STUDIES TOWARDS THE CHEMOENZYMATIC SYNTHESES OF THIOPEPTIDES AND THIOMARINOL AND VISIBLE LIGHT CATALYZED *O*-GLYCOSYLATION OF THIOGLYCOSIDES

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences in the Doctoral Program of the UNC Eshelman School of Pharmacy (Division of Chemical Biology and Medicinal Chemistry).

Chapel Hill
2016

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ABSTRACT

Walter Jose Wever: Studies Towards The Chemoenzymatic Syntheses Of Thiopeptides And Thiomarinol And Visible Light Catalyzed *O*-Glycosylation Of Thioglycosides
(Under the direction of Albert A. Bowers)

Over 2 million people in the United States, and millions more worldwide, are affected by antibiotic resistant bacterial infections. The discovery of new antibiotics has been stalled by the lack of incentives from pharmaceutical companies and academia from both an economic and regulatory standpoint. The advent of next-generation DNA sequencing and gene annotation through homology models has provided a vast pool of biosynthetic clusters from which new and old bioactive molecules can be predicted. This research focuses on the characterization of the enzymes TclM, TmlU, and HolE and the visible light activated *O*-glycosylation of thioglycosides.

We have characterized the enzyme TclM in the thiocillin biosynthetic pathway from *Bacillus cereus* ATCC 14579 as a pyridine synthase by ligating a leader peptide recognition sequence to a semi-synthetic core substrate through native chemical ligation and subsequent conversion of cysteine side chains to dehydroalanines by elimination of a trialkylated tetrahydrothiophene.

We have additionally characterized the enzymes TmlU and HolE from the thiomarinol biosynthetic pathway from *Pseduoalteromonas* spp. SANK7339 and found them to be responsible for ligating the components pseudomonic acid C-coenzyme A thioester and holothin, respectively, to create the hybrid antibiotic. Structure-activity-relationship studies

were done on the dithiolopyrrolone holothin and found group promiscuity at the endocyclic and exocyclic amides but bioactivity was lost when the disulfide was oxidized to the thiosulfinate moiety. Further reactivity studies showed the ability of dioxoholomycin to oxidize small molecule thiols and redox-sensitive cysteines in the proteins YwlE and AtAPSK.

We have also designed a visible light mediated *O*-glycosylation of thioglycosides by utilizing blue LEDs as a light source in the presence of an Ir (III) catalyst. It was found that 1^{o}, 2^{o}, and 3^{o} alcohols react and serve as acceptors. Mechanistic studies showed the need for a bromine-trihalogenated carbon bond for the initiation step, but that subsequent propagation relies on the *p*-methoxythiophenol disulfide, formed through dimerization from the activated starting material.

To the loving memory of my father.

ACKNOWLEDGEMENTS

My journey through graduate school has not been a lonely one. There are many people who have had a strong influence on the path. First, I would like to thank God for giving me the strength to walk this path, and for guiding me through this stage in my life. Second, I want to thank my wife Jennifer; she is my everyday inspiration, and all that I do is for our future. Thank you for being there in both the good and the bad times. You are an amazing person, and I am very lucky to have you by my side.

I would like to thank my advisor, Dr. Albert Bowers, for his guidance throughout my doctoral studies. You have taught me with your dedication and perseverance how to be successful, and I am glad that I had the opportunity to learn from you. Your passion for science is undeniable, and I hope I can take that passion with me wherever I go.

I would also like to acknowledge people in my committee. Thank you Dr. Scott Singleton for agreeing to be my committee chair and providing valuable discussion during my seminars and research proposal. I would also like to thank Dr. Tim Willson for allowing me to do pull-down experiments with his group and for providing valuable discussion. Thank you Dr. Robert Nicholas for helping me in my graduate career by testing the dithiolopyrrolone compounds against *Neisseria gonorrhoeae* and for allowing me to have great conversations with you. Lastly, I would like to thank Dr. Eric Brustad for allowing me to work with his group and to learn about protein engineering.

Thank you to all who have been part of the Bowers laboratory: Mark Westbroek, Maris Cinelli, Scott Allen, Rachel Bleich, Paul Himes, Nicoleta Economou, Jonathan Bogart, Swapnil Ghodge, Kelly Bird, Steven Fleming, Sarah Jane Bassett, Sungwon Hwang, and Caitlin Lee. I learned a lot from each one and I hope that I can leave by having a positive impact in your scientific careers. A special thanks to Dr. Bo Li and her laboratory for allowing me to collaborate with them in the dithiolopyrrolone project and for providing with valuable information and input regarding my projects. Thank you to Dr. Karl Koshlap for his assistance with NMR experiments.

Finally, I would like to thank my family for their support. To my grandfather Roberto and my grandmother Lesvia, thank you for preparing me during my early formative years to be successful. To my mom, Ana Lucrecia, and my brothers, Christian and Fernando, I would like to thank you for your continual support. To my uncle Estuardo, my aunt Regina, and my cousins Maria Regina and Estuardo, thank you for always reminding me that love is a big part of family. Most of all, I would like to thank my uncle Roberto, my aunt Veronica, and my cousin Ximena for giving the opportunity to follow my dream and to pursue a career in the chemistry field. Without your help, I would not be where I am. Thank you for welcoming me to your immediate family and for believing in me.

Thank you all for everything you have done to make this happen: “It is good to have an end to journey toward; but it is the journey that matters, in the end” (Ursula K. Le Guin, author).

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LIST OF ABBREVIATIONS

1D NMR	One dimensional nuclear magnetic resonance
2D NMR	Two dimensional nuclear magnetic resonance
ATP	Adenosine 5'-triphosphate
Boc	*tert*-butyloxycarbonyl
$CHCl_3$	Chloroform
CoA	Coenzyme A
DCM	Dichloromethane
DHA	Dehydroalanine
DHB	Dehydrobutyrine
DIPEA	*N*,*N*-Diisopropylethyl amine
DMAP	4-Dimethylamino pyridine
DME	1,2-Dimethoxyethane
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
dqf-COSY	Double quantum filtered correlation spectroscopy
DTP	Dithiolopyrrolone
EBP	Ethyl bromopyruvate
EDC*HCl	*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride
EF-G	Elongation factor G
EF-Tu	Elongation factor thermo unstable
EtOAc	Ethyl acetate
EtOH	Ethanol

Fmoc	9-Fluorenylmethoxycarbonyl
Gn	Guanidine
GSH	L-Glutathione, reduced
GSSG	L-Glutathione, oxidized
HATU	*N*-[(Dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HCl	Hydrochloric acid
HEPES	N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
Hex	Hexanes
HFIP	1,1,1,3,3,3-Hexafluoroisopropanol
HMBC	Heteronuclear Multiple Bond Correlation
HOBt	1-Hydroxybenzotriazole
HOSu	*N*-Hydroxysuccinimide
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Correlation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LC/MS	Liquid chromatography–mass spectrometry
LED	Light-emitting diode
LIC	Ligation independent cloning
LP	Leader peptide
M-KO	*tclM* knockout
mBBr	Monobromobimane
MBP	Maltose binding protein

MeCN	Acetonitrile
MeOH	Methanol
MESNa	Sodium 2-mercaptoethanesulfonate
MIC	Minimum inhibitory concentration
MLCT	Metal to ligand charge transfer
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS/MS	Tandem mass spectrometry
NaOH	Sodium hydroxide
NaP_i	Sodium phosphate
NCL	Native chemical ligation
NMM	*N*-methylmorpholine
NMR	Nuclear magnetic resonance
NRPS	Non-ribosomal peptide synthesis
ORF	Origin of replication fork
PAA	Pseudomonic acid A
PAB	Pseudomonic acid B
PAC	Pseudomonic acid C
PAC-holothin	Pseudomonyl C holothinamide
PKS	Polyketide synthase
PMBSH	*p*-Methoxybenzylthiol
PMP	*p*-Methoxyphenyl
PyBOP	(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
RiPPs	Ribosomally synthesized and post-translationally modified peptides

RRE	RiPPs recognition element
RS	Recognition sequence
SET	Single electron transfer
SILAC	Stable isotope labeling by amino acids
SLIC	Sequence and ligase independent cloning
SOMO	Singly occupied molecular orbital
SPPS	Solid phase peptide synthesis
TBTU	*O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate
TCEP	Tris(2-carboxyethyl)phosphine
TEV	Tobacco etch virus
TFA	Trifluoroacetic acid
TFAA	Trifluoroacetic anhydride
TFE	2,2,2-Trifluoroethanol
THF	Tetrahydrofuran
THZ	Thiazole
TLC	Thin layer chromatography

CHAPTER I

INTRODUCTION

1.1 Antibiotic Resistance

With over 3.2 billion people worldwide at risk for common infectious diseases like malaria and pneumonia, the need for new antibiotics is imperative.[1,2] Each year in the United States, at least 2 million people become infected with antibiotic resistance bacteria, and at least 23,000 of those infected die from the disease.[3] The rapid emergence of resilient strains threatens the efficacies of current therapeutics that have saved millions of lives thus far.[4] Since the discovery of penicillin by Sir Alex Fleming, Howard Florey, and Ernst Chain[5,6], resistant strains have been identified not too far from the time they are introduced (**Figure 1.1**).

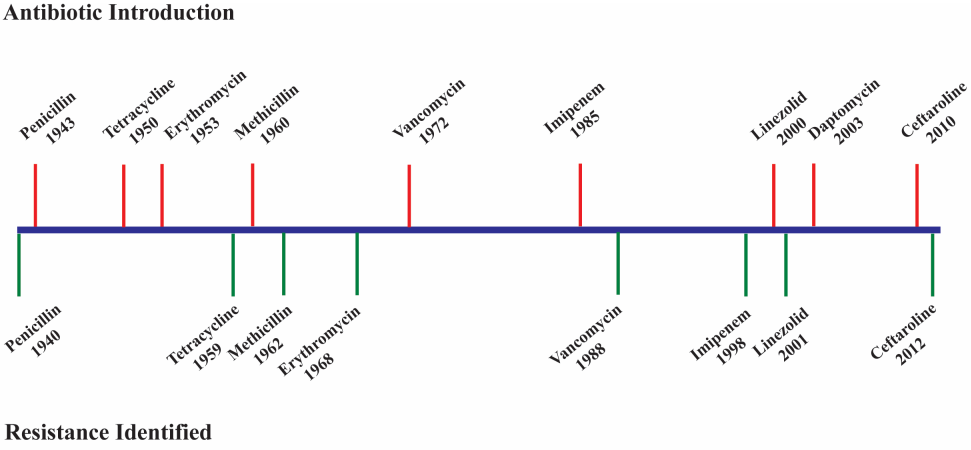

Figure 1.1. Timeline depicting bacterial resistance.

Natural products are at the forefront of drug discovery. Their privileged scaffolds have been extensively utilized as antibiotics or as antibiotic leads[7,8] for the treatment of common pathogens such as *Staphylococcus epidermidis, Streptococcus pyogenes*, and *Mycobacterium tuberculosis*.[9,10] The isolation of natural products is highly dependent on the ability of scientists to grow the specific bacterium in conditions in which it can produce the molecule of interest in sufficient quantities to be able to detect it and isolate in enough quantity for characterization. Furthermore, the constant fight for resources within microbial communities have selected for production of these natural products under the right environments and ecosystems.[11] Thus, a method that would allow for the prediction of these compounds would greatly enhance the rate of discovery.

The overuse and inappropriate prescription of antibiotics has increased bacterial resistance.[12] This trend has led many pharmaceutical companies to abandon exploring new antibacterials due to the increasing financial and regulatory obstacles, which compromise a good return on investment. Moreover, of the 18 largest pharmaceutical companies, 15 have abandoned the antibiotic field in order to focus on more chronic diseases such as diabetes and asthma.[13] In parallel, the high incidence of rediscovery of old scaffolds has limited the potential of harnessing bacteria for more bioactive molecules.[14] Yet, recent advances in next-generation DNA sequencing and bioinformatics have provided scientists with a broad pool of untapped resources for annotating gene clusters. These clusters can produce bioactive molecules based on sequence similarity of previously established ones.[15] Scientists can now harbor these biosynthetic machineries and utilize them to characterize new active biomolecules.[16] Moreover, it has provided a foundation from which chemists can take advantage of years of evolution and design compounds that are analogous to those found in

nature in order to replicate and/or enhance bioactivity. Among the most well characterized biosynthetic clusters are the polyketide synthases, the non-ribosomal peptide synthesis, and the ribosomally-encoded and post-translationally modified peptides. These biosynthetic machineries produce secondary metabolites with various degrees of complexity and bioactivities.

Polyketide synthases (PKS) have an architecture in which successive modules catalyze carbon-carbon linear extensions through Claisen condensations of malonyl, methylmalonyl, or keto-functionalities processing reactions on intermediates covalently tethered to carrier domains.[17] They constitute about a third of currently available pharmaceuticals,[18] and key members in this family are the pseudomonic acids, erythromycin, and amphotericin b (**Figure 1.2**).[19] Erythromycin is a commonly utilized antibiotic for the treatment of Gram-positive and Gram-negative infections and is involved in disrupting protein synthesis by blocking the 50S polypeptide export tunnel.[20] Like PKS, non-ribosomal peptide synthesis (NRPS) molecules are produced by a modular architecture in which small peptides are covalently linked to core domains that serve as carriers by which a set of tailoring domains modify the core structure. NRPS have the advantage of being able to access a wide chemical space due to modification of amino acid side chains or of the termini.[21] An important NRPS is gramicidin (**Figure 1.2**), an antibiotic used in the treatment of eye infections. In contrast to a modular-like assembly, the ribosomally-encoded and post-translationally modified peptides (RiPPs) biosynthetic pathways take advantage of gene-encoded polypeptides that are extensively post-translationally modified.[22] RiPPs present the advantage of being able to modify the expected mature product by genomic mutagenesis. Patellamide A (**Figure 1.**2) is an example of a RiPP that has been characterized as active

towards the inhibition of tumor cell growth.[23] With this great arsenal of chemical modifications at their disposal, it is no surprise that bacteria are constantly evolving new means of defense against foreign molecules, and hence why antibiotic resistance presents a great challenge.

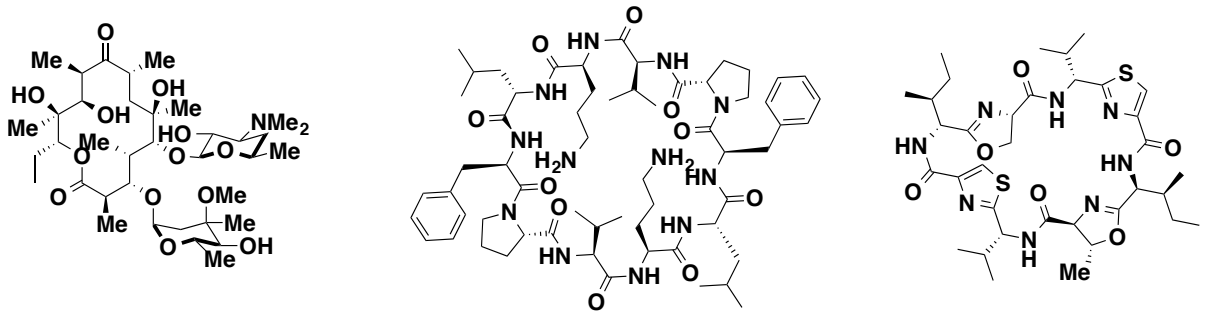

Figure 1.2. Bioactive molecules from three biosynthetic machineries.

New venues in drug discovery have focused on utilizing the nucleotide sequence of enzymes from the PKS, NRPS, and RiPPs pathways to predict new biosynthetic pathways from metagenomic sequencing of bacteria. This has facilitated the annotation of the clusters responsible for producing known antibiotics and reducing the rediscovery rate. In addition, scientists now have the ability to clone these new sets of genes and heterologously express them in more manageable bacterial systems or yeast,[24] opening a new era of innovative drug discovery of these orphan clusters.

Moreover, the advent of new enzymology and drug delivery systems have the advantage of coupling moieties that can enhance targeted drug delivery. For instance, the attachment of a rhamnose cap in the delivery of doxorubicin greatly enhanced its efficacy in murine liver tumor cells by taking advantage of the high density of lectins (carbohydrate

binding proteins) in eukaryotic cell surfaces.[25] The use of liposomes and other nanoparticles, often coated with sugars at the surface, have also been very successful,[26] suggesting a strong role for carbohydrates in drug delivery.

A recent study by the PEW charitable trust reported the need for the discovery of antibiotics from natural products.[27] There have been no new classes of antibiotics registered for therapeutic use since 1984; hence, the antimicrobial agents that have become commercially available tend to be just slight variants of previously established antibiotics. A main problem with this approach stems from the potential resistance to a family of antibiotics by individual resistance to one of the introduced variants. Moreover, natural product antibiotics have evolved for an extensive number of years to be enticing to the targeted microorganism. Thus, the discovery of new natural products classes is essential for antibiotic development.

In the present work, the design of new antibiotics will be explored by characterizing key transformation in enzymes involved in recently annotated gene clusters. More specifically, we will explore the enzymes involved in the formal [4+2] pyridine construction in the biosynthetic pathways of thiocillin and thiomuracin. A solid-phase peptide synthesis method for exploring substrate promiscuity and core sequence requirements will be developed. Additionally, we will utilize organic syntheses to produce analogues of the dithiolopyrrolone family of natural products for probing the mechanism of action and understanding their involvement in redox cycling in bacterial cells. We will further explore an *O*-glycosylation method for coupling carbohydrates into alcohol acceptors through visible light catalysis to establish a method for coupling into natural products.

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CHAPTER II

CHEMOENZYMATIC SYNTHESES OF THIAZOLYL PEPTIDES

2.1 Introduction

Natural products have provided scientists a vast library of compounds for drug discovery. Their isolation has not only led to their use as therapeutics, but also to advancements in our understanding of the chemistry found in natural sources.[1,2] Unfortunately, since the "golden era" of natural product discovery in the 1950s, high frequencies of bacterial resistance and molecule rediscovery[3] have dissuaded advancement in this area. Moreover, the usually complex structures of these compounds make them difficult to synthesize in quantities amenable for high-throughput screenings and biological testing.[4,5]

Historically, bacteria have been a rich source to discover bioactive compounds.[6] Their competition for resources have caused them to evolve secondary metabolites in order to gain a fitness advantage over their competitors, which has allowed scientists to grow bacteria, extract these metabolites, and utilize them as lead compounds for drug discovery.[7] This strategy has an important limitation: growing the bacteria under the right conditions to produce the desired natural products. Advancements in genome sequencing during the 21st century has allowed the identification of gene clusters that produce new compounds not been previously seen in the laboratory setting,[8,9]

RiPPs are a class of natural products derived from a linear precursor peptide that is heavily post-translationally modified to create the mature compound (**Figure 2.1**).[10,11] The

precursor peptide consists of a primary sequence of amino acids subdivided into two components: the leader peptide and the core peptide. It is the leader peptide that provides the recognition sequence by which the modifying enzymes can act upon the core peptide;[12] the leader peptide is subsequently cleaved during a late stage modification, or by a protease encoded in the gene cluster, and the mature compound is released. Common modifications found in RiPPs include the dehydration of serine and/or threonine side chains to their corresponding dehydroalanine (Dha) and dehydrobutyrine (Dhb) analogs,[13] thioether cross-linkages,[14] oxazoles and/or thiazoles from serine, threonine and cysteine residues,[15] and sactionine thioether linkages[16] among many others.[10]

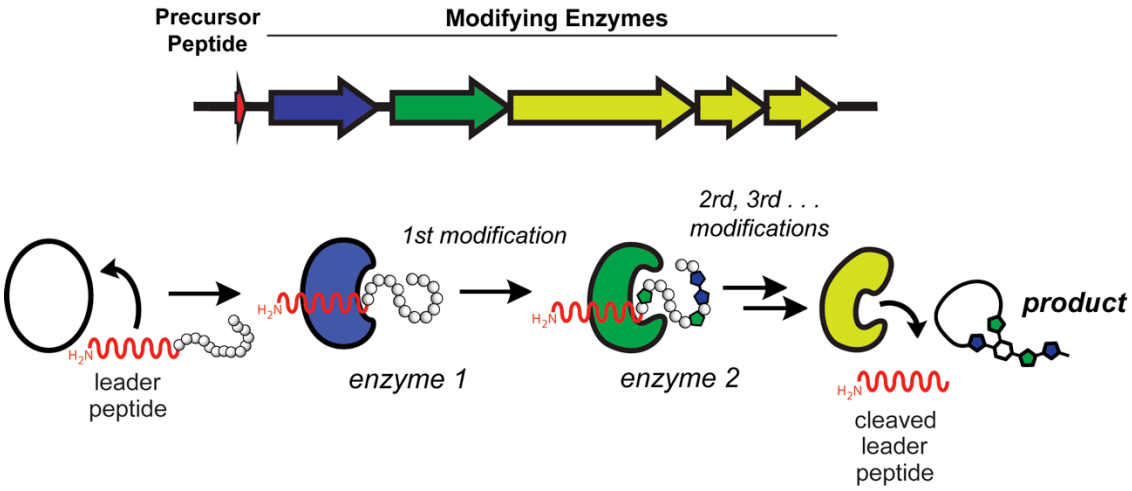


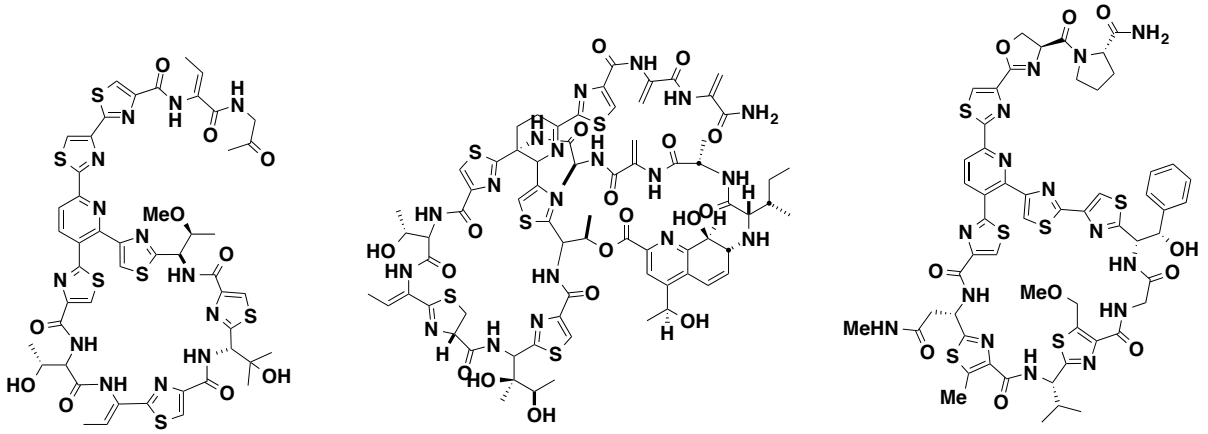
Figure 2.1. Overview of ribosomally encoded and post-translationally modified peptides.

The pathways involved in RiPP biosynthesis have opened the door for bioengineering from these clusters due to the direct correlation of gene to product.[17] The modifying enzymes in RiPPs tend to be promiscuous towards different cores if the leader peptide sequence remains intact.[18,19] This allows for modifications at the DNA level by simple molecular

cloning techniques such as site-directed mutagenesis. In theory, one can envision mutating the individual amino acid components of the core peptide with other proteinogenic ones to create compound libraries from a single encoded gene. This elegant utilization of amino acids has the evolutionary advantage of providing the producing species a reliable source of building blocks with great structural variety. The complexity of RiPP products are diverse and can vary from simple dehydrations and thioether linkages,[20] to very complex thiazoles, pyridine rings, and methyl-indole groups, such as the ones found in nosiheptide.[21] Amongst the most complex RiPP products are the ones belonging to the family of thiazolyl peptides.

Thiazolyl peptides feature a unique combination of functionalities that include thiazoles, thiazolines, dehydroalanines, dehydrobutyrines, and a ring-closure, 6-membered *N*-containing heterocycle, often dehydropiperidine or pyridine (**Figure 2.2**). They have potent activity against both Gram-positive and Gram-negative bacteria,[22] and even against methicillin resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci*.[23,24] There are over 100 known members in the family of these structurally complex, and high molecular weight compounds (typically >1000 Da). Their associated bacterial target is based on their architecture. Thiocillin (**2-1**), a 26-member macrocycle when counted by the shortest atom travel inside the ring, targets a crevice in the 50S ribosomal subunit between the 23 sRNA and protein L11, halting translation by inhibiting binding of elongation-factor G (EF-G) and subsequent translocation of *t*RNA.[25] Another member is thiostrepton (**2-2**); it has two macrocycles in its core structure and, while it also targets EF-G, it has been shown to be involved in inhibition of the proteasome and the oncogenic transcription factor FoxM1.[26] Additionally, GE2270A, a 29-atom macrocycle, has been extensively studied and found to

target the ribosomal co-chaperone EF-Tu by inhibiting its ability to deliver amino-acyl *t*RNA.[27]



Thiocillin
***B. cereus* ATCC 14579**
Target: EF-G
2-1

Thiostrepton
S. laurentii
Targets: EF-G, Proteasome, FoxM1
2-2

GE2270A
P. rosea
Target: EF-Tu
2-3

Figure 2.2. Members of the thiazolyl peptide family.

The thiazolyl peptides have great chemical characteristics that make them highly desirable as potential leads. They are rigid and thus conformationally stable to degradation by proteases; they are derived from canonical amino acids, which allows for the examination of substitutions at various positions inside the ring to probe activity. However, members of the thiazolyl peptides have traditionally shown poor pharmacological profiles due to poor solubility and low bioavailability. Overcoming these limitations would require a modular synthetic approach that allows for functional group interchange. While total syntheses of thiazolyl peptides have been reported,[28,29] the most successful semi-synthetic derivative has come from a natural source. Functional group modifications in GE2270A (**2-3**) through removal of C-terminal groups, and subsequent introduction of the solubilizing urethane moiety, yielded the compound LFF571. This compounds was until recently in phase II

clinical trials for the treatment of *Clostridium difficile* infections in the upper gastrointestinal tract.[30]

The thiocillin biosynthetic gene cluster from *Bacillus cereus* ATCC 14579 has been shown to produce the mature compound from a cascade of fourteen post-translational modifications from the precursor peptide (**Figure 2.3**),[31] which is found in four copies on the gene cluster (*tclE-H*). The precursor peptide consists of a 38 amino acid leader peptide linked to a 14 amino acid core. Genetic manipulation experiments have demonstrated that the pathway can be promiscuous to site-directed mutagenesis about the core peptide, allowing for a biosynthetic library of thiocillin analogues with various degrees of antibiotic activity.[32] Interestingly, all the gene products can be functionally annotated based on sequence homology to other known enzyme functions, except for *tclM*. A *tlcM* knockout (M-KO) of the producer strain *Bacillus cereus* ATCC 14579 was accomplished by homologous recombination of a plasmid containing sequence homology to the areas adjacent to, but not containing, *tclM*.[33] After standard expression conditions of thiocillin, cell extractions, and characterization by HPLC and LC/MS showed a post-translationally modified linear form of the core peptide, where only a few amino acids from the leader peptide remained. This suggests that the enzyme TclM is involved in the putative [4+2] cyclization of the central pyridine core and that most other modifications precede the ring closure step. Moreover, when *tclM* was reintroduced into the M-KO on a plasmid, reconstitution of the central pyridine was accomplished. This function would categorize TclM as a new class of enzyme catalyzing a formal heteroannulation between two dehydroalanines. Because initial attempts to obtain a linear substrate for TclM *in vivo* proved to be unsuccessful due to leader peptide cleavage,[33] we hypothesized that if we built a linear analogue of thiocillin with its leader

peptide and subjected it to TclM *in vitro*, we could effect the cyclization to the mature thiazolyl peptide analogue.

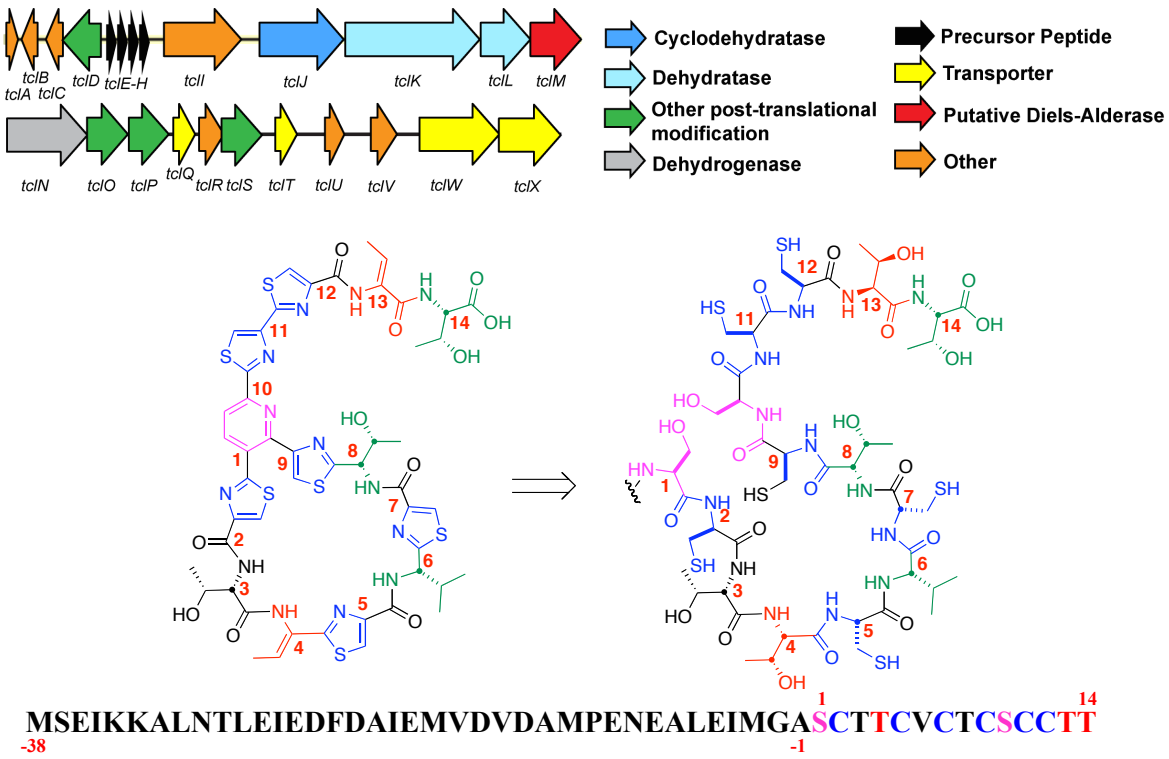


Figure 2.3. Thiocillin biosynthetic pathway.

While enzymes such as SpnF[34] and VstJ[35] are known as natural occurring Diels-Alderases, they only enhance the rates of already spontaneous reactions. These Diels-Alderases utilize the required 4π diene and a 2π dienophile in order to make two new carbon-carbon bonds with the formation of a cyclohexene ring. TclM, however, would be the first example of an enzyme catalyzing a formal [4+2] cycloaddition when the reaction is non-spontaneous (**Figure 2.4**), as the starting Dha residue would have to tautomerize to its imidic acid form to yield the 4π partner and make the tetrahydropyridine core. A previous report by Moody and co-workers demonstrated that they needed to trap the imidic acid as the imidate

ethyl ester and irradiate with microwave at 120 °C to effect the cyclization with a small molecule Dha, and ensuing aromatization, in only 33% yield at an early stage during the total synthesis of the thiazolyl peptide amythiamycin.[36] This report emphasizes the harshness needed for the pyridine formation, and the need for a mild, late stage catalytic system to overcome this limitation.

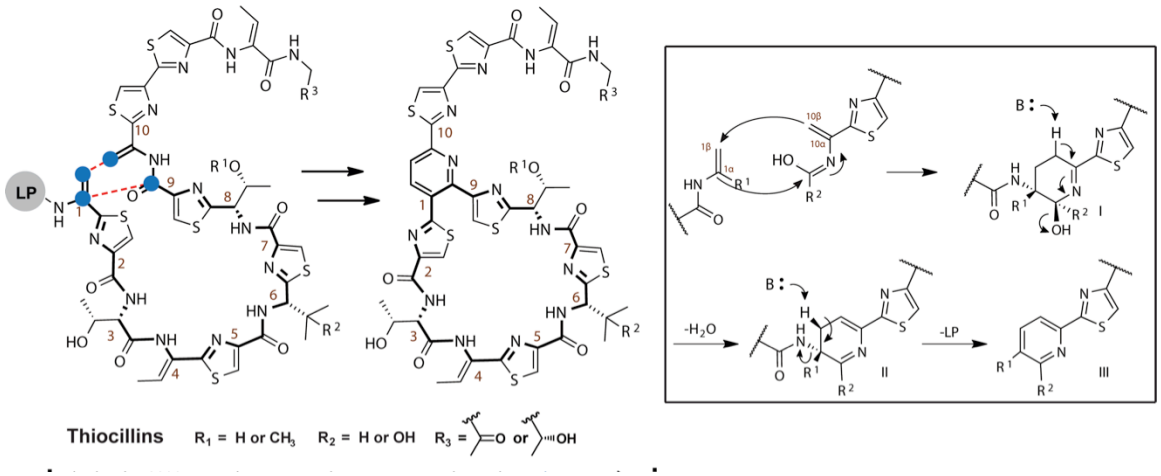


Figure 2.4. Suggested mechanism for pyridine ring formation in thiocillins.

2.2 Isolation of TclM

Our initial efforts focused on obtaining pure TclM. Thus, the gene *tclM* was amplified from genomic DNA of *Bacillus cereus* ATCC 14579 and cloned into the ligation independent cloning (LIC)-vector pMCSG9, which encodes for an *N*-terminal 6xHis-maltose binding protein (MBP) linked through a tobacco etch virus (TEV) cutting sequence to TclM to improve solubility during the isolation process.[37] The construct was transformed into *E. coli* RIPL cells for heterologous expression. 6xHis-MBP-TEV-TclM was obtained after nickel-affinity purification and size exclusion chromatography. Removal of the *N*-terminal tag was accomplished by treating the construct with 1% (w/w) TEV for 12 h at 4 °C.

Subtractive purification of our cleavage reaction yielded 300 μL of TclM as an 8.09 mg/mL stock in over 90% purity.

2.3 Synthesis of Thiazolyl Peptide Analogue of Thiocillin

With our enzyme purified, we next focused on the rational design of a TclE-H analogue that would allow us to test the *in vitro* function of TclM. Building the 52 amino acid linear substrate by solid phase peptide synthesis (SPPS) would challenge the limits of the method. Thus, our initial efforts took us to explore different avenues. We surveyed the idea of linking the unmodified leader peptide sequence with the highly modified core peptide through a ligation strategy such as native chemical ligation (NCL, **Figure 2.5**). NCL provides for a convergent synthesis of multiple cores that we can subsequently ligate to the leader peptide.

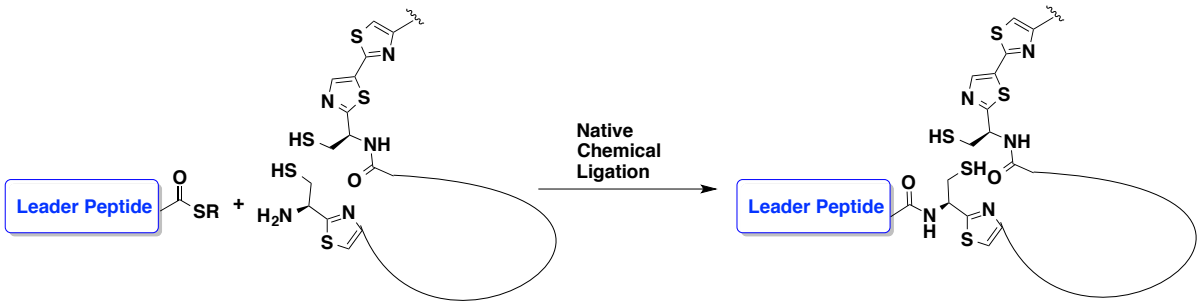


Figure 2.5. Strategy for substrate preparation for TclM.

2.3.1 Isolation of TclE-H's Leader Peptide

TclE's leader peptide (LP) was amplified from genomic DNA of *Bacillus cereus* ATCC 14579 and cloned into the sequence and ligase independent cloning (SLIC)-vector pETXSH; this construct allowed expressing the short leader peptide with a C-terminal intein

from *Mycobacterium xenopi* that contains a 6xHis-tag at the C-terminus. In the presence of excess thiol, this intein protein self-excises to yield C-terminal thioesters.[38] The construct was transformed into *E. coli* RIPL cells for heterologous expression. Upon isolation of the LP-Intein-6xHis construct by nickel-affinity purification, our initial attempt at the formation of the LP-thioester by addition of 2-mercaptoethanesulfonate sodium salt (MESNa) to a final concentration of 0.5 M yielded a truncated version of the thioester, with removal of the terminal two amino acids (Gly and Ala). Quickchange mutagenesis of the C-terminus allowed us to add an extra Gly-Ala sequence, and now the desired thioester was obtained upon MESNa treatment (**Figure 2.6**). Isolation of the LP-MESNa thioester was performed by removal of the excised intein through nickel-affinity purification.

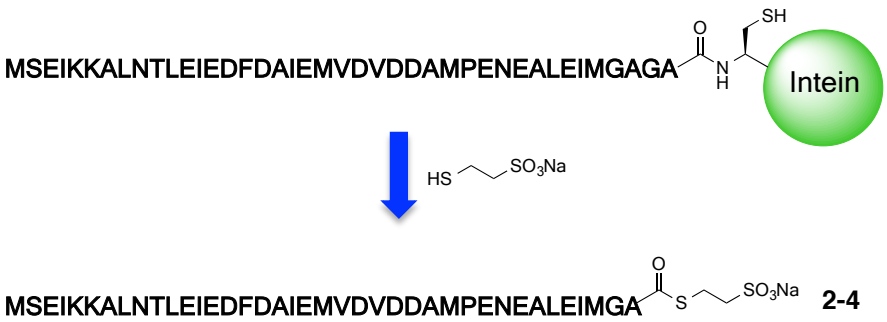


Figure 2.6. Isolation of TclE-H's leader peptide as a C-terminal thioester.

2.3.2 Synthesis of Thiazolyl Peptide Analogue Core

Having one of the coupling partners for native chemical ligation, we then focused on the synthesis of an analogue of the highly modified core. Over 65 mutations through site-directed mutagenesis of *in vivo* processing of TclE-H had shown that mutations inside the macrocycle are well tolerated, but mutations of the residues around the pyridine ring are not.[39] Previous mutagenesis results are summarized in **Figure 2.7**.

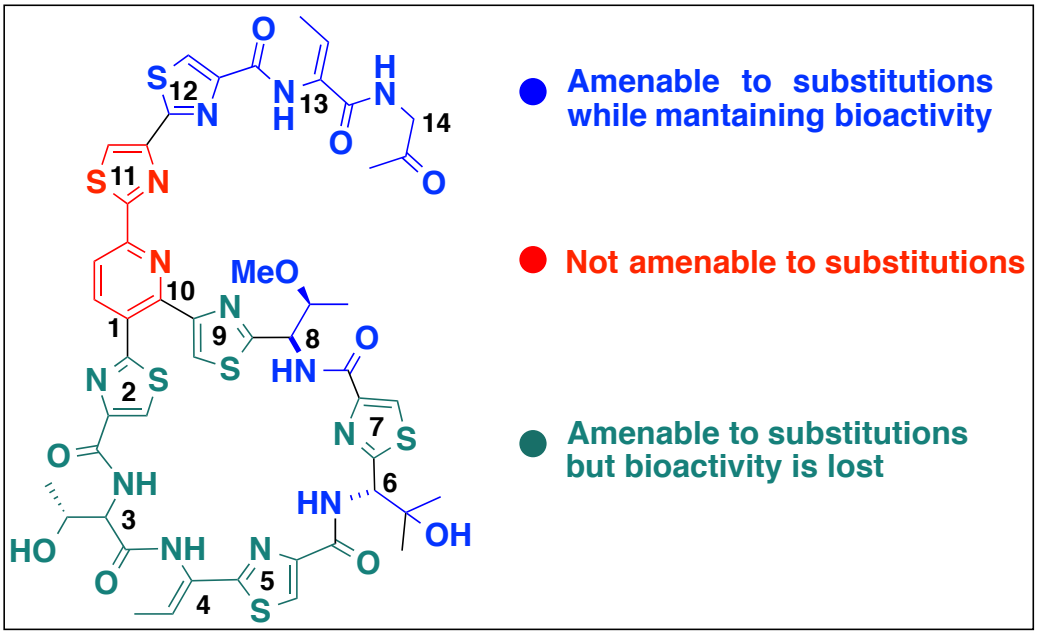
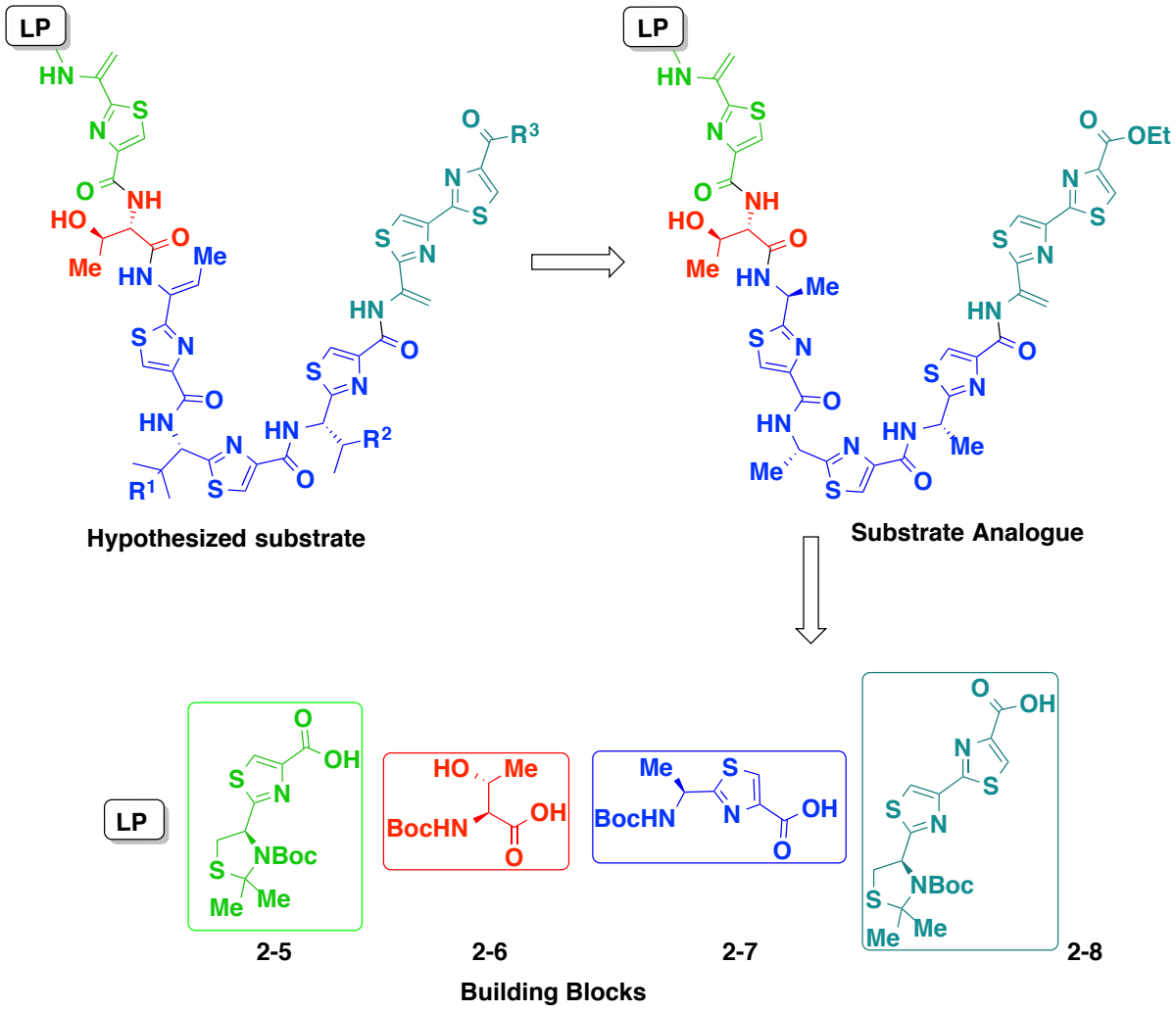


Figure 2.7. Effects of substitutions in thiocillin.

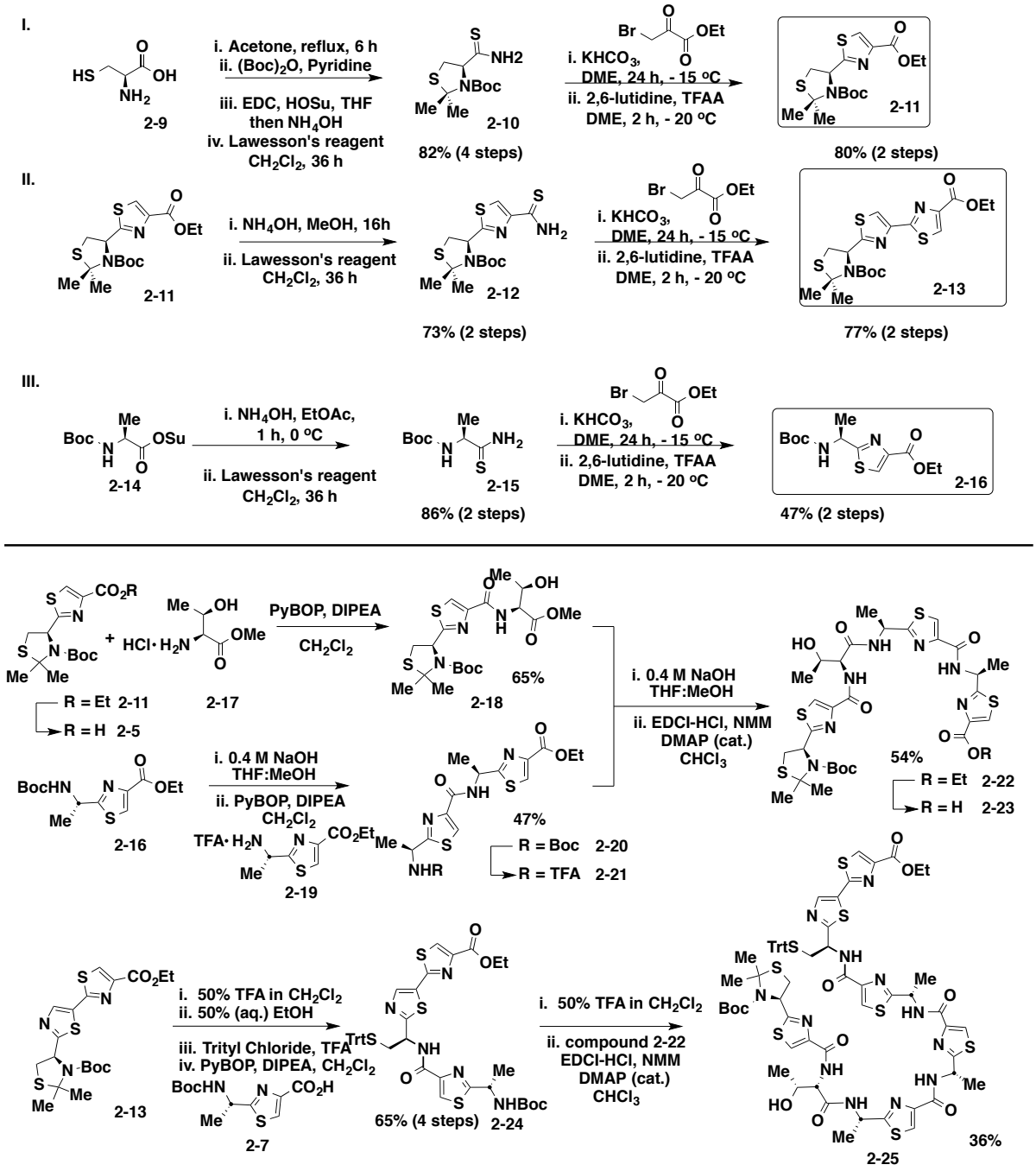
We envisioned a modular synthetic approach to a simplified core through peptide couplings of thiazole-containing amino acids (**Scheme 2.1**). Our simplified analogue would substitute Thr4, Val6, and Thr8 with alanine side chains and truncate the C-terminus after Cys12 to the ethyl ester; furthermore, it was crucial to keep the Dha moieties at positions 1 and 10 and the thiazoles at positions 2, 9, and 11 in order to keep a core that TclM would be active towards. We also decided to keep Thr3 since it is an unmodified residue and it is commercially available. Our analysis led us to synthesize the building blocks containing thiazoles by utilizing a known method[28c-d] from *tert*-butyloxycarbonyl (Boc)-protected amino acids. More specifically, from *N*-Boc- and *N,S*-dimethylthiazolidine protected cysteine thiazole (**2-5**), *N*-Boc-alanine thiazole (**2-7**), and *N*-Boc- and *N,S*-dimethylthiazolidine protected cysteine *bis*-thiazole (**2-8**).



Scheme 2.1. Retrosynthetic analysis of thiocillin core analogue.

We began our synthesis by building from L-cysteine (**2-9**) the *N,S*-dimethylthiazolidine, *N*-Boc thioamide (**2-10**) in 4 steps (**Scheme 2.2**). Cyclization of **2-10** with ethylbromopyruvate (EBP) and subsequent elimination with trifluoroacetic anhydride (TFAA) in the presence of 2,6-lutidine yielded the building block **2-11**. **2-11** was split and hydrolyzed to **2-5** with 0.4 M NaOH and utilized to build a second thiazole by amidation with ammonium hydroxide and thiolation with Lawesson's reagent to afford **2-12** in 73% yield. Cyclization with EBP and TFAA afforded the *bis*-thiazole **2-13**. In parallel, amidation

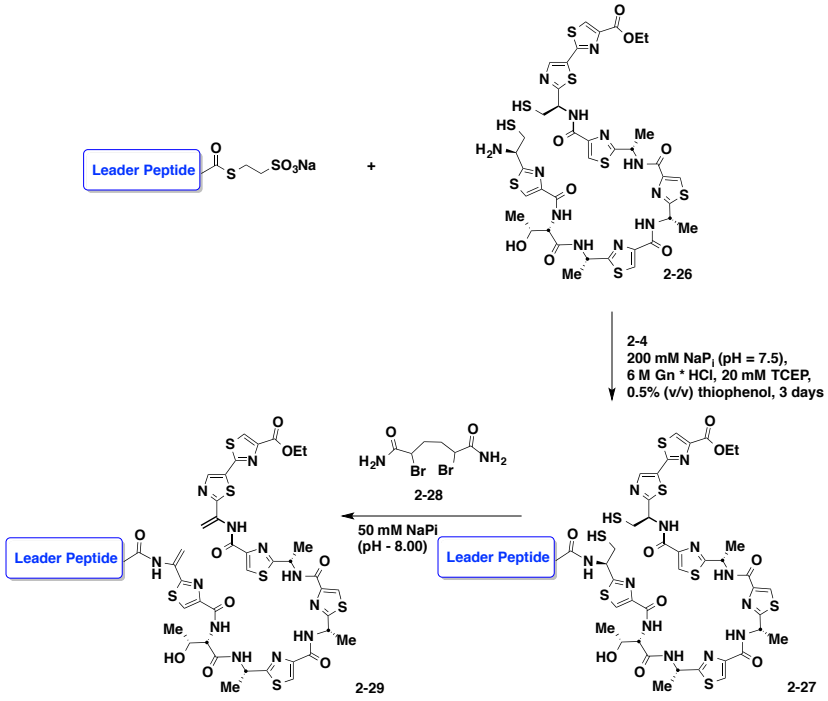
and thiolation of L-Boc-alanine succinimide ester (**2-14**) afforded the Boc-alanine thioamide **2-15**, which was cyclized with EBP and TFAA to yield the Boc-alanine thiazole ethyl ester **2-16** in 47% yield. Having all the three main building blocks, **2-5** was coupled to L-threonine methyl ester hydrochloride (**2-17**) with **(**Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) to make the dipeptide **2-18** in 65% yield; separately, **2-16** was hydrolyzed to the corresponding acid and coupled to the trifluoroacetate salt of Boc-alanine ethyl ester **2-19** to yield the dipeptide **2-20**. We next hydrolyzed the ester **2-18** and coupled it to the fully deprotected **2-21** with N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC*HCl) to afford the trithiazole, tetrapeptide **2-22**, which was hydrolyzed to its corresponding acid **2-23**. The last coupling block was made by hydrolyzing **2-16** to **2-7** with 0.4 M NaOH and coupling it to the trifluoroacetate salt of L-cysteine-(S-trityl)-thiazole ethyl ester with PyBOP to make dipeptide **2-24**. Removal of the Boc-protecting group of **2-24** and coupling with EDC*HCl to **2-23**, allowed us to synthesize the protected hexathiazole **2-25** in 36% yield.



Scheme 2.2. Synthesis of the core analogue of thiocillin. HOSu = *N*-hydroxysuccinimide. TFAA = trifluoroacetic anhydride. DME = 1,2-dimethoxyethane. PyBOP = (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate. EDC = N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride. DMAP = 4-dimethylaminopyridine. NMM = *N*-methylmorpholine.

2.3.3 Native Chemical Ligation and Dehydroalanine formation

Final deprotections with trifluoroacetic acid (TFA) in the presence of triisopropyl silane and removal of the *N,S*-dimethylthiazolidine moiety with 50% (aq.) ethanol of **2-25** allowed us to obtain the hexathiazole-ethyl ester coupling partner **2-26** for native chemical ligation. Buffer exchanging the LP-MESNa thioester **2-4** to a native chemical ligation buffer consisting of 200 mM sodium phosphate (pH = 7.5), 6 M guanidinium, 20 mM *tris*(2-carboxyethyl)phosphine, and 0.5% (v/v) thiophenol[40] and reacting it with excess **2-26** for 3 days at room temperature showed full conversion to the ligated product **2-27** (**Scheme 2.3**). Elimination of the cysteine side chains to their corresponding Dha was accomplished with excess *meso*-2,5-dibromohexanediamide (**2-28**),[41] and the final substrate for TclM was produced **2-29** upon dialysis of excess **2-28**.



Scheme 2.3. Preparation of TclM substrate. NaP_i = sodium phosphate. Gn = guanidine. TCEP = *tris*(2-carboxyethyl)phosphine.

2.4 TclM Assay

Having TclM and its putative linear substrate **2-29**, we tested to see if we could effect the formal [4+2] cycloaddition in a 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer with 150 mM sodium chloride (**Figure 2.8**). A characteristic absorbance of the tri-substituted pyridine is observed at λ = 350 nm; hence, we monitored the reaction over the course of 16 h at 4 h intervals and noticed an increase in the signal at this wavelength (**Figure 2.8a**) over time. Additionally, analytical HPLC shows complete consumption of starting material (rt = 7.8 min) after 20 h and the appearance of a new peak at (rt = 9.0 min).

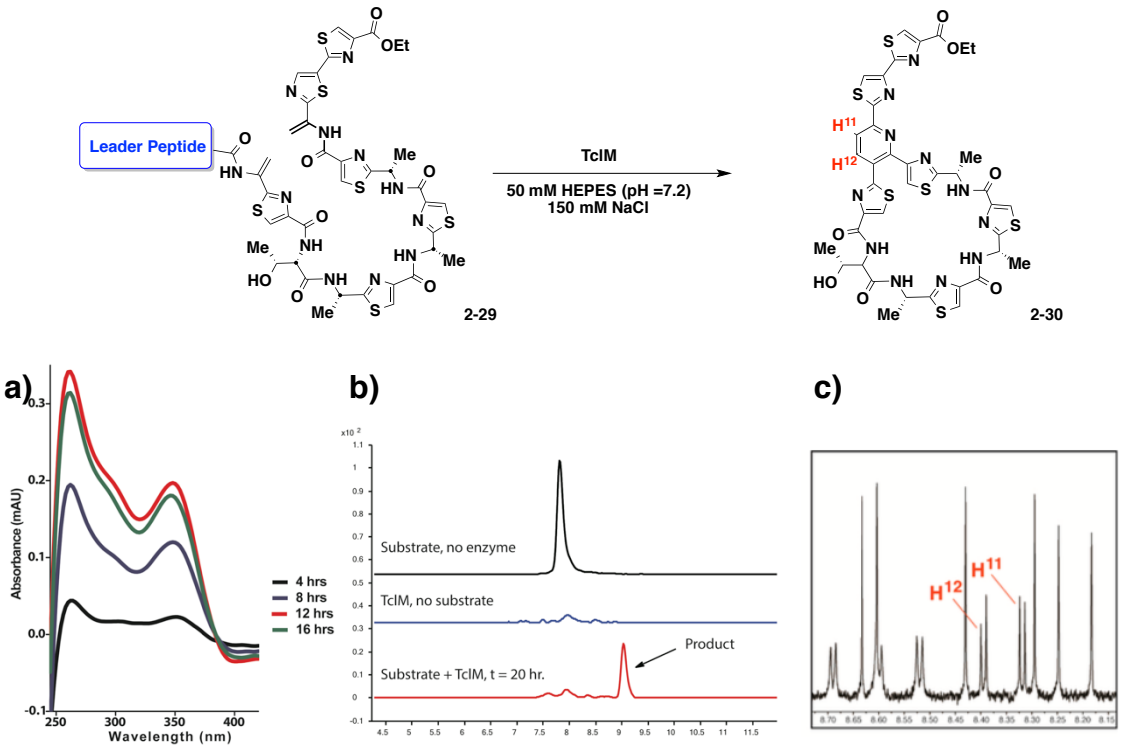


Figure 2.8. Formation of thiocillin analogue by TclM. a) UV-Vis absorbance from 250 nm to 450 nm. b) Analytical HPLC traces monitored at λ = 254 nm. c) ^{1}H NMR showing pyridine protons.

LC/MS analysis of the newly formed product showed a mass corresponding to an *m/z* of 962.1171, which is within instrumental error of the expected $[M+H]^+$ of **2-30** (*m/z* 962.1118). Further characterization by MS/MS showed the expected pattern of the cyclized thiazolyl peptide; but in order to unequivocally assign the product as the pyridine core, we scaled the assay to isolate milligram quantities of the product for NMR analysis. ^{1}H NMR showed the appearance of the expected two doublets from the aromatic protons of the pyridine core (**Figure 2.8c**) along with the six singlets from the thiazole scaffolds. Additional 1D and 2D NMR analyses (^{13}C, dqf-COSY, coupled-HSQC, and HMBC) confirmed the structure of the thiocillin analogue **2-30**. Intriguingly, the search for intermediates or the ultimate fate of the leader peptide in our LC/MS runs did not yield any intermediates or side products. This suggests that the intermediates are either short-lived or TclM itself catalyzes both the formal [4+2] cycloaddition and aromatization. The lack of presence of the leader peptide from our worked-up assays intimates that it might be strongly held by TclM or that it might aggregate with the surface of TclM.

2.5 Mutagenesis Studies of TclM

Identifying the key residues for TclM will be important to understand and propose plausible mechanism for enzymatic transformation. Nonetheless, TclM is an enzyme with very low homology to proteins of known function. Even within the family of thiazolyl peptides, TclM homologues have low sequence similarity. We began our studies by attempting to crystallize apo-TclM, TclM + leader peptide, and TclM with thiocillin. Unfortunately, our efforts proved to be unsuccessful. Hence, we decided to focus on identifying potential key residues that might be important for catalysis and trying to obtain

biochemical information from their assays. A sequence alignment of TclM and its homologues in other thiazolyl peptide biosynthetic pathways (**Figure 2.9a**) allowed us to identify eight conserved amino acids at the C-termini. Quickchange mutagenesis facilitated mutating these resides to alanines, yielding the TclM mutants E128A, F155A, Y173A, H242A, F261A, R265A, R285A, and E297A. These constructs and the native TclM sequence were introduced to the M-KO strain of *Bacillus cereus* ATCC 14579, and the bacteria grown with thiocillin producing conditions and 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce expression from the plasmid utilized, pHT01. Pellet extraction with methanol, followed by LC/MS analyses of the extracts showed that the mutants E128A, F155A, Y173A, F261A, and E297A produced equal amounts of thiocillin when compared to unmodified TclM. Interestingly, the mutants H242A and R285A had great reduction in thiocillin production (<50% for H242A and <15% for R285A when compared to unmodified TclM, **Figure 2.9b**), while the mutant R265A completely halted thiocillin production *in vivo*. These results lead us to modify our TclM alignments to further explore other residues that might be important for function. An alignment of only TclM-homologues that produce a pyridine core were considered. Six more conserved residues were identified and mutated to alanines and tested for thiocillin production as done for the previous mutants. The six new mutants, S147A, S150A, H151A, S239A, Y272A, and C289A, did not have a substantial change in thiocillin production when compared to the wild type enzyme (data not shown).

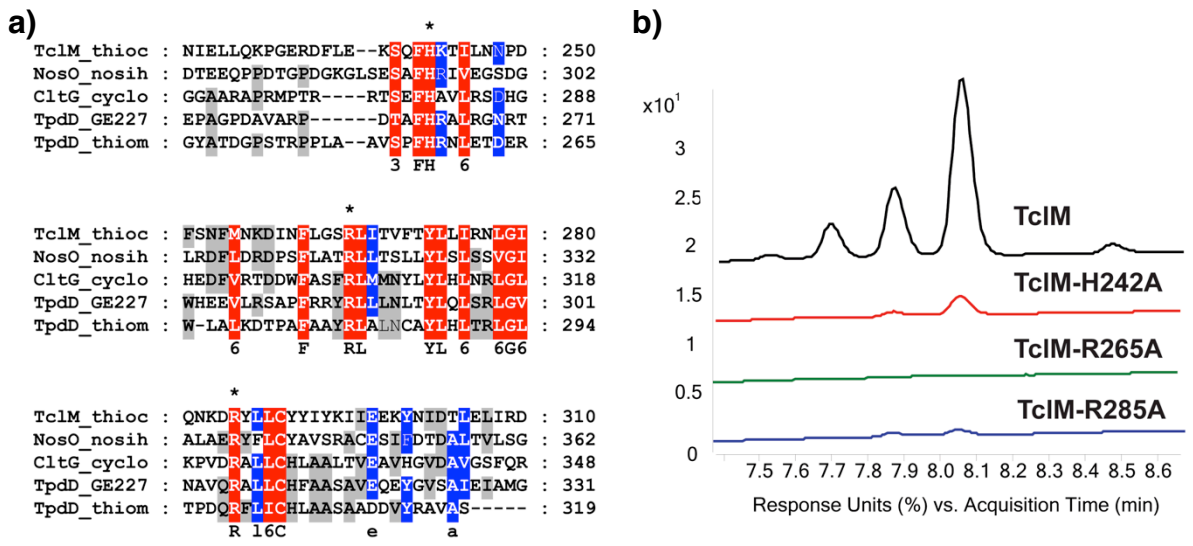


Figure 2.9. Probing conserved residues in TclM and its homologues. a) Alignment of residues 231-310 from TclM with its homologues. b) *In vivo* production of thiocillins by TclM and TclM-mutants at 350 nm.

We next cloned the mutants H242A, R265A, and R285A into our TclM expression vector pMCSG9 and heterologously expressed them in *E. coli* RIPL cells. Purifications follow the same procedure as for wild type TclM. Subjecting substrate **2-29** to our standard TclM-assay conditions with our three mutants showed that conversion was poorer for all three (**Figure 2.10**). The *in vivo* result from R265A can be correlated *in vitro*, but H242A and R285A cannot, although no production of cyclized product was observed for H242A. It is noteworthy that the substrate utilized is not the native linear one, which might explain the lack of correlation for the latter two mutants. We hoped that the decrease in efficiency would allow us to visualize by LC/MS analyses potential intermediates, but we were again unsuccessful at detecting new species.

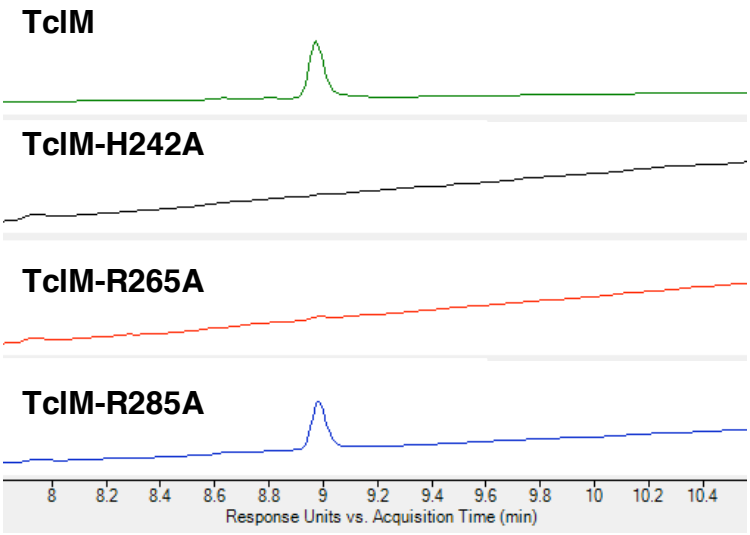


Figure 2.10. *In vitro* production of thiocillins by TclM and TclM-mutants at 350 nm.

2.6 Probing The Leader Peptide Requirement of TclM

The LP requirement for TclM was probed by truncating the *N*-terminal peptide sequence of our linear thiazolyl peptide until no cyclization could be observed by LC/MS analysis. The LP sequences are described in **Table 2.1** and show a gradual decline in the the number of amino acid residues in the peptide chain. Substrate containing the ΔN18 truncate was prepared according to our previously described method in **Section 2.3**. The ΔN18 truncate did not require the addition of an extra Gly-Ala for MESNa mediated intein cleavage. Substrates with LP truncations ΔN28, ΔN30, ΔN31, and ΔN32 were prepared by solid phase peptide synthesis (described in further detail in **Section 2.7**) utilizing H-Rink-Amide ChemMatrix®.

Table 2.1. Leader peptide truncations of TclE-H

Leader Peptide (amino acids)	Leader Peptide Sequence
ΔN18 (19-38)	MIEMVDVDAMPENEALEIMGA
ΔN28 (29-38)	ENEALEIMGA
ΔN29 (30-38)	NEALEIMGA
ΔN30 (31-38)	EALEIMGA
ΔN31 (32-38)	ALEIMGA
ΔN32 (33-38)	LEIMGA

Interestingly, when we subjected the truncated substrates with TclM, all tested versions yielded the cyclized compound except for the ΔN32 truncate (**Figure 2.11**). The ΔN31 and ΔN30-LPs yielded very low amounts of product, while the ΔN29 and ΔN28-LPs were similar in yield to the ΔN18. It is noteworthy that the ΔN28-LP provided cleaner conversion than the smaller constructs; the smaller ones tended to yield even further truncated versions of the substrate, presumably due to low efficiency and background hydrolysis of the Dha moieties. This result has the great advantage of allowing for a modular combinatorial synthesis of thiopeptide cores that can be readily tested for cyclization by attaching a more amenable 10-residue LP, in contrast to the native 38-residue LP, by chemical methods such as solid phase peptide synthesis.

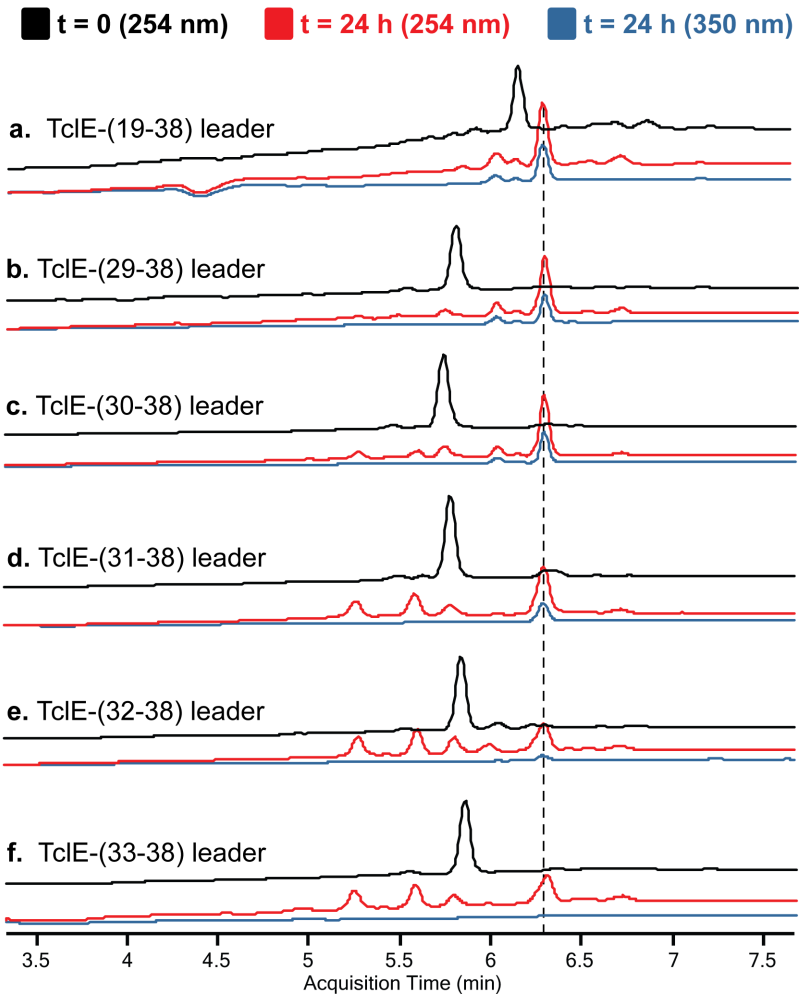


Figure 2.11. Production of thiocillin analogue by truncated leader peptide sequences.

2.7 Probing TclM and TbtD by Solid Phase Peptide Synthesis of Thiazolyl Peptides

We wanted to take advantage of the shorter 10-residue recognition sequence (RS) by TclM to probe the substrate promiscuity of the enzyme in the residues adjacent to the Dhas that will become the pyridine ring. To expedite the process, we wanted to take advantage of a modular synthesis by fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS, **Figure 2.12**). The cores could be coupled by successive microwave assisted SPPS (**step 1**, **Figure 2.12**) with *N*-Fmoc protected amino acids thiazoles. Similar to our solution

phase synthesis, cores would have an *N*-terminal cysteine thiazole to facilitate Native Chemical Ligation. We explored the literature for NCL procedures on-bead and found a method by Dawson *et al.* where they made use of a *N*-methylbenzimidazolidinone strategy as thioester surrogates (**step 2**, **Figure 2.12**) to ligate two fragments.[42] This provides the advantage of making the LP independently from the cores, and it would allow us to build core libraries on-bead and ligate them to the LP in a single step. Subsequent cleavage of the precursor peptides and desulfhydrylation of cysteine side chains to Dhas would provide substrates for TclM (**steps 3-4**, **Figure 2.12**). Two key transformations during the synthesis included the selective deprotection of the *S*-trityl groups without peptide cleavage from the resin with 0.1 N HCl in hexafluoroisopropanol (HFIP)[43] of the N-terminal cysteine thiazole from the cores in order to liberate them for NCL, and the desulfhydrylation with methyl 2,5-dibromopentanoate (**2-31**) as the desulfhydrylation agent [44] to avoid intramolecular "stapling." With a versatile synthesis in hand, we decided to extend our probes to synthesize cores that would resemble the one for the thiopeptide thiomuracin. The pyridine synthase from the thiomuracin biosynthetic pathway, TbtD, has recently been characterized;[45] it will allow us to test the formation of 29-atom macrocycles, complementing the 26-atom macrocycle from thiocillin. Just like with TclM, we anticipated that a similar 10-residue LP from the precursor peptide, TbtA, would be sufficient for TbtD to cyclize linear substrates.

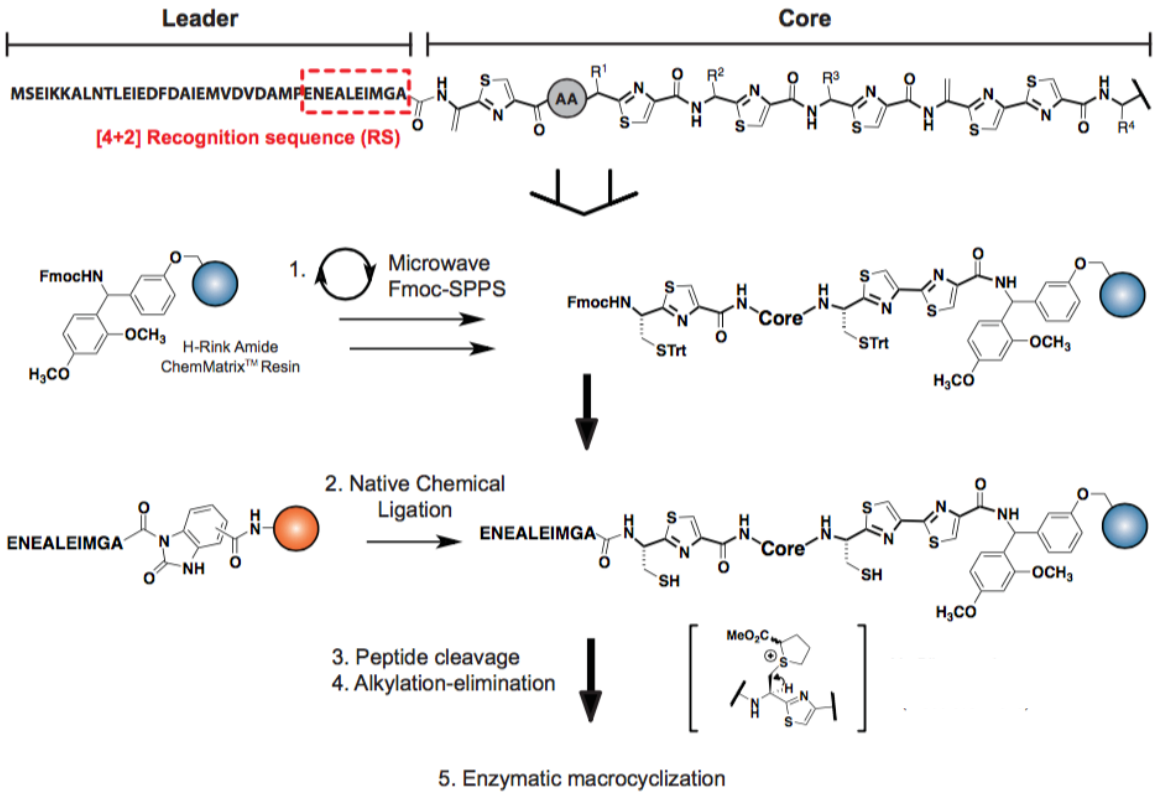


Figure 2.12. Overall SPPS scheme for thiazolyl peptide analogues.

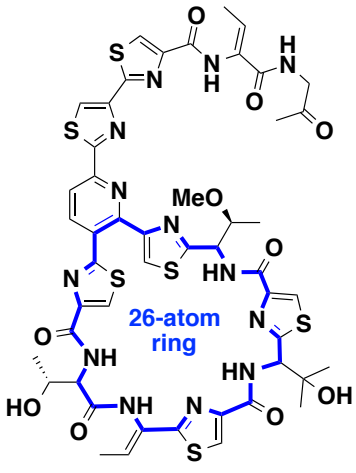
By this strategy, a total of eleven cores and their sequences are summarized in **Table 2.2**. There are interesting trends that can be discerned from these results: a 10-LP from TbtA is sufficient for TbtD to perform the cyclization (entries 6,8, and 10), albeit the conversion is much poorer and most of the starting material remains (data not shown). We hypothesize that there is a [4+2] recognition sequence near the C-terminus of thiazolyl peptides LPs, but additional experiments are needed to confirm this trend. Additionally, the environment surrounding the downstream 4π-system involved in the pyridine formation seems to be important. TclM can tolerate expanding the macrocycle to a 29-atom macrocycle (entry 5), but when the exocyclic bisthiazole is changed to a monothiazole, the cyclization does not occur (entry 4). Likewise, TbtD needs a C-terminal Dha in order to cyclize the core (entry 6

vs. entry 7), but, surprisingly, it can also cyclize in the presence of both an endocyclic and exocyclic bisthiazole moieties (entry 10). Finally, while TclM was able to expand the macrocycle, TbtD could not tolerate ring compression when the tyrosine residue was removed from the core (entry 11).

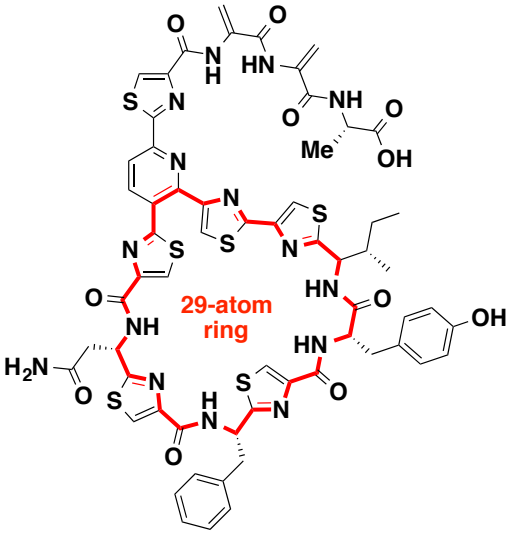
Table 2.2. Results of constructed cores by SPPS with TclM and TbtD

		Cyclization	
Entry	Core	TclE(29-38)/TclM	TbtA(25-34)/TbtD
1	Dha Thz Thr Dha Thz Ala Thz Ala Thz Dha Thz Thz Ala	✓	✗
2	Dha Thz Thr Ala Thz Ala Thz Ala Thz Dha Thz Thz	✓	✗
3	Dha Thz Thr Ala Thz Ala Thz Ala Thz Dha Thz Dha	✗	✗
4	Dha Thz Thr Ala Thz Ala Thz Ala Thz Dha Thz	✗	✗
5	Dha Thz Thr Ala Thz Ala Thz Ala Ala Thz Dha Thz Thz	✓	✗
6	Dha Thz Asn Thz Phe Thz Tyr Val Thz Thz Dha Thz Dha	✗	✓
7	Dha Thz Asn Thz Phe Thz Tyr Val Thz Thz Dha Thz	✗	✗
8	Dha Thz Asn Thz Val Thz Tyr Val Thz Thz Dha Thz Dha	✗	✓
9	Dha Thz Asn Thz Val Thz Tyr Val Thz Thz Dha Thz	✗	✗
10	Dha Thz Asn Thz Phe Thz Tyr Val Thz Thz Dha Thz Thz	✗	✓
11	Dha Thz Asn Thz Phe Thz Val Thz Thz Dha Thz Thz Dha	✗	✗

Dha = dehydroalanine. Thz = thiazole. Asn = *N*-methyl asparagine.



Thiocillin
2-1

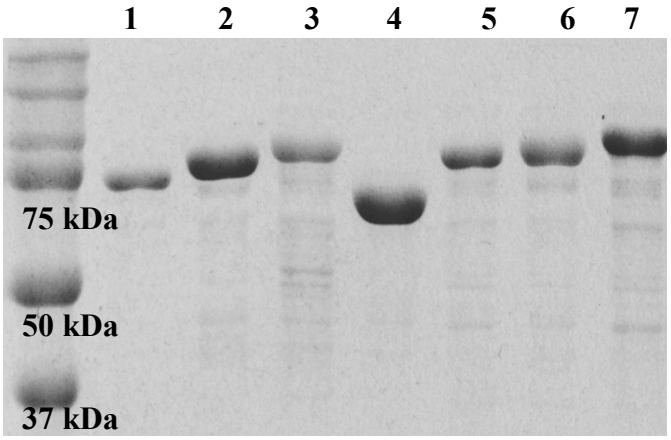


Thiomuracin GZ
2-32

2.8 TclM Homologues

Recently, Mitchell *et al*. described the presence of a RiPPs-recognition element (RRE) found in many of the prokaryotic RiPP biosynthetic pathways;[46] these domains have homology to PqqD, an accessory protein needed for leader peptide binding of the precursor PqqA in the pyrroloquinoline quinone biosynthesis. We queried the amino acid sequences of TclM and TbtD for the presence of the RRE through the homology predictor HHpred, but did not find one. Our studies suggest that there is a recognition sequence (RS) at the C-terminus of the LPs in the precursor peptides. Thus, the putative RS can be a new RRE involved in the recognition of the C-terminal sequence of LPs, along with the presence of the highly modified core, by TclM and its homologues as a late stage effector of thiazolyl peptide maturation and release.

We set up to clone, overexpress, and purify homologues from the family of pyridine/tetrahydropyridine synthases from known thiazolyl peptides. As with TclM, all enzymes were cloned into the LIC vector pMCSG9 and expressed as N-terminal 6xHis-MBP-TEV-constructs (**Figure 2.13**) to enhance solubility and to assist with purification. Having all these enzymes would allows us to extend our knowledge of leader peptide requirements and provide access to different sizes of macrocycles in thiopeptides with the ultimate goal of finding new avenues to make them bioavailable for use in therapeutics.



Entry	Enzyme	Producer	Thiopeptide
1	TclM	*B. cereus* ATCC 14579	Thiocillins
2	TbtD	*T. bispora* DSM 4483	Thiomuracin GZ
3	NosO	*S. actuosus*	Nosiheptide
4	GetF	*S.* ATCC 55365	GE37468
5	CltD	*S. hygroscopicus* 10-22	Cyclothiazomycin
6	BerD	*S. bernensis*	Berninamycin
7	TsrE	*S. laurentii*	Thiostrepton

Figure 2.13. Purified TclM and homologues as 6xHis-MBP-TEV-Fusions.

2.9 Summary

We have characterized the function of TclM as a formal [4+2] heterocyclase from the thiocillin biosynthetic pathway in *B. cereus* ATCC 14579. We obtained a linear, highly modified substrate through ligation of the leader peptide thioester to a synthetic hexathiazole core through native chemical ligation and subsequent conversion of cysteine side chains to dehydroalanines. The thiocillin analogue was characterized by 1D and 2D NMR, LC/MS, and MS/MS analyses. Residues H242, R265, and R285 in TclM are important for thiocillin production *in vivo* and *in vitro*. Leader peptide truncation from the TclE precursor peptide showed a minimal 7-residue sequence needed to effect cyclization of linear thiopeptides. A

modular solid phase peptide synthesis was established to probe for requirements around the 4π system in the downstream dehydroalanine involved in the formation of the central pyridine core in thiocillin and thiomuracin GZ. Homologues of TclM were cloned and purified for future studies.

2.10 Experimental

2.10.1 General Methods

All reactions were carried out in an oven-dried round-bottomed-flask under an inert nitrogen atmosphere with stirring. Solvents, reagents, and chemicals were purchased through Fisher Scientific and used as received unless otherwise noted. Amino acids and their protected derivatives, 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), and 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC*HCl) were purchased from ChemPep. *N,N*-diisopropylethyl amine (DIPEA), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), and 1-Hydroxybenzotriazole hydrate (HOBt*H_2O) were purchased from Sigma-Aldrich. Spectra for ^{1}H and ^{13}C NMR were recorded at room temperature, unless otherwise noted, with a Varian Inova 400 (400 MHz and 100 MHz, respectively) or Varian Inova 500 (500 MHz and 125 MHz, respectively) spectrometers. Chemical shifts are reported in δ (ppm) relative units to residual solvent peaks $CDCl_3$ (7.26 ppm for ^{1}H and 77.0 ppm for ^{13}C) and DMSO-*d*6 (2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C). Splitting patterns are assigned as s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), multiplet (m), dd (doublet of doublets), and td (triplet of doublets). LC/MS measurements were recorded using an Agilent 6520 Accurate-Mass Q-TOF ESI positive in high-resolution mode, 350 ^{o}C temperature and

250 V fragmentor, with a 100 x 2.10 mm Kinetex® 2.6 m C8 100 Å column or a 50 x 2.1 mm Kinetex® 2.6 m C18 100 Å column; both were purchased from Phenomenex. MS-MS was performed with a collision energy of 45 V or 60 V. Predicted masses were extracted to ± 5 ppm. Two methods were utilized:

Method A:

Time	**% Solvent B**
0 - 2 min	2%
2 - 5 min	2 - 45%
5 – 22 min	45 - 60%
22 - 23 min	60 – 95%
23 - 24 min	95%
24 – 25 min	95 - 2%

Method B:

Time	**% Solvent B**
0 -2 min	2%
2- 13 min	2 - 98%
13– 15 min	98%

Microwave assisted peptide couplings were programmed and synthesized in a Biotage Initiator + Alstra™ with Rink Amide ChemMatrix purchased from Biotage. Solutions

utilized were freshly prepared just before synthesis. Couplings were monitored utilizing the Kaiser test. Flash chromatography was performed on a Biotage Isolera™ system with a hexanes/ethyl acetate solvent system or a dichloromethane/methanol system. Thin-layer-chromatography glass plates were purchased from Sorbent Technologies, Inc.® with a fluorescent indicator at l = 254 nm. Preparatory HPLC was performed in a Shimadzu UFLC CBM-20A with a dual channel wavelength detector at 220 nm and 254 nm with a LUNA 10 m C18(2) 100 Å, AXIA (Phenomenex) semi-preparatory column with a 15 mL/min flow rate. Purifications were carried out with a two solvent system (solvent A = 0.1% trifluoroacetic acid in water; solvent B = 0.1% trifluoroacetic acid in acetonitrile).

Method A:

Time	**% Solvent B**
0 -2 min	5%
2- 8 min	5 - 35%
8 – 23 min	35-55%
23- 25 min	55 – 100%
25 – 28.5 min	100%
28.5 – 30 min	100 – 5%
30 – 32 min	5%

Method B:

Time	**% Solvent B**
0 -2 min	5%

2- 25 min	5 - 95%
25 - 27 min	95%
27 - 28 min	95 – 5%
28 – 30 min	5%

2.10.2 Synthesis

Ethyl-(*S*)-2-(3-(*tert*-butoxycarbonyl)-2,2-dimethylthiazolidin-4-yl)thiazole-4-carboxylate (2-11). Compound **2-11** was prepared as a white solid according to a literature procedure from L-cysteine[28c] (4.54 g, 24% overall yield, 5 steps). ^{1}H is in agreement with that previously reported.[1] ^{1}H NMR (400 MHz, DMSO-*d*6, 70 ^{o}C): δ 8.37 (s, 1H), 5.64 (d, *J* = 6.4 Hz, 1H), 4.31 (q, *J* = 7.2 Hz, 2H), 3.65 (dd, *J* = 6.8, 12.4 Hz, 1H), 3.09 (d, *J* = 12.4 Hz, 1H), 1.88 (s, 3H), 1.80 (s, 3H), 1.35 (s, 9H), 1.32 (t, *J* = 7.2 Hz, 3H). HR-MS (ESI): Calculated $C_{16}H_{25}N_2O_4S_2^+$ $[M + H]^+$ = 373.1250; found $[M + H]^+$ = 373.1255. $[a]_D^{20.4}$ = -44.76^o (c = 0.1, $CHCl_3$).

***tert*-Butyl-(*S*)-4-(4-carbamothioylthiazol-2-yl)-2,2-dimethylthiazolidine-3-carboxylate (2-12).** Compound **2-11** (0.50 g, 1.34 mmol) is dissolved in MeOH (11.5 mL) and cooled to 0 ^{o}C. 30% NH_4OH (6.9 mL) is added dropwise and solution is allowed to warm to rt and stir for 16 h under N_2. Reaction is concentrated to dryness under reduced pressure. Crude mixture is dissolved in DCM and Lawesson's reagent (0.27 g, 0.671 mmol) is added. Reaction is allowed to stir for 36 h at rt under N_2. Mixture is evaporated to dryness and purified by flash chromatography with silica gel (~60 mesh) with a hexanes (Hex) / ethyl acetate (EtOAc) mixture (R_f = 0.25, 30 % EtOAc in Hex) to afford the corresponding thioamide as a pale

yellow solid (**2-12**, 0.35 g) in 73% yield (2 steps). ^{1}H NMR (400 MHz, DMSO-*d*6, 70 ^{o}C): δ 9.71 (s, br, 1H), 9.16 (s, br, 1H), 8.34 (s, 1H), 5.61 (d, *J* = 6.4 Hz, 1H), 3.63 (dd, *J* = 6.0, 12.2 Hz, 1H), 3.27 (d, *J* = 12.4 Hz, 1H), 1.87 (s, 3H), 1.80 (s, 3H), 1.37 (s, 9H). ^{13}C NMR (100 MHz, DMSO-*d*6, 70 ^{o}C): δ 189.21, 173.27, 152.71, 151.34, 126.26, 80.22, 71.77, 70.68, 64.97, 32.69, 28.87, 27.56. HR-MS (ESI): Calculated $C_{14}H_{22}N_3O_2S_3^+$ $[M + H]^+$ = 360.0869; found $[M + H]^+$ = 360.0851. $[a]_D^{20.4}$ = -50.98^{o} (c = 0.1, $CHCl_3$).

Ethyl(*S*)-2'-(3-(*tert*-butoxycarbonyl)-2,2-dimethylthiazolidin-4-yl)-[2,4'-bithiazole]-4-carboxylate (2-13). Compound **2-12** (0.50 g, 1.40 mmol) is dissolved in DME (8.0 mL) and cooled to -40 ^{o}C. Anhydrous $KHCO_3$ (0.56 g, 5.59 mmol) is added followed by dropwise addition of 80-85% ethylbromo pyruvate (0.85 mL, 5.59 mmol) and reaction stirred for 24 h at -15 ^{o}C. Precipitate is removed by vacuum filtration and washed with DME (2 mL). Solution is cooled to -20 ^{o}C and 2,6-lutidine (1.13 mL, 9.66 mmol) is added dropwise followed by TFAA (0.643 mL, 4.63 mmol). Reaction is stirred for 2 h at this temperature. Mixture is evaporated to dryness and partitioned between $CHCl_3$ (10 mL) and H_2O (10 mL). Collected organic layer and extracted aqueous layer two more times with $CHCl_3$ (5 mL each). $CHCl_3$ layer is washed with 10% $CuSO_4$ (2 x 10 mL), 0.05 M citric acid (2 x 10 mL), and brine (10 mL). Mixture is purified by flash chromatography with silica gel (~60 mesh) with a hexanes (Hex) / ethyl acetate (EtOAc) mixture (R_f = 0.30, 25 % EtOAc in Hex) to afford the corresponding *bis*-thiazole ethyl ester as a white solid (**2-13**, 0.493 g) in 77% yield. ^{1}H NMR (400 MHz, DMSO-*d*6, 70 ^{o}C): δ 8.48 (d, *J* = 0.8 Hz, 1H), 8.25 (d, *J* = 0.8Hz, 1H), 5.70 (d, *J* = 6.4 Hz, 1H), 4.35 (q, *J* = 6.8 Hz, 2H), 3.67 (dd, *J* = 6.4, 12.4 Hz, 1H), 3.16 (t, *J* = 15.6 Hz, 1H), 1.91 (s, 3H), 1.81 (s, 3H), 1.36 (s, 9H), 1.35 (t, *J* = 7.2 Hz,

3H) . ^{13}C NMR (100 MHz, DMSO-*d*6, 70 ^{o}C): δ 174.68, 162.18, 160.33, 146.85, 146.54, 128.63, 117.76, 80.09, 70.62, 70.58, 64.86, 60.42, 32.99, 28.90, 27.74, 27.55, 13.78. HR-MS (ESI): Calculated $C_{19}H_{26}N_3O_4S_3^+$ $[M + H]^+$ = 456.1080; found $[M + H]^+$ = 456.1099. $[a]_D^{20.4}$ = -69.38^{o} (c = 0.1, $CHCl_3$).

***tert*-Butyl (*S*)-(1-amino-1-thioxopropan-2-yl)carbamate (2-15).** Boc-Ala-OSu (10.0 g, 34.9 mmol) is dissolved in EtOAc (450 mL) and cooled to 0 ^{o}C 30% NH_4OH (12 mL) is added dropwise over a 15 min period and reaction stirred at 0 ^{o}C for 1 h. 200 mL of sat. (aq.) $NaHCO_3$ are added and the organic layer collected, dried with Na_2SO_4, and concentrated to dryness under reduced pressure. Crude reaction mixture is dissolved in DCM (250 mL) and Lawesson's reagent (7.17 g, 17.7 mmol) is added and reaction stirred for 36 h at rt under N_2. Mixture is evaporated to dryness and purified by flash chromatography with silica gel (~60 mesh) with a hexanes (Hex) / ethyl acetate (EtOAc) mixture (R_f = 0.65, 50 % EtOAc in Hex) to afford the corresponding thioamide as a pale yellow solid (**2-15**, 6.01 g) in 82% yield (2 steps). ^{1}H NMR (400 MHz, DMSO-*d*6, 26 ^{o}C): δ 9.54 (s, br, 1H), 9.08 (s, br, 1H), 6.79 (d, *J* = 6.4 Hz, 1H), 4.22-4.27 (m, 1H), 1.37 (s, 9H), 1.23 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-*d*6, 26 ^{o}C): δ 209.37, 154.52, 78.13, 55.62, 28.16, 21.07. HR-MS (ESI): Calculated $C_8H_{16}N_2O_2SNa^+$ $[M + Na]^+$ = 227.0830; found $[M + H]^+$ = 227.0838. $[a]_D^{20.4}$ = -48.14^{o} (c = 0.1, $CHCl_3$).

Ethyl(*S*)-2-(1-((*tert*-butoxycarbonyl)amino)ethyl)thiazole-4-carboxylate (2-16). Compound **2-15** (4.27 g, 20.9 mmol) is dissolved in DME (113 mL) and cooled to -40 ^{o}C. Anhydrous $KHCO_3$ (8.37 g, 83.6 mmol) is added followed by dropwise addition of 80-85%

ethylbromo pyruvate (13.1 mL, 83.6 mmol) and reaction stirred for 24 h at -15 °C. Precipitate is removed by vacuum filtration and washed with DME (20 mL). Solution is cooled to -20 °C and 2,6-lutidine (16.82 mL, 144 mmol) is added dropwise followed by TFAA (9.62 mL, 69.2 mmol). Reaction is stirred for 2 h at this temperature. Mixture is evaporated to dryness and partitioned between $CHCl_3$ (10 mL) and H_2O (10 mL). Collected organic layer and extracted aqueous layer two more times with $CHCl_3$ (5 mL each). $CHCl_3$ layer is washed with 10% $CuSO_4$ (2 x 10 mL), 0.05 M citric acid (2 x 10 mL), and brine (10 mL). Mixture is purified by flash chromatography with silica gel (~60 mesh) with a hexanes (Hex) / ethyl acetate (EtOAc) mixture (R_f = 0.30, 25 % EtOAc in Hex) to afford the corresponding alanine-thiazole ethyl ester as a white solid (**2-16**, 2.97 g) in 47% yield. ^{1}H NMR (400 MHz, DMSO-*d*6, 26 °C): δ 8.38 (s, 1H), 7.82 (d, *J* = 7.2 Hz, 1H), 4.85-4.88 (m, 1H), 4.30 (t, *J* = 6.8 Hz, 2H), 1.46 (d, *J* = 7.2 Hz, 3H), 1.40 (s, 9H), 1.29 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-*d*6, 26 °C): δ 176.65, 160.69, 155.00, 145.79, 128.59, 78.49, 60.59, 48.58, 28.11, 20.39, 14.14. HR-MS (ESI): Calculated $C_{13}H_{21}N_2O_4S^+$ $[M + H]^+$ = 301.1217; found $[M + H]^+$ = 301.1244. $[a]_D^{20.4}$ = -28.10° (c = 0.1, $CHCl_3$).

General Procedure A

Corresponding ester (1.0 equiv.) is dissolved in THF (9.43 mL/mmol) and MeOH (7.86 mL/mmol) and cooled to 0 °C. 0.4 M NaOH (3.13 equiv.) is added dropwise and reaction stirred for 4 h at rt. Concentrated until about a third of solvent is left and the aqueous layer is acidified with 0.5 M citric acid until pH = 3.0. Extracted compound with ethyl acetate, unless otherwise noted, (3 x 12 mL/mmol). Washed with H_2O (12 mL/mmol) and brine (12

mL/mmol). Dried with Na_2SO_4 and concentrated to dryness under reduced pressure to afford the corresponding acid, which was utilized without further purification.

General Procedure B

Corresponding Boc-protected amine (1.0 equiv.) is dissolved in DCM (3.45 mL/mmol) and cooled to 0 ^oC. Trifluoroacetic acid (3.45 mL/mmol) is added dropwise over a 10 min period and reaction warmed to rt over a 2 h period. Mixture is concentrated to dryness under reduced pressure. EtOAc is added (5 mL) and reaction concentrated to dryness. Crude amine salt was utilized without further purification.

General Procedure C

Corresponding acid (1.05 equiv.) is dissolved in DCM (9.32 mL/mmol). PyBOP (1.05 equiv.) and *N*,*N*-diisopropylethylamine (2.5 equiv.) are added and mixture stirred for 15 min at rt. Meanwhile, amine salt (1.0 equiv.) is dissolved in DCM (1.43 mL/mmol) and MeCN (1.43 mL/mmol) and added dropwise to the above acid solution at 0 ^oC. Reaction is monitored by TLC until complete consumption of starting amine. Reaction is concentrated to dryness under reduced pressure and purified by silica gel (~60 mesh).

General Procedure D: Adapted from a literature procedure.[28c] Compound (1.0 equiv.) is dissolved in methanol (MeOH, 7.8 mL/mmol) and tetrahydrofuran (THF, 9.4 mL/mmol) and cooled to 0 ^oC. 0.4 M NaOH (3.13 equiv.) is added dropwise and reaction stirred for 4 hrs at rt. Organics are removed by rotary evaporation under reduced pressure. Aqueous layer is acidified with 1 N HCl until pH = 3.0. Acid derivative is extracted with ethyl acetate (EtOAc,

3 x 10 mL/mmol), unless otherwise noted, dried with Na_2SO_4 and concentrated to dryness. Crude acid is dissolved in dichloromethane (DCM, 3.7 mL/mmol) and cooled to 0 ^oC; trifluoroacetic acid (TFA, 3.7 mL/mmol) is added dropwise and reaction stirred for 2 h at 0 ^oC and 1h at rt. Concentrated. 50% (aq.) ethanol (3.7 mL/mmol) is added and removed by concentration; repeated this process 3 more times. Dried under vacuum to dryness. Crude compound is dissolved in TFA (4.6 mL/mmol) and trityl chloride (1.0 equiv.) is added and reaction stirred for 10 min at rt. Concentrated. Added DCM (9.2 mL/mmol) and concentrated; repeated three more times. Added DCM (18.4 mL/mmol) and washed with H_2O (3.7 mL/mmol). Dried with Na_2SO_4 and concentrated to dryness. Crude compound is dissolved in THF (3.7 mL/mmol) and H_2O (3.7 mL/mmol) and cooled to 0 ^oC. $NaHCO_3$ (2.5 equiv.) is added scoop wise, followed by Fmoc-OSu (1.0 equiv.) over a 20 min period. Reaction is stirred for 20 h at rt. Concentrated to remove THF. Extracted with EtOAc (14.8 mL/mmol), unless otherwise noted, and collected organic layer; repeated the extraction one more time. Dried organics with Na_2SO_4 and concentrated. Desired compound was purified by flash chromatography with silica gel.

General Procedure E: Compound (1.0 equiv.) is dissolved in methanol (MeOH, 7.8 mL/mmol) and tetrahydrofuran (THF, 9.4 mL/mmol) and cooled to 0 ^oC. 0.4 M NaOH (3.13 equiv.) is added dropwise and reaction stirred for 4 hrs at rt. Organics are removed by rotary evaporation under reduced pressure. Aqueous layer is acidified with 1 N HCl until pH = 3.0. Acid derivative is extracted with ethyl acetate (EtOAc, 3 x 10 mL/mmol), unless otherwise noted, dried with Na_2SO_4 and concentrated to dryness. Crude acid is dissolved in dichloromethane (DCM, 3.7 mL/mmol) and cooled to 0 ^oC; trifluoroacetic acid (TFA, 3.7

mL/mmol) is added dropwise and reaction stirred for 2 h at 0 ^{o}C and 1h at rt. Concentrated. Extracted with EtOAc (14.8 mL/mmol), unless otherwise noted, and collected organic layer; repeated the extraction one more time. Dried organics with Na_2SO_4 and concentrated. Desired compound is purified by flash chromatography with silica gel.

General Procedure F: Adapted from a literature procedure.[47] Compound (1.0 equiv.) is suspended in benzene (2.5 mL/mmol) and Lawesson's reagent is added (0.5 equiv.); reaction is refluxed for 1 h and then cooled to rt. EtOAc is added (5 mL/mmol) and organics washed with sat. NH_4Cl (aq., 2 x 1.25 mL/mmol), sat. $NaHCO_3$ (2.4 mL/mmol), and brine. Dried organics with Na_2SO_4 and concentrated. Crude mixture is dissolved in 1,2-dimethoxyethane (DME, 3.6 mL/mmol) and cooled to -40 ^{o}C. $KHCO_3$ (4.0 equiv.) is added followed by dropwise addition of ethylbromopyruvate (4.0 equiv.). Reaction is let to react for 16 hrs while warming to -20 ^{o}C then to rt. Solid is filtered and washed with DME. Filtrate is cooled to -15 ^{o}C and 2,6-lutidine added (6.91 equiv.), followed by trifluoroacetic anhydride (3.31 equiv.) over a 20 min period. Reaction is allowed to stir at this temperature for 3 hrs. Reaction is concentrated to dryness and EtOAc added (4 mL/mmol). Organic layer is washed with 0.1 N HCl (4 x 2 mL/mmol) and brine. Dried organic layer with Na_2SO_4 and concentrated. Desired compound is purified by flash chromatography on silica gel.

***tert*-Butyl-(*S*)-4-(4-(((2*S*,3*R*)-3-hydroxy-1-methoxy-1-oxobutan-2-yl)carbamoyl)thiazol-2-yl)-2,2-dimethylthiazolidine-3-carboxylate (2-18).** Acid of compound **2-11** was prepared according to General Procedure A. Coupling of acid and amine hydrochloride salt of L-threonine methyl ester was done according to General Procedure C for 2 h. Mixture was

purified by flash chromatography with silica gel (~60 mesh) with a hexanes (Hex) / ethyl acetate (EtOAc) mixture (R_f = 0.45, 50 % EtOAc in Hex) to afford the corresponding coupled product as a white solid (**2-18**, 0.34 g) in 65% yield. ^{1}H NMR (400 MHz, DMSO-*d*6, 70 ^{o}C): δ 8.21 (s, 1H), 7.89 (d, *J* = 8.4 Hz, 1H), 5.68 (d, *J* = 6.4 Hz, 1H), 4.47 (dd, *J* = 2.8, 8.6 Hz, 1H), 4.23-4.27 (m, 1H), 3.68 (s, 3H), 3.65 (d, *J* = 6.8 Hz, 1H), 3.14 (d, *J* = 12.4 Hz, 1H), 1.90 (s, 3H), 1.80 (s, 3H), 1.35 (s, 9H), 1.13 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-*d*6, 70 ^{o}C): δ 174.07, 170.38, 160.20, 151.25, 148.01, 123.90, 80.10, 70.60, 65.83, 64.87, 57.42, 51.54, 51.50, 32.93, 28.83, 27.54, 19.94. HR-MS (ESI): Calculated $C_{19}H_{30}N_3O_6S_2^+$ $[M + H]^+$ = 460.1571; found $[M + H]^+$ = 460.1585. $[a]_D^{20.4}$ = -44.76^o (c = 0.1, $CHCl_3$).

Ethyl-2-((*S*)-1-(2-((*S*)-1-((*tert*-butoxycarbonyl)amino)ethyl)thiazole-4-carboxamido)ethyl)thiazole-4-carboxylate (2-20)

Acid of compound **2-16** was prepared according to General Procedure A and extracted with chloroform. Amine salt of compound **2-16** was prepared according to General Procedure B. Coupling of acid and amine salt was done according to General Procedure C for 3 h. Mixture was purified by flash chromatography with silica gel (~60 mesh) with a hexanes (Hex) / ethyl acetate (EtOAc) mixture (R_f = 0.6, 50 % EtOAc in Hex) to afford the corresponding coupled product as a pale yellow solid (**2-20**, 0.43 g) in 49% yield. ^{1}H is in agreement with that previously reported.[48] ^{1}H NMR (400 MHz, DMSO-*d*6, 70 ^{o}C): δ 9.03 (t, 11.6 Hz, 1H), 8.41 (d, *J* = 0.8 Hz, 1H), 8.22 (s, 1H), 7.84 (d, *J* = 6.4 Hz, 1H), 5.38-5.45 (m, 1H), 4.86-4.93 (m, 1H), 4.29 (q, *J* = 7.2 Hz, 2H), 1.65 (d, *J* = 6.8 Hz, 3H), 1.50 (d, *J* = 6.8 Hz, 3H), 1.41 (s, 9H), 1.30 (t, *J* = 7.2 Hz, 3H). HR-MS (ESI): Calculated $C_{19}H_{27}N_4O_5S_2^+$ $[M + H]^+$ = 455.1417; found $[M + H]^+$ = 455.1416. $[a]_D^{20.4}$ = -27.94^o (c = 0.1, $CHCl_3$).

Ethyl 2'-((*S*)-1-(2-((*S*)-1-((*tert*-butoxycarbonyl)amino)ethyl)thiazole-4-carboxamido)-2-(tritylthio)ethyl)-[2,4'-bithiazole]-4-carboxylate (2-24). Compound **2-13** (0.50 g, 1.10 mmol) is dissolved in DCM (6 mL) and cooled to 0 ^{o}C. Trifluoroacetic acid (6 mL) is added dropwise and reaction stirred for 4 h at rt. Reaction is concentrated under reduced pressure. 50 % (aq.) EtOH (5 mL) is added and mixture concentrated again; repeated four more times. Dried under vacuum. Crude mixture is dissolved in trifluoroacetic acid (5 mL) and trityl chloride (0.306g, 1.10 mmol) is added and reaction stirred for 10 min at rt. Concentrated. Added 10 mL of DCM and concentrated. Repeated three more times. Added 10 mL of DCM and 5 mL of H_2O. Collected organic layer, dried with Na_2SO_4 and concentrated to dryness under reduced pressure. Compound was utilized without any further purification.

The coupling to the acid of compound **2-16**, prepared via General Procedure A, was performed according to General Procedure C for 3 h. Mixture was purified by flash chromatography with silica gel (~60 mesh) with a hexanes (Hex) / ethyl acetate (EtOAc) mixture (R_f = 0.65, 50 % EtOAc in Hex) to afford the corresponding coupled product as a yellow solid (**2-24**, 0.44 g) in 65% yield (4 steps). ^{1}H NMR (400 MHz, DMSO-*d*6, 70 ^{o}C): δ 8.81 (d, *J* = 8.0 Hz, 1H), 8.47 (s, 1H), 8.22 (s, 1H), 8.20 (s, 1H), 7.53 (s, br, 1H), 7.30-7.38 (m, 12 H), 7.22-7.26 (m, 3H), 5.21-5.27 (m, 1H), 4.93-4.99 (m, 1H), 4.35 (t, *J* = 6.8 Hz, 2H), 3.18 (dd, *J* = 8.8, 12.6 Hz, 1H), 2.95 (dd, *J* = 5.2, 12.8 Hz, 1H), 1.55 (d, *J* = 6.8 Hz, 3H), 1.41 (s, 9H), 1.34 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-*d*6, 70 ^{o}C): δ 176. 13, 171.72, 161.95, 160.28, 159.95, 154.56, 148.30, 146.83, 143.93, 128.79, 128.70, 127.61, 126.42, 124.06, 118.04, 78.25, 66.60, 60.38, 50.63, 48.58, 35.30, 27.84, 20.26, 13.75. HR-MS (ESI):

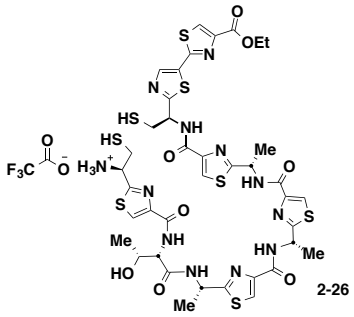
Calculated $C_{41}H_{41}N_5O_5S_4^+$ $[M + H]^+$ = 812.2063; found $[M + H]^+$ = 812.2064. $[a]_D^{20.4}$ = -33.88° (c = 0.1, $CHCl_3$).

Ethyl-2-((*S*)-1-(2-((*S*)-1-((2*S*,3*R*)-2-(2-((*S*)-3-(*tert*-butoxycarbonyl)-2,2-dimethylthiazolidin-4-yl)thiazole-4-carboxamido)-3-hydroxybutanamido)ethyl)thiazole-4-carboxamido)ethyl)thiazole-4-carboxylate (2-22). Acid of compound **2-18** was prepared according to General Procedure A. Amine salt of compound **2-20** was prepared according to General Procedure B. Amine salt (0.198 g, 0.423 mmol) is dissolved in $CHCl_3$ (2 mL) and cooled to 0 °C. *N*-methylmorpholine (0.146 mL, 1.33 mmol) is added very slowly dropwise. Meanwhile, acid (0.237 g, 0.532 mmol) is dissolved in $CHCl_3$ (4 mL) and THF (0.75 mL) and added to the above cooled solution dropwise. EDC*HCl (0.102 g, 0.532 mmol) is added followed by 2 crystals of *N,N*-4-dimethylaminopyridine (DMAP) and reaction stirred for 18 h at rt. Reaction is washed with 0.05 M citric acid (2 x 5 mL), dried with Na_2SO_4, and concentrated. Mixture was purified by flash chromatography with silica gel (~60 mesh) with a hexanes (Hex) / ethyl acetate (EtOAc) mixture (R_f = 0.50, 100% EtOAc) to afford the corresponding coupled product as a white solid (**2-22**, 0.275 g) in 54% yield. ^{1}H NMR (400 MHz, DMSO-*d*6, 70 °C): δ 8.72 (d, *J* = 7.6 Hz, 1H), 8.62 (d, *J* = 8.0 Hz, 1H), 8.36 (s, 1H), 8.21 (s, 1H), 8.19 (s, 1H), 7.92 (d, *J* = 8.4 Hz, 1H), 5.68 (d, *J* = 6.4 Hz, 1H), 5.40-5.47 (m, 1H), 5.25-5.32 (m, 1H), 4.46 (dd, *J* = 3.2, 8.2, 1H), 4.32 (q, *J* = 7.2 Hz, 2H), 4.17-4.22 (m, 1H), 3.67 (dd, *J* = 6.4, 12.4 Hz, 1H), 3.12 (d, *J* = 12.4 Hz, 1H) 1.90 (s, 3H), 1.80 (s, 3H), 1.68 (d, *J* = 7.2 Hz, 3H), 1.60 (d, *J* = 6.8 Hz, 3H), 1.35 (s, 9H), 1.32 (t, *J* = 7.2 Hz, 3H), 1.12 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-*d*6, 70 °C): δ 174.21, 173.95, 173.87, 169.31, 160.36, 160.06, 159.90, 148.48, 148.40, 145.47, 128.25, 124.07, 123.57, 80.14, 70.62, 66.36,

64.91, 60.23, 57.79, 46.79, 46.76, 46.72, 32.93, 28.81, 27.78, 27.55, 20.15, 19.87, 19.82, 13.76. HR-MS (ESI): Calculated $C_{32}H_{44}N_7O_8S_4^+$ $[M + H]^+$ = 782.2129; found $[M + H]^+$ = 782.2120. $[a]_D^{20.4}$ = -46.34° (c = 0.1, $CHCl_3$).

Ethyl-2'-((*S*)-1-(2-((*S*)-1-(2-((*S*)-1-(2-((*S*)-1-((2*S*,3*R*)-2-(2-((*S*)-3-(*tert*-butoxycarbonyl)-2,2-dimethylthiazolidin-4-yl)thiazole-4-carboxamido)-3-hydroxybutanamido)ethyl)thiazole-4-carboxamido)ethyl)thiazole-4-carboxamido)ethyl)thiazole-4-carboxamido)-2-(tritylthio)ethyl)-[2,4'-bithiazole]-4-carboxylate (2-25). Acid of compound **2-22** was prepared according to General Procedure A. Amine salt of compound **2-24** was prepared according to General Procedure B. Amine salt (0.26 g, 0.326 mmol) is dissolved in $CHCl_3$ (2 mL) and cooled to 0 °C. *N*-methylmorpholine (0.11 mL, 1.00 mmol) is added very slowly dropwise. Meanwhile, acid (0.25 g, 0.332 mmol) is dissolved in $CHCl_3$ (4 mL) and added to the above cooled solution dropwise. EDC*HCl (63 mg, 0.328 mmol) is added followed by 2 crystals of *N,N*-4-dimethylaminopyridine (DMAP) and reaction stirred for 20 h at rt. Mixture was purified by flash chromatography with silica gel (~60 mesh) with a dichloromethane (DCM) / methanol (MeOH) mixture (R_f = 0.30, 5% MeOH in DCM) to afford the corresponding coupled product as a white solid (**2-25**, 0.165 g) in 36% yield. ^{1}H NMR (400 MHz, DMSO-*d*6, 70 °C): δ 8.85 (d, *J* = 8.4 Hz, 1H), 8.80 (d, *J* = 7.6 Hz, 1H), 8.74 (d, *J* = 7.6 Hz, 1H), 8.64 (d, *J* = 7.2 Hz, 1H), 8.48 (s, 1H), 8.24 (s, 1H), 8.22 (s, 1H), 8.212 (s, 1H), 8.207 (s, 1H), 8.20 (s, 1H), 7.95 (d, *J* = 8.0 Hz, 1H), 7.30-7.37 (m, 12H), 7.23-7.26 (m, 3H), 5.68 (d, *J* = 6.0 Hz, 1H), 5.42-5.53 (m, 2H), 5.28-5.33 (m, 1H), 5.20-5.26 (m, 1H), 4.47 (dd, *J* = 3.6, 8.2 Hz, 1H), 4.35 (q, *J* = 6.8 Hz, 2H), 4.18-4.23 (m, 1H), 3.67 (dd, *J* = 6.4, 12.4 Hz, 1H), 3.18 (t, *J* = 9.2 Hz, 1H), 3.12 (d, *J* = 12.4

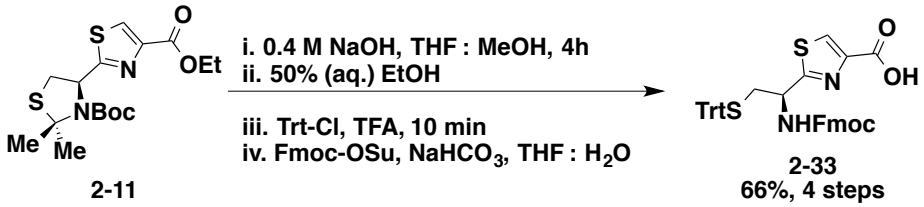
Hz, 1H), 2.94 (dd, *J* = 5.2, 12.8 Hz, 1H), 1.89 (s, 3H), 1.80 (s, 3H), 1.74 (d, *J* = 6.8 Hz, 3H), 1.72 (d, *J* = 8.8 Hz, 3H), 1.60 (d, *J* = 7.2 Hz, 3H), 1.35 (s, 9H), 1.33 (t, *J* = 4.4 Hz, 3H), 1.14 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-*d*6, 70 ^{o}C): δ 174.29, 174.17, 174.03, 173.96, 171.75, 169.34, 161.96, 160.30, 160.08, 160.01, 159.99, 159.93, 151.29, 148.60, 148.48, 148.42, 148.23, 146.82, 146.79, 143.94, 128.80, 127.65, 126.46, 124.40, 124.08, 123.58, 118.10, 80.14, 70.61, 66.62, 66.38, 64.90, 60.41, 57.80, 50.70, 46.93, 46.80, 35.29, 32.93, 28.84, 27.80, 27.56, 20.20, 19.94, 19.87, 19.84, 19.80, 13.77. HR-MS (ESI): Calculated $C_{66}H_{71}N_{12}O_{10}S_8^+$ $[M + H]^+$ = 1447.3176; found $[M + H]^+$ = 1447.3216. $[a]_D^{20.4}$ = -50.98^{o} (c = 0.1, $CHCl_3$).



(*S*)-1-(4-(((2*S*,3*R*)-1-(((*S*)-1-(4-(((*S*)-1-(4-(((*S*)-1-(4-(((*S*)-1-(4-(ethoxycarbonyl)-[2,5'-bithiazol]-2'-yl)-2-mercaptoethyl)carbamoyl)thiazol-2-yl)ethyl)carbamoyl)thiazol-2-yl)ethyl)carbamoyl)thiazol-2-yl)ethyl)amino)-3-hydroxy-1-oxobutan-2-yl)carbamoyl)thiazol-2-yl)-2-mercaptoethan-1-aminium 2,2,2-trifluoroacetate (2-26). Compound **2-25** (49.2 mg, 34.6 mmol) is dissolved in DCM (1 mL) and cooled to 0 ^{o}C. Trifluoroacetic acid (1 mL) is added dropwise followed by triisopropylsilane (7.5 mL, 34.6 mmol). Reaction is stirred for 4 h at rt. Mixture is concentrated *in vacuo* and 50% (aq.) EtOH is added (3 mL) and concentrated again. Repeated 3 more times. Dried overnight *in vacuo*

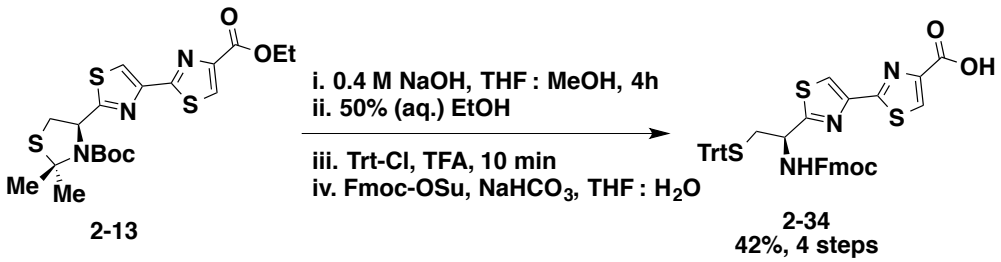
and dissolved mixture in 346.5 mL of *N,N*-dimethylformamide (DMF) to make a 0.10 M stock.

2,5-Dibromohexanediamide (2-28). Compound **2-28** was prepared as a white solid according to a literature procedure from adipic acid.[41] ^{1}H is in agreement with that previously reported. ^{1}H NMR (600 MHz, DMSO-*d*6): δ 7.69 (s, 2H), 7.31 (s, 2H), 4.33 (q, *J* = 4.8 Hz, 2H), 2.01-2.06 (m, 1H), 1.88-2.00 (m, 2H), 1.79-1.84 (m, 1H).



(S)-2-(1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-(tritylthio)ethyl)thiazole-4-carboxylic acid (2-33). Compound was prepared from compound 2-11 according to General Procedure D. Compound was purified by flash chromatography with silica gel (~60 mesh) in a dichloromethane (DCM) / methanol (MeOH) mixture (R_f = 0.4, 5 % MeOH in DCM) to afford the corresponding Fmoc-protected amino acid as a pale tan solid (2-33) in 66% yield (4 steps). ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.30 (d, *J* = 8.5 Hz, 1H), 8.26 (s, 1H), 7.88 (d, *J* = 7.5 Hz, 2H), 7.70 (d, *J* = 7.5 Hz, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.31-7.40 (m, 14H), 7.24-7.28 (m, 4H), 4.51-4.55 (m, 1H), 4.34 (d, *J* = 7.0 Hz, 2H), 4.23 (t, *J* = 7.0 Hz, 1H), 2.83 (dd, *J* = 9.5, 12.25 Hz, 1H), 2.67 (dd, *J* = 5.0, 12.5 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-*d*6): δ 171.62, 162.06, 155.45, 144.15, 143.64, 140.69, 129.08, 128.12, 127.60, 127.03, 127.01, 126.85, 125.19, 125.16, 120.08, 66.46, 65.67, 52.48, 46.62, 35.43. HR-MS (ESI): Calculated

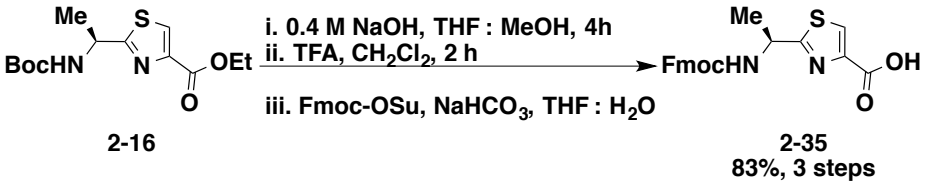
$C_{40}H_{32}N_2NaO_4S_2^+$ [M + Na]$^+$ = 691.1696; found [M + Na]$^+$ = 691.1689. [a]$_D^{22.4}$ = +16.0^o (c = 0.1, MeOH).



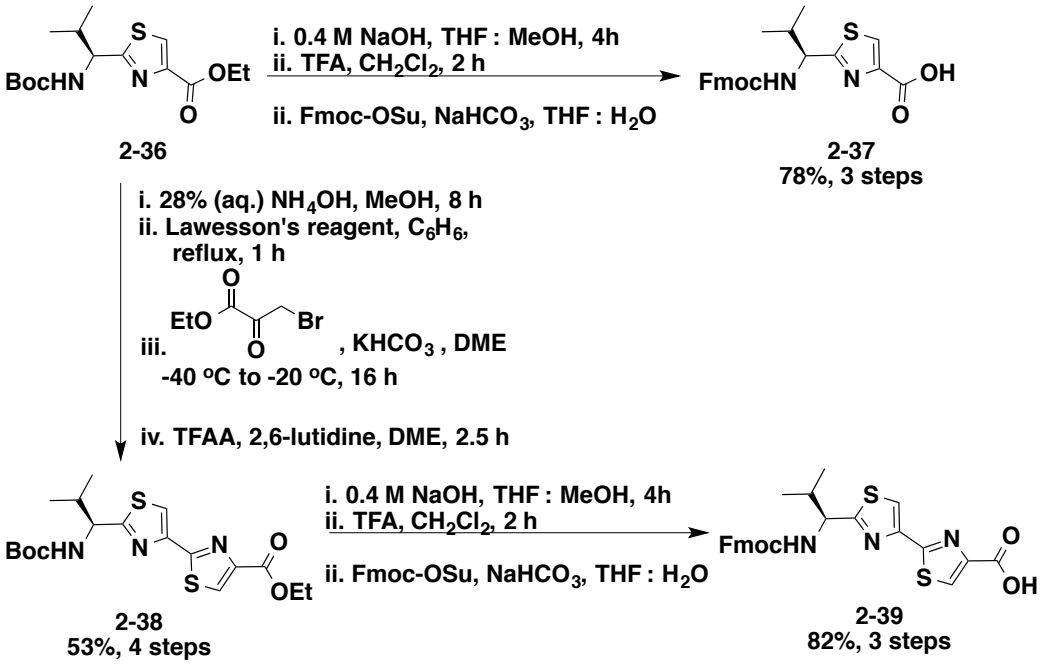
2-13

2-34
42%, 4 steps

(*S*)-2'-(1-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-2-(tritylthio)ethyl)-[2,4'-bithiazole]-4-carboxylic acid (2-34). Compound **2-34** was prepared from compound **2-13** according to General Procedure D. Compound was purified by flash chromatography with silica gel (~60 mesh) in a dichloromethane (DCM) / methanol (MeOH) mixture (R_f = 0.1, 5 % MeOH in DCM) to afford the corresponding Fmoc-protected amino acid pale tan solid (**2-34**) in 42% yield (4 steps). ^{1}H NMR (500 MHz, DMSO-*d*6): δ 13.15 (s, br, 1H), 8.47 (s, 1H), 8.40 (d, *J* = 8.5 Hz, 1H), 8.19 (s, 1H), 7.87 (d, *J* = 7.5 Hz, 2H), 7.72 (d, *J* = 5 Hz, 2H), 7.32-7.40 (m, 15H), 7.25-7.30 (m, 4H), 4.55 (q, *J* = 6 Hz, 1H), 4.43 (t, *J* = 6 Hz, 1H), 4.38 (t, *J* = 7 Hz, 1H), 4.26 (t, *J* = 6.5 Hz, 1H), 2.94 (t, *J* = 6.5 Hz, 1H), 2.69 (dd, *J* = 3.5, 12.5 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-*d*6): δ 173.42, 162.02, 155.61, 148.09, 147.46, 144.17, 143.65, 140.74, 129.13, 128.24, 128.12, 127.62, 127.06, 126.86, 125.19, 125.12, 120.09, 118.05, 66.77, 65.68, 53.05, 46.70, 35.74, 25.23. HR-MS (ESI): Calculated $C_{43}H_{33}N_3NaO_4S_3^+$ [M + Na]$^+$ = 774.1525; found [M + Na]$^+$ = 774.1526. [a]$_D^{22.8}$ = +14.0^o (c = 0.1, MeOH).



(*S*)-2-(1-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)ethyl)thiazole-4-carboxylic acid (2-35). Compound **2-35** was prepared from compound **2-16**according to General Procedure E. Compound was purified by flash chromatography with silica gel (~60 mesh) in a dichloromethane (DCM) / methanol (MeOH) mixture (R_f = 0.35, 5 % MeOH in DCM) to afford the corresponding Fmoc-protected amino acid as a white solid (**2-35**) in 83% yield (3 steps). ^{1}H NMR (500 MHz, DMSO-*d*6): δ 13.0 (s, br, 1H), 8.35 (s, 1H), 8.28 (d, *J* = 8 Hz, 1H), 7.88 (d, *J* = 7.5 Hz, 2H), 7.72 (d, *J* = 8 Hz, 2H), 7.41 (t, *J* = 7.5 Hz, 2H), 7.33 (t, *J* = 8 Hz, 2H), 4.96 (t, *J* = 7.5 Hz, 1H), 4.44 (t, *J* = 7 Hz, 1H), 4.34 (t, *J* = 6.5 Hz, 1H), 4.24 (t, *J* = 6.5 Hz, 1H), 1.51 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-*d*6): δ 175.52, 162.11, 155.67, 147.00, 143.79, 143.73, 140.78, 128.36, 127.66, 127.10, 125.21, 125.14, 120.13, 65.61, 49.04, 46.75, 20.53. HR-MS (ESI): Calculated $C_{21}H_{19}N_2O_4S^+$ $[M + H]^+$ = 395.1060; found $[M + H]^+$ = 395.1055. $[a]_D^{22.5}$ = +24. 0^o (c = 0.1, MeOH).



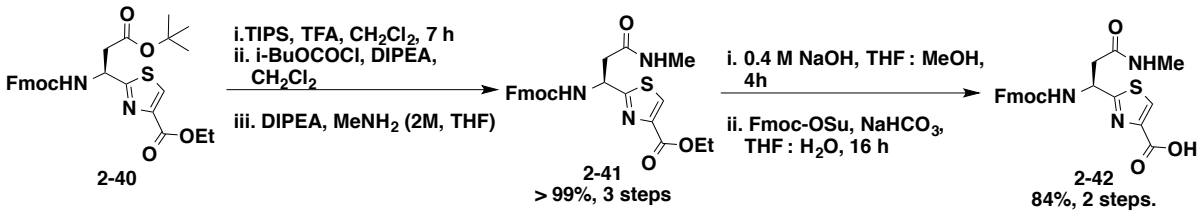
Ethyl (*S*)-2-(1-((*tert*-butoxycarbonyl)amino)-2-methylpropyl)thiazole-4-carboxylate (2-36). Compound **2-36** was prepared according to a literature procedure[28c] from $_L$-Boc-Val-OH as a white solid (3.02 g, 75% overall yield, 3 steps). ^{1}H is in agreement with that previously reported.[28c] ^{1}H NMR (400 MHz, DMSO-*d*6): δ 8.40 (s, 1H), 7.70 (d, *J* = 8.4 Hz, 1H), 4.62 (t, *J* = 7.6 Hz, 1H), 4.29 (q, *J* = 7.2 Hz, 2H), 2.18-2.24 (m, 1H), 1.39 (s, 9H), 0.89 (d, *J* = 6.8 Hz, 3H), 0.84 (d, *J* = 7.2 Hz, 3H). HR-MS (ESI): Calculated $C_{15}H_{25}N_2O_4S^+$ $[M + H]^+$ = 329.1530; found $[M + H]^+$ = 329.1524. $[a]_D^{22.8}$ = -14.0° (c = 0.1, MeOH).

(*S*)-2-(1-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-2-methylpropyl)thiazole-4-carboxylic acid (2-37). Compound **2-37** was prepared from compound **2-36** according to General Procedure D. Compound was purified by flash chromatography with silica gel (~60 mesh) in a dichloromethane (DCM) / methanol (MeOH) mixture (R_f = 0.4, 5% MeOH in DCM) to afford the corresponding Fmoc-protected amino acid as a pale tan solid (**2-37**) in

78% yield (3 steps). ^{1}H NMR (500 MHz, DMSO-*d*6): δ 12.98 (s, br, 1H), 8.36 (s, 1H), 8.23 (d, *J* = 8.5 Hz, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.72 (d, *J* = 6.0 Hz, 2H), 7.41 (d, *J* = 6.5 Hz, 2H), 7.32 (t, *J* = 7.5 Hz, 2H), 4.67 (t, *J* = 8 Hz, 1H), 4.31-4.37 (m, 2H), 4.24 (t, *J* = 7 Hz, 1H), 2.23-2.28 (m, 1H), 0.93 (d, *J* = 7 Hz, 3H), 0.86 (d, *J* = 7 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-*d*6): δ 173.67, 162.08, 156.14, 146.85, 143.72, 140.71, 128.34, 127.63, 127.03, 125.24, 120.10, 65.66, 58.99, 46.69, 32.18, 19.18, 18.21. HR-MS (ESI): Calculated $C_{23}H_{23}N_2O_4S^+$ $[M + H]^+$ = 423.1373; found $[M + H]^+$ = 423.1352. $[a]_D^{22.8}$ = -20.0^o (c = 0.1, MeOH).

Ethyl (*S*)-2'-(1-((*tert*-butoxycarbonyl)amino)-2-methylpropyl)-[2,4'-bithiazole]-4-carboxylate (2-38). Compound **2-36** (4.08 g, 12.4 mmol) is dissolved in MeOH (75 mL) and cooled to 0 ^{o}C. 28% (aq.) NH_4OH (50 mL) is added dropwise and reaction stirred for 8 hrs at rt. Methanol is removed under reduced pressure and DCM is added (75 mL). Collected organic layer, dried with Na_2SO_4, and concentrated to dryness to afford the amide derivative that was subjected to General Procedure F to make compound **2-38**. Compound was purified by flash chromatography with silica gel (~60 mesh) in a hexanes (Hex) / ethyl acetate (EtOAc) mixture (R_f = 0.3, 20% EtOAc in Hex) to obtain **2-38** (2.71 g, 53% yield, 4 steps). ^{1}H is in agreement with that previously reported.[36] ^{1}H NMR (400 MHz, DMSO-*d*6): δ 8.53 (s, 1H), 8.26 (s, 1H), 7.76 (d, *J* = 8.4 Hz, 1H), 4.66 (t, *J* = 7.2 Hz, 1H), 4.33 (q, *J* = 6.8 Hz, 2H), 2.22-2.29 (m, 1H), 1.41 (s, 9H), 1.32 (t, *J* = 6.8 Hz, 3H), 0.93 (d, *J* = 6.4 Hz, 3H), 0.89 (d, *J* = 6.8 Hz, 3H). HR-MS (ESI): Calculated $C_{18}H_{26}N_3O_4S_2^+$ $[M + H]^+$ = 412.1359; found $[M + H]^+$= 412 1354. $[a]_D^{22.6}$ = -27.0^o (c = 0.1, MeOH).

(*S*)-2'-(1-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-2-methylpropyl)-[2,4'-bithiazole]-4-carboxylic acid (2-39). Compound **2-39** was prepared from compound **2-38** according to General Procedure E. Compound was purified by flash chromatography with silica gel (~60 mesh) in a dichloromethane (DCM) / methanol (MeOH) mixture (R_f = 0.35, 5% MeOH in DCM) to afford the corresponding Fmoc-protected amino acid as a pale tan solid (**2-39**) in % yield (3 steps). ^{1}H NMR (400 MHz, DMSO-*d*6): δ 8.32 (s, br, 1H), 8.26 (d, *J* = 8.8 Hz, 1H), 8.22 (s, 1H), 7.89 (d, *J* = 7.6 Hz, 2H), 7.72-7.74 (m, 2H), 7.39-7.43 (m, 2H), 7.32 (t, *J* = 7.6 Hz, 2H), 4.70 (t, *J* = 7.6 Hz, 1H), 4.36 (quint., *J* = 7.6 Hz, 2H), 4.24 (t, *J* = 7.4 Hz, 1H), 2.24-2.31 (m, 1H), 0.96 (d, *J* = 6.4 Hz, 3H), 0.90 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-*d*6): δ 174.97, 162.21, 162.02, 156.19, 148.10, 147.31, 143.71, 140.72, 128.91, 127.64, 127.04, 125.24, 125.18, 120.10, 117.77, 65.67, 58.95, 46.72, 32.30, 19.31, 18.25. HR-MS (ESI): Calculated $C_{26}H_{24}N_3O_4S_2^+$ $[M + H]^+$ = 506.1203; found $[M + H]^+$ = 506.1202. $[a]_D^{22.8}$ = -29.0 ^{o} (c = 0.1, MeOH).



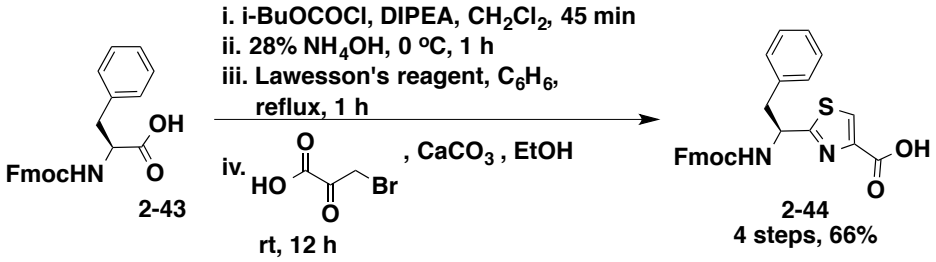
Ethyl (*S*)-2-(1-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(*tert*-butoxy)-3-oxopropyl)thiazole-4-carboxylate (2-40). Compound **2-40** was prepared as a white solid according to a literature method[36] from Fmoc-Asp(OtBu)-OH (4.41 g, 58% overall yield, 5 steps). ^{1}H is in agreement with that previously reported.[49] ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.44 (s, 1H), 8.33 (d, *J* = 8 Hz, 1H), 7.89 (d, *J* = 8 Hz, 2H), 7.70 (d, *J* = 7.5 Hz, 2H), 7.41 (t, *J* = 7 Hz, 2H), 7.32 (t, *J* = 7 Hz, 2H), 5.22 (q, *J* = 6.5 Hz, 1H), 4.36-4.46 (m, 2H), 4.23-4.31

(m, 3H), 2.99 (dd, *J* = 6, 15.8 Hz, 1H), 2.77 (dd, *J* = 9, 15.8 Hz, 1H), 1.37 (s, 9H), 1.29 (t, *J* = 7.5 Hz, 3H). HR-MS (ESI): Calculated $C_{28}H_{31}N_2O_6S^+$ $[M + H]^+$ = 523.1897; found $[M + H]^+$ = 523.1903. $[\alpha]_D^{22.8}$ = -2.0° (c = 0.1, MeOH).

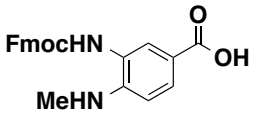
Ethyl (*S*)-2-(1-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(methylamino)-3-oxopropyl)thiazole-4-carboxylate (2-41). Compound **2-40** (4.41 g, 8.44 mmol) is dissolved in DCM (60 mL) and cooled to 0 °C. Triisopropylsilane (1.94 mL, 9.28 mmol) is added followed by dropwise addition of TFA (40 mL). Reaction is stirred for 7 h at rt. Reaction is concentrated; DCM (50 mL) is added and solution concentrated. DCM is added (80 mL) and solution cooled to 0 °C; DIPEA (4.41 mL, 25.3 mmol) is added, followed by dropwise addition of *iso*-butylchloroformate (3.28 mL, 25.3 mmol). Reaction is allowed to stir for 40 min at 0 °C, then additional DIPEA is added (7.35 mL, 42.2 mmol) followed by a dropwise addition of a 2 M $MeNH_2$ solution (in THF, 21.1 mL, 42.2 mmol). Reaction is allowed to stir for 40 min at 0 °C and at rt for 80 min. Mixture is partitioned between 1 N HCl & 10% MeOH in DCM. Organics are dried with Na_2SO_4 and concentrated. Mixture was purified by flash chromatography with silica gel (~60 mesh) with a dichloromethane (DCM) / methanol (MeOH) mixture (R_f = 0.45, 5% MeOH in DCM) to afford the corresponding coupled product as a white solid (**2-41**, 4.05 g) in > 99% yield (3 steps). ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.41 (s, 1H), 8.23 (d, *J* = 8.5 Hz, 1H), 7.87-7.90 (m, 3H), 7.69 (t, *J* = 5.5 Hz, 2H), 7.41 (t, *J* = 7 Hz, 2H), 7.32 (t, *J* = 7 Hz, 2H), 5.25 (q, *J* = 8 Hz, 1H), 4.39 (t, *J* = 7 Hz, 1H), 4.27-4.34 (m, 3H), 4.23 (t, *J* = 6.5 Hz, 1H), 2.85 (dd, *J* = 5.5, 15 Hz, 1H), 2.69 (dd, *J* = 8.5, 15.2 Hz, 1H), 2.57 (d, *J* = 4.5 Hz, 3H), 1.29 (t, *J* = 7 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-*d*6): δ 173.88, 168.89, 160.64, 155.50, 145.67, 143.75, 143.60, 140.70, 129.16, 127.60, 127.06,

127.03, 125.15, 125.11, 120.09, 120.03, 65.62, 60.67, 50.25, 46.65, 25.52, 14.18. HR-MS (ESI): Calculated $C_{25}H_{26}N_3O_5S^+$ $[M + H]^+$ = 480.1588; found $[M + H]^+$ = 480.1570. $[\alpha]_D^{22.7}$ = -11.2° (c = 0.1, MeOH).

(*S*)-2-(1-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(methylamino)-3-oxopropyl)thiazole-4-carboxylic acid (2-42). Compound **2-41** (1.83 g, 3.82 mmol) is dissolved in MeOH (23.9 mL) and THF (28.7 mL) and cooled to 0 °C. 0.5 M NaOH (23.9 mL, 11.96 mmol) is added dropwise and reaction stirred for 4 hrs at rt. THF and MeOH are removed by rotary evaporation and the pH of the aqueous phase is acidified to 7 with 1N HCl. Concentrated to dryness. Crude mixture is dissolved in THF (25 mL) and H_2O (25 mL) and cooled to 0 °C. $NaHCO_3$ (1.12 g, 13.4 mmol) is added scoop-wise followed by Fmoc-OSu (1.29 g, 3.82 mmol) over a 20 min period. Reactions is let to react for 16 hrs at rt. THF is removed by rotary evaporation and water layer is adjusted to pH = 3 with 1 N HCl to form a ppt. Solid is collected by filtration and washed with 10% MeOH in DCM (2 x 50 mL) to afford the corresponding Fmoc-protected amino acid as a white solid (**2-42**) in 84% yield (2 steps). ^{1}H NMR (500 MHz, DMSO-*d*6): δ 12.98 (s, br, 1H), 8.35 (s, 1H), 8.23 (d, *J* = 8 Hz, 1H), 7.89 (d, *J* = 7 Hz, 2H), 7.84 (s, br, 1H), 7.69 (t, *J* = 8 Hz, 2H), 7.41 (t, *J* = 7 Hz, 2H), 7.32 (t, *J* = 7 Hz, 2H), 5.26 (q, *J* = 8 Hz, 1H), 4.39 (t, *J* = 8 Hz, 1H), 4.32 (t, *J* = 6.5 Hz, 1H), 4.24 (t, *J* = 7 Hz, 1H), 2.86 (dd, *J* = 5, 15.25 Hz, 1H), 2.70 (dd, *J* = 9, 15 Hz, 1H), 2.57 (t, *J* = 4.5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-*d*6): δ 173.59, 168.99, 162.01, 155.54, 146.82, 143.78, 143.63, 140.73, 128.71, 127.63, 127.10, 127.06, 125.19, 125.15, 120.12, 65.65, 50.28, 46.67, 25.55. HR-MS (ESI): Calculated $C_{23}H_{22}N_3O_5S^+$ $[M + H]^+$ = 452.1275; found $[M + H]^+$ = 452.1266. $[\alpha]_D^{22.7}$ = -25.0° (c = 0.1, MeOH).

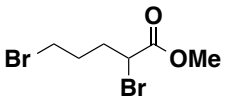


(*S*)-2-(1-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-2-phenylethyl)thiazole-4-carboxylic acid (2-44). Compound **2-44** was made as a white solid according to a literature procedure[50] from Fmoc-Phe-OH in 66% yield (4 steps). ^{1}H is in agreement with that previously reported.[50] ^{1}H NMR (400 MHz, 40 °C, DMSO-*d*6): δ 13.00 (s, br, 1H), 8.37 (s, 1H), 8.34 (d, *J* = 8.4 Hz, 1H), 7.88 (d, *J* = 7.6 Hz, 2H), 7.61 (d, *J* = 7.6 Hz, 2H), 7.41 (t, *J* = 7.6 Hz, 2H), 7.31-7.34 (m, 3H), 7.26-7.30 (m, 3H), 7.20 (t, *J* = 7.2 Hz, 1H), 5.02-5.08 (m, 1H), 4.20-4.30 (m, 2H), 4.15 (t, *J* = 6.8 Hz, 1H), 3.38 (d, *J* = 4.4 Hz, 1H), 3.34 (d, *J* = 4.4 Hz, 1H), 3.03-3.09 (m, 1H). HR-MS (ESI): Calculated $C_{27}H_{23}N_2O_4S^+$ $[M + H]^+$ = 471.1373; found $[M + H]^+$ = 471.1371 . $[a]_D^{22.8}$ = -12.0° (c = 0.1, MeOH).



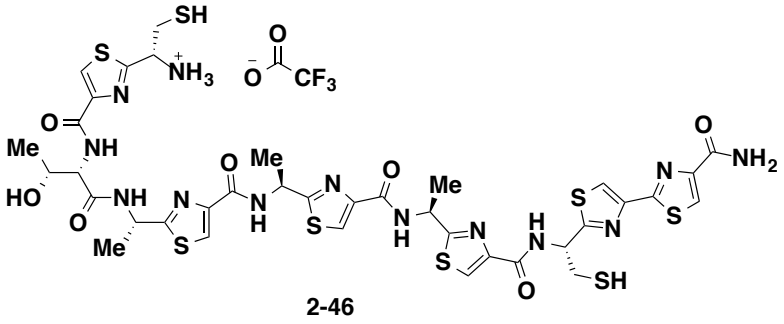
2-45
82%, 3 steps

3-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-4-(methylamino)benzoic acid (2-45). Compound **2-45** was made as a brown solid according to a literature procedure[42] from 4-fluoro-3-nitrobenzoic acid in 83% yield (3 steps). ^{1}H is in agreement with that previously reported.[42] ^{1}H NMR (400 MHz, 40 °C, DMSO-*d*6): δ 12.11 (s, br), 8.62 (s, br), 7.89 (d, *J* = 7.6 Hz, 2H), 7.69 (t, *J* = 7.6 Hz, 3H), 7.42 (t, *J* = 7.6 Hz, 2H), 7.33 (t, *J* = 7.6 Hz, 2H), 6.61 (d, *J* = 8.4 Hz, 1H), 4.37 (d, br, *J* = 4.4 Hz, 2H), 4.27 (d, br, *J* = 6 Hz, 1H), 2.79 (s, 3H).



2-31
22%, 2 steps

Methyl 2,5-dibromopentanoate (2-31). Compound **2-31** was made as an oil according to a literature procedure[44] from δ-valerolactone in 22% yield (2 steps). ^{1}H is in agreement with that previously reported.[44] ^{1}H NMR (500 MHz, DMSO-*d*6): δ 4.66 (dd, *J* = 2.4, 8.2 HZ, 1H), 3.72 (s, 3H), 3.58 (td, *J* = 1.6, 6.4 Hz, 2H), 2.10-2.19 (m, 1H), 1.81-2.06 (m, 3H).



2-46

(*S*)-1-(4-(((2*S*,3*R*)-1-(((*S*)-1-(4-(((*S*)-1-(4-(((*S*)-1-(4-(((*S*)-1-(4-carbamoyl-[2,4'-bithiazol]-2'-yl)-2-mercaptoethyl)carbamoyl)thiazol-2-yl)ethyl)carbamoyl)thiazol-2-yl)ethyl)carbamoyl)thiazol-2-yl)ethyl)amino)-3-hydroxy-1-oxobutan-2-yl)carbamoyl)thiazol-2-yl)-2-mercaptoethan-1-aminium 2,2,2-trifluoroacetate (2-46). Compound **2-46** was built on solid-phase support utilizing RinkAmide Resin. Detailed procedure can be found in **Section 2.10.3**. ^{1}H NMR (500 MHz, DMSO-*d*6): δ 9.14 (d, *J* = 8.5 Hz, 1H), 9.10 (d, *J* = 8.0 Hz, 1H), 9.04 (d, *J* = 8.0 Hz, 1H), 8.96 (d, *J* = 7.5 Hz, 1H), 8.83 (s, br, 3H), 8.46 (s, 1H), 8.28 (t, *J* = 3 Hz, 2H), 8.24-8.26 (m, 2H), 8.01 (d, *J* = 8.5 Hz, 1H), 7.81 (s, 1H), 7.67 (s, 1H), 5.42-5.49 (m, 3H), 5.25 (quint, *J* = 7.5 Hz, 1H), 4.94-4.95 (m, 1H), 4.52 (dd, *J* = 4, 8.8 Hz, 1H), 4.16-4.20 (m, 1H), 3.24-3.35 (m, 2H), 3.09-3.15 (m, 2H), 2.82-2.88

(m, 1H), 2.66 (t, *J* = 8.5 Hz, 1H), 1.72 (d, *J* = 7.5 Hz, 3H), 1.70 (d, *J* = 7.5 Hz, 3H), 1.58 (d, *J* = 7 Hz, 3H), 1.14 (d, *J* = 6 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-*d*6): δ 174.96, 174.90, 174.74, 172.84, 169.60, 164.79, 162.14, 161.49, 160.73, 160.39, 160.34, 159.96, 158.20 (q, *J* = 36 Hz), 151.12, 148.81, 148.77, 148.72, 148.59, 148.57, 147.48, 126.69, 124.99, 124.76, 124.75, 124.49, 119.27, 118.19, 116.95, 114.62, 112.29, 66.94, 57.91, 53.80, 53.19, 47.27, 47.21, 47.08, 27.62, 27.18, 20.66, 20.61, 20.20. HR-MS (ESI): Calculated $C_{37}H_{42}N_{13}O_7S_8^+$ $[M + H]^+$ = 1036.1090; found $[M + H]^+$ = 1036.1091. $[a]_D^{20.4}$ = +447^o (c = 0.1, MeOH).

2.10.3 Solid Phase Peptide Synthesis

General Microwave SPPS Method 1:

Resin (50 mg, 0.47 mmol peptide/g resin) is initially swollen in DMF (1.5 mL) for 20 min at 70°C.

(i) Coupling: Fmoc-AA-OH (5.0 equiv., 0.5 M in DMF), HATU (5.0 equiv., 0.5 M in DMF), and DIEA (10.0 equiv., 1.0 M in DMF) were added to the swollen resin in that order. The resulting suspension was heated under microwave irradiation: 5 min at 75 °C. The reaction vessel is then drained and resin is thoroughly washed with DMF four times.

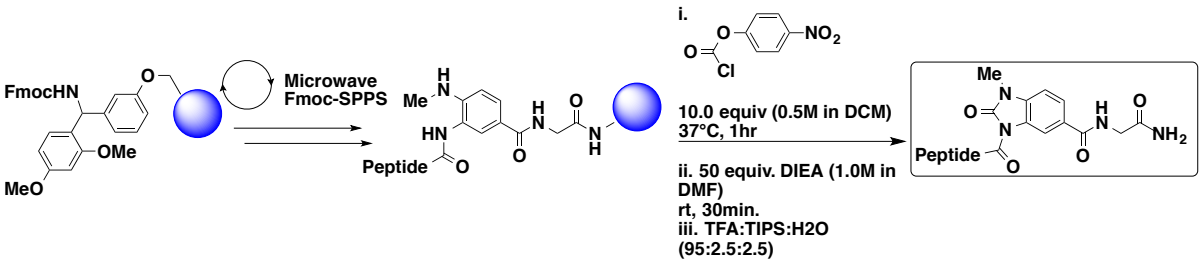
(ii) Fmoc removal: Following coupling, excess 20% piperidine is added to the reaction vessel and allowed to incubate for 3 min while stirring at rt. The reaction vessel is drained, washed with DMF and excess 20% piperidine is introduced again and allowed to stir at rt for 10 min. The reaction vessel is drained and resin is thoroughly washed with DMF four times.

General Microwave SPPS Method 2:

(i) Coupling: Fmoc-AA-OH (5.0 equiv., 0.5 M in DMF), TBTU (5.0 equiv., 0.5 M in DMF), HOBt (5.0 equiv., 0.5 M in DMF) and DIEA (10.0 equiv., 1.0 M in DMF) were added to the resin in that order. The resulting suspension was heated under microwave irradiation: 5 min at 75 °C. The reaction vessel is then drained and resin is thoroughly washed with DMF four times.

(ii) Fmoc removal: Same as Fmoc removal in Method 1.

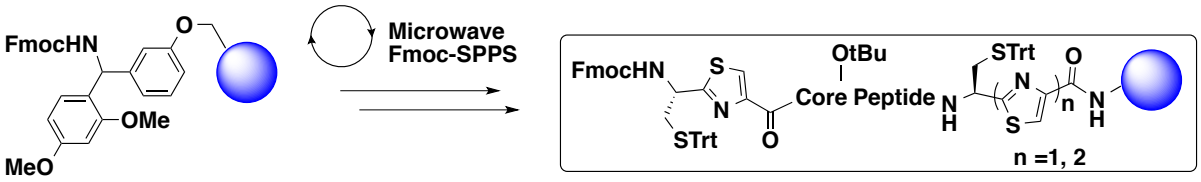
A. Leader Peptides



Procedure has been adapted from a literature one.[42] The linear synthesis of minimal the leader peptides were carried out on a ChemMatrix solid support (0.47 mmol/g) on a 0.188 mmol scale. To facilitate higher loading of Fmoc-MeDbz-OH, a glycine or alanine spacer was coupled to the resin first using Method 1. Coupling of the Fmoc-MeDbz-OH moiety was accomplished using Method 2. All following Fmoc-AA-OH were coupled using Method 1. All couplings and deprotections were monitored by Kaiser Tests. Last coupling is done with the Boc-AA-OH derivative. Subsequently, the peptide was acylated with a solution of *p*-nitrophenyl chloroformate (10.0 equiv, 0.5 M in DCM) over 1 hr at 37 °C. The resin was filtered, washed with DCM, and treated with a solution of DIEA (0.5 M in DMF) for 30 min.

After washing the resin with DMF and DCM, the resin was dried and cleaved using the standard cleavage cocktail (TFA/TIS/H_2O, 95:2.5:2.5) to yield the LP-MeNbz.

B. Core Peptides



The linear syntheses of the core peptides were carried out on a ChemMatrix solid support (0.47 mmol/g) on a 0.01175 mmol scale. With the exception of **2-34,** all building blocks were coupled using Method 1. Compound **2-34** was coupled using Method 2. All couplings and deprotections were monitored by Kaiser test. After washing the resin with DMF and DCM, the resin was dried and cleaved using the standard cleavage cocktail (TFA/TIS/H_2O, 95:2.5:2.5) to yield the core peptides.

C. On-Resin Native Chemical Ligation

Core Deprotection Protocol (0.5mg resin, 0.00235mM scale):

(i) Fmoc-protected resin-bound cores were swollen in DMF (0.5mL) for 30min at 37°C. Fmoc removal was accomplished using 20% Piperidine in DMF (0.5mL) for 4min at room temperature. This procedure was repeated twice. Resin was washed 5 times with DMF (1.0mL), then 5 times with DCM (1.0mL).

(ii) Selective trityl group deprotections were done according to a literature procedure[43] using 0.1 N HCl in HFIP + 1% TIPS (0.2 mL) for 1min at room temperature. This procedure was repeated once. Resin was washed 5 times with DMF (1.0mL), then 5 times with DCM (1.0mL)

(iii) Reduced cysteines were maintained by incubating the deprotected resin in 1:1 DMF:H_2O with 1.0 equiv. of *tris*(2- carboxyethyl)phosphine hydrochloride (TCEP) at 37°C for 30 min.

Ligations:

All ligations were performed at 37°C in ligation buffer, made fresh before use: 200 mM sodium phosphate (pH = 7.20), 6 M guanidinium HCl, 200 mM 4-mercaptophenylacetic acid (MPAA), 400 mM TCEP. 3.0 equiv. of crude LP-MeNbz-AAs were solubilized in buffer to a concentration of 3.5 mM (relative to the resin-bound core peptide) and pH was adjusted back to 7.2 with NaOH (aq). Ligations were incubated for 9 hrs at 37 °C and mixture decanted. Resin was washed 3 times with buffer, 5 times with DMF, then 5 times with DCM. Ligated products were then cleaved with standard cleavage cocktail and purified by semi-preparatory HPLC utilizing Method B. Fractions containing ligated products were collected and lyophilized. Peptides were fully characterized by LC/MS and MS/MS.

2.10.4 Cloning of *tclM* and *tbtD* into Ligation Independent Cloning pMCSG9 vector

The gene *tclM* was amplified from *B. cereus* ATCC 14579 genomic DNA. The *E. coli* codon optimized gene *tbtD* was purchased from Integrated DNA Technologies (IDT). Genes were amplified by PCR using Q5® High-Fidelity Master Mix with HF-buffer (NEB) with GC-enhancer following the manufacturer's manual. Primers utilized:

tclM

Forward (5'→ 3'): - TAC TTC CAA TCC AAT GCG ATG GAG CAG TAT CAT AAA ATT– 3'

Reverse (5'→ 3'): - TTA TCC ACT TCC AAT GCG CTA TCA ATA TCT TTG TAA GTC– 3'

tbtD

Forward (5'→ 3'): TAC TTC CAA TCC AAT GCG ATG GCA GCA GGT GAA CGT TGG TGG

Reverse (5'→ 3'): TTA TCC ACT TCC AAT GCG CTA TTA ACG GGT CGC AAC TTC TTC ATG TGC

The purified PCR products were phosphorylated with T4-PNK, and then treated with T4-DNA Polymerase to create LIC-overhangs. pMCSG9 was linearized with SSp1 and dephosphorylated with Antarctic Phosphatase, and then treated with T4-DNA Polymerase to create LIC-overhangs. Treated PCR product and vector were combined in a 1:1 ratio and transformed into One-Shot® Top10 and BL21 (DE3) Competent Cells (Agilent).

2.10.5 Cloning of *tclE-H's* Leader Peptide into Sequence and Ligase Independent Cloning pETXSH vector

A. Full Length Leader Peptide (38 amino acids)

The leader peptide sequence from *tclE* was amplified from *B. cereus* ATCC 14579 genomic DNA by PCR using the Phusion ® High-Fidelity Master Mix with HF-buffer (NEB) following the manufacturer's manual. Primers utilized:

Forward (5'→ 3'): - TTT AAG AAG GAG ATA TAC CAA TGA GTG AAA TTA AAA AAG C– 3'

Reverse (5'→ 3'): – AGT GCA ATC TCC CGT GAT ACA CGC TCC CAT AAT TTC AAG C– 3'

The purified PCR product was treated with T4-DNA Polymerase to create SLIC-overhangs. pETXSH was linearized with NsiI and dephosphorylated with Antarctic Phosphatase, and then treated with T4-DNA Polymerase to create SLIC-overhangs. Treated PCR product and vector were combined and transformed into One-Shot® Top10 and BL21-CodonPlus (DE3)-RIPL Competent Cells (Agilent).

B. Quickchanges into pETXSH containing TclE's FLeader Peptide

Due to the removal of the C-terminus GlyAla during cleavage of the intein-tag with sodium 2-mercaptoethane sulfonate (MESNa), addition of another GlyAla to the C-terminus afforded the desired cleaved leader peptide-thioester. These additions were accomplished by first introducing an additional Gly to the C-terminus followed by an addition of Ala. Quichanges were performed using Pfu-Turbo (Agilent) according to manufacturer's manual. Primers utilized are as follows:

Glycine addition:

Forward (5'→ 3'): GAA ATT ATG GGA GCG GGA TGT ATC ACG GGA GAT

Reverse (5' → 3'): ATC TCC CGT GAT ACA TCC CGC TCC CAT AAT TTC

Alanine addition after glycine addition:

Forward (5'→ 3'): ATT ATG GGA GCG GGA GCA TGT ATC ACG GGA GAT

Reverse (5' → 3'): ATC TCC CGT GAT ACA TGC TCC CGC TCC CAT AAT

C. ΔN18 Leader Peptide (20 amino acids)

PCR procedure is the same as in **A**.

Forward (5'→ 3'): TAC TTC CAA TCC AAT GCG ATG GCA GCA GGT GAA CGT TGG TGG

Reverse (5'→ 3'): TTA TCC ACT TCC AAT GCG CTA TTA ACG GGT CGC AAC TTC TTC ATG TGC

2.10.6 Protein Expression and Purification

A. TclM

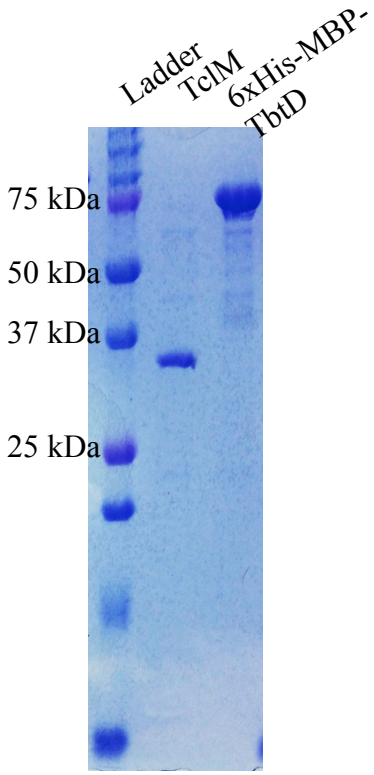
TclM protein was heterologously expressed in *E. coli* RIPL cells. 1 L of Luria-Bertani (LB) medium, supplemented with ampicillin (100 mg/mL) and chloramphenicol (34 mg/mL), was inoculated with 5 mL of an overnight culture and grown to an OD_{600} between 0.6-0.8 at 37 ^{o}C, at which point 200 mL of a 1 M stock of IPTG was added and culture grown for 18 h at 16 ^{o}C. Cells were pellet and resuspended in 40 mL of lysis/binding buffer (50 mM $KHPO_4$ at pH = 7.00, 250 mM KCl, 10 mM imidazole, and 10% glycerol) supplemented with 250 mg/mL lysozyme, 2 mM PMSF, and 1-protease inhibitor tablet (ROCHE®). Cells were lysed by sonicating twice with a 30% maximum amplitude intermittent pulses for 1:30 min. Cell debris was removed by centrifugation (40 min at 15,000 rpm at 4 ^{o}C) and supernatant collected and cold filtered through a 0.44 mm filter. Supernatant was loaded into a 5-mL HisTrap (Ni^{2+}) IMAC column and washed (4 CV) to remove non-specific binding. 6xHis-MBP-TclM was eluted with an elution buffer gradient (50 mM $KHPO_4$ at pH = 7.00, 250 mM KCl, 500 mM imidazole, and 10% glycerol) from 0 – 100% (5 CV). Fractions containing eluted protein are pooled and concentrated to 5 mL utilizing a Centricon (30,000

Da MWCO) concentrator (EMD Millipore®). The resulting concentrated protein was buffer-exchanged into 50 mM $KHPO_4$ at pH = 7.00, 250 mM KCl, and 10% glycerol utilizing a PD-10 column (GE Healthcare Life Sciences®) to obtain 7 mL of 6xHis-MBP-TclM. Removal of the MBP-tag was accomplished with 1% (w/w) TEV-protease and cleavage was left overnight at 4 ^{o}C. Supernatant was loaded into a 5-mL HisTrap (Ni^{2+}) IMAC column and washed with same buffer (3 CV) to elute the cleaved protein. Concentration of the pooled fractions containing pure TclM (10,000 Da MWCO) to ~300 mL afforded 8.09 mg/mL of protein which was utilized for enzymatic assays.

B. TbtD

Similarly, TbtD protein was heterologously expressed in *E. coli* BL21 (DE3) cells. 1 L of Luria-Bertani (LB) medium, supplemented with ampicillin (100 mg/mL) was inoculated with 5 mL of an overnight seed and grown to an OD_{600} between 0.6-0.8 at 37 ^{o}C, at which point 200 mL of a 1 M stock of IPTG was added and culture grown for 18 h at 16 ^{o}C. Cells were pellet and resuspended in 40 mL of lysis/binding buffer (50 mM $KHPO_4$ at pH = 7.00, 250 mM KCl, 10 mM imidazole, and 10% glycerol) supplemented with 250 mg/mL lysozyme, 2 mM PMSF, and 1-protease inhibitor tablet (ROCHE). Cells were lysed by sonicating twice with a 20% maximum amplitude intermittent pulses for 1:30 min. Cell debris was removed by centrifugation (20 min at 15,000 rpm at 4 ^{o}C) and supernatant collected and cold filtered through a 0.44 mm filter. Supernatant was loaded into a 5-mL HisTrap (Ni^{2+}) IMAC column and washed (4 CV) to remove non-specific binding. 6xHis-MBP-TbtD was eluted with an elution buffer gradient (50 mM $KHPO_4$ at pH = 7.00, 250 mM KCl, 500 mM imidazole, and 10% glycerol) from 0 – 100% (5 CV). Fractions

containing eluted protein are pooled and concentrated to 5 mL utilizing a Centricon (30,000 Da MWCO) concentrator (EMD Millipore). The resulting concentrated protein was buffer-exchanged into 50 mM $KHPO_4$ at pH = 7.00, 250 mM KCl, and 10% glycerol utilizing a PD-10 column (GE Healthcare Life Sciences) to obtain 7 mL of 6xHis-MBP-TbtD, which was concentrated (30,000 Da MWCO concentrator) to 1 mL to yield a 9.8 mg/mL solution of protein which was utilized for enzymatic assays.



C. Leader Peptide Thioesters

LP-intein protein was heterologously expressed in *E. coli* RIPL cells. 1 L of Luria-Bertani (LB) medium, supplemented with ampicillin (100 mg/mL) and chloramphenicol (34 mg/mL), was inoculated with 5 mL of an overnight culture and grown to an OD_{600} between 0.6-0.8 at 37 ^{o}C, at which point 200 mL of a 1 M stock of IPTG was added and culture grown

for 18 h at 16 ^{o}C. Cells were pellet and resuspended in 40 mL of lysis/binding buffer (50 mM $NaHPO_4$ at pH = 7.00, 250 mM NaCl, and 10 mM imidazole) supplemented with 250 mg/mL lysozyme, 2 mM PMSF, and 1-protease inhibitor tablet (ROCHE®). Cells were lysed by sonicating twice with a 30% maximum amplitude intermittent pulses for 1:30 min. Cell debris was removed by centrifugation (40 min at 15,000 rpm at 4 ^{o}C) and supernatant collected and cold filtered through a 0.44 mm filter. Supernatant was loaded into a 5-mL HisTrap (Ni^{2+}) IMAC column and washed (4 CV) to remove non-specific binding. LP-intein was eluted with an elution buffer gradient (50 mM $NaHPO_4$ at ph = 7.00, 250 mM NaCl, and 500 mM imidazole) from 0 – 100% (5 CV). Fractions containing eluted protein are pooled and concentrated to 5 mL utilizing a Centricon (10,000 Da MWCO) concentrator (EMD Millipore®). The resulting concentrated protein was buffer-exchanged into 50 mM HEPES at pH = 7.50 and 150 mM NaCl utilizing a PD-10 column (GE Healthcare Life Sciences®) to obtain 7 mL of LP-intein. Removal of the intein-tag was accomplished by adding sodium 2-mercaptoethane sulfonate (MESNa) to 0.5 M. Mixture was left overnight to react at room temperature. Dialysis was performed to remove excess MESNa with a 2,000 Da MWCO cassette in 2 L of 50 mM HEPES at pH = 7.50 and 150 mM NaCl; after 2 hours, the buffer was exchanged for fresh one and dialyzed for 6 h more. Supernatant was loaded into a 5-mL HisTrap (Ni^{2+}) IMAC column and washed with same buffer (3 CV) to elute LP-MESNa thioester (14). Pooled fractions containing peptide (positive dot blot test), concentrated using a 1 kDa MWCO concentration (PALL®) and utilized for next step as it is. Obtained:

Full length LP-MESNa: 10.5 mL of 0.464 mg/mL.

ΔN18 LP-MESNa: 2 mL of 2.5 mg/mL.

Peptides were characterized by LC/MS and MS/MS.

2.10.7 Native Chemical Ligations

LP-thioesters are buffer exchanged to 3.5 mL of buffer (200 mM $NaHPO_4$, pH = 7.50, 6 M guanidinium hydrochloride, 20 mM *tris*(2-carboyxethyl) phosphine, and 0.5% (v/v) thiophenol. Meanwhile, 20 μL of core of **2-26** or **2-46** (0.10 M in DMF) was added and mixture allowed to incubate for 3 days at room temperature. Crude mixture was purified by semi-preparatory HPLC utilizing Method A. Peptides were characterized by LC/MS and MS/MS.

2.10.8 Desulhydrylation of Cysteine Side Chains to Dehydroalanines

A. When 2-28 was utilized

Native chemical ligation product was buffered exchanged using a PD-10 into 50 mM $NaHPO_4$ (pH = 8.00). A 500-fold excess of 2,5-dibromohexanediamide (0.50 M in DMF) was added and mixture incubated at room temperature for 2 h and at 37 ^oC for 3.5 h. Precipitate was removed by centrifugation (1 min at 15,000 rpm) and supernatant was dialyzed into 2 L of 50 mM $NaHPO_4$ (pH = 8.00) utilizing a dialysis cassette (2,000 Da MWCO from THERMO®) for 2h, after which the buffer was replaced with fresh one and dialysis continued overnight at 4 ^oC. Supernatant was collected.

B. When 2-31 was utilized

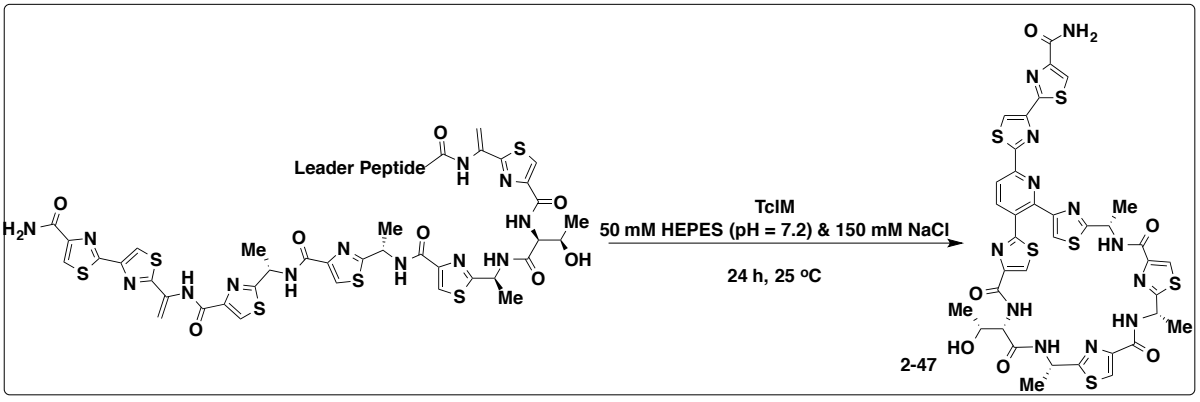
Adapted from utilized a previously described method.[44] Briefly, in a 1.7 mL microcentrifuge tube, cysteine-containing substrate is dissolved to a final concentration of 0.63 μM in a solution containing 50% (aq.) DMF, 1.26 mM TCEP, and 63 mM K_2CO_3. Mixture is allowed to incubate for 15 min at 37 ^oC, after which compound **2-31** is added to a

final concentration of 63 mM. Reaction is allowed to stir at 37 °C for 3 h. Excess **2-31** is removed by washing with diethyl ether (Et_2O) twice: Et_2O (300 μL / 50 μL reaction) is added, mixture vortexed for 3 s, and centrifuged for 10 s at 6,000 rpm. Carefully, top ether layer is removed. Microcentrifuge tube is set at 37 °C with the cap open to remove excess Et_2O for 10 min. Substrates were utilized without further purification.

2.10.9 Cyclization Assays

Assays were performed in a 50 mM HEPES (pH = 7.20) and 150 mM NaCl buffer with final concentrations of 50 μM substrate and 10 μM of the corresponding enzyme (TclM or MBP-TbtD) in a total volume of 100 mL. Reactions were incubated at 25 °C and 350 rpm for 20 h, after which 200 μL of methanol were added, incubated at rt for 3 min, and precipitated protein removed by centrifugation (3 min at 15,000 rpm) and the supernatant collected. The supernatants were analyzed by LC/MS with the C18 Kinetex® column by injecting 30 μL/sample utilizing Method B.

2.10.10 Leader Peptide Truncations



The leader peptide (LP) requirement for TclM was probed by truncating the *N*-terminal peptide sequence of our linear thiazolyl peptide until no product production could be observed by LC/MS analysis. Substrates containing the full-length leader peptide and ΔN18 truncate were prepared according to the method described in **Section 2.10.6c**, **Section 2.10.7**, and **Section 2.10.8b** . Substrates with LP truncation ΔN28, ΔN29, ΔN30, ΔN31, and ΔN32 were prepared by solid phase peptide synthesis (**Section 2.10.3** and **Section 2.10.8b**). Peptides were characterized by LC/MS and MS/MS.

In a final volume of 100 μL, substrate (10.5 μM) was incubated with TclM (10.5 μM) at 25 ^{o}C for 24 h. 200 μL of methanol are added and reactions incubated for 3 min at rt; precipitated protein is removed by centrifugation (3 min at 15,000 rpm) and supernatant collected. Crude supernatant is analyzed by injecting 30 μL into the LC/MS with the Kinetex® C8 column utilizing Method A.

2.10.11 Expression of Thiocillin

5 mL of Luria-Bertani (LB) broth in 10 mL culture tubes were individually inoculated with 5 μL of an overnight seed of *Bacillus cereus* ATCC 14579, *Bacillus cereus* ATCC 14579 *tclM*-KO, or *Bacillus cereus* ATCC 14579 *tclM*-KO with pHT01 containing the desired mutant and grown for three days at 30 ^{o}C and 250 rpm using a rotary incubator. Grow-ups containing pHT01 were grown with 5 μg/mL of chloramphenicol and induced after one day with 0.2 mM IPTG. Cells are pelleted and supernatant removed. 500 μL of methanol are added and pellet extracted for 30 min. Cell debris is removed by centrifugation (5 min at 15,000 rpm), and supernatant collected. Samples for LC/MS analyses were prepared by diluting 10 μL of supernatant into 90 μL of 50% (aq.) acetonitrile.

2.10.12 Quickchange Mutagenesis of *tclM*-mutants in pHT01 or pMCSG9 and Transformation into *B. cereus* ATCC 14579 *tclM*-KO

Quichanges were performed using Pfu-Turbo (Agilent) according to manufacturer's manual. Primers utilized:

Plasmid	Mutation	Sequence (5' → 3') Forward	Sequence (5' → 3') Reverse
pHT01	E128A	ATAGAATTTGCGCTAGCTATA ATGTTAATTTCA	TGAAATTAACATTATAGCT AGCGCAAATTCTAT
pHT01	S147A	AAAAAAGGCTACTTAGCTTAT GCATCTCATGTA	TACATGAGATGCATAAGCT AAGTAGCCTTTTTT
pHT01	S150A	GCTACTTATCCTATGCAGCTC ATGTAAATGGATTT	AAATCCATTTACATGAGCT GCATAGGATAAGTAGC
pHT01	H151A	CTTATCCTATGCATCTGCTGTA AATGGATTTTTT	AAAAAATCCATTTACAGCA GATGCATAGGATAAG
pHT01	F155A	TCTCATGTAAATGGAGCTTTT ACTAGATGGAAA	TTTCCATCTAGTAAAAGCT CCATTTACATGAGA
pHT01	Y173A	GATATATTTCATAAGAACGCT TTAAATAACAAAGAGTAC	GTACTCTTTGTTATTTAAAG CGTTCTTATGAAATATATC
pHT01	S239A	GATTTTCTAGAAAAAGCTCAG TTTCATAAGACG	CGTCTTATGAAACTGAGCT TTTTCTAGAAAATC
pHT01, pMCSG9	H242A	GAAAAAAGTCAGTTTGCTAAG ACGATATTAAAT	ATTTAATATCGTCTTAGCA AACTGACTTTTTTC
pHT01	F261A	AATAAAGATATAAACGCTTTA GGTTCACGCCTA	TAGGCGTGAACCTAAAGCG TTTATATCTTTATT
pHT01, pMCSG9	R265A	AACTTTTTAGGTTCAGCTCTA ATAACTGTATTT	AAATACAGTTATTAGAGCT GAACCTAAAAAGTT

pHT01	Y272A	CGCCTAATAACTGTATTTACG GCTTTATTAATACGAAAT	ATTTCGTATTAATAAAGCC GTAAATACAGTTATTAGGC G
pHT01, pMCSG9	R285A	ATTCAAAATAAAGATGCTTAT CTACTTTGCTAC	GTAGCAAAGTAGATAAGCA TCTTTATTTTGAAT
pHT01	C289A	GATAGATATCTACTTGCTTAC TATTCATATAAA	TTTATAGATATAGTAAGCA AGTAGATATCTATC
pHT01	E297A	CTATATCTATAAAATTATTGC TGAAAAATATAATATAG	CTATATTATATTTTTCAGCA ATAATTTTATAGATATAG

Transformations into *B. cereus* ATCC 14579 *tclM*-KO were done according to a literature procedure.[51] Cells were recovered in 1 mL of LB for 1 h and then pelleted by centrifugation for 5 min at 3,500 rpm. 900 μL of supernatant are removed and cells resuspended in 100 μL. 50 μL were plated in an LB-agar plate containing 5 μg/mL chloramphenicol for 36 h at 30 ^{o}C.

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CHAPTER III

CHEMOENZYMATIC SYNTHESIS OF THIOMARINOL AND STRUCTURE-ACTIVITY-RELATIONSHIP OF ITS DITHIOLOPYRROLONE CONSTITUENT

3.1 Introduction

Antibiotic resistance has become a great concern worldwide, and the search for new antibacterial compounds or natural products can be time-consuming. This process can be shortened by taking advantage of known natural products and create hybrid antibiotics in which two bioactive molecules are covalently linked to each other. This strategy has the advantage of improving bioactivity against bacterial strains that are resistant to one of the individual components[1] and by improving pharmacokinetics and pharmacodynamics.[2,3] A potentially useful class of naturally occurring hybrid antibiotics is the thiomarinols (**3-5**, **Figure 3.1**). Thiomarinols were first discovered from *Pseduoalteromonas* spp. SANK7339[4,5] and they consist of two scaffolds, linked via an octanoyl fatty chain, related to two individual antibiotic moieties. First, it possesses the monic acid side chain of the molecule mupirocin, an FDA approved antibacterial mixture of pseudomonic acids under the name Bactroban (GlaxoSmithKline), which is known to be active against methicillin-resistant *S. aureus* (MRSA) by inhibiting isoleucyl *t*RNA synthetase. Second, the thiomarinols contain a holothin core from the dithiolopyrrolone (DTP) family of natural products, bioactive molecules shown to have activity against rifamycin-resistant bacteria and MRSA.[6,7]

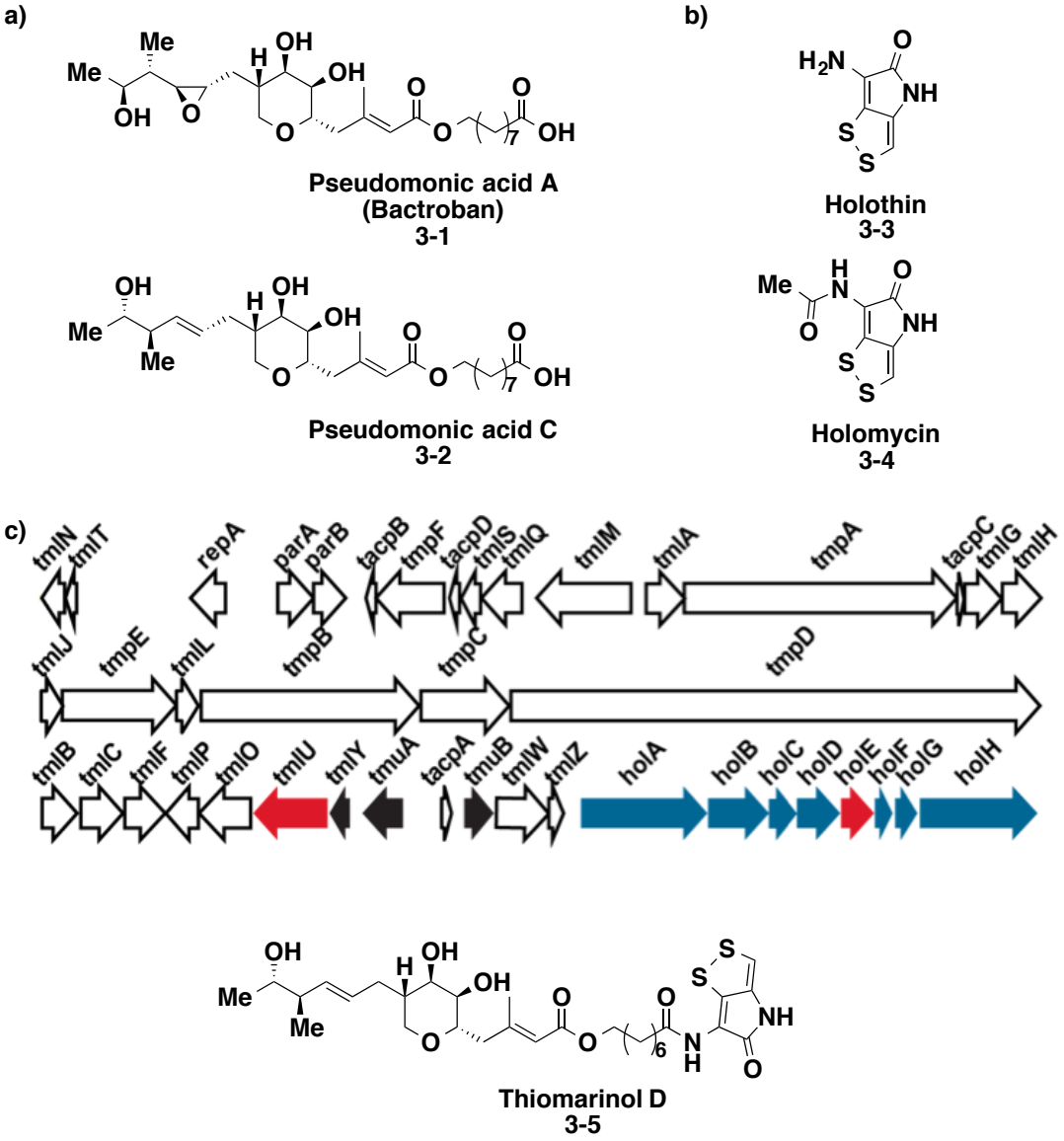


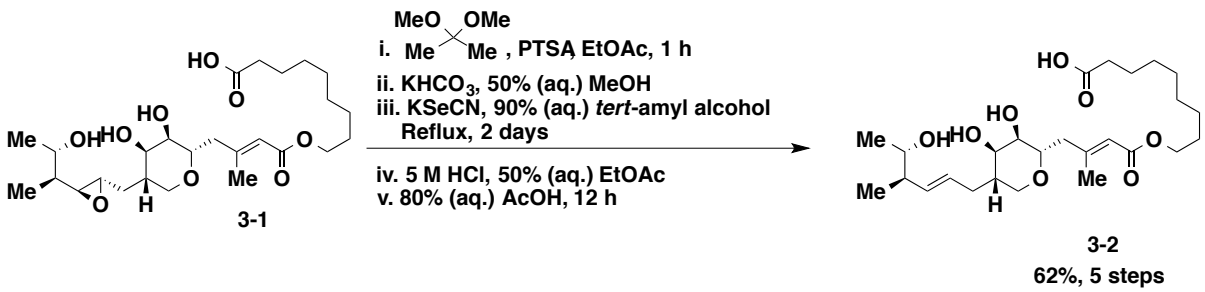
Figure 3.1. Structures related to thiomarinol biosynthesis. a) Structure of pseudomonic acids. b) Structure of dithiolopyrrolones. c) Thiomarinol biosynthetic cluster from *P. spp.* SANK73390. Open arrows indicate ORFs with significant homology to the mupirocin pathway; blue ORFs are homologous to DTP biosynthetic genes; black ORFs are unique to the thiomarinol pathway, and the red ORFS, TmlU and HolE, have counterparts in the mupirocin and holomycin pathway, respectively, and predicted to be involved in the ligation of the two moieties.

The thiomarinol gene cluster was discovered by whole genome sequencing from *P.* SANK73390 and found to be encoded in a 97 kb plasmid comprising of genes related to non-ribosomal peptide synthesis (NRPS), which produce the holothin core, and polyketide

synthesis (PKS), which produces the marinolic acid scaffold.[5] Gene knockout experiments showed that the enzyme TmlU was the one responsible for linking holothin to marinolic acid.[5,8] TmlU was assigned as an amide ligase based on sequence identity with enzymes involved in the biosynthesis of aminocoumarins by linking different functionalities to the coumarin core through amide linkages. The ligase utility of TmlU could be harnessed *in vitro* to test for substrate promiscuity. Moreover, it would provide a platform by which structure-activity-relationship models can be pursued for both the marinolic acid and holothin moieties.

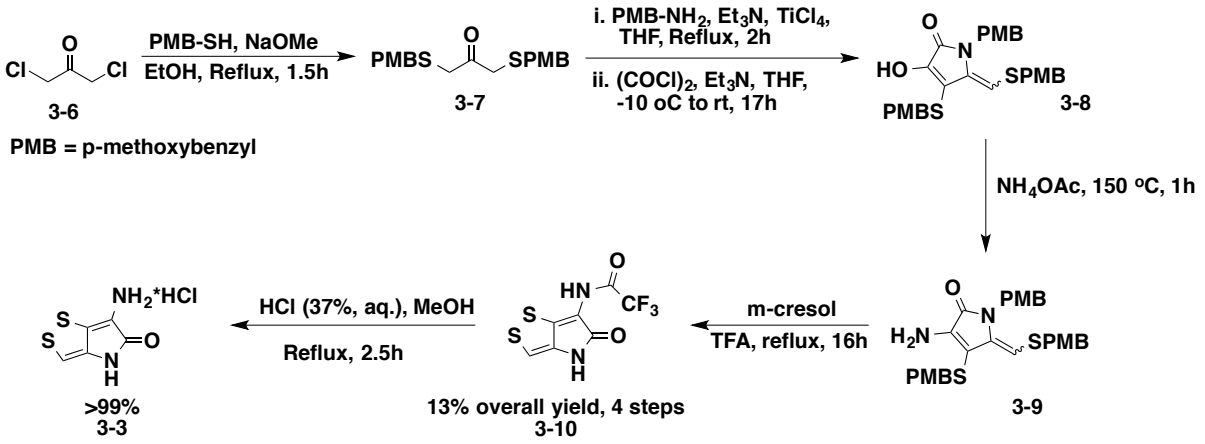
3.2 Synthesis of Pseudomonic acid C, Holothin, and Pseudomonyl C Holothinamide

In order to test TmlU *in vitro*, we needed to access the individual components of thiomarinol. The marinolic acid component is not readily available, but a closely related compound, the antibiotic mupirocin, is commercially available. It differs to the marinolic acid moiety in thiomarinol by having one extra methylene group in the octanoyl linker. Thus, in our subsequent studies, we focused on utilizing the pseudomonic acid moiety over the marinolic acid. Mupirocin consists mostly of pseudomonic acid A (PAA, >90%) with small amounts of pseudomonic acid B (PAB), and C (PAC), yet PAC is the predominant species found in thiomarinol. We utilized a known method by King *et al.*[9] to convert PAA to PAC (**Scheme 3.1**) in 62% overall yield. They key transformation relied on the use of potassium selenocyanate in the reduction of the epoxide of PAA to yield the *trans*-alkene in PAC.



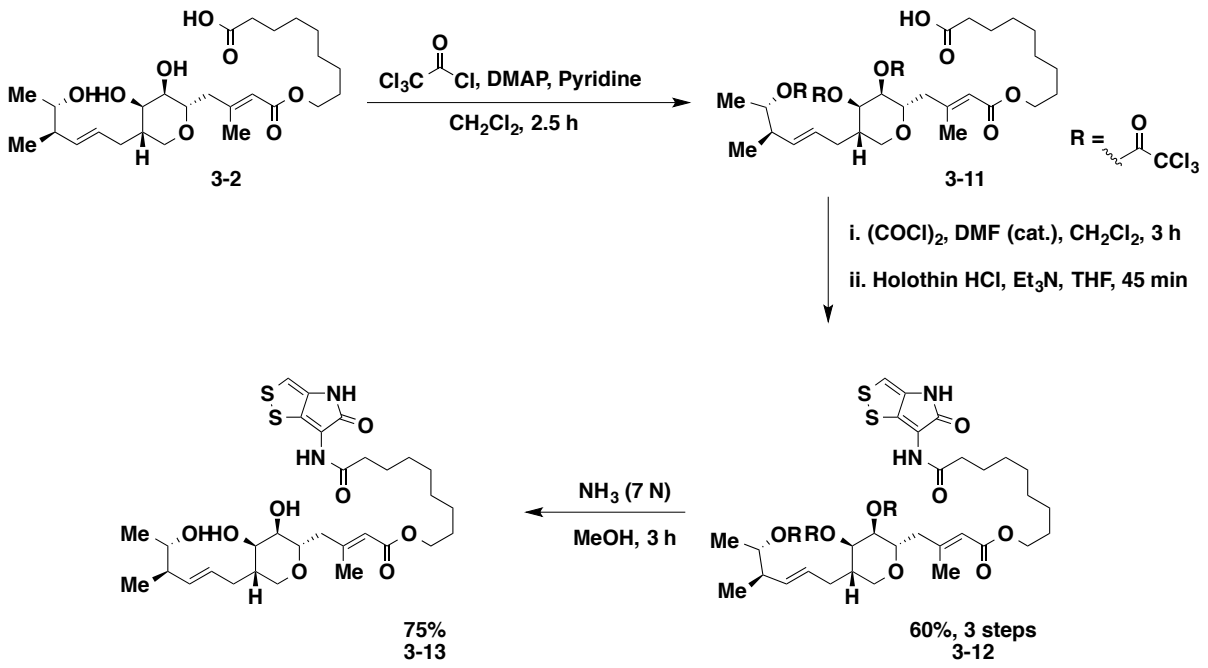
Scheme 3.1. Synthesis of pseudomonic acid C from pseudomonic acid A. PTSA = *p*-toluene sulfonic acid. EtOAc = ethyl acetate. AcOH = acetic acid.

The second component, thiolutin, was accessed by utilizing a previously reported method in the expedient total synthesis of dithiolopyrrolones (**Scheme 3.2**).[10] Starting from 1,3-dichloroacetone (**3-6**), displacement with *p*-methoxybenzylthiol (PMBSH) of the chlorides yields compound **3-7**. Condensation with PMB-NH_2 and cyclization with oxalyl chloride yields the pyrrolone ring **3-8**. Aminolysis with ammonium acetate and deprotection of the PMB-protecting groups with refluxing trifluoroacetic acid (TFA) in the presence of *m*-cresol yielded the trifluoroacetyl holothin **3-10**. The trifluoroacetyl group was removed by addition of concentrated HCl in refluxing methanol to afford holothin as the hydrochloride salt **3-3**. All subsequent reactions were done with this salt of holothin in order to improve aqueous solubility and compound stability.



Scheme 3.2. Synthesis of holothin hydrochloride. PMB = *p*-methoxybenzyl. EtOH = ethanol. TFA = trifluoroacetic acid.

We additionally synthesized the ligated product pseudomonyl C holothinamide (PAC-holothin) **3-13** (**Scheme 3.3**) to obtain a standard for our enzymatic assays. The hydroxyl groups at position 6,7, and 13 in PAC (**3-2**) were protected with trichloroacetyl chloride to obtain **3-11**. Formation of the acyl chloride with oxalyl chloride allowed the coupling of holothin in 60% yield for the 3 steps. Removal of the protecting groups with 7 N NH_3 (MeOH) afforded our standard **3-13**. This standard was provided to our collaborators in Dr. Bo Li's laboratory in the department of Chemistry at the University of North Carolina at Chapel Hill to test the chemoenzymatic ligation of holothin (**3-3**) with PAC (**3-2**). Their results are summarized in **Figure 3.2**. It was found that TmlU acted as an acetyl coenzyme A ligase to yield the CoA thioester of PAC (**3-14**), and HolE ligated the thioester to holothin.



Scheme 3.3. Synthesis of pseudomonyl C holothinamide. DMAP = 4-dimethylamino pyridine. DMF = *N,N*-dimethylformamide. THF = tetrahydrofuran. MeOH = methanol.

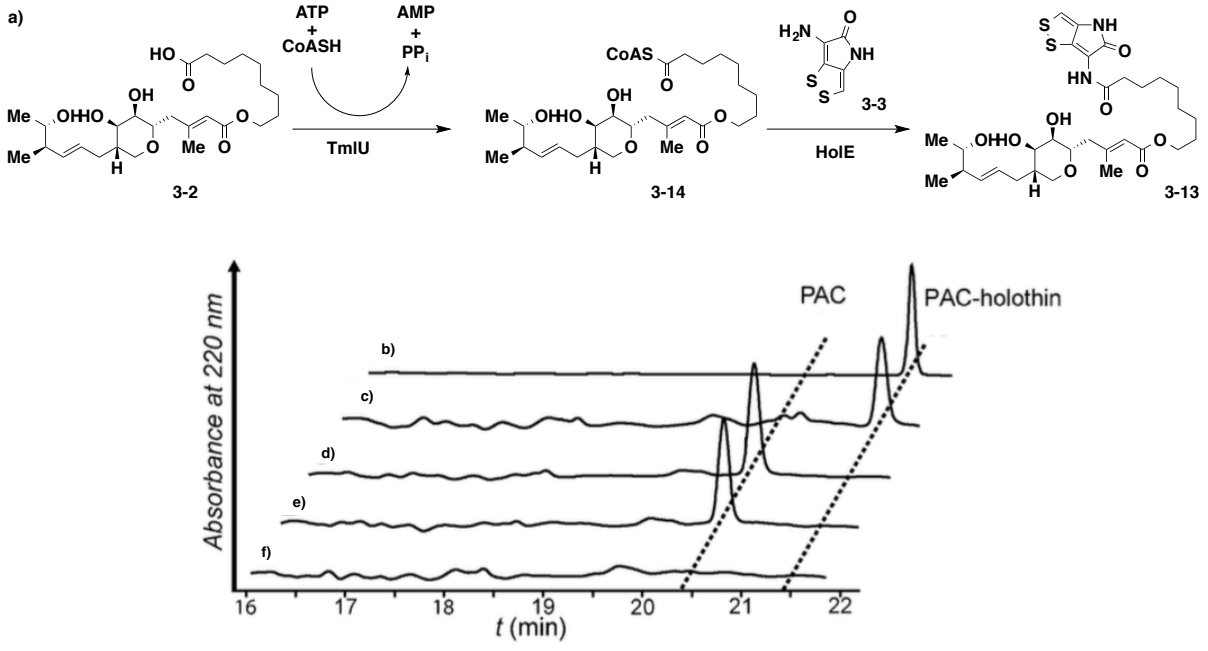


Figure 3.2. Pseudomonic acid C and holothin ligation by TmlU and HolE. a) Hypothesized ligation mechanism. b) Standard **3-13**. c) Ligation with TmlU and HolE. d) No CoASH. e) No TmlU. f) No HolE. ATP = adenosine 5'-triphosphate. AMP = adenosine monophosphate. PP_i = pyrophosphate. Assay conditions: 20 mM $KHPO_4$ (pH = 7.5), 1 mM ATP, 2 mM $MgCl_2$, 1 mM CoASH, 0.1 mM PAC, 0.1 mM holothin, 1 μM TmlU, and 1 μM HolE.

3.3 Dithiolopyrrolone Natural Products

Of the two constituents found in thiomarinol, we became interested in exploring the holothin moiety as a standalone antibiotic. Holothin belongs to the family of natural products called dithiolopyrrolones (DTPs), first discovered in the early 1940s. These antibiotics contain a unique pyrrolone structure fused to a 5-membered ene-disulfide ring.[11] Within this class of compounds, thiolutin (**3-15**),[12] isolated from *Streptomyces luteosporeus*, and holomycin (**3-4**),[13] isolated from *Streptomyces clavuligerus*, are two of the most well characterized; other congeners include aureothricin (**3-16**), xenorhabdin I (**3-17**), tigloyl holothin (**3-18**, **Figure 3.4**). Recently, it has been shown that *Photobacterium halotolerans*[14] and *Yersinia ruckeri*[7] produce holomycin biosynthetically as well, making it somewhat of an ubiquitous natural product found in both marine and soil systems.

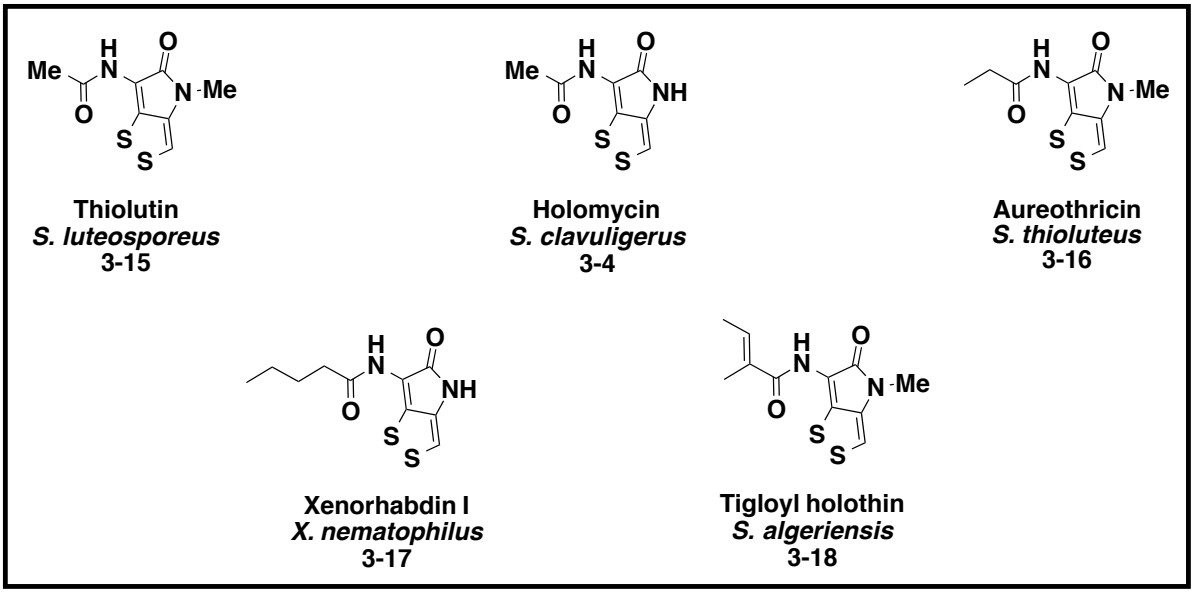


Figure 3.3. Representative members of the dithiolopyrrolone family of natural products.

DTPs gained interest from the research community by showing activity against Gram-positive and Gram-negative bacteria,[15] with important bioactivity against *Neisseria gonorrhoeae* and MRSA.[6,7] Moreover, DTPs have been extensively tested against cancer cell lines; they were found to be involved in many biological pathways such as effecting phosphorylation levels of Hsp27 in cell adhesion, and inhibition of angionesis and tumor growth in endothelial cells.[16] The exact mechanism of action of DTPs has not been well established. One pathway that has been suggested involves the arrest in production of mRNA and proteins by interacting with DNA-dependent RNA polymerases.[17, 18] Experiments have shown that in vivo inhibition of RNA synthesis cannot be correlated in vitro, suggesting a mode of action where DPTs can act as prodrugs and need to be modified by living systems.[18] In contrast, the holomycin producing species *Y. ruckeris* encodes for an RNA methyltransferase that confers self-resistance for the antibiotic itself, and when the gene encoding for this protein is knocked-out, the bacterium becomes susceptible towards the compound.[7] The mechanism by which *Y. ruckeris* protects itself from holomycin suggests a pathway in which DTPs interact with important RNA nucleotides and can lead to disruption of ribosomal related processes such as translation.

3.4 Syntheses and Biological Activities of Dithiolopyrrolone Analogues

We have envisioned that changing the substituents and electronics of the bicyclic system in DTPs would allow us to further study solubility, susceptibility towards oxidation, possible disulfide exchange, and metal chelation properties (**Figure 3.5**). Holomycin and thiolutin are sparingly soluble in water, thus finding substitution patterns that enhance aqueous solubility are important; we envision that the introduction of functional groups such

as a mesyl moiety would assist with this property. Additionally, we wanted to synthesize analogs similar to those found in nature to probe their physical properties. We further look to determine the possibility of having a Michael acceptor to determine if there are any nucleophiles that might interact with this functional group and mimic possible covalent bonds or transient interactions with protein partners *in vivo*. Next, we wanted to change the steric factors involved with the *N*-endocyclic amide and *N*-exocyclic amide; we speculated that introducing bulky groups would allow for a structure-activity-relationship model to be identified since these natural products differ only by different *N*-acylations and/or *N*-methylation.

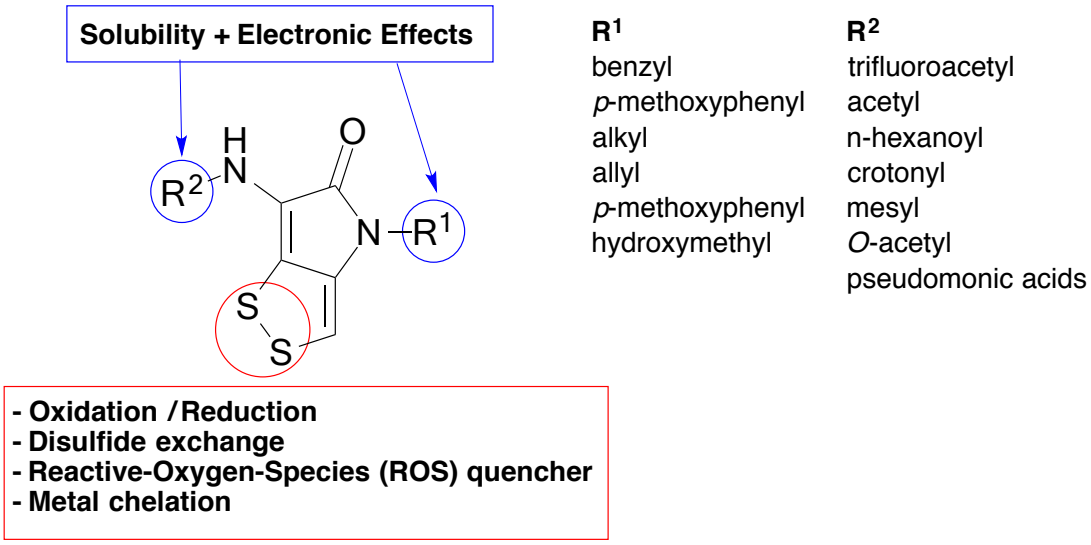
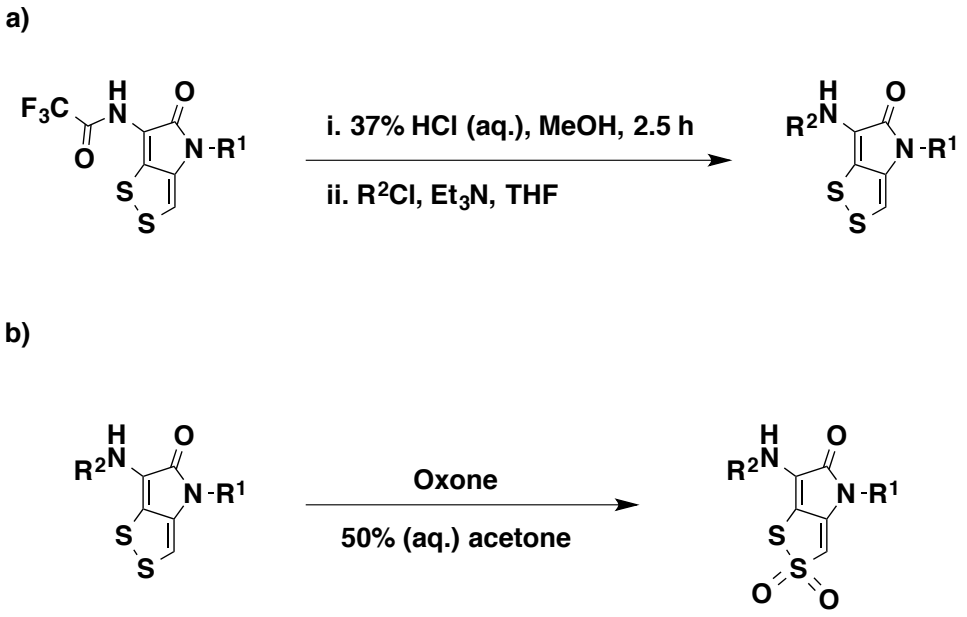


Figure 3.4. Rational design of dithiolopyrrolone analogs.

We took advantage of the previously described synthesis of holothin (**Scheme 3.2**) to access analogues by acylating at the exocyclic amine (**Scheme 3.4a**). All acylating partners were either commercially available or could be easily accessed by conversion of their carboxylic acid to their acyl chloride by treatment with oxalyl chloride. Making substitutions

at the endocyclic amide proved to be limited to electron rich arylated amines due to displacement of the alkylated amine moiety during the aminolysis step with ammonium acetate (**Scheme 3.2**) with ammonia to yield the pyrrolone without *N*-substitution at the endocyclic amide. Likewise, the presence of the disulfide moiety led us to speculate the redox potential and/or metal chelating properties of this scaffold since earlier reports have shown that the oxidized thiosulfinate from holothin can be found in thiomarinol B and has comparable bioactivity to non-oxidized ones.[19] Thus, we synthesized oxidized analogs through the use of oxone (**Figure 3.4b**) as previously shown to occur.[20]

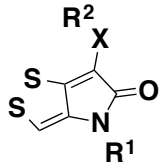


Scheme 3.4. Syntheses of dithiolopyrrolone derivatives. a) Acylating strategy from trifluoroacetyl intermediate. b) Oxidation strategy for thiosulfinate production.

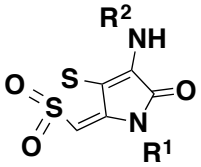
The derivatives synthesized were probed for antibiotic activity against *Neisseria gonorrhoeae* FA19 and for cytotoxicity in HeLa cells. Disk-diffusion-assays on *N. gonorhoeae* FA19 have shown that most derivatives tested thus far are active against this highly resistant strain (**Table 3.1**), with the exception of those analogues in which the

exocyclic amide is replaced by an ester and where the disulfide is oxidized to the thiosulfinate. Unfortunately, no structure-activity-relationship could be extracted from our dataset since no clear pattern can be discerned.

Table 3.1. Biological activities against HeLa cells and *N. gonorrhoeae* FA19 of DTPderivatives.



Entries 1-17



Entries 18-19

Entry	Compound #	X	R^1	R^2	CC_{50}[a,b] (μM)	Activity Diameter[c] (mm) 1 μg
1	**3-4**	NH	acetyl	acetyl	5.05	21
2	**3-15**	NH	methyl	acetyl	1.15	17
3	**3-17**	NH	H	pentanoyl	2.50	17
4	**3-18**	NH	H	tigloyl	ND	20
5	**3-19**	NH	H	mesyl	26.8	10
6	**3-20**	NH	H	6-heptynoyl	5.75	21
7	**3-21**	NH	H	5-hexynoyl	19.7	21
8	**3-22**	NH	H	hexanoyl	18.5	24
9	**3-23**	NH	H	octanoyl	6.05	21
10	**3-24**	NH	H	crotonyl	ND	21
11	**3-25**	NH	H	methacryloyl	ND	22
12	**3-26**	NH	H	3,3-dimethylacryloyl	ND	21

13	**3-27**	NH	H	6-azidohexanoyl	ND	22
14	**3-28**	NH	Bn	acetyl	ND	19
15	**3-29**	O	PMP	acetyl	ND	8
16	**3-30**	NH	H	pivaloyl	ND	ND
17	**3-31**	NH	H	benzoyl	ND	ND
18	**3-32**	NH	H	acetyl	ND	6
19	**3-33**	NH	H	trifluoroacetyl	ND	6

[a]The cytotoxicity values correspond to HeLa cells in which ATP contents equals 50% of the vehicle controls. [b]Average of two separate determinations. [c]Activity on *Neisseria gonorrhoeae* FA19 on disk diffusion assays. Disk diameter is 6 mm. ND = not determined. Bn = benzyl. PMP = *p*-methoxyphenyl.

3.5 Pull-down Assay with Stable Isotope Labeling by Amino Acids

While DTPs have been studied since the 1940s, their mechanism of action is still not well understood, in part because the *in vivo* results cannot be correlated *in vitro*. Hence, we designed a pull-down experiment where we could be able to detect binding partners and obtain a better understanding of the type of interactions these small molecules can have with potential targets. We decided to utilize the Stable Isotope Labeling by Amino acids (SILAC) technique[21] because it provides a robust, quantitative approach for measuring protein binders by mass spectrometry. We immobilized 6-azidohexanoyl holothin (**Table 3.1**, entry 13) on a cyclooctyne-containing bead through click-chemistry. We then grew two sets of *E. coli* MG1655 cultures with defined MOPS media to early stationary phase: the first culture contained all standard amino acids, while the second culture contained heavy L-lysine ($^{13}C6$) and heavy L-arginine ($^{13}C6^{15}N4$). Cultures were lysed and protein concentration standardized to 2 mg/mL according to protein concentration standardization by bicinchoninic acid assay (BCA). Next, we incubated 6-azidohexanoyl with the "light" lysate (i.e. no heavy amino

acids utilized), followed by the beads containing our immobilized compound (**Figure 3.6**). After three rounds of bead washing, elution of binding proteins was accomplished by boiling the beads and running them on 12% SDS PAGE. Proteins bands were cut, trypsinized, and analyzed by LC/MS-MS.

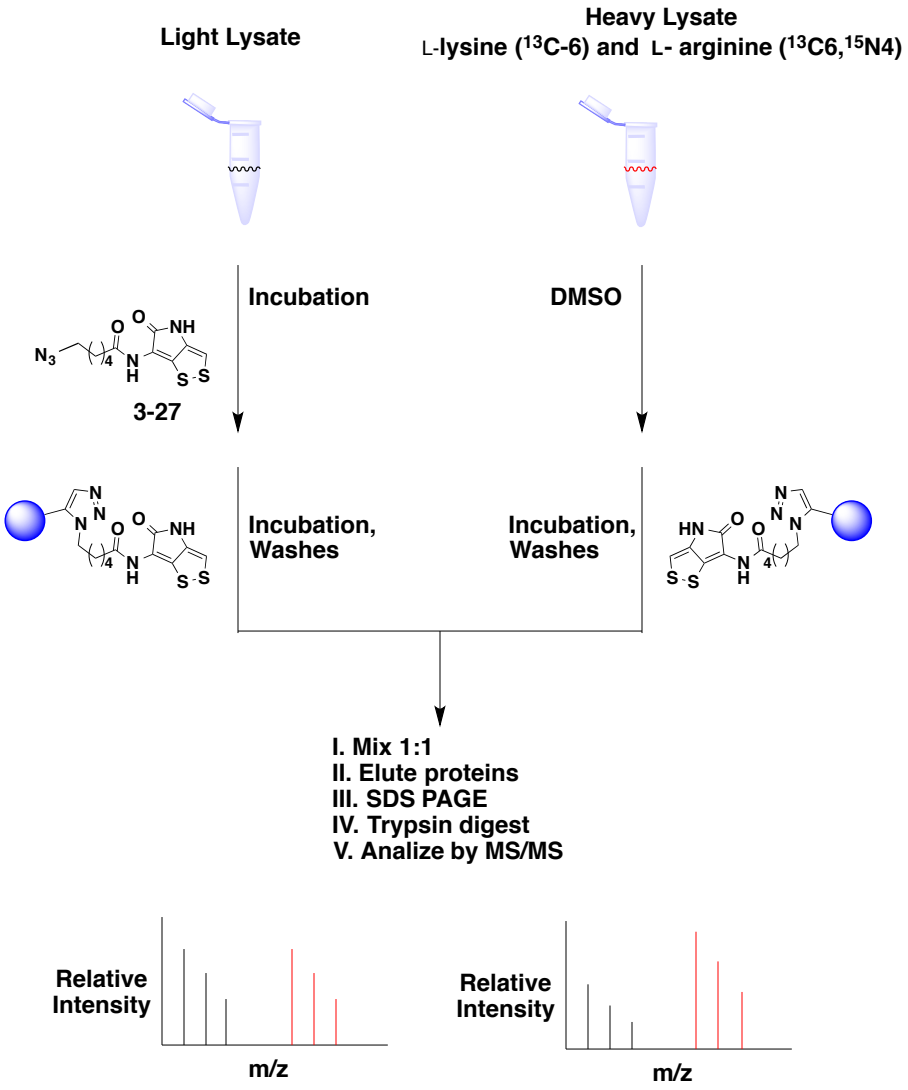


Figure 3.5. Workflow for SILAC pull-down assay with dithiolopyrrolones.

Interestingly, our pull-down returned the association of our DTP with a cyclodioxygenase (**Figure 3.7**), although a secondary test such as isothermal titration

calorimetry is needed to confirm the specific binding affinity. This result can be attributed to the presence of the disulfide moiety being recognized by enzymes that oxidize sulfides to sulfonic acids. Moreover, the disulfide bond can be reduced inside the cell and provide the putative iron-chelating properties of thiols, which cyclodioxygenases need to add oxygen to their oxidizing species. Upon close inspection of the holomycin biosynthetic cluster, we find that the origin of replication fork (ORF) 3493 from *S. clavuligerus* encodes for a cupin like protein; cupins are known oxidases of cysteine side chains and cysteamine to their metabolic products.[22]

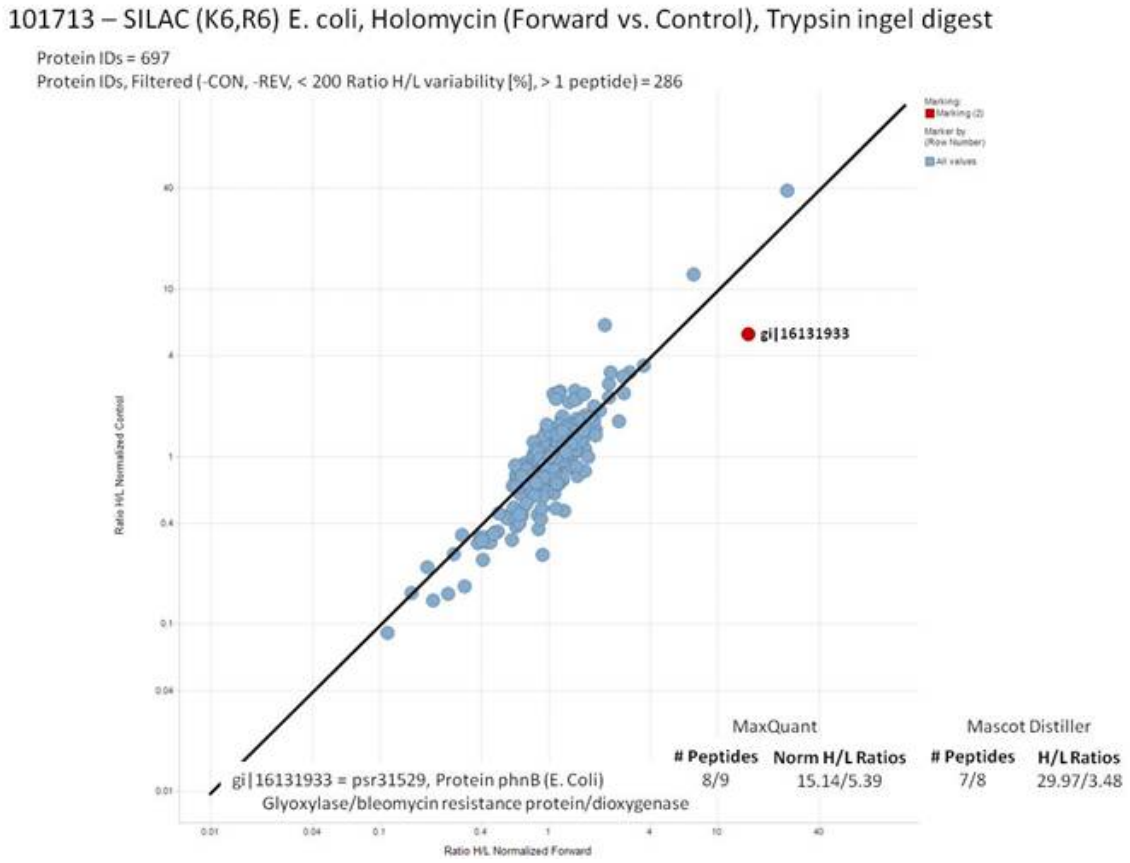


Figure 3.6. SILAC pull-down results.

3.6 Oxidized Holomycin Reactivity

The reactivity of thiosulfinate and thiosulfonate derivatives of ester-substituted DTP analogs has been explored by Schachtner *et al.*,[20] and they found them to be reactive to nucleophiles; however, holomycin and thiolutin are much more electron-rich, and similar reactivity was not examined. With the possibility that the thiosulfinate version of DTPs can be important, we made use of dioxoholomycin (**Table 3.1**, entry 18) and exposed it to the thiol nucleophile *N*-acetylcysteine in sodium phosphate buffer at various pHs. Surprisingly, we did not detect adducts were there was nucleophilic displacement or disulfide mixing, yet we did see the appearance of what appeared to be *N*-acetyl cysteamine and holomycin. We further monitor this reaction by UV-Vis due to the characteristic λ_{max} = 300 nm of dioxoholomycin and λ_{max} = 390 nm for holomycin. Our studies showed that, indeed, there is a shift from 300 nm to 390 nm over our time-course experiment (**Figure 3.8**), and that this change is strongly pH-dependent, with acidic conditions promoting the oxidation. This effect was also observed when reduced-glutathione was used.

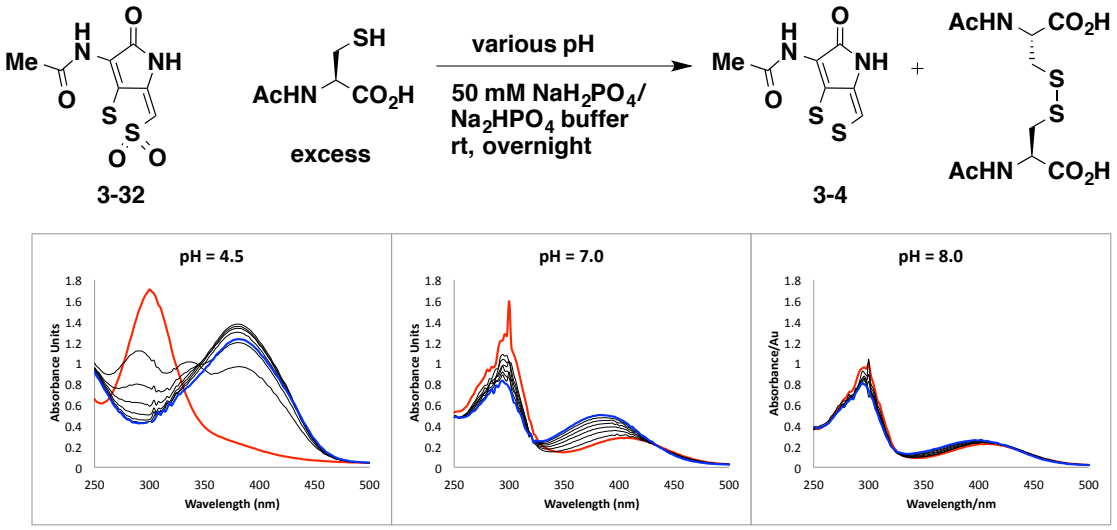


Figure 3.7. Dioxoholomycin reactivity towards thiols. Red line corresponds to t = 0 h. Blue line corresponds to t = 14 h. Intermediate lines are separated by 2 h.

Oxidative-sensitive cysteines are important for regulatory processes in the cell.[23] They can act as on/off switches when in a certain oxidation state. We searched the literature for proteins sensitive to oxidative conditions to test whether **3-32** could oxidize cysteines in proteins. Recent reports focusing on the arginine phosphatase YwlE[24] and the adenosyl-5'-phosphosulfate kinase AtAPSK[25] have been shown, by simple analytical tools, how these proteins contain cysteines that are labile towards redox conditions. We found that the arginine phosphatase YwlE is inactivated by a redox-active inhibitor that promotes the intramolecular disulfide formation between its Cys9 and Cys14, which are the only two cysteines in the protein, and thus leading to its inactivation. We heterologously expressed and purified YwlE and subjected it to our standard reaction conditions with **3-32** and found by mass spectrometry a mass change of -2 Dalton, corresponding to the disulfide (**Figure 3.9a**). Likewise, AtAPSK has been shown to dimerize at appropriate redox conditions, and thus inactivating the enzyme by linking Cys86 in one monomer to Cys119 in the other monomer. Ravilious *et al* have expressed a truncated form of the protein AtAPSK (AtAPSKΔ77) and

shown that its oxidation state is affected by the presence of the oxidant L-glutathione, dimer (GSSG) or the reductant L-glutathione, reduced (GSH).[25] AtAPSKΔ77 has the advantage of being able to be visualized by simple SDS-PAGE by monitoring the bands at 22 kDa (monomer, reduced sate) and 44 kDa (dimer, oxidized state). Our results confirm the GSSG/GSH redox effect (**Figure 3.9b**) and show that oxo-holomycin can oxidize the protein into its dimeric isoform; moreover, the introduction of GSH and oxo-holomycin prevents AtAPSK from being fully oxidized.

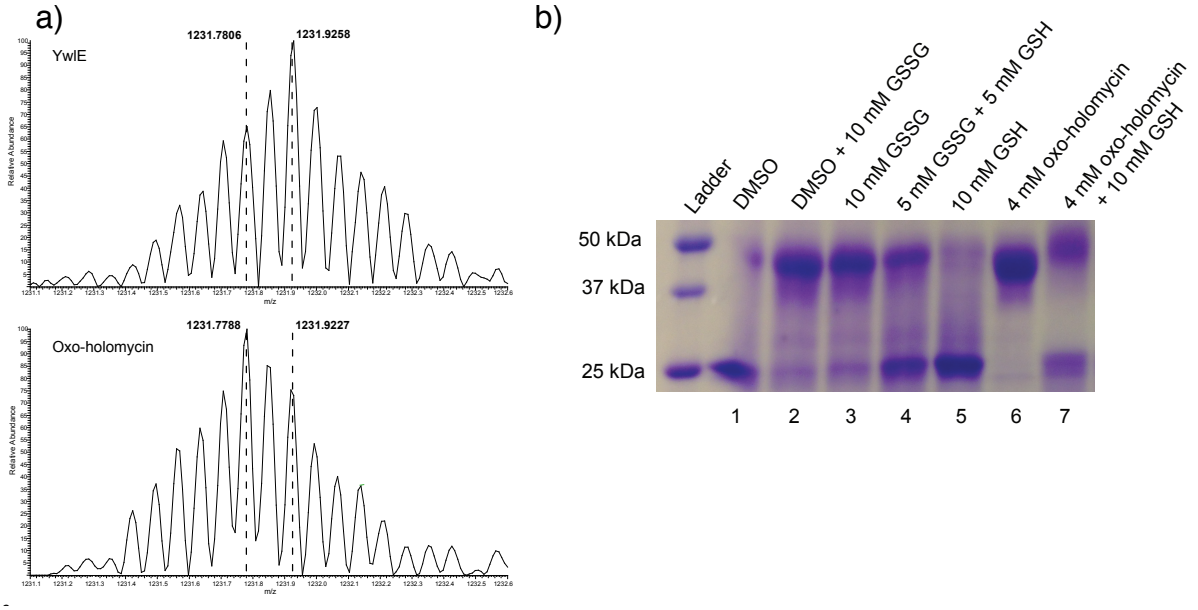
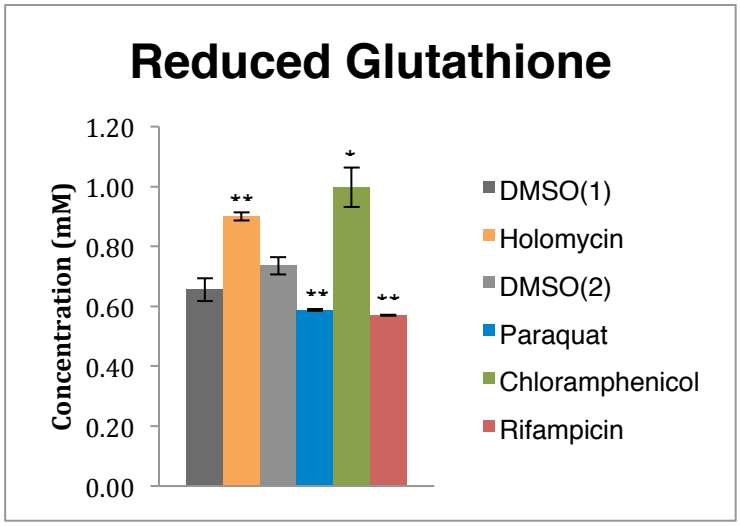
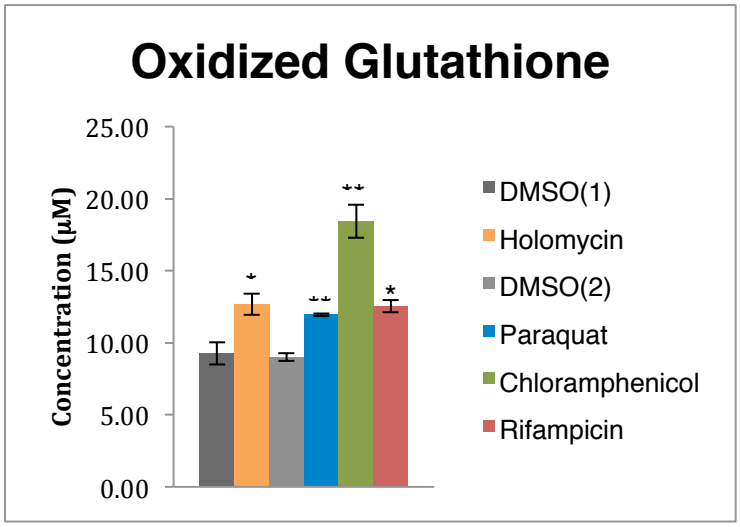


Figure 3.8. Dioxoholomycin oxidation of YwlE and AtAPSKΔ77: a) YwlE at z = 14 (reduced YwlE = 17233 Da ; oxidized YwlE= 17231 Da) and b) AtAPSKΔ77 (reduced monomer = 22 kDa, oxidized dimer = 44 kDa).

3.7 *In vivo* Reduced Glutathione Analysis of Holomycin Treated in *Escherichia coli*

The SILAC pull-down experiment and our oxidation studies on the dioxoholomycin (**3-32**) presented evidence that DTPs can be involved in redox cycling inside the cells. Hence, we sought to test this hypothesis by measuring the *in* vivo effect of holomycin in GSH/GSSG levels in *E. coli* MG1655 at the literature MIC of 2 μg/mL.[18] We decided to utilize holomycin and not dioxoholomycin because the latter lacked antibiotic activity in our disk diffusion assays previously described. To complement our experiment, we utilized the known redox active compound Paraquat as a positive control, chloramphenicol due to its effect on glutathione,[26] and rifampicin as an inhibitor of glutathione biosynthesis.[27] *E. coli* cultures were grown to half log-phase (OD_{600} = 0.3) at 37 ^{o}C, and the controls or antibiotics were added to the following final concentrations: 0.5% DMSO, 2 μg/mL holomycin, 300 μg/mL Paraquat, 5 μg/mL chloramphenicol, and 10 μg/mL rifampicin. Cells were incubated for 1h afterwards and then pelleted and lyophilized. Dried cells were utilized to measure glutathione content by using monobromobimane according to a literature procedure.[28] As expected, Paraquat, and rifampicin did increase the amount of GSSG with respect to their control by lowering the amount of GSH (**Figure 3.10**). Similarly, chloramphenicol greatly increased the GSSG content, but the amount of GSH increased significantly as well, possibly due to the fact that glutathione is used to detoxify this antibiotic by dechlorinating it.[26] Holomycin, on the other hand, did not have an effect on the ratio of GSH to GSSG when compared to its control, although, like chloramphenicol, the actual concentrations of both were higher.





Ratio	DMSO(1)	Holomycin	DMSO(2)	Paraquat	Chloramphenicol	Rifampicin
GSH	77	77	82	49	54	46
GSSG	1	1	1	1	1	1

Figure 3.9. Quantifying reduced and oxidized glutathione in treated *E. coli* MG1655. DMSO(1) corresponds to DMSO control for holomycin treated cells; DMSO(2) corresponds to DMSO control for Paraquat, chloramphenicol, and rifampicin. $^{*}p < 0.05$. $^{**}p < 0.01$.

3.8 Summary

TmlU and HolE from the thiomarinol biosynthetic pathway are the enzymes that ligate individual components of the hybrid antibiotic; TmlU activates pseudomonic acid C by converting it to the CoA thioester, and HolE is the transferase that ligates holothin to the CoA thioester. TmlU does not show high substrate promiscuity, with preference for long, complex alkyl chains. HolE does allow for multiple CoA thioesters as coupling partners, but not the use of aryl amines. Multiple analogs of holothin were synthesized and tested for biological activity against *N. gonorrhoeae* and found to be active, except for the oxidized thiosulfinates. SILAC pull-down experiments showed that DTPs can associate with iron-dependent cyclodioxygenases. Dioxoholomycin can oxidize thiols from small molecules as well as from redox-sensitive cysteines in proteins. Holomycin does not have an effect on the redox status of *E. coli* MG1655 *in vivo*, although the concentration of GSH and GSSG are increased overall. The reactivity of dioxoholomycin reveals a plausible mechanism of action of dithiolopyrrolones where redox homeostasis can be disturbed through thiol oxidation or iron sequestration.

3.9 Experimental

3.9.1 General Methods

All reactions were carried out in an oven-dried round-bottomed-flask under an inert nitrogen atmosphere. Solvents and reagents were used as received unless otherwise noted. Mupirocin was purchased from Applichem through Fisher scientific. Thiolutin was purchased from commercial sources. Spectra for ^{1}H and ^{13}C NMR were recorded at room temperature with a Bruker Avance^{III} (500 MHz and 125 MHz or 600 MHz and 150 MHz,

respectively) or a Varian Inova 400 (400 MHz and 100 MHz, respectively). Chemical shifts are reported in δ (ppm) relative units to residual solvent peaks $CDCl_3$ (7.26 ppm for ^1H and 77.0 ppm for ^{13}C) and DMSO-*d*6 (2.50 ppm for ^1H and 39.5 ppm for ^{13}C). Splitting patterns are assigned as s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), multiplet (m), dd (doublet of doublets), and td (triplet of doublets). Mass spectrometry measurements were recorded using an Agilent 6520 Accurate-Mass Q-TOF ESI positive in high-resolution mode. Predicted masses were extracted to $\pm$ 5 ppm.

3.9.2 Synthesis

9-(((*E*)-4-((2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-((4*R*,5*S*,*E*)-5-hydroxy-4-methylhex-2-en-1-yl)tetrahydro-2*H*-pyran-2-yl)-3-methylbut-2-enoyl)oxy)nonanoic acid (3-2, Pseudomonic acid C). Pseudomonic acid C was prepared according to a literature method.[9] Mupirocin (3-1, 1.02 g, 2.04 mmol) was dissolved in 2,2-dimethoxypropane (20 mL) and ethyl acetate (20 mL). *p*-Toluenesulfonic acid monohydrate (102 mg, 0.54 mmol) was added and reaction stirred at rt for 1 h. Mixture was washed with brine (40 mL), dried with anhydrous Na_2SO_4, and concentrated under reduced pressure to dryness. The resulting 6,7-*O*-isopropylidenepseudomonic acid A was dissolved in 50 % aqueous methanol (40 mL) and converted to its potassium salt by addition of potassium bicarbonate (0.204 g, 2.04 mmol). After concentration to dryness under reduced pressure, the resulting potassium salt was dissolved in 90 % aqueous *tert*-amylalcohol (30 mL) and potassium selenocyanate (0.88 g, 6.11 mmol) was added. The resulting mixture was refluxed for 2 days under nitrogen. After cooling to rt, the reaction was filtered with celite and washed with additional 90 % aqueous *tert*-amylalcohol (20 mL) and concentrated to dryness to afford. The crude compound was

dissolved in water (40 mL) and ethyl acetate was added (40 mL); 5 M HCl was added, while stirring, until pH = 2. The organic layer was collected and the aqueous layer further extracted with ethyl acetate (2 x 20 mL). The combined extracts were dried with Na_2SO_4 and evaporated to dryness. The crude oil was dissolved in 80 % acetic acid (20 mL) and stirred for 12 h at rt. Mixture was evaporated to dryness and purified by silica gel (60 mesh) with a 0 – 6 % methanol gradient in chloroform (R_f = 0.30, 5 % MeOH in $CHCl_3$) to afford pseudomonic acid C (#, 0.61 g) in 62% overall yield. ^{1}H and ^{13}C NMRs are in agreement with those previously reported.[9] HR-MS (ESI): Calculated $C_{26}H_{44}NaO_8^+$ $[M + Na]^+$ = 507.2928; found $[M + Na]^+$ = 507.2911.

2,2,2-trifluoro-*N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)acetamide (3-10 Trifluoroacetyl holothin) was prepared according to a literature method.[10] The residue was purified by flash column chromatography (Hex/EtOAc 1:2, R_f = 0.40) to obtain a yellow solid (5 steps, 13% overall yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 7.32 (s, 1H), 10.92 (s, 1H), 11.60 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 112.0, 112.2, 115.4 (q, J = 285 Hz), 133.5, 140.3, 153.8 (q, J = 37.5 Hz), 167.5. HR-MS (ESI): Calculated $C_7H_4F_3N_2O_2S_2^+$ $[M + H]^+$ = 268.9661; found $[M + H]^+$ = 268.9664.

6-amino-[1,2]dithiolo[4,3-*b*]pyrrol-5(4*H*)-one hydrochloride (3-3, holothin hydrochloride)

3-10 (5.00 mg, 18.6 mmol) was dissolved in MeOH (1.0 mL) and 37% HCl (25 mL) was added dropwise. Reaction was refluxed for 2.5 h, cooled to rt, and concentrated to dryness

under reduced pressure to afford holothin hydrochloride (**3-3**) as a yellow solid which was utilized without any further purification.

(2*S*,3*S*,4*R*,5*S*)-2-((*E*)-2-methyl-4-oxo-4-((9-oxo-9-((5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)amino)nonyl)oxy)but-2-en-1-yl)-5-((4*R*,5*S*,*E*)-4-methyl-5-(2,2,2-trichloroacetoxy)hex-2-en-1-yl)tetrahydro-2*H*-pyran-3,4-diyl bis(2,2,2-trichloroacetate) (3-12). Pseudomonic acid C (0.267g, 0.55 mmol) was dissolved in CH_2Cl_2 (9.2 mL) and cooled to 0 ^oC. Trichloroacetyl chloride (0.31 mL, 2.75 mmol) and pyridine (0.19 mL, 2.38 mmol) were added dropwise, followed by addition of 4-*N,N*-dimethylaminopyridine (6.7 mg, 0.055 mmol). Reaction was warmed to rt and stir for 2.5 h. Mixture was diluted with CH_2Cl_2 (10 mL) and washed with 1N HCl (2 x 5 mL). Organic layer was dried with Na_2SO4 and concentrated to dryness under reduced pressure to afford **S9**, which was utilized without any further purification.

Crude compound (0.385g, 0.42 mmol) was dissolved in CH_2Cl_2 (7.0 mL) and cooled to 0 ^oC. Oxalyl chloride (71 mL, 0.84 mmol) was added dropwise followed by one drop of *N,N*-dimethylformamide. Reaction was stirred at rt for 3 h and concentrated to dryness under reduced pressure. The resulting crude acyl chloride was utilized without any further purification.

Crude acyl chloride was dissolved in THF (2.13 mL) and added dropwise to a 0 ^oC suspension of holothin hydrochloride (87.1 mg, 0.418 mmol) in THF (19 mL). Et_3N (0.13 mL, 0.931 mmol) was added dropwise to the mixture and reaction stirred at rt for 45 min. Mixture was concentrated to dryness under reduced pressure. Silica gel (60 mesh) purification with a methanol gradient (0-5%) in chloroform (R_f = 0.75, 5% MeOH in $CHCl_3$) afforded **3-12** (0.271g, 0.252 mmol) as a yellow foam in 60 % overall yield. ^1H NMR (600

MHz; $CDCl_3$) δ 8.77 (s, 1H), 7.61 (s, 1H), 6.81 (s, 1H), 5.73 (s, 1H), 5.55 (m, 2H), 5.48 (t, *J* = 3.0 Hz, 1H), 4.98 (m, 1H), 4.92 (dd, *J* = 3.0, 9.9 Hz, 1H), 4.07 (t, *J* = 6.9 Hz, 2H), 3.87 (dd, *J* = 3.0, 12.0 Hz, 1H), 3.71 (d, *J* = 12.0 Hz, 1H), 2.50 (m, 1H), 2.45 (dd, *J* = 2.4, 14.4 Hz, 1H), 2.34 (m, 5H), 2.20 (d, *J* = 1.2 Hz, 3H), 2.03 (m, 1H), 1.69 (m, 2H), 1.63 (m, 3H), 1.32 (m, 9H), 1.25 (m, 2H), 1.11 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (150 MHz; $CDCl_3$) δ 171.65, 168.47, 166.41, 161.41, 160.83, 160.80, 154.13, 134.44, 133.06, 127.68, 118.74, 114.39, 112.06, 90.29, 89.53, 89.05, 79.85, 74.40, 73.53, 72.55, 65.56, 63.95, 42.69, 41.92, 40.01, 36.42, 32.01, 29.71, 29.16, 29.07, 29.05, 28.65, 25.92, 25.32, 19.04, 16.76, 16.31. Calculated $C_{37}H_{44}Cl_9N_2O_{11}S_2^+$ $[M + H]^+$ = 1070.9578; found $[M + H]^+$ = 1070.9887. $[a]_D^{20.4}$ = +9.54° (c = 0.1, $CHCl_3$).

9-oxo-9-((5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)amino)nonyl (*E*)-4-((2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-((4*R*,5*S*,*E*)-5-hydroxy-4-methylhex-2-en-1-yl)tetrahydro-2*H*-pyran-2-yl)-3-methylbut-2-enoate (3-13). 3-12 (0.230 g, 0.214 mmol) was dissolved in MeOH (4 mL) and cooled to 0 °C. NH_3 (7 N in MeOH, 0.245 mL, 1.72 mmol) was added dropwise. Reaction was stirred for 3 h at rt and concentrated to dryness under reduced pressure. Silica gel (60 mesh) purification with a methanol gradient (0-6%) in chloroform (R_f = 0.5, 5% MeOH in $CHCl_3$) afforded **3-13** (0.103g, 0.161 mmol) as an orange-yellow solid in 75 % yield. ^1H NMR (600 MHz; $CDCl_3$) δ 9.17 (s, 1H), 8.01 (s, 1H), 6.88 (s, 1H), 5.76 (s, 1H), 5.45 (m, 2H), 4.07 (t, *J* = 6.3 Hz, 2H), 3.92 (t, *J* = 3.3 Hz, 1H), 3.79 (dd, *J* = 3.0, 11.4 Hz, 1H), 3.73 (td, *J* = 3.0, 9.0 Hz, 1H), 3.64 (m, 2H), 3.56 (m, 1H), 3.51 (dd, *J* = 1.8, 11.4 Hz, 1H), 3.47 (dd, *J* = 3.0, 8.4 Hz, 1H), 2.62 (dd, *J* = 2.4, 15.0 Hz, 1H), 2.44 (s, br, 3H), 2.36 (t, *J* = 7.5 Hz, 2H), 2.28 (dd, *J* = 8.1, 14.7 Hz, 1H), 2.23 (m, 1H),

2.20 (d, *J* = 0.6 Hz, 3H), 2.16 (m, 1H), 2.09 (m, 1H), 1.86 (m, 1H), 1.69 (m, 1H), 1.62 (m, 1H), 1.32 (m, 8H), 1.16 (d, *J* = 6.0 Hz, 3H), 0.99 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (150 MHz; $CDCl_3$) δ 171.93, 168.49, 166.79, 156.76, 134.38, 133.14, 129.44, 117.51, 113.95, 112.49, 74.85, 71.24, 70.53, 70.37, 68.92, 64.82, 63.74, 44.71, 42.88, 41.73, 36.30, 32.32, 28.81, 28.75, 28.70, 28.40, 25.73, 25.56, 20.41, 19.22, 16.71. Calculated $C_{31}H_{47}N_2O_8S_2^+$ $[M + H]^+$ = 639.2768; found $[M + H]^+$ = 639.2765. $[a]_D^{20.4}$ = +21.74^o (c = 0.1, $CHCl_3$).

General Procedure for the preparation of dithiolopyrrolone analogs: Trifluoroacetyl holothin (**3-10**, 0.050g, 0.186mmol) is dissolved in MeOH (7.25 mL) and HCl (37% aqueous, 0.24 mL) is added dropwise. Reaction is refluxed for 2.5 h. Mixture is allowed to cool to room temperature and concentrated *in vacuo* yielding compound **3-3** as a yellow-orange solid that is used without further purification (R_f = 0, Hex/EtOAC 1:2). The residual solid is suspended in THF (16.5 mL) and cooled to 0 ^{o}C. The corresponding acid chloride is added dropwise (0.231 mmol) followed by Et_3N (57.8 μL, 0.415mmol). Reaction is allowed to warm to room temperature and stirred for 30 minutes. Mixture is concentrated *in vacuo* and purified by flash column chromatography.

***N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)acetamide (3-4, Holomycin)**. Prepared according to the general procedure. Acetyl chloride (16.4 μL, 0.231 mmol) was utilized as the acid chloride source. Flash column chromatography (R_f = 0.67, EtOAc/MeOH 9:1) yielded compound **3-4** as an orange solid (57 % yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 2.00 (s, 3H), 7.04 (s, 1H), 9.89 (s, 1H), 10.71 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ

22.4, 110.7, 115.4, 133.7, 134.0, 167.9, 168.9. HR-MS (ESI): Calculated $[M + H]^+$ = 214.9943; found $[M + H]^+$ = 214.9946.

***N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)pentanamide (3-17, Pentanoyl Holothin)**. Prepared according to the general procedure. Pentanoyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.10, Hex/EtOAc 1:2) yielded compound as a yellow solid (84% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 0.86 (t, J = 7.0 Hz, 3H), 1.22-1.30 (m, 2H), 1.46-1.52 (quint, J = 7.5 Hz, 2H), 2.33 (t, J = 7.0 Hz, 2H), 7.04 (s, 1H), 9.84 (s, 1H), 10.71 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 13.7, 21.7, 27.2, 34.4, 110.5, 115.3, 133.7, 133.9, 167.9, 171.8. HR-MS (ESI): Calculated $[M + H]^+$ = 257.0413; found $[M + H]^+$ = 257.0418.

(*E*)-2-methyl-*N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)but-2-enamide (3-18, Tigloyl Holothin). Prepared according to the general procedure. Tigloyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.35, Hex/EtOAc 1:2) yielded compound as an orange solid (66% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.73 (dd, J = 1.0 and 7.0 Hz, 3H), 1.80 (t, J = 1.0 Hz, 3H), 6.53 (qq, J = 1.0 and 7.0 Hz, 1H), 7.09 (s, 1H), 9.07 (s, 1H), 10.77 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 12.2, 13.9, 110.8, 115.3, 130.1, 132.9, 133.7, 134.9, 166.9, 168.0. HR-MS (ESI): Calculated $[M + H]^+$ = 255.0256; found $[M + H]^+$ = 255.0267.

***N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)methanesulfonamide (3-19, Mesyl Holothin).**

Prepared according to the general procedure. Mesyl chloride (22.2μL, 0.231 mmol) was utilized as the acid chloride source. Flash column chromatography (R_f = 0.5, EtOAc/MeOH 9:1) yielded compound as a brown solid (15% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 2.99 (s, 3H), 7.19 (s, 1H), 9.36 (s, 1H), 10.83 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 40.8, 111.4, 113.1, 133.9, 141.8, 168.7. HR-MS (ESI): Calculated $[M + H]^+$ = 250.9613; found $[M + H]^+$ = 250.9633.

***N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)hept-6-ynamide (3-20, 6-Heptynoyl Holothin)**. Prepared according to the general procedure. 6-heptynoyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.15 Hex/EtOAc 1:2) yielded compound as a brown solid (24% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.42 (quint, J = 7.5 Hz, 2H), 1.59 (quint, J = 7.5 Hz, 2H), 2.16 (td, J = 2.5 and 7.0 Hz, 2H), 2.34 (t, J = 7.5 Hz, 2H), 2.77 (t, J = 2.5 Hz, 1H), 7.05 (s, 1H), 9.87 (s, 1H), 10.72 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 17.9, 24.7, 27.9, 34.5, 71.8, 84.8, 111.1, 115.8, 134.1, 134.5, 168.4, 172.1. HR-MS (ESI): Calculated $[M + H]^+$ = 281.0413; found $[M + H]^+$ = 281.0423.

***N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)hex-5-ynamide (3-21, 5-Hexynoyl Holothin)** Prepared according to the general procedure. 5-hexynoyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.5, Hex/EtOAc 1:2) yielded compound as a brown-orange solid (16% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.68 (quint, J = 7.0 Hz, 2H), 2.16 (td, J = 2.5 and 7.0 Hz, 2H), 2.43 (t, J = 7.0 Hz, 2H), 2.78 (t, J = 3 Hz, 1H), 7.04 (s, 1H), 9.88 (s, 1H), 10.68 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 17.3,

24.0, 71.6, 83.9, 110.5, 115.3, 133.7, 134.1, 167.9, 171.1. HR-MS (ESI): Calculated $[M + H]^+ = 267.0256$; found $[M + H]^+ = 267.0260$.

***N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)hexanamide (3-22, Hexanoyl Holothin).** Prepared according to the general procedure. Hexanoyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.60, EtOAc) yielded compound as a yellow solid (77% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 0.85 (t, J = 7.0 Hz, 3H), 1.19-1.31 (m, 4H), 1.50 (quint, J = 7.0 Hz, 2H), 2.32 (t, J = 7.5 Hz, 2H), 7.04 (s, 1H), 9.84 (s, 1H), 10.71 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 13.8, 21.8, 24.7, 30.8, 34.6, 110.5, 115.3, 133.7, 133.9, 167.9, 171.8. HR-MS (ESI): Calculated $[M + H]^+ = 271.0569$; found $[M + H]^+ = 271.0590$.

***N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)octanamide (3-23, Octanoyl Holothin)**. Prepared according to the general procedure. Octanoyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.45, Hex/EtOAc 1:2) yielded compound as a yellow solid (77% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 0.84 (t, J = 7.0 Hz, 3H), 1.18-1.29 (m, 8H), 1.50 (quint, J = 7.0 Hz, 2H), 2.32 (t, J = 7.5 Hz, 2H), 7.04 (s, 1H), 9.83 (s, 1H), 10.70 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 13.9, 22.0, 25.1, 28.4, 28.5, 31.1, 34.6, 110.5, 115.4, 133.7, 133.9, 167.9, 171.8. HR-MS (ESI): Calculated $[M + H]^+ = 299.0882$; found $[M + H]^+ = 299.0887$.

(*E*)-*N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)but-2-enamide (3-24, Crotonyl Holothin). Prepared according to the general procedure. Crotonyl chloride was utilized as the

acid chloride source. Flash column chromatography (R_f = 0.20, Hex/EtOAc 1:2) yielded compound as an orange solid (39% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.81 (dd, J = 1.5 and 7.0 Hz, 3H), 6.33 (dd, J = 1.5 and 15.2 Hz, 1H), 6.74 (dq, J = 6.5 and 15.5 Hz, 1H), 7.08 (s, 1H), 9.97 (s, 1H), 10.75 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 17.7, 111.0, 115.3, 124.2, 133.8, 134.4, 163.5, 167.9. HR-MS (ESI): Calculated $[M + H]^+$ = 241.0100; found $[M + H]^+$ = 241.0103.

***N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)methacrylamide (3-25, Methacryloyl Holothin)**. Prepared according to the general procedure. Methacryloyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.15, Hex/EtOAc 1:2) yielded compound as an orange solid (39% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.92 (s, 3H), 5.50 (t, J = 1.0 Hz, 1H), 5.89 (s, 1H), 7.12 (s, 1H), 9.34 (s, 1H), 10.80 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 18.4, 111.0, 114.9, 121.8, 133.7, 135.7, 138.1, 166.1, 168.0. HR-MS (ESI): Calculated $[M + H]^+$ = 241.0100; found $[M + H]^+$ = 241.0103.

3-methyl-*N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)but-2-enamide (3-26, 3,3-Dimethylacryloyl Holothin) Prepared according to the general procedure. 3,3-dimethylacryloyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.4, Hex/EtOAc 1:2) yielded compound as an orange solid (54% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.81 (d, J = 1.0 Hz, 3H), 2.12 (d, J = 1.0 Hz, 3H), 6.07-6.09 (m, 1H), 7.04 (s, 1H), 9.73 (s, 1H), 10.72 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6)

δ 19.8, 27.2, 110.5, 115.5, 117.5, 133.7, 133.8, 152.8, 164.6, 168.0. HR-MS (ESI): Calculated $[M + H]^+$ = 255.0256; found $[M + H]^+$ = 255.0266.

6-azido-*N*-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)hexanamide (3-27, 6-Azidohexanoyl Holothin) Prepared according to the general procedure. 6-azidohexanoyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.3 Hex/EtOAc 1:2) yielded compound as an orange solid (19%). ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.26-1.33 (m, 2H), 1.53 (quint, J = 7.0 Hz, 4H), 2.34 (t, J = 7.5 Hz, 2H), 3.31 (t, J = 7.0 Hz, 2H), 7.05 (s, 1H), 9.86 (s, 1H), 10.71 (s). ^{13}C NMR (125 MHz; DMSO-d_6) δ 24.6, 25.7, 27.9, 34.5, 50.5, 110.6, 115.3, 133.7, 134.0, 167.9, 171.7. HR-MS (ESI): Calculated $[M + H]^+$ = 312.0583; found $[M + H]^+$ = 312.0592.

***N*-(4-benzyl-5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)acetamide**. **(3-28).** Flash column chromatography (R_f = 0.45, Hex/EtOAc 1:1) yielded compound as an orange solid (61% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 2.02 (s, 3H), 4.95 (s, 2H), 7.21-7.27 (m, 3H), 7.29 (s, 1H), 7.30-7.34 (m, 2H), 10.07 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 22.3, 44.0, 111.8, 114.5, 127.2, 127.4, 128.6, 133.4, 134.6, 137.2, 166.0, 168.8. HR-MS (ESI): Calculated $[M + H]^+$ = 305.0413; found $[M + H]^+$ = 305.0414.

***tert*-Butyl (5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-b]pyrrol-6-yl)carbamate (3-30, Pivaloyl Holothin)**. Prepared according to the general procedure. Pivaloyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.10, Hex/EtOAc 1:2) yielded compound as a yellow solid (77% yield). ^{1}H NMR (400 MHz; DMSO-d_6) δ 1.20 (s, 9H),

7.08 (s, 1H), 8.73 (s, 1H), 10.76 (s, 1H). ^{13}C NMR (100 MHz; DMSO-d_6) δ 26.94, 110.70, 115.11, 133.61, 134.87, 168.05, 176.60.

N-(5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-b]pyrrol-6-yl)benzamide (3-31, Benzoyl Holothin). Prepared according to the general procedure. Pivaloyl chloride was utilized as the acid chloride source. Flash column chromatography (R_f = 0.10, Hex/EtOAc 1:2) yielded compound as a yellow solid (72% yield). ^{1}H NMR (400 MHz; DMSO-d_6) δ 7.16 (s, 1H), 7.47-7.51 (m, 2H), 7.56-7.60 (m, 1H), 7.97-99 (m, 2H), 10.02 (s, 1H), 10.81 (s, 1H).

***N*-(2,2-dioxido-5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)acetamide (3-32, Dioxyholomycin)**. **3-4** (64.1 mg, 0.299 mmol) is dissolved in a mixture of acetone (12.8 mL) and water (12.8 mL) and cooled to 0 ^{o}C. Oxone® is added scoopwise (0.46 g, 1.49 mmol) and solution stirred at this temperature for 30 minutes. Reaction is quenched by addition of three drops of a saturated (aqueous) $NaHCO_3$ solution and stirred for an additional 30 minutes. Solids are filtered off and filtrate is concentrated. Flash column chromatography (R_f = 0.75, EtOAc/MeOH 9:1) yielded compound as a light yellow solid (40% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 2.08 (s, 3H), 7.27 (s, 1H), 10.57 (s, 1H), 11.35 (s, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 22.6, 109.5, 115.2, 123.3, 143.2, 165.8, 170.6. Calculated $[M + H]^+$ = 246.9842; found $[M + H]^+$ = 246.9843.

***N*-(2,2-dioxido-5-oxo-4,5-dihydro-[1,2]dithiolo[4,3-*b*]pyrrol-6-yl)-2,2,2-trifluoroacetamide (3-33, Dioxytrifluoroacetyl holothin)**. **3-10** (0.050 g, 0.186 mmol) is dissolved in a mixture of acetone (10 mL) and water (5 mL) and cooled to 0 ^{o}C. H_2O_2 (1.02

mmol), 30% solution in H_2O, is added dropwise and reaction stirred at 0 ^oC for 5 min, followed by 45 min at rt. Reaction is quenched by addition of two drops of a saturated (aqueous) $NaHCO_3$ solution and stirred for an additional 5 minutes. Concentrated. Purification of the mixture by flash column chromatography (R_f = 0.45, Hex/EtOAc 1:2) yielded compound as a light yellow solid (64% yield, b.r.s.m.). ^1H NMR (500 MHz; DMSO-d_6) δ 7.46 (s, 1H), 11.53 (s, 1H), 12.24, (s, broad, 1H). ^{13}C NMR (125 MHz; DMSO-d_6) δ 111.6, 117.3 (q, J = 285 Hz), 121.5, 123.8, 142.1, 155.5 (q, J = 38.8 Hz), 165.0. Calculated $[M + H]^+$ = 300.9559; found $[M + H]^+$ = 300.9563.

3.9.3 Ywle Expression, Purification, and Assay

E. coli BL21, transformed with pET21a(+) plasmid containing the YwlE gene, are grown in 1.0 L of LB broth supplemented with 100 μg/mL of ampicillin to an OD_{600} = 0.8 at 37 ^oC. Induced with 0.5mM IPTG for 2 h at 37 ^oC. Centrifuged cells at 4,000rpm for 15 min and removed supernatant. Resuspended cells in 35 mL lysis buffer (50mM Tris, pH = 7.5, and 300mM NaCl) supplemented with 2 mM PMSF, 10 mM $MgCl_2$, 250 μg/mL lysozyme, 2 U/mL DNase I, and 2 EDTA-free protease inhibitor tablets from ROCHE. Disrupted cells by sonicating twice for 1.5 min at 30% max burst intensity intervals. Centrifuged debris for 1 h at 15,000rpm. Lysate was filtered with a 0.45μm sterile filter and loaded into a Ni^{2+} column, previously equilibrated with lysis buffer. Washed off non-specific proteins by washing with lysis buffer first, followed by lysis buffer with 100mM imidazole. Eluted YwlE protein with lysis buffer supplemented with 250mM imidazole. Phosphatase-containing fractions were loaded on a Superdex®-75 column equilibrated with 20mM Tris (pH = 7.5) and 100mM NaCl. Fractions containing YwlE were pooled and concentrated to 9.25 mg/mL using a

Centricon concentrator with a 10kDa nominal molecular weight mass cutoff. Buffer-exchanged protein using a PD-10 desalting column (GE Healthcare) to 50mM NH_4HCO_3 (pH = 8.0).

Enzymatic assays were carried out in 50 mM NH_4HCO_3 (pH = 4.5) with final volumes of 100 μL:

1. 3.5 mg/mL YwlE + DMSO
2. 3.5 mg/mL YwlE + 10mM Oxo-holomycin

Incubated for 1 h at rt. Analyzed protein redox state with the LTqFT-ESI^+ high-resolution mass spectrometer.

3.9.4 AtAPSK Expression, Purification, and Assay

E. coli BL21, transformed with pET28a(+) plasmid containing the AtAPSKΔ77 gene, were grown in 1.0 L of terrific broth supplemented with 50 μg/mL of kanamycin to an OD_{600} = 0.8 at 37 ^oC. Induced with 1.0mM IPTG overnight at 20 ^oC. Centrifuged cells at 4,500rpm and remove supernatant. Resuspend cells in 35 mL of lysis buffer (50mM Tris, pH = 8.0, 500mM NaCl, 20 mM imidazole, 1mM 2-mercaptoethanol, 10% (v/v) glycerol, 1% Tween-20, 2 mM PMSF, 10 mM $MgCl_2$, 250 μg/mL lysozyme, 2 U/mL DNase I, and 2 protease inhibitor tablets from ROCHE). Disrupted cells by sonicating twice for 1.5 min with 30% of max burst intensity. Centrifuged debris for 1 h at 15,000rpm. Lysate is filtered with a 0.45 μm sterile filter and loaded into the Ni^{2+} column, previously equilibrated with equillibration buffer (lysis buffer without 1% Tween-20). Washed off non-specific proteins by washing with equilibration buffer. Eluted YwlE protein with equilibration buffer with 250mM imidazole. Kinase-containing fractions are loaded on a Superdex-200 column equilibrated

with 25mM Hepes, pH = 7.5, 200mM KCl, 5mM 2-mercaptoethanol, 5% (v/v) glycerol, Fractions containing AtAPSKΔ77 are pooled and concentrated to 1.3 mg/mL using a Centricon concentrator with a 3 kDa nominal molecular weight mass cutoff. Buffer exchanged protein using a PD-10 column to 50mM NaH_2PO4/Na_2HPO_4 (pH = 4.5) and concentrated to a final concentration of 2.01 mg/mL. Enzymatic reactions are carried out in 22.8 μM AtAPSKΔ77 in 50 mM sodium phosphate buffer (pH = 4.5). Final volume of reactions is 100 μL. Reactions are stirred for 1 h at rt while stirring. Oxo-holomycin stock is 200 mM in DMSO. $_L$-Glutathione, reduced, stock is 200 mM in H_2O. $_L$-Glutathione dimer stock is 100mM in H_2O. H_2O_2 stock is 0.3% in H_2O (98 mM).

3.9.5 Dioxoholymycin Reactivity with *N*-Acetylcysteine

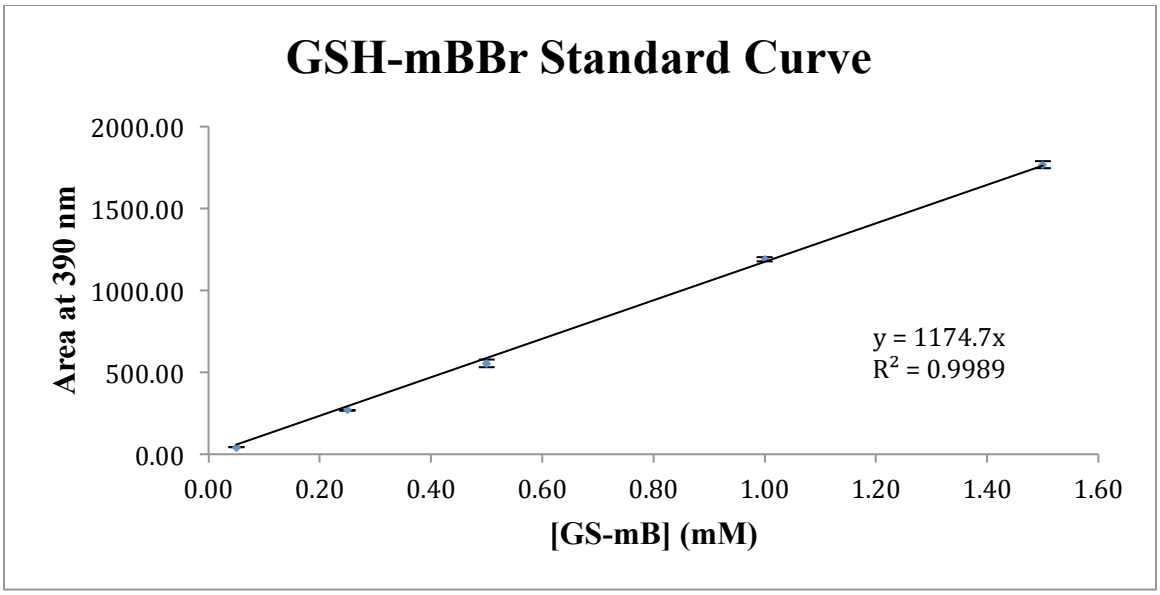
To a 2 mL screw-cap HPLC vial with septum was added 50 mM $NaHPO_4$ buffer, at the indicated pH, followed by **3-32** from a freshly prepared 10 mM DMSO stock, and *N*-Acetylcysteine from a freshly prepared 10 mM pH = 7.0 $NaHPO_4$ stock, such that the total volume was 1 mL and the concentrations of oxo-holomycin and *N*-Acetylcysteine were 0.5 mM and 1.0 mM, respectively. Reactions were monitored with UV/Vis in 384-well Greiner UV-star plates and were analyzed using a TECAN M1000 Infinite Pro microplate reader.

3.9.6 Measuring Glutathione Content in *E. coli* MG1655

Each experiment was done by growing 1.5 L of freshly prepared Luria-Bertani (LB) broth, in 2.8 L baffled-flasks with 1.5 mL inoculation from overnight seed, with *E. coli* MG1655 and 2 drops of antifoam at 37 ^oC until half log-phase was reached (OD_{600} = 0.3, ~ 3.5 h). Then, DMSO, holomycin (**3**-4, DMSO, final concentration 2 mg/mL), Paraquat (H_2O,

final concentration 300 mg/mL), chloramphenicol (MeOH, final concentration 5 mg/mL), or rifampicin (DMSO, final concentration 10 mg/mL) solutions were added. Cells were grown for one more hour at 37 ^{o}C and cells pelleted for 15 min at 4,500 rpm. Pellets were lyophilized to obtain dry cells.

Each pellet was split into 6 equal portions of ~50 mg residual dry weight each. Three cell pellets were extracted with 1 ml of pre-warmed (60 °C) 50% aqueous-acetonitrile containing 2 mM monobromobimane (mBBr) and 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 8.0. These thiol samples were incubated at 60 °C for 15 min, cooled on ice and acidified with 5 µl of 5M methanesulfonic acid. The cell debris was removed by centrifugation (13,000*g* for 10 min). The other three cell pellets were extracted with 1 ml of pre-warmed (60 °C) 50% aqueous-acetonitrile containing 5 mM N-ethylmaleimide (NEM) and 20 mM HEPES pH 8.0. These disulfide samples were incubated at 60 °C for 15 min and cooled on ice. The cell debris was removed by centrifugation (same as above). A 0.8 mL aliquot of the supernatant was mixed with 2.5 mM dithiothreitol (DTT) to react with residual NEM. The 0.8 mL supernatant was reduced with 2 mM DTT for 20 min at 23 °C and mBBr was added to a final concentration of 6 mM and reacted in the dark for an additional 10 min. The labeling reaction was quenched with 5 µl 5 M methanesulfonic acid. Analysis was done on LC/MS by injecting 15 µL of non-NEM treated samples and 30 µL of NEM-treated samples. Concentrations were calculated according to a previously made standard curve:


GSH-mBBr Standard Curve
Area at 390 nm
2000.00
1500.00
1000.00
500.00
0.00
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
[GS-mB] (mM)
y = 1174.7x
R² = 0.9989

3.10 REFERENCES

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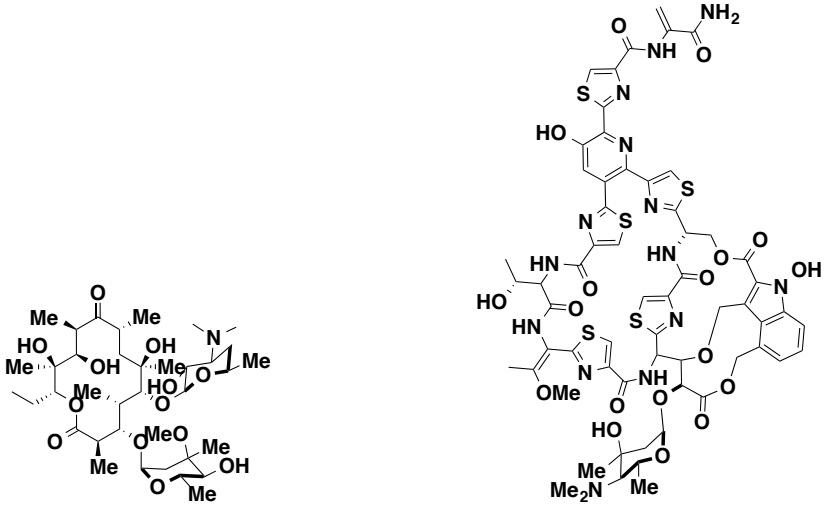
CHAPTER IV

VISIBLE LIGHT *O*-GLYCOSYLATION OF THIOGLYCOSIDES

4.1 Introduction

Natural products containing *O*-sugar linkages are often found in nature,[1] with aminoglycosides being the first class of naturally occurring antibiotics discovered through systematic screening of naturally occurring scaffolds.[2] Erythromycin is amongst the most well studied antibiotics containing a sugar moiety and having strong activity against *Staphylococcus aureus and Streptococcus pyogenes*[3] (**Figure 4.1**). It is often the substitute for patients possessing allergy to penicillin. Moreover, the thiopeptide nocathiacin I from *Nocardia* sp. ATCC 202099 has been found to be active against Gram-positive bacteria and a multi-drug resistant strain of *Enterococcus faecium* (**Figure 4.1**).[4] Sugar moieties can often increase solubility and aid in targeted drug delivery by allowing recognition of specific interactions within the cell surface.[5] Therefore, a method by which sugars could be linked to natural products is highly desirable. We envisioned the design of a method that would be mild and selective for activating glycosides in the presence of other functional groups, and then trapping it with an alcohol-containing acceptor molecule. The field of visible light catalysis has provided an avenue in which to pursue the glycosylation reaction.



Erythromycin

Nocathiacin I

Figure 4.1. *O*-Linked sugar containing antibiotics.

Visible light catalysis has found increasing use in organic chemistry for the activation of key moieties towards nucleophilic trapping by utilizing substoichiometric photoredox catalysts[6,7] involved in the formation of carbon-carbon, carbon-hydrogen, carbon-oxygen, and carbon-halogen bonds. This is best exemplified by the catalysts involved in singly occupied molecular orbital (SOMO) enantioselective additions into chiral 3π imidazolidinones developed by the MacMillan group.[8,9] In parallel, visible light catalysis has been harnessed for light activated metal-ligand complexes to access radical pathways that activate redox cycles by single-electron-transfer (SET) from a co-reductant (reductive quenching) or co-oxidant (oxidative quenching, **Figure 4.2**).[10,11]

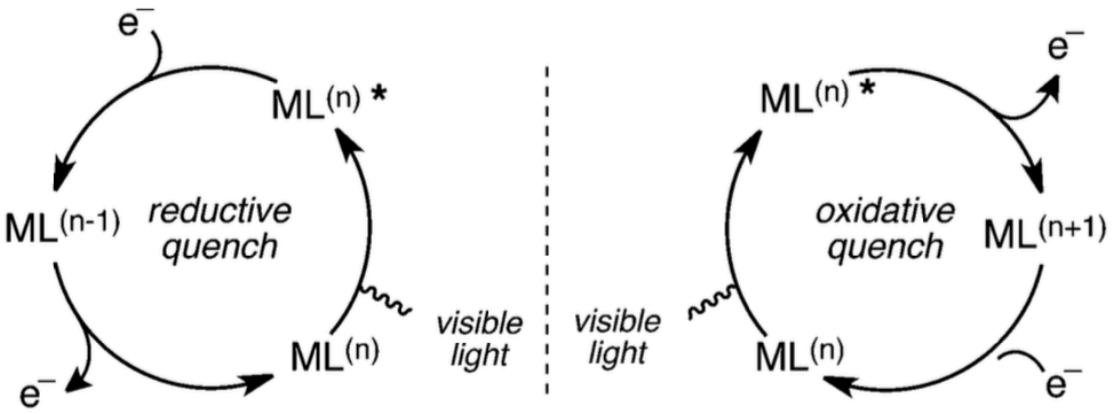
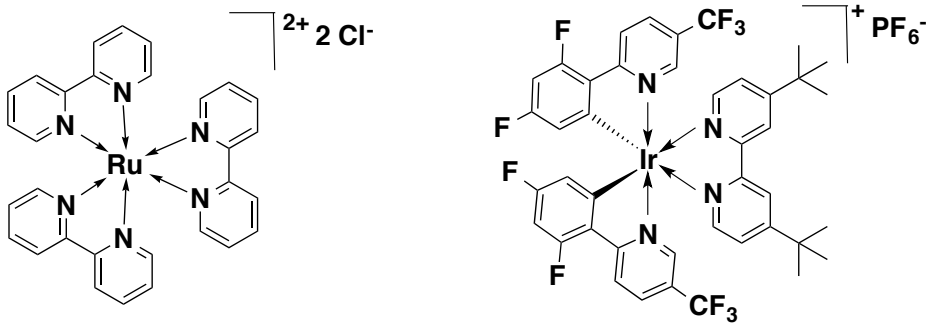


Figure 4.2. Commonly utilized visible light photoredox cycles. M = metal. L = ligand.

Visible light catalysis has allowed for transformation of alcohols to alkyl halides[12], formation of iminium intermediates for the Strecker reaction[7] and other nucleophilic additions, C-H activation at benzylic positions,[13] alkyl halide addition to olefins[14], and many other transformations. Due to the ability of photocatalysts to both receive and donate electrons, they can be fine-tuned to access various excitation wavelengths by the ligands associated with the metal center, allowing for the utilization of various visible light resources to promote redox cycling.

Amongst the most well studied photocatalysts are $Ru(bpy)_3Cl_2$ (**4-1**) and $Ir[dF(CF_3)ppy]_2$-(dtbbpy)PF_6 (**4-2**, **Figure 4.3**). These compounds have the advantage of having long-lived photoexcited states and inherent stability. Moreover, the Ru (II) and Ir (III) centers are able to take part in metal to ligand charge transfer (MLCT), resulting in a species where the metal center is formally oxidized while the ligand is formally reduced. Rapid intersystem crossing between the excited singlet state to the triplet state, allows for a relative long-lived state that can relay electrons since its decay to the singlet ground state is spin forbidden. [15]



4-1

$Ru(bpy)_3Cl_2$

4-2

$Ir[dF(CF_3)ppy]_2(dtbbpy)PF_6$

Figure 4.3. Commonly utilized visible light catalysts.

A key area where visible light catalysis can be extremely useful is in glycosylation chemistry. Selective activation of redox active moieties can be readily advantageous in the production of key synthetic methods by complementing protection/deprotection strategies. Thioglycosides, important starting blocks in carbohydrate chemistry,[16] can be considered ideal candidates for visible light catalysis. Preliminary work by Yoshida *et al*[17] has utilized this moiety in electron relay cycles for the activation of the anomeric position in electrochemical cells, showing the potential for thioglycosides to be activated towards oxidation and hence promising compatibility with visible light catalysis.

The complexity of oligosaccharides found in nature has spurred a great interest in developing chemical transformations that can link one or more carbohydrates together. For many years, chemical transformations have relied on the premise that the anomeric position of an acceptor glycoside can become active upon having an electronic demanding moiety that polarizes the σ* of the bond by which the oxygen in the pyran ring can promote dissociation and form an oxocarbenium ion, an elusive intermediate that has just been recently observed in superacid media[18] by nuclear magnetic resonance (NMR). An incoming nucleophile donor

can then trap the oxocarbenium ion (**Figure 4.4a)**. The use of rationally designed protecting groups for selective protection/deprotection at specific positions in the ring (**Figure 4.4b)** when the glycosylation does not involve the anomeric position is of equal importance. We envisioned a method by which we could combine both visible light catalysis and oxocarbenium chemistry to develop a mild method for *O*-glycosylation of thioglycosides that would be amenable for synthetic glycochemistry.

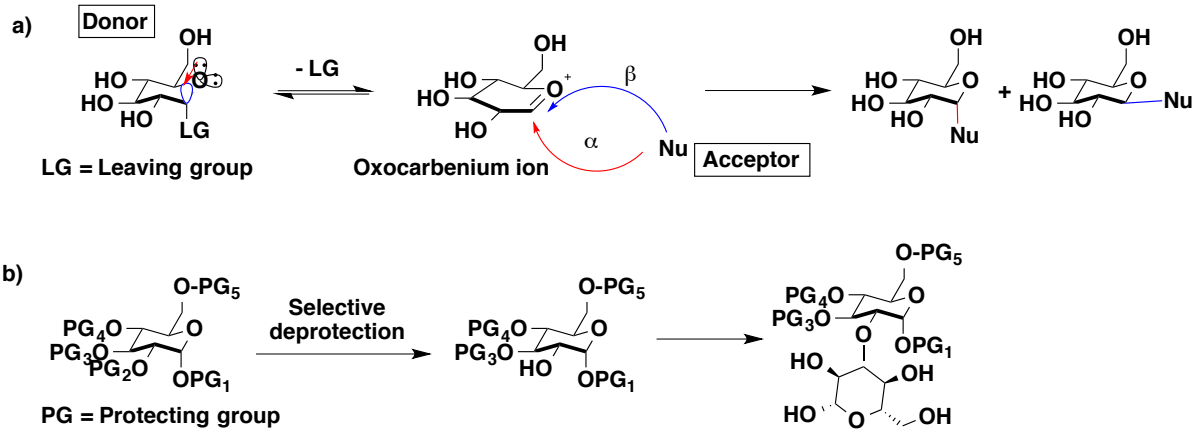


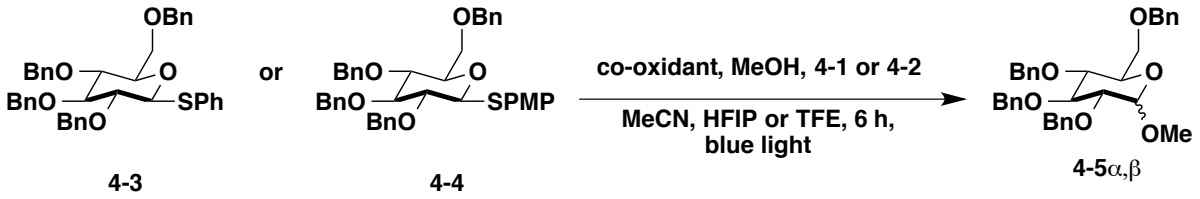
Figure 4.4 Glycochemistry reactivity. a) Formation of oxocarbenium ion and trapping by nucleophile at either α or β anomeric position. b) Representation of selective glucopyranoside reaction with different protecting groups at each hydroxyl position.

4.2 Visible Light Activation of Thioglycosides

We speculated that catalysts **4-1** and **4-2** would be able to generate oxocarbenium ions from electron rich thioglycosides through MLCT; these reactive intermediates could then be trapped with an alcohol donor. Our initial studies led us to synthesize *S*-phenyl (SPh, **4-3**) and *S*-*p*-methoxyphenyl (SPMP) β-glucose tetra-*O*-benzyl-thioglycoside (**4-4**) and to effect their activation with blue visible light irradiation in the presence of the co-oxidant $BrCCl_3$, catalyst **1** or **2**, and methanol as the trapping nucleophile under the conditions described in **Table 4.1**. Ru-catalyst **4-1** did not effect the activation of either SPh (**4-3)** or SPMP (**4-4**, entries 1 and 2) and trapping by methanol, but the Ir-catalyst **4-2** did allow for

the production of methoxylated product (**4-5)** when **4-4** was employed as substrate (**Table 4.1**, entries 4-10). The differential activity of the two thioglycoside donors can be attributed to the oxidation potentials of **4-3** and **4-**4 measured at 1.31 eV and 1.16 eV (vs Ag/AgCl), respectively, and the oxidation potential of **4-2** at 1.21 eV (vs Ag/AgCl).

Table 4.1. Optimizing glycosidation conditions*.



Entry	Substrate	MeOH (equiv.)	Catalyst (5 mol%)	Co-oxidant (equiv.)	Additive	Yield
1	**4-3**	10	**4-1**	$BrCCl_3$ (2.0)	--	0
2	**4-4**	10	**4-1**	$BrCCl_3$ (2.0)	--	0
3	**4-3**	10	**4-2**	$BrCCl_3$ (2.0)	--	0
4	**4-4**	10	**4-2**	$BrCCl_3$ (2.0)	--	96
5	**4-4**	2	**4-2**	$BrCCl_3$ (2.0)	--	12
6	**4-4**	2	**4-2**	$BrCCl_3$ (2.0)	TFE	37
7	**4-4**	2	**4-2**	$BrCCl_3$ (2.0)	HFIP	51
8	**4-4**	2	**4-2**	CBr_4 (2.0)	HFIP	58
9	**4-4**	2	**4-2**	CBr_4 (0.25)	HFIP	65
10	**4-4**	2	**4-2**	$BrCCl_3$ (0.25)	HFIP	61

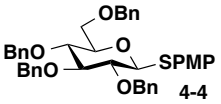
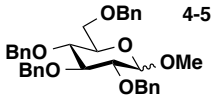
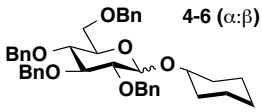
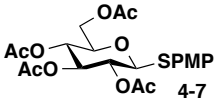
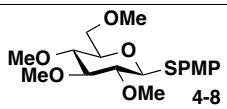
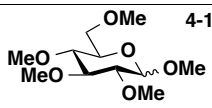
*Reactions were carried in 0.1 M MeCN and 10 equiv. of additive.

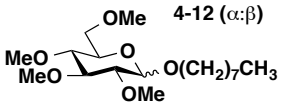
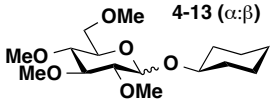
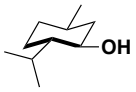
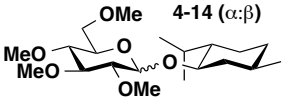
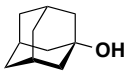
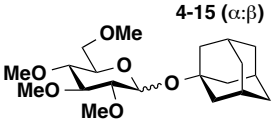
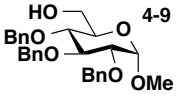
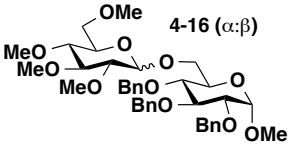
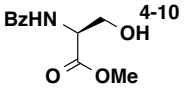
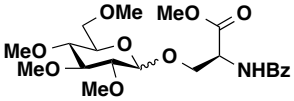
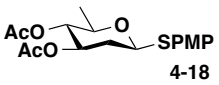
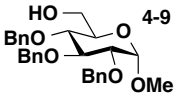
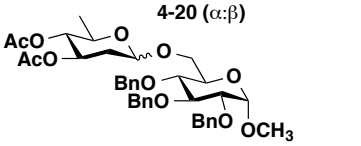
The equivalents of alcohol were reduced from 10.0 equiv. to 2.0 equiv. to make it more amenable for future glycosidic reactions, but the reaction yield decreased substantially (**Table 4.1**, entry 5). Previous work has shown the positive influence of adding a polar, protic

acid to aid in solvation of the charge transfer complex;[19] hence, we utilized 2,2,2-trifluoroethanol (TFE) as an additive and found that although the yield did increase, we would see trapping of the oxocarbenium ion by TFE (**Table 4.1**, entry 7). We next tried the additive 1,1,1,3,3,3,-hexafluoroisopropanol (HFIP) and found that no trapping could be seen by this alcohol and the yield increased to 51% (**Table 4.1**, entry 7). We further explored the use of CBr_4 as co-oxidant (**Table 4.1**, entry 8) and found it to be efficient in promoting the reaction. Notably, the use of substoichiometric amounts of co-oxidant led to similar yields (**Table 4.1**, entries 9-10), suggesting a pathway where the X_3CBr species acts as an initial electron relay compound but it is not needed to propagate the reaction. All subsequent experiments were run with conditions in entries 9 and 10 from **Table 4-1**.

The substrate scope of the reaction was further explored and the use of primary, secondary, and tertiary alcohols as trapping agents were examined as well as different protecting groups on the glycosyl donor.

Table 4.2. Substrate scope and compatibility for glycosylation.

Entry	Donor	Acceptor (R'OH)	Product	% Yield (α:β)
1	 **4-4**	CH_3OH	 **4-5** (α:β)	65% [a] (0.9:1)
2			 **4-6** (α:β)	66% [a] (1:1.5)
3	 **4-7**	CH_3OH	N/A	NR
4	 **4-8**	CH_3OH	 **4-11** (α:β)	72% [a] (1.6:1)

5		$CH_3(CH_2)_7OH$	 4-12 (α:β)	71% [a,c] (0.75:1)
6			 4-13 (α:β)	87% [a] (1.6:1)
7			 4-14 (α:β)	71%[b,d] (1.1:1)
8			 4-15 (α:β)	71% [b,e] (1.6:1)
9		 4-9	 4-16 (α:β)	23% [b] (1.2:1)
10		 4-10	 4-17 (α:β)	61% [b,f] (1.6:1)
11	 4-18	CH_3OH	 4-19 (α:β)	82% [b] (1:4.1)
12		 4-9	 4-20 (α:β)	42% [b] (1:2.9)

Reactions carried out at 0.1M thioglycoside with 5 mol% catalyst and irradiated for 6 h with blue LEDs unless otherwise stated. Yields are reported for isolated products. [a] CBr_4 used. [b] $BrCCl_3$ used. [c] Irradiated for 23 h. [d] 18h. [e] 9h. [f] 10h. NR = No reaction.

Primary, secondary, and tertiary alcohols were all permissive nucleophiles for this glycosylation chemistry and afforded the desired products as a mixture of anomers with high yields. Our initial conditions proved to be amenable towards activating thioglycosides with electron rich *O*-protecting groups and 2-deoxyrhamnal (**Table 4.2**, entries 1, 4, and 11). The use of greasy, alkyl alcohols are allowed (**Table 4.2**, entry 3). The secondary alcohols cyclohexanol and (-)-mentol were also tolerated (**Table 4.2**, entries 2, 6, and 7). The tetra-*O*-acetyl protected thioglycoside **4-7** is not active towards our photocatalyst, presumably due to

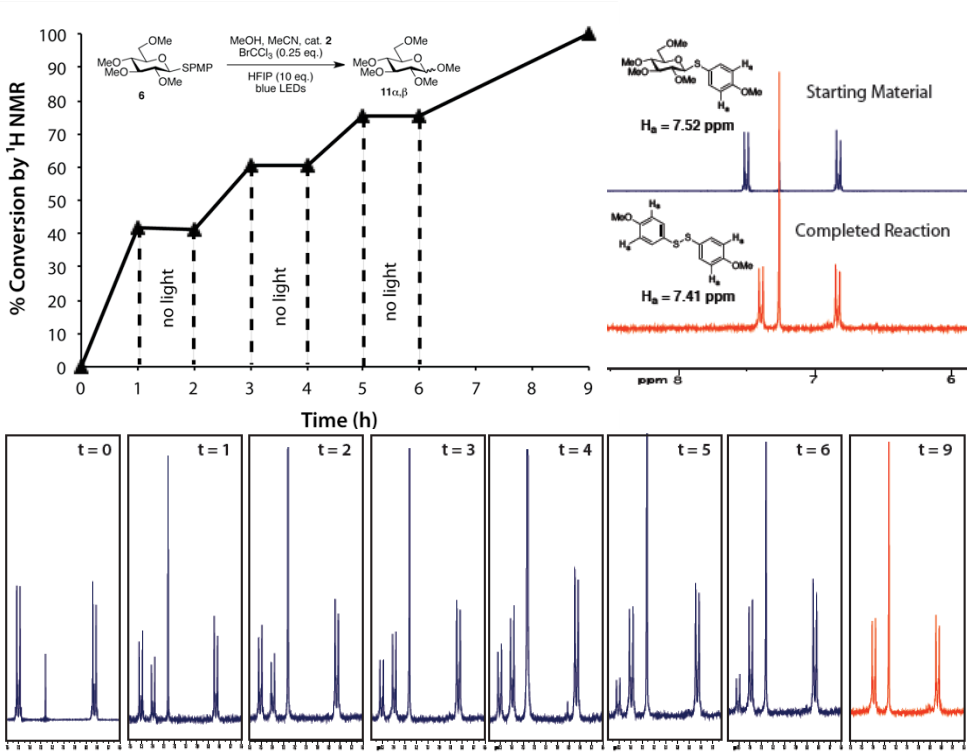
a higher oxidation potential due to the inactivating nature of the protecting group (**Table 4.2**, entry 3). Yet, acetyl groups are in fact tolerated in the redox cycle as exemplified by the use of 2-deoxy-L-rhamnothioglycoside **4-18** (**Table 4.2**, entries 11-12). Most of our studies were done with the more electron rich tetra-*O*-methyl thioglycoside **4-8**, which allowed for conversions to expand into bulky, tertiary alcohols (**Table 4.2**, entry 8), and an enolizable stereocenter without loss of chirality (**Table 4.2**, entry 10). It is noteworthy that glycosyl acceptors bearing aryl protecting group are amenable to this chemistry and 1,6-linkages can be readily accessed (**Table 4.2**, entries 9 and 12).

4.3 Mechanistic Studies

Having established conditions for thioglycoside activation and intermediate trapping, we wanted to investigate the individual components of the reaction by using thioglycoside **4-4** and methanol. When the catalyst is removed from the reaction, there is no glycosylation product; furthermore, when the co-oxidant alkyl halide is removed, the reaction does proceed, albeit at a much slower rate (only ~21% after 4 h of irradiation). The dependence of the reaction towards exposure to blue light was monitored by ^{1}H NMR. The symmetric disulfide byproduct can be recovered quantitatively after the reaction is complete, and it can serve as a measuring point for time-dependent conversion of starting material to product. We were able to visualize the disappearance of the starting SPMP aryl thiol peak at 7.52 ppm and the appearance of a new peak corresponding to the symmetric disulfide at 7.41 ppm. As seen in **Figure 4.5a**, the reaction is completely dependent on blue light as no reaction progression is seen when the blue LEDs are turned off, even after 1 h. Interestingly, the reaction can be readily turned off and on intermittently until full consumption of starting material. Further

NMR studies focused on the role of HFIP probed by ^{19}F NMR to ensure that no HFIP is consumed during the reaction. Trifluorotoluene was utilized as an internal standard to compare and quantify the amount of additive before and after blue light irradiation for 6 h (**Figure 4.5b**); ^{19}F NMR showed that no HFIP is expended during the process, enhancing its role as a stabilizer of the CLMT complex.

a)



b)

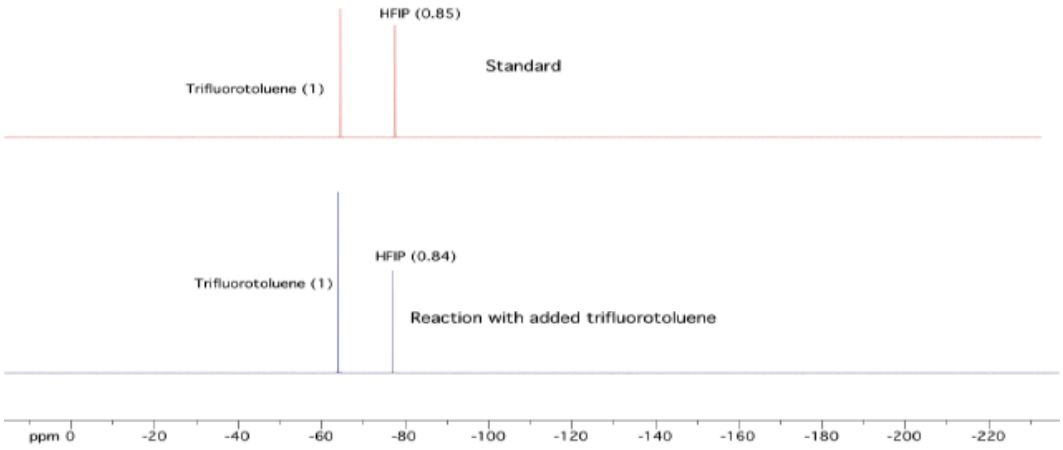


Figure 4.5. Mechanistic studies probed by NMR. a) Monitoring of blue light irradiance on thioglycoside activation by ^{1}H NMR. b) Monitoring of HFIP consumption by ^{19}F NMR with trifluorotoluene as standard.

An intriguing observation of this reaction is the lack of anomeric selectivity seen upon completion. We expected to see a "nitrile effect" where the solvent acetonitrile stabilized the oxocarbenium cation by stabilizing the α-position, thus obtaining β-selective addition. Indeed, pronounced β-selectivity can be seen early but degrades over the course of the reaction (**Figure 4.6**). This isomerization can be attributed to the low pH of the finalized reaction (pH = 4.0) with the expected formation of acidic byproducts from co-oxidant consumption and the 10.0 equiv. of HFIP. Efforts at utilizing non-nucleophilic bases during the reaction such as *N,N*-diisopropylethyl amine, 2,6-di-*tert*-butyl-4-methyl pyridine, and 2,4,6-tri-*tert*-butylpyridine to neutralize acidic byproducts were unsuccessful due to inhibition of reaction progression from the electron rich bases.

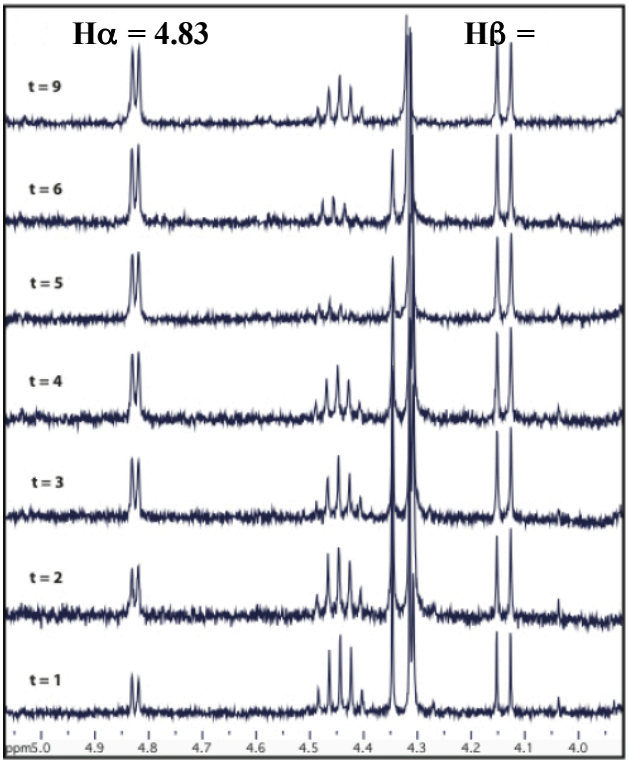


Figure 4.6. Loss of anomeric selectivity during reaction progress. t = time in hours.

Consideration of the possible mechanism suggested that H2 may be produced as a by-product of the reaction. To test this hypothesis, we scaled the reaction 200-fold and attached a H-sensing polymer[20] to the cap of the reaction vessel. A positive test was observed only when the reaction was treated with light (**Figure 4.7**).

Figure 4.7. Hydrogen detection during reaction. A = Untreated polymer. B = Reaction, no light. C = H_2-treated polymer. D = Reaction with light. E = Reaction with light, no catalyst.

4.4 Proposed Mechanism

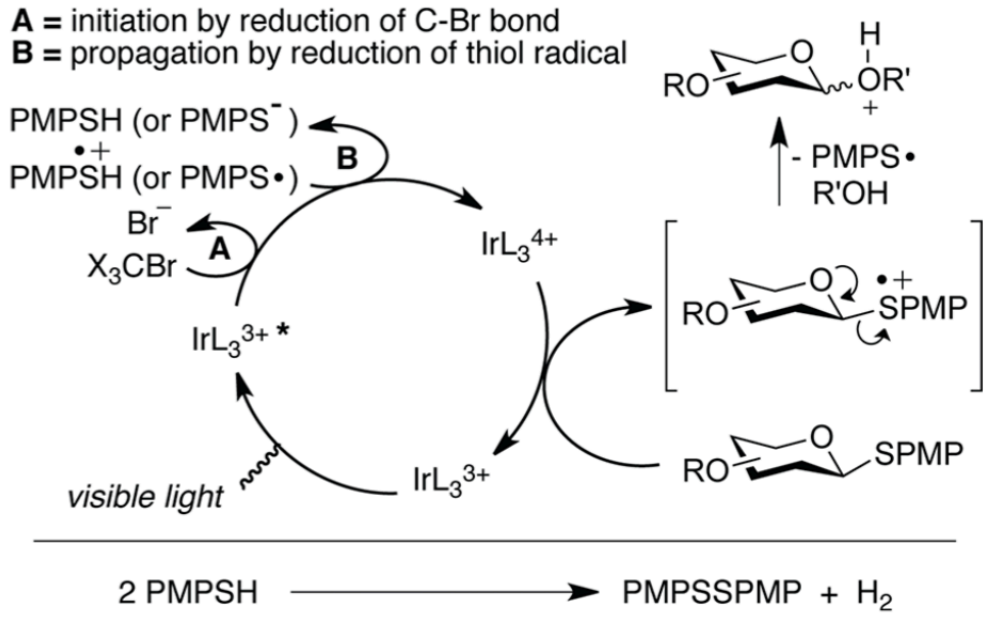


Figure 4.8. Proposed mechanism for *O*-glycosylation of thioglycosides mediated by visible light catalysis.

With the data in hand regarding the mechanistic studies in our SET system activation of thioglycosides, we have proposed the mechanism shown in **Figure 4.8**. The initial step involves excitation with visible light of our Ir-catalyst and transfer of an electron from it to the C-Br bond. The oxidized catalyst can now be oxidatively quenched by the SPMP moiety in the thioglycoside, forming a radical cation. This radical cation can be released from the anomeric position assistance from the oxygen pyran and an oxocarbenium ion is produced, which can be trapped by our alcohol nucleophiles. The key propagation step relies on the through the formation of an SPMP disulfide, that serves as the co-oxidant after the initial X_3CBr has been consumed, through release of hydrogen.

4.5 Summary

We have designed method by which *O*-glycosides can be accessed from thioglycoside donors *via* visible-light catalysis. We found that the catalyst $Ir[dF(CF_3)ppy]_2$ (dtbbpy)PF_6 achieves the condensation of primary, secondary, and tertiary alcohols with *O*-protected *S*-*p*-methoxyphenyl (SPMP) thioglycosides in the presence of a substoichiometric amount of the co-oxidant bromotrichloromethane, the protic co-solvent hexafluoroisopropanol (HFIP), and activation with blue light in acetonitrile. The reaction proceeds *via* the formation of an oxocarbenium ion during a single-electron-transfer (SET) event and subsequent breakdown of the carbon-SPMP bond. We see a preference for making the β-anomer over the α-anomer early in the reaction. The anomeric selectivity can be attributed to the solvent nitrile effect stabilizing the oxocarbenium ions, but the selectivity erodes over the course of the reaction presumably due to the increasing acidity of the reaction from byproducts. Future experiments

will allow testing this methodology in creating linkages between thioglycosides and natural products containing alcohol moieties.

4.6 Experimental

4.6.1 General Methods

Solvents and reagents were purchased from commercial vendors and were used without any further purification, except for 1-adamantanol, which was purified by column chromatography and recrystallization from EtOAC/hexanes and (–)-menthol, which was azeotroped with toluene twice and crystallized under vacuum prior to use. ^{1}H NMR spectra were recorded at 300 MHz, ^{13}C NMR spectra were recorded at 75 MHz, and ^{19}F NMR spectra were recorded at 282 MHz, all using a Bruker ARX300 spectrometer with a QNP probe. ^{13}C NMR spectra for mechanistic studies were recorded at 125 MHz, both using a Bruker Avance 500 (BBO probe). All NMR spectra were recorded in $CDCl_3$. Mass spectral analyses were performed at the Purdue University Campus-Wide Mass Spectrometry Center. ESIMS was performed using a FinniganMAT LCQ Classic mass spectrometer system. GCMS was performed using an Agilent 9575 GC-MS with a db5 column, heating from 40 to 320 ˚C; analysis was performed in electron impact mode. Optical rotations were determined using an Autopol I automatic polarimeter (Rudolph Research Analytical) in a polarimeter cell with a path length of 50 mm. Silica gel flash chromatography was performed using 200-400 mesh (40-75 μm) silica gel. Preparative thin-layer chromatography was performed on Analtech silica gel G1200 uM glass plates. Compounds **4-3**,[21] **4-4**,[21] **4-7**,[21] **4-9**,[22] and **4-10**[23] were prepared according to literature methods. Ir[dF(CF_3)ppy]$_2$(dtbbpy)PF_6 (**4-2**) was prepared by the method of Lowry et al.[24] and was purified by flash column chromatography

prior to use, eluting with a gradient of 10% EtOAc in hexanes to 25% hexanes in EtOAc. Alternatively, the catalyst can be purified by recrystallization from acetone-hexane vapor diffusion. Percent conversion was calculated by ^{1}H NMR using the formula [Integral value for disulfide produced]/[Combined integral values for disulfide and starting S-PMP protons] x 100. Hydrogen sensing was performed using a catalyzed molybdenum tri-oxide pigment (100 μm) embedded in a silicone matrix deposited on a transparent PET substrate (Element One Inc., Boulder, CO).[6-7] Reactions were performed in sealed screw-cap vials where the polymer was affixed to the inside of the cap.

4.6.2 Experimental Procedures

***p*-Methoxyphenyl 2,3,4,6-tetra-*O*-methyl-1-thio-β-D-glucopyranoside (4-8).**[25]

Acetyl thioglycoside **4-7** (1.4 g, 2.97 mmol) was diluted in MeOH (30 mL), and the mixture was cooled to 0 ˚C. NaOMe (0.160 g, 2.97 mmol) was added, and the mixture was warmed to room temperature and stirred for 1 h, after which it was neutralized by the addition of DOWEX 50WX8-100 ion-exchange resin. The mixture was filtered and the filtrate was concentrated to yield an orange semisolid, confirmed as the intermediate tetraol by ^{1}H NMR. The semisolid was diluted in anhydrous DMF (20 mL) and the mixture was cooled to 0 ˚C. NaH (0.713 g, 17.82 mmol, 60% dispersion) was added in portions, and the mixture was stirred for 15 min before methyl iodide (2.11 g, 14.8 mmol) was added. The mixture was allowed to warm to room temperature and was stirred for 18 h before it was quenched by the addition of 5 mL MeOH. Concentration afforded a residue that was partitioned between EtOAc and H_2O (100 mL of each). The organic layer was separated and the aqueous layer was extracted with EtOAc (2 x 50 mL). The combined organic layers were washed with brine

(50 mL), dried over anhydrous sodium sulfate, and concentrated. The residue was purified by flash column chromatography (SiO_2), eluting with 3:1 hexanes: EtOAc to yield the title compound as a clear syrup (1.02 g, 96%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ ^{1}H NMR (300 MHz; $CDCl_3$): δ 7.53-7.48 (m, 2 H), 6.86-6.81 (m, 2 H), 4.33 (d, *J* = 9.8 Hz, 1 H), 3.80 (s, 3H), 3.64 (s, 3H), 3.60 (d, *J* = 2.1 Hz, 1 H), 3.57 (d, *J* = 4.3 Hz, 1H), 3.52 (s, 3 H), 3.39 (s, 3H), 3.26-3.09 (m, 3H), 2.98 (dd, *J* = 9.7, 8.4 Hz, 1H); ^{13}C NMR δ 159.6, 135.1, 123.6, 114.2, 88.6, 88.0, 82.4, 79.3, 78.7, 71.3, 60.9, 60.7, 60.4, 59.3, 55.2; $[\alpha]^{26.7}_D$ (*c* 0.83, $CHCl_3$): -24 °. ESIMS *m/z* (rel. intensity) 381 (MNa^+, 97/100).

***p*-Methoxyphenyl 3,4-*O*-acetyl-2,6-dideoxythio-β-L-arabino-hexopyranoside (4-18).**[26] L-Rhamnal (1.5 g, 7.00 mmol) was diluted in anhydrous toluene (12 mL) and cooled to 0 °C. A solution of dry gaseous HCl was bubbled through the mixture for 30 min, and the mixture was stirred an additional 30 min at 0 °C. The mixture was purged with dry nitrogen for 45 min and then concentrated. The resultant white solid was dissolved in anhydrous toluene (12 mL) and 4-methoxythiophenol (1.46 g, 10.5 mmol) was added, followed by DIEA (1.35 g, 10.5 mmol). The mixture was stirred at room temperature for 22 h and then diluted with CH_2Cl_2, washed with H_2O, dried over anhydrous sodium sulfate, and concentrated. The residue was purified by flash column chromatography, eluting with 4:1 hexanes: EtOAc to yield the title compound as a colorless semisolid (1.4 g, 56%), contaminated with trace amounts of the α-anomer.

General Procedure for Visible Light-Mediated Glycosylation. Thioglycoside **4-3**, **4-4**, **4-8**, **4-7**, or **4-18** (0.050 g-0.080 g, 0.0754-0.223 mmol), or a mixture of thioglycoside and

glycosyl acceptors (sugar alcohol **4-9,** 1-adamantanol, or (–)-menthol 0.150-0.446 mmol, 1.5-2 eq.) was diluted with anhydrous acetonitrile (1-2 mL). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, 0.754-2.23 mmol), $Ir[dF(CF_3)ppy]_2(dtbbpy)PF_6$ (5 mol%) and either CBr_4 (0.25 mol% to stoichiometric, for preparing compounds **4-5α,β, 4-6α,β, 4-11α,β** and **4-13α,β**) or $CBrCl_3$ (0.25 mol%, for examples **4-12α,β, 4-14α,β, 4-15α,β, 4-16α,β, 4-17α,β**, and **4-20α,β,**) were added. To those reaction mixtures containing only thioglycoside, the glycosyl acceptor (methanol, *n*-octanol, or cyclohexanol, 0.151-0.446 mol, preparation of compounds **4-5α,β, 4-6α,β, 4-11 - 4-13α,β**) was added. The mixture was placed under an argon atmosphere, sealed securely with a rubber septum, and with stirring at room temperature, was irradiated using blue LEDs (~452 nm) for 6 h (9 h for adamantanol, 18 h for menthol, 10 h for the amide **4-10** and 23 h for *n*-octanol). Reactions were judged as complete by ^{1}H NMR. The crude mixtures were concentrated and the resulting residues were purified as described under the headings for individual compounds.

Methyl 2,3,4,6-tetra-*O*-benzyl-D-glucopyranoside (4-5α and **4-5β).** Using thioglycoside **4-4** (0.050 g, 0.0754 mmol) and MeOH (0.151 mmol), the general procedure afforded the title compound after flash column chromatography (10:1 hexanes:EtOAc- 6:1 hexanes:EtOAc), as a mixture of the α-anomer **4-5α** (yellow syrup, 0.013 g) and β-anomer **4-5β** (yellow syrup, 0.014 g). A total of 0.027 g (65%) of products (α:β ratio: 0.9:1) was obtained. The ^{1}H NMR data for **4-5α** and **4-5β** are in good agreement with the literature values.[27]

Cyclohexyl 2,3,4,6-Tetra-*O*-benzyl-D-glucopyranoside (4-6α and **4-6β).** Using thioglycoside **4-4** (0.080 g, 0.1206 mmol) and cyclohexanol (0.2413 mmol), the general

procedure afforded the title compound after flash column chromatography (15:1 hexanes:EtOAc- 10:1 hexanes:EtOAc), as a mixture of inseparable anomers (colorless syrup). A total of 0.050 g (66%) of products (α:β ratio: 1:1.5, estimated by ^{1}H NMR) was obtained. The ^{1}H NMR data for **4-6α** and **4-6β** are in good agreement with the literature values.[28]

Methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside (4-11α and **4-11β).** Using thioglycoside **4-8** (0.080 g, 0.223 mmol) and MeOH (0.446 mmol), the general procedure afforded the title compound after purification by flash column chromatography (8:1 hexanes:EtOAc-3:1 hexanes:EtOAc) as a mixture of the β-anomer **4-11β** (yellow syrup, 0.015 g) and α-anomer **4-11α** (yellow syrup, 0.025 g). A total of 0.040 g (72%) of products (α:β ratio: 1.6:1) was obtained. The ^{1}H NMR data for **4-11α** are in good agreement with the literature values;[29] **4-11β** is also known.[30] ^{1}H NMR (300 MHz; $CDCl_3$ **11β**): δ 4.13 (d, *J* = 7.7 Hz, 1H), 3.65-3.60 (m, 1 H), 3.61 (s, 4 H), 3.57-3.53 (m, 1 H), 3.56 (s, 4 H), 3.52 (s, 3 H), 3.51 (s, 3 H), 3.40 (s, 3 H), 3.29-3.24 (m, 1H), 3.16-3.13 (m, 2 H), 3.00-2.94 (m, 1H).

***n*-Octyl 2,3,4,6-Tetra-*O*-methyl-D-glucopyranoside (4-12α** and **4-12β).**[31] Using thioglycoside **4-8** (0.080 g, 0.223 mmol) and *n*-octanol (0.446 mmol), the general procedure afforded the title compound after purification by flash column chromatography (10:1 hexanes:EtOAc-5:1 hexanes:EtOAc) as a mixture of the **4-12β** anomer (yellow syrup, 0.034 g) and **4-12α** anomer (yellow syrup, 0.025 g). A total of 0.059 g (77%) of products (α:β ratio: 0.75:1) was obtained. ^{1}H NMR (300 MHz; $CDCl_3$, **4-12α**): δ 4.91 (d, *J* = 3.6 Hz, 1 H), 3.64-3.41 (m, 5 H), 3.62 (s, 3 H), 3.53 (s, 3 H), 3.47 (s, 3 H), 3. 40 (s, 3 H), 3.21-3.15 (m, 2

H), 1.66-1.57 (m, 2 H), 1.34-125 (m, 10 H), 0.86 (t, J = 6.8 Hz, 3 H). ^{1}H NMR (300 MHz; $CDCl_3$, **4-12β**): δ 4.19 (d, J = 7.7 Hz, 1 H), 3.88 (dt, J = 9.4, 6.5 Hz, 1 H), 3.60 (s, 3 H), 3.60-3.51 (m, 2 H), 3.56 (s, 3 H), 3.51 (s, 3 H), 3.44 (dt, J = 9.4, 6.9 Hz, 1 H), 3.38 (s, 3H), 3.27-3.22 (m, 1 H), 3.17-3.07 (m, 2 H), 2.99-2.94 (m, 1 H), 1.62-1.54 (m, 2 H), 1.38-1.21 (m, 10 H), 0.86 (t, J = 6.7 Hz, 3 H).

Cyclohexyl 2,3,4,6-Tetra-*O*-methyl-D-glucopyranoside (4-13α and **4-13β).** Using thioglycoside **4-8** (0.080 g, 0.223 mmol) and cyclohexanol (0.446 mmol), the general procedure afforded the title compound after purification by flash column chromatography (10:1 hexanes:EtOAc-7:1 hexanes:EtOAc) as a mixture of the β-anomer **4-13β** (yellow syrup, 0.024 g) and α-anomer **4-13α** (yellow syrup, 0.038 g). A total of 0.052 g (87%) of products (α:β ratio: 1.6:1) was obtained. The ^{1}H NMR data for **13α** and **13β** are in good agreement with the literature values. [32]

(–)-Menthyl 2,3,4,6-Tetra-*O*-methyl-D-glucopyranoside (4-14α and **4-14β).** Using thioglycoside **4-8** (0.080 g, 0.223 mmol) and (–)-menthol (0.446 mmol), the general procedure afforded the title compound after purification by flash column chromatography (11:1 hexanes:EtOAc - 6:1 hexanes:EtOAc) as a mixture of the β-anomer **4-14β** (yellow syrup, 0.028 g) and α-anomer **4-14α** (yellow syrup, 0.032 g). A total of 0.060 g (71%) of products (α:β ratio: 1.1:1) was obtained. ^{1}H NMR (300 MHz; $CDCl_3$, **4-14α**) δ 5.00 (d, J = 3.7 Hz, 1 H), 3.76 (ddd, J = 10.1, 3.4, 2.3 Hz, 1 H), 3.61 (s, 3 H), 3.52 (s, 3 H), 3.57-3.45 (m, 3 H), 3.42 (s, 3 H), 3.39 (s, 3 H), 3.31 (td, J = 10.6, 4.4 Hz, 1 H), 3.22-3.18 (m, 1H), 3.12 (dd, J = 9.7, 3.7 Hz, 1 H), 2.40-2.30 (m, 1 H), 2.09 (br dtd, J = 9.5, 4.9, 2.5 Hz, 1H), 1.61 (br

dt, *J* = 12.9, 3.1 Hz, 2 H), 1.42-1.27 (m, 2 H), 1.06-0.84 (m, 9 H), 0.76 (d, *J* = 6.9 Hz, 3 H); ^{13}C NMR (75 MHz; $CDCl_3$) δ 97.9, 82.8, 82.2, 81.0, 79.5, 71.0, 69.8, 60.5, 60.3, 59.1, 58.5, 48.6, 42.9, 34.2, 31.7, 24.6, 22.9, 22.2, 21.1, 16.0; $[\alpha]^{21.6}_D$ (*c* 1.32, $CHCl_3$): 50.4°; ESIMS *m/z* (rel. intensity) 771 ($2M+Na^+$, 100), 397 (MNa^+, 52). ^{1}H NMR (300 MHz; $CDCl_3$, **4-14β**): δ 4.27 (d, *J* = 7.8 Hz, 1H), 3.61 (s, 3 H), 3.57-2.55 (m, 2 H), 3.56 (s, 3 H), 3.52 (s, 3 H), 3.44-3.36 (m, 1 H), 3.38 (s, 3 H), 3.24-3.09 (m, 3 H), 2.94 (td, *J* = 7.3, 1.9 Hz, 1 H), 2.34-2.24 (m, 1 H), 2.10-2.03 (m, 1 H), 1.62 (dt, *J* = 9.6, 3.1 Hz, 2 H), 1.38-1.16 (m, 3 H), 0.99-0.86 (m, 8 H), 0.76 (d, *J* = 6.9 Hz, 3 H); ^{13}C NMR (75 MHz; $CDCl_3$) δ 100.9, 86.6, 83.6, 79.4, 78.0, 74.6, 71.5, 60.7, 60.45, 60.34, 59.4, 47.9, 40.7, 34.4, 31.5, 25.0, 23.0, 22.2, 21.1, 15.5; $[\alpha]^{21}_D$ (*c* 1.16, $CHCl_3$): -46.5°; ESIMS *m/z* (rel. intensity) 397 (MNa^+, 100).

1′-Adamantyl 2,3,4,6-Tetra-*O*-methyl-D-glucopyranoside (4-15α and **4-15β).** Using thioglycoside **4-8** (0.070 g, 0.223 mmol) and 1-adamantanol (0.446 mmol), the general procedure afforded the title compound after purification by flash column chromatography (10:1 hexanes:EtOAc-3:1 hexanes:EtOAc) as the α-anomer **4-15α** (yellow syrup, 0.033 g) and the β-anomer **4-15β.** Compound **4-15β** co-eluted with unreacted adamantanol, which was removed by trituration of the obtained crude solid with cold hexanes (4 x 1 mL). Concentration of the filtrate afforded pure **4-15β** (yellow syrup, 0.021 g). A third fraction (0.005 g) containing a 1.4:1 mixture of **4-15β**:**4-15α** was also collected; a total of 0.059 g (71%) of products (overall α:β ratio: 1.6:1) was obtained. ^{1}H NMR (300 MHz; $CDCl_3$, **4-15α**): δ 5.31 (d, *J* = 3.7 Hz, 1 H), 3.80 (dt, *J* = 10.1, 2.7 Hz, 1 H), 3.61 (s, 3 H), 3.57-3.55 (m, 1H), 3.53 (s, 3 H), 3.52-3.47 (m, 2 H), 3.45 (s, 3 H), 3.39 (s, 3 H), 3.22 (dd, *J* = 10.0, 8.9 Hz, 1 H), 3.14 (dd, *J* = 9.7, 3.7 Hz, 1 H), 2.12 (br s, 3 H), 1.86-1.76 (m, 6 H), 1.65-1.56 (m, 6 H).

^{13}C NMR (75 MHz; $CDCl_3$) δ 89.1, 83.0, 81.7, 79.6, 74.3, 80.0, 69.3, 60.7, 60.3, 59.1, 58.2, 42.3, 36.2, 30.6; $[\alpha]^{24.7}_D$ (*c* 1.15, $CHCl_3$): 97°; ESIMS *m/z* (rel. intensity) 393 (MNa^+, 100); ^{1}H NMR (300 MHz; $CDCl_3$, **4-15β**): δ 4.49 (d, *J* = 7.8 Hz, 1 H), 3.62 (s, 3 H), 3.60-3.50 (m, 2 H), 3.57 (s, 3 H), 3.51 (s, 3 H), 3.38 (s, 3 H), 3.27-3.21 (m, 1 H), 3.16-3.03 (m, 2 H), 2.95 (t, *J* = 8.3 Hz, 1H), 2.14 (s, 3 H), 1.81 (br q, *J* = 16.3 Hz, 7 H), 1.61 (br s, 6 H). ^{13}C NMR δ 95.8, 86.6, 83.8, 79.6, 74.9, 74.3, 71.7, 60.7, 60.5, 60.3, 59.3, 42.5, 36.2, 30.6; $[\alpha]^{25}_D$ (*c* 1.08, $CHCl_3$): 3.7°; ESIMS *m/z* (rel. intensity) 393 (MNa^+, 100).

Methyl-2,3,4-tri-*O*-benzyl-6-*O*-(2,3,4,6-Tetra-*O*-methyl-D-glucopyranosyl)-D-glucopyranoside (4-16α and 4-16β). Using thioglycoside **6** (0.080 g, 0.223 mmol) and methyl glycoside **4-9** (0.130 g, 0.446 mmol), the general procedure afforded (**4-16α** and **4-16β**) after purification by chromatotron (2:1 hexanes:EtOAc-1:1 hexanes:EtOAc). 0.022 g of **4-16α** eluted first, followed by 0.018 g **4-16β** (23% yield, 1.2:1 anomeric ratio). Spectral data closely matched previously reported values.[33]

***O*-Methyl-(*N*-benzenecarboxamido)-L-serinyl 2,3,4,6-Tetra-*O*-methyl-D-glucopyranoside (4-17α and 4-17β).** Using thioglycoside **6** (0.070 g, 0.223 mmol) and serine derivative **4-10** (0.334 mmol), the general procedure afforded the title compound after after purification by flash column chromatography (3:1 hexanes:EtOAc-1.5:1 EtOAc:Hexanes) as the α-anomer **4-17α** (yellow syrup, 0.037 g) and the β-anomer **4-17β**. Compound **4-17β** was further purified by preparative TLC (2% MeOH in CH_2Cl_2) and was obtained as a pale yellow syrup (0.023 g). A total of 0.060 g (61%) of products (overall α:β ratio: 1.6:1). ^{1}H NMR (300 MHz; $CDCl_3$, **4-17α**) δ 7.88-7.85 (m, 2 H), 7.66 (br d, *J* = 8.3

Hz, 1 H), 7.53-7.40 (m, 3 H), 4.96 (dt, *J* = 8.3, 3.2 Hz, 1 H), 4.90 (d, *J* = 3.8 Hz, 1 H), 4.34 (dd, *J* = 11.5, 3.4 Hz, 1 H), 3.95 (dd, *J* = 11.5, 3.1 Hz, 1 H), 3.76 (s, 3 H), 3.61-3.59 (m, 1 H) 3.59 (s, 3 H), 3.52-3.49 (m, 2 H), 3.49 (s, 3 H), 3.44 (s, 3 H), 3.38-3.29 (m, 1 H), 3.22-3.15 (m, 2 H). ^{13}C NMR (75 MHz; $CDCl_3$) δ 170.6, 167.1, 133.6, 131.7, 128.5, 127.3, 98.7, 83.0, 81.4, 79.0, 70.9, 70.7, 70.6, 60.7, 60.4, 59.0, 58.7, 53.1, 52.6; $[\alpha]^{22.1}_D$ (*c* 1.54, $CHCl_3$): 108°; ESIMS *m/z* (rel. intensity) 464 (MNa^+, 100). A total of 0.060 g (61%) of products (overall α:β ratio: 1.6:1). ^{1}H NMR (300 MHz; $CDCl_3$, **4-17β**) δ (m, 2 H), 7.55-7.41 (m, 3 H), 7.35 (br d, *J* = 8.2 Hz, 1 H), 4.96 (dt, *J* = 8.1, 3.1 Hz, 1H), 4.47 (dd, *J* = 11.0, 2.8 Hz, 1 H), 4.25 (d, *J* = 7.8 Hz, 1 H), 3.92 (dd, *J* = 11.1, 3.5 Hz, 1H), 3.77 (s, 3 H), 3.62-3.60 (m, 3 H), 3.56 (s, 3 H), 3.57-4.54 (m, 2 H), 3.51 (s, 3 H), 3.37 (s, 3 H), 3.20-3.11 (m, 3 H), 3.01-2.96 (m, 1 H). ^{13}C NMR (75 MHz; $CDCl_3$): δ 170.4, 167.0, 133.6, 131.8, 128.5, 127.2, 104.3, 86.5, 83.5, 79.0, 74.6, 70.8, 70.5, 60.9, 60.7, 60.4, 59.2, 53.2, 52.6; $[\alpha]^{25.3}_D$ (*c* 0.97, $CHCl_3$): 25.2°; ESIMS *m/z* (rel. intensity) 442 (MH^+, 100).

Methyl 3,4-di-O-acetyl-2,6-dideoxy-L-arabino-hexopyranoside (4-19α and 4-19β). Using thioglycoside **4-18** (0.081 g, 0.229 mmol) and methanol (0.460 mmol), the general procedure afforded methyl glycosides **4-19α** and **4-19β** after flash column chromatography (10:1 hexanes:EtOAc). **4-19α** (0.009 g, 16% yield) eluted first, followed by **4-19β** (0.037 g, 66% yield). The ^{1}H and ^{13}C NMR data for **4-19α** and **4-19β** are in good agreement with the literature values.[34]

Methyl-2,3,4-tri-*O*-benzyl-6-*O*-(3,4-di-O-acetyl-2,6-dideoxy-L-arabino-hexopyranosyl)-D-glucopyranoside (4-20α and **4-20β).** Thioglycoside **8** (0.080 g, 0.226 mmol) was

coupled to acceptor **9** (0.19 mmol) by the general procedure to yield **4-20α** and **4-20β** after chromatoton (eluting in 4:1 hexanes:EtOAc). 0.016 g **4-20α** (11% yield) eluted first, followed by 0.048g **4-20β** (31% yield). Spectral data for **4-20α** matched literature reports.[35] A total of 0.060 g (61%) of products (overall α:β ratio: 1.6:1). ^{1}H NMR (300 MHz; $CDCl_3$, **4-20β**) δ 7.25-7.42 (m, 15H), 5.21-5.30 (m, 1H), 5.00 (d, *J* = 10.8 Hz, 1H), 4.90 (d, *J* = 11.1 Hz, 1H), 4.81 (d, *J* = 11.4 Hz, 2H), 4.66-4.75 (m, 3H), 4.58 (d, *J* = 3.6 Hz, 1H), 4.54 (d, *J* = 11.1 Hz, 1H), 4.00 (t, *J* = 9.3 Hz, 1H), 3.74-3.87 (m, 3H), 3.55 (dd, *J* = 3.6, 9.6 Hz, 1H), 3.43-3.49 (m, 2H), 3.38 (s, 3H), 2.14-2.21 (m, 1H), 2.05 (s, 3H), 2.00 (s, 3H), 1.73 (m, 1H), 1.12 (d, *J* = 6.3 Hz, 3H). ^{13}C NMR (75 MHz; $CDCl_3$): δ 170.2, 138.6, 138.1, 128.4, 128.3, 128.1, 128.0, 127.9, 127.8, 127.6, 97.9, 97.0, 82.1, 80.1, 77.7, 77.2, 75.8, 75.0, 74.7, 73.4, 69.9, 68.9, 66.2, 65.5, 55.1, 35.2, 21.0, 20.9, 17.4 $[\alpha]^{25}_{D}$ (*c* 1., $CHCl_3$): -37; ESI-MS *m/z* (rel. intensity) 680 (MH^+, 100).

^{19}F NMR Mechanistic Studies. The reaction was performed using thioglycoside **4-4** (0.030 g, 0.045 mmol), 9.6 μL $CBrCl_3$ (2 eq.), $Ir[dF(CF_3)ppy]_2(dtbbpy)PF_6$ (5 mol%) and 51 μL HFIP (0.484 mmol, 10.7 eq.). Water (1.8 μL, 2 eq.) was the nucleophile employed. The reaction was conducted in CD_3CN (0.6 mL), and after 6 h of irradiation, trifluorotoluene (119 μL, 0.969 mmol) was added to the complete reaction. ^{19}F NMR spectra were obtained (at 282 MHz) and compared to a standard consisting of HFIP (51 μL, 0.484 mmol) and trifluorotoluene (119 μL, 0.969 mmol) in 0.6 mL of CD_3CN. The ratio of signals from trifluorotoluene to HFIP was 1:0.85 in the standard, and 1:0.84 in the completed reaction.

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CHAPTER V

CONCLUSION

We have developed chemoenzymatic strategies to probe for the function of enzymes involved in the production of the antibiotic thiocillin and the hybrid antibiotic thiomarinol, with specific focus on the dithiolopyrrolone antibiotic component for the latter. We have utilized chemical syntheses to construct natural product variants to test for substrate promiscuity and mechanism of action. We have further developed a mild *O*-glycosylation strategy from thioglycosides that will allow us to construct more structural variants of natural products.

In the present work, we have utilized the enzyme TclM from the thiocillin biosynthetic pathway to show that it catalyzes a formal [4+2] cycloaddition between two dehydroalanines in a linear precursor peptide analogue, prepared by native chemical ligation of the leader peptide thioester to a N-terminal cysteine core, of its natural substrate. Further studies showed a seven amino acid minimal leader peptide to induce the cyclization. A solid-phase peptide synthesis method was developed to construct thiopeptide variants. With this strategy, we were able to show that TclM cyclized variants where the ring core was both expanded and mutated, and where the C-terminus was expanded as well. We extended our studies to the TclM homologue TbtD from the thiomuracin GZ biosynthetic pathway. We found that a C-terminal dehydroalanine or thiazole at the +1 position of the 4π partner is

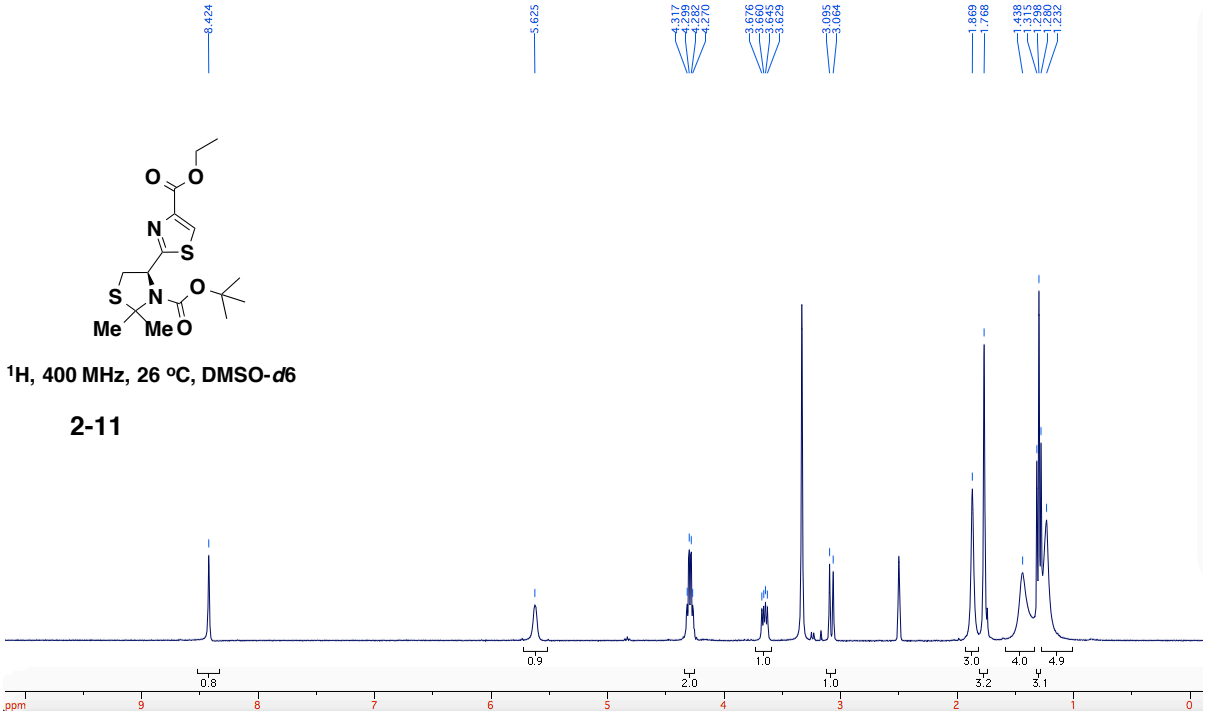
essential for cyclization to occur and that ring contractions is not permitted when a tyrosine is removed from the macrocycle.

We have also showed that the thiomarinol analogue pseudomonyl C holothinamide can be constructed with the enzymes TmlU and HolE from the thiomarinol biosynthetic pathway. Additional studies on the dithiolopyrrolone constituent showed promiscuity towards different acyl groups in the exocyclic amide while maintaining activity against *N. gonorrhoeae*. The oxidized analogue dioxoholomycin can readily oxidize thiols into disulfides in small molecules and in proteins, providing a putative mechanism of action for the dithiolopyrrolone family of antibiotics.

Furthermore, we have developed a technique in which we utilize visible light catalysis and an Ir (III) catalyst to activate thioglycosides and achieve *O*-glycosylation. We showed that 1^{o}, 2^{o}, and 3^{o} alcohols readily trap the activated oxocarbenium ion. We envision utilizing this coupling strategy in the linkage of carbohydrate moieties in alcohol-containing natural products to aid in solubility and in targeted drug delivery.

The advent of next-generation DNA sequencing has yielded a broad pool of biosynthetic gene clusters from bacterial whole genome sequencing from which new antibiotics can be discovered. Natural products have evolved for many years by the producing organisms to be enticing to their bacterial targets; thus, strategies to harness the biosynthetic pathways involved in producing the molecules is of high importance. Likewise, finding avenues for natural product modification by organic syntheses can enhance the understanding of the reactivities associated with these molecules and provide a strategy by which variants can be constructed with better physical properties to combat antibiotic resistance.

APPENDIX A. SPECTRA FOR CHAPTER 2



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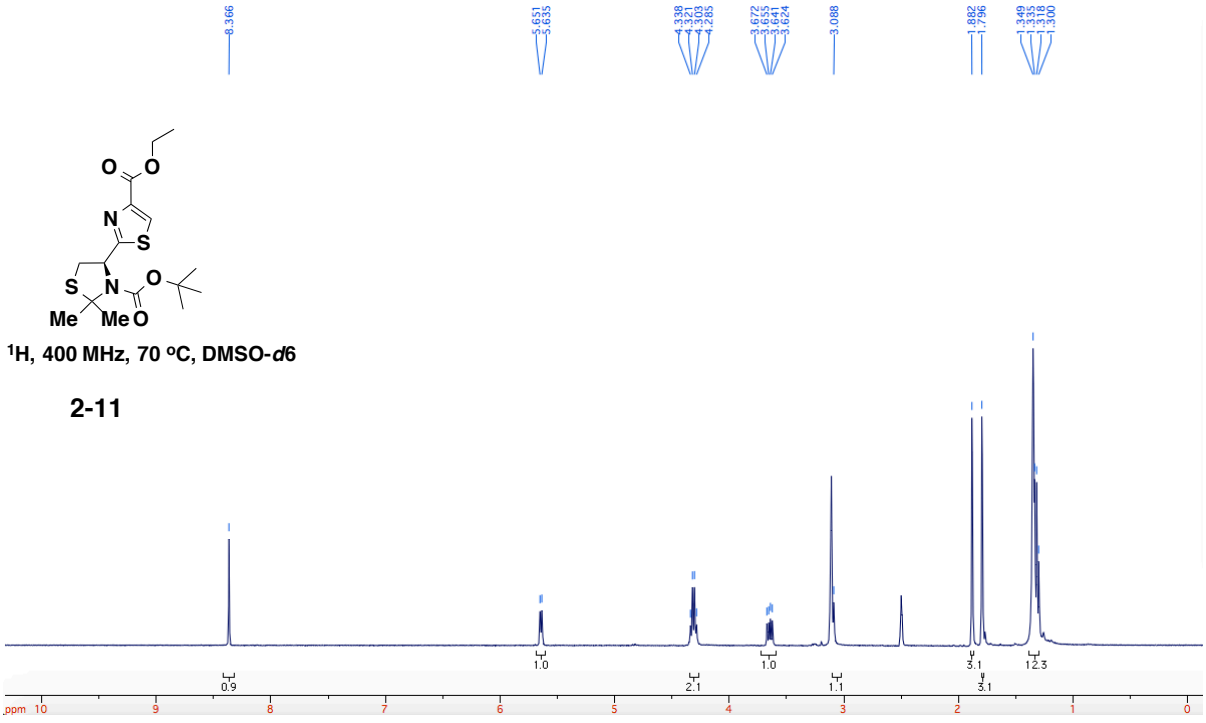
2-11



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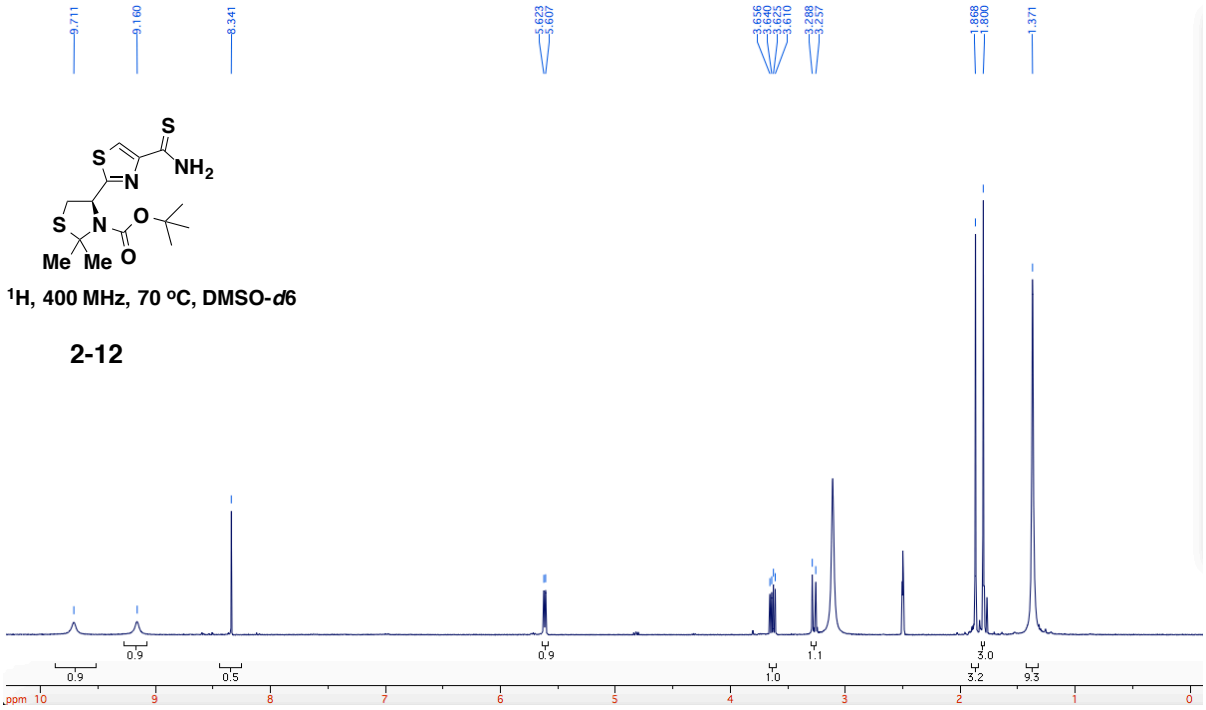
2-11



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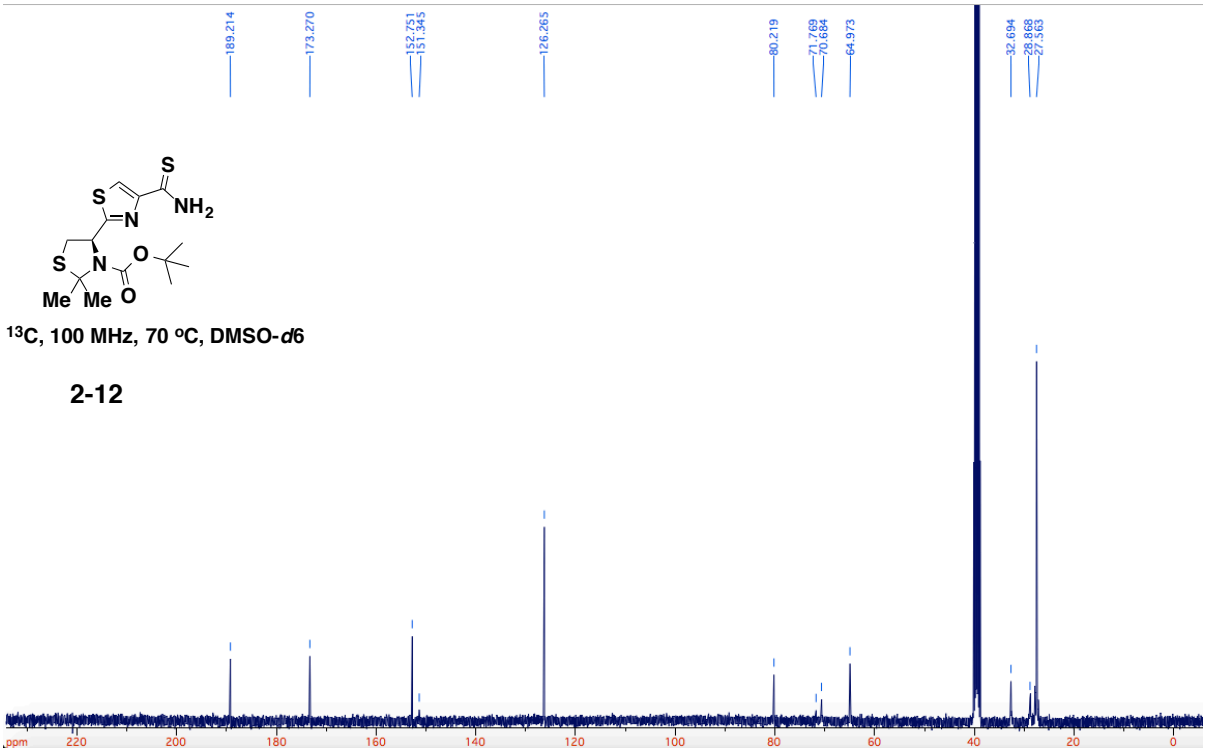
2-12



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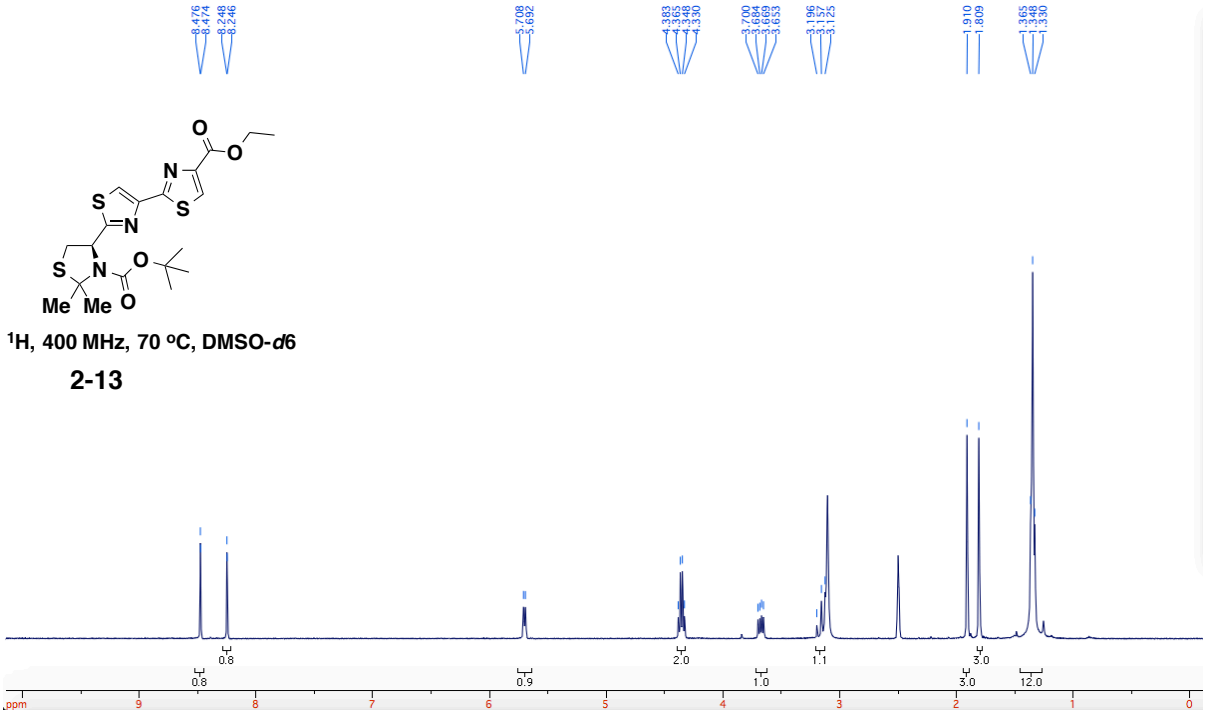
2-12



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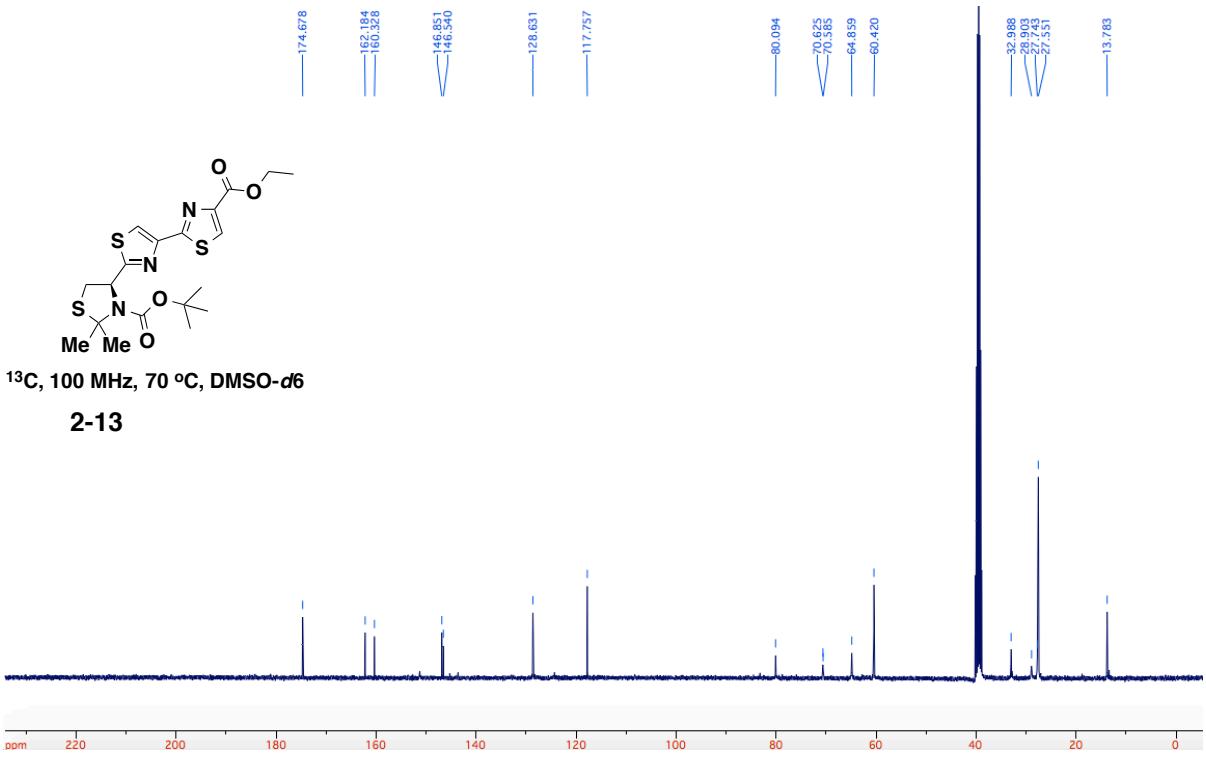
2-13



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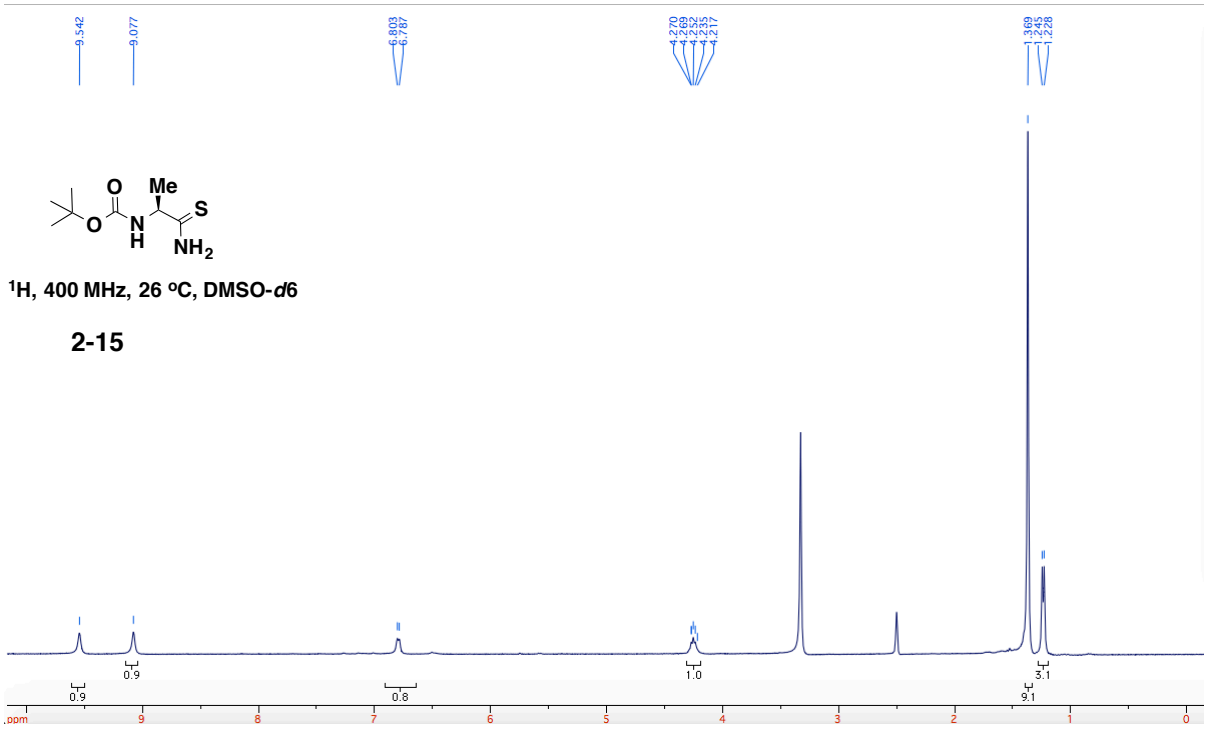
2-13



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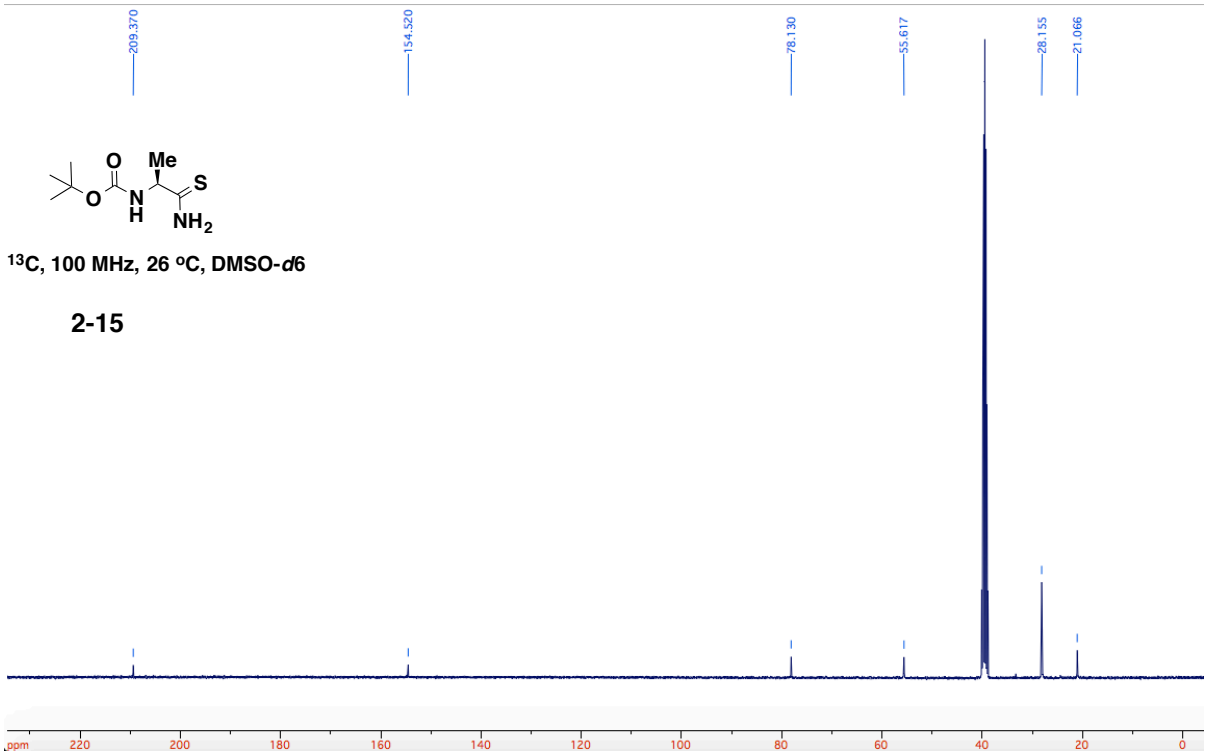
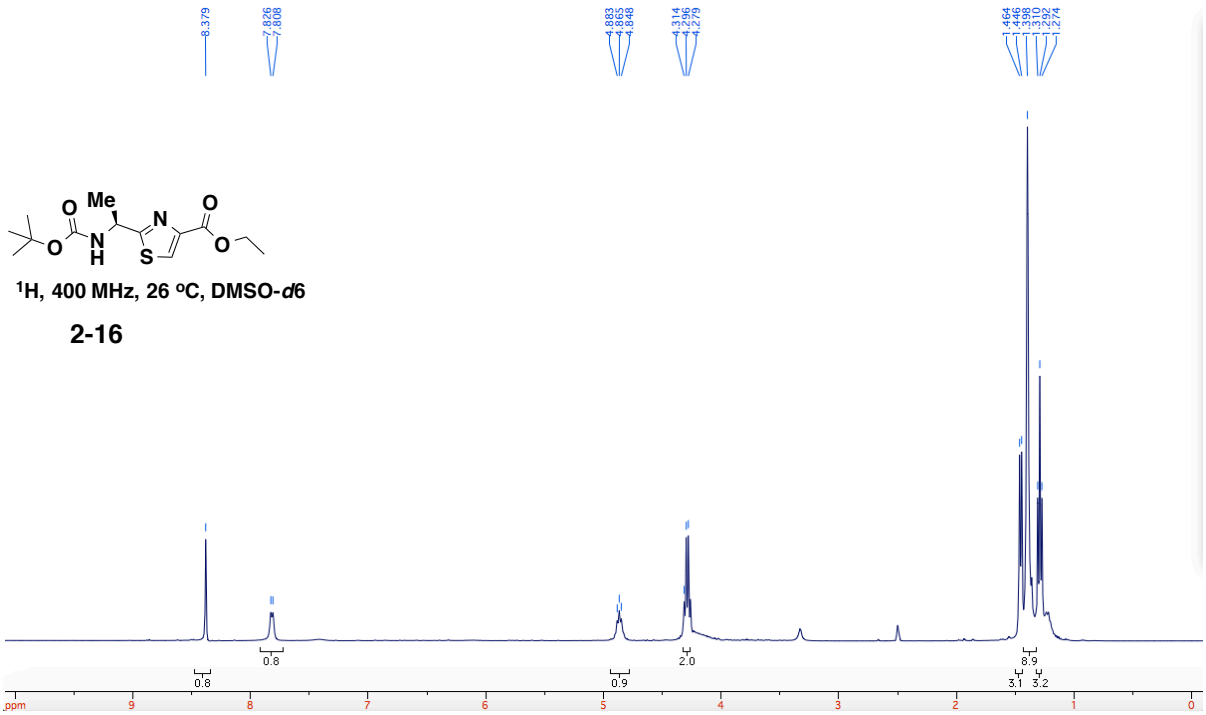
2-15



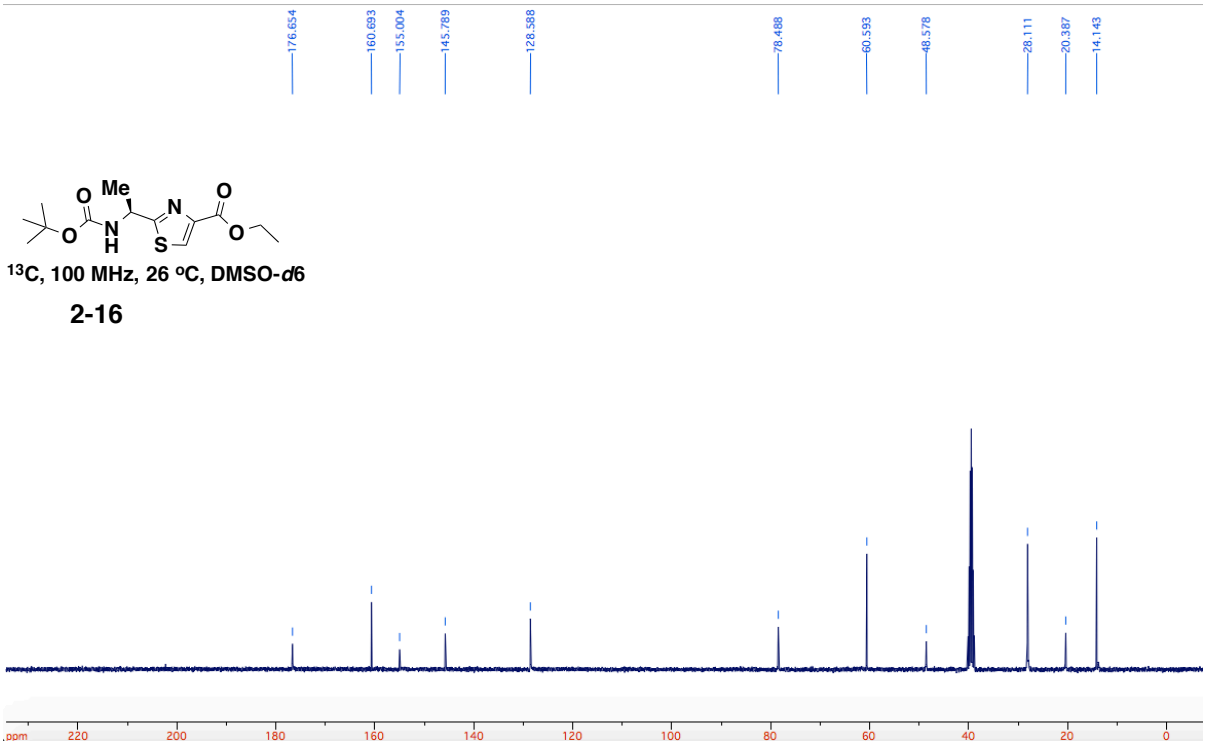
^{13}C, 100 MHz, 26 ^{o}C, DMSO-*d*6

2-15



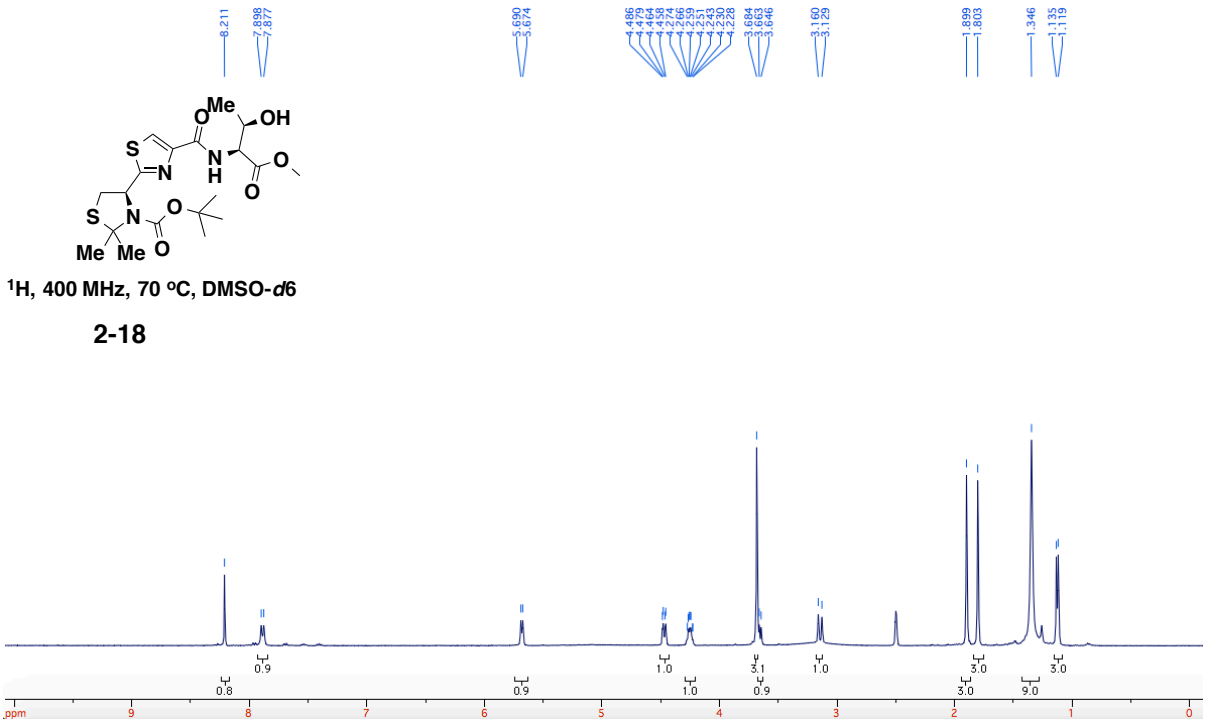
^{1}H, 400 MHz, 26 ^{o}C, DMSO-*d*6

2-16



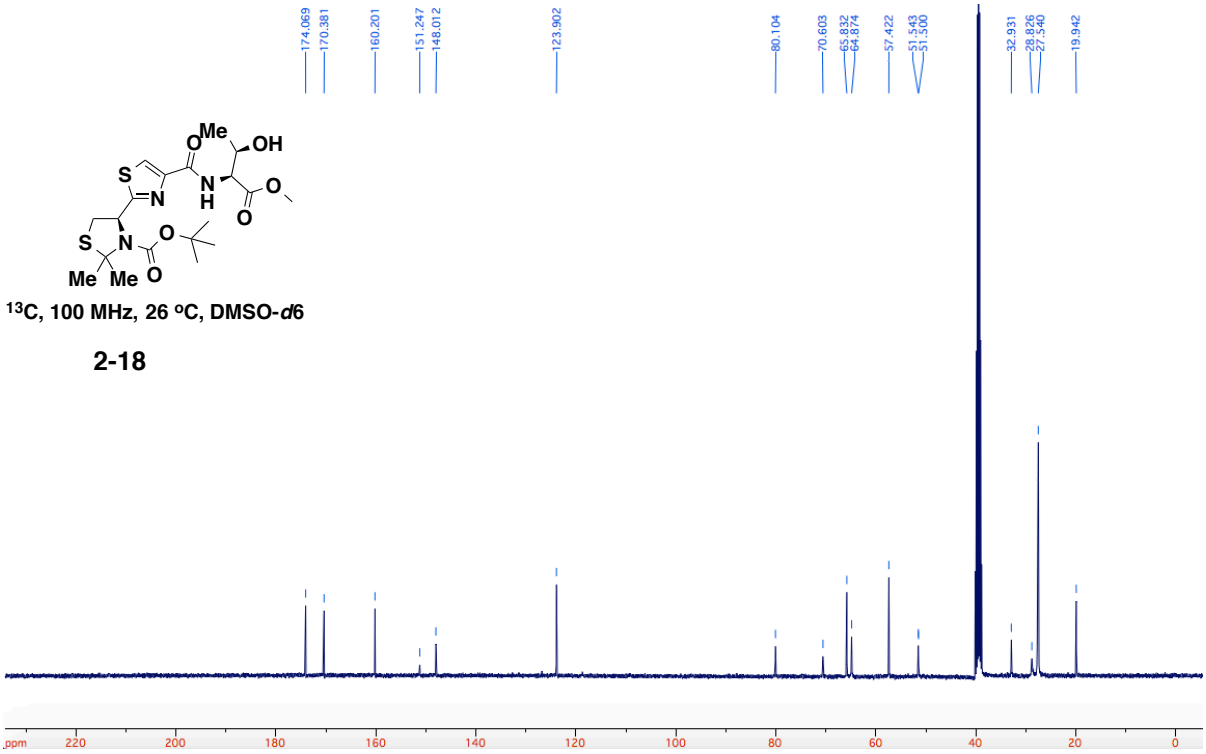
^{13}C, 100 MHz, 26 ^{o}C, DMSO-*d*6

2-16



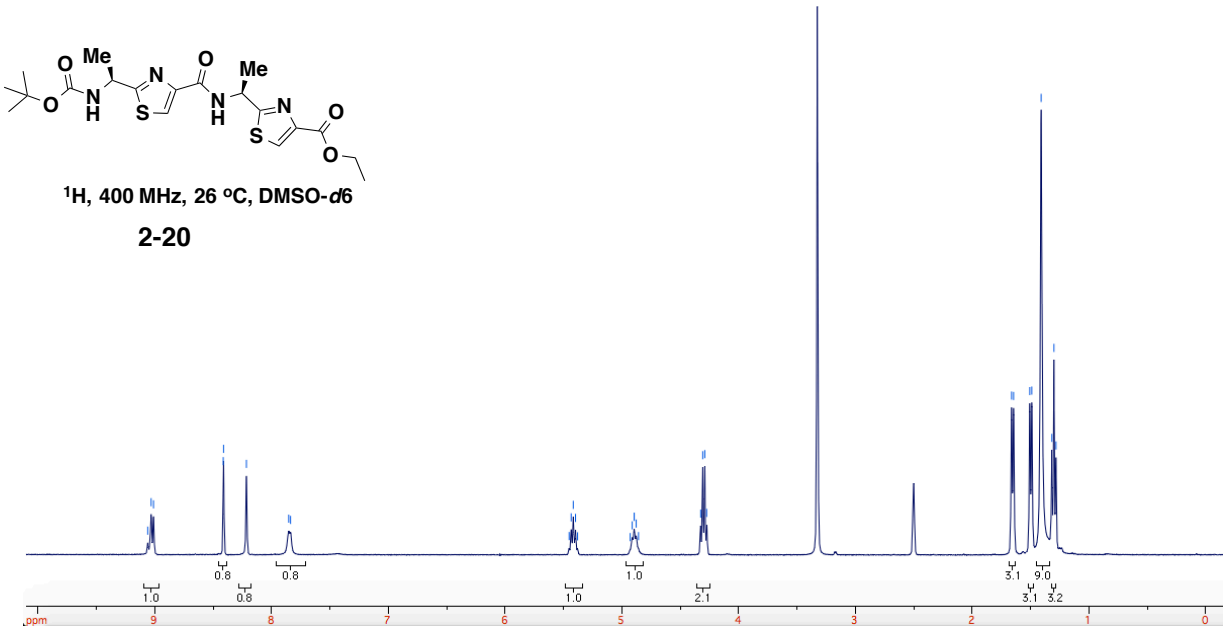
^{1}H, 400 MHz, 70 ^{o}C, DMSO-*d*6

2-18



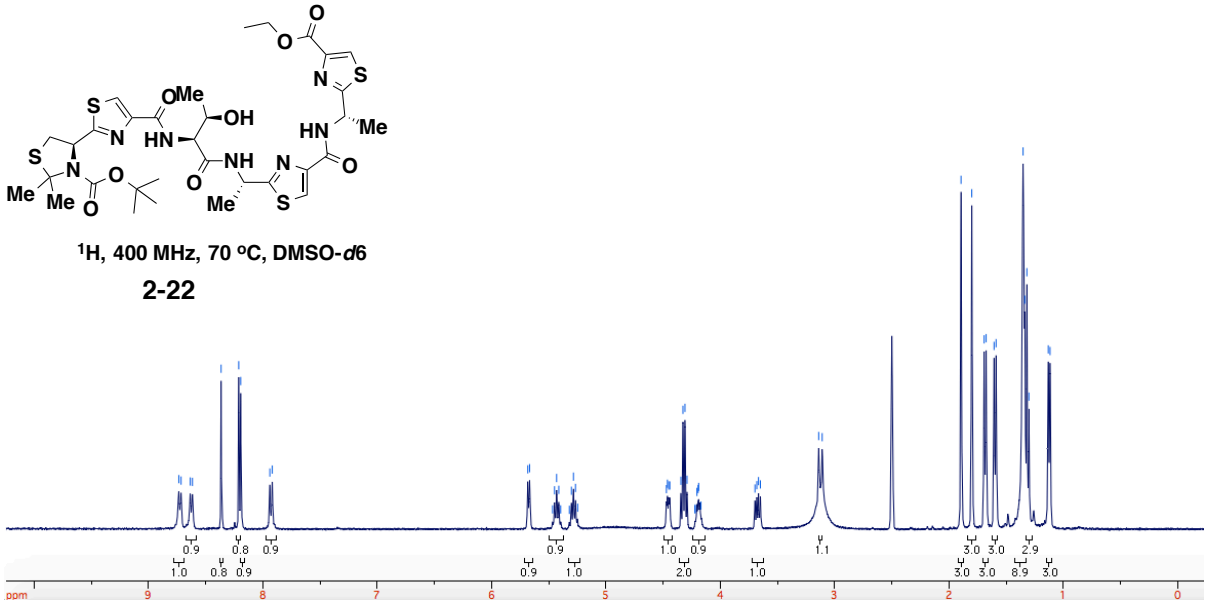
^{13}C, 100 MHz, 26 ^{o}C, DMSO-*d*6

2-18



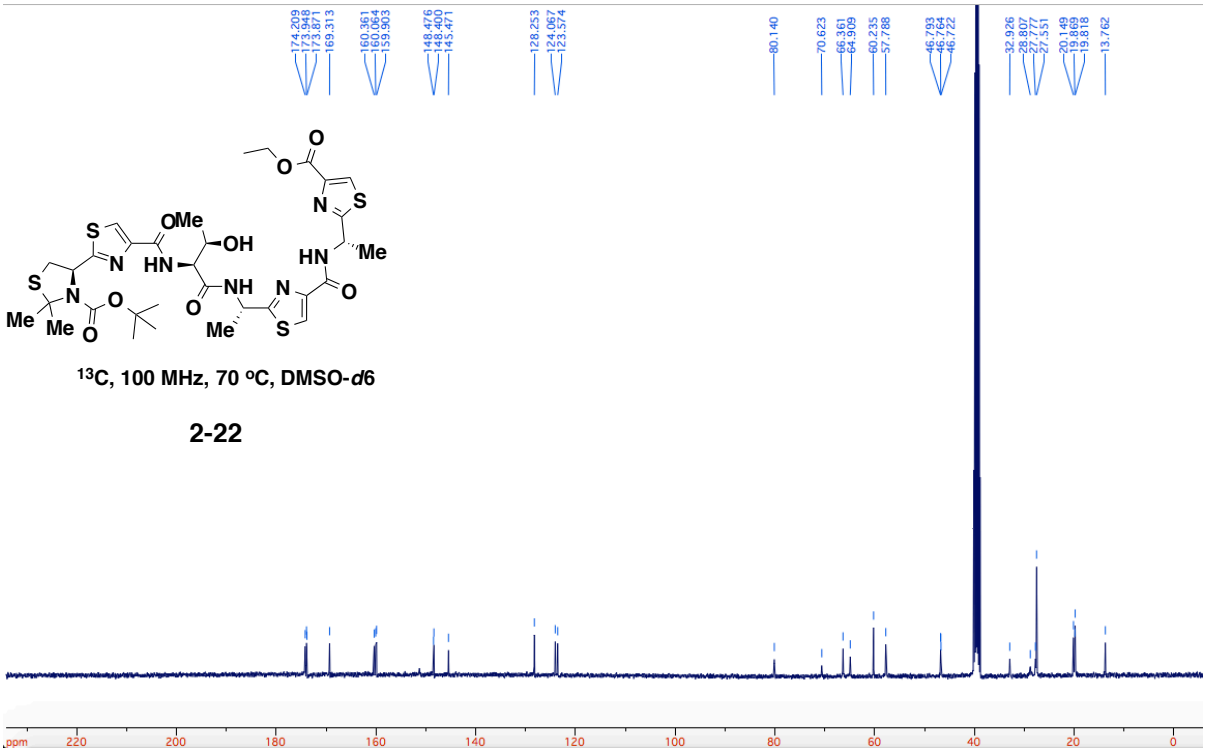
^{1}H, 400 MHz, 26 ºC, DMSO-*d*6

2-20



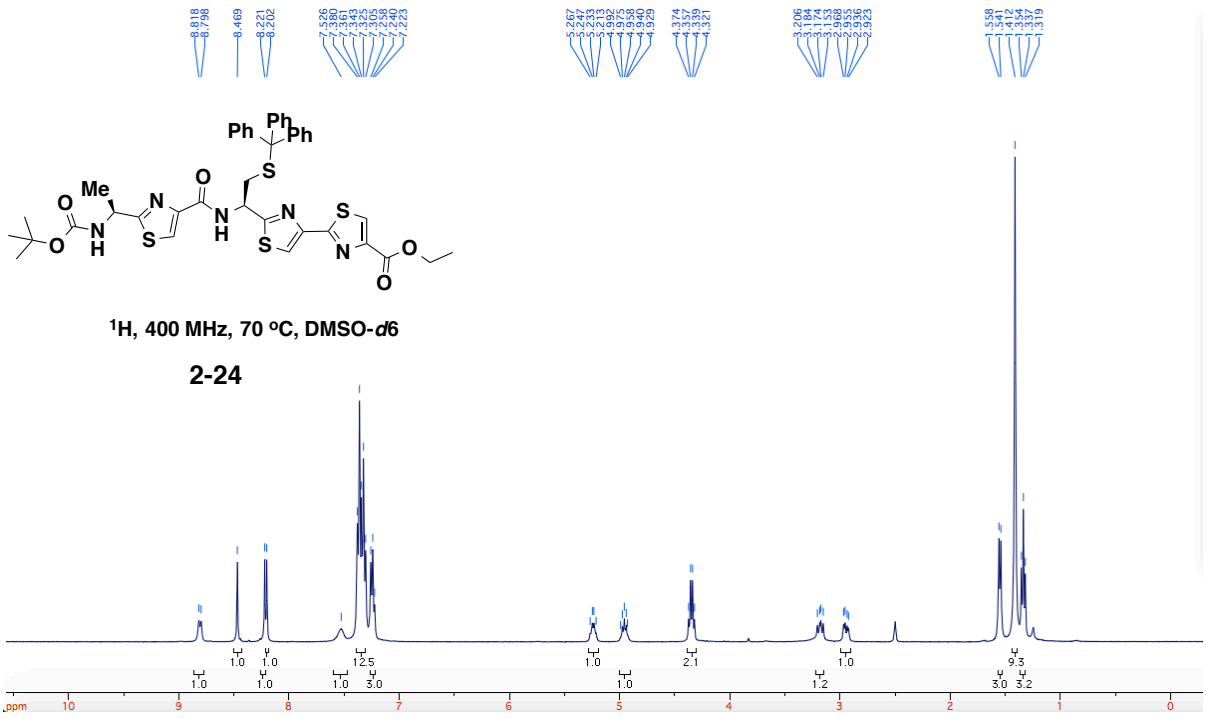
^{1}H, 400 MHz, 70 ºC, DMSO-*d*6

2-22



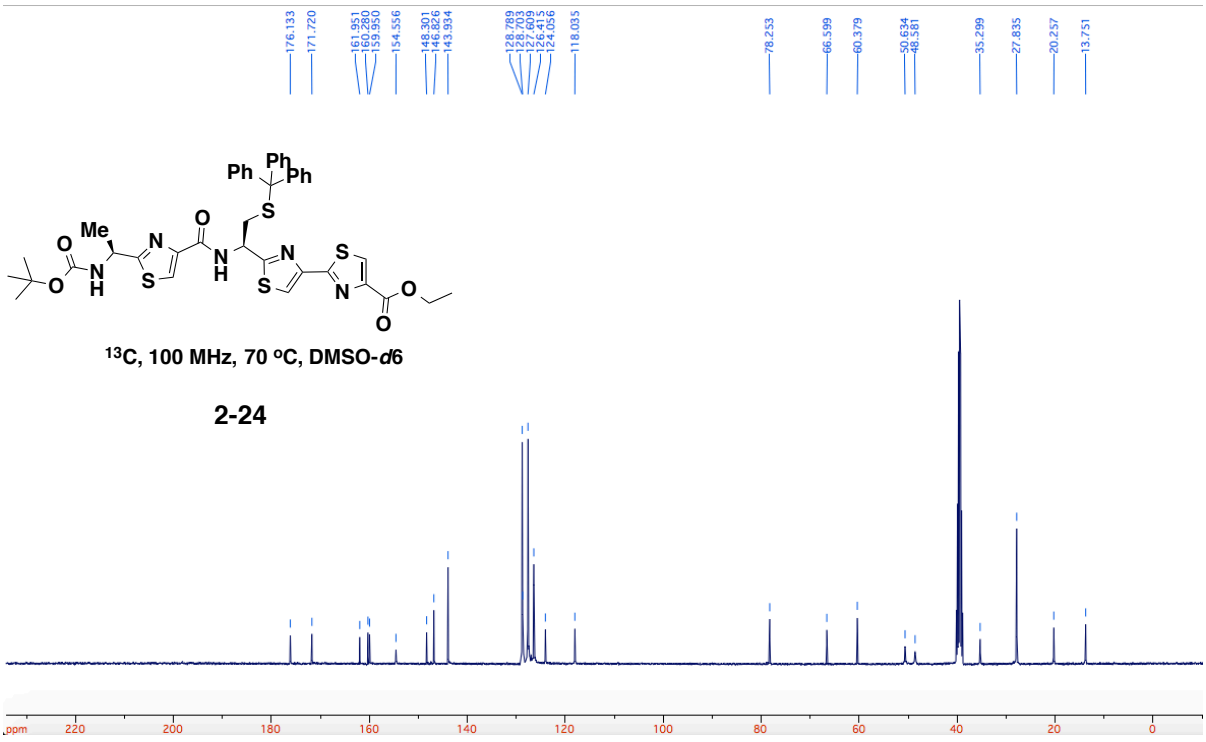
^{13}C, 100 MHz, 70 °C, DMSO-*d*6

2-22



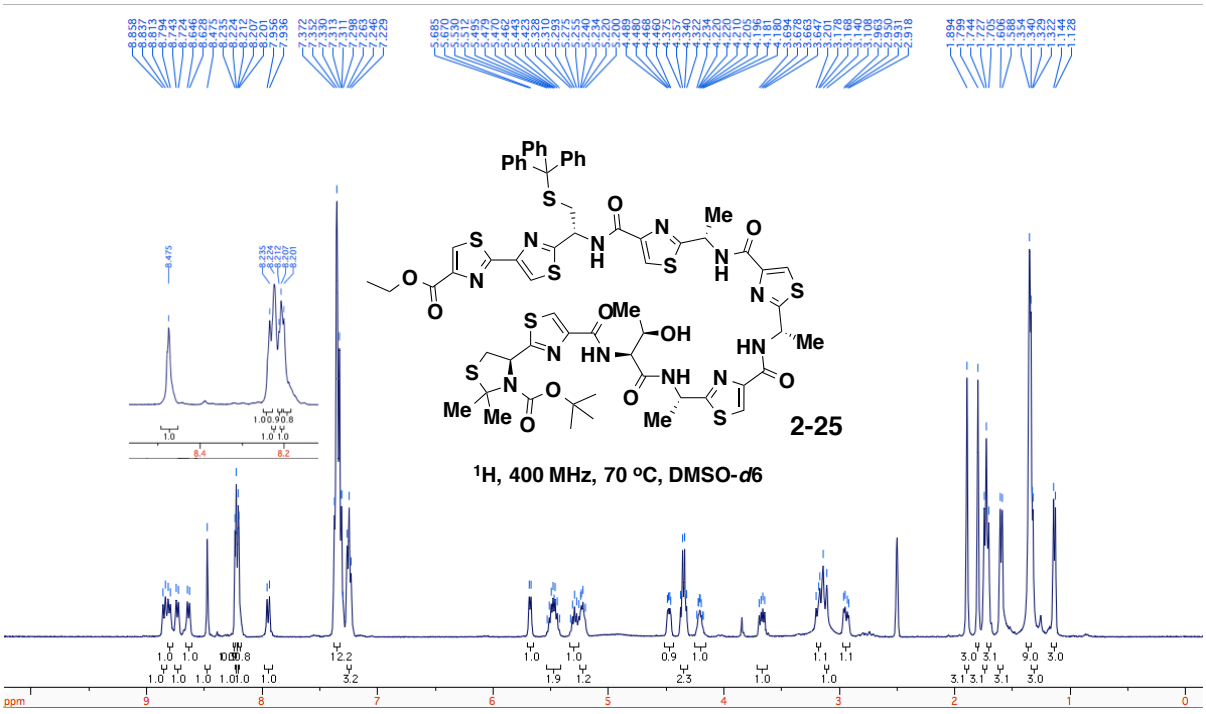
^{1}H, 400 MHz, 70 °C, DMSO-*d*6

2-24



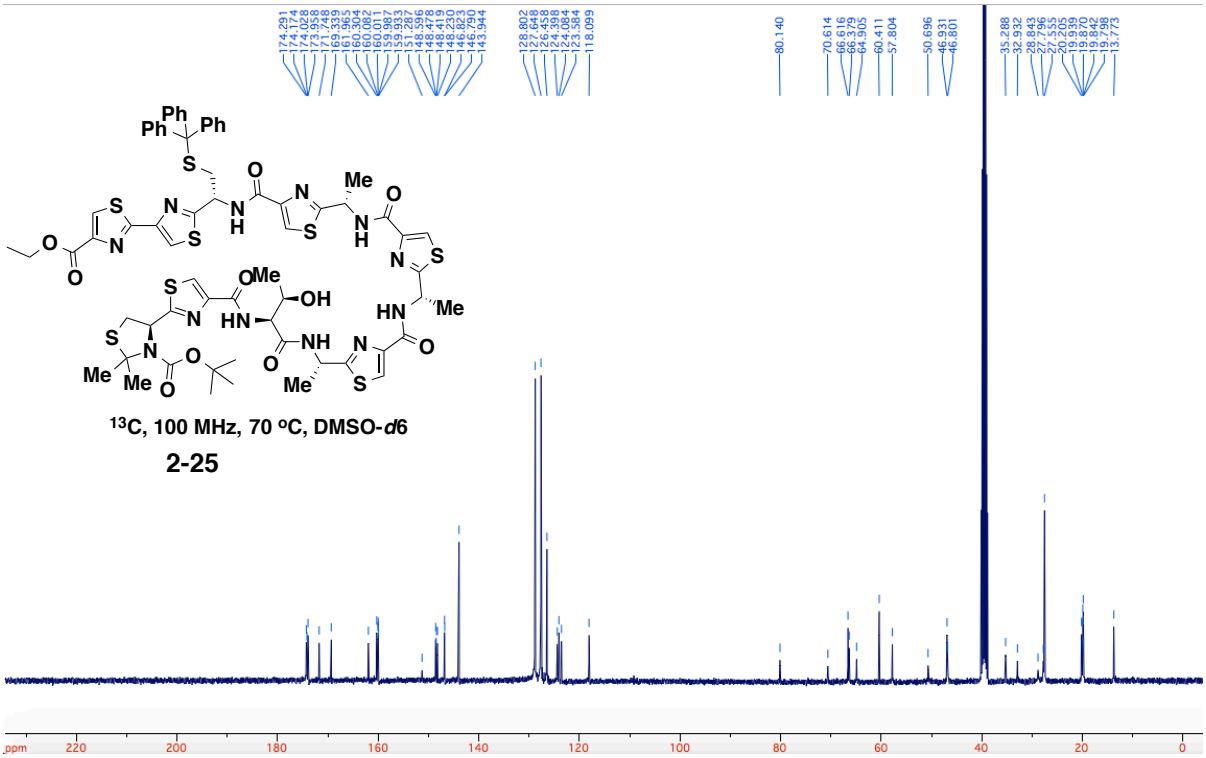
^{13}C, 100 MHz, 70 ºC, DMSO-*d*6

2-24



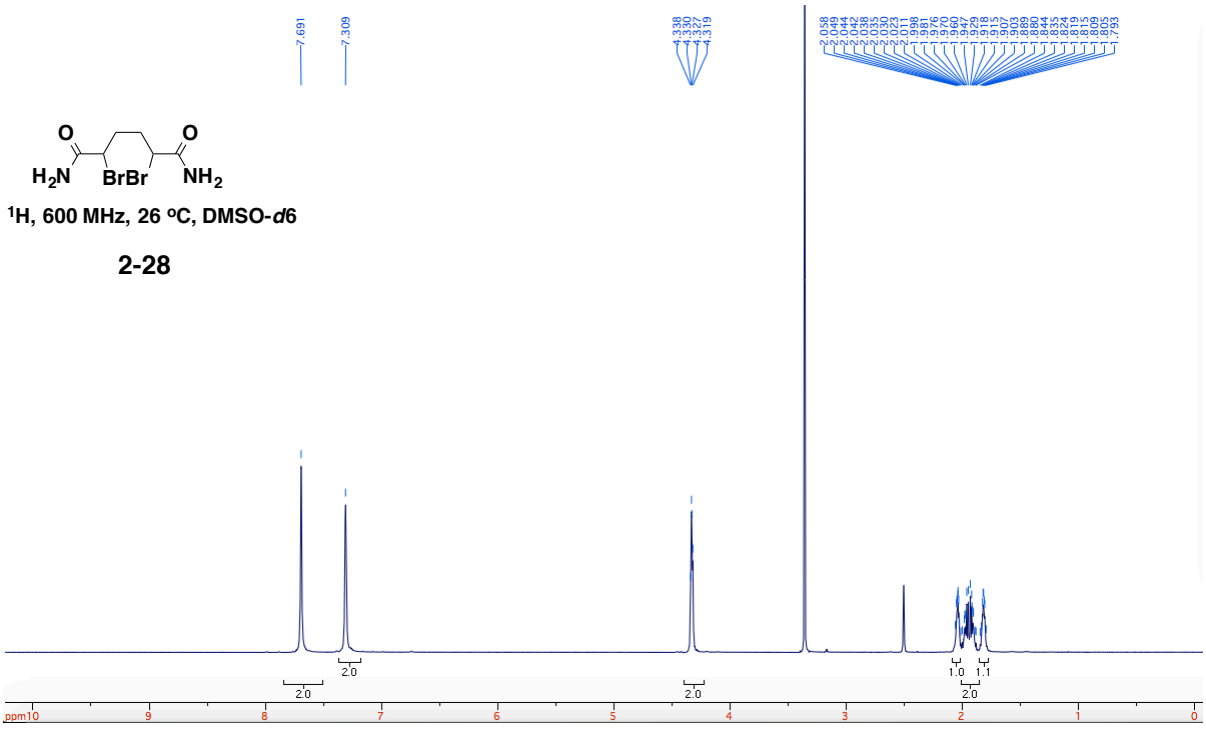
^{1}H, 400 MHz, 70 ºC, DMSO-*d*6

2-25



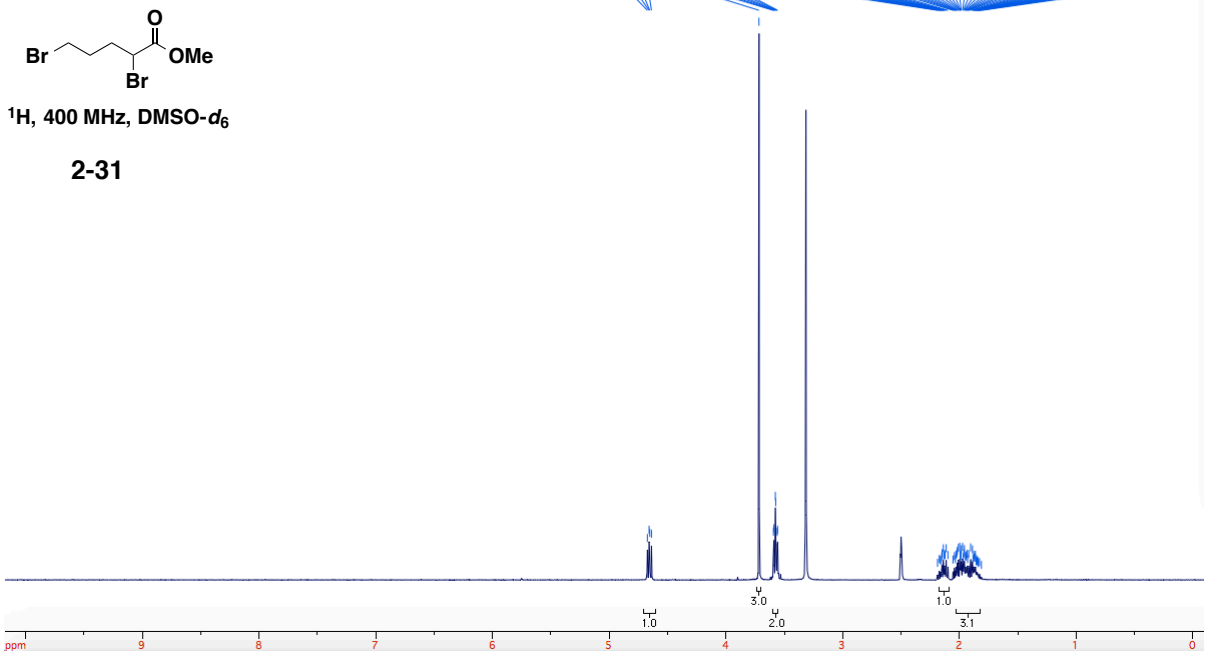
^{13}C, 100 MHz, 70 °C, DMSO-*d*6

2-25



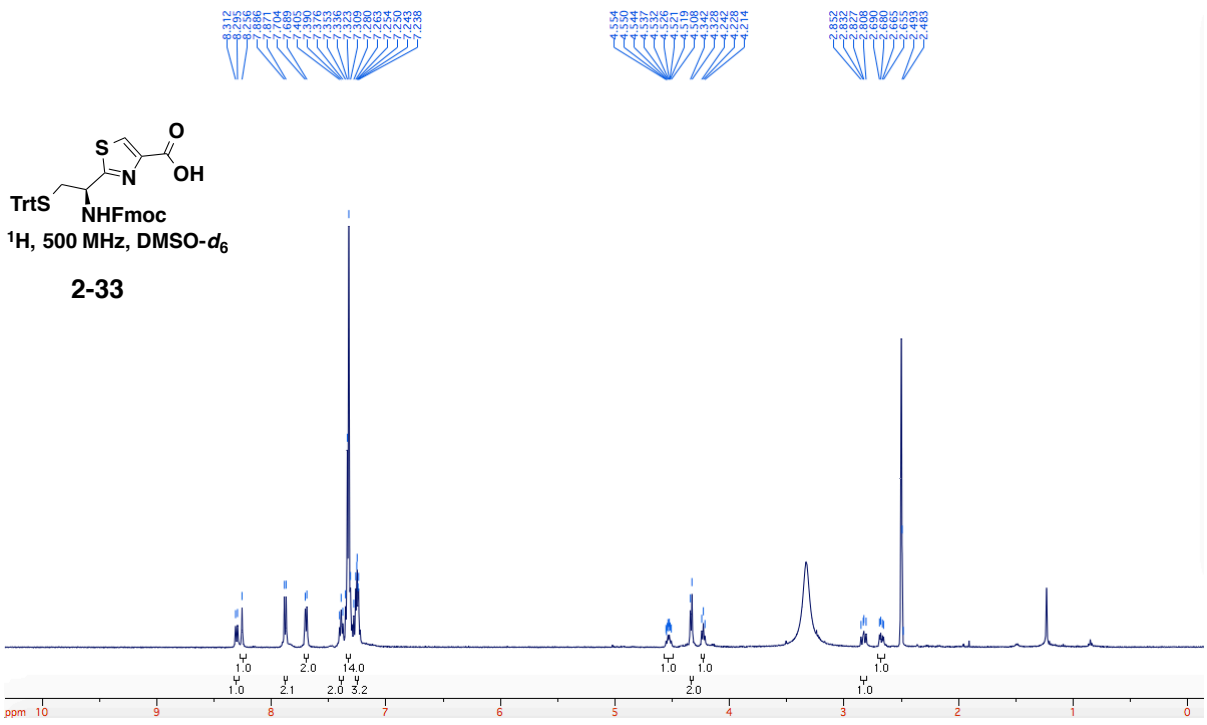
^{1}H, 600 MHz, 26 °C, DMSO-*d*6

2-28



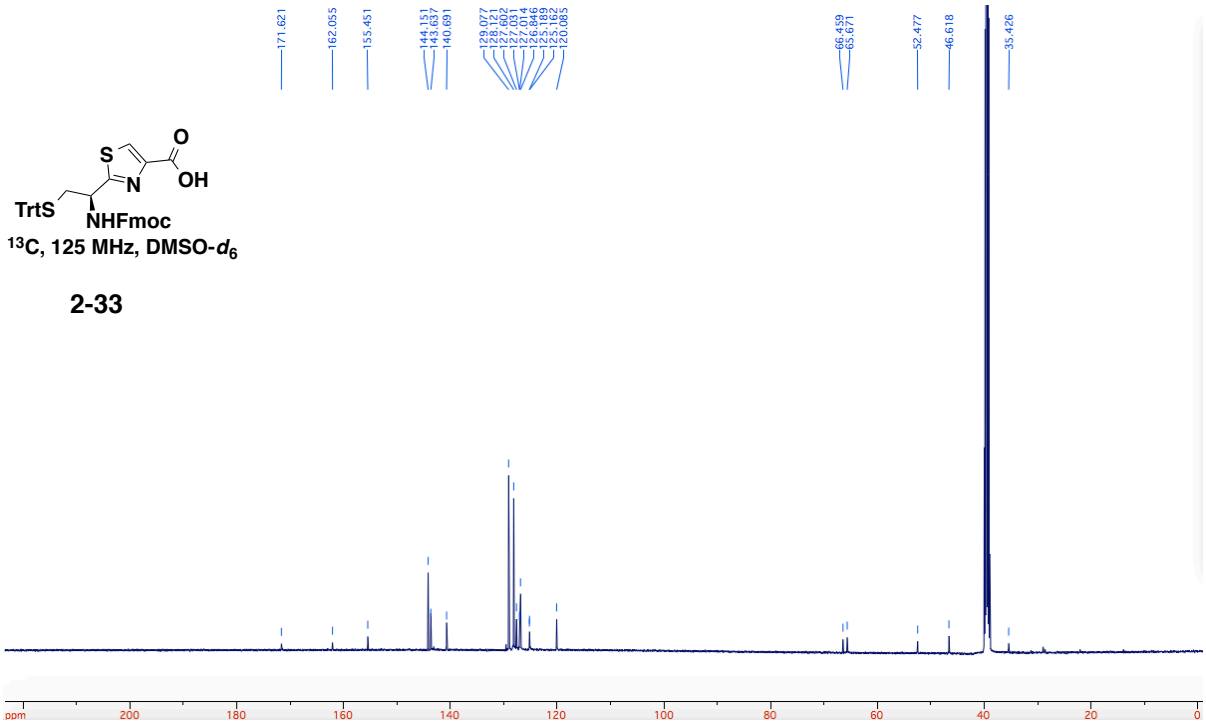
^{1}H, 400 MHz, DMSO-d_6

2-31



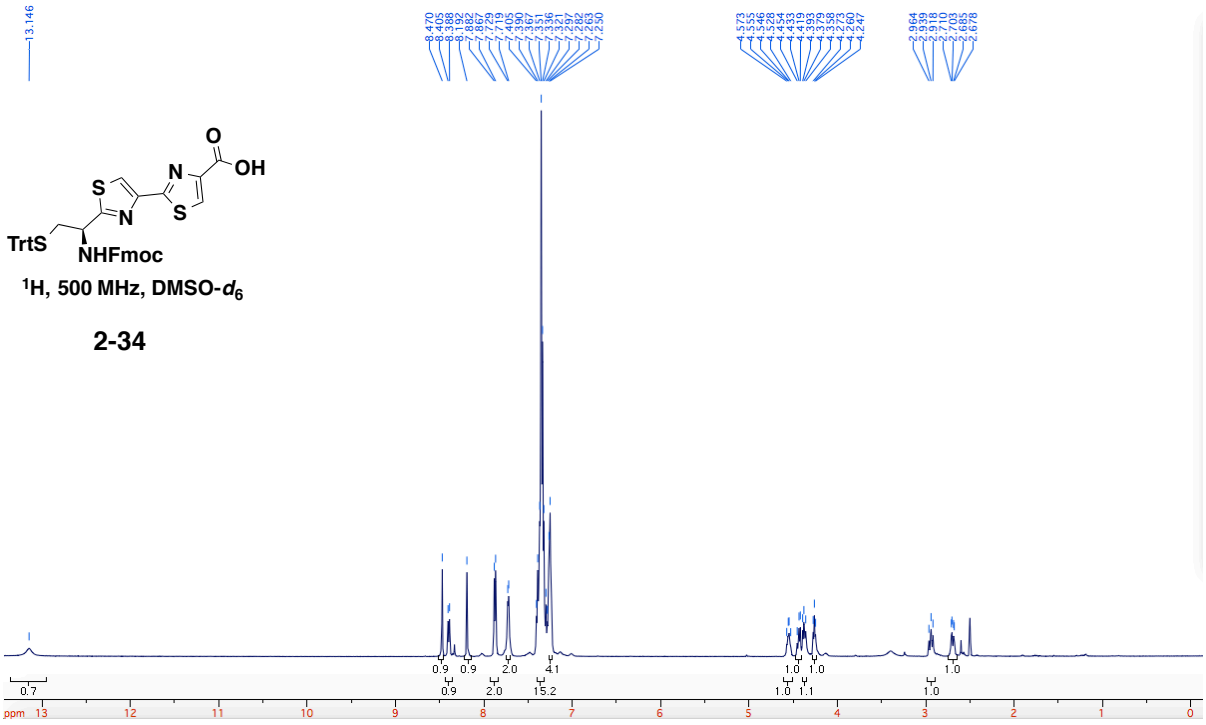
^{1}H, 500 MHz, DMSO-d_6

2-33



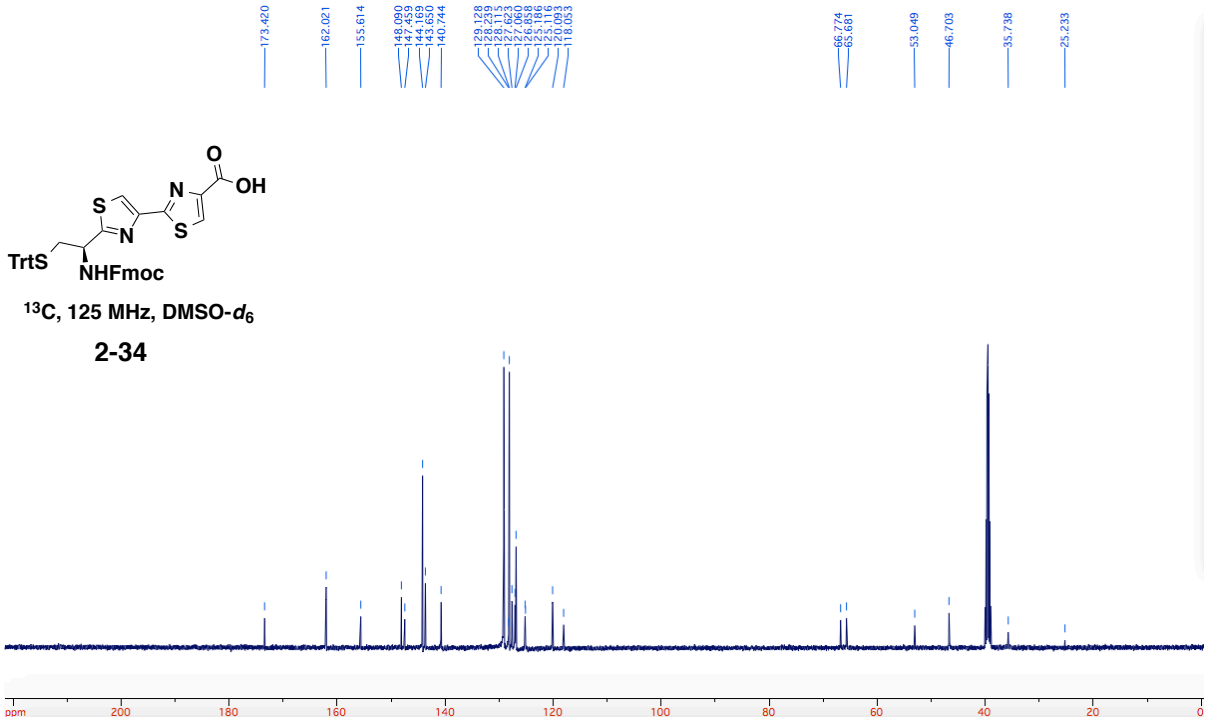
^{13}C, 125 MHz, DMSO-d_6

2-33



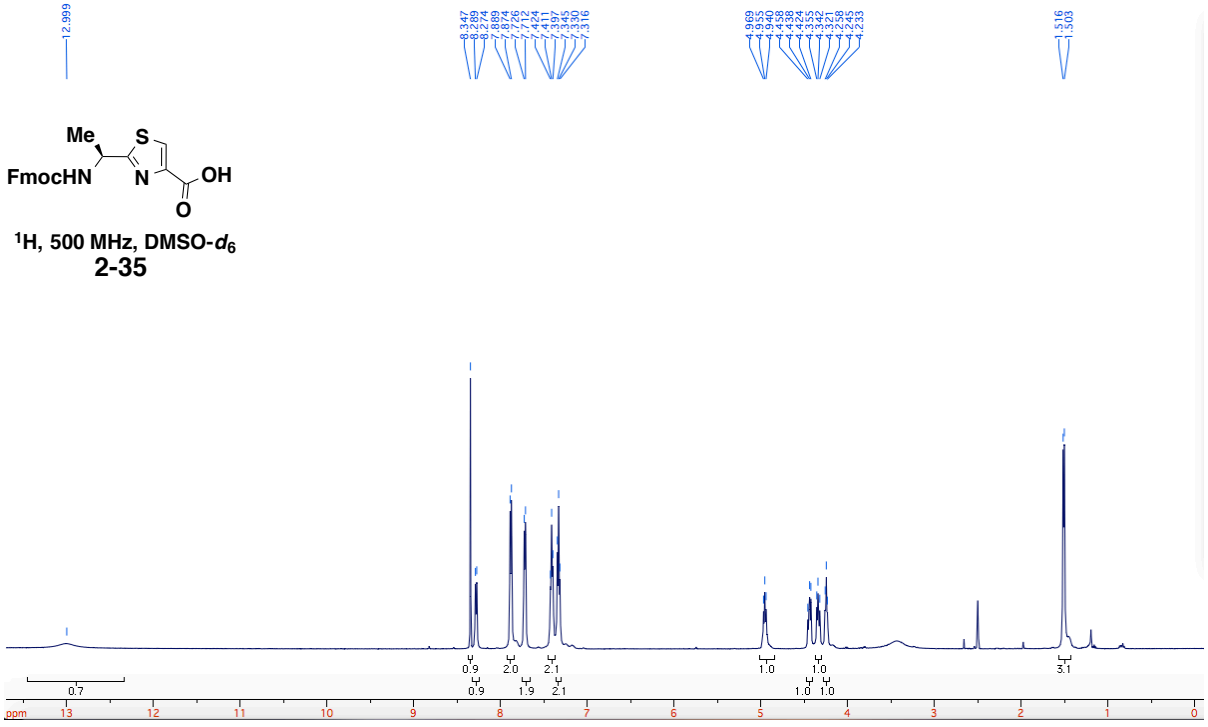
^{1}H, 500 MHz, DMSO-d_6

2-34



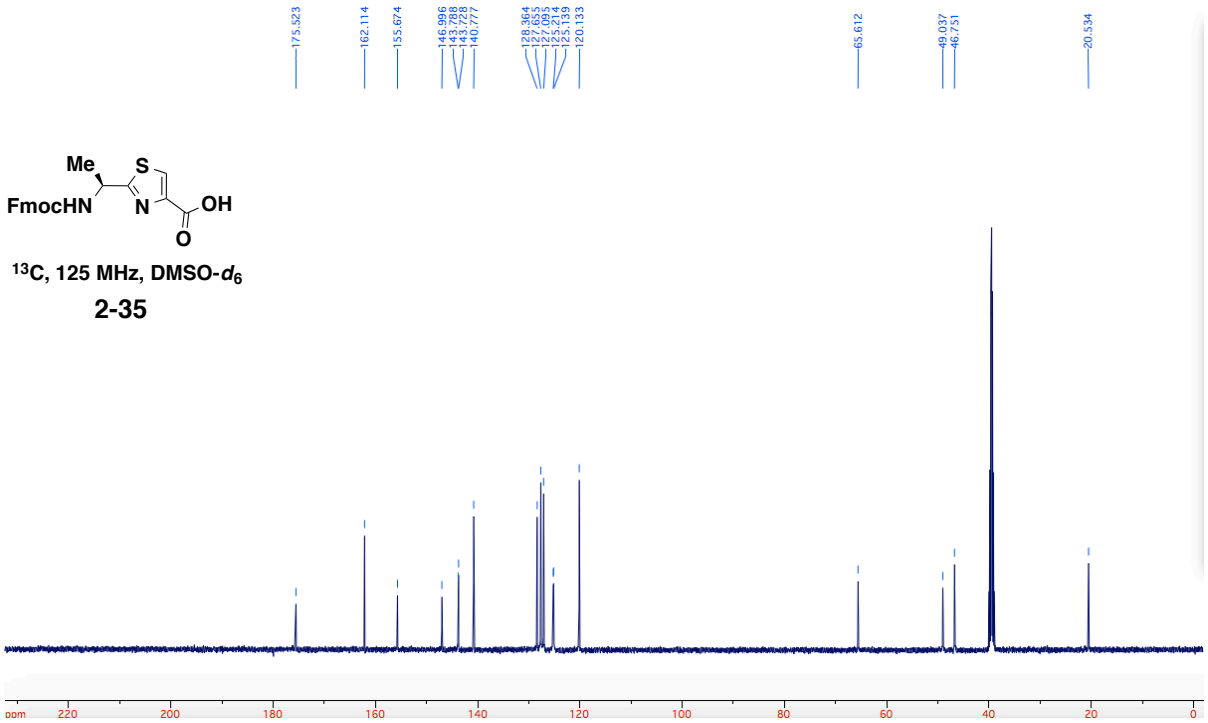
^{13}C, 125 MHz, DMSO-d_6

2-34



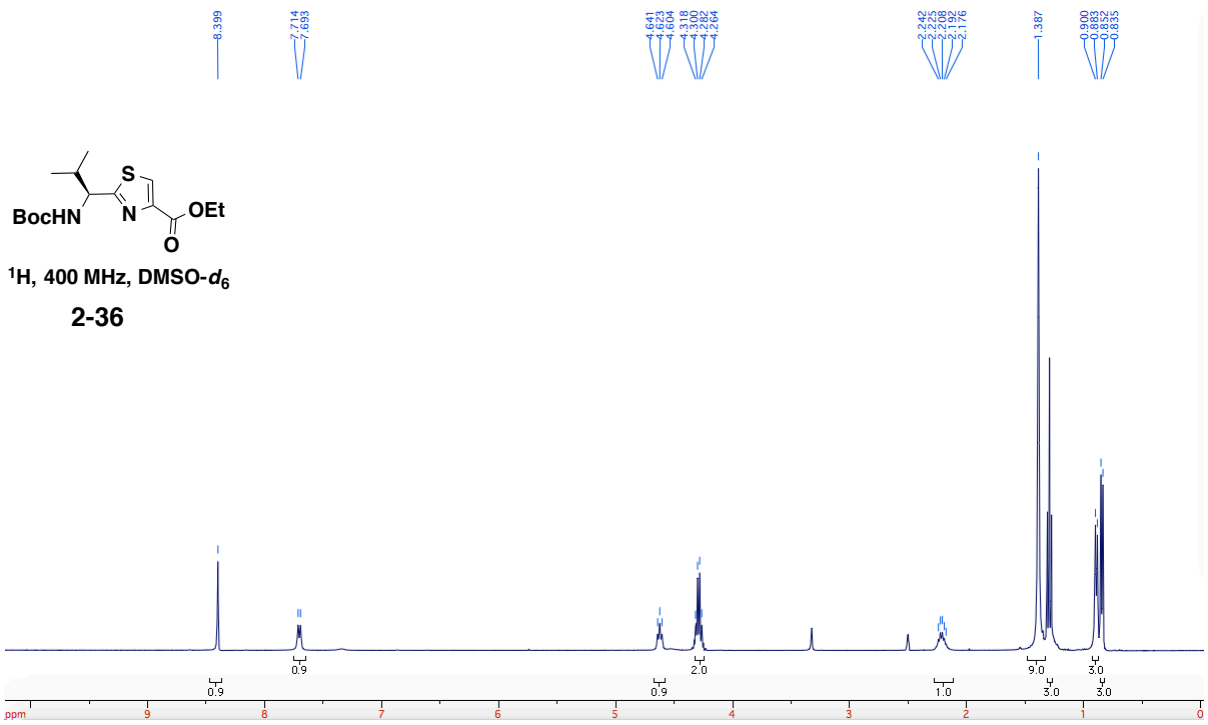
^{1}H, 500 MHz, DMSO-d_6

2-35



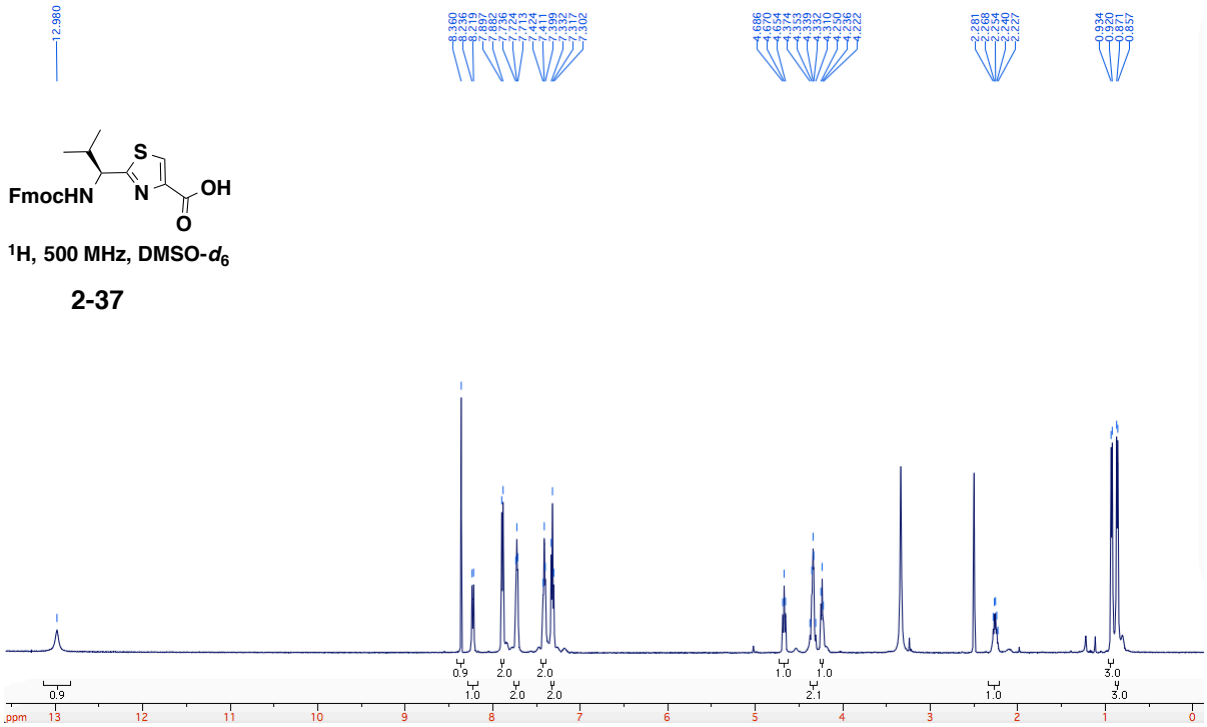
^{13}C, 125 MHz, DMSO-d_6

2-35



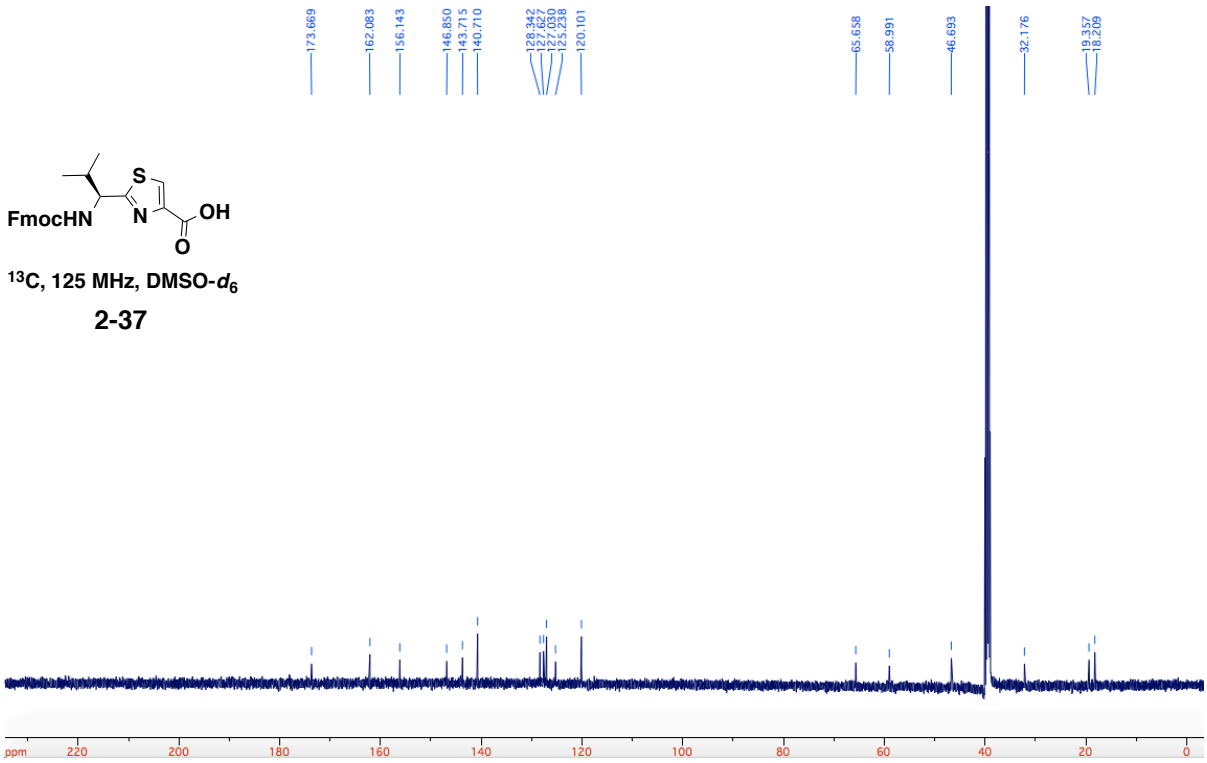
^{1}H, 400 MHz, DMSO-d_6

2-36



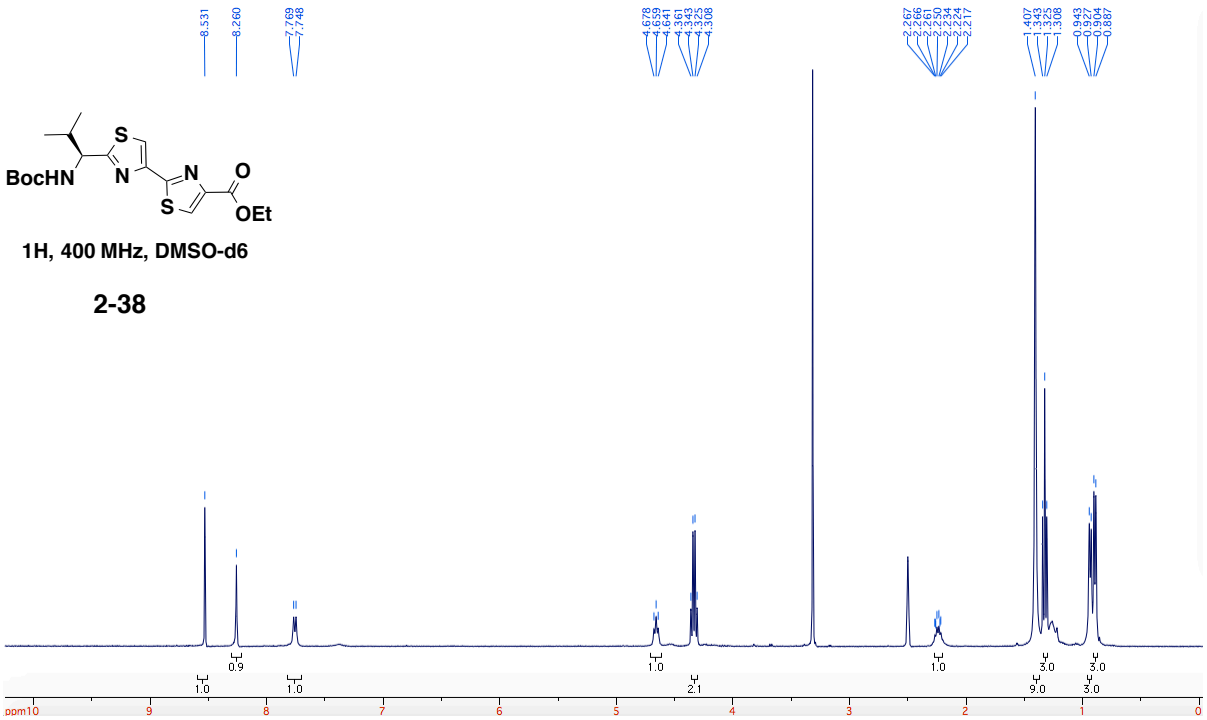
^{1}H, 500 MHz, DMSO-d_6

2-37



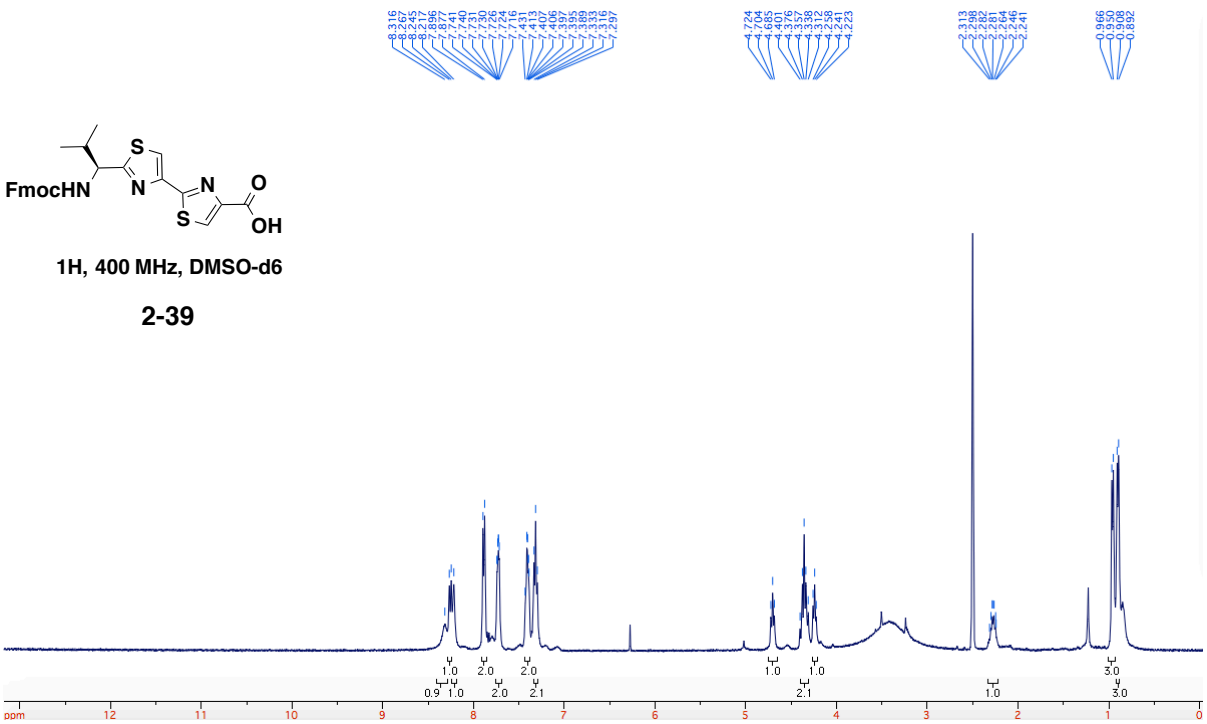
^{13}C, 125 MHz, DMSO-d_6

2-37



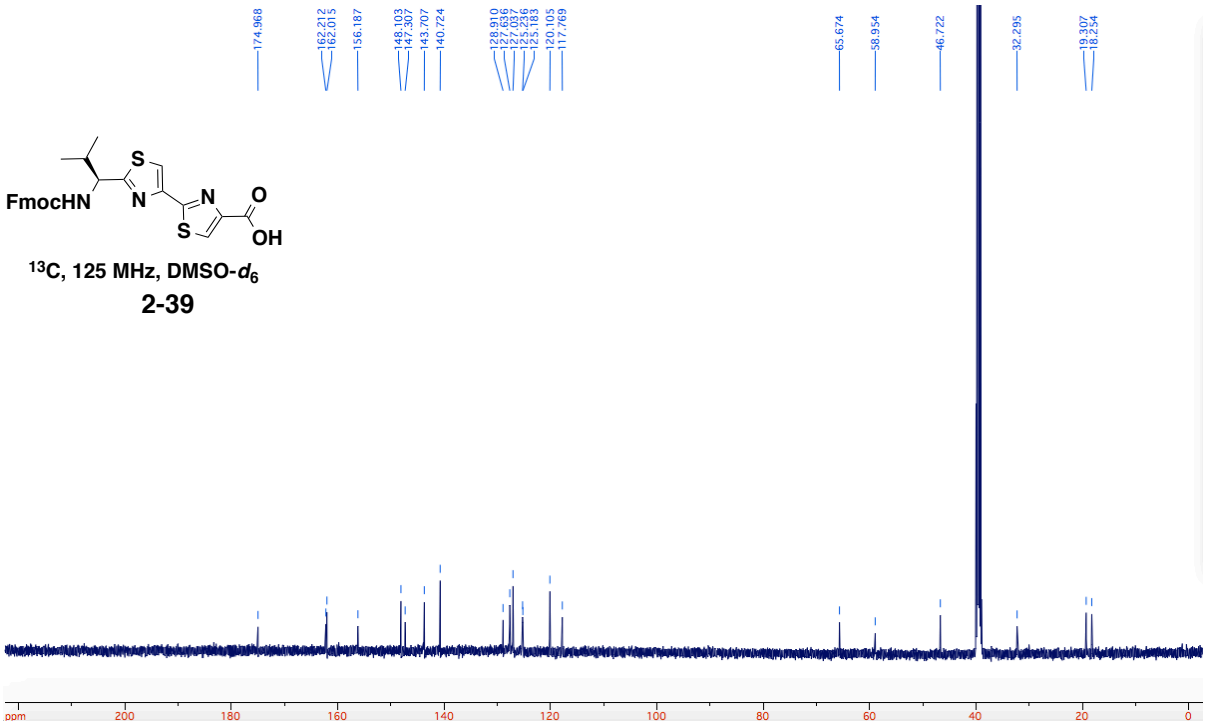
1H, 400 MHz, DMSO-d6

2-38



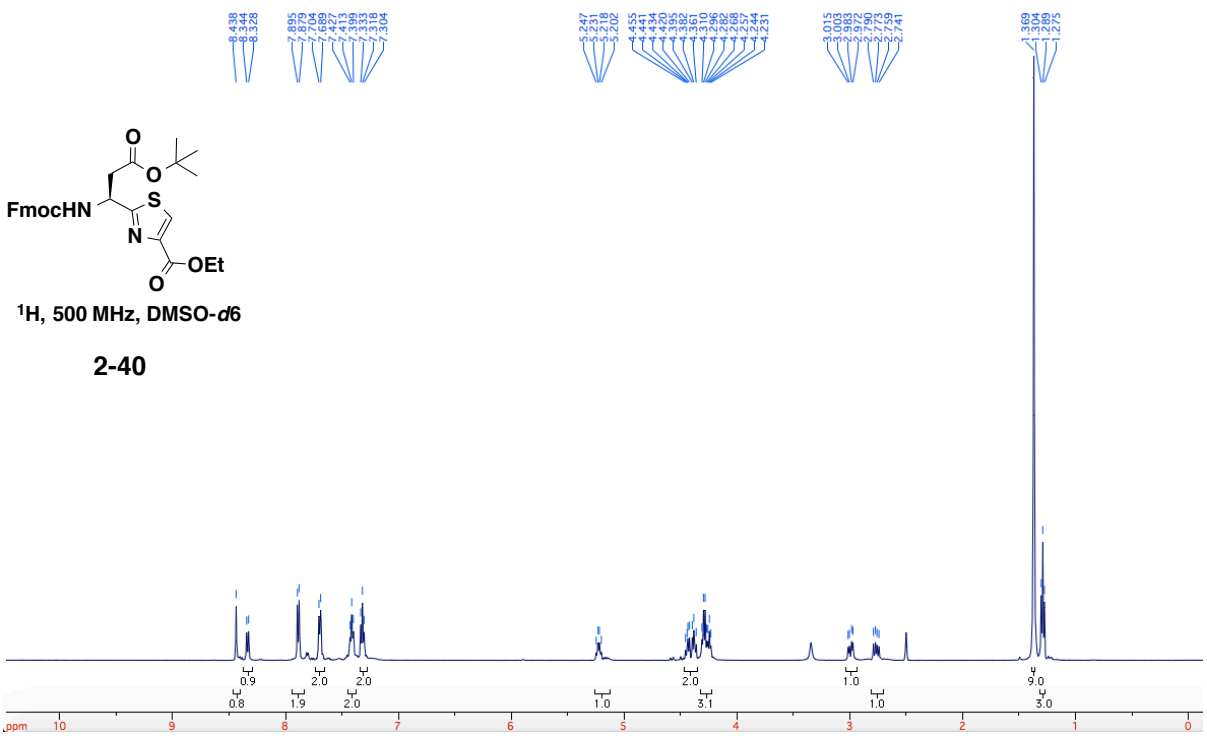
1H, 400 MHz, DMSO-d6

2-39



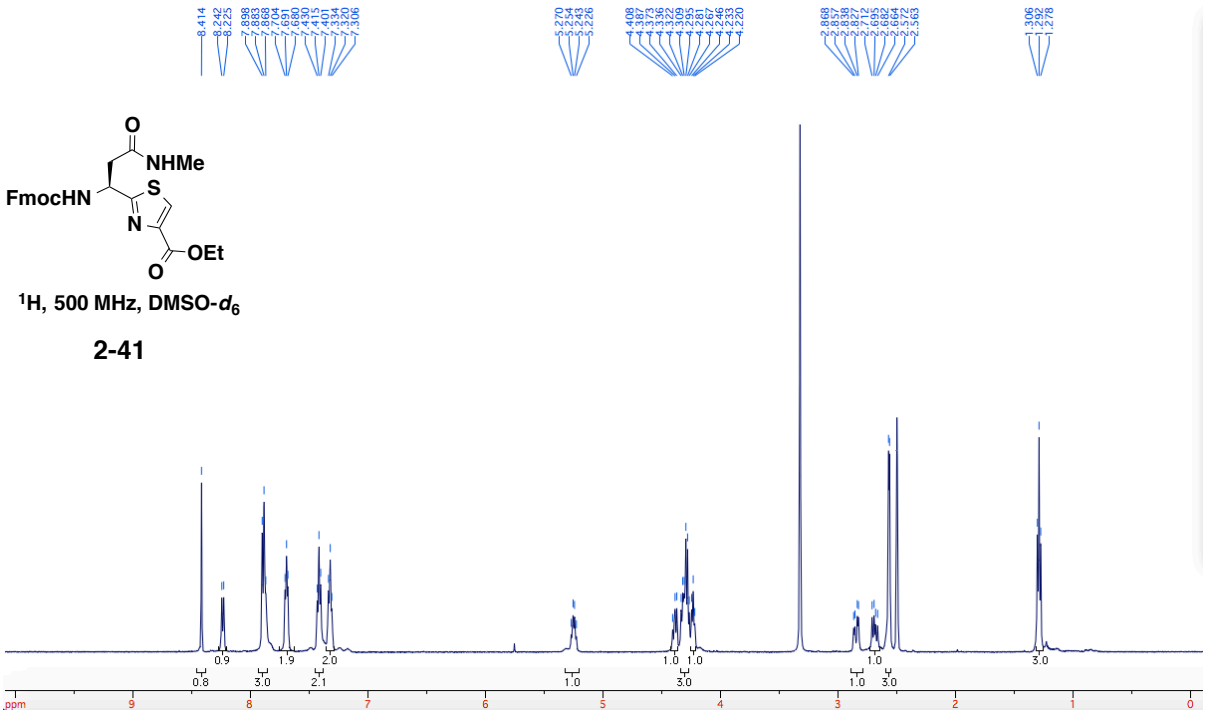
^{13}C, 125 MHz, DMSO-d_6

2-39



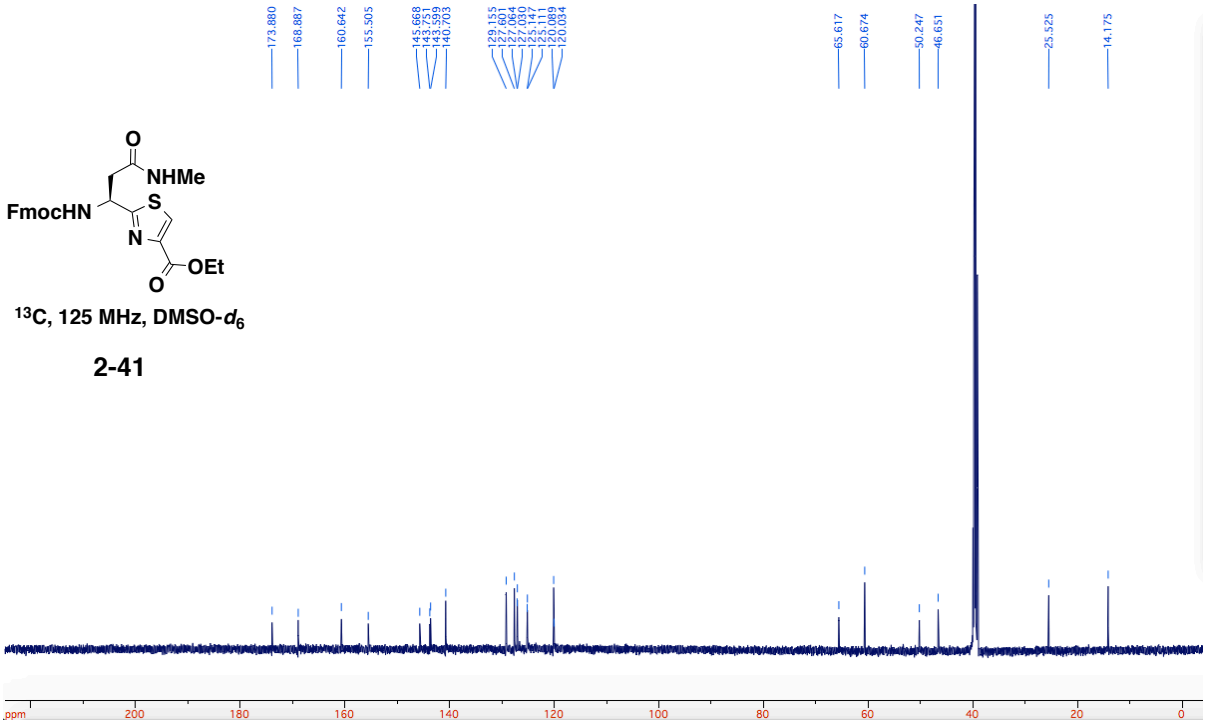
^{1}H, 500 MHz, DMSO-*d*6

2-40



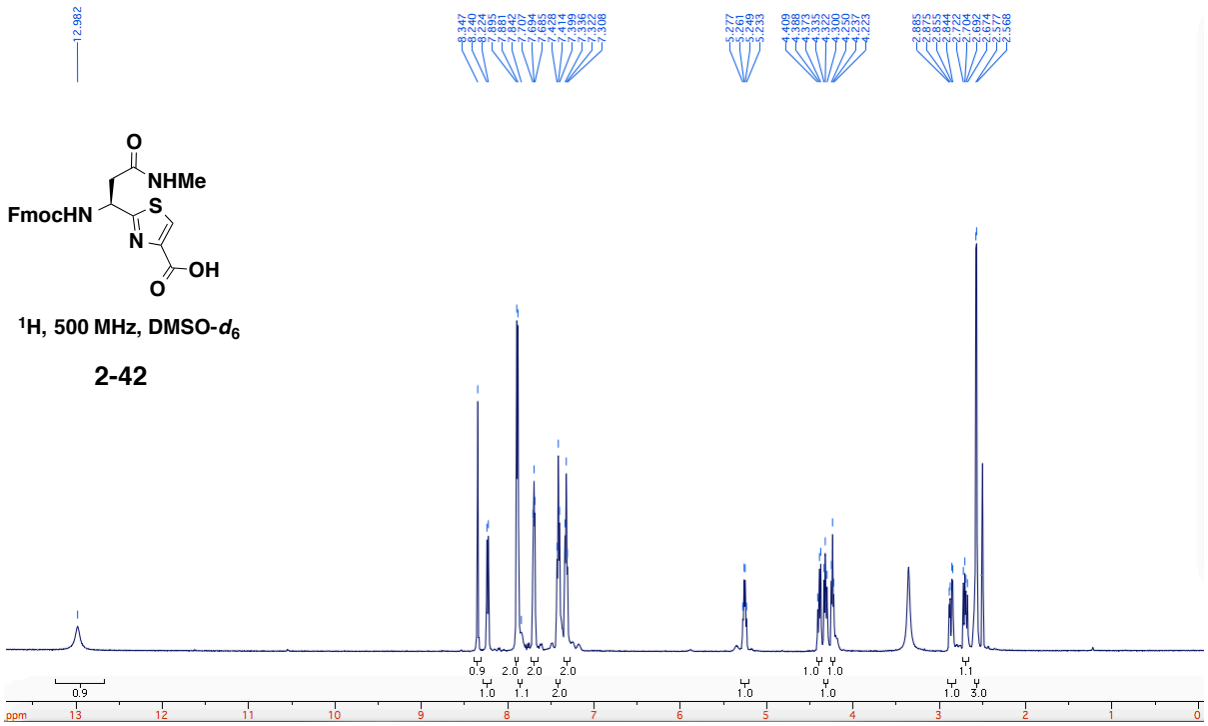
^{1}H, 500 MHz, DMSO-d_6

2-41



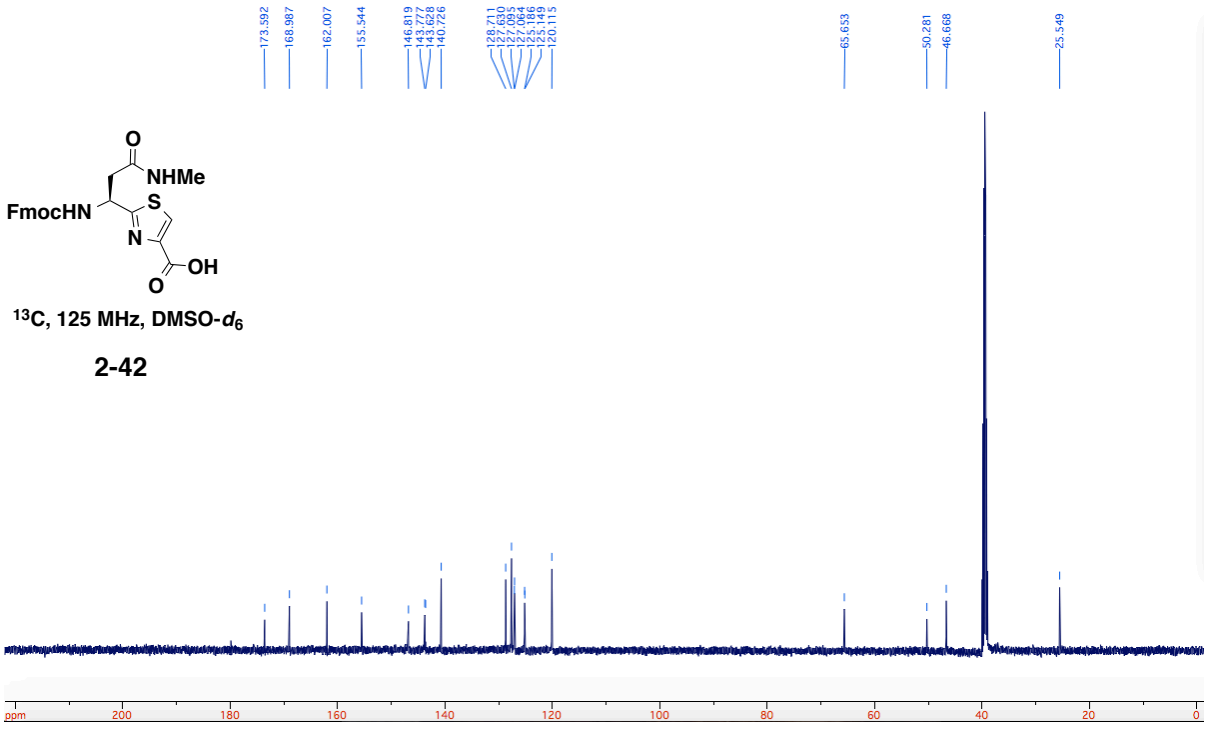
^{13}C, 125 MHz, DMSO-d_6

2-41



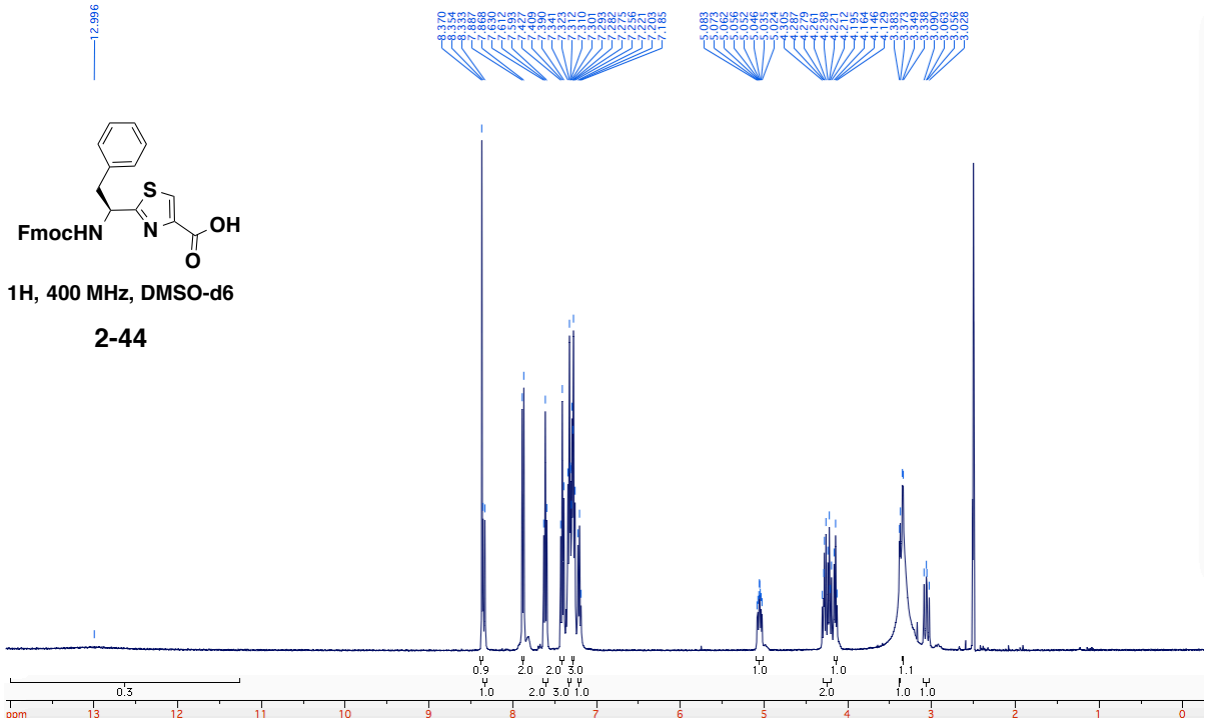
^{1}H, 500 MHz, DMSO-d_6

2-42



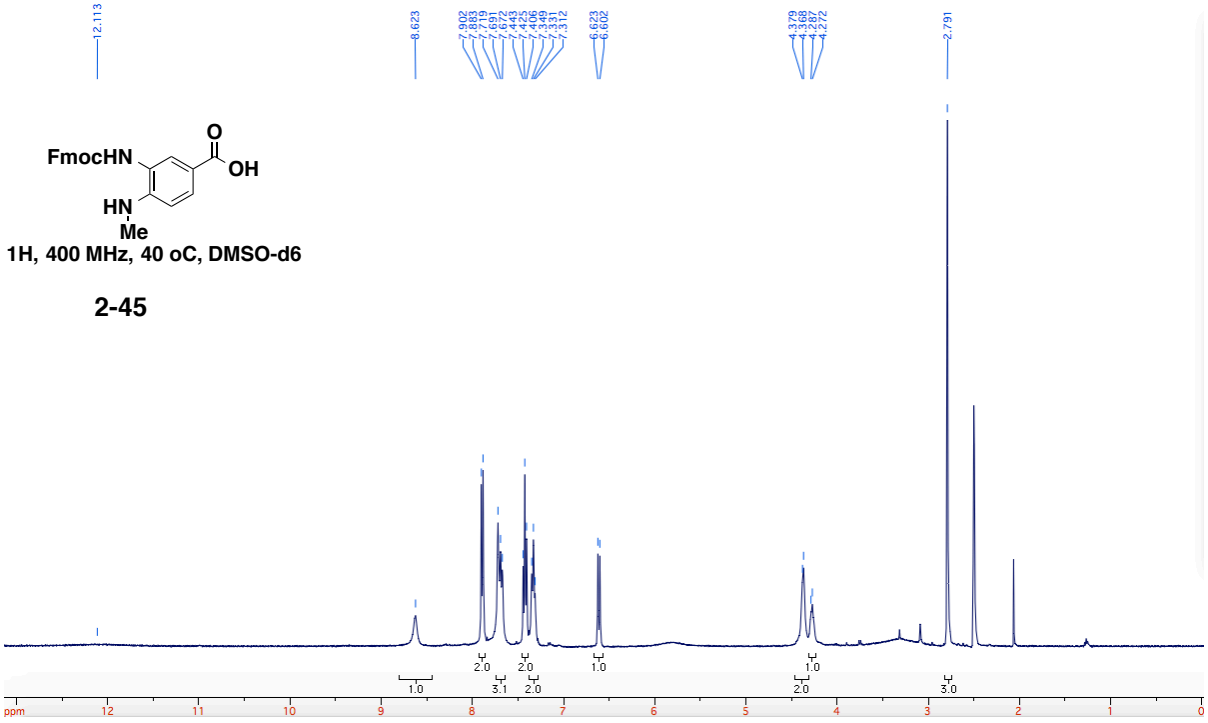
^{13}C, 125 MHz, DMSO-d_6

2-42



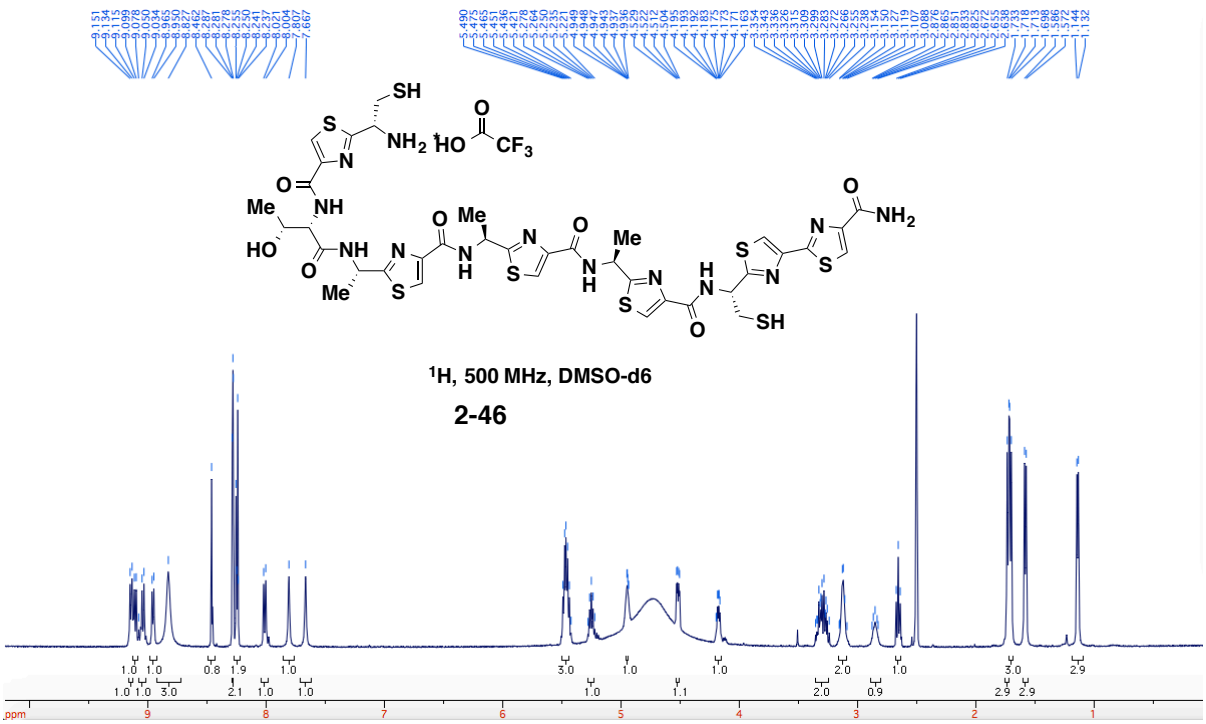
1H, 400 MHz, DMSO-d6

2-44



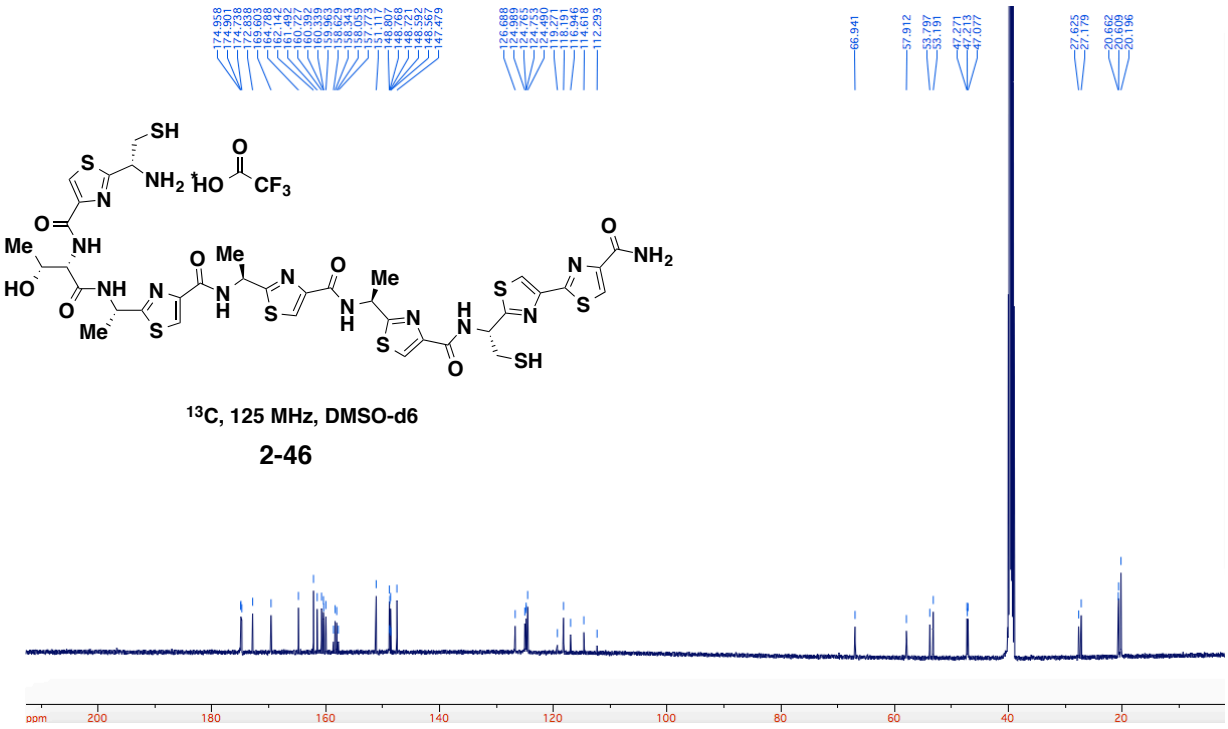
1H, 400 MHz, 40 oC, DMSO-d6

2-45



^{1}H, 500 MHz, DMSO-d6

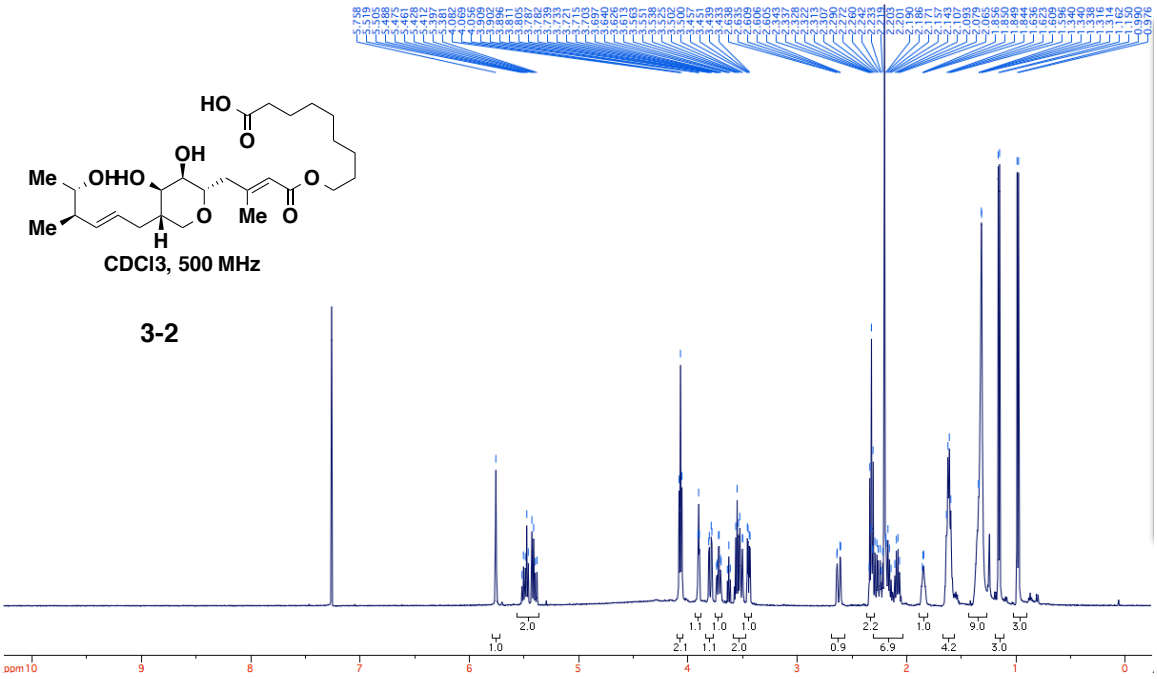
2-46

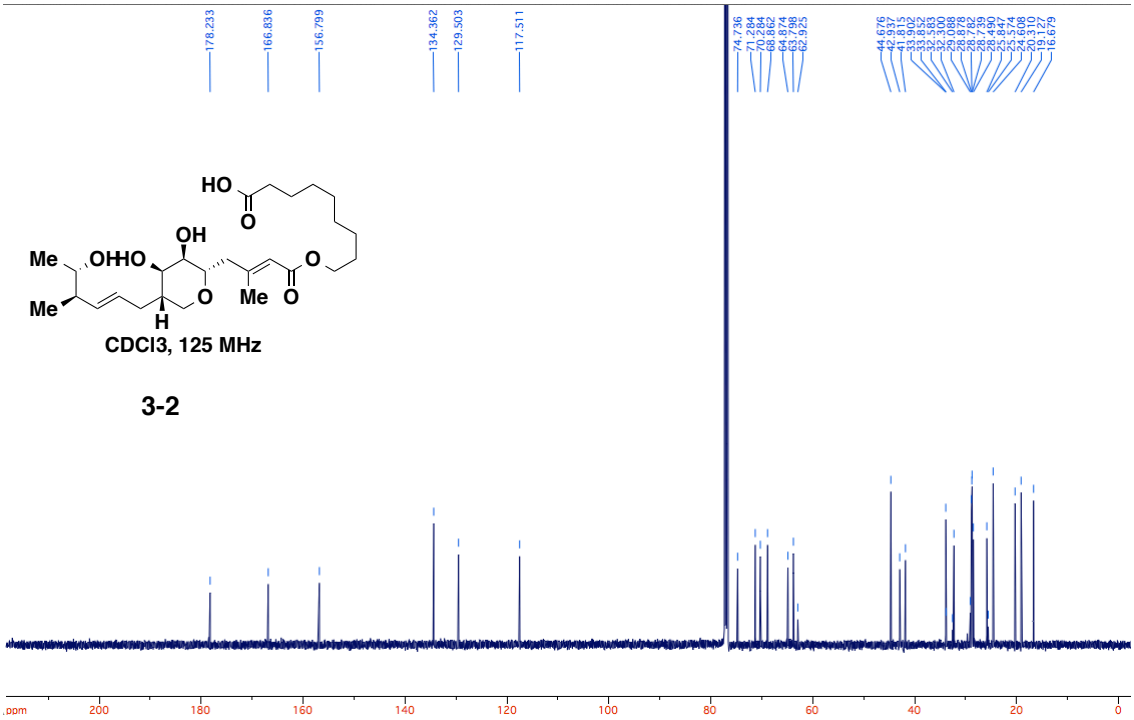


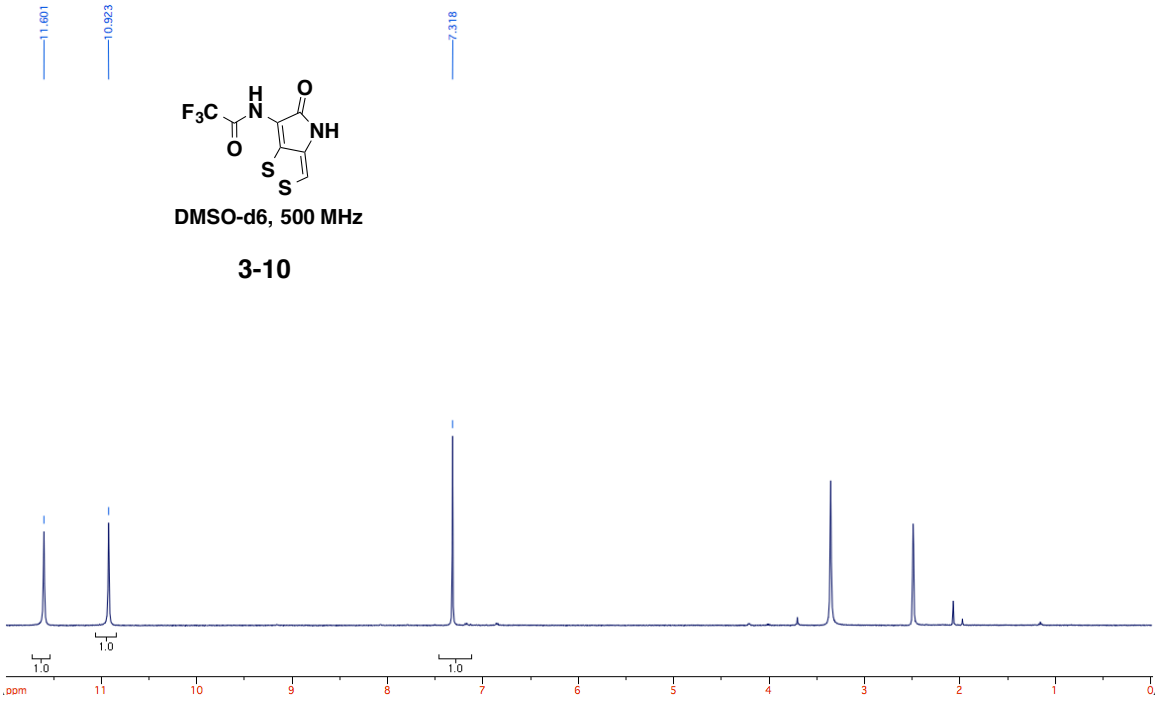
^{13}C, 125 MHz, DMSO-d6

2-46

APPENDIX B. SPECTRA FOR CHAPTER 3

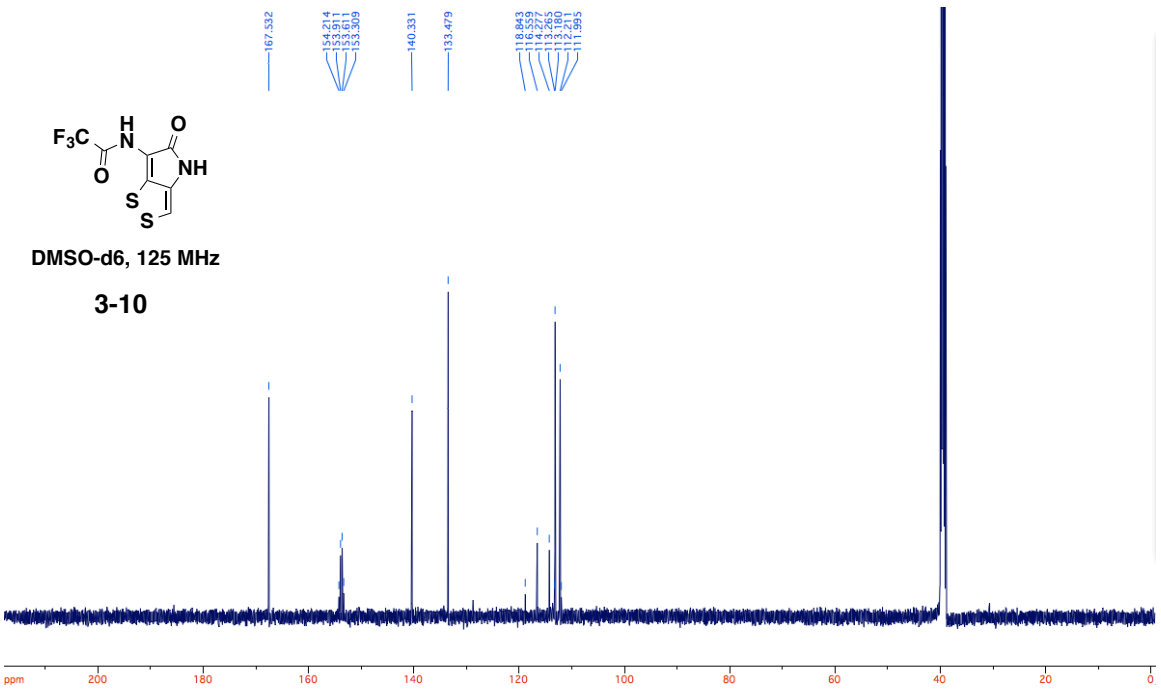






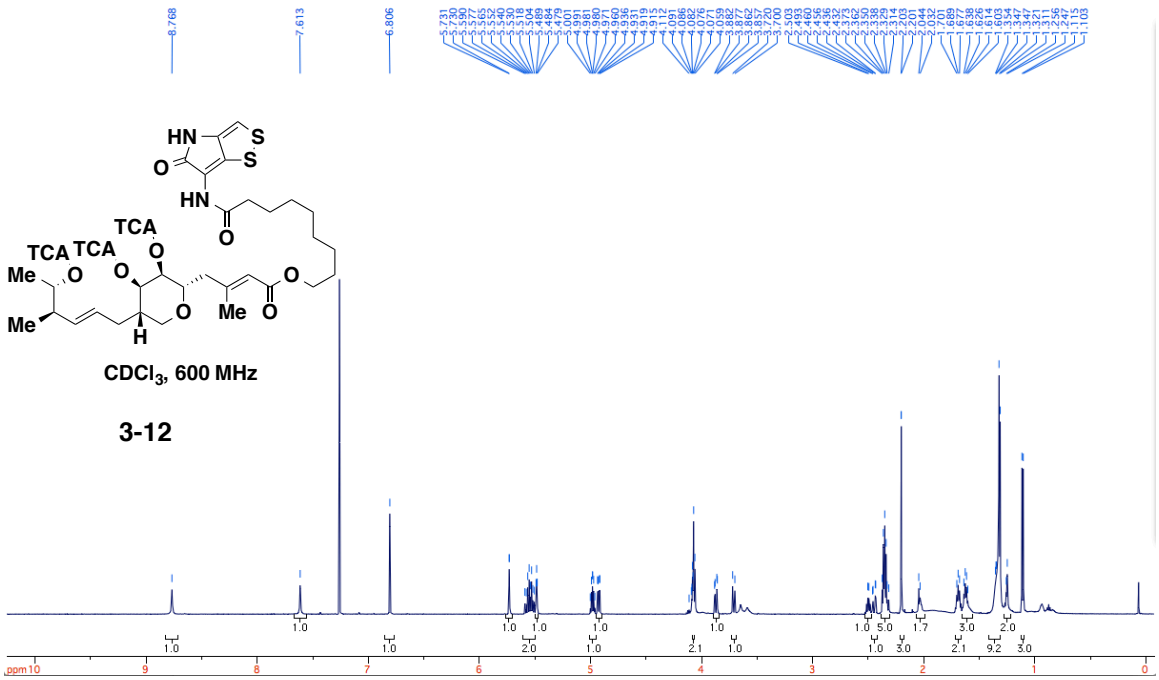
DMSO-d6, 500 MHz

3-10



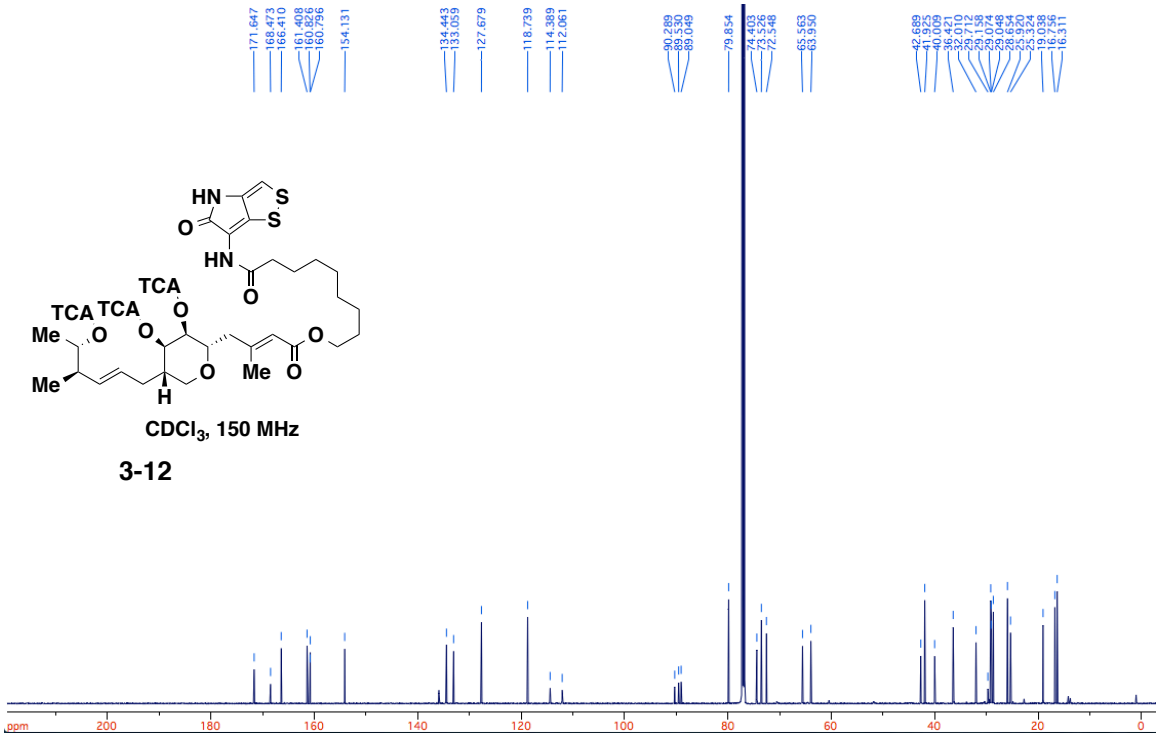
DMSO-d6, 125 MHz

3-10



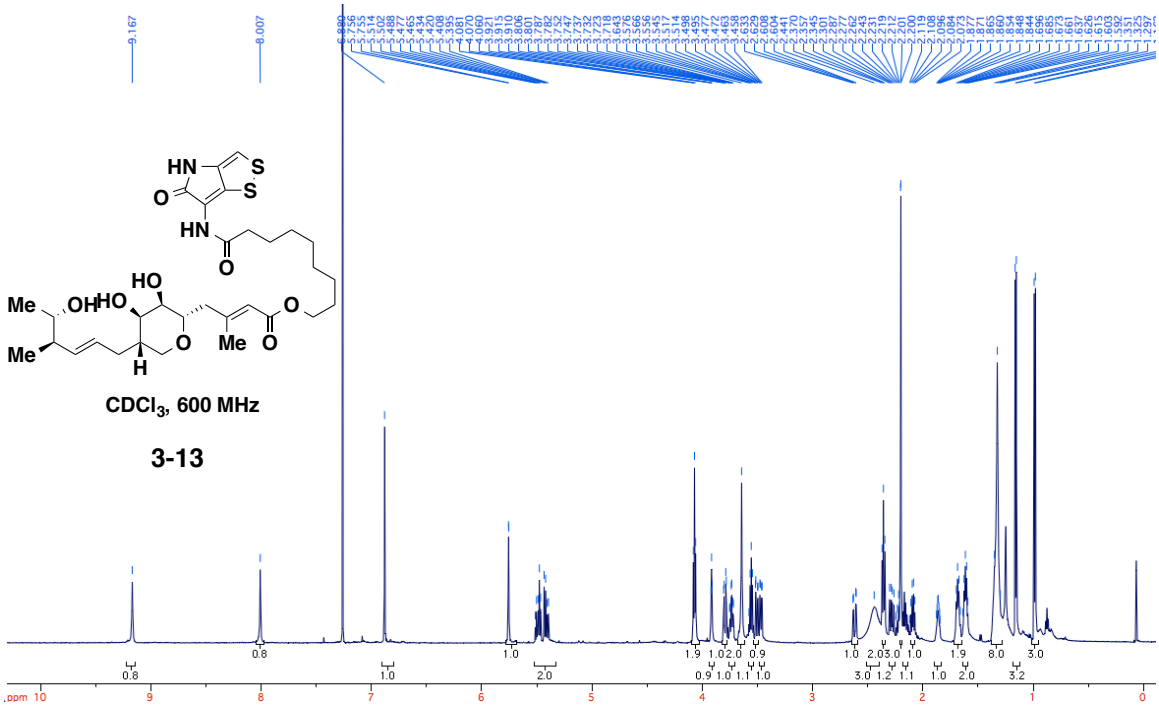
$CDCl_3$, 600 MHz

3-12



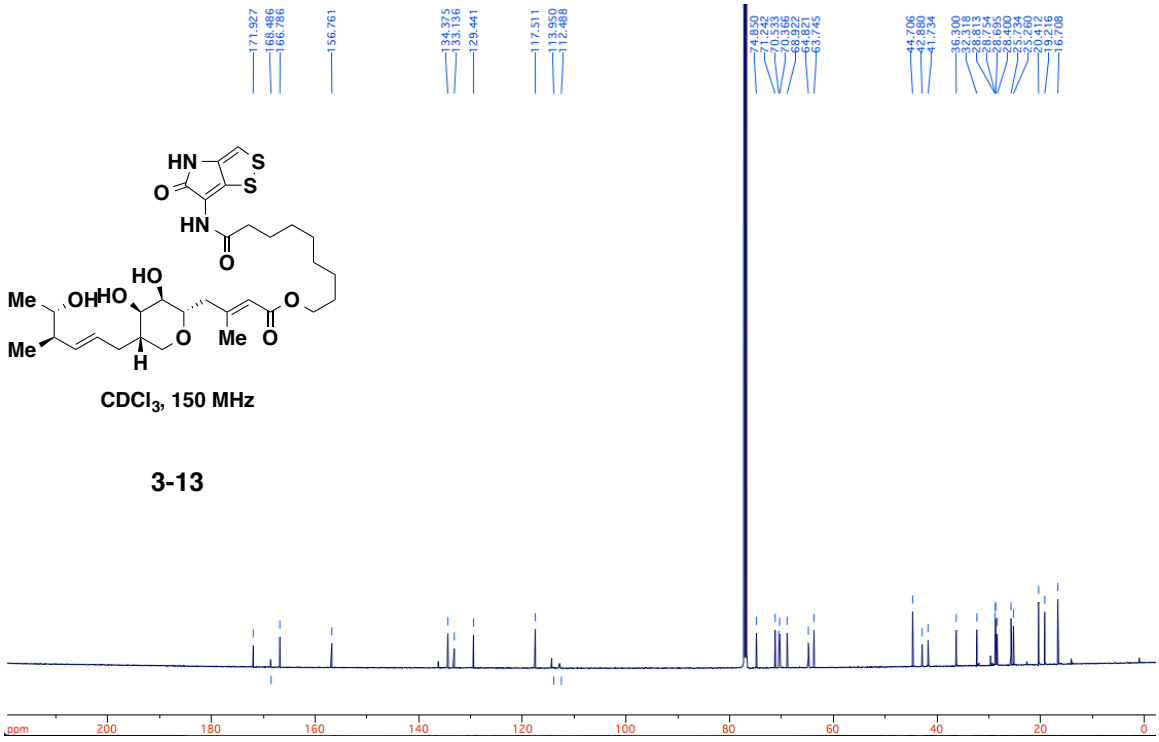
$CDCl_3$, 150 MHz

3-12



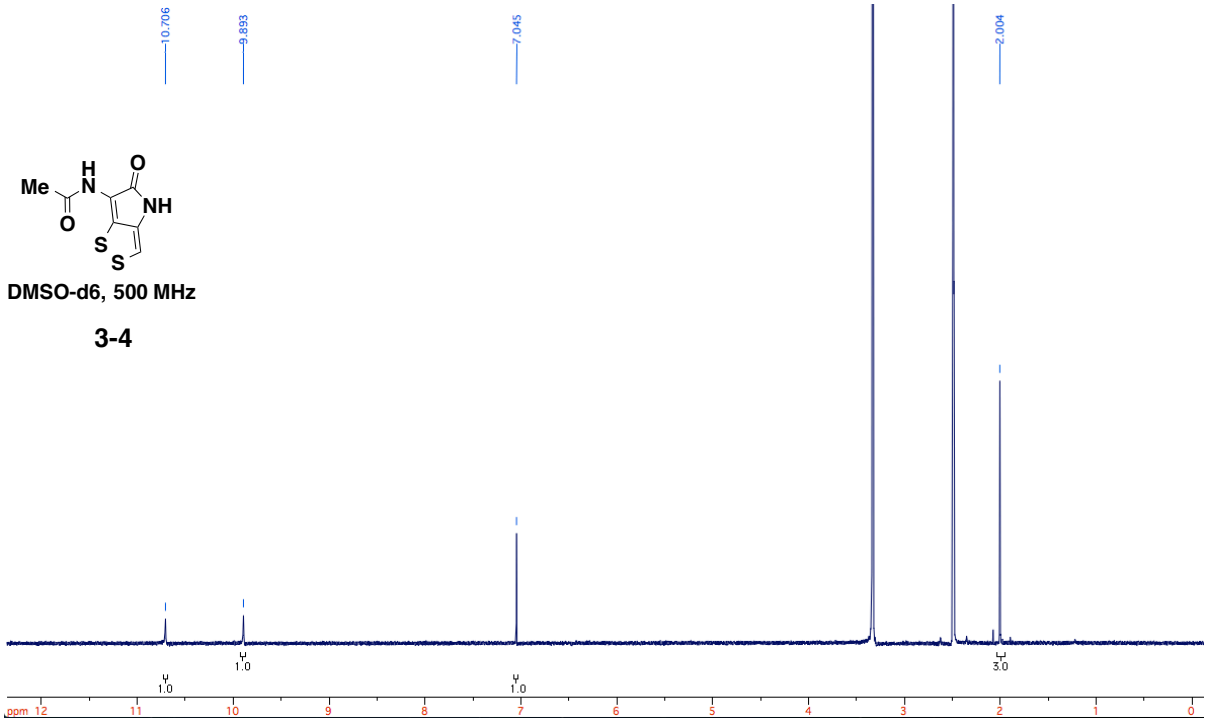
$CDCl_3$, 600 MHz

3-13



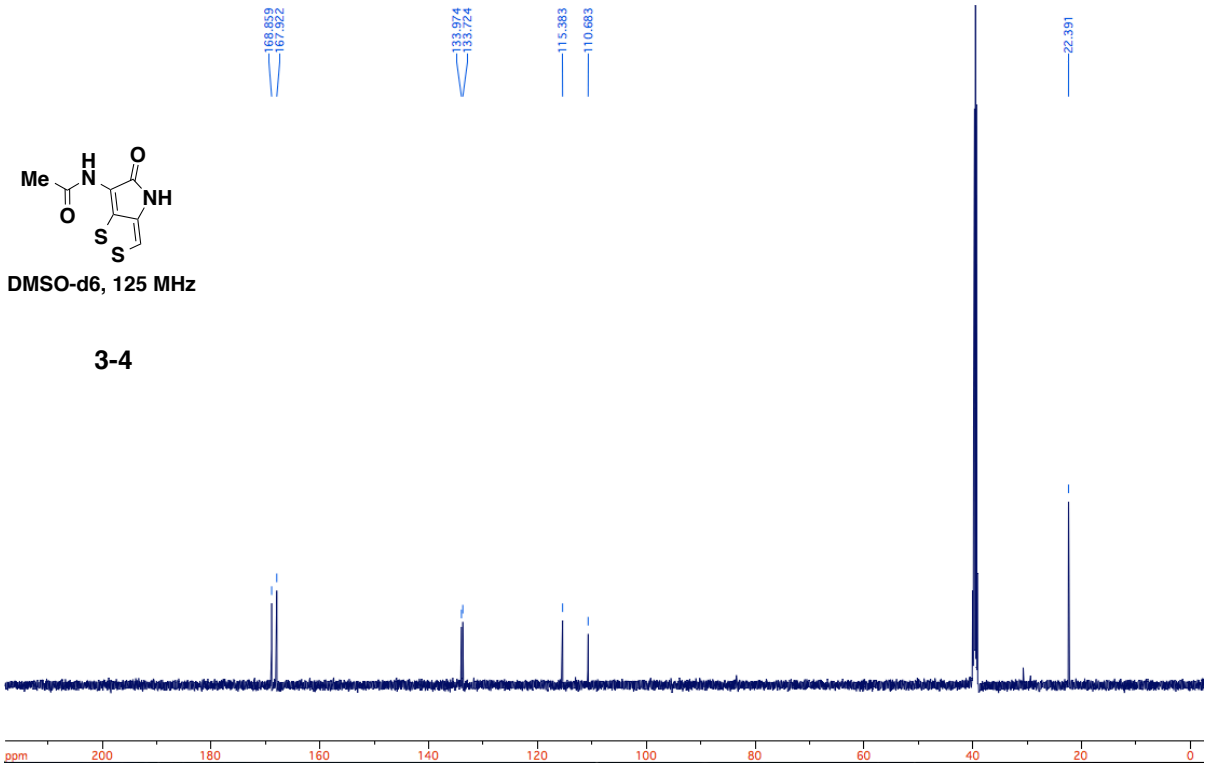
$CDCl_3$, 150 MHz

3-13



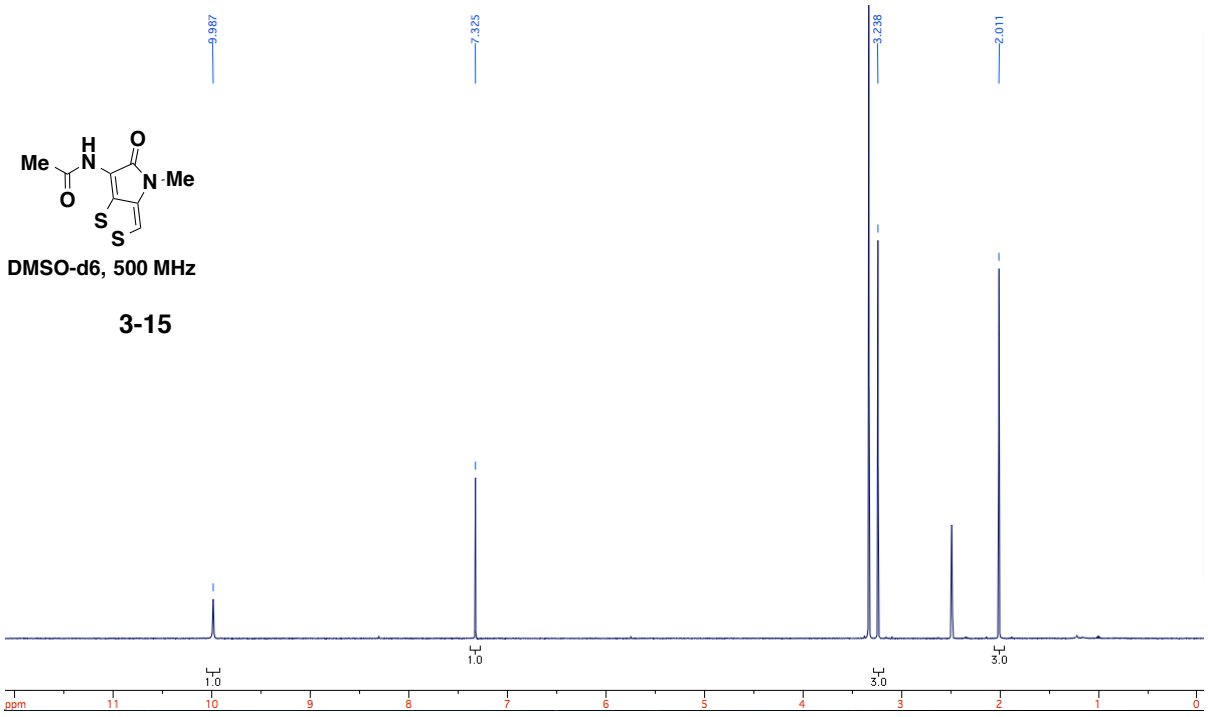
DMSO-d6, 500 MHz

3-4



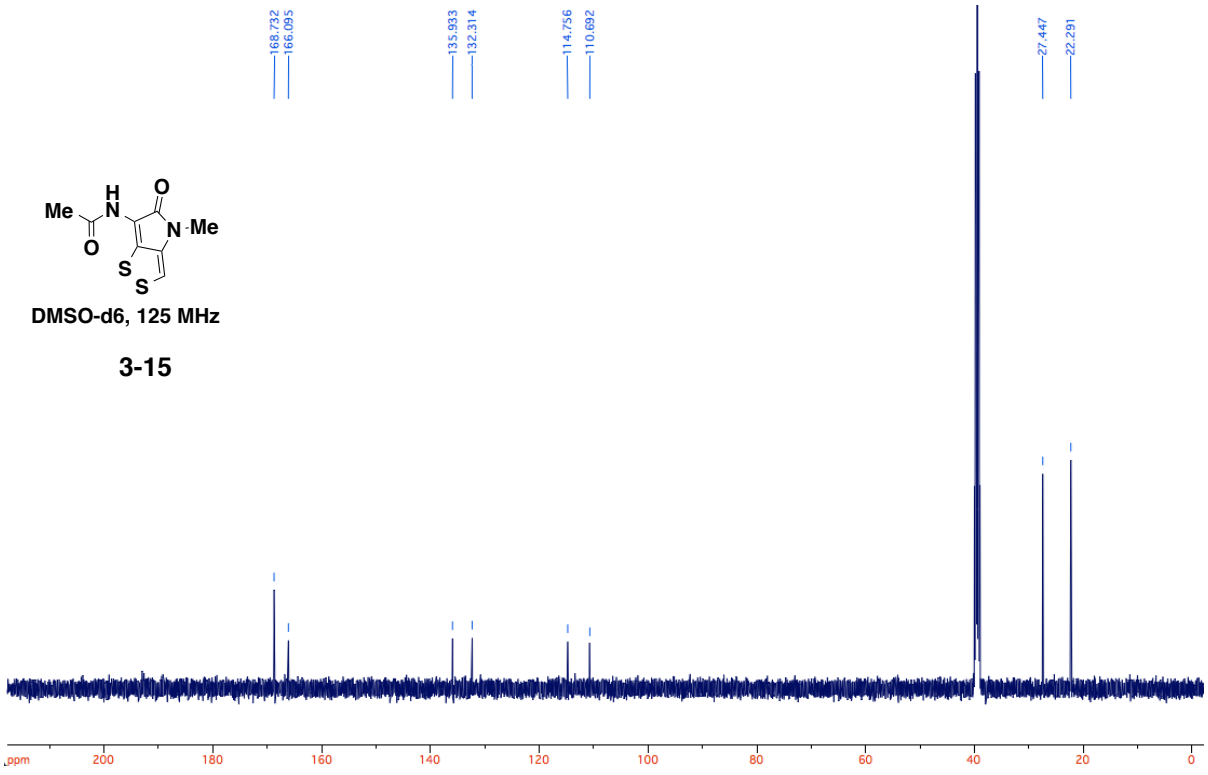
DMSO-d6, 125 MHz

3-4



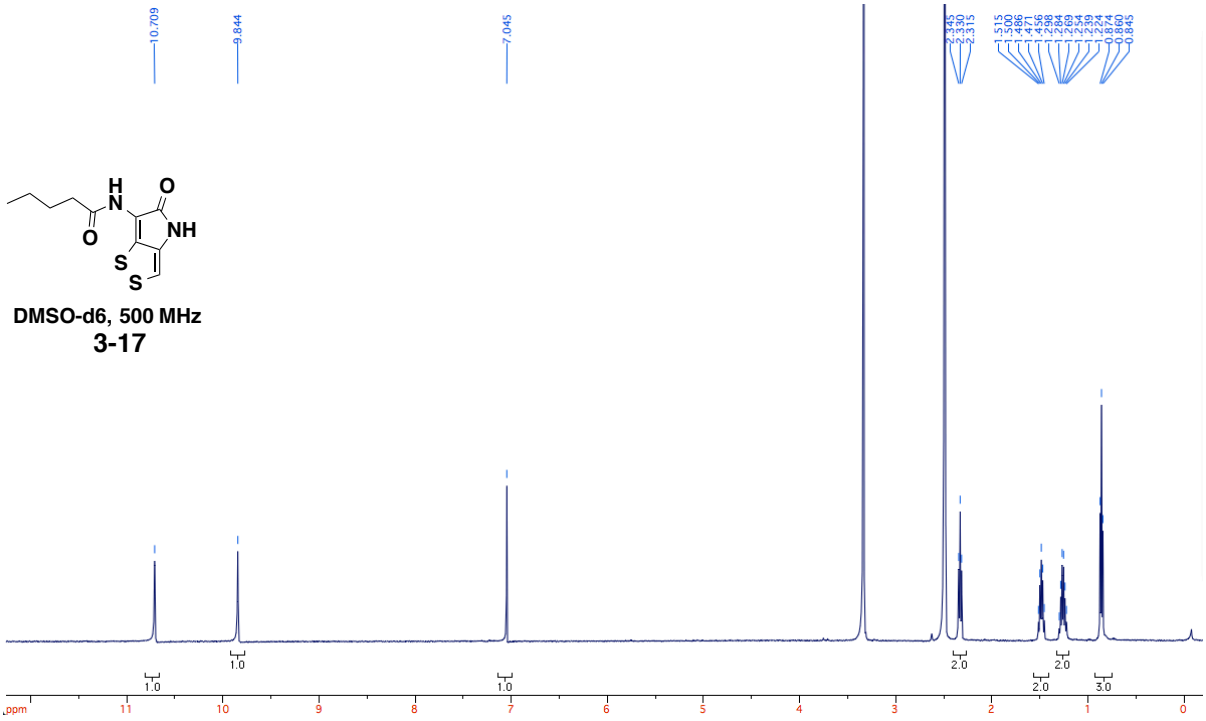
DMSO-d6, 500 MHz

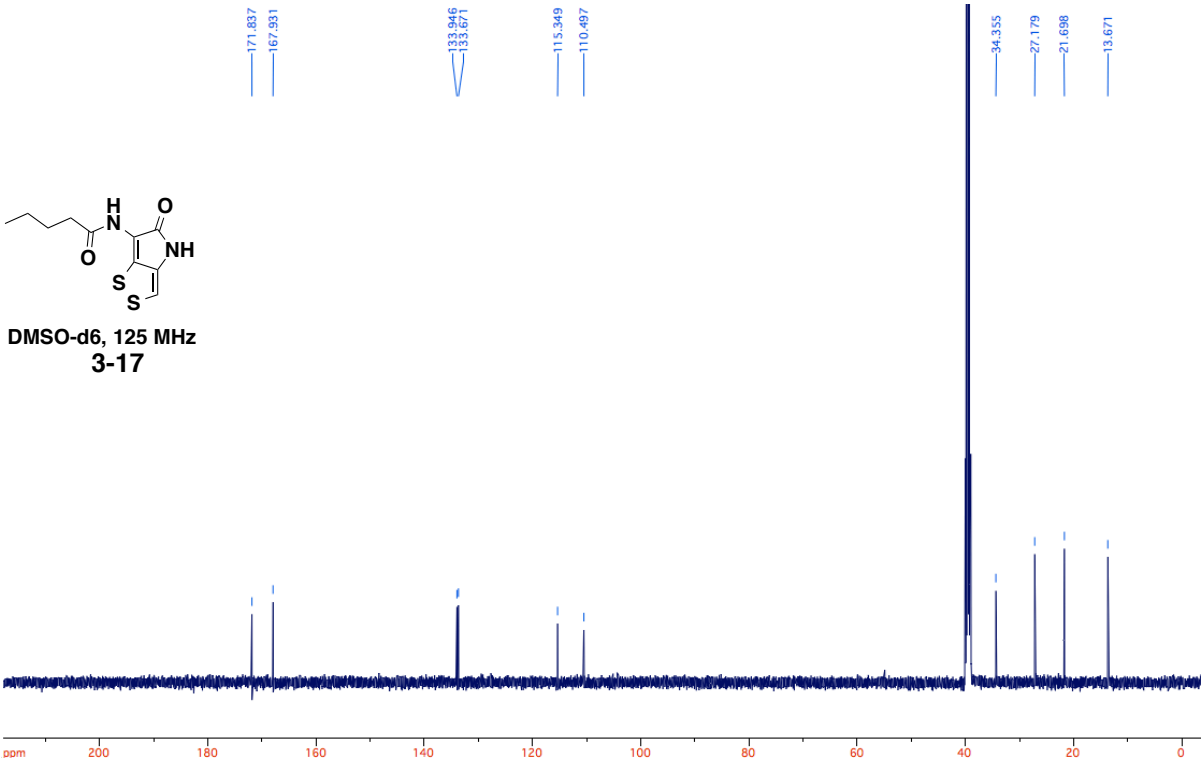
3-15

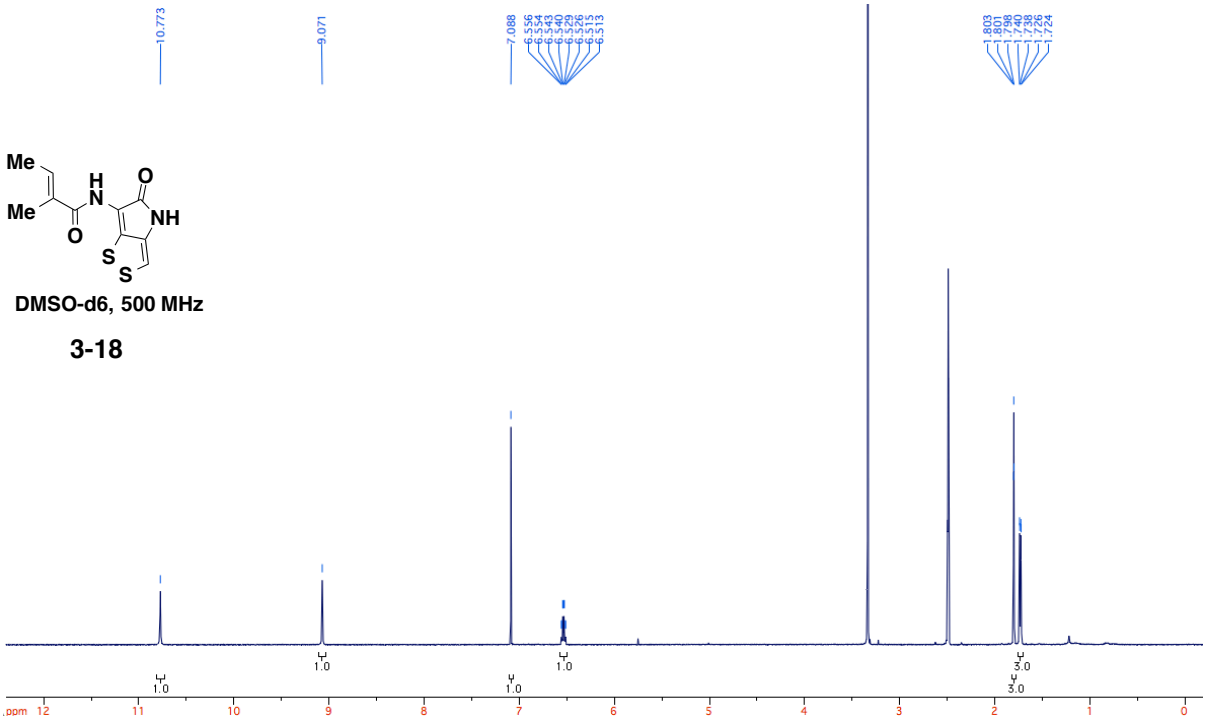


DMSO-d6, 125 MHz

3-15

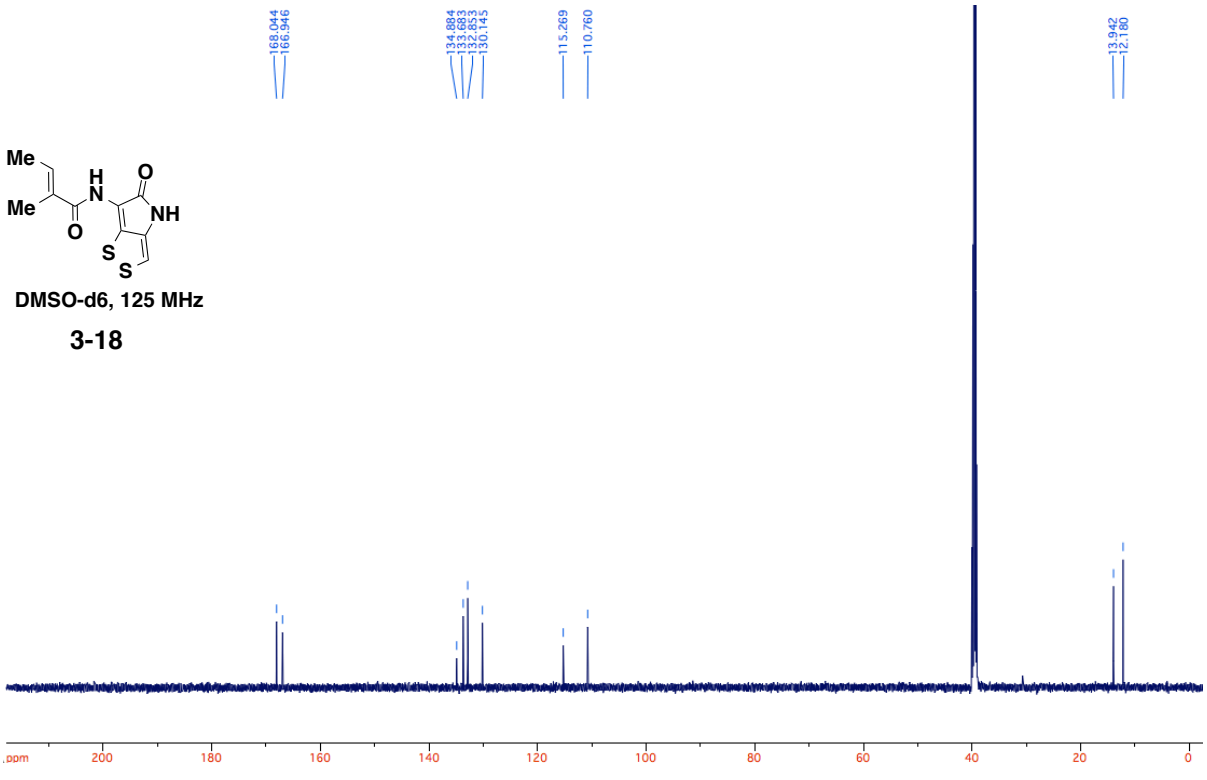

10.709
9.844
7.045
2.345
2.330
2.315
1.515
1.500
1.486
1.471
1.456
1.298
1.284
1.269
1.254
1.239
1.224
0.874
0.860
0.845
H
N
O
O
NH
S
S
DMSO-d6, 500 MHz
3-17
1.0
1.0
1.0
2.0
2.0
3.0
ppm
11
10
9
8
7
6
5
4
3
2
1
0


171.837
167.931
133.946
133.671
115.349
110.497
34.355
27.179
21.698
13.671
H
N
O
O
NH
S
S
DMSO-d6, 125 MHz
3-17
ppm
200
180
160
140
120
100
80
60
40
20
0



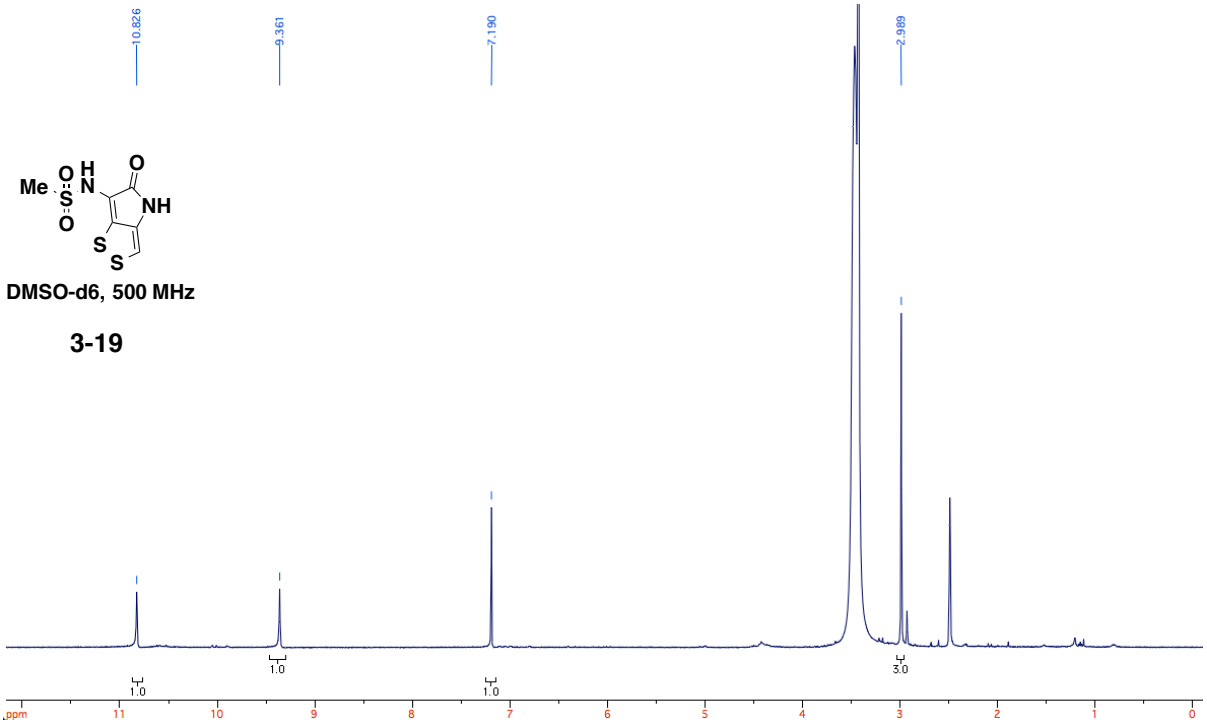
DMSO-d6, 500 MHz

3-18



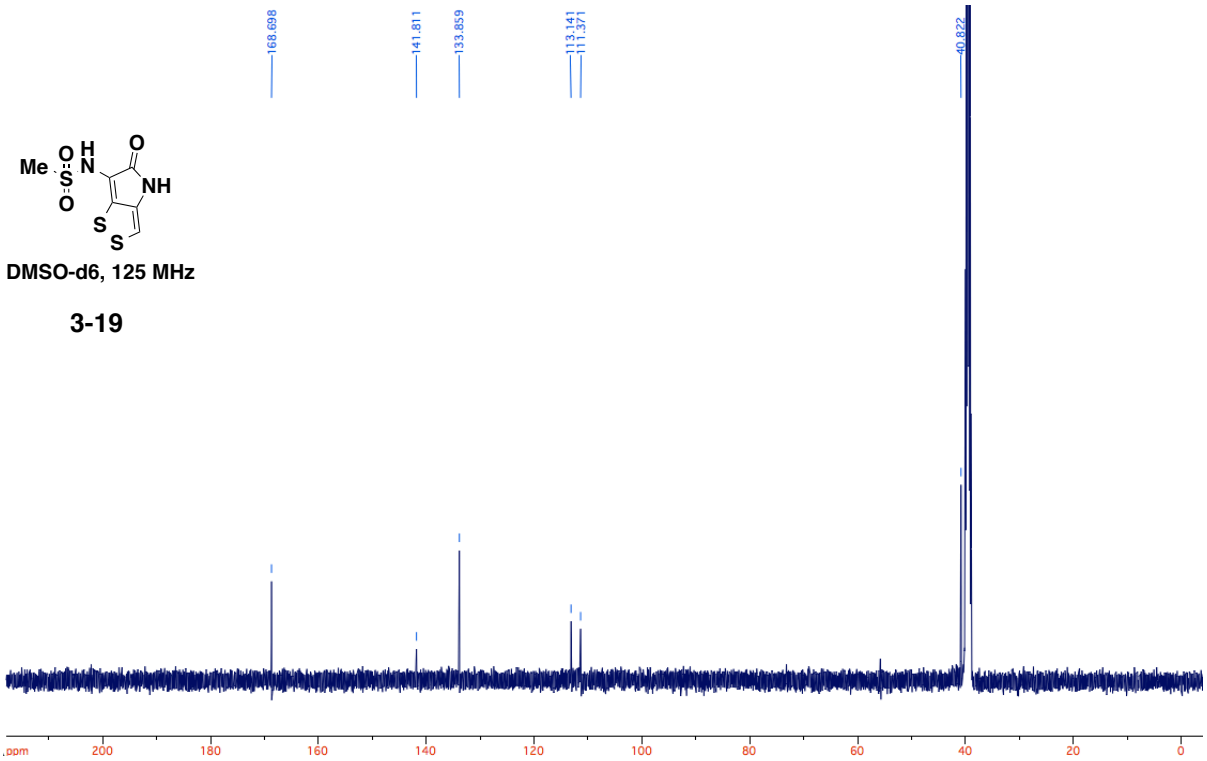
DMSO-d6, 125 MHz

3-18



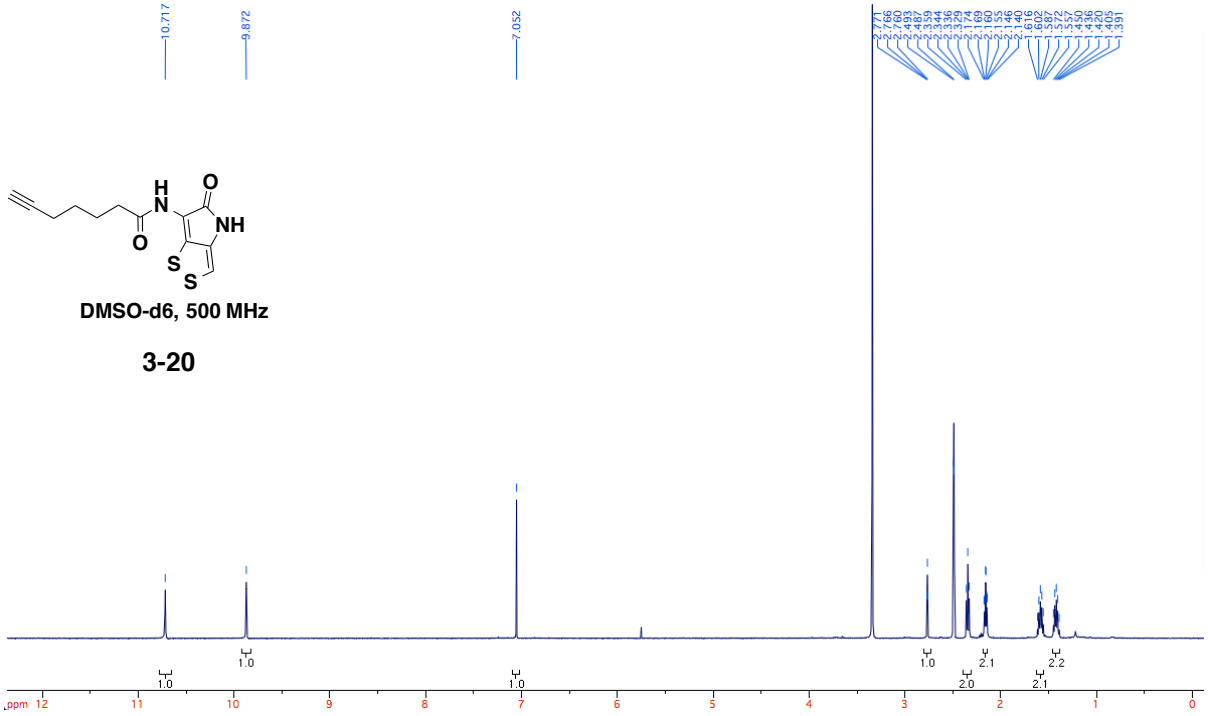
DMSO-d6, 500 MHz

3-19



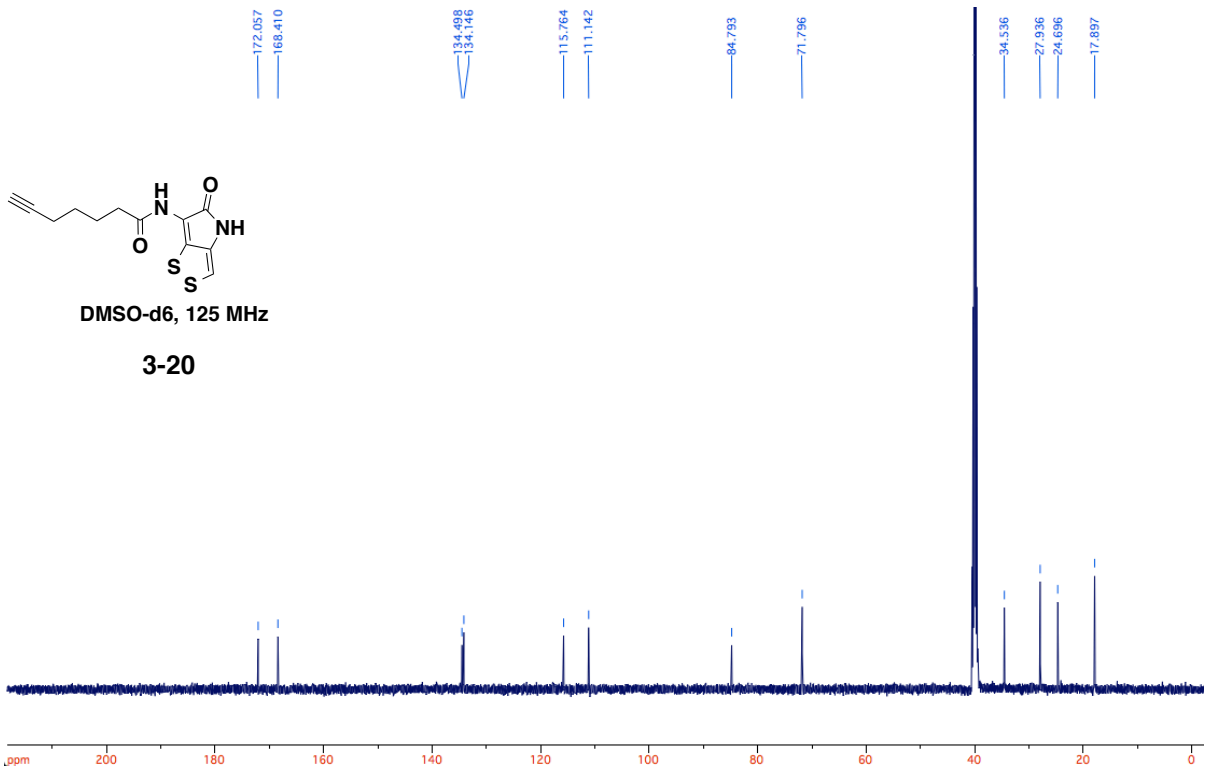
DMSO-d6, 125 MHz

3-19



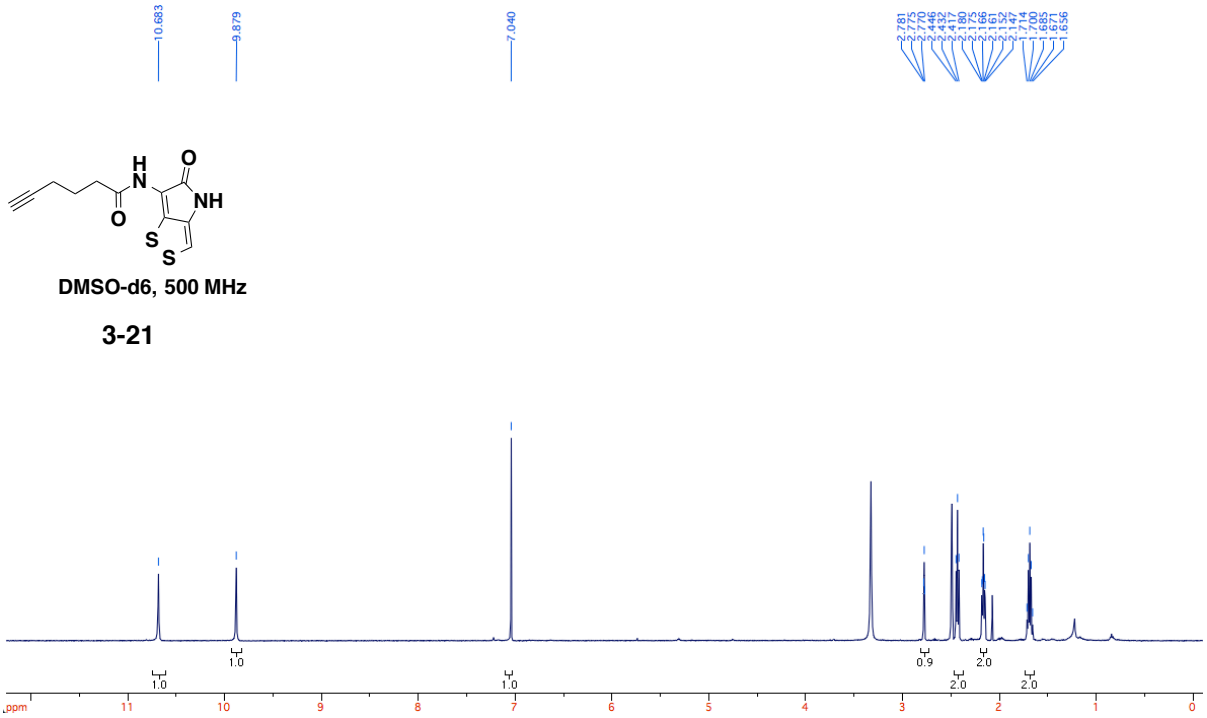
DMSO-d6, 500 MHz

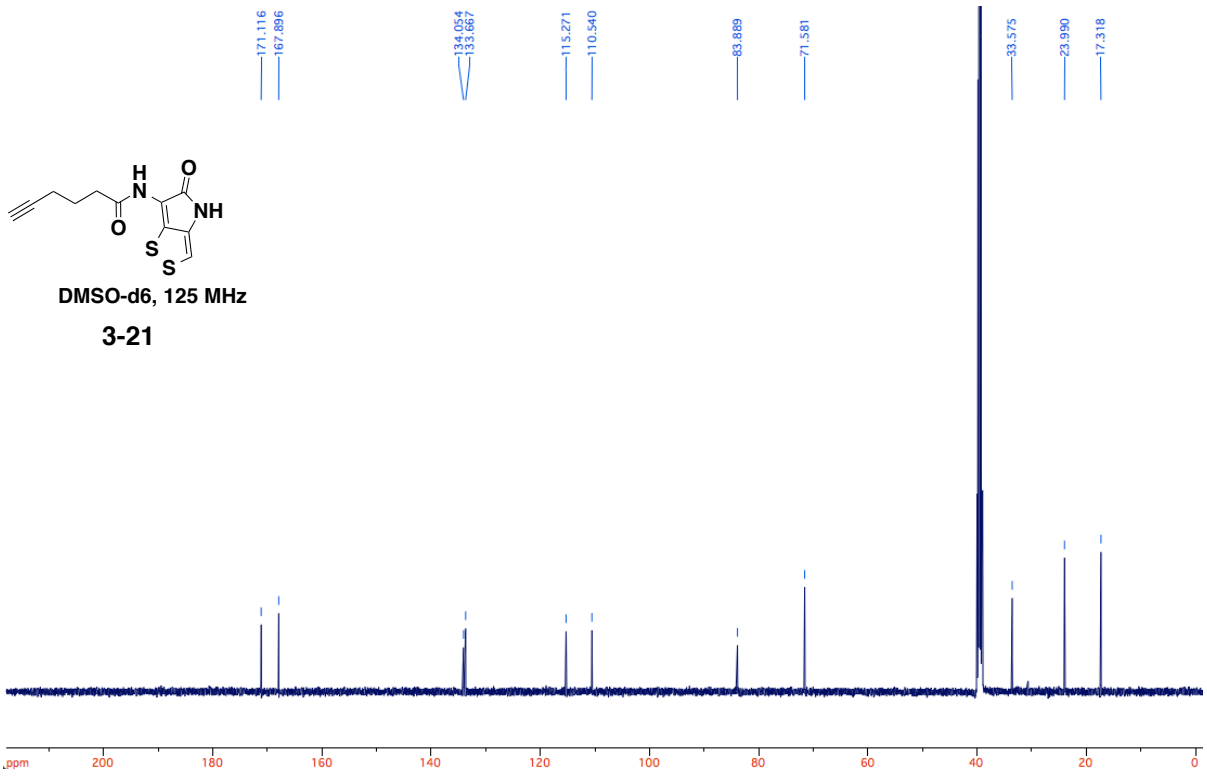
3-20



DMSO-d6, 125 MHz

3-20



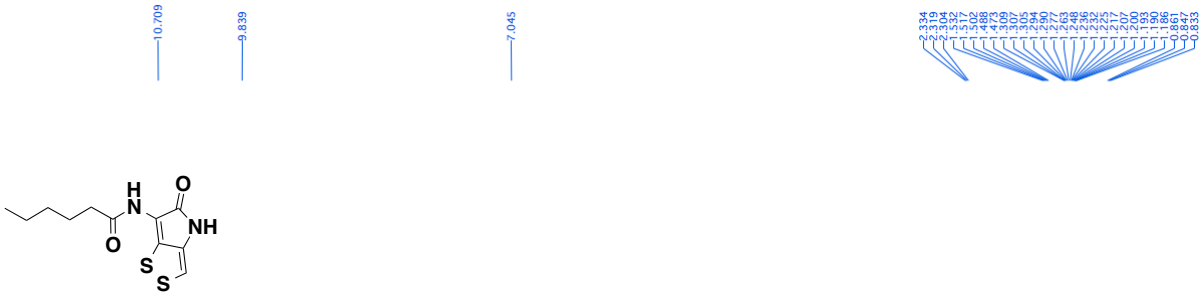


DMSO-d6, 500 MHz

3-21

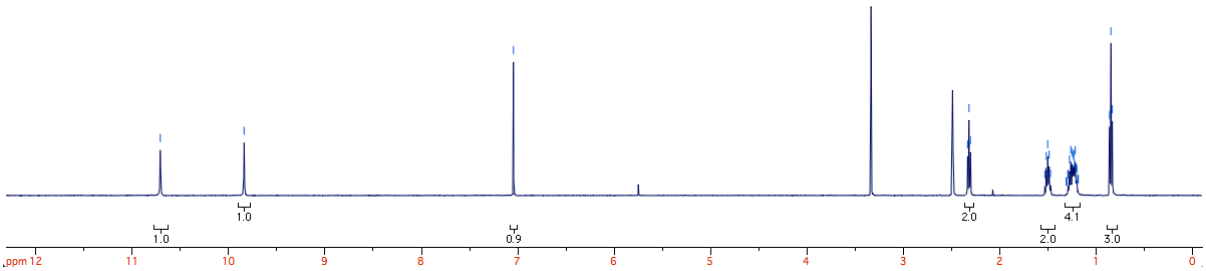
DMSO-d6, 125 MHz

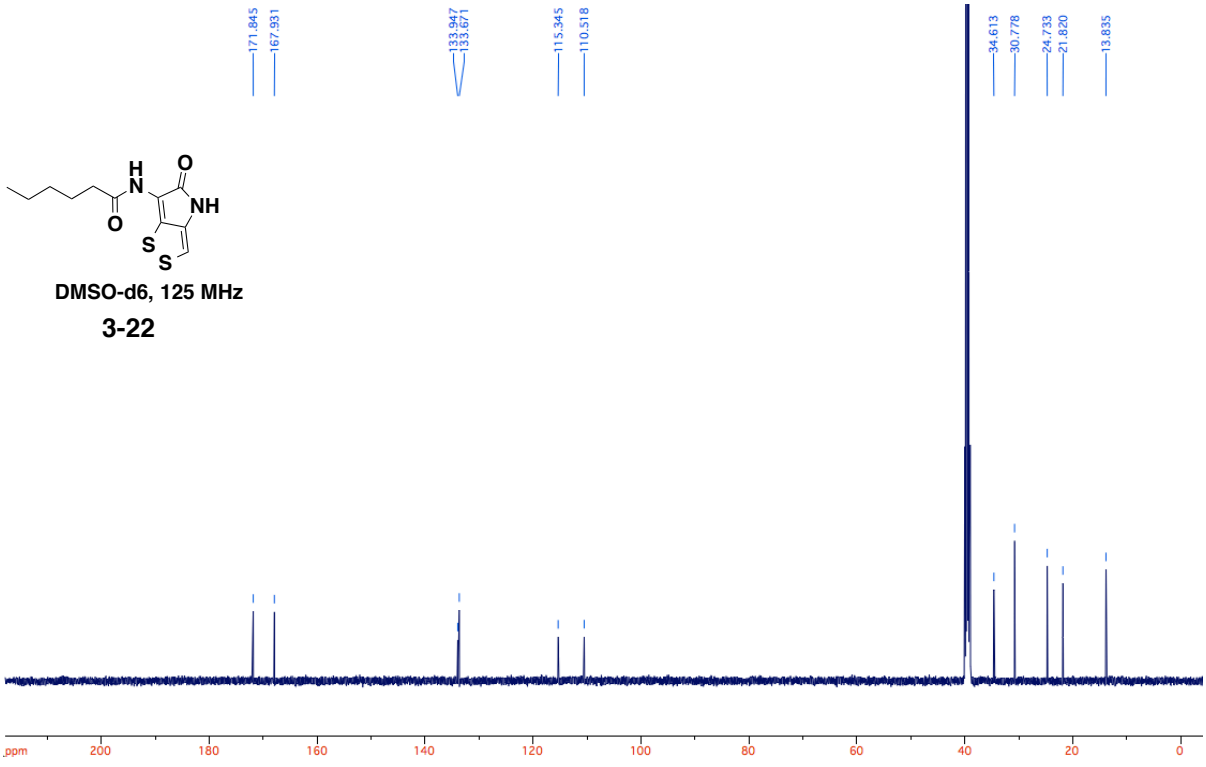
3-21



DMSO-d6, 500 MHz

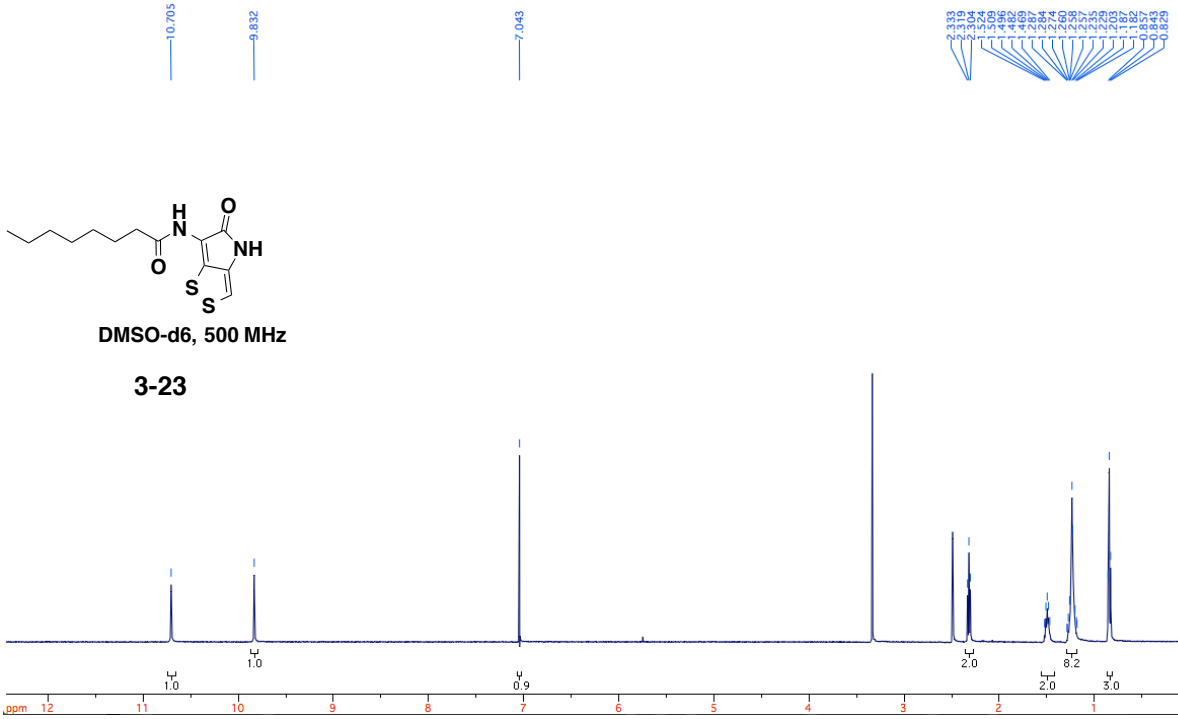
3-22





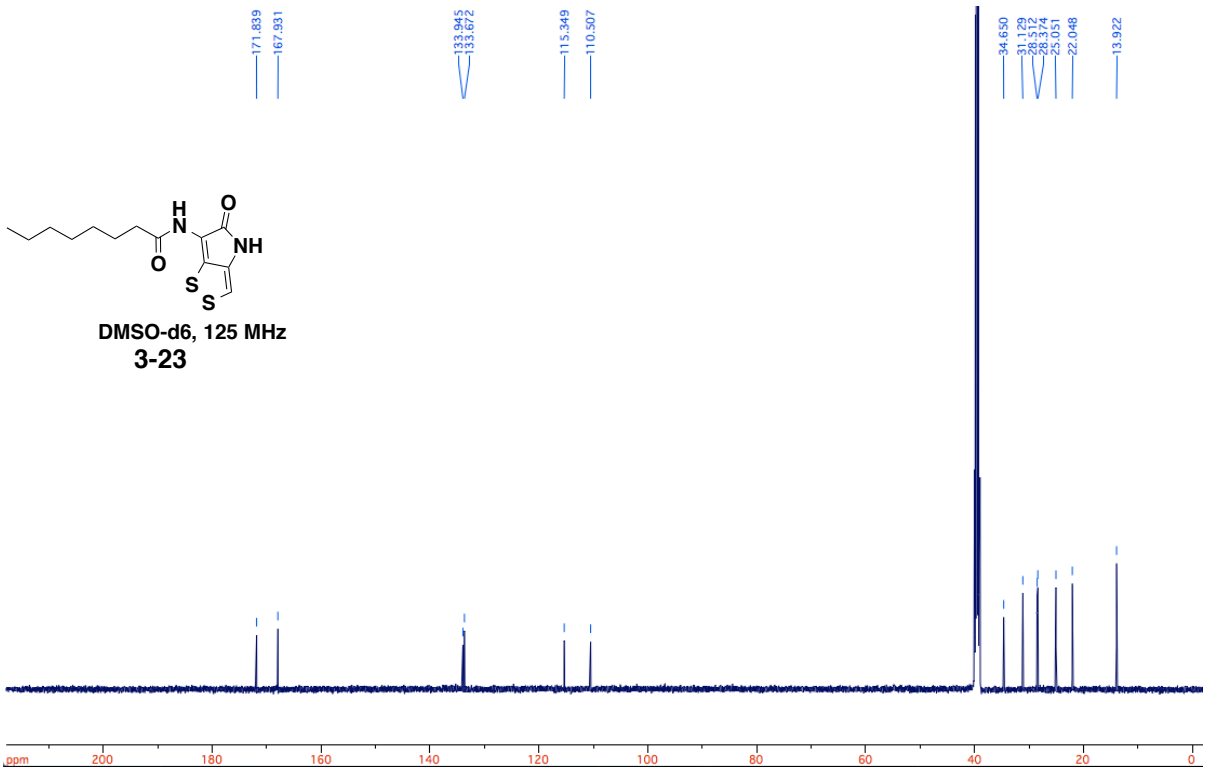
DMSO-d6, 125 MHz

3-22



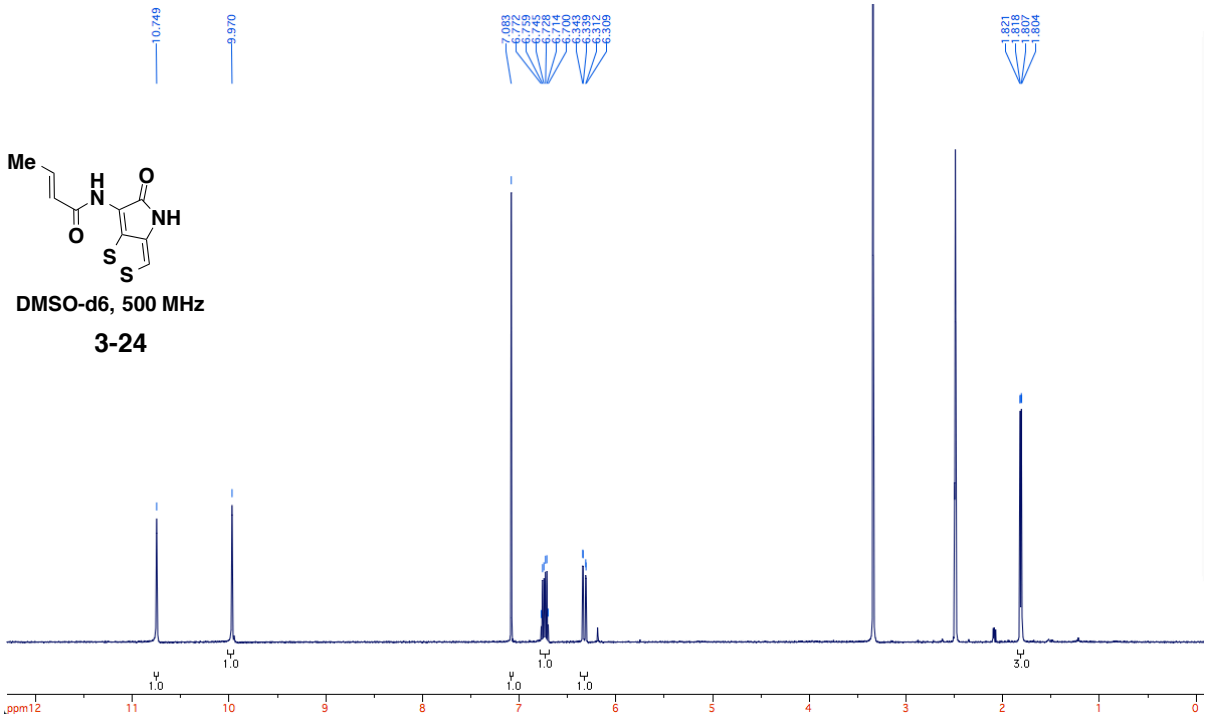
DMSO-d6, 500 MHz

3-23



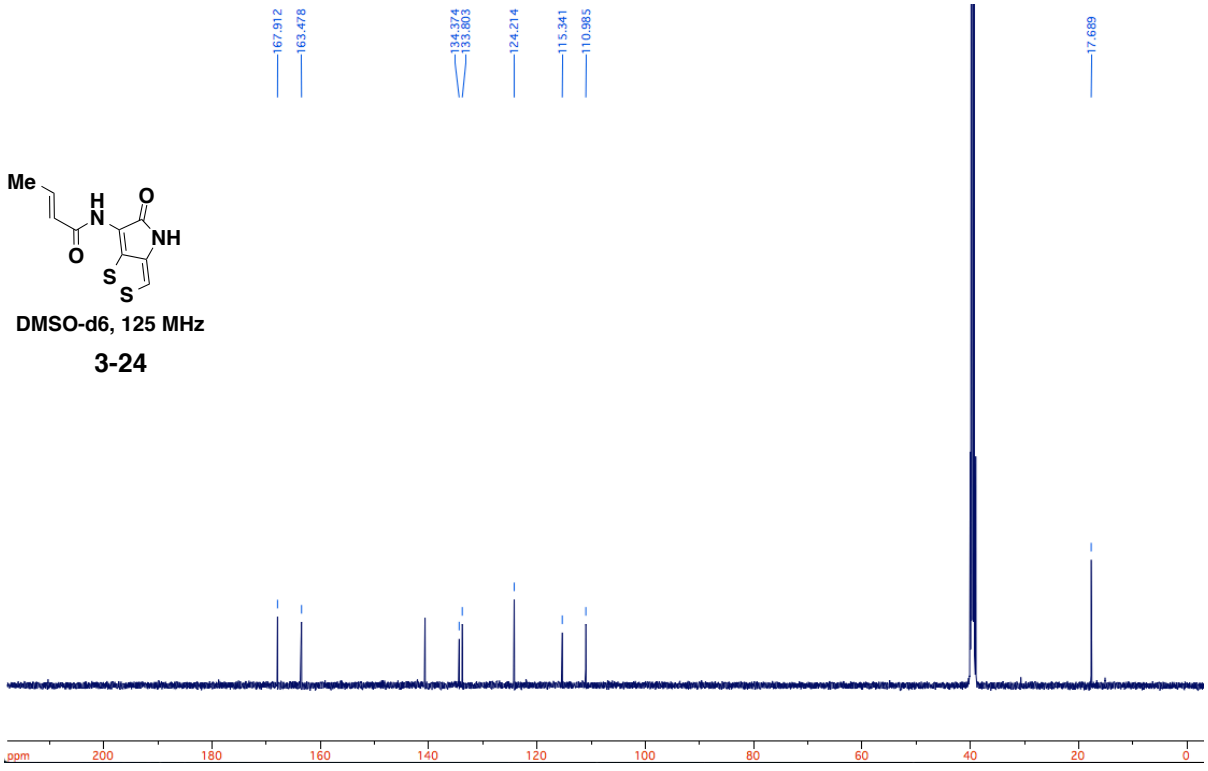
DMSO-d6, 125 MHz

3-23



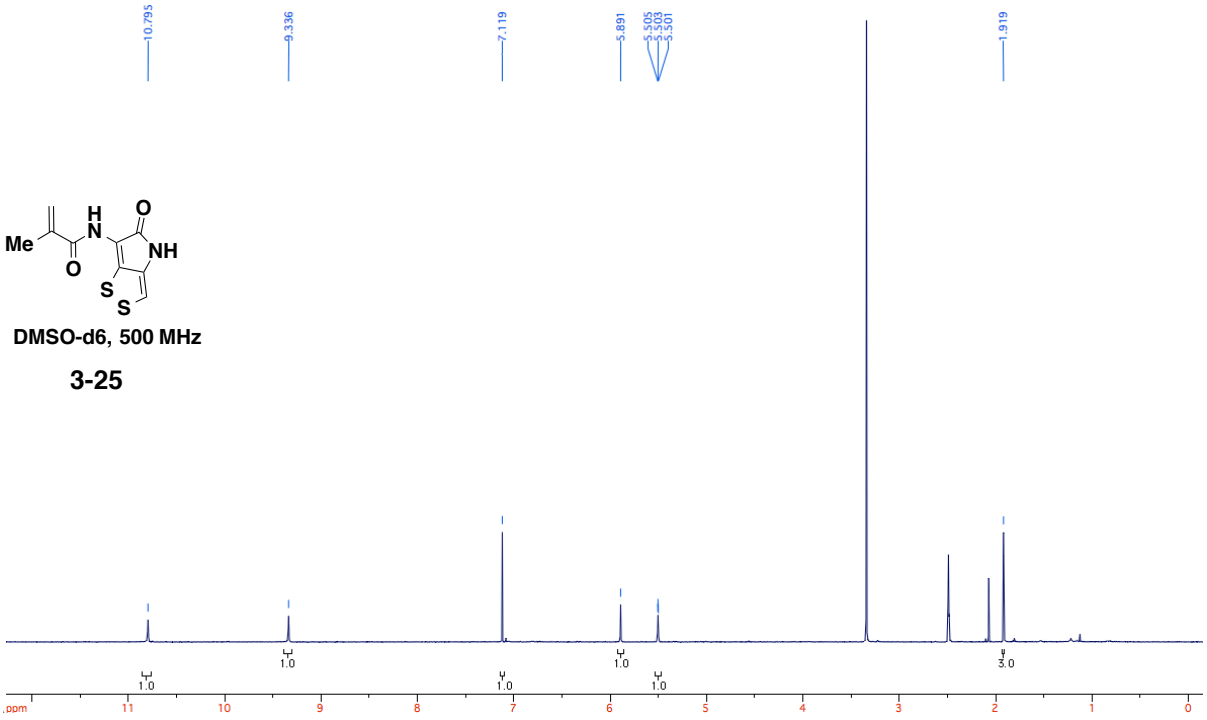
DMSO-d6, 500 MHz

3-24



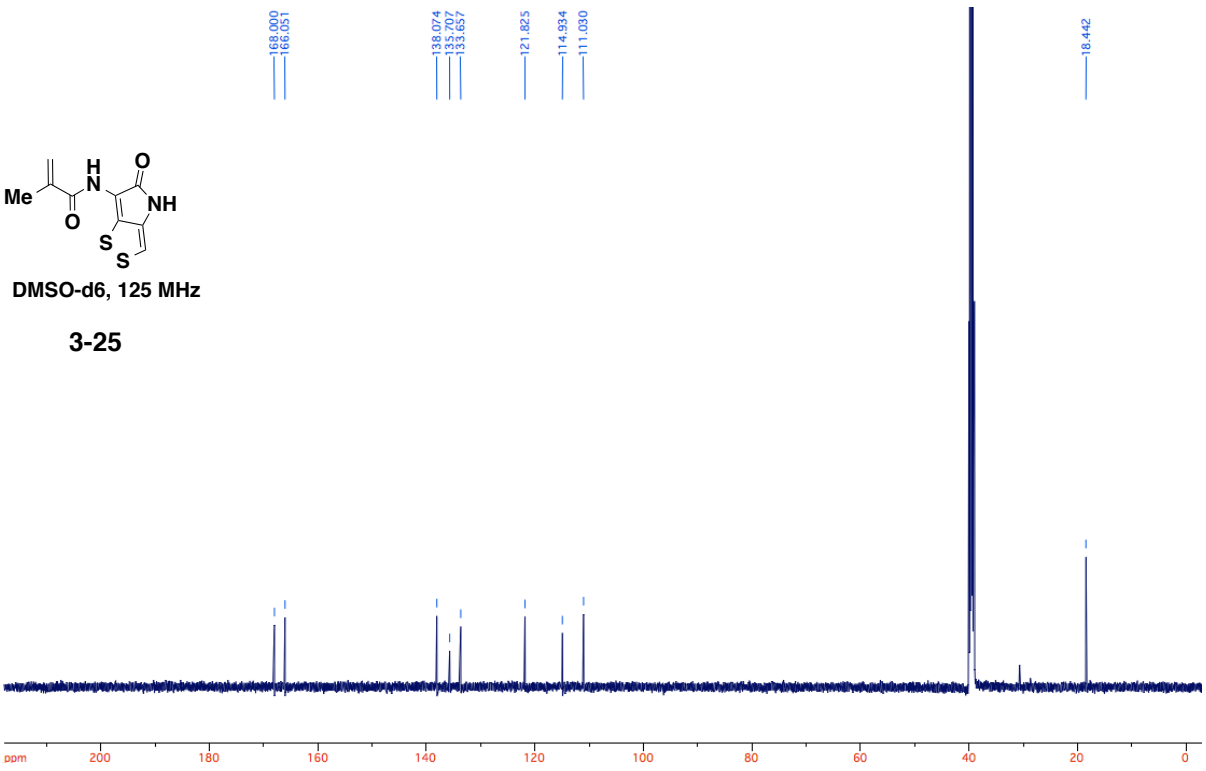
DMSO-d6, 125 MHz

3-24



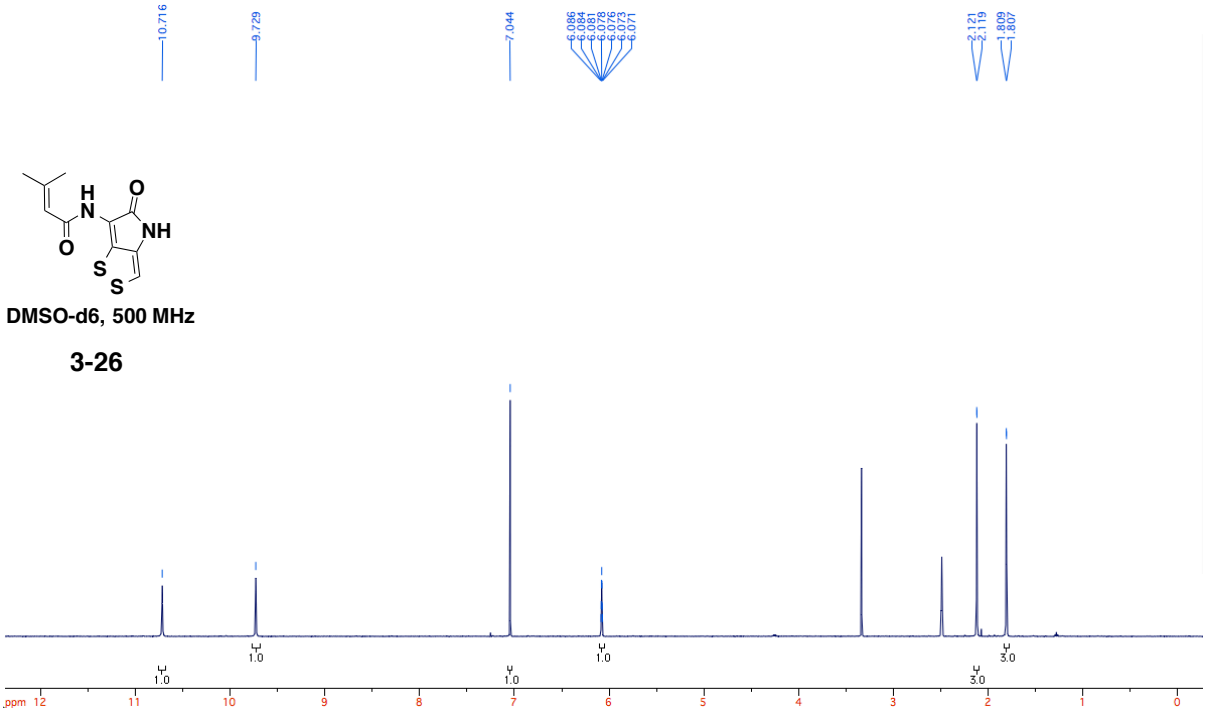
DMSO-d6, 500 MHz

3-25



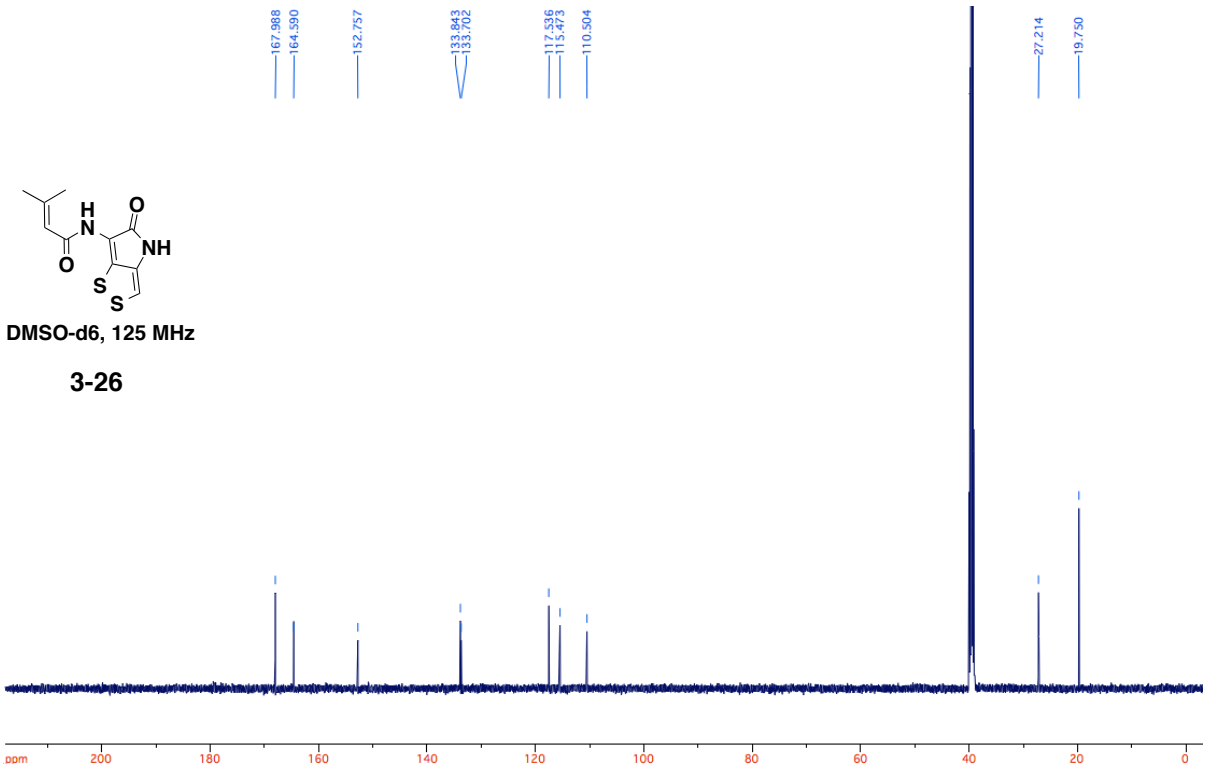
DMSO-d6, 125 MHz

3-25



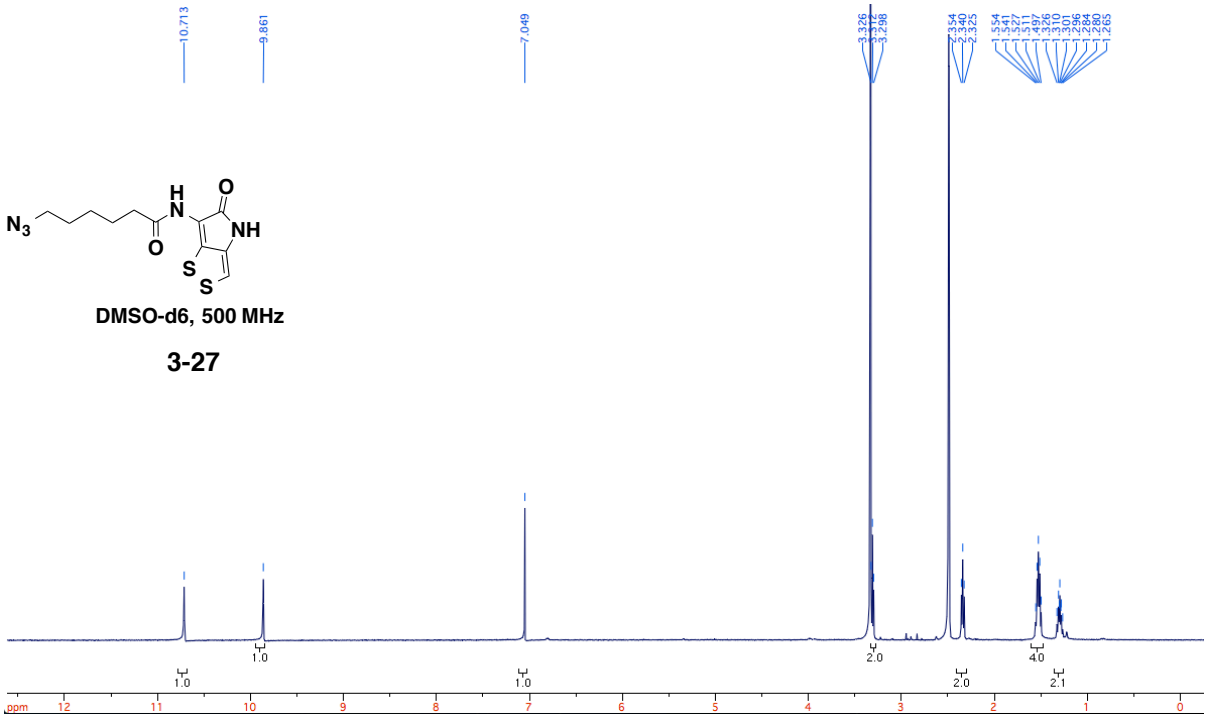
DMSO-d6, 500 MHz

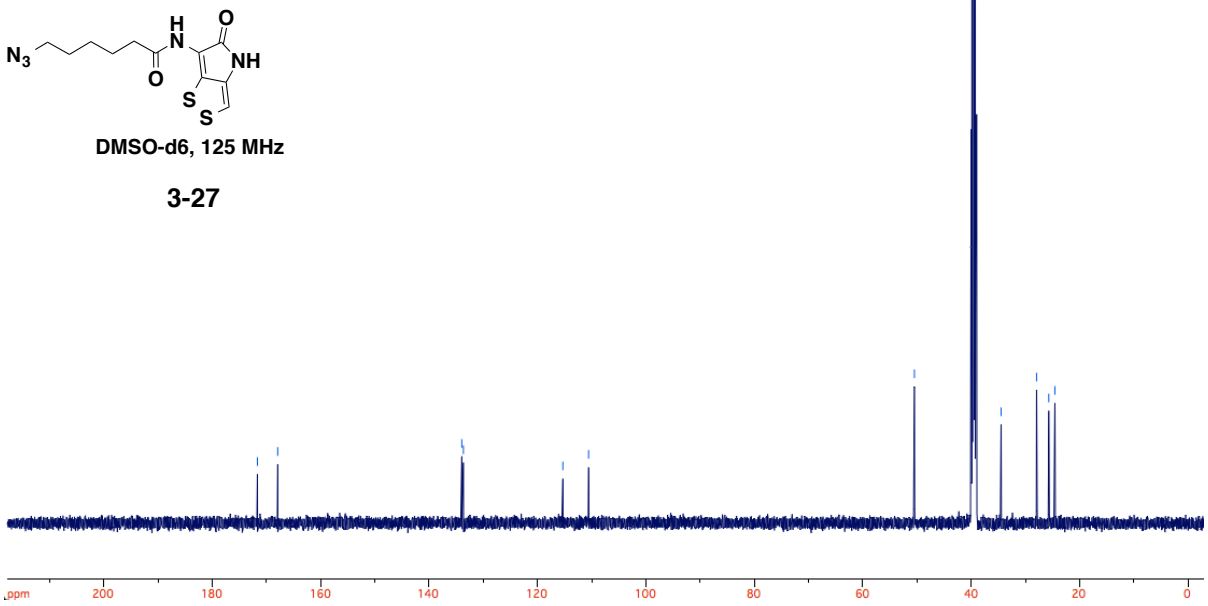
3-26



DMSO-d6, 125 MHz

3-26



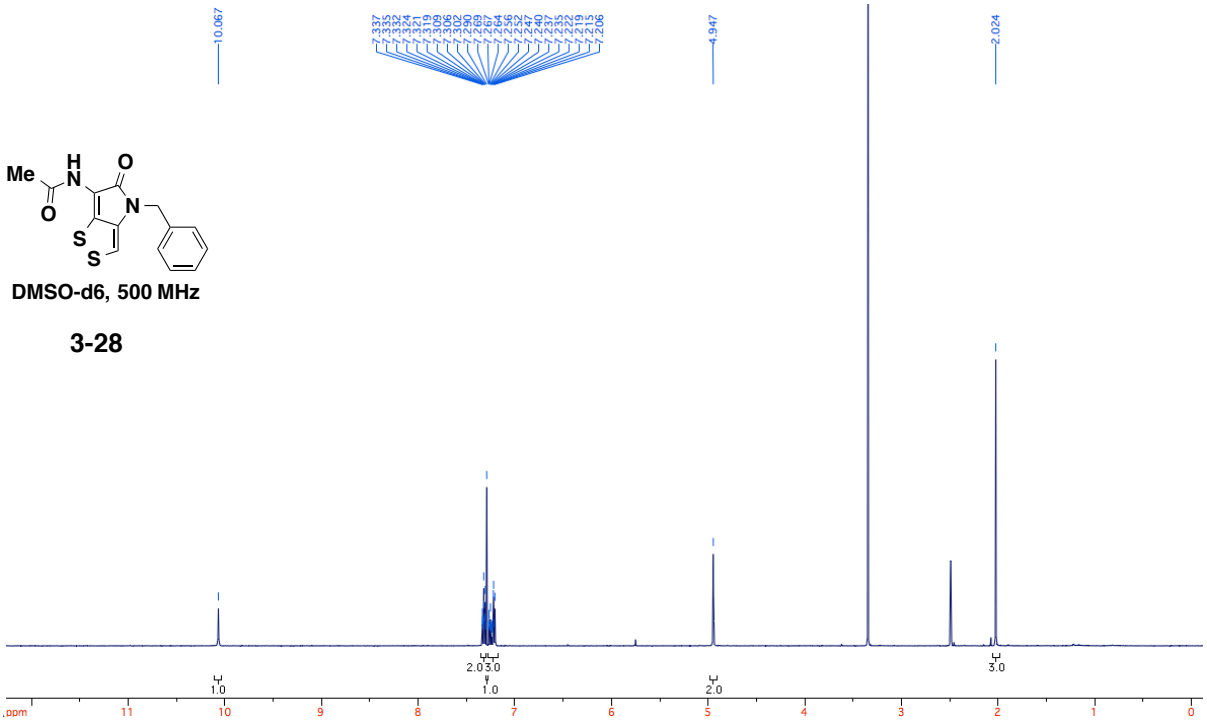


DMSO-d6, 500 MHz

3-27

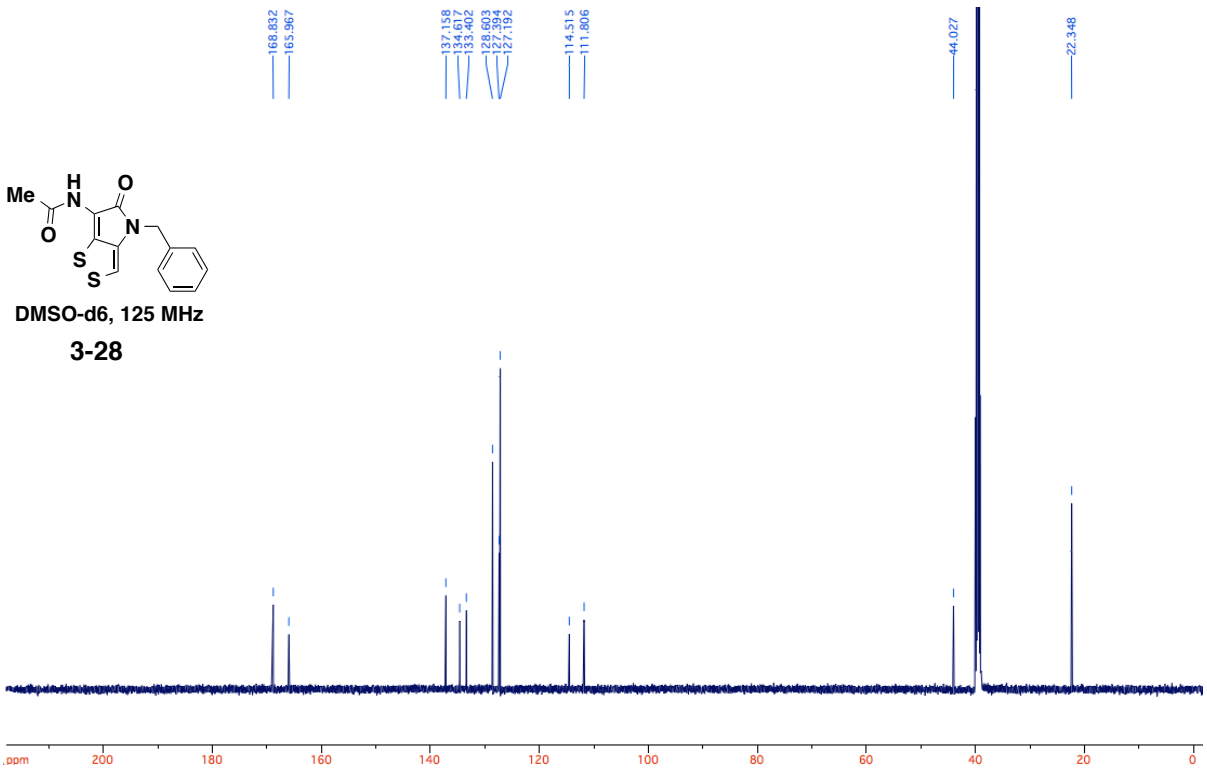
DMSO-d6, 125 MHz

3-27



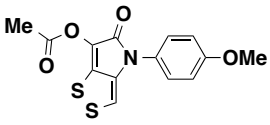
DMSO-d6, 500 MHz

3-28



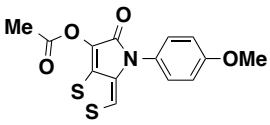
DMSO-d6, 125 MHz

3-28



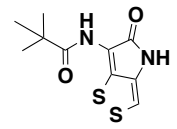
DMSO-d6, 500 MHz

3-29



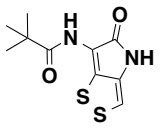
DMSO-d6, 125 MHz

3-29



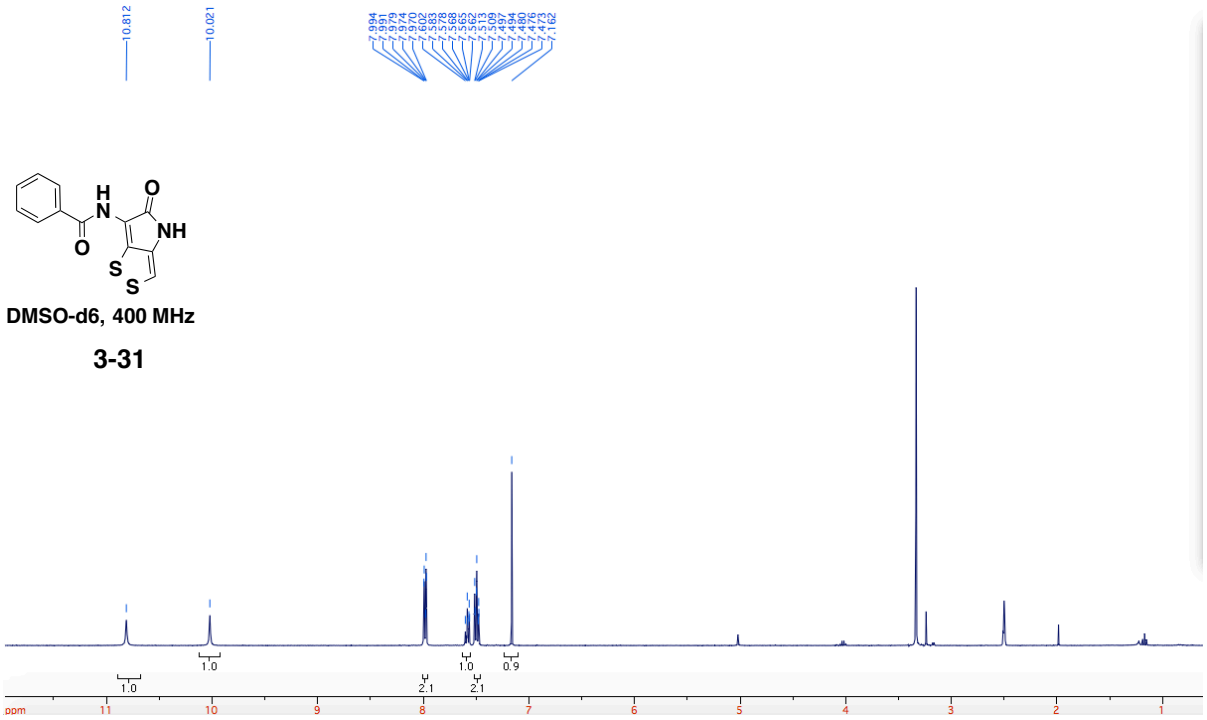
DMSO-d6, 400 MHz

3-30



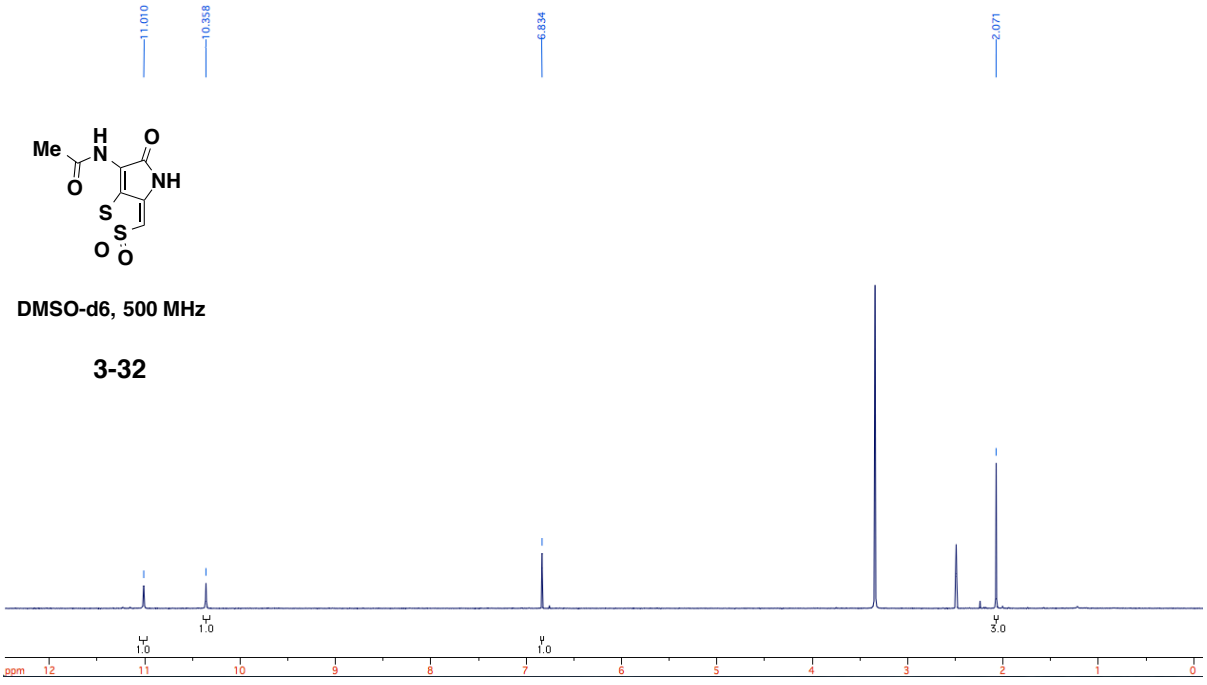
DMSO-d6, 100 MHz

3-30



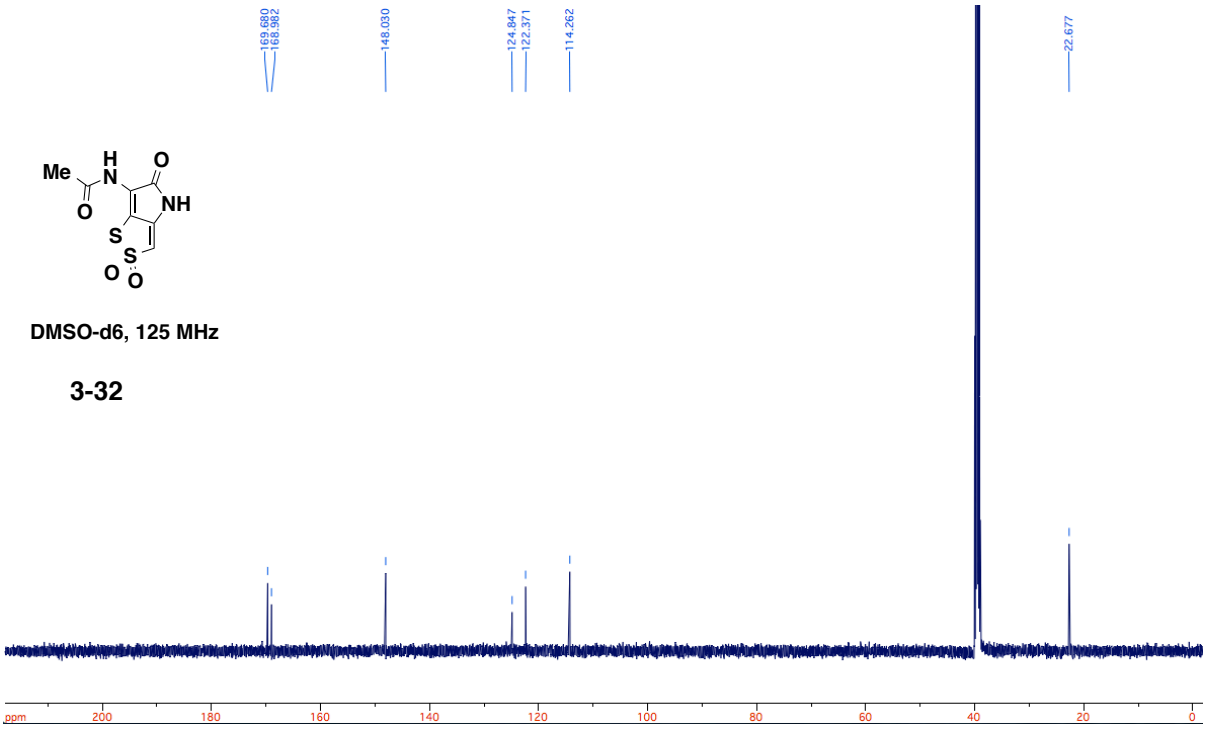
DMSO-d6, 400 MHz

3-31



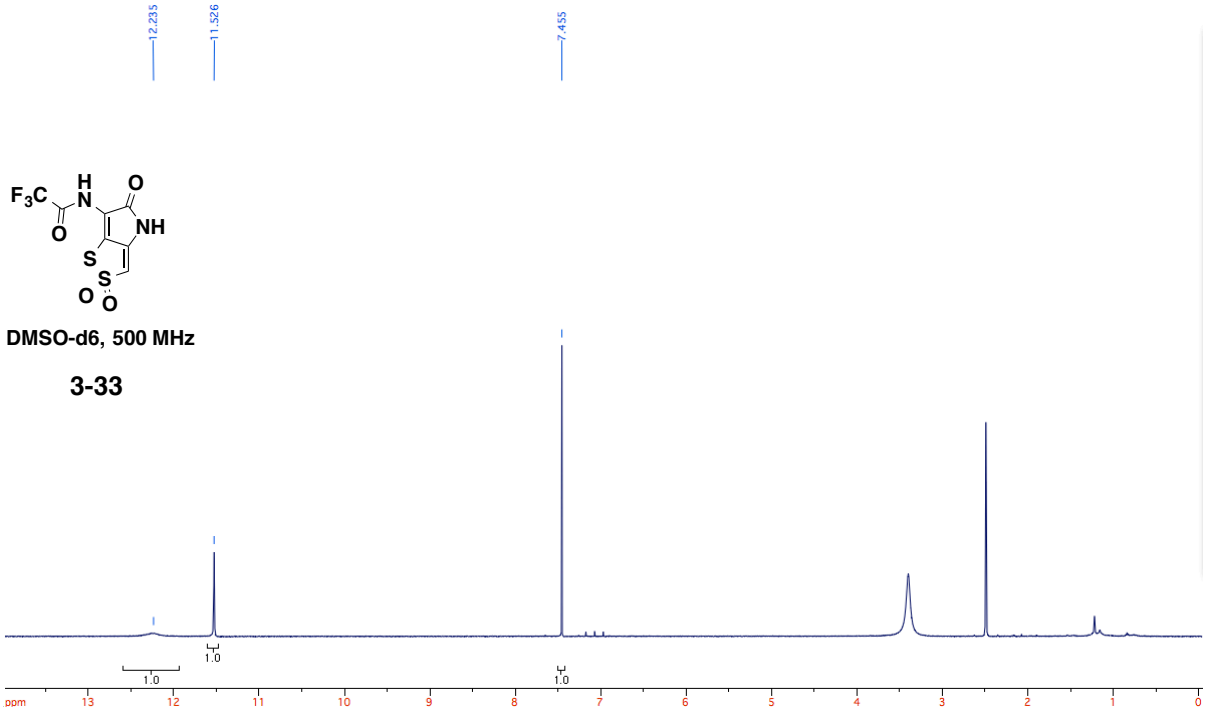
DMSO-d6, 500 MHz

3-32



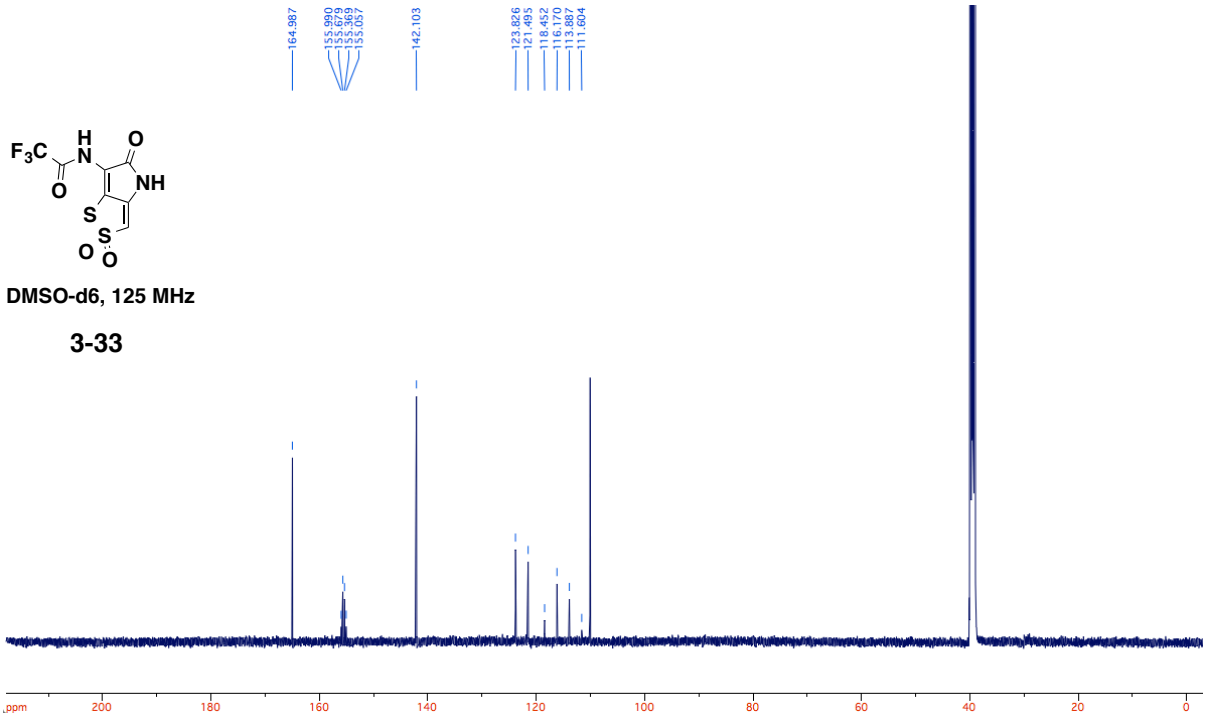
DMSO-d6, 125 MHz

3-32

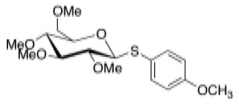


DMSO-d6, 500 MHz

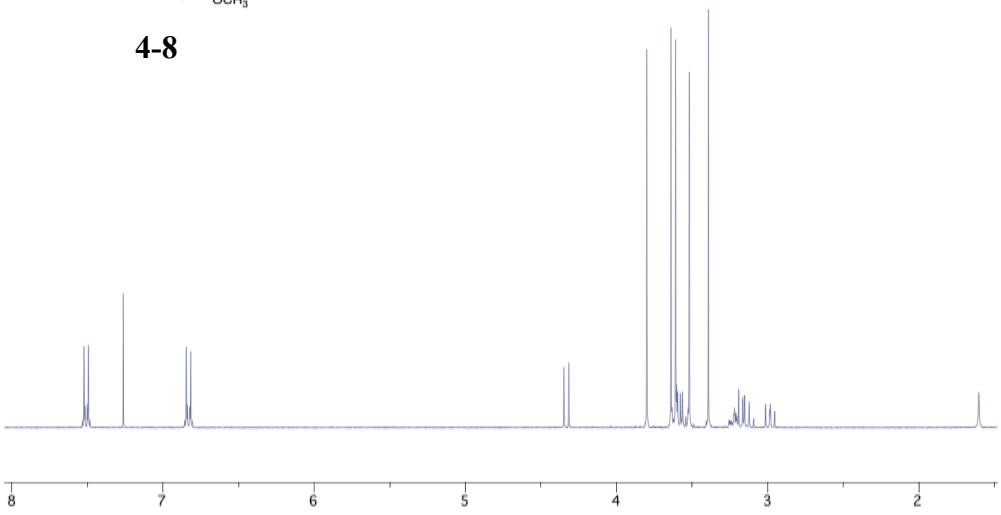
3-33

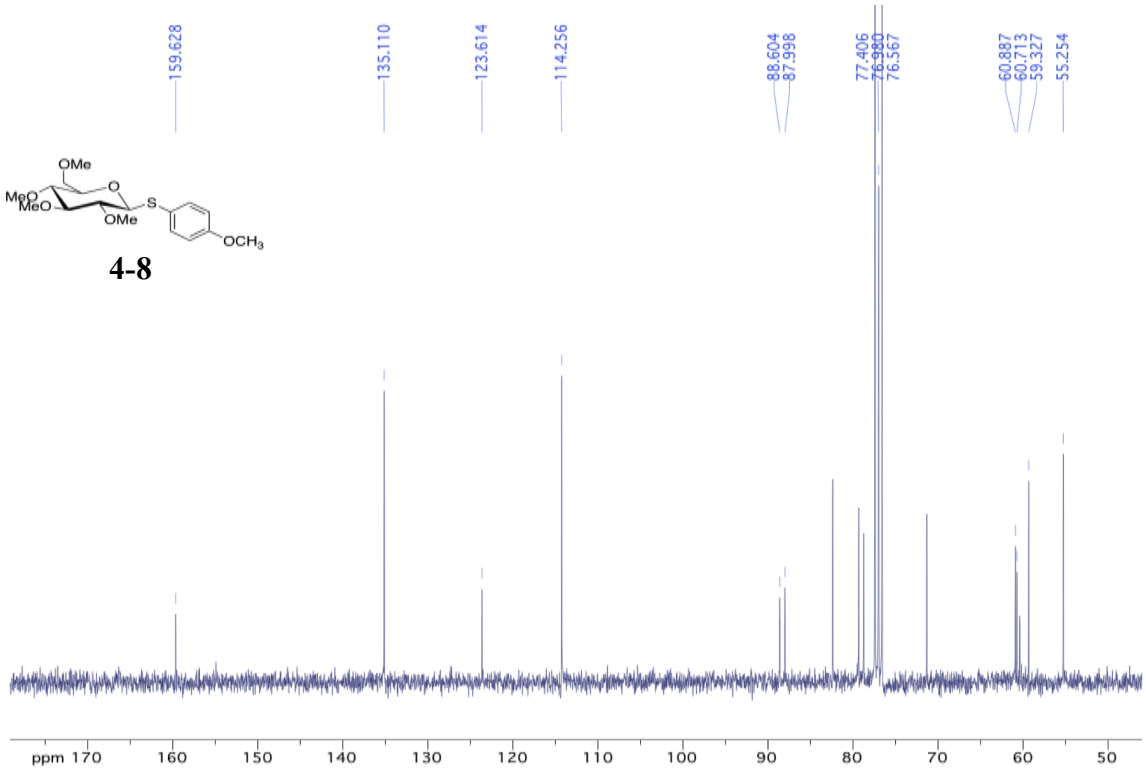

164.987
155.990
155.679
155.368
155.057
142.103
123.826
121.495
118.452
116.170
113.887
111.604
F3C
H
N
O
O
NH
S
S
O
O
DMSO-d6, 125 MHz
3-33
ppm
200
180
160
140
120
100
80
60
40
20
0

APPENDIX C. SPECTRA FOR CHAPTER 4

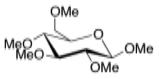


4-8

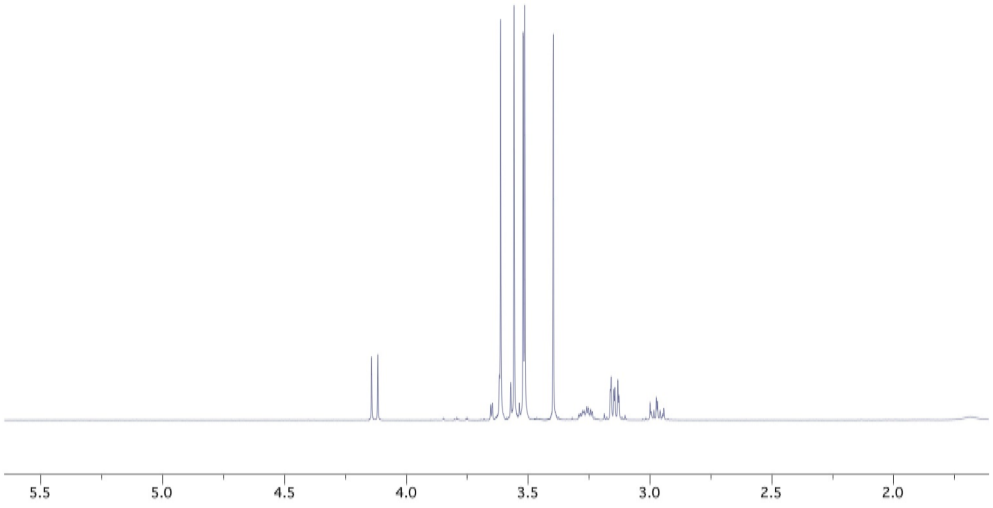


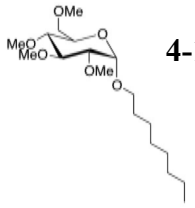


4-8


OMe
MeO
MeO
O
OMe
OMe

4-11β

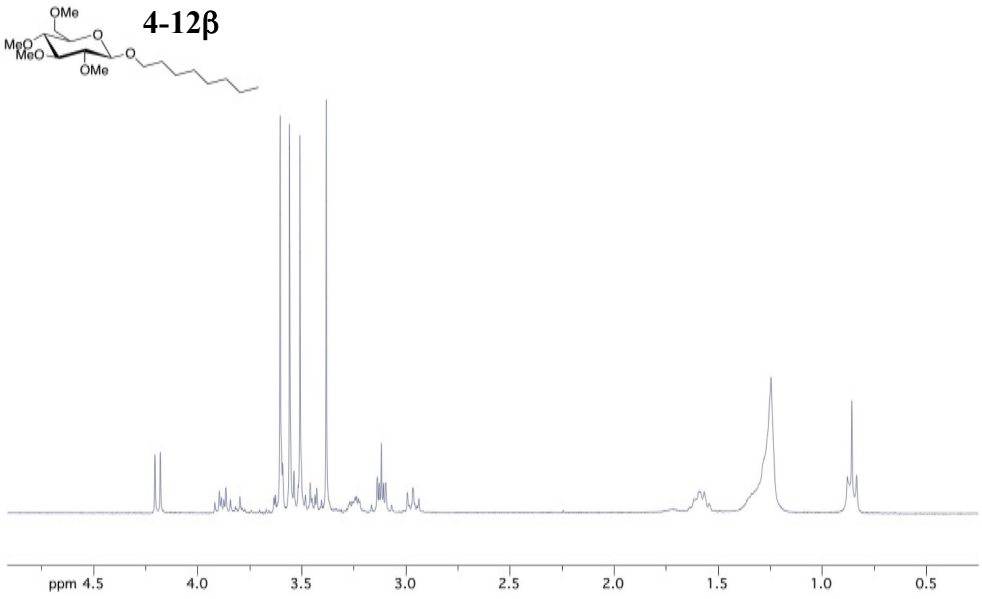

5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0


OMe
MeO
MeO
O
OMe
O

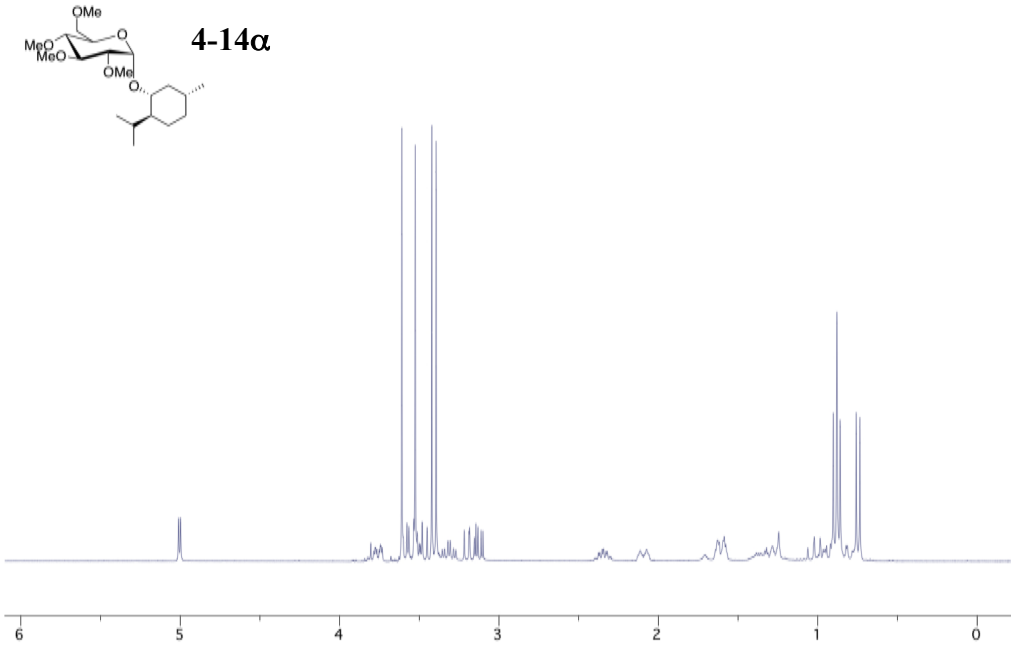
4-12α

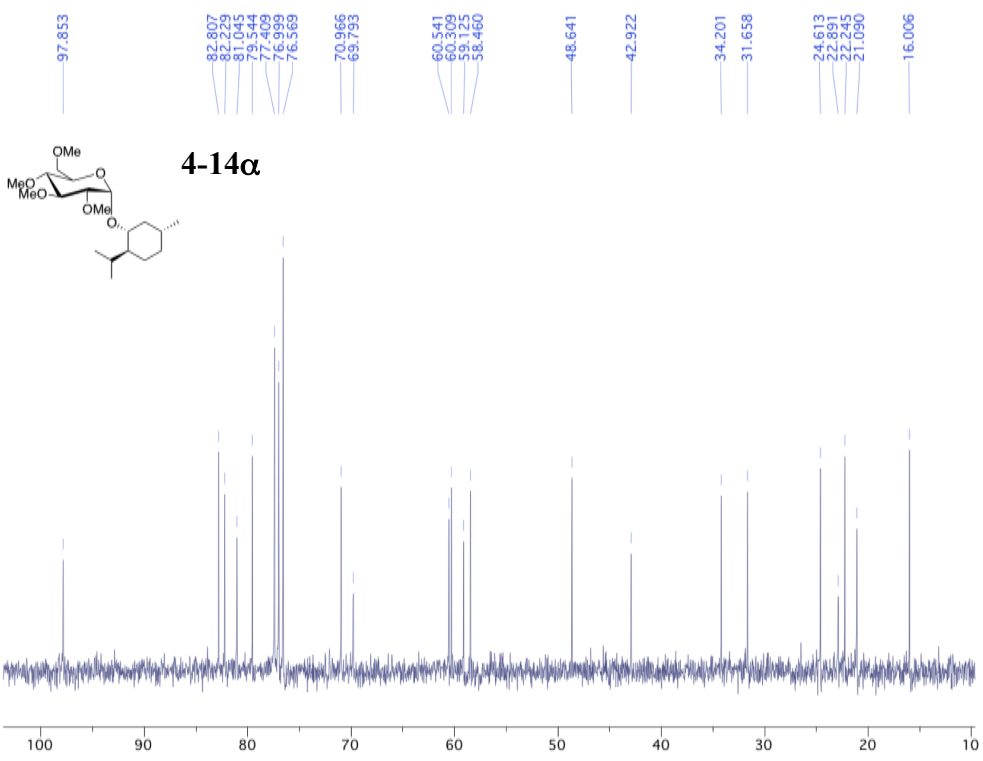
ppm 6
5
4
3
2
1

4-12β

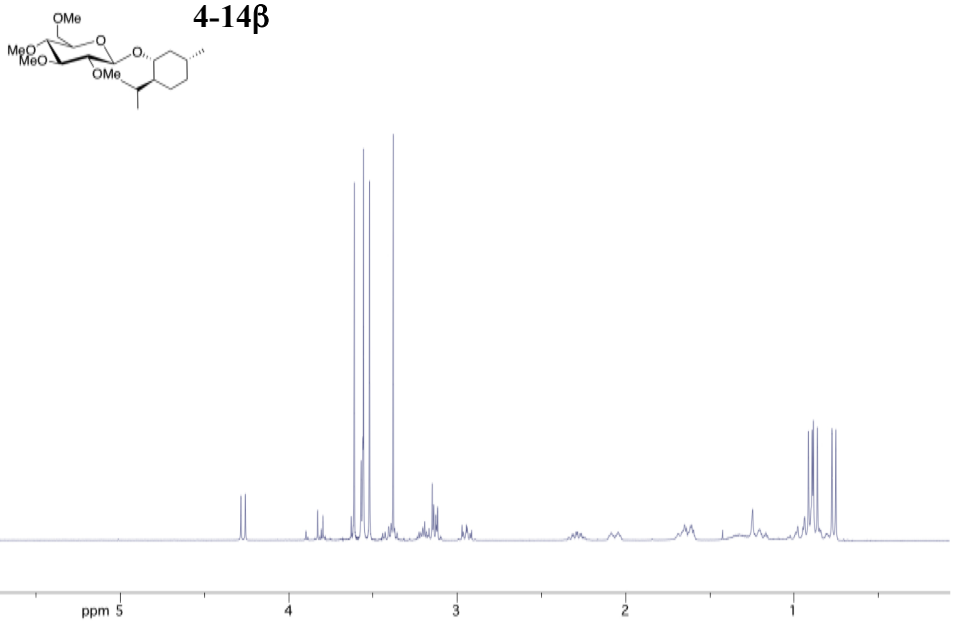


4-14α

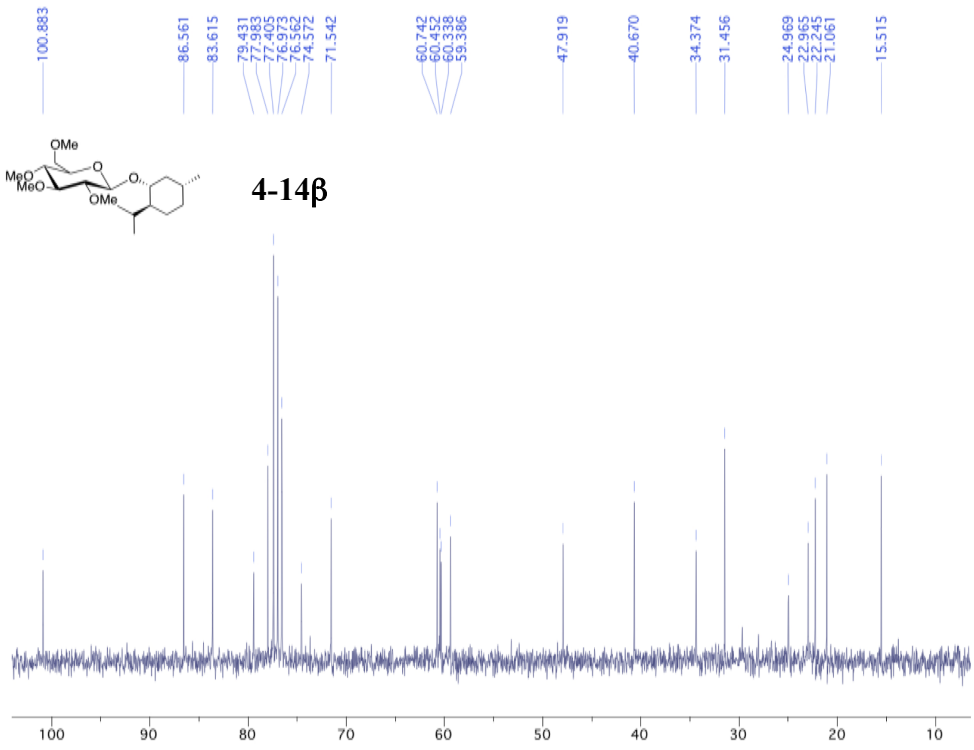


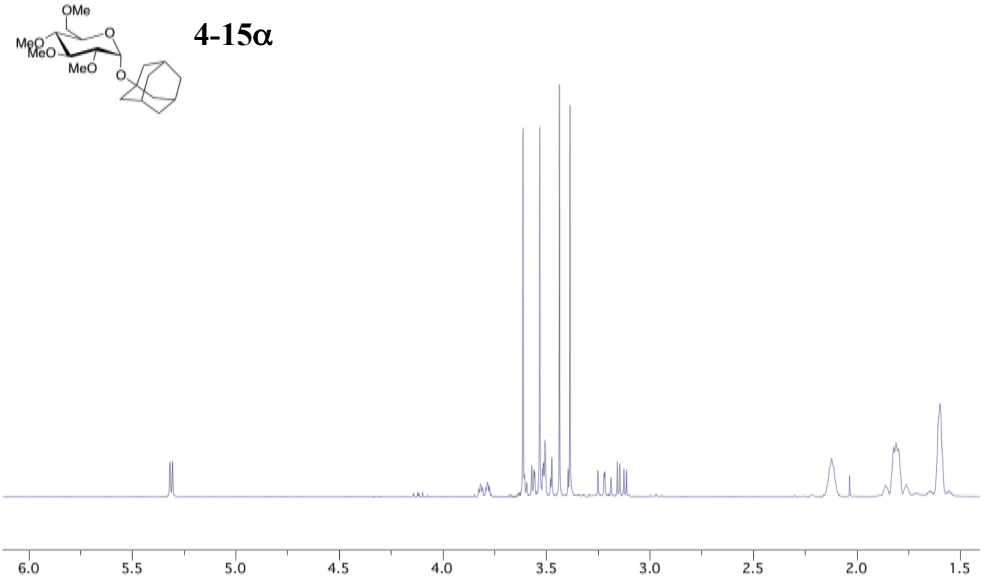

97.853
82.807
82.229
81.045
79.544
77.409
76.999
76.569
70.966
69.793
60.541
60.309
59.125
58.460
48.641
42.922
34.201
31.658
24.613
22.891
22.245
21.090
16.006
OMe
MeO
MeO
OMe
O
100
90
80
70
60
50
40
30
20
10

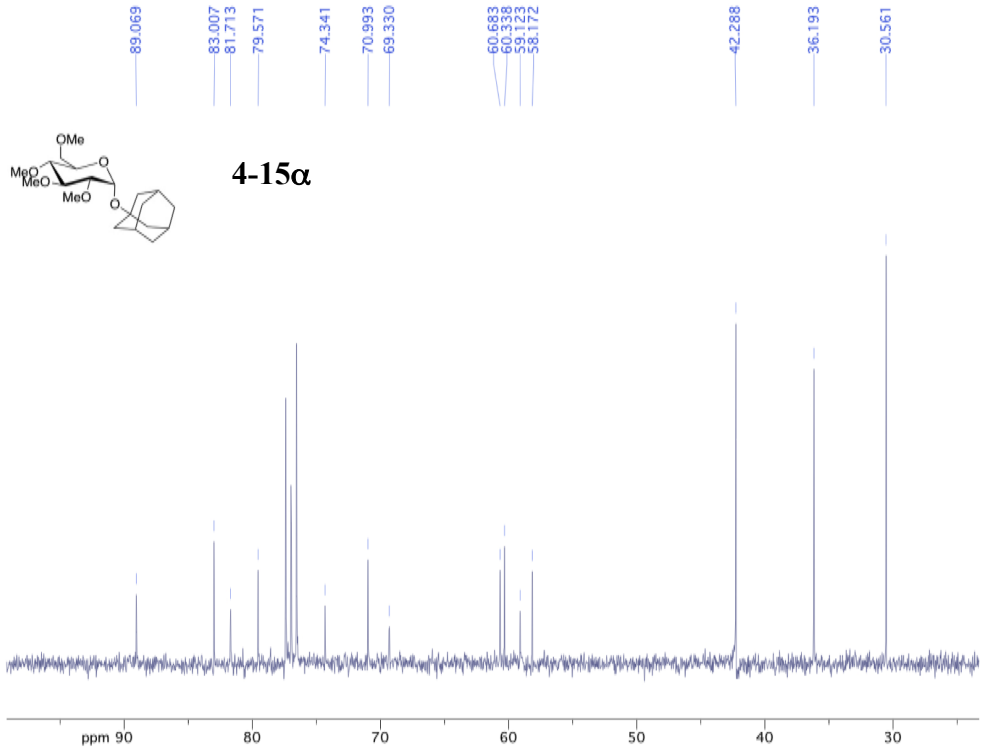
4-14α


OMe
MeO
MeO
OMe
O
ppm 5
4
3
2
1

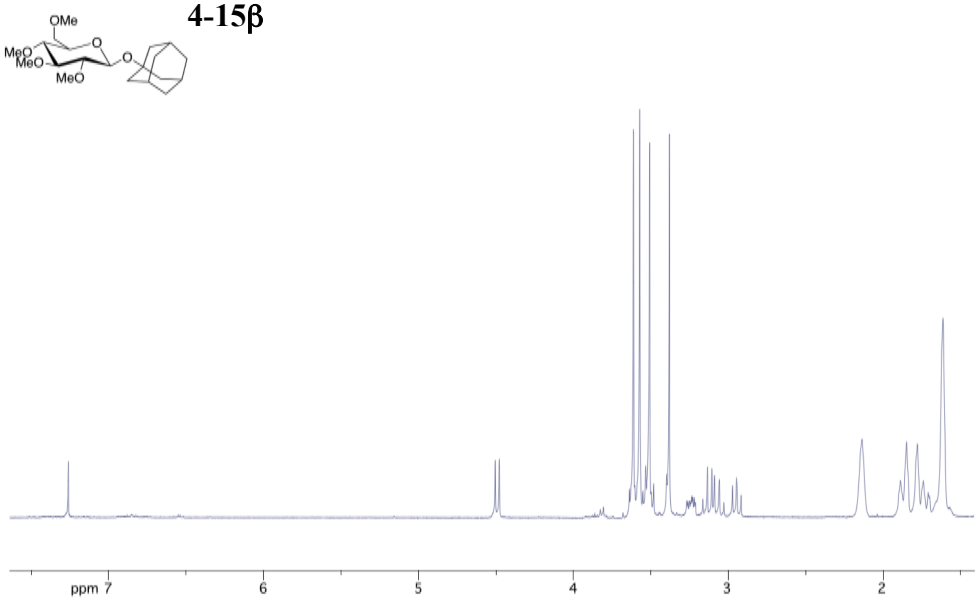
4-14β


100.883
86.561
83.615
79.431
77.983
77.405
76.973
76.562
74.572
71.542
60.742
60.452
60.338
59.386
47.919
40.670
34.374
31.456
24.969
22.965
22.245
21.061
15.515
OMe
MeO
MeO
OMe
O
4-14β
100
90
80
70
60
50
40
30
20
10

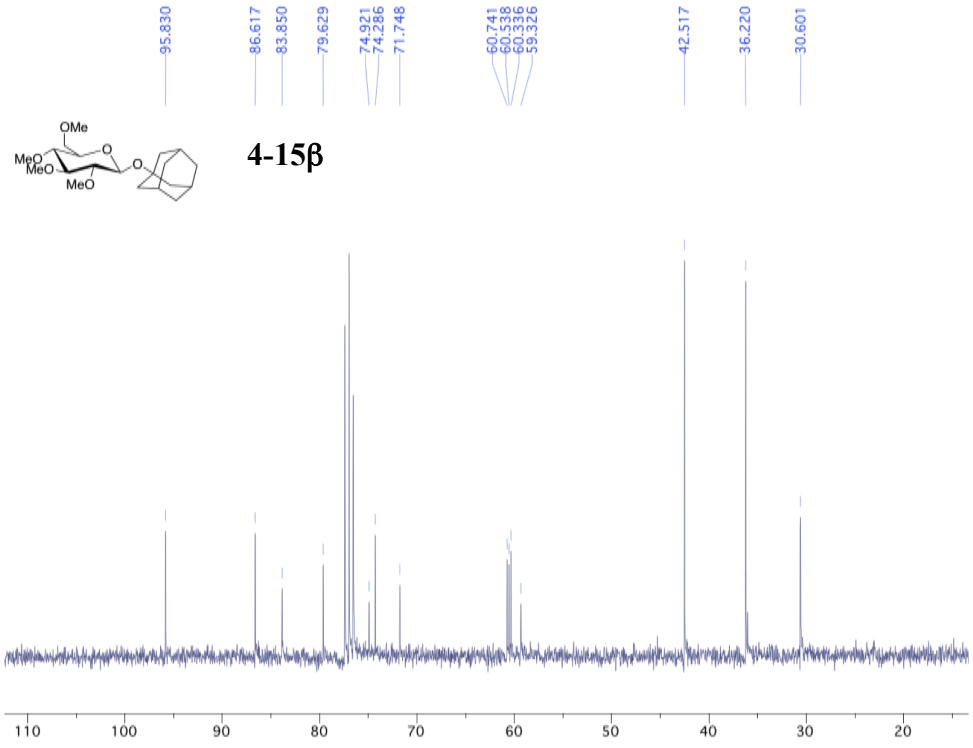

OMe
MeO
MeO
MeO
O
4-15α
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5


89.069
83.007
81.713
79.571
74.341
70.993
69.330
60.683
60.338
59.123
58.172
42.288
36.193
30.561
OMe
MeO
MeO
MeO
O
O
ppm 90
80
70
60
50
40
30

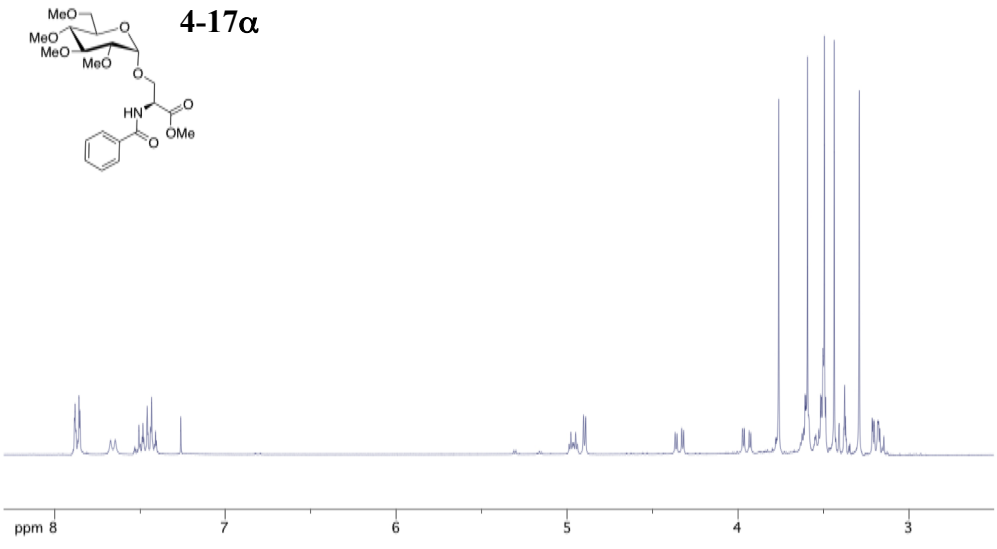
4-15α


OMe
MeO
MeO
MeO
O
O
ppm 7
6
5
4
3
2

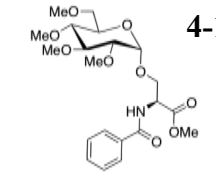
4-15β


95.830
86.617
83.850
79.629
74.921
74.286
71.748
60.741
60.538
60.336
59.326
42.517
36.220
30.601
OMe
MeO
MeO
MeO
O
O
110
100
90
80
70
60
50
40
30
20

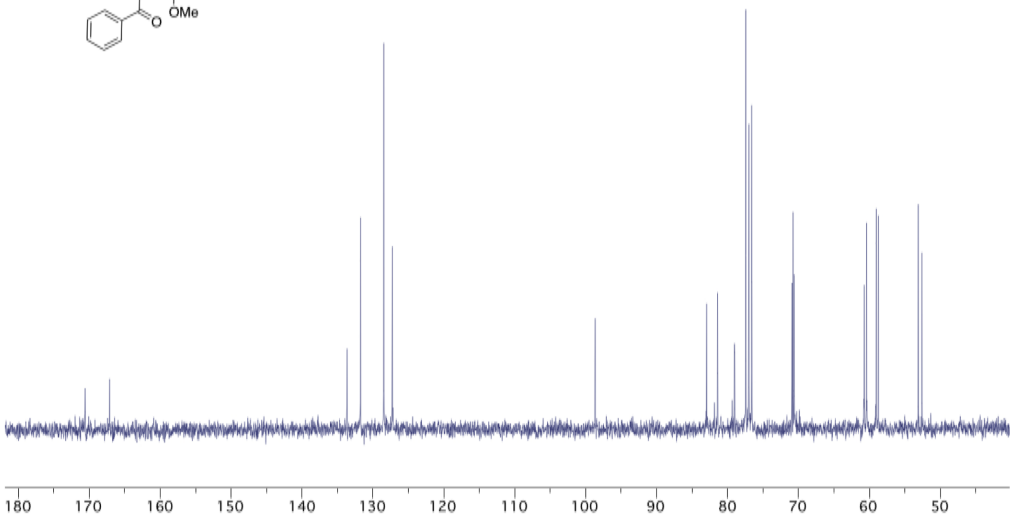
4-15β

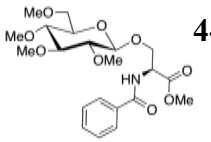

MeO
MeO
MeO
MeO
O
O
HN
O
O
OMe
ppm 8
7
6
5
4
3

4-17α

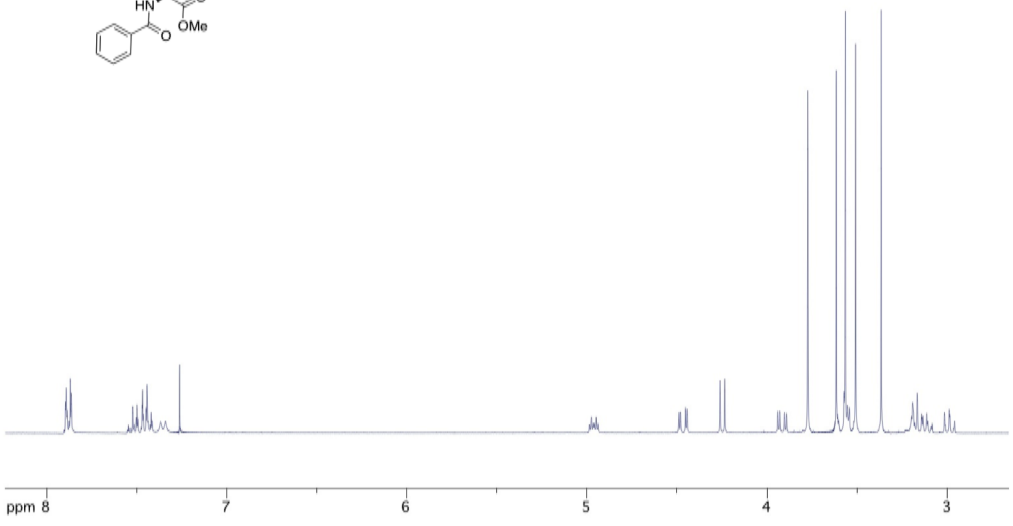

MeO
MeO
MeO
MeO
O
HN
O
OMe
O

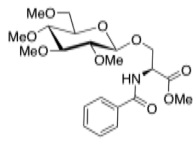
4-17α


180
170
160
150
140
130
120
110
100
90
80
70
60
50


MeO
MeO
MeO
OMe
O
HN
O
OMe

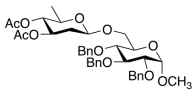
4-17β


ppm 8
7
6
5
4
3


MeO
MeO
MeO
OMe
HN
O
OMe
O
O

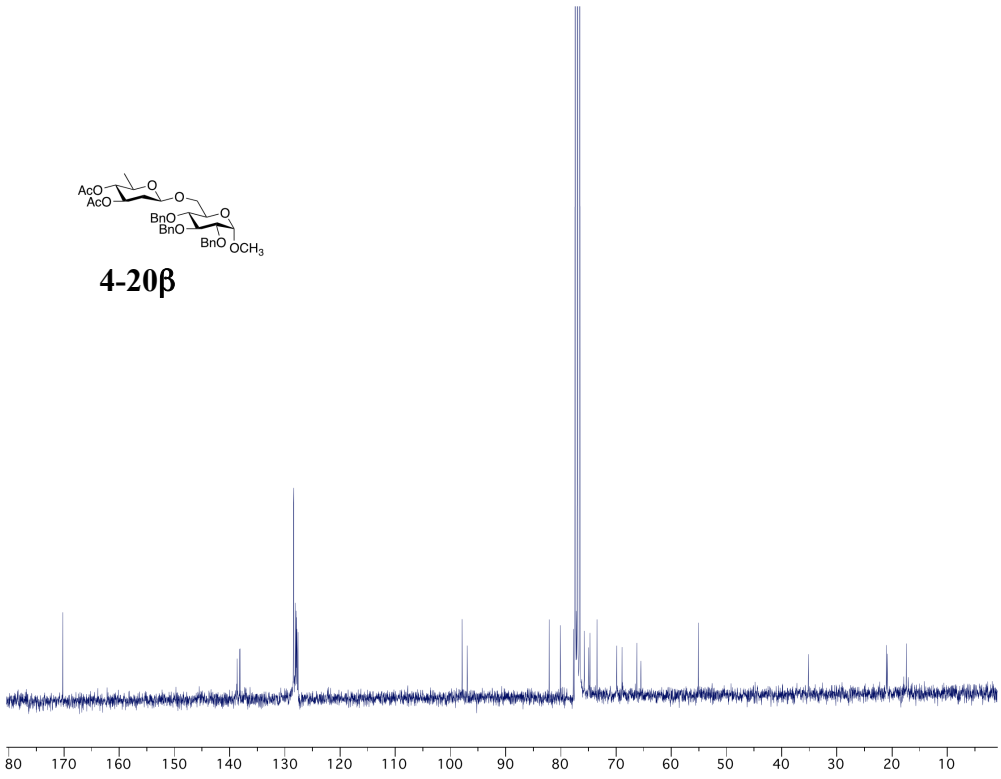
4-17β

ppm 170
160
150
140
130
120
110
100
90
80
70
60
50


AcO
AcO
BnO
BnO
BnO
OCH3

4-20β

ppm 8
7
6
5
4
3
2
1


AcO
AcO
O
O
BnO
BnO
O
BnO
OCH3
80
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10

4-20β