UNIVERSITY OF CINCINNATI

Date: 06/26/06

I, Rebecca Marie Vermillion, hereby submit this work as part of the requirements for the degree of:
Masters of Science in:
Chemistry
It is entitled:
Synthesis of multivalent glycoconjugates for the Detection of pathogens

This work and its defense approved by:

Chair: Dr. Suri Iyer
Dr. James Mack
Dr. Carl Seliskar
Synthesis of multivalent glycoconjugates for the detection of pathogens

A thesis submitted to the

Division of Research & Advanced Studies

of the University of Cincinnati

in partial fulfillment of the requirements for the degree of

Masters of Science

in the Department of Chemistry

of the College of Arts & Sciences

2006

By

Rebecca Marie Vermillion

B.S., University of Cincinnati, 2004

Committee Chair: Suri S. Iyer, Ph.D.
Abstract

To meet the need for speed and accuracy in detecting and analyzing a biothreat incident, oligosaccharides have been synthesized that are stable under ambient conditions. They can be used as a fingerprint system for specific pathogens by integrating them into various biosensor systems. Amine containing multivalent saccharides can be directly attached to a fluorescent dye or can be extended with several sugar residues to create a detection system which can differentiate between types of pathogens that are of interest to the nation’s defense program.
Acknowledgements

I would like to thank my advisor, Dr. Suri S. Iyer, for giving me the opportunity to be a part of his research and for directing me throughout this project. Also, I sincerely appreciate the help of our post doctoral fellow, Dr. Ramesh R. Kale for his guidance and our director, Dr. Thomas Ridgway for his encouragements.

I would also like to thank my committee members, Dr. James Mack and Dr. Carl Seliskar, for their continued support throughout my years at UC.

I appreciate the assistance of Dr. Stephen Macha in running my mass spectroscopy samples and Dr. Elwood Brooks for use of the NMR spectrometer and help in the interpretation of the results.

Lastly, I would like to thank my family: Dr. Karl Vermillion and Dr. Joan Furilla, Christine and D. Scott Vermillion, Joshua D. Lederer, and Christopher M. Haun for their heartfelt support through all aspects of my degree, both academically and emotionally.
Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>6</td>
</tr>
<tr>
<td>List of Schemes</td>
<td>7</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>8</td>
</tr>
<tr>
<td>I. Introduction</td>
<td>10</td>
</tr>
<tr>
<td>A. Botulinum toxin</td>
<td>14</td>
</tr>
<tr>
<td>1. Incidence and routes of infection</td>
<td>15</td>
</tr>
<tr>
<td>2. Detection</td>
<td>16</td>
</tr>
<tr>
<td>3. Prophylaxis</td>
<td>17</td>
</tr>
<tr>
<td>4. Biological activity</td>
<td>18</td>
</tr>
<tr>
<td>B. Human influenza virus</td>
<td>21</td>
</tr>
<tr>
<td>1. Incidence and routes of infection</td>
<td>21</td>
</tr>
<tr>
<td>2. Detection</td>
<td>24</td>
</tr>
<tr>
<td>3. Prophylaxis</td>
<td>27</td>
</tr>
<tr>
<td>4. Biological activity</td>
<td>30</td>
</tr>
<tr>
<td>C. References</td>
<td>34</td>
</tr>
<tr>
<td>II. Scaffold synthesis</td>
<td>38</td>
</tr>
<tr>
<td>A. Dimeric scaffold for Botulinum toxin</td>
<td>38</td>
</tr>
<tr>
<td>B. Tetrameric scaffold for Influenza virus</td>
<td>39</td>
</tr>
<tr>
<td>C. Experimental</td>
<td>41</td>
</tr>
<tr>
<td>D. References</td>
<td>43</td>
</tr>
<tr>
<td>III. Sugar synthesis</td>
<td>44</td>
</tr>
<tr>
<td>A. Sugar derivatives</td>
<td>44</td>
</tr>
<tr>
<td>B. Coupling the sugars</td>
<td>47</td>
</tr>
<tr>
<td>C. Scaffold attachment</td>
<td>49</td>
</tr>
<tr>
<td>D. Experimental</td>
<td>50</td>
</tr>
<tr>
<td>E. References</td>
<td>55</td>
</tr>
<tr>
<td>IV. Future work</td>
<td>56</td>
</tr>
<tr>
<td>V. Appendix</td>
<td>58</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Structures of monomeric and oligomeric Pk inhibitors of Shiga-like toxins. 1, Pk trisaccharide methyl glycoside; 2, dimeric bridged Pk trisaccharide; 3, decameric</td>
<td>13</td>
</tr>
<tr>
<td>Figure 2</td>
<td>1,3-dipolar cycloaddition to a microtiter plate well</td>
<td>14</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Three-dimensional structure of BoNT type A. The molecule is comprised of three functional domains: the carboxyterminus of the heavy chain (orange), the aminoterminus of the heavy chain (green), and the light chain (blue)</td>
<td>20</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Structure of Influenza virus and its life cycle through a human cell</td>
<td>35</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Numbering scheme for sialic acid.</td>
<td>47</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Numbering scheme for lactose.</td>
<td>48</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Sample SDS-PAGE result</td>
<td>58</td>
</tr>
</tbody>
</table>
## List of Schemes

<table>
<thead>
<tr>
<th>Scheme</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheme 1: Synthesis of dimeric scaffold</td>
<td>40</td>
</tr>
<tr>
<td>Scheme 2: Synthesis of tetrameric scaffold</td>
<td>41</td>
</tr>
<tr>
<td>Scheme 3: Synthesis of linker</td>
<td>46</td>
</tr>
<tr>
<td>Scheme 4: Synthesis of sialic acid derivative</td>
<td>47</td>
</tr>
<tr>
<td>Scheme 5: Synthesis of lactose derivative</td>
<td>48</td>
</tr>
<tr>
<td>Scheme 6: Synthesis of trisaccharide</td>
<td>49</td>
</tr>
<tr>
<td>Scheme 7: Attachment of trisaccharide to dimeric scaffold</td>
<td>50</td>
</tr>
<tr>
<td>Scheme 8: Attachment of trisaccharide to tetrameric scaffold</td>
<td>57</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Ac</td>
<td>acetate</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>Boc</td>
<td>t-Butoxycarbonyl</td>
</tr>
<tr>
<td>BoNT</td>
<td>Botulinum neurotoxin</td>
</tr>
<tr>
<td>bz</td>
<td>benzoyl</td>
</tr>
<tr>
<td>CDMT</td>
<td>2-Chloro-4,6-dimethoxy-1,3,5-triazine</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMAP</td>
<td>Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>H</td>
<td>heavy chain of BoNT</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HANNA</td>
<td>Handheld Advanced Nucleic Acid Analyzer</td>
</tr>
<tr>
<td>L</td>
<td>in experimental section, liter</td>
</tr>
<tr>
<td>L</td>
<td>in introduction, light chain of BoNT</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>ml</td>
<td>milliter</td>
</tr>
<tr>
<td>mol</td>
<td>mole</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>NMM</td>
<td>N-methylmorpholine</td>
</tr>
</tbody>
</table>
### Abbreviations (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTNH</td>
<td>Nonhemagglutinin</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBDMS</td>
<td>t-Butyldimethylsilyl</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TIPS</td>
<td>Triisopropyl phosphite</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
</tbody>
</table>
I. Introduction

Recent terrorist events have led to an increased interest in developing environmental monitoring systems and portable sensors capable of detecting harmful pathogens quickly. Several new technologies could potentially be developed for hand held biosensors. For example, the Handheld Advanced Nucleic Acid Analyzer (HANAA) is a hand held biosensor that can run several samples simultaneously or conduct four tests on each sample in less than 30 minutes. However, it requires PCR amplification and the first responder must have some insight of the pathogen which is to be detected so that the correct fluorescent DNA probe is used.\(^1\) Obviously, this makes the HANNA undesirable while doing fieldwork. In an effort to recognize and quarantine an aerial attack, the United States employs a program called BioWatch. It was developed by US National Laboratories to collect aerosol samples from existing EPA air quality monitoring stations and test them for potential threats to public safety. However, these samples must be shipped to a central laboratory for analysis; therefore this early warning system is not a true “real time” biosensor.\(^2\) Additionally, there is a paramount need to detect pathogens from other media besides air, such as serum, blood, drinking water, and food. Other more conventional methods, such as mass spectroscopy and surface Raman spectroscopy, are emerging. However, these technologies require sophisticated personnel and equipment and are incredibly bulky.

Currently, antibodies are the standard recognition element for hand held biosensors. However, these antibody-based sensors face many caveats that expose the need for a faster, more reliable system. These downfalls include decay of antibodies under mild temperature and pH changes. Also, antibodies are highly cross-reactive, leading to a decrease in the sensitivity and selectivity of the sensor. Most antibody-based tests require trained personnel to run and interpret the results, thus making this type of test slow and costly. Recognizing these problems, research into small molecule receptors, such as peptoids and carbohydrates as antibody alternatives has emerged over the past few years.\(^3\) In particular, carbohydrates are attractive because they are natural receptors for many cellular processes. Additionally, carbohydrates are very robust and can withstand temperature and pH changes.

In the past, carbohydrates have not been the receptors of choice due to their low single site binding affinities as compared to antibodies. Nature compensates for this barrier by increasing the valency of carbohydrate-protein interactions.\(^4\) In essence, a cluster of different sugar residues is
presented to a multimeric receptor. The multiple binding events raise both the effective binding affinities and the specificity. Since each pathogen has several hundred binding sites, binding events are maximized when there are several sites available. Biomimetic approaches using sugar-based scaffolds of tethered individual sugar residues on a central molecule or polymer backbone have already been used by several groups. These groups have noticed that “random multivalency” is extremely inefficient. However, recent studies had shown that multivalent ligands are critical for recognition, though they must be tailored instead of random. This strategy of rational structure based ligands was successfully demonstrated for Shiga-like toxins by Bundle and coworkers in 2000 (see Figure 1, below). It was established that the inhibitory activity of multivalent carbohydrate ligands were 10-million-fold higher than the univalent trisaccharide for these Shiga-like toxins.5

![Figure 1: Structures of monomeric and oligomeric Pk inhibitors of Shiga-like toxins. 1, Pk trisaccharide methyl glycoside; 2, dimeric bridged Pk trisaccharide; 3, decameric STARFISH](image)

Carbohydrate microarrays are ideal for pathogen detection because they present carbohydrate ligands on a surface that mimics interactions at cell interfaces. Since carbohydrate-based ligands imitate particular sugars on the surface of the cell, this approach can potentially be exceptionally specific and sensitive. However, when compared to DNA or protein microarrays, carbohydrate microarrays are still undeveloped. This is primarily because the synthesis of complex sugars is not trivial nor is it easy to isolate pure sugars from natural sources. Nevertheless, “proof of concept” carbohydrate microarrays...
have appeared recently.\textsuperscript{6} For example, Mrkisch and coworkers covalently immobilized a microarray of ten monosaccharides by conjugation to self-assembling monolayers of alkenethiols on a gold surface using a high yielding Diels-Alder cycloaddition product. The monosaccharides were evaluated by profiling the binding specificities of labeled plant lectins known to bind to different monosaccharides. The saccharides were covalently immobilized onto separate wells on a microplate and each well was carefully observed for binding. Five microplates were tested against five lectins and each plate showed selective binding of the lectin to the carbohydrate for which it was known to bind.\textsuperscript{7} Alternatives to this fabrication process have also been reported. Wong and coworkers noncovalently immobilized a long-chain aliphatic alkyne at specific locations on a microtiter surface and used 1,3-dipolar addition to couple carbohydrates bearing azide groups to the alkynes (see Figure 2, below). Three different tetrasaccharides were tested, one which contained fucose and two that did not. They were tested against an enzyme that was specific for fucose and it was observed that only the fucose-containing sugar tested positive for binding with the enzyme.\textsuperscript{8}

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure2.png}
\caption{1,3-dipolar addition to a microtiter plate well.\textsuperscript{8}}
\end{figure}
Polysaccharides obtained from natural sources have also been utilized in microarrays. Wang and coworkers engineered a high-precision robotic cDNA based arrayer to spot dextrans derived from different strains of bacteria on nitrocellulose coated glass slides. After air drying, the model system was incubated with monoclonal antibodies specific to different micro structures of the polysaccharide. The results showed that the immunological properties of the dextrans were not compromised. In addition, human serum from 20 patients was applied to the microarray to detect human serum antibodies. The assays were successful as 12 distinct specificities of IgM antibodies and 35 IgG anti-carbohydrates were identified. A similar approach was described by Willets et al. using polysaccharides derived from plant cell walls. The glycoarray was probed with hybridoma antibodies and the predicted patterns of antibody binding were observed using labeled secondary antibodies. The major concerns related to obtaining polysaccharides from plant or animal sources is that there is lot to lot variation and potential contamination because polysaccharides are heterogeneous and complex in nature.

The results of these carbohydrate-based microarrays show tremendous potential, but they are nothing more than "proof of concept" experiments. They do not address the problem of non-specific binding, so they cannot take advantage of the libraries of previously characterized protein-carbohydrate interactions. Likewise, they do not differentiate between closely related moieties.

By synthesizing ligands with certain pathogens in mind, it is possible to tailor these microarrays for a few pathogens only. Human influenza virus has specialized proteins on their surface that enable them to bind to specific sugars. Botulinum toxin also binds specifically to sialic acid residues. Both target the same sialic acid on the surface of the cell. Therefore, similar ligands can be used to detect and differentiate both of these pathogens, however small changes in the structure and density of the ligands will be used to differentiate between the two pathogens. These two pathogens will be the main focus. The same ligands can later be used in detection of other pathogens.
**A. Botulinum Toxin**

Botulinum neurotoxin (BoNT) is a uniquely potent substance. For decades, it has been described as “the most poisonous of poisons”.\(^{11}\) In fact, the minimum concentration needed to disrupt cell functionality has not been determined.\(^{12}\) It is estimated that a single gram of crystalline toxin, evenly distributed and inhaled, can kill more than one million people. It is this poisonous nature that makes BoNT so distinctive. Because BoNT is lethal, accessible, and easy to transport, it has become a major bioterrorism threat.\(^{13}\) On the other hand, BoNT is currently medically licensed for treatment of the genetic neurological movement disorder called dystonia. Dystonia is often characterized by the invocation of involuntary, sustained facial muscle contractions and intramuscular injections of small amounts of BoNT can return these muscles to their normal working condition.\(^{14}\) Still, there are many naturally occurring cases involving BoNT, not due to malicious or medical intents, but rather due to careless handling of food or contamination of wounds.

BoNT is synthesized by the bacteria *Clostridium botulinum*, *Clostridium baratii* and *Clostridium butyricum*. Different types of *C. botulinum* produce antigenically distinguishable neurotoxins which have been labeled A through G, although only types A,B,E and F, cause illness in humans. The toxin types are defined by their absence of cross-neutralization (i.e. the antitoxin for type A does not neutralize types B-G, and so on). Type A is most common in the Rocky Mountain area of the United States. It causes more severe and longer lasting paralysis than type B, which is widespread throughout the Eastern seaboard. Types C and D effect animals, most notable amongst birds (“Avian botulism” or “limber neck”) and horses (“Shaker Foal Syndrome”). The most frequent outbreaks of Type E is throughout the Great Lakes and Alaskan areas, as it is found in freshwater sediment and is often associated with the ingestion of these indigenous fish. Type G has recently been identified, and has not yet been confirmed as a cause of illness in humans or animals.\(^{15}\)

BoNT type A has been the first bacterial toxin to be FDA approved as a drug.\(^{13}\) In 1989, Oculinum was licensed to treat two eye conditions (blepharospasm and strabismus) characterized by excessive muscle contractions that often inhibit the vision ability of the patient. The toxin is now marketed under the trade name Botox. Small doses of the purified toxin are injected into the affected muscles. As with botulism, the toxin binds to the nerve endings, blocking acetylcholine release, which would otherwise
signal the muscle to contract. The toxin paralyzes the muscle, leaving other muscles unaffected. Normal function is often restored.  

1. Incidence and routes of infection

BoNT acts preferentially on peripheral cholinergic nerve endings to block the release of acetylcholine (ACh), a neurotransmitter that signals muscles to contract. This causes a serious paralytic illness called botulism. Botulism incubates for anywhere between 2 hours and 1 week. Symptoms are mostly physical effects caused by the inhibition of acetylcholine, such as vision problems, constipation, and slurred speech. If untreated, these symptoms may progress to cause paralysis of the arms, legs, trunk, and respiratory muscles, ultimately causing the patient to stop breathing.

There are four main kinds of botulism, distinguished by the manner in which they are contracted: wound, foodborne, infant, and airborne. Wound botulism is very rare. *C. botulinum* bacteria produce the toxin directly in an infected wound. It is almost exclusively found in California and is associated with intravenous drug users. Wound botulism has a relatively low mortality rate (5-10%), but the symptoms can continue for more than one year.

Foodborne botulism is cause from ingestion of the toxin itself by consumption of contaminated food. Because BoNT thrives in anaerobic environments, it is often a consequence of improperly home-canned foods. Specifically, foods with low acid content offer a natural harbor for the bacteria (asparagus, green beans, and corn). Another widespread source of the toxin is freshwater fish, as spores have been found in marine sediment that the fish live in and eat.

Infant botulism differs from foodborne botulism in that the toxin itself is not ingested. Instead, *C. botulinum* spores swallowed by the infant germinate and produce the toxin in the favorable environment of the baby’s intestines. Because the spores are present everywhere in nearly every environment, adults and children regularly ingest them. The general populous rarely suffers illness because their gastrointestinal tracts quickly destroy the spores. The spores flourish in gastrointestinal tracts where the normal flora has not been established, such as infants. Occasionally, adults who have had intestinal surgery or whose intestinal tracts have otherwise been altered have contracted the disease this way.
Interestingly, the main source of infant botulism is honey. The FDA estimates that about 10% of store-bought honey contains *C. botulinum* spores.\textsuperscript{15}

A fourth, man-made form of botulism results from the inhalation of aerosolized BoNT. Primarily, this mode of transmission is limited to bioterrorism attempts; although in 1962, three German veterinary personnel were accidentally infected by reaerosolized BoNT. Aerosolized BoNTs are of particular interest to bioterrorists due to its availability and its ability to infect large populations simultaneously. Inhalation is the most direct route of the toxin to the bloodstream, thus making it possibly a weapon of choice. BoNT is not a threat to public water systems. No instance of waterborne infection as ever been reported. BoNT is rapidly inactivated by standard potable water treatments (chlorination and aeration).\textsuperscript{13}

Any type of suspected botulism represents a potential public health emergency because of the possibility that a contaminated food remains in circulation to others or that a toxin has been deliberately released. In these cases, prompt intervention by the medical community and civil authorities is needed to prevent additional infections.

2. Detection

According to the Center for Disease Control, an average of 110 cases of botulism are reported each year.\textsuperscript{15} However, many cases of botulism go undetected because often it is misdiagnosed. It is easily confused with strokes, Guillain-Barre syndrome, chemical and bacterial food poisoning, chemical intoxication and diphtheria. In infant botulism, meningitis, mineral-electrolyte imbalance, and Reye syndrome are often incorrectly established as the causes for illness. If there is a large outbreak, the diagnosis is not difficult because it is suspected. However, since most cases are isolated the initial diagnosis poses a problem.

Routine laboratory studies are not helpful in confirming the suspicion of botulism. Renal and liver function tests, complete blood tests, urinalysis and electrocardiograms are not good indicators, unless a secondary complication has occurred. Special tests, such as MRIs, cerebrospinal fluid examinations, and nerve conduction tests help in the diagnosis of botulism; however these tests are not always accurate. The purpose of these tests is to exclude the common misdiagnosed conditions, not to test positive for botulism.\textsuperscript{18} The only direct way to confirm botulism is to isolate BoNT in the patient’s serum or stool and
then inject the sample into mice. If the mice exhibit signs of botulism, then the patient is considered positive for botulism. However, this test can only be done at the Center for Disease control or at a handful of state department laboratories and this test does not confirm a negative result. Also, these laboratory tests take several days to complete once the samples are received.\textsuperscript{15} The diagnosis of botulism often cannot await the results of the laboratory, especially in response to a bioterrorist attack with BoNT.

3. Prophylaxis

The administration of an equine antitoxin is the only therapy available for botulism. It is only effective if given within 24 hours of infection.\textsuperscript{12} It is not a “cure-all,” but will instead lower the fatality risk and shorten the duration of illness. Use of this antitoxin is limited by its scarcity; it is only available through the Center for Disease Control. The antitoxin is administered for adults with suspected botulism. Under no circumstances is the antitoxin given to infants, for fear of fatal complications.

The antitoxin is trivalent, with antibodies for each of the three most common types of BoNT (A, B, and E). However, a heptavalent antitoxin is under investigation that would treat A, B, C, D, E, F, and G. It is unlikely that it would be useful in an outbreak due to the time it takes to identify the serotype of the toxin in a patient and subsequently acquire and administer the heptavalent antitoxin.\textsuperscript{15}

The presence of neutralizing antibodies in the bloodstream can prevent botulism. Because the incidence of botulism in humans is low, no reason has existed for immunizing large parts of the population. However, it is useful to have a vaccine available, especially for laboratory personnel who might come in contact with BoNT and the military in the shadow of bioterrorist activities. An oral vaccine is currently being researched, based on the extensive research done on the most immunogenic parts of BoNT. There is hope that this will lead to a parental vaccine. Because the heavy chain itself does not invoke toxicity when distributed intranasally, it was tested as a potential inhalation vaccine. In animal studies performed by Simpson and coworkers, 100\% of the animals who received the preliminary vaccine survived, whereas 0\% of the animals that did not receive the vaccine died within 1 hour of infection.\textsuperscript{19}

Immunity can be provided by either the antitoxin or a vaccine of BoNT.\textsuperscript{13} Therefore, it is imperative to develop drug candidates for the neutralization of BoNT.
4. Biological activity

*C. botulinum* is a spore-forming, obligate anaerobe which produces BoNT, a 150kDa polypeptide. In specific, *C. botulinum* consists of four genetically different groups that share nothing in common except for the common characteristic of producing BoNT. The organisms that produce the toxin possess a protease that nicks the molecule to create a dichain structure of a heavy chain (H, 100kDa) and a light chain (L, 50kDa) linked by a single disulfide bond. This dichain form is the active form of the toxin. Figure 3 (below) shows the three-dimensional structure of BoNT type A. The C-terminal region (orange) of the H chain binds to the surface of target nerve cells. The N-terminal region (green) of the H chain translocates the L chain (blue) across membranes. The L chain contains a sequence of amino acids common to metalloproteinases. A tetrahedral zinc binding motif has been established for the L chain. It has been proposed that the L chain itself is a substrate for proteolytic cleavage that could terminate toxin action. The actual BoNT, depending on the serotype, has 30-40% sequence homology to tetanus toxin.

![Figure 3: Three-dimensional structure of BoNT type A. The molecule is comprised of three functional domains: the carboxyterminus of the heavy chain (orange), the aminoterminus of the heavy chain (green), and the light chain (blue).](image)

The toxin is released from bacteria as part of a noncovalent multimeric complex. This complex may help protect the toxin at lower pHs (such as the GI tract) and spontaneously dissociates at physiologic pH. Auxiliary proteins to the toxin include hemagglutinins (HA) and nonhemagglutinin
(NTNH). These auxiliary proteins are critical to the process of oral poisoning. When HA and NTNH are intertwined, they are extraordinarily resistant to the harsh conditions of enzymes and low pH in the stomach. However, there is evidence that these proteins are not essential to poisoning. Most cases of poisoning occur orally, although as previously stated, patients have occasionally contracted the disease through inhalation and contaminated wounds. This may simply be explained that the respiratory system does not exhibit the harsh conditions that the stomach does. Thus, auxiliary proteins are not essential to poisoning.²²

For oral poisoning, the small intestine has been determined as the major site of absorption, with the stomach and colon being minor absorption sites. Epithelial cells at these sites are the actual transport cells. However toxin molecules are too large for any significant diffusion to occur. Therefore, it is thought that the toxin binds exploitatively to a receptor that is linked to an efficient transport process. BoNT binds to the apical surface of epithelial cells and then undergoes receptor-mediated endocytosis and transcytosis, which ultimately delivers the toxin to the basolateral surface of the cells. In other words, the epithelial cells carry the toxin from the lumen of the gut to the interstitial fluid and then the general circulation.²⁵

For inhalational poisoning, the exact location of absorption has not yet been determined. Two schools of thoughts have come from studies done to determine this. One thought is that BoNT binds to alveolar epithelial cells in the lungs.²⁶ Another thought is that the toxin uptake occurs in the nasal epithelium. Either way, HA and NTNH are not seen as important for cells to transport the toxin to the general circulation.²²

Once in the general circulation, BoNT must exit the vasculature to reach the extracellular space in the vicinity of its target organs (cholinergic nerve cells).²⁷ As of this point, there have been no studies that describe toxin exit from general circulation. The phenomenon has been thought to be either an active transcellular process or simply a paracellular movement. However, it is well known that the toxin does not penetrate the blood-brain barrier.²²

Once the toxin reaches peripheral cholinergic nerve endings, there is a sequence of membrane-penetrating events. BoNT does not typically cause cell death; therefore it does not cross membrane barriers by killing cells in its path. Alternatively, the toxin can recognize and exploit transport processes
that are used by the cell. Initially, the toxin binds to the surface of plasma membranes. The substrates for the seven serotypes of the toxin have been determined, as well as the specific cleavage sites. BoNT types A and E act on synaptosomal-associated proteins of about 25kDa called SNAP-25; types B, D, F, and G act on vesicle-associated membrane proteins called VAMP (also called synaptobrevin); serotype C acts mainly on syntaxin, although it can also cleave SNAP-25. Each implicates two sialic acid binding sites.28,28

Binding is followed by receptor-mediated endocytosis and pH-induced translocation across the endosome membrane. BoNT has a hydrophobic domain (the H chain) that is inaccessible at normal pH but is exposed when the pH is lowered.30 The H chain, once exposed, inserts into the endosome membrane, creating a channel that the L chain can cross.31 The disulfide bond is then reduced, which allows the separation of H and L chains and restoration of the L chain from an acidic environment in the endosome membrane to the neutral environment of the cytosol. When L chain reaches the cytosol, it acts as a zinc-dependant endoprotease to cleave the polypeptides that are essential for exocytosis.32

Blockage of transmitter release accounts for the limp and drooping paralysis that is characteristic of the disease botulism. Although the toxin acts preferentially on cholinergic nerve endings, it does have the ability to block exocytosis from other nerve endings as well – such as norepinephrine and serotonin.

Both types A and B block spontaneous release of acetylcholine, but serotype A reduces the frequency of spontaneous release by approximately two orders of magnitude more than type B.33 Both types A and B block evoked transmitter release.34 Type A blocks an explosive release of acetylcholine in nerve endings by alpha-Latrotoxin, however type B does not.35 Serotypes C and E act similarly to type A, whereas types D and F act similar to type B.36

The duration of BoNT varies with serotype. Type A has the longest duration, lasting from many weeks to many months.37 No one yet knows the mechanism that accounts for termination of toxin action, although it is likely that the L chain is either diffused or metabolized by the cells.

The creation of a dimeric scaffold with two sialic acid residues is based on the research that has been done on the mechanism of action of BoNT, more specifically the cleavage locations.
B. Human influenza virus

Influenza, commonly known as the “flu” or the “grippe”, is a very contagious disease of the upper respiratory system. It is notorious for rapidly spreading around the world in seasonal epidemics, resulting in between three and five million cases of severe illness and between 250,000 and 500,000 deaths each year. Several health organizations, such as the World Health Organization and the Center for Disease Control, have specific programs for dealing with the epidemics of influenza that occur nearly every year during the winter months in the United States. It is estimated that 5-15% of the population are affected with this upper respiratory tract infection each year and 71-167 million USD is spent on hospitalizations, health care costs, and loss of productivity.  

There are three types of influenza which are distinguished by serological responses to their internal proteins, labeled A, B and C. Influenza A viruses can be further subtyped according to the reactivity of their surface antigens, haemagglutinin (HA) and neuraminidase (NA). To date, 15 HA subtypes (H1-H15) and 9 NA (N1-N9) subtypes have been found. The natural hosts for influenza A viruses are avian species, particularly aquatic birds. All of the HA and NA subtypes are found in avian species, whereas only three of the HA subtypes (H1, H2 and H3) and two of the NA subtypes (N1 and N2) have historically been associated with illness in humans. The two subtypes that are particularly harmful to humans are H3N2 and H1N1. Influenza A causes the most serious illness in humans due to its antigenic variability, which changes the immune response from the host. As the variability of the antigen changes, the virus can escape neutralizing antibodies and thus evade human immunity. Less antigenic variability is seen in influenza B and C viruses. Influenza B and C viruses circulate in humans, but are not divided into any subtypes. Rarely, influenza C can found in pigs. In particular, influenza C causes a less severe illness than influenza types A and B, similar to the common cold. Influenza C affects mainly children and elderly persons.

1. Incidence and routes of infection

Influenza is a high infectious disease that attacks the respiratory system of humans. Birds and waterfowl are the natural hosts for influenza. Usually, birds are asymptomatically infected but excrete high concentrations of influenza A in their feces. This serves as a reservoir of the disease for other
populations, such as humans, horses and pigs. This is especially true in Asia, where birds, pigs, and humans live in close proximity. Occasionally, highly pathogenic strains of the virus cause disease and high fatality rates in birds. The species barrier between birds and humans has been considered too great for direct transmission. More recently evidence suggests that the avian to human transmission may occur more frequently than previously thought, and the limitation to spread of infection may relate to the ability of the particular strain of virus to adapt to a mammalian host. There is little evidence for nonhuman reservoirs for influenza B and C viruses; therefore they do not pose a pandemic threat.40

The infectious nature of influenza causes periodic seasonal epidemics. Influenza A and B are responsible for these recurrent annual epidemics. Influenza A and B are antigenically distinct and do not exhibit cross-immunity, nor do they undergo genetic recombination. These viruses co-circulate and either may predominate in a particular influenza season. An increased incidence of influenza B frequently follows a peak of influenza A activity. In recent years, B viruses have tended to predominate every 2-3 years.

Influenza A viruses have undergone major genetic mutations in the H-component, resulting in global pandemics. This has happened three times in the last century. For definition purposes, the term pandemic will be reserved for instances in which outbreaks have been noted worldwide. The most infamous was the “Spanish Flu,” which is thought to have killed at least 40 million people between 1918 and 1919. More recently, two other influenza A pandemics occurred. In 1957 the “Asian Flu” (H1N1) and in 1986 “Hong Kong Influenza” (H3N2) caused significant morbidity and mortality globally. In contrast to current influenza epidemics, these pandemics were associated with severe outcomes among healthy younger populations. Although the names of these pandemics indicate that they hit Asia specifically, they were devastating to the entire world, including the US. Currently, limited outbreaks of a new influenza subtype H5N1 directly transmitted from birds to humans have occurred in Hong Kong Special Administrative Region of China in 1997 and 2003.42,43 Since only a few subtypes are established in humans, the population is especially vulnerable to severe infection by antigenically novel viruses.

As previously stated, genetic changes of influenza A are responsible for the different subtypes and increased threat. There are two types of antigenic change: drift and shift. Antigenic drift occurs when the genes that encode the viral surface antigens (NA and HA) undergo stepwise mutation. This
could potentially happen every time the virus replicates. Eventually, the surface antigens become sufficiently different that host antibodies are unable to neutralize the virus, resulting in a variant capable of causing illness through evasion of the host’s immune system. The second type of antigenic change is antigenic drift. It occurs far less frequently than antigenic drift. Shifting occurs when two different viruses, possibly each from a different host species, co-infect a single host, which then acts as a ‘mixing vessel. By reassortment of genome segments, a new virus is created that has elements from both the original viruses. This process results in unpredictable pathogenicity of the new virus, which may lack the requisite virulence factors or may possess full virulence for humans.39

Antigenic drift continues to produce epidemics of influenza in the years between pandemics. Influenza epidemics nearly always occur during winter months. The cold, damp conditions favor virus survival outside the host airway. Also, behavioral influences of the winter months may help fuel an epidemic, such as people spending longer amounts of time together indoors. Schoolchildren are also thought to play a central role in the spread of influenza due to their contact with other children and their undeveloped hygienic habits. Equatorial climates may not have such defined seasonality.44

Influenza is shed onto respiratory secretions, which are expelled into the air by coughing or sneezing. The microdroplets are then transmitted to the next host. Influenza infections range in severity from asymptomatic to serious illness with systematic features. Acute infection is preceded by a one- to two- day incubation period including symptoms of headache, malaise, myalgia, fever, and respiratory symptoms such as non-productive cough and sore throat of varied intensity. Most patients recover within one to two weeks without requiring any medical treatment. However, high risk groups, such as young children, elderly, and the chronically ill, suffer the biggest threat from the complications of influenza.45 Pneumonia and myocardial infection deaths during pandemics are over eight times as high as compared to years where there are no epidemics.42 Sometimes, populations of elderly appear to have a sort of immunity, which is thought to stem from exposure to previously circulated strains. This return of a previously “known” subtype to circulation is referred to as antigenic recycling.46 Children under 6 months old are spared from community-based respiratory infections, although the annual frequency of respiratory illness rises during the second year of life. The frequency rises again during the child-bearing years of life
as parents are exposed to children in the age groups that have the greatest burden of respiratory illnesses.\textsuperscript{43}

2. Detection

There is an old saying amongst doctors that says “Flu is easy to diagnose if you know that flu is going around.” In other words, if the physician has a real time detection method for the flu, he can, based on clinical symptoms alone, make the correct decision concerning treatment with a high degree of reliability. However, rapid detection of when the influenza is “going around” is fairly difficult.\textsuperscript{47} During an outbreak, brief delays in starting prophylaxis could expose many people to influenza.\textsuperscript{48} Recent studies have demonstrated that neuraminidase inhibitors effectively reduce the duration of illness caused by influenza by one to two days and can significantly reduce the rate of complication.\textsuperscript{49} Thus, there have been many efforts to correctly detect and diagnose influenza, especially in high risk groups. There have been several recent innovations in the real time detection of influenza, as laboratory-based virus cultures produce results too late for therapeutic intervention.\textsuperscript{50}

Three commercially available real time influenza detection tests are QuickVue Influenza (Quidel, USA), Directigen Flu A/Directigen Flu A + B (Becton Dickinson Diagnostic Systems, USA), and Zstat Flu (ZymeTX Inc, USA). Each system has a different process of detection, but each system has the same output: a color change. Also, each system has the same caveats: they are not sensitive and most give high false positives. Moreover, they are insufficient for testing for avian influenza.

QuickVue is a lateral flow immunoassay which detects both influenza A and B viruses but does not differentiate between the two. First, the surface antigens of influenza A and B are removed via detergents. The patient sample is the extracted, exposing internal viral proteins. After extraction, a test strip is introduced, where the specimen nucleoproteins are allowed to react with the antigens of influenza A and B that were removed with detergents. If the extracted specimen contains influenza viruses A or B, a pink or red line is displayed along with the blue control line. The test is read after 10 minutes. Studies with a pediatric population have shown that QuickVue is only 75% accurate. For comparison, a qualitative diagnosis of influenza from a doctor based off of symptoms of fever and cough alone with no other input has a sensitivity of 67-79%.\textsuperscript{51} In a study performed by the California Animal Health and Food
Safety Laboratory System, zero of 39 chicken embryo samples that tested positive for avian influenza by the “golden standard” cell cultures tested positive under the QuickVue assay.\textsuperscript{52}

Directigen Flu A and Directigen Flu A + B are membrane based enzyme immunoassays which differentiate between influenza viruses A and B. A drop of the patient specimen is diluted and put directly onto each of the wells on the provided assay. Subsequently, specific conjugate, washing buffer, and substrate solutions are added within a 10 minute time window. The results are read after 5 more minutes, and a control dot must be present for a determinate diagnosis.\textsuperscript{51} The results are a visible color change. Directigen, regardless of which specific test is completed, has an average sensitivity of 78%.\textsuperscript{53} In the same study done by the California Animal Health and Food Safety Laboratory System for avian influenza, five of 29 samples tested positive for avian influenza under Directigen that tested positive under a cell culture.\textsuperscript{52}

Zstat Flu is different from the previous two rapid diagnostic tools because instead of utilizing an enzyme immunoassay directed at the viral antigen, it is based upon the neuraminidase activity of the influenza viruses. Like the other tests, the patient sample is diluted. Zstat utilizes chemiluminescence; at its core, it exploits a chemiluminescent sialidase. This sialidase is washed over the sample. The chemiluminescence is detected by a Polaroid High Speed Detector Film that can pick up an image after just 1 minute of exposure. Depending on what is displayed on the exposed film, a positive or negative result can be determined. At 20 degrees Celsius, Zstat gave no false positives, but at 30 degrees Celsius it gave 50% false positives. For the most accurate result, the test needs to be done at or below 0 degrees Celsius. When operated in a cold, narrow range of temperatures, Zstat has a sensitivity of around 80%.\textsuperscript{54} The California Animal Health and Food Safety Laboratory System tested Zstat for activity with avian influenza and found zero detection with it.\textsuperscript{52}

A new rapid test (FLU OIA, Biostar, USA) was designed for guiding the prescription of antiviral treatment of patients with flu-like illnesses. The FLU OIA is an optical immunoassay based on alteration of the reflected light path by influenza antigen-antibody complexes formed on the optical surface. This alteration is directly visible as a color change. The FLU OIA is capable of distinguishing influenza A from influenza B and produces a result in 16 minutes. A major disadvantage of this rapid detection method is that a trained technician must be available and to run and interpret the results of this test. Also, the FLU
OIA shows extremely low sensitivity. In a Swiss study done in 2001 with 400 patients, one of four patients with influenza was falsely classified with a negative result. In a practitioner’s office or a general clinical setting where a trained technician is not available, the sensitivity and specificity would be much lower. The authors of this test state that “the FLU OIA is not suitable for use as a decision-making tool when evaluating the application of antiviral treatment.”

The first internationally standardized, rapid influenza surveillance system, RealFlu (Hoffman-La Roche, Germany), was established in 2004. It operates during the winter months for each hemisphere of the world. It provides information in real-time, including a baseline from previous year’s data. Primary care physicians and pediatricians record daily patient visits and influenza like illnesses. Each doctor is trained to use Influenza A/B Rapid Test. The Influenza A/B Rapid test is based on the Roche Gold-Labeled Optical Read Immunoassay technology. It detects the viral nucleoprotein associated with the viral nucleic acid. It is not commercially available and is used only for surveillance purposes only. While the sensitivity of 70% is not suitable for individual diagnosis, it is adequate for providing timely results for surveillance purposes. Dedicated servers and applications are available for the physicians to enter their data daily. A program automatically consolidates all data and performs all calculations. The program itself is simulated from the course of an influenza outbreak, with pre-epidemic activity, epidemic activity onset, increase and peak, and decline to a post-epidemic level. Data is available on the city, state, national, and global level as over 16 countries use this system as well. The system is equipped with three levels of activity and alerts the corresponding health officials when levels of outbreak follow those of an epidemic. However, this system is only in its pilot project phase.

All of the rapid diagnostic tests are inaccurate or show a high level for false positive/false negative results. Therefore, there exists a great need to develop technologies that can detect flu precisely and quickly.

3. Prophylaxis

The viruses causing human influenza were discovered in the early 1930s. Attempts to develop vaccines for the prevention and treatment of influenza were initiated soon thereafter. Inactivated influenza virus vaccines were introduced to medical practice in the 1940s, and the first specific antiviral
for influenza followed not long after in 1960. The main antivirals available for influenza treatment can be classified into two categories: the adamantanes and the neuraminidase inhibitors.\textsuperscript{55}

The antiviral activity of the adamantanes is limited to influenza A. This type of antiviral contains the drugs amantadine (Symmetrel\textsuperscript{®}) and rimantadine (Flumadine®). These agents inhibit viral replication by blocking the ion channel activity of influenza A. Both amantadine and rimantadine are effective for the treatment of established influenza A infection, reducing viral shedding and duration of symptoms if begun within 48 hours of the onset of symptoms.

There are some important differences between amantadine and rimantadine. Although rimantadine is somewhat more expensive than amantadine, it has the added benefit of lowered CNS adverse affects (only 1.9% of patients on rimantadine noticed these side effects). The most common CNS toxicity with amantadine was confusion, however it has been known to increase incidence of seizures and gait difficulties.\textsuperscript{56} Also, there are significant differences in the management of dosage adjustments between the two drugs. Due to the excretion of unchanged amantadine in the urine of patients, it is necessary to determine a creatinine clearance prior to administration of amantadine, especially among elderly patients where renal insufficiency and risk of serious toxicity are more likely. Rimantadine is metabolized by both renal and hepatic routes; however dose adjustment is required only for severe renal or hepatic insufficiency. Amantadine is approved for prophylaxis of influenza A in individuals >1 years of age. Rimantadine is approved for the treatment of influenza in people aged 14 and above. For maximum benefit, it is important to begin these antivirals within 48 hours of the onset of symptoms.\textsuperscript{51} Both of these drugs have the advantage of having a long shelf life, even at room temperature.\textsuperscript{58} Yet, there are several limitations to the clinical usefulness of adamantanes. As mentioned earlier, they only have activity against influenza A. Furthermore, resistance to the adamantanes develops frequently and rapidly. Viral resistant strains have been isolated in patients within two to seven days of treatment. Rimantadine-resistant strains are completely cross-resistant to amantadine.\textsuperscript{59}

Another class of influenza antivirals is the neuraminidase inhibitors, which include the drugs zanamivir (Relenza\textsuperscript{®}) and oseltamivir (Tamiflu\textsuperscript{®}). These drugs block the propagation of influenza virus by complexing with the sialic acid binding site of the viral neuraminidase. These drugs are synthetic derivatives of sialic acid, which bind irreversibly to the sialic acid binding site of neuraminidase and inhibit
its function. When the neuraminidase is impaired, clusters of viral particles can be seen to accumulate at the cell surface on electron microscopy. Numerous studies have show that these agents reduce the duration of viral shedding and provide significant clinical benefits in treatment of naturally occurring influenza, both types A and B. Zanamivir is administered by oral inhalation using a specifically designed inhalation device. A study done involving over 1100 patients taking zanamivir once daily was 67% effective in preventing illness. In general, adverse effects of zanamivir are minor; however patients with asthma and obstructive pulmonary disease are not advised to take zanamivir as it can increase respiratory distress. Presumably, adverse complications of zanamivir are a reflection of the route of administration.

Oseltamivir is administered orally, with results similar to those with zanamivir. However, there are some salient differences between zanamivir and oseltamivir. Dosage adjustment is recommended for patients with renal failure. Although the adverse effects are very mild, oseltamivir can cause greater nausea and vomiting than zanamivir. Thus far, oseltamivir has not been reported to cause heightened respiratory insufficiency. On the basis of several trials in which oseltamivir has shown 89% effectiveness in preventing illness, it has been approved for prophylaxis of influenza A and B.

Resistance to neuraminidase inhibitors has been documented in vitro and in treated patients. Resistance can be mediated either by mutations in the neuraminidase or HA proteins or both. Resistance to zanamivir treatment has not been found, moreso for the lack of research. In a clinical trial of oseltamivir, 4% of individuals were found to develop resistance. Influenza strains resistant to neuraminidase inhibitors frequently have reduced infectivity in mice or ferrets, although their infectivity in humans is unknown. There has been no evidence thus far of transmission of neuraminidase inhibitor resistant strains to other patients, though more experience with these drugs is necessary to determine whether resistance will be a clinically important problem in the future.

The overall antiviral activity and clinical effectiveness of the current anti-influenza agents appears to be similar, however there have not been any trials to compare the adamantanes with the neuraminidase inhibitors. The neuraminidase inhibitors have clear advantages over the adamantanes, including lower incidence and intensity of adverse effects, activity for influenza A and B, and reduced
incidence for resistance. However, the adamantanes cost considerably less. The least costly medication is amantadine, but it is the most toxic.

Currently available antiviral agents target relatively few aspects of the viral life cycle (i.e. replication) and thus new efforts are being put forth to make antiviral agents that focus on other vulnerable stages of the life cycle. Inhibition of HA activity is an emerging antiviral therapy. Inhibition of the binding of HA to epithelial cells could be an efficient means of blocking infection. Also, proteolytic cleavage of the HA is another critical step in the life cycle that is probably vulnerable to antiviral intervention.\textsuperscript{60} Defensins are low molecular weight antimicrobial peptides produced by phagocytes and in various epithelial locations, including the lung and the trachea.\textsuperscript{69} It is thought that defensins could inhibit infectivity of influenza through disruption of the viral envelope.\textsuperscript{70} These antivirals are still in their infancy.

Of these four different antiviral drugs, amantadine and rimantadine definitely do not show activity against avian influenza. More research needs to be done to determine if the neuramidase inhibition drugs will work against avian influenza.\textsuperscript{71}

Perhaps the secret lies not in treating influenza but in preventing it. Vaccination is currently the most effective and economical method of controlling influenza. Extensive surveillance and careful analysis are required to successfully identify the strains that will be prevalent each year so that sufficient quantities of vaccine can be produced in advance of the influenza season. For influenza, trivalent intramuscular, killed virus vaccines are most common. The vaccine includes the most prevalent influenza strains (usually one each of H1N1 and H3N2 subtypes) and an influenza B virus strain. Use of this vaccine has been shown to significantly reduce the rate of hospitalization from complications of influenza.\textsuperscript{24}

There are two major limitations to this vaccine. First, the vaccine has reduced effectiveness for inducing protective immunity in high risk groups (like the elderly).\textsuperscript{72} Second, immunity induced by the current vaccine is specific for the viral strains included in the vaccine. Alternative vaccine preparations and routes of administration are under study in an attempt to overcome these problems. Most noteworthy include administering live, attenuated influenza vaccines through the intranasal route.\textsuperscript{73} Also, research is ongoing in an effort to induce heterosubtypic immunity (immunity against variant strains of influenza).\textsuperscript{74}
Currently, there is no vaccination for avian influenza. However, the CDC and NIAID have been researching this problem since April 2005 to prevent against an outbreak of the two avian-human crossover strains of influenza (H5N1 and H9N2).

Clearly, there is a great need to detect influenza because there currently is no reliable method for doing so. One goal is to prevent distribution of medicines to those with lowered immune systems, therefore preventing complications. Looking to the future, it will be important to differentiate between avian and human influenza in order to hinder the spread of the virus and to ensure that the right medications are being given to the right people.

4. Biological activity

Influenza viruses A, B, C, and Thorgotovirus are the only members of the orthomyxovirus family, having seven or eight (influenza A and B, influenza C and Thorgotovirus respectively) single-stranded, segmented, negative-sense RNA genomes. The virions are enveloped, being 50-120nm in diameter. The envelope has surface projections composted of viral receptors (HA and N) and tetramers of neuraminidase. Orthomyxoviruses are subject to high rates of mutations and reassortments (antigenic drifts and shifts) because they lack the proofreading enzymes that maintain the continuity of DNA replication. As previously stated, influenza has two major surface glycoproteins, HA and N. N accounts for 5 to 10% of the total influenza virus protein (see Figure 3, below).

Influenza is inhaled into the upper respiratory tract by droplet transmission. At this point, infection could be prevented by antibodies. Influenza virus infection is initiated by attachment to sialated oligosaccharides on target epithelial cell membranes by means of HA spikes on its lipid envelope. The sialic acid is a terminal sugar on the cell surface proteins and lipids. The HA glycoprotein is essential for viral attachment and cellular penetration, by an endocytic pathway. If this process is blocked by the presence of anti-HA antibodies, the infection does not occur. The other viral surface glycoprotein, NA, is thought to act by changing the normal properties of the mucus, permitting viral access to target epithelial cells. NA also prevents viral aggregation. NA specific immunoglobulins tend to reduce the intensity of infection so that illness does not occur or is substantially reduced.
The HAs of avian influenza viruses contain Gln226 and Gln228 residues, which form a narrow receptor binding pocket for a preferential binding of (2-3)-linked sialic acids. However, human species usually contain Leu226 and Ser228, which form a broad pocket that prefers binding with the terminal (2-6)-linkage. Recent studies have shown that (2-6) linked sialic acids are mainly expressed on nonciliated cells of the human airway epithelium, whereas (2-3) linked glycoconjugates are expressed on ciliated cells to allow entry and replication of avian viruses. Ciliated cells therefore most likely serve as primary target cells in those rare cases where avian viruses cause human disease. Studies by Mastrovich et al. found that epidemic influenza A and B viruses specifically target nonciliated (2-6)-linked cells and this clearly indicates that ciliated cells are suboptimal for influenza virus replication and/or transmission in humans. This could explain why avian influenza viruses usually fail to cause disease in humans and why human-to-human transmission of avian viruses is inefficient. The ability of both types of virus to infect pigs is considered a result of the presence of both types of linkages in cell surface molecules in the pig trachea. In order for influenza to jump the species barrier, it must undergo a change in preferential binding site.

As a prerequisite for infection of epithelial cells, the viral HA must also undergo cleavage into HA1 and HA2 subunits, which are linked by a single disulfide bond. The cleavage is necessary to activate the membrane potential of HA. Cleavage is mediated by host proteases present in the airway. Recent studies have indicated that the ability of a given influenza viral strain to bind to host proteases is an important virulence factor. The HAs of human influenza strains are principally cleaved by extracellular process, whereas the HAs of some virulent avian strains are cleaved by ubiquitous intracellular proteases.

The virus generally enters the cell via endocytosis. As the endosome enters the cytoplasm, the pH within the endosome lowers. Due to this pH change, the viral core is then released from the endosome when the viral HA protein undergoes a conformational change. This exposes a domain that induces fusion between the viral and endosomal membranes that then allows viral ribonucleoproteins to dissociate by acting as an ion channel through the viral membrane. The viral ribonucleoproteins then translocate to the host nucleus.
Once the host cell is occupied by the viral contents, the virus undergoes replication, assembly, and release. Viral mRNA enters the cytoplasm and viral protein synthesis takes place. The viral proteins return to the nucleus where they are assembled into new ribonucleoproteins which are then transported out of the nucleus for assembly of new viral particles. Viral envelope proteins are produced on the endoplasmic reticulum and Golgi apparatus and expressed on the luminal surface of the epithelial cell membrane. Newly formed viral particles then bud off the cell surface. The viral surface neuraminidase protein must enzymatically cleave terminal sialic acids off viral and cell surface carbohydrates at this stage to allow the viral particle to bud freely off the cell. A single cell replication cycle takes approximately four to six hours. Within 24 to 48 hours, enough cells have been killed or damaged to generate the classical symptoms of influenza. Epithelial death and damage in the upper airways and trachea is the main cause of local symptoms, such as sore throat and dry cough. Figure 4, below, shows the basic life cycle of the influenza virus including such mechanisms as noted above.
The selectivity of the virus to certain linked sialic acids are the foundation for the synthesis of (2-3)-linked sialic acids.
C. References


51. Ruest, A.; Michaud, S.; Deslandes, S. and Frist, E.H. 2003. Comparison of the Directigen Flu A+B Test, the QuickVue Influenza Test, and clinical case definition to viral culture and
72. CDC. January 14, 2006. CDC recommends against the use of amantadine and rimantadine for the treatment or prophylaxis of influenza in the US during the 2005-6 influenza season. Health Alert Network.


II. Synthesis of Scaffolds

Two different scaffolds have been synthesized for the attachment of a 2,6-linked sialic acid sugar residue. Each scaffold has been created to maximize the multivalent binding affinities of each pathogen with terminal alkynes used as attachment points for the sugar residues. The dimeric scaffold, has two terminal alkynes, whereas the tetrameric scaffold has four terminal alkynes.

A. Dimeric Scaffold for BoNT

A dimeric scaffold is necessary for binding to BoNT, as the toxin has 2 sites for binding to sialic acid. A sialic acid residue is attached to each “arm” of the scaffold. 5-amino isopthalic acid was used as a starting material for an inflexible scaffold, as it is commercially available and easy to work with. The acid functionalities are in the meta position, so the possible formation of a lactone byproduct is greatly reduced. The amine is essential so that it can later be alkylated for surface attachment or further extended by reaction with a carboxylic acid that possesses a biotin or fluorophore. These extensions can be used in different types of bioassays. For example, the alkyl chain can be tethered to a HM5 BIACORE chip, creating a tight fit between the surface and the scaffold as the alkyl chain will interact with the
hydrophobic surface of the chip. The acid groups are necessary for extension to terminal alkynes, which are desired as they can be used in 1,3-dipolar cycloaddition (click chemistry) for the attachment of the sugar residues. Click chemistry can be achieved in a water mixture, which overcomes solubility issues of the highly polar end product. Briefly, the free amine is extended and protected with a CBz group, which is stable to the basic conditions used to extend the acid groups once the protection is complete. NMM and CDMT are used to activate the acid groups, which are then reacted with two equivalents of propargyl amine to yield the final product. The product has two terminal alkynes which can be attached via click chemistry to any azide bearing carbohydrate.¹

B. Tetrameric Scaffold for Influenza virus

Scheme 2: Synthesis of tetrameric scaffold
A tetrameric scaffold is necessary for optimal binding of neuraminidase, which is the surface glycoprotein of interest on various types and subtypes of influenza virus. The synthesis of the tetrameric scaffold is similar to the synthesis of the dimeric scaffold. Again, 5-amino isopthalic acid was used as a starting material. The free amine was protected via a t-Boc group. After that, the acid groups were extended into terminal alkynes by activation with CDMT and NMM, followed by an addition of propargyl amine to introduce the terminal alkynes. These terminal alkynes are used for click chemistry of the azide-containing sugar residues. Once the alkynes are attached, the Boc group is cleaved under acidic conditions and the free amine is extended and protected once again. The amine is not highly reactive as the lone pair of electrons on the nitrogen is directly conjugated to the benzene ring, so an extension is necessary. However, the amine reacts readily with activated carboxylic acids, such as acid chlorides. Thus, reaction with chloroacetyl chloride in the presence of a suitable base, such as triethyl amine results in the extended product, which has a chloride. The chloride was reacted with excess ammonia to yield a molecule (8) bearing two terminal alkynes and a free amine. The free amine is reacted with a dimeric molecule that has 2 carboxylic acid functionalities.

In a similar manner, the amine group of 5 amino isopthalic acid was extended with bromoacetyl bromide and subsequent reaction with excess ammonia to yield a di-acid bearing a primary amine. The free amine was protected with a suitable protecting group, a carboxybenzyl group. Activation of the free acids and subsequent reaction with 2.2 equivalents of 3 in the presence of CDMT and NMM resulted in 10, a tetrameric compound with four terminal alkynes, in appreciable yields. Thus, the sequence of convergent steps of this multivalent reaction has yielded a dimeric and a tetrameric scaffold.

The alkynes can be clicked with any azide bearing molecule such as a carbohydrate, an antibody, or a peptide.
C. Experimental

1. General

All chemical reagents were of analytical grade, used as supplied without further purification unless indicated. Acetic anhydride and acetyl chloride were distilled under an inert atmosphere and stored under argon. Analytical thin layer chromatography (TLC) was conducted on silica gel. Plates were visualized under UV light and/or treatment with phosphomolybdic acid or p-anisaldehyde followed by heating. Low pressure chromatography was completed using silica gel (230-400 mesh from EMD Chemicals) at flow rates of 1-10 ml min\(^{-1}\).

\(^1\)H and \(^{13}\)C NMR spectra were recorded on a Bruker AMX 400 MHz spectrometer. Chemical shifts are reported in ppm units using \(^{13}\)C and residual \(^1\)H signals from deuterated solvents as references. Spectra were analyzed with Mest-Re-C Lite (Mestrelab Research) and/or XWinPlot (Bruker-Biospin).

Electrospray ionization mass spectra were recorded on a Micromass Q-Tof 2 (Waters) and data were analyzed with MassLynx 4.0 (Waters) software.

2. Synthesis of scaffolds

**Dimeric Scaffold**

Synthesis of 5-(2-Chloroacetylamino)-isopthalic acid (1) – 10g (0.0552 mol) of 5-amino isophthalic acid was dissolved in 50ml of 4M NaOH and the solution was cooled to 0°C. 16ml of chloroacetyl chloride was added in 4ml portions every 10 min for 40 min. The reaction was left for an additional 30 min. The product was precipitated with dilute aqueous HCl until the pH reached 2. The solid product was filtered and washed with cold deionized water. The product was concentrated \textit{in vacuo} to give 11.54g (81%) yield.

Synthesis of 5-(2-Amino-acetylamino)-isopthalic acid (2) – 11.54g (0.0448 mol) of starting material (1) was dissolved in 200ml aqueous ammonia solution and stirred at RT overnight. 160ml of solvent was removed in \textit{vacuo} and 30ml of EtOH was added. The mixture was put on ice and a white precipitate was formed. The precipitate was filtered and dried to give 12.71g (84%) yield.
Synthesis of 5-(2-Benzoyloxycarbonylamino-acetylamin o)-isopthalic acid (3) – To the same flask, 12.71g of starting material (2) were added to 38.11g sodium carbonate and dissolved in 150ml of water. The mixture was cooled to 0°C. 9.03ml of benzyl chloroformate was added in 2 portions 10 min apart from each other. The mixture warmed up to RT and was left overnight. Saturated sodium bicarbonate was added to the reaction mixture. 100ml of diethyl ether was added. The aqueous layer was removed and gradually acidified with HCl, forming a bulky white precipitate. The precipitate was filtered, washed with cold deionized water, and dried in vacuo. The yield was 13.415g (68%).

Synthesis of (4) – Dissolved 3.0g (0.00805 mol) of starting material (3) in 45ml THF. Cooled to 0°C. Added 3.11g CDMT and let stir for 20 min before adding 1.9ml NMM. Stirred for 12 h. Added 1.13ml propargyl amine and 1.9ml NMM to 6ml of 5:1 THF:DMF. Added this mixture to the reaction mixture and let stir for 24h, warming up to RT. 1N HCl was added until acidic, and added 50ml diethyl ether. Extracted organic layer and washed aqueous layer twice with diethyl ether. Combined the organic layers and dried over sodium sulfate. Concentrated product in vacuo. The product was purified via column chromatography in ethyl acetate to give 3.081g (89%).

Tetrameric Scaffold

Synthesis of (5) – To 93ml of 1:1 DMF:water containing 10g (0.0553 mol) of 5-amino isophthalic acid and 4.8g NaOH at 0°C was added 12.09g t-butyl dicarbonate. The reaction was allowed to come to RT and stirred for 30h. The reaction mixture was acidified slowly with 3N HCl and diluted with an additional 100ml of water. The precipitated was filtered, washed with water, and dried to give 8.35g (54%) yield.

Synthesis of (6) – Dissolved 3g (0.0106 mol) of starting material (5) in 45ml THF and cooled to 0°C. Added 4.107g CDMT and let stir for 20 min before adding 2.57ml of NMM. Let stir for 12h. Added 1.49ml propargyl amine and 2.57ml of NMM to 6ml of 5:1 THF:DMF. This was added to the reaction mixture at 0°C and let stir for 24h, warming up to RT. The solvents were removed in vacuo. Ethyl acetate and water were added and the organic layer was removed and dried. The product was purified via column chromatography in 75/25 ethyl acetate/hexane to give 3.82g (100%).
Synthesis of (7) – Added 3.0g (0.00845 mol) of starting material (6) to 50ml of methylene chloride, followed by 1.74ml of TIPS. Cooled to 0°C and added 6.24ml trifluoroacetic acid dropwise. Let warm up to RT and stirred for 10h. Removed solvents *in vacuo* and purified via column chromatography in 75/25 ethyl acetate/hexane. The yield was 2.102g (99%).

Synthesis of (8) – Dissolved 2.102g (0.0840 mol) of starting material (7) in 45ml acetonitrile. Added 0.828g dry Na₂CO₃ under argon and cooled to 0°C. Added 0.534ml bromoacetyl bromide to 5ml acetonitrile under argon and added this mixture to the reaction mixture. Let stir overnight. Added 100ml ethyl acetate to reaction mixture and gravity filtered the solution, reserving liquid. Diluted the liquid with another 100ml of ethyl acetate and passed through a filter column. The product was concentrated *in vacuo* to give 2.135g (68%).

Synthesis of (9) – To a clean, dry flask added 60ml of 0.7M ammonia in MeOH. Cooled to 0°C. In another flask, added 2.135g (0.00567 mol) of starting bromide (8) in 15ml THF and dropwise added the contents to the ammonia/MeOH mixture. Let stir overnight. Solvents were removed *in vacuo*. The product was purified via column chromatography in 30/70 methanol/ethyl acetate to give 1.31g (74%).

Synthesis of (10) – Added 0.526g (0.00141mol) of acid (3) to a clean, dry, flask followed by 0.5462g CDMT and cooled to 0°C. In another flask, added 0.342ml NMM to 5ml THF then added the contents of this flask to the flask with the acid mixture in it. Let stir overnight at 0°C. Added 0.342ml NMM to 1.0g (0.00311 mol) starting amine (9) in 12ml 5:1 THF:DMF. Cooled to 0°C and added the amine mixture to the reaction mixture. Let stir for 36 hours. Purified via column chromatography in 10/90 MeOH/ethyl acetate to give 0.217g (16%).

D. References

III. Carbohydrate Synthesis

A trisaccharide, more specifically a (2,6)-linked sialic acid to a lactose bearing an oligoethylene glycol based linker has been synthesized. Tetraethylene glycol was chosen as an appropriate linker for initial studies. It should specifically bind to human influenza (versus avian influenza) and also should be active to botulinum toxin as well.

A. Sugars

The first step was the synthesis of the tetraethylene glycol based linker.

1. Linker

Although in this synthesis a tetraethylene glycol unit was used as the starting material, the linker can be made of various lengths and thus should have a differential impact on the binding of the pathogen, based on the orientation and spacing of the binding site. Tetraethylene glycol was mesylated with 1.1 equivalents of mesyl chloride to activate one of the hydroxyl groups, after which it was reacted with sodium azide to give a 20/80 bis/mono azide product. The mono azide product is what is attached to the lactose part of the sugar residue, and the azide serves as the attachment point to the scaffold. Again, the azide is necessary for click chemistry in water.
2. Sialic Acid

Sialic acid is the binding point of each pathogen to the tailored ligand. Its position on the ligand is determined by the structure of the lactose. However, sialic acid must also be manipulated to be attached at the 2 position (see Figure 5, below).

First, the carboxylic acid group is protected by esterification with methanol and acid resin, protecting it from the inversion chemistry that will take place at the 2 position. Then, all of the hydroxyl groups are protected by acylation, after which the configuration at the 2 position is inverted when the acetate group is replaced by a chlorine atom via an SN2-like pathway. The 2 position is then inverted again by the introduction of an S-acetate group, which is used to couple it to the lactose derivative. A study done by von Itzstein and coworkers shows that sulfur linked sialidases do not undergo hydrolysis.
like oxygen linked sialidases do. The study was done with various sialidases, both S- and O-linked, in D₂O and monitored by NMR. After just 120 minutes, almost all of the O-linked sialosides were cleaved, yet after 14 days the S-linked sialosides remained unchanged. Since stable glycoside linkages are required for various applications, an S-glycoside was synthesized instead of an O-glycoside. The study done by von Itzstein and coworkers served as a good foundation for this decision.

3. Lactose Derivative

Each sialic acid derivative was attached to the 6 position on lactose. This is primarily for the detection of human over avian influenza. Below, Figure 6 shows the numbering scheme for lactose.
Lactose is used because it is cheap and commercially available. Unpublished studies by other group members show that influenza does bind to sialic acid but that the lactose base is needed for better selectivity. First, all of the hydroxyl groups are acylated. Then, only the anomeric hydroxyl (1' position) is deprotected and turned into the corresponding imidate. The imidate is then activated by an acid source and the linker is attached. All of acetate groups are then removed and a protecting group was added to selectively protect the 4 and 6 position. At first, the 6 position was protected by a dibutyltinoxide group as the primary hydroxyl (position 6) is the most reactive. However, due to extremely low yields (less than 5% for 2 attempts), another route was taken. Instead, a benzylidene group was used to protect both the 4 and 6 positions. Once the protection was in place, the rest of the hydroxyl groups were protected by acylation again, as acetate groups are stable to acid. The benzylidene group was then cleaved under acidic positions and a selectively deprotected 6 primary hydroxyl was created.

**B. Coupling the sugars**

![Scheme 6: Synthesis of triasaccharide](image)
Under anhydrous conditions, the primary alcohol is activated via triflate. Then a strong base (triethyl amine) is added, along with the S-Ac sialic acid, to create a 2-S-linked sialic acid in the 6 position of lactose.
C. Scaffold attachment

The sugar residue was attached to the dimer scaffold in water by 1,3-dipolar cycloaddition followed by removal of all the acetate groups to give the final product.
D. Experimental

1. General

All chemical reagents were of analytical grade, used as supplied without further purification unless indicated. Acetic anhydride and acetyl chloride were distilled under an inert atmosphere and stored under argon. Analytical thin layer chromatography (TLC) was conducted on silica gel XXXXX. Plates were visualized under UV light and/or treatment with phosphomolybdic acid or p-anisaldehyde followed by heating. Low pressure chromatography was completed used with silica gel (230-400 mesh from EMD Chemicals) at flow rates of 1-10 ml min⁻¹.

¹H and ¹³C NMR spectra were recorded on a Bruker AMX 400 MHz spectrometer. Chemical shifts are reported in ppm units using ¹³C and residual ¹H signals from deuterated solvents as references. Spectra were analyzed with Mest-Re-C Lite (Mestrelab Research) and/or XWinPlot (Bruker-Biospin).

Electrospray ionization mass spectra were recorded on a Micromass Q-Tof 2 (Waters) and data were analyzed with MassLynx 4.0 (Waters) software.

2. Synthesis of linker and sugar derivatives

Linker Synthesis

Synthesis of Methanesulfonic 2-(2-(2-hydroxyethoxy)-ethoxy)-ethoxy)-ethyl ester (11). To a clean, dry flask, 19.65g (0.113 mol) of silver oxide was added and under argon. 15g (0.103 mol) of tetraethylene glycol was added via syringe, followed by 150ml of dichloromethane via cannula. To a different flask, 7.1ml (0.124 mol) of mesyl chloride was added via syringe. 50ml of DCM was added under argon via cannula. The mesyl chloride-DCM mixture was added to the silver oxide mixture. This flask was covered in aluminum foil and stirred at RT for 48h. DCM was used to wash the product as it was filtered through a vacuum to remove the excess silver oxide. The solvent was then removed in vacuo. This mixture was taken to the next step without any further purification. The reaction formed two products, mono- and bis-mesylates. The overall mono-product yield was 16.2g (80%) and the bis-product yield was 4.05g (18%).

Synthesis of 2-(2-(2-(2-azidoethoxy)-ethoxy)-ethanol (12). To a clean, dry flask, 20.26g (0.071 mol) of the starting material (11) was added. The flask was flushed with argon. 200ml of DMF was added via
cannula. 11.21g (0.292 mol) of sodium azide was added quickly and the mixture was brought to 100°C for 2h. The DMF was then removed in vacuo. The product (12) was purified via column chromatography in 95/5 DCM/MeOH to yield 13.98g (85%) mono product.

**Sialic Acid Derivative Synthesis**

*Synthesis of 5-Acetylamino-2-carboxylic-2,4-dihydroxy-6-(1,2,3-trihydroxy-propyl)-tetrahydropyranose (13).* To a clean, dry flask 1.0g (0.0032 mol) of sialic acid was added, followed by 250mg of Amberlite H⁺ resin. The flask was flushed with argon and stoppered. A cannula was used to transfer 30ml of MeOH. The mixture was refluxed at 80°C for 2 hours under argon. The resin was filtered off and the solvent was removed in vacuo to give a quantitative yield.

*Synthesis of (14).* To 1.0g (0.0032 mol) starting material (13), 0.075g of DMAP was added in a clean, dry flask. 20ml of pyridine was added and the mixture was cooled to 0°C. 15ml (0.064 mol) of acetic anhydride was added via cannula and the reaction stirred for 18h at 0°C. The pyridine and acetic anhydride were removed in vacuo, using toluene to form an azeotrope. Column chromatography with 100% ethyl acetate resulted in a white foam. The yield was 0.974g (70%).

*Synthesis of (15).* 1.0g (0.0023 mol) of the starting material (14) was added to a clean, dry flask. Via syringe, 3ml (excess) of acetyl chloride was added and the mixture was stirred. HCl gas was bubbled through the reaction mixture for 9 min. The reaction was left overnight at 0°C. The solvent was removed in vacuo, using toluene to remove the excess acetyl chloride. The yield was 1.05g (96%). The crude material was used directly in the next step.

*Synthesis of (16).* To 1.0g (0.0021 mol) of starting material (15) to a clean, dry flask 5ml of ethyl acetate was added. The mixture was cooled to 0°C and 0.704g (0.0021 mol) of tetrabutylammonium hydrogen sulfate and 1.177g (0.011 mol) of potassium thioacetate was added. 5ml of sodium carbonate was added dropwise via syringe. The ice bath was removed and the reaction was stirred for 30 min. The organic layer was separated and washed with water. The water layer was extracted with 2 25-ml portions of ethyl
acetate. All organic layers were combined and dried over sodium sulfate. The solvent was removed \textit{in vacuo}. The final product (16) was purified with column chromatography in 90/10 ethyl acetate/hexane to give 0.843g (73%).

**Lactose Derivative Synthesis**

\textit{Synthesis of (17).} In an clean, dry flask, 10.0g (0.029 mol) of lactose and 0.5g of DMAP were added. The flask was flushed with argon and stoppered. 100ml of pyridine was added via cannula, followed by 60ml (0.582 mol) of acetic anhydride. The mixture was stirred at RT for 36h. After quenching with ice, the organic layer was washed with dilute HCl, saturated sodium bicarbonate, and brine. The organic layer was dried over sodium sulfate and the solvent was removed \textit{in vacuo} using toluene to create an azeotrope. The product was purified via column chromatography in 40/60 ethyl acetate/hexane to produce 15.47g (80%) yield.

\textit{Synthesis of (18).} To a clean, oven dried flask was added 19.71g (0.029 mol) of starting material (17), followed by 150ml THF via cannula. The reaction flask was cooled to 0°C. Quickly, 3.74g (0.029 mol) of hydrazine acetate was added. The ice bath was removed after 1h and the reaction was left to stir for 21h at RT. 100ml of cold deionized water was added to the reaction flask, followed by 100ml of ethyl acetate. The extracted organic layer was washed twice with 100ml each saturated sodium bicarbonate, 100ml of brine, and 100ml of deionized water. The organic phase was dried over sodium sulfate and the product was concentrated \textit{in vacuo}. The product was purified via column chromatography in 70% ethyl acetate/30% hexane to give a yield of 17.89g (97%).

\textit{Synthesis of (19).} To a clean, dry flask was added 15.5g (0.0281 mol) of starting material (18), followed by 17.90g (0.112 mol) dried potassium carbonate. 200ml of dichloromethane was added via cannula. 28.21ml (0.281 mol) of trichloroacetonitrile was added dropwise under argon. The reaction was stirred at RT for 48h. The reaction was quenched with ice and diluted with 100ml of ethyl acetate. 100ml of cold deionized water was also added. The organic layer was extracted and the water layer was washed 3 times with 100ml of ethyl acetate. The organic layers were combined and dried over sodium sulfate. The
product was then concentrated in vacuo. Purification was obtained via column chromatography in 50% hexane/50% ethyl acetate to give 17.21g (78.5%) yield.

**Synthesis of (20).** To a clean, dry flask was added 6.4g (0.0082 mol) of the imidate product (19) and dissolved in 50ml of DCM. 1.88g (0.0086 mol) of linker product (12) was dissolved in 10ml of DCM in a separate flask. The flask containing the linker (12) solution was added to the reaction flask containing the imidate product. The solution of imidate (19) and the linker (12) was cooled to 0°C and then 0.745ml of 0.22M trimethylsilyltriflate in DCM was added dropwise via syringe. The reaction was stirred at 0°C for 1.5h. The reaction was quenched with 100ml of cold saturated sodium bicarbonate. 100ml of ethyl acetate was added and the organic layer was extracted. The aqueous layer was washed twice with 100ml each of ethyl acetate. The organic layers were combined, dried over sodium sulfate and concentrated in vacuo. The product was purified via column chromatography in 30% hexane/70% ethyl acetate to give 4.02g (52%) yield.

**Synthesis of (21).** To a clean, dry flask was added 8.4g (0.00885 mol) of starting material (20), followed by 150ml of MeOH. The reaction flask was cooled to 0°C and 1.77ml of 0.5M sodium methoxide in MeOH was added dropwise under argon. The reaction was stirred for 0°C for 1h and then stirred for 2.5h at RT. The reaction was quenched with DOWEX resin until the pH reached 5. The resin was filtered and the product was concentrated in vacuo. The product was purified by column chromatography in 15% methanol/ 85% ethyl acetate to give 4.37g (91%) yield.

**Synthesis of (22).** To a clean, dry flask was added 2.34g (0.0043 mol) of starting material (21), followed by 75ml dry acetonitrile. 1.43ml (0.0095 mol) benzaldehyde dimethyl acetal was added under argon RT. Triethylamine was added to quench the reaction. The solution was concentrated in vacuo. The product was purified via column chromatography in 7% methanol/93% methylene chloride to give 2.69g (97%) yield.
Synthesis of (23). To a clean, dry flask was added 1.92g (0.00303 mol) of the benzilidine protected starting material (22), followed by a catalytic amount of DMAP and 50ml of pyridine. The solution was cooled to 0°C. Added 3.83ml (0.0378 mol) of dry acetic anhydride under argon. The ice bath was removed and the reaction was let to warm to RT and stirred overnight. Toluene was added and the product was concentrated in vacuo. The product was then purified via column chromatography in 30% hexane/70% ethyl acetate to give 2.24g (88%) yield.

Synthesis of (24). To a clean, dry flask was added 2.2g (0.00261 mol) of starting material (23), followed by 50ml DCM. The solution was cooled to 0°C. 55ml (3:2) of trifluoroacetic acid in water was added dropwise under argon. The ice bath was removed for 1h. The reaction mixture was diluted with 50ml DCM. The reaction was quenched with 100ml of cold saturated sodium bicarbonate. The organic layer was extracted. The aqueous layer was washed twice with 50ml each DCM. The organic layers were combined and dried over sodium sulfate. The product was concentrated in vacuo. It was further purified via column chromatography in 90% ethyl acetate/10% hexane to give 1.46g (75%) yield.

Trisaccharide synthesis

Synthesis of (25). To a clean, dry flask was added 0.263g (0.000348 mol) of diol starting material (24) followed by 2ml DCM and 0.28ml ( 0.00348 mol) pyridine. The solution was cooled to -78°C. To this solution, 0.083ml (0.000487 mol) triflic anhydride was added dropwise under argon. The reaction was stirred 2.5h at -78°C. The reaction was diluted with 10ml DCM. 10ml of cold deionized water was added and the organic phase was extracted. The aqueous phase was washed twice with 5ml each DCM. The organic phases were combined and concentrated in vacuo. The product was directly used in the next step without any further purification.

Synthesis of (26). To a clean, dry flask containing 0.000348 mol of the triflic product (25) was added 0.211g (0.000382 mol) of sialic acid derivative (16), followed by 2.5ml of DMF. The solution was cooled to 0°C and 0.75ml diisopropyl amine was added. The reaction was stirred 4.5h at 0°C. The solvent was
removed in vacuo. The product was purified via column chromatography in 5% methanol/95% ethyl acetate.

**Attachment to scaffold**

*Synthesis of (27).* To a clean, dry flask containing 0.0171g (0.00003986 mol) of the dimeric scaffold (4) was added 0.10900g (0.00007477 mol) of trisaccharide (24), 0.1194g (0.00004078 mol) CuSO₄, and 0.01895g (0.00008157 mol) sodium ascorbate. 3 ml of 1:1 THF/H₂O was then added via syringe. The reaction was stirred at RT for 24h. The solvent was removed in vacuo and the product was purified via column chromatography in 10/90 MeOH/ethyl acetate to give 0.040g (36%).

*Synthesis of (28).* To a clean, dry flask containing 0.027g (0.00000951 mol) of protected dimer product was added 5ml of methanol and 0.01ml of 0.5M sodium methoxide in methanol. The reaction was stirred for 48h at RT and the solvent was removed in vacuo. The product was purified via column chromatography in methanol on a C-18 column to give 0.015g (73%).

**E. References**

IV. Future Work

The dimeric structure was completed; however the tetrameric structure still needs to be synthesized. The syntheses of the tetrameric scaffold and trisaccharide have already been completed but they need to be coupled as in Scheme 8, below.

Scheme 8: Attachment of trisaccharide to tetrameric scaffold
The ligands will be tested in an assay against the two pathogens. Specifically, sodium dodecyl sulfate polyacrylamide will be the assay used. It will definitely display the difference between a ligand that shows binding to the pathogen and a ligand that does not. In SDS-PAGE, the distance of migration through the gel can be assumed to be directly related to only the size of the molecule. Therefore, it is expected that a pathogen bound to the ligand will appear higher in the gel than a ligand that does not bind. Also, it will be possible to observe partial binding. Figure 7 (below) shows a sample SDS-PAGE.

![Sample SDS-PAGE result](image)

Once the biological studies are complete and show specific binding to the pathogens of interest, the ligands can be attached to the microarrays and nanoparticles coated with alkynes. A fingerprint pattern can then be determined for each pathogen using ligands created by other Iyer group members.
V. Appendix

1. Spectroscopic Data for compounds
Compound 6

\[
\begin{align*}
\text{HN} & \quad \text{C} \quad \text{C} \quad \text{NH} \\
\text{HN} & \quad \text{O} \quad \text{NH} \\
\text{NHBoc} & \quad 6
\end{align*}
\]
Compound 7

[Chemical structure image of Compound 7]
Compound 8

\[
\begin{align*}
\text{HN} & \quad \text{C} & \quad \text{O} \\
\text{HN} & \quad \text{C} & \quad \text{O} \\
\text{Br} & \quad \text{O} & \quad \text{N} \\
\end{align*}
\]
Compound 11

Mono and bis mixture
Compound 14

\[
\begin{align*}
&\text{O} \quad \text{AcO} \\
&\text{CO}_2\text{Me} \\
&\text{OAc} \\
&\text{AcO} \\
&\text{AcHN} \\
&\text{OAc} \\
&\text{OAc}
\end{align*}
\]
Compound 16

\[
\begin{align*}
\text{AcO} & \quad \text{OAc} \\
\text{AcO} & \quad \text{CO}_2\text{Me} \\
\text{AcHN} & \quad \text{AcO} \\
\text{SAc} & \quad 16
\end{align*}
\]
Compound 17

\[
\begin{array}{c}
\text{OAc} \\
\text{AcO} \\
\text{OAc} \\
\text{OAc} \\
\text{OAc}
\end{array}
\]
Compound 19
Compound 20
Compound 21
Compound 22
Compound 24

\[
\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{AcO} & \quad \text{OAc} \\
\text{OAc} & \quad \text{AcO} \\
\text{O} & \quad \text{OAc} \\
\text{O} & \quad \text{AcO} \\
\text{N}_3 & \quad 24
\end{align*}
\]
Compound 24

Top – Calculated, Middle – (Na+M)+, Bottom – Sample
Compound 26
Compound 26
Compound 26

Top – (M+Na)+ calculated, Bottom – Sample
Compound 27
Compound 28
Compound 28

Top – Calculated (M+2H)²⁺, Bottom – Sample (M+2H)²⁺