1.57 (m, 2H), 1.26 (m, 12H).

S-(2-acetamidoethyl) (E)-13-hydroxytridec-2-enethioate (**2.7**)



To a solution of 104 mg LiBr (1.2 mmol, 5 eq.) in dry THF was added 144 mg **2.11** (0.485 mmol, 2 eq.) under argon, the solution was stirred for 10 minutes at room temperature. 210 μL NEt₃ (1.5 mmol, 6 eq.) was slowly added and the solution was stirred 10 more minutes. 97 mg **2.10** (0.323

mmol) was added over 30 minutes and the solution was stirred for 16 h. The solution was quenched with NH_4Cl , extracted with EtOAc, dried over MgSO_4 and concentrated. The crude product was purified by column chromatography (50:50 EtOAc/hexanes) and subsequently dissolved in acetonitrile, 331 μL HF (28.0 M, 40 eq.) was slowly added and the solution was stirred for 10 minutes. The reaction was quenched with sat. NH_4Cl , extracted with EtOAc, dried over MgSO_4 and concentrated to yield 63 mg pure **2.7** (87%) as a white solid.

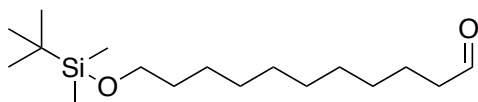
$R_f = 0.14$ (75:25 EtOAc/hexanes)

$^1\text{H NMR}$ (400 MHz, DMSO-d_6) δ 8.03 (s, 1H), 6.88 – 6.73 (dt, $J = 15.6, 7.0$ Hz, 1H), 6.18 (d, $J = 15.6$ Hz, 1H), 3.32 (t, $J = 6.6$ Hz, 2H), 3.14 (dd, $J = 12.8, 6.5$ Hz, 2H), 2.91 (t, $J = 6.8$ Hz, 2H), 2.15 (q, $J = 7.0$ Hz, 2H), 1.75 (s, 3H), 1.36 (m, 4H), 1.20 (m, 12H).

$^{13}\text{C NMR}$ (100 MHz, CD_3OD) δ 189.62, 172.12, 146.17, 128.12, 61.60, 38.87, 32.26, 31.72, 29.29, 29.19, 29.17, 29.05, 28.85, 27.74, 27.46, 25.54, 21.02.

HRMS (ESI+) calc. for $\text{C}_{17}\text{H}_{31}\text{NO}_3\text{SNa}$ ($\text{M}+\text{Na}$) 352.1922; obsd. 352.1917

11-((*tert*-butyldimethylsilyl)oxy)undecanal (**2.10**)



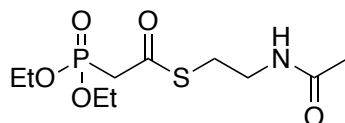
400 mg of Amberlyst cation exchange resin was added to 900 mg of 11,11-dimethoxyundecan-1-ol (3.88 mmol) in an Acetone/water solution (60:1) and stirred overnight. The solution was filtered, dried over MgSO_4 and concentrated to yield 720 mg of 11-hydroxyundecanal. The crude product was dissolved in DMF, followed by the addition of 310 mg imidazole (4.55 mmol, 1.2 eq.), 31 mg DMAP (0.254 mmol, 0.05 eq.), and 800 mg TBSCl (5.3 mmol, 1.4 eq.). The solution was stirred for 18 h and quenched with sat. NH_4Cl . The aqueous mixture was extracted with Et_2O , dried over MgSO_4 and concentrated. The crude mixture was purified by column chromatography (5:95

EtOAc/hexanes) to yield 900 mg **2.10** (77% over 2 steps) as a colorless oil. Characterization is consistent with previously reported data.¹⁹

$R_f = 0.45$ (5:95 EtOAc/hexanes)

¹H NMR (400 MHz, CDCl₃) δ 9.74 (t, $J = 1.9$ Hz, 1H), 3.57 (t, $J = 6.7$ Hz, 2H), 2.40 (td, $J = 7.3$, 1.9 Hz, 2H), 1.60 (m, 2H), 1.48 (m, 2H), 1.26 (m, 12H), 0.89 – 0.85 (s, 9H), 0.10 – -0.06 (s, 6H).

S-(2-acetamidoethyl) 2-(diethoxyphosphoryl)ethanethioate (**2.11**)

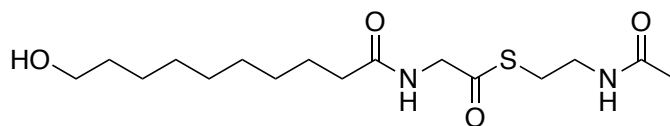


To a solution of 1.22 g diethyl phosphonoacetic acid (6.22 mmol) in DCM at 0°C was added 1.48 mg EDC (7.74 mmol, 1.3 eq.), 82 mg DMAP (0.672 mmol 0.1 eq.), and 888 mg of N-acetyl cysteamine. The reaction was stirred overnight at room temperature. Upon completion the reaction was quenched with sat. NH₄Cl. The organic layer was separated, and the aqueous layer extracted with DCM. The organic layers were washed with sat. NaHCO₃, Brine, dried over MgSO₄, and concentrated. The crude product was purified by column chromatography (5:95 MeOH/DCM) yielding 422 mg of **2.11** (23%) as a colorless oil. Characterization is consistent with previously reported values.²⁰

$R_f = 0.17$ (5:95 methanol/DCM)

¹H NMR (400 MHz, CDCl₃) δ 6.15 – 5.99 (s, 1H), 4.15 (q, $J = 7.1$, 4H), 3.45 (dd, $J = 12.2$, 6.1 Hz, 2H), 3.22 (s, 2H), 3.14 – 3.02 (m, 2H), 1.95 (s, 3H), 1.34 (t, $J = 7.1$ Hz, 6H).

S-(2-acetamidoethyl) 2-(10-hydroxydecanamido)ethanethioate (**2.12**)



0.5 g of Boc-glycine-N-acetyl cysteamine (1.923 mmol) was dissolved in dioxane under argon and cooled to 0°C. 4 mL of 4.0 HCl/dioxane was added to the solution on ice. The solution was stirred for 30 minutes and the precipitated pure salt was filtered and suspended in dry DCM and cooled to 0°C. 244 mg EDC (1.27 mmol, 1.5 eq.), 350 μ L NEt₃ (2.51 mmol, 3 eq.), and 160 mg 10-hydroxydecanoic acid (0.955 mmol) was added and the solution was stirred at room temperature for 3 h. NH₄Cl was added, and the solution was extracted with DCM, washed with NaHCO₃, Brine, dried over MgSO₄, and concentrated. The crude product was purified by column chromatography (50:50 EtOAc/hexanes) to yield 40 mg **2.12** (14%) as a white powder.

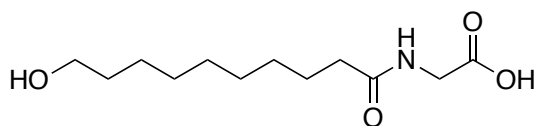
R_f = 0.11 (50:50 EtOAc/hexanes)

¹H NMR (400 MHz, DMSO-d₆) δ 8.46 (t, *J* = 5.1 Hz, 1H), 8.00 (s, 1H), 4.29 (t, *J* = 5.1 Hz, 1H), 3.92 (d, *J* = 6.0 Hz, 2H), 3.36 – 3.31 (m, 2H), 3.15 – 3.05 (m, 2H), 2.83 (t, *J* = 7.0 Hz, 2H), 2.11 (t, *J* = 7.4 Hz, 2H), 1.74 (s, 3H), 1.47 (m, 2H), 1.36 (m, 2H), 1.21 (m, 10H).

¹³C NMR (100 MHz, CD₃OD) δ 197.50, 175.53, 172.06, 61.60, 48.62, 38.58, 35.38, 32.26, 29.19, 29.12, 28.96, 28.86, 27.34, 25.52, 25.32, 21.09.

HRMS (ESI+) calc. for C₁₆H₃₀N₂O₄SNa (M+Na) 369.1824; obsd. 369.1805

(10-hydroxydecanoyl)glycine (**2.13**)



To a solution of 685 mg ethyl glycinate (4.91 mmol, 1.5 eq.) in DCM was added 1.1 g EDC (3.19 mmol, 1.6 eq.) and 1.37 mL NEt₃ (9.8 mmol, 3 eq.), the solution was then stirred for 10 minutes. 600 mg 10-hydroxydecanoic acid (3.19 mmol) was added to the solution at 0°C and stirred for 14 h at room temperature. The solution was quenched with NH₄Cl, extracted with EtOAc, dried over MgSO₄, and concentrated. The crude product was dissolved in MeOH/THF/H₂O (6:10:6), followed by the addition of 845 mg LiOH (36.7 mmol, 10 eq.) and subsequently stirred at 60°C for 4 h. The solution was acidified to pH = 2, extracted with EtOAc, dried over MgSO₄, and concentrated. The crude product was purified by column chromatography (80:20 EtOAc) to yield 600 mg **2.13** (77%) as a white powder.

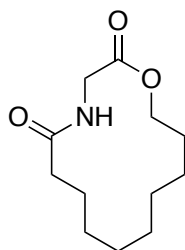
R_f = 0.2 (80:20 EtOAc/hexanes)

¹H NMR (400 MHz, DMSO-d₆) δ 8.02 (s, 1H), 3.66 (d, *J* = 5.9 Hz, 2H), 3.33 (m, 2H), 2.06 (t, *J* = 7.4 Hz, 2H), 1.50 – 1.30 (m, 4H), 1.20 (m, 10H).

¹³C NMR (100 MHz, DMSO-d₆) δ 173.02, 171.92, 61.18, 40.99, 35.52, 33.01, 29.46, 29.39, 29.25, 29.07, 25.96, 25.65.

HRMS (ESI+) calc. for C₁₂H₂₃NO₄Na (M+Na) 268.1525; obsd. 268.1536

1-oxa-4-azacyclotetradecane-2,5-dione (**2.15**)



100 mg **2.4a** (0.41 mmol) was dissolved in dry DCM and added to a solution of 705 g DMAP (5.8 mmol, 30 eq.) and 407 mg PyBOP (0.8 mmol, 4 eq.), over a 10-hour period at room temperature

to a final concentration of 0.004 M. The solution was stirred an additional 2 h, quenched with NH_4Cl , extracted with EtOAc, dried over MgSO_4 and concentrated. The crude product was purified by column chromatography (30:70 EtOAc/hexanes) to yield 56 mg **2.15**(56%) as a white solid.

$R_f = 0.3$ (30:70 EtOAc/hexanes)

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ 5.92 – 5.76 (s, 1H), 4.22 – 4.15 (m, 2H), 4.08 (d, $J = 6.3$ Hz, 2H), 2.30 – 2.22 (m, 2H), 1.73 – 1.59 (m, 4H), 1.36 (m, 10H).

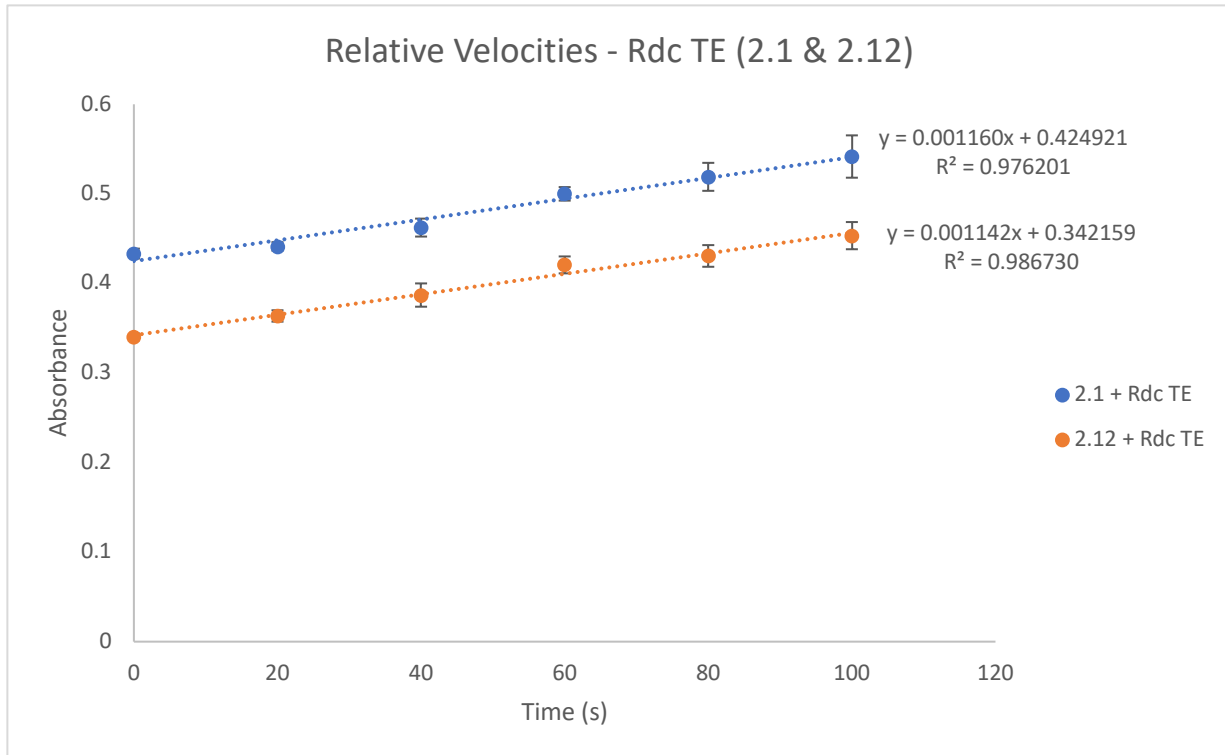
$^{13}\text{C NMR}$ (100 MHz, CD_3OD) δ 172.91, 169.16, 64.57, 41.86, 35.50, 26.76, 26.30, 26.01, 25.85, 25.25, 24.89, 23.16.

HRMS (ESI+) calc. for $\text{C}_{12}\text{H}_{21}\text{NO}_3\text{Na}$ ($\text{M}+\text{Na}$)250.1419; obsd. 250.1407

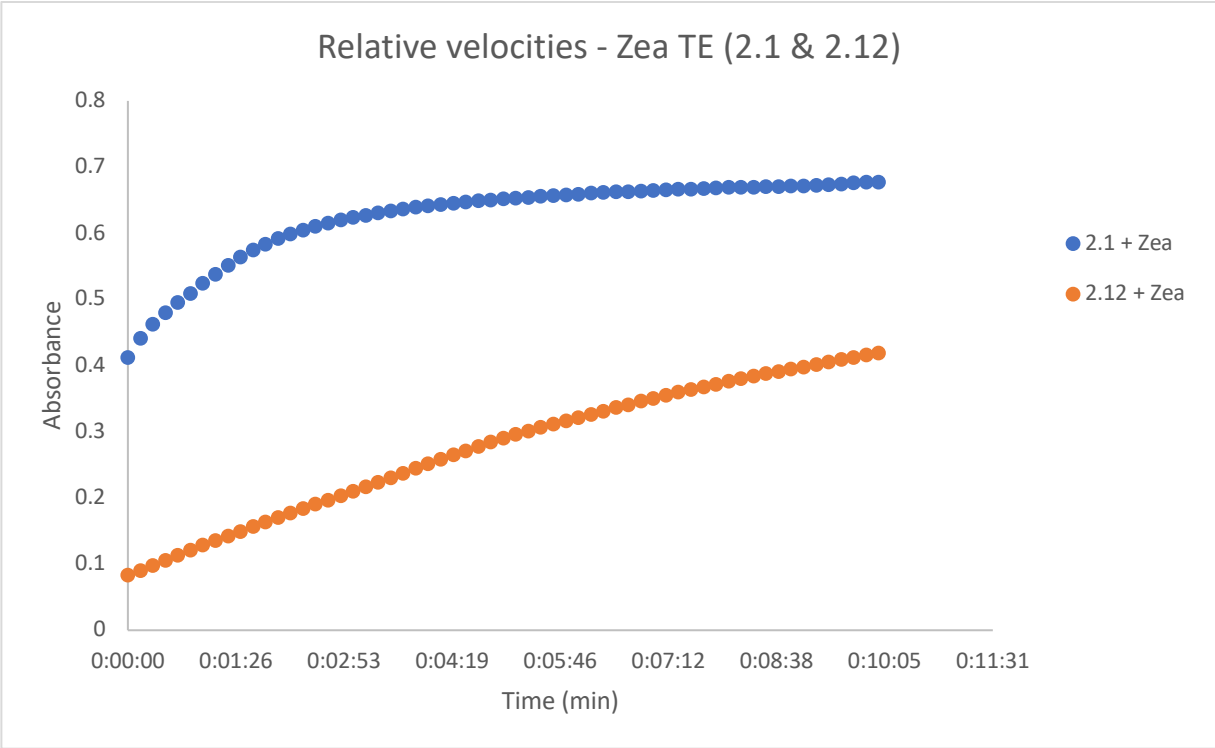
2.5.3 Enzymatic Protocols

Assays were run at a total volume of 50 μL containing 50 mM phosphate buffer (pH 7.4), 1.0 mM SNAC substrate, 5 – 25 μM enzyme (Rdc or Zea), and 10% v/v DMSO. All reactions were quenched with an equal volume of 0.5% formic acid in acetonitrile prior to HPLC analysis. HPLC analysis was conducted with an Agilent 1260 Modular system using a Dikima Leapsil 2.7 μm C18, 100 x 2.1 mm column. HPLC conditions: Flow rate 0.4 mL/min, 0 to 100% B over 30 min. (A: H_2O B: ACN). Kinetic activity was established via a continuous Ellman's assay run at a total volume of 100 μL containing 50 mM phosphate buffer (pH 7.4), 1.0 mM SNAC substrate, 5 μM Zea, 10% v/v DMSO, and 50 μL 8% DTNB (Ellman's reagent). DTNB has been shown to inactivate Rdc TE in real-time assays, therefore a discontinuous assay was run by taking 5 μL aliquots of the assay and quenching with 5 μL DTNB. 2 μL of this mixture was loaded onto a nanodrop 2000 podium and recorded at 412 nm.

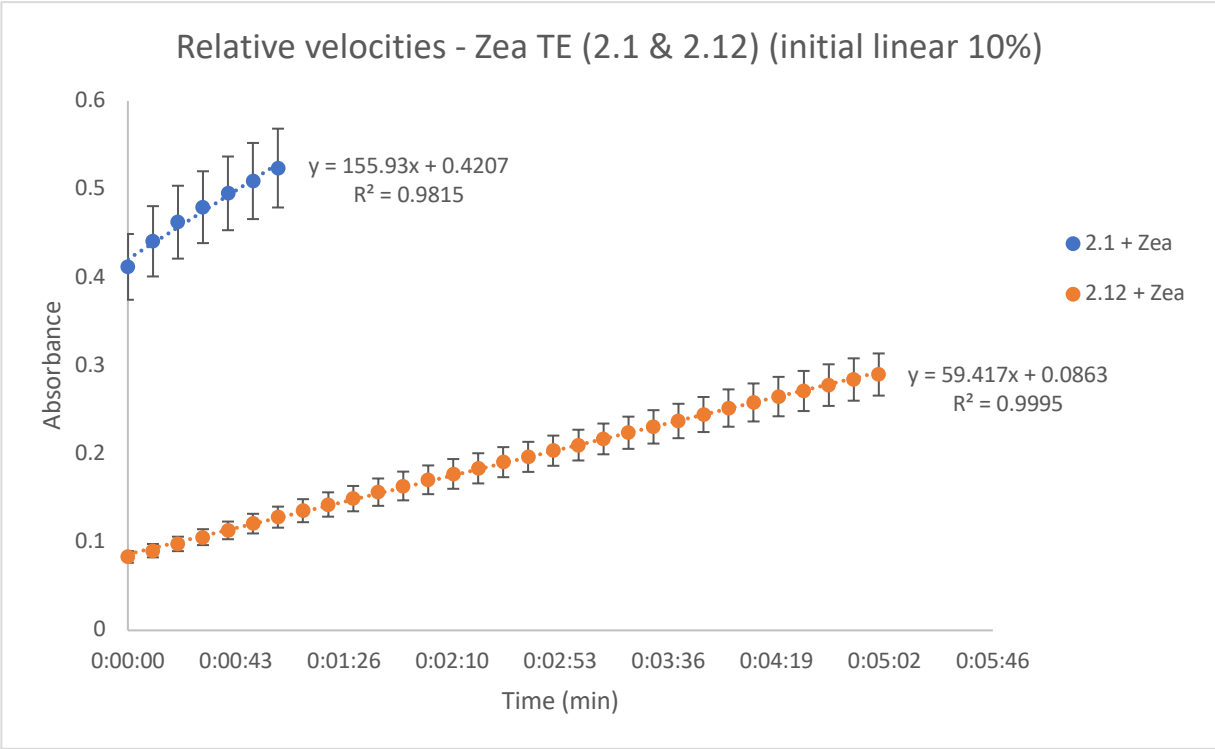
2.5.4 Relative Velocity Curves



Initial velocities of substrate 2.1 and 2.12 with Rdc TE.



Initial velocities of substrate 2.1 and 2.12 with Zea TE.



Initial velocities of substrate 2.1 and 2.12 with Rdc TE (Corrected for linear portion).

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Chapter 3: Depsipeptide Macrocyclization Using Two Resorcylic Acid Lactone Thioesterases.

3.1 Introduction:

3.1.1 Depsipeptides

The term depsipeptide refers to an amino acid containing compound that contains at least one ester bond in the peptide chain. There are several examples of macrocyclic NRPs that are cyclized through the formation of a depsipeptide. Daptomycin, cryptophycin-1, and valinomycin are examples where NRPS TEs catalyze esterification, forming cyclic depsipeptides (Fig 3.1).¹⁻³ Ester bonds in peptides can be favoured over amides because they reduce the polarity of the peptide. With an ester there is no longer an H-bond donor and this can help with cell permeability.⁴ The increased nucleophilicity of an amine would seemingly favour lactam formation, lactone formation is sometimes preferred for its lack of reactivity. With the less nucleophilic alcohol there is a smaller chance of forming undesired by-products, upstream of the thioesterase.

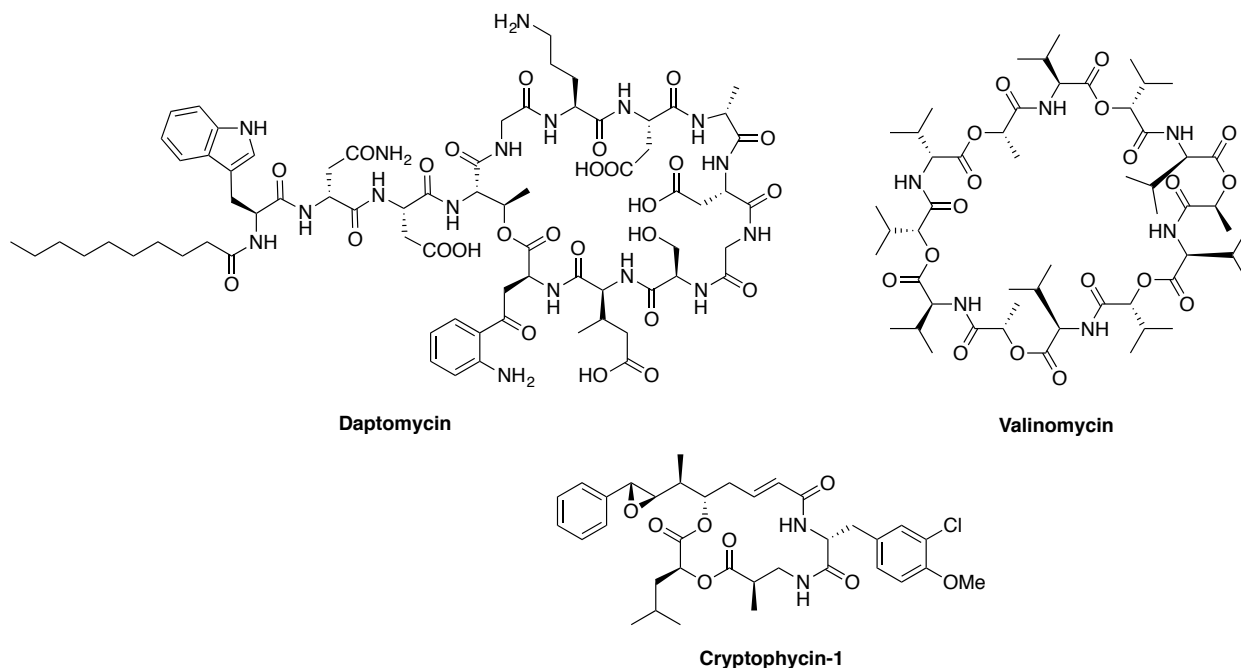


Figure 3. 1 Three cyclic depsipeptides.

Compounds like Cryptophycin-1 employ the use of two classes of biosynthetic machinery to produce hybrid natural products. Lactonization by the thioesterase in these biosynthetic pathways can occur either on the NRPS or PKS proteins depending on which proteins initiate and terminate the biosynthesis. For example epothilone C biosynthesis is terminated on the PKS protein and utilizes the Epo TE found on the PKS⁵, whereas cryptophycin-1 is lactonized by the Crp TE found on the NRPS.² These examples suggest that PKS TEs could possibly tolerate peptide containing compounds and vice versa. A study by Wang *et al.* tested peptide containing substrates for hydrolysis with three PKS TEs (DEBS, Epo, and Pim). The results show that in certain cases the peptidyl substrates were more efficiently hydrolyzed than the more native acyl substrates.⁶ These results show increasing promise for the development of a universal thioesterase.

3.2 Results and Discussion

3.2.1 Hypotheses about depsipeptide formation with Zea and Rdc TEs.

The results observed in chapter 2 solicited the question of “how tolerant are these TEs when it comes to forming cyclic depsipeptides?”. An in depth look into this question was warranted because this is the first example of a strictly PKS TE efficiently cyclizing an amino acid containing non-native substrate. Although there are examples of PKS TEs macrocyclizing depsipeptides, these are native to the biosynthetic pathway. In the case of zearalenone and radicicol these TEs would have never been introduced to amino acids throughout their biosynthetic pathway evolution. Herein we highlight the enzymatic results of a synthesized library of amino acid containing substrates. These substrates were designed to answer particular questions about the depsipeptide tolerance of these RAL TEs.

3.2.2 Library of macrocyclic depsipeptide precursors.

This library consists of 6 unique amino acid containing compounds that were synthesized and tested on both the zearalenone and radicicol thioesterases (Fig. 3.2).

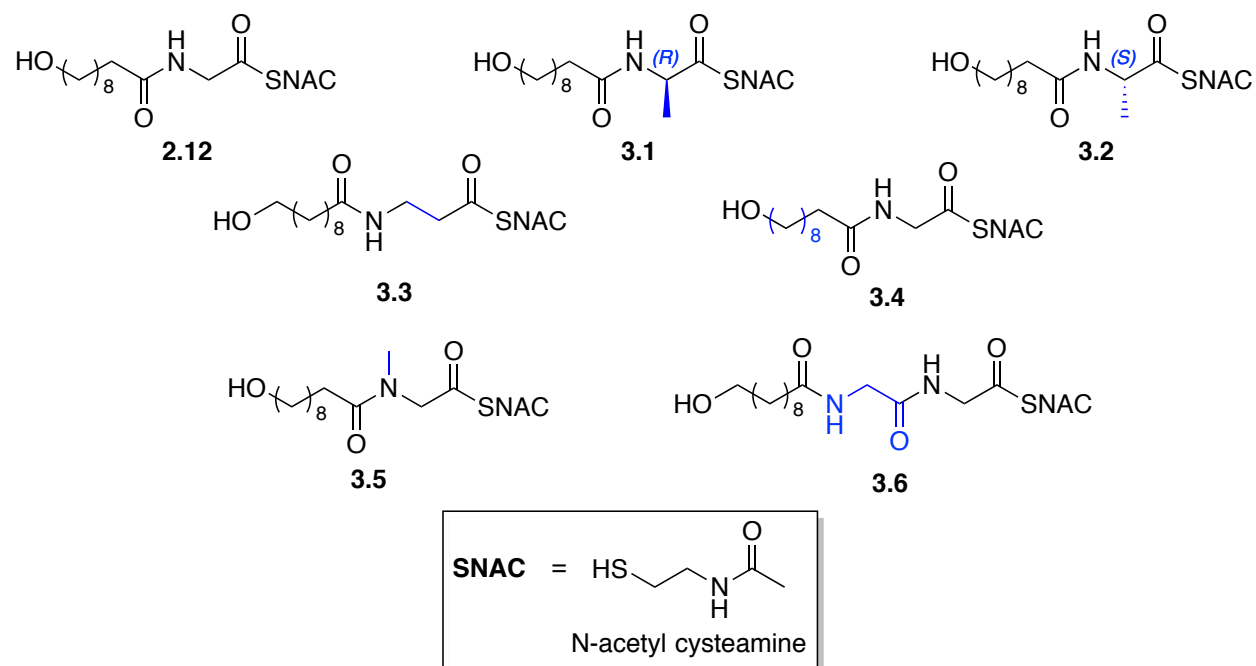
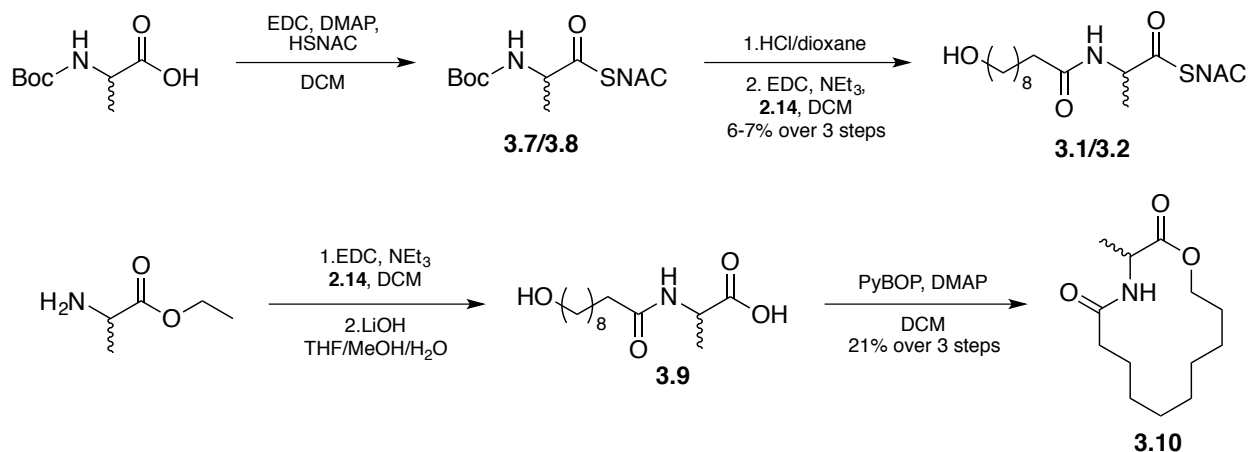


Figure 3. 2 Library of amino acid containing compounds.

All substrates were modelled after compound **2.12**, linear carbon chains containing an amino acid, adjacent to the SNAC thioester, with a terminal hydroxyl nucleophile. Compounds **3.1** and **3.2** have alanine insertions, both D- and L-configurations. This is to probe whether the amino acid configuration affects thioesterase loading and/or product distribution. Compound **3.3** contains a β -alanine amino acid, increasing the carbon chain between the amide and thioester from one carbon **2.12** to two carbons **3.3**. **3.4** was synthesized as a control for compound **3.3**. Since **3.3** forms a 15-membered macrocycle, we wanted to confirm that any activity/lack of activity was due to the amide shift, as oppose to the different ring size. **3.5** contains a sarcosine amino acid insertion. The methylated amide significantly changes the conformation of the amide, as well as removes the H-bond donation from the substrate amide. Lastly, **3.6** contains a glycine-glycine insertion allowing us to probe for the formation of macrocyclic didepsipeptide.

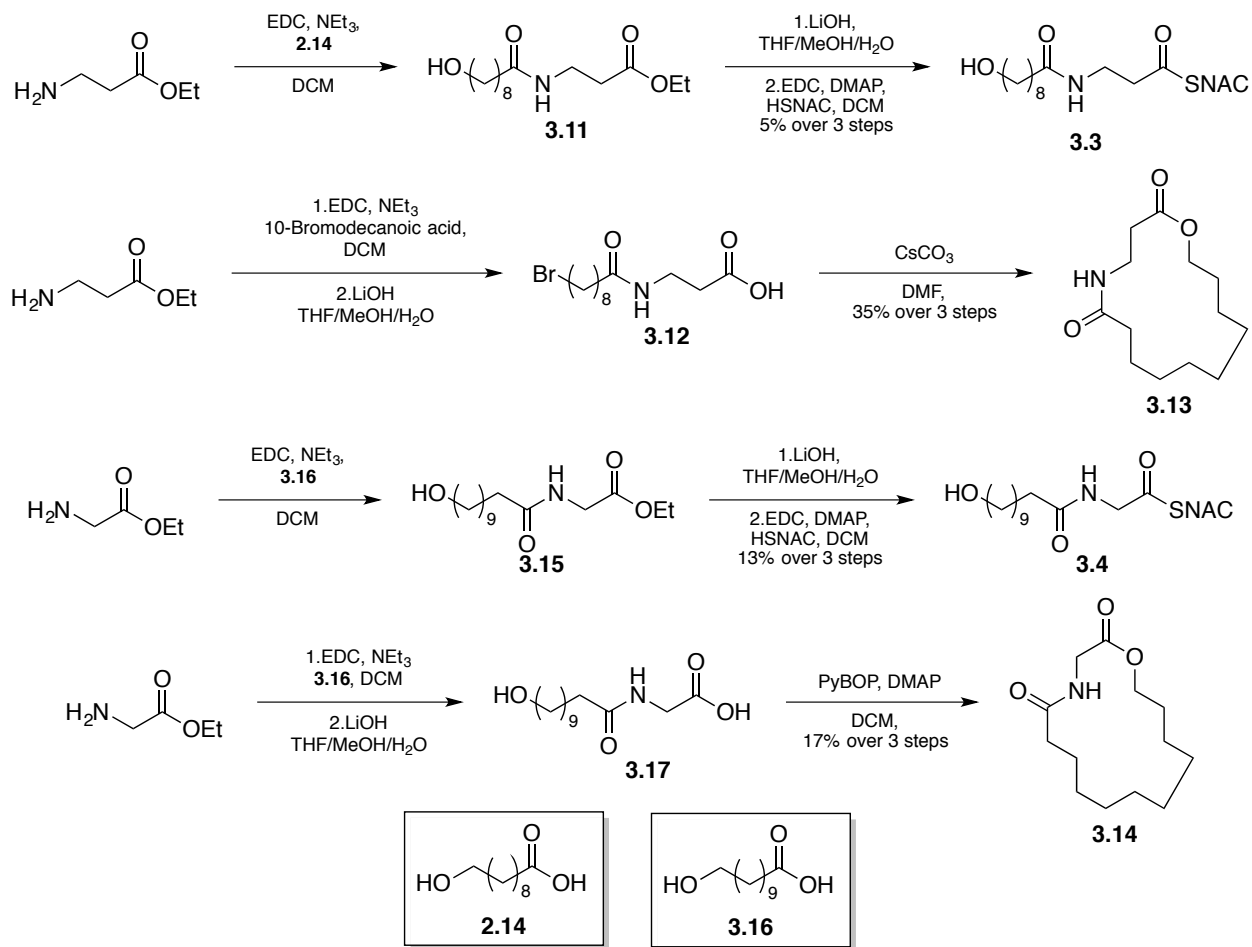
3.2.3 Synthesis of depsipeptide library and macrocyclic standards.

Compound **3.1** and **3.2** were synthesized analogous to each other. SNAC was coupled to the Boc protected amino acid (D- & L-alanine) using EDC, and HOBt. Although Boc amino acids rarely undergo epimerization when activating the C-terminal acid, HOBt was used to further suppress any epimerization. The SNAC Boc amino acids **3.7/3.8** were deprotected with an HCl/dioxane mixture and subsequently coupled with 10-hydroxy decanoic acid using EDC to afford both compounds **3.1** and **3.2**. Synthesis of the macrocyclic standard was carried out using **3.9**, in a PyBOP, DMAP macrocyclization analogous to **2.15** synthesis (Scheme 3.1).



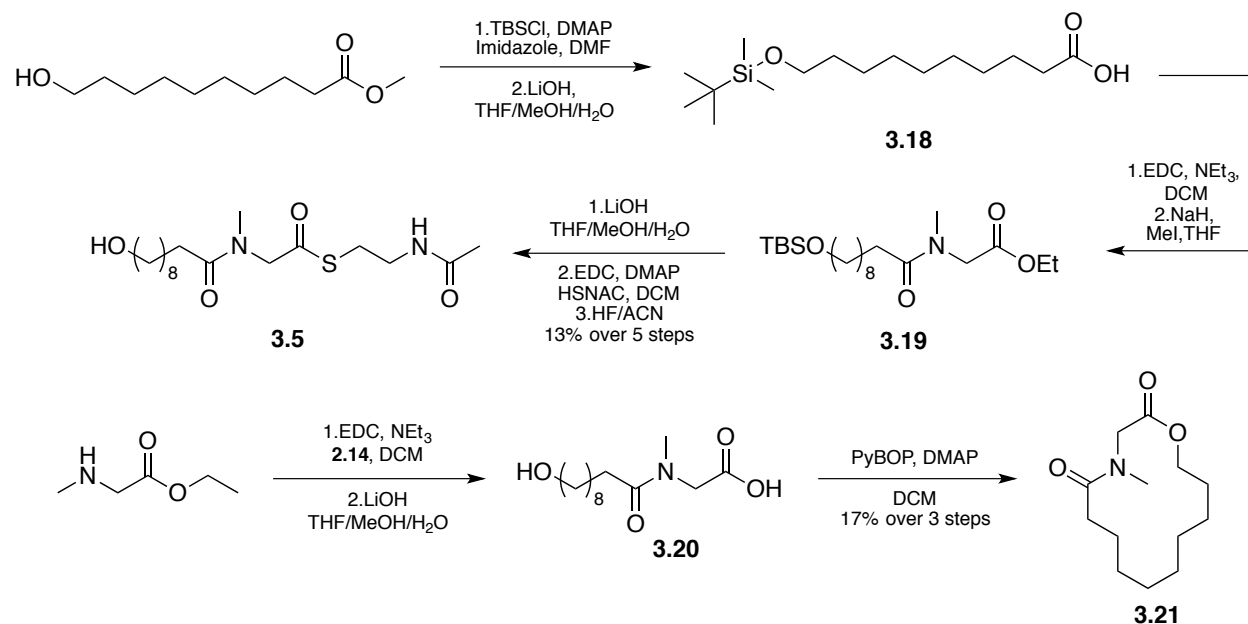
Scheme 3.1 Synthesis of compounds **3.1**, **3.2**, and macrocycle standard **3.10**.

Compound **3.3** was synthesized starting with an amide coupling between β -alanine ethyl ester and 10-hydroxydecanoic acid, using EDC, to form **3.11**. Hydrolysis using LiOH, followed by an EDC/DMAP thioester coupling with SNAC formed compound **3.3**. Substrate **3.4** was synthesized analogously using glycine ethyl ester and 11-hydroxydecanoic acid. The macrocycle standard **3.13** synthesis started with an amide coupling of β -alanine and 10-bromodecanoic acid using EDC, followed by hydrolysis to form **3.12**. The macrocyclization step was completed using CsCO₃ in DMF to yield macrocycle **3.13**. **3.14** was synthesized analogously to **3.10** using *seco*-acid **3.17** (Scheme 3.2).



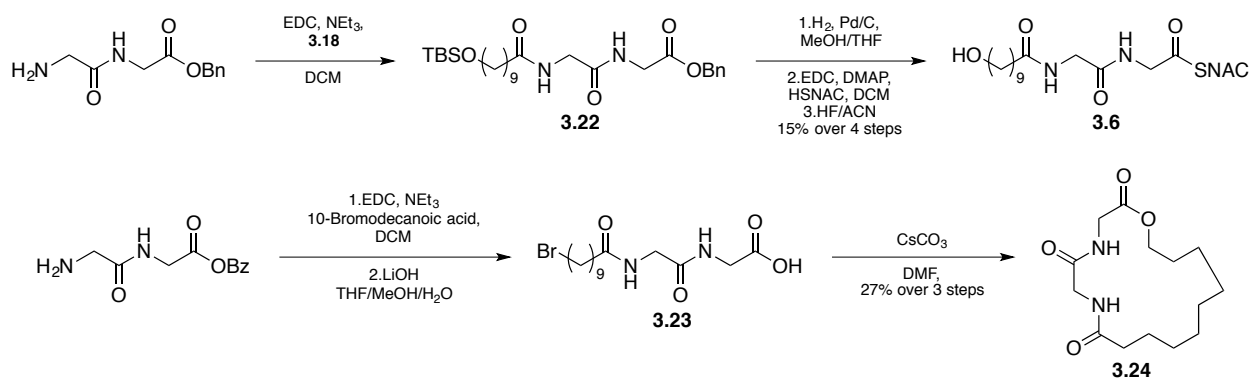
Scheme 3. 2 Synthesis of compounds 3.3, 3.4, 3.13, and 3.14.

For compound **3.5**, the 10-hydroxydecanoic acid had to first be TBS protected at the hydroxyl due to the harsh methylating conditions of the following step. 10-hydroxydecanoic acid was TBS protected using TBSCl, then coupled with glycine ethyl ester via EDC, followed by an amide methylation using NaH and MeI to afford **3.19**. The ethyl ester was hydrolyzed using LiOH, SNAC was coupled to the free acid using EDC & DMAP, and lastly, the TBS group was removed using aqueous HF to provide compound **3.5**. The macrocycle standard was formed by coupling sarcosine to 10-hydroxydecanoic acid, hydrolyzing the ethyl ester and macrocyclizing the *seco*-acid using PyBOP and DMAP to afford compound **3.21** (Scheme 3.3).



Scheme 3. 3 Synthesis of compound 3.5 and 3.21.

Synthesis for **3.6** began with an amide coupling of the TBS protected 10-hydroxydecanoic acid **3.18** and benzyl protected glycine-glycine peptide to afford compound **3.22**. The benzyl group was removed by hydrogenolysis with H₂ and Pd/C, SNAC was coupled to the free acid using EDC, and lastly the TBS group was removed with aqueous HF to make **3.6**. Compound **3.24** was synthesized analogously to **3.13**, by making the bromo-acid **3.23**, and subsequently cyclizing it using CsCO₃ to provide macrocycle **3.24** (Scheme 3.4).



Scheme 3.4 Synthesis of compound **3.6** and **3.24**.

Difficulties with PyBOP/DMAP cyclization with *seco*-acid versions of compounds **3.3** and **3.6** due to poor solubility, lead to the CsCO₃ cyclization of bromo-acid analogs to form macrocycles **3.13** and **3.24**.

3.2.4 Zea & Rdc TE enzymatic assays with depsipeptide library.

Enzymatic assays performed with this library of 6 compounds were done to illustrate the loading ability of these enzymes with the amino acid containing substrates, but also to observe the product distribution of the release step. The utility of these TEs as biocatalysts relies more heavily on their ability to form macrocycle product as opposed to hydrolysis or oligomerization. Previous work with the DEBS TE has shown that TEs show some variability when it comes to loading substrates but macrocyclization suffers greatly when there is deviation from the native substrate.⁷⁻

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Initial assays done at 5 μM showed significantly less activity for the depsipeptide substrates than the phenyl-containing compounds. For this reason, all enzymatic assays were run with 25 μM enzyme to better observe product distribution. These assays were done over an 18 h incubation period at room temperature followed by an HPLC comparison to the starting substrate and macrocycle standard.

D-alanine (3.1)

The D-alanine containing substrate **3.1** showed >90% consumption with the Rdc TE (Fig. 3.3), with a product distribution around 50:50 hydrolysis to macrocycle. Incubation of **3.1** with the Zea TE showed much starting material consumption (approximately 60%) with a product distribution of 40:60 hydrolysis to macrocycle. These data indicates that both RAL thioesterases are capable of chemoenzymatically macrocyclizing substrate **3.1**, with radicicol being more substrate tolerant (>90% consumption), and zearalenone having a higher tendency to macrocyclize (40:60 hydrolysis/macrocycle).

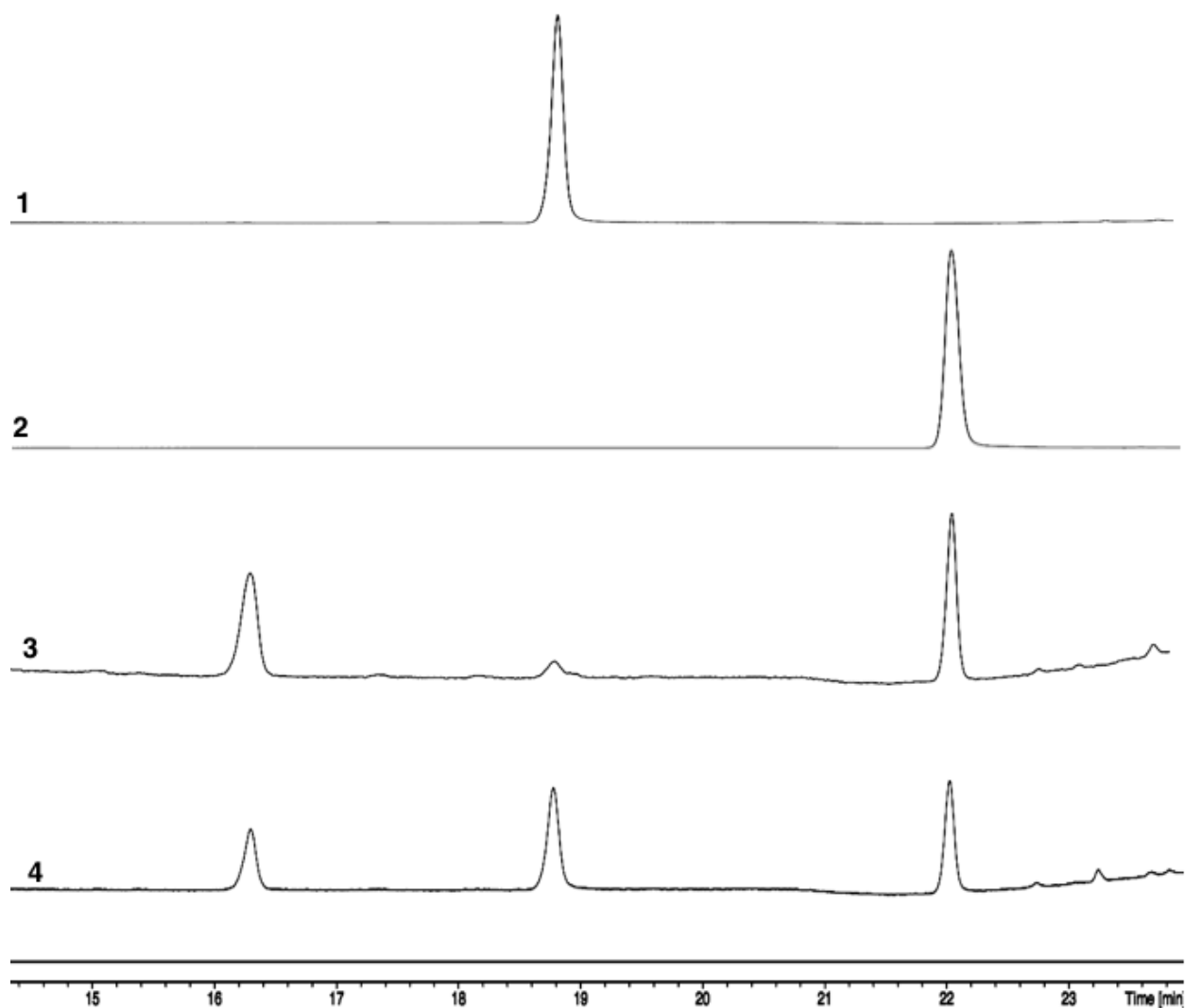


Figure 3. 3 HPLC traces of enzymatic assays of Zea and Rdc TEs with substrate 3.1.

1) substrate standard **3.1**. 2) macrocycle standard **3.10**. 3) 1.0 mM substrate, 25.0 μ M Rdc TE, 24 h. 4) 1.0 mM standard. 25.0 μ M Zea TE, 24 h. (Peak at 16.3 is hydrolysis product)

L-alanine (3.2)

~70% consumption of substrate **3.2** was observed upon incubation with the Rdc TE (Fig. 3.4). The product distribution was around 30:70 hydrolysis to macrocycle. When **3.2** was incubated with Zea TE, <20% substrate consumption was observed, with a product distribution of >95% macrocyclization. The radical thioesterase, again showed a greater tendency to load the SNAC substrate, but at the cost of partial product hydrolysis. Zearealenone TE showed limited forward progress but favoured macrocyclization over hydrolysis.

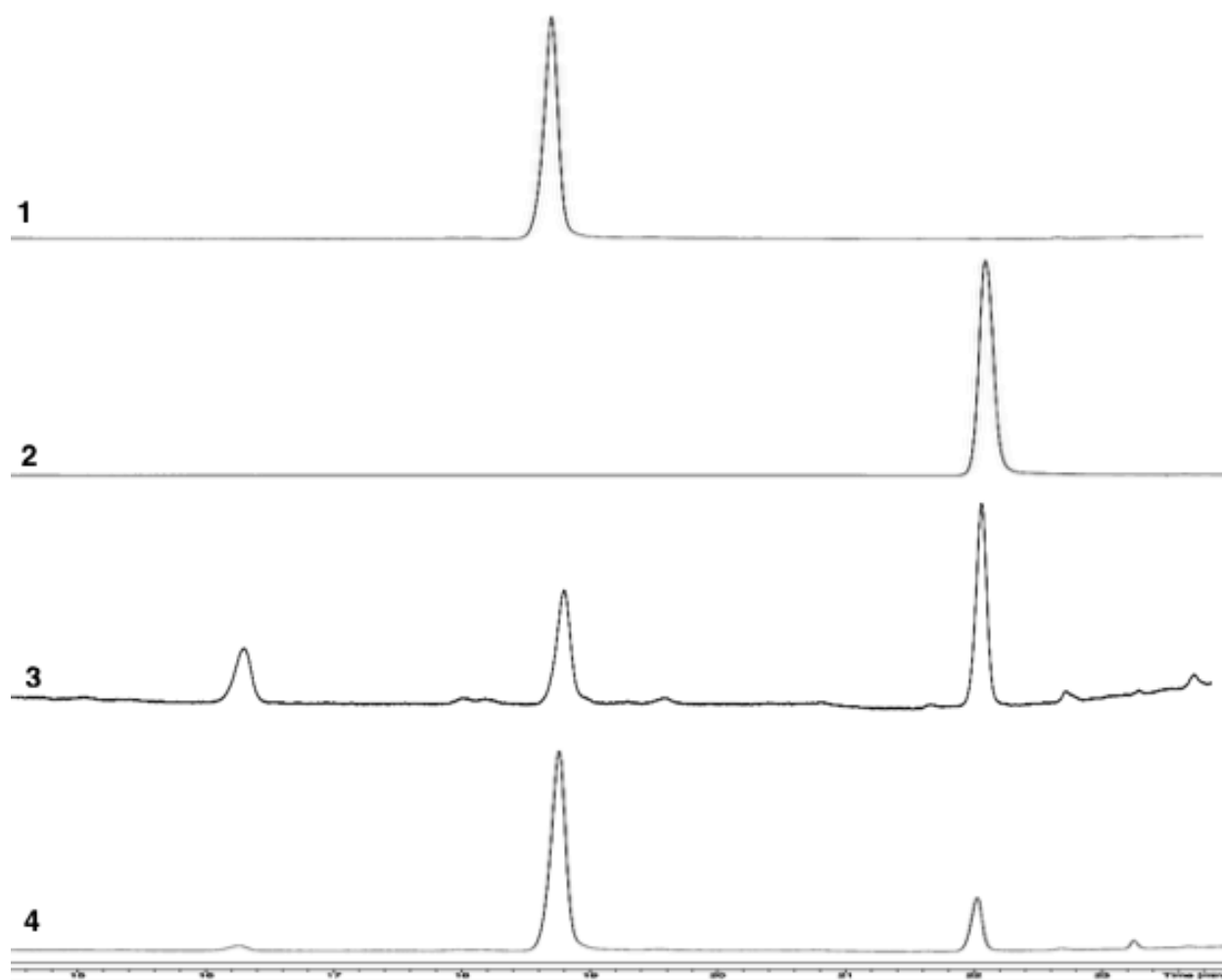


Figure 3. 4 HPLC traces of enzymatic assays of Zea and Rdc TEs with substrate 3.2.

1) substrate standard **3.2**. 2) macrocycle standard **3.10**. 3) 1.0 mM substrate, 25.0 μ M Rdc TE, 24 h. 4) 1.0 mM standard. 25.0 μ M Zea TE, 24 h. (Peak at 16.3 is hydrolysis product)

The enzymatic data for these two substrates suggest three things. The D-alanine substrate was consumed faster than the L configured substrate, the comparison with both TE's illustrates this (Figure 3.2 & 3.3). This means both RAL TEs show preference for D-amino acids. The zearalenone TE is much more substrate sensitive during the loading step, whereas radicicol shows more promiscuity. Lastly, radicicol is much more prone to hydrolysis, zearalenone is more efficient at macrocycle processing once the substrate has loaded.

β -alanine (3.3)

Interestingly, when the β -alanine substrate was assayed, there was almost negligible substrate consumption with both TEs (Fig. 3.5). In both cases about ~1% macrocycle product can be detected, but at such high enzyme concentrations (25 μ M) and long incubation period, this is a negligible result. The lack of activity with this substrate does tell us quite a bit about what structural features are essential for enzyme activity. The data collected from compounds **2.2**, **2.7**, and **3.3**, suggest that functionality within the first two carbons adjacent to the SNAC thioester is essential for reactivity. This further reinforces the theory that type I iterative PKS thioesterases rely on a "logic gate" to initiate macrocyclization activity.⁹

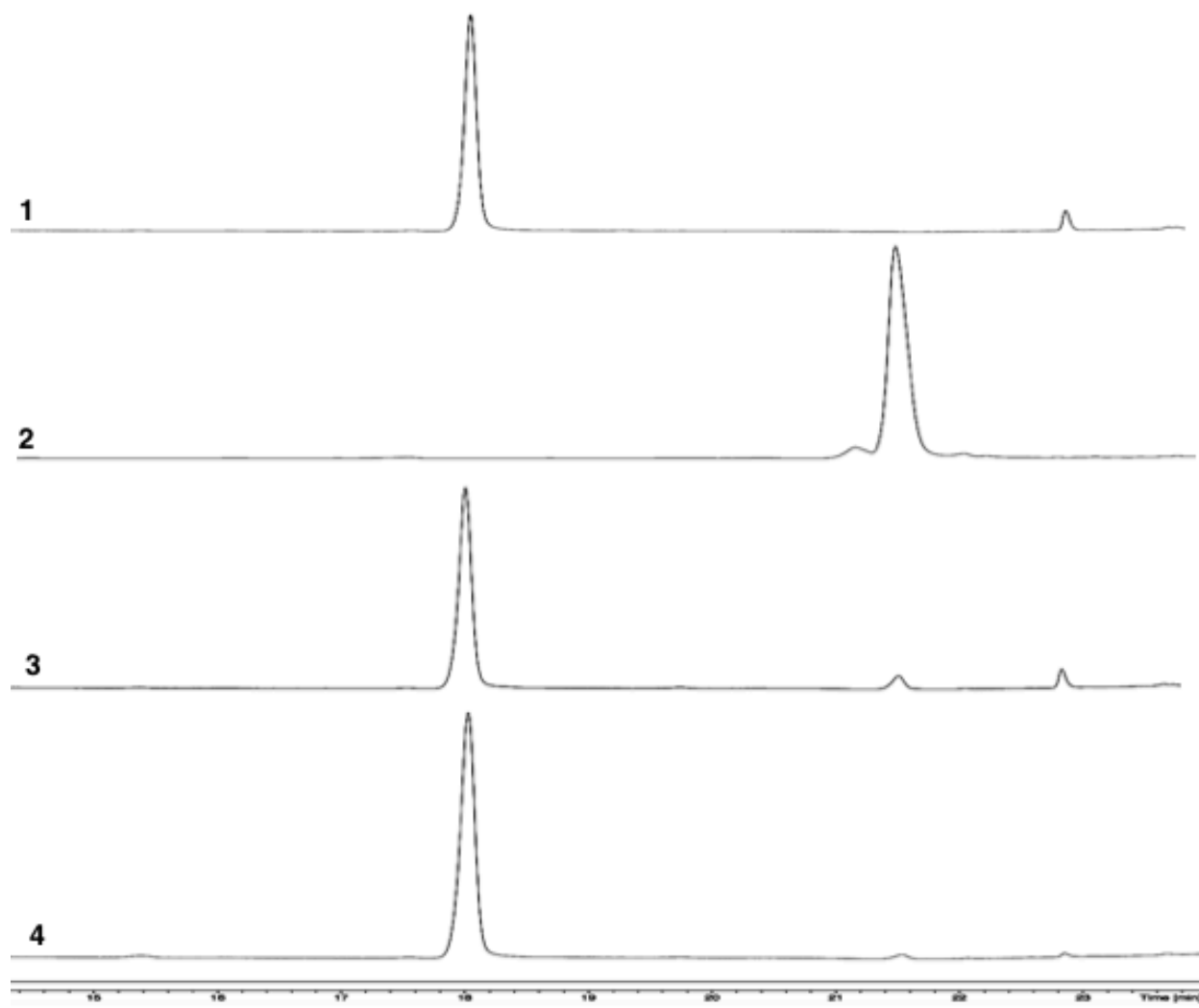


Figure 3. 5 HPLC traces of enzymatic assays of Zea and Rdc TEs with substrate 3.3.

1) substrate standard **3.3**. 2) macrocycle standard **3.13**. 3) 1.0 mM substrate, 25.0 μ M Rdc TE, 24 h. 4) 1.0 mM standard. 25.0 μ M Zea TE, 24 h.

Gly-15-membered (**3.4**)

To confirm that the lack of activity with compound **3.3** was due to the carbon spacer between the amide and thioester, and not due to the increased length of the linear substrate from 14 to 15, substrate **3.4** was assayed. It is clearly seen with both TEs that there is no starting material left indicating 15-membered substrates can be efficiently processed by the thioesterase (Fig. 3.6).

Although with the 15-membered substrate we do see a higher percentage of hydrolysis to macrocyclization with both Rdc and Zea TEs, the complete consumption of substrate supports that the β -alanine insertion in substrate **3.3** is responsible for abolished activity rather than the different ring size of the product.

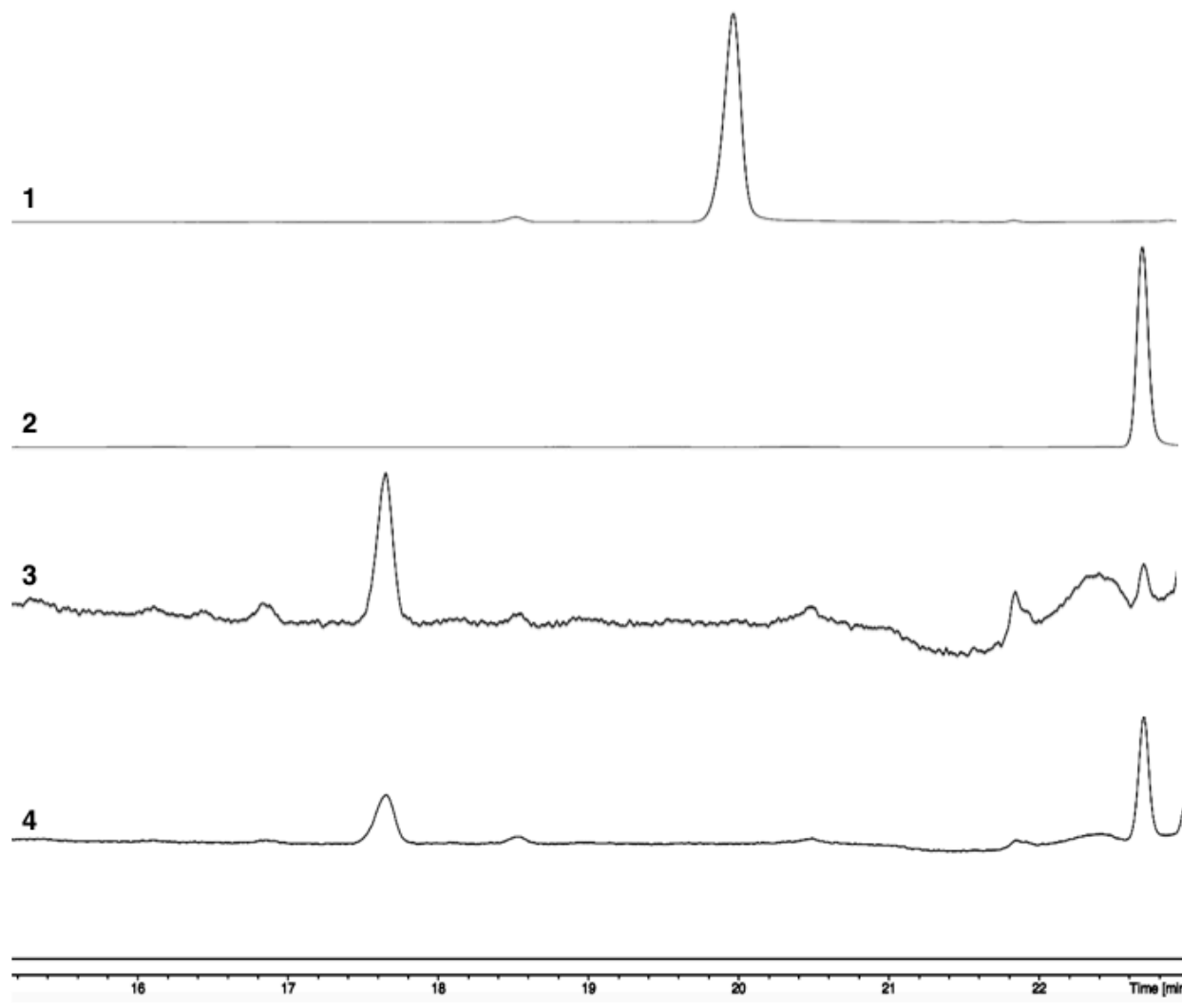


Figure 3. 6 HPLC traces of enzymatic assays of Zea and Rdc TEs with substrate 3.4.

1) substrate standard **3.4**. 2) macrocycle standard **3.14**. 3) 1.0 mM substrate, 25.0 μ M Rdc TE, 24 h. 4) 1.0 mM standard. 25.0 μ M Zea TE, 24 h. (Peak at 17.7 is hydrolysis product)

Gly-N-Me (**3.5**)

Substrate **3.5** is analogous to **2.12** (14-membered glycine insertion) but contains a methylated amide. Since we know that the functionality of the amide in the substrates is crucial for activity, we wanted to see if alkylating the amide changes enzyme binding.

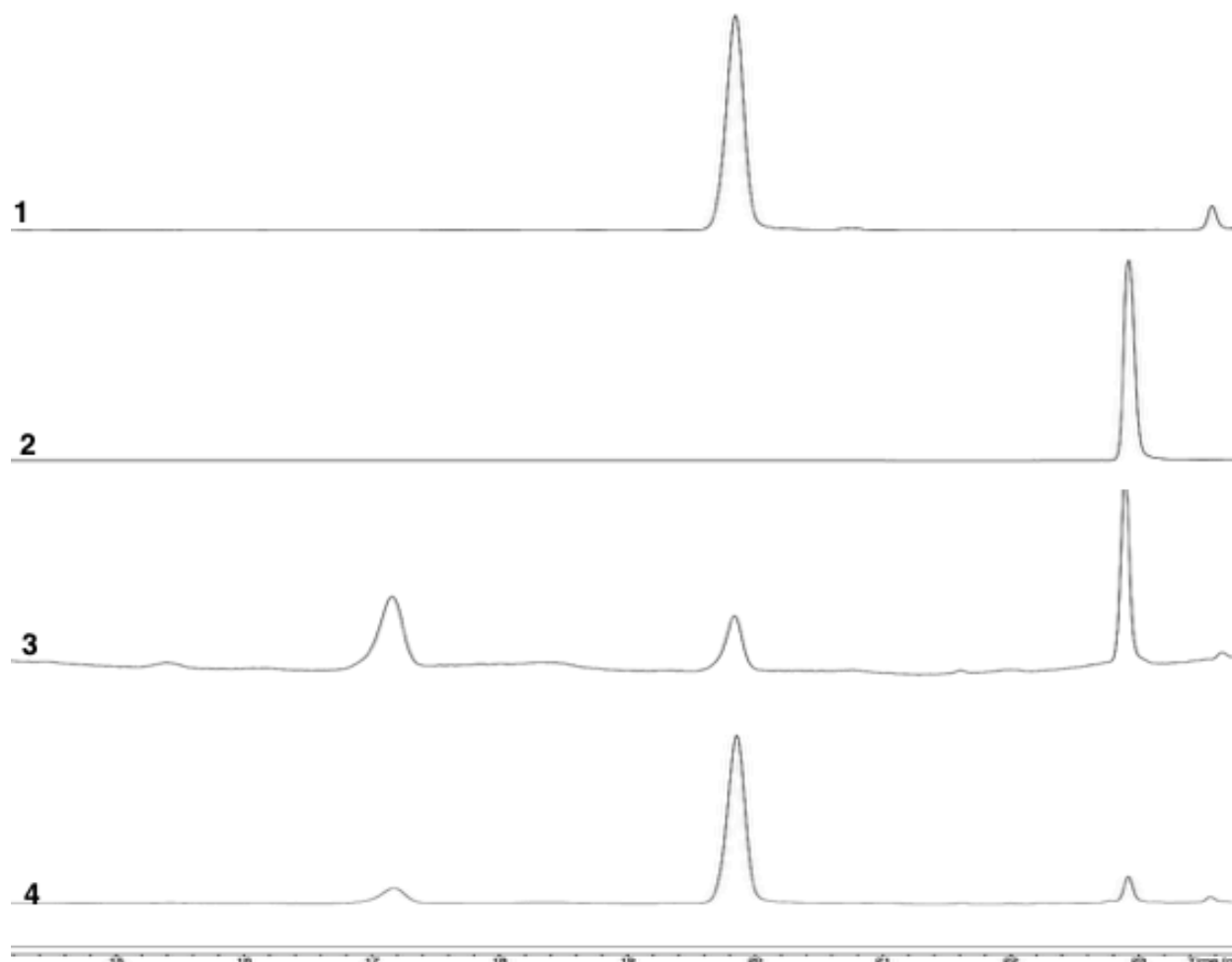


Figure 3.7 HPLC traces of enzymatic assays of Zea and Rdc TEs with substrate **3.5**.

1) substrate standard **3.5**. 2) macrocycle standard **3.21**. 3) 1.0 mM substrate, 25.0 μ M Rdc TE, 24 h. 4) 1.0 mM standard. 25.0 μ M Zea TE, 24 h. (Peak at 17.1 is hydrolysis product)

The HPLC trace for the Rdc assay (Fig. 3.7) is very similar to that observed for the non-methylated glycine substrate (**2.12**, figure **2.15**). Loading of the substrate is impeded considering the depsipeptide assays were run with 25 μ M enzyme oppose to 5 μ M used for compound **2.12**. That being said a very similar product distribution is observed meaning the methylated amide likely

affects the rate of loading of the Rdc TE but does not impact the release step compared to a non-methylated amide.

The assay run with the Zea TE shows a significant decrease in substrate consumption. Less than 20% of the starting material was loaded onto the TE compared to compound **2.12**, which showed almost 100% substrate consumption. It is interesting that when the α -carbon to the thioester is methylated as is with substrate **3.1**, Zea had moderate loading capacity but when the methylation is moved down one more atom almost complete loss of activity is observed. This could mean the amide proton is stabilizing the substrate in the binding pocket via an H-bond, but it could also be a result of the changed geometry of the amide bond in the binding pocket.

Glycine-glycine (3.6)

The final depsipeptide substrate tested contained a dipeptide (glycine-glycine) insertion adjacent to the SNAC thioester. This was done simply to test if higher order peptides are suitable for these two thioesterases. Neither TE showed any significant amount of macrocycle or hydrolysis formed (Fig. 3.8). The added functionality of a second amide further down the substrate likely affected the TE loading. Both native substrates modified by these two TEs have very limited functionality in their carbon chain so it is plausible that this amide is hindering access to the binding pocket. Since this substrate forms a 17-membered ring, activity loss may be attributed to ring size, but previous research done by the Boddy lab has shown that even 18-membered rings can be efficiently cyclized.¹⁰ On the upside there are trace amounts of macrocycle observed for both TEs. Control experiments run in the absence of enzyme indicate that the trace macrocycle is due to TE activity, meaning dipeptides can in fact be cyclized with these two thioesterases although at low efficiencies.

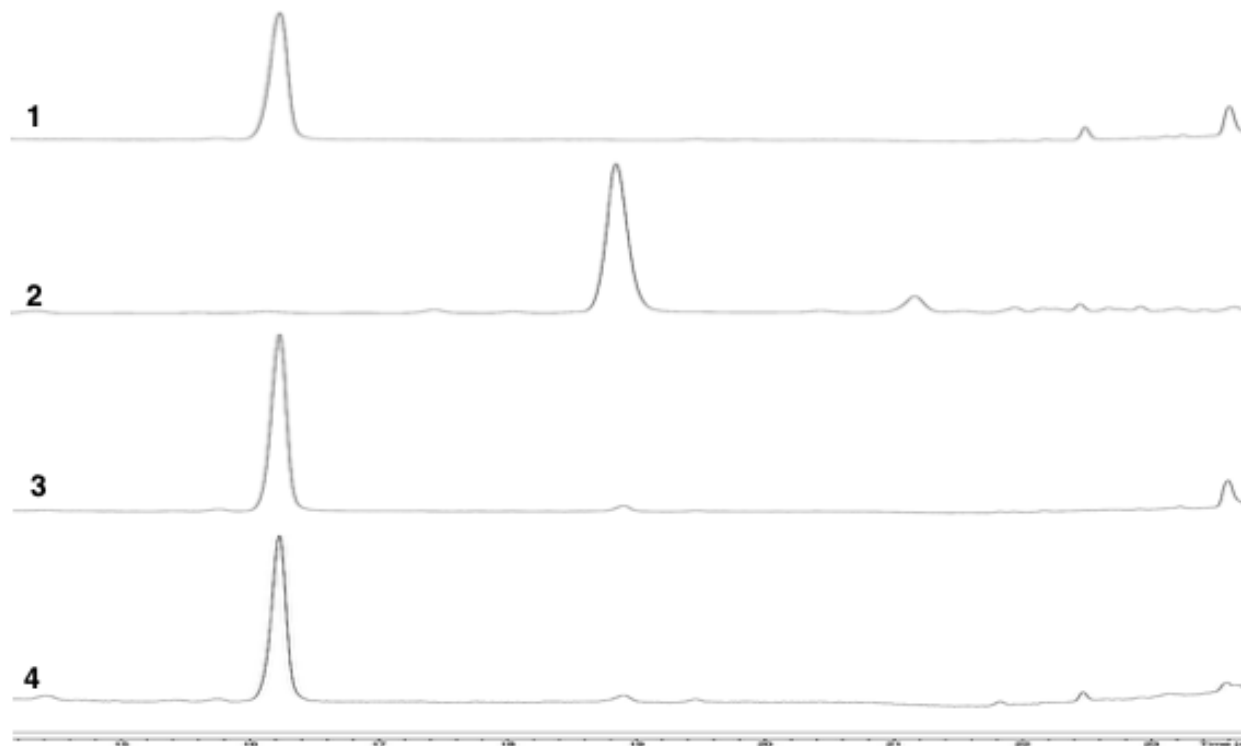


Figure 3. 8 HPLC traces of enzymatic assays of Zea and Rdc TEs with substrate 3.6.

1) substrate standard **3.6**. 2) macrocycle standard **3.24**. 3) 1.0 mM substrate, 25.0 μ M Rdc TE, 24 h. 4) 1.0 mM standard. 25.0 μ M Zea TE, 24 h.

3.2.5 Relative velocities of depsipeptide substrates.

The enzymatic assays run for the depsipeptide substrates showed substrate consumption at a fixed time and enzyme concentration and corresponding product distribution but did not provide any kinetic information. To provide some kinetic characterization on all the substrates with both TEs, relative velocities were recorded. Relative velocities were recorded by comparing initial velocities of thioesterase loading using an Ellman's assay. Parameters used were analogous to those in chapter 2.

Substrate	Zea TE v_{rel}	Rdc TE v_{rel}
14-membered 2.1	0.100 ± 0.03	0.66 ± 0.02
Glycine 2.12	0.39 ± 0.03	0.65 ± 0.09
D-alanine 3.1	0.04 ± 0.02	0.07 ± 0.02
L-alanine 3.2	0.10 ± 0.03	0.06 ± 0.01
β -alanine 3.3	0.02 ± 0.01	0.03 ± 0.02

Glycine-15-membered 3.4	0.17 ± 0.03	0.12 ± 0.04
Gly-N-Me 3.5	0.04 ± 0.02	0.13 ± 0.03
Glycine-glycine 3.6	N/A	0.03 ± 0.01

Table 3. 1 Relative velocities of depsipeptide substrates.

The relative velocities fit accordingly with the HPLC traces in terms of substrate that was loaded onto the two thioesterases. Radicol tends to show slightly more efficient substrate acylation, and the general trend of the substrate tolerance is followed. A higher velocity was recorded for substrate **3.4**, and then as the amino acids are manipulated the loading efficiency lowers.

3.3 Conclusions

In conclusion the substrate scope of both Rdc and Zea TEs has been further expanded on to include a variety of depsipeptides. Both TEs were able to cyclize amino acid containing substrates with both (*R*) and (*S*) configurations. The D-alanine substrate **3.1** saw more activity with both TE's specifically with the loading step. With respect to product distribution the configuration of the amino acid does not seem to play a part. It is clear that amide methylation is tolerable with Rdc but not so much with Zea, but in both cases macrocycle was formed concluding that amide H-bonding and configuration is not a necessity for macrocyclization. The two compounds showing negligible TE loading define two key limitations of the native Rdc and Zea TE's. The logic gate of loading requires functionality around the α - β carbons to the SNAC thioester (sub. **2.1**, **2.12**). Native-like phenyl rings and β -amide insertions show high catalytic activity but a one carbon shift of the amide (sub. **3.3**), or complete removal of any functionality (sub. **2.2**, **2.7**) abolishes any activity. Lastly, the lack of activity with the dipeptide brings about the conclusion that functionality further down the carbon chain (eg. glycine amide, sub. **3.6**) greatly affects the ability of these thioesterases to load the desired substrate. Although the product distribution of the Rdc TE shows more hydrolysis compared to the Zea TE, the lack of catalytic loading with Zea makes Rdc the more practical candidate as a biocatalyst.

This chapter illustrates the diversity of these fungal polyketide thioesterases. Their ability to macrocyclize non-native substrates with amino acids incorporated into their linear structure makes these TEs the most substrate tolerant PKS thioesterases to date, making them ideal candidates for biocatalysts.

3.4 Experimental

3.4.1 General Synthetic Methods

All reagents were purchased from Sigma-Aldrich or Oakwood chemicals at the highest available purity and used without further purification. All solvents were purchased from Fisher Scientific. All reactions were conducted using dry solvents under an argon atmosphere unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) and carried out on 0.25 mm E. Merck silica gel plates using UV light (254 nm) as a visualizing agent and phosphomolybdic acid stain and heat as developing agent. NMR spectroscopy was performed with a Bruker Avance II, operating 400 MHz for ^1H spectra, and 100 MHz for ^{13}C spectra. All chemical shifts are reported in parts per million (δ), integration and coupling constants in Hz and corrected using the solvent residual peak as internal standard. High-resolution mass spectroscopy (HRMS) was conducted on a Micromass Q-TOF I for ESI measurements (John L. Holmes Mass Spectroscopy).

3.4.2 Synthetic Protocols

General Procedure 1 for *N*-acetyl cysteamine thioester coupling

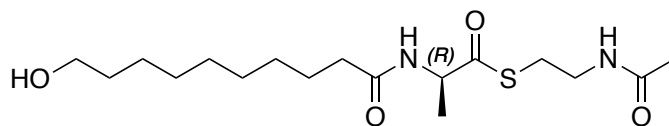
A carboxylic acid (1.0 equiv.) was dissolved in dry DCM under argon and cooled to 0 °C. EDC (1.5 equiv.) and DMAP (0.1 equiv.) were added and the mixture was stirred for 10 min at 0 °C. *N*-acetyl cysteamine (1.2 equiv.) was added, the mixture was stirred overnight at ambient

temperature. The solution was concentrated *in vacuo*, re-dissolved in EtOAc, and quenched with sat. NH₄Cl. The organic layer was separated, and the aqueous layer was extracted twice with EtOAc. The combined organic layers were washed with sat. NaHCO₃, brine, dried over MgSO₄, and concentrated. The crude product was purified by silica chromatography.

General Procedure 2 for amide coupling

An amine (1.2 equiv.) was suspended in dry DCM under argon and cooled to 0 °C. NEt₃ (3.0 equiv.), EDC (1.5 equiv.) and a carboxylic acid (1.0 equiv.) were added and the solution was stirred for 4 hours at ambient temperature. The solution was concentrated *in vacuo*, re-dissolved in EtOAc, and quenched with sat. NH₄Cl. The organic layer was separated, and the aqueous layer was extracted twice with EtOAc. The combined organic layers were washed with sat. NaHCO₃, brine, dried over MgSO₄, and concentrated. The crude product was purified by silica chromatography.

***S*-(2-acetamidoethyl) (*R*)-2-(10-hydroxydecanamido)propanethioate (3.1)**



835 mg of Boc-D-alanine (4.4 mmol, 1.0 equiv.) was dissolved in dry DCM under argon and cooled to 0 °C. 1.27 g of EDC (6.0 mmol, 1.5 equiv.) and 744 mg of HOBt (4.9 mmol, 1.2 equiv.) were added and the solution was stirred for 10 min at 0 °C. 550 mg of *N*-acetyl cysteamine (4.62 mmol, 1.1 equiv) was added and the solution was stirred at room temperature overnight. The mixture was concentrated *in vacuo*, re-dissolved in EtOAc, and quenched with the addition of sat. NH₄Cl. The aqueous layer was extracted with EtOAc, and the organic layers were washed with sat. NaHCO₃, brine, dried over MgSO₄ and concentrated. The crude product was purified by column chromatography (80:20 EtOAc/Hex) to afford 554 mg of **3.7** (45%) as a white solid. **3.7** was dissolved in dry DCM and cooled to 0 °C. 5 ml of 4.0 M HCl/dioxane was added and the solution was stirred at room temperature for 2 hours. The solvent was evaporated to afford the

crude amine HCl salt. General procedure 2 was then used to afford 100 mg of compound **3.1** (7% over 3 steps) as a white solid.

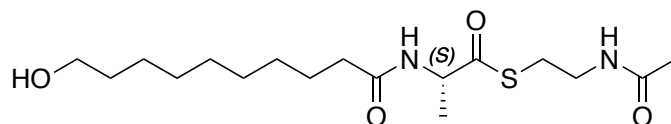
$R_f = 0.1$ (5:95 MeOH/DCM)

$^1\text{H NMR}$ (400 MHz, DMSO- d_6) δ 8.41 (d, $J = 7.2$ Hz, 1H), 7.98 (t, $J = 5.1$ Hz, 1H), 4.39 – 4.22 (m, 2H), 3.33 (dd, $J = 11.8, 6.5$ Hz, 2H), 3.08 (dd, $J = 12.8, 6.3$ Hz, 2H), 2.85 – 2.71 (m, 2H), 2.09 (td, $J = 7.7, 1.6$ Hz, 2H), 1.74 (s, 3H), 1.53 – 1.40 (m, 2H), 1.39 – 1.29 (m, 2H), 1.27 – 1.05 (m, $J = 7.0$ Hz, 13H).

$^{13}\text{C NMR}$ (100 MHz, MeOD) δ 201.31, 175.00, 172.01, 61.60, 55.17, 38.59, 35.35, 32.28, 29.22, 29.15, 28.98, 28.92, 27.48, 25.54, 25.31, 21.15, 16.38.

HRMS (ESI+) calc. for $\text{C}_{17}\text{H}_{32}\text{N}_2\text{O}_4\text{SNa}$ ($M+\text{Na}$)383.1980; obsd. 383.1981

***S*-(2-acetamidoethyl) (*S*)-2-(10-hydroxydecanamido)propanethioate (**3.2**)**



The same procedure used for **3.1** was followed starting with 420 mg Boc-L-alanine (2.2 mmol). 50 mg of compound **3.2** (6.5% over 3 steps) was isolated as a white solid.

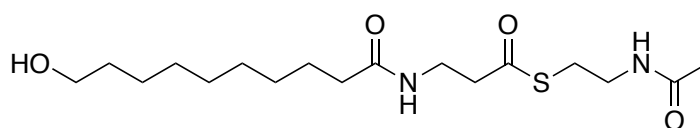
$R_f = 0.1$ (5:95 MeOH/DCM)

$^1\text{H NMR}$ (400 MHz, DMSO- d_6) δ 8.40 (d, $J = 7.2$ Hz, 1H), 7.97 (t, $J = 5.8$ Hz, 1H), 4.35 – 4.25 (m, 2H), 3.33 (dd, $J = 11.7, 6.5$ Hz, 2H), 3.08 (dd, $J = 13.4, 7.0$ Hz, 2H), 2.84 – 2.76 (m, 2H), 2.09 (td, $J = 7.5, 1.8$ Hz, 2H), 1.74 (s, 3H), 1.51 – 1.43 (m, 2H), 1.39 – 1.32 (m, 2H), 1.27 – 1.16 (m, $J = 7.2$ Hz, 13H).

^{13}C NMR (100 MHz, CD_3OD) δ 201.31, 175.02, 172.02, 61.60, 55.16, 38.58, 35.34, 32.26, 29.19, 29.13, 28.96, 28.90, 27.46, 25.52, 25.29, 21.10, 16.34.

HRMS (ESI+) calc. for $\text{C}_{17}\text{H}_{32}\text{N}_2\text{O}_4\text{SNa}$ ($\text{M}+\text{Na}$)383.1980; obsd. 383.1982

S-(2-acetamidoethyl) 3-(10-hydroxydecanamido)propanethioate (3.3)



239 mg of β -alanine ethyl ester (1.56 mmol, 1.4 equiv.) was suspended in dry DCM under argon and cooled to 0 °C. 470 μL NEt_3 (3.38 mmol, 3.0 equiv.), 320 mg EDC (1.68 mmol, 1.5 equiv.), and 200 mg **2.14** (1.06 mmol, 1.0 equiv.) was added and the solution was stirred for 3 hours at room temperature. The solution was concentrated and quenched with sat. NH_4Cl . The aqueous layer was extracted with EtOAc, washed with NaHCO_3 , brine, dried over MgSO_4 and concentrated. The crude product was purified by column chromatography (50:50 EtOAc/Hex) to yield 103 mg of compound **3.11** (37%) as a white powder. **3.11** was then dissolved in THF/MeOH/ H_2O (3:3:5) and heated to 50 °C. 202 mg of LiOH (3.59 mmol, 10 equiv.) was added and the mixture was allowed to stir for 3 h. Upon completion 1.0 M HCl was added and the mixture was extracted with EtOAc, washed with brine, dried over MgSO_4 , and concentrated. The crude *seco*-acid was then thioesterified following general procedure **1** to yield 23 mg of compound **3.3** (5% over 3 steps) as a white solid.

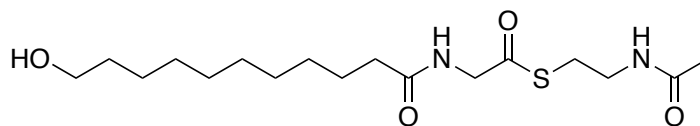
R_f = 0.15 (7:93 MeOH/DCM)

^1H NMR (400 MHz, CD_3OD) δ 3.53 (t, J = 6.6 Hz, 2H), 3.44 (t, J = 6.5 Hz, 2H), 3.32 (t, J = 4.4 Hz, 8H), 3.01 (t, J = 6.7 Hz, 2H), 2.78 (t, J = 6.5 Hz, 2H), 2.15 (t, J = 7.5 Hz, 2H), 1.92 (s, J = 7.7 Hz, 3H), 1.62 – 1.45 (m, 4H), 1.39 – 1.23 (m, 11H).

^{13}C NMR (100 MHz, DMSO) δ 196.95, 172.31, 169.31, 60.72, 48.61, 38.12, 35.29, 34.97, 32.56, 29.00, 28.94, 28.77, 28.61, 28.13, 25.50, 25.23, 22.53.

HRMS (ESI+) calc. for $\text{C}_{17}\text{H}_{32}\text{N}_2\text{O}_4\text{SNa}$ (M+Na)383.1980; obsd. 383.1969

S-(2-acetamidoethyl) 2-(11-hydroxyundecanamido)ethanethioate (3.4)



154 mg of glycine ethyl ester (1.2 mmol, 1.2 equiv.) was suspended in dry DCM under argon and cooled to 0 °C. 320 μL NEt_3 (3.0 mmol, 3.0 equiv.), 233 mg EDC (1.4 mmol, 1.4 equiv.) and 145 mg **3.16** (1.0 mmol, 1.0 equiv.) was added and the solution was stirred at room temperature for 3.5 h. The solution was concentrated, re-dissolved in EtOAc, and quenched with sat. NH_4Cl . The aqueous layer was extracted with EtOAc, washed with sat. NaHCO_3 , brine, dried over MgSO_4 , and concentrated. The crude solid was purified by column chromatography (50:50 EtOAc/Hex) to afford 120 mg of **3.15** (40%) as a white solid. **3.15** was hydrolyzed with 200 mg LiOH (4.76 mmol, 10 equiv.) in THF/MeOH/ H_2O (3:3:5) by stirring at 50 °C for 4 hours. The reaction was acidified with 1.0 M HCl, extracted with EtOAc, washed with brine, dried over MgSO_4 , and concentrated. The resulting *seco*-acid was thioesterified using general procedure 1 to afford 50 mg of compound **3.4** (13% over 3 steps) as a white powder.

R_f = 0.19 (8:92 MeOH/DCM)

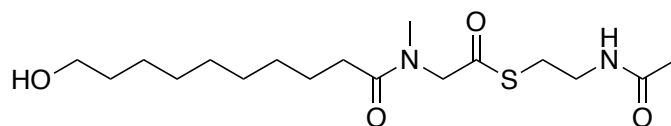
^1H NMR (400 MHz, DMSO- d_6) δ 8.46 (t, J = 6.1 Hz, 1H), 8.00 (t, J = 5.5 Hz, 1H), 4.28 (t, J =

5.2 Hz, 1H), 3.92 (d, $J = 6.0$ Hz, 2H), 3.37 – 3.27 (m, 2H), 3.09 (dd, $J = 12.8, 6.9$ Hz, 2H), 2.83 (t, $J = 7.0$ Hz, 2H), 2.10 (t, $J = 7.4$ Hz, 2H), 1.74 (s, $J = 8.5$ Hz, 3H), 1.51 – 1.43 (m, 2H), 1.39 – 1.31 (m, 2H), 1.26 – 1.14 (m, 12H).

^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ 198.95, 173.42, 169.70, 61.19, 49.27, 38.62, 35.53, 33.02, 29.54, 29.42, 29.38, 29.23, 29.09, 27.90, 25.98, 25.50, 22.97.

HRMS (ESI+) calc. for $\text{C}_{17}\text{H}_{32}\text{N}_2\text{O}_4\text{SNa}$ ($\text{M}+\text{Na}$)383.1980; obsd. 383.1985

***S*-(2-acetamidoethyl) 2-(10-hydroxy-*N*-methyldecanamido)ethanethioate (3.5)**



266 mg glycine ethyl ester HCl salt (1.9 mmol, 1.3 equiv.) was suspended in dry DCM and cooled to 0 °C under argon. 600 μL NEt_3 (4.3 mmol, 3.0 equiv.), 397 mg EDC (2.07 mmol, 1.5 equiv.), and 450 mg **3.18** (1.5 mmol, 1.0 equiv.) were added, the solution was stirred for 4 hours at room temperature. The solution was concentrated, re-suspended in EtOAc and quenched with NH_4Cl . The aqueous layer was extracted with Et_2O , washed with NaHCO_3 , brine, dried over MgSO_4 , and concentrated. The crude product was dissolved in dry THF under N_2 and cooled to 0 °C. 110 mg NaH (2.75 mmol, 2.0 equiv.) was added and the solution was stirred for 1 h. 230 μL MeI (3.69 mmol, 3.0 equiv.) was added and the solution stirred overnight. Water was added, and the solution was extracted with EtOAc, washed with brine and dried over MgSO_4 to yield 150 mg **3.19** (33% over 2 steps) as a colourless oil. **3.19** was hydrolyzed using LiOH in $\text{MeOH}/\text{H}_2\text{O}$. General procedure **1** was used to form the *N*-acetyl cysteamine thioester which was subjected to aqueous HF for 30 min to yield to remove the TBS protecting group. The crude product was purified using column chromatography (6:94 MeOH/DCM) to yield 25 mg of pure **3.5** (8% over 4 steps) as a colourless oil.

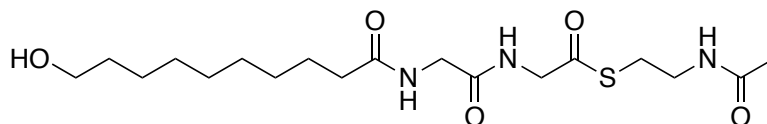
$R_f = (6:94 \text{ MeOH/DCM})$

$^1\text{H NMR (600 MHz, DMSO-}d_6)$ (major rotamer) δ 8.05 (s, 1H), 4.33 (td, $J = 5.2, 2.2$ Hz, 1H), 4.23 (d, $J = 10.3$ Hz, 2H), 3.39 – 3.35 (m, 2H), 3.15 (dd, $J = 13.3, 6.4$ Hz, 2H), 3.05 (s, 3H), 2.90 (t, $J = 7.0$ Hz, 2H), 2.35 (t, $J = 7.4$ Hz, 2H), 1.79 (s, $J = 1.4$ Hz, 3H), 1.53 – 1.43 (m, 2H), 1.42 – 1.36 (m, 2H), 1.32 – 1.19 (m, 10H). (minor rotamer) δ 8.05 (s, 1H), 4.39 (d, $J = 12.1$ Hz, 2H), 4.33 (td, $J = 5.2, 2.2$ Hz, 1H), 3.39 – 3.35 (m, 2H), 3.19 (dd, $J = 12.8, 6.6$ Hz, 2H), 2.95 (t, $J = 6.8$ Hz, 2H), 2.85 (s, 3H), 2.21 (t, $J = 7.4$ Hz, 2H), 1.79 (s, 3H), 1.53 – 1.43 (m, 2H), 1.42 – 1.36 (m, 2H), 1.32 – 1.18 (m, 10H).

$^{13}\text{C NMR (150 MHz, DMSO-}d_6)$ (major rotamer) δ 197.50, 173.49, 169.70, 61.28, 57.72, 38.70, 36.97, 33.01, 32.67, 29.41, 29.34, 29.24, 29.13, 28.05, 25.95, 24.85, 22.93. (minor rotamer) δ 198.11, 173.28, 169.70, 61.28, 59.56, 38.50, 36.97, 33.01, 32.41, 29.41, 29.34, 29.24, 29.13, 28.39, 25.95, 25.04, 22.93.

HRMS (ESI+) calc. for $\text{C}_{17}\text{H}_{32}\text{N}_2\text{O}_4\text{SNa}$ ($\text{M}+\text{Na}$) 383.1980; obsd. 383.1965

S-(2-acetamidoethyl) 2-(2-(10-hydroxydecanamido)acetamido)ethanethioate (3.6)



612 mg of dipeptide glycine-glycine benzyl ester (1.55 mmol, 1.2 equiv.) was suspended in dry DCM under argon and cooled to 0 °C. 470 μL NEt_3 (3.38 mmol, 3.0 equiv.), 319 mg EDC (1.67 mmol, 1.5 equiv.), and 193 mg **3.19** (1.03 mmol, 1.0 equiv.) were added and the solution was stirred for 3.5 hours. The mixture was concentrated, re-dissolved in EtOAc and quenched with sat. NH_4Cl . The aqueous layer was extracted with EtOAc and the combined organic layers were

washed with sat. NaHCO_3 , brine, dried over MgSO_4 and concentrated. The crude solid was purified by column chromatography (50:50 EtOAc/Hex) to yield 663 mg of **3.22** (75%) as a white solid. **3.22** was dissolved in 20 ml (10:10 THF/MeOH), 200 mg Pd/C, was added and the solution was stirred at room temperature, overnight under an H_2 atmosphere. The solution was filtered through celite and concentrated. The crude product was thioesterified following general procedure **1**, the product was subsequently dissolved in 7 mL of acetonitrile in a 15 mL falcon tube. 300 μL of HF (28.0 M) was added and the solution was stirred for 20 min, filtered and dried to afford 40 mg **3.6** (15% over 4 steps) as a white solid.

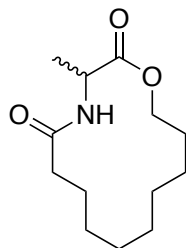
$R_f = 0.25$ (10:90 MeOH/DCM)

$^1\text{H NMR}$ (400 MHz, DMSO-d_6) δ 8.46 (t, $J = 6.0$ Hz, 1H), 8.08 – 7.96 (m, 2H), 4.28 (t, $J = 5.2$ Hz, 1H), 3.96 (d, $J = 6.0$ Hz, 2H), 3.69 (d, $J = 5.8$ Hz, 2H), 3.36 – 3.31 (m, 2H), 3.10 (dd, $J = 13.2, 6.3$ Hz, 2H), 2.84 (t, $J = 7.0$ Hz, 2H), 2.08 (t, $J = 7.5$ Hz, 2H), 1.75 (s, 3H), 1.50 – 1.40 (m, 2H), 1.40 – 1.31 (m, 2H), 1.26 – 1.14 (m, 10H).

$^{13}\text{C NMR}$ (100 MHz, DMSO-d_6) δ 198.42, 173.08, 170.44, 169.74, 61.19, 49.26, 42.21, 38.62, 35.63, 33.01, 29.45, 29.40, 29.26, 29.16, 27.95, 25.96, 25.55, 22.98.

HRMS (ESI+) calc. for $\text{C}_{18}\text{H}_{33}\text{N}_3\text{O}_5\text{SNa}$ ($\text{M}+\text{Na}$) 426.2039; obsd. 426.2040

3-methyl-1-oxa-4-azacyclotetradecane-2,5-dione (**3.10**)



240 mg of alanine ethyl ester HCl salt (1.57 mmol, 1.4 equiv.) was suspended in dry DCM at 0 °C. 320 mg EDC (1.68 mmol, 1.5 equiv.), 470 μ L NEt₃ (3.38 mmol, 3.0 equiv.), and 200 mg of **2.14** (1.06 mmol, 1.0 equiv.) was added and the solution was stirred for 3 h at room temperature. The solution was evaporated, re-suspended in EtOAc, quenched with NH₄Cl, extracted with EtOAc, washed with NaHCO₃, Brine, dried over MgSO₄, and concentrated. The crude product was dissolved in THF/MeOH/H₂O (3:3:5). 200 mg LiOH (4.81 mmol, 10.0 equiv.) was added and the solution was stirred for 1 hour at 50 °C. The solution was acidified with 1.0 M HCl, extracted with EtOAc, washed with brine, dried over MgSO₄ and concentrated to yield 103 mg of **3.9** (33%) as a white solid. 46 mg of **3.9** (0.17 mmol, 1.0 equiv.) was dissolved in 8 mL of dry DMF. 672 mg of DMAP (5.51 mmol, 30 equiv.) and 420 mg PyBOP (0.81 mmol, 4 equiv.) was dissolved in 45 mL of dry DCM under Argon. The solution of **3.9** was added to the DMAP/PyBOP solution over 12 h using a syringe pump (0.004 M of **3.9**). The solution was stirred an additional 2 h. The solution was concentrated re-dissolved in EtOAc, washed with NH₄Cl twice, brine 3 times, dried over MgSO₄ and concentrated. The crude product was purified by column chromatography (40:60 EtOAc/Hex) to yield 25 mg of **3.10** (64%) as a white solid.

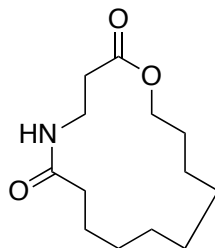
R_f = 0.19 (40:60 EtOAc/hexanes)

¹H NMR (400 MHz, CDCl₃) δ 5.69 (d, *J* = 6.5 Hz, 1H), 4.74 (dq, *J* = 8.5, 7.3 Hz, 1H), 4.16 (t, *J* = 6.6 Hz 2H), 2.34 (ddd, *J* = 14.5, 6.9, 3.5 Hz, 1H), 2.12 (ddd, *J* = 14.3, 10.9, 3.1 Hz, 1H), 1.95 – 1.80 (m, 1H), 1.73 – 1.54 (m, 2H), 1.53 – 1.18 (m, 13H).

¹³C NMR (100 MHz, CDCl₃) δ 172.22, 172.18, 77.33, 77.01, 76.69, 64.47, 48.05, 35.52, 27.05, 26.02, 25.97, 25.80, 25.17, 24.83, 23.11, 18.08.

HRMS (ESI+) calc. for C₁₃H₂₃NO₃Na (M+Na)264.1576; obsd. 264.1583

1-oxa-5-azacyclopentadecane-2,6-dione (3.13)



500 mg of β -alanine ethyl ester HCl salt (3.26 mmol, 1.2 equiv.) was suspended in dry DCM at 0 °C. 826 mg EDC (3.91 mmol, 1.5 equiv.), 1.1 ml NEt_3 (7.82 mmol, 3.0 equiv.), and 680 mg of 10-bromodecanoic acid (2.67 mmol, 1.0 equiv.) was added and the solution was stirred for 3 h at room temperature. The solution was evaporated, re-suspended in EtOAc, quenched with NH_4Cl , extracted with EtOAc, washed with NaHCO_3 , Brine, dried over MgSO_4 , and concentrated. The crude product was dissolved in THF/MeOH/ H_2O (6:6:10). 400 mg LiOH (9.62 mmol, 10.0 equiv.) was added and the solution was stirred for 1 hour at 50 °C. The solution was acidified with 1.0 M HCl, extracted with EtOAc, washed with brine, dried over MgSO_4 and concentrated to yield 500 mg of **3.12** (40%) as a white solid. 50 mg **3.12** (0.17 mmol, 1.0 equiv.) and 60 mg CsCO_3 (0.19 mmol, 1.1 equiv.) was dissolved in 40 mL of dry DMF (0.004 M). The solution was stirred at 40 °C for 8 h. The solution was quenched by 50% sat. NaCl, extracted with Et_2O , washed with brine 3 times, dried over MgSO_4 , and concentrated to yield 30 mg of **3.13** (87%) as a white solid.

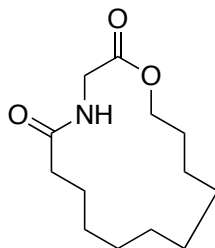
R_f = 0.13 (30:70 EtOAc/hexanes)

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ 5.96 (s, 1H), 4.27 – 4.18 (m, 2H), 3.48 (dd, J = 11.0, 6.0 Hz, 2H), 2.64 – 2.55 (m, 2H), 2.18 – 2.09 (m, 2H), 1.67 – 1.56 (m, 4H), 1.40 – 1.32 (m, 2H), 1.31 – 1.24 (m, 6H), 1.22 – 1.14 (m, 2H).

$^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 173.55, 172.89, 63.27, 36.48, 35.35, 33.90, 28.14, 27.45, 26.98, 26.55, 25.87, 25.63, 23.91.

HRMS (ESI+) calc. for C₁₃H₂₃NO₃Na (M+Na)264.1576; obsd. 264.1565

1-oxa-4-azacyclopentadecane-2,5-dione (3.14)



154 mg of glycine ethyl ester HCl salt (1.1 mmol, 1.2 equiv.) was suspended in dry DCM at 0 °C. 233 mg EDC (1.22 mmol, 1.5 equiv.), 320 μ l NEt₃ (2.31 mmol, 3.0 equiv.), and 145 mg of **3.16** (1.1 mmol, 1.0 equiv.) was added and the solution was stirred for 3 h at room temperature. The solution was evaporated, re-suspended in EtOAc, quenched with NH₄Cl, extracted with EtOAc, washed with NaHCO₃, Brine, dried over MgSO₄, and concentrated. The crude product was dissolved in THF/MeOH/H₂O (2:2:4). 200 mg LiOH (4.81 mmol, 10.0 equiv.) was added and the solution was stirred for 1 hour at 50 °C. The solution was acidified with 1.0 M HCl, extracted with EtOAc, washed with brine, dried over MgSO₄ and concentrated to yield 84 mg of **3.17** (31%) as a white solid. 41 mg of **3.17** (0.19 mmol, 1.0 equiv.) was dissolved in 8 ml of dry DMF. 720 mg of DMAP (5.9 mmol, 30 equiv.) and 450 mg PyBOP (0.86 mmol, 4 equiv.) was dissolved in 45 mL of dry DCM under Argon. The solution of **3.17** was added to the DMAP/PyBOP solution over 12 h using a syringe pump (0.004 M of **3.17**). The solution was stirred an additional 2 h. The solution was concentrated re-dissolved in EtOAc, washed with NH₄Cl twice, brine 3 times, dried over MgSO₄ and concentrated. The crude product was purified by column chromatography (40:60 EtOAc/Hex) to yield 20 mg of **3.14** (56%) as a white solid.

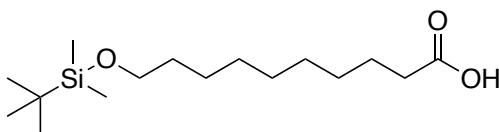
R_f = 0.22 (40:60 EtOAc/hexanes)

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ 5.93 (s, 1H), 4.20 – 4.13 (m, 2H), 4.07 (d, $J = 6.0$ Hz, 2H), 2.28 – 2.21 (m, 2H), 1.75 – 1.59 (m, 4H), 1.40 – 1.24 (m, 12H).

$^{13}\text{C NMR}$ (100 MHz, CDCl_3) δ 173.04, 169.39, 65.78, 41.94, 35.40, 27.66, 27.56, 26.72, 26.61, 26.56, 26.08, 25.20, 24.31.

HRMS (ESI+) calc. for $\text{C}_{13}\text{H}_{23}\text{NO}_3\text{Na}$ ($\text{M}+\text{Na}$)264.1576; obsd. 264.1581

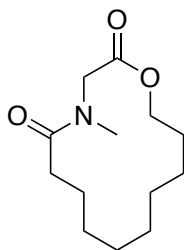
10-((*tert*-butyldimethylsilyl)oxy)decanoic acid (**3.18**)



1.6 g of methyl 10-hydroxydecanoate (8.0 mmol, 1.0 equiv.) was dissolved in dry DMF along with 661 mg imidazole (9.72 mmol, 1.2 equiv.), and 55 mg of DMAP (0.45 mmol, 0.05 equiv.). The solution was cooled to 0 °C, 1.58 g TBSCl (10.49 mmol, 1.3 equiv.) was added and the solution was stirred at room temperature for 2 h. The solution was quenched with sat. NH_4Cl , extracted with Et_2O , washed with brine, dried over MgSO_4 , and concentrated. The crude product was dissolved in a mixture of THF/MeOH/ H_2O (3:3:5), 3.0 g of LiOH (71.5 mmol, 10 equiv.) was added and the solution stirred for 3 h at 50 °C. The mixture was acidified with 1.0 M HCl, extracted with EtOAc, washed with brine, dried over MgSO_4 and concentrated to give 680 mg of compound **3.18** (70% over 2 steps) as a colorless oil. Characterization is consistent with previously reported data.¹¹

¹H NMR (400 MHz, CDCl₃) ¹H NMR (400 MHz, CDCl₃) δ 3.57 (t, *J* = 6.6 Hz, 2H), 2.33 (t, *J* = 7.5 Hz, 2H), 1.66 – 1.53 (m, 2H), 1.54 – 1.42 (m, 2H), 1.36 – 1.24 (m, 10H), 0.87 (s, 9H), 0.03 (s, 6H).

4-methyl-1-oxa-4-azacyclotetradecane-2,5-dione (3.21)



197 mg of sarcosine HCl salt (1.41 mmol, 1.2 equiv.) was dissolved in DCM and cooled to 0 °C. 302 mg EDC (1.58 mmol, 1.3 equiv.), 500 μL NEt₃ (3.59 mmol, 3.0 equiv.), and 300 mg of 10-bromodecanoic acid (1.2 mmol, 1.0 equiv.) was added and the solution was stirred for 3 h. The solution was evaporated, re-dissolved in EtOAc, and quenched with NH₄Cl. The aqueous solution was extracted with EtOAc, washed with NaHCO₃ and brine, dried over MgSO₄ and concentrated. The crude solution was hydrolyzed using 60 mg LiOH (1.42 mmol, 10 equiv.) in a 50/50 MeOH/H₂O mixture. The product was dissolved in 30 mL of DMF, 40 mg CsCO₃ (0.12 mmol, 1.1 equiv.) were added and the solution was stirred overnight. The solution was quenched with brine, extracted with ether, dried over MgSO₄ and concentrated. The crude product was purified by column chromatography (20:80 EtOAc/Hex) to yield 17.5 mg of **3.21** (17% over 3 steps) as a white solid.

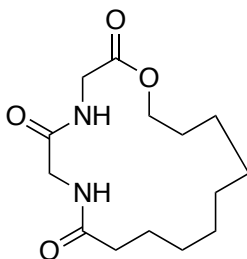
R_f = 0.17 (20:80 EtOAc/hexanes)

¹H NMR (400 MHz, CDCl₃) (minor rotamer) δ 4.27 – 4.22 (m, 2H), 4.05 (s, 2H), 3.00 (s, 3H), 2.24 (t, *J* = 7.1 Hz, 2H), 1.72 – 1.57 (m, 4H), 1.42 – 1.20 (m, 10H). (major rotamer) δ 4.19 – 4.08 (m, 4H), 3.10 (s, 3H), 2.41 (t, *J* = 7.1 Hz, 2H), 1.75 – 1.55 (m, 4H), 1.42 – 1.20 (m, 10H).

¹³C NMR (100 MHz, CDCl₃) (minor rotamer) δ 173.32, 169.41, 65.82, 52.33, 35.23, 31.24, 26.75, 26.51, 25.74, 25.01, 24.89, 23.30, 22.93. (major rotamer) δ 173.43, 169.55, 64.39, 50.68, 37.07, 30.67, 27.16, 25.86, 25.50, 25.39, 25.15, 24.26, 23.18.

HRMS (ESI+) calc. for C₁₃H₂₃NO₃Na (M+Na)264.1576; obsd. 264.1570.

1-oxa-4,7-diazacycloheptadecane-2,5,8-trione (3.24)



Macrocycle **3.24** was synthesized analogous to **3.13** starting with glycine-glycine benzyl ester as opposed to β-alanine ethyl ester.

R_f = 0.22 (20:80 EtOAc/hexanes)

¹H NMR (400 MHz, CDCl₃) δ 6.94 (s, 1H), 6.44 (s, 1H), 4.19 – 4.14 (m, 2H), 3.99 (dd, *J* = 9.0, 5.6 Hz, 4H), 2.29 – 2.24 (m, 2H), 1.73 – 1.66 (m, 2H), 1.62 – 1.57 (m, 2H), 1.31 – 1.24 (m, 10H).

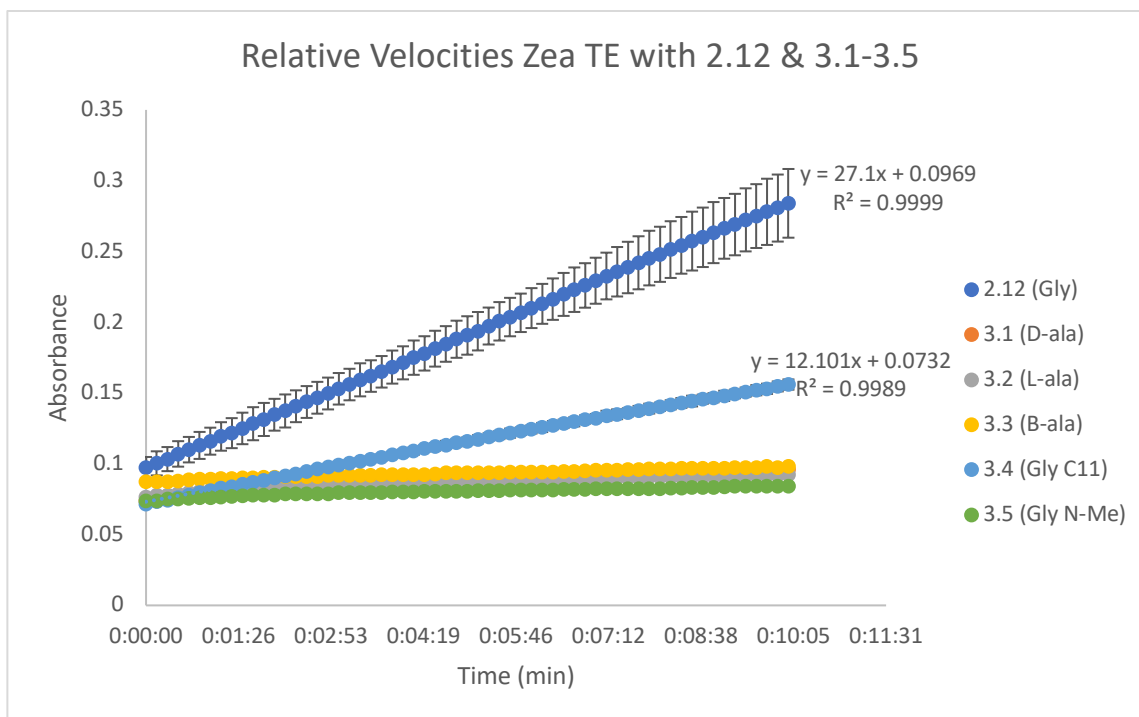
¹³C NMR (100 MHz, CDCl₃) δ 174.37, 169.69, 169.04, 65.42, 43.53, 42.09, 35.86, 28.79, 28.54, 27.87, 27.21, 26.97, 25.27, 25.12.

HRMS (ESI+) calc. for C₁₄H₂₄N₂O₄Na (M+Na)307.1634; obsd. 307.1623.

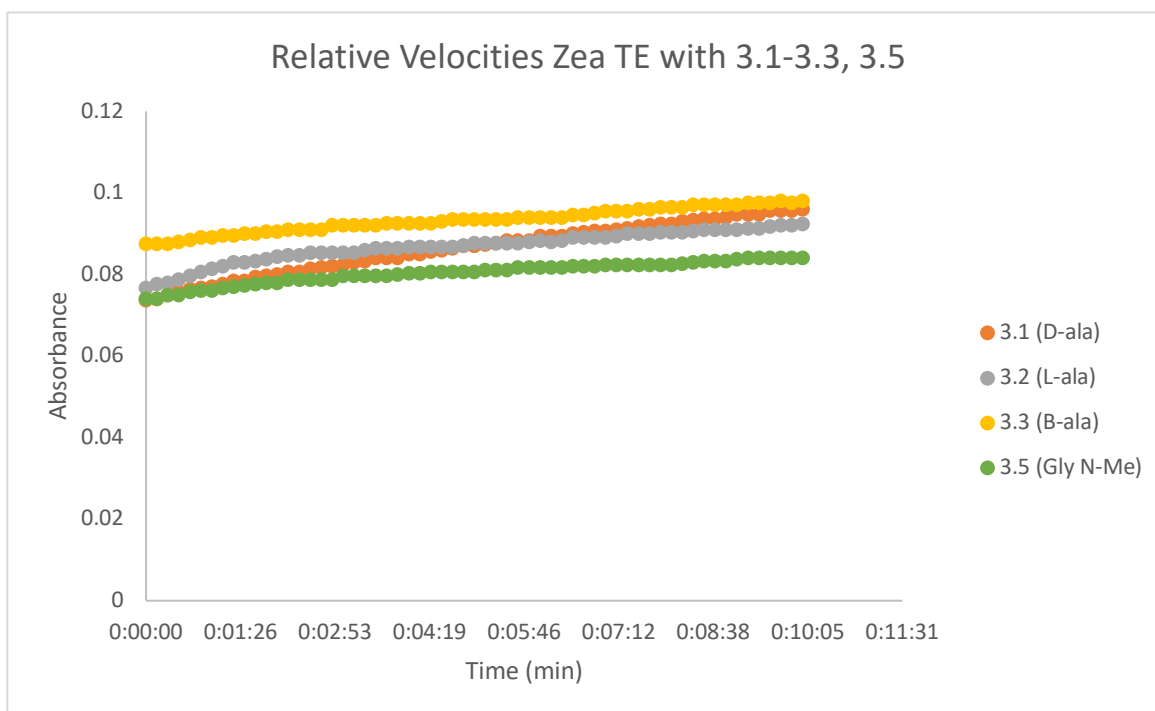
3.4.3 Enzymatic Protocols

Assays were run at a total volume of 50 μL containing 50 mM phosphate buffer (pH 7.4), 1.0 mM SNAC substrate, 5 – 25 μM enzyme (Rdc or Zea), and 10% v/v DMSO. All reactions were quenched with an equal volume of 0.5% formic acid in acetonitrile prior to HPLC analysis. HPLC analysis was conducted with an Agilent 1260 Modular system using a Dikima Leapsil 2.7 μm C18, 100x2.1 mm column. HPLC conditions: Flow rate 0.4 mL/min, 0 to 100% B over 30 min. (A: H₂O B: ACN). Kinetic activity was established via a continuous ellman's assay run at a total volume of 100 μL containing 50 mM phosphate buffer (pH 7.4), 1.0 mM SNAC substrate, 5 μM Zea or Rdc, 10% v/v DMSO, and 50 μL 8% DTNB (ellman's reagent).

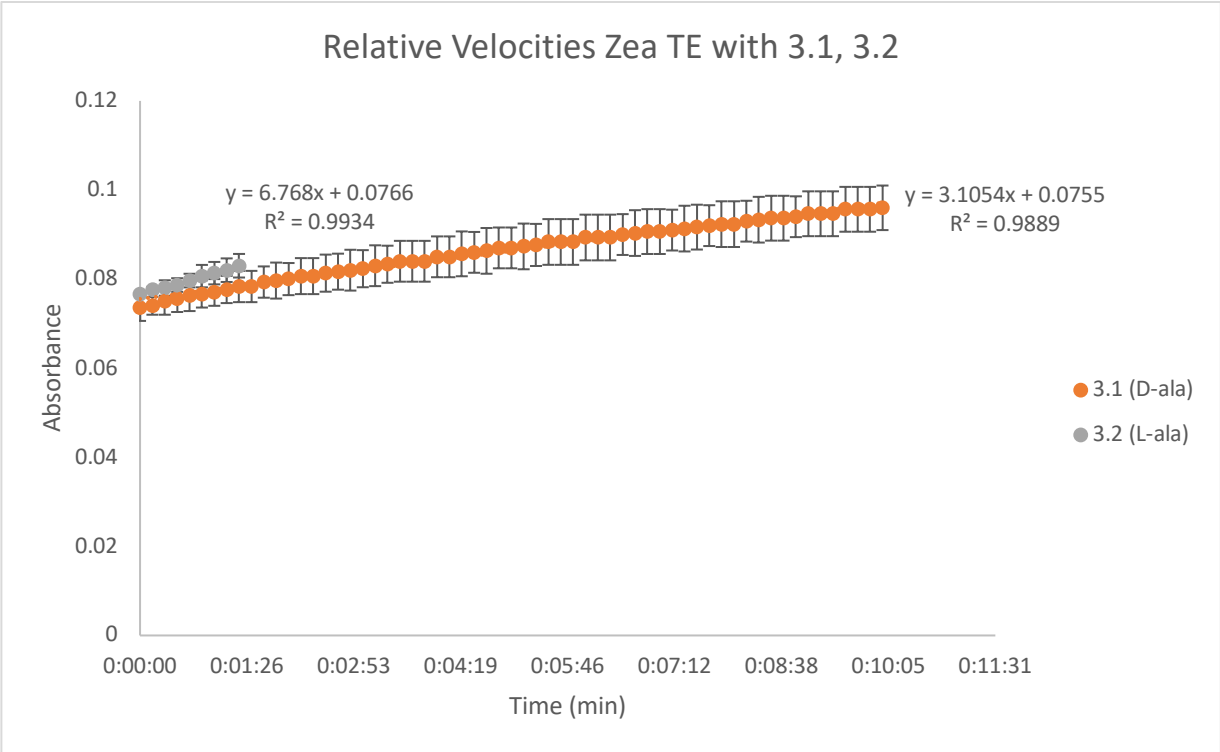
3.4.4 Relative Velocity Curves



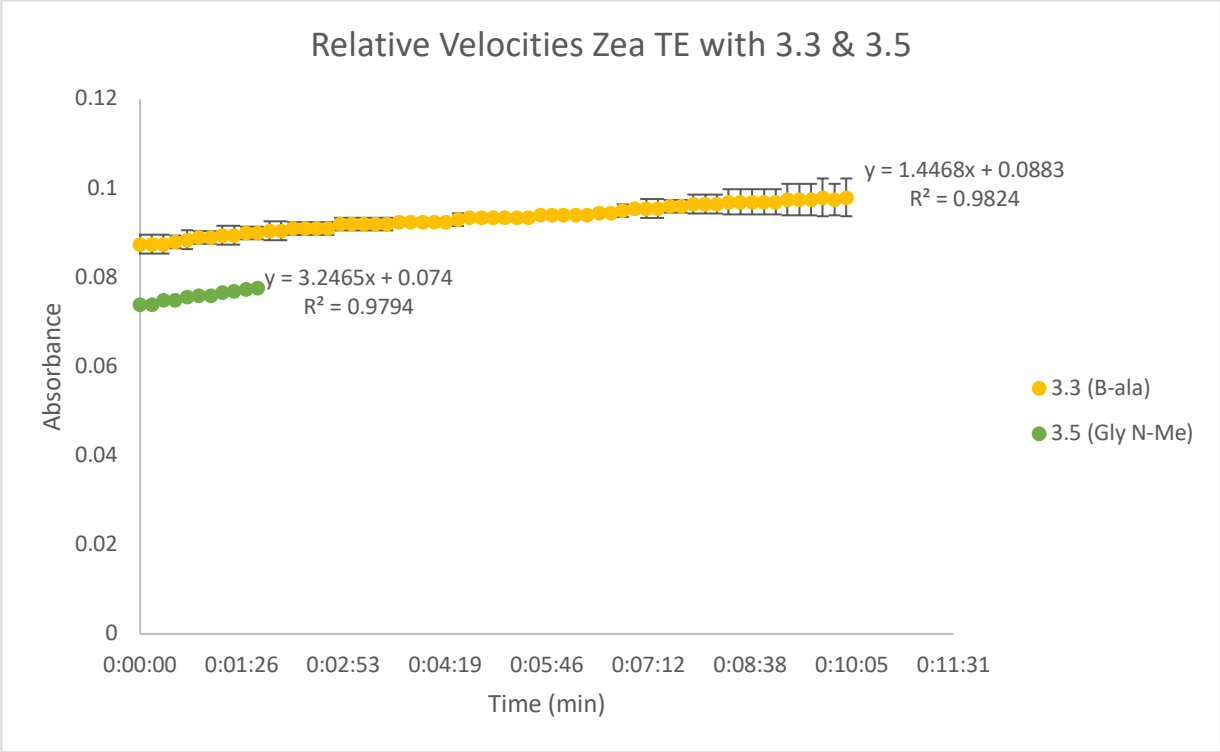
Initial velocities of substrates 2.12 and 3.1-3.5 with Zea TE.



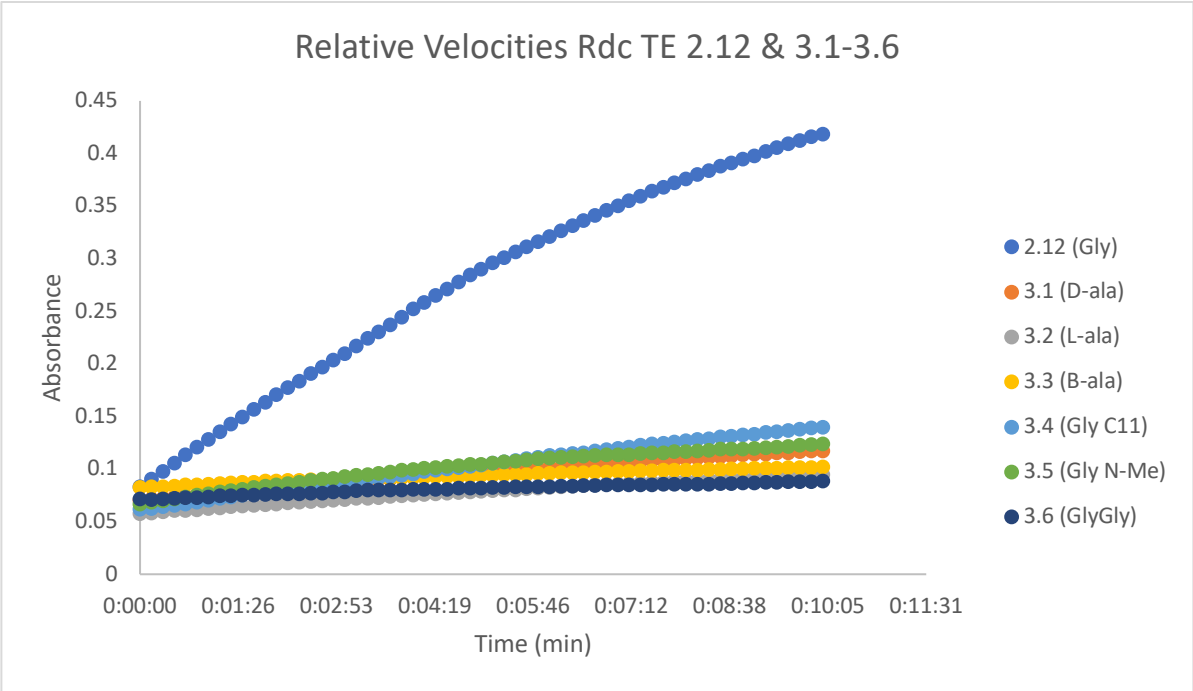
Initial velocities of substrates 3.1-3.3 and 3.5 with Zea TE.



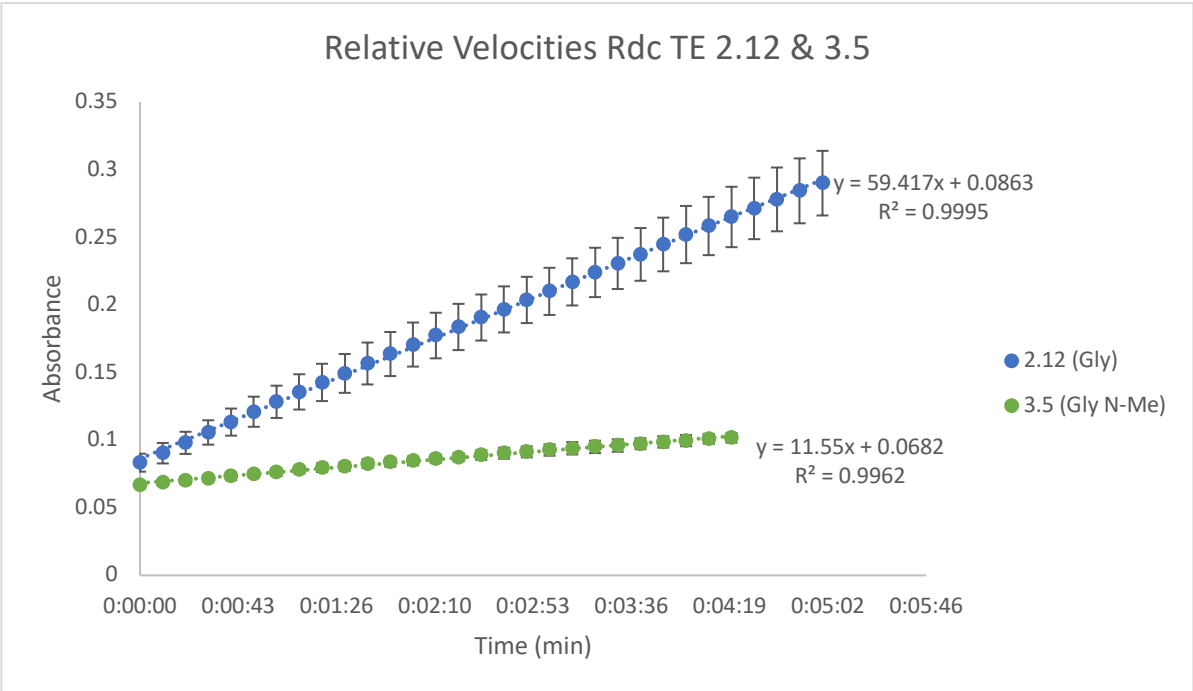
Initial velocities of substrates 3.1 and 3.2 with Zea TE. (Corrected for linear portion).



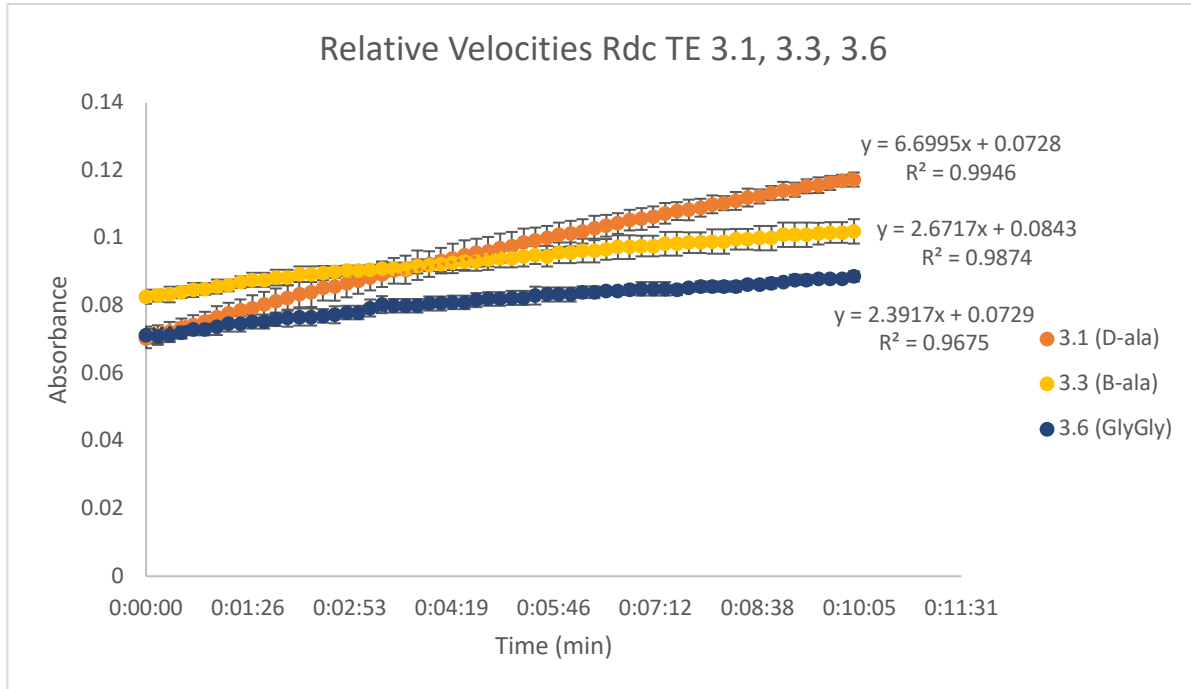
Initial velocities of substrates 3.3 and 3.5 with Zea TE. (Corrected for linear portion).



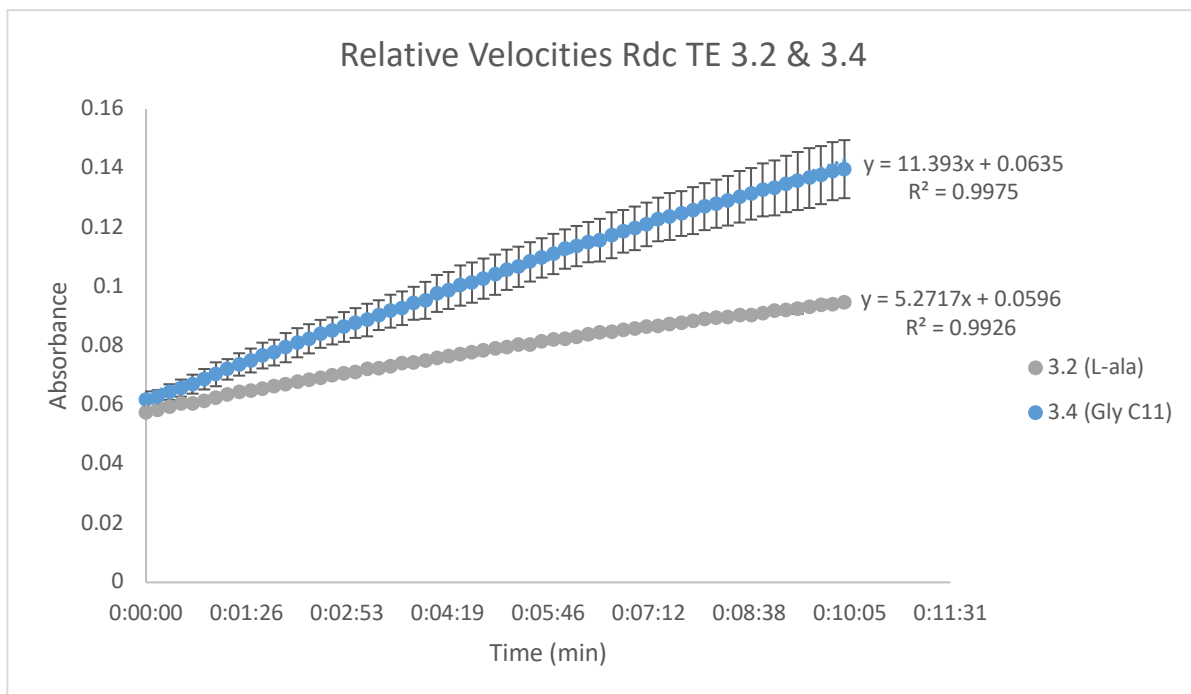
Initial velocities of substrates 2.12 and 3.1-3.6 with Rdc TE.



Initial velocities of substrates 2.12 and 3.5 with Rdc TE. (Corrected for linear portion).



Initial velocities of substrates 3.1, 3.3 and 3.6 with Rdc TE. (Corrected for linear portion).



Initial velocities of substrates 3.2 and 3.4 with Rdc TE. (Corrected for linear portion).

3.5 References

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Chapter 4: Advances Towards the Chemoenzymatic Synthesis of Asperterrestide A

4.1 Introduction

Asperterrestide A (Fig. 4.1) is a cyclic 13-membered tetrapeptide natural product. It was first isolated from *Aspergillus terreus* by Fei He *et al* in 2013.¹ The unique 13-membered macrocycle contains 3 D-amino acids and an *o*-amino benzoic acid (ABA) moiety. Structure elucidation done by Fei He reported an *o*-amino benzoic acid, D-alanine, 2*R*,3*S*-N-Me-phenylserine, and either D-allo-isoleucine or D-isoleucine (absolute configuration could not be achieved using Marfey's method)¹. Asperterrestide A has been shown to be moderately cytotoxic toward several lines of human carcinoma, as well it exhibits inhibitory effects on several strains of influenza virus. Although there has been no work done to elucidate its biosynthetic pathway, the structural characteristics suggest a NRPS type synthesis that likely culminates with a thioesterase macrocyclization.

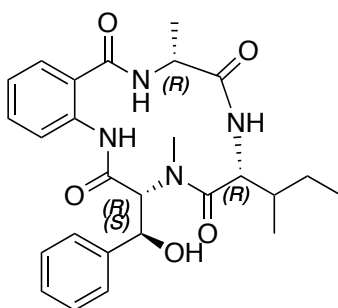


Figure 4. 1 Structure of Asperterrestide A.

4.2 Results and Discussion

The purpose of this research with asperterrestide A is to chemoenzymatically synthesize this natural product using the RAL TEs to form the 13-membered cyclic tetrapeptide (Fig. 4.2). Illustrating that these TEs can cyclize amino acid substrates led us to attempt the first chemoenzymatic synthesis of cyclic non-ribosomal peptide using a completely non-native thioesterase (Rdc and/or Zea TEs). There are several characteristics that make asperterrestide A a sufficient candidate for these TEs. The 13-membered structure fits into the scope of macrocycle size tolerance found with Zea and Rdc along with the macrolactamization ability.² The ABA group is analogous to the resorcylate moiety found in both radicicol and zearalenone showing potential for macrolactamization at the ABA-D-alanine peptide bond.^{3,4}

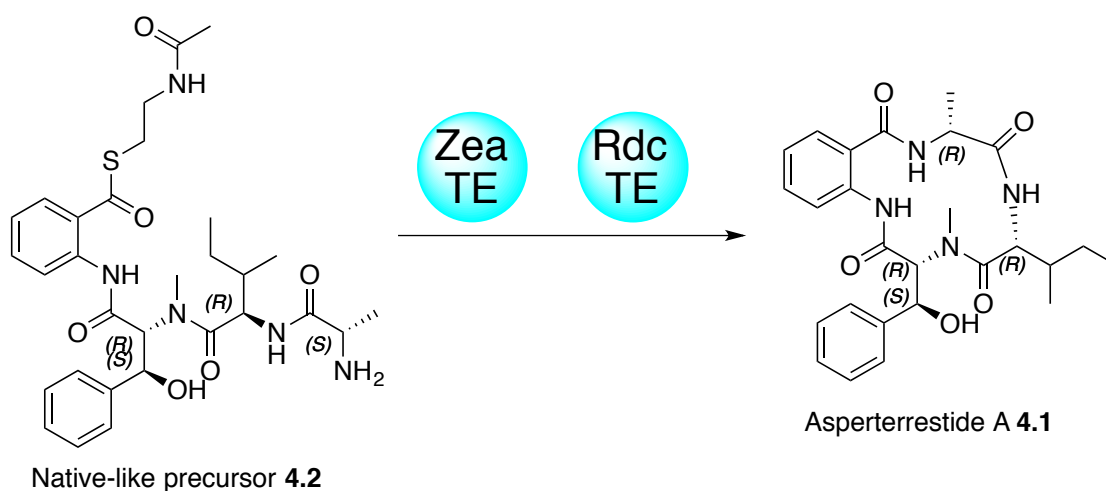


Figure 4. 2 Chemoenzymatic synthesis of asperterrestide A from 4.2.

4.2.1 Initial approach at the chemoenzymatic synthesis of asperterrestide A.

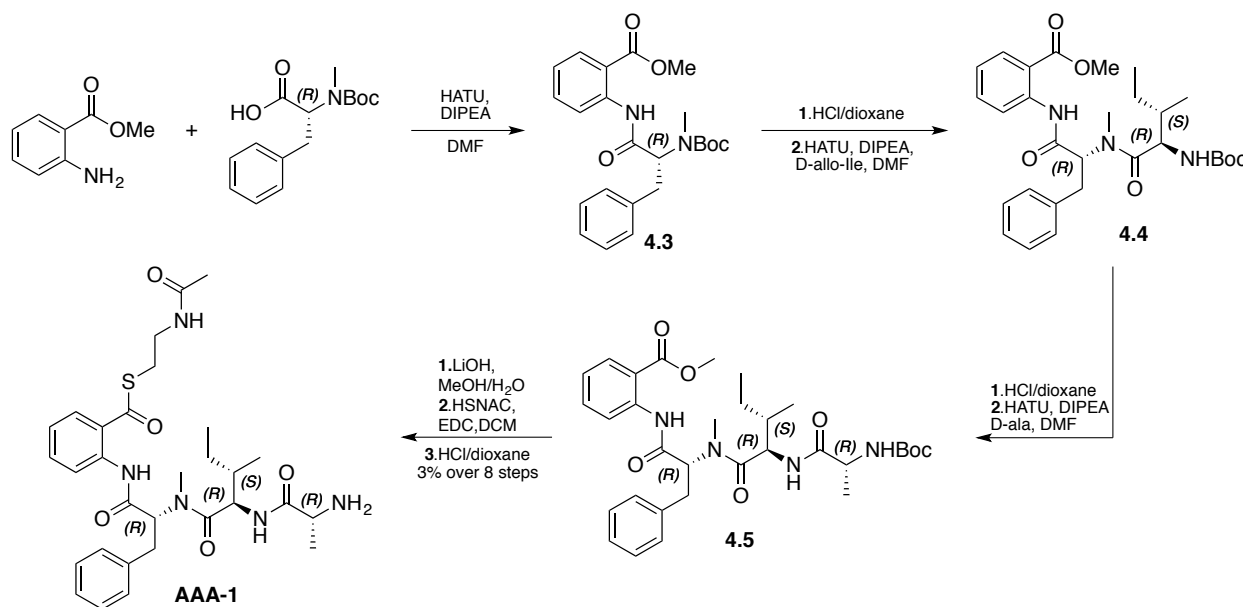
The initial chemoenzymatic route to asperterrestide A included an enzymatic macrolactamization with the amide bond formation between the ABA group and the D-alanine moiety. With the ABA-SNAC thioester resembling the native-like compound **2.1**, we believed this would provide the best opportunity at forming the macrocycle. The nucleophilic amine on the D-alanine is also much less sterically hindered than the amine on D-allo-Ile, and theoretically should simplify lactam formation.⁵

A key hurdle in the synthesis of the linear tetrapeptide precursor, is the formation of the 2*R*,3*S*-N-Me-phenylserine. This non-proteinogenic amino acid contains two stereocentres in the

syn-configuration. Due to the complexity of the 2*R*,3*S*-*N*-Me-phenylserine as well as the uncertainty that macrocyclization can be achieved with the RAL TEs, a simplified analog approach was used. By replacing the 2*R*,3*S*-*N*-Me-phenylserine with the commercially available Boc-*N*-Me-*D*-Phe, the synthesis would be simplified while still providing a similar macrocyclization system to the native linear precursor.

4.2.2 Synthesis of asperterrestide A precursor analog AAA-1

The simplified linear tetrapeptide was synthesized in eight steps starting from methyl anthranilate (Scheme 1). The 4 amino acids used to form the linear tetrapeptide were all commercially available, making the synthesis just a series of peptide coupling and Boc deprotection reactions.⁶



Scheme 4. 1 Synthesis of compound AAA-1.

The most challenging step of the synthesis was the coupling of methyl anthranilate to the Boc-*N*-Me-*D*-Phe, arising from the deactivated aniline nitrogen. This step was optimized using a HATU, DIPEA amide coupling with excess methyl anthranilate to afford dipeptide 4.3. The dipeptide was Boc-deprotected and subsequently coupled to Boc-*D*-allo-Ile using the same HATU coupling

procedure. Tripeptide **4.4** was deprotected using an HCl/dioxane mixture and coupled to Boc-D-alanine using HATU to produce **4.5**. The methyl ester on the anthranilate amino acid was hydrolyzed via LiOH, and SNAC was coupled to the free acid using EDC. The final step was another HCl/dioxane boc-deprotection to yield compound **AAA-1**.

4.2.3 Enzymatic Assays of AAA-1 with Zea and Rdc TEs.

Enzymatic assays were analogous to those in chapter 3 with both thioesterases. 24 h incubation of 1.0 mM substrate with 25 μ M enzyme at RT in 50 mM phosphate buffer. HPLC traces for both TEs as well as the no enzyme control show a large amount of hydrolysis (Fig. 4.4).

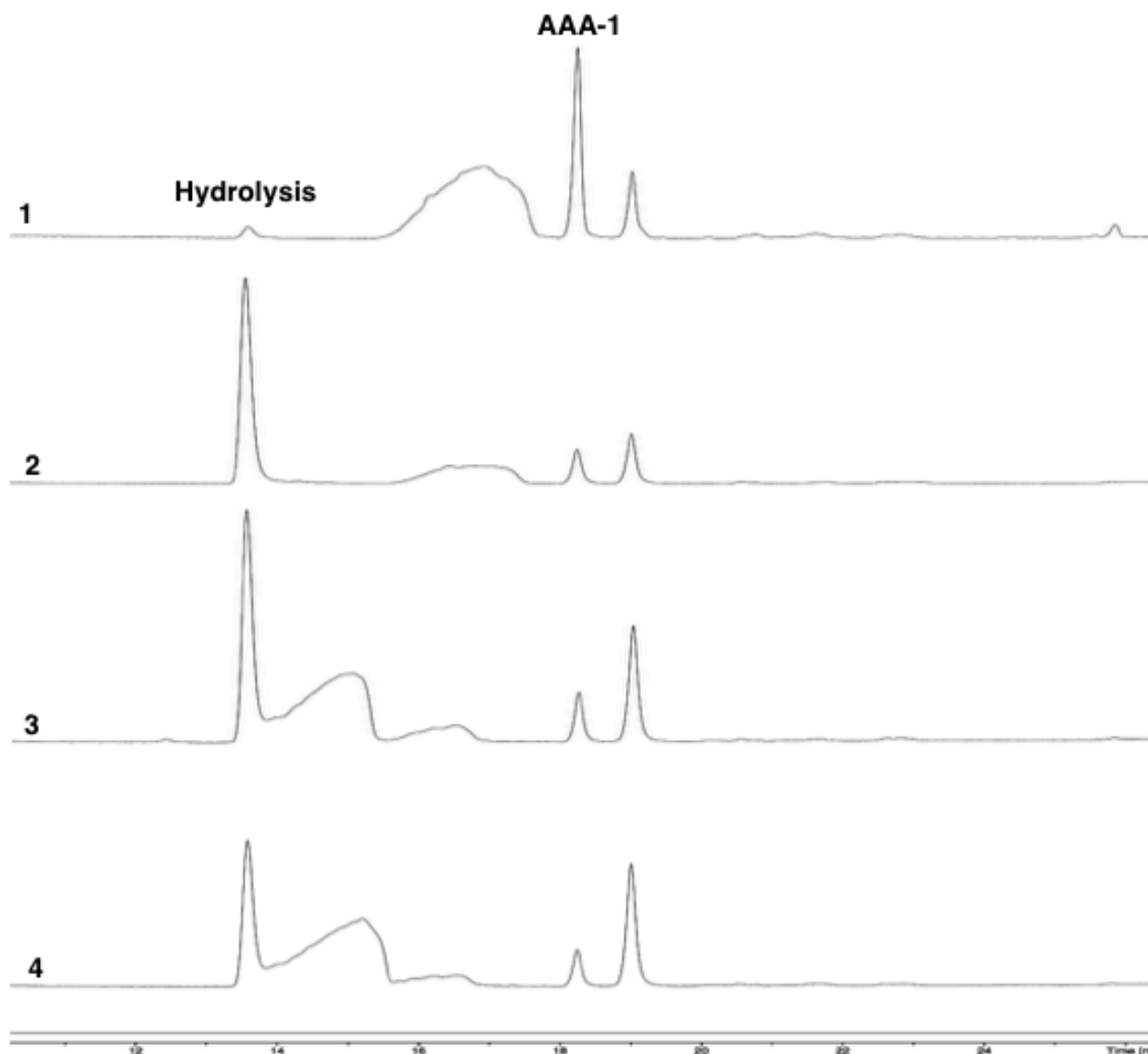


Figure 4. 3 HPLC traces of enzymatic assays of Zea and Rdc TEs with substrate AAA-1.

- 1) substrate standard **AAA-1**.
- 2) no enzyme control.
- 3) 1.0 mM substrate, 25.0 μ M Rdc TE, 24 h.
- 4) 1.0 mM standard. 25.0 μ M Zea TE, 24 h.

It seemed that the SNAC thioester was spontaneously hydrolyzing in the aqueous buffer.⁷ Since these assays were run over 24 h we could not confirm whether the hydrolysis seen in the enzymatic assays was completely due to aqueous buffer hydrolysis, or if the enzyme was also catalyzing hydrolysis. A time course assay was run with analysis run at 1, 3, and 5 h. The HPLC data showed the same hydrolysis in the no enzyme control as was seen in the TE assays. Our conclusion was that the rate of buffer hydrolysis was much greater than the loading step with the thioesterases.

An issue that was observed during the synthesis of **AAA-1** was the formation of a bicyclic intermediate during the reaction to access the SNAC thioester. While activating the *o*-amino benzoic acid moiety, nucleophilic attack from the adjacent amide carbonyl formed a bicyclic by-product (Fig. 4.4).

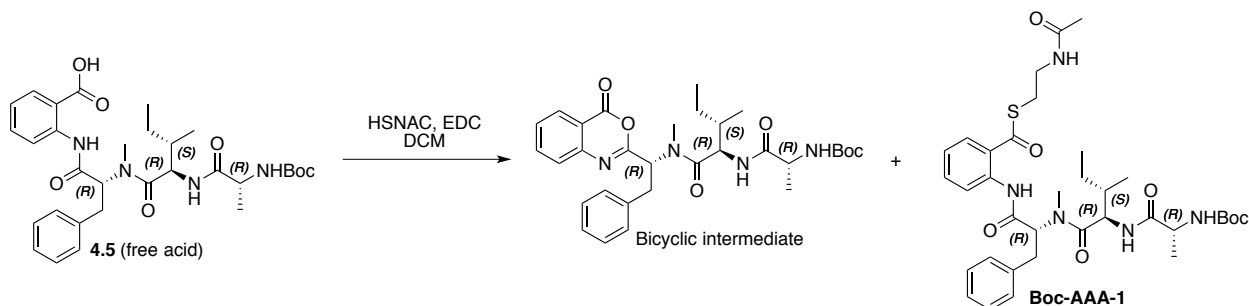


Figure 4. 4 Bicyclic intermediate formation during SNAC thioesterification.

This side-product was converted into the desired product during synthesis by increasing the concentration of SNAC.⁷ Examination of our enzymatic assays suggested that this bicyclic intermediate was being generated under these reaction conditions as well. The thioester bond being labile to nucleophilic attack, was forming this intermediate rapidly and then hydrolyzing in the aqueous environment. As this side reaction occurred in the no-enzyme control as well, it was clearly not TE-mediated. Potentially, adjustment of the pH could help suppress the formation of

hydrolysis product, but we would likely still observe the premature release of SNAC and therefore lose the ability to load onto the thioesterases.

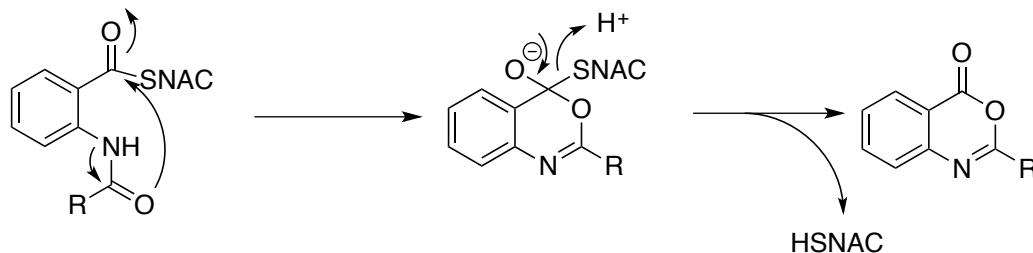


Figure 4. 5 Mechanism of bi-cycle formation.

4.2.4 New Approach to the Chemoenzymatic Synthesis of Asperterrestide A

The lack of thioesterase loading due to the bicycle formation lead to a modification in asperterrestide A precursor design. By changing the location of macrolactamization from the ABA-D-ala bond to the D-ala-D-allo-Ile bond, we can eliminate formation of this problematic intermediate. Referring back to chapter 3, macrocyclization with both TEs was observed with the D-alanine containing substrate **3.1**, providing evidence that we may be able to obtain macrocyclization at the D-alanine-D-allo-Ile bond (Fig. 4.6).

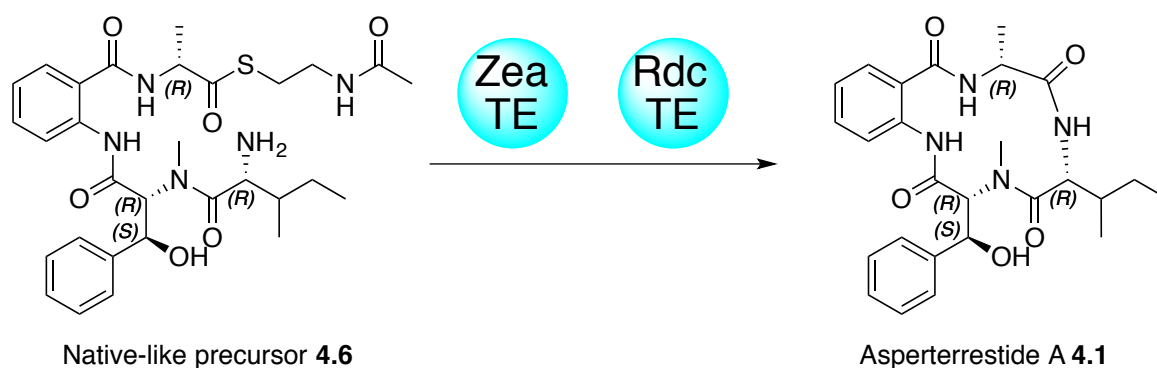


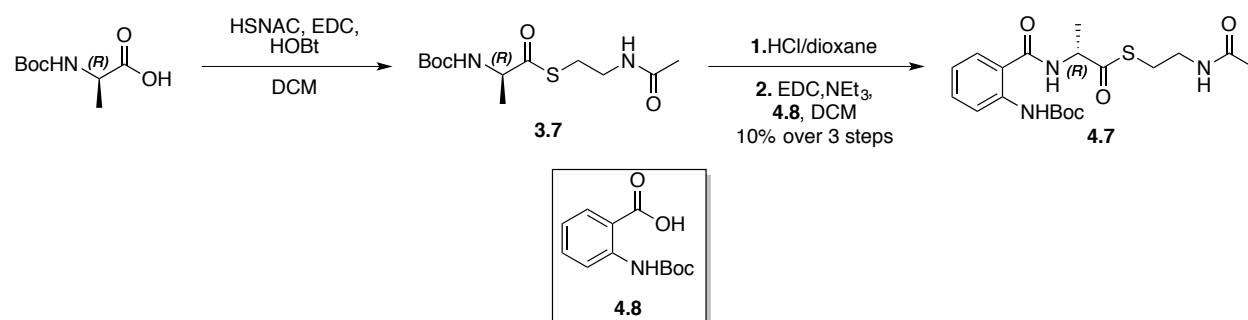
Figure 4. 6 Chemoenzymatic synthesis of asperterrestide A from 4.6

Although **AAA-1** had a relatively straight forward synthesis, it still required eight steps to complete. Had a simple dipeptide containing SNAC been tested, the buffer hydrolysis could have been observed without completing the whole synthesis. To observe whether the TEs would load

the substrate containing the SNAC thioester at the D-alanine, a simplified dipeptide **4.7** was synthesized and tested.

4.2.5 Synthesis of Asperterrestide analog **4.7**.

Synthesis of compound **4.7** took three steps starting with SNAC and Boc-D-alanine. An EDC/HOBt coupling was used to produce the thioester **3.7**. This was followed by an HCl/dioxane deprotection of the boc-group and a subsequent EDC coupling with Boc-anthranilic acid **4.8**, to produce compound **4.7** (Scheme 4.2).



Scheme 4. 2 Synthesis of compound 4.7

Compound **4.7** was then assayed with the Rdc TE to determine whether the D-ala-SNAC thioester can sufficiently load onto the thioesterase.

4.2.6 Enzymatic Assays of AAA-1 with Zea and Rdc TEs.

Enzymatic assays with **4.7** were performed analogous to those with **AAA-1**. Since compound **4.7** did not contain a nucleophile, the hydrolysis product is what was expected.

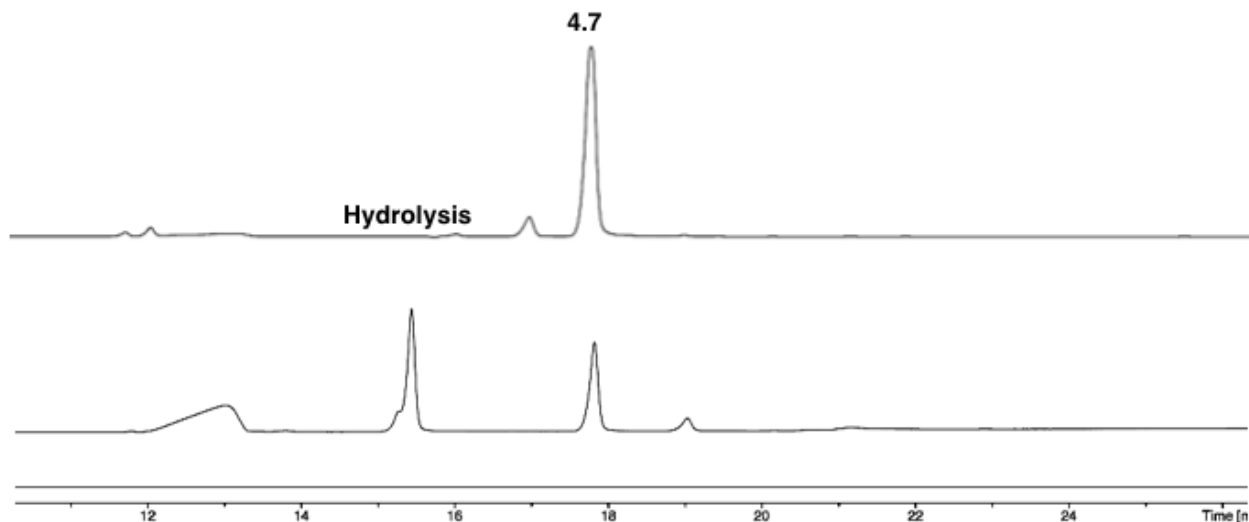


Figure 4. 7 HPLC traces of enzymatic assays of Rdc TE with substrate 4.7.

1) no enzyme control. 2) 1.0 mM substrate, 25.0 μ M Rdc TE, 24 h.

The enzymatic assay with **4.7** and Rdc TE showed a moderate to high amount of substrate loading (Fig. 4.7). Comparing to the no-enzyme blank, there was no hydrolysis observed. This indicates that the Rdc TE is loading the substrate and then hydrolyzing it off due to the lack of internal nucleophile. These results reassure the possibility of macrocyclization at the D-ala-D-allo-Ile amide. The major limitation with the initial approach was overcome by changing the site of macrocyclization. Due to the similarities of the Rdc and Zea TEs, the zearalenone thioesterase was not tested with **4.7**.

4.3 Conclusions

The purpose of this research was to investigate whether these RAL TEs are capable of chemoenzymatically synthesizing asperterrestide A. **4.1**, although it is a non-ribosomal peptide, it shares characteristics with the native products of these TEs. The initial approach to cyclize at the *o*-amino benzoic acid site failed due to the formation of a bi-cyclic intermediate (Fig. 4.5). This intermediate formation forced us to change the cyclization site on the linear precursor. The new strategy had the SNAC thioester linked to the D-alanine moiety thereby negating the possibility of nucleophilic attack by a neighbouring amide carbonyl. Initial tests with compound **4.7** and the radical thioesterase show abolishment of bi-cycle formation as well as thioesterase loading. The

data with **4.7** and Rdc TE shows promise that once the complete linear precursor **4.6** is synthesized, it will load onto the RAL TEs and possibly macrocyclize. Preliminary ground work has been established on the chemoenzymatic synthesis of asperterrestide A using two RAL TEs. Initial data shows promise that a non-ribosomal peptide can be formed chemoenzymatically using a PKS TE.

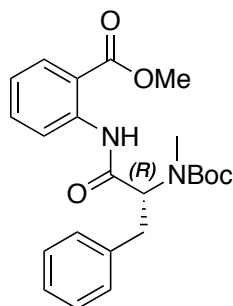
4.4 Experimental

4.4.1 General Synthetic Methods

All reagents were purchased from Sigma-Aldrich or Oakwood chemicals at the highest available purity and used without further purification. All solvents were purchased from Fisher Scientific. All reactions were conducted using dry solvents under an argon atmosphere unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) and carried out on 0.25 mm E. Merck silica gel plates using UV light (254 nm) as a visualizing agent and phosphomolybdic acid stain and heat as developing agent. NMR spectroscopy was performed with a Bruker Avance II, operating 400 MHz for ¹H spectra, and 100 MHz for ¹³C spectra. All chemical shifts are reported in parts per million (δ), integration and coupling constants in Hz and corrected using the solvent residual peak as internal standard. High-resolution mass spectroscopy (HRMS) was conducted on a Micromass Q-TOF I for ESI measurements (John L. Holmes Mass Spectroscopy).

4.4.2 Synthetic Protocols

methyl (*R*)-2-(2-((*tert*-butoxycarbonyl)(methyl)amino)-3-phenylpropanamido)benzoate (4.3)

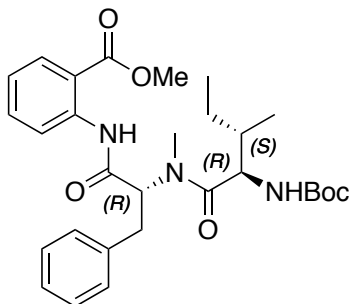


751 mg Boc-N-Me-D-Phe (2.86 mmol, 1.0 equiv.) was dissolved in DMF and cooled to 0 °C under an inert atmosphere. 2.71 g HATU (7.12 mmol, 2.5 equiv.) and 5.1 ml DIPEA (28.0 mmol, 10 equiv.) were added and the solution was stirred for 5 minutes. 2.6 ml methyl anthranilate (20.0 mmol, 7.0 equiv.) was added and the solution was stirred overnight at 45 °C. The solution was quenched with brine, extracted with EtOAc, washed with NH₄Cl, and additionally with brine. The solution was dried over MgSO₄ and concentrated. The crude product was purified by column chromatography (30:70 EtOAc/Hex) to yield 385 mg of **4.3** (34%) as a colourless oil. Characterization is consistent with previously reported data.⁸

R_f = 0.19 (30:70 EtOAc/Hex)

¹H NMR (400 MHz, CDCl₃) δ 11.57 (s, 1H), 8.79 – 8.70 (m, 1H), 8.07 – 7.93 (m, 1H), 7.57 – 7.47 (m, 1H), 7.31 – 7.24 (m, 3H), 7.23 – 7.14 (m, 2H), 7.12 – 7.02 (m, 1H), 5.25 – 4.58 (m, 1H), 3.86 (s, *J* = 5.1 Hz, 3H), 3.59 – 3.41 (m, 1H), 3.04 – 2.94 (m, 1H), 2.77 (s, 3H), 1.33 (s, 9H).

methyl 2-((*R*)-2-((2*R*,3*S*)-2-((*tert*-butoxycarbonyl)amino)-*N*,3-dimethylpentanamido)-3-phenylpropanamido)benzoate (4.4)



385 mg **4.3** (0.974 mmol, 1.0 equiv.) was dissolved in dry DCM, cooled to 0 °C and 5 ml of 4.0 M HCl/dioxane was added, the solution was stirred for 3 h. The solvent was removed under vacuum and the crude product was dissolved in DMF and cooled to 0 °C. 690 mg HATU (1.815 mmol, 1.6 equiv.) and 530 μ l DIPEA (2.96 mmol, 3.0 equiv.) was added and the solution was stirred for 5 min. 360 mg Boc-D-allo-Ile (1.56 mmol, 1.5 equiv.) was added and the solution was stirred at room temperature for 2 h. Solution was quenched with brine, extracted with EtOAc, washed with NH_4Cl , and washed again with brine. Solution was dried over MgSO_4 and concentrated under vacuum. The crude product was purified via column chromatography (12:88 EtOAc/Hex) to yield 350 mg **4.4** (70%) as a yellow oil.

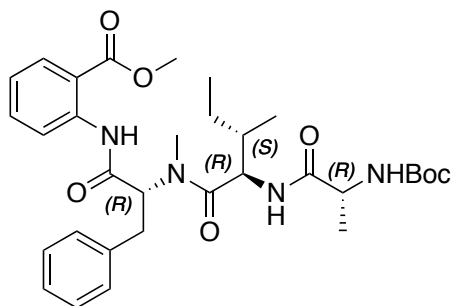
$R_f = 0.13$ (12:88 EtOAc/Hex)

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ 11.31 (s, 1H), 8.69 (d, $J = 8.3$ Hz, 1H), 7.98 (d, $J = 6.4$ Hz, 1H), 7.56 – 7.48 (m, 1H), 7.32 – 7.25 (m, 2H), 7.23 – 7.14 (m, 3H), 7.12 – 7.03 (m, 1H), 5.48 (dd, $J = 9.2, 6.4$ Hz, 1H), 4.98 (d, $J = 9.5$ Hz, 1H), 4.52 (dd, $J = 9.5, 4.1$ Hz, 1H), 3.85 (s, 3H), 3.53 (dd, $J = 14.3, 6.3$ Hz, 1H), 3.07 – 2.97 (m, 4H), 1.85 – 1.73 (m, 1H), 1.47 – 1.37 (m, 10H), 1.19 – 1.13 (m, 1H), 0.91 (t, $J = 7.3$ Hz, 3H), 0.82 (d, $J = 6.7$ Hz, 3H).

^{13}C NMR (100 MHz, CDCl_3) δ 173.13, 168.94, 168.30, 155.64, 141.02, 137.22, 134.57, 130.84, 129.10, 128.46, 126.67, 122.80, 120.45, 115.30, 79.25, 60.72, 53.43, 52.39, 37.14, 34.09, 28.34, 27.82, 26.93, 13.73, 11.92.

HRMS (ESI+) calc. for $\text{C}_{29}\text{H}_{39}\text{N}_3\text{O}_6\text{Na}$ ($\text{M}+\text{Na}$)548.2737; obsd. 548.2726.

methyl 2-((6*R*,9*R*,12*R*)-12-benzyl-9-((*S*)-*sec*-butyl)-2,2,6,11-tetramethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-amido)benzoate (**4.5**)



240 mg **4.4** (0.445 mmol, 1 equiv.) was dissolved in dry DCM cooled to 0 °C, 2 ml of 4.0 M HCl/dioxane were added and the solution was stirred for 2 h. The solution was concentrated and dissolved in DMF and cooled to 0 °C. 302 mg HATU (0.794 mmol, 1.6 equiv.) and 635 μl DIPEA (1.34 mmol, 3.0 equiv.) were added and the solution was stirred for 10 min. 130 mg Boc-D-alanine (0.69 mmol, 1.4 equiv.) was added and the solution was stirred for 4 h. The solution was quenched with brine, extracted with EtOAc, washed with NH_4Cl , brine, dried over MgSO_4 and concentrated. The crude product was purified using column chromatography (30:70 EtOAc/Hex) to yield 73 mg **4.5** (27%) as a colourless oil.

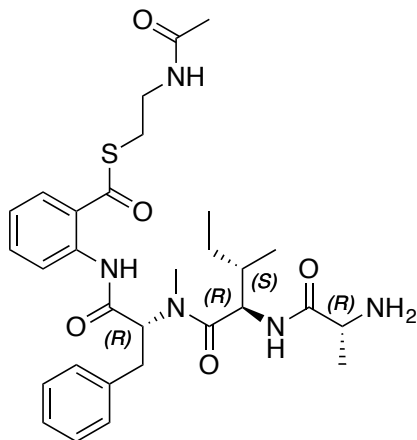
$R_f = 0.26$ (30:70 EtOAc/Hex)

^1H NMR (400 MHz, CDCl_3) δ 11.39 (s, 1H), 8.72 (d, $J = 8.5$ Hz, 1H), 8.03 (dd, $J = 8.0, 1.6$ Hz, 1H), 7.61 – 7.51 (m, 1H), 7.38 – 7.31 (m, 2H), 7.27 – 7.19 (m, 3H), 7.11 (t, $J = 7.7$ Hz, 1H), 6.50 (d, $J = 9.2$ Hz, 1H), 5.39 (dd, $J = 9.5, 6.1$ Hz, 1H), 5.08 – 4.98 (m, 1H), 4.90 (dd, $J = 9.0, 3.7$ Hz, 1H), 4.10 – 3.97 (m, 1H), 3.90 (s, $J = 1.6$ Hz, 3H), 3.56 (dd, $J = 14.4, 6.0$ Hz, 1H), 3.11 – 3.02 (m, 4H), 1.91 – 1.78 (m, 1H), 1.49 – 1.40 (m, 10H), 1.24 (d, $J = 7.0$ Hz, 3H), 1.19 – 1.12 (m, 1H), 0.94 (t, $J = 7.3$ Hz, 3H), 0.87 (d, $J = 6.7$ Hz, 3H).

^{13}C NMR (75 MHz, CDCl_3) δ 172.30, 172.06, 168.69, 168.37, 141.01, 137.19, 134.58, 130.84, 129.05, 128.47, 126.68, 122.80, 120.38, 115.22, 61.27, 52.46, 52.07, 51.88, 37.77, 37.37, 34.00, 32.89, 28.27, 27.05, 13.74, 11.92.

HRMS (ESI+) calc. for $\text{C}_{32}\text{H}_{44}\text{N}_4\text{O}_7\text{Na}$ ($\text{M}+\text{Na}$)619.3108; obsd. 619.3101.

***S*-(2-acetamidoethyl)-2-((*R*)-2-((2*R*,3*S*)-2-((*R*)-2-aminopropanamido)-*N*,3-dimethylpentanamido)-3-phenylpropanamido)benzothioate (AAA-1)**



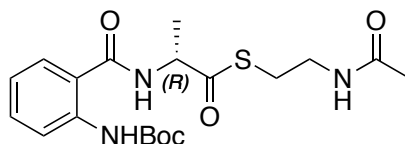
74 mg **4.5** (0.12 mmol, 1.0 equiv.) was dissolved in a 50:50 mixture of MeOH/H₂O, followed by the addition of 49 mg LiOH (1.16 mmol, 10.0 equiv.). The solution was stirred for 3 h, quenched with 1.0 M aq. HCl, and extracted with EtOAc. The organics were washed with brine, dried over MgSO₄ and concentrated. The crude product was dissolved in dry DCM under N₂. 30 mg N-acetyl cysteamine (0.25 mmol, 1.6 equiv.) was added, followed by the addition of 30 mg EDC (0.16 mmol, 1.4 equiv.). The solution was stirred overnight and quenched with NH₄Cl. The aqueous layer was extracted with EtOAc, washed with brine and concentrated. The crude product was purified using column chromatography and subsequently dissolved in dry DCM. To the solution was added 1 mL 4.0 M HCl/dioxane and the solution was stirred for 3 h. the solution was concentrated, and the crude product was purified using column chromatography (10:90 MeOH/DCM) to yield 19 mg **AAA-1** (25% over 3 steps) as a milky white oil.

¹H NMR (400 MHz, CDCl₃) δ 11.18 (s, 1H), 8.69 (d, *J* = 8.6 Hz, 1H), 8.04 (d, *J* = 6.8 Hz, 1H), 7.64 (d, *J* = 10.1 Hz, 1H), 7.57 (t, *J* = 7.3 Hz, 1H), 7.34 – 7.27 (m, 2H), 7.25 – 7.18 (m, 3H), 7.13 (t, *J* = 7.6 Hz, 1H), 7.03 – 6.96 (m, 1H), 5.17 (s, 1H), 4.97 (dd, *J* = 9.7, 4.5 Hz, 1H), 3.67 – 3.47 (m, 3H), 3.40 (q, *J* = 7.0 Hz, 1H), 3.32 – 3.21 (m, 1H), 3.16 (t, *J* = 6.9 Hz, 2H), 3.12 – 3.05 (m, 1H), 2.98 (s, 3H), 1.98 (s, 3H), 1.82 – 1.73 (m, 1H), 1.41 – 1.38 (m, 1H), 1.25 – 1.24 (m, 3H), 1.15 – 1.11 (m, 1H), 0.92 (t, *J* = 7.3 Hz, 3H), 0.86 (d, *J* = 6.7 Hz, 4H).

^{13}C NMR (100 MHz, CDCl_3) δ 195.61, 175.38, 172.39, 170.67, 168.81, 138.29, 137.59, 135.08, 129.84, 129.04, 128.49, 126.62, 123.08, 122.52, 120.80, 51.94, 50.73, 39.20, 37.28, 33.83, 29.70, 28.68, 27.15, 23.08, 21.74, 14.20, 14.06, 11.96.

HRMS (ESI+) calc. for $\text{C}_{30}\text{H}_{41}\text{N}_5\text{O}_5\text{SNa}$ (M+Na)606.2726; obsd. 606.2732.

***S*-(2-acetamidoethyl) (*R*)-2-(2-((*tert*-butoxycarbonyl)amino)benzamido)propanethioate (4.7)**



690 mg Boc-D-alanine (3.64 mmol, 1.0 equiv.) was dissolved in dry DCM. 1.05 g EDC (4.10 mmol, 1.2 equiv.), 619 mg HOBt (4.04 mmol, 1.2 equiv.), and 500 mg N-acetyl cysteamine (5.49 mmol, 1.5 equiv.) were added and the solution was stirred for 6 h. NH_4Cl was added to quench the reaction and the aqueous layer was extracted with EtOAc. The organics were washed with NaHCO_3 , brine, dried over MgSO_4 and concentrated to yield 707 mg of **3.7** (67%) as a white solid. **3.7** was dissolved in DCM and cooled to 0 °C. 7 ml of 4.0 M HCl/dioxane was added and the solution stirred for 3 h. The solvents were removed under vacuum and the crude solid was added to a mixture of 546 mg Boc-anthranilic acid (2.3 mmol, 1.0 equiv.), 674 mg EDC (3.5 mmol, 1.5 equiv.), and 1.0 mL NEt_3 (7.19 mmol, 3.0 equiv.) in DCM. The solution was stirred at RT for 4 h. The reaction was quenched with NH_4Cl , extracted with EtOAc, washed with NaHCO_3 , brine, dried over MgSO_4 and concentrated. The crude product was purified using column chromatography (50:50 EtOAc/Hex) to yield 150 mg of **4.7** (10% over 3 steps) as a colourless oil.

¹H NMR (400 MHz, CDCl₃) δ 9.96 (s, 1H), 8.31 (d, *J* = 8.5 Hz, 1H), 7.56 (dd, *J* = 7.9, 1.4 Hz, 1H), 7.48 – 7.35 (m, 1H), 7.14 (d, *J* = 7.0 Hz, 1H), 7.05 – 6.91 (m, 1H), 6.11 (s, 1H), 4.79 (p, *J* = 7.2 Hz, 1H), 3.45 – 3.29 (m, 2H), 3.06 – 2.96 (m, 2H), 1.91 (s, *J* = 5.6 Hz, 3H), 1.52 – 1.46 (m, 12H).

¹³C NMR (100 MHz, CDCl₃) δ 201.13, 170.56, 168.92, 153.00, 140.40, 132.95, 127.19, 121.48, 119.87, 118.89, 80.47, 55.69, 39.16, 28.47, 28.32, 23.11, 18.24.

HRMS (ESI+) calc. for C₁₉H₂₇N₃O₅SNa (M+Na)432.1569; obsd. 432.1567.

4.4.3 Enzymatic Protocols

Assays were run at a total volume of 50 μL containing 50 mM phosphate buffer (pH 7.4), 1.0 mM SNAC substrate, 25 μM enzyme (Rdc or Zea), and 10% v/v DMSO. All reactions were quenched with an equal volume of 0.5% formic acid in acetonitrile prior to HPLC analysis. HPLC analysis was conducted with an Agilent 1260 Modular system using a Dikima Leapsil 2.7 μm C18, 100x2.1 mm column. HPLC conditions: Flow rate 0.4 mL/min, 0 to 100% B over 30 min. (A: H₂O B: ACN).

4.5 References

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Chapter 5: Conclusions and Future Perspective

5.1 Conclusions

In conclusion we have demonstrated that RAL TEs Zea & Rdc are exceptionally substrate tolerant with few limitations. Swapping the phenyl-ring found in the native substrate, for a glycine moiety still allows for efficient macrocyclization with both the RAL TEs. Removal of the phenyl-ring abolishes activity, which coincides with the hypothesis that the phenyl-ring acts as a logic gate for these fungal thioesterases.

Testing done with amino acid containing depsipeptide substrates showed macrocycle formation is possible with most substrates tested with both TEs. It is quite remarkable that a PKS TE can efficiently macrocyclize peptidyl substrates. Adding to the ring-size and stereochemical tolerance of these RALs, they are currently the most tolerant PKS thioesterases to date and show great potential as biocatalysts.

Work towards the chemoenzymatic synthesis of asperterrestide A shows promise with the radical thioesterase. The precursor dipeptide showed efficient enzyme loading, indicating a good affinity with the Rdc TE. If macrocyclization of this NRP is accomplished, it will be the first time a PKS TE has macrocyclized a non-ribosomal peptide. This would pave the way for more research into fungal TEs, specifically RALs.

Catalytic efficiencies and product distribution for substrates tested in this thesis remain much too low to label Rdc and Zea TEs as viable biocatalysts. The idea behind this research is having a TE that can moderately catalyze a large subset of compounds, will simplify the optimization process in terms of protein engineering. Here we believe we have found a thioesterase that is far superior than any to date.

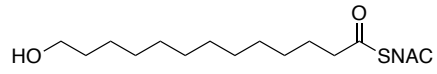
5.2 Future Perspective

Moving forward the total chemoenzymatic synthesis of asperterrestide A is underway. The bottle-neck of this synthesis being the formation of the 2*R*,3*S*-N-Me-Phenylserine. Current attempts at this synthesis have been successful. Once the SNAC tetrapeptide precursor **4.6** is complete enzymatic assays will hopefully produce macrolactam product.

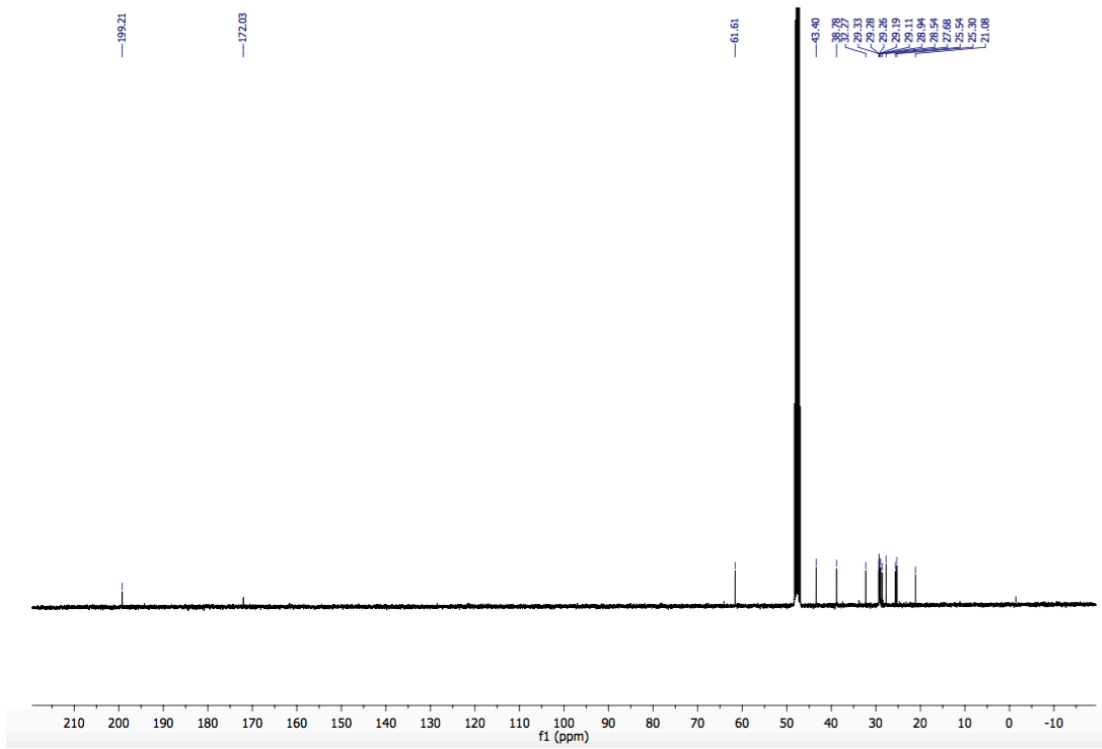
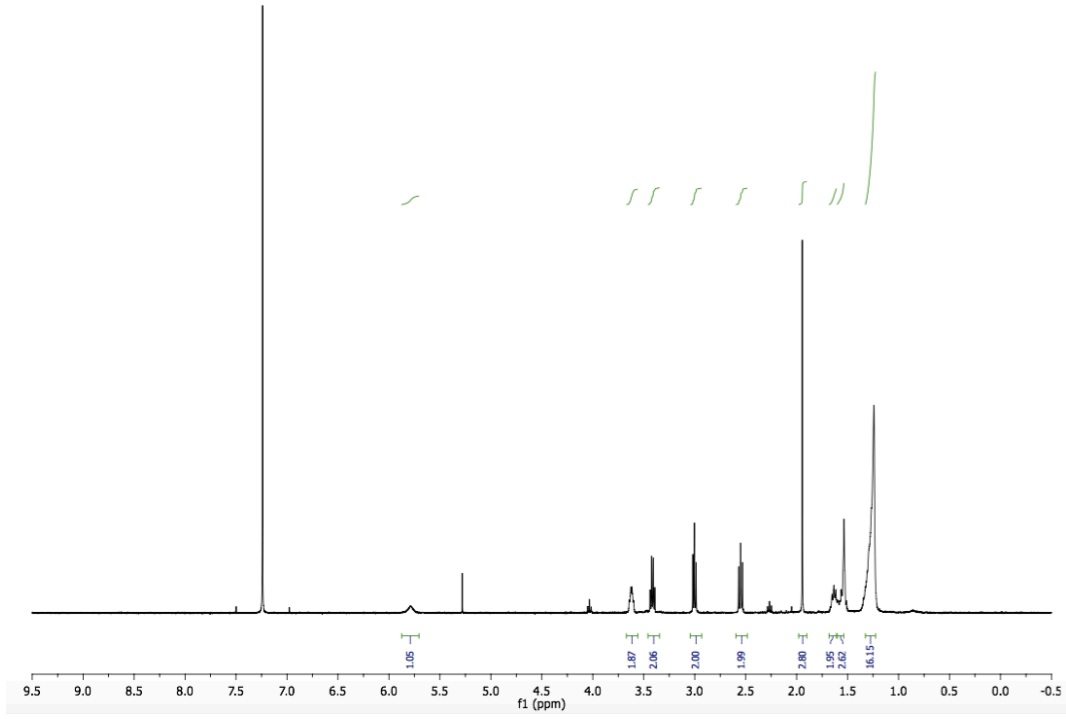
A further understanding of these enzymes is required in terms of biocatalyst development. Protein engineering experiments with the radical thioesterase are underway using a random mutagenesis approach. This will help identify key amino acid mutations that can increase efficiencies with the non-native like substrates. Lastly, attempts at obtaining a crystal structure of the Rdc TE are also being done. A crystal structure would greatly improve the understanding we have of these thioesterases and aid in trying to optimize for non-native substrates.

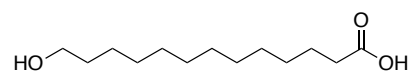
Appendix A: NMR Spectra

Chapter 2

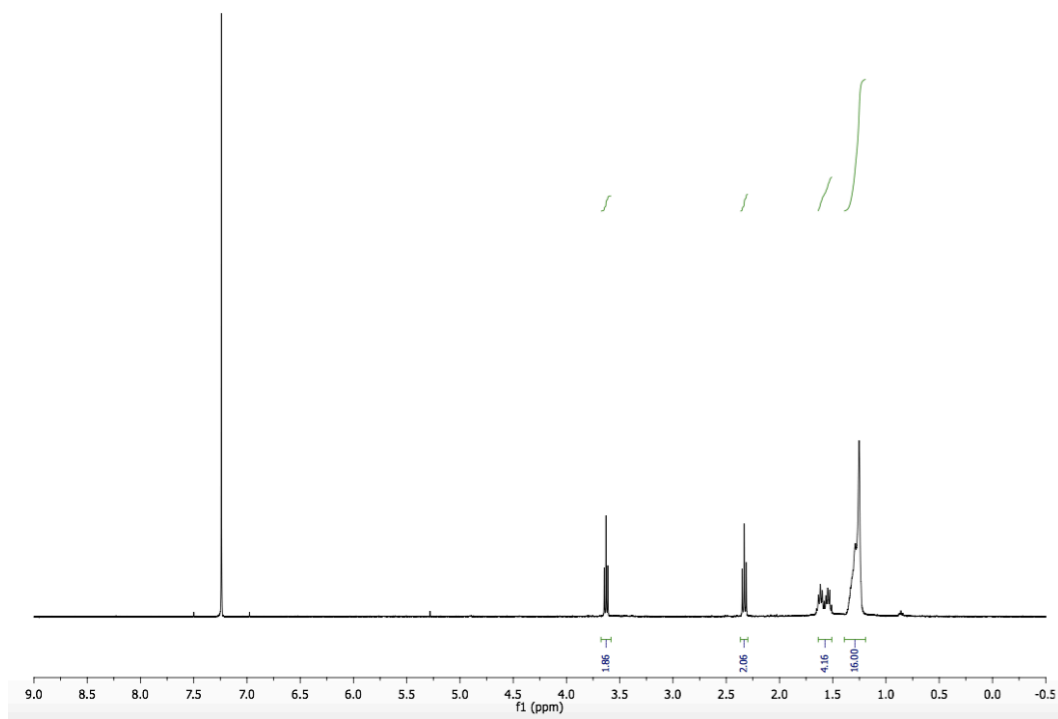


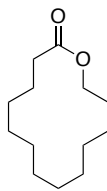
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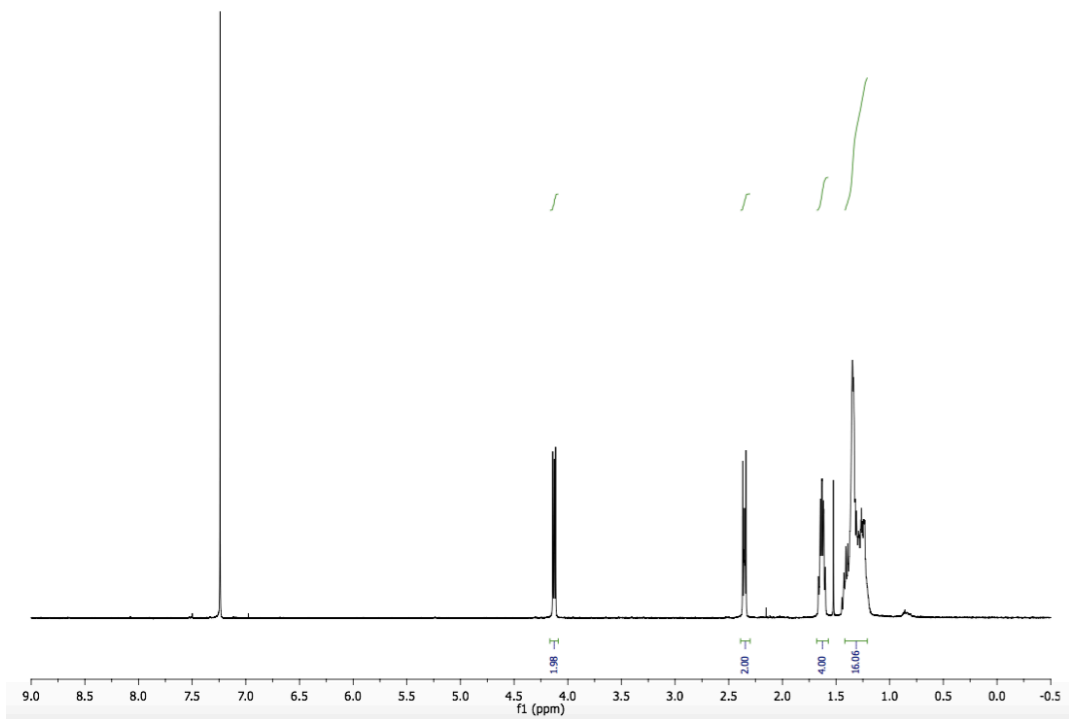


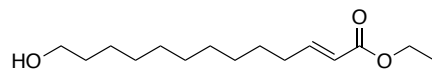
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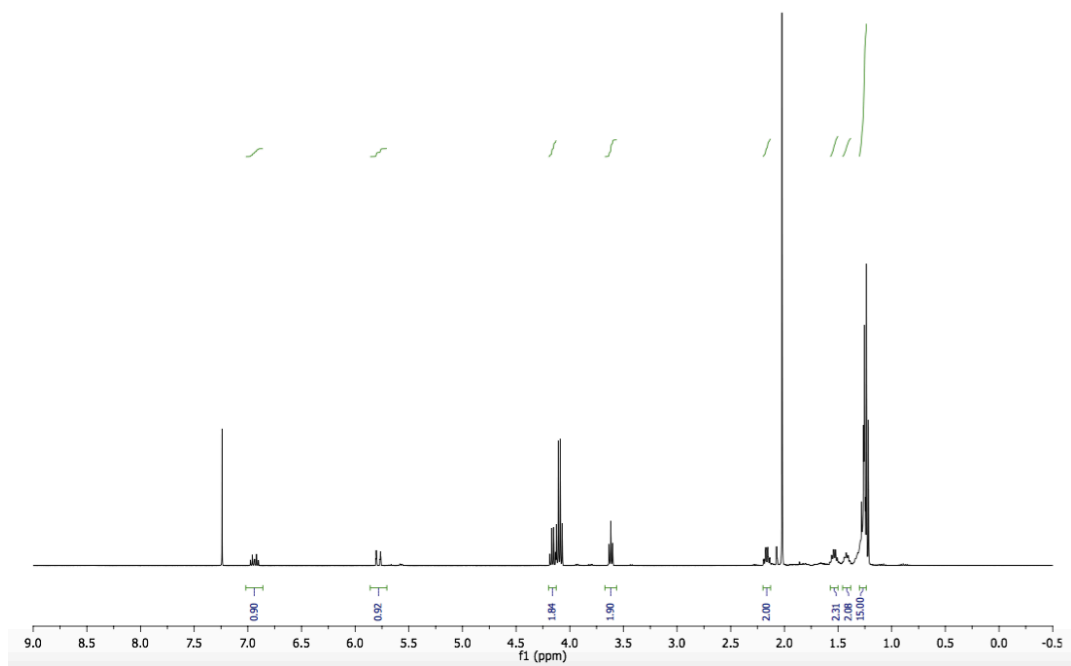


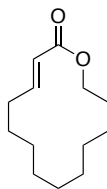
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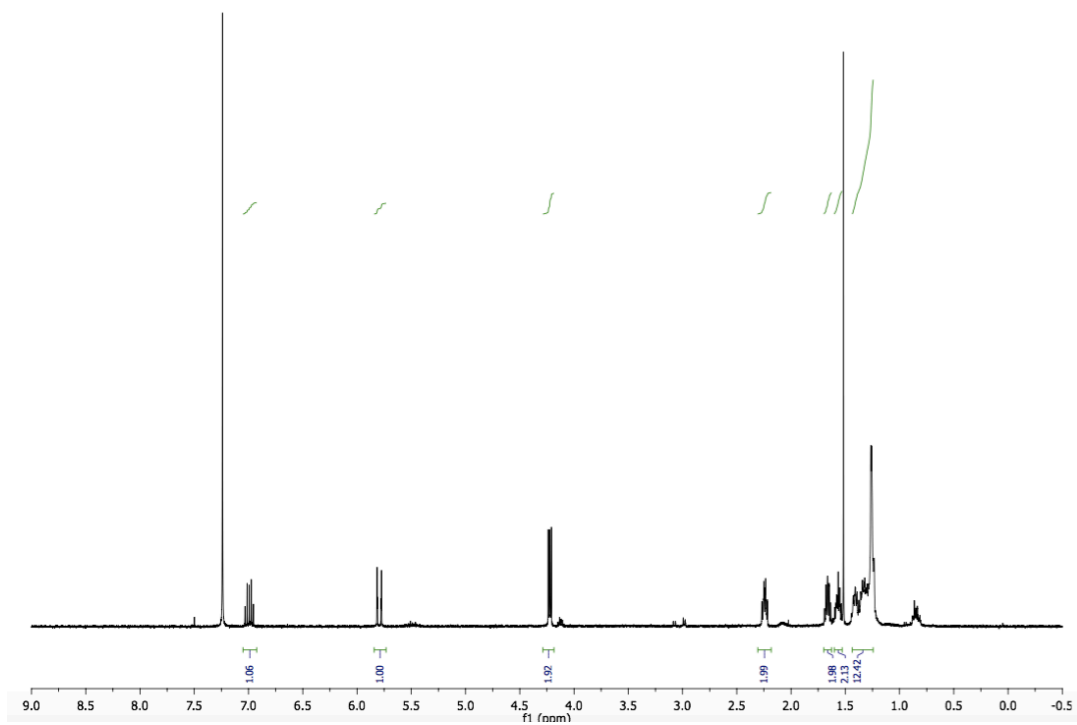


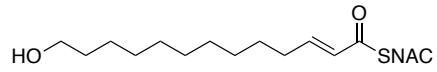
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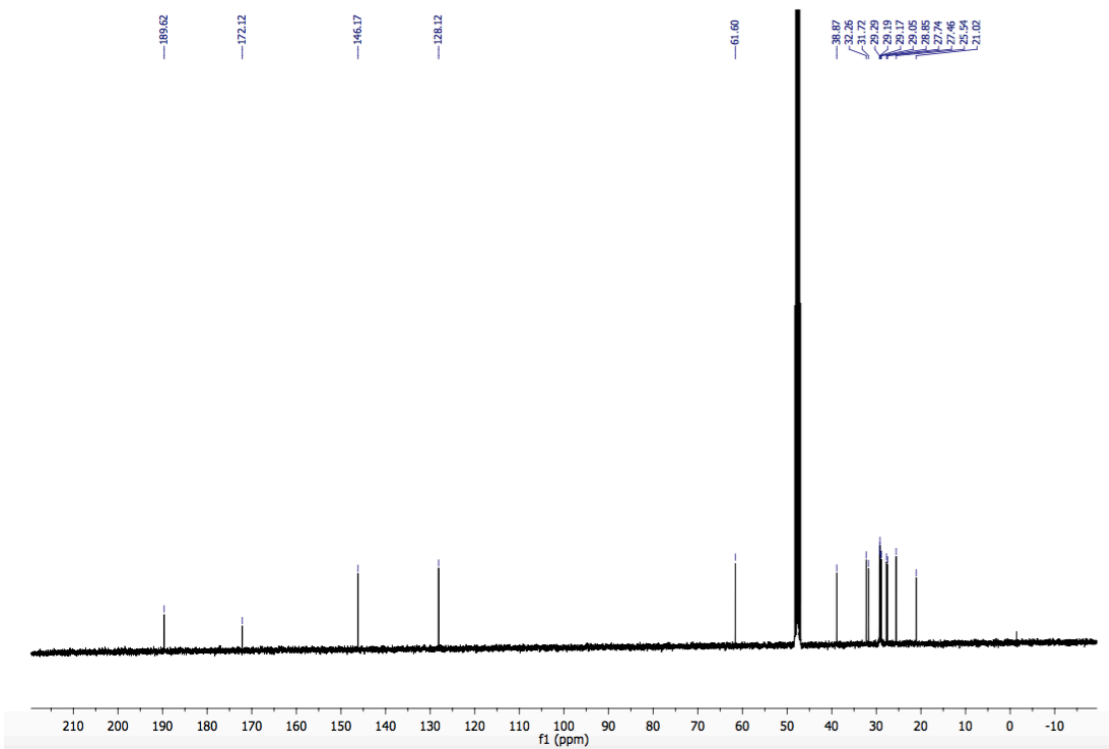
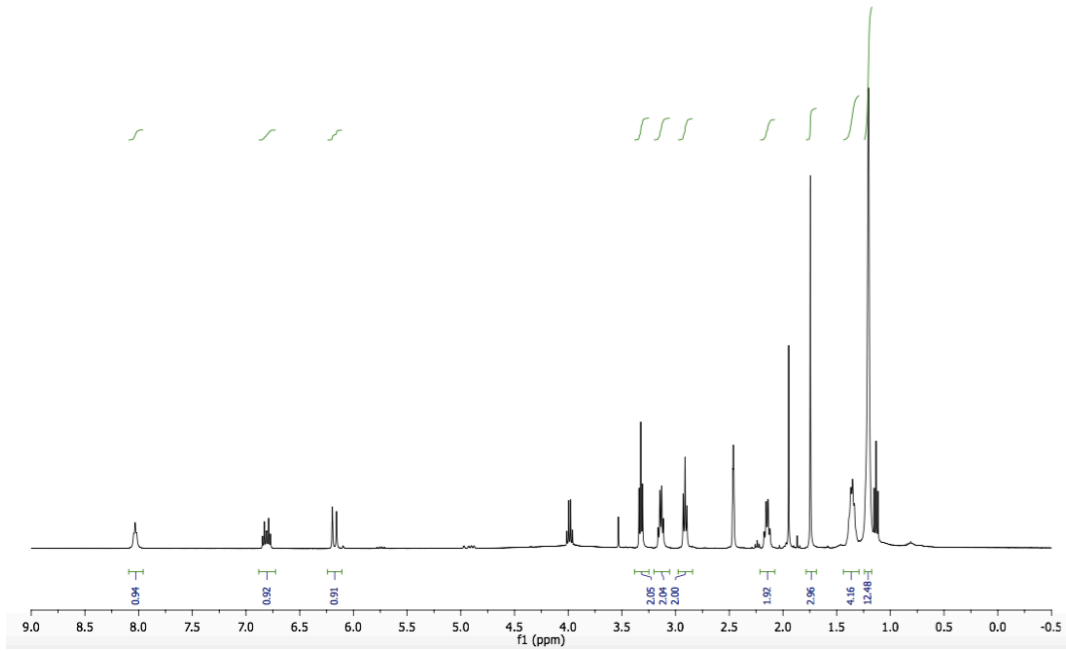


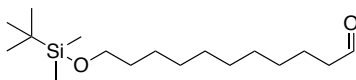
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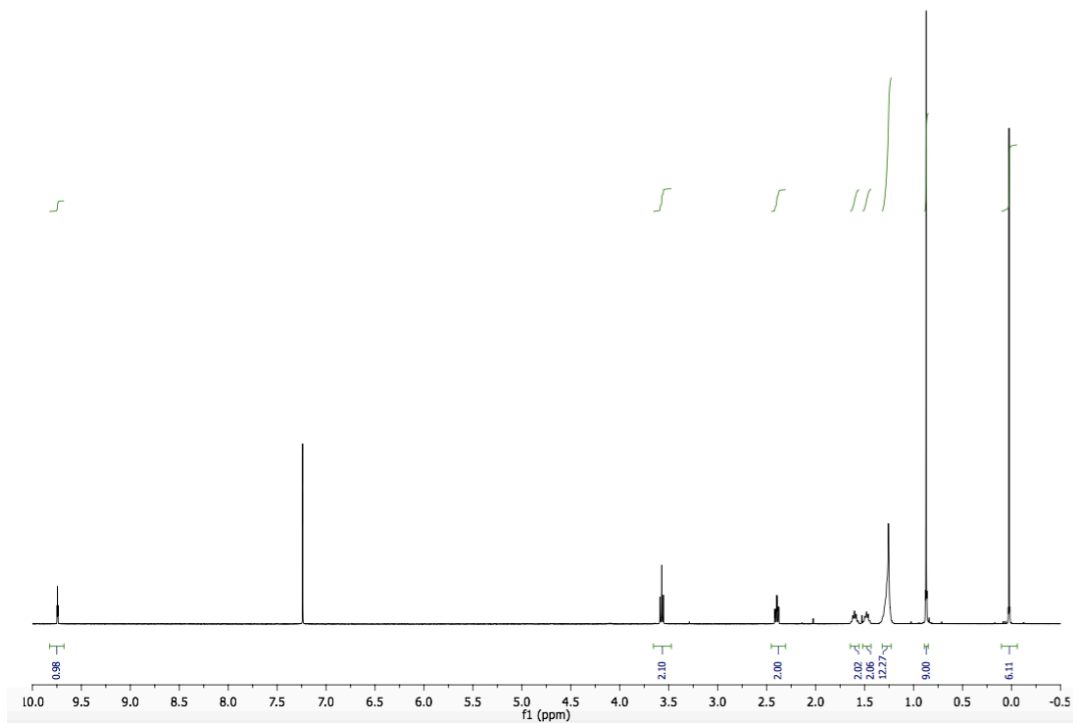


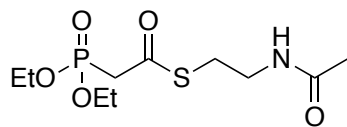
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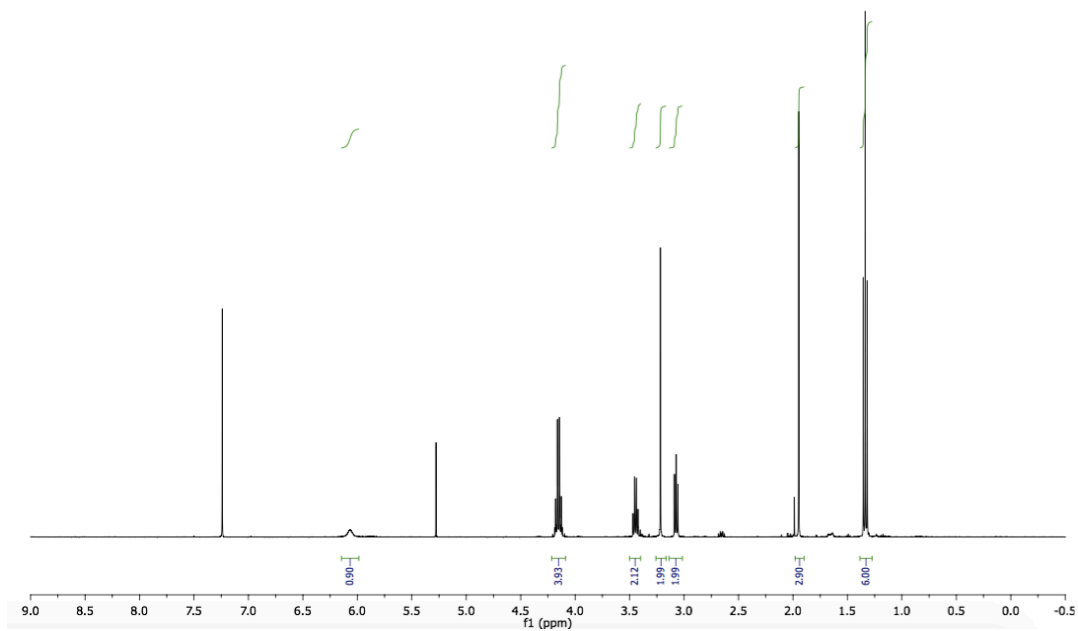


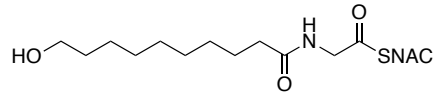
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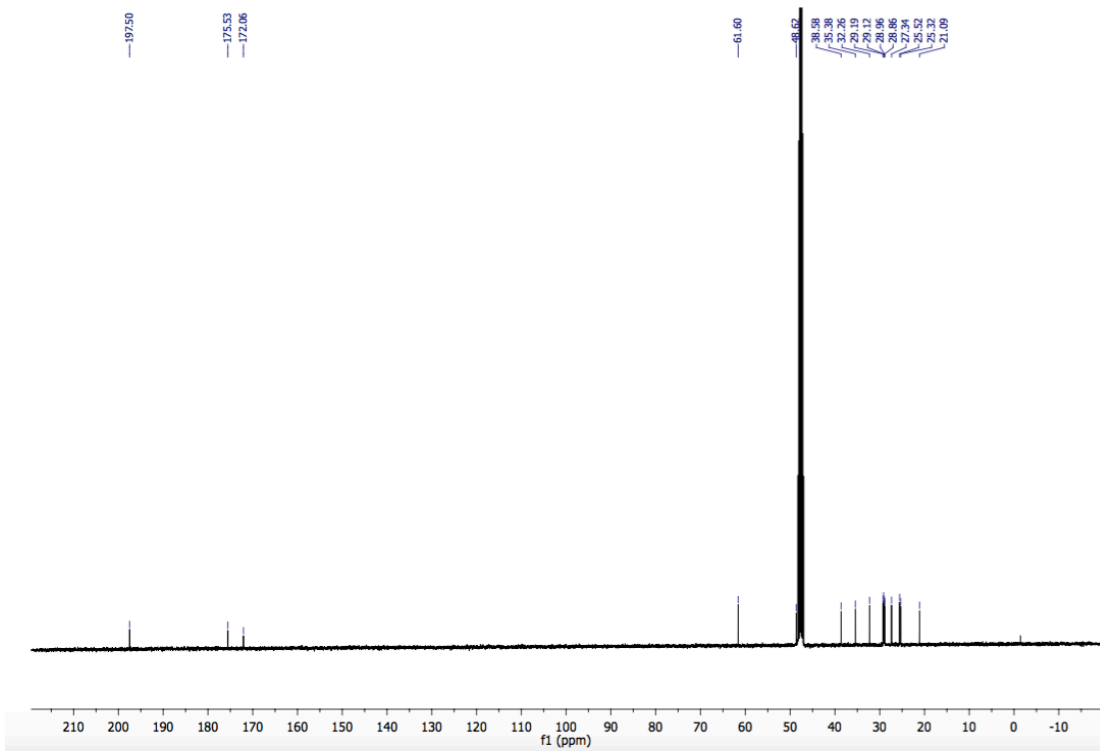
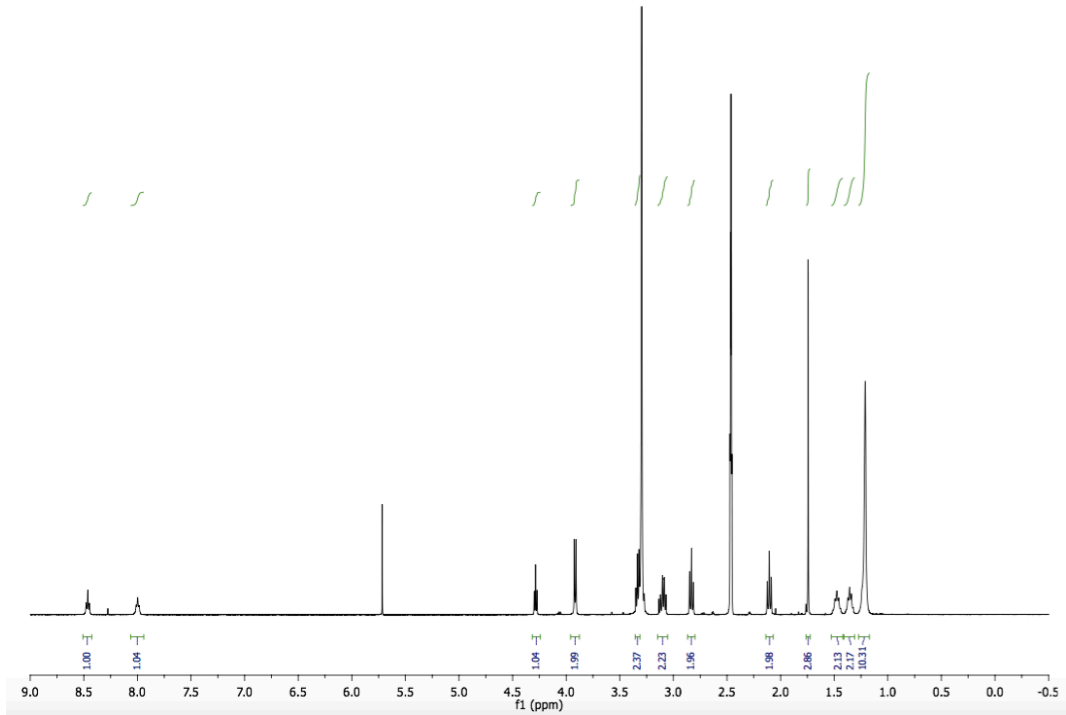


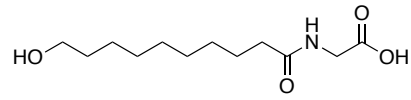
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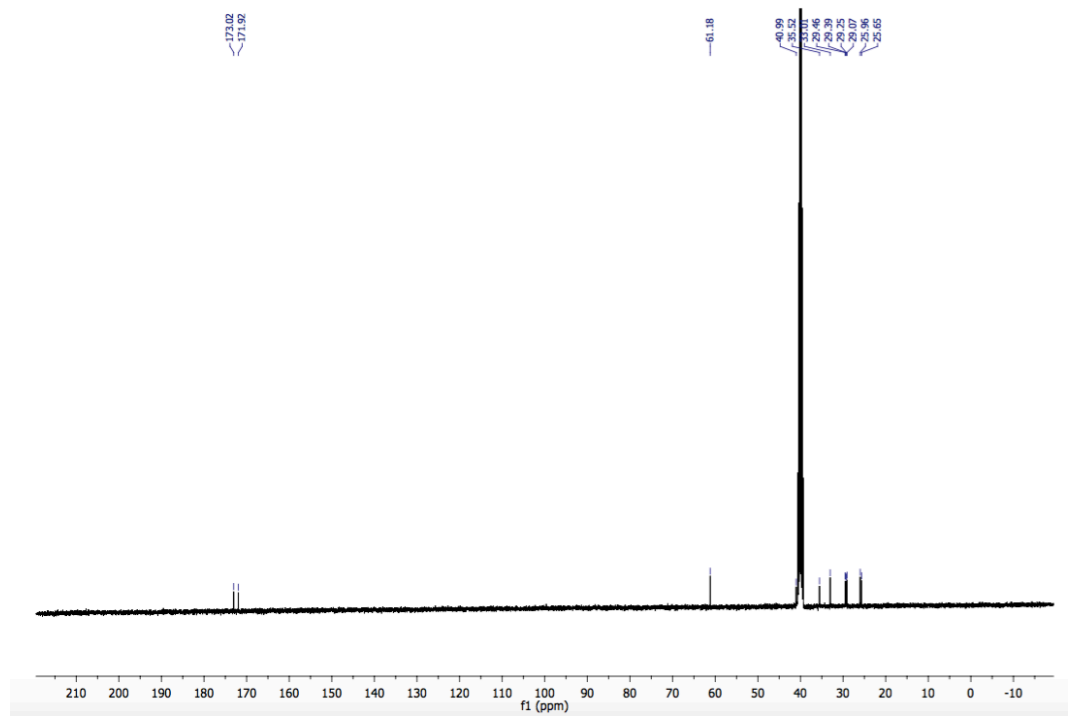
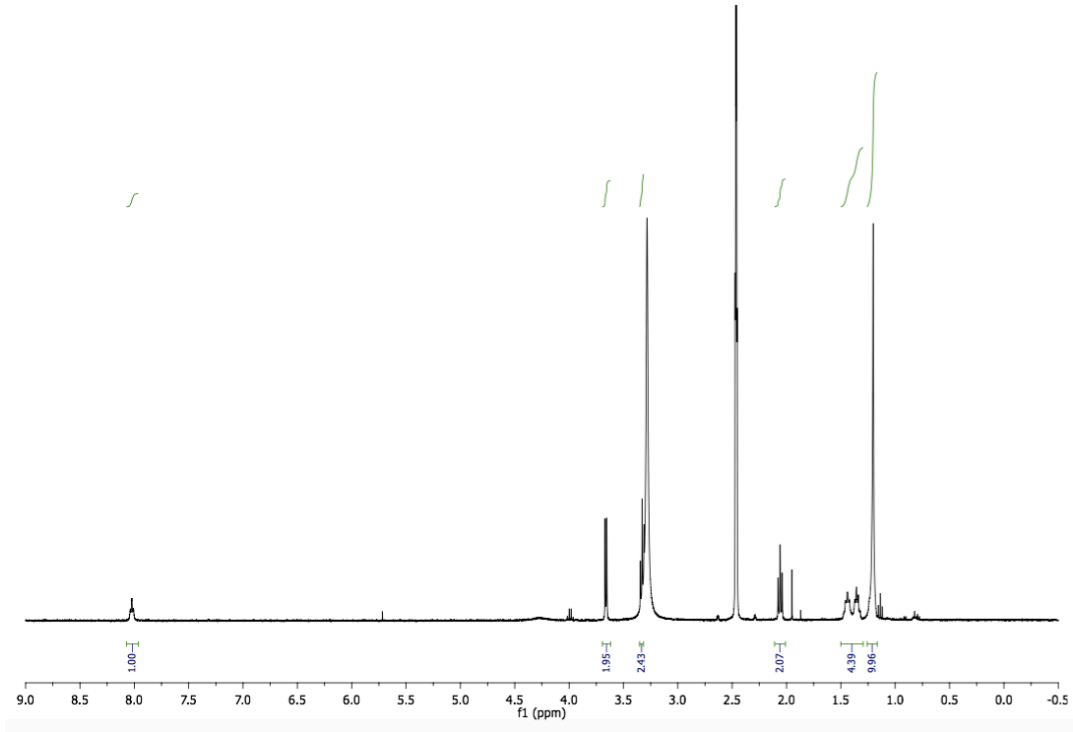


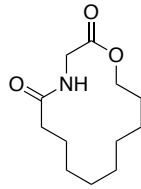
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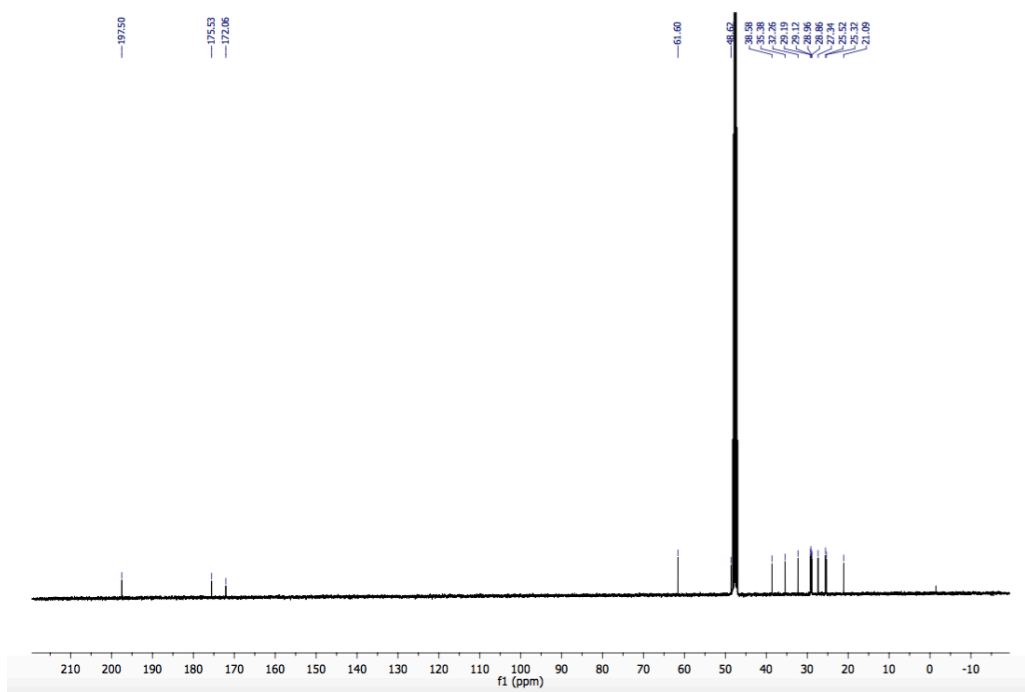
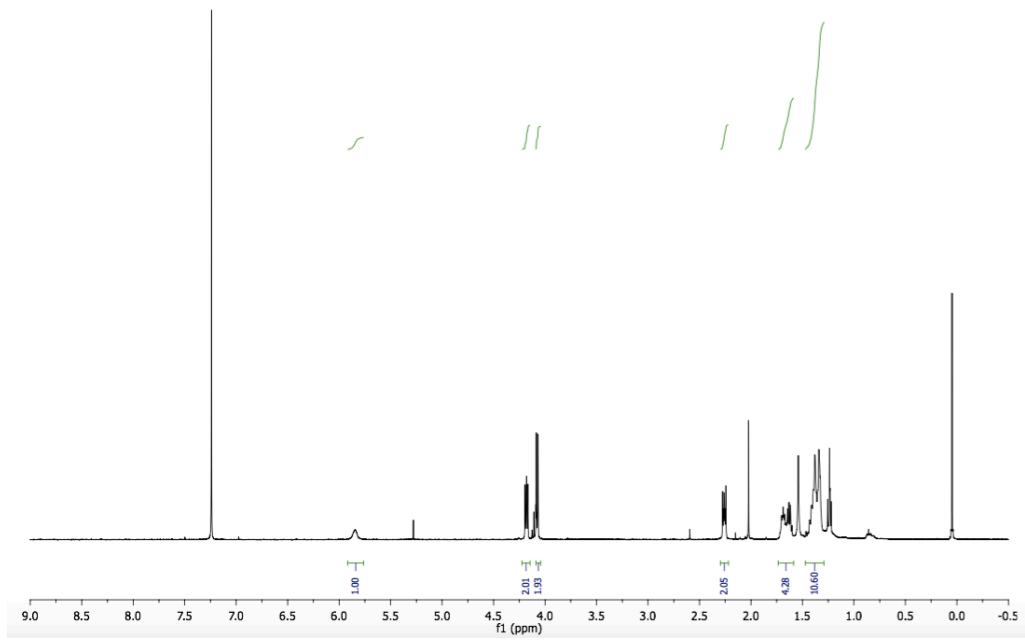


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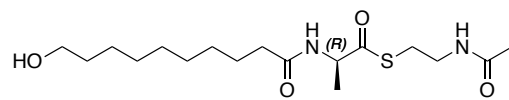




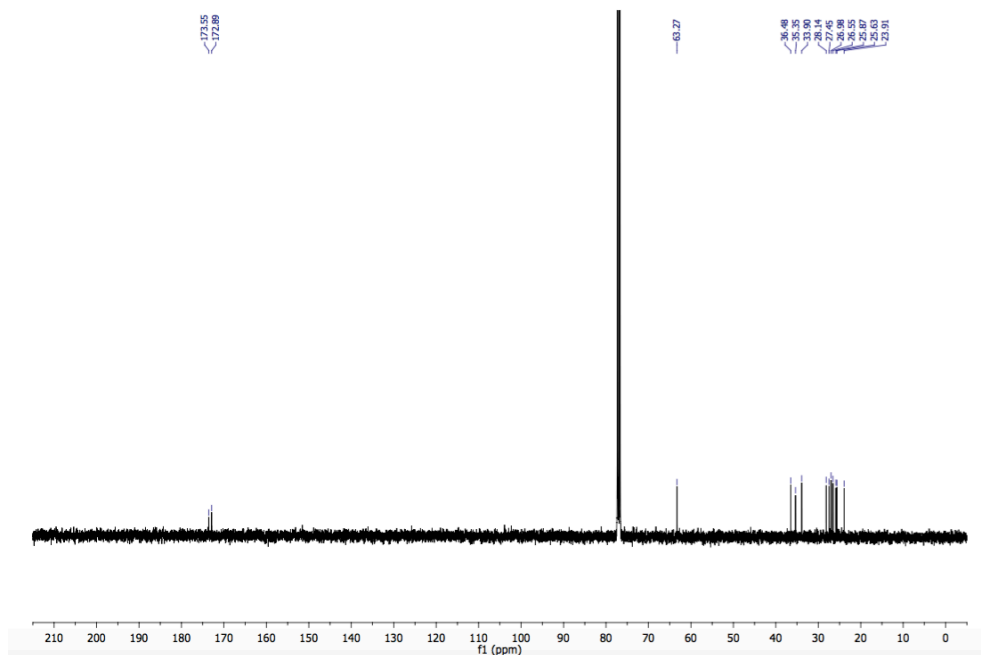
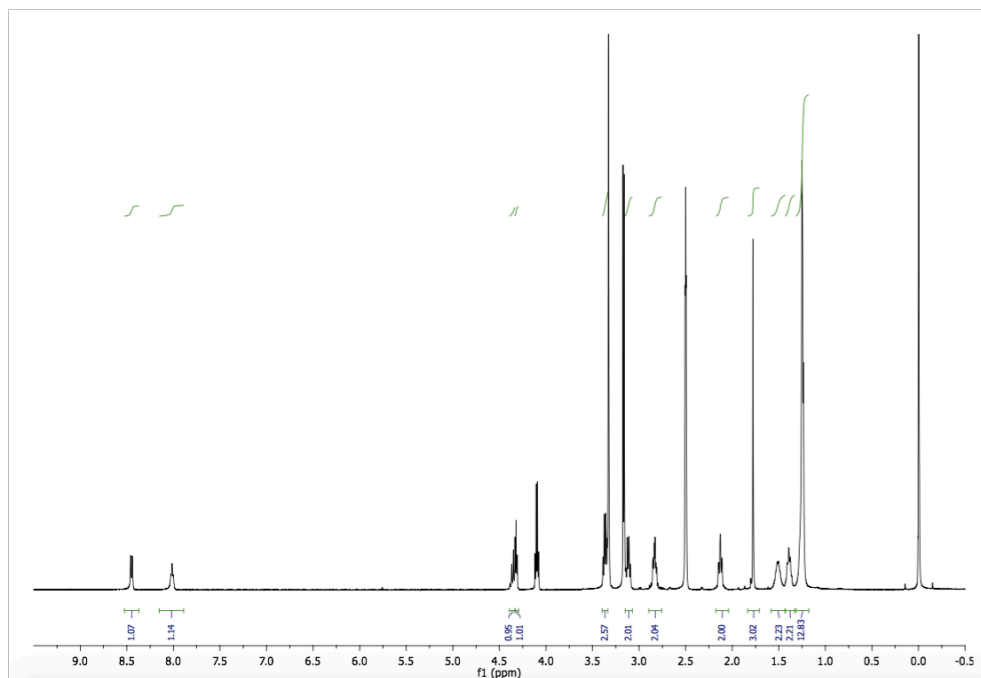
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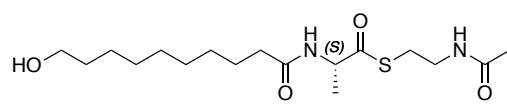


Chapter 3

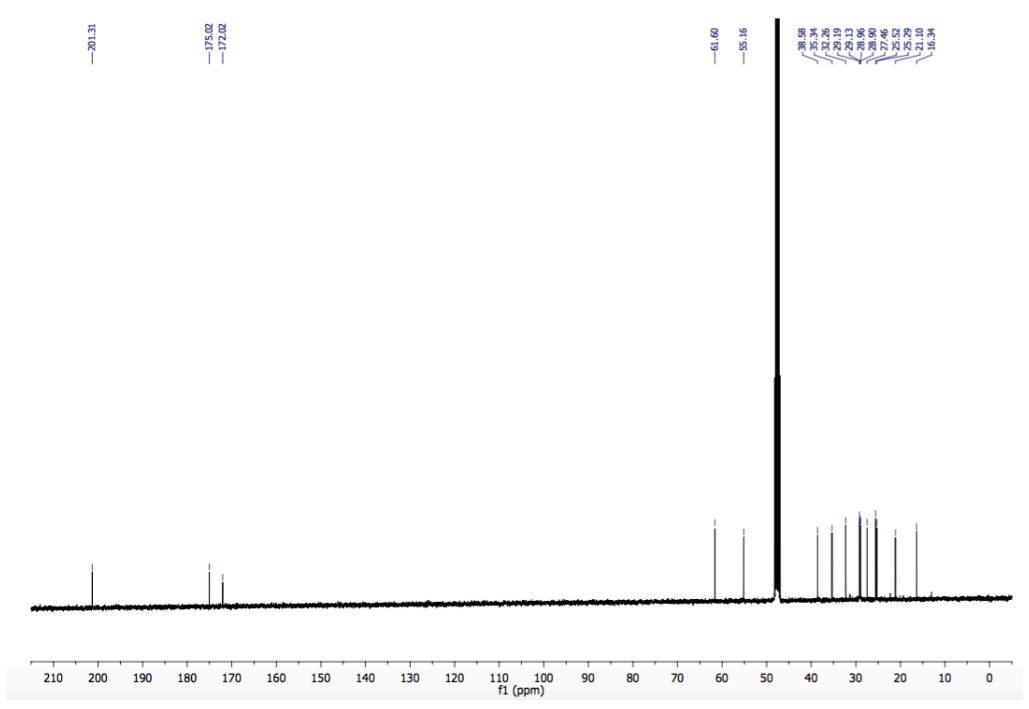
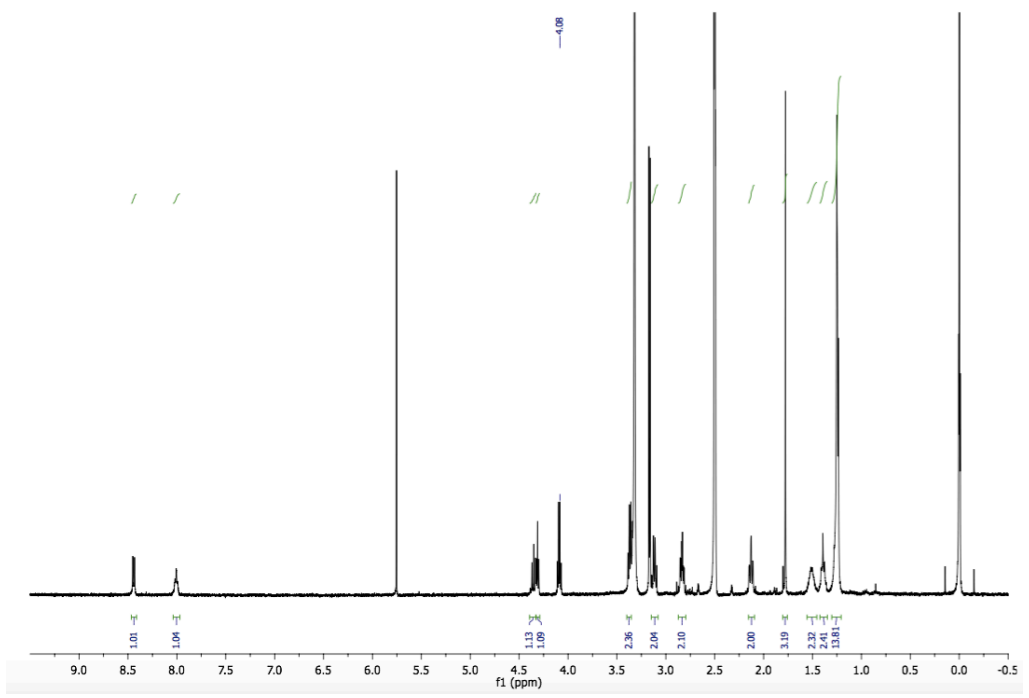


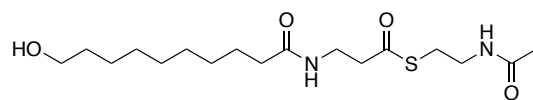
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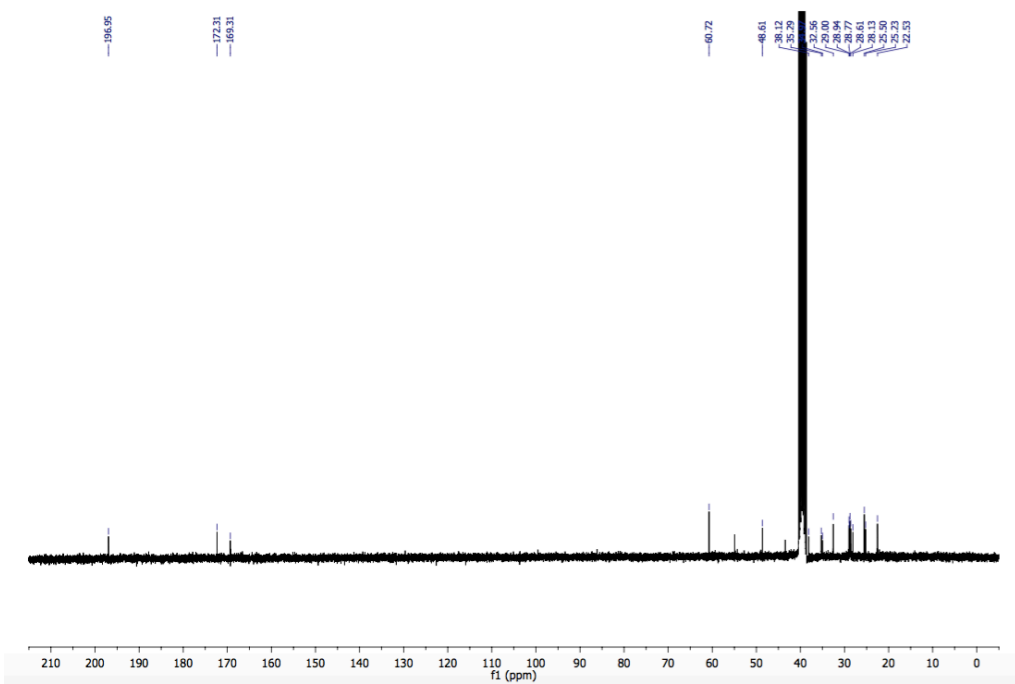
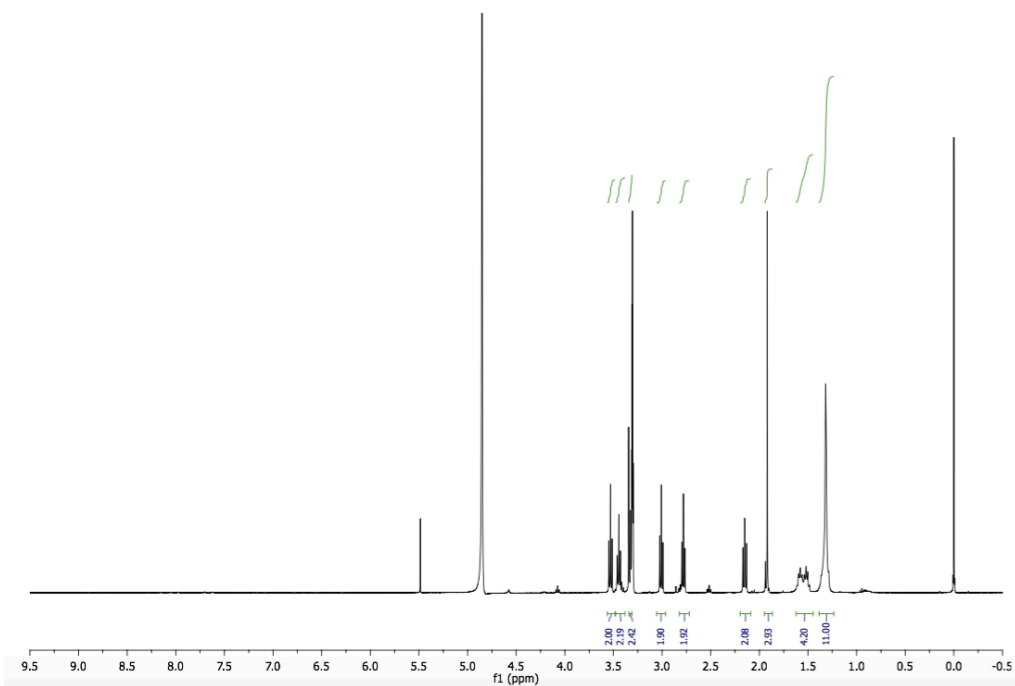


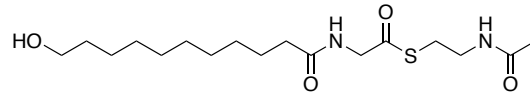
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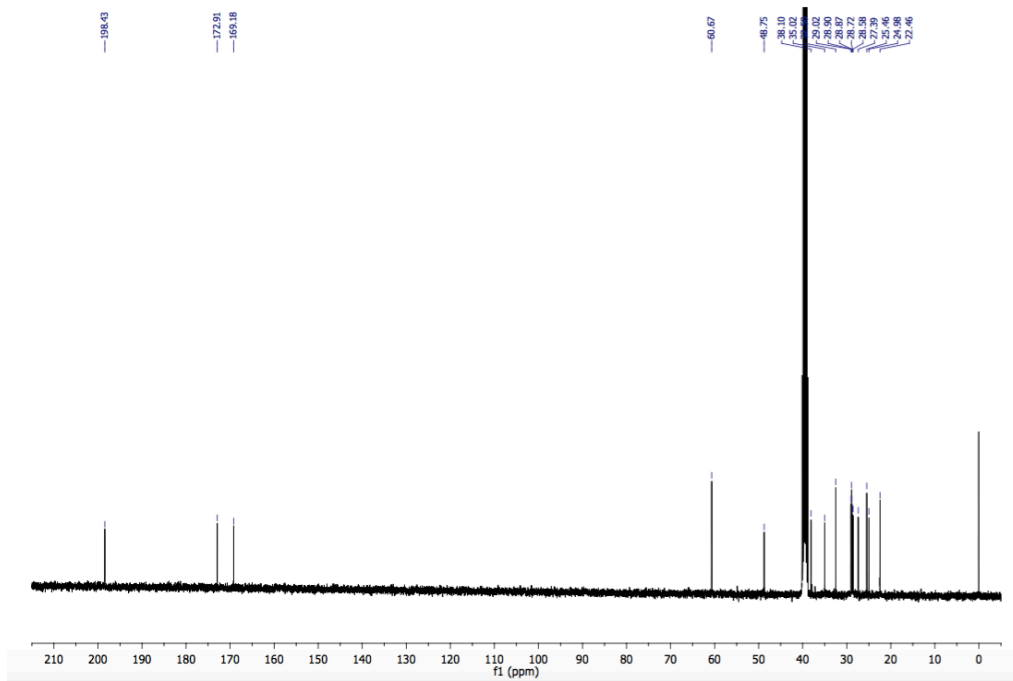
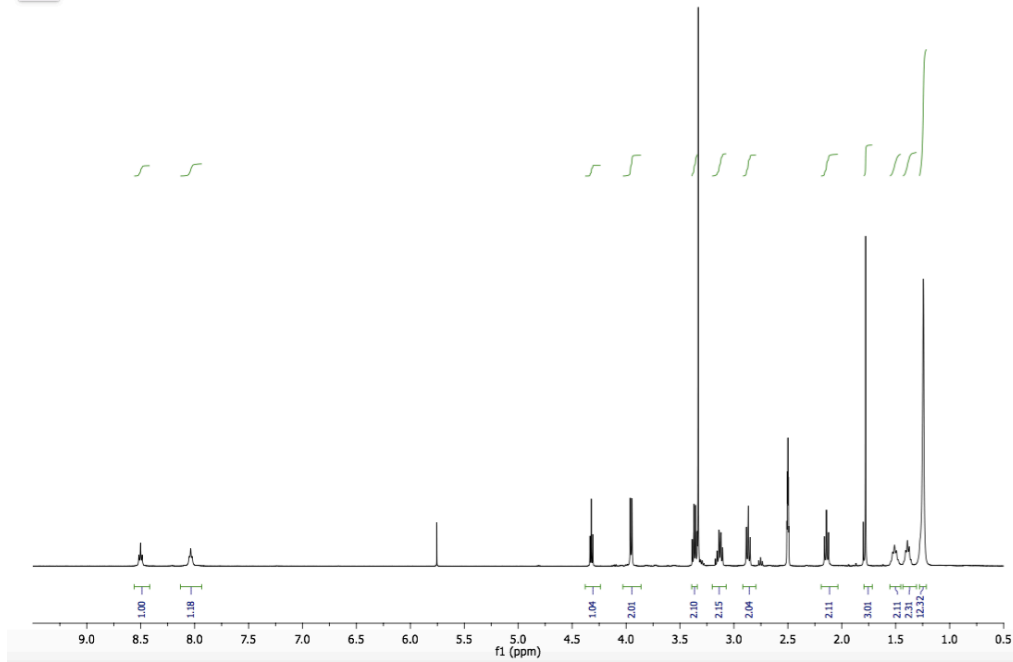
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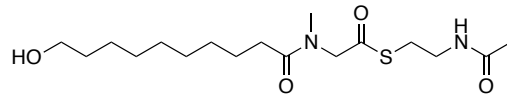




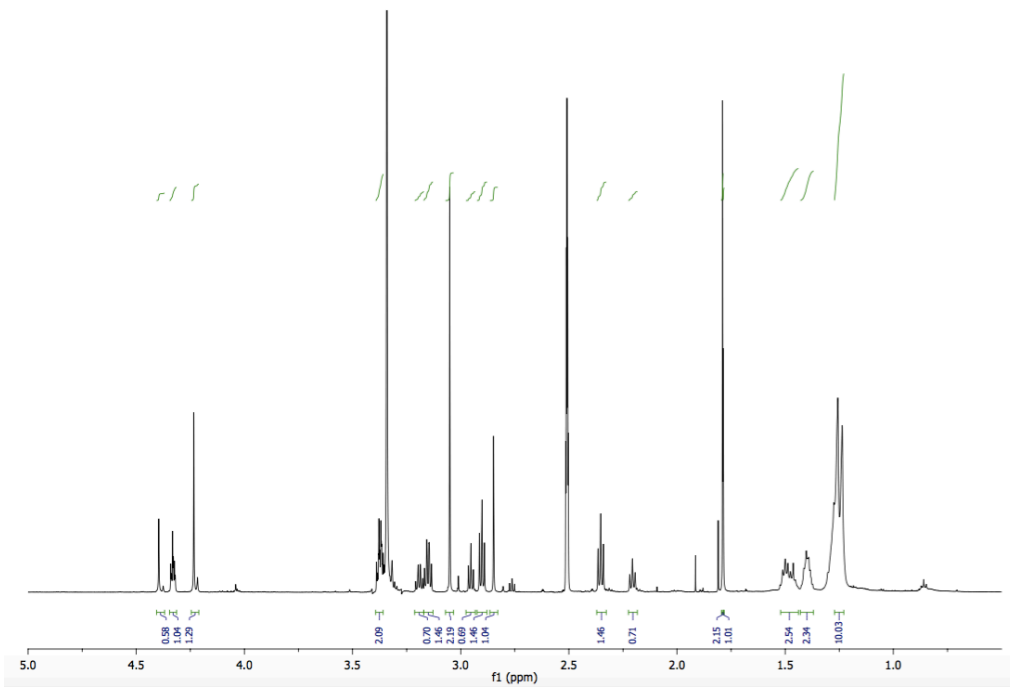
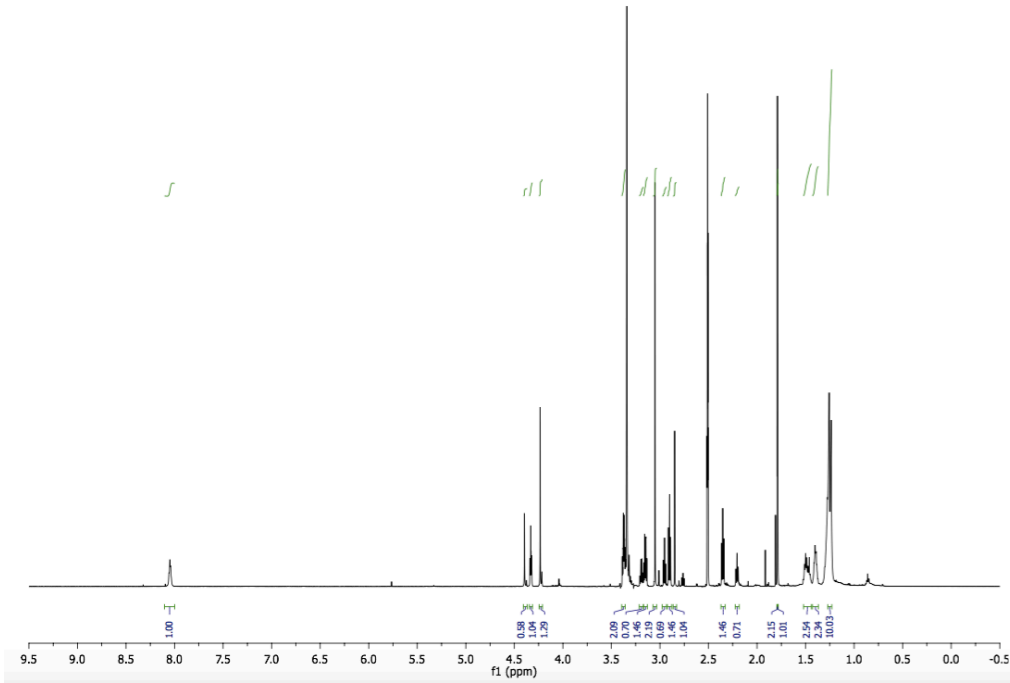
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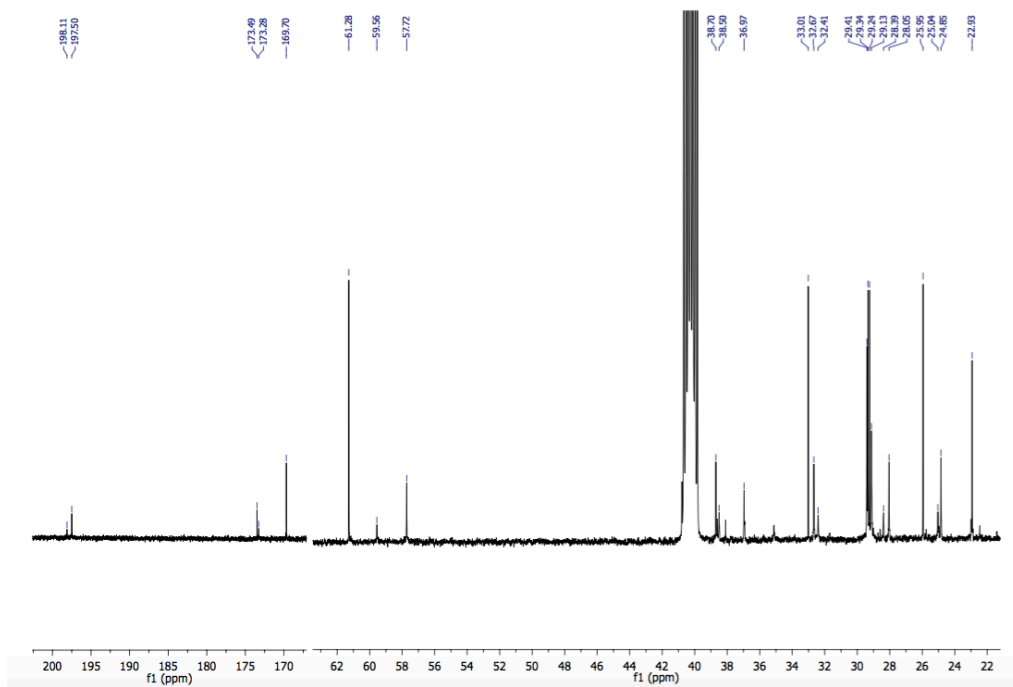
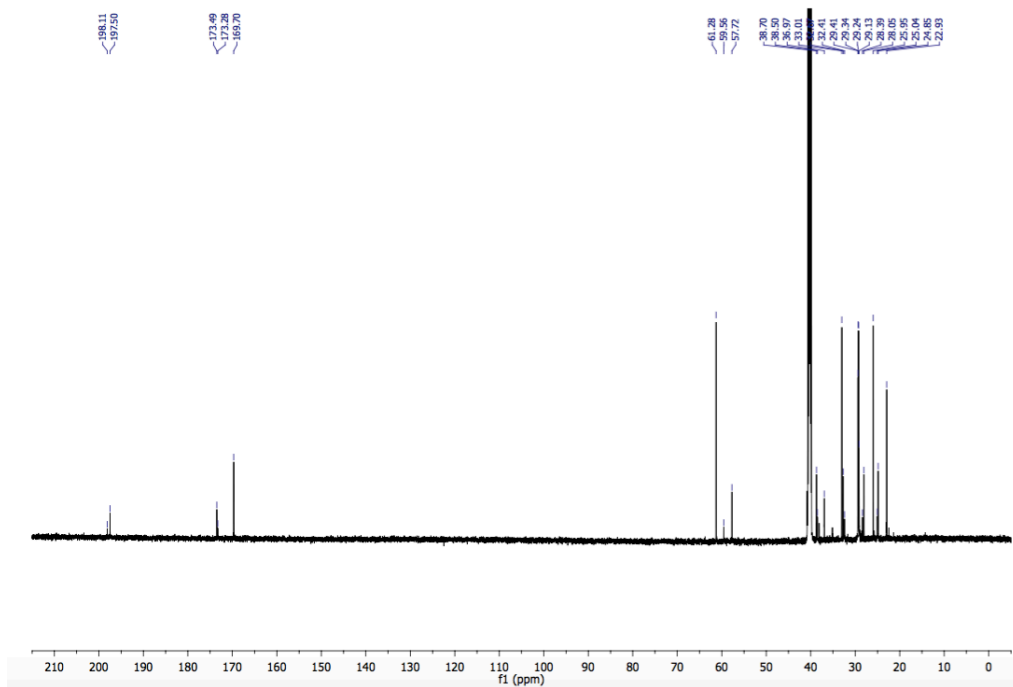
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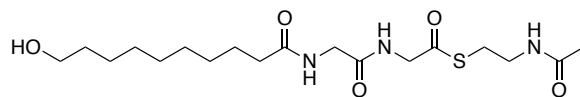




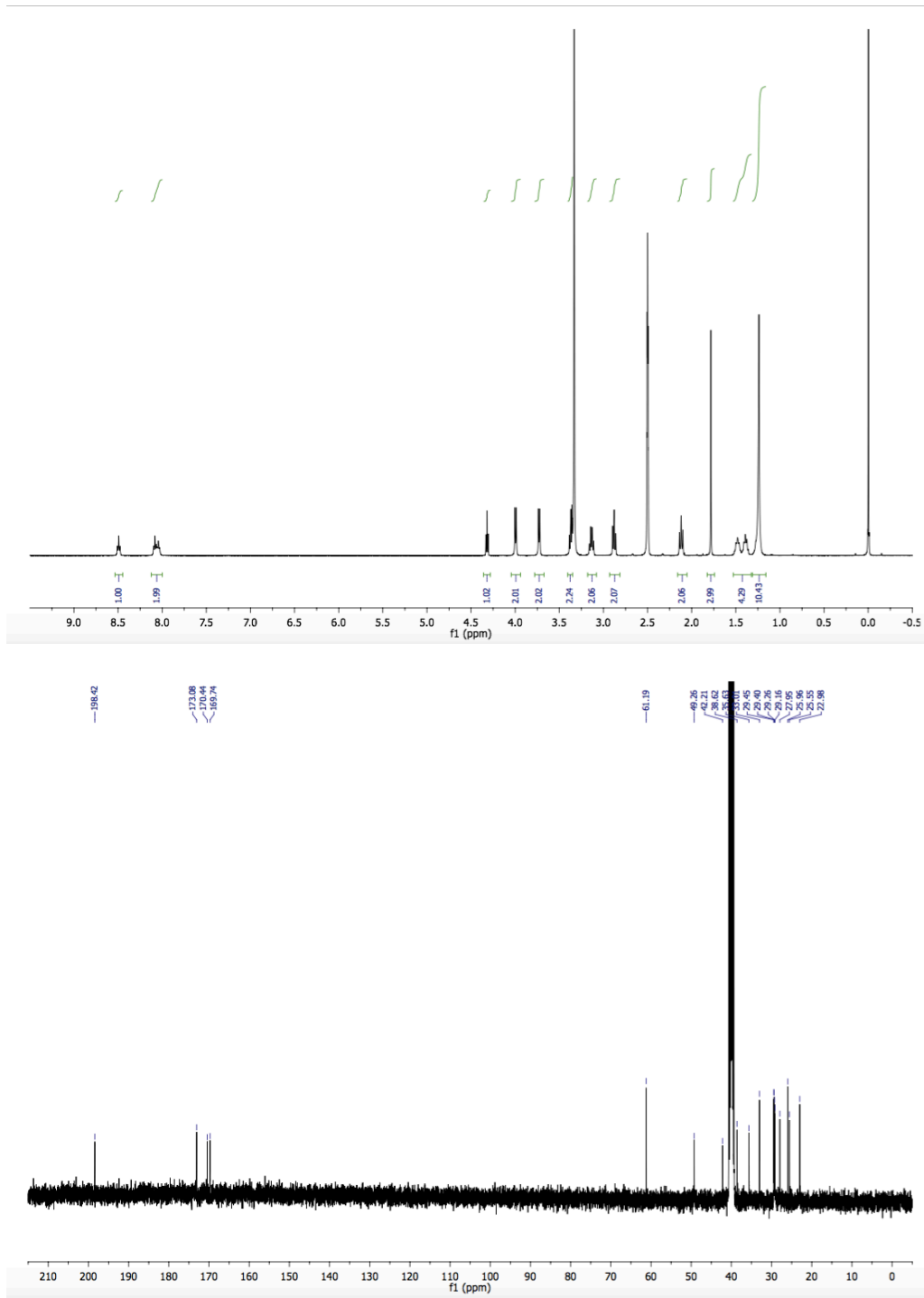
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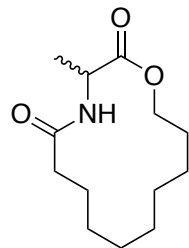




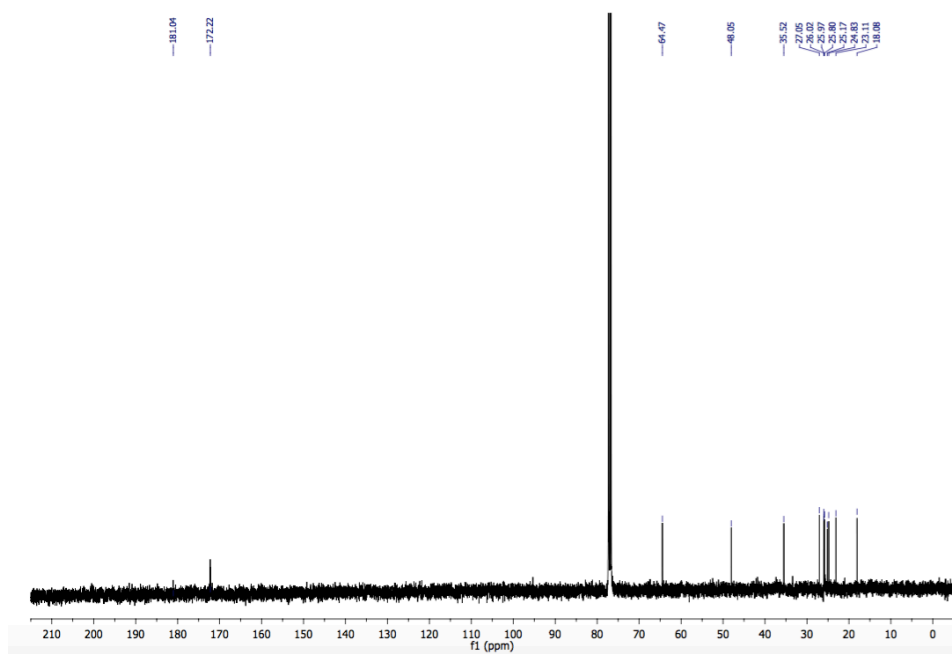
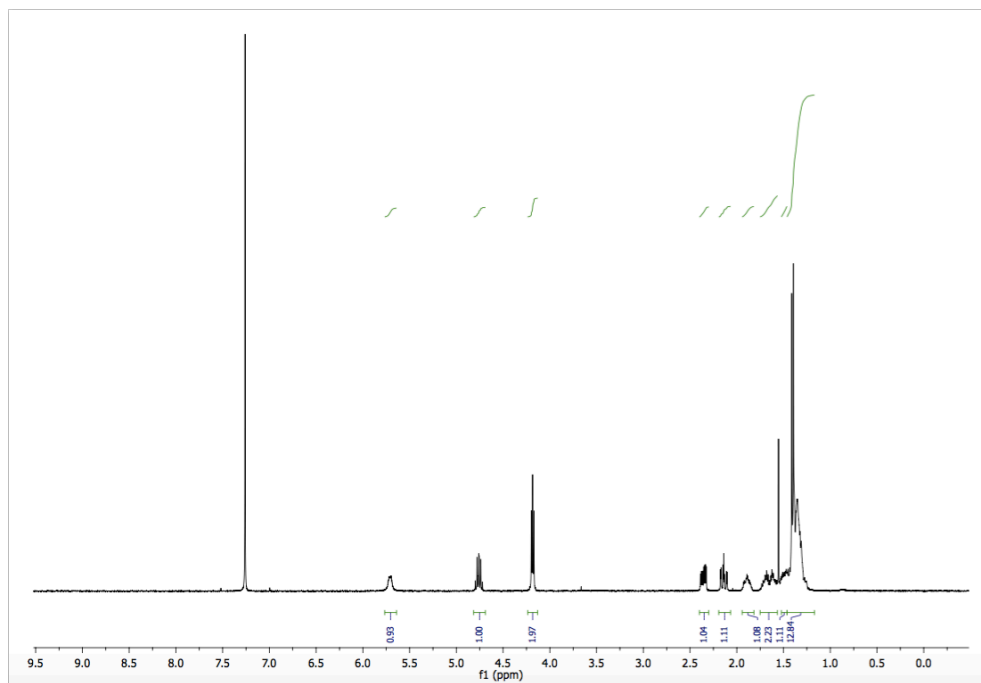


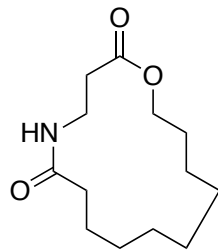
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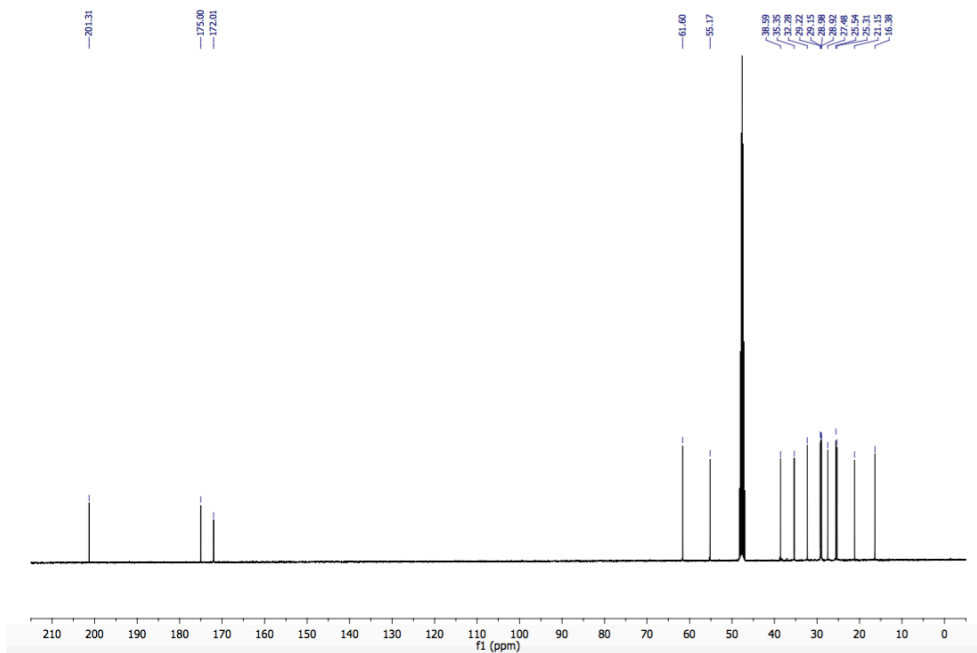
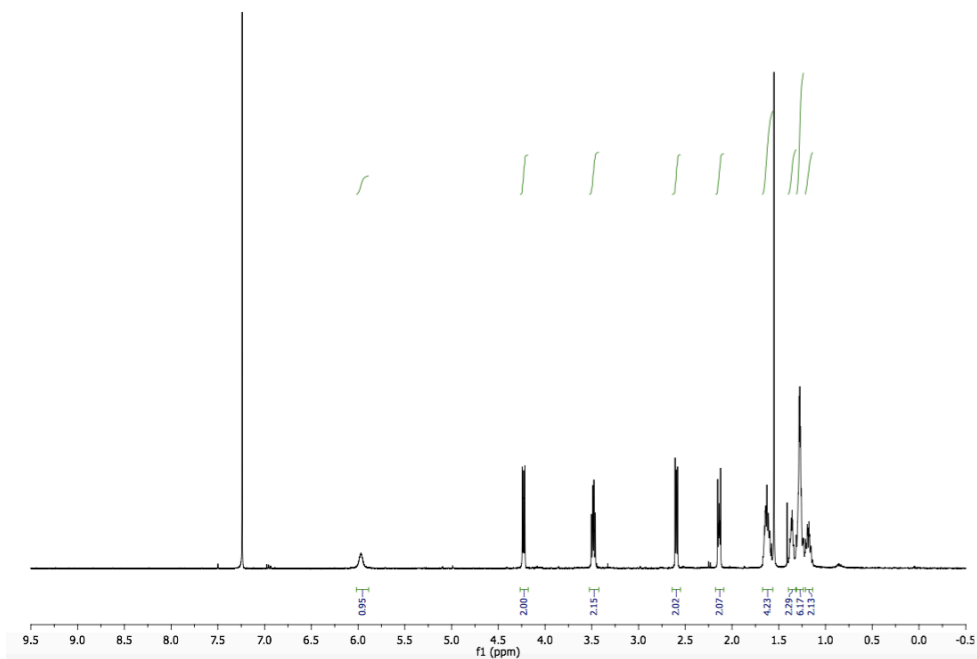


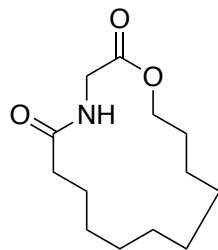
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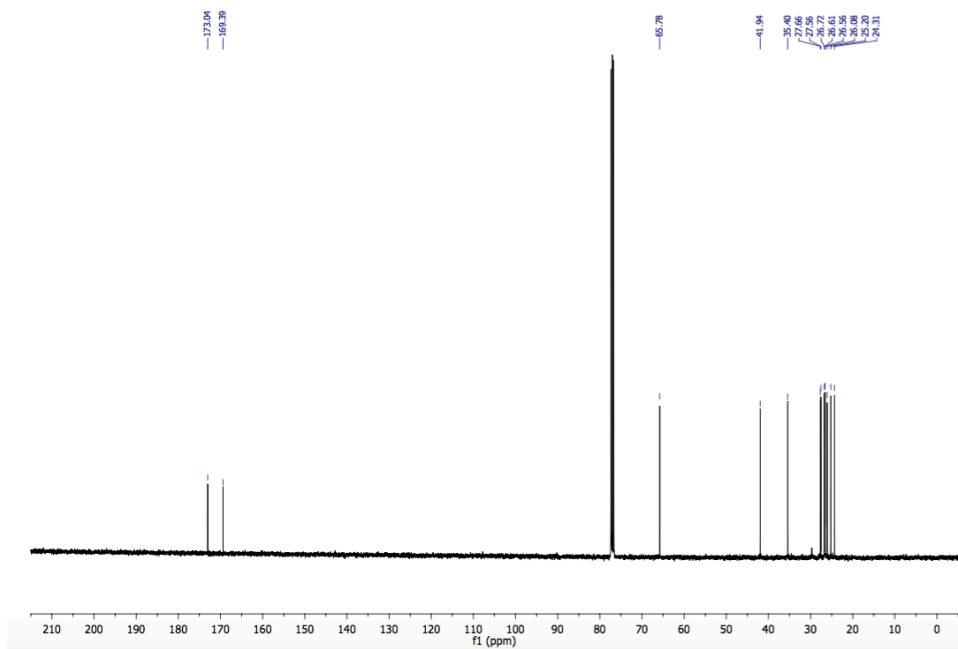
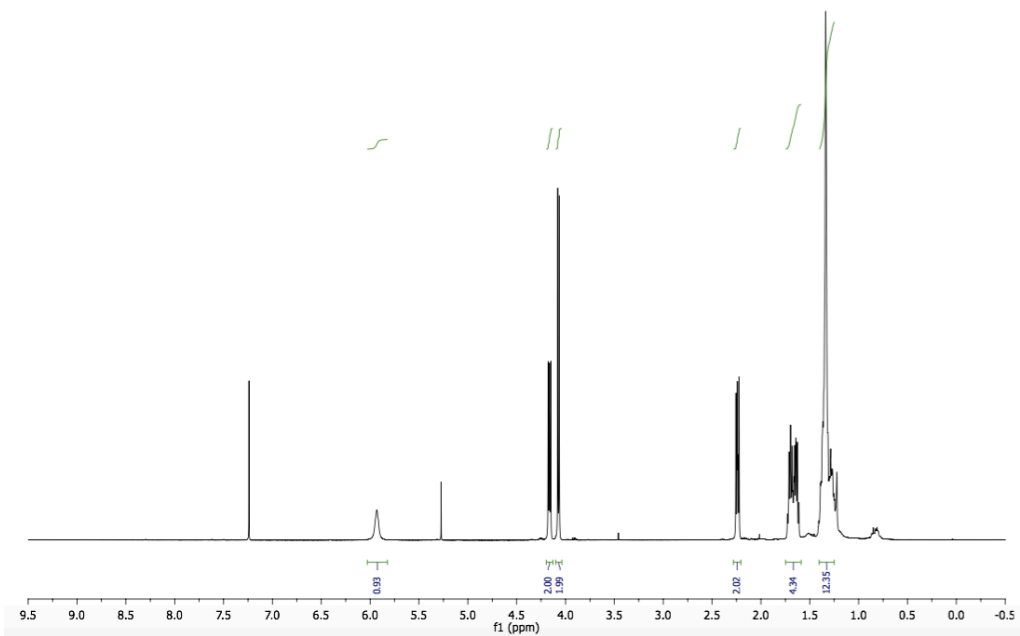


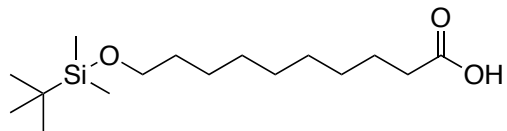
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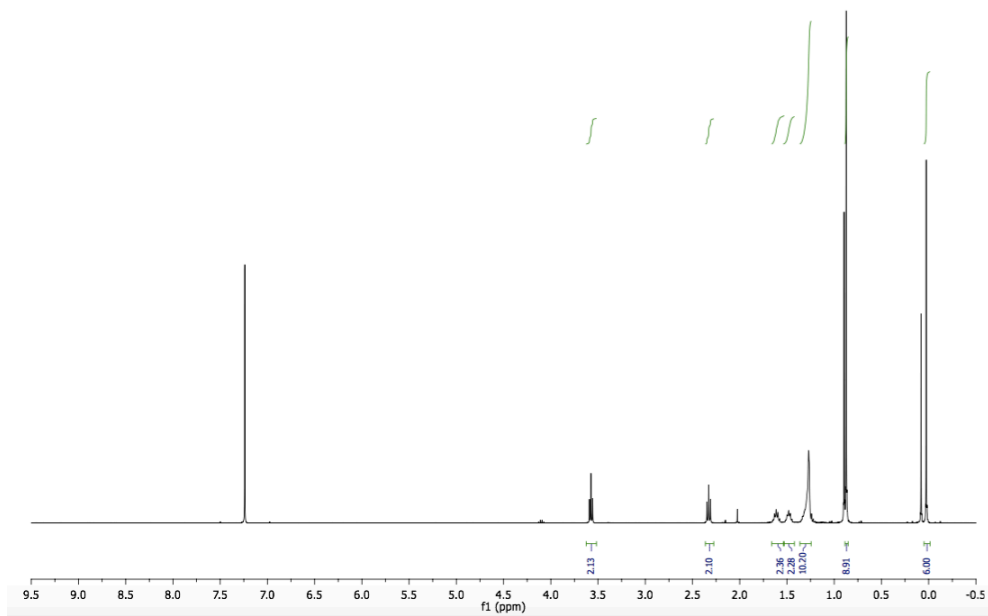


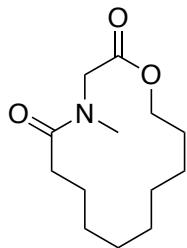
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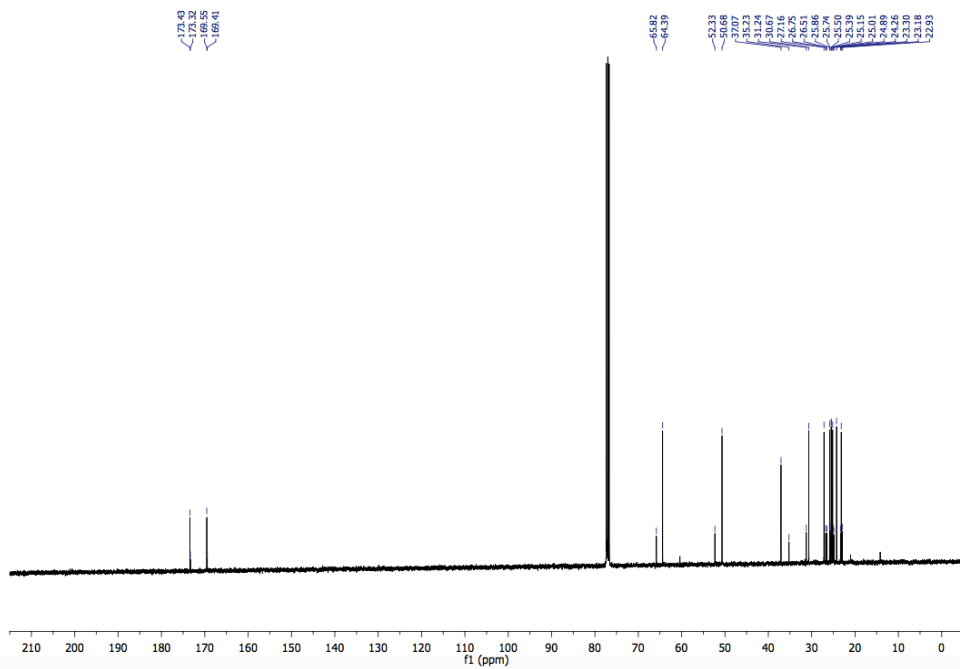
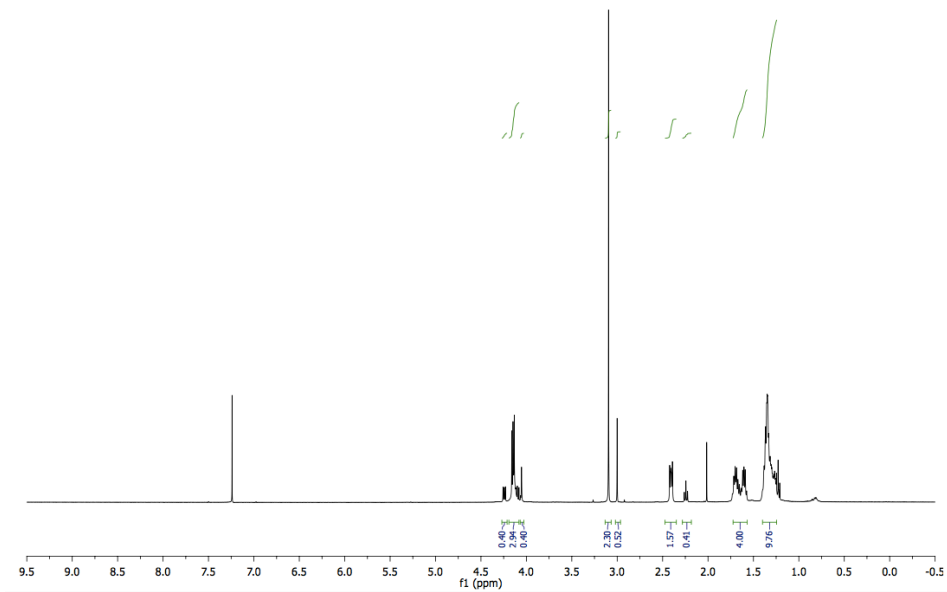


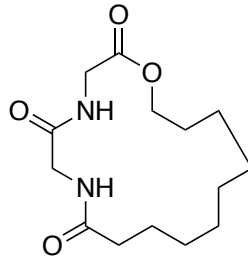
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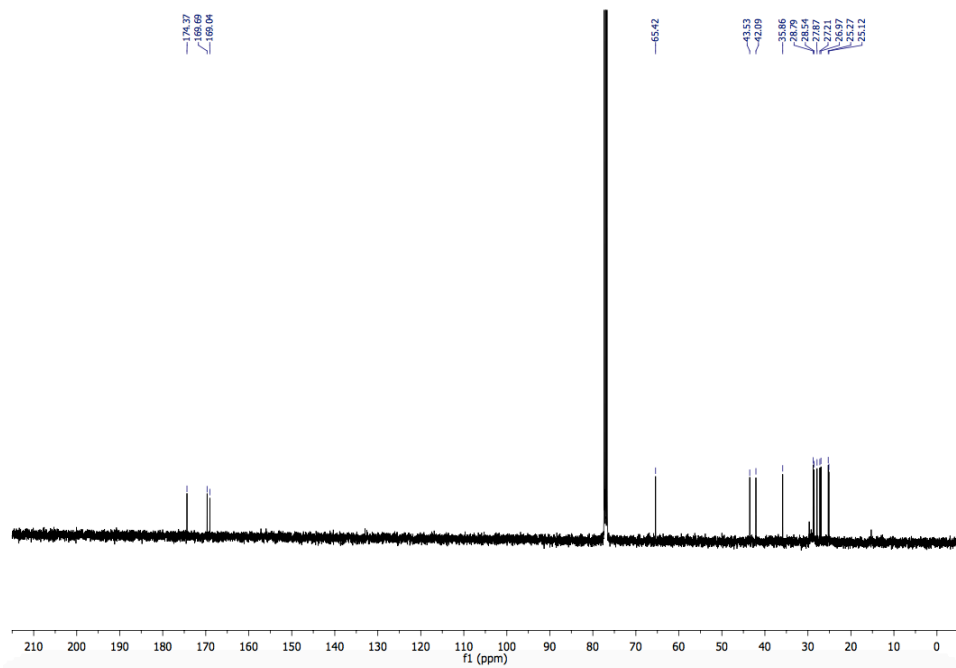
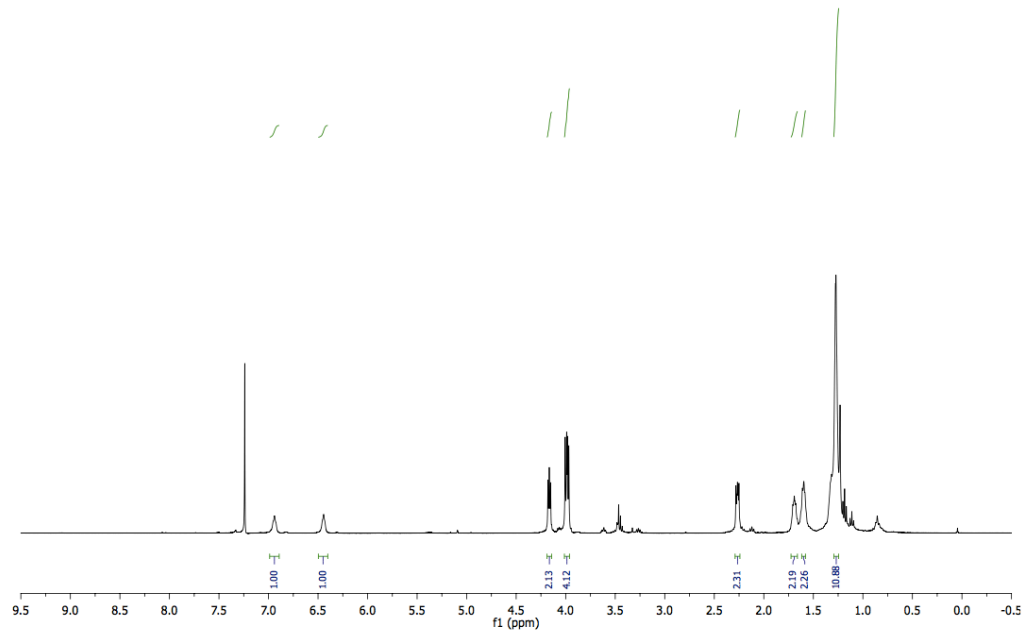


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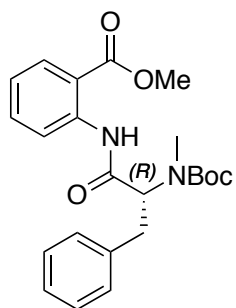




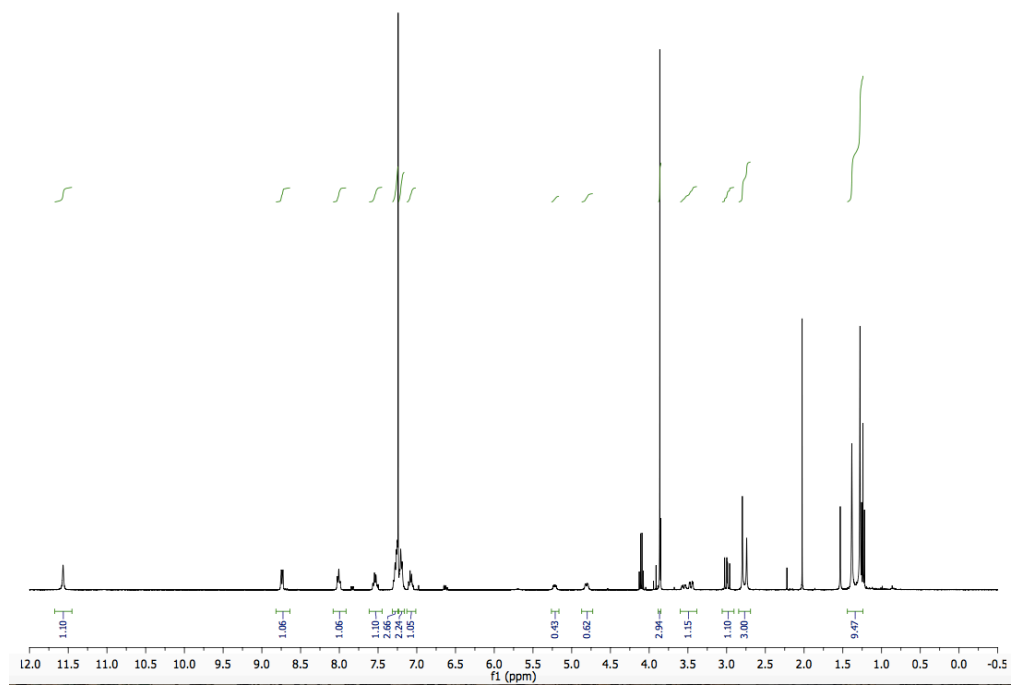
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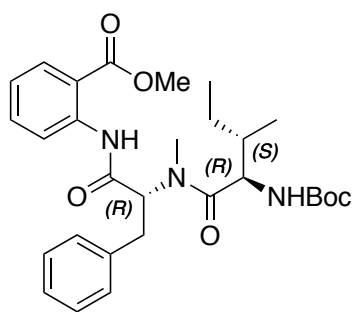


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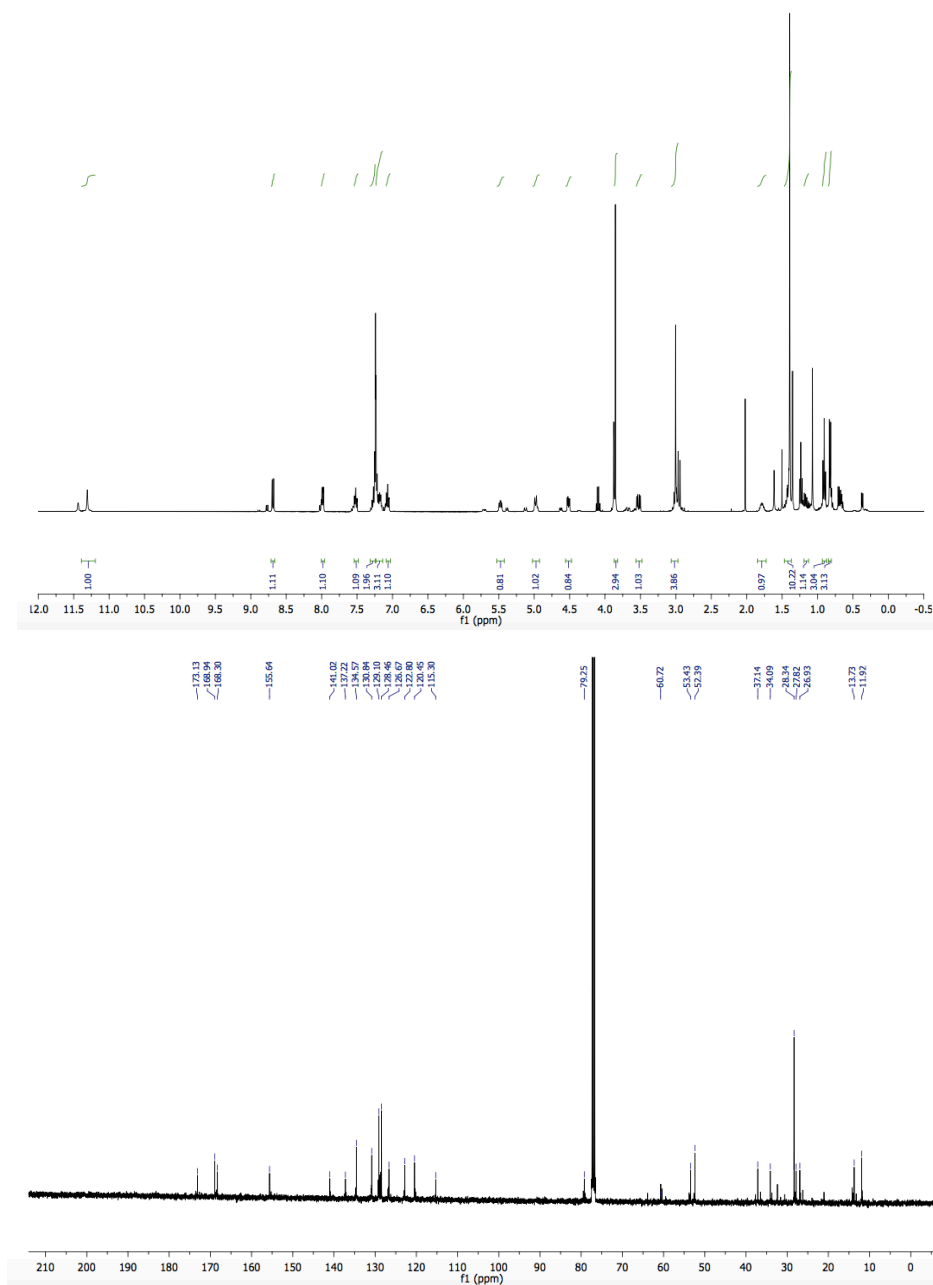


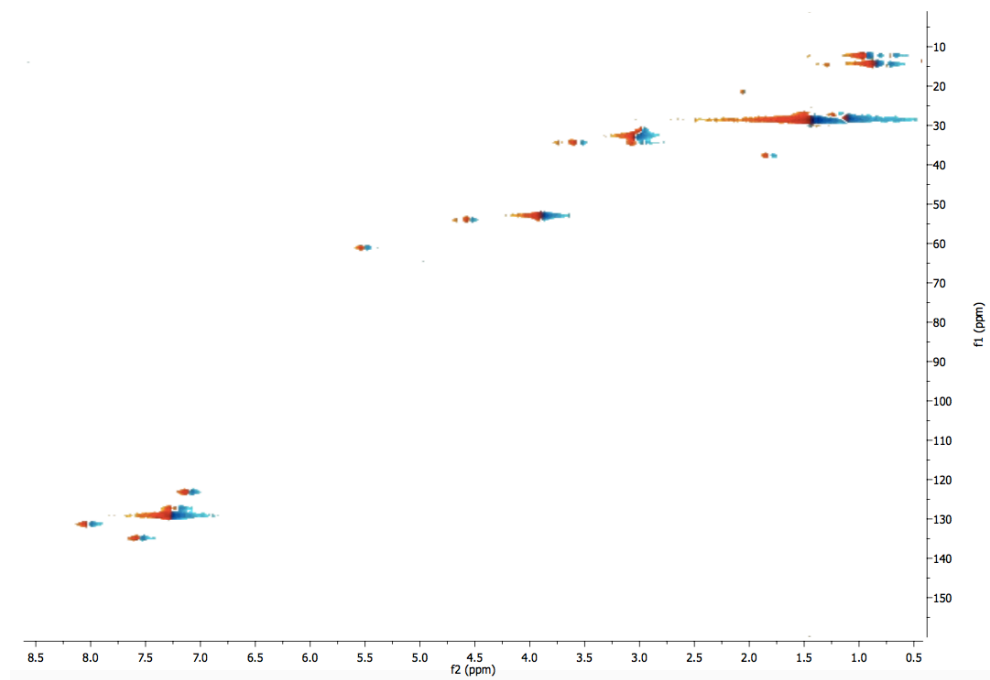
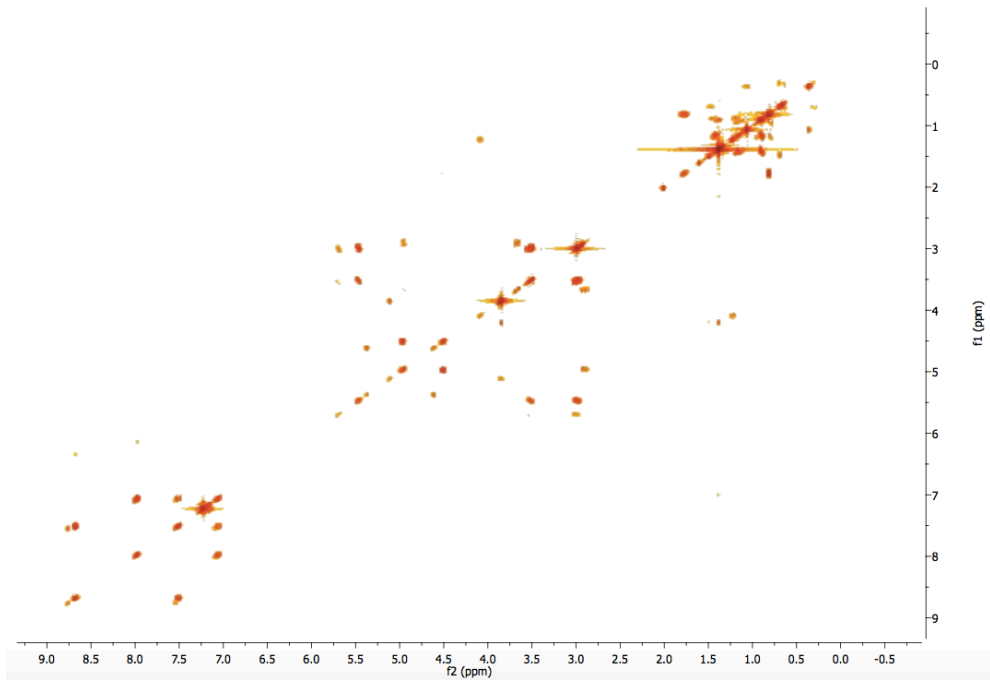
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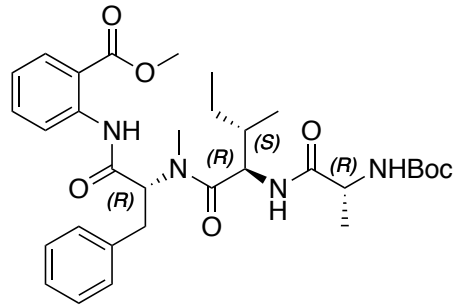




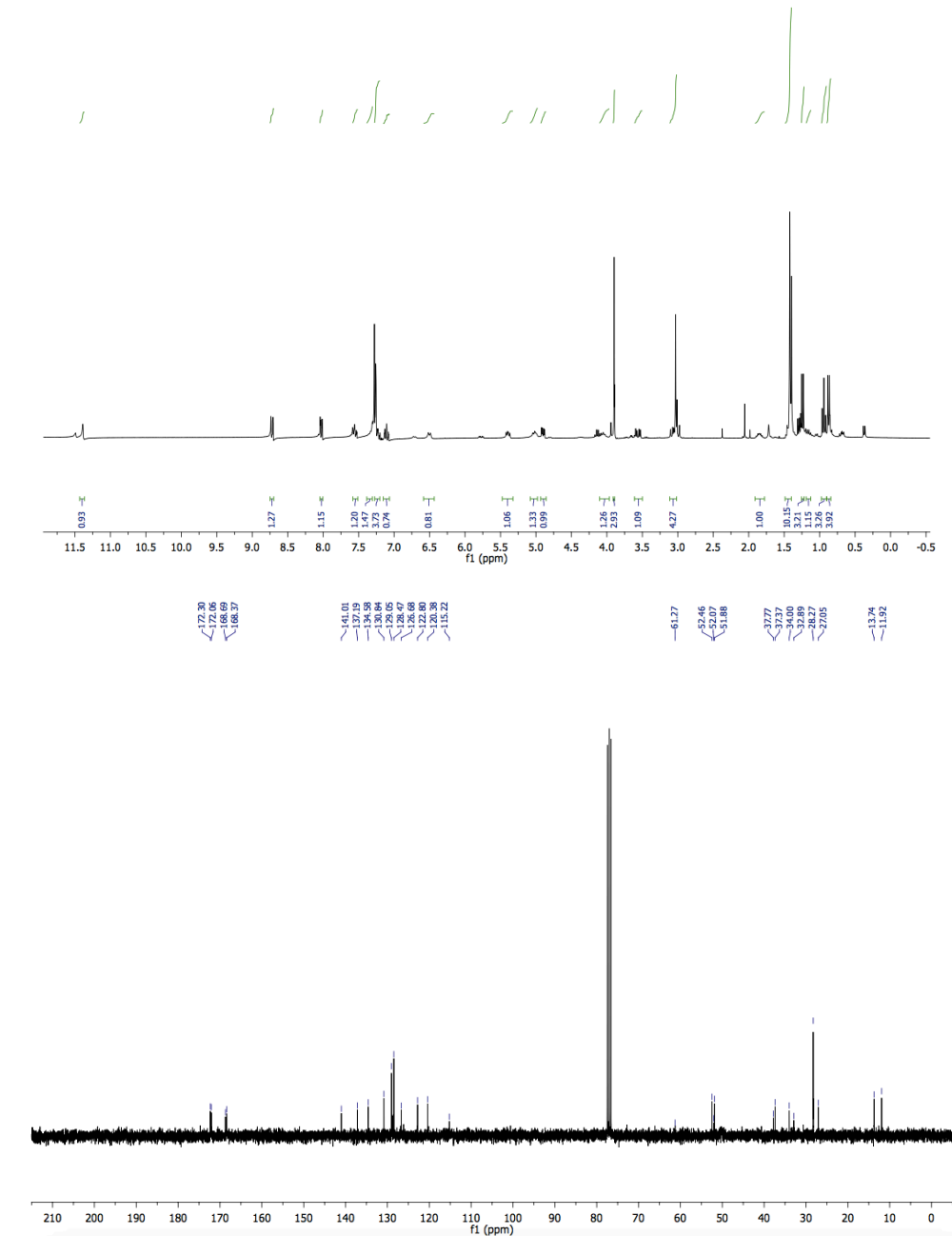
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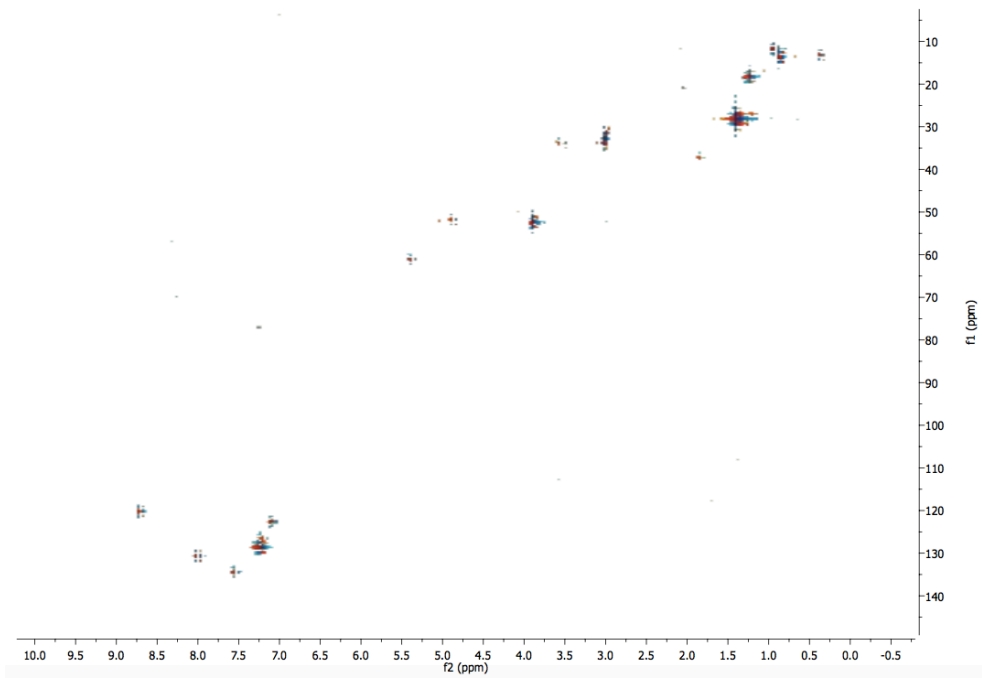
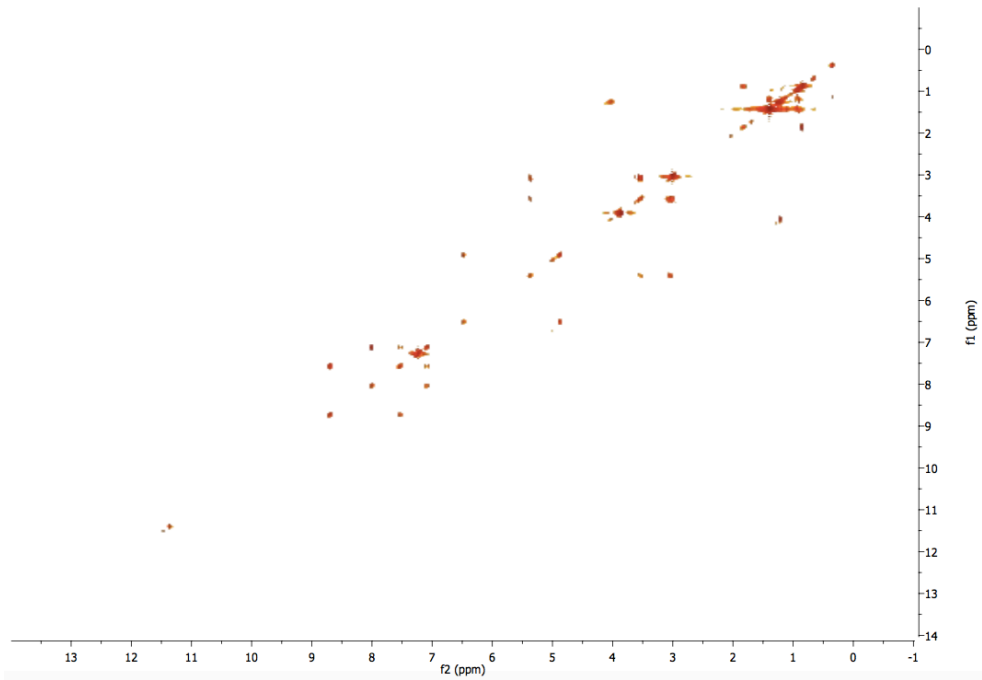


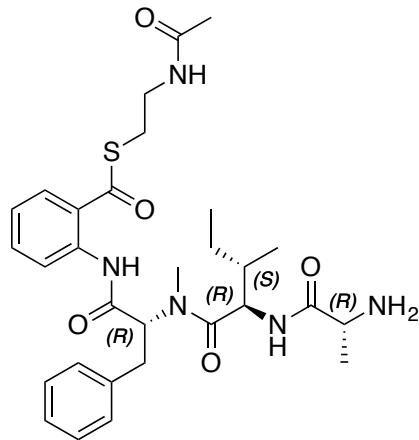




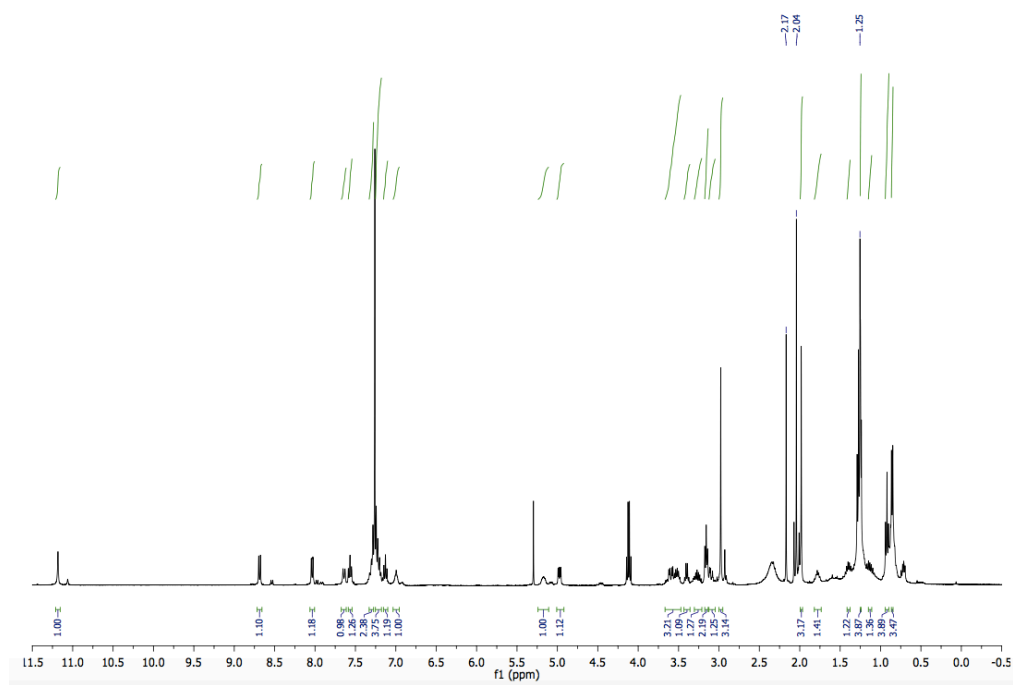
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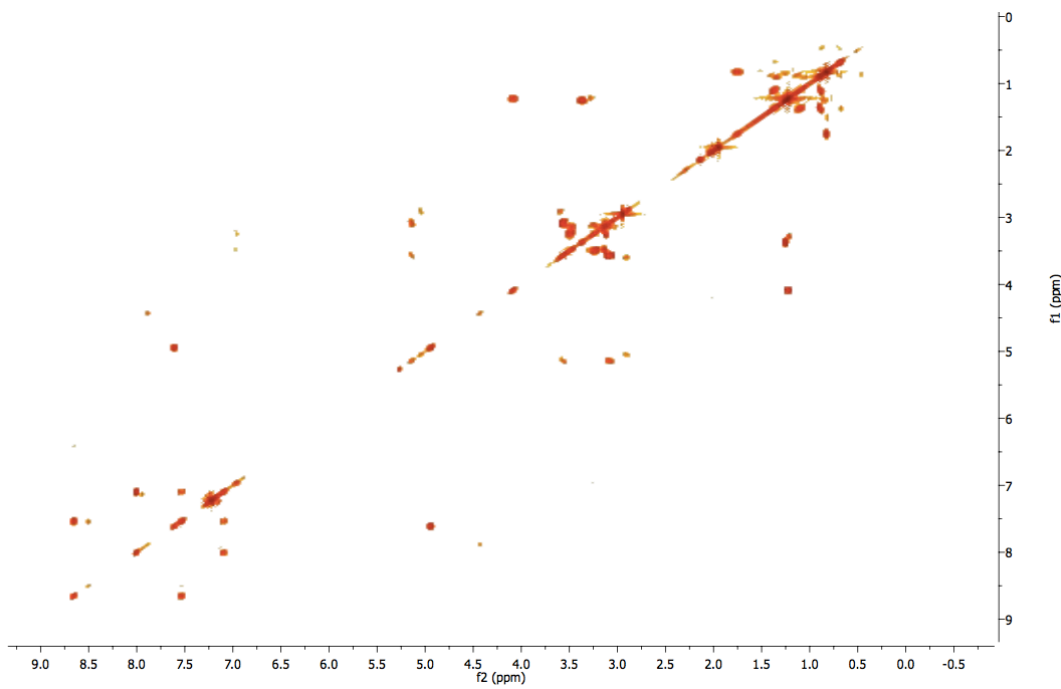
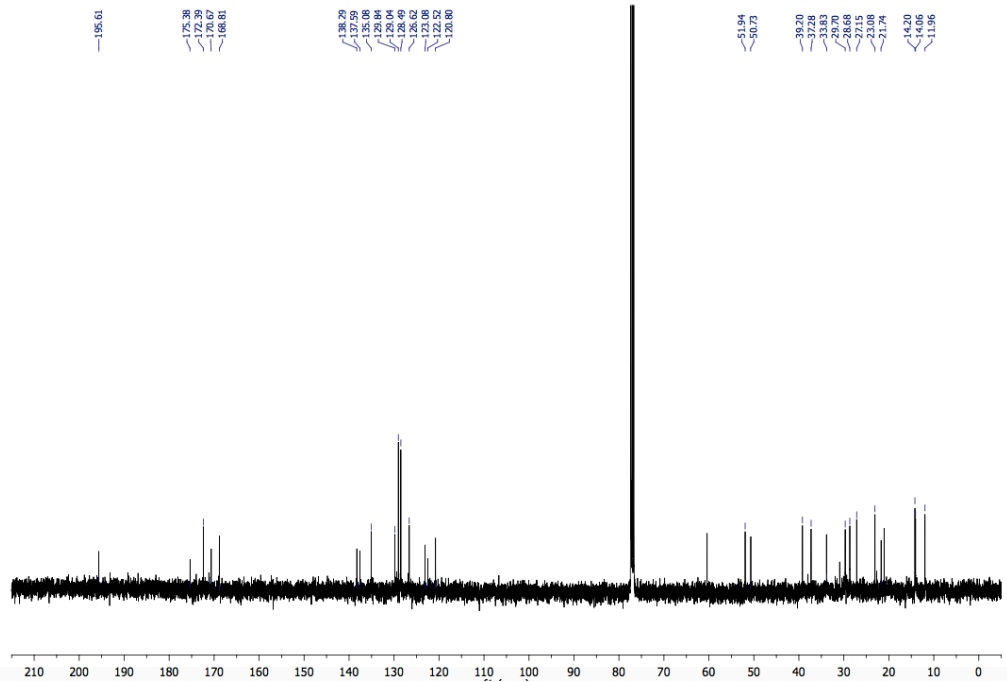


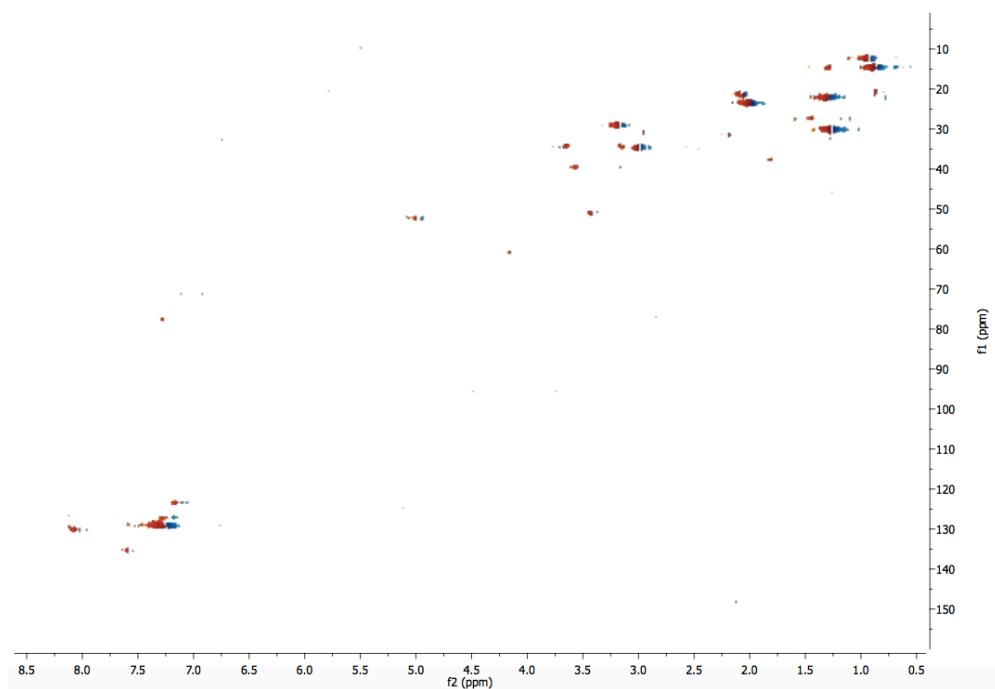


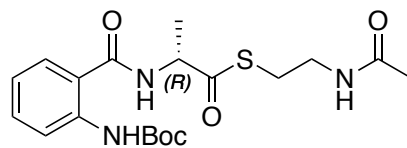


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