UNIVERSITY OF CALIFORNIA,
IRVINE

Expanding the Scope of Collision-Induced Dissociation-Cleavable Protein Cross-Linkers

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Chemistry

by

Eric James Novitsky

Dissertation Committee:
Professor Scott D. Rychnovsky, Chair
Professor James S. Nowick
Professor David L. Van Vranken

2017

DEDICATION

To my family – past, present, and future

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LIST OF ABBREVIATIONS

Å	Angstroms
Ac	Acetyl
Atm	Atmosphere
Bn	Benzyl
Boc	tert-butoxycarbonyl
Bp	Boiling point
Bu	Butyl
BuLi	Butyllithium
°C	Degree Celsius
cat.	Catalytic
CSA	Camphorsulfonic acid
CI	Chemical ionization
CMC	*N*-Cyclohexyl-*N′*-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate
d	day(s)
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
δ	Chemical shift
DIBAL-H	Diisobutylaluminum hydride
DMAP	4-Dimethylaminopyridine
DMF	*N*,*N*-Dimethylformamide
DMP	Dess-Martin periodinane
DMPU	1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone
DMSO	dimethyl sulfoxide
dppf	1,1'-bis(diphenylphosphino)ferrocene
d.r.	Diastereomeric ratio
ee	Enantiomeric excess
EI	Electron-impact ionization
e.r.	Enantiomeric ratio
eq.	Equation
equiv.	Equivalents

ESI	Electrospray ionization
Et	Ethyl
GC	Gas chromatography
h	hour(s)
HMPA	*N*,*N*,*N*',*N*',*N*'',*N*''-hexamethylphosphoramide
HRMS	High resolution mass spectrometry
Hz	Hertz
i	iso
IR	Infrared spectroscopy
J	Coupling constant
KHMDS	Potassium hexamethyldisilazide
LAH	Lithium aluminium hydride
LiDBB	Lithium di-tert-butylbiphenylide
LDA	Lithium diisopropylamide
LiHMDS	Lithium hexamethyldisilazide
μ	micro
m	milli
M	Molar
m-CPBA	3-Chloroperoxybenzoic acid
min	minute(s)
Me	Methyl
MHz	Megahertz
MS	Mass spectrometer
n	nano
NMR	Nuclear magnetic resonance
nOe	Nuclear Overhauser Effect
Ph	Phenyl
ppm	parts per million
rt	room temperature
sec	second(s)
t	tert

TBAF	tetra-*n*-butylammonium fluoride
TBS	*t*-butyldimethylsilyl
TBDPS	*t*-butyldiphenylsilyl
TES	triethylsilyl
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
THF	Tetrahydrofuran
THP	Tetrahydropyran
TIPS	Triisopropylsilyl
TLC	Thin layer chromatography
TMS	Trimethylsilyl
Ts	4-Toluenesulfonyl
TsOH	4-Toluenesulfonic acid

ACKNOWLEDGMENTS

"Was it worth it?" A question often asked after an individual accomplishes a hard task, many people envision the situation unfolding followed by a positive, reassuring "Yes!", affirming the past decisions and difficult times. This makes practical sense, since after successfully completing any difficult challenge, it seems almost wrong to think otherwise. Scientists are trained to think critically about any scenario and to look at the facts with pure objectivity, regardless of whether this is a research project in which countless hours have been invested or a personal endeavor that has taken years to accomplish. Thus, after reviewing the facts in my life, I do not think obtaining a doctoral degree was worth the sacrifices I have made regarding my personal time, money, and most importantly, my physical and mental health.

This conclusion I have reached was in no way a fault of the faculty and staff that have mentored and supported me throughout my time at UC Irvine – they have been nothing short of excellent. First, my research advisor Professor Scott Rychnovsky is the reason why I am still here to write this thesis. Scott, thank you so much. I could not have actually finished my degree had I not been in your group. You have allowed me the flexibility to continue pursuing my passion and goal of teaching which has kept me relatively sane during my time here. You always have great advice for any type of question, and you have given me tremendous freedom to pursue the research pathways that I feel will be the most fruitful. I have tried my hardest to perform the best research I could do and publish papers so you can continue your goal of maintaining an excellent research group.

I thank Professor James Nowick for entrusting the entire chemistry outreach program to me for a short period of time, and allowing me to have to freedom to work together with a small team of dedicated teachers (Greg Suryn and Mike Morris) to build a program that reaches

hundreds of students every month. James, I admire your longstanding commitment to outreach at a large R1 university. Greg, thanks for working with me for the better part of a year and reinvigorating/saving the program. Mike, thanks for taking over the project and looking over it while Greg and I move on.

I thank Professor Dave Van Vranken for many helpful discussions about my orals research, orals proposal, and thesis research. Thank you for guiding me during advancement, and for always pushing me to patent my linkers. After almost four years, we have finally submitted all the paperwork for a full patent containing many of my linkers, and your constant reminders definitely helped keep the ball rolling.

I thank the past and present members of the cross-linking project. Wynne, thanks for helping me start this project as I arrived at UCI. Sarah, thanks for continuing the project, and more importantly for being the best grad student that I could have ended up working and sharing a work space with. I thank my collaborators (Professor Lan Huang, Clinton, Craig, Alex) for the incredible amount of work you have done with the cross-linkers I have handed off.

I thank Professor Steven Zimmerman for accepting me into his group as an undergraduate at the University of Illinois at Urbana Champaign, and for always taking the time to help and mentor me early in my career. I thank Dr. Cyrus Anderson, my graduate mentor while I was an undergrad, for the amount of time and dedication he spent training me to be a synthetic organic chemist. The relationship we had between graduate student mentor and undergrad mentee shaped the way I conducted myself as I because a graduate student. Most importantly, thank you for teaching me that you can have a tremendous influence on someone and never know the magnitude of your impact.

I thank the faculty at the Center for Engaged Instruction (De, Danny, and Brenda) for offering all of the pedagogical support that they do, and for working with me during many teaching projects and endeavors. I thank the residents of bay 3003 (Justin, Greg, and Sarah) for some fun times and making bad days better. I also thank the past and present members of the Rychnovsky group for helping me with questions, editing, and general advice. I thank my undergrad mentee, Monica, for your enthusiasm performing research in lab. Spending time teaching you about synthetic organic chemistry has influenced the way I think about chemistry as well. I hope our time together has been mutually beneficial.

Last and most importantly, I thank my family for all the support over the years. Mom and Dad, I thank you for the literally never-ending encouragement and positive thoughts. I thank my wife, Andrea, for being there even when I was at my lowest. You always kept a small part of me permanently outside of grad school, and without that I would have lost myself. I could not have done this without you.

CURRICULUM VITAE

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EDUCATION

University of California - Irvine Oct. 2012 – June 2017
Ph.D., Organic Chemistry
Advisor: Professor Scott Rychnovsky
Dissertation Title: "Expanding the Scope of Collision-Induced Dissociation-Cleavable Protein Cross-Linkers"

University of Illinois at Urbana-Champaign Aug. 2008 – May 2012
B. S. in Chemistry, High Distinction
Advisor: Professor Steven Zimmerman
Undergraduate Thesis Title: "Bisureido and Diamido Naphthyridines as Reversible Quadruple Hydrogen Bonding Motifs"

TEACHING EXPERIENCE

Santa Ana College Aug. 2016 – Present
Student Intern

- Designed and taught a unit on electrochemistry to a class of 16 students.
- Helped students with calculations, questions, and homework in general chemistry, in both laboratory and lecture classes.

University of California - Irvine Jan. 2016 – Present
Pedagogical Fellow

- Designed a twelve hour workshop for incoming first-year graduate teaching assistants focused on honing their pedagogical skills.
- Participated in a search committee identifying and selecting future pedagogical fellows.

University of California - Irvine, Rychnovsky Group May 2015 – Present
Undergraduate Mentor

- Taught one undergraduate student with no laboratory experience the basic techniques of synthetic organic chemistry.
- Developed and guided a research project synthesizing a new protein cross-linker.
- Current position: third-year undergrad student at the University of California – Irvine.

University of California - Irvine Jan. 2014 – June 2014
Chemistry Tutor

- Created worksheets and review materials in addition to helping with homework and exam preparation.
-

University of California - Irvine Oct. 2012 – July 2014
Teaching Assistant

- Taught general chemistry, organic chemistry, and organic chemistry labs.
- Prepared worksheets, taught discussion sections, created and graded exams.
- Taught a total of four quarters and one summer session.

University of Illinois at Urbana-Champaign Aug. 2010 – May 2012
Teaching Assistant

- Taught general chemistry for non-majors, both on- and off-sequence.
- Prepared worksheets, taught discussion sections, and proctored exams.
- Taught a total of four semesters and one summer session.

University of Illinois at Urbana-Champaign Jan. 2009 – May 2012
Chemistry Tutor

- Tutored students individually, in small groups, and at a tutoring center, helping with both general and organic chemistry.

COURSES TAUGHT

Chemistry 51LC, second quarter organic chemistry laboratory. Summer 2014

- One section of 18 students.

Chemistry 1C, third quarter general chemistry. Spring 2014

- Taught all of the discussion sections for four weeks of the quarter while the other teaching assistant took a vacation, total class size of eight sections of approximately 45 students each.

Chemistry 51C, third quarter organic chemistry. Spring 2013

- Three sections of approximately 30 students each.

Chemistry 51LB, first quarter organic chemistry laboratory. Winter 2013

- Two sections of 20 students each.

Chemistry 1A, first quarter general chemistry. Fall 2012

- Four sections of approximately 35 students each.

Chemistry 104, second semester general chemistry. Fall 2010 – Spring 2012

- Four sections of approximately 30 students each, per semester.
- Taught a total of four semesters and one summer session.

SERVICE

University of California - Irvine Oct. 2012 – Present
Chemistry Outreach Program

- Prepared and performed chemistry demonstrations for elementary school students in the Orange County area.
- Oversaw the operations of the program in-between grant applications when there was no individual to head the program.

University of California - Irvine Sept. 2016
Pedagogical Fellows Program

- Conducted a twelve hour workshop for incoming first-year graduate teaching assistants focused on honing their pedagogical skills.

University of Illinois at Urbana-Champaign Aug. 2009 – May 2012
Laboratory Assistant

- Prepared and performed chemical demonstrations, ordered chemicals, and set up laboratory equipment for general chemistry labs and lectures.
- Trained three lab assistants.

University of Illinois at Urbana-Champaign Jan. 2009 – May 2012
Chemistry Outreach Program

- Prepared and performed chemistry demonstrations for elementary and middle school students in the Urbana- Champaign community.

RESEARCH EXPERIENCE

University of California - Irvine, Rychnovsky Group Oct. 2012 – Present
Graduate Student Researcher

- Synthesized fourteen new protein cross-linkers for proteomics utilizing tandem mass spectrometry.

University of Illinois, Zimmerman Group May 2010 – May 2012
Undergraduate Research Assistant

- Synthesized four small molecules and appended them to polystyrene to study the influence of supramolecular interactions on interfacial adhesion.
- Synthesized three photo-responsive molecules to study the influence of light on intermolecular interactions.

FELLOWSHIPS

UCI Pedagogical Fellowship — Jan. 2016 – Present
NIH Chemical and Structural Biology Graduate Training Grant — Aug. 2014 – Present

PUBLICATIONS

1. Wang, X.; Cimermancic, P.; Yu, C.; Schweitzer, A.; Chopra, N.; Engel, J. L.; Greenberg, C. H.; Huszagh, A. S.; Beck, F.; Sakata, E.; Yang, Y.; Novitsky, E. J.; Leitner, A.; Nanni, P.; Kahraman, A.; Guo, X.; Dixon, J. E.; Rychnovsky, S. D.; Aebersold, R.; Baumeister, W.; Sali, A.; Huang, L. *Mol. Cell. Proteomics.* **2017**, *in press*.

2. Yu, C.; Huszagh, A.; Viner, R.; Novitsky, E. J.; Rychnovsky, S. D.; Huang, L. *Anal. Chem.* **2016**, *88*, 10301 – 10308.

3. Gutierrez, C. B.; Yu, C.; Novitsky, E. J.; Huszagh, A. S.; Rychnovsky, S. D.; Huang, L. *Anal. Chem.* **2016**, *88*, 8315 – 8322.

4. Yu, C.; Mao, H.; Novitsky, E. J.; Tang, X.; Rychnovsky, S. D.; Zheng, N.; Huang, L. *Nature Communications*. **2015**, *6*:10053.

5. Burke, A. M.; Kandur, W.; Novitsky, E. J.; Kaake, R. M.; Yu, C.; Kao, A.; Vellucci, D.; Huang, L.; Rychnovsky, S. D. *Org. Biomol. Chem*. **2015**, *13*, 5030 – 5037.

6. Kaake, R. M.; Wang, X.; Burke, A.; Yu, C.; Kandur, W.; Yang, Y.; Novitsky, E. J.; Second, T.; Duan, J.; Kao, A.; Guan, S.; Vellucci, D.; Rychnovsky, S. D.; Huang, L. *Mol. Cell. Proteomics*. **2014**, *13*, 3533 – 3543.

7. Anderson, C. A.; Jones, A. R.; Briggs, E. M.; Novitsky, E. J.; Kuykendall, D. W.; Sottos, N. R.; Zimmerman, S. C. *J. Am. Chem. Soc.* **2013**, *135*, 7288–7295.

INVITED PRESENTATIONS AND WORKSHOPS

University of California - Irvine — Feb. 2017
Workshop Leader
- Orchestrated a workshop to graduate students on effective grading practices.

University of California - Irvine — Feb. 2016
Guest Lecturer
- Delivered two lectures on general chemistry to a class of approximately 350 students.

University of California - Irvine Summer 2015

Inside UCI Summer Session

- Delivered six lectures about the chemistry of sugar to incoming freshmen and transfer students.

University of Illinois at Urbana-Champaign Feb. 2012

Guest Lecturer

- Delivered two lectures on general chemistry to a class of approximately 400 students.

AWARDS

Certificate in Teaching Excellence	Spring 2016
NSF GRFP Honorable Mention	Winter 2013
Carl S. Marvel Award	Spring 2012
John E. Gieseking Scholarship	Fall 2011 and Spring 2012

ABSTRACT OF THE DISSERTATION

Expanding the Scope of Collision-Induced Dissociation-Cleavable Protein Cross-Linkers

By

Eric James Novitsky

Doctor of Philosophy in Chemistry

University of California, Irvine, 2017

Professor Scott D. Rychnovsky, Chair

The dissertation describes the efforts towards synthesizing new collision-induced dissociation-cleavable protein cross-linkers (CID-XLs). Chapter one describes the background of CID-XLs and the various fragmentation pathways molecules undergo during collision-induced dissociation. Chapter two details the design and synthesis of new lysine-reactive, cysteine-reactive, and carboxylic acid-reactive sulfoxide-containing cross-linkers. A mechanism for fragmentation during tandem mass spectroscopy is proposed, and the limits of the sulfoxide functional group prompted investigation into a different CID-XL functional group. Chapter three investigates the trioxane core as a novel cleavable functional group, and two trioxane-containing CID-XLs were synthesized. Chapter four focuses on appending affinity purification to both a sulfoxide-containing and trioxane-containing CID-XL.

Chapter 1

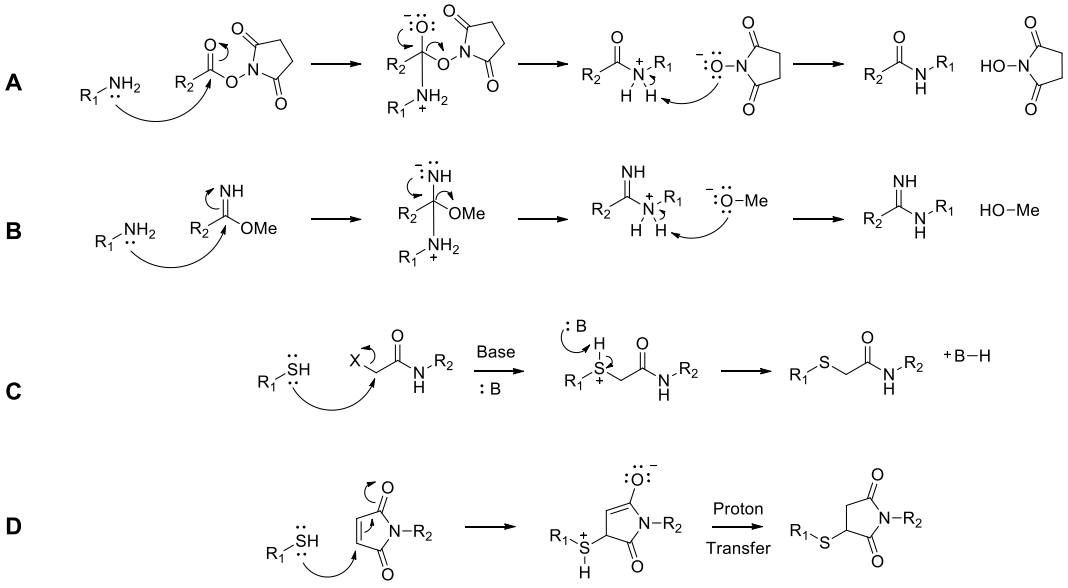
Collision-Induced Dissociation-Cleavable Protein Cross-Linkers in Proteomics

I. Introduction

Elucidating proteasome structure by utilizing both *in vitro* and *in vivo* techniques is paramount for gaining insight into the mechanistic pathways in which proteasomes manipulate and fold smaller proteins. Roughly 40 percent of protein families have no member with a known three-dimensional structure, and many large protein structures are based primarily on smaller, homology modeling.[1,2] A robust method to obtain information about the tertiary and quaternary structure of large proteins will greatly aid in completing the protein database. There are a number of current methods to aid determination of protein structure: nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography, cryo-electron microscopy (Cryo-EM), and mass spectrometry.[3–5] Unfortunately, not all of these techniques are effective for large proteasomes. Analyzing entire proteins in their native environment results in a large quantity of signals in NMR spectroscopy, making analysis difficult because many signals overlap. Difficulties in crystallizing proteins with a high molecular weight is often difficult and removes the protein from its native environment, which hinders the utility of x-ray crystallography.[6] Cryo-EM can be used to analyze the proteasome in its native assembly; however, the resolution of the image is too low to unambiguously identify the conformation of individual amino acids which comprise the tertiary protein structure.[7]

Chemical cross-linkers can provide valuable insight into the three-dimensional structure of a proteasome by reacting with the proteasome in its native environment and trapping any transient interactions or intermediate structures. Cross-linkers are designed to chemoselectively target a specific amino acid in a protein, with each cross-linking unit containing two or more reactive sites.[8] The two most common amino acids targeted with cross-linkers are lysine and cysteine (Scheme 1.1).

Scheme 1.1. Model lysine and cysteine reactive functional groups. R_1 refers to lysine residues on the protein complex and R_2 refers to the arm of the cross-linker.



Lysine residues are typically targeted by *N*-hydroxysuccinimide (NHS) esters or by imidoesters, and cysteine residues are typically targeted with maleimides or haloacetyls. After treatment with a cross-linker, a cross-linked protein can be enzymatically digested, purified, and analyzed by inducing collision-induced dissociation (CID) in tandem mass spectrometry (MS/MS) to identify the amino acid sequences directly bound and in close proximity to the cross-linker.[6,8,9] The fixed length of the cross-linker allows for the determination of the distances between amino acids in the natural conformation of the protein.

II. Background of CID MS/MS

A large cross-linked proteasome can be analyzed with MS/MS to elucidate tertiary protein structure. When there is more than one analyzer in the mass spectrometer, MS experiments can be performed in tandem. The main feature in CID MS/MS is a device called a collision cell, which is placed between mass analyzers to serve as a vessel where fragmentation of ions can occur. To induce dissociation, the analyte ions are accelerated towards the collision cell with translational energy. In the collision cell, the charged ions collide with neutral atoms that have a very high ionization potential (e.g. argon). When the charged ion collides with a neutral molecule, a portion of the ion's kinetic energy is converted into excess vibrational or electronic energy, a process which is called collisional activation. When the energy acquired during the collision is enough to break a chemical bond, then the analyte is said to have undergone CID. The CID process is cumulative because the ions enter the collision cell with all of the energy initially given to the analyte, and once in the cell, some ions accumulate additional energy incrementally through multiple collisions with other ions in the collision cell until they fragment into a variety of product ions. CID only occurs for the protonated molecules, since sodiated species are more stable during MS/MS.

For protein sequencing and analysis, two mass spectrometry experiments are performed (Figure 1.1). First, the large cross-linked protein is enzymatically digested to produce smaller peptides. Then, the first mass spectrometry experiment (MS1) is performed. The exact mass of each peptide is identified; peptides are then selected and subjected to the second mass spectrometry experiment (MS2). MS2 is where CID happens, and it is performed at a specific collision energy in order to cleave peptide bonds and sequence the protein. CID generates many fragments corresponding to sections of the peptide, since CID breaks the peptide backbone

between the N and C termini of linked amino acids. The exact masses of these fragments can be analyzed by computer programs to determine the sequence of the small peptide. The high resolution and accuracy of mass spectrometry can separate sequences of amino acids that are only a fraction of a Dalton apart.[8]

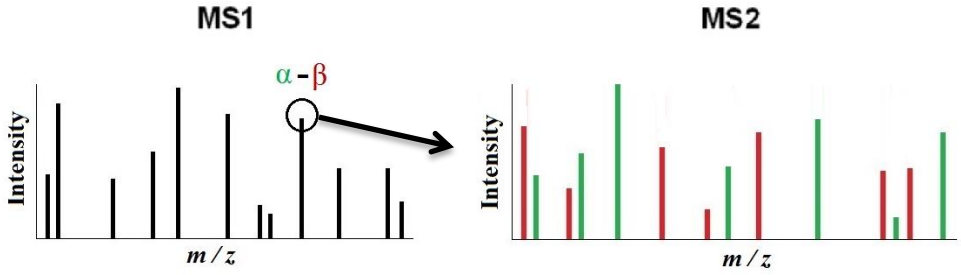


Figure 1.1. MS/MS analysis of a cross-linked peptide (α-β).

For large proteins and protein complexes, there is a large amount of data to analyze, and it often difficult to sort through, even with the help of computer software. To help with this problem, cross-linkers can be designed with specific sites in them which will fragment during CID. By incorporating CID-labile sites into the cross-linkers, which can later be fragmented inside the mass spectrometer during MS/MS, data analysis can be simplified (Figure 1.2).[10] In MS1, the exact mass of the cross-linked peptide is identified, selected, and subjected to MS2. MS2 is where CID of the CID-cleavable cross-linker happens, and it is performed at a specific collision energy. This collision energy should be high enough to cleave a specifically designed bond in the cross-linker selectively, but at an energy low enough to keep the peptide backbone intact. This "tags" each peptide with a specific cross-linker fragment, and allows each half of the cross-linked peptide to be sequenced individually. In a third mass spectrometry experiment (MS3), each half of the peptide can be selected individually, and the CID is performed at an even higher collision energy in order to cleave peptide bonds and sequence the protein. For a CID-

cleavable cross-linker (CID-XL) to be effective, it must cleave at a lower collision energy than the peptide backbone in MS/MS analysis.

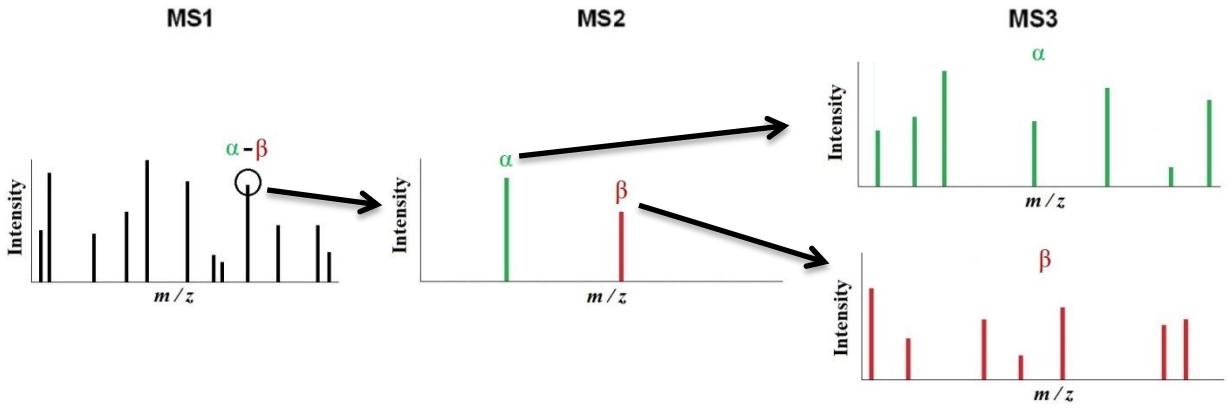


Figure 1.2. MS/MS analysis of a peptide cross-linked with a CID-cleavable cross-linker.

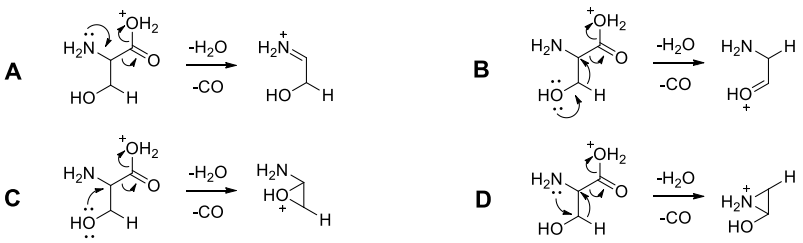
III. CID-Cleavable Mechanisms

There are often multiple fragmentation mechanisms for molecules subjected to CID-cleavage during MS/MS. This is due to the high energy obtained when the molecules are in the collision cell. There have been a number of both computational and experimental studies that attempt to shed light on plausible mechanisms. The primary difficulty in proposing mechanisms during CID is that molecules with relatively high kinetic energy (usually 10 - 100 V) generate highly energetic intermediates in the collision cell that have lifetimes too short for analysis, around 10 - 100 nanoseconds. Additionally, the fragmentation pathway cannot be monitored easily during the course of the reaction (e.g., as opposed to kinetic isotope experiments via NMR) and the only way to analyze the products is by their *m*/*z* ratio after fragmentation. The only evidence available in analyzing MS data is fragments of an original molecule, and similar structures could have the same *m*/*z* ratio. This problem is further complicated by multiple fragmentation pathways that produce a variety of ions, providing evidence of multiple

mechanisms and often leading to a complex mixture of products. The high energy CID collisions lead to behavior that is often difficult to predict. Some research groups have used hydrogen and deuterium substitution to shed light on this problem, but hydrogen/deuterium exchange was observed in the high energy gas phase CID collision cell, complicating analysis.[11,12] There are also other variables that further complicate studies of the mechanistic process, such as the magnitude of energy that can be transferred during a collision based on the orientation of the collision, the distribution of the transferred energy, and the variability of the charge distribution within the molecule if rearrangement of the atoms occurs over multiple mechanistic steps.[13,14]

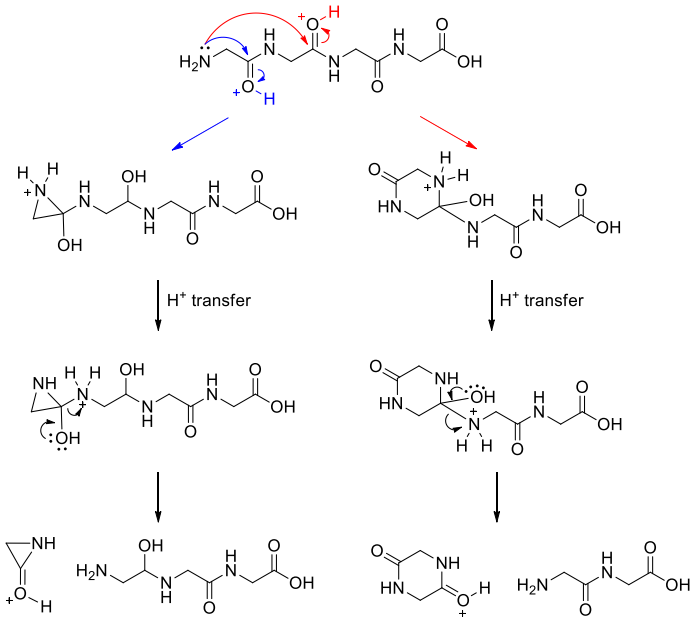
One way to attempt to account for all of the variables in the CID process is to utilize high level electronic structure calculations to compute transition states, reaction intermediates, and energy minimums for the various mechanistic pathways. A computational study done by Rogalwicz *et al*. studied the fragmentation pathways for serine during CID (Scheme 1.2).[15] In the computational study they found the iminium ion fragment (Scheme 1.2, pathway A) to be the most stable, with the basicity of the nitrogen as well as the absence of a three-membered ring contributing to the stability of the fragment. This study perfectly illustrates the problem proposing CID mechanisms because all of the resulting product ions have the same m/z, so the experimental data cannot be used to distinguish between the fragments and the only evidence supporting the most plausible mechanism is computational data.

Scheme 1.2. Modeled fragments for the fragmentation of serine during CID.



The amount of confidence placed in theoretical data is undermined when it does not correlate with the experimental data. Even with calculated energy minima, higher energy fragments are observed experimentally during CID. Reid *et al*. subjected tetraglycine to CID and observed multiple fragmentation ions (Scheme 1.3)[16].

Scheme 1.3. CID fragmentation of tetraglycine. The blue arrows denote the aziridine formation pathway and the red arrows denote the 2,5-diketopiperazine formation pathway.



Even though the aziridine fragment is much higher in energy than the 2,5-diketopiperazine fragment, both were observed in the resulting spectrum. This result was not exclusive to the peptide studied, as other research groups have observed the higher energy aziridine fragment for similar oligopeptide experiments.[17] Many additional experimental and computational studies have replicated studies and evidence towards the hypothesis that there are different fragmentation pathways and a variety of ions observed for seemingly simple small oligopeptide chains.[18,19] The inconsistent fragmentation adds to the complexity of elucidating CID mechanisms and designing CID-XLs. The complexity of CID mechanisms can also be seen in the computational and experimental studies of aliphatic fatty acids, which contain only two

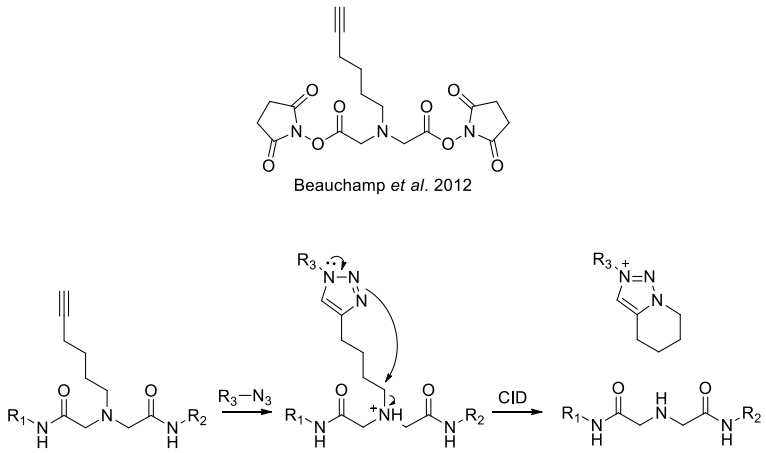
heteroatoms in the carboxyl group. Deuterium studies, theoretical calculations, and experimental evidence shows no consensus can be reached on a single fragmentation mechanism, with many mechanisms proposed to account for the variety of fragments.[20–23]

When designing CID-cleavable cross-linkers, the possibility for multiple mechanisms poses a challenge to design a site that will preferentially cleave. It is important to consider all of the possible fragmentation mechanisms, even those that result from high energy intermediates, in order to design a cross-linker which will have one bond or "fragmentation site" in the linker that will preferentially cleave during CID. Most importantly, the CID-XL must cleave during CID before the peptide backbone, so each half of the cross-linked peptide can be sequenced individually. Many groups have designed cross-linkers that will fragment during MS/MS.

IV. Previously Developed CID-XLs

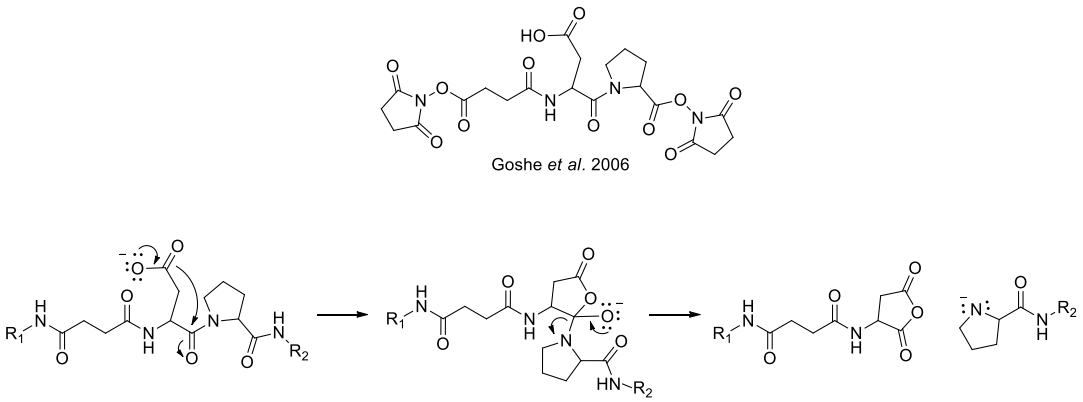
Many CID-cleavable cross-linkers are designed to undergo unimolecular fragmentation after reaction with the respective amino acids. To date, lysine reactive CID-cleavable cross-linkers are the most common.[24,25] Beauchamp *et al*. designed a CID-XL, CXL, which fragmented during CID after reacting with biotin-$(PEG)_3$-azide beads in a "click" reaction.[26] The electrons in the triazone were proposed to displace the amine in the main portion of the cross-linker, cleaving the affinity tag from the cross-linker and giving the cross-linked protein a marker ion of an exact mass (Scheme 1.4). This ~6.6 Å CID-XL was shown to work well linking residues in HEK 293 cells and the affinity purification employed greatly increased ease of data analysis, which previously had complicated analysis when working with cross-linked mixtures of HEK 293 cells.

Scheme 1.4. Proposed CID-XL fragmentation mechanism. R_1 and R_2 refer to lysine residues on the protein complex which have reacted with the NHS esters, and R_3 is $(PEG)_3$-Biotin.



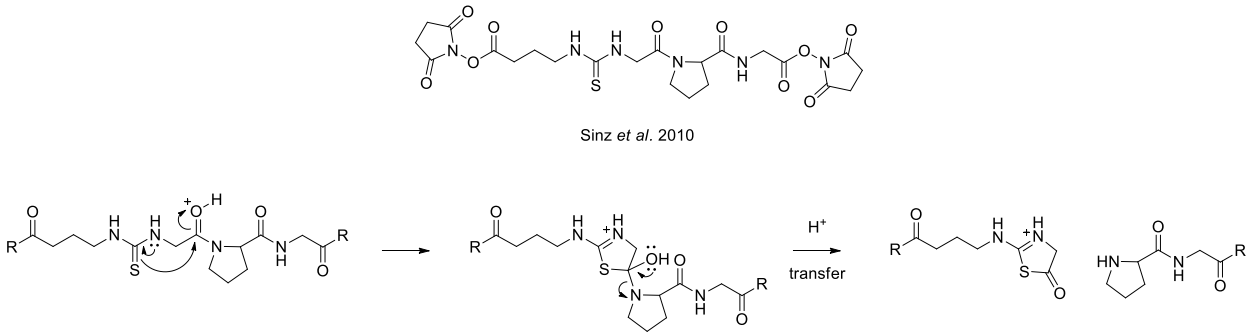
The fragmentation products from most CID-XLs are two fragments resulting from an intramolecular cleavage. Goshe *et al*. designed a lysine-reactive CID-XL (SuDP), which is proposed to form a succinic anhydride fragment during CID (Scheme 1.5).[27] The ~23.9 Å linker was found to effectively cross-link monomeric BSA. The authors reported a very low abundance of ions when positive mode CID was used, so they employed negative mode CID which afforded many characteristic fragments. Similar compounds that do not produce many ions in positive mode have been shown to be much more effective when subjected to negative mode CID.[28,29] Additional experimental and theoretical studies provided supporting evidence for the illustrated CID cleaving mechanism of SuDP.[30]

Scheme 1.5. Structure and proposed fragmentation mechanism of SuDP. R_1 and R_2 refer to lysine residues on the protein complex which have reacted with the NHS esters.



Sinz *et al*. designed a CID-XL called NHS-BuTuGPG-NHS which fragments similar to SuDP, except instead of producing a succinic anhydride portion, it gives a thiazolidinone moiety (Scheme 1.6). The ~16.7 Å linker, although quite structurally similar to SuDP, worked well in positive mode CID and was found to effectively cross-link the 14.3 kDa protein lysozyme.

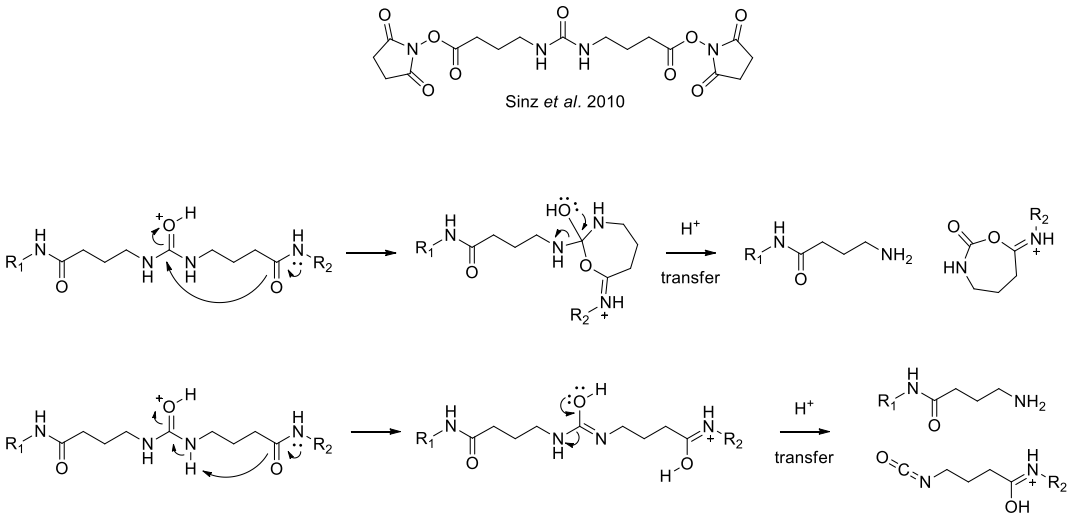
Scheme 1.6. Structure and proposed CID-cleavage mechanisms of NHS-BuTuGPG-NHS. R_1 and R_2 refer to lysine residues on the protein complex which have reacted with the NHS esters.



Sinz *et al.* have developed a number of other CID-XLs, one of which is DSBU.[31] The central urea portion of the cross-linker could cleave through two different mechanisms, both of which give the same m/z of the resulting fragments (Scheme 1.7).[25,31] This 12.5 Å linker was found to effectively cross-link peptides Munc13-1 and PPARα, and the authors commented on the improvement of the quality of the data obtained when DSBU was employed over a non-

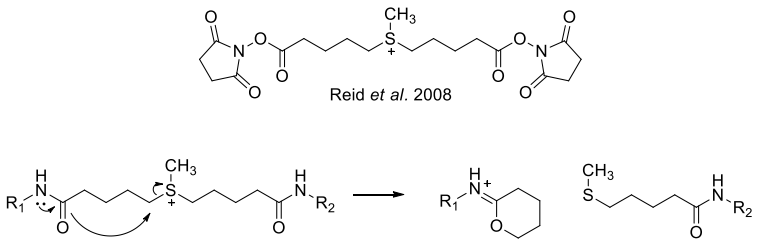
cleavable linker, with a noticeable increase in the cross-links obtained as well as a decrease in the noise in the resulting spectra.

Scheme 1.7. Structure and proposed CID-cleavage mechanisms of DSBU. R_1 and R_2 refer to lysine residues on the protein complex which have reacted with the NHS esters.



Reid *et al*. developed a sulfonium-based CID-XL with a simple proposed mechanism for fragmentation (Scheme 1.8).[32] The cross-linker was found to effectively cross-link model proteins neurotensin and angiotensin II at a high efficiency that was hypothesized to arise from the excellent aqueous solubility of the sulfonium ion designed in the CID-XL.

Scheme 1.8. Structure and proposed CID-cleavage mechanism for the sulfonium CID-XL. R_1 and R_2 refer to lysine residues on the protein complex which have reacted with the NHS esters.



Borchers *et al.* designed two isotopic cross-linkers (CBDPS and CBDPS-d_8) which differed by eight mass units, with one CID-XL containing eight hydrogens while another structurally similar CID-XL contained eight deuteriums.[33] Using a pair of isotopically labeled

cross-linkers can simplify data analysis. When the MS/MS spectra has many impurities or a lot of noise, a 1:1 ratio of deuterated to non-deuterated CID-XL can be used to give a doublet in the resulting spectra. The authors did not propose a mechanism for the fragmentation of their cross-linker, but rather proposed structures for the CID fragmentation (Figure 1.3). They did mention that there was some experimental data which did not align with their proposed fragmentation, which they explained by:

> "…proton/deuterium exchange often occurs between the two portions of the cross-linker but can only be observed in the deuterated form of the cross-linker where it leads to an unusual but characteristic isotopic distribution which contains an (M − 1 Da) peak for the fragment that does not include the nitrogen-containing ring and a higher than expected (M + 1 Da) peak in the corresponding cleaved portion of the cross-link that includes the nitrogen-containing ring."

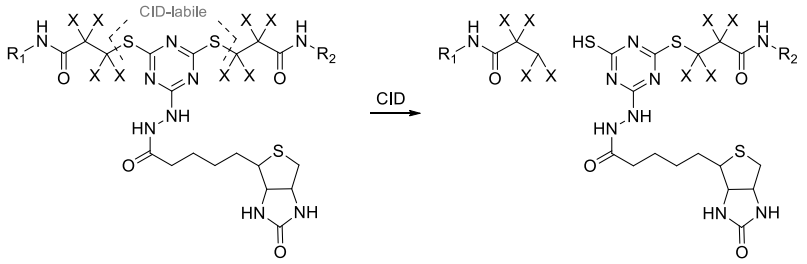
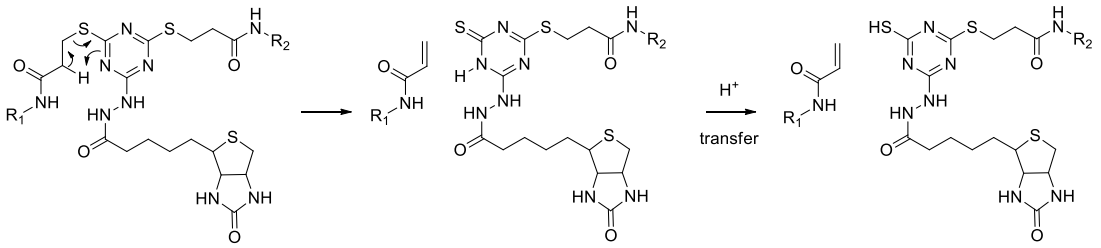


Figure 1.3. Structure and proposed fragments of CBDPS.

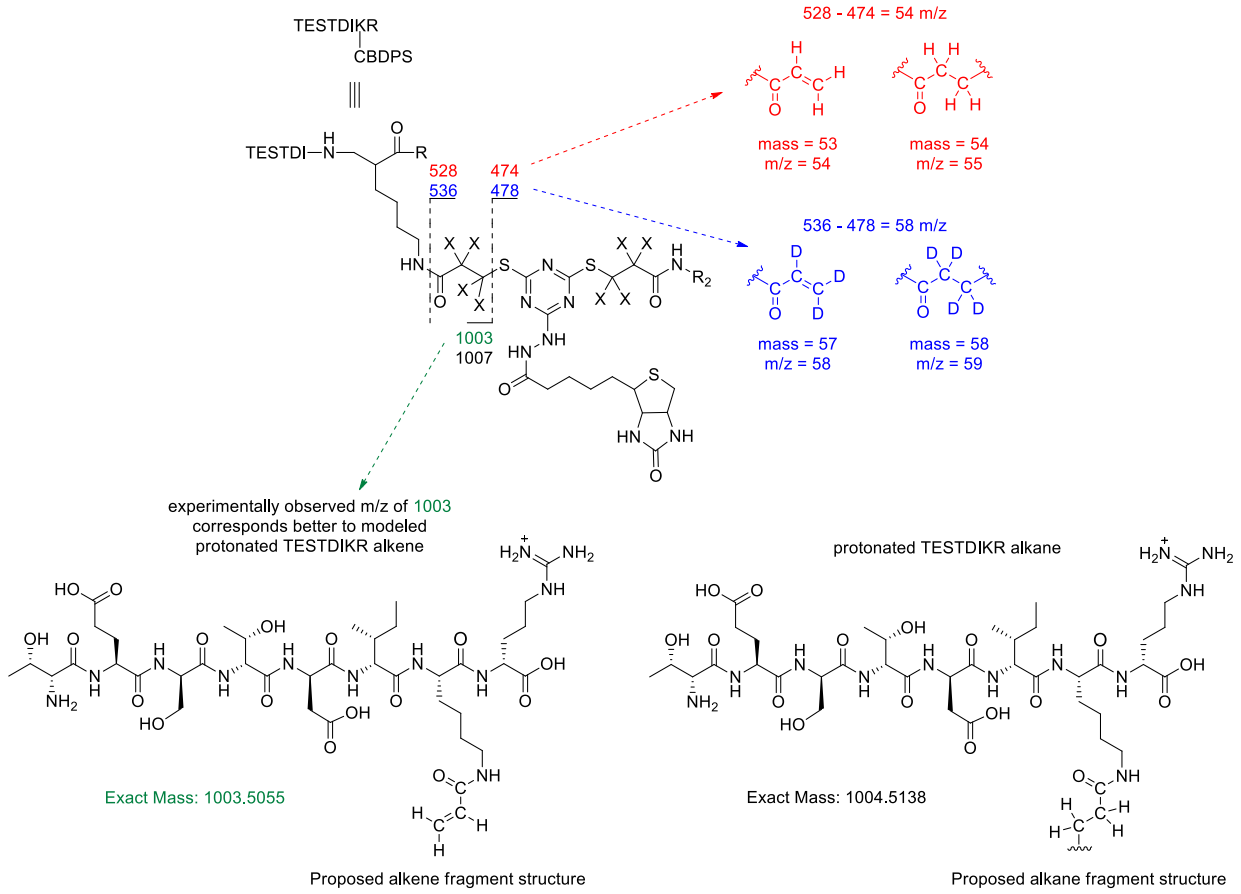
After reviewing the experimental data in the paper, the following revised fragmentation structures and fragmentation mechanism is proposed (Scheme 1.9).

Scheme 1.9. Proposed fragmentation mechanism for CBDPS.



This proposed mechanism would account for the deuterium exchange between the two fragments, with a pericyclic-like reaction generating a thiol fragment and an alkene fragment. This proposed mechanism matches up well with the experimental data (Scheme 1.10). The difference between the fragments corresponding to 528 *m*/*z* and 474 *m*/*z* is 54 *m*/*z*, aligning with the authors proposed fragment. However, this is not taking into account the extra proton which must be present to protonate the molecule and make it able to fly in mass spectrometry. Additionally, the source of the two protons that appear on both halves of the fragmented molecule is unknown. The alkene fragment, with a mass of 53, would give a 54 *m*/*z* mass difference when protonated.

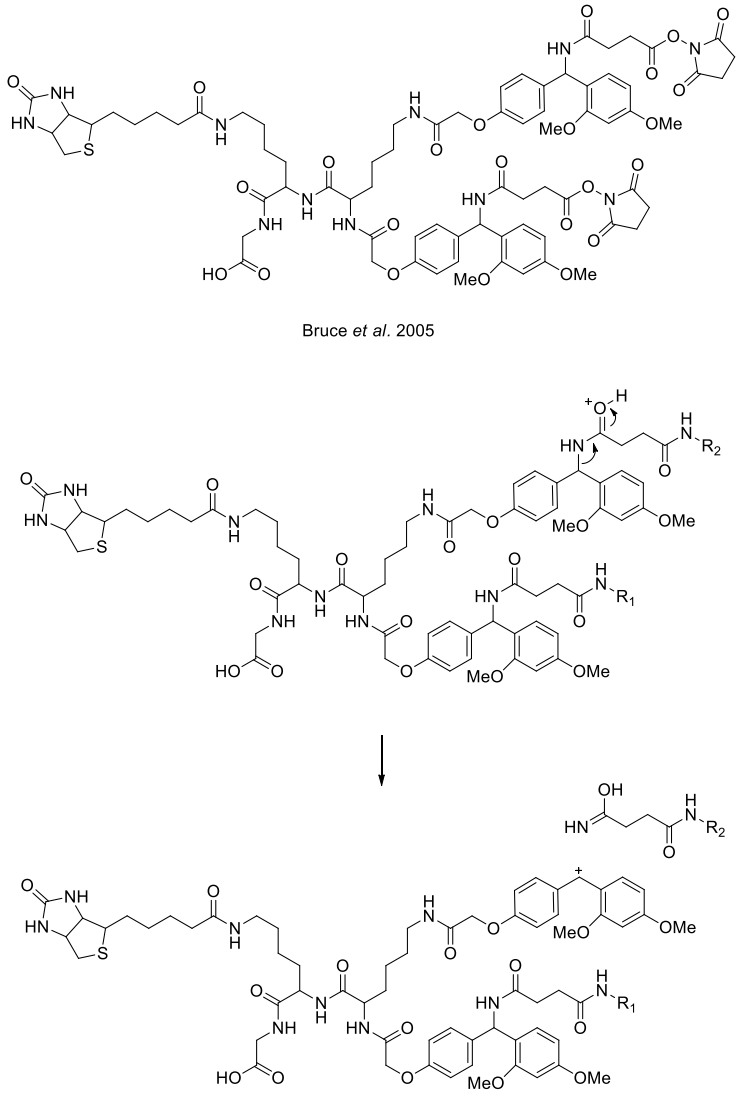
Scheme 1.10. Proposed fragments of CBDPS after cross-linking with TESTDIKR, and modeled TESTDIKR fragments.



The same pattern of reactivity is seen with the deuterated fragment as well, except with the deuterated fragment there is a mass difference of 57 with the alkene fragment, which when protonated generates the observed 58 mass difference. When the full TESTDIKR cross-linked fragment was modeled, a *m*/*z* corresponding to the alkene hypothesized mechanism was closer to the experimentally observed results. (Scheme 1.10). Regardless, the 14 Å CBDPS was shown to effectively cross-link the heterodimer of HIV reverse transcriptase.

Bruce *et al*. designed a longer, 43 Å CID-XL abbreviated PIR. The linker was found to effectively cross-link RNase S and also perform well *in vivo* cross-linking proteins in *S. oneidensis*. PIR was used to discover protein-protein interactions in *S. oneidensis* without the need for specific antibodies or genetic manipulations of the system.[34,35] The proposed fragmentation mechanism shows cleavage of the protonated amine, resulting in a stabilized doubly benzylic carbocation (Scheme 1.11).

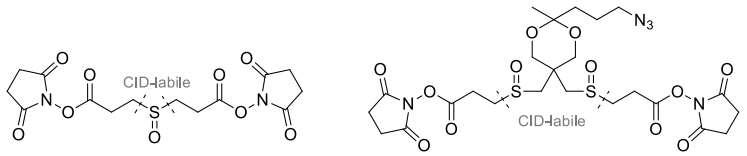
Scheme 1.11. Structure and proposed CID-cleavage mechanism for the CID-XL, PIR. R_1 and R_2 refer to lysine residues on the protein complex which have reacted with the NHS esters. After the initial fragmentation, the CID-XL can fragment a second time on the other 'arm' of the XL.



V. The Rychnovsky Group's Approach to CID-XLs

With the variety of CID-XLs in the literature, it was envisioned that one could be synthesized which had a predictable fragmentation from a novel functional group, and that it would be also inexpensive and easy to synthesize. The Rychnovsky group has studied various functional groups and reaction types which might be susceptible to cleavage in MS/MS,

including sulfoxides, sulfones, retro Diels-Alder and retro Michael additions.[36] Of these, the sulfoxide functional group was found to fragment at the lowest collision energy. The first reported CID-cleavable cross linker by the Rychnovsky group was sulfoxide **1-1** (DSU) which was designed to react with lysine residues (Figure 1.4).[10,37,38] A second CID-cleavable cross-linker was developed to explore if affinity purification would simplify MS/MS data. Disulfoxide **1-2** (Azide-Bis) was designed with an acid labile ketal functionalized with a pendant azide handle for affinity purification.[39] The cross-linked protein could be reacted with alkyne beads in a "click" reaction, allowing for the removal of any unmodified protein.[40] After the beads had been washed, they were treated with acid so that the cross-linked proteins could be detached from the beads and analyzed using MS/MS. Azide-Bis was found to be beneficial for proteins where additional purification was necessary.[39] The cross-linking experiments, purification, and MS/MS analysis are performed by the Huang group in the Department of Developmental and Cell Biology at UC Irvine.[10,38]



1-1 1-2

Figure 1.4. Previously synthesized CID-cleavable cross-linkers in the Rychnovsky group.

Currently, there are not many commercially available CID-cleavable cross-linkers. Out of the 92 total cross-linking reagents offered by Thermo Scientific, there are no selective tyrosine, serine, glutamic acid, or aspartic acid reactive cross-linkers, and additionally only a few CID-cleavable linkers.[41] The research described herein will detail our efforts to construct a library of CID-cleavable cross-linkers that have different lengths, different chemoselectivities, and novel cleaving functional groups.

VI. References

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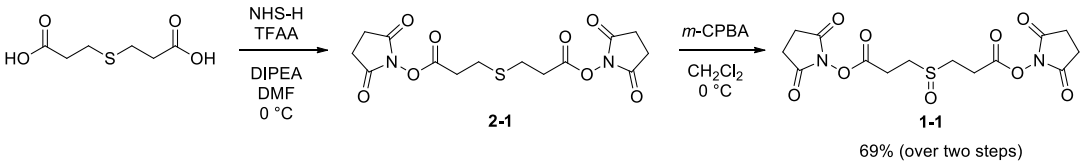
Chapter 2

Sulfoxide-Containing Collision-Induced Dissociation-Cleavable Protein Cross-Linkers

I. Optimization of Previously Developed Lysine-Reactive CID-XLs

DSU and Azide-Bis were found to be effective cross-linkers, so optimization of the synthesis for multi-gram scale was investigated (Scheme 2-1). Previously, sulfide **2-1** was purified by washing with cold hexanes and diethyl ether.[1] Gram scale preparation of DSU would generate unnecessary waste using this purification method. It was found that trituration of the crude material with minimal diethyl ether (2 mL per mmol, repeated three times) afforded crude **2-1**. Subsequent oxidation afforded DSU in a 69% yield over two steps, an improvement over the previous two-step yield of 45%.[1] Without triturating **2-1**, the yield over two steps was 25%. DSU has been employed successfully in many cross-linking experiments in the Rychnovsky-Huang collaboration, and is currently commercially available.[2–4]

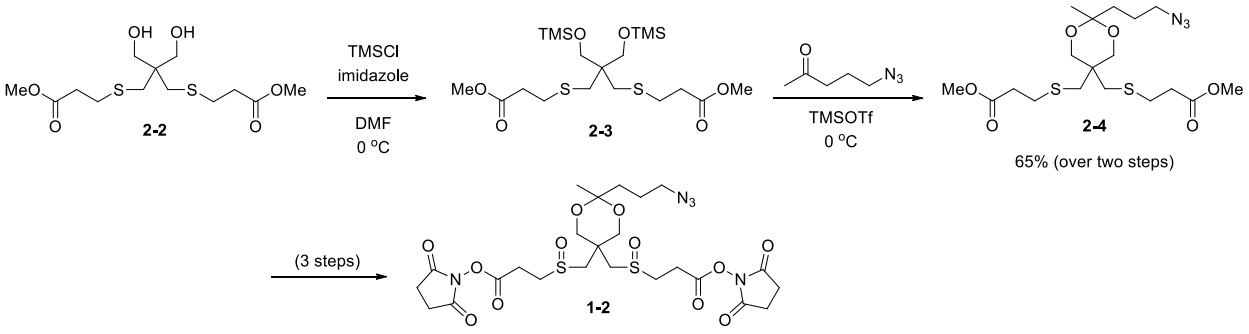
Scheme 2.1. Synthesis of DSU.



The optimization of the synthesis of Azide-Bis was investigated next. Despite a previously reported 75% yield,[5] heating diol **2-2** for 24 h at 115 ºC with 5-azidopentan-2-one, camphorsulfonic acid, and a Dean-Stark trap only afforded ketal **2-4** in 25% yield after column chromatography. Alternatively, trimethylsilyl chloride (TMSCl) protection of both alcohols, immediately followed by ketal formation with 5-azidopentan-2-one and trimethylsilyl triflate

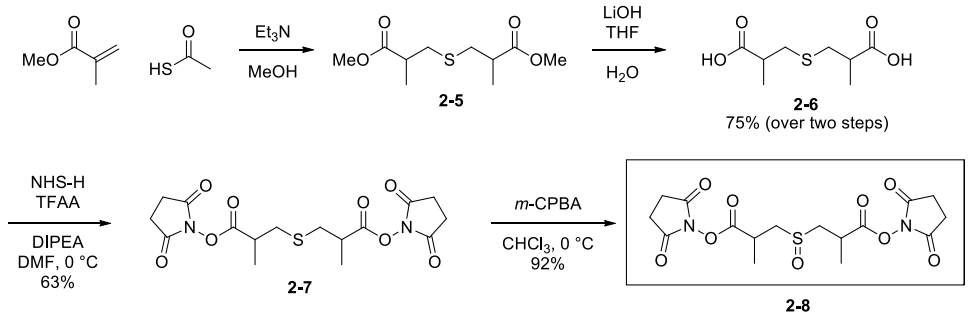
(TMS-OTf), afforded the desired intermediate **2-4** in 65% yield over two steps with one purification (Scheme 2.2).[6–8] The remaining steps of the synthesis were previously optimized.[9]

Scheme 2.2. Partial synthesis of Azide-Bis.



The synthesis of a previously published pair of cross-linkers was also improved (Scheme 2.3).[10] Previously, the yield of diacid **2-6** was 36% over two steps. In an attempt to improve the yield and reduce waste generated for one gram scale-up, the concentration of the Michael addition was increased by 10 fold.[5] After heating methyl methacrylate and thioacetic acid at 60 °C for 6 h, additional methyl methacrylate was added dropwise to drive the reaction to completion. After workup, crude **2-5** was taken forward without purification, and after basic hydrolysis the yield of **2-6** was improved to 75% over two steps.

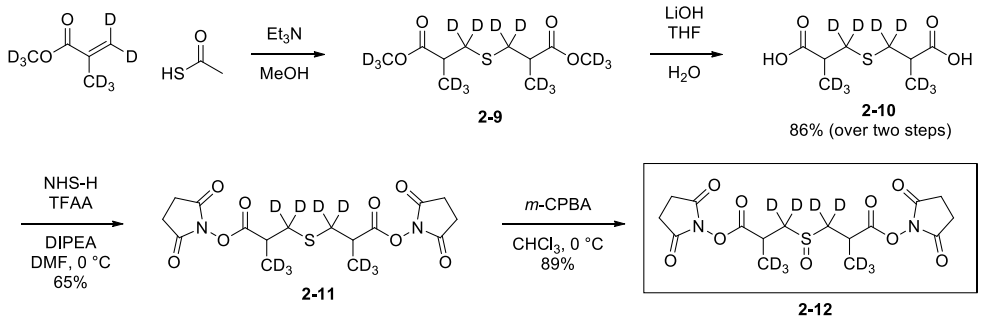
Scheme 2.3. Synthesis of non-deuterated cross-linker **2-8**.



The purification of bis-NHS-ester **2-7** previously involved column chromatography. Alternative methods of purification were investigated for a four gram reaction scale. Sonication of the crude reaction mixture using a minimal volume of 1:4 ethyl acetate:hexanes (2 mL per

mmol, repeated three times) afforded **2-7** as a white solid. The solutions from sonication were combined, concentrated in vacuo to a black paste, and recrystallized using 1:4 ethyl acetate:hexanes to afford additional **2-7**. Attempts to recrystallize the crude reaction with a variety of solvent mixtures without first using sonication resulted in lower yields. Subsequent oxidation using *m*-CPBA afforded **2-8** in high yield. The deuterated version of **2-8** was accomplished in an identical manner, with the exception of using d_8-methyl methacrylate to form **2-9** (Scheme 2.4).[11]

Scheme 2.4. Synthesis of deuterated cross-linker **2-12**.

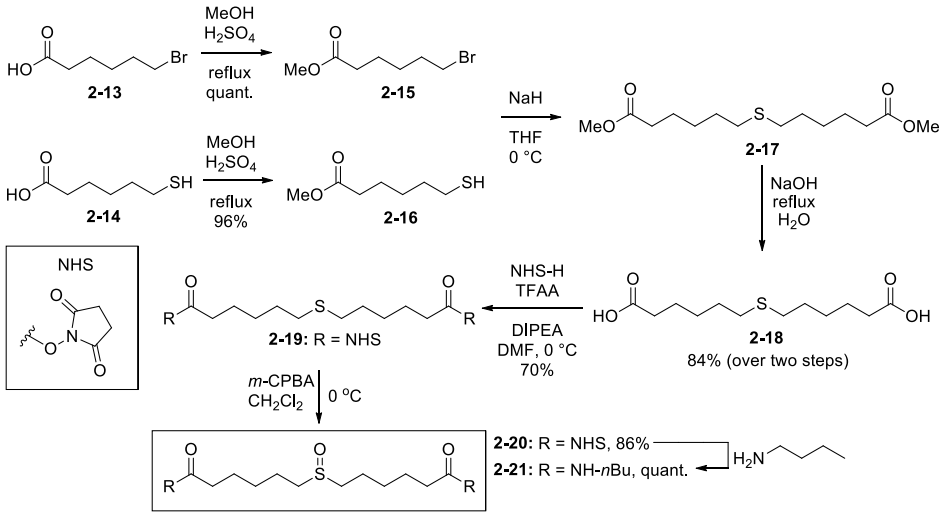


II. Synthesis of New Lysine-Reactive CID-XLs

Utilizing the effectiveness of the sulfoxide as a good CID-cleavable functional group, a cross-linker with a longer linear carbon chain than DSU was pursued (Scheme 2.5). The longer length of the cross-linker would make it possible to link lysines that are further away, potentially providing additional information about the spacial relativity of amino acids in a protein. It was hypothesized that esterification of both **2-13** and **2-14** could facilitate formation of **2-17** by rendering the carboxylic acids inert under basic conditions. Thankfully, after esterification of both **2-13** and **2-14**[12], addition of thiol ester **2-16** to a solution of bromo-ester **2-15** with sodium hydride in THF afforded sulfide **2-17** exclusively.[13] Heating **2-17** in sodium hydroxide provided diacid **2-18** in 84% yield over two steps. *N*-Hydroxysuccinimide (NHS) ester formation of

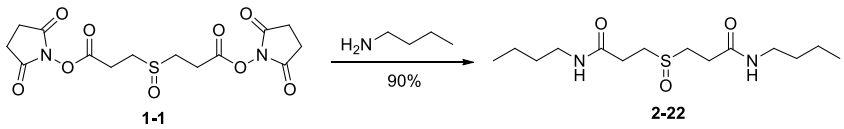
sulfide **2-18** proceeded in 70% yield after column chromatography, and subsequent oxidation with *m*-CPBA afforded sulfoxide **2-20**.[14] The synthesis of **2-20** was accomplished in 49% yield over five convergent steps with only one chromatographic purification.

Scheme 2.5. Synthetic pathway to form sulfoxide **2-20**.



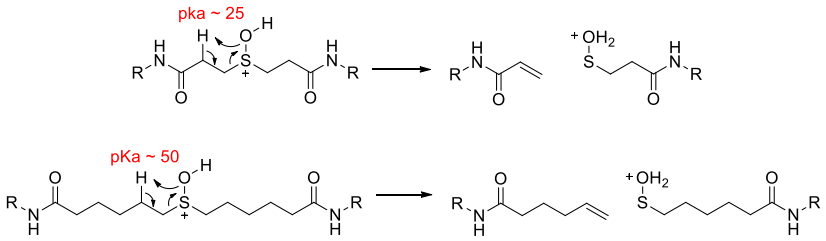
To probe the voltage required for cleavage of the cross-linker, bis-NHS-ester **2-20** was treated with *n*-butylamine to afford diamide **2-21**, a model system to simulate the reaction between an NHS ester and a lysine in a protein. When subjected to MS/MS analysis at voltages of 5, 10, 15, and 20 V, diamide **2-21** cleaved at the same collision energy as the peptide backbone (Appendix Figure A.1). As a control, DSU was also reacted with *n*-butylamine and then subjected to MS/MS analysis at voltages of 5, 10, 15, and 20 V (Scheme 2.6 and Appendix Figure A.2).

Scheme 2.6. Formation of diamide **2-22.**

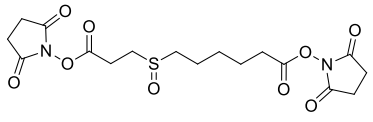


It was found that diamide **2-22** cleaved at 10 V, which was well below the threshold of the peptide backbone. With **2-21** cleaving at the same energy as the peptide backbone, it was decided that **2-20** would not be an effective cross linker. We hypothesized that the acidity of the sulfoxide core β-hydrogens had an effect on the collision energy required to cleave during MS/MS (Scheme 2.7). Less acidic β-hydrogens appeared to require higher collision energies to initiate sulfoxide cleavage.

Scheme 2.7. Proposed cleaving mechanism of the sulfoxide functional group.



It was envisioned that cross-linkers could be designed to maintain the cleaving ability of DSU on one side, while altering the length of the other arm. The target sulfoxide **2-23** was designed to test this hypothesis. (Figure 2.1). Sulfoxide **2-23** was designed to be three carbons longer than DSU on one side, keeping the relationship of five methylenes in-between the sulfoxide and carbonyl similar to **2-20**, while the opposite side would keep the same relationship of two methylenes in-between the sulfoxide and carbonyl as seen as DSU.



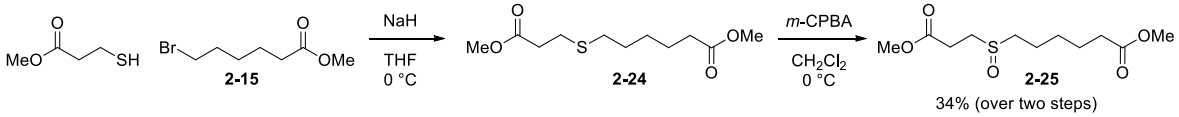
2-23

Figure 2.1. Target cross-linker **2-23.**

To probe the cleavage energy of the new sulfoxide linker system, a diester of **2-23** was first prepared (Scheme 2.8). The diester was used as a surrogate for the NHS-ester because the diester could be quickly prepared in two steps. Addition of the thiol to a solution of sodium

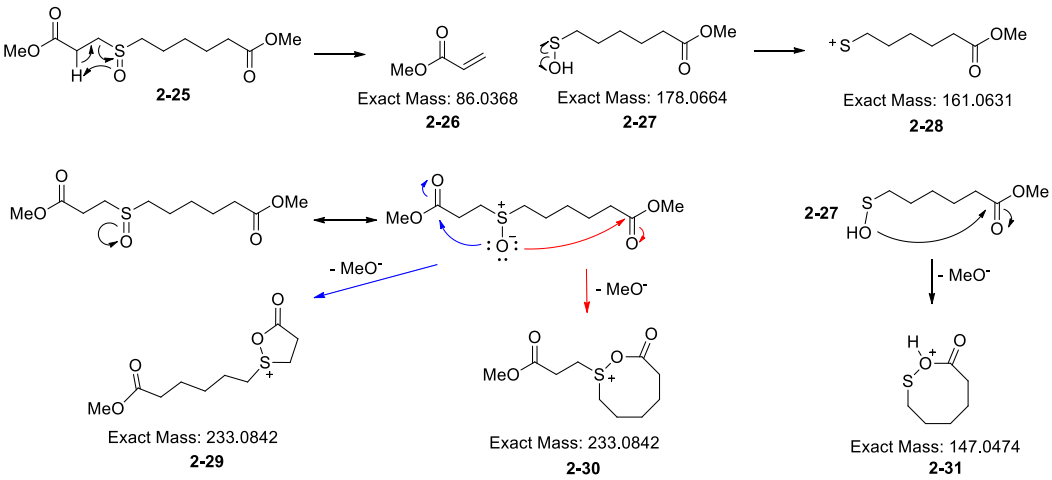
hydride and bromide **2-15** provided sulfide **2-24**, and subsequent oxidation with *m*-CPBA afforded the sulfoxide ester **2-25**.[13]

Scheme 2.8. Synthetic pathway to form sulfoxide ester **2-25.**



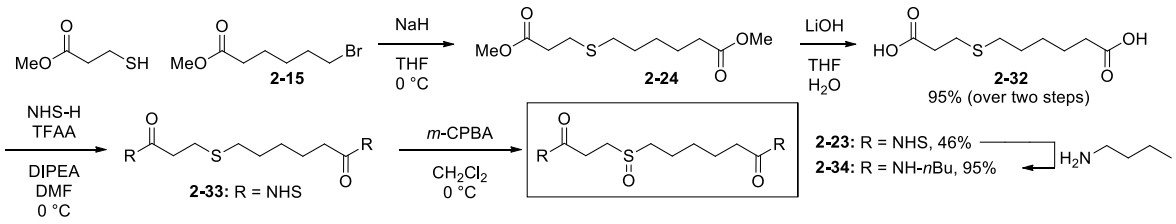
MS/MS analysis for sulfoxide **2-25** gave the expected α-β unsaturated carbonyl **2-26** as well as the hydroxythio-fragment **2-27** (Scheme 2.9 and Appendix Figure A.3).[15] The small peak at 160.91 *m*/*z* is hypothesized to arise from the loss of hydroxyl radical from **2-27**, giving fragment **2-28**. However, the largest peak in the MS/MS spectrum was at 232.97 *m*/*z*. This is hypothesized to arise from addition of the sulfoxide oxygen into either carbonyl, followed by displacement of the methoxy group to give **2-29** or **2-30**. The peak at 146.91 *m*/*z* is hypothesized to arise from attack of **2-27** into the carbonyl, followed by displacement of the methoxy group, giving **2-31**. Hypothesized ring fragments **2-29**, **2-30**, and **2-31** hindered an accurate approximation of when **2-23** would fragment, which prompted pursuing the full synthesis of sulfoxide **2-23.**

Scheme 2.9. Hypothesized fragmentation mechanisms for sulfoxide **2-25.**



To synthesize sulfoxide **2-23**, hydrolysis of **2-24** using lithium hydroxide afforded diacid **2-32**. NHS ester formation followed by subsequent oxidation of sulfide **2-33** with *m*-CPBA afforded sulfoxide **2-23**, which was reacted with *n*-butylamine to yield diamide **2-34** (Scheme 2.10). Diamide **2-34** was subjected to MS/MS and was found to cleave completely around 10 V, yielding only the expected fragments with no observed cyclization intermediates. Even at a collision energy of 25 V, the major fragments correspond to cleavage on only the side of **2-34** which contained two methylenes in-between the sulfoxide and carbonyl, suggesting that **2-23** is an effective cross-linker (Appendix Figure A.4).

Scheme 2.10. Synthetic pathway to form sulfoxide **2-23.**



With evidence suggesting **2-23** as an effective cross-linker, it was handed off to collaborators for *in vitro* testing. While biological experiments were being conducted, three additional lysine-reactive CID-XLs were synthesized. Utilizing the effectiveness of the sulfoxide as a good CID-cleavable functional group, sulfoxides **2-35**, **2-36**, and **2-37** were envisioned (Figure 2.2). As each cross-linker acts as a mini "ruler" to measure approximate distances between lysines, cross-linkers of different lengths could provide additional information about the spacial relativity of lysines in a protein. Sulfoxides **2-35** and **2-36** were designed to be shorter than **2-23**, while sulfoxide **2-37** was designed to be slightly longer**.**

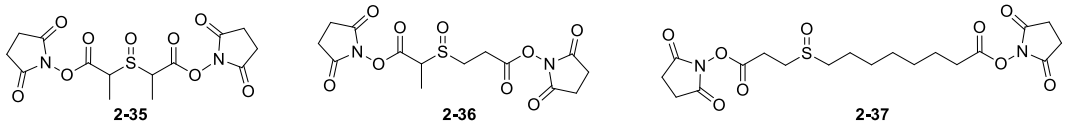
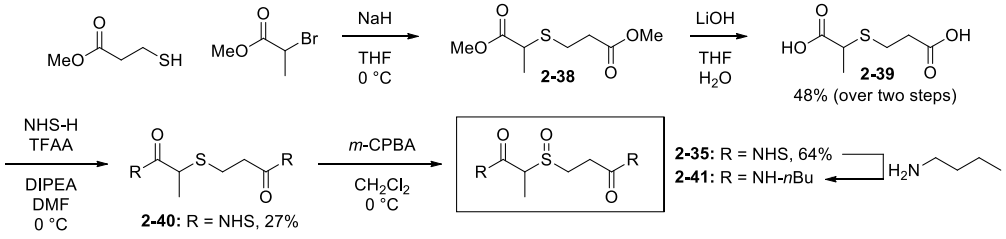


Figure 2.2. Target mixed length cross-linkers**.**

The synthesis of sulfoxides **2-35**, **2-36**, and **2-37** were all accomplished in a similar manner. Addition of the thiol to a solution of sodium hydride and the respective bromide provided the sulfide, which was hydrolyzed to afford the diacid. NHS ester formation, followed by subsequent oxidation of the sulfide with *m*-CPBA, afforded the corresponding sulfoxide.[14] In order to mimic the biological system when a lysine reacts with an NHS-ester, the sulfoxides were reacted with *n*-butylamine to yield diamides. Each of the diamides was subjected to MS/MS analysis. Diamide **2-41** was subjected to MS/MS and complete cleavage was observed before 15 V, yielding the expected fragments (Scheme 2.11, Appendix Figure A.5). It was not known at which side of the sulfoxide that **2-41** was cleaving, as cleavage on either side would produce fragments of identical mass. Regardless of the side at which **2-41** cleaves, the MS/MS data suggested **2-35** would be a potentially effective cross-linker.

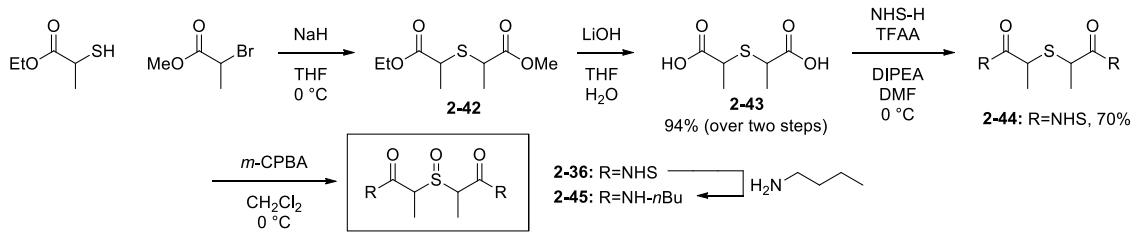
Scheme 2.11. Synthesis of sulfoxide **2-35**.



After the oxidation of NHS-ester **2-44** with *meta*-chloroperoxybenzoic acid (*m*-CPBA), the byproduct of the oxidation (*meta*-chlorobenzoic acid) could not be separated from the desired product despite different workup techniques and column chromatography conditions. Additional attempts of oxidation using a variety of oxidants did not afford the desired sulfoxide, but instead either cleaved the NHS-ester or over-oxidized the sulfur to the sulfone.[16,17] Nevertheless, the crude reaction mixture from the oxidation of **2-44** with *m*-CPBA was taken forward to synthesize diamide **2-45** (Scheme 2.12). Even though *meta*-chlorobenzoic acid still remained as an impurity before MS/MS analysis, **2-45** was identifiable as a separate ion during MS/MS

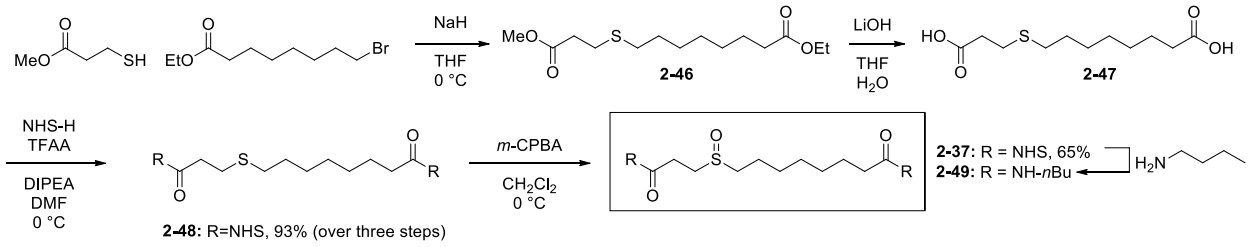
experiments. Diamide **2-45** was subjected to MS/MS, and only showed partial cleavage at 15 V (Appendix Figure A.6). Complete cleavage was observed at 20 V. Incomplete cleavage below 15 V, along with the difficulty in purification, suggested that **2-36** would not be an effective cross-linker.

Scheme 2.12. Synthesis of sulfoxide 2-**36**.



Diamide **2-49** was subjected to MS/MS and complete cleavage was observed before 15 V, producing only the expected fragments. Even at a collision energy of 25 V, the major fragments correspond to cleavage towards the shorter side which contained two methylenes in-between the sulfoxide and carbonyl, suggesting that **2-37** is an effective cross-linker (Scheme 2.13, Appendix Figure A.7). With evidence suggesting **2-35** and **2-37** are effective cross-linkers, they were handed off to collaborators for *in vitro* testing.

Scheme 2.13. Synthesis of sulfoxide **2-37**.



Preliminary results for the *in vitro* testing of the mixed length cross-linkers **2-23**, **2-35**, and **2-37** showed that they did cross-link lysine residues, but at a slower rate than commercially available lysine-reactive cross-linkers. The structure of commercially available cross-linkers

differed from the mixed length linkers by containing many *poly*-ethylene glycol (PEG) units as well as an oxygen gamma to the NHS ester (Figure 2.3).

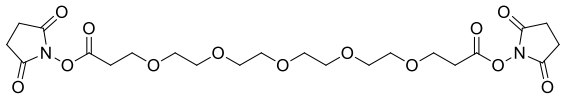


Figure 2.3. One example of a commercially available cross-linker. Other similar cross-linkers contain varying amounts of PEG units (e.g. "BS(PEG)9" contains 9 PEG units).

It was hypothesized that the added oxygenation in the commercially available cross-linkers improved the solubility of the cross-linkers in aqueous buffer. The three CID-XLs synthesized all contained grease-like aliphatic chains, which possibly hindered aqueous solubility and would result in a lower effective cross-linker concentration, lowering reactivity during *in-vitro* testing. Therefore, modified sulfoxide cross-linkers **2-50** and **2-51** were designed (Figure 2.4).

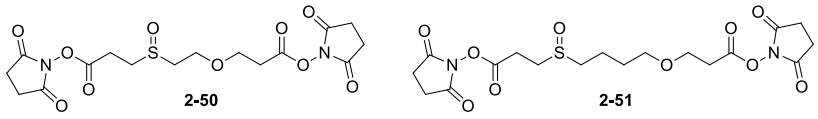
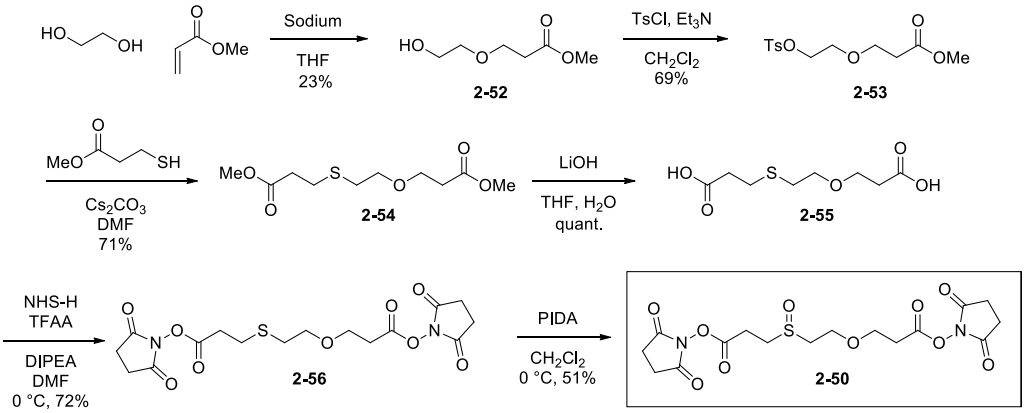


Figure 2.4. Modified oxygenated sulfoxide cross-linkers **2-50** and **2-51**.

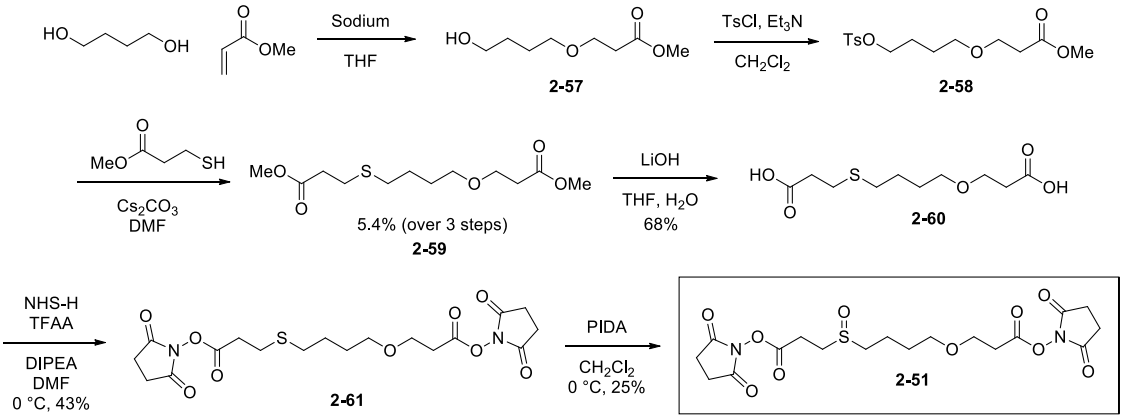
The synthesis of cross-linker **2-50** began with an oxa-Michael addition of ethylene glycol into methyl acrylate to generate ester **2-52.** Tosylation of the primary alcohol, followed by nucleophilic displacement by 3-mercaptopropinoate afforded diester **2-54**. Basic hydrolysis and then NHS ester formation gave sulfide **2-56**. Finally, oxidation to the sulfoxide yielded the desired cross-linker **2-50** (Scheme 2.14).

Scheme 2.14. Synthesis of new oxygenated cross-linker **2-50.**



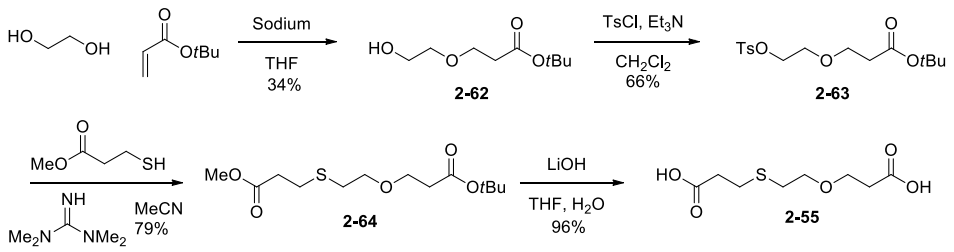
The synthesis of sulfoxide cross-linker **2-51** proceeded in an almost identical manner (Scheme 2.15). However, the oxa-Michael addition of 1,4-butanediol into methyl acrylate afforded an unidentified side product in addition to alcohol **2-57** which co-eluted during column-chromatography. The mixture was subjected to tosylation conditions to afford **2-58** and an unknown side product. Despite multiple chromatography conditions, **2-58** could not be separated from the unknown byproduct. Pushing forward, after coupling tosylate **2-58** to methyl 3-mercaptopropanoate, the pure thiol **2-59** was isolable after column chromatography. Saponification, subsequent NHS ester formation, and final sulfur oxidation afforded oxygenated cross-linker **2-51**.

Scheme 2.15. Synthesis of new oxygenated linker **2-51.**



Preliminary results from *in vitro* testing obtained by our collaborators suggested that these new oxygenated cross-linkers were more reactive than the non-oxygenated counterparts. This prompted investigation into improving the overall yield of **2-50** and **2-51**, especially the initial oxa-Michael addition. The low yield was due mostly to the competing *trans*-esterification reaction, so it was hypothesized that switching the Michael acceptor from methyl acrylate to *tert*-butyl acrylate would slow the competing reaction. Utilizing *tert*-butyl acrylate greatly increased the yield of the 1,4-butanediol addition, and also resulted in a single separable product after column chromatography. The yield of the ethylene glycol addition was only slightly increased, and *trans*-esterification still accounted for the majority of product. Neither mono-protection of the alcohol, switching the base, nor varying the concentration of the reaction had any improvement on the yield of the desired Michael addition product. Regardless, due to the inexpensive starting materials and ease of purification of compound **2-62**, the synthesis was carried forward (Scheme 2.16).

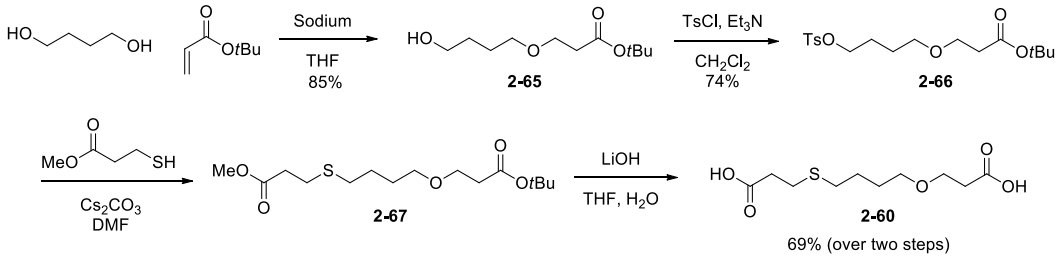
Scheme 2.16. Improved synthesis of cross-linker **2-55.**



The synthesis starting from *tert*-butyl acrylate was very similar to the synthesis starting from methyl acrylate. Instead of cesium carbonate as the base used during the coupling reaction, a brief base screen was conducted, and 1,1,3,3-tetramethylguanidine was found to produce **2-64** in the highest yield. The overall yield for **2-50** increased from 0.4% to 0.6% over six steps. The synthesis of **2-51** was also executed in a similar fashion, however the yield of the first step

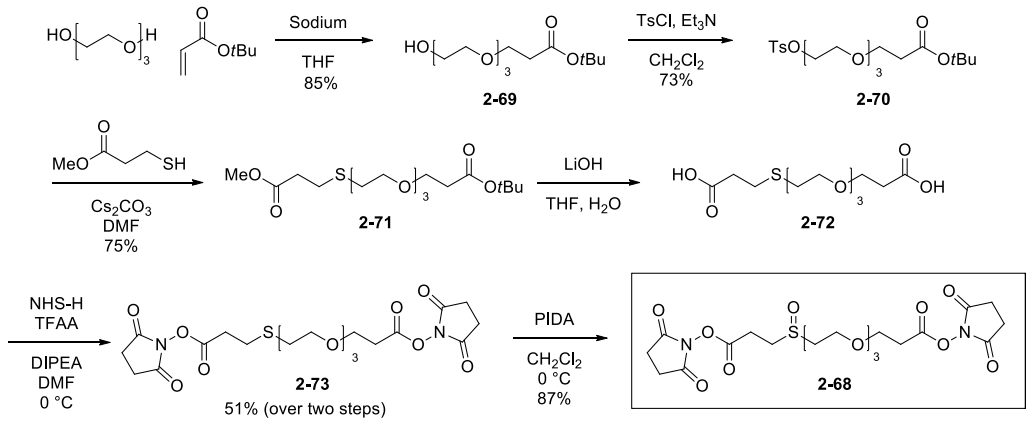
greatly increased (Scheme 2.17). The overall yield for **2-51** increased from 0.7% to 9% over six steps.

Scheme 2.17. Improved synthesis of cross-linker **2-60.**



In addition to oxygenated linkers **2-50** and **2-51**, a longer oxygenated linker **2-68** was also synthesized (Scheme 2.18). *tert*-Butyl acrylate was used initially for the oxa-Michael addition to afford *tert*-butyl ester **2-69** in high yield. Tosylation followed by thiol coupling gave **2-71**. Hydrolysis and subsequent NHS-ester formation afforded **2-73**. Finally, sulfur oxidation afforded **2-68** in an overall 21% yield over six steps. Both *in-vitro* and *in-vivo* cross-linking experiments are currently being conducted for CID-XLs **2-50**, **2-51**, and **2-68**.

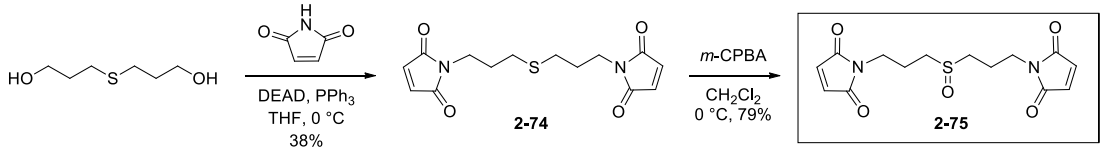
Scheme 2.18. Synthesis of longer oxygenated cross-linker **2-68.**



III. Synthesis of Maleimide-Reactive CID-XLs

With no CID-XLs currently containing cysteine reactive functional groups, it was envisioned to appended maleimides to a sulfoxide chain, which would serve as a Michael-acceptor for free cysteines (Scheme 2.19).[18] Thus, the synthesis of **2-75** was developed to allow quick access to a potential cysteine-reactive CID-XL. Commercially available 3,3'-thiodipropanol and maleimide were subjected to Mitsunobu conditions to afford the dimaleimide. Despite different reaction times, reaction temperatures, and reagent equivalents, the maximum yield of **2-74** obtained was 38%.[19–21] Subsequent oxidation afforded sulfoxide **2-75** in 79% yield. MS/MS analysis of **2-75** indicated complete fragmentation around 15 V, which was very close to the energy required to fragment the peptide backbone (Appendix Figure A.8). It was unknown whether **2-75** could fragment completely before the peptide backbone during CID. *In-vitro* testing confirmed **2-75** fragmented at the same collision energy as the peptide backbone, demonstrating that **2-75** was not an effective CID-XL.

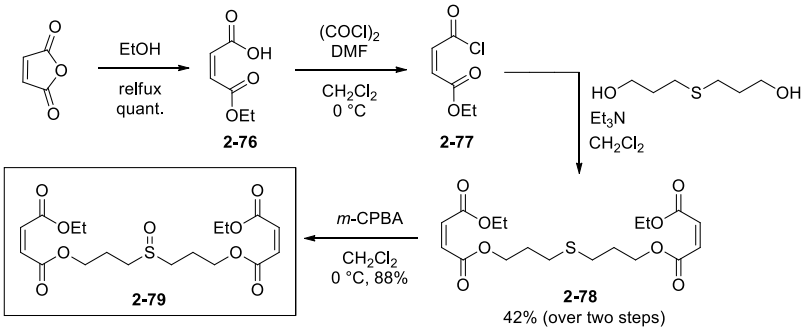
Scheme 2.19. Synthesis pathway of cysteine reactive cross-linker **2-75.**



It has been shown that maleimides can be susceptible to hydrolysis during *in vitro* cross-linking.[22] Additionally, the Huang group had experimentally observed partial hydrolysis of commercially available maleimide-containing cross-linkers. This inspired investigating a Michael acceptor that had a low rate of hydrolysis yet still had a quick rate of reaction with cysteine.[18,22,23] Diethyl maleate was discovered to possess these desired qualities, and was incorporated into a potential CID-XL **2-79** (Scheme 2.20). To synthesize sulfoxide **2-79**, maleic anhydride was opened to give **2-76** in quantitative yield by heating at reflux in ethanol.[24]

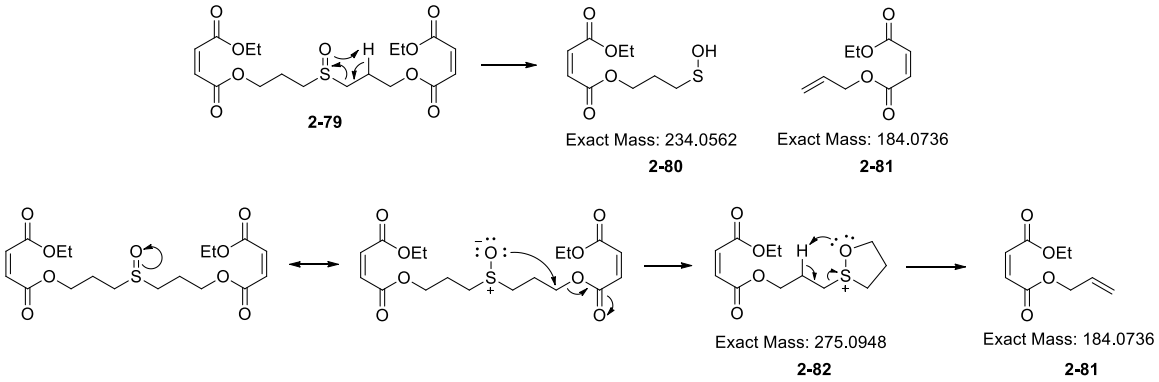
Formation of acid chloride **2-77** proceeded smoothly, which was reacted immediately with 3,3'-thiodipropanol and triethylamine to yield **2-78** in 42% yield after column chromatography over two steps.[25–27] Subsequent oxidation to the sulfoxide proceeded in high yield to afford **2-79** in an overall 37% yield over four steps with only one chromatographic purification.

Scheme 2.20. Synthesis of the Michael acceptor sulfoxide **2-79**.



MS/MS analysis of **2-79** indicated almost complete fragmentation at a collision energy of 10 V (Appendix Figure A.9). However, the expected hydroxythio-fragment **2-80** was not observed at 10 V, suggesting that **2-79** is not cleaving in the expected manner. Instead, a peak was observed at 275 *m*/*z*, which is hypothesized to arise from the sulfoxide oxygen displacing the maleate fragment, yielding the five-membered ring **2-82** (Scheme 2.21). Intermediate **2-82** is hypothesized to fragment further to give alkene **2-81**, which is seen at a collision energy of 15 V. Fragment **2-82** was suggested to hinder the expected fragmentation pathway, raising the energy that is needed to completely fragment the cross-linker. Initial *in-vitro* cross-linking experiments confirmed that CID-XL **2-79** did not fragment completely before the peptide backbone, and therefore it was concluded that **2-79** was not an effective CID-XL.

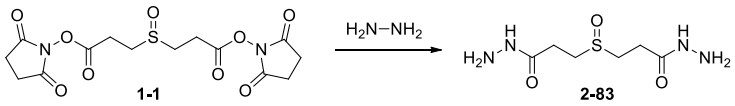
Scheme 2.21. Proposed fragmentation mechanisms for sulfoxide **2-79.**



IV. Synthesis of Carboxylic Acid-Reactive CID-XLs

With less than five carboxylic acid-reactive cross-linkers in the literature and none of them containing a CID-cleavable functional group, CID-XL **2-83** was designed (Scheme 2.22).[28] It was hypothesized that subjecting DSU to hydrazine would afford **2-83** with the byproduct of the reaction, *N*-hydroxysuccinimide, able to be easily removed from the reaction by trituration. Despite triturations in many solvents, as well as attempted recrystallizations, pure **2-83** could not be isolated.

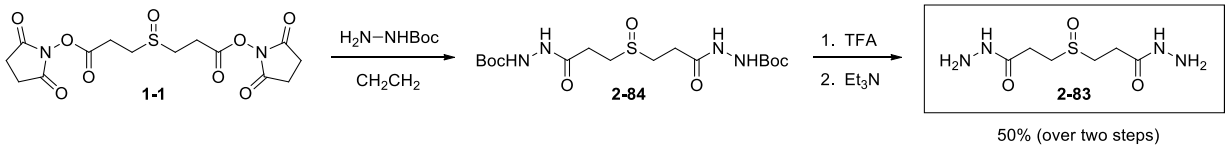
Scheme 2.22. Initial synthesis plan of carboxylic acid-reactive CID-XL **2-83.**



A revised synthesis of **2-83** was developed that employed a Boc-protected hydrazine, as similar systems in the literature benefited from a two-step protected hydrazine addition followed by deprotection, rather than a one-step unprotected hydrazine addition.[29] Thankfully, addition of *tert*-butyl hydrazinecarboxylate to DSU resulted in crude **2-84** which was immediately deprotected and subsequently basified, generating the dihydrazide (Scheme 2.23). Fortunately,

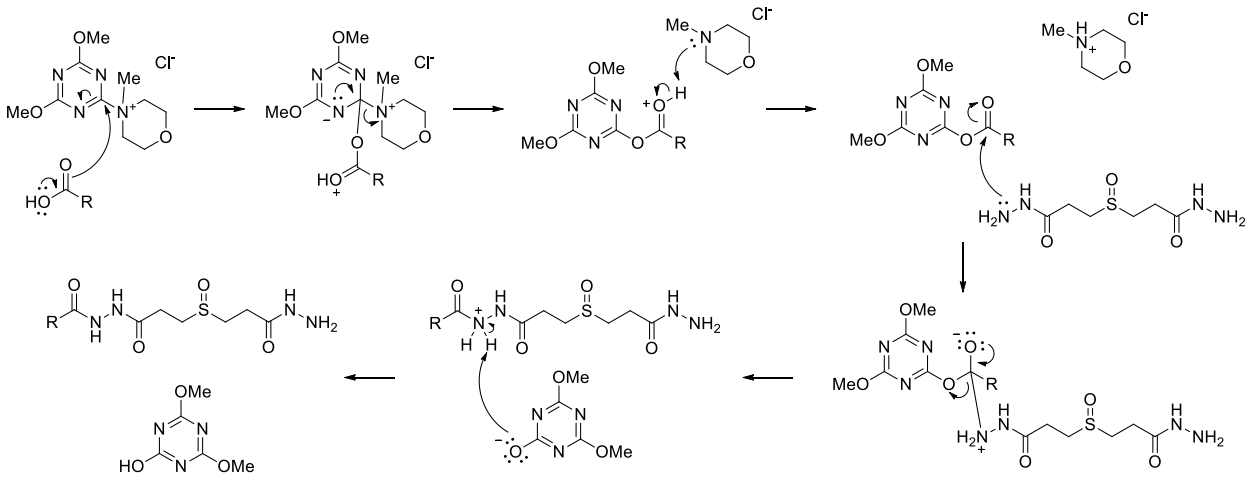
2-83 was found to be sparingly soluble in methanol, so repeated trituration of the crude reaction mixture with cold methanol afforded **2-83**.

Scheme 2.23. Successful synthesis of **2-83.**



When cross-linking acidic residues in proteins, a coupling agent is required to activate either the acidic residue or the cross-linker. The coupling reagent used during *in-vitro* experiments was 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium chloride (DMTMM), which activates either glutamic or aspartic acid before cross-linking with **2-83** (Scheme 2.24).[30] CID-XL **2-83** was found to cross-link model proteins when used with DMTMM, and *in-vivo* experiments are currently underway to discover the utility of the first carboxylic acid-reactive CID-XL.[31]

Scheme 2.24. Proposed mechanism for the activation of carboxylic acid residues followed by cross-linking with **2-83.**



V. Conclusions

Improved gram-scale syntheses of four previously developed CID-XLs were developed. A longer sulfoxide-containing CID-XL was synthesized, and although it did not prove to be effective for biological testing, it did provide data which contributed to formulation of a CID cleavage mechanism for the sulfoxide functional group. Six other lysine-reactive CID-XLs were synthesized, three of which are showing promise in biological testing. Two cysteine-reactive CID-XLs were synthesized, but failed to provide any useful data during biological testing. The first carboxylic acid-reactive CID-XL was synthesized, and was shown to effectively cross-link model proteins and aid in their sequencing.

VI. General Experimental Details

All chemicals were purchased from Sigma-Aldrich, Acros Organics, Alfa Aesar, TCI, Advanced ChemTech, or Fisher and used without further purification unless otherwise noted. Zinc (II) chloride was flame dried under vacuum prior to use. Ethanol was purchased from Gold Shield. Solvents were of reagent grade and used as without further purification except as follows: *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), tetrahydrofuran (THF), and diethyl ether (ether) were degassed and then passed through anhydrous neutral alumina A-2 before use, according to the procedure described by Grubbs.[32] Methanol was dried over activated 3Å molecular sieves prior to use. Triethylamine was distilled over calcium hydride and stored over activated 3Å molecular sieves prior to use. Diisopropylethylamine (DIPEA) was distilled over calcium hydride prior to use. Trifluoroacetic anhydride (TFAA) and trimethylsilyl triflate (TMS-OTf) were distilled prior to use. Reported reaction temperatures refer to the temperature of the heating medium. Reactions were performed in flame- or oven-dried

glassware under an atmosphere of dry argon using standard Schlenk techniques unless otherwise noted. Room temperature (rt) refers to 25 ± 3 ºC. Reactions were monitored by thin-layer chromatography (TLC) using EMD Chemicals Inc. silica gel 60 F_{256} plates. Flash chromatography was performed using Ultra Pure SiliaFlash P60, 230-400 mesh (40-63 μm) silica gel (SiO_2) following the general procedure by Still and co-workers.[33]

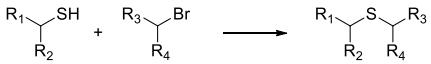
VII. Instrumentation

Proton NMR spectra measurements were acquired using either a Bruker DRX500 with a cryoprobe, Bruker GN500, or a Bruker AVANCE600 spectrometer, at 500 MHz, 500 MHz, and 600 MHz, respectively. Carbon NMR spectra were obtained on a Bruker DRX500 with a cryoprobe at 125 MHz. Proton NMR chemical shifts (δ) are reported in parts per million (ppm) and referenced to the residual solvent peak at 7.26 ppm for deuterated chloroform ($CDCl_3$) and 2.50 for deuterated dimethylsulfoxide (DMSO-d_6).[34] Carbon NMR chemical shifts (δ) are reported in parts per million (ppm) and referenced to the residual solvent peak at 77.16 ppm for deuterated chloroform and 39.52 for deuterated dimethylsulfoxide.[34] All NMR spectra were processed using MestReNova (Mestrelab Research). NMR data are reported in the following manner: chemical shift, multiplicity, (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, br = broad, app = apparent), coupling constants (J) in hertz (Hz), and integration. High resolution mass spectrometry (HRMS) accurate mass experiments were ran by the University of California, Irvine mass spectrometry laboratory. Infrared (IR) spectroscopy data were acquired on a Shimadzu IRAffinity-1 Spectrophotometer with a MIRacle 10 single reflection ATR accessory. Melting points (mp) were acquired on a Mel-Temp melting point

apparatus and are uncorrected. Tandem mass spectroscopy (MS/MS) analysis was performed on a Waters Quattro Premier XE mass spectrometer by Dr. John Greaves.

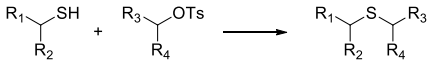
VIII. Detailed Experimental Procedures for Compounds in Chapter 2

General Procedure 2.1: Formation of sulfides with sodium hydride.



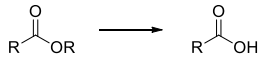
To a cooled (0 ºC) solution of the alkyl bromide (1 equiv) and sodium hydride (60 % dispersion in mineral oil, 1.5 equiv) in THF (0.072 M) was added a solution of the sulfide (1.1 equiv) in THF (0.065 M) dropwise, slowly. The grey solution was let warm to rt. After the reaction was complete by TLC, the crude reaction mixture was partitioned between DCM and water. The layers were separated, and the aqueous layer was extracted with DCM (2×). The combined organic layers were washed with water (2×) and brine (1×), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo.

General Procedure 2.2: Formation of sulfides with cesium carbonate.



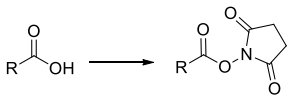
To a solution of the tosylated alcohol (1.1 equiv) and cesium carbonate (2.0 equiv) in DMF (0.40 M) was added the sulfide (1.0 equiv) dropwise, slowly. The milky solution was let stir overnight at rt. After the reaction was complete by TLC, the crude reaction mixture was diluted in sufficient DCM. The organic layer was washed with water (3×) and brine (1×), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo.

General Procedure 2.3: Diacid formation from hydrolysis of diesters.



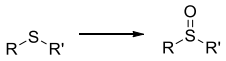
To the diester (1 equiv) in a solution of THF (0.1 M) was added a solution of lithium hydroxide monohydrate (10 equiv) in an equal volume of H_2O. The reaction was let stir at rt until determined complete by TLC (longer reaction times were observed for the *t*-butyl ester, up to three days). The crude reaction was cooled (0 ºC) and then carefully acidified to a pH of 1 (monitored by pH paper) with sulfuric acid (18 M). The acidified aqueous layer was extracted with ethyl acetate (3×). The organic layers were combined, washed with water (2×), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo**.**

General Procedure 2.4: *N*-hydroxysuccinimide ester formation from diacids.[14]



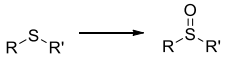
To a cooled (0 ºC) mixture of the diacid (1 equiv), *N*-hydroxysuccinimide (4 equiv), and DIPEA (8 equiv) in DMF (0.2 M) was added TFAA (4 equiv) dropwise, slowly. The light orange solution was allowed to warm to rt and stir until determined complete by TLC, after which it was partitioned between ethyl acetate and hydrochloric acid (1 M). The layers were separated, and the acidic aqueous layer was extracted with ethyl acetate (2×). The organic layers were combined, washed with sodium bicarbonate solution (1 M, 3×), water (1×), and brine (1×). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo.

General Procedure 2.5: Oxidation of sulfides using *m*-CPBA.[35]



To a cooled (0 ºC) solution of the sulfide (1 equiv) in DCM (0.6 M) was added a solution of *m*-CPBA (1 equiv) in DCM (0.6 M) dropwise, slowly. After stirring for 10 min, the reaction mixture was diluted in additional DCM and washed with sodium bicarbonate solution (1 M, 3×) and water (3×). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo.

General Procedure 2.6: Oxidation of sulfides using phenyliodonium diacetate (PIDA).

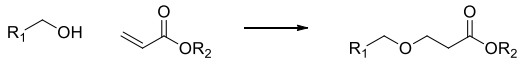


To a solution of the sulfide (1 equiv) in DCM (0.06 M) was added PIDA (1.2 equiv) in one portion. After stirring open to air for 24 hours, the crude reaction mixture was concentrated in vacuo.

General Procedure 2.7: Amide formation of NHS sulfoxides.

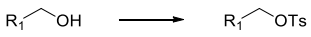
To the sulfoxide (1 equiv) was added *n*-butylamine (4 equiv). The resulting solution was let stir for 1 h, after which it was diluted in hexanes (20 mL). The resulting precipitate was collected by filtration, triturated with hexanes (4×), and dried in vacuo.

General Procedure 2.8: Formation of ethers from an oxa-Michael reaction.[36]



To a solution of the alcohol (2.84 equiv) in THF (0.660 M) was added sodium metal (1 mol%). The resulting mixture was let stir at rt for 2 h, after which the acrylate (1 equiv) was added dropwise, slowly. The reaction was let stir overnight, after which any excess sodium was quenched by adding HCl (1M). After adding sufficient water, the mixture was extracted with DCM (3×). The combined organic layers were washed with brine (1×), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo.

General Procedure 2.9: Tosylation of primary alcohols.



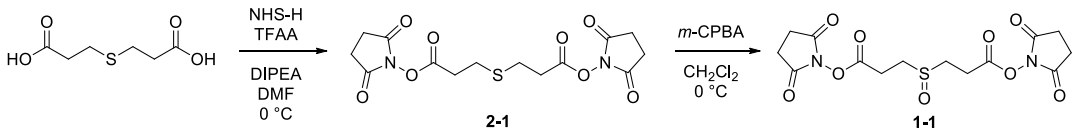
To a stirred solution of the primary alcohol (1 equiv) in DCM (0.33 M) was added triethylamine (1.75 equiv), followed by tosyl chloride (1.5 equiv). The reaction was let stir overnight, after which it was quenched with saturated sodium bicarbonate. The organic layer was separated, and then diluted with sufficient DCM. The organic layer was washed with water (2×), brine (1×), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo.

General Procedure 2.10. Purification of compounds via normal-phase chromatography using an automated column chromatography instrument.

Automated column chromatography (ISCO purification) was performed on a Teledyne Isco CombiFlash® Rf+ instrument. The following parameters were the same for all purifications. Initial waste: 0.0 column volumes (CV). Air purge: 0.0 minutes. Peak tube volume: max. Non-peak tube volume: max. Loading type: solid. Wavelength range for

detection: 200-400 nm, threshold 0.10 AU. All other parameters are detailed in each compound's experimental procedure.

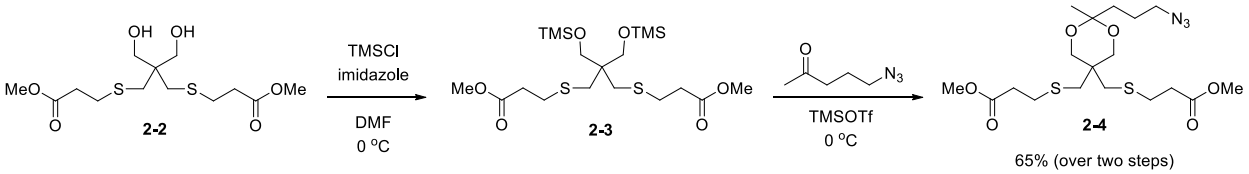
Bis(2,5-dioxopyrrolidin-1-yl)-3,3'-sulfinyldipropanoate (1-1)



3,3'-Thiodipropionic acid (1.00 g, 5.61 mmol) was subjected to general procedure 2.4 to afford an orange solid. The crude product was triturated with diethyl ether (3 × 12 mL) and dried in vacuo to afford crude **2-1** as a tan solid which was used immediately without further purification.

To a cooled (0 ºC) solution of crude **2-1** (0.900 g) in DCM (22 mL per mmol of crude **2-1,** sonication for 10 minutes at rt needed to dissolve **2-1** completely) was added a solution of *m*-CPBA (mixture of 77% *m*-CPBA by weight, 0.569 g) in DCM (3 mL per mmol of *m*-CPBA) dropwise, slowly. After stirring for 1 h, the reaction mixture was vacuum filtered, washed with ice cold chloroform (20 mL), and dried in vacuo to afford **1-1** as a white solid (0.650 g, 69% over two steps). ^{1}H and ^{13}C NMR spectra were consistent with those previously reported for this compound.[1]

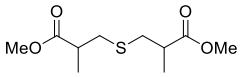
Dimethyl 3,3'-(((2-(3-azidopropyl)-2-methyl-1,3-dioxane-5,5-diyl)bis(methylene))bis(sulfanediyl))dipropanoate (2-4)



To a stirred solution of **2-2**[35] (0.108 g, 0.318 mmol) and imidazole (0.148 g, 2.17 mmol) in DMF (4 mL) was added TMSCl (1 M solution in THF, 1.8 mL) resulting in a yellow solution. After stirring for 12 h, the reaction mixture was quenched with water (40 mL) and extracted with ethyl acetate (3 × 30 mL). The combined organic portions were washed with water (3 × 30 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to afford crude **2-3** as an orange oil which was used immediately without further purification: ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.71–3.67 (m, 10H), 2.78 (t, *J* = 7.5 Hz, 4H), 2.61 (t, *J* = 7.5 Hz, 4H), 2.57 (s, 4H), 0.08 (s, 18H).

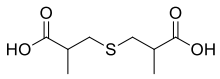
To a cooled (–78 ºC) solution of crude **2-3** (1.00 g) and 5-azidopentan-2-one[5] (0.262 g, 2.06 mmol) was added TMS-OTf (50 μL, 0.1 mmol). The solution was stirred for 12 h, over which it gradually warmed to room temperature. The reaction mixture was quenched with two drops of pyridine, and then diluted in ethyl acetate (100 mL). The organic layer was washed with water (2 × 100 mL) and brine (100 mL), dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo* to give crude **2-4** as a black oil. The crude product was purified via column chromatography (1:3 ethyl acetate:hexanes) to afford **2-4** as an orange oil (0.651 g, 65% over two steps). ^{1}H and ^{13}C NMR spectra were consistent with those previously reported for this compound.[5]

Dimethyl 3,3'-thiobis(2-methylpropanoate) (2-5)



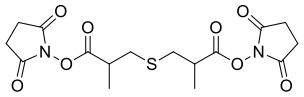
Methyl methacrylate (0.57 mL, 5.3 mmol) and thioacetic acid (0.47 mL, 6.8 mmol) were combined in a flame-dried vial under argon, which was sealed and heated to 80 °C for 6 h. The vial was allowed to cool to rt and the mixture was evaporated to remove any excess thioacetic acid. To the crude reaction mixture was added methanol (135 mL) and triethylamine (4.2 mL, 30 mmol), after which methyl methacrylate (1.9 mL, 18 mmol) was added dropwise. The reaction was let stir for 24 h, after which the solution was evaporated to remove methanol and triethylamine. The crude mixture was taken on to the next step assuming a quantitative yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.71 (s, 6H), 2.87–2.82 (m, 2H), 2.68–2.63 (m, 2H), 2.60–2.55 (m, 2H), 1.28–1.24 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.6, 51.9, 40.2, 36.0, 16.8; IR (thin film) 2976, 2953, 2936, 1730 cm^{-1}; HRMS (ESI) *m / z* calcd for $C_{10}H_{18}O_4SNa$ $(M+Na)^+$ 257.0823, found 257.0820.

3,3'-Thiobis(2-methylpropanoic acid) (2-6)



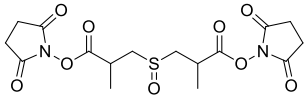
Diester **2-5** was subjected to general procedure 2.3 to afford **2-6** as a white solid (0.83 g, 75% over two steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.27 (br s, 2H), 2.84 (dd, *J* = 9.5, 12.2, 2H), 2.79–2.77 (m, 2H), 2.60 (dd, *J* = 4.4, 12.2, 2H), 1.28 (app d, *J* = 6.7, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 182.0, 40.1, 34.6, 17.5; IR (thin film) 2920, 2354, 1724, 1697, 1449 cm^{-1}; HRMS (ESI) *m / z* calcd for $C_8H_{14}O_4SNa$ $(M+Na)^+$ 229.0511, found 229.0507.

Bis(2,5-dioxopyrrolidin-1-yl) 3,3'-thiobis(2-methylpropanoate) (2-7)



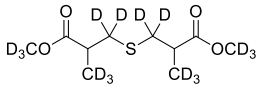
Diacid **2-6** (4.13 g, 20.0 mmol) was subjected to general procedure 2.4. Sonication of the crude product in 1:4 ethyl acetate:hexanes (3 × 40 mL) afforded **2-7** (4.01 g, 50%) as a white powder. Concentration of the trituration solvents in vacuo and further recrystallization in minimal 1:4 ethyl acetate:hexanes afforded additional **2-7** (1.04 g, 13%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.06–2.96 (m, 4H), 2.83 (br s, 8H), 2.78–2.72 (m, 2H), 1.43 (s, 3H), 1.42 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.5, 170.5, 169.2, 169.1, 38.4, 38.3, 35.7, 35.7, 25.8, 16.8, 16.6; IR (thin film) 2918, 1811, 1781, 1741, 1070 cm^{-1}; HRMS (ESI) *m / z* calcd for $C_{16}H_{20}N_2O_8SNa$ $(M+Na)^+$ 423.0838, found 423.0834.

Bis(2,5-dioxopyrrolidin-1-yl) 3,3'-sulfinylbis(2-methylpropanoate) (2-8)



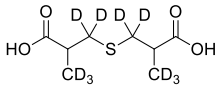
NHS ester **2-7** (0.063 g, 0.16 mmol) was subjected to general procedure 2.5, using ethyl acetate instead of DCM as the solvent, affording **2-8** as a white powder (0.058 g, 92%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.56–3.41 (m, 2H), 3.35–3.27 (m, 1.4H), 3.22 (dd, *J* = 9.2, 13.3, 0.6H), 2.90 (dd, *J* = 9.0, 13.2, 1H), 2.85 (s, 8H), 1.61–1.56 (m, 4H), 1.55 (s, 3H); IR (thin film) 2849, 1812, 1776, 1710, 1201 cm^{-1}; HRMS (ESI) *m / z* calcd for $C_{16}H_{20}N_2O_9SNa$ $(M+Na)^+$ 439.0787, found 439.0777.

Deuterated analog of dimethyl 3,3'-thiobis(2-methylpropanoate) (2-9)



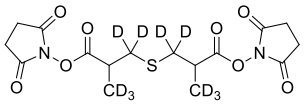
A similar procedure to preparation of **2-5** was performed starting with d_8-methyl methacrylate (1.00 g, 9.25 mmol) and thioacetic acid (0.720 mL, 10.2 mmol). The second step in the one-pot procedure was conducted with d_8-methyl methacrylate (1.00 g, 9.25 mmol), methanol (3.25 mL), and triethylamine (3.85 mL, 27.8 mmol). After evaporation, the crude mixture was taken on to the next step assuming a quantitative yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.64 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.6, 40.0; HRMS (ESI) *m / z* calcd for $C_{10}H_2D_{16}O_4SNa$ $(M+Na)^+$ 273.1827, found 273.1819.

Deuterated analog of 3,3'-thiobis(2-methylpropanoic acid) (2-10)



Diester **2-9** was subjected to general procedure 2.3 to afford **2-10** as a white solid (1.72 g, 86% over two steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.32 (br s, 2H), 2.72 (s, 1H), 2.71 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 181.9, 181.7, 40.0, 39.9; IR (thin film) 2919, 2544, 2229, 1696, 1421 cm^{-1}; HRMS (ESI) *m / z* calcd for $C_8H_3D_{10}O_4S$ $(M-H)^-$ 215.1162, found 215.1171.

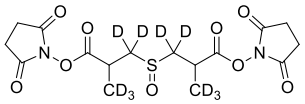
Deuterated analog of bis(2,5-dioxopyrrolidin-1-yl) 3,3'-thiobis(2-methylpropanoate) (2-11)



Diacid **2-10** (0.37 g, 1.7 mmol) was subjected to general procedure 2.4. Sonication of the crude product in 1:4 ethyl acetate:hexanes (3 × 4 mL) afforded **2-11** (0.365 g, 52%) as a white powder.

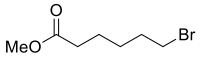
Concentration of the trituration solvents in vacuo and further recrystallization in minimal 1:4 ethyl acetate:hexanes afforded additional **2-11** (0.091 g, 13%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.99 (s, 1H), 2.98 (s, 1H), 2.83 (br s, 8H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.5, 169.2, 38.01, 37.97, 25.8; IR (thin film) 2924, 1812, 1782, 1737, 1065 cm^{-1}; HRMS (ESI) *m / z* calcd for $C_{16}H_{10}D_{10}N_2O_8SNa$ $(M+Na)^+$ 433.1465, found 433.1455.

Deuterated analog of bis(2,5-dioxopyrrolidin-1-yl) 3,3'-sulfinylbis(2-methylpropanoate) (2-12)



NHS ester **2-11** (0.22 g, 0.54 mmol) was subjected to general procedure 2.5, using $CHCl_3$ instead of DCM as the solvent, affording **2-12** as a white powder (0.20 g, 89%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.50–3.39 (m, 2H), 2.84 (s, 8H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.9, 33.0, 32.9, 31.9, 31.2, 29.9, 25.8; HRMS (ESI) *m / z* calcd for $C_{16}H_{10}D_{10}N_2O_9SNa$ $(M+Na)^+$ 449.1415, found 449.1412.

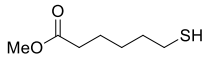
Methyl 6-bromohexanoate (2-15)



To a stirred solution of 6-bromohexanoic acid (1.04 g, 5.33 mmol) in methanol (20 mL) was added sulfuric acid (18 M, 3 drops). The colorless solution was heated at reflux for 12 h, after which it was let cool to rt. The solution was diluted in DCM (100 mL), then washed with water (2 × 100 mL) and brine (100 mL). The organic layer was dried over anhydrous sodium sulfate,

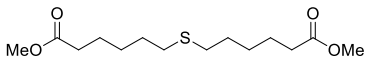
filtered, and concentrated in vacuo to afford **2-15** as a colorless oil (1.10 g, quant.). ^{1}H and ^{13}C NMR spectra were consistent with those previously reported for this compound.[37]

Methyl 6-mercaptohexanoate (2-16)



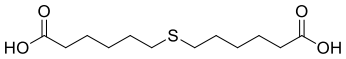
To a stirred solution of **2-14** (1.02 g, 6.88 mmol) in methanol (20 mL) was added sulfuric acid (18 M, 3 drops). The colorless solution was heated at reflux for 12 h, after which it was let cool to rt. The solution was diluted in DCM (100 mL), then washed with water (2 × 100 mL) and brine (100 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to afford **2-16** as a colorless oil with a pungent odor (1.06 g, 96%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.67 (s, 3H), 2.53 (q, *J* = 7.2 Hz, 2H), 2.32 (t, *J* = 7.5 Hz, 2H), 1.68–1.59 (m, 4H), 1.45–1.38 (m, 2H), 1.33 (t, *J* = 7.8 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.2, 77.4, 77.2, 76.9, 51.7, 34.0, 33.7, 27.9, 24.5, 24.5; IR (thin film): 2931, 1734, 1435, 1197, 1168, 1004 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_7H_{18}NO_2S$ $[M + NH_4]^+$ 180.1053, found 180.1067.

Dimethyl 6,6'-thiodihexanoate (2-17)



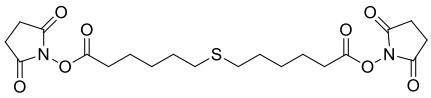
Bromide **2-15** (0.089 g, 0.43 mmol) and sulfide **2-16** (0.076 g, 0.47 mmol) were subjected to general procedure 2.1 to afford crude **2-17** as a colorless oil which was used without further purification: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.67 (s, 6H), 2.55–2.45 (d, *J* = 7.3 Hz, 4H), 2.32 (t, *J* = 7.5 Hz, 4H), 1.71–1.51 (m, 8H), 1.44–1.39 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.2, 51.6, 34.1, 32.1, 29.4, 28.5, 24.7; IR (thin film): 2930, 1736, 1435, 1195, 1168, 1124 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{14}H_{26}O_4SNa$ $[M + Na]^+$ 313.1444, found 313.1441.

6,6'-Thiodihexanoic acid (2-18)



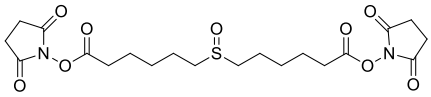
A stirred solution of **2-17** (0.085 g, 0.29 mmol) in sodium hydroxide (12 M, 5 mL) was heated at reflux for 16 h, after which it was let cool and acidified to a pH of 1 (monitored by pH paper) with sulfuric acid (18 M). The acidified aqueous layer was extracted with ethyl acetate (3 × 30 mL), and the combined organic layers were washed with water (2 × 50 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to afford **2-18** as a white solid (0.064 g, 84% over two steps): mp 102–106 ºC; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.99 (s, 2H), 2.46 (t, J = 7.3 Hz, 4H), 2.19 (t, J = 7.4 Hz, 4H), 1.55–1.43 (m, 8H), 1.35–1.31 (m, 4H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 174.5, 33.6, 31.0, 28.9, 27.8, 24.1; IR (thin film): 3080, 2931, 1687, 1433, 1408, 1278, 1190, 916 cm^{-1}; HRMS (ESI) m / z calcd for $C_{12}H_{21}O_4S$ $[M - H]^-$ 261.1166, found 261.1168.

Bis(2,5-dioxopyrrolidin-1-yl)-6,6'-thiodihexanoate (2-19)



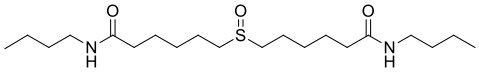
Diacid **2-18** (0.074 g, 0.28 mmol) was subjected to general procedure 2.4 to afford an orange oil. The crude product was purified by column chromatography (1:1:4 ethyl acetate:DCM:hexanes) to afford **2-19** as an orange solid (0.090 g, 70%): mp 90–91 ºC; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.82 (s, 8H), 2.60 (t, J = 7.3 Hz, 4H), 2.50 (t, J = 7.2 Hz, 4H), 1.84–1.66 (m, 4H), 1.66–1.55 (m, 4H), 1.54–1.46 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.3, 168.6, 31.9, 30.9, 29.2, 28.1, 25.7, 24.3; IR (thin film): 2926, 1716, 1640, 1549, 1208, 1062, 644 cm^{-1}; HRMS (ESI) m / z calcd for $C_{20}H_{28}N_2O_8SNa$ $[M + Na]^+$ 479.1464, found 479.1447.

Bis(2,5-dioxopyrrolidin-1-yl)-6,6'-sulfinyldihexanoate (2-20)



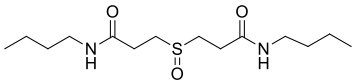
Sulfide **2-19** (0.027 g, 0.059 mmol) was subjected to general procedure 2.5 to afford **2-20** as a yellow solid (0.024 g, 86%): mp 111–112 ºC; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.82 (s, 8H), 2.74–2.59 (m, 8H), 1.85–1.72 (m, 8H), 1.62–1.52 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.2, 168.4, 52.1, 30.8, 28.0, 25.7, 24.3, 22.3; IR (thin film): 2926, 1813, 1782, 1728, 1365, 1207, 1045, 808, 646; HRMS (ESI) *m* / *z* calcd for $C_{20}H_{28}N_2O_9SNa$ $[M + Na]^+$ 495.1408, found 495.1411.

6,6'-Sulfinylbis(*N*-butylhexanamide) (2-21)



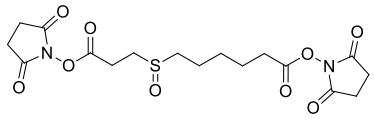
Sulfoxide **2-20** (0.003 g, 0.006 mmol) was subjected to general procedure 2.7 to afford **2-21** as a white solid (0.003 g, quant.): mp 159–161 ºC; ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.73 (s, 2H), 3.01 (q, *J* = 6.4 Hz, 4H), 2.74–2.67 (m, 2H), 2.65–2.55 (m, 2H), 2.05 (t, *J* = 7.3 Hz, 4H), 1.66–1.58 (m, 4H), 1.57–1.46 (m, 4H), 1.39–1.31 (m, 8H), 1.29–1.23 (m, 4H), 0.86 (t, *J* = 7.3 Hz, 6H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 171.7, 51.0, 38.0, 35.2, 31.3, 27.8, 25.0, 22.0, 19.6, 13.7; IR (thin film): 3317, 2918, 2868, 1635, 1542, 1464, 1101, 682 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{20}H_{40}N_2O_3SNa$ $[M + Na]^+$ 411.2657, found 411.2648.

3,3'-Sulfinylbis(*N*-butylpropanamide) (2-22)



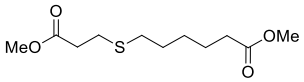
Sulfoxide **1-1** (0.010 g, 0.026 mmol) was subjected to general procedure 2.7 to afford **2-22** as a white solid (0.007 g, 90%): mp 110 ºC (dec); ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.98 (s, 2H), 3.04 (q, J = 6.6 Hz, 4H), 3.01–2.94 (m, 2H), 2.80–2.73 (m, 2H), 2.46 (t, J = 7.5 Hz, 4H), 1.41–1.32 (m, 4H), 1.29–1.22 (m, 4H), 0.86 (t, J = 7.1 Hz, 6H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.6, 46.8, 38.3, 31.2, 27.9, 19.6, 13.7; IR (thin film): 3268, 2958, 1635, 1540, 1432, 1029, 717 cm^{-1}; HRMS (ESI) m / z calcd for $C_{14}H_{28}N_2O_3SNa$ $[M + Na]^+$ 327.1718, found 327.1709.

2,5-Dioxopyrrolidin-1-yl 6-((3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropyl)sulfinyl)hexanoate (2-23)



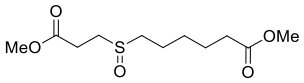
Sulfide **2-33** (0.050 g, 0.12 mmol) was subjected to general procedure 2.5 to afford **2-23** as a tan solid as a mix of diastereomers (0.030 g, 57%) (*indicates minor diastereomer): mp 117–120 ºC; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.25–2.92 (m, 4H), 2.84 (s, 8H), 2.81–2.67 (m, 2H), 2.64 (t, J = 7.3 Hz, 2H), 1.85–1.76 (m, 4H), 1.62–1.54 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.33*, 169.29, 168.9, 168.8*, 168.46, 168.40*, 167.3, 166.6*, 53.1*, 52.3, 47.4*, 45.8, 30.8, 30.6*, 27.9, 27.4*, 26.0*, 25.71, 25.69, 24.9*, 24.2, 24.0*, 22.3, 21.6*; IR (thin film): 2916, 1784, 1736, 1365, 1205, 1066, 644 cm^{-1}; HRMS (ESI) m / z calcd for $C_{17}H_{22}N_2O_9SNa$ $[M + Na]^+$ 453.0944, found 453.0928.

Methyl 6-((3-methoxy-3-oxopropyl)thio)hexanoate (2-24)



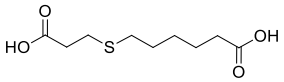
Bromide **2-15** (0.20 g, 0.96 mmol) and methyl 3-mercaptopropanoate (0.126 g, 1.05 mmol) were subjected to general procedure 2.1 to afford crude **2-24** as a colorless oil which was used without further purification: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.70 (s, 3H), 3.66 (s, 3H), 2.77 (t, *J* = 7.4 Hz, 2H), 2.60 (t, *J* = 7.4 Hz, 2H), 2.53 (t, *J* = 7.5 Hz, 2H), 2.31 (t, *J* = 7.5 Hz, 2H), 1.68–1.56 (m, 4H), 1.45–1.38 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.1, 172.5, 51.9, 51.6, 34.8, 34.0, 32.0, 29.2, 28.3, 27.0, 24.6; IR (thin film): 2949, 1734, 1435, 1356, 1195, 1016 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{11}H_{20}O_4SNa$ $[M + Na]^+$ 271.0980, found 271.0974.

Methyl 6-((3-methoxy-3-oxopropyl)sulfinyl)hexanoate (2-25)



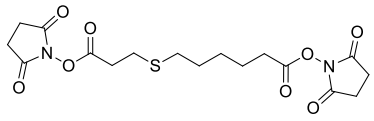
Sulfide **2-24** (0.0075 g, 0.030 mmol) was subjected to general procedure 2.5 to afford **2-25** as a white solid (0.0023 g, 34%): mp 45 ºC; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.73 (s, 3H), 3.67 (s, 3H), 3.06–2.98 (m, 1H), 2.90–2.81 (m, 3H), 2.78–2.63 (m, 2H), 2.34 (t, *J* = 7.4 Hz, 2H), 1.88–1.74 (m, 2H), 1.73–1.63 (m, 2H), 1.57–1.41 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.0, 172.0, 52.6, 52.4, 51.7, 47.0, 33.8, 28.4, 27.1, 24.6, 22.5; IR (thin film): 2950, 1732, 1437, 1361, 1211, 1172, 1028 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{11}H_{20}O_5SNa$ $[M + Na]^+$ 287.0929, found 287.0933.

6-((2-Carboxyethyl)thio)hexanoic acid (2-32)



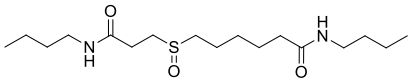
Crude diester **2-24** (2.1 g, 8.4 mmol) was subjected to general procedure 2.3 to afford **2-32** as a white solid (1.5 g, 95% over two steps): mp 78–86 ºC; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.13 (s, 2H), 2.65 (t, J = 6.9 Hz, 2H), 2.55–2.45 (m, 4H), 2.20 (t, J = 6.9 Hz, 2H), 1.58–1.47 (m, 4H), 1.38–1.30 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 174.5, 173.1, 34.6, 33.6, 30.9, 28.8, 27.8, 26.4, 24.1; IR (thin film): 3026, 2931, 1685, 1425, 1267, 1184, 920, 651 cm^{-1}; HRMS (ESI) m / z calcd for $C_9H_{15}O_4S$ $[M - H]^-$ 219.0691, found 219.0698.

2,5-Dioxopyrrolidin-1-yl 6-((3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropyl)thio)hexanoate (2-33)



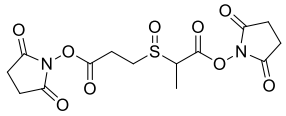
Diacid **2-32** (0.040 g, 0.18 mmol) was subjected to general procedure 2.4 to afford an orange oil. The crude product was purified by column chromatography (2:1 ethyl acetate:hexanes) to afford **2-33** as a white solid (0.056 g, 69%): mp 116–122 ºC; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.96–2.72 (m, 12H), 2.62 (t, J = 7.3 Hz, 2H), 2.58 (t, J = 7.3 Hz, 2H), 1.78 (quin, J = 7.9 Hz, 2H), 1.65 (quin, J = 7.9 Hz, 2H), 1.61–1.55 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.3, 169.1, 168.6, 167.3, 32.2, 32.0, 30.9, 29.0, 27.9, 26.4, 25.74, 25.73, 24.3; IR (thin film): 2955, 1811, 1781, 1729, 1362, 1200, 1063, 645 cm^{-1}; HRMS (ESI) m / z calcd for $C_{17}H_{22}N_2O_8SNa$ $[M + Na]^+$ 437.0995, found 437.0977.

***N*-butyl-6-((3-(butylamino)-3-oxopropyl)sulfinyl)hexanamide (2-34)**



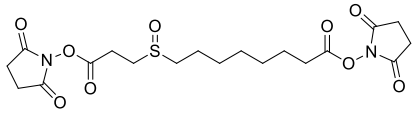
Sulfoxide **2-23** (0.013 g, 0.030 mmol) was subjected to general procedure 2.7 to afford **2-34** as a white solid as a mixture of rotamers: mp 105 ºC (dec); ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.98 (br s, 0.5H), 7.84–7.68 (m, 1.5H), 3.28–3.24 (m, 0.5H), 3.10–2.98 (m, 4.5H), 2.82–2.56 (m, 1.5H), 2.56–2.43 (m, 2.5H), 2.34–2.21 (m, 2H), 2.21–2.10 (m, 2H), 2.10–2.00 (m, 1H), 1.72–1.56 (m, 1H), 1.56–1.45 (m, 1H), 1.43–1.19 (m, 10H), 0.86 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (126 MHz, DMSO-d_6): δ 171.7, 170.8, 169.6, 168.5, 51.7, 50.9, 48.0, 46.8, 41.0, 38.3, 38.2, 38.1, 35.2, 35.0, 31.31, 31.27, 31.18, 30.6, 27.95, 27.86, 27.84, 25.0, 22.0, 19.58, 19.56, 19.52, 13.9, 13.71, 13.68; IR (thin film): 3317, 2918, 1635, 1542, 1011, 682 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{17}H_{34}N_2O_3SNa$ $[M + Na]^+$ 369.2188, found 369.2183.

2,5-Dioxopyrrolidin-1-yl 3-((1-((2,5-dioxopyrrolidin-1-yl)oxy)-1-oxopropan-2-yl)sulfinyl)propanoate (2-36)



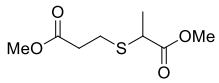
NHS-ester **2-40** (0.072 g, 0.190 mmol) was subjected to general procedure 2.5 to afford **2-36** as a sticky film as a mixture of inseparable diastereomers (0.048 g, 64%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.11 (q, *J* = 7.2 Hz, 0.4H), 3.93 (q, *J* = 7.2 Hz, 0.6H), 3.55–3.44 (m, 0.8H), 3.34–3.14 (m, 3.2H), 2.85 (s, 4H), 2.82 (s, 4H), 1.73 (d, *J* = 7.2 Hz, 2H), 1.69 (d, *J* = 7.2 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.90, 168.89, 168.72, 168.68, 166.91, 166.87, 164.3, 163.4, 58.4, 55.9, 45.1, 43.3, 25.7, 25.1, 24.1, 11.7, 8.6; IR (thin film): 3001, 1802, 1735, 1727, 1703, 1361, 1200, 1120 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{14}H_{16}N_2O_9SNa$ $[M + Na]^+$ 411.0474, found 411.0469.

2,5-Dioxopyrrolidin-1-yl 8-((3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropyl)sulfinyl)octanoate (2-37)



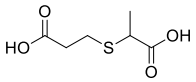
NHS-ester **2-48** (0.25 g, 0.57 mmol) was subjected to general procedure 2.5 to afford **2-37** as a white solid (0.168 g, 65%): mp 105–107 ºC; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.21–3.09 (m, 3H), 3.02–2.91 (m, 1H), 2.84 (br s, 8H), 2.80–2.64 (m, 2H), 2.61 (t, *J* = 7.3 Hz, 2H), 1.85–1.70 (m, 4H), 1.56–1.30 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.3, 168.9, 168.7, 167.3, 52.6, 45.8, 31.0, 28.6, 28.5, 28.4, 25.73, 25.70, 24.5, 24.3, 22.5; IR (thin film): 2941, 1805, 1730, 1358, 1196, 1057 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{19}H_{26}N_2O_9SNa$ $[M + Na]^+$ 481.1257, found 481.1247.

Methyl 3-((1-methoxy-1-oxopropan-2-yl)thio)propanoate (2-38)



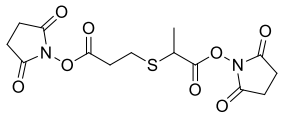
Methyl 2-bromopropanoate (0.20 g, 1.20 mmol) and methyl 3-mercaptopropanoate (0.158 g, 1.310 mmol) were subjected to general procedure 2.1 to afford crude **2-38** as a colorless oil which was used without further purification: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.74 (s, 3H), 3.70 (s, 3H), 3.44 (q, *J* = 7.1 Hz, 1H), 2.97–2.82 (m, 2H), 2.62 (td, *J* = 7.3, 3.3 Hz, 2H), 1.45 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.6, 172.2, 52.5, 52.0, 41.2, 34.5, 26.4, 17.2; IR (thin film): 2953, 1732, 1437, 1248, 1161, 1070, 605 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_8H_{14}O_4SNa$ $[M + Na]^+$ 226.0511, found 229.0520.

3-((1-Carboxyethyl)thio)propanoic acid (2-39)



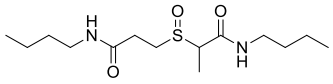
Diester **2-38** (1.9 g, 9.2 mmol) was subjected to general procedure 2.3 to afford **2-39** as a white solid (0.72 g, 48% over two steps): mp 87–89 ºC; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.43 (s, 2H), 3.42 (q, J = 7.0 Hz, 1H), 2.80–2.75 (m, 2H), 2.59–2.44 (m, 2H), 1.29 (d, J = 7.2 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 174.1, 172.9, 40.4, 34.2, 26.0, 17.3; IR (thin film): 3053, 2926, 1684, 1404, 1293, 1239, 920, 666 cm^{-1}; HRMS (ESI) m / z calcd for $C_6H_9O_4S$ $[M - H]^-$ 177.0222, found 177.0216.

2,5-Dioxopyrrolidin-1-yl 3-((1-((2,5-dioxopyrrolidin-1-yl)oxy)-1-oxopropan-2-yl)thio)propanoate (2-40)



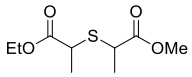
Diacid **2-39** (0.085 g, 0.480 mmol) was subjected to general procedure 2.4 to afford an orange oil. The crude product was purified by column chromatography (3:1 ethyl acetate:hexanes) to afford **2-40** as a yellow oil (0.048 g, 27%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.77 (q, J = 7.1 Hz, 1H), 3.22–2.98 (m, 2H), 2.98–2.94 (m, 2H), 2.84 (s, 4H), 2.82 (s, 4H), 1.57 (d, J = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.1, 168.6, 167.1, 38.4, 31.6, 25.8, 25.72, 25.68, 16.8; IR (thin film): 3161, 1784, 1709, 1201, 1066, 814, 646 cm^{-1}; HRMS (ESI) m / z calcd for $C_{14}H_{16}N_2O_8SNa$ $[M + Na]^+$ 395.0525, found 395.0522.

_N_-butyl-3-((1-(butylamino)-1-oxopropan-2-yl)sulfinyl)propanamide (2-41)



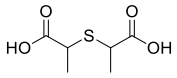
Sulfoxide **2-35** (0.013 g, 0.033 mmol) was subjected to general procedure 2.7 to afford a mixture of diamide **2.41** and *n*-butylamine in approximately a 1:1 ratio by proton NMR: ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.19 (s, 2H), 3.59 (q, *J* = 6.9 Hz, 1H), 3.32–3.15 (m, 4H), 2.84 (t, *J* = 7.3 Hz, 4H), 1.64–1.40 (m, 7H), 1.40–1.28 (m, 4H), 0.92–0.82 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.4, 170.1, 58.4, 42.5, 40.1, 39.7, 39.5, 31.8, 31.6, 31.4, 25.5, 20.2, 20.0, 13.9; IR (thin film): 3262, 2943, 1636, 1554, 1220, 1082 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{14}H_{28}N_2O_3SNa$ $[M + Na]^+$ 327.1718, found 327.1724.

Ethyl 2-((1-methoxy-1-oxopropan-2-yl)thio)propanoate (2-42)



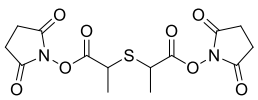
Methyl 2-bromopropanoate (0.50 g, 3.00 mmol) and ethyl 2-mercaptopropanoate (0.44 g, 3.30 mmol) were subjected to general procedure 2.1 to afford crude **2-42** as a colorless oil as a mixture of inseparable diastereomers which was used without further purification: ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.26–4.12 (m, 2H), 3.76 (s, 1.7H), 3.73 (s, 1.3H), 3.71–3.54 (m, 2H), 1.48 (dd, *J* = 7.2, 4.3 Hz, 3H), 1.42 (dd, *J* = 7.3, 4.3 Hz, 3H), 1.29 (td, *J* = 7.1, 3.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.5, 173.4, 173.0, 172.9, 61.5, 61.40, 52.54, 52.51, 41.7, 41.49, 41.46, 41.3, 18.00, 17.97, 17.2, 17.1, 14.3, 14.2; IR (thin film): 2984, 1729, 1455, 1256, 1160 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_9H_{16}O_4SNa$ $[M + Na]^+$ 243.0667, found 243.0662.

2,2'-Thiodipropanoic acid (2-43)



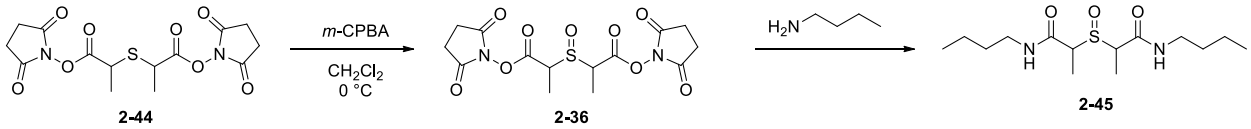
Crude diester **2-42** (0.90 g, 4.10 mmol) was subjected to general procedure 2.3 to afford **2-43** as a white solid as a mixture of inseparable diastereomers (0.50 g, 94% over two steps): mp 94–97 ºC; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.64 (s, 2H), 3.59–3.51 (m, 2H), 1.35 (d, J = 7.1 Hz, 2.5H), 1.29 (d, J = 7.2 Hz, 3.5H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.9, 173.8, 41.1, 40.6, 17.9, 17.4; IR (thin film): 2900, 1685, 1413, 1233, 1079 cm^{-1}; HRMS (ESI) m / z calcd for $C_6H_9O_4S$ $[M - H]^-$ 177.0222, found 177.0216.

Bis(2,5-dioxopyrrolidin-1-yl) 2,2'-thiodipropanoate (2-44)



Diacid **2-43** (0.33 g, 1.90 mmol) was subjected to general procedure 2.4 to afford an orange oil. The crude product was purified by column chromatography (3:1 ethyl acetate:hexanes) to afford **2-44** as a yellow oil as a mixture of inseparable diastereomers (0.49 g, 70%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.32 (q, J = 7.1 Hz, 1.25H), 3.94 (q, J = 7.3 Hz, 0.25H), 2.85 (s, 8H), 1.69 (d, J = 7.3 Hz, 2H), 1.57 (d, J = 7.1 Hz, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.1, 168.9, 168.5, 168.4, 38.7, 38.5, 25.7, 18.0, 15.9; IR (thin film): 2943, 1802, 1727, 1362, 1209, 1064 cm^{-1}; HRMS (ESI) m / z calcd for $C_{14}H_{16}N_2O_8SNa$ $[M + Na]^+$ 395.0525, found 395.0527.

2,2'-Sulfinylbis(*N*-butylpropanamide) (2-45)

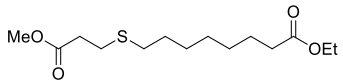


2-44 2-36 2-45

NHS-ester **2-44** (0.021g, 0.070 mmol) was subjected to general procedure 2.5 to afford **2-36** and 3-chlorobenzoic acid contaminant in a 1:1 ratio as a yellow solid which was used immediately in the next reaction without any further purification: ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.94 (q, *J* = 7.2 Hz, 0.7H), 4.37 (d, *J* = 7.2 Hz, 0.7H), 4.25 (d, *J* = 7.2 Hz, 0.5H), 2.87 (s, 8H), 1.88 (d, *J* = 7.2 Hz, 2.1H), 1.85 (d, *J* = 7.2 Hz, 2.1H), 1.76 (d, *J* = 7.2 Hz, 1.8H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.6, 165.1, 164.7, 162.1, 57.2, 56.7, 54.8, 25.7, 13.0, 12.3, 9.8.

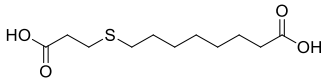
To the crude reaction mixture of **2-36** and 3-chlorobenzoic acid in deuterated chloroform (0.7 mL) inside an NMR tube was added *n*-butylamine (1 drop, ca. 50 μL). The resulting solution was gently mixed by hand, after which proton and carbon NMR data were immediately acquired. Accurate mass measurements confirmed the formation of diamide **2-45:** ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.32 (q, *J* = 6.9 Hz, 2H), 3.10–2.98 (m, 4H), 1.75–1.58 (m, 5H), 1.58–1.44 (m, 5H), 1.42–1.31 (m, 4H), 0.98–0.88 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.5, 168.3, 167.4, 166.9, 60.9, 57.3, 56.9, 40.0, 39.9, 39.8, 31.6, 31.44, 31.39, 29.8, 25.5, 20.24, 20.18, 20.11, 19.8, 14.1, 13.84, 13.79, 13.65, 12.8, 12.2; IR (thin film): 3226, 2957, 1717, 1653, 1483, 1379, 1217 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{14}H_{28}N_2O_3SNa$ $[M + Na]^+$ 327.1718, found 327.1717.

Ethyl 8-((3-methoxy-3-oxopropyl)thio)octanoate (2-46)



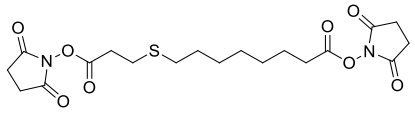
Ethyl 8-bromooctanoate (1.0 g, 4.0 mmol) and methyl 3-mercaptopropanoate (0.53 g, 4.4 mmol) were subjected to general procedure 2.1 to afford crude **2-46** as a colorless oil which was used without further purification: ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.12 (q, *J* = 7.1 Hz, 2H), 3.70 (s, 3H), 2.78 (t, *J* = 7.4 Hz, 2H), 2.61 (t, *J* = 7.3 Hz, 2H), 2.57–2.48 (m, 2H), 2.28 (t, *J* = 7.5 Hz, 2H), 1.64–1.54 (m, 4H), 1.42–1.29 (m, 6H), 1.25 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.9, 172.6, 60.3, 51.9, 34.8, 34.5, 32.3, 29.6, 29.1, 29.0, 28.8, 27.1, 25.0, 14.4; IR (thin film): 2927, 1733, 1435, 1224, 1165 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{14}H_{26}O_4SNa$ $[M + Na]^+$ 313.1450, found 313.1443.

8-((2-Carboxyethyl)thio)octanoic acid (2-47)



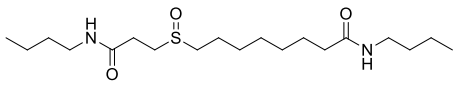
Crude diester **2-46** (1.36 g, 4.68 mmol) was subjected to general procedure 2.3 to afford **2-47** as a white solid which was used without further purification: ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.12 (br s, 2H), 2.68 (t, *J* = 14.7 Hz, 2H), 2.52–2.48 (m, 4H), 2.20 (t, *J* = 7.3 Hz, 2H), 1.53–1.47 (m, 4H), 1.42–1.22 (m, 6H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 174.5, 173.1, 34.58, 33.7, 31.0, 29.0, 28.5, 28.3, 28.1, 26.4, 24.5; IR (thin film): 2931, 1686, 1426, 1287, 1182 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{11}H_{19}O_4S$ $[M - H]^-$ 247.1004, found 247.1010.

2,5-Dioxopyrrolidin-1-yl 8-((3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropyl)thio)octanoate (2-48)



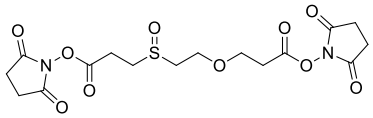
Diacid **2-47** (0.72 g, 2.90 mmol) was subjected to general procedure 2.4 to afford an orange oil. The crude product was purified by column chromatography (3:1 ethyl acetate:hexanes) to afford **2-48** as a tan solid (1.19 g, 93% over three steps): mp 110 ºC (dec); ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.96–2.73 (m, 12H), 2.59 (t, *J* = 7.5 Hz, 2H), 2.54 (t, *J* = 7.3 Hz, 2H), 1.73 (quin, *J* = 7.4 Hz, 2H), 1.59 (quin, *J* = 7.4 Hz, 2H), 1.43–1.32 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.4, 169.1, 168.8, 167.3, 32.20, 32.18, 31.0, 29.4, 28.65, 28.64, 28.5, 26.4, 25.70, 25.69, 24.6; IR (thin film): 2931, 1816, 1715, 1360, 1168, 1054 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{19}H_{26}N_2O_8SNa$ $[M + Na]^+$ 465.1308, found 465.1297.

***N*-butyl-8-((3-(butylamino)-3-oxopropyl)sulfinyl)octanamide (2-49)**



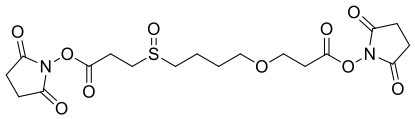
Sulfoxide **2-37** (0.015 g, 0.033 mmol) was subjected to general procedure 2.7 to afford a mixture of diamide **2-49** and *n*-butylamine in approximately a 1:1 ratio by proton NMR: ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.10–6.85 (br s, 2H), 3.24–3.20 (m, 4H), 2.89–2.76 (m, 4H), 2.70–2.67 (m, 2H), 2.13 (t, *J* = 7.4 Hz, 2H), 1.76–1.70 (m, 2H), 1.62–1.54 (m, 2H), 1.57–1.39 (m, 6H), 1.37–1.26 (m, 8H), 0.98–0.79 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.0, 170.1, 52.7, 47.5, 39.8, 39.6, 39.3, 36.8, 31.9, 31.7, 31.1, 29.2, 29.0, 28.9, 28.6, 25.7, 25.4, 22.7, 20.2, 20.0, 13.89, 13.85; IR (thin film): 3306, 2932, 1727, 1668, 1547, 1238, 1076 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{19}H_{38}N_2O_3SNa$ $[M + Na]^+$ 397.2501, found 397.2504.

2,5-Dioxopyrrolidin-1-yl 3-((2-(3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropoxy)ethyl)sulfinyl)propanoate (2-50)



Sulfide **2-56** (0.075 g, 0.18 mmol) was subjected to general procedure 2.6 to afford a white powder. The crude product was purified by column chromatography. For sulfoxides that contained an oxygen gamma to the NHS-ester, a column no taller than 9 cm was required for purification, or else the percent yield decreased. All impurities were flushed off with 1:3 acetonitrile:ethyl acetate, followed by elution of the desired product using a solvent system of 1:1 acetonitrile:ethyl acetate affording **2-50** as a sticky white film (0.040 g, 51%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.96 – 3.93 (m, 1H), 3.91–3.87 (m, 1H), 3.84 (t, *J* = 5.8 Hz, 2H), 3.31–3.23 (m, 1H), 3.18–3.10 (m, 4H), 2.97–2.91 (m, 1H), 2.87–2.79 (m, 10H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.3, 168.9, 167.3, 166.9, 66.1, 63.5, 52.3, 46.6, 32.4, 25.8, 25.7, 24.5; IR (thin film): 2947, 1809, 1777, 1730, 1198, 1065 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{16}H_{20}N_2O_{10}SNa$ $[M + Na]^+$ 455.0736, found 455.0739.

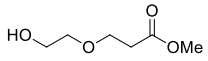
2,5-Dioxopyrrolidin-1-yl 3-((4-(3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropoxy)butyl)sulfinyl)propanoate (2-51)



Sulfide **2-61** (0.0750 g, 0.169 mmol) was subjected to general procedure 2.6 to afford an orange oil. The crude product was purified by column chromatography. For sulfoxides that contained an oxygen gamma to the NHS-ester, a column no taller than 9 cm was required for purification, or

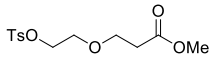
else the percent yield decreased. All impurities were flushed off with 1:3 acetonitrile:ethyl acetate, followed by elution of the desired product using a solvent system of 1:1 acetonitrile:ethyl acetate affording **2-51** as a sticky white film (0.0368 g, 47%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.76 (t, *J* = 5.9 Hz, 2H), 3.51 (t, *J* = 5.7 Hz, 2H), 3.18–3.09 (m, 3H), 3.03–2.94 (m, 1H), 2.90–2.74 (m, 12H), 1.91–1.82 (m, 2H), 1.76–1.72 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.3, 168.9, 167.4, 167.1, 70.7, 65.5, 52.1, 45.3, 32.5, 28.5, 25.74, 25.70, 24.3, 19.9; IR (thin film): 2914, 1812, 1780, 1730, 1365, 1200 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{18}H_{24}N_2O_{10}SNa$ $[M + Na]^+$ 483.1049, found 483.1040.

Methyl 3-(2-hydroxyethoxy)propanoate (2-52)



Ethylene glycol (17.5 mL, 0.253 mol) and methyl acrylate (10.0 mL, 0.122 mol) were subjected to general procedure 2.8 to afford a colorless oil. The crude product was purified by column chromatography (3:1 ethyl acetate:hexanes) to afford **2-52** as a colorless oil (3.75 g, 23%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.74 (t, *J* = 6.1 Hz, 2H), 3.71–3.65 (m, 5H), 3.57–3.53 (m, 2H), 2.65 (s, 1H), 2.58 (t, *J* = 6.1 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.5, 72.1, 66.2, 61.6, 51.9, 34.8; IR (thin film): 3443, 2951, 2878, 1731, 1177, 1117 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_6H_{12}O_4Na$ $[M + Na]^+$ 171.0633, found 171.0637.

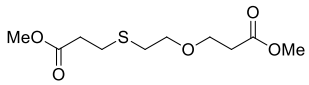
Methyl 3-(2-(tosyloxy)ethoxy)propanoate (2-53)



Alcohol **2-52** (1.00 g, 6.75 mmol) was subjected to general procedure 2.9 to afford an orange oil. The crude product was purified by column chromatography (1:1 ethyl acetate:hexanes) to afford

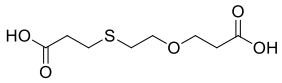
2-53 as a colorless oil (1.39 g, 69%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.78 (d, *J* = 8.3 Hz, 2H), 7.34 (d, *J* = 8.0 Hz, 2H), 4.18–4.07 (m, 2H), 3.70–3.65 (m, 5H), 3.65–3.62 (m, 2H), 2.51 (t, *J* = 6.4 Hz, 2H), 2.44 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.9, 145.0, 133.1, 129.9, 128.1, 69.2, 68.5, 66.8, 51.9, 34.8, 21.8; IR (thin film): 2881, 1735, 1354, 1172, 918 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{13}H_{18}O_6SNa$ $[M + Na]^+$ 325.0722, found 325.0714.

Methyl 3-((2-(3-methoxy-3-oxopropoxy)ethyl)thio)propanoate (2-54)



Tosylated alcohol **2-53** (0.100 g, 0.331 mmol) and methyl 3-mercaptopropanoate (0.0314 g, 0.262 mmol) were subjected to general procedure 2.2 to afford an orange oil. The crude product was purified by column chromatography (step gradient from 1:2 ethyl acetate:hexanes to 1:1 ethyl acetate:hexanes) to afford **2-54** as a colorless oil (0.056 g, 71%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.73 (t, *J* = 6.4 Hz, 2H), 3.70–3.68 (m, 6H), 3.63 (t, *J* = 6.7 Hz, 2H), 2.82 (t, *J* = 7.4 Hz, 2H), 2.70 (t, *J* = 6.6 Hz, 2H), 2.65–2.56 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.4, 172.0, 70.9, 66.4, 51.9, 51.8, 34.9, 34.8, 31.5, 27.5; IR (thin film): 2952, 1729, 1365, 1246, 1156 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{10}H_{18}O_5SNa$ $[M + Na]^+$ 273.0773, found 273.0779.

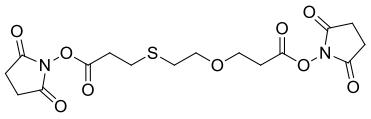
3-((2-(2-Carboxyethoxy)ethyl)thio)propanoic acid (2-55)



Diester **2-54** (0.050 g, 0.20 mmol) was subjected to general procedure 2.3 to afford **2-55** as a white solid (0.044 g, quant.): mp 70–72 ºC (dec); ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.22 (s, 2H), 3.73 (t, *J* = 6.2 Hz, 2H), 3.65 (t, *J* = 6.4 Hz, 2H), 2.86–2.77 (m, 2H), 2.71 (t, *J* = 6.4 Hz, 2H),

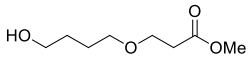
2.69–2.57 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.1, 177.7, 71.3, 66.1, 35.0, 34.9, 31.6, 27.2; IR (thin film): 3015, 2905, 2669, 1687, 1106 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_8H_{13}O_5S$ $[M - H]^-$ 221.0484, found 221.0484.

2,5-Dioxopyrrolidin-1-yl 3-((2-(3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropoxy)ethyl)thio)propanoate (2-56)



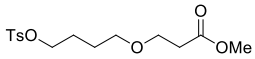
Diacid **2-55** (0.20 g, 0.90 mmol) was subjected to general procedure 2.4 to afford an orange oil. The crude product was dissolved in ethyl acetate (2 mL), and then hexanes (18 mL) was carefully layered on top of the ethyl acetate layer. The biphasic solution was carefully placed in the freezer for 72 h, after which a white solid had precipitated. The precipitate was collected by filtration, after which it was washed with ice cold hexanes (2 × 10 mL) and dried in vacuo to afford **2-56** as white solid (0.24 g, 72%): mp 74–76 ºC (dec); ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.83 (t, *J* = 6.2 Hz, 2H), 3.69 (t, *J* = 6.2 Hz, 2H), 2.98–2.92 (m, 4H), 2.88 (t, *J* = 6.2 Hz, 2H), 2.83 (s, 8H), 2.76 (t, *J* = 6.2 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.21, 169.15, 167.4, 167.0, 71.8, 65.8, 32.4, 32.3, 31.6, 27.1, 25.74, 25.72; IR (thin film): 2983, 1809, 1781, 1735, 1362, 1197, 1046 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{16}H_{20}N_2O_9SNa$ $[M + Na]^+$ 439.0787, found 439.0774.

Methyl 3-(4-hydroxybutoxy)propanoate (2-57)



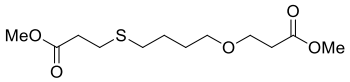
1,4-butanediol (13.9 mL, 0.157 mol) and methyl acrylate (5.00 mL, 0.0552 mol) were subjected to general procedure 2.8 to afford a colorless oil. The crude product was purified by column chromatography (3:1 ethyl acetate:hexanes) to afford **2-57** and an unidentified side product (which co-eluted with **2-57**) as a colorless oil. The semi-purified mixture was carried forward without any further purification: IR (thin film): 3442, 2944, 2870, 1732, 1176, 1064 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_8H_{16}O_4Na$ $[M + Na]^+$ 199.0946, found 199.0953.

Methyl 3-(4-(tosyloxy)butoxy)propanoate (2-58)



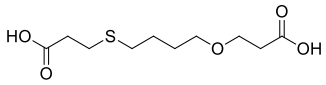
The semi-purified mixture of alcohol **2-57** and an unknown byproduct (0.986 g) was subjected to general procedure 9 to afford an orange oil. The crude product was purified by column chromatography (1:1 ethyl acetate:hexanes) to afford **2-58** and an unidentified side product (which co-eluted with **2-58**) as a colorless oil. A small amount (90 mg) of the mixture was subjected to general procedure 2.10 in a further attempt to separate the products. A 4 g RediSep silica column was used. The flow rate was 18 mL/min. The equilibration volume was 5.0 CV. The solvent system was a linear gradient of 0 – 20% methanol in DCM over 30 CV. Column fractions from 22 CV to 23 CV were concentrated in vacuo. Unfortunately, **2-58** and the unknown side product had co-eluted. The semi-purified mixture was carried forward without any further purification: IR (thin film): 2877, 1734, 1355, 1172, 1072 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{15}H_{22}O_6SNa$ $[M + Na]^+$ 353.1035, found 353.1038.

Methyl 3-((4-(3-methoxy-3-oxopropoxy)butyl)thio)propanoate (2-59)



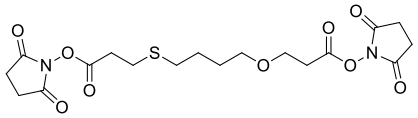
The semi-purified mixture of tosylated alcohol **2-58** and an unknown byproduct (0.210 g) and methyl 3-mercaptopropanoate (0.0702 g, 0.584 mmol) were subjected to general procedure 2.2 to afford an orange oil. The crude product was purified by column chromatography (1:2 ethyl acetate:hexanes) to afford **2-59** as a colorless oil (0.0900 g, 5.4% over three steps): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.70–3.65 (m, 8H), 3.43 (t, *J* = 6.0 Hz, 2H), 2.76 (t, *J* = 7.5 Hz, 2H), 2.60–2.51 (m, 6H), 1.68–1.57 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.5, 172.2, 70.6, 66.2, 51.9, 51.8, 35.0, 34.8, 32.0, 28.8, 27.0, 26.3; IR (thin film): 2868, 1732, 1435, 1244, 1070 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{12}H_{22}O_5SNa$ $[M + Na]^+$ 301.1086, found 301.1094.

3-((4-(2-Carboxyethoxy)butyl)thio)propanoic acid (2-60)



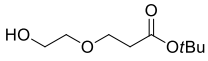
Diester **2-59** (0.090 g, 0.34 mmol) was subjected to general procedure 2.3 to afford **2-60** as a white solid (0.055 g, 68%): mp 61–62 ºC (dec); ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.32 (s, 2H), 3.72–3.64 (m, 2H), 3.47 (t, *J* = 5.9 Hz, 2H), 2.77 (t, *J* = 5.3 Hz, 2H), 2.67–2.58 (m, 4H), 2.54 (t, *J* = 7.0 Hz, 2H), 1.67–1.63 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.1, 177.6, 70.7, 65.9, 35.1, 34.9, 32.0, 28.6, 26.7, 26.3; IR (thin film): 2940, 2866, 1688, 1406, 1256, 1111 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{10}H_{17}O_5SNa$ $[M - H]^-$ 249.0797, found 249.0807.

2,5-Dioxopyrrolidin-1-yl 3-((4-(3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropoxy)butyl)thio)propanoate (2-61)



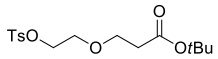
Diacid **2-60** (0.050 g, 0.20 mmol) was subjected to general procedure 2.4 to afford an orange oil. The crude product was purified by column chromatography (step gradient from 1:1 ethyl acetate:hexanes to 2:1 ethyl acetate:hexanes) to afford **2-61** as white solid (0.16 g, 43%): mp 55 ºC (dec); ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.76 (t, *J* = 6.3 Hz, 2H), 3.50–3.45 (m, 2H), 2.96–2.75 (m, 14H), 2.60–2.54 (m, 2H), 1.69–1.63 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.12, 169.08, 167.3, 166.9, 70.7, 65.2, 32.3, 32.1, 31.9, 28.5, 26.11, 26.08, 25.63, 25.62; IR (thin film): 2945, 1813, 1782, 1731, 1205, 1067 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{18}H_{24}N_2O_9SNa$ $[M + Na]^+$ 467.1100, found 467.1097.

***tert*-Butyl 3-(2-hydroxyethoxy)propanoate (2-62)**



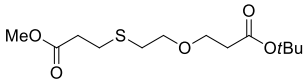
Ethylene glycol (11.0 mL, 0.197 mol) and *tert*-butyl acrylate (10.0 mL, 0.0683 mol) were subjected to general procedure 2.8 to afford a colorless oil. The crude product was purified by column chromatography (3:2 ethyl acetate:hexanes) to afford **2-62** as a colorless oil (4.38 g, 34%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.73–3.67 (m, 4H), 3.58–3.54 (m, 2H), 2.62 (s, 1H), 2.49 (t, *J* = 6.1 Hz, 2H), 1.44 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.4, 81.0, 72.0, 66.5, 61.7, 36.2, 28.2; IR (thin film): 3429, 2930, 1725, 1366, 1156, 1118 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_9H_{18}O_4Na$ $[M + Na]^+$ 213.1103, found 213.1103.

***tert*-Butyl 3-(2-(tosyloxy)ethoxy)propanoate (2-63)**



Alcohol **2-62** (0.770 g, 4.05 mmol) was subjected to general procedure 2.9 to afford an orange oil. The crude product was purified by general procedure 2.10. A 24 g RediSep silica column was used. The flow rate was 35 mL/min. The equilibration volume was 2.5 CV. The solvent system was a linear gradient of 0 – 10% methanol in DCM over 13 CV. Column fractions from 10 CV to 12 CV were concentrated in vacuo to afford **2-63** as a colorless oil (0.915 g, 66%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.79 (d, *J* = 8.3 Hz, 2H), 7.34 (d, *J* = 8.0 Hz, 2H), 4.15–4.12 (m, 2H), 3.65–3.61 (m, 4H), 2.44 (s, 3H), 2.41 (t, *J* = 6.4 Hz, 2H), 1.43 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.8, 144.9, 133.1, 129.9, 128.1, 80.8, 69.3, 68.5, 67.1, 36.3, 28.2, 21.8; IR (thin film): 2974, 1735, 1348, 1165, 918 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{16}H_{24}O_6SNa$ $[M + Na]^+$ 367.1191, found 367.1185.

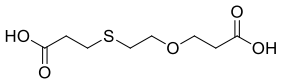
***tert*-Butyl 3-(2-((3-methoxy-3-oxopropyl)thio)ethoxy)propanoate (2-64)**



Tosylated alcohol **2-63** (5.24 g, 15.2 mmol), methyl 3-mercaptopropanoate (3.78 g, 31.5 mmol), 1,1,3,3-tetramethylguanidine (2.70 g, 23.5 mmol), and acetonitrile (95 mL) were combined in a scintillation vial with stir bar, under argon. The pale yellow reaction was let stir for 12 h, after which it had turned dark yellow. The crude reaction mixture was diluted in DCM (300 mL), then washed with water (3 × 100 mL) and brine (100 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to afford an orange oil. The crude product was purified by column chromatography (step gradient from 1:7 ethyl acetate:hexanes to 1:5 ethyl acetate:hexanes) to afford **2-64** as a colorless oil (3.52 g, 79%): ^{1}H NMR (500 MHz,

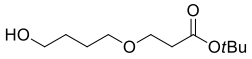
$CDCl_3$): δ 3.71–3.65 (m, 5H), 3.60 (t, *J* = 6.6, 2H), 2.81 (t, *J* = 7.2 Hz, 2H), 2.69 (t, *J* = 6.7 Hz, 2H), 2.60 (t, *J* = 7.4 Hz, 2H), 2.47 (d, *J* = 6.5, 2H), 1.43 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.5, 170.9, 80.7, 70.9, 66.7, 51.9, 36.4, 34.9, 31.6, 28.2, 27.6; IR (thin film): 2976, 1730, 1365, 1246, 1156 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{13}H_{24}O_5SNa$ $[M + Na]^+$ 315.1242, found 315.1239.

3-((2-(2-Carboxyethoxy)ethyl)thio)propanoic acid (2-55)



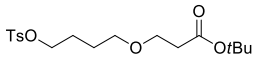
Diester **2-64** (3.18 g, 10.8 mmol) was subjected to general procedure 2.3 to afford a yellow solid. The crude product was purified by column chromatography (1% acetic acid in ethyl acetate) affording a colorless oil. Upon standing overnight, **2-55** crystallized as a white solid (2.32 g, 96%): The characterization data was identical to compound **2-55** above.

***tert*-Butyl 3-(4-hydroxybutoxy)propanoate (2-65)**



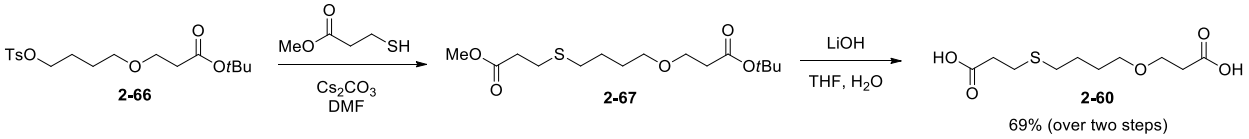
1,4-butanediol (2.60 mL, 29.3 mmol) and *tert*-butyl acrylate (1.50 mL, 10.2 mmol) were subjected to general procedure 2.8 to afford a colorless oil. The crude product was purified by column chromatography (step gradient from 1:4 ethyl acetate:hexanes to 1:1 ethyl acetate:hexanes to 100% ethyl acetate) to afford **2-65** as a colorless oil (1.9 g, 85%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.66 (t, *J* = 6.4 Hz, 2H), 3.64–3.59 (m, 2H), 3.47 (t, *J* = 5.7 Hz, 2H), 2.47 (t, *J* = 6.4 Hz, 2H), 2.26 (s, 1H), 1.71–1.57 (m, 4H), 1.43 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.1, 80.8, 71.1, 66.5, 62.7, 36.3, 30.1, 28.2, 26.6; IR (thin film): 3442, 2919, 1732, 1177, 1056 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{11}H_{22}O_4Na$ $[M + Na]^+$ 241.1416, found 241.1415.

***tert*-Butyl 3-(4-(tosyloxy)butoxy)propanoate (2-66)**



Alcohol **2-65** (1.83 g, 8.38 mmol) was subjected to general procedure 2.9 to afford an orange oil. The crude product was purified by column chromatography (1:3 ethyl acetate:hexanes) to afford **2-66** as a colorless oil (2.30 g, 74%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.77 (d, *J* = 8.2 Hz, 2H), 7.33 (d, *J* = 8.1 Hz, 2H), 4.02 (t, *J* = 6.4 Hz, 2H), 3.58 (t, *J* = 6.4 Hz, 2H), 3.36 (t, *J* = 6.2 Hz, 2H), 2.49–2.35 (m, 5H), 1.76–1.64 (m, 2H), 1.59–1.51 (m, 2H), 1.42 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.0, 144.8, 133.2, 129.9, 128.0, 80.6, 70.5, 70.1, 66.5, 36.4, 28.2, 25.9, 25.7, 21.7; IR (thin film): 2976, 1726, 1357, 1174, 1097 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{18}H_{28}O_6SNa$ $[M + Na]^+$ 395.1504, found 395.1495.

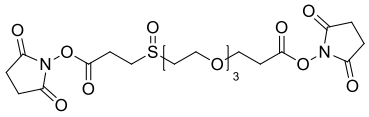
3-((4-(2-Carboxyethoxy)butyl)thio)propanoic acid (2-60)



Tosylated alcohol **2-66** (2.0 g, 5.4 mmol) and methyl 3-mercaptopropanoate (0.55 mL, 4.9 mmol) were subjected to general procedure 2.2 to afford crude diester **2-67** as an colorless oil which was used without further purification: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.68 (s, 3H), 3.62 (t, *J* = 6.5 Hz, 2H), 3.42 (t, *J* = 5.7 Hz, 2H), 2.75 (t, *J* = 7.4 Hz, 2H), 2.58 (t, *J* = 7.5 Hz, 2H), 2.52 (t, *J* = 6.9 Hz, 2H), 2.45 (t, *J* = 6.5 Hz, 2H), 1.67–1.58 (m, 4H), 1.43 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.5, 171.1, 80.6, 70.5, 66.5, 51.9, 36.4, 34.8, 32.0, 28.8, 28.2, 27.0, 26.3; IR (thin film): 2976, 1729, 1365, 1245, 1154 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{15}H_{28}O_5SNa$ $[M + Na]^+$ 343.1555, found 343.1550.

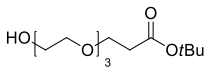
Crude diester **2-67** (0.293 g) was subjected to general procedure 2.3 to afford a light brown solid. Trituration with hexanes (4 × 20 mL) afforded afford **2-60** as a white solid (0.136 g, 69% over two steps). The characterization data was identical to compound **2-60** above.

Bis(2,5-dioxopyrrolidin-1-yl) 4,7,10-trioxa-13-sulfinylhexadecane-1,16-dioate (2-68)



NHS ester **2-73** (0.140 g, 0.277 mmol) was subjected to general procedure 2.6 to afford an orange oil. The crude oil was dissolved in ethyl acetate (1 mL), and then hexanes (19 mL) were carefully layered on top. The mixture was let sit in the freezer for 24 h, after which a sticky orange residue had precipitated. The solvent was decanted and then the residue was dried in vacuo. This process was repeated a second time affording **2-68** as a sticky orange semi-solid (0.126 g, 87%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.99–3.87 (m, 1H), 3.87–3.81 (m, 2H), 3.78–3.68 (m, 1H), 3.66–3.61 (m, 8H), 3.24–3.05 (m, 4H), 2.99–2.88 (m, 4H), 2.87–2.77 (d, 8H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.3, 169.0, 167.3, 167.0, 70.84, 70.80, 70.62, 70.56, 65.8, 63.4, 52.5, 46.1, 32.3, 25.7 (unresolved methylene carbons), 24.3; IR (thin film): 2943, 1811, 1778, 1724, 1367, 1196 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{20}H_{28}N_2O_{12}SNa$ $[M + Na]^+$ 543.1260, found 543.1248.

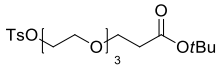
***tert*-Butyl 3-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)propanoate (2-69)**



Triethylene glycol (11.8 mL, 88.5 mmol) and *tert*-butyl acrylate (4.50 mL, 30.7 mmol) were subjected to general procedure 2.8 to afford a colorless oil. The crude product was purified by

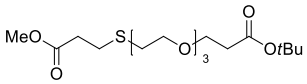
general procedure 2.10. A 330 g RediSep silica column was used. The flow rate was 200 mL/min. The equilibration volume was 1.0 CV. The solvent system was a linear gradient of 0 – 30% methanol in DCM over 5 CV. Column fractions from 2.5 CV to 3.5 CV were concentrated in vacuo to afford **2-69** as a colorless oil (6.21 g, 85%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.77–3.54 (m, 15H), 2.50 (t, *J* = 6.6 Hz, 2H), 1.43 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.1, 80.7, 72.6, 70.8, 70.6, 70.50, 70.47, 67.0, 61.9, 36.3, 28.2; IR (thin film): 3451, 2870, 1726, 1366, 1156 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{13}H_{26}O_6Na$ [M + Na]$^+$ 301.1627, found 301.1631.

***tert*-Butyl 3-(2-(2-(2-(tosyloxy)ethoxy)ethoxy)ethoxy)propanoate (2-70)**



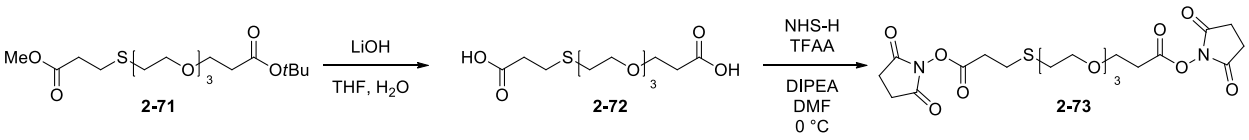
Alcohol **2-69** (1.00 g, 3.59 mmol) was subjected to general procedure 2.9 to afford an orange oil. The crude product was purified by general procedure 2.10. A 40 g RediSep silica column was used. The flow rate was 40 mL/min. The equilibration volume was 2.0 CV. The solvent system was a linear gradient of 0 – 20% methanol in DCM over 11 CV. Column fractions from 7 CV to 8 CV were concentrated in vacuo to afford **2-70** as a colorless oil (1.12 g, 73%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.79 (d, *J* = 8.2 Hz, 2H), 7.34 (d, *J* = 8.0 Hz, 2H), 4.18–4.13 (m, 2H), 3.73–3.54 (m, 12H), 2.49 (t, *J* = 6.5 Hz, 2H), 2.44 (s, 3H), 1.44 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.0, 144.9, 133.1, 129.9, 128.1, 80.6, 70.9, 70.66, 70.65, 70.5, 69.4, 68.8, 67.0, 36.4, 28.2, 21.8; IR (thin film): 2871, 1726, 1356, 1175, 1096 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{20}H_{32}O_8SNa$ [M + Na]$^+$ 455.1716, found 455.1700.

1-*tert*-Butyl 16-methyl 4,7,10-trioxa-13-thiahexadecane-1,16-dioate (2-71)



Tosylated alcohol **2-70** (5.03 g, 11.6 mmol) and methyl 3-mercaptopropanoate (1.30 g, 10.8 mmol) were subjected to general procedure 2.2 to afford an orange oil. The crude product was purified by column chromatography (1:2 ethyl acetate:hexanes) to afford **2-71** as a colorless oil (3.08 g, 75%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.72–3.66 (m, 5H), 3.65–3.56 (m, 10H), 2.81 (t, *J* = 7.4 Hz, 2H), 2.70 (t, *J* = 6.8 Hz, 2H), 2.60 (t, *J* = 7.3 Hz, 2H), 2.48 (t, *J* = 6.5 Hz, 2H), 1.42 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.4, 171.0, 80.6, 71.1, 70.67, 70.65 – 70.63 (unresolved methylene carbons), 70.5, 67.0, 51.9, 36.4, 34.9, 31.6, 28.2, 27.5; IR (thin film): 2868, 1729, 1365, 1247, 1155 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{17}H_{32}O_7SNa$ [M + Na]$^+$ 403.1766, found 403.1751.

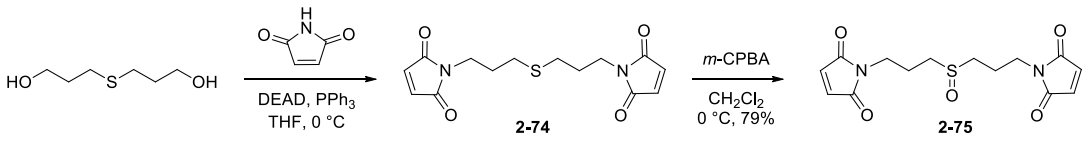
Bis(2,5-dioxopyrrolidin-1-yl) 4,7,10-trioxa-13-sulfinylhexadecane-1,16-dioate (2-73)



Diester **2-71** (1.71 g, 4.50 mmol) was subjected to general procedure 2.3 to afford crude **2-72** as a dark orange oil that was used in the next reaction without further purification: ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.63 (s, 2H), 3.76 (t, *J* = 6.2 Hz, 2H), 3.70–3.56 (m, 10H), 2.83 (t, *J* = 7.1 Hz, 2H), 2.73 (t, *J* = 6.6 Hz, 2H), 2.67 (t, *J* = 7.1 Hz, 2H), 2.63 (t, *J* = 6.2 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.4, 176.9, 71.1, 70.64, 70.56, 70.46, 70.41, 66.5, 35.1, 35.0, 31.6, 27.3; IR (thin film): 2910, 1687, 1423, 1218, 1108 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{12}H_{22}O_7SNa$ [M + Na]$^+$ 333.0984, found 333.0987.

Crude diacid **2-72** (1.50 g, 4.83 mmol) was subjected to general procedure 2.4 to afford a black oil. The crude product was purified by column chromatography (5:1 ethyl acetate:hexanes) to afford **2-73** as a light orange solid (1.24 g, 51% over two steps): mp 195–198 ºC (dec); ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.83 (t, *J* = 6.4 Hz, 2H), 3.70–3.57 (m, 10H), 2.97–2.86 (m, 6H), 2.82 (s, 8H), 2.74 (t, *J* = 6.5 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.21, 169.19, 167.4, 166.9, 71.24 (unresolved methylene carbons), 70.8, 70.7, 70.6, 70.5, 65.8, 32.2, 31.8, 27.0, 25.67 (unresolved methylene carbons); IR (thin film): 2872, 1812, 1780, 1730, 1200, 1064 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{20}H_{28}N_2O_{11}SNa$ $[M + Na]^+$ 527.1312, found 527.1312.

1,1'-(Sulfinylbis(propane-3,1-diyl))bis(1H-pyrrole-2,5-dione) (**2-75**)

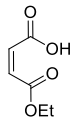


To a stirred solution of 3 3'-thiodipropanol (0.053 g, 0.35 mmol) in THF (5 mL) was added triphenylphosphine (0.207 g, 0.790 mmol) and maleimide (0.079 g, 0.81 mmol). The resulting colorless solution was let stir for 5 min and then placed in an ice bath for 15 min. Diethyl azodicarboxylate (0.125 mL, 0.797 mmol) was added dropwise through the septum, resulting in a light orange solution. The solution was let stir for 12 h, after which it was dark orange. The crude reaction mixture was diluted in DCM (60 mL), then washed with water (3 × 30 mL) and brine (1 × 30 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo* to give an orange oil. The crude product was immediately purified by column chromatography (ethyl acetate) to obtain **2-74** and diethyl hydrazine-1,2-dicarboxylate contaminant in a 2:3 ratio as a yellow oil. The mixture was used in the next reaction without any

further purification: ^{1}H NMR (500 MHz; $CDCl_3$): δ 6.70 (s, 4H), 6.41 (s, 2H), 4.21 (q, *J* = 7.1 Hz, 4H), 3.62 (t, *J* = 7.1 Hz, 4H), 2.49 (t, *J* = 7.4 Hz, 4H), 1.86 (quin, *J* = 7.1, 4H), 1.28 (t, *J* = 7.9 Hz, 6H).

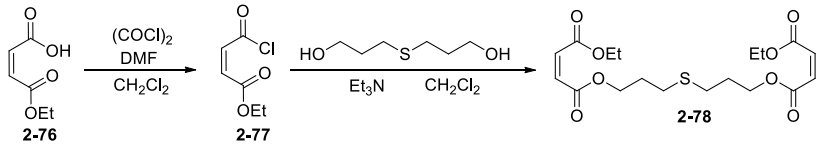
Sulfide **2-74** and diethyl hydrazine-1,2-dicarboxylate contaminant (0.035 g, approximately 40% **2-74** by ^{1}H NMR) were subjected to general procedure 2.5 to afford the crude product as a yellow oil. The crude mixture was subjected to column chromatography (1:3 acetone:ethyl acetate) followed by sonication in hexanes to afford **2-75** as a yellow solid (9.0 mg, 79%): mp 80 ºC (dec); ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.72 (s, 4H), 3.68 (td, *J* = 6.6, 2.3 Hz, 4H), 2.65 (dq, *J* = 13.1, 5.5 Hz, 4H), 2.09 (quin, *J* = 7.6, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.8, 134.4, 50.0, 36.9, 22.5; IR (thin film): 2939, 1697, 1408, 1136, 1022, 827, 694 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{14}H_{16}N_2O_5SNa$ $[M + Na]^+$ 347.0672, found 347.0673.

(*Z*)-4-Ethoxy-4-oxobut-2-enoic acid (**2-76**)



Maleic anhydride (3.07 g, 31.3 mmol) and absolute ethanol (30 mL) were combined in a round bottom flask and heated at 60 ºC for 2 h. Afterwards, the reaction was let cool, and the solvent was removed in vacuo affording **2-76** as a colorless oil (4.50 g, quant.). ^{1}H and ^{13}C NMR spectra were consistent with those previously reported for this compound.[24]

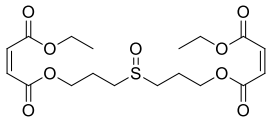
(*Z*)-Diethyl *O*,*O*'-(thiobis(propane-3,1-diyl)) dimaleate (2-79)



To a cooled (0 ºC) solution of **2-76** (0.501 g, 3.48 mmol) in DCM (1.9 mL) and DMF (1 drop) was added oxalyl chloride (0.9 mL, 10.5 mmol) dropwise, slowly, resulting in light bubbling. After 15 min, the bubbling had stopped and the solution had turned yellow. The reaction was let stir for additional 2 h, after which the excess solvent was removed *in vacuo* to afford **2-77** as a dark yellow oil which was used immediately without purification: ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.99 (q, *J* = 15.4 Hz, 2H), 4.30 (q, *J* = 7.1 Hz, 2H), 1.34 (t, *J* = 7.1 Hz, 3H).

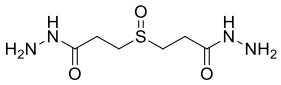
To a cooled (0 ºC) solution of 3,3'-thiodipropanol (0.24 mL, 1.7 mmol) and crude **2-77** (0.55 g, 3.4 mmol) in DCM (9 mL) was added triethylamine (0.49 mL, 3.5 mmol) slowly, dropwise. Fuming was observed, and the yellow solution was let stir for 16 h, after it was diluted in DCM (100 mL). The organic layer was washed with water (3 × 50 mL), brine (1 × 50 mL), dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo* to an orange oil, which was immediately purified by column chromatography (1:3 hexanes:ethyl acetate) to afford **2-78** as a colorless oil (0.285 g, 42% over two steps): ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.84 (s, 4H), 4.29 (t, *J* = 6.3 Hz, 4H), 4.25 (q, *J* = 7.2 Hz, 4H), 2.60 (t, *J* = 7.2 Hz, 4H), 1.95 (quin, *J* = 6.9 Hz, 4H), 1.30 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 164.9, 134.0, 133.3, 63.8, 61.4, 28.49, 28.45, 14.2; IR (thin film): 2995, 1715, 1294, 1255, 1151, 1025, 976, 773; HRMS (ESI) *m* / *z* calcd for $C_{18}H_{26}O_8SNa$ $[M + Na]^+$ 425.1246, found 425.1230.

(*Z*)-Diethyl *O*,*O*'-(sulfinylbis(propane-3,1-diyl)) dimaleate (2-79)



Sulfide **2-78** (0.131 g, 0.33 mmol) was subjected to general procedure 2.5 to afford **2-79** as a white paste (0.121 g, 88%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.84 (s, 4H), 4.85–4.31 (m, 4H), 4.24 (q, *J* = 7.1 Hz, 4H), 2.85–2.69 (m, 4H), 2.26–2.12 (m, 4H), 1.30 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 164.8, 134.4, 132.9, 63.7, 61.5, 49.2, 22.3, 14.1; IR (thin film): 2956, 1715, 1296, 1258, 1153, 1024, 774; HRMS (ESI) *m* / *z* calcd for $C_{18}H_{26}O_9SNa$ $[M + Na]^+$ 441.1190, found 441.1197.

3,3'-Sulfinyldi(propanehydrazide) (2-83)



To **1-1** (1.41 g, 4.17 mmol) in DCM (50 mL) was added *tert*-butyl carbazate (1.10 g, 8.32 mmol). The resulting yellow solution was let stir at rt for 12 h, after which trifluoroacetic acid (2.20 mL, 28.7 mmol) was added. The resulting orange solution was let stir for 72 h before removing the solvent in vacuo. The resulting orange oil was dissolved in methanol (40 mL), and then triethylamine (6 mL) was added. The resulting mixture was let stir for 20 mins, after which a white solid had precipitated. The solid was collected via centrifuge, and then stirred with fresh methanol (40 mL) for 20 mins. The solid was collected via centrifuge again, and this process of stirring with fresh methanol was repeated another two times. Drying the isolated white solid in vacuo afforded **2-83** (0.375 g, 46%): mp 159–162 ºC; ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.13 (s, 2H), 4.24 (s, 4H), 3.0 –2.97 (m, 2H), 2.83–2.75 (m, 2H), 2.43 (t, *J* = 7.5 Hz, 4H); ^{13}C NMR (125

MHz, DMSO-d_6): δ 169.3, 46.7, 26.2; IR (thin film): 3308, 3044, 1631, 1449, 1297, 1032 cm^{-1};

HRMS (ESI) *m* / *z* calcd for $C_6H_{15}N_4O_3S$ $[M + H]^+$ 223.0865, found 223.0857.

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Chapter 3

Trioxane-Containing Collision-Induced Dissociation-Cleavable Protein Cross-Linkers

I. Utilizing the Trioxane Core in a CID-XL.

In addition to sulfoxides, the trioxane core was investigated as a potential CID-cleavable cross-linker. Trioxanes are known to degrade under acidic or thermal conditions, so it was hypothesized that the trioxane core could cleave in CID during MS/MS.[1,2] The cross-linker design includes two cross-linking arms and one non-cross-linking substitution (Figure 3.1). This non-cross-linking substituent could have a low molecular weight and thus not contribute to complexity in the MS/MS data. Alternatively, it could contain a functional group such as biotin for affinity purification.

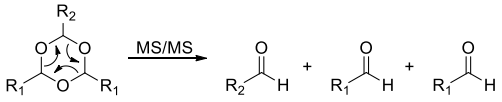


Figure 3.1. Proposed fragmentation mechanism during MS/MS, with R_1 = cross-linking arms, and R_2 = non-cross-linking substituent.

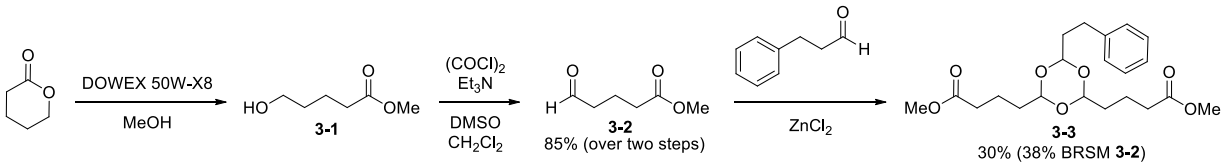
Another benefit to the trioxane design is that the two cross-linking arms could be designed to be of equal mass, so it would not matter which orientation the cross-linker reacted with the protein as the two expected fragments of the cross-linking arms would have the exact mass.

II. Synthesis of Trioxane CID-XLs.

To test the fragmentation hypothesis of the trioxane core in MS/MS, model substrate **3-3** was synthesized (Scheme 3.1). In order to eventually synthesize a lysine-reactive cross-linker,

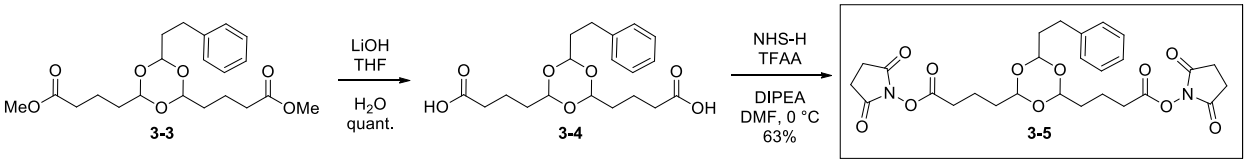
an ester moiety was employed for two of the three arms of the trioxane. Acid catalyzed ring opening of *delta*-valerolactone in methanol provided methyl ester **3-1**, which was converted to **3-2** via Swern oxidation in an 85% yield over two steps.[3] Treatment of two equivalents of **3-2** and one equivalent of 3-phenylpropanal with 10 mol% zinc-(II) chloride afforded a nearly statistical distribution of trimerization products, as well as small amounts of starting materials.[4] Trioxane **3-3** was isolated from the mixture by column chromatography in 30% yield.

Scheme 3.1. Synthesis of test trioxane **3-3.**



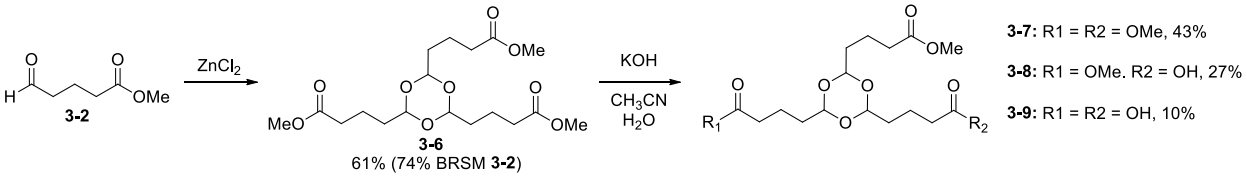
Trioxane **3-3** was subjected to MS/MS; however, despite utilizing a solvent of 20% formic acid in methanol, **3-3** was only detected as the sodium salt ($M + Na^+$). The CID process is only effective for protonated molecules ($M + H^+$), as an increase in stability is observed in the sodiated species, hindering an accurate approximation of the fragmentation energy (Appendix Figure A.10).[5] Therefore, the MS/MS data for **3-3** could not be compared to the MS/MS data of the diamidated lysine-reactive sulfoxide CID-XLs (e.g. **2-22**) where the cross-linker was detected with a proton. Thus, the synthesis of the trioxane cross-linker **3-5** was carried out (Scheme 3.2). Saponification of **3-3** with lithium hydroxide afforded **3-4** in quantitative yield, and NHS-ester formation yielded the desired trioxane **3-5** in 63% yield after column chromatography. During cross-linking experiments by the Huang group, **3-5** was discovered to cleave before the peptide backbone, suggesting that it is an effective cross-linker.

Scheme 3.2. Synthesis of trioxane **3-5.**



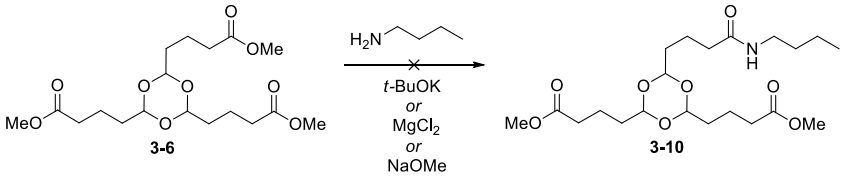
With knowledge that the trioxane moiety is susceptible in MS/MS, attempts were made to improve the overall yield of the trioxane cross-linker. As an alternative to the low yielding trimerization step to form **3-3**, it was envisioned that trioxane **3-6** could be mono-functionalized. This would allow one 'arm' of the trioxane to be modified separately from the other arms. Initially, mono-hydrolysis was investigated (Scheme 3.3). Considering that concentration may be a significant factor, the reaction was run under dilute conditions in an attempt to diminish the competing di- and tri-hydrolysis reactions.[6,7] Despite running the mono-hydrolysis at 0.01 M with a slow addition of potassium hydroxide, the maximum yield of the mono-hydrolysis product was 27%.

Scheme 3.3. Mono-hydrolysis of trioxane **3-6.**



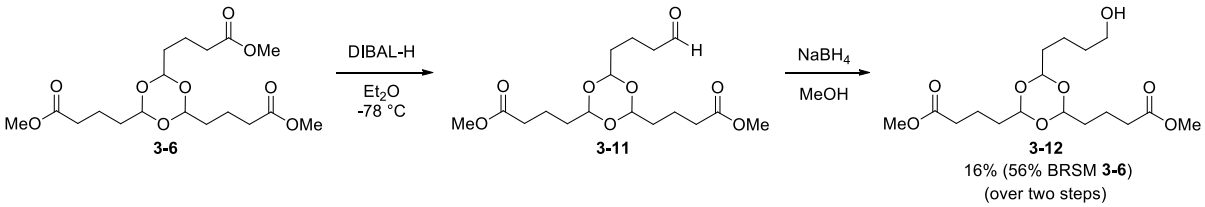
Alternatively, it was envisioned that mono-amidation could be applied to a symmetric triester system. Mono-amidation of symmetric diesters has been achieved in high yield with a number of different catalysts.[8–10] Unfortunately, efforts to mono-amidate **23** using *n*-butylamine with various bases and catalysts did not afford any desired product (Scheme 3.4).

Scheme 3.4. Attempts to mono-amidate trioxane **3-6.**



Mono-reduction of diesters with diisobutylaluminium hydride (DIBAL-H) has also been reported to proceed in high yield.[11,12] After the reaction of **3-6** with DIBAL-H, trioxane aldehyde **3-11** was found to be inseparable from starting trioxane **3-6**, so the crude reaction mixture was subjected to sodium borohydride reduction conditions to afford alcohol **3-12** (Scheme 3.5). Although trioxane **3-6** was recovered by column chromatography, the overall yield of desired trioxane alcohol **3-12** was low.

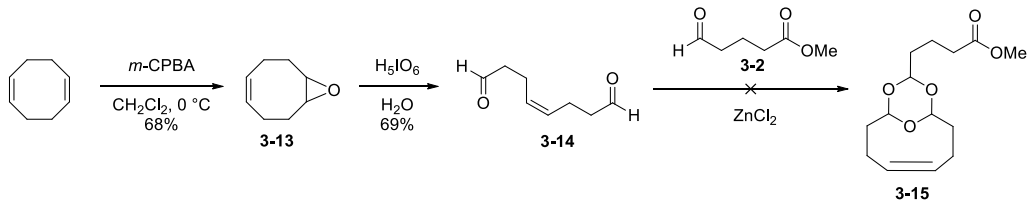
Scheme 3.5. Mono-reduction of trioxane **3-6.**



A different attempt to synthesize the trioxane core was also investigated (Scheme 3.6). It was envisioned that a bicyclic compound such as **3-15** could be synthesized, after which the double bond would be oxidatively cleaved to afford a bi-functional cross-linker. Mono-oxidation of commercially available 1,5-cyclooctadiene afforded epoxide **3-13**, which was followed by oxidative cleavage to yield alkene **3-14**.[13,14] Unfortunately, attempts to cyclize **3-14** with **3-2** were unsuccessful. Within five minutes of starting the reaction, the solution turned black, and upon workup followed by column chromatography only starting materials were recovered in a near quantitative amount. It was hypothesized that the zinc-(II) chloride was coordinating to the alkene in **3-14**, impeding the reaction.[15] Upon adding additional equivalents

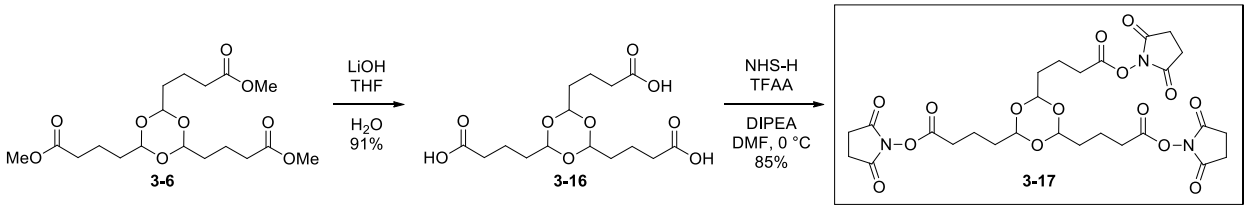
of the Lewis acid, the reaction still failed to afford **3-15**, and still resulted in recovery of the starting materials in a near quantitative amount.

Scheme 3.6. Ring closing/opening plan to synthesize the trioxane core.



The unsuccessful mono-functionalization routes prompted the synthesis of a symmetrical trioxane (Scheme 3.7). Hydrolysis of symmetrical tri-ester **3-6** afforded tri-acid **3-16**, which was subsequently hydrolyzed to yield symmetrical tri-NHS-ester **3-17**.

Scheme 3.7. Synthesis of symmetric trioxane **3-17.**



III. Conclusions

The trioxane functional group was found to cleave during MS/MS before the peptide backbone, suggesting that it is an effective scaffold to incorporate into CID-XLs for peptide sequencing. Trioxane CID-XL **3-5** was synthesized and worked well in initial biological testing. This inspired the synthesis of trioxane **3-17**, which is currently being used in *in-vitro* and *in-vivo* testing.

IV. General Experimental Details

All chemicals were purchased from Sigma-Aldrich, Acros Organics, Alfa Aesar, TCI, Advanced ChemTech, or Fisher and used without further purification unless otherwise noted. Zinc (II) chloride was flame dried under vacuum prior to use. Ethanol was purchased from Gold Shield. Solvents were of reagent grade and used as without further purification except as follows: *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), tetrahydrofuran (THF), and diethyl ether (ether) were degassed and then passed through anhydrous neutral alumina A-2 before use, according to the procedure described by Grubbs.[16] Methanol was dried over activated 3Å molecular sieves prior to use. Triethylamine was distilled over calcium hydride and stored over activated 3Å molecular sieves prior to use. Diisopropylethylamine (DIPEA) was distilled over calcium hydride prior to use. Trifluoroacetic anhydride (TFAA) and trimethylsilyl triflate (TMS-OTf) were distilled prior to use. Reported reaction temperatures refer to the temperature of the heating medium. Reactions were performed in flame- or oven-dried glassware under an atmosphere of dry argon using standard Schlenk techniques unless otherwise noted. Room temperature (rt) refers to 25 ± 3 ºC. Reactions were monitored by thin-layer chromatography (TLC) using EMD Chemicals Inc. silica gel 60 F_{256} plates. Flash chromatography was performed using Ultra Pure SiliaFlash P60, 230-400 mesh (40-63 μm) silica gel (SiO_2) following the general procedure by Still and co-workers.[17]

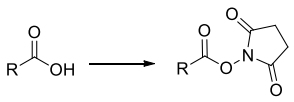
V. Instrumentation

Proton NMR spectra measurements were acquired using either a Bruker DRX500 with a cryoprobe, Bruker GN500, or a Bruker AVANCE600 spectrometer, at 500 MHz, 500 MHz, and 600 MHz, respectively. Carbon NMR spectra were obtained on a Bruker DRX500 with a

cryoprobe at 125 MHz. Proton NMR chemical shifts (δ) are reported in parts per million (ppm) and referenced to the residual solvent peak at 7.26 ppm for deuterated chloroform ($CDCl_3$) and 2.50 for deuterated dimethylsulfoxide (DMSO-d_6).[18] Carbon NMR chemical shifts (δ) are reported in parts per million (ppm) and referenced to the residual solvent peak at 77.16 ppm for deuterated chloroform and 39.52 for deuterated dimethylsulfoxide.[18] All NMR spectra were processed using MestReNova (Mestrelab Research). NMR data are reported in the following manner: chemical shift, multiplicity, (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, br = broad, app = apparent), coupling constants (J) in hertz (Hz), and integration. High resolution mass spectrometry (HRMS) accurate mass experiments were ran by the University of California, Irvine mass spectrometry laboratory. Infrared (IR) spectroscopy data were acquired on a Shimadzu IRAffinity-1 Spectrophotometer with a MIRacle 10 single reflection ATR accessory. Melting points (mp) were acquired on a Mel-Temp melting point apparatus and are uncorrected. Tandem mass spectroscopy (MS/MS) analysis was performed on a Waters Quattro Premier XE mass spectrometer by Dr. John Greaves.

VI. Detailed Experimental Procedures for Compounds in Chapter 3

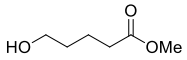
General Procedure 3.1: *N*-hydroxysuccinimide ester formation from diacids.[19]



To a cooled (0 ºC) mixture of the diacid (1 equiv), *N*-hydroxysuccinimide (4 equiv), and DIPEA (8 equiv) in DMF (0.2 M) was added TFAA (4 equiv) dropwise, slowly. The light orange solution was allowed to warm to rt and stir until determined complete by TLC, after which it was partitioned between ethyl acetate and hydrochloric acid (1 M). The layers were

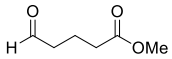
separated, and the acidic aqueous layer was extracted with ethyl acetate (2×). The organic layers were combined, washed with sodium bicarbonate solution (1 M, 3×), water (1×), and brine (1×). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo.

Methyl 5-hydroxypentanoate (3-1)



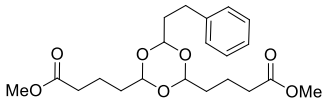
^{1}H and ^{13}C NMR spectra matched those previously reported for this compound.[20]

Methyl 5-oxopentanoate (3-2)



^{1}H and ^{13}C NMR spectra matched those previously reported for this compound.[20]

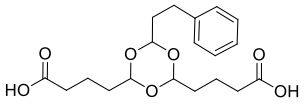
Dimethyl 4,4'-(6-phenethyl-1,3,5-trioxane-2,4-diyl)dibutanoate (3-3)



To zinc (II) chloride (0.009 g, 0.066 mmol) was added **3-2** (0.05 g, 0.38 mmol) and 3-phenylpropanal (0.025 g, 0.190 mmol) simultaneously. The resulting cloudy solution was let stir at rt for 16 h, after which it was diluted in ethyl acetate (100 mL). The reaction mixture was washed with water (3 × 30 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to a colorless oil. The crude reaction mixture was chromatographed (1:1 ethyl acetate:heaxanes) to afford **3-3** as a colorless oil (0.023 g, 30%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.30–7.25 (m, 2H), 7.19–7.13 (m, 3H), 4.88–4.82 (m, 3H), 3.67 (s, 6H), 2.73 (t, *J* = 7.7 Hz, 2H), 2.36 (t, *J* = 7.0 Hz, 4H), 1.99 (q, *J* = 6.6 Hz, 2H), 1.78–1.70 (m, 8H); ^{13}C NMR (125 MHz,

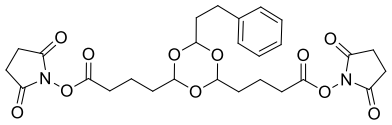
$CDCl_3$): δ 174.0, 141.4, 128.6, 128.5, 126.1, 101.1, 100.7, 51.7, 35.7, 33.8, 33.7, 29.7, 19.2; IR (thin film): 2848, 2359, 2340, 1735, 1435, 1361, 1128, 698 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{21}H_{30}O_7Na$ $[M + Na]^+$ 417.1889, found 417.1882.

4,4'-(6-Phenethyl-1,3,5-trioxane-2,4-diyl)dibutanoic acid (3-4)



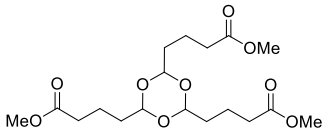
To a stirred solution of **3-3** (0.03 g, 0.79 mmol) in THF (2 mL) and water (2 mL) was added LiOH (98%, 0.06 g, 2.50 mmol). The resulting cloudy solution was let stir for 12 h, after which it was acidified to pH 1 (monitored by pH paper) with hydrochloric acid (6 M). The aqueous layer was extracted with ethyl acetate (3 ×10 mL). The organic layers were combined, washed with water (3 × 15 mL), brine (1 × 15 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to afford **3-4** as a colorless oil (0.028 g, quant.): ^1H NMR (600 MHz, $CDCl_3$): δ 10.98 (br s, 2H), 7.30–7.25 (m, 2H), 7.19–7.13 (m, 3H), 4.87 (t, *J* = 4.8 Hz, 2H), 4.84 (t, *J* = 5.3 Hz, 1H), 2.79–2.68 (m, 2H), 2.41 (t, *J* = 7.1 Hz, 4H), 2.05–1.95 (m, 2H), 1.86–1.68 (m, 8H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 180.0, 141.4, 128.58, 128.57, 128.54, 126.1, 101.0, 100.7, 35.7, 33.8, 33.5, 29.7, 18.9; IR (thin film): 3126, 2930, 1709, 1128, 1070 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{19}H_{25}O_7$ $[M - H]^-$ 365.1600, found 365.1607.

Bis(2,5-dioxopyrrolidin-1-yl) 4,4'-(6-phenethyl-1,3,5-trioxane-2,4-diyl)dibutanoate (3-5)



Diacid **3-4** (0.028 g, 0.076 mmol) was subjected to general procedure 3.1 to afford a colorless oil. The crude product was subjected to column chromatography (3:1 ethyl acetate:hexanes) to afford **3-5** as a colorless oil (0.027 g, 63%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.28–7.25 (m, 2H), 7.22–7.15 (m, 3H), 4.90 (t, *J* = 4.8 Hz, 2H), 4.84 (t, *J* = 5.2 Hz, 1H), 2.83 (br s, 8H), 2.77–2.71 (m, 2H), 2.68 (t, *J* = 7.4 Hz, 4H), 2.04–1.96 (m, 2H), 1.89–1.82 (m, 4H), 1.81–1.70 (m, 4H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.3, 168.5, 141.4, 128.57, 128.52, 126.0, 100.65, 100.63, 35.7, 33.1, 30.7, 29.6, 25.7, 18.7; IR (thin film): 2931, 1811, 1736, 1363, 1203, 1066 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{14}H_{16}N_2O_9SNa$ $[M + Na]^+$ 583.1904, found 583.1921.

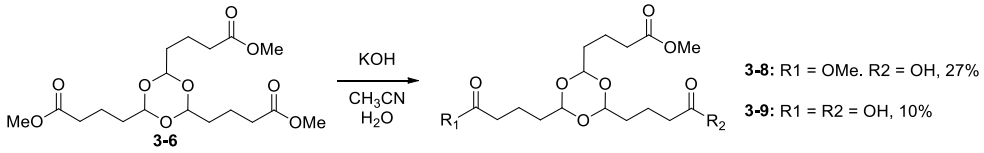
Trimethyl 4,4',4''-(1,3,5-trioxane-2,4,6-triyl)tributanoate (3-6)



To zinc (II) chloride (0.077 g, 0.560 mmol) was added **3-2** (0.49 g, 3.80 mmol). The resulting cloudy solution was let stir at rt for 12 h, after which it was diluted in ethyl acetate (60 mL). The reaction mixture was washed with water (3 × 30 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to a colorless oil. The crude reaction mixture was subjected to column chromatography (1:3.5:14 acetonitrile:ethyl acetate:heaxanes) to afford **3-6** as a colorless oil (0.300 g, 61%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.86 (t, *J* = 4.9 Hz, 3H), 3.67 (s, 9H), 2.35 (t, *J* = 7.3 Hz, 6H), 1.83–1.64 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.9, 101.1,

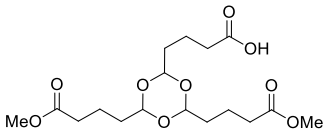
51.7, 33.8, 33.7, 19.1; IR (thin film): 2955, 2863, 1734, 1710, 1436, 1170, 1129 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{18}H_{30}O_9Na$ $[M + Na]^+$ 413.1787, found 413.1782.

Attempted mono-hydrolysis of trimethyl 4,4',4''-(1,3,5-trioxane-2,4,6-triyl)tributanoate (3-6)



Triester **3-6** (0.053 g, 0.14 mmol) was dissolved in acetonitrile (0.25 mL), after which water (10 mL) was added. To the cooled (0 °C) solution was added potassium hydroxide dropwise (0.05 M solution, 2.7 mL) via syringe pump over 1 h. The resulting colorless solution was let stir for 1 h, after which the cooled (0 °C) crude reaction was carefully acidified to a pH of 1 (monitored by pH paper) with sulfuric acid (18 M). The acidified aqueous layer was extracted with ethyl acetate (4 × 10 mL). The organic layers were combined, washed with water (1 × 20 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to a colorless oil. The crude product was subjected to column chromatography (1:6:12 acetonitrile:ethyl acetate:heaxanes, with 1% glacial acetic acid) to afford **3-8** (0.014 g, 27%) as a colorless oil and **3-9** (0.005 g, 10%) as a colorless oil.

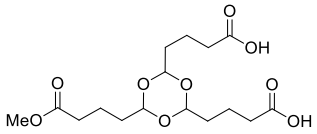
4-(4,6-Bis(4-methoxy-4-oxobutyl)-1,3,5-trioxan-2-yl)butanoic acid (3-8)



^{1}H NMR (500 MHz, $CDCl_3$): δ 9.77 (br s, 2H), 4.88–4.85 (m, 3H), 3.66 (s, 6H), 2.40 (t, *J* = 7.0 Hz, 2H), 2.35 (t, *J* = 7.1 Hz, 4H), 1.84–1.60 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 179.0,

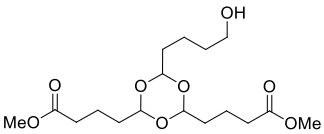
174.0, 101.08, 101.05, 51.72, 51.70, 33.8, 33.6, 19.1, 18.9, 18.8; IR (thin film): 2955, 2359, 1733, 1436, 1167, 1127 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{17}H_{28}O_9Na$ $[M + Na]^+$ 399.1631, found 399.1645.

4,4'-(6-(4-Methoxy-4-oxobutyl)-1,3,5-trioxane-2,4-diyl)dibutanoic acid (3-9)



^1H NMR (500 MHz, $CDCl_3$): δ 10.07 (br s, 2H), 5.00–4.79 (m, 3H), 3.67 (s, 3H), 2.40 (t, *J* = 6.8 Hz, 4H), 2.35 (t, *J* = 7.2 Hz, 2H), 1.91–1.57 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 179.7, 174.1, 101.1, 101.0, 53.6, 51.7, 33.8, 33.6, 33.4, 19.1, 19.0; IR (thin film): 2954, 2861, 2363, 1734, 1436, 1167 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{16}H_{26}O_9Na$ $[M + Na]^+$ 385.1475, found 385.1479.

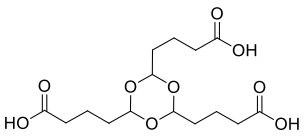
Dimethyl 4,4'-(6-(4-hydroxybutyl)-1,3,5-trioxane-2,4-diyl)dibutanoate (3-12)



To a cooled (–78 °C) solution of **3-6** (0.113 g, 0.289 mmol) in ether (5 mL) was added diisobutylaluminium hydride (1M solution in hexanes, 0.70 mL) slowly, dropwise. The reaction was let stir 45 min at –78 °C, after which water (0.5 mL) was added. The reaction was let warm to rt, after which it was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to a colorless oil. The oil was dissolved in methanol (2 mL), after which THF (0.5 mL) and sodium borohydride (0.04 g, 1.06 mmol) were added. The colorless solution was let stir 12 h, after which it was partitioned between hydrochloric acid (0.25M, 60 mL) and ethyl acetate (60

mL). The layers were separated, and the aqueous layer was extracted with ethyl acetate (60 mL). The combined organic layers were washed with water (1 × 40 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to a colorless oil. The crude product was subjected to column chromatography (3:2 ethyl acetate:heaxanes) to afford **3-12** as a colorless oil (0.017 g, 16% over two steps): ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.87–4.84 (m, 3H), 3.67–3.61 (m, 8H), 2.34 (t, *J* = 7.1 Hz, 4H), 1.82–1.64 (m, 8H), 1.59–1.55 (m, 4H), 1.53–1.41 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.0, 101.4, 101.1, 62.8, 51.7, 34.1, 33.8, 33.7, 32.5, 19.8, 19.2; IR (thin film): 2954, 2358, 1733, 1436, 1249, 1160 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{17}H_{30}O_8Na$ $[M + Na]^+$ 385.1838, found 385.1843.

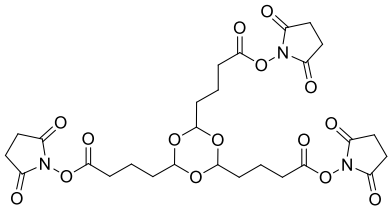
4,4',4''-(1,3,5-Trioxane-2,4,6-triyl)tributanoic acid (3-16)



To triester **3-6** (0.460 g, 1.18 mmol) in a solution of THF (30 mL) was added a solution of lithium hydroxide monohydrate (1.6 g, 38 mmol) in an equal volume of H_2O (30 mL). The cloudy reaction was let stir vigorously at rt overnight. In the morning, the crude reaction was cooled (0 ºC) and then carefully acidified to a pH of 1 (monitored by pH paper) with sulfuric acid (18 M). The acidified aqueous layer was extracted with ethyl acetate (3 × 125 mL). The organic layers were combined, washed with water (50 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to give an off-white solid. Trituration in hexanes (10 mL) afforded **3-16** as a white solid (0.375 g, 91%): mp 129–130 ºC; ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.02 (s, 3H), 4.95 (t, *J* = 3.9 Hz, 3H), 2.23 (t, *J* = 6.4 Hz, 6H), 1.60–1.50 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.3, 100.2, 33.2, 33.2, 18.7; IR (thin film): 3394, 2960, 1703, 1407,

1293, 1126 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{15}H_{24}O_9Na$ $[M + Na]^+$ 371.1318, found 371.1327.

Tris(2,5-dioxopyrrolidin-1-yl) 4,4',4''-(1,3,5-trioxane-2,4,6-triyl)tributanoate (3-17)



Triacid **3-16** (1.5 g, 4.3 mmol) was subjected to general procedure 3.1 to give a dark orange oil. Many triturations and recrystallizations were attempted with various mixtures of hexanes and ethyl acetate, but no pure product was obtained. Thus, column chromatography (4:1 ethyl acetate:heaxanes) afforded **3-17** as a light orange solid (2.53 g, 85%): mp 115–117 ºC; ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.92 (t, *J* = 4.7 Hz, 3H), 2.81 (s, 12H), 2.66 (t, *J* = 7.3 Hz, 6H), 1.94–1.82 (m, 6H), 1.81–1.74 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.3, 168.5, 100.6, 33.0, 30.7, 25.7, 18.7; IR (thin film): 2946, 1779, 1729, 1360, 1200 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{27}H_{33}N_3O_{15}Na$ $[M + Na]^+$ 662.1809, found 662.1795.

VII. References

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Chapter 4

Efforts Toward Incorporating a Tandem-Mass-Tag into Collision-Induced Dissociation-Cleavable Protein Cross-Linkers

I. Introduction

Affinity purification is often employed in chemical cross-linking experiments to remove unwanted impurities after cross-linking experiments, which improves the signal-to-noise ratio in the resulting MS/MS spectra. The two most common affinity purification techniques are biotin conjugation with avidin/streptavidin beads or covalent trapping using click reactions of azide and alkyne functional groups.[1] In recent years, Tandem-Mass-Tag (TMT) affinity purification has gained popularity for both reducing the amount of impurities present in MS/MS data as well as quantifying MS/MS data.[2] TMT affinity purification utilizes *cis*-2,6-dimethylpiperidine in a manner analogous to biotin where *cis*-2,6-dimethylpiperidine binds non-covalently to specifically designed antibody beads. After washing the TMT-antibody beads with solvents that solubilize impurities, the TMT-containing cross-linked peptide can be eluted from the beads using a variety of tertiary amines (e.g. a solution containing DIPEA or *cis*-2,6-dimethylpiperidine). The tertiary amine solution competes for binding to the antibody beads with the TMT reagent and releases the TMT-containing cross-linked peptide.[3] Thermo has developed a series of TMT reagents that can label lysine residues (Figure 4.1).[4,5] The TMT labeling strategy had been successfully implemented for a number of model proteins, so the feasibility of a TMT-containing CID-XL was investigated.

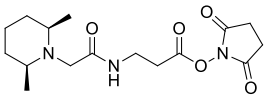


Figure 4.1. Commercially available TMT reagent for labeling lysine residues.

II. Synthesis of TMT-Labeled Cross-Linkers

It was envisioned that TMT CID-XL **4-1** could be prepared from piperidine intermediate **4-2** and the Azide-Bis intermediate **2-4** (Figure 4.2).

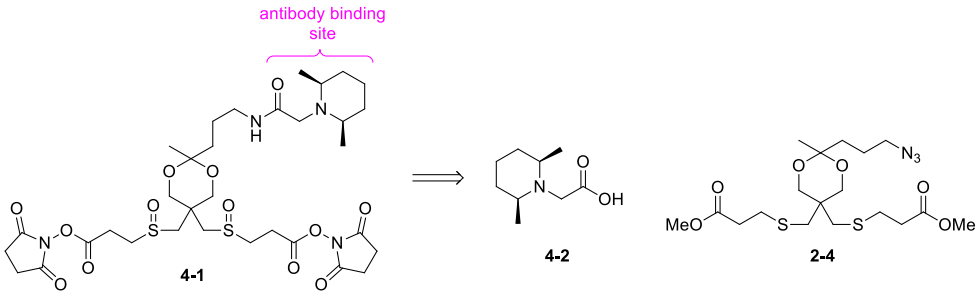
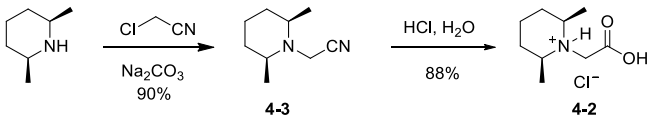


Figure 4.2. Initial retrosynthetic plan to synthesize TMT-containing cross-linker **4-1.**

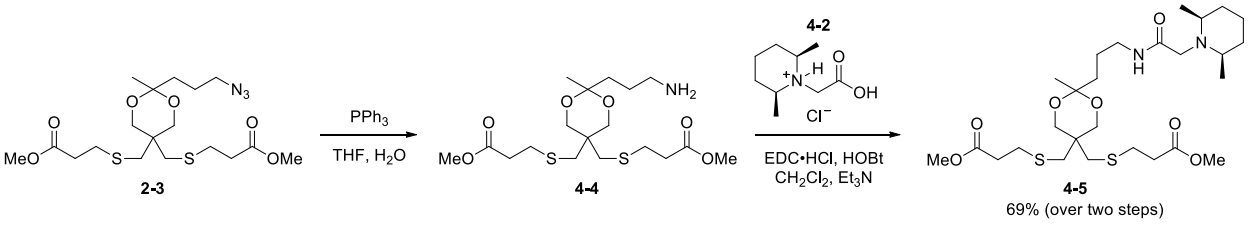
Starting from commercially available *cis*-2,6-dimethylpiperidine, an S_N2 reaction with 2-chloroacetonitrile afforded **4-3** in excellent yield, and further treatment of the nitrile under strongly acidic conditions afforded **4-2**, which was isolated as the HCl salt.[6,7]

Scheme 4.1. Synthesis of the HCl salt of amino acid **4-2.**



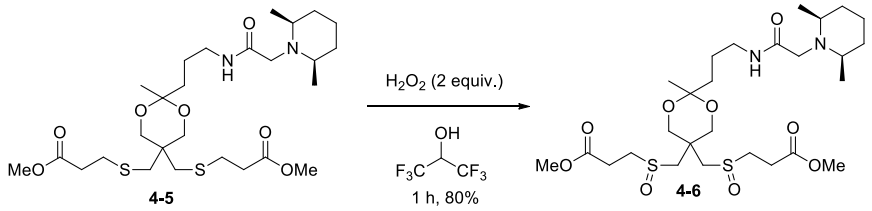
With the amine portion of the envisioned TMT fragment in hand, the sulfide portion was synthesized next. Taking previously synthesized **2-3** and treating it with Staudinger reduction conditions afforded the amine **4-4**, which was dried and immediately subjected to EDC coupling conditions to yield **4-5** in 69% over two steps (Scheme 4.2).[8,9]

Scheme 4.2. Synthesis of the TMT intermediate **4-5.**



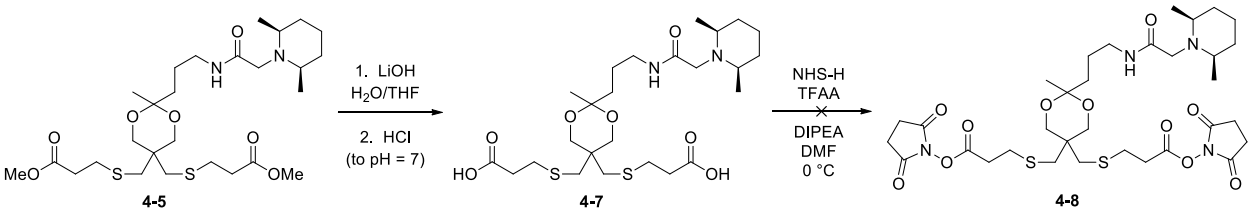
Oxidation of sulfur to sulfoxide in cross-linkers previously synthesized was primarily done with *m*-CPBA. It has been shown that *N*-oxidations of piperidines with *m*-CPBA occurs readily at 0 °C,[10,11] so with **4-5** in hand, an alternative method of oxidation with hexafluoroisopropanol (HFIP) and hydrogen peroxide was tried to avoid oxidation of the tertiary amine.[12] Thankfully, treatment of **4-5** with HFIP and hydrogen peroxide afforded **4-6** with no *N*-oxidation (Scheme 4.3).

Scheme 4.3. Test oxidation of **4-5** with HFIP and hydrogen peroxide.

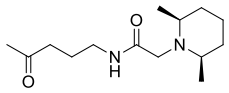


The intermediate **4-5** was taken forward in an attempt to synthesize **4-1**. Basic hydrolysis of **4-5** with lithium hydroxide afforded **4-7** as the dianion (Scheme 4.3). The reaction was deemed complete by monitoring via mass spectrometry to observe complete consumption of **4-5** and formation of the dianion.

Scheme 4.3. Attempted formation of the NHS ester **4-8.**



In an attempt to isolate **4-7** as the HCl salt by acidification to pH = 1, the acetal group was cleaved completely. To avoid acetal group cleavage, the pH was controlled during workup with careful addition of HCl to neutralize the solution to pH ≈ 7, and then the aqueous layer was removed via lyophilization. The resulting mixture was very hygroscopic, so after lyophilization the mixture was rigorously kept under argon and immediately subjected to NHS formation. Unfortunately, standard NHS ester conditions did not produce any **4-8**, but instead produced by-product **4-10** (Figure 4.3).

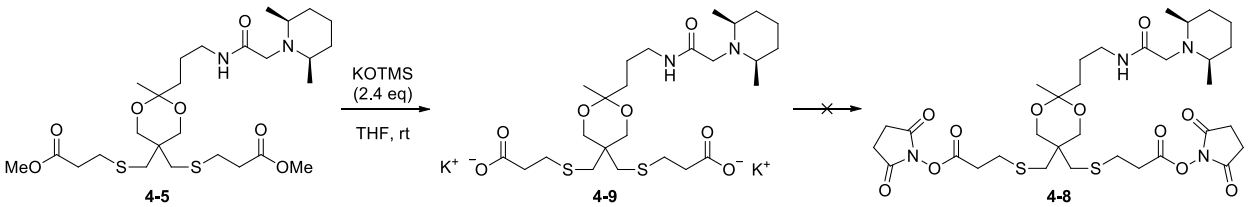


4-10

Figure 4.3. By-product of the attempted ester hydrolysis/NHS formation sequences.

The difficulty handling of compound **4-7** prompted investigation into a water-free hydrolysis of **4-5**. It has been shown that methyl esters can be cleaved with potassium trimethylsilanolate (KOTMS) under anhydrous conditions, and the resulting methoxytrimethylsilane along with the reaction solvent can be removed in vacuo, generating the potassium salt of the ester (Scheme 4.4).[13–15]

Scheme 4.4. Hydrolysis of **4-5** with potassium trimethylsilanolate followed by attempted NHS formation.



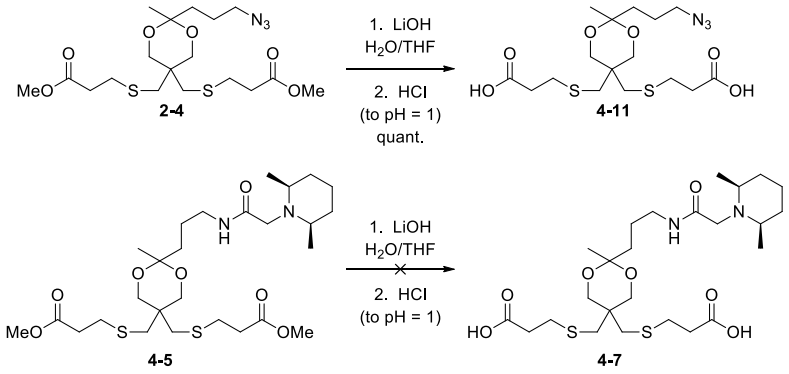
4-5 **4-9** **4-8**

After treating **4-5** with KOTMS in THF, the resulting reaction was monitored by mass spectrometry to monitor formation of the dianion. Complete consumption of the starting material was observed after two days, and a large peak corresponding to **4-9** was observed. The

NHS reaction to form **4-8** was attempted with multiple different coupling conditions, but no desired product was observed, rather the by-product **4-10** was produced.

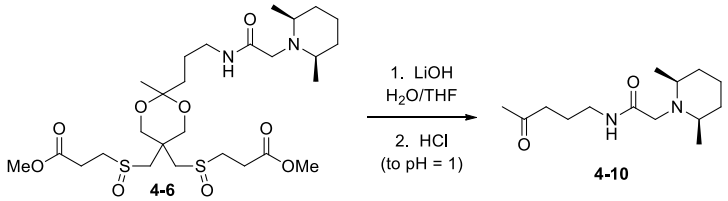
Formation of byproduct **4-10** led to the investigation about the stability of the new TMT CID-XL **4-1**. Previously, when diester **2-4** was subjected to basic hydrolysis followed by acidic workup, diacid **4-11** was isolated in quantitative yield. However, when diester **4-5** was subjected to the same workflow, no product was observed (Scheme 4.5).

Scheme 4.5. Products observed for basic hydrolysis followed by acidic workup in two different sequences**.**



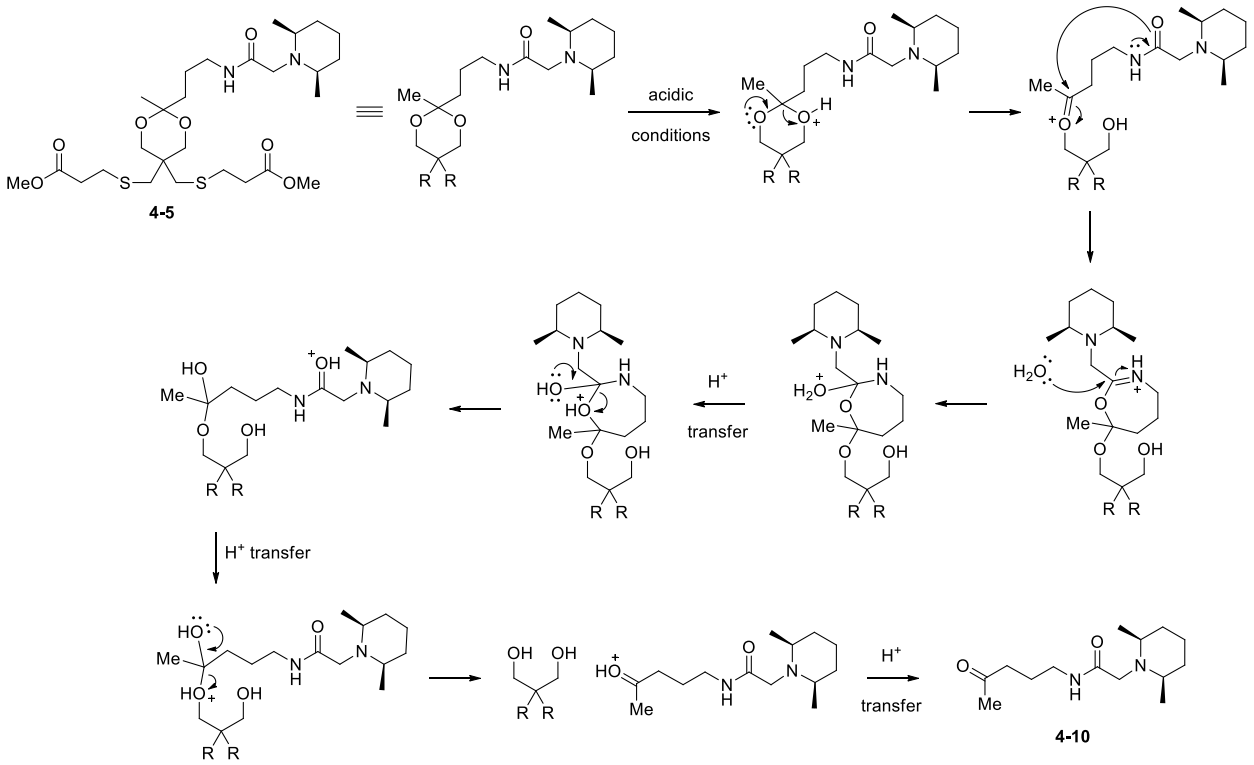
The oxidized diester **4-6** was subjected to the same basic hydrolysis/acidic workup conditions as **2-4** and **4-5** in order to determine the possibility of the TMT group participating in an unwanted side-reaction (Scheme 4.6). After acidic workup, it was found that the ketal had cleaved to afford **4-10**.

Scheme 4.6. Test reaction for **4-6.**



The TMT group is proposed to contribute to the formation of byproduct **4-10** by aiding in acetal cleavage. The basicity (or more electronegative character) of the nitrogen in the amide stabilizes the 7-membered intermediate in the proposed mechanism (Scheme 4.7).

Scheme 4.7. Proposed mechanism for the formation of byproduct **4-10.**



The instability of the acetal group with the TMT reagent arm inspired investigation into a different TMT CID-XL without that functionality. The trioxane was an appealing choice because one of the "arms" of the trioxane could be designed with the TMT tag and the other two arms could be functionalized with NHS esters to react with lysine residues (Figure 4.4). Thus, the synthesis of **4-12** was carried out.

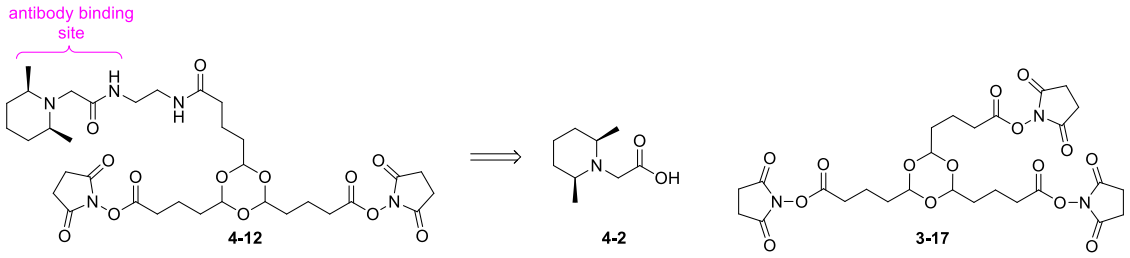
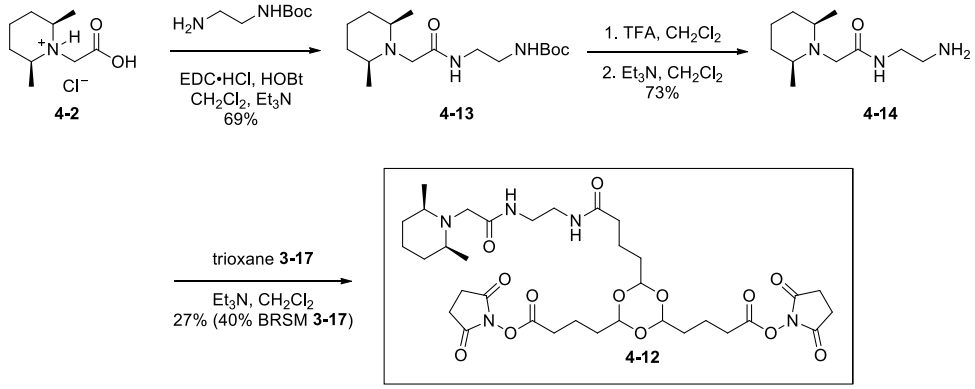


Figure 4.4. Retrosynthesis of target trioxane TMT cross-linker **4-12.**

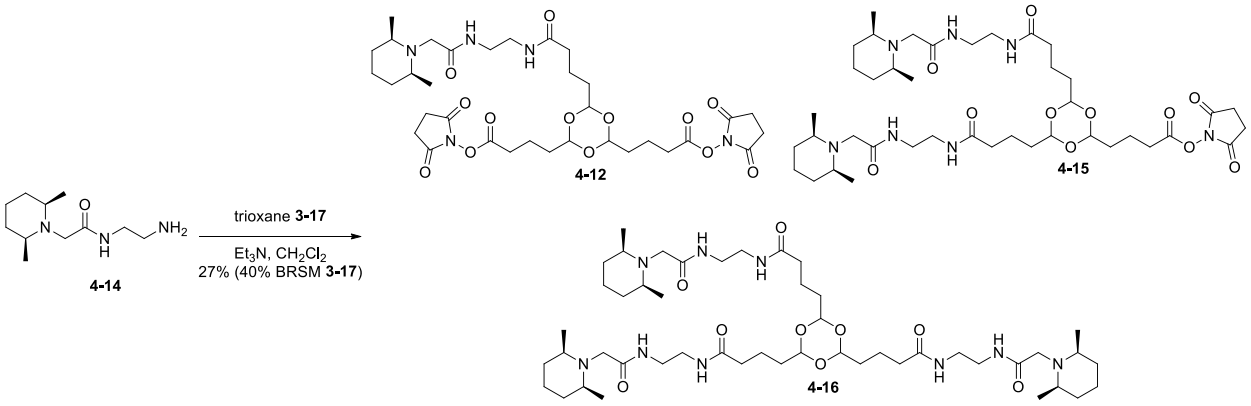
Common intermediate **4-2** was coupled with *tert*-butyl (2-aminoethyl)carbamate[16] using EDC coupling conditions to afford **4-13** in 69% yield (Scheme 4.8). Deprotection of the Boc group, followed by basic workup with 6 M sodium hydroxide yielded **4-14** as the freebase amine in 73% yield.

Scheme 4.8. Synthesis of trioxane TMT cross-linker **4-12.**



Finally, coupling **4-14** with trioxane **3-17** afforded the TMT trioxane **4-12**. This final coupling step proved problematic, with a statistical mixture of unmodified **3-17**, desired mono-addition product **4-12**, di-addition product **4-15**, and tri-addition product **4-16**, despite the low reaction concentration (0.01 M) and slow addition of **4-14** to the reaction mixture (0.008 mmol/min) (Scheme 4.9).

Scheme 4.9. Mixture of products obtained during coupling of **4-14** with trioxane **3-17.**



Initial biological tests with model proteins showed the TMT-trioxane **4-12** to be a promising cross-linker, and while **4-12** was used in *in-vitro* testing, a synthesis of a new sulfoxide containing TMT reagent was designed (Figure 4.5). It was envisioned that a bis-sulfoxide cross-linker could be synthesized without an acetal group, since the acetal group of **4-1** proved problematic in the previous synthesis.

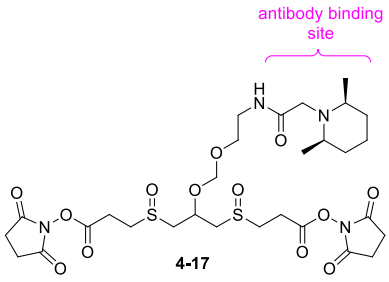
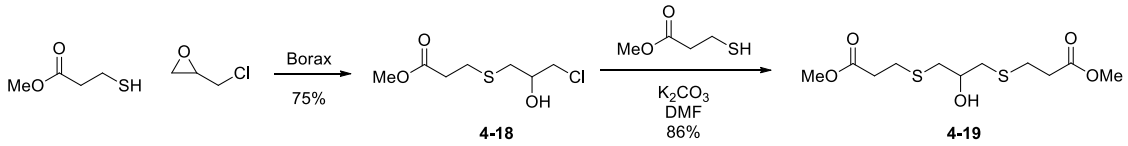


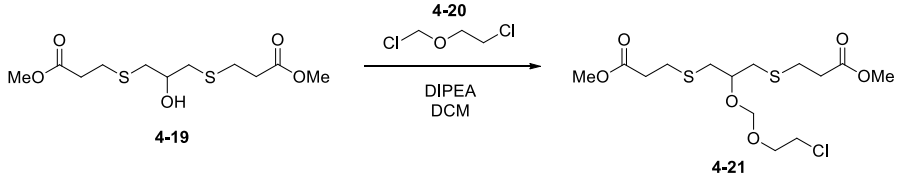
Figure 4.5. Structure of the new TMT-containing sulfoxide cross-linker **4-17.**

Without any common intermediates to utilize, the synthesis of **4-17** began with by treating epichlorohydrin with methyl 3-mercaptopropanoate and borax, affording **4-18** (Scheme 4.10).[17] S_N2 displacement of the chloride with another equivalent of methyl 3-mercaptopropanoate gave the dithiol **4-19**.[18]

Scheme 4.10. Formation of dithiol **4-19.**



Coupling 1-chloro-2-(chloromethoxy)ethane (**4-20**) with **4-19** via S_N2 displacement proved sluggish (Table 4.1).[19] The highest yield of ether **4-21** was with 5.5 equivalents of **4-20** and 2 equivalents of DIPEA. Higher conversion might have been achieved with a greater amount of equivalents of **4-20**, but was not pursued due to the high cost of **4-20**.



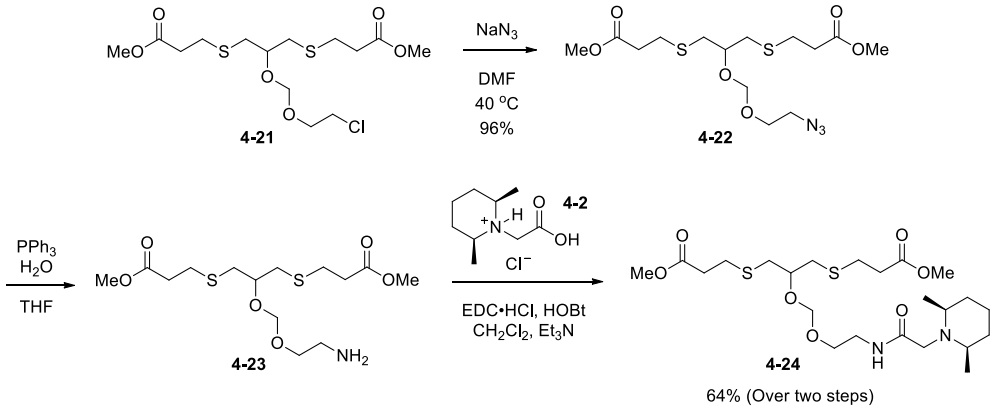
4-19 (equiv.)	**4-20** (equiv.)	DIPEA (equiv.)	Product Yield	DCM (M)
1	2.35	2.5	58% (87% BRSM **4-19**)	1.9
1	3.75	2.5	41% (53% BRSM **4-19**)	1.9
1	1.1	2.0	---[a]	0.2
1	5.5	2.0	79% (90% BRSM **4-19**)	0.2

[a] <5% conversion after 24 h by ^{1}H NMR.

Table 4.1. Optimization of the S_N2 displacement of **4-20.**

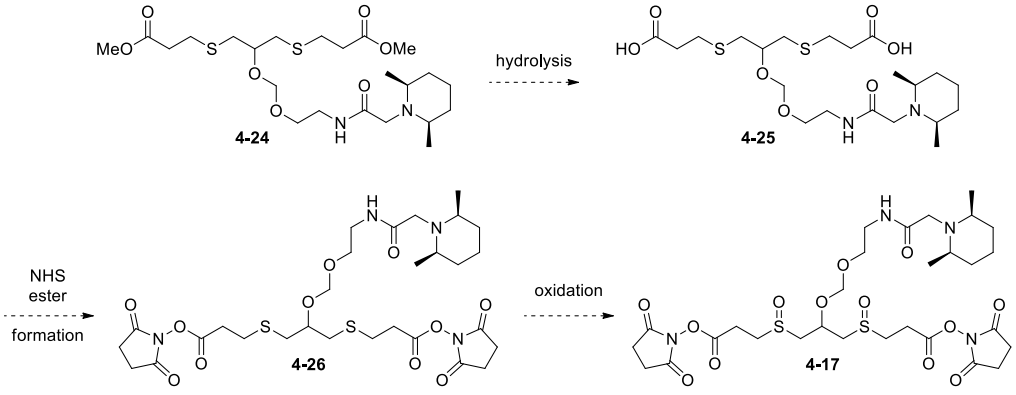
With the ether intermediate in hand, the synthesis was continued by lightly heating ether **4-21** in DMF with sodium azide to afford azide **4-22** in excellent yield (Scheme 4.11). Staudinger reduction to afford amine **4-23** followed by coupling to **4-2** gave the TMT sulfide diester intermediate **4-24**.

Scheme 4.11. Synthesis of the TMT intermediate **4-24**.



With intermediate **4-24** in hand, TMT CID-XL **4-17** could potentially be finished in three steps (Scheme 4.12). Hydrolysis of diester **4-24**, followed by NHS ester formation and subsequent dioxidation would theoretically yield the fully elaborated bis-sulfoxide TMT compound **4-17**. This project is currently being pursued by Ph.D. candidate Sarah Block in the Rychnovsky laboratory.

Scheme 4.12. Envisioned end-game for the completion of TMT CID-XL **4-17.**



III. Conclusions

TMT CID-XL **4-12** was successfully synthesized and was subjected to biological testing. The instability of acetal-containing CID-XLs was investigated, and contributed to the improved

design of a sulfoxide containing CID-XL, the synthesis of which is currently underway. Future directions involve completing the synthesis of **4-17**.

IV. General Experimental Details

All chemicals were purchased from Sigma-Aldrich, Acros Organics, Alfa Aesar, TCI, Advanced ChemTech, or Fisher and used without further purification unless otherwise noted. Zinc (II) chloride was flame dried under vacuum prior to use. Ethanol was purchased from Gold Shield. Solvents were of reagent grade and used as without further purification except as follows: *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), tetrahydrofuran (THF), and diethyl ether (ether) were degassed and then passed through anhydrous neutral alumina A-2 before use, according to the procedure described by Grubbs.[20] Methanol was dried over activated 3Å molecular sieves prior to use. Triethylamine was distilled over calcium hydride and stored over activated 3Å molecular sieves prior to use. Diisopropylethylamine (DIPEA) was distilled over calcium hydride prior to use. Trifluoroacetic anhydride (TFAA) and trimethylsilyl triflate (TMS-OTf) were distilled prior to use. Reported reaction temperatures refer to the temperature of the heating medium. Reactions were performed in flame- or oven-dried glassware under an atmosphere of dry argon using standard Schlenk techniques unless otherwise noted. Room temperature (rt) refers to 25 ± 3 ºC. Reactions were monitored by thin-layer chromatography (TLC) using EMD Chemicals Inc. silica gel 60 F_{256} plates. Flash chromatography was performed using Ultra Pure SiliaFlash P60, 230-400 mesh (40-63 μm) silica gel (SiO_2) following the general procedure by Still and co-workers.[21]

V. Instrumentation

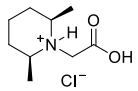
Proton NMR spectra measurements were acquired using either a Bruker DRX500 with a cryoprobe, Bruker GN500, or a Bruker AVANCE600 spectrometer, at 500 MHz, 500 MHz, and 600 MHz, respectively. Carbon NMR spectra were obtained on a Bruker DRX500 with a cryoprobe at 125 MHz. Proton NMR chemical shifts (δ) are reported in parts per million (ppm) and referenced to the residual solvent peak at 7.26 ppm for deuterated chloroform ($CDCl_3$) and 2.50 for deuterated dimethylsulfoxide (DMSO-d_6).[22] Carbon NMR chemical shifts (δ) are reported in parts per million (ppm) and referenced to the residual solvent peak at 77.16 ppm for deuterated chloroform and 39.52 for deuterated dimethylsulfoxide.[22] All NMR spectra were processed using MestReNova (Mestrelab Research). NMR data are reported in the following manner: chemical shift, multiplicity, (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, br = broad, app = apparent), coupling constants (J) in hertz (Hz), and integration. High resolution mass spectrometry (HRMS) accurate mass experiments were ran by the University of California, Irvine mass spectrometry laboratory. Infrared (IR) spectroscopy data were acquired on a Shimadzu IRAffinity-1 Spectrophotometer with a MIRacle 10 single reflection ATR accessory. Melting points (mp) were acquired on a Mel-Temp melting point apparatus and are uncorrected. Tandem mass spectroscopy (MS/MS) analysis was performed on a Waters Quattro Premier XE mass spectrometer by Dr. John Greaves.

VI. Detailed Experimental Procedures for Compounds in Chapter 4

General Procedure 4.1: Purification of compounds via reverse-phase chromatography using an automated column chromatography instrument.

Automated column chromatography (ISCO purification) was performed on a Teledyne Isco CombiFlash® Rf+ instrument. The following parameters were the same for all purifications. Initial waste: 0.0 column volumes (CV). Air purge: 0.0 minutes. Peak tube volume: max. Non-peak tube volume: max. Loading type: solid. Wavelength range for detection: 200-400 nm, threshold 0.10 AU. All other parameters are detailed in each compound's experimental procedure.

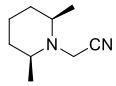
(2*S*,6*R*)-1-(Carboxymethyl)-2,6-dimethylpiperidin-1-ium chloride (4-2)



To hydrochloric acid (12 M, 10.0 mL, 0.120 mmol) was added **4-3** (0.195 g, 1.28 mmol), resulting in a light yellow solution. The reaction mixture was heated at reflux for 14 h, after which the reaction was let cool to 40 °C. The volume of the reaction was reduced to 1 mL by gentle heating, after which yellow crystals had precipitated. The mixture was let cool to rt, after which acetone (10 mL) was added, precipitating slightly more crystals. The crystals were collected via vacuum filtration and thoroughly washed with acetone (5 × 10 mL). Drying in vacuo afforded **4-2** as white crystals as a mixture of two stereoisomers in a 2:1 ratio by NMR (* denotes major stereoisomer) (0.235 g, 88%): mp 136–137 ºC; ^{1}H NMR (500 MHz, D_2O) δ 4.10 (s, 0.62H), *3.82 (s, 1.32H), *3.60–3.51 (m, 1.33H), 3.49–3.41 (m, 0.63H), 1.98–1.92 (m, 0.62H), *1.85–1.43 (m, 5H), 1.34 (d, *J* = 6.4 Hz, 1.52H), *1.22 (d, *J* = 6.7 Hz, 3.60H); ^{13}C NMR

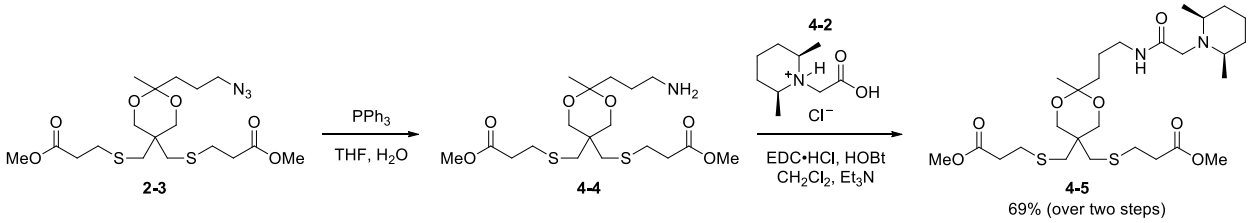
(125 MHz, D_2O) δ *170.8, 169.8, 63.1, *61.5, 52.9, *42.2, 31.5, *25.3, *22.0, 21.6, 17.7, *16.4; IR (thin film): 3227, 2939, 1978, 1693, 1419, 1322, 1270 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_9H_{18}NO_2$ $[M + H]^+$ 172.1338, found 172.1342.

2-((2*S*,6*R*)-2,6-Dimethylpiperidin-1-yl)acetonitrile (4-3)



To (2*S*,6*R*)-2,6-dimethylpiperidine (5.4 mL, 40 mmol) and sodium carbonate (8.5 g, 79 mmol) was added 2-chloroacetonitrile (3.0 mL, 47 mmol), resulting in a light cloudy solution. The reaction mixture was vigorously stirred and heated at 85 °C for 20 h, after which it had turned brown. The reaction was let cool, after which ethyl acetate (200 mL) was added. The organic layer was washed with water (4 × 25 mL), removing the sodium carbonate and most of the brown color, and then washed with brine (25 mL), dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo to a dark oil. The crude reaction mixture was chromatographed (1:1 ethyl acetate:heaxanes) to afford **4-3** as a light yellow oil (5.5 g, 90%): ^1H NMR (500 MHz, $CDCl_3$) δ 3.78 (s, 2H), 2.53–2.37 (m, 2H), 1.74–1.59 (m, 3H), 1.48–1.23 (m, 3H), 1.12 (d, *J* = 6.3 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 114.5, 55.7, 37.8, 35.1, 24.3, 21.0; IR (thin film): 2930, 2598, 2229, 1455, 1429, 1320, 1757, 1095 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_9H_{16}N_2Na$ $[M + Na]^+$ 175.1211, found 175.1217.

Dimethyl 3,3'-(((2-(3-(2-((2*R*,6*S*)-2,6-dimethylpiperidin-1-yl)acetamido)propyl)-2-methyl-1,3-dioxane-5,5-diyl)bis(methylene))bis(sulfanediyl))dipropanoate (4-5)

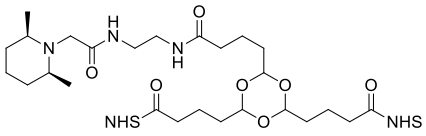


To a stirred solution of **2-3** (0.401 g, 0.892 mmol) in THF (12 mL) and deionized water (1.6 mL) was added triphenylphosphine (0.234 g, 0.892 mmol). The resulting orange solution was let stir open to air for 36 h, after which it was transferred to a 200 mL round bottom flask with additional THF (100 mL). A large stir bar was added to the flask along with enough anhydrous magnesium sulfate to fill the flask up approximately half-way, but still allowing for vigorous stirring. The flask was lightly capped and let stir vigorously for 10 min, after which the solution was gravity filtered and concentrated in vacuo to an orange oil. The crude product was used without any further purification.

To a stirred solution of the crude product of the Staudinger reduction and the triphenylphosphine oxide (0.640 g total crude mass) in DCM (10 mL) was added **4-2** (0.154 g, 0.741 mmol) and triethylamine (0.200 mL, 1.43 mmol). After cooling to 0 °C, EDC•HCl (0.374 g, 1.95 mmol) and HOBt (0.125 g, 0.925 mmol) were added. The resulting cloudy solution was let gradually warm to rt and stir for a total of 14 h, after which it was chromatographed immediately (1:9:90 triethylamine:methanol:DCM, 30 cm column height), yielding a dark orange oil (0.760 crude mass). This crude product was purified by general procedure 4.1. A 50 g RediSep C18 reverse-phase column was used. The flow rate was 40 mL/min. The equilibration volume was 5.0 CV. The solvent system was 10% acetonitrile in water with 0.1% TFA over 2 CV, followed by a

linear gradient of 10–70% acetonitrile in water with 0.1% TFA over 23 CV. Column fractions from 13 CV to 17 CV were combined in a separatory funnel. Saturated aqueous sodium bicarbonate (25 mL) was added, and the mixture was extracted with DCM (4 × 75 mL). The combined organic layers were washed with brine (50 mL), dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo to afford **4-5** as a pale yellow oil as a mix of diastereomers (0.296 g, 69% over two steps): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (s, 1H), 3.77–3.63 (m, 10H), 3.29–3.20 (m, 2H), 3.04 (s, 2H), 2.85–2.72 (m, 6H), 2.64–2.55 (m, 6H), 2.50–2.37 (m, 2H), 1.81–1.72 (m, 1H), 1.72–1.49 (m, 6H), 1.37–1.29 (m, 3H), 1.27–1.13 (m, 3H), 1.03–0.93 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8, 173.5, 172.6, 172.32, 172.31, 99.5, 66.0, 65.6, 58.9, 51.96, 51.93, 51.91, 44.9, 40.9, 39.0, 38.3, 38.1, 35.79, 35.76, 35.67, 34.9, 34.8, 28.97, 28.94, 28.7, 24.3, 24.0, 23.9, 21.6, 20.2; IR (thin film): 3340, 2918, 1733, 1656, 1524, 1358, 1245, 1196 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{27}H_{49}N_2O_7S_2$ $[M + H]^+$ 577.2981, found 577.2982.

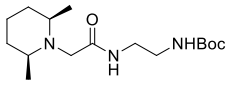
Bis(2,5-dioxopyrrolidin-1-yl) 4,4'-(6-(4-((2-(2-((2*R*,6*S*)-2,6-dimethylpiperidin-1-yl)acetamido)ethyl)amino)-4-oxobutyl)-1,3,5-trioxane-2,4-diyl)dibutanoate (4-12)



A solution of **4-14** (0.150 g, 0.703 mmol) in DCM (15 mL) was added via syringe pump at a rate of 0.17 mL/min to a stirred solution of **3-17** (0.450 g, 0.704 mmol) in DCM (200 mL). The resulting light yellow solution was let stir for 14 h, after which it was concentrated in vacuo to an orange oil. The crude product was purified by general procedure 4.1. A 50 g RediSep C18 reverse-phase column was used. The flow rate was 40 mL/min. The equilibration volume was

5.0 CV. The solvent system was a linear gradient of 10–20% acetonitrile in water with 0.1% TFA over 2 CV, followed by 20% acetonitrile in water with 0.1% TFA over 5 CV, followed by a linear gradient of 20–70% acetonitrile in water with 0.1% TFA over 30 CV. Column fractions from 11 CV to 17 CV were combined in a separatory funnel. Saturated aqueous sodium bicarbonate (25 mL) was added, and the mixture was extracted with DCM (4 × 75 mL). The combined organic layers were washed with brine (50 mL), dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo to afford **4-12** as a sticky pale yellow foam (0.138 g, 27%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.84 (s, 2H), 6.41 (s, 1H), 4.91 (t, *J* = 4.8 Hz, 2H), 4.87 (t, *J* = 4.7 Hz, 1H), 3.44–3.29 (m, 4H), 3.04 (s, 2H), 2.83 (s, 8H), 2.66 (t, *J* = 7.3 Hz, 4H), 2.43 (br s, 2H), 2.19 (t, *J* = 7.2 Hz, 2H), 1.93–1.82 (m, 4H), 1.81–1.62 (m, 8H), 1.58–1.52 (m, 2H), 1.38–1.11 (m, 4H), 0.97 (d, *J* = 6.3 Hz, 6H); (Note: for the ^{13}C of compounds **4-12**, **4-13**, and **4-14**, the ethylene unit in-between the two nitrogen atoms did not show in the spectra acquired); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.1, 173.3, 169.4, 168.5, 101.2, 100.6, 58.9, 40.5, 38.6, 36.2, 33.6, 33.0, 30.7, 29.8, 25.7, 24.3, 21.5, 19.8, 18.7; IR (thin film): 3389, 2927, 1779, 1701, 1650, 1550, 1217, 1075 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{34}H_{52}N_5O_{13}$ $[M + H]^+$ 738.3561, found 738.3545.

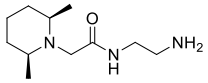
tert-Butyl (2-(2-((2*S*,6*R*)-2,6-dimethylpiperidin-1-yl)acetamido)ethyl)carbamate (4-13)



To a stirred solution of **4-2** (0.125 g, 0.602 mmol) in DCM (6 mL) was added *tert*-butyl (2-aminoethyl)carbamate (0.109 mg, 0.680 mmol) and triethylamine (0.120 mL, 0.861 mmol). The resulting light yellow solution was cooled to 0 °C, after which EDC•HCl (0.153 g, 0.798 mmol) and HOBt (0.101 g, 0.861 mmol) were added. The light yellow solution was let gradually warm to rt and stir for a total of two days. Then, DCM (15 mL) was added and the crude reaction

mixture was chromatographed immediately (1:14:85 triethylamine:methanol:DCM, 30 cm column height) to afford **4-13** as a yellow solid after drying in vacuo (0.129 g, 69%): mp 68–70 ºC; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (s, 1H), 5.01 (s, 1H), 3.34 (q, *J* = 6.1 Hz, 2H), 3.25–3.18 (m, 2H), 3.04 (s, 2H), 2.42 (br s, 2H), 1.67–1.57 (m, 1H), 1.56–1.48 (m, 2H), 1.38 (s, 9H), 1.32–1.24 (m, 1H), 1.24–1.12 (m, 2H), 0.96 (d, *J* = 6.5 Hz, 6H); (Note: for the ^{13}C of compounds **4-12**, **4-13**, and **4-14**, the ethylene unit in-between the two nitrogen atoms did not show in the spectra acquired); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.3, 156.2, 79.3, 58.8, 40.9, 38.9, 28.5, 24.2, 21.4; IR (thin film): 3371, 2967, 1690, 1655, 1518, 1246, 1166 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{16}H_{32}N_3O_3$ $[M + H]^+$ 314.2444, found 314.2447.

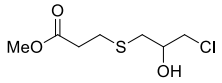
_N_-(2-Aminoethyl)-2-((2*S*,6*R*)-2,6-dimethylpiperidin-1-yl)acetamide (4-14)



To a stirred solution of **4-13** (2.02 g, 6.44 mmol) in DCM (80 mL) was added TFA (4.00 mL, 52.2 mmol). The resulting light yellow solution was stirred at rt for 2 days, after which mass spectrometry indicated complete consumption of **4-13**. Triethylamine (10 mL) was added slowly, dropwise, resulting in voluptuous clouds of white vapor. After the white clouds had subsided, aqueous sodium hydroxide (1 M, 30 mL) was added, and the mixture was extracted with DCM (3 × 100 mL). The combined organic layers were washed with water (3 × 100 mL), brine (2 × 75 mL), dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo to afford **4-14** as a pale yellow oil (0.998 g, 73%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (s, 1H), 3.27 (q, *J* = 6.2 Hz, 2H), 3.01 (s, 2H), 2.76 (t, *J* = 6.1 Hz, 2H), 2.39 (br s, 2H), 1.66–1.57 (m, 3H), 1.54–1.45 (m, 2H), 1.34–1.23 (m, 1H), 1.21–1.11 (m, 2H), 0.95 (d, *J* = 6.5 Hz, 6H); (Note: for the ^{13}C of compounds **4-12**, **4-13**, and **4-14**, the ethylene unit in-between the two

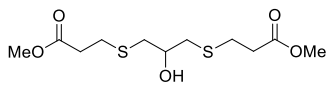
nitrogen atoms did not show in the spectra acquired); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.9, 58.8, 41.87, 41.84, 24.2, 21.5; IR (thin film): 3357, 3291, 2923, 1652, 1590, 1464, 1202 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{11}H_{24}N_3O$ $[M + H]^+$ 214.1919, found 214.1926.

Methyl 3-((3-chloro-2-hydroxypropyl)thio)propanoate (4-18)



To a stirred solution of methyl 3-mercaptopropanoate (0.500 mL, 4.49 mmol) and 2-(chloromethyl)oxirane (0.500 mL, 6.38 mmol) was added borax (0.176 mg, 0.461 mmol) and deionized water (0.5 mL). The resulting cloudy solution was let stir at rt overnight, after which it was transferred to a separatory funnel and diluted with DCM (75 mL). The organic layer was washed with water (30 mL), brine (30 mL), dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo to a colorless oil. The crude reaction mixture was chromatographed (1:3 ethyl acetate:heaxanes) to afford **4-18** as a colorless oil (0.716 g, 75%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.95 (dquint, *J* = 7.1, 5.0 Hz, 1H), 3.70 (s, 3H), 3.68–3.60 (m, 2H), 2.88–2.78 (m, 4H), 2.73–2.67 (m, 1H), 2.63 (t, *J* = 7.1 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.4, 70.2, 52.1, 48.0, 36.5, 34.7, 27.6; IR (thin film): 3443, 2952, 1726, 1435, 1358, 1247, 1043 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_7H_{13}ClO_3SNa$ $[M + Na]^+$ 235.0172, found 235.0184.

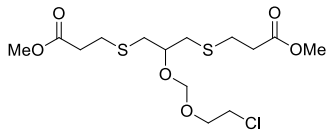
Dimethyl 3,3'-((2-hydroxypropane-1,3-diyl)bis(sulfanediyl))dipropanoate (4-19)



To a stirred solution of **4-18** (0.201 g, 0.944 mmol) in DMF (4.6 mL) was added methyl 3-mercaptopropanoate (0.210 mL, 1.89 mmol) and potassium carbonate (0.149 g, 1.08 mmol). The

resulting chunky white solution was let stir 36 h, after which it had become milky white and homogeneous. The reaction mixture was diluted in DCM (60 mL), and washed thoroughly with a dilute brine solution (9:1 water:saturated aqueous sodium chloride, 10 × 10 mL) to remove the DMF. The organic layer was dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo to a yellow oil. The crude reaction mixture was chromatographed (1:2 ethyl acetate:heaxanes) to afford **4-19** as a colorless oil (0.240 g, 86%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.84 (dquint, *J* = 7.5, 4.5 Hz, 1H), 3.70 (s, 6H), 2.84 (t, *J* = 7.3 Hz, 4H), 2.79–2.74 (m, 2H), 2.67–2.59 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.4, 69.4, 52.0, 38.4, 34.7, 27.6; IR (thin film): 2951, 1731, 1435, 1354, 1240 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{11}H_{20}O_5S_2Na$ [M + Na]$^{+}$ 319.0650, found 319.0649.

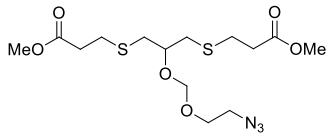
Dimethyl 3,3'-((2-((2-chloroethoxy)methoxy)propane-1,3-diyl)bis(sulfanediyl))dipropanoate (4-21)



To a stirred solution of **4-19** (0.0960 g, 0.324 mmol) in DCM (0.30 mL) was added 1-chloro-2-(chloromethoxy)ethane (0.185 mL, 1.84 mmol) and DIPEA (0.120 mL, 0.689 mmol). The resulting colorless solution was let stir at rt overnight, after which saturated aqueous sodium bicarbonate (15 mL) was added. The aqueous layer was extracted with DCM (3 × 25 mL), and the combined organic layers were dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo to a yellow oil. The crude reaction mixture was chromatographed (step gradient from 1:2 ethyl acetate:heaxanes to 2:3 ethyl acetate:heaxanes) to afford **4-21** as a colorless oil (0.099 g, 79%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.77 (s, 2H), 3.91–3.80 (m, 3H),

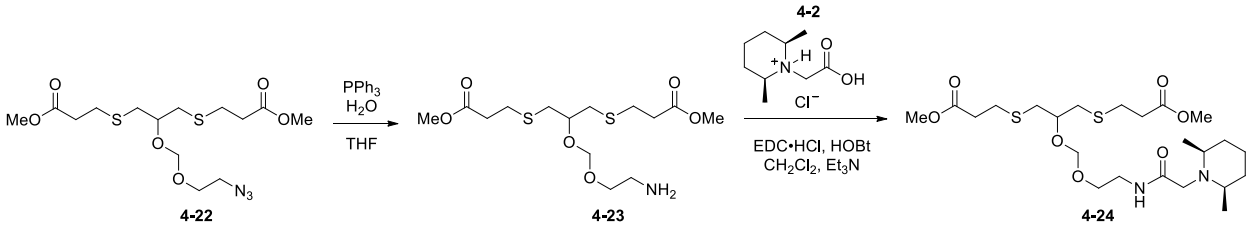
3.69–3.60 (m, 8H), 2.87–2.69 (m, 8H), 2.58 (t, J = 7.4 Hz, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.2, 94.8, 76.6, 68.4, 51.9, 43.2, 35.7, 34.7, 27.9; IR (thin film): 2951, 1731, 1495, 1357, 1245, 1026 cm^{-1}; HRMS (ESI) m / z calcd for $C_{14}H_{25}ClO_6S_2Na$ $[M + Na]^+$ 411.0679, found 411.0696.

Dimethyl 3,3'-((2-((2-azidoethoxy)methoxy)propane-1,3-diyl)bis(sulfanediyl))dipropanoate (4-22)



To a stirred solution of **4-21** (8.30 g, 21.3 mmol) in DMF (100 mL) was added sodium azide (4.20 g, 64.6 mmol). The resulting cloudy solution was let stir at 40 0 °C for 48 h, after which it was let cool. Saturated aqueous sodium bicarbonate (50 mL) and deionized water (150 mL) were added, and the aqueous layer was extracted with DCM (6 × 50 mL). The combined organic layers were washed thoroughly with a dilute brine solution (9:1 water:saturated aqueous sodium chloride, 15 × 50 mL) to remove the DMF. The organic layer was dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo to a yellow oil. The crude reaction mixture was chromatographed (2:3 ethyl acetate:heaxanes) to afford **4-22** as a yellow oil (8.10 g, 96%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.77 (s, 2H), 3.86 (quintet, J = 5.6 Hz, 1H), 3.79–3.72 (m, 2H), 3.66 (s, 6H), 3.47–3.34 (m, 2H), 2.88–2.69 (m, 8H), 2.59 (t, J = 7.4 Hz, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.3, 94.7, 76.6, 67.0, 51.9, 50.9, 35.7, 34.7, 27.9; IR (thin film): 2933, 2096, 1732, 1435, 1245, 1164, 1035 cm^{-1}; HRMS (ESI) m / z calcd for $C_{14}H_{25}N_3O_6S_2Na$ $[M + Na]^+$ 418.1082, found 418.1083.

Methyl 1-((2*R*,6*S*)-2,6-dimethylpiperidin-1-yl)-9-(((3-methoxy-3-oxopropyl)thio)methyl)-2-oxo-6,8-dioxa-11-thia-3-azatetradecan-14-oate (4-24)



To a stirred solution of **4-22** (0.303 g, 0.766 mmol) in THF (4 mL) and deionized water (0.35 mL) was added triphenylphosphine (0.212 g, 0.807 mmol). The resulting orange solution was let stir open to air for 36 h, after which it was transferred to a 200 mL round bottom flask with additional THF (100 mL). A large stir bar was added to the flask along with enough anhydrous magnesium sulfate to fill the flask up approximately half-way, but still allowing for vigorous stirring. The flask was lightly capped and let stir vigorously for 10 min, after which the solution was gravity filtered and concentrated in vacuo to crude **4-23** as an orange oil. The crude product was used without any further purification.

To a stirred solution of the crude product of the Staudinger reduction and triphenylphosphine oxide (0.517 g total crude mass) in DCM (7.5 mL) was added **4-2** (0.275 g, 0.1.33 mmol) and triethylamine (0.240 mL, 1.72 mmol). After cooling to 0 °C, EDC•HCl (0.191 g, 0.998 mmol) and HOBt (0.130 g, 0.965 mmol) were added. The resulting cloudy solution was let gradually warm to rt and stir for a total of 14 h, after which it was chromatographed immediately (1:9:90 triethylamine:methanol:DCM, 30 cm column height), yielding a dark orange oil (0.602 crude mass). This crude product was purified by general procedure 4.1. A 50 g RediSep C18 reverse-phase column was used. The flow rate was 40 mL/min. The equilibration volume was 5.0 CV. The solvent system was 10% acetonitrile in water with 0.1% TFA over 2 CV, followed by a

linear gradient of 10–20% acetonitrile in water with 0.1% TFA over 21 CV, followed by a linear gradient of 20–40% acetonitrile in water with 0.1% TFA over 10 CV, followed by a linear gradient of 40–60% acetonitrile in water with 0.1% TFA over 2 CV, followed by a linear gradient of 60–100% acetonitrile in water with 0.1% TFA over 6 CV. Column fractions from 25 CV to 35 CV were combined in a separatory funnel. Saturated aqueous sodium bicarbonate (25 mL) was added, and the mixture was extracted with DCM (5 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over anhydrous sodium sulfate, gravity filtered, and concentrated in vacuo to afford **4-24** as a pale yellow oil (0.262 g, 66% over two steps): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80 (s, 1H), 4.74 (s, 2H), 3.9 –3.79 (m, 1H), 3.67 (s, 6H), 3.63 (t, *J* = 5.3 Hz, 2H), 3.48–3.42 (m, 2H), 3.04 (s, 2H), 2.90–2.69 (m, 8H), 2.60 (t, *J* = 7.3 Hz, 4H), 2.43 (s, 2H), 1.68–1.60 (m, 1H), 1.58–1.47 (m, 2H), 1.35–1.26 (m, 1H), 1.24–1.11 (m, 2H), 1.02–0.92 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.6, 172.3, 94.9, 76.6, 67.6, 61.5, 58.8, 51.9, 38.7, 35.8, 34.74, 28.0, 25.1, 24.3, 21.5, 14.4; IR (thin film): 3351, 2926, 1733, 1669, 1517, 1434, 1244, 1029 cm^{-1}; HRMS (ESI) *m* / *z* calcd for $C_{23}H_{42}N_2O_7S_2Na$ $[M + Na]^+$ 545.2331, found 545.2325

VII. References

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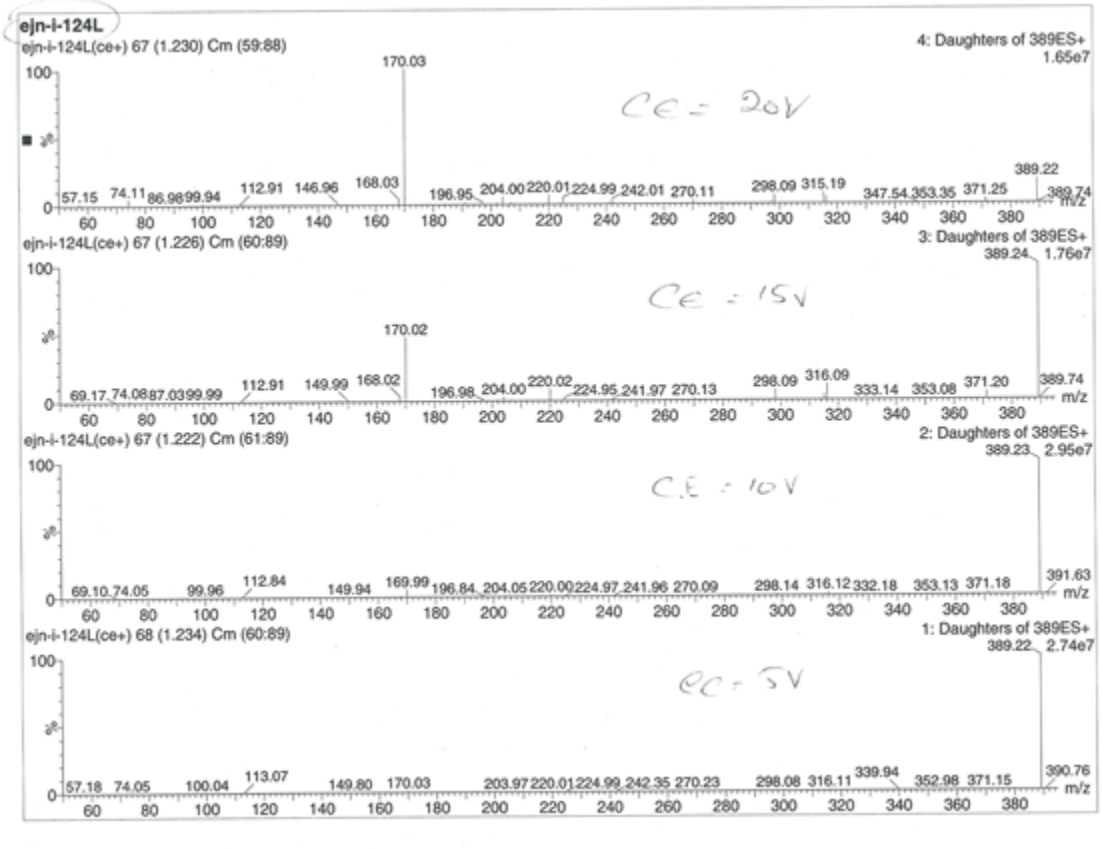
Appendix

Tandem Mass Spectrometry (MS/MS) Data

To probe the approximate voltage required for cleavage of CID-XLs, the CID-XL was treated with *n*-butylamine to afford the diamide model system which was used to simulate the reaction between an NHS ester and a lysine in a protein. Then, the diamine was subjected to MS/MS analysis at stepwise, increasing voltages, and fragments from CID were observed. Exact masses calculated by ChemDraw Ultra 12.0. Theoretical masses for fragments are off by ~0.17 m/z across all measurements for all fragments.



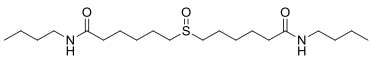
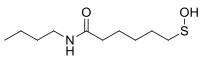
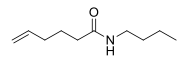
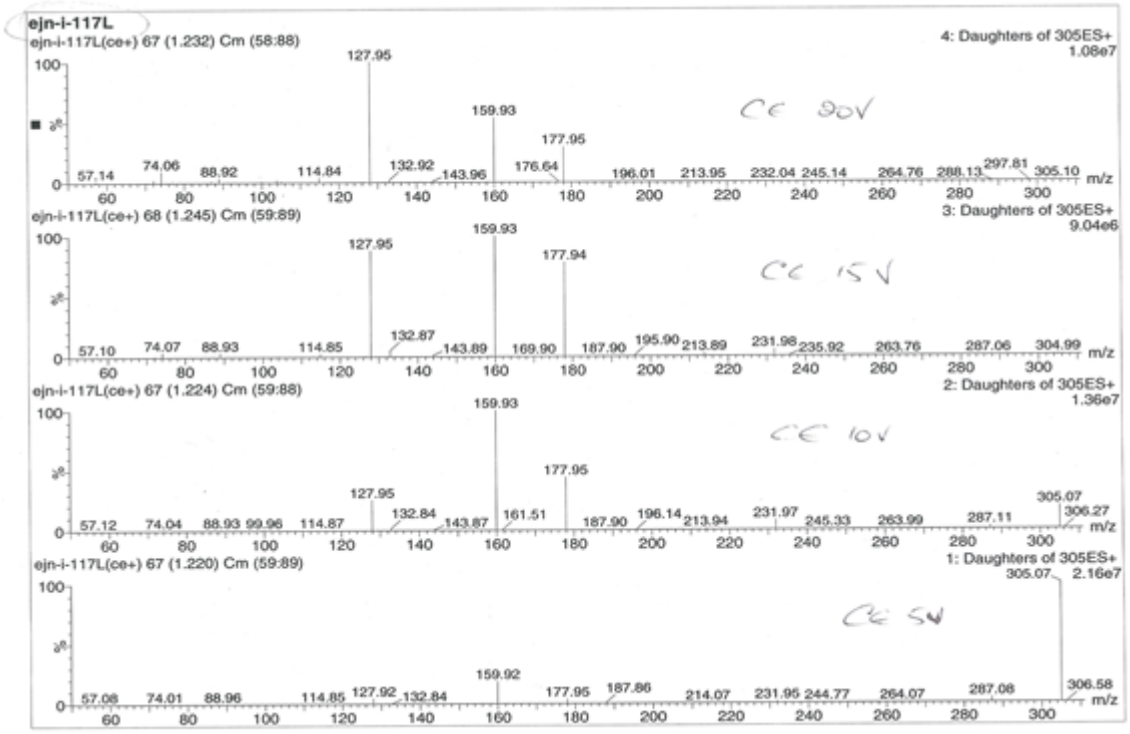
	Expected and Observed Fragments		
Collision Energy (V)	 Exact Mass: 388.2760	 Exact Mass: 219.1293	 Exact Mass: 169.1467
5	✓		
10	✓		✓
15	✓	✓	✓
20	✓	✓	✓

Figure A.1. MS2 data for **2-21.** (From the bottom spectrum) Collision energy 5, 10, 15, 20 V.



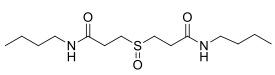
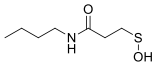
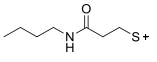
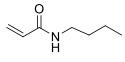
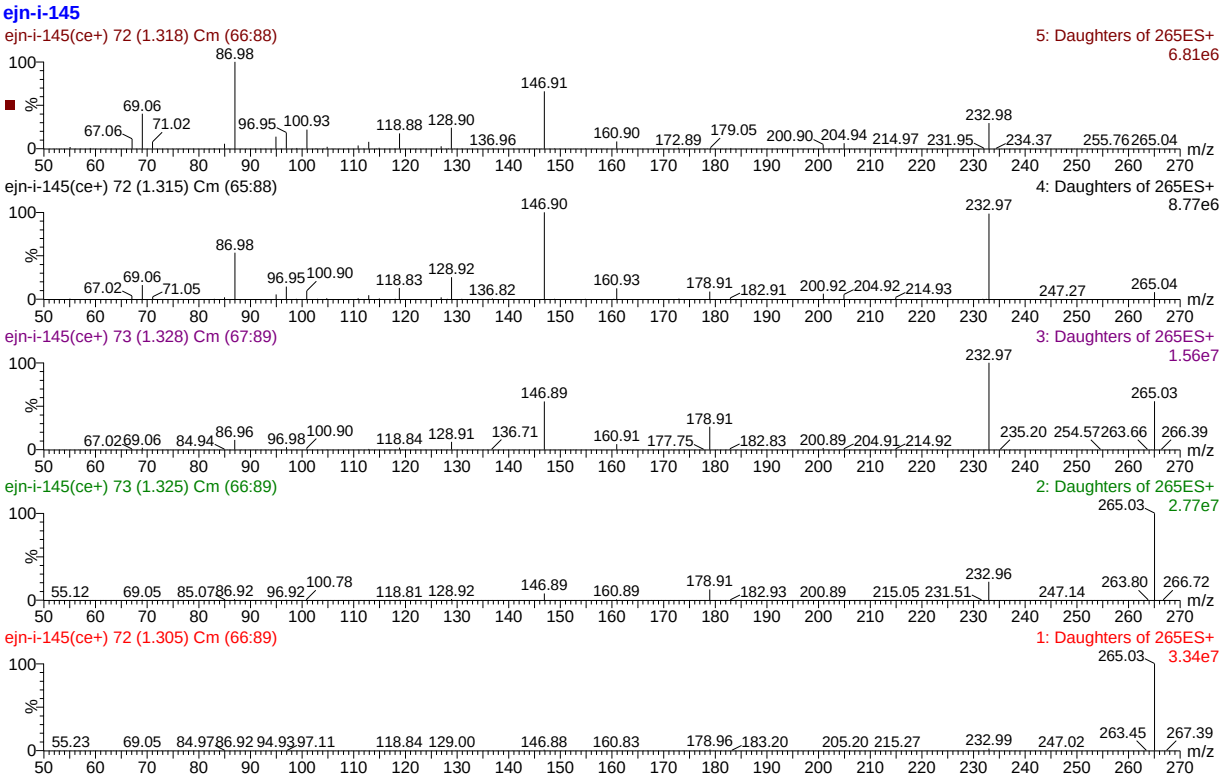
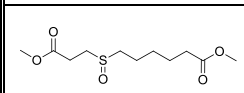
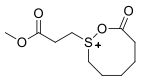
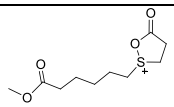
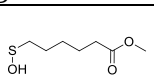
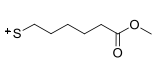
	Expected and Observed Fragments			
Collision Energy (V)	 Exact Mass: 304.1821	 Exact Mass: 177.0823	 Exact Mass: 160.0791	 Exact Mass: 127.0997
5	✓		✓	
10	✓	✓	✓	✓
15		✓	✓	✓
20		✓	✓	✓

Figure A.2. MS2 data for **2-22**. (From the bottom spectrum) Collision energy 5, 10, 15, 20 V.



Collision Energy (V)	Expected and Observed Fragments			
	 Exact Mass: 264.1031	 Exact Mass: 233.0842 or  Exact Mass: 233.0842	 Exact Mass: 178.0664	 Exact Mass: 161.0631
5	✓			
10	✓	✓	✓	
15	✓	✓	✓	✓
20	✓	✓	✓	✓
25		✓		✓

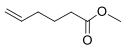
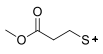
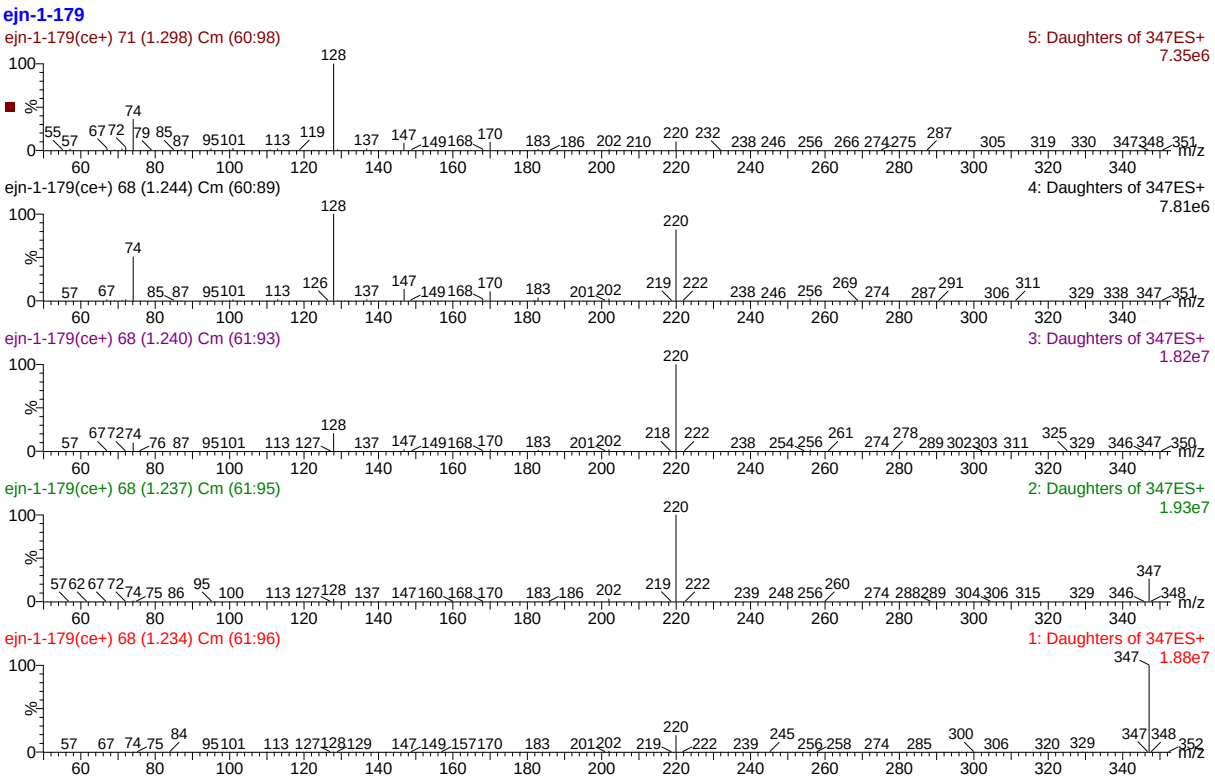
Collision Energy (V)	Expected and Observed Fragments			
	 Exact Mass: 147.0474	 Exact Mass: 128.0837	 Exact Mass: 119.0161	 Exact Mass: 86.0368
5				
10				
15	✓	✓		✓
20	✓	✓	✓	✓
25	✓	✓	✓	✓

Figure A.3. MS2 data for **2-25.** (From the bottom spectrum) Collision energy 5, 10, 15, 20, 25 V.



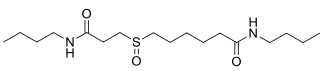
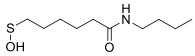
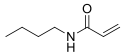
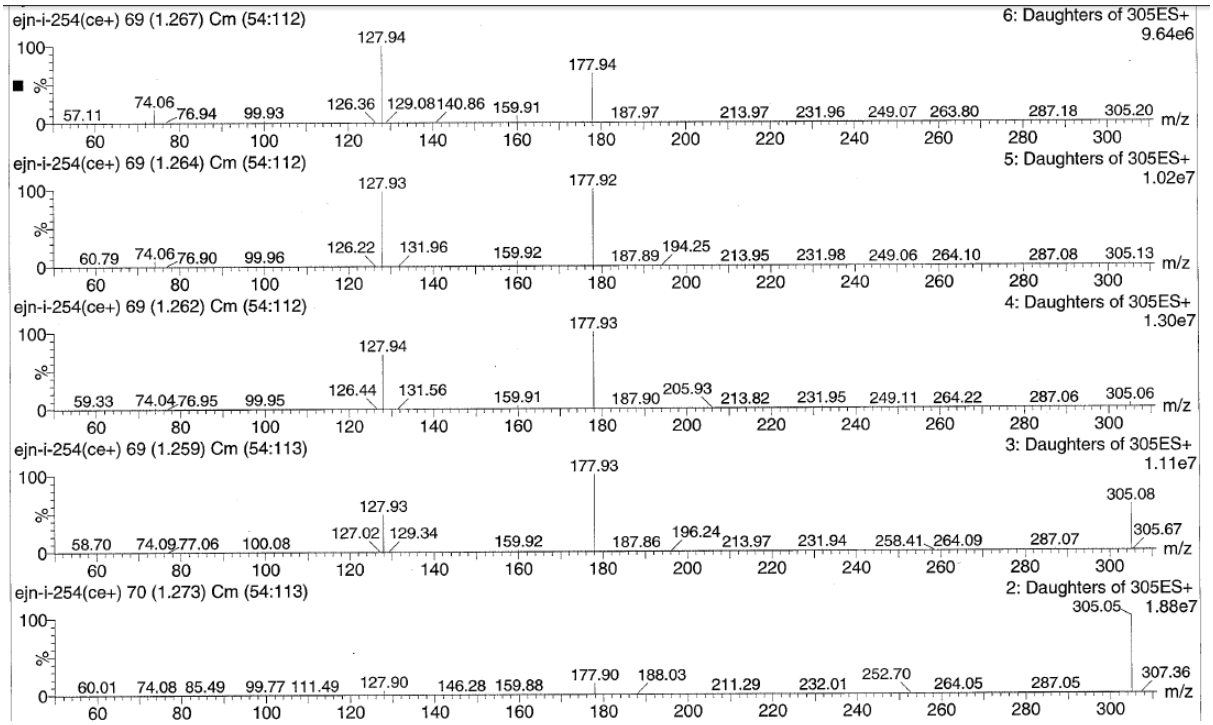
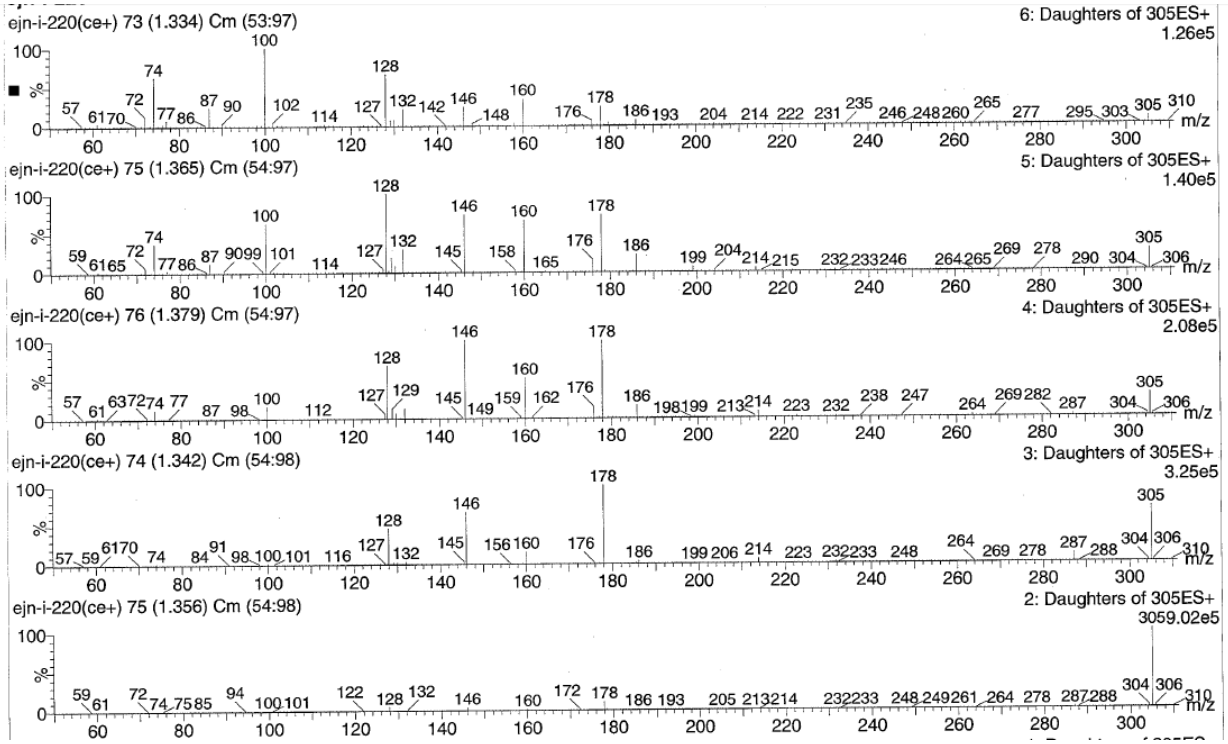
Collision Energy (V)	Expected and Observed Fragments		
	 Exact Mass: 346.2290	 Exact Mass: 219.1293	 Exact Mass: 127.0997
5	✓	✓	
10	✓	✓	
15		✓	✓
20		✓	✓
25		✓	✓

Figure A.4. MS2 data for **2-34.** (From the bottom spectrum) Collision Energy 5, 10, 15, 20, 25 V.



	Expected and Observed Fragments			
Collision Energy (V)	Exact Mass: 304.1821	Exact Mass: 177.0823 or Exact Mass: 177.0823	Exact Mass: 127.0997	Exact Mass: 73.0891
5	✓			
10	✓	✓	✓	
15		✓	✓	
20		✓	✓	
25		✓	✓	✓

Figure A.5. MS2 data for **2-41.** (From the bottom spectrum) Collision energy 5, 10, 15, 20, 25 V.



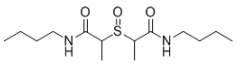
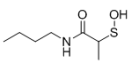
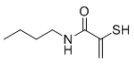
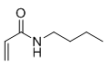
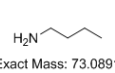
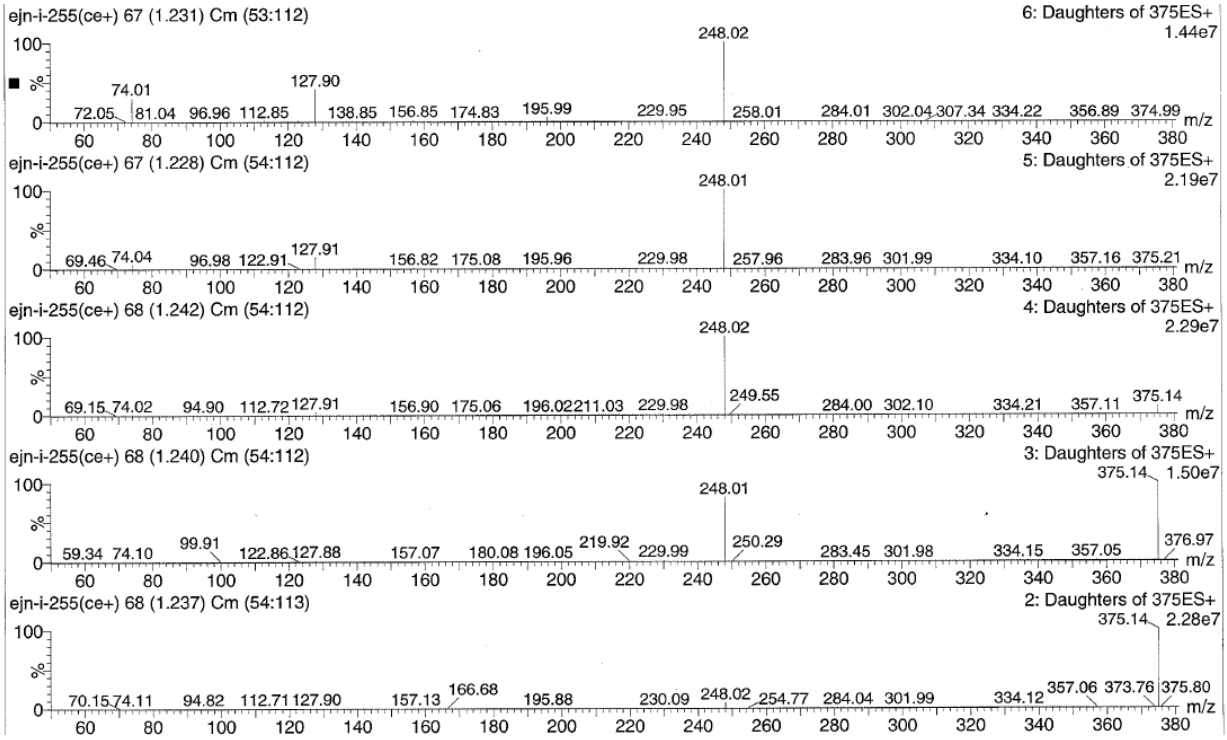
	Expected and Observed Fragments				
Collision Energy (V)	 Exact Mass: 304.1821	 Exact Mass: 177.0823	 Exact Mass: 159.0718	 Exact Mass: 127.0997	 Exact Mass: 73.0891
5	✓				
10	✓	✓	✓	✓	
15	✓	✓	✓	✓	
20	✓	✓	✓	✓	✓
25		✓	✓	✓	✓

Figure A.6. MS2 data for **2-45.** (From the bottom spectrum) Collision energy 5, 10, 15, 20, 25 V.



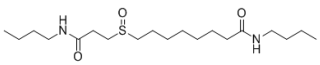
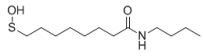
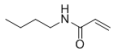
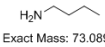
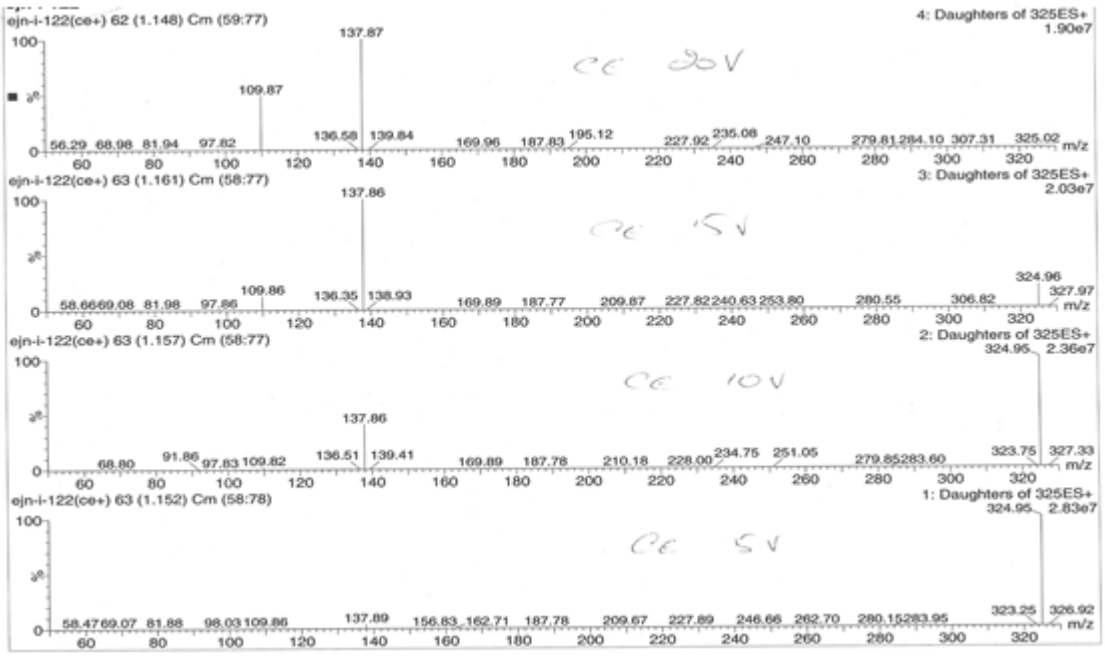
	Expected and Observed Fragments			
Collision Energy (V)	 Exact Mass: 374.2603	 Exact Mass: 247.1606	 Exact Mass: 127.0997	 Exact Mass: 73.0891
5	✓			
10	✓	✓		
15		✓		
20		✓	✓	
25		✓	✓	✓

Figure A.7. MS2 data for **2-49.** (From the bottom spectrum) Collision energy 5, 10, 15, 20, 25 V.



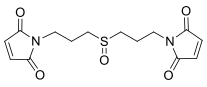
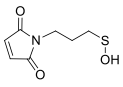
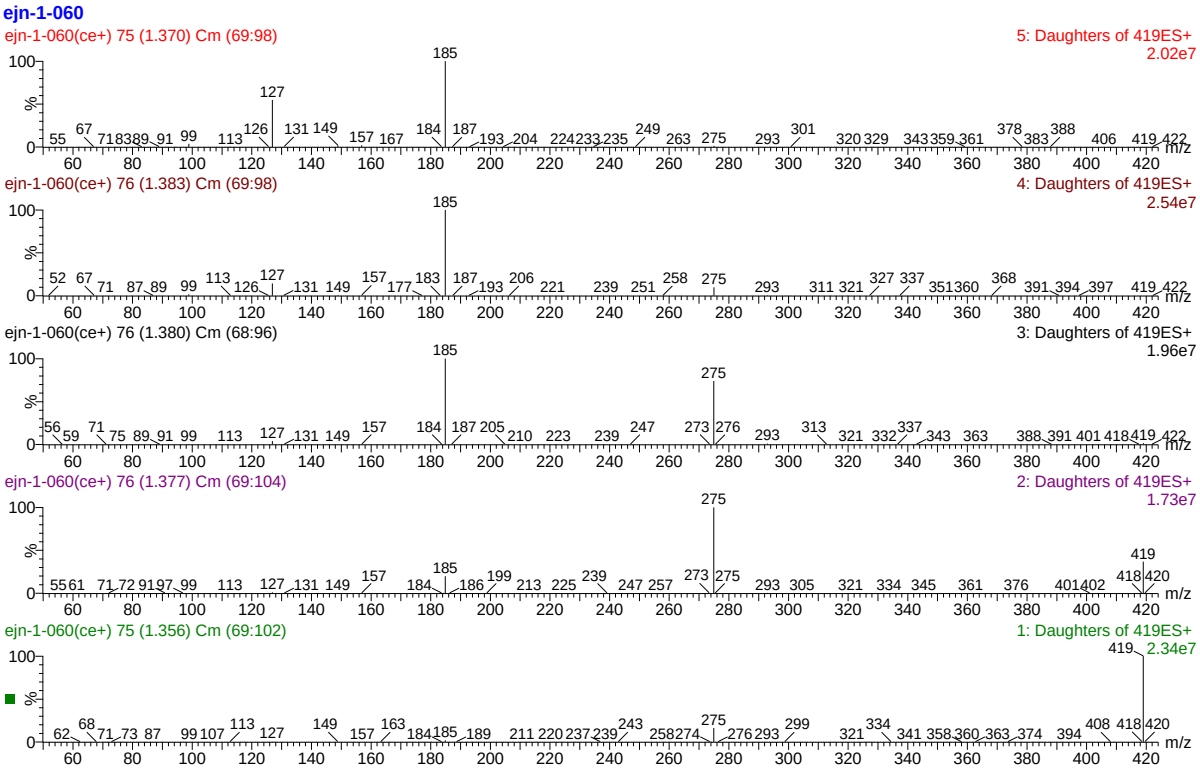
	Expected and Observed Fragments			
Collision Energy (V)	 Exact Mass: 324.08	 Exact Mass: 187.03	 Exact Mass: 137.05	 Exact Mass: 110.0231
5	✓			
10	✓		✓	
15	✓		✓	✓
20			✓	✓

Figure A-8. MS2 data for **2-75.** (From the bottom spectrum) Collision Energy 5, 10, 15, 20 V.



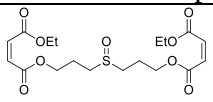
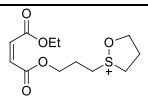
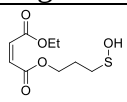
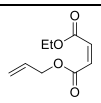
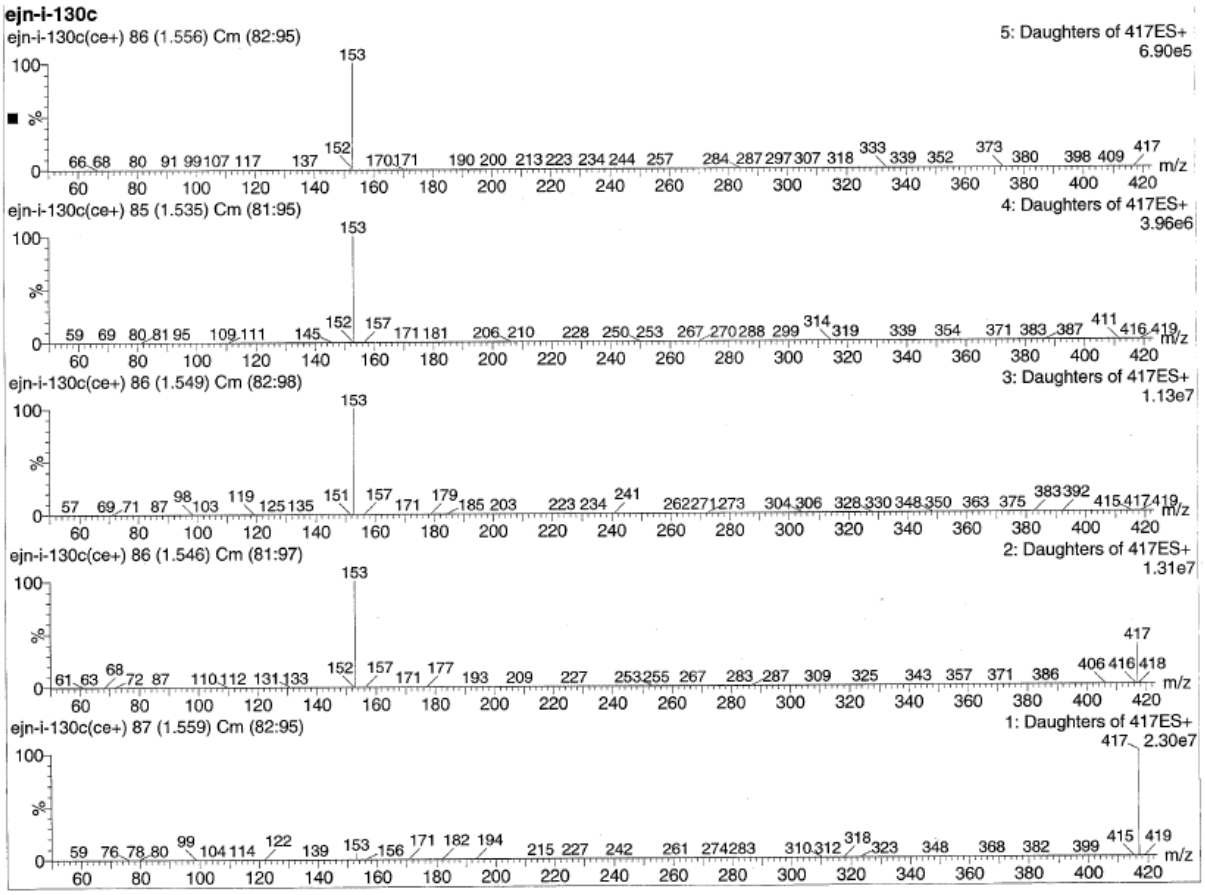
Collision Energy (V)	Expected and Observed Fragments			
	 Exact Mass: 418.1298	 Exact Mass: 275.0948	 Exact Mass: 234.0562	 Exact Mass: 184.0736
5	✓			
10	✓	✓		✓
15		✓		✓
20				✓
25				✓

Figure A-9. MS2 data for **2-79.** (From the bottom spectrum) Collision Energy 5, 10, 15, 20, 25 V.



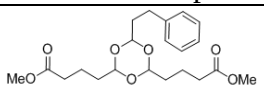
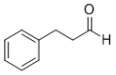
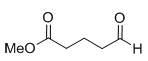
	Expected and Observed Fragments		
Collision Energy (V)	 Exact Mass: 394.1992	 Exact Mass: 134.0732	 Exact Mass: 130.0630
10	✓		
20	✓		✓
30			✓
40			✓
50			✓

Figure A.10. MS2 data for **3-5.** (From the bottom spectrum) Collision energy 10, 20, 30, 40, 50 V.