

ABSTRACT

Title of Dissertation: DESIGN, SYNTHESIS, AND ANTIBODY BINDING STUDIES OF HIV-ASSOCIATED CARBOHYDRATE ANTIGENS

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The development of an effective prophylactic human immunodeficiency virus (HIV) vaccine is a critical global health priority. However, to-date, all efforts to design an HIV vaccine have been met with a paucity of success. The design of an effective HIV vaccine is challenging, however, the recent isolation of potent broadly neutralizing antibodies (bNAbs) capable of neutralization across multiple HIV strains suggests that a properly designed HIV immunogen could develop into an effective vaccine. The work presented here describes the synthesis of a series of HIV antigens for functional studies as well as potential immunogens. Five projects are described herein, probing both the role of carbohydrates in defining the epitopes of anti-HIV carbohydrate reactive proteins, as well as approaches to reconstitute these carbohydrate-based epitopes in a synthetic format. In Chapter 2, a series of oligomannose-virus-like particle (VLP) conjugates representing the bNAb 2G12 epitope were synthesized and used to probe the specific binding preferences of 2G12.

The synthetic glycan-protein conjugates were highly antigenic toward 2G12. Chapter 3 describes a general method developed to chemoenzymatically synthesize differentially glycosylated HIV-related glycopeptides in a site-defined manner. The method was used to synthesize a series of glycopeptide antigens of the bNAb PG9. The method developed was applied in Chapter 4 toward the synthesis of multivalently displayed glycopeptides on a VLP scaffold as PG9 immunogens. The multivalent glycopeptide display significantly enhanced the antigenicity compared to monomeric glycopeptides. In Chapter 5 the synthesis of an oligomannose library containing all intermediate oligomannose glycans $\text{Man}_{1-9}\text{GlcNAc}_2$ is described, including the associated glycan-protein conjugates. Preliminary binding studies against a panel of mannose-reactive lectins and anti-HIV bNAbs revealed binding preferences consistent with the given oligomannose display. Similarly, in Chapter 6, a library of highly-branched, bisected Galactose/N-acetylglucosamine terminal glycan-protein conjugates were synthesized. The conjugates were designed to present potential cryptic N-glycan HIV epitopes for use in serum screening studies to identify novel glycan binding proteins. The work presented has yielded important information regarding the reconstitution of HIV glyco-epitopes using synthetic protein conjugates. Together, these insights should facilitate the rational design of immunogens that are better able to mimic the native gp120 epitopes.

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ASSOCIATED CARBOHYDRATE ANTIGENS

by

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Dedication

I dedicate this work to my beautiful wife Amy. You have been so patient during this entire process. Your support helped me to complete this dissertation.

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I would like to sincerely thank my advisor, Dr. Lai-Xi Wang, for his help and support during my dissertation research. I learned so much under his mentorship. I would also like to thank the members of the Wang research group for their scientific mentorship.

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List of Abbreviations

9-Fluorenylmethyl	Fmoc
Acquired immunodeficiency syndrome	AIDS
2-Aminobenzamide	2-AB
2-Aminobenzoic acid	2-AA
Antibodies	Abs
Antibody-dependent cytotoxicity	ADCC
C-C Chemokine receptor type 5	CCR5
Antigen	Ag
Bovine serum albumin	BSA
Broadly neutralizing antibodies	bNAbs
Calnexin	CXN
Calreticulin	CRT
Cluster of differentiation 4	CD4
Complimentarity-determining region	CDR
Copper(I) catalyzed alkyne-azide cycloaddition	CuAAC
C-X-C Chemokine receptor type 4	CXCR4
Dendritic cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin	DC-SIGN
Distortionless enhancement by polarization transfer	DEPT
Electrospray ionization-Mass spectrometry	ESI-MS
Endoplasmic reticulum	ER
Enzyme-linked immunosorbent assay	ELISA
Fourier transform ion cyclotron resonance	FTICR
Germinal center	GC
Heteronuclear single quantum coherence spectroscopy	HSQC
Highly active antiretroviral therapy	HAART
Human immunodeficiency virus	HIV
Inferred germline	iGL
Intrinsic mannose patch	IMP
Keyhole limpet hemocyanin	KLH
Matrix-assisted laser desorption/ionization-Time-of-flight	MALDI-TOF
Membrane-proximal external region	MPER
Natural killer cells	NK
Neutralizing antibodies	NAbs
Nuclear magnetic resonance	NMR
Outer membrane protein complex	OMPC
Ovalbumin	OVA
Polyamidoamine	PAMAM
Polymerase chain reaction	PCR
Potential N-glycosylation site	PNGS
Proton-proton correlation spectroscopy	¹ H- ¹ H-COSY
Selection with modified aptamers	SELMA

Solid-phase peptide synthesis	SPPS
Somatic hypermutations	SHM
Toll-like receptor 4	TLR4
Immunoglobulin G	IgG
Trimer-associated mannose patch	TAMP
Unmutated common ancestor	UCA
Virus-like particles	VLPs
Soybean agglutinin	SBA

Chapter 1: Introduction

1.1 Human Immunodeficiency Virus (HIV)

The Human immunodeficiency virus (HIV) remains one of the most tenacious infectious diseases confronting modern science. The global HIV pandemic has infected over 60 million and claimed the lives of over 25 million people since the discovery that HIV is the cause of acquired immunodeficiency syndrome (AIDS) over 30 years ago. As of 2014, it is estimated that there are 37 million people globally living with HIV infection (Figure 1.1). In addition to the staggering cost of human life, recent antiretroviral therapy options that keep the virus in check, also extend the lives of those infected, leading to enormous health care costs to care for the secondary infections and associated symptoms of the increasing number of people living with HIV.

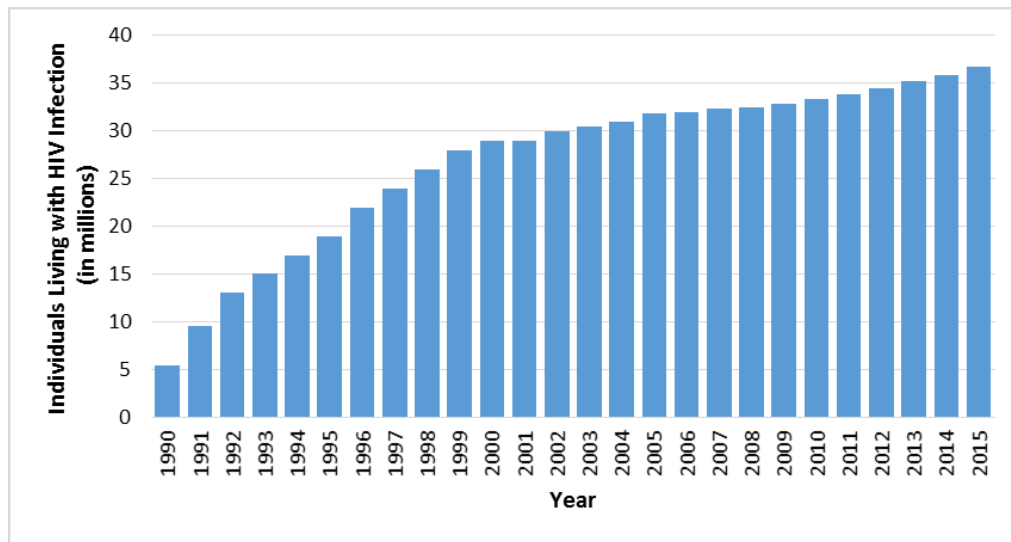


Figure 1.1. The number of individuals living with HIV infection has steadily risen over the past 25 years.¹

There are two broad epidemiological patterns that are observed in the global distribution of HIV-1 infection. In *concentrated* HIV-1 epidemics, occurring in the majority of countries, HIV-1 infection is detected in specific at-risk groups (i.e. sex workers and injection drug users). In contrast to concentrated HIV-1 epidemics, the epidemics in many sub-Saharan countries are *generalized* in that they are self-sustaining in the population (i.e. between married couples and mother to child infections).²

One of the major obstacles in treating HIV is its high degree of genetic variability. There are two known HIV types that are capable of infecting humans HIV-1 and HIV-2. It is widely assumed that HIV-1 arose from a cross-species (i.e. zoonotic) transmission of a chimpanzee virus to humans.³⁻⁴ Similarly, HIV-2 is very closely related to the simian immunodeficiency virus endemic to sooty mangabeys.⁵ HIV-2 infection is highly localized, especially in comparison to globally prevalent HIV-1, infecting individuals in primarily West Africa and India. Additionally, for unknown reasons, HIV-2 is far less pathogenic than HIV-1, characterized by a slower progression to immune deficiency and lower transmission efficiency between individuals.⁶ The limited range and slower progression of HIV-2 has driven research efforts for focus primarily on HIV-1. HIV-1 is characterized by three distinct groups, marked by distinct genomes, labelled M, N, and O, where group M is the most common. Group M is further divided into 9 distinct subtypes called clades (A-D, F-H, J, and K). The genomes between the clades generally differ by 15-20%.⁷ The prevalence of a particular HIV-1 clade is regional, with each geographic region

dominated by one or more clade. Increasingly, recombinant strains are also being detected.

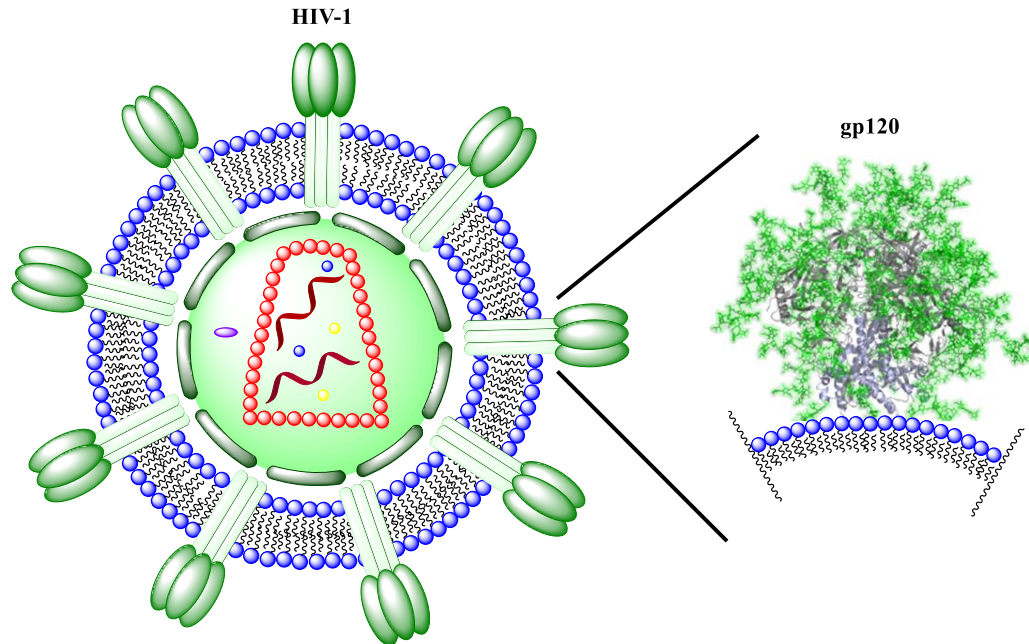
1.2 HIV-1 Infectivity

Transmission of HIV, irrespective of the route of infection, is predicated on two variables, the infectiousness of the “index case,” or the person transmitting the virus, and the idiosyncratic susceptibility of the naïve host.⁸ There is a direct correlation between viral burden in the blood and infectiousness. It has been generally observed that the infectiousness of the index case is greatest in the acute stages (first 3 months after the transmission event), as the immune system is still naïve and can offer no resistance, and in late stage infections, due to the effects of attrition on the immune system such that the infected host can no longer attempt to clear the virus.⁹ Late stage infections are also a co-indication of low CD4 counts, which similarly indicates high viral burden in the host. In contrast to the relative constancy of the factors of infectiousness, there is little uniformity in human susceptibility to HIV-1 infection. The factors impacting HIV-1 susceptibility are diverse and in large part, poorly understood. Resistance to infection appears to reflect a combination of genetic factors, innate resistance, and potentially even acquired resistance. The best characterized example of the impact of genetic factors in conferring resistance to infection is the deletion mutation of the gene that encodes the C-C chemokine receptor type 5 (CCR5). CCR5 is a co-receptor critical for cell entry in the early stages of infection, with majority of HIV-1 clades utilizing CCR5 macrophage-tropic

receptors (see section **1.3**).¹⁰ People that are homozygous for the CCR5 deletion display a profound resistance to HIV-1 infection, presumably due to the inability of HIV to enter the target host cells and initiate the infection cycle. Subsequently the converse is also true: higher expression of CCR5 co-receptors have been associated with higher susceptibility to HIV infection.¹¹ Individuals homozygous for the CXCR4 deletion display a similar resistance to HIV infection.¹² Other mutations conferring resistance to HIV-1 have been reported, however, the extent of protection is lower and the reasons for resistance are largely unclear.¹³

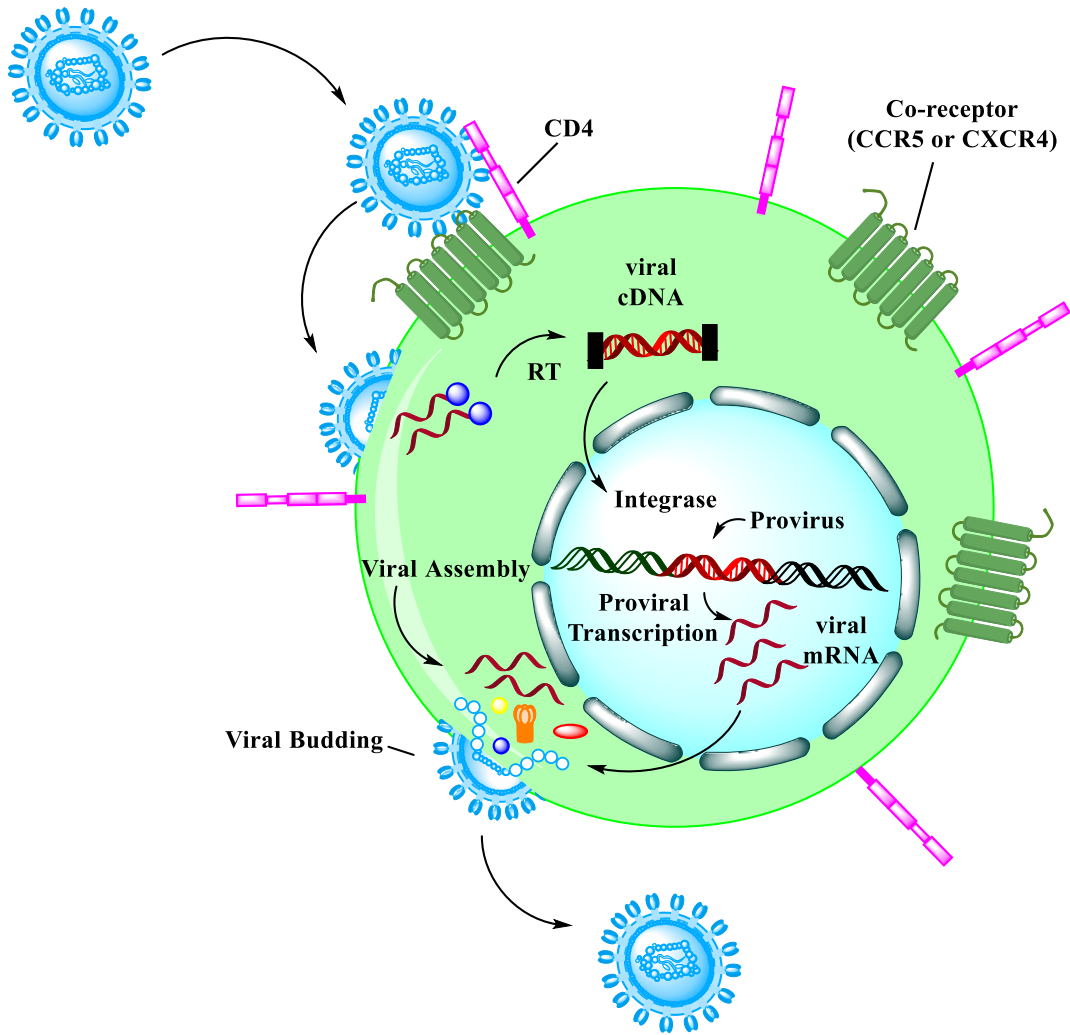
1.3 HIV Structure and Genome

Like all viruses, the structure of HIV is quite simple. HIV-1 relies on the host cell for many of its structural requirements, leading to a limited number of requisite transcribed biological structures. HIV is a single stranded RNA, enveloped lentivirus. Composed of a small number of proteins, the two copies of single stranded RNA are the entire viral genome. The genome only encodes three major polypeptide gene products, Gag, which provides the structural requirements for HIV (i.e. viral matrix, capsid, and the nucleocapsid).¹⁴ Pol contains the information to express viral enzymes (including protease, reverse transcriptase and integrase).¹⁵ The final gene product, Env, forms the viral spike, a transmembrane protein that decorates the outer surface of the virus and is incorporated into the lipid bilayer envelop during viral budding (see Scheme **1.1**).^{14 15}



Scheme 1.1 Cross section of HIV. The main surface antigen and target of vaccine design on HIV-1 is gp120 (gp120 structure adapted with permission from the publisher).¹⁶

The viral spike of HIV, the major antigenic determinant of the virus, is composed of a heterodimer of trimers, a membrane-spanning portion, gp41, and surface protein gp120. The initial Env protein begins as a single pro-protein gp160, and the mature viral spike forms upon furin cleavage. Trimeric gp120 mediates viral entry into host cells.¹⁷



Scheme 1.2 HIV Life Cycle

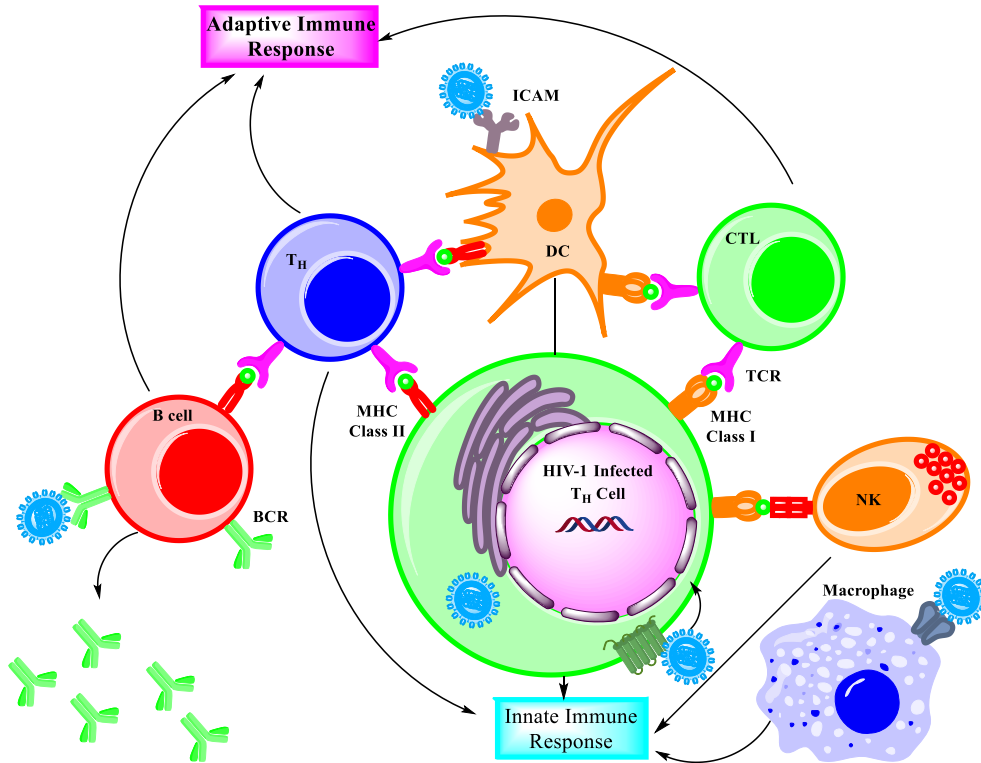
Cluster of differentiation 4 (CD4) is the main receptor for HIV. HIV-1 tropism is further defined by two co-receptors C-C cytokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4).¹⁸⁻²⁰ CD4 is expressed on a number of immunological cells including T lymphocytes, dendritic cells, and macrophages.²¹ All three types of cells subsequently contribute to propagating the HIV-1 infection. Cell entry by the virus is mediated by the interaction of the gp120 subunits binding to CD4 on CD4⁺ T cells. A significant conformational change in the viral spike is

observed during binding.²² The membrane spanning protein, gp41 initiates fusion of the viral and host cell membranes, releasing the viral capsid into the host cell cytoplasm.²³ Within the cytoplasm, the viral reverse transcriptase (RT) produces double stranded DNA from the viral RNA template. After import of the viral DNA into the host cell nucleus, the viral integrase splices the viral DNA into the host-genome creating a provirus.²³ Upon incorporation of the viral DNA into the host cell genome, the host cell's translational machinery is exploited to produce viral proteins, enabling the formation of new virions.²⁴ The newly packaged virions bud from the cell surface, gaining the final envelop, and undergo maturation leading to fully functional viruses.²⁴ Depending on the host conditions, the HIV-1 can persist in memory T cells as a provirus for extended periods, avoiding eradication by anti-retroviral therapy.²⁵⁻²⁶ It has been demonstrated that proviral reservoirs are preferentially derived from quiescent T cells, rather than active T cells undergoing clonal expansion.²⁷

1.4 HIV Transmission and Pathogenesis

HIV-1 transmission occurs primarily via intravenous exposure and via entry through the vaginal or rectal mucosa. The mucosa acts as a barrier stymying HIV-1 access to host cells susceptible to infection. HIV penetrates the mucosal layer by several means, including endocytosis, transcytosis, and migration through epithelial junctures.²⁸ The initial HIV-1 infection is usually established by a single founder virus; this creates an immunological bottleneck, and a successful vaccine would primarily be required to induce an immune response capable of eliminating the initial virus before escape variants arise.²⁹⁻³⁰ Subsequent to the initial exposure, HIV

infection follows a series of phases with defined characteristics. During the eclipse phase of the HIV infection (the first 8-12 days following exposure), there is no detectable viral RNA present in the plasma.³¹ However, immediately following this ostensibly quiescent phase, there is an exponential increase in viral levels, termed the acute phase, with the viral load peak between 21-28 days. The acute phase is characterized by a pronounced adaptive immune response.³¹ CD8⁺ T cells are activated, the humoral immune response ramps up and seroconversion of antibodies occurs. There is also a measurable loss of CD4⁺ T cells during the acute phase.³⁰ The activation of the immune response leads to influenza-like symptoms that assist the virus in spreading to the lymph nodes, rich in CXCR4⁺/CCR5⁺/CD4⁺ T lymphocytes.³² After the virus reaches the lymph nodes, a significant loss of T lymphocytes is observed. At the close of the acute phase, viral levels decline until a viral set point is reached corresponding to the pressure from the rising adaptive immune response. As the infection reaches this final stage, the virus is difficult to detect and only very slowly targets CD4⁺ T lymphocytes, which nearly return to pre-infection levels. During the final stage of infection, HIV-1 continually evades immune surveillance by developing escape variants. The gradual attrition of CD4⁺ T lymphocytes eventually weakens the immune system, leaving the host vulnerable to opportunistic infections, which indicates the progression to AIDS.³²



Scheme 1.3 Innate and Adaptive immune responses to HIV-1 infection.

1.5 Host Immune Response to HIV Infection

Innate resistance in HIV-1 infection has been a considerable challenge to characterize. The degree of resistance to infection during the initial exposure is thought to reflect both the native host microbial flora and the immunologic mucosal defenses. There are two major cellular contributors to the HIV-1 innate immune response, dendritic cells (DCs) and natural killer cells (NK). During the initial transmission event, HIV-1 must pass through the mucosa and breach the robust epithelial wall to contact the target host cells (CD4⁺ T cells).³³ The mucosal barrier is rich with dendritic cells that capture HIV-1. DCs express pattern recognition receptors that act as viral sensors (Scheme 1.3). Viral recognition by DCs leads to the

upregulation of chemokine production, inducing inflammation and recruiting activated HIV-1 target cells, which leads to local amplification of the infection.³⁴ DCs also act as antigen presenting cells, which accelerate the adaptive immune response to HIV-1 infection. In rare individuals that are functionally non-infected due to immune control over viral production (i.e. exposed uninfected individuals, long-term non-progressors, and elite-controllers), DCs have decreased pro-inflammatory cytokine production, and increased antigen-presenting properties.³⁵ These anomalous features in so-called 'elite-controllers' are thought to contribute to the slow or absent progression of the HIV-1 infection. Natural killer cells are also found in the sites of HIV-1 infection and have been found, in conjunction with DCs, to be a critical checkpoint in shaping the adaptive immune response.³⁶ The interactions between NKs and DCs is multidimensional. There is a so-called cross-talk between NKs and DCs with the ratio of NKs/DCs determining the nature of the cross-talk.³⁶ At high NKs concentration, NKs kill immature DCs, concentrating the DC response to just matured DCs, therefore only mature DCs fully present HIV-1 antigens to licensed T cells, leading to a directed immune response to HIV-1, rather than one that is diluted by immature DCs.³⁷ Conversely, at low NK concentrations, DCs activate NKs, priming them to kill HIV-1 infected CD4⁺ T cells.³⁷ In this way, even with a weak T cell response, the infection can be controlled early and confined to the site of the transmission event. In the case of an HIV-1 progressor, the cross-talk between NKs and DCs is thought to be lost, resulting in an amplification of inflammation and progression of the infection at the site due to high levels of immature DCs (not regulated by NK response).³⁷ Moreover, aberrations in antigen presentation by DCs

could lead to ineffective T cell responses, given the failure of NKs to kill immature DCs due to inappropriate NK/DC cross-talk.³⁸ Other smaller contributions by the innate immune system have also been implicated in long-term HIV-1 control, however, the sources of viral suppression are largely unclear.

In addition to the innate response, the adaptive immune system plays a critical role in HIV infection. During an effective immune response to a viral infection, both neutralizing (NAbs) and non-neutralizing Abs (non-NAbs) are simultaneously stimulated. In the context of HIV, a NAb is defined as an Ab that binds specifically to a critical feature of HIV-1 Env, preventing viral transmission to its cellular CD4 receptor. A non-NAb is defined as an Ab that binds specifically to non-critical sites of HIV-1 proteins, which is unable to prevent viral binding to CD4.³⁹ These two classes of Abs work synergistically to contribute to antiviral immunity by four major pathways. First, the NAbs neutralize free virus particle, preventing cellular uptake. Second, both classes of Abs stimulate the complement system leading to complement-mediated lysis of both free virus particles and infected host cells.³⁹ Third, both classes of Abs are critical for opsinization-mediated phagocytosis by both macrophages and other cells. Finally, both NAbs and non-NAbs trigger viral destruction via Ab-dependent cellular cytotoxicity (ADCC).³⁹ HIV-1 is able to escape complement-mediated destruction facilitated by NAbs/non-NAbs, by harnessing an immunotolerance mechanism, the incorporation of a host derived membrane-bound hCD59 receptor during viral budding, which regulates complement response against host cells.⁴⁷

Beyond activation of critical Ab-facilitated antiviral immunity, non-NAbs are unable to prevent viral entry into target cells. However, it has been repeatedly anomalously observed that the major titers of anti-HIV Abs raised upon viral challenge are non-NAbs.⁴⁰ This paradoxical observation obfuscates typical anti-viral immune rationale, given the obvious superiority of NAbs, which can activate extraneous anti-viral pathways while simultaneously blocking viral entry into target cells. The key to this unusual observation lies in the heterogeneity of the Env protein on the surface of HIV capsid. Successful viral entry into a target cell requires intact trimeric gp120. However, it has been well established that gp120 monomers can be shed from the HIV capsid, resulting in a heterogeneous presentation to the immune system.⁴⁰ It was initially thought that only gp120 monomer was sufficiently labile to dissociate, leaving trimeric gp41 stumps with varying degrees of gp120 association. The presumed stability of the gp41 trimer was based on the strength of the association in the pre-fusion state, however, it has been shown that the stability of gp41 trimer association decreases in the post-cleavage state.⁴¹ Studies have shown that upon gp120 monomer shedding, the gp41 stalks are able to dissociate and move freely through the membrane as trimers, dimers, or monomers, with or without an associated gp120 monomer (Figure 1.2). The observed heterogeneity present on the viral envelop has important implications in the development of the humoral immune response. The heterogeneity gives rise to the observed non-NAbs (raised against “junk” Env proteins) as well as the NAbs (raised against intact trimeric Env protein). This observation also explains the source of non-NAbs inability to prevent viral transmission. The non-NAbs are raised against the non-neutralizing face of gp120

(trimer-associated face) which is normally occluded in the trimeric context. These highly antigenic non-neutralizing epitopes are accessible only on monomeric and dimeric forms of gp120, which are non-functional and cannot facilitate host-cell entry. Conversely, NAb target the solvent exposed neutralizing face of gp120 and, depending on the quaternary preferences (requirements) of a given epitope, are largely focused on intact Env proteins (i.e. trimeric gp120). Moreover, the dense glycan shield (discussed at length in section 1.8) on the neutralizing face (solvent exposed) of gp120 obscures access to the conserved peptide epitopes on the gp120 surface. The limited access to peptide epitopes focuses the neutralizing humoral response against largely carbohydrate-based epitopes. During B-cell priming, there is a selection process in which the viral particle is viewed as a single target. Affinity selection of germ-line B-cells mandates the amplification of higher-affinity clones in preference to those of lower affinity. As a corollary, the development of Ab responses toward each of the antigens on the viral capsid surface is contingent on their relative accessibility to Ab binding. Therefore, the high accessibility of the non-functional “junk” forms of Env may explain the ostensible immunodominance.⁴² Finally, the initial NAb response is strain-specific, and the neutralizing epitopes are subsequently lost upon the emergence of viral escape variants.

1.6 Current therapeutic options (HAART Therapy): Advantages and Limitations

Within a few years of the discovery of the virus, the first antiretroviral drug against HIV-1, azidothymidine, was approved for therapeutic use.⁴³ The high rate of HIV-1 mutation to overcome anti-retroviral therapy has led to the rapid development of a host of drug-resistant HIV strains. The development of drug resistance in HIV

has necessitated the coincidental development of a large number of inhibitors. The inhibitors can be divided into six basic classes, categorized according to their mechanism of action, nucleoside-analog reverse transcriptase (RT) inhibitors, non-nucleoside RT inhibitors, integrase inhibitors, protease inhibitors, fusion inhibitors, and entry inhibitors.⁴⁴ HIV is characterized by an error prone reverse transcriptase, leading to a high rate of mutation and the selection of drug-resistant escape mutants.⁴⁵⁻⁴⁶ The selection of escape mutants (referred to as quasi-species) quickly overcomes single therapeutic treatment, and has therefore been replaced by the use of a cocktail of at least three inhibitors, called highly active antiretroviral therapy (HAART).⁴⁷ HAART therapy has revolutionized HIV treatment, making a previously fatal disease treatable. In certain cases, HAART therapy has increased the life-expectancy to near normal levels.⁴⁸

Unfortunately, despite the successes of HAART therapy, it is far from a cure and there are a number of significant limitations. There are a high number of side-effects associated with HAART therapy that, when combined with the complex daily treatment regimens, often lead to patient compliance issues. Discontinuing HAART therapy results in an almost immediate relapse to initial viral load levels, as HAART therapy is unable to eradicate latent viral reservoirs.⁴⁹ Additionally, there are a number of chronic health problems associated with long-term HAART therapy. A major pathology associated with long-term HAART therapy is chronic liver disease. Indeed, end-stage liver disease is a major cause of death in patients receiving long-term HAART therapy.⁴⁹ While HAART-related toxicities are identified as the main cause of liver damage, chronic HIV infection, even when suppressed by HAART, has

been shown to contribute to the pathogenesis of liver fibrosis via activation by gp120 of the Toll-like Receptor 4 (TLR4).⁴⁹ TLR4 is thought to form a co-cluster receptor with CCR5, expanding the repertoire of cellular proteins propagating the gp120 signals. TLR4 activation with CCR5 co-clustering by gp120 signals a cascade event leading to proinflammatory and profibrogenic signals. The ability of HIV to maintain persistent immune activation and the associated accelerated liver fibrosis progression even with the intervention of HAART remains a major obstacle to effective HAART therapy.⁵⁰

1.7 Structure and Function of HIV-1 Env Protein

One of the most effective evasive defenses of HIV-1 is the host-derived lipid envelope that assembled virions obtain during viral budding from the host cell's plasma membrane. During the budding process, various glycolipids can be incorporated into the viral lipid bilayer, and, as they are host-derived, the ability of B cells to respond to these antigens is severely limited due to antigen self-tolerance mechanisms.⁵¹⁻⁵² In addition to masking the virions, some of the host-derived glycolipids can facilitate viral infection. For example, sialylated viral envelope glycolipids (gangliosides) have been shown to interact with cellular lectin receptors, sialic acid-recognizing Ig superfamily lectins-1 (Siglec-1), on DCs, potentially enhancing the infection.⁵³⁻⁵⁴ An interesting exception to the largely protective role of host-derived glycans in HIV immune evasion, is the observation that during transmission, HIV-1 primary virions can carry non-self ABO blood group antigens from the infected individual.⁵⁵⁻⁵⁶ Therefore, viruses from A or B donors show sensitivity to anti-A or anti-B antibodies from donors that were discordant with the

recipients blood group.^{55 56} Despite this minor exception, the host-derived glycans (both glycolipids and glycoproteins) are an extremely effective shield that protects HIV.

Beyond the host-derived glycolipids, the major antigenic determinant on the surface of HIV is the viral spike, composed of the glycoproteins gp41/gp120. Both components of the metastable viral spike in its pre-fusion state are involved in CD4 receptor binding. Upon CD4 binding, the pre-fusion state undergoes a dramatic conformational change that instigates fusion of the viral and host cell membranes. Gp120 has a highly variable surface composed of five variable loops (V1-V5) dispersed among five constant regions.^{19, 57-61} While gp41, responsible for fusion mediation, is far more conserved and simple. Gp41 is composed of only four components, an ectodomain, which interacts with gp120, a membrane proximal external region (MPER), the transmembrane domain, and a cytoplasmic tail.⁶² The conformationally dynamic nature of Env, together with the high level of N-glycosylation (carbohydrates comprise ~50% of the total weight gp120, making it one of the most highly glycosylated proteins to date) has made elucidating the structure of Env a challenge.⁵⁹ Nevertheless, Peter Kwong's group reported the first X-ray crystal structure of CD4-complexed monomeric gp120 nearly 20 years ago.⁵⁷ Subsequently, a number of studies using both X-ray crystallography and cryo-electron microscopy (cryo EM) have generated a number of structures of gp120, both free and receptor bound, and monomeric and trimeric.^{58 59-61, 63-69} The structural studies have yielded unprecedented access to the fine-details of gp120 structure and have helped facilitate informed antigen design. A major innovation in characterizing the fine-details of the

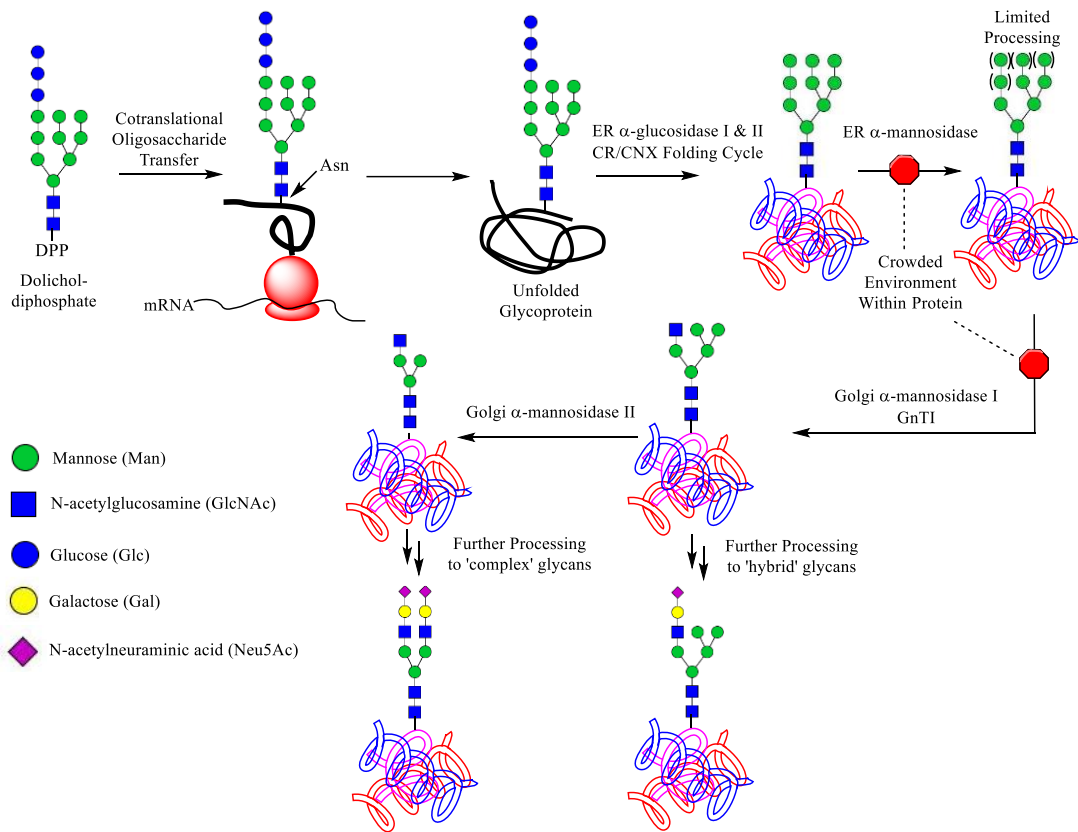
trimeric gp120 structure has been the development of recombinant trimer mimics (BG505 SOSIP.664), with mutations introduced that stabilize the structure while maintaining the native conformation and antigenic properties.⁷⁰⁻⁷¹

The structural studies of monomeric gp120 have revealed a common fold consisting of an N-terminal inner domain and a C-terminal outer domain.⁵⁹ The inner and outer domains are connected by a bridging sheet, composed of four-strands forming an anti-parallel β -sheet.⁵⁹ A number of significant structural changes occur to the viral spike, induced by CD4 receptor binding, a process characterized by a large unfavorable change in entropy.⁷²⁻⁷³ Receptor binding results in the formation and stabilization of the co-receptor binding site within the viral spike, triggering the insertion of the hydrophobic gp41 N-terminal fusion peptide into the target cells lipid membrane. Rearrangement of two gp41 α -helix proteins into a hairpin-like domain within each of the three gp41 subunits creates a six-helical bundle, driving membrane fusion.⁶²

1.8 Env Glycan Shield

Env glycosylation plays a dual role in the viral life cycle. The glycan structures can contribute to disease transmission by interacting with host cell receptors (i.e. lectins on DC-SIGN shuttle the virus to CD4⁺ T lymphocyte rich lymph nodes). The glycan structures also act as a shield to hide the highly antigenic protein surface from immune surveillance. The profound ability of HIV to withstand the host humoral immune system, despite the presence of high-titer anti-HIV antibodies, is largely attributed to an evolving glycan shield, together with the high-rate of mutation, characteristic of a small RNA virus.⁶⁵ The extraordinary level of N-linked

glycosylation, with an average of 93 N-linked glycans on certain trimer strains, makes a formidable immunological defense.⁷⁴ Indeed, studies have shown that only 19% of the gp120 protein surface is solvent-accessible, with only 3% accessible to the Fab domain of an IgG Ab, the rest of the surface is occluded by glycans.⁶⁹ Each N-glycan is encoded by a characteristic tripeptide sequence Asn-X-Ser/Thr (where X can be any amino acid except proline), referred to as the N-glycan sequon.⁷⁵



Scheme 1.4 N-glycan processing. Glycan maturation pathway.

HIV glycosylation is derived from host glycosylation machinery, facilitated by en bloc transfer of a dolicol-linked $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ precursor glycan onto nascent polypeptides by oligosaccharyltransferase as they extrude from the ribosome into the

lumen of the endoplasmic reticulum (ER) (Scheme 1.4). During the calnexin (CXN)/calreticulin (CRT) folding cycle, the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ precursor is trimmed down to $\text{Man}_9\text{GlcNAc}_2$.⁷⁶ Glycan processing within the ER by α -mannosidase ordinarily yields $\text{Man}_5\text{GlcNAc}_2$; however, the high spatial density of glycans on gp120 results in stymied glycan processing, yielding a mix of $\text{Man}_{5-9}\text{GlcNAc}_2$ glycans (Scheme 1.4). For non-congested N-glycosylation sites, further glycan processing occurs in the Golgi via trimming by glycosidases and modifications by glycosyltransferases, resulting in a mixed display of various forms of oligomannose glycans, as well as highly processed complex and hybrid-type glycans.⁷⁷ Because the N-linked glycosylation on Env is host-derived, the glycan shield is largely privileged from Ab recognition through immune tolerance mechanisms. For example, Ab cross-reactivity of viral N-glycans with host N-glycans (self-recognition) would lead to anergy or apoptosis of the glycan reactive B cell.⁷⁸ Although host-glycans are immunologically silent, Abs are capable of recognizing non-self glycosylation (i.e. blood-group antigens).⁷⁹ HIV gp120 glycosylation is heterogeneous and depends on the strain, stage of HIV infection, the type of cell producing the virus,⁷⁷ and the oligomeric form (i.e. monomer versus trimer).⁸⁰ Moreover, there are several unusual features to gp120 glycosylation that enhance the antigenicity and support the notion that the glycan shield is a viable target for vaccine design. HIV glycosylation is generally divided into three distinct regions. First, in the regions of gp120 that are close to the trimeric interface (the interprotomer region), the steric inaccessibility of glycans to glycosidase enzymes at the trimer interface results in an enrichment of high-mannose-type glycans (referred to as the ‘trimer-associated mannose patch’

(TAMP)). The second region is related and referred to as the intrinsic high-mannose patch (IMP). The spatial distribution of potential N-glycosylation (PNG) sites within the IMP is very close, resulting in dense glycosylation, and steric hindrance to glycan processing, yielding mostly unprocessed oligomannose glycans. This site is centered around the N332, N334, N392, and N295 PNG sites.^{81 82} The remaining portions of gp120, outside of the protomer interface and regions of dense glycosylation, have been shown to bear more highly processed complex or hybrid-type glycans. The preponderance of oligomannose glycans on gp120 show a lower structural variation when compared to host-cell glycoproteins. Moreover, the oligomannose glycans tend to form clusters on the Env surface that are immunologically distinctive from host glycosylation. Broadly speaking, the display of the host-derived glycans in a viral protein context can yield a modicum of differentiation and serve as the basis for targeting the Env glycan shield.^{83 84}

1.9 HIV Glycan Role in Immune Escape

The Env glycan shield is dynamic and it is constantly evolving in different strains to avoid recognition by newly produced NABs.⁸⁵ The degree of PNGS plasticity for trimeric spike is limited by the unique glycan structural feature, particularly the IMP, TAMP, and the non-glycosylated CD4-binding site. These features of the Env architecture, including the relative number of PNGSs, are surprisingly maintained in spite of years of viral evolution. Indeed studies have indicated that in certain strains up to 60% of N-linked glycans are highly conserved.⁸⁶ Oligomannose-type glycans are the most highly conserved PNGSs, suggesting an essential structural role of these glycans in the viral life cycle. Significantly, the

escape mutants display non-random mutations in the PNGS, conserving both the spatial distribution, and the specific identity of the glycan, suggesting conservation within the glycan shield and further supporting the notion of targeting the glycan shield for antigen design.⁸⁵ The N332 PNGS, the so-called ‘super-site of vulnerability,’ bearing almost exclusively a Man₉GlcNAc₂ glycan, is known to be vulnerable to migration. Studies following the pathogenesis of a single infection have shown that the virus responds to immunological recognition of this site by shifting the position from N332 to N334. After two years of infection, the virus was shown to have escaped immune recognition again simply by migrating the N334 position back to N332.⁸⁷ The persistence of HIV to maintain a spatially consistent glycan shield is highly significant and suggests a more fundamental structural role of the glycan shield beyond immune evasion.

1.10 Non-carbohydrate-mediated HIV immune evasion

Beyond the ability of HIV to evade immune surveillance by decorating the functional Env in a dense carbohydrate shield, HIV has been shown to exhibit the ability to induce gp120-mediated immunosuppressive responses from dendritic cells.⁸⁸ The immunosuppressive ability is predicated on gp120 mannose moieties via IL-10 expression.⁸⁹ The exact mechanism of IL-10 upregulation by gp120 mannose moieties has not been elucidated.⁹⁰ IL-10 is a pleiotropic cytokine and suppresses immune activation by downregulating the expression of pro-inflammatory cytokines.⁸⁸ This discovery indicates that the glycan shield of HIV promotes pathogenesis, not only by preventing recognition by the immune system, but also by actively participating in host immune-suppression.

Elite controllers (EC) are thought to limit HIV infection in part due to the high antibody-dependent cell-mediated cytotoxicity (ADCC) activity that characterizes EC individuals. ADCC is therefore expected to be an integral feature in controlling HIV infection. It has been recently reported that HIV has developed a mechanism to suppress ADCC-mediated immune clearance via the intervention of the accessory viral protein Nef. Nef is thought to downregulate CD4, affecting the conformation of Env. The epitope required for ADCC-mediated recognition is only exposed in the CD4-bound conformation, therefore in the unbound conformation, the site is unavailable, and HIV is able to evade recognition by NK cells.⁹¹

Subsequent to gp160 cleavage, the heterodimer of homodimers is held intact by non-covalent forces. Consequently, the gp120-gp41 complex can dissociate to varying extents yielding a mixture of functional (i.e. trimeric) and non-functional Env proteins (see Scheme **1.5A**). The non-functional Env protein presents a number of

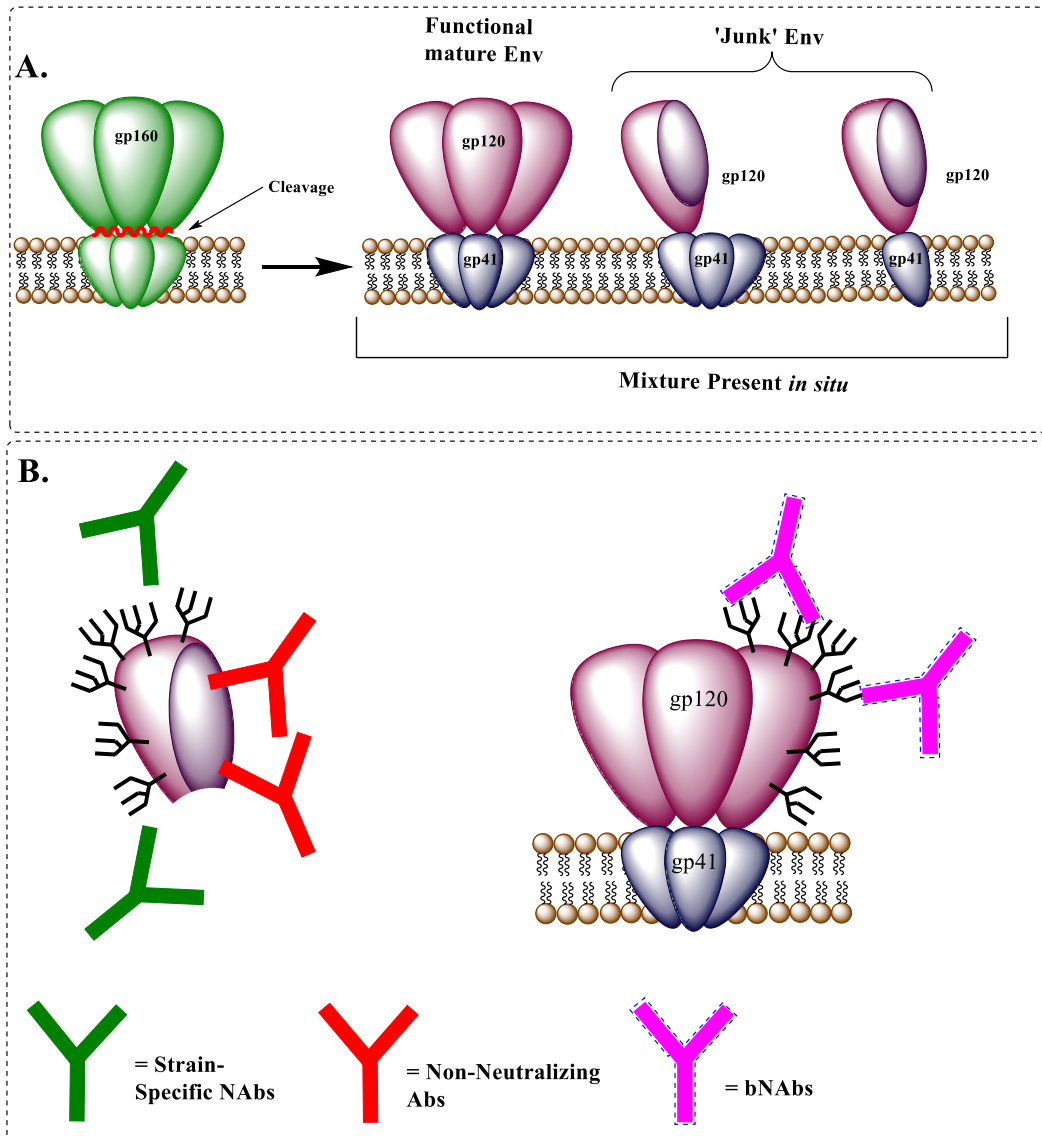


Figure 1.2 **A)** After furin cleavage of gp160, the Env complex exists as a heterogeneous mixture of functional and non-functional proteins within the HIV milieu. **B)** Non-neutralizing and strain-specific Abs do not lead to a sustained inhibitory response. The conserved epitopes of bNAbs ensure neutralization capacity across a wide range of HIV-1 strains.

highly antigenic epitopes on the protomer-associated face that is occluded in functional trimers. The non-neutralizing epitopes raise a strong non-neutralizing immune response, however, as the non-trimeric Env protein cannot mediate cell entry, the response is unable to impede HIV pathogenesis. Similarly, the Env protein displays a limited number of strain-specific neutralizing epitopes capable of eliciting strain-specific NAbs (Scheme **1.5B**). However, as the epitope is strain-specific, the neutralizing capacity is lost upon the emergence of the first escape variants that arise in response to the mounting immune pressure. The ability of broadly neutralizing Abs (bNAbs) to recognize conserved epitopes on Env across strains make them attractive targets for vaccine design (Scheme **1.5B**).

1.11 The Env glycan shield as an immunological target: bNAbs

Glycosylation play a critical dual role in viral pathogenesis. Exposed glycans on the viral surface often interact with host cell receptors and mediate disease transmission. The densely displayed, dynamic, bulky glycans also prevent access by the host immune system to the variable antigenic viral protein surface. The glycan shield of HIV is incredibly effective at mediating the multi-step relationship between host and virus during the first stages of infection, as well as masking the virus after the infection has been established. HIV also uses mutations in the glycan shield to create viral escape variants that continually evade host immune surveillance. Despite the sophisticated application of the glycan shield by HIV to facilitate viral pathogenesis, the discovery of broadly neutralizing Abs (bNAbs) that largely incorporate the glycan shield as a critical component of their neutralizing epitope suggests that the glycan shield is a viable target for vaccine design. The host immune

system is capable of discriminating between host glycans and host-derived viral glycans as the HIV glycan shield has several unusual features that deviate from host-glycosylation patterns. First, the spacing between glycans on gp120 is unusually close in comparison to glycan spacing on mammalian proteins, placing a conformational constraint on the glycans, increasing the rigid presentation.⁹² This rigid presentation is bolstered by a network of intermolecular hydrogen bonds, which stabilizes the glycans structures, partially locking them in a non-self conformation, forming the basis for immunological discrimination.⁶⁵

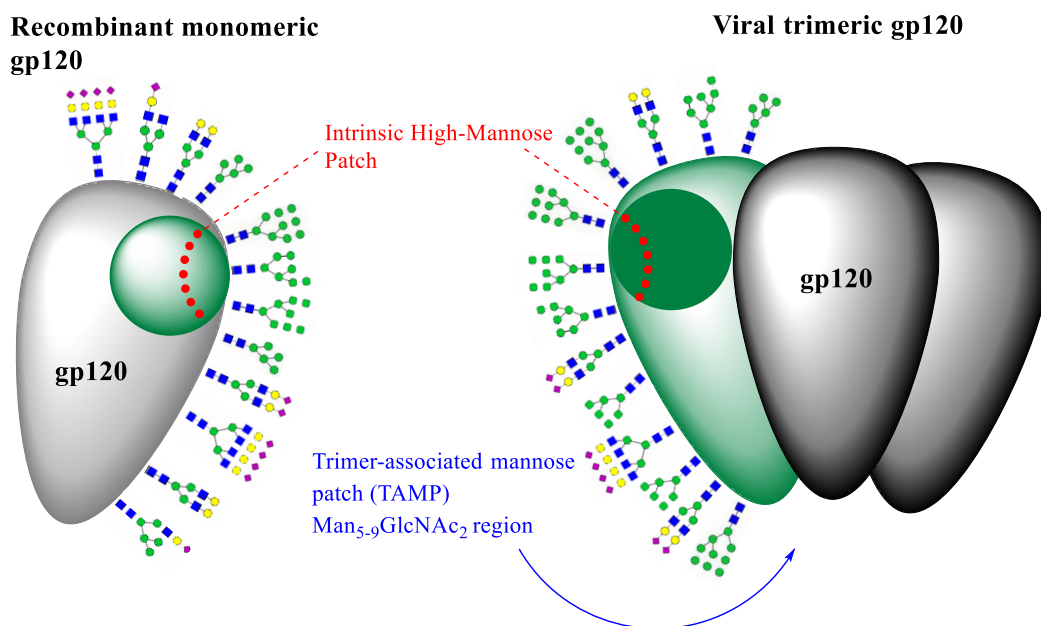


Figure 1.3 In the context of quaternary structure, gp120 glycosylation exhibits lower glycan processing and therefore less complex-type glycan than recombinant monomeric gp120.

Second, the unusually high degree of glycosylation that is characteristic of HIV Env protein stymies glycan processing (supra vide) yielding an abundance of under-

processed oligomannose-type glycans (Scheme **1.6-right**). The underprocessed oligomannose glycans are primarily concentrated in sterically constrained regions, including regions with the highest numbers of PNGSs (i.e. IMP), and the interface between gp120 protomers (i.e. TAMP).⁹³ The under-processed glycans display lower structural variation compared to host cellular glycosylation, a distinction that is recognized by the innate immune system through mannose-binding lectins (MBLs).⁹⁴⁻⁹⁵ Unfortunately, HIV takes advantage of the recognition by dendritic cells (i.e. Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin [DC-SIGN]) via MBLs, which shuttle the virus from the site of infection (usually mucosal tissue) to CD⁺ T cell-rich lymphoid tissue, thereby propagating the infection.^{94, 96} The regional delineation of oligomannose glycans results in clustered arrangements of oligomannose glycans in congested regions, as well as clustered arrangements of highly processed complex-type glycans to regions that are more accessible to host glycosylation machinery, typically the region near the gp120-gp41 interface.¹⁶ The predominance of oligomannose glycans on trimeric Env is limited to a quaternary context. Recombinant gp120, expressed in a variety of cells, results in a significantly different glycosylation profile with considerably higher quantities of complex-type glycans (Scheme **1.6-left**), although oligomannose glycans are still the dominate species.⁹³

Given the paucity of antigenic features on the surface of the HIV, Env is the most promising feature of HIV for targeted antigen design. Traditional vaccination attempts using monomeric gp120 have been largely unsuccessful. Four approaches to vaccination have been tested previously, each incorporating monomeric gp120 with

different priming/booster strategies.^{97 98 99 100} In all cases, the primary humoral immune response was largely limited to non-neutralizing Abs with no discernable protection against HIV. The only vaccine trial that has achieved a modicum of success, the RV144 trial in Thailand, was found to provide ~30% protection from HIV infection.¹⁰⁰ The source of the protection is attributed to a high titer of weakly neutralizing V2-directed Abs. As discussed in section 1.5, the expose trimer associated face of gp120 is highly antigenic and humoral responses raised against this non-neutralizing epitope dominate (Scheme **1.5A**).¹⁰¹ The first NAbs elicited target strain-specific epitopes and are unable to subsequent escape variants (Scheme **1.5B**).¹⁰²⁻¹⁰³ In contrast, several classes of anti-gp120 antibodies targeting diverse strains with strong, neutralizing capacity have been recently described. These so-called broadly neutralizing Abs (bNAbs) target conserved regions accessible on the gp120 trimer, providing clues regarding HIV's vulnerability to neutralization (Scheme **1.5B**). Moreover, an adequate understanding of the bNAb epitope can facilitate synthetic antigen/vaccine design targeting a conserved, neutralizing epitope. A common feature among the vast majority of anti-gp120 bNAbs is the incorporation of the HIV-1 N-glycan shield as a critical component of the individual epitopes.¹⁰⁴ bNAbs that target self-glycans as an epitope component initially gave rise to concerns that many of these bNAbs were self-reactive. Such self-reactivity would render them poor targets for vaccine design as self-reactivity would likely lead to their elimination from the B cell pool, making them extremely difficult to induce.¹⁰⁵ However, subsequent studies have revealed that the induction of bNAbs during HIV-1 infection is not as rare as originally thought, as ~30% of HIV-1 patients develop bNAb-like

humoral responses within two years of infection, with some bNAb responses arising within a year of infection.¹⁰⁶⁻¹⁰⁸ Given the relatively slow development of these bNAbs, the patients that develop such NAbs are not protected by them, rather the viral load and strain-diversity is too high by the time the mature bNAbs appear.¹⁰⁹ The slow rate of development of bNAbs is likely due to their development at the edge of immune tolerance mechanisms, and their reliance on unusual structural features to allow strong antigen binding.

There is a characteristic interdependence between HIV-1 changes and bNAb sequence variation due to parallel evolution pathways.¹¹⁰⁻¹¹¹ This interdependence has far reaching consequences for the affinity maturation ceiling within the germinal center (GC). Normally, the GC reaction is limited due to the Ab affinity ceiling.¹¹²⁻¹¹³ However, the increased pressure on the immune system due to the diversity and swift development of HIV-1 escape variants that can escape immune surveillance via mutation and selection, results in a continually evolving antigen that produces a persistent co-evolving immune response. The persistence of the immune response raises the affinity maturation ceiling, resulting in unusual exotic bNAb structures that can only arise during chronic infection.¹¹⁴⁻¹¹⁵

The majority of bNAbs are poly-reactive and display a modicum of promiscuity that allows them to accommodate a variety of HIV strains. The endogenous promiscuity of bNAbs expands the level of sequence variation tolerated from glycan shifts in the PNGSs that often characterize viral escape variants. Provided the glycan shift is spatially conserved, the bNAb can often accommodate the alteration of the native epitope, albeit with reduced binding affinity.¹¹⁶ The ability

of bNAbs to neutralize not only the founder virus, but the subsequent escape variants as well, make bNAbs an ideal immunological template for vaccine design. Indeed, numerous studies have shown the ability of passive immunization with bNAbs to provide complete protection from viral challenge with SHIV (chimeric simian/human immunodeficiency virus) in non-human primates.¹¹⁷⁻¹²¹ The ability of these bNAbs to provide protection in passive circulation justifies the efforts to develop a rationally designed vaccine to elicit such bNAbs.

To date, there have been ~100 bNAbs isolated and characterized.¹²² The described bNAbs generally target defined, conserved regions of vulnerability on the HIV-1 Env glycoprotein, including the IMP, the CD4 binding site, the membrane-proximal external region (MPER) of gp41, glycopeptide epitopes on the V1/V2 and V3 variable loops, and the bridging region at the gp120-gp41 interface (Scheme 1.4).¹²³⁻¹²⁴ These bNAbs primarily incorporate N-linked glycans as a component of their neutralizing epitopes, proving that the dense HIV-1 glycan shield contains immunological vulnerabilities that can be used as a basis for discrimination by the host humoral immune response. Carbohydrate-reactive bNAbs primarily target either the N332 glycan (and the associated V3 loop), the N160 glycan (and the associated V1/V2 loop), or the glycans associated with the gp120-gp41 interface.¹²⁵ The first carbohydrate reactive bNAb described, 2G12, recognizes an exclusively carbohydrate epitope composed of a cluster of oligomannose glycans within the IMP, including N295, N332, N339, and N392.^{84, 126-127} 2G12 achieves remarkably high-binding to a carbohydrate epitope through an unusual domain-exchanged structure affording high avidity binding via the formation of a multivalent surface.¹²⁸ Subsequent

carbohydrate-reactive bNAbs have not shared the unusual domain-exchange structure characteristic of 2G12, but in all cases have a typical Y-type Ab structure, with integrated epitopes consisting of both protein and glycan components.¹²⁹ Compared to 2G12, newer classes of carbohydrate-reactive bNAbs have exhibited higher breadths and potencies. For example, while 2G12 has been shown to neutralize 32% of viral strains with a median IC₅₀ of 2.38 µg/mL, it has been demonstrated that PGT128 can neutralize 72% of HIV-1 strains with a median IC₅₀ of 0.02 µg/mL.^{92, 130-131} There are two major classes of bNAbs that target an integrated epitope, the PGT-series and the PG-series. Members of the PGT-series target several regions of the glycan shield, including the gp120-gp41 interface (PGT151), targeting complex-type glycans on gp41 and a protein epitope on gp120,¹³² and the glycan at N160 on the V1/V2 loop (PGT145) (Scheme 1.7).¹³¹ Other members of the PGT-series target the V3 loop, and specifically incorporate a high-mannose glycan at N332 as a component of the epitope (Scheme 1.7). The supersite of vulnerability N332, although incorporated in multiple bNAb epitopes (2G12, PGT series) has been shown to be non-critical as many of these bNAbs display a modicum of promiscuity allowing them to bind to other glycans in the absence of N332 and thereby recover the epitope.¹¹⁶ The other major family, the PG-series, targets the V1/V2 loop at the trimer apex and specifically makes contacts with glycans at the N160 and N156/173 positions.¹³³

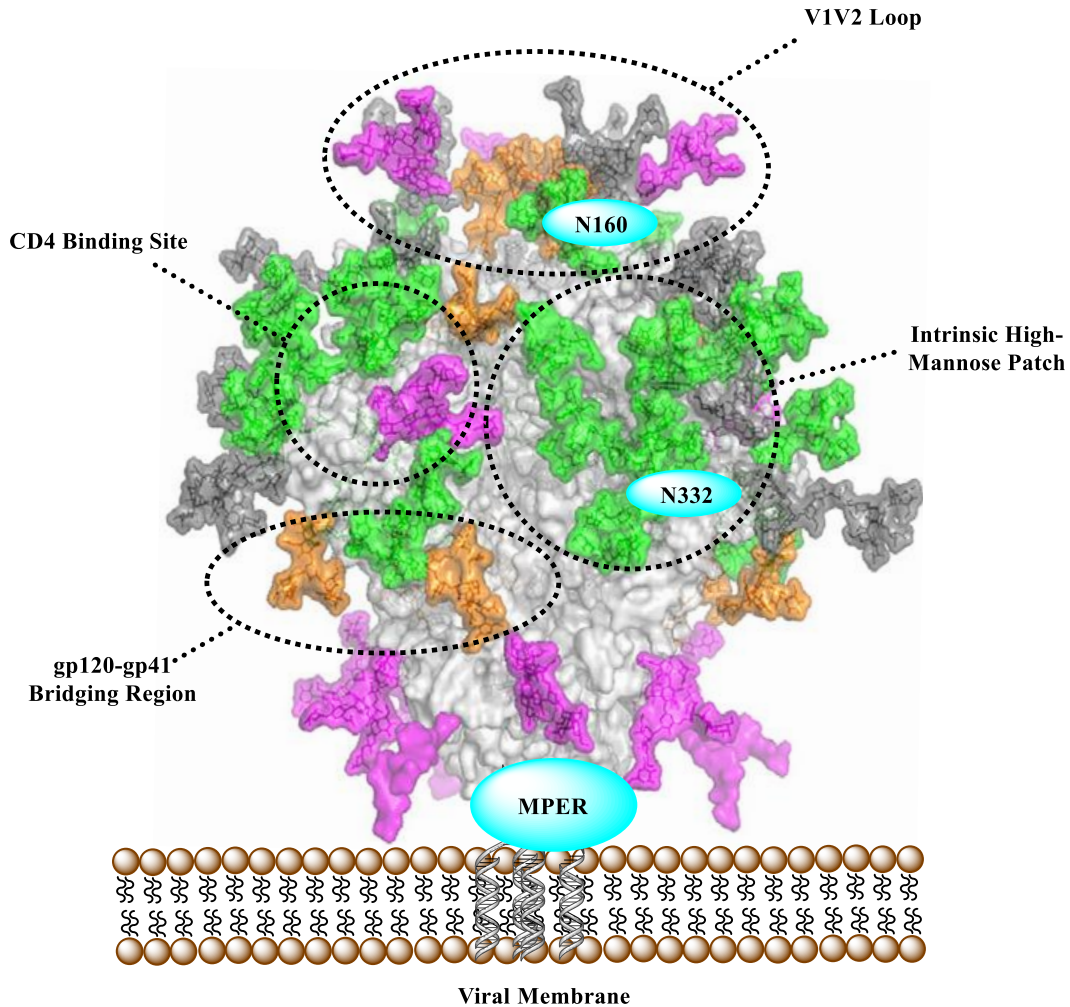


Figure 1.4 bNAb epitopes are scattered across the silent face of gp120, and almost exclusively incorporate N-glycans as an epitope component (gp120 structure adapted with permission from the publisher).¹⁶

There are five major structural characteristics that define the majority of bNAbs that deviate from more traditional IgG Abs, including extended heavy chain third complementarity-determining regions (CDRH3), unusual post-translational modifications (i.e. tyrosine sulfation), domain-exchange (2G12 only), auto-/poly-reactivity, and extensive somatic hypermutations. For bNAbs that target an integrated

epitope consisting of N-glycans and the underlying protein sequence, long, extended CDRH3 regions are common, usually containing charged sequences within the CDRH3. For example, while the average length of a typical human B cell CDRH3 is 14 residues,¹³⁴ PG9 and PG16 both contain 30 residues, while PGT145 contains 33 residues.¹³⁵⁻¹³⁷ The extended CDRH3 facilitates penetration through the dense glycan shield that extends ~20 Å from the protein surface allowing contact with the otherwise occluded protein surface. The long CDRH3 in certain cases even provides sufficient length to accommodate secondary structure within the loop, allowing this feature of the paratope to “fit” the epitope more closely. An example of this is the “hammerhead” shape of the PG9/PG16 CDRH3, permitting penetration of the glycan shield and extensive contact with glycan and protein targets. The high incidence of unusual PTMs often works in conjunction with other characteristic structural features to enhance binding affinity. For example, tyrosine sulfation has been reported on the CDRH3 of certain bNAbs, and is known to interact with cationic residues within the protein component of the epitope.^{133, 135, 138} A subset of bNAbs have the remarkable characteristic of polyreactivity, in which a single Ab can recognize more than one antigen.¹³⁹⁻¹⁴⁰ Many of these bNAbs recognize two specific binding sites on the Env surface, a high-affinity anti-HIV-1 binding site and a low-affinity self-antigen (i.e. host-derived N-glycan) that is present on the Env surface. The polyreactivity of these bNAbs most likely mediates this bivalent heteroligation, overcoming the low affinity of the second binding site and increasing the apparent affinity of the bNAb to HIV-1. Polyreactivity, normally deselected from typical B cell lines, plays a critical role in overcoming the HIV-1 defenses, which is most clearly evident in the prevalence of

specific memory B cells that are polyreactive in HIV-1 infected individuals. Indeed, 75% of memory B cells in HIV-1 positive individuals are polyreactive, compared to just 5% of the B cells in non-infected individuals.¹⁴⁰ The exotic and unusual nature of bNAbs necessitates extensive rounds of mutations within the GC, requiring extensive somatic hypermutations (SHM) during affinity maturation.¹⁴¹⁻¹⁴³ While non-HIV-1 Abs typically undergo 5-15% changes in the heavy chain during affinity maturation, some bNAbs accumulate up to 36% of changes.¹⁴¹ The majority of SHMs are limited to the CDR regions, however, certain HIV-1 targeting bNAbs have also undergone SHMs within the conserved framework region (FWR) of the Ab as well.¹⁴⁴

1.12 HIV-1 Vaccine Design

There have been a number of attempts to vaccinate against HIV infection, largely following four major vaccination strategies, protein subunit vaccines, recombinant adenovirus vectors, canarypox vector prime, followed by a protein subunit boost, and a DNA prime followed by a recombinant adenovirus vector boost.¹⁴⁵ Early attempts using recombinant gp120 mixed with the adjuvant alum (AIDSVAX), elicited only non-neutralizing Abs, and no protection was observed.⁹⁷⁻⁹⁸ Two vaccine attempts, the Step trial and the Phambili trial, investigated three types of recombinant, attenuated adenovirus vectors expressing three HIV-1 related proteins (i.e. Gag, Pol, and Nef). Both studies observed a stimulated CD8⁺ T cell response to the viral vectors, however, the vaccination yielded no protective benefits, and the trials were cut short due to concerns that HIV-1 infection risk was actually enhanced post-vaccination.^{99, 146-148} The reasons for the enhanced susceptibility to HIV-1 infection post-vaccination are not well understood. The most successful trial

to-date was the RV144 trial in Thailand (so-called ‘Thai trial’), which incorporated a canarypox viral vector that expressed gp120, Gag, and Pol. The recombinant viral vector was used as the prime, which was followed by two sequential booster injections of the previously reported AIDSVAX preparation.¹⁰⁰ Significantly, the combined vaccination strategy provided a small but noteworthy protective effect, with 30% fewer infections among vaccinated patients.¹⁰⁰ The source of the protective effect is somewhat unclear, but it is thought to be due in part to the elicitation of V2-directed NAbs. Most recently, a combined DNA/viral vector vaccination strategy has been applied, incorporating a DNA prime, composed of plasmids encoding HIV-related proteins from several strains, and an adenovirus vector boost.¹⁴⁹ This trial (referred to as the HVTN 505 trial) resulted in no observable protective effects, and was also cut short due to concerns over increased risk of infection among the vaccinated.¹⁴⁹

A recent alternative vaccination approach is the design of recombinant trimer mimics. The design of a stable, soluble gp140 trimer eschews the challenges associated with vaccination with gp120 monomer by occlusion of the immunodominant non-neutralizing epitopes, solely focusing presentation of the neutralizing epitopes as immunological targets. A number of Env constructs have been reported, however, the structures correspond to uncleaved trimers (i.e. gp160), which are not representative of the native trimeric gp120 conformation.⁶⁴ Recently, a new recombinant trimeric gp140 Env protein has been developed that incorporates subtle modifications that generate a stable and soluble trimer mimic. These ‘SOSIP’ Env proteins introduce a single disulfide bond between the ectodomain of a truncated

(removal of the MPER and transmembrane domain) gp41 and gp120 (SOS) as well as a point mutation (isoleucine to proline-IP).⁷⁰ The development of these SOSIP trimers has led to a number of strain-specific variants, many of which have demonstrated native-like structural properties and antigenicity.^{71, 150} Immunization studies using these soluble, native-like Env SOSIP trimers have successfully elicited Abs against autologous tier 2 virus,¹⁵¹ however it was later demonstrated in a mouse study that there is an immunodominant non-neutralizing epitope near the trimer base that limits the utility of these gp140 constructs as immunogens.¹⁵²

Despite the extensive progress that has been made by the development of soluble, native-like trimers, there is a significant possibility that a heavily glycosylated recombinant protein will not be sufficiently immunogenic to induce the type of strong, sustained Ab response that will be required by an effective vaccine. An optimized vaccine will likely require repetitive presentation of the antigen, perhaps through particulate presentation, to initiate a sustained immune response. Particulate presentation of epitopes improves Ab responses through a variety of mechanisms. First, repeated presentation of the epitope allows the immunogen to cross-link B cell receptors, improving B cell activation.¹⁵³⁻¹⁵⁴ Second, circulating IgMs are capable of binding to repetitively displayed antigens via high-avidity interactions, simultaneously activating the complement system and lowering the threshold necessary for B cell receptor activation.¹⁵³ Third, the affinity of naïve B cell receptors for bNAb epitopes is likely to be low; a repetitive array of low-affinity epitopes increases avidity, raising the likelihood that the right B cell will be activated.¹⁵⁵ This assertion is supported by the observation that nanoparticle-displayed antigens

frequently induce Abs against additional epitopes compared to antigen alone, increasing the neutralization breadth.¹⁵⁶⁻¹⁵⁹ Fourth, host self-antigens usually don't contain highly repetitive antigenic structures and recurring epitopes are regarded as non-self antigens by B cells.¹⁶⁰ The use of repetitive self-derived epitopes may also rescue autoreactive B cell progenitors that would otherwise be deleted during Ab development expanding the potential repertoire of germline B cells available. In such a case, the autoreactive B cell progenitors would be tuned to recognize self-antigens only in the context of highly repetitive epitopes.¹⁶⁰ Finally, studies have suggested that highly repetitive and recurring epitopes are capable of inducing long-lived Abs.^{154, 157} This is particularly relevant for strategies that are alternatives to Env based vaccines, as both monomeric and trimeric gp120 based vaccines usually induce short-lived Ab responses.^{100, 151, 161}

1.13 Challenges of Carbohydrate-based Vaccine Design

Protein-protein and protein-carbohydrate interactions are both mediated by a high degree of hydrogen bonding, hydrophobic interactions, van der Waals forces, and electrostatic interactions. Similarly, Ab binding to both carbohydrates and proteins results in a favorable enthalpy contribution to the free energy of the interaction.¹⁶² However, for carbohydrates, the favorable enthalpy contribution is significantly offset due to an entropic penalty incurred during binding. The entropic penalty arises from the disruption of the solvation shell and subsequent solvent rearrangement upon binding, together with the loss of oligosaccharide conformational flexibility upon complex formation.^{163 164} The entropic penalty incurred during protein-oligosaccharide binding can significantly abrogate Ab affinity for an

oligosaccharide epitope. The low-affinity binding interactions necessitate the reliance of glycan-protein interactions on avidity effects enabled through multivalent interactions.

In contrast to protein/peptide antigens, purely carbohydrate antigens are thymus-independent type 2 (TI-2) antigens and therefore activate B cell responses in the absence of T cell assistance.^{165 166} B cell receptor cross-linking through binding repetitive motifs (i.e. repetitive carbohydrate antigens) activates antigen specific B cells independent of CD4⁺ helper T cells.¹⁶⁷ The failure to recruit the full complement of the immune response necessitates several limitations in the efficacy of the response. First, largely low-affinity IgM antibodies are produced by the B cells upon stimulation by purely carbohydrate antigens.¹⁶⁸ These low affinity IgM antibodies lack both affinity maturation and isotype switching, severely hampering the specificity achieved by these Abs for the particular antigen.¹⁶⁹ The generalized immune response lacks the necessary features to distinguish the antigens, resulting in a short-lived, weak immune response. Finally, Ab responses cannot even be induced in newborns and children up to 2 years old when vaccinated with a purely carbohydrate antigen.¹⁷⁰ In contrast, proteins typically generate a CD4⁺ helper T cell-dependent response, resulting in the generation of high affinity, class-switched Abs.^{171 172} In order to overcome the short-lived immune response typical of the vast majority of carbohydrate antigens, and generate long-lived antibody-mediated protection, exogenous CD4⁺ helper T cell epitopes are typically required in the vaccine design, usually in the form of a carrier protein.^{171 173} An additional challenge to targeting carbohydrate epitopes for vaccine design is the inherent heterogeneity

endemic to glycoproteins, such heterogeneity can dilute the efficacy of glycan specific Ab responses. Therefore, the multivalent display of *defined* carbohydrate epitopes on a protein carrier is necessary for successful carbohydrate-based vaccines.

1.14 Antibody-guided approach to vaccine design

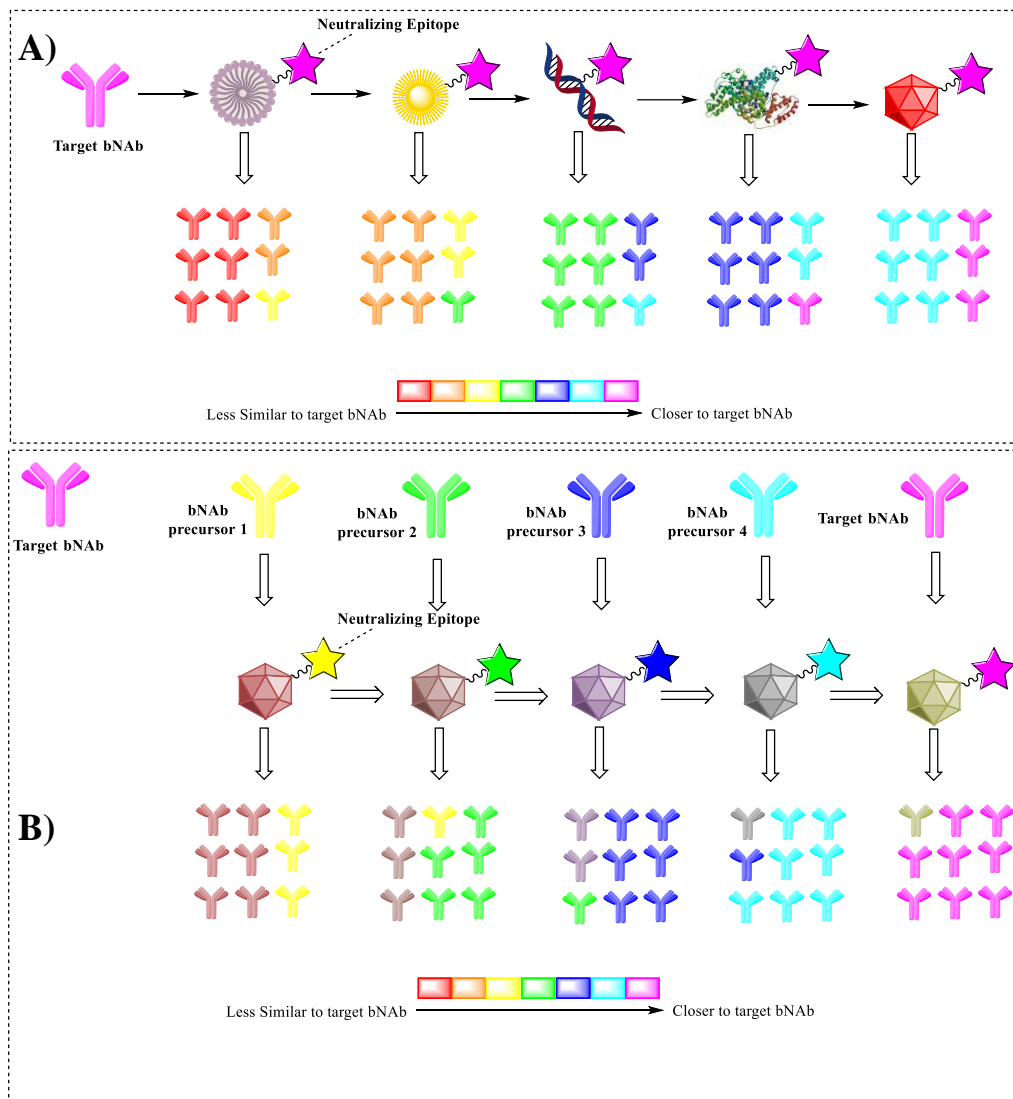
The most promising strategy towards a rationally designed HIV-1 immunogen is the so-called ‘reverse vaccine engineering’ strategy, also known as the ‘antibody-guided’ strategy. The reverse vaccine engineering strategy uses known bNAbs to define neutralizing epitopes that can be used as targets to induce a similarly potent and broad B cell response.^{174 175} The antibody-guided approach has been applied to design several minimal, putative, carbohydrate-based mimics of bNAb epitopes. The epitope of 2G12 for example, has been the target of intensive effort. 2G12 recognizes a cluster of α 1,2-linked mannose within the IMP, many groups have designed chemically synthesized multivalent oligomannose antigens on a variety of scaffolds, including proteins, peptides, dendrimers, carbohydrates, DNA, steroid, and gold nanoparticles. While no construct to date has been capable of stimulating HIV neutralizing activity post-vaccination, many of these constructs have reported excellent antigenicity results, indicating a close similarity to the native epitope. A number of glycopeptide-based mimics of the PG9/PG16 epitope have also been reported, exhibiting excellent antigenicity.

1.15 Long-term Vaccination Strategies

There is much speculation regarding the ability of the immune system to stimulate de novo bNAbs from a single properly designed immunogen. Evidence

suggests that the target bNAbs may need to be elicited via stepwise vaccination using multiple immunogens. Initial strategies were centered around targeting the inferred germ-line (iGL) precursors of the target bNAbs. However, it has been consistently demonstrated that iGL precursors have little to no affinity for HIV-1 Env antigens and totally lack neutralizing activity.¹⁷⁶⁻¹⁷⁸ This observation suggests that activation of specific germline bNAb-expressing B cells will require specific antigens that are designed to target an epitope that is considerably different from the mature target epitope (i.e. a germline prime). The stochastically dynamic model of the immune response to a rapidly evolving viral target makes rational design of a vaccination strategy extremely difficult. One proposed method that has been supported by in vivo and in silico studies is the combination of different antigen variants containing the same neutralizing epitope (Scheme **1.5A**). The major benefit of this method is the induction of cross-reactive Abs that are focused on the same shared epitope.^{179 180-181} The initial antigen primes the B cell responses to the desired epitope, while the other epitope scaffold conjugates, displaying the same epitope as the prime, selectively stimulate memory B cells that are specific for the shared antigenic determinant. This strategy narrows the response to the antigenic determinant alone, regardless of the epitope scaffold. Another design, proposed by Hayes et al., referred to as the B cell lineage immunogen design, targets the unmutated common ancestors (UCAs) of the target bNAb.^{182 124 183} An immunogen that can stimulate the precursor bNAbs may lead the immune system to elicit the target bNAb (see Scheme **1.5B**). A major challenge associated with this strategy is the observed lack of binding between bNAb UCAs and the antigen of the mature bNAb.^{144, 184-185} Such a strategy therefore

requires the design of sequential serial immunogens with decreasing epitope modifications that can target the germline bNABs, the mature bNAB, as well all intermediate species.¹⁸⁶ This method has been tested in the past with limited success.¹⁸⁷ Recently, this strategy has been applied to generate VRC01-like bNABs,¹⁸⁹ as well as the V3-loop-directed PGT121.¹⁹⁰⁻¹⁹¹ Both studies relied on a reductionist sequential immunization strategy in which immunogens were strategically mutated to remove selected sites, generating a panel of potential vaccine primes that were tested for binding against germline bNABs.



Scheme 1.5 A) Sequential immunization strategy: same neutralizing epitope presented on different scaffolds may promote affinity maturation of cross-reactive antibodies that focus on the shared epitope. B) B-cell lineage immunogen design.

The concentration of the antigen(s) at the time vaccination is another major determinant in the efficacy of a vaccine candidate. The dose of the antigen needs to be sufficiently high, but not too high. If an Ag dose is too low, B cells within the GC are not likely to be selected, and the GC will collapse without the production of Abs.¹⁹² If the Ag dose is too high the B cell selection is very rapid, however, due to the lack of competition between B cells, the GC is overwhelmed by low-affinity clones, rather than the highly specific clones that are desirable.¹⁹²

1.16 Conclusion

The significant global health impact of the HIV-1 pandemic necessitates the development of a means to eradicate the virus. A prophylactic vaccine capable of inducing even modest titers of NAbs could be sufficient to provide circulating protection from HIV infection. Despite the tremendous advances in our understanding of HIV-1 structure and function, the development of an effective protective HIV-1 vaccine continues to present a significant challenge. The series of failed vaccination studies do not define the current prognosis. The discovery of bNAbs that are potent at concentrations achievable by vaccination regimens, provides a basis for optimism that a properly designed immunogen(s) combined with a proper vaccine regimen could lead to a functional vaccine. The exotic structures that characterize many of these bNAbs are a result of extensive somatic

hypermutations, and are, most likely, a result of the length of time required for bNAb development, rather than a strict requirement for substantial mutations. Recent reports highlighting efforts to ‘walk’ B cell development from distant germline bNAb precursors to bNAb-like phenotypes via sequential immunization demonstrate that a direct path to eliciting bNAbs is possible without long-term mutations. A successful vaccine will likely need to overcome the self-tolerance mechanisms to host-derived glycans in the glycan shield via non-self presentation of the epitopes. One of the most promising methods to overcome self-tolerance mechanisms, without disrupting the careful immunological balance, is the repetitive presentation of the epitopes on antigenic scaffolds. The repetitive presentation of host-derived antigens is viewed as foreign by the immune system, thus potentially overcoming the ability of HIV to hide from immune recognition. One of the most pressing needs for the development of an effective anti-HIV vaccine is the design of antigens that present the native-like epitope outside of the context of the greater gp120 structure to focus the development of a neutralizing response against neutralizing epitopes only. Such a strategy will avoid dilution of the immune response by co-presentation of non-neutralizing epitopes. The focus of the work presented here is the development of synthetic antigens intended to mimic the epitopes of host-generated anti-gp120 proteins. The construction of these complex biomolecules relies extensively on the tools of chemical biology.

The work presented here seeks to address several of these pressing needs in HIV research, including optimized immunogen design and a means to identify carbohydrate-reactive proteins that could be used as a template for downstream

vaccine design. In chapter 2, I developed a series of immunogens targeting 2G12-like bNAbs that will probe the exact glycan preference of 2G12 towards developing an optimized 2G12 vaccine. In chapter three, I describe the development of a general chemoenzymatic method to facilitate site-selective addition of two unique N-glycans on a polypeptide. The method was used to construct a series of differentially glycosylated glycopeptides that serve as minimal putative mimics of the V1/V2 loop of HIV. In chapter 4, the method developed in chapter 3 was applied to design and construct a series of glycopeptide-VLP protein conjugates that present the bNAb PG9 epitope in a multivalent format, thereby enhancing the antigenicity. The second half of my dissertation describes the synthesis of two novel N-glycan libraries that can be applied to serum screening of HIV⁺ serum. In chapter 5, the construction of a chemoenzymatically derived oligomannose N-glycan library is presented. The individual oligomannose glycans were conjugated to a protein carrier and tested against a panel of mannose-reactive proteins in a microarray format. Finally, in chapter 7, the design and synthesis of a naturally-derived N-glycan library containing highly branched, bisected tri-/tetra-/penta-antennary glycans is described. The presentation of these unusual Gal/GlcNAc terminal glycans on a carrier protein can be used to probe HIV⁺ serum for proteins that recognize clustered, highly branched N-glycans that may be present on the HIV virion itself, identifying potentially novel immunological targets.

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Chapter 2: 2G12-Epitope Vaccine Design: Synthesis of Triazole-Linked Oligomannose-Bacteriophage (Q β) Conjugates to Probe the Specific Contribution of the Chitobiose Core on 2G12 Vaccine Serum Recognition of gp120.

2.1 Introduction

Despite over thirty years of intensive HIV research, an effective prophylactic vaccine, widely considered the best hope to combat the global HIV-1 pandemic, continues to evade all design efforts. To date no vaccine that induces a sustained neutralizing antibody (NAb) response across multiple HIV-1 strains has been effectively designed.¹⁻⁴ The sole target for vaccine design, the unstable heterodimeric trimer consisting of the homotrimeric glycoproteins gp120 and gp41 composing the viral envelop spike (Env)—required for viral infectivity, is extremely effective at masking conserved regions of the immunological target. The viral spike is a very dynamic structure with conserved epitopes recessed, transiently exposed, or else occluded by both an extremely dense glycan shield and immunodominant loops that are characterized by a high level of sequence variability.⁵⁻⁸ These formidable defenses contribute to the difficulties in the elicitation of neutralizing Abs to the masked, conserved epitopes located on the Env protein. Traditional vaccine approaches incorporating recombinantly expressed monomeric gp120 have been consistently unsuccessful, despite extensive efforts⁹⁻¹⁰⁻¹² in part due to the formidable evasive defenses endogenous to HIV Env protein. A major break-through in HIV

vaccine design arose from the observation that certain HIV-infected individuals remained asymptomatic in the absence of treatment. Subsequent investigations revealed that certain individuals produce rare and unusual Abs that effectively neutralize a broad range of HIV-1 isolates.¹³ These broadly neutralizing Abs (bNAbs) achieve this remarkable ability by targeting conserved epitopes within the glycan shield itself. The discovery of these bNAbs suggests that a cross-reactive NAb response against HIV-1 is possible. Therefore, the very defenses that HIV employs for immune evasion (i.e. the glycan shield) form the basis for immunological neutralization. The discovery of monoclonal bNAbs provides exciting tools for facilitating informed vaccine design, as elucidation of their neutralizing epitopes adumbrates conserved elements within the immunological “armor” of Env protein that can be exploited to generate a more effective vaccine that targets neutralizing HIV-1 epitopes.

Within the past five years there has been an explosion in the number of bNAbs discovered and characterized.^{14-21 22} The vast majority of the described bNAbs incorporate components of the gp120 glycan shield as a critical feature of their neutralizing epitope. An interesting corollary to this observation is that, though highly variable, the pattern and spatial distribution of glycosylation on gp120 is highly conserved.²³⁻²⁵

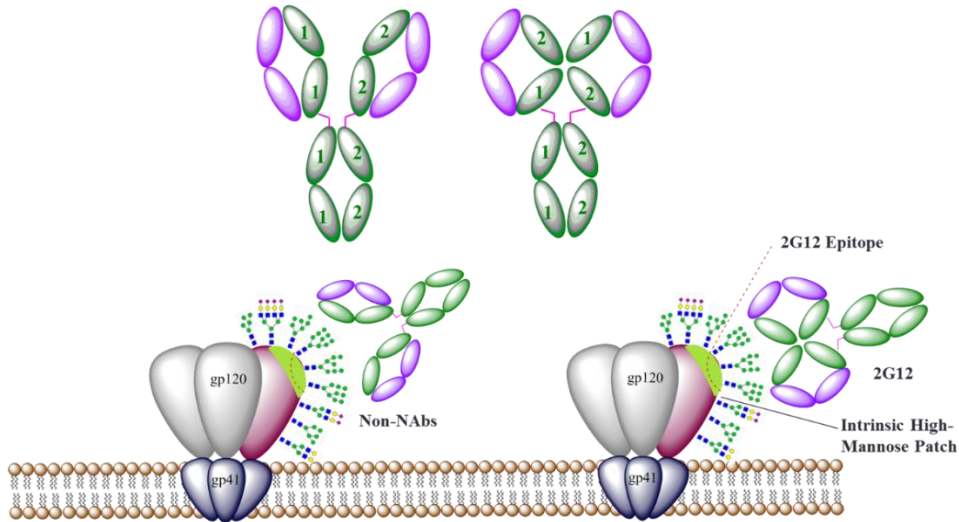


Figure 2.1 Structural differences between 2G12 and typical IgG Abs.

The prototypical carbohydrate-reactive anti-HIV-1 bNAb is 2G12. 2G12 was the second bNAb to be described²⁶ and the first to target HIV glycans.²⁶ 2G12 targets the gp120 “glycan shield” binding to a cluster of oligomannose glycans located within the intrinsic high-mannose patch, specifically, N-linked glycans on N295, N332, N339, and N392 (Figure 2.1).^{27 28 29}

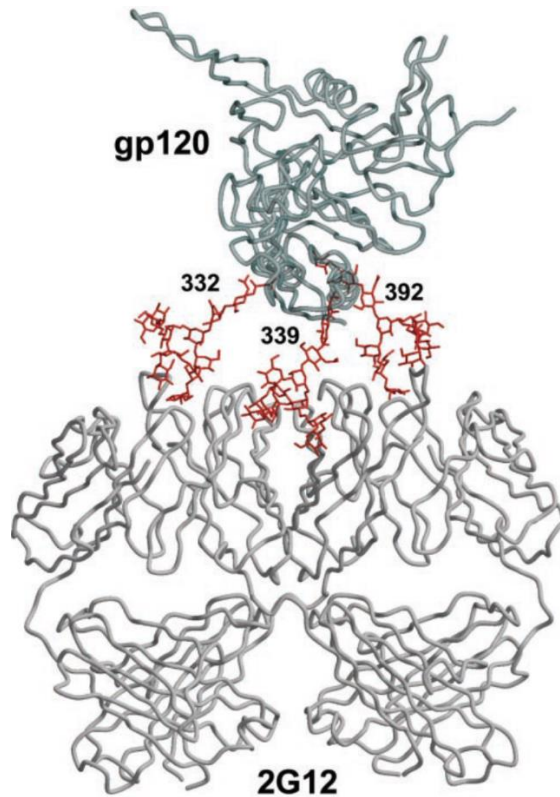


Figure 2.2 Model of 2G12 glycan recognition of gp120. ¹⁴

Based on the model of 2G12 co-crystallized with $\text{Man}_9\text{GlcNAc}_2$ and overlaid on gp120 (Figure 2.2), three separate oligomannose moieties (shown in red) interact with 2G12. Two $\text{Man}_9\text{GlcNAc}_2$ glycans interact with the two primary combining sites, and a third interacts with the newly created combining site at the V_H/V_H' interface formed by domain-exchange (see Figure 2.2). Additional oligomannose glycans within the cluster are also critical for maintaining the epitope conformation. ^{27 28}

The “glycan shield” of HIV is composed of host-derived glycans and it is therefore difficult to overcome the immunotolerance mechanisms to target these glycans. The presentation of the oligomannose glycans within the 2G12 epitope as a dense cluster is thought to form the basis of immunological discrimination of this

epitope. Clustering of oligomannose glycans on mammalian glycoproteins is extremely rare, and therefore viewed as “non-self” by the human immune system. The ability of 2G12 to bind with neutralizing capacity to a solely carbohydrate epitope is thought to be a result of the extremely unusual domain-exchanged architecture of the 2G12 Fab (see Figure 2.1).^{14 30} The variable heavy chain subunit (V_H) of each antibody arm is exchanged with the V_L domain on the opposite branch creating a compact multivalent binding surface, providing a means to bind the solely carbohydrate epitope with high affinity, an affinity that is unachievable by a typical Y-shaped Ab. Indeed, mutagenesis studies that eliminate the domain-exchanged structure, abrogates 2G12 binding to gp120.^{31 32} The large antigen recognition surface is capable of high avidity interactions with multiple tightly packed mannose residues. For several years, 2G12 was the only known carbohydrate-reactive bNAb, in addition, the relatively straight-forward nature of the 2G12 epitope (i.e. 2-4 tightly clustered oligomannose glycans) has made reconstitution of the epitope an attractive target. Initial attempts at 2G12 antigen design centered around constructing densely clustered oligomannose glycans. The strategies can be loosely grouped into two categories: immunogens which incorporated natural oligomannose glycans containing the chitobiose core, and immunogens that utilized synthetic, mannose-only glycans.

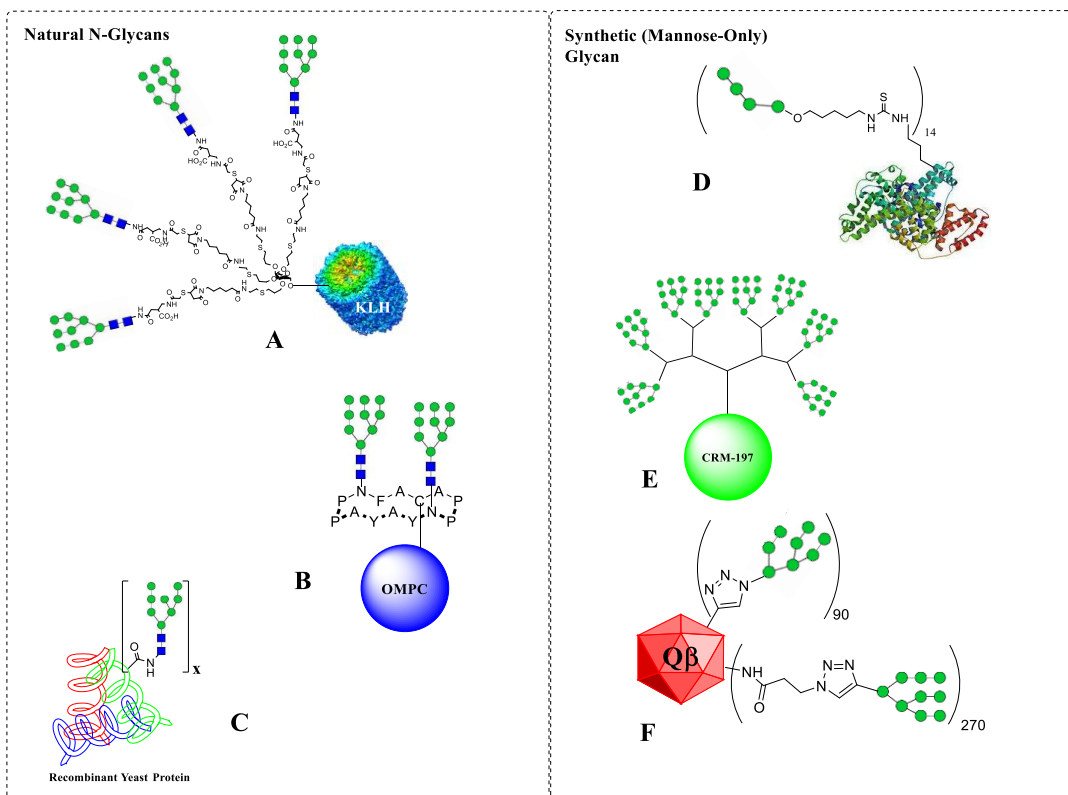


Figure 2.3 Selected multivalent high-mannose glycan cluster antigens. A) Tetraivalent $\text{Man}_9\text{GlcNAc}_2$ on a galactoside scaffold conjugated to KLH.^{33 34 35} B) Divalent cyclic peptide conjugated to OMPC.³⁶⁻³⁷ C) Recombinant yeast proteins bearing $\text{Man}_8\text{GlcNAc}_2$ glycans.^{38-39 40 41} D) Man_4 -conjugated to BSA carrier.^{42 43} E) Man_9 dendrimers on a CRM-197 carrier.^{44 45} F) Heterogeneous multivalent display of Man_9 and Man_8 on a Q β phage particle.⁴⁶

The Wang group was the first to report a synthetic 2G12 antigen³⁵ constructed from a cluster of $\text{Man}_9\text{GlcNAc}_2$ glycans on a galactoside scaffold (Figure 2.3A). 2G12 exhibited markedly higher affinity for the tetra-valent cluster versus the individual $\text{Man}_9\text{GlcNAc}_2$ subunits. This initial design was modified by utilizing a rigid cholic acid scaffold with tri-valent display of $\text{Man}_9\text{GlcNAc}_2$.³³ The rigid

scaffold reduces the entropic penalty that is incurred upon 2G12 binding to a dynamic, heavily solvated epitope. The cholic acid cluster again demonstrated higher affinity for 2G12. Both studies highlighted the importance of glycan clustering in 2G12 binding. In an alternative design, Danishefsky and co-workers utilized a functionalized cyclized peptide as a scaffold to construct oligomannose glycan clusters to further characterize the 2G12 epitope (Figure **2.3B**).³⁶ The cyclic peptide scaffolds displayed up to three Man₉GlcNAc₂ glycans and again showed enhanced recognition by 2G12 as compared to individual Man₉GlcNAc₂ glycans. The limitations of antigenicity toward host-derived “self-glycans” were considered in the design of oligomannose glycan clusters that incorporated synthetic non-self sugars within the cluster. A design by Wang and co-workers utilized a similar cyclic peptide scaffold strategy to that reported by Danishefsky, however, the glycan clusters attached to one face of the cyclic peptide were composed of non-natural fluorinated Man₄ glycans that mimic the D1 arm of Man₉ while remaining non-self due to the fluorination.⁴⁷ The other face of the cyclic peptide was used to attach two T-helper peptides. Surface plasmon resonance indicated the enhanced affinity of these glycan clusters over individual glycan subunits.

Despite the clear enhancement in 2G12 affinity of multivalent glycan display exhibited by the reported clusters. The highest affinity of the synthetic glycan clusters (μM level) was still far lower than the affinity of 2G12 for gp120 (low nM level), its native epitope. It was speculated that the level of multivalent display was still too low to properly mimic the 2G12 epitope on gp120. In order to reach the required threshold for oligomannose display, Wong and co-workers devised a strategy using a

dendrimeric skeleton scaffold to attach multiple copies of synthetic Man₄ or Man₉ glycans. The dendrimers were found to bind 2G12 with extremely high affinity, comparable with gp120 itself.⁴⁴ The promise of the strategy instigated the development of glycodendrimers as candidate vaccines using diverse carriers, including polyamidoamine (PAMAM)⁴⁵ and gold nanoparticles allowing measurable changes in the density of loading (Figure **2.3E**).⁴⁸ An elegant approach reported by Krauss and co-workers, used a directed evolution approach using a DNA scaffold with random insertions of alkyne-modified base-pairs followed by click-chemistry with synthetic Man₉ glycan.⁴⁹ The DNA-scaffold glycoconjugates were screened using a 2G12-affinity column, and the most promising candidates were sequenced and multiplied by PCR selection with modified aptamers (SELMA).⁵⁰ A second generation of the directed evolution SELMA approach uses RNA-DNA duplex. The associated peptide was translated and tagged with oligomannose glycans via click chemistry yielding a peptide screened to bind to 2G12.⁵¹⁻⁵² Despite excellent antigenicity results, no immunization data has been reported.

The antigenicity studies focusing on the design of multivalent oligomannose display indicated that clustered synthetic oligomannose antigens are capable of binding 2G12 with neutralizing affinity. However, given the poor immunogenicity of carbohydrates antigens, an effective carbohydrate-based immunogen usually requires conjugation of the antigen to a T-cell helper epitope (i.e. a carrier protein). Such a conjugate is more effective at triggering the T cell-dependent immune response capable of eliciting IgG Abs that is usually required for a successful vaccine candidate. Many of the oligomannose clusters that yielded promising antigenicity

results were reformulated with a carrier protein and tested as possible HIV immunogens for raising anti-mannose Abs with a shared epitope with 2G12. The galactoside-based tetravalent $\text{Man}_9\text{GlcNAc}_2$ cluster reported by Wang and co-workers was conjugated to a key-hole limpet hemocyanin (KLH) carrier protein via maleimide linkages (Figure **2.3A**).³⁴ Rabbits were immunized with the glycoconjugates and analysis of the antisera revealed moderate anti-carbohydrate Abs that were weakly cross-reactive with gp120. Unfortunately, the Abs raised were unable to neutralize HIV. Similarly, Danishefsky and co-workers conjugated their previously described $\text{Man}_9\text{GlcNAc}_2$ clusters on a cyclic peptide scaffold to the protein outer membrane protein complex (OMPC) isolated from *Neisseria meningitidis* (Figure **2.3B**).³⁷ Analysis of the antisera from the associated immunogenicity study, obtained from both guinea pigs and rhesus macaques, indicated that the glycoconjugate was capable of eliciting high-titers of anti-carbohydrate Abs. The Abs were weakly cross-reactive with recombinant gp160, but were unable to neutralize viral isolates. In a more recent study, Angrawal-Gamse and co-workers expressed a library of recombinant yeast proteins produced in the presence of kifunisin, which blocked the glycan processing pathway resulting in proteins bearing $\text{Man}_8\text{GlcNAc}_2$ glycans alone. (Figure **2.3C**). Screening of the expressed proteins (via a 2G12 affinity column) isolated a single candidate that was recognized by 2G12 with high affinity. The candidate was sequenced and cloned (via PCR). Immunization with the yeast-derived glycoproteins elicited carbohydrate-reactive Abs that were cross-reactive with gp120 and capable of neutralizing a panel of virions.^{39 41} However, it should be noted that

only virions expressing exclusively high-mannose glycans were vulnerable to neutralization.

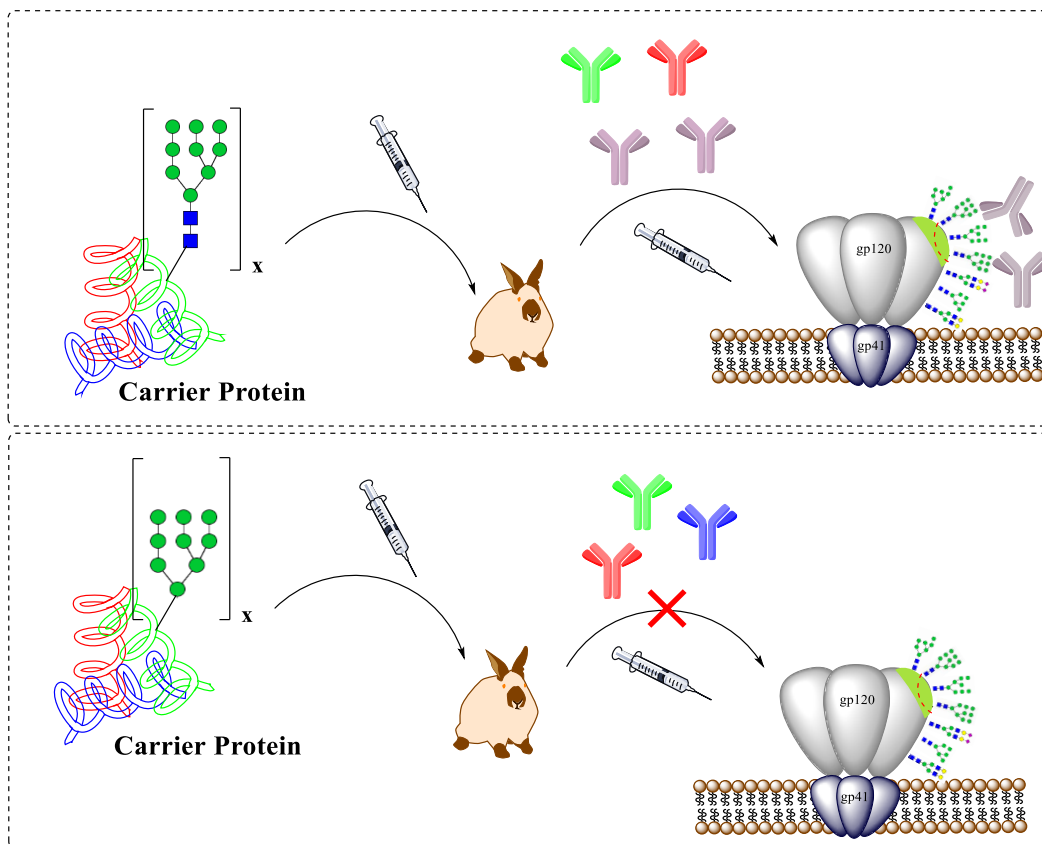


Figure 2.4. Immunogens incorporating natural $\text{Man}_9\text{GlcNAc}_2$ glycans elicit gp120-cross-reactive Abs. Abs raised by immunogens that use synthetic (mannose-only) glycans are not cross-reactive with gp120.

A number of immunogens incorporating synthetic (mannose-only) oligomannose glycans have been reported, also largely constructed from previously described antigenicity studies. The dendrimer-based oligomannose clusters bearing either Man_4 or Man_9 glycans were conjugated to a non-toxic mutant protein derived from diphtheria toxin (Figure 2.3E).⁴⁵ In a similar study, a conjugate bearing Man_4 glycans

on a bovine serum albumin (BSA) scaffold was tested as an immunogen (Figure 2.3D).⁴² In both studies, while capable of raising anti-mannose Abs, the antisera derived from either of these glycoconjugates was unable to recognize high-mannose glycans in the context of gp120, indicating that the immune response was directed against a different mannose-only epitope on the immunogens. Several groups have explored virus-like particles (VLPs) as carrier proteins for glycoconjugate vaccines. Finn, Burton, and co-workers tested a side-by-side comparison of two VLPs (Cowpea Mosaic Virus [CPMV] and bacteriophage Q β) by construction of glycoconjugate immunogens using click-chemistry to attach synthetic Man₄, Man₈, and/or Man₉ glycans (Figure 2.3F). The Q β scaffold was found to be superior, displaying multiple copies of high-mannose glycans in a highly organized presentation.⁴⁶ The researchers incorporated alkyne groups in a two-step process (i.e. during protein expression and acylation of the exposed surface lysine groups) allowing two-step glycosylation, providing greater control in investigating the effects of varying the number, identity, and geometry of oligomannose display. The strategy allowed for manipulation of individual parameters to fine-tune the glycan presentation. The Q β conjugates carrying Man₄ and Man₉ glycans were found to bind 2G12 with high affinity, and a heterogeneous mixed conjugate containing Man₈ and Man₉ showed the highest 2G12 recognition. Despite the promising antigenicity results, the antisera from the associated immunogenicity studies yielded high-titers of anti-Man₄ and anti-Man₉ IgG Abs, however, none of the Abs isolated from the antisera could cross-react with gp120. In addition, the antisera displayed no HIV neutralization activity. In a similar design strategy, Davis and co-workers conjugated (via click-chemistry) methylated

Man₄ to a Q β scaffold. The use of methylated Man₄ glycans provided a non-self sugar mimic, intended to have enhanced antigenicity and overcome immune tolerance mechanisms.⁵³ The associated immunogenicity studies produced antisera that was not cross-reactive with gp120. Moreover, the antisera totally lacked HIV neutralization activity. Interestingly, glycan microarray analysis of the antisera indicated a higher antigenicity toward 2G12 when compared to unmodified Man₄, supporting the notion that non-self sugar mimics are capable of improving immunogenicity. A similar rationale was utilized by Pantophlet and co-workers, who incorporated a bacterial lipooligosaccharide as a non-self oligomannose analog. The study utilized heat-killed bacteria displaying the lipooligosaccharide as an immunogen. Interestingly, the antisera obtained from the immunization studies was found to interact moderately with monomeric gp120, however, the antisera was unable to neutralize HIV virions.⁵⁴

In spite of enormous effort, to date, no effective 2G12-targeted vaccine has proven successful. However, the results of these studies have yielded interesting and important observations. Without exception, glycoconjugates containing natural, full-length, chitobiose core containing oligomannose N-glycans (i.e. Man₉GlcNAc₂) have been capable of raising Abs that are moderately cross-reactive with HIV-1 gp120 (Figure 2.4). The inability of glycoconjugates containing synthetic mannose-only oligomannose glycans to elicit Abs capable of cross-reactivity with gp120 may be due, in part, to the lack of a chitobiose core. Importantly, these synthetic mannose glycoconjugates are capable of eliciting carbohydrate-reactive Abs, however, this reactivity is lost in the context of gp120. It is clear that the synthetic glycoconjugates

are not able, thus far, to mimic the orientation of the glycans on gp120. Taken together, the observations suggest that the chitobiose core may contribute to modulating the orientation of the oligomannose glycans. The orientation of the glycans appears to be critical for enhancing the antigenicity and especially the immunogenicity of the glycoconjugates, and may be required for a faithful reconstitution of the 2G12 epitope in a glycoconjugate context. In order to determine the specific role of the chitobiose core in defining the immunogenicity of a given glycoconjugate, it is necessary to compare two conjugates side-by-side (i.e. one containing the chitobiose core and the other lacking the chitobiose core). The subsequent differences in the immunogenicity of the antisera will directly indicate the specific requirement of the core GlcNAc. Herein we designed several glycoconjugates using a Q β scaffold that display oligomannose clusters intended to represent the principal epitope of 2G12. The oligomannose clusters each incorporate an alternative glycan presentation intended to yield information regarding the specific glycan preferences of 2G12, including a side-by-side comparison of high-mannose Man₉ glycans with and without the chitobiose core. Utilizing different glycan presentations on the glycoconjugates provided a means to fine-tune the presentation of oligomannose glycans to maximize the binding affinity to 2G12.

The well-characterized icosahedral viral particle bacteriophage Q β capsid was chosen as the platform for presentation. Q β self-assembles into a virus-like particle engulfing random cellular RNA upon recombinant expression in *Escherichia coli*. Q β is pathogenic to *E. coli*, and presents no risk of infectivity in mammalian cells. Q β has been extensively studied and found to be safe and immunogenic, as well as an

excellent polyvalent scaffold for the display of various macromolecules.^{55 56-59 60} Q β capsid particles self-assemble from 180 small (14 kDa) subunits. The assembly from multiple small subunits results in a VLP with a smooth capsid surface, and regular, geometrically displayed reactive amines (lysine residues and the N-termini of the Q β subunit) on the surface of the capsid.⁶¹ The reactive surface amines that act as the attachment sites for the oligomannose glycans are spaced at distances roughly correspondent with those required for 2G12 recognition.^{56, 60}

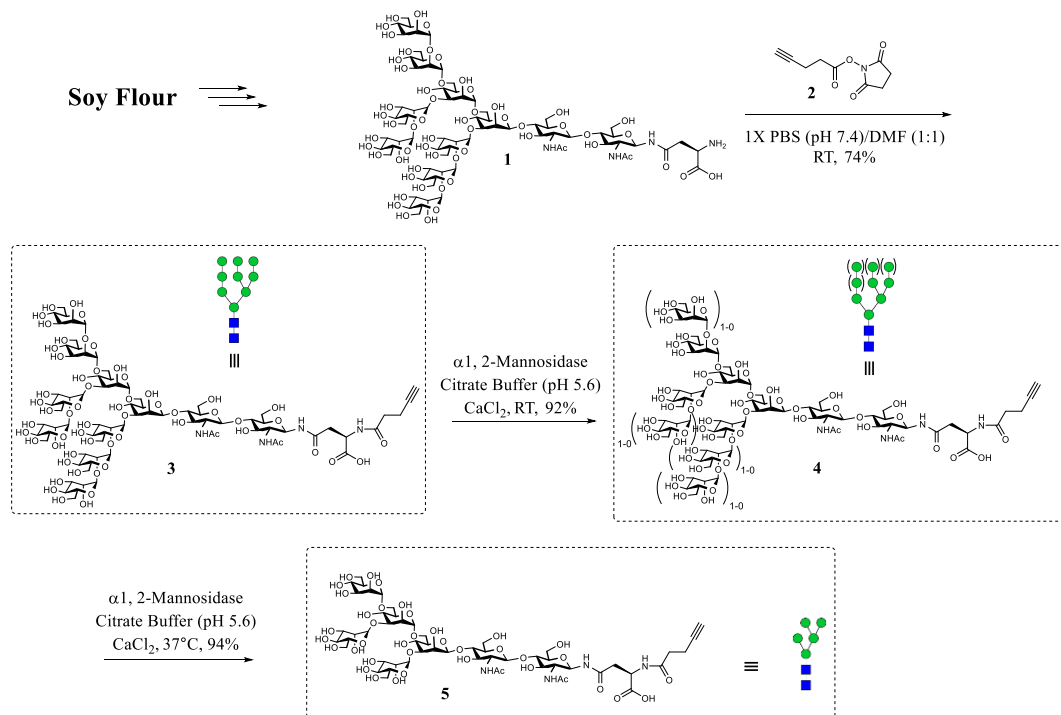
Several glycoconjugates displaying Man₉GlcNAc₂, Man₉, Man₅₋₉GlcNAc₂, and Man₅GlcNAc₂ on a Q β scaffold were synthesized, allowing us to probe the specific glycan preferences of 2G12, as well as the specific impact of the chitobiose core on glycan orientation. The linkers and method of conjugation to the protein are shared among the glycoconjugates, and therefore any differences in 2G12 affinity can be attributed to differences in the glycan preference of 2G12. The immunogenicity studies are ongoing, and will be informative as to the specific role of the chitobiose core on defining the 2G12 epitope.

2.2 Results and Discussion

In the design of these novel oligomannose glycan clusters, we were seeking to probe several aspects regarding the specific glycan preferences of 2G12. The major question that we are seeking to answer, namely, what is the specific contribution of the chitobiose core in defining the immunogenicity of oligomannose clusters, informed the initial design of the project. This specific question was addressed by a side-by-side comparison of two classes of Man₉ glycan, one that incorporates the

chitobiose core, and one lacking the chitobiose core. The subsequent questions were related to optimizing glycan presentation to mimic the native epitope with the highest possible fidelity. Specifically, sufficient latitude must be extended to the heterogeneity of gp120 glycosylation. Given recent reports,^{23, 62-64 65} the traditional picture of the 2G12 epitope consisting of 2-3 homogeneous Man₉GlcNAc₂ glycans is clearly an artifice and doesn't accurately represent the complicated pattern of glycosylation on gp120. It is probable that the actual 2G12 epitope on native gp120 consists of a heterogeneous mixture of oligomannose glycans at varying stages of glycan processing, likely consisting of a mixture of Man₅₋₉GlcNAc₂ glycans. We therefore chose to incorporate a mixed building block that consists of a heterogeneous mixture of Man₅₋₉GlcNAc₂ glycans within the ensemble of oligomannose building blocks. The mixed oligomannose glycan presentation by this building block will hopefully mirror the actual native epitope. The final building block chosen to complete the ensemble consists of Man₅GlcNAc₂. 2G12 has been shown in previous studies to be unable to recognize Man₅GlcNAc₂³⁵ presumably due to the lack of distal α 1, 2-Man-Man linkages that ostensibly make up the 2G12 epitope. Supporting this was the finding that treating gp120 with α 1, 2-mannosidase abrogates 2G12 binding to gp120.²⁸ We speculated that the incorporation of a Man₅GlcNAc₂ building block will yield interesting results regarding the linkage specificity of the Abs raised by the immunogen. Specifically, it will indicate whether the Abs raised by the immunogens are mannose-reactive only, or share a similar linkage preference to 2G12, suggesting degeneracy in the mode of oligomannose recognition.

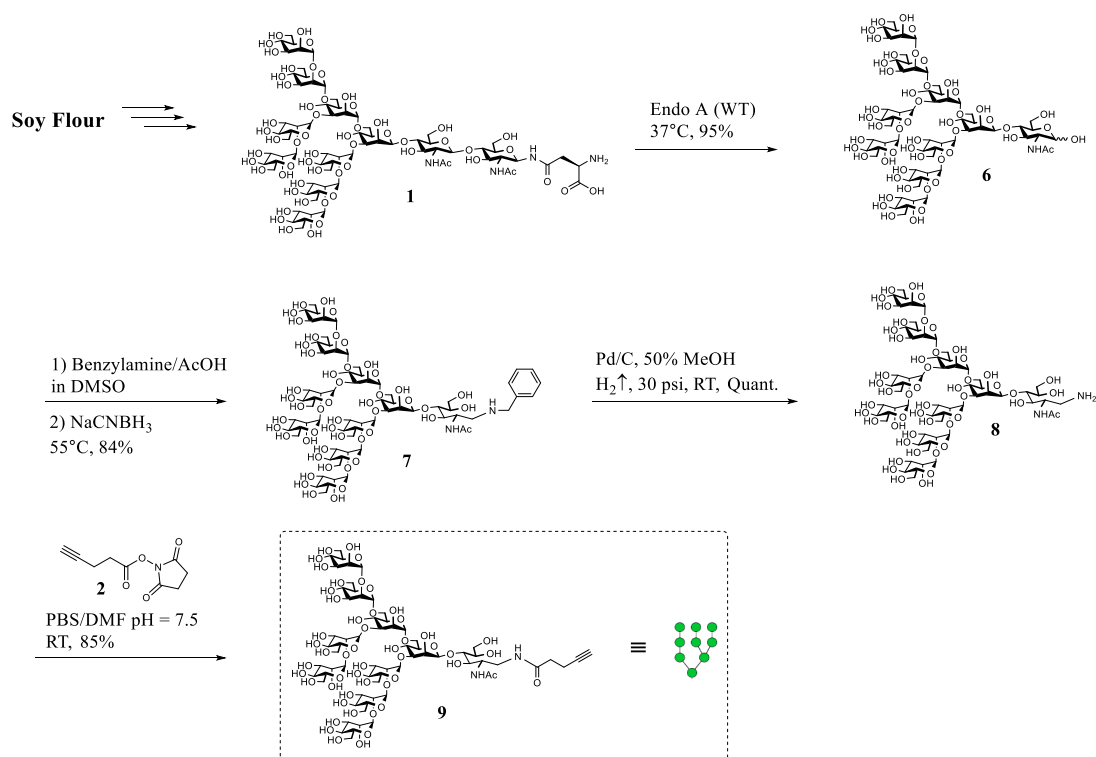
The synthesis of the alkyne tagged sugar building blocks containing the chitobiose core was achieved via a top-down method utilizing naturally derived $\text{Man}_9\text{GlcNAc}_2\text{Asn}$ as the common starting material. All subsequent final compounds were derived by a chemoenzymatic synthetic strategy.



Scheme 2.1 Top-down chemoenzymatic synthesis of alkyne-tagged chitobiose-core containing oligomannose glycan building blocks.

The precursor of the chitobiose core containing N-linked oligomannose glycans bearing an alkyne group **1** ($\text{Man}_9\text{GlcNAc}_2\text{Asn}$) was isolated from soybean agglutinin (SBA) by a modified method recently reported by our group.^{35, 66-67} Compound **3** ($\text{Man}_9\text{GlcNAc}_2$) was prepared by acylating the amino-terminus of asparagine of **1** using a large excess of 4-pentynoic acid succinimidyl ester (**2**). The other two chitobiose-containing alkyne-sugar precursors were prepared by enzymatic digestion subsequent to acylation. Compounds **4** and **5** were subjected to different degrees of

digestion by α 1, 2-mannosidase. While all α 1, 2-mannose linkages in compound **5** (Man₅) were hydrolyzed by the enzyme, **4** (Man₅₋₉) was digested in a controlled manner, affording access to a mixture of high-mannose glycans particularly enriched with Man₇GlcNAc₂ (see Appendix Figure A.2.1). Compound **1** (Man₉Asn) served as the common starting material for both types of precursor oligomannose building blocks, with and without the chitobiose core.



Scheme 2.2 Top-down chemoenzymatic synthesis of an alkyne-tagged oligomannose building block lacking the chitobiose core.

The second class of alkyne-sugar building block lacking the chitobiose core (**9**) was prepared by digestion of **1** with Endo A (WT) yielding the reducing sugar **6**. A reductive amination strategy was employed which both installed a reactive amine group at the reducing terminus, while simultaneously breaking the ring yielding the

acyclic glycan which served functionally as a linker (Scheme 2.6). An amine group was added at the reducing terminus using a modified benzylamination strategy similar to that reported previously by Y.C. Lee et al.⁶⁸ We chose to use a masked, benzyl-protected amine, given the reported superiority of the method over the direct installation of an amine to prevent the formation of dimers. Initially, we tested the reported reducing conditions (i.e. BH₃-Pyridine), however, we discovered the formation of a side-product corresponding to dehydration of the target compound, leading us to test alternative, more mild reducing strategies. We found an optimized condition incorporating sodium cyanoborohydride (NaCNBH₃) in DMSO to be a more effective method, as the mild reducing agent eschewed the formation of the dehydration product. The conditions used are identical to those used for the well-established 2-aminobenzamide (2-AB)/2-aminobenzoic acid (2-AA) method for glycan labelling.⁶⁹⁻⁷²

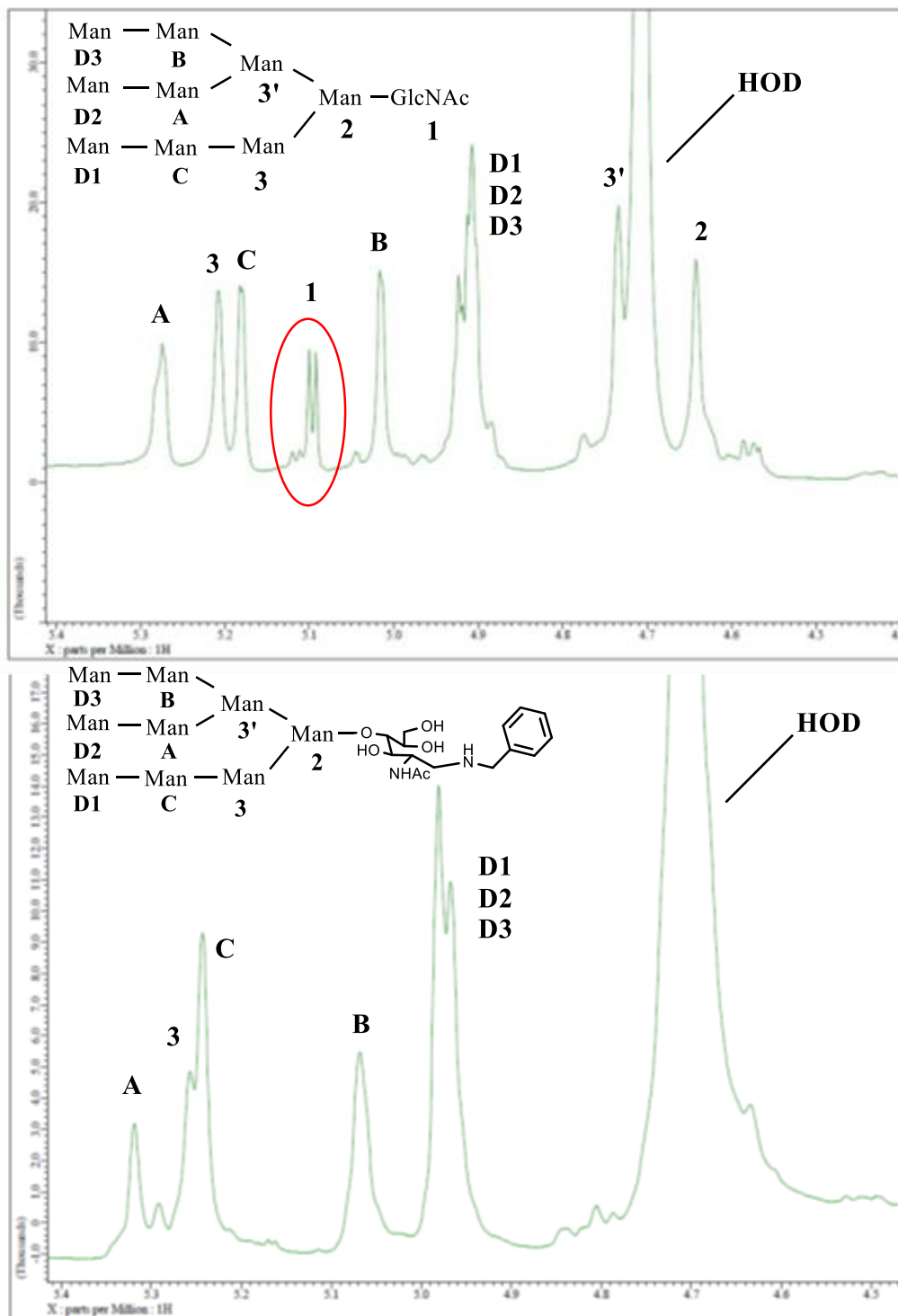
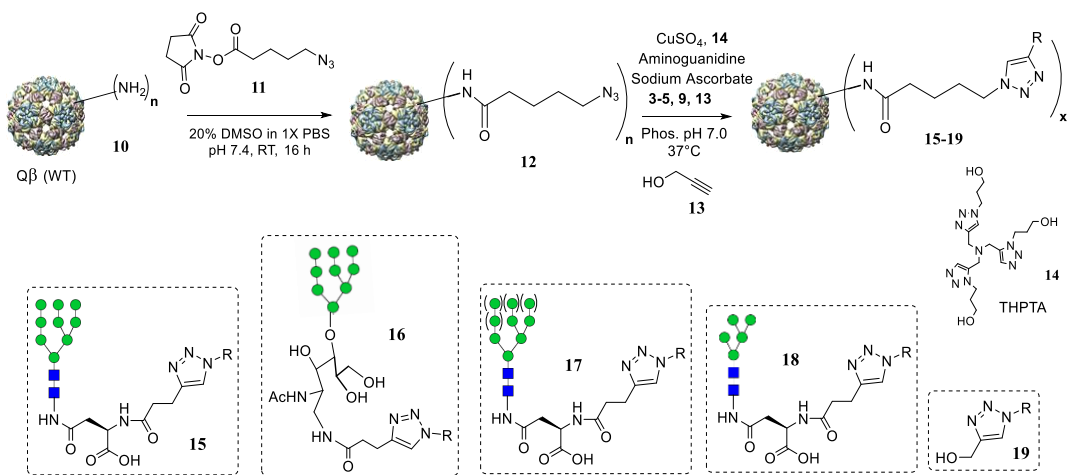


Figure 2.5 ¹H-NMR comparison of Man₉GlcNAc (6) and Man₉(Acyclic-GlcNAc)-Benzylamine (7) clearly demonstrates loss of the anomeric GlcNAc proton signal at

~5.15 ppm, indicating acyclic structure. Anomeric proton signals for protons 3' and 2 from **7** are hidden under the HOD peak.

The open-ring form of the reducing GlcNAc was confirmed by ¹H-NMR. The anomeric proton signal from the terminal GlcNAc was clearly lost upon the acyclization/reduction of Man₉GlcNAc (Figure 2.5). In comparison to the starting material, which had a doublet corresponding to the anomeric GlcNAc proton at 5.1 ppm, the benzyl-tagged Man₉ with an acyclic GlcNAc lost the signal at 5.1 ppm (Figure 2.5). In addition, two-dimensional NMR spectra, Heteronuclear single quantum coherence spectroscopy-distortionless enhancement by polarization transfer (¹H-¹³C-HSQC-DEPT) and proton-proton correlation spectroscopy (¹H-¹H-COSY) was used to analyze the samples. Through HSQC-DEPT, the other two anomeric proton signals of two Man residues, obscured by the water peak in the ¹H-NMR, were clearly observed and assigned for **7** (see appendix for 2D-NMR data). The result was further confirmed using high-resolution mass spectrometry (MALDI-FTICR). Subsequent to the reductive amination reaction with benzylamine, the benzyl group was removed using palladium-catalyzed hydrogenation. De-N-benylation by low-pressure hydrogenation proved ineffective, as the N-benzyl group was obdurate to removal. Complete unmasking of the amine group required the use of a catalytic hydrogenator apparatus under high pressure (35 psi) hydrogen atmosphere. The unmasked amine-bearing oligomannose sugar, lacking the chitobiose core (**8**) was tagged with an alkyne group by direct acylation with **2** in a single step, yielding the target compound **9**.

Subsequent to the synthesis of the oligomannose building blocks, careful consideration was given to the type of carrier protein scaffold that would be most appropriate for the given application. Multivalent display of oligomannose clusters is requisite for the proper reconstitution of the 2G12 epitope, and the chosen scaffold needed to accommodate dense loading of oligomannose glycans while incorporating proper spacing. The scaffold needed to display oligomannose glycans in a rigid manner so that the binding interaction between 2G12 and the immunogen is not stymied by the flexibility of the antigenic glycans. The spacing between the antigenic oligomannose glycans in the 2G12 epitope have been reported as 5.8 Å (N295-N332), 20.3 Å (N332-N392), and 23.6 Å (N295-N392). The presentation of the oligomannose glycans on the synthetic immunogen should, at least roughly, approximate these distances. Bacteriophage Q β has spacing of the solvent-exposed surface reactive amine groups that roughly approximate the spacing estimated between the oligomannose glycans on gp120.



Scheme 2.3 Copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) synthesis of viral glycoconjugates.

High density display of oligomannose glycans on Q β capsid surface by chemical conjugation requires a conjugation reaction that is sufficiently efficient to overcome the unfavored steric constraints intrinsic to the attachment of bulky, dynamic glycan structures within a small surface area. Click-chemistry has been used for a similar purpose by our group and others^{47, 73-75}⁴⁶ and is an attractive strategy given its high efficiency and bioorthogonality. In order to install an orthogonal reaction partner for the alkyne-tagged building blocks, the surface amino groups of Q β (**10**) were acylated using a large excess of succinimidyl 5-azidovalerate ester **11** (Scheme **2.7**). Following purification to remove the excess linker from the particle, the alkyne-tagged oligomannose glycans were attached to Q β with the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. The use of the CuAAC click reaction provided a means to couple these stable, orthogonal reaction partners at low concentrations to reproducibly yield high loading conjugates. The click-chemistry conditions used to construct the glycoconjugates were similar to those recently reported by Huang and co-workers.⁷⁴ The copper source was pre-complexed with the ligand tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) reported previously (**14**).⁷⁶ In addition, propargyl alcohol (**13**) was also used as a reaction partner yielding **19**, displaying “naked” triazole linkage which will indicate the immunogenicity of the linker itself. The resulting constructs **15-19** were characterized by size-exclusion chromatography (SEC), polyacrylamide gel electrophoresis (SDS-PAGE), and matrix-assisted laser desorption ionization with time-of-flight analysis (MALDI-TOF).

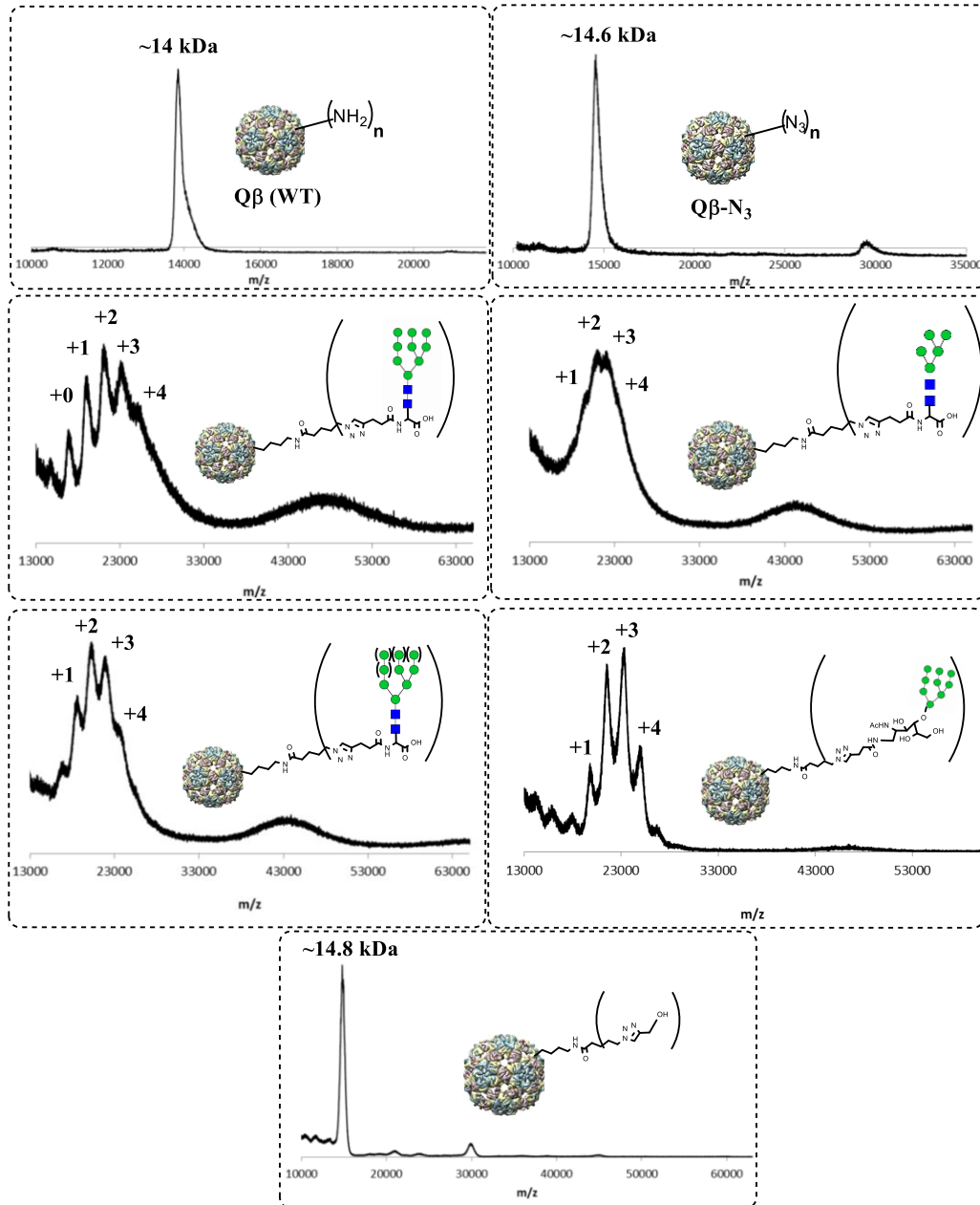


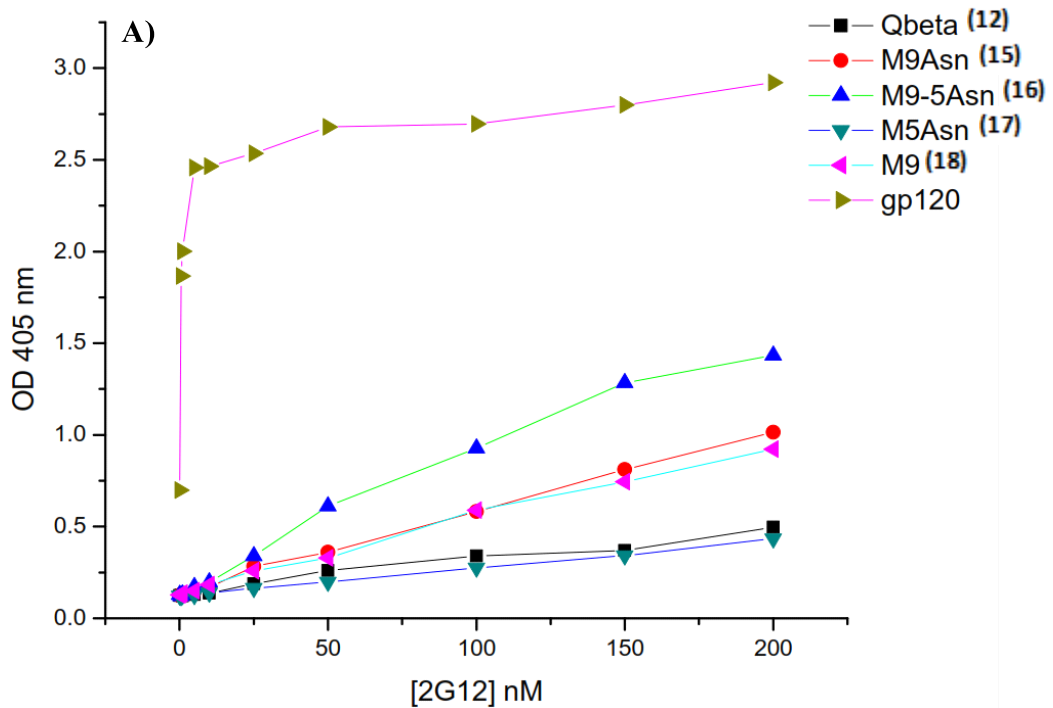
Figure 2.6 MALDI-TOF analysis of oligomannose-Q β conjugates.

The number of triazole-tethered oligomannose glycans on each subunit of the VLP was estimated by MALDI-TOF and SDS-PAGE, and found to be an average of 2-3

oligomannose glycans per subunit, approximately 450 oligomannose glycans attached to each Q β particle (Figure 2.6).

Interaction of Q β glycoconjugates with 2G12

The ability of the neoglycoconjugates to interact with 2G12, was first estimated using a conventional enzyme-linked immunosorbent assay (ELISA), in which serially diluted 2G12 was incubated with antigens directly coated onto wells. The binding assay provided a means by which the affinity of 2G12 for the capsid conjugates could be directly compared to the affinity of 2G12 for monomeric gp120_(BAL).



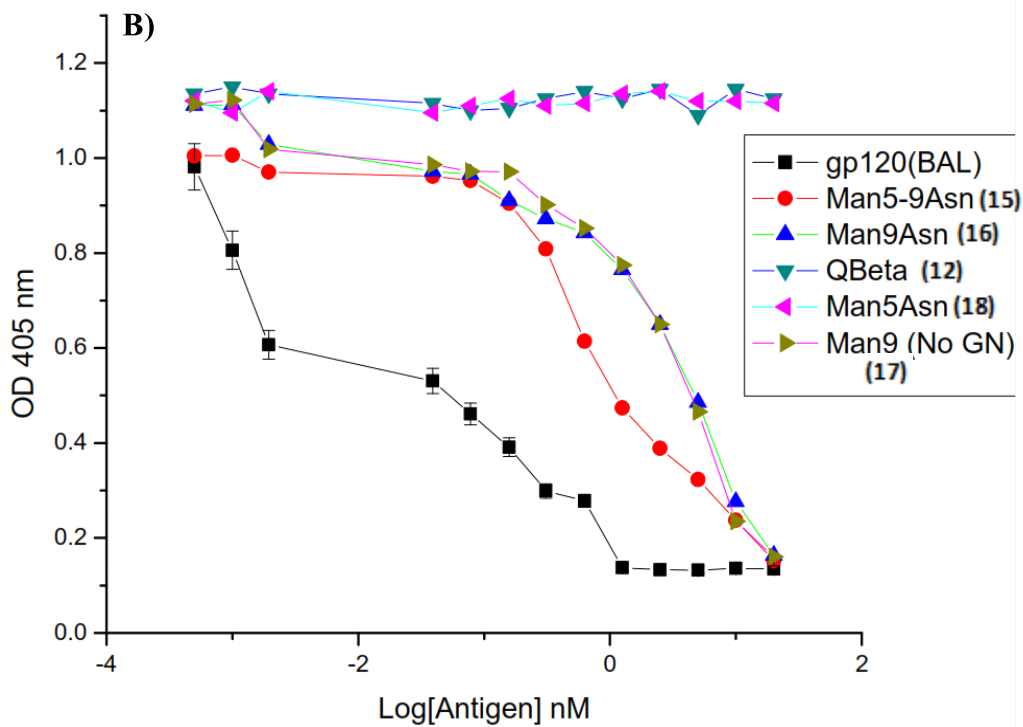


Figure 2.7 Affinity of 2G12 for oligomannose-Q β conjugates versus gp120. **A)** Conventional ELISA. **B)** Competitive ELISA.

As expected, compounds **15** and **16**, both bearing Man₉ glycans, showed similar affinity for 2G12. Unexpectedly, compound **17**, bearing a heterogeneous mixture of Man₅₋₉ exhibited the highest affinity for 2G12, a nearly 2-fold increase compared to the conjugate bearing Man₉. Neither compound **18**, nor the azide-tagged Q β particle (**12**) displayed significant affinity for 2G12. Despite following the expected trends, the conventional ELISA results were disappointing, as the response from the highest 2G12 dilution in the series was still ~2-fold lower than gp120 response at the midpoint concentration (Figure **2.7A**). Previous reports for similar compounds⁷⁷ have suggested that the highly glycosylated nature of the glycoconjugates can stymie their adsorption onto the plastic wells of a 96-well plate, particularly for particles

displaying larger glycans. Therefore, a conventional ELISA format potentially underestimates the affinity of 2G12 for the described Q β -glycoconjugates. To address the possibility, the synthetic glycoconjugates were examined for their capacity for competitive inhibition of 2G12 binding to gp120. In the new competitive ELISA format, monomeric gp120_(BAL) was immobilized to the plate, and a fixed concentration of 2G12 was added to each well, containing a serial dilution of the glycoconjugate antigens. The competitive ELISA data followed the same trends as conventional ELISA, however it revealed much higher binding potencies between the Q β glycoconjugates and 2G12, with IC₅₀ values for compounds **15-17** of 641 nM, 653 nM, and 605 nM respectively. The observed superior binding of **17** to 2G12 is in agreement with prior reports where a mixed display of Man₉/Man₈ yielded the strongest antigen, even compared to a similarly loaded Man₉ analog.⁴⁶ A possible explanation is that the mixed display of heterogeneous glycans is a more faithful representation of the native 2G12 epitope on gp120. That compounds **15** and **16** exhibited nearly identical affinity toward 2G12 was not surprising, as both conjugates bore similar loading and linker lengths with the overall effect of a similar oligomannose presentation. The conjugates with the highest affinity were still ~50-fold less potent than gp120 at 2G12 inhibition. As expected, the non-glycosylated Q β particle **19** and compound **18** both lacked detectable 2G12 inhibition (see Figure **2.7B**). As discussed above, 2G12 targets an epitope consisting of Man- α 1, 2-Man glycans, so it is expected that Man₅GlcNAc₂, which lacks α 1,2-Man linkages, would be a poor antigen, displaying no affinity for 2G12.

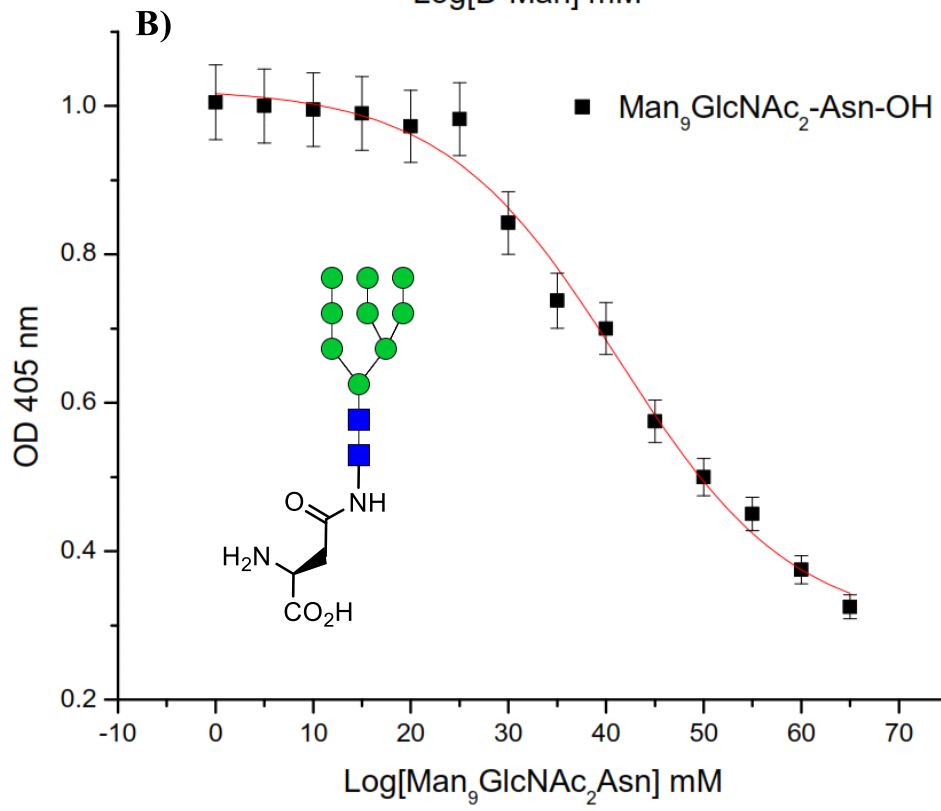
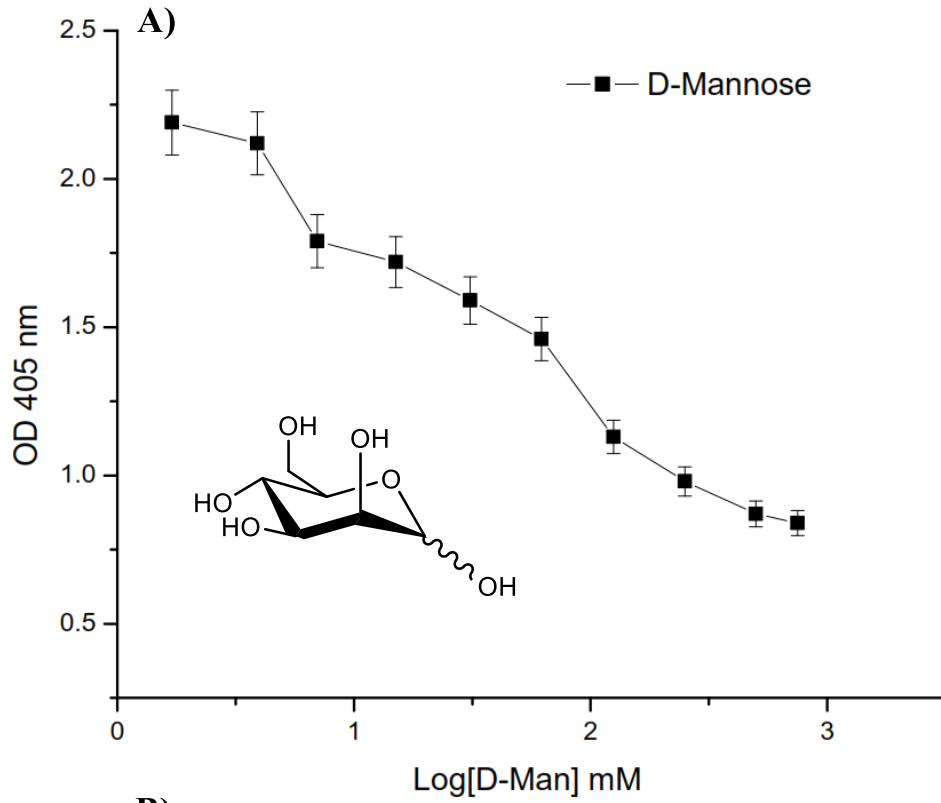


Figure 2.8 Inhibition of 2G12 binding to gp120_{BAL} by monovalent mannose display.

A) D-mannose inhibition of 2G12-gp120 binding. B) Man₉GlcNAc₂-Asn inhibition of 2G12-gp120 binding.

The significance of multivalent glycan display was reiterated by competitive ELISA using both monosaccharide D-mannose and Man₉GlcNAc₂Asn (monomeric display) (Figure 2.8). The IC₅₀ values were in the high μ M range for monosaccharide mannose. While the display of three mannose arms increased the potency, the IC₅₀ for Man₉GlcNAc₂ was in the μ M range. When the oligomannose glycans are arranged in a multivalent display, the potency dramatically increases. Immunization studies are currently underway by our collaborator (Dr. Denong Wang, Stanford University). The results will be informative regarding the specific role of the chitobiose core in defining oligomannose-VLP conjugate immunogenicity.

2.3 Conclusion

The numerous strategies that have been reported to design a synthetic 2G12 epitope have met with limited success, presumably due to the inability to reconstitute the fine-details of the epitope in a synthetic format. The overall lack of success suggests that there is still much to learn about what is required to elicit 2G12-like Abs that target Man α 1, 2-Man-linked glycans in the context of gp120. A particular challenge is targeting specific mannose reactive Abs that recognize a specific oligomannose cluster motif on HIV, without cross-reactivity with “self” glycosylation. The use of VLPs provides a promising strategy to overcome these

obstacles by virtue of their well-defined chemistry, rigid structures, and atomic level understanding of the surface composition providing unprecedented access to previously inaccessible design aspects. These features afford access to tailored antigens at a level heretofore impossible. The ability to manipulate the fine details of antigen presentation is crucial for targeting carbohydrate antigens, which are typically poorly immunogenic. The VLP scaffold affords greater flexibility for tuning the fine-details to create an immunogenic epitope even from host-derived carbohydrate antigens. The ongoing immunogenicity studies will be informative as to the specific role contributed by manipulations to the oligomannose display. In particular, the results will indicate the specific role of the chitobiose core in defining the *immunogenicity* of the carbohydrate antigens. Moreover, despite the well-known observation that strong antigenicity doesn't necessarily translate into strong immunogenicity, the enhanced 2G12 affinity of the mixed oligomannose display in compound **17**, may be indicative of an improvement in the glycan display, making the synthetic epitopes closer in shape and orientation to the actual 2G12 epitope on gp120.

2.4 Experimental Section

General Procedures. ESI-MS spectra were obtained using a ThermoFisher Q Exactive LC/MS orbitrap mass spectrometer. MALDI-TOF analysis was performed using a Bruker UltrafleXtreme (UTX) mass spectrometer with TOF/TOF detection. MALDI-FTICR analysis was performed using a Bruker Solarix ESI/MALDI-12T FT-ICR mass spectrometer. MALDI-FTICR analysis was performed by Dr. Claire

Carter (UMB). 1D/2D-NMR was performed using a Bruker AVIII-600MHz NMR. Catalytic hydrogenation was performed using a Catalytic Hydrogenation Apparatus (Parr Instruments, Moline, IL) under 35 psi hydrogen pressure. α 1,2-Mannosidase was expressed from *E. coli* by Dr. Kevin Li as previously reported.⁷⁸ 2G12 and monomeric gp120_{BAL} were both obtained from the National Institutes of Health (NIH) through the AIDS Reagent Program. Bacteriophage Q β was produced in *E. coli* by Dr. Qiang Yang according to a previously published procedure.⁷⁹ Purification of the particles was achieved by size-exclusion chromatography using Sepharose CL-4B column. Only the center elution band was taken to ensure high purity material. The yield of the recovered WT Q β was 65%, with 95% purity, according to SDS-PAGE analysis. The particles were >95% intact, according to analytical size-exclusion fast protein liquid chromatography (FPLC) using a Superose-6 column.

Modification of VLP Surface Chemistry

Q β _{WT} (**10**) in 1X PBS (pH 7.4) was added to compound **11** (13 mM, 5 equivalents with respect to the Q β asymmetric subunit, 1.25 eq. per reactive amine) in DMSO such that the final DMSO concentration was 20%. The final protein concentration was 2 mg/mL. The solution was shaken overnight at room temperature on a rotisserie shaker. The excess NHS-azide and DMSO were removed by repeated centrifugal filtration using Millipore 10 KDa MWCO filters against PBS buffer. Total protein concentration was determined by Quick Start Bradford Reagent (BioRad), using bovine serum albumin as the standard for the concentration curve, with a recovery of 70%. Average loading of surface azido groups was estimated by MALDI-TOF analysis. On average, all four reactive amines were tagged with azido groups.

General CuAAC Reaction Conditions

Synthesis of oligomannose-Q β conjugates (**15-19**) was achieved using the CuAAC click reaction, with conditions similar to those reported by M.G. Finn and co-workers.⁷³ All solvents were degassed before use. In general, the following reagents were combined in the following order, 14 μ L 500 mM phosphate buffer, pH 7.0 (final 80 mM), 50 μ g **12** (from 7 mg/mL stock), alkyne-tagged oligomannose glycans (**3-5, 9, 13**) [10 eq./subunit, approximately 2.5 eq./reactive azide]. Pre-mixed Cu-THPTA solution (CuSO₄, 50 mM in water [3.4 eq.] + THPTA ligand (**14**) 50 mM in water, 5-fold excess compared to Cu), aminoguanidine (100 mM in water, 100 eq. compared to **12**), and sodium ascorbate (100 mM in water, 100 eq. compared to **12**). After the addition of sodium ascorbate, the reaction tube was flushed with argon and sealed. The mixture was shaken at 37°C overnight. The reaction was quenched by the addition of 500 mM EDTA (10 mM final). Purification of the glycopeptide-Q β conjugates was performed by centrifugal filtration against PBS.

Mass Spectrometry of derivitized Q β particles

20 μ g antigen was mixed with a solution of 8 M urea (30 μ L) containing 1M dithiothreitol (DTT) (5 μ L). The mixture was incubated at 37°C for 1 h. 50 μ L 0.5 M iodoacetamide (IAA) was added and the solution was incubated for an additional 1 h at 37°C. The excess IAA was reacted with 1 M DTT (10 μ L), and incubated at 37°C for 10 min. The mixture was lyophilized and dissolved in 10 μ L water containing 0.2% trifluoroacetic acid (TFA) and purified using C4 ZipTips (EMD Millipore) according to the manufacturer's instructions. The purified antigen (1 μ L) was mixed 1:1 with matrix (sinapic acid) on the MALDI plate.

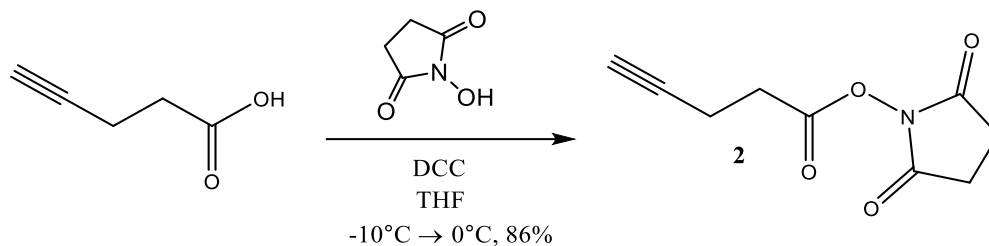
Enzyme-linked immunosorbant assay (ELISA)

Conventional ELISA Each well of a high binding Ultra Cruz 96-well assay plate (Santa Cruz Biotech.) was first coated with 100 μ L PBS containing either gp120_{BAL} or the oligomannose-Q β antigens (250 ng). The plates were incubated overnight at 4°C. The plate was then washed three times with PBS/0.05% Tween-20 (PBS-T), followed by the addition of 100 μ L 3% (w/v%) BSA in PBS to each well. The plate was incubated at RT for 1 h. The plate was again washed with PBS-T (3x). Next, 2G12 was diluted in blocking buffer (PBS/0.05% Tween-20/1% (w/v %) BSA [PBS-BT]) to a maximum concentration of 200 nM, followed by a 1.5-fold dilution series. For each 2G12 antibody dilution, a total of 100 μ L was added to the appropriate wells. The plate was incubated with 2G12 for 2 h at RT and then washed with PBS-T (3x). Next, a 1:1000 dilution of alkaline phosphatase (AP)-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratory) was made in PBS-BT, with 100 μ L of this diluted secondary antibody added to each well. The plates were incubated with the secondary antibody for 1 h at RT then washed with PBS-T (3x). Finally, the wells were developed with 50 μ L p-nitrophenyl phosphate liquid substrate system (Sigma) for 5 min at RT before quenching the reaction with 30 μ L of 3 M NaOH. The optical density was then measured at a wavelength of 405 nm.

Competitive ELISA General assay conditions, procedures, and buffers solutions were as described above, except as follows. All ELISA wells were coated with gp120_{BAL} (250 ng) overnight, washed and blocked. Serial dilutions of the oligomannose antigens in 50 μ L PBS-BT were added to each well in a 2-fold dilution series. A fixed concentration of 2G12 (0.0625 nM), as determined by ELISA titration,

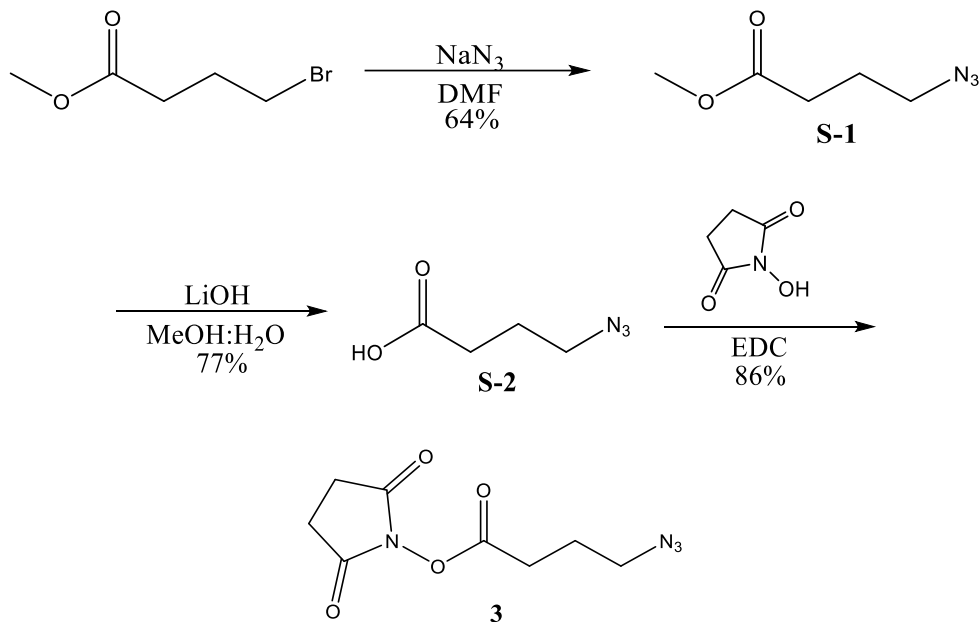
was added to each well (50 μ L/well). The competition ELISA was incubated at RT for 2 h, followed by another series of washes and probed with secondary Ab (alkaline phosphatase (AP)-conjugated goat anti-human IgG antibody) for an additional 1 h. After a final series of washes, the plate was developed as above. 0.0001025

Synthesis of chitobiose-core containing alkyne-linked building blocks



Scheme 2.4 Synthesis of pent-4-ynoic acid 1-oxysuccinimidyl ester (**2**)

4-Pentynoic acid succinimidyl ester (2**).** Compound **2** was prepared according to a previously reported procedure.⁸⁰ Briefly, 4-pentynoic acid (400 mg, 4.0 mmol) and N-hydroxysuccinimide (470 mg, 4.0 mmol) were dissolved in THF (14 mL) at -10°C . N, N'-dicyclohexylcarbodiimide (DCC) (842 mg, 4.0 mmol) was added and the mixture was stirred at -10°C for 2 h. The reaction was stirred at $-10^{\circ}\text{C} \rightarrow 0^{\circ}\text{C}$ overnight. The DCU that formed during the reaction was removed by filtration over a celite pad and the solvent was evaporated under reduced pressure. The residue was dissolved in cold (-20°C) ethyl acetate (10 mL), filtered again, washed with saturated NaHCO_3 (10 mL x 3) and brine (10 mL). The organic layer was dried over Na_2SO_4 , filtered, and the solvent removed under vacuum, affording **2** as a white solid (687 mg, 88%). $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ 2.90 (t, $J = 7.5$, 2H), 2.86 (s, 4H), 2.63 (td, $J = 7.2$ Hz, 2H), 2.08 (t, $J = 2.6$, 1H). $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz): δ 169.43, 167.49, 81.31, 70.48, 30.69, 25.99, 14.49.



Scheme 2.5 Synthesis of succinimidyl-4-azidobutyrate (**3**).

Methyl 4-azidobutyrate (S-1). Compound **S-1** was prepared according to a previously reported procedure.⁸¹ Briefly, to a solution of methyl 4-bromobutyrate (3.0 g, 16.7 mmol) in DMF (4 mL) was added sodium azide (2.2 g, 33.4 mmol). The mixture was vigorously stirred at 80°C overnight. The reaction mixture was diluted with ethyl acetate (50 mL) and washed with saturated NaHCO₃ (20 mL x 3), water (20 mL x 3), and brine. The organic layer was dried over anhydrous Na₂SO₄, filtered, and the solvent was removed under vacuum, yielding **S-1** as a pale yellow liquid (1.53 g, 64%). ¹H-NMR (CDCl₃, 400 MHz): δ 3.68 (s, 3H), 3.35 (t, J = 6.92 Hz, 3H), 2.41 (t, J = 7.4 Hz, 2H), 1.89 (m, 2H). ¹³C-NMR (CDCl₃, 125 MHz): δ 173.51, 52.09, 50.98, 31.26, 24.60.

5-Azidovaleric acid (S-2). Compound **S-2** was prepared according to a previously reported procedure.⁸¹ Briefly, to a solution of LiOH (20 mmol in 5 mL water) and

MeOH (15 mL), **S-1** (1.5 g, 10.48 mmol) was added. The suspension was vigorously stirred overnight at RT. The solvent was removed under vacuum and the remaining residue was diluted in ethyl acetate (50 mL) and washed with 1.0 M HCl (20 mL x 3), water (20 mL x 3), and brine (20 mL). The organic layer was dried over anhydrous Na₂SO₄ and evaporated to dryness under vacuum, yielding **S-2** as a pale yellow liquid (1.05 g, 77%). ¹H-NMR (CDCl₃, 400 MHz): δ 11.12 (s, 1H), 3.39 (t, J = 6.64 Hz, 3H), 2.49 (t, J = 7.2 Hz, 2H), 1.93 (m, 2H). ¹³C-NMR (CDCl₃, 125 MHz): δ 179.67, 50.86, 31.35, 24.33.

Succinimidyl 5-Azidovalerate (11). Compound **11** was prepared according to a previously reported procedure.⁸¹ Briefly, to a mixture of **S-2** (800 mg, 6.2 mmol) and N-hydroxysuccinimide (785 mg, 6.8 mmol) in CH₂Cl₂ (40 mL), 1-(3-dimethylaminopropyl)-3 ethylcarbodiimide hydrochloride (EDC-HCl) (1.31 g, 6.8 mmol) was added. The mixture was stirred at RT overnight. Water (40 mL) was added, and the organic layer was washed with water (40 mL x 3) and brine (40 mL). The organic layer was dried over Na₂SO₄ and evaporated under vacuum, affording **11** as a white solid (1.15 g, 82%). ¹H-NMR (CDCl₃, 400 MHz): δ 3.45 (t, J = 6.96, 2H), 2.84 (s, 4H), 2.73 (t, J = 7.8 Hz, 2H), 2.01 (m, 2H). ¹³C-NMR (CDCl₃, 125 MHz): δ 169.46, 168.35, 50.37, 28.49, 25.98, 24.52.

Synthesis of Man₉GlcNAc₂-Asn-NH₂ (1) The precursor Man₉GlcNAc₂-Asn-NH₂ (**1**) was obtained by enzymatic digestion of soybean agglutinin from soybean flour, followed by chromatographic purification using a method reported previously by our group and others.^{66 35} HRMS (MALDI-FTICR) *m/z* [M + Na]⁺ Calcd for C₇₄H₁₂₅N₄O₅₈Na: 2019.67742; found: 2019.67983.

Synthesis of Man₉GlcNAc₂-Asn-Alkyne (3) To a solution of **1** (20 mg, 10.02 μmol) in 1X PBS (0.2 mL, pH 7.4), was added Alkyne-OSu (20 mg, 102.5 μmol) in DMF (0.2 mL). The reaction mixture was vigorously shaken at RT overnight. The solvent was removed under vacuum and the residue was dissolved in 0.1 M acetic acid and applied to a Sephadex G-10 size exclusion column and purified. The elution was monitored by the phenol-sulfuric acid test and carbohydrate-positive fractions were pooled and concentrated (to ~1 mL) under vacuum. The semi-pure glycans were applied to a C18 solid-phase extraction column (8.5 x 2 cm) and washed with water (3 CVs). The alkyne-tagged glycans were eluted with 5:1 water:MeOH. The pooled eluted glycans were lyophilized yielding **3** as a white powder (15.4 mg, 74%). HRMS (MALDI-FTICR) *m/z* [M + Na]⁺ Calcd for C₇₉H₁₂₉N₄O₅₉Na: 2099.70363; found: 2099.70483.

Synthesis of Man₅₋₉GlcNAc₂-Asn-Alkyne (4). To a solution of **3** (10 mg, 4.82 μmol, final 2 mM solution) in citrate buffer (50 mM, pH 5.6) containing 5 mM CaCl₂, was added α1,2-mannosidase (final 2 ng/μL). The enzymatic digestion was incubated at RT for 30 min while monitoring reaction progress using HPAEC-PAD. When Man₇GlcNAc₂-Asn-Alkyne was the major component, the reaction was quenched by the addition of 0.1 M acetic acid, and the reaction mixture was applied to a G-10 size-exclusion column and eluted with 0.1 M acetic acid. The carbohydrate positive fractions were pooled and lyophilized, yielding **4** as a white powder (7.8 mg, 92%). MALDI-TOF *m/z* [M + Na] **Man₉** Calcd for C₇₉H₁₂₈N₄O₅₉Na: 2099.70363, found: 2099.601. **Man₈** Calcd for C₇₃H₁₁₈N₄O₅₄Na: 1937.65081, found: 1937.629. **Man₇** Calcd for C₆₇H₁₀₈N₄O₄₉Na: 1775.59799, found: 1775.546. **Man₆** Calcd for

C₆₁H₉₈N₄O₄₄Na: 1613.54516, found: 1613.532. **Man₅** Calcd for C₅₅H₈₈N₄O₃₉Na: 1451.49234, found: 1451.471.

Synthesis of Man₅GlcNAc₂-Asn-Alkyne (5). To a solution of **3** (10 mg, 4.82 μmol, final 2 mM) in citrate buffer (50 mM, pH 5.6) containing 5 mM CaCl₂, was added α1,2-mannosidase (final 1 μg/μL). The enzymatic digestion was incubated at 37°C overnight. The reaction was monitored by HPAEC-PAD, and quenched with 0.1 M acetic acid upon complete hydrolysis of the α1,2-Man-Man linkages. The reaction mixture was applied to a G-10 size-exclusion column and eluted with 0.1 M acetic acid. The carbohydrate positive fractions were pooled and lyophilized, yielding **5** as a white powder (6.5 mg, 94%). HR-MS (MALDI-FTICR) *m/z* [M + Na]⁺ Calcd for C₅₅H₈₈N₄O₃₉Na: 1451.49234; found: 1451.49284.

Synthesis of Man₉GlcNAc (6).

Compound **1** (20 mg, 10.02 μmol) was treated with *Arthrobacter* endo-β-N-acetylglucosaminidase (EndoA (WT)) in acetate buffer (50 mM, pH 6.6) at 37°C overnight. Subsequent Sephadex G-10 size-exclusion chromatography and C18 SPE yielded pure Man₉GlcNAc as a white powder (15.9 mg, 95%). HRMS (MALDI-FTICR) *m/z* [M + Na]⁺ Calcd for C₆₂H₁₀₅NO₅₈Na: 1702.55512, found: 1702.55472. ¹H-NMR (400 MHz, D₂O; HOD, δ 4.70): δ 5.28 (s, 1H, H-1, Man), 5.21 (s, 1H, H-1, Man), 5.18 (s, 1H, H-1, Man), 5.10 (d, J = 3.6, 1H, H-1, GlcNAc), 5.02 (s, 1H, H-1, Man), 4.92-4.90 (m, 3H, Man x 3), 4.73 (s, 1H, H-1, Man), 4.64 (s, 1H, H-1, Man), 4.11-3.42 (m, 63H, H-2, 3, 4, 5, 6 of Man and GlcNAc), 1.91 (s, 3H, GlcNAc).

Synthesis of Man₉-Benzylamine (7). A solution of benzylamine (64 mg, 0.601 mmol) and glacial acetic acid (35 mg, 0.601 mmol) was pre-mixed and added in one

portion to a solution of **6** (20 mg, 11.91 μmol) in anhydrous DMSO (1.08 mL). The mixture was heated to 55°C in a water bath for 30 min, and NaCNBH₃ (40 mg, 0.640 mmol) was added in one portion. The reaction was heated at 55°C for 1 h, with LC/MS monitoring. At the completion of the reaction, the mixture was diluted with 0.1 M acetic acid and applied to a Sephadex G-10 column, eluted by 0.1 M acetic acid. Carbohydrate positive fractions were applied to a C18 SPE cartridge and eluted first with water, followed by 5:1 water/MeOH. The purified product was obtained as a white powder (17.7 mg, 84%). (using a modified method from that reported by Lee and co-workers.⁶⁸ HRMS (MALDI-FTICR) m/z [M + Na]⁺ Calcd for C₆₉H₁₁₅N₂O₅₀Na: 1793.63370; found: 1793.63315. ¹H-NMR (400 MHz, D₂O; HOD, δ 4.71): δ 7.35-7.25 (m, 5H, Benzyl), 5.32 (s, 1H, H-1, Man), 5.26 (s, 1H, H-1, Man), 5.25 (s, 1H, H-1, Man), 5.07 (s, 1H, H-1, Man), 4.99-4.96 (m, 3H, Man x 3), 4.74 (s, 1H, H-1, Man), 4.65 (s, 1H, H-1, Man), 4.10-3.47 (m, 67H, H-2, 3, 4, 5, 6 of Man and acyclic GlcNAc), 1.92 (s, 3H, acyclic GlcNAc), 1.79 (s, 2H, -NH-CH₂-Benzyl).

Synthesis of Man₉-NH₂ (8). To a solution containing **7** (10 mg, 5.7 μmol) in 50% MeOH (10 mL) was added a catalytic amount of 10% Pd/C. The benzyl group was removed under hydrogen atmosphere (35 psi) using a catalytic hydrogenation apparatus at RT for 2 h. The reaction mixture was filtered and the solvent removed under vacuum. The resulting residue was dissolved in water and lyophilized. The resulting white powder (9.6 mg, quant.) and used without further purification. HRMS (MALDI-FTICR) m/z [M + Na]⁺ Calcd for C₆₂H₁₀₈N₂O₅₀Na: 1703.58675; found: 1703.58788.

Synthesis of Man9-Alkyne (9). To a solution of 8 (8 mg, 4.8 μmol) in 1X PBS (0.1 mL, pH 7.4), was added 2 (8 mg, 41 μmol) in DMF (0.1 mL). The reaction mixture was vigorously shaken at RT overnight. The solvent was removed under vacuum and the residue was dissolved in 0.1 M acetic acid and applied to a Sephadex G-10 size-exclusion column and purified. The elution was monitored by the phenol-sulfuric acid test and carbohydrate-positive fractions were pooled and concentrated (to ~1 mL) under vacuum. The semi-pure glycans were applied to a C18 solid-phase extraction column (8.5 x 2 cm) and washed with water (3 CVs). The alkyne-tagged glycans were eluted with 5:1 water/MeOH. The pooled, eluted glycans were lyophilized yielding **9** as a white powder (7.3 mg, 85%). HRMS (MALDI-FTICR) m/z $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{67}\text{H}_{112}\text{N}_2\text{O}_{51}\text{Na}$: 1783.61297; found: 1783.61258.

2.5 References

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Chapter 3: Site-Selective Chemoenzymatic Glycosylation of an HIV-1 Polypeptide Antigen with Two Distinct N-Glycans via an Orthogonal Protecting Group Strategy

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3.1 Introduction

Glycosylation can profoundly affect a protein's structure, stability, intracellular trafficking, and biological functions.¹⁻⁴ In the case of the human immunodeficiency virus type 1 (HIV-1), the heavy N-linked glycosylation of the outer envelope glycoprotein gp120 represents a major defensive mechanism for the virus to evade host immune attack. The N-glycans assembled by the host's synthetic machinery are viewed as "self" and are weakly immunogenic.^{5,6} Nevertheless, the recent discovery of a new class of broadly neutralizing antibodies (bNAbs) that recognize both conserved N-glycans and a segment of peptide in the variable (V1V2 and V3) regions of gp120 as an integrated epitope strongly suggest that the defensive glycan shield of the virus and, in particular, the unique HIV-1 glycopeptide antigens, can serve as important targets for HIV-1 vaccine design.⁷⁻¹¹ PG9 is a broadly neutralizing antibody (bNAbs) isolated from HIV-1 infected patients that can neutralize HIV-1 primary strains with significant breadth and potency. Mutational, biochemical and structural studies suggest that PG9 recognizes a strand of peptide and two conserved N-glycans in the V1V2 domain.^{12,13} The PGT series neutralizing antibodies, including PGT128 and PGT121, also follow a similar antibody-antigen

recognition mode involving unique N-glycans and a protein segment centered at the V3 region as targets.¹⁴⁻¹⁶ These discoveries have stimulated great interest in the chemical and chemoenzymatic synthesis of the putative HIV-1 glycopeptide epitopes, aiming at fine characterization and reconstitution of the precise neutralizing epitopes for HIV vaccine design.¹⁷⁻¹⁹

Major progress has been made in recent years in the total chemical synthesis of large glycopeptides and even homogeneous glycoproteins.^{18,20,21} Nevertheless, each complex glycopeptide target could present a special challenge that may require significant optimization of the synthetic strategies in terms of the coupling efficiency for critical ligation steps and the compatibility of protecting group manipulations. On the other hand, the chemoenzymatic approach that exploits the endoglycosynthase-catalyzed transglycosylation for transfer of large oligosaccharide *en bloc* to a GlcNAc-peptide or protein using a glycan oxazoline as donor substrate is emerging as a promising method for expeditious synthesis of complex glycopeptides and for glycosylation remodeling of glycoproteins as well.²²⁻³⁰ This method is highly convergent and permits independent manipulations of the glycan and polypeptide portions. We have recently applied this chemoenzymatic method for the synthesis of a series of complex HIV-1 V1V2 glycopeptides that enabled the characterization of the glycan specificity of the bNAb PG9.¹⁷ However, construction of complex glycopeptides carrying two or more different N-glycans by this method remains a difficult task, as the endoenzymes are unable to distinguish between the GlcNAc acceptors at different sites in a polypeptide. As a result, a careful HPLC separation of the partially glycosylated intermediates was required in order to introduce two

different N-glycans at the pre-determined sites,¹⁷ which was tedious and would be difficult to generalize for other peptides.

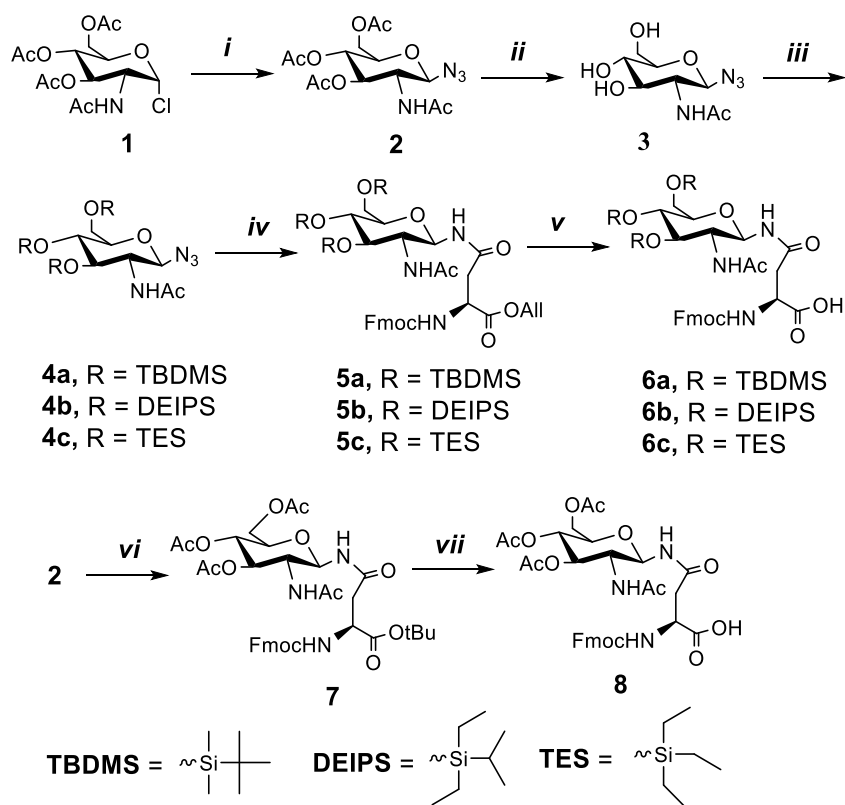
To address this fundamental problem, we describe in this paper an orthogonal protecting group strategy for construction of glycopeptides carrying two distinct N-glycans. We reasoned that introduction of two orthogonally protected GlcNAc-Asn building blocks during automated solid-phase peptide synthesis (SPPS) would allow selective deprotection of the GlcNAc primers at different stages, such that different N-glycans could be sequentially installed in a polypeptide by glycosynthase-catalyzed transglycosylation. We found that a GlcNAc-Asn building block, temporarily protected by O-diethylisopropylsilyl (DEIPS) groups, was particularly efficient. The protecting group strategy was stable during synthesis but could be readily deprotected simultaneously during acidic global deprotection and retrieval of the peptide from the resin to introduce a free GlcNAc-Asn primer. We demonstrate that the combined use of the DEIPS-protected and O-acetylated GlcNAc-Asn building blocks, coupled with the enzymatic sequential glycosylation, enables a highly efficient and quick synthesis of an array of HIV-1 V1V2 glycopeptides carrying distinct N-glycans.

3.2 Results and Discussion

Synthesis of orthogonally protected GlcNAc-Asn building blocks. We envisioned that a GlcNAc-Asn building block carrying an acid-sensitive protecting group such a silyl group could be combined with the common O-acetyl protected GlcNAc-Asn building block to achieve site-selective glycosylation, as an O-silyl group

could be simultaneously removed during the global peptide deprotection to provide the free GlcNAc acceptor for the attachment of the first N-glycan via enzymatic transglycosylation. The O-acetyl protected GlcNAc could be unmasked to allow a second glycosylation with a different N-glycan. To test the feasibility of this approach, we first synthesized the GlcNAc-Asn building blocks in which the GlcNAc moiety was protected with three different types of silyl groups that are supposed to possess variable acidic sensitivity (Scheme 3.1). The β -glycosyl azide (**3**) was prepared by treatment of the α -glycosyl chloride (**1**)³¹ with sodium azide under a phase-transfer catalysis conditions, followed by de-O-acetylation with a catalytic amount of MeONa in MeOH. Protection of the free hydroxy groups in **3** with three types of silyl groups was achieved by treatment of **3** with *tert*-butyldimethylsilyl triflate (TBDMSOTf), diethylisopropylsilyl triflate (DEIPSOTf), or triethylsilyl triflate (TESOTf), respectively, giving the corresponding silyl protected glycosyl azide derivatives (**4a-c**) in quantitative yield. It should be noted that, given the highly hindered nature of the protecting groups, we found that using the highly active silyl triflate was essential for high-yield conversions, as the use of the less active silyl chloride derivatives resulted in incomplete reactions on the secondary hydroxy groups.³² Conversion of the anomeric azide group of **4a-c** into a primary amine was accomplished via palladium-catalyzed reduction under a hydrogen atmosphere to give the corresponding β -glycosyl amine, which was coupled *in situ* with Fmoc-Asp-OAll using HATU/DIPEA as the coupling reagent to afford the silyl-protected derivatives (**5a-c**) in moderate to excellent yields. We observed that the TES-protected derivative (**5c**) was much less stable than bulky silyl derivatives (**5a** and **5b**), as partial deprotection of the TES groups occurred

during the coupling reactions (a by-product that missed one TES group was isolated in 33% yield). De-O-allylation at the C-terminal carboxyl groups of **5a-c** was achieved under the catalysis of Pd(PPh₃)₄/PhSiH₃ to give the glycosylamino acid building blocks (**6a-c**) in 96%, 95%, and 90% isolated yields, respectively. It should be noted that silica gel purification, while successful for **6a** and **6b**, proved to be too acidic for the TES protected building block (**6c**). Thus, **6c** was purified by LH-20 size-exclusion chromatography using 2:1 DCM/MeOH as the eluent. All glycosylamino acid building blocks and intermediates were characterized by NMR and MS analysis. We observed that the J_{1,2} coupling constants (~5 Hz) of the anomeric protons of the silyl-protected β-glycosyl azides (**4a-c**) were significantly smaller than a typical J_{1,2} coupling constant (usually > 9 Hz) found in the acetylated β-glycosyl azide (**2**). This data suggests that the bulky silyl ether protecting groups may induce a conformational change in the sugar ring structures from a typical ⁴C₁ chair conformation to a twisted half-chair or a skew-boat conformation. This observation is consistent with previously reported results for similar silylated glycopyranoside derivatives.³³ On the other hand, the O-acetyl protected building block (**8**) was synthesized by direct coupling of the glycosyl azide (**2**) and an aspartic acid derivative (Fmoc-Asp-OtBu) with triethyl phosphine to give **7**, followed by removal of the t-butyl ester group with formic acid (Scheme **3.1**).



Scheme 3.1 Synthesis of GlcNAc-Asn building blocks carrying silyl (acid-labile) and acetyl (base-labile) protecting groups. Reaction conditions: i. NaN_3 , water/ CH_2Cl_2 , Bu_4NHSO_4 , RT; 95%; ii. 1) cat. MeONa , MeOH ; 2) Dowex H^+ , RT, quantitative in two steps; iii. Silyl triflate, 2,6-lutidine, 40°C , quant.; iv. 1) Pd/C , H_2 , MeOH , RT; and 2) Fmoc-Asp-OAll, HATU, DIPEA, DMF, RT, 74% (**5a**), 73% (**5b**), and 38% (**5c**); v. $\text{Pd}[\text{P}(\text{Ph})_3]_4$, PhSiH_3 , CH_2Cl_2 , RT, 94% (**6a**), 95% (**6b**), and 93% (**6c**); vi. Fmoc-Asp-OtBu, $\text{P}(\text{Et})_3$, CH_2Cl_2 , -78°C to RT, 81%; vii. 96% formic acid, RT, 100%.

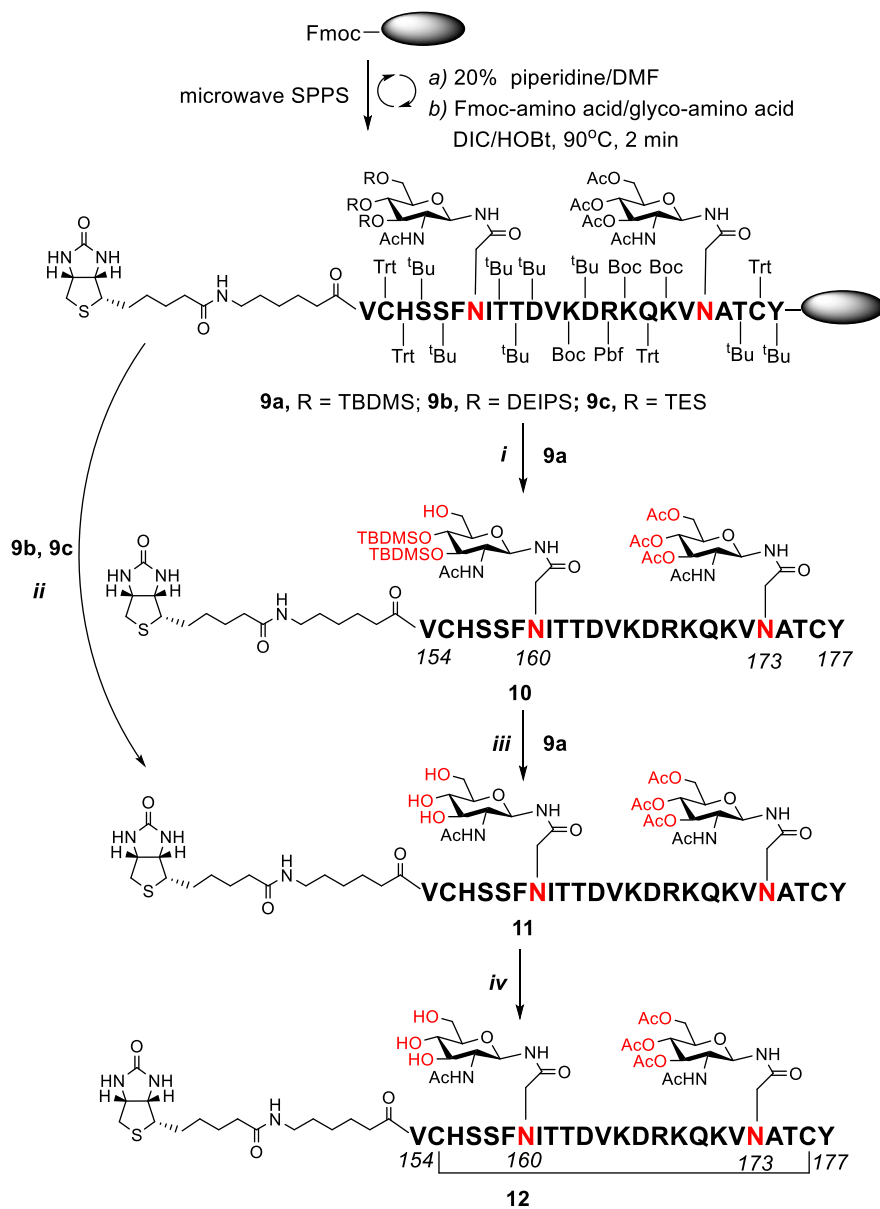
Use of the GlcNAc-Asn building blocks for glycopeptide synthesis. The usefulness of the orthogonally protected GlcNAc-Asn building blocks (**6** and **8**) for the synthesis of complex glycopeptides carrying different N-glycans was examined

by the synthesis of several HIV-1 V1V2 glycopeptide antigens that were proposed as the neutralizing epitope for the bNAb PG9.^{13,17} We have recently designed and synthesized an array of cyclic 24-mer V1V2 glycopeptides bearing different glycan structures at the N160 and N173 glycosylation sites.¹⁷ The synthesis, coupled with antibody binding studies, has enabled the characterization of the fine glycan specificity of antibody PG9 in antigen recognition. That is, a Man₅GlcNAc₂ glycan at the N160 site and a sialylated complex type N-glycan at the N173 glycosylation site (for the ZM109 strain) coupled with a segment of V1V2 peptide, constitutes the minimal epitope of PG9. However, the previous chemoenzymatic synthesis of these glycopeptide epitopes had to rely on tedious separation of the mono-glycosylated intermediates in order to introduce two distinct N-glycans.¹⁷ Here we sought to add the acid-labile silyl-protected GlcNAc-Asn building block at N160 site first and then the base-labile acetylated GlcNAc-Asn building block at the N173 site to achieve sequential glycosylations with distinct N-glycans at the two sites. Thus, automated solid-phase peptide synthesis (SPPS) incorporating the two distinctly protected GlcNAc-Asn building blocks led to the assembling of the resin-bound fully protected peptide (**9a-c**) (Scheme 3.2).

A biotin-tag was introduced at the N-terminus to facilitate detection and immobilization for binding studies. The sensitivity of the silyl protecting groups during global peptide deprotection was first examined with cocktail R (TFA/thioanisole/EDT/anisole, 90/5/3/2, v/v), a common cocktail reagent for global deprotection and retrieval of the polypeptide from the resin. Aliquots were taken at intervals and the product was analyzed by HPLC. It was observed that treatment of

the TBDMS protected glycopeptide-resin (**9a**) with cocktail R with an extended reaction time (up to 4 h) led to only partial de-silylation to give a glycopeptide product with only one of the three TBDMS group being removed (assessed by LC-MS analysis), which was assumed to be glycopeptide **10** with the deprotection at the 6-position. In contrast, deprotection of the DEIPS- and TES-protected glycopeptide-resin, **9b** and **9c**, respectively, was readily achieved using cocktail R or even the milder cocktail K (TFA/phenol/H₂O/thioanisole/EDT, 82.5/5/5/5/2.5, v/v) within 2h to obtain the desired glycopeptide intermediate (**11**), in which a free GlcNAc was installed at the N160 site while the O-acetyl protected GlcNAc at the N173 site was still intact. The resistance of the O-TBDMS groups in **9a** to acid-catalyzed deprotection was unexpected, as previous reports have shown that the TBDMS groups on a corresponding disaccharide moiety could be efficiently removed by a similar acid treatment during peptide deprotection.^{32,33} To verify whether the phenomenon was special for the peptide context, we treated the TBDMS-protected GlcNAc-Asn building block (**6a**) with cocktail R and found that the O-TBDMS groups could be completely removed within 1 h. This result suggests that the resistance of the O-TBDMS groups in the glycopeptide-resin (**9a**) to acid-catalyzed deprotection is likely specific for the polypeptide context. The reason is not clear. Interestingly, complete de-silylation of **10** was readily achieved by its treatment with TBAF in pyridine/AcOH to give **11** in excellent yield. Taken together, the experimental data suggest that the DEIPS protecting group, which is much more stable than the TES group during building block synthesis and can be readily removed during the global polypeptide deprotection, is the best among the three for

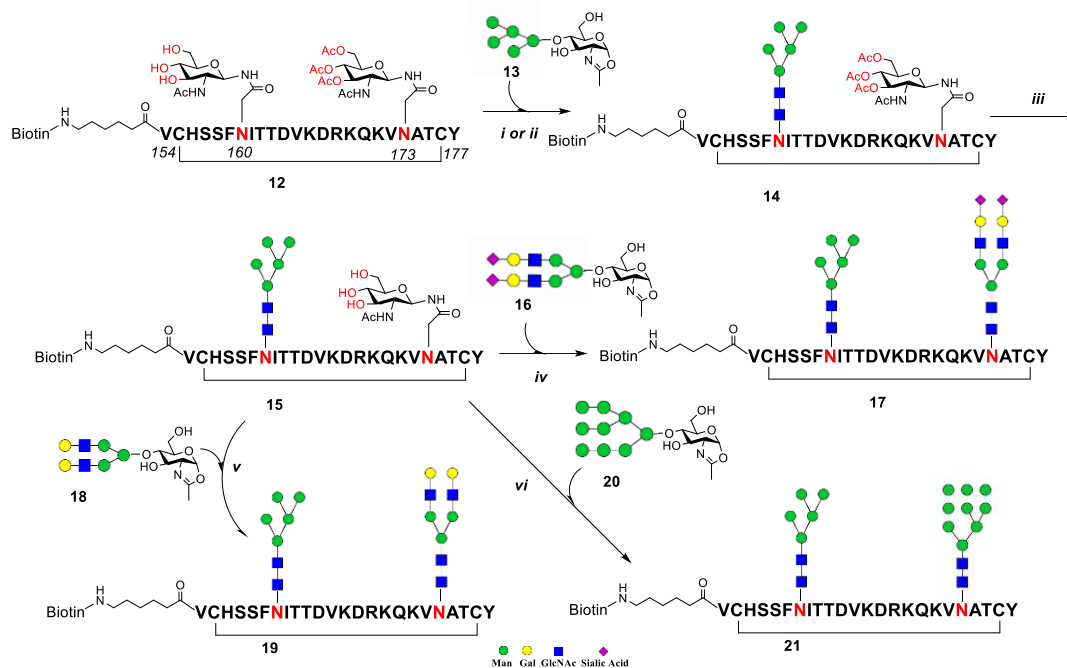
introducing a free GlcNAc-Asn building block in SPPS. Finally, cyclization of the linear glycopeptide (**11**) was achieved by treatment with 20% aqueous DMSO to give the cyclic glycopeptide (**12**), where a free GlcNAc was located at N160 and a temporarily acetyl-protected GlcNAc moiety is installed at the N173 site (Scheme 3.2).



Scheme 3.2 Synthesis of GlcNAc-peptide precursor using the orthogonally protected GlcNAc-Asn building blocks. Reaction conditions: i. Cocktail R, 4h, 39% (total isolated yield, only one TBDMS removed); ii. Cocktail R, 2h, 41% (total isolated yield, complete removal of all three silyl ether groups), or Cocktail K, 38% (total isolated yield, complete removal of all three silyl ether groups); iii. Bu₄NF, pyridine/AcOH; iv. phosphate buffer (pH, 7.2), 20% DMSO, 94%.

Convergent synthesis of HIV-1 V1V2 glycopeptide epitopes of antibody PG9.

With the key intermediate (**12**) at hand, we first tested the transfer of a Man₅GlcNAc glycan to the GlcNAc moiety at N160 using mutant endoglycosidase D (N332Q) from *Streptococcus pneumoniae*.³⁴ Unexpectedly, incubation of **12** with Man₅GlcNAc oxazoline (**13**) proceeded very inefficiently to give only trace amounts of the transglycosylation product (**14**) (Scheme **3.3**). This observation was surprising as the N322Q mutant of Endo-D was previously found to be highly efficient to transfer Man₅GlcNAc oxazoline (**13**) to a similar cyclic glycopeptide carrying two free GlcNAc moieties to give a doubly glycosylated peptide.¹⁷ In contrast, we found that glycosynthase mutants of Endo-M from *Mucor hiemalis*,^{35,36} such as the N175Q mutant, were efficient to catalyze the transfer of a Man₅GlcNAc glycan to the free GlcNAc moiety in acceptor **12**, giving glycopeptide **14** in excellent yield (Scheme **3.3**).



Scheme 3.3 Convergent chemoenzymatic installation of two distinct N-glycans in the V1V2 cyclic peptide. Reaction conditions: i. EndoD-N322Q, donor/acceptor = 8:1, Tris buffer (80 mM, pH 7.2), RT, <5%; ii. EndoM-N175Q, donor/acceptor = 8:1, Tris buffer (80 mM, pH 7.2), RT, 85%; iii. 2.5 % aq. hydrazine, RT; iv. EndoM-N175Q, donor/acceptor = 4:1, Tris buffer (80 mM, pH 7.2), RT, 92%; v. EndoM-N175Q, donor/acceptor = 4:1, Tris buffer (80 mM, pH 7.2), RT, 95%; vi. EndoM-N175Q, donor/acceptor = 4:1, Tris buffer (80 mM, pH 7.2), RT, 91%.

In order to test if a proximal acetylated GlcNAc moiety caused the low yield in enzymatic transglycosylation with the Endo D mutant (N322Q), we synthesized the corresponding linear peptide (**A 3.1**, see appendix) by reduction of **12** with DTT followed by alkylation with iodoacetamide (Scheme **A 3.1**, Figure **A 3.1**, appendix). Interestingly, we found that both EndoD-N322Q and EndoM-N175Q mutants were able to efficiently glycosylate the unmasked GlcNAc moiety in the linear peptide (**A**

3.1) to give the corresponding glycopeptide (**A 3.2**) (Scheme **A 3.1** and Figure **A 3.2**, appendix). This result suggests that the O-acetylated GlcNAc moiety present in the cyclic peptide (**12**) may provide steric hindrance and/or an unfavorable hydrophobic interaction with the Endo D mutant, leading to a much reduced catalytic activity, while the Endo M mutant seems more flexible for the presence of the bulky/hydrophobic Ac₃GlcNAc-Asn moiety proximal to the free GlcNAc acceptor in enzymatic transglycosylation.

After the installation of the first N-glycan (Man₅GlcNAc₂) at the N160 site, the O-acetyl protecting groups on the GlcNAc moiety at the N173 site were then removed by treatment with 2.5% aqueous hydrazine to give glycopeptide **15**, in which the free GlcNAc moiety at the N173 site is now ready for introduction of a different N-glycan. Installation of a sialylated complex type N-glycan at the N173 site was achieved by EndoM-N175Q catalyzed transglycosylation of **15** with sialylated glycan oxazoline **16** to obtain glycopeptide **17**. Transglycosylation of **15** with glycan oxazoline **18**, which lacks the sialic acids, gave the corresponding asialylated glycopeptide (**19**). Moreover, enzymatic transglycosylation with a high mannose type N-glycan oxazoline, the Man₉GlcNAc-oxazoline (**20**), gave the glycopeptide (**21**). Thus, this convergent approach permits a quick construction of various glycopeptides from a common precursor. In all the cases, the enzymatic reaction went smoothly and a quantitative conversion was achieved by using an excess of glycan oxazolines, which could be recovered as free N-glycans during HPLC purification and could be converted back into the glycan oxazoline substrate in one step.^{37,38} The

transglycosylation products were purified by HPLC, and the purity and identity were confirmed by ESI-MS analysis (Figure 3.1).

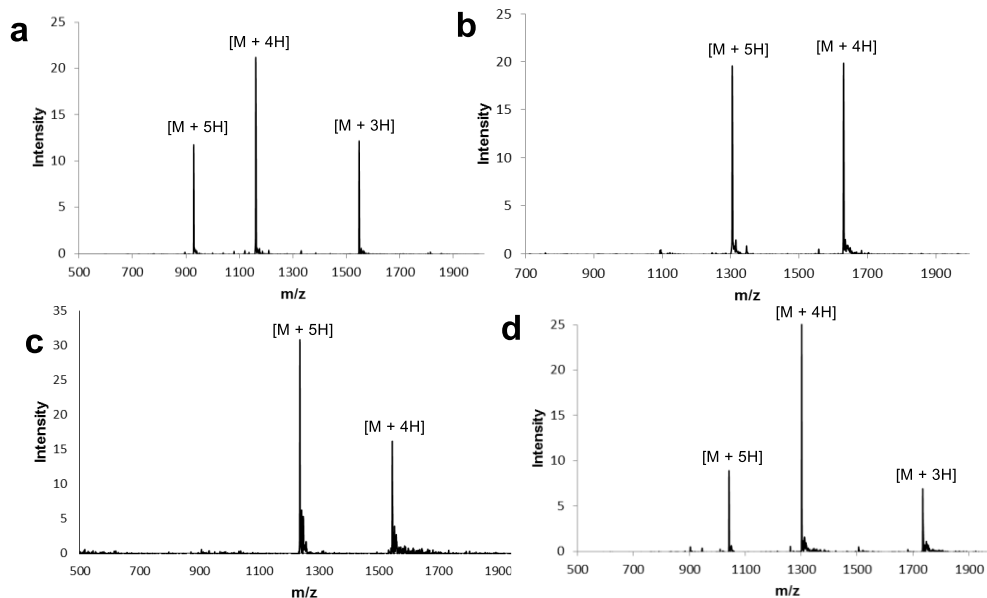


Figure 3.1 ESI-MS Characterization of doubly glycosylated glycopeptides. a) Compound **15**, b) Compound **17**, c) Compound **19**, d) Compound **21**.

SPR analysis of the interactions between antibody PG9 and the synthetic glycopeptides. The binding of the synthetic glycopeptides with antibody PG9 was probed by surface plasmon resonance (SPR) analysis through immobilization of the biotinylated glycopeptides on a streptavidin chip. The results are summarized in Figure 3.2. The data indicate that the glycopeptide **17**, which carries a $\text{Man}_5\text{GlcNAc}_2$ glycan at the N160 and a sialylated N-glycan at N173 site, has the highest affinity among all the synthetic V1V2 glycopeptides, with an apparent K_D of 67 nM. Removal of the sialic acid residues in **17** resulted in more than 39-fold decrease of the affinity for antibody PG9, as demonstrated by a significant increase of the K_D (2.65

μM) for the asialylated glycopeptide (**19**). Interestingly, installation of a full-size, non-sialylated high-mannose glycan at the secondary glycosylation site, as demonstrated by glycopeptide **21**, resulted in drastic decrease of the affinity for PG9 ($K_D = 996 \text{ nM}$), suggesting that a bulky N-glycan at the secondary site may conflict the interaction of the antibody with the neutralizing epitope. These results are consistent with the previously reported affinity data where the PG9 antibody Fab fragment was used for the binding analysis,¹⁷ and the more recent crystal structural analysis of PG9 and PG16 in complex with sialylated V1V2 domain.³⁹ The experimental data indicated that sialylation of the secondary N-glycan is essential for the high-affinity PG9-glycopeptide epitope interactions.

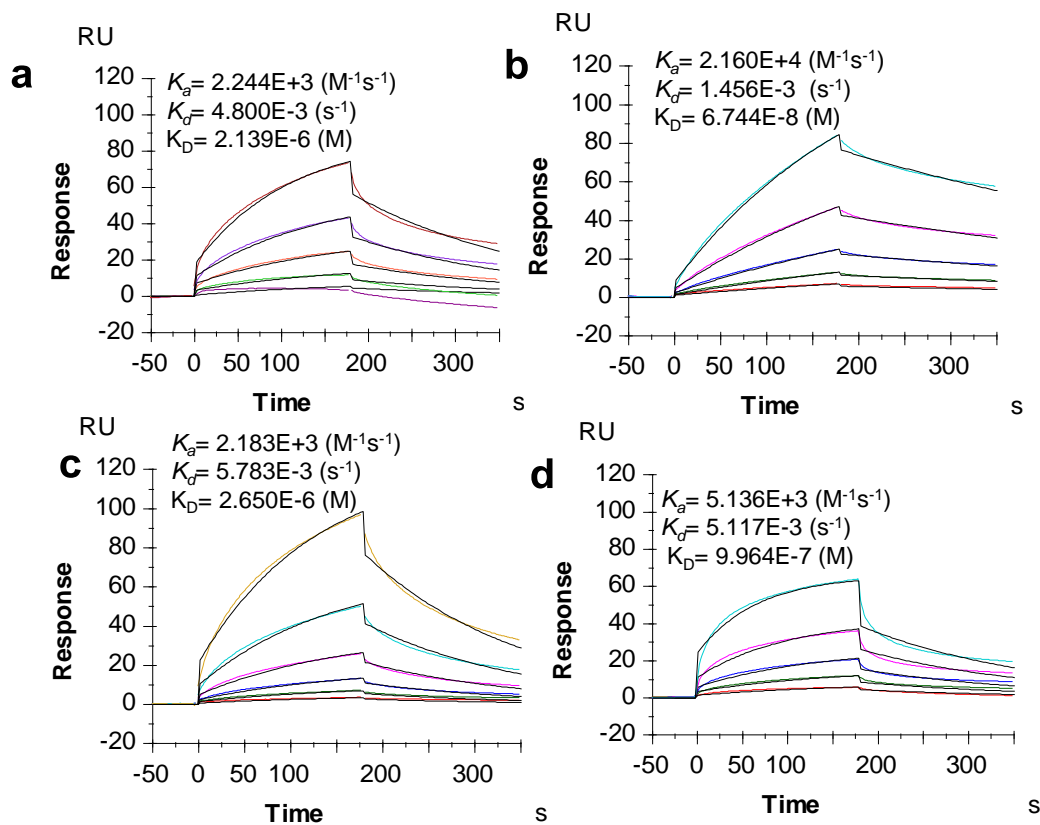


Figure 3.2 SPR analysis of PG9 IgG recognition of synthetic V1V2 glycopeptides. Biotinylated glycopeptides were immobilized on streptavidin-coated chips and whole-IgG PG9 was flowed through as the analyte. a) Compound **15** (N160 = M5 / N173 = GN) b) Compound **17** (N160 = M5 / N173 = SCT) c) Compound **19** (N160 = M5 / N173 = CT) d) Compound **21** (N160 = M5 / N173 = M9).

3.3 Conclusion

A facile chemoenzymatic strategy that permits sequential enzymatic glycosylations to introduce distinct N-glycans into a polypeptide is described. This method exploits two orthogonally protected GlcNAc-Asn building blocks in SPPS. Among several acid-sensitive silyl protecting groups tested, the diethylisopropylsilyl (DEIPS) protecting group was found to be the most efficient, which is stable during building block synthesis but can be easily removed during the global peptide deprotection step, while the O-acetyl protecting groups can be selectively removed at a later stage. The usefulness of this method was exemplified by the efficient synthesis of the HIV-1 V1V2 glycopeptide neutralizing epitopes carrying two distinct N-glycans. The uniqueness of this synthetic strategy is its high convergence and efficiency. This method should be equably applicable for convergent synthesis of other complex glycopeptides carrying multiple distinct N-glycans.

3.4 Acknowledgements

C.L. Toonstra performed all experiments and associated data collection. M.N. Amin assisted with SPR data collection.

3.5 Experimental

General Methods and Materials

Endo D^{N322Q} and Endo M^{N175Q} mutants were produced by the reported procedures^{34,35}. ¹H and ¹³C NMR spectra were collected on either a 500 MHz or 400 MHz NMR spectrometer. The chemical shifts (δ) were assigned in parts per million (ppm) rounded to the nearest 0.01 for ¹H NMR and 0.1 for ¹³C NMR. Complete proton assignment was determined by two-dimensional correlational experiments (COSY (¹H-¹H)). The ¹³C NMR was measured at 100 MHz. Analytical and preparative reverse-phase HPLC (RP-HPLC) purifications were carried out using an HPLC system equipped with a UV detector, using a C18 column at either a flow rate of 0.5 mL/min (analytical) or 4 mL/min (preparative) using MeCN containing 0.1% TFA at 40 °C. Glycopeptides were detected at two wavelengths (214 and 280 nm). Mass spectrometry data for peptides was collected using either a single quadrupole mass spectrometer or a LTQ- LC/MS tandem quadrupole mass spectrometer. High-resolution (HR) mass spectra were collected with an electrospray ionization time-of-flight instrument (ESI-TOF) equipped with an ESI source and a time-of-flight (TOF) detector. Glycopeptides were analyzed using an analytical C18 column at a flow rate of 0.4 mL/min at 50°C using MeCN containing 0.1% formic acid at a gradient of 5-95%B in 10 min. Peptide synthesis was performed using a microwave-assisted peptide synthesizer. Surface plasmon resonance (SPR) analysis was performed at 25 °C. Biotinylated glycopeptides were immobilized using a streptavidin-bound sensor chips in a solution of 0.1 M HEPES, 0.15 M NaCl, 0.5% v/v surfactant P20, pH 7.4. The substrates were immobilized manually by injecting the samples until 20-30 RU

was achieved (low-loading). PG9 was injected over four cells at two-fold increasing concentrations at a flow rate of 50 $\mu\text{L}/\text{min}$ for 3 min. Dissociation was allowed to occur over a period of 5 min. The chip was regenerated by injection of 3 M MgCl_2 at a flow rate of 50 $\mu\text{L}/\text{min}$ for 3 min followed by injection of the running buffer for 5 min. Data was collected at a rate of 10 Hz. SPR evaluation software was used to generate the data, applying a 1:1 Langmuir binding model.

2-Acetamido-3, 4, 6-tri-O-acetyl-1, 2-deoxy- β -D-glucopyranosyl azide (2). A solution of **1**³¹ (50 g, 137 mmol) in DCM (50 mL) was mixed with a solution of sodium azide (25 g, 391 mmol) and tetrabutylammonium hydrogen sulfate (133 g, 391 mmol) in saturated sodium bicarbonate (50 mL). The biphasic mixture was stirred vigorously for 2 h. Upon completion of the reaction as indicated by TLC, the organic layer was separated, dried with Na_2SO_4 , and filtered. The filtrate was concentrated and the residues was crystallized from ethanol/hexane to give **2**³¹ (50 g, quant. yield) as a white crystal. ^1H NMR (500 MHz, CDCl_3): δ 1.99, 2.03, 2.04, 2.10 (s each, 3H each, 3 \times OAc and NHAc), 3.84 (m, 1H, H-5), 3.95 (m, 1H, H-2), 4.16-4.29 (m, 2H, H-6), 4.85 (d, 1H, $J = 9.2$ Hz, H-1), 5.10 (t, 1H, $J = 7.9$ Hz, H-3), 5.12 (t, 1H, $J = 8.2$ Hz, H-4), 6.22 (d, 1H, $J = 9.0$ Hz, NH). ^{13}C NMR (100 MHz, CDCl_3): δ 171.0, 170.9, 170.8, 169.5, 88.6, 74.1, 72.4, 68.4, 62.1, 54.3, 23.4, 20.9, 20.8, 20.8. MS (ESI) m/z $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{20}\text{N}_4\text{O}_8$: 372.13; Found 373.23. The ^1H and ^{13}C NMR data were consistent with the reported data³¹.

2-Acetamido-1, 2-deoxy- β -D-glucopyranosyl azide (3). A solution of **2** (10 g, 27 mmol) in MeOH (20 ml) containing a catalytic amount of MeONa (79 mg, 2.7 mmol) was stirred at 25 °C for 2 h. Then Dowex (H⁺ form) was added, and the mixture was filtered. The filtrate was concentrated to dryness to afford **3** (6.65 g, quant. yield) as a white solid. The product was used for the next step without further purification. ¹H NMR (500 MHz, CDCl₃): δ 1.99 (s, 3H, -NHAc), 3.30-3.36 (m, 3H, H-5, H-6), 3.45 (t, J = 8.5 Hz, 1H, H-3), 3.65-3.72 (m, 2H, H-2, H-4), 3.89 (d, J = 12.3 Hz, 1H, H-1), 4.50 (d, J = 9.3 Hz, 1H, -NHAc), 4.84 (s, 3H, 3 \times OH). ¹³C NMR (100 MHz, CDCl₃): δ 23.0, 56.9, 62.8, 71.9, 75.9, 80.5, 90.3, 173.9. MS (ESI) *m/z* [M + H]⁺ Calcd for C₈H₁₄N₄O₅: 246.10; Found 246.52. The ¹H and ¹³C NMR data are consistent with the previously reported data ⁴⁰.

2-Acetamido-3, 4, 6-tri-O-tert-butyldimethylsilyl-1, 2-deoxy- β -D-glucopyranosyl azide (4a). To a solution of **3** (1 g, 41 mmol) in 2, 6-lutidine (4 mL) at 0 °C was added *tert*-butyldimethylsilyl trifluoromethanesulfonate (1.8 g, 7.4 mmol). The solution was stirred for 1 h on ice, then heated to 40°C and stirred overnight. The solvent was removed and the product was purified on silica (1:1 hexane: ethyl acetate), yielding **4a** (2.4 g, quant.) as a white solid. ¹H NMR (500 MHz, CDCl₃): δ 0.03-0.12 (m, 18H, 6 Si-CH₃), 0.87-0.91 (m, 27H, 3 *t*-Bu), 1.93 (s, 3H, NHAc), 3.68 (m, 1H, H-3), 3.75-3.84 (m, 2H, H-6a,b), 3.93 (m, 1H, H-4), 3.98 (m, 1H, H-2), 4.20 (t, J = 9.4 Hz, 1H, H-5), 5.04 (d, J = 5.0 Hz, 1H, H-1), 6.67 (d, J = 9.2 Hz, 1H, -NHAc). ¹³C NMR (100 MHz, CDCl₃): δ 18.1, 18.2, 18.5, 23.6, 25.9, 26.0, 26.1, 50.4,

63.3, 68.6, 70.8, 80.1, 87.5, 168.9. HRMS (ESI-TOF) m/z $[M + Na]^+$ Calcd for $C_{26}H_{56}N_4O_5Si_3Na$: 611.3456; Found 611.3447.

2-Acetamido-3, 4, 6-tri-O-diethylisopropylsilyl-1, 2-deoxy- β -D-glucopyranosyl azide (4b). To a solution of **3** (1 g, 41 mmol) in 2, 6-lutidine (4 mL) at 0 °C was added diethyl(isopropyl)silyltrifluoromethanesulfonate (2.1 g, 7.4 mmol). The solution was stirred for 1 h on ice, then heated to 40°C and stirred overnight. The solvent was removed and the product purified on silica (1:1 hexane: ethyl acetate), yielding **4b** (2.6 g, quant.) as a white solid. 1H NMR (500 MHz, $CDCl_3$): δ 0.621-0.720 (m, 15H, Si- CH_2 , Si-CH), 0.976-1.056 (m, 36H, 4 Si- CH_3), 1.964 (s, 3H, $NHAc$), 3.801 (m, 1H, H-3), 3.835-3.923 (m, 2H, H-6), 4.036 (m, 1H, H-4), 4.052 (m, 1H, H-2), 4.289 (t, $J = 9.35$ Hz, 1H, H-5), 5.09 (d, $J = 5.0$ Hz, 1H, H-1), 6.77 (d, $J = 9.35$ Hz, 1H, $-NHAc$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 3.1, 3.2, 3.4, 3.4, 3.5, 4.5, 6.7, 6.9, 6.9, 6.9, 7.0, 12.6, 12.63, 12.7, 13.3, 17.0, 17.2, 17.2, 17.3, 17.3, 23.3, 50.5, 63.1, 68.5, 70.6, 80.1, 87.4, 168.7. HRMS (ESI-TOF) m/z $[M + Na]^+$ calcd for $C_{29}H_{62}N_4O_5Si_3Na$: 653.3926; Found 653.3927.

2-Acetamido-3, 4, 6-tri-O-triethylsilyl-1, 2-deoxy- β -D-glucopyranosyl azide (4c).

To a solution of **3** (1 g, 41 mmol) in 2, 6-lutidine (4 mL) at 0°C was added triethylsilyltrifluoromethanesulfonate (1.9 g, 7.4 mmol). The solution was stirred for 1 h on ice, then heated to 40°C and stirred overnight. The solvent was removed and the product purified on silica (1:1 hexane: ethyl acetate), yielding **4c** as a white solid (2.4 g, quant.). 1H NMR (500 MHz, $CDCl_3$): δ 0.58-0.68 (m, 18H, 6 Si- CH_2CH_3), 0.94-

1.00 (m, 27H, 9 Si-CH₂CH₃), 1.96 (s, 3H, NHAc), 3.74 (t, *J* = 6.4 Hz, 1H, H-3), 3.79-3.81 (m, 2H, H-6), 3.92 (m, 1H, H-4), 3.94 (m, 1H, H-2), 4.16 (t, *J* = 10.8 Hz, 1H, H-5), 5.02 (d, *J* = 5.1 Hz, 1H, H-1), 6.59 (d, *J* = 9.2 Hz, 1H, -NHAc). ¹³C NMR (100 MHz, CDCl₃): δ 4.6, 4.8, 4.8, 6.9, 6.9, 6.9, 23.6, 51.5, 62.8, 68.9, 71.4, 80.2, 87.6, 169.1. HRMS (ESI-TOF) *m/z* [M + Na]⁺ Calcd for C₂₆H₅₆N₄O₅Si₃Na: 611.3456; Found 611.3448.

N^o-(2-Acetamido-3,4,6-tri-O-tert-butyldimethylsilyl-2-deoxy-β-D-glucopyranosyl-N^o-(9-fluorenylmethyloxycarbonyl)-L-asparagine-allyl ester

(5a). To a solution of **4a** (1 g, 1.7 mmol) in MeOH (40 mL) was added palladium on carbon (8.5 mg). The flask was evacuated and flushed with hydrogen three times, and the mixture was stirred under hydrogen for 1 h at RT, until the reaction was complete as indicated by TLC. The sample was filtered through celite. The filtrate was concentrated and the resulting clear oil was used immediately in the next step without further purification. The clear oil was dissolved in DMF (25 mL) and Fmoc-Asp-OAll (0.752 g, 1.9 mmol) was added. Diisopropylethylamine (0.66 g, 5.1 mmol) in DMF (1 M) was added with vigorous stirring under argon. HATU (1.94 g, 5.1 mmol) in DMF (0.5 M) was added dropwise. The reaction was stirred for 1 h at RT, until the reaction was complete as indicated by TLC. The reaction was diluted with EtOAc, and washed with *aq.* NaHCO₃. The aqueous layer was extracted with EtOAc (3x). The organic extracts were combined, dried over Na₂SO₄, filtered. The filtrate was concentrated. The product was purified by flash silica gel chromatography (20-50% EtOAc in hexane) to give **5a** (1.18 g, 74%) as a white solid. ¹H NMR (500 MHz,

CDCl₃): δ 0.04-0.16 (m, 18H, 6 Si-CH₃), 0.88-0.95 (t, J = 8.2 Hz, 27H, 9 t-Bu), 2.04-2.06 (s, 3H, -NHAc), 2.75-3.02 (dd, 2H, Asn- β -CH₂), 3.84-3.87 (m, 2H, H-6), 3.88-3.90 (m, 1H, H-5), 3.95-3.98 (m, 1H, H-2), 4.24-4.47 (m, 5H, H-3, H-4, Fmoc-CH, Fmoc-CH₂), 4.63-4.64 (m, 1H, α -CH-Asn), 4.65-4.69 (d, J = 4.8 Hz, 2H, O-CH₂-CH=CH₂), 5.19-5.22 (d, J = 10.5 Hz, 1H, OCH₂-CH=CH_{cis}H_{trans}), 5.33 (d, J = 17.2 Hz, 1H, OCH₂-CH=CH_{cis}H_{trans}), 5.55 (d, J = 7.75 Hz, 1H, H-1), 5.87-5.94 (m, 1H, OCH₂-CH=CH₂), 6.04 (d, J = 8.5 Hz, 1H, Asn- α -NH), 6.82 (d, J = 7.6 Hz, 1H, NH-Asn), 7.19 (d, J = 8.7 Hz, 1H, -NHAc), 7.30-7.76 (m, 8H, Fmoc-Ar). ¹³C NMR (100 MHz, CDCl₃): δ 171.5, 170.9, 170.0, 156.4, 144.1, 141.5, 131.9, 127.9, 127.4, 125.5, 120.1, 118.6, 81.7, 72.7, 71.0, 68.2, 67.4, 66.5, 61.5, 51.5, 50.7, 47.3, 38.1, 25.9, 23.8, 4.7. HRMS (ESI-TOF) *m/z* [M + H]⁺ Calcd for C₄₈H₇₈N₃O₁₀Si₃: 940.4995; Found 940.4994.

N^o-(2-Acetamido-3,4,6-tri-O-diethylisopropyl-2-deoxy- β -D-glucopyranosyl-N^o-(9-fluorenylmethyloxycarbonyl)-L-asparagine-allyl ester (5b). To a solution of **4b** (1 g, 1.6 mmol) in MeOH (40 mL), and palladium on carbon (8 mg) was added. The flask was evacuated and flushed with hydrogen three times, and stirred under hydrogen for 1 h at RT, until the reaction was complete by TLC. The sample was filtered through celite. The filtrate was concentrated and the resulting clear oil was used immediately in the next step without further purification. The clear oil was dissolved in DMF (25 mL) and Fmoc-Asp-OAll (0.709 g, 1.8 mmol) was added. Diisopropylethylamine (0.62 g, 4.8 mmol) in DMF (1 M) was added with vigorous stirring under argon. HATU (1.94 g, 4.8 mmol) in DMF (0.5 M) was added slowly,

dropwise. The reaction was stirred for 1 h at RT until the reaction was complete by TLC. After completion, the reaction was diluted with EtOAc, and washed with 50% NaHCO₃ (aq.). The aqueous layer was extracted with EtOAc 3x. The organic extracts were combined, dried over Na₂SO₄, filtered, and evaporated *in vacuo*. The compound was purified by flash silica gel chromatography (20-50% EtOAc in hexane), yielding **5b** (1.15 g, 73%) as a white solid. ¹H-NMR (500 MHz, CDCl₃): δ 0.63-0.73 (m, 15H, Si-CH₂, Si-CH), 0.98-1.06 (m, 36H, Si-CH₃), 2.06 (s, 3H, -NHAc), 2.77-3.03 (dd, 2H, Asn-β-CH₂), 3.86-3.89 (m, 1H, H-5), 3.93-3.98 (m, 2H, H-6), 4.0-4.03 (m, 1H, H-2), 4.25-4.41 (m, 5H, H-3, H-4, Fmoc-CH, Fmoc-CH₂), 4.63-4.64 (m, 1H, α-CH-Asn), 4.67-4.70 (d, J = 5.4 Hz, 2H, O-CH₂-CH=CH₂), 5.23 (d, J = 10.5 Hz, 1H, OCH₂-CH=CH_{cis}H_{trans}), 5.34 (d, J = 17.2 Hz, 1H, OCH₂-CH=CH_{cis}H_{trans}), 5.56 (d, J = 8.0 Hz, 1H, H-1), 5.92 (m, 1H, OCH₂-CH=CH₂), 6.09 (d, J = 8.6 Hz, 1H, Asn-α-NH), 6.84 (d, J = 8.0 Hz, 1H, 1-NH), 7.27-7.78 (m, 9H, Fmoc-Ar, -NHAc). ¹³C NMR (100 MHz, CDCl₃): δ 171.5, 170.9, 169.9, 156.4, 144.1, 144.1, 141.5, 131.9, 131.9, 127.8, 127.3, 125.5, 125.4, 120.1, 118.6, 118.5, 81.9, 72.0, 70.9, 68.2, 67.5, 66.5, 61.5, 51.7, 50.8, 47.3, 38.1, 23.7, 21.2, 17.6, 17.5, 17.4, 17.4, 12.8, 12.8, 12.8, 7.2, 7.2, 7.2, 7.1, 7.1, 3.6, 3.6, 3.6, 3.5. HRMS (ESI-TOF) *m/z* [M + H]⁺ Calcd for C₅₁H₈₄N₃O₁₀Si₃: 982.5465; Found 982.5466.

N^o-(2-Acetamido-3,4,6-tri-O-triethylsilyl-2-deoxy-β-D-glucopyranosyl-N^α-(9-fluorenylmethoxycarbonyl)-L-asparagine-allyl ester (5c). Compound **4c** (1 g, 1.7mmol) was dissolved in methanol (42 mL), and palladium on carbon (8.5 mg) was added. The flask was evacuated and flushed with hydrogen three times, and stirred

under hydrogen for 1 h at RT, until the reaction was complete by TLC. The sample was filtered through celite. The filtrate was concentrated and the resulting clear oil was used immediately in the next step without further purification. The clear oil was dissolved in DMF (25 mL) and Fmoc-Asp-OAll (0.752 g, 1.9 mmol) was added. Diisopropylethylamine (0.66 g, 5.1 mmol) in DMF (1 M) was added with vigorous stirring under argon. HATU (1.94 g, 5.1 mmol) in DMF (0.5 M) was added slowly, dropwise. The reaction was stirred for 1 h at RT, until the reaction was complete by TLC. After completion, the reaction was diluted with EtOAc, and washed with 50% NaHCO₃ (aq.). The aqueous layer was extracted with EtOAc. The organic extracts were combined, dried over Na₂SO₄, filtered, and evaporated in vacuo. The compound was purified by flash silica gel chromatography (20-50% EtOAc in hexane), yielding **5c** (0.61 g, 38%) as a white solid. ¹H NMR (500 MHz, CDCl₃): δ 0.88-1.03 (m, 18H, -Si-CH₂-CH₃), 0.53-0.71 (m, 27H, -Si-CH₂-CH₃), 2.02-2.05 (s, 3H, -NHAc), 2.76-3.03 (dd, 2H, Asn-β-CH₂), 3.81-3.98 (m, 3H, H-2, 5, 6), 4.22-4.43 (m, 5H, H-3, 4, Fmoc-CH, Fmoc-CH₂), 4.62-4.66 (m, 1H, α-CH-Asn), 4.68 (d, J = 5.4 Hz, 1H, OCH₂-CH=CH₂), 5.21 (d, J = 10.5 Hz, 1H, OCH₂-CH=CH_{cis}H_{trans}), 5.32 (d, J = 17.2 Hz, 1H, OCH₂-CH=CH_{cis}H_{trans}), 5.58 (d, J = 7.95 Hz, 1H, H-1), 5.87-5.94 (m, 1H, OCH₂-CH=CH₂), 6.07 (d, J = 8.65 Hz, 1H, NH-Asn), 6.86 (d, J = 7.95 Hz, 1H, 1-NH), 7.29 (d, J = 8.05 Hz, 1H, -NHAc), 7.27-7.77 (m, 8H, Fmoc-Ar). ¹³C NMR (100 MHz, CDCl₃): δ 171.5, 170.9, 169.9, 156.3, 144.1, 141.4, 131.9, 129.4, 127.8, 127.2, 125.4, 120.8, 120.0, 81.8, 71.7, 70.9, 68.3, 67.9, 67.4, 66.4, 61.0, 51.6, 47.2, 37.9, 23.7, 6.9, 6.9, 4.6, 4.6, 4.5. HRMS (ESI-TOF) *m/z* [M + H]⁺ Calcd for C₄₈H₇₈N₃O₁₀Si₃: 940.4995; Found 940.4999.

N^o-(2-Acetamido-3,4,6-tri-O-tert-butyldimethylsilyl-2-deoxy-β-D-glucopyranosyl-N^α-(9-fluorenylmethoxycarbonyl)-L-asparagine (6a). To a solution of **5a** (1 g, 1.1 mmol) in DCM (50 mL) were added tetrakis(triphenylphosphine)palladium(0) (25.4 mg, 0.02 mmol) and phenylsilane (238 mg, 2.2 mmol). The resulting mixture was stirred at RT, under argon, for 45 min. After completion of reaction as indicated by TLC, water (2 mL) was added to quench the reaction, and the biphasic mixture was vigorously stirred for 30 min. The mixture was concentrated to dryness and the residue was purified by flash silica gel column chromatography (0-15% MeOH in DCM containing 0.5% acetic acid) and the crude product was further purified by size-exclusion chromatography (Sephadex LH-20, 2:1 CH₂Cl₂/MeOH) to afford **6a** (0.93 g, 94% yield) as a white powder. ¹H NMR (500 MHz, CDCl₃): δ 0.11-0.16 (m, 18H, Si-Me), 0.88-0.96 (m, 27H, Si-tBu), 2.06 (s, 3H, -NHAc), 2.85-2.98 (m, 2H, Asn-β-CH₂), 3.39-3.48 (m, 3H, H-4, H-6), 3.81 (m, 1H, H-5), 4.06 (m, 1H, H-2), 4.19-4.35 (m, 3H, Fmoc-CH, Fmoc-CH₂), 4.49 (t, J = 11.9 Hz, 1H, H-3), 4.65 (m, 1H, α-CH-Asn), 5.58 (d, J = 6.35 Hz, 1H, H-1), 6.22 (d, J = 6.60 Hz, 1H, Asn-α-NH), 7.27-7.74 (m, 10H, Fmoc-Ar, -NHAc, 1-NH). ¹³C NMR (100 MHz, CDCl₃): δ 173.7, 173.1, 172.8, 156.4, 144.1, 144.0, 141.4, 127.8, 127.3, 125.5, 120.1, 81.3, 71.9, 69.9, 69.3, 67.5, 58.5, 51.0, 47.3, 25.9, 23.5, 18.1, 4.8. HRMS (ESI-TOF) *m/z* [M + H]⁺ Calcd for C₄₅H₇₄N₃O₁₀Si₃: 900.4682; Found 900.4694.

N^o-(2-Acetamido-3,4,6-tri-O-diethylisopropylsilyl-2-deoxy-β-D-glucopyranosyl-N^α-(9-fluorenylmethoxycarbonyl)-L-asparagine (6b). To a solution of **5b** (1 g,

1.0 mmol) in DCM (50 mL), were added tetrakis(triphenylphosphine)palladium(0) (25.4 mg, 0.02 mmol) and phenylsilane (238 mg, 2.2 mmol). The resulting mixture was stirred at RT, under argon, for 45 min. After completion of reaction as indicated by TLC, water (2 mL) was added to quench the reaction, and the biphasic mixture was vigorously stirred for 30 min. The mixture was concentrated to dryness and the residue was purified by flash silica gel chromatography (0-15% MeOH in DCM containing 0.5% acetic acid), and the crude product was further purified followed by size exclusion chromatography (Sephadex LH-20, 2:1 CH₂Cl₂/MeOH) to afford **6b** (0.89 g, 95% yield) as a white powder. ¹H NMR (500 MHz, CDCl₃): δ 0.59-0.69 (m, 15H, Si-CH₂, Si-CH), 0.94-1.01 (m, 36H, Si-CH₃), 2.03 (s, 3H, -NHAc), 2.77-2.88 (m, 2H, Asn-β-CH₂), 3.87-3.96 (m, 5H, H-3, H-4, Fmoc-CH, Fmoc-CH₂), 3.98-4.01 (m, 1H, H-2), 4.17-4.33 (m, 3H, H-5, H-6), 4.59-4.61 (m, 1H, α-CH-Asn), 5.58 (d, J = 7.5 Hz, 1H, H-1), 6.09 (d, J = 6.5 Hz, 1H, Asn-α-NH), 7.19 (d, J = 7.0 Hz, 1H, 1-NH), 7.28 (d, J = 7.0 Hz, 1H, -NHAc), 7.34-7.73 (m, 8H, Fmoc-Ar). ¹³C NMR (100 MHz, CDCl₃): δ 173.2, 172.7, 171.2, 155.9, 143.9, 141.2, 127.6, 127.1, 125.3, 119.8, 81.7, 71.7, 71.2, 67.9, 67.2, 61.1, 51.5, 50.5, 47.1, 23.4, 17.3, 12.6, 12.6, 12.5, 6.9. HRMS (ESI-TOF) *m/z* [M + H]⁺ Calcd for C₄₈H₈₀N₃O₁₀Si₃: 942.5152; Found 942.5167.

N^o-(2-Acetamido-3,4,6-tri-O-triethylsilyl-2-deoxy-β-D-glucopyranosyl-N^o-(9-fluorenylmethyloxycarbonyl)-L-asparagine (6c). To a solution of **5c** (1 g, 1.1 mmol) in DCM (50 mL) were added tetrakis(triphenylphosphine)palladium(0) (25.4 mg, 0.02 mmol) and phenylsilane (238 mg, 2.2 mmol). The resulting mixture was

stirred at RT, under argon, for 45 min. After completion of reaction by TLC, water (2 mL) was added to quench the reaction, and the biphasic mixture was vigorously stirred for 30 min. The mixture was concentrated to dryness and purified by flash silica gel column chromatography size exclusion chromatography (0-15% MeOH in DCM containing 0.5% acetic acid), the crude product was further purified by size-exclusion chromatography (Sephadex LH-20, 2:1 CH₂Cl₂/MeOH) to afford **6c** (0.92 g, 97% yield) as a white powder (0.92 g, 97% yield). ¹H NMR (500 MHz, CDCl₃): δ 0.54-0.69 (m, 18H, -Si-CH₂-CH₃), 0.89-0.99 (m, 27H, -Si-CH₂-CH₃), 1.95 (s, 3H, -NHAc), 2.63-2.90 (m, 2H, Asn-β-CH₂), 3.57 (m, 1H, H-5), 3.68-3.71 (m, 2H, H-6), 3.82-3.85 (m, 2H, Fmoc-CH₂), 3.95 (m, 1H, H-2), 4.17-4.19 (m, 1H, Fmoc-CH), 4.25-4.29 (m, 1H, H-4), 4.35-4.48 (m, 1H, H-3), 4.58-4.59 (m, 1H, α-CH-Asn), 5.14 (d, J = 8.0 Hz, 1H, H-1), 6.24 (d, J = 8.2 Hz, 1H, NH-Asn), 6.58 (d, J = 9.2 Hz, 1H, 1-NH), 7.19 (d, J = 8.4 Hz, 1H, -NHAc), 7.28-7.29 (d, J = 7.35 Hz, 2H, Fmoc-Ar), 7.35-7.38 (t, J = 8.7 Hz, 2H, Fmoc-Ar), 7.57-7.61 (t, J = 8.6 Hz, 2H, Fmoc-Ar), 7.73-7.74 (d, J = 7.6 Hz, 2H, Fmoc-Ar). ¹³C NMR (100 MHz, CDCl₃): δ 173.6, 172.0, 171.2, 162.9, 156.4, 141.2, 132.4, 132.2, 128.6, 128.5, 127.1, 119.9, 79.3, 67.4, 62.6, 50.5, 47.1, 36.5, 31.5, 23.2, 6.9, 4.4, 4.0. HRMS (ESI-TOF) *m/z* [M + H]⁺ Calcd for C₄₅H₇₄N₃O₁₀Si₃: 900.4682; Found 900.4689.

N^α-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-N^α-(9-fluorenylmethoxycarbonyl)-L-asparagine tert-butyl ester (7). A solution of **2** (5 g, 13.4 mmol) and Fmoc-Asp-OtBu (4.96 g, 12.1 mmol) in DCM (10 mL) was cooled to -10 °C under an argon atmosphere. A catalytic amount of triethylphosphine (PEt₃)

(31.9 mg, 0.27 mmol) was added, and the reaction mixture was stirred to RT overnight. The product (**7**) precipitated as a white solid.² The solid was collected and recrystallized from warm CH₂Cl₂ to afford **7** (8.03 g, 81% yield). ¹H NMR (500 MHz, CDCl₃): δ 1.45 (s, 9H, t-Bu), 1.96 (s, 3H, -NHAc), 2.04, 2.05, 2.07 (three s, 9H, 3 × OAc), 2.69-2.87 (m, 2H, Asn-β-CH₂), 3.74-3.76 (m, 1H, H-5), 4.05-4.24 (m, 3H, H-2, 6), 4.28-4.44 (m, 3H, Fmoc-CH₂, Fmoc-CH), 4.52 (m, 1H, Asn-α-CH), 5.06-5.14 (m, 3H, H-1, 3, 4), 5.98 (d, J = 8.5 Hz, 1H, Asn-α-NH), 6.19 (d, J = 8.0 Hz, 1H, -NHAc), 7.22 (d, J = 10 Hz, 1H, 1-NH), 7.30 (t, J = 8.4 Hz, 2H, Fmoc-Ar), 7.39 (t, J = 8.2 Hz, 2H, Fmoc-Ar), 7.60 (d, J = 7.4 Hz, 2H, Fmoc-Ar), 7.75 (t, J = 7.4 Hz, 2H, Fmoc-Ar). ¹³C NMR (100 MHz, CDCl₃): δ 172.8, 172.2, 171.5, 171.1, 170.4, 169.7, 156.6, 144.2, 141.7, 141.6, 128.1, 127.5, 125.6, 120.4, 80.7, 80.5, 73.9, 73.2, 68.1, 67.6, 62.1, 53.8, 51.4, 47.5, 38.6, 28.3, 23.5, 21.1, 21.00, 13.9, 0.4. MS (ESI) *m/z* [M + H]⁺ Calcd for C₃₇H₄₅N₃O₁₃: 739.30; Found 740.74. The ¹H and ¹³C NMR data were consistent with the reported data ⁴¹.

N^o-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-N^α-(9-fluorenylmethyloxycarbonyl)-L-asparagine (8). Compound **7** (1 g, 1.35 mmol) was dissolved in neat formic acid. The mixture was stirred at RT until complete removal of the tBu group, as indicated by TLC. Formic acid was removed in vacuo to provide **8** (0.922 g, quant.). ¹H NMR (400 MHz, (CD₃)₂SO): δ 1.72 (s, 3H, NHAc), 1.89 (s, 3H, OAc), 1.96 (s, 3H, OAc), 1.99 (s, 3H, OAc), 2.62-2.67 (m, 2H, Asn-β-CH₂), 3.79-3.95 (m, 3H, H-5, 6), 4.16-4.29 (m, 4H, H-2, Fmoc-CH₂, Fmoc-CH), 4.35-4.39 (m, 1H, α-CH-Asn), 4.79-4.84 (t, J = 8.8 Hz, 1H, H-4), 5.07-5.19 (m, 2H, H-1, 3),

7.33 (t, J = 8.5 Hz, 2H, Fmoc-Ar), 7.41 (t, J = 8.1 Hz, 2H, Fmoc-Ar), 7.51 (d, J = 8.40 Hz, 1H, Asn- α -NH), 7.71 (d, J = 7.60 Hz, 2H, Fmoc-Ar), 7.89 (m, 3H, -NHAc, Fmoc-Ar), 8.61 (d, J = 9.2 Hz, 1H, 1-NH). ^{13}C NMR (100 MHz, $(\text{CD}_3)_2\text{SO}$): δ 173.9, 170.9, 170.7, 170.4, 170.4, 170.2, 163.2, 156.7, 144.7, 144.7, 141.6, 129.8, 128.5, 127.9, 126.2, 126.1, 120.9, 78.9, 74.2, 73.1, 69.2, 66.6, 62.7, 52.9, 50.9, 47.5, 37.8, 36.7, 31.6, 23.5, 21.4, 21.3, 21.2. MS (ESI) m/z $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{37}\text{H}_{45}\text{N}_3\text{O}_{13}$: 683.23; Found 683.24. The ^1H and ^{13}C NMR data were consistent with the reported data ⁴¹.

Synthesis of the precursor glycopeptides incorporating the GlcNAc-Asn building blocks. All the three precursor glycopeptides were synthesized on a 0.1 mmol scale using 5-fold excess of reagents [0.2 M amino acid solution (in DMF)] with 1 M DIC (in DMF) and 0.5 M HOBt (in DMF). Low-loading Fmoc-PAL-PEG-PS resin (0.17 mmol/g, Life Technologies) was used for the synthesis of the peptides. The glycosylamino acid building blocks (**6a-c** and **8**) were coupled to the growing peptide at 90 °C with a 50 Hz MW power for 10 min, Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH were coupled at 50°C with a 50 Hz MW power for 2 min. Fmoc-Arg(Pbf)-OH was double coupled (RT without MW for 25 min, followed by 90 °C with 50 Hz MW power for 2 min). All other amino acids were coupled at 90 °C with 50 Hz MW power for 2 min. The deblocking reagent used was 20% piperidine with 0.1 M HOBt (deblocking occurred at 50 °C and 50 Hz MW power). The N-terminus was capped with a biotin tag by treatment with biotin-LC-N-hydroxysuccinimide (3 mol. equivalent) in DIPEA/DMF. Resin cleavage and global peptide deprotection was

achieved after washing the resin with DCM (3x) and adding freshly prepared cocktail R (TFA/thioanisole/1,2-EDT/anisole, 90/5/3/2) and shaking for 2 h at RT. The resin was filtered onto ice-cold ethyl ether for precipitation in a 50 mL centrifuge tube.

The crude glycopeptides were purified by RP-HPLC and the purity and identity were confirmed by analytical HPLC and LC-MS analysis. Glycopeptide (**10**): Analytical RP-HPLC, $t_R = 26$ min. (gradient, 0-90% aq. MeCN containing 0.1% TFA for 30 min; flow rate = 0.5 mL/min.); ESI-MS: Calcd M = 3856.50; found: 771.53 [M + 5H]⁵⁺, 964.24 [M + 4H]⁴⁺, 1285.43 [M + 3H]³⁺. Deconvolution mass: 3856.03 ± 0.15. Glycopeptide (**11**): Analytical RP-HPLC, $t_R = 21$ min. (gradient, 5-25% aq. MeCN containing 0.1% FA for 30 min; flow rate, 1 mL/min.); ESI-MS: Calcd M = 3628.37; found: 726.55 [M + 5H]⁵⁺, 908.18 [M + 4H]⁴⁺, 1210.57 [M + 3H]³⁺, 1815.35 [M + 2H]²⁺. Deconvolution mass: 3628.65 ± 0.28.

Synthesis of glycopeptide (12): Compound **11** (50 mg) was dissolved in DMSO (5 mL) and then the solution was diluted with water (20 mL) (final concentration of DMSO, 20%). The solution was shaken overnight at RT. After cyclization was complete (by HPLC analysis), the mixture was lyophilized and the resulting residue was purified by preparative HPLC, yielding **12** (43 mg, 85%) as a white powder. Analytical RP-HPLC, $t_R = 33$ min. (gradient, 15-30% aq. MeCN containing 0.1% TFA for 30 min; flow rate, 0.5 mL/min.); ESI-MS: Calcd M = 3626.21; found: 726.61 [M + 5H]⁵⁺, 908.04 [M + 4H]⁴⁺, 1210.08 [M + 3H]³⁺, 1789.79 [M + 2H]²⁺. Deconvolution mass: 3626.63 ± 0.96.

Preparation of Man₅GlcNAc and Man₉GlcNAc oxazolines (13 and 20).

Man₉GlcNAc₂Asn was isolated from soy bean flour, using the method previously reported⁴². For the synthesis of Man₅GlcNAc oxazoline (**13**), a solution of Man₉GlcNAc₂Asn (10 μmol) was dissolved in 50 mM citrate buffer (pH = 5.6) containing 5 mM calcium chloride. α1,2-mannosidase from *Bacteroides thetaiotaomicron* was added to a final concentration of 0.02 mg/mL, the enzymatic reaction was incubated at 37 °C for 8 h, while monitoring by HPAEC-PAD analysis. Once complete conversion was observed, the pH of the solution was adjusted to 7.0, and enzyme Endo-A was added to convert Man₅GlcNAc₂Asn to Man₅GlcNAc via enzymatic cleavage of the chitobiose core. Man₅GlcNAc was purified by gel filtration on a Sephadex G-15 (GE Healthcare) column. The carbohydrate positive fractions (assessed by phenol-sulfuric acid analysis) were pooled and lyophilized to obtain the free glycan Man₅GlcNAc (9 mg, 87%). The identity and purity of the Man₅GlcNAc was confirmed by MALDI-TOF and HPAEC-PAD (data not shown). The sugar oxazoline of Man₅GlcNAc was prepared by treatment of a solution of Man₅GlcNAc (9 mg, 87.3 μmol) in water (0.45 mL) with triethylamine (3.9 mmol) and 2-chloro-1,3-dimethylimidazolium chloride (DMC) (1.3 mmol). The reaction was incubated on ice for 30 min, while monitored by HPAEC-PAD analysis. The glycan oxazoline product was purified by SEC (Sephadex G-10) eluting with 0.1% triethylamine. The carbohydrate containing fractions were pooled and lyophilized to give **13** (8 mg, 91%). The sugar oxazoline of Man₉GlcNAc (**20**) was prepared from soy flour in a similar manner as **13**.

Enzymatic transglycosylation: Synthesis of glycopeptide 14 bearing a Man₅GlcNAc₂ moiety. A solution of the GlcNAc-containing peptide **12** (1 mg, 0.28 μmol) and Man₅GlcNAc-oxazoline (**13**) (2.3 mg, 2.24 μmol) in a Tris buffer (40 μL, 80 mM, pH 7.2) was incubated with EndoM-N175Q (final concentration, 0.2 μg/μL) at RT. The reaction was monitored by RP-HPLC. After 30 min, the reaction was quenched by addition of 0.1% aq. TFA. The transglycosylation product was purified by RP-HPLC to afford **14** (1.1 mg, 85%) as a white powder after lyophilization. Analytical RP-HPLC, $t_R = 31$ min. (gradient, 15-30% aq. MeCN containing 0.1% TFA for 30 min; flow rate = 0.5 mL/min.); ESI-MS: Calcd M = 4639.56; found: 929.31 [M + 5H]⁵⁺, 1161.43 [M + 4H]⁴⁺, 1548.71 [M + 3H]³⁺. Deconvolution mass: 4640.32 ± 0.84.

Deprotection of glycopeptide 15. Compound **14** (0.95 mg, 0.21 μmol) was dissolved in 2.5% aqueous hydrazine (final concentration 2 mg/mL). The mixture was shaken at RT for 30 min. When the reaction was complete as indicated by RP-HPLC, the solution was neutralized by addition of glacial acetic acid. The product was purified by RP-HPLC yielding **15** (0.54 mg, 58%) as a white powder. Analytical RP-HPLC, $t_R = 28$ min. (gradient, 15-30% aq. MeCN containing 0.1% TFA for 30 min; flow rate = 0.5 mL/min.); ESI-MS: Calcd M = 4514.63; found: 904.11 [M + 5H]⁵⁺, 1129.53 [M + 4H]⁴⁺, 1506.03 [M + 3H]³⁺. Deconvolution mass: 4514.94.

Enzymatic transglycosylation: Synthesis of glycopeptide 17 bearing a Man₅GlcNAc₂ at N160 and a sialylated glycan at N173 sites. A solution of the

GlcNAc-containing peptide (**15**) (1 mg, 0.22 μmol) and (NeuGalGlcNAc)₂Man₃GlcNAc-oxazoline (**16**) (2 mg, 0.9 μmol) in Tris buffer (40 μL , 80 mM, pH 7.2) was incubated with EndoM-N175Q (final concentration, 0.2 $\mu\text{g}/\mu\text{L}$) at RT. The reaction was monitored by RP-HPLC. After 30 min, the reaction was quenched by addition of 0.1% aq. TFA. The transglycosylation product was purified by RP-HPLC yielding **17** (1.32 mg, 92%) as a white powder. Analytical RP-HPLC, t_{R} = 22 min. (gradient, 15-30% aq. MeCN containing 0.1% TFA for 30 min; flow rate = 0.5 mL/min.); ESI-MS: calcd M = 6517.30; found: 1304.66 [M + 5H]⁵⁺, 1630.41 [M + 4H]⁴⁺. Deconvolution mass: 6516.21 \pm 1.45.

Synthesis of glycopeptide 19. The enzymatic transglycosylation of **15** with glycan oxazoline (**18**) under the catalysis of EndoM-N175Q was carried out in the same manner as for the preparation of **17**, giving **19** (95% yield based on HPLC estimation). Analytical RP-HPLC, t_{R} = 24 min. (gradient, 15-30% aq. MeCN containing 0.1% TFA for 30 min; flow rate = 0.5 mL/min.); ESI-MS: Calcd M = 5933.01; found 996.55 [M + 6H]⁶⁺, 1188.28 [M + 5H]⁵⁺, 1484.88 [M + 4H]⁴⁺, 1978.93 [M + 3H]³⁺. Deconvolution mass: 5934.49 \pm 1.67.

Synthesis of glycopeptide 21. The enzymatic transglycosylation of **15** with glycan oxazoline (**20**) under the catalysis of EndoM-N175Q was carried out in the same way as for the preparation of **17**, giving **21** in 95% yield (based on HPLC estimation). Analytical RP-HPLC, t_{R} = 25 min. (gradient, 15-30% aq. MeCN containing 0.1% TFA

for 30 min; flow rate = 0.5 mL/min.); ESI-MS: Calcd M = 6175.37; found: 1236.54 [M + 4H]⁴⁺, 1735.28 [M + 3H]³⁺. Deconvolution mass: 6175.76 ± 0.81.

Synthesis of the linear glycopeptide (S-1). Compound **12** (0.5 mg, 0.14 μmol) was incubated with 0.5 mM dithiothreitol (DTT) for 1 h at 37°C. Iodoacetamide was added to a final concentration of 180 mM, and the reaction was incubated for 30 min at 37°C. The reaction was monitored by HPLC. The reaction was purified by HPLC yielding **S-1** (0.47 mg, 90%) as a white powder. Analytical RP-HPLC, t_R = 30 min. (gradient, 15-30% aq. MeCN containing 0.1% TFA for 30 min; flow rate = 0.5 mL/min.); ESI-MS: calcd M = 3740.71; found: 748.38 [M + 5H]⁵⁺, 935.78 [M + 4H]⁴⁺, 1246.93 [M + 3H]³⁺, 1870.61 [M + 2H]²⁺. Deconvolution mass: 3740.82 ± 0.92.

Transglycosylation of linear glycopeptide S-1 by the Endo D mutant. Compound **S-1** (0.5 mg, 0.13 μmol) and Man₅GlcNAc-oxazoline (**13**) (1.1 mg, 1.05 μmol) in Tris buffer (20 μL, 80 mM, pH 7.2) was incubated with EndoD-N322Q (final concentration, 40 ng/μL) at RT. The reaction was monitored by RP-HPLC. After 30 min, the reaction was quenched by addition of 0.1% aq. TFA. The transglycosylation product was purified by RP-HPLC yielding **S-2** (0.57 mg, 92%) as a white powder. Analytical RP-HPLC, t_R = 26 min. (gradient, 15-30% aq. MeCN containing 0.1% TFA for 30 min; flow rate = 0.5 mL/min.); ESI-MS: Calcd M = 4736.06; found: 947.94 [M + 5H]⁵⁺, 1184.88 [M + 4H]⁴⁺, 1578.62 [M + 3H]³⁺. Deconvolution mass: 4736.71 ± 0.65.

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Chapter 4: Synthesis and binding studies of an anti-HIV PG9 neoglycoprotein immunogen mimic of gp120 quaternary structure incorporating synthetic V1V2 loops.

4.1 Introduction

The past five years have yielded an enormous amount of information regarding the vulnerability of HIV-1 to neutralization by carbohydrate-reactive antibodies. Initial attempts at HIV vaccine design resulted in a vast number of designed constructs^{1-3 4 5 6-10} aimed at simulation of Env architecture, none of which have been able to raise a sustaining neutralizing humoral immune response. The functional Env is a trimer of heterodimers composed of the receptor binding protein gp120, and a membrane-spanning fusion protein gp41. These heterodimers, constructed from homotrimers (gp41 and gp120), assemble into the non-covalently associated viral spike. The trimeric structure is inherently unstable, with a pronounced lability resulting in heterogeneous surface display of viral spikes at different levels of composition. The major issue in these early designs was the use of gp120 monomer, which exposed the trimer-associated face that is normally occluded in functional Env protein. The bulk of the immune response was therefore directed against the highly antigenic trimer-associated face, while the solvent exposed face was protected by the endogenous dense glycosylation (i.e. the “glycan shield”). Therefore, any vaccination attempt using monomeric gp120 yields a strong non-neutralizing humoral response

against non-functional Env protein, distracting the response from the solvent exposed face on functional trimeric Env proteins and consequently yielding no vaccine-induced protection from HIV-1. This “glycan shield,” comprising ~50% of gp120 by weight, once considered one of HIV’s most effective tools for immune evasion is now viewed as the most promising target for vaccine design. Indeed, the discovery of carbohydrate-reactive broadly neutralizing antibodies (bNAbs) has revolutionized the field of HIV vaccine design by providing a number of partially defined and potent epitopes on the so-called glycan shield. The first of these epitopes to be discovered was that of the bNAb 2G12. 2G12 recognizes an oligomannose cluster within the HIV glycan shield, with three epitopes per trimeric Env protein. The epitope of 2G12 is unique in that it consists solely of carbohydrate, comprised of 2-3 oligomannose glycans, with 2G12 binding to the distal α 1, 2-linked Man-Man glycans. After the initial description of the 2G12 epitope, many mimics of the gp120 oligomannose cluster were generated using a wide variety of scaffolds in the hope of eliciting 2G12-like Abs (see Chapter 2). Despite very impressive antigenicity studies with 2G12 recognition of synthetic antigens comparable¹¹⁻¹³ to more potent¹⁴ than the affinity of 2G12 for its natural substrate, gp120, to date, none of the synthetic antigens have been successful as immunogens. Among the multifarious reasons often cited for the lack of success in inducing 2G12-like bNAbs,¹⁵ most likely the problem is exacerbated by the unusual domain-exchanged arrangement of the Ab heavy chains. The domain-exchanged combining sites, providing greater surface area for binding to carbohydrate antigens, accounting for its unique mode of antigen recognition, is most likely difficult to induce by vaccination.¹⁶

Until 2009, the only reported anti-HIV-1 carbohydrate-reactive bNAb was 2G12. Since that time, thanks to aggressive high-throughput screening of B cell clones from elite-neutralizer patients, a number of carbohydrate-reactive bNAbs have been described.¹⁷⁻²⁰ These new classes of carbohydrate-reactive bNAbs, isolated from several infected donors, all have a typical Y-shaped IgG architecture, in contrast to the very unusual domain-exchanged structure of 2G12.^{18 17, 21 22-23 24} It was initially thought that bNAbs were extremely rare, only arising in a small number of infected individuals after an extended period of HIV infection. It is now known that approximately 10-30% of HIV-1 infected individuals develop bNAbs within 3 years of infection, with some arising after just one year.²⁵ In particular, two series of bNAbs, the PG and PGT-series, are exceptionally potent and have very broad neutralizing capacity across multiple HIV clades. The first antibodies to be described in this new class were PG9 and the closely related PG16, both of which neutralize HIV-1 with extremely high potency and breadth, particularly in comparison to 2G12. For example, when tested against a 162-pseudovirus panel, PG9 was shown to neutralize 77% of HIV-1 strains with an IC₅₀ of 0.23 µg mL⁻¹ (compared to 32% and 2.4 µg mL⁻¹ for 2G12).¹⁷ The PG-series of bNAbs is able to achieve such a high potency and neutralization breadth in spite of its conventional Y-shaped architecture. The PG-series (as well as the PGT-series) has several unusual features not found on more typical IgGs, most notably extended, heavily tyrosine-sulfated CDRH3 loops. The extended CDRH3 loops protruding from the Fab forms a “hammer-head” conformation, penetrating the glycan shield and contacting the N160 and N156/173 glycans and interacting with the peptide surface of the V1 loop of gp120.^{21, 26-27}

Specifically, the HCDRH3 hammerhead mediates largely charged interactions with a beta-strand (bearing the cationic sequence KQKKD) on the V1V2 region of gp120, while the glycans at N160 and N156/173 interact with the surrounding Ab paratope.²¹ Thus the PG-series bNAb targets an integrated epitope, augmenting the binding mode and enhancing the overall potency versus the solely anti-carbohydrate Ab 2G12. Cryoelectron microscopy studies have suggested that the PG-series of bNAbs are trimer-preferring, targeting an epitope at the apex of the gp120 trimer that spans across two monomers, as a second N160 glycan on a neighboring protomer was involved in PG9 recognition (Figure 4.1A). Such findings suggest that PG9 immunogen design must be guided within the context of quaternary structure, as the quaternary epitope spans two V1V2 loops straddling two gp120 protomers (Figure 4.1B).²⁶

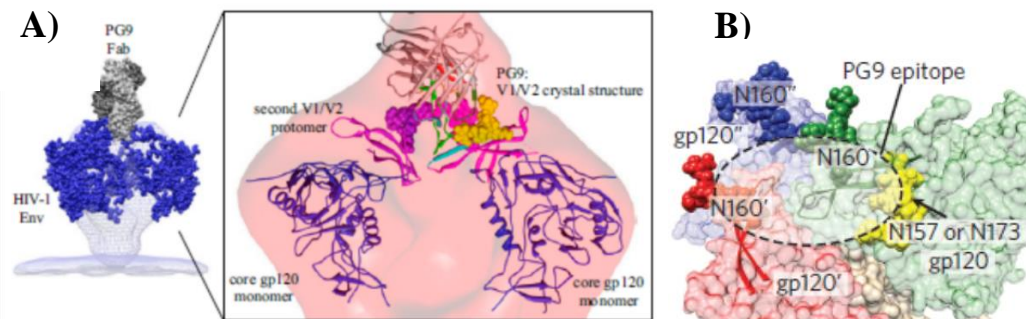


Figure 4.1. **A)** Electron microscopy crystal structure of a PG9 Fab bound to trimeric gp120 spanning two protomers. **B)** Detail of putative PG9 quaternary epitope (images reproduced by permission of the publishers).^{26, 28}

The breadth and potency of PG9 have made the bNAb an attractive target for vaccine design. The initial efforts have been informed by structural studies based on

the bNAbs in complex with gp120. Mutational analysis indicated the critical role of a PNG site at N160 for PG9 binding.²⁹ Indeed, X-ray crystallographic studies of PG9 bound to a segment of the V1V2 region of gp120 ectopically grafted onto a scaffold protein clearly showed that PG9 makes extensive contacts with a Man₅GlcNAc₂ glycan at N160. Lower, but significant contact was also made with PNG sites at the spatially-related N156/N173 positions. Unfortunately, the most informative structural studies were performed using V1V2 constructs that were produced in cell lines deficient of N-acetylglucosaminyltransferase I (GnTI), giving rise to homogeneous Man₅GlcNAc₂ glycoforms, limiting the information obtained regarding the specific glycan requirements of PG9.²¹ Moreover, from the crystal structure obtained, the identity of the glycan at N156/173 was unclear, although the results indicated the precise fitting of Man₅GlcNAc₂ at the N160 site. To clarify the glycan requirement for optimized PG9 recognition, a number of studies have been performed by our group and others.³⁰⁻³² Our group was the first to interrogate the glycan requirement of PG9 using a chemoenzymatic method involving “random-glycosylation” to synthesize a library of glycopeptides that were tested for PG9 recognition.³⁰ The findings supported the observed requirement from structural studies for an oligomannose Man₅GlcNAc₂ glycan at N160. The study also found that a biantennary sialylated complex-type glycan at N156/173 was critical for optimal recognition, exhibiting 20-fold stronger binding affinity compared to an analogous peptide bearing Man₅GlcNAc₂ at both positions. The importance of a sialylated glycan at N156/173 was supported by a STD-NMR study.²⁷ Independently, Danishefsky’s group synthesized a V1V2 peptide mimic of the PG9 epitope incorporating a peptide dimer

intended to roughly approximate the quaternary requirements of the bNAbs.^{33 31} The peptide was glycosylated with either two Man₅GlcNAc₂ glycans or two Man₃GlcNAc₂ glycans (bearing four glycans total). The mimics yielded the strongest antigenicity results for PG9 to date, in spite of the non-optimal glycosylation pattern, presumably due to the incorporation of quaternary-like elements to the peptide antigens. The study also incorporated full-length PG9 IgGs in the SPR affinity studies, which, when compared to the PG9 Fab alone in the study by Wang and co-workers, artificially enhances the observed recognition. Subsequent analysis by our group of the strongest binding cyclic glycopeptide (ZM strain where N160 = Man₅ and N173 = SCT), probed with PG9 whole IgG, revealed similar affinity to that reported by Danishefsky and co-workers, minimizing the enhancement due to dimerization. However, the very strong recognition of the non-optimal glycosylation pattern in Danishefsky's peptides (i.e. two Man₅ glycans rather than Man₅/SCT) suggests an enhancement in recognition due dimerization, even in the absence of a sialylated glycan at N173.

A major innovation in the overall understanding of the structure and conformation of gp120 has been the recent successes of recombinantly expressed, covalently conjugated gp140 trimer (BG505 SOSIP.664) proteins.³⁴ Despite mutational modifications to the gp120/gp41 structure to introduce covalent attachments to maintain the trimeric structure, the well-folded BG505 SOSIP.664 gp140 trimer has proven to be an excellent antigenic³⁵⁻³⁷ and structural^{19, 38 39} mimic of the native viral spike. These well-folded SOSIP gp140 trimers have been tested as trimer immunogens⁴⁰ alone as well ectopically expressed on various VLP

nanoparticle scaffolds.^{41 42 43} The gp140 trimers alone have proven to be unsuccessful as immunogens,⁴⁰ however, the VLP-scaffolded gp140 immunogens have shown great promise as a potential path toward a successful anti-HIV-1 vaccine. One of the major limitations to the design, however, is the presentation of multiple non-natural immunodominant epitopes that can direct the host immune response toward epitopes that do not exist in a native context. For example, the multifaceted systematic immunization strategy recently report by Iyer et al. is extremely encouraging, however, a major antigenic determinant in the study was the presence of the cytoplasmic tail of gp41, normally occluded in the native context within the interior of the virus.⁴³ Burton and co-workers made the first attempt to address this issue by the design of self-assembling nanoparticle proteins that ectopically expressed the trimeric V1V2 epitope of PG9 of the ZM HIV-1 strain.⁴² In this comprehensive and very elegant study, the trimeric V1V2 domain was based on the BG505 SOSIP gp140 trimer. The sole expression of the V1V2 epitope abrogates the issue of alternative immunodominant epitopes on the immunogen. A major limitation of this strategy, however, is the lack of control over glycosylation, as the nanoparticles were expressed in N-acetylglucosaminyltransferase I-negative (GN TI^{-/-}) HEK 293S cells, yielding mostly Man₅GlcNAc₂ glycans. Although PG9 has remarkable promiscuity, accommodating different glycans in the N173 position, PG9 has a specific preference for a biantennary sialylated complex-type glycan at the N173 position. The ability to define the glycosylation at both sites would be a major advantage in tuning the selectivity of the immunogen for PG9. The presentation of the sole-epitope of PG9

with defined glycosylation in a quaternary context will potentially lead to a more directed response solely against the neutralizing epitope.

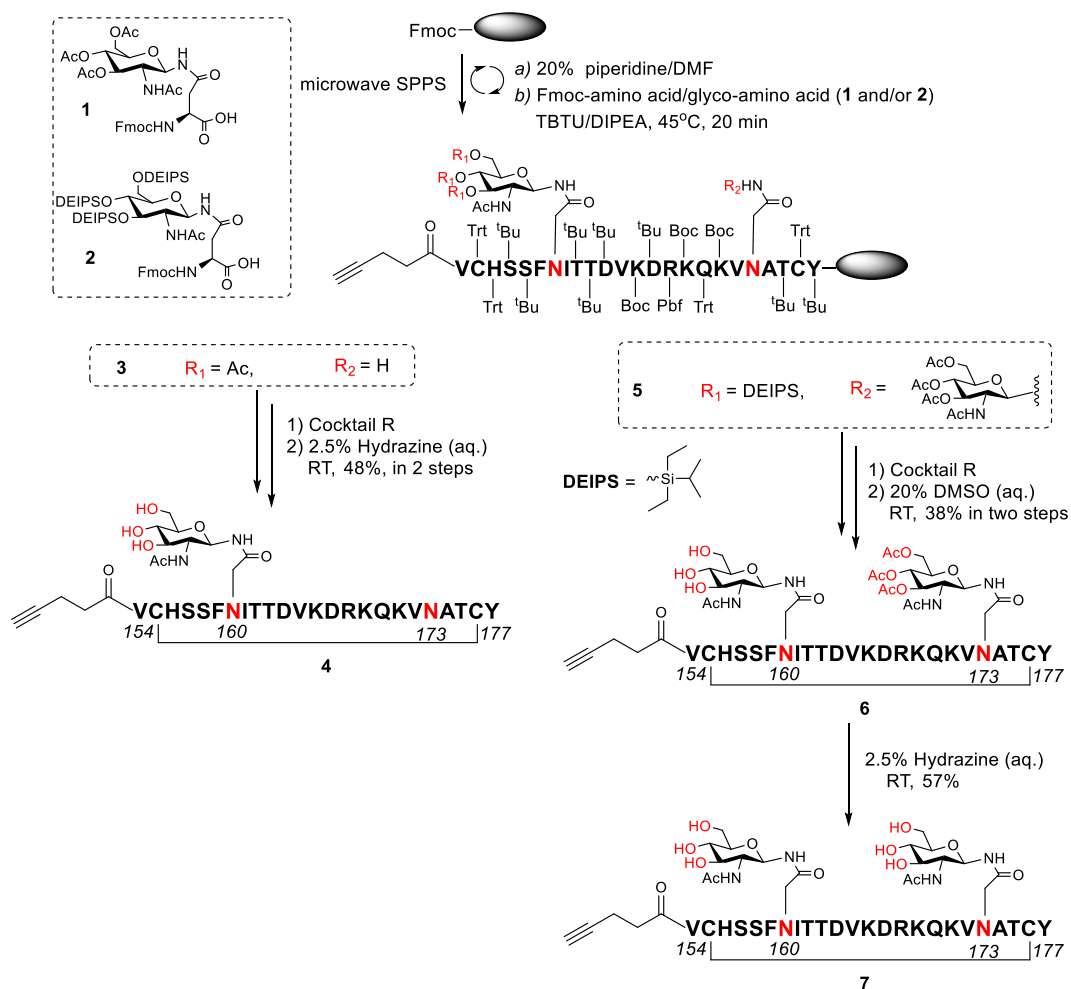
The goal of the present work is to combine successful features from the previous studies in a trimer-like context to measure any improvement in antigenicity. The study incorporated peptides that were previously demonstrated to be potent antigens for PG9 in a monomeric context. The quaternary mimicry is achieved through the multi-valent presentation of V1V2 peptide antigens, which, when presented to PG9, roughly approximates quaternary structure. The use of peptides allows selective control over tuning the pattern of glycosylation, and represents an alternative strategy towards a quaternary-like HIV-1 immunogen to the recombinant approaches previously reported.

4.2 Results and Discussion

The crystal structure of PG9 in complex with the ectopically expressed monomeric V1V2 region revealed a Greek key motif with the epitope consisting of a short segment centered at the N160 position. Two glycans critical for PG9 binding are located on two of the strands. Our approach began with the observation, reported by our group and others^{30-31, 44} that short, properly designed peptide mimics of the V1V2 loop were potent antigens for PG9. We reasoned that multivalent display of manifold, glycan-defined, glycopeptides on an appropriate scaffold could approximate gp120 quaternary structure. Our choice of the self-assembling virus-like particle (VLP) bacteriophage Q β was informed by recent reports of its superior antigenicity and immunogenicity properties within the context of protein conjugate vaccines.⁴⁵⁻⁴⁸ The

use of a VLP scaffold presents advantages from several angles. First, the particles are stable, uniform, and provide a smooth surface for highly repetitive antigen presentation with well-defined surface chemistry.⁴⁵ Second, the application of a viral-based scaffold incorporates a virus-like presentation of the antigens that differs from mammalian glycoprotein presentation, which helps to overcome immune tolerance mechanisms. Third, the encapsulation of random bacterial RNA during expression/self-assembly delivers an internal adjuvant that can stimulate a more pronounced and sustained immune response during vaccination.

We chose to use a minimal V1V2 peptide sequence that contained the critical features of the epitope, including both glycans that are critical for binding, as well as the cationic peptide sequence (KQKKD). The sequence chosen has been used by our group previously with excellent results.^{30 44}

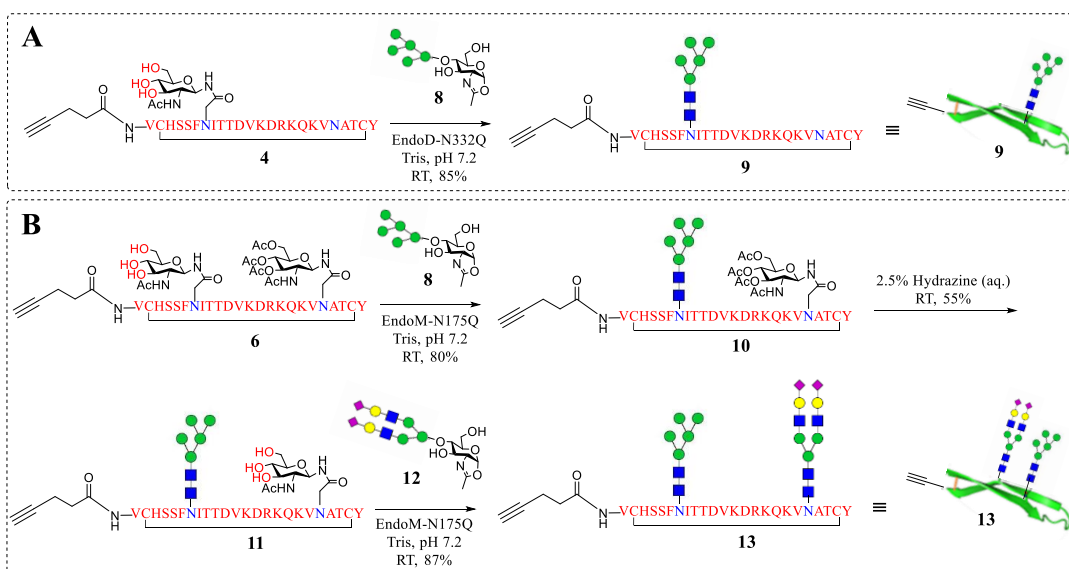


Scheme 4.1 Synthesis of GlcNAc-containing peptides using orthogonally protected glycosylaminoacid building blocks.

The precursor glycopeptides that served as the building blocks for the final complex antigens were synthesized using microwave-assisted solid-phase peptide synthesis (MW-SPPS) in excellent yields (Scheme 4.1). To introduce a defined N-glycan at a pre-determined site, selectively protected GlcNAc-moieties were installed at pre-determined locations on the peptide, the N160 and N173 (for the doubly glycosylated peptide only) positions. Two types of glycopeptides were chosen as

building blocks for the antigens, one that includes only a single GlcNAc at the N160 position, and another that includes two glycans, one at N160 and the other at N173. During the synthesis of the second glycopeptide containing two GlcNAc acceptor sites, two selectively protected building block were incorporated, with an acid-labile Fmoc-Asn(DEIPS₃GlcNAc)-OH glycosylamino acid installed at the N160 position and a base-labile Fmoc-Asn-(Ac₃GlcNAc)-OH glycosylamino acid installed at the N173 position (5). This method followed the site-selective orthogonal protection strategy recently reported by our group.⁴⁴ The method provided a path to selectively glycosylate the glycopeptide with two distinct N-glycans at pre-determined sites. An alkyne tag with a short (3 carbon) linker was introduced at the N-terminus of the glycopeptides to facilitate biorthogonal attachment to the protein scaffold. After global peptide deprotection and peptide release using cocktail R, the GlcNAc at position N160 was selectively unmasked, while the acetylated GlcNAc at N173 remained protected. Both glycopeptides were cyclized after release from the resin. The glycopeptide bearing a single acetylated GlcNAc was cyclized concomitantly with deacetylation of the GlcNAc by treatment with 2.5% aqueous hydrazine. The double glycosylated glycopeptide was cyclized using a very mild method of 20% DMSO in water to maintain the acetyl protecting groups on the N173 position.⁴⁹ After cyclization, the glycopeptides were appropriate substrates for endoglycosidase-mediated transglycosylation. Given the specific substrate preferences of the endoglycosidase mutants, different endoglycosidase mutants were used for each substrate. Our group previously reported the high efficiency of the endoglycosidase D N332Q (EndoD-N332Q) mutant for the transfer of Man₅GlcNAc oxazoline to diverse

substrates,^{30, 50} we therefore chose to use the glycosynthase EndoD-N332Q to catalyze the transfer of Man₅GlcNAc (**8**) to the N160 position of the singly glycosylated peptide (**4**). We have previously shown that a cyclic glycopeptide bearing an acetylated GlcNAc is a poor substrate for transglycosylation catalyzed by EndoD-N332Q mutant. This result was supported when **6** was tested as a substrate for transfer of a Man₅GlcNAc glycan from its oxazoline form by EndoD-N332Q to give compound **10**. Unfortunately, EndoD-N332Q was found to be very inefficient at transfer, yielding <5% transglycosylation product.



Scheme 4.2. A) Transglycosylation (Man₅ox) of single GlcNAc acceptor peptide **4** at N160. **B)** Transglycosylation of orthogonally protected peptide **6**.

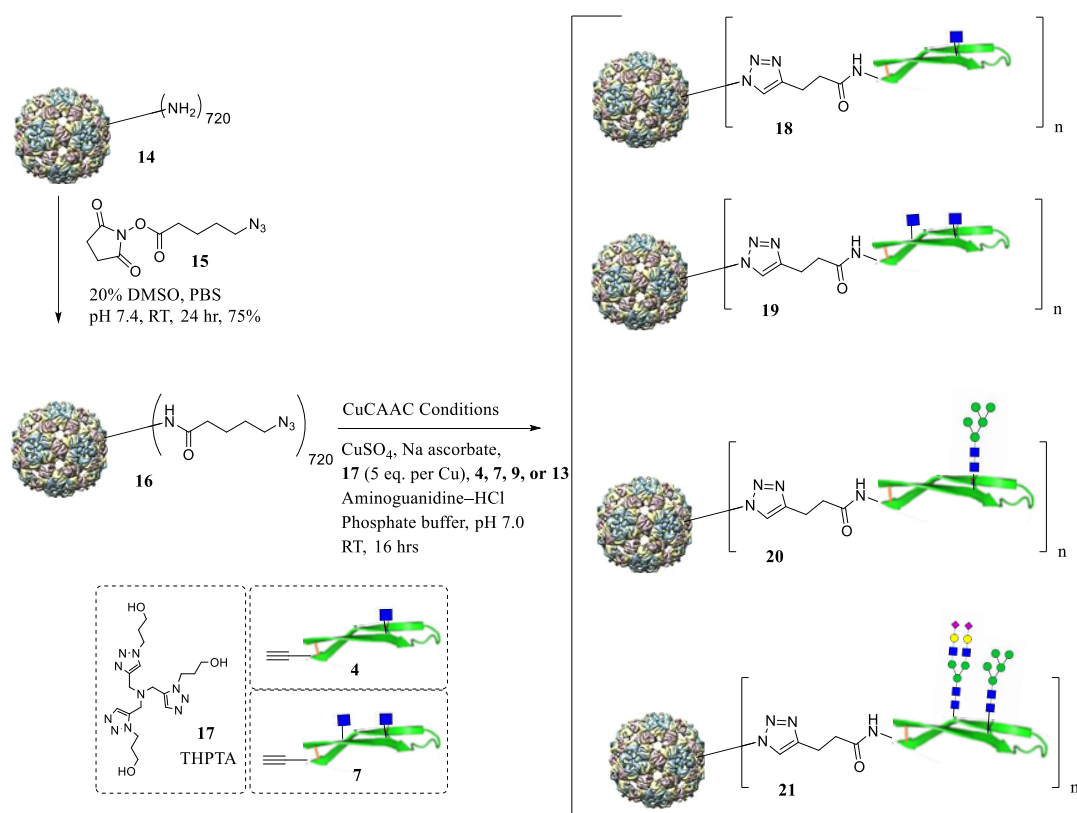
We therefore chose to use the glycosynthase EndoM-N175Q mutant to transfer Man₅GlcNAc oxazoline to the N160 position of the doubly glycosylated peptide **6** (Scheme **4.2B**), as our previous work indicated that the acetylated GlcNAc was well tolerated by EndoM-N175Q, giving the product **10** in excellent yield. For the

glycopeptide bearing a single GlcNAc, lacking the second unfavorable acetylated GlcNAc, EndoD-N332Q was highly efficient at transferring a Man₅GlcNAc oxazoline glycan, yielding the target glycopeptide building block **9** in excellent yield (Scheme **4.2A**). Both the purity and identity of the V1V2 glycopeptides was confirmed by HPLC and LC/MS analysis (see appendix).

Synthesis and Characterization of V1V2 Glycopeptide-Q β Conjugates

In order to achieve the multivalent display of the glycopeptides that we believed would be required to mimic the quaternary epitope of PG9, an appropriate carrier needed to be selected to provide a repetitive clustered pattern that will enhance the immunogenicity. An ideal carrier should also contain multiple T helper cell epitopes to be able to induce strong IgM and IgG responses, as well as potentiate these Ab responses. We again turned to virus-like particles (VLPs) as synthetic PG9-epitope delivery platforms. VLPs are composed of subunit proteins that self-assemble into highly ordered (usually) icosahedral structures. VLPs offer a promising approach to vaccine development, as VLPs, while non-infectious to human or animal hosts, are highly immunogenic. The high immunogenicity of VLPs can be attributed to their sizes, <100 nm diameter, which promotes trafficking to lymph nodes, as well as uptake by antigen presenting cells (APCs), their repetitive subunit display, which promotes B cell recognition, and their ability to cross-link B cell receptors, promoting a stronger, more sustained immune response. In addition, during recombinant bacterial expression, VLPs package bacterial RNA within the capsid, which acts as an internal adjuvant for activating Toll-like receptors during immunization. In addition, amorphous carriers traditionally used for vaccine conjugates (i.e. keyhole limpet

hemocyanin (KLH), tetanus toxoid (TT), etc.) cannot achieve the highly organized antigen geometry that is intrinsic to VLP scaffolds. The VLP bacteriophage Q β was used by Huang, X. and co-workers in a number of studies with great success as a carrier protein for the multivalent display of short peptide-based tumor associated carbohydrate antigens (TACAs).⁴⁶⁻⁴⁸ Q β has also been used in a number of anti-HIV vaccine candidates associated with reconstitution of the 2G12 epitope.⁵¹⁻⁵⁵ The Q β particle is a self-assembling protein composed of 180-identical protein subunits (~14 kD each). The subunits organize into an icosahedral structure that is 28 nm in diameter, and has excellent stability toward chemical modifications to its surface. We therefore chose to use the Q β particle as scaffold for multivalent clustered display of the V1V2 glycopeptides.



Scheme 4.3 Synthesis of Q β conjugates 17-20.

The ligation of the alkyne-tagged glycopeptides to the Q β capsid was achieved via the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction using biorthogonal reaction conditions similar to those described in Chapter 2. The CuAAC reaction is highly efficient and allowed high density loading of bulky glycopeptides onto a relatively small area. The decision to incorporate a highly stable covalent bond (i.e. the triazole linkage) between the glycopeptide and the Q β surface was informed by recent reports that the covalent linkage ensures that the antigen is processed with Q β and both are presented together to B cells.⁵²⁻⁵³ Such a manner of presentation will ensure potent B cell stimulation by matching activated T helper cells. The Q β particle **14** was derivitized with azido groups following the method described in Chapter 2. The alkyne-modified V1V2 glycopeptides were added to the azide-bearing Q β **16** in the presence of the copper catalyst, using THPTA (**17**) as the ligand (Scheme **4.3**). In order to ensure high loading of the glycopeptides onto Q β (i.e. 2-3 glycopeptides/subunit), a large excess of alkyne-tagged glycopeptides was found to be necessary. When 10 equivalents of alkyne-glycopeptide was added per subunit (2.5 equivalents per reactive azide) the loading was excellent for compounds **18-20** with an average of 2-3 glycopeptides per subunit. Conditions were tested with less alkyne-glycopeptide (5 equivalents, or 1.25 eq. per reactive azide) and loading was correspondingly diminished to an average of 1-2 glycopeptides per subunit. The excess unreacted glycopeptides could be recovered from the reaction mixture by HPLC. The doubly glycosylated alkyne peptide **13** proved difficult to load fully onto the Q β capsid (**21**), with an average of 1-2 peptides per subunit even in the presence

of excess alkyne-linked glycopeptides. The obstinacy toward high loading is likely a result of the high steric hindrance of these bulky glycopeptides bearing two large conformationally dynamic glycans. The reactive amines on the surface of Q β are well-dispersed in a repetitive pattern, thus the glycopeptide display is expected to be uniformly dispersed as repetitive clusters, potentially mimicking the display of the trimeric PG9 epitope. The glycopeptide-Q β conjugates were analyzed by MALDI-TOF analysis and SDS-PAGE to assess loading. For compounds **21** and **22** with significant quantities of unmodified subunits, MALDI-TOF underestimated the glycopeptide loading. The MALDI-TOF profiles of **21** and **22** indicate the presence of mostly unmodified subunits, while SDS-PAGE analysis indicates significant (i.e. 1-2 glycopeptides/subunit) loading (Figure 4.2). This apparent discrepancy is most likely attributable to the use of C4 ZipTips for protein purification prior to MALDI sample preparation. The hydrophobic (C4) stationary phase of the ZipTip most likely preferentially bound free subunits without glycosylation, as the large hydrophilic glycans have a low affinity for the hydrophobic C4 resin, thus artificially inflating the presence of free subunits by enrichment during purification. For glycopeptide-Q β conjugates with no significant portion of unmodified subunits, only subunits with glycosylation were present, yielding a profile that is much more consistent with the SDS-PAGE profile (Figure 4.2).

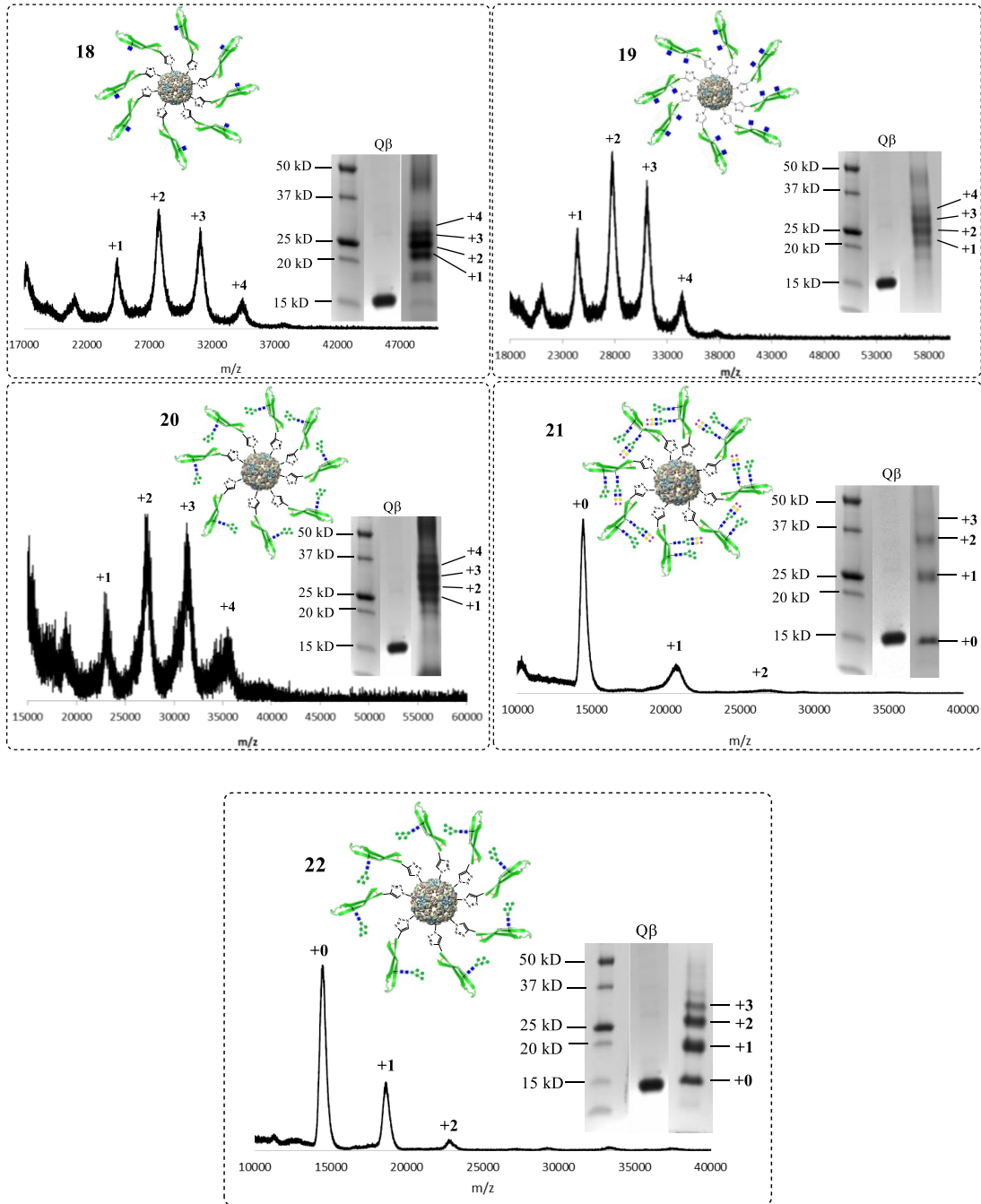


Figure 4.2 MALDI-TOF and SDS-PAGE analysis of glycopeptide-Q β conjugates **18-22**.

Antigenicity Studies

In order to assess the ability of PG9 to interact with the glycopeptide-conjugates as well as estimate the enhancement in binding affinity due to multivalent display, a conventional ELISA format was used. As a control, monomeric gp120 from clade M, strain M.CON-S (gp120_{M.CON-S}) was used. PG9 can only bind to monomeric gp120 from a select number of strains, as the binding affinity is frequently too low outside of the quaternary context. The glycopeptide conjugate antigens were immobilized on the plate and probed by a serial dilution of PG9. As a comparison, the monovalent glycopeptides were included.

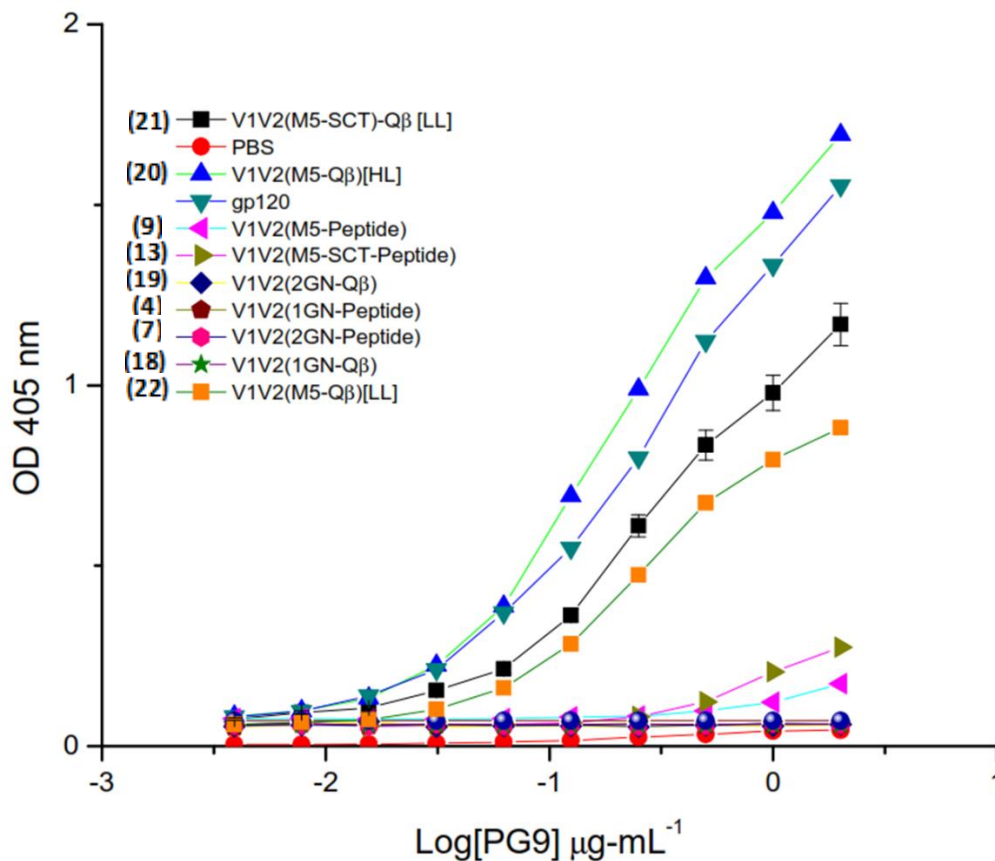


Figure 4.3. Conventional ELISA binding of V1V2 antigens to apex-directed PG9 bNAbs. LL = low-loading (1-2 peptides/subunit); HL = high-loading (2-3 peptides/subunit).

The antigenicity of the conjugates was found to be highly dependent upon glycosylation, as protein conjugates bearing GlcNAc (either one or two) peptides alone yielded no recognition by PG9 (see Figure 4.3), whereas the addition of a single Man₅GlcNAc₂ glycan substantially increased the binding affinity. The results suggest that the peptide component of the epitope alone, containing the cationic KQKKD sequence, is insufficient for significant binding affinity by PG9, even in a multivalent context. The finding is in agreement with the lack of affinity observed between PG9 and monovalent peptides containing GlcNAc alone previously reported by our group and others.^{30-31, 44} Clearly the epitope requires two or more components (i.e. cationic peptide sequence and one or more critical glycans), and even the presentation of multiple cationic peptide sequences in close proximity is not sufficient to produce high avidity binding in the absence of a glycan component. The addition of a single Man₅GlcNAc₂ glycan (construct **20**) was sufficient to significantly increase the binding affinity. Of note, the binding affinity of compound **20** for PG9 was significantly higher than for monomeric gp120_{M.CON-S} monomer, suggesting that the multivalent display provides enhanced binding, even in the absence of a second glycan at N173, when compared to *monomeric* gp120. In contrast, the monovalent glycopeptide **9**, bearing a single Man₅GlcNAc₂ glycan displayed significantly lower binding affinity for PG9 even at the same level of immobilization, the multivalent

display produced a ~18-fold enhancement compared to the monovalent peptide. Likewise, compound **21**, bearing two N-glycans, exhibited a similar enhancement in binding affinity, due to multivalent display, for the glycopeptide conjugate, versus the monovalent doubly glycosylated glycopeptide **13** producing a ~4-fold enhancement due to multivalency. As expected, the double glycosylated monovalent peptide (**13**) displayed higher binding affinity for PG9 than the corresponding singly glycosylated monovalent peptide (**9**). The lower affinity binding of **21** compared to **20** (~3-fold lower) with PG9 is striking as it is anticipated that the addition of a sialylated glycan at the N173 position should enhance binding. The lower binding affinity suggests that proper reconstitution of the quaternary PG9 epitope requires dense peptide loading onto the VLP surface. The loading of compound **21** (1-2 glycopeptides/subunit) is low compared to high-loading compound **20** (2-3 glycopeptides/subunit), and in spite of the sub-optimal glycosylation pattern, the higher glycopeptide loading is enough to significantly enhance recognition by PG9. In order to confirm that high-loading was critical for optimal PG9 binding, a low-loading glycopeptide-Q β conjugate bearing the V1V2 peptide with a single Man₅GlcNAc₂ glycan at N160 was prepared. Low glycopeptide loading was achieved by reducing the quantity of alkyne-tagged V1V2 glycopeptide added to the CuAAC reaction to 5 equivalents. The low-loading glycopeptide-Q β conjugate **22** exhibited ~4-fold reduced binding affinity for PG9 compared to the high-loading analog (**20**). Notably, the low-loading conjugate **22** also bound PG9 with reduced affinity compared to the double-glycosylated-Q β conjugate **21**. As the level of glycopeptide loading is the same for both conjugates **22** and **21**,

the superior binding affinity of compound **21** can be attributed to the presence of a sialylated glycan at the N173 position.

4.3 Conclusion

Recent innovations in the knowledge of trimeric gp120 epitope presentation have propelled the field of HIV-1 vaccine design forward and have emphasized the significance of quaternary structure in the proper orientation of bNAb epitopes on gp120. The recent report of low-level induction of neutralizing Abs by native-like envelope trimers⁴⁰ suggests that soluble trimers alone are not sufficient as immunogens and other factors, including proper multivalent display of bNAb epitopes, may be required to stimulate the type of response needed for an effective immunogen. Similarly, the multivalent display of stabilized gp120-like trimers on a nanoparticle scaffold was insufficient as both an antigen and immunogen to target the type of responses necessary for an effective vaccine. One promising approach is the multivalent display of specific bNAb epitopes. The display of the epitopes alone, outside of the context of gp120, can potentially direct the response toward induction of bNAbs, and prevent the dilution of the humoral response by the presentation of multiple antigenic regions, as on gp140. In this study, we sought to determine the effect of multivalent display on a VLP scaffold on recognition by PG9 to determine the ability to reconstitute the trimeric quaternary PG9 epitope using strategically designed glycopeptides. The use of glycopeptides gave us control over the specific pattern of glycosylation to determine the influence of glycan identity on PG9

antigenicity. Our results clearly indicate the ability of multivalent glycopeptide clustering to mimic the quaternary context of the V1V2 loops on gp120; indicating that the level of glycopeptide loading was directly connected to reconstitution of the PG9 epitope. In summary, the rational design and antigenic profiling of these V1V2 trimer-like glycopeptide clusters on a VLP scaffold clearly indicate the importance of multivalent display, and merit a detailed investigation into the immunogenic properties of these conjugates as potential vaccine candidates.

4.4 Experimental

General Procedures. Analytical reverse-phase HPLC was performed on a Waters Alliance[®] e2695 HPLC system equipped with a dual absorbance 2489 UV/Vis detector. Separations were performed using a C18 column (either Waters XBridge[®] Shield, 4.6 X 250 mm, 3.5 μ m; or Thermo Scientific Hypersil Gold[®], 4.6 X 250 mm, 3 μ m) at a flow rate of 0.5 mL/min. The column was eluted using a linear gradient of either 5-25% MeCN (Waters) in 30 min or 5-50% MeCN in 30 min (Thermo) with both solvents containing 0.1% TFA. ESI-MS spectra were obtained using either a Waters SQ Detector 2 single quadrupole mass spectrometer, or a ThermoFisher Q Exactive LC/MS orbitrap mass spectrometer. MALDI-TOF analysis was performed using a Bruker UltrafleXtreme (UTX) mass spectrometer with TOF/TOF detection. Preparative RP-HPLC was performed on a Waters 600 HPLC system equipped with a dual absorbance UV detector using a C18 column (Waters XBridge, Prep Shield, 10 X 250 mm, 5 μ m) at a flow-rate of 4 mL/min. The column was eluted using a linear gradient of 15-25% MeCN containing 0.1% TFA over 30 min. Expression and surface derivatization of bacteriophage Q β particles is described in chapter 2. Sugar

oxazolines (**8** and **12**) were obtained using the method described previously by our group.^{30, 54} PG9 and monomeric gp120_{MCON.S} were both obtained from the National Institutes of Health (NIH) through the AIDS Reagent Program.

General CuAAC Reaction Conditions

Synthesis of glycopeptide-Q β conjugates (**18-21**) was achieved using the CuAAC click reaction, with conditions similar to those reported by M.G. Finn and co-workers.⁴⁶ All solvents were degassed before use. In general, the following reagents were combined in the following order, 14 μ L 500 mM phosphate buffer, pH 7.0 (final 80 mM), 50 μ g **16** (from 7 mg/mL stock), alkyne-tagged glycopeptides (**6-7, 9, 13**) [10 eq./subunit, approximately 2.5 eq./reactive azide]. Pre-mixed Cu-THPTA solution (CuSO₄, 50 mM in water [3.4 eq.] + THPTA ligand (**17**) 50 mM in water, 5-fold excess compared to Cu), aminoguanidine (100 mM in water, 100 eq. compared to **16**), and sodium ascorbate (100 mM in water, 100 eq. compared to **16**). After the addition of sodium ascorbate, the reaction tube was flushed with argon and sealed. The mixture was shaken at 37°C overnight. Compound **22** was synthesized by a similar method, however, 5 eq./subunit (1.25 eq./reactive azide) of **9** was added to achieve low-loading. The reaction was quenched by the addition of 500 mM EDTA (10 mM final). Purification of the glycopeptide-Q β conjugates was performed by centrifugal filtration against PBS.

Peptide Synthesis. Peptide synthesis was performed under microwave-enhanced conditions using a CEM Liberty Blue microwave-assisted peptide synthesizer.

Peptide synthesis was based on Fmoc chemistry, using Fmoc-PAL-PEG-PS resin (18 mmol/g) on a 0.1 mmol scale. Synthesis was performed in DMF using a 6-fold excess

of both Fmoc-protected amino acids and TBTU, and a 12-fold excess of DIPEA (in NMP). The couplings were performed at 45°C for 20 min. The selectively protected glycosylamino acid building blocks ((DEIPS₃GlcNAc)-Asn-OH and (Ac₃GlcNAc)-Asn-OH) were introduced at pre-determined sites. The selectively protected glycosylamino acid building blocks were synthesized following methods recently reported by our group.⁴⁴ Fmoc deblocking was performed in 20% piperidine in DMF containing 0.1 M HOBt. After synthesis, the resin was washed with DMF (3x) and DCM (3x), dried, and then coupled with 5-alkynoic acid (3 eq.) in DMF using TBTU (3 eq.) and DIPEA (6 eq.) at RT. The resin was again washed with DMF/DCM and global peptide cleavage was performed using cocktail R (TFA/Thioanisole/Ethanedithiol/Anisole [90/5/3/2]) for 2 h. The peptide was separated from the resin by filtration and the peptide precipitated onto cold (-20°C) diethyl ether. The crude peptide was dried and dissolved in glacial acetic acid, followed by lyophilization. The doubly, differentially glycosylated peptide (**5**) was cyclized in 20% DMSO (aq.) at RT. The singly glycosylated peptide (**6**) was simultaneously cyclized and de-O-acetylated at the GlcNAc by treatment with a 2.5% hydrazine (aq.) solution at RT. All crude peptides were purified on preparative RP-HPLC to afford the purified glycopeptides.

Synthesis of cyclic peptide 6, bearing a single GlcNAc at Asn160. The precursor resin (0.1 mmol) was cleaved using cocktail R (*supra vide*). The crude, acyclic, released peptide was treated with an aqueous solution of 2.5% hydrazine (final peptide concentration = 2 mg/mL) at RT for 2h. After complete de-O-acetylation and cyclization as determined by RP-HPLC, the reaction was quenched by the addition of

glacial acetic acid. The crude material was purified by preparative RP-HPLC, yielding the target peptide **6** as a white powder (147.31 mg, 48% overall yield). ESI-MS: Calcd. M = 3039.22; found (m/z): 608.92 [M + 5 H]⁵⁺, 760.84 [M + 4 H]⁴⁺, 1014.44 [M + 3 H]³⁺, 1520.57 [M + 2 H]²⁺. Deconvolution mass: 3037.4. RP-HPLC retention time, t_R = 18.06 min.

Synthesis of cyclic peptide 5, bearing a GlcNAc at Asn160 and an acetyl-protected GlcNAc at Asn173. The precursor resin (0.1 mmol) was cleaved using cocktail R (supra vide). The crude, acyclic released peptide was cyclized using 20% DMSO (aq.) (2 mg/mL final peptide concentration) at RT overnight with shaking. The crude reaction mixture was lyophilized and purified by preparative RP-HPLC yielding glycopeptide **5** as a white powder (129.23 mg, 38% overall yield). ESI-MS: Calcd. M = 3369.35; found (m/z): 674.72 [M + 5 H]⁵⁺, 843.18 [M + 4 H]⁴⁺, 1123.87 [M + 3 H]³⁺, 1685.30 [M + 2 H]²⁺. Deconvolution mass: 3369.4. RP-HPLC retention time, t_R = 29.58 min.

Synthesis of glycopeptide 7, bearing a GlcNAc at Asn160 and a GlcNAc at Asn173. Glycopeptide **5** (50 mg, 14.8 μmol) was treated with an aqueous mixture of 2.5% hydrazine (final peptide concentration = 2 mg/mL) at RT for 1 h. The reaction was quenched by the addition of glacial acetic acid, and the crude mixture was purified by preparative RP-HPLC yielding glycopeptide **7** as a white powder (27.41 mg, 57%). ESI-MS: Calcd. M = 3242.32; found (m/z): 649.29 [M + 5 H]⁵⁺, 811.36 [M + 4 H]⁴⁺, 1081.50 [M + 3 H]³⁺, 1621.73 [M + 2 H]²⁺. Deconvolution mass: 3241.0. RP-HPLC retention time, t_R = 16.96 min.

Chemoenzymatic synthesis of homogeneous alkyne-tagged V1V2-glycopeptides.

Synthesis of glycopeptide 9, bearing a single Man₅GlcNAc₂ glycan at Asn160.

Glycopeptide **6** (4 mg, 1.32 μ mol) was incubated with excess Man₅-oxazoline (8.02 mg, 7.92 μ mol) at RT in the presence of EndoD-N332Q (40 ng/ μ L final) in phosphate buffer (80 mM, pH 7.2, 200 μ L). After completion of the enzymatic reaction after 1 h, as indicated by RP-HPLC analysis, the reaction was quenched by addition of 0.1% TFA (aq.). The product was purified by preparative RP-HPLC to give glycopeptide **9** as a white powder (4.82 mg, 90%). ESI-MS: Calcd. M = 4052.56; found (m/z): 811.37 [M + 5 H]⁵⁺, 1014.13 [M + 4 H]⁴⁺, 1351.71 [M + 3 H]³⁺ Deconvolution mass: 4051.8. RP-HPLC retention time, t_R = 13.53 min.

Synthesis of glycopeptide 10, bearing a Man₅GlcNAc₂ glycan at Asn160 and an

acetylated GlcNAc at Asn173. Glycopeptide **5** (5 mg, 1.48 μ mol) and Man₅-oxazoline (12.03 mg, 11.87 μ mol) were incubated at RT with EndoM-N375Q (0.2 μ g/ μ L) in phosphate buffer (80 mM, pH 7.2, 200 μ L). After completion of the transglycosylation reaction in 30 min (RP-HPLC monitoring), the reaction was quenched by the addition of 0.1% TFA (aq.). The product was purified by preparative RP-HPLC yielding glycopeptide **10** as a white powder (5.52 mg, 84%). ESI-MS: Calcd. M = 4380.69; found (m/z): 877.17 [M + 5 H]⁵⁺, 1096.44 [M + 4 H]⁴⁺, 1461.58 [M + 3 H]³⁺. Deconvolution mass: 4380.8. RP-HPLC retention time, t_R = 29.58 min.

Synthesis of glycopeptide 11 bearing a Man₅GlcNAc₂ glycan at Asn160 and a

GlcNAc at Asn173. Glycopeptide **10** (5.5 mg, 1.26 μ mol) was treated with aqueous 2.5% hydrazine to remove the O-acetyl protecting groups (final concentration = 2 mg/mL) at RT. The reaction mixture was neutralized by the addition of glacial acetic acid, and the product was purified using preparative RP-HPLC to give the

glycopeptide **11** as a white powder (3 mg, 56%). ESI-MS: Calcd. $M = 4254.66$; found (m/z): 851.96 $[M + 5 H]^{5+}$, 1064.96 $[M + 4 H]^{4+}$, 1419.92 $[M + 3 H]^{3+}$. Deconvolution mass: 4254.7. RP-HPLC retention time, $t_R = 28.32$ min.

Synthesis of glycopeptide 13 bearing a $Man_5GlcNAc_2$ glycan at Asn160 and a sialylated complex-type glycan (SCT) at Asn173. Glycopeptide **11** was dissolved in phosphate buffer (80 mM, pH 7.2, 150 μ L) with SCT-oxazoline (8.5 mg, 4.23 μ mol) in the presence of EndoM-N375Q (0.2 μ g/ μ L) at RT. After completion of the reaction as determined by RP-HPLC, the enzymatic reaction was quenched by the addition of 0.1% TFA (aq.). The product was purified by preparative RP-HPLC, yielding the target glycopeptide **13** as a white powder (3.1 mg, 70%). ESI-MS: Calcd. $M = 6256.35$; found (m/z): 1252.29 $[M + 5 H]^{5+}$, 1565.41 $[M + 4 H]^{4+}$. Deconvolution mass: 6257.0. RP-HPLC retention time $t_R = 24.68$ min.

Enzyme-linked immunosorbant assay (ELISA)

Each well of a high binding Ultra Cruz 96-well assay plate (Santa Cruz Biotech.) was first coated with 100 μ L PBS containing the V1V2 antigens (5 μ g/mL). The plates were incubated overnight at 4°C. The plate was then washed three times with PBS/0.05% Tween-20 (PBS-T), followed by the addition of 100 μ L 3% (w/v%) BSA in PBS to each well. The plate was incubated at RT for 1 h. The plate was again washed with PBS-T (3x). Next, PG9 was diluted in blocking buffer (PBS/0.05% Tween-20/1% (w/v %) BSA [PBS-BT]) to a maximum concentration of 2 mg/mL, followed by a 2-fold dilution series. For each PG9 antibody dilution, a total of 100 μ L was added to the appropriate wells. The plate was incubated with PG9 for 2 h at RT and then washed with PBS-T (3x). Next, a 1:2000 dilution of alkaline phosphatase

(AP)-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratory) was made in PBS-BT, with 100 μ L of this diluted secondary antibody added to each well. The plates were incubated with the secondary antibody for 1 h at RT then washed with PBS-T (3x). Finally, the wells were developed with 50 μ L p-nitrophenyl phosphate liquid substrate system (Sigma) for 5 min at RT before quenching the reaction with 30 μ L of 3 M NaOH. The optical density was then measured at a wavelength of 405 nm.

4.5 References

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Chapter 5: Chemoenzymatic synthesis of diverse high-mannose N-glycan library and related neoglycoproteins through top-down enzymatic trimming of a natural N-glycan

5.1 Introduction

Among the three major classes of asparagine-linked (N-linked) glycans, high-mannose (oligomannose) glycans contribute to the glycome in remarkably diverse and important roles, being implicated in such disparate functions as mannose binding protein interactions, HIV-related immunogenicity (via DC-SIGN, 2G12, etc.), neuronal cellular outgrowth, serum clearance rates of human IgG, etc. In particular, the well-known enrichment of oligomannose glycans on gp120 presents an interesting opportunity for vaccine design. Dense oligomannose glycan clusters are not common in mammalian systems ¹ and their extensive presence on gp120 represents a significant aberration in the glycosylation pattern when compared to host glycoproteins. The incorporation of predominantly oligomannose glycans within the glycan shield is a result of the enormous level of glycosylation that characterizes gp120. The dense glycosylation prevents access by host glycosylation machinery, stalling the glycan processing pathway, and resulting in significant levels of underprocessed glycans. ²⁻³ Moreover, within any region of the gp120 trimer that is highly congested (i.e. at the

protomer interface), higher levels of oligomannose glycans are observed.⁴⁻⁵ The preponderance of oligomannose glycans are critical both for gp120 structure and function. There are both long-range and short-range glycan stabilization effects that have been observed, and any perturbation results in a redistribution of Man₉GlcNAc₂/Man₈GlcNAc₂ glycans within the glycan shield.³ In addition, perturbations in the infectivity of the virus have been observed upon loss of glycosylation sites at N262, N386, and N392.^{6 7-8} Although HIV uses deletion of potential N-glycosylation sites (PNGS) as a means of escape from immunological recognition, the virus usually compensates for the loss of glycans by the addition of glycans at other sites, thereby maintaining a relatively constant glycosylation pattern within the glycan shield.⁹ Many bNAbs have been shown to bind promiscuously with gp120 and are able to accommodate the shifting PNGSs, as the glycans are usually spatially conserved.¹⁰ Many of the most promising bNAbs described to-date incorporate oligomannose glycans as a key component of their neutralizing epitope.¹¹ Finally, the interaction between HIV virions and dendritic cells homozygous for SIGN expression (i.e. DC-SIGN) is mediated by oligomannose glycans on gp120.¹² The mannose-binding proteins on DC-SIGN bind to the virions at the infection site and shuttle the active virus to the CD4⁺ T cell-rich lymphatic tissue, facilitating HIV infection.¹³ The reliance of HIV on N-glycans for both immune evasion and infectivity creates a weak point in the HIV armor that can be exploited by vaccine design that incorporates glycan epitopes. Targeting proteins (NAbs and others) that bind to oligomannose glycans on gp120 could provide a template for vaccine design. Such an approach has been used for the oligomannose-reactive bNAb 2G12.¹⁴⁻¹⁶

Despite the significance of oligomannose glycans in mediating HIV infection there is a conspicuous lack of access to the intermediate oligomannose isoforms, creating a major limitation in probing the exact glycan preferences of the immunological participants. Facile access to defined individual oligomannose isoforms (i.e. an oligomannose N-glycan library) would represent a major advancement in facilitating our understanding of the specific functions of these glycans in the corresponding biological processes. There have been a number of previous studies toward the development of a oligomannose N-glycan library, primarily utilizing a purely synthetic approach. An elegant solution to this problem was the development of a top-down chemoenzymatic strategy for the synthesis of a complete oligomannose N-glycan library including all possible regioisomers, recently reported by Ito et al.^{17 18} A limitation of this method is the scale, as well as the inaccessibility and time required for the synthesis of each component. The primary purpose of this work is the development of a chemoenzymatic method that allows facile access to a complete oligomannose library derived from natural sources consisting of Man₉GlcNAc₂-Asn down to the smallest constituent member Man₁GlcNAc₂-Asn and including all intermediate glycans from Man₈GlcNAc₂-Asn to Man₂GlcNAc₂-Asn. Such a library of defined oligomannose glycans could be used to reconstitute bNAb epitopes and selectively incorporate the specific oligomannose glycan that is optimal for the gp120-NAb interaction, together with many other applications that will facilitate our understanding of HIV-host interactions. In addition, the conjugation of defined oligomannose glycans to a carrier protein will yield antigens that can be applied to serum screening studies to identify novel

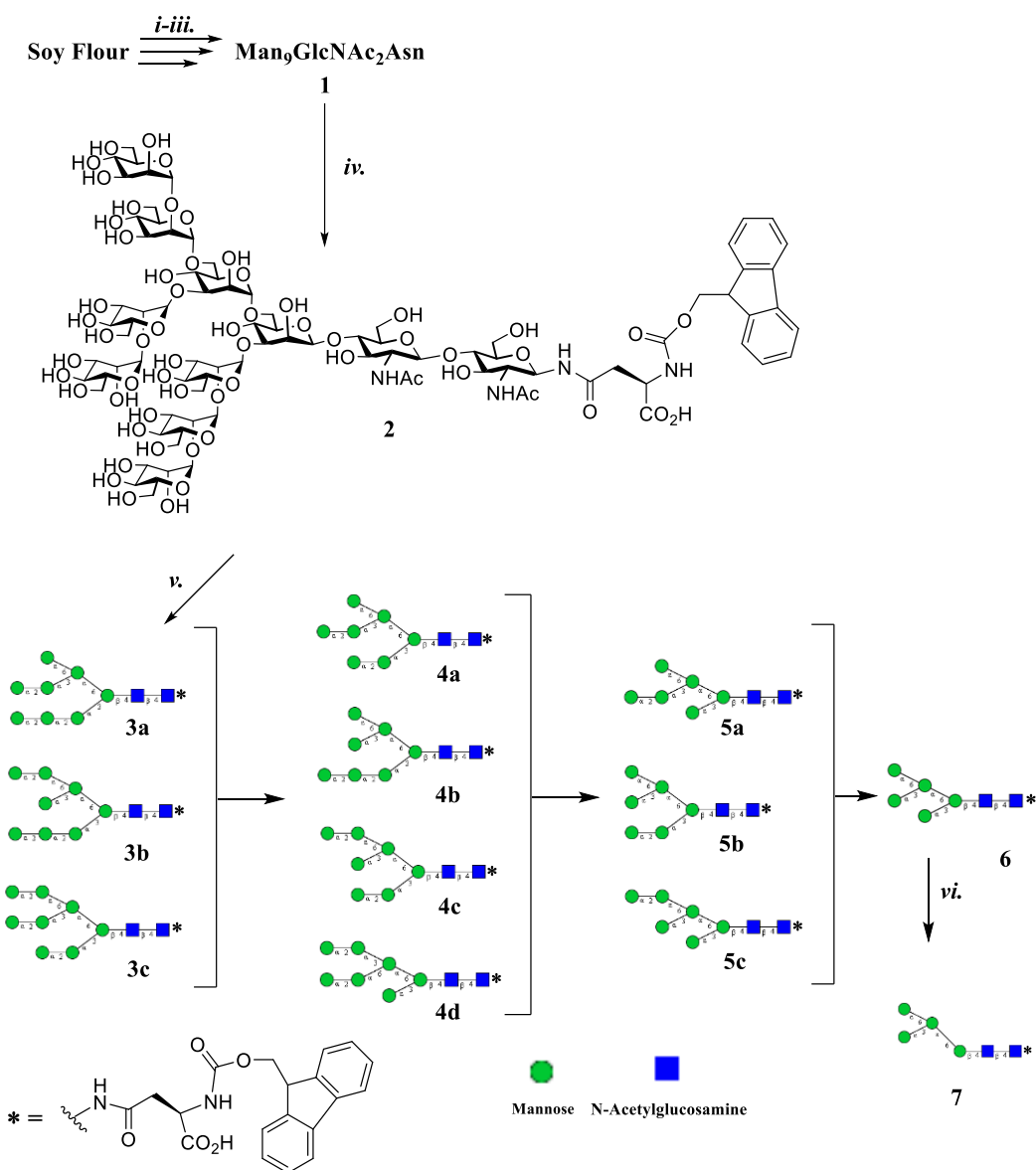
mannose-reactive proteins, with specific mannose-preferences, that could be used as a template for vaccine design. We here report the top-down chemoenzymatic synthesis of a complete oligomannose library, as well as the synthesis of oligomannose-protein conjugates. The associated binding studies demonstrate the utility of the reported method.

5.2 Results and Discussion

In the construction of the oligomannose library, speed and efficiency were of paramount importance. Given the rigor and time consuming nature of synthetic carbohydrate chemistry, we opted to obtain all necessary precursors from natural sources; chemoenzymatically modifying the precursors as needed. For the construction of the complete library, two precursors are critical starting materials, including Man₉GlcNAc₂-Asn (M9), for the construction of glycans Man₉₋₄GlcNAc₂-Asn, and sialylated complex-type glycan (SCT), for glycans Man₃₋₁GlcNAc₂-Asn. Two natural sources were identified for isolation of the precursors. The plant lectin soybean agglutinin (SBA) is well-characterized as a source for Man₉GlcNAc₂-Asn and has been used extensively by our group and others.¹⁹⁻²¹ Hen egg yolk is known to contain a myriad of useful glycoproteins and glycopeptides, in particular here, we isolated SCT-Asn from sialylated glycopeptide (SGP) obtained from hen egg yolk.

Each of the precursors, M9 and SCT-Asn, were released from the associated protein via exhaustive proteolytic digestion. SBA was first digested to peptidic fragments using proteolytic digestion with pepsin from porcine gut mucosa that eschewed an additional denaturing step, streamlining the process. The resulting

peptides were more easily digested with pronase down to the corresponding glycosylamino acid than starting with the parent protein. After release, the precursor glycan was purified by size exclusion chromatography and the oligosaccharide-Asn rich fractions were tagged with a 9-fluorenylmethyl (Fmoc) chemical handle to facilitate further purification as well as downstream isomer separation following a strategy similar to that reported by Kajihara et al.²² Fmoc was selected due to its ease of attachment/removal, its excellent UV-activity, and high degree of hydrophobicity, which affords better differentiation of individual glycans in the context of high-performance liquid chromatography (HPLC) separation.



i. 0.9% NaCl, 0.02% NaN₃, 30-60% ammonium sulfate; **ii.** Pepsin, pH 2.0; **iii.** Pronase, tris buffer, pH 8.0, 5 mM CaCl₂, 0.02% NaN₃; **iv.** 10X PBS pH 7.4, dimethylformamide (1:1), Fmoc-OSu; **v.** α-1-2-mannosidase, sodium citrate, calcium chloride, pH 5.6; **vi.** α-1-2,3-mannosidase, sodium citrate, calcium chloride, pH 6.0.

Scheme 5.1 Top-down chemoenzymatic synthesis starting from $\text{Man}_9\text{GlcNAc}_2\text{Asn}$ yields oligomannose glycans $\text{Man}_{4-9}\text{GlcNAc}_2\text{Asn-Fmoc}$.

Chemoenzymatic construction of a complete oligomannose library

In order to facilitate the chemoenzymatic construction of a functional oligomannose library, a variety of specific exoglycosidase enzymes were required. A major challenge in the synthesis of the $\text{Man}_{5-9}\text{GlcNAc}_2\text{Asn-Fmoc}$ (**2-6**) isoforms is the presence of four $\alpha 1, 2\text{-Man-Man}$ linkages. These linkages are *chemically* indistinguishable by the bacterial human gut symbiont enzyme $\alpha 1, 2\text{-mannosidase}$ (*Bacteroides Thetaiotaomicron*) reported by Davies et al.,²³ although the enzyme appears to have specific preferences based on substrate accessibility as dictated by the *steric* demands. Analysis of the mannosidase digestion by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) revealed the presence of two regioisomers each for Man_6 , Man_7 , and $\text{Man}_8\text{GlcNAc}_2\text{Asn-Fmoc}$ (Figure **5.1**) despite the possibility of three regioisomers each for Man_8 and Man_6 , and four isomers possible for Man_7 (Scheme **5.1**). The results of the HPAEC-PAD analysis demonstrate a clear preference by the enzyme for particular arms of each oligomannose glycan. In an uncontrolled reaction, all four $\alpha 1, 2\text{-Man}$ linkages will be hydrolyzed, yielding $\text{Man}_5\text{GlcNAc}_2$ as the sole product. We sought to capture the intermediate oligomannose glycans by controlling the enzyme concentration and carefully monitoring the progress of reaction. We discovered that at very low enzyme concentrations (i.e. 2 ng/mL), the intermediate HM-glycans $\text{Man}_{8-6}\text{GlcNAc}_2$ could be captured yielding a mixture of glycans particularly enriched with the intermediate glycans Man_{6-8} (Figure **5.1**). $\text{Man}_4\text{GlcNAc}_2\text{Asn-Fmoc}$ (**7**) was selectively obtained from $\text{Man}_5\text{GlcNAc}_2\text{Asn-Fmoc}$ (**6**) by exploiting the preference of $\alpha 1\text{-2, 3-mannosidase}$ for unbranched substrates, as the enzyme quickly removed the

linear α 1, 3-mannose, while removal of the branched α 1, 6-mannose proceeded extremely slowly, affording a modicum of selectivity (Scheme **5.1**).²⁴

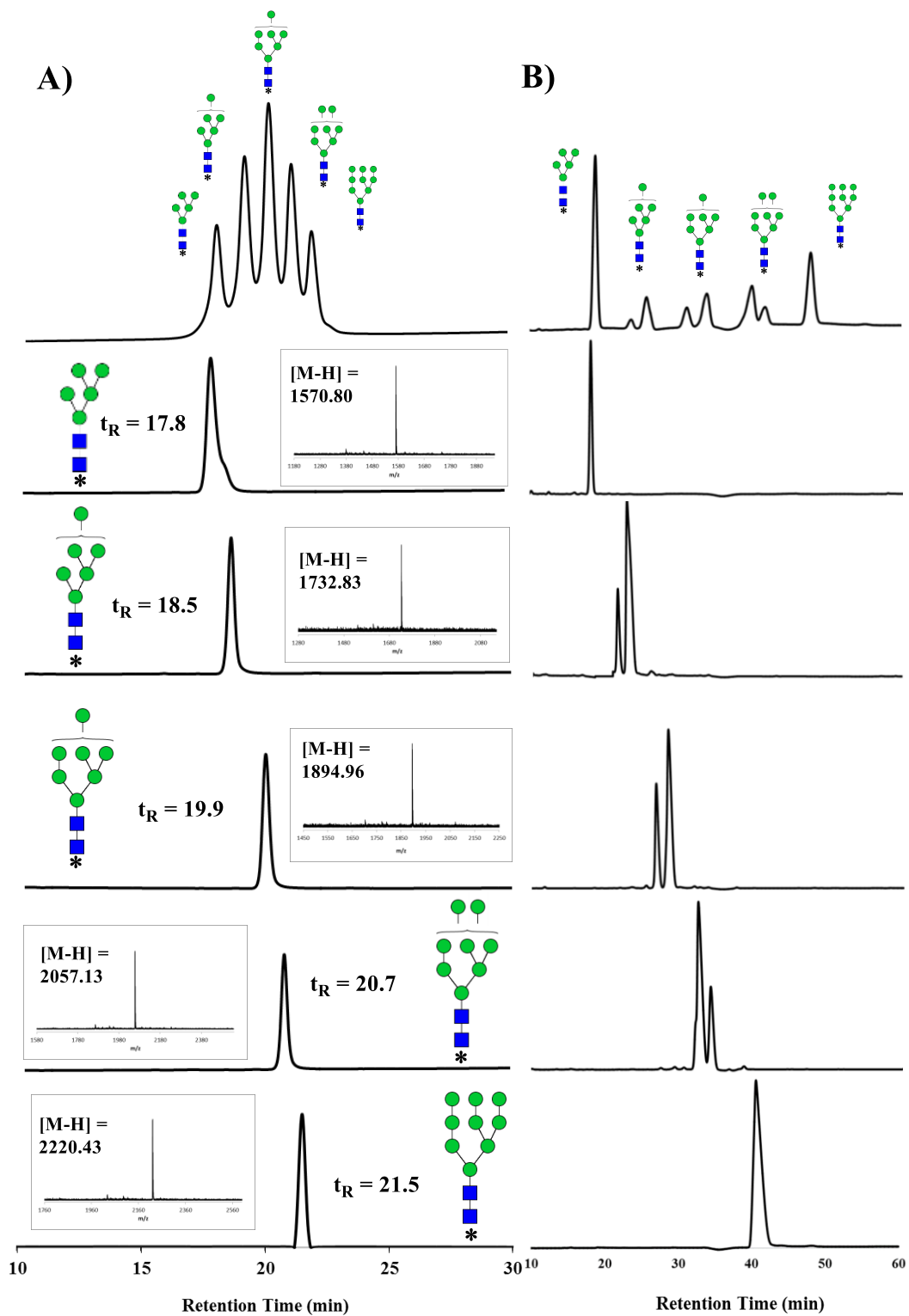
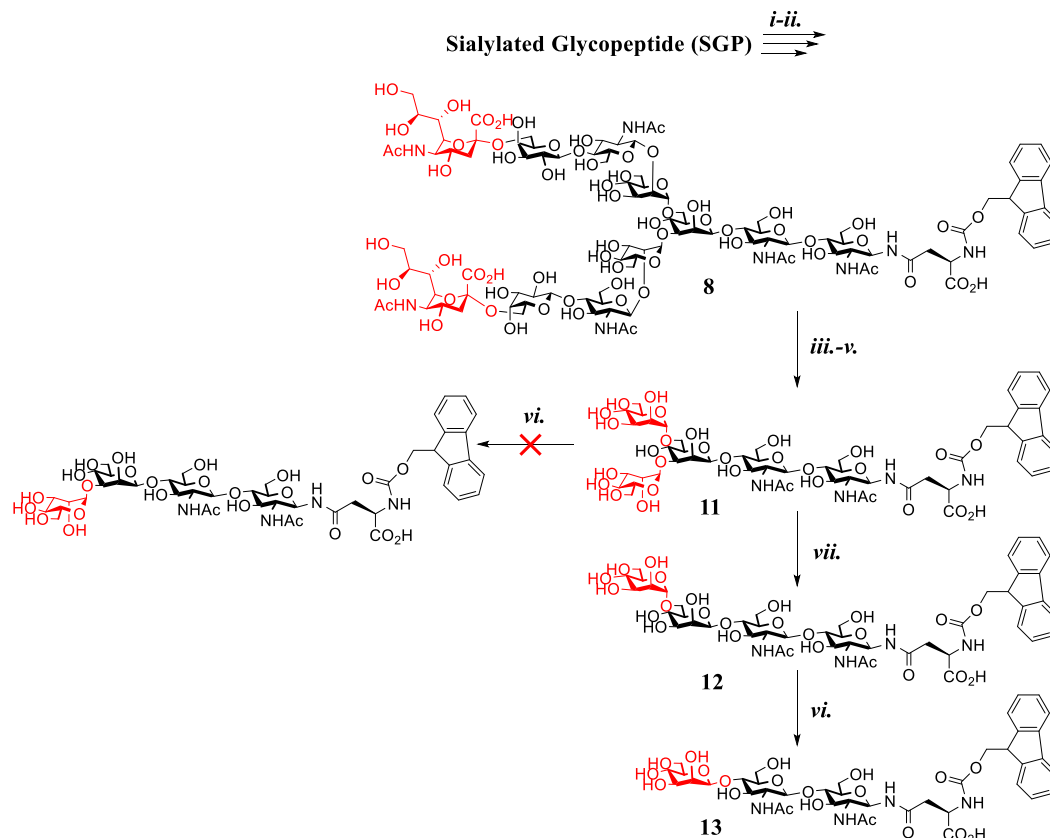


Figure 5.1 **A)** Separation of oligomannose glycans **2-6** was well resolved via 2D-HPLC. **B)** HPAEC-PAD analysis of $\text{Man}_{5-9}\text{GlcNAc}_2\text{Asn}$ reveals the formation of two regioisomers each for Man_6 , Man_7 , and $\text{Man}_8\text{GlcNAc}_2\text{Asn}$.

The construction of the pauci-mannose glycan library required the use of more diverse exoglycosidase enzymes including α 2-4,6,8-neuraminidase, β 1,4-galactosidase, β -N-acetyl-glucosaminidase, α 1,2,3-mannosidase, and α 1,6-mannosidase (Scheme **5.2**). Digestion of the SCT glycan, up to the Man_3 core could be conveniently performed as a one-pot reaction following a modified procedure from that reported by Huang et al.²⁵ The pauci-mannose glycans $\text{Man}_2\text{GlcNAc}_2\text{Asn-Fmoc}$ (**10**) and $\text{Man}_1\text{GlcNAc}_2\text{Asn-Fmoc}$ (**11**) were synthesized from $\text{Man}_3\text{GlcNAc}_2\text{Asn-Fmoc}$ (**9**) using α 1-2, 3-mannosidase and α 1, 6-mannosidase respectively. It should be noted that it was necessary for the Man_2 species to contain an α 1, 6-linked mannose, given the linear substrate requirement of α 1,6-mannosidase.²⁴ Therefore, enzymatic synthesis of the corresponding α 1,3-linked isomer was not possible (Scheme **5.2**).



i. Tris buffer, pH = 8.0, 5 mM CaCl₂, 0.02% NaN₃; **ii.** 10X PBS/dimethylformamide (1:1), Fmoc-OSu; **iii.** Neuraminidase, sodium acetate, CaCl₂, pH = 5.5; **iv.** β1-4-Galactosidase, pH = 4.5; **v.** β-N-acetylglucosaminidase, 100 μg/mL BSA, pH = 5.5; **vi.** α1-6-Mannosidase; **vii.** α1-2,3-Mannosidase.

Scheme 5.2 Digestion of sialylated complex-type glycan yields pauci-mannose glycans Man₁₋₃GlcNAc₂Asn-Fmoc.

Oligomannose glycan separation

Careful 2D-HPLC was necessary for separation of both the Man₅₋₉ glycans. The Fmoc-tagged glycans were first purified using reverse-phase (RP)-HPLC, followed by normal-phase (NP)-HPLC using an amide-bonded column. The oligomannose glycans were well resolved and collected as individual glycans (Figure 5.1). The individual oligomannose glycans were characterized by NP-HPLC and MALDI-TOF analysis to confirm the homogeneity of the separated glycans (Figure 5.1A, inset).

While analysis by NP-HPLC and MALDI established the purity and homogeneity of each individual glycan, HPAEC-PAD analysis revealed the presence of isomers for Man_{6-8} that could not be resolved on conventional NP-HPLC (Figure 5.1B).

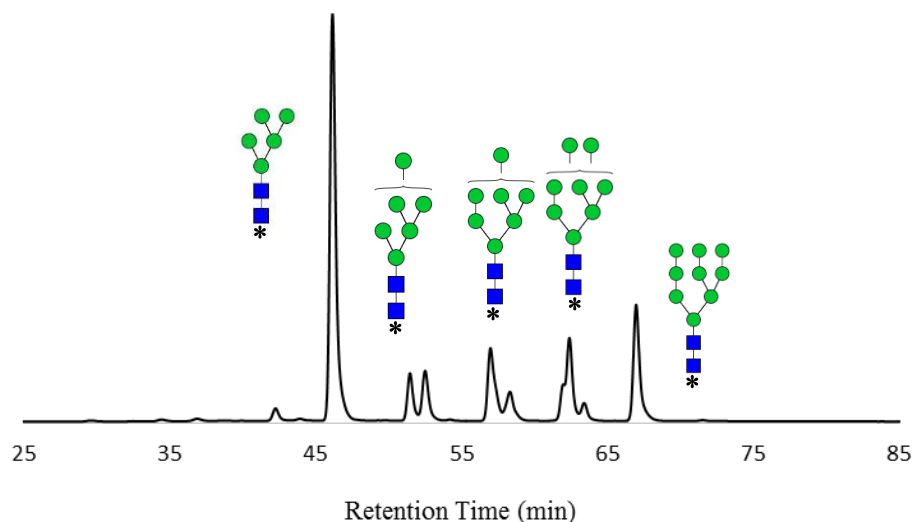
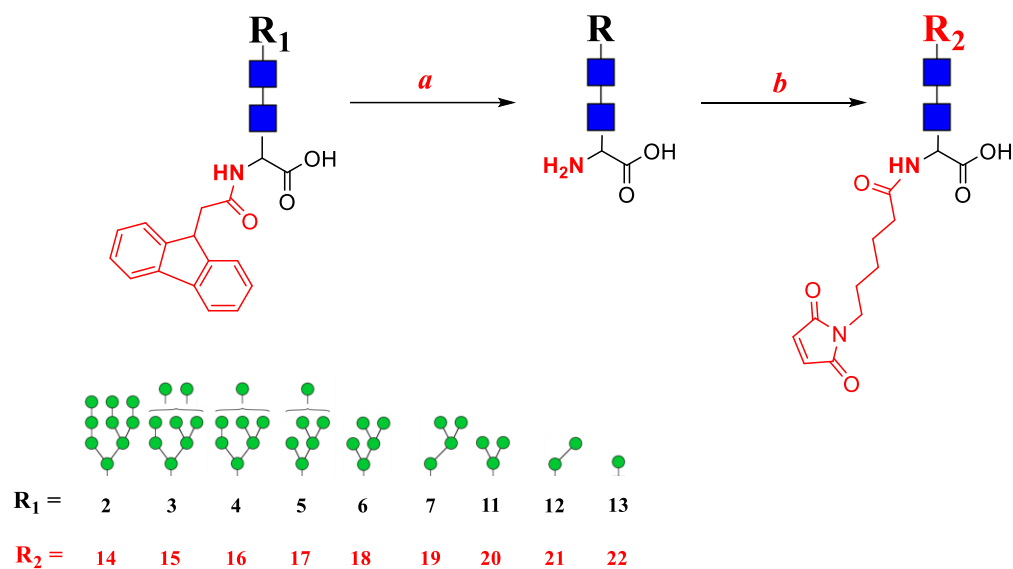


Figure 5.2 HILIC-HPLC separation of $\text{Man}_{5-9}\text{GlcNAc}_2\text{Asn-Fmoc}$ and the associated regioisomers.

While the Man_{6-8} regioisomers were impossible to separate using conventional NP-HPLC, the regioisomers were resolved using careful hydrophilic liquid interaction chromatography (HILIC)-HPLC. Figure 5.2 reveals near baseline separation between isomers, and the isomer pattern is in agreement with the HPAEC-PAD data. The ability to separate the isomers using HILIC-HPLC is highly significant as it provides access to functional quantities of very precious oligomannose isomers previously accessible only by chemical synthesis.

Oligomannose glycan functionalization: maleimide derivitization

Subsequent to the separation of the oligomannose glycans, the Fmoc tag was removed during a deblocking step using 20% piperidine in DMF/water. The unmasked primary amine was tagged using a heterobifunctional linker bearing both N-hydroxysuccinimide and maleimide functionalities (Scheme 5.3). The maleimide functionalized oligomannose glycans were purified by RP-HPLC yielding defined, conjugatable oligomannose glycans that facilitate downstream binding studies with a compatibly derivitized carrier protein.



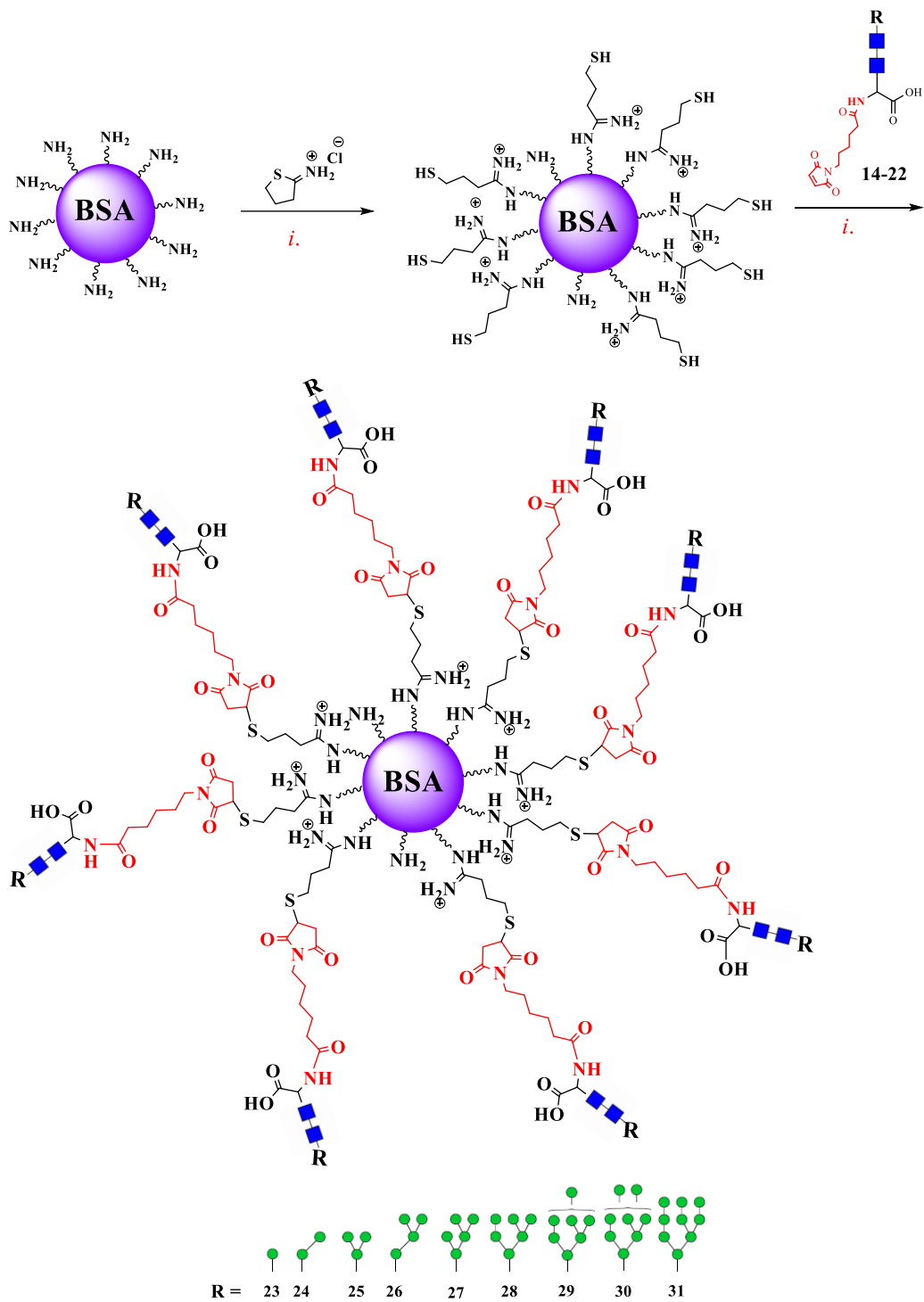
^a20% Piperidine, DMF:water (9:1). ^bEMCS, phosphate buffer, pH 7.5, 20% ACN.

Scheme 5.3 Deblocking the Asn amino-terminus to exchange tags with maleimide.

Synthesis of oligomannose-BSA conjugates.

Bovine serum albumin (BSA) was chosen as the carrier protein for serum screening studies. BSA is an optimal carrier for this context given its well-understood surface chemistry and resolved antigenicity. BSA was derivitized using Traut's reagent, which tagged solvent-exposed lysine groups with sulfhydryl groups (Scheme

5.4). Incubation of sulfhydryl-derivitized BSA with maleimide-tagged oligomannose ligands, yielded oligomannose glycan-BSA conjugates, with an average loading of 10 oligomannose glycans per BSA carrier (Scheme **5.4**). The strategy employed was reported previously by our group.²⁶⁻²⁷ The oligomannose glycan loading was quantified using the phenol-sulfuric acid test against a monosaccharide standard curve. The oligomannose-BSA conjugates were purified by size-exclusion chromatography and used directly in the microarray serum screening studies.



i. 50 mM Phosphate Buffer, 5 mM EDTA, pH 7.5

Scheme 5.4 Sulphydryl functionalization of native BSA and subsequent formation of the oligomannose-BSA conjugates.

Glycan Microarray Serum Screening Studies

In order to verify the compositions of the structures as well as illustrate the utility of the described method, the oligomannose-BSA conjugates were tested against various oligomannose binding proteins in an ELISA format. The oligomannose-BSA conjugates were directly printed onto the microarray plate, and the oligomannose-binding proteins were incubated with each conjugate. The oligomannose-binding proteins chosen included two lectins, Narcissus pseudonarcissus lectin (NPA) and Galanthus nivalis lectin (GNA), as well as the broadly-neutralizing antibody (bNAb) 2G12, which targets the high-mannose patch on the envelop glycoprotein gp120 of HIV. The two lectins, NPA and GNA, are specific for terminal $\text{Man}\alpha 1\rightarrow 6$ moieties and $\text{Man}\alpha 1,3\text{Man}/\text{Man}\alpha 1,6\text{Man}$ linkages respectively. 2G12 recognizes the terminal $\text{Man}\alpha 1,2$ linkages on the three arms of Man_9 . Positive controls included native gp140, which is heavily glycosylated with oligomannose glycans, and a tetravalent galactose scaffold bearing four Man_9 glycans conjugated to a Keyhole lymphocyte hemocyanin (KLH) carrier [(M9)₄-TH] reported previously by our group.²⁰ The background (Bg) signal served as the negative

control.

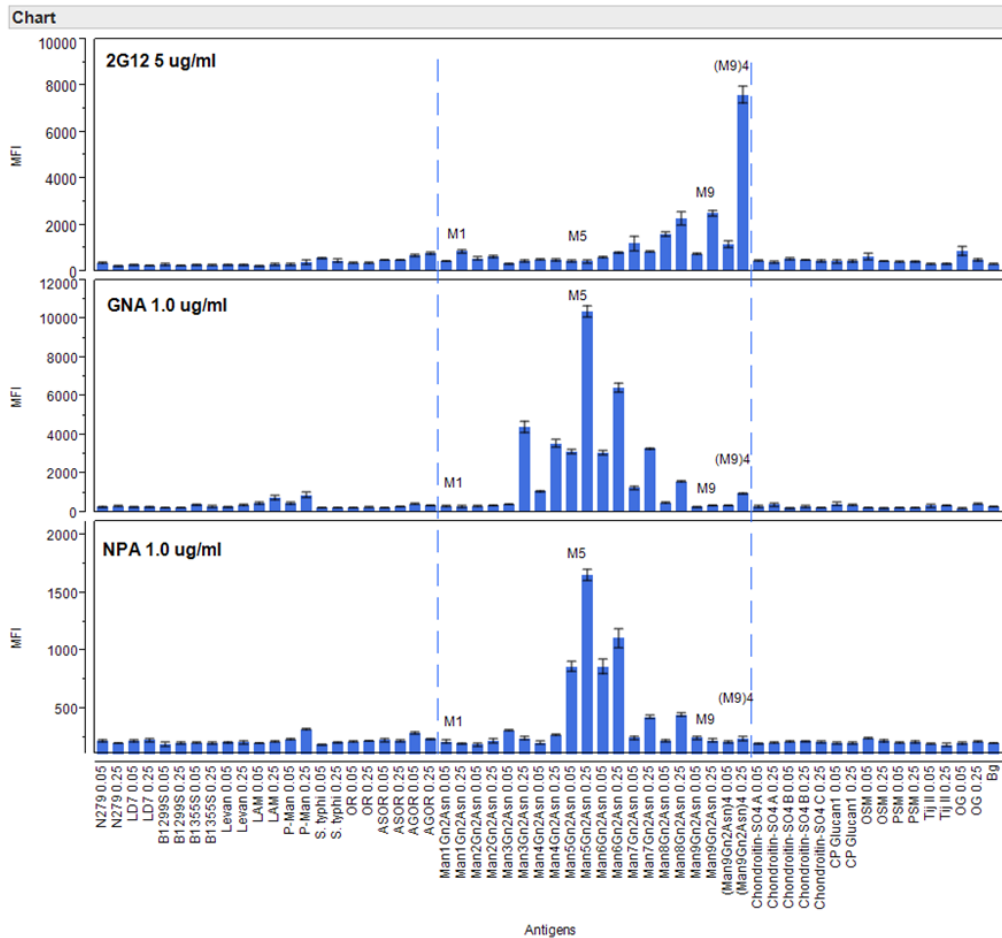


Figure 5.3 Binding of mannose-reactive proteins 2G12, GNA, and NPA to oligomannose-BSA conjugates

The binding data was in agreement with the expected preferences of the oligomannose-binding proteins. The intensity of the 2G12 recognition of the oligomannose-BSA conjugates roughly corresponded to the number of Man α 1,2 linkages present. The affinity of 2G12 for the oligomannose-BSA conjugates decreased for conjugates bearing fewer Man α 1, 2 linkages. As expected, the gp120 and (M9)4-TH controls exhibited very strong binding, while among the BSA

conjugates, compounds **31** and **30** (M9 and M8-BSA) showed the best recognition (Figure **5.3**). The far lower binding of the BSA conjugates as compared to the controls can be attributed to the mode of oligomannose glycan presentation. The high-mannose patch of gp120 and the tetravalent (M9)₄-TH conjugate present the oligomannose glycans in a dense, clustered arrangement optimal for 2G12 binding. In the BSA conjugates, the glycan is dispersed across the face of the protein resulting in a lower density non-clustered presentation and decreased 2G12 recognition. The oligomannose-BSA conjugates with higher access to the α 1, 6-linked mannose generally bound more strongly to NPA, with compound **27** displaying the highest affinity (Figure **5.3**). Recognition of the BSA conjugates by GNA appeared to be dependent upon the accessibility of the target Man α 1, 3-Man linkages. GNA had little affinity for compound **31** (M9-BSA), however, recognition improved as access to the core Man α 1, 3-Man linkages became more available. It follows that the two smallest pauci-mannose constituents (M1 and M2) had no affinity for GNA as they both lack Man α 1, 3-Man linkages (Figure **5.3**).

5.3 Conclusions

The method described entails a novel approach giving unprecedented access to preparative quantities of critical oligomannose glycan intermediates that can be used for functional and analytical studies. The utility of this method is emphasized by the epitope mapping studies, highlighting the different characteristics between the oligomannose glycans. This facile method introduces a powerful tool for probing the specific glycan preferences of oligomannose-dependent biological events. Access to

the library also allows the specific contribution of each individual glycan type to be measured, optimizing the specific oligomannose glycan chosen for functional studies. The ability of the mannose-binding proteins used in this study to discriminate between the oligomannose glycans conjugated to BSA adumbrates the use of these glycan-protein conjugates for identifying novel mannose-binding proteins that may be present in the serum of HIV infected individuals via serum screening studies. The identification of new mannose-binding proteins could define new epitopes on gp120, yielding new templates for vaccine design and expanding the options for HIV eradication.

5.4 Acknowledgements

C.L. Toonstra carried out all experimental procedures. The binding studies were performed by Dr. Denong Wang of Stanford University.

5.5 Experimental

Untoasted soy flour was obtained from Archer-Daniel-Midlands (ADM) (Chicago, IL). α 1-2-mannosidase was isolated from *bacteroides thetaiotaomicron* as reported.²³ Neuraminidase, α 1-2,3-mannosidase, α 1-6-mannosidase, and β -N-acetylglucosaminidase were purchased from New England Biolabs. β 1-4-galactosidase was purchased from Sigma. Reverse phase analytical high performance liquid chromatography was performed using a Waters Alliance e2956 HPLC system using a Waters XBridge C18 column (4.6 x 250 mm, 3.5 μ m). Solvent A was water containing 0.1% trifluoroacetic acid (TFA). Solvent B was acetonitrile containing

0.1% TFA. Normal phase HPLC was performed with a YMC Amide-bonding NH₂ column (4.6 x 250 mm, 3.5 μm). Preparative HPLC was performed using a Waters (600e) HPLC system. The RP preparative column used was a Waters XBridge Shield C18 column. HILIC-HPLC was performed using a Waters BEH Glycan column (4.6 x 250 mm, 3 μm), with 100 mM ammonium formate (pH 4.6) [solvent A] and acetonitrile (solvent B). A linear gradient was used for HILIC-HPLC from 22→44%A in 80 min. Electrospray ionization mass spectrometry was performed using a Thermo Q Exactive LC/MS. Matrix-assisted laser desorption ionization with time-of-flight detection was performed using a Bruker UltrafleXtreme MALDI TOF/TOF Mass Spectrometer with a dihydroxybenzoic acid/dimethylamide (DHB/DMA) matrix. High-performance anion-exchange chromatography with pulsed-amperometric detection (HPAEC-PAD) was performed using a Dionex 9000 system equipped with a CarboPac PA200 anion-exchange column (Thermo). Fast Protein Liquid Chromatography (FPLC) was performed using a General Electric (GE) Akta Explorer equipped with a GE Sephadex G-25 column.

Isolation of SBA from Untoasted Soy Flour and Subsequent Man₉GlcNAc₂Asn

Release and Fmoc Labelling.

Man₉GlcNAc₂Asn was prepared by enzymatic digestion of soybean agglutinin (SBA) and subsequent size-exclusion chromatographic purification. Crude SBA was obtained from untoasted soy flour following a modified method from that reported by our group and others.^{19 16} Briefly, 0.8 kg untoasted soy flour was suspended in 4 L 0.9% saline solution containing 0.02% sodium azide. The pH of the suspension was

adjusted to 4.6 using 2 M hydrogen chloride, and vigorously stirred at 4°C for 2 hrs. The suspension was centrifuged at 8,000 RPM for 20 min, and to the supernatant was added 30% (NH₄)₂SO₃ and the solution was stirred for 2 hrs at 4°C. Again the solution was centrifuged (8,000 RPM for 20 min), and ammonium sulfate was added to the supernatant to a final concentration of 60%. The solution was stirred vigorously overnight (RT) and centrifuged at 8,500 RPM for 30 min. The pellet was dissolved in 400 mL water, and the crude SBA was dialyzed against 0.2% saline with 3 changes of dialysis solution (2 hr for each). The SBA was dialyzed against running water overnight. The dialyzed protein was centrifuged (8,000 RPM/20 min) and the pH of the supernatant was adjusted to 2. Pepsin from porcine gut mucosa was added (1:20 protein to enzyme as assessed by Nanodrop estimation) and the solution was shaken at 37°C overnight. After determination of the complete protein digestion (Bradford Assay), the pH of the solution was adjusted to pH 8 with 2 M NaOH. The solvent was reduced in vacuo to 60 mL, and CaCl₂ and NaN₃ was added to final concentrations of 5 mM and 0.02% respectively. Pronase was added (1:50 protein to enzyme ratio) and the solution was incubated overnight at 55°C. The release of Man₉GlcNAc₂Asn was monitored by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The enzyme digestion was lyophilized and purified by size-exclusion chromatography (Sephadex G50) with monitoring by the phenol-sulfuric acid test (for carbohydrates). The carbohydrate positive fractions were pooled and lyophilized to yield semi-pure Man₉GlcNAc₂Asn. Semi-pure Man₉GlcNAc₂Asn was further purified by C18 solid phase extraction (SPE) eluted by water. To a solution of Man₉GlcNAc₂Asn (20 mg, 10.02 μmol) in PBS (200 μL, pH

7.4) was added Fmoc-OSu (20 mg, 59.29 μmol) dissolved in dimethylformamide (200 μL). The solution was shaken at RT for 4 hrs, and the target was precipitated by the addition of a 10-fold excess of cold (-20°C) acetone. The crude product **2** was dissolved in water and purified by RP-HPLC (0-90% B in 30 min, $t_{\text{R}} = 16$ min), yielding the target compound **2** (19.6 mg, 88% yield). MALDI-TOF: m/z : $[\text{M} + \text{H}] = 2221.17$ (Calculated = 2220.14).

Isolation of sialylated glycopeptide (SGP) from hen egg yolk, and subsequent sialylated complex-type (SCT) glycan release and Fmoc labelling (8).

SGP was isolated from hen egg yolk using a method previously reported by our group.²⁸ The purified SGP was dissolved in 80 mM Tris buffer (pH 8.0) containing 5 mM CaCl_2 and pronase was added (final concentration 0.1 mg/mL), and the digestion was incubated at 55°C overnight. The released SCT-Asn glycan was purified by G50 SEC, monitored by the phenol-sulfuric acid test. The carbohydrate positive fractions were pooled and lyophilized. Semi-pure SCT-Asn was further purified by C18 solid phase extraction (SPE) eluted by water. To a solution of SCT-Asn (30 mg, 12.8 μmol) in PBS (300 μL , pH 7.4) (pH adjusted to 7.5 with 0.5 M NaOH) was added Fmoc-OSu (30 mg, 88.93 μmol) dissolved in dimethylformamide (300 μL). The solution was shaken at RT for 4 hrs, and the target was precipitated by the addition of a 10-fold excess of cold (-20°C) acetone. The crude product **8** was dissolved in water and purified by RP-HPLC (0-90% B in 30 min, $t_{\text{R}} = 15$ min), yielding the target compound **8** (29.1 mg, 89% yield). ESI-Orbitrap: m/z : $[\text{M} + 3\text{H}] = [\text{M} + 2\text{H}] = [\text{M} + \text{H}] = 2559.34$ (Calculated = 2559.90).

Synthesis of high-mannose glycans (3-6) from Man₉GlcNAc₂ (2) precursor

To a 2 mM solution of **2** (27 mg, 12.2 μmol) in sodium citrate buffer (50 mM, pH 5.6) containing CaCl₂ (10 mM) was added α1, 2-mannosidase (final concentration = 2 ng/μL). The solution was incubated at RT for 30 min, while monitoring by HPAEC-PAD. The deblocked HPAEC-PAD samples were prepared by incubating 1 μL of the enzymatic digestion with 1 μL 0.5 NaOH at 60 °C for 5 min. After achieving optimal digestion, the reaction was quenched by the addition of excess 0.1% TFA. The mixture of oligomannose glycans was purified by RP-HPLC and the individual oligomannose glycans were separated by NP-HPLC, using a gradient of 22-41%B in 40 min. The individual isomers were desalted using RP-HPLC, yielding each isomer in milligram quantities (**2**, 3 mg, 1.40 μmol, 16%; **3**, 4 mg, 1.95 μmol, 21%; **4**, 5 mg, 2.64 μmol, 26%; **5**, 4 mg, 2.31 μmol, 21%; **6**, 3 mg, 1.91 μmol, 16%; 19 mg total recovered, 82% estimated yield [based on Man₇]).

Compound 3 (Man₈GlcNAc₂Asn-Fmoc)

NP-HPLC: t_R = 20.7 min. MALDI-TOF: m/z: [M + H] = 2057.13 (Calculated = 2057.71). HPAEC-PAD: t_R = 35.67 min.

Compound 4 (Man₇GlcNAc₂Asn-Fmoc)

NP-HPLC: t_R = 19.9 min. MALDI-TOF: m/z: [M + H] = 1894.96 (Calculated = 1895.66). HPAEC-PAD: t_R = 29.4 min.

Compound 5 (Man₆GlcNAc₂Asn-Fmoc)

NP-HPLC: t_R = 18.5 min. RN-MALDI-TOF: m/z: [M - H] = 1732.83 (Calculated = 1732.59). HPAEC-PAD: t_R = 22.7 min.

Compound 6 (Man₅GlcNAc₂Asn-Fmoc)

NP-HPLC: $t_R = 17.8$ min. RN-MALDI-TOF: m/z : $[M - H] = 1570.80$ (Calculated = 1570.45). HPAEC-PAD: $t_R = 15.9$ min.

Synthesis of Man₄GlcNAc₂Asn-Fmoc (7) from Glycan Precursor Man₅Asn-Fmoc (6)

To a solution of **6** (10 mg, 6.37 μ mol) in sodium citrate buffer (50 mM, pH 6.0) containing CaCl₂ (10 mM) was added α 1-2,3-mannosidase (final concentration = 1 μ g/ μ L). The enzymatic reaction mixture was incubated at 37°C overnight. The complete conversion of Man₅ to Man₄ was monitored by HPAEC-PAD and ESI-MS. The product was purified by RP-HPLC (0-90%B, $t_R = 16$ min), yielding the target compound **7** (8.1 mg, 90% yield). MALDI-TOF: m/z : $[M + H] = 1409.67$ (Calcd. = 1409.54).

Synthesis of pauci-mannose library (11-13)

The starting material **8** (20 mg, 7.8 μ mol) was digested to the largest common precursor **11**, following a modified version of the one-pot enzymatic procedure reported by Huang et al.²⁵ Briefly, **8** (20 mg, 7.8 μ mol) was dissolved in 50 mM sodium acetate buffer (50 mM, pH 5.5, pH adjusted with 0.5 M NaOH). Neuraminidase was added (60 U), after complete removal of sialic acid (determined by HPAEC-PAD/ESI-MS), the pH was adjusted to 4.5 with 1 M HCl and β -galactosidase was added (32 U), the solution was incubated at 37°C overnight. After removal of terminal galactose, the pH was adjusted to 5.5 and β -N-Acetylglucosaminidase was added (0.2 U) and the solution was again incubated overnight at 37°C. The mannose-terminal compound was purified by RP-HPLC yielding the

pauci-mannose precursor **11** (9.1 mg, 89%). MALDI-TOF: m/z: [M +H] = 1247.92 (Calcd. = 1247.17).

Synthesis of Man₂GlcNAc₂-Asn-Fmoc (12) from compound 11.

To a solution of **11** (7 mg, 5.6 μ mol) in sodium citrate buffer (50 mM, pH 6.0) α 1-2,3-mannosidase was added (960 U) and the solution was incubated overnight at 37°C. The target compound was purified by RP-HPLC yielding the target compound **12** (6 mg, 94% yield). MALDI-TOF: m/z: [M +H] = 1085.79 (Calcd. = 1085.03).

Synthesis of Man₁GlcNAc₂-Asn-Fmoc (13) from compound 12.

To a solution of **12** (3 mg, 2.7 μ mol) in sodium citrate buffer (50 mM, pH 6.0) was added α 1-6-mannosidase (1200 U) and the solution was incubated overnight at 37°C. The target was purified by RP-HPLC yielding the target compound **13** (2.3 mg, 92% yield). ESI-MS: m/z: [M +H] = 923.10 (Calcd. = 922.89).

General Procedure for Deblocking N-Terminus of Asparagine on Oligomannose Library

Fmoc-tagged compounds **2-10** were dissolved in a small amount of water. Piperidine in DMF was added to give a final concentration of 20% in 1:9 water/DMF. The reaction mixture was shaken at RT for 30 minutes while monitoring by HPLC. The reaction mixture was neutralized with glacial acetic acid and lyophilized. The product was precipitated using cold 80% acetone. The white precipitate was collected, dried and used in the next step without further purification.

Maleimide Conjugation of Oligomannose Glycans (14-22)

General conditions: The individual deblocked oligomannose glycan was dissolved in 1X PBS (final concentration = 25 mg/mL), 8 equivalents of the heterobifunctional linker N- ϵ -maleimidocaproyl-oxysuccinimide ester (EMCS) dissolved in acetonitrile was added, giving a final acetonitrile concentration of 20%. The solution was shaken at RT for 2 hrs, while monitoring with RP-HPLC (0-50%B, 214/302 nm, t_R = 15 min). Once the reaction was complete, the solution was diluted with solvent A and purified by RP-HPLC, giving the target compound as a white powder.

Compound 14 (Man₁GlcNAc₂Asn-Maleimide)

From **13** (3 mg, 4.2 μ mol), recovered yield (**14**): 3.2 mg, 84%. RP-HPLC: t_R = 14.8 min. MALDI-TOF: m/z: [M + H] = 894.14 (Calculated = 894.86).

Compound 15 (Man₂GlcNAc₂Asn-Maleimide)

From **12** (3 mg, 3.4 μ mol), recovered yield (**15**): 3.0 mg, 83%. RP-HPLC: t_R = 14.9 min. MALDI-TOF: m/z: [M + H] = 1057.64 (Calculated = 1057.00).

Compound 16 (Man₃GlcNAc₂Asn-Maleimide)

From **11** (3 mg, 2.9 μ mol), recovered yield (**16**): 3.0 mg, 86%. RP-HPLC: t_R = 14.9 min. MALDI-TOF: m/z: [M + H] = 1219.47 (Calculated = 1219.14).

Compound 17 (Man₄GlcNAc₂Asn-Maleimide)

From **7** (3 mg, 2.5 μ mol), recovered yield (**17**): 3.1 mg, 89%. RP-HPLC: t_R = 15.0 min. MALDI-TOF: m/z: [M + Na] = 1402.12 (Calculated = 1402.26).

Compound 18 (Man₅GlcNAc₂Asn-Maleimide)

From **6** (3 mg, 2.2 μ mol), recovered yield (**18**): 3.0 mg, 88%. RP-HPLC: t_R = 15.0 min. MALDI-TOF: m/z: [M + Na] = 1564.23 (Calculated = 1564.40).

Compound 19 (Man₆GlcNAc₂Asn-Maleimide)

From **5** (3 mg, 1.9 μ mol), recovered yield (**19**): 2.7 mg, 84%. RP-HPLC: t_R = 15.1 min. MALDI-TOF: m/z: [M + Na] = 1726.88 (Calculated = 1726.54).

Compound 20 (Man₇GlcNAc₂Asn-Maleimide)

From **4** (3 mg, 1.7 μ mol), recovered yield (**20**): 2.9 mg, 90%. RP-HPLC: t_R = 15.1 min. MALDI-TOF: m/z: [M + Na] = 1888.79 (Calculated = 1888.68).

Compound 21 (Man₈GlcNAc₂Asn-Maleimide)

From **3** (3 mg, 1.6 μ mol), recovered yield (**21**): 2.8 mg, 84%. RP-HPLC: t_R = 15.2 min. MALDI-TOF: m/z: [M + Na] = 2050.12 (Calculated = 2050.82).

Compound 22 (Man₉GlcNAc₂Asn-Maleimide)

From **2** (3 mg, 1.5 μ mol), recovered yield (**22**): 2.9 mg, 88%. RP-HPLC: t_R = 15.2 min. MALDI-TOF: m/z: [M + Na] = 2212.78 (Calculated = 2212.96).

Sulfhydryl Derivatization of Bovine Serum Albumin

Sulfhydryl groups were installed on the surface of BSA following a procedure previously reported by our group.²⁶⁻²⁷ In brief, 20 mg of BSA was dissolved in 1X PBS containing 5 mM EDTA. 20 equivalents (5 mg) of Traut's reagent was added, and the mixture was shaken at RT for 2 hrs. The derivitized protein was purified by FPLC (G25). The protein positive fractions (Bradford assay) were pooled and concentrated by a protein concentrator. The quantity of recovered protein (18 mg) was estimated by Nanodrop analysis. Free sulfhydryl groups loaded on the surface of BSA was quantified by Ellman's reagent (9 μ mol free sulfhydryl groups), against an L-cysteine standard curve. The sulfhydryl-containing BSA was used within hours to prevent disulfide formation and protein aggregation.

Conjugation of Maleimide-Tagged Oligomannose Glycans with Sulfhydryl-Derivatized BSA

Sulfhydryl-derivatized BSA (**21**) (4 mg, 2 μ mol sulfhydryl groups) was diluted in phosphate buffer (20 mM, pH 7.2) containing EDTA (5 mM). Maleimide-tagged glycan (1 μ mol) was added and the solution was shaken at RT for 3 hrs and purified by FPLC (G-25) using 10 mM phosphate buffer (pH 6.6). Protein positive fractions were pooled, and carbohydrate-loading was estimated by phenol-sulfuric acid assay, quantified against a mannose monosaccharide standard curve, yielding an average loading of 12% carbohydrate by weight (see appendix **Table A 5.1**).

Complete characterization of all reported compounds can be found in the appendix.

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Chapter 6: Expanding the N-glycan library: Chemoenzymatic Synthesis of a Highly-Branched Bisected Gal and GlcNAc Terminal Tri-/Tetra-/and Penta-antennary N-glycan library

6.1 Introduction

Since its discovery over thirty years ago, the human immunodeficiency virus (HIV) has proven to be one of the most intractable virological targets encountered. Multifarious aspects of HIV account for the challenge of vaccine design, including the high rate of mutation, owing to an error-prone reverse-transcriptase, the ability of the virus to remain latent for extended periods as a pro-virus, the profound rate of viral-tropism, allowing HIV adapt to the evolving immune system avoiding recognition. Also, the variability in the HIV isolates themselves, manifested as different clades and subtypes, has obfuscated efforts to make a universal HIV vaccine.¹ Moreover, the viral spike of HIV is one of the most heavily glycosylated proteins ever described. The extensive glycan shield is dynamic, allowing changes in both the type and occupancy of the potential N-glycosylation sites (PNGSs).² The formation of escape variants, together with the other evasive tactics, have been so successful that HIV has, to-date, largely eschewed extensive vaccine efforts. The surface of the HIV capsid consists of bare phospholipid, with the exception of the presence of the viral spike composed of the non-covalently associated homotrimers gp120/gp41, and several host-derived (non-antigenic) membrane-bound proteins.³ As gp41 is membrane-associated, the majority of vaccine efforts have focused on gp120.

³ Each monomer of gp120 consists of two faces, the heavily-glycosylated solvent-exposed, so-called silent face, and the trimer associated face. As the glycosylated solvent-exposed face is the primary feature of HIV that is presented to the host immune system, nearly all efforts to target gp120 have incorporated glycans as a point of focus. As viruses are produced by host-cellular machinery, the HIV glycan shield is derived from “self-glycans” that effectively mask HIV from host-immune recognition. ⁴ The reliance of HIV on glycosylation for immune evasion creates a tenuous predicament for vaccine design. Targeting host-derived glycans by overcoming immunological tolerance mechanisms to host glycan structures has the potential of inducing auto-reactive Abs, creating a serious immunological challenge. ⁵ The incorporation of “self-glycans” on HIV has forced efforts to be directed against deviations from host-glycosylation patterns. As such, many efforts have focused on specific features of the humoral immune system raised against specific glycan-based gp120 epitopes, representing aberrations in host-glycosylation patterns. Examples include the high-density of mainly oligomannose glycans (i.e. 2G12) and integrated glycan-peptide epitopes (i.e. PG and PGT-series bNAbs). An alternative strategy is to target unusual cryptic N-glycans that are either unique to gp120 or at least under-represented in host-glycosylation. The presence of aberrant cryptic N-glycans on gp120 with a non-native presentation would presumably produce a humoral immune response in the form of monoclonal antibodies (mAbs). Unusual N-glycan structures rarely seen in host glycoproteins provide a natural point of deviation to target as a weak point in the HIV glycan shield. While obviously predicated on the potency,

mAbs raised against a cryptic HIV N-glycans would be a valuable addition to the repertoire of anti-HIV Abs.

HIV gp120 glycosylation is well known to contain a paucity of highly branched galactose and GlcNAc terminating tri-/tetra-/penta-antennary glycans with and without core fucose/terminal sialylation.⁶⁻⁸ As these glycans are present to a limited extent in host glycosylation, there is a major question regarding host tolerance mechanisms to these glycans in a viral context. Targeting such glycans requires a delicate balance between host tolerance and auto-reactivity. The use of cryptic N-glycans as immunological targets has a virological precedent as such glycans were found to be humoral immune targets in severe acute respiratory syndrome corona virus (SARS-CoV).⁹⁻¹⁰ Further support for the notion of targeting highly branched Gal terminal glycans as potential N-glycan epitopes on HIV is drawn from the report by Wang and co-workers that a leucoagglutinin lectin from phaseolus vulgaris (red kidney bean) (PHA-L), specific for terminal N-acetylgalactosamine and galactose glycans, was moderately cross-reactive with native HIV.¹⁰ Indeed, previous studies incorporating tetra-antennary complex-type glycans have indicated a moderate degree of HIV-1 antigenicity, as indicated by recognition by carbohydrate specific bNAbs in a glycan microarray format.¹¹

The majority of N-glycosylation on gp120 is known to be oligomannose, with the average 25 potential N-linked glycosylation sites (PNGSs) largely occupied by oligomannose glycans present as a mosaic cluster.^{8,12} The overwhelming presence of under-processed oligomannose-type glycans is thought to be a result of the dense glycosylation, as well as steric hinderance near the gp120 protomer interface.¹³⁻¹⁴

Both glycan and protein congestion contribute to limiting access by host-glycosylation machinery, restricting the extent of glycan processing at sterically hindered sites.¹⁵ The result of the limited glycan processing at highly hindered sites is the presence of oligomannose clusters near the protomer interface, within the intrinsic mannose patch (IMP), and other regions that are highly congested.¹⁶ Glycan/protein congestion at specific locations suggests that processed complex-type glycans are limited to regions that are minimally congested, resulting in an enrichment of complex-type glycans in defined locations. Confining complex-type glycans to low-congestion regions could potentially give rise to a clustered arrangement of complex-type glycans that are immunologically distinct from host glycosylation. Given that highly-branched (i.e. tetra-antennary) galactose-terminal glycans constitute a non-trivial component of gp120 glycosylation (~10%), these heavily processed glycans are likely present at only the least congested regions and potentially present as clusters.⁸

The availability of defined N-glycan libraries through synthetic and chemoenzymatic approaches have provided essential standards and probes for MS analysis and glycan microarray studies of various carbohydrate binding proteins.¹⁷⁻¹⁹²⁰ The goal of this work was to develop an N-glycan library of highly-branched bisected GlcNAc-/Gal-terminal glycans on a protein scaffold for N-glycan microarray analysis. The strategy employed in this work was the synthesis of glycan-protein conjugates that incorporate various defined cryptic highly branched N-glycans that can be used to screen serum derived from HIV⁺ patients (compared to the serum of healthy subjects). Positive hits against these glycan-protein conjugates will suggest

anti-cryptic N-glycan mAbs and provide a basis of support for targeting such glycans in vaccine design. The cryptic N-glycans were obtained from ovalbumin after a series of intensive enrichments and careful NP-HPLC isolation. The highly-branched N-glycans were remodeled by treatment with either an exoglycosidase or glycosyltransferase yielding two libraries of GlcNAc or Gal-terminal N-glycans.

6.2 Results and Discussion

A number of studies have reported synthetic routes toward the chemical synthesis of highly congested N-glycans. For example, Danishefsky and co-workers reported the synthesis of a sialylated triantennary complex-type glycan.^{21 22} Wong and co-workers recently reported a modular synthetic strategy to produce bi-, tri-, and tetra-antennary complex-type glycans.¹¹ Similarly, Paulson's group reported the synthesis of multi-sialylated glycans.²³ Boons and co-workers reported a very elegant synthetic strategy to chemoenzymatically construct a library of asymmetric multiantennary N-glycans.²⁴ The synthesis of a bisected pentaantennary glycan has also been reported in a landmark study by Unverzagt and co-workers.²⁵ The previous efforts toward the design of highly-branched complex-type glycan libraries have all relied on extremely difficult synthetic routes, usually performed on an analytical scale. In our approach to a complex-type library, we envisioned a chemoenzymatic strategy relying on precursor glycans obtained from a natural source; the precursor N-glycans could then be chemoenzymatically remodeled yielding the target glycans on a preparative (i.e. milligram) scale. In the design of the N-glycan library, it was necessary to identify a source (i.e. glycoprotein) that contains a significant abundance

of highly branched N-glycans. The N-glycosylation pattern of hen ovalbumin (*Gallus gallus*) has been extensively studied,²⁶⁻²⁸ and is well-known to contain bisected tri-/tetra-/and penta-antennary glycans with and without terminal galactose at the sole N-glycosylation site (N292). We therefore chose ovalbumin as the source for highly-branched N-glycans. The impact of the presence of a bisected GlcNAc on biological recognition will presumably be negligible, as the dense presentation of terminal Gal and GlcNAc will likely be largely unaffected. Ovalbumin has been shown to incorporate a very diverse variety of N-glycans, including pauci-mannose and oligomannose glycans, as well as bisected complex-type glycans with two to five branches. In addition, there are significant amounts of hybrid-type glycans containing the Man₄/Man₅ core with the addition of a bisected GlcNAc and terminal GlcNAc/Gal branching (see Figure 6.1).

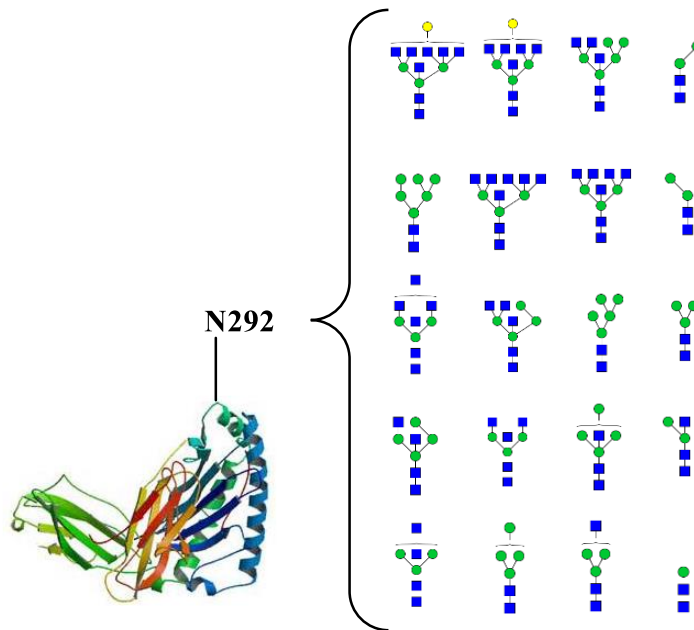
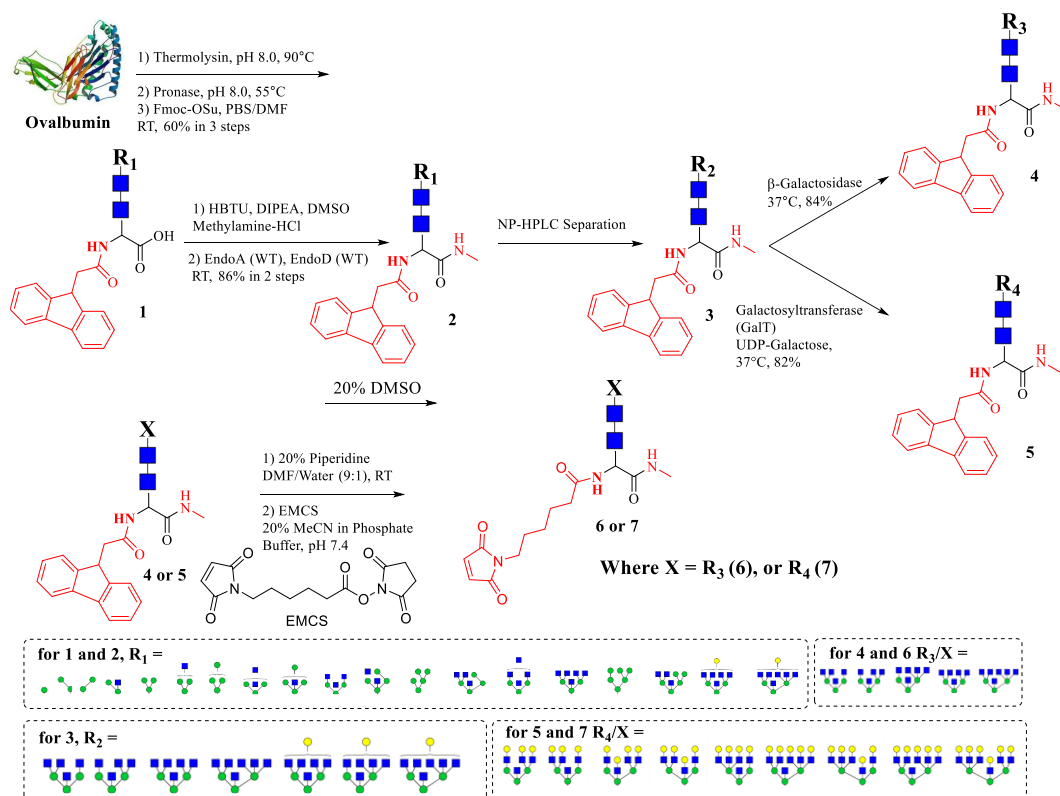


Figure 6.1. Diversity of N-glycans on ovalbumin.

A major limitation of ovalbumin as a natural source for specific N-glycans is the extensive glycan heterogeneity. The construction of a library of highly-branched N-glycans required the selective enrichment of target glycans by removal of non-target glycans.



Scheme 6.1 Chemoenzymatic synthesis of a library of bisected highly-branched N-glycans from ovalbumin.

The total N-glycans on ovalbumin were released via a two-step proteolytic digestion using thermolysin first, followed by pronase. The use of thermolysin (from *geobacillus stearothermophilus*) eschewed the need to denature the protein prior to digestion by incubating at 90°C, which unfolded the protein sufficiently to digest into glycopeptides. The individual N-glycans were released as asparagine-linked sugars

from the glycopeptides by exhaustive pronase (from *streptomyces griseus*) digestion (Scheme 6.1). The N-glycans were purified by size-exclusion chromatography and solid-phase C18 extraction. The N-terminus of the asparagine of the purified glycans was tagged with 9-Fluorenylmethyl *N*-succinimidyl carbonate (Fmoc-OSu), to facilitate further purification as well as down-stream separation, and purified by RP-HPLC yielding compound **1**. Initial attempts to separate the N-glycans via normal-phase HPLC were complicated by the presence of a free carboxylic acid group at the C-terminus of asparagine, resulting in longer retention times and the inability to resolve individual glycans. The C-terminus was therefore protected by N-acylation with methylamine forming an amide bond, after activation of the carboxylic acid by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) yielding **2**. The protection of the C-terminus yielded enhanced resolution of the N-glycans by NP-HPLC (Figure 6.2).

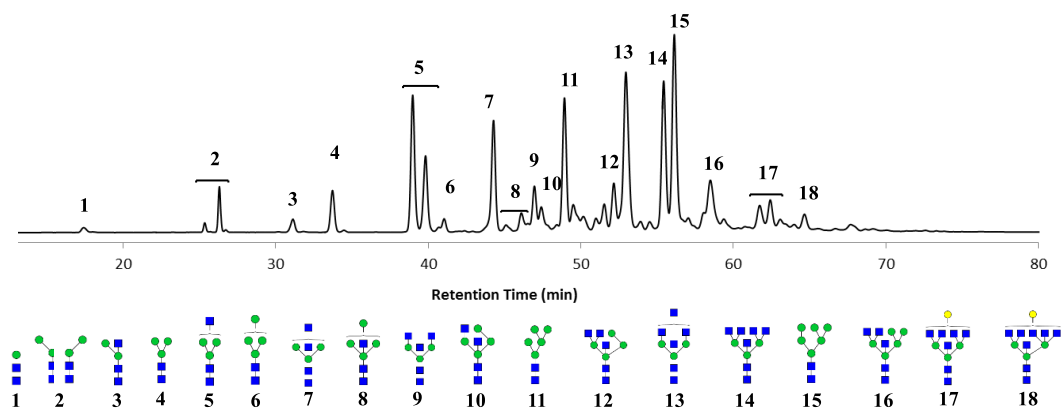


Figure 6.2 Hydrophilic interaction chromatography (HILIC)-HPLC profile of released N-glycans (**2**) from ovalbumin.

The released N-glycans were characterized by hydrophilic interaction chromatography (HILIC)-HPLC and MALDI-TOF analysis, revealing a diverse number of asparagine-linked glycans (see Figures 6.2 and 6.3).

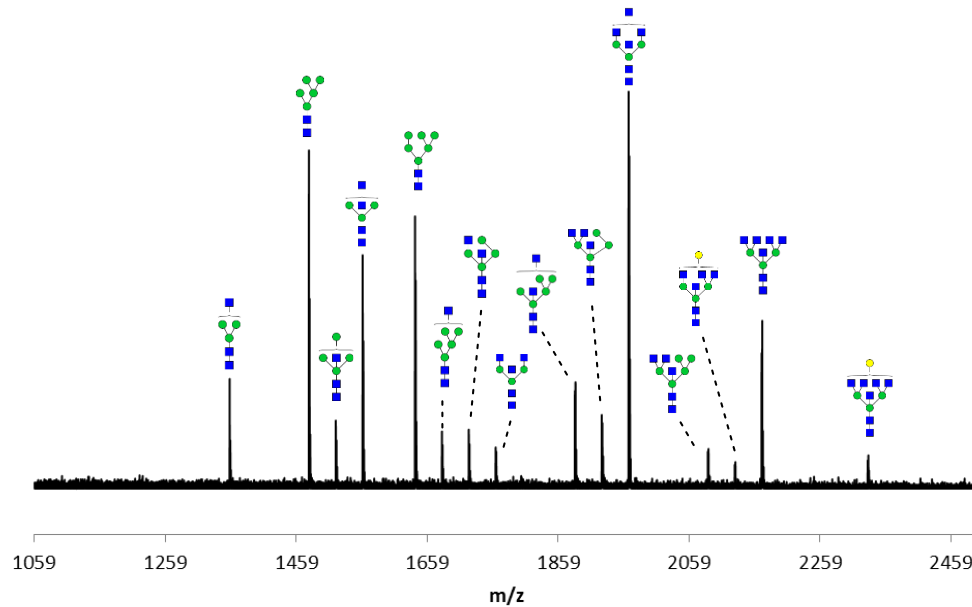


Figure 6.3 MALDI-TOF analysis of total released ovalbumin glycans indicates the predominance of oligomannose and hybrid-type glycans.

A major component of the released N-glycans was comprised of oligomannose glycans, both high- and pauci-mannose.

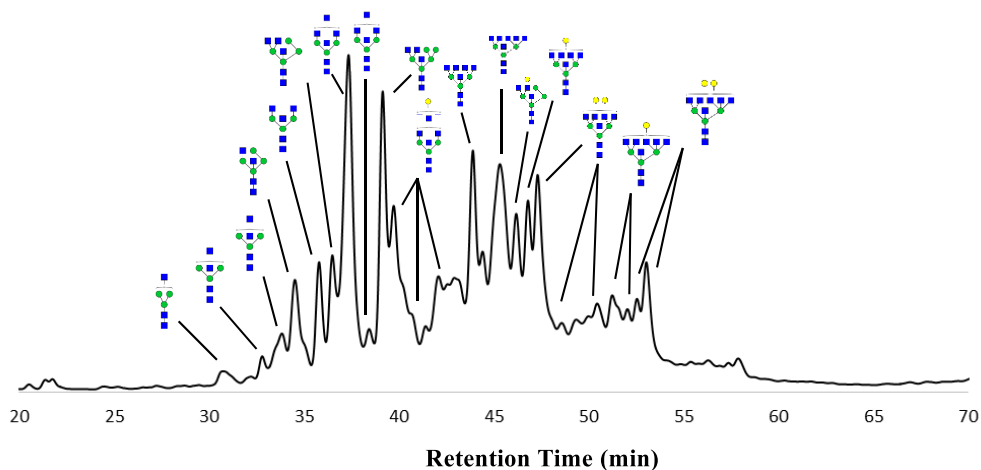


Figure 6.4 HILIC-HPLC analysis of endoglycosidase-cocktail enriched OVA glycans
3.

In order to remove the dominant oligomannose glycans and enrich the lower quantity, highly-branched glycans, the modified N-glycan mixture was treated with an endoglycosidase cocktail containing EndoA (WT) and EndoD (WT). EndoA (WT) is selective for high-mannose glycans, and was used to cleave the prevalent Man₅ and Man₆ species. EndoD (WT) is selective for pauci-mannose glycans, and removed Man₁₋₃ glycans. Endoglycosidase treatment hydrolyzed the chitobiose core, leaving behind GlcNAc-Asn(N-Fmoc)(C-ONMe), which was easily removed from the Fmoc tagged glycans by RP-HPLC. The released GlcNAc-Asn(N-Fmoc)(C-ONMe) was retained longer on RP-HPLC ($t_R = 26$ min) compared to the Fmoc tagged glycans ($t_R = \sim 24$ min), providing a means to monitor reaction progress. Endoglycosidase

treatment yielded enhanced access to the lower abundance glycans **3**, in particular the highly-branched bisected glycans (Figure **6.4**).

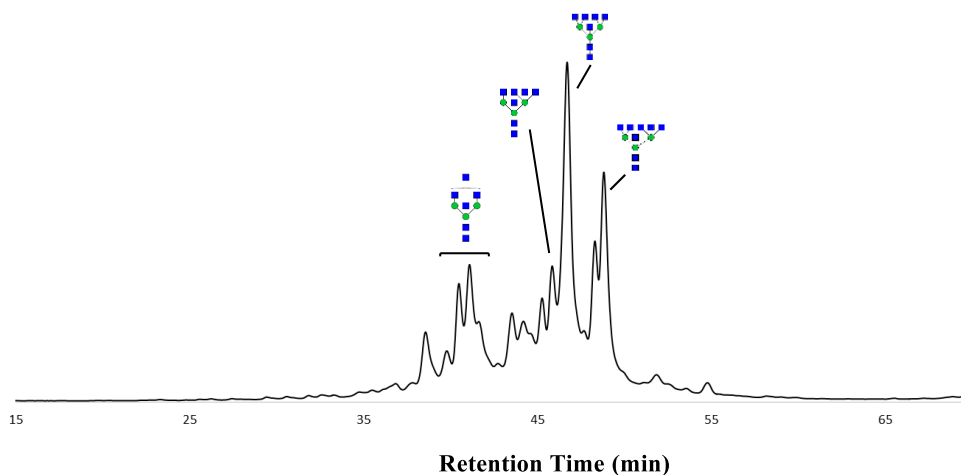


Figure 6.5 HILIC-HPLC analysis of GlcNAc-terminal glycans **4**.

The target bisected tri-/tetra-/penta-antennary glycans were isolated from bisected bi-antennary complex-type glycans, as well as bisected hybrid-type glycans by NP-HPLC (Figure **6.4**). The target highly-branched glycans with and without low-level galactosylation were thus isolated and used as the precursor building blocks for chemoenzymatic remodeling. One portion of the mixture of tri-/tetra-/penta-antennary glycans (**3**) was treated with β -galactosidase (from *aspergillus oryzae*) an exoglycosidase specific for β 1,4-Gal linkages, yielding a mix of GlcNAc-terminating bisected tri-/tetra-/penta-antennary glycans **4** (Figure **6.5**). The second portion of partially galactosylated bisected tri-/tetra-/penta-antennary glycans **3** was treated with

β 1,4-galactosyltransferase (B4GALT) (from *Neisseria meningitides*) in the presence of uridine-diphosphate (UDP)-Gal. B4GALT is known to efficiently transfer UDP-Gal to an appropriate acceptor.²⁹ The B4GALT treatment yielded a mix of Gal-terminating bisected tri-/tetra-/penta-antennary glycans (**5**). The use of excess UDP-Gal yielded nearly complete galactosylation of the branched GlcNAc, and even a small quantity of galactose added to the bisected GlcNAc (Figure 6.6).

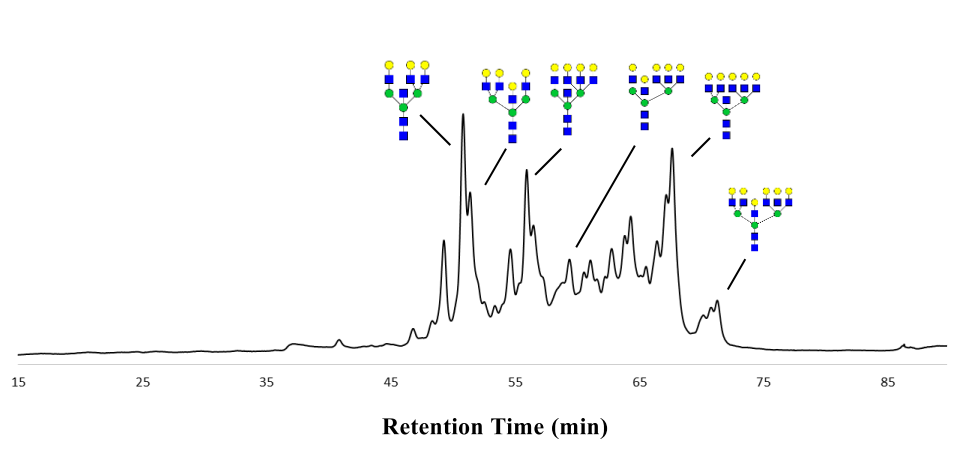


Figure 6.6 HILIC-HPLC analysis of Gal-terminal OVA glycans **5**.

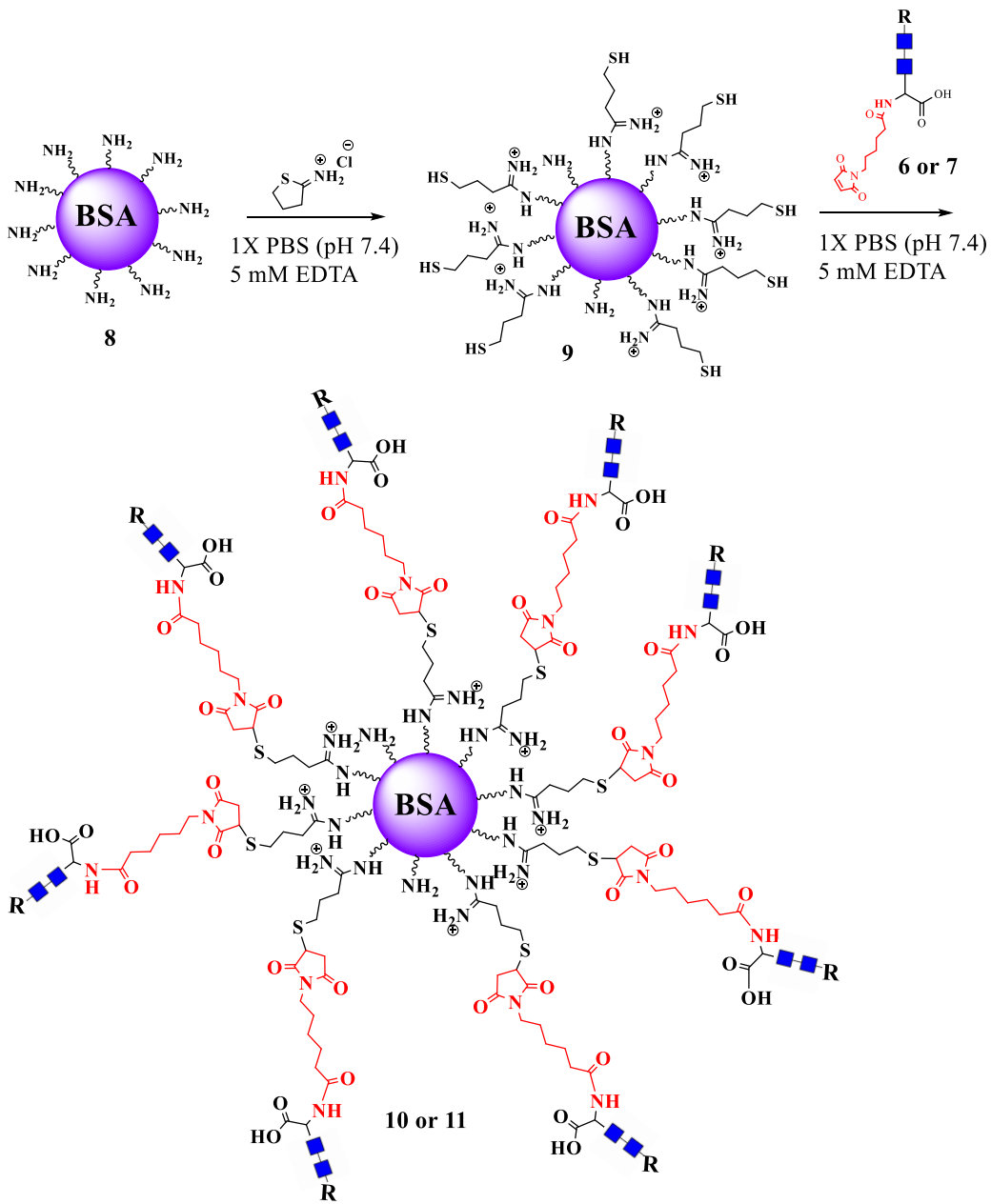
Synthesis of highly-branched bisected N-glycan neoglycoproteins

The functional purpose of the highly-branched complex-type N-glycan library is to screen human serum to identify carbohydrate binding proteins that recognize highly-branched GlcNAc and Gal terminal glycans. Such an identification could potentially yield new viral epitopes that can be targeted as templates through antibody-based vaccine design. Proper multivalent presentation of the glycans in an ELISA or microarray format is critical for optimal protein-carbohydrate binding interactions. Previous screening studies utilizing glycans covalently attached to a

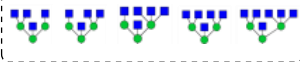
protein scaffold have yielded interesting and impressive screening results.^{30 10, 31-32}

We therefore chose to adopt a similar strategy, beginning with the identification of an appropriate carrier protein. Bovine serum albumin (BSA) was chosen as the protein scaffold, as it is a commonly used scaffold for the multivalent presentation of ligands, including oligosaccharides.³³⁻³⁴ BSA contains numerous chemically accessible Lys residues (the theoretical loading capacity is 30-35 ligands/BSA molecule), and is

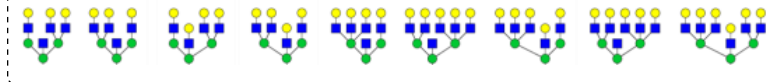
characterized by a well-defined, stable three dimensional structure.³³



for 10, R =



for 11, R =



Scheme 6.2. Synthesis of neoglycoproteins based on highly-branched bisected GlcNAc/Gal-terminal N-glycans.

In order to introduce a chemically complimentary group onto BSA that would facilitate conjugation of the maleimide-linked sugars, BSA (**8**) was treated with Traut's reagent, which reacted with the exposed surface lysine groups, installing free sulfhydryl groups, yielding compound **9**. The addition of maleimide-linked N-glycans (**6** or **7**) resulted in two neoglycoproteins bearing 15% and 14% glycosylation (an average of ~10 glycans per BSA molecule) respectively. The presentation of the glycan portion of these neoglycoproteins is currently being evaluated by our collaborators (Dr. Denong Wang, Stanford University) by binding to a series of carbohydrate binding proteins. Subsequent to the evaluation, the neoglycoproteins will be incorporated into serum screening studies.

6.3 Conclusion

The sophisticated ability of HIV to evade immune recognition necessitates the use of multifaceted immunological approaches to develop an effective vaccine. Targeting unusual glycans that may be enriched regionally on HIV Env protein represents a potentially lucrative approach by exploiting aberrations from host-glycosylation. Capturing carbohydrate-reactive proteins in the serum of infected individuals that may be elicited by these cryptic N-glycan epitopes could yield valuable templates for vaccine design. The work presented here demonstrates the development of a successful top-down chemoenzymatic strategy towards the efficient synthesis of an N-glycan library consisting of highly-branched, bisected glycans. The

combination of enzymatic modifications (both glycan extension and glycan trimming) with extensive 2D-HPLC separation provided access to these unusual highly-branched glycans on a multi-milligram scale. The entire method was predicated on the use of robust glycosyltransferase/exoglycosidase enzymes. Conjugation of these highly-branched glycans to a BSA protein carrier yielded two unique antigens bearing GlcNAc or Gal-terminal glycans that can be used for serum screening capture of serum proteins that recognize these cryptic N-glycans. In summary, the top-down chemoenzymatic method described here provides a practical approach for the rapid production of highly-branched, bisected glycans with defined, non-reducing termini. The method described has the potential to become a general approach to introduce access to other unusual glycans.

6.4 Experimental

General Procedures. All chemical reagents, unless otherwise noted were purchased from Sigma-Aldrich. Analytical reverse-phase HPLC was performed on a Waters Alliance[®] e2695 HPLC system equipped with a dual absorbance 2489 UV/Vis detector. Separations were performed using a C18 column (Waters XBridge[®] Shield, 4.6 X 250 mm, 3.5 μ m). The column was eluted using a linear gradient of 5-25% MeCN containing 0.1% TFA in 30 min. Analytical normal-phase HPLC was performed using an amide-bonded column (YMC America, Pack-NH2, 4.6 X 250 mm, 5 μ m), using a linear gradient of 15-50%A with a flow rate of 1 mL/min, where solvent A = 100 mM ammonium formate (pH 4.5) and solvent B = 100% acetonitrile. Preparative normal-phase HPLC was performed using an amide-bonded column

(YMC America, Pack-NH₂, 20 X 250 mm, 5 μm), using a linear gradient of 15-50% A with a flow-rate of 12 mL/min. Analytical hydrophilic interaction chromatography-HPLC was performed using a HILIC analytical column (Waters, XBridge[®] BEH Glycan, 4.6 X 250 mm, 3.5 μm), using a linear gradient of 22-44% A with a flow rate of 1 mL/min. All HILIC-HPLC samples were prepared in 80% acetonitrile (solvent B) and 20% solvent A. ESI-MS spectra were obtained using a ThermoFisher Q Exactive LC/MS orbitrap mass spectrometer. MALDI-TOF analysis was performed using a Bruker UltrafleXtreme (UTX) mass spectrometer with TOF/TOF detection. Preparative RP-HPLC was performed on a Waters 600 HPLC system equipped with a dual absorbance UV detector using a C18 column (Waters XBridge, Prep Shield, 10 X 250 mm, 5 μm) at a flow-rate of 4 mL/min. The column was eluted using a linear gradient of 15-25% MeCN containing 0.1% TFA over 30 min. Ovalbumin was obtained from Sigma-Aldrich as a crystallized powder (grade III). Thermolysin (*geobacillus stearothermophilus*) was obtained as a lyophilized powder from Sigma-Aldrich. Pronase (*Streptomyces griseus*) was obtained through Roche. β_{1,4}-galactosidase (*aspergillus oryzae*) was obtained as a lyophilized powder from Sigma-Aldrich. β_{1,4}-galactosyltransferase (B4GALT) (*neisseria meningitides*) was expressed from *E. coli* by Dr. Joseph Lomino. UDP-Gal was purchased through CalBioChem.

Proteolytic release of N-glycans from ovalbumin

Ovalbumin (grade III) (5 g) was dissolved in 50 mM Tris-HCl buffer (pH = 8.0), CaCl₂ was added to a final concentration of 5 mM. Thermolysin (20 mg, 1:0.004 protein:enzyme ratio) was added and the enzymatic digestion was incubated at 90°C

overnight with periodic shaking. After complete digestion of the protein into peptides (as assessed by Bradford assay), the reaction was cooled to 37°C and pronase (40 mg, 1:0.008 protein:enzyme ratio) was added. The reaction was incubated at 55°C while monitoring with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). When the peaks corresponding to released Asn-linked glycans were highest (usu. 1-2 days), the mixture was lyophilized and applied to a Sephadex G-25 size-exclusion column, eluting with 0.1 M acetic acid. The carbohydrate positive fractions (as determined by phenol-sulfuric acid monitoring) were pooled and applied to a C18 solid-phase extraction column (column was previously washed with one-column volume MeCN, one column volume MeOH, and equilibrated with three column volumes of water). The carbohydrate positive fractions were pooled and lyophilized. The released OVA N-glycans were obtained as a white powder (117 mg, 78% recovery, based on 3% OVA glycosylation weight).

Derivatization of Asn N-terminus with a lipophilic Fmoc tag (1)

The released Asn-linked OVA N-glycans (100 mg) were dissolved in 1 mL 0.3 M NaHCO₃ (pH 8.0). Fmoc-OSu (100 mg) dissolved in 1 mL DMF was added at once to the solution, and the mixture was vigorously mixed at RT overnight. The glycans were precipitated by the addition of 10-fold excess cold (-20°C) acetone, which simultaneously removed excess Fmoc-OSu. The acetone layer was discarded and the white pellet dissolved in water and purified by RP-HPLC yielding the Fmoc-tagged glycans **1** as a white powder (80 mg).

N-acylation of Asn C-terminus with methylamine (2)

Fmoc-tagged glycans **1** (70 mg) were dissolved in dry DMSO (3 mL). Methylamine-HCl (20 eq.) was added to the solution, followed by diisopropylethylamine (DIPEA) (3.5 eq.) and HBTU (3.5 eq.). The reaction was shaken at RT for 1 h, while monitoring with analytical HPLC. After the reaction was complete, the mixture was purified by RP-HPLC yielding the product **2** as a white powder (62 mg).

Enrichment of low-abundance bisected N-glycans via endoglycosidase digestion (3)

The modified N-glycans **2** (60 mg) were dissolved in phosphate buffer (50 mM, pH 7.0). EndoA (WT) (10 µg) and EndoD (WT) (5 µg) were added and the reaction was shaken at 37°C overnight. The reaction was monitored by RP-HPLC. After the reaction was complete, the Fmoc-tagged glycans were purified by RP-HPLC yielding the enriched low-abundant hybrid-/complex-type Fmoc-tagged glycans as a white powder (33 mg). The highly-branched bisected glycans were further enriched by NP-HPLC purification. The fractions containing tri-/tetra-/penta-antennary bisected glycans were separated from the hybrid-type glycans, pooled and lyophilized. The resulting mix of glycans was desalted by RP-HPLC yielding the target glycans as a fluffy white powder (**3**) (15 mg).

Enzymatic synthesis of bisected highly-branched terminal GlcNAc glycans (4)

The partially galactosylated highly-branched glycans (**4**) (5 mg) were dissolved in sodium acetate (50 mM, pH 4.5) and β1,4-galactosidase (20:1 glycan:enzyme ratio) was added. The reaction was shaken overnight at 37°C. After complete removal of Gal (as determined by NP-HPLC and LC/MS), the mixture was purified by RP-HPLC, yielding the GlcNAc-terminal glycans (**4**) as a white powder (4 mg).

Enzymatic synthesis of bisected highly-branched terminal Gal glycans (5)

The partially galactosylated highly-branched glycans (**4**) (5 mg) were dissolved in Tris-HCl buffer (50 mM, pH = 8.0). MnCl₂ was added to a final concentration of 20 mM, along with UDP-Gal (20 eq.). β 1,4-Galactosyltransferase was added to a final concentration of 1.5 mg/mL and the enzymatic reaction was shaken at 37°C overnight. After nearly complete addition of terminal Gal (as determined by NP-HPLC and LC/MS), the mixture was purified by RP-HPLC, yielding the Gal-terminal glycans (**5**) as a white powder (4.1 mg).

General Procedure for Deblocking N-Terminus of Asparagine on highly-branched bisected complex-type N-glycans

Fmoc-tagged compounds **4** and **5** were dissolved in a small amount of water. Piperidine in DMF was added to give a final concentration of 20% in 1:9 water/DMF. The reaction mixture was shaken at RT for 30 min while monitoring by HPLC. The reaction mixture was neutralized with glacial acetic acid and lyophilized. The product was precipitated using cold 80% acetone. The white precipitate was collected, dried and used in the next step without further purification.

Maleimide Conjugation of highly-branched bisected complex-type N-glycans (6 and 7)

General conditions: The deblocked highly-branched bisected N-glycans were dissolved in 1X PBS (final concentration = 25 mg/mL), 8 equivalents of the heterobifunctional linker N- ϵ -maleimidocaproyl-oxysuccinimide ester (EMCS) dissolved in acetonitrile was added, giving a final acetonitrile concentration of 20%. The solution was shaken at RT for 2 h, while monitoring with RP-HPLC (0-50%B,

214/302 nm, $t_R = 15$ min). Once the reaction was complete, the solution was diluted with solvent A and purified by RP-HPLC, giving the target compounds **6** and **7** as white powders.

Compound 6 (GlcNAc-terminal-Maleimide)

From **4** (3 mg), RP-HPLC: $t_R = 15.1$ min. Product (**6**) obtained as a white powder (2.5 mg).

Compound 7 (Gal-terminal-Maleimide)

From **5** (3 mg), RP-HPLC: $t_R = 14.9$ min. Product (**7**) obtained as a white powder (2.7 mg).

Sulfhydryl Derivatization of Bovine Serum Albumin (9)

Sulfhydryl groups were installed on the surface of BSA following a procedure previously reported by our group. In brief, 20 mg of BSA (**8**) was dissolved in 1X PBS containing 5 mM EDTA. 20 equivalents (5 mg) of Traut's reagent was added, and the mixture was shaken at RT for 2 h. The derivitized protein was purified by FPLC (G25). The protein positive fractions (Bradford assay) were pooled and concentrated by a protein concentrator. The quantity of recovered protein (18 mg) was estimated by Nanodrop analysis. Free sulfhydryl groups loaded on the surface of BSA was quantified by Ellman's reagent (9 μ mol free sulfhydryl groups), against an L-cysteine standard curve. The sulfhydryl-containing BSA (**9**) was used within hours to prevent disulfide formation and protein aggregation.

Conjugation of Maleimide-Tagged Highly-Branched Bisected Glycans with

Sulfhydryl-Derivatized BSA (10 and 11)

Sulfhydryl-derivitized BSA (**9**) (4 mg, 2 μ mol sulfhydryl groups) was diluted in phosphate buffer (20 mM, pH 7.2) containing EDTA (5 mM). Maleimide-tagged glycan (compounds **6** or **7**) (1 μ mol) was added and the solution was shaken at RT for

3 h and purified by FPLC (G-25) using 10 mM phosphate buffer (pH 6.6), yielding compounds **10** and **11**. Protein positive fractions were pooled, and carbohydrate-loading was estimated by phenol-sulfuric acid assay, quantified against a mannose monosaccharide standard curve, yielding an average loading of 15% carbohydrate by weight.

6.5 *References*

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Chapter 7: Conclusion and Future Perspectives

7.1 Conclusion

A successful HIV vaccine must either completely prevent viral transmission, or prevent the establishment of a latent pool of HIV infected cells by destroying the initial round of infected CD4⁺ T cells. Such a vaccine cannot rely on memory immune responses as the virus will establish the infection before an immune challenge, and will instead require high levels of protective immunity at the time of contact with the virus. The de novo synthesis of an optimized HIV-1 antigen leading to an effective vaccine is challenging and will likely require a multifaceted approach involving a number of antigens combinations on different carriers. The work that I have presented here represents a small but significant step forward in our understanding of rational HIV-1 antigen design.

Epitope-based immunogen design targets induction of bNAb B cell lineages via Env epitope immunogens that mimic the native Env epitopes on HIV virions, while minimizing (or removing) non-/restricted-NAb epitopes.¹⁻² Such a strategy concentrates the immune focus on the conserved neutralizing epitope. The epitope-based immunogen design strategy was utilized in the construction of the immunogens in Chapters 2 and 4. In Chapter 2, the oligomannose-VLP conjugates were designed to mimic the 2G12 epitope. The diverse forms of oligomannose glycan that were incorporated into the design probe specific questions regarding the fine-details of the

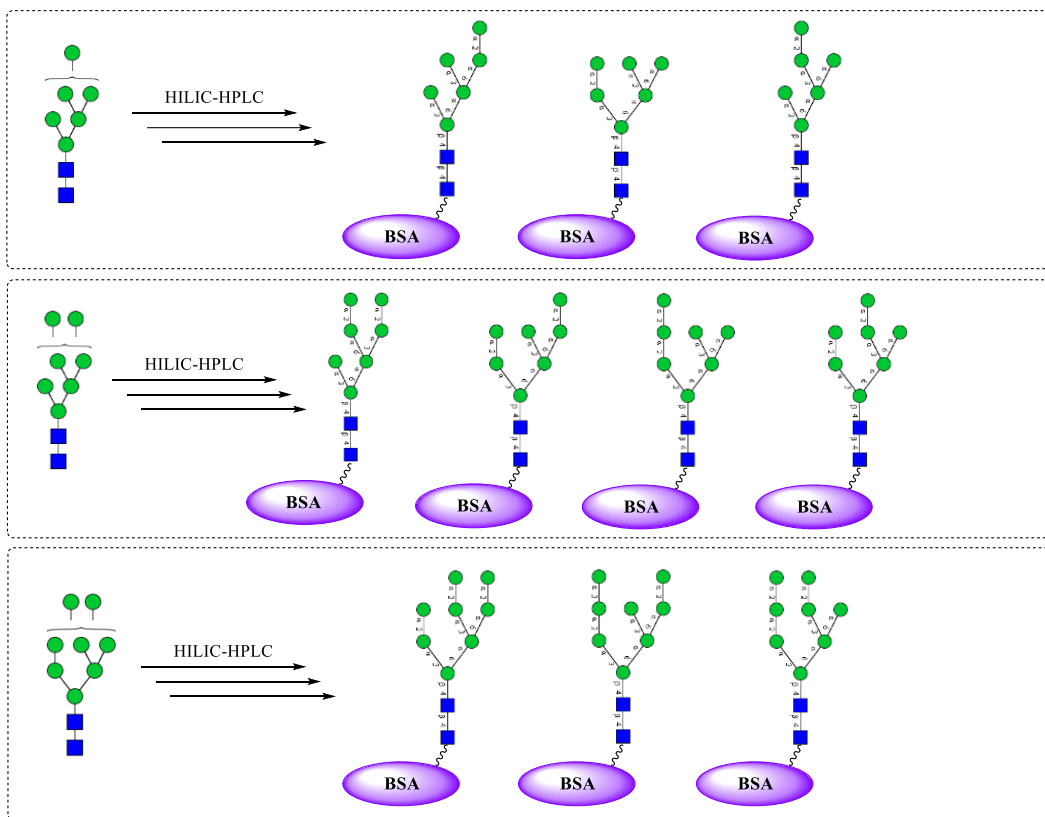
epitope. The characterization of the conjugates, as well as the antigenicity results were very promising, and the ongoing vaccination study will be extremely informative regarding the contribution of the chitobiose core in proper orientation of the oligomannose 2G12 epitope. Similarly, the immunization results of the mixed-display Man₅₋₉ conjugate, which demonstrated the highest affinity for 2G12, will potentially indicate the preference of 2G12 for heterogeneous oligomannose display, in contrast to homogeneous Man₉ only.

The dramatic preference of PG9 for multivalent peptide display in contrast to monovalent, as described in chapter 4, is an exciting finding. The preference suggests the ability to mimic gp120 quaternary structure via multivalent peptide display. Such an ability could be harnessed to design a PG9 immunogen that displays the neutralizing epitope with higher fidelity than monovalent glycopeptide alone. The prospect is extremely exciting; if the multivalent display truly mimics trimeric gp120, then a “native-like” neutralizing epitope can be presented to the immune system without the complications of the presence of non-neutralizing epitopes. It will be necessary to perform vaccination studies to determine the extent to which these glycopeptide-VLP conjugates are able to mimic the actual trimeric PG9 epitope.

The orthogonal protection strategy, developed in Chapter 3, used to synthesize the differentially glycosylated glycopeptides in Chapter 4, is broadly applicable to any glycopeptide containing two or more unique glycans. The method is highly valuable for determining specific glycan preferences in a variety of biological interactions, and can be used to probe the specific glycan preference at a given site. The strategy is currently being employed by other members of the Wang research

group to synthesize differentially glycosylated glycopeptides that mimic the neutralizing epitopes of various V3-directed bNAbs.

The design of N-glycan libraries containing unusual glycans that are otherwise difficult to obtain, provides unprecedented access to the glycan preferences of carbohydrate reactive proteins, beyond the canonical N-glycans (i.e. Man₉GlcNAc₂, Man₅GlcNAc₂, biantennary complex-type, etc.) that are more readily available. Moreover, the unusual glycans can be used to screen for novel carbohydrate-reactive proteins that target atypical glycans that can be used as a basis of discrimination in host-pathogen interactions. Such unusual glycans are potentially enriched in a pathological state, adumbrating a path to novel vaccine design. The top-down chemoenzymatic synthesis of an oligomannose (Man₅₋₉GlcNAc₂) library is not new,³⁴ however, the use of naturally derived Man₉GlcNAc₂ from soybean agglutinin, as described in Chapter 5, grants access to the full suite of oligomannose glycans with unprecedented speed and scale. The top-down chemoenzymatic method provided a means to synthesize oligomannose-BSA conjugates on a multi-milligram scale for serum-screening studies. The results of preliminary binding studies with oligomannose-reactive proteins (i.e. lectins and IgG Abs) indicated binding preferences corresponding with the known glycan targets of the proteins. Vaccination studies are underway to raise anti-oligomannose Abs against these glycan-protein conjugate antigens. The long-term goal of these conjugates will be the identification of novel oligomannose-reactive proteins from the serum of HIV⁺/viral infected individuals. A preference for a specific intermediate oligomannose glycan could potentially represent a unique viral epitope.

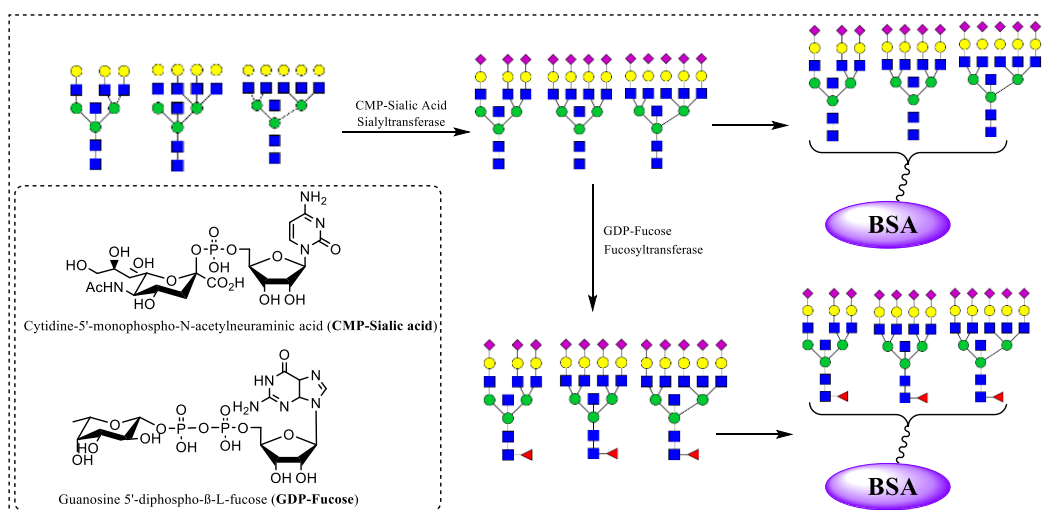


Scheme 7.1. Construction of oligomannose-BSA conjugates bearing regioisomers will complete the oligomannose N-glycan library.

Another long-term goal is the isolation of intermediate ($\text{Man}_6\text{-}_8\text{GlcNAc}_2$) oligomannose glycan regioisomers and the associated BSA-conjugates to complete the oligomannose library including all possible regioisomers (Scheme 7.1). Such a project would likely require multiple mannosidase enzymes from a variety of sources to accommodate the specific branching preferences of the exoglycosidases.

The chemoenzymatic construction of a naturally-derived library of highly-branched complex-type N-glycans, affords access to unusual, inaccessible glycans that may have a significant role in viral pathology. Due to the enormous complexity of these glycans, relatively little work has been reported towards the synthesis of

these interesting oligosaccharides, and the work that has been reported is limited to very small scale.⁵⁻⁸ The method reported in Chapter 6, regarding the synthesis of an N-glycan library of highly-branched complex-type glycans represents the first study of its kind on a preparative scale. The identification of ovalbumin as a source for highly-branched N-glycans was critical, as was the enzymatic enrichment process to remove the dominant N-glycans. The use of 2D-HPLC to separate the enriched glycans on a multi-milligram scale was invaluable for facilitating the study design.



Scheme 7.2. Expanding the highly-branched complex-type N-glycan library.

Future work should be directed toward expanding the library of these highly-branched N-glycans. Glycan analysis of trimeric gp120 has indicated the presence of highly-branched complex-type glycans on HIV, particularly at the gp120-gp41 interface,^{9 10-11} as well as the four PNGSs on gp41.¹²⁻¹³ The results have shown both sialylated and non-sialylated glycans, as well as the presence and absence of a core fucose.⁹ Expanding the library described in Chapter 6 to include terminal sialylation and core fucosylation will tailor the glycans to be more representative of the highly-

branched glycans found on HIV virions (Scheme 7.2). In addition, the existing highly-branched, GlcNAc/Gal-terminal glycan-BSA conjugates should be applied to serum screening studies to search for novel carbohydrate reactive proteins that may recognize previously unknown cryptic N-glycan HIV-1 epitopes. The discovery of serum proteins that recognize enriched highly-branched glycans on BSA could suggest novel non-self epitopes on gp120 that could be exploited for vaccine design.

Given the current status of HIV-1 vaccine design, it seems evident that an effective vaccine will require a sequential vaccination strategy to “walk” the immune system toward production of bNAbs. The immunogens described in this work concentrate the immune focus on neutralizing epitopes and could therefore become useful vaccine candidates. To be successful, these immunogens (i.e. oligomannose/V1V2-glycopeptide-Q β conjugates) will likely need to incorporate antigenic elements that allow binding to germline precursors, as well as the intermediate Ab precursors to the mature bNAb of interest. One method that has proven successful for recombinant immunogens is the glycan deletion method, in which deletion of certain glycans from gp120 allow recognition by germline bNAbs.^{14 15} The initial step will require testing the existing immunogens for binding to germline bNAbs. In order to achieve a useful breadth of protection, it will likely be necessary to design multivalent immunogens that target multiple bNAbs. A final future direction for this research is the design of multivalent immunogens that bear multiple putative, minimal epitopes for a variety of bNAbs to stimulate a broad response targeting all major HIV-1 strains.

The development of an effective vaccine against HIV-1 continues to present a formidable challenge. A combination of epitope masking and the rapid formation of escape variants have helped HIV evade all previous vaccine strategies. The discovery of bNAbs together with the recent advances that indicate that it is possible to elicit such highly modified IgG Abs by proper vaccination strategies has given renewed hope to the field. Synthetic HIV-1 antigens such as those presented in this work will be key to the development of an effective HIV-1 vaccine.

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Appendices

A.2.1 Chapter 2: Supplementary Information

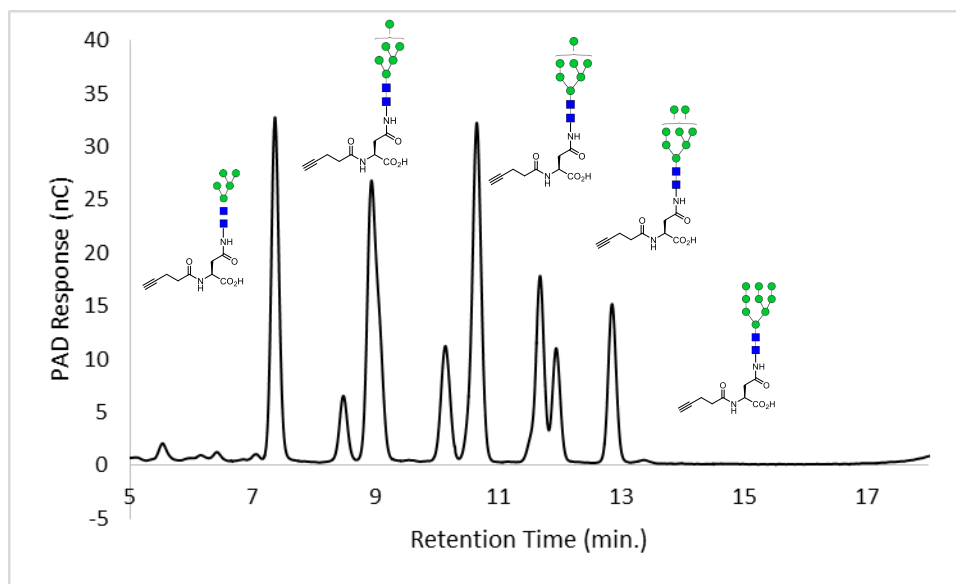


Figure A 2.1. HPAEC-PAD analysis of Alkyne-tagged Man₅₋₉GlcNAc₂-Asn Building block 4.

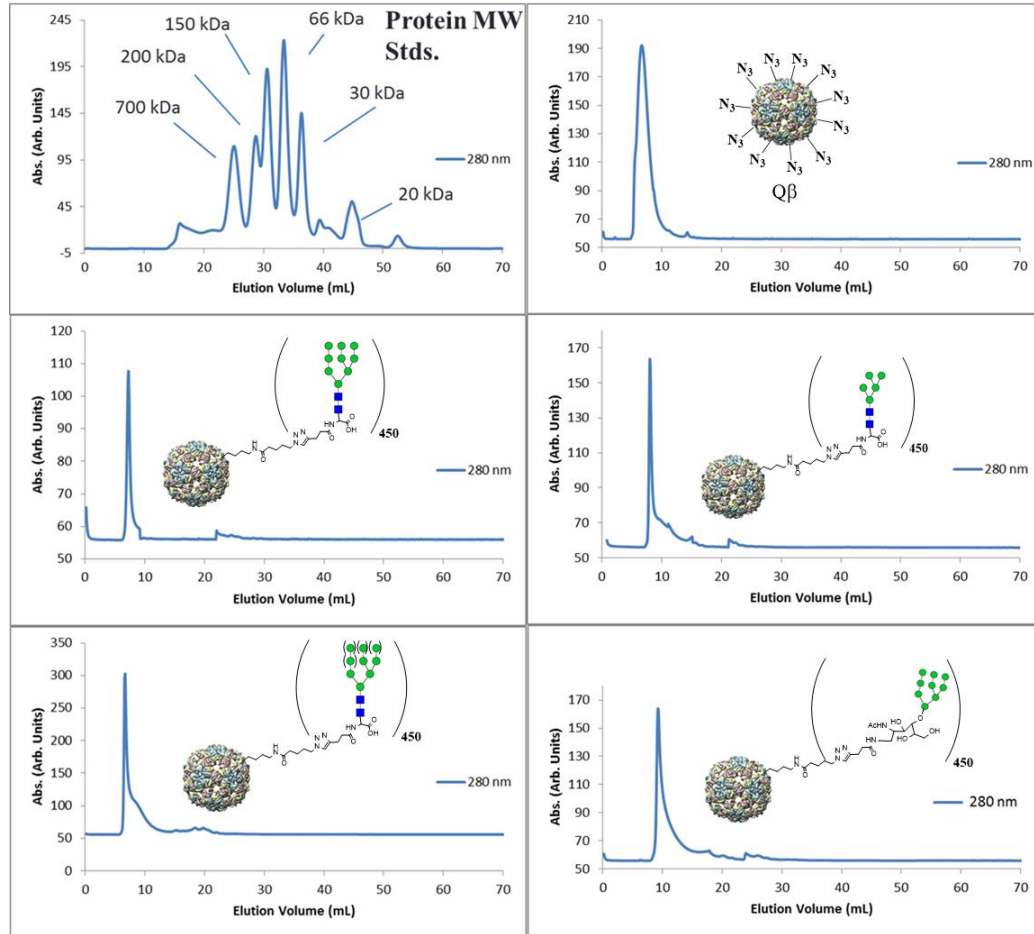


Figure A 2.2 Size-exclusion analysis of oligomannose-Q β conjugates using a Superose 6 (GE Health Science) column reveals that the particles remain intact after surface modifications.

NMR

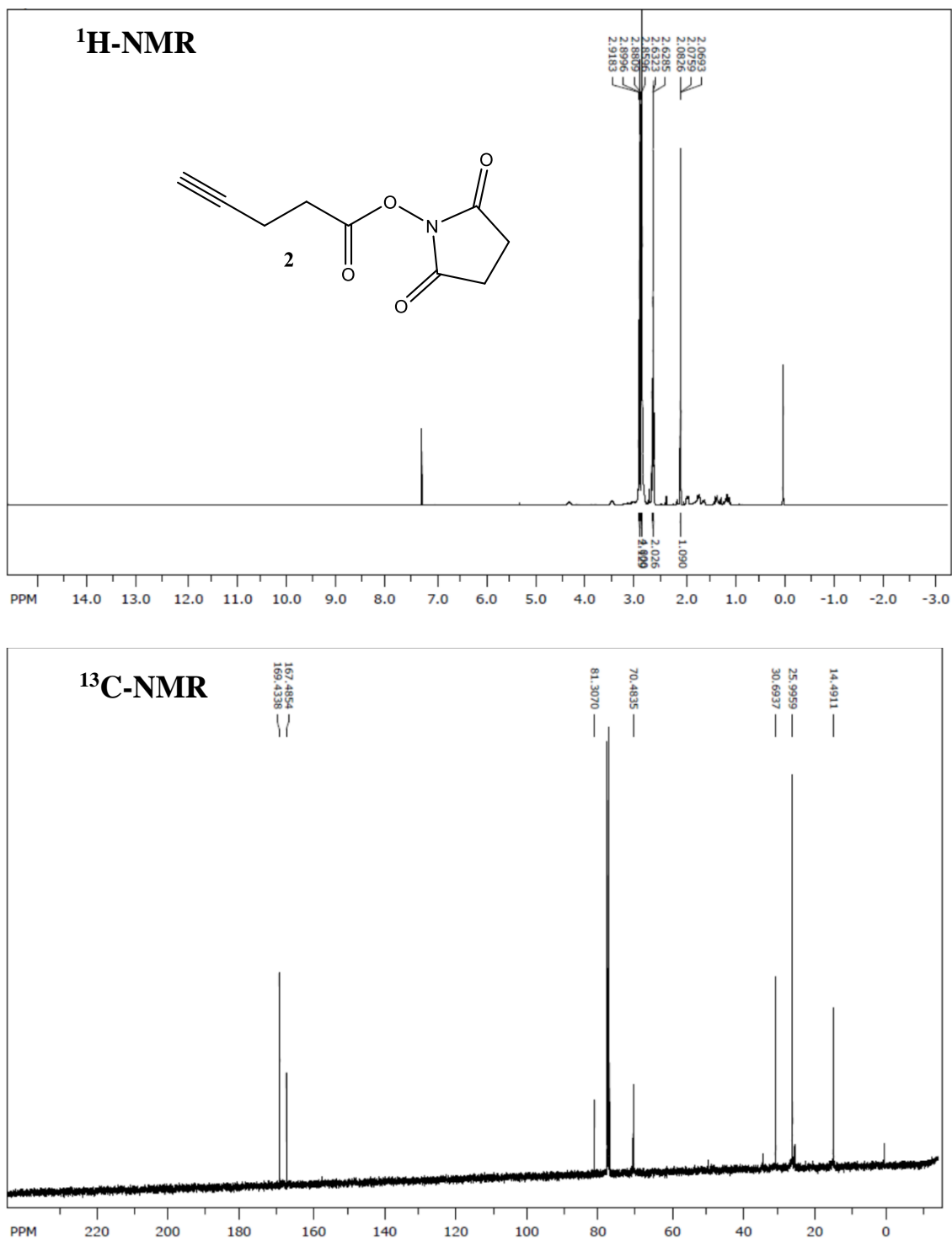


Figure A 2.3 Proton and Carbon NMR Spectra for compound 2.

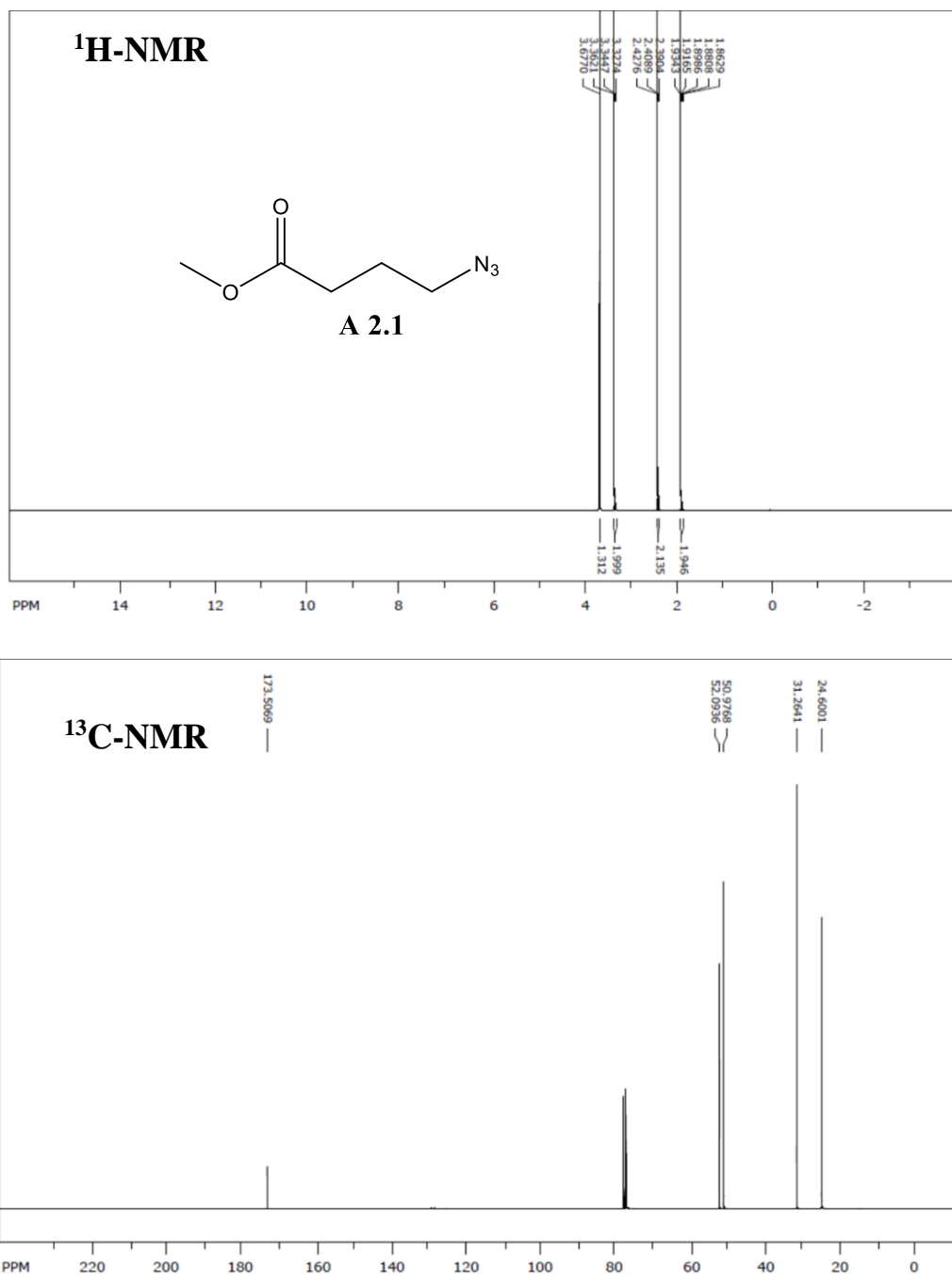


Figure A 2.4 Proton and Carbon NMR Spectra for compound **A 2.1**.

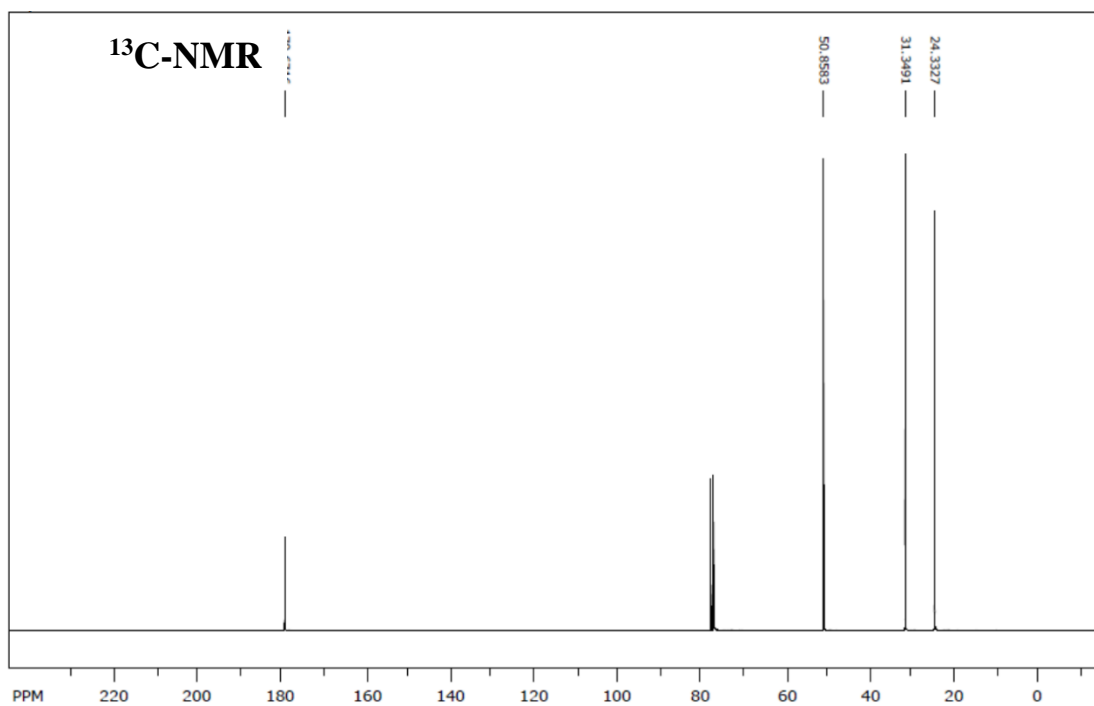
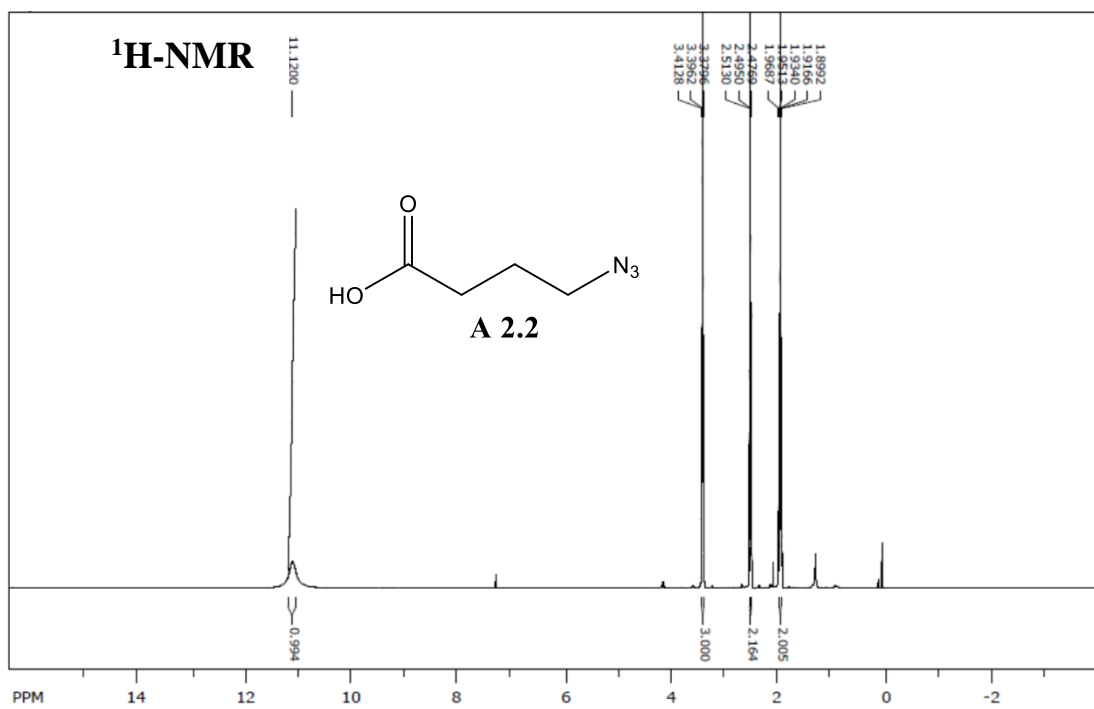


Figure A 2.5 Proton and Carbon NMR Spectra for compound **A 2.2**.

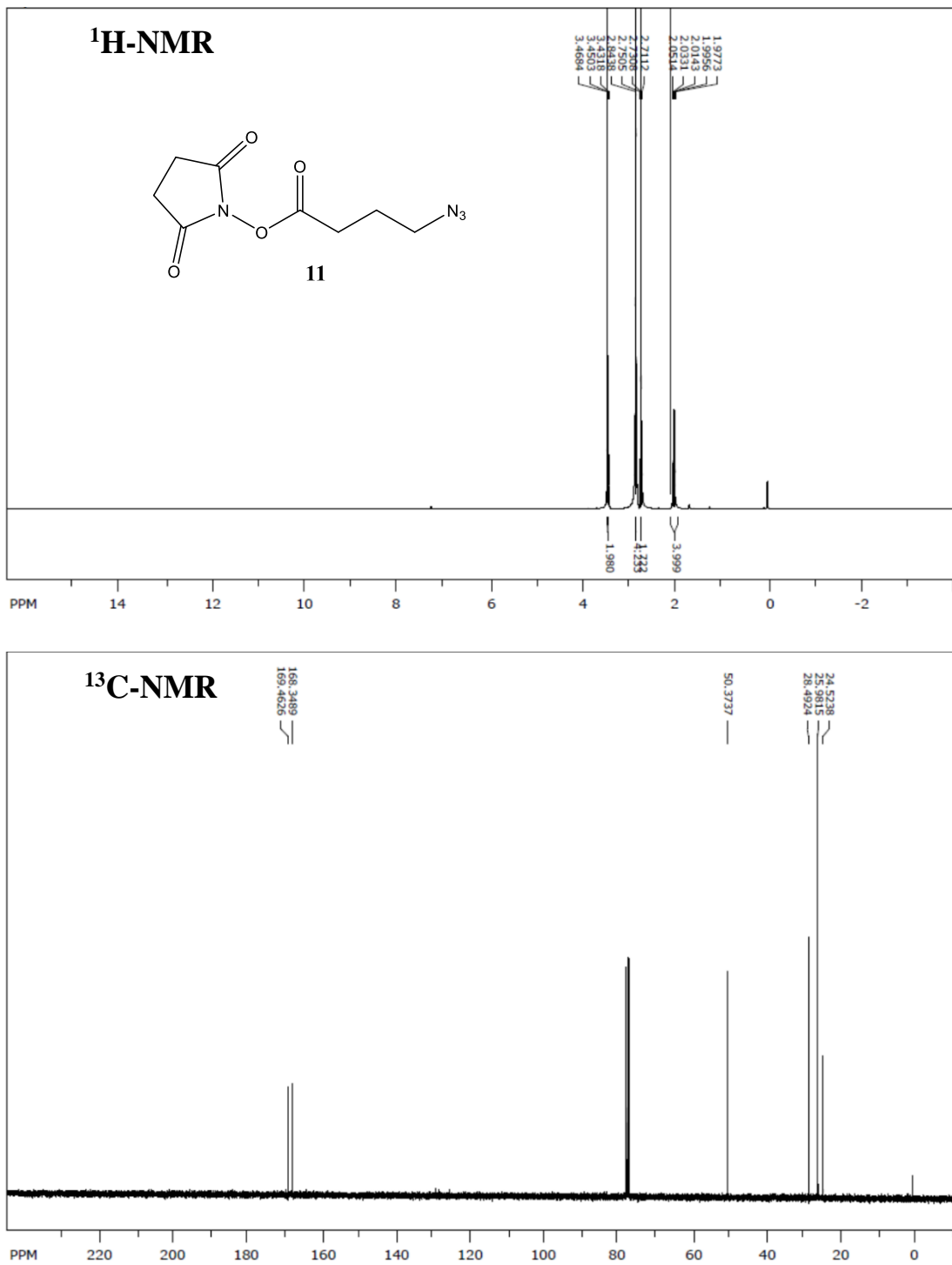
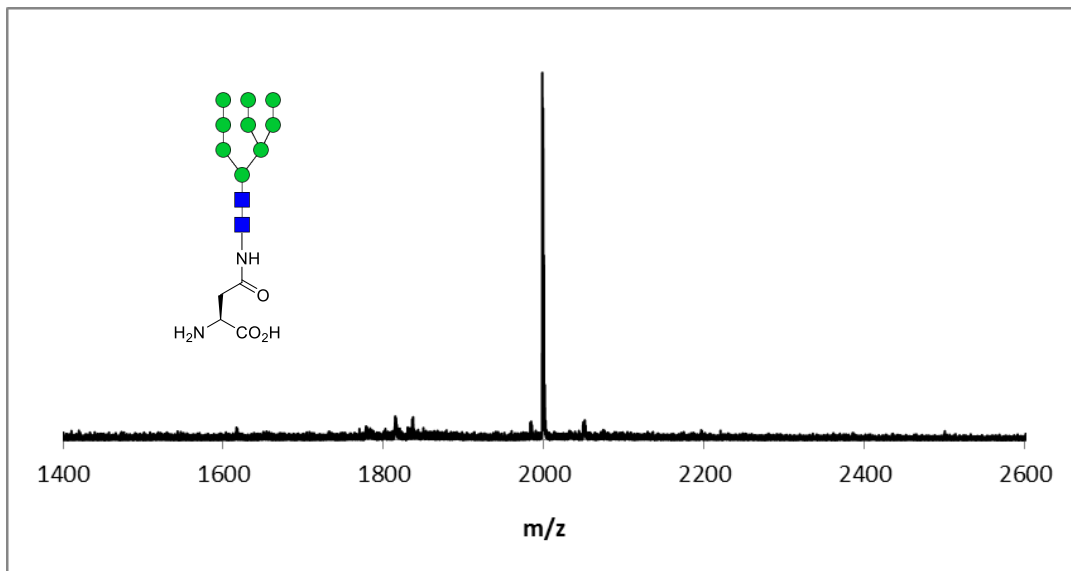


Figure A 2.6 Proton and Carbon NMR Spectra for compound **11**.



A 2.7 MALDI-TOF analysis of compound **1**.

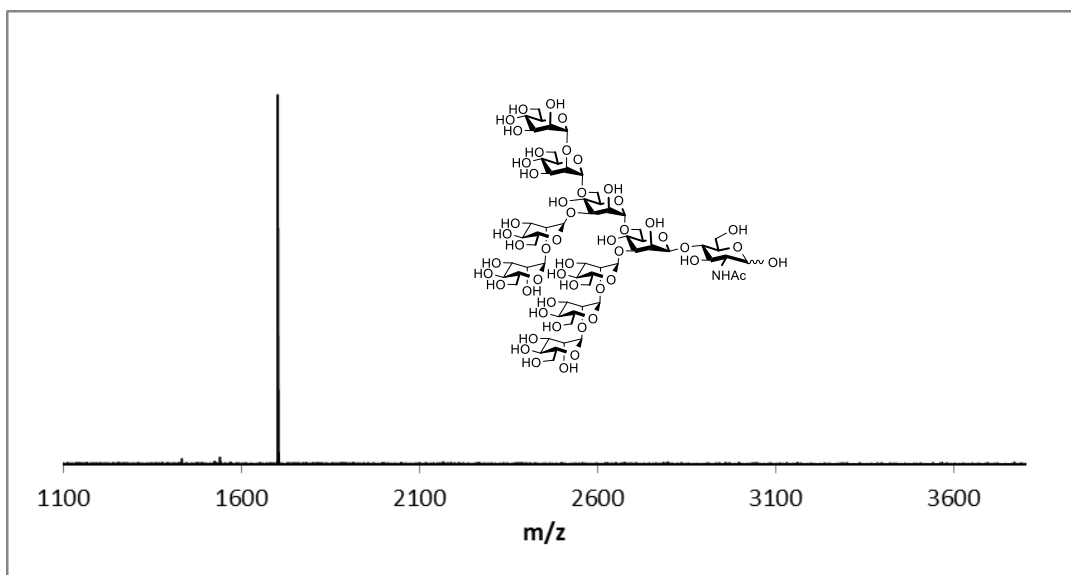


Figure A 2.8 MALDI-TOF analysis of compound **6**.

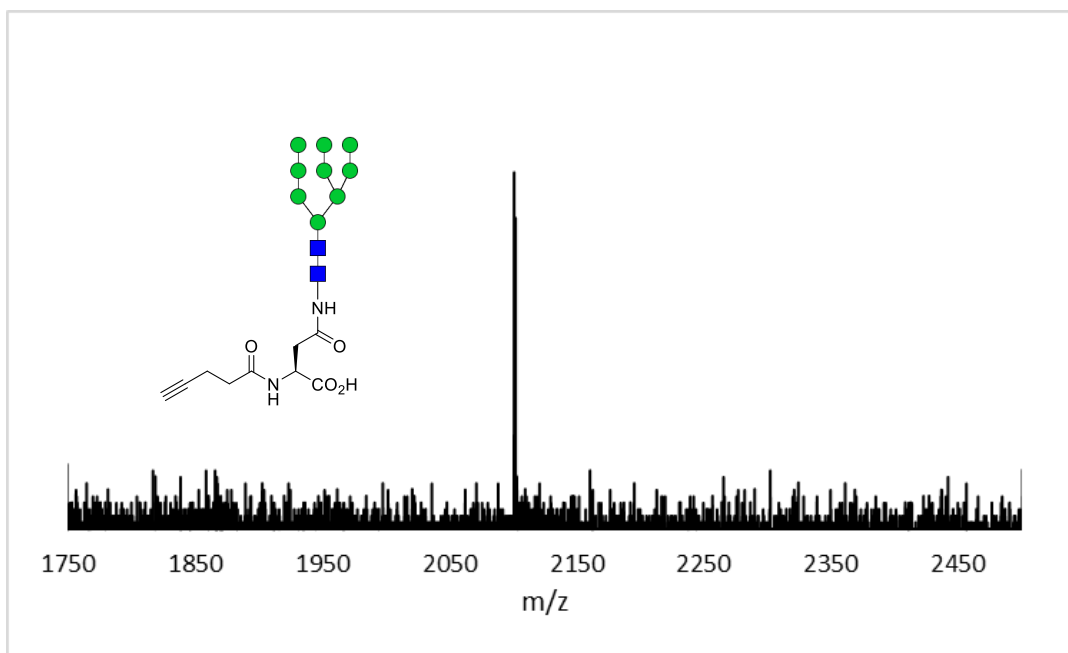


Figure A 2.9 MALDI-TOF analysis of compound 3.

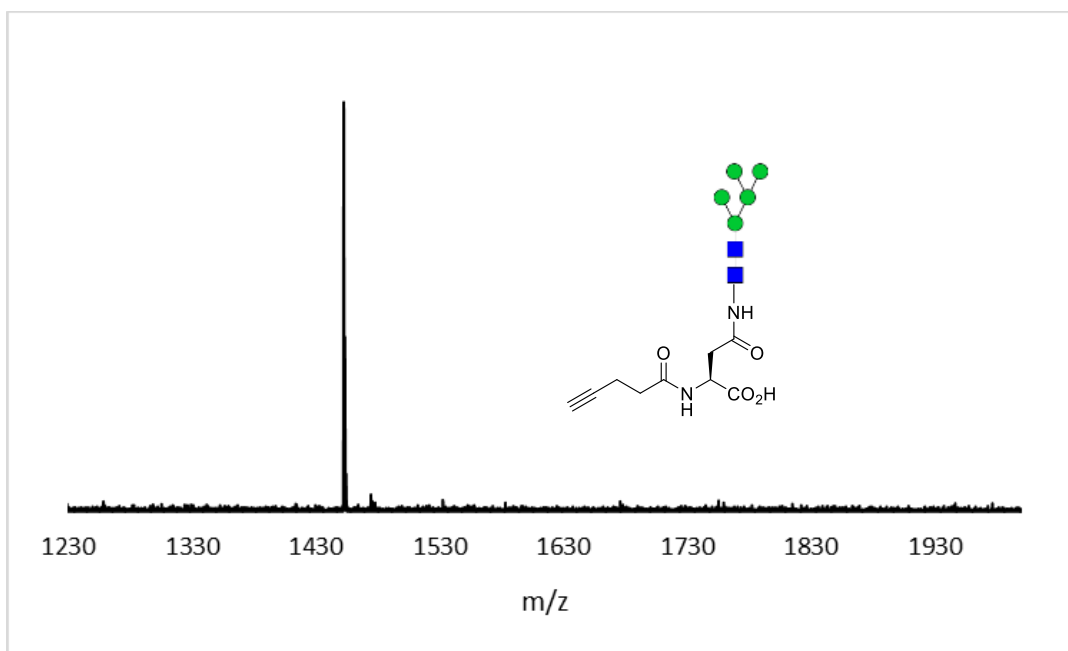


Figure A 2.10 MALDI-TOF analysis of compound 5.

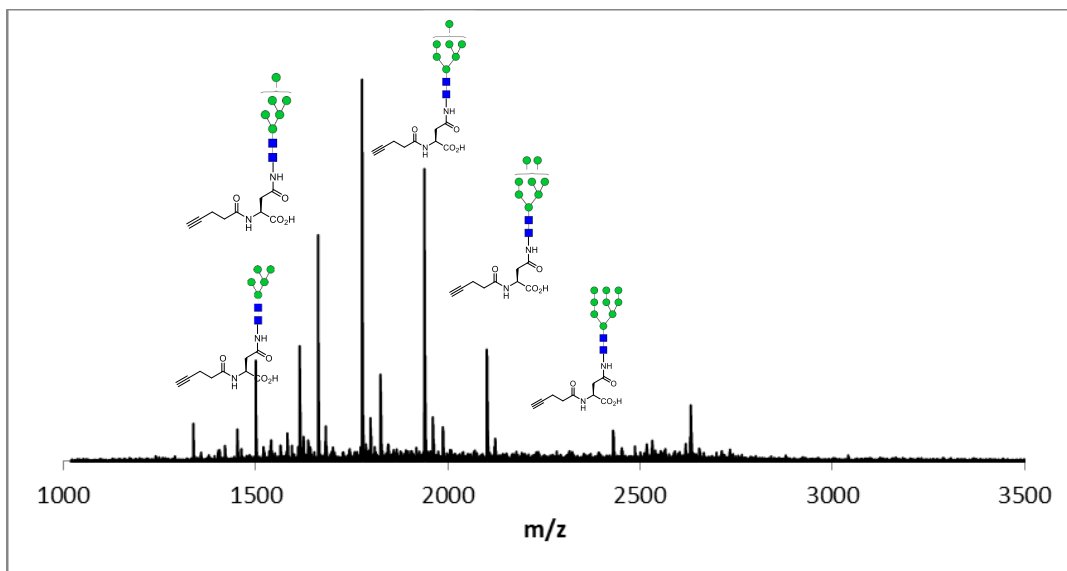


Figure A 2.11 MALDI-TOF analysis of compound 4.

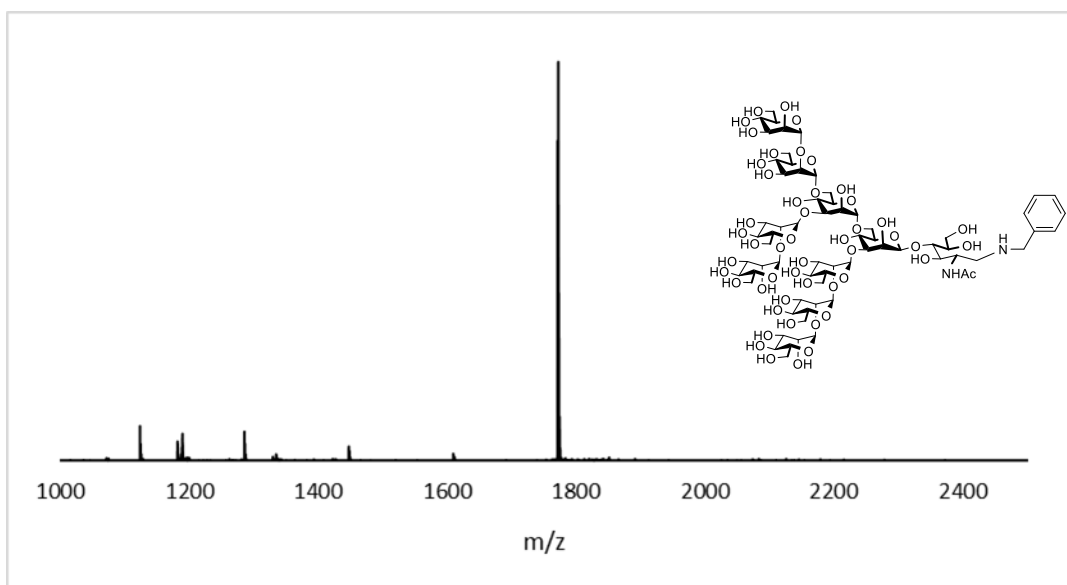


Figure A 2.12 MALDI-TOF analysis of compound 7.

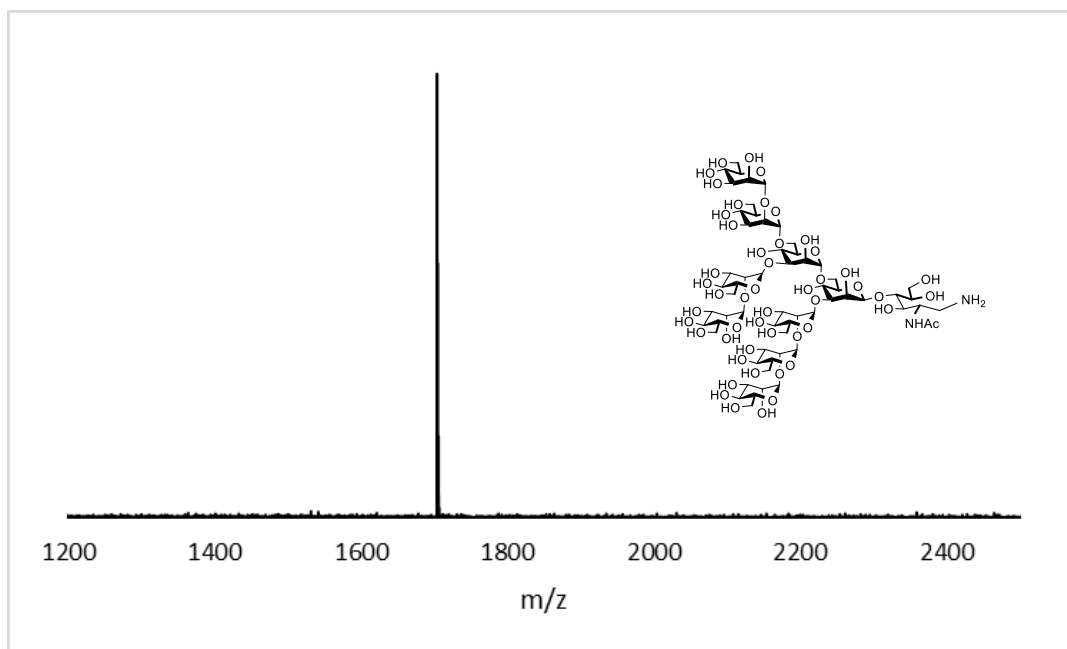


Figure A 2.13 MALDI-TOF analysis of compound **8**.

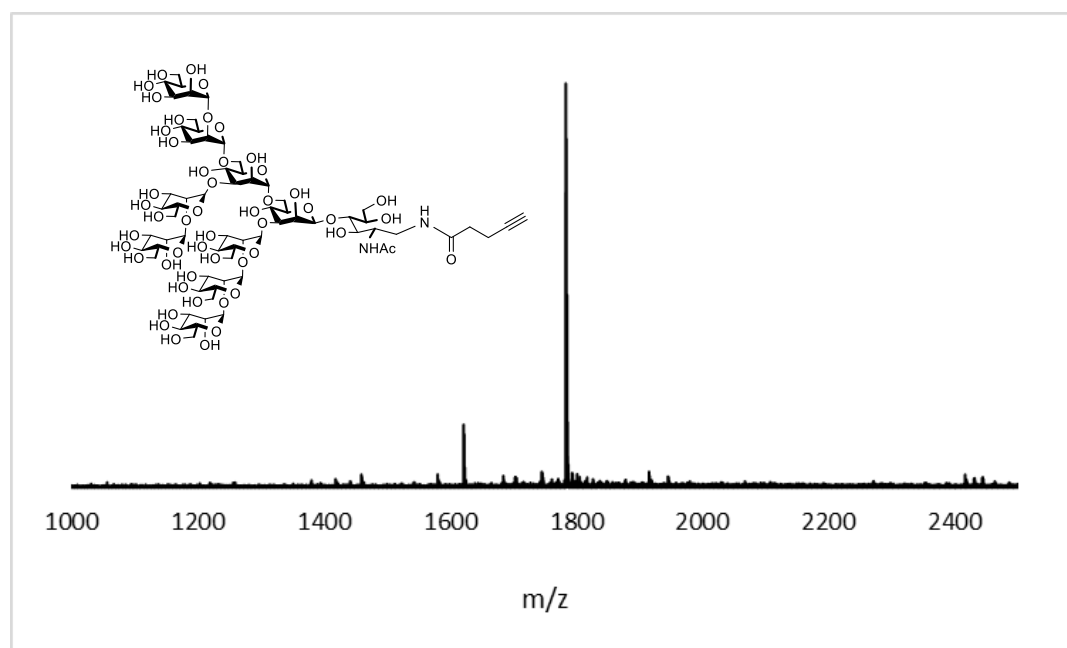


Figure A 2.14 MALDI-TOF analysis of compound **9**.

Figure A 2.16 ^1H NMR (D_2O , 400 MHz) compound **7**.

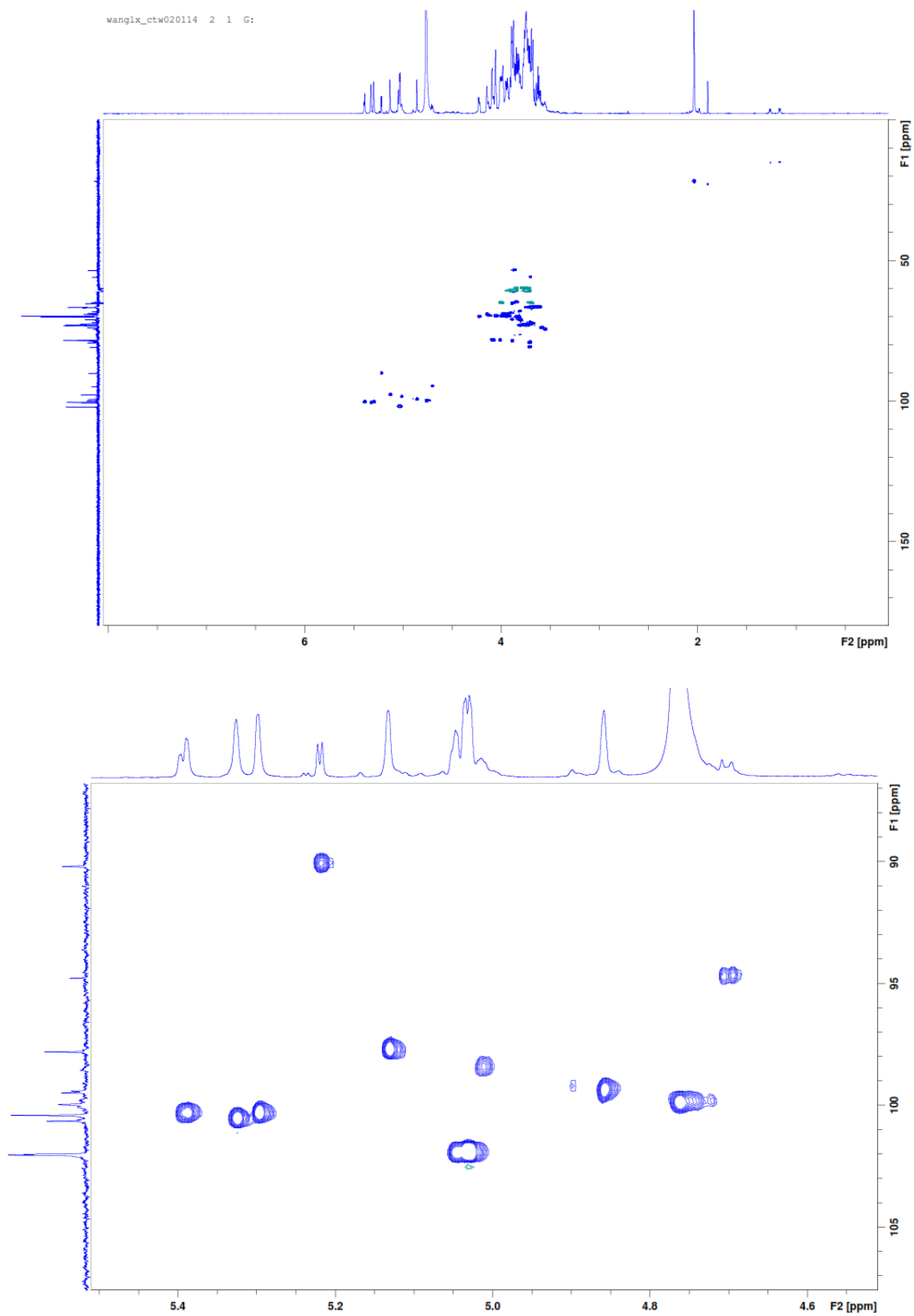


Figure A 2.17 ^1H - ^{13}C HSQC NMR (D_2O , 600 MHz) (bottom: anomeric region)
compound **6**.

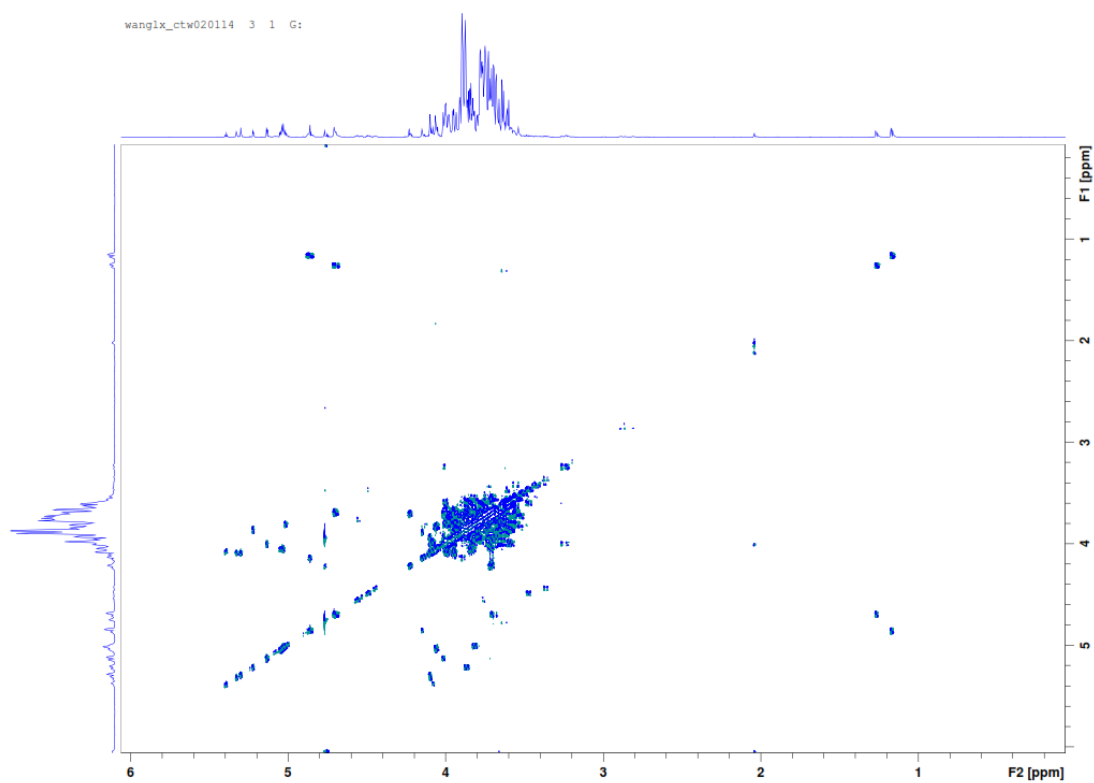
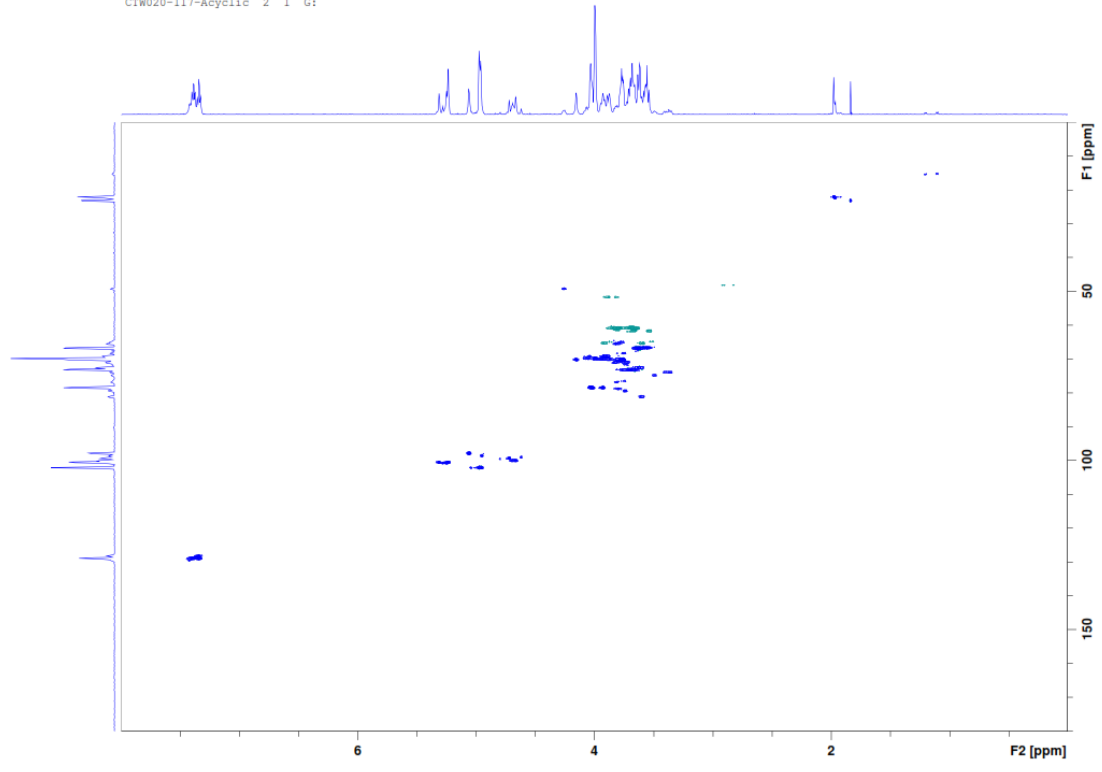


Figure A 2.18 ^1H - ^1H COSY NMR (D_2O , 600 MHz) compound **6**.

CIW020-117-Acyclic 2 1 G:



CIW020-117-Acyclic 2 1 G:

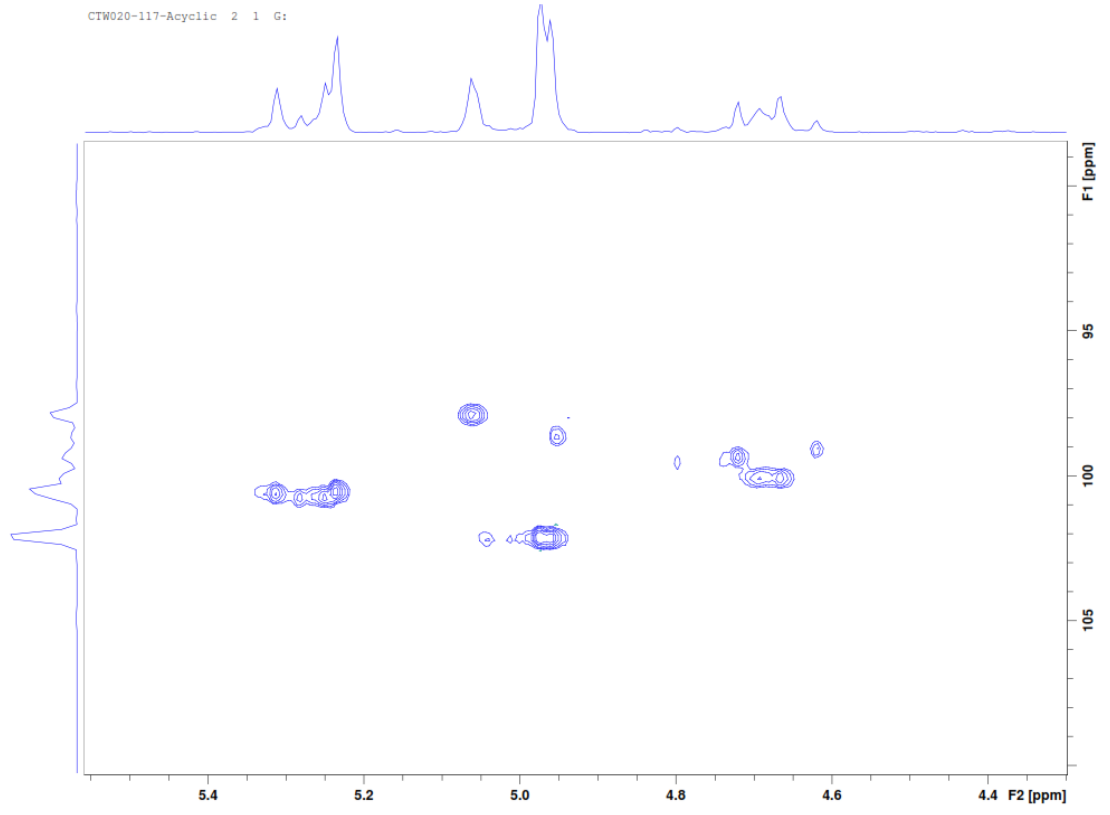


Figure A 2.19 ^1H - ^{13}C HSQC NMR (D_2O , 600 MHz) (bottom: anomeric region) compound **7**.

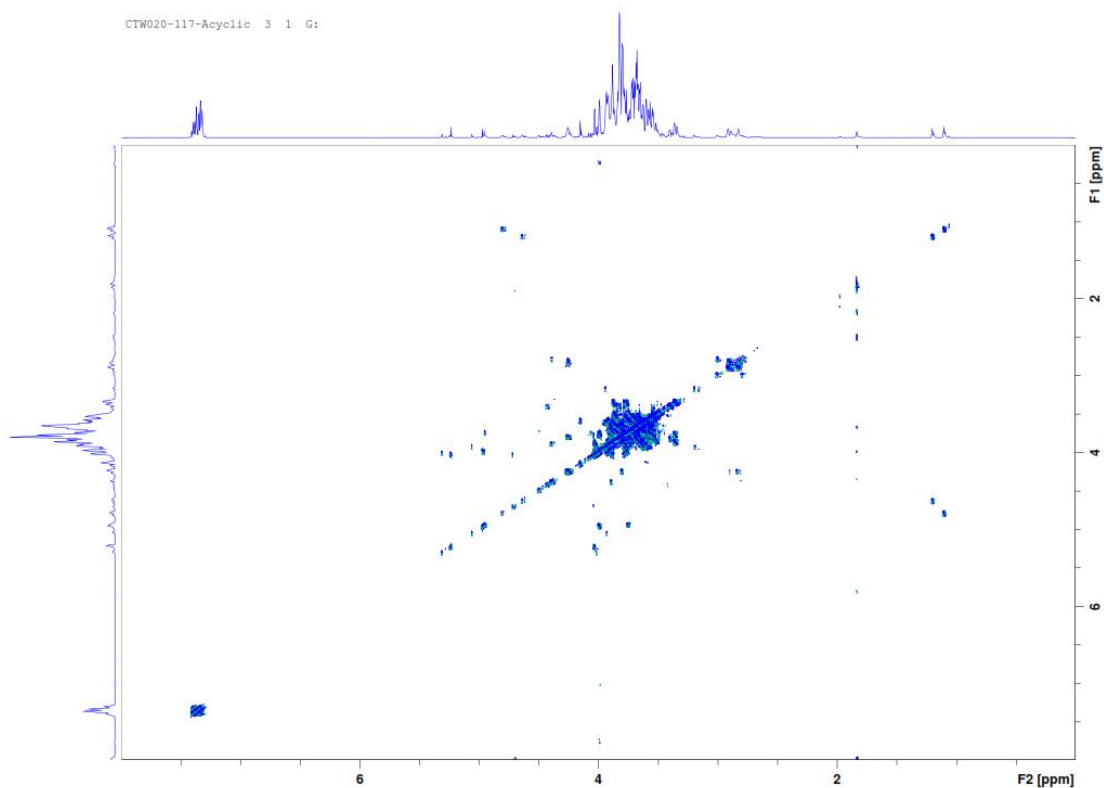


Figure A 2.20 ^1H - ^1H COSY NMR (D_2O , 600 MHz) compound **7**.

A.3.1 Chapter 3: Supplementary Information

Scheme A 3.1. Synthesis and evaluation of a linear version of GlcNAc-peptide (**11**) in EndoD-N322Q catalyzed transglycosylation.

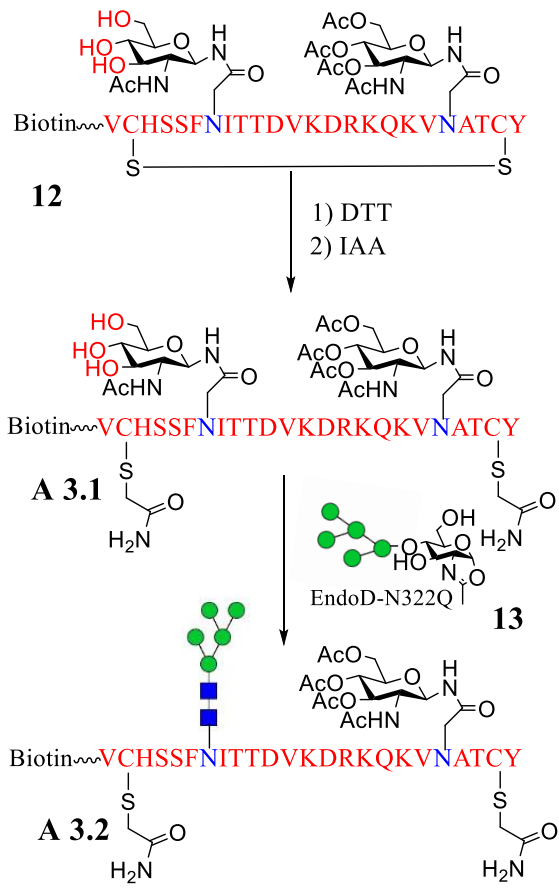
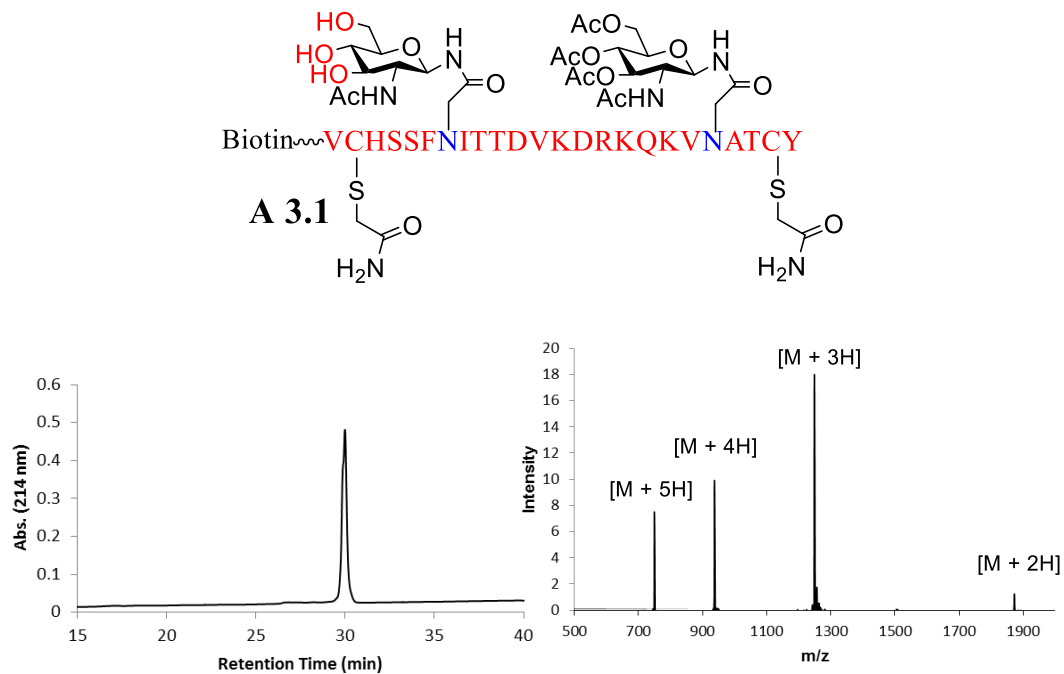
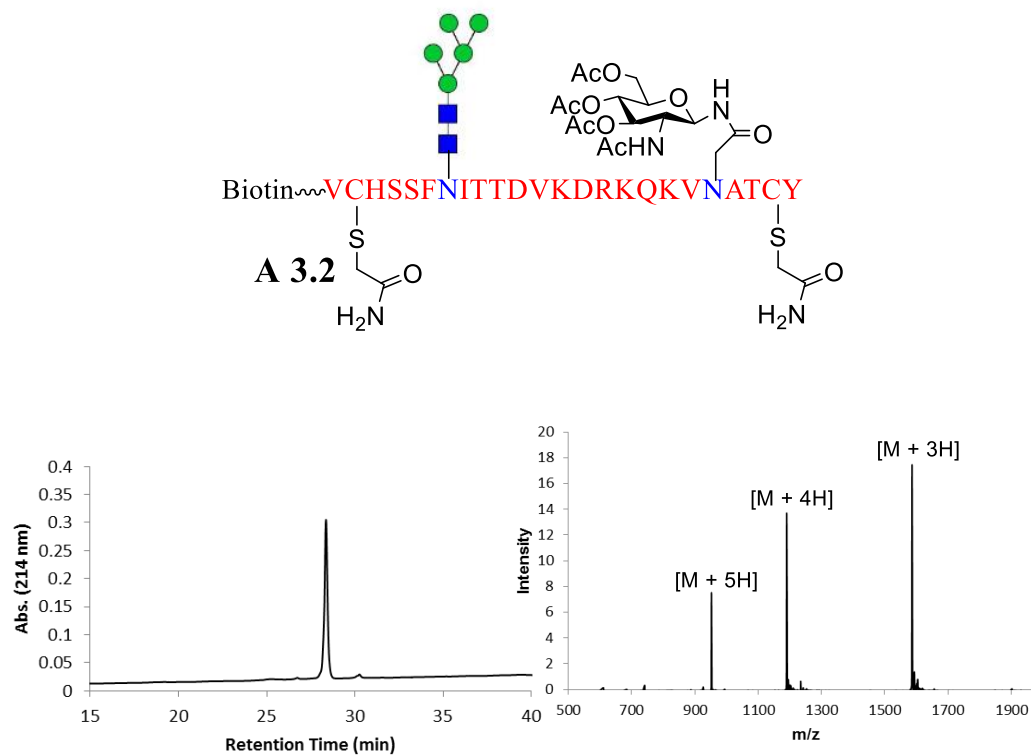


Figure A 3.1 HPLC and ESI-MS characterization of linear glycopeptide **A 3.1**^a



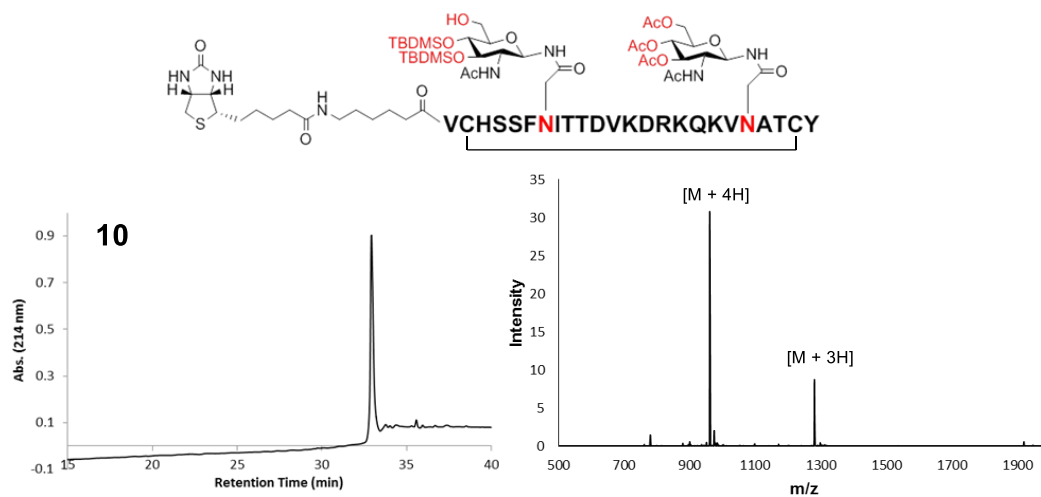
^aHPLC runs were performed on a C18 column with a linear gradient of 15-30% MeCN in 30 min.

Figure A 3.2. HPLC and ESI-MS characterization of linear glycopeptide **A 3.2**^a



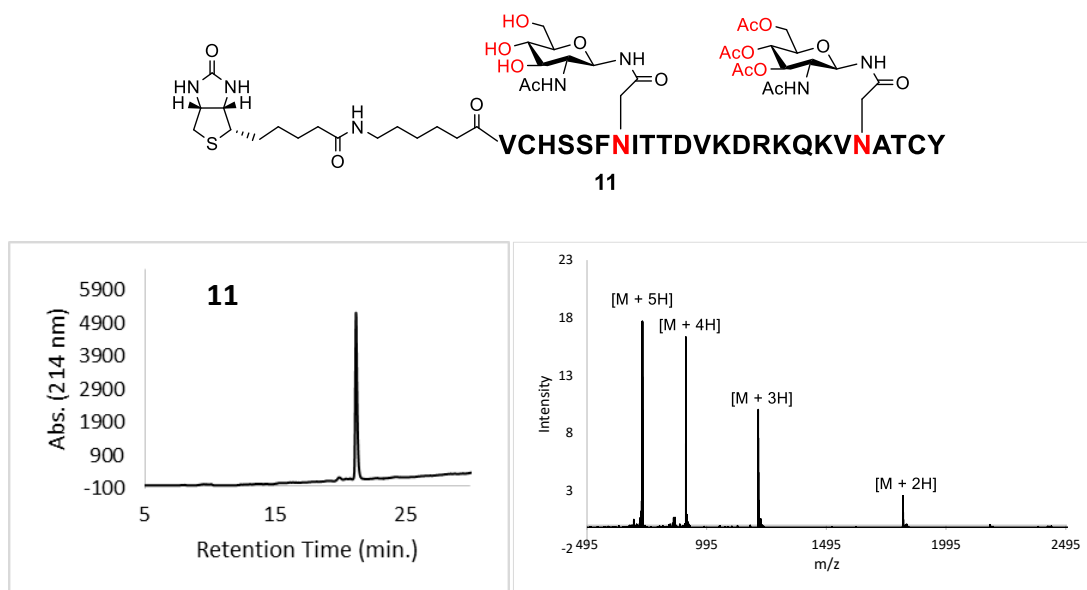
^aHPLC runs were performed on a C18 column with a linear gradient of 15-30% MeCN in 30 min.

Figure A 3.3 HPLC and ESI-MS characterization of glycopeptide **10**^a



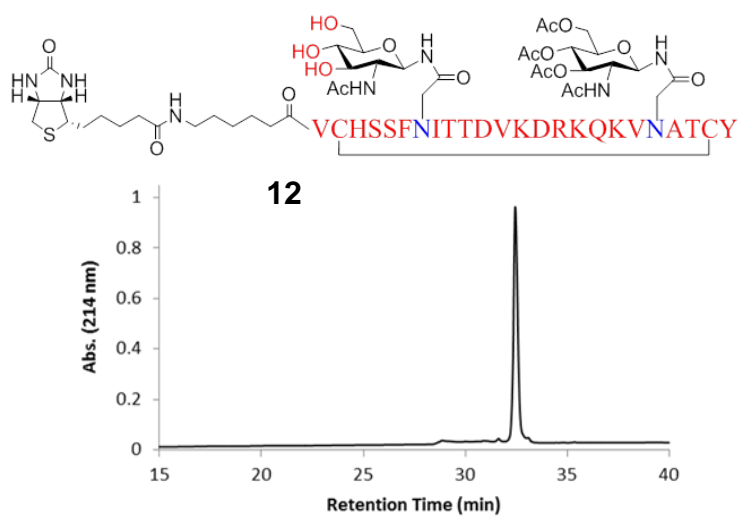
^aCompound **10** purified on a Thermo Acclaim C18 column (4.6 × 150 mm, 3.5 μm), 0-30% B in 30 min.

Figure A 3.4 HPLC and ESI-MS characterization of acyclic glycopeptide **11**^a



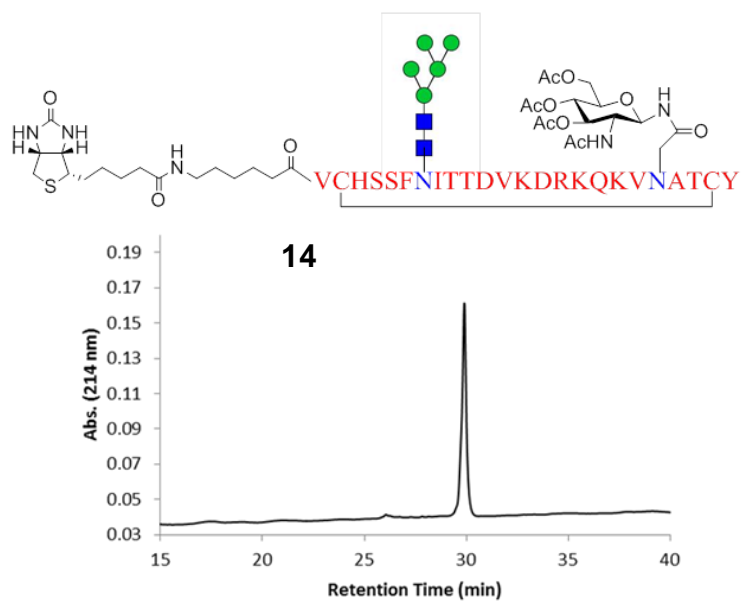
^aCompound **11** purified on a XBridge[®] Shield BEH C18 column (Waters, 3.5 μ m, i.d. 4.6 \times 250 mm), 5-25% B in 30 min.

Figure A 3.5 HPLC characterization of glycopeptide **12**^a



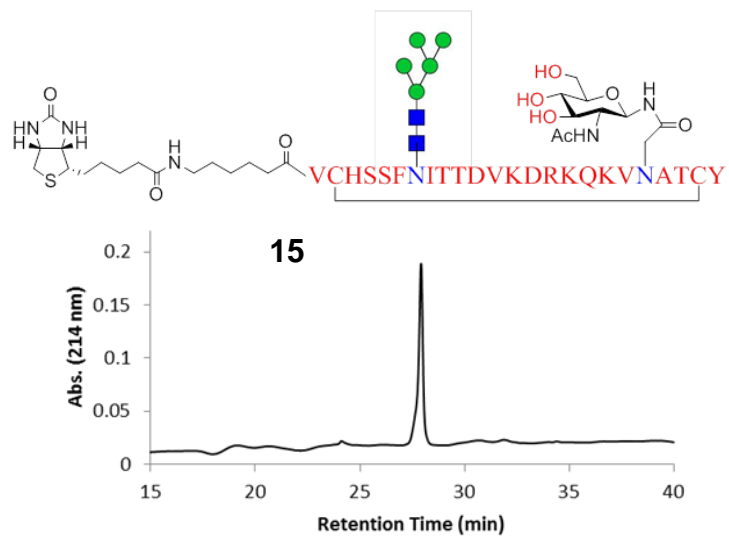
^aHPLC runs were performed on a C18 column with a linear gradient of 15-30% MeCN in 30 min.

Figure A 3.6 HPLC characterization of glycopeptide **14**^a



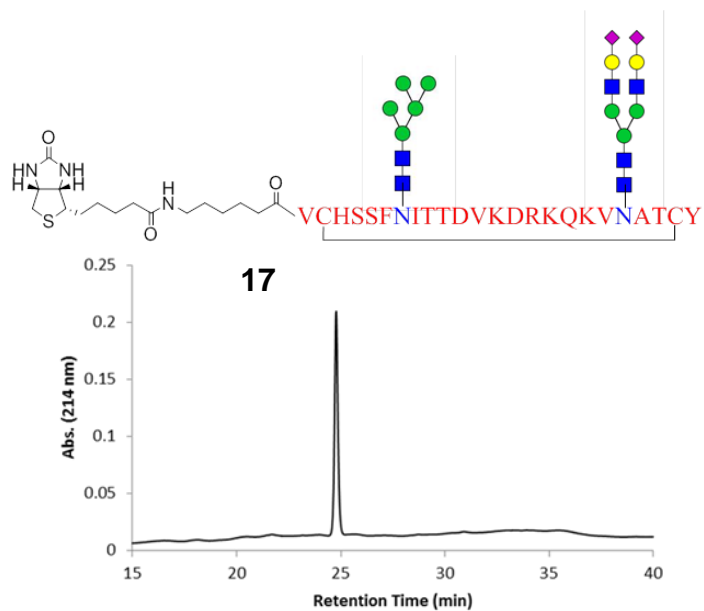
^aHPLC runs were performed on a C18 column with a linear gradient of 15-30% MeCN in 30 min.

Figure A 3.7 HPLC characterization of glycopeptide **15**^a



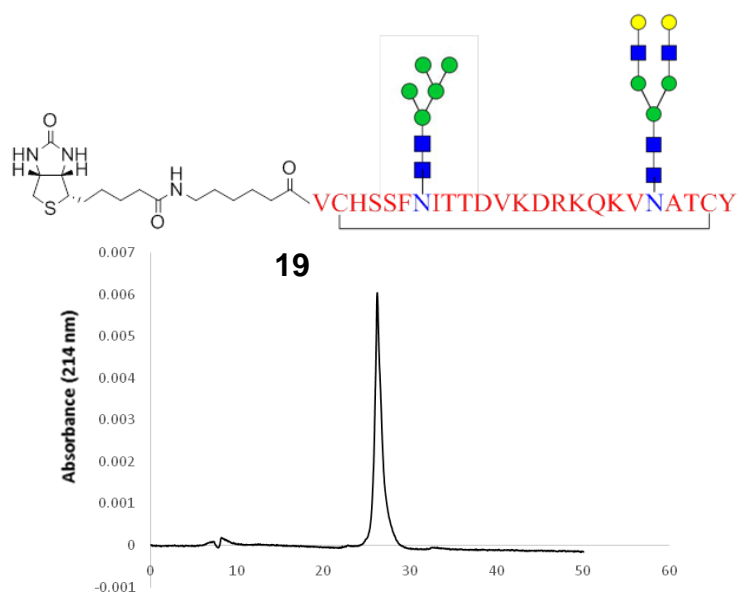
^aHPLC runs were performed on a C18 column with a linear gradient of 15-30% MeCN in 30 min.

Figure A 3.8 HPLC characterization of glycopeptide **17**^a



^aHPLC runs were performed on a C18 column with a linear gradient of 15-30% MeCN in 30 min.

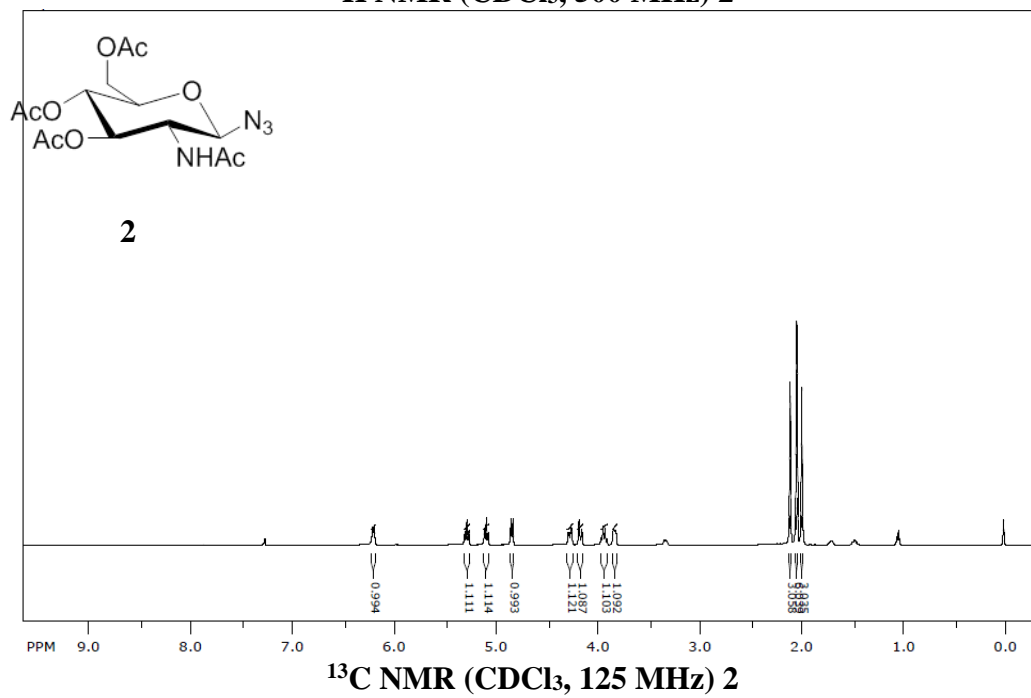
Figure A 3.9 HPLC characterization of glycopeptide **19**^a



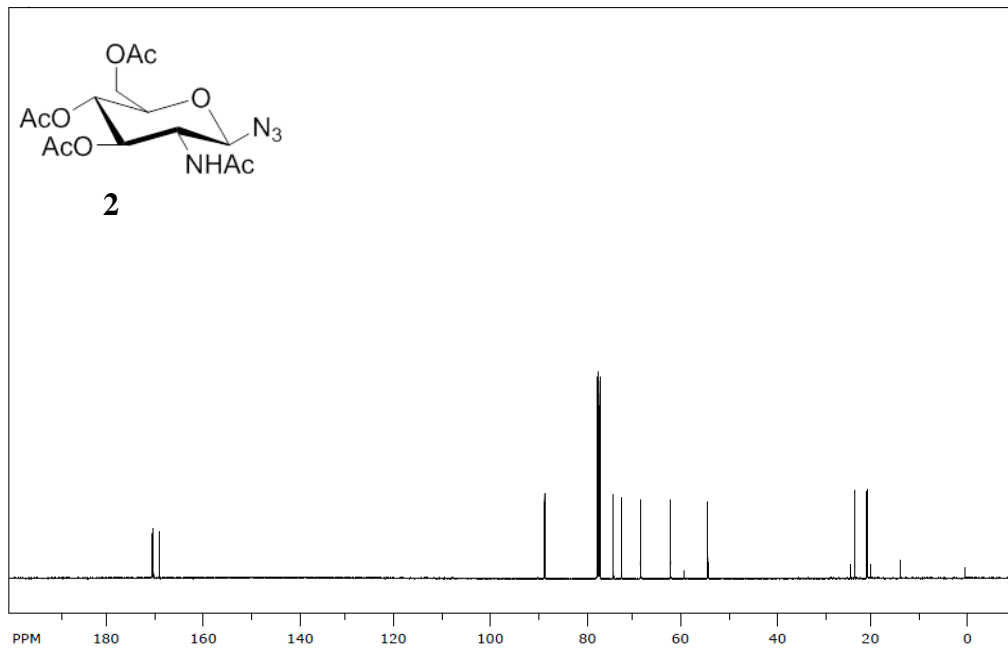
^aHPLC runs were performed on a C18 column with a linear gradient of 15-30% MeCN in 30 min.

NMR Spectra

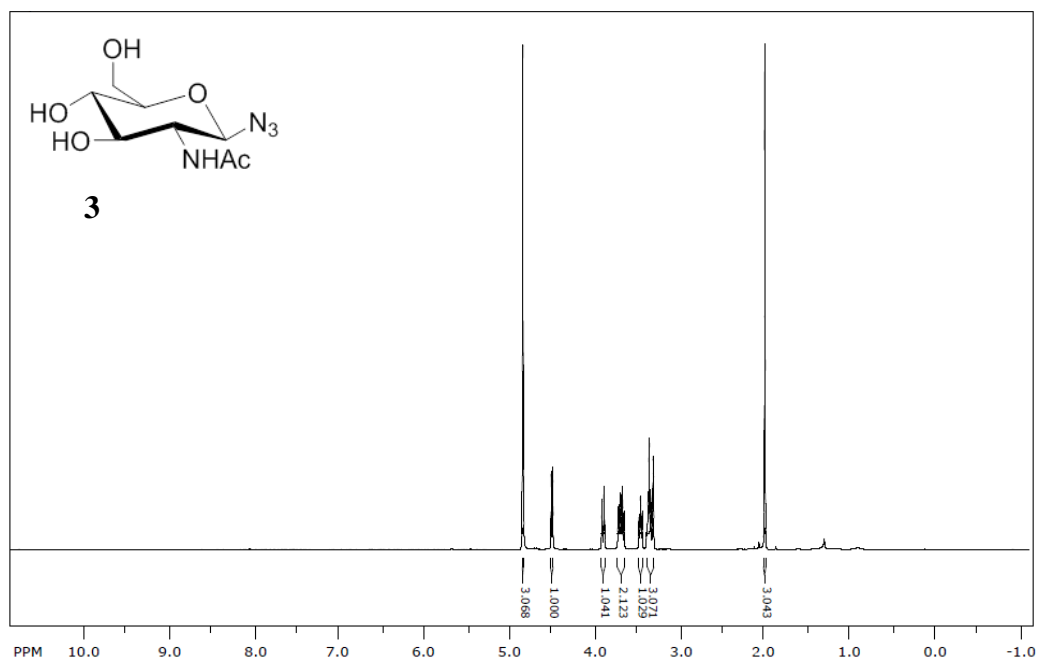
^1H NMR (CDCl_3 , 500 MHz) 2



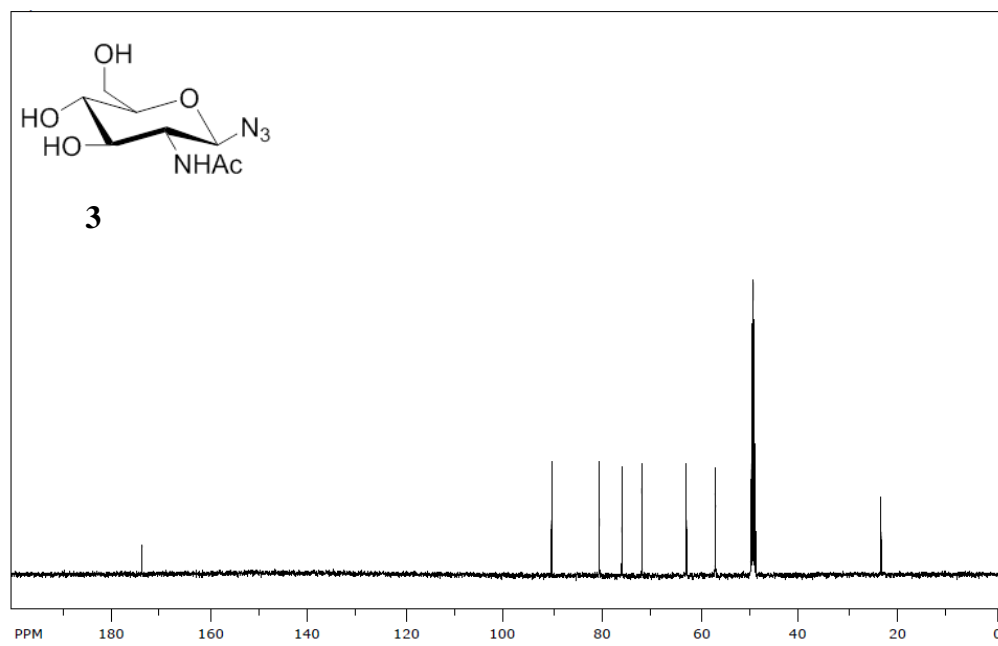
^{13}C NMR (CDCl_3 , 125 MHz) 2



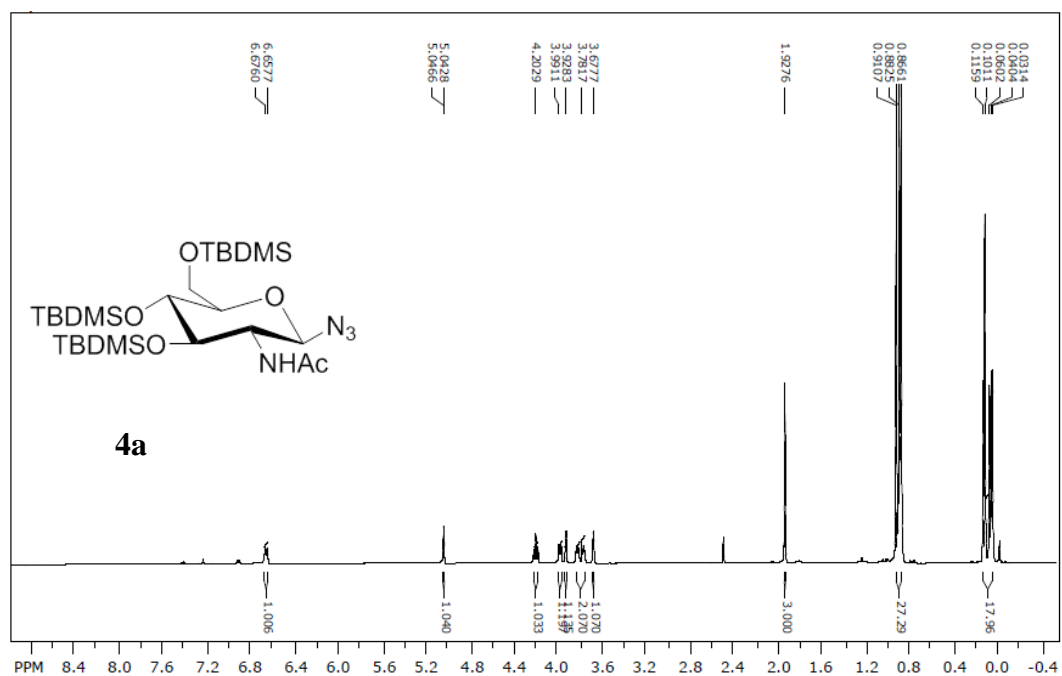
^1H NMR (CDCl_3 , 500 MHz) 3



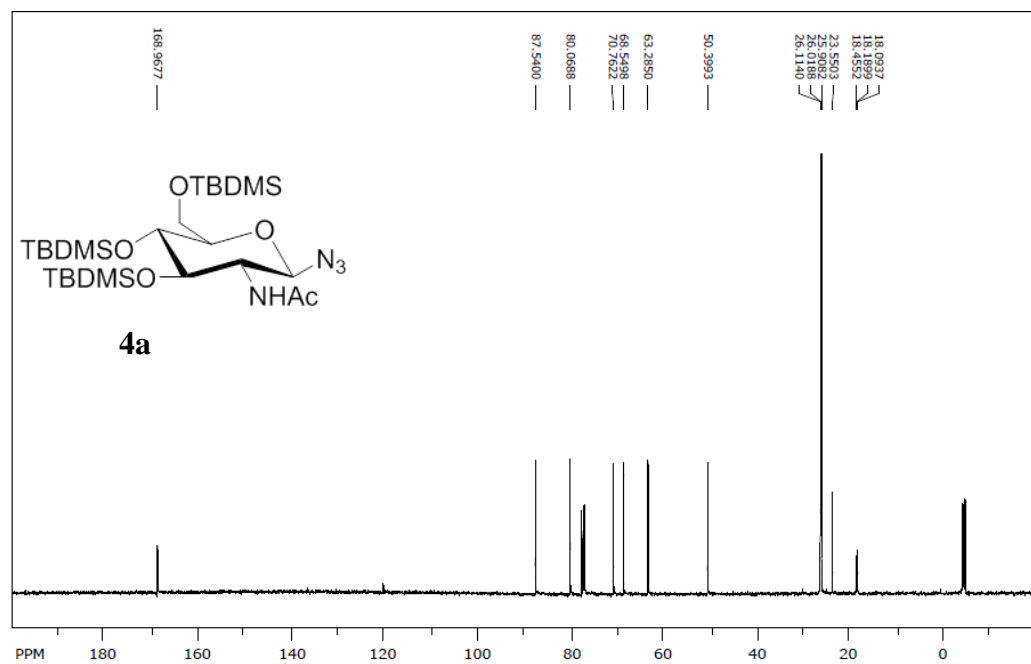
^{13}C NMR (CDCl₃, 125 MHz) **3**



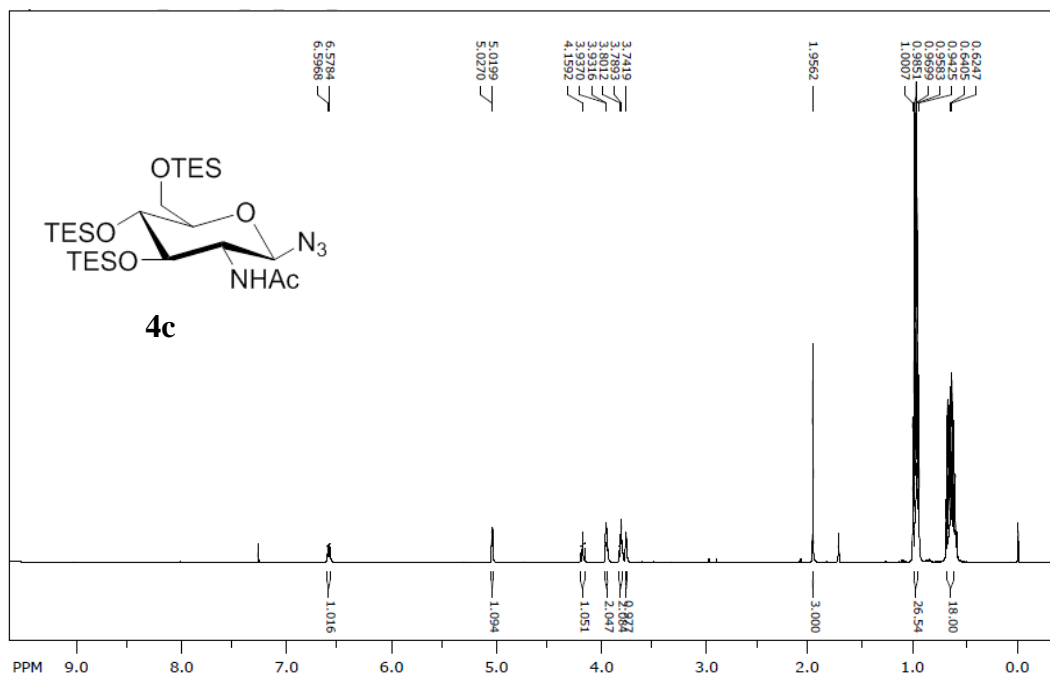
^1H NMR (CDCl₃, 500 MHz) **4a**



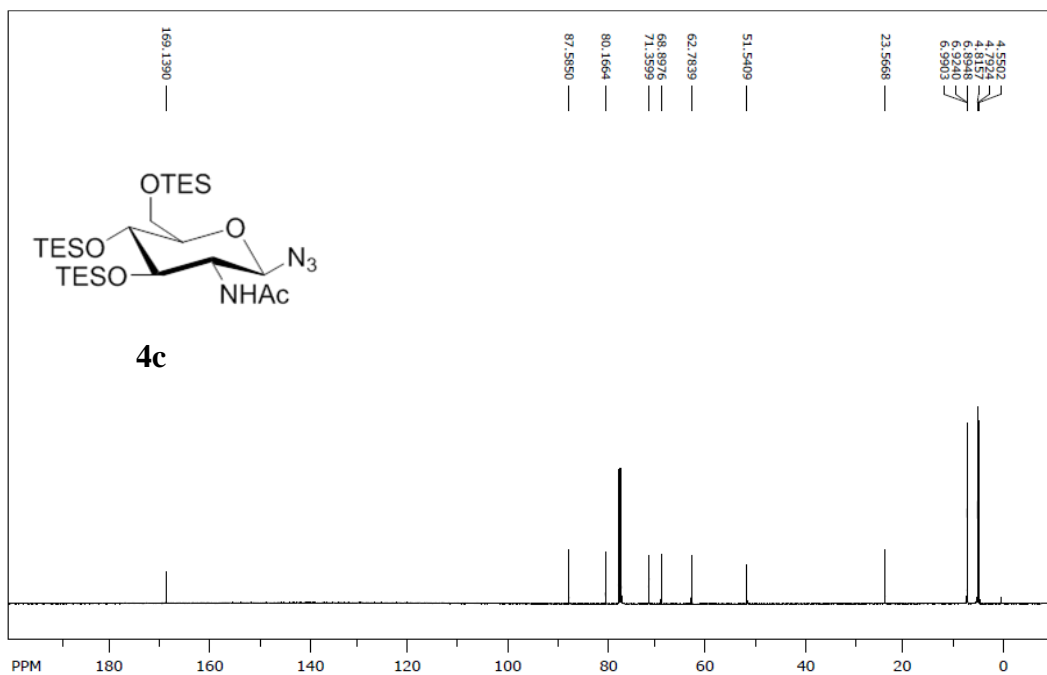
¹³C NMR (CDCl₃, 125 MHz) 4a



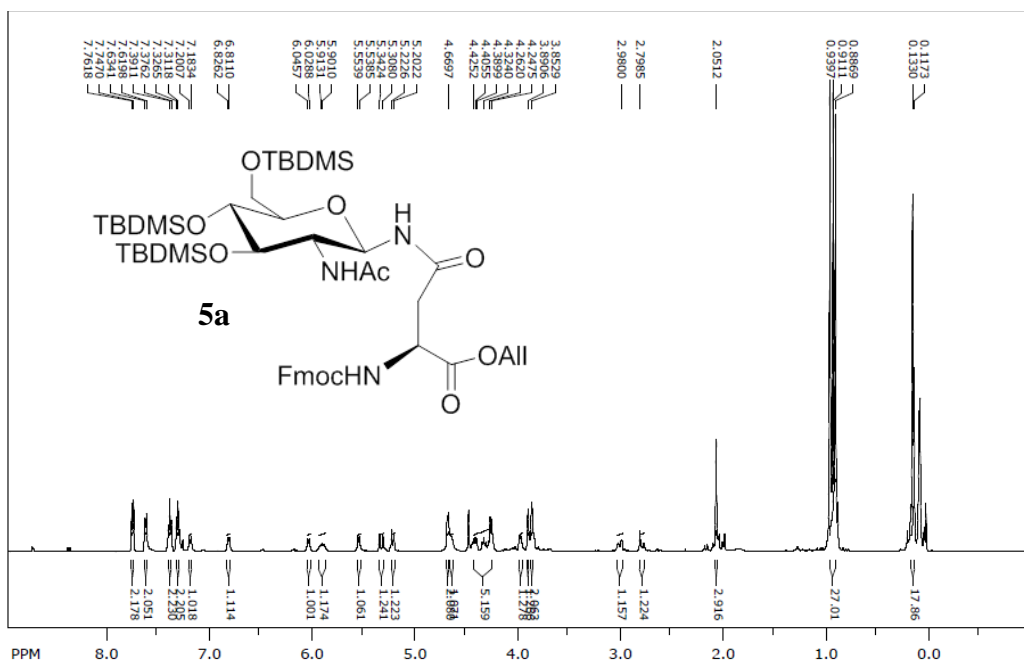
¹H NMR (CDCl₃, 500 MHz) 4b



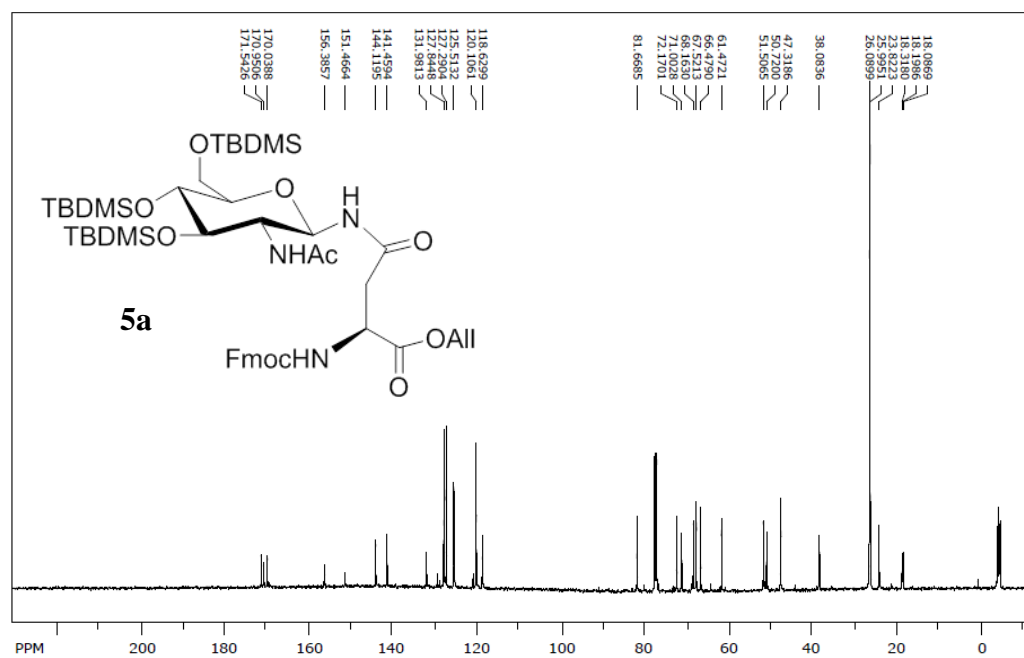
¹³C NMR (CDCl₃, 125 MHz) 4c



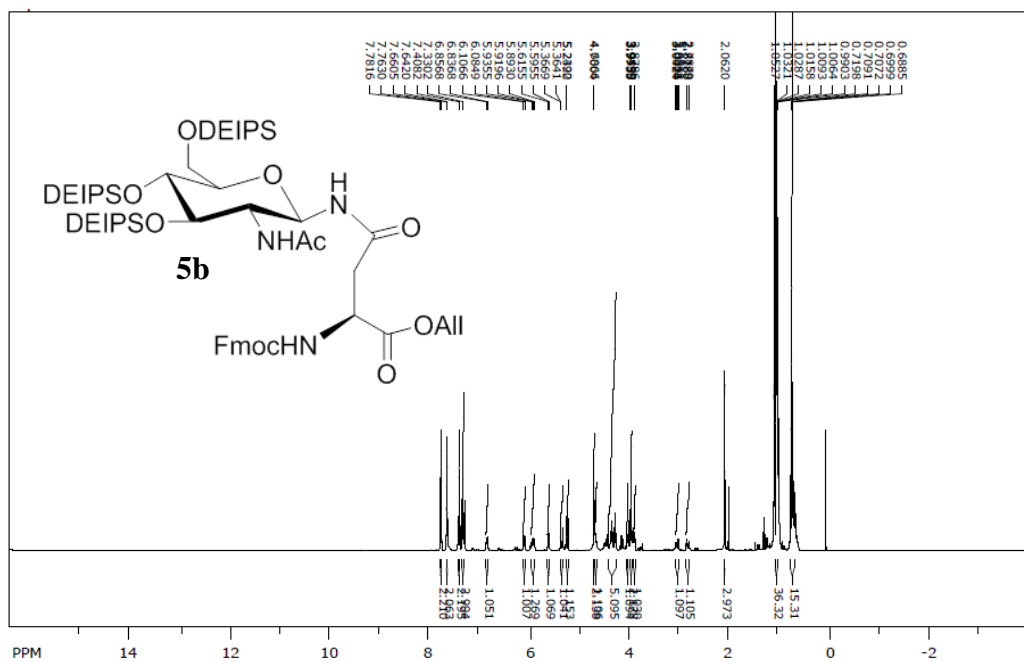
¹H NMR (CDCl₃, 500 MHz) 5a



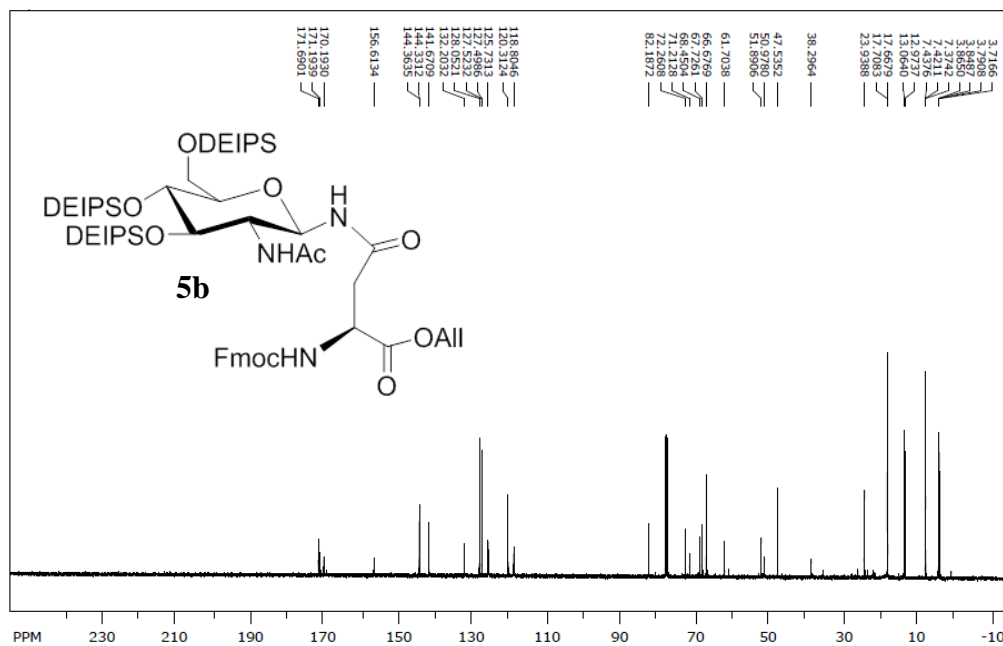
¹³C NMR (CDCl₃, 125 MHz) 5a

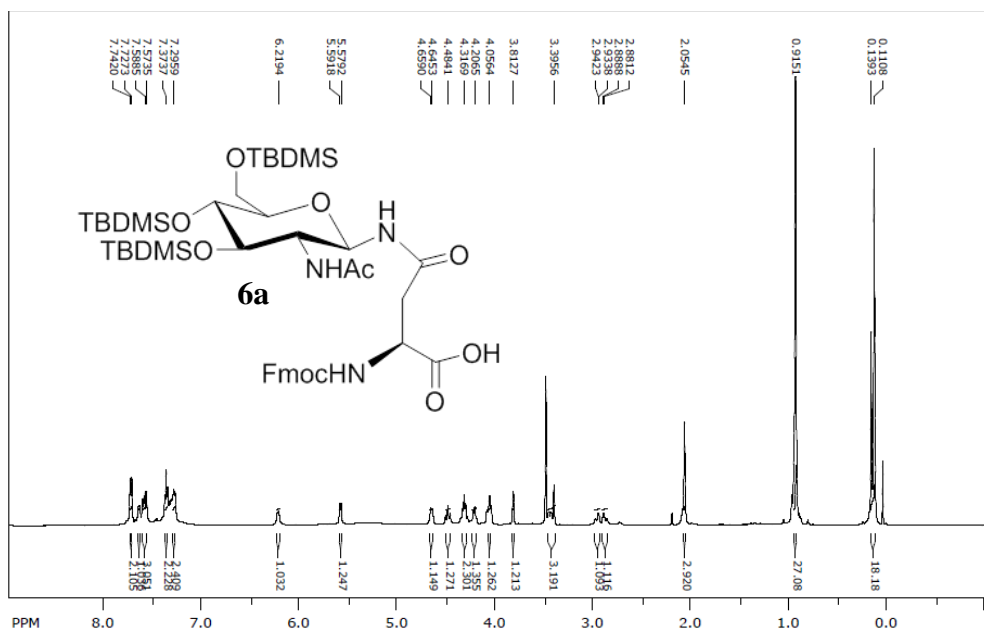


¹H NMR (CDCl₃, 400 MHz) 5b

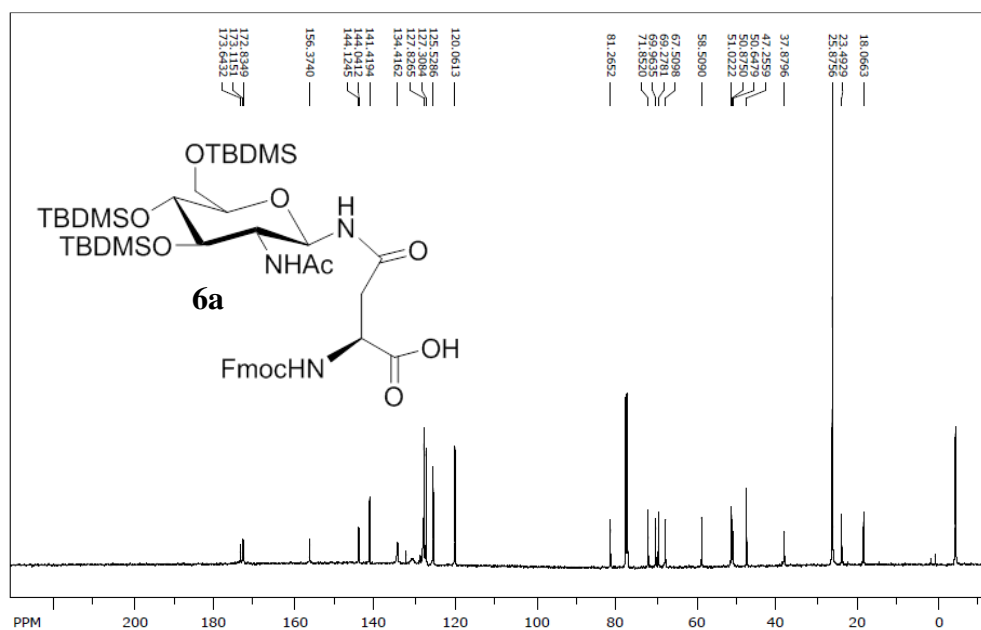


¹³C NMR (CDCl₃, 125 MHz) 5b

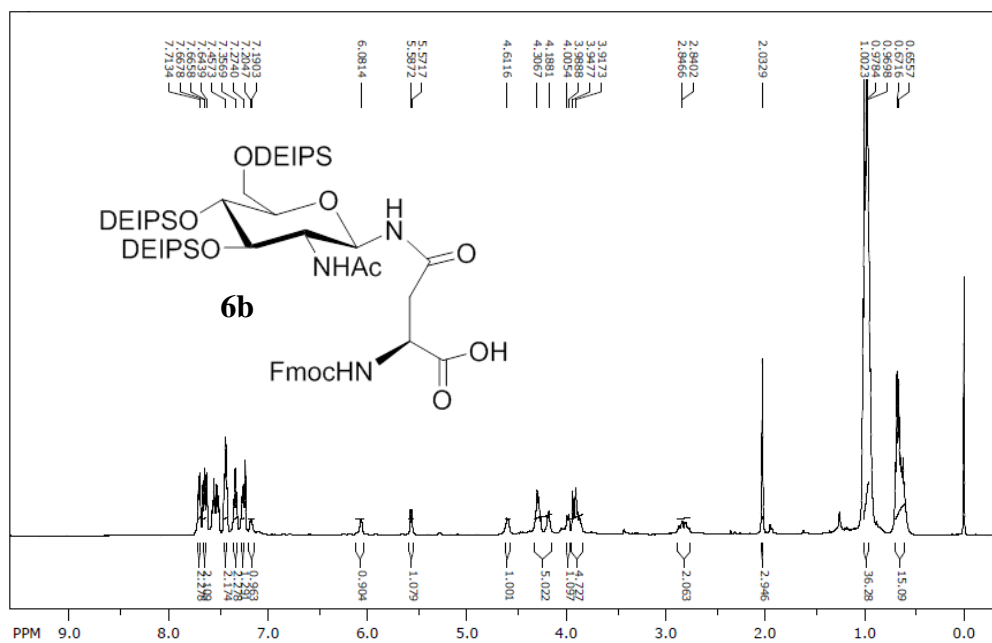




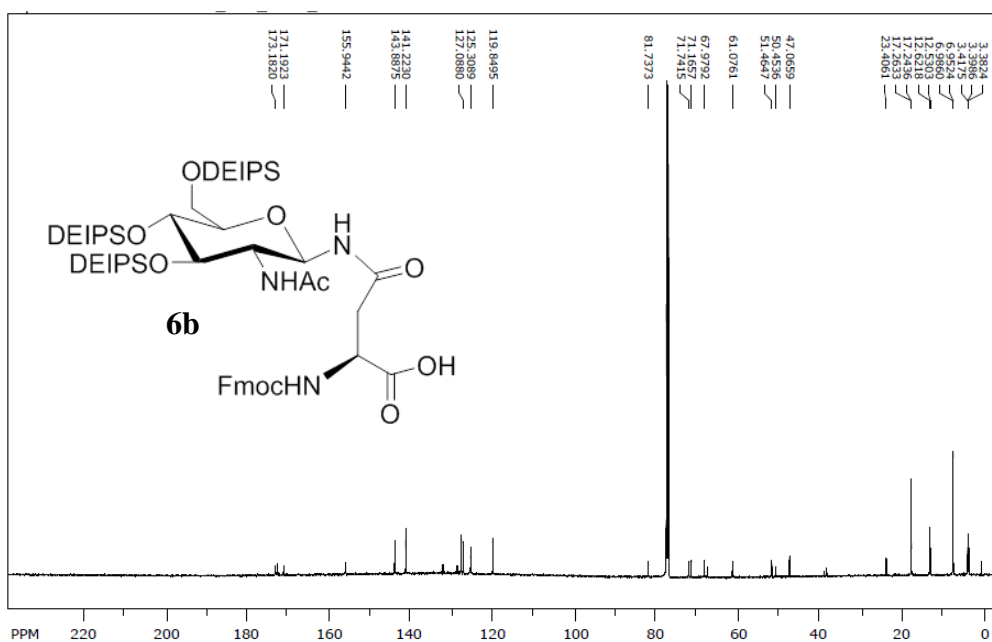
¹³C NMR (CDCl₃, 125 MHz) 6a



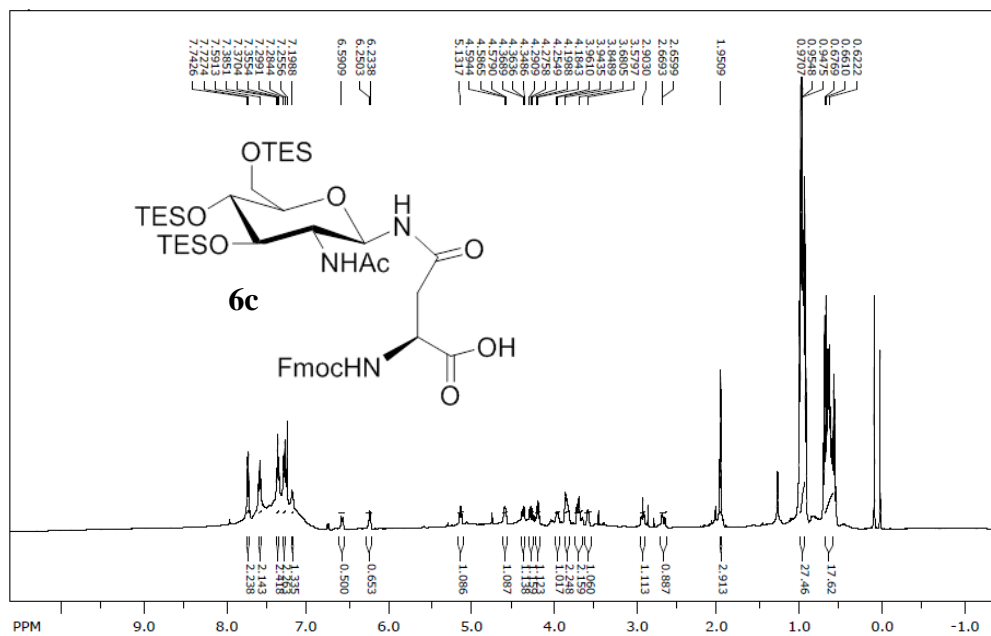
¹H NMR (CDCl₃, 500 MHz) 6b



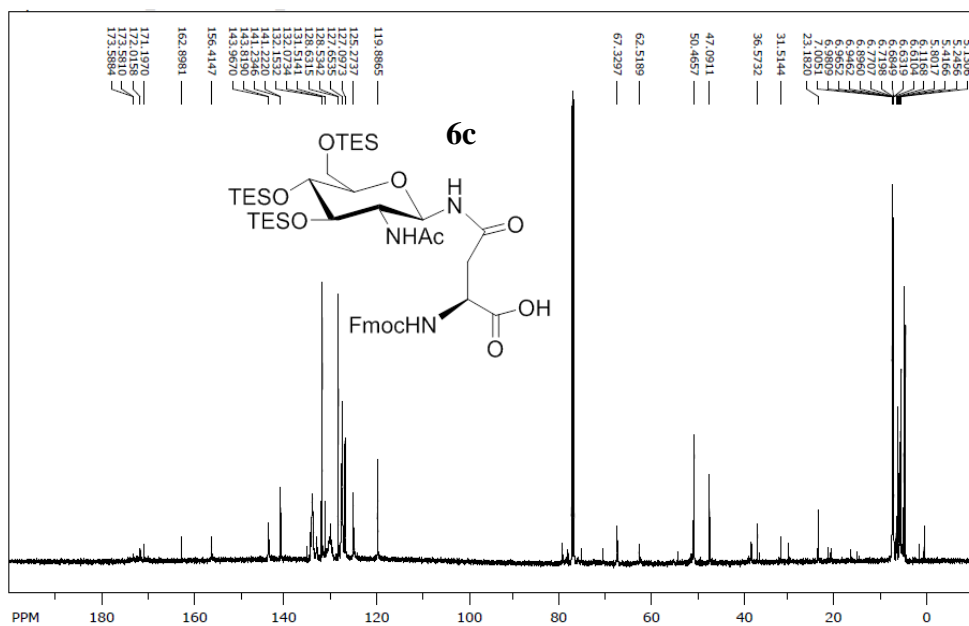
¹³C NMR (CDCl₃, 125 MHz) 6b



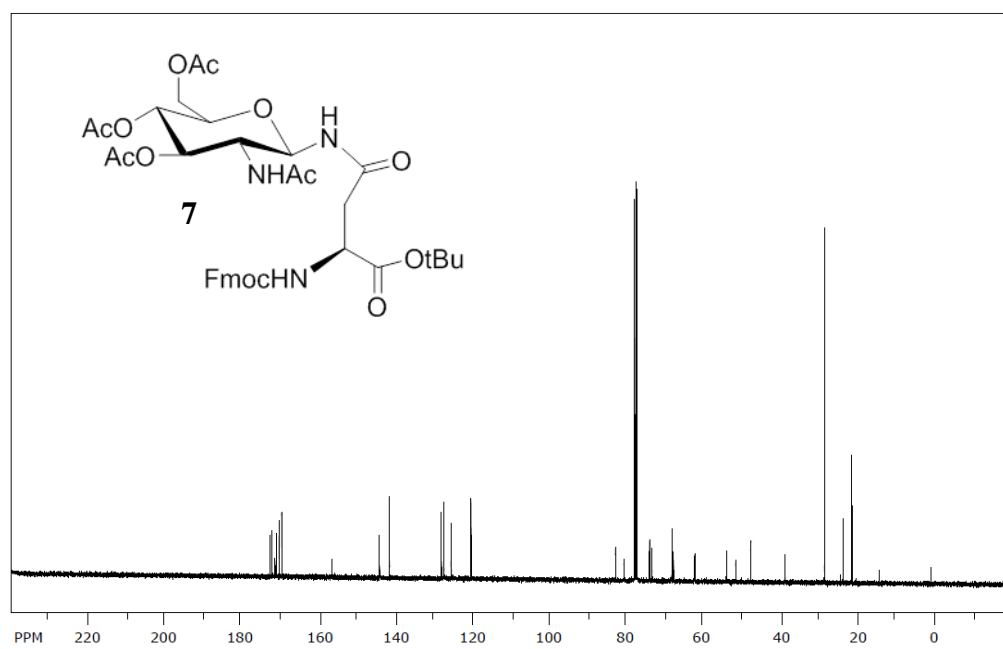
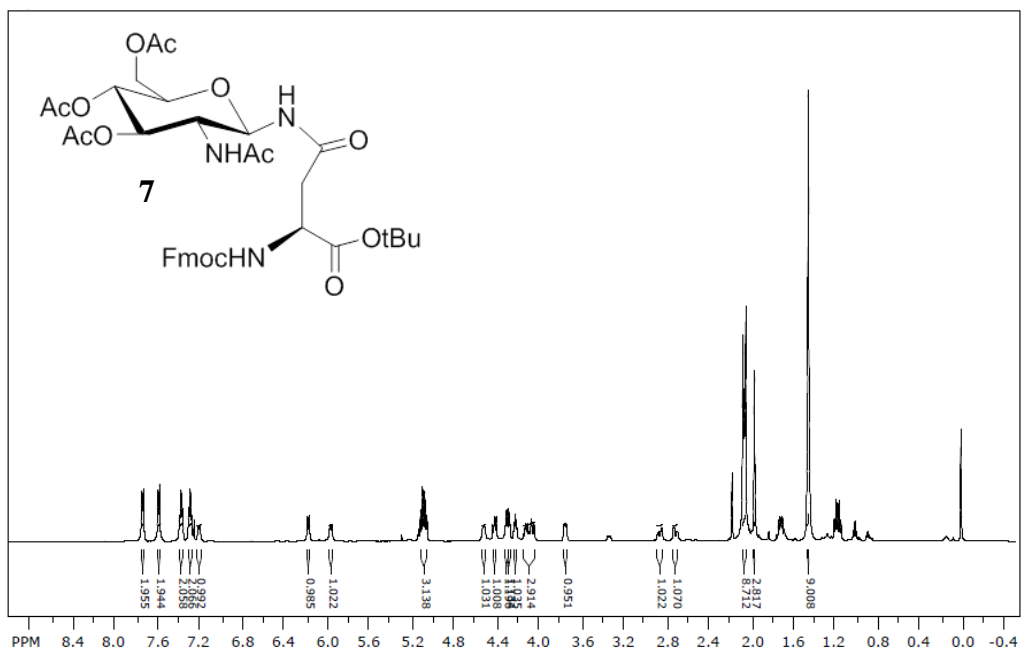
¹³C NMR (CDCl₃, 125 MHz) 6c

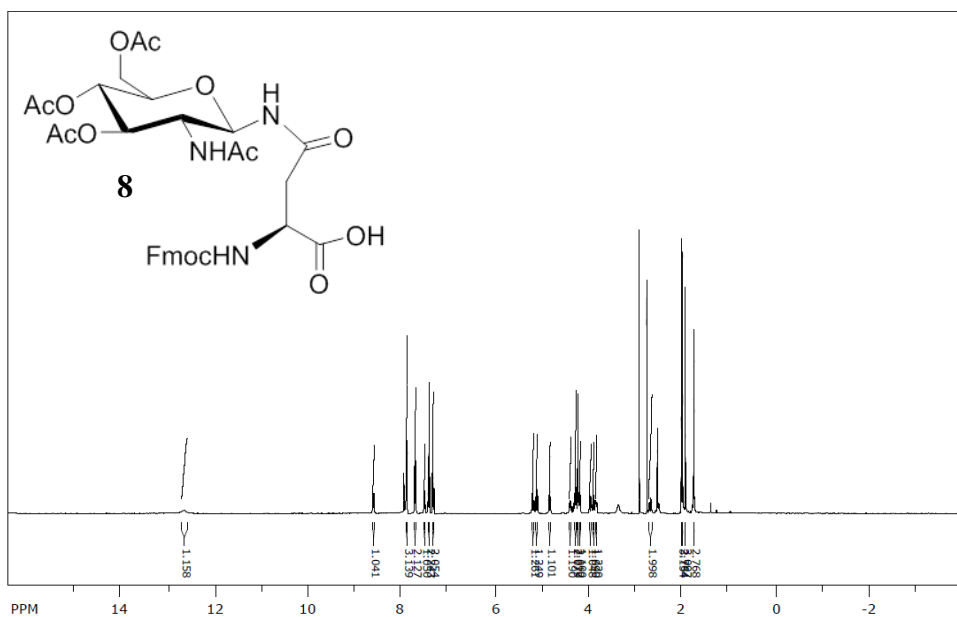


¹³C NMR (CDCl₃, 125 MHz) 6c

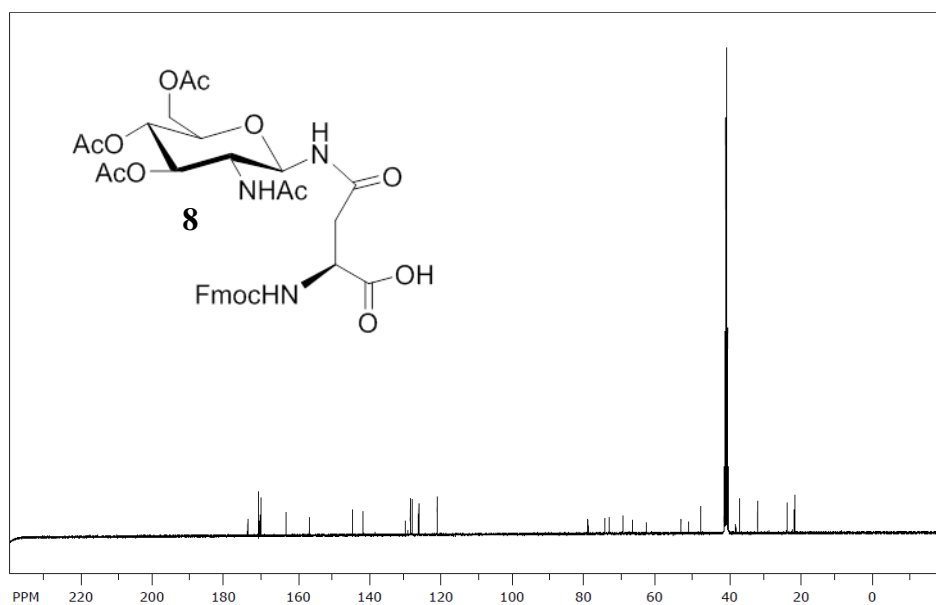


¹H NMR (CDCl₃, 400 MHz) 7



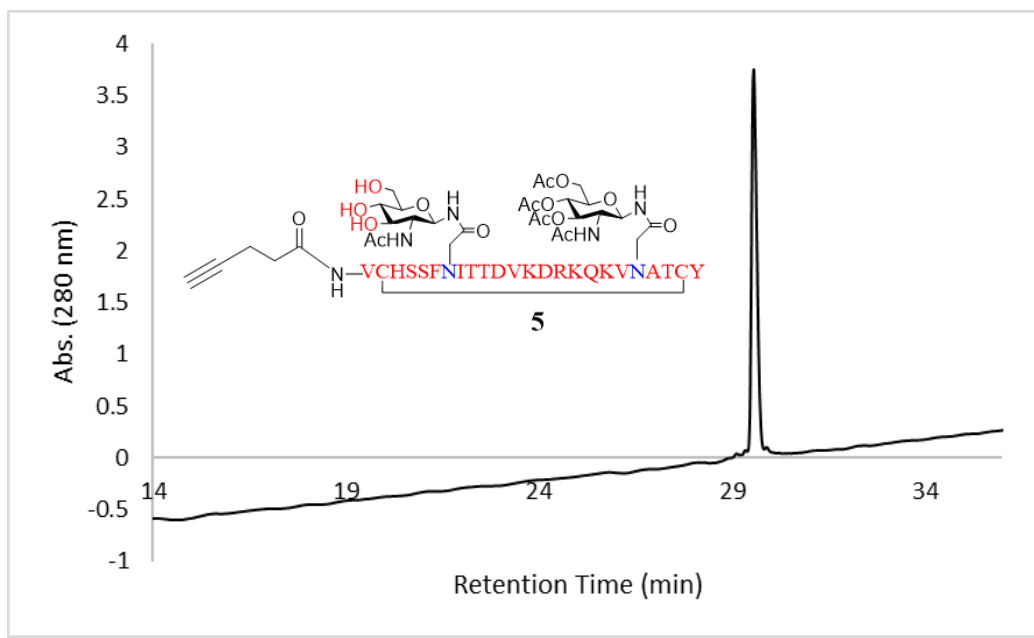


¹³C NMR ((CD₃)₂SO, 125 MHz) **8**



A.4.1 Chapter 4: Supplementary Material

Figure A 4.1 HPLC and ESI-MS characterization of linear glycopeptide **5^a**



^aHPLC runs were performed on a C18 column with a linear gradient of 5-50% MeCN in 30 min.

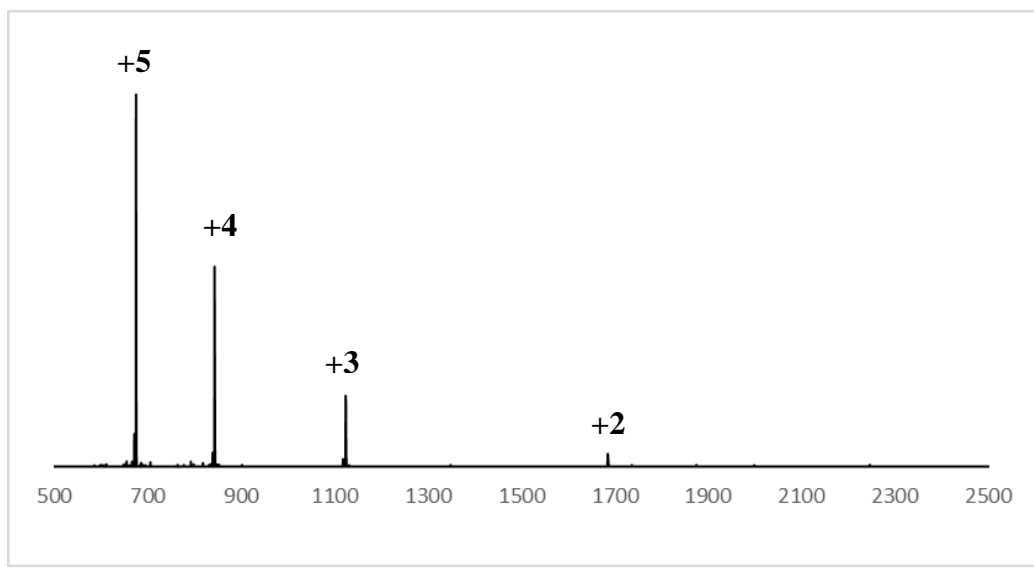
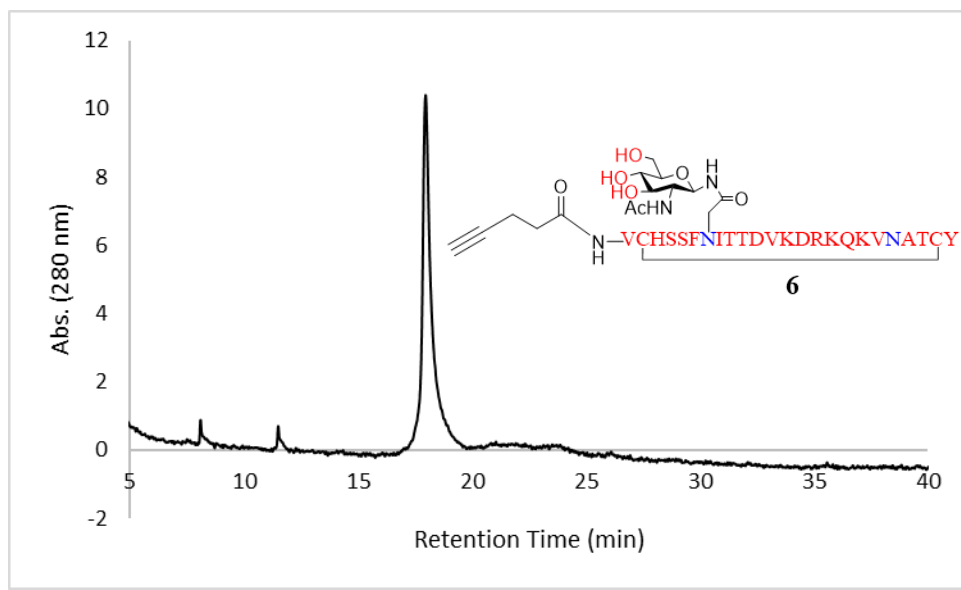


Figure A 4.2 HPLC and ESI-MS characterization of linear glycopeptide **6^a**



^aHPLC runs were performed on a C18 column with a linear gradient of 5-25% MeCN in 30 min.

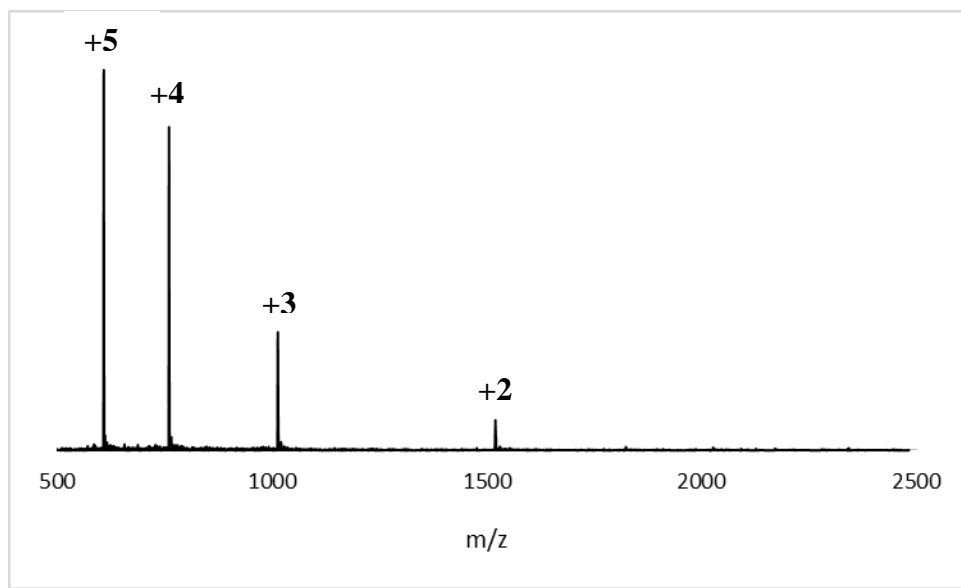
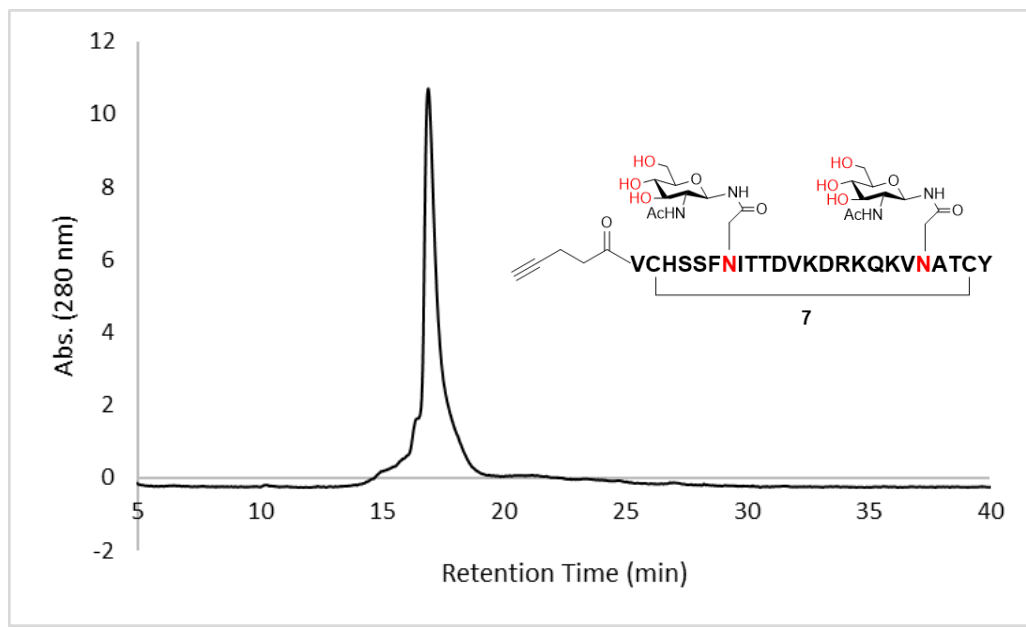


Figure A 4.3 HPLC and ESI-MS characterization of linear glycopeptide **7^a**



^aHPLC runs were performed on a C18 column with a linear gradient of 5-25% MeCN in 30 min.

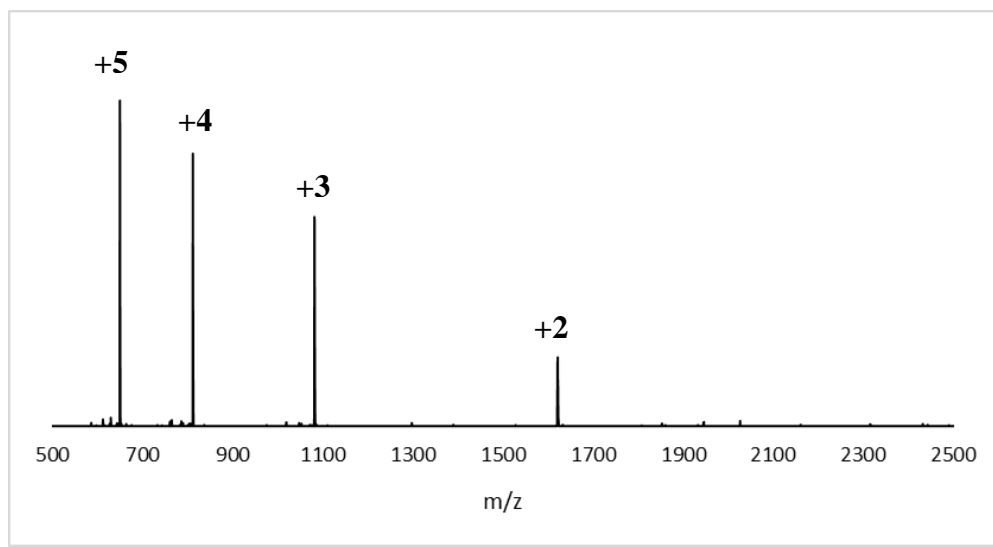
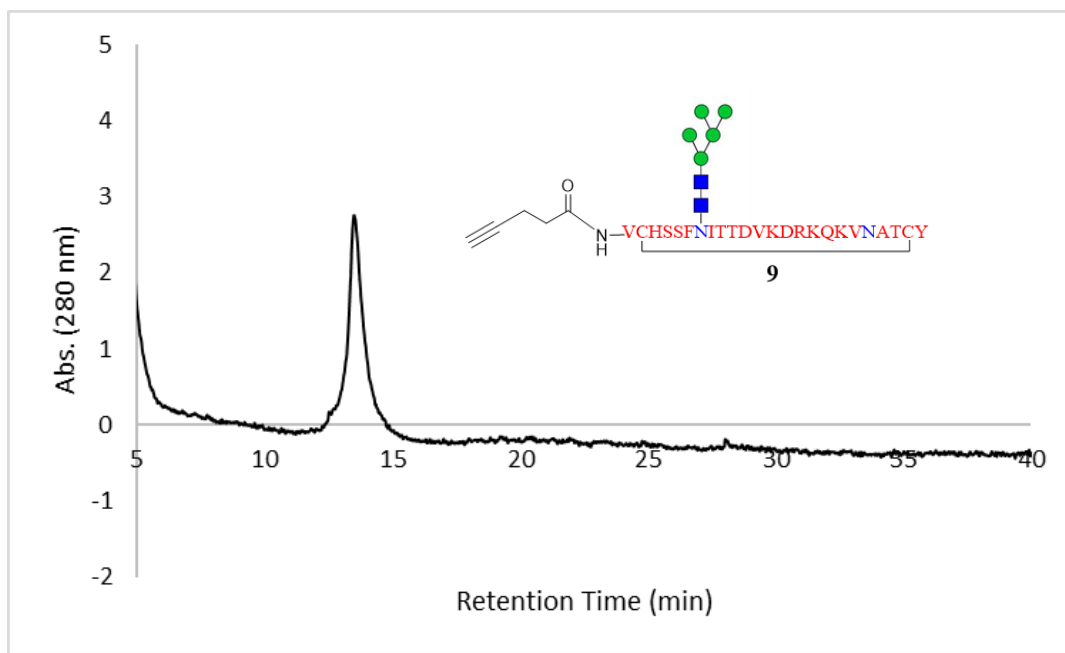


Figure A 4.4 HPLC and ESI-MS characterization of linear glycopeptide **9^a**



^aHPLC runs were performed on a C18 column with a linear gradient of 5-25% MeCN in 30 min.

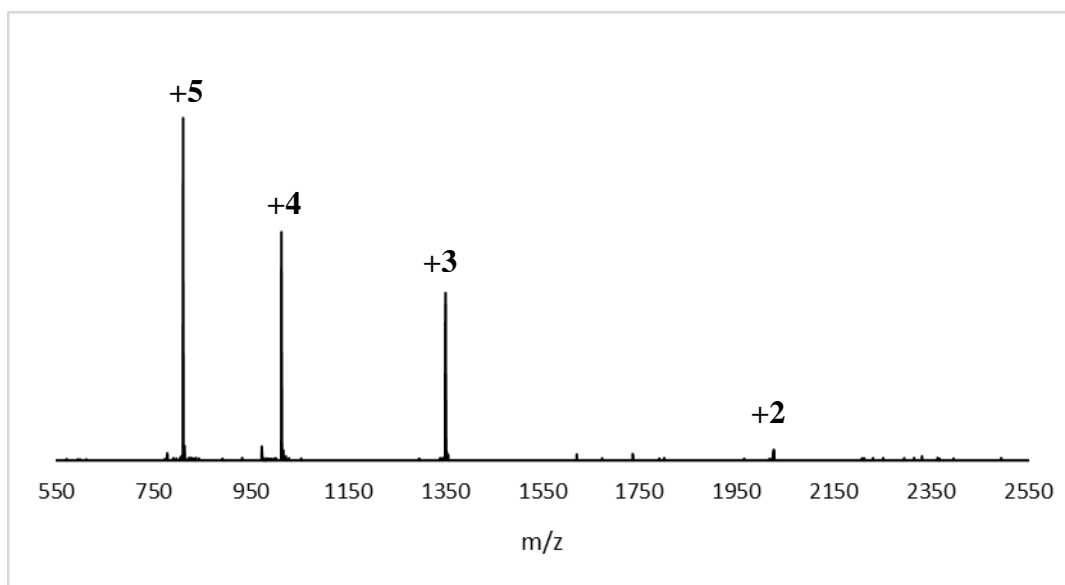
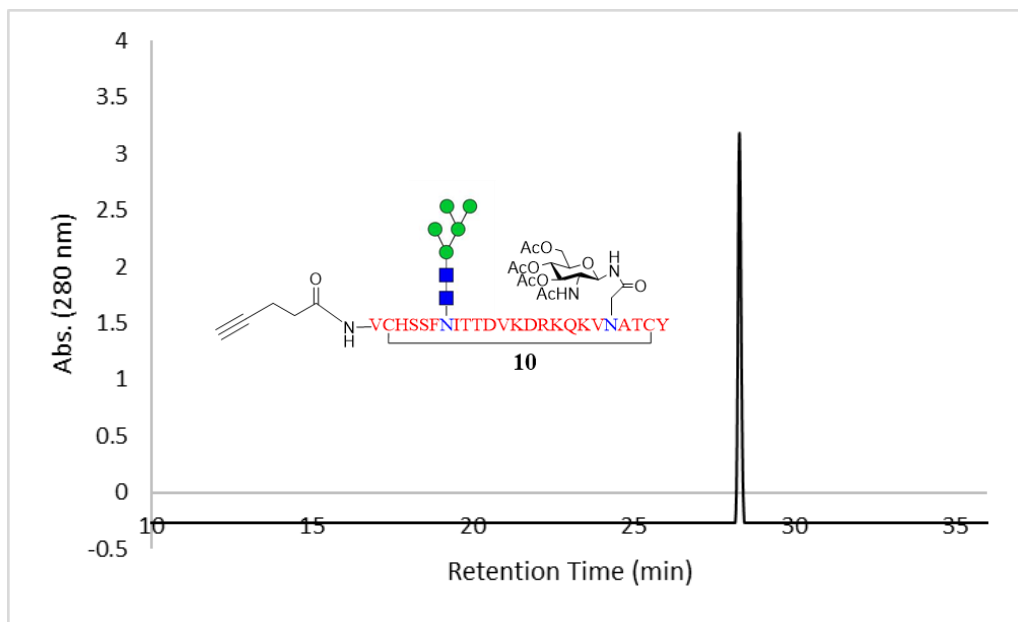


Figure A 4.5 HPLC and ESI-MS characterization of linear glycopeptide **10^a**



^aHPLC runs were performed on a C18 column with a linear gradient of 5-50% MeCN in 30 min.

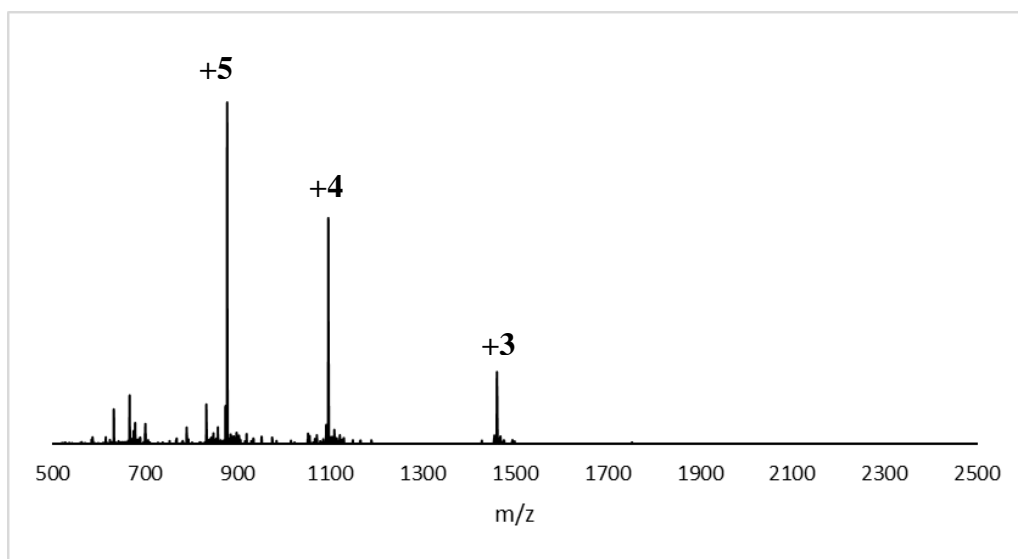
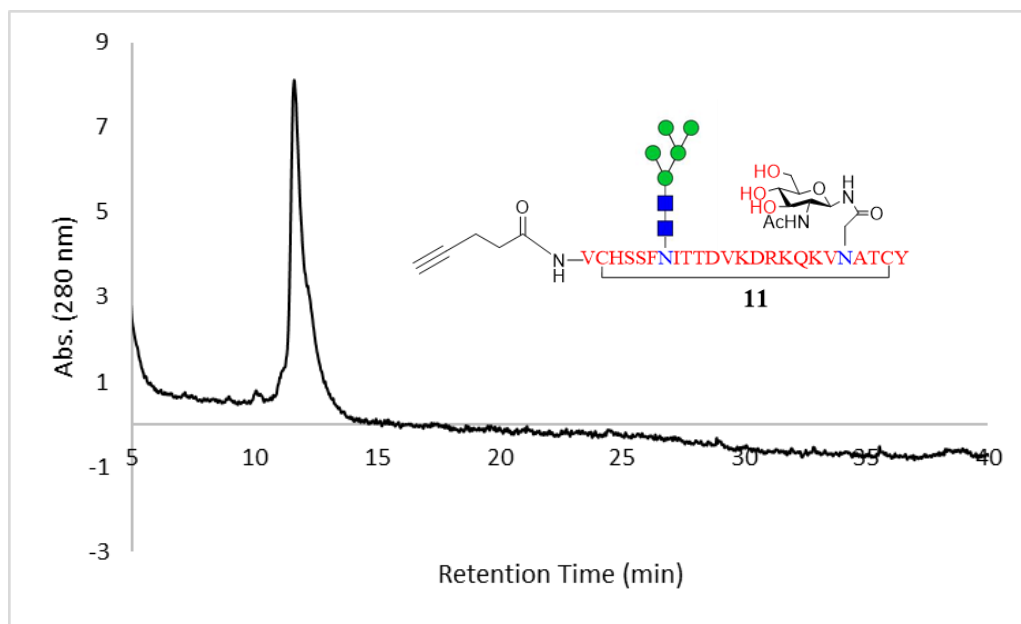


Figure A 4.6 HPLC and ESI-MS characterization of linear glycopeptide **11^a**



^aHPLC runs were performed on a C18 column with a linear gradient of 5-25% MeCN in 30 min.

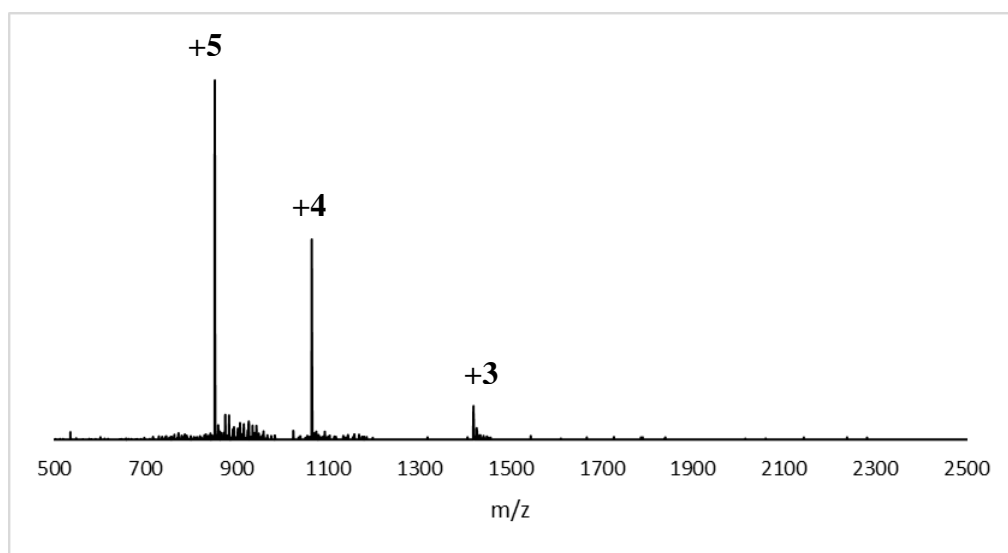
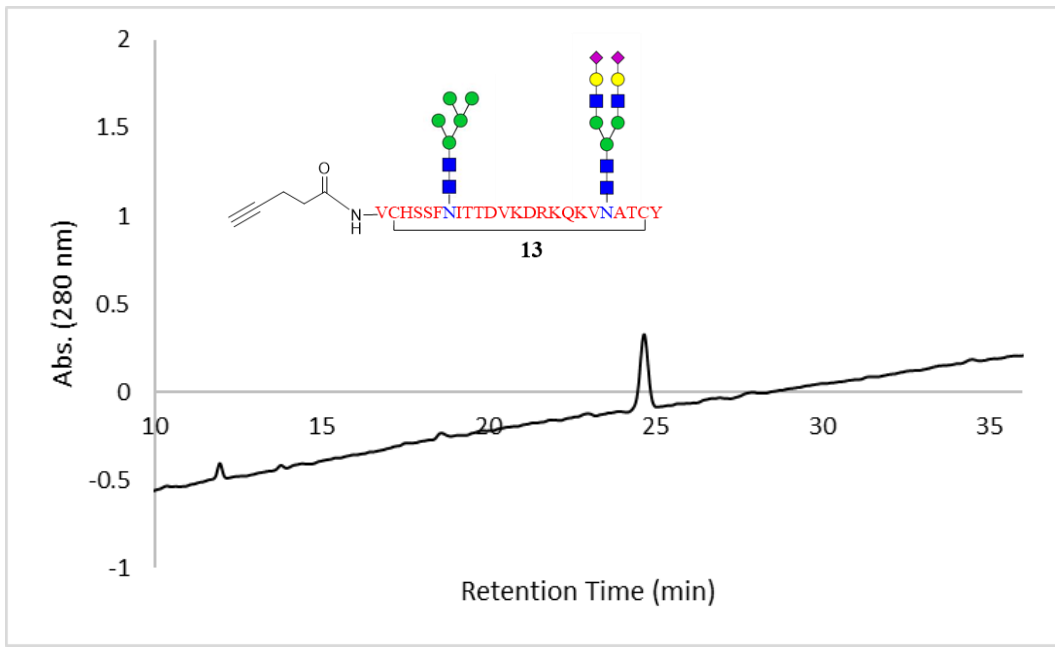
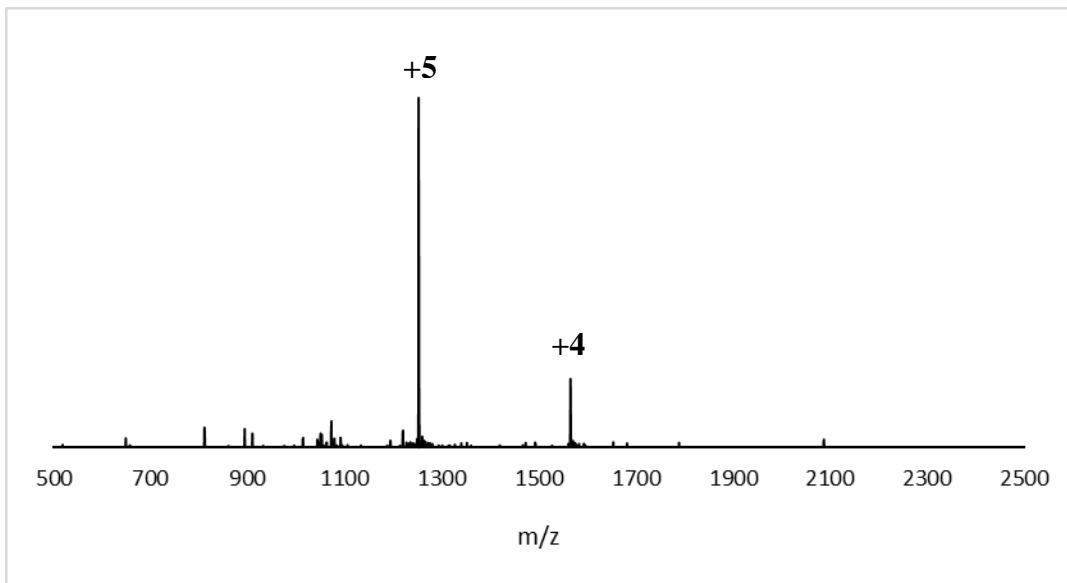


Figure A 4.7 HPLC and ESI-MS characterization of linear glycopeptide 13^a



^aHPLC runs were performed on a C18 column with a linear gradient of 5-50% MeCN in 30 min.



A.5.1 Chapter 5: Supplementary Material

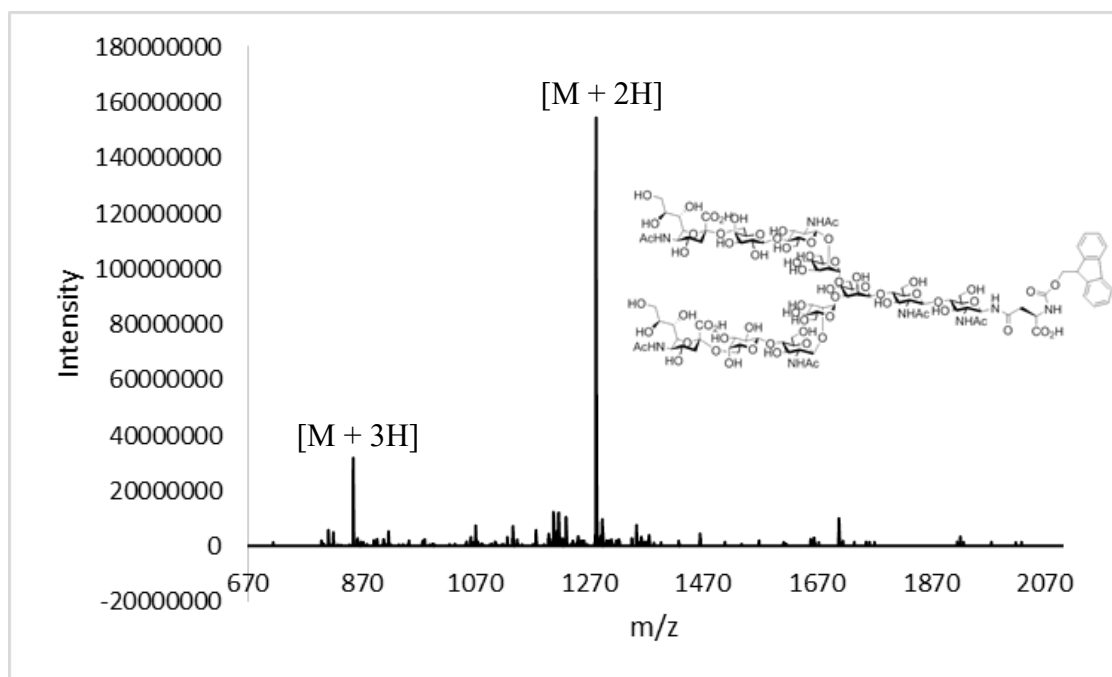


Figure A 5.1 MALDI-TOF analysis of compound **8**.

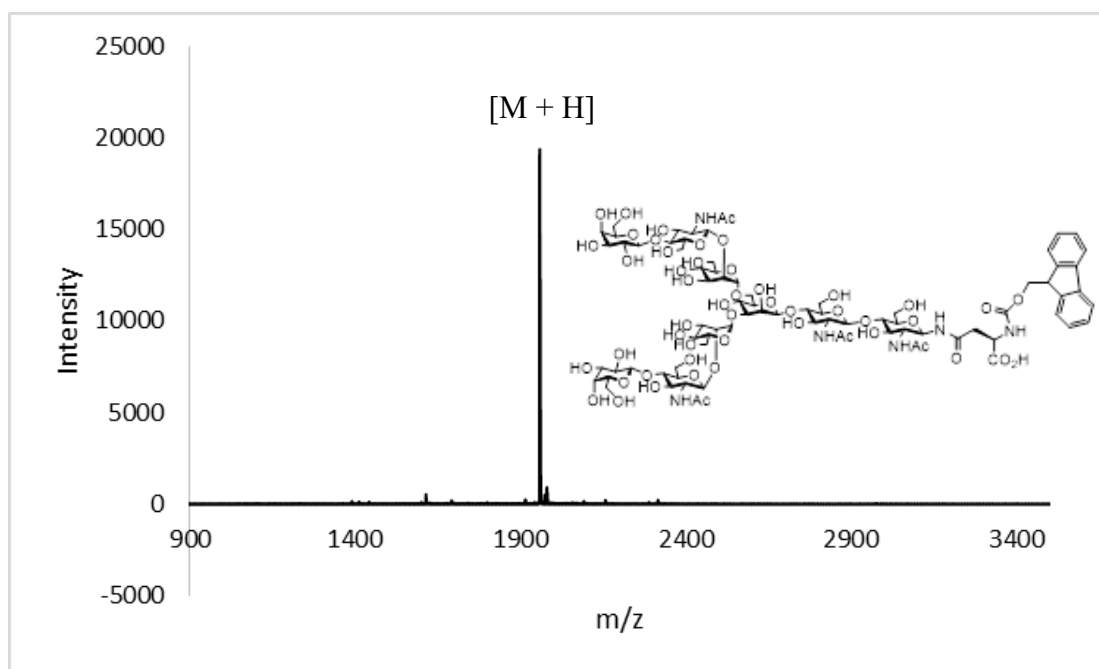


Figure A 5.2 MALDI-TOF analysis of compound **9**.

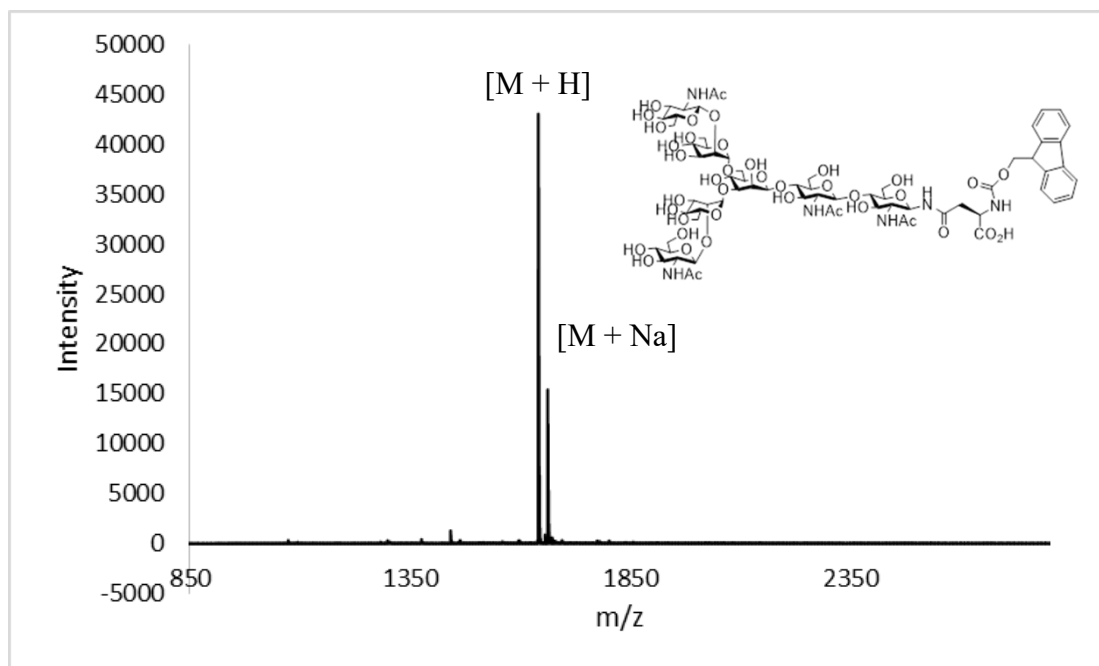


Figure A 5.3 MALDI-TOF analysis of compound **10**.

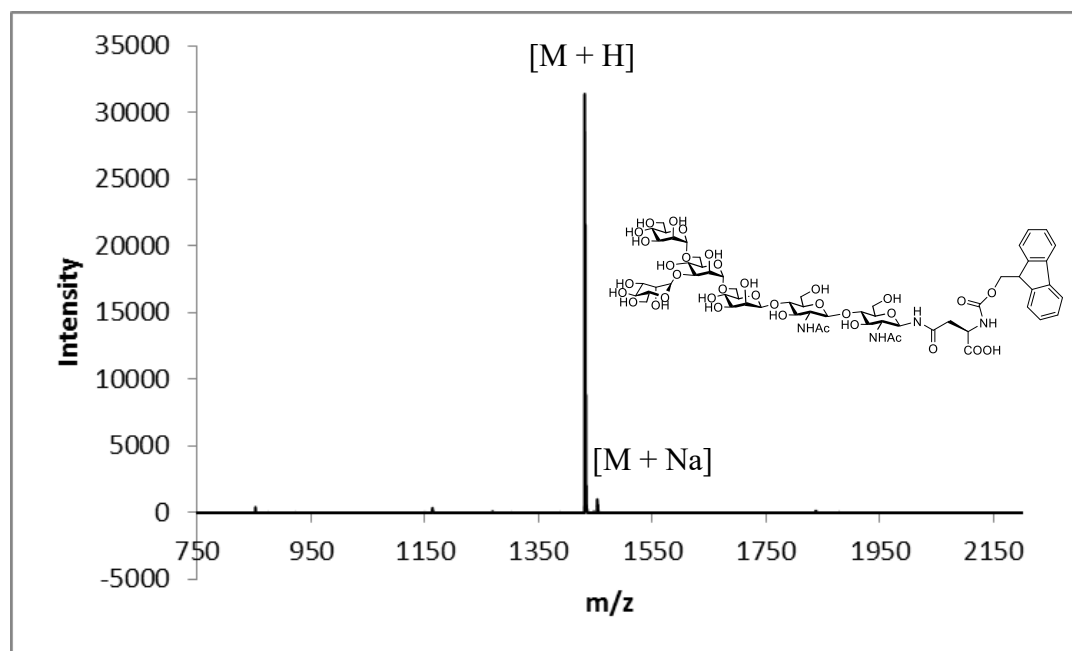


Figure A 5.4 MALDI-TOF analysis of compound **11**.

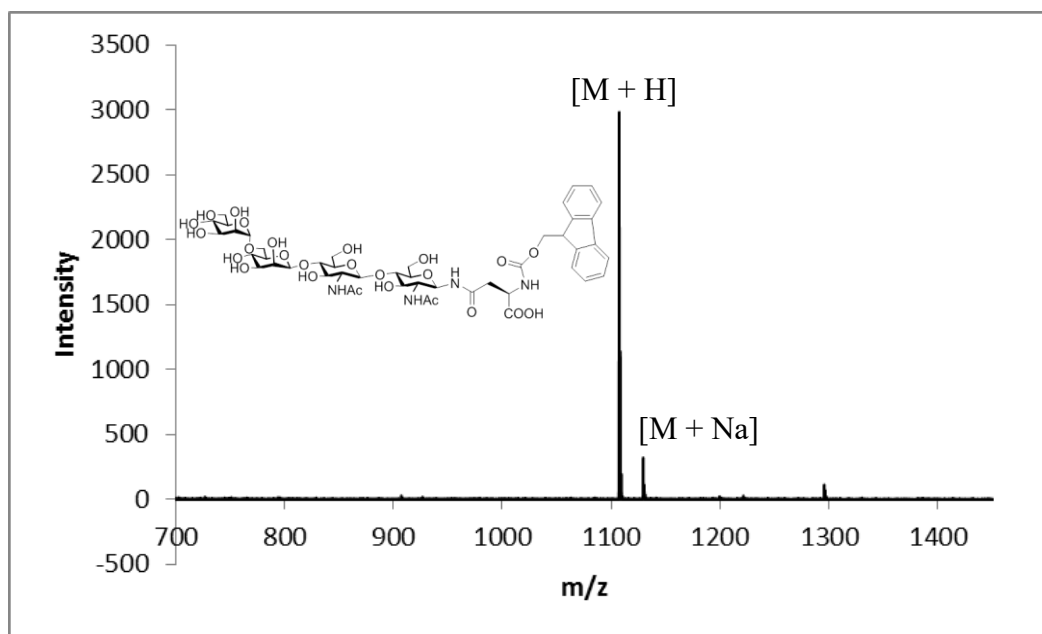


Figure A 5.5 MALDI-TOF analysis of compound 12.

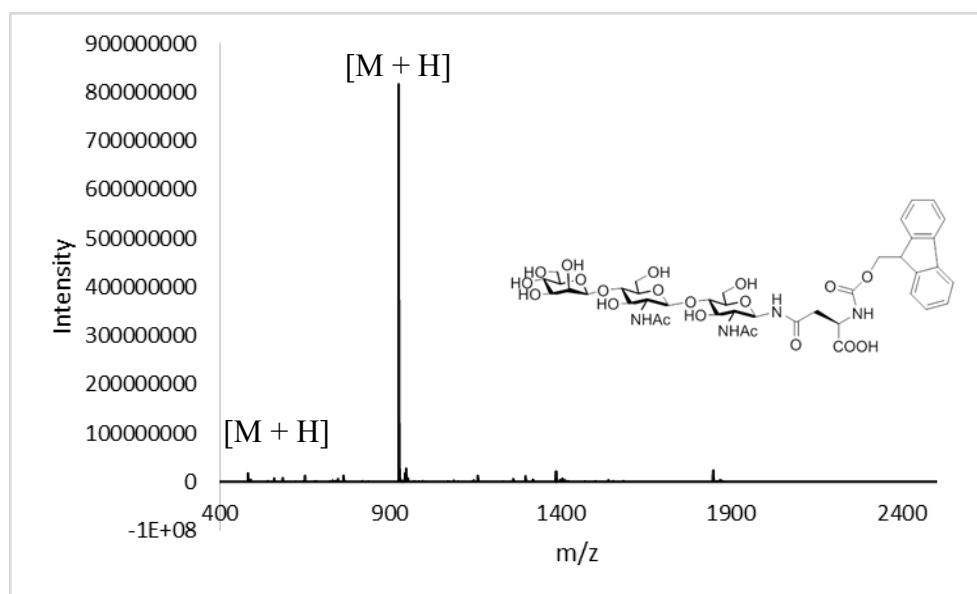


Figure A 5.6 MALDI-TOF analysis of compound 13.

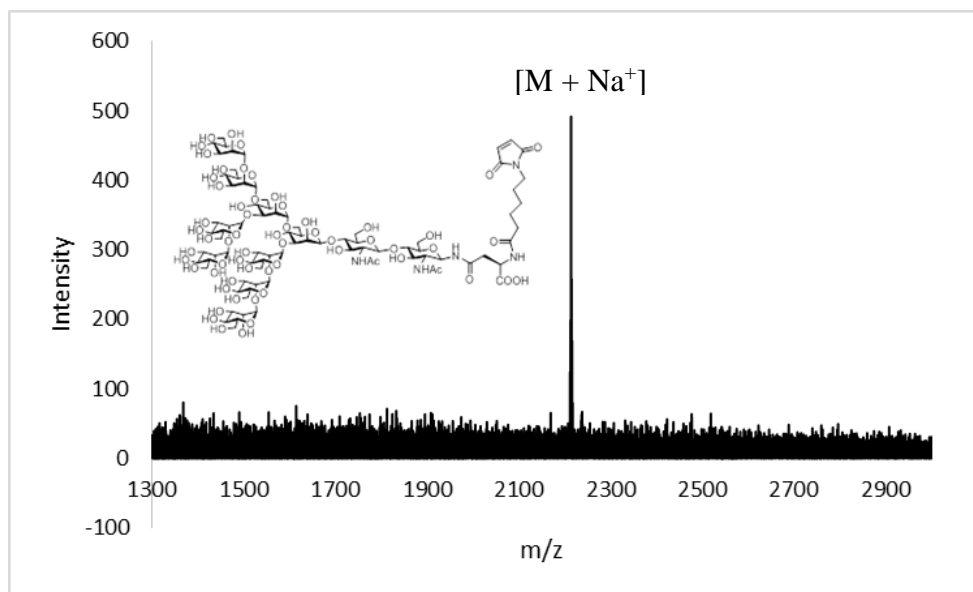


Figure A 5.7 MALDI-TOF analysis of compound **14**.

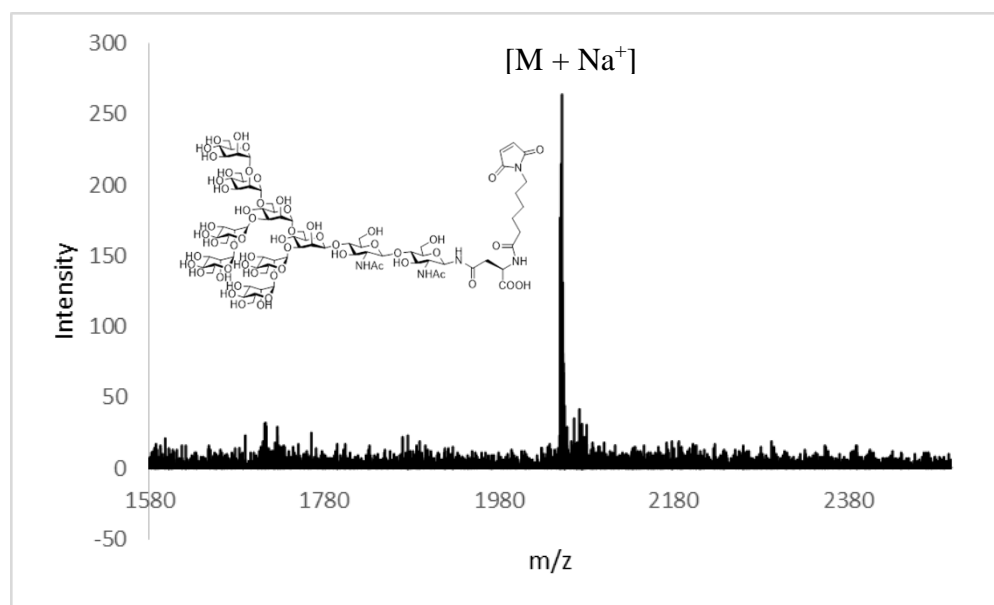


Figure A 5.8 MALDI-TOF analysis of compound **15**.

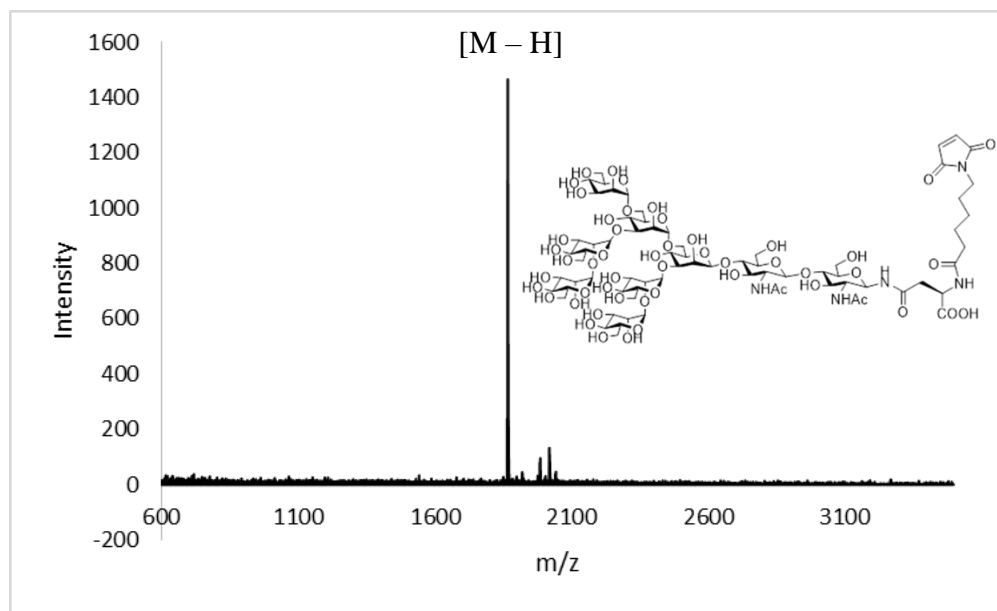


Figure A 5.9 MALDI-TOF analysis of compound 16.

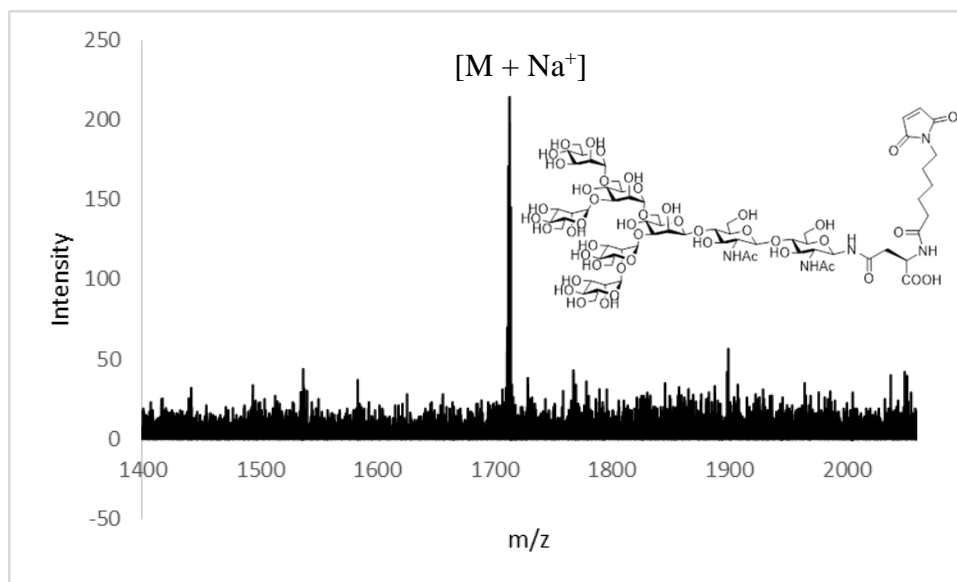


Figure A 5.10 MALDI-TOF analysis of compound 17.

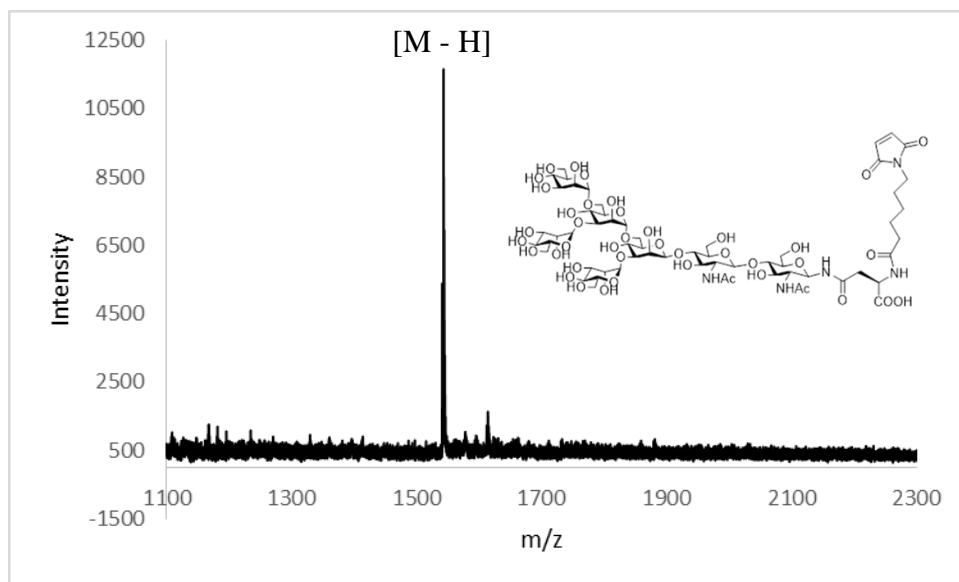


Figure A 5.11 MALDI-TOF analysis of compound **18**.

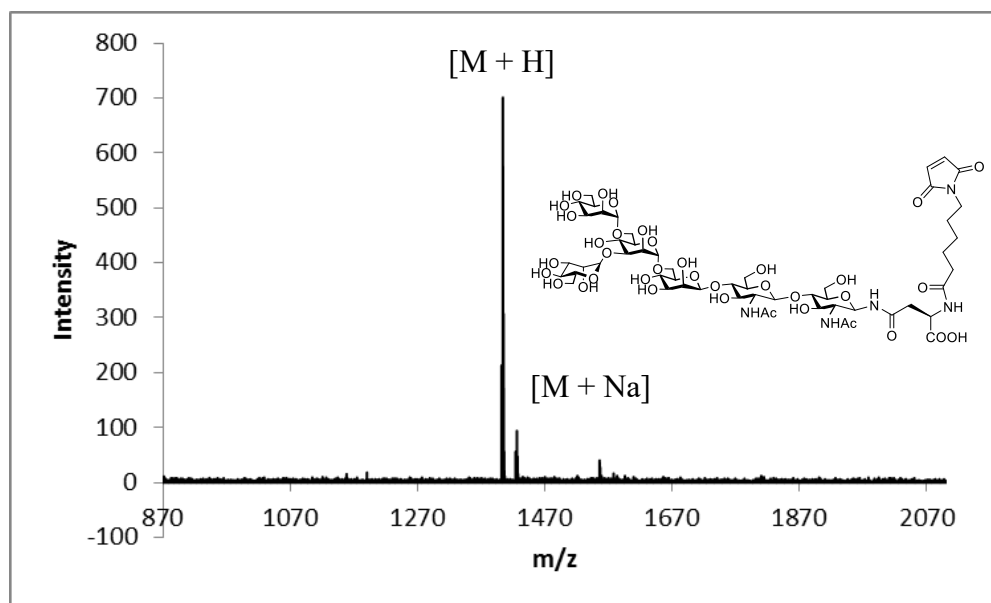


Figure A 5.12 MALDI-TOF analysis of compound **19**.

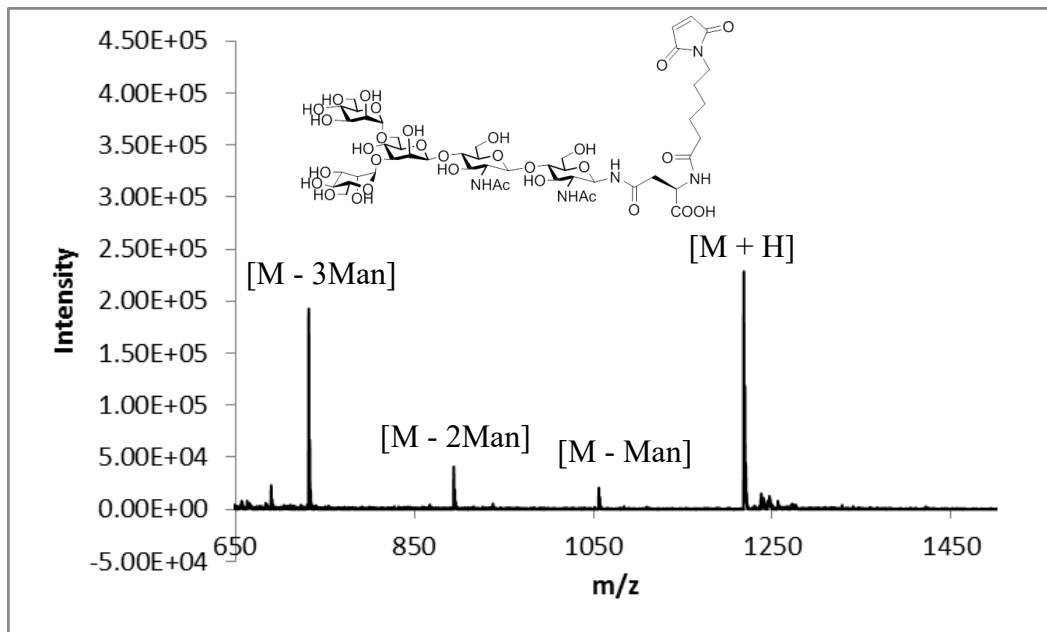


Figure A 5.13 MALDI-TOF analysis of compound 20.

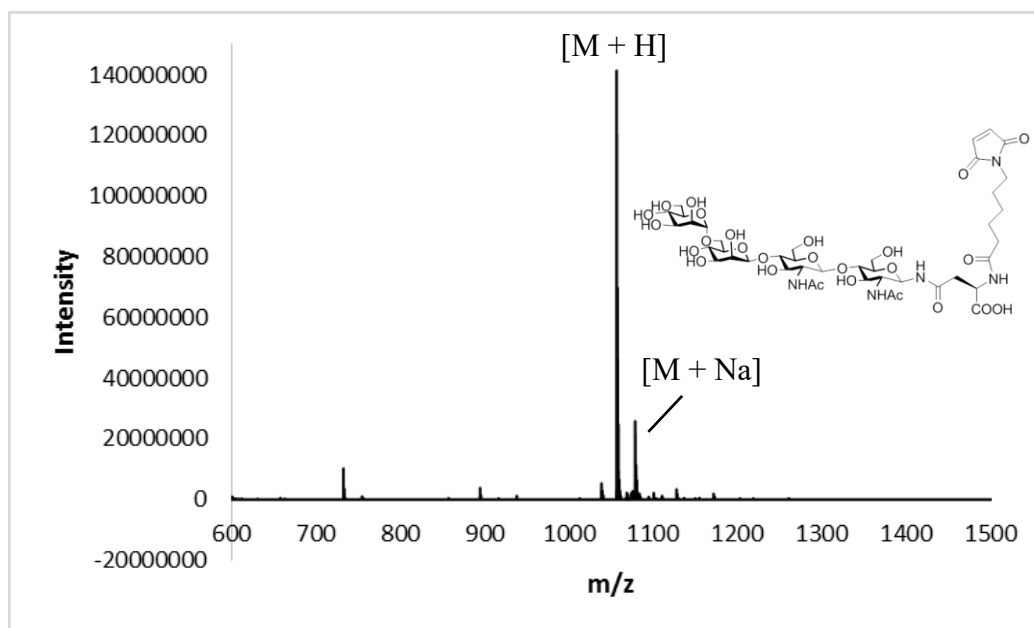


Figure A 5.14 MALDI-TOF analysis of compound **21**.

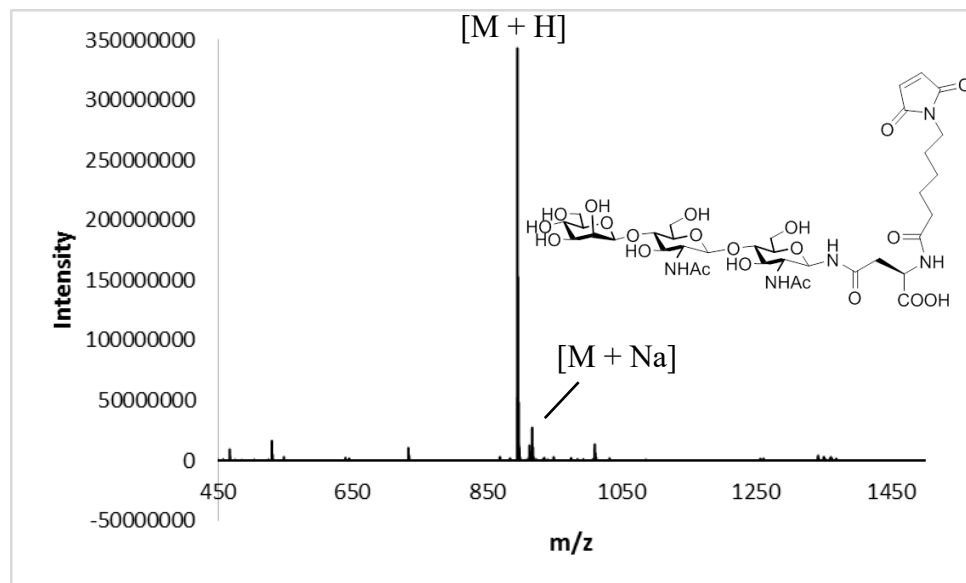
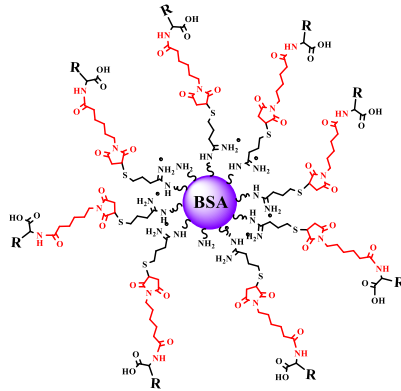


Figure A 5.15 MALDI-TOF analysis of compound **22**.



Glycan Composition	23	24	25	26	27	28	29	30	31
R =									
% Glycan Loading on BSA	14%	14%	14%	15%	11%	13%	12%	13%	13%

Table A 5.1 Quantification of oligomannose glycan loading on BSA-glycan conjugates.

A.6.1 Chapter 6: Supplementary Material

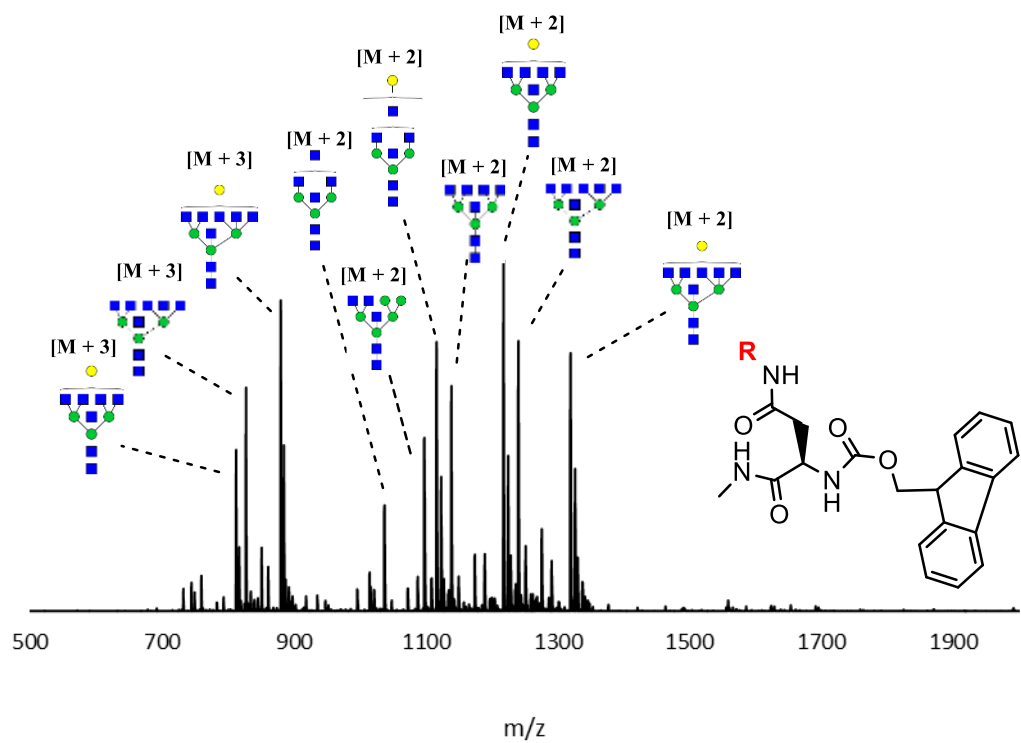


Figure A 6.1 ESI-MS (Orbitrap) Profile of enriched, Fmoc-tagged highly-branched ovalbumin glycans.

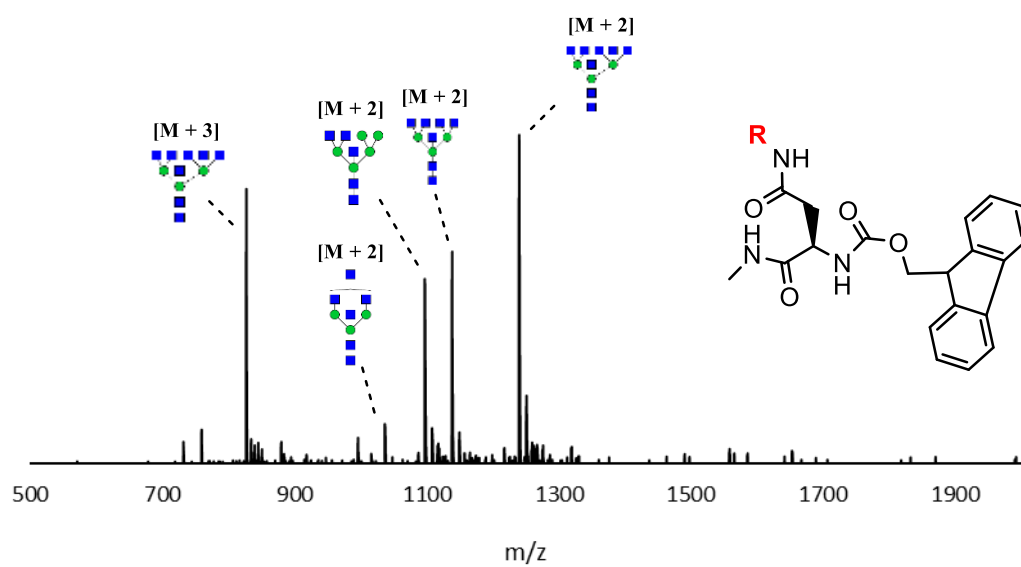


Figure A 6.2 ESI-MS (Orbitrap) Profile of Fmoc-tagged GlcNAc-terminal highly-branched ovalbumin glycans.

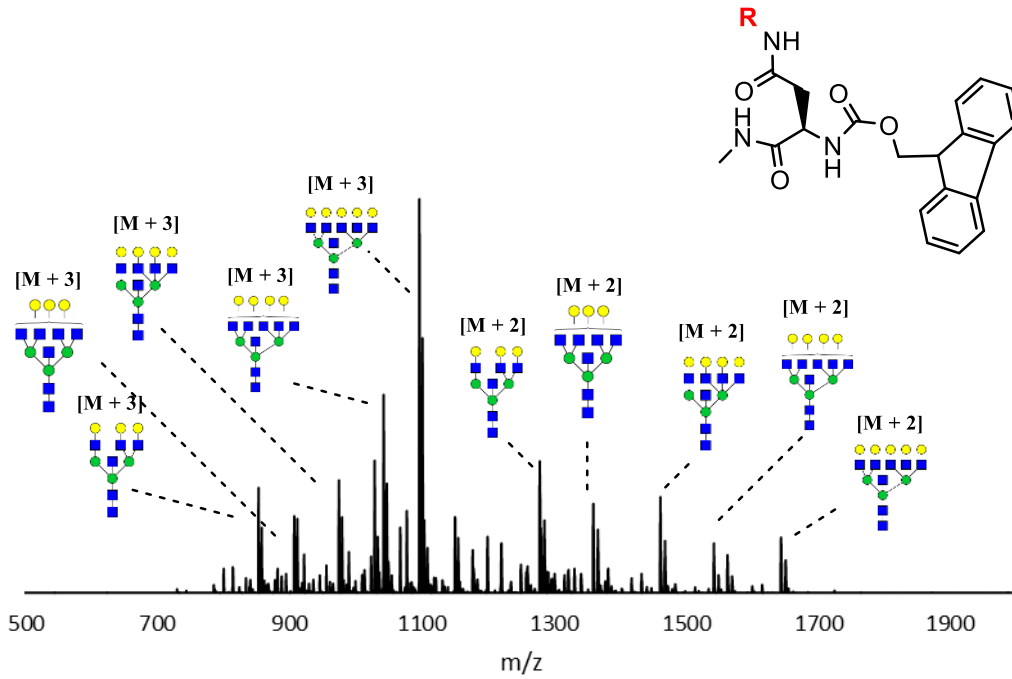


Figure A 6.3 ESI-MS (Orbitrap) Profile of Fmoc-tagged Gal-terminal highly-branched ovalbumin glycans.

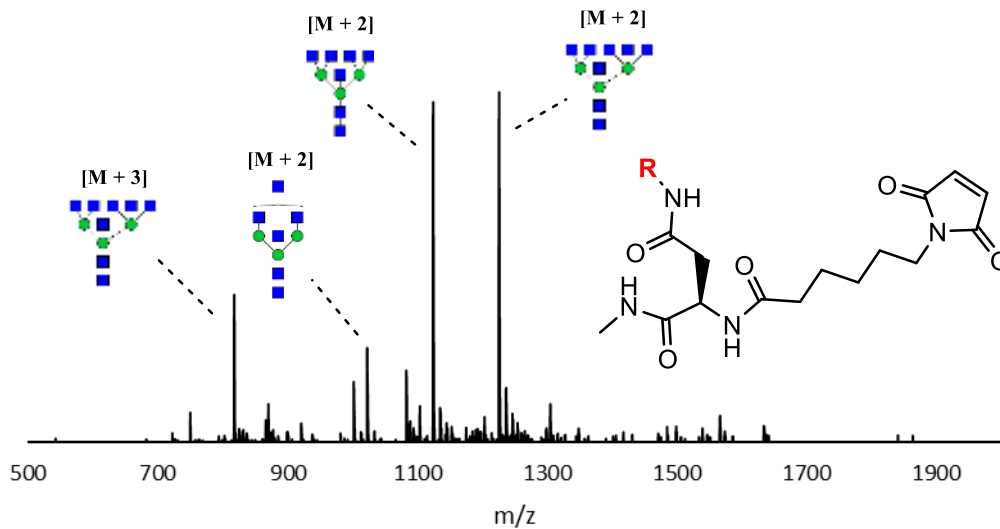


Figure A 6.4 ESI-MS (Orbitrap) Profile of Maleimide-tagged GlcNAc-terminal highly-branched ovalbumin glycans.

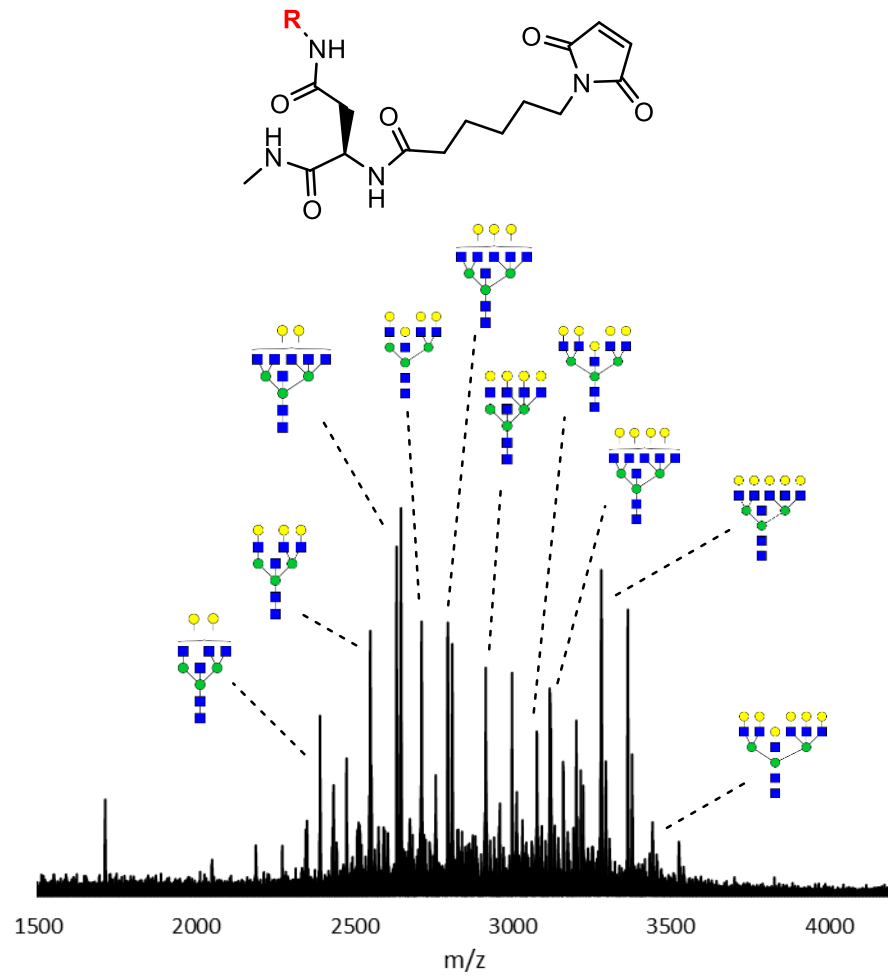


Figure A 6.5 MALDI-TOF Profile of Maleimide-tagged Gal-terminal highly-branched ovalbumin glycans.

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Chapter 1

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Chapter 2

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Chapter 3

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