Development of Coupling Partners for Bioorthogonal Reactions with N-Hydroxylamines

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Bioorthogonal chemistry enables biological chemists to study the dynamics of complex biological systems. Bioorthogonal chemistry allows the selective reaction of an orthogonal tag, which is incorporated on the molecule of interest via biosynthetic or biochemical pathway, and its reactive partner without interfering with native biochemical processes. A number of reactions have been developed in the past two decades. However, there is still a need to develop new chemistries to widen the scope and power of these chemistries to elucidate the biological systems. Therefore, we developed a metal-free bioorthogonal reaction between $N$-hydroxylamine (orthogonal tag) and a bifunctional probe with aldehyde and alkene (reactive partner) to form a stable isoxazolidine cycloadduct. We showed that glucose could be modified with $N$-hydroxylamine in four steps. In addition, we designed and synthesized probes that condense then cyclize at 37 °C within 24 hours to yield the cycloadduct, thus making this reaction a viable chemistry to use in biological systems.
Acknowledgments

Graduate school has been an amazing experience. I will cherish all the people I’ve met during my journey. Thank you for the friendship, encouragement, guidance, and support. I was only here for a year and a half but I can truly say that I am a much better person and scientist as I was when I started.

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Also, I would like to thank everybody in the Nitz group and the people I met in the Chemistry department. My initial plan was just to get my MSc degree as fast as I can and get out there as soon as I can. But they made me feel at home from the moment I started. I can’t believe how lucky I am to have met these amazing people. They encouraged me to do my best in spite all the stress. They gave me unlimited advise on how to succeed in grad school. They taught me lab techniques that a naïve first year would not know. They let me be silly and goofy all time. They were the reason why I looked forward to coming in to work everyday.

Lastly, I would like to thank my family for their never-ending love and support. To my parents, Rene and Aliw Santos, thank for always being there for me, for always telling me everything will be okay, for setting good examples for me and my siblings, and for everything!
# Table of Contents

ACKNOWLEDGMENTS...................................................................................................................... III  

TABLE OF CONTENTS................................................................................................................... IV  

LIST OF TABLES ............................................................................................................................ VI  

LIST OF FIGURES ........................................................................................................................... VII  

LIST OF SCHEMES ......................................................................................................................... VIII  

LIST OF EQUATIONS ..................................................................................................................... IX  

LIST OF GRAPHS ........................................................................................................................... X  

CHAPTER 1 ...................................................................................................................................... 1  

1 BIOORTHOGONAL CHEMISTRY .................................................................................................. 1  

1.1 BACKGROUND .......................................................................................................................... 1  

1.2 EXAMPLES OF BIOORTHOGONAL REACTIONS ................................................................... 2  

1.2.1 Azide-alkyne cycloaddition ................................................................................................. 2  

1.2.2 Aldehyde/ketone condensation with heteroatom-bound amines ........................................ 5  

1.2.3 Tetrazine-alkene ligation .................................................................................................... 6  

1.2.4 Nitrone-strained cyclooctyne cycloaddition ......................................................................... 7  

1.3 SCOPE OF THESIS .................................................................................................................. 9  

CHAPTER 2 ...................................................................................................................................... 11  

2 PROOF OF CONCEPT ................................................................................................................. 11  

2.1 INTRODUCTION ....................................................................................................................... 11  

2.1.1 Glucose modified with N-hydroxylamine ......................................................................... 11  

2.1.2 First generation probe ....................................................................................................... 12  

2.2 RESULTS AND DISCUSSION ................................................................................................. 13  

2.2.1 Glucose modified with N-hydroxylamine ......................................................................... 13  

2.2.2 Reaction of glucose modified with N-hydroxylamine with aldehydes then maleimides .... 13  

2.2.3 Reaction of probe 6 with glucose modified with N-hydroxylamine ............................... 15  

2.2.4 Reaction of probe 6 with N-methylhydroxylamine ........................................................... 16  

2.2.5 Reaction of probe 6 with N-phenylhydroxylamine ............................................................ 18  

2.3 SUMMARY ............................................................................................................................... 19
2.4 EXPERIMENTAL ................................................................................................................................. 20

2.4.1 Synthesis of glucose modified with N-hydroxylamine ............................................................... 20

2.4.2 Reaction of glucose modified with N-hydroxylamine with aldehydes then maleimides .......... 22

2.4.3 Synthesis of first-generation intramolecular probe ..................................................................... 23

CHAPTER 3 ............................................................................................................................................... 26

3 DESIGN AND SYNTHESIS OF SECOND GENERATION PROBES ............................................. 26

3.1 INTRODUCTION ................................................................................................................................. 26

3.1.1 Design of the second-generation probes .................................................................................... 26

3.2 RESULTS AND DISCUSSION ............................................................................................................ 28

3.3 SUMMARY ......................................................................................................................................... 32

3.4 EXPERIMENTAL ............................................................................................................................... 32

3.4.1 Synthesis of second-generation probes ....................................................................................... 32

3.4.2 Procedure for monitoring the reactivity of second generation probes using $^1H$ NMR .......... 37

CHAPTER 4 ............................................................................................................................................... 39

4 SUMMARY AND FUTURE DIRECTIONS ......................................................................................... 39

4.1 SUMMARY ......................................................................................................................................... 39

4.2 FUTURE DIRECTIONS ....................................................................................................................... 41

REFERENCES ............................................................................................................................................ 43

APPENDIX I ............................................................................................................................................. 47
List of Tables

**TABLE 1.** APPROXIMATE SECOND-ORDER RATE CONSTANT OF STRAINED-CYCLOOCTYNE DERIVATIVES WHEN REACTED WITH AZIDE.............................................................................................................4

**TABLE 2.** SECOND-ORDER RATE CONSTANTS OF ENDOCYCLIC AND ACYCLIC NITRONES AND AZIDE WITH DIFFERENT STRAINED CYCLOOCTYNE .............................................................................................................8

**TABLE 3.** SUMMARY OF THE YIELDS WHEN MODIFIED GLUCOSE 4 WAS REACTED WITH THE CORRESPONDING ALDEHYDES FOLLOWED BY N-METHYLMALEIMIDE TO FORM 5A-E .............................................................................................................14
List of Figures

**Figure 1.** General scheme of bioorthogonal reaction .......................................................................................... 1

**Figure 2.** Aldehydes that were reacted with glucose modified with N-hydroxylamine 4 ......................... 12

**Figure 3.** $^1$H NMR spectra of the two products isolated from the reaction of probe 6 with glucose modified with N-hydroxylamine 4 .......................................................................................... 16

**Figure 4.** $^1$H NMR spectra measuring the reaction of probe 6 with N-methylhydroxylamine ............... 17

**Figure 5.** $^1$H NMR spectra measuring the reaction of probe 6 and N-phenylhydroxylamine ............... 19

**Figure 6.** Isoxazolidine construct. .................................................................................................................. 27

**Figure 7.** $^1$H NMR spectra of the reaction of probe 15 with N-methylhydroxylamine at time 0, 3, 6, and 24 hours. ........................................................................................................ 30

**Figure 8.** Third-generation probes ............................................................................................................... 41
List of Schemes

**Scheme 1.** Condensation of aldehyde/ketone with alkoxyamines or hydrazines/hydrazides ...............5

**Scheme 2.** Reaction of an aldehyde with a tryptamine via Pictet-Spengler reaction .................................6

**Scheme 3.** General reaction scheme of tetrazine-alkene ligation ...............................................................6

**Scheme 4.** Protein modification via strain-promoted alkyne-nitrone cycloaddition ........................................9

**Scheme 5.** Proposed general reaction of N-hydroxylamine and a probe with both aldehyde and alkene moieties. .............................................................................................................................................10

**Scheme 6.** Synthetic procedure to install N-hydroxylamine to glucose at the C-2 position .........................12

**Scheme 7.** Synthesis of the first-generation intramolecular probe 6. .........................................................13

**Scheme 8.** General reaction of modified glucose 4 with an aldehyde then with N-methylmaleimide to yield 5a-e ..................................................................................................................................................14

**Scheme 9.** Reaction of probe 6 with N-hydroxylamine modified glucose ...................................................15

**Scheme 10.** Reaction of probe 6 with N-methylhydroxylamine .................................................................16

**Scheme 11.** Reaction of probe 6 with N-phenylhydroxylamine .................................................................18

**Scheme 12.** Proposed mechanism of second-generation probe that forms a bicyclo[3.2.1]octane cycloadduct (bridged) ..................................................................................................................................27

**Scheme 13.** Proposed mechanism of second-generation probe that forms a bicyclo[3.3.0]octane system (fused) ..................................................................................................................................................28

**Scheme 14.** Reaction of probes 13-16 with N-methylhydroxylamine ........................................................29

**Scheme 15.** General synthetic scheme of dihydroxyacetamide .....................................................................34

**Scheme 16.** Schematic of the synthesis of sialic acid modified with N-hydroxylamine ..............................42
List of Equations

**Equation 1.** Calculation of percentage conversion of cycloadducts of second-generation probes with N-methylhydroxylamine ................................................................. 37
List of Graphs

GRAPH 1. THE PERCENTAGE CONVERSION OF THE SECOND-GENERATION PROBES ..................................................31
Chapter 1

1 Bioorthogonal Chemistry

1.1 Background

Biological systems are complex. There are numerous events happening simultaneously. Thus, it is very challenging to elucidate combination of events that leads to an observed phenotype. There are many components present, such as, proteins, DNAs, RNAs, glycans, lipids, and small molecule metabolites. They all play significant roles in cellular processes. Each of these components exhibits its own sets of chemical characteristics such as polarity, nucleophilicity, electrophilicity, etc. Understanding the localization and mechanistic processes of multiple metabolites is a painstakingly slow process with standard biochemical approaches.

Figure 1. General scheme of bioorthogonal reaction. The target molecule (green box) is selectively modified with an orthogonal tag (red circle) to react selectively with its reactive partner (blue half circle).

The discovery and further development of green fluorescent protein (GFP) and additional fluorescent proteins was a milestone in providing tools to understand protein dynamics in living systems\(^1\)\(^-\)\(^3\). The use of fluorescent proteins has the advantage of genetic encoding. Thus, scientists can readily fuse fluorescent proteins to their protein of interest (POI) to track it in a
living system. However, this approach is limited to studying proteins, and is ill suited for use with biomolecules as fluorescent proteins are quite large and can interfere with the activity of small molecules or small proteins.

Bioorthogonal chemistry has been developed to overcome the challenges of tracking non-protein molecules in biological systems. A functional group unique to the biological system, also known as an orthogonal tag, is installed on the molecule of interest via biosynthetic or biochemical pathway. Subsequently, the tag reacts selectively and readily with a second reagent (reactive partner), which can be functionalized with a fluorescent probe or affinity handle, to form a stable adduct. The reaction between the tag and the reagent occurs selectively – in spite of all the other functional groups present in the biological system (Figure 1).

Ideally, the reaction between the orthogonal tag and its reactive partner should display “click” reaction characteristics as described by Kolb, Finn, and Sharpless: fast kinetics, high yields, broad solvent compatibility (especially water), lack of undesired side products, and readily available starting material(s).

Using an orthogonal tag-reactive partner approach is powerful because it enables scientists to study biomolecules that are not genetically encoded. There are a number of advantages over the genetically fused fluorescent proteins. First, the modification of the molecule of interest is minimal in terms of size and potential interactions with other molecules unlike fluorescent protein modifications which are few kDAs in size. Second, there have been a number of reactive partners that have been developed in the past two decades that give chemical biologists the flexibility to choose which is most useful in the system they are trying to study. Third, the orthogonal tag is tolerated by other biomolecules by hijacking their native biosynthetic pathway, which is very important with post-translational modified structures such as glycosylation and lipidation. Lastly, due to the multiple reactivities available, scientists can quantify, track, and/or monitor multiple sites simultaneously (multi-labeling).

1.2 Examples of Bioorthogonal Reactions

1.2.1 Azide-alkyne cycloaddition

The reaction between azides and alkynes is arguably one of the most widely used bioorthogonal reaction. This was first reported by Michael in 1893. However, it was not until the 1960s that
Huisgen and coworkers studied this reaction extensively\textsuperscript{15,16}. Subsequently, Sharpless and Meldel discovered independently that using copper (I) as a catalyst improved the kinetics of the reaction dramatically\textsuperscript{17,18}.

The thermal addition of azides and unactivated alkynes to form 1,2,3-triazoles is very exergonic. However, this reaction requires high temperature and/or pressure to proceed since the activation energy is approximately 26 kcal/mol. Transition metal catalysis (copper (I)) improves the kinetics of the reaction and lowers the activation energy. Moreover, it allows the reaction to proceed at room temperature\textsuperscript{19}. However, the requirement to use copper metal limits the application of the reaction in living systems. Thus, the development of strained cyclooctyne as the reaction partner for azides emerged, which does not require a metal catalyst.

The reaction of azides with strained cyclooctynes became attractive to many chemical biologists. Wittig and Krebs were the first ones to demonstrate that cyclooctynes react with azides at room temperature\textsuperscript{20}. The ring has roughly 18 kcal/mol ring strain that is released at the transition state of the reaction. However, the reaction is much slower than the copper-catalyzed reaction counterpart.

A lot of modifications were developed since the first discovery of this reaction. It has been found that changing the electronics of the ring by installing an electron-withdrawing group adjacent to the triple bond increases the rate of the reaction. In addition, adding more ring strain by appending a cyclopropane also increases the rate of the reaction. A summary of some notable strained-cyclooctyne derivatives can be found below (Table 1).
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Approximate rate constant (M⁻¹ s⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclooctyne (OCT)</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>0.0012</td>
<td>[Agard, 2004]²¹</td>
</tr>
<tr>
<td>Aza-dimethoxycyclooctyne (DIMAC)</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>0.0030</td>
<td>[Sletten, 2008]²²</td>
</tr>
<tr>
<td>Difluorocyclooctyne (DIFO)</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>0.076</td>
<td>[Baskin, 2007]²³</td>
</tr>
<tr>
<td>Bicyclo[6.1.0]nonyne (BCN)</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>0.14</td>
<td>[Dommerholt, 2010]²⁴</td>
</tr>
<tr>
<td>Biarylazacyclooctynone (BARAC), dibenzocyclooctyne (DIBO), aza-dibenzocyclooctyne (DIBAC)</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>0.17-0.96</td>
<td>[Jewett, 2010]²⁵</td>
</tr>
<tr>
<td>6-tetramethyl-thiacycloheptynone (TMTH)</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>4.0</td>
<td>[Almeida, 2012]²⁶</td>
</tr>
</tbody>
</table>

**Table 1.** Approximate second-order rate constant of strained-cyclooctyne derivatives when reacted with azide.
1.2.2 Aldehyde/ketone condensation with heteroatom-bound amines

The condensation of aldehyde/ketone was one of the first bioorthogonal reactions reported\(^{27}\). However, a number of biomolecules present potential reactive functional groups (e.g. amines). Imine formation is reversible and thermodynamically unfavorable in aqueous environment. However, the use of heteroatom-bound amine shifts the equilibrium towards the product due to the increase of nucleophilicity of the amine (Scheme 1). This phenomenon is called \(\alpha\)-effect, where the nucleophilicity of an atom is increased due to the adjacent (alpha) atom with lone pair of electrons.

There are a number of ways to install aldehydes/ketones in the molecule of interest: periodate oxidation of cell surface diols (i.e. sialic acids\(^ {28}\)), biosynthetic incorporation of unnatural amino acids\(^ {29}\), and enzymatic oxidation of alcohol or thiols in endogenous biomolecules\(^ {30}\).

The reaction is significantly faster under mildly acidic conditions. The condensation of the heteroatom-bound amines is most efficient at pH 3-6\(^ {27}\). Aniline-based nucleophilic catalysts accelerate the reaction at physiological pH. However, high concentration of the catalyst may be needed and toxicity of the additive is a concern\(^ {28,31}\).

![Scheme 1](image)

**Scheme 1.** Condensation of aldehyde/ketone with alkoxyamines or hydrazines/hydrazides to form oximes or hydrazones, respectively.

The formation of an oxime or hydrazine under aqueous conditions is reversible. Oximes are more stable than hydrazines\(^ {32}\). Fortunately, the stability of the adduct can be increased by modifying the substituents of the heteroatom-bound amine reagents. Particularly, Bertozzi and coworkers recently developed a reaction that exploits amino-trypamines (Scheme 2). The condensation of the aldehyde with amino-trypamine results in the formation of an oxyiminium intermediate, which reacts readily to form a C-C bond with the indole nucleophile; ultimately forming a stable oxycarboline adduct\(^ {33}\).
Scheme 2. Reaction of an aldehyde with a trypamine via Pictet-Spengler reaction.

An obvious limitation of aldehyde/ketone condensation with heteroatom is that aldehyde/ketone is not completely orthogonal to biological systems. There are molecules (e.g. glucose and other metabolites) that possess aldehyde/ketone functional group in their structures. The use of this method is generally only applicable on cell surfaces.

1.2.3 Tetrazine-alkene ligation

A major advancement in the field of bioorthogonal chemistry was the development of the tetrazine ligation. This reaction proceeds extremely quickly with the second-order rate constant up to $10^4$ M$^{-1}$ s$^{-1}$ depending on the reactive partner. Tetrazines undergo rapid inverse electron-demand Diels-Alder reaction with strained alkenes and alkynes. The strained alkene or alkyne initially forms an adduct that readily loses nitrogen gas to produce dihydropyridizine which then tautomerizes to yield a stable adduct (Scheme 3); consequently allowing efficient and quantitative conversion to product with low concentrations of reagents. The tetrazine moiety is also a photoquencher via fluorescence resonance energy transfer (FRET). When a fluorophore-conjugated tetrazine is unreacted, there is no fluorescent signal. Once the tetrazine reacts with a strained alkene or alkyne, quenching is relieved, thus liberating a fluorescent signal.

Scheme 3. General reaction scheme of tetrazine-alkene ligation.

There were three groups that independently studied tetrazine ligation in late 2008 to early 2009: Fox and coworkers, Hilderbrand, Weissleder and coworkers, and Pipkorn and coworkers. First, Fox and coworkers were the first ones to develop trans-cyclooctene with tetrazines. They
demonstrated that the ligation between the two reagents proceed with rate constant of $10^3 \text{M}^{-1} \text{s}^{-1}$, which was the fastest bioorthogonal chemical reaction ever reported then. However, tetrazines are only useful in simple biological systems. Although tetrazine selectively reacts with the cyclooctene, it can also react with amines, thiols, and water, giving a significant signal to noise ratio$^{36}$. Second, Hilderbrand, Weissleder and coworkers used norbornene-tetrazine ligation to label antibodies of live cells. This reaction was slower than what Fox and coworkers reported ($\sim 1 \text{ M}^{-1} \text{s}^{-1}$). However, it was comparable to the fastest cyclooctyne reagent for Cu-free click chemistry$^{35}$. Lastly, Pipkorn and coworkers demonstrated a method that ligates a peptide to a drug called temozolomide (TMZ). They showed that using tetrazine ligation method, the efficiency and pharmacological effects of the drug were dramatically enhanced, even in a 10-fold lower concentration than normally applied$^{40}$.

1.2.4 Nitrone-strained cyclooctyne cycloaddition

Strain promoted cyclooctyne-nitrone cycloaddition (SPANC) is a 1,3-dipolar cycloaddition to for a stable isoxazoline adduct. Pezacki and van Delft independently reported that nitrone reacts readily with strained cyclooctynes more rapidly than with azides. Both of them reported that even with the intensive effort to improve the reaction of azides with differently activated cyclooctynes, the kinetics were still very poor$^{41,42}$. The advantage of looking into the reactivity of the nitrone versus the azide is that the chemical reporter itself can be activated.

Pezacki and coworkers examined the difference between the reactivity of the acyclic and endocyclic nitrones. They found that acyclic aromatic nitrones with electron-withdrawing groups react more rapidly than nitrones with electron-donating groups$^{41}$. The nitrones with electron-donating groups have higher second-order rate constants that is found in azides and biarylazacyclooctyne (BARAC)$^6$. On the other hand, endocyclic nitrones have rate constants about 5 times faster than the reaction of azides to BCN; about 25 times faster than the reaction of azides to DIBO and BARAC$^6,41$ (Table 2). A summary of the second-order rate constants of endocyclic and acyclic nitrones and azide with different strained cyclooctyne can be found below (Table 2).
<table>
<thead>
<tr>
<th>Orthogonal tag/secondary reactive partner</th>
<th><img src="image" alt="Orthogonal tag" /></th>
<th><img src="image" alt="Secondary reactive partner" /></th>
<th><img src="image" alt="Reactive partner" /></th>
</tr>
</thead>
<tbody>
<tr>
<td>MeO-Benzyl-N^+O^-</td>
<td>0.0011</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>O_2N-Benzyl-N^+O^-</td>
<td>0.029</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Ph-Benzyl-N^+O^-</td>
<td>0.0029</td>
<td>0.088</td>
<td>2.8</td>
</tr>
<tr>
<td>Ph-Benzyl-N^+O^-</td>
<td>---</td>
<td>---</td>
<td>58.8</td>
</tr>
<tr>
<td>Ph-Methyl-N^+O^-</td>
<td>---</td>
<td>0.13</td>
<td>6.8</td>
</tr>
<tr>
<td>MeO-Benzyl-Methyl-N^+O^-</td>
<td>---</td>
<td>---</td>
<td>4.9</td>
</tr>
<tr>
<td>O_2N-Benzyl-Methyl-N^+O^-</td>
<td>---</td>
<td>---</td>
<td>10.1</td>
</tr>
<tr>
<td>Nitrone moiety</td>
<td>0.65</td>
<td>1.5</td>
<td>22.4</td>
</tr>
<tr>
<td>Ratios</td>
<td>0.14</td>
<td>0.062</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Table 2. Second-order rate constants of endocyclic and acyclic nitrones and azide with different strained cyclooctyne.\(^{6,41,43,44}\)

Van Delft and coworkers showed that the nitrone moiety could be installed in monosaccharides, peptides, and proteins. They demonstrated that peptides and proteins could have a nitrone moiety via one-pot synthesis. They first installed an N-terminal serine to a peptide or protein. Then, the
peptide or protein is treated with (1) sodium periodate and \( p\)-MeOC\(_6\)H\(_4\)SH for an hour, (2) \( p\)-MeOC\(_6\)H\(_4\)NH\(_2\) for 2 hours, and finally (3) MeNHOHHCl for another 21 hours to generate the nitrone moiety (Scheme 4). They have successfully shown that peptides and proteins can be tagged with strained-cyclooctyne. However, when they tried to tag nitrone modified monosaccharides, they did not see any labeling\(^{42}\).

![Scheme 4](image)

**Scheme 4.** Protein modification via strain-promoted alkyne-nitrone cycloaddition\(^{6,42}\).

Van Delft and coworkers tried to metabolically label monosaccharides modified with nitrone. They incubated Jurkat cells in the presence of nitrone modified D-mannosamine, sialic acid, and D-galactose. However, when they tried to label the cells with dibenzocyclooctyne-biotin and tagged with avidin-fluorescein isothiocyanate (FITC), they did not observe any labeling. They postulated that either the biosynthetic glycosylation machinery did not accept the nitrone modification or that the nitrones undergo intracellular hydrolysis in acidic compartments\(^{42}\).

For the past six years, nitrone-strained alkyne reaction has been an interest due to its fast kinetics to forming a stable isoxazoline adduct, making it an ideal reaction\(^{41,42}\). In addition, Pezacki and coworkers recently showed that unnatural amino acids with endocyclic nitrones, together with azides, could be used to multi-label peptidoglycan\(^{45}\). These reasons suggest nitrone bioorthogonal reaction is an interesting chemistry for further development.

**1.3 Scope of Thesis**

For my thesis project, we aim to use \( N\)-hydroxylamine as a new orthogonal tag. This functional group is similar in size and polarity to a hydroxyl group. Therefore, unlike tetrazine and its reactive partner, an \( N\)-hydroxylamine is small in size and can be easily put in monosaccharides like azides, thus minimally perturbing to the biosynthetic glycosylation machinery.
The current method to tag proteins bioorthogonally using nitroso is three steps, not including the insertion of N-terminal serine. This method requires more time and multiple incubations. With our proposed method, we designed our reactive partner probes that has both an aldehyde and alkene components. The idea is that the condensation of the aldehyde will form the nitroso. Subsequently, the alkene component of the probe will react intramolecularly to form the isoxazolidine adduct (Scheme 5).

**Scheme 5.** Proposed general reaction of N-hydroxylamine and a probe with both aldehyde and alkene moieties.

To determine if N-hydroxylamine is an effective bioorthogonal chemical reporter, we did some preliminary studies. We installed N-hydroxylamine functional group on a monosaccharide. We reacted the N-hydroxylamine modified monosaccharide with a number of different aldehydes, then, with a maleimide to determine if it will form the isoxazolidine adduct (Chapter 2). As the last part of our proof of concept, we synthesized our first-generation probe with both the aldehyde and alkene components. Subsequently, we reacted our first-generation probe with (a) N-hydroxylamine modified monosaccharide (b) N-alkyl hydroxylamine and (c) N-aryl hydroxylamine to determine the effectiveness of the probe (Chapter 2). In addition, we designed and synthesized our second-generation probes to improve the kinetics of the intramolecular cycloaddition (Chapter 3).
2 Proof of Concept

2.1 Introduction

There are many bioorthogonal tools available\textsuperscript{4,6,7}. However, there is a need to develop new chemistries to broaden the scope and power of these chemistries to study biological systems.

As discussed in the first chapter, tetrazine ligation has the fastest kinetics of the currently available chemistries. However, it produces nitrogen gas as a side product, which limits its use intracellularly. Hence, developing a bioorthogonal reaction that is comparable with this reaction is ideal, especially for multi-labeling purposes.

We propose to use $N$-hydroxylamine as a new orthogonal tag as mentioned in Chapter 1. As the first part of our proof of concept, we installed $N$-hydroxylamine function group on glucose skeleton. Second, we synthesized our first-generation probe that both have the aldehyde and alkene components and react it with a number of $N$-hydroxylamines.

2.1.1 Glucose modified with $N$-hydroxylamine

We modified glucose with $N$-hydroxylamine at the second position. Glucosamine hydrochloride was used as a convenient starting material, which could be oxidized to the desired 2-deoxy-2-$N$-hydroxylamine (Scheme 6).
Scheme 6. Synthetic procedure to install $N$-hydroxylamine to glucose at the C-2 position.

To the modified monosaccharide 4, different aldehydes (Figure 2) were added to form the nitrone intermediate. Subsequent reaction with maleimide formed the isoxazolidine adduct (Scheme 8).

Figure 2. Aldehydes that were reacted with glucose modified with $N$-hydroxylamine 4.

2.1.2 First generation probe

We synthesized our probe 6 according to literature (Scheme 7)\textsuperscript{46}. This compound was chosen as a target as it was stated in the literature that “condensation of [probe 6] with $N$-phenylhydroxylamine in absolute ethanol resulted in the transient formation of [the nitrone intermediate] which immediately cyclized to give [the isoxazolidine adduct].”\textsuperscript{47} This statement suggested that reaction of the compound with our nitrones should be sufficiently fast to enable bioorthogonal reactions.
We first reacted probe 6 with compound 4, then with an alkyl (N-methylhydroxylamine) and with an aryl (N-phenylhydroxylamine) hydroxylamine. We observed the reaction of probe 6 with N-hydroxylamines using $^1$H NMR.

### 2.2 Results and Discussion

#### 2.2.1 Glucose modified with N-hydroxylamine

We synthesized the modified glucose with and N-hydroxylamine functional group via a 4 step sequence. The imine formation step yielded comparable results to the literature. However, the benzyl protection step has lower yield compared to literature (65% vs. 56%). This was potentially due to the humidity, thus, not having a truly anhydrous reaction. The epoxidation with mCPBA also gave us lower yield compared to the literature. However, we used different protecting groups (TES vs. Bn) and we conducted our experiment on a smaller scale. Finally, the oxaziridine was methanolized in a step the formation of 1,2-dihydroxylaminecyclohexane hydrochloride.

#### 2.2.2 Reaction of glucose modified with N-hydroxylamine with aldehydes then maleimides

The modified monosaccharide 4 was reacted with different aldehydes (Figure 2) then with N-methylmaleimide to form the isoxazolidine adduct (Scheme 8). The nitrone intermediate formed rapidly when the aldehydes were reacted with modified glucose with N-hydroxylamine (~5-40 mins, using TLC). However, when N-methylmaleimide was added, there was no cycloadduct observed (using TLC) even after 18 hours at room temperature. The reactions were then heated to 85 °C. After three hours, the reactions were stopped and were purified using flash column chromatography.
Scheme 8. General reaction of modified glucose 4 with an aldehyde then with N-methylmaleimide to yield 5a-e.

The reaction conditions we used to form the isoxazolidine adducts from a nitrone intermediate and maleimide were similar to what was in the literature. They used high temperatures (80-100 °C) in a number of different solvents, including toluene. In addition, the reaction was incubated for extended periods, up to 4 days.

As expected, the nitrone formed from para-nitrophenyl benzaldehyde gave the highest yield of cycloadduct. On the other hand, the nitrone with para-methoxyphenyl electron-donating group gave the lowest yield of the cycloadduct. The branched alkyl aldehyde (Figure 2d) did not give any of the desired cycloadduct. This maybe due to steric hindrance introduced by the branch, as the unbranched alkyl aldehyde (Figure 2e) yielded 45% of the cycloadduct (Table 3).

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>Nitrone intermediate (%)</th>
<th>Cycloadduct (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a*</td>
<td>&gt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>b*</td>
<td>&gt;65</td>
<td>&lt;35</td>
</tr>
<tr>
<td>c</td>
<td>16</td>
<td>84</td>
</tr>
<tr>
<td>d</td>
<td>100</td>
<td>n/a</td>
</tr>
<tr>
<td>e</td>
<td>55</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 3. Summary of the yields when modified glucose 4 was reacted with the corresponding aldehydes followed by N-methylmaleimide to form 5a-e. *at least one of the diastereomers was not isolated completely from the nitrone intermediate.
There were two diastereomers of the cycloadduct isolated from aldehydes a-c. However, we were only able to separate a single diastereomer from the mixture of aldehydes a and b to give clean $^1$H NMR spectra. The other diastereomers co-eluted with their corresponding nitrone intermediate (Appendix I). We were able to isolate both diastereomers from aldehyde c (See experimental section).

Our goal is to use $N$-hydroxylamine as an orthogonal tag in biological settings, thus having to heat to 85 °C is not ideal. Next, we synthesized our first bifunctional probe containing an aldehyde and an alkene with the goal of improving the reaction kinetics.

2.2.3 Reaction of probe 6 with glucose modified with $N$-hydroxylamine

Scheme 9. Reaction of probe 6 with $N$-hydroxylamine modified glucose.

Probe 6 was condensed with the $N$-hydroxylamine modified glucose 4 in DCM at room temperature. After leaving the reaction overnight, we did not observe (using TLC) the formation of the desired cycloadduct. Subsequently, we attempted to do the reaction again following the procedure described in Section 2.4.3.1. Surprisingly, even after heating up the reaction to 100 °C for 18 hours, the major product after isolation was still the nitrone intermediate (Scheme 9). We were not able to obtain a clean $^1$H NMR spectra of the isolated products but we were confident that the major product obtained was the nitrone intermediate and the minor product obtained was the cycloadduct 6a (Figure 3). Note, both the nitrone and the cycloadduct have the same molecular mass which was consistent with both isolated products. In Figure 3, the top spectrum (minor product) has no imine peak and only has traces of the alkene peaks and we assume this is a diastereomeric mixture of cycloadducts. The bottom spectrum (major product) has fewer observable alkyl peaks and has absorbance characteristic of alkenes, we assume this product is the nitrone.
Figure 3. $^1$H NMR spectra of the two products isolated from the reaction of probe 6 with glucose modified with N-hydroxylamine 4. The top spectrum is of the minor product: cycloadduct 6a. The bottom spectrum is of the major product: nitrone intermediate of the reaction (Scheme 8).

2.2.4 Reaction of probe 6 with N-methylhydroxylamine

Scheme 10. Reaction of probe 6 with N-methylhydroxylamine hydrochloride in deuterated methanol at room temperature.

To determine the reason for the observed reduced reactivity in comparison to the “immediate” reaction stated in the literature, we reacted probe 6 with N-methylhydroxylamine hydrochloride in deuterated methanol at room temperature and monitored the reaction using $^1$H NMR (Scheme 10). Similarly to what was observed with monosaccharide 4, even after four days at room
temperature, the reaction only showed trace amounts of the desired isoxazolidine adduct (Figure 4). This result suggests that the differences in steric hindrance between 4 and N-methylhydroxylamine had little to no effect and the reduced reaction rate was more likely due to electronic effects.

![Chemical structures]  

**Figure 4.** $^1$H NMR spectra measuring the reaction of probe 6 with N-methylhydroxylamine over 4 days at room temperature to form compound 6b (Scheme 10). Peaks in the orange box indicate alkene peaks. Peaks in the blue box indicate the appearance of new (sp$^3$ hybridized) peaks.
2.2.5 Reaction of probe 6 with \( N \)-phenylhydroxylamine

\[
\begin{align*}
\text{Scheme 11. Reaction of probe 6 with } N \text{-phenylhydroxylamine in deuterated methanol at room temperature.}
\end{align*}
\]

Revisiting the literature, we carried an \(^1\)H NMR experiment to monitor the reaction of probe 6 with \( N \)-phenylhydroxylamine in deuterated methanol at room temperature (Scheme 11). We confirmed that after an hour, the formation of the isoxazolidine adduct was observed. However, after three hours, there was still a significant amount of the alkene containing starting material present (Figure 5). This result suggests that the formation of the isoxazolidine adduct is affected by the electronics of the nitrone intermediate.
Figure 5. $^1$H NMR spectra measuring the reaction of probe 6 and $N$-phenylhydroxylamine over 3 hours at room temperature to form compound 6c (Scheme 11). Peaks in the orange box indicate alkene peaks. Peaks in the blue box indicate the appearance of new (sp$^3$ hybridized) peaks.

2.3 Summary

In conclusion, as part of our proof of concept to use $N$-hydroxylamine as new bioorthogonal tag, we were able to synthesize glucose modified with $N$-hydroxylamine 4 in four steps. We showed that this modified glucose reacts with different aldehydes to form the corresponding nitrones. Subsequently, these nitrones can be reacted with $N$-methylmaleimide to form the isoxazolidine cycloadduct. In addition, we synthesized our first-generation probe 6 that has both an aldehyde and alkene components as a reactive partner. Unfortunately, probe 6 does not form the isoxazolidine adduct at room temperature with alkyl nitrones. Next we have designed and synthesized our second-generation probes to improve the reaction kinetics further (Chapter 3).
2.4 Experimental

2.4.1 Synthesis of glucose modified with N-hydroxylamine

2-N-(4-methoxybenzylidene)amino-2-deoxy-D-glucose (1)$^{48}$

$p$-anisaldehyde (1.13 g, 10.2 mmol) added to a solution of D-glucosamine hydrochloride (2.0 g, 9.3 mmol) in 5 M NaOH (2.0 mL). The mixture was stirred at room temperature for 5 mins. The product precipitated and the solution was stored at 4 °C for an additional 21 hours. The solid was collected by filtration and was washed with (25 mL x 2) diethyl ether:hexanes (2:1) to afford the product (2.5 g, 93%). The product was carried to the next step without further purification.

1,3,4,6-tetra-O-benzyl-2-N-(4-methoxybenzylidene)amino-2-deoxy-D-glucose (2)$^{48}$

Benzylbromide (10.262 g, 60.0 mmol) was added to a solution of 1 (3.47 g, 11.7 mmol) in dry DMF (40 mL). The solution was cooled to 0 °C and sodium hydride (60% dispersion in mineral oil) (2.72 g, 68.0 mmol) was added to the solution in 5 portions over 30 mins. The solution was stirred at room temperature for 20 hours. After 20 hours, the reaction was quenched with EtOAc (40 mL). The mixture was evaporated in vacuo and was resuspended in EtOAc. The mixture was washed with water and sat. NaCl. The organic layer was dried with MgSO$_4$, filtered and evaporated in vacuo. The crude yellow oil was purified by flash chromatography using 20% EtOAc in pentane + 5% Et$_3$N (rf = 0.5) to afford the product (4.247 g, 56%). 1H NMR (500 MHz, Chloroform-d) $\delta$ 8.29 (s, 1H), 7.75 – 7.67 (m, 2H), 7.41 – 7.07 (m, 24H), 7.00 – 6.93 (m, 2H), 4.88 (dd, J = 11.8, 9.1 Hz, 2H), 4.75 (d, J = 7.9 Hz, 1H), 4.70 – 4.52 (m, 7H), 3.93 – 3.68 (m, 8H), 3.62 (ddd, J = 9.8, 4.7, 2.0 Hz, 1H), 3.37 (dd, J = 9.5, 7.8 Hz, 1H).
1,3,4,6-tetra-\textit{O}-benzyl-2-(3-(4-methoxyphenyl)-1,2-oxaziridin-2-yl)-2-deoxy-\textit{\beta}-D-glucose (3)\textsuperscript{49}

mCPBA (<77%) (0.281 g, 1.14 mmol) was added to a solution of 2 (0.499 g, 0.759 mmol) in DCM and sat. NaHCO\textsubscript{3} (0.825 mL). The mixture was stirred vigorously. After 5 hours, the layers were separated. The aqueous layer was washed with DCM. The organic layers were combined, washed with water, and sat. NaCl. The organic layer was dried using MgSO\textsubscript{4}, filtered and evaporated \textit{in vacuo}. The crude mixture was purified by flash chromatography using 30% EtOAc in pentane + 1% Et\textsubscript{3}N (rf = 0.5) to afford the product (185 mg, 36%). 1H NMR (500 MHz, Chloroform-d) \(\delta\) 8.32 – 8.27 (m, 2H), 7.43 (s, 1H), 7.40 – 7.09 (m, 24H), 7.03 – 6.97 (m, 2H), 5.20 (d, \(J = 7.8\) Hz, 1H), 4.91 (dd, \(J = 23.0, 11.4\) Hz, 2H), 4.78 (d, \(J = 10.5\) Hz, 1H), 4.72 – 4.56 (m, 6H), 3.90 (s, 3H), 3.86 – 3.75 (m, 4H), 3.66 (ddd, \(J = 9.9, 3.7, 2.6\) Hz, 1H).

1,3,4,6-tetra-\textit{O}-benzyl-2-hydroxyamino-2-deoxy-\textit{\beta}-D-glucose (4)\textsuperscript{50}

HCl (1 M in MeOH, 1.13 mL, 1.13 mmol) was added to a solution of 3 (202 mg, 0.299 mmol) and O-benzylhydroxylamine hydrochloride (96 mg, 0.601 mmol) in dry MeOH. The solution was stirred at room temperature for an hour. The solution was evaporated \textit{in vacuo}. The mixture was redissolved in EtOAc and washed with distilled water and sat. NaCl. The organic layer was dried using MgSO\textsubscript{4}, filtered and evaporated \textit{in vacuo}. The crude mixture was purified by flash chromatography using 30% EtOAc in pentane (rf = 0.65) to afford the product (90 mg, 54%). 1H NMR (500 MHz, Chloroform-d) \(\delta\) 7.41 – 7.17 (m, 20H), 4.93 (dd, \(J = 20.5, 11.6\) Hz, 2H), 4.79 (dd, \(J = 36.7, 11.2\) Hz, 2H), 4.68 – 4.50 (m, 5H), 4.01 (s, 1H), 3.87 – 3.66 (m, 4H), 3.45 (ddd, \(J = 9.7, 4.2, 2.5\) Hz, 1H), 2.79 (dd, \(J = 10.2, 8.1\) Hz, 1H).
2.4.2 Reaction of glucose modified with N-hydroxylamine with aldehydes then maleimides

*General reaction procedure of glucose modified with N-hydroxylamine with aldehydes then maleimides*

The appropriate aldehyde a-e (1.1 eq.) and acetic acid (0.1 eq.) were added to a solution of compound 4 (1 eq.) in DCM (0.1 M). The solution was stirred at room temperature for an hour. The solution was evaporated *in vacuo* and was redissolved in toluene (0.1 M). The solution was stirred and heated to 85 °C. After 3 hours, the reaction was cooled to room temperature and was evaporated *in vacuo*. The crude mixture was purified using flash column chromatography.

**Compound (5a)**

![Chemical structure of compound 5a]

$^1$H NMR (500 MHz, Chloroform-$d$) δ 7.46 – 7.13 (m, 25H), 4.98 – 4.87 (m, 3H), 4.84 (d, $J = 7.6$ Hz, 1H), 4.68 – 4.47 (m, 7H), 4.27 (dd, $J = 9.9$, 9.0 Hz, 1H), 3.72 – 3.57 (m, 3H), 3.49 (td, $J = 7.6$, 0.5 Hz, 1H), 3.44 (ddd, $J = 9.6$, 4.1, 2.5 Hz, 1H), 2.97 (dd, $J = 9.9$, 8.0 Hz, 1H), 2.72 (s, 1H). Crude $^1$H NMR of the other diastereomer can be found in Appendix I.

**Compound (5b)**

![Chemical structure of compound 5b]

$^1$H NMR (500 MHz, Chloroform-$d$) δ 7.44 – 7.15 (m, 20H), 7.10 (ddd, $J = 5.3$, 2.9, 1.3 Hz, 2H), 6.81 – 6.76 (m, 2H), 4.96 – 4.89 (m, 2H), 4.83 (dd, $J = 8.7$, 7.6 Hz, 2H), 4.70 – 4.47 (m, 7H), 4.26 (dd, $J = 9.9$, 9.1 Hz, 1H), 3.78 (s, 3H), 3.69 – 3.57 (m, 3H), 3.50 – 3.41 (m, 2H), 2.95 (dd, $J = 9.9$, 8.0 Hz, 1H), 2.71 (s, 3H). Crude $^1$H NMR of the other diastereomer can be found in Appendix I.

**Compound (5c – major)**

![Chemical structure of compound 5c]

$^1$H NMR (500 MHz, Chloroform-$d$) δ 7.95 (d, $J = 8.7$ Hz, 1H), 7.44 – 7.22 (m, 9H), 7.17 – 7.10 (m, 2H), 5.23 (d, $J = 8.8$ Hz, 1H), 5.05 (d, $J = 7.9$ Hz, 1H), 4.94 (dd, $J = 23.3$, 11.6 Hz, 1H), 4.80 – 4.66 (m, 2H), 4.62 – 4.55 (m, 1H), 4.53 – 4.46 (m, 1H), 3.93 (dd, $J =$
10.2, 8.4 Hz, 1H), 3.77 – 3.60 (m, 1H), 3.55 – 3.31 (m, 2H), 2.82 – 2.66 (m, 2H).

**Compound (5c – minor)**

\[
\text{1H NMR (500 MHz, Chloroform-}d\text{) } \delta 8.10 – 8.01 (m, 2H), 7.57 – 7.48 (m, 2H), 7.45 – 7.19 (m, 20H), 5.02 – 4.96 (m, 3H), 4.85 (d, } J = 7.6 \text{ Hz, 1H), 4.69 – 4.47 (m, 7H), 4.28 (dd, } J = 9.9, 9.0 \text{ Hz, 1H), 3.69 (d, } J = 3.2 \text{ Hz, 2H), 3.62 (dd, } J = 9.6, 9.0 \text{ Hz, 1H), 3.43 (dt, } J = 9.7, 3.3 \text{ Hz, 1H), 3.39 (t, } J = 7.6 \text{ Hz, 1H), 2.84 (dd, } J = 9.9, 8.1 \text{ Hz, 1H), 2.74 (s, 3H).}
\]

**Compound (5d)**

This compound was not observed.

**Compound (5e)**

\[
\text{1H NMR (500 MHz, Chloroform-}d\text{) } \delta 7.36 – 7.20 (m, 16H), 7.15 – 7.08 (m, 4H), 4.93 (d, } J = 12.4 \text{ Hz, 1H), 4.85 (d, } J = 11.6 \text{ Hz, 1H), 4.71 – 4.59 (m, 5H), 4.58 – 4.51 (m, 3H), 4.18 – 4.08 (m, 1H), 3.81 – 3.65 (m, 4H), 3.47 (ddd, } J = 9.5, 3.9, 3.0 \text{ Hz, 1H), 3.32 – 3.27 (m, 1H), 3.08 (dd, } J = 9.4, 7.8 \text{ Hz, 1H), 2.68 (s, 3H), 1.85 – 1.73 (m, 1H), 1.59 – 1.48 (m, 1H), 1.37 – 1.21 (m, 4H), 0.87 (t, } J = 7.2 \text{ Hz, 3H).}
\]

2.4.3 Synthesis of first-generation intramolecular probe

2'-vinyl-[1,1'-biphenyl]-2-carbaldehyde (6)\textsuperscript{52}

2-vinylphenylboronic acid (1.5 eq.) in ethanol and sodium carbonate (2.0 eq.) in water were added to a solution of tetrakis(triphenylphosphine)palladium (0.3 % mol) and 2-bromobenzaldehyde (1 eq.) in DME. The solution was stirred and
refluxed. After 18 hours, the solution was cooled to room temperature and was diluted with diethyl ether. The mixture was washed with water and brine. The organic layer was dried using MgSO₄, filtered and evaporated in vacuo. The crude mixture was purified by flash chromatography using 100% diethyl ether. ¹H NMR (500 MHz, Chloroform-d) δ 9.71 (d, J = 0.8 Hz, 1H), 8.03 (dd, J = 7.8, 1.4 Hz, 1H), 7.69 – 7.59 (m, 2H), 7.52 (tt, J = 7.6, 1.1 Hz, 1H), 7.43 (td, J = 7.5, 1.3 Hz, 1H), 7.39 – 7.31 (m, 2H), 7.24 (dd, J = 7.5, 1.4 Hz, 1H), 6.40 (dd, J = 17.5, 11.0 Hz, 1H), 5.67 (dd, J = 17.5, 1.1 Hz, 1H), 5.17 (dd, J = 11.0, 1.1 Hz, 1H).

2.4.3.1 Reaction of probe 6 with N-hydroxylamines

**Reaction procedure of probe 6 with compound 4 (6a)**

Probe 6 (1.1 eq.) and acetic acid (0.1 eq.) were added to a solution of 4 (1 eq.) in DCM (0.1 M). The solution was stirred at room temperature for an hour. The solution was evaporated in vacuo and was redissolved in toluene (0.1 M). The solution was stirred and refluxed. After 18 hours, the reaction was cooled to room temperature and was evaporated in vacuo. The crude mixture was purified using flash column chromatography with 30% EtOAc in pentane. There were two spots isolated but both did not give clean ¹H NMR spectra: (A) nitrone intermediate (B) cycloadduct 6a (Scheme 9, Figure 3).

**General reaction procedure of probe 6 with alkyl and aryl N-hydroxylamines**

Sodium acetate (2.2 eq.) and an N-hydroxylamine were added to a solution of probe 6 (1 eq.) in deuterated methanol (0.1 M) in a half-dram vial. The solution was mixed by pipetting five times. The solution was transferred to a 3 mm NMR tube and kept at room temperature. ¹H NMR was taken at time 0, 1, and 3 hours.

**Compound (6b)**

This compound was not isolated. Crude ¹H NMR spectrum can be found in Appendix I.
**Compound (6c)**

This compound was not isolated. Crude $^1$H NMR spectrum can be found in Appendix I.
Chapter 3

3 Design and Synthesis of Second Generation Probes

3.1 Introduction

Our first attempt to design intramolecular nitrone based probe were only successful with aryl hydroxylamines. Further analysis of the literature suggested other approaches that might improve the reaction kinetics with our target N-alkyl hydroxylamines. From our experiments, it was clear that the poor kinetics were due to the electronics of the nitrone intermediates: alkyl nitrone vs. aryl nitrone (Chapter 2).

For the design of our second-generation of probes, we made the assumption that the rate-limiting step was the intramolecular cycloaddition. We performed our experiments under slightly acidic conditions, where the rate of condensation of the aldehyde to form the nitrone is fast at concentrations that the equilibrium lies towards the product\textsuperscript{27}. Thus, for this chapter, we focused on designing probes to improve the kinetics of the intramolecular cycloaddition.

3.1.1 Design of the second-generation probes

Nitrone-alkene reaction is a 1,3-dipolar cycloaddition. Huisgen and coworkers have extensively studied the mechanism of 1,3-dipolar cycloaddition\textsuperscript{15,16,53}. The majority of their results point to a highly ordered transition state. The reaction rate is not dependent on solvent polarity\textsuperscript{54}. In addition, this reaction is stereospecifically suprafacial, with small activation energies\textsuperscript{55}, and has very negative entropies of activation\textsuperscript{56}. For these reasons, we designed our probes based on their predicted transition states.

According to the frontier orbital theory, decreasing the gap between the participating HOMO and LUMO of the reactants can increase the relative reactivity of a 1,3-dipole towards a dipolarophile. In addition, the energy of the HOMO can be increased by the presence of an electron-donating group (including alkyl). The LUMO energy can be decreased by the presence of an electron-withdrawing group and conjugating substituents\textsuperscript{55}. 
Regioselectivity of the 1,3-dipolar cycloaddition is influenced by both electronic and steric
effects of the concerted transition state\textsuperscript{15,53}. Reaction between the termini with the largest
coefficients in the HOMO and LUMO of the 1,3-dipole and dipolarophile will be the favored
cycloadduct to form\textsuperscript{57}. As a general rule, isoxazolidine C-5 substituted results in the maximum
overlap of the LUMO of the nitrene (especially when activated with an electron-withdrawing
group) with the HOMO of the alkene partner (especially when activated with an electron-
donating group) (Figure 6)\textsuperscript{51}.

**Figure 6.** Isoxazolidine construct.

Pezacki and coworkers have shown that activating the acyclic nitrones with an electron-
withdrawing group on the carbon atom would increase the rate of the reaction of nitrones
towards azides\textsuperscript{41,45,44}. We decided to construct of our second-generation of probes
(intramolecular probes) to have an oxoacetamide functional group. The beta carbonyl should
condense with the hydroxylamine to form the nitrone intermediate with acetamide on the carbon
atom.

**Scheme 12.** Proposed mechanism of second-generation probe that forms a bicyclo[3.2.1]octane
cycloadduct (bridged).

The presence of the acetamide on the nitrone carbon atom of the intermediate decreases the
energy of the LUMO of the 1,3-dipole. To further increase the reactivity, we added an electron-
donating group on the alkene to increase the energy of the its HOMO\textsuperscript{51,55,58}. We planned to
synthesize probes with different functionalized alkenes: phenyl, \( p \)-methoxyphenyl, and \( p \)-
hydroxyphenyl.
Scheme 13. Proposed mechanism of second-generation probe that forms a bicyclo[3.3.0]octane system (fused).

In addition to activating both the nitrone and the alkene, we also added an extra substituent on the amide to pre-position the transition state towards the cis-amide. We synthesized the methyl and isopropyl derivatives to determine if the extra steric bulk improved the reactivity. Moreover, we investigated probes that had the potential to form either a bicyclo[3.3.0]octane system (fused) or bicyclo[3.2.1]octane (bridged) cycloadducts (Scheme 12-13).

3.2 Results and Discussion

We synthesized probes 13 and 15 in five steps with one purification (not including extractions); and probes 14 and 16 in six steps with one purification (not including extractions) (3.3 Experimental). The last step of the synthesis of the second-generation probes was purified using extraction. The organic layers were dried with MgSO₄, evaporated in vacuo, and further dried in high vacuum for at least 24 hours. However, the proposed oxoacetamide functional group of the second-generation probes is hydrated as dihydroxyacetamide functional group. We carried out our preliminary studies of the reactivity of probes 13-16 as dihydroxyacetamide.

Preliminary studies of the reactivity of our second-generation probes were carried out by ¹H NMR. For simplicity of interpreting the spectra, we tried the reaction with N-methylhydroxylamine, instead of the glucose modified monosaccharide 4 (Scheme 14). Each of the second-generation probes was reacted one equivalence of N-methylhydroxylamine and two equivalence sodium acetate in deuterated methanol. Each reaction was incubated at 37 °C for 24 hours. Each reaction was monitored using ¹H NMR at time 0, 3, 6, and 24 hours.
Scheme 14. Reaction of probes 13-16 with N-methylhydroxylamine to yield compounds 17-20. (a) N-MeNHOH-HCl (1.1 eq.), NaOAc (2.2 eq.), CD₃OD, 37 °C, 24 hrs.

The reactivity of probes 13-16 were monitored by calculating the percentage conversion to their corresponding cycloadduct 17-20 (Scheme 14). We monitored the ratio of the disappearance of the alkene peaks relative to the appearance of the new sp³ hybridized peaks that do not overlap with other peaks (Figure 7). The procedure to determine percentage conversion of the second-generation probes with N-methylhydroxylamine to yield their corresponding cycloadducts can be found in 3.3 Experimental. The reaction of probe 15 with N-methylhydroxylamine is discussed below as the representative of the second-generation probes.
Figure 7. $^1$H NMR spectra of the reaction of probe 15 with $N$-methylhydroxylamine to monitor the formation of cycloadduct 19 at time 0, 3, 6, and 24 hours.

The reaction of probe 15 with $N$-methylhydroxylamine to form cycloadduct 19 was monitored at time 0, 3, 6 and 24 hours using $^1$H NMR (Figure 7). We focused our attentions at the alkene (orange box, Figure 7) and alkyl (blue box, Figure 7) regions of the $^1$H NMR spectra to monitor the intramolecular cycloaddition. At time 0 hour, there were two alkene peaks ($H_1$ and $H_2$) present and there were no alkyl peaks. At time 3 hours, there was a noticeable decrease on the alkene peaks’ signals and there were appearances of five alkyl peak signals ($H_3$ – $H_7$) (Figure 7). However, the percentage conversion calculated was only 67% (Graph 1). At time 6 hours, there were still alkene peak signals but were almost not noticeable compared to the alkyl peak signals (Figure 7). The percentage conversion calculated was 86% (Graph 1). After 24 hours, there were no alkene peak signals, thus the percentage conversion was 100% at this time (Figure 7). The results suggest that probe 15 could be used as a reactive partner for bioorthogonal reaction with $N$-hydroxylamines.
We found that after 3 hours, the percentage conversion of probes 13-16 were 72%, 65%, 67%, and 58%, respectively. The difference between the percentage conversions of the second-generation probes was not significant. The same could be said for the percentage conversion of the second-generation probes at time 6 hours. The percentage conversion of probes 13-16 were 84%, 83%, 86%, and 80%, respectively (Graph 1). These results were unexpected. There is no preference on either forming the bicyclo[3.3.0]octane system (fused) or bicyclo[3.2.1]octane (bridged). Moreover, the pre-positioning of the intermediate with either an isopropyl or a methyl substituent did not make a significant difference to the reactivity of the probes.

Gratifyingly, our second-generation probes have better reactivity compared to the biphenyl derivative first explored. They all formed their corresponding cycloadduct at 37 °C within 24 hours. This result shows that the second-generation probes are good candidates as reactive partners of N-hydroxylamine as orthogonal tag, according to the characteristics of bioorthogonal reactions discussed in Chapter 1. The reaction between N-methylhydroxylamine and second-generation probes have relatively fast kinetics (in biological time scale) and were high yielding with no undesirable side products (t = 24 hours of ¹H NMR experiments in Appendix I). We
were only able to perform the reaction in deuterated methanol. Further studies will be done to determine if the \([3 + 2]\) cycloaddition of \(N\)-hydroxylamine with the probes is a viable as a bioorthogonal reaction.

### 3.3 Summary

Our first attempt at designing a simple first-generation probe 6 with both the aldehyde and the alkene moiety did not give suitable reaction kinetics with \(N\)-alkyl hydroxylamines as the reaction did not form the isoxazolidine adduct at room temperature. Thus, we designed our second-generation probe more rationally. We designed our probes based on their predicted transition states.

First, we activated the nitrone intermediate to have an electron-withdrawing group (acetamide), thus decreasing the energy the LUMO of the 1,3-dipolar. Second, we further increased the cycloaddition reactivity by activating the alkene with an electron-donating group, thus increasing the energy of its HOMO. By FMO theory, the activation of the nitrone and the alkene should increase their reactivity towards each other to form the isoxazolidine adduct. Lastly, we added an extra substituent on the amide to pre-position the transition state towards the cis-amide.

We synthesized probes 13-16 and monitored their reaction with \(N\)-methylhydroxylamine using \(^1H\) NMR. They all formed their corresponding cycloadducts at 37 °C after 24 hours, thus making \(N\)-hydroxylamine a good candidate as a new orthogonal tag.

### 3.4 Experimental

#### 3.4.1 Synthesis of second-generation probes

\((E)\)-\(N\)-isopropyl-3-phenylprop-2-en-1-amine (7)

In a 50 mL RBF with acetonitrile (6 mL), 10 eq. (1.32 mL) of isopropylamine was added and stirred at room temperature for 5 mins. 3-bromo-1-phenylpropene (0.250 mL, 1 eq.) was added dropwise to the solution and stirred at room temperature for 1.5 hours. The solution was diluted with EtOAc, washed with sat. NaHCO₃, distilled water, and sat. NaCl. The organic layer was dried using MgSO₄, filtered and evaporated \textit{in vacuo}. The crude solid obtained was purified by flash chromatography using 10% MeOH in DCM (Rf = 0.2) as eluent to afford the product (220 mg,
76%) as yellow oil. $^1$H NMR (500 MHz, Chloroform-d) $\delta$ 7.37 (ddd, J = 8.4, 1.3, 0.5 Hz, 2H), 7.33 – 7.27 (m, 2H), 7.23 – 7.19 (m, 1H), 6.52 (dt, J = 15.8, 1.5 Hz, 1H), 6.31 (dt, J = 15.9, 6.4 Hz, 1H), 3.41 (dd, J = 6.4, 1.5 Hz, 1H), 2.89 (hept, J = 6.2 Hz, 1H), 1.40 (s, 1H), 1.10 (d, J = 6.3 Hz, 6H).

(3-bromoprop-1-en-2-yl)benzene (8)

$N$-bromosuccinimide (1.506 g, 8.461 mmol) added in four portions over 20 mins and p-TsOH (660 mg) was added to a solution of alpha-methyl styrene (1 mL, 7.69 mmol) in 20 mL dry THF. The solution was cooled to 0 °C and was allowed to stir at room temperature for 15 mins. The solution was refluxed 4 hrs. The solution was then cooled to room temperature and diluted with diethyl ether. The solution was washed with distilled water three times, and then with sat. NaCl. The organic layer was dried using MgSO$_4$, filtered and evaporated in vacuo. The crude oil was used without further purification.

$N$-isopropyl-2-phenylprop-2-en-1-amine (9)

Crude of 6 (252 mg, 1.28 mmol) dissolved in MeCN (2 mL) was added dropwise to a solution of isopropylamine (1.1 mL, 12 mmol) in dry MeCN (6 mL). The solution was stirred at room temperature for an hour. The solution was diluted with EtOAc, washed with sat. NaHCO$_3$, distilled water, and sat. NaCl. The organic layer was dried using MgSO$_4$, filtered and evaporated in vacuo. The crude solid obtained was purified by flash chromatography using 100% EtOAc (Rf = 0.2) as eluent to afford the product (174 mg, 74%) as yellow oil. $^1$H NMR (500 MHz, Chloroform-d) $\delta$ 7.46 – 7.41 (m, 2H), 7.37 – 7.32 (m, 2H), 7.31 – 7.26 (m, 1H), 5.39 (dt, J = 1.4, 0.7 Hz, 1H), 5.24 (q, J = 1.4 Hz, 1H), 3.66 (dd, J = 1.4, 0.7 Hz, 2H), 2.85 (hept, J = 6.3 Hz, 1H), 1.49 (s, 1H), 1.06 (d, J = 6.2 Hz, 6H).

(E)-$N$-methyl-3-phenylprop-2-en-1-amine (10)

3-bromo-1-phenylpropene (2.0 g, 10 mmol) dissolved in THF (5 mL) was added dropwise to a 2.0 M solution of methylamine (25 mL, 50 mmol) in THF was. The solution was allowed to stir at room temperature for 2 hours. The solution was evaporated in vacuo and was redissolved in EtOAc. The solution was washed with sat. NaHCO$_3$, distilled water, and sat. NaCl. The organic layer was dried using MgSO$_4$, filtered
and evaporated in vacuo. The crude solid obtained was purified by flash chromatography using 100% EtOAc (Rf = 0.4) as eluent to afford the product (1.1 g, 72%) as yellow oil. $^1$H NMR (500 MHz, Chloroform-d) $\delta$ 7.41 – 7.37 (m, 2H), 7.34 – 7.29 (m, 2H), 7.25 – 7.21 (m, 1H), 6.58 – 6.52 (m, 1H), 6.31 (dt, J = 15.9, 6.8 Hz, 1H), 3.23 (dd, J = 6.7, 1.3 Hz, 2H), 2.51 (s, 1H), 2.32 (s, 3H).

$N$-methyl-2-phenylprop-2-en-1-amine (11)

Crude of compound 6 (1.64 g, 8.34 mmol) dissolved in THF (5 mL) added dropwise to a 2.0 M solution of methylamine (24.0 mL, 48.0 mmol) in THF. The solution was allowed to stir at room temperature for 2 hours. The solution was evaporated in vacuo and was redissolved in EtOAc. The solution was washed with sat. NaHCO$_3$, distilled water, and sat. NaCl. The organic layer was dried using MgSO$_4$, filtered and evaporated in vacuo. The crude solid obtained was purified by flash chromatography using 10% MeOH in DCM (Rf = 0.1) as eluent to afford the product (394 mg, 44%) as yellow oil. $^1$H NMR (500 MHz, Chloroform-d) $\delta$ 7.45 – 7.41 (m, 2H), 7.37 – 7.32 (m, 2H), 7.31 – 7.27 (m, 1H), 5.43 (dt, J = 1.2, 0.6 Hz, 1H), 5.27 (q, J = 1.3 Hz, 1H), 3.68 – 3.64 (m, 2H), 2.80 (s, 1H), 2.44 (s, 3H).

(3R,4R)-2,5-dioxotetrahydrofuran-3,4-diyl diacetate (12)

In a 150 mL RBF cooled to 0 °C, (L)-(+)–tartaric acid (5.08 g, 33.8 mmol) was suspended in 12 mL acetic anhydride. Four drops of concentrated sulfuric acid was added to the solution and stir for 5 mins at 0 °C. The mixture was refluxed for 15 mins. The solution was cooled down to room temperature then -10 °C to recrystallize. The fine white crystals were filtered, washed with toluene then diethyl ether to afford the product (5.84 g, 80%). $^1$H NMR (500 MHz, Chloroform-d) $\delta$ 5.68 (s, 2H), 2.23 (s, 6H).

**General procedure to synthesize oxoacetamide from secondary amine**

1) HNRR’, DCM, 35 °C, 1.5 hrs
2) NaOMe, MeOH, rt, 3 hrs
3) NaIO$_4$, THF/H$_2$O, rt, 10 mins

**Scheme 15.** General synthetic scheme of dihydroxyacetamide.
The secondary amine (1.0 eq.) was added to a solution of 12 (1.1 eq.) in 0.3 M dry DCM cooled to 0 °C. The solution was stirred for an additional 15 mins at room temperature. The solution was refluxed. After 1.5 hours, the solution was cooled to room temperature and diluted with DCM. The solution was washed with sat. NaHCO₃ twice. The aqueous layers were combined and acidified using 1 M HCl until pH ~5. The solution was washed with EtOAc twice. The organic layers were combined and washed distilled with water three times, and then with sat. NaCl. The organic layer was dried using MgSO₄, filtered and evaporated in vacuo. The product was redissolved in dry MeOH and then 25% (w/w) NaOMe (2.0 eq.) was added and stirred for 3 hours. After 3 hours, the pH of the solution was neutralized to ~7 pH using Dowex ® 50WX8 hydrogen form. The resin was filtered and the solution was evaporated in vacuo to afford the diol. The diol was redissolved in THF:H₂O (2:1) (0.2 M). Sodium periodate (1.1 eq.) was added and was stirred at room temperature for 10 mins. The solution was diluted with EtOAc and washed with distilled water, sat. NaHCO₃, distilled water, and sat. NaCl. The organic layer was dried using MgSO₄, filtered and evaporated in vacuo to afford the product.

**N-cinnamyl-2,2-dihydroxy-N-isopropylacetamide (13)**

![Structure](image)

**1H NMR** (500 MHz, Chloroform-d) δ 7.52 – 7.11 (m, 5H), 6.75 – 6.38 (m, 1H), 6.28 – 6.08 (m, 1H), 5.87 – 5.27 (m, 1H), 4.76 – 3.84 (m, 3H), 1.35 – 1.09 (m, 6H).

**2,2-dihydroxy-N-isopropyl-N-(2-phenylallyl)acetamide (14)**

![Structure](image)

**1H NMR** (500 MHz, Chloroform-d) δ 7.53 – 7.13 (m, 5H), 5.95 – 5.02 (m, 3H), 5.02 – 3.85 (m, 3H), 1.33 – 1.15 (m, 6H).

**N-cinnamyl-2,2-dihydroxy-N-methylacetamide (15)**

![Structure](image)

**1H NMR** (500 MHz, Methanol-d₄) δ 7.42 – 7.16 (m, 5H), 6.55 (dd, J = 16.0, 6.7 Hz, 1H), 6.18 (ddt, J = 31.9, 15.9, 6.2 Hz, 1H), 5.16 (d, J = 7.2 Hz, 1H), 4.18 (ddd, J = 5.8, 4.2, 1.5 Hz, 1H), 4.13 (ddd, J = 17.8, 6.3, 1.5 Hz, 1H), 3.00 (d, J = 66.0 Hz, 3H).
2,2-dihydroxy-N-methyl-N-(2-phenylallyl)acetamide (16)

\[ \text{H NMR (500 MHz, Methanol-}d_4 \text{) } \delta 7.45 - 7.40 (m, 2H), 7.37 - 7.24 (m, 3H), 5.47 (dq, } J = 4.0, 0.9 \text{ Hz, 1H), 5.18 - 5.04 (m, 2H), 4.62 - 4.34 (m, 2H), 2.96 (d, } J = 21.1 \text{ Hz, 3H).} \]

5-isopropyl-1-methyl-3-phenylhexahydro-6H-pyrrolo[3,4-c]isoxazol-6-one (17)

This compound was not isolated. \(^1\)H NMR spectrum of crude in Appendix I.

1,5-dimethyl-3-phenylhexahydro-6H-pyrrolo[3,4-c]isoxazol-6-one (18)

This compound was not isolated. \(^1\)H NMR spectrum of crude in Appendix I.

(1S,5S)-3-isopropyl-7-methyl-5-phenyl-6-oxa-3,7-diazabicyclo[3.2.1]octan-2-one (19)

This compound was not isolated. \(^1\)H NMR spectrum of crude in Appendix I.

(1S,5S)-3,7-dimethyl-5-phenyl-6-oxa-3,7-diazabicyclo[3.2.1]octan-2-one (20)

This compound was not isolated. \(^1\)H NMR spectrum of crude in Appendix I.
3.4.2 Procedure for monitoring the reactivity of second generation probes using $^1$H NMR

3.4.2.1 Preparation of the reaction in NMR tubes

Sodium acetate (2.2 eq) and $N$-methylhydroxylamine hydrochloride (1.1 eq.) were added to a solution of the probe (~10 mg, 1 eq.) in deuterated methanol (25 mM) in a half-dram vial. The solution was mixed by pipetting five times. The solution was transferred to a 3 mm NMR tube and kept at 37 °C. $^1$H NMR was taken at time 0, 3, 6, and 24 hours.

3.4.2.2 Calculation of percentage conversion of cycloadducts

The average of the integration of new sp$^3$ hybridized H’s that do not overlap with any other signal divided by the sum of the average of the integration of new sp$^3$ hybridized H’s and the average integration of the sp$^2$ hybridized H’s at a given time (Equation 1).

$$\frac{(\text{average of new sp}^3\text{ hybridized peaks})}{(\text{average of new sp}^3\text{ hybridized peaks}) + (\text{average alkene peaks})} \times 100 = \% \text{ conversion}$$

Equation 1. Calculation of percentage conversion of cycloadducts of second-generation probes with $N$-methylhydroxylamine.

3.4.2.3 Example of calculation of percentage conversion of cycloadducts

The reaction of probe 15 with $N$-methylhydroxylamine is used as an example of the calculation of percentage conversion (Table 3). Time 3 hours and 6 hours were the only time points used. Time 0 hour has no new sp$^3$ hybridized peaks. On the other hand, time 24 hours has no alkene peaks. Percentage conversion of probe 15 with $N$-methylhydroxylamine to yield cycloadduct 19.
<table>
<thead>
<tr>
<th>Probe 15</th>
<th>$sp^2$ peaks integration</th>
<th>$sp^3$ peaks integration</th>
<th>% conversion</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t = 3$ hrs</td>
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<td>1.02</td>
<td>2.62</td>
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<tr>
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<td>67</td>
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<td>---</td>
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</tr>
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<td>5.30</td>
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<tr>
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<td>5.65</td>
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<tr>
<td>$t = 6$ hrs ave</td>
<td>1.01</td>
<td>5.39</td>
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</tbody>
</table>

**Table 3.** Calculation of percentage conversion of second-generation probes (probe 15 as example). Integration and average value of alkene peaks under $sp^2$ column at a given time. Integration and average value of new $sp^3$ hybridized H’s under $sp^3$ column at a given time. Calculated percentage conversion of probe 15 under column % conversion at a given time using Equation 1.
Chapter 4

4 Summary and Future Directions

4.1 Summary

Bioorthogonal chemistry is a key concept in the field of biological chemistry. It enables scientists to study the nature and kinetics of events in living biological systems. Bioorthogonal chemistry requires incorporation of a unique functional group (orthogonal tag) on the molecule of interest via biosynthetic or biochemical pathway. Later, this tag reacts selectively and readily with its reactive partner, which can be functionalized with a fluorescent probe or affinity handle to form a stable adduct, ultimately tagging the molecule of interest.

A number of orthogonal tags have been developed over the past two decades. This includes azide-alkyne cycloaddition, aldehyde/ketone condensation, tetrazine ligation, and nitrone-strained cyclooctyne cycloaddition, which were discussed in Chapter One.

We proposed to use $N$-hydroxylamine as a new orthogonal tag and a bifunctional probe with aldehyde and alkene as its reactive partner which could form a stable isoxazolidine cycloadduct. This reaction (a) does not require metal catalysis and the orthogonal tag could be activated (azide-alkyne cycloaddition); (b) is not reversible (aldehyde/ketone condensation); (c) does not produce undesired side product (e.g. nitrogen gas) (tetrazine ligation); and (d) does not require multiple incubations (nitrone-strained cyclooctyne cycloaddition).

With our proposed method, we designed our reactive partner probes that have both an aldehyde and alkene components. We showed that a hydroxylamine functional group could be installed on glucose skeleton (compound 4) in 4 steps. With this construct, we found that intermolecular cycloaddition of nitrones with an alkene (maleimide) needed heating for the reaction to occur over a reasonable time period, suggesting that this reaction had a high activation energy. Heating the reaction to 85 °C was necessary to observe the isoxazolidine adduct in an overnight incubation (Chapter Two). Thus, intermolecular cycloaddition of nitrone and alkene would not be valuable for biological uses.
We synthesized a simple biphenyl derivative probe 6 as our first-generation intramolecular hydroxylamine coupling partner. After the condensation of glucose derivative 4 with probe 6 to form the nitrone, the [3 + 2] cycloaddition was not observed at room temperature. Heating the reaction to 100 °C was necessary to observe the formation of the cycloadduct. But even after heating the reaction for 18 hours, the major product isolated was the nitrone intermediate. To determine if it was due to steric hindrance, probe 6 was reacted with N-methylhydroxylamine. The reaction also did not form the cycloadduct at room temperature even after 4 days. However, the reaction of N-phenylhydroxylamine and biphenyl probe 6 resulted in the formation of the cycloadduct at room temperature, however, the reaction was not complete after 3 hours. Thus, we determined that the formation of the isoxazolidine adduct is determined by the electronics of the 1,3-dipole and the dipolarophile. Based on our results presented in Chapter Two, we designed and synthesized our second-generation probes.

We designed our second-generation probes based on the predicted transition state of the cycloaddition. Initially, we designed our probes to have an oxoacetamide. The rational was two-fold. First, the nitrone formed after the condensation of the beta-carbonyl would result in an activated nitrene from the resulting acetamide, lowering the energy of the LUMO of the 1,3-dipole. Second, the acetamide enables us to add an extra bulky N-substituent to bias the ground state conformation towards the cis-amide. In addition, we activated the alkene with a phenyl group to increase the energy of the LUMO of the dipolarophile (Schemes 12-13).

We synthesized probes 13-16 and reacted them with N-methylhydroxylamine. We monitored their reactions to form the isoxazolidine adduct using ¹H NMR. Gratifyingly, the [3 + 2] cycloaddition was observed from all four probes at room temperature. The reaction was done within 24 hours. However, surprisingly there were no significant differences in the observed addition rates between all four probes (Chapter Three).

We therefore conclude that N-hydroxylamine is a good candidate to be a new orthogonal tag and a bifunctional probe with aldehyde and alkene as its reactive partner to form a stable isoxazolidine cycloadduct. The reaction has relatively fast kinetics (in biological time scale). It is also high yielding with no undesirable side products.
4.2 Future Directions

Future work will focus on verifying that $N$-hydroxylamine is a viable orthogonal tag in biological settings. Our plan has two parts. First, we plan to synthesize our third-generation probes, derived from our second-generation probes, which are functionalized with fluorophores. Second, we plan to modify sialic acid with $N$-hydroxylamine and subsequently feed this derivative to the glycosylation machinery of cells.

For the first part of our plan, we will initially synthesize the unactivated alkene derivative of our second-generation probes to determine if the phenyl substituent on the alkene does improve the kinetics of its $[3 + 2]$ cycloaddition with the nitrone. We previously synthesized the unactivated alkene derivative with methyl as the extra amide substituent (Figure 8 (a)). However, we failed to purify this compound due to its extreme polarity (partially soluble in water) to perform our $^1$H NMR experiment with $N$-methylhydroxylamine. Thus, we propose to synthesize the benzyl derivative (Figure 8 (b)) to decrease the polarity.

![Chemical structures](image)

**Figure 8.** Third-generation probes. (a) polar unactivated alkene derivative with $N$-methyl of second-generation probes (b) less polar proposed unactivated alkene derivative with $N$-benzyl of second-generation probes (c) unbranched third-generation probe (d) branched-third generation probe.

In addition, we will perform experiments to determine the stability of our second-generation probes and their corresponding cycloadducts. Depending on the results that we would gather from these experiments, we will decide to either synthesize the unbranched (Figure 8 (c)) or the branched (Figure 8 (d)) as our third-generation probe.
For the second part our plan, we will synthesize sialic acid (Neu5Ac to be more specific) modified with \( N \)-hydroxylamine as per Scheme 15. We only have to perform the last step of the synthesis for we have the OTs-derivative sialic acid in hand.

\[
\begin{align*}
\text{HO} & \quad \text{COOH} \\
\text{HO} & \quad \text{AcHN} \\
\text{HO} & \quad \text{OH} \\
\end{align*}
\rightarrow

\begin{align*}
\text{HO} & \quad \text{COOMe} \\
\text{AcHN} & \quad \text{OH} \\
\text{HO} & \quad \text{OH} \\
\end{align*}
\rightarrow

\begin{align*}
\text{TsO} & \quad \text{COOMe} \\
\text{HO} & \quad \text{AcHN} \\
\text{HO} & \quad \text{OH} \\
\end{align*}
\rightarrow

\begin{align*}
\text{HOHN} & \quad \text{COOMe} \\
\text{AcHN} & \quad \text{OH} \\
\end{align*}

**Scheme 16.** Schematic of the synthesis of sialic acid modified with \( N \)-hydroxylamine.

In eukaryotic cells, all cell surfaces are decorated with glycans. There are nine kinds of monosaccharide, including sialic acids. Sialic acids often reside on the outermost ends of these glycan chains. There are more than 50 chemically diverse sialic acids on cell surfaces. The two main ones are \( N \)-acetylneuraminic acid (Neu5Ac) and \( N \)-glycolyneuraminic acid (Neu5Gc)\(^{59}\).

As proposed above, we plan to synthesize Neu5Ac modified with \( N \)-hydroxylamine. We will culture cells in the presence of the sialic acid \( N \)-hydroxylamine derivative to introduce the unnatural functional group on cell surfaces by hijacking the glycosylation machinery. Subsequently tagging cells with our third-generation probes. This is similar to what van Delft and coworkers\(^{42}\) tried with nitrone modified monosaccharides but were unsuccessful as discussed in Chapter One.
References


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Appendix I

$^1$H NMR of 2
$^1$H NMR of 3

$^1$H NMR of 4
$^1$H NMR of 5a

Crude $^1$H NMR of the other diastereomers of 5a
$^{1} \text{H NMR of 5b}$

Crude $^{1} \text{H NMR of the other diastereomers of 5a}$
$^1$H NMR of 5c - major

COSY of 5c - major
NOESY of 5c - major

NOESY of 5c – major (zoomed)
$^1$H NMR of 5c – minor

COSY of 5c - minor
NOESY of 5c - **minor**

![NOESY of 5c - minor](image1)

NOESY of 5c – **major** (zoomed)

![NOESY of 5c - major](image2)
$^1$H NMR of 5e

$^1$H NMR of 6
$^1$H NMR spectra of probe 6 with $N$-methylhydroxylamine at time 0, 1, and 3 hours and 4 days (from bottom to top) of to form $6b$

$^1$H NMR spectra of probe 6 with $N$-phenylhydroxylamine at time 0, 1, and 3 hours (from bottom to top) of to form $6c$
$^1$H NMR of 7

$^1$H NMR of 9
$^1$H NMR of 10

$^1$H NMR of 11
$^1$H NMR of 12

$^1$H NMR of 13
$^1$H NMR of 14

$^1$H NMR of 15
$^1$H NMR of 16

$^1$H NMR of crude 17
$^1$H NMR spectra of probe 13 with N-methylhydroxylamine at time 0, 3, 6, and 24 hours (from bottom to top) of to form 17

Zoomed $^1$H NMR spectra of probe 13 with N-methylhydroxylamine at time 0, 3, 6, and 24 hours (from bottom to top) of to form 17
$^1$H NMR of crude 14

$^1$H NMR spectra of probe 14 with N-methylhydroxylamine at time 0, 3, 6, and 24 hours (from bottom to top) of to form 18
Zoomed $^1$H NMR spectra of probe 14 with N-methylhydroxylamine at time 0, 3, 6, and 24 hours (from bottom to top) of to form 18

$^1$H NMR of crude 19
$^1$H NMR spectra of probe 15 with N-methylhydroxylamine at time 0, 3, 6, and 24 hours (from bottom to top) of to form 19

Zoomed $^1$H NMR spectra of probe 15 with N-methylhydroxylamine at time 0, 3, 6, and 24 hours (from bottom to top) of to form 19
$^1$H NMR spectrum of crude 16

$^1$H NMR spectra of probe 16 with N-methylhydroxylamine at time 0, 3, 6, and 24 hours (from bottom to top) of to form 20
Zoomed $^1$H NMR spectra of probe 16 with N-methylhydroxylamine at time 0, 3, 6, and 24 hours (from bottom to top) of to form 20