CHEMICAL TOOLS FOR BIOLOGY

by

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ABSTRACT

ADAM BRADLEY FESSLER. Chemical tools for biology. (Under the direction of Dr. CRAIG OGLE)

The ability to adapt compounds for specific applications in biological systems is crucial for the development of new pharmaceuticals and biotechnologies. The field of bioconjugation has rapidly evolved with the increasing prevalence of targeted pharmaceuticals such as antibody drug conjugates and biological assays for the screening of diseases. These conjugates must be robust and well understood for application in these systems.

This project has involved the expansion and application of an isatoic anhydride based platform for bioconjugation. Biotinylation precursors were prepared and used to prepare a set of final biotinylation reagents. These reagents were tested against traditional biotinylation protocols. Additionally, a set of thiol reactive reagents were prepared utilizing two separate established click mechanisms for reactivity. Asymmetric reagents were prepared, producing a "mix and match scheme" that adds additional heterobifunctional capabilities to the reagents.

Applications of the isatoic anhydride platform were demonstrated through the surface modification of biomolecules. These applications presented an opportunity to improve the reagents and develop a labeling protocol. Anion exchange was performed, producing highly soluble reagents. This work increased utility of the reagents by unifying labeling protocol, showing sensitivity of isatoic anhydride reagents and the ability to control surface character through use of specialized reagents or stepwise additions.

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LIST OF ABBREVIATIONS

Ab	antibody
ACN	acetonitrile
amu	Atomic mass unit
BSA	bovine serum albumin
CDI	carbonyldiimidazole
DCM	dichloromethane
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DOL	degree of labeling
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ESI	electrospray ionization
EtOAc	ethyl acetate
EtOH	ethanol
Fab	Antigen binding fragment
GPC	gel permeation chromatography
HABA	4’-hydroxyazobenzene-2-carboxylic acid
HPLC	high-performance liquid chromatography
HRP	Horseradish peroxidase
IPA	isopropyl alcohol
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry

ε	molar extinction coefficient
MeOH	methanol
MS	mass spectrometry
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PEG	polyethylene glycol
SPR	surface plasmon resonance
Sulfo-NHS	N-hydroxysulfosuccinimide
RNA	ribonucleic acid
RT	room temperature
TEA	triethylamine
THF	tetrahydrofuran
UV-vis	ultraviolet visible

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CHAPTER 1: INTRODUCTION

1.1 Bioconjugation

Biomolecules have found uses in assays, drug delivery, immunotherapeutics, and numerous other technologies; helping to create the ever-expanding field of biotechnology.[1] Utilization of natural products for these technologies often requires modification of the biomolecule's native state before the substance is suitable use in the desired application. This change is often done through the addition of non-native features to the surface through covalent modifications. Bioconjugation is a process in which chemical functionality is added to a biomolecule through the formation of a bond to the biomolecule. Covalent attachment of non-native functionalities increases the scope of applicability by producing a conjugate with a unique feature directly bound to the surface. Bioconjugation reagents must contain reactive functionality that is able to react with a biomolecule in inoffensive conditions to avoid denaturation.

Bioconjugate technologies tend to follow one of two general pathways, where some bioconjugates may be used directly after the chemical modification and others require additional modifications before use. A stepwise addition of functionality that reacts selectively with the original modification site results in the production of the final bioconjugate (Figure 1-1). The single step method will be performed with a reagent containing a single reactive moiety, where the multistep method will utilize a reactive functionality to install a non-native reactive group which is then used for the addition of

the next piece. The path chosen is typically determined by the end goal of the bioconjugate technology and the type of functionality being added.[2] Many assays are based off a single step to form the bioconjugate species of interest, whereas drug delivery systems are often based on stepwise addition of functionality. Advances in bioconjugation technologies have given researchers the ability to utilize biomolecules in ways that are inaccessible in their native state. The application of bioconjugates cover multiple disciplines with applications spanning from cancer research to assay technology.[3]

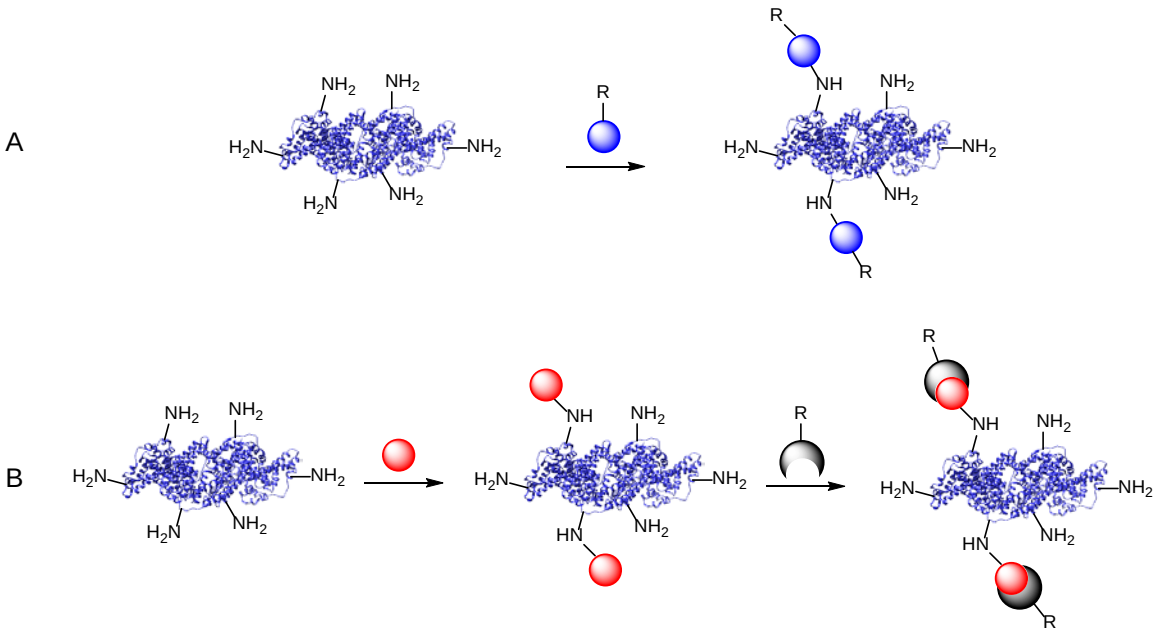


Figure 1-1: General overview of bioconjugation processes, one step method (A) and multistep method (B)

The addition of non-native functionality through covalent linkage is playing a critical role in expanding pharmaceutical technologies. Bioconjugation of antibodies (Ab)

for pharmaceutical application has offered new methodologies to utilize highly cytotoxic payloads Antibodies are “Y” shaped proteins produced by the immune system that recognize sites called antigens and binds to the site tightly. This binding acts as a signal that can trigger an immune response which may result in the destruction of the substance.[1] Since Ab target a particular biological occurrence, attachment of a drug or fluorophore directly to an Ab through the use of bioconjugation strategies allows utilization of the targeting capabilities. Attachment of a powerful drug directly to an antibody produces a guided therapeutic that will have an increased delivery to the site of that occurrence. Since the drug is targeted, there is an increase in delivery compared to traditional methods resulting in advantages such as reduced dose size and minimized off target effects. Applications to chemotherapeutics has helped produce more robust medicines since many anti-cancer drugs have poor circulation half-life with high rates of renal clearance as a small molecule and are highly cytotoxic if taken up by a healthy cell.[4] The systemic distribution of a drug in the blood stream leads to off target death of healthy cells that take up to the drug, causing nonspecific cell death. The antibody acts as a targeting system that can bind to the cell surface receptors of a cancerous cell and be internalized prior to releasing the payload. The application of Ab to drug delivery has also increased the efficiency of treatment while simultaneously avoiding some of the serious side effects of therapeutics through a localized treatment.[1] Bioconjugation of drugs, imaging agents and other functionalities to antibodies are currently used in several top selling pharmaceuticals and will continue to be applied as new bioconjugate therapeutics are passed through clinical trials.[3]

Bioconjugates have been applied to numerous biotechnologies such as biosensors, assays and separation techniques.[5] For application to these technologies, a biomolecule must retain its activity as in its natural, unmodified state after the bioconjugation event. That requires the attachment of functionality to not disrupt the natural function or structure of the biomolecule. This ability to modify a biomolecule without the loss of function is critical for a bioconjugate to be used in an application which is based off the biomolecules native activity or structure. For example, antibodies require the use of their antigen-binding fragment (Fab) region for binding to a target, a bioconjugation event on this portion of the antibody may result in a loss of function and the inability to bind to the receptor. This produces a bioconjugate which is no longer suitable for the intended purpose. The inactivation is a difficulty numerous bioconjugation strategies face since many are based off of conjugation to lysine residues that are found throughout a protein.[6] Targeting of specific, non-native functionalities or use of disulfide bridges can overcome the some of the nonspecific labeling problems but cannot fully eliminate these issues.[7] When using a lysine reactive moiety it has been shown that reducing the amount of conjugated functionality on the surface will reduce the frequency of producing inactive conjugates.[1] Since the labeling reaction is non-specific, simply reducing the number of labels on the surface reduces the likelihood of labeling the active region.

Many bioconjugation techniques are performed through a reaction with a lysine residue found on the surface of a biomolecule using a select set of chemical crosslinking agents.[8] This is commonly done through the formation of an activated ester; most frequently through the reaction of a carboxylic acid with N-hydroxysuccinimide (NHS). NHS esters react rapidly with amines but offer poor solubility in aqueous solutions,

because of the poor solubility, reactive reagents can be designed utilizing a sulfo-NHS strategy which uses a sodium salt of the succinimide to help facilitate water solubility. Sulfo-NHS reagents are much more expensive than the traditional NHS ester and offer no solubility benefit to the final bioconjugate since the solubility is imparted through the NHS group that is displaced during the amidation.

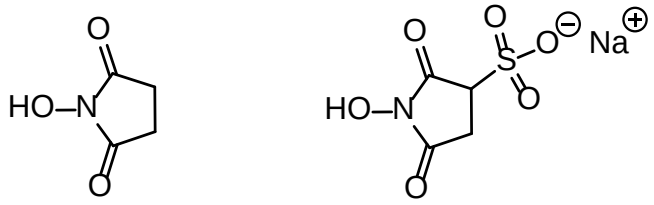


Figure 1-2: N-hydroxysuccinimide (NHS) and N-hydroxysulfosuccinimide (Sulfo-NHS) sodium salt

Nucleophilic lysine residues on the surface of a substrate displace NHS through the reaction with the activated carbonyl forming an amide bond between the substrate and chemical functionality being incorporated. NHS ester reagents are susceptible to hydrolysis in basic conditions, re-forming the carboxylic acid of the bioconjugate reagent.[9] This hydrolysis results in the inactivation of the carbonyl and results in the inability to covalently link the reagent in its intended manner. In order to use lysine residues for bioconjugation the terminal amine must be neutral, this requires bioconjugation reactions to be performed in basic conditions since lysine residues are protonated at neutral pH.[8] This competitive process requires an excess of the NHS based bioconjugation reagent to be used to ensure each biomolecule will contain functionality. The amount of reagent will lead to variable amounts of functionality on an individual

biomolecule, thus making accurate quantification of the number of functional sites important to create an effective conjugate.

Bioconjugate technologies have drastically improved assay technologies by increasing the sensitivity and reducing waste through amplification techniques. Bioconjugates based on the addition of a biotin moiety have been found advantageous in many applications, becoming one of the most ubiquitous technologies within the biotechnology fields.[5, 10] Bioconjugate technologies have resulted in the rapid increase in biotechnologies and the ability to diagnose disease states and proteins.

1.2 Biotin Background

Biotin is a natural product with a unique structure that has a high affinity for binding with a pocket on avidin or streptavidin, homotetrameric proteins with four non-cooperative binding sites (Figure 1-3). Each protomer consists of a β-barrel structure where the first and last barrels are adjacent and held together through hydrogen bonding.[11] The noncovalent interaction between biotin and the binding pocket is a result of hydrogen bonds with the carbonyl oxygen and the ureido nitrogens. Four tryptophan residues are in contact with the biotin molecule, adding additional van der Waals forces helping to stabilize the binding. Cooperative structural changes lead to a small conformational change of the protein that induces a squeezing of the ligand.[12] The tight fit and numerous hydrogen bonds results in an incredibly strong noncovalent interaction with a picomolar dissociation constant for biotin and streptavidin, $K_d \approx 10^{-15}$ M.

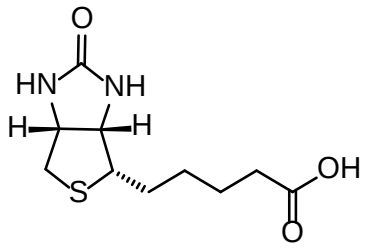


Figure 1-3: Structure of biotin (vitamin B7)

Avidin and streptavidin have 2 binding sites found on opposite faces located about 20 Å from one another.[11] Avidin is found in egg whites and streptavidin is isolated from *Streptomyces avidinii*, the two proteins possess similar structures with a shared affinity for biotin. Streptavidin lacks glycosylation resulting in a lower solubility and isoelectric point (pI) than that of avidin. This lack of carbohydrate reduces non-specific binding and background noise that can arise due to nonspecific binding with carbohydrates, making streptavidin more applicable for binding of dilute or dirty samples.[5] The two proteins can be used interchangeably in many technologies although streptavidin is notably more expensive than avidin, resulting in preferential use of avidin.

The addition of biotin functionality to a biomolecule allows the use of well-developed technologies. The affinity constant between avidin and biotin has produced a number of technologies that range from purification to signal amplification. The interaction between avidin and biotin relies on the fused rings of biotin to fit deep into binding pocket to utilize the strong interaction (Figure 1-4).[11] The ligand fits tightly into the hydrophobic binding pocket with the carboxylate of the valeric acid side chain remaining exposed outside of the pocket.

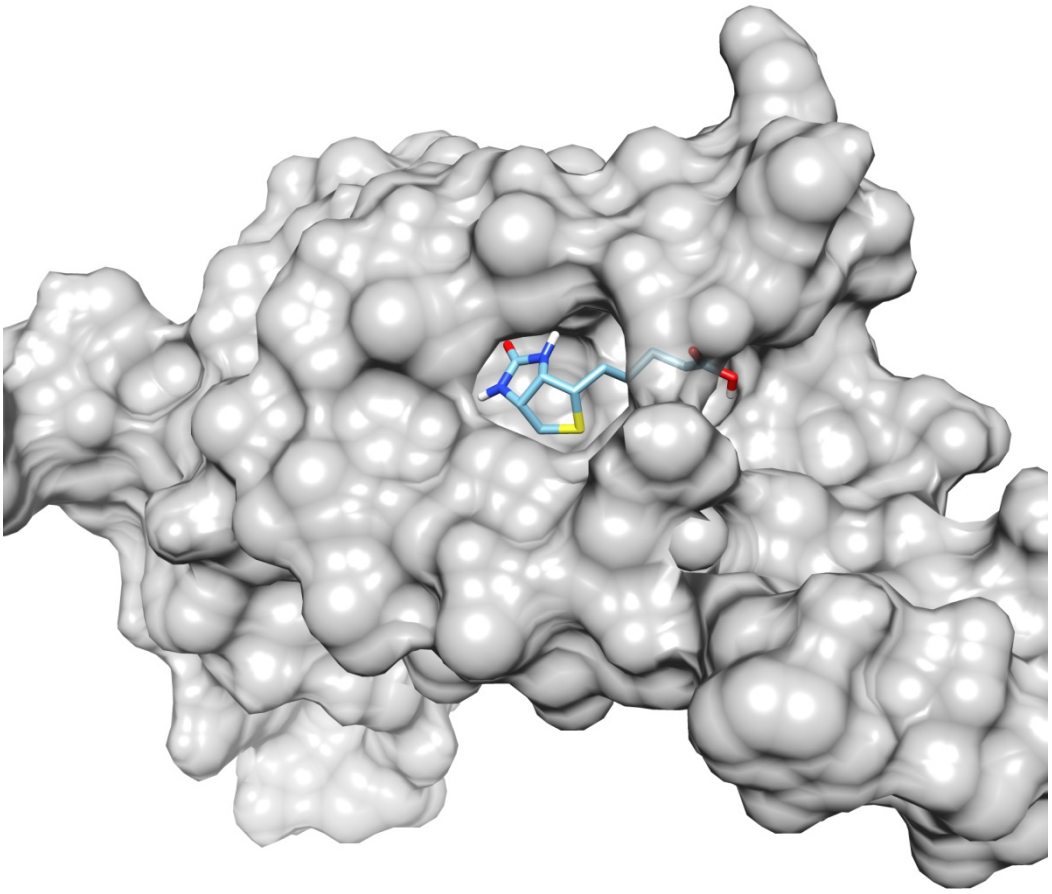


Figure 1-4: Computational biotin- streptavidin interaction using AutoDoc Vina[6]

The attachment of the biotin functionality to a substrate through a covalent bond is typically performed by activation of the carboxylic acid on the valeric acid side chain. NHS is used to form reactive species through the use of a carbodiimide reagent, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). EDC adds to the carboxylic acid to form a reactive O-acylisourea intermediate, an activated carbonyl, which is quickly

displaced by NHS in solution to form the amine reactive NHS ester (Figure 1-5). NHS is water soluble but upon formation of the ester, the water solubility is drastically reduced by the reduction in polarity. NHS-biotin has poor solubility in aqueous conditions; this is not ideal for application to biological samples which are often used in aqueous buffers. The use of a small amount of a water miscible organic co-solvent such as DMF or DMSO helps solubilize the NHS based reagents. Mixed aqueous and organic solvent systems can result in the loss of biological function for the substrate through irreversible conformational changes so use of water soluble reagents is advantageous.[13] Water soluble derivatives of NHS esters are possible through the use of sulfo-NHS which incorporates a salt onto the reagent, increasing the water solubility of the reagent and the ester. The reagents using sulfo-NHS can be solubilized in neutral and buffered conditions but the cost of the reagent is typically over ten times as much as the NHS derivative. This is one major factor preventing the widespread application of the sulfo-NHS derivatives. Since NHS-esters possess an activated carbonyl they are prone to hydrolysis in aqueous buffers. Hydrolysis reforms biotin as the free carboxylic acid and NHS, this requires samples used for bioconjugation to be made fresh to ensure reproducibility.[9]

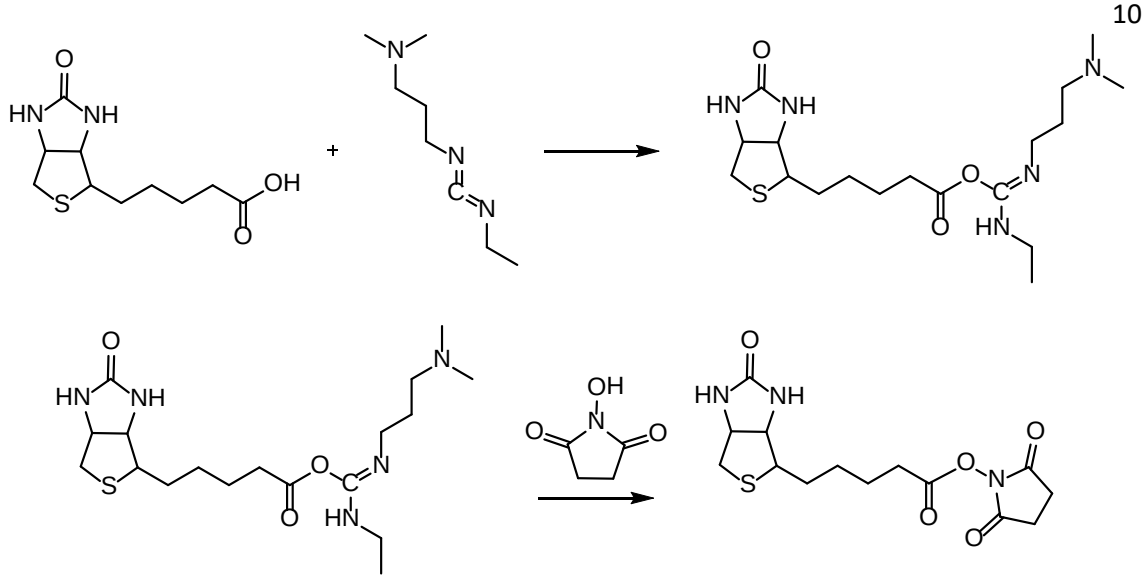


Figure 1-5: Synthesis of the NHS ester of Biotin

To utilize the strong binding affinity, biotin must fit in the deep binding pocket; this can be difficult when the biotinylated substrate possesses a bulky character. Direct biotinylation of a substrate can result in failed binding due to unfavorable steric interactions that reduce the binding affinity or prevent binding. When compared to the hydrodynamic radius of a protein, the valeric acid chain of biotin does not offer much length for free movement. A common reagent used for increasing the distance between the fused rings of biotin and the substrate is known as long chain NHS-biotin (NHS-LC-biotin). This reagent has a short 7 atom spacer added between the valeric acid and the reactive NHS functionality (Figure 1-6). This spacer offers a flexible linkage that can allow the biotinylated substrate to minimize the steric interference during binding. The addition of a short spacer arm has been shown to be useful for most applications to proteins, antibodies and cell surface studies.[10]

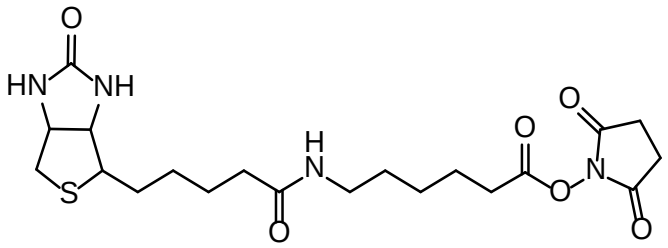


Figure 1-6: NHS-LC-Biotin

The avidin/streptavidin-biotin affinity results in its use in highly impactful methods for characterization of biological materials and interactions. Some of these tools include; enzyme linked immunosorbent assays (ELISA), Western blotting, cell-surface labeling, and affinity purification. Immobilization of biotin on a surface allows for use of the avidin-biotin interaction for biological sensors, a monolayer of streptavidin can be added to the layer of biotin, leaving two binding sites exposed. These then can be then be used to immobilize biotinylated samples to be examined by surface plasmon resonance (SPR).[14] Immobilization of biotin on a surface allows for a wide variety of substrates to be detected using the ELISA technique. Using biotinylated antibodies to detect substrate bound to the plate has become a frequent strategy allowing for the production of assays for virtually any analyte.[3]

Along with uses for immobilization of substrate on a surface, the biotin-avidin interaction is commonly used for signal amplification. Avidin complexed with biotinylated horseradish peroxidase (HRP) will bind to a biotinylated substrate or antibody to deliver multiple copies of the enzyme per biotin molecule. This technique of signal amplification utilizing HRP is another example of a commonly applied ELISA technique, using a biotinylated antibody to bind to the analyte followed by the addition

of streptavidin bound to HRP (Figure 1-7). HRP catalyzes the formation of brightly colored complexes from colorless substrates, producing a strong signal even at low concentrations increasing the limits of detection of trace analytes. Signal amplification using biotinylation drastically increases the sensitivity and speed of the assay since the strong interaction between the complementary pair helps increase the rate of complexation while also reducing the amount of materials needed to perform the assay since washing the wells will not result in washing off the substrate. The signal can be amplified with a direct Antibody/HRP conjugate but increasing the amount of HRP conjugated to the antibody but this increases the likelihood of an inactive bioconjugate by conjugation to the antigen binding region.

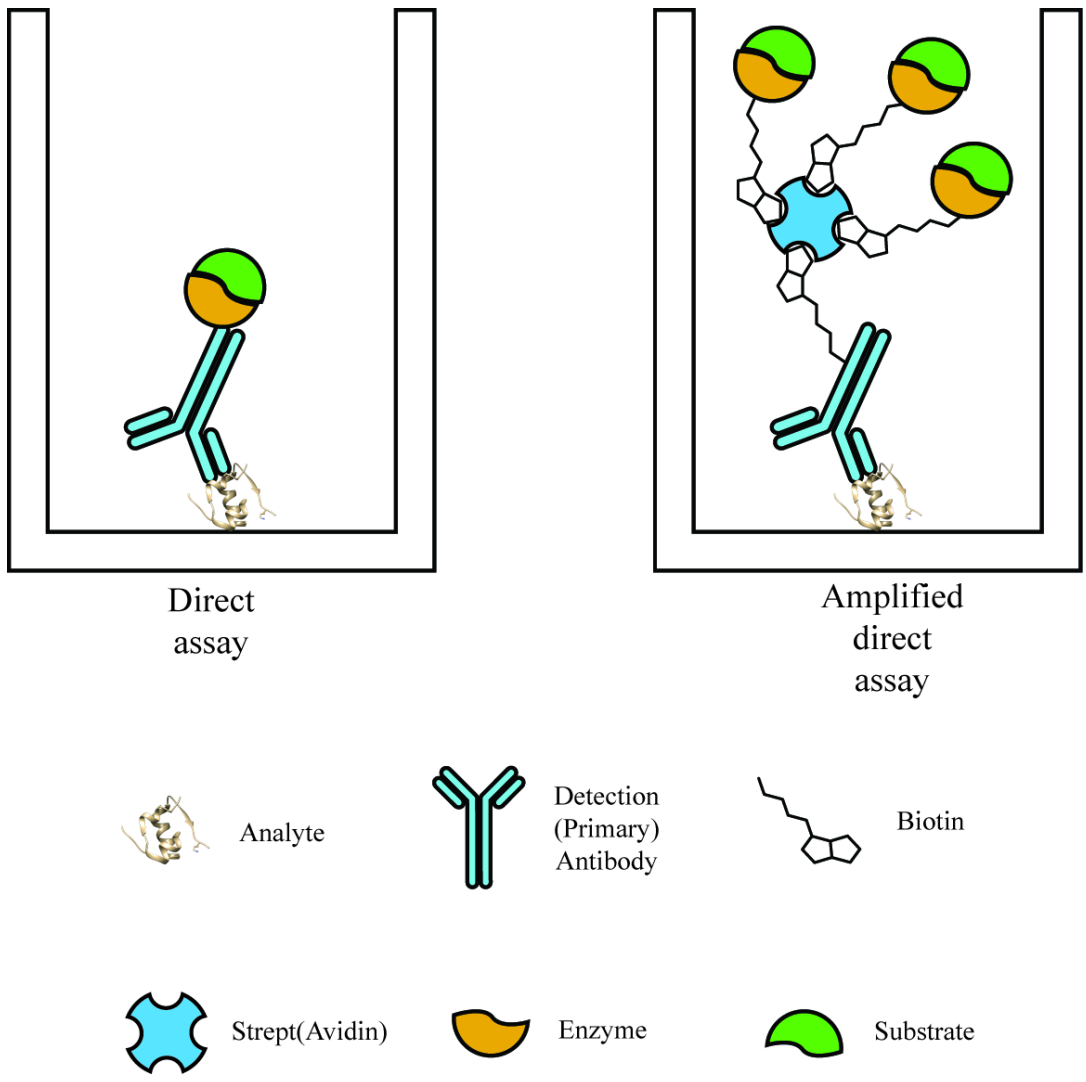


Figure 1-7: Direct assay for ELISA using two different bioconjugate antibodies, materials are not to scale to help with clarity

When using biotin for signal amplification in ELISA, knowing the amount of biotin on the antibody is critical for calculating the amount of substrate that has been detected by the assay. Determination of the degree of biotinylation is performed using a secondary assay in which biotin is quantified by the displacement of a dye from avidin during the binding event. 4'-hydroxy-azobenzene-2-carboxylic acid (HABA) is loosely bound to each of the binding sites of avidin, this complex has a known absorbance at 500

nm. When biotin binds the pocket, the absorbance at 500 nm is reduced due to solvochromatic effects shifting the wavelength of maximum absorbance of the dye. This method has the assumption that all biotin molecules on the substrate must be able to bind streptavidin. The HABA assay is the most commonly utilized method for determination of the degree of biotinylation but this assay results in loss of value added product (Figure 1-8). This small loss is significant when utilizing expensive reagents or materials that are only known in trace amounts and the assay must be performed for each labeling reaction.

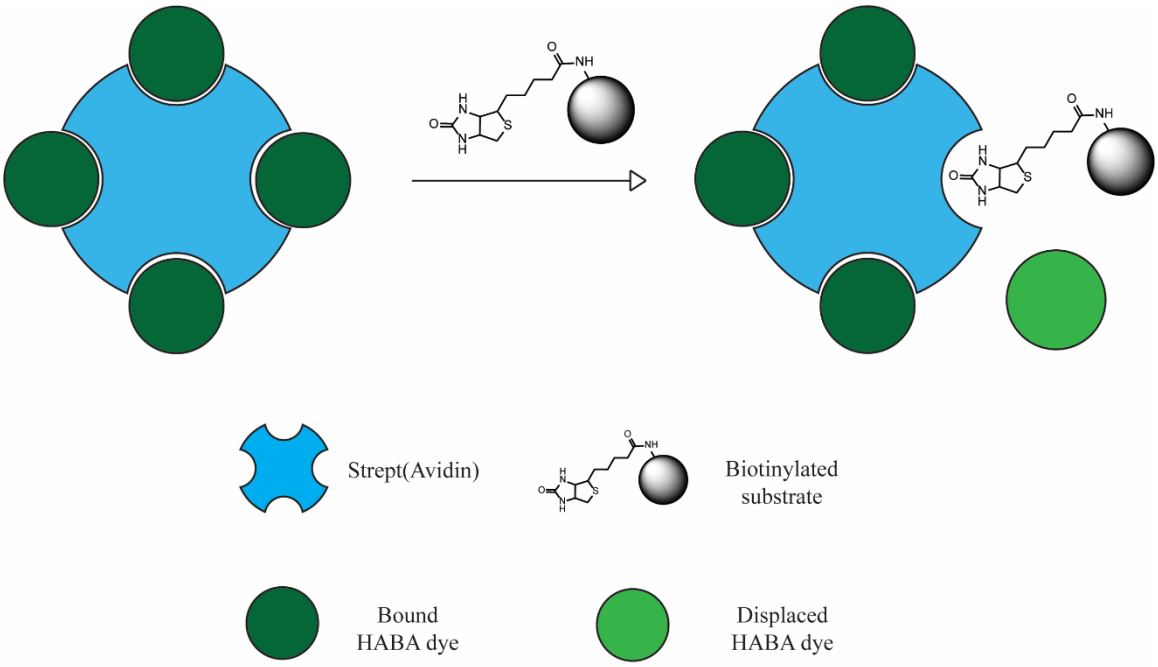


Figure 1-8: Determination of biotinylation through HABA assay

Affinity labeling allows for the study a broad scope of materials. Biotinylated cell lysates or proteins can be quickly purified utilizing a streptavidin column followed by a specific set of elution conditions.[10] This form of capture based purification is commonly

used since the biotin/streptavidin interaction is so strong. Biotinylated materials will remain bound to the streptavidin coated column as other materials are washed out. The purification of biotinylated samples can also be done by systems that disrupt the binding or more often through the addition of cleavable linkages. This is commonly done through the addition of a disulfide bond to the spacer arm. Disulfide bonds can be reducibly cleaved, thus making them ideal for application to affinity purification, which can purify the biotinylated substrate then be cleaved to elute the sample, giving high purity and recovery.

1.3: Thiol Conjugation

Many bioconjugation applications focus around protein modifications, this allows a researcher the ability to envision reactions with other residues in an amino acid sequence than lysine residues or terminal amines. Applications with reagents that react with cysteine residues on a protein have gained popularity in recent years. These reagents offer an increased specificity by reacting with cysteine residues rather than through conjugation to common lysine residues. Much like that of conjugation through amines, there are multiple methods of bioconjugation to a sulfur atom.

Two main strategies of reactions with thiols have become the most utilized strategies. These are reactions are both based off the addition of a thiol across a double bond although through different mechanisms. These reagents use either a maleimide or an alkene. Maleimide reagents react with a thiol through a Michael addition of a thiol across the alkene of the maleimide. The alkene reagents react with a thiol in what is termed a thiol-ene reaction, a radically initiated addition of a sulfhydryl group across the double bond (Figure 1-8). Both chemistries have found a home within bioconjugation

although maleimide chemistry is the more frequently applied since the addition occurs at neutral pH without requiring the generation of radical species.

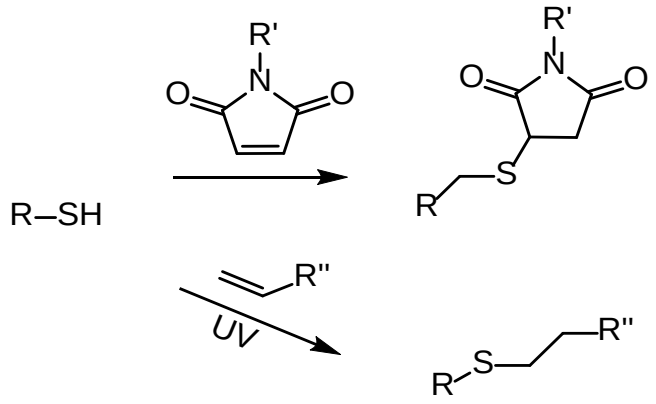


Figure 1-9: General maleimide and thiol-ene chemistries

Targeting of cysteine residues increases that specificity of the final bioconjugate since the occurrence of cysteine residues as free thiols are less common than that of lysine. Most cysteine residues within a biomolecule are not solvent exposed but rather are critical to the structure and shape of the protein, forming disulfide bridges that help add strength to a folded conformation. When labeling a biomolecule the amount of free thiol can be theoretically determined through examining the 3D structure to see if there are solvent exposed residues and then experimentally determined through the use of an Ellman's test.[15] The Ellman's reagent is reduced by free sulfhydryl groups on a protein which results in the formation of a brightly colored product that can be used to determine the concentration of the free cysteine residues. Since there are few free thiols on a protein surface, use of strategies selective for thiols increases the control of both the site and

number of labels during bioconjugation. These then gives rise to an increased likelihood for the production of active bioconjugates.

Maleimide chemistry has found application in numerous papers throughout the literature as it is a convenient method for modification of free sulfhydryl groups on a substrate.[6, 8, 16] This reaction is typically performed at pH 6.5-7.5 as increasing pH has shown to result in increase in byproducts as amines are more common and are nucleophilic in basic conditions.[17] The R group of the maleimide plays a key role in the stability and reactivity of the reagent (Figure 1-10).[18] This reactivity has been tuned to synthesize reagents that inhibit retro-Michael addition through a ring opening hydrolysis which results in an increased stability of the thioether.[19]

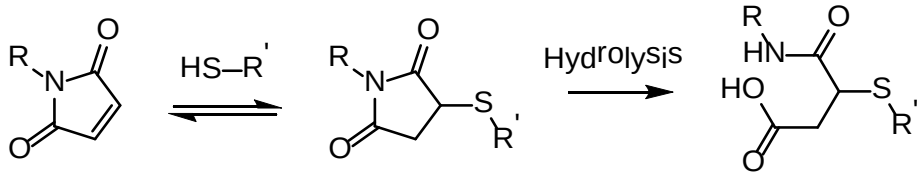


Figure 1-10: Maleimide side chain effects

Reagents capable of this “locking” action tend to be N-aryl maleimides, where the R group consists of an aromatic ring, drastically increasing the reactivity of the maleimide. This increased reactivity also results in poor reagent stability in solution and an overall increase in side reactions with other nucleophiles on a peptide. Alkyl maleimides are less susceptible to hydrolytic cleavage of the maleimide which results in an increased deconjugation through reverse Michael addition. Short incubations in basic

conditions have been shown to help facilitate the hydrolysis of the thiosuccinimide of alkyl maleimides, increasing the stability of the conjugate in buffered conditions.[19]

Thiol-ene "click" chemistry has found application in many fields, more recently it was investigated as a new method for bioconjugation.[20-21] One outstanding feature of thiol-ene chemistry is that it is radically initiated yet proceeds rapidly in the presence of water and oxygen. Additionally, the reaction is selective for the addition of a thiol radical to an alkene in near quantitative yields. Thiol-ene chemistry is efficient with non-sterically hindered electron rich alkenes or to strained alkenes, reacting much quicker than with electron poor alkenes.[22]

These radical thiols are based off the weak S-H bond of a thiol. Using UV light as an initiator with a trace amount of a photoinitiator produces a thiyl radical. The properties of the alkene play a key role in the reaction rates. If high concentrations of the alkenes are used then the radical generation may result in the polymerization of the alkene. For applications in bioconjugation where concentrations tend to be very low, polymerization of the substrate is not as critical of a concern. Proteins are free of any olefins so this technique is selective for the cysteine residues in an amino acid sequence.[22]

1.4: Isatoic Anhydride

Reactive anhydrides can be used as a bioconjugation tool through the formation of amide bonds. Much like NHS esters, anhydrides possess active carbonyl's which are susceptible to nucleophilic attack from amines such as lysine residues found on proteins. When applying anhydride based reagents for application in bioconjugation it is important they possess qualities that make them viable for reproducible results. The anhydride must be reasonably stable in the basic conditions required to keep lysine residues deprotonated

making them nucleophilic and must also react more rapidly with nucleophilic residues than with hydroxide in basic water. Anhydrides, like NHS esters, are susceptible to hydrolysis which results in the inactivation of the anhydride. This hydrolysis is a competitive process to the nucleophilic cleavage of the anhydride and must be considered when using anhydride based reagents.

The carbonyls of anhydrides are activated towards nucleophilic acyl substitution making them suitable for bioconjugation. Symmetrical anhydrides waste one half of the reagent since the substitution occurs on only one of the carbonyls. With typical unsymmetrical anhydrides there are two different sets of products possible (Figure 1-11). The goal for anhydride based conjugation is to utilize the reactive moiety to produce a single, uniform product, without wasting half the reagent. For this reason, the anhydride platform chosen for this work was isatoic anhydride, an asymmetric anhydride which is locked in a ring possessing two carbonyls with much different electronics, allowing the selection of only one of the carbonyls.

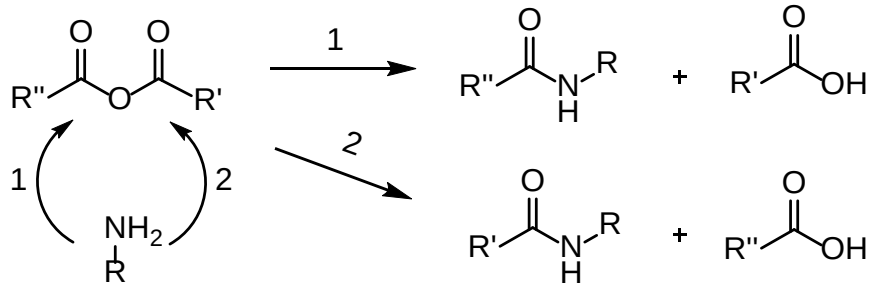


Figure 1-11: Nucleophilic attack on asymmetric anhydride to form amide bond and mixture of products

The synthesis, application, and derivation of isatoic anhydride has been extensively studied throughout the literature.[23] The anhydride is susceptible to attack at the carbonyl closest the aromatic ring. Hydrolysis results in the formation of anthranilic acid, an amino acid, whereas attack from a nucleophilic amine results in the formation of a covalent amide bond (Figure 1-12). The reaction of this anhydride with an amine results in the formation of a single product, forming the amide product with gaseous CO_2 as the only byproduct.

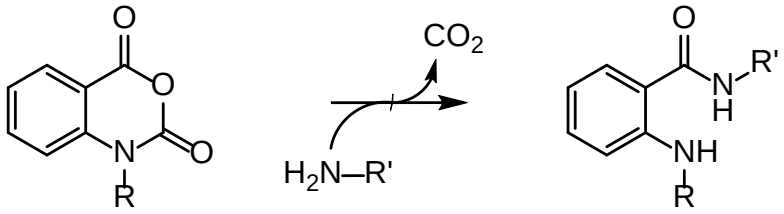
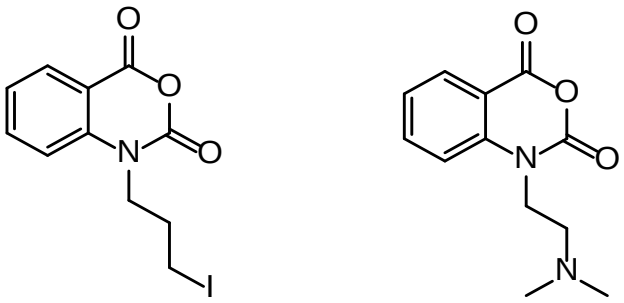


Figure 1-12: Isatoic anhydride amide bond formation

Further derivation of isatoic anhydride affords a broader scope of synthetic possibilities; previous work on the platform narrowed the focus to two main reactive precursor molecules. The 1-(3-iodopropyl)-2*H*-3,1-Benzoxazine-2,4(1*H*)-dione and the 1-[2-(dimethylamino)ethyl]-2*H*-3,1-Benzoxaine-2,4(1*H*)-dione, herein referred to as **1a** and **1b** respectively, are the penultimate reagents for the synthetic platform (Figure 1-13). As such, these reagents have little intrinsic value for bioconjugation but serve as a broad platform from which a variety of functionalities can quickly be installed through similar chemistries.



1a **1b**

Figure 1-13: **1a** and **1b** derivatives of isatoic anhydride

Derivative **1a** being a bis-electrophile and **1b** an electrophile and nucleophile, are using similar but complementary methodology for installation of functionality. The final bioconjugation reagents are produced through the formation of a quaternary ammonium salt. These two derivatives of isatoic anhydride require the addition of reagents with converse functionality (figure 1-14). The **1b** derivative requires the addition of a reagent possessing an activated bromide or a primary iodide for the quaternization of the tertiary amine. Primary bromides and chlorides are fairly unreactive towards the tertiary amine, requiring higher temperatures and extended reaction times which result in discoloration and degradation. Highly elevated temperatures also increase the likelihood of hydrolysis of the anhydride by trace moisture held by the solvent.

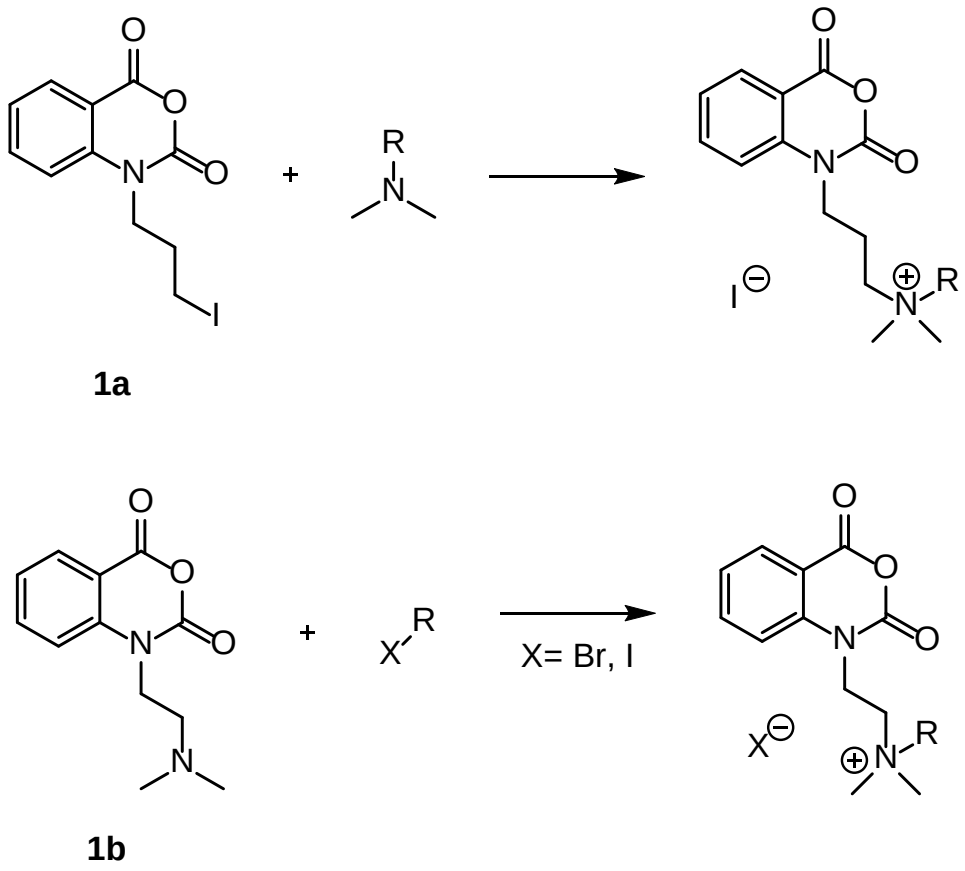


Figure 1-14: Quaternization of **1a** and **1b** reagents

Previous work studying the reactivity of isatoic anhydride based reagents has shown that the rate of nucleophilic substitution with a primary amine vastly exceeds the rate of hydrolysis in water even when the concentration of water greatly exceeds that of the amine. Even in dilute conditions, the reaction of the anhydride with a primary amine is favored over neutral water. As basicity of the solution is increased, the concentrations of the reagent and amine become more important as hydrolysis becomes a competitive process, but still several orders of magnitude slower than that of amidation.

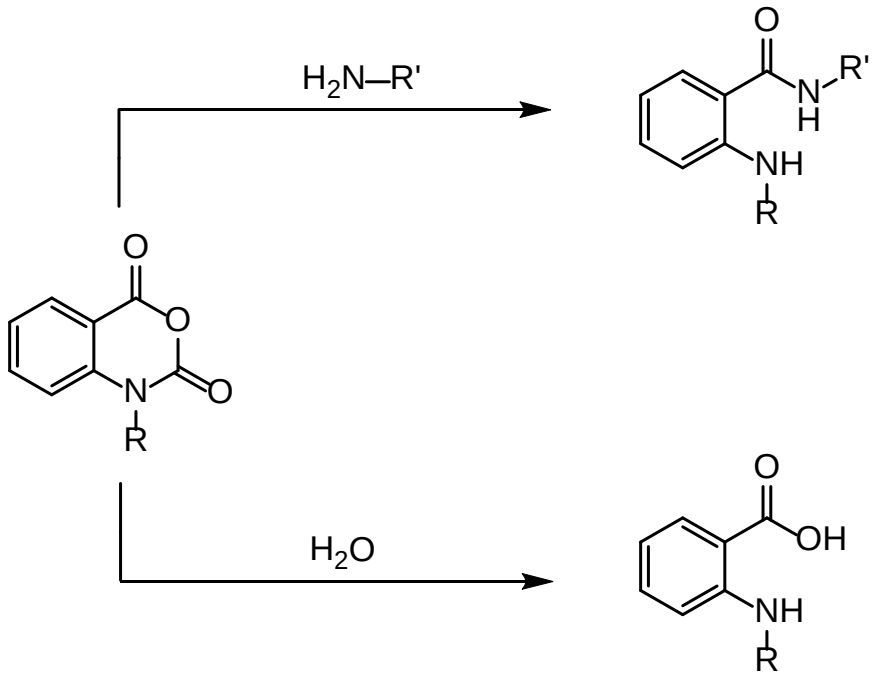


Figure 1-15: Two competing processes for the reaction of isatoic anhydride reagents

A goal for bioconjugation is minimizing changes in solvent systems as it is shown there are irreversible changes that occur in the secondary structure of peptides when solvated in mixed aqueous and organic solvent systems.[13] This means there are inherent benefits to being able to perform bioconjugation reactions in aqueous solutions. This makes the reactivity of isatoic anhydride near ideal for the application of the reagents in typical media used for bioconjugation. These isatoic anhydride reagents generally do not require organic co-solvents because they involve the around the formation of a permanently charged quaternary ammonium salt, this leads to innate water solubility of the compounds (Figure 1-16).

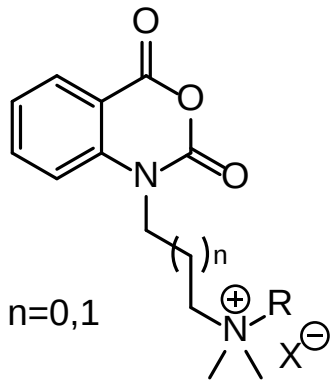


Figure 1-16: Isatoic anhydride quaternary ammonium salt platform general structure

The water solubility of each reagent is based upon the solvation of the salt pair, dependent both on the anion and cation. The cations for each of the reagents are similar but would need to be engineered to change the solubility from this part of the salt. Since R contains the desire functionality, a positively charged ammonium ion is formed during the nucleophilic attack on the organohalide. The anion is dependent on which derivative used, the **1a** derivatives will be iodide and the **1b** can be either a bromide or iodide. These anions are subject to change after creation of the reagent by an exchange reaction.

Since there is an intrinsic chromophore/fluorophore built into the isatoic anhydride platform, the reagents can be tracked both through fluorescence at 420 nm and absorbance at 330 nm. Most biomolecules have the maximum absorbance at 280 nm with very little absorbance at higher wavelengths, making the absorbance of the chromophore just outside the biological range. This is one of the advantages of using this particular anhydride as a platform for bioconjugation technology. Unlike NHS based bioconjugation reagents that do not contain a method of direct quantification, derivatives of isatoic anhydride allow for direct determination of the degree of labeling, equation 1-1.

This is highly useful as many bioconjugation techniques do not have a secondary assay to determine the amount of reagent covalently attached. This uncertainty can lead to poor batch to batch control, reducing reproducibility since there is added variability without an accurate method of quantification.

$$DOL = \frac{A_{max}\varepsilon_{protein}}{(A_{280} - A_{max}C_{280})\varepsilon_{max}}$$

Equation 1-1: Degree of labeling (DOL)

Where A_{max} is the absorbance at the wavelength of maximum absorbance of the chromophore used for labeling, $\varepsilon_{protein}$ is the molar extinction coefficient of the protein at 280 nm in $M^{-1}cm^{-1}$, A_{280} is the absorbance at 280 nm, C_{280} is the correction factor of the chromophore at 280 nm, and ε_{max} is the molar extinction coefficient of the chromophore at the wavelength of maximum absorbance in $M^{-1}cm^{-1}$. For isatoic anhydride based reagents ε_{max} is 330 nm, correction factors are required since the reagents have a small contribution to the absorbance at 280 nm and must be subtracted to get a true DOL. This calculation results in an output of labels per protein through the spectrophotometric properties of the reagent.

Chapter 2: PROJECT OVERVIEW AND OBJECTIVES

2.1 Project Overview

This project was focused around the further development of an isatoic anhydride based platform for bioconjugation by the addition of new functionalities, specifically biotinylation and thiol reactive reagents. The platform was further tested through the applications of functionalization of protein. The project dealt with the synthesis and testing of new reagents for bioconjugation, multiple synthetic routes were taken to produce reagents of with the desired function. Additionally, improvements to previously synthesized reagents were made by increasing the water solubility of poorly soluble reagents.

2.2 Biotinylation Reagents

A set of biotinylation reagents were prepared by the attachment to the isatoic anhydride platform, this linkage renders these reagents water soluble. Since the reagents can be traced through their UV-vis absorbance at 330 nm, biotinylation can be directly quantified when utilizing these reagents without the need for an expensive secondary assay. These reagents were synthesized utilizing a general reaction scheme involving the synthesis of linear arms of varying composition and length. These linkers were added onto the valeric acid side chain of biotin then attached to the isatoic anhydride platform through the formation of a quaternary ammonium salt. This forms the reactive anhydride reagent, acting much like the activated carbonyl of an NHS ester, that offers water

solubility and mitigates the need for organic co-solvents for the delivery of the bioconjugation reagent.

2.3 Thiol Reactive Reagents

A set of thiol reactive reagents were prepared and added to the isatoic anhydride platform. This added new reactive moieties to the platform, increasing the scope of applicability through the ability to target a cysteine residue on a protein. Two separate methods for reaction with the sulfhydryl group were prepared. Additionally, reagents possessing differentiated bifunctional sulfhydryl reactive groups were prepared alongside a bifunctional, thiol reactive biotin reagent.

2.4 Platform Improvements

The platform based technology was improved by increasing the water solubility of the reagents through ion exchange. Additionally, the platform was simplified by moving to a single penultimate reagent. Existing reagents were tested and improved during the transition to the unified platform. Labeling experiments to show relative reactivity of the reagents were performed to show that the functionality added to the platform does not drastically alter the rate of conjugations. An assay was developed to show the effect that labeling an enzyme may have on its catalytic functionality.

CHAPTER 3: BIOTINYLATION

3.1 Short-arm Biotin

The biotin-avidin interaction is dependent on the biotin being able to fit into the binding pocket on avidin. The binding pocket is recessed deep into the protein; this feature aids in the remarkable strength of the interaction but also requires consideration of the linker arm length when binding to a substrate. When native biotin is added to avidin, only the carboxylate at the end of the valeric acid arm from the side of the fused ring is exposed to the solvent. This makes utilization of a zero-length spacer such as NHS-biotin susceptible to steric blockage and electrostatic repulsion.

For this reason, many technologies favor the use of biotin derivatives that add an additional spacer between the valeric acid sidechain and the surface of the modified substrate, minimizing the risk of negative steric interactions (Figure 3-1). NHS-LC-biotin is commonly used for an additional 6 atom spacer that doubles the length of the biotin side chain, making it a “medium length” spacer arm. Longer linkers are available such as NHS-PEG4-biotin which incorporates a PEG-4 spacer, imparting additional length while also aiding in the water solubility of the reagent and the final bioconjugate (Figure 3-2). This is important to the function of bioconjugation reagent as changing the surface chemistry of a protein can change the solubility and stability of the bioconjugate. The addition of multiple hydrophobic reagents onto the surface of a protein can result in changes to the biomolecule, which may result in a loss of native function. Most NHS-

biotin derivatives are hydrophobic and result in alteration of the peptides solubility in aqueous conditions.[3]

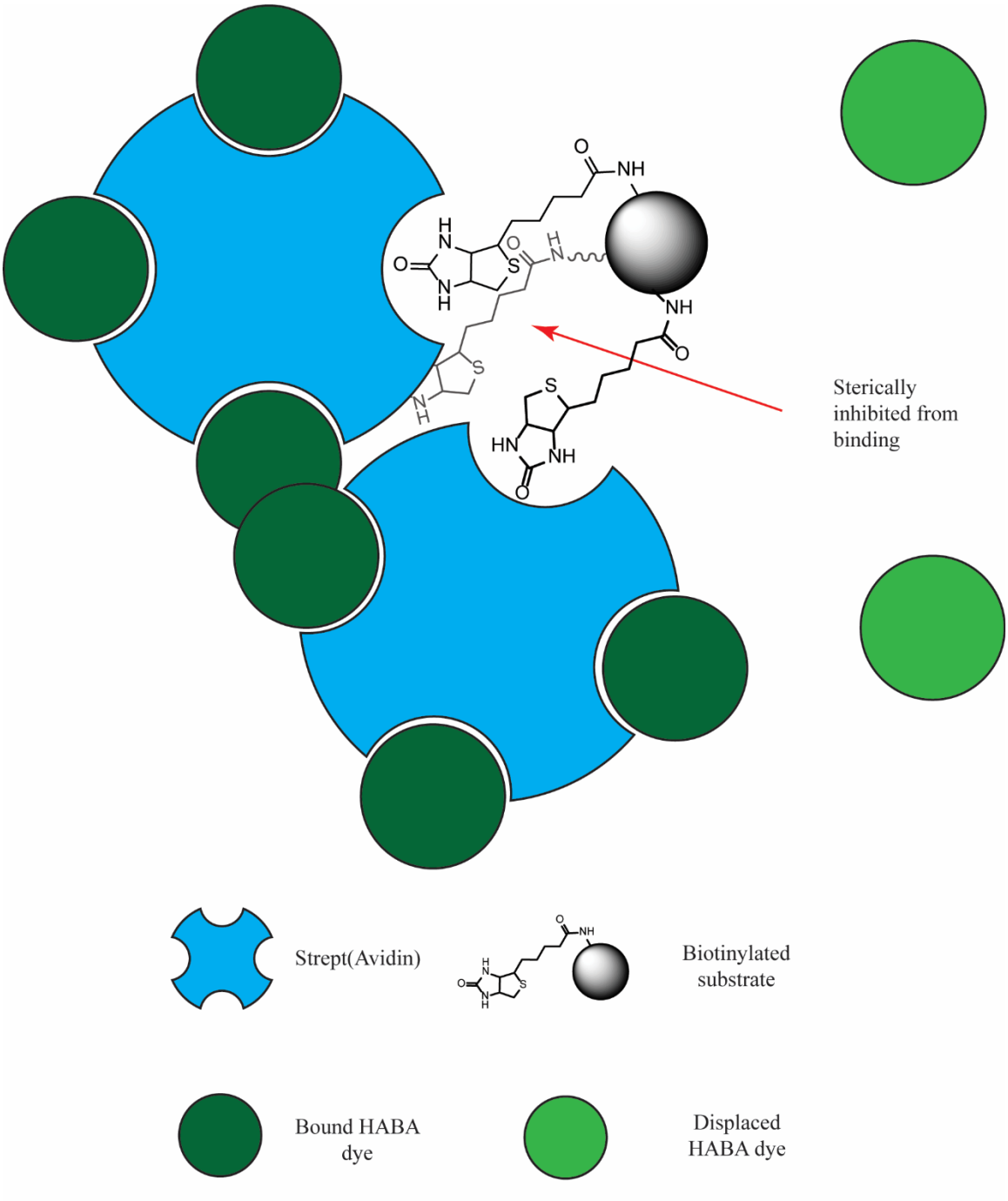


Figure 3-1: Inhibited streptavidin binding of biotin due to steric bulk

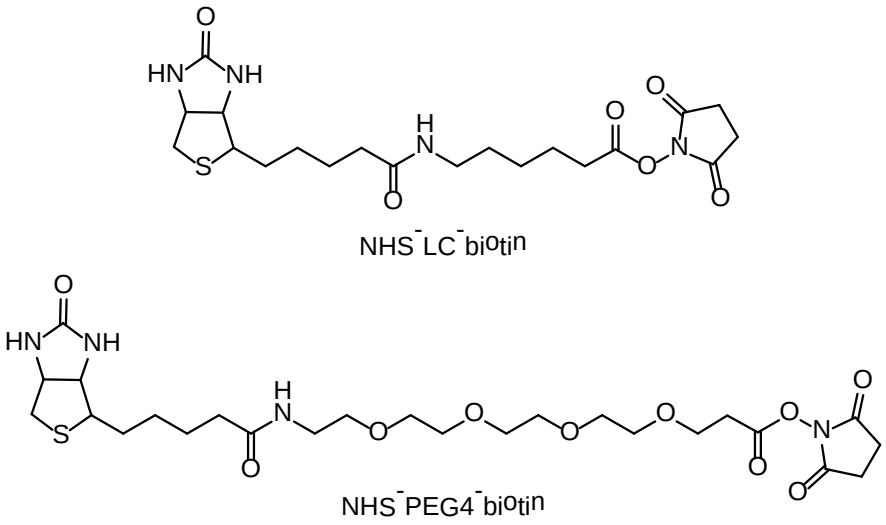


Figure 3-2: Commercially available biotinylation reagents with variable spacer arms

Commercial reagents have found methods for solubilizing biotin but few reagents offer a method to quantify the degree of functionality. Biotin is lacking a chromophore, making isolation of the biotinylated substrate after an affinity column or other purification method difficult.[5] The addition of a chromophore to biotin would offer an increased ease in the utilization of the biotin-strept/avidin technologies while also eliminating the need for a secondary HABA assay. With the commercially available biotin reagents in mind, the goal for adding a biotinylation reagent to the isatoic anhydride platform was to synthesize a reagent that possessed a linker of adequate length while remaining water soluble and traceable. This was done through the use of NHS biotin, **3**, and a short, commercial available linker, N,N-dimethyl-1,3-propanediamine, **2a**. The primary amine displaces NHS forming the **3a** derivative of biotin with a short 5 atom spacer added to the valeric acid side chain. The crude product is triturated with ethyl ether from DMF then purified via column chromatography. This reagent now

possesses the necessary features for attachment to the isatoic anhydride platform through the reaction with the **1a** derivative (Figure 3-3).

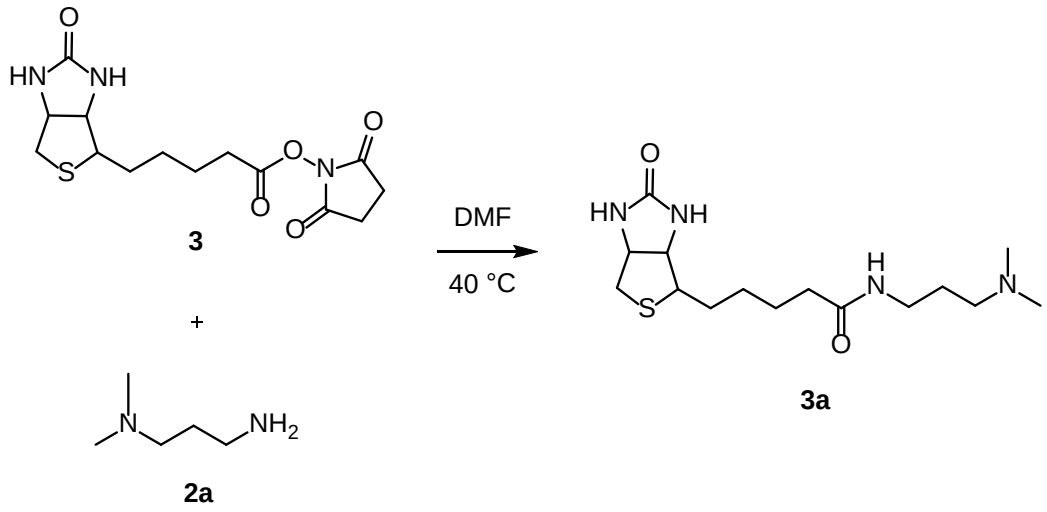


Figure 3-3: Preparation of **3a** an amide derivative of biotin

Unlike many reagents added to the isatoic anhydride platform, biotin reagents exhibit poor solubility in acetone, meaning a modified strategy must be taken for the formation and purification of the final quaternary ammonium salts. Biotin derivatives must be dissolved in polar aprotic solvents, mainly DMF and DSMO, both of which the final quaternary ammonium salts are highly soluble in. A series of solvent system experiments were performed to determine the conditions that did not result in precipitation of the **3a** derivative too. A mixture 40% DMF and 60% acetone mixture was determined to produce highest purity reagent and did not result in precipitation of **3a**. The high solubility of the **4a** reagent in DMF required trituration from the DMF/acetone mixture with ethyl ether. The reaction must be allowed to proceed until the **3a** has been quaternized prior to the trituration step as both **3a** and **4a** in solution will precipitate

during trituration. The final quaternary reagent, **4a**, was collected as an oil which was then sonicated with acetone, concentrated and placed on high vacuum, producing a pale yellow solid.

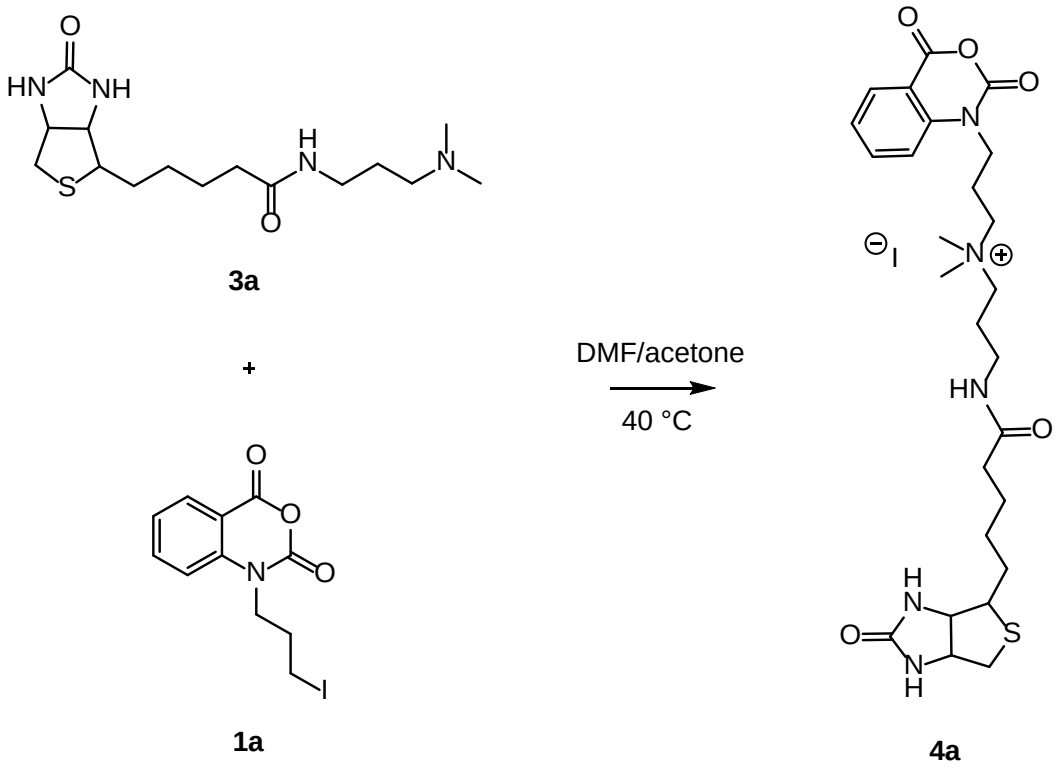


Figure 3-4: Quaternization of **3a** with **1a**, forming a biotinylation reagent

The **4a** derivative was the focus of many of the biotinylation studies; this reagent was utilized to determine labeling conditions for biotinylation of BSA. This was performed via several tests, varying either the reagent concentration or the protein concentration. Since the reaction is second order, varying the concentrations of both the labeling reagent and the protein will alter the rate of reaction and offer control for the degree of which functionality is incorporated. The labeling studies were done and the

resulting conjugates were quantified using the HABA assay to determine if the reagents efficiently bind streptavidin with the short additional linker added to the valeric acid side chain.

Comparison back to the HABA assay is performed using the properties of the anthranilic amide chromophore. Taking a measure of the absorbance from the chromophore with a known molar extinction coefficient, the exact concentration of the reagent can be determined. The concentration of the labeled protein can also be determined if the molar extinction coefficient has been accurately determined. This ratio between the two concentrations produces the statistical degree of labeling, an average of the functionality of the sample. The DOL studies gave evidence for a shortcoming when utilizing the HABA assay. At medium degrees of functionality, both forms of measurement produced results with good agreement (Figure 3-5).

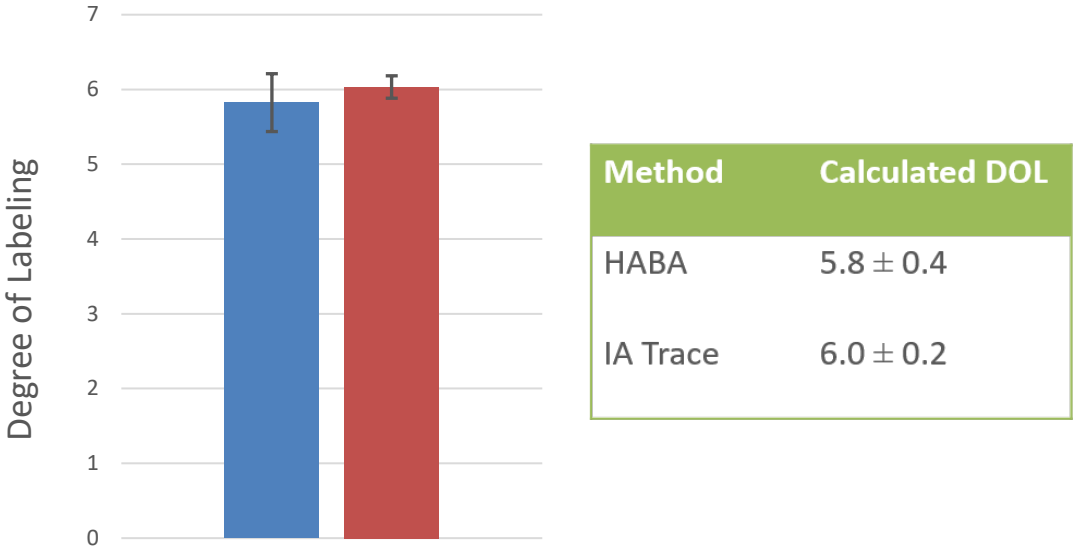
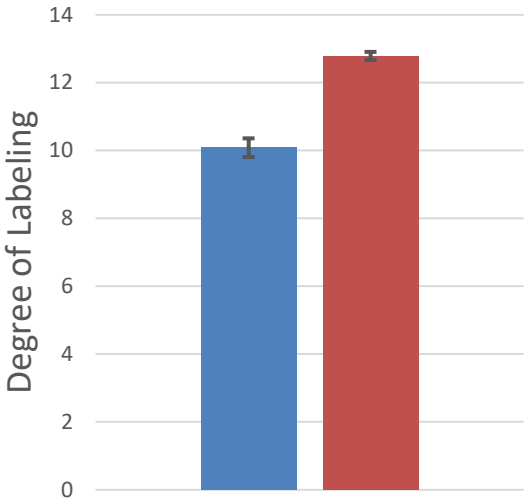


Figure 3-5: Comparison of HABA assay (blue) and isatoic anhydride trace (red) for a sample of mildly biotinylated BSA

A second sample of biotinylated BSA was prepared using higher ratios of **4a** to produce a bioconjugate with a increased degree of biotinylation. The determination of the amount of biotin was done using the HABA assay vs the single point UV-vis measurement. The results were interesting although not surprising (Figure 3-6). The single point measurement showed the degree of biotinylation to be significantly higher than that of the HABA results. This is likely a reflection of the steric factors becoming important. At higher DOL's, the biotins on the surface become closer together, making some sterically unable to bind avidin. When the degree of biotinylation of BSA high, the HABA assay failed to quantify the reagent accurately, leading to a substantial underestimation of the amount of conjugated functionality. This underestimation may be less severe with an increased assay time, but for this experiment the assay was not allowed to proceed for over 30 minutes since the ΔAbs at 500nm had become stable



Method	Calculated DOL
HABA	10.1 ± 0.3
IA Trace	12.8 ± 0.1

Figure 3-6: Comparison of HABA results (blue) to isatoic anhydride trace (red) for a sample of highly biotinylated BSA

The **4a** compound served as the first biotinylation reagent isolated as an isatoic anhydride derivative. Previous work within the group had synthesized an in-situ example but was unable to isolate the product. This reagent represents one of the few examples of a water soluble, traceable biotinylation reagent with a reactive group built in. The **3a** reagent includes a 7.2 Å spacer arm added to the length of the 6.2 Å biotin valeric acid side chain. When quaternized with the **1a** reagent, an additional 7.2 Å spacer is added through the additional 7 atom spacer built into the **1a** reagent. Visualization of the reagent within the binding pocket of a streptavidin monomeric unit was done using AutoDoc Vina with the anhydride reacted with n-butylamine as a chemical isostere of the lysine side chain (Figure 3-7).[6] These computations aided in assuring the short additional linker should be free to rotate away from the binding pocket to avoid reduction in binding affinity due to steric interference.

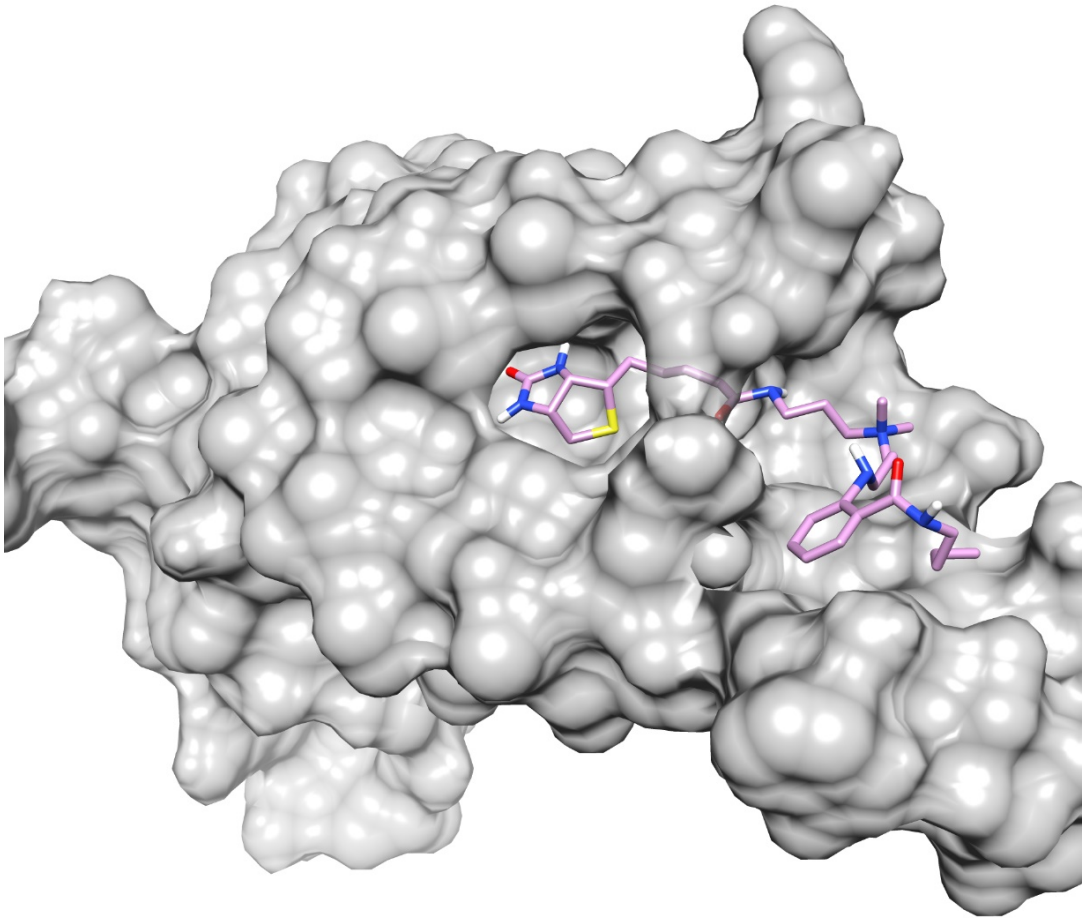


Figure 3-7: **4a** derivative bound within the binding pocket of streptavidin monomer

These computational models show the short addition of length should give the reagent sufficient space to avoid steric clashes compared to NHS-biotin reagents. Additionally, the quaternary ammonium provides a charge that does not have a direct interaction with the surface but rather is preferentially solubilized by the solvent. The computational models were compared to the binding of native biotin and were found to

bind in similar orientations with minimal differences in binding affinity or access to the binding pocket.[6]

3.2 Variable linkers

Noting the variety of linker lengths of the commercially available biotinylation reagents, a set of varying linkers were synthesized to gain additional linker lengths. This route will provide additional length to remove the biotin moiety from the surface of a bioconjugate, increasing the scope of substrate sizes. These linkers were prepared through a common starting point as few commercial compounds are available with the required α-N,N-dimethyl amine and an Ω primary amine. All reagents start from a short 3-dimethylamino propanol, **2**, which is reacted with CDI to form a reactive urethane for the subsequent steps. This urethane is slowly added to an excess of a diamine to give the final product from which is the reaction mixture is removed through bulb to bulb distillation in vacuo.

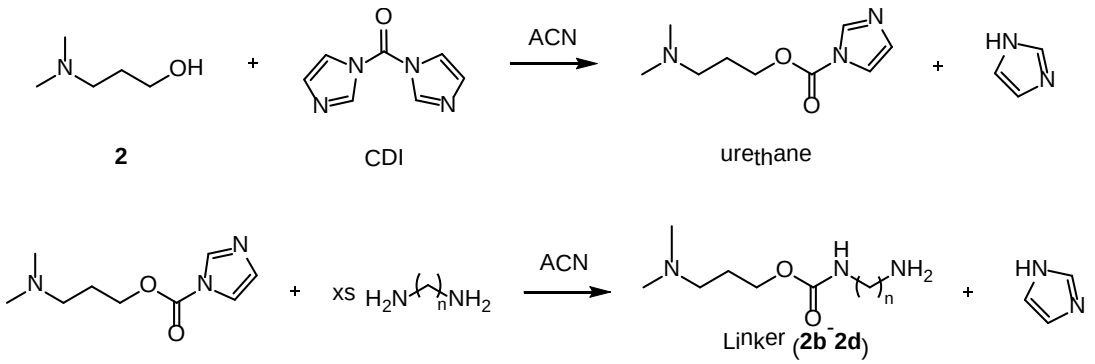
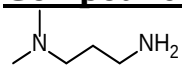
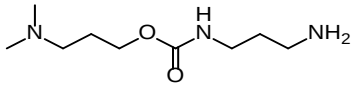
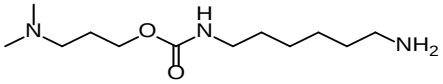
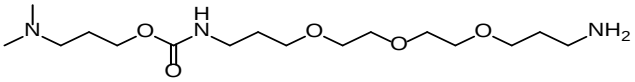
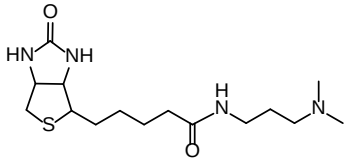
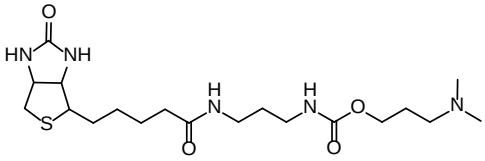
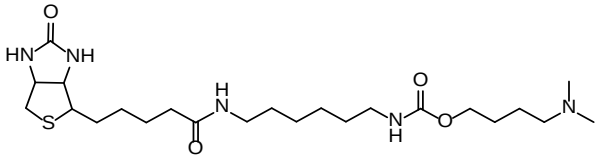
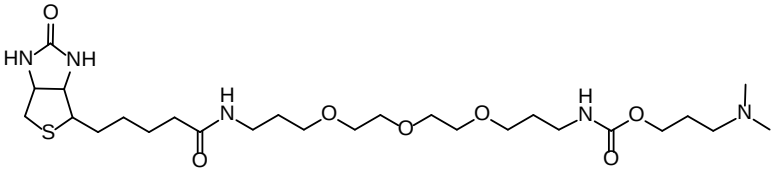


Figure 3-8: General method for preparation of spacer **2b-2d**

This process was followed to synthesize three linkers of varying length and properties (Table 3-1). Each reagent was combined with NHS biotin in DMF to yield the

N,N-dimethyl biotin derivatives which were then purified via column chromatography. The isolated product was concentrated under reduced vacuum and lyophilized in 10% ACN/H_2O yielding off-white powder which was stored under argon.

Table 3-1: Linker and biotin derivative details

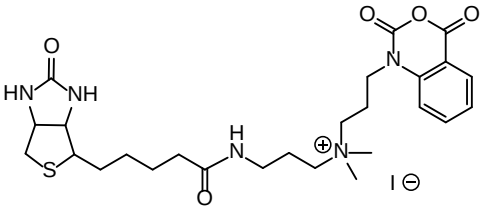
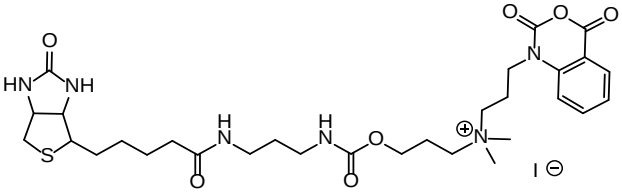
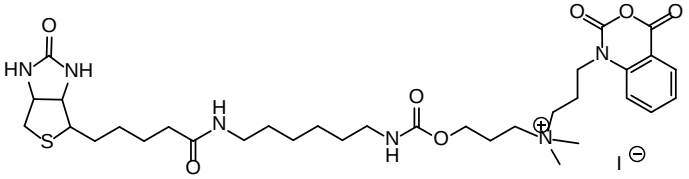
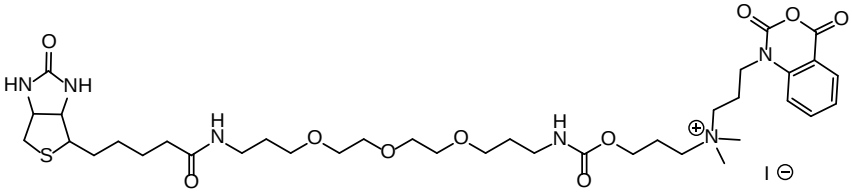
Compound	Yield	#
	*	2a
	70%	2b
	70%	2c
	50%	2d
	82%	3a
	60%	3b
	60%	3c
	50%	3d

*Purchased commercially

These were utilized in the quaternization reaction with the **1a** derivative to give the final reagents (Table 3-2). Following the process of the shorter reagent, trituration from the acetone/DMF mixture resulted in a light yellow oil which was placed under

vacuum to remove residual solvents, then lyophilized in acetonitrile diluted to 10% v/v in water to prevent the reagent from precipitating out. This process was repeated until all DMF had been removed, yielding a hygroscopic powder.

Table 3-2: Quaternary biotinylation reagents

Compound	Yield	Arm (Å)	#
	65%	20.6	4a
	70%	26.8	4b
	65%	30.6	4c
	50%	38.7	4d

Linker **2d** proved difficult to purify using bulb to bulb distillation as the diamine used was high boiling. Early attempts to purify the **2d** linker resulted in brown material which formed during the removal of excess diamine during bulb to bulb distillation. Synthesis of **4d** also proved problematic as lyophilization of **3d** resulted in a yellow solid

which proved to be highly hygroscopic. The **4b** and **4c** reagents were isolated and required minimal effort to prevent the hygroscopic nature of the salt from sequestering moisture. Lyophilization of the **4b-c** reagents resulted in a pale yellow, flocculent material which remained flocculent for extended periods under inert atmosphere at room temperature. These materials showed negligible hydrolysis when stored at 4 °C under inert atmosphere; the storage stability of the dry materials proved excellent.

The hygroscopic nature of the **4d** reagent is due to the addition of the short diethylene glycol unit. PEG is commonly used to increase the water solubility and stability of materials in physiological conditions; this is due to ability to accept hydrogen bonds, helping to coordinate water into solvation. As expected, **4d** offered a high solubility in mg/mL. Storage of the final quaternary salt proved difficult as it retained a highly hygroscopic nature. The rationale behind the addition of the short peg unit rather than alkyl chains was to prevent reduction in solubility from the long alkyl chain as we observed using the **2c** linker. Although the **2d** linker offered the longest spacing arm, the **4c** reagent was more experimentally useful due to the lack of hygroscopic behavior.

The addition of the 3 extra carbon units in the diamine chosen resulted in a substantial decrease in mg/mL solubility and a much greater decrease in molar solubility as the reagent gained amu. This reduction in solubility is due to the addition of long hydrophobic alkyl chains onto the reagent. Water solubility of the reagent is directly linked to the properties of the linker. Additional length of linker arms must account for this fact, long alkyl chains should be avoided in favor for long chains with periodic heteroatoms that are able to hydrogen bond and increase the hydrophilicity of the linker compared to hydrocarbon. Additional functionality can be designed into the linkers

through the addition of features such as cleavable linkages including disulfide bond. Compounds **2c-2d** served as sample linkers to show the ability to add additional length to the platform through the use of variable spacer arms.

3.3 Application

Several techniques were utilized to determine if the reagents would perform in the same manner as traditional biotinylation reagents. The shortest biotinylation reagent, **4a**, derivative was used for most experiments since it is accessed by a commercially available diamine that is purchased in large quantities for reasonable prices and high purity. This also served as the benchmark since it possessed the shortest spacer arm of the reagents produced. Theoretically if the reagent worked, the extended reagents should follow the same trend. The **4a** reagent was used for a set of experiments to determine the ability to detect trace concentrations of proteins. This was performed by labeling BSA in a variety of concentrations and ratios.

Labeling studies consisted of several methods to determine the function of the bioconjugate reagents. The effect on the DOL of concentration on both biomolecule and reagent were tested. Since the reaction is second order, ratio of both the reactive anhydride and the nucleophilic amines are critical to the degree of functionality. Prior work with the reagents has shown that a majority of the functionality is added in the first 15 minutes but for ease of reproducibility, all labeling reactions were performed at room temperature for a standard time of 60 minutes at RT. Experiments were performed both varying the concentration of the reagent, **4a**, and of BSA.

For the variable protein concentration experiments, a few important characteristics were determined, protein concentration and DOL are inversely

proportional when reagent is held constant (Figure 3-8). Additionally, increasing the amount of protein improves the efficiency of the labeling reaction. Increase in efficiency of the reagent results in a reduced waste of the labeling reagent. The increase in efficiency is attributed to the increase in the nucleophilic residues in solution as the concentration of the protein is increased.

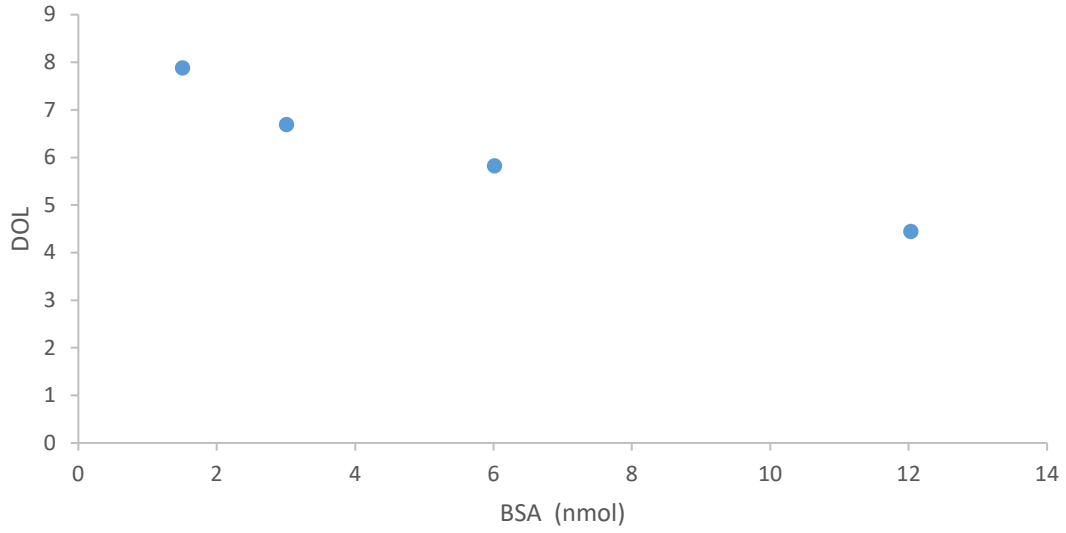


Figure 3-9: Degree of labeling of **4a** vs moles of protein in solution with reagent held constant, 300nmol **4a** reagent delivered to each reaction. BSA concentration of 0.1875, 0.375, 0.75, and 0.15 mM from left to right.

Since the reaction is second order, the protein and reagent concentration both affect the bioconjugate formed. To determine the effect that the amount of labeling reagent has on the reaction a second experiment was performed. Three varying labeling amounts of reagent were added to the protein and the total volume of all three reactions was then adjusted during the addition of the labeling reagent to keep protein concentration equal despite variable additions of reagent. This resulted in a reduced

concentration of the protein compared to the typical labeling conditions used for this work. The data from the experiment agreed with the expectation that the amount of the labeling reagent would result directly affect the amount of functionality added to the biomolecule (Figure 3-10). As the amount of in solution is increased lysine residues that are more sterically incumbent are able to react with the reagents. This results in a bioconjugate with an increased DOL.

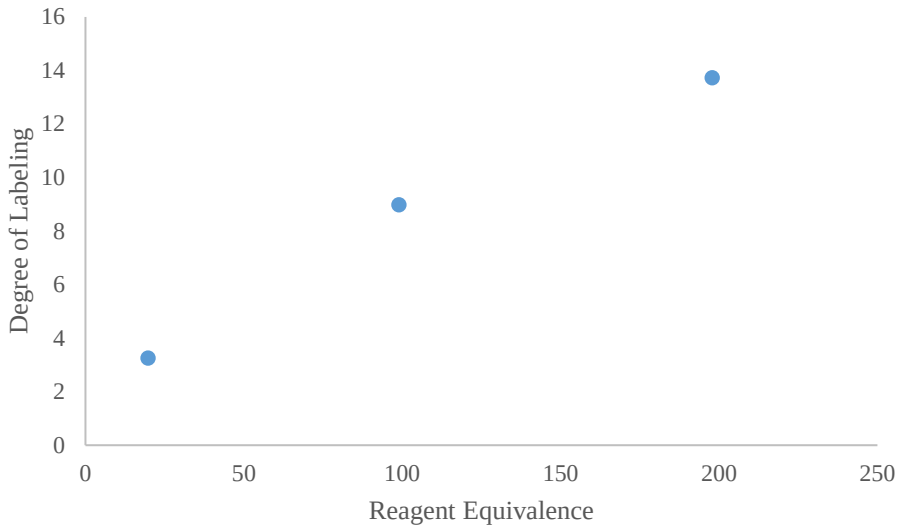


Figure3-10: Degree of labeling vs Reagent **4a** equivalence, final BSA concentration of 50 µM used in order to accommodate volume of H_2O required to solubilize reagent.

Using a BSA with a DOL of 14 of **4a**, the sensitivity of the isatoic anhydride fluorophore was examined using a Molecular Devices SpectraMax M5 plate reader. This was done through a set of serial dilution of the sample. The linear dynamic range of the reagent was found to cover greater than 3 orders of magnitude of concentrations (Figure 3-11). The instrument was able to detect concentrations of 30 nM BSA, meaning reagent

concentrations of roughly 500 nM are able to be quantified using the fluorescence properties of isatoic anhydride. The fluorescent properties of the reagent allows for identification of tracking of trace biomolecules. Additionally, the fluorescence was adequate for use in typical SDS PAGE experiments.

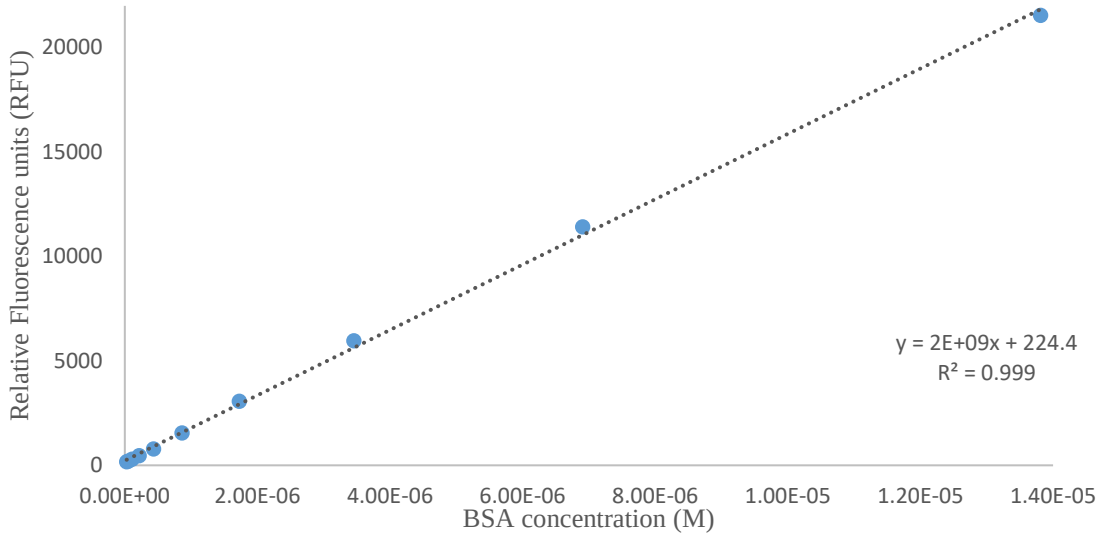


Figure 3-11: Fluorescent properties of a biotinylated BSA sample (DOL=14) at various concentrations

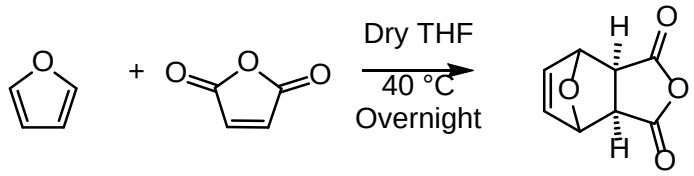
CHAPTER 4: THIOL REACTIVE

4.1 Maleimide

In order to add a thiol reactive maleimide to the isatoic anhydride platform the necessary α-N,N-dimethyl-Ω-diamine functionality must be added. This requires for the addition of a short alkyl chain to the maleimide functionality. For this addition to work, the reactive alkene of the maleimide needs to be protected through a Diels-Alder [4+2] cycloaddition. The protected maleimide must also undergo a reverse Diels-Alder reaction to deprotect and expose the reactive alkene after the functionality has been added. As the maleimide, the reagent is expensive and required the use of a strong base such as NaH to remove the proton from the nitrogen which then is reacted with an alkyl halide containing the necessary N,N-dimethyl amine.

Since the route to the final maleimide requires protection of the alkene, it seemed advantageous to avoid the use of both the expensive starting reagent and NaH. Instead, maleic anhydride was utilized as a maleimide isostere to which was added furan to undergo the [4+2] cycloaddition on the alkene (Figure 4-1). This offered a protected alkene rather than the electron poor alkene found in a maleimide. The Diels-Alder reaction is performed in mild conditions in anhydrous THF resulting in a crystalline precipitate which was purified with several rinses with THF, followed by sonication then centrifugation of the product. After the excess furan was rinsed away, solvent was

removed under reduced pressure. This gave a white crystalline powder in high purity which was used directly for the next step.



5'

Figure 4-1: Maleic anhydride [4+2] cycloaddition with furan

The anhydride adduct forms as the exo adduct which has been previously described[24] and can then be reacted with an α-N,N-dimethyl-Ω-diamine in refluxing MeOH which ring opens the anhydride forming an amide and a carboxylic acid. Under the reaction conditions[25] this then dehydrates to form the imide (Figure 4-2). This method accesses the required functional protected maleimide reagents while circumventing the use of expensive starting materials. The maleimide is formed through a retro Diels-Alder reaction which requires heating the adduct for extended periods under slightly reduced pressure encouraging the removal of furan from the reaction vesicle. This is necessary as the Diels-Alder adduct would reform slowly if all the furan is not removed from the reaction.

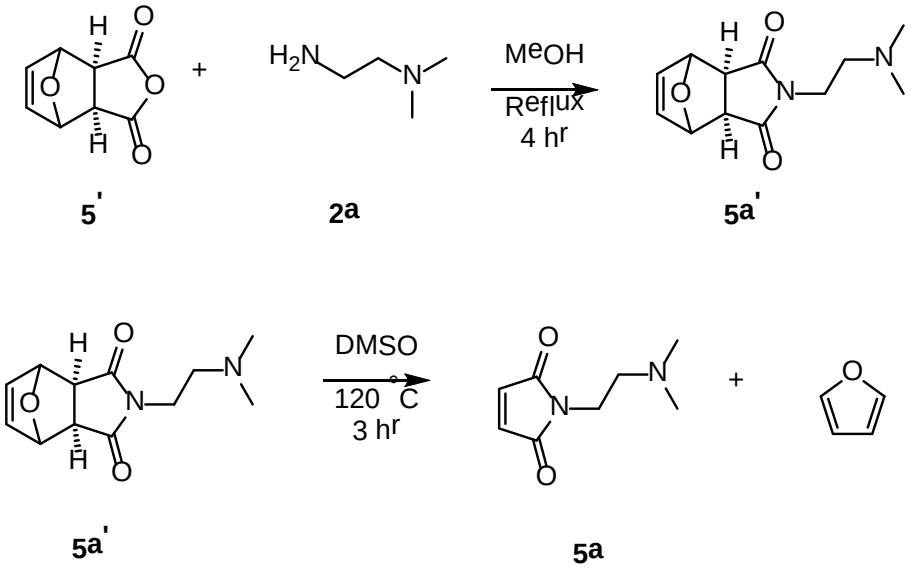
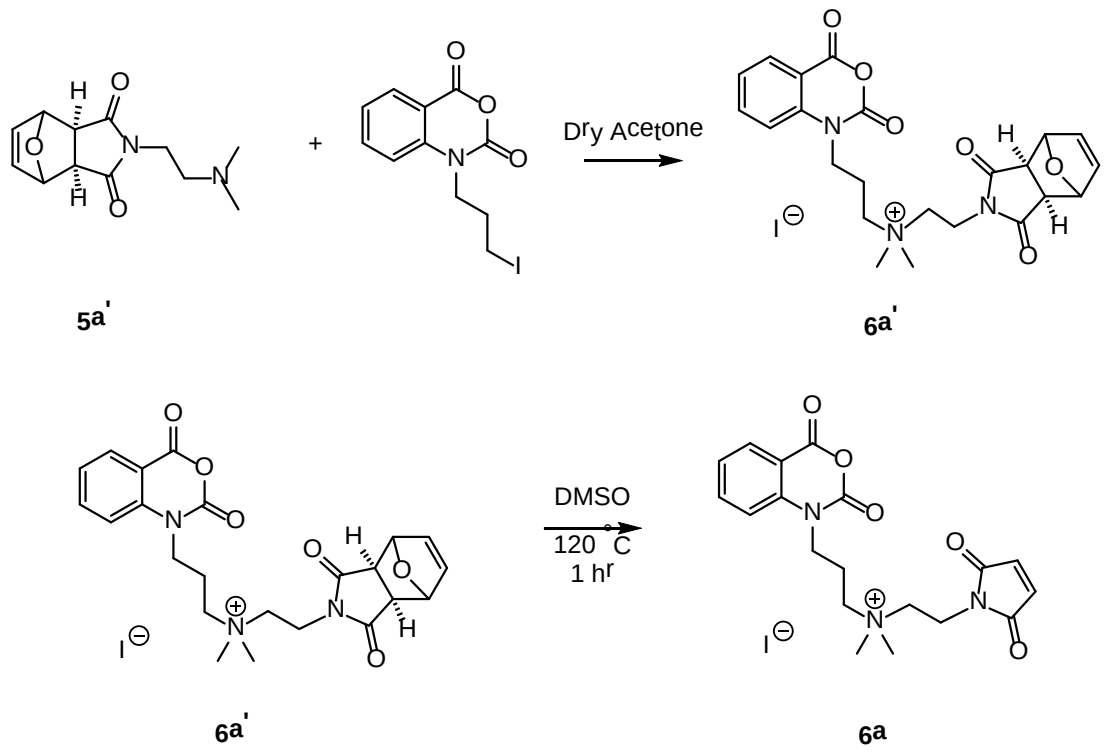


Figure 4-2: Conversion of the anhydride adduct of maleic anhydride to a functional maleimide reagent

The deprotection of **5a'** was performed in deuterated DMSO and monitored by NMR. The disappearance of singlets corresponding to the bridgehead protons were compared to the maleimide alkene protons growing in to determine the deprotection. The deprotection of **5a'** to **5a** was demonstrated as shown in figure 4-2 one time but deprotection of **6a'**was of more interest (Figure 4-3). This would allow for storage of a protected reagent rather than as the free maleimide. The protected reagents are more robust and thus better for storage at room temperature. The protected **5a'** was quaternized with **1a** to give the **6a'** derivative, this was deprotected following the same procedure as that **5a'** reagent and was monitored by NMR. For this method to work, the anhydride must not be hydrolyzed by trace moisture in the DMSO. The sample was prepared carefully under N_2 before being transferred to be heated under reduced vacuum. DMSO was removed through reduced pressure with a LN_2 trap.



5a' **6a'** **6a'** **6a**

Figure 4-3: Deprotection of maleimide reagent as quaternary salt, producing heterobifunctional thiol/amine reactive reagent

Since this reagent was accessed without the use of maleimide starting materials, the short 2 carbon spacer can be replaced with any N,N-dimethyl diamine, such as reagents **2b-2d**, the variable linkers used for the synthesis of biotinylation reagents, Section 3-2. This allows for the uses of interchangeable spacer arms to tailor the reagent for specific applications.

4.2 Thiol-ene

The use of radical addition of thiols to double bonds has grown popular in polymer applications.[20] The application of said chemistries to the fields of bioconjugation have shown and made inroads for direct conjugation to cysteine residues without the

possibility of a reverse reaction which results in the deconjugation of functionality, leading to destruction of the conjugate.[17] This, along with the rapid reaction rates and high specificity of thiol-ene chemistry has generated interest in its application to biotechnologies.

Thiol-ene reactions are dependent on the properties of the alkene, including the local environment. Substrates in close proximity to quaternary ammonium salts have been previously demonstrated and tested. The choice of a dimethyl quaternary salt compared to a diethyl derivative increases the reactivity of the alkenes since there is a reduced steric bulk. The spacer arm of the alkene from the quaternary salt is also important since the electronics of the quaternary salt play a role in the thiol-ene chemistry. A single carbon spacer arm from an allyl generally results in poorer reaction yields.

Previous work has prepared an allyl and a styrene derivative of **1b**, both of which are not ideal for thiol-ene chemistry for differing reasons. For this work, the **1b** derivative was utilized for the development of a thiol-ene reaction by adding additional length to the spacer arm. This was done by using 4-bromo-1-butene and the **1b** derivative in standard quaternization conditions (Figure 4-4). The choice of the butenyl spacer removes the alkene from the positively charged nitrogen, increasing the chance of a successful thiol-ene radical reaction with increased yield.

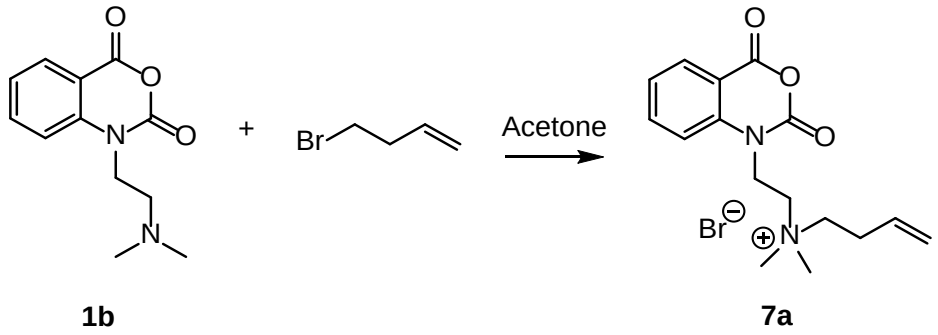


Figure 4-4: Formation of the quaternary thiol-ene reaction

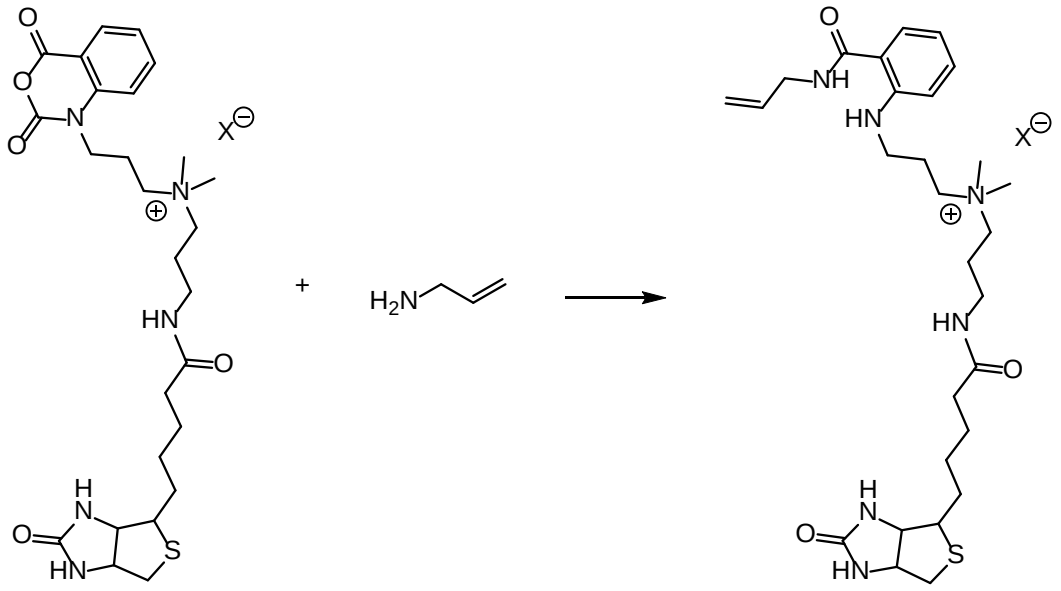
Numerous reagents for application to thiol-ene chemistry can be envisaged through the addition of an alkene to the isatoic anhydride platform. Interestingly, strained alkenes are shown to be excellent substrates for thiol-ene chemistry.[21] Norborenyl substrates show some of the highest reactivies for the radical addition. This suggests that the **6a'**, with its norborene like structure, might be a good reagent for a thiol-ene reaction.

4.3 Heterobifunctional Reagents

As synthesized, most quaternary reagent of the isatoic anhydride platform are heterobifunctional, with an amine reactive anhydride and a second reactive functionality added through the formation of the quaternary ammonium salt. To increase the scope of the heterobifunctional reagents, the modification of the amine reactive portion of the anhydride could result in an expansion of the reagent scope. To test this, a few heterobifunctional reagents based off of thiol reactive species were prepared.

To ease the synthesis of the heterobifunctional reagents, reagents were chosen in a fashion which both optimized the properties while minimizing the difficulties of purification. A thiol reactive derivative of biotin was prepared through the reaction of **4a** with allylamine (Figure 4-5). The formation of the amide bond results in the inactivation

of the derivative for lysine conjugation, but instead adding an alkene capable of undergoing thiol-ene reactions. This reagent was reacted and monitored in deuterated DMSO, after the reaction with the anhydride, the reagent is no longer sensitive to heat or water. This enables purification by simply concentrating the solution under reduced pressure, removing the excess amine. This reagent shows the ability for a simple but effective method for the increase in functionality using isatoic anhydride as a basis for bioconjugation.



4a **4a-1**

Figure 4-5: Preparation of a thiol reactive biotin **4a-1**.

Based off of previously published work[22]; the use of a short allyl spacer from an amide results in a sufficiently reactive alkene for thiol-ene chemistry. A heterobifunctional thiol reactive compound was envisaged by utilizing two separate thiol

reactive chemistries. The **6a'** reagent was taken and reacted with excess allylamine in deuterated DMSO which resulted in a reaction with the anhydride. This is cleaned up at reduced pressures to remove the excess allylamine, the solution is then heated to 120 °C for 1 hour, resulting in the deprotection of the maleimide, generating the **6a-1** derivative (Figure 4-6).

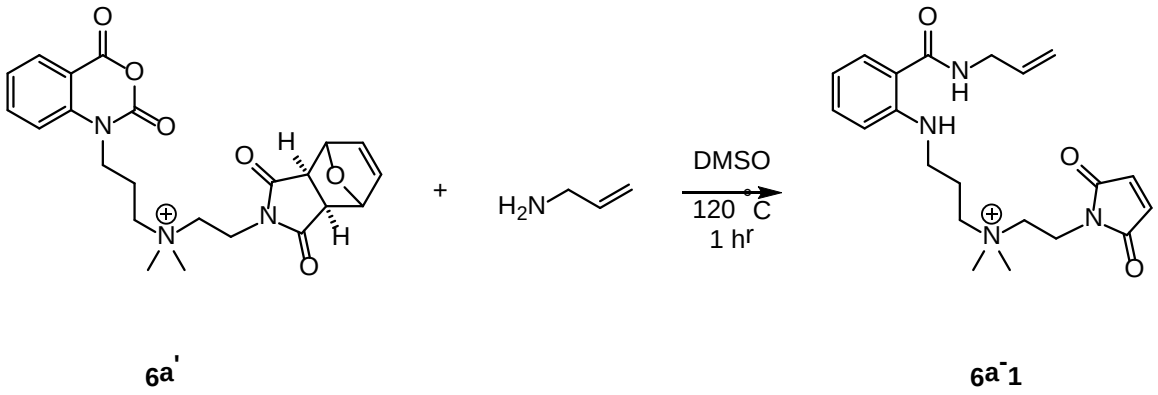


Figure 4-6: A differentiated bifunctional thiol reactive reagent possessing differing reactivities.

Although both active functionalities on the reagent are thiol reactive, they do so through different mechanisms and conditions. That allows for reactions with different thiol substrates by using stepwise addition. The maleimide will react in the presence of a sulfhydryl, meaning it will have to be the first chemistry to be utilized. This can then be purified and reacted with another sulfhydryl after the addition of a photoinitiator and UV light. The **6a-1** reagent allows for the production of unsymmetrical bisthioethers.

CHAPTER 5: PLATFORM IMPROVEMENT

5.1 Solubility

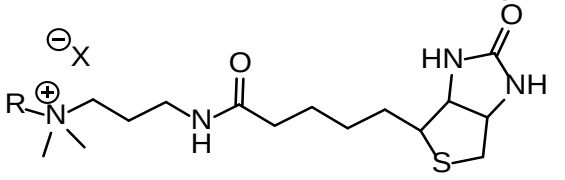
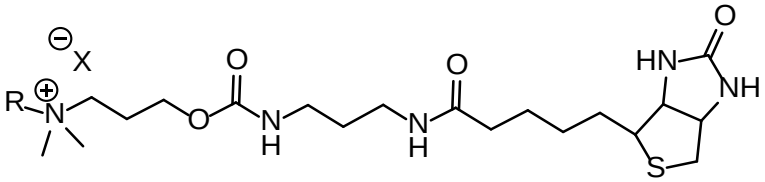
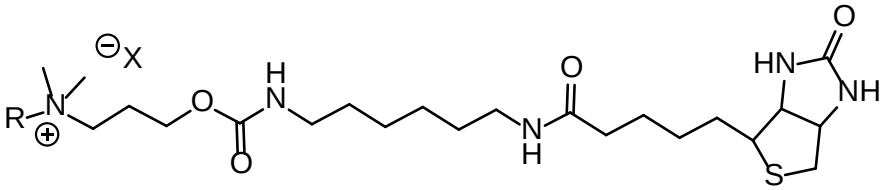
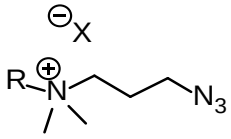
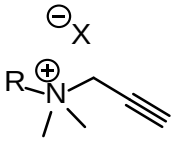
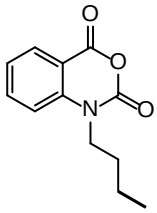
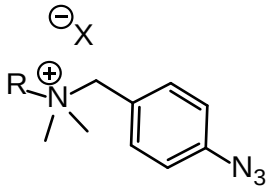
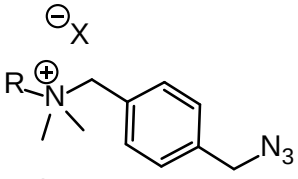
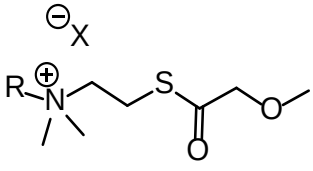
As synthesized, all reagents prepared from the **1a** derivative of are salts with the anion being iodine. Although this is advantageous for the ease of synthesis of the quaternary reagent, these iodide salts tend to discolor with age as well as exhibit reduced solubility when compared to reagents synthesized from the **1b** derivatives and the required activated bromide. For this reason, it was hypothesized that the poor water solubility of some reagents could be mitigated through anion exchange from iodide to chloride salts.

The ion exchange was performed on reagents of modest to poor solubility. The exchange was performed using Dowex marathon OH beads which had been exchanged for chloride ions. Reagents were dissolved into 10% ACN in ice cold DI H_2O and stirred over the beads for 10 minutes before removing the beads via frit and lyophilizing the solution. The ion exchange was optimized by monitored the disappearance of the iodide using ESI in negative mode. This allowed for determination of an optimized process which minimized the risk of hydrolysis of the reagent as the water began to warm.

Exchange from iodide to chloride results in a drastically increase in the solubility for most reagents. This exchange was performed on several poorly soluble compounds and gave substantial increase in solubility for all reagents (Table 5-1). The increase in

solubility further enhances in molar solubility since the anion is chloride rather than an iodide.

Table 5-1: Solubility of reagents as I^- or Cl^- salt.

Reagent	I^-	Cl^-
	15	≥40
	5	≥40
	2.5	≥40
	1.0	≥40
	1.0	≥40
R=  	<1.0	≥40
	<1.0	≥40
	1.2	≥40

As shown in previous chapters, increasing the concentration of a bioconjugation reagent makes it possible to dramatically increase the efficiency of the labeling reaction. The exchange to chloride has two important advantages. It allows bioconjugation reactions to be carried out at higher concentrations, which increases the reaction rate of the labeling which leads to an increase in efficiency. Secondly, the highly soluble reagents do not require an organic co-solvent, such as DMSO, which can lead to the denaturation of proteins. [13]

5.2 Reagent Properties

Isatoic anhydride serves as a useful platform for bioconjugation due to its unique physiochemical properties. The asymmetric anhydride results in the formation of an amide bond when reacting with a nucleophilic lysine or N terminal amine of the protein. The aromatic core gives rise to the photochemical properties of the reagents which give rise to the traceability of reagents through absorbance at 330 nm or fluorescence at 420 nm, outside the biological range.

The final quaternary ammonium salts have a permanent electrostatic charge, aiding in solubility of the reagents but also results in no change to the net charge of the peptide in physiological pH since the reaction is with a lysine residue, pKa ≈ 8.5. Typical bioconjugation through lysine results in a loss of the native charge of the residue during the formation of the neutral amide bond. This results in a change of the surface charge of the protein. With the isatoic anhydride reagents, the quaternary salt takes the place of the lysine's positive charge resulting in little change in the electrostatic surface charge unlike conjugation through typical NHS-esters which replace the positive charge with a neutral amide. At physiological pH and below, modification using a quaternary salt results in the

preservation of the native isoelectric point and retains the microenvironment which may help retain the native structure and activity.[2] Using quaternary ammonium derivatives of isatoic anhydride results in no change to the charge and thus minimizes the electronic effect of conjugation.

The incorporation of the various functionalities as a quaternary ammonium salt occurs several angstroms from the reactive carbonyl of the anhydride, as such suggests that the various reagents should have similar reaction rates with the lysine residues. This hypothesis was tested through labeling BSA, if the reagents reactivity are to be similar, reaction at the same concentration and same amount of reagent should result in similar degrees of functionality. Three reagents, **4a**, **8a**, and **8b** were selected to determine the effect of the functionality on the DOL (Figure 5-1). **8a** and **8b** were selected as they are complementary pairs for CuAAC click chemistry that possess similar molecular weights but different properties, and **4a** since it has a much greater molecular weight than the other reagents. All reagents were prepared from the **1a** platform followed by ion exchange to a chloride, this was done so all reactions could be performed at the same reagent concentration, prior to ion exchange, **8a** and **8b** exhibited too poor of solubility for this application. The reagents were prepared at 15 mM and BSA at 10 mg/mL (0.15 mM), 100 uL BSA solution was combined with 30 uL of the reagent solution and vortexed vigorously. At the 60 minute point, 5 µL of NH_4OH was added to halt the reaction by hydrolyzing remaining anhydride reagent.

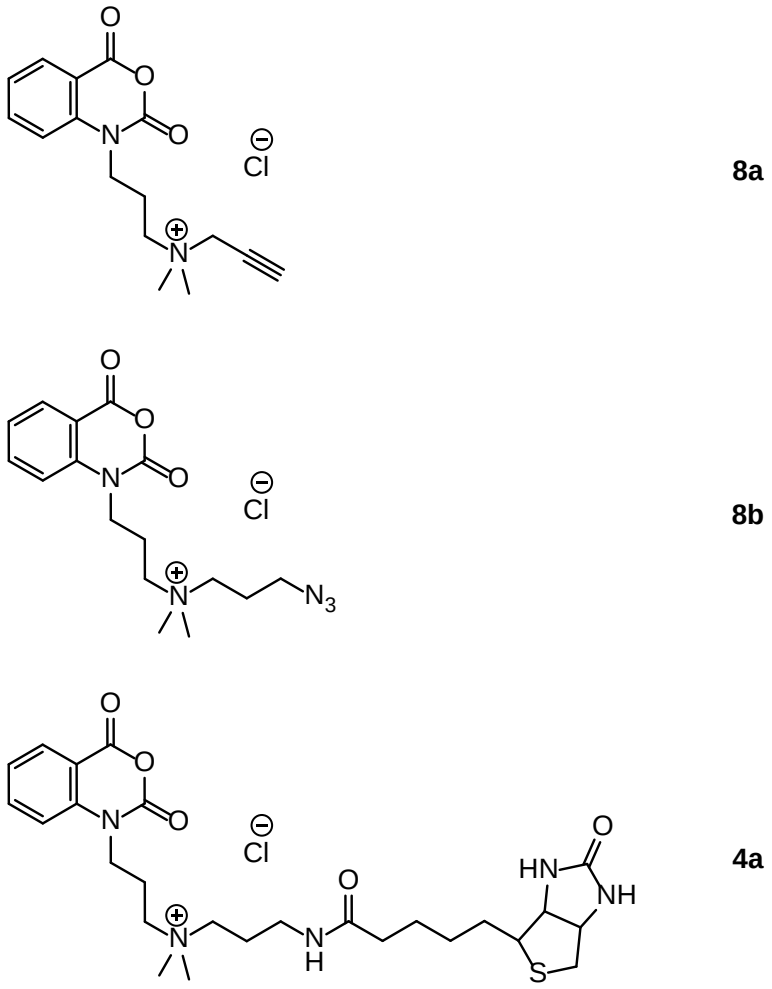


Figure 5-1: **8a**, **8b**, and **4a** isatoic anhydride derivatives used for evaluating the effects of R on the reactivity of the bioconjugate.

The solution was filtered through a gel permeation column (Princeton Separations) to remove any unreacted small molecules. The DOL was determined via the absorbance of the isatoic anhydride chromophore on a Molecular devices Spectramax M5 plate reader in a 500 µL cuvette. The samples were run in triplicate to ensure reproducibility. The results from the three reagents were all within the standard deviation of one another where **8a** gave a DOL of 8.1 ± 0.2, **8b** gave a DOL of 8.0 ± 0.2, and **4a** gave a DOL of 8.0 ± 0.4 (Figure 5-2). These results show that the functionality seems to

be far enough removed from the reactive anhydride to have little if any effect on of the anhydride. This is important for method development which can allow for a general protocol to be used to produce similar results. Additionally, this shows that reactivity of the anhydride is not negatively affected, even in when a large, hydrophobic functionality is utilized.

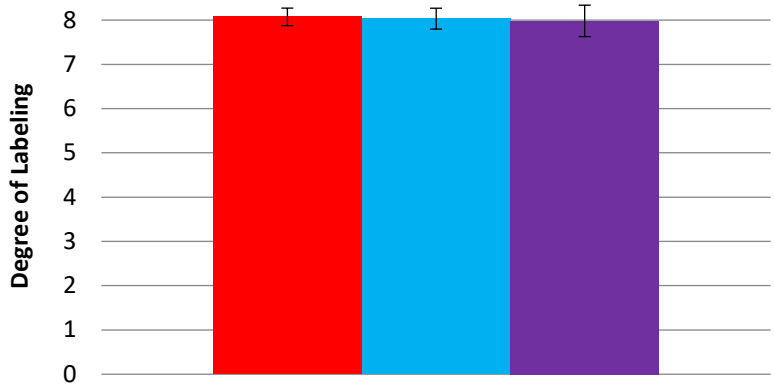


Figure 5-2: Degree of labeling of **8a** (red), **8b** (blue), and **4a** (purple)

Another key feature for bioconjugates is that the addition of functionality must not disrupt the natural function of a biomolecule. The use of an enzymatically active protein, HRP, as the substrate for modification gives the ability to determine if activity is conserved after modification. For this experiment, an HRP based assay was developed in which the amount of HRP could be determined through the formation of a colored complex from o-phenylenediamine which was measured at 450 nm. A commercially purchased bottle of HRP expressing 50-150 units/mg enzymatic activity was used for the assay. Conditions were screened to determine the sensitivity of the assay, finding the assay to be linear over a select range of moles of HRP. This assay was developed as a

mimic of an assay which would utilize biotinylated HRP to determine the amount of a trace substrate which has been detected through use of an antibody bioconjugated with streptavidin.

For use in an application such as this, the HRP enzymatic function must be retained, to test this; a calibration curve of unmodified HRP was prepared. HRP has 6 free lysine residues on the protein and is known to quickly modify 1-3 lysine residues on the peptide. LYS-232 tends to be found as the first residue modified, two other residues are often found as intermittently modified leaving 3 residues unmodified.[26] This is likely due to the three residues being the only surface accessible amines, the remaining three are inaccessible.

The assay was tested using 6.0 nM HRP as a control, 6.0 nM HRP was modified with biotin and purified by GPC. The sample was then used beside the control to determine the HRP content of the sample. The control was found to be 5.9 ± 0.3 nM and the biotinylated HRP was found at 5.6 ± 0.1 nM. One outlier was removed from the modified HRP as it showed modification to have led to an increase the concentration. The assay served as a simple method to show that chemical modification with an isatoic anhydride based reagent to active enzyme does not drastically alter the native function. The addition of the biotin handle does not disrupt the native function of the enzyme.

5.3 Unified platform: **1a** vs **1b**

In the beginning the bioconjugation platform utilized the two complementary penultimate reagents, **1a** vs **1b**, as entries to these bioconjugation reagents. Although it is sensible concept, prior work has shown the difficulty of utilizing the **1b** derivative as it is difficult to prepare and purify and requires reactive bromides or iodides. When compared

to **1a**, which itself is an activated iodide, is synthesized through a similar process but in much higher yields and is easily recrystallized from IPA, all together making **1b** less attractive. The **1a** derivative is subject to a slow decay which can be determined visually through an increase in the coloration, producing a yellowed appearance. This is a slow process which simply requires recrystallization of the parent material in IPA after several months of sitting. The recrystallized material possesses a faint yellow color and exhibits high solubility in acetone. This derivative tends to form the final quaternary salt in 24-48 hrs at 40 °C under typical concentrations depending on the reagent used.

The **1a** derivative is the more advantageous derivative from the synthetic point of view as it is produced in higher yields and expressed greater solubility in acetone than the **1b** derivative. Prior to quaternization, **1a** or **1b** will be heated into acetone and filtered through a 0.2 μm filter at a concentration of 100 mg/mL. Once in solution, the **1a** derivative stays solubilized even as equilibration to room temperature proceeds. **1b** slowly forms amorphous solids that must be heated back into the solution. This is problematic when the solution is being filtered as a cold filter will result in the precipitation of solid, making exact ratios of the reagents more difficult to achieve.

Reagents similar to those prepared from **1b** can be produced from **1a** through a simple reaction with dimethylamine to displace the activated halide (Figure 5-3). This offers the chemical handle required for the formation of the quaternary ammonium using the **1a** derivative. This is advantageous since the **1a** derivative is obtained more readily in much higher yields and purity.

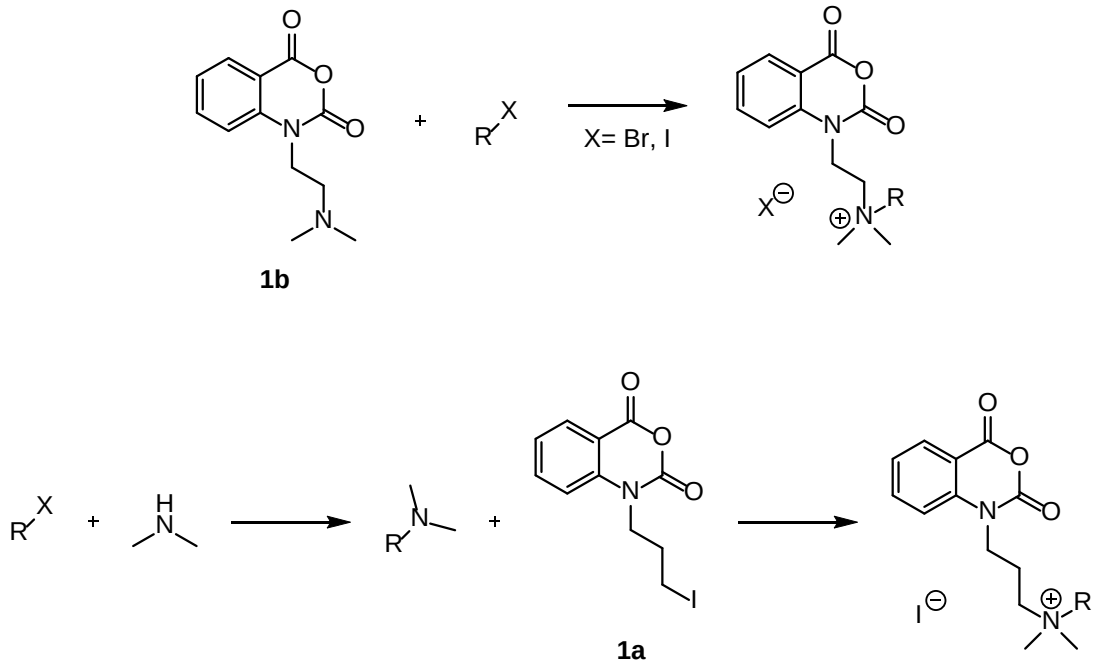


Figure 5-3: Unification of the platform onto the **1a** derivative through the modification of the active halide to dimethyl amine functionality

The S_N2 reaction of dimethylamine and a primary halide proceeds rapidly in polar aprotic solvents. Dimethylamine is used in excess and is removed under reduced pressure. This can be performed on most of the reagents without modification, affording a direct method to move to the **1a** platform. Although reagents based on the **1b** platform tend to exhibit an increased water solubility compared to that of **1a**, the use of ion exchange eliminates this discrepancy, minimizing the slight drawback of the **1a** quaternary salts. Additionally, reagents with the desired functionality for addition to the **1a** platform can be easily accessed through the modification of both alcohols and carboxylic acids. Both of these are common starting materials for organic syntheses, this allows a method to access a number of materials through the use of these common features (Figure 5-4).

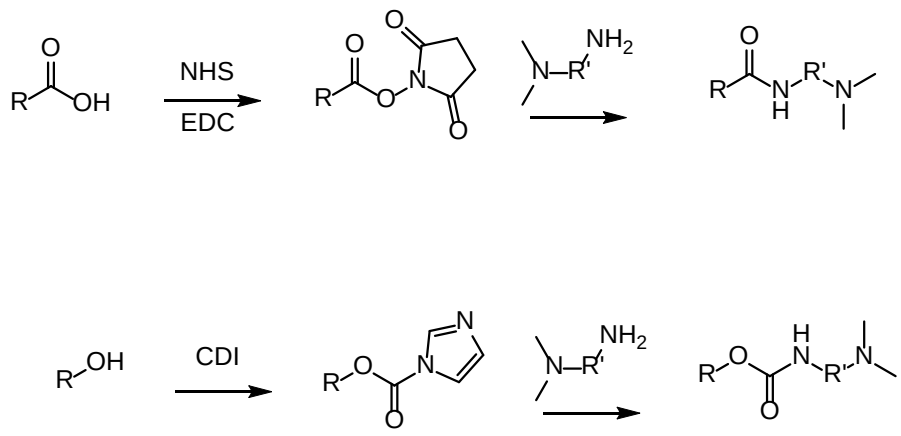


Figure 5-4: Methods for the access of N,N-dimethyl derivatives for reaction with **1a**

CHAPTER 6: CONCLUSIONS

A set of biotinylation reagents were synthesized that are water soluble and traceable. They were prepared with the ability to adjust the linker arm through the use of variable linkers which are synthesized through common synthetic pathways, allowing increased control of the reagents. These reagents have been tested and shown to be effective biotinylation reagents. The degree of labeling for these reagents can be quickly accessed through using the UV handle of the reagent, eliminating the need for a secondary assay for determination of the functionality. This method of determination is advantageous since it is not subjected to the steric constraints of the HABA assay and does not result in the loss of valuable sample.

The biotinylation reagents, **4a-4d** represent novel reagents possessing water solubility, traceability and while simultaneously retaining the natural charge of a biomolecule. Compounds possessing these properties are not commercially available. Additionally, the permanent electrostatic charge will help to remove the reagents from the surface of the protein through preferential interactions with a solvation shell of water. This property should minimize the likelihood of the bioconjugated functionality from being trapped in a hydrophobic pocket on the surface of the labeled biomolecule, increasing the likelihood for a successful bioconjugate.

Thiol reactive derivatives were added to the platform through both maleimide and thiol-ene chemistry. These derivatives are also water soluble and offer the ability to trace

the reagents through the use of the UV absorbance of the aromatic ring. Interestingly the **6a'** maleimide derivative has promise for thiol-ene chemistry through offering a strained alkene substrate for radical chemistry. This offers a unique method for the use of a single starting point which can be utilized for two separate click chemistries through a simple modification using heat. A unique heterobifunctional sulfhydryl reactive reagent was synthesized, offering a handle for both maleimide and thiol-ene chemistry. This showed the ability to quickly alter the amine reactive anhydride to another kind of chemistry, this represents the ability to produce mix and match reagents.

The solubility of the quaternary reagents was increased through ion exchange. This improvement allows for the expansion of bioconjugation possibilities since long aliphatic chains or aromatic reagents should not suffer from poor solubility. This solubility will be upheld even after conjugation since the reagent contains a permanent electrostatic charge. The high solubility of the reagents will help to avoid biomolecule precipitation; this is useful for applications in which a high surface density of functionality is required. Additionally, increasing solubility of reagents allows for the unification of the labeling procedures since reagents which may have previously suffered from poor solubility can exhibit increased solubility.

The activity of a bioconjugate post modification was demonstrated through the modification of HRP. For this experiment, biotinylation of the HRP showed little effect on the enzymes ability to catalyze the reaction of OPD to a brightly colored complex. This was compared to native HRP and offered similar results, offers a positive result showing the addition of the quaternary salt does not result in drastic alteration of the native function.

Typically, both **1a** and **1b** reagents have been used for the formation of quaternary ammonium salts. This was forgone in the favor of a unified platform for bioconjugation. This allows for the production of a single, cheaper, more readily accessed isatoic anhydride derivative, **1a**. The ability to successfully modify substrates that can react with **1b** to react with **1a** is simplistic and readily purified. Derivatives previously accessed through **1b** can be quickly moved to **1a**, making it a more ideal reagent.

CHAPTER 7: EXPERIMENTAL

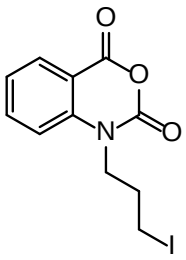
Instrumentation.

NMR data was obtained on a 500 MHz JEOL EC500 NMR

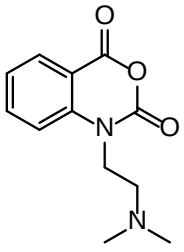
ESI-MS data was obtained using a PerSeptive Biosystems: Mariner Biospectrometry Workstation model using Mariner Instrument Control Panel v. 4.0.0.0 software and Data Explorer v. 4.0.0.1 software for data analysis

Molecular Devices Spectramax M5 plate reader

Chemicals. All chemicals were purchased commercially and used without further purification unless otherwise stated.

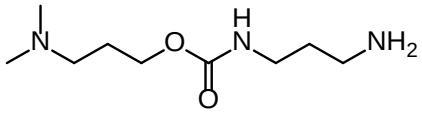


1a. This reagent was prepared as previously detailed.[27]

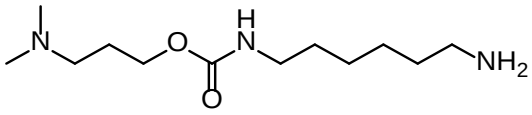


1b. This reagent was prepared as previously detailed.[27]

General Procedure 1. To a round bottom flask flushed with N_2 was added 3-dimethylamino-1-propanol (5.0g, 48.5 mmol). To this was added 0.8 eq N,N'-carbonyldiimidazole (6.3g, 38.8 mmol) in dry ACN (40 mL). This solution was stirred for 1 hour then was taken into a syringe. The solution was slowly added to 10 eq. of a diamine (388 mmol) vigorously stirring under N_2. This mixture is stirred for 4 hours then concentrated under reduced pressure. The resulting oil is purified by bulb to bulb distillation for 4 hours are 120 °C, removing excess diamine and imidazole, leaving the product in the round bottom. The purified liquid is allowed to cool prior to removal from the distillation as to avoid charring of the sample.



2b. General procedure 1 was followed for the preparation of this reagent. Final product was collected as a yellow oil. (5.52g, 70%) $C_9H_{21}N_3O_2$ (203.29) ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.03 (t, 1H), 3.90 (t, 2H), 2.97 (q, 2H), 2.48 (t, 2H), 2.19 (t, 2H), 2.07 (s, 6H), 1.61 (p, 2H), 1.40 (p, 2H) ppm, ^{13}C NMR (500 MHz, DMSO-d_6): δ 156.9, 62.6, 56.2, 45.7, 39.9, 38.5, 33.9 ppm. MS (ESI): Calc. 203.16; Found: 203.71 (M^++H) m/z.



2c. General procedure 1 was followed for the preparation of this reagent. Final product was collected as an off-white solid. (6.66g, 70%) $C_{12}H_{27}N_3O_2$ (245.37) ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.02 (t, 1H), 3.89 (t, 2H), 2.90 (q, 2H), 2.47 (t, 2H), 2.46 (t, 2H), 2.19 (t, 2H), 2.07 (s, 6H), 1.61 (p, 2H), 1.33 (m, 2H),1.28 (m, 2H), 1.20 (m, 4H) ppm, ^{13}C NMR (500 MHz, DMSO-d_6): δ 156.2, 61.9, 55.6, 45.1, 41.5, 40.0, 33.3, 29.4, 26.9, 26.1, 26.0 ppm. MS (ESI): Calc. 245.21; Found: 246.09 (M^++H) m/z.

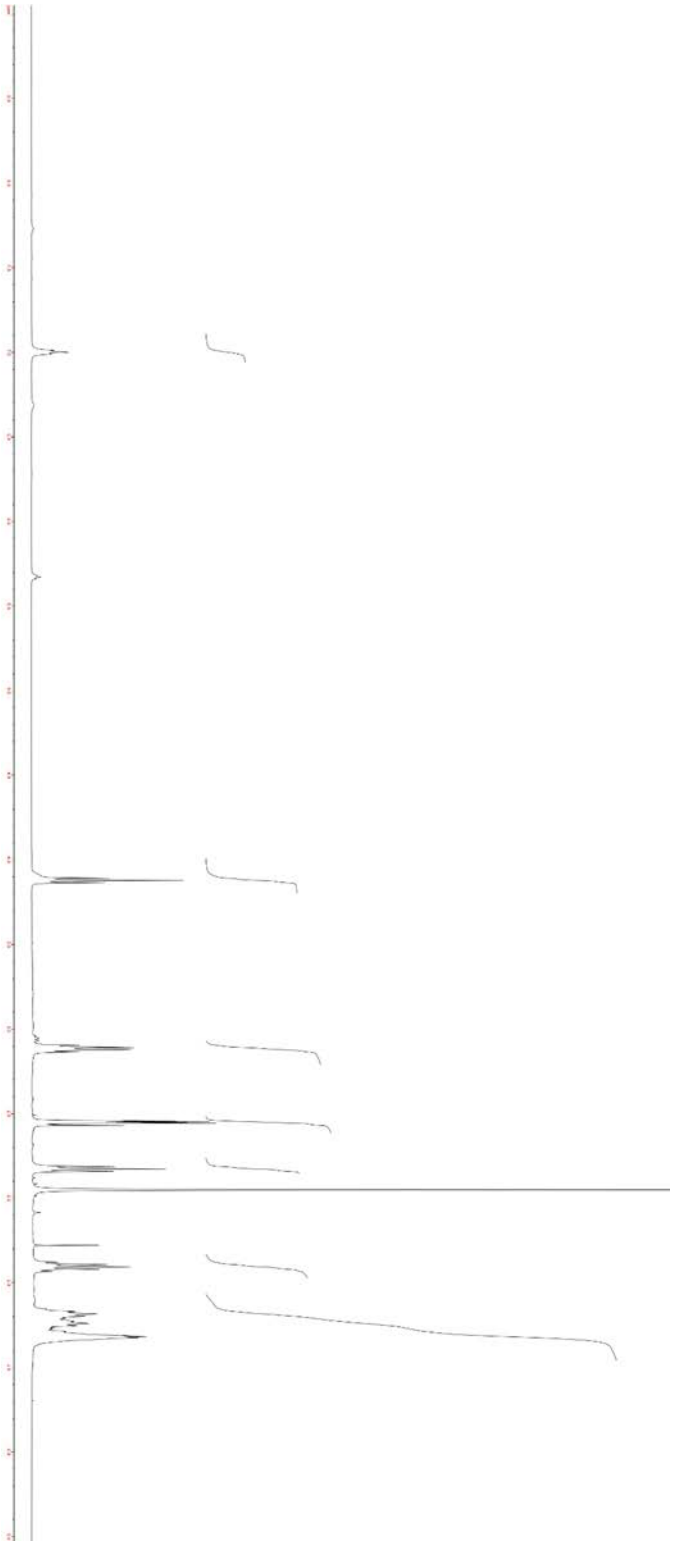
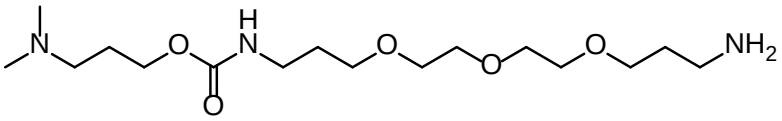
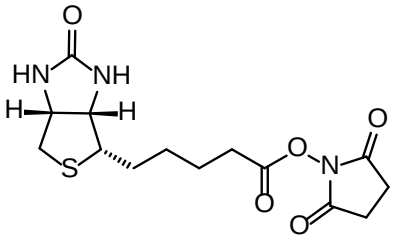


Figure 7-1: NMR spectra of **2c** in acetone



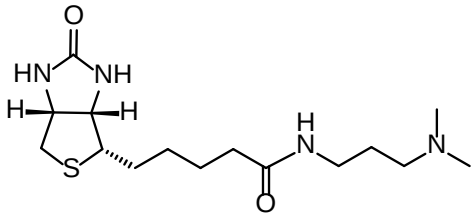
2d. General procedure 1 was followed with the following modifications for the preparation of this reagent. Reaction was scaled down to 5 mmol CDI starting material and bulb to bulb distillation was performed for 6 hrs to remove excess diamine. Final product was isolated as a yellow oil (0.87 g, 50%). $C_{16}H_{35}N_3O_5$ (349.47), ^{1}H NMR (500 MHz, DmMSO-d_6): δ 4.15 (t, 2H), 3.30 (m, 16H), 2.58 (t, 2H), 2.12 (t, 2H), 1.96 (s, 6H), 1.65 (p, 2H), 1.56 (p, 2H), 1.48 (p, 2H) ppm, MS (ESI): Calc. 349.26; Found: 350.07 (M^++H) m/z.



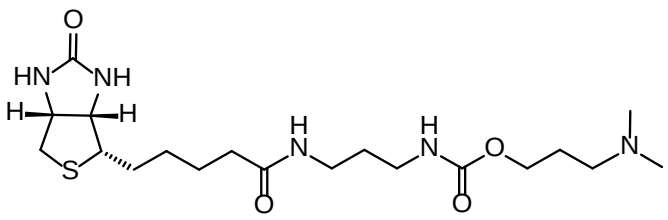
3. To a round bottom containing biotin (3 g, 12.3 mmol) stirring in dry DMF (100 mL) under N_2, was added 1.2 eq 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.8g, 14.7 mmol). To this solution was added 1.25 eq N-hydroxysuccinimide (1.8 g, 15.5 mmol). This solution was stirred overnight then poured into a 1000mL beaker containing ice (500 g) resulting in the precipitation of a white solid. Solid is collected through filtration over glass frit then washed with chilled methanol. Solid is collected and placed under vacuum overnight. Final product was collected as a white powder (3.35 g, 80%). $C_{14}H_{19}N_3O_5S$ (341.38), ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.41 (s, 1H), 6.35 (s, 1H), 4.27 (t, 1H), 4.11 (t,1H), 3.07 (q, 1H), 2.80 (t, 1H), 2.78 (s, 4H), 2.64 (t, 2H) 2.54 (s, 1H), 1.45 (m, 6H)

ppm, ^{13}C NMR (500 MHz, DMSO-d_6): δ 170.3, 168.9, 162.7, 60.9, 59.1, 55.2, 40.8, 29.9, 27.8, 27.5, 25.4, 24.3 ppm. MS (ESI): Calc. 341.10; Found: 341.34 (M^++H) m/z.

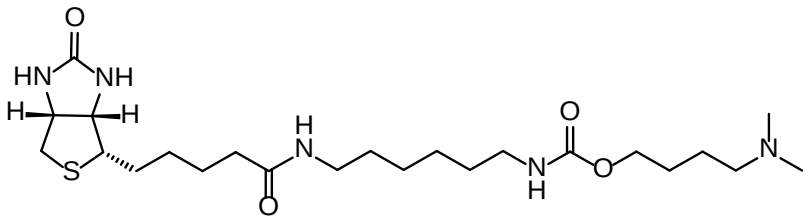
General Procedure 2. To a round bottom flask under N_2 was added 3 eq. α-N,N-dimethyl-Ω-diamine (**2a-2d**) in 20 mL dry DMF (3.6 mmol). To this was added a suspension of **3** in 20 mL dry DMF (0.4g, 1.2 mmol). The suspension was stirred vigorously at 30° C until the cloudy solution went clear, turning pale yellow. The reaction was stirred at RT for 8 hrs then concentrated under reduced vacuum. Product was purified via column chromatography over flash silica 70-90μm, 60 Å pore size, using 2 CV 95:5 DCM/MeOH then 10 CV 5:95 DCM/MeOH.



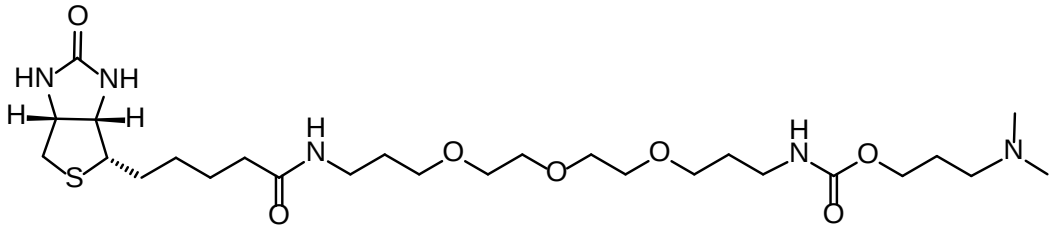
3a. General procedure 3 was followed for the preparation of this reagent. Product was isolated as a pale yellow solid (323 mg, 82%). $C_{15}H_{28}N_4O_2S$ (328.48) ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.75 (t,1H), 6.42 (s, 1H), 6.35 (s, 1H), 4.30 (t, 1H), 4.12 (m, 1H), 3.09 (m, 1H), 3.03 (d, 2H), 2.82 (dd, 1H), 2.87 (t, 4H), 2.18 (t, 2H), 2.11 (s, 6H), 2.04 (t, 2H), 1.45 (m, 8H) ppm, ^{13}C NMR (500 MHz, DMSO-d_6): δ 173.2, 172.1, 163.0 61.3, 59.5, 56.9, 55.8, 45.4, 37.0, 35.5, 28.5, 28.4, 27.5, 25.5 ppm. MS (ESI): Calc. 328.19; Found: 330.05 (M^++H) m/z.



3b. General procedure 3 was followed for the preparation of this reagent. Final product was isolated as a pale yellow solid (309 mg, 60%). $C_{19}H_{35}N_5O_4S$ (429.58), ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.743 (t, 1H), 7.02 (t, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.30 (t, 1H) 4.12 (t, 1H) 3.94 (t, 2H), 3.09 (m, 1H) 3.02 (q, 2H), 2.95, (q, 2H), 2.82 (dd, 1H), 2.57 (d, 1H), 2.23 (t, 2H), 2.10 (s, 6H), 2.04 (t, 2H) 1.35 (m, 12H) ppm, ^{13}C NMR (500 MHz, DMSO-d_6): δ 172.2, 162.9, 156.5, 62.4, 61.3, 59.5, 55.9, 55.7, 45.4, 38.3, 36.3, 35.5, 29.8, 28.5, 28.3, 27.2, 25.6 ppm, MS (ESI): Calc. 429.24; Found: 429.96 (M^++H) m/z.



3c. General procedure 3 was followed for the preparation of this reagent. Product was isolated as a pale yellow solid (339 mg, 60%). $C_{22}H_{41}N_5O_4S$ (471.66) ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.71 (t, 1H), 7.03 (t, 1H), 6.42 (s, 1H), 6.35 (s, 1H), 4.30 (t, 2H), 4.12 (t, 1H), 3.93 (t, 2H). 3.09 (m, 1H), 3.00 (q, 2H), 2.93 (q, 2H), 2.82 (d, 1H), 2.57 (d, 1H), 2.22 (t, 2H), 2.10 (s, 6H), 2.04 (t, 2H), ^{13}C NMR (500 MHz, DMSO-d_6): δ 172.2, 163.2, 156.9, 62.4, 61.5, 59.6, 56.1, 55.9, 45.6, 38.8, 35.7, 29.9, 29.6, 28.7, 28.5. 27.4, 26.5, 26.4, 25.8 ppm, MS (ESI): Calc. 471.29; Found: 471.08 (M^++H) m/z.



3d. General procedure 3 was followed for the preparation of this reagent with the following modification, 2.2 mmol **2d** and 0.7 mmol **3**. Product was isolated as a yellow

oil. (200 mg, 50%) $C_{26}H_{49}N_5O_7S$ (575.77), ^{1}H NMR (500 Mz, MeOD): δ 4.99 (t, 1H) 4.299 (q, 1H), 4.10 (t, 2H), 3.51 (m, 6H), 3.19 (m, 8H) 2.92 (q,1H), 2.81, (s,6H), 2.70 (d, 1H), 2.19 (t, 2H), 1.75 (m, 8H), 1.42 (p, 2H), 1.30 (t, 4H), 1.16 (t, 2H), ^{13}C NMR (500 Mz, MeOD): δ 175.8,165.9, 158.5, 71.5, 71.2, 69.9, 69.7, 63.3, 62.6, 61.6, 58.3, 56.9, 56.5, 47.8, 43.8, 41.1, 39.1, 37.8, 36.8, 30.8, 30.4, 29.8, 26.3, 18.4, 9.3 ppm. MS (ESI): Calc. 575.34; Found: 575.84 (M^{+}+H, 100%) m/z.

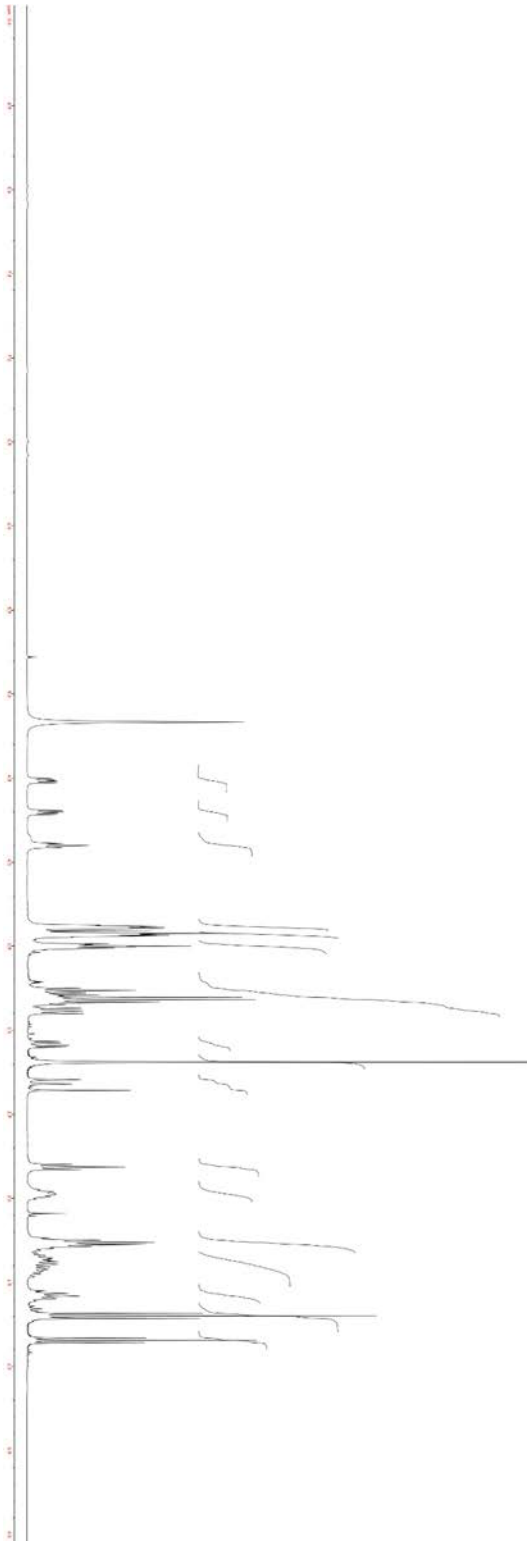
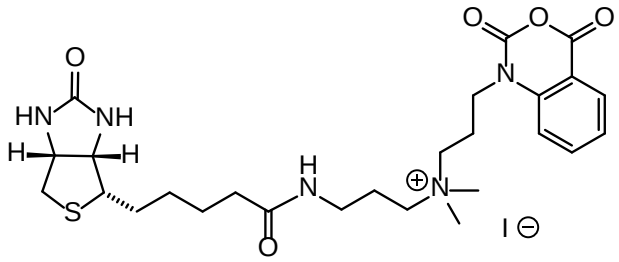
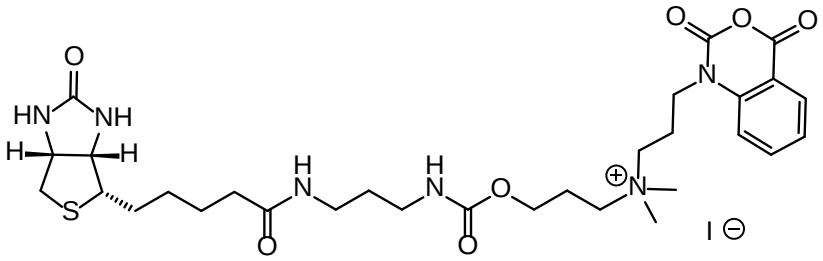


Figure 7-4: NMR spectra of **3d** in DMSO

General Procedure 4. To a 1.5 mL vial flushed with N_2 was added **1a** (100 mg, 0.3 mmol) in 750 µL dry acetone through a 0.2 µm filter. To this was added 0.9 eq of biotin derivative (**3a-3d**) (0.27 mmol) in 750 µL dry DMF. The vial is sealed and placed at 40° C for 24 hrs; the solution was transferred to a dry centrifuge vial. To this was added diethyl ether until the solution became turbid (~5 mL), the solution was mixed thoroughly until clear. To this additional ethyl ether was added quickly (~5mL), vortexed vigorously then centrifuged to collect precipitated solid. The solution was carefully decanted and the solid was placed in vacuo for 48 hours.



4a. General procedure 4 was followed for the preparation of this reagent. Product was isolated as a pale yellow solid. (115 mg, 65%) $C_{26}H_{38}IN_5O_5S$ (659.58) ^{1}H NMR (500 Mz, DMSO-d_6): δ 8.06 (d, 1H), 7.89 (t, 1H), 7.53 (d, 1H), 7.30 (t, 1H), 6.39 (s, 1H), 6.36 (s, 1H), 4.31 (t, 1H), 4.12 (m, 2H), 3.43 (m, 2H), 3.22 (m, 2H), 3.11 (m, 3H), 2.97 (s, 6H), 2.81 (d, 1H), 2.57 (t, 2H), 2.06 (m, 4H), 1.80 (m, 2H), 1.60 (m,2H), 1.50 (m, 3H), 1.30 (m, 2H) ppm, ^{13}C NMR (500 Mz, DMSO-d_6): δ 173.3, 162.7, 159.8, 137.6, 130.2, 124.2, 114.5, 62.9, 61.6, 61.4, 59.8, 55.4, 50.7, 42.8, 41.6, 40.2, 35.6, 35.2, 28.0, 25.2, 23.0, 20.7 ppm, Calc. 532.26; Found: 532.54 (M^+) m/z.



4b. General procedure 4 was followed for the preparation of this reagent. Product was isolated as a pale yellow solid. (143 mg, 70%) $C_{30}H_{45}IN_6O_7S$ (760.68), ^1H NMR (500 Mz, DMSO-d_6): δ 8.03 (d, 1H), 7.85 (t, 1H) 7.72 (t, 1H), 7.51 (d, 1H), 7.34 (d, 1H), 7.02 (t, 1H), 6.36 (s, 1H), 6.32 (s, 1H), 4.27 (t, 1H), 4.08 (t, 3H), 3.95 (t, 2H), 3.42 (m, 2H), 2.97 (m, 12H), 2.78 (d, 2H), 2.06 (m, 2H) 2.01 (t, 2H), 1.95 (m, 2H), 1.57 (m, 2H), 1.48 (m, 5H) 1.25 (m, 2H) ppm, ^{13}C NMR (500 Mz, DMSO-d_6): δ 172.3, 162.9, 159.2, 156.0, 148.4, 141.4, 137.5, 130.0, 124.1, 114.9, 112.1, 61.2, 61.1, 60.9, 60.7, 59.5, 55.6, 50.4, 41.6, 36.3, 35.3, 34.6, 29.8, 28.3, 25.6, 22.3, 20.5 ppm, Calc. 633.31; Found: 633.67 (M^+) m/z.

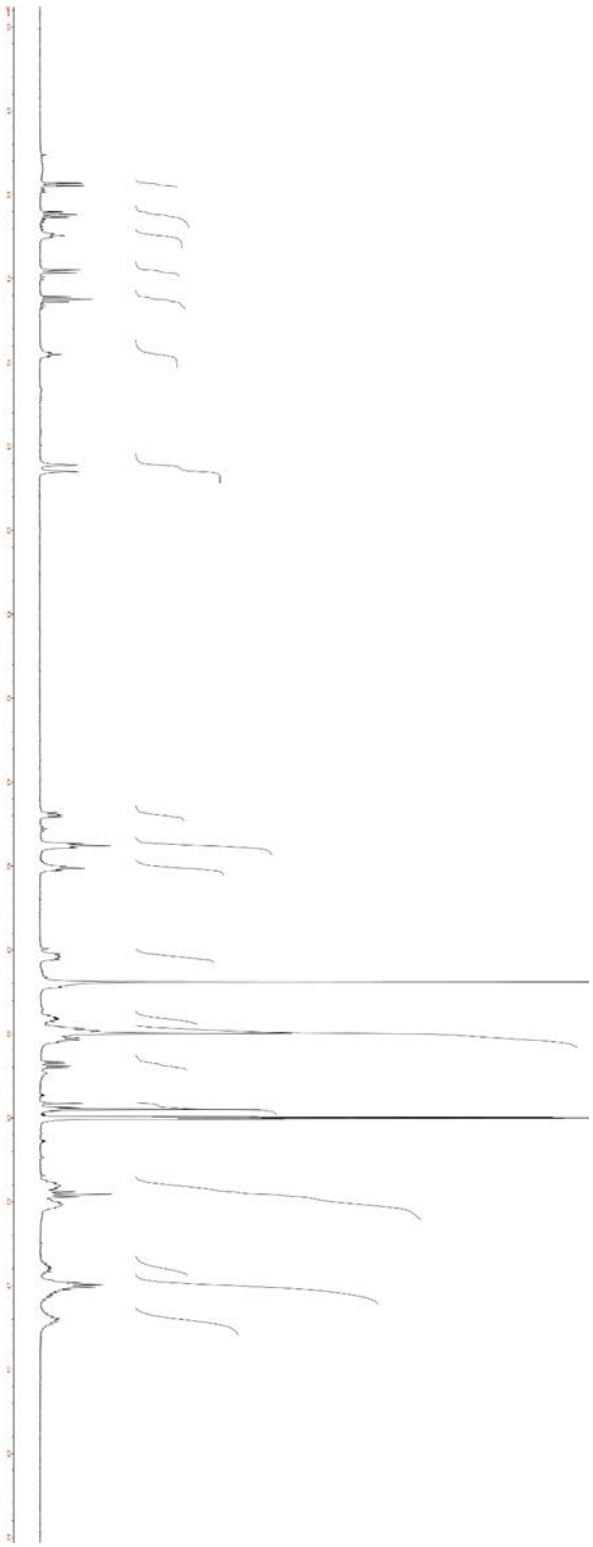
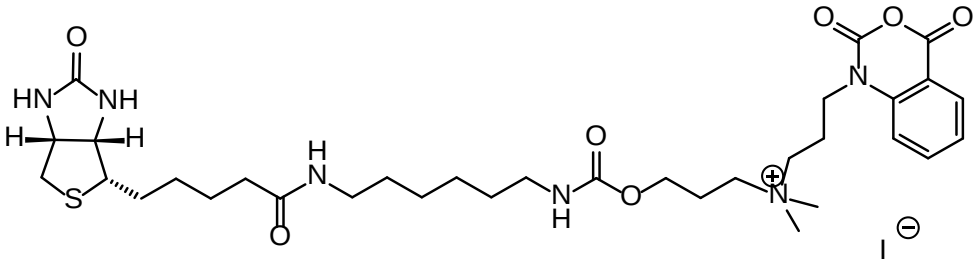
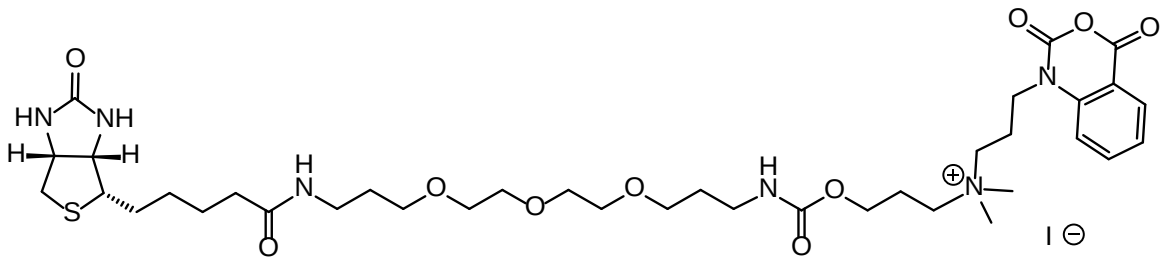


Figure 7-3: NMR spectra of **4b** in DMSO

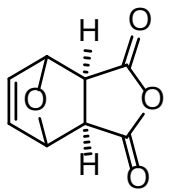


4c. General procedure 4 was followed for the preparation of this reagent. Product was isolated as a pale yellow solid. (140 mg, 65%) $C_{33}H_{51}IN_6O_7S$ (802.76) ^{1}H NMR (500 MHz, DMSO-d_6): 8.06 (d, 1H), 7.88 (t, 1H), 7.71 (t, 1H), 7.54 (d, 1H), 7.37 (t, 1H), 7.02 (t, 1H),640 (s, 1H), 6.35 (s, 1H),4.30 (t, 1H), 4.11 (t, 2H), 3.97 (t, 2H), 3.46 (m, 2H), 3.08 (m, 2H), 3.00 (s, 8H), 2.94 (q, 2H), 2.80 (t, 2H),2.09 (m, 2H), 2.03 (t, 2H), 1.98 (m, 2H), 1.60 (m, 1H), 1.49 (m, 3H), 1.25 (m, 12H) ppm, ^{13}C NMR (500 Mz, DMSO-d_6): 171.9, 162.9, 159.1, 156.0, 148.0 141.2, 137.4, 129.9, 123.9, 114.8, 112.0, 61.2, 61.03, 61.0, 60.6, 59.3, 55.6, 50.3, 41.5, 38.4, 35.4, 34.6, 29.5, 29.2, 28.4, 28.2, 26.3, 26.0, 25.5, 22.5, 20.4 ppm. Calc. 675.35; Found: 675.67 (M^+) m/z.

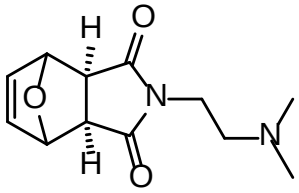


4d. General procedure 4 was followed for the preparation of this reagent. Product was isolated as a deep yellow oil. (122 mg, 50%) $C_{37}H_{59}IN_6O_{10}S$ (906.87), ^{1}H NMR (500 Mz, DMSO-d_6): δ 8.07 (d,1H), 7.88 (t, 1H), 7.73 (t,1H), 7.54 (t, 1H), 7.37 (t, 1H), 7.08 (t, 1H), 6.40 (s, 1H), 6.35 (s, 1H), 4.30 (t, 1H), 4.12 (t,3H), 3.98 (t, 2H), 3.51 (m, 5H), 3.47 (m, 6H), 3.38 (m, 5H), 3.06 (q, 4H), 3.00 (s, 6H), 2.55 (m, 4H), 2.04 (m, 6H), 1.61 (m,

5H), 1.61 (m, 5H), 1.48 (m, 3H), 1.29 (m, 2H) ppm, Calc. 779.40; Found: 779.68 (M^+) m/z.



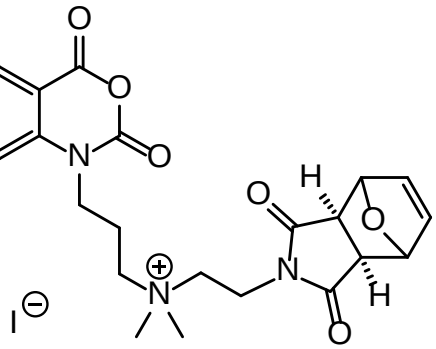
5'.[24] In a round bottom flushed with N_2 was added maleic anhydride (5 g, 51 mmol). To this was added furan (5.02 g, 76 mmol) as both the solvent and reactant. The reaction was performed at RT with no stirring, resulting in the production of a white powered product which was purified by removal of solvent under reduced pressure (6.4 g, 75 %). $C_8H_6O_4$ (166.13), ^{1}H NMR (500 Mz, Acetone-d_6): δ 6.65 (s, 2H), 5.38 (s, 2H), 3.38 (s, 2H) ppm, ^{13}C NMR (500 Mz, Acetone-d_6): 206.3, 137.8, 83.1, 50.0 ppm. MS (ESI): Calc. 166.03; Found: 166.52 (M^++H) m/z.



5a'.[28] To a round bottom was added **5'** (3.0 g, 18 mmol). To this was added MeOH (20mL) and N,N-dimethylethylenediamine (2.4 g, 27 mmol). The reaction is held at reflux overnight then allowed to cool. The product is concentrated under reduced pressure, dissolved into ethyl ether and washed 3x with DI H_2O. The solution is dried over sodium sulfate then concentrated under reduced pressure. The final product is isolated as a white solid (3.0 g, 70%).$C_{12}H_{16}N_2O_3$ (236.27), ^{1}H NMR (500 MHz, Acetone-d_6): δ 6.57 (t, 2H), 5.12 (t, 2H), 3.49 (t, 2H), 2.90 (s, 2H), 2.35 (t, 2H), 2.17 (s,

6H) ppm, ^{13}C NMR (500 MHz, Acetone-d_6): δ 206.1, 137.3, 81.7, 56.9, 48.2, 45.6, 37.2 ppm. MS (ESI): Calc. 236.12; Found 236.66 (M^++H) m/z.

General Procedure 5. To a 1.5 mL vial flushed with N_2 was added **1a** (100 mg, 0.3 mmol) in 750 µL dry acetone through a 0.2 µm filter. To this was added 0.9 eq of N,N-dimethyl-derivative of selected functionality (0.27 mmol) in 750 µL dry acetone. The vial is sealed and placed at 40° C for 24 hrs; reaction is placed at RT and allowed to precipitate the quaternary reagent. Vial is washed 5x with acetone then residual solvent is removed under reduced pressure. Headspace of vial is purged with argon prior to storage.



6a'. General procedure 5 was followed for the synthesis of the reagent. Product was isolated as a pale yellow solid. (122 mg, 80 %). $C_{23}H_{26}IN_3O_6$ (567.38), ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.06 (d, 1H), 7.89 (t, 1H). 7.56 (d, 1H), 7.38 (t, 1H), 6.57 (s, 2H), 5.14 (s, 2H), 4.13 (t, 2H), 3.79 (t, 2H), 3.54 (p, 2H), 3.36 (t, 2H), 3.07 (s, 6H). 2.98 (s, 2H), 2.11 (p,2H) ppm, ^{13}C NMR (500 MHz, DMSO-d_6): δ 176.5, 137.6, 136.6, 130.0, 124.1, 118.1, 110.1, 80.7, 60.9, 59.0, 50.6, 47.7, 41.6, 31.9, 20.6 ppm, MS (ESI): Calc. 440.47, Found 439.07 (M^+) m/z.

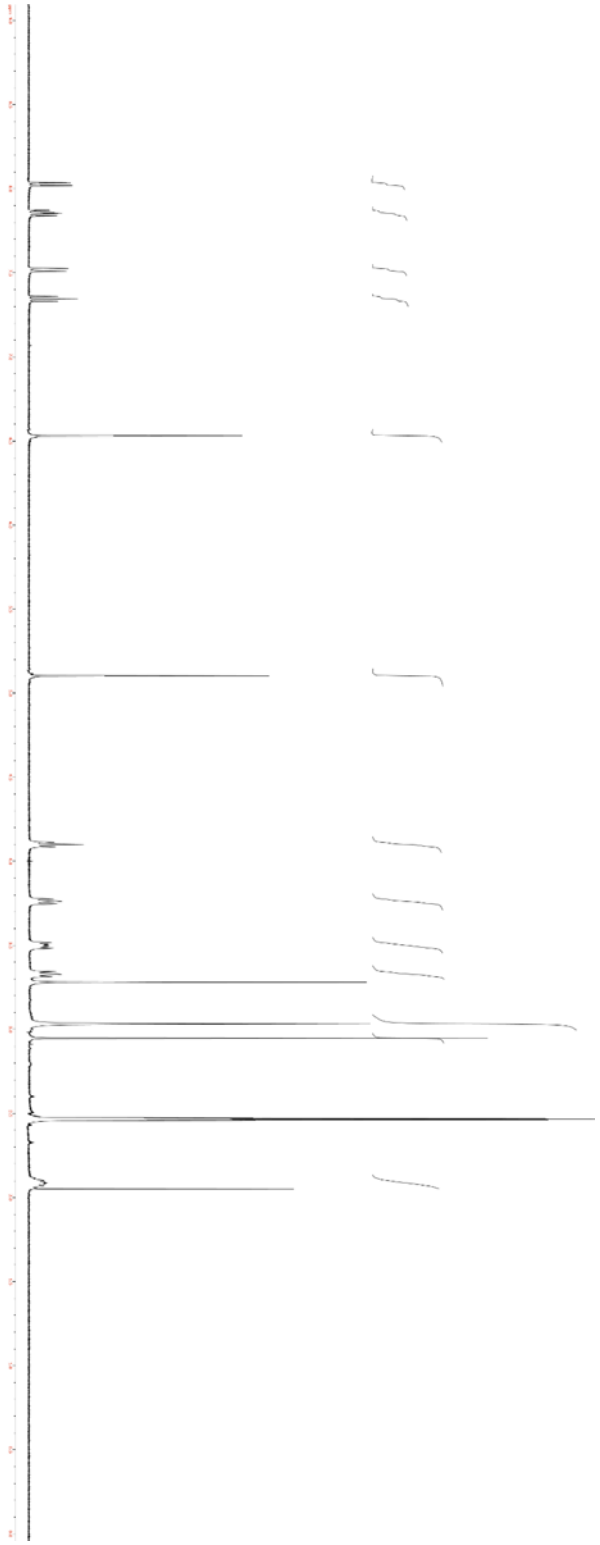
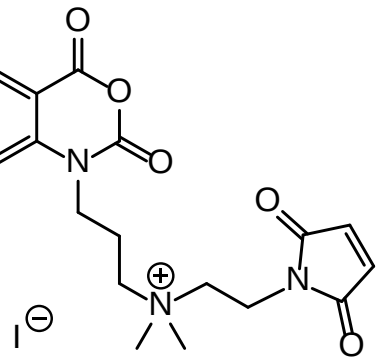


Figure 7-4: NMR spectra of **6a'** in DMSO



6a. 20 mg of **6a'** (0.035 mmol) dissolved into DMSO-d_6 and heated a 120 °C for 2 hours under reduced pressure. Solution is concentrated by removal of DMSO by cryogenic pumping, resulting in yellow oil. The oil is diluted into cold water then lyophilized to give a flocculent yellow solid. (16 mg, 95%) ($C_{19}H_{22}IN_3O_5$ (499.31), ^{1}H NMR (500 MHz, DMSO-d_6): 8.06 (d, 1H), 7.89 (t, 1H), 7.55 (d, 1H), 7.38 (t, 1H), 7.10 (s, 2H), 4.14 (t, 2H), 3.83 (t, 2H), 3.55 (m, 2H), 3.43 (t, 2H), 3.07 (s, 6H), 2.12 (d, 2H). MS (ESI): Calc. 372.39, Found 372.68 (M^+) m/z.

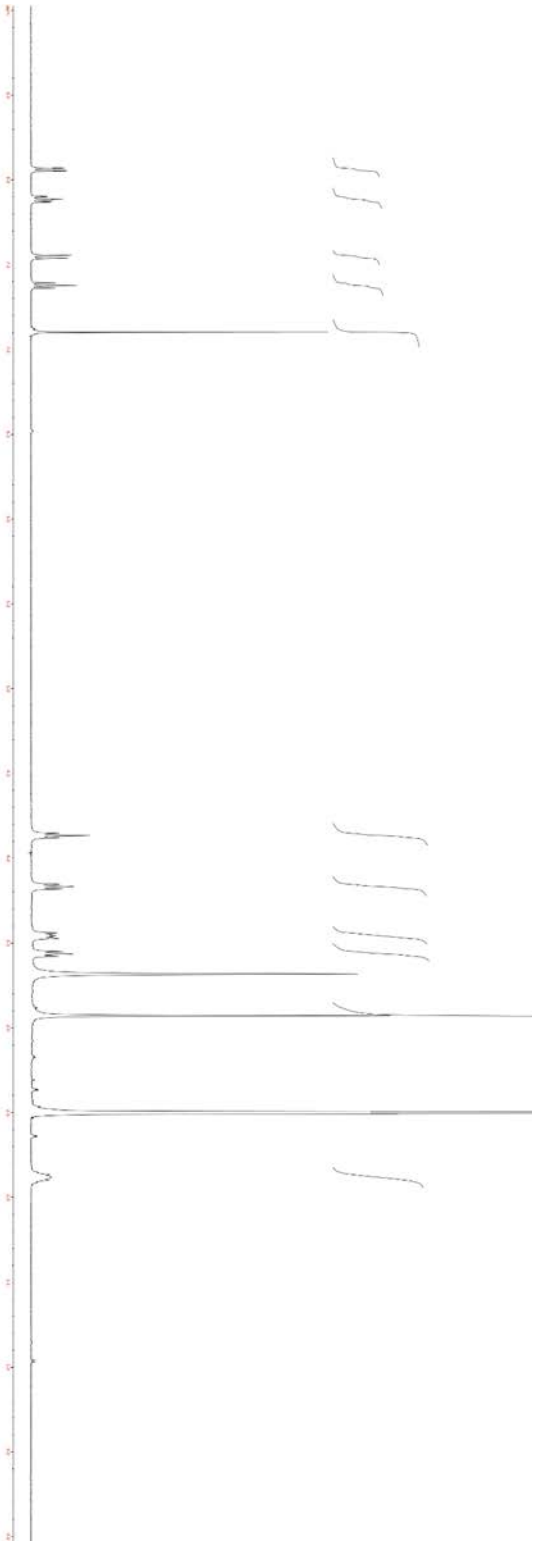
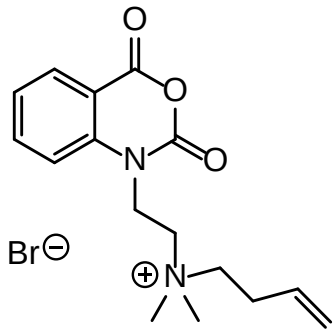


Figure 7-5: NMR spectra of **6a** in DMSO



7a. General procedure 5 was followed with the following modification, **1a** was substituted for **1b**. Product was collected as an off-white solid (84 mg, 85%). $C_{16}H_{21}BrN_2O_3$ (369.26), ^{1}H NMR (500 MHz, DMSO-d_6):8.06 (d, 1H), 7.90 (t, 1H), 7.59 (d, 1H), 7.39 (t, 1H), 5.78 (m, 1H), 5.25 (d, 1H), 5.17 (d, 1H), 4.5 (t, 2H), 3.64 (t, 2H), 3.55 (t, 2H), 3.22 (s, 6H), 2.55 (p,2H), ^{13}C NMR (500 MHz, DMSO-d_6): 159.0, 148.0, 141.1, 137.8, 133.1, 129.9, 124.4, 118.8, 114.9, 112.3, 62.6, 58.6, 50.8, 38.0, 26.7 ppm, MS (ESI): Calc. 289.35, Found 288.47 (M^+) m/z.

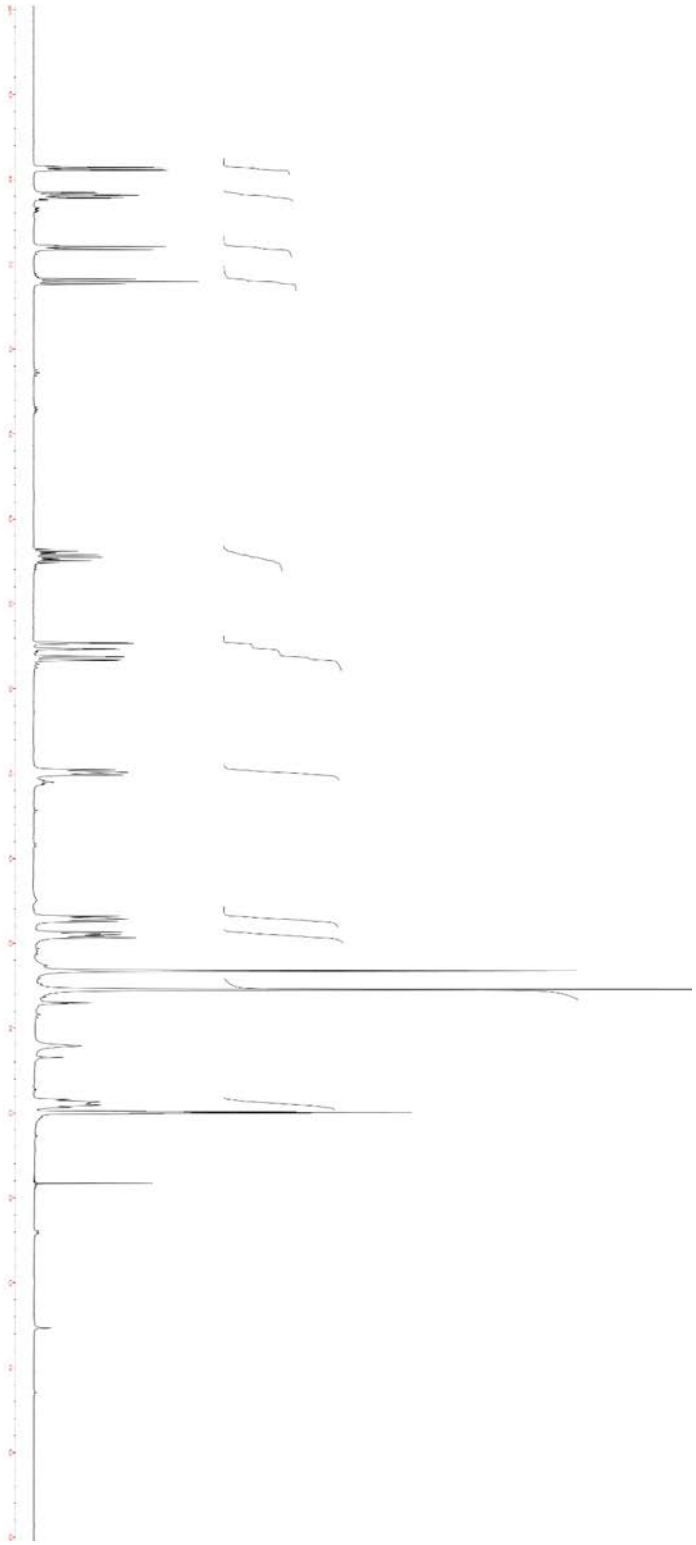
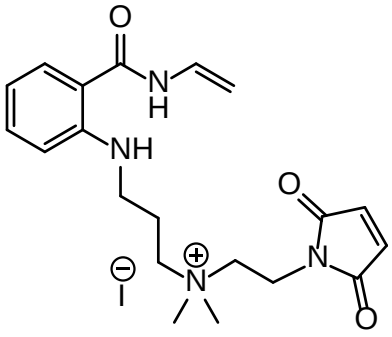
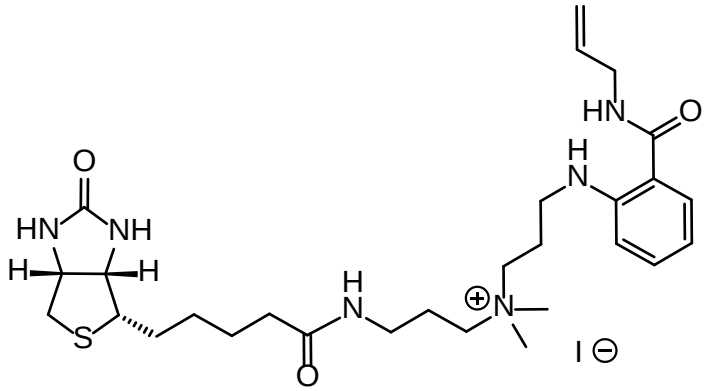


Figure 7-6: NMR spectra of **7a** in DMSO



6a-1. 20 mg **6a'** (0.035 mmol) combined with 10 eqs allylamine (0.35 mmol) in DMSO-d_6. Reaction was monitored via NMR, once completed, excess allylamine is removed under reduced pressure. Sample is then heated to 120 °C for 2 hours under reduced pressure. (17 mg, 95%) $C_{20}H_{27}IN_4O_3$ (498.36). ^{1}H NMR (500 MHZ, DMSO-d_6): δ 8.48 (t, 1H), 7.85 (t, 1H), 7.56 (d, 1H), 7.26 (t, 1H), 7.07 (s, 2H), 5.54 (m, 1H), 5.12 (q, 1H), 5.04 (q, 1H), 3.81 (t, 4H), 3.48 (q, 4H), 3.16 (d, 2H), 3.07 (s, 6H), 1.97 (m, 2H) ppm, ^{13}C NMR (500 MHZ, DMSO-d_6): δ 176.6, 170.8, 169.2. 149.2, 136.7, 135.9, 132.8, 128.6, 115.4, 114.9, 111.3, 80.8, 61.9, 59.7, 50.7, 47.7, 41.4, 30.9, 22.5 ppm. MS (ESI): Calc. 385.48, Found 385.75 (M^+) m/z.



4a-1. 20 mg **4a** (0.030 mmol) combined with 10 eqs allylamine (0.3 mmol) in DMSO at RT for 30 min. Excess allylamine is removed under reduced pressure. (19 mg, 95%) $C_{28}H_{45}IN_6O_3S$ (672.66). MS (ESI): Calc. 545.33, Found 544.45 (M^+) m/z.

General Procedure 6. 50 mg of quaternary reagent is weighed into a 50 mL centrifuge tube. To this was added 5.0 mL dry acetonitrile. The suspension is sonicated to maximize surface area of the salt. To this is added 45 mL cold deionized water, vortexed vigorously until the solid completely dissolved. The solution is transferred to a beaker containing 10 g of ion exchange resin for 10 min. The solution is passed through a course glass frit then flash frozen in liquid nitrogen for lyophilization.

General Procedure 7. 100 µL of 0.15 mM BSA in 25 mM bicarbonate buffer added to Eppendorf tube to this was added appropriate volume of 15 mM reagent freshly dissolved in DI H_2O. Solution is vortexed vigorously then allowed to react for 1 hr at RT. Reaction is quenched with the addition of 5µL NH_4OH. Reaction mixture is purified trough gel permeation chromatography with a Princeton Separations CS-800 Pro Spin GPC column. DOL is determined on a Molecular devices Spectramax M5 plate reader by taking 10 µL of purified sample and diluting with 490 µL 25mM bicarbonate buffer and transferring to a 500 µL quartz cuvette to read absorbance (250-450 nm).

General Procedure 8. HABA/Avidin reagent purchased from Sigma Aldrich, powder was resuspended according to protocol. 120 µL of solution was transferred to Corning 96-well half area plate. The absorbance at 500 was taken until stabilized. To this was added 14.1 µL of 3 µM BSA solution and the absorbance at 500 nm was recorded until the reading became stable. DOL was calculated following the supplied procedure for quantification.

General Procedure 9. Stock solutions of 6 nM HRP in 25 mM bicarbonate buffer (pH 8.40), 120 mM H_2O_2 in DI H_2O, and 125.6 mM O-phenylenediamine (OPD) in PBS (pH 7.0) are prepared fresh. Calibration curve is prepared using 0-4.5 µL HRP stock in 0.5 µL steps, adding directly to the well of a Corning 96-well half area plate. PBS (pH 7.0) is added to each well to give a final volume of 73 µL. To this was added 75 µL OPD solution, followed by 2 µL H_2O_2 solution. The wells are mixed then placed in the dark. Measurement of the HRP assay was performed on a Molecular devices Spectramax M5 plate reader reading from 350-550 nm after the reaction had progressed 30 minutes. HRP labeled with **4a** through use of general procedure 7, using HRP in place of BSA. Cleaned biotinylated HRP is then diluted to 6nM. A fresh batch of HRP stock was prepared at 6 nM. The 2 µL of the solutions were tested to determine the amount of HRP delivered in triplicate.

General Procedure 10. Roughly 5 mg of quaternary reagent weighed out and dissolved into 1000 µL DMSO-D_6. Three aliquots of 300 µL were taken. To this was added 10 equivalences of n-butylamine. The reaction was vortexed vigorously and allowed to sit for 1 hr. To this was added 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt in D_2O (33 mM) as an internal standard and 300 µL DMSO-D_6. 500 µL were placed in an NMR tube for analysis. Absorbance data was obtained using a Molecular Devices SpectraMAx M5 plate reader (250-450 nm), using 25 µL of the reaction mixture diluted into 1975 µL bicarbonate buffer (pH 8.4) in a 2 mL quartz cuvette. ε values were determined for 330 nm and NMR data was used to correct for water content of the hygroscopic salts.

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