University of Richmond
UR Scholarship Repository

Honors Theses | Student Research

2024

Exploration of Sulfonamides and Benzothiazoles as Peptide-Based Cleavable Linkers

Abigail Dalton

Follow this and additional works at: https://scholarship.richmond.edu/honors-theses

 Part of the Chemistry Commons

Recommended Citation

Dalton, Abigail, "Exploration of Sulfonamides and Benzothiazoles as Peptide-Based Cleavable Linkers" (2024). *Honors Theses*. 1746.
https://scholarship.richmond.edu/honors-theses/1746

This Thesis is brought to you for free and open access by the Student Research at UR Scholarship Repository. It has been accepted for inclusion in Honors Theses by an authorized administrator of UR Scholarship Repository. For more information, please contact scholarshiprepository@richmond.edu.

Exploration of Sulfonamides and Benzothiazoles as Peptide-Based Cleavable Linkers

by

Abigail Dalton

Honors Thesis

Submitted to:

Chemistry Department

University of Richmond

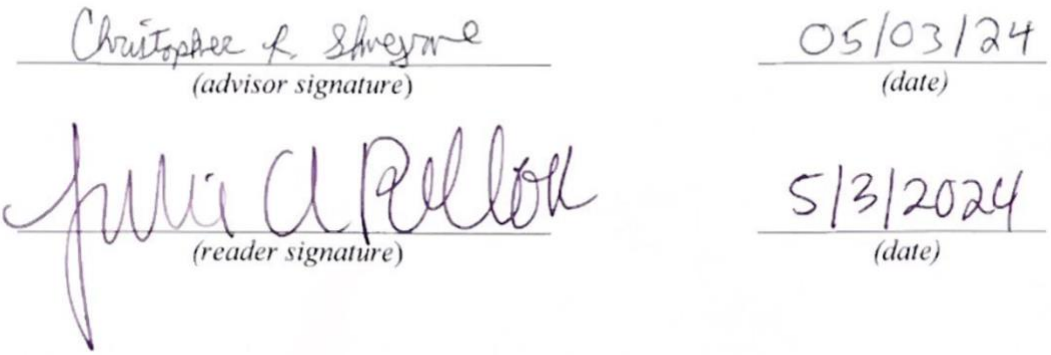
Richmond, VA

April 18, 2024

Advisor: Dr. Christopher Shugrue

This thesis has been accepted as part of the honors

requirements in the Chemistry Department.



Christopher L. Shreyne — *(advisor signature)* — 05/03/24 — *(date)*

Julia A. Pollock — *(reader signature)* — 5/3/2024 — *(date)*

Table of Contents.

Acknowledgements

I would like to thank my research advisor, Dr. Christopher Shugrue for his mentorship and guidance in lecture and in lab. Without his leadership and passion for his students' futures, I would not be as secure in my career and life aspirations as chemist after undergraduate. I would also like to thank my other lab members in the Shugrue Lab, especially Ashly Avila and John Blobe for their contributions to the projects, occasionally working alongside each other conducting the repeated reactions. I gratefully write this thesis utilizing the tools I have learned from many of the respected faculty members, staff, and students of the chemistry department.

Abstract

Cleavable linkers have demonstrated great potential in various applications of medicinal organic chemistry, such as in modern therapeutic development. Linkers are stable compounds that cleave in specific conditions to release molecular cargo. We have developed cleavable linkers based on nucleophilic aromatic substitution reactions on sulfonamide and benzothiazole substrates in small molecule and in peptide studies. Sulfonamides, commonly with an electron-withdrawing group, reacted in high conversion of starting material to the sulfide product in small molecule studies, but was unable to successfully cleave the sulfonamide linker on peptide in mild conditions. Next, a benzothiazole sulfone substrate was analyzed and optimized in small molecule studies with the addition of an electron-withdrawing group. The benzothiazole sulfone was readily synthesized on peptide and complete cleavage could be performed in mild nucleophilic conditions. Future explorations include expanding the variety of peptide residues that are compatible with cleavage conditions.

Background & Introduction

Cleavable linkers are a promising innovation in chemistry that has many applications in the medicinal field, such as its use in proteomics or in antibody-drug conjugate therapies (ADC's).[1] These linkers are encouraging advances through their ability to separate two or more molecules in specific conditions. A cleavable linker is a stable compound that is triggered to quickly release molecular cargo in controlled, mild conditions. There are many types of linkers currently in research, ranging from disulfides to hydrazones, that are utilized in modern medical interventions.[2] Cleavable linkers can also be integrated onto peptide residues to increase the scope of chemical applications.[3] The disulfide linkers commonly react through reductive conditions to produce thiol derivatives, and hydrazones are cleaved through a hydrolysis reaction. Although there are many successful cleavable linkers, there are many cellular environments that have diverse demands in which continued research in cleavage-based reactions can address.

Many cleavable linker therapies take advantage of the target area's environment, such as an increase in concentration of a certain compound, like mild nucleophiles.[4] Thiols are one such mild nucleophile that is found naturally throughout the body. Additionally, high thiol concentrations can exist amongst certain cellular environments that may be desired targets.[5] The difference in abundance offers an opportunity for organic chemists to develop cleavable linkers to react in thiol-like nucleophilic conditions.

Thiols engage in nucleophilic aromatic substation reactions (or S_NAr reactions) and are reactive with sulfones. Sulfonamides and benzothiazole sulfones were selected to be studied due to the compounds' aromatic stability in most conditions (Figure 1). However, the substrates are still reactive under thiol-like conditions. The general stability of the reactants, but reactive in

selective environments are desired characteristics in cleavable linker developments, and therefore, we began the project with these substrates.

Sulfonamide Benzothiazole

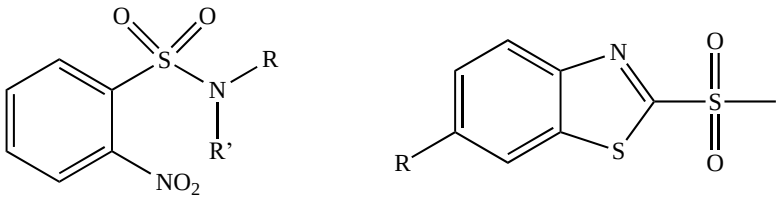


Figure 1. General sulfonamide sulfone and benzothiazole sulfone analyzed in cleavage studies.

Additionally, the generation of the cleavable linkers, a sulfone bonded to an aryl group, were relatively facile, undergoing a series of substitutions and oxidations to produce the starting material (Procedures 1-12).

The synthesized cleavable linker was then tested in small molecule studies for its reactivity utilizing a nucleophilic aromatic substitution to facilitate the cleavage. The observed production of the resulting sulfide was accurately predicted utilizing a proposed mechanism, seen in an example of the benzothiazole cleavage (Figure 2).

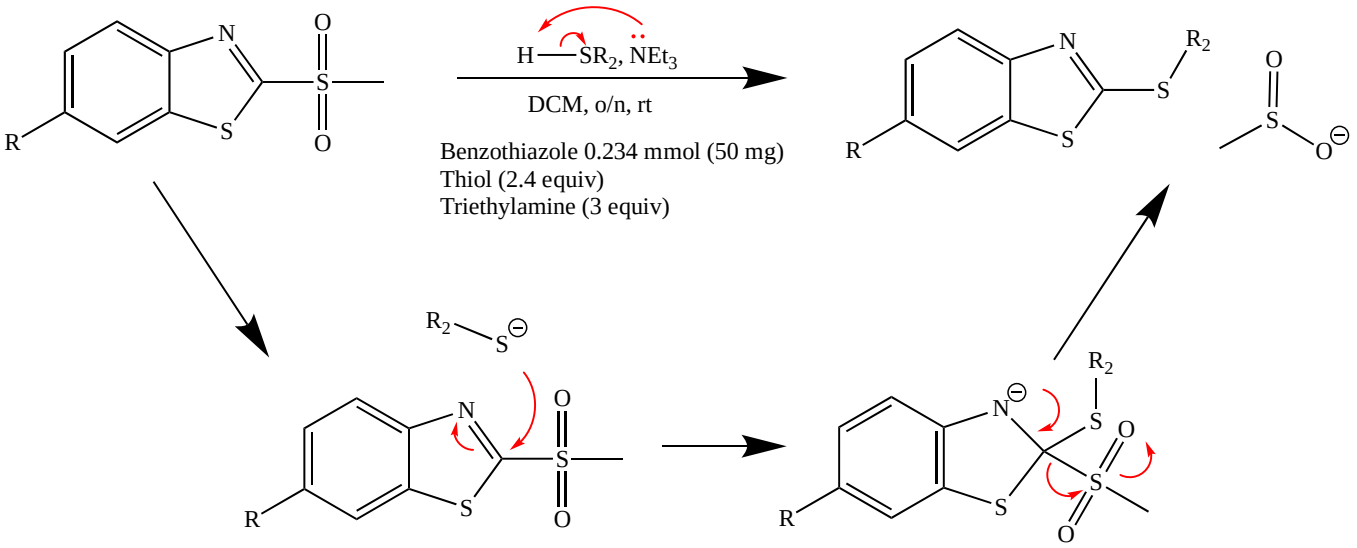


Figure 2. Mechanism of nucleophilic aromatic substitution on benzothiazole sulfone substrate.

Substituents of varying electrostatic properties were introduced on the substrate in addition to testing other nucleophiles to optimize the conversion of starting material to product (Figure 3). All of the benzothiazole substituents are shown, however, only the two best and two worst conversion substituents are shown displayed. A total of nine various substituents were analyzed in the sulfonamide small molecule studies.

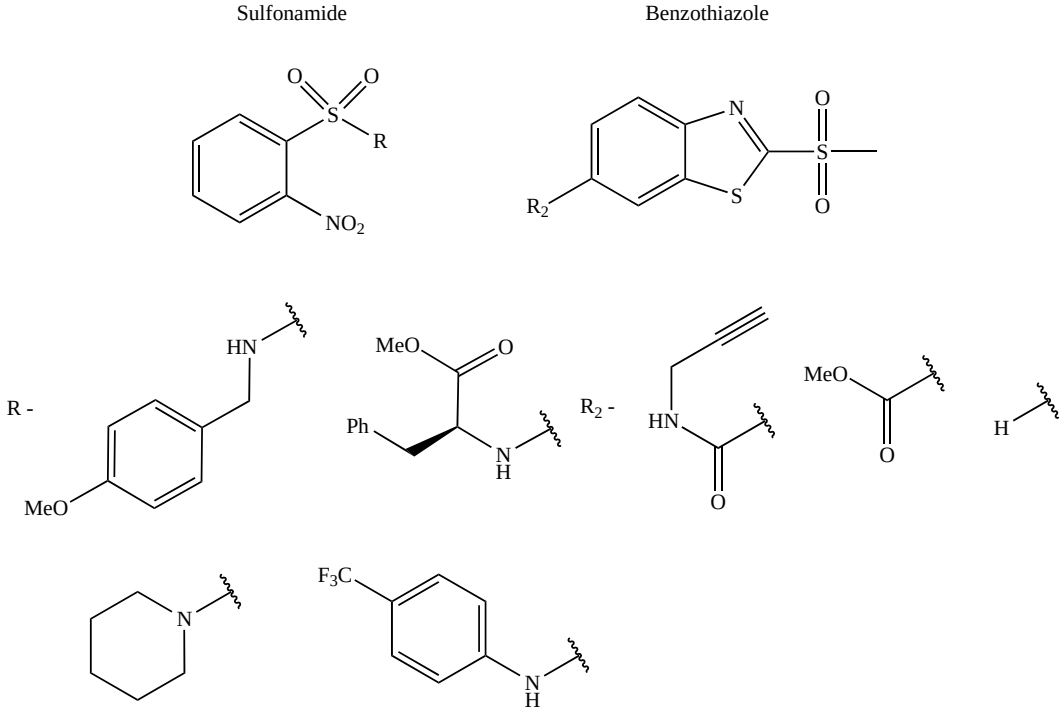


Figure 3. Various substituents on sulfonamide and benzothiazole cleavable linker tested to optimize the reaction parameters.

The substrate with the highest conversion percentage, determined through diagnostic peak picking in ^{1}H NMR spectra (Typical Procedure I), was then subjected to similar synthesis procedures to attach the linker to peptide (Procedure 14). If attachment to the peptide is successful, the small molecule conditions are similarly repeated in to observe the reactivity of the linker on peptide. The reaction was consequently analyzed by MALDI spectra.

Results & Discussion

Sulfonamides: Small Molecule Studies

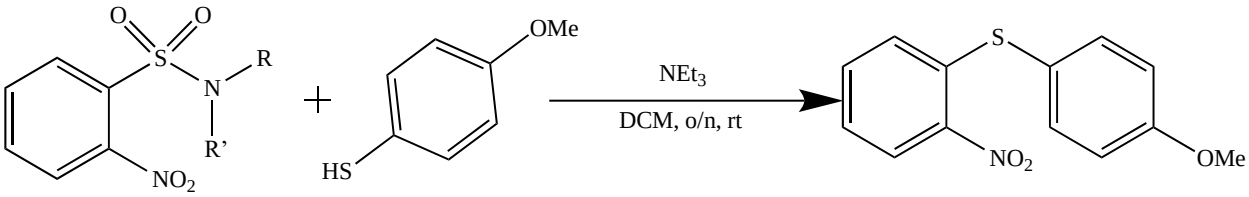
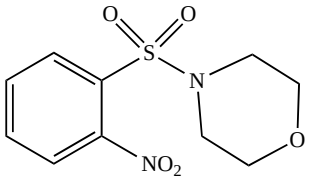
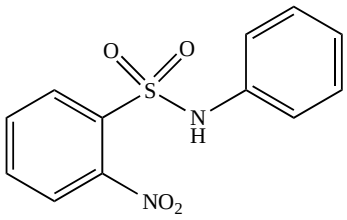
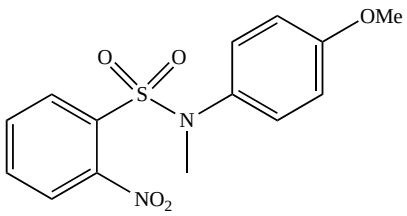
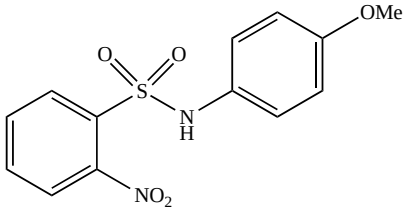
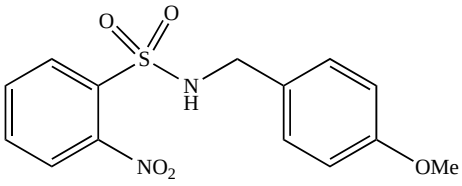


Figure 4. Sulfonamide cleavage scheme in small molecule studies.

The general reaction scheme is shown above, generating a sulfide product (Figure 4.) The results of each cleavage reaction and its corresponding starting materials are shown below (Table 1). Electron-withdrawing substituents appeared to have greater conversion rates in sulfonamide reactivity studies, however there is no definitive evidence that the electrostatics are the dominating influence in these reactions. The highest conversion rate was obtained from a reaction between N-[(4-Nitrophenyl)sulfonyl]-D-phenylalanine methyl ester (ACI) and 4-methoxybenzenethiol as the nucleophile. An average of 87% starting material was converted into product, a successful reaction in small molecule studies.

Sulfonamide Derivative	Conversion %
4-[(2-Nitrophenyl)sulfonyl]morpholine (ACl) 	26%*

2-Nitro-N-phenylbenzene-1-sulfonamide 	27%*
N-(4-Methoxyphenyl)-N-methyl-2-nitrobenzenesulfonamide (ACl) 	25%
N-(4-Methoxyphenyl)-2-nitrobenzenesulfonamide (ACl) 	62%
N-(4-Methoxybenzyl)-2-nitrobenzenesulfonamide 	68%***

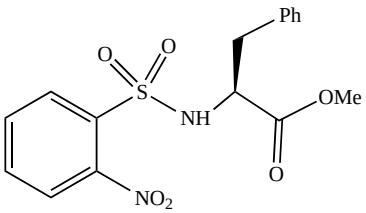
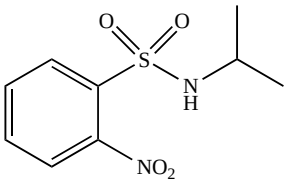
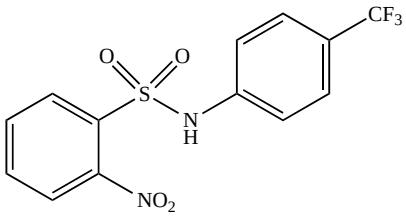
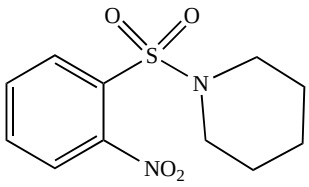
N-[(4-Nitrophenyl)sulfonyl]-D-phenylalanine methyl ester (ACI) 	86%*
N-Isopropyl-2-nitrobenzenesulfonamide 	33%
2-Nitro-N-[4-(trifluoromethyl)phenyl]benzenesulfonamide (ACl) 	2%
1-(2-Nitrobenzenesulfonyl)piperidine 	12%

Table 1. Various sulfonamide substrates and their conversion percentage of starting material to products. * indicates that the experiment was repeated once, and the conversion is an average of the two. ** indicates that the experiment was repeated twice, and the conversion is an average of the three.

Sulfonamides: Peptide Studies

The phenylalanine methyl ester substrate was selected to be attached to H_2N-FCPFGLLYGR-$CONH_2$; however, the reaction was unsuccessful. No product was made and thus, the second highest substrate was selected to undergo peptide reactivity studies. The cleavable linker was able to successfully attach to H_2N-GRPPGFSPFR-$CONH_2$ (Figure 5).

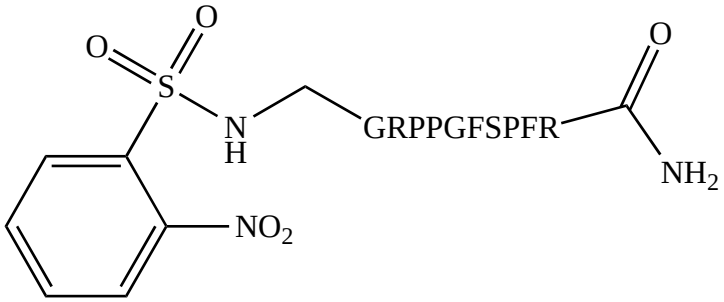


Figure 5. Sulfonamide cleavable linker successfully attached to peptide chain.

With similar conditions, the linker attached to a peptide (1 mM) was reacted with 4-methoxybenzene thiol as the nucleophile (5mM), pH 8 phosphate (100 mM), and TCEP (20 mM), which is a reagent that prevents disulfides from forming (Typical Procedures II). However, the spectrum was extremely messy and unable to read.The sulfonamide project had explored many reactions in small molecule studies; however, the sulfonamide substrate was not compatible with reactions on peptide. Therefore, the project shifted towards another substrate: benzothiazoles.

Benzothiazoles: Small Molecule Studies

Initial probes into benzothiazoles included a reaction with 2-(Methylsulfonyl) benzothiazole to test the reactivity in nucleophilic conditions that are described in Typical Procedure 1 (Figure 6).

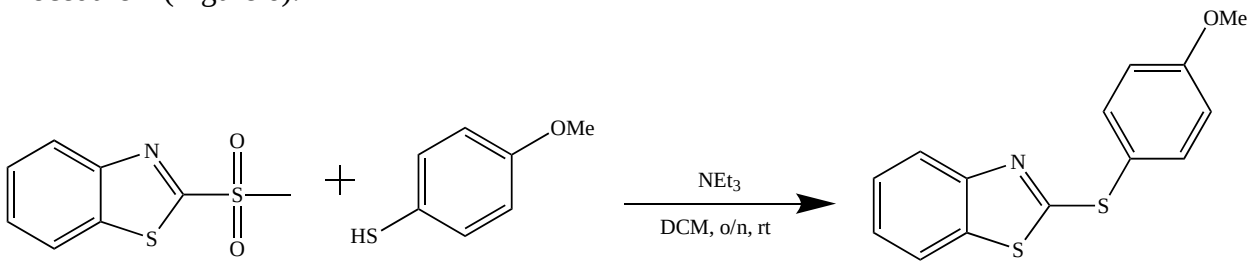


Figure 6. Benzothiazole sulfone cleavage scheme in small molecule studies.

Unlike the relatively high conversion rates found in the sulfonamide small molecule studies, this benzothiazole linker had consistently low conversion to the sulfide product. Therefore, electrostatics substituents were incorporated into the substrate in anticipation that it would facilitate more favorable forward reactions. A propargyl group was added to the benzothiazole linker and the conversion increased to an observable amount to quantify, 37.86% (Figure 7).

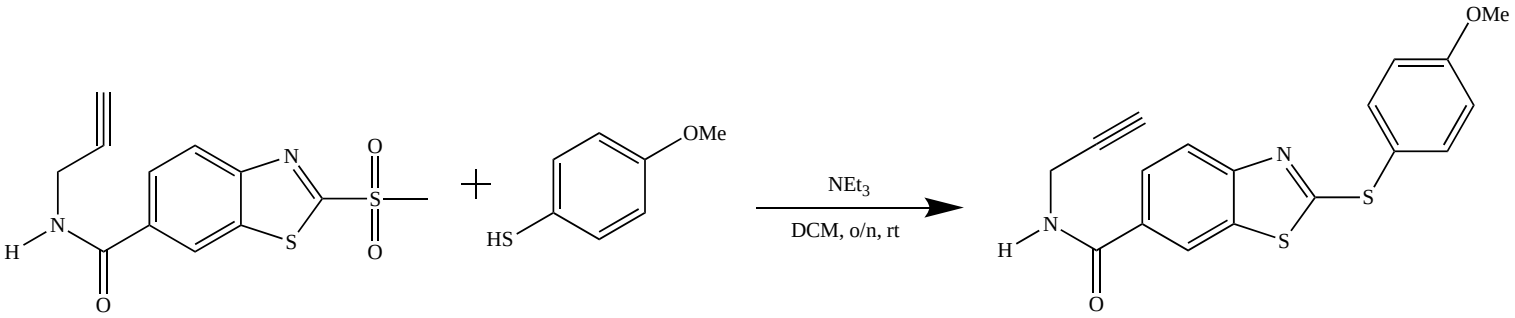


Figure 7. Reaction scheme of 2-(Methylsulfonyl)-*N*-2-propyn-1-yl-6-benzothiazolecarboxamide (ACI) and 4-methoxybenzenethiol to produce the sulfide product.

Although the conversion was not as high as would be satisfactory, the cleavable linker had been synthesized in enough quantity to observe reactivity in peptide studies.

Benzothiazoles: Peptide Studies

The benzothiazole with the propargyl amide substituent was successfully added to the peptide sequence H_3N-RGKLLGFPCF-resin (Procedure 14). The peptide was cleaved from resin (Procedure 15) and was subjected to reactions to determine the optimal conditions for complete cleavage of linker to product. The general reaction scheme is shown below (Figure 8).

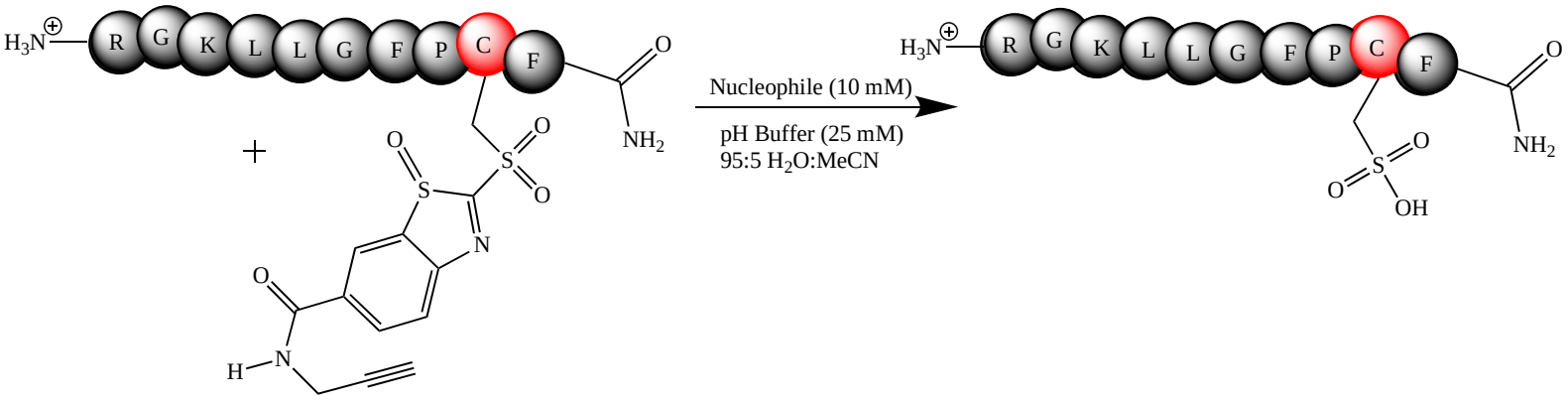


Figure 8. Reaction scheme for peptide cleavage reactions.

Many reagents including beta-mercaptoethanol (BME), tris(2-carboxyethyl)phosphine (TCEP), ethane-1,2-dithiol (EDT), and various pH buffers: pH 7 & 8 phosphate buffers and pH 7 tris buffer. The cleanest spectrum on average appeared to be the reaction of TCEP and pH 8 phosphate buffer in concentration conditions detailed in Typical Procedure II. TCEP was initially added to prevent oxidation of the thiol to a disulfide, but through experimentation, we observed that TCEP was a better nucleophile compared to the previous conditions tried. Complete conversion of starting material (1398.67 m/z) to product (1183.62 m/z) was observed in a 3 hour reaction at room temperature (Procedure 16). The MALDI spectra may appear to be off by +2 m/z. This could potentially be due to the oxidation conditions on resin, and further studies to explain this phenomenon are underway.

In mild nucleophilic conditions, the optimized benzothiazole linker on peptide has been shown to fully cleave and, therefore, has the potential to efficiently aid in the release of more

complex biological concerns. The project has succeeded in producing an efficient, reagent-controlled cleavable linker, and plans to expand the variety of peptides that continues to produce a successful reaction (Figure 9).

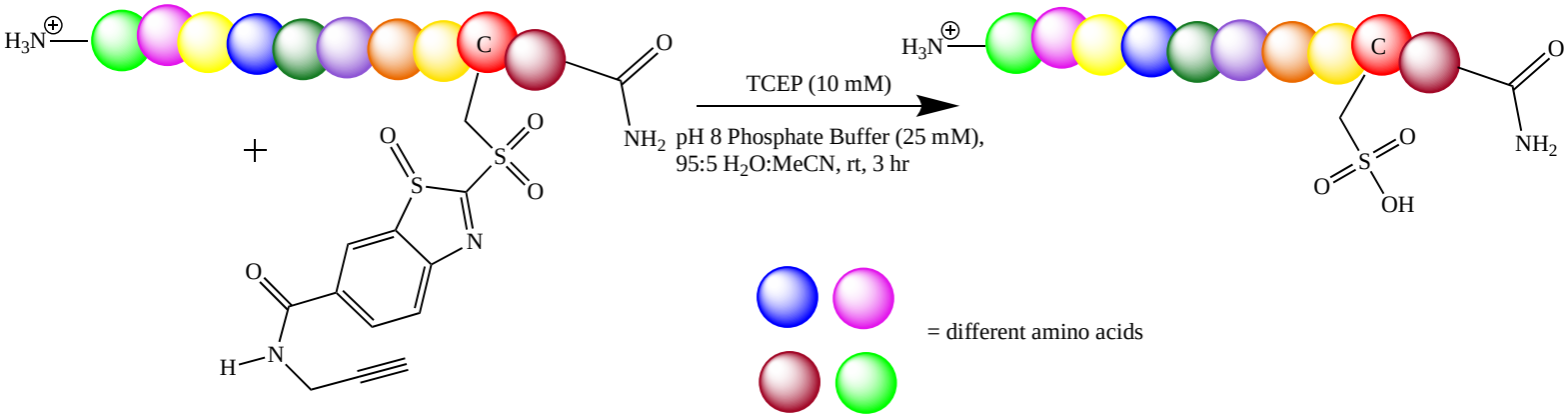


Figure 9. Visual representation of exchanging amino acids to test reactivity with optimized conditions.

Conclusion

Sulfonamides proved to be successful in small molecule cleavage conditions but produced low conversion in peptide cleavage reactions. The benzothiazole sulfone can achieve complete conversion through a S_NAr reaction in both small molecule and peptide cleavage studies. The benzothiazole sulfone linker on peptide is continued to be explored through expanding peptide compatibility.

Experimental

Typical Procedure I: Reagent-Controlled Cleavage in Small Molecule Studies.

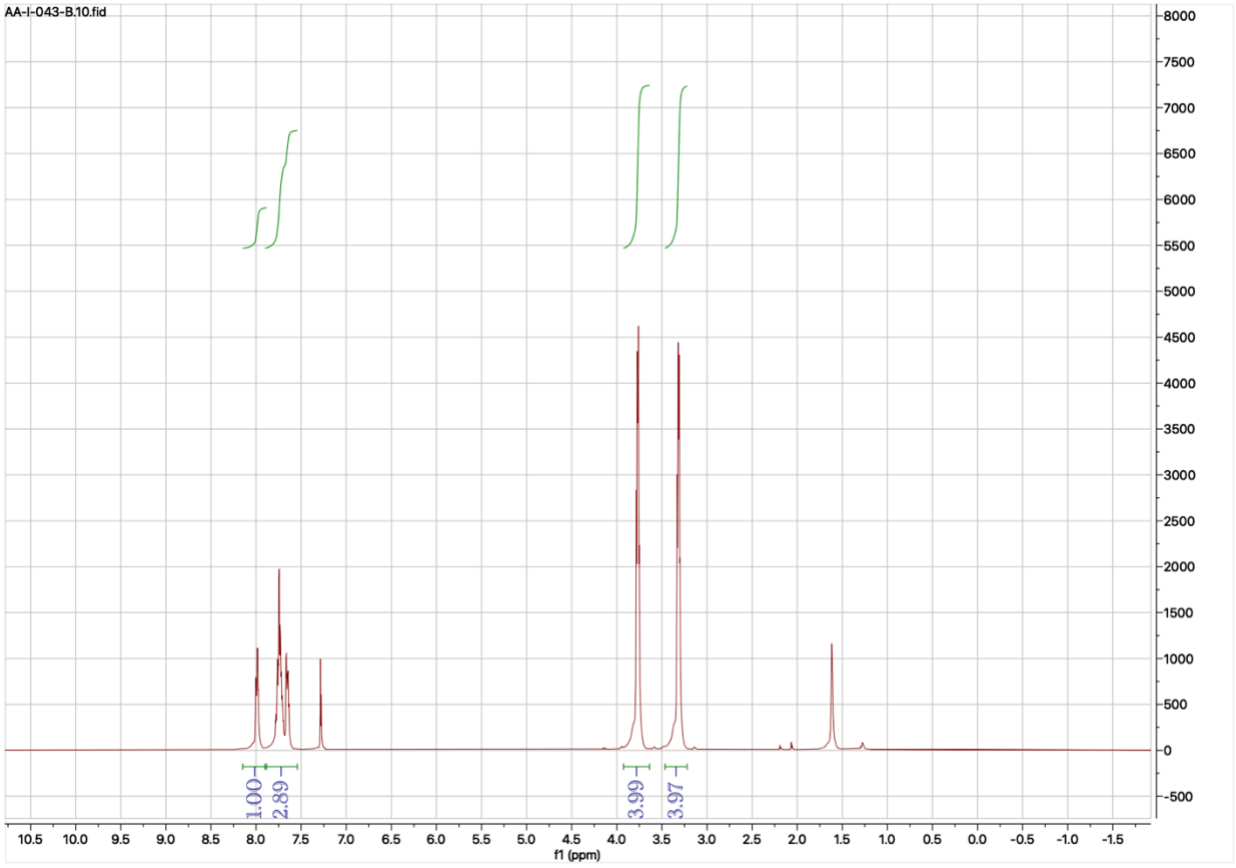
In a 25 mL rbf, 0.234 mmol of sulfonamide or benzothiazole was dissolved in 1.17 mL of DCM. The mild nucleophile (2.4 equiv) and the base, triethylamine (3 equiv), were added, and the reaction stirred between 15 min to o/n. Reaction was subjected to liquid-liquid extraction, utilizing both an acidic and basic aqueous wash. Organic layer was extracted from separatory funnel and dried over Na_2SO_4. Product was dried of solvent through rotary evaporation and high vacuum conditions for 10 minutes minimum. The following product was then characterized through ^1H NMR spectroscopy. If necessary, subsequent purifications were conducted through flash-chromatography utilizing a gradient of hexanes and ethyl acetate. Diagnostic peak picking determined the conversion rates through identification of a ^1H peak that was not present in the reactant but is present in the product and one peak that follows the opposite. The crude NMR spectrum's determined peaks were compared through the calculated integration values. The integration of the product peak divided by the sum of the integrations of the reactant peak and the product peak. This computes a ratio of product made compared to how much starting material was available at the start of the reaction.

Typical Procedure II: Reagent-Controlled Cleavage in Peptide Studies.

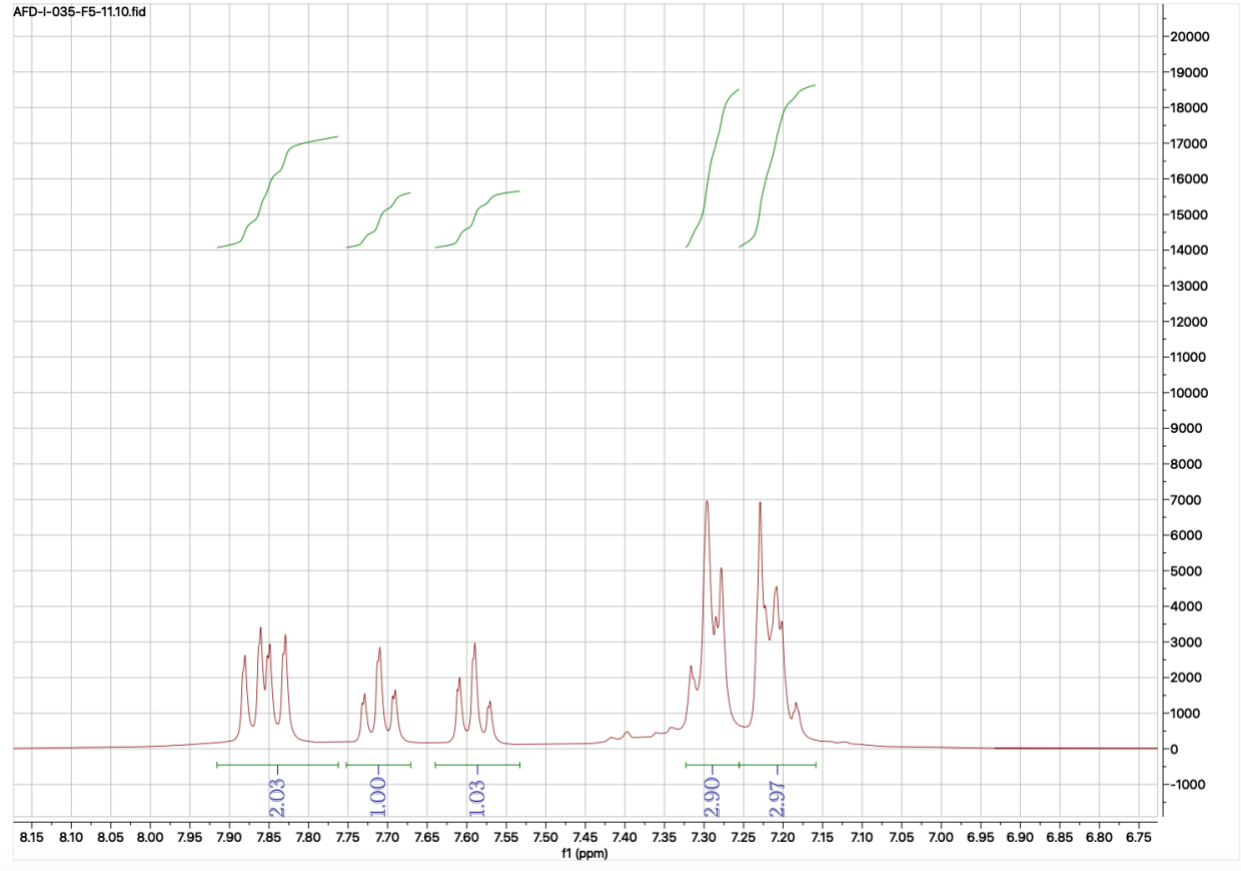
The cleavable linker on peptide, nucleophile, and pH buffer were added simultaneously to a microcentrifuge tube at a respective final concentrations of 0.5 mM, 10 mM, and 25 mM in a 95:5 H_2O:MeCN 20 μL reaction. The reaction was shaken between 15 minutes to o/n at rt or 37°C. The reaction is then spotted on the MALDI plate, utilizing conventional instructions to conduct MALDI analysis. The product m/z peak and reactant m/z peak can be compared to give relative conversion rates. If necessary, purification measures can be administered through

performing a zip-tip C18 procedure of the resulting reaction and subsequent MALDI spectra can be acquired.

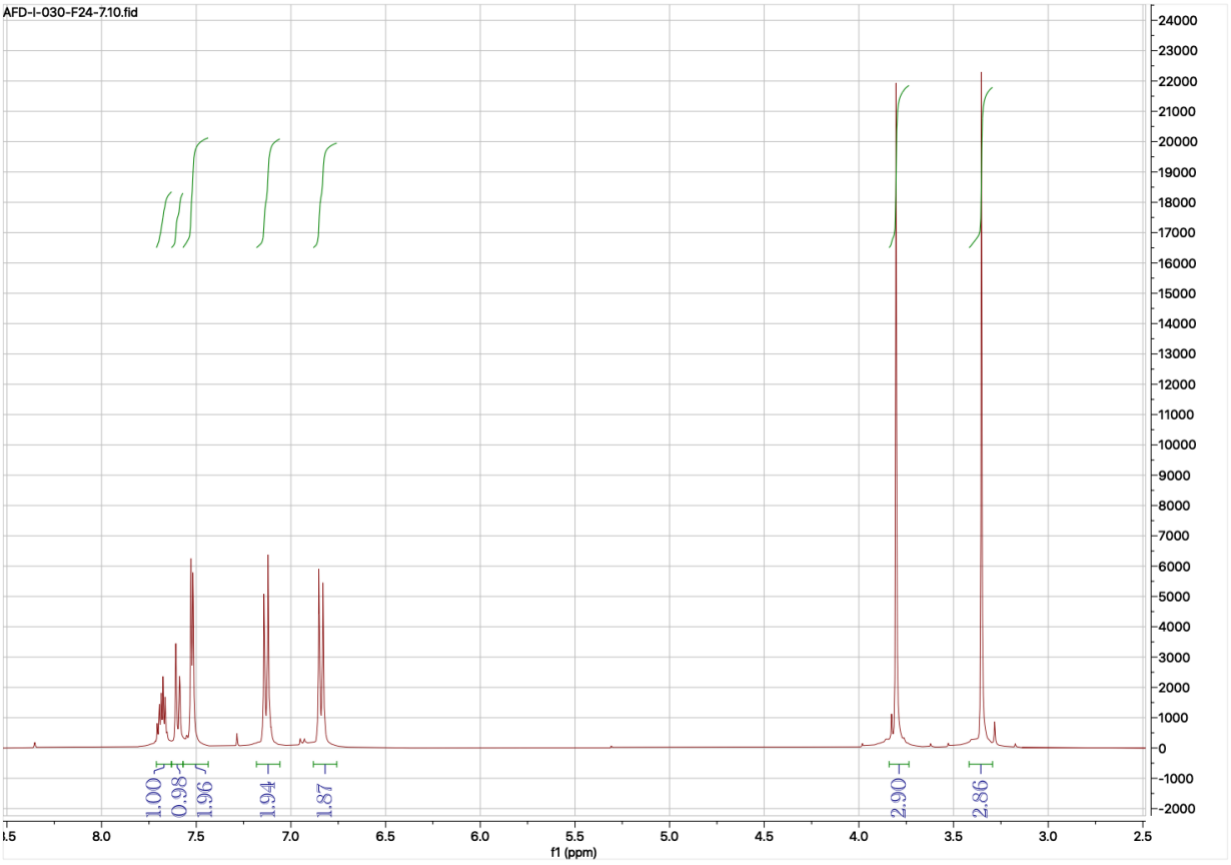
Procedure 1. 4-[(2-Nitrophenyl)sulfonyl]morpholine (ACl). In a 25 mL rbf, 1.62 mmol of morpholine and 3.57 mmol of K_2CO_3 was dissolved in 4.96 mL of DCM and mixed for 15 minutes. The solution was cooled to 0°C in an ice-water bath, and a solution of 1.44 mL of DCM and 0.43 g of 2-nitrobenzenesulfonyl chloride was added dropwise to the chilled solution. The reaction was mixed o/n at rt and subsequently washed with water and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^{1}H NMR spectroscopy. 41.6% yield.



Procedure 2. 2-Nitro-N-phenylbenzene-1-sulfonamide. In a 25 mL rbf, 1.62 mmol of aniline and 3.57 mmol of K_2CO_3 was dissolved in 4.96 mL of DCM and mixed for 15 minutes. The solution was cooled to 0°C in an ice-water bath, and a solution of 1.44 mL of DCM and 0.43 g of 2-nitrobenzenesulfonyl chloride was added dropwise to the chilled solution. The reaction was mixed o/nt at rt and subsequently washed with water and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^1H NMR spectroscopy. Subsequent purifications were conducted through flash-chromatography. 44.0% yield.

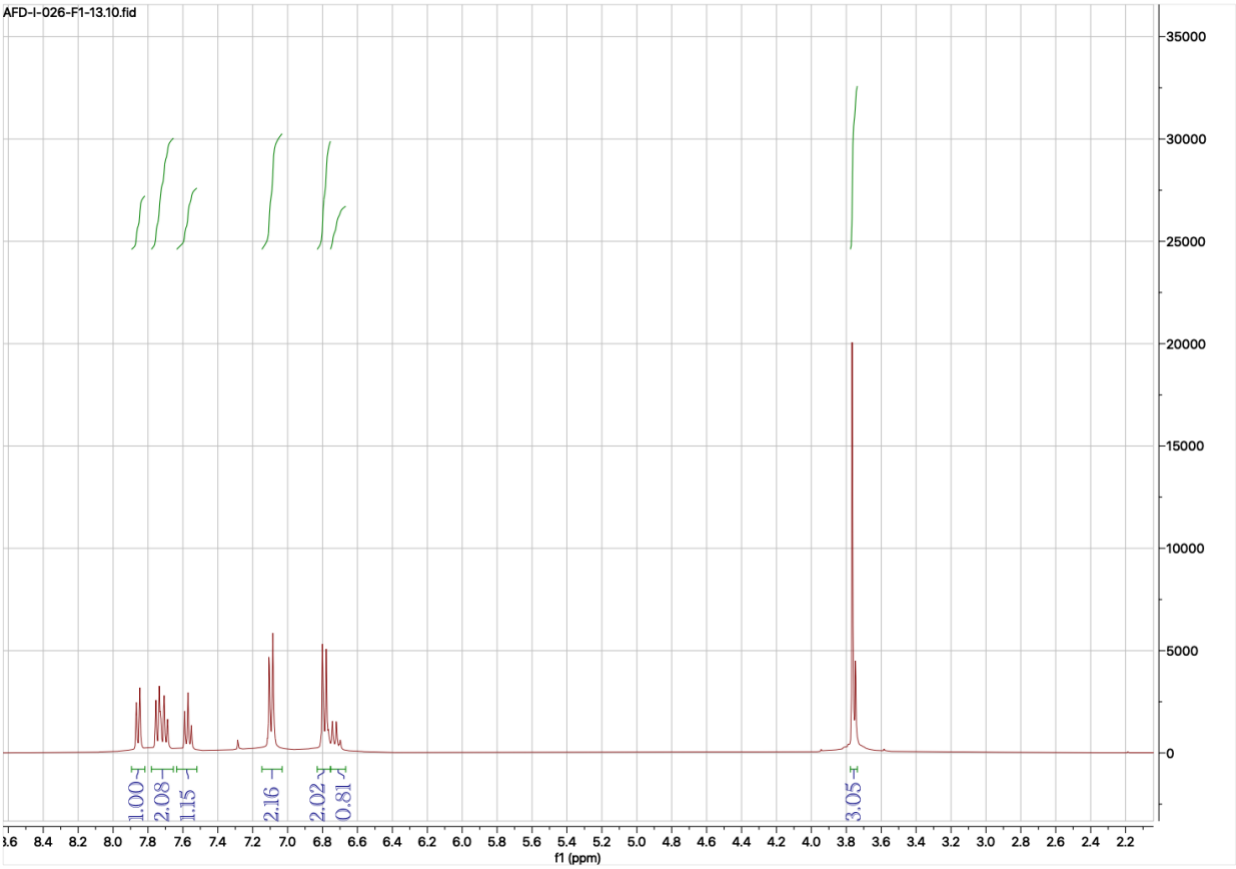


Procedure 3. N-(4-Methoxyphenyl)-N-methyl-2-nitrobenzenesulfonamide (ACl). In a 25 mL rbf, 1.62 mmol of 4-methoxy-N-methyl aniline and 3.57 mmol of K_2CO_3 was dissolved in 4.96 mL of DCM and mixed for 15 minutes. The solution was cooled to 0°C in an ice-water bath, and a solution of 1.44 mL of DCM and 0.43 g of 2-nitrobenzenesulfonyl chloride was added dropwise to the chilled solution. The reaction was mixed o/n at rt and subsequently washed with water and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^1H NMR spectroscopy. Subsequent purifications were conducted through flash-chromatography. 46.2% yield.



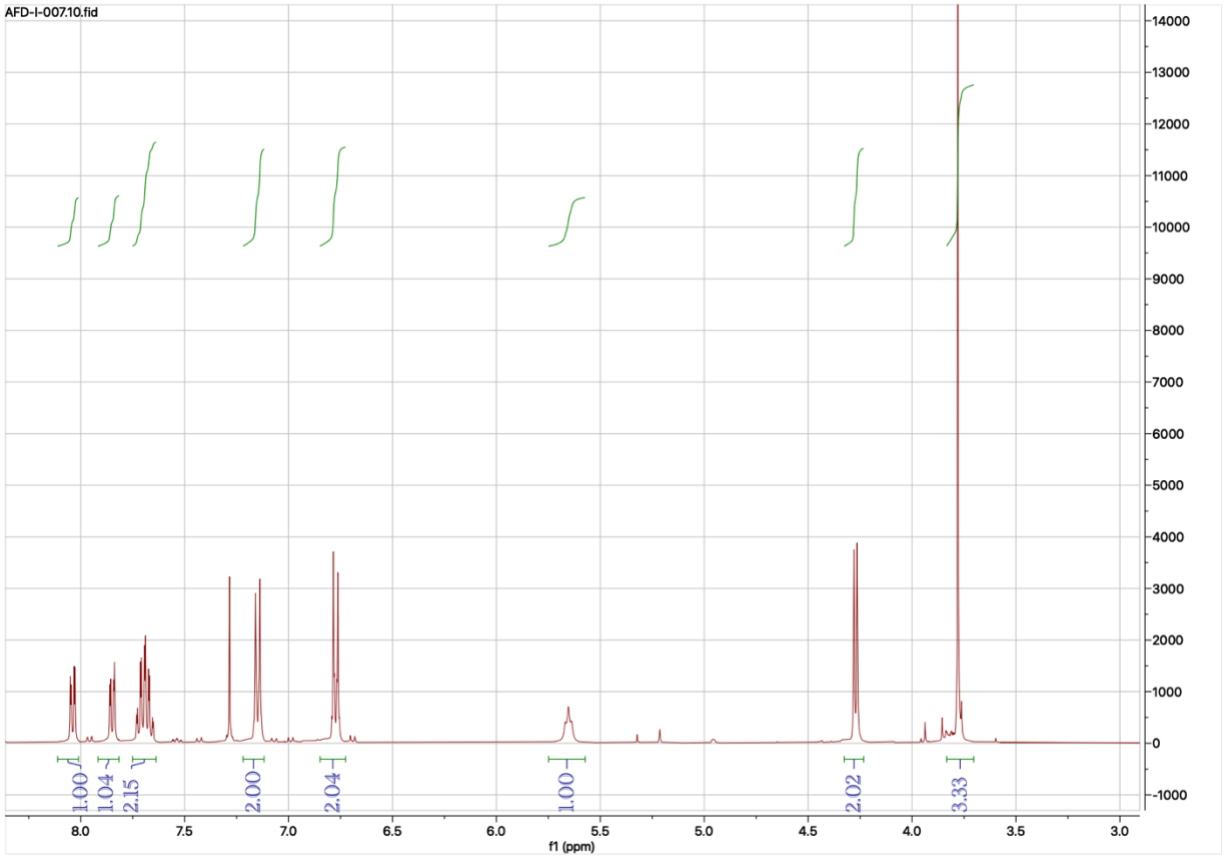
Procedure 4. N-(4-Methoxyphenyl)-2-nitrobenzenesulfonamide (ACl). In a 25 mL rbf, 1.62 mmol of 4-methoxyaniline and 3.57 mmol of K_2CO_3 was dissolved in 4.96 mL of DCM and

mixed for 15 minutes. The solution was cooled to 0°C in an ice-water bath, and a solution of 1.44 mL of DCM and 0.43 g of 2-nitrobenzenesulfonyl chloride was added dropwise to the chilled solution. The reaction was mixed o/n at rt and subsequently washed with water and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^{1}H NMR spectroscopy. Subsequent purifications were conducted through flash-chromatography. 40.6% yield.



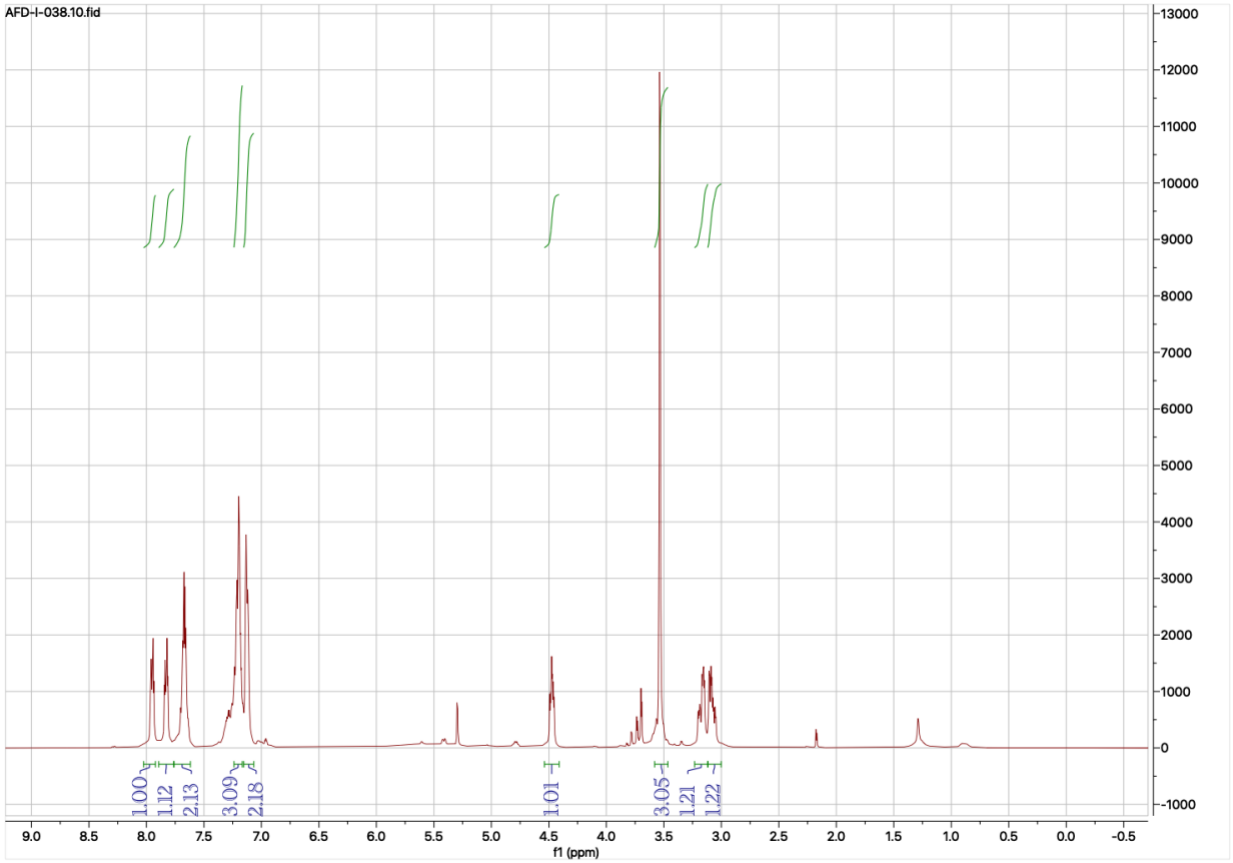
Procedure 5. N-(4-Methoxybenzyl)-2-nitrobenzenesulfonamide. In a 25 mL rbf, 1.55 mmol of 4-methoxybenzylamine and 2.9 mmol of K_2CO_3 was dissolved in 4.03 mL of DCM and mixed for 15 minutes. The solution was cooled to 0°C in an ice-water bath, and a solution of 1.17 mL of DCM and 0.35 g of 2-nitrobenzenesulfonyl chloride was added dropwise to the chilled

solution. The reaction was mixed o/nt at rt and subsequently washed with water and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^{1}H NMR spectroscopy. 82.4% yield.

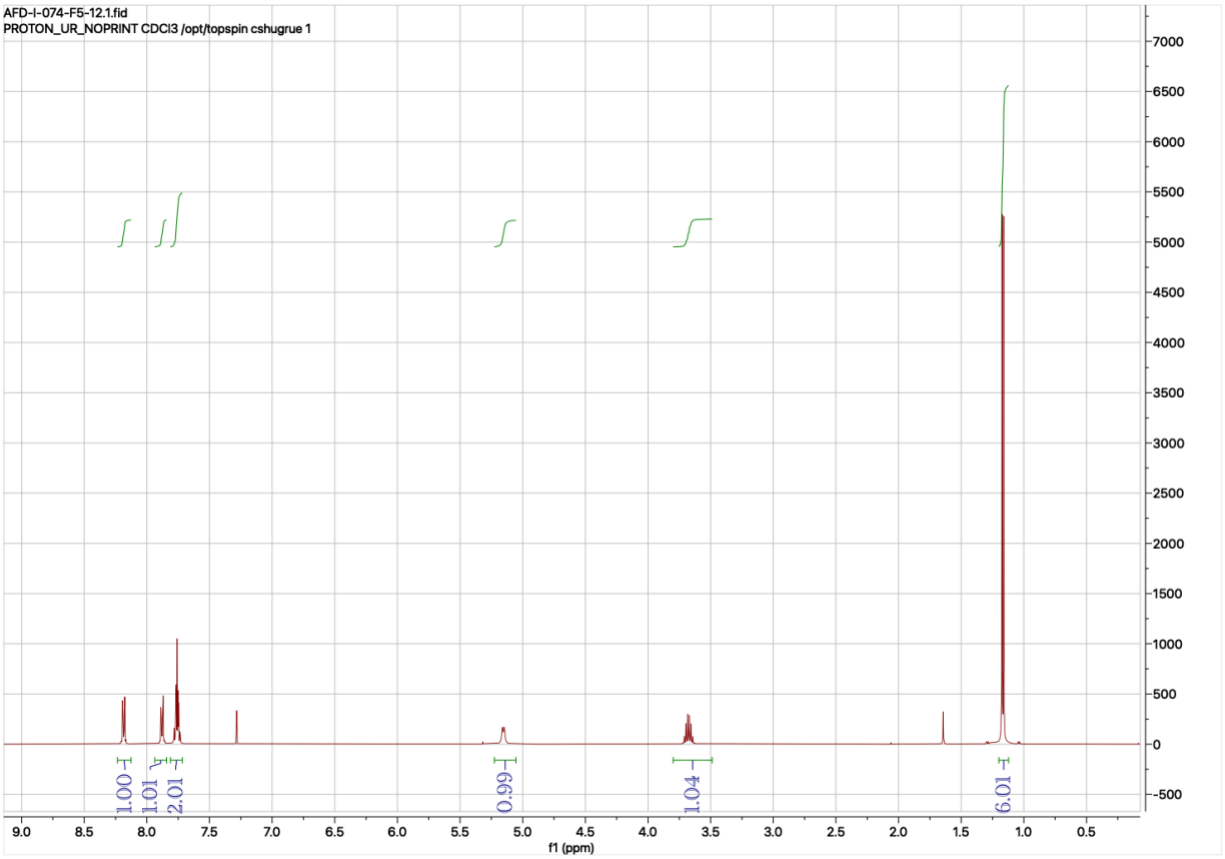


Procedure 6. N-[(4-Nitrophenyl)sulfonyl]-D-phenylalanine methyl ester (ACI). In a 25 mL rbf, 1.62 mmol of phenylalanine methyl ester HCl and 5.18 mmol of K_2CO_3 was dissolved in 4.96 mL of DCM and mixed for 15 minutes. The solution was cooled to 0°C in an ice-water bath, and a solution of 1.44 mL of DCM and 0.43 g of 2-nitrobenzenesulfonyl chloride was added dropwise to the chilled solution. The reaction was mixed o/n at rt and subsequently washed with water and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten

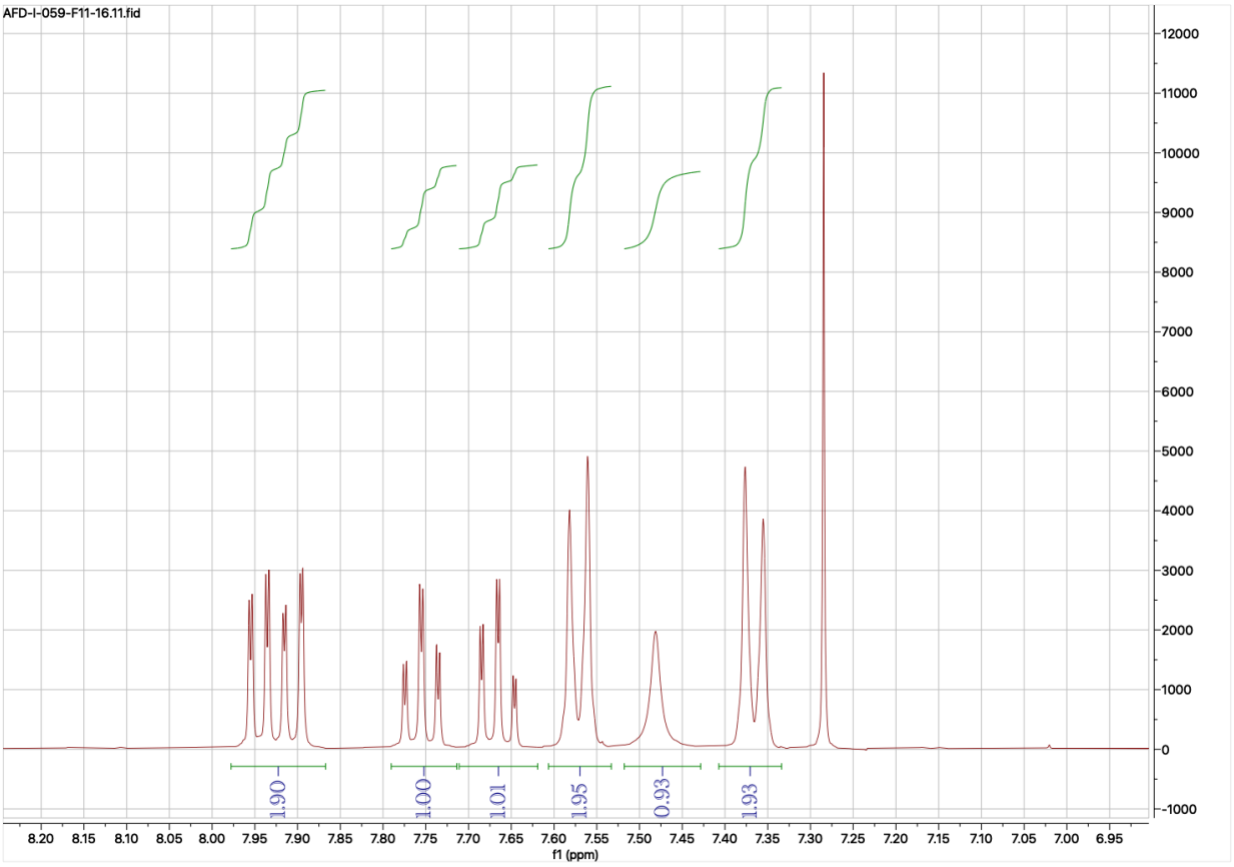
minutes. The resulting product was then characterized through ^{1}H NMR spectroscopy. Subsequent purifications were conducted through flash-chromatography. 27.1% yield.



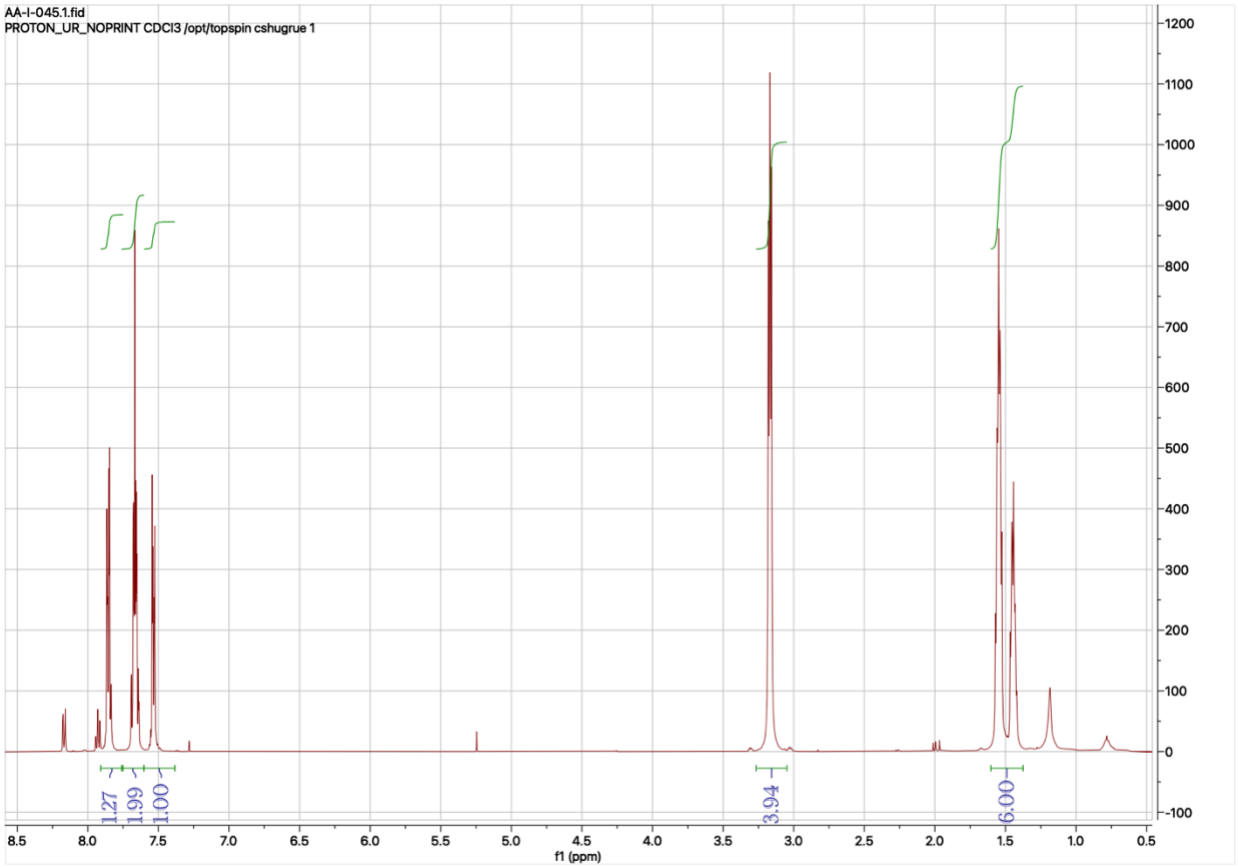
Procedure 7. N-Isopropyl-2-nitrobenzenesulfonamide. In a 25 mL rbf, 1.62 mmol of isopropylamine and 3.57 mmol of K_2CO_3 was dissolved in 4.96 mL of DCM and mixed for 15 minutes. The solution was cooled to 0°C in an ice-water bath, and a solution of 1.44 mL of DCM and 0.43 g of 2-nitrobenzenesulfonyl chloride was added dropwise to the chilled solution. The reaction was mixed o/n at rt and subsequently washed with water and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^{1}H NMR spectroscopy. Subsequent purifications were conducted through flash-chromatography. 16.9% yield.



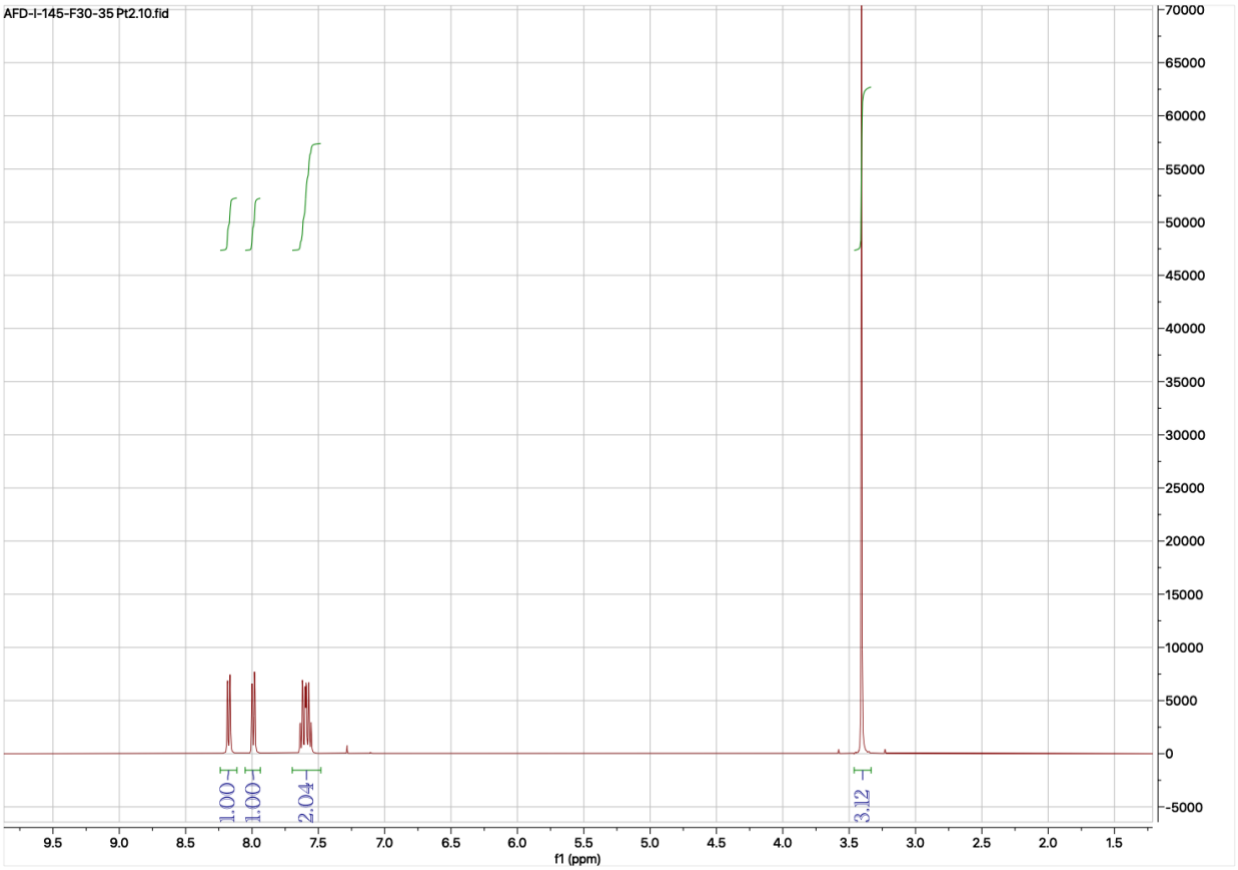
Procedure 8. 2-Nitro-N-[4-(trifluoromethyl)phenyl]benzenesulfonamide (ACl). In a 25 mL rbf, 1.62 mmol of 4-aminobenzotrifluoride and 3.57 mmol of K_2CO_3 was dissolved in 4.96 mL of DCM and mixed for 15 minutes. The solution was cooled to 0°C in an ice-water bath, and a solution of 1.44 mL of DCM and 0.43 g of 2-nitrobenzenesulfonyl chloride was added dropwise to the chilled solution. The reaction was mixed o/n at rt and subsequently washed with water and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^{1}H NMR spectroscopy. Subsequent purifications were conducted through flash-chromatography. 4.9% yield.



Procedure 9. 1-(2-Nitrobenzenesulfonyl)piperidine. In a 25 mL rbf, 1.62 mmol of piperidine and 3.57 mmol of K_2CO_3 was dissolved in 4.96 mL of DCM and mixed for 15 minutes. The solution was cooled to 0°C in an ice-water bath, and a solution of 1.44 mL of DCM and 0.43 g of 2-nitrobenzenesulfonyl chloride was added dropwise to the chilled solution. The reaction was mixed o/n at rt and subsequently washed with water and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^1H NMR spectroscopy. Subsequent purifications were conducted through flash-chromatography. 73.1% yield.



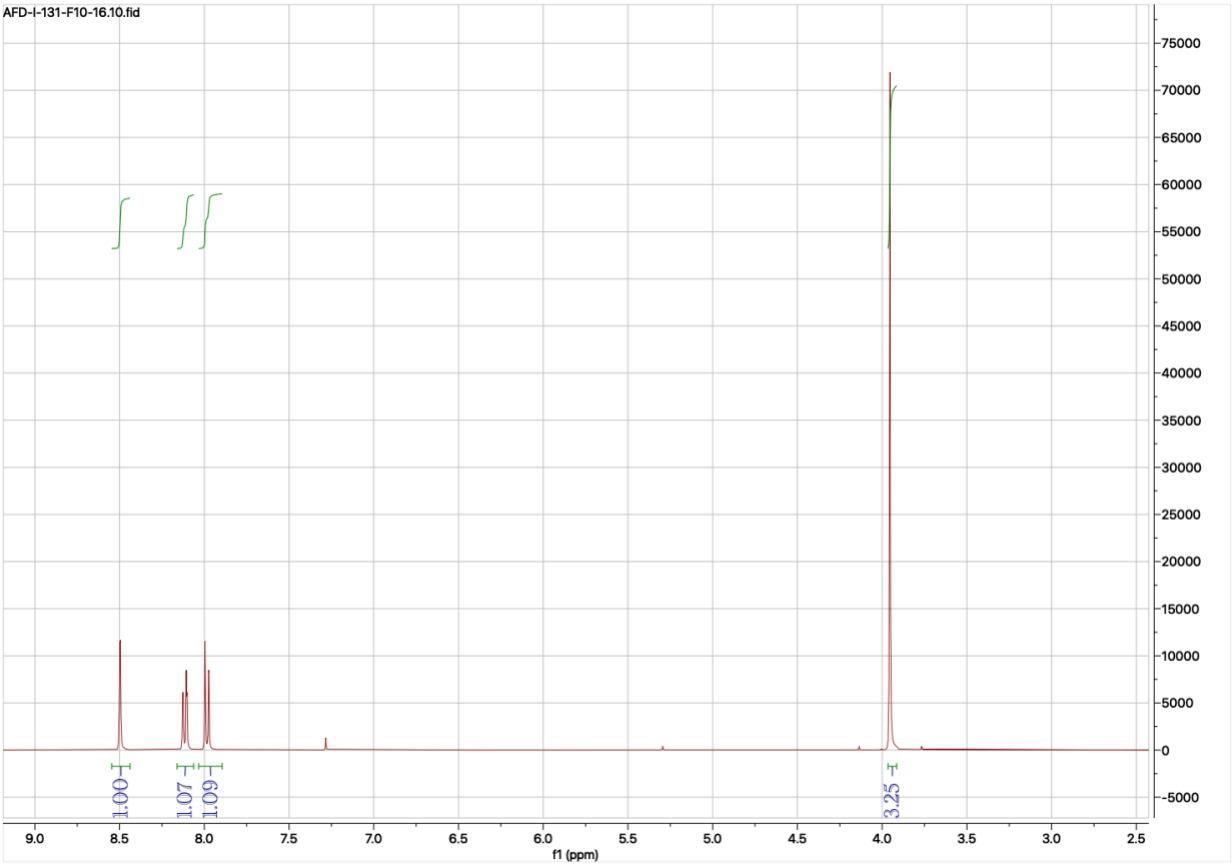
Procedure 10. 2-(Methylsulfonyl)benzothiazole. To a 250 mL rbf, 1g of 2-(methylthio)benzothiazole was dissolved in 60.85 mL of DCM. The solution was cooled to 0°C in an ice-water bath. 16.54 mmol of mCPBA (77%) was added slowly to the chilled solution and mixed for 48 hours at rt. The reaction was quenched with $Na_2S_2O_3$ (approximately 1/3 of reaction volume). The solution was then washed with water, $NaHCO_3$, and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^1H NMR spectroscopy. Subsequent purifications were conducted through hand-column chromatography. 56.3% yield.



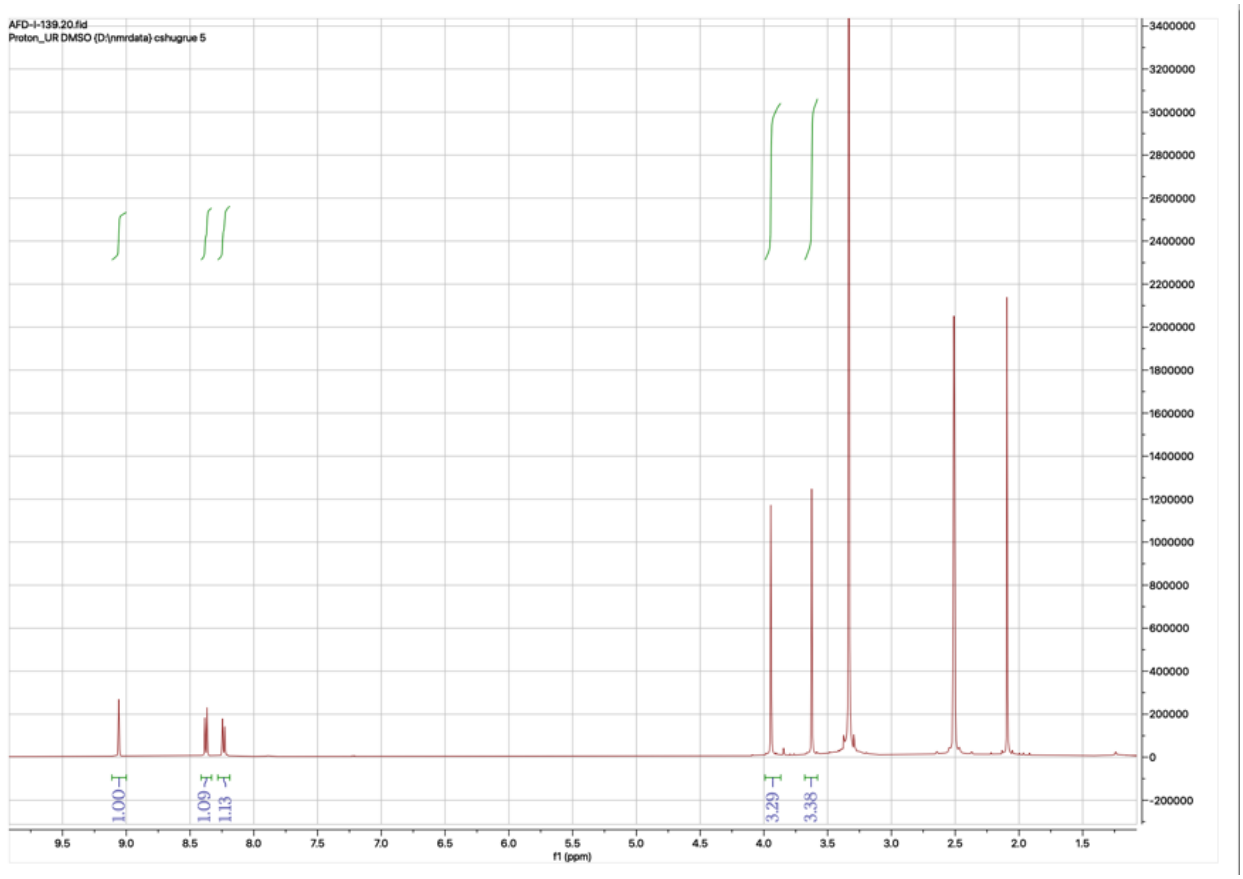
Procedure 11. Methyl 2-(methylsulfonyl)-6-benzothiazolecarboxylate. A) To a 100mL rbf, 0.5 g of 2-bromo-6-benzothiazole, 3.87 mmol of EDC HCl, 3.87 mmol of HOBt H_2O, and 19 mL of MeOH was added. The reaction was mixed o/n and concentrated through rotary evaporation. The reaction was then washed with ethyl acetate, $NaHCO_3$, citric acid 10%, and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^1H NMR spectroscopy. Subsequent purifications were conducted through hand-column chromatography. 28.9% yield. **B)** 0.51 g of Procedure 11 A product and 2.2 mL of DMF was added to a 10 mL rbf. 0.76 mmol of $MeSO_2Na$ was mixed into solution and reacted for 3.5 hours at 70°C. The resulting reaction was concentrated through rotary evaporation and added to a separatory funnel with 250 mL of LiCl and 250 mL of ethyl acetate. The organic layer was

extracted and underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^{1}H NMR spectroscopy. 103.7% yield – solvent added to yield as spectrum, seen below, is clean of any impurities.

A)



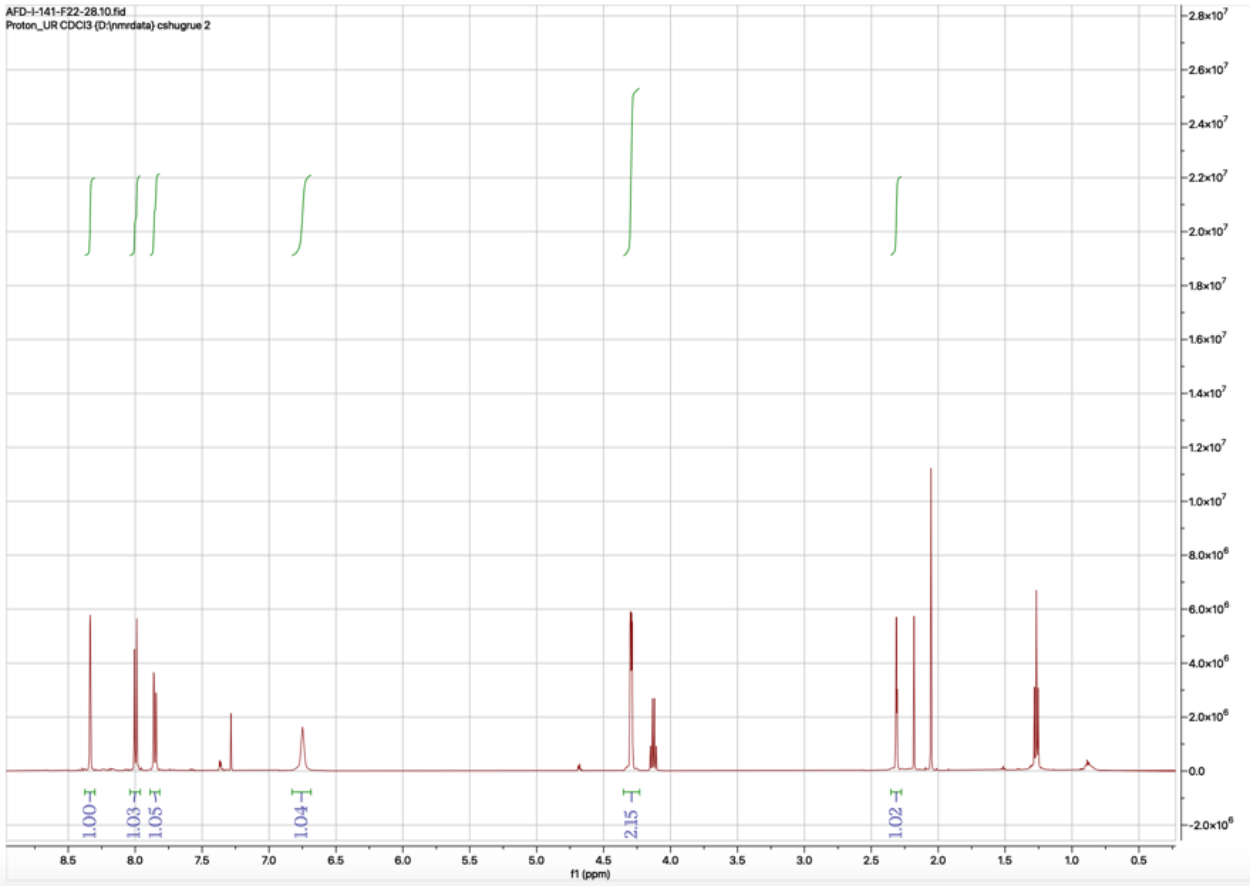
B)



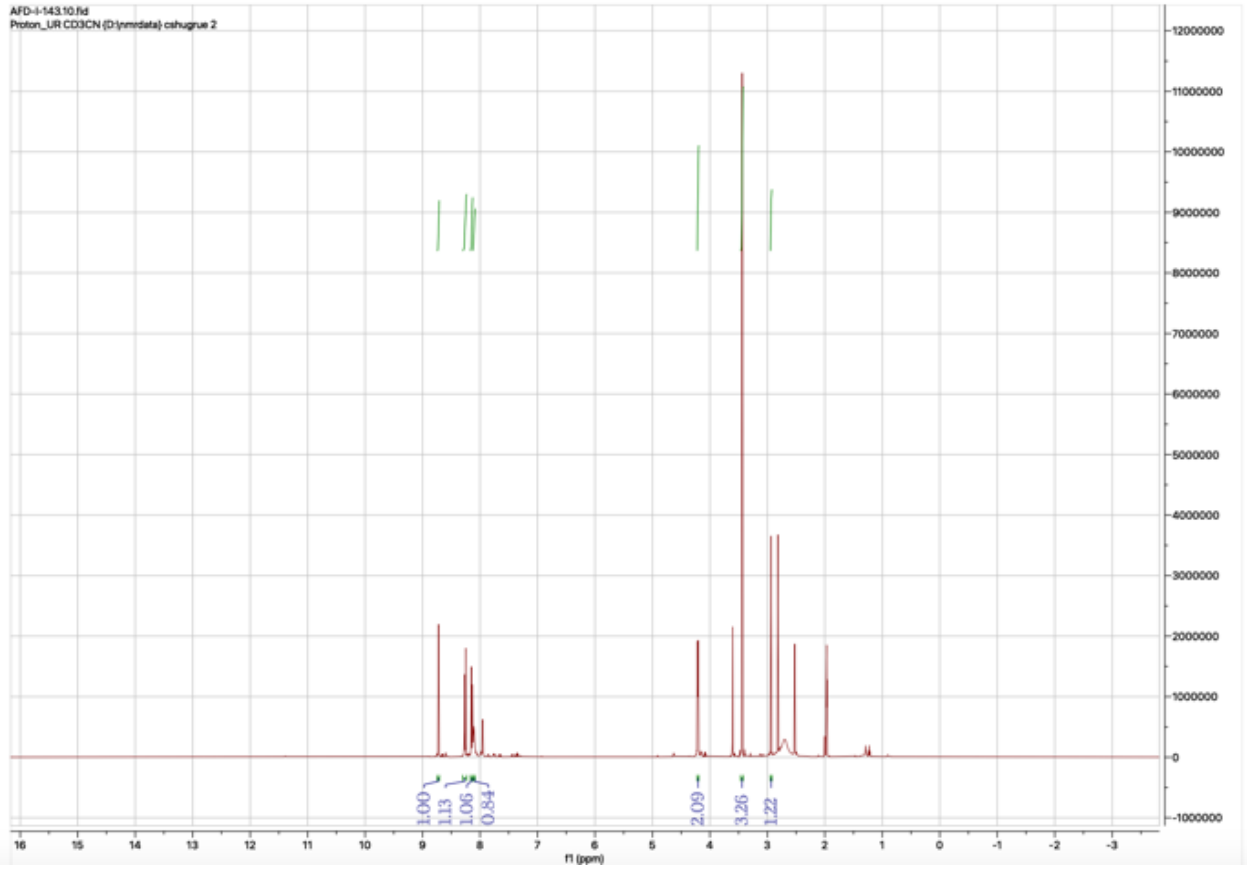
Procedure 12. 2-(Methylsulfonyl)-*N*-2-propyn-1-yl-6-benzothiazolecarboxamide. A) To a 100mL rbf, 0.5 g of 2-bromo-6-benzothiazole, 2.33 mmol of EDC HCl, and 2.33 mmol of HOBt H_2O was added. A solution of 10 mL of DCM, 4.27 mmol of DIPEA, and 2.13 mmol of propargylamine was added to the reaction. The mixture reacted o/n and concentrated through rotary evaporation. The reaction was then washed with ethyl acetate, $NaHCO_3$, citric acid 10%, and brine. The organic layer was then dried over sodium sulfate. The solution underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^{1}H NMR spectroscopy. Subsequent purifications were conducted through hand-column chromatography. 34.7% yield. **B)** 0.51 g of Procedure 12 A product and 2.2 mL of DMF was added to a 10 mL rbf. 0.76 mmol of $MeSO_2Na$ was mixed into

solution and reacted o/n at 70°C. The resulting reaction was concentrated through rotary evaporation and added to a separatory funnel with 250 mL of LiCl and 250 mL of ethyl acetate. The organic layer was extracted and underwent rotary evaporation and dried on a high-powered vacuum for a minimum of ten minutes. The resulting product was then characterized through ^{1}H NMR spectroscopy. 103.7% yield – solvent added to yield as spectrum, seen below, is clean of any impurities.

A)



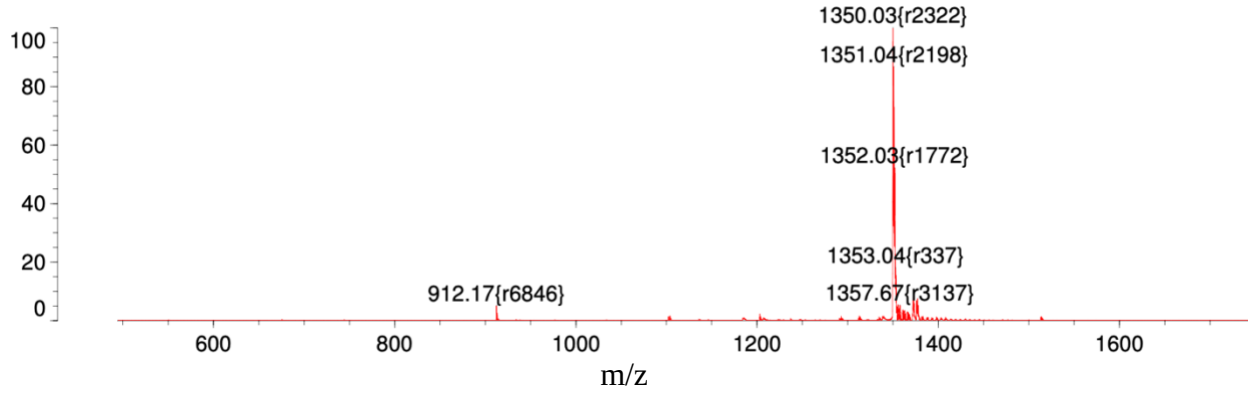
B)



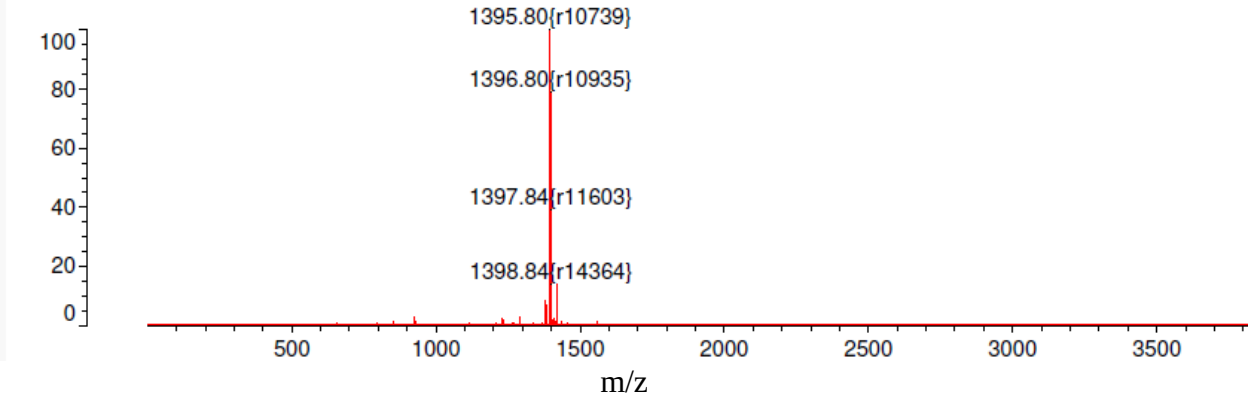
Procedure 13. Solid-phase peptide general synthesis. A collection of Fmoc-protected amino acids are deprotected and consecutively reacted to elongate the peptide chain. Sufficient quantity of HATU:DMF solution was mixed: 2.5 mL per each amino acid. 0.2 g of rink amide resin was swelled with DMF for five minutes in a fritted syringe. At one amino acid at a time, micropipette 2.5 mL of HATU solution into falcon tube with 0.1 mmol of amino acid. 500 µL of DIPEA was added to the amino acid falcon tube immediately before amino acid solution was poured into fritted syringe for 15 minutes. Drained syringe and washed with DMF 5x. Fmoc deprotection – 5 mL of 20% piperidine in DMF was added to resin for 5 minutes. Solution was drained and resin washed with DMF. Repeat with each amino acid until peptide is constructed and wash with DCM 3x.

Procedure 14. Attachment of 2-(Methylsulfonyl)-*N*-2-propyn-1-yl-6-benzothiazolecarboxamide to peptide. A) The peptide, H_3N-RGKLLGFPCys(S-tBu)F-resin, is reduced from a disulfide bond to a thiol product. 0.2g of resin was swelled with 5 mL of DMF for 10 minutes in a fritted syringe. In an alternate flask, 5 mL of DMF, 2.0 mmol of dithiothreitol, and 2.0 mmol of NEt_3 was mixed together. Resin was drained of DMF, and the DMF/DTT/NEt_3 solution was added and reacted for 1hour. Washed resin with DMF 5x and stored in 0°C.[6] **B)** The thiol is reacted to produce a sulfide bond between the linker and the peptide. The thiol, at 0.05 g of resin, was reacted for one hour with 2.5 mL of DMF, 0.47 mmol of NEt_3 and 0.26 mmol of benzothiazole linker after resin had swelled in DMF for ten minutes. The resulting resin was washed with DMF 5x. Small portion was cleaved from resin and analyzed with MALDI (Procedure 15). Product weighed 1350.67 m/z. **C)** The sulfide is oxidized to produce the sulfone cleavable linker. The resin, 0.05 g, was swelled for 15 minutes with dioxane in a fritted syringe and subsequently drained. mCPBA, 0.25 mmol, was added to 1.19 mL of dioxane and mixed into the fritted syringe. Washed with DMF 2x, 1:1 DMF:H_2O 2x, DMF 2x, and DCM 2x. Small portion was cleaved from resin and analyzed with MALDI (Procedure 15). Product weighed 1397.64 m/z.

B)

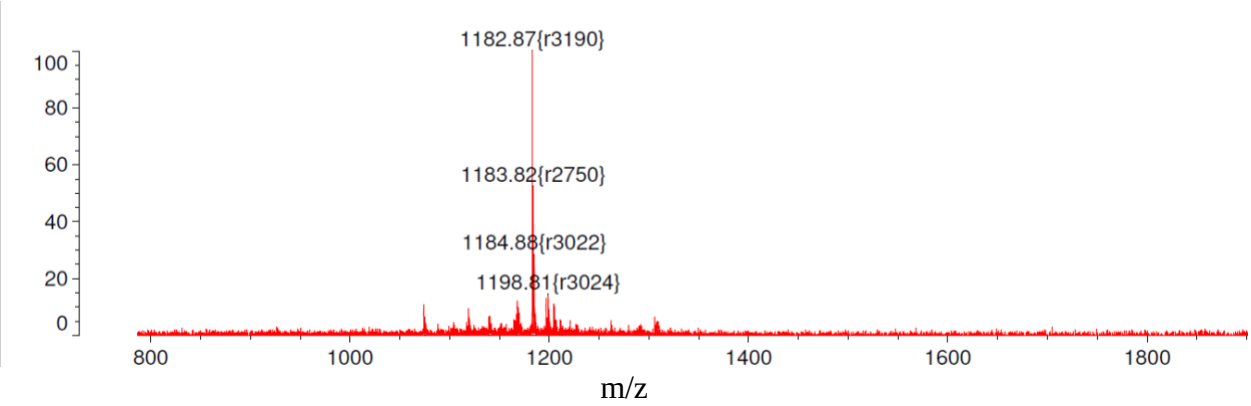


C)



Procedure 15. Cleavage of peptide from resin. In a falcon tube, 0.2 g of resin was mixed with 4.7 mL of trifluoroacetic acid (TFA), 125 µL of H_2O, and 50 µL of triisopropyl silane (TIPS) for 2 hours. The resin was then dried with nitrogen gas to neutralize the TFA in solution, and 40 mL of 0°C ether was added. The falcon tube was subjected to the centrifuge, and the solvent layer was decanted off the peptide chains. The resulting solids were then washed with 2 mL of 95:5 H_2O:MeCN where the solvent now contains the generated peptide in solution. Conduct MALDI analysis and store in freezer. If necessary, subsequent purification can be achieved through reverse-phase flash chromatography.

Procedure 16. Cleavage of benzothiazole on peptide. Reactant has been cleaved from resin at this stage (Procedure 15). The benzothiazole reacted to produce sulfonic acid on peptide. 2 µL benzothiazole peptide (1398.67 m/z) in 95:5 H_2O:MeCN, was mixed with 1 µL of 200 mM of TCEP, 0.5 µL of 1 M pH 8 phosphate in a total volume of 20 µL for 3 hours at rt. The reaction was then analyzed utilizing the MALDI spectra. Sulfonic acid product weighed 1183.62 m/z.



[1] Yang, Y., Fonović, M., & Verhelst, S. H. (2017). Cleavable linkers in chemical proteomics applications. *Methods in molecular biology (Clifton, N.J.)*, *1491*, 185–203. https://doi.org/10.1007/978-1-4939-6439-0_14

[2] Sheyi, R., de la Torre, B. G., & Albericio, F. (2022). Linkers: an assurance for controlled delivery of antibody-drug conjugate. *Pharmaceutics*, *14*(2), 396. https://doi.org/10.3390/pharmaceutics14020396

[3] Dubowchik, G. M., & et. al. (2002). Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and antigen-specific in vitro anticancer activity. *Bioconjugate chemistry*, *13*(4), 855–869. https://doi.org/10.1021/bc025536j

[4] Wu, G., Fang, Y. Z., & et. al. (2004). Glutathione metabolism and its implications for health. *The Journal of nutrition*, *134*(3), 489–492. https://doi.org/10.1093/jn/134.3.489

[5] Liu, R., Wang, R. E., & Wang, F. (2016). Antibody-drug conjugates for non-oncological indications. *Expert Opinion on Biological Therapy*, *16*(5), 591–593. https://doi.org/10.1517/14712598.2016.1161753

[6]Tuang, S., & et. al. (2021). A reactive peptide interface for site-selective cysteine bioconjugation. *Royal Society of Chemistry*. 4.