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Modified Carbohydrate Cancer Vaccines for Breaking Immune Self-Tolerance

by

Isaac Andrew Miller

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Chemistry

in the

Graduate Division

of the University of California, Berkeley

Committee in charge:

Professor Carolyn R. Berozzi, Chair
Professor Matthew B. Francis
Professor David H. Raulet

Fall 2010

Abstract

Modified Carbohydrate Cancer Vaccines for Breaking Immune Self-Tolerance

by

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Doctor of Philosophy in Chemistry

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Professor Carolyn R. Bertozzi, Chair

The unusual glycosylation patterns on cancer cells, which harbor carbohydrate structures that are rarely seen on healthy tissue, provide a tempting target for the design of anti-cancer vaccines for immunotherapy. Unfortunately, carbohydrates tend to be poor immunogens. Much of the work in the carbohydrate-based cancer vaccine field is focused on boosting the immune system's response to these antigens. Scientists have had moderate success in this endeavor with strategies that include adding T-cell epitopes to the antigens, incorporating adjuvants in the vaccine injection mixture or directly conjugated to the vaccine, and priming immune cells outside of the body and re-injecting them, but no breakthrough strategy has emerged. Despite all this effort, to date no carbohydrate-based cancer vaccine has won approval from the Food and Drug Administration, and only one peptide-based cancer vaccine has been approved.

Chapter 1 describes some of the strategies that have been pursued in the quest to develop effective cancer immunotherapies, particularly those based on

carbohydrate antigens. Later in the chapter, I introduce a new strategy to break immune self-tolerance based on vaccines built from modified carbohydrate antigens, and discuss some of the precedent established by our laboratory and others that suggests the new strategy is viable. In Chapter 2, I describe the synthesis of a panel of four vaccine constructs based on the disaccharide sialyl Tn. Then, I discuss the immune response elicited against the vaccines in rabbits based on ELISA analysis of their polyclonal antisera.

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*This dissertation is dedicated to my daughter*

*Lillian Marie Miller*

*who made it oh so difficult to write*

*and to my wife*

*Elise Miller*

*who is always right*

**Modified Carbohydrate Cancer Vaccines for Breaking Immune Self-Tolerance**

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# Chapter 1: Vaccines and cancer

## Introduction

Since 1796 when Edward Jenner first demonstrated that inoculating patients with cowpox virus protects them against smallpox, the power of vaccines has been proven time and again. By harnessing the killing power of the immune system and directing it against dangerous pathogens, vaccines have virtually eliminated many once great scourges such as smallpox and polio. Other pathogens such as human immunodeficiency virus (HIV) have largely thwarted vaccination attempts by their ability to quickly reconfigure themselves to avoid immune detection.[1] Still, along with antibiotics, vaccines stand at the top of the list of the greatest successes in medicine.

While vaccines have found most of the their success against disease-causing pathogens, scientists have recognized their potential to treat other diseases including cancer. In principle, if cancer cells could be specifically targeted and killed by the immune system, a process called immunotherapy, disease could be eliminated while healthy tissues are left unharmed. Current cancer treatments such as surgery, chemotherapy, and radiation inevitably kill healthy tissues along with cancerous ones, causing side effects that can be painful and even debilitating. At the same time, these treatments often leave some cancerous cells alive, which can cause recurrences of the disease. The immune system can potentially overcome both of these problems. It is capable of remarkable specificity and is able to distinguish

between subtle changes in antigens. Antibodies and immune cells pervade virtually the entire body, so the immune system could potentially eliminate every cancer cell, including metastases far away from the main tumor site. Unfortunately, these capabilities have not yet come anywhere close to fruition. To date, the Food and Drug Administration (FDA) has approved only one true active specific vaccine that treats cancer: Provenge (sipuleucel-T) made by Dendreon (Figure 1-1).[2] Provenge consists of a patient's own autologous peripheral-blood mononuclear cells (PBMCs), which include antigen presenting cells (APCs), that have been treated *ex vivo* with a fusion protein of prostatic acid phosphatase (PAP), a prostate antigen, and granulocyte-macrophate colony-stiumulating factor (GM-CSF), an immune activator. Clinical trials indicate that Provenge adds a median of four months to the lives of certain therapy-resistant prostate cancer patients,[3] and costs $93,000 for 3 treatments.[2]

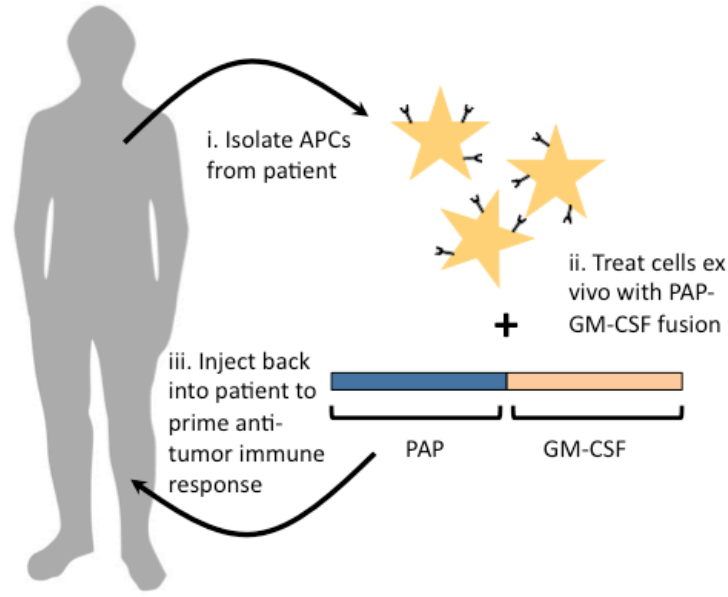



**Figure 1-1.** Provenge is the first FDA approved active cancer immunotherapy. It consists of i. isolation of PBMCs from a patient, which contain antigen presenting cells (APCs), then ii. *ex vivo* treatment of those cells with PAP-GM-CSF, a prostate antigen-immune activator fusion protein, and finally iii. injection of those cells back into the patient.

There are many reasons why, after years of research, only one moderately effective cancer vaccine has been approved by the FDA and only in the few months before the filing of this dissertation. One difficulty is that cancer cells have found ways to attenuate the immune system in order to avoid being killed.[4] Another problem is that many cancer patients who have been given experimental immunotherapy have had their immune systems ravaged by disease and cancer therapies. Most significantly, targeting cancer cells over healthy cells is particularly challenging. While cancers cells are rogues whose built-in growth checks have failed, they still originate from the body's own cells. Only a few of the antigens on the surfaces of cancer cells are specific to cancer, and most of these can be found in

small amounts on healthy tissue. Because of this, the immune system may recognize these antigens as "self" and so not attack the cancer cells bearing them. Much of the work in the cancer vaccine field focuses on overcoming this problem of self-tolerance.

## Cancer immunotherapy

### *Considerations in antigen selection*

The first step in a cancer vaccine strategy is to choose an antigen or antigens to target. An ideal antigen for immunotherapy is found exclusively on cancer cells, its expression cannot be turned off by the tumor to avoid recognition, and it is capable of eliciting a potent immune response. The antigen can be a protein, as in the case of Provenge, or a carbohydrate. In Provenge, the target PAP is a prostate-specific enzyme that is found in increased levels on prostate cancers.[2] PAP is not exclusively a tumor-associated antigen (TAA) since it is found on healthy prostate tissue. In practice, however, PAP acts as an exclusive TAA since, in the case of prostate cancer, the entire gland is often removed surgically, leaving any antigen specific to the prostate as an exclusive TAA. While studies have shown that certain cancer patients have humoral and cellular immune recognition of PAP, it is not on its own capable of eliciting an effective immune response.[5] This may be due to tolerance of "self" antigens or to the cancer modulating the immune response, but to achieve an effective immune response, researchers muct fuse it to GM-CSF and load it *ex vivo* onto a patient's own APCs.

*Carbohydrates as specific cancer antigens*

Unlike with prostate cancer, we do not have the luxury of targeting any organ-specific antigen with most other cancers, because most organs cannot be removed from a patient. Since autoimmune attack against the whole organ must be avoided, finding suitable targets that are exclusive to cancer cells becomes more of a challenge. One class of molecules that has shown promise for the selective targeting of cancer is carbohydrates.[6] The surface of mammalian cells is decorated with structurally diverse glycans that can be attached to lipids and proteins. These glycans play important physical roles, such as maintaining a slippery hydrated surface in epithelial cells, as well as chemical roles like mediating cell-cell adhesion and ligand-receptor binding.[7] Glycosylation is the most common post-translational modification, and it has been estimated that over 50 percent of proteins are glycosylated.[8] Glycans are formed in cells by a series of glycosyltransferases and glycosidases that act on the growing glycan. Unlike proteins and nucleic acids, which are built from templates, glycans are made by enzymes arranged spatially in the endoplasmic reticulum and Golgi compartments. Because of the way they are assembled, glycans often change as the cellular state changes. An important example of the phenomenon is with cancer.

The first report of the change in glycan structure on cancer cells came in 1969 when Wu et al., using a radiographic labeling technique, discovered that cells transformed with an oncogenic virus had different ratios of sugars on membrane-

bound proteins than the non-transformed cells.[9] Since then, multiple studies have shown that cancer cells have aberrant glycosylation. Commonly, carbohydrates on the cell surface are upregulated in general.[10] Certain glycan structures seen on cancer cells are not seen in healthy tissues, or are seen only in small amounts or at certain stages of development.[6] These antigens are known as tumor-associated carbohydrate antigens (TACAs). One sugar in particular that is a component of many of these TACAS and is often found upregulated in cancer cells is sialic acid. Sialic acid levels have been found to be elevated in colon,[11,12] pancreatic,[13] gastric,[14,15] breast,[16] liver,[17] kidney,[18] prostate,[19] and lung cancer,[20] as well as in leukemia.[21,22] Further, the degree of sialylation has been correlated to the metastatic potential of the cancer.[23,24] Studies have shown that these changes in glycan structure can be functional to the cells, which may indicate that turning off the expression of TACAs could be detrimental to the cancer cell. Glycans have long been known to play both structural[25,26] and signaling[27,28] roles in cells. The increase in cell surface carbohydrates on cancer cells may actually help that cell avoid immune detection, since sugars do not tend to invoke strong immune responses.[29] These changes in surface carbohydrates may also be involved in metastasis, making the cells more “slippery” at their site of origin, but facilitating extravasation through blood vessels at a distant site through interactions of specific carbohydrate structures with recognition proteins on blood vessels.[30,31]

*Carbohydrates in vaccines*

The idea of using carbohydrates as vaccine components likely began sometime around 1923 when it was discovered that the antigens of pneumococcus targeted by the immune system were capsular polysaccharides.[32] In 1929, it was shown that immunization with pneumococcal polysaccharides could elicit antibodies that would recognize a range of pneumococci species,[33] and later in 1950, that this immunization could lead to persistent immunity against the bacteria.[34] Despite this finding, it took another 33 years before the first carbohydrate-based vaccine would become commercially available.

This long delay between the observations that carbohydrates can be targeted by the immune system and the development of carbohydrate-based vaccines could be attributable to a combination of the overwhelming interest in antibiotics during this period, which took some of the focus away from vaccine research, along with some of the difficulties in creating vaccines from carbohydrates.[10] The greatest difficulty in developing a carbohydrate-based vaccine is that carbohydrates tend to elicit poor antibody responses. This is partly due to the inability of carbohydrates to activate $CD4^+$ (helper) T-cells.[6] When a typical immunogenic protein antigen is detected by the immune system, it is engulfed by APCs and processed into fragments that are packaged onto the APC's surface major histocompatibility complex (MHC) molecules (Figure 1-2). These MHC molecules require a peptide antigen. Once bound, these fragments are able to activate $CD4^+$ T-cells whose T-cell receptor recognizes that particular fragment. When these T-cells are activated, they can

subsequently activate B-cells that recognize the same immunogenic protein. Activation of B-cells by helper T-cells changes the B-cells into antibody-spewing plasma cells, and this augments the production of specific antibodies against the antigen. Activation of helper T-cells also directs the immune system to begin producing more of a class of antibodies called immunoglobulin G (IgG). For these reasons, activation of helper T-cells is critical to the establishment of a potent immune response. Carbohydrates that cannot activate them often evoke only a comparatively weak immune response characterized mostly by immunoglobulin M (IgM) antibodies. This type of response tends to be short-lived and ineffective compared to a full blown $CD4^+$ mediated immune response characterized by high concentration of specific IgG antibodies.[10]

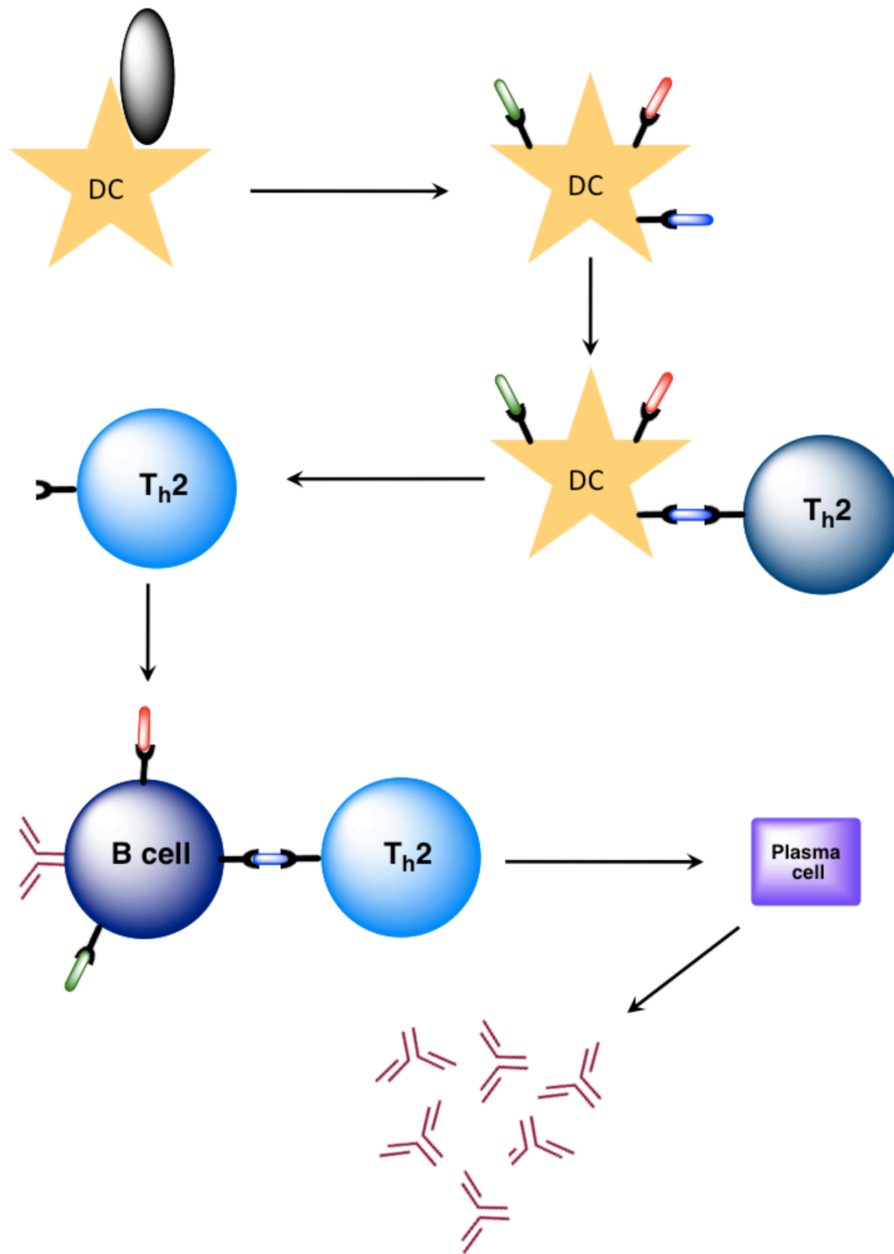



**Figure 1-2.** Mechanism of humoral immunity activation in a typical response to an immunogenic protein. The protein is first ingested by a dendritic cell (DC), which processes it to peptides that the DC displays on MHC molecules on its surface. These peptides are recognized by the T-cell receptor of a helper ($T_h2$) cell, which becomes activated. The activated T-cell can activate a B cell whose antibody recognizes the same peptide, converting it to an antibody-producing plasma cell. With few exceptions, free carbohydrates cannot activate this pathway because they cannot be displayed by DCs.

While it is difficult to coax the immune system to mount a strong response against a carbohydrate antigen, it is not impossible. Certain zwitterionic polysaccharides are able to bind to MHC complexes without a peptide component,[35] and other methods exist to augment an immune response. It was first shown in 1939 that conjugating a carbohydrate to a protein can increase its immunogenicity.[36] In 1983, Merck debuted PneumoVax™, the first commercially available carbohydrate-based vaccine. Pneumovax is a capsular polysaccharide-based pneumonia vaccine that is still available today in an updated format. Since that first introduction, numerous carbohydrate-based vaccines have been approved to prevent infectious disease by the FDA or are undergoing trials (Tables 1-1 and 1-2).

| Pathogen | Composition | Manufacturer |
|---|---|---|
| Haemophilus influenzae type b (Hib) | Glycoconjugugate, polysaccharide with tetanus toxoid (TT) | Sanofi Pasteur (ActHIB); GlaxoSmithKline Biologicals (Hiberix) |
| | Diphtheria toxoid (DT), TT and acellular pertussis adsorbed, inactivated poliovirus and Hib–TT conjugate vaccine | Sanofi Pasteur (Pentacel) |
| | Hib conjugate (meningococcal protein conjugate) | Merck & Co (PedvaxHIB) |
| | Hib conjugate (meningococcal protein conjugate) and hepatitis B (recombinant) vaccine | Merck & Co (Comvax) |
| Neisseria meningitidis A, C, Y and W-135 | Glycoconjugate, meningococcal polysaccharide with DT | Sanofi Pasteur (Menactra) |
| | Meningococcal polysaccharide | Sanofi Pasteur (Menomune-A/C/Y/W-135) |
| Salmonella typhi | Vi capsular polysaccharide | Sanofi Pasteur (TYPHIM Vi) |
| Streptococcus pneumoniae 4, 6B, 9V, 14, 18C, 19F and 23F | Pneumococcal polysaccharide 7-valent–CRM197 conjugate | Wyeth Pharmaceuticals (Prevnar) |
| Streptococcus pneumoniae 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F | Pneumococcal polysaccharide, 23-valent | Merck & Co (Pneumovax 23) |

**Table 1-1.** FDA approved carbohydrate-based vaccines.

| Pathogen | Composition | Phase |
|---|---|---|
| Enterohaemorrhagic Escherichia coli | O-specific polysaccharide–protein conjugate | Phase I |
| Group A Streptococcus spp. | Glycoconjugate of Group A polysaccharide with TT | Preclinical |
| Group B Streptococcus spp. | Glycoconjugates of type Ia, Ib, II, III and V polysaccharides linked to carrier proteins | Phase II |
| Haemophilus influenzae (non-typeable) | Subunit-detoxified lipooligosaccharide conjugate | Preclinical |
| Pseudomonas aeruginosa | Octavalent glycoconjugate of O-polysaccharide with toxin A | Phase III |
| Salmonella typhi | rEPA–Vi conjugate vaccine | Phase III |
| Shigella dysenteriae | O-specific polysaccharide–protein conjugate | Preclinical |
| Shigella flexneri | O-specific polysaccharide–protein conjugate | Phase II |
| Shigella sonnei | O-specific polysaccharide–protein conjugate | Phase III |
| Streptococcus pneumoniae | Glycoconjugates of synthetic 6B polysaccharide motifs | Preclinical |
| Vibrio cholerae | Lipopolysaccharide–protein conjugate | Phase I |
| Aspergillus fumigatus | β-Glucan–CRM197 conjugate | Preclinical |
| Candida albicans | Cell surface oligomannosyl epitope (various conjugates) | Preclinical |
| | β-glucan–CRM197 conjugate | Preclinical |
| Cryptococcus neoformans | Glycoconjugate of capsular polysaccharide with TT | Phase I |
| | β-glucan–CRM197 conjugate | Preclinical |
| Leishmania spp. | Lipophosphoglycan | Preclinical |
| | Lipophosphoglycan conjugates | Preclinical |
| Plasmodium falciparum | Glycosylphosphotidylinositol–KLH conjugate | Preclinical |
| HIV-1 | Man α(1→2)Man oligomannosyl epitope (various conjugates, engineered yeast strains and modified glycoproteins) | Preclinical |
| Breast cancer | Unimolecular hexavalent conjugates (Globo H–GM2–Lewis$^{y}$–sTn–TF–Tn–R) | Preclinical |
| | sTn(c)–KLH plus QS-21 as adjuvant | Phase I |
| Epithelial cancer | Globo H–GM2–Lewis$^{y}$–MUC1-32(aa)–sTn(c)–TF(c)–Tn(c)–KLH conjugate vaccine plus QS-21 as adjuvant | Phase I |
| Melanoma | GM3NPhAc–KLH | Preclinical |
| Prostate cancer | Unimolecular hexavalent conjugates (Globo H–GM2–Lewis$^{y}$–sTn–TF–Tn–R) | Preclinical |
| | TF(c)–KLH plus QS-21 as adjuvant | Phase I |
| | Tn(c)–KLH and Tn(c)–palmitic acid | Phase I |
| | Globo H–GM2–Lewis$^{y}$–MUC1-32(aa)–TF(c)–Tn(c)–KLH conjugate vaccine plus QS-21 as adjuvant | Phase II |

**Table 1-2.** Carbohydrate vaccines currently in clinical trials.

## Carbohydrate-based cancer vaccines

Despite the success of carbohydrate-based vaccines for infections diseases, scientists have struggled to effectively enlist the immune system to eliminate cancer cells in the body. There have been few great successes, but the small successes have been enough to keep researchers interested in the topic. The challenges are great: cancers have mechanisms to evade immune responses, cancer patients often have severely degraded immune systems due to the effects of the disease or its treatment, and just one lingering cancer cell is enough to bring about recurrence of the disease. Perhaps the greatest challenge to cancer immunotherapy is the fact that cancer cells are not foreign invaders, but rather the body's own cells, and the immune system develops in each individual so as to protect the individuals own cells. Breaking this self-tolerance, which is the long-range goal of the work described here, requires somehow making a cancer antigen appear foreign to the immune system.

### *The usual approach to breaking immune self-tolerance*

The typical approach to breaking the immune system's tolerance of antigens on cancer cells is to present the antigen in an immunogenic context (Figure 1-3). In this strategy, a tumor-associated carbohydrate antigen (TACA) is coupled via a linker to an immunogenic carrier protein such as keyhole limpet hemocyanin (KLH). This carrier protein serves the dual purpose of initiating an inflammatory response and also providing a peptide scaffold so the carbohydrate can serve as a T-cell antigen and enlist helper T-cells. This TACA-protein conjugate is injected into a

subject, usually with an adjuvant that serves to further stimulate the inflammatory response. The hope is that by presenting cancer antigens to the immune system in the context of immune challenge, the body will come to recognize the antigen as undesired, and mount a strong response.

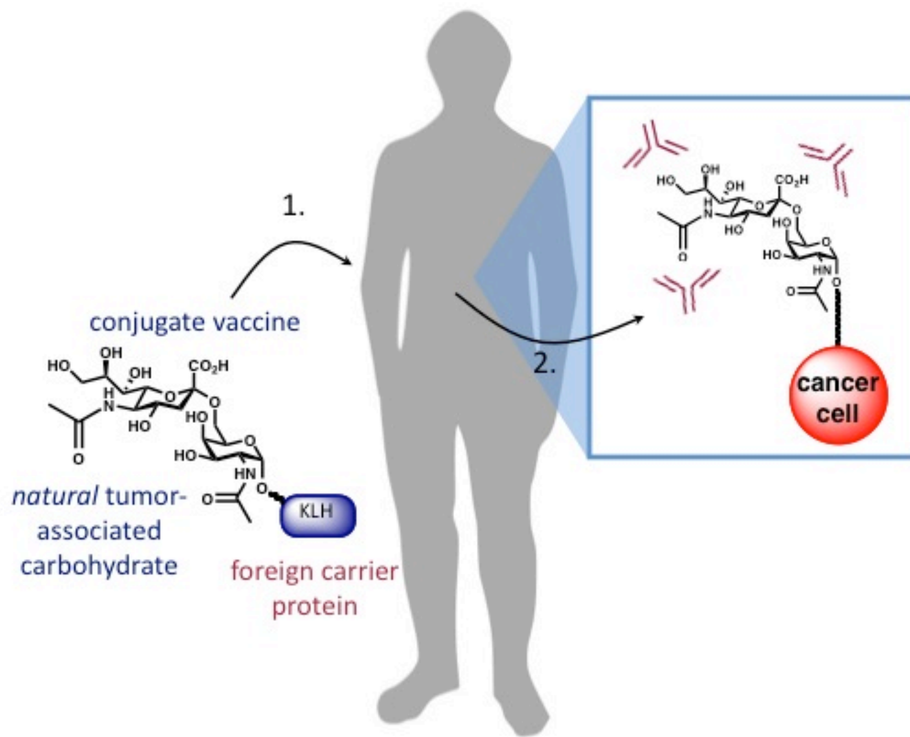



**Figure 1-3.** Traditional cancer vaccine strategy. In this typical cancer vaccine strategy, a cancer antigens such as sialyl Tn is conjugated to an immunogenic proteins such as KLH and injected into a patient. The protein serves as a T-cell epitope and elicits an inflammatory response, which augments the humoral immune response against the antigen.

*Monomeric cancer vaccines*

The first attempts at executing this strategy for cancer immunotherapy consisted of different variations of a monomeric vaccine; that is, many copies of a single TACA were conjugated to an immunogenic carrier protein and used in combination with an adjuvant to immunize mice or people. These studies explored the combinations of carrier protein and adjuvant in order to maximize the antibody response. Many different carbohydrate antigens have been built into carbohydrate vaccines including GM2,[37,38] GD2,[39] and GD3,[40,41] Tn,[42] Tf,[43,44] and globo H,[45] and sialyl Lewis A[46] (Figure 1-4). While these constructs were all capable of eliciting an antibody response to some degree, none has proved itself to be remarkably effective in the clinic.

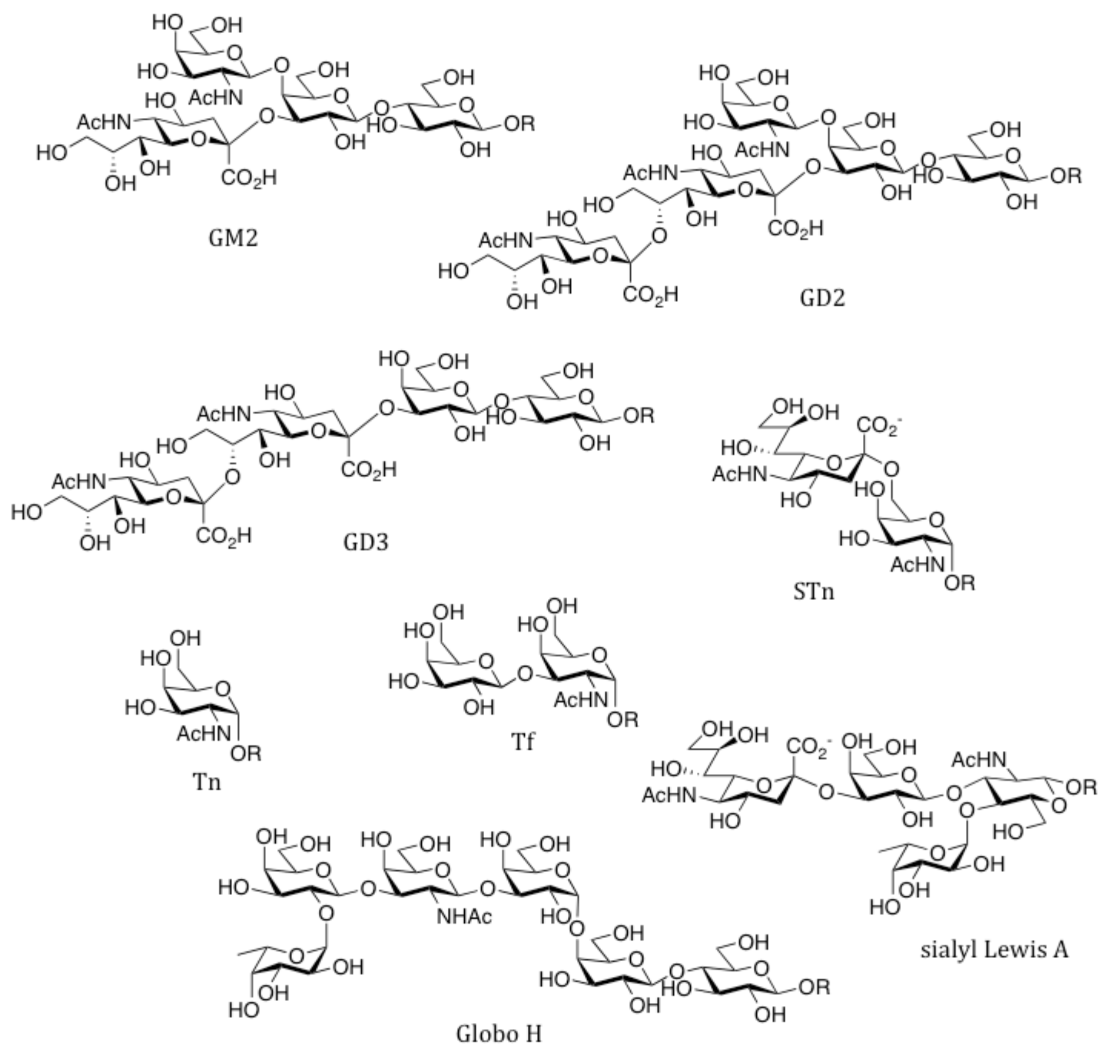
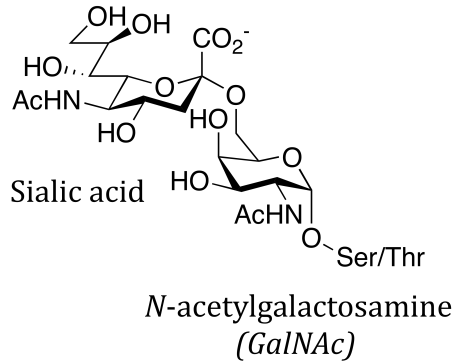



**Figure 1-4.** Carbohydrate cancer antigens that have been used as the basis for experimental vaccines

One particularly attractive target that has been explored as a monomeric vaccine is sialyl Tn (STn) (Figure 1-5). Sialyl Tn is a dissacharide consisting of a sialic acid linked α(2-6) to an *N*-acetylgalactosamine (GalNac) which is α-linked to a serine or threonine residue on a protein. It is found in an assortment of epithelial

cancers, including colon,[47,48] pancreatic,[49] gastric,[50] and ovarian cancers,[51] but is very rarely seen in healthy tissue. Administration of a radiolabelled α–STn monoclonal antibody results in specific localization into tumors.[52] STn expression in epithelial cancers has been shown to correlate to a more aggressive phenotype and a poorer prognosis for the patient.[53] High levels of α–STn antibodies in a patient, on the other hand, correlate with a more favorable prognosis.[54] Because STn seems like such a good candidate as an antigen for cancer immunotherapy, much effort has been directed toward developing it as vaccine.[55] Biomira Corporation developed a vaccine of STn linked to KLH named Theratope™ that it pushed through a series of clinical trials.[56] While the construct was able to elicit a mild humoral immune response in cancer patients, no statistical increase in average lifespan was seen over the control group.




sialyl Tn (STn)

**Figure 1-5.** Sialyl Tn is a tumor-associated carbohydrate antigen found on cancers of epithelial origin. It consists of a sialic acid linked ($\alpha$2-6) to an ($\alpha$) serine- or threonine-linked *N*-acetylgalactosamine.

*Polyvalent cancer vaccines*

One explanation for the failure of monomeric vaccines in the clinic is that the presentation of the carbohydrate in the vaccine does not match that on the cancer cell. On the surface of cells, carbohydrates tend to be presented in heterogeneous clusters, especially carbohydrates found in a class of heavily glycosylated proteins known as mucins.

In an effort to more closely model the presentation of sugars in a vaccine on the actual presentation in a cell,[57] Danishefsky and others have synthesized several conjugate vaccines with multimeric presentation of the carbohydrate antigens.[58] These range in complexity from relatively simple conjugates of three-mers of the same glycan to a hexavalent conjugate vaccine containing multimeric presentation of six different complex antigens that are directed toward epithelial cancers (Figure

1-6). These vaccines represent heroic synthetic effort, and seem to provide a better antibody response than their monomeric counterparts, but they still have not solved the problem of the relatively low immunogenicity of carbohydrates.

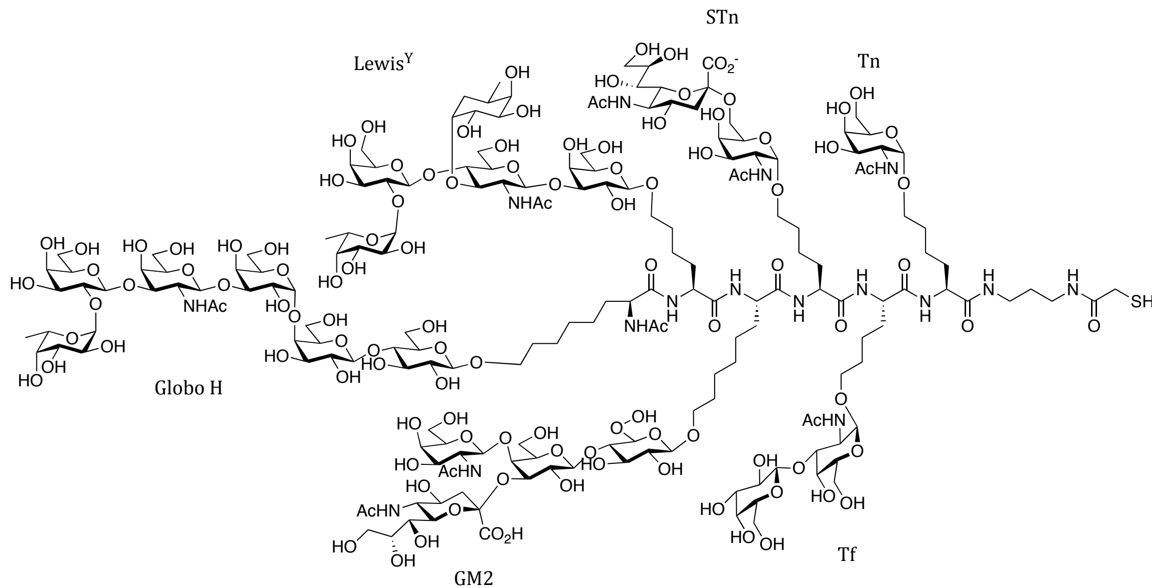



**Figure 1-6.** A polyvalent antigen containing a cluster of six different TACAs contained on a polypeptide chain. This molecule is an example of "clustering" carbohydrate antigens in an attempt to build vaccines that more faithfully represent the structure seen on a cell's surface.[57]

*Lymphocyte tolerance of self-antigens*

The ineffective immune response seen in patients receiving carbohydrate-based cancer vaccines such as the monomeric STn vaccine Theratope™ may result from immune tolerance of self-antigens. While some carbohydrate antigens appear to be found nearly exclusively on cancer cells, they are usually considered self-antigens since they may be seen during development or in small, difficult to detect quantities on healthy tissues. As a B-cell matures in the bone marrow, it is tested for

reactivity against self-antigens (Figure 1-7). If it is strongly self-reactive, its maturation is halted, and it will not be allowed to produce its self-recognizing antibody. That B-cell may be deleted, its receptor may undergo further rearrangement, or it may become anergic. Similarly, as part of their maturation process in the thymus, immature T-cells are allowed sample self-antigens bound in MHC complexes. If the binding to these antigens is too weak, the T-cells are deemed unable to effectively bind the APC receptors, and so are deleted. If the binding is too strong, the T-cells are also deleted because they pose the threat of causing an autoimmune response. Because TACAs are still self-antigens, they may not be able to elicit strong humoral and cellular immune responses due to tolerance mechanisms of B- and T-cells, respectively.

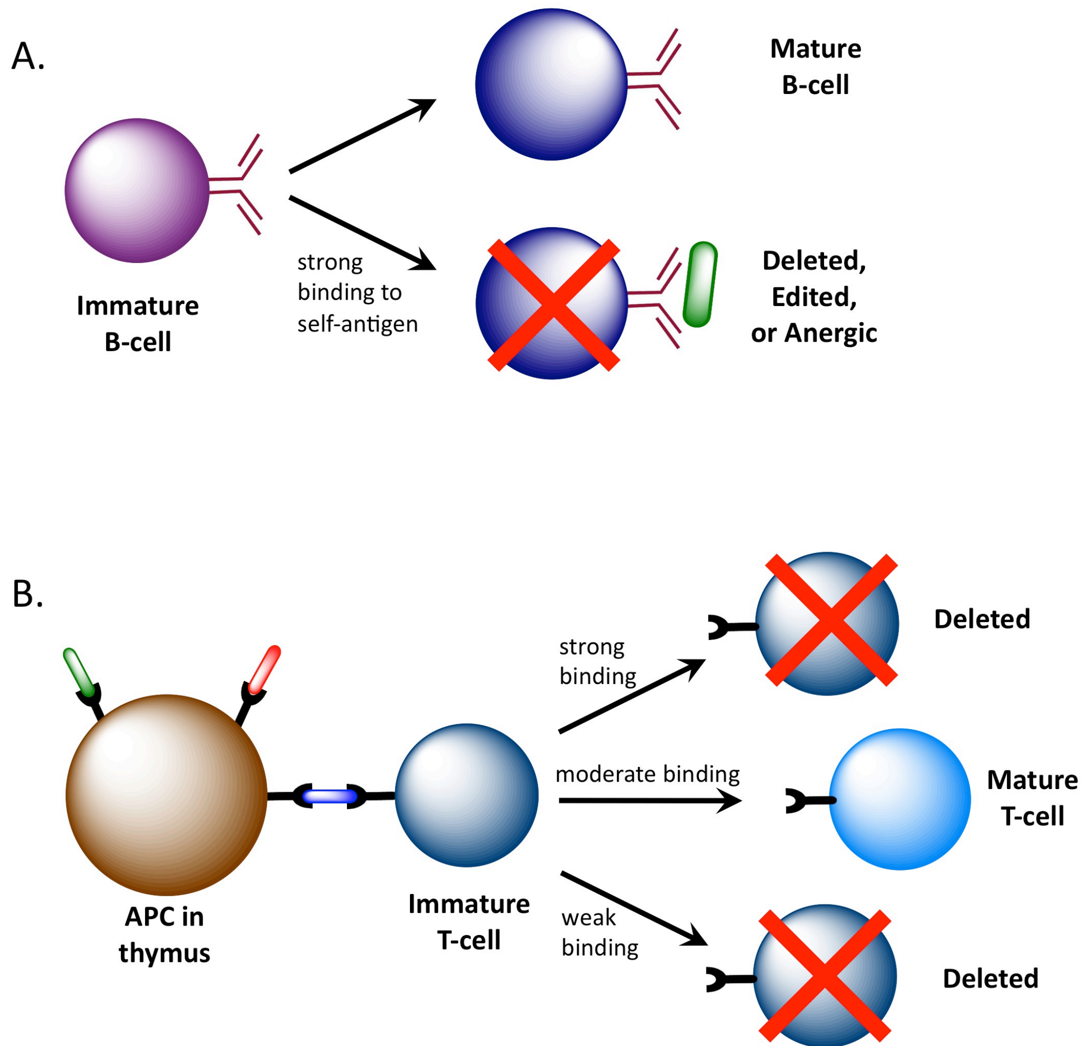



**Figure 1-7.** Self-tolerance mechanisms of lymphocytes. A. If an immature B-cell that is developing in the bone marrow strongly binds a self-molecule, that B-cell may be deleted, undergo a process to re-shuffle its receptor binding site, or become anergic. B. During their development in the thymus, T-cells that bind too strongly or weakly to presented self-antigens are deleted to prevent autoimmune problems and destroy ineffective T-cells, respectively. T-cells that show a moderate amount of binding are allowed to live, as they should not be strongly auto-immunogenic, but are capable of binding to MHC complexes, and so can respond in the event of an immune challenge.

*Combination vaccine*

One particularly interesting attempt at breaking immune self-tolerances comes from the Boons lab.[59] Researchers there have built a unimolecular three-component vaccine that combines a TACA, a promiscuous T-cell epitope, and a toll-like receptor 2 (TRL2) agonist (Figure 1-8). This combination vaccine elicits a humoral immune response in mice that is much greater than simply injecting all three unlinked components at the same time. While it is not clear how this approach overcomes the problem of B-cell tolerance, it appears to boost the immune response based on some other mechanism. This work suggests that linking the various components necessary to an immune response in one large molecule may be an effective way to boost immune response.

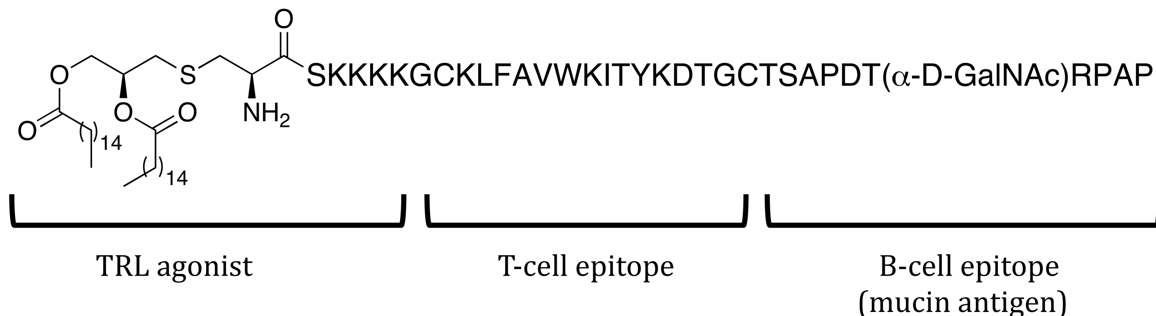



**Figure 1-8.** A three-part single-molecule vaccine consisting of a mucin antigen (B-cell epitope), MHC-binding peptide (T-cell epitope), and immune system activator (TRL agonist). Having all three components connected as a single molecule is key to improving efficacy.[59]

*Immune recognition of glycan perturbation*

Another method to break immune tolerance of self-antigens involves the modification of the antigens to render them "foreign." The immune system is capable of recognizing very small changes in glycans. For example, the blood group A antigen is a tetrasaccharide with a terminal GalNAc residue. Replacing the GalNAc with galactose, a change of just one *N*-acetate group to a hydroxyl group, gives blood group B antigen (Figure 1-9). This subtle change makes type A blood immunogenic to type B carriers and vice versa.

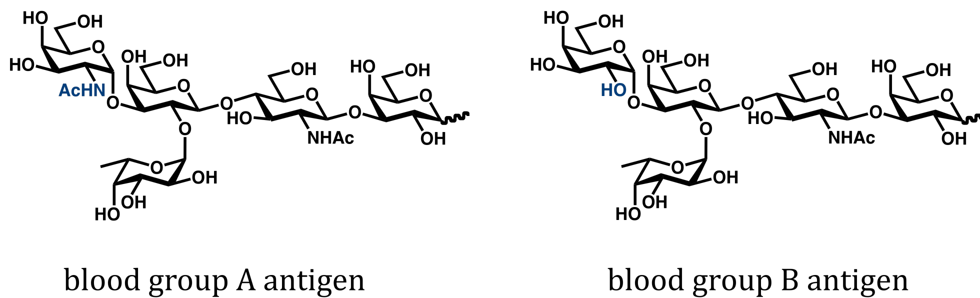



**Figure 1-9.** The blood group antigens A and B. The antigens that define blood types A and B differ only in their terminal GalNAc (A) or galactose (B) residues, but elicit strong immune responses in subjects whose blood is of the other type.

Small changes in sialic acids have been also been shown to result in immune recognition. Sialic acids undergo species-specific modification such as *O*-acetylation, *O*-methylation, *N*-deacetylation, hydroxylation, phosphorylation, or sulfation. [60] When these species-specific sialic acids are ingested or injected into another species, specific antibodies against the foreign sialic acids can be isolated. Thus, while a species may be tolerant of its own particular forms of sialic acid, slightly

modified foreign forms are sufficient to invoke an immune response. Further, structural modifications of sialic acids in oligosaccharide carbohydrate vaccines have been shown to increase their immunogenicity. For instance, esterifying, amidating, and reducing the sialic acids in ganglioside-based vaccines lead to a stronger antibody response.[61,62]

These examples suggest that small structural modifications of carbohydrate tumor antigens may be sufficient to overcome the problem of immune self-tolerance of cancer antigens. While enzymatic and synthetic methods allow the modification of antigens in vaccine construction, this does not solve the problem that the surface antigens of the cancer cells themselves are still self-antigens.

One approach would be to hope that the strong immune response to the slightly modified antigen would “bleed over” and provide some response against the unmodified antigens. This method may be viable since helper T cells, which are more restrictive than B cells, would be activated by protein component of the modified antigen. These T cells may be able to activate B cells whose antibody recognizes the natural structure. A better approach, however, would be to modify the tumor surface antigens *in situ* such that the surface of the tumor matches the structure in the vaccine. This can be accomplished by metabolic oligosaccharide engineering.

*Metabolic oligosaccharide engineering and vaccines*

Metabolic incorporation of a wide range of unnatural molecules into live cells and animals has been achieved and exploited for various purposes. In these strategies, analogs of the natural molecules are incorporated into larger molecules or structures using the endogenous biosynthetic machinery. This strategy relies on the promiscuous substrate specificity of endogenous enzyme. The specific modification depends both on the tolerance of the enzyme and the intended application. Examples include selenomethionine incorporation in proteins,[63] bromodeoxyuridine in nucleic acids,[64] and deoxy sugars into carbohydrates.[65]

A remarkable step forward for metabolic engineering in mammalian glycans came when Reutter and coworkers first showed in 1992 that unnatural analogs of sialic acid can be incorporated in cells by the incorporation and processing of *N*-acyl analogs of its biosynthetic precursor *N*-acetylmannosamine (ManNAc) (Figure 1-10).[66] This discovery meant that each of the five enzymes in the pathway was tolerant to certain *N*-acyl perturbations. In 1997, Lara Mahal and others extended this methodology by incorporating a functional group that could be subsequently modified chemically.[67] They showed that a ketone-containing sialic acid analog could be incorporated onto cell surface glycans and subsequently labeled with hydrazine or aminooxy-containing detection reagents. Since that time, other carbohydrate synthetic pathways have proved vulnerable to metabolic

oligosaccharide engineering, including the pathway for mucin core residue *N*-acetylgalactosamine[68] and cytosolic *N*-acetylglucosamine.[69]

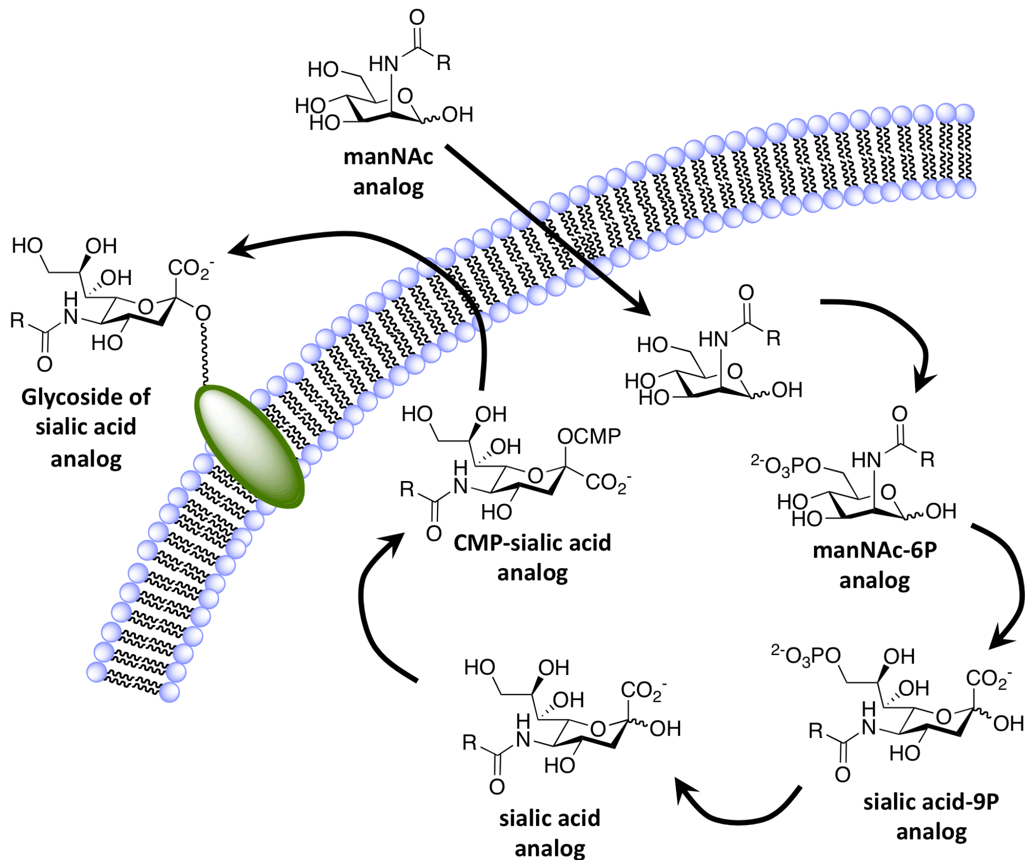



**Figure 1-10**. Cell surface sialic acids are amenable to engineering by providing suitably modified metabolic precursors. Many *N*-acyl analogs of ManNAc are capable of transiting the five enzymatic conversions to become *N*-acyl analogs of surface sialic acids.

Importantly, metabolic oligosaccharide engineering is achievable in live organisms, including vertebrates. Prescher and coworkers showed that injecting acetylated ManNAc or GalNAc analogs into mice results in the incorporation of these

unnatural sugars into glycans in a broad range of their tissues.[70] This combined work shows that glycans can be modified in living animals. By applying metabolic engineering to carbohydrate cancer antigens, these typically non-immunogenic antigens can be made to appear foreign to an organism's own immune system.

*A new strategy for cancer immunotherapy*

We set out to overcome this self-tolerance of carbohydrate cancer antigens by introducing a new strategy for cancer immunotherapy. In the traditional strategy like that used by Biomira, the natural antigen-carrier protein complex is injected into the patient at intervals, and the immune system is left to mount a response against the self-antigen as best as it can manage. In the new strategy, the antigen is first chemically modified away from its natural state (Figure 1-11). This unnatural analog of the carbohydrate cancer antigen is then coupled to a carrier protein and injected into a patient as in the traditional strategy. Because the analog is a completely foreign molecule the immune system, the patient should be able to mount a much more powerful immune response against the modified antigen. The response, however, is directed against the modified antigen, and not the one that naturally occurs on cancer cells. In order to direct the immune response against the cancer cells, this strategy requires a new step: modified metabolic precursors to the modified antigen are injected into the patient. The endogenous biosynthetic machinery processes these unnatural metabolites to display their corresponding modified sugars on the surface of cells. While most cells will be induced to display

unnatural sugars on their surface, only the cancer cells express high levels of the TACA that makes up the vaccine. Thus, while azidosugars will be found on most cells, healthy and cancerous, only the cancer cells will have high levels of the modified higher order antigen found in the vaccine. As long as the immune response is specific to the entire antigen and not the monosaccharide components, then the immune response would selectively target the cancer cells.

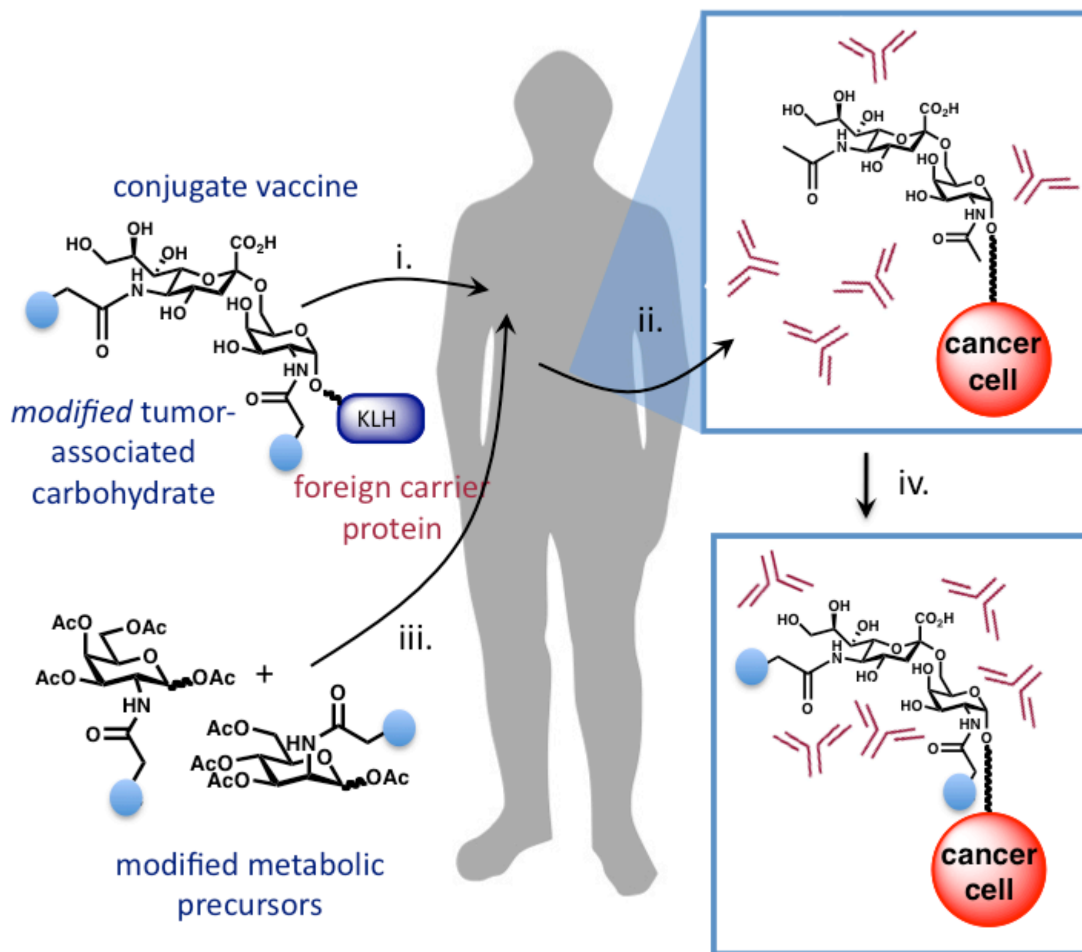



**Figure 1-11.** A new strategy for cancer immunotherapy. In this strategy, the traditional approach of boosting immunogenicity of a TACA by conjugating it to a carrier protein (i. and ii.) is combined with the use of a modified carbohydrate antigen. The modification represented by the blue circles makes the antigen foreign to the immune system, which may allow the immune system to mount a more potent immune response than against the natural antigen. Since the immune response is directed against the modified antigen, the cancer antigen must be engineered by supplying the appropriate metabolic precursors (iii.), which can induce the appearance of the vaccine antigen on cancer cells (iv.).

This strategy takes advantage of the ability to modify the chemical structure of carbohydrate moieties *in vivo* to render cancer antigens significantly more immunogenic than they typically would be as essentially self-antigens. A potential pitfall of this strategy is that many normal carbohydrate structures would be made unnatural, possibly leading to an autoimmune response. Preliminary work in our lab has not indicated autoimmune responses in mice given unnatural sugars, however, more exploration is warranted. The potential benefits of the strategy include, primarily, a much stronger immune response to the now "foreign" cancer antigen. Further, the susceptibility of the cancer cells to the immune response should be modifiable by controlling the supply of the metabolic precursors. While this temporal control of the immune response may not provide a benefit to the cancer treatment, it provides a safety release should the patient develop symptoms of an unwanted autoimmune response against healthy tissues.

*Proof of concept with SiaLev*

Previous work in the laboratory served as a proof-of-concept for this strategy using a monomeric vaccine (Figure 1-12).[71] Specifically, an N-levulinoyulsialic acid (SiaLev)-KLH construct was injected along with an adjuvant into rabbits. This inoculation resulted in a high-titer antibody response, while rabbits receiving a natural sugar-KLH conjugate showed a much lower response. The antibodies were shown to be specific to the modified form of the sugar, but did not recognize natural sialic acid in an enzyme-linked immunosorbent assay (ELISA). Further, the

antibodies were capable of recruiting cell-killing factors from immune "complement" and directing the lysis of cells that had been treated with *N*-levulinoylmannosamine (ManLev), the metabolic precursor of SiaLev, but not those that had not received ManLev. This work using a monomeric antigen demonstrates both that structural modification of an antigen can result in a strong immune response, and that this response is specific to the modified antigen. Most importantly, it shows metabolic engineering can be used to make cells vulnerable to the immune response by forcing them into displaying the modified antigen on their surface. Because monomeric antigens such as ManNAc are ubiquitous on cells, this strategy must be applied using a TACA in order to be viable.

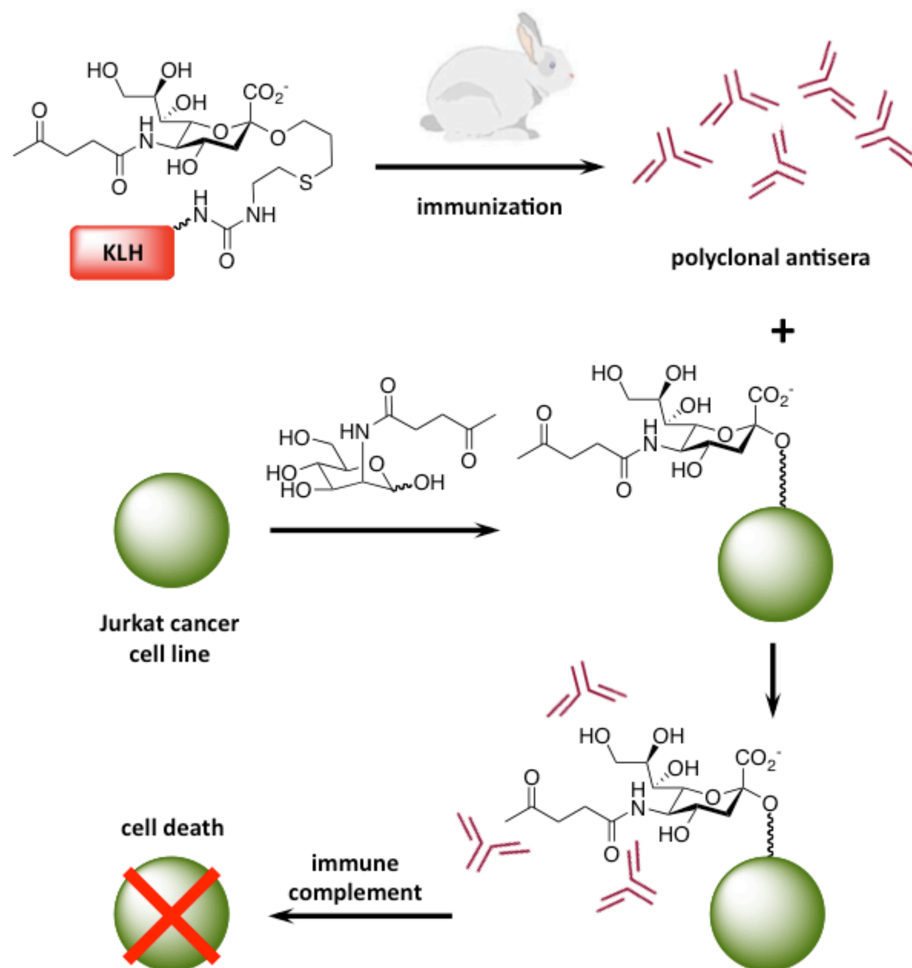



**Figure 1-12.** SiaLev elicits a strong and specific immune response. Polyclonal antisera from rabbits inoculated with SiaLev-KLH can specifically induce complement-mediated cell death ManLev treated Jurkat cells but not untreated Jurkats.

*Passive immunization*

Jennings and coworkers demonstrated this strategy in a "passive immunization" approach with the TACAs polysialic acid (PSA).[72] They showed that

treatment with a monoclonal antibody raised against poly-*N*-propanoyl sialic acid inhibits tumor growth and slows metastasis of implanted tumor cells in mice when both the cells and the mice had been treated with the metabolic precursor of poly-*N*-propanoyl sialic acid ManNProp. They applied this strategy with similar success to the ganglioside GD3.[73]

*Modified sialyl Tn antigens*

During the completion of this work, the Guo group from Case Western Reserve and later Wayne State University published the results of related experiments performed by their group. They modified *N*-acyl position of the sialic acid moiety in STn by replacing the acetyl group with a panel of structural analogs: ethylacetyl, isopropylacetyl, and phenylacetyl. They showed that, especially with the largest phenylacetyl modification, the unnatural STn analogs were significantly more immunogenic than their natural counterparts.[74]

**Dissertation overview**

The rest of this dissertation details work to synthesize a panel of STn-based conjugate cancer vaccines. I will present the synthesis of a panel of four STn analogs that are modified at the sialic acid position, the GalNAc position, both, or neither. These analogs were coupled to the carrier protein KLH and injected into rabbits for the production of antisera. These antisera were tested in an ELISA to determine the

contribution of modification at each position to the strength and specificity of the resulting humoral immune response.

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74 Wu, J. and Guo, Z. (2006). Improving the Antigenicity of sTn Antigen by Modification of Its Sialic Acid Residue for Development of Glycoconjugate Cancer Vaccines. *Bioconj. Chem.* ***17,*** 1537-1544.

## Chapter 2: Synthesis and evaluation of a panel of carbohydrate cancer vaccine

### Introduction

Often the most difficult step in vaccine development is obtaining the antigen in the first place. Vaccine antigens can be obtained by natural product isolation or by synthesis. Natural product isolation has the benefit that it requires no synthesis, but the resulting antigens tend to be mixtures of many different compounds, and they are limited to those produced at isolable levels in natural systems. In some cases, having a mixture of antigens may be desirable, but other times, more control over the composition of the target antigen is desired. Pure, structurally defined antigens are best attained through synthesis. Synthesis allows complete control of structure and purity and provides access to non-isolable or unnatural structures, but it is often a complex undertaking. Protein antigens can be "synthesized" by cloning and expression in bacteria or yeast, and this allows access to unnatural and fusion versions of the antigen. Unnatural carbohydrate antigens, however, are more difficult to come by. They are typically obtainable only by isolation after engineering the natural system, modification of natural products, or direct chemical or enzymatic synthesis. Carbohydrate synthesis is a classically difficult undertaking due to the chemical similarity of the many hydroxyl groups in the molecules as well as the difficulty in forming and controlling the stereochemistry of glycosidic linkages. Despite these challenges, sophisticated protecting group strategies and

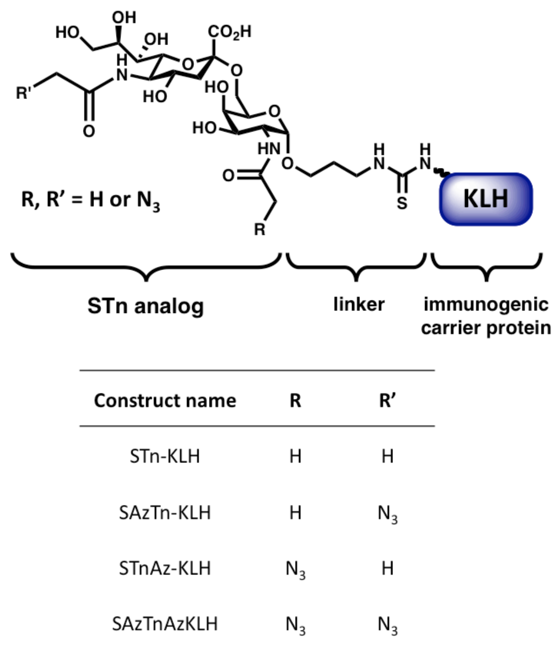
advances in the chemistry for forming glycosidic linkages has allowed for heroic syntheses of complex multicomponent carbohydrates such as globo H.[1]

This chapter details the synthesis of a panel of cancer vaccine constructs as well as the use of the vaccine to develop antisera in rabbits, which is evaluated by ELISA assay to determine the relative strength and specificity of the antisera.

## Target constructs

I set out to synthesize a panel of four constructs based on STn. The constructs were designed with a natural or modified carbohydrate antigen attached to a carrier protein *via* a linker (Figure 2-1). Based on work published by Helling and others,[2] I chose KLH as my immunogenic carrier protein. This protein has proven a successful immunogenic carrier protein for other carbohydrate antigens, including gangliosides like GM2 and mucin antigens including STn. The linker was chosen to be minimally perturbing, but allow convenient linking to KLH and a natural alpha *O*-glycoside linkage to the sugar. The sugars were modified at one, both, or neither of the *N*-acetyl positions of the sialic acid and GalNAc residues of STn. Modifications at these positions were chosen because these positions are modifiable *in vivo* by metabolic oligosaccharide engineering. Thus, cancer antigens can be modified on tumor cells simply by providing metabolic precursors to the patient. This panel allows comparison of the antibody response between the unnatural and natural STn antigen, as well as permits the comparison of the contribution to the immune response by modification at the sialic acid and GalNAc

positions. For the modification, I chose the azide, giving the panel of four target structures shown in Figure 2.1.




| Construct name | R | R' |
| --- | --- | --- |
| STn-KLH | H | H |
| SAzTn-KLH | H | $N_3$ |
| STnAz-KLH | $N_3$ | H |
| SAzTnAzKLH | $N_3$ | $N_3$ |

**Figure 2-1.** Vaccine targets. A panel of four target cancer vaccines constructs consisting of a modified or natural STn antigen a-linked via a short linker to KLH was .

*The azide modification*

While any modification of the natural structure should contribute to the increased immunogenicity of the antigen, the azide was chosen for several reasons. First, the biosynthetic pathways for surface GalNAc and SiaNAc are both tolerant to azide substitutions at the N-acyl positions, giving surface *N*-azidoacetylgalactosamine (GalNAz) and *N*-azidoacetylsialic acid (SiaNAz), respectively.[3,4] Our lab has considerable experience exploiting this tolerance to

install azides on cell surface glycans via metabolic engineering both in culture and in vivo. Second, while not exhaustively studied, we have not noticed any obvious immune response in mice to the injection of the azidosugar metabolic precursors. Finally, our lab has developed the Staudinger[5] and copperless click[6] reactions to modify azido sugars *in vivo* (Figure 2-2). While this is not necessary to the modified antigen vaccine strategy, it allows convenient chemical detection of modified glycans and also provides a chemical handle to further modify an antigen *in vivo*. This might be useful if the azide proved an insufficiently large modification to augment the immune response, and the glycan biosynthetic machinery was intolerant of larger modifications.

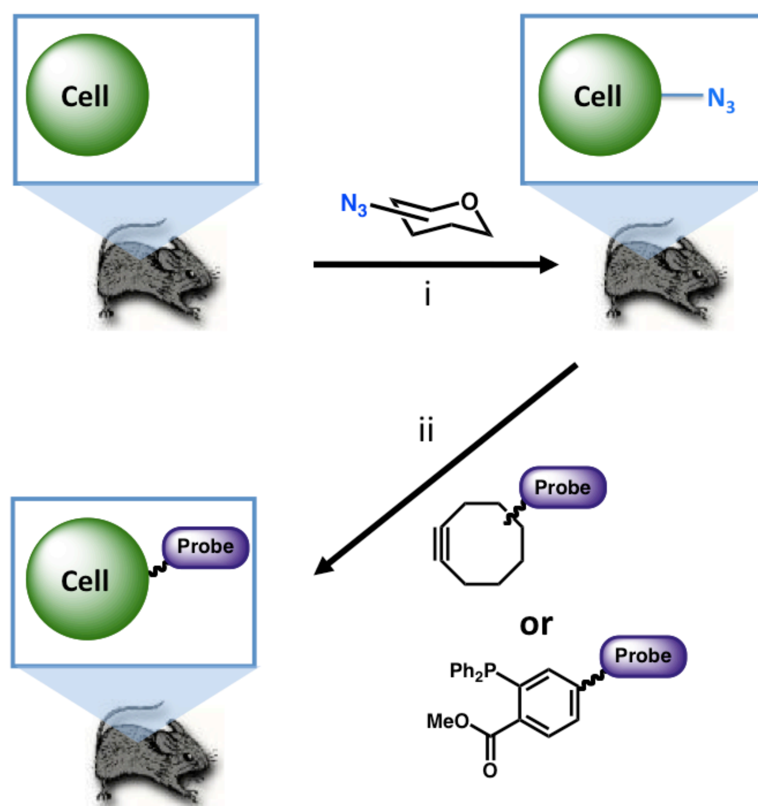


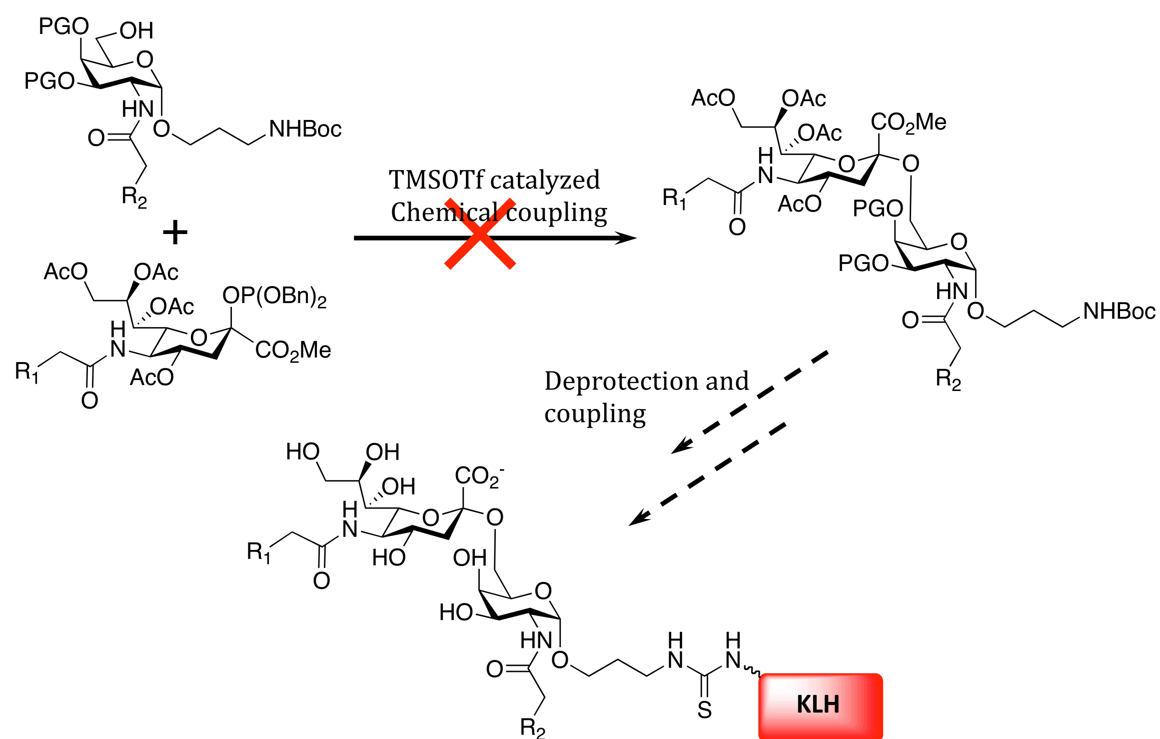

**Figure 2-2.** The azide modification. The azide was chosen as the modification both because azidosugar metabolic intermediates can lead to azide-containing glycans on cells surfaces *in vivo* (i) and because those surfaces can be labeled or further remodeled via a strain-promoted click reaction or Staudinger ligation (ii).

## Synthesis

*Initial chemical approach*

To assemble the constructs in Figure 2-1, I first attempted a fully synthetic strategy (Scheme 2-1) where a protected galactosyl acceptor with linker was coupled to an activated sialyl donor, followed by deprotection and coupling to KLH. Sialation chemistry is notorious difficult because the structure of sialic acid complicates traditional strategies for forming glycosidic bonds (Figure 2-3).[7,8] The

congested quaternary center at the 2-position leads to a hindered tertiary oxocarbenium intermediate that slows coupling reactions, allowing competing reaction pathways to direct material to unwanted products. Adding to this, the lack of a directing group at C-3 makes controlling the stereochemistry of the glycosidic linkage particularly difficult.[9] Finally, the electron withdrawing carboxlylic acid group at C-1 coupled with the lack of substituion at C-3 promotes 2,3-elimination to form a glycal. Because of these limitations, traditional donors for direct glycosylation such as halides and thiols tend to give poor yields and selectivity with all but the simplest primary alcohol acceptors. Some chemists have found success by introducing participating groups at C-3[10,11] and more recently at C-4[12,13] and C-5[14,15], but these syntheses add several steps to append and then remove the participating moieties. Notably, sialylation reactions are finicky, and a general method has not been found that provides adequate yield and selectivity in all reactions. For direct glycosylations with more hindered secondary and tertiary acceptors, the most success has been found with phosphite donors introduced independently by Schmidt[16] and Wong[17]. Phosphite donors are popular because the phosphites are relatively easy to synthesize compared to many other donors, are stable to purification, but are extremely reactive in the presence of catalytic trimethylsilyltriflate.[9]




**Scheme 2-1.** The chemical synthesis route. Chemical synthesis of the vaccine constructs from Figure 2-1 failed at the difficult sialylation step.

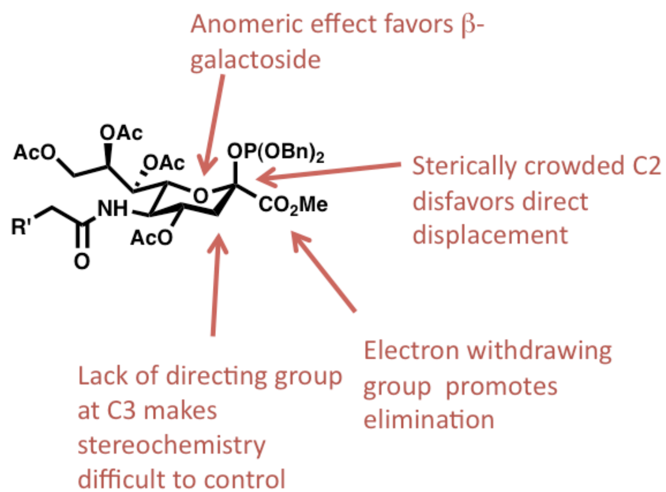



**Figure 2-3.** Sialylation reactions are notoriously difficult due to the unusual structure of sialic acid.

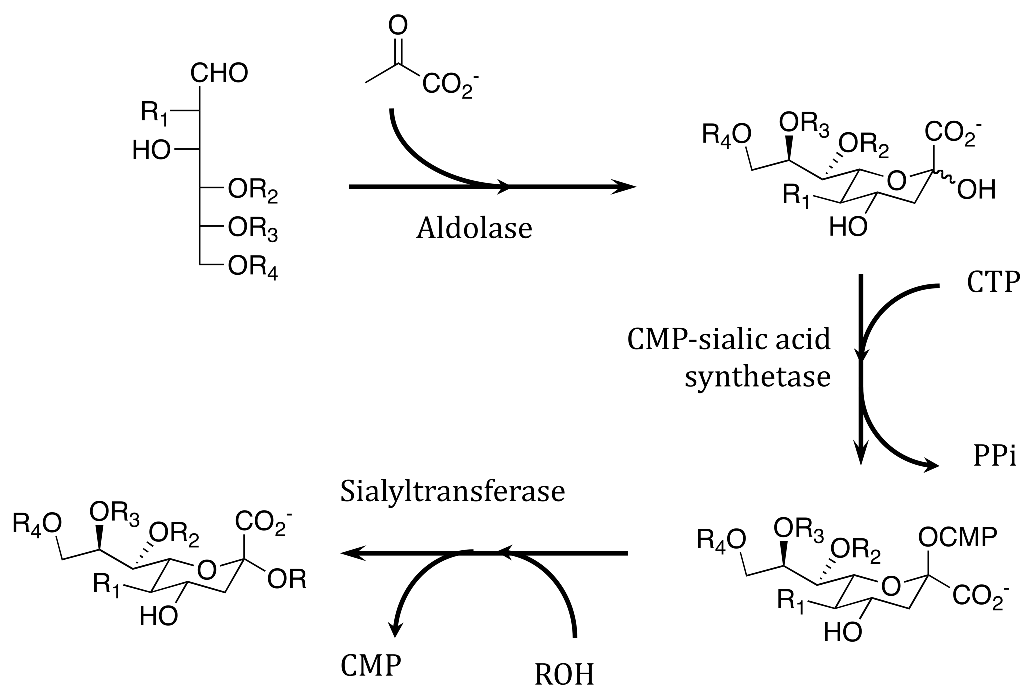
I chose to use the dibenzyl phosphites shown in Scheme 2-1 as donors, and I attempted to couple those with different protect *N*-acetylgalactosamine analogues containing a Boc-protected α-linked protected linker. Synthesis of the galactosyl acceptor was problematic, though after a few fruitless routes, I achieved the desired differentially protected acceptor. Under carefully controlled anhydrous conditions using catalytic TMS-OTf, the donor could be coaxed react with the phosphite acceptor to give a very low yield of the desired dissacharide, however, there was no selectivity for the desired α-anomer, and the mixture proved impossible to separate. After many failed attempts to separate the anomers or deprotect the mixture of anomers, I abandoned the attempt and decided to approach the synthesis via a chemoenzymatic route.

*Chemoenzymatic and biological synthesis of sialosides*

Scientists similarly dissatisfied with chemical sialylation have looked to biology to achieve this linkage for them. Typically, one of two strategies is used to harness biology to synthesize sialic acids. Either whole cells are genetically engineered to have the required machinery to synthesize large quantities of the desired sialoside and all the requisite intermediates and cofactors, or the enzymes are purified and enzymatic synthesis is performed *in vitro*. While there are impressive examples or huge yields of sialosides produced by engineered cells, the *in vitro* enzymatic strategy is better suited to smaller scale production of novel sialosides.

The eukaryotic enzymes involved in sialoside synthesis tend to have quite rigid substrate requirements that prohibit the formation of unnatural sialoside analogs. Bacterial enzymes on the other hand, particularly those from pathogenic bacteria, tend to have significantly relaxed substrate requirements.[18] A recent boom in the cloning and characterization of bacterial enzymes has greatly simplified and expanded enzymatic synthesis of unnatural sialosides. In particular, Xi Chen at UC Davis has developed a three-enzyme one-pot system that allows researchers to access sialosides starting with an acceptor, ManNAc analogs, cytidine triphosphate (CTP), and pyruvate (Figure 2-4).[19] In this reaction, sialic acid is formed by a sialidase whose typical function is to break down sialic acid into ManNAc and pyruvate. However, by using a large excess of pyruvate, the enzyme can be used in

the reverse direction to form sialic acid or an analog from the appropriate *N*-acyl mannosamine. In order to activate the sialic acid analog for coupling to the acceptor, it must be converted to the cytosine monophosphate (CMP) sugar. This is achieved using a bacterial CMP-sialic acid synthetase (CSS) that has relaxed substrate specifity. This CMP-sialic acid analog can then be used to form an unnatural sialoside with the supplied acceptor using a third enzyme, a bacterial sialyltransferase. Control of regio- and stereochemistry is achieved by selection of the sialyltransferase. For instance, the same acceptor and sialic acid pair could be combined with an $\alpha$2-6 or $\alpha$2-3 linkage from the same starting materials simply by using a different sialyltransferase enzyme.



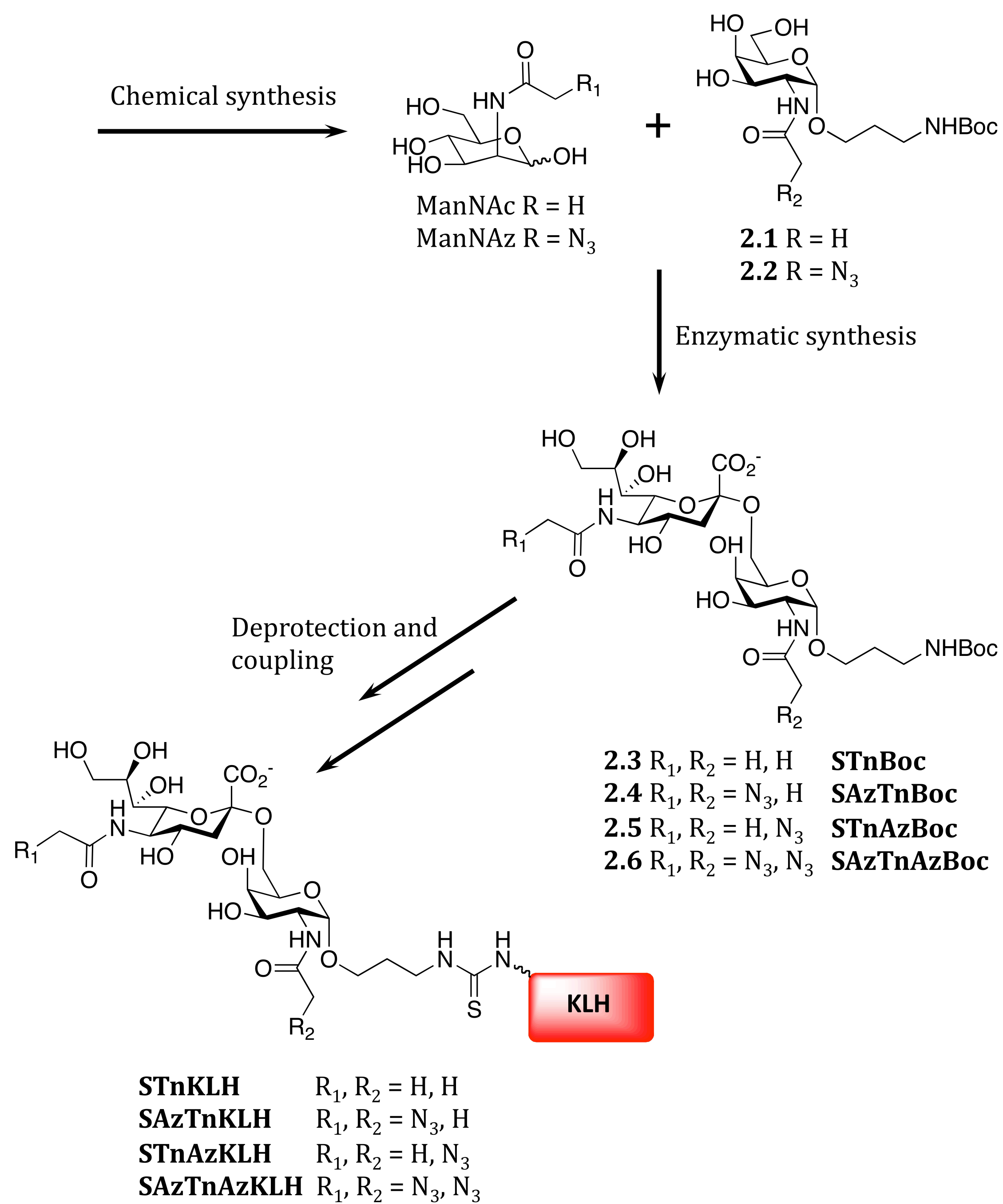

| confirmed stuctural tolerances | | |
|---|---|---|
| R 1 | = | NHAc, OH, NHGc or other |
| R2 | = | H, Ac, or other |
| R3 | = | H, Ac, SO3H, Me or other |
| R4 | = | H, Ac, Lactyl |
| R | = | Molecule with terminal Gal(Nac) |

**Figure 2-4**. A One-pot three-enzyme method for making modified sialosides. Xi Chen developed a one-pot system for sialoside synthesis that starts from synthetically tractable ManNAc analogs and a glycosyl donor.[19]

There are many benefits to chemoenzymatic synthesis. Foremost is the ease in achieving the desired stereochemistry of the glycosyl linkage. As long as an enzyme is available, it does all the work. Second is the ability to begin with *N*-acyl mannosamine analogs instead of sialic acid analogs, which greatly reduces the number of required chemical synthesis steps. The yields tend to be higher and the reactions less finicky than chemical sialylation. And purification from the crude enzymatic preparation tends to be simpler than from cellular production and is often simpler even than crude preparations from chemical synthesis since there is usually no need to remove an undesired stereoisomer. Recent advances in chemoenzymatic synthesis of sialosides has greatly expanded the number of unnatural sialosides that are easily available and have lead to combinatorial synthesis of sialosides for high throughput screening applications.[20] While chemoenzymatic synthesis is a powerful tool, there are still several limitations. Preparative syntheses require large amounts of expensive CTP, and purification can be difficult depending on the exact structure of the desired sialoside. Most restrictively, access to structures is limited by the availability of enzymes. Many of the bacterial sialic acid biosynthetic enzymes have promiscuous selectivity, however, these enzymes are still only tolerant of relatively small changes in structure. Chemical synthesis is still necessary for many linkages for which enzymes do not exist or for substrates that are modified so as to render them unavailable to any of the enzymes in the pathway.

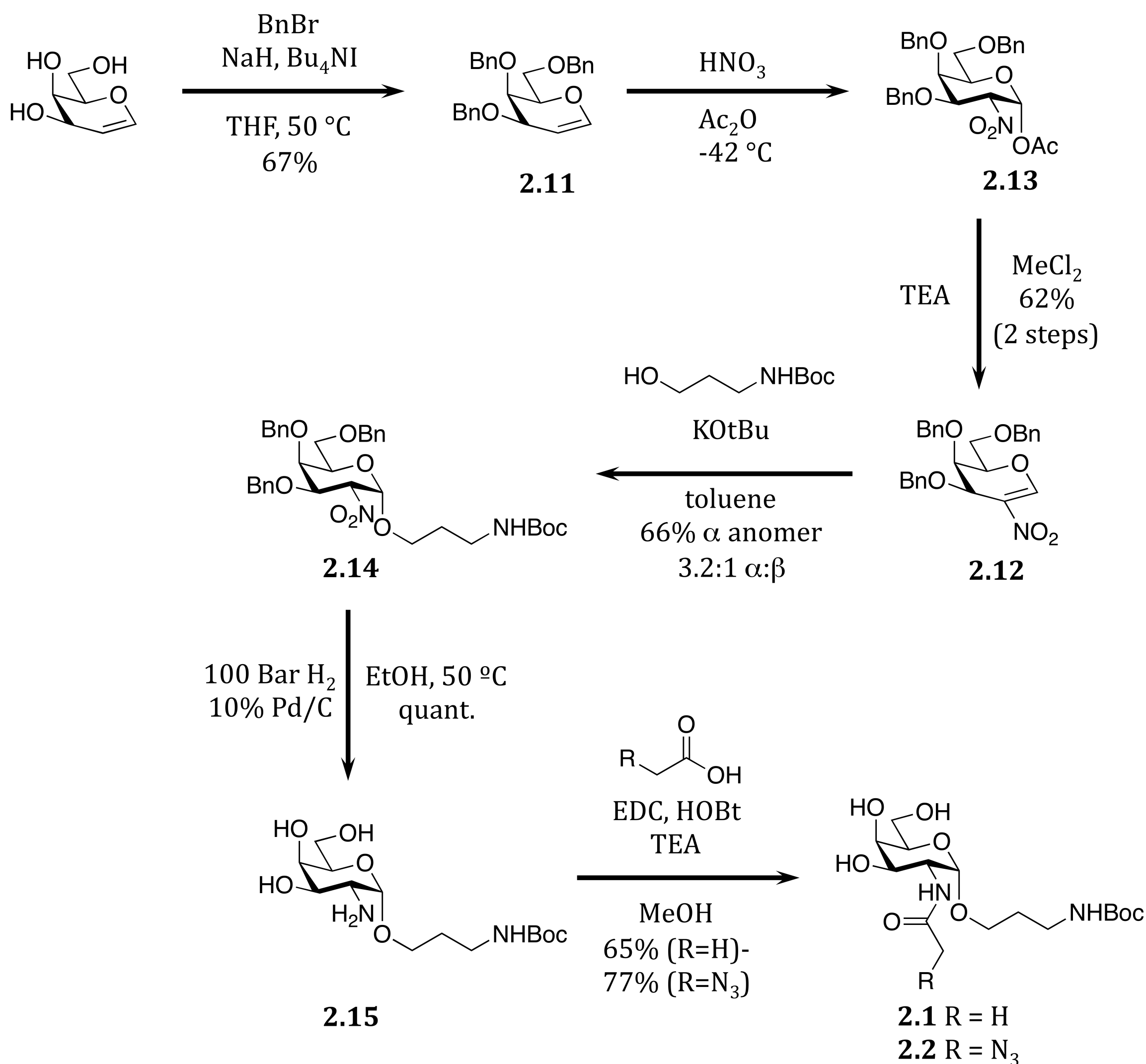
*Chemoenzymatic synthesis of vaccine antigen panel*

Despite the limitations, Chen's system is very well suited to synthesis of moderate quantities of sialosides with only moderate changes from the natural state. The system seemed particularly well suited for my structures, which are similar to those that can be formed by mammalian enzymes, which are very restrictive in their substrate tolerance. So looking to take advantage of Chen's one-pot three-enzyme reaction, I tried a new strategy illustrated in Scheme 2-2. In this strategy, I would chemically synthesize Boc-protected acceptors **2.1** and **2.2**. Using Chen's one-pot system with ManNAc and *N*-acetylmannosamine (ManNAz), I would enzymatically synthesize STn analogs with protected linker **2.3**-**2.6**. These would be deprotected to form analogs with a free amine **2.7-10**. Finally, these constructs would be coupled to KLH for completion of the vaccine constructs.




**Scheme 2-2.** A chemoenzymatic approach to modified cancer vaccine synthesis.

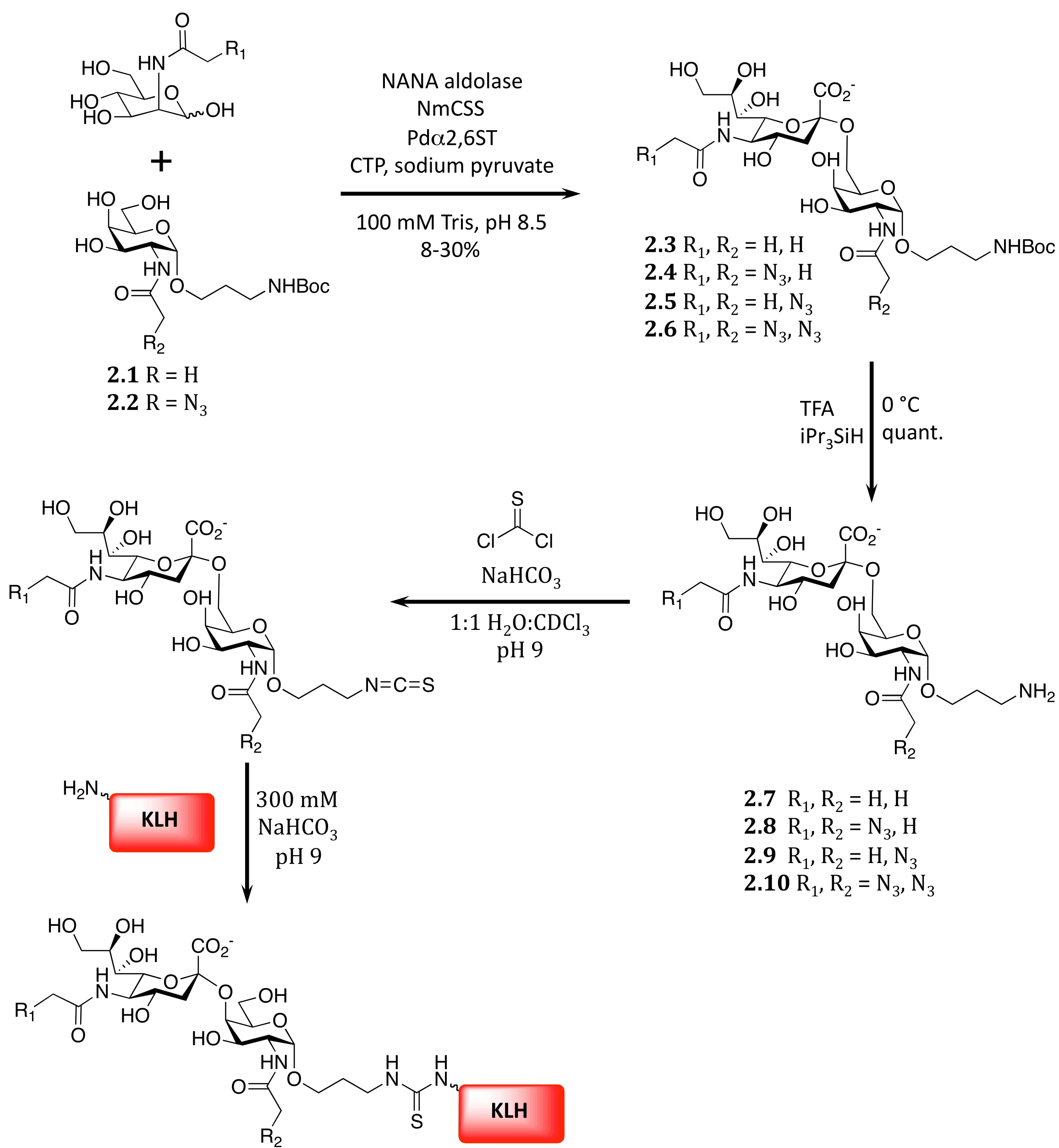
I synthesized acceptors **2.1** and **2.2** starting from galactal (Scheme 2-3). Galactal was perbenzylated by treating with benzyl bromide and sodium hydride in warm THF with catalytic tetrabutylammonium iodide to give protected galactal **2.11**. Perbenzylated galactal was converted to the nitrogalactal **2.12** following the two-step nitroglycal concatenation method of Schmidt and others.[21] First, nitroacetate formed *in situ* with cold nitric acid and acetic anhydride was added regioselectively across the galactal to give the anomeric acetate **2.13**. Acetic acid was then eliminated from the crude intermediate using triethylamine in dry methylene chloride to give nitrogalactal **2.12**. Once the nitro group was installed, the protected linker was added by Michael addition of 3-(boc-amino)-1-propanol into the nitrogalactal, giving protected nitrogalactoside **2.14**. High pressure hydrogenation ethanol in a Parr bomb with palladium hydroxide on carbon gave aminogalactoside **2.15** in quantitative yield, though the reaction was inconsistent, and was particularly difficult at larger scale. **2.15** was reacted with either acetic acid or azidoacetic acid in methanol with triethylamine, hydroxybenzotriazole, and *N*-(3-dimethylaminopropyl)-N'-ethylcarbodiimide to give protected acceptors **2.1** and **2.2** respectively.




**Scheme 2-3.** Chemical synthesis of galactosyl acceptors.

To synthesize the complete vaccine constructs, I allowed each acceptor **2.1** and **2.2** to react overnight at 37 °C with either ManNAc or ManNAz with CTP, sodium pyruvate, and Chen's 3-enyzme cocktail including sialidase, CSS, and an α2-6-sialyltransferase (Scheme 2-4). The resulting four protected constructs **2.3**-**2.6** were purified by high performance liquid chromatography (HPLC) (Figure 2-6),

though protected disaccharide **2.4** was unable to be completely separated from **2.1**. The disaccharides were deprotected in neat TFA to yield the final constructs **2.7**-**2.10** after HPLC purification using an amino column.




**Scheme 2-4.** Chemoenzymatic synthesis of final vaccine constructs.

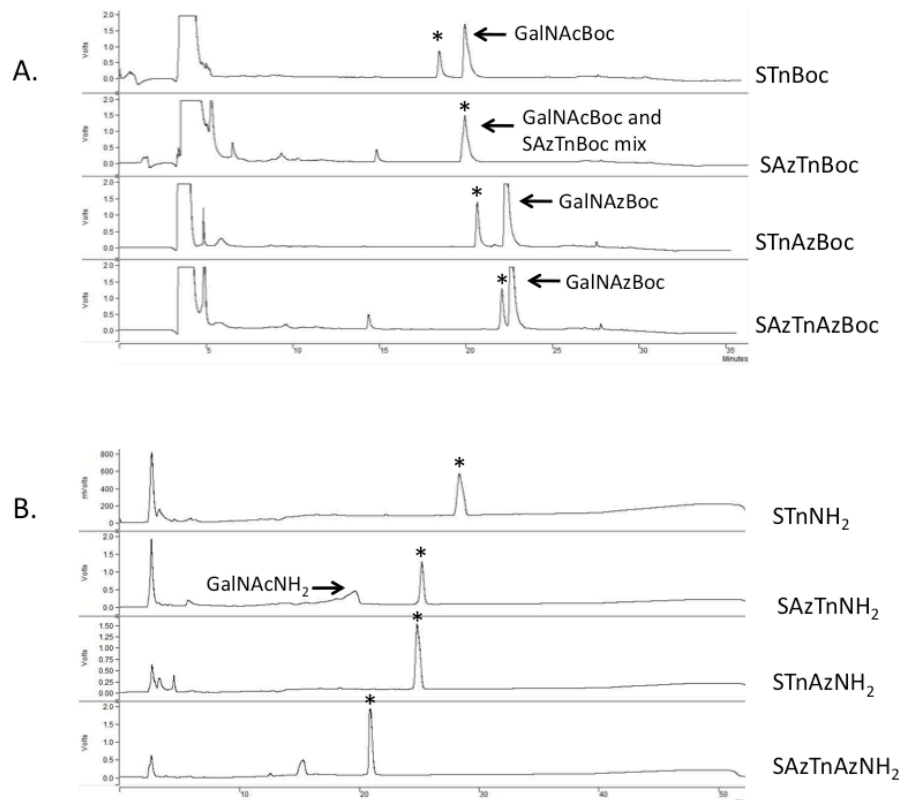


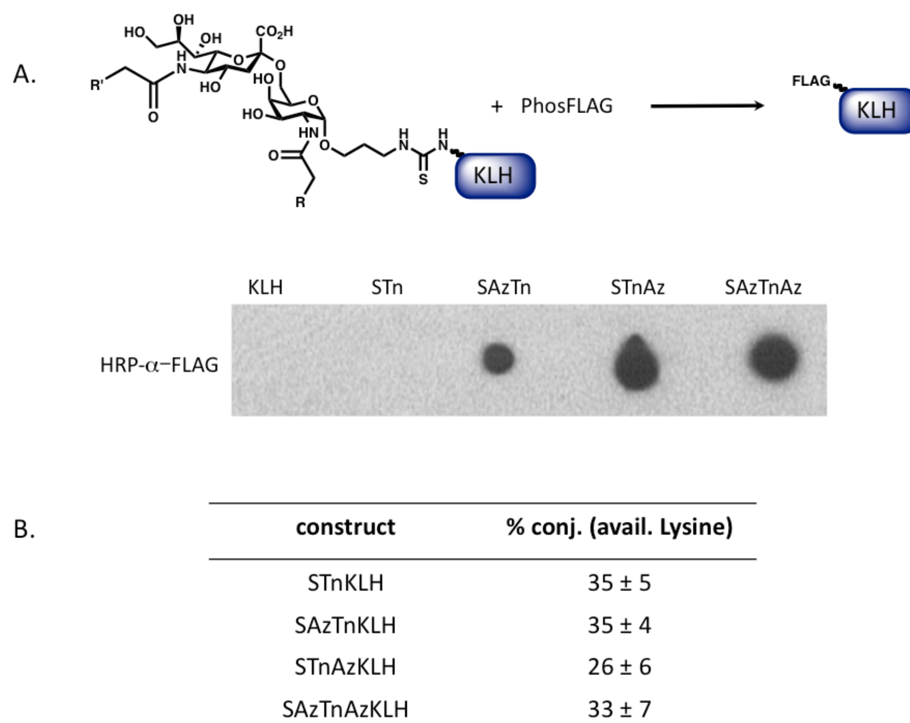

**Figure 2-5.** HPLC purification of disaccharides. Peaks containing desired product are marked with an asterisk.

*Conjugation to KLH*

Completed dissacharides **2.7**-**2.10** were conjugated nonspecifically to free lysines on KLH via a thiourea linkage (Scheme 2.4). Constructs **2.7-2.10** were reacted with thiophosgene until all starting material had been consumed according to TLC, converting their free amine termini to isothiocyanates. The resulting crude isothiocyanates were reacted overnight with KLH in carbonate buffer, yielding the final vaccine constructs after purification by buffer exchange.

Conjugate of azide-containing molecules to KLH was demonstrated by dot blot (Figure 2-6.A). The hapten-KLH constructs were reacted with phosphine-FLAG,

an azide-specific reagent with a peptide affinity flag. Only azide-containing KLH constructs should become labeled with the FLAG antigen. After purification by buffer exchange, aliquots of the labeled KLH conjugates were spotted onto nitrocellulose and labeled with an HRP-$\alpha$FLAG antibody. Detection of HRP by fluorescent substrate indicated that the SAzTn-, STnAz-, and SAzTnAz-KLH constructs all contained azides as expected. This assay allows direct detection of azides on KLH, but since this assay is qualitative not quantitative, and success of the STn-KLH conjugate reaction could not be determined this way, I turned to an assay that would indirectly indicate successful conjugation by quantitating the loss of free amines from KLH lysines.




| construct | % conj. (avail. Lysine) |
| --- | --- |
| STnKLH | 35 ± 5 |
| SAzTnKLH | 35 ± 4 |
| STnAzKLH | 26 ± 6 |
| SAzTnAzKLH | 33 ± 7 |

**Figure 2-6.** Conjugagtion to KLH. A. To test qualitiatively whether KLH conjugation was successful, KLH conjugates were treated with PhosFLAG, then washed, spotted on nitrocellulose, and visualized with an HR-α-FLAG antibody. Since STn does contain a reactive azide, its conjuagtion cannot be confirmed with this assay. B. Degree of modification. A fluorescent assay was used for each construct to determine the percentage of available lysines that were modified.

In order to maximize the presentation of the glycans to the immune system in a vaccination strategy, a significant portion of available lysines on KLH should be modified with carbohydrates, but the literature indicates a relatively wide range of modification to be acceptable, with successful results ranging from 8-70% of available lysines being modified (Figure 2-6.B).[22,23] By coupling available amines in modified or umodified KLH with a fluorogenic substrate and looking for the loss in resulting KLH fluorescence in the conjugated molecules, the degree of modification

can be quantitated. Using a trinitrobenzene sulfonic assay developed by Sashidhar and others,[24] I determined that my degree of modification ranged from 25-35% of available lysines—approximately 25 to 60 copies per KLH molecule–which is well within the range of useful conjugation efficiency.

**Antisera generation**

To evaluate the potential of the modified vaccine constructs to elicit an immune response, antisera were generated in rabbits and subsequently analyzed by ELISA. I sent my vaccine constructs to an outside company, ProSci, Inc. for antibody generation. A total of eight rabbits, two rabbits per construct, were immunized according to the schedule in shown in Table 2-1. Each received a total of four immunizations consisting of a hapten-KLH construct and complete or incomplete Freund's adjuvant over six weeks with periodic production bleeds extending to eight weeks. The initial dose of Freund's complete adjuvant, which contains fragments of mycobacteria, serves to augment the immunogenicity of the vaccine by eliciting a strong inflammatory response from the rabbit immune system. Booster immunizations are contains Freund's incomplete adjuvant, basically a suspension of mineral oil in water, which also serves to augment the immune response, but without the same degree of inflammatory side effects as the complete adjuvant.

| Week | Procedure | V/rabbit |
|---|---|---|
| 0 | collect pre-immune serum | 5 mL |
| 0 | immunize 200 μg/rabbit with complete Freund's adjuvant | |
| 2 | immunize 100 μg/rabbit with incomplete Freund's adjuvant | |
| 4 | immunize 100 μg/rabbit with incomplete Freund's adjuvant | |
| 5 | bleed 1 | 20-25 mL |
| 6 | immunize 100 μg/rabbit with incomplete Freund's adjuvant | |
| 7 | bleed 2 | 20-25 mL |
| 8 | bleed 3 | 20-25 mL |

**Table 2-1.** Schedule for antisera generation in rabbits.

**ELISA analysis of antisera**

With antisera generated from each member of my four variant panel, I wanted to answer several questions about my vaccines. First, I wanted to know whether my modified vaccines were more immunogenic than the vaccine based on natural STn and whether the location of the modification or the presence of a dual modification made a significant difference in the immune response. Second, I

wanted to ensure that the IgG antibody response increased over time. Third, I wondered if the antisera were specific to the structure of the antigen against which they were raised. I was most interested in the IgG response, since this is a more mature antibody type than IgM and so would be a better predictor of the ability of the vaccine to elicit an effective immune response. To answer these questions, I employed an enzyme-linked immunosorbent assay (ELISA) diagrammed in Figure 2-7. In this assay, bovine serum albumin (BSA) conjugates of the disaccharide structures are allowed to adhere to the surface of an adherent plate. Dilutions of antisera in buffer are applied and allowed to bind, then the IgG content of the bound antisera is measured using a horseradish peroxidase-conjugated $\alpha$Rabbit IgG antibody, which can then be detected with a fluoregenic reagent. The disaccharide components of the vaccines are appended to bovine serum albumin (BSA) instead of KLH to confine the readout to those antibodies that recognize the sugar portion of the vaccine. Because I was also interested on whether the antisera recognized the individual components of the vaccine in addition to the disaccharide, I also appended the core GalNAc or GalNAz moiety to BSA using the same linker as with the disaccharides.

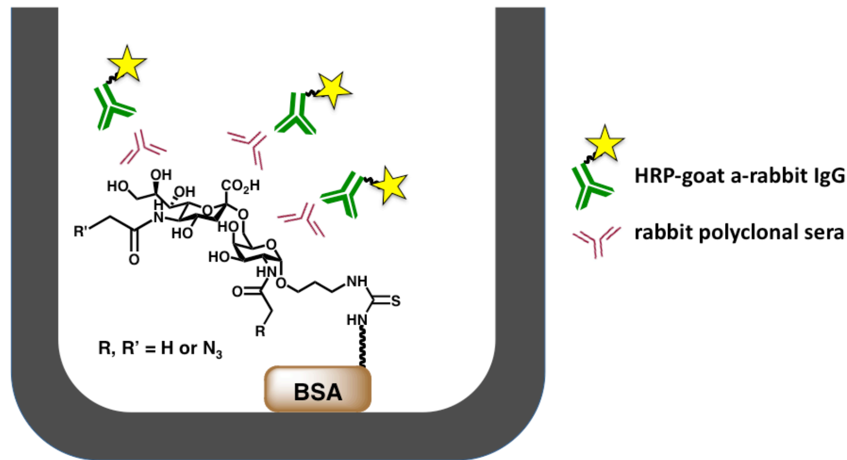



**Figure 2-7.** ELISA analysis schematic. The antisera were analysed by an ELISA assay that utilized BSA constructs of each disaccharide as well as the monosaccharides GalNAc and GalNAz. BSA was used to avoid the detection of antibodies that recognized KLH. In the assay, poloclonal antisera are allowed to bind with the STn-BSA constructs in microtiter wells, then the IgG antibodies that stick are detected with HRP-conjugated goat-a-rabbit IgG.

To determine the relative immunogenicity of the components, antisera generated from each KLH construct were tested against the BSA construct of the same carbohydrate antigen. For example, αSAzTn antisera were tested in a well containing SAzTn-BSA and αSTn antisera were tested in a well with STn-BSA (Figure 2-8). The resulting titers ranged from 750,000 to over 2,000,000, which are surprisingly high for polyclonal antisera. These titers are more than ten times greater than those seen in mice in similar studies by the Guo lab.[22]

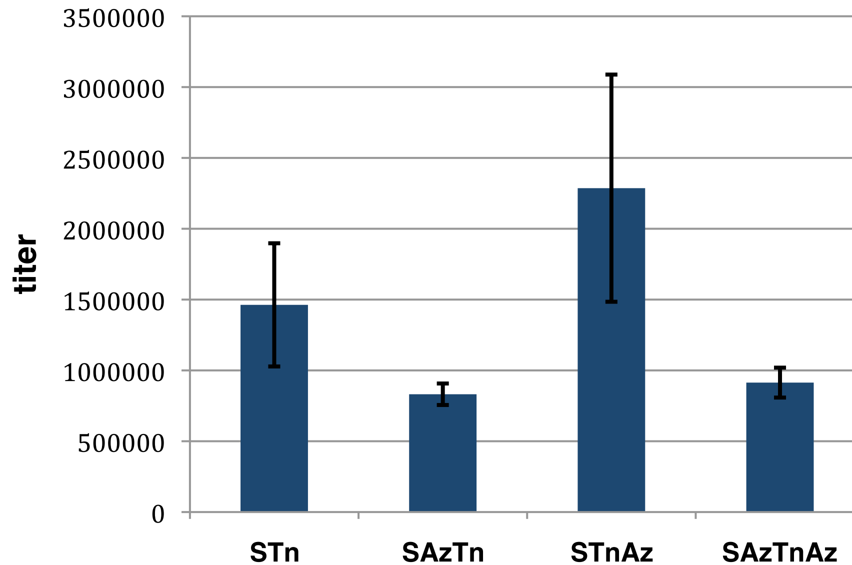



**Figure 2-8.** Antigen-specific antibody titer.

In addition to being unexpectedly high, the antigen-specific antibody titer is surprisingly insensitive to the structure of the antigen. The polyclonal antisera raised against STnAz is the most immunogenic substrate followed, by STn, the unmodified construct, then the other two constructs. This result could be interpreted in several ways. It could be that azide modification of STn is not a great enough modification to break immune self-tolerance and generate a more potent immune response. Perhaps rabbits find natural STn to be particular immunogenic, and so do not respond further to a modified antigen. It is difficult to compare these results to other studies since most of those were performed in mice. Another possibility is that the linker is the most immunogenic portion of the molecule, and

the antisera response is mostly measuring the number of antibodies that recognize the linker portion of the molecule.

While modifying the GalNAc residue of STn does increase the specific IgG response in rabbits, modifying the GalNAc residue of SAzTn leads to only a slight increase. Also, modifying the sialic acid residue of STn or STnAz does not augment the immune response. In fact, it appears to decrease the response. The main conclusion to be drawn from these data are that all four constructs lead to similar immune responses in rabbits; the small differences between the levels of IgG antibodies may not be particularly meaningful, especially given a sample size of only two rabbits.

To investigate whether the IgG response increases over time, antisera raised against SAzTnAzKLH taken at either 5 or 8 weeks were assayed against BSA conjugates of each disaccharide (Figure 2-9). The 8-week antisera were better able to detect all four antigens than the 5-week sera. Futher, the selectivity of the 8-week sera for the doubly-modified antigen against which they were raised was much higher than the 5-week sera. In fact, the 5-week sera showed an unexpected preference to bind SAzTnBSA over SAzTnAzBSA, but that preference was reversed in the more mature sera.

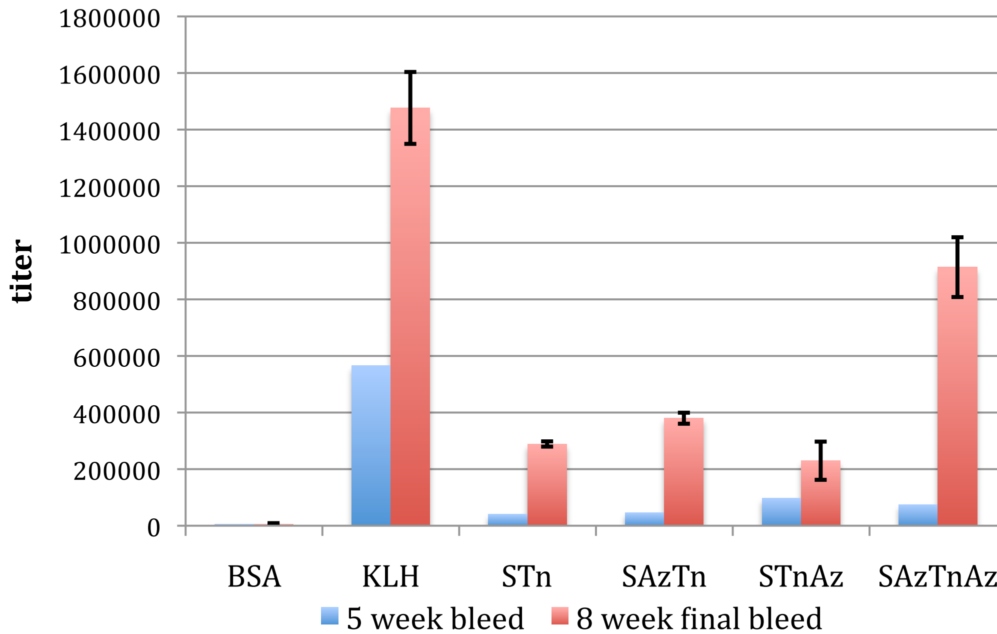



**Figure 2-9.** Antisera maturation. 5-week and 8-week α-SAzTnAz polysera mature with time, becoming more potent and specific.

To further investigate the specificity of the immune response to the, I tested 8-week antisera raised against each construct against BSA conjugates of each disaccharide, GalNAc, and GalNAz (Figure 2-10). αSTnKLH sera showed only a slight decrease in selectivity when the GalAc residue was modified, and virtually no decrease when the sialic acid residue was modified. It was, however, selective for the disaccharide over the monosaccharides. αSAzTnKLH sera showed a more pronounced preference for disaccharides without a modified GalNAc residue, though virtually no preference for modification of the sialic acid residue. Interestingly, while the sera preferred the disaccharides to the monosaccharides, they preferred the GalNAz conjugate to the GalNAc conjugate. This is surprising since the KLH conjugate the antisera were raised against contained the unmodified

GalNAc component. αSTnAzKLH sera show modest selectivity for the BSA conjugate of STnAz, and amongst the other dissacharides, showed a very slight preference for the GalNAz containing conjugate. Unlike the previous antisera, however, αSTnAzKLH sera showed a strong response against the BSA conjugate of GalNAz, though not against that of GalNAc. Finally, αSAzTnAzKLH sera showed better specificity for the doubly modified disaccharide than any of the other antisera showed against their own antigen. Modification of either sialic acid or GalNAc component on its own showed very little (sialic acid) or no (GalNAc) increase in relevant IgG. Both components must be modified to see the increase in specificicty. However, like the αSTnAzKLH sera, the αSAzTnAzKLH sera showed significant cross-reactivity with the BSA conjugate of GalNAz, though not with GalNAc.

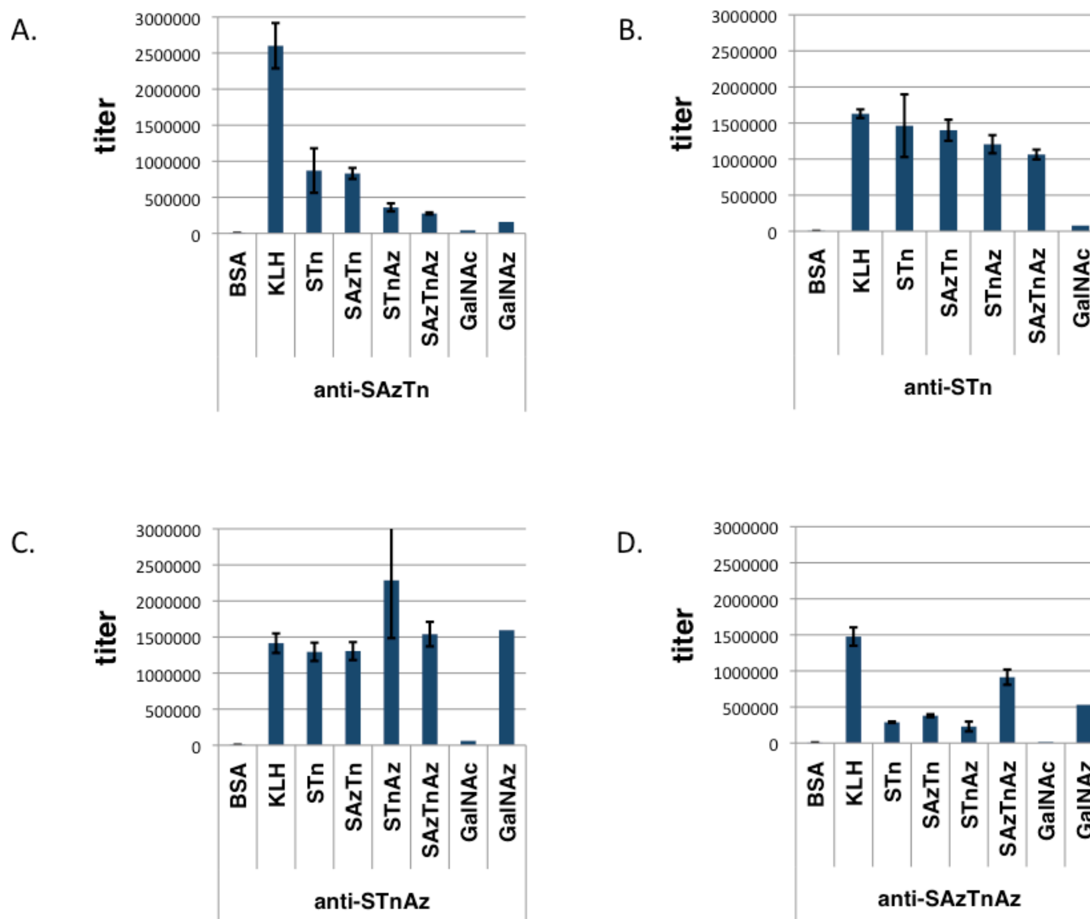



**Figure 2-10.** Antisera cross-reactivity. Polyclonal antisera raised against SAzTn (A), STn (B), STnAz (C) and SAzTnAz are tested against BSA constructs of each disaccharides as well as GalNAc and GalNAz, as well as unconjugated BSA and KLH as negative and positive controls, respectively.

## Conclusion

Four versions of the STn disaccharide were synthesized and conjugated to KLH to produce a panel of four "natural" and "unnatural" carbohydrate cancer vaccine constructs. 8 rabbits were immunized with these constructs along with

adjuvant, and antisera were collected from these rabbits at various time points. Theses antisera were analyzed by ELISA to determine the IgG response against the "natural" and "modified" cancer antigens.

Antisera from every rabbit showed strong IgG recognition of the cancer antigens in the ELISA, indicating that the immunization procedure produced a strong antibody response in the rabbits. Modifying the antigens to render them "unnatural," however, showed almost no benefit. A very modest increase in immunogenicity appeared to correlate with the modification of the GalNAc portion of STn. Given the small nature of the increase, however, and coupled with the fact that modification of the sialic acid portion appeared to *decrease* the specific IgG response, the benefit to the strength of the immune response of modifying the either component with an azide is likely negligible in rabbits.

While modifying the vaccine did not lead to a significant increase in the immune response, it did lead to a modest increase in the selectivity of the antisera, particularly with the doubly modified antigen. In general, however, the antisera were fairly promiscuous to STn analogs, and the antisera raised against KLH constructs with modified GalNAc components were also able to recognize the GalNAz monosaccharides.

Due to competing benefits, the importance of the selectivity of the antisera to other versions of the cancer antigen is not entirely clear. On one hand, a promiscuous response would make cancer cells with antigens that had not been modified by metabolic engineering vulnerable the immune response. This would be

beneficial since it generally increases the number of effective antigens for the immune response. Further, some cancer cells may be in tissues that are poorly accessible to the metabolic engineering precursor, but are accessible to antibodies and immune cells. For these reasons, a response that is promiscuous might actually be better suited to eliminating cancer cells than a more specific response, particularly for eliminating metastatic cells that can spread into various tissues and environments in the body. On the other hand, a response that recognizes the natural antigen would not be temporally controllable by the supply of metabolic precursors. A possible benefit of the cancer immunotherapy strategy described in this dissertation is that it allows cessation of the metabolic engineering portion of treatment at any time, after which the unnatural sugars would gradually be metabolized away as glycans are turned over in the cells. The benefit would be for those patients who show harmful autoimmune symptoms—perhaps if they happen to express more STn on healthy tissues than most patients. In those cases, a specific immune response could be halted by ending supply of sugar metabolic precursors. While the antibodies and immune cells recognizing the modified antigens would still be there, the antigens they recognized would be gone. However, if the antigens and immune cells recognize both the modified and natural antigens, the response could not be turned off.

The general lack of specificity is a further indication that the presence of the azide modification was not sufficient to make the STn constructs seem "foreign" to the immune system in rabbits. The strategy was based on the theory that natural

STn could not strongly recognized by relevant immune cells, and so modification was necessary to overcome this problem. Since, for the most part, the natural antigen was recognized to a similar degree as the modified antigens, it appears that the azido modification is not turning on a capability that is lacking for the natural antigen.

More worrisome than the fact that the immune response is promiscuous toward the various modifications of disaccharide antigen is that the immune response elicited by the vaccine constructions containing the GalNAz component were able to recognize the GalNAz monosaccharide. This is particularly worrisome since tissues that are healthy as well as cancerous would be expressing various GalNAz containing glycans when a patient is provided sugar metabolic precursors. Targeting of the response to cancer cells requires that the response is directed against the full antigen and not its components, which are found ubiquitously in cells. Since none of the vaccines lead to a significant response against the natural monosaccharide GalNAc, it appears this possible autoimmune susceptibility could be overcome by halting the supply of the precursors, but for this to one day be an effective strategy for cancer treatment, the immune response must be specific to the structure of the cancer antigen.

**Future outlook**

The work presented in this dissertation indicates that, in rabbits, azide modification of STn antigen does not appear to be sufficient to break immune self

tolerance and lead to a powerful immune response that can be directed against cancer cells. However, this does not mean that the strategy in not viable or should no longer be pursued. The doubly modified antigen presented here shows some promise given the selectivity of the immune response it provoked, so the strategy of multiple modifications seems worth pursuing. Work performed in the Guo lab has indicted that, in mice, the size of the modification is important to how much the immune system is stimulated.[15] This suggests that future work should focus on optimizing the modifications of natural antigens such that self-tolerance can be overcome while still allowing for *in vivo* modification of the antigen on cancer cells by metabolic engineering. The use of the azide provides the option of further *in vivo* elaboration of the tumor antigen after metabolic engineering by selective reaction with a bioorthoganol azide-reactive reagent. This would greatly expand the extent of modification that could be accessed on cancer cells, however, it would also lead to large modifications of the sugar analogs in healthy cells, which might lead to autoimmune complications. This illustrates the overarching conundrum of the modified cancer vaccine approach: the modification must be large enough to render a cancer antigen "foreign" and susceptible to attack by an immune system primed with a vaccine, however, the modification can not be so great as to cause an harmful immune response outside the context of a vaccine.

Even with optimization of the modification, this vaccination strategy may never reach its full potential without combining the antigen modification strategy with other strategies currently being pursued by other researchers. Using a more

natural amino acid-like linker and presenting the antigens as mixed clusters as Danishefsky and coworkers do makes good sense and will likely lead to a better vaccine. Perhaps conjugating a TLR2 agonist to the modified TACA and using a promiscuous T-cell epitope instead of KLH like the researchers in the Boons lab will result in better activation of the immune system. And maybe presentation of the vaccine directly to APCs *ex vivo* like with Provenge will be necessary to avoid the immune suppressing effects orchestrated by the tumors *in vivo*. Cancer vaccines are beginning to make a tiny dent in cancer, and that dent will only grown with time, but cancer is a complex disease, and the answer to it must likewise be complex.

## Materials and methods

### *General methods*

Dulbecco's phosphate-buffered saline (PBS), α-FLAG M2-peroxidase (HRP-α-FLAG), keyhole limpet hemocyanin (KLH), and bovine serum albumin (BSA) were from Sigma. HRP-goat-α-rabbit IgG antibody was from Promega. NeuAc aldolase, CMP-sialic acid synthetase and α2,6-sialyltransferase enzymes were a gracious gift from Xi Chen's laboratory at UC Davis.

All chemical reagents obtained from commercial suppliers were used without further purification. All reactions were performed in flame-dried glassware with the exception of reactions performed in aqueous media. Water was obtained from a Milli-Q purification system for use in all manipulations. Thin layer chromatography was performed on glass-backed Analtech Uniplate® silica gel plates. Plates were

visualized with UV light or by staining with either 5% sulfuric acid in ethanol or CAM stain. Flash chromatography was performed using Merck 60 Å 230-400 mesh silica gel. Reversed-phase HPLC was performed on a Varian PrepStar system with a Varian ProStar 325 UV-Vis detector on Microsorb C-18 preparative (21.4 x 250 mm) and super-preparative (41.4 x 250 mm) columns at a flow rate of 20 mL/min and 80 mL/min, respectively. Normal phase HPLC was performed on analytical Agilent Zorbax NH2 amino column at a flow rate of 1 mL/min with a Varian ProStar system with a Varian 345 UV-Vis detector. NMR spectra were obtained on Bruker AV-600, DRX-500, AVB-400 and AVQ-400 MHz spectrometers at the UC-Berkeley College of Chemistry NMR Facility. $^{1}$H chemical shifts are reported as δ referenced to solvent, and coupling constants (*J*) are reported in Hz. $^{13}$C resonances are unassigned and reported as observed. High resolution mass spectrometry was performed at the UC Berkeley Mass Spectrometry Laboratory.

*Synthetic procedures*

**(2*R*,3*R*)-3,4-bis(benzyloxy)-2-(benzyloxymethyl)-3,4-dihydro-2*H*-pyran (2.11).** **2.11** was prepared according to a literature procedure.[25]

**(2*R*,3*R*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-(benzyloxymethyl)-3-nitrotetrahydro-2*H*-pyran-2-yl acetate (2.13).** **2.13** was prepared according to a literature procedure.[21]

**(2*R*,3*R*)-3,4-bis(benzyloxy)-2-(benzyloxymethyl)-5-nitro-3,4-dihydro-2*H*-pyran (2.12).** **2.12** was prepared according to a literature procedure.[21]

***tert*-butyl 3-((2*S*,3*S*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-(benzyloxymethyl)-3-nitrotetrahydro-2*H*-pyran-2-yloxy)propylcarbamate (2.14).** **2.12** (878 mg, 1.9 mmol) and *tert*-butyl 3-hydroxypropylcarbamate (427 mg, 2.4 mmol) were dissolved in dry toluene and cooled to 0 °C. Potassium t-butoxide (21 mg, 0.19 mmol) was added and the mixture was stirred and allowed to warm to room temperature overnight. Acetic acid was added dropwise until the solution became just acidic. The reaction mixture was concentrated *in vacuo* and purified by flash chromatography on silica gel (3:7 EtOAc:hexanes) to yield 800 mg (62%) of a clear yellow oil. Rf = 0.67 (2:1 hexanes:ethyl acetate, UV). $^1$H NMR ($CDCl_3$, 500 MHz): δ 1.45 (s, 9H), 1.70-1.79 (m, 2H), 3.07-3.25 (m, 2H), 3.45 (dd, 1H, *J* = 10.5, 5.5 Hz), 3.48 (dd, 1H, *J* = 15.0, 6.0 Hz), 3.55, (app t, 1H, *J* = 7.5)3.75-3.82 (m, 1H), 3.95-4.03 (m, 2H), 4.41-4.49 (m, 3H), 4.54 (d, 1H, *J* = 12.0 Hz), 4.74 (d, 1H, *J* = 11.0 Hz), 4.77 (d, 1H, *J* = 10.5 Hz), 4.84 (d, 1H, *J* = 11.0 Hz), 4.95 (br s, 1H), 5.02 (dd, 1H, *J* = 10.5, 4.0 Hz), 5.31 (d, 1H, *J* = 4.0 Hz), 7.20-7.39 (m, 15 H). $^{13}$C NMR ($CDCl_3$, 125 MHz): δ 28.37, 29.24, 37.52, 66.39, 68.40, 69.86, 73.03, 73.10, 73.45, 74.93, 75.21, 78.86 84.24, 96.21, 127.84, 128.04, 128.12, 128.16, 128.30, 128.41, 128.48, 137.20, 137.50, 137.76, 155.96. HRMS (ESI): Calcd for $C_{35}H_{44}N_2O_9$ $[M+Li]^+$ 643.3201, found 643.3203.

***tert*-butyl 3-((2*S*,3*R*,4*R*,5*R*,6*R*)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy)propylcarbamate (2.1)** and ***tert*-butyl 3-((2*S*,3*R*,4*R*,5*R*,6*R*)-3-(2-azidoacetamido)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy)propylcarbamate (2.2). 2.1** and **2.2** were synthesized by an identical procedure. For **2.2** as a representative synthesis, **2.12** (242 mg, 0.380 mmol) was dissolved in THF (4 mL) and placed in a Parr bomb. Palladium hydroxide on carbon [Pd($OH_2$)/C] (106 mg) was added and the bomb was sealed and charged with 100 bar $H_2$. The reaction was stirred at 50 °C overnight, then the bomb was opened and the reaction was diluted with methanol. Pd($OH_2$)/C was removed from the mixture by filtration through celite, and the crude amine product (**2.13**) was concentrated *in vacuo* and dried by coevaporation with toluene. A portion of the crude amine (12 mg, 0.036 mmol) was dissolved in dry methanol (0.5 mL). Azidoacetic acid (acetic acid for **2.1**, 3.5 mg, .035 mmol) and triethylamine (.02 mL, .05 mmol) were added, and the solution was cooled to 0°C. 1-hydroxybenzo-triazole (HOBt, 5.3 mg, .037) and N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 15 mg, .074) were added. The mixture was stirred at 0 °C for one half hour then allowed to warm to room temperature. After 2 hours, the reaction mixture was concentrated *in vacuo* and purified by flash column chromatography (5% MeOH in $CH_2Cl_2$) to yield 6.3 mg (77%) of a clear oil.

**2.2** Rf = 0.77 (3:2 MeOH: $CH_2Cl_2$, $H_2SO_4$ stain). $^1$H NMR (MeOD, 400 MHz): δ 1.42 (s, 9H), 1.72-1.82 (m, 2H), 3.04-3.21 (m, 2H), 3.37-3.55 (m, 1H), 3.68-3.83 (m, 6H),

3.88-3.91 (m, 1H), 3.99 (s, 2H), 4.28 (dd, 1H, *J* = 10,8, 3.6 Hz), 4.88 (d, 1H, *J* = 3.6 Hz). $^{13}$C NMR (MeOD, 100 MHz): δ 28.92, 30.92, 38.46, 51.77, 52.97, 62.98, 66.24, 69.94, 70.51, 72.72, 80.07, 98.87, 158.68, 170.85. HRMS (ESI): Calcd for $C_{16}H_{29}N_5O_8$ $[M+Li]^+$ 426.2171, found 426.2167.

**2.1** 65% yield. Rf = 0.74 (3:2 MeOH: $CH_2Cl_2$, $H_2SO_4$ stain). $^1$H NMR (MeOD, 400 MHz): δ 1.43 (s, 9H), 1.71-1.76 (m, 2H), 2.01 (s, 3H), 3.07-3.23 (m, 2H), 3.38-3.43 (m, 1 H), 3.66-3.80 (m, 6H), 3.87-3.90 (m, 1H), 4.25 (dd, 1H, *J* = 10.8, 3.6 Hz), 4.85 (dd, 1H, *J* = 3.6 Hz). $^{13}$C NMR (MeOD, 100 MHz): δ 22.85, 28.91, 30.89, 38.48, 51.73, 63.00, 66.17, 70.03, 70.53, 72.63, 80.05, 98.92, 158.68, 174.25. HRMS (ESI): Calcd for $C_{16}H_{30}N_2O_8$ $[M+Li]^+$ 385.2157, found 385.2163.

*Enzymatic synthesis of dissacharides **2.3-2.6**.*

Enzymatic synthesis was peformed as described.[19] Briefly, an acceptor (**2.1** or **2.2**, 0.023 mmol) , a donor (ManNAc or ManNAz, 0.035 mmol), sodium pyruvate (13 mg, 0.12 mmol), and CTP ( 20 mg, 0.035 mmol) were dissolved in Tris buffer (2 mL, 100 mM, pH 8.8, 20 mM $MgCl_2$). An enzyme cocktail containing aldolase (2 U), sialic acid synthetase (2U), and (α)2-6-sialyltranserase (1U) was added, and the reaction was placed in a 37 °C shaker overnight. Then, ethanol was added (1 mL) and the mixture was incubated on ice for 30 minutes, then spun on a table top centrifuge to pellet precipitate. The supernatant was collected and the precipitate was washed with water (1 mL), and pelleted again. The supernatants were

combined, and the solvents were removed *in vacuo*. **2.3-2.6** were purified by reverse phase HPLC (1% to 30% acetonitrile in water, 0.1% TFA), leaving white solid after lyophilization (8-30% yield). Compound **2.4** was unable to be separated from **2.1** by HPLC, so was carried through as a mixture of those two compounds and purified after the next step. **2.3**, **2.5**, and **2.6** were isolated in small quantities such that $^{13}$C analysis was unable to detect all of the highly substituted carbon atoms, even with extended (12 hour) collection times on a 600 MHz instrument.

**2.3**. Rf = 0.28 (4:1:0.1 acetonitrile:water:TFA, CAM stain). $^{1}$H NMR (MeOD, 400 MHz): δ 1.43 (s, 9H), 1.5 (m, 3H), 1.99 (s, 3H), 2.01 (s, 3H) 2.70 (dd, 1H, *J* = 13.2, 4.0 Hz), 3.15-3.22 (m, 1H), 3.33-3.45 (m, 1H), 3.48-3.57 (m, 2H), 3.51-3.67 (m, 1H), 3.69-3.79 (m, 6H), 3.82-3.86 (m, 2H), 3.88-3.91 (m, 3H), 4.24 (dd, 1H, *J* = 7.2, 2.4), 4.75 (d, 1H, *J* = 2.8). $^{13}$C NMR (MeOD, 150 MHz): δ 22.79, 28.85, 28.95, 38.67, 41.93, 49.78, 51.71, 54.07, 64.65, 64.93, 66.54, 69.07, 69.58, 69.91, 70.44, 71.28, 73.21, 75.03, 99.40, 173.99, 175.64. HRMS (ESI): Calcd for $C_{27}H_{47}N_3O_{16}$ [M+Na+]$^+$ 692.2849, found 692.2847.

**2.4** Rf = 0.30 (4:1:0.1 acetonitrile:water:TFA, CAM stain). HRMS (ESI): Calcd for $C_{27}H_{46}N_6O_{16}$ [M+Na+]$^+$ 733.2863, found 733.2845.

**2.5** Rf = 0.30 (4:1:0.1 acetonitrile:water:TFA, CAM stain). $^{1}$H NMR (MeOD, 500 MHz): δ 1.43 (s, 9H), 1.72-1.78 (m, 3H), 1.99 (s, 3H), 2.70 (dd, 1H, *J* = 8.5, 4.5 Hz), 3.04-3.22

(m, 1H), 3.36-3.41 (m, 1H), 3.49-3.60 (m, 2H), 3.60-3.67 (m, 1H), 3.69-3.92 (m, 11H), 3.94 (s, 2H), 4.24-4.30 (m, 1H), 4.77 (d, 1H, *J* = 3.5 Hz). $^{13}$C NMR (MeOD, 150 MHz): δ 22.62, 28.98, 42.03, 49.72, 51.95, 53.00, 53.94, 64.14, 64.73, 66.07, 69.13, 69.96, 70.05, 70.12, 71.35, 73.05, 75.64, 80.86, 99.10. HRMS (ESI): Calcd for $C_{27}H_{46}N_6O_{16}$ $[M+Na+]^+$ 733.2863, found 733.2858.

**2.6** Rf = 0.33 (4:1:0.1 acetonitrile:water:TFA, CAM stain). $^1$H NMR (MeOD, 400 MHz): δ 1.43 (s, 9H), 1.71-1.80 (m, 3H), 2.71 (dd, 1H, *J* = 12.8, 4.4 Hz), 3.09-3.20 (m, 2H), 3.35-3.40 (m, 2H), 3.48-3.52 (m, 1H), 3.62-3.98 (m, 16H), 4.30 (m, 1H), 4.80 (d, 1H, *J* = 4.0 Hz). $^{13}$C NMR (MeOD, 150 MHz) δ 28.93, 29.13, 38.37, 41.95, 49.72, 51.35, 52.78, 52.84, 54.37, 64.49, 64.72, 66.35, 69.18, 69.55, 69.90, 70.08, 70.24, 71.28, 73.31, 74.70, 99.86. HRMS (ESI): Calcd for $C_{27}H_{45}N_9O_{16}$ $[M+Na+]^+$ 774.2876, found 774.2869.

*Deprotection of dissacharides*

Disaccharides **2.3**-.**2.6** were placed in a vial over ice and 1 droplet of isopropylsilane was added. Approximately 0.5 mL of TFA was added, and the mixture was mixed by pipetting the mixture up and down repeatedly. After one minute, the reaction mixture was diluted with cold toluene, and the TFA was removed by coevaporation with toluene to give crude **2.7**-**2.10**. **2.7**-**2.10** were purified by normal phase HPLC on an amino column (5-100% 10mM $NH_4OAc$, pH 5.5, in acetonitrile). The quantities of **2.3**-**2.6** isolated were insufficient to allow for $^{13}$C analysis, even after

extended (12 hours) data collection with a 600 MHz instrument.

**2.7.** Rf = 0.12 (3:1:1 acetonitrile:water:acetic acid). $^{1}$H NMR (MeOD, 500 MHz): δ 1.59 (t, 1H, *J* = 12.5 Hz), 1.94-1.99 (m, 2H), 1.97 (s, 3H), 2.00 (s, 3H), 2.79 (dd, 1H, *J* = 12.5, 4.5 Hz), 2.96-3.12 (m, 2H), 3.42-3.47 (m, 1H), 3.51-3.68 (m, 5H), 3.74-3.90 (m, 6H), 3.96-4.0 (m, 2H), 4.23 (dd, 1H, *J* = 11.0, 4.0 Hz), 4.79 (d, 1H, *J* = 4.0 Hz). HRMS (ESI): Calcd for $C_{22}H_{39}N_3O_{14}$ $[M+Li+]^+$ 576.2587 found 576.2580.

**2.8** Rf = 0.14 (3:1:1 acetonitrile:water:acetic acid). $^{1}$H NMR (MeOD, 500 MHz): δ 1.57–1.64 (m, 1H), 1.94–1.98 (m, 2H), 1.97 (s, 3H), 2.80 (dd, 1H, *J* = 11.5, 3.5), 2.98-3.14 (m, 2H), 3.45-3.49 (m, 1H), 3.53-3.62 (m, 3H), 3.71-4.00 (m, 12H), 4.23 (dd, 1H, *J* = 11.0, 3.5 Hz), 4.79 (d, 1H, *J* = 3.5 Hz). HRMS (ESI): Calcd for $C_{22}H_{38}N_6O_{14}$ $[M+Li+]^+$ 617.2601, found 617.2592.

**2.9** Rf = 0.14 (3:1:1 acetonitrile:water:acetic acid). $^{1}$H NMR (MeOD, 500 MHz): δ 1.60 (t, 1H, *J* = 11.5 Hz), 1.93-1.99 (m, 2H), 2.00 (s, 3H), 2.80 (dd, 1H, *J* = 12.0, 4.5 Hz), 2.97-3.12 (m, 2H), 3.42-3.71 (m, 6H), 3.75-4.02 (m, 10H), 4.25 (dd, 1H, *J* = 11.0, 4.0 Hz), 4.81 (d, 1H, *J* = 3.5 Hz). HRMS (ESI): Calcd for $C_{22}H_{38}N_6O_{14}$ $[M+Li+]^+$ 617.2601, found 617.2591.

**2.10** Rf = 0.15 (3:1:1 acetonitrile:water:acetic acid). $^{1}$H NMR (MeOD, 400 MHz): δ 1.59-1.64 (m, 1H), 1.94-2.02 (m, 2H), 2.79 (dd, 1H, *J* = 11.5, 4.0 Hz), 2.99-3.14 (m, 2

H), 3.53-3.64 (m, 3H), 3.70-3.98 (m, 15H), 4.26 (dd, 1H, *J* = 11.0, 4.0 Hz), 4.82 (d, 1H, *J* = 4.0 Hz). HRMS (ESI): Calcd for $C_{22}H_{37}N_9O_{14}$ $[M+Li+]^+$ 658.2615, found 658.2607.

*Protein conjugation of dissacharides*

**2.7-2.8** (.0032 mmol) were dissolved individually in $CDCl_3$ (15 μL) , and each was added to a mass spectroscopy vial fitted with a stir bar. A 2% solution of thiophosgene in $CDCl_3$ was added (15 μL), and the mixture was stirred at room temperature. After 3 hours, the solvents were removed *in vacuo* and the crude isothiocyanate construct was resuspended in $NaHCO_3$ buffer (38 μL, 300 mM, pH 9) in a 0.5 mL Eppendorf tube. KLH (1 mg KLH in approximately 4 mg lyophilized power) or BSA (1 mg) dissolved in the same buffer (38 μL,) was added, and the solution was shaken at 400 RPM at room temperature overnight. The conjugated proteins were purified by buffer exchange using 30 kD MW cut-off centrifugal concentrators (4 x approx. 50 μL diluted to 4 mL), and were stored at 4 °C at approximately 10 mg/mL concentration.

*Dot blot*

STn analog-KLH conjugates were incubated with 250 μM azide-reactive phosphine-FLAG in PBS for 1 hour at room temperature. The conjugates were purified by buffer exchange by spin concentrator (30 kD MW cutoff, approx. 80x dilution repeated 4 times) and 4 μL of each conjugate was spotted on nitrocellulose blotting paper. Blot was blocked with 5% milk for 1 hour, then incubated with HRP-α-FLAG

(1:10,000) in 5% milk for 1 hour.  Blot was washed with PBS + 0.05% Tween 20 (3 x 10') and PBS (1 x 5'), then visualized with SuperSignal West Pico visualization kit.

*TNBS assay*

Serial dilutions of lysine and glutamic acid (0.01-0.1 mg/mL) in BSA were prepared and loaded in triplicate (10 μL each) into the wells of  a 96-well assay plate.  Also added to the plate were samples of free or STn analog-conjugated KLH at a standard concentration in PBS (target concentration 0.1 - 1 mg/mL).  To each well was added 2.2% $NaHCO_3$ buffer (90 μL, pH 8.5) and 0.01% trinitrobenzene sulfonic acid (TNBS, 50 μL).  The plate was incubated at 42 °C for 2 hours, then 10% SDS (50 μL) and 1N HCl (25 μL) were added, and the plate absorbance was read at 335 nm.  A standard curve was generated from difference between the lysine and glutamic acid absorbances, and this curve was used to estimate the number of lysines in each sample (N).  The percent of available lysines that were conjugated was calculated with the formula: $\%conj = 100\% * N_{sam} / N_{KLH}$ , where $N_{sam}$ is the number of lysines found to be available on the KLH conjugate of interest and $N_{KLH}$ is the number found on unmodified KLH.

*Antisera generation*

Antisera generation was carried out by ProSci Corporation in rabbits according to their standard protocol (Table 2-1).

*ELISA*

Into each well of a 96-well ELISA plate was added 100 μL of STn analog-BSA conjugate, KLH (positive control), or unconjugated BSA (negative control) at 40 μg/mL concentration in 50 mM $NaHCO_3$ buffer. The plate was covered and incubated at room temperature for 24 hours. The plate was emptied and washed 3x with water. Blocking buffer (200 μL, 0.1% BSA in PBS) was added and allowed to incubate for 2 hours. After discarding buffer, serial dilutions of antisera in blocking buffer (125 μL) were added and incubated for one hour at room temperature. The plate was emptied and washed twice with wash buffer (200 μL, 0.05% Tween 20 in PBS) and once with water (200 μL). HRP-goat-α-rabbit IgG was added (100 μL, 1:50,000), and the plate incubated for one hour. Plates were washed again with wash buffer (2 x 200 μL) and water (1 x 200 μL), then developed with TMB substrate and read by absorbance at 450 nm.

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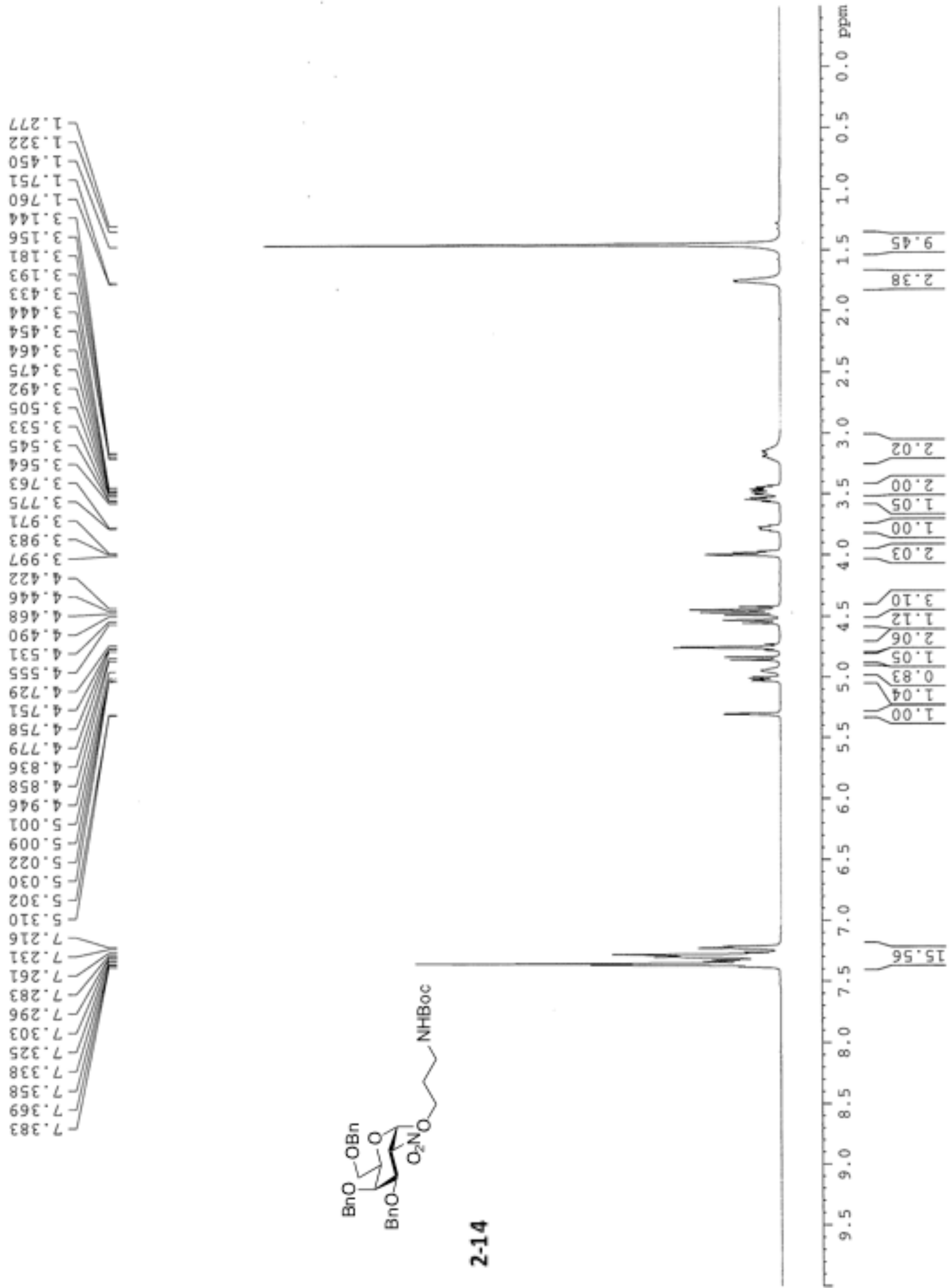
# Appendix

## $^{1}H$ and $^{13}C$ NMR Spectra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BnO
OBn
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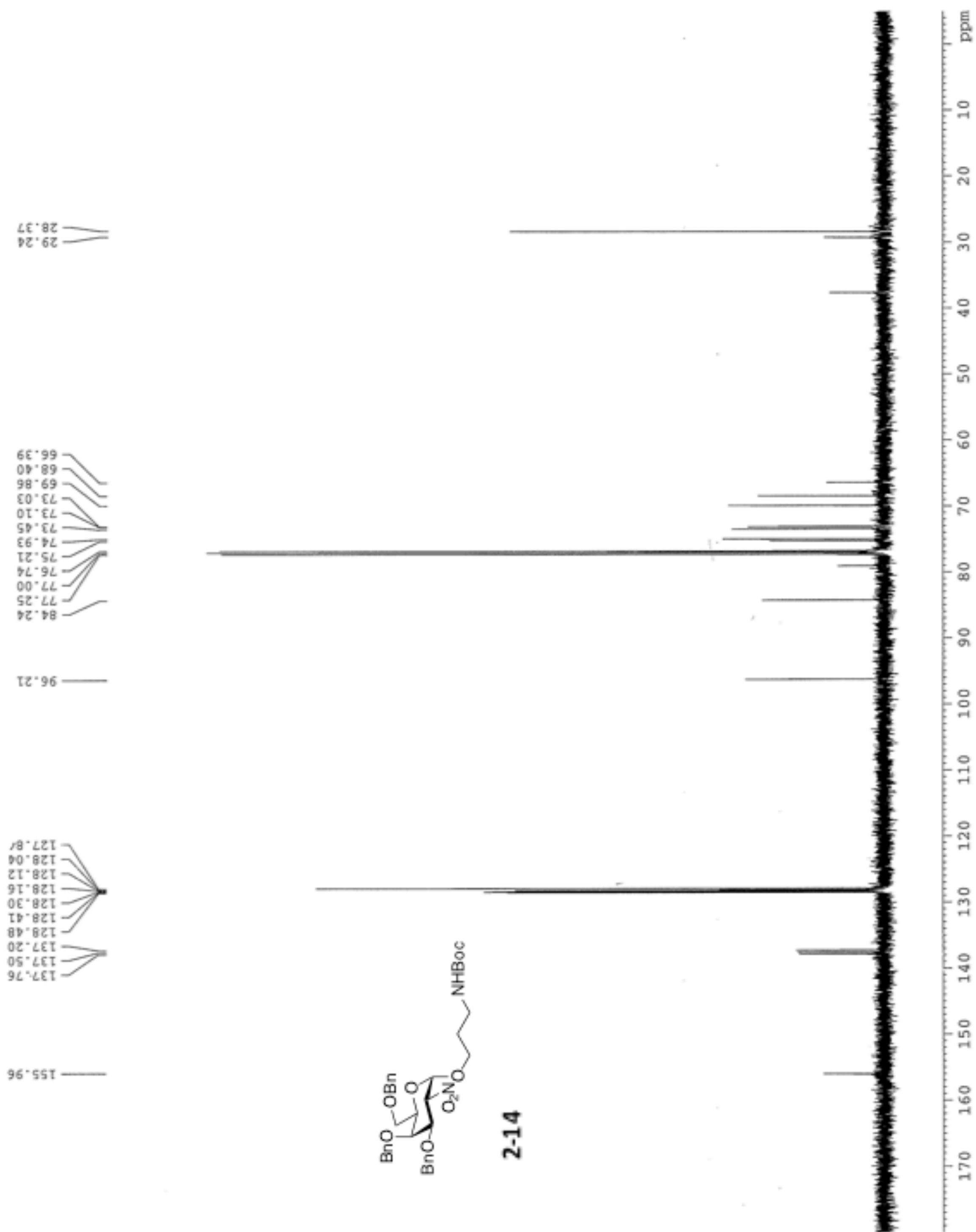

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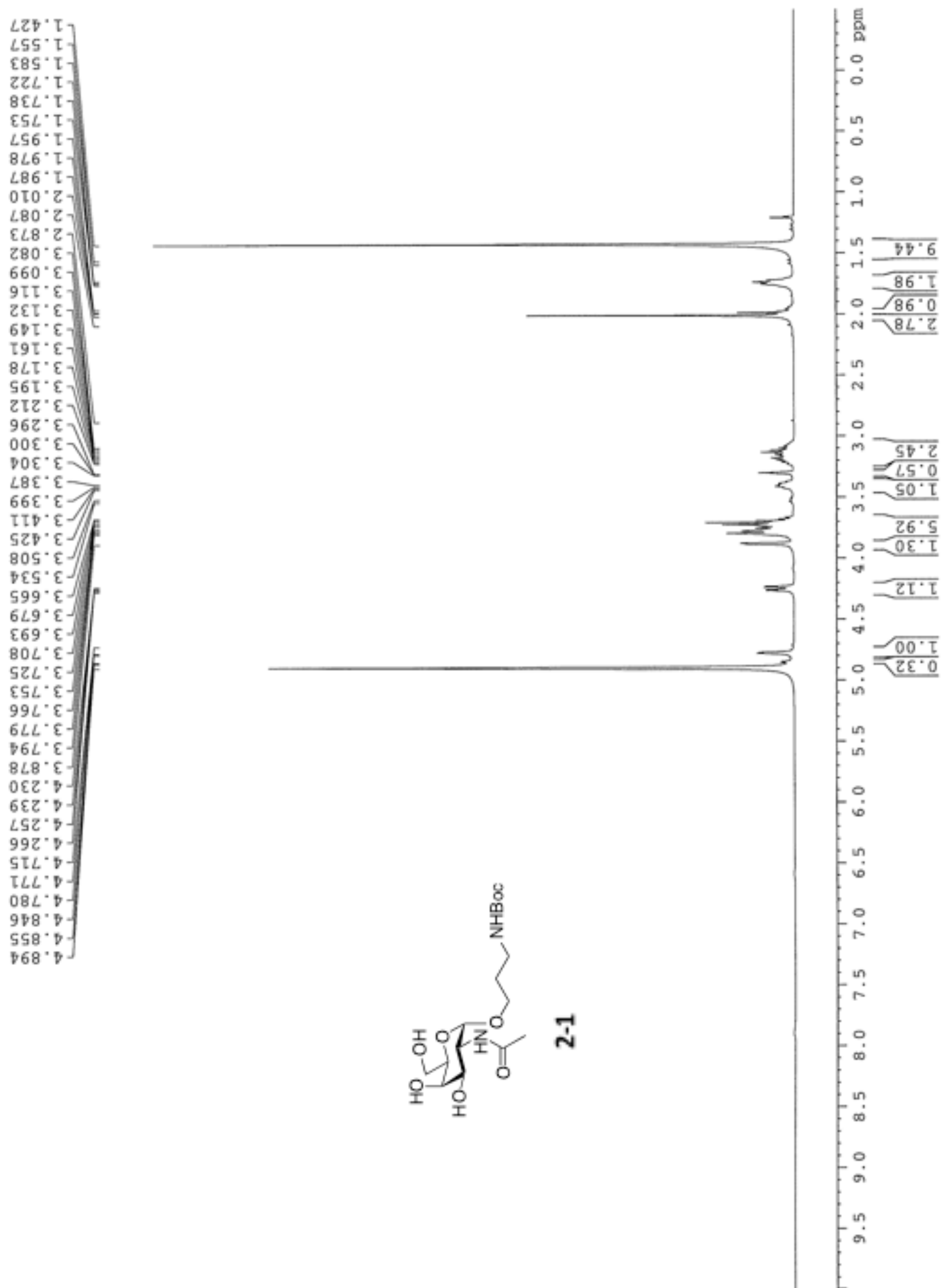
29.24
66.39
68.40
69.86
73.03
73.10
73.45
74.93
75.21
76.74
77.00
77.25
84.24
96.21
127.87
128.04
128.12
128.16
128.30
128.41
128.48
137.20
137.50
137.76
155.96
NHBoc
2-14
ppm



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1.753
1.957
1.978
1.987
2.010
2.087
2.873
3.082
3.099
3.116
3.132
3.149
3.161
3.178
3.195
3.212
3.296
3.300
3.304
3.387
3.399
3.411
3.425
3.508
3.534
3.665
3.679
3.693
3.708
3.725
3.753
3.766
3.779
3.794
3.878
4.230
4.239
4.257
4.266
4.715
4.771
4.780
4.846
4.855
4.894
HO
OH
HO
O
HN
O
O
NHBoc
2-1
9.44
1.98
0.98
2.78
2.45
0.57
1.05
5.92
1.30
1.12
1.00
0.32
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
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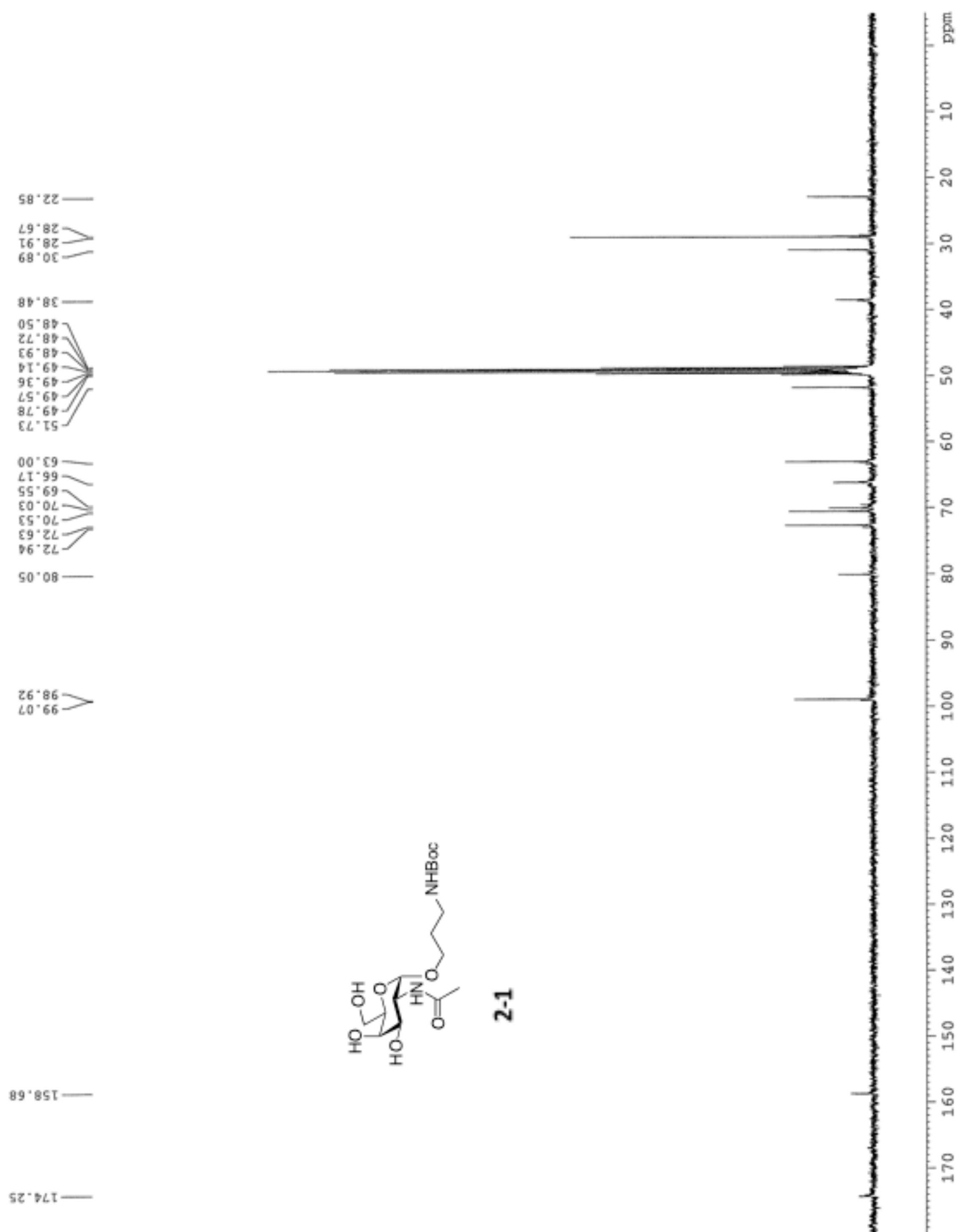
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm



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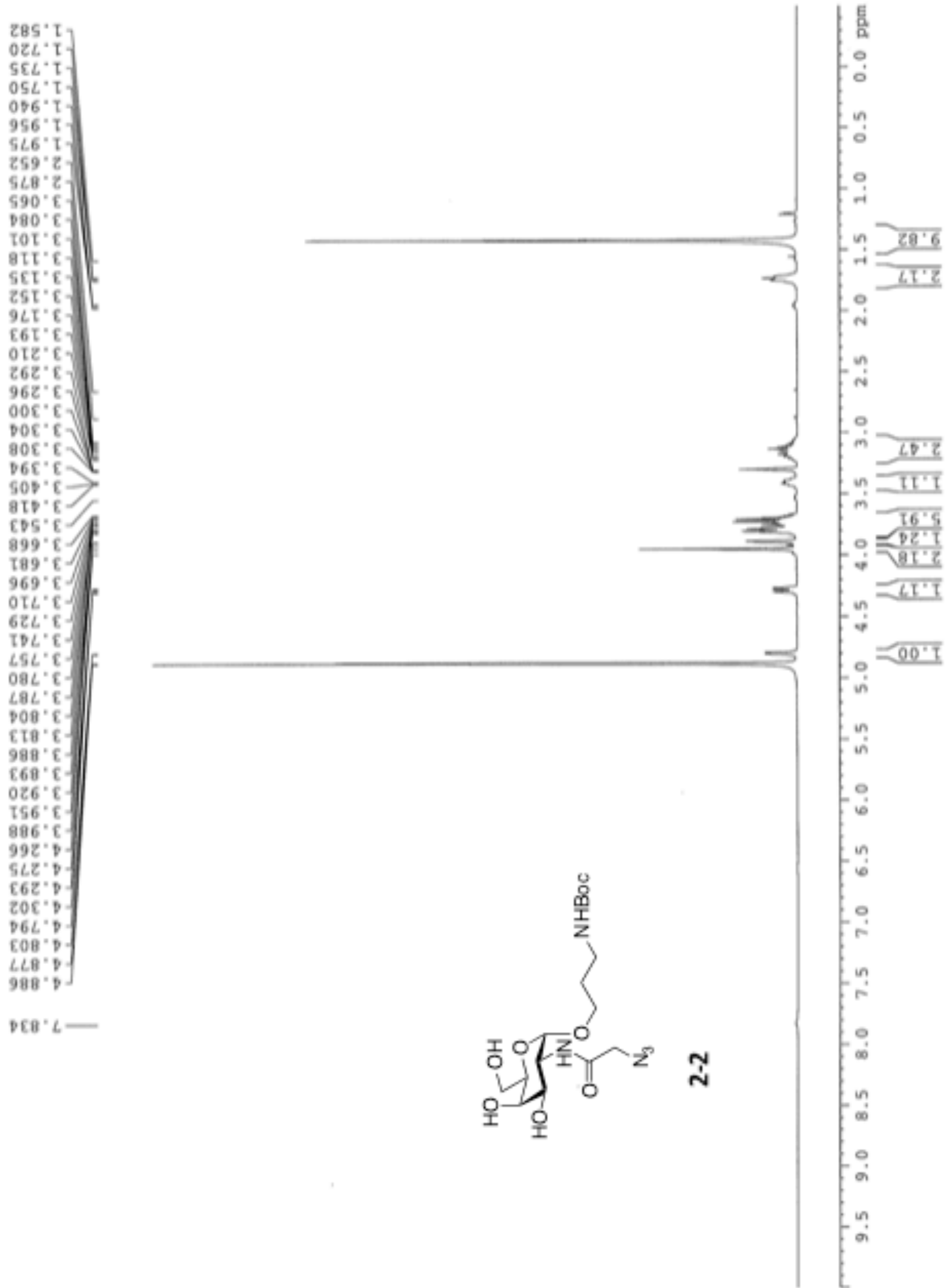
28.67
28.91
30.89
38.48
48.50
48.72
48.93
49.14
49.36
49.57
49.78
51.73
63.00
66.17
69.55
70.03
70.53
72.63
72.94
80.05
98.92
99.07
158.68
174.25
HO
OH
O
HO
HN
O
NHBoc
2-1
ppm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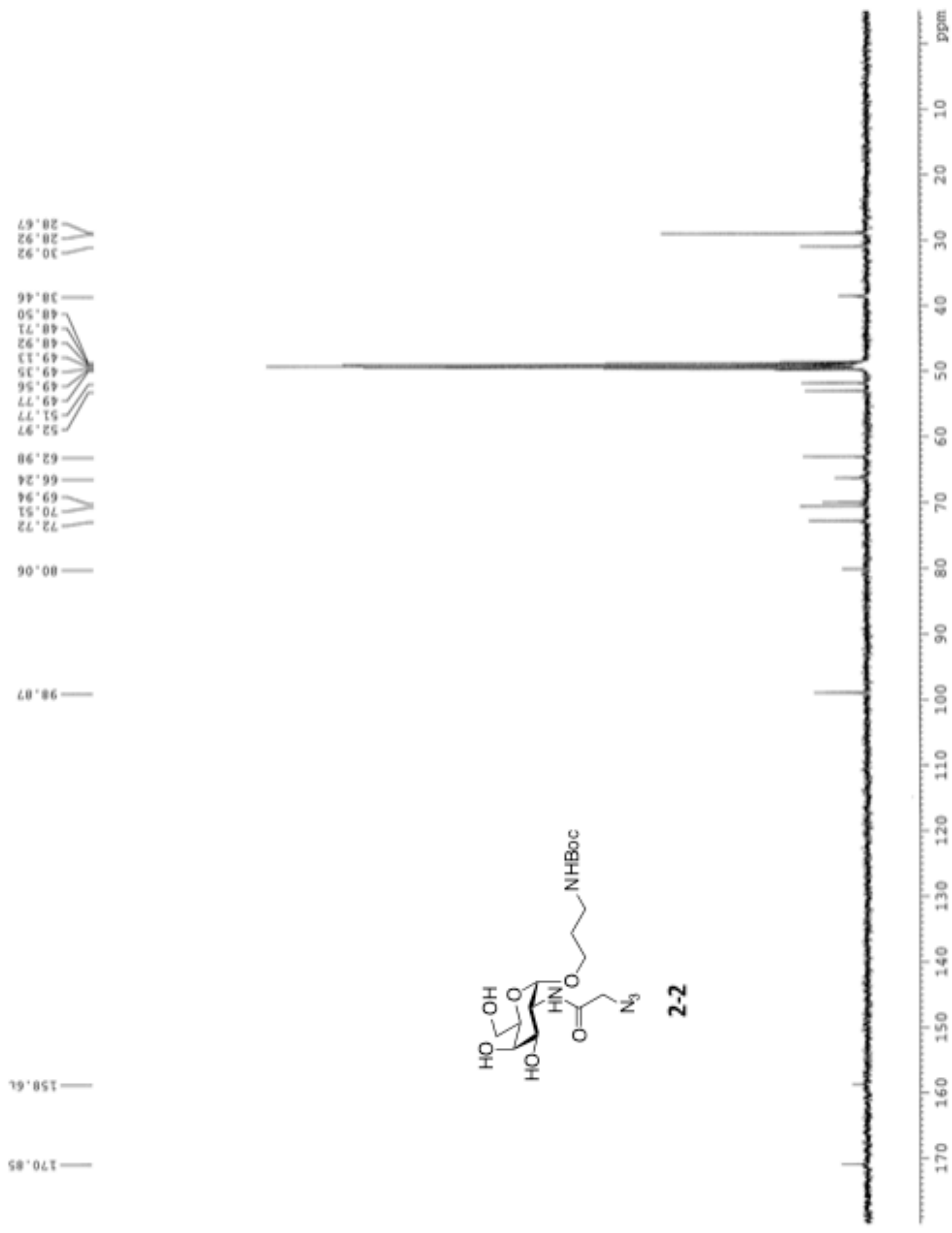
ppm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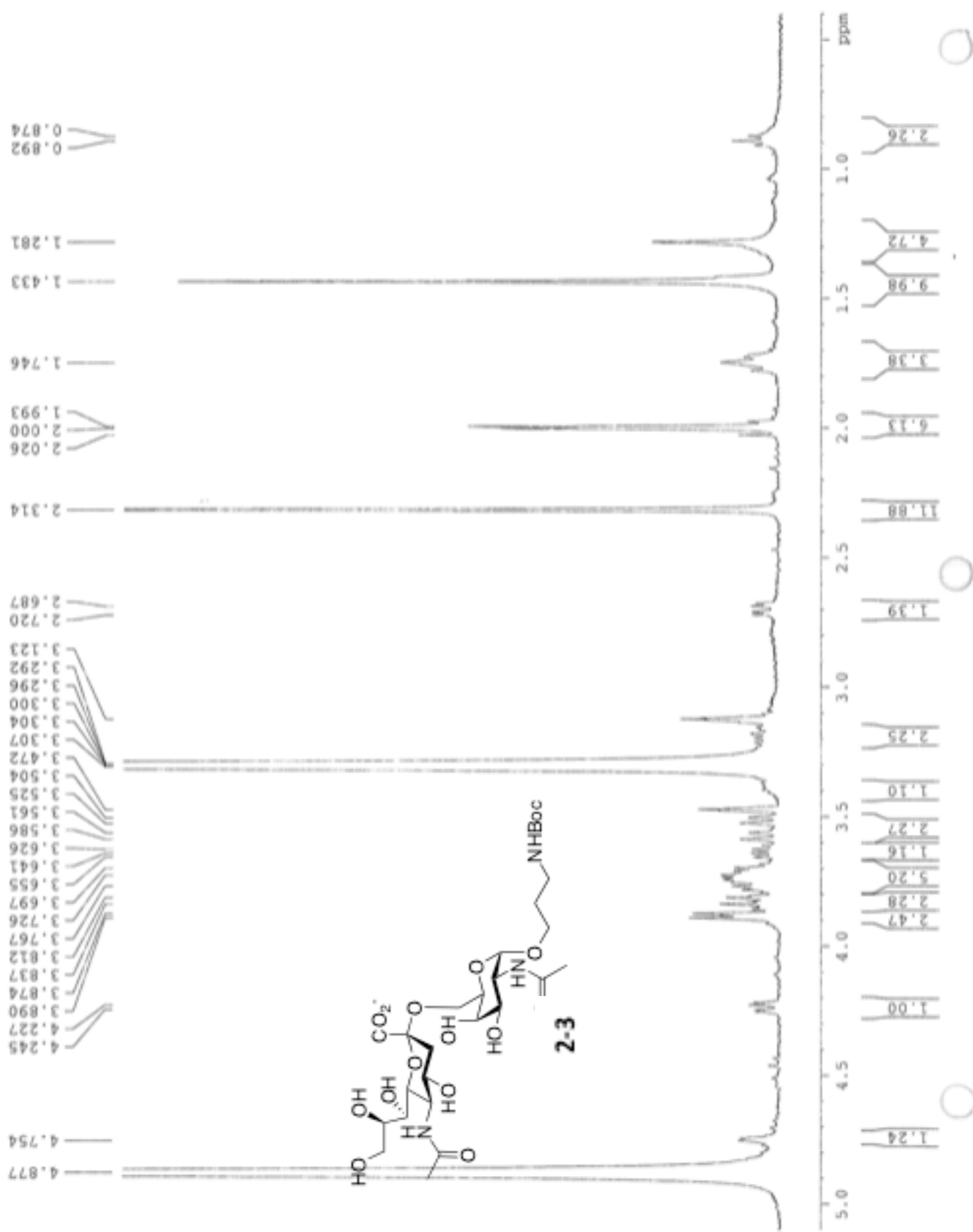
52.97
62.98
66.24
69.96
70.51
72.72
80.06
98.87
158.61
170.85
NHBoc
HO
OH
HO
O
HN
O
$N_3$
2-2
ppm
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170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3.292
3.296
3.300
3.304
3.307
3.472
3.504
3.525
3.561
3.586
3.626
3.641
3.655
3.697
3.726
3.767
3.812
3.837
3.874
3.890
4.227
4.245
4.754
4.877
NHBoc
$CO_2^-$
2-3
ppm
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1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
2.26
4.72
9.98
3.38
6.13
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5.20
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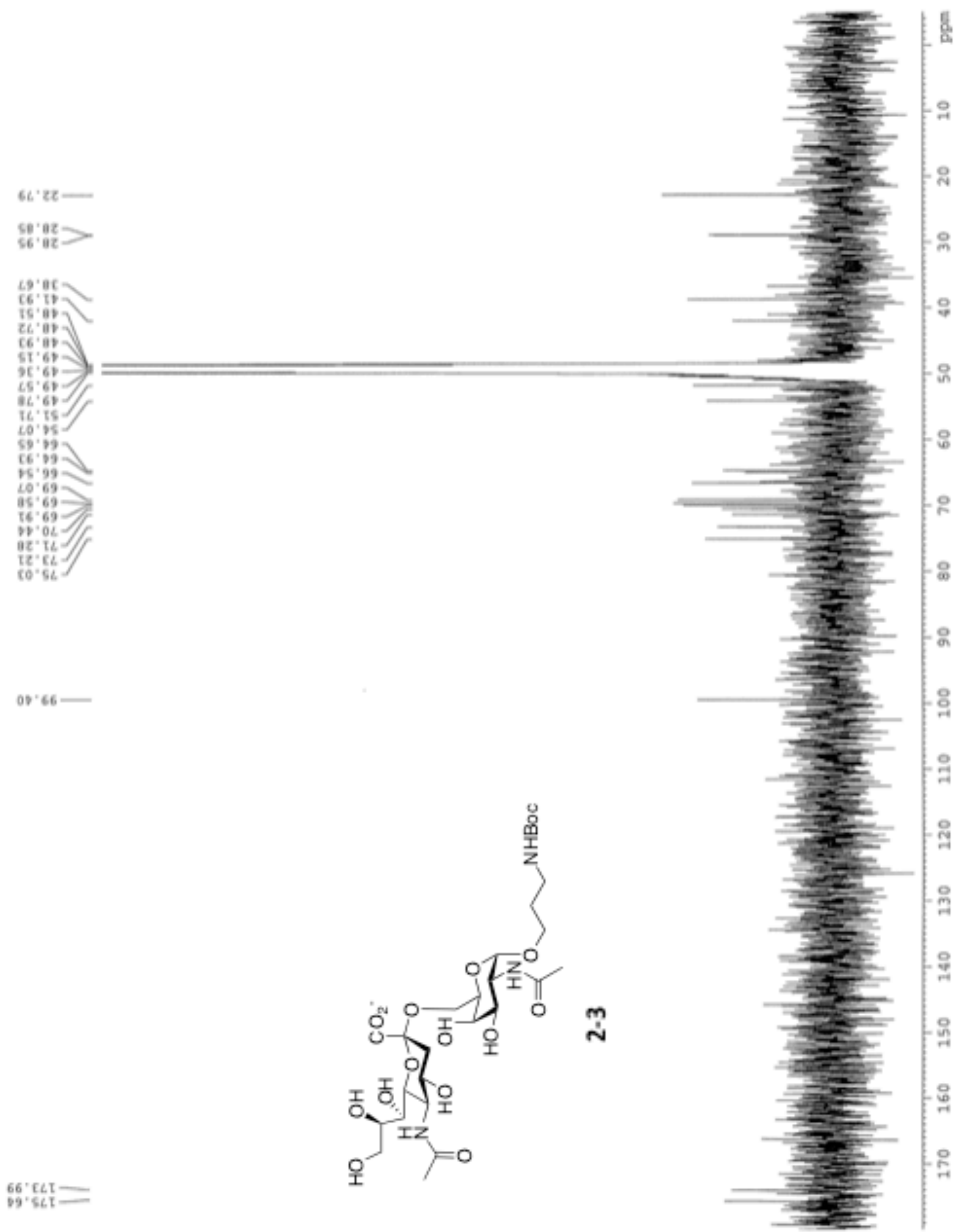
2.47
1.00
1.24



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64.65
64.93
66.54
69.07
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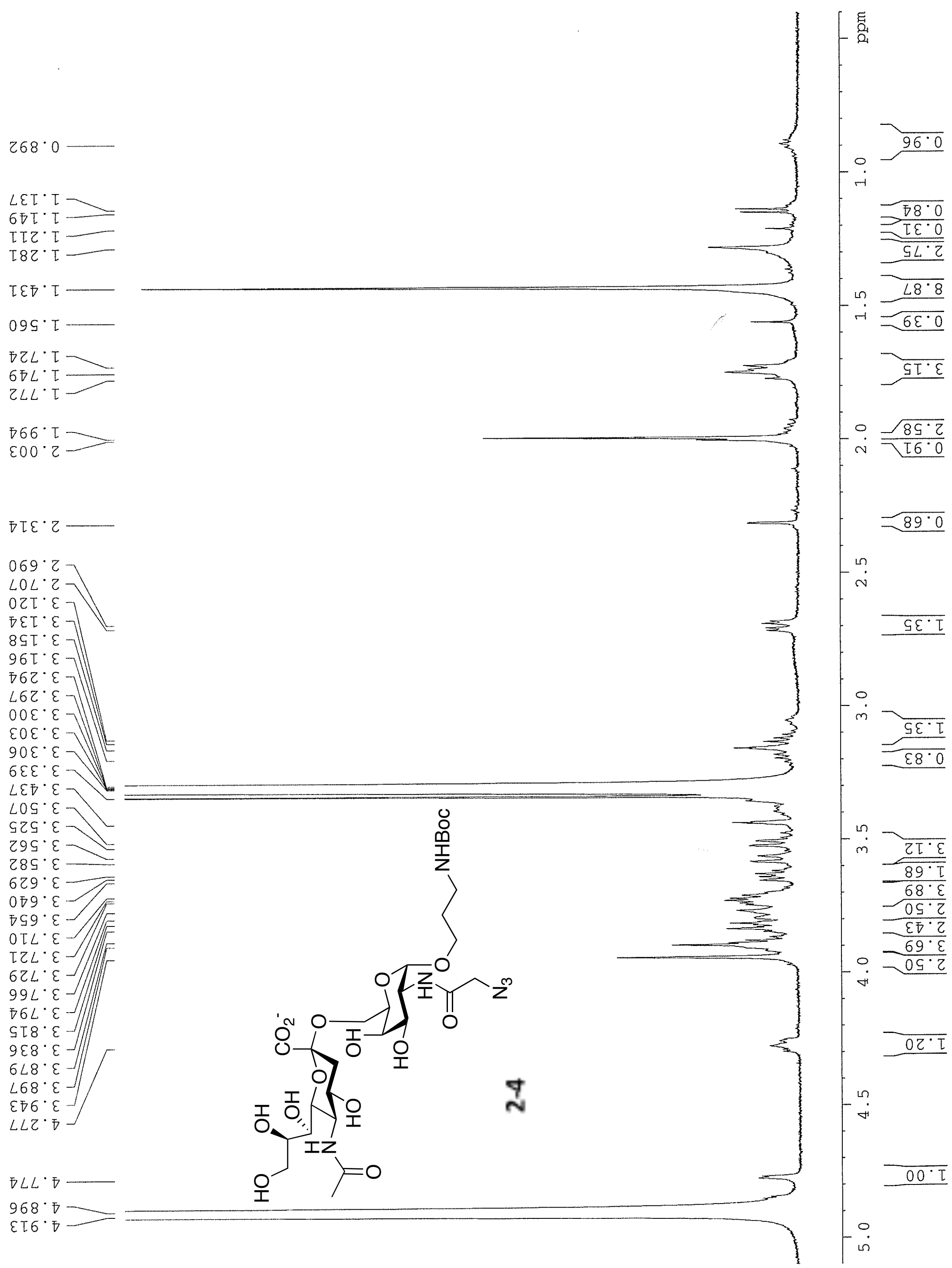
NHBoc
2-3
ppm



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3.120
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3.158
3.196
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3.297
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3.306
3.339
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3.507
3.525
3.562
3.582
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3.640
3.654
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3.721
3.729
3.766
3.794
3.815
3.836
3.879
3.897
3.943
4.277
4.774
4.896
4.913
NHBoc
HN
O
$N_3$
HO
OH
$CO_2^-$
HN
2-4
0.96
0.84
0.31
2.75
8.87
0.39
3.15
2.58
0.91
0.68
1.35
1.35
0.83
3.12
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3.89
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2.43
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ppm
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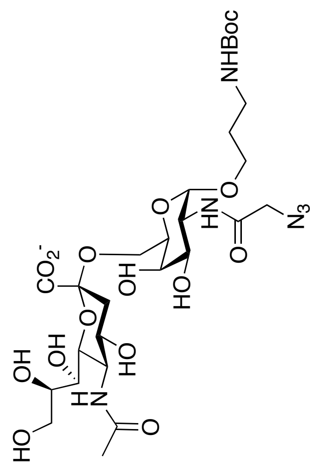


2-4

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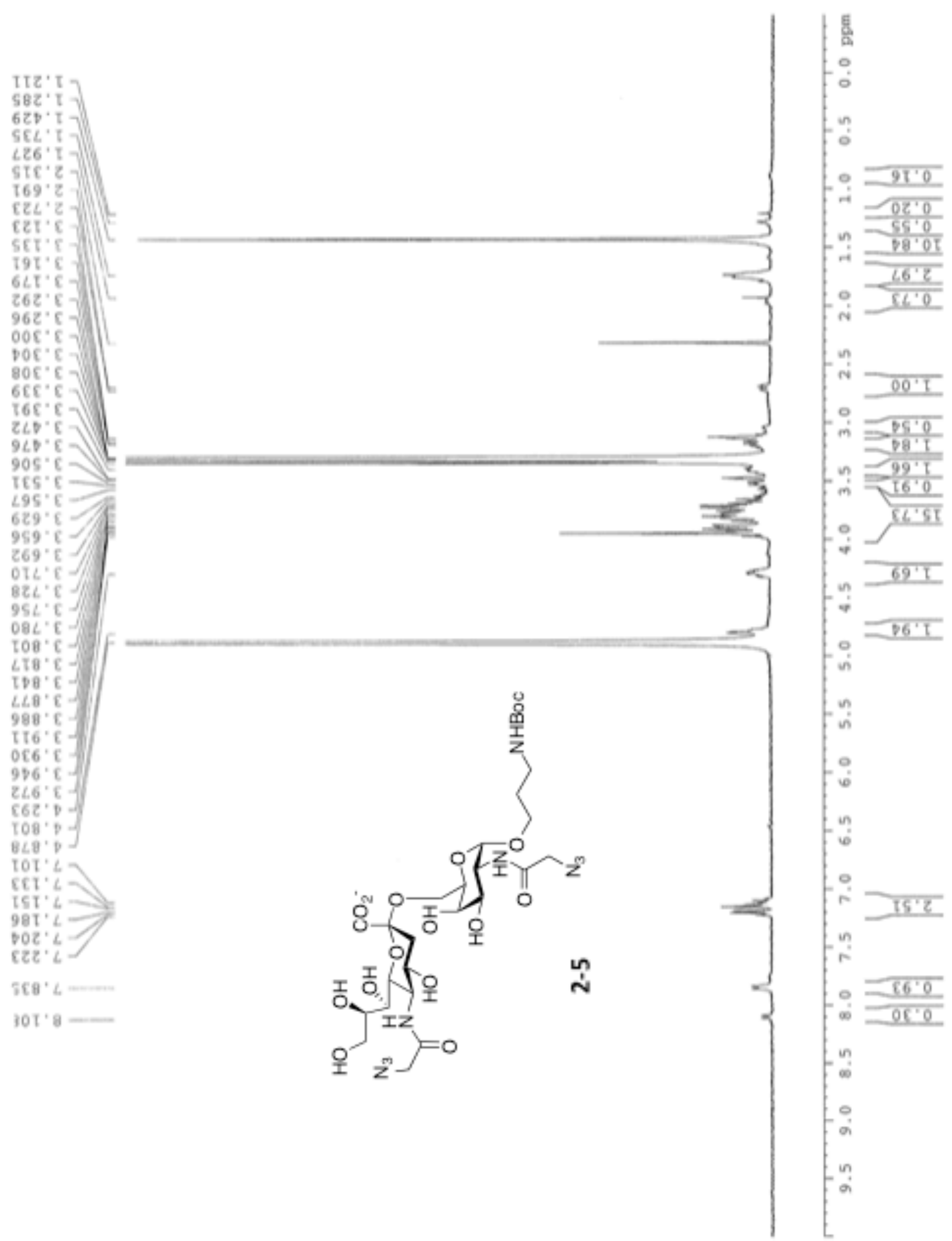
49.72

ppm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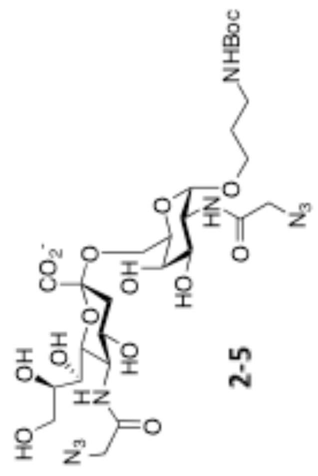
ppm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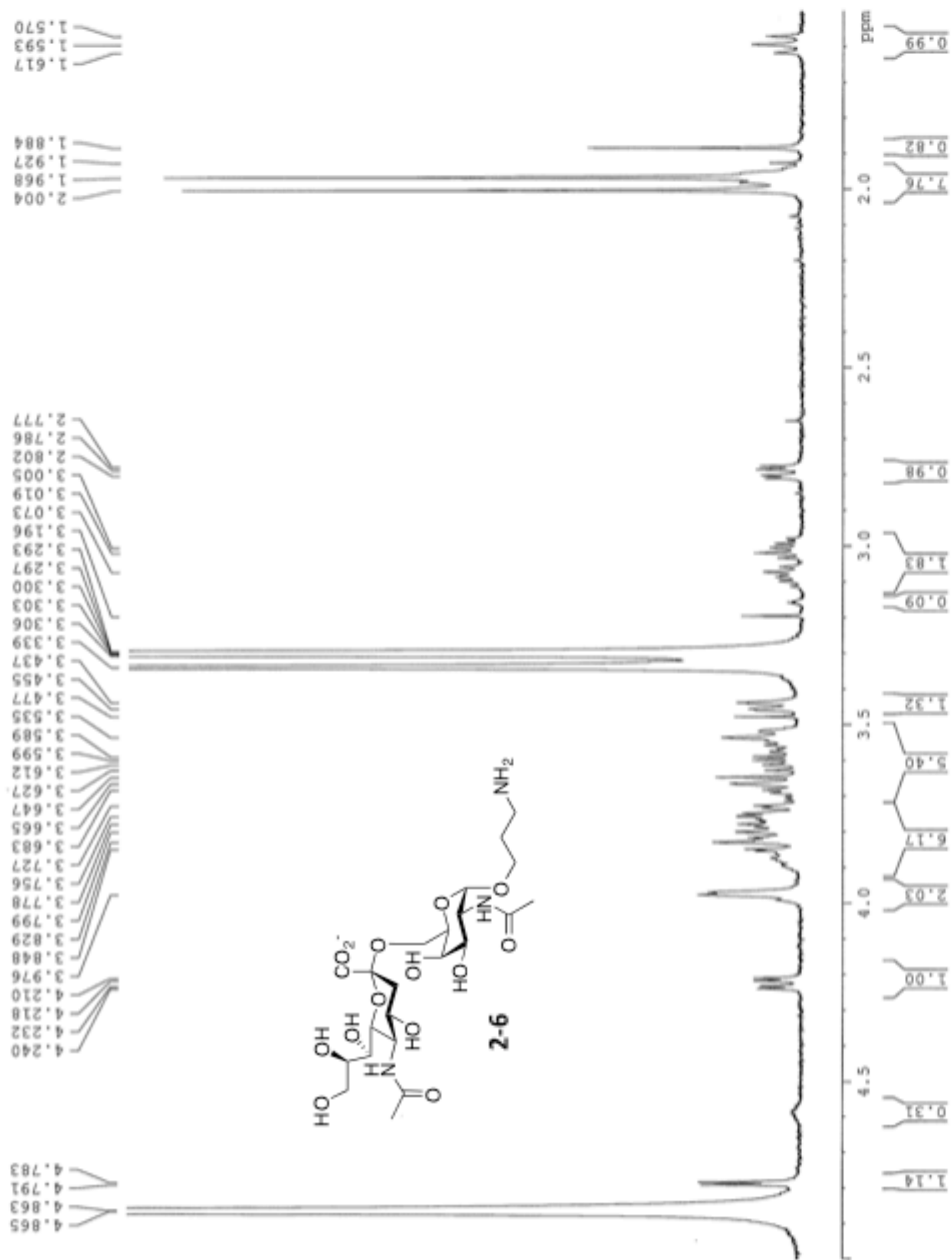
$N_3$



2-6
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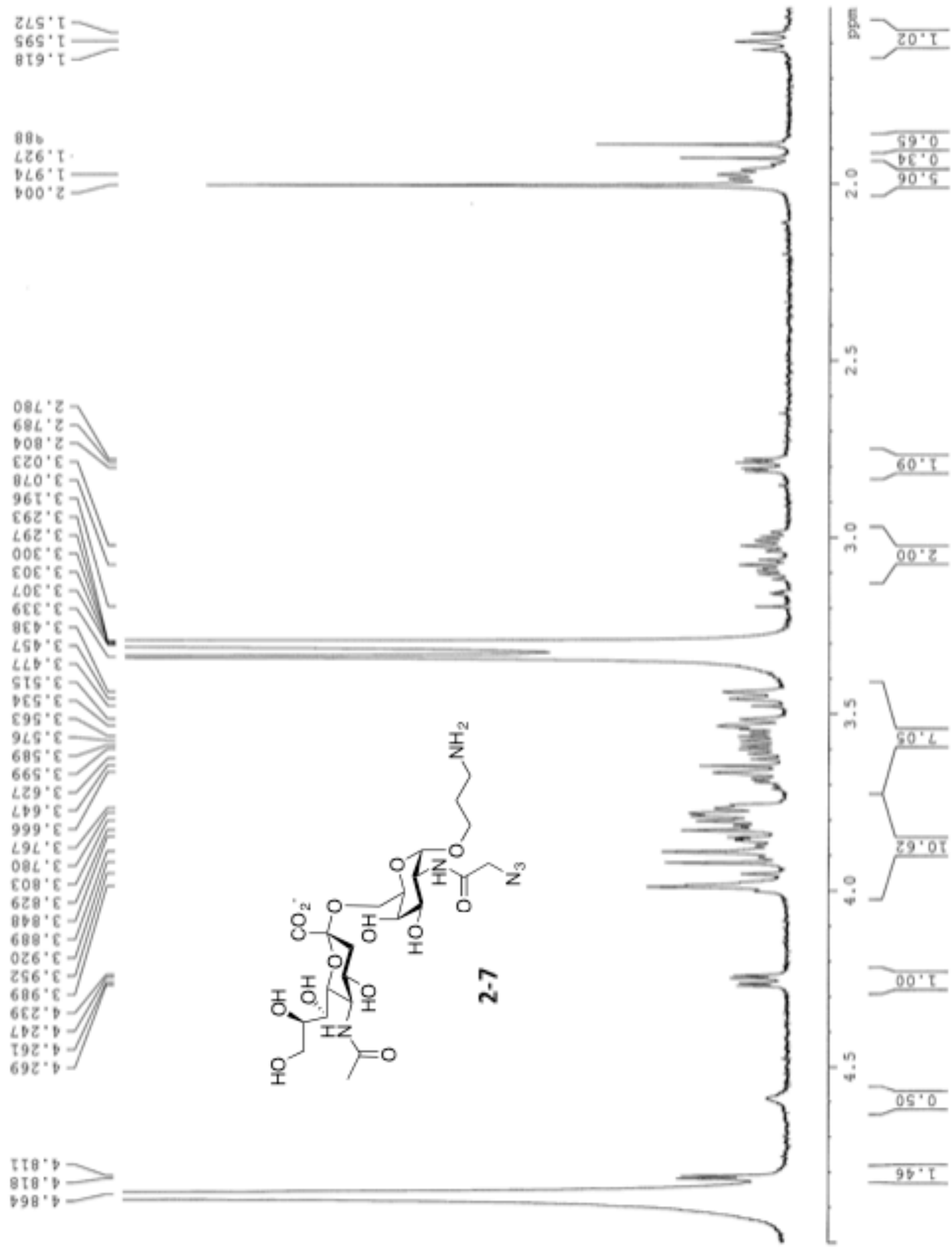
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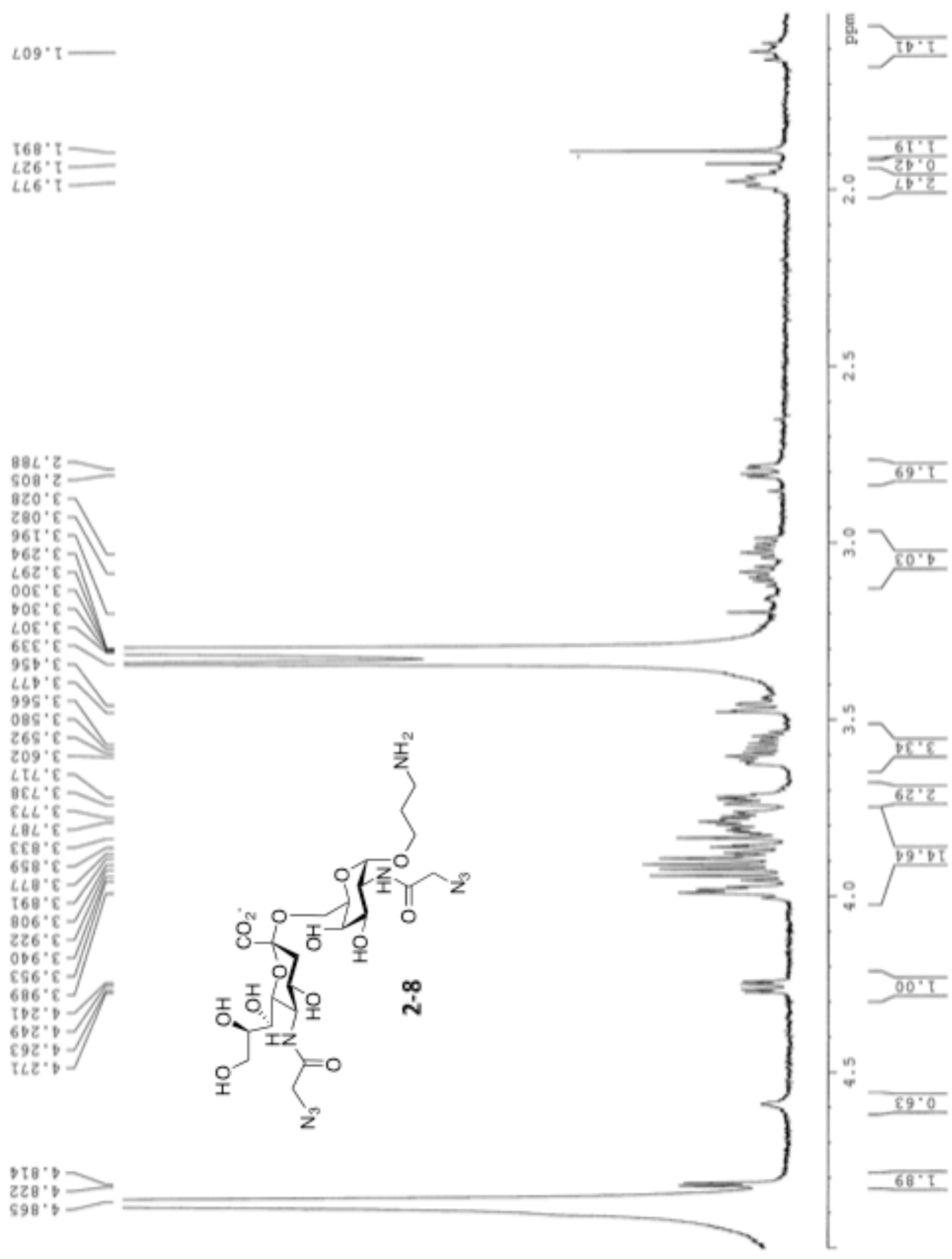
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2-8
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