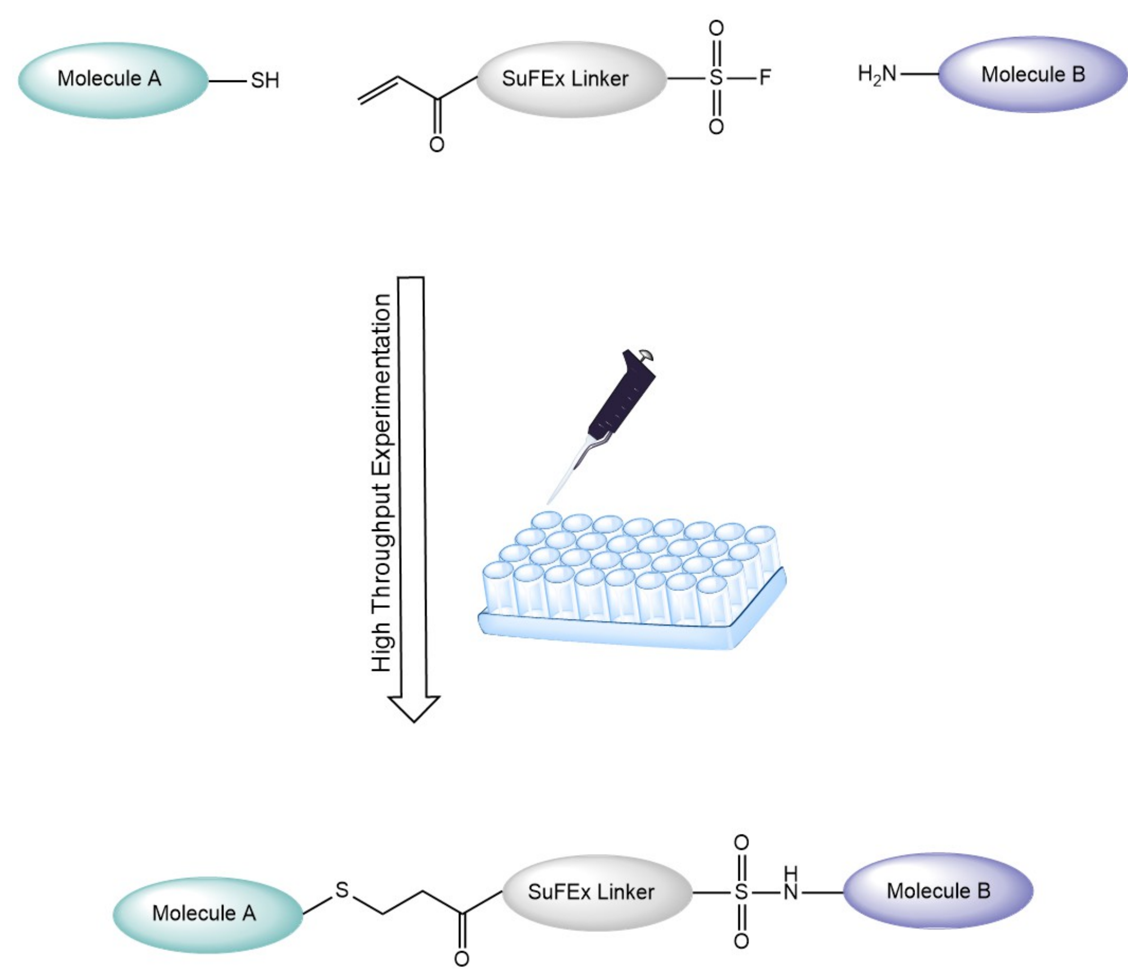

Conjugation of biologically relevant molecules using SuFEx reagents

Master's thesis in Material's Chemistry

MALIN FUHRMAN

DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2025
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MASTER'S THESIS 2025

Conjugation of biologically relevant molecules using SuFEx reagents

Malin Fuhrman

Department of Chemistry and Chemical Engineering
Division of Chemistry and Biochemistry
CHALMERS UNIVERSITY OF TECHNOLOGY
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Conjugation of biologically relevant molecules using SuFEx reagents:
Malin Fuhrman

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Supervisor: Dr. Maximilian Häfner, AstraZeneca
Examiner: Prof. Jerker Mårtensson, Department of Chemistry and Chemical Engineering

Master's Thesis 2025
Department of Chemistry and Chemical Engineering
Division of Chemistry and Biochemistry

Chalmers University of Technology
SE-412 96 Gothenburg
Telephone: +46 31 772 1000

Cover: Linking together molecule A and molecule B using SuFEx linkers in a high throughput format.

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Malin Fuhrman
Department of Chemistry and Chemical Engineering
Chalmers University of Technology

Abstract

Sulfur fluoride exchange (SuFEx) chemistry is a type of click chemistry utilizing the reactivity of the S(VI)-F bond, where nucleophiles can selectively attack the sulfur center and exchange with fluoride upon activation with e.g., Lewis acids. SuFEx chemistry has the potential to be a simple and easy strategy to create new molecular connections by forming covalent S-N, S-O and S-C bonds. In this project, bi-functional SuFEx linkers have been investigated with the purpose of creating novel conjugates of biologically relevant molecules.

Six novel bi-functional SuFEx linkers were synthesized in the project. All contain a SuFEx group for reaction with amines, and another functionality for reaction with thiols. The SuFEx linkers were screened in a high throughput format where a two-step, one-pot reaction was performed, using the linker to join together a model thiol and model amines. Two self-synthesized and two commercially available SuFEx linkers were compared in different reaction conditions. The results showed proof of concept, where all screened linkers successfully formed the thiol intermediate and the SuFEx product, however, the SuFEx reaction is highly dependent on the choice of reactions conditions and the properties of the amine.

SuFEx linkers were finally used to form conjugates between biologically relevant molecules containing either a thiol or an amine. All but one linker successfully yielded the thiol intermediate, however, all failed to produce the SuFEx product. Further research is required to elucidate the reasons behind this and to find and improve better reaction conditions.

Keywords: Click Chemistry, SuFEx, HTE, Conjugation

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Malin Fuhrman, Gothenburg, June 2025

Table of contents

List of Abbreviations **2**

1 Introduction **3**
1.1 Aim and Importance . . . 6
1.2 Scope and Limitations . . . 8

2 Theory **9**
2.1 SuFEx Linkers . . . 9
2.1.1 The SuFEx group . . . 9
2.1.2 The thiol accepting group . . . 10
2.2 High Throughput Experimentation . . . 11
2.2.1 Reactions and Mechanisms . . . 12
2.3 Purification . . . 12
2.3.1 Column chromatography . . . 12
2.3.2 Aqueous work-up . . . 13
2.4 Analysis . . . 14
2.4.1 TLC . . . 14
2.4.2 LC-MS . . . 14
2.4.3 NMR . . . 15

3 Results and Discussion **17**
3.1 Synthesis of SuFEx linkers . . . 17
3.2 High Throughput Experimentation . . . 21
3.2.1 Determination of conditions for thiol addition . . . 23
3.2.2 Determination of conditions for the SuFEx reaction . . . 24
3.2.3 Heat Maps . . . 24
3.2.3.1 Thiol Addition . . . 25
3.2.3.2 SuFEx reaction . . . 26
3.3 Linker Screen . . . 29
3.3.1 Thiol addition . . . 30
3.3.2 SuFEx reaction . . . 34
3.4 Conjugation . . . 39

4 Conclusion and Outlook **51**

5 Experimental **54**
5.1 Synthesis of SuFEx linkers . . . 54
5.2 High Throughput Experimentation . . . 59
5.3 Linker Screen . . . 62

5.4 Synthesis of starting materials for conjugation 63
5.5 Synthesis of conjugates 68

References 71

A First appendix A-1
A.1 SuFEx Linkers A-1
A.2 Conjugation A-17

List of Abbreviations

DABCO	1,4-diazabicyclo[2.2.2]octane
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DIPEA	*N,N*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EtOAc	Ethyl Acetate
iPrOH	Isopropanol
MeCN	Acetonitrile
Me-THF	2-Methyltetrahydrofuran
***t*-AmylOH**	*Tert*-Amyl alcohol
TCEP	Tris(2-carboxyethyl)phosphine
TEA	Triethylamine
TFA	Trifluoroacetic acid

1. Introduction

Click chemistry is a synthetic strategy enabling easy formation of carbon-heteroatom and heteroatom-heteroatom linkages [1], and was first coined by Sharpless in 2001 as a response to the synthetic challenges posed by many drug candidates in the pharmaceutical industry. The idea was to explore different types of linking chemistry that could be used to build molecules in a simple way, in order to decrease time and costs during the drug discovery process [2].

In order for a reaction to be classified as click chemistry, it must fulfill a list of criteria. The most important requirements are that the reaction must be simple to perform and generate high yields of the product in a reliable manner. Purification steps should be limited and the reaction should have applications in many areas and use only commercially available starting materials [2]. There are four main categories of click reactions, namely cycloaddition reactions (e.g., Diels-Alder cycloadditions), nucleophilic substitution reactions, (e.g., ring-opening of strained rings such as epoxides and aziridines), carbonyl condensation reactions and addition reactions (e.g., Michael addition) [3]. The first reaction to be described as click chemistry in 2001 was the cycloaddition between an alkyne and an azide, catalyzed by Cu(I) (the 'CuAAC' reaction) [2]. This reaction is regarded as *the* click reaction [1].

Figure 1.1: Cu(1) catalyzed cycloaddition of an alkyne and an azide.

Being biocompatible, click chemistry is an excellent strategy for use in drug discovery and is also heavily used in medicinal chemistry [4]. For example, click chemistry in combination with high throughput strategies can accelerate the drug discovery process by forming libraries of drug candidates to be screened for e.g., targets or cellular assays [5]. The CuAAC reaction has been used to form peptide-peptide conjugates [5] and conjugates of oligodeoxyribonucleotides (ODNs) and small fluorescent molecules for the purpose of labeling [6] (figure 1.2). It has also been used in library syntheses of small molecule enzyme inhibitors such as protein tyrosine phosphatasesenzyme (PTP) warheads and protein kinases inhibitors [5]. Furthermore, the Diels-Alder click reaction has been used to synthesize many drugs such as Bisantrene (to treat leukemia), Benzoctamine (against anxiety) and Oselmivir (influenza medication) but has also been used in bioconjugation and in the synthesis of biologially active molecules [4].

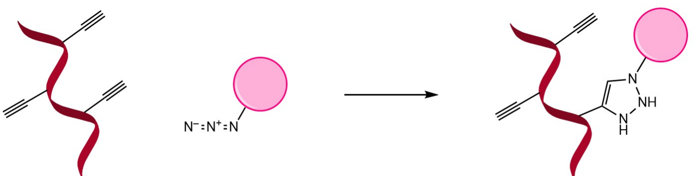


Figure 1.2: Modifying ODNs with fluorescent small molecules using CuAAC click chemistry [6].

Sulfur fluoride exchange (SuFEx) chemistry was first described by Sharpless et al.~ in 2014 [7] and is a type of click chemistry utilizing the stability and reactivity of the S(VI)-F bond. The S(VI)-F bond is thermodynamically stable and resistant to acidic and basic conditions and compared to its S-Cl sibling, it is also resistant to reduction and to nucleophilic attack. The reactivity is however unlocked when fluoride, a poor leaving group and strongly bonded to sulfur, is activated by coordination to H^+ or R_3Si^+ [7] or by addition of a Lewis acid or base [8]. This allows for substitution to occur upon collision with a nucleophile, and due to the high electronegativity of fluorine, nucleophilic attack occurs selectively at the sulfur center resulting in exchange of fluoride [7].

Figure 1.3: Reactivity of sulfur fluoride exchange (SuFEx).

SuFEx molecules contain an S(VI)-F bond that can undergo SuFEx reactions. Different variations exist, including sulfuryl fluoride (SO_2F_2), sulfonyl fluoride (RSO_2F) and sulfamoyl fluoride ($R_1R_2NSO_2F$).

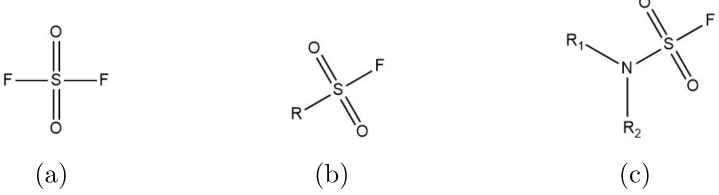


Figure 1.4: Examples of common SuFEx functional groups: (a) sulfuryl fluoride, (b) sulfonyl fluoride and (c) sulfamoy fluoride.

Sulfuryl fluoride is a harmful gas normally used as fumigant [9], and displays high reactivity towards phenols and secondary amines [10]. **Sulfonyl fluorides** react with oxygen, nitrogen or carbon-based nucleophiles, whilst **sulfamoyl fluorides** are less explored and have only been reported to react with nitrogen-based nucleophiles [8].

SuFEx connectors can thus be used to create molecular connections by forming covalent S-O, S-N and S-C bonds between molecules. There is an increasing number

of applications in bio-conjugation [11], medicinal chemistry [12], polymer chemistry [13], organic synthesis and drug discovery [9], to name a few. Sulfonyl fluoride groups on drug candidates can inhibit enzyme function by covalently link to amino acids on the active sites [1], and the use of sulfuryl fluoride for late stage functionalization of phenols showed improved anticancer activities for certain compounds [14]. The fact that the SuFEx reaction proceeds under metal–free conditions is especially important for applications in biology and drug discovery [1].

Thionyl fluoride (SOF_4) derived reagents can react with primary amines on the protein bovine serum albumin as a way to modify proteins via bio-conjugation [11], and the sulfonyl fluoride group has been shown to act as covalent warhead for the inhibition of the Glutathione S-transferase (GST) Pi enzyme, which is present to a great extent on various types of cancer cells [15].

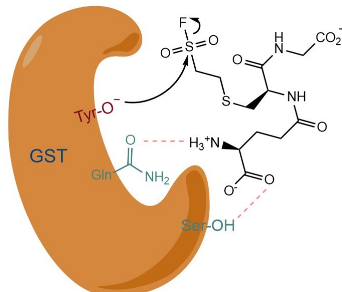


Figure 1.5: Sulfonyl fluoride as a covalent warhead binding to GST Pi enzyme [15].

Furthermore, sulfonyl fluoride groups on stapled alpha-helical peptides are used to target His252 on hMcl-1, another protein that is abundantly expressed on cancer cells, by covalently bind to the imidazole on histidine via a SuFEx reaction [16].

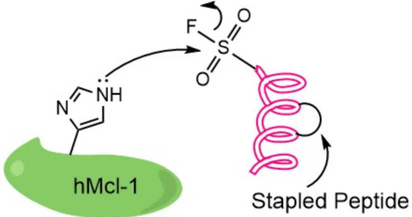


Figure 1.6: Stapled alpha-helical peptides used to target His252 on hMcl-1 via a SuFEx reaction [16].

SuFEx molecules containing another type of functionality can serve as bi-functional linkers. An example is ethene sulfonyl fluoride (ESF), which apart from its SuFEx moiety contains an activated double bond to which nucleophiles can add.

Figure 1.7: Ethene sulfonyl fluoride (ESF).

The advantage compared to other SuFEx molecules is that the bi-functional linkers contain different types of reactivity that can be used to form molecular connections [1]. In this project, different bi-functional SuFEx linkers have been investigated. All contain a SuFEx functionality (sulfonyl fluoride or sulfamoyl fluoride) to which amines can exchange with fluoride, and one functionality with which thiols can react, either via conjugate addition or via substitution.

1.1 Aim and Importance

The aim of the project was to compare different bi-functional SuFEx linkers for the purpose of linking together biologically relevant molecules. The project was divided into three parts, where the first part consisted of synthesizing bi-functional SuFEx linkers containing a SuFEx group and a functionality to which thiols can add (the thiol accepting group). In the second part of the project, synthesized and commercially available SuFEx linkers were screened for amine and thiol reactivity in a high throughput format. The SuFEx linkers were compared whilst also exploring different reaction conditions. Product yield was used to determine the optimal conditions for an extended comparison between additional linkers. The final part of the project consisted of conjugating biologically relevant molecules using the results from the screening processes. The amine functionality is abundant in drug molecules and is therefore a good target for conjugation [17], and primary and secondary amines and anilines on commercial small molecule drugs were used to react with the SuFEx group on the linker. Thiols on various drug modalities could add to the linker via conjugate addition or via substitution.

SuFEx linkers provide a simple route to create molecular connections [9] and to the best of my knowledge the linkers investigated in this project are under–explored in the scientific community. The demand for fast, efficient and physiologically compatible methods to form chemical conjugations increases, and versatile approaches to building complex molecules are therefore essential in order to meet these demands [11]. Investigating whether the SuFEx linkers in this project could be used to conjugate biologically relevant molecules is thus important as this can expand the current linker chemistry available. The drug modalities included in this project were a peptide, a dye, lipids, a biotin derivative and an E3 ligase ligand. Each of these classes of molecules play important roles in science and drug discovery.

Peptide therapy has become increasingly important in medicine as peptides usually exhibit good binding to molecular targets with high specificity and potency. By conjugating a peptide to a small molecule drug, the aqueous solubility, metabolism and cell permeability of the drug can improve whilst also allowing targets to be reached inside the cell, something that previously has been a limitation to peptide-based therapeutics. Additionally, the peptide can provide targeted delivery to the right cells, which can increase the local concentration of the drug, and also decrease accumulation at healthy cells and thus limit toxicity. Some examples of peptide-based therapeutics include glucagon-like peptide-1 agonists (GLP-1) and somatostatins (growth hormone-inhibiting hormone). Somastostatin conjugated with small molecule

radioisotope ^{177}Lu displayed higher efficacy and improved safety for cancer treatment compared to the individual constituents alone [18].

Figure 1.8: A peptide conjugated to a small molecule drug via a linker.

Proteolysis-targeting chimeras, or PROTACs, are bi-functional small molecules intended to degrade protein and consist of two small molecule ligands connected by a linker. One ligand binds to the protein target whilst the other (the **E3 ligase ligand**) binds to an E3 ubiquitin ligase. The small molecule PROTAC enables protein degradation by the E3 ligase due to the close proximity of the two entities [19]. PROTACs are an interesting group of new modality recently reaching clinical trial in several occasions, and improving the linker part of the molecule is an important part of the extensive optimization process required due to the increased complexity of the larger molecule [20].

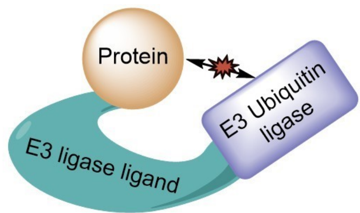


Figure 1.9: The mechanism of protein degradation via E3 ligase ligand binding.

Small molecules conjugated to **fluorescent dyes** enables imaging of drug candidates to elucidate transport and distribution pathways on cellular levels *in vitro* and *in vivo*, thus aiding in the time-consuming process of drug optimization [21]. To modify drug properties, small molecule drugs can be conjugated to **lipids** in order to make them more lipophilic. This can have positive effects such as improved bioavailability via increased cell membrane interaction, and increased blood–brain barrier penetration for compounds that have to be lipophilic in order to pass. It can also alter drug release profiles to allow release for an extended period of time with fewer dose administrations as a consequence [22]. **Biotinylation** is the process of attaching biotin (vitamin B_7) to biologically active molecules, and it can be used for small molecules drugs to elucidate interactions with the protein target [23]. Biotinylation has also showed increased uptake of small molecule drugs, especially in cancer cells [24] as they have an increased number of biotin uptake systems [25].

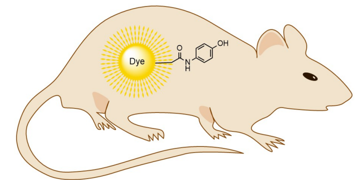


Figure 1.10: A fluorescent dye elucidating the pathway of a drug.

1.2 Scope and Limitations

The overall goal of the project was to produce final conjugates using SuFEx linkers synthesized in the project. Sub-goals included successfully synthesizing SuFEx linkers and testing them in a high throughput format. To ensure that this would be possible in the 20 weeks that the project progressed, the time allowed for linker synthesis was limited to 10 weeks. The synthesized SuFEx reagents were limited to sulfamoyl fluoride linkers as this SuFEx moiety can be attached using a solid imidazolium salt and an amine, a procedure already described in literature. High throughput experiments (HTE) were limited to 96 reactions in order to fit in one reaction plate, and the number of HTE screens performed was limited to one. The number of conjugates was limited to a maximum of five, including five small molecule drugs, five different drug modalities and five different linkers. Each molecule was part of only one conjugate.

2. Theory

This chapter covers the theory behind the synthesis and reactions of the different SuFEx linkers used in the project, as well as of the analysis and purification methods used.

2.1 SuFEx Linkers

The first part of the project consisted of synthesizing the SuFEx linkers that were to be used to conjugate biologically available molecules. The SuFEx linkers in this project contained a SuFEx group and an thiol accepting group (for thiol addition) connected by a linker backbone. Figure 2.1 displays a representative example of such a linker, where the SuFEx group is the sulfamoyl fluoride and the thiol accepting moiety is the activated double bond of the acrylate.

Figure 2.1: A representative example of a SuFEx linker synthesized in the project.

2.1.1 The SuFEx group

The SuFEx group on the linkers used in the project were of two types: sulfamoyl fluorides and sulfonyl fluorides.

Sulfamoyl fluorides
The SuFEx group on the synthesized linkers were in most cases of the sulfamoyl fluoride type ($R_1R_2NSO_2F$). A common method to synthesize sulfamoyl fluorides is to use sulfuryl fluoride (SO_2F_2) but because this reagent is both harmful and a gas, it comes with some challenges [8]. Laboratories require a special setup to handle harmful gas, and there might be regulatory limitations in the use of sulfuryl fluoride. Additionally, the reaction between sulfuryl fluoride and amines to yield sulfamoyl fluorides are not as straightforward as with phenols due to slow reaction times, complicated reaction conditions and undesired elimination reactions. To avoid these problems, a solid imidazolium salt was developed by Sharpless and co-workers in 2018 [26], which possesses a greater reactivity towards nitrogen nucleophiles and give good yields of sulfamoyl fluorides without the addition of base or activating agents, something that is required when using sulfuryl fluoride. Other advantages

include shorter reaction times and the fact that the imidazolium salts are solid, which makes the synthesis simpler [26]. For these reasons, this is the method used in this project. The overall reaction is displayed in figure 2.2 below.

Figure 2.2: The overall reaction of making sulfamoyl flourides using imidazolium salts.

Sulfonyl fluorides
The SuFEx group on the commercially available linkers used in this project were of the sulfonyl fluoride type (RSO_2F). Sulfonyl fluorides can be synthesized using gaseous sulfuryl fluoride or sulfur dioxide, however solid reagents are desired due to practical reasons. For example, DABSO forms sulfonyl fluorides by reaction with Grignard reagents and sulfonyl chlorides can be converted to sulfonyl fluorides by reaction with KHF_2, [8]. Below is an example of a SuFEx linker with a sulfonyl fluoride group.

Figure 2.3: Ethene sulfonyl fluoride (ESF) bearing a sulfonyl fluoride group.

2.1.2 The thiol accepting group

The thiol-accepting group on the SuFEx linkers were Michael acceptors for conjugate addition of the thiol, and alkyl bromides for substitution reactions. The different thiol accepting groups installed on the molecules were chosen based on what is commonly used in bio-conjugation of thiols [27], [28], [29]. They were installed on the compounds via amide formation, using different reagents for different linkers. A representative reaction is shown below, where amide formation is successful using acryloyl chloride.

Figure 2.4: A representative example of installing the thiol accepting group via amide formation.

2.2 High Throughput Experimentation

The second part of the project consisted of screening successfully synthesized SuFEx linkers for amine and thiol reactivity in a high throughput format. High Throughput Experimentation (HTE) is a method used to run a large number of reactions simultaneously to allow for fast experimentation [30]. HTE was originally a technique used in biology but is currently used in chemistry for e.g., research on novel synthetic strategies, optimization of reaction conditions such as solvents or catalysts [31] or to quickly synthesize many compounds for use in drug discovery [32]. By systematically planning and performing reactions in a high throughput format, scientists can gain a better understanding of e.g., specific reaction steps, reaction mechanisms or reagent scope by running reactions in an extensive array of conditions. The small scale results in minimal use of reagents, and the lack of purification steps and work-up generates results quickly [31].

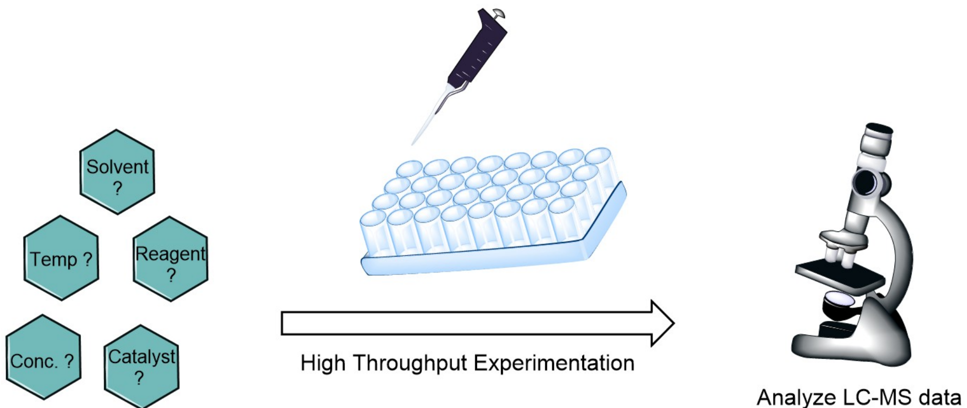


Figure 2.5: High Throughput Experimentation.

The purpose of using the SuFEx linkers in HTE was to connect an amine with a thiol using the two different types of reactivities of the linker. The general reaction is displayed below using ethene sulfonyl fluoride (ESF) as SuFEx linker and a model thiol and amine.

Figure 2.6: The general reaction performed in HTE.

The reaction is a two-step reaction, where the thiol is added before the amine. As the amine can react both with the SuFEx group and the alkyl bromide/Michael acceptor, it must be added after the thiol has had time to completely add to the thiol accepting moiety, leaving only the SuFEx group available for attack by the amine.

2.2.1 Reactions and Mechanisms

Thiols can add to the SuFEx reagents via a substitution mechanism, where the thiol substitutes a bromide.

Thiols can add to the SuFEx reagents via conjugate addition to an activated alkene or alkyne.

Adding an amine to the SuFEx group goes via a substitution mechanism, and is the same for primary and secondary amines and anilines.

2.3 Purification

After synthesis, compounds need to be purified to obtain the pure product. The different methods for purification that were used during this project are described below.

2.3.1 Column chromatography

Column chromatography is a method used to separate compounds from a chemical mixture in order to isolate the desired product. The sample is injected into a column filled with a stationary phase, and a mobile phase is passed through the column. In this project, liquid chromatography was used, where the mobile phase is a liquid.

There are two options: 1) polar stationary phase and nonpolar mobile phase (normal phase chromatography) and 2) nonpolar stationary phase and polar mobile phase (reversed phase chromatography). As the stationary phase and the mobile phase differ in polarity, compounds will interact with varying strength to either phase depending on the polarity of the compound itself. Due to differences in polarity, compounds in the chemical mixture retain on the column to varying extents and will elute at different times, which makes separation possible [33].

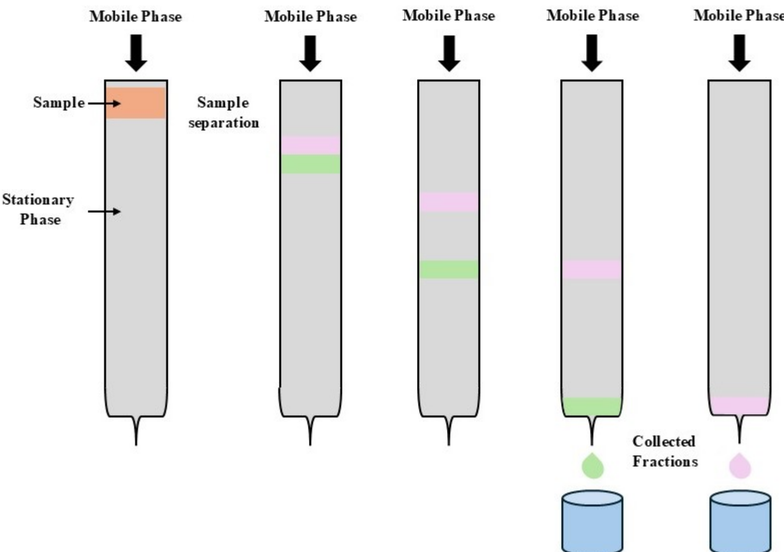


Figure 2.7: A schematic overview of separation via column chromatography.

In this project, Biotage® flash column chromatography was used. For normal phase chromatography, the stationary phase then consists of silica spheres and for reversed phase C_{18} or C_8 carbon chains [34]. For normal phase chromatography, the mobile phase can consist of e.g., ethyl acetate in heptane and for reversed phase of acetonitrile in water [35]. A gradient system is used, where the composition of the mobile phase is adjusted during the separation to allow for compounds with different polarity to elute in a reasonable time frame [33].

2.3.2 Aqueous work-up

Work-up is the process of isolating the product from the reaction mixture and include for example filtration, distillation and extraction. When the reaction mixture contains compounds that are soluble in aqueous and organic media, an aqueous work-up can be performed to separate aqueous compounds from organic products using their differences in solubility. To the reaction mixture is added an aqueous solvent (such as water) and the mixture is transferred to a separating funnel and extracted with an organic solvent (such as ethyl acetate). Organic compounds will prefer the organic layer, and aqueous compounds will stay in the aqueous layer. Addition of acid and/or base can make molecules such as organic amines or acids more water soluble and thereby exclude them from the organic layer. The extraction can be done in steps to exclude one or more compounds at a time until only the

product dissolves in the organic layer. A drying agent can finally be used to remove residual water from the organic layer [36].

2.4 Analysis

To analyze reactions mixtures and products throughout the project, the following methods were used.

2.4.1 TLC

Thin Layer Chromatography, TLC, is a type of liquid chromatography that uses a plate with a thin ($\sim$ 0.25 mm) layer of absorbing material as the stationary phase [37]. The mobile phase is a liquid in which the plate is placed. TLC is used to separate compounds in a chemical mixture by the difference in polarity between them. In this project, the stationary phase was polar silica gel and the mobile phase was usually a mixture of ethyl acetate and heptane. The sample is placed on the TLC plate, which is slightly submerged in the mobile phase allowing the molecules in the mixture to interact with both phases [38], as they move up the plate due to capillary forces [37]. Polar molecules will interact to a greater extent with the polar silica gel than with the less polar mobile phase and will therefore retain on the plate close to the starting point. Less polar compounds will on the other hand display a stronger interaction with the mobile phase than the stationary phase, and will therefore migrate farther up the plate together with the mobile phase [38]. After the plate is taken out of the mobile phase and the solvent has evaporated, spots of different components from the mixture can be detected by e.g., UV detection or by staining. In this project, potassium permanganate ($KMnO_4$) was used as stain the majority of the time. Permanganate oxidizes functional groups such as alcohols, alkenes, ethers, amines, amides, esters etc. to form MnO_2 which shows up as yellow spots on the TLC plate upon heating [39].

2.4.2 LC-MS

LC-MS stands for Liquid Chromatography with Mass Spectrometry and is an analytical method that separates compounds from a chemical mixture and subsequently identifies them based on their mass. In this project, the separation is provided by reversed phase High Performance Liquid Chromatography (HPLC). The stationary phase is nonpolar and consists of a column covered with C_8 or C_{18} carbon chains bound to porous silica particles. The particles are small (3-10 µm) and tightly packed to increase the surface area of the stationary phase. Smaller particles create smaller interstitial spaces for the mobile phase to occupy, and thus a high pressure is required to push the mobile phase through the column. In reversed phase HPLC, the mobile phase is polar and can for example be a mixture of water and acetonitrile. Less polar compounds will remain on the stationary phase to a greater extent and will elute at a later time than polar molecules that prefer to interact with the mobile phase rather than with the stationary phase. The composition of the mobile phase can be adjusted during the separation to form a gradient, going from more polar to less

polar during the separation. The varying polarity will ensure that compounds with different polarity still elute in a reasonable time frame [33].

The mass spectrometer ionizes the compounds eluted from the column, and can also cause fragmentation of the molecules into smaller parts. The ionization makes it possible to separate them based on their mass-to-charge ratio using a magnetic or electrical field, and a detector counts the number of ions to obtain both quantitative and qualitative information [33]. There are different types of ion sources and mass analyzers. In Electron Impact Ionization, the sample is evaporated and passed through an electron beam forming positive ions. The ions are unstable and fragment, and the method is classified as a hard ionization method [40]. Electrospray Ionization converts the sample into a charged spray of droplets that will ionize the compounds as solvent molecules are evaporated. This method does not impact the molecules with much energy and they are therefore not very fragmented [41]. The Time of Flight (TOF) mass analyzers measure the distance that ions travel after being accelerated to reach the same kinetic energy. Their mass-to-charge ratio gives them different velocities which makes it possible to separate them by mass [42]. Quadrupole analyzers on the other hand use a set of four parallel metal rods through which molecules with a certain mass-to-charge ratio can travel as the voltage is varied [41].

2.4.3 NMR

Nuclear Magnetic Resonance (NMR) utilizes the quantum property of spin angular momentum of protons and neutrons in atomic nuclei. The spins of the elementary particles couple to create a net spin, which exist for all atomic nuclei with an odd atomic number. The nuclear spins give rise to a magnetic field [43] and because spin is a quantum property, the spin states of atomic nuclei are quantized with discrete energy levels. The most common nuclei for NMR are ^{1}H and ^{13}C, and ^{1}H NMR will be discussed for conceptual purposes. The hydrogen nucleus has two spin states, and the lack of an external magnetic field will result in two degenerate states with the same energy [44]. However, once an external magnetic field is applied to the nucleus, the energy levels split and become separated by an energy difference. Nuclei can align with the magnetic field and occupy a lower energy level or align against the external field and occupy a higher energy level [43]. During an NMR experiment, an oscillating magnetic field supplies the energy required to excite nuclei in the lower energy level to the upper [44], and the frequency at which the transition occurs is called the resonance frequency [43]. The excited nuclei will eventually relax back to the lower energy level and in doing so they emit energy corresponding to the energy difference between the two spin states. The NMR machine records the frequency at which the relaxation occurs and measures the intensity to produce a signal [43].

The reason NMR can be used to characterize molecules is that different nuclei resonance at different frequencies depending on their chemical environment. The chemical environment of a nucleus is determined by the electron cloud surrounding it, which in turn is affected by electron densities on neighboring atoms [44]. Electron densities generate their own magnetic fields that work opposite the applied magnetic

field and thus cause the nuclei to experience a smaller net magnetic field. This effect of subtracting from the applied magnetic field is called *shielding* and the extent at which it will occur depends on the electronic environment around each nucleus, giving rise to different resonance frequencies. However, for nuclei close to electron withdrawing substituents, the opposite occurs. These substituents will pull electron density from the nucleus and decrease the shielding effect, and they are said to be *de-shielding*. The protons will therefore experience a larger net magnetic field than shielded protons and the resonance frequencies become higher [43]. The frequency at which resonance occurs is called the chemical shift, and is reported relative to a reference [44].

For ^{1}H NMR, an additional feature is present. For a CH_2 proton, there are four combinations of spin states for the two nuclei. Both nuclei can occupy the upper energy level, the lower energy level state or occupy different energy levels. Because there are two alternatives of the two protons occupying different energy levels, the signal intensity of them doing so will be twice the intensity of both of them occupying different levels, and the intensity ratio is thus 1:2:1. A proton on an adjacent carbon will therefore interact with the protons on the CH_2 in three different ways, as there are three spin combinations to interact with. Its signal will thus be split into three according to the 1:2:1 ratio. The result is called signal splitting, and affects protons that are able to interact with protons on adjacent carbons. The integrated peak area intensities for each signal correspond to the number of protons giving rise to the signal, and can be used to deduce which nuclei correspond to which peak in the NMR spectrum [44].

3. Results and Discussion

This chapter covers the results and discussion on the different parts of the project: linker synthesis, high throughput experimentation, linkers screen and conjugation.

3.1 Synthesis of SuFEx linkers

Six SuFEx linkers were successfully synthesized in the project, and are presented in table 4.1. L1, L2, L4 and L6 were synthesized via a three step synthesis where the SuFEx group was installed first using a solid imidazolium salt according to a procedure described in litearture [26]. The starting materials were Boc-protected amines and the resulting SuFEx groups were thus sulfamoyl fluorides. Two Boc-protected amines were used, which allowed the SuFEx linkers to have different degree of flexibility. After the SuFEx group had been installed, the Boc-group was deprotected and the thiol accepting group was added to the compound via amide formation. L3 and L5 were each synthesized in one step; L3 via amide formation to create a sulfonyl fluoride linker, and L5 via installation of the sulfamoyl fluoride group using the imidazolium salt. The different thiol accepting groups installed on the molecules were chosen based on what is commonly used in bio-conjugation of thiols [27], [28], but also based on what was commercially available.

Table 3.1: SuFEx linkers synthesized in the project.

Name	Structure	Molecular Weight	Label
4-(but-2-ynoyl)piperazine-1-sulfonyl fluoride		234.245 g/mol	L1
(3-(2-bromoacetamido) propyl) sulfamoyl fluoride		277.108 g/mol	L2
3-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl) carbamoyl)benzene sulfonyl fluoride		326.298 g/mol	L3
4-acryloylpiperazine-1-sulfonyl fluoride		222.234 g/mol	L4
(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)sulfamoyl fluoride		222.190 g/mol	L5
4-(2-bromoacetyl) piperazine-1-sulfonyl fluoride		289.119 g/mol	L6

Adding the sulfamoyl fluoride group to the Boc-protected amine **2** using **1** gave the SuFEx product **3** in good yield.

Subsequently adding the thiol accepting group was first attempted with the NHS-esters **5** and **6**, but in both cases it formed the undesired addition and substitution products **7** and **8**, respectively.

The reason is assumed to be the bulkiness of the secondary amine, making it sterically more accessible to attack the activated double bond or the alkyl bromide rather than the carbonyl of the NHS-esters **5** and **6**. L6 was also attempted by reacting piperazine **4** with bromoacetyl bromide **9**, but despite the relief in steric hindrance, the reaction was unsuccessful.

However, using the less sterically hindered reagent acryloyl chloride **10** to install the acrylate on piperazine **4** gave L4 in good yield upon scaling up the reaction to 1 g of piperazine **4**. At smaller scale ($\sim$ 100 mg), the yield from the same reaction was about 30 %.

Butynoic acid **11** was reacted with **4** using EDC as an attempt to install the alkyne, however this yielded product in low yield ($\sim$ 10%). As the reason for this could have been the choice of coupling agent, T3P was explored as an alternative in the reaction. T3P proved to be very efficient for the amide coupling and furthermore provided pure material after aqueous work-up, making further purification unnecessary.

The success of amide formation using T3P motivated the attempt to synthesize product L6 again, using T3P and bromoacetic acid **12**, and this reaction formed the product in good yield. However, bromoacetyl chloride, a reagent ordered as bromoacetyl bromide **9** did not work, also proved to be a good reagent for the formation of L6. The increased reactivity in using the acetyl chloride instead of the acetyl bromide might be needed due to the bulkiness of the amine.

Attaching the SuFEx group onto amine **13** using **1** was also a successful reaction.

However, installing the thiol accepting groups on Boc-deprotected amine **15** came with some challenges. For example, linker **16** was obtained in extremely low yield of about 5% upon reacting **15** with acryloyl chloride **10**. This is surprising as acryloyl chloride **10** formed product of significant yield when reacted with piperazine **4**. The reason could be an intramolecular cyclization of the amine, or a polymerization reaction. The same product was also attempted by reacting **15** with the NHS-ester of the acrylate **5**, but this also yielded product only in low yield (6%). A final attempt was performed using T3P and acrylic acid, but despite the success of T3P in previous reactions, this reaction yielded no product.

As for piperazine **4**, reaction of amine **15** with butynoic acid **11** using EDC as coupling reagent was unsuccessful, and for amine 10 the reaction yielded no product at all. Perhaps EDC is not a suitable coupling reagent for these types of compounds. Amine **15** was only successful in forming a SuFEx linker when reacted with the NHS-ester of bromoacetate **6**, yielding 23% of product. The fact that this reaction worked but not with the acrylate equivalent **5** is surprising considering both reagents

are sterically comparable. The difference might be due to the conjugated system of the acrylate, which increases electron density at the carbonyl and thus decreases the electrophilicity.

As attaching the thiol accepting group had proven to be problematic, the synthesis was attempted backwards with Boc-protected amine **15** and acryloyl chloride **10**, first attaching the thiol accepting group followed by Boc-deprotection and subsequent installation of the SuFEx group. The first step proved to be a successful reaction and gave product in 40% yield. However, upon attaching sulfamoyl fluoride on **17** using **1**, no product was obtained after purification, despite the fact that **16** was UV-active in LC-MS.

The maleimide-based linker L5 was synthesized in one step forming an amide between amine **18** and carboxylic acid **19**, using T3P as coupling reagent. As for most other reactions using T3P, L3 was produced in high yield requiring only aqueous work-up to form the pure product.

However, attaching the sulfamoyl fluoride on amine **18** using the imidazolium salt **1** proved to be less successful, which is surprising considering the high yields in reactions of **1** with amines **2** and **13**. This reaction was attempted four times, with three different equivalents of base, however the highest yield recorded was only 22%.

The SuFEx linkers in this project are small and some of them are not UV-active, which make them difficult to detect in LC-MS analysis and after purification by column chromatography. For example, compound **20** was attempted twice with imidazolium salt **1** to install the sulfamoyl fluoride, but failed both times. The compound ionizes well and was clearly visible in the LC-MS analysis of the reaction mixture, however, it is not very UV-active and could not be detected after column chromatography. Another attempted linker is the polyethylene glycol (PEG) based linker **21** shown below, however due to time limitations, installation of the thiol

accepting group was never attempted. Addition of the SuFEx group was however successful and different PEG-linkers could thus be explored in future studies.

The sulfonyl fluorides presented in table 3.2 are commercially available and were used as SuFEx linkers in the project.

Table 3.2: Commercially available SuFEx linkers used in the project.

Name	Structure	Molecular Weight	Label
4-(bromomethyl) benzenesulfonyl fluoride		253.085 g/mol	L7
2-phenylvinylsulfonyl fluoride		186.20 g/mol	L8
Ethylenesulfonyl fluoride		110.102 g/mol	L9

3.2 High Throughput Experimentation

Reactions in high throughput format were performed in a 96 well plate containing 1 mL glass vials. Two subsequent reactions were performed: thiol addition (Michael addition/alkylation) and SuFEx reaction. The same 96 well plate was used for both reactions and the solvent was evaporated between steps. Due to time limitations the thiol addition reaction was performed over the weekend, while the SuFEx reaction was performed overnight. Both steps were performed at room temperature.

Two synthesized (L3 and L4) and two commercial (L7 and L8) linkers were tested in a high throughput format. The linkers were chosen based on two things: 1) to have four different thiol accepting groups: a maleimide, an acrylate, an alkyl bromide and a vinyl sulfonyl fluoride, 2) based on the amount of linker available. Each of the four linkers used in HTE were reacted with one model thiol in the first step, and with three model amines in the second step (the SuFEx reaction). The requirements on the model compounds were that they should exhibit UV activity but not have their nucleophilic group affected by other functional groups, as to be able to examine only thiol and amine reactivity. Furthermore, three amines were chosen to allow SuFEx reactions with 1) a primary amine, 2) a secondary amine and 3) an aniline. The structures of the model compounds are presented in table 3.3 below.

Table 3.3: Model thiol and amines used in HTE.

Name	Structure	Molecular Weight	Label
2-phenylethane-1-thiol		138.228 g/mol	S1
phenylmethanamine		121.18 g/mol	A1
N-Methyl-N-phenethylamine		135.21 g/mol	A2
4-aminobenzenesulfonyl fluoride		171.22 g/mol	A3

The plate layout for the first step, the thiol addition, is presented in figure 3.1 below. Using four linkers in one 96 well plate allows for 24 reactions per linker. The same conditions were used for all reactions using L4, L3 and L8 as these are all Michael acceptors. Other conditions were used for the first step using L7 as this is an alkyl bromide. Determination of conditions was done according to the procedure described in section 3.2.1 below.

	L4			L3			L8				L7		
	S1	S1	S1	S1	S1	S1	S1	S1	S1		S1	S1	S1
MeCN DMAP	A1	A2	A3	A4	A5	A6	A7	A8	A9	MeCN KI K_2CO_3	A10	A11	A12
MeCN DMAP	B1	B2	B3	B4	B5	B6	B7	B8	B9	MeCN KI K_2CO_3	B10	B11	B12
MeCN DMAP	C1	C2	C3	C4	C5	C6	C7	C8	C9	MeCN KI K_2CO_3	C10	C11	C12
MeCN DMAP	D1	D2	D3	D4	D5	D6	D7	D8	D9	MeCN KI K_2CO_3	D10	D11	D12
MeCN DMAP	E1	E2	E3	E4	E5	E6	E7	E8	E9	MeCN KI K_2CO_3	E10	E11	E12
MeCN DMAP	F1	F2	F3	F4	F5	F6	F7	F8	F9	MeCN KI K_2CO_3	F10	F11	F12
MeCN DMAP	G1	G2	G3	G4	G5	G6	G7	G8	G9	MeCN KI K_2CO_3	G10	G11	G12
MeCN DMAP	H1	H2	H3	H4	H5	H6	H7	H8	H9	MeCN KI K_2CO_3	H10	H11	H12

Figure 3.1: Plate Layout of the thiol addition reaction. The colors represent the four different linkers.

The plate layout for the second step, the SuFEx reaction, is presented in figure 3.2 below. The four linkers produce four thiol intermediates from the first step, which are all reacted with the three amines. In a 96 well plate, this allows for 8 different SuFEx reactions per linker–amine combination to take place, and thus 8 different conditions were tried. Determination of conditions was done according to

the procedure described in section 3.2.2 below.

	L4+Thiol			L3+Thiol			L8+Thiol			L7+Thiol		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
Me-THF DABCO Ca(NTf$_2$)$_2$	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
DMSO DABCO Ca(NTf$_2$)$_2$	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
MeCN:H$_2$O 1:1 DABCO Ca(NTf$_2$)2	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
t-AmylOH DABCO Ca(NTf$_2$)$_2$	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
iPrOH DABCO Ca(NTf$_2$)$_2$	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
MeCN DIPEA	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
DMSO DIPEA	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
MeCN:H$_2$O 1:5 TEA MgO	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Figure 3.2: Plate Layout of the SuFEx reaction. The colors represent the four different thiol intermediates.

All solid materials were dispensed automatically into the reaction plate using Chronect®XPR Robotic Dispensing. Stock solutions of all liquid reagent combinations were prepared manually and were then automatically dispensed into the reaction plate. The liquid dispensing was performed by Andrew+ Alliance Pipetting Robot. Removal of solvent after the thiol addition was performed using Genevac EZ-2 Elite Evaporator.

Analysis was performed using plate LC-MS, where area integration was done by the software with manual editing when necessary. Product conversion was determined by analyzing product peak area in relation to total peak area of product and remaining starting material as

$$\text{Conversion} = \frac{\text{Product Peak Area}}{\text{Product Peak Area} + \text{Starting Material Peak Area}}. \tag{3.1}$$

3.2.1 Determination of conditions for thiol addition

To determine which conditions would be suitable for thiol addition in a high throughput format, test reactions were performed. Conditions to test in these reactions were based on experiments reported in literature.

Alkylation of alkyl bromide **L7** (1 eq) was performed in three solvents; acetonitrile,

DMSO and butanone, all with the addition of thiol **S1** (1.5 eq), KI (1 eq) and a base (2 eq; cesium carbonate for acetonitrile and DMSO and potassium carbonate for butanone). Conversion to the alkylated products was difficult to determine in the reactions with acetonitrile and DMSO due to poor ionization in LC-MS. The primary amine **A1** (1.5 eq) was therefore added to all reaction mixtures, together with DIPEA (2 eq) and $Ca(NTf_2)_2$ (1 eq), and the conversion to the SuFEx product was determined by LC-MS. As there was no significant difference in yield, acetonitrile was chosen as solvent as it is accessible and easy to evaporate. Potassium carbonate was chosen as base as this was already available in the dispensing robot.

The conjugate addition of the thiol to Michael acceptor **L8** (1 eq) was performed with thiol **S1** (1 eq) in acetonitrile using three different activating agents; DBU (1 eq), TCEP (0.05 eq) and DMAP (0.05 eq). Conversion to the addition product could not be determined as the product did not ionize in LC-MS. The primary amine **A1** (1.5 eq) and $Ca(NTf_2)_2$ (1 eq) were therefore added to the reaction mixtures with TCEP and DMAP and conversion to the SuFEx product could be determined by LC-MS. A crude NMR of the reaction with DBU was measured which showed the addition product, but the reaction was considerably slower than the others. Because the LC-MS of the reaction with DMAP was cleaner, it was decided to use it as activating agent in HTE.

3.2.2 Determination of conditions for the SuFEx reaction

To determine which conditions would be suitable for the SuFEx reaction in a high throughput format, a literature review was done. Using DABCO as base and $Ca(NTf_2)_2$ as lewis acid is reported to give excellent yields of SuFEx products in various solvents [45], [46], [10]. Other sets of conditions such as MgO in MeCN:H_2O [7] and TEA in MeCN [26], as well as with aqueous buffer [11] are also reported. Solvents, bases and lewis acids were combined to form both new and existing combinations to be used in HTE. One objective in determining these combinations was to be able to test the reactions in greener conditions.

3.2.3 Heat Maps

Below are heat maps of the 96 well plate in which the thiol addition and SuFEx reaction were performed. Values come from LC-MS analyses. Peak integration was done by the LC-MS software and additional editing was done manually when needed. Among the 96 samples, green indicates the most desired results and red indicates the most undesired results, where the color intensity represents the degree to which a value is positive or negative in relation to the rest. For the heat maps showing product peak area and conversion, green represents high values as a large amount of product and high conversion are desired. For the heat maps showing starting material peak area and impurity peak area, green represent low values, as the presence of remaining starting material or impurities is undesired.

3.2.3.1 Thiol Addition

	L4			L3			L8			L7		
	1	2	3	4	5	6	7	8	9	10	11	12
A	28.7	31.2	27.9	74.8	76.8	77.3	65.9	65.4	65.5	69.2	75.7	75.1
B	24.9	7.3	29.2	63.1	71	76.6	64.8	64	64.8	72	67.5	65.6
C	19.9	23.9	31.6	76	75.8	76.5	63	64.4	63.7	76.6	65.5	59.9
D	30.2	28.5	31.2	71.7	75.5	69.8	64.5	61	62.2	68.1	66.9	66.1
E	29.5	30.3	28.5	59.8	66.4	60.5	61.4	60.6	61.8	74	63.9	66.8
F	11	29	28.1	65.9	66.7	66.4	61.7	61.3	61.3	64.3	54.3	65.8
G	27	26.8	21.5	65.3	66.1	66.4	61.9	65	62.1	63.3	12	42.3
H	30.8	12.7	30.8	62.2	61.1	61.8	56.4	56.5	56.7	51.5	48.8	45.5

(a)

	L4			L3			L8			L7		
	1	2	3	4	5	6	7	8	9	10	11	12
A	25.1	19.1	21.4	0	0	0	0	0	0	0	0	0
B	24.2	57.6	20.2	0	0	0	0	0	0	0	0	0
C	33.7	27.8	15.6	0	0	0	0	0	0	0	0	0
D	21.5	23	21.4	0	0	0	0	0	0	0	0	0
E	22.5	21.2	23.1	0	0	0	0	0	0	0	0	0
F	49.4	20.4	23.4	0	0	0	0	0	0	0	0	0
G	21	24.9	32	0	0	0	0	0	0	0	0	0
H	17.3	42.7	15.3	0	0	0	0	0	0	0	0	0

(b)

	L4			L3			L8			L7		
	1	2	3	4	5	6	7	8	9	10	11	12
A	22.7	26.9	26.4	0	0	0	0	0	0	0	0	0
B	22.7	17.1	23.6	0	0	0	0	0	0	0	0	0
C	23.8	23.9	27.5	0	0	0	0	0	0	0	0	0
D	21.1	23.2	21.2	0	0	0	0	0	0	0	0	0
E	20.5	21.1	21.6	0	0	0	0	0	0	0	0	0
F	16.9	23.8	21.8	0	0	0	0	0	0	0	0	0
G	25.2	22.1	21.7	0	0	0	0	0	0	0	0	0
H	20.1	19.2	22	0	0	0	0	0	0	0	0	0

(c)

	L4			L3			L8			L7		
	1	2	3	4	5	6	7	8	9	10	11	12
A	53.4	62.1	56.6	100	100	100	100	100	100	100	100	100
B	50.7	11.3	59.1	100	100	100	100	100	100	100	100	100
C	37.1	46.2	66.9	100	100	100	100	100	100	100	100	100
D	58.4	55.4	59.3	100	100	100	100	100	100	100	100	100
E	56.7	58.8	55.3	100	100	100	100	100	100	100	100	100
F	18.1	58.8	54.6	100	100	100	100	100	100	100	100	100
G	56.3	51.8	40.2	100	100	100	100	100	100	100	100	100
H	64	22.9	66.7	100	100	100	100	100	100	100	100	100

(d)

Figure 3.3: Heat maps from the thiol addition reaction showing (a) product peak area (%), (b) starting material peak area (%), (c) impurity peak area (%) and (d) conversion (%).

According to figure 3.3, all linkers except L4 performed well in the thiol addition reaction. L4 did convert to product but also formed an impurity which is believed to be the DMAP-intermediate, where DMAP has added to the double bond to form an

intermediate that does not react further with the thiol. The assumption is based on the detected mass in LC-MS analysis. Additionally, there was starting material left in the reaction mixture. With only one experiment, it is difficult to draw any clear conclusions about the usefulness of L4. The reactivity of the thiol might be too low in combination with this linker, and other activating agents could perhaps have resulted in more of the thiol intermediate. Additionally, further exploration of conditions could result in a more successful reaction for this linker.

For the other linkers, no starting material remained and no linker-specific impurities had formed. However, a general impurity was present in all reaction mixtures, both from the first and second step, which in some reaction mixtures constituted a significant amount. This impurity is the main reason that Product Peak Area (hereon PPA) is not 100% for the linkers that converted to product. The impurity did not ionize and therefore does not show a mass in the LC-MS spectra, but because it is present in all reactions it is assumed to be some kind of thiol-impurity as the thiol is the only common denominator in all reactions, perhaps the disulfide of the thiol. However, to ascertain what compound it is it would be necessary to purify the reaction mixtures and isolate the different components in order to measure NMR. This is something that was not manageable in the time frame of the project.

Even though all 24 reactions per linker were performed in the same conditions for the first step, there are some differences in PPA. For example, A4, A5 and A6 yielded a higher PPA value than, say, E4, E5 and E6. Such random differences could be attributed to mistakes in the solid or liquid dispensing, either in preparing the reaction or the analysis plate. Reagents could be heterogeneously distributed in the vials and for example get stuck on the walls due to movement or from stirring which would yield an uneven result.

3.2.3.2 SuFEx reaction

Looking at the heat maps from the SuFEx reaction presented in figure 3.4, there are significantly more variations in the appearance compared to the first step. To begin with, there is a major difference in PPA between the aniline (A3, columns 3, 6, 9 and 12) and the primary and secondary amines. PPA is especially low for A3 in L4, and this is believed to be partly because the linker did not completely convert to the thiol intermediate in the first step. However, the reactivity of the aniline probably also suffers from the electron withdrawing nature of the sulfonyl substituent in the para position, pulling electron density from the nitrogen and making it less reactive.

	L4			L3			L8			L7		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
	1	2	3	4	5	6	7	8	9	10	11	12
A	17.5	9.4	0	30.4	41.1	31.3	25.8	31.7	20	35.2	33.8	21.5
B	3.4	2.6	0	33.3	39	2	6.3	10.7	4.5	19	16.6	1.8
C	5.5	5	0	40	35.6	9.6	8.6	13.2	1.3	20	24.4	10.2
D	24.7	28.8	3	24.4	31.8	24.3	18.5	29.4	7.5	45.5	45.2	38.9
E	30.8	15.8	1	23	41.6	25.9	12	17.9	1.5	63.5	71.6	31.2
F	1.1	0	0	42.6	35.6	0	14.8	20.5	10	30.3	7.9	0
G	1.5	0	0	40	22.1	0	6.9	9.6	0	14.2	4.8	0
H	2.3	0	0	5.2	5.3	0	19.6	2.2	5.5	25.5	21.2	0

(a)

	L4			L3			L8			L7		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
	1	2	3	4	5	6	7	8	9	10	11	12
A	19.2	15.7	18.1	2	0	11.8	1.1	3.2	8.8	4.6	3.4	10.8
B	27.7	23.7	27.6	2	1.6	15.7	0	1.6	6.1	20.9	19.5	21.8
C	23.7	18.3	19.3	0	0	13.9	0	0	10.3	1.1	0	0
D	8	4.6	14.2	18.5	13.5	0	0	0	6.6	3.6	2.5	13.3
E	10.5	13.2	18.5	10.4	0	12.3	0	0	0	3.4	0	16.9
F	38.2	39.1	43.1	10.5	18.5	51.8	5.5	8.2	31.2	44.7	59.1	74.6
G	41.5	38.5	40.6	7.4	21.5	51.2	2.8	5.2	29.6	20.5	20.6	48.8
H	56.1	41.3	53.6	0	0	0	0	0	0	1.2	0	0

(b)

	L4			L3			L8			L7		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
	1	2	3	4	5	6	7	8	9	10	11	12
A	46.8	53.7	64.3	53.8	41.2	39.7	63.7	54.4	59	56.9	56.7	61.6
B	40.1	48.4	52.2	45.1	31.9	73.8	81.3	76.4	75.9	55.2	57.7	67.4
C	30.9	35	59.7	21.2	49.1	66	86.2	78.9	83.4	74.9	69.9	87.7
D	34.5	40.4	29.3	42.3	39	25.8	73.2	55	71.3	48.3	46	37.9
E	34.6	44.9	62.8	50.1	15.1	54.8	62.7	59.6	40.9	26.5	20.5	39.2
F	54.1	46.7	46.9	26.9	28.1	28.9	65.7	57.7	32.4	17.2	22.3	16.3
G	48.4	46.6	50.2	29.7	35.4	30.5	63.3	63.4	30.4	58	62	39.2
H	18.3	38.1	35.6	82.4	89.1	92.5	72.6	90.8	90.7	70.1	74	97.9

(c)

	L4			L3			L8			L7		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
	1	2	3	4	5	6	7	8	9	10	11	12
A	47.7	37.5	0	93.8	100	72.6	96	90.8	69.4	88.5	90.8	66.6
B	10.9	9.8	0	96.5	96.2	11.3	100	87.3	42.3	47.6	45.9	7.7
C	18.7	21.3	0	100	100	40.9	100	100	11.4	95	100	100
D	75.6	86.3	17.3	56.8	70.2	100	100	100	53.2	92.7	94.8	74.5
E	74.6	54.5	5.2	68.9	100	67.8	100	100	100	94.9	100	64.9
F	2.8	0	0	80.2	65.8	0	73.1	71.5	24.2	40.4	11.8	0
G	3.5	0	0	84.4	50.7	0	71.2	64.8	0	41	19	0
H	4	0	0	100	100	-	100	100	100	95.5	100	-

(d)

Figure 3.4: Heat maps from the SuFEx reaction showing (a) product peak area (%), (b) starting material peak area (%), (c) impurity peak area (%) and (d) conversion (%).

The reason that this specific aniline was chosen is mainly because it is a solid, which the naked aniline is not, and because it was commercially available. As it turned out that stock solutions of all liquid reagents had to be made before HTE could proceed, it did not matter whether reagents are solids or liquids. Future high throughput experiments could incorporate the naked aniline instead as this might be more reactive.

As can be seen in figure 3.4 (a), the aniline performed especially poor in conditions F, G and H across all linkers, which are the conditions not using $Ca(NTf_2)_2$ and DABCO, a successful combination of reagents reported in literature [45], [46], [10], [47], [48]. However, the results probably have more to do with the reactivity of the aniline rather than the conditions themselves, as both F and G yielded the SuFEx product for the primary and secondary amine in similar amounts as in conditions A through E, which all used $Ca(NTf_2)_2$ and DABCO.

Condition H turned out to be a poor condition for any amine and linker, however this is not too surprising considering it is reported in literature to yield the SuFEx product when stirred at reflux [7]. Using only one reaction plate, it was not possible to attempt the reaction at different temperatures. However, looking at the heat map of starting material and impurities for condition H it can be seen that, at least for L2-L4, there is no starting material left but a lot of impurities have formed. The condition is thus giving rise to some kind of reaction that consumes the thiol-intermediate. The kind of impurities formed cannot be deduced from the LC-MS data, and purification would be necessary to ascertain what has occurred.

Conditions A, D and E seemed to work best across all linkers and amines, with D and E yielding product in all reactions, although with varying success. These conditions also gave the highest value of PPA obtained out of the 96 reactions. The two conditions are similar as the solvents used are both alcohols. Condition D using *t*-AmylOH is reported in literature [47], [48], [46], however, condition E using iProH is not. These results are especially exciting as iProH is considered a green solvent. The PPA appearance from condition A is less varying and generally works better for the aniline, giving the highest PPA values among all conditions for that amine.

It is important to note that the general conditions are not necessarily the best conditions for every linker-amine combination. For example, L3 performed the best with A1 in condition F, which is interesting considering only base is added in this reaction. Condition G, with only DMSO and DIPEA, and C, using MeCN:H_2O, $Ca(NTf_2)_2$ and DABCO also yielded a good amount of product. Both are important; DMSO is a common solvent for storage of compounds and MeCN:H2O is a common solvent for e.g., reactions with peptides.

Regarding the linkers, it is clear, as for the first step, that L4 performed poorly compared to the others. Conditions F, G and H were especially poor, and this might be due to the fact that these conditions are the ones not using $Ca(NTf_2)_2$ and DABCO. However, conditions B and C were also unsuccessful for L4 and the same fact does not apply here. As only conditions A, D and E yielded product in any significant amount, the success of the SuFEx reaction using L4 is therefore assumed to be heavily dependent on the choice of conditions. However, the fact that the linker did not convert completely to the thiol intermediate in the first step probably affects the results from the SuFEx reaction and makes it difficult to draw any conclusions.

L3 and L7 were the most successful linkers in the SuFEx reaction and yielded product in a significant amount across most conditions, implying that they are not as strongly dependent on the choice of conditions to produce the SuFEx product. However for L7, conditions E gave a significant increase in product formation, and seems to be to optimal conditions for this linker. L8 on the other hand yielded product in variable success. The problem for this linker was not the SuFEx reaction but the fact that the SuFEx product did retro-Michael reaction and eliminated the thiol to form an impurity consisting of the linker–amine molecule (figure 3.5), owing to the high values of Impurity Peak Area.

Figure 3.5: Retro-Michael reaction of linker L8.

3.3 Linker Screen

After high throughput experimentation, a linker screen was performed using the synthesized and commercially available linkers that were not part of the 96 well plate reactions. Four synthesized linkers (L1, L2, L5 and L6) and one commercial linker (L9) were tested in a thiol addition and SuFEx reaction, using thiol **S1** for thiol addition and amine **A1** for the SuFEx reaction. The amount of linker used in thiol addition was decided based on a theoretical amount of product yield after the SuFEx reaction of about 30 mg in case it would be necessary to isolate the product. In total, 10 reactions were performed. The conditions used in the first step were the same as during HTE and two sets of conditions were used for the SuFEx reactions. Condition E from HTE, with iPrOH, was used because of the successful results from HTE and because iPrOH is a green solvent. The other condition for the SuFEx reaction, MeCN:H_2O, DABCO and $Ca(NTf_2)_2$ in 40 °C, was chosen because it resembles physiological conditions, which is interesting for bio-conjugation. The reactions were performed in fume hoods using one 1.5 mL vial per reaction. All solid and liquid dispensing were done manually, and the solvent was removed between steps under a stream of N_2. Figure 3.6 gives an overview of the reactions performed in the linker screen.

Reaction	Linker	Structure	Conditions Thiol addition	Conditions SuFEx reaction
1	L9		MeCN DMAP RT	iPrOH DABCO $Ca(NTf_2)_2$ RT
2	L5		MeCN DMAP RT	iPrOH DABCO $Ca(NTf_2)_2$ RT
3	L1		MeCN DMAP RT	iPrOH DABCO $Ca(NTf_2)_2$ RT
4	L2		MeCN KI K_2CO_3 RT	iPrOH DABCO $Ca(NTf_2)_2$ RT
5	L6		MeCN KI K_2CO_3 RT	iPrOH DABCO $Ca(NTf_2)_2$ RT
6	L9		MeCN DMAP RT	$MeCN:H_2O$ 1:1 DABCO $Ca(NTf_2)_2$ 40° C
7	L5		MeCN DMAP RT	$MeCN:H_2O$ 1:1 DABCO $Ca(NTf_2)_2$ 40° C
8	L1		MeCN DMAP RT	$MeCN:H_2O$ 1:1 DABCO $Ca(NTf_2)_2$ 40° C
9	L2		MeCN KI K_2CO_3 RT	$MeCN:H_2O$ 1:1 DABCO Ca(NTf2)2 40° C
10	L6		MeCN KI K_2CO_3 RT	$MeCN:H_2O$ 1:1 DABCO Ca(NTf2)2 40° C

Figure 3.6: Reactions performed in the linker screen.

3.3.1 Thiol addition

The peak appearances from LC-MS spectra from the thiol addition reactions 1–10 are presented below. The numbers by each structure represents the molecular weight (g/mol) of that compound.

Reactions 1 and 6

138.228 + 110.102 → 248.034

Figure 3.7 shows the LC-MS spectra from reactions 1 and 6, respectively. None of the peaks contain a mass that corresponds to either thiol intermediate mass or linker mass, however, this is assumed to be because these compounds do not ionize and therefore cannot be identified. It is thus unclear whether the thiol addition worked or not.

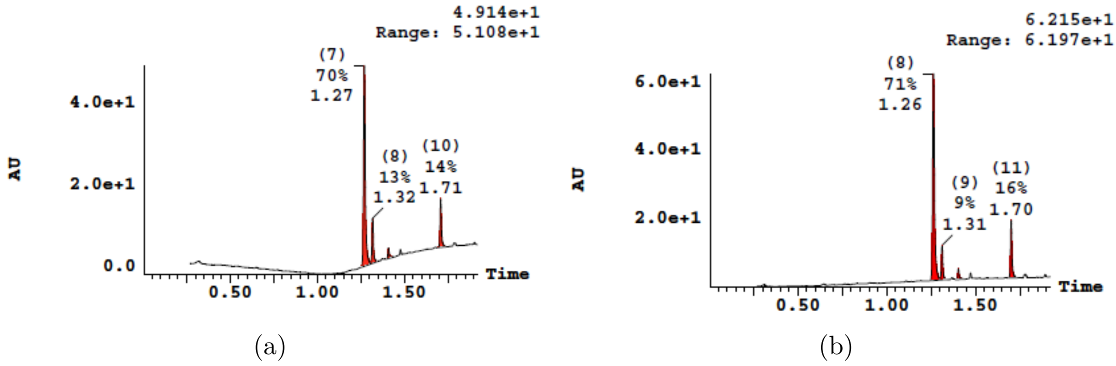


Figure 3.7: LC-MS peak appearance from thiol addition reactions (a) 1 and (b) 6.

Reactions 2 and 7

138.228 + 222.190 → 360.061

Figure 3.8 and figure 3.9 show the LC-MS spectra from reactions 2 and 7, respectively.

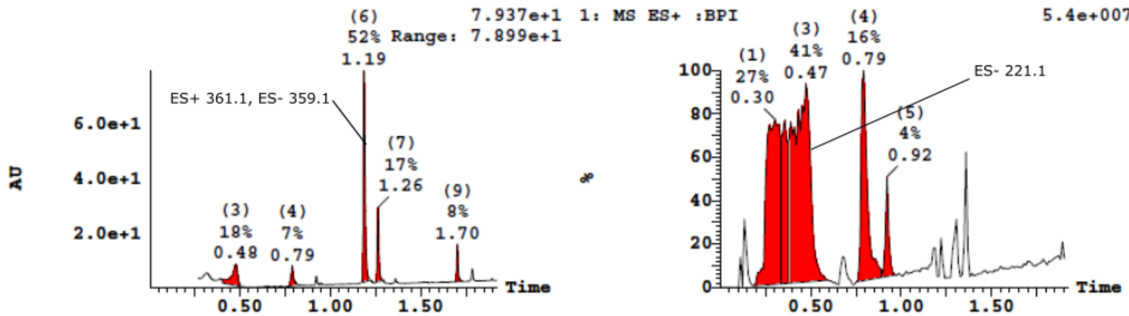


Figure 3.8: LC-MS peak appearance from thiol addition 2.

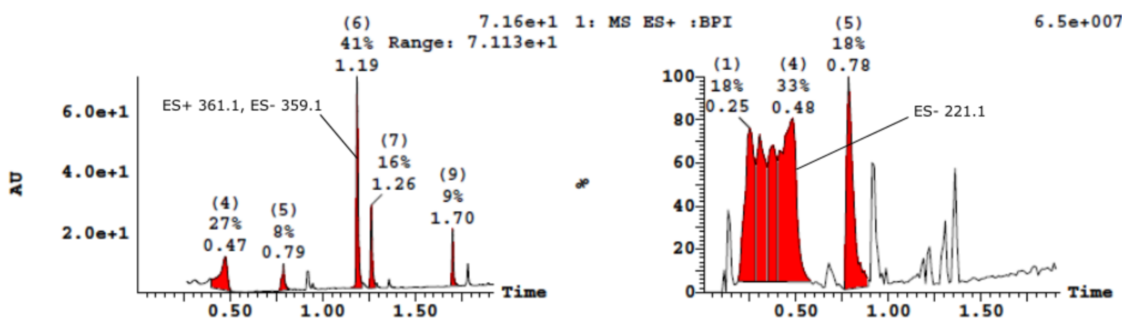


Figure 3.9: LC-MS peak appearance from thiol addition 7.

How much linker remains in the reaction mixtures of reactions 2 and 7 is difficult to estimate. The linker itself is not very UV-active according to the LC-MS spectra, and it could be assumed that there is barely any linker remaining in the mixture. However, from the looks of the ionization spectra it is possible that there is a significant amount left. Purification would be necessary to obtain a quantitative result.

Reactions 3 and 8

SH + →

138.228 234.245 372.098

Figure 3.10 and 3.11 show the LC-MS spectra from reactions 3 and 8, respectively.

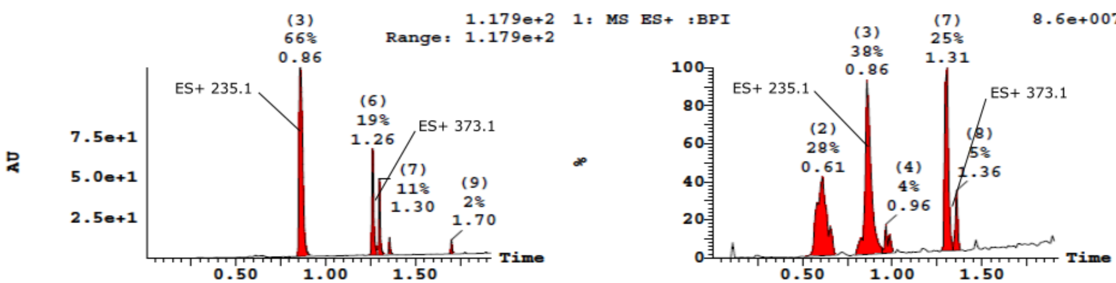


Figure 3.10: LC-MS peak appearance from thiol addition 3.

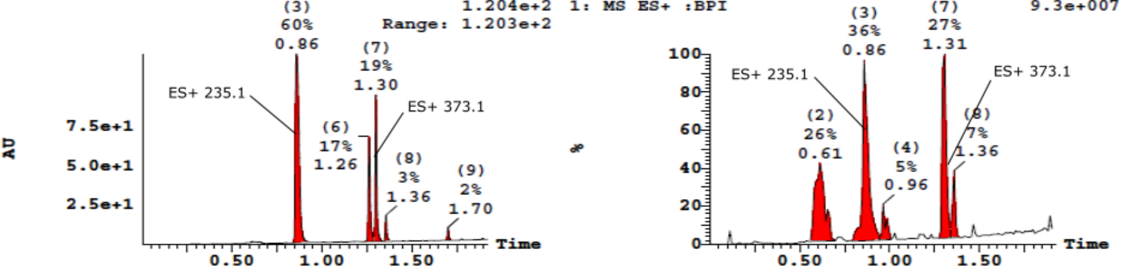


Figure 3.11: LC-MS peak appearance from thiol addition 8.

As for reactions 2 and 7, it is difficult to estimate how much linker is unreacted. Having the phenyl ring on the thiol intermediate should make it more UV-active than the linker and the UV-signal might therefore be misleading. The ionization spectra of the two compounds might display a more accurate result, a give a lower value percentage of linker remaining.

The mass corresponding to the mass of the thiol intermediate from reactions 2 and 7 is also present in the LC-MS spectra from reactions 3 and 8 (at 1.19, 361.1 in positive mode; 359.1 in negative mode). There is a possibility of contamination between the vials, but due to the amount detected it is probably more likely that this mass does not correspond to the mass of the thiol intermediate from reaction 2 and 7 but to another compound with the same mass, or some adduct or impurity from the LC-column. This assumption is backed up by the fact that the masses 357.1 and 361.1 are also present in negative mode in both LC-MS spectra from reactions 2 and 7, something that the thiol intermediate would not give rise to.

Reactions 4 and 9

138.228 + 277.108 → 334.082

Figure 3.12 shows the LC-MS spectra from reaction 4 and 9, respectively. The peak at 0.65 is assumed to be thiol intermediate peak due to the mass of 333.1 being detected in negative mode. However, the mass of 331.1 is more prevalent and should not correspond to the thiol intermediate, and might correspond to something else in the reaction mixture.

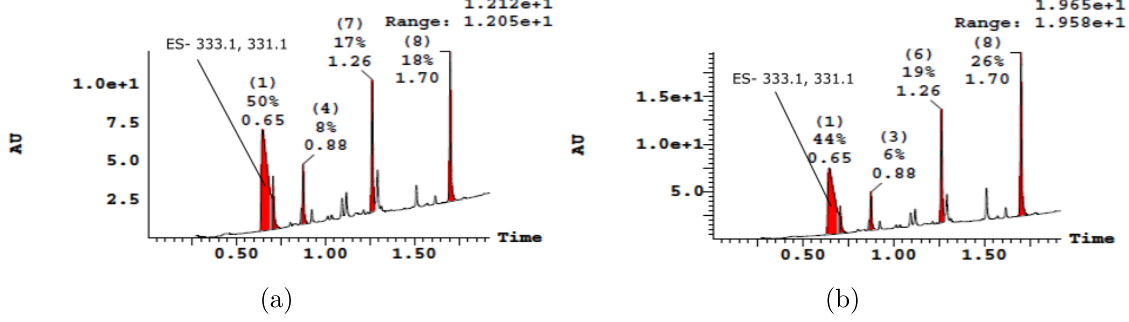


Figure 3.12: LC-MS peak appearance from thiol addition (a) 4 and (b) 9.

Reactions 5 and 10

138.228 + 289.119 → 346.082

Figure 3.13 shows the LC-MS spectra from reaction 5 and 10, respectively.

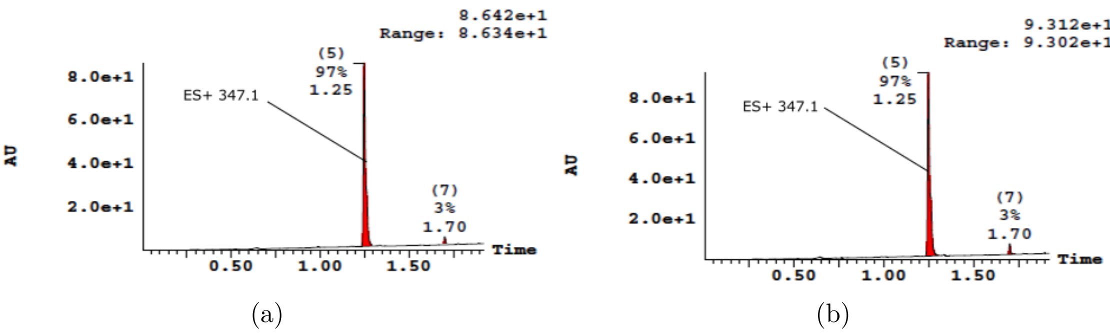


Figure 3.13: LC-MS peak appearance from thiol addition (a) 5 and (b) 10.

Only in reactions 4, 9, 5, and 10 is there thiol intermediate without remaining linker, however for reactions 4 and 9 there are several other masses detected as well. More importantly, the thiol intermediate peak contains another mass, which might mean that something else is giving rise to the signal. The spectra from reactions 5 and 10 on the other hand show almost only thiol intermediate in UV and represent the cleanest results out of all reactions. Both linkers should ionize and be visible in the ionization spectra, and with no traces of remaining linker, L2 and L6 are considered the best in the first step of the linker screen. The trend in the results reveals that the alkyl bromides performed better than the Michael acceptors. However, as the conditions used in the first step have not been optimized, the reason might not be the reactivity of the linkers but the fact that there are other conditions that are more suitable for reaction with Michael acceptors.

3.3.2 SuFEx reaction

The peak appearances from LC-MS spectra from the SuFEx reactions are presented below. The LC-MS analysis for the second step of the linker screen was performed using the same instrument as for the analysis of the HTE plate, with the aim to better compare the data. Reactions 1–5 were performed in iPrOH at room temperature, and reactions 6–10 were performed in MeCN:H_2O 1:1 at 40 °C.

Reactions 1 and 6

Figure 3.14 shows the LC-MS spectra from reactions 1 and 6, respectively. No product is detected, however this is assumed to be because the product does not ionize very well, as was the problem for the first step. Only an impurity at 1.77 is detected, which is the same as the impurity detected in all reactions in HTE. Again, this compound does not ionize and therefore does not show a mass in LC-MS. Purification and NMR analysis would be necessary to elucidate which structure is giving rise to the signal.

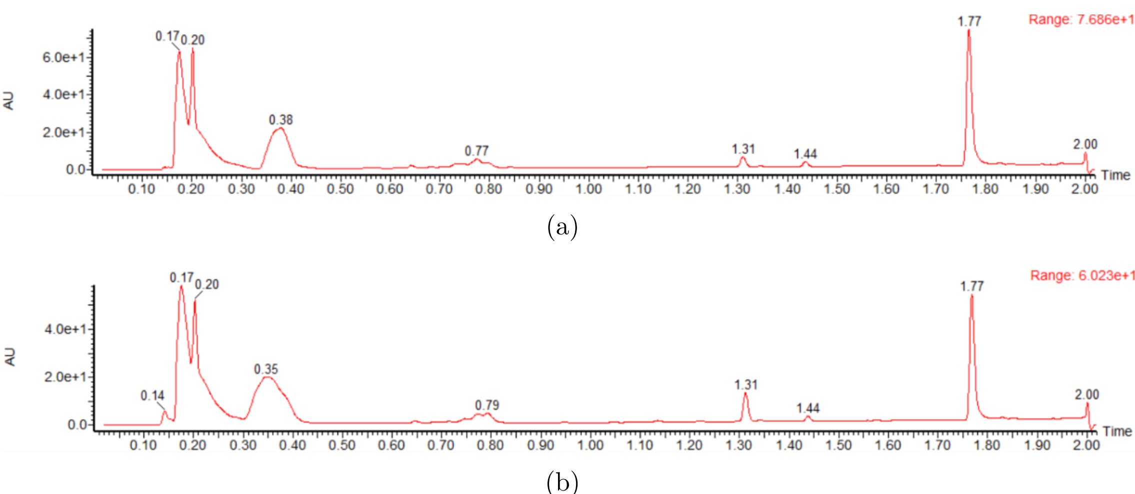
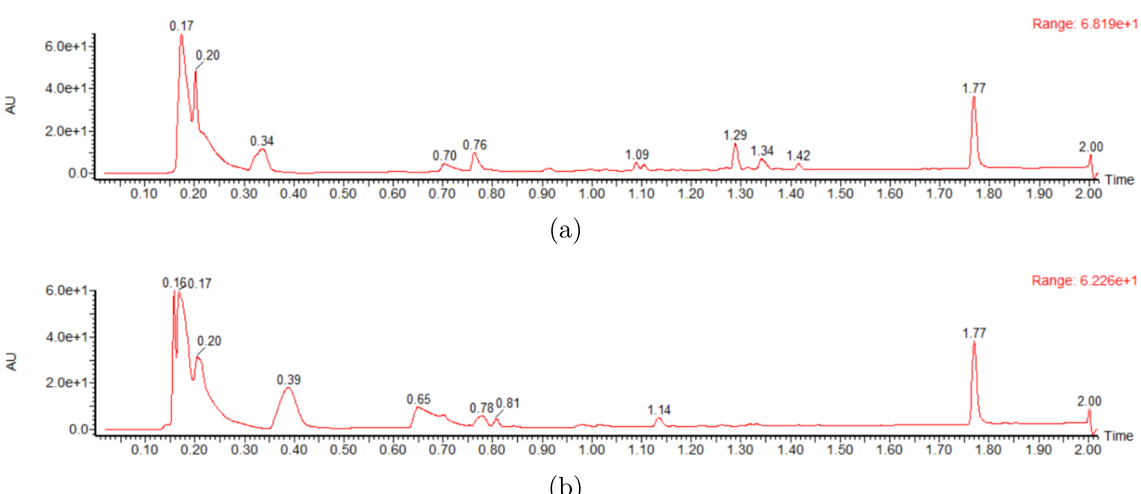


Figure 3.14: LC-MS peak appearance from SuFEx reaction (a) 1, in iPrOH and (b) 6, in MeCN:H_2O 1:1.

Reactions 2 and 7

Figure 3.15 shows the LC-MS spectra from reactions 2 and 7, respectively. The results show the impurity peak at 1.77, but also several other peaks, as opposed to the LC-MS data from reactions 1 and 6. However, none of the peaks contain a mass corresponding to the mass of the SuFEx product. With promising LC-MS data from the first step, this either means that the thiol intermediate is consumed in an undesirable partway or that the mass detected from the first step did not arise as a result of thiol intermediate formation. The fact that other masses were detected in the thiol intermediate peak from the first step and that the same mass was detected in other reactions mixtures support the latter theory.



(a)

(b)

Figure 3.15: LC-MS peak appearance from SuFEx reaction (a) 2, in iPrOH and (b) 7, in MeCN:H_2O 1:1.

Reactions 3 and 8

Figure 3.16 shows the LC-MS spectra from reactions 3 and 8, respectively. Due to L1 being an alkyne, there are two isomers of the thiol intermediate and two isomers of the SuFEx product. They all have different retention times (in the area 1.36–1.45) but the retention time of one of the isomers of the thiol product is similar enough to the retention time of one isomer of the SuFEx product to make these peaks overlap. This makes it difficult to determine a conversion, however, with the peak at 1.36 corresponding to thiol intermediate and 1.46 corresponding to product, it can be concluded that there is more thiol intermediate remaining than the amount of product formed. A 4 minute method was run for this sample as an attempt to separate the peaks, however this did not help. A comparison between the two conditions reveals that the reaction run in MeCN:H_2O resulted in more product formation.

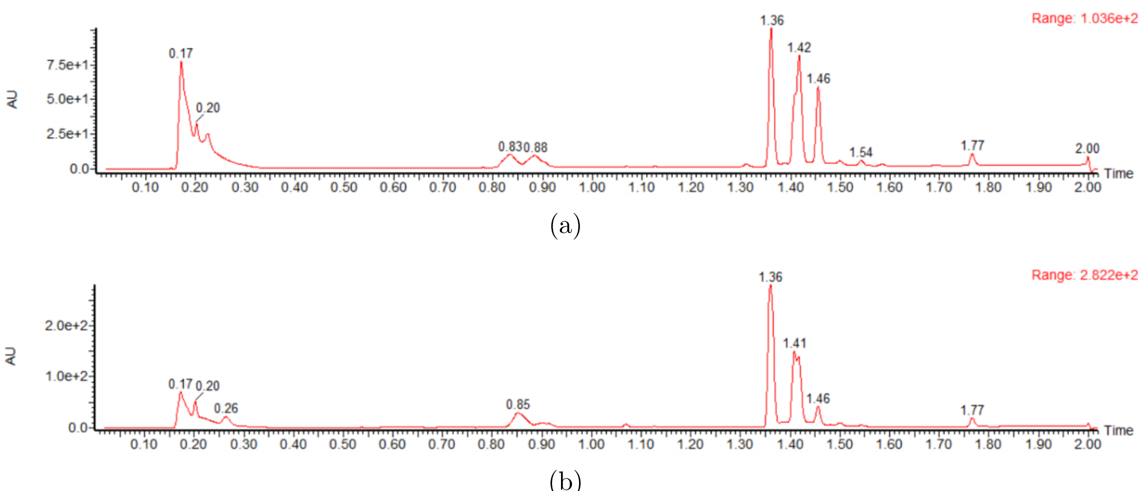


Figure 3.16: LC-MS peak appearance from SuFEx reaction (a) 3, in iPrOH and (b) 8, in MeCN:H_2O 1:1.

Reactions 4 and 9

Figure 3.17 shows the LC-MS spectra from reactions 4 and 9, respectively. As for reactions 1 and 6, the results show nothing but the impurity at 1.77. This is surprising considering the thiol intermediate was visible in the LC-MS spectra from the first step. The change of instruments should not be the reason for this discrepancy, and the SuFEx product should exhibit a significant UV-activity due to its two aromatic groups. Perhaps the masses detected from the first step did not arise as a result of thiol intermediate formation but correspond to the mass of an impurity in the reaction mixture or on the LC-column. The fact that the mass 331.1 was most prevalent in the thiol intermediate peak from the first step supports this theory. If this is the case, it is impossible to deduce whether the problem is the first or the second step, and the reactions would have to be remade, followed by purification and NMR analysis in order to elucidate this.

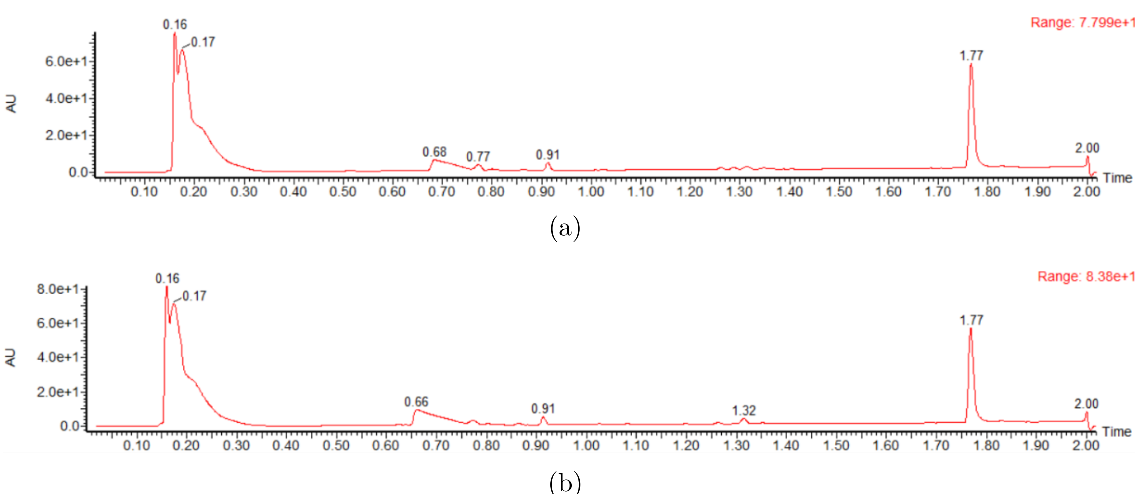


Figure 3.17: LC-MS peak appearance from SuFEx reaction (a) 4, in iPrOH and (b) 9, in MeCN:H_2O 1:1.

Reactions 5 and 10

Figure 3.18 shows the LC-MS spectra from reactions 5 and 10, respectively. The spectra show a clear product (1.31) and thiol intermediate (1.36) peak, representing the cleanest results out of all reaction even if there is more thiol intermediate than product in the reaction mixtures. A rough calculation of conversion reveals that the reaction performed in MeCN:H_2O 1:1 yielded more product (47% conversion) than the reaction performed in iPrOH (27% conversion).

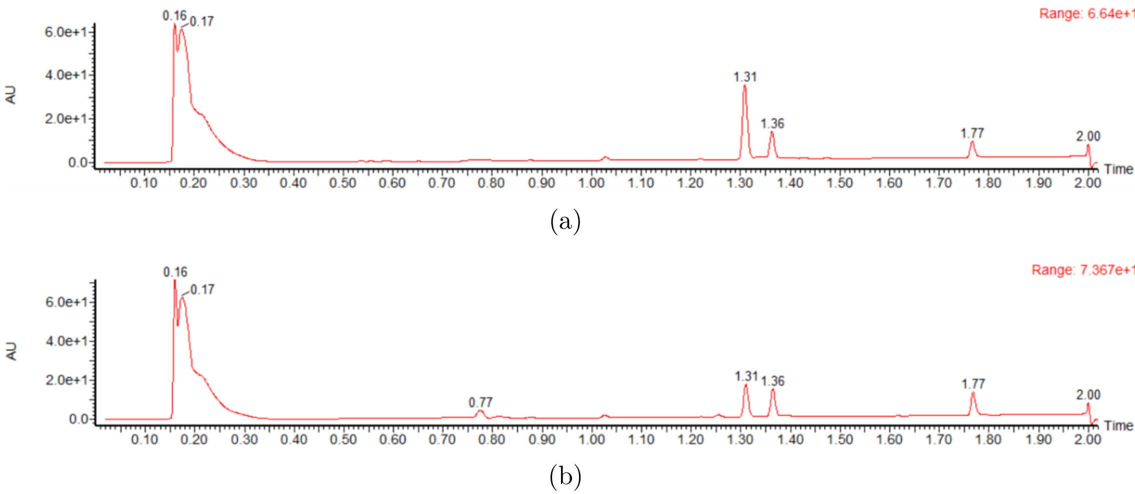


Figure 3.18: LC-MS peak appearance from SuFEx reaction (a) 5, in iPrOH and (b) 10, in MeCN:H_2O 1:1.

In summary, the results from the SuFEx reaction showed that only reactions 3, 5, 8 and 10 (L1 and L6) gave the SuFEx product. As opposed to the linker success from the first step, the best linkers in the SuFEx reaction are both Michael acceptors. With significant variability in the results using different types of linkers, a more extensive screen of linkers, conditions and reagents is needed to elucidate if there is an actual difference between Michael acceptor linkers and alkyl bromide linkers. For the four reactions in which product formed, there is a difference in conditions favoring MeCN:H_2O 1:1 at 40 °C.

There is a common impurity in all reactions with the same retention time (1.77) as the impurity found in all reactions in HTE. Again, this compound does not ionize and can therefore not be identified via LC-MS analysis, however it is assumed to be some kind of thiol-adduct as the thiol is the only common denominator in all reactions; perhaps the disulfide of the thiol. Purification and NMR analysis would be necessary to deduce which compound is giving rise to the signal, however this was not possible in the time frame of the project. For some reactions, it constituted a significant amount and future work to elucidate the structure would be helpful in further understanding the reactions.

There were some problems handling the small vials and small amounts used in the linker screen. For example, when removing the solvent from the first step under a stream of N_2, some of the reaction mixtures were blown out by the pressure from the nitrogen stream. For reaction 10, at least half the reaction mixture was blown out, and for reaction 7, a smaller amount was removed along with the solvent. This did not have any apparent effect on the results from the SuFEx reaction, however, influences are impossible to ascertain as the reactions were performed only once.

When adding the amounts of reagents to the vials in the linker screen (in both steps), all solids were weighted out separately. In hindsight, it would have been more accurate to prepare stock solutions of reagents that were added in amounts less than $\sim$ 5 mg, such as DMAP. This solution would also have made the reaction preparation less laborious.

3.4 Conjugation

Using the results from the high throughput experimentation and the linker screen, the aim was to form conjugates of biologically relevant molecules using SuFEx linkers from the project. The biologically relevant molecules used in conjugation are presented in table 3.5 and the attempted conjugates are presented in table 3.6. Some of the starting materials for the conjugations had to be synthesized before conjugations could proceed. Reagents used in the synthesis of these starting materials are presented in table 3.4 below.

The synthesis of conjugates proceeded in a one-pot, two-step reaction as for HTE and the linker screen. The thiol addition was performed in the first step, followed by SuFEx reaction with the corresponding amines. For all conjugates except C2

and C3, using a peptide, condition E from HTE was used, i.e., iPrOH, $Ca(NTf_2)_2$ and DABCO. The conjugates with the peptide used MeCN:H_2O 1:1, $Ca(NTf_2)_2$ and DABCO for solubility reasons.

Table 3.4: Reagents used in the synthesis of starting materials for conjugation.

Name	Structure	Molecular Weight	Label
4-((2-aminoethyl) amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione hydrochloride	O, N, O, NH, O, HN, NH2, HCl	316.317 g/mol	R6
2,5-dioxopyrrolidin-1-yl 3-(pyridin-2-yldisulfaneyl) propanoate	O, N, O, O, O, S, S, N	312.358 g/mol	R7
3',6'-dihydroxy-3-oxo-3H-spiro[isobenzo furan-1,9'-xanthene]-5-carboxylic acid	HO, O, OH, O, O, OH, O	376.320 g/mol	R8
2-(tritylthio)ethan-1-amine	H2N, S	319.466 g/mol	R9
2,5-dioxopyrrolidin-1-yl 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate	O, N, O, O, O, H, HN, O, NH, S, H	341.382 g/mol	R10
tert-butyl (2-(2-(2-aminoethoxy) ethoxy) ethyl)carbamate	H2N, O, O, N, H, O, O	248.323 g/mol	R11

Table 3.5: Biologically relevant molecules used in conjugation.

Molecule	Structure	Molecular Weight	Type	Label
Thiocholesterol		402.725 g/mol	Thiol	T1
Peptide [1]		1554.853 g/mol	Thiol	T2
E3 ligase ligand		404.441 g/mol	Thiol	T3
Dye		435.450 g/mol	Thiol	T4
Biotin derivative		462.624 g/mol	Thiol	T5
Niraparib		320.396 g/mol	Amine	N1
Sulfaphenazole		314.363 g/mol	Amine	N2
Hexadecanamine		241.463 g/mol	Amine	N3
Flecainide		414.348 g/mol	Amine	N4
Mirabegron		396.509 g/mol	Amine	N5
Norfloxacin		319.336 g/mol	Amine	N6

[1] Sequence: Ac-R-F-Pen-T-G-H-F-G-Sar-NMeLeu-Y-P-C-NH_2

Table 3.6: Conjugates attempted in the project.

Label	Thiol	Linker	Amine	Molecular Weight Thiol Product	Molecular Weight SuFEx Product
C1				574.898 g/mol	875.288 g/mol
C2				1879.764 g/mol	2173.842 g/mol
C3				1879.764 g/mol	2101.035 g/mol
C4				626.163 g/mol	1020.294 g/mol
C5				669.695 g/mol	1046.198 g/mol
C6				658.820 g/mol	958.150 g/mol

Below are the peak appearances from selected LC-MS spectra obtained from analyzing the reaction mixtures from the conjugates.

Conjugate 1

Figure 3.19 shows the LC-MS spectrum from the thiol addition reaction of conjugate C1. None of the peaks contain a mass corresponding to the mass of either starting material or the thiol intermediate. The thiol intermediate is assumed not to ionize, and would therefore not be visible in the LC-MS spectrum even if it was present in the reaction mixture. It is thus impossible to say whether the first step worked or not. In hindsight, it would probably have been more productive to choose an ionizable linker in order to increase the chances of detecting the thiol intermediate.

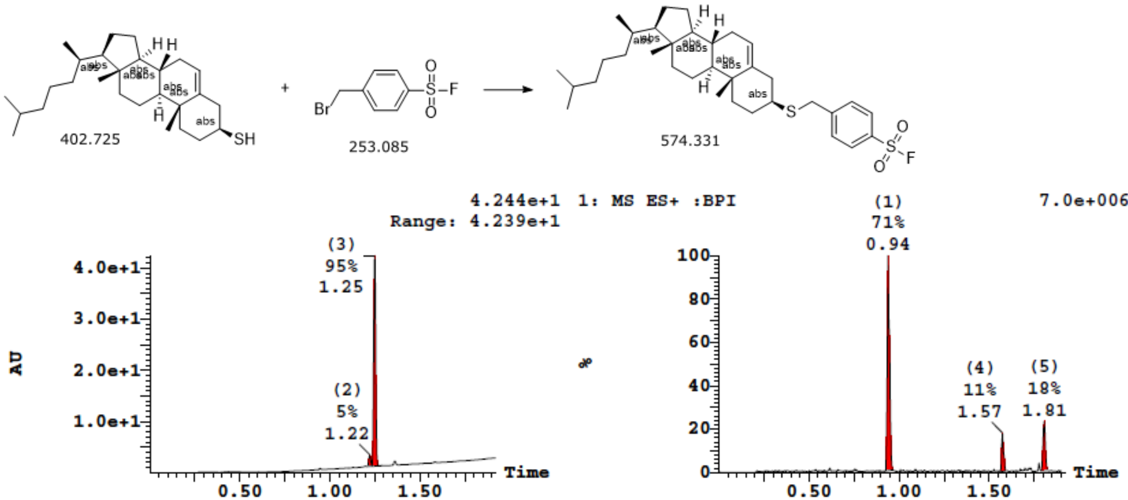


Figure 3.19: LC-MS peak appearance of the thiol addition reaction of conjugate C1, run in an acidic method.

Figure 3.20 shows the LC-MS spectrum from the SuFEx reaction of conjugate C1. Because the amine, Niraparib, is both UV-active and ionizes well according to the LC-MS spectra, the SuFEx product would too if it had formed. No mass corresponding to the mass of the SuFEx product is found and it is thus assumed that it is not present in the reaction mixture.

The linker (L7) was successful in yielding both the thiol intermediate and the SuFEx product in HTE and should not be the major problem in forming the SuFEx conjugate. The fact that Niraparib did not dissolve in the reaction mixture is probably a major reason that the SuFEx reaction did not work, as it is unclear how much of the amine was actually available for reaction with the thiol intermediate. However, as the results from the first step does not show any thiol intermediate at all, there might also be little or no thiol intermediate in the reaction mixture to begin with.

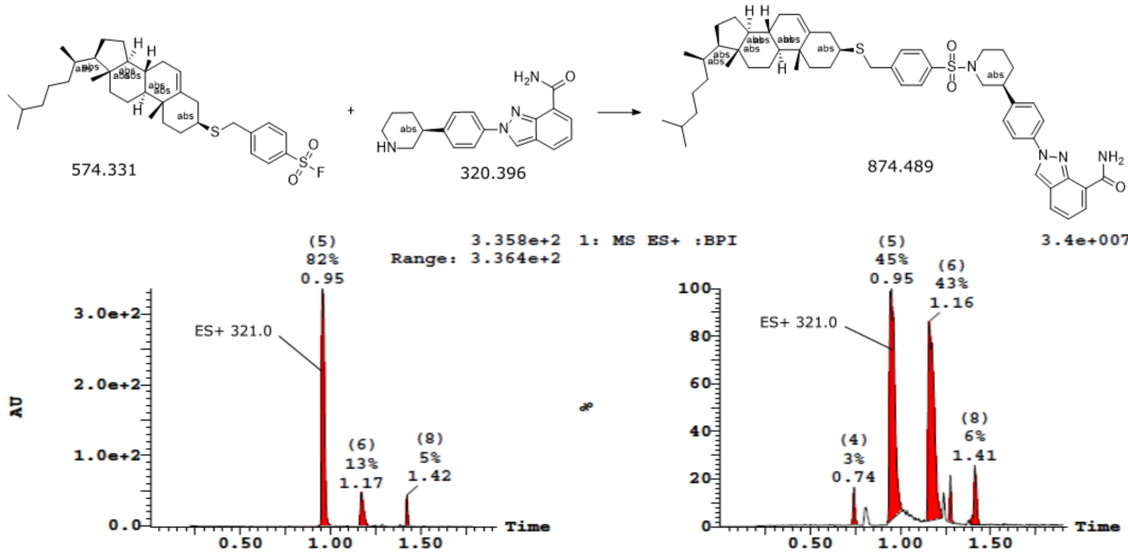


Figure 3.20: LC-MS peak appearance of the SuFEx reaction of conjugate C1, run in a basic method.

Conjugate 2 and 3

Figure 3.21 shows the LC-MS spectrum from the thiol addition reaction of conjugate C2 and C3, which have identical first steps. The thiol intermediate formation for C2 and C3 is the most successful among all conjugates, with 82% purity and no remaining starting materials.

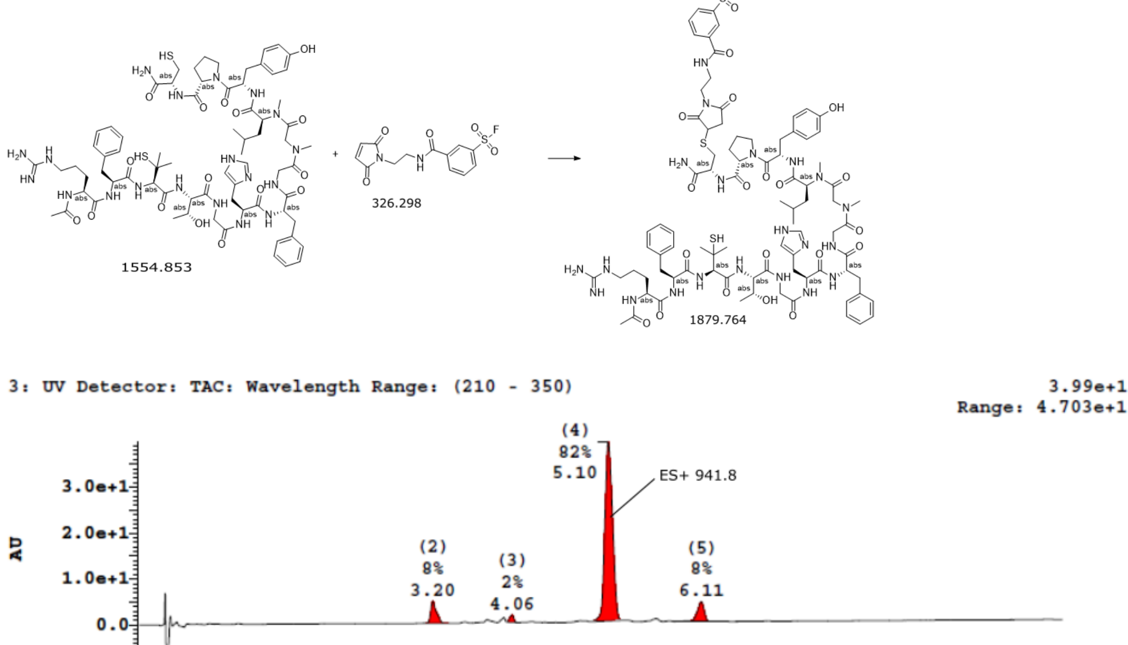


Figure 3.21: LC-MS peak appearance of the thiol addition reaction of conjugate C2 and C3, run in an acidic method.

Figure 3.22 shows the LC-MS spectrum from the SuFEx reaction of conjugate C2 and C3; these were performed in the same vial. No peaks contain a mass corresponding to the mass of either SuFEx products. C2 was attempted first and when it did not yield the SuFEx product, C3 was attempted in the same reaction vial by adding the corresponding amine. The peak of Sulfaphenazole was the most prominent peak throughout the reaction. Eventually, a small peak contained a mass that corresponds to the mass of the cyclized peptide product, meaning that an intramolecular SuFEx reaction had occurred.

Increased complexity, such as the number of functionalities of the larger molecule, make side reactions more probable than for small model compounds used in HTE. However, the major reason why the desired SuFEx reactions were unsuccessful is believed to be connected to the amines. As for C1, none of the amines dissolved in the reaction mixture and the amount available for reaction is thus difficult to estimate. The linker and peptide should not be fully responsible for the failed reaction considering the success of the linker (L3) in previous experiments and the fact that another, although undesired, SuFEx reaction occurred between linker and peptide.

C2

1879.764 + 314.363 → 2173.842

C3

1879.764 + 241.463 → 2101.035

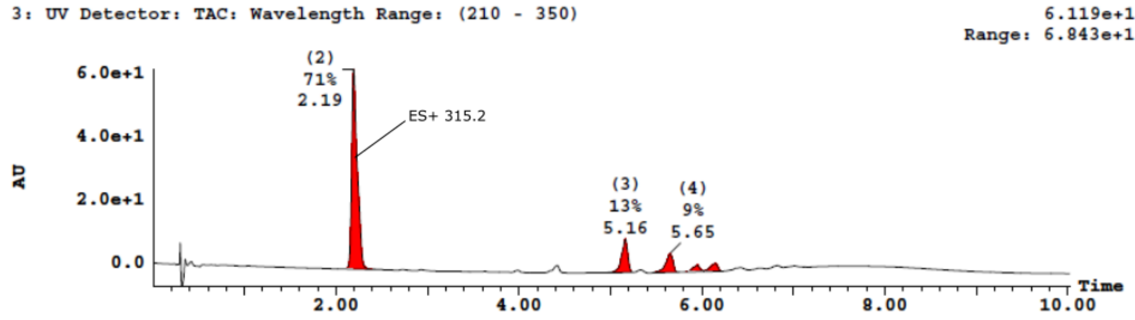


Figure 3.22: LC-MS peak appearance of the SuFEx reaction of conjugate C2 and C3, run in an acidic method.

Conjugate 4

Figure 3.23 show the LC-MS spectra from the thiol addition reaction of conjugate C4 before and after addition of base. By the look of the UV-spectra, there is significantly more starting material than thiol intermediate in figure 3.23 (a), however the opposite is true after addition of base. The thiol intermediate should be as UV-active as the E3 ligase ligand and the UV-spectrum can thus be assumed to give an accurate picture of what has occurred in the vial. It is therefore clear that base is needed for the reaction to proceed.

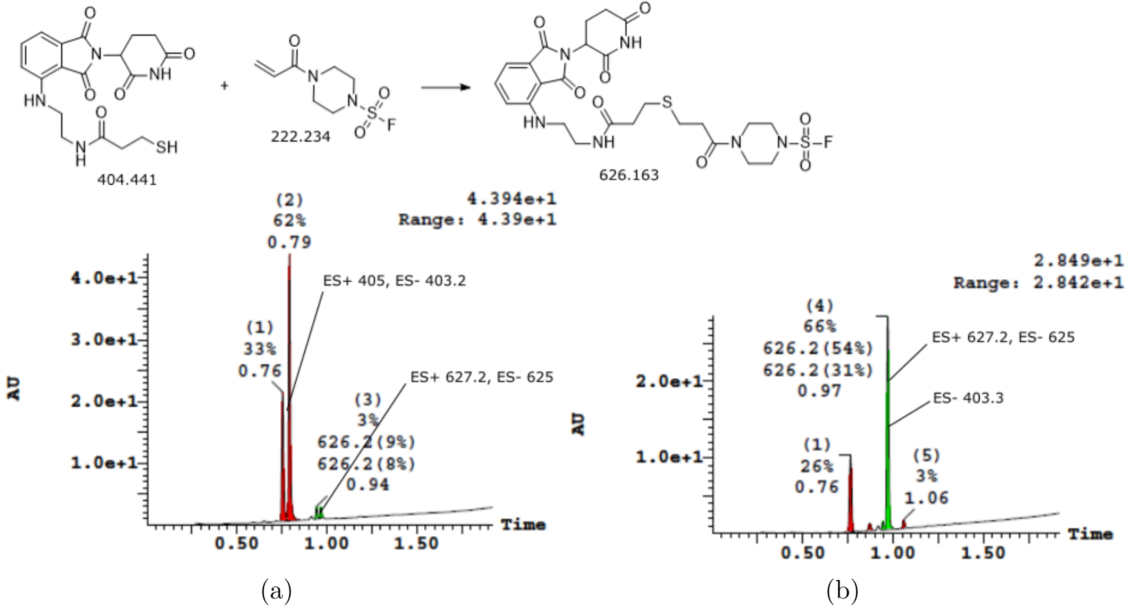


Figure 3.23: LC-MS peak appearance of the thiol addition reaction of conjugate C4 (a) before addition of base and (b) after addition of base, both run in an acidic method.

Figure 3.24 shows the LC-MS spectrum from the SuFEx reaction of conjugate C4. No peak contains a mass that corresponds to the mass of the product. The fact that Fleacinide did not dissolve in the reaction mixture and that the linker (L4) was

slightly problematic in HTE (especially in the first step), can be reasons that the SuFEx reaction was unsuccessful for C4.

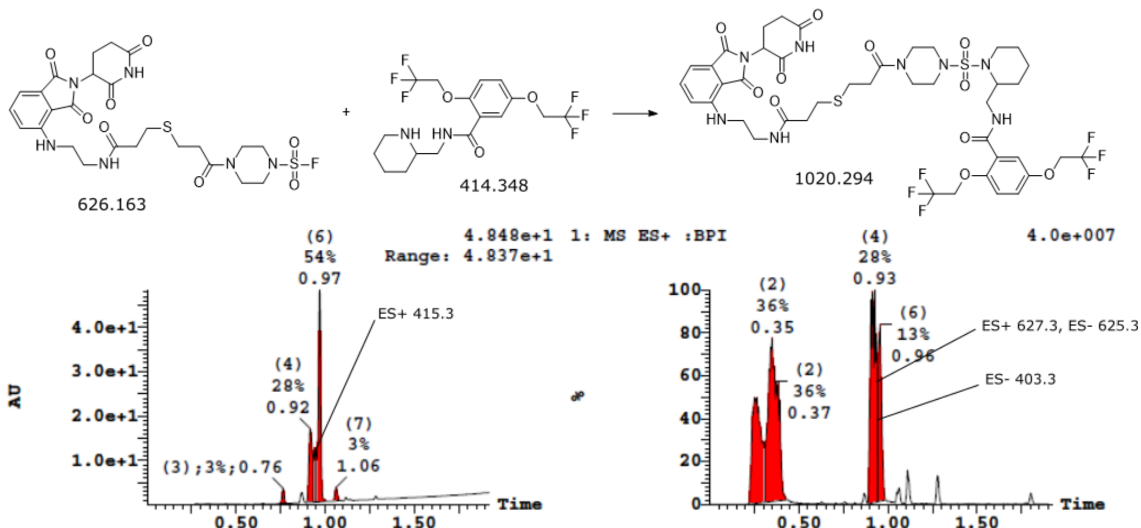


Figure 3.24: LC-MS peak appearance of the SuFEx reaction of conjugate C4, run in an acidic method.

Conjugate 5

Figure 3.25 shows the LC-MS spectrum from the thiol addition reaction of conjugate C5, before and after addition of base. Both starting material and thiol intermediate are assumed to be equally UV-active and the UV-spectra shown in figure 3.25 are assumed to accurately represent the reaction status. As for the first step of C4, it is clear that this reaction requires base in order to proceed.

It is interesting to note that even though the linkers used for C2 and C3 (L3), C4 (L4) and C5 (L1) are all Michael acceptors and should display similar trends, the first step of C3 proceeded without addition of base, and gave the purest result out of all conjugates. Previous experiments also show a superior behavior of L3 compared to L4 and L1. This might be attributed to the reactivity of the linker, with L3 being a maleimide and L4 and L1 being activated double- and triple bonds. L4 and L1 might have been more successful in previous experiments if base had been added to the reaction mixtures.

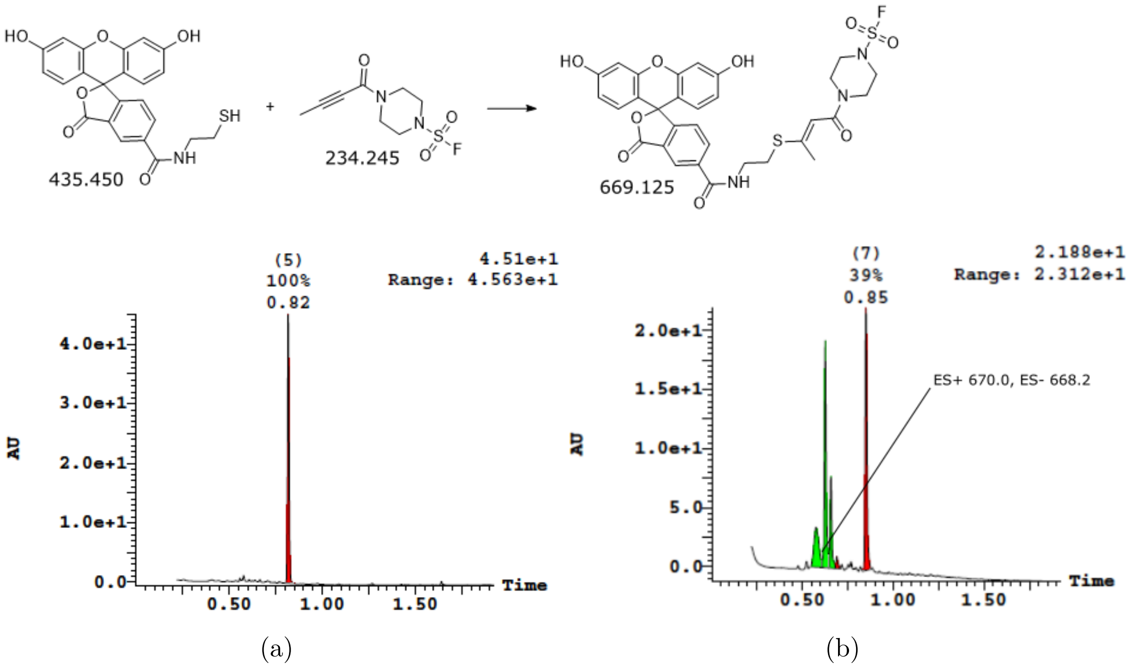


Figure 3.25: LC-MS peak appearance of the thiol addition reaction of conjugate C5 (a) before addition of base and (b) after addition of base, both run in a basic method.

Figure 3.26 shows the LC-MS spectrum from the SuFEx reaction of conjugate C5 after having stirred at 60 °C over the weekend. The second step of C5 also did not yield the SuFEx product, however, the linker (L1) should not be responsible for this. Even though it was a bit problematic during the first step of the linker screen, it was one of two linkers that yielded the SuFEx product at all. Also in this reaction mixture, the amine did not dissolve, which makes it difficult to assess whether there is a problem with reactivity or simply a need for optimizing reaction conditions. Some changes to the reaction conditions were attempted for C1, C3 and C5 during the reactions, such as increased temperature and addition of other solvents, however without positive results.

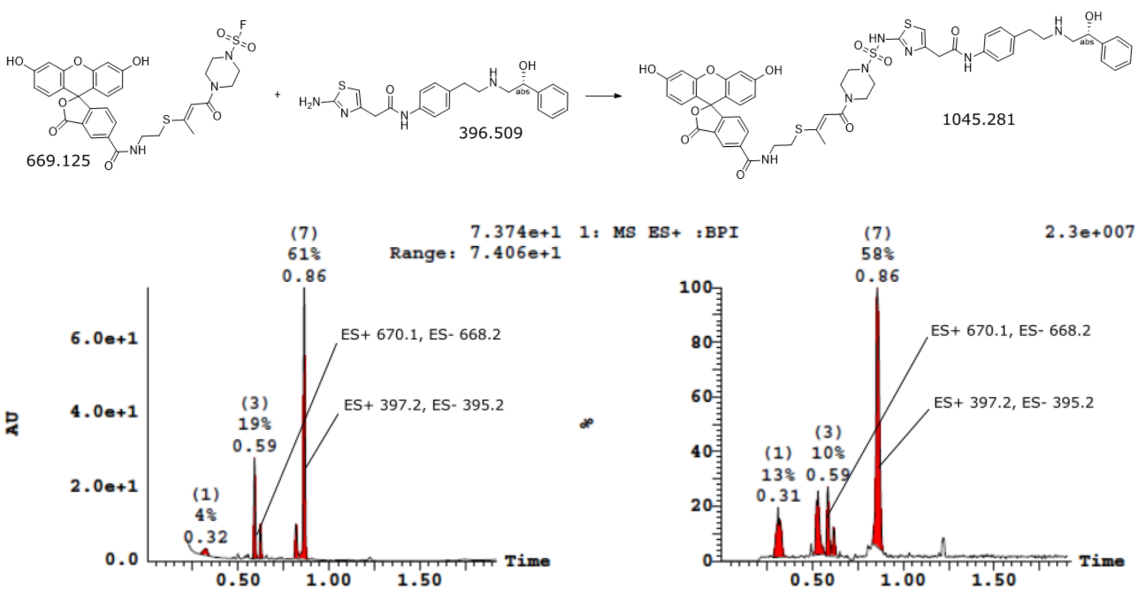


Figure 3.26: LC-MS peak appearance of the SuFEx reaction of conjugate C5, run in a basic method.

Conjugate 6

Figure 3.27 shows the LC-MS spectrum from the thiol addition reaction of conjugate C6. Due to time limitations, the first step of C6 could only run for approximately 5 hours, and to be able to perform the SuFEx reaction at all, it had to be started regardless of whether the results from the thiol addition showed the thiol intermediate in LC-MS analysis or not. Luckily, the thiol intermediate had formed already after 1 hour, however, the biotin derivative still remained in the reactions mixture.

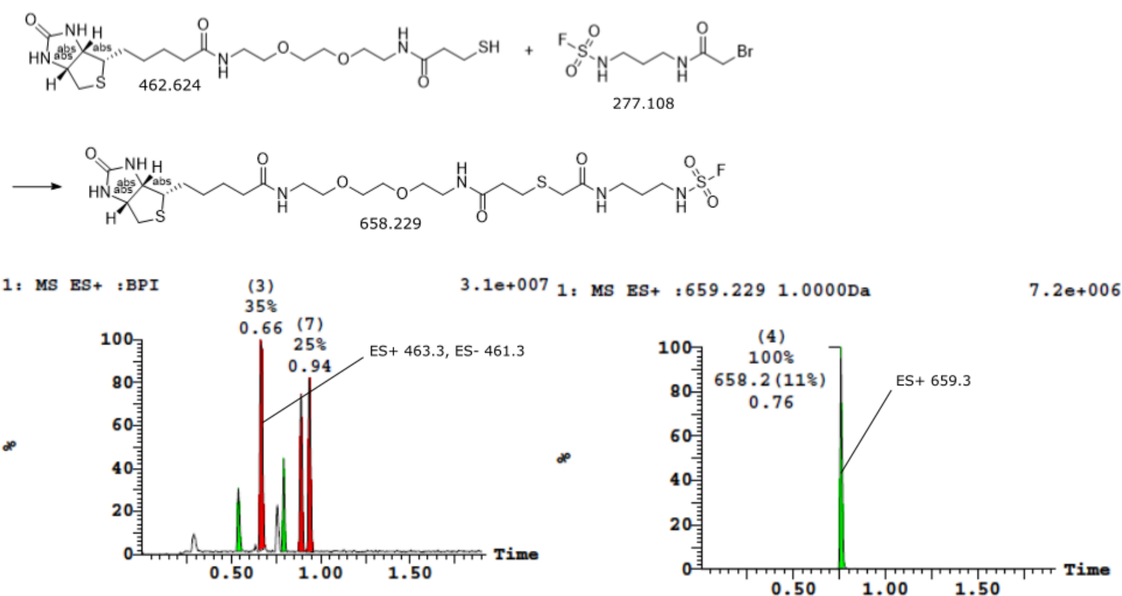


Figure 3.27: LC-MS peak appearance of the thiol addition reaction of conjugate C6, run in an acidic method.

Figure 3.28 shows the LC-MS spectrum from the SuFEx reaction of conjugate C6. As for the other conjugates, the SuFEx reaction yielded no product. The thiol intermediate is however no longer visible in LC-MS after the seconds step, which could mean that it was consumed in an undesired pathway. The linker used for the final conjugate has not produced a SuFEx product in a previous experiment as the results from the linker screen showed nothing but the impurity at 1.77 minutes. It was however successful in the corresponding first step, and it is therefore improbable that it would be the major problem in the SuFEx reaction.

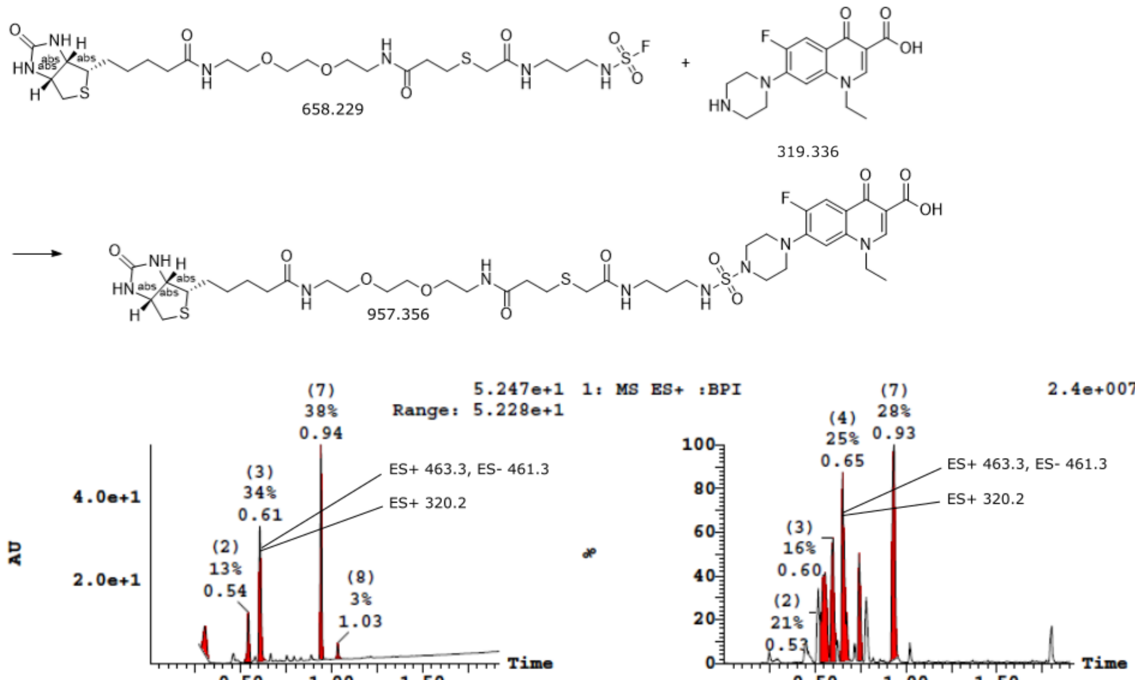


Figure 3.28: LC-MS peak appearance of the SuFEx reaction of conjugate C6, run in a basic method.

In summary, LC-MS spectra of the first step showed the thiol intermediate for all conjugates except C1, but did not show the desired SuFEx product for any conjugates. One reason that the SuFEx reactions were unsuccessful could be solubility issues. The solvents did not dissolve all components in any reaction during the second step, and the amines were especially difficult, together with $Ca(NTf_2)_2$. Small amounts of reagents and low solubility increase the uncertainty of the actual amounts available for reaction with the thiol intermediate. Additionally, the increasing complexity that comes with larger molecules, such as more functionalities, makes side reactions and intramolecular reactions more probable, as with the SuFEx cyclization of the peptide. Furthermore, the reactions were only attempted once at small scale, and other solvents, bases or temperatures might have been more successful. Some changes to the conditions were attempted, e.g., by addition of other solvents or by increasing the temperature, however this had no positive results. This is something that could be further explored in future studies.

4. Conclusion and Outlook

Six sulfamoyl fluoride linkers with different thiol accepting groups were successfully synthesized in this project using a solid imidazolium salt for installation of the sulfamoyl fluoride group. The linker backbones were both rigid and flexible and the thiol accepting groups were of two kinds: Michael acceptors and alkyl bromides, where the Michael acceptors included an acrylate, maleimides, an alkyne and an alkene. The linkers are presented in table 4.1.

Table 4.1: SuFEx linkers synthesized in the project.

Label	Structure	Label	Structure
L1		L4	
L2		L5	
L3		L6	

Synthesized SuFEx linkers were compared with commercially available linkers in a high throughput format which provided proof of concept: the SuFEx linkers successfully yield a thiol intermediate and a SuFEx product with a model thiol and three model amines. The general reaction performed in HTE is displayed in chapter 4. However, the success of the reactions depended on linker, amine and the conditions used. The commercially available linkers used in HTe are presented in chapter 4. Linkers L3 and L7 were the best linkers across all amines and conditions. L4 did not convert to thiol intermediate completely during the first step, and L8 did retro-Michael reaction of the thiol.

Figure 4.1: The general reaction performed in HTE.

(a) (b)

Figure 4.2: Commercially available SuFEx linkers used in HTE; (a) L7 and (b) L8.

A linker screen was performed, which revealed that the synthesized linker L6 was the only linker yielding the thiol intermediate without remaining linker and simultaneously yielding the SuFEx product. L1 was the only other linker yielding the SuFEx product, however this linker did not completely convert to the thiol intermediate during the first step. No linkers converted to SuFEx product completely.

SuFEx linkers successfully yielded the thiol intermediate for four different conjugates of biologically relevant molecules. However, no SuFEx reactions yielded the desired SuFEx conjugates between the thiol intermediates and corresponding amines. This is assumed to be due to solubility issues and sub-optimal reaction conditions.

Further research needs to be done to elucidate why the SuFEx reaction was unsuccessful for all conjugates. Investigating the reactivity of the different components in simpler environments could elucidate if there is a problem with certain compounds. An HTE screen of only the SuFEx reaction using a simple SuFEx molecule, phenylsulfonyl fluoride, with some of the amines used in conjugation is planned (figure 4.3). Additionally, purifying and analyzing the different components in the reaction mixtures from the HTE and linker screens would give valuable insight in whether or not side reactions occurred, and would also allow for a quantitative result on how much product formation there was.

Figure 4.3: Exploring only the SuFEx step in a planned HTE screen.

It is possible that optimization of reaction conditions could provide a solution, considering the solubility issues during the experiment. Conditions using nucleophilic catalysis such as HOBT in DMSO have been reported and could potentially be tested in the future [49], [50].

Figure 4.4: Possible conditions to try in future SuEFx reactions [49], [50].

4. Conclusion and Outlook

A recent paper show how similar SuFEx linkers were used in covalent targeting of proteins via a SuFEx reaction, indicating that modification of conditions and linkers can lead to a positive conclusion [51].

5. Experimental

5.1 Synthesis of SuFEx linkers

Below are the procedures of the synthesis of all SuFEx linkers. NMR summaries are attached, but complete NMR data with spectra can be found in appendix B.

tert-butyl 4-(fluorosulfonyl)piperazine-1-carboxylate I1
In a 100 mL round bottom flask tert-butyl piperazine-1-carboxylate **AM1** (1.26 g, 6.78 mmol) was dissolved in acetonitrile (25 mL). 1-(fluorosulfonyl)-2,3-dimethyl-1H-imidazol-3-ium trifluoromethanesulfonate **SM1** (2.67 g, 8.14 mmol) was added and the solution was stirred overnight at room temperature. Water (100 mL) was added and the mixture was extracted with EtOAc (3x150 mL). The combined organic phase was washed with water (100 mL) with some added brine and with brine (100 mL), dried over Na_2SO_4 and filtered. The solvent was removed din vacuo to yield tert-butyl 4-(fluorosulfonyl)piperazine-1-carboxylate **I1** (1.72 g, 95 %) as a colorless solid.

LCMS: product did not ionize.

1H NMR (500 MHz, CDCl3, 25°C) δ 1.47 (s, 9H), 3.39–3.45 (m, 4H), 3.54–3.6 (m, 4H).
13C NMR (126 MHz, CDCl3) δ 154.2, 81.1, 47.0, 42.7, 28.5.
19F NMR (471 MHz, CDCl3) δ 39.7.

piperazine-1-sulfonyl fluoride, I2a
In a 10 mL round bottom flask tert-butyl 4-(fluorosulfonyl)piperazine-1-carboxylate (100 mg, 0.37 mmol) **I1** was dissolved in Hydrogen chloride solution 4.0 M in Dioxane (2 mL, 57.60 mmol) and the solution was stirred at room temperature for 1 hour. The solvent was removed in vacuo and the residue was used in the next step without further treatment.

LCMS (m/z, ES+) mass found = 169.0 [M+H]+, expected = 169,04 [M+H]+ for $[C_4H_{10}FN_2O_2S^+]$.

piperazine-1-sulfonyl fluoride, I2b

In a 100 mL round bottom flask (3-acrylamidopropyl)sulfamoyl fluoride (1.00 g, 3.73 mmol) was dissolved in DCM (15 mL) and 2,2,2-trifluoroacetic acid (15 mL, 194 mmol) was added. The solution was stirred at room temperature for 3 hours. The solvent was removed in vacuo and the residue was used in the next step without further treatment.

LCMS (m/z, ES+) mass found = 169.1 [M+H]+, expected = 169,04 [M+H]+ for $[C_4H_{10}FN_2O_2S^+]$.

tert-butyl (3-((fluorosulfonyl)amino)propyl)carbamate, I3

In a 50 mL round bottom flask tert-butyl (3-aminopropyl)carbamate **AM2** (1.5 mL, 8.61 mmol) was dissolved in acetonitrile (25 mL) and the solution was cooled down to 0° C. 1-(fluorosulfonyl)-2,3-dimethyl-1H-imidazol-3-ium trifluoromethanesulfonate **SM1** (3.39 g, 10.33 mmol) was added and the solution was stirred overnight while slowly warming up to room temperature. Water (100 mL) was added and the mixture was extracted with EtOAc (3x150 mL). The combined organic phase was washed with water (100 mL) with some added brine and with brine (100 mL), dried over Na_2SO_4 and filtered. The solvent was removed in vacuo to yield tert-butyl (3-((fluorosulfonyl)amino)propyl)carbamate **I3** (2.19 g, 99 %) as a colorless oil.

LCMS (m/z, ES-) mass found = 255.1 [M-H]-, expected = 255,08 [M-H]- for $[C_8H_{16}FN_2O_4S^-]$.

1H NMR (500 MHz, CDCl3, 25°C) δ 1.44 (s, 9H), 1.72 (p, J = 5.9 Hz, 2H), 3.30 (ddt, J = 14.7, 12.1, 6.3 Hz, 4H), 4.78 (s, 1H), 7.09 (s, 1H).
13C NMR (126 MHz, CDCl3) δ 157.9, 80.7, 41.0, 36.5, 30.2, 28.4.
19F NMR (471 MHz, CDCl3) δ 52.1.

(3-aminopropyl)sulfamoyl fluoride, I4

In a 100 mL round bottom flask tert-butyl (3-((fluorosulfonyl)amino)propyl)carbamate **I2** (1.00 g, 3.90 mmol) was dissolved in DCM (15 mL) and 2,2,2-trifluoroacetic acid (15 ml, 194 mmol) was added. The solution was stirred at room temperature for 3 hours. The solvent was removed in vacuo and the residue was used in the next step without further treatment.

4-(but-2-ynoyl)piperazine-1-sulfonyl fluoride, L1
In a 10 mL round bottom flask piperazine-1-sulfonyl fluoride **I2** (62.7 mg, 0.37 mmol) and but-2-ynoic acid **R2** (34.5 mg, 0.41 mmol) were dissolved in DCM (3 mL) and the mixture was cooled down to 0 °C. N-ethyl-N-isopropylpropan-2-amine (0.33 mL, 1.86 mmol) was added, followed by a dropwise addition of 2,4,6-tripropyl-1,3,5,2,4,6-trioxatriphosphinane 2,4,6-trioxide (0.28 mL, 0.93 mmol). The solution was stirred at 0 °C for 10 minutes and then at room temperature for 2 hours. Sat. $NaHCO_3$ (20 mL) was added and the mixture was extracted with EtOAc (3x20 mL). The combined organic phase was washed with 1M HCl solution (20 mL) and with brine (20 mL), dried over Na_2SO_4 and filtered. The solvent was removed in vacuo to yield 4-(but-2-ynoyl)piperazine-1-sulfonyl fluoride **L1** (72.5 mg, 83 %) as a colorless solid.

LCMS (m/z, ES+) mass found = 235.2 [M+H]+, expected = 235,05 [M+H]+ for [$C_8H_{12}FN_2O_3S^+$].

1H NMR (500 MHz, CDCl3, 25°C) δ 2.04 (d, J = 0.6 Hz, 3H), 3.42–3.52 (m, 4H), 3.76–3.92 (m, 4H).
13C NMR (126 MHz, CDCl3) δ 153.2, 91.3, 72.3, 47.3, 46.7, 45.7, 40.2, 4.3.
19F NMR (471 MHz, CDCl3) δ 40.5.

(3-(2-bromoacetamido)propyl)sulfamoyl fluoride, L2
In a 100 mL round bottom flask (3-aminopropyl)sulfamoyl fluoride **I4** (609 mg, 3.90 mmol) was dissolved in acetonitrile (30 mL). 2,5-dioxopyrrolidin-1-yl 2-bromoacetate **R1** (1.84 g, 7.80 mmol) was added, followed by a slow addition of N-ethyl-N-isopropylpropan-2-amine (1.36 mL, 7.80 mmol). The solution was stirred at room temperature overnight. The solvent was removed in vacuo and the residue was purified on flash column chromatography using a gradient of 12-100% ethyl acetate in heptane. Fractions containing the product were combined and the solvent was removed in vacuo. (3-(2-bromoacetamido)propyl)sulfamoyl fluoride **L2** (247 mg, 22.85 %) was obtained as a colorless oil.

LCMS (m/z, ES-) mass found = 274.9 [M-H]-, expected = 274,95 [M-H]- for [$C_5H_9BrFN_2O_3S^-$].

1H NMR (500 MHz, DMSO, 25°C) δ 1.67 (p, J = 7.1 Hz, 2H), 3.09–3.21 (m, 4H), 3.83 (s, 2H), 8.31 (d, J = 5.9 Hz, 1H), 9.35 (q, J = 5.5 Hz, 1H).
13C NMR (126 MHz, CDCl3) δ 167.9, 41.1, 36.7, 29.4, 28.8.
19F NMR (471 MHz, DMSO) δ 50.9.

3-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)carbamoyl)benzene sulfonyl fluoride, L3

In a 25 mL round bottom flask 1-(2-aminoethyl)-1H-pyrrole-2,5-dione hydrochloride **AM3** (100 mg, 0.57 mmol) and 3-(fluorosulfonyl)benzoic acid **R4** (127 mg, 0.62 mmol) were dissolved in DCM (5 mL) and the mixture was cooled down to 0 °C. N-ethyl-N-isopropylpropan-2-amine (0.49 mL, 2.83 mmol) was added, followed by a dropwise addition of 2,4,6-tripropyl-1,3,5,2,4,6-trioxatriphosphinane 2,4,6-trioxide (0.42 mL, 1.42 mmol). The solution was stirred at 0 °C for 10 minutes and then at room temperature for 1 hour. Sat. $NaHCO_3$ (40 mL) was added and the mixture was extracted with EtOAc (3x30mL). The combined organic layer was washed with 1M HCl solution (30 mL) and brine (30 mL), dried over Na_2SO_4 and filtered. The solvent was removed in vacuo to yield 3-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)carbamoyl)benzenesulfonyl fluoride **L3** (170 mg, 92 %) as a colorless solid.

LCMS (m/z, ES+) mass found = 327.2 [M+H]+, expected = 327,04 [M+H]+ for [$C_{13}H_{12}FN_2O_5S^+$].

1H NMR (500 MHz, DMSO, 25°C) δ 3.4–3.47 (m, 2H), 3.61 (dd, J = 6.5, 4.9 Hz, 2H), 7.02 (s, 2H), 7.90 (t, J = 7.9 Hz, 1H), 8.25–8.33 (m, 2H), 8.41 (t, J = 1.8 Hz, 1H), 9.01 (t, J = 5.9 Hz, 1H).
13C NMR (126 MHz, DMSO) δ 171.1, 164.1, 136.1, 135.1, 134.6, 132.0, 131.8, 130.9, 130.8, 126.6, 37.9, 36.9.
19F NMR (471 MHz, DMSO) δ 66.4.

4-acryloylpiperazine-1-sulfonyl fluoride, L4

In a 100 mL round bottom flask piperazine-1-sulfonyl fluoride **I2b** (627 mg, 3.73 mmol) was dissolved in DCM (30 mL) and the solution was cooled down to 0 °C. acryloyl chloride **R3** (0.30 mL, 3.73 mmol) was added, followed by a slow addition of N-ethyl-N-isopropylpropan-2-amine (1.30 mL, 7.46 mmol). The solution was stirred at 0 °C for 30 minutes and was then allowed to slowly warm up to room temperature. The solution was stirred until completion (1.5 hours). The solvent was removed in vacuo and the residue was purified on flash column chromatography using a gradient of 12-100% ethyl acetate in heptane. Fractions containing the product were combined and the solvent was removed in vacuo. 4-acryloylpiperazine-1-sulfonyl fluoride **L4** (534 mg, 64.5 %) was obtained as a colorless solid.

LCMS (m/z, ES+) mass found = 223.1 [M+H]+, expected = 223,05 [M+H]+ for [$C_7H_{12}FN_2O_3S^+$].

1H NMR (500 MHz, DMSO, 25°C) δ 3.44–3.48 (m, 4H), 3.65–3.75 (m, 4H), 5.74 (dd, J = 10.5, 2.3 Hz, 1H), 6.14 (dd, J = 16.7, 2.3 Hz, 1H), 6.79 (dd, J = 16.7, 10.5 Hz, 1H).
13C NMR (126 MHz, DMSO) δ 164.5, 128.1, 127.9, 46.9, 46.5, 43.9.
19F NMR (471 MHz, DMSO) δ 41.2.

(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)sulfamoyl fluoride, L5
In a 25 mL round bottom flask1-(2-aminoethyl)-1H-pyrrole-2,5-dione hydrochloride **AM1** (177 mg, 1 mmol) was dissolved in dichloromethane (5 mL) and 1-(fluorosulfonyl)-2,3-dimethyl-1H-imidazol-3-ium trifluoromethanesulfonate **SM1** (492 mg, 1.50 mmol) was added in one portion. triethylamine (0.14 mL, 1.00 mmol) was added drop-wise and the mixture was stirred for 1 hour at room temperature. The solvent was evaporated and the residue was dissolved in EtOAc (20 mL) and the mixture was washed with 1 M HCl (3x20 mL) and brine (1x20 mL), dried over Na_2SO_4, and filtered. The solvent was evaporated and the residue was dissolved in DCM and purified by flash column chromatography using a gradient of 12-100% EtOAc in heptane. Fractions containing the product were combined and the solvent was evaporated in vacuo. (2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)sulfamoyl fluoride **L5** (48.9 mg, 22.01 %) was obtained as a colorless solid.

LCMS (m/z, ES-) mass found = 221.0 [M-H]-, expected = 221,00 [M+H]+ for [$C_6H_6FN_2O_4S^-$].

1H NMR (500 MHz, CDCl3, 25°C) δ 3.5–3.57 (m, 2H), 3.77–3.84 (m, 2H), 5.61 (s, 1H), 6.79 (s, 2H).
13C NMR (126 MHz, CDCl3) δ 170.9, 134.6, 43.9, 37.3.
19F NMR (471 MHz, CD3CN) δ 50.0.

4-(2-bromoacetyl)piperazine-1-sulfonyl fluoride, L6
In a 25 mL round bottom flask piperazine-1-sulfonyl fluoride **I2a** (125 mg, 0.74 mmol) and 2-bromoacetic acid **R5** (0.059 mL, 0.82 mmol) were dissolved in DCM (5 mL) and the mixture was cooled down to 0 °C. N-ethyl-N-isopropylpropan-2-amine (0.65 mL, 3.72 mmol) was added, followed by a dropwise addition of 2,4,6-tripropyl-1,3,5,2,4,6-trioxatriphosphinane 2,4,6-trioxide (0.55 mL, 1.86 mmol). The solution was stirred at 0 °C for 10 minutes and then at room temperature for 1.5 hours. Sat. $NaHCO_3$ (40 mL) was added and the mixture was extracted with EtOAc (3x40 mL). The combined organic phase was washed with 1M HCl solution (40 mL) and brine (40 mL), dried over Na_2SO_4 and filtered. The solvent was removed in vacuo to yield 4-(2-bromoacetyl)piperazine-1-sulfonyl fluoride **L6** (141 mg, 65.8 %) as a colorless solid.

LCMS (m/z, ES+) mass found = 289.1 [M+H]+, expected = 288,97 [M+H]+ for [$C_6H_{11}BrFN_2O_3S^+$].

1H NMR (500 MHz, CDCl3, 27°C) δ 3.45–3.59 (m, 4H), 3.66–3.82 (m, 4H), 4.08 (s, 2H).
13C NMR (126 MHz, CDCl3) δ 165.4, 47.0, 46.8, 45.4, 41.0, 40.5.
19F NMR (471 MHz, CDCl3) δ 40.5.

5.2 High Throughput Experimentation

The reaction in HTE was a two-step, one-pot reaction where the first step consisted of adding the thiol to the thiol accepting group of the linker. The second step was the SuFEx reaction, where the amines did a SuFEx reaction with the linker. A 96 well plate was used for both reactions. LC-MS plate analysis was used to analyze the two reactions.

Thiol Addition

The amount of reagents in columns 1 through 9 were: linker (1 eq, 40 µmol), thiol **S1** (1.5 eq, 60 µmol) and DMAP (0.05 eq, 2 µmol). The total volume was 200 µl.

The amount of reagents in columns 10 through 12 were: linker (1 eq, 40 µmol), thiol **S1** (1.5 eq, 60 µmol), K_2CO_3 (2 eq, 80 µmol) and KI (1 eq, 40 µmol). The total volume was 200 µl.

SuFEx reaction

The amount of reagents per reaction (in relation to linker added in the first step) were: amines A1, A2 and A3 (1.2 eq. 48 µmol) DABCO (1.5 eq, 60 µmol), $Ca(NTf_2)_2$ (1.1 eq, 44 µmol), DIPEA (1.5 eq, 60 µmol), TEA (1.5 eq, 60 µmol), MgO (2.5 eq, 100 µmol). The total volume was 200 µl.

Below are the masses found in LC-MS analysis of the different SuFEx products.

N-phenethyl-4-(3-(phenethylthio)propanoyl)piperazine-1-sulfonamide

LCMS (m/z, ES+) mass found = 462.3 [M+H]+, expected = 462.19 [M+H]+ for [$C_{23}H_{32}N_3O_3S_2^+$].

N-methyl-N-phenethyl-4-(3-(phenethylthio)propanoyl)piperazine-1-sulfon amide

LCMS (m/z, ES+) mass found = 476.3 [M+H]+, expected = 476.20 [M+H]+ for [$C_{24}H_{34}N_3O_3S_2^+$].

N-(2-(2,5-dioxo-3-(phenethylthio)pyrrolidin-1-yl)ethyl)-3-(N-phenethyl sulfamoyl)benzamide

LCMS (m/z, ES+) mass found = 566.2 [M+H]+, expected = 566.18 [M+H]+ for [$C_{29}H_{32}N_3O_5S_2^+$].

N-(2-(2,5-dioxo-3-(phenethylthio)pyrrolidin-1-yl)ethyl)-3-(N-methyl-N-phenethylsulfamoyl)benzamide

LCMS (m/z, ES+) mass found = 580.1 [M+H]+, expected = 580.19 [M+H]+ for [$C_{30}H_{34}N_3O_5S_2^+$].

N-(2-(2,5-dioxo-3-(phenethylthio)pyrrolidin-1-yl)ethyl)-3-(N-(4-(methyl sulfonyl)phenyl)sulfamoyl)benzamide

LCMS (m/z, ES-) mass found = 614.2 [M-H]-, expected = 614.11 [M-H]- for [$C_{28}H_{28}N_3O_7S_3^-$].

N-phenethyl-2-(phenethylthio)-2-phenylethane-1-sulfonamide

LCMS (m/z, ES+) mass found = 448.3 [M+Na], expected = 448.14 [M+Na] for [$C_{24}H_{27}NNaO_2S_2$].

N-methyl-N-phenethyl-2-(phenethylthio)-2-phenylethane-1-sulfonamide

LCMS (m/z, ES+) mass found = 478.3 [M+K], expected = 478.13 [M+K] for [$C_{25}H_{29}KNO_2S_2$].

N-(4-(methylsulfonyl)phenyl)-2-(phenethylthio)-2-phenylethane-1-sulfonamide

LCMS (m/z, ES-) mass found = 474.2 [M-H]-, expected = 474.09 [M-H]- for [$C_{23}H_{24}NO_4S_3^-$].

N-phenethyl-4-((phenethylthio)methyl)benzenesulfonamide

LCMS (m/z, ES+) mass found = 412.3 [M+H]+, expected = 412.14 [M+H]+ for [$C_{23}H_{26}NO_2S_2^+$].

N-methyl-N-phenethyl-4-((phenethylthio)methyl)benzenesulfonamide

LCMS (m/z, ES+) mass found = 426.3 [M+H]+, expected = 426.16 [M+H]+ for [$C_{24}H_{28}NO_2S_2^+$].

N-(4-(methylsulfonyl)phenyl)-4-((phenethylthio)methyl)benzenesulfon amide

LCMS (m/z, ES+) mass found = 460.0 [M-H]-, expected = 460.07 [M-H]- for [$C_{22}H_{22}NO_4S_3^-$].

5.3 Linker Screen

Thiol addition of Michael acceptors L1, L5 & L9
To a vial containing linker (1 eq, 0.07 mmol) and DMAP (0.05 eq, 3.62 µmol) was added MeCN (300 μl), followed by addition of thiol **S1** (1.5 eq, 0.11 mmol). The solution was stirred at room temperature overnight. The solvent was removed under a stream of N_2 and the residue was used in the next step without further treatment.

Thiol addition of alkyl bromides L2 & L6
To a vial containing linker (1 eq, 0.07 mmol), KI (1 eq, 0.07 mmol) and K_2CO_3 (2 eq, 0.14 mmol) was added MeCN (300 μl), followed by addition of thiol **S1** (1.5 eq, 0.11 mmol). The mixture was stirred at room temperature overnight. The solvent was removed under a stream of N_2 and the residue was used in the next step without further treatment.

SuFEx reaction in iPrOH
To a vial containing the residue from thiol addition was added $Ca(NTf_2)_2$ (1.1 eq, 0.08 mmol), DABCO (1.5 eq, 0.11 mmol) and iPrOH (300 μl). Amine **A1** (1.2 eq, 0.08 mmol) was added and the mixture was stirred at room temperature overnight.

SuFEx reaction in H_2O:MeCN 1:1
To a vial containing the residue from thiol addition was added $Ca(NTf_2)_2$ (1.1 eq, 0.08 mmol), DABCO (1.5 eq, 0.11 mmol) and H_2O:MeCN 1:1 (300 μl). Amine **A1** (1.2 eq, 0.08 mmol) was added and the mixture was stirred at at 40 °C overnight.

5.4 Synthesis of starting materials for conjugation

N-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethyl)-3-(pyridin-2-yldisulfaneyl)propanamide, I5

In a 50 mL round bottom flask 4-((2-aminoethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione hydrochloride **R6** (100 mg, 0.28 mmol) was dissolved in DCM (5 mL) and N-ethyl-N-isopropylpropan-2-amine (0.12 mL, 0.71 mmol) was added, followed by addition of 2,5-dioxopyrrolidin-1-yl 3-(pyridin-2-yldisulfaneyl)propanoate**R7** (93 mg, 0.30 mmol). The solution was stirred at room temperature overnight. The solvent was removed in vacuo and the residue was purified on flash column chromatography using a gradient of 12-100% EtoAc:EtOH 3:1 in heptane. Fractions containing the product were retained and the solvent was removed in vacuo. The residue was washed with EtOAc:H_2O 1:1 (10 mL). The organic layer was dried over Na_2SO_4, filtered and the solvent was removed in vacuo to yield N-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethyl)-3-(pyridin-2-yldisulfaneyl)propanamide **I5** (113 mg, 77 %) as a yellow gum.

LCMS (m/z, ES+) mass found = 514.3 [M+H]+, expected = 514.12 [M+H]+ for [$C_{23}H_{24}N_5O_5S_2{}^+$].

1H NMR (500 MHz, CDCl3, 27°C) δ 2.09–2.14 (1H, m), 2.64 (2H, t), 2.69–2.8 (2H, m), 2.85–2.88 (1H, m), 3.07–3.13 (2H, m), 3.51 (2H, d), 3.53–3.58 (2H, m), 4.87–4.93 (1H, m), 7.00 (1H, dd), 7.10 (1H, dd), 7.14 (1H, ddd), 7.22 (1H, s), 7.46–7.52 (1H, m), 7.61 (1H, dt), 7.66 (1H, ddd), 8.02 (1H, s), 8.16 (1H, s), 8.42 (1H, ddd)

N-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethyl)-3-mercaptopropanamide, I6

In a 5 mL round bottom flask containing N-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethyl)-3-(pyridin-2-yldisulfaneyl)propanamide **I5** (20 mg, 0.04 mmol), 2-propanol (1 ml) was added followed by addition of 3,3',3"-phosphane triyltripropionic acid hydrochloride (86 µl, 0.04 mmol). The solution was stirred at room temperature until completion (2 hours) and was then used in the next step without further treatment.

LCMS (m/z, ES+) mass found = 405.3 [M+H]+, expected = 405.12 [M+H]+ for [$C_{18}H_{21}N_4O_5S^+$].

3’,6’-dihydroxy-3-oxo-N-(2-(tritylthio)ethyl)-3H-spiro[isobenzofuran -1,9’-xanthene]-5-carboxamide, I7

In a 100 mL round bottom flask 3’,6’-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9’-xanthene]-5-carboxylic acid **R8** (300 mg, 0.80 mmol) was dissolved in dry DMF (30 mL) under an atmosphere of nitrogen. 1-(bis(dimethylamino)methylene)-1H-[1,2,3]triazolo[4,5-b]pyridine-1-ium 3-oxide hexafluorophosphate(V) (288 mg, 0.76 mmol) was added, followed by addition of N-ethyl-N-isopropylpropan-2-amine (0.29 mL, 1.59 mmol). The solution was stirred for 5 min and then 2-(tritylthio)ethan-1-amine **R9** (242 mg, 0.76 mmol) was added. The solution was stirred at room temperature overnight. The solvent was removed in vacuo and the residue was purified on flash column chromatography using a gradient of 12-100% EtoAc:EtOH 3:1 in heptane. Fractions containing the product were retained and the solvent was removed in vacuo. 3’,6’-dihydroxy-3-oxo-N-(2-(tritylthio)ethyl)-3H-spiro[isobenzofuran-1,9’-xanthene]-5-carboxamide **I7** (177 mg, 32.7 %) was obtained as a yellow solid.

LCMS (m/z, ES+) mass found = 678.2 [M+H]+, expected = 678.19 [M+H]+ for [$C_{42}H_{32}NO_6S^+$].

Analytical data were in accordance to those reported earlier [52].

3’,6’-dihydroxy-N-(2-mercaptoethyl)-3-oxo-3H-spiro[isobenzofuran -1,9’-xanthene]-5-carboxamide, T4

In a 50 mL round bottom flask 3’,6’-dihydroxy-3-oxo-N-(2-(tritylthio)ethyl)-3H-spiro[isobenzofuran-1,9’-xanthene]-5-carboxamide **I8** (153 mg, 0.23 mmol) was dissolved in 2,2,2-trifluoroacetic acid (6.96 mL, 90.22 mmol), water (185 µL, 10.27 mmol), triisopropylsilane (74 µL, 0.36 mmol) and 2,2’-(ethane-1,2-diylbis(oxy))bis(ethane-1-thiol) (185 µL, 1.14 mmol) and the solution was stirred at room temperature overnight. The solution was concentrated to appr. 1 mL under a stream of N_2 and was then recrystallized at -20 °C (60 mL Et_2O:pentane 2:1). The product was washed with Et_2O (3 mL), dissolved in MeCN and water and freeze dried. 3’,6’-dihydroxy-N-(2-mercaptoethyl)-3-oxo-3H-spiro[isobenzofuran-1,9’-xanthene]-5-carboxamide **T4** (20.7 mg, 21.10 %) was obtained as an orange solid.

LCMS (m/z, ES+) mass found = 436.2 [M+H]+, expected = 436.08 [M+H]+ for [$C_{23}H_{18}NO_6S^+$].

Analytical data were in accordance to those reported earlier [52].

tert-butyl (2-(2-(2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethoxy)ethoxy)ethyl)carbamate, I9

2,5-dioxopyrrolidin-1-yl 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate **R10** (500 mg, 1.46 mmol) was dissolved in DMF (20 mL) and tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate **R11** (0.52 mL, 2.20 mmol) was added, followed by addition of triethylamine (0.41 mL, 2.93 mmol). The solution was stirred at room temperature overnight and then the solvent was removed in vacuo. Water (25 mL) and ethyl acetate (25 mL) were added and the mixture was shaken to remove residual DMF. The aqueous phase was extracted with ethyl acetate (25 mL) and the combined organic phase was dried over Na_2SO_4, filtered and the solvent was removed in vacuo. The residue was dissolved in DMSO and purified on preparative HPLC using a gradient of 20-60% acetonitrile in water. Fractions containing the product were combined and freeze dried. tert-butyl (2-(2-(2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethoxy)ethoxy)ethyl)carbamate **I9** (144 mg, 20.70 %) was obtained as a colorless solid.

LCMS (m/z, ES-) mass found = 473.4 [M-H]-, expected = 473.24 [M-H]- for [$C_{21}H_{37}N_4O_6S^-$].

1H NMR (500 MHz, DMSO, 27°C) δ 1.23–1.34 (2H, m), 1.37 (9H, s), 1.4–1.54 (3H, m), 1.56–1.66 (1H, m), 2.06 (2H, t), 2.57 (1H, d), 2.82 (1H, dd), 3.06 (2H, q), 3.08–3.12 (1H, m), 3.18 (2H, q), 3.38 (4H, dt), 3.49 (4H, s), 4.12 (1H, ddd), 4.30 (1H, dd), 6.34 (1H, s), 6.40 (1H, s), 6.75 (1H, s), 7.81 (1H, t).
13C NMR (126 MHz, CDCl3) δ 173.18, 163.38, 156.18, 79.58, 70.34, 70.07, 61.93, 60.28, 55.38, 40.66, 40.53, 39.33, 36.00, 28.59, 28.23, 28.18, 25.60.

N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide, I10

In a 10 mL round bottom flask tert-butyl (2-(2-(2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethoxy)ethoxy)ethyl)carbamate (100 mg, 0.21 mmol) **I9** was dissolved in DCM (2 ml) and 2,2,2-trifluoroacetic acid (1.00 ml, 12.96 mmol) was added. The solution was stirred at room temperature for 3 hours. The solvent was removed in vacuo and the residue was used in the next step without further treatment.

LCMS (m/z, ES+) mass found = 375.1 [M+H]+, expected = 375.21 [M+H]+ for [$C_{16}H_{31}N_4O_4S^+$].

5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(2-(2-(2-(3-(pyridin-2-yldisulfaneyl)propanamido)ethoxy)ethoxy)ethyl) pentanamide, I11

In a 5 mL round bottom flask [N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide **I10** (79 mg, 0.21 mmol) was dissolved in DCM (3 ml) and 2,5-dioxopyrrolidin-1-yl 3-(pyridin-2-yldisulfaneyl) propanoate **R7** (69.5 mg, 0.22 mmol) was added, followed by addition of N-ethyl-N-isopropylpropan-2-amine (55 µL, 0.32 mmol). The solution was stirred at room temperature overnight. N-ethyl-N-isopropylpropan-2-amine (0.15 ml, 0.84 mmol) was added and the solution was stirred at room temperature overnight. N-ethyl-N-isopropylpropan-2-amine (0.15 ml, 0.84 mmol) was added again and the solution was stirred at room temperature for 4 hours. The solvent was removed in vacuo and the residue was purified on flash column chromatography using a gradient of 20-60% acetonitrile in water (0.1% FA). The fraction containing the product was freeze dried. 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(2-(2-(2-(3-(pyridin-2-yldisulfaneyl)propanamido)ethoxy)ethoxy)ethyl)pentanamide **I11** (39.8 mg, 33.0 %) was obtained as a colorless solid.

LCMS (m/z, ES+) mass found = 572.2 [M+H]+, expected = 572.20 [M+H]+ for [$C_{24}H_{38}N_5O_5S_3^+$].

1H NMR (500 MHz, CDCl3, 27°C) δ 1.6–1.78 (4H, m), 2.22 (2H, t), 2.65 (2H, t), 2.73–2.78 (1H, m), 2.92 (1H, dd), 3.05–3.13 (4H, m), 3.16 (1H, td), 3.35–3.43 (1H, m), 3.45–3.49 (2H, m), 3.55–3.62 (7H, m), 3.67 (2H, p), 4.33 (1H, ddd), 4.52 (1H, dd), 5.24 (1H, s), 6.10 (1H, s), 6.47 (1H, t), 7.12 (2H, tdd), 7.61–7.75 (2H, m), 8.47 (1H, ddd).

N-(2-(2-(2-(3-mercaptopropanamido)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide, T5

In a 50 mL falcon tube 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(2-(2-(2-(3-(pyridin-2-yldisulfaneyl)propanamido)ethoxy)ethoxy)ethyl) pentanamide **I11** (31.4 mg, 0.05 mmol) was dissolved in 2-propanol (1 mL) and 3,3',3"-phosphane triyltripropionic acid hydrochloride (0.12 mL, 0.06 mmol) was added. The solution was stirred at room temperature for 1.5 hours and was then used in the next step without further treatment.

LCMS (m/z, ES+) mass found = 463.4 [M+H]+, expected = 463.20 [M+H]+ for [$C_{19}H_{35}N_4O_5S_2^+$].

(S)-1-(N-(N-((R)-2-((S)-2-((S)-2-acetamido-5-guanidinopentanamido)-3-phenyl propanamido)-3-mercapto-3-methylbutanoyl)-L-threonylglycyl-L-histidyl-L-phenylalanyl glycyl-N-methylglycyl)-N-methyl-L-leucyl-L-tyrosyl)-N-((R)-1-amino-3-mercapto-1-oxopropan-2-yl)pyrrolidine-2-carboxamide (Ac-R-F-Pen-T-G-H-F-G-Sar-NMeLeu-Y-P-C-NH2) T2
Automated Fmoc SPPS, Biotage® Initiator+ Alstra^{TM} automated microwave peptide synthesizer. Rink Amide MBHA resin (final loading 0.68 mmol/g) was swollen in EtOAc:DMSO (7:3) for 15 min and then the solvent was drained. The Fmoc N-protecting group was removed with 20% piperidine in EtOAc:DMSO (3:7) (2 x 10 min) at room temperature. The amino acids (4 equiv.) dissolved in DMF (0.2 M) were repeatedly coupled with DIC (4 equiv.) in DMF (2 M) and OXYMA (4 equiv.) in DMF (0.5 M) at 40 °C for 20 min. Washing of the resin between the coupling steps was performed with EtOAc:DMSO (7:3). The resin was finally washed with IPA (2 x 9 mL) and CPME (1 x 9 mL). After the final Fmoc deprotection, a solution of EtOAc:DMSO (7:3): acetic anhydride: 2,6 Lutidine 89:5:6 (5 mL) was added to the resin and stirred at room temperature for 60 min before the mixture was drained. This procedure was repeated twice, and the resin was washed with DMSO/EtOAC 1:9 (2 x 4 mL). A solution of TFA/water/DODT/TIS 90:2.5:2.5:5 (10 mL) was added to the dry resin. The reaction mixture was shaken at room temperature for 3 h. The filtrate was collected into Et_2O. The precipitate was placed in the freezer -20° C for 5 min, centrifuged at 13 rpm for 2 min and then decanted, and dried in vacuo to yield the crude as a white solid. The peptide was purified on flash column chromatography using a gradient of 10-50% acetonitrile in water (0.1% TFA). Fractions containing the product were combined and freeze dried. The product (Ac-R-F-Pen-T-G-H-F-G-Sar-NMeLeu-Y-P-C-NH2) was obtained as a colorless solid.

LCMS (m/z, ES+) mass found = 778.6 [M+2H]2+, expected = 777.87 [M+2H]2+ (m/z: 777.87 (100.0%), 778.37 (79.7%), 778.87 (36.0%), 778.87 (14.9%), 779.37 (13.1%)) for [$C_{72}H_{105}N_{19}O_{16}S_{22}^{+}$].

5.5 Synthesis of conjugates

C1

In a 5 mL round bottom flask (3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta [a]phenanthrene-3-thiol **T1** (18.9 mg, 0.05 mmol) and 4-(bromomethyl)benzene sulfonyl fluoride **L7** (11.9 mg, 0.05 mmol) were dissolved in 2-propanol (1 ml) and N-ethyl-N-isopropylpropan-2-amine (16 µL, 0.09 mmol) was added. The mixture was stirred at room temperature for 24 hours. (S)-2-(4-(piperidin-3-yl)phenyl)-2H-indazole-7-carboxamide **N1** (15 mg, 0.05 mmol), calcium bis((trifluoromethyl)sulfonyl) amide (30.9 mg, 0.05 mmol) and 140 µl of a 0.5 M stock solution of DABCO in iPrOH were added and the mixture was stirred at room temperature overnight. 2-propanol (1 ml) was added and the mixture was shaken at 40° C overnight. N-ethyl-N-isopropylpropan-2-amine (16 µL, 0.09 mmol) was added and the mixture was stirred at room temperature overnight. DMSO (200 µL) was added and the mixture was stirred at 60 °C for°C over the weekend and then at room temperature overnight. tAmylOH (1 mL) was added, together with N-ethyl-N-isopropylpropan-2-amine (41 µL, 0.23 mmol) and a spoonful of calcium bis((trifluoromethyl)sulfonyl)amide, and the mixture was stirred at room temperature overnight.

C2 and C3

In a 5 mL round bottom flask (S)-1-(N-(N-((R)-2-((S)-2-((S)-2-acetamido-5-guanidino pentanamido)-3-phenylpropanamido)-3-mercapto-3-methylbutanoyl)-L-threonylglycyl-L-histidyl-L-phenylalanylglycyl-N-methylglycyl)-N-methyl-L-leucyl-L-tyrosyl)-N-((R)-1-amino-3-mercapto-1-oxopropan-2-yl)pyrrolidine-2-carboxamide **T2** (23.9 mg, 0.02 mmol) and 3-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)carbamoyl)benzene sulfonyl fluoride **L3** (5 mg, 0.02 mmol) were dissolved in acetonitrile (250 µL) and water (250 µL). The solution was stirred at room temperature for 24 hours. 4-amino-N-(1-phenyl-1H-pyrazol-5-yl)benzenesulfonamide **N2** (4.82 mg, 0.02 mmol), calcium bis((trifluoromethyl)sulfonyl)amide (10.1 mg, 0.02 mmol) and 80 µl of a 0.25 M stock solution of DABCO in acetonitrile:water 1:1 were added and the solution was stirred at room temperature overnight. acetonitrile (250 µL) and water (250 µL) were added and the solution was shaken at 40° C overnight. hexadecan-1-amine **N3** (3.70 mg, 0.02 mmol) was added and the mixture was shaken at room temperature for 4 hours. hexadecan-1-amine **N3** (3.70 mg, 0.02 mmol), calcium bis((trifluoromethyl)sulfonyl)amide (10.1 mg, 0.02 mmol) and 80 µl of a 0.25 M stock solution of DABCO in acetonitrile:water 1:1 were added and the mixture was stirred at room temperature overnight.

LCMS (m/z, ES+) mass found = 627.0 [M+H]+, expected = 627.17 [M+H]+ for [$C_{25}H_{32}FN_6O_8S_2^+$].

LCMS (m/z, ES+) mass found = 931.8 [M+H]+, expected = 930.88 [M+H]+ (m/z: 930.88 (100.0%), 931.39 (94.1%), 931.89 (50.5%), 932.39 (22.0%), 931.88 (21.2%), 932.38 (14.3%), 931.38 (10.2%)) for [$C_{85}H_{115}N_{21}O_{21}S_{32}^+$].

C4

In a 5 mL round bottom flask containg N-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoiso indolin-4-yl)amino)ethyl)-3-mercaptopropanamide **T3** (15.8 mg, 0.04 mmol) in 2-propanol (1 ml), 4-acryloylpiperazine-1-sulfonyl fluoride **L4** (8.65 mg, 0.04 mmol) was added and the solution was stirred at room temperature overnight. N-ethyl-N-isopropylpropan-2-amine (6.8 µl, 0.04 mmol) was added and the solution was stirred for another 4 hours. N-ethyl-N-isopropylpropan-2-amine (6.8 µl, 0.04 mmol) was added again and the solution was stirred at room temperature overnight. N-(piperidin-2-ylmethyl)-2,5-bis(2,2,2-trifluoroethoxy)benzamide **N4** (16.1 mg, 0.04 mmol), calcium bis((trifluoromethyl)sulfonyl)amide (25.7 mg, 0.04 mmol) and 120 µl of a 0.5 M stock solution of DABCO in iPrOH were added together with 2-propanol (200 µL) and the solution was stirred at room temperature overnight. DMSO (200 µL) was added and the mixture was stirred at 60° C for 4 hours. 2-methyl tetrahydrofuran (500 µL) was added and the mixture was stirred at 60 °C over the weekend and then at room temperature overnight. DMSO (1.000 ml) was added together with N-ethyl-N-isopropylpropan-2-amine (34 µl, 0.19 mmol) and a spoonful of calcium bis((trifluoromethyl)sulfonyl)amide and the mixture was stirred at room temperature overnight.

LCMS (m/z, ES+) mass found = 941.8 [M+2H]2+, expected = 941.39 [M+2H]2+ (m/z: 941.39 (100.0%), 940.89 (97.2%), 941.89 (42.5%), 941.89 (26.8%), 942.39 (18.1%), 942.39 (16.7%)) for [$C_{85}H_{116}FN_{21}O_{21}S_{32}{}^{+}$].

C5

In a 5 mL round bottom flask 3',6'-dihydroxy-N-(2-mercaptoethyl)-3-oxo-3H-spiro[iso benzofuran-1,9'-xanthene]-5-carboxamide **T4** (20.4 mg, 0.05 mmol) and 4-(but-2-ynoyl)piperazine-1-sulfonyl fluoride **L1** (11.0 mg, 0.05 mmol) were dissolved in 2-propanol (1 ml) and the mixture was stirred at room temperature overnight. N-ethyl-N-isopropylpropan-2-amine (8.2 µl, 0.05 mmol) was added and the mixture was stirred for another 4 hours. N-ethyl-N-isopropylpropan-2-amine (16 µl, 0.09 mmol) was added and the solution was stirred at room temperature overnight. Color change from yellow to orange upon addition of base. (R)-2-(2-aminothiazol-4-yl)-N-(4-(2-((2-hydroxy-2-phenylethyl)amino)ethyl)phenyl)acetamide **N5** (18.6 mg, 0.05 mmol), calcium bis((trifluoromethyl)sulfonyl)amide (30.9 mg, 0.05 mmol) and 140 µl of a 0.5 M stock solution of DABCO in iPrOH were added, together with 2-propanol (300 µL). Color change from orange to reddish. The solution was stirred at room temperature overnight. DMSO (200 µL) was added and the mixture was stirred at 60° C for 4 hours. 2-methyl tetrahydrofuran (500 µL) was added and the mixture was stirred at 60 °C over the weekend and then at room temperature overnight. DMSO (500 ul) and tAmylOH (500 ul) were added together with N-ethyl-N-isopropylpropan-2-amine (41 µl, 0.23 mmol) and a spoonful of calcium bis((trifluoromethyl)sulfonyl)amide and the mixture was stirred at room temperature overnight.

LCMS (m/z, ES+) mass found = 670.0 [M+H]+, expected = 670.13 [M+H]+ for [$C_{31}H_{29}FN_3O_9S_2{}^{+}$].

C6

In a 50 mL falcon tube containing N-(2-(2-(2-(3-mercaptopropanamido)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide **T5** (25.4 mg, 0.05 mmol) in 2-propanol (1 ml) a solution of (3-(2-bromoacetamido)propyl)sulfamoyl fluoride (15.2 mg, 0.05 mmol) in 2-propanol (100 µL) was added, followed by addition of N-ethyl-N-isopropylpropan-2-amine (19 µl, 0.11 mmol). The solution was stirred at room temperature for 5 hours. 1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid **N6** (17.5 mg, 0.05 mmol) was added together with calcium bis((trifluoromethyl)sulfonyl)amide (36.3 mg, 0.06 mmol), 2-propanol (200 µL) and 160 ul of a 0.5 M solution of DABCO in iPrOH and the mixture was stirred at room temperature overnight.

LCMS (m/z, ES+) mass found = 659.3 [M+H]+, expected = 659.24 [M+H]+ for [$C_{24}H_{44}FN_6O_8S_3^+$].

References

[1] A. S. Barrow *et al.*, “The growing applications of SuFEx click chemistry,” *Chemical Society Reviews*, vol. 48, no. 17, pp. 4731–4758, 2019.

[2] H. C. Kolb, M. G. Finn, and K. B. Sharpless, “Click Chemistry: Diverse Chemical Function from a Few Good Reactions,” *Angewandte Chemie International Edition*, vol. 40, no. 11, pp. 2004–2021, Jun. 2001. DOI: 10.1002/1521-3773(20010601)40:11<2004::AID-ANIE2004>3.0.CO;2-5. (visited on 2025-04-21).

[3] J. Kaur, M. Saxena, and N. Rishi, “An Overview of Recent Advances in Biomedical Applications of Click Chemistry,” *Bioconjugate Chemistry*, vol. 32, no. 8, pp. 1455–1471, Aug. 2021. DOI: 10.1021/acs.bioconjchem.1c00247. (visited on 2025-05-29).

[4] P. Bishnoi, B. Saroha, S. Kumar, *et al.*, “Click Chemistry: An overview and recent updates in the medicinal attributes of click-derived heterocycles,” *Molecular Diversity*, Feb. 2025. DOI: 10.1007/s11030-025-11110-z. (visited on 2025-06-07).

[5] P. Thirumurugan, D. Matosiuk, and K. Jozwiak, “Click Chemistry for Drug Development and Diverse Chemical–Biology Applications,” *Chemical Reviews*, vol. 113, no. 7, pp. 4905–4979, Jul. 2013. DOI: 10.1021/cr200409f. (visited on 2025-06-07).

[6] J. Gierlich, G. A. Burley, P. M. E. Gramlich, D. M. Hammond, and T. Carell, “Click Chemistry as a Reliable Method for the High-Density Postsynthetic Functionalization of Alkyne-Modified DNA,” *Organic Letters*, vol. 8, no. 17, pp. 3639–3642, Aug. 2006. DOI: 10.1021/ol0610946. (visited on 2025-05-29).

[7] J. Dong *et al.*, “Sulfur(VI) fluoride exchange (SuFEx): Another good reaction for click chemistry,” vol. 53, p. 36, 2014.

[8] D. Serbetci, L. Marraffa, P. Natho, M. Andresini, and R. Luisi, “A Practical Guide to SuFEx Chemistry: An Overview of S(VI)-SuFEx Linkers and Their Reactivity,” *Synthesis*, vol. 57, no. 09, pp. 1569–1582, May 2025. DOI: 10.1055/a-2410-2039. (visited on 2025-04-21).

[9] D. Zeng *et al.*, “Advances in the construction of diverse SuFEx linkers,” *National Science Review*, vol. 10, no. 6, Nov. 2023.

[10] M. Chrominski *et al.*, “Introducing SuFNucs: Sulfamoyl-fluoride-functionalized nucleosides that undergo sulfur fluoride exchange reaction,” *Organic Letters*, vol. 24, no. 27, pp. 4977–4981, 2022.

[11] F. Liu *et al.*, “Biocompatible SuFEx click chemistry: Thionyl tetrafluoride (SOF4)-Derived connective hubs for bioconjugation to DNA and proteins,” vol. 58, p. 24, Mar. 2019.

[12] S. Kitamura *et al.*, “Sulfur(VI) fluoride exchange (SuFEx)-Enabled high-throughput medicinal chemistry,” *Journal of the American Chemical Society*, vol. 142, no. 25, pp. 10 899–10 904, 2020.

[13] H. Wang *et al.*, “SuFEx-Based polysulfonate formation from ethenesulfonyl Fluoride–Amine adducts,” vol. 56, p. 37, Mar. 2017.

[14] Z. Liu *et al.*, “SuFEx click chemistry enabled late-stage drug functionalization,” *Journal of the American Chemical Society*, vol. 140, no. 8, pp. 2919–2925, 2018.

[15] Y. Shishido *et al.*, “A covalent G-site inhibitor for glutathione S-transferase Pi (GSTP1-1),” *Chemical Communications*, vol. 53, no. 81, pp. 11 138–11 141, 2017.

[16] G. Alboreggia *et al.*, “Histidine-covalent stapled alpha-helical peptides targeting hMcl-1,” *Journal of Medicinal Chemistry*, vol. 67, no. 10, pp. 8172–8185, 2024.

[17] A. M. Kaufmann and J. P. Krise, “Lysosomal Sequestration of Amine-Containing Drugs: Analysis and Therapeutic Implications,” *Journal of Pharmaceutical Sciences*, vol. 96, no. 4, pp. 729–746, Apr. 2007. DOI: `10.1002/jps.20792`. (visited on 2025-06-07).

[18] R. He, B. Finan, J. P. Mayer, and R. D. DiMarchi, “Peptide Conjugates with Small Molecules Designed to Enhance Efficacy and Safety,” *Molecules*, vol. 24, no. 10, p. 1855, May 2019. DOI: `10.3390/molecules24101855`. (visited on 2025-04-10).

[19] T. Ishida and A. Ciulli, “E3 ligase ligands for PROTACs: How they were found and how to discover new ones,” *SLAS Discovery*, vol. 26, no. 4, pp. 484–502, 2021.

[20] M. P. Plesniak *et al.*, “Rapid PROTAC discovery platform: Nanomole-scale array synthesis and direct screening of reaction mixtures,” *ACS Medicinal Chemistry Letters*, vol. 14, no. 12, pp. 1882–1890, 2023.

[21] W. Lang, C. Yuan, L. Zhu, *et al.*, “Recent advances in construction of small molecule-based fluorophore-drug conjugates,” *Journal of Pharmaceutical Analysis*, vol. 10, no. 5, pp. 434–443, Oct. 2020. DOI: `10.1016/j.jpha.2020.08.006`. (visited on 2025-04-10).

[22] D. Irby, C. Du, and F. Li, “Lipid–Drug Conjugate for Enhancing Drug Delivery,” *Molecular Pharmaceutics*, vol. 14, no. 5, pp. 1325–1338, May 2017. DOI: `10.1021/acs.molpharmaceut.6b01027`. (visited on 2025-04-10).

[23] P. C. Trippier, “Synthetic Strategies for the Biotinylation of Bioactive Small Molecules,” *ChemMedChem*, vol. 8, no. 2, pp. 190–203, Feb. 2013. DOI: `10.1002/cmdc.201200498`. (visited on 2025-04-10).

[24] A. Pandeya, L. Yang, O. Alegun, *et al.*, “Biotinylation as a tool to enhance the uptake of small molecules in Gram-negative bacteria,” *PLOS ONE*, vol. 16, no. 11, H. W. Van Veen, Ed., e0260023, Nov. 2021. DOI: `10.1371/journal.pone.0260023`. (visited on 2025-04-10).

[25] R. Tripathi, A. Guglani, R. Ghorpade, and B. Wang, "Biotin conjugates in targeted drug delivery: Is it mediated by a biotin transporter, a yet to be identified receptor, or (an)other unknown mechanism(s)?" *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 38, no. 1, p. 2 276 663, Dec. 2023. DOI: 10.1080/14756366.2023.2276663. (visited on 2025-04-10).

[26] T. Guo, G. Meng, X. Zhan, *et al.*, "A New Portal to SuFEx Click Chemistry: A Stable Fluorosulfuryl Imidazolium Salt Emerging as an "F-SO_2 +" Donor of Unprecedented Reactivity, Selectivity, and Scope," *Angewandte Chemie International Edition*, vol. 57, no. 10, pp. 2605–2610, Mar. 2018. DOI: 10.1002/anie.201712429. (visited on 2025-04-21).

[27] P. Ochtrop and C. P. R. Hackenberger, "Recent advances of thiol-selective bioconjugation reactions," *Current Opinion in Chemical Biology*, vol. 58, pp. 28–36, Oct. 2020. DOI: 10.1016/j.cbpa.2020.04.017. (visited on 2025-06-07).

[28] M. H. Stenzel, "Bioconjugation Using Thiols: Old Chemistry Rediscovered to Connect Polymers with Nature's Building Blocks," *ACS Macro Letters*, vol. 2, no. 1, pp. 14–18, Jan. 2013. DOI: 10.1021/mz3005814. (visited on 2025-06-07).

[29] M. Ahangarpour, I. Kavianinia, and M. A. Brimble, "Thia-Michael addition: The route to promising opportunities for fast and cysteine-specific modification," *Organic & Biomolecular Chemistry*, vol. 21, no. 15, pp. 3057–3072, 2023. DOI: 10.1039/D2OB02262A. (visited on 2025-06-16).

[30] S. M. Mennen, C. Alhambra, C. L. Allen, *et al.*, "The Evolution of High-Throughput Experimentation in Pharmaceutical Development and Perspectives on the Future," *Organic Process Research & Development*, vol. 23, no. 6, pp. 1213–1242, Jun. 2019. DOI: 10.1021/acs.oprd.9b00140. (visited on 2025-06-04).

[31] M. Shevlin, "Practical High-Throughput Experimentation for Chemists," *ACS Medicinal Chemistry Letters*, vol. 8, no. 6, pp. 601–607, Jun. 2017. DOI: 10.1021/acsmedchemlett.7b00165. (visited on 2025-06-04).

[32] T. Cernak, N. J. Gesmundo, K. Dykstra, *et al.*, "Microscale High-Throughput Experimentation as an Enabling Technology in Drug Discovery: Application in the Discovery of (Piperidinyl)pyridinyl-1 *H* -benzimidazole Diacylglycerol Acyltransferase 1 Inhibitors," *Journal of Medicinal Chemistry*, vol. 60, no. 9, pp. 3594–3605, May 2017. DOI: 10.1021/acs.jmedchem.6b01543. (visited on 2025-06-04).

[33] D. Harvey, *Modern Analytical Chemistry*. Boston: McGraw-Hill, 2000.

[34] Biotage®, "Biotage® Sfär Columns," Brochure PPS497.v2, 2020.

[35] Biotage®, "Successful Flash Chromatography," White Paper PPS490.V.5, 2018.

[36] *Aqueous Workup*, https://chemtl.york.ac.uk/techniques/reaction-techniques/workup/theory. (visited on 2025-05-01).

[37] A. D. Pucker and J. J. Nichols, "Analysis of Meibum and Tear Lipids," *The Ocular Surface*, vol. 10, no. 4, pp. 230–250, Oct. 2012.

[38] "Thin Layer Chromatography," in *Methods in Enzymology*. Elsevier, 2013, pp. 303–324. DOI: 10.1016/b978-0-12-420067-8.00024-6. (visited on 2025-05-01).

[39] *Thin Layer Chromatography Stains*, https://www.reachdevices.com/TLC_stains.html. (visited on 2025-05-01).

[40] P. M. V. Raja and A. R. Barron, *2.2: Mass Spectrometry*, https://chem.libretexts.org/Courses/Un

[41] J. J. Pitt, "Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry," *The Clinical Biochemist. Reviews*, vol. 30, no. 1, pp. 19–34, Feb. 2009.

[42] J. T. Watson and O. D. Sparkman, *Introduction to Mass Spectrometry: Instrumentation, Applications, and Strategies for Data Interpretation*, 4th ed. Hoboken (N.J.): J. Wiley & sons, 2007.

[43] X. Liu, *Organic Chemistry I* (Open Textbook Library). British Columbia: Kwantlen Polytechnic University, 2021.

[44] J. A. Koutcher and C. T. Burt, "Principles of Nuclear Magnetic Resonance," vol. 25, no. 1, pp. 101–111, Jan. 1984.

[45] T. D. Yassa *et al.*, "Lewis acid-catalyzed sulfur fluoride exchange," *Organic Letters*, vol. 26, no. 46, pp. 9897–9902, 2024.

[46] S. Mahapatra *et al.*, "SuFEx activation with Ca(NTf2)2: A unified strategy to access sulfamides, sulfamates, and sulfonamides from S(VI) fluorides," *Organic Letters*, vol. 22, no. 11, pp. 4389–4394, 2020.

[47] D.-D. Liang, N. Lional, B. Scheepmaker, *et al.*, "Enantiospecific Synthesis of Aniline-Derived Sulfonimidamides," *Organic Letters*, vol. 25, no. 30, pp. 5666–5670, Aug. 2023. DOI: 10.1021/acs.orglett.3c02132. (visited on 2025-06-03).

[48] P. Mukherjee, C. P. Woroch, L. Cleary, *et al.*, "Sulfonamide Synthesis via Calcium Triflimide Activation of Sulfonyl Fluorides," *Organic Letters*, vol. 20, no. 13, pp. 3943–3947, Jul. 2018. DOI: 10.1021/acs.orglett.8b01520. (visited on 2025-06-03).

[49] D. Deans, S. Iqbal, Z. Zhong, *et al.*, *Catalytic SuFEx Reactivity of Sulfonimidoyl Fluorides with Functionalized Amines with Automation Applicable Conditions*, May 2025. DOI: 10.26434/chemrxiv-2025-rjn5d. (visited on 2025-06-08).

[50] M. Wei, D. Liang, X. Cao, *et al.*, "A Broad-Spectrum Catalytic Amidation of Sulfonyl Fluorides and Fluorosulfates**," *Angewandte Chemie International Edition*, vol. 60, no. 13, pp. 7397–7404, Mar. 2021. DOI: 10.1002/anie.202013976. (visited on 2025-06-08).

[51] X. Xia, W. Gao, X. Yang, W. Huang, X.-X. Xia, and D. Yan, "Chemically Engineered Affinity Protein Drugs for Covalent Targeted Cancer Therapy," *Journal of the American Chemical Society*, jacs.5c02212, May 2025. DOI: 10.1021/jacs.5c02212. (visited on 2025-06-08).

[52] M. Buhl, M. Tesch, S. Lamping, J. Moratz, A. Studer, and B. J. Ravoo, "Preparation of Functional Alternating Polymer Brushes and Their Orthogonal Surface Modification through Microcontact Printing," *Chemistry – A European Journal*, vol. 23, no. 25, pp. 6042–6047, May 2017. DOI: 10.1002/chem.201603565. (visited on 2025-06-16).

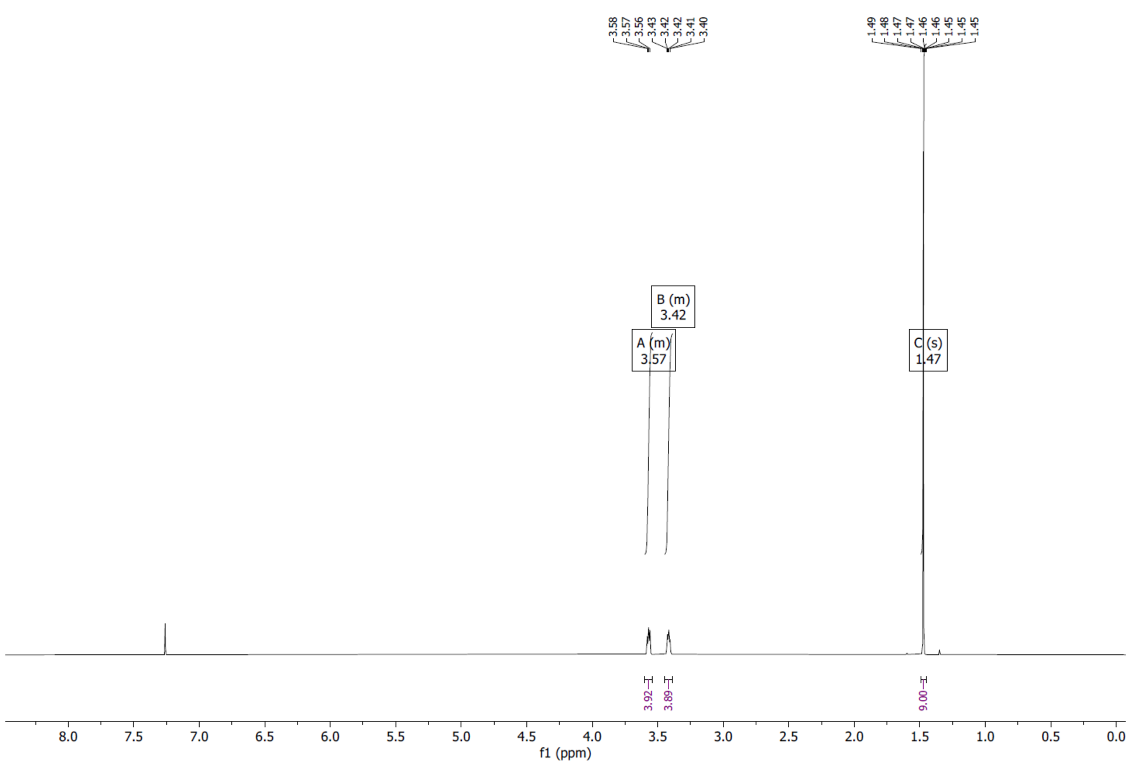
A. First appendix

Below are NMR spectra of compounds synthesized in the project. Non-purified linkers sometimes contain a peak in the 19F spectra in the region -73 – -78; these peaks correspond to residual triflate.

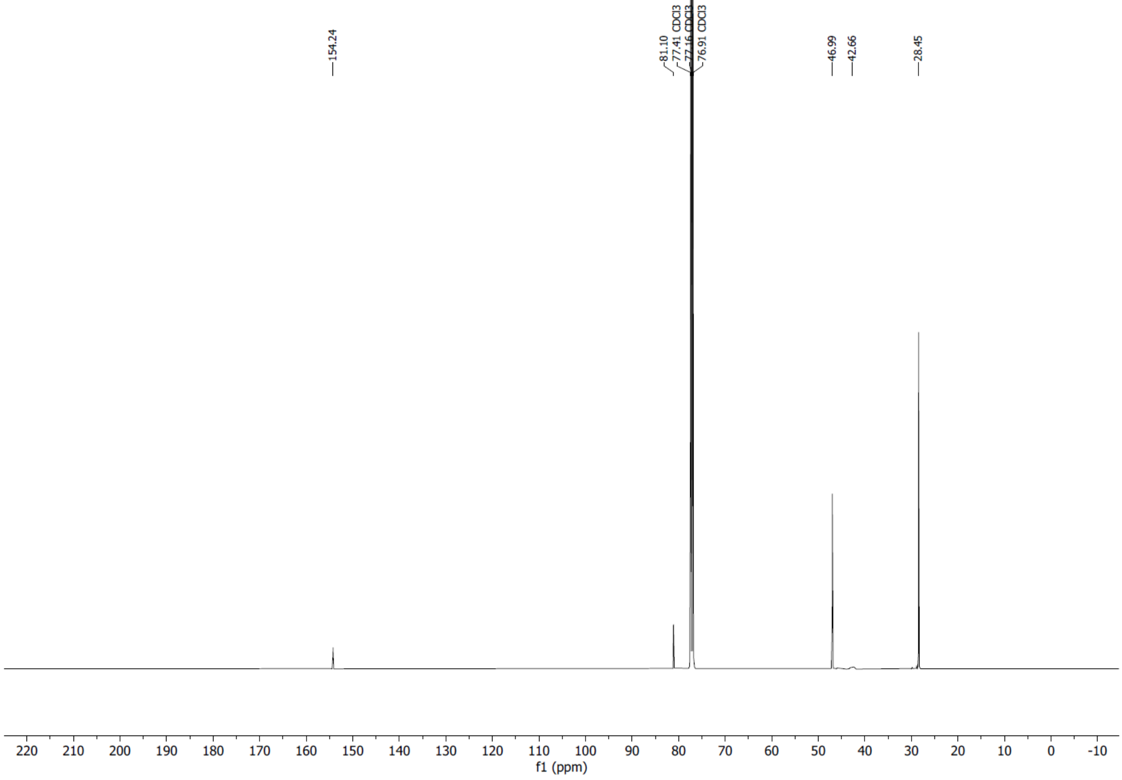
A.1 SuFEx Linkers

tert-butyl 4-(fluorosulfonyl)piperazine-1-carboxylate I1

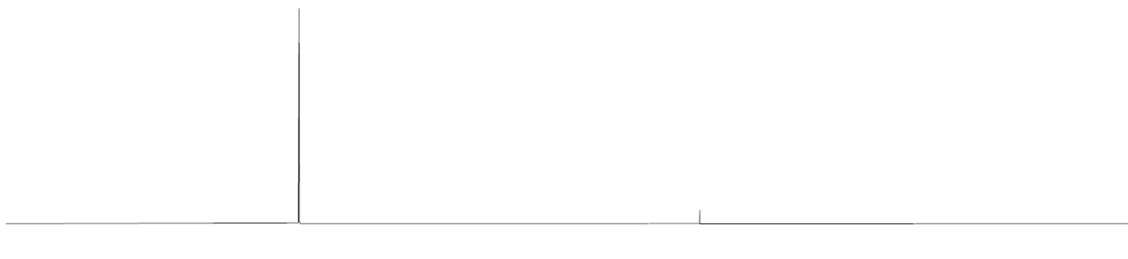
1H NMR (500 MHz, CDCl3, 25°C) δ 1.47 (s, 9H), 3.39–3.45 (m, 4H), 3.54–3.6 (m, 4H).



13C NMR (126 MHz, CDCl3) δ 154.2, 81.1, 47.0, 42.7, 28.5.

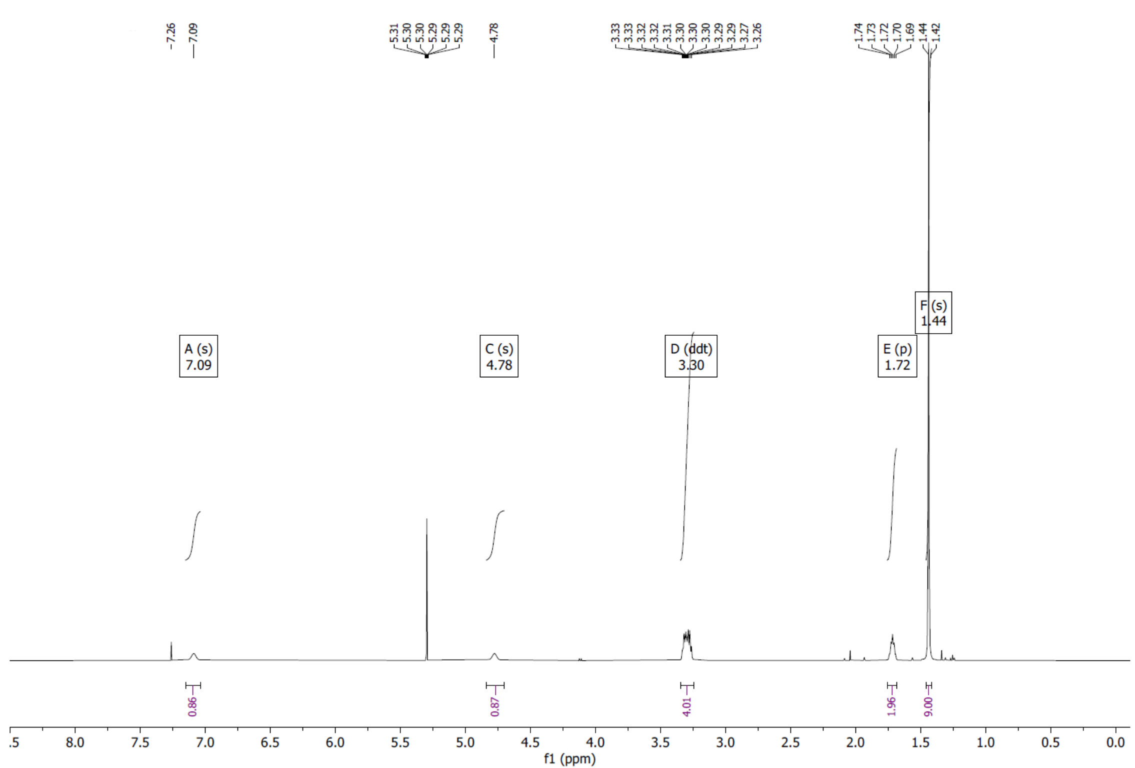


19F NMR (471 MHz, CDCl3) δ 39.7, -78.4.

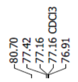


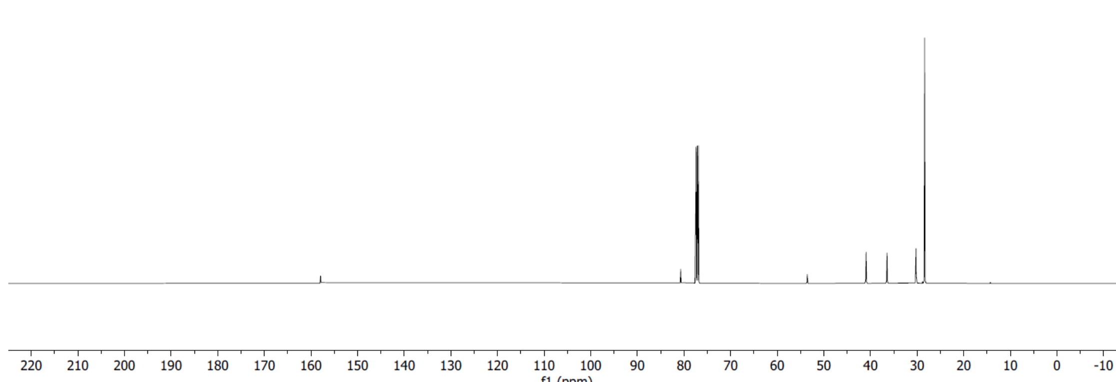
tert-butyl (3-((fluorosulfonyl)amino)propyl)carbamate, I3

1H NMR (500 MHz, CDCl3, 25°C) δ 1.44 (s, 9H), 1.72 (p, J = 5.9 Hz, 2H), 3.30 (ddt, J = 14.7, 12.1, 6.3 Hz, 4H), 4.78 (s, 1H), 7.09 (s, 1H).

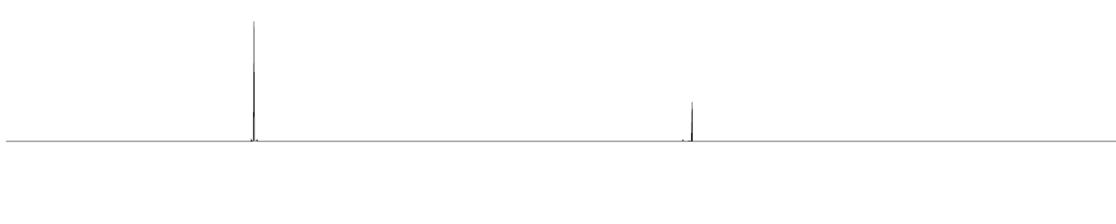


13C NMR (126 MHz, CDCl3) δ 157.9, 80.7, 41.0, 36.5, 30.2, 28.4.



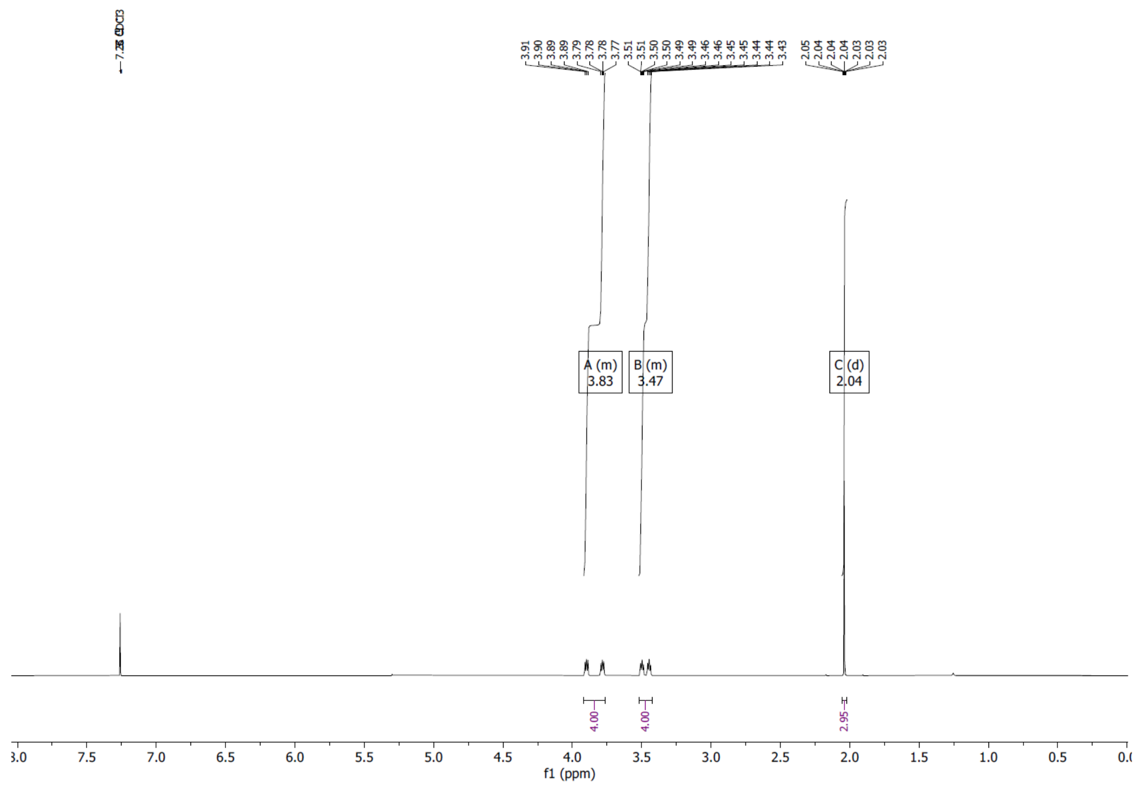


19F NMR (471 MHz, CDCl3) δ 52.1, 52.1, -78.5.

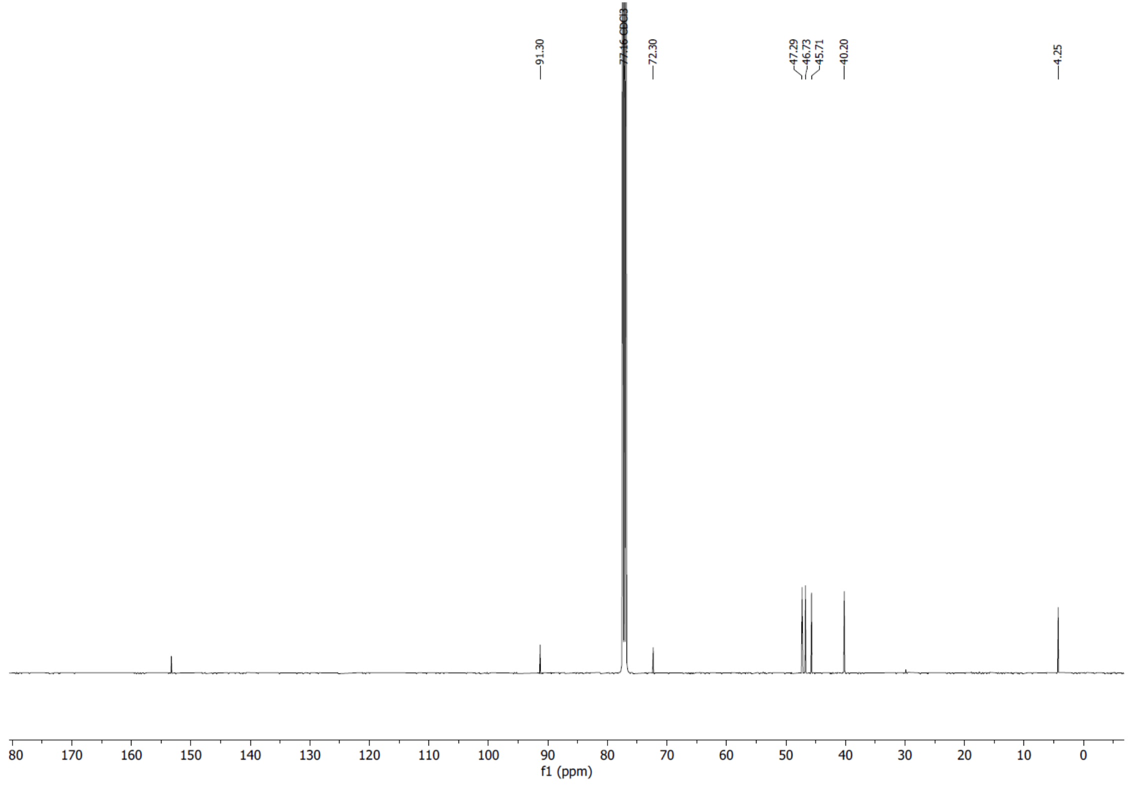


4-(but-2-ynoyl)piperazine-1-sulfonyl fluoride, L1

1H NMR (500 MHz, CDCl3, 25°C) δ 2.04 (d, J = 0.6 Hz, 3H), 3.42–3.52 (m, 4H), 3.76–3.92 (m, 4H).



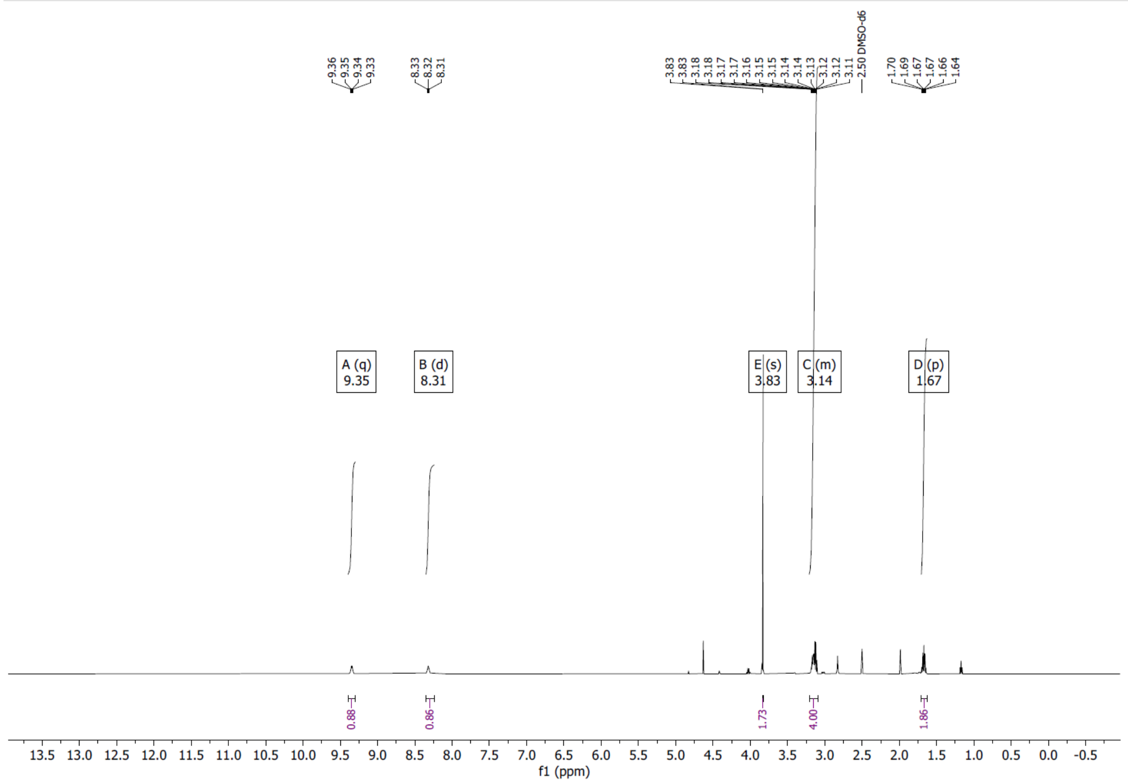
13C NMR (126 MHz, CDCl3) δ 153.2, 91.3, 72.3, 47.3, 46.7, 45.7, 40.2, 4.3.



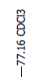
19F NMR (471 MHz, CDCl3) δ 40.5, -75.8, -78.3.

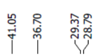
(3-(2-bromoacetamido)propyl)sulfamoyl fluoride, L2

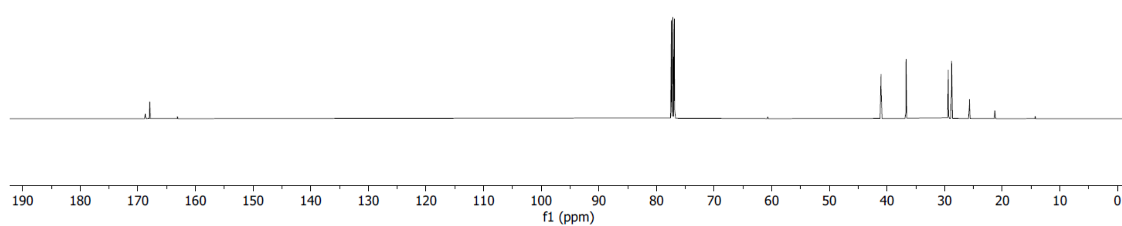
1H NMR (500 MHz, DMSO, 25°C) δ 1.67 (p, J = 7.1 Hz, 2H), 3.09–3.21 (m, 4H), 3.83 (s, 2H), 8.31 (d, J = 5.9 Hz, 1H), 9.35 (q, J = 5.5 Hz, 1H).



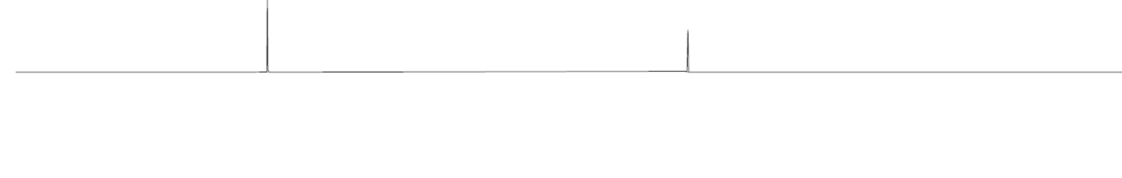
13C NMR (126 MHz, CDCl3) δ 167.9, 41.1, 36.7, 29.4, 28.8.





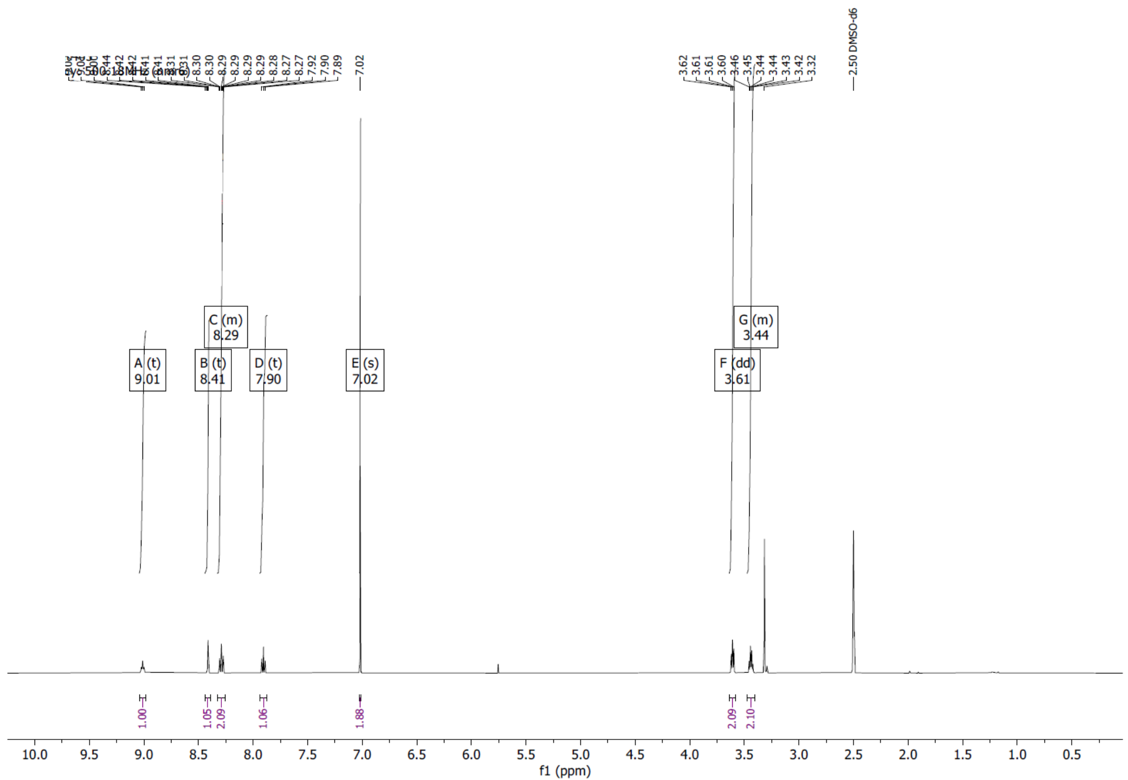


19F NMR (471 MHz, DMSO) δ 50.9, -74.6.

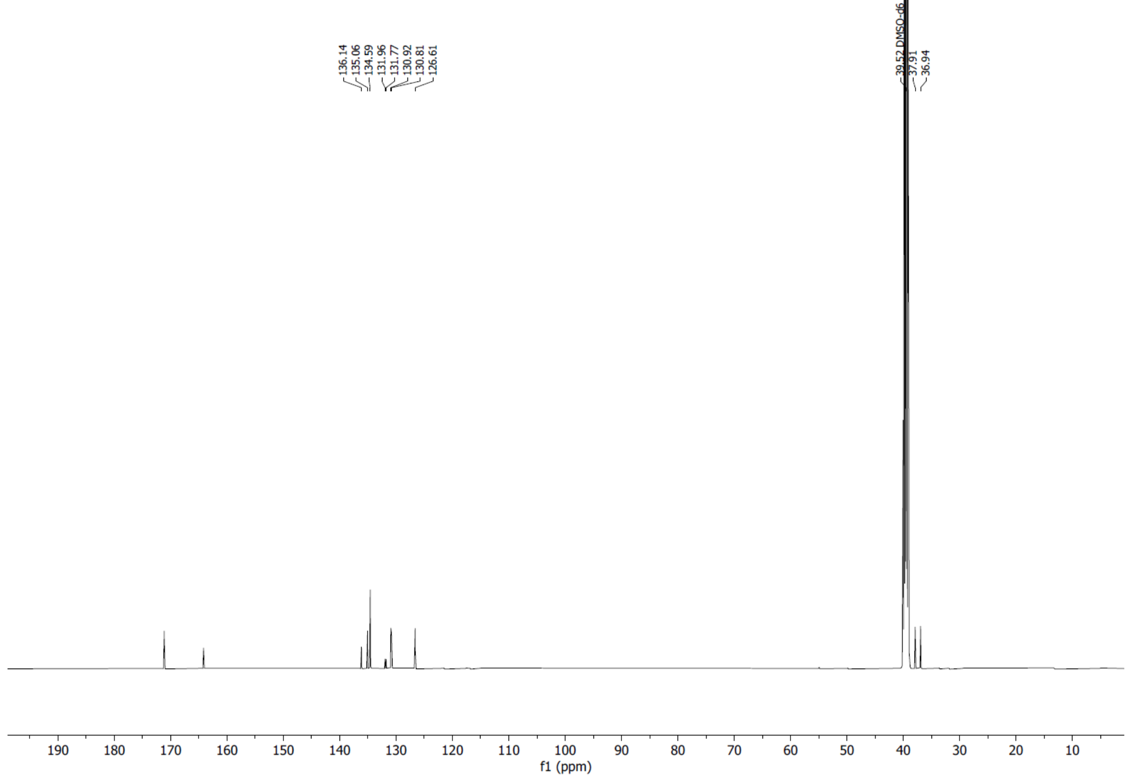


3-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)carbamoyl)benzene sulfonyl fluoride, L3

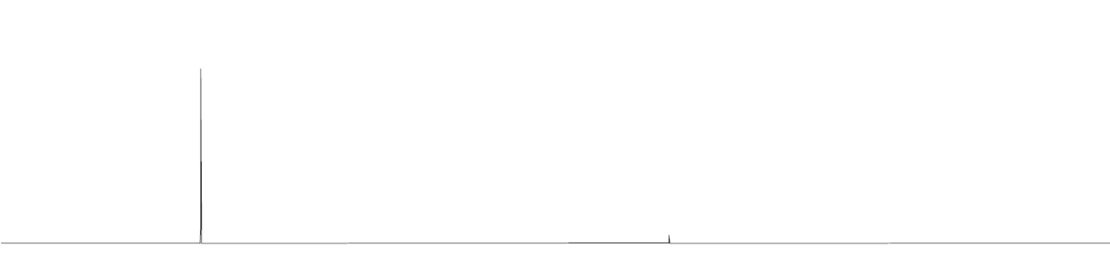
1H NMR (500 MHz, DMSO, 25°C) δ 3.4–3.47 (m, 2H), 3.61 (dd, J = 6.5, 4.9 Hz, 2H), 7.02 (s, 2H), 7.90 (t, J = 7.9 Hz, 1H), 8.25–8.33 (m, 2H), 8.41 (t, J = 1.8 Hz, 1H), 9.01 (t, J = 5.9 Hz, 1H).



13C NMR (126 MHz, DMSO) δ 171.1, 164.1, 136.1, 135.1, 134.6, 132.0, 131.8, 130.9, 130.8, 126.6, 37.9, 36.9.

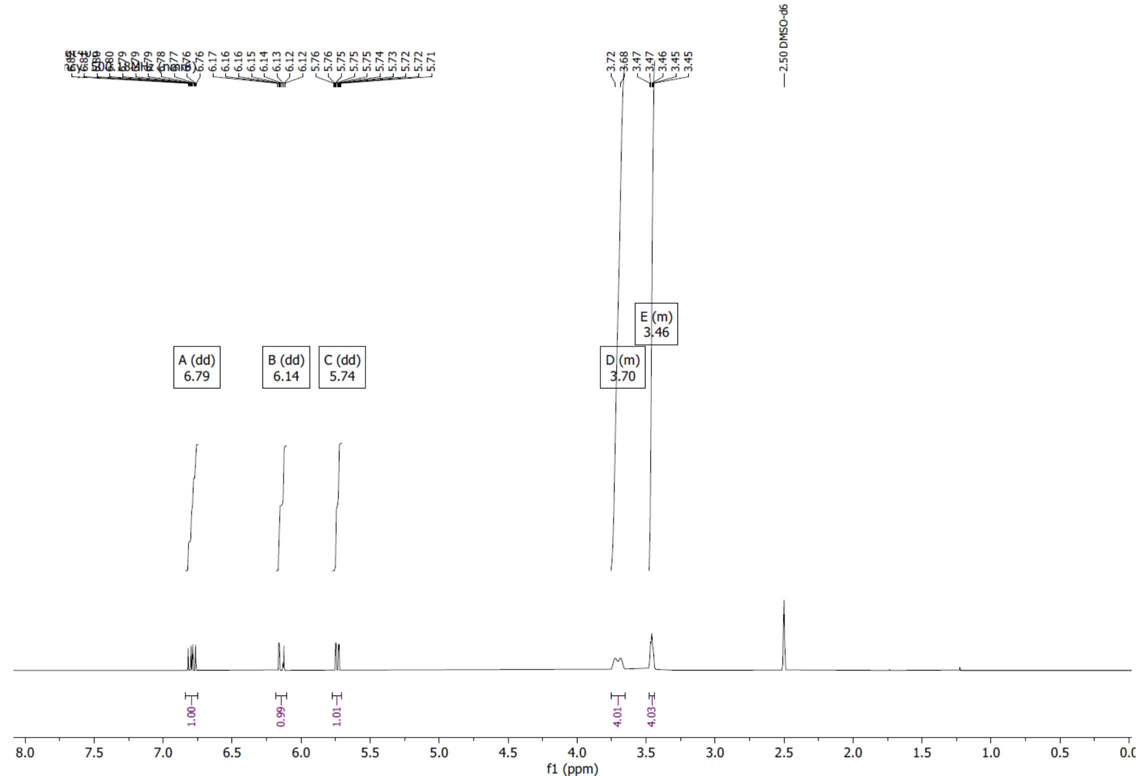


19F NMR (471 MHz, DMSO) δ 66.4, -73.4.

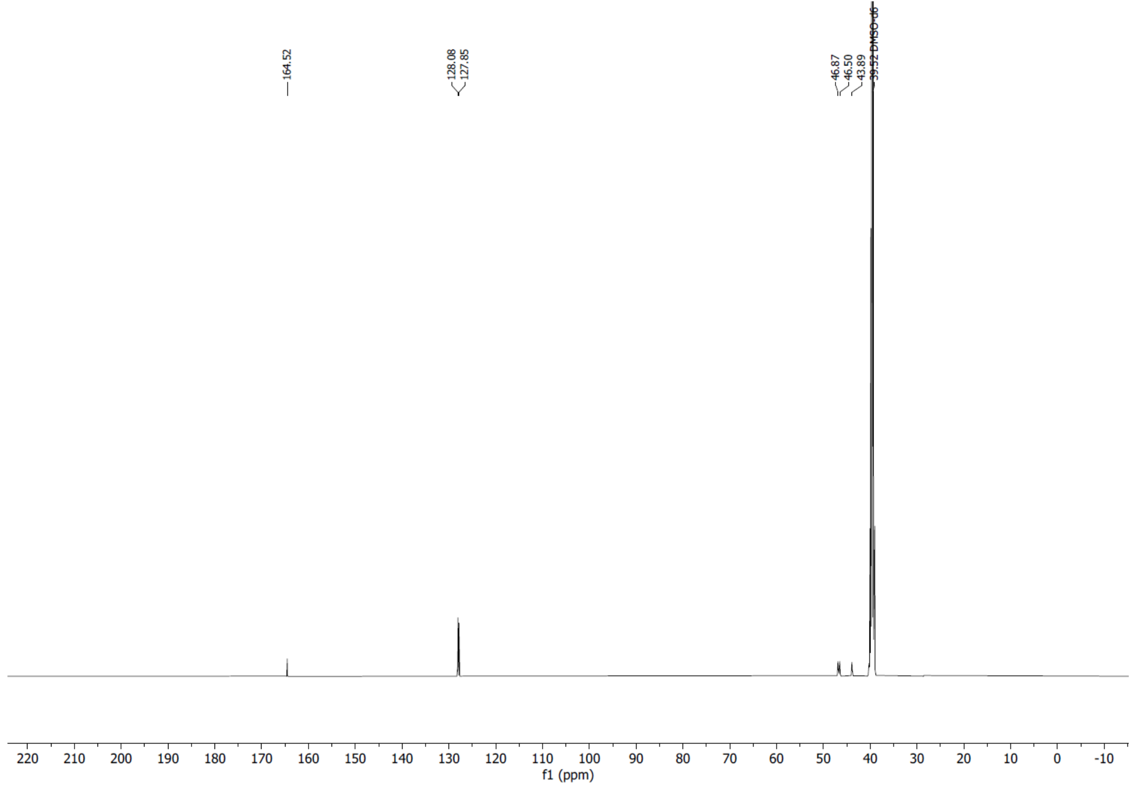


4-acryloylpiperazine-1-sulfonyl fluoride, L4

1H NMR (500 MHz, DMSO, 25°C) δ 3.44–3.48 (m, 4H), 3.65–3.75 (m, 4H), 5.74 (dd, J = 10.5, 2.3 Hz, 1H), 6.14 (dd, J = 16.7, 2.3 Hz, 1H), 6.79 (dd, J = 16.7, 10.5 Hz, 1H).



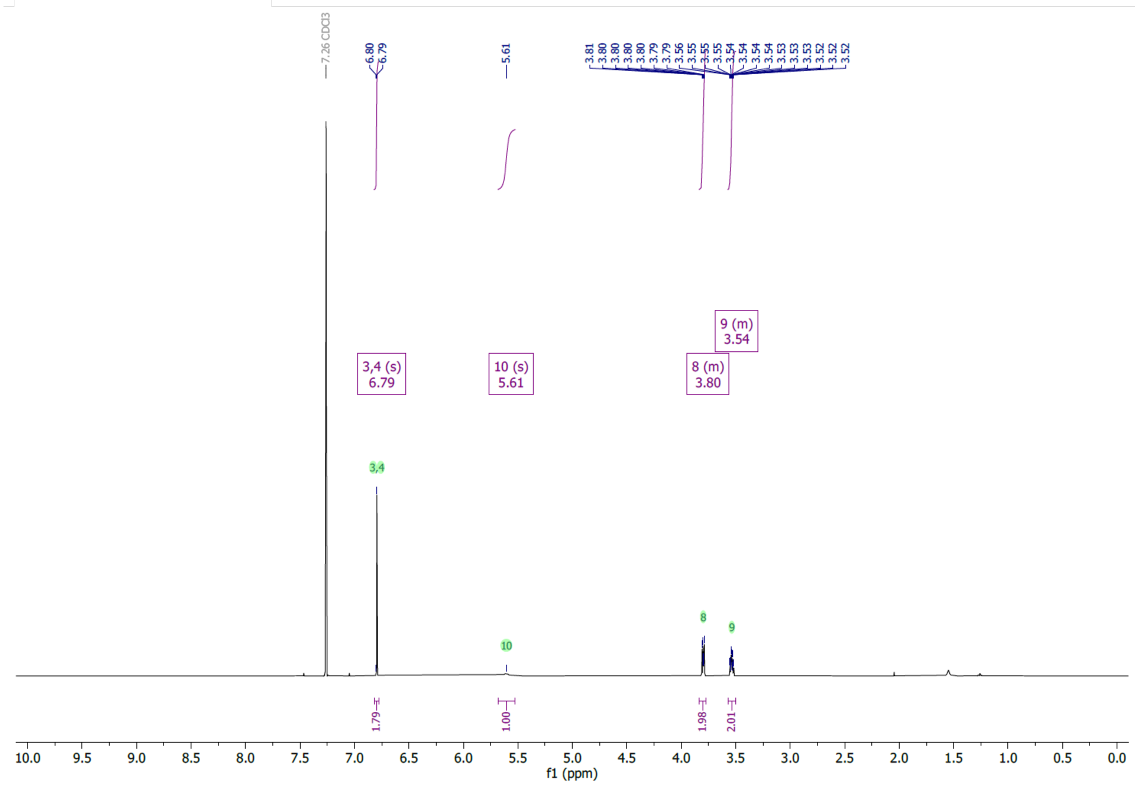
13C NMR (126 MHz, DMSO) δ 164.5, 128.1, 127.9, 46.9, 46.5, 43.9.



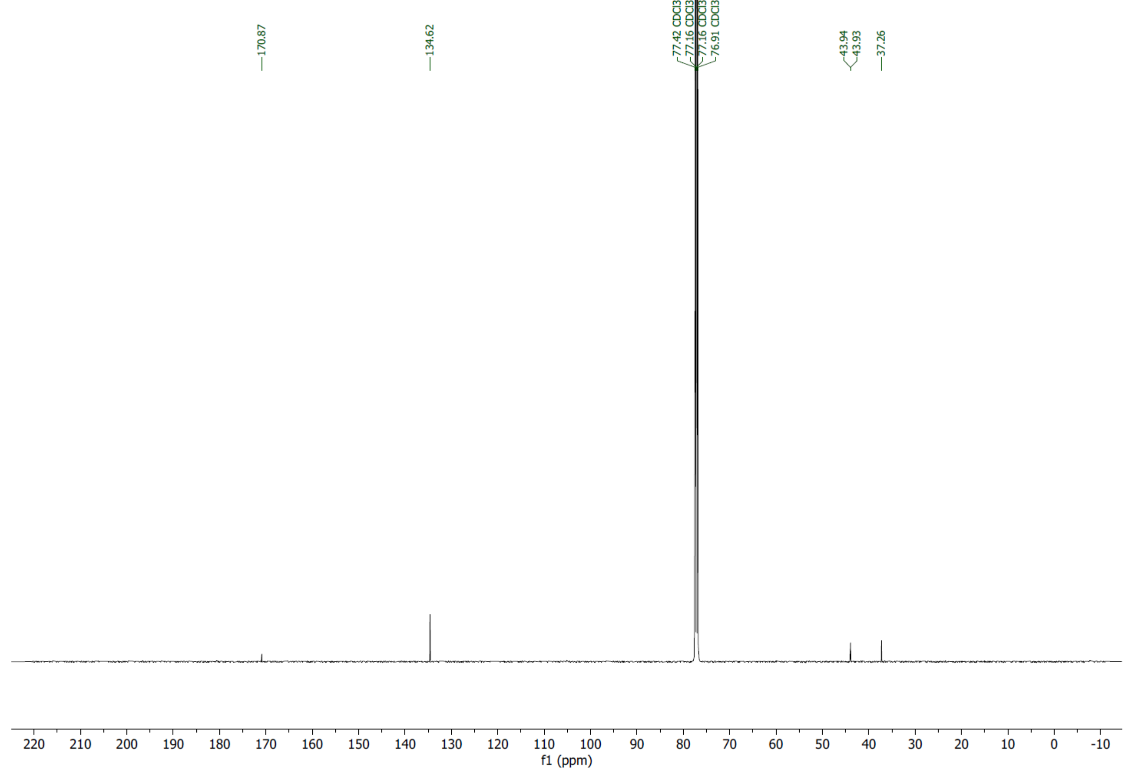
19F NMR (471 MHz, DMSO) δ 41.2, -74.1.

(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)sulfamoyl fluoride, L5

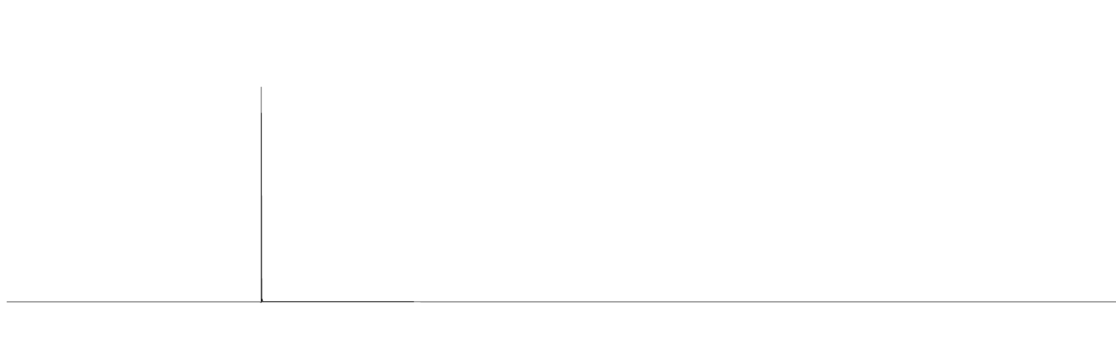
1H NMR (500 MHz, CDCl3, 25°C) δ 3.5–3.57 (m, 2H), 3.77–3.84 (m, 2H), 5.61 (s, 1H), 6.79 (s, 2H).



13C NMR (126 MHz, CDCl3) δ 170.9, 134.6, 43.9, 37.3.

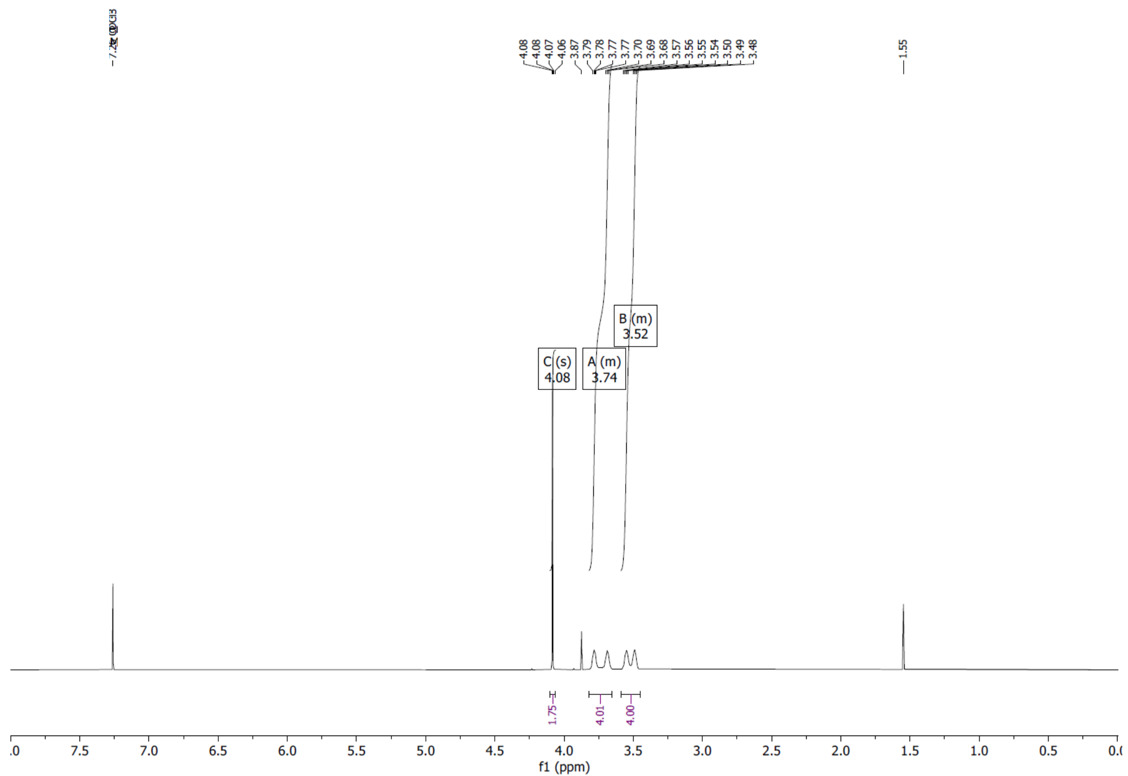


19F NMR (471 MHz, CD3CN) δ 50.0.

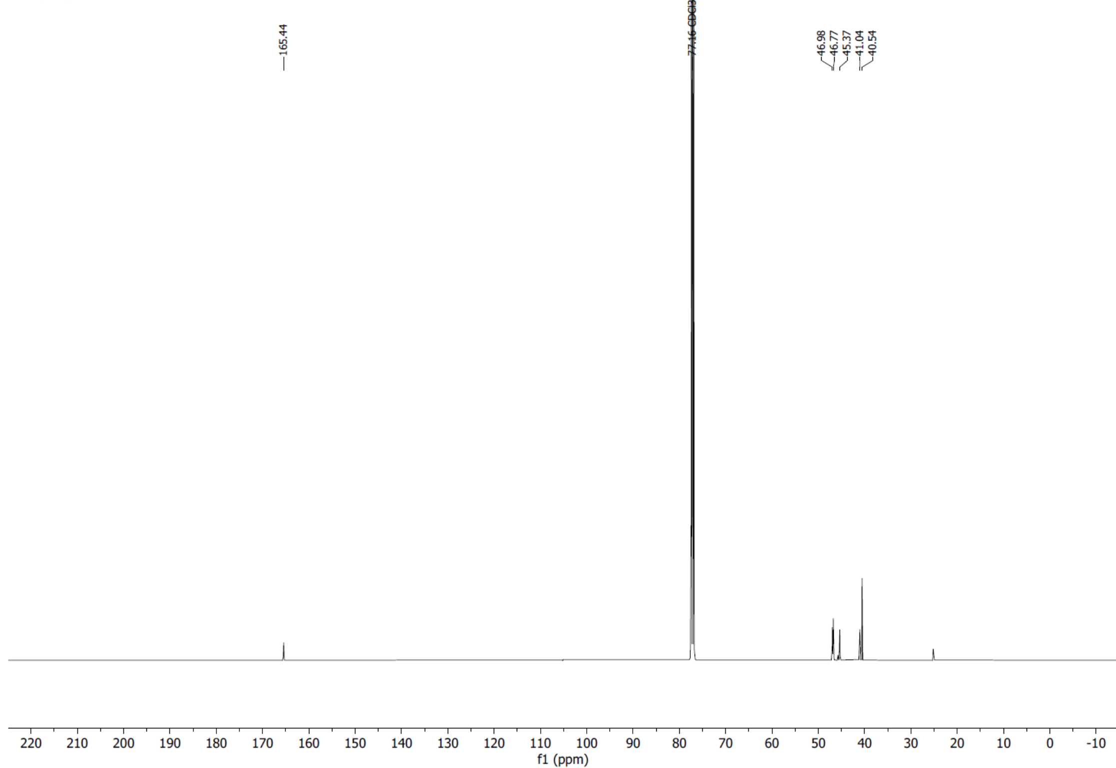


4-(2-bromoacetyl)piperazine-1-sulfonyl fluoride, L6

1H NMR (500 MHz, CDCl3, 27°C) δ 3.45–3.59 (m, 4H), 3.66–3.82 (m, 4H), 4.08 (s, 2H).



13C NMR (126 MHz, CDCl3) δ 165.4, 47.0, 46.8, 45.4, 41.0, 40.5.

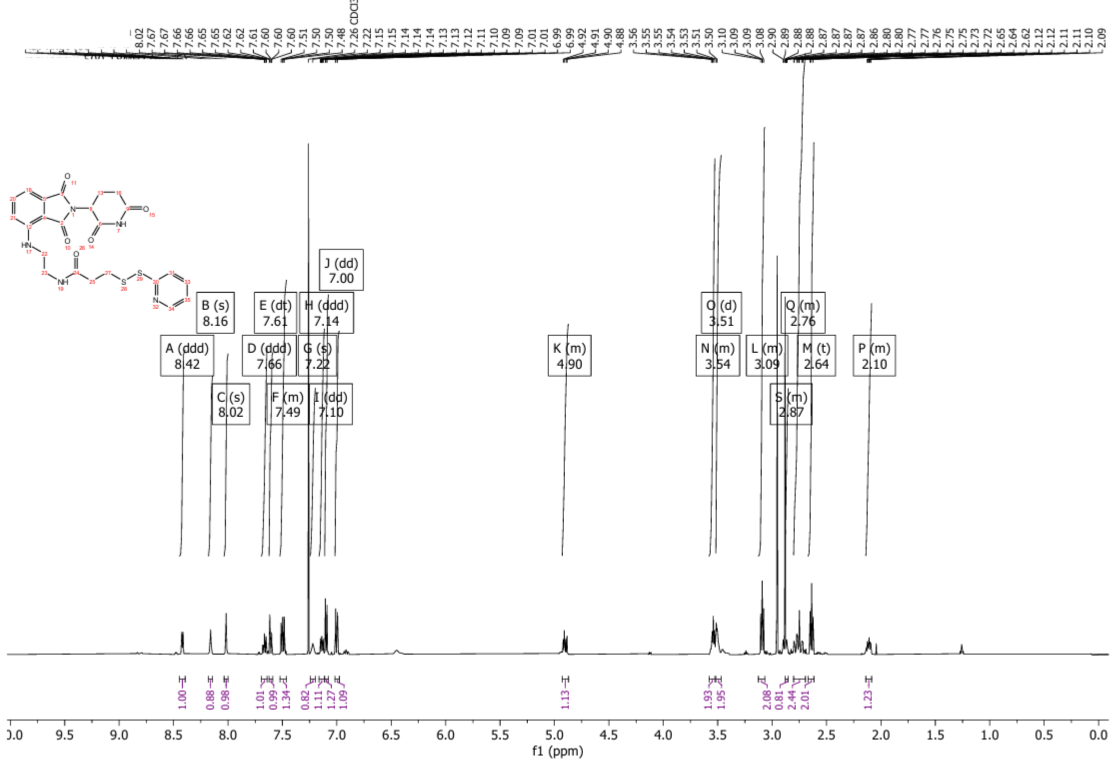


19F NMR (471 MHz, CDCl3) δ 40.5.

A.2 Conjugation

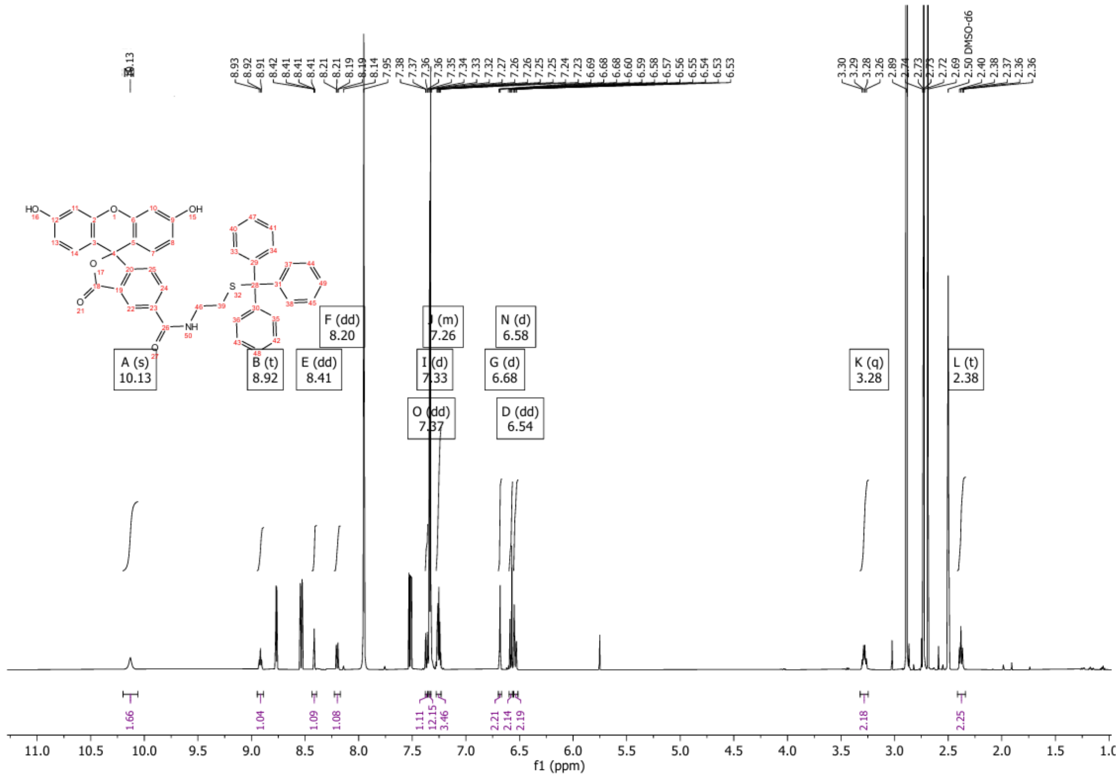
N-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethyl)-3-(pyridin-2-yldisulfaneyl)propanamide, I5

1H NMR (500 MHz, CDCl3, 27°C) δ 2.09–2.14 (1H, m), 2.64 (2H, t), 2.69–2.8 (2H, m), 2.85–2.88 (1H, m), 3.07–3.13 (2H, m), 3.51 (2H, d), 3.53–3.58 (2H, m), 4.87–4.93 (1H, m), 7.00 (1H, dd), 7.10 (1H, dd), 7.14 (1H, ddd), 7.22 (1H, s), 7.46–7.52 (1H, m), 7.61 (1H, dt), 7.66 (1H, ddd), 8.02 (1H, s), 8.16 (1H, s), 8.42 (1H, ddd)



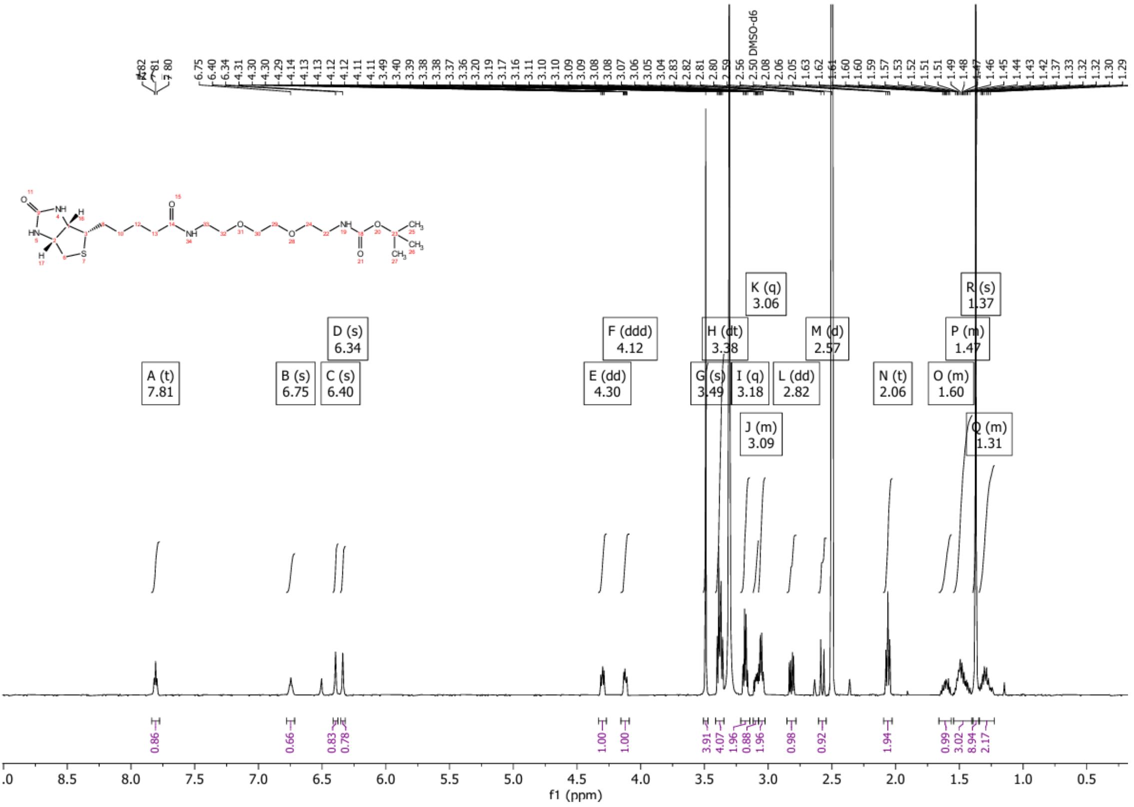
3’,6’-dihydroxy-3-oxo-N-(2-(tritylthio)ethyl)-3H-spiro[isobenzofuran -1,9’-xanthene]-5-carboxamide, I7

1H NMR (500 MHz, DMSO, 27°C) δ 2.38 (t, J = 7.1 Hz, 2H), 3.28 (q, J = 6.6 Hz, 2H), 6.54 (dd, J = 8.7, 2.3 Hz, 2H), 6.58 (d, J = 8.7 Hz, 2H), 6.68 (d, J = 2.3 Hz, 2H), 7.23–7.28 (m, 3H), 7.33 (d, J = 4.2 Hz, 12H), 7.37 (dd, J = 8.1, 0.7 Hz, 1H), 8.20 (dd, J = 8.1, 1.6 Hz, 1H), 8.41 (dd, J = 1.6, 0.7 Hz, 1H), 8.92 (t, J = 5.6 Hz, 1H), 10.13 (s, 2H).

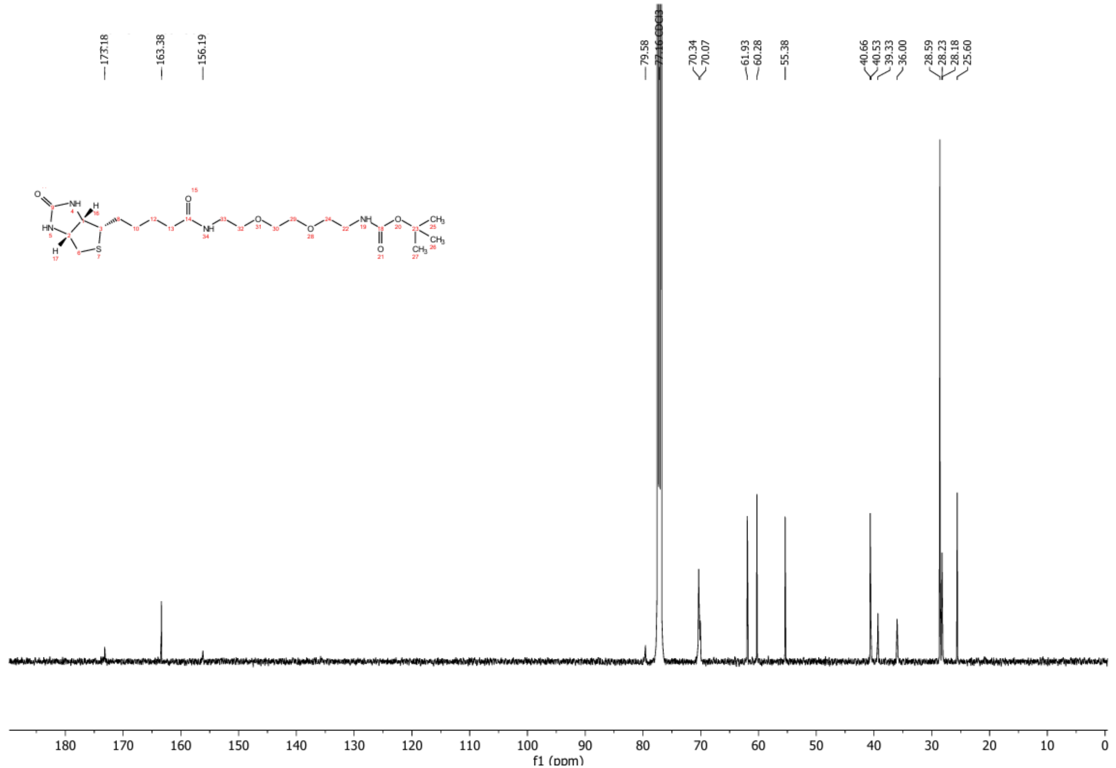


tert-butyl (2-(2-(2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethoxy)ethoxy)ethyl)carbamate, I9

1H NMR (500 MHz, DMSO, 27°C) δ 1.23–1.34 (2H, m), 1.37 (9H, s), 1.4–1.54 (3H, m), 1.56–1.66 (1H, m), 2.06 (2H, t), 2.57 (1H, d), 2.82 (1H, dd), 3.06 (2H, q), 3.08–3.12 (1H, m), 3.18 (2H, q), 3.38 (4H, dt), 3.49 (4H, s), 4.12 (1H, ddd), 4.30 (1H, dd), 6.34 (1H, s), 6.40 (1H, s), 6.75 (1H, s), 7.81 (1H, t).

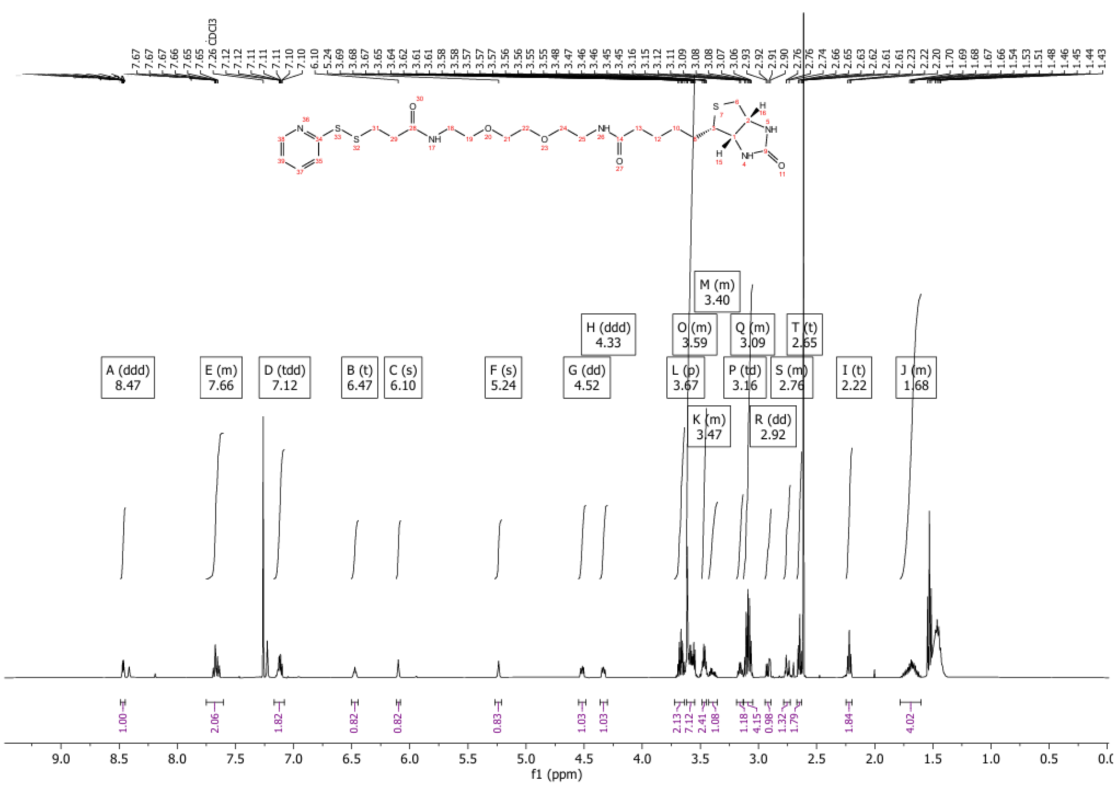


13C NMR (126 MHz, CDCl3) δ 173.18, 163.38, 156.18, 79.58, 70.34, 70.07, 61.93, 60.28, 55.38, 40.66, 40.53, 39.33, 36.00, 28.59, 28.23, 28.18, 25.60.



5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(2-(2-(2-(3-(pyridin-2-yldisulfaneyl)propanamido)ethoxy)ethoxy)ethyl) pentanamide, I11

1H NMR (500 MHz, CDCl3, 27°C) δ 1.6–1.78 (4H, m), 2.22 (2H, t), 2.65 (2H, t), 2.73–2.78 (1H, m), 2.92 (1H, dd), 3.05–3.13 (4H, m), 3.16 (1H, td), 3.35–3.43 (1H, m), 3.45–3.49 (2H, m), 3.55–3.62 (7H, m), 3.67 (2H, p), 4.33 (1H, ddd), 4.52 (1H, dd), 5.24 (1H, s), 6.10 (1H, s), 6.47 (1H, t), 7.12 (2H, tdd), 7.61–7.75 (2H, m), 8.47 (1H, ddd).



DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden
www.chalmers.se