Synthesis of Small Molecules for Diagnostics and Therapeutics of Influenza Virus

Hieu T. Dinh

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SYNTHESIS OF SMALL MOLECULES FOR DIAGNOSTICS AND THERAPEUTICS OF
INFLUENZA VIRUS

by

Hieu T. Dinh

Under the Direction of Suri S. Iyer, PhD
Abstract

Influenza infection remains constant threat to human health and results in huge financial loss annually. Rapid and accurate detection of influenza can aid health officials monitor influenza activity and take measurements when necessary. In addition, influenza detection in a timely manner can help doctors make diagnosis and provide effective treatment. Additionally, novel inhibitors of influenza virus are in high demand because circulating strains have started to develop resistance to currently available anti-viral drugs.

Influenza virus has two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA), which play important roles in the influenza infection. The binding of HA to sialic acid-containing carbohydrates on cell surface initiates virus internalization, while cleavage of terminal sialic acid by NA facilitates viral particle release. In this dissertation, we focus on the development of glycan microarray that is comprised of a panel of NA resistant sialosides, and demonstrate the application of microarray to capture influenza virus at ambient temperature without the addition of NA inhibitors

INDEX WORDS: Influenza, sialosides, microarray, NA inhibition
SYNTHESIS OF SMALL MOLECULES FOR DIAGNOSTICS AND THERAPEUTICS OF
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by

Hieu T. Dinh

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctorate of Philosophy

in the College of Arts and Sciences

Georgia State University

2015
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College of Arts and Sciences

Georgia State University

August 2015
DEDICATION

This dissertation is dedicated to my loving family; especially to my mother and father. I could not imagine making it this far without your prayers and support mom and dad. I hope with this dissertation, you will always be proud.
ACKNOWLEDGEMENTS

I would like to acknowledge everyone who has supported me in this journey. Most of all, I would like to acknowledge my Ph.D. advisor Dr. Suri S. Iyer, who has always believed in me even when I did not believe in myself. I also would like to thank all my current and past group members; Dr. Yang Yang, Dr. Yun He, Dr. Abasaheb Dhawane, Dr. Bharat Gurale, Joyce Sweeney, Amrita Das, Xiaohu Zhang and Xikai Cui.
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1.1 Abbreviation

Hemagglutinin; HA, Neuraminidase; NA, N,N Dimethyl formamide, DMF; Ethyl acetate, EtOAc; Trifluoroacetic acid, TFA; Acetonitrile, CH$_3$CN; Azidotrimethylsilane, TMSN$_3$; Diethylamine DEA; Trimethylsilyl trifluoro methanesulfonate, TMSOTf; p-Toluene sulfonyl, Tos; Di-tert-butyl dicarbonate, (Boc)$_2$O; tert-Butyl alcohol, t-BuOH; Methanesulfonyl chloride, MsCl; Methanol, MeOH; Tetrahydrofuran, THF; Dichloromethane, DCM; Hydrochloric acid, HCl; Triphenylphosphine, PPh$_3$; Sodium sulfate, Na$_2$SO$_4$; Sodium azide, NaN$_3$; N-methyl morpholine, NMM; 2-Chloro-4,6-DiMethoxy-1,3,5-Triazine, CDMT; Sodium methoxide, NaOMe; Cupric sulfate, CuSO$_4$; Et$_3$N: Triethylamine, TEA. Azidotrimethylsilane, TMSN$_3$; Tetrabutylammonium bisulfate, TBAB; p-Toluene sulfonyl, Tos; Di-tert-butyl dicarbonate, (Boc)$_2$O; Methanesulfonyl chloride, MsCl.
2

Introduction
2.1 History of Influenza

Influenza is a respiratory illness that affects millions of people annually. In addition to seasonal influenza, pandemics can cause considerable harm. The first major influenza pandemic was reported in 1918 (H1N1), also known as the Spanish flu, where approximately one third of the world’s population was infected, and deaths were estimated up to 50 million.\(^1\) After the 1918 pandemic, there were no major pandemics for ~30 years. In 1957, the Asian influenza (H2N2) outbreak led to 250,000 deaths in Hong Kong in a very short period of time. The Asian influenza strain (H2N2) had a short life span and disappeared in only 11 years.

It is not until another decade later that another influenza pandemic infected a large number of people. The outbreak of 1968 (H3N2) in Southeast Asia and migrated to Europe and the Americas. This strain caused severe illness in the United States. Nonetheless, in Western Europe, including United Kingdom, low death rates accompanied the severe illness. It took another decade for the 1977 pandemic (H1N1) to arrive, but this strain was a juvenile age-restricted strain. The most recent pandemic occurred in 2009 (H1N1, swine flu). The first reported case in the United States was reported in April 2009 and by June 2009, 18,000 new cases were reported.\(^2\) It is not until August of 2010 that the World Health Organization (WHO) declared an end to the H1N1 flu pandemic of 2009.
Table 1: Deaths and infection rate associated with major pandemics. Table was adapted from multiple sources. 

Table 1  Deaths and infections rate associated with major pandemics

<table>
<thead>
<tr>
<th>Pandemics</th>
<th>Deaths</th>
<th>Fatality Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1918-1919 (H1N1)</td>
<td>20-100 million</td>
<td>2.0%</td>
</tr>
<tr>
<td>1957-1958 (H2N2)</td>
<td>1-1.5 million</td>
<td>0.13%</td>
</tr>
<tr>
<td>1968-1969 (H3N2)</td>
<td>0.75-1.0 million</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>2009-2010 (H1N1)</td>
<td>18,000-284,500</td>
<td>0.03%</td>
</tr>
</tbody>
</table>
Figure 1: Influenza is classified into three different types; A, B and C.
2.2 Classification of Influenza

Influenza virus is classified into three types; A, B and C. Types A and B are most important due to their ability to infect humans. Type A influenza virus can be further broken into subtypes based on their two surface proteins; HA and NA. The virus was first isolated from swine in the late 1920s, followed by the first virus isolation from humans in 1933. Influenza type A is a member of the Orthomyxoviridae family and is characterized by the presence of eight segmented negative strand RNA. These single stranded RNA encoded for about 10 viral proteins. Amid the 8 single-stranded RNA, the two shortest are associated with nucleoproteins (NP) and all three P proteins (PB1, PB2 and PA). These proteins are viral machinery for RNA replications and transcriptions.

Influenza virus (type A and B) contain three important surface proteins: Hemagglutinin (HA), Neuraminidase (NA), and a M2 ion transporter. HA and NA are glycoproteins anchored on the lipid bilayers of the virus. They are very important in the replication process of the influenza virus. Extensive research has shown that HA and NA binds to N-acetyl neuraminic acid (sialic acid) on glycoproteins and glycolipids of host cells. HA is implicated in the early stage of infection by recognizing the sialic residues on cell surface. This initial step is followed by endocytosis of viral particles into the cells. 16 HA subtypes and nine NA subtypes that had been identified. These subtypes are conserved in type A influenza virus in aquatic birds population. Influenza type A, like many other viruses, goes through frequent evolutionary mutations at multiple levels. These mutations is resulted from a number of mechanisms: point mutations (antigenic drift), gene re-assortment (genetic shift), defective interfering particles, and RNA recombinations. Arise in drug resistant strains of influenza virus is the result of these frequent mutations.
Figure 2 Life cycle of the influenza virus

**Figure 2:** *Life cycle of the influenza virus* A. Influenza A viruses composed of eight single stranded RNA and have three major proteins on the cells surfaces: HA, NA and M2 ion pumps. B. The influenza virus infection is initiated by the binding of HA to the host receptors which will induce endocytosis. Once inside the cells, pH changes causes the fusion of viral envelope and endosome which will cause the release of vRNA.\(^1\) (Figure was taken with permission from publisher)
2.3 Life Cycle of Influenza

The cycle of influenza begins when HA binds to sialic acid, which is the cellular receptor. These sialic acids are the terminal carbohydrates bound to glycolipids or glycoproteins located on the cell surfaces. In humans, the infections begin in the upper respiratory track. The binding leads to the fusion process to bring the viral particles into the endosome via endocytosis. M2 proteins respond to the low pH of the endosome by opening and allowing protons flux into the viral particles. The low pH causes the viral RNA to dissociate from the matrix proteins and the fusion of viral and endosome membranes. The fusion of viral and endosome results in the release of viral RNA into the cytoplasm. When viral RNA is released, they must travel into the nucleus where they can replicate and transcribed into a positive sense RNA, which travel back into the cytoplasm to be translated into proteins. Once the RNA has been replicated, the packaging and processing occurs and the viral progeny leaves the cell to infect other cells. Influenza viruses leave the cells via a process call budding. The influenza virus is an envelope virus, therefore, in the process of budding it uses the plasma membrane of the host cells to form its envelope. The surface of these newly formed viral particles contains all the required proteins to leave the host cells. As the virus emerges, residual sialic acids on the dying mammalian cell bind to the viral progeny presenting the virus from infecting other cells. The virus overcomes this problem by using the surface glycoprotein, NA. The function of NA is to cleave terminal sialic acid from glycoproteins and glycolipids to release the newly formed viral particles to go and infect other cells. Details of the major influenza proteins are described in detail below.

Influenza viral proteins

There are a number of essential proteins important for the efficient replication of influenza viruses. We highlight the essential proteins.
2.4 Hemagglutinin (HA)

HA is a receptor-binding glycoprotein that are composed of three identical subunits and is anchored on the surface of the influenza virus. HA proteins are classified into 16 different subtypes. Their roles are to recognize sialic acid present on the surface of host cells and to initiate the endocytosis process. Sialic acid is conjugated to the 3 or 6 position of galactose present on glycolipids and glycoproteins. HAs from different viral strains prefer to bind to different oligosaccharides i.e. the nature of the penultimate saccharide and internal saccharides can dictate the binding preferences. For example, HA’s from the avian lineage prefers NeuAc α2, 3 Gal linkages, while HA’s (H1, H2, and H3) from human lineage prefers NeuAcα2, 6-Gal linkages. On the other hand, HA’s from the swine lineage exhibits similar binding preferences to NeuAcα2, 3 Gal and NeuAcα2, 6 Gal linkages.
Figure 3 HA host transmission, homotrimer structure and receptors

**Figure 3:** HA host transmission, homotrimer structure and receptors  
A) Schematic showing the relationship between HA’s from different lineage. Avian HA’s prefers binding to NeuAcα2, 3 Gal linked over NeuAcα2, 6 Gal linkages. On the other hand, human HA’s preferred NeuAcα2, 6 Gal linked over NeuAcα2, 3 Gal linkages. Swine HA’s are able to recognize and bind to both substrates.  
B) Side and overhead view of the 1918's H1 HA.  
C) Receptors recognized by HA’s of different lineage.
2.5 Neuraminidase (NA)

NA is the second major surface glycoprotein of both influenza A and B viruses. NA’s exist as tetramer that consist of four identical subunits with the molecular weight of approximately 240kD.¹⁷ The role of NA is to facilitate mobility of the influenza viruses’ particles. NA catalyzes the enzymatic cleavage of both NeuAα-2-3, and NeuAα 2-6 glycosidic bonds. The cleavage of these terminal sialic acids gives several advantages to the replication cycle. First, NA allows viral particles to move through mucin by cleaving sialic acid from host cells and preventing other influenza virus from infected already infected cells. Second, NA can remove both sialic acid from cellular glycoproteins, glycolipids, and even from both virus surface proteins. Thus, NA prevents newly formed viruses from aggregating.

![Figure 4: Three dimensional structure of NA](image)

**Figure 4:** Three dimensional structure of Neuraminidase. A) Overhead view of NA tetramer. B) Overhead view of a single unit of NA with binding site.¹⁴ (Figure was taken Gamblin S.J. et al with permission)
NA is considered an exo-glycohydrolase; it hydrolyzes terminal sialic acid residues from glycoproteins and glycoconjugates. Cleavage of terminal sialic acids is initiated with the binding of to the active sites of NA. The stable chair conformation is forced into a less stable boat conformer due to the highly conserved triarginyl cluster (Arg-118, Arg-292, and Arg-371). Conformational changes facilitate the departure of aglycon residue through the formation of an oxocarbocation ion intermediate (sialosyl cation). The sialosyl cation undergoes a nucleophilic attack to form a glycosyl-enzyme intermediate. The glycosyl-enzyme intermediate breaks to form a sialosyl cation, where it goes under another stereo-selective nucleophilic attack by a water molecule to form an α-Neu5aAc as the first product to release for the active site. The α-Neu5Ac product isomerizes into β-Neu5Ac, the more thermodynamically favored product.
Figure 5: Mechanism of Neuraminidase for cleaving terminal sialic acid

Mechanism of Neuraminidase for cleaving terminal sialic acids. Figure was adapt from Nature publisher with permission.7
2.6 M2 Proton Channel

M2 protein of type A and B influenza virus are homotetrameric, type III integral membrane proteins that are important to the life cycle of the influenza virus. M2 proteins are responsible for the dissociation of the viral particles to release RNA materials. At the later stage of replication, M2 is responsible for maintaining the high pH of the trans-Golgi network to prevent premature folding of HA. M2 works as a proton gate where they have an open and close mechanism. Researchers believe that the open and close mechanism is based on a single transmembrane domain residue of tryptophan (Trp41).

Figure 6: Three dimensional representation of tetrameric forms of M2 trans-membrane proteins.

Figure 6: Three dimensional representation of the tetrameric forms of M2 trans-membrane proteins. A) Side view of the proteins B) Overhead view of the proteins.
2.7 Influenza Viral Polymerase

Influenza polymerase is a heterotrimer, which is composed of three subunits call PA, PB1, and PB2 with the molecular weight of approximately 250k Da. All three subunits are required for the transcription and replications of viral RNA. Each unit has a distinct role in the transcription process. The initiation step for viral RNA transcription is where the PB2 subunit recognizes cap structure of host pre-mRNA in the nucleus. The short capped oligomers (10-13 nucleotides) is cleaved by an endonuclease subunit of PA and is used to prime viral mRNA by the subunit of PB1. The transcription process ends when the polymerase recognizes the polyadenylation signal, a oglio-U sequence located near the 5' end. Cusack et. al. was successful in obtaining X-ray crystals of the complete structure of influenza A and B polymerase including PA, PB1, PB2 and viral RNA promoter. The crystal structure of both influenza A and B polymerase is expected to lead to potential therapeutics in the future.
Figure 7: Influenza viral RNA replication with polymerase. Polymerase composed of three subunits PB1, PB2 and PA.\textsuperscript{30} (Figure was taken with permission from Boivin S. \textit{et al})
2.7.1 Endonuclease (PA-Polymerase Acidic)

The role of PA unit in influenza polymerase was not well defined until recently. There are several clues that support PA with several roles in the transcription and replication processes. These roles range from endonuclease, protease activities, and cap binding. Recent publications have confirmed that the PA subunit main function is endonuclease. PA was cleaved into two subunits by proteolysis studies labeled as N-terminal (25-kDa) and C-terminal (55-kDa). The larger fragment is understood to be a binding site to PB1 during complex formations and serve as a nuclear transport. The N-terminal domain is responsible for the cleavage of the CAP peptides from the host pre mRNA.

2.7.2 PB1-Polymerase Basic 1

Influenza polymerase has a very unique initiation step mechanism for viral RNA replication. The combination of PA, PB1 and PB2 helps successfully “snack” the cap from cellular pre mRNA to prime its own RNA for replication. In this process, PB1 has the most important role. It plays the central role in the RNA replication by acting as the backbone of the polymerase and holds the catalytic site for the RNA polymerase. PB1 has several highly conserve regions of amino acid (motifs I, II, III and IV) and they range from 4-13 amino acid residues. These highly conserved motifs participate in the active site of the polymerase. Cusack et al have reported the crystal structure for influenza A and B polymerase with all three subunits; PA, PB1 and PB2.

2.7.3 PB2-Polymerase Basic 2

Polymerase Basic 2 serves as an initiator for vRNA transcriptions and replications. It is also considered a major viral determinant for adaptation. PA2 binds to host-cell pre-mRNAs via the 5’ cap (10-13 amino acid units) and is cleaved by the endonucleolytic activity of PA subunit. The recognition of capped oligoribonucleotides by PB2 is stimulated by the binding of the 5’ end
of the viral RNA template to PB1 unit.\textsuperscript{35} The structure FluA PB\textsubscript{2} with 318-483 amino acid units containing cap-binding pocket complex with guanosine triphosphate (GTP) was recently determined. \textsuperscript{36} Zheng et. al. has determined the structure for FluB\textsubscript{cap} with 318-484 amino acid units containing cap-binding pocket complex with guanosine diphosphate (GDP).
Figure 8: Three dimensional structure of influenza virus polymerase. Polymerase consists of three subunits PA, PB1 and PB2. (Figure was taken from A. Pflug et al with permission).
Controlling the transmission of influenza viruses

Influenza is a deadly pathogen and therefore, a number of methods have been employed to arrest the spread of this disease. These methods include proper hygiene, vaccinations, antivirals and rapid diagnostic tests for appropriate countermeasures. These different methods have been shown to arrest the spread of the disease, however as the virus has a high mutation rate, some of these methods are no longer effective.

2.8.1 Vaccines

Influenza affects millions annually and thus the need for preventions are in high demand. Antiviral drugs assist in treating the infected and also arrest the spread of the viruses. However, the excessive use of one kind of antiviral (Oseltamivir) has resulted in a number of resistant virus strains. Annual vaccinations against the influenza virus is a good method to control the spread. Vaccination generates antibodies that mainly targets HA surface glycoproteins. As influenza antibodies mainly recognized specific regions on the globular head of the HA surface proteins, their efficiency relies on the similarities between the HA of the vaccines and the HA of the currently seasonal strains. Recently, a trivalent influenza vaccine was produced that induced antibodies against H1 and H3 in human A virus and one single HA phylogentic lineage of influenza B virus. However, at the beginning of the 2013 influenza season, quadrivalent, live-attenuated (FluMist Quadrivalent, MedImmune) and inactivated (Fluarix Quadrivalent, GlaxoSmithKline, and Fluzone, Sanofi Pasteur) influenza vaccines were available. Despite these advances, there are problems. It is not easy to predict the emergence of new variant strains for an upcoming season, which becomes problematic to produce vaccines and vaccinate millions in time for the new influenza season. There have been efforts to develop a “universal” vaccine for a broad spectrum of influenza strains and this effort remains an active area of research.
2.8.2 Current Anti-Influenza Drugs

A second method to control the spread of influenza and decrease the severity and time of illness, anti virals have been developed. These are given below.

2.8.3 First Class of Anti-Influenza (M2 Inhibitors)

As mentioned above, the influenza virus particles have three major surface proteins. Among the three surface proteins, one is an ion channel (M2). The M2 ion channel is responsible for transporting protons to facilitate dissociation of viral particle and the release of viral genome. This process is crucial for viral infections and therefore, was among one of the first proteins targeted for antiviral drugs. The first class of antivirals that was used and most effective for the treatment of influenza are adamantane-based drugs. Amantadine and ramantadine target M2 channel and were used as first-choice antiviral against outbreak of the influenza A virus. The antivirals work by direct pore-blocking mechanism. However, as the influenza virus mutate, these older antiviral drugs has proven ineffective. Over 90% of the influenza viral A strains has shown resistance to this class of antivirals. A more recent strain of H1N1 related to the 2009 pandemic outbreak, has also shown to be resistant to the adamantane-based drugs. Therefore, these drugs are no longer recommended in the treatment of influenza in the United States since 2005.
Figure 9: Structures of adamantane based antiviral drugs used against the influenza virus. These class of inhibitors inhibit the M2 channel on the influenza virus.

2.8.4 Second Class of Anti-Influenza (NA Inhibitors)

The next generation of antivirals that are more effective and less resistant drugs are inhibitors of NA. High resolution of X-ray crystallographic data have given insight into the active site of NA to develop two transition state analog inhibitors. Zanamivir\textsuperscript{©} is a sialic acid analog (2-deoxy-\(\alpha\)-D-Nacetylneuraminc acid) that has a guanidine functionality at the 4\textsuperscript{th} position of 2-deoxy-\(\alpha\)-D-Nacetylneuraminc acid. Oseltamivir\textsuperscript{©} is a cyclohexene derivative that contains an amino functionality at the 4\textsuperscript{th} position of the cyclohexene ring.
**Figure 10:** Structures of current NA inhibitors. A) Native sialic acid with numeric system for assigning carbon. B) Deoxy form of sialic acid with hydroxy group at the 4th position. C) Zanamivir<sup>©</sup> is a deoxy form of sialic acid with a guanidine functional group at the 4th position. D) Oseltamivir<sup>©</sup> derivative of cyclonexane that mimics the transition state of sialic acid with an amino functional group on the 4th position.

Zanamivir and Oseltamivir both were approved by the FDA in 1999 and very few strains of virus has shown to be resistant. Zanamivir is licensed under the trade name of Relenza is administered through an inhaler (10mg/dose) and Oseltamivir is licensed under Tamiflu. Oseltamivir is administered orally (75mg/dose).<sup>37</sup> Oseltamivir is the more widely used in comparison to Zanamivir. This is because of it’s good bioavailability as an oral drug and because most patients prefer oral medication over an inhaler.<sup>42</sup> Unlike Oseltamivir, Zanamivir is administered by an inhaler device and must be administered twice a day for five days. Unfortunately, the more recent community outbreak in 2007-2008 of A(H1N1) has shown to have more Oseltamivir-resistant strains. Fortunately, most of the Osteltamivir-resistant strains are not resistant to Zanamivir. However, since patients prefer oral drugs and there is a strong possibility that resistance to Zanamivir may develop, there is an urgent need to develop better analogs.
Figure 11: Peramivir is a NA inhibitors recently approved by FDA for the treatment of influenza in 2012.

With the demand for more effective drugs to combat the annual influenza outbreak and potential pandemics, there are new drugs that were approved in 2012. Peramivir (Rapiacta®, Peramiflu®, BioCryst Pharmaceuticals, Research Triangle Park, North Carolina, USA) is among one of the drugs that was in phase III clinical trials and was approved for use during the 2009 H1N1 influenza outbreak under the Emergency Investigational New Drug (eIND) regulations. Peramivir, unlike traditional NA inhibitors, it has a unique structure different from known NA inhibitors. This NA inhibitor exhibits high binding affinity that last up to 24 hours and has a slow dissociation rate. Premavir has been approved for the treatment of influenza in Japan and South Korea. Unlike Zanamivir and Oseltamivir, Peramivir is administered through IV with the dosage of approximately 12mg/kg.
Figure 12: Laninamivir and its pro-drug structure. The prodrug form has shown to have an half-life of 41.4 hours in healthy adults.

Laninamivir, like Zanamivir, is administered through an inhaler and was approved for use in Japan in 2010. Researchers have also developed a pro-drug form of Laninamivir by introducing an 8-carbon chain at the 9-position via an ester bond. The pro-drug form of Laninamivir is currently under clinical trials and have shown to have a half-live of approximately 41.4 hours in mice. Other studies with healthy volunteers have demonstrated that Laninamivir pro-drug was slowly excreted from the body in a span of six days from one inhalation in healthy human volunteers. The long excretion time is due to the body hydrolyzing the long carbon chain to activate the pro-drug to its’ active form Laninamivir.

2.9 Drugs in Clinical Trials.

There are a number of influenza antivirals in clinical trials. We highlight some of the promising antivirals.
### 2.9.1 Fludase

DAS181 (Fludase® Ansun Biopharma Inc., San Diego, California, USA) is currently in clinical trials in the United States. Fludase is composed of bacterial sialidase catalytic domain taken from *Actinomyces viscosus* which is amphiregulin tag that is required for effective for targeting epithelial cells.\(^{47}\) The enzymatic mechanism of Fludase cleaves both α2,3- and α2,6-SA receptors on the host cells surface. With the removal of these receptors, it renders the influenza virus entry into the cells. Fludase is currently in phase II study and had shown to reduced viral load and significantly shorter time to reduced viral shedding.\(^{48}\) However, the use of Fludase has some concerns, one of which is systemic distribution and its effect on normal sialic acid function.

### 2.9.2 Favipiravir

Favipiravir (T-705) is another class of anti-influenza drugs that is currently in clinical trials. Unlike many anti-influenza drugs, Favipiravir mechanism of action works by inhibiting influenza viral RNA polymerase. The anti-viral drug acts as a prodrug that is phosphor-ribosylated in cells to the active form, favipiravir-RTP, which inhibits viral replications. The active form of Favipiravir, Favipiravir-ribofranosyl-5'-triphosphate (RTP), acts as purine mimic and is incorporated into the nascent RNA strand and inhibits strand extension.\(^{49}\) Favipiravir have been shown to have effect on a range of types and subtypes of influenza including NA-inhibitors resistant strains.\(^{50}\) Favipiravir is currently in stage II clinical trials in the United States and has been approved in Japan for pandemics.\(^{38}\)
Figure 13: Favipiravir is metabolized in the cells to first introduce a ribose sugar then it gets phosphorylated into the active form of the drug as an inhibitor against viral polymerase.

2.9.3 Nitazoxanide and Tizoxanide

2-(acetyloxy)-N-(5-nitro-2-thiazolyl) benzamide or Nitazoxanide was first developed as an antiparasitic in the 1970s. Later studies have shown that nitazoxanide is metabolized in the body to convert it into tizoxanide which have a range of therapeutic properties against a number of different diseases, such as: influenza, Hepatitis B virus, Hepatitis C virus, human immunodeficiency virus (HIV) and even tuberculosis (TB). Nitazoxanide is the first class of antiviral drugs that has a broad spectrum of treatments for multiple diseases. Therefore, it has been licensed in the United States for the treatment of intestinal infections. Further, Nitazoxanide has been in clinical trials for the treatment of influenza. In clinical trials, it has been shown to inhibit the replications of 16 strains of influenza A/H1N1, H3N2, N3N2, H3N8, H5N9, H7N1 and one strain of influenza B virus. Studies have shown that nitazoxanide works by preventing the maturation of viral HA. Nitazoxanide is currently in phase III clinical trials and will be the largest clinical trial conducted for the treatment of acute uncomplicated influenza.
2.9.4 Current Influenza Detection Methods

Early, rapid and accurate detection can also help in arresting the spread of influenza. There are several diagnostic tests for the accurate detection of influenza. These techniques can range from viral isolations (most inexpensive), rapid diagnostic test kits (inexpensive but not accurate) and real-time reverse transcriptase polymerase chain reaction (RT-PCR) (expensive, highly accurate). Although cell culture has been the golden standard for detection of respiratory virus, it requires a laboratory environment and takes days to confirm the infection. PCR has always been the most sensitive and most accurate method of detection, however, traditional PCR it required a long turnaround time and required highly trained technicians to operate. Newer point of care PCR detection systems are faster, but requires expensive instrumentation and the right primers. Details of the different techniques are given below.

2.9.5 Viral Isolation

Among all techniques used for the detection of the influenza virus, viral culture is considered the “gold standard” and is one of the most well established route. As the results of many clinical samples may have low virus titers and thus, amplification of viruses are required for accurate detection. The amplification process can be done with a numbers of different cell lines; cell lines such as primary epithelial cells or human adenoid, Vero cells, MRC-5, primary monkey kidney cells and Madin-Darby canine kidney (MDCK) cells have been used for
amplification and isolation of influenza virus. However, MDCK cells are most commonly used cell lines in the case of influenza virus because it can support the growth of both influenza A and B.\textsuperscript{55} Traditionally, results of viral amplification can be observed based on cytopathic effect (CPE). The concentration of viruses in a clinical samples are usually unknown and requires multiple passage before any CPE can be observed. In order to determine if the sample is negative or not, it must has at least two passages and this can result in long turn overtime (can last up to 4-10 days).\textsuperscript{56} After viral culture, immunoassay can be done to characterize the viruses.\textsuperscript{56}

\textbf{2.9.6 Polymerase Chain Reaction (PCR)}

Polymerase Chain Reaction is a technique developed by Kary B Mullis in 1985 for the amplification of DNA materials. This technique has been accepted as the gold standard for influenza virus detection because it is highly accurate and can differentiate the subtypes of influenza.\textsuperscript{57} \textsuperscript{58} However, PCR is limited due to the availability of primers for new emerging virus strains and also it requires specialized training. In recent years, since the 2009 outbreak, the need for point of care detection kit has increase dramatically. Due to the time constraint associated with traditional PCR, new emerging PCR based technology has emerged.\textsuperscript{59} These new technologies utilize the PCR based which are highly selective and highly accurate to detect influenza within four hours. Currently, there are multiple PCR based assays that are approved by the FDA for the diagnostic of influenza. The first of this class is Prodesse Proflu\textsuperscript{+} by Hologic which was approved by the FDA in 2008.\textsuperscript{60}

\textbf{2.9.7 Rapid Diagnostic Tool (RDT)}

The first detection method, enzyme immunoassays technology (EIA), was first approved by the FDA in 1970’s and followed by many more in the late 1990’s. There are currently 16 Food and Drugs Administration (FDA) approved rapid diagnostic tool (RDT). The basis for these
rapid diagnostic tools is antigen detection that target either the HA or NA of influenza.\textsuperscript{61} However, many RDT’s exhibit poor sensitivities.\textsuperscript{61}
Table 2: Compilations for the sensitivities of RDT's for the detection of influenza (Table was taken from FDA website without permission)

Table 2: Compilations for the sensitivities of RDT's for the detection of influenza
<table>
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<tr>
<th>Manufacturer</th>
<th>Device Names</th>
<th>Date of FDA Clearance</th>
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<th>B (%)</th>
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2.9.8 Glycan based diagnostics

To overcome issues with current diagnostics, we have developed a receptor based approach for influenza viruses. Briefly, the virus requires HA and NA to bind to the cell surface sialic acids. These two influenza glycoproteins are excellent targets for capturing influenza virus for use in diagnosis, particularly because there are approximately 200-300 copies of HA and 50-100 copies of NA on a single viral particle, respectively. The same molecules can be used to inhibit the glycoproteins. Therefore, we developed sialic acid analogs as recognition molecules. The details of our approach, synthesis and biological studies are given in the following chapters.

2.10 Summary

Influenza virus affect millions worldwide annually. It is well known that influenza viruses have several copies of HA and NA that are good targets for intervention. The first class of antiviral is adamantane based, which block M2 protons pump proteins. However, most strains of influenza viruses exhibit resistance to this class of antivirals. The second generation of anti-viral for influenza are NA inhibitors. Oseltamivir, an orally available antiviral is widely used, but resistance to this antiviral is on the rise. Thus, the need for newer drugs that exhibit less resistance is urgent. We are developing sialic acid analogs that can be used to inhibit HA and NA. Our approach, design and synthesis are described in Chapter 3.

Early detection of influenza viruses is also an important need to arrest the spread of this deadly disease. However, detections methods currently on the market are inadequate because they are expensive, require trained personnel, equipment, or exhibit poor sensitivities. The lack of good detection tests is problematic as most physicians prescribe antivirals based on symptoms. We have developed a receptor based method that uses sialic acid analogs to detect influenza viruses and determine drug susceptibility. The approach, synthesis and initial biological studies are described in Chapter 3.
3

Glycan Based Detection and Drug Susceptibility of Influenza Viruses

This work was published in ACS Analytical Chemistry 2014, 86, 8238-44. A second manuscript is under preparation,
3.1 Abstract

Influenza viruses have three surface proteins, two of which are glycoproteins called HA and NA. HA functions as an initiator for cellular infections by binding to highly specific ligands to gain entry into the cell. HA is known to bind to N-Acetyl neuraminic acid (NeuAc or sialic acid) conjugated to the 3 or 6 hydroxyl position of galactose. NA is an enzyme that facilitates the release of viral progeny that adheres to sialic acid of the dying cell. NA are known to cleave O-linked sialic acid residues on both viral and non-viral glycoproteins. We have designed a panel of small molecules that can bind to these viral glycoproteins as recognition molecules to detect the influenza viruses. These glycans were printed onto glass slides to evaluate their ability to capture the influenza viruses via NA. We demonstrate that the glycans bind to three influenza viruses with high sensitivity. We also demonstrate drug susceptibility using FDA approved antivirals, Zanamivir and Oseltamivir. Finally, we synthesized a novel molecule that can be used as a reporter molecule instead of antibodies as reporters.
3.2 Introduction

Influenza virus is a respiratory disease that causes 200,000 hospitalizations and over 30,000 deaths in the United States annually.\textsuperscript{63} Pandemics, like the 1918 H1N1 “Spanish flu”, can cause millions of infections and deaths in addition to disruption of economic activity.\textsuperscript{64, 65} Early detection is the key in arresting the spread of this disease, because most antivirals lose their efficacy if it is not administered within 24-48 hours upon infection.\textsuperscript{63} Thus, inexpensive point of care diagnostic devices that are capable of detecting the virus rapidly would be very useful to mitigate the effects of the virus and possibly arrest its spread. One recent example where a rapid detection device was needed was the 2009 H1N1 “swine flu” pandemic. The virus spread across the globe within ten days and resulted in an influx of patients at local clinics and hospitals.\textsuperscript{66, 67, 68} This fast moving pandemic took the world by surprise and most medical facilities were overwhelmed. There are a number of available methods for detecting influenza - polymerase chain reaction (PCR), antibody based tests and cell culture. PCR based detection is the most accurate and definitive, however PCR is expensive and may require highly trained personnel to run samples. Antibody based tests are the basis of all point of care diagnostic kits. These tests are can give results within 15-30 minutes, however, they are typically only used for surveillance due to their low sensitivity.\textsuperscript{69, 70} Cell culture traditionally has always been the golden standard for detecting respiratory diseases, however, it requires a long turnaround time and required trained personnel.

Influenza virus is known to bind to terminal residues of N-acetyl neuraminic acid (or sialic acid) of glycoproteins and glycolipids to induce cell entry. Thus, sialic acid can be used to capture influenza viruses as a receptor based approach.\textsuperscript{7, 71, 72} Glycan based receptors for diagnostics is a relatively new area of research. Synthetically, glycans can be synthesized in large quantities with good economic yield. Glycans are highly robust under different conditions.
and do not require refrigeration. Glycans are small molecules and are much smaller than antibodies or affibodies, therefore they can potentially be used in nanobiosensor applications, where a nano-glycan conjugate would not perturb the overall size of the nanoparticle. An added advantage with synthesized glycans is that we can introduce different functional groups to modify the glycans. A panel or library of glycans can be used to create a microarray, which can be used to study binding patterns of viruses. Indeed, other researchers have also developed glycan microarrays to capture influenza virus via HA using microarrays.

3.2.1 Microarray Technology

Microarray technology has been around for several decades. Recently, this technology has been applied to study glycan-protein interactions. Microarray technology has allowed researchers to investigate hundreds of glycan-binding proteins interactions on a single chip. Therefore, glycan microarrays are a good tool for the analysis of the specificity of ligands to influenza surface proteins. It also has been shown to be a helpful tool for both intact viruses and recombinant HA.
**Figure 15**: Microarray based assays have the ability to test the specificity of hundreds of glycan-binding protein interactions on a single strip. Glycans can also be printed onto activated glass slides that will allow glycans to be molecularly attached onto the surface and to investigate the specificity of glycans to intact influenza virus and recombinant HA. (Figure was taken from Nature Publishing Group with permission).
Figure 16: Antibody based microarray assays as reporters

**Figure 16:** Microarray assays are traditionally done with antibodies as a tool for reporting the effectiveness of the assay. However, there are some drawbacks when antibodies are used; first, they are hard to generate, second they are not stable if not stored at the proper conditions. (Figure taken from Nature Publishing Company with permission)
Using microarray technology allows us to understand the fundamentals of virus-receptor relations; glycan microarray using O-sialosides have been used to explain the preference between 2,3 linkage and 2,6 linkage for different types of influenza viruses.\textsuperscript{86, 85, 87} However, one concern is that in order to use the same microarray with intact viruses is that NA cleaves O-sialosides rapidly.\textsuperscript{88} Therefore, most published papers have performed assays at low temperature (4°C) and/or in the presence of NA inhibitors to avoid the NA from cleaving O-sialosides.\textsuperscript{89, 90, 91} In order to overcome this issues, former lab members Dr. Yun He and Dr. Yang Yang designed and synthesized a panel of S-, N- or C-linked (\textbf{Figure 16}) that can be used to capture virus via HA at ambient temperature and without NA inhibitors.
Figure 17: First generation capture molecules (synthesized by Dr. Yun He and Dr. Yang Yang).
### 3.2.2 First Generation Capture Molecules

Our first generation capture molecules were designed and synthesized by former lab members Dr. Yun He and Dr. Yang Yang. These molecules are not susceptible to cleavage by NA as they do not have O-linkages\(^7\), therefore, they can be used with intact viruses. The first generation capture molecules were able to capture influenza viruses based on our microarray assays and successfully generated a binding pattern for individual virus strains. These molecules were tested against six influenza strains. The results are shown below. The H1N1 A/Puerto Rico/8/34 strain, for example, has higher affinity to compound SC6 rather than H1N1 A/Brisbane/59/2007 that has a higher infinity to SC8. In comparing the H1N1 A/New Caledonia/20/99 and H1N1 A/Solomon Islands/59/07, the binding patterns are not completely identical. For H1N1 A/New Caledonia/20/99, compound SC3 and SC7 have a higher affinity in comparison to H1N1 A/Solomon Islands/59/99 where SC8 has the highest affinity. In terms of the pandemic strains, H1N1 A/California/7/09, SC3, SC4 and SC8 have the highest affinity for this viral strain. Based on these results, we can conclude that binding affinities for different ligands are completely different and a pattern of recognition can be potentially used to type the strain.
Figure 18: Binding profiles of different strains of MDCK cell adapted influenza viruses. (Figure was taken from Dr. Yun He’s dissertation).
3.2.3 Second Generation Capture Molecules

The second generation molecules were designed based on transition state analogs and the native sialic acid (SA). These molecules were designed to target NA. Based on the X-ray crystal structures, a number of NA inhibitors have been developed to mimic the transition state of the cleavage reaction. These inhibitors are among some of the current anti-viral drugs on the market (Oseltamivir and Zanamivir). The rational design is based on the crystal structure (Figure 19) where there are three amino acid residues in the active site interacting with the fourth position of SA, therefore, if a positively charge group, such as a guanidine group, is introduced the binding affinity will increase. Our design for this class of capture molecules was to introduce a linker to the 7 position of Zanamivir as modifications at this position do not affect the binding to NA. A second class of capture molecules were also developed. We introduced the guanidine or amino group at the fourth position to the native structure of sialic acid and we introduced a sulfur linkage (S-linked) at the second position. We also hypothesized that if we increase the number of ligands, the binding affinity will increase due the multivalency. Thus, we introduced a dimeric scaffold with two terminal alkyne functionalities that allow us to utilize azide-alkyne Huisgen 1,3-dipolar cycloaddition to attach the glycan monomers to the scaffold. The dimeric scaffold also helps in separating the glycan from the surface, giving it more mobility and accessibility to the NA.
Figure 19 Active site of influenza NA

**Figure 19:** Active site of influenza NA. Figure was taken with permission from Nature Publisher.
3.2.3.1 1,3 Huisgen Cycloaddition (Click-chemistry)

Click-chemistry, also known as Huisgen 1,3-dipolar cycloaddition, involves an azido group reacting with an alkyne to form a five membered ring system.\textsuperscript{95} The reaction occurs due to the $\pi$ electrons between the two systems, where it occurs in a concerted, pericyclic shift, therefore, it is considered a stereo conservative addition. The stereo selectivity can be controlled using appropriate catalysts. Thus, this type of chemistry is highly versatile and has broad range of applications.\textsuperscript{95, 96, 97} Click chemistry can also be used for our system, because we can use a copper (I) catalyzed reaction to selectively introduced our carbohydrate ligands onto the scaffold that is trans stereo specific.\textsuperscript{95}
Figure 20: Cis and trans conformations for the formation of the triazole ring can be controlled with different catalysts. Trans-formation can be achieved with copper catalyst and Cis-conformation can be controlled by ruthenium catalyst.
3.2.4 *Synthesis of Second Generation Capture Molecules*

The structures of the eight molecules used in the development of our microarray based assays are shown in Figure 7. There are several features of the designed molecules. First, all eight molecules are derived from natural receptors (sialic acid). Second, the molecules have a free amine at the end of the spacer, which is important for immobilizing it onto the glass slides that contains the activated carboxylic acid groups. Third, the molecules also have an amine or guanidine group at the fourth position, thus this will result in high specificity binding to the influenza NA, that has been shown by crystal structures, but will not fit into human NA. Fourth, we have designed two types of molecules; SC1-4 are sialic acid analogs that has a spacer attached to the second position via a thiol linkage, the other, SC5-SC8, are Zanamivir analogues that are attached to a spacer at the seventh position. Finally, we recently have demonstrated that molecules similar to SC1-4 and bivalent molecules thereof, inhibited two H1N1 and H3N2 strains at low nanomolar concentrations, here we designed monovalent and divalent derivatives. We have designed a divalent scaffold for two reasons; first, the divalent scaffold provides additional distance from the microarray surface for viral NAs’ to bind. Second, NA exist in tetramer with four binding sites from one single NA tetramer or, fit into the pockets of two adjacent NA tetramers on a single viral particle. When these mono and divalent molecules are tethered on a surface, the overall binding affinity of multiple glycans with the influenza viruses are expected to increase exponentially.

The synthesis of the molecules is shown in Scheme 1. For SC1-4, the synthesis begins with commercially available sialic acid from Carbosynth and is esterified by suspending compound 1 in MeOH and the pH was adjusted with DowX H⁺ resin to 2, then allowed to stir overnight to yield compound 2 in quantitative yield. Compound 2 was then subjected to acetic anhydride in pyridine to convert the free hydroxyl groups into acetate groups with quantitative yield to give compound 3. An azido group was introduced at the fourth position by a two-step one pot synthesis starting with compound 3 in anhydrous EtOAc and TMSOTf at 40°C for hour.
hour, then after work up and being dried, it was dissolved in t-BuOH and TMSN$_3$ was refluxed overnight to yield compound 4 with an overall yield of 90%. Compound 6 was made by dissolving compound 4 in CH$_3$CN with LiCl and HCl gas was bubbled through for five days and neutralized to give compound 5. Compound 5 was dissolved in a DCM:H$_2$O system with TBAB and KSAc which was allowed to stir overnight to yield compound 6. Compound 7 was synthesized by reacting compound 6 with 6-chlorohexyl 4-methylbenzenesulfonate in anhydrous DMF and TEA to yield 7 in 90% yield. With compound 7 in hand, the reduction of compound 7 with triphenyl phosphine in THF:H$_2$O (1:1) system and the amine was protected with di-t-butyl carbonate anhydride and TEA in THF resulted in 85% yield of compound 8a. Compound 8b was made in a similar fashion, where compound 7 was reduced and reacted with 1,3-bis(tert-butoxy-carbonyl)-2-methyl-2-thiopseudourea, HgCl and TEA in anhydrous DCM with 85% yield. Compound 9a and 10b were made in similar fashion; compound 7a or 7b was dissolved in DMF, then NaN$_3$ was added and heated at 60°C to give 95% and 96% yield, respectively. Zemplén deprotection to remove the acetates and methyl ester was followed by acidic removal of the tert-butoxy groups and reduction of the azide using hydrogenation conditions yielded the monovalent compounds SC$_1$ and SC$_2$ which had an amine and a guanidine group at the four position of the sialic acid, respectively, from compound 8a,b. The key intermediate compounds are 8a and 8b, because they can be “clicked” to our divalent scaffold. Copper(I) catalyzed 1,3 dipolar addition of 8a,b with a dimeric scaffold, 10 bearing two alkyne groups yielded the fully protected divalent compounds, 10a,b in good yields.
Figure 21: Structures of synthesized molecules for the capture of influenza viruses. Figure taken from Analytical Chemistry with permission.98
Figure 22: a) Dowex H+ Resin, MeOH.  b) Acetic anhydride, pyridine.  c) i. TMSOTf, EtOAc, 40°C, 4h, ii. TMSN₃, t-BuOH, reflux. d) LiCl, HCl(g) CH₃CN.  e) KSAc, TBAHS, DCM:H₂O.  f) 6-chlorohexyl 4-methylbenzenesulfonate, TEA, DMF. g) i. Ph₃P, THF:H₂O, ii. 1,3-Bis-(tert-butoxycarbonyl)-2-methyl-2-thiopseudoharnstoff, HgCl, TEA, DCM.  h) i. Ph₃P TFH:H₂O, ii. di-
t-butyl carbonate anhydride, TEA, THF. i) NaN₃, DMF. j) i. NaOMe, MeOH  ii. 50mM NaOH  iii. TFA:DCM. k) Sodium ascorbate, CuSO₄, THF:H₂O (1:1).

The synthesis of SC⁵⁻⁸ was done by Xiaohu Zhang and is shown in Scheme 2. Compound ⁴ was taken and the azido group was reduced via hydrogenation with Lindlar catalyst in EtOH to give compound ¹⁰ with quantitative yield. Compound ¹⁰ was subjected to based induced deprotection of the acetate groups and acetonide protection of the 8,9 hydroxyl groups leaving the 7 hydroxyl group open for conjugation to the spacer. Compound ¹₂ was obtained by converting the free hydroxyl group at the ⁷th position of compound ¹¹ into an activated ester via 4-nitrophenyl chloroformate with DMAP and pyridine. The active ester was then reacted with ⁷-azidoheptan-1-amine in THF and TEA to yield compound ¹₂ with the yield of 89%. The acetonide group was removed under mild acidic conditions to produce ¹₃ᵃ. The guanidine derivative was synthesized from compound ¹₃ᵃ, the tert butoxy group was removed and a suitably protected guanidine group was attached to the free amine to yield ¹₃ᵇ in significant amounts. This strategy of installing the guanidine group at this later stage was more successful in our hands as opposed to introducing the guanidine group early in the synthesis, the latter gave us undesirable products and variable results. Global deprotection of ¹₃ᵃ,ᵇ was performed as described for SC¹⁻² to yield SC⁵⁻⁶ in good yields. The final compounds were purified using size exclusion chromatography using Biogel P2 and the appropriate fractions containing the compounds were freeze dried to produce colorless foamy material.
Figure 22: Synthesis scheme of Zanamivir analogs

Figure 23:  l) Lindlar Catalyst, H2, quantitative yield  m) NaOMe, MeOH, quantitative yield
ii. H+ resin, acetone, 12 h, 88%.  n) 4-nitrophenyl chloroformate, DMAP, pyridine, 80% yield,
ii. 7-azidohexan-1-amine, TEA, THF, 89% yield.  o) TFA:DCM (1:1), 1,3-Bis-(ter-
butoxycarbonyl)-2-methyl-2-thiapseudohernstoff, HgCl, TEA, DCM, 84% for 14b.  j) NaOH
50mM ii. TFA:DCM (1:1) quantitative yield.  k) Na-Lascorbate, Copper sulfate. THF/H2O, 12h,
78% for 16a; 80% for 16b. (Work was done by Xiaohu Zhang)
3.3 Alternative to Antibody-Based Reporting Microarrays

As reported earlier, we have developed a number of bivalent sialic acid scaffolds that have the ability to capture the influenza virus via NA; SC3, SC4, SC7, SC8. We have utilized the same scaffold with the same substrate, and we have installed a biotin handle at the free primary amine that is located on the spacer (Figure 23). The biotin handle will allow us to use labeled streptavidin as a reporter. Biotin-streptavidin dissociation constant (\( K_d \)) is extremely low, and there have been many applications designed using this concept.\(^99\) Furthermore, streptavidin is highly stable that exists in a homo-tetrameric form and thus, multiple biotinylated compounds can bind to streptavidin increasing the multivalency.\(^100\) Taken together, the stability, multivalency, and low \( K_d \) allow biotin-streptavidin to be used as a handle for highly specific detection.
Figure 23  Synthesized biotinylated reporter molecules

Figure 24: SC3, SC4, SC7, SC8 were used and we introduced a biotin handle to allow us to use fluorescently labeled streptavidin as a reporting method.
3.3.1 Biotin-Streptavidin

The protein, streptavidin, was first discovered to cause nutritional deficiency in rats due to the formation of a highly water soluble, non-covalent complex with biotin (vitamin B7). The affinity between biotin-streptavidin is exceptionally high ($K_d = 10^{-15}$ M) and therefore has very specific binding. Streptavidin exists in a tetrameric form with four identical subunits with a molecular weight of 52.8 KDa. The crystal structure was first reported in 1989 by two independent groups. Biotin-streptavidin interaction have been applied to numerous assays such as enzyme linked immunosorbert assay (ELISA), immunohistochemistry (IHC), Western, Northern, and Southern blotting, immune precipitation, cell-surface labeling, affinity purification, fluorescence-activated cell sorting (FACS) and electromobility shift assays (EMSA).
Figure 24: Three dimensional structure of streptavidin

**Figure 25:** Three dimensional structure of Streptavidin. Streptavidin exists as a tetramer with four identical subunits. Biotin-streptavidin binding affinity has one of the highest interactions known in biological systems. Structure was taken from Pugliese, L. *et al* with permission.100
Figure 25: Microarray assays designed with labeled streptavidin

Figure 26: Schematic of our assay; we will be immobilizing our glycans on glass slides, described in a previous chapter, and will use biotinylated compounds coupled with labeled streptavidin as a method for quantifying our results.
Figure 26: Synthesis of biotinylated S-linked sialic acid

Figure 27: 

a) i. DCM:TFA, ii) 7-(9H-fluoren-9-yl)methoxy)carbonyl)amino)heptanoic acid, CDMT, NMM, THF 80%. 
b) i. DMF:Piperidine, ii. (+)-Biotin N-hydroxysuccinimide ester, TEA, DMF 62% over two steps. 
c) Sodium ascorbate, CuSO4, THF:H2O:t-BuOH (1:1:1) 60-65%. 
d) i. NaOMe, MeOH, ii. NaOH (50mM), iii. DCM:TFA (1:1) 70-75%.
Dimeric scaffold 17 was first subjected to acidic removal of the tert-butoxy groups yielding the intermediate amine. The amine was then react with known 7-aminoheptanoic acid (NH-Fmoc protected) by CDMT-NMM coupling condition to give compound 18 with high yield. Fmoc protecting group were used because we want to be able to selectively deprotect this amino group to react with an activated biotin compound. Compound 18 was subjected to Fmoc deprotection in pyperidine and DMF system (1:1) to yield an amine intermediate. The amine intermediate is then reacted with (+)-Biotin N-hydroxysuccinimide ester in DMF and TEA to yield compound 19 in quantitative yield. Copper(I) catalyzed 1,3 dipolar addition of 8a,b with a dimeric scaffold, 19 bearing two alkyne groups yielded the fully protected divalent compounds, 20a,b in good yields. Zemplén deprotection to remove the acetates and methyl ester was followed by acidic removal of the tert-butoxy groups yielded the divalent compounds SAR and SGR.

3.4 Biological Assays

All final compounds were printed onto commercially available glass slides with activated carboxyl groups for the conjugation of the free amine from SC1-8 to produce a microarray. Microarray printing was done with various concentrations; the optimized concentration of 200 µM for all assays gave the best signal to noise ratio. We printed amine terminal PEG as a negative control. The microarray assay was done using three different influenza strains; two H1N1 A/Brisbane/59/2007 & A/Solomon Island/3/2006 and an H3N2 strain A/Aichi/2/1968H3N2. The strains were incubated at room temperature for 1h to allow attachment of viral particles to printed compounds, followed by incubation of the appropriate primary and fluorescently labeled secondary antibody. The slides were washed at every stage extensively before scanning. The slides were scanned with Genepix scanner at 532 or 635 nm. Fluorescence image is reproduced in Figure 14 where the printed compounds were exposed to
10^5 PFU/ml (plaque forming units) of H1N1 Influenza virus A/Brisbane/59/2007. As seen in the figure, bivalent compounds, SC3,4,7,8 capture the virus very well. However, almost all the monovalent compounds, SC1,2,5,6 have weak binding to this strains, suggesting there should be a minimal distances between the glass surfaces and the glycans for sufficient binding to occur. The same compounds were assays against an H3N2 (A/Aichi/2/1968) strain shows similar results, however when assayed against H1N1 (A/Solomon Island/3/2006) all compounds show excellent binding. We also observed differentiation of the binding patterns for these compounds to different strains of influenza viruses. For example, A/Aichi/2/1968 strain exhibits similar binding patterns for all bivalent compounds, whereas A/Brisbane/59/2007 strain binds better to the bivalent compounds SC4 and SC8. Compounds SC4 and SC8 are compounds that have a guanidine groups at the 4th position and the best binding is SC4. Next, we determined the analytical sensitivities of our microarray assays using different concentrations of the different strains from 10^6 to 10^1 PFU/ml. Figure 15 shows all bivalent compounds, SC3,4,7,8, bind well at higher concentration of viral particles according to fluorescence intensities. Further, at low concentration, 10^1 PFU/ml, all bivalent compounds bind H1N1 A/Brisbane/59/2007 and A/Solomon Island/3/2006, however only SC3 binds to the H3N2 A/Aichi/2/1968 strains at this concentration. Finally, we tested the microarray for susceptibility to FDA approved antivirals, Zanamivir and Oseltamivir (Figure 7). We first premixed each of the three strains of viruses with either one of the antivirals and exposed this mixture to the microarray. After sufficient washing we detect using the appropriate primary antibodies and subsequent secondary antibodies. The results supported our hypothesis. When we introduced NA inhibitors they blocked the binding sites, which leads to no signal. However, among our eight compounds, there are two outliers SC3 and SC7. In the presence of antivirals, SC3 binds to H3N2 A/Aichi2/1968 strains and SC7 binds to both H1N1 strains. Presumably, these molecules have some binding affinity to HA and therefore were able to capture some viral particles.
Figure 27  Influenza binding studies

**Figure 28:** Influenza binding studies **A.** Fluorescence image of microarray containing eight glycans (SC1-SC8) after exposure to $10^5$ PFU/ml of H1N1 Influenza A/Brisbane/59/2007, followed by ferret hyperimmune sera and anti-ferret rhodamine labeled secondary antibody for A/Brisbane/59/2007 and scanned by Genepix scanner at 532 nm. **B.** Fluorescence detection of two H1N1 (A/Brisbane/59/2007 and A/Solomon Islands/3/2006) and one H3N2 (A/Aichi/2/1968) influenza A viruses using synthetic glycans. Glycans, PEG (negative control) and biotin (positive control) were printed at 200µM. All virus concentrations were $10^6$ plaque forming units (PFU). Fluorescence intensity was measured by the Genepix scanner using ferret hyperimmune sera to influenza A/Brisbane/59/2007 (H1N1) and anti-ferret rhodamine labeled antibody; ferret hyperimmune sera to influenza A/Solomon Islands/3/2006 (H1N1), and anti-ferret rhodamine labeled antibody; polyclonal antiserum chicken to A/Aichi/2/1968 (H3N2), and Alexa Fluor® 633 labeled anti-chicken. A/Brisbane/59/2007 and A/Solomon
Islands/3/2006 were scanned at 532nm and A/Aichi/2/1968 at 635nm. All experiments were performed in triplicate. (Experiments were done by Joyce Sweeney).
Figure 28: Analytical studies using synthesized molecules

Figure 29: Analytical studies. A. H1N1 Influenza A/Brisbane/59/2007. B. H1N1 Influenza A/Solomon Islands/3/2006. C. H3N2 Influenza A/Aichi/2/1968. Fluorescence intensity was measured as previously described for figure 14. All experiments were performed in triplicate. (Experiments performed by Joyce Sweeney).
Figure 29 Drug susceptibility test using synthesized molecules

**Figure 30:** Drug susceptibility test. A. H1N1 Influenza A/Brisbane/59/2007. B. H1N1 Influenza A/Solomon Islands/3/2006. C. H3N2 Influenza A/Aichi/2/1968. 10 ng of antivirals Zanamivir® or Oseltamivir® were premixed with the strains at $10^5$ PFU for 30 min at rt and subsequently added to the microarray. Fluorescence intensity was measured as previously described for figure 14. All experiments were performed in triplicate. (Experiments performed by Joyce Sweeney).
3.5 Summary

We have successfully synthesized eight glycans, printed them onto glass slides and demonstrated their ability to capture three influenza strains at different concentrations. Through the microarray assays, we have determined limit of detection for our platform to be $10^1$ PFU, which is extremely low in terms of sensitivity. We have also performed drug susceptibility on the FDA approved antivirals, Zanamivir and Oseltamivir, by premixing the antiviral with the viruses and then performing the assays. Therefore, our platform can rapidly be performed within hours using minimal instruments. Our microarray assays detection platform has several advantages over traditional methodologies, where our platform does not required highly trained personnel, specialized equipment and long turnaround times. We are currently developing second generation capture molecules where they can bind to more strains. Optimization of these molecules, testing different conditions and biosensor platforms. This assay has the potential to be translated to a rapid diagnostic test.
3.6 Chemical synthesis and characterization

6-S-[Methyl 5-acetamido-7, 8, 9-tri-O- acetyl-4-azido-3, 4, 5-trideoxy -Dglycer-o-D-galactono-non-2-ulopyranosyl) onate]-1-chloro-hexane

![Chemical structure](image)

To a stirring solution of compound 1 (500 mg, 0.94 mmol) was dissolved in DMF (1.0 mL) and TEA (0.95, 9.4 mmol) was added. The mixture was allowed to stir for 10 minutes then 6-chlorohexyl 4-methylbenzenesulfonate (350.0 mg, 1.1 mmol) was added and the reaction was allowed to stir overnight. The reaction mixture was poured into a brine solution and extracted with ethyl acetate (3x). Organic layers were combed and washed with HCl solution (1M, 1x). The organic layer was dried over sodium sulfate and concentrate on vacuo. Compound was purified via column chromatography with hexane and acetone (3:1 ratio) as eluent to yield yellow oil (80% yield). ¹H-NMR (400 MHz, CDCl₃) δ ppm 5.57 (d, J = 8 Hz, 1H), 5.39-5.36 (m, 1H), 5.31 (d, J = 7.6 Hz, 1H), 4.34-4.30 (m, 1H), 4.25-4.19 (m, 1H), 4.11 (d, J = 10.8 Hz, 1H), 4.06-4.02 (m, 1H), 3.83 (s, 3H), 3.56 (t, J = 6.4, 13.2 Hz, 2H), 3.33-3.25 (m, 1H), 2.82-2.73 (m, 2H), 2.59-2.53 (m, 1H), 2.18 (d, J = 3.2 Hz, 6H), 2.06 (s, 3H), 2.01 (s, 3H), 1.81-1.73 (m, 3H), 1.546-1.39 (m, 6H). ¹³C-NMR (100 MHz, CDCl₃) δ ppm 170.8, 170.7, 170.6, 170.0, 168.3, 82.9, 72.8, 68.3, 68.0, 62.0, 58.0, 52.3, 45.0, 38.2, 32.4, 29.1, 28.7, 28.0, 26.3, 23.45, 21.1, 21.0, 20.7. HRMS (ESI): Calculated for; C₂₄H₃₁ClN₄O₁₀S 608.1919, found; 609.1975 (M+H).
6-S-[Methyl 5-acetamido-7, 8, 9-tri-O- acetyl-3, 4, 5-trideoxy -4-(N-tert-butyloxy carbonyl)-amino-D-glycero-α-D-galacto-non-2-ulopyranosyl]onate]-1-azidoheptane

To a stirring solution of compound 2 (0.20 g, 0.33 mmol) in a mixture of THF: H2O (5.0 ml, 1:1), PPh3 (0.10 g, 0.39 mmol) was added and the reaction was heated at 40 °C for 12 h. The solvent was removed in vacuo and the residue purified by column chromatography to obtain a clear oil. This compound was directly taken to the next step without further purification. To a stirring solution of the crude product (0.18 g, 0.31 mmol) in THF (0.010 L) and TEA (31 mg, 0.31 mmol), di-t-butyl carbonate anhydride (0.10 g, 0.47 mmol) was added. Reaction mixture was stirred for 12h at rt. The white solid was filtered and the residue was concentrated, dissolved in DCM (25 ml) and washed with HCl (25 ml, 1M, 1x). The organic layer was dried over Na2SO4 and concentrated in vacuo. Product was purified via column chromatography with hexane: acetone (5:1) to yield a clear yellow oil (0.13 g, 60% yield over two steps). 1H NMR (400 MHz, CDCl3) δ 5.46 (s, 1H), 5.33 (s, 2H), 4.75 (d, J = 8.8 Hz, 1H), 4.28 (d, J = 12 Hz, 1H), 4.09 (d, J = 8.8 Hz, 1H), 3.88 (d, J = 10.4 Hz, 1H), 3.79-3.73 (m, 5H), 3.52 (t, J = 6.4, 3H), 2.72 (d, J = 11.6 Hz, 2H), 2.54-2.48 (m, 1H), 2.15 (s, 3H), 2.11 (s, 3H), S-4 2.03 (s, 3H), 1.88 (s, 4H), 1.77-1.71 (m, 4H), 1.38 (s, 19H). 13C NMR (100 MHz, CDCl3) δ 171.0, 170.7, 170.0 169.9, 168.7, 156.0, 83.4, 80.0, 68.5, 67.5, 62.2, 52.9, 50.2, 50.0, 45.0, 39.2, 32.4, 29.1, 28.7, 28.3, 26.4, 23.2, 21.2, 20.8, 20.7. HRMS (ESI): Calculated for C29H47ClN2O12S: 682.2538; Found: 683.2613 (M+H).

To a stirring solution of the chloro derivative (71 mg, 0.10 mmol) in DMF (1.0 ml) NaN3 (65 mg, 1.04 mmol) was added and stirred at 60 °C. After 12 hr, the reaction mixture was quenched with DI water (25 ml) and extracted with DCM (25 ml, 3x). The organic layers were collected and washed with brine (25 ml, 1x), dried over Na2SO4 and concentrated in vacuo, to
yield a clear oil (64 mg, 90% yield). 1H NMR (400 MHz, CDCl3) δ 5.47 (d, J = 12 Hz, 1H), 5.33 (s, 2H), 4.75 (d, J = 8 Hz, 1H), 4.29 (d, J = 12 Hz, 1H), 4.09 (d, J = 8 Hz, 1H), 3.87 (m, 1H), 3.79 (s, 3H), 3.60 (m, 1H), 3.52 (vt, J = 12, 8.0 Hz, 2H), 2.73 (d, J = 12 Hz, 2H), 2.54-2.48 (m, 1H), 2.15 (s, 3H), 2.11 (s, 3H), 2.03 (s, 3H), 1.88 (s, 3H), 1.77-1.74 (m, 3H), 1.5-1.40 (m, 2H), 1.38 (s, 14H). 13C NMR (100 MHz, CDCl3) δ 171.0, 170.7, 170.1, 170.0, 168.7, 156.0, 83.4, 80.0, 68.5, 67.4, 62.2, 52.9, 50.2, 50.0, 45.0 39.2, 32.4, 28.3, 26.4, 23.2, 21.2, 20.84, 20.80. HRMS (ESI): Calculated for C29H47N5O12S: 689.2942; Found: 690.3004 (M+H).

6-S-[Methyl 5-acetamido-7, 8, 9-tri-O-acetyl-3, 4, 5-trideoxy-4-(bis-N, N'-tert-butyloxycarbonyl)-guanidino-D-glycero-α-D-galacto-non-2-ulopyranosyl]onate]-hexane

To a stirring solution of compound 2 (0.11 g, 0.20 mmol) in a mixture of THF and H2O (2.0 ml, 1:1), PPh3 (62 mg, 0.24 mmol) was added and heated at 40 °C for 12 hr. The solvent was removed in vacuo and purified by column chromatography using DCM:MeOH (10:1) and used in the next step without purification. This compound (0.10 g, 0.16 mmol) in DCM (0.01 L), TEA (0.3ml, 1.6 mmol) was added and stirred for 10 min at rt. 1,3-bis(tert-butoxy-carbonyl)-2-methyl-2-thiopseudourea (55 mg, 0.19 mmol) and HgCl2 (51 S-5 mg, 0.19 mmol) was added and stirred for 12 h. Next, the reaction mixture was washed with H2O (25 ml, 1x) and brine (25 ml, 1x). The organic layers were separated, dried over Na2SO4 and concentrated in vacuo. Product was purified using column chromatography with hexane:acetone (3:1 ratio) to yield a clear oil (0.12 g, 80% yield). 1H NMR (400 MHz, CDCl3) δ 11.30 (s, 1H), 8.35 (d, J = 8.0 Hz, 1H), 6.05 (d, J = 8.0 Hz, 1H), 5.37-5.29 (m, 2H), 4.36-4.33 (m, 1H), 4.06 (s, 3H), 3.54 (t, J = 12, 4.0 Hz,
To a stirring solution of the chloro compound (47 mg, 0.06 mmol) in DMF (1.0 ml), NaN₃ (39 mg, 0.60 mmol) was added and stirred at 60 °C. After 12 h, the reaction mixture was quenched with H₂O and extracted with DCM (25 ml, 3x). The organic layers were collected and washed with brine (25 ml, 1x), dried over Na₂SO₄ and concentrated in vacuo, to yield a clear oil (40 mg, 85% yield). 1H NMR (400 MHz, CDCl₃) δ 11.31 (s, 1H), 8.40 (d, J = 7.0 Hz, 1H), 6.03 (d, J = 8.7 Hz, 1H), 5.33 (t, J = 15.6 Hz, 4H), 4.35 (d, J = 12.4 Hz, 2H), 4.14 – 3.94 (m, 4H), 3.91 – 3.69 (m, 7H), 3.27 (t, J = 6.8 Hz, 4H), 2.99 – 2.47 (m, 9H), 2.17 (s, 5H), 2.15 (s, 5H), 2.05 (s, 6H), 1.84 (s, 5H), 1.60 (d, J = 6.3 Hz, 6H), 1.49 (s, 22H), 1.39 (s, 10H), 1.26 (s, 8H). 13C NMR (100 MHz, CDCl₃) δ 170.8, 170.6, 170.1, 168.8, 162.9, 156.8, 152.7, 83.8, 83.3, 79.5, 75.4, 69.0, 67.8, 62.4, 52.9, 50.4, 49.7, 38.8, 29.7, 29.1, 28.7, 28.3, 28.0, 26.2, 23.0, 21.2, 21.0, 20.8. HRMS (ESI): Calculated for C₃₅H₅₇ClN₄O₁₄S: 824.3281; Found: 825.3348 (M+H).

8-((tert-butoxycarbonyl)amino)octanoic acid

To a stirring solution of compound 13 (1046 mg, 7.2 mmol) in NaOH solution (34.0 mL, 1M) and dioxane (34.0 mL). Reaction flask was placed in an ice bath and allowed to stir at 0°C for 10 minutes then di-tert-butyl carbonate (2354 mg, 10.8 mmol) was added in dropwise. Reaction mixture was allowed to warm up to room temperature and continued to stir overnight. Reaction mixture was placed on vacuo to remove all dioxane, and neutralized using HCl solution
(1M) to pH 7. Compound was extracted with ethyl acetate (3x), dried over sodium sulfate and concentrate on vacuo to yield a white solid (95% yield). No purification was required. NMR matched as reported compound.

tert-butyl(7-((3,5-bis(prop-2-yn-1-ylcarbamoyl)phenyl)amino)-7-oxoheptyl)carbamate

To a stirring solution of the known spacer (64 mg, 0.26 mmol) in THF (5.0 ml), CDMT (91 S-6 mg, 0.52 mmol) and NMM (52 mg, 0.52 mmol) was added at 0 °C for 12 hr. In a separate flask, the known dimeric scaffold2 was dissolved in THF (5 ml) with NMM (52 mg, 0.52 mmol) at 0 °C and added to the activated acid and stirred for 12 hr. The reaction mixture was quenched with H2O, extracted with EtOAc (10 ml, 3x), dried over Na2SO4 and concentrated in vacuo. The compound was purified using flask chromatography with hexane: acetone (3:1) to yield a white solid (96 mg, 80% yield). 1H NMR (400 MHz, CDCl3) δ 9.39 (s, 1H), 8.27 (s, 2H), 8.04 (s, 1H), 7.75 (s, 2H), 4.80 (s, 1H), 4.17 (s, 4H), 3.00 (d, J = 6.1 Hz, 2H), 2.25 (m, 5H), 1.42 (s,15H). 13C NMR (100 MHz, CDCl3) δ 173.0, 166.7, 159.4, 139.4, 134.6, 121.8, 79.4, 77.4, 77.0, 76.7, 71.7, 56.0, 37.0, 31.6, 30.9, 29.8, 28.6, 28.4, 26.3, 25.2, 22.6, 14.1. HRMS (ESI) Calculated for C26H34N4O5: 482.2529; Found: 505.2404 (M+Na).
Compound 5a:

To a stirring solution of 3a (40 mg, 0.06 mmol) in THF/water (1 ml, 1:1), 4 (9.6 mg, 0.02 mmol) was added. CuSO₄ (10 mg, 0.04 mmol) was added along with sodium L-ascorbate (7.9 mg, 0.04 mmol) and the reaction was stirred at rt for 12 hr. Solvent was removed in vacuo and product was purified using flash column chromatography with DCM:MeOH (10:1) to yield 5a (33 mg, 60% yield). ¹H-NMR (400 MHz, CDCl₃) δ ¹H-NMR (400 MHz, CDCl₃) δ 9.21 (s, 1H), 8.35 (s, 3H), 8.07 (s, 3H), 6.08 (s, 2H), 5.53 (d, J = 8 Hz, 2H), 5.36-5.31 (m, 5H), 4.70 (s, 5H), 4.32-4.29 (m, 6H), 4.07 (dd, J = 4, 12 Hz, 2H), 3.99-3.92 (m, 2H), 3.78-3.75 (m, 3H), 3.71 (s, 6H), 3.55 (d, J = 8 Hz, 2H), 3.10 (d, J = 4 Hz, 2H), 2.72-2.65 (m, 6H), 2.55-2.51 (m, 2H), 2.38 (s, 2H), 2.19 (m, 3H), 2.13 (s, 6H), 2.02 (s, 6H), 1.98 (s, 6H), 1.93 (s, 6H), 1.45 (s, 12H), 1.36 (s, 27H), 1.27 (s, 12H). ¹³C NMR (100 MHz, CDCl₃) δ 210.8, 172.3, 171.3, 170.7, 170.1, 170.0, 168.8, 166.6, 156.3, 156.1, 139.7, 135.0, 134.9, 123.1, 121.5, 121.44, 121.4, 121.3, 83.5, 79.7, 79.13, 79.11, 77.9, 77.6, 77.5, 77.2, 76.6, 76.5, 74.4, 69.5, 68.4, 68.2, 68.0, 62.4, 53.8, 52.9, 51.0, 50.3, 50.2, 50.15, 50.08, 50.00, 40.5, 40.4, 38.8, 37.2, 37.1, 35.5, 35.4, 31.9, 31.8, 29.9, 29.8, 29.78, 29.7, 29.6, 29.5, 29.4, 29.3, 28.8, 28.7, 28.73, 28.69, 28.65, 28.62, 28.5, 28.4, 28.3, 28.2, 28.18, 28.14, 27.3, 27.2, 26.4, 25.9, 25.6, 25.5, 25.4, 25.3, 25.2, 23.3, 23.2, 22.7, 22.6, 21.1, 21.0, 20.8, 20.7, 14.1. HRMS (ESI) Calculated for C₈₄H₁₃₈N₁₄O₂₉S₂: 1860.8413, found; 1923.6098 (M+ 3Na).
Compound 5b:

To a stirring solution of compound 3b (26 mg, 0.04 mmol) in THF and water mixture (1 ml, 1:1), 4 (8.6 mg, 0.017 mmol) was added. CuSO4 (7.5 mg, 0.38 mmol) was added with sodium L-ascorbate (6.7 mg, 0.28) and the reaction was stirred for 12hr at rt. Solvent was removed in vacuo and the product was purified using flash column chromatography with DCM:MeOH (10:1) to yield compound 5b (84 mg, 65% yield). 1H NMR (400 MHz, CDCl3) δ 11.30 (s, 2H), 8.50 (d, J = 27.2 Hz, 3H), 8.38 – 8.19 (m, 2H), 7.86 – 7.59 (m, 6H), 6.25 (s, 2H), 5.33 (m, 5H), 4.72 (m, 6H), 4.34 (m, 7H), 4.21 – 4.01 (m, 9H), 3.88 – 3.69 (m, 10H), 3.11 (d, J = 6.1 Hz, 1H), 2.87 – 2.61 (m, 5H), 2.58 – 2.44 (m, 2H), 2.38-1.87 (m, J = 7.3 Hz, 22H), 1.57 – 1.14 (m, 67H), 13C NMR (101 MHz, CDCl3) δ 170.9, 170.7, 170.2, 168.7, 166.3, 162.9, 156.8, 152.5, 144.6, 134.9, 122.9, 121.3, 114.1, 83.5, 77.3, 77.2, 77.0, 76.7, 75.0, 68.8, 67.8, 62.5, 56.1, 53.8, 52.9, 50.2, 35.5, 31.9, 29.8, 29.7, 29.4, 29.3, 28.6, 28.4, 28.2, 27.9, 27.5, 25.8, 23.0, 22.7, 21.2, 20.9, 20.8, 14.1. HRMS (ESI) Calculated for: C_{96}H_{148}N_{18}O_{33}S_{2}; 2144.9898, found; 2145.9985 (M + H).
Compound SC1.

To a stirring solution of compound 3a (10 mg, 16 µmol) in MeOH (1.0 mL) sodium methoxide (5.4 M, 250 µL) was added. The reaction was allowed to stir for 1 hr, neutralized with H resin. The resin was filtered out, solvent was removed via vacuo and residue was re-dissolved DCM:TFA (1:1) solution and allowed to stir for 1 hr, then solvent was removed via vacuo. The residue was then re-dissolved in EtOH with Pd(OH)₂ and allowed to stir for 8 hr. Pd(OH)₂ was filtered and solvent was removed via vacuo, re-dissolved in MeOH (1.0 mL) and sodium hydroxide solution (10 mM, 1.0 mL) was added and allowed to stirred for 1 hour. Acid resin was used to neutralize to pH 7 and solvent was removed and compounds were purified with Bio-Gel P-2 Gel with filtered water as solvent (yield 75%). ¹H NMR (400 MHz, D₂O) δ 3.72 (t, J = 11.4 Hz, 1H), 3.60 – 3.42 (m, 2H), 3.22 (s, 1H), 2.87 (t, J = 7.6 Hz, 1H), 2.74 – 2.45 (m, 2H), 1.92 (s, 1H), 1.52 (dd, J = 16.9, 8.7 Hz, 2H), 1.28 (s, 2H). ¹³C NMR (101 MHz, D₂O) δ 181.47, 174.45, 86.58, 75.30, 72.14, 68.18, 62.58, 51.81, 50.00, 40.85, 39.39, 29.43, 29.08, 27.40, 26.49, 24.91, 23.27, 21.99. HRMS (ESI) Calculated for: C₁₇H₃₃N₃O₇S 423.2039, found: 422.1962 (M-1).
Compound SC2.

To a stirring solution of compound 3a (5 mg, 16 µmol) in MeOH (1.0 mL) sodium methoxide (5.4 M, 250 µL) was added. The reaction was allowed to stir for 1 hr, neutralized with ¹H resin. The resin was filtered out, solvent was removed via vacuo and residue was re-dissolved in ethanol with Pd(OH)₂ and allowed to stir for 8 hr. Pd(OH)₂ was filtered and solvent was removed via vacuo, re-dissolved in MeOH (1.0 mL) and sodium hydroxide solution (10 mM, 1.0 mL) was added and allowed to stirred for 1 hour. Acid resin was used to neutralize to pH 7 and solvent was removed and compounds were purified with Bio-Gel P-2 Gel with filtered water as solvent. ¹H NMR (400 MHz, D₂O) δ 4.25-4.08 (m, 3H), 3.99 (m, 3H), 3.58-3.55 (m, 1H), 3.36-3.34 (m, 1H), 3.12-3.07 (m, J = 8.2 Hz, 2H), 2.95-2.78 (m, 1H), 2.44-2.35 (m, 3H), 2.15 (s, 1H), 2.03 (s, 1H), 1.87 (s, 5H), 1.69-1.54 (m, 3H). HRMS (ESI) Calculated for: C₁₈H₃₅N₅O₇S 465.2257, found: 464.1987 (M⁻, negative ion).
Compound SC3.

To a stirring solution of compound 5a (4 mg, 2.6µmol) in MeOH (1.0 mL) sodium methoxide (5.4 M, 250 µL) was added. The reaction was allowed to stir for 1 hr, neutralized with 1H resin. The resin was filtered out, solvent was removed via vacuo and residue was redissolved in DCM and TFA (1.0 mL, 1:1 ratio) and allowed to stir for 1 hr. Solvent was removed via vacuo, re-dissolved in MeOH (1.0 mL) and sodium hydroxide solution (10 mM, 1.0 mL) was added and allowed to stir for 1 hr. Acid resin was used to neutralize to pH 7 and solvent was removed and compounds were purified with Bio-Gel P-2 Gel with filtered water as solvent (yield 80%). 1H-NMR (400 MHz, D2O) δ 7.83-7.75 (m, 5H), 4.21 (t, J = 4 Hz, 2H), 4.01-3.95 (m, 1H), 3.71-3.51 (m, 11H), 3.24-3.17 (m, 2H), 2.86 (t, J = 8 Hz, 2H), 2.75 (dd, J = 4, 12 Hz, 2H), 2.60-2.52 (m, 3H), 2.42-2.39 (m, 2H), 2.26 (t, J = 8 Hz, 2H), 1.92 (s, 6H), 1.8-1.74 (m, 2H), 1.65 (t, J = 8 Hz, 3H), 1.55-1.49 (m, 3H), 1.36-1.02 (m, 15H). 13C-NMR (100 MHz, D2O) 175.5, 175.0, 173.2, 168.5, 144.4, 138.2, 134.6, 123.9, 123.8, 123.9, 122.6, 122.5, 121.8, 100.0, 85.9, 74.7, 72.0, 67.7, 62.4, 62.3, 50.8, 50.3, 47.9, 39.4, 36.4, 35.0, 29.2, 26.6, 25.1, 22.1, 13.2. HRMS (ESI) Calculated for C55H88N14O17S2 is 1280.5893, found 641.3013 (M÷2).
Compound SC4

To a stirring solution of compound 5b in MeOH (1.0 mL) sodium methoxide (5.4 M, 250 µL) was added. The reaction was allowed to stir for 1 hr, neutralized with ¹H resin. The resin was filtered out, solvent was removed via vacuo and residue was re-dissolved in DCM and TFA (1.0 mL, 1:1 ratio) and allowed to stir for 1 hr. Solvent was removed via vacuo, re-dissolved in MeOH (1.0 mL) and sodium hydroxide solution (50 mM, 1.0 mL) was added and allowed to stir for 1 hr. Acid resin was used to neutralize to pH 7 and solvent was removed and compounds were purified with Bio-Gel P-2 Gel with filtered water as solvent, yield white solid (70% yield). ¹H-NMR (400 MHz, D₂O) δ 7.88-7.79 (m, 5H), 4.69 (s, 1H), 4.28 (d, J = 8 Hz, 1H), 4.05 (s, 1H), 3.87-3.79 (m, 3H), 3.71-3.60 (m, 11H), 3.52-3.42 (m, 11H), 2.86-2.84 (m, 10H), 2.70 (s, 2H), 2.61-2.55 (m, 6H), 2.40-2.31 (m, 4H), 1.87 (s, 7H), 1.75-1.56 (m, 11H), 1.29 (m, 9H), 1.14-1.09 (m, 10H). HRMS (ESI) Calculated for C₅₇H₉₂N₁₈O₁₇S₂ is 1364.6329, founded 1365.6420 (M+H).
Scheme 2. Reagents and conditions. k. DCM, TFA, 1 hr. quant. l. 1,3-Bis(tert-butoxycarbonyl) -2- methyl -2-thiopseudourea. HgCl₂, Et₃N, DCM, overnight, 84%. j. Na-Lascorbate, copper sulfate. THF/H₂O, overnight, 78%. g. NaOH, MeOH. h. DCM/TFA.70%. i. H₂, Lindlar Catalyst,乙醇, 4 hrs. quant.
**Compound 12:** 5-Acetylamino-4- N-tert-butyloxy carbonyl-6- (1,2,3-triacetoxy-propyl) -5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester.

To a solution of compound 4 (1.50 g, 3.3 mmol) in EtOH (25 mL), Lindlar catalyst (.15 g, .1 equivalent) was added. Hydrogen gas was bubbled to the solution and stirred at rt for 12 hr. After filtering using celite, the filtrate was collected and solvent removed in vacuo to give a white product (1.42 g, quant.). To this compound (530 mg, 1.23 mmol) in THF (20 mL), Et$_3$N (150 mg, 1.48 mmol) was added. The solution was stirred rt for 30 min and Boc$_2$O (538 mg, 2.46 mmol) was added and reaction stirred for 12 hr at rt. Upon completion, THF was removed in vacuo. The residue was washed using HCl (1M, 25 ml) and extracted by DCM (30 ml, 3x), the organic phases were combined and dried over Na$_2$SO$_4$. DCM was removed in vacuo and the reaction mixture was purified using column chromatography with hexane: acetone (3:1) as eluent to give a white product. (561 mg, 86%). $^1$H NMR (400 MHz, CDCl$_3$) δ 6.46 (d, J = 9.0 Hz, 1H), 5.94 (s, 1H), 5.43 (s, 1H), 5.27 (s, 1H), 4.63 (d, J = 12.4 Hz, 1H), 4.44 (d, J = 10.0 Hz, 1H), 4.35 (d, J = 8.9 Hz, 1H), 4.15 (dd, J = 12.3, 7.1 Hz, 1H), 3.95 (d, J = 9.2 Hz, 1H), 3.77 (s, 3H), 2.14 (s, 9H), 2.10 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.95 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 170.9, 170.6, 170.3, 170.0, 161.8, 156.2, 144.6, 111.0, 80.2, 71.4, 67.8, 62.2, 60.4, 52.4, 50.1, 47.3, 28.2, 23.1, 21.0, 20.9, 20.8, 20.7, 14.2. HRMS. Calculated for C$_{25}$H$_{34}$N$_2$O$_{12}$, 530.2112. Found: 531.2181 (M+H)
**Compound 13:** 5-Acetylamino-4-N-tert-butyloxycarbonyl-6-[(2,2-dimethyl-[1,3]dioxolan-4-yl) 5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester.

To a solution of compound 7 (560 mg, 1.06 mmol) in MeOH (10 mL), MeONa (0.05 eq) was added. The solution was stirred at rt for 5 hr. The progress of the reaction was monitored by TLC. Upon completion, the reaction mixture was neutralized by H+ resin, the suspension was filtered. The liquid phase was collected and dried in vacuo to give a colorless compound. To a solution of this compound (425 mg, 1.05 mmol) in dry acetone (10 mL), H+ resin was added to adjust the pH to 4. The solution was stirred at rt for 12 hr. The suspension was filtered, acetone was removed in vacuo, the residue was washed by saturated NaHCO3, extracted by DCM (3 × 20 mL), the organic phases were combined and dried over Na2SO4. DCM was removed in vacuum and the reaction mixture was purified by flash column chromatography using hexane : acetone (4:1) to give the product 3. (410 mg, 88%). 1H NMR (400 MHz, CDCl3) δ 6.65 (d, J = 6.4 Hz, 1H), 5.79 (s, 1H), 5.08 (d, J = 4.2 Hz, 1H), 4.82 (d, J = 9.0 Hz, 1H), 4.59 (t, J = 9.4 Hz, 1H), 4.38 (dd, J = 13.5, 5.3 Hz, 1H), 4.26 – 4.06 (m, 2H), 4.01 (d, J = 10.6 Hz, 1H), 3.91 (td, J = 10.1, 6.7 Hz, 1H), 3.76 (s, 3H), 3.50 (dd, J = 8.3, 4.3 Hz, 1H), 2.03 (s, 3H), 1.44 (s, 9H), 1.40 (s, 3H), 1.36 (s, 3H). 13C NMR (400 MHz, CDCl3) δ 173.9, 162.0, 157.2, 146.3, 109.2, 107.8, 81.0, 78.3, 77.3, 77.0, 76.7, 74.0, 69.7, 67.3, 52.4, 52.1, 48.7, 28.2, 27.1, 25.3, 23.0. HRMS (ESI) Calculated for C20H32N2O9, 444.2108. Found: 445.2180 (M+H).
**Compound 9:** 5-Acetylamino-4-N-tert-butyloxycarbonyl-6-[(2,2-dimethyl-[1,3]dioxolan-4-yl)- (4-nitro-phenoxy carbonyloxy)-methyl]-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester.

![Chemical Structure](image)

To a solution of compound 3 (35mg, 0.079 mmol) in pyridine (10 ml), DMAP (19mg, 0.16 mmol) was added. The solution was stirred at rt for 30 min and 4-nitrophenylchloroformate (31.7mg, 0.157 mmol) was added. The reaction was stirred at rt for 16 hr. The reaction mixture was washed by HCl (1 M, 25 ml) and extracted with DCM (3 × 20 mL), the organic phases were combined and dried over Na$_2$SO$_4$. DCM was removed in vacuo and the product was purified by column chromatography using hexane: acetone (3:1) to give 4. (39mg, 80%). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.27 (d, $J = 8.9$ Hz, 2H), 7.50 (d, $J = 8.9$ Hz, 2H), 5.97 (d, $J = 9.6$ Hz, 1H), 5.90 (s, 1H), 5.31 (t, $J = 5.7$ Hz, 1H), 4.81 (d, $J = 9.6$ Hz, 1H), 4.54 (t, $J = 9.7$ Hz, 1H), 4.47 – 4.29 (m, 2H), 4.24 (dd, $J = 8.9$, 5.7 Hz, 2H), 4.17 – 4.05 (m, 1H), 3.79 (s, 3H), 1.95 (s, 3H), 1.42 (s, 9H), 1.41 (s, 3H), 1.38 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.3, 161.6, 156.4, 155.7, 152.5, 145.6, 144.8, 125.2, 122.3, 110.5, 108.9, 80.6, 77.3, 77.0, 76.7, 75.1, 74.2, 65.5, 52.5, 49.5, 48.1, 28.2, 26.4, 25.5, 23.2. HRMS (ESI) Calculated for C$_{27}$H$_{35}$N$_3$O$_{13}$, 609.2170. Found: 632.2057 (M+Na).
**Compound 15a:** 5-Acetylamino-4-N-tert-butyloxycarbonyl-6-[(6-azido-hexyl car bamoyloxy)-(2,2-dimethyl-[1,3]dioxolan-4-yl)-methyl]-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester.

![Image of compound 15a](image-url)

To a solution of 6-azidohex-1-amine (19 mg, 0.13 mmol) in CH$_3$CN (8 mL), Et$_3$N (20 mg, 0.19 mmol) was added. The solution was stirred at rt for 30 min. 9 (39 mg, 0.064 mmol) in CH$_3$CN (2 mL) was added. The reaction was stirred at rt for 3 hr. The progress of the reaction was monitored by TLC. Upon completion, CH$_3$CN was removed in vacuo and the reaction mixture was washed by HCl (1 M, 25 ml), extracted by DCM (3 × 20 mL), the organic phases were combined and dried over Na$_2$SO$_4$. DCM was removed in vacuo and the product was purified by column chromatography using hexane : EtOAc (1:1) to give 5. (35 mg, 89%). ¹H NMR (400 MHz, CDCl$_3$) δ 6.15 (d, J = 9.4 Hz, 1H), 5.90 (s, 1H), 5.24 (d, J = 3.7 Hz, 1H), 5.01 – 4.83 (m, 2H), 4.51 (t, J = 8.9 Hz, 1H), 4.33 (dd, J = 13.6, 7.8 Hz, 2H), 4.19 – 4.04 (m, 2H), 4.04 – 3.92 (m, 2H), 3.76 (s, 3H), 3.73 (s, 1H) 3.24 (t, J = 6.9 Hz, 2H), 3.11 (dt, J = 20.7, 6.6 Hz, 2H), 1.93 (s, 3H), 1.66 – 1.53 (m, 2H), 1.48 (m, 2H), 1.41 (m, 2H), 1.39 (s, 9H), 1.34(s, 3H), 1.23(s, 3H). ¹³C NMR (101 MHz, CDCl$_3$) δ 170.8, 162.1, 156.2, 155.5, 144.4, 111.4, 108.9, 80.1, 74.9, 69.7, 65.9, 52.4, 51.3, 50.0, 47.6, 41.1, 29.6, 28.7, 28.3, 26.4, 26.3, 26.3, 25.4, 23.2. HRMS (ESI) Calculated for C$_{27}$H$_{44}$N$_6$O$_{10}$, 612.3119. Found: 635.3006 (M+Na).
**Compound 15b:** 5-Acetylamino-4-[2,3-bis(tert-butoxycarbonyl)guanidine]-6-[(6-azido-hexyl carboxamidoyloxy)-2,3 dihydroxy propyl]-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester.

![Chemical structure of Compound 15b]

To a solution of compound 10a (40mg, 0.084 mmol) in THF (3 ml), TFA (3 ml) was added, the reaction was stirred at rt for 1 hr. THF was removed in vacuo, Et₃N (26 µl, 0.25 mmol) was added. The solution was stirred at rt for 30 mins. HgCl₂ (27 mg, 0.1mmol) and 1,3-Bis(tert-butoxycarbonyl)-2-methyl-2-thio-pseudourea (29 mg, 1.2mmol) was added. The reaction was stirred at rt for 12 hr. The reaction mixture was washed with HCl (1M, 25 ml), extracted with DCM (3 x 10 ml), DCM was removed in vacuo and the product was purified by column chromatography using DCM : MeOH (25 :1) to give the product 10b. (50 mg, 84%). ¹H NMR (400 MHz, CDCl₃) δ 11.40 (s, 1H), 8.54 (d, J = 8.7 Hz, 1H), 7.39 (s, 1H), 7.28 (s, 1H), 6.85 (s, 1H), 5.89 (s, 1H), 5.32 (s, 1H), 5.20 (t, J = 9.5 Hz, 1H), 5.03 (t, J = 5.7 Hz, 1H), 4.82 (d, J = 9.4 Hz, 1H), 4.49 (d, J = 10.5 Hz, 1H), 4.37 (dd, J = 19.7, 10.0 Hz, 1H), 4.14 (dd, J = 14.2, 7.1 Hz, 1H), 4.05 (d, J = 9.2 Hz, 1H), 3.80 (s, 3H), 3.68 (dd, J = 24.9, 12.7 Hz, 1H), 3.27 (t, J = 6.8 Hz, 2H), 3.17 (ddd, J = 19.4, 13.4, 6.8 Hz, 2H), 1.95 (d, J = 10.0 Hz, 3H), 1.60 (dd, J = 13.8, 6.8 Hz, 2H), 1.50 (s, 18H), 1.38 (s, 4H), 1.27 (dd, J = 7.7, 6.5 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 171.0, 162.8, 162.4, 162.2, 157.1, 157.1, 152.5, 152.5, 144.9, 118.2, 115.3, 110.5, 83.7, 79.7, 69.4, 68.7, 62.4, 53.5, 52.31, 51.26, 49.4, 47.1, 45.7, 41.2, 29.4, 28.7, 28.3, 28.2, 28.1, 27.9, 27.9, 26.3, 26.2, 22.7, 8.4. HRMS (ESI) Calculated for C₃₀H₅₀N₈O₁₂, 714.3548. Found: 715.3627 (M+H).
To a solution of 16a (54 mg, 0.088 mmol) in THF/H2O (1 ml, 1:1), 4 (18 mg, 0.036 mmol) was added. CuSO4 (15 mg, 0.1 mmol) was added along with sodium L-ascorbate (20 mg, 0.1 mmol). The reaction was stirred at rt for 12 h. Upon completion by TLC, solvent was removed in vacuo and the product was purified by column chromatography using EtOAc:MeOH (30:1) to give 11a (48 mg, 78%). 1H NMR (400 MHz, CDCl3) δ 8.23 (s, 3H), 7.83 (s, 2H), 6.67 (s, 2H), 5.95 (s, 2H), 5.64 (s, 1H), 5.24 (s, 3H), 4.60 (m, 6H), 4.33 (s, 8H), 4.12 (s, 8H), 3.98 (s, 6H), 3.04 (d, J=33.2 Hz, 4H) 2.40 (s, 1H), 2.19 (s, 2H), 2.09 (s, 2H), 2.06 (s, 2H), 1.90 (s, 10H), 1.68 (s, 2H), 1.44 (s, 14H), 1.26–1.36 (m, 42H). HRMS (ESI) Calculated for C80H126N16O25, 1706.8767. Found: 1707.8838 (M+H).
Compound 11b:

To a solution of 16b (40 mg, 0.056 mmol) in THF/H2O (1 ml, 1:1), 4 (13 mg, 0.025 mmol) was added. CuSO₄ (14 mg, 0.088 mmol) was added with sodium L-ascorbate (17mg, 0.088mmol). The reaction was stirred at rt for 12 h. Upon completion, solvent was removed in vacuo and the product was purified by column chromatography using EtOAc : MeOH (20:1) to give 9. (38 mg, 80%). ¹H NMR (400 ¹H NMR (400 MHz, CDCl₃) δ 11.38 (s, 1H), 9.74 (s, 1H), 8.19 (m, 5H), 7.28 (s, 2H), 5.89 (s, 2H), 5.31 (s, 6H), 5.21 – 5.05 (m, 2H), 4.78 (s, 2H), 4.52 (s, 5H), 4.17 – 3.90 (m, 4H), 4.09-3.85 (m, 4H), 3.80-3.74 (m, 8H), 3.54 (s, 2H), 3.36 (s, 1H), 3.30 – 2.72 (m, 6H), 2.35 (d, J = 13.9 Hz, 2H), 2.05 (s, 2H), 1.81 (s, 7H), 1.54 (m, 22H), 1.45 – 1.33 (m, 12H), 1.33 – 0.99 (m, 28H), 0.90-0.84 (m, 10H). HRMS (ESI) Calculated for C₈₆H₁₃₄N₂₀O₂₉, 1910.9626. Found: 1912.0572(M+H)
**Compound SC5:**

To a solution of compound 10a (1.8 mg, .29 µmol) in MeOH (5 mL), NaOH (0.5 M, 1 ml) was added. The solution was stirred at rt for 2 h. The progress of the reaction was monitored by TLC. Upon completion, the reaction mixture was neutralized by H+ resin, the suspension was filtered. The filtrate was collected and dried in vacuo. The residue was added to DCM/TFA (5 ml, 1:1), the mixture was stirred at rt for 1 h. DCM was removed in vacuo, the product was dissolved in EtOH (5 mL), Lindlar catalyst (10%) was added. The hydrogen was bubbled to the solution and stirred at rt for 12 h. The suspension was filtered and the filtrate, which contained product SC5, was collected and crude product was purified by P2 column to give pure SC5 (1.0 mg, 76%). 1H NMR (400 MHz, D2O) δ 5.73 (s, 1H), 4.34 – 4.23 (m, 2H), 4.16 – 4.04 (m, 2H), 3.71 – 3.57 (m, 1H), 3.39 (dt, J = 13.3, 6.8 Hz, 2H), 3.02 (s, 4H), 1.98 (s, 3H), 1.55 – 1.30 (m, 4H), 1.23-1.18 (m, 4H). HRMS(ESI) Calculated for C18H32N4O8, 432.2220. Found:433.2292 (M+H).
Compound SC6:

SC6 was synthesized in a manner similar to that of SC5 using 10b (1.6 mg, .22 µmol) and the crude product was purified by P2 column to give the SC6 (.75 mg, 72%). 1H NMR (400 MHz, D2O) δ 5.63 (s, 1H), 4.38 – 4.18 (m, 2H), 4.10 – 3.87 (m, 2H), 3.74 – 3.61 (m, 2H), 3.38 (t, J = 7.2 Hz, 1H), 3.02 (s, 4H), 1.93 (s, 3H), 1.52 – 0.99 (m, 8H). HRMS (ESI) Calculated for C19H34N6O8, 474.2438. Found: 475.2516 (M+H).

Compound SC7:

To a solution of compound 11a (4.5 mg, .26 µmol) in MeOH (5 ml), NaOH (0.5M, 1 ml) was added. The solution was stirred at rt for 2 hr. The progress of the reaction was monitored by TLC. Upon completion, the reaction mixture was neutralized by H+ resin and the suspension
was filtered. The filtrate was dried in vacuo and DCM/TFA (1:1, 5mL) was added to the residue and was stirred at rt for 1 hr. After removal of solvent, the crude product was purified by P2 column to give SC7. (2.4mg, 70%). \( ^1H \) NMR (400 MHz, D$_2$O) \( \delta \) 7.89 (s, 2H), 7.80 (s, 3H), 5.91 (s, 2H), 4.84 (d, J = 9.4 Hz, 2H), 4.54 (s, 2H), 4.47 (d, J = 10.5 Hz, 2H), 4.29 (s, 3H), 4.25 – 4.08 (m, 3H), 3.96 (s, 4H), 3.53 (d, J = 9.8 Hz, 2H), 3.37 (dd, J = 11.9, 6.4 Hz, 2H), 2.88 (d, J = 7.4 Hz, 4H), 1.90 (s, 6H), 1.76 (s, 2H), 1.57 (s, 2H), 1.30 (s, 6H), 1.13-1.05 (m, 14H), 0.70-0.63 (m, 6H). HRMS (ESI) Calculated for C$_{57}$H$_{86}$N$_{16}$O$_{19}$, 1298.6255. Found: 1299.6329(M+H).

**Compound SC8:**

SC8 was synthesized in a manner similar to SC7 using 11b (3.5 mg, .18 µmol) and the crude product was purified by P2 column to give SC8. (2.0 mg, 79%). \( ^1H \) NMR (400 MHz, D$_2$O) \( \delta \) 7.89 (s, 5H), 5.80 (d, J = 17.5 Hz, 2H), 4.44 (d, J = 10.1 Hz, 2H), 4.38 (d, J = 8.5 Hz, 2H), 4.34 – 4.20 (m, 4H), 4.16 (dd, J = 26.4, 10.1 Hz, 2H), 4.01 (dt, J = 13.9, 6.9 Hz, 4H), 3.62 (d, J = 8.8 Hz, 2H), 3.51 (d, J = 11.6 Hz, 1H), 2.99 – 2.79 (m, 5H), 2.29 (d, J = 7.5 Hz, 2H), 1.96 (s, 2H), 1.95 – 1.85 (m, 5H), 1.82 (d, J = 14.9 Hz, 2H), 1.71 (s, 3H), 1.54 (s, 4H), 1.27 (s, 8H), 1.20 – 1.11 (m, 6H), 1.06 (s, 6H). HRMS (ESI) Calculated for C$_{57}$H$_{86}$N$_{16}$O$_{19}$, 1382.6691. Found: 1383.6761(M+H).
To an overnight stirring solution of compound 2.1 (1.0 g, 2.7 mmol) in THF at 0°C with CDMT (0.95 g, 5.4 mmol) and NMM (0.59 g, 5.4 mmol), compound 2 (1.4 g, 1.5 mmol) in THF was added dropwise. Reaction mixture was allowed to stir overnight while warming to room temperature. Water was added to quench reaction, reaction mixture was placed on rota vap to remove majority of the THF and extracted with DCM (3x). Organic layers were combined and washed with brine (1x), 1M HCl (1x) then dried over NaSO₄, concentrated on vacuo and purified using flash chromatography with DCM and MeOH as solvent (10:1), yield yellow/white solid (80%). ¹H NMR (400 MHz, MeOD) δ 8.88 (s, 1H), 8.17 (s, 2H), 7.97 (s, 1H), 7.75 (d, J = 7.7 Hz, 2H), 7.61 (d, J = 7.6 Hz, 3H), 7.32 (dt, J = 31.7, 7.6 Hz, 5H), 4.32 (d, J = 6.8 Hz, 1H), 4.16 (d, J = 2.2 Hz, 5H), 3.97 (s, 1H), 3.09 (t, J = 7.0 Hz, 2H), 2.63 (s, 2H), 2.39 (t, J = 7.7 Hz, 3H), 1.77 – 1.61 (m, 3H), 1.55 – 1.44 (m, 2H), 1.43 – 1.25 (m, 7H). ¹H NMR (400 MHz, MeOD) δ 8.9, 8.2, 8.0, 7.8, 7.7, 7.6, 7.5, 7.4, 7.3, 7.29, 7.28, 7.26, 4.3, 4.3, 4.2, 4.1, 4.0, 3.3, 3.2, 3.1, 3.0, 2.6, 2.5, 2.4, 2.3, 2.2, 2.1, 1.7, 1.6, 1.6, 1.5, 1.4, 1.47, 1.36, 1.30, 1.2.

To a stirring solution of compound 2.2 (107 mg, 0.3 mmol) was dissolved in DMF:Piperidine (1:1 mixture) and allowed to stir for approximately 1 hr and thin layer
To a stirring solution of compound 8a (26 mg, 0.04 mmol) in THF and water mixture (1 ml, 1:1), compound 2,3 (8.6 mg, 0.017 mmol) was added. CuSO4 (7.5 mg, 0.38 mmol) was added with sodium L-ascorbate (6.7 mg, 0.28) and the reaction was stirred for 12 hr at rt.
Solvent was removed in vacuo and the product was purified using flash column chromatography with DCM:MeOH (10:1) to yield compound 2.4a (84 mg, 65% yield). $^1$H NMR (400 MHz, MeOD) $\delta$ 8.29 – 7.80 (m, 1H), 6.49 (d, $J$ = 9.3 Hz, 1H), 5.34 (s, 1H), 4.46 (d, $J$ = 33.0 Hz, 1H), 4.31 (d, $J$ = 12.0 Hz, 1H), 4.18 (s, 1H), 4.05 (d, $J$ = 12.1 Hz, 1H), 3.89 – 3.72 (m, 2H), 3.50 (d, $J$ = 9.9 Hz, 1H), 3.19 (d, $J$ = 5.7 Hz, 1H), 2.93 (dd, $J$ = 12.8, 4.5 Hz, 1H), 2.71 (d, $J$ = 11.5 Hz, 1H), 2.55 (d, $J$ = 6.4 Hz, 1H), 2.42 (s, 1H), 2.16 (s, 1H), 2.08 (s, 1H), 2.00 (s, 1H), 1.86 (s, 1H), 1.81 – 1.59 (m, 2H), 1.53 – 1.30 (m, 8H). $^{13}$C NMR (101 MHz, MeOD) $\delta$ 171.1, 170.4, 170.1, 168.9, 83.3, 78.9, 74.0, 68.1, 67.5, 61.9, 60.3, 55.6, 52.0, 47.9, 47.6, 47.4, 47.2, 47.0, 39.7, 39.0, 38.5, 36.5, 35.5, 29.1, 28.5, 28.4, 28.2, 27.7, 27.3, 26.3, 25.6, 25.3, 21.5, 20.0, 19.5, 19.4.

To a stirring solution of compound 8b (26 mg, 0.04 mmol) in THF and water mixture (1 ml, 1:1), compound 2.3 (8.6 mg, 0.017 mmol) was added. CuSO$_4$ (7.5 mg, 0.38 mmol) was added with sodium L-ascorbate (6.7 mg, 0.28) and the reaction was stirred for 12 hr at rt. Solvent was removed in vacuo and the product was purified using flash column chromatography with DCM:MeOH (10:1) to yield compound 2.4b (84 mg, 65% yield). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 11.31 (s, 1H), 9.39 (d, $J$ = 47.5 Hz, 1H), 8.42 (d, $J$ = 21.5 Hz, 2H), 8.22 (s, 1H), 8.08 (s, 1H), 7.74 (s, 1H), 6.71 (d, $J$ = 36.5 Hz, 1H), 6.25 (d, $J$ = 60.8 Hz, 1H), 5.46 – 5.24 (m, 2H), 4.66
To a stirring solution of compound 2.4a in MeOH (1.0 mL) sodium methoxide (5.4 M, 250 µL) was added. The reaction was allowed to stir for 1 hr, neutralized with H resin. Solvent was removed via vacuo, re-dissolved in MeOH (1.0 mL) and sodium hydroxide solution (50 mM, 1.0 mL) was added and allowed to stir for 1 hour then neutralized with acid resin. The resin was filtered out, solvent was removed in vacuo and the residue was re-dissolved in DCM and TFA (1.0 mL, 1:1 ratio) and allowed to stir for 1 hr. Solvent was removed and compounds were dissolved in DI water then purified with Bio-Gel P-2 Gel with filtered water as solvent, yield white solid (70% yield). 1H NMR (400 MHz, D2O) δ 7.98 – 7.81 (m, 5H), 4.27 (s, 2H), 4.06-4.03 (m, 2H), 3.74-3.66 (m, 7H), 3.56 (d, J = 8.6 Hz, 4H), 3.34-3.23 (m, 4H), 3.05 (s, 3H), 2.74-2.70
(m, 6H), 2.64-2.58 (m, 4H), 2.53 – 2.40 (m, 2H), 2.31-2.32 (d, J = 6.8 Hz, 2H), 1.95 (s, 6H), 1.74 (s, 4H), 1.45-1.39 (m, 14H), 1.26 – 1.03 (m, 16H).

To a stirring solution of compound 2.4b in MeOH (1.0 mL) sodium methoxide (5.4 M, 250 µL) was added. The reaction was allowed to stir for 1 hr, neutralized with `H resin. Solvent was removed via vacuo, re-dissolved in MeOH (1.0 mL) and sodium hydroxide solution (50 mM, 1.0 mL) was added and allowed to stir for 1 hour then neutralized with acid resin. The resin was filter out, solvent was removed in vacuo and residue was re-dissolved in DCM and TFA (1.0 mL, 1:1 ratio) and allowed to stir for 1 hr. Solvent was removed and compounds were dissolved in DI water then purified with Bio-Gel P-2 Gel with filtered water as solvent, yielding a white solid (75% yield). 1H NMR (400 MHz, D2O) δ 8.02 – 7.82 (m, 5H), 4.06 – 3.65 (m, 4H), 3.51 (dd, J = 19.5, 11.7 Hz, 3H), 3.14 (t, J = 18.8 Hz, 2H), 2.83 (d, J = 82.0 Hz, 1H), 2.63 (s, 1H), 2.55 (d, J = 11.9 Hz, 1H), 2.34 (d, J = 18.5 Hz, 1H), 2.12 (d, J = 20.9 Hz, 1H), 1.83 (s, 3H), 1.61 (s, 2H), 1.43 (s, 1H), 1.31 (d, J = 21.8 Hz, 4H), 1.07 (s, 1H).

Acknowledgements. We are grateful for NIH-NIAID (R01-AI089450) for funding. We also thank BEI Resources, NIAID, NIH for the viral strains and the antibodies. We also thank Dr. Binghe Wang, GSU for use of the microarray printing facilities.
Synthesis of Thiosialosides for the Inhibition of Influenza Neuraminidase

4.1 Abstract

We have synthesized a panel of bivalent S-sialoside analogues along with six S-sialosides norbornene based polymers, with modifications at the 4 position, as inhibitors of influenza virus. These first generation compounds show IC50 values ranging from low micromolar to high nanomolar in enzyme inhibition and plaque reduction assays with two intact viruses, Influenza H1N1 (A/California/07/2009) and H3N2 (A/Hongkong/8/68).
4.2 Introduction

N-acetyl neuraminic acid or sialic acid is a known substrate that will bind to influenza HA and NA. In the case of HA, only NeuAcα2, 3 Gal and NeuAcα2, 6 Gal linked are recognized to facilitate the endocytosis process. The variation in binding can varies between 2,3 and 2,6glycosidic links depending on the lineage of the HA.15 NA on the other hand, its function is to cleave O-linked sialic acid residues and therefore will recognize sialic acid residues regardless where the glycosidic bond is located. In combatting the spread of influenza, scientists have developed several FDA approved transition state analogs that inhibit NA from cleaving the sialic acid on infected cell surface.102 Oseltamivir and Zanamivir are transition state analogs that essentially arrest the virus progeny from escaping the cells surface. However, some strains of influenza have been shown to developed resistant to both Oseltamivir and Zanamivir.103 In recent years, a new class of NA inhibitors have been reported to have high inhibition properties against broad spectrum of influenza strains, including resistant strains.49, 67 Unlike the transition state analogs, these compounds resemble the natural state sialic acid, where the 3rd and 4th position were modified to enhance inhibition properties.

Labeling experiments have shown that influenza viruses normally have approximately 200-300 copies of trimeric HA and 50-100 copies of tetrameric NA.104, 105 Thus, multiple strategies have been developed to target both HA and NA. Similar inhibitors were developed following Nature’s method using multivalency to target HA and preventing viral entry. The innate human immune system respond in a very similar fashions by producing mucin, an endogenous sialylate proteins released by respiratory epithelial cells. Mucin capture viral particles by virtue of their multiple sialic acid residues that will bind to both HA and NA, and purged them via a natural process known as sneezing or coughing.106, 107, 108 Similarly, glycopolymers and glycodendrimers of sialic acid have been generated to capture influenza
viruses. It has been shown that the increase in valency of sialic acid increase the inhibitory effect significantly. The IC$_{50}$ values of mono/di/tri saccharide can improve from micromolar to submicromolar range.$^{109, 110, 111}$ Hence, we want to take advantage of the multivalent effects and introduced dimeric and polymeric sialic acid analogs to investigate the IC$_{50}$ value of these analogs.
Figure 30 Structure of N-acetyl neuraminic acid analogs

Figure 31: Structures of N-acetyl neuraminic acid (sialic acid) analogs. (A) Sialic acid with the numbering scheme. (B) Oseltamivir (C) Zanamivir , (D) Premavir (E–F)Fluorogenic substrate of sialic acid for obtaining IC$_{50}$ values.
4.3 Design and Synthesis of Bivalent S-linked sialic acid

In previous the chapter, we have reported our sialic acid analogs are able to capture the influenza viral particles with biological sensitivities. Based on previous studies, we want to further investigate these analogs for potential NA inhibition properties. However, as inhibitors these molecules do not require any functionalities use for immobilization, therefore the dimeric scaffold is not required. First, we want to investigate the effects of the spacer length for our dimeric analogs. Second, after optimization of the appropriate spacer, we want to attach these analogs onto a handle that will allow us to easily polymerize these compounds to increase the multivalency. The design and synthesis shown in Scheme 1 and 2. The synthesis begin with known azido compound 4, hydrogen chloride was added across the double bond, following by SN2 type replacement of the chloride by a thioacetate to introduce the sulfur moiety at the anomeric center with good yield. \(^1\)H and \(^13\)C NMR spectroscopy were done to confirmed that the \(\alpha\)-anomeric conformation in compound 23 before pursuing forward. Reaction of compound 23 with hydrophobic six carbon space was done in the present of diethylamine to yield compound 24. Next, azido compound 25 was reduced via trephenyl-phosphine to yield amine 3.2, which is subsequently protected with t-butylcarbonyl group or reacted with a suitable protected guanidinium group to yield 26 and 27 respectively. Removal of esters group under basic conditions follows by the removal of t-butylcarbonyl group with acidic condition to afford SA and SG in good yield.
**Figure 31** Synthesis route for monomeric S-linked sialic acid

**Figure 32:** Synthesis route for monomeric sialic acid. a) HCl, KCl, CH$_3$CN, 5 days  b) KSAc, TBTAS, DCM:H$_2$O(1:1) 50% after 2 steps  c) S-(6-(tert-butyldimethylsilyl)hexyl) ethanethioate, DMF, DEA 65%  d) PPh$_3$, THF:H$_2$O, 40% overnight  e) (Boc)$_2$O, TEA, THF 65% over two steps  f) MeS(=NBoc)NHBoc, HgCl$_2$, DCM 80% over two steps  g) i. KOH, THF:H$_2$O:MeOH 2 hrs, ii. TFA:DCM, 1hr.
We have used a series of activated homobifunctional hydrophobic and hydrophilic linkers to produce the dimeric compounds, shown in Scheme 2. Multiple spacers were used because the bivalent molecules can interact with viral NA in three ways. The bivalent molecules can crosslink adjacent NAs on the same tetramer or crosslink two NAs from two different NA tetramers on the same viral particle or crosslink NA’s from two different viral particles. The minimal required distances between NA’s in all three cases are approximately 16, 30, 50 Å, respectively.\textsuperscript{112} Thus, by using a series of activate hydrophilic and hydrophobic spacers, we generated a panel of Gemini compounds 28-32 with decent yield. The removal of ester groups from azido compounds 28-32 was done in basic conditions and were reduced via palladium catalyzed hydrogenation to yield bivalent compound amine at the 4\textsuperscript{th} position. The deprotection and reductions resulted in final amine compounds with good yield (C6-SA, C12-SA, TetraEG-SA, PentaEG-SA, HexaEG-SA). The azido intermediate 28-32 were reduced via palladium catalyzed hydrogenation to introduce the amine at the 4\textsuperscript{th} position and was directly protected guinidinium group to yield compounds 33-37 with decent yield. The removal of the ester from compounds 33-37 were done in basic conditions and follow by the removal of t-butylcarbonate in acid condition to afford final compounds in good yield (C6-SG, C12-SG, TetraEG-SG, PentaEG-SG, HexaEG-SG). All intermediates and final compounds were confirmed with mass spectroscopy, \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR spectroscopy.
Figure 32: Synthesis of bivalent analogs of S-linked Sialosides

Figure 33: Reagents and Conditions: a) DEA, DMF; 76–85%; (b) (i) NaOH/CH3OH (aq), (ii) H2/Pd(OH)2; 90–92% over 2 steps; (c) i. NaOMe, MeOH  ii. NaOH (50mM) iii. DCM:TFA
4.4 Design and Synthesis of S-linked Sialosides Polymers

In earlier chapters we have shown that our compounds can act as capture molecules to capture the influenza virus through assay.\textsuperscript{98} Further we have also shown our dimeric sialoside analogs have potential therapeutics against two influenza strains H1N1 (A/California/07/2009) and H3N2 (A/HongKong/8/68).\textsuperscript{93} Here we report the synthesis of sialoside polymers and their inhibition properties. Previous studies have shown that our compounds have the abilities to bind to the influenza neuraminidase.\textsuperscript{93,98} Thus, it was logical for us to investigate their inhibition property against more influenza virus strains. We have also shown that these sialoside analogs can inhibit intact viral particles via plaque reduction assays, therefore we want to see if increasing the multivalency of these analogs would decrease the IC\textsubscript{50}. According to our previous studies, the IC\textsubscript{50} value for our dimeric sialoside compounds are in high nanomolar range.\textsuperscript{93} Therefore, we are hypothesizing that by increasing the sialoside units on one molecules increase the inhibition properties and thus decrease the IC\textsubscript{50} values due to the multivalent effects.\textsuperscript{113} We have molecularly attached compound \textbf{1.9a} and \textbf{1.9b} to an exo-5-norbornenecarboxylic acid as the building blocks for our polymers.

The advancement of ring-opening metathesis polymerization (ROMP) has made it the most widely use polymerization method.\textsuperscript{114} Well defined olefin-metathesis catalysts such as; \textbf{[(tBuO)\textsubscript{2}(ArN)Mo=CH(tBu)](38)} and \textbf{[(PCy\textsubscript{3})\textsubscript{2(Cl)\textsubscript{2}Ru=CHPh]} (Cy-cyclohexyl) (39)} has aided in controlling the growth of the living polymer, which has also made ROMP a novel method to synthesize polymers with various functional
groups.\textsuperscript{115, 116} Second generation Grubb’s catalyst (40) exhibits high polymerization activities and also high tolerance to varieties of functional groups better than compound 41.\textsuperscript{117, 118, 119} Therefore, we have selected second generation Grubbs’ catalyst (42) as our catalyst to carry out ROMP.

![Grubb's second generation catalyst for ROMP](image)

Figure 33 Grubb's second generation catalyst for ROMP (43)

Carbon-carbon double bond (C=C) is the starting point for many organic reactions. Among which, olefin metathesis reaction allows the formation and the breaking of carbon-carbon double bonds. The process is mediated by a metal carbene catalyst \textbf{Figure 4}.\textsuperscript{119} The mechanism occurs when the olefin binds to the metal carbene catalyst, which results in the formation of metalloyclobutane.\textsuperscript{119} The metalloycyclic intermediate species can either form new olefin and the metal carbene or revert back to the original species (\textbf{Figure 35a}). ROMP is a type of olefin metathesis and driven by the release of ring strain inherent in the monomers (\textbf{Figure 35b}).
Figure 34: Mechanism of olefin metathesis

Figure 35: A) Olefin metathesis mechanism. B) ROMP mechanism with initiation, propagation and termination steps.\textsuperscript{120} Initiation steps involves the coordination of the cyclic olefin to an alkylidene metal complex. Subsequently forms a metallocyclobutane intermediate via a \([2+2]\) cycloaddition. The metallocyclic intermediate undergoes a cycloreversion to yield a new metal alkylidene. The cycle continues until equilibrium is reach or the reaction is quenched.
4.5 Procedures

Norbornene was selected as our monomer building blocks due to their ability to easily undergo ROMP and polymerization is easy to control.\textsuperscript{115, 121} Thus, our synthesis begin with commercially available compound 38, where the free acid group was activated with CDMT/NMM and reacted with 1.1 equivalent of propargyl amine to yield compound 39 in good yield.\textsuperscript{72} Next, compound 1.9a and 1.9b can be introduced onto compound 39 via copper (I) catalyzed 1,3 dipolar addition to yield compound 40 and 41 respectively with good yield. Zemplén deprotection to remove the acetates and methyl ester was followed by acidic removal of the tert-butoxy groups to yield the monovalent compounds SA and SG which had an amine and a guanidine group at the four position of the sialic acid, respectively from compound 40 and 41.
Figure 35 Synthesis of sialoside polymers

**Figure 36:** Reagent and conditions; a) Propargyl amine, CDMT, NMM, THF 90% b) 1.9a or 1.9b sodium ascorbate, CuSO4, THF:H2O:t-BuOH (1:1:1) 57%, 70% respectively c) Ru-initiator (4.3, 1:25, 1:10, 1:5 molar ratio) anhydrous THF (quant.) d) i. LiOH, H2O ii. TFA:DCM (SA-25, 10, 5 – 80%, 75%, 80%, SG-25, 10, 5 – 75%, 85%, 60%).
Polymerization of the glycomonomer 40 and 41 with a second generation olefin metathesis Ru-initiator 43\textsuperscript{122, 119, 123} in anhydrous tetrahydrofuran (THF) for 6 hours followed by chain termination with ethyl vinyl ether afforded desired polymers (42 a,b,c and 43a, b, c). The molecular equivalent ratio of initiator to glycomonomers used were; 1:25, 1:10, 1:5. Polymerization proceeded efficiently, the process was monitored by TLC. Fully protected glycopolymers 42a, b, c and 43a, b, c were subjected to global deprotection of acetates and methyl esters via Zemplén methods and then removal of tert-butoxy groups by acidic conditions. The global deprotections of acetates, methyl esters and tert-butoxy groups resulted in a pale white foamy solid after lyophilization for all six glycopolymers (SA-25, SA-10, SA-5, SG-25, SG-10, SG-5).

4.6 Biological Assays

Next, we subjects these NA’s inhibitors to a number of assays. First, we use commercially available 2’-(4-methyllumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) as substrate with water soluble viral NA before testing with intact virus.Initial binding component is necessary for inhabitation, we premix the compounds with NA and following the cleavage of the substrate over two hours to obtain the IC\textsubscript{50} values using the linear slope between 0-35 minutes. The IC\textsubscript{50} values with three independent experiments for each compound are given in Table 4 and the selected raw data is presented in the Supplemental data. We used commercial available drugs, Zanamivir and Oseltamivir as our controls. The results confirmed our expected values for sialic acid (millimolar inhibition) and the antivirals (nanomolar inhibition). Introduction of an amine group at the 4th position decrease the IC\textsubscript{50} value by at approximately 1000 fold to the micromolar range for both influenza viral enzyme N1 and N2; the IC\textsubscript{50} values
for SA range from 60-180 µM. The bivalent compounds (C6-SA, C12-SA, TetraEG-SA, PentäEG-SA and HexaEG-SA) all exhibit higher inhibition activity, approximately four to five times increase in inhibition property, especially N1 enzyme when compared to the monovalent compound. However, there were exception, the compounds with the longer spacer (PentaEG-SA and HexaEG-SA), were not that efficacious compared to the monomeric compounds. Similar observation were seen with N2 enzyme, where the bivalent compounds exhibiting a twofold higher inhibition compared to the monomeric amine containing compound and the longer spacer compounds are less effective. The effectiveness of the inhibitors were enhanced with the introduction of the guanidine group. The IC₅₀ value ranging from 0.2-2.5 µM for SG, C6-SG, C12-SG, TetraEG-SG, PentäEG-SG and HexaEG-SG. The IC₅₀ values for the bivalent compounds, irrespective of the spacers, are similar to SG, which is the monomeric compounds and the values for both enzymes are similar, in the submicromolar range. This is due to the soluble NA's not being part of the membrane and some present as a mixture of monomers, dimers, trimers and some are tetramers. In summary, these experiments confirm that the compounds with amine at the 4th position (SA, C6-SA, C12-SA, TetraEG-SA, PentäEG-SA and HexaEG-SA) are better inhibitors than the nature receptor. Compounds with the guanidine group at the 4th position (SG, C6-SG, C12-SG, TetraEG-SG, PentäEG-SG and HexaEG-SA) are better inhibitors than compounds with the amine at the same position. We also confirmed that both classes of NA, NA1 with the more open binding pocket and are more flexible loop that closes upon the introduction of sialic acid or an inhibitors and NA2, which has a preset binding pocket, are inhibited very well by the synthetic compounds.
<table>
<thead>
<tr>
<th>Compounds Name</th>
<th>H3N2 (µM)</th>
<th>H1N1 (µM)</th>
<th>H5N1 (µM)</th>
<th>H3N2 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>64±14</td>
<td>56±6</td>
<td>127±29</td>
<td>1778 ± 20</td>
</tr>
<tr>
<td>C6-SA</td>
<td>11.6±3.5</td>
<td>9.9±1.8</td>
<td>20±5</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>C12-SA</td>
<td>15±9</td>
<td>11±1</td>
<td>31±7</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>TetraEG-SA</td>
<td>11±5</td>
<td>9.8±2.4</td>
<td>28±3</td>
<td>39 ± 14</td>
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Table 3  IC50 values from MUNANA based assays
<table>
<thead>
<tr>
<th>Compound</th>
<th>21±3</th>
<th>36±4</th>
<th>64±14</th>
<th>178 ± 20</th>
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<tbody>
<tr>
<td>PentaEG-SA</td>
<td>33±15</td>
<td>45±1</td>
<td>85±12</td>
<td>216 ± 56</td>
</tr>
<tr>
<td>HexaEG-SA</td>
<td>0.55±0.02</td>
<td>0.8±0.1</td>
<td>0.1 ± 0.002</td>
<td>1 ± 0.1</td>
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<tr>
<td>C6-SG</td>
<td>0.25±0.05</td>
<td>0.26±0.003</td>
<td>0.2 ± 0.002</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>C12-SG</td>
<td>0.5±0.3</td>
<td>0.8±0.006</td>
<td>0.73 ± 0.003</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>TetraEG-SG</td>
<td>1.78±1.3</td>
<td>2.4±0.4</td>
<td>2.5 ± 0.1</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>PentaEG-SG</td>
<td>1±0.06</td>
<td>1.2±0.2</td>
<td>1.1 ± 0.1</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>HExaEG-SG</td>
<td>0.5±0.6</td>
<td>0.9±0.1</td>
<td>0.57 ± 0.006</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Zanamivir</td>
<td>≈ 0.001</td>
<td>≈ 0.01</td>
<td>≈ 0.04</td>
<td>≈ 0.04</td>
</tr>
<tr>
<td>Oseltamivir</td>
<td>≈ 0.008</td>
<td>≈ 0.01</td>
<td>≈ 0.05</td>
<td>≈ 0.05</td>
</tr>
</tbody>
</table>

Our compounds were able to inhibit water soluble NA’s, we performed the same assay with two intact viruses, Influenza H1N1 (A/California/07/2009) and H3N2 (A/HongKong/8/68) to ensure that the compounds are able to inhibit the transmembrane NAs present on the viruses. A similar trend was observed for soluble NA, SA the compounds with the amine at the 4th position, exhibit an IC$_{50}$ value of 60 µM for both strains. The bivalent comlecules, C6-SA, C12-SA, TetraEG-SA, PentaEG-SA and HexaEG-SA exhibit a 5-fold decrease in the IC$_{50}$ compared to the monomer to ≈ 10 µM. The lone exception is the compound with the longest oligoethylene glycol spacer, which did not have a similar effect as the other
bivalent molecules, presumably because of the spacer is too flexible to exhibit a bidentate effect. In the case of the guanidine containing compounds, the monomer $\text{SG}$ has an IC$_{50}$ of $\approx 1 \mu M$ for both strains, which is a 50-fold decrease when compared to $\text{SA}$ compound. The bivalent compounds, $\text{C6-SG}$, $\text{C12-SG}$, $\text{TetraEG-SG}$, $\text{PentaEG-SG}$, and $\text{HexaEG-SG}$ all show significant decrease in the IC$_{50}$ values, ranging from 250 nM to 2.4 $\mu M$ for both viruses, with $\text{C6-SG}$ the best inhibitor of all compounds. However, since the values for all bivalent copounds are similar, it is not clear if the compounds bind to the NA’s on the same viron or different virons. What is clear, is that our hypothesis increasing the intrinsic binding affinity of an individual sialoside analogs coupled with bivalency decrease the IC$_{50}$ values from the millimolar ranging by 10,000 fold to nanomolar range.

<table>
<thead>
<tr>
<th>Compounds Name</th>
<th>H$_3$N$_2$ (µM)</th>
<th>H$_1$N$_1$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{SA}$</td>
<td>10-100</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>$\text{C6-SA}$</td>
<td>1-10.0</td>
<td>$&gt;100$</td>
</tr>
</tbody>
</table>

Table 4  IC$_{50}$ values from plaques based assays
We were also interested in testing the efficacy of these compounds to inhibit the virus in cell base systems. Toward that end, we performed a plaque assay for all the compounds. The viruses was introduced to the cells for 30 min, followed by addition of the compounds to arrest the spread of the viruses. IC$_{50}$ values based on 50% plaque reduction in plaque size are given in Table 2. We were please to observe a similar trend for all compounds as was seen with the cell free system. The SA compound with an amine at the 4$^{\text{th}}$ position exhibit inhibition in the 10-
1000 µM range for both H1N1 and H3N2 strains and the dimers **C6-SA, C12-SA, TetraEG-SA, PentaEG-SA** and **HexaEG-SA**, exhibiting a 10-fold decrease in the IC$_{50}$ values (range from 1-100 µM) in comparison to **SA**. The bivalent derivatives **C6-SG, C12-SG, TetraEG-SG, PentaEG-SG** and **HexaEG-SG**, all exhibiting lower IC$_{50}$ value (10-1000 nM) than **SG**.

4.7 Summary

In summary, we have combined robustness of S-sialosides, increased intrinsic affinity of a single nit and used bivalency to produce a panel of compounds that inhibit two different strains of influenza viruses. The IC$_{50}$ values range from the low micromolar to high nanomolar. We have also identified the appropriate length of the spacer for attachment of an appropriate scaffold, longer spacers such as a pentra or hexa ethylene glycol do not seem to increase the effectiveness of the inhibitors. Finally, we note that these compounds are not as efficacious as Zanamivir or Oseltamivir. However, we are currently making structural modifications, for example introducing a fluorine at the 3 position and tethering the molecules polymeric/dendrimeric scaffold. We anticipate that these modifications will enhance the IC$_{50}$ values to produce highly potent inhibitors.

4.8 Experimental

Scheme 1. Synthesis of SA and SG. Reagents and Conditions: a) HCl (g), LiCl, CH₃CN, 6 days; b) KSAc, TBAB (Tetrabutylammonium bisulfate), CH₂Cl₂/H₂O, overnight, 50% over 2 steps; c) TosOC₆H₄SO₃H, DEA, DMF, 65%; d) PPh₃, THF/H₂O, 12h; e) (Boc)₂O, TEA, THF, 60% over 2 steps f) MeS-(C=NBoc)NHBOc, HgCl₂, TEA, CH₂Cl₂, 80% over 2 steps; g) MeOH/NaOH (aq), 1 h; h) TFA/DCM (1:1). 90% over two steps for SA and SG, respectively.

Methyl (5-acetamido-7, 8, 9-tri-O-acetyl-4-azido-2-chloro-3, 4, 5-trideoxy -β-D-glycerol -D-galacto-2-nonulopyranosid)onate 2. Anhydrous HCl was bubbled through a solution of glycal 1 (1g, 2.1 mmol) in CH₃CN (20 ml) containing LiCl (500 mg, 11.7 mmol) for 30 min. The reaction mixture was stirring at room temperature for 4 days. HCl gas was bubbled again for 30 min through the solution again and the reaction was stirred for 2 more days. The solvent was evaporated to dryness, suspended in CH₂Cl₂, washed with ice-cold saturated NaHCO₃, dried over Na₂SO₄, concentrated to dryness. The crude material was directly used in next step.
Methyl (5-acetamido- 7, 8, 9-tri-O-acetyl-4-azido-2-thioacetyl-3, 4, 5-trIDEOxy -α-D-glycero-D-galacto-2-nonulopyranosid) onate 3. KSAC (1.25 g, 0.01 mmol, 5eq based on 1) and TBAB (740 mg, 2.2 mmol, 1eq based on 1) were dissolved in water; a solution of crude chloride in CH₂Cl₂ was added dropwise. The two phase reaction system was stirred for 12 h. The organic phase was extracted by CH₂Cl₂ and washed with water, dried over Na₂SO₄. The solvent was removed in vacuum to give a residue, which was purified by flash chromatography to afford the title compound (580 mg, 50%) and 1 as a mixture (molar ratio 1:1 determined by NMR).

6-S-[Methyl 5-acetamido-7, 8, 9-tri-O-acetyl-4-azido-3, 4, 5-trIDEOxy -D-glycero-α-D-galacto-non-2-ulopyranosyl] onate]-1-thiolacety -hexane 4. Thioacetate 3 (200 mg, 0.37 mmol) and TosOC₆H₁₂SAc (88.6 mg, 0.3 mmol) were dissolved in dry DMF (8 ml). DEA (312 µL, 3 mmol) was added dropwise and the reaction mixture was stirred for 2 h at room temperature. The solvent was removed in vacuo and the residue was purified by flash column chromatography to give the product as a colorless oil (215 mg, 65%). ¹H NMR (400 MHz, CDCl₃) δ 5.64 (d, J = 8.3 Hz, 1H, -NHAc), 5.35-5.29 (m, 2H, H-7, H-8), 4.31 (d, J = 11.4 Hz, 1H), 4.20 (dd, J = 12.5, 4.3 Hz, 1H), 4.09 (d, J = 10.7 Hz), 3.99 (br, 1H), 3.83 (d, J = 10.5 Hz, 3H), 3.32-3.31 (m, 1H), 2.87 (t, J = 7.3 Hz, 2H), 2.82 - 2.63 (m, 2H, -SChA, H-3eq), 2.62 - 2.45 (m, 1H, -SChA), 2.33, 2.17, 2.05, 2.00 (4s, 12H), 1.86 – 1.32 (m, 8H). ¹³C NMR (101 MHz, CDCl₃) δ 195.9, 170.9, 170.5, 170.1, 169.8, 168.3, 162.5, 82.9, 77.5, 77.2, 76.8, 73.5, 68.5, 67.4, 62.0, 58.7, 52.7, 50.3, 38.0, 36.5, 31.4, 30.5, 29.2, 29.0, 28.9, 28.6, 28.1, 28.0, 23.1, 21.0, 20.8, 20.6. ESI-HRMS calcd for C₂₆H₄₀N₄O₁₁Sc₂: 649.2213, found: m/z 649.2230 [M + H]⁺; C₂₆H₄₀N₄O₁₁Sc₂Na: 671.2038, found: m/z 671.2033 [M + Na]⁺.

6-S-[Methyl 5-acetamido-7, 8, 9-tri-O-acetyl-3, 4, 5-trIDEOxy-4-(bis-N, N'-tert-butyloxycarbonyl)-guanidino-D-glycero-α-D-galacto-non-2-ulopyranosyl] onate] -
1-thioacetyl-hexane 7. A solution of azide 4 (200 mg, 0.33 mmol) and PPh₃ (0.4 mmol) in THF/H₂O (1:1, 10 ml) was stirred overnight. The solvent was removed in vacuo. The crude product 5 was dissolved in anhydrous CH₂Cl₂, and TEA was added dropwise to obtain a clear solution. Then 1, 3-bis (tert-butoxycarbonyl)-2-methylthioseadourea (115 mg, 0.4 mmol) and HgCl₂ (109 mg, 0.4 mmol) was added under 0 °C. The solution was warmed to room temperature and stirred for 12 h to obtain a suspension. The reaction was filtered. The filtrate was concentrated and purified by silica gel chromatography to obtain title compound 7 as a colorless amorphous solid (135 mg, 80% over two steps). ¹H NMR (400 MHz, CDCl₃) δ 11.29 (s, 1H, NH⁻Boc), 8.39 (1H, d, J=6.8 Hz, -NHBoc), 6.01 (1H, d, J=8.8 Hz, -NHAc), 5.39-5.30 (m, 2H, H-7, H-8), 4.34 (dd, 1H, J=2.0, 12.0 Hz, H-9a), 4.10-4.00 (m, 3H, H-9b, H-6, H-4), 3.83 (s, 3H, -OMe), 3.81-3.76 (m, 1H, H-5), 3.54 (t, 2H, J=6.8 Hz, -CH₂SAC), 2.82-2.72 (m, 2H, -SCH₂-), 2.58-2.53 (m, 1H, -SCH₃), 2.17, 2.14, 2.04, 1.86 (4s, 4×3H, 4×-CH₃CO), 1.89-1.21 (m, 26H); ¹³C NMR (101 MHz, CDCl₃) δ 195.9, 170.7, 170.6, 170.1, 168.7, 162.9, 156.7, 152.6, 83.8, 83.3, 79.4, 77.3, 77.0, 76.7, 75.4, 69.0, 67.7, 62.3, 52.8, 50.4, 49.7, 38.8, 30.6, 29.3, 29.2, 29.1, 29.0, 28.7, 28.3, 28.2, 28.2, 28.0, 23.0, 21.2, 20.9, 20.7. ESI-HRMS calcd for C₃₇H₆₁N₄O₁₅S₂: 865.3575, found: m/z 865.3590 [M + H]+.

6-S-[Methyl 5-acetamido-7, 8, 9-tri-O-acetyl-3, 4, 5-trideoxy-4-(N-tert-butloxyxycarbonyl)-amino-D-glycero-α-D-galacto-non-2-ulpynanosyl]onate] 1-thioacetyl-hexane 6. The crude product 5 (1 mmol) was dissolved in THF, (Boc)₂O (218 mg, 1 mmol) was added and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was concentrated under reduced pressure. The crude product was purified by silica gel chromatography to obtain title product as a colorless amorphous solid (134 mg, 60%). ¹H NMR (400 MHz, CDCl₃) δ 5.60 – 5.21 (m, 3H), 4.70 (d, J = 8.4 Hz, 1H), 4.31 (d, J = 12.0 Hz, 1H), 4.11 (dd, J = 12.4, 4.2 Hz, 1H), 4.03 – 3.70 (m, 5H), 3.53-3.52 (m, 1H), 2.86 (t, J = 7.3 Hz, 1H), 2.79 – 2.70 (m, 2H), 2.55-2.48 (m,1H), 2.32, 2.16, 2.12, 2.04, 1.90 (5s, 5OAc), 1.75-
1.69 (m, 1H), 1.56 – 1.26 (m, 17H); $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 195.9, 170.9, 170.6, 169.9, 168.6, 155.9, 83.4, 79.9, 77.3, 77.0, 76.7, 74.8, 68.5, 67.4, 62.1, 52.9, 50.2, 50.1, 39.2, 30.6, 29.3, 29.1, 29.0, 28.7, 28.3, 28.2, 28.2, 23.2, 21.1, 20.7. ESI-HRMS calcd for C$_{31}$H$_{50}$N$_2$O$_{13}$S$_2$Na: 745.2652, found: m/z 745.2657 [M + Na]$^+$.

6-S-[5-acetamido-3, 4, 5-trideoxy-4- amino-D-glycero-\alpha-D-galacto-enonic acid] -1-thiol -hexane SA. 6 was treated with MeOH and 1 M NaOH. After stirred for 1 h, the solution was neutralized to pH 7 with Dowex 50W X 8 (H$^+$) resin, filtered and evaporated to dryness. Then TFA/DCM (1:1) was added, and the reaction mixture was stirred for 2 h at room temperature. The solution was evaporated to dryness. The residue was lyophilized to get a colorless amorphous solid (90%). 'H NMR (400 MHz, MeOD) $\delta$ 4.19-4.14 (m, 1H); 3.85-3.61 (m, 5H), 3.44-3.32 (m, 1H); 2.90-2.67 (m, 5H, HSCH$_2$, -SCH$_2$, H$_3$no), 2.02 (s, 3H, -NHAc), 1.57-1.28 (m, 8H); $^{13}$C NMR (100 MHz, MeOD) $\delta$ 174.8, 172.5, 75.5, 71.5, 68.2, 62.9, 50.6, 38.1, 36.2, 28.9, 28.8, 28.6, 28.1, 27.5, 21.5. ESI-HRMS calcd for C$_{17}$H$_{33}$N$_2$O$_7$S$_2$: 441.1729, found: m/z 440.1639 [M + H]$^+$.

6-S-[5-acetamido-4-guanidino-3, 4, 5-trideoxy -D-glycero-\alpha-D -galacto-non-2 -ulopyranosyl) onate]-1-thiol-hexane SG.

The guanidine compound 7 was deprotected with the same procedure described above to get a colorless amorphous solid (90%). 'H NMR (400 MHz, D$_2$O) $\delta$ 3.72-3.30 (m, 7H), 2.64-2.51 (m, 5H, HSCH$_2$, -SCH$_2$, H$_3$no), 1.81 (s, 3H, -NHAc), 1.57-1.28 (m, 8H); $^{13}$C NMR (125 MHz, D$_2$O) $\delta$ 174.8, 174.5, 162.7, 158.4, 117.8, 114.9, 86.3, 75.5, 72.0, 68.2, 62.5, 52.1, 50.8, 39.7, 38.2, 29.5, 29.2, 28.2, 27.6, 27.1, 22.0. ESI-HRMS calcd for C$_{18}$H$_{35}$N$_4$O$_7$S$_2$: 483.1946, found: m/z 483.1947 [M + H]$^+$.
General Protocol for the synthesis of oligoethylene glycol dibromide

\[
\text{HOCH}_2\text{CH}_2(\text{OCH}_2\text{CH}_2)_m\text{OCH}_2\text{CH}_2\text{OH} \xrightarrow{i) \text{MsCl/Py}} \text{BrCH}_2\text{CH}_2(\text{OCH}_2\text{CH}_2)_m\text{OCH}_2\text{CH}_2\text{Br} \xrightarrow{ii) \text{LiBr/Acetone}}
\]

\[m=2, 3, 4\]

**Scheme 2.** Synthesis of oligoethylene glycol dibromides. *Reagent and conditions* i) MsCl/Py; ii) LiBr/Acetone.

To a solution of the diol (1.0 eq) in pyridine, methanesulfonyl chloride (3.0 eq) was added dropwise at 0°C. The reaction mixture was stirred at room temperature for 3 h. The reaction was washed with HCl (1 M) and NaHCO\(_3\) (aq), extracted with CH\(_2\)Cl\(_2\) and dried with Na\(_2\)SO\(_4\). The solvent was removed in vacuo and the residue was dissolved in acetone. Lithium bromide (4 eq) was added. The reaction mixture was stirred and heated up to reflux overnight. The reaction was concentrated in vacuo and the crude product was purified by chromatography to obtain the product.

**Tetraethylene glycol dibromide** (**m=2**) was prepared by the procedure described above as a yellow oil (87%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 3.81 (t, \(J = 6.0\) Hz, 4H, BrCH\(_2\) CH\(_3\)O), 3.67 (s,
8H, OCH₂CH₂O), 3.48 (t, J = 6.0 Hz, 4H, BrCH₂CH₂O); ¹³C NMR (101 MHz, CDCl₃) δ 71.2, 70.7, 70.5, 30.4.

**Pentaethylene glycol dibromide (m=3)** was prepared by the procedure described above as a yellow oil (85%).¹H NMR (400 MHz, CDCl₃) δ 3.81 (t, J = 6.0 Hz, 4H, BrCH₂CH₂O), 3.67 (s, 12H, OCH₂CH₂O), 3.48 (t, J = 6.0 Hz, 4H, BrCH₂CH₂O); ¹³C NMR (101 MHz, CDCl₃) δ 71.2, 70.7, 70.6, 70.5, 30.4.

**Hexaethylene glycol dibromide (m=4)** was prepared by the procedure described above as a yellow oil (85%).¹H NMR (400 MHz, CDCl₃) δ 3.80 (t, J = 6.0 Hz, 4H, BrCH₂CH₂O), 3.67 (s, 16H, OCH₂CH₂O), 3.45 (t, J = 6.4 Hz, 4H, BrCH₂CH₂O); ¹³C NMR (101 MHz, CDCl₃) δ 71.2, 70.7, 70.6, 70.5, 30.4.

**b. Synthesis of S-sialodimers.**

**General Protocol for the synthesis of S-sialodimers.**

Thioacetate 3 (2.2 eq) and dibromide (1eq) were dissolved in dry DMF. DEA was added dropwise and the reaction was stirred for 3 h at room temperature. Then the reaction mixture was washed with 1 M HCl (aq), extracted with CH₂Cl₂, dried with Na₂SO₄. The solution was concentrated and purified by column chromatography.
1, 6-Di-S-[Methyl 5-acetamido-7, 8, 9-tri-O- acetyl-4-azido-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl] onate]-hexane 8. Thioacetate 3 and 1, 6-dibromohexane were used as described above. The crude product was purified by column chromatography to give the title compound as colorless oil (86%). 1H NMR (400 MHz, CDCl3) δ 5.74 (d, J = 8.3 Hz, 2H, -NHaC), 5.32-5.28 (m, 4H, H-7, H-8), 4.30 (d, J = 12.4 Hz, 2H, H-9a), 4.17 (dd, J = 12.4, 4.0 Hz, 2H, H-9b), 4.06 (d, J = 10.5 Hz, 2H, H-6), 3.91-3.90 (m, 2H, H-4, 3.80 (s, 6H, -OMe), 3.35-3.34 (m, 2H, H-5), 2.76-2.73 (m, 4H, -SCH2- H3eq), 2.62 - 2.42 (m, 2H, -SCH2-), 2.34 - 1.90 (m, 24H, -OAc), 1.75 (t, J = 12.6 Hz, 2H, H-3ax), 1.37 (dd, J = 53.7, 48.4 Hz, 6H); 13C NMR (101 MHz, CDCl3) δ 170.80, 170.73, 170.60, 170.00, 168.31, 82.97, 77.37, 77.05, 76.74, 72.69, 68.42, 67.77, 62.04, 57.77, 52.93, 51.68, 38.22, 29.10, 28.78, 28.35, 23.47, 21.12, 20.92, 20.73.

1, 12-Di-S-[Methyl 5-acetamido-7, 8, 9-tri-O- acetyl-4-azido-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl] onate]-dodecane 9. Thioacetate 3 and 1, 12-dibromo dodecane were used as described above. The crude product was purified by column chromatography to give the title compound as colorless oil (76%). 1H NMR (400 MHz, CDCl3) δ 5.86 (br, 2H, -NHaC), 5.31-5.28 (m, 4H, H-7, H-8), 4.30 (dd, J = 12.4, 2.0 Hz, 2H, H-9a), 4.17 (d, J = 12.4 Hz, 2H, H-9b), 4.02 (d, J = 6.4 Hz, 2H, H-6), 3.92-3.79 (m, 2H, H-4), 3.79 (s, 6H, -OMe), 3.36-3.34 (m, 2H, H-5), 2.78-2.71 (m, 4H, -SCH2- H3eq), 2.57-2.52 (m, 2H, -SCH2-), 2.29 - 1.90 (m, 24H), 1.75 (t, J = 12.5 Hz, 1H, H3ax), 1.58 - 1.18 (m, 20H); 13C NMR (101 MHz, CDCl3) δ 170.82, 170.62, 170.03, 168.35, 83.00, 77.38, 77.07, 76.75, 72.87, 68.55, 67.76, 62.05, 57.94, 53.45, 52.91, 51.46, 38.20, 29.54, 29.45, 29.26, 29.19, 28.90, 28.76, 23.44, 21.12, 20.91, 20.74.

Di-S-[Methyl 5-acetamido-7, 8, 9-tri-O- acetyl-4-azido-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl] onate]-tetraethylene glycol 10. Thioacetate 3 and
tetraethylene glycol dibromide were used as described above. The crude product was purified by column chromatography to give the title compound as colorless oil (80%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.86 (br, 2H, -NHAc), 5.31-5.28 (m, 4H, H-7, H-8), 4.29 (d, $J = 12.4$ Hz, 2H, H-9a), 4.23 - 4.09 (m, 2H, H-9b), 4.03 (d, $J = 10.4$ Hz, 2H, H-6), 3.89-3.85 (m, 2H, H-4), 3.80 (s, 6H, OMe), 3.61-3.50 (m, 12H), 3.37-3.36 (m, 2H, H-5), 3.05 – 2.87 (m, 2H, -SCH$_3$), 2.86 – 2.70 (m, 4H, m, 4H, -SCH$_3$, H$_{3eq}$), 2.39 – 1.89 (m, 24H), 1.77 (t, $J = 12.6$ Hz, 2H, H$_{3ax}$); $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 170.87, 170.65, 170.12, 168.36, 82.78, 77.39, 77.07, 76.75, 72.82, 70.44, 70.24, 68.35, 67.69, 62.09, 57.80, 53.05, 51.54, 38.09, 28.93, 23.43, 21.12, 20.90, 20.72.

**Di-S-[Methyl 5-acetamido-7, 8, 9-tri-O-acetyl-4-azido-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl] onate]-pentaethylene glycol 11.** Thioacetate 3 and pentaethylene glycol dibromide were used as described above. The crude product was purified by column chromatography to give the title compound as colorless oil (87%).$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.81 (d, $J = 8.3$ Hz, 2H, -NHAc), 5.29-5.27 (m, 4H, H-7, H-8), 4.30 (d, $J = 12.3$ Hz, 2H, H-9a), 4.14 (dd, $J = 12.4$, 4.2 Hz, 2H, H-9b), 4.05 (d, $J = 10.5$ Hz, 2H, H-6), 3.98 – 3.87 (m, 2H, H-4), 3.86 – 3.77 (m, 8H, H-6, OMe), 3.61-3.60 (m, 16H), 3.32 (d, $J = 9.3$ Hz, 2H, H-5), 3.05 – 2.87 (m, 2H, -SCH$_3$), 2.86 – 2.70 (m, 4H, m, 4H, -SCH$_3$, H$_{3eq}$), 2.39 – 1.89 (m, 24H), 1.77 (t, $J = 12.6$ Hz, 2H, H$_{3ax}$); $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 170.74, 170.62, 170.48, 169.98, 168.41, 82.84, 77.35, 77.03, 76.71, 72.82, 70.57, 70.43, 70.24, 68.50, 67.87, 62.11, 57.69, 52.92, 51.78, 38.20, 28.99, 23.35, 21.01, 20.80, 20.60.

**Di-S-[Methyl 5-acetamido-7, 8, 9-tri-O-acetyl-4-azido-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl] onate]-hexaethylene glycol 12.** Thioacetate 3 and hexaethylene glycol dibromide were used as described above. The crude product was purified by
column chromatography to give the title compound as colorless oil (85%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.88 (s, br, 2H, -NHAc), 5.29-5.27 (m, 4H, H-7, H-8), 4.27 (d, $J$ = 12.0 Hz, 2H, H-9a), 4.13 (d, $J$ = 9.6 Hz, 2H, H-9b), 4.00 (d, $J$ = 9.3 Hz, 2H, H-4), 3.83-3.78 (M, 8H, H-6, OMe), 3.61-3.60 (m, 20H), 3.38-3.37 (m, 2H, H-5), 3.03 – 2.85 (m, 2H, -SCH$_\alpha$), 2.85 – 2.65 (m, 4H, -SCH$_\beta$, H$_{3eq}$), 2.36 – 1.88 (m, 24H), 1.75 (t, $J$ = 12.6 Hz, 1H, H-3ax ); $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 170.83, 170.62, 170.05, 168.33, 82.73, 77.41, 77.09, 76.77, 72.82, 70.55, 70.52, 70.39, 70.21, 68.24, 67.63, 62.07, 57.82, 53.47, 53.02, 51.42, 38.09, 28.93, 23.42, 21.12, 20.90, 20.72.

**General protocol for the deprotection of the azide.**

The azide compound was treated with MeOH and 1 M NaOH (1:1 v/v). After stirred for 1 h, the solution was neutralized to pH 7 with Dowex 50W X 8 (H$^+$) resin, filtered and evaporated to dryness. Then the residue in EtOH/EtOAc (1:1) with 2 drop of acetic acid was hydrogenated with Pd(OH)$_2$/C under atmosphere of hydrogen (3 psi) for 12 h. Then the mixture was filtered and washed with MeOH. The filtrate was concentrated to dryness, purified by Bio-Gel® (P-2 Polyacrylamide Gel column) and lyophilized to get a colorless amorphous solid.
Scheme 2. Synthesis of bivalent analogs of S-Sialosides. Reagents and Conditions: a) DEA, DMF; 76-85%; b) i) NaOH/CH₃OH (aq), ii) H₂/Pd(OH)₂; 90-92% % over 2 steps; c) i) H₂/Pd(OH)₂, ii) MeS-(C=NBoc)NH-Boc, HgCl₂, TEA, CH₂Cl₂. 85-89% % over 2 steps; d) NaOH/CH₃OH (aq), 1 h; i) TFA/DCM (1:1), 80-85% over 2 steps.

1, 6-Di-S-[5-acetamido- 4-amino-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-enonic acid]-hexane C6-SA. ¹H NMR (500 MHz, D₂O) δ 3.89-3.47 (m, 14H), 2.78-2.63 (m, 4H, -SCH₃, H₃O), 2.58 – 2.53 (m, 2H, -SCH₂), 1.94 (s, 6H, -NH₂), 1.46-1.24 (m, 8H); ¹³C NMR (125 MHz, D₂O) δ 174.5, 174.3, 170.4, 83.0, 74.3, 70.3, 67.5, 67.3, 62.3, 58.3, 53.1, 49.3, 36.8, 28.5, 28.1, 26.9, 21.6, 21.5. ESI-HRMS calcd for C₂₈H₅₁N₅O₄S₂: 730.2843, found: m/z 730.2868 [M + H]⁺.
1, 12-Di-S-[5-acetamido-4-amino-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-enonic acid]-dodecane C_{12}SA. \(^1\)H NMR (500 MHz, D$_2$O) \(\delta\) 3.90-3.74 (m, 14H), 2.71-2.61 (m, 4H, -SCH$_2$, H$_{3eq}$), 2.58 – 2.53 (m, 2H, -SCH$_3$), 1.95 (s, 6H, -NHAc), 1.73-1.71 (m, 1H, H$_{3ax}$) 1.49-1.07 (m, 20H); \(^{13}\)C NMR (125 MHz, D$_2$O) \(\delta\) 174.2, 172.4, 84.3, 74.4, 71.0, 67.4, 67.2, 62.3, 61.9, 58.9, 49.4, 37.5, 28.9, 28.7, 28.4, 28.1, 21.6. ESI-MS calcd for C$_{34}$H$_{51}$N$_4$O$_{14}$S$_2$: 815.3782, found: m/z 815.3787 [M + H]$^+$. 

Di-S-[5-acetamido-4-amino-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-enonic acid]-tetraethylene glycol TetraEG-SA. \(^1\)H NMR (500 MHz, D$_2$O) \(\delta\) 3.76-3.48 (m, 26H), 2.85-2.78 (m, 6H, -SCH$_2$, H$_{3eq}$), 1.93 (s, 6H, -NHAc), 1.87-1.85 (m, 1H, H$_{3ax}$); \(^{13}\)C NMR (125 MHz, D$_2$O) \(\delta\) 174.5, 172.4, 84.9, 74.1, 71.4, 69.2, 68.9, 68.8, 67.2, 62.1, 61.9, 50.3, 46.9, 36.1, 28.5, 21.6, 21.5. ESI-MS calcd for C$_{36}$H$_{55}$N$_4$O$_{17}$S$_2$: 807.3004, found: m/z 807.3022 [M + H]$^+$. 

Di-S-[5-acetamido-4-amino-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-enonic acid]-pentaethylene glycol PentaEG-SA. \(^1\)H NMR (500 MHz, D$_2$O) \(\delta\) 3.76-3.48 (m, 30H), 2.85-2.78 (m, 6H, -SCH$_2$, H$_{3eq}$), 1.93 (s, 6H, -NHAc), 1.87-1.85 (m, 1H, H$_{3ax}$); \(^{13}\)C NMR (125 MHz, D$_2$O) \(\delta\) 174.5, 174.2, 173.2, 172.5, 85.4, 85.0, 74.4, 74.1, 71.5, 71.4, 69.3, 69.2, 69.1, 68.9, 68.8, 68.4, 67.4, 67.2, 66.1, 62.0, 61.9, 59.3, 50.3, 49.2, 47.0, 37.4, 36.1, 28.5, 21.6, 21.5. ESI-MS calcd for C$_{38}$H$_{59}$N$_4$O$_{18}$S$_2$: 851.3266, found: m/z 851.3258 [M + H]$^+$. 

Di-S-[5-acetamido-4-amino-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-enonic acid]-hexaethylene glycol HexaEG-SA. \(^1\)H NMR (500 MHz, D$_2$O) \(\delta\) 3.76-3.41 (m, 34 H), 2.85-2.71 (m, 6H, -SCH$_2$, H$_{3eq}$), 1.93 (s, 6H, -NHAc), 1.71-1.69 (m, H$_{3ax}$); \(^{13}\)C NMR (125 MHz, D$_2$O) \(\delta\) 174.5, 174.2, 173.2, 85.4, 74.1, 71.4, 69.4, 69.1, 68.9, 68.7, 68.4, 67.4, 66.1, 62.0, 59.4,
50.3, 49.2, 37.5, 28.5, 22.7, 21.5, 21.5. ESI-HRMS calcd for C_{34}H_{63}N_{4}O_{19}S_{2}: 895.3528, found: m/z 895.3521 [M + H]^+.

**General protocol for the changing of the azide to guanidine.**

The azide in EtOH/EtOAc (1:1) was hydrogenated with Pd(OH)$_2$/C under atmosphere of hydrogen (3 psi) for 12 h. Then the mixture was filtered and washed with MeOH. The filtrate was concentrated to dryness. The guanidine compound was synthesized according to the procedure described above for the synthesis of the monoer guanidino product as colorless syrup (85-89%).

1, 6-Di-S-[Methyl 5-acetamido-7, 8, 9-tri-O- acetyl-3, 4, 5-trideoxy-4-(bis-N, N'-tert-butyloxycarbonyl) -guanidino-D-glycero-α-D-galacto-non-2-ulopyranosyl) onate]-hexane 13. $^1$H NMR (400 MHz, CDCl$_3$) δ 11.30 (s, 2H, -NH$_2$), 8.39 (d, $J = 7.1$ Hz, 2H, -NH$_2$Boc), 6.03 (d, $J = 8.7$ Hz, 2H, -NHAc), 5.36-5.29 (m, 4H, H-7, H-8), 4.35 (d, $J = 12.3$ Hz, 1H, H-9a), 4.12-3.99 (m, 6H, H-9b, H-4, H-5), 3.95 - 3.67 (m, 8H, OMe, H-6), 2.81 - 2.70 (m, 4H, -SCH$_3$, H$_{eq}$), 2.67 – 2.47 (m, 2H, -SCH$_3$), 2.16, 2.14, 2.04, 1.83 (4s, 24H, -Ac), 1.69 – 1.14 (m, 34H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 170.81, 170.65, 170.13, 168.79, 162.99, 156.79, 152.70, 83.81, 83.36, 79.50, 77.35, 77.03, 76.71, 75.46, 69.19, 67.83, 62.35, 52.89, 50.46, 49.69, 38.82, 29.69, 29.09, 28.83, 28.43, 28.26, 28.02, 23.02, 21.21, 20.98, 20.81.

1, 12-Di-S-[Methyl 5-acetamido-7, 8, 9-tri-O- acetyl-3, 4, 5-trideoxy-4-(bis-N, N'-tert-butyloxycarbonyl) -guanidino-D-glycero-α-D-galacto-non-2-ulopyranosyl)
onate]-dodecane 14. $^1$H NMR (400 MHz, CDCl$_3$) δ 11.30 (s, 2H, -NHBOc), 8.39 (d, $J$ = 7.2 Hz, 2H, -NHBOc), 6.05 (d, $J$ = 9.1 Hz, 2H, -NHAc), 5.63 – 5.16 (m, 4H, H-7, H-8 ), 4.35 (dd, $J$ = 12.4, 2.6 Hz, 1H, H-9a), 4.12-3.99 (m, 6H, H-9b, H-4, H-5), 3.95 – 3.70 (m, 8H, OMe, H-6), 2.81 – 2.70 (m, 4H, -SCH$_2$), 2.67 – 2.47 (m, 2H, -SCH$_2$), 2.18, 2.16, 2.03, 1.82 (4s, 24H, -Ac), 1.68 – 1.19 (m, 56H); $^{13}$C NMR (101 MHz, CDCl$_3$) δ 170.81, 170.65, 170.17, 168.81, 162.98, 156.79, 152.70, 83.80, 83.38, 79.49, 77.36, 77.04, 76.73, 75.51, 69.32, 67.88, 62.35, 52.85, 50.47, 49.69, 38.84, 29.61, 29.51, 29.27, 28.95, 28.85, 28.25, 28.02, 23.02, 21.20, 20.96, 20.80.

Di-S-[Methyl 5-acetamido-7, 8, 9-tri-O- acetyl-3, 4. 5-trideoxy-4-(bis-N, N’-tert-butyloxycarbonyl)-guanidino-D-glycero-α-D-galacto-non-2-ulopyranosyl] onate]-tetraethylene glycol 15. $^1$H NMR (400 MHz, CDCl$_3$) δ 11.31 (s, 2H, -NHBOc), 8.40 (d, $J$ = 6.8 Hz, 2H, -NHBOc), 6.06 (d, $J$ = 8.6 Hz, 2H, -NHAc), 5.35-5.27 (m, 4H, H-7, H-8), 4.33 (d, $J$ = 12.3 Hz, 2H, H-6), 4.21 – 3.95 (m, 6H, H-9a, H-9b, H-4), 3.85 (s, 6H, -OMe), 3.76 (d, $J$ = 9.9 Hz, 1H, H-5), 3.67-3.63 (m, 12H), 3.03-2.82 (m, 6H, -SCH$_2$-H$_{3eq}$-SCH$_2$-), 2.18, 2.17, 2.04, 1.89 (4s, 12H, -Ac), 1.90-1.87 (m, 1H, H-3ax), 1.49 (s, 36H); $^{13}$C NMR (101 MHz, CDCl$_3$) δ 170.82, 170.19, 168.78, 162.97, 156.81, 152.70, 83.84, 83.15, 79.53, 77.35, 77.03, 76.71, 75.47, 70.45, 70.30, 70.22, 68.98, 67.76, 62.41, 52.97, 50.42, 49.63, 38.73, 30.92, 28.93, 28.26, 28.03, 23.02, 21.22, 20.98, 20.80.

Di-S-[Methyl 5-acetamido-7, 8, 9-tri-O- acetyl-3, 4. 5-trideoxy-4-(bis-N, N’-tert-butyloxycarbonyl)-guanidino-D-glycero-α-D-galacto-non-2-ulopyranosyl] onate]-pentaethylene glycol 16. $^1$H NMR (400 MHz, CDCl$_3$) δ 11.28 (s, 2H, -NHBOc), 8.38 (d, $J$ = 6.8 Hz, 2H, -NHBOc), 6.06 (d, $J$ = 8.6 Hz, 2H, -NHAc), 5.34-5.26 (m, 4H, H-7, H-8), 4.33 (d, $J$ =
$12.3$ Hz, 2H, H-6), 4.07 – 3.97 (m, 6H, H-9a, H-9b, H-4), 3.81 (s, 6H, -OMe), 3.76-3.73 (d, $J = 9.9$ Hz, 1H, H-5), 3.63-3.61 (m, 16H), 2.94-2.82 (m, 6H, -SCH$_{eq}$, H$_{3eq}$, -SCH$_{b}$), 2.18, 2.17, 2.04, 1.89 (4s, 12H, -Ac), 1.90-1.87 (m, 1H, H-3ax), 1.49 (s, 36H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 170.3, 170.2, 169.7, 169.6, 168.3, 162.4, 156.3, 152.1, 83.3, 82.6, 79.1, 74.9, 70.0, 69.9, 69.8, 69.7, 68.4, 67.2, 61.9, 52.3, 49.8, 49.1, 38.1, 28.4, 27.7, 27.5, 22.5, 20.7, 20.4, 20.3,

Di-S-[Methyl 5-acetamido-7, 8, 9-tri-O-acetyl-3, 4, 5-trideoxy-4-(bis-N, N'-tert-butyloxy carbonyl)-guanidino-D-glycero-α-D-galacto-non-2-ulopyranosyl) onate]-hexaethylene glycol 17. $^1$H NMR (400 MHz, CDCl$_3$) δ 11.29 (s, 2H, -NH-Boc), 8.39 (d, $J = 6.8$ Hz, 2H, -NH-Boc), 6.05 (d, $J = 8.8$ Hz, 2H, -NHAc), 5.35-5.26 (m, 4H, H-7, H-8), 4.33 (d, $J = 12.3$ Hz, 2H, H-6), 4.09 – 3.98 (m, 6H, H-9a, H-9b, H-4), 3.82 (s, 6H, -OMe), 3.77-3.74 (d, $J = 9.9$ Hz, 1H, H-5), 3.64-3.62 (m, 20H), 2.95-2.78 (m, 6H, -SCH$_{eq}$, H$_{3eq}$, -SCH$_{b}$), 2.15, 2.13, 2.03, 1.83 (4s, 12H, -Ac), 1.90-1.87 (m, 1H, H-3ax), 1.48 (s, 36H); $^{13}$C NMR (101 MHz, CDCl$_3$) δ 170.8, 170.7, 170.2, 168.8, 162.9, 156.8, 152.6, 83.8, 83.1, 79.5, 77.3, 77.0, 76.7, 75.4, 70.5, 70.4, 70.2, 70.2, 68.9, 67.7, 62.4, 52.9, 50.3, 49.6, 38.7, 28.9, 28.2, 28.0, 23.0, 21.2, 20.9, 20.7.

**General protocol for the deprotection of the guanidine.**

The guanidine compound was treated with MeOH and 1 M NaOH. After stirred for 1 h, the solution was neutralized to pH 7 with Dowex 50W X 8 (H$^+$) resin, filtered and evaporated to dryness. Then TFA/DCM (1:1) was added, and the reaction mixture was stirred for 2 h at room temperature. The solution was evaporated to dryness. The residue was purified by Bio-Gel® (P-2 Polyacrylamide Gel column) and lyophilized to get a colorless amorphous solid.
1, 6-Di-S-[5-acetamido-4-guanidino-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-enonic acid]-hexane C6-SG. $^1$H NMR (500 MHz, D$_2$O) δ 3.90-3.49 (m, 14H), 2.68-2.62 (m, 4H, -SCH$_2$, H$_{3eq}$), 2.58 – 2.54 (m, 2H, -SCH$_3$), 1.88 (s, 6H, -NHAc), 1.79 (t, J= 10, 15 Hz, H$_{3ax}$), 1.46-1.24 (m, 8H); $^{13}$C NMR (125 MHz, D$_2$O) δ 174.5, 172.8, 162.3, 156.1, 84.7, 74.3, 71.2, 67.4, 51.4, 49.3, 37.8, 29.0, 28.5, 28.9, 28.4, 27.0, 21.3. ESI-HRMS calcd for C$_{30}$H$_{55}$N$_8$O$_{14}$S$_2$: 815.3279, found: m/z 815.3282 [M + H]$^+$.  

1, 12-Di-S-[5-acetamido-4-guanidino-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-enonic acid]-dodecane C12-SG. $^1$H NMR (500 MHz, D$_2$O) δ 3.95-3.55 (m, 14H), 2.69-2.57 (m, 6H, -SCH$_2$, H$_{3eq}$, SCH$_3$), 1.90 (s, 6H, -NHAc), 1.49-1.07 (m, 20H); $^{13}$C NMR (125 MHz, D$_2$O) δ 174.1, 170.0, 162.5, 162.2, 156.2, 117.0, 114.7, 85.4, 82.7, 74.5, 70.4, 67.6, 62.2, 53.2, 50.4, 49.5, 37.3, 29.0, 28.9, 28.6, 28.4, 28.1, 21.4. ESI-HRMS calcd for C$_{36}$H$_{67}$N$_8$O$_{14}$S$_2$: 899.4218, found: m/z 899.4221 [M + H]$^+$. 

Di-S-[5-acetamido-4-guanidino-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-enonic acid]-tetraethylene glycol TetraEG-SG. $^1$H NMR (500 MHz, D$_2$O) δ 3.87-3.48 (m, 26H), 2.85-2.78 (m, 6H, -SCH$_2$, H$_{3eq}$), 1.93 (s, 6H, -NHAc), 1.77 (t, J= 10, 15 Hz, H$_{3ax}$), $^{13}$C NMR (125 MHz, D$_2$O) δ 173.9, 173.0, 156.2, 85.0, 74.4, 71.4, 69.3, 69.0, 68.9, 68.8, 67.4, 61.9, 51.5, 49.4, 37.8, 28.3, 21.3. ESI-HRMS calcd for C$_{32}$H$_{59}$N$_8$O$_{17}$S$_2$: 891.9843, found: m/z 815.9832 [M + H]$^+$.  

Di-S-[5-acetamido-4-guanidino-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-enonic acid]-pentaethylene glycol PentaEG-SG. $^1$H NMR (500 MHz, D$_2$O) δ 3.90-3.51 (m, 30H), 2.85-2.68 (m, 6H, -SCH$_2$, H$_{3eq}$), 1.93 (s, 6H, -NHAc), 1.79 (t, J= 10, 15 Hz, H$_{3ax}$), $^{13}$C NMR (125 MHz, D$_2$O) δ 173.9, 173.1, 156.1, 85.0, 74.4, 71.4, 69.3, 69.0, 68.9, 68.8,
Di-S-[5-acetamido-4-guanidino-3, 4, 5-trideoxy-D-glycero-α-D-galacto-non-2-enonic acid]-hexaethylene glycol HexaEG-SG. $^1$H NMR (500 MHz, D$_2$O) δ 3.89-3.48 (m, 34 H), 2.84-2.68 (m, 6H, -SCH$_2$-, H$_{3eq}$), 1.88 (s, 6H, -NHAc), 1.77 1.79 (t, J = 10, 15 Hz, H$_{3ax}$); $^{13}$C NMR (125 MHz, D$_2$O) δ 173.9, 173.3, 156.1, 85.4, 74.1, 71.5, 69.4, 69.1, 68.9, 68.7, 67.4, 61.9, 51.7, 49.4, 37.9, 28.4, 21.3. ESI-HRMS calcd for C$_{36}$H$_{66}$N$_8$O$_{19}$S$_2$: 979.3964, found: m/z 979.3976 [M + H]$^+$. 

To a stirring solution of compound 1 (1.1 g, 8.0mmol) in tetrahydrofuran (THF), 2-chloro-4,6-dimethoxy-1,3,5-triazine (1.5 g, 8.7mmol) and 4-methylmorpholine (0.885 g, 8.7mmol) was added. Reaction mixture was placed in an ice bath and allowed to stir overnight. Compound 2 was dissolved in THF with 4-methylmorpholine (0.482 g, 8.7mmol) was added into the reaction mixture dropwise at 0°C. The reaction mixture was allowed to stir overnight. The reaction was quenched with water, extracted with ethyl acetate (3x), dried over sodium sulfate and concentrate invacuo. Compound was purified using flash chromatography with solvent system of hexane and acetone (10:1 ratio) to yield a golden yellow solid compound 2 (90% yield).$^1$H NMR (400 MHz, CDCl$_3$) δ 6.15 (s, 1H), 6.11 (s, 1H), 6.03 (s, 1H), 4.12 – 4.01 (m, 2H), 2.94 (d, J = 11.3 Hz, 2H),
2.27 – 2.19 (m, 1H), 2.03 (dd, J = 8.5, 4.3 Hz, 1H), 1.93 (dd, J = 7.8, 3.2 Hz, 1H), 1.71 (d, J = 7.8 Hz, 1H), 1.41 – 1.28 (m, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 175.4, 138.3, 135.9, 79.9, 71.4, 47.1, 46.3, 44.1, 41.6, 30.5, 29.3. HRMS (ESI) Calculated for C$_{11}$H$_{13}$NO 175.0997, found: 176.1064 (M+H).

To a stirring solution of compound 3 (220 mg, 0.32mmol) in THF, water and t-butyl alcohol mixture (1:1:1 ratio), then compound 2 (50.0 mg, 0.29mmol) was added. Copper sulfate (87 mg, 0.34mmol) was added along with sodium ascorbate (67 mg, 0.34) and argon as was bubbled through for 10 minutes then the reaction was allowed to stir over night at room temperature. Solvent were removed via vacuo and product were purified using flash column chromatography with hexane:acetone (2:1 ratio) solvent system to yield desired product, 150 mg (57% yield). $^1$H NMR (400 MHz, CDCl$_3$) δ 6.04 (d, J = 13.5 Hz, 1H), 5.88 (d, J = 9.6 Hz, 1H), 5.32 (s, 1H), 5.28 (s, 1H), 5.07 (d, J = 8.9 Hz, 1H), 4.37 (s, 1H), 4.28 (d, J = 12.2 Hz, 1H), 4.06 (s, 1H), 3.85 (s, 1H), 3.75 (d, J = 15.5 Hz, 2H), 3.52 (d, J = 9.6 Hz, 1H), 2.89 (d, J = 11.7 Hz, 1H), 2.75 – 2.64 (m, 1H), 2.61 (s, 1H), 2.47 (dd, J = 12.5, 6.9 Hz, 1H), 2.12 (s, 1H), 2.05 (s, 1H), 1.99 (s, 2H), 1.86 (s, 2H), 1.75 (t, J = 12.6 Hz, 1H), 1.65 (s, 1H), 1.53 – 1.42 (m, 1H), 1.36 (s, 5H), 1.23 (s, 1H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 171.0, 170.7, 170.0, 169.9, 168.7, 156.1, 137.2, 135.2, 83.5, 79.8, 77.5, 77.1, 76.8, 74.7, 68.5, 67.6, 62.3, 53.5, 52.9, 50.2, 49.9, 47.1, 46.2, 41.57, 39.1, 30.5, 29.8, 29.3,
28.9, 28.6, 28.3, 27.9, 26.0, 23.1, 21.2, 20.8. HRMS (ESI) Calculated for C_{40}H_{60}N_{6}O_{13}S 864.3939, found 865.3996 (M+H).

To a stirring solution of compound 3 (220 mg, 0.22 mmol) in THF, water and t-butyl alcohol mixture (1:1:1 ratio), then compound 2 (34.8 mg, 0.20 mmol) was added. Copper sulfate (35.1 mg, 0.22 mmol) was added along with sodium ascorbate (43.0 mg, 0.22) and argon as was bubbled through for 10 minutes then the reaction was allowed to stir over night at room temperature. Solvent were removed via vacuo and product were purified using flash column chromatography with hexane:acetone (2:1 ratio) solvent system to yield compound 4, 150 mg.

$^1$H NMR (400 MHz, CDCl$_3$) δ 11.26 (s, 1H), 8.34 (d, J = 6.6 Hz, 1H), 6.18 – 5.96 (m, 3H), 5.28 (dt, J = 18.0, 6.0 Hz, 3H), 4.48 (s, 1H), 4.28 (d, J = 12.4 Hz, 3H), 4.07 – 3.91 (m, 3H), 3.77 (s, 4H), 2.86 (d, J = 7.0 Hz, 2H), 2.80 – 2.73 (m, 1H), 2.73 – 2.64 (m, 1H), 2.59 (s, 1H), 2.51 – 2.42 (m, 1H), 2.13 (s, 1H), 2.11 (s, 3H), 2.08 (s, 3H), 2.02 (s, 1H), 1.97 (s, 3H), 1.84 (d, J = 10.1 Hz, 3H), 1.78 (s, 2H), 1.62 (t, J = 12.6 Hz, 1H), 1.43 (d, J = 2.1 Hz, 20H), 1.39 – 1.33 (m, 3H), 1.28 (d, J = 5.1 Hz, 4H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 170.6, 170.5, 169.9, 168.6, 162.8, 156.5, 152.5, 137.9, 135.9, 83.6, 83.1, 79.2, 77.3, 77.0, 76.7, 75.1, 68.6, 67.5, 62.2, 52.7, 50.3, 50.0, 49.6, 46.9, 46.1, 44.2, 41.3, 38.6, 34.8, 30.3,
General Procedures for Polymerization

To a stirring solution of monomer 4 (150 mg, 0.17 mmol) in anhydrous THF at rt, Grubbs catalyst II (2.9 mg, 0.003 mmol) and reaction mixture was allowed to stirred overnight. Reaction was monitored with thin layer chromatography until starting materials has all gone. After the starting materials are gone, the reaction was quenched with vinyl ethyl ether (0.1 ml) and allowed to stir for 30 minutes then hexane (10 mL) was added to the reaction mixture. The solid was filtered and wasted with hexane:THF (10:1 ratio, 3x) to yield a grayish tan solid.

Polymer Type I (NHBoc)

5:1 (Ratio)

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.68 (s, 1H), 5.93 (s, 1H), 5.26 (d, $J = 75.7$ Hz, 5H), 4.31 (d, $J = 10.7$ Hz, 4H), 4.11 (s, 2H), 3.93 – 3.64 (m, 7H), 3.56 (s, 2H), 2.71 (s, 3H), 2.53 (s, 3H), 2.24 – 2.01 (m, 11H), 1.98 – 1.75 (m, 10H), 1.34 (d, $J = 46.0$ Hz, 20H). 13C NMR (101 MHz, CDCl$_3$) $\delta$ 171.1, 170.7, 170.1, 170.0, 168.8, 156.1, 83.5, 79.8, 74.8, 68.6, 68.0, 67.6, 62.3, 53.0, 50.2, 50.0, 39.1, 34.7, 34.5, 31.6, 30.1, 29.1, 28.6, 28.3, 28.1, 26.0, 25.6, 25.3, 23.2, 22.7, 21.2, 20.9, 20.7, 18.8, 14.1, 11.4.

10:1 (Ratio)
$^1$H NMR (400 MHz, CDCl$_3$) δ 7.72 (s, 1H), 5.97 (s, 1H), 5.36 (s, 2H), 5.18 (s, 1H), 4.32 (d, J = 11.7 Hz, 3H), 4.10 (d, J = 10.7 Hz, 1H), 3.81 (dt, J = 9.0, 7.9 Hz, 6H), 3.58 (s, 1H), 2.72 (s, 4H), 2.23 – 1.76 (m, 14H), 1.34 (d, J = 52.1 Hz, 11H).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 171.1, 170.7, 170.1, 170.0, 168.8, 156.1, 83.5, 79.8, 74.8, 74.0, 68.6, 68.0, 67.6, 62.3, 53.0, 50.2, 39.1, 34.8, 30.1, 29.0, 28.6, 28.3, 28.1, 26.0, 25.6, 23.2, 21.3, 20.9.

25:1 (Ratio)

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.70 (s, 1H), 5.98 (s, 1H), 5.36 (s, 3H), 5.18 (s, 2H), 4.31 (d, J = 11.4 Hz, 5H), 4.09 (d, J = 10.4 Hz, 1H), 3.90 (d, J = 9.2 Hz, 1H), 3.77 (d, J = 6.6 Hz, 4H), 3.57 (s, 1H), 2.68 (d, J = 26.2 Hz, 3H), 2.48 (d, J = 37.9 Hz, 2H), 2.21 – 1.98 (m, 10H), 1.88 (d, J = 12.3 Hz, 6H), 1.62 – 1.47 (m, 3H), 1.47 – 1.25 (m, 18H).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 171.1, 170.74, 170.1, 170.0, 168.8, 156.1, 83.5, 79.8, 74.8, 68.6, 68.0, 67.6, 62.3, 53.0, 50.2, 50.0, 39.1, 34.7, 34.5, 31.6, 30.1, 29.1, 28.6, 28.3, 28.1, 26.0, 25.6, 25.3, 23.2, 22.7, 21.2, 20.9, 20.7, 18.8, 14.1, 11.4.

Polymer Type II (NHC=NBocNHBoc)

5:1 (Ratio)

$^1$H NMR (400 MHz, CDCl$_3$) δ 11.31 (s, 4H), 8.39 (d, J = 6.2 Hz, 4H), 7.63 (s, 3H), 6.14 (s, 4H), 5.33 (d, J = 13.8 Hz, 13H), 4.32 (s, 13H), 4.18 – 3.94 (m, 13H), 3.83 (s, 8H), 3.75 (t, J = 6.4 Hz, 11H), 2.15 (d, J = 10.4 Hz, 28H), 2.03 (s, 11H), 1.93 – 1.80 (m, 32H), 1.46 (d, J = 20.7 Hz, 71H), 1.36 – 1.23 (m, 63H), 0.98 (s, 13H), 0.93 – 0.82 (m, 55H).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 170.8, 170.7, 170.2, 168.7, 163.0, 156.7, 152.7, 83.8, 83.3, 79.5, 75.3,
68.9, 68.0, 67.8, 62.4, 52.9, 50.3, 49.7, 38.8, 36.1, 34.7, 31.6, 29.1, 28.3, 28.0, 25.6, 25.3, 22.6, 20.6, 18.8, 14.1, 11.4.

10:1 (Ratio)

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 11.30 (s, 10H), 8.38 (d, $J = 6.5$ Hz, 10H), 7.60 (d, $J = 28.2$ Hz, 10H), 6.16 (s, 10H), 5.32 (t, $J = 10.0$ Hz, 30H), 5.18 (s, 10H), 4.32 (d, $J = 10.0$ Hz, 50H), 4.11 – 3.95 (m, 20H), 3.82 (s, 10H), 3.74 (s, 45H), 2.14 (d, $J = 10.6$ Hz, 30H), 2.02 (s, 20H), 1.90 – 1.81 (m, 30H), 1.48 (s, 30H), 1.30 – 1.23 (m, 280H), 0.86 (ddd, $J = 8.7, 8.1, 3.8$ Hz, 30H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 170.8, 170.6, 170.1, 168.7, 163.0, 156.7, 152.7, 135.1, 130.1, 83.7, 83.3, 79.4, 75.3, 68.9, 67.9, 67.7, 62.4, 52.9, 50.2, 49.8, 38.8, 36.1, 34.6, 34.5, 31.6, 30.13, 29.0, 28.7, 28.3, 28.0, 26.9, 26.0, 25.6, 25.3, 23.0, 22.6, 21.2, 21.0, 20.8, 20.7, 18.7, 14.1, 11.4.

25:1 (Ratio)

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 11.30 (s, 25H), 8.38 (d, $J = 6.2$ Hz, 24H), 7.58 (d, $J = 30.4$ Hz, 22H), 6.10 (s, 25H), 5.31 (t, $J = 11.0$ Hz, 100H), 4.32 (d, $J = 11.1$ Hz, 100H), 4.18 – 3.92 (m, 100H), 3.85 – 3.76 (m, 100H), 2.90 – 2.65 (m, 75H), 2.53 (d, $J = 5.9$ Hz, 50H), 2.15 (s, 75H), 2.13 (s, 75H), 2.02 (s, 100H), 1.83 (s, 125H), 1.54 – 1.23 (m, 425H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 170.9, 170.7, 170.1, 168.6, 162.3, 156.5, 152.6, 84.1, 83.3, 79.9, 75.2, 68.8, 67.7, 62.3, 52.9, 50.3, 49.9, 45.6, 38.8, 30.1, 29.1, 28.7, 28.2, 28.0, 26.0, 23.0, 21.2, 21.0, 20.9, 8.5.

General Procedures for Deprotection
To a stirring solution of appropriate polymer in THF (0.5 mL) lithium hydroxide (10.0 mg, 3 mL MeOH:H$_2$O 3:1) was added. The reaction was allowed to stir for 5 hrs, neutralized with $^+$H resin. The resin was filtered out, solvent was removed via vacuo and residue was re-dissolved in DCM and TFA (1.0 mL, 1:1 ratio) and allowed to stir for 1 hr. Solvent was removed in vacuo and redissolved in H$_2$O and lipolyze overnight to give a dark pale foamy solid (70-90% yield).

**SA**

$^1$H NMR (400 MHz, D$_2$O) δ 7.79 (s, 1H), 6.11 (dd, $J = 7.9$, 2.9 Hz, 1H), 4.33 (dd, $J = 17.9$, 11.0 Hz, 1H), 4.06 (t, $J = 10.8$ Hz, 1H), 3.81 − 3.65 (m, 3H), 3.55 (dd, $J = 12.4$, 6.7 Hz, 2H), 3.29 (td, $J = 12.1$, 4.4 Hz, 1H), 2.81 (dd, $J = 13.2$, 4.4 Hz, 3H), 2.71 − 2.46 (m, 3H), 2.12 (dd, $J = 9.6$, 4.9 Hz, 1H), 1.96 (s, 3H), 1.92 − 1.74 (m, 4H), 1.70 − 1.58 (m, 1H), 1.48 (dt, $J = 14.4$, 7.3 Hz, 3H), 1.39 (d, $J = 8.5$ Hz, 2H), 1.34 − 1.19 (m, 5H), 1.15 (dd, $J = 15.4$, 7.4 Hz, 3H). $^{13}$C NMR (101 MHz, D$_2$O) δ 175.1, 138.3, 136.2, 123.7, 109.8, 74.6, 71.8, 67.7, 62.4, 51.5, 50.4, 47.5, 46.5, 45.9, 43.9, 41.3, 34.5, 30.1, 29.0, 25.0, 22.1, 18.8. HRMS (ESI) Calculated for C$_{28}$H$_{44}$N$_6$O$_8$S 624.2941, found: 625.3017 (M+1).

**SG**

$^1$H NMR (400 MHz, D$_2$O) δ 7.80 (s, 1H), 6.11 (dd, $J = 7.9$, 2.9 Hz, 1H), 4.35 (d, $J = 21.1$ Hz, 1H), 3.91 (d, $J = 10.5$ Hz, 1H), 3.73 (dt, $J = 18.6$, 6.3 Hz, 3H), 3.59 − 3.46 (m, 4H), 2.93 − 2.90 (m, 1H), 2.84 (d, $J = 5.1$ Hz, 2H), 2.74 − 2.61 (m, 2H), 2.60 − 2.45 (m, 1H), 2.12 (dd, $J = 9.7$, 4.8 Hz, 1H), 1.91 (s, 3H), 1.85 − 1.75 (m, 3H), 1.69 − 1.60 (m, 1H), 1.54 − 1.36 (m, 4H), 1.32 − 1.20 (m, 5H), 1.15 (dd, $J = 16.0$, 6.9 Hz, 3H). $^{13}$C NMR (101 MHz,
\( \text{D}_2\text{O}) \delta 175.1, 138.3, 136.2, 123.7, 109.8, 74.6, 71.8, 67.7, 62.4, 51.5, 50.4, 47.5, 46.5, 45.9, 43.9, 41.3, 34.5, 30.1, 29.0, 25.0, 22.1, 18.8. \) HRMS (ESI) Calculated for \( \text{C}_{28}\text{H}_{44}\text{N}_6\text{O}_8\text{S} \) 666.3159, found: 667.3232 (M+1).

**SA-25** NH\(_2\) Polymer 25:1

\( ^1\text{H NMR} \) (400 MHz, \( \text{D}_2\text{O} \)) \( \delta \) 7.93 (d, \( J = 162.0 \) Hz, 8H), 4.13 (s, 22H), 3.93 – 3.65 (m, 64H), 3.62 – 3.26 (m, 56H), 2.94 – 2.39 (m, 75H), 1.96 (s, 75H), 1.72 (s, 4H), 1.55 – 0.72 (m, 15H).

**SA-10**

\( ^1\text{H NMR} \) (400 MHz, \( \text{D}_2\text{O} \)) \( \delta \) 8.13 (s, 1H), 7.72 (s, 8H), 4.13 (s, 23H), 3.86 – 3.46 (m, 106H), 2.85 (s, 21H), 2.59 (d, \( J = 47.4 \) Hz, 38H), 1.96 (s, 71H), 1.72 (s, 32H), 1.54 – 1.27 (m, 50H), 1.18 (dd, \( J = 14.3, 6.8 \) Hz, 77H).

**SA-5**

\( ^1\text{H NMR} \) (400 MHz, \( \text{D}_2\text{O} \)) \( \delta \) 7.68 (s, 1H), 4.10 (s, 6H), 3.61 (dt, \( J = 96.0, 45.8 \) Hz, 42H), 2.83 – 2.44 (m, 19H), 1.93 (s, 38H), 1.71 (s, 13H), 1.46 – 0.89 (m, 42H).

**SG-25** NHC=\( \text{NHNH}_2\) Polymer 25:1

\( ^1\text{H NMR} \) (400 MHz, \( \text{D}_2\text{O} \)) \( \delta \) 6.97 (s, 1H), 4.09 (s, 18H), 3.68 (dd, \( J = 51.7, 30.9 \) Hz, 55H), 3.30 (s, 9H), 2.65 (d, \( J = 127.4 \) Hz, 22H), 1.93 (s, 41H), 1.69 (s, 23H), 1.40 (s, 18H), 1.13 (s, 89H).

**SG-10**

\( ^1\text{H NMR} \) (400 MHz, \( \text{D}_2\text{O} \)) \( \delta \) 7.73 (s, 1H), 7.00 (s, 1H), 5.28 (s, 1H), 3.65 (d, \( J = 53.2 \) Hz, 9H), 2.60 (s, 4H), 2.22 (d, \( J = 22.6 \) Hz, 2H), 1.87 (s, 6H), 1.41 (s, 1H), 1.17 (s, 5H).

**SG-5**
$^1$H NMR (400 MHz, D$_2$O) δ 7.86 – 7.53 (m, 1H), 7.04 (s, 3H), 4.28 (s, 4H), 4.12 (s, 16H), 3.74 – 3.40 (m, 40H), 2.62 (dd, $J = 87.1, 81.1$ Hz, 15H), 2.25 (d, $J = 20.5$ Hz, 20H), 2.02 – 1.60 (m, 53H), 1.46 – 0.95 (m, 60H).
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## 6 SUPPLEMENTAL INFORMATION

![Chemical Structure]

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