SYNTHESIS AND IMMUNOTHERAPEUTIC STUDIES OF CARBOHYDRATE-BASED CANCER VACCINES

by

PAMELA S. THOMPSON

(Under the Direction of GEERT-JAN BOONS)

ABSTRACT

Cancer remains a major cause of death throughout the world. Current treatment of cancer has primarily relied on a combination of therapies, in many cases surgical reduction of the tumor, followed by chemotherapy and radiation, which targets rapidly dividing cells. These treatments, however, do not only affect tumor cells, but also normal cells, resulting in severe side effects. As research has unraveled more details of the intrinsic underlying biological and immunological mechanisms of cancer, new approaches such as angiogenesis inhibitor therapy, gene therapy, and immunotherapy have emerged as possible treatments.

The identification of tumor-associated antigens has made it possible to develop antigen-specific vaccines. It has been established that aberrant glycosylation is closely associated with a majority of human cancers. The low antigenicity of tumor-associated carbohydrate antigens signifies a hurdle in vaccine development. In this research, we have examined a three-component vaccine candidate which is able to break tolerance and induce humoral and cellular immune responses against the tumor-associated glycoprotein MUC1, generating CTLs and ADCC-mediating antibodies. This vaccine candidate, which is composed of the tumor-associated antigen MUC1, a promiscuous T-helper peptide derived from the polio virus, and a built-in adjuvant, the TLR2 ligand Pam_3CysSK_4, demonstrated a superior therapeutic anti-tumor effect in a mouse model of breast cancer.

The synthesis of these glycolipopeptide vaccine candidates represents a formidable challenge due to the unique properties of the individual components. In this research, we have developed a highly efficient microwave-assisted liposome-mediated native chemical ligation protocol to obtain cancer vaccine candidates. In our efforts to further streamline the synthesis, we have successfully exploited microwave-assisted solid-phase peptide synthesis (MW-SPPS) for the linear construction of these glycolipopeptides. We applied this technology towards the synthesis of vaccine candidates which contain Pam_3CysSK_4 and aberrantly glycosylated long MUC1 peptide sequences. Immunization with these vaccine constructs resulted in the production of glycopeptide-specific IgG antibody responses, demonstrating that glycopeptide sequences from MUC1 can be processed and presented to MHC-II. Finally, a strategically protected sialyl-Tn antigen was synthesized in a stereoselective manner and was utilized during the linear assembly of a vaccine candidate via the newly developed MW-SPPS protocol.

INDEX WORDS: cancer, vaccine, carbohydrate, glycopeptide, glycolipopeptide, multi-component, microwave

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DEDICATION

When you want something, all the universe conspires in helping you to achieve it.

-Paulo Coelho, *The Alchemist*

To

My parents

For their continuous encouragement and unwavering belief in all my endeavors

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ABBREVIATIONS

Å	Angstrom
Ac	Acetyl
Acm	Acetamidomethyl
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen-presenting cell
Ar	Aromatic
BCG	Bacillus Calmette-Guerin
BF_3•Et_2O	Borontrifluoride diethyletherate
Bn	Benzyl
Boc	tert-Butyloxycarbony
BSA	Bovine serum albumin
Bz	Benzoyl
ACN	Acetonitrile
CDC	Complement-dependent cytotoxicity
CFA	Complete Freund's adjuvant
CpG-ODN	Phosphate-guanine-containing oligodeoxynucleotide
CSA	Camphorsulfonic acid
CTL	Cytotoxic T-lymphocyte
d	Doublet
Da	Dalton
DBU	1,8-Diazabiccycloundec-7-ene
DC	Dendritic cell
DCM	Dichloromethane

DDQ	2,3-Dicyano-5,6-dichloro quinone
DEIPS	Diethylisopropyl silyl
DIPEA	*N,N*-Diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMF	*N,N*-Dimethylformamide
DNA	Deoxyribonucleic acid
DPC	Dodecylphosphocholine
EDTA	Ethylenediamine tetraacetic acid
EL	Empty liposomes
ELISA	Enzyme-linked immunosorbent assay
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethyl alcohol
Fmoc	Fluorenylmethyloxycarbonyl
Gn	Guanidine
GnT-1	Glucosylaminyl transferase 1
GPI	Glycosylphosphatidylinositol
H	Hour
HATU	*O*-(7-Azabenzotriazol)- 1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HBTU	*O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate
HBV	Hepatitus B virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HOAc	Acetic acid

HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	Hydroxybenzotriazole
HPLC	High pressure liquid chromatography
HR-MALDI	High resolution-matrix assisted laser desorption/ionization
Hz	Hertz
IC	Inhibitory concentration
IFN	Interferon
Ig	Immunoglobulin
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IL	Interleukin
KLH	Keyhole limpet hemocyanin
Lev	Levulinoyl
Le^x	$Lewis^x$
Le^y	$Lewis^y$
LPS	Lipopolysaccharide
m	Minutes
m/z	Mass to charge ratio
MAb	Monoclonal antibody
MAG	Multi-antigenic glycopeptide
MeOH	Methanol
MESNa	Sodium 2-mercaptoethanesulfonate
MHC	Major histocompatibility complex
MI	Maleimide
mM	Millimolar

µM	Micromolar
mmol	Millimole
MMT	Mouse mammary tumor
MP	p-Methoxyphenyl
MPAA	4-Mercaptophenyl acetic acid
MS	Molecular sieves
MUC1	Mucin 1
MW	Microwave
MW-SPPS	Microwave-assisted solid-phase peptide synthesis
NaH	Sodium hydride
NAP	Naphthyl
NBS	*N*-Bromosuccinimide
NCI	National Cancer Institute
NCL	Native chemical ligation
NIS	*N*-Iodosuccinimide
NK	Natural killer
NMP	*N*-methyl pyrrolidone
NMR	Nuclear Magnetic Resonance
Pam	Palmitoyl
PAMP	Pathogen-associated molecular pattern
PEG	Polyethylene glycol
q	Quartet
RAFT	Regioselectively addressable functionaized template
Rf	Retention factor
RNA	Ribonucleic acid

RP-HPLC	Reversed-phase high performance liquid chromatography
rt	Room temperature
s	Singlet
SAda	Thioadamantyl
SAMA-Opfp	*S*-Acetylthioglycolic acid pentafluorophenyl ester
SePh	Selenophenyl
sLe^x	Sialyl $Lewis^x$
SPPS	Solid-phase peptide synthesis
STn	Sialyl-Tn
SUV	Small unilamellar vesicles
t	Triplet
TACA	Tumor-associated carbohydrate antigen
TBAF	Tetrabutyl ammoniumfluoride
t-BuOH	tert-Butyl alcohol
TCEP	Tris(2-carboxyethyl)phosphine
TDS	Thexyl dimethyl silyl
TEA	Triethylamine
TESOTf	Triethylsilyl trifluoromethanesulfonate
TF	Thomsen-Friedenreich
TFA	Trifluoroacetic acid
TfOH	Trifluoromethanesulfonic acid
Tg	Transgenic
Th1	T-helper-1
Th2	T-helper-2

THF	Tetrahydrofuran
TIPS	Triisopropyl silane
TLC	Thin layer chromatography
TLR	Toll-like receptor
TMS	Trimethylsilyl
TMSOTf	Trimethylsilyl trifluromethanesulfonate
TNF	Tumor necrosis factor
TT	Tetanus toxoid
Troc	Trichloroethoxy carbonyl
VNTR	Variable number of rtandem repeat units
Z	Benzyloxycarbonyl

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Cancer and the Immune System

Cancer remains a major cause of death throughout the world. Current treatment of cancer has primarily relied on a combination of therapies, in many cases surgical reduction of the tumor, followed by chemotherapy and radiation, which targets rapidly dividing cells. These treatments, however, do not only affect tumor cells, but also normal cells, resulting in severe side effects which can limit treatment. As research has unraveled more details of the intrinsic underlying biological and immunological mechanisms of cancer, new approaches such as angiogenesis inhibitor therapy, gene therapy, and biological or immunotherapy have emerged as possible treatments.[1-7]

The theory of developing immunotherapy to control cancer growth has now been shown to be partially correct, as strong immune responses against cancer cells are difficult to generate. This is because cancer cells have developed numerous ways to evade the immune system. For example, cancerous cells shed certain types of molecules that inhibit the ability of the body to attack cancer cells. As a result, cancers become less “visible” to the immune system.[8] Given today’s knowledge of most cell types of the immune system and their functions, it is clear that cooperation between innate and adaptive arms of the immune system is vital to the initiation of signaling processes and immunological memory against invading cancerous cells.[8-11] One example of immunotherapy is the passive administration of antibodies, which has been shown

to mediate tumor regression in certain patients (Figure 1.1).[12-14] Currently, Rituxan® (Genentech biogen idec) and Herceptin® (Genentech oncology) are two monoclonal antibody (mAbs) drugs that are used for the treatment of non-Hodgkin's lymphoma and breast cancer, respectively. [13]

1.1.1 Cancer Vaccines

Classical vaccine theory entails a prophylactic vaccination against a specific pathogen that will evoke an immune response, which will provide protection against subsequent encounters with the pathogen. Cancer vaccines, on the other hand, are to be regarded as therapeutic vaccines, as they need to evoke an immune response that is capable of eradicating an already existing disease.[8, 15-18] Typically, a cancer vaccine can be used to treat minimal residual disease and to protect against relapses, once a tumor has been de-bulked by surgery or chemotherapy.

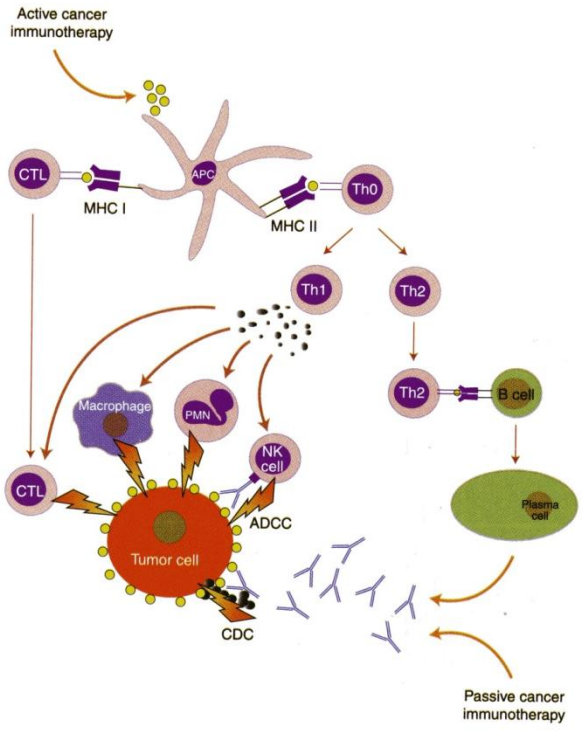


Figure 1.1. Principles of active and passive cancer immunotherapy.[19]

There are several key steps in the immunological response to cancer cells which are important in the development of cancer vaccines. The immune system recognizes tumor-specific antigens through the help of antigen-presenting cells (APCs) such as dendritic cells (Figure 1.1). Dendritic cells are pivotal for the initiation of T-cell anti-cancer responses by processing and presenting tumor-associated antigens to T lymphocytes. After their initial exposure to the antigen, dendritic cells can be stimulated via co-stimulatory molecules (CD40L or agonistic anti-CD40), as well as adjuvants, to induce maturation and a potent anti-cancer immune response. Natural killer (NK) cells are also important in cancer immunotherapy, as they are capable of directly killing cancer cells.

Cells of the innate immune system bridge to the adaptive immune system by the activation and presentation of antigens to T- and B-cells. Unlike the innate immune system, T- and B-lymphocytes are capable of developing immunological memory, which is attractive for cancer immunotherapy. T-cells are activated by direct recognition of tumor antigens or antigens that are presented by APCs. Activated T-cells in turn produce cytokines and chemokines, which mediate the killing of tumor cells. The primary function of B-cells, on the other hand, is to differentiate into plasma cells, which produce antibodies that can facilitate the eradication of tumor cells by complement-dependent cytotoxicity (CDC) and/or antibody-dependent cell-mediated cytotoxicity (ADCC) performed by NK cells and macrophages.

Cancer vaccines are emerging as a treatment for cancer.[1, 2, 7, 8, 15-18] Today, two types of prophylactic cancer vaccines have been approved for human use. These vaccines protect individuals against contracting viral infections that have been associated with an increased chance of developing certain types of cancers. These vaccines are aimed at the hepatitis B virus (HBV), associated with certain liver cancers (Engerix-B® and Twinrix®, GlaxoSmithKline Biologicals), and the human papilloma virus (HPV), which is closely linked to cervical carcinomas (Gardasil®: Merck and Company; Cervarix®: GlaxoSmithKline Biologicals). The

HPV vaccine protects against two types of virus, HPV 16 and 18, which are the cause of 70% of cervical cancer cases.[20]

Most experimental cancer vaccines are therapeutic vaccines, *i.e*. vaccines that are administered to an already diseased person.[15, 21] The concept of these vaccines is that upon administration, the vaccine will trigger an immune response that can reduce the tumor size or target metastasized tumor cells that may have evaded surgery or other primary therapies. A pre-requisite of a cancer vaccine is the ability to distinguish tumor cells from normal cells. Therefore, an ideal target antigen should be expressed exclusively, or in abundance, by malignant cells and be accessible to the immune system on the cell surface. Several therapeutic cancer vaccines, which are aimed at for example breast, lung, colon and prostate cancer, have reached evaluation in clinical trials.

Cancer vaccines derived from whole tumor cells, which were irradiated to render them unable to replicate, were among the first therapeutic cancer vaccines tested.[22] An advantage of using tumor cells is that they express multiple antigens, and thus a specific antigen does not need to be identified. The primary tumor and metastases may display different antigens and therefore cell-based vaccines may reduce the possibility of antigenic escape. However, tumor cells can possess properties that enable immune evasion and as a result may have limited immunogenicity. This has prompted the use of gene transfer strategies to tumor cell lines to enhance the immune response by providing immuno-stimulatory molecules.[23-27] One such vaccine based on genetically modified prostate cancer cell-lines, GVAX (Cell Genesys), has reached Phase 3 clinical trials.[28]

The identification of tumor-associated antigens has made it possible to develop antigen-specific vaccines. Such vaccines offer the distinct benefit of providing methods for monitoring and evaluating specific immune responses. A pre-requisite for the success of antigen-specific vaccination is to find antigens that are expressed exclusively or abundantly on tumor-cells and

not on normal cells. Tumor antigens may be mutated and over- or aberrantly expressed. Although many tumor-specific mutations have been identified in intra-cellular proteins, a significant number have been observed in the extra-cellular domain of membrane proteins. Consequently, several cancer vaccines have been designed that are aimed at exploiting cellular immune responses towards this type of antigen.[29-31]

Aberrant glycosylation is closely associated with a majority of human cancers.[32-35] Tumor cells may display an over-expression of carbohydrates, truncated versions of oligosaccharides, unusual terminal oligosaccharide sequences, and an increase in sialylation of their cell-surface glycolipids and *O*- and *N*-linked glycoproteins. This is the result of up- and/or down-regulation of glycosyl transferases and changes in the elongation of core oligosaccharides, which then serve as acceptors for capping glycosyl transferases. Truncated glycosylation of a glycoprotein also renders part of the peptide backbone that is normally shielded by the glycan more accessible to the immune system. Apart from being membrane bound, many tumor-associated carbohydrate antigens (TACAs) are secreted into the blood by the tumor-cells. Thus, these antigens provide viable targets for the development of both diagnostics and tumor-selective or tumor-specific carbohydrate-based vaccines.[36-43]

1.2 Cell-surface carbohydrates

In the recent years, the importance of carbohydrates has become increasingly significant; they have been identified in various physiological and pathological processes. The cell surface is decorated with carbohydrates; however, carbohydrates differ among cell types and are regulated in development and differentiation.[44-47] On the cell surface, carbohydrates are present in the form of glycoconjugates, such as glycolipids and glycoproteins. Glycolipids are oligosaccharides that are covalently attached to a lipid moiety. The lipid portion is anchored into the hydrophobic cell membrane, thereby exposing the oligosaccharide to the extracellular

environment.[48-50] Glycoproteins are composed of oligosaccharides that are covalently attached to a polypeptide backbone. There are two types of protein glycosylation: *N*-glycosylation, wherein the glycan is attached to an asparagine residue, and *O*-glycosylation, wherein the glycans is attached to a hydroxylated amino acid, most commonly serine, threonine, or hydroxyproline residues. For *O*-linked glycoproteins, there is no common core, while *N*-linked glycoproteins have an identical pentasaccharide core.

Cell surface carbohydrates are integral in a wide range of biological processes, such as cell-cell recognition and adhesion, cell proliferation and differentiation, cell development, and fertilization (Figure 1.2). Carbohydrates also play important roles in the immune system; they are involved in the invasion and attachment of pathogens (bacteria and virus), inflammation (leukocyte migration), cancer metastasis, and blood group immunology. Thus, it is necessary to understand and probe the roles of these carbohydrates in specific processes and diseases in order to develop novel therapies and vaccines.[51]

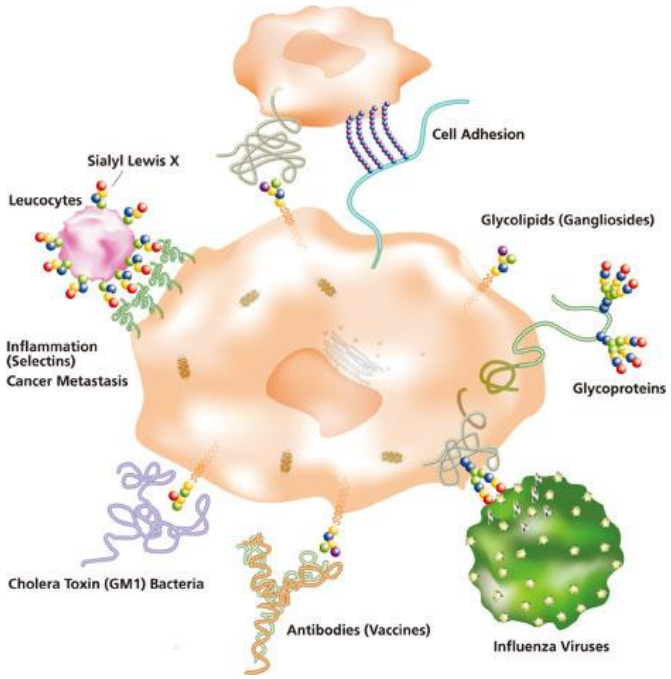


Figure 1.2. Cell surface oligosaccharides involved in various biological processes.[52]

Over the past decade, advances in genomics, proteomics, and mass spectrometry have enabled the identification of specific glycan structures with disease states, revealing the functional implication of disease-associated changes in glycosylation. The site of glycosylation is critical to protein expression and folding and increases thermal and proteolytic stability. While there have been significant advances toward understanding the effect of glycosylation in various biological processes, progress toward a realization of specific roles of glycan structures is limited due to their complexity and heterogeneity.[38]

1.3 Tumor-associated carbohydrate antigens (TACAs)

Protein- and lipid-bound oligosaccharides found on the surface of cells are involved in many essential processes impacting eukaryotic biology and disease, and thus it is not surprising that malignant cells, which display differences in cell adhesion and cell motility, also display altered cell-surface glycosylation.[44, 49, 53] The abnormal glycosylation has been shown to play a key role in the induction of invasion and metastasis and there is a wealth of evidence suggesting that abnormal glycosylation in primary tumors is closely correlated with the survival rate of cancer patients.[54]

Tumor-associated carbohydrates can be linked to lipids such as gangliosides or to proteins such as mucins. Glycolipid TACAs include GM2, GD2, GD3, fucosyl-GM1, Globo-H, and Lewis^y (Le^y), and the glycoprotein TACAs include the truncated Tn-, TF-, and sialylated Tn (STn)-antigens as well as Globo-H and Le^y (Figure 1.3).

The glycosphingolipids GM2, GD2, and GD3 are implicated in human melanomas and have been the target of extensive vaccine research.[40] Although detectable on normal cells, they are highly expressed on malignant cells. Globo-H, also known as the MBr-1 antigen, was isolated from human breast cancer cells using a monoclonal antibody MBr-1 and has since also been identified as a tumor-associated antigen for ovary, colon, prostate, lung, and small-cell lung cancers.[55-58]

Several tumor-associated glycosphingolipids have been identified as adhesion molecules, and, consequently, these compounds have been shown to promote tumor-cell invasion and metastasis.[59] For example, the Lewis antigens sialyl Lewis^a (SLe^a), SLe^x, SLe^x-Le^x, and Le^y have been identified as tumor-associated antigens (Figure 1.3).[60, 61] The Le^y tetrasaccharide is over-expressed on a range of carcinomas including ovary, breast, colon, prostate, and non-small cell lung cancers. The KH-1 antigen, which displays the heterodimeric Le^y-Le^x heptasaccharide, was isolated from human colonic adenocarcinoma cells.[62] This antigen has only been found on the surface of these cells and has never been isolated from normal colonic tissue, thus providing a highly specific marker for malignancies.[63, 64]

The blood group precursors, Tn-, STn-, and TF-antigens, are the result of incomplete O-glycan synthesis. The Tn-antigen, α-GalNAc-Thr/Ser, results from the lack of core 1 β-3-galactosyl transferase (T-synthase). Recently it has been shown that the expression of T-synthase is regulated by a key molecular chaperone, Cosmc, which resides in the ER. Mutations that lead to the loss of function of Cosmc lead to the loss of T-synthase activity.[65,66] These antigens are not expressed in normal tissue, but are found immunereactive in the majority of carcinomas, thus representing excellent targets for cancer vaccine development.

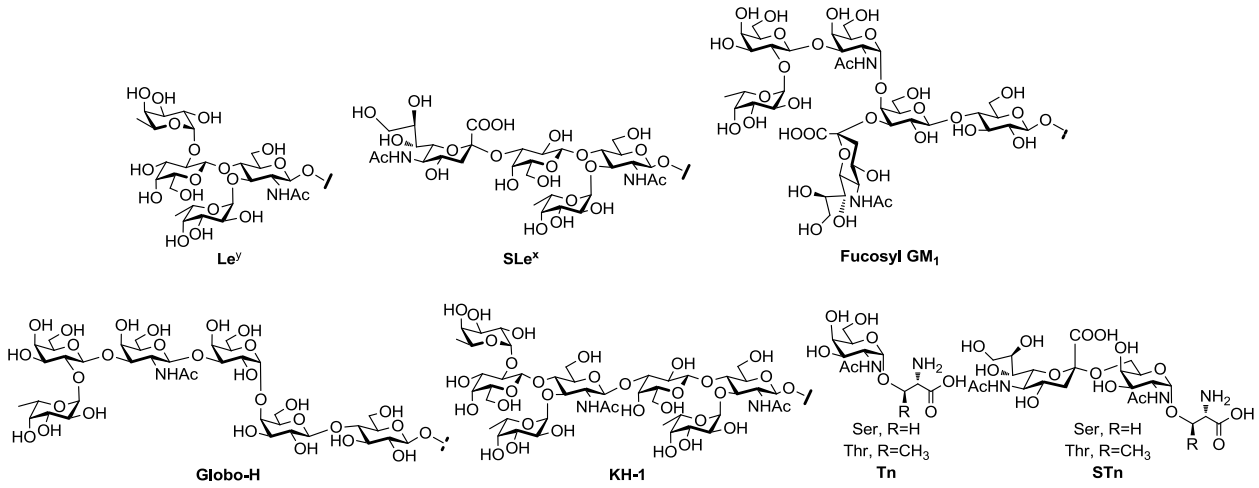


Figure 1.3. Human tumor-associated carbohydrate antigens.

1.3.1 Mucins

Mucins are high molecular weight glycoproteins containing numerous *O*-linked carbohydrate side chains, and are found at the apical surface of epithelial cells or as extracellular secreted glycoproteins (Figure 1.4). Twenty different human mucins are known, which posses a similar overall architecture with an *N*-terminal region followed by a region containing a variable number of tandem repeat units (VNTR). The tandem repeats of the different mucins, which are rich in threonine, serine, and proline residues, contain 8 to 23 amino acids. The cell membrane tethered mucins are involved in diverse functions ranging from shielding the airway epithelium against pathogenic infection to regulating cellular signaling and transcription.[65]

Many of the mucins have been implicated in disease such as pulmonary diseases and cancer. For example, MUC1 (Polymorphic epithelial mucin, PEM) is found over-expressed in more than 90% of breast carcinomas and is also found over-expressed in patient sera and have, as a consequence, found clinical use as a marker (CA15-3, Truquant, CASA) for breast cancer.[66-68] MUC1 has also been associated with other carcinomas such as ovarian, lung, colon, and pancreatic carcinomas. MUC1 is a transmembrane protein with a large and highly glycosylated extra-cellular domain consisting of multiple 20 amino acid repeating units (HGVTSAPDTRPAPGSTAPPA), of which each repeat has five potential sites for *O*-glycosylation.[69] In cancer cells, MUC1 is over-expressed and deficiently glycosylated due to a down regulation of glucosylaminyl transferase 1 (GnT-1) (Figure 1.4).[70-72] As a result, tumor associated MUC1 carries the antigens Tn (αGalNAc-Thr), STn (αNeu5Ac-(2,6)-αGalNAc-Thr) and the Thomsen-Friedenreich (TF or T) antigen (αGal(1,3)-αGalNAc-Thr) (Figure 1.3).[69, 73-75] Recently, the NCI Translational Research Working Group prioritized cancer vaccine targets based on therapeutic function, immunogenicity, role of the antigen in oncogenicity, specificity, expression level, stem cell expression, percentage of patients with antigen-positive cancer, and

cellular location.[76] MUC1 was ranked second of 75 tumor-associated antigens. In this respect, MUC1 displays nearly ubiquitous expression in a wide variety of tumor types.

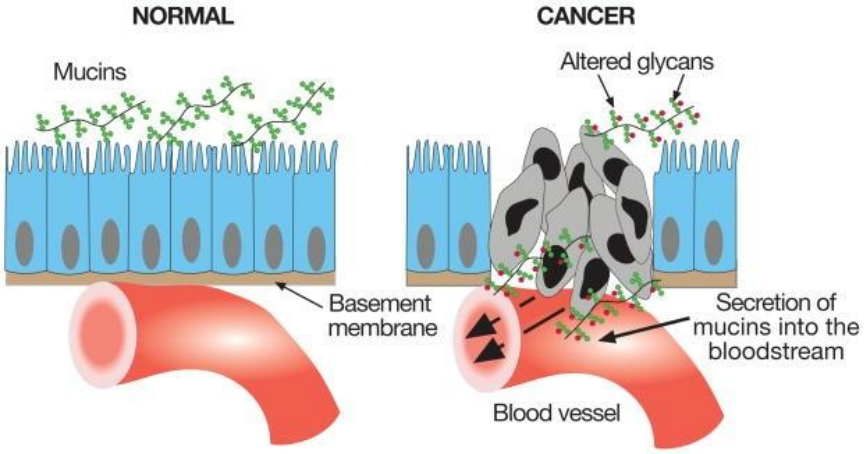


Figure 1.4. Mucins in normal and cancer cells .[77]

Humoral responses to MUC1 have been observed in benign diseases and carcinoma patients and it has been found that the presence of circulating antibodies against MUC1 at the time of cancer diagnosis correlates with a favorable disease outcome in breast cancer patients.[78] Antibodies induced by MUC1 isolated from tumor tissues have identified the PDTRP peptide motif as the immuno-dominant domain of the MUC1 tandem repeat.[79, 80] The specificity of these anti-MUC1 antibodies has been verified employing synthetic Tn- and T-antigens.[81-84] Furthermore, conformational studies by NMR complemented by light scattering measurements have indicated that de-glycosylation of MUC1 results in a less extended and more globular structure.[85] Similar studies using MUC1 related *O*-glycopeptides have shown that the carbohydrate moieties exert conformational effects, which may rationalize differences observed in antibody binding of MUC1 related glycopeptides and peptides.[85]

1.4 Difficulties in carbohydrate vaccine development

The development of carbohydrate-based cancer vaccines is by no means a trivial task and several hurdles need to be overcome. The heterogeneity of cell surface glycosylation

makes the isolation of tumor-associated carbohydrate antigens in well-defined forms and reasonable amounts an almost impossible task. Fortunately, this obstacle can be addressed by synthetic organic chemistry, which can provide homogeneous oligosaccharide antigens of high purity, indisputable structural integrity, and in relatively large amounts. Recent advances in organic synthesis of oligosaccharides has equipped chemists with more sophisticated tools, yet, the preparation of many of these large complex antigens still represents a considerable undertaking.

1.4.1 Immune response to carbohydrates

The low antigenicity of tumor-associated carbohydrate antigens signifies an additional hurdle. This observation is not surprising as tumor-associated saccharides are 'self-antigens' and consequently receive tolerance by the immune system. This immune-tolerance is further reinforced as the growing tumor sheds these antigens into the bloodstream. As a consequence, induction of high affinity IgG antibodies against tumor-associated carbohydrate antigens has proven to be more challenging than the induction of similar antibodies against viral and bacterial carbohydrate antigens. Indeed, high titers of IgG antibodies have been referred to as the "holy grail" for carbohydrate-based cancer vaccines.[86] The question thus posed is how to trick the immune system to break tolerance and induce a response against these tumor-associated antigens. The inherently T-cell independent nature of oligosaccharides further complicates carbohydrate-based cancer vaccine development. The production of high affinity IgG antibodies requires antigen recognition by B- and T-lymphocytes and dendritic cells. Consequently, the inability of carbohydrates to activate T-lymphocytes results in formation of exclusively low affinity IgM antibodies and lack of immunological memory.

1.4.2 Humoral response to carbohydrates

Antibodies that target tumor-related carbohydrate and glycopeptide antigens have been shown in preclinical and clinical settings to have the ability to eliminate circulating tumor cells.[8, 87-89] The antibodies can be acquired naturally, by passive immunization, or induced by active specific immunization with a vaccine containing a carbohydrate epitope. The antibodies mediate elimination of tumor cells by complement-dependent cytotoxicity (CDC) and by antibody-dependant cellular cytotoxicity (ADCC) conferred by effector cells such as natural killer cells and macrophages (Figure 1.5).

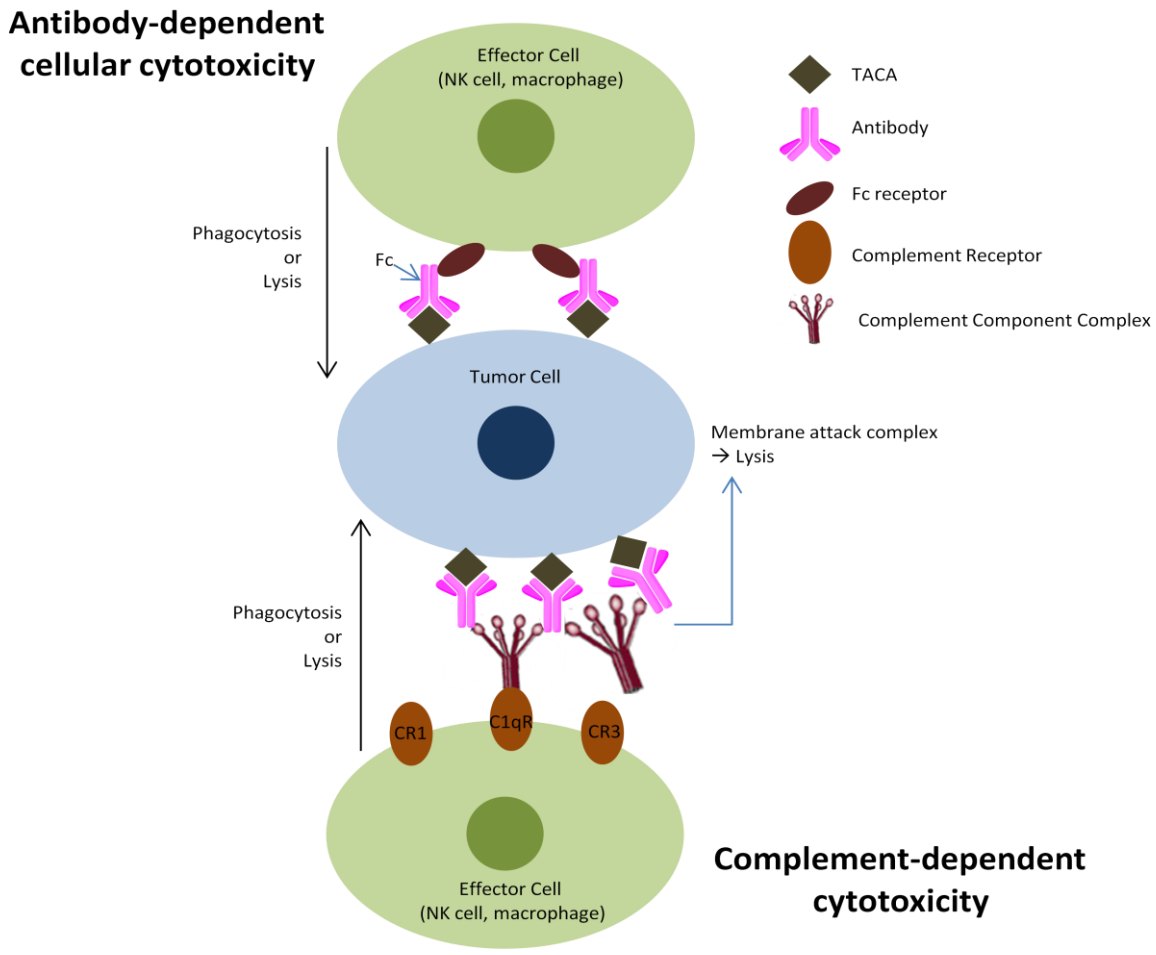


Figure 1.5. Antibodies against tumor-associated carbohydrate and glycopeptide antigens (TACA) have the ability to eliminate tumor cells, by antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC).

Antibodies are produced by B-cells that have been activated with their cognate antigen. The B-lymphocytes carry membrane-bound Ig proteins that can recognize a wide variety of compounds. Carbohydrates, for example, can bind to receptors of B-lymphocytes, induce cross-linking of the Ig proteins, which will lead to activation of the B-cell and production of low affinity IgM antibodies.[90] To achieve a class switch to high affinity IgG antibodies, the B-cells need to interact with helper T-cells (Figure 1.6).[91,92] Activation of helper T-cells requires, in turn, the involvement of antigen-presenting cells (APCs). The most highly specialized APCs are dendritic cells, which are capable of capturing protein antigens that, after internalization and proteolytic cleavage into peptides, are presented on the surface of the APC as a complex with class II major histocompatibility complex (MHC) molecules. Subsequently, the APCs will migrate to the lymph nodes where the peptide complexed to class II MHC will interact with the T-cell receptors of naïve T-lymphocytes, resulting in their activation.[93, 94] A similar type of interaction via MHC class II exists between B-cells and T-cells. Naïve B- and helper T-cells reside in different compartments of the lymphatic system and are induced to migrate towards one another only after activation by an antigen, ensuring that the cells come together only when needed. Thus, activation of naïve T-cells induce migration to the T-cell zone where the T-helper cell will interact with B-cells.[95] The class II MHC-peptide complex presented by a B-cell will mediate an interaction with the helper T-cells, which will lead to expression of co-stimulatory proteins, further augmenting the interaction between the two cell types. Activated helper T-cells express CD40L, which will bind with CD40 on the B-cell resulting in cytokine production by the T-cell.[96] A combination of binding to CD40 and cytokine signaling will stimulate the B-cell to proliferate and differentiate into antibody-secreting cells. In addition, memory B-cells will be formed that live for a long time and respond rapidly to subsequent exposures of antigen by differentiating into high-affinity (IgG) antibody secretors.[97]

MHC molecules show a relatively broad specificity for peptide binding and the fine specificity of antigen recognition resides largely in the antigen receptor of the T-lymphocyte. However, peptides that can bind to MHC share many structural features that promote binding interactions. In general, class II MHC requires peptides of 12-20 amino acids for optimum binding. MHC genes are polymorphic with more than 250 alleles for some of these genes in the population. As a result, different persons recognize different peptides as T-helper epitopes. Structural studies have, however, identified peptide sequences that are recognized by many individuals. These peptides are named universal or promiscuous peptide T- helper epitope and have garnered attention for vaccine development.

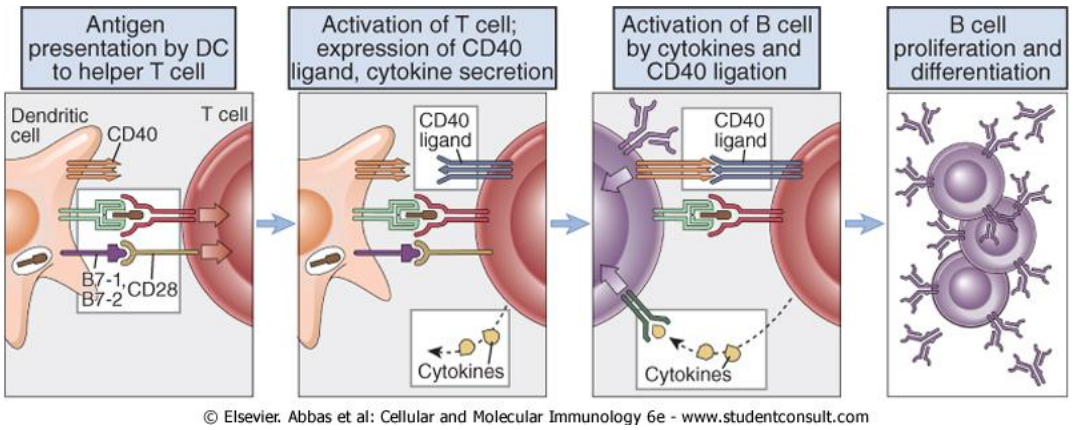


Figure 1.6. Mechanism of T-cell and B-cell activation.[98]

1.4.3 MHC-mediated response to glycopeptides

$CD8^+$ and $CD4^+$ T cells recognize protein antigens presented as peptides bound to MHC class I and II molecules, respectively (Figure 1.7). Extracellular protein antigens are recognized by APCs which generate MHC class II restricted peptide epitopes through a multi-step process. The process culminates in transport of a MHC class II-peptide complex to the cell surface and presentation of the peptide antigen by MHC class II to $CD4^+$ helper T cells. MHC class I molecules, on the other hand, form a complex with peptides derived from intracellular microbial proteins. When this complex is presented on the cell surface the peptide antigen can be

recognized by $CD8^+$ cytotoxic T-cells, which leads to their activation and proliferation resulting in killing of infected cells.

Contrary to previous understanding, it is now apparent that glycopeptides can mediate classical MHC-mediated immune responses.[99-102] In addition to the peptide backbone that provides the binding motif for the MHC molecule, the glycan moiety can facilitate the recognition of T-cells and stimulate immune responses specific for a carbohydrate antigen. For example, this feature has been shown in studies employing glycopeptides derived from type II collagen and the HIV envelope glycoprotein.[103,104] These studies revealed that the peptide backbone binds to the MHC class II groove and that the sugar moiety was recognized by T-cell receptor on T-cells. Studying endosomal processing of MUC1 by APCs showed that the carbohydrates survive the cellular processing of the glycoprotein by dendritic cells (DCs) for presentation of the generated glycopeptide antigens to MHC II.[105]

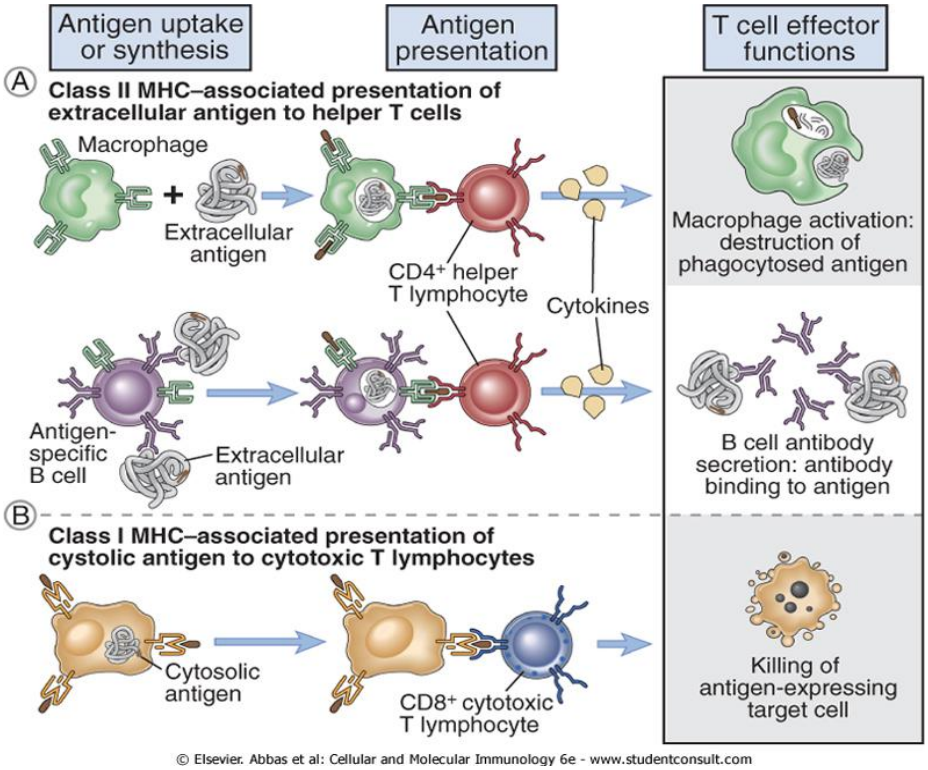


Figure 1.7. MHC class I and II antigen presentation.[98]

DCs have long been known to activate helper T-cells through the MHC-II pathway. On the other hand, it was believed that MHC class I presentation of endogenous peptide antigens is performed by nucleated somatic cells. However, it is now apparent that in addition to class II restricted epitopes, DCs can acquire exogenous antigens and generate MHC class I restricted peptides and present these to $CD8^+$ cytotoxic T cells in a process termed "cross-presentation".[106-108] Adenocarcinomas express only low levels of MHC class II molecules and in addition, $CD4^+$ helper T cells are not expected to react with tumor cells. Cytotoxic T-lymphocytes (CTL) on the other hand, are expected to have a direct effect on tumor cells and represent an opportunity for the development of glycopeptide-based cancer vaccines.[109] Native MUC1 glycopeptides have been shown to bind to MHC class I molecules both *in vitro* and *in vivo.*[110] The binding affinity was higher for the glycopeptide compared to peptide alone. MUC1 glycopeptides carrying the TF- or Tn-antigen designed to have a high affinity for MHC class I molecules, were used to induce a carbohydrate-specific cytotoxic T-cell response in mice.[111] It was found that the CTL lines generated with TF- or Tn-antigen, cross-reacted with both Tn- and TF-, which led to the conclusion that the GalNAc residue is highly immunogenic and is recognized by the large majority of T-cell receptors. However, helper T-cells are required to sustain $CD8^+$ cells and to ensure the development of memory CD8+ cells.[95, 112] Therefore, Gendler and co-workers designed and immunologically elucidated a di-epitope vaccine candidate that incorporates a MUC1 CTL epitope and a universal helper T-cell epitope derived from the Hepatitis B core antigen sequence.[113] The vaccine was administered in combination with GM-CSF and phosphate-guanine-containing oligodeoxynucleotides (CpG-ODN) as adjuvants. The vaccine was tested in a therapeutic and prophylactic setting in a mouse tumor model, using MC38 colon cancer cells, and led to a reduction of tumor burden and complete tumor rejection, respectively.

1.4.4 Toll-like receptors and innate and adaptive immunity

In addition to activation of B- and T-lymphocytes, adaptive immune responses require danger signals which are provided by the innate immune system (Figure 1.8). This mode of activation is called the two-signal hypothesis for lymphocyte activation and ensures that immune responses are not induced against harmless substances or self-antigens. In a vaccine setting, an adjuvant is included to provide the necessary danger signals.

The innate immune system is an evolutionarily ancient system designed to detect the presence of microbial invaders and activate protective responses.[114] The innate immune system responds rapidly to families of highly conserved compounds, which are integral parts of pathogens and perceived danger signals by the host. Recognition of these pathogen-associated molecular patterns (PAMPs) is mediated by sets of highly conserved receptors, whose activation results in acute inflammatory responses. [115] These transmembrane receptor proteins are referred to as Toll-like receptors (TLRs). The responses mediated by TLRs include direct local attack against the invading pathogen and the production of a diverse set of cytokines and chemokines.[116-118] Apart from possessing antimicrobial properties, cytokines also activate and regulate the adaptive component of the immune system.[119, 120] Thus, while the innate and adaptive components of the immune system are often depicted as being distinct entities, in effect they complement and compensate for each other.

The discovery of the TLRs less than a decade ago has advanced our understanding of early events in microbial recognition and response, and the subsequent development of an adaptive immune response.[121-127] To date, eleven members of the mammalian TLR family have been identified, each potentially recognizing a discrete class of PAMPs.[128] For example, lipopeptides such as Pam_2Cys are recognized by TLR2/6, Pam_3Cys derivatives by TLR1/2, lipopolysaccharide (LPS) by TLR4/MD2, bacterial flagellin by TLR5, double-stranded RNA by TLR3, and bacterial DNA by TLR9.[129]

There is emerging evidence that cytokines, produced by activation of TLRs, play crucial roles in the initiation and control of adaptive immune responses. For example, antigen presenting cells and naïve T-cells need to be stimulated to produce a number of co-stimulatory proteins for optimum interaction between T-helper cells and B- and antigen presenting cells.[130, 131] Other cytokines are important for directing the effector T-cell response towards a T helper-1 (Th-1) or T-helper-2 (Th-2) phenotype.[132] Several studies have indicated that the B7-1 signal preferentially promotes the development of Th-1 cells, which leads to the production of pro-inflammatory cytokines including interferon gamma (IFN-γ) and tumor necrosis factor β (TNF-β) and stimulates the production of cytotoxic T-lymphocytes. The B7-2 signal leads to the development of Th-2 cells which produce interleukin-4 (IL-4) and IL-5 cytokines which favor antibody production and class switching (Figure 1.8).[133]

During the last few years, it has become clear that many adjuvants used for immunization contain ligands for TLRs. For example, the active component of complete Freund's adjuvant (CFA) is heat killed mycobacterium tuberculosis that has several ligands for TLRs. Recently, TLR9 agonists have demonstrated potential for treatment of cancer both as monotherapy and in combination with other immunotherapies such as therapeutic vaccines.[134] The agonists induce activation and maturation of dendritic cells, which initiate activation of natural killer cells and the expansion of Th-1 cells and CTLs. TLR9 agonists also enhance the differentiation of B-cells into antibody secreting cells. A range of synthetic CpG-ODNs are currently in clinical trials. Their use as vaccine adjuvants have shown to enhance antibody titers and antigen-specific $CD8^+$ T-cells. As previously discussed, a MUC1 glycopeptide vaccine co-administered with CpG-ODN was shown to reduce the tumor burden in mice.[113]

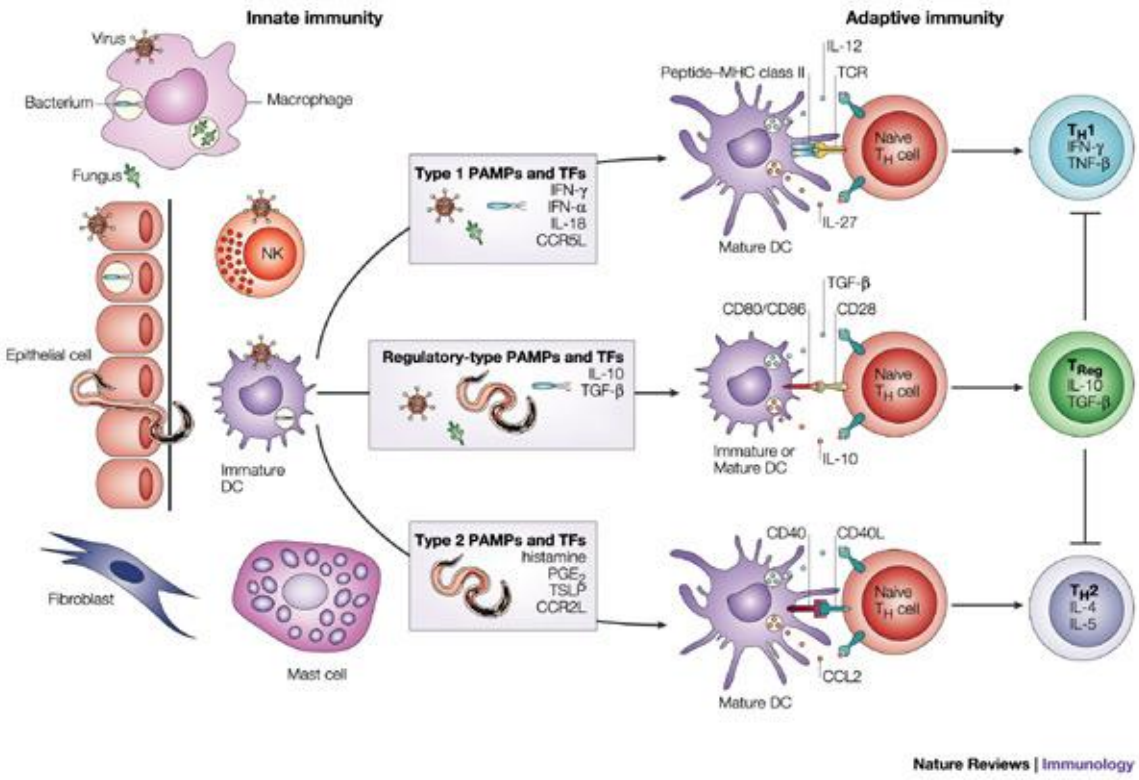


Figure 1.8. Bridging innate and adaptive immunity.[135]

1.5 Immunotherapy for cancer: carbohydrate-based cancer vaccines

1.5.1 Classical approach to carbohydrate-based vaccines

Classical carbohydrate-based cancer vaccines follow the successful approach used for bacterial carbohydrate antigens, involving the conjugation of a carbohydrate antigen to a carrier protein such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), or tetanus toxoid (TT).[43, 136] The carrier protein provides helper T-epitope peptides (12-15 amino acids), which are presented on the surface of an APC in complex with MHC after internalization and proteolysis. As a result, a class switch from low affinity IgM to high affinity IgG antibodies can be accomplished. The protein carrier thus enhances the presentation of the carbohydrate antigen and induces activation of helper T-cells. Proteins can also possess mitogenic and adjuvant-like properties that stimulate the innate immune response to provide cytokines. In addition,

conjugate vaccines are often administered with an immunoadjuvant such as BCG, Detox, QS-21, GPI-0100 or MOLA to further stimulate the innate immune response.

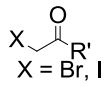
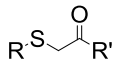
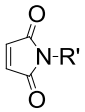
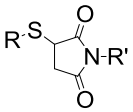
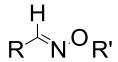
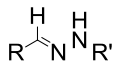
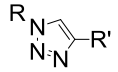
An important issue for carbohydrate-protein conjugate cancer vaccine development is the use of appropriate conjugation chemistry to attach the carbohydrate antigen to the carrier protein. Carbohydrates isolated from natural sources are typically conjugated to a protein carrier by reductive amination through the aldehyde functionality of the reducing end sugar. This might destroy the vital recognition elements, especially in the case of short oligosaccharides, resulting in a decrease or complete loss of immunogenicity. Synthetic oligosaccharides, on the other hand, can be designed to incorporate a linker that has a functional group with unique reactivity for selective conjugation to a carrier protein in a manner that does not interfere with the antigenic epitope.

1.5.2 Conjugate vaccines using synthetic carbohydrate antigens

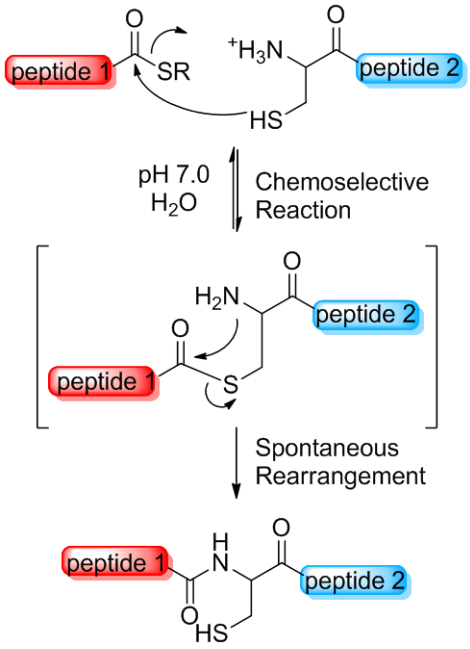
The power of organic synthesis has made it possible to prepare highly complex tumor-associated carbohydrate antigens. Efficient synthetic methods are critical for the development of carbohydrate-based vaccines and although considerable improvements have been made in this field,[137-145] the construction of oligosaccharides and glycopeptides remains a challenging task due to the combined demands of elaborate procedures for glycosyl donor and acceptor preparation and the requirements of regio-and stereo-selectivity in glycoside bond formation. Many new leaving groups for the anomeric center have been developed, which can be introduced under mild reaction conditions and are sufficiently stable for purification and storage for a considerable period of time. The most commonly employed glycosyl donors include anomeric fluorides, trichloroacetimidates, and thioglycosides.[146-148] These approaches, under the appropriate reaction conditions, can give high yields and anomeric ratios. The glycal assembly strategy, the use of anomeric sulfoxides, and dehydrative glycosylation protocols are

also emerging as attractive tools for the assembly of complex oligosaccharides.[149-154] Furthermore, these leaving groups can be activated under mild reaction conditions and guarantee high yields and good anomeric ratios. Convergent synthetic strategies that enable the convenient assembly of complex oligosaccharides from properly protected building blocks involving a minimum number of synthetic steps have become available. In particular, one-pot-multi-step approaches for oligosaccharide preparation are being pursued, which do not require immediate work-up and purification steps, hence speed up the process of chemical synthesis considerably.[155-157] Several research groups have demonstrated that chemoselective, orthogonal, and iterative glycosylation strategies, which exploit differential reactivities of anomeric leaving groups, allow several selected glycosyl donors to react in a specific order, resulting in a single oligosaccharide product.[158-162] Methods for solid phase oligosaccharide synthesis have been reported and these procedures shorten oligosaccharide synthesis by removing the need to purify intermediate derivatives.[163-165]

Table 1.1. Conjugation chemistry for ligation of a peptide epitope and an oligosaccharide (or glycopeptide).

Reaction	Functional Group 1	Functional Group 2	Product
Thioalkylation	R-SH	 X = Br, I	
Thiol addition	R-SH		
Disulfide formation	R-SH	HS-R'	
Oxime formation			
Hydrazone formation			
Huisgen cycloaddition Triazole formation	$R-N_3$		

A crucial step in the chemical synthesis of glycopeptide vaccine candidates is the merger of carbohydrate and peptide chemistry.[138, 142] Different synthetic approaches can be envisaged for the preparation of glycopeptides. For example, a protected (or unprotected) oligosaccharide can be linked to the side chain of an amino acid and then be incorporated by solid-phase glycopeptide synthesis. Alternatively, an unprotected oligosaccharide equipped with a proper functional group can be conjugated to a peptide using well-established conjugation chemistry, such as disulfide and thioether formation and oxime chemistry (Table 1.1). Recently, native chemical ligation and “click” chemistry (Cu(I)-mediated Huisgen cycloaddition) have emerged as powerful tools for chemoselective ligations.[166-168] In the Huisgen cycloaddition, an azide and an alkyne group reacts, typically in the presence of Cu(I) to form a triazole moiety. Although attractive, it should be noted that the click reaction introduces a rigid triazole moiety, which may be immunogenic and thus further suppress the low immunogenicity of a tumor-associated carbohydrate antigens. Native chemical ligation (NCL), on the other hand, is a chemoselective reaction that results in the formation of an amide bond (Scheme 1.1).



Scheme 1.1. Schematic presentation of the mechanism of native chemical ligation (NCL)

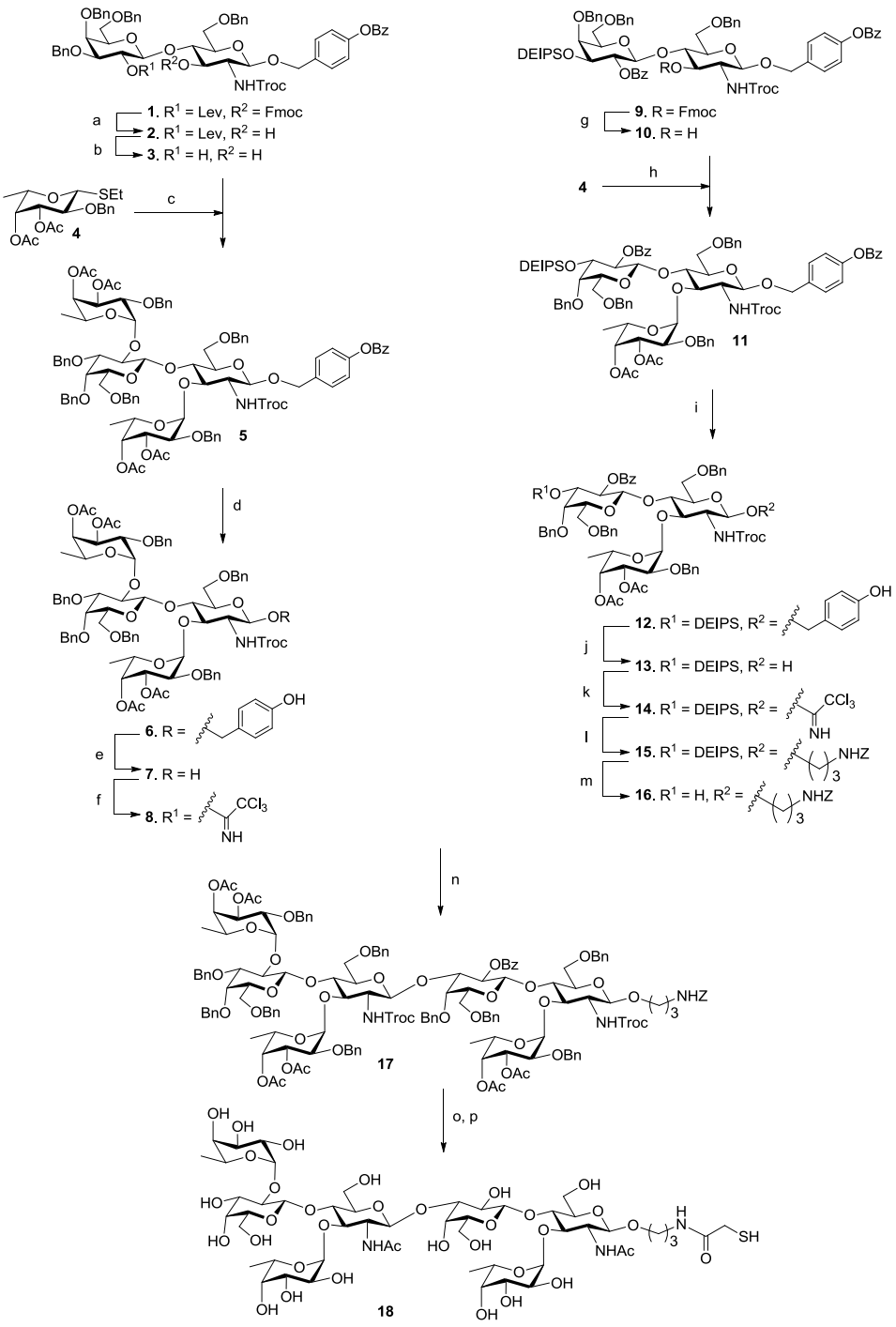
Research teams led by Livingston and Danishefsky at Memorial Sloan-Kettering Cancer Center have made notable contributions to the field of carbohydrate-based cancer vaccine development, but several other research groups have also reported elegant syntheses and immunological evaluations of these antigens.[144, 169-186] The Livingston-Danishefsky team has reported the synthesis of Globo-H, Le^y, Le^x, Le^b, KH-1, MUC1, and the Tn, STn, and TF antigens.[187-194] Several of the antigens have also been synthesized in a clustered configuration in an attempt to improve immunogenicity. The rationale behind the clustered presentation of TACAs is that in the humoral immune response, after a B-cell recognizes its cognate antigen, antigen-induced clustering of the B-cell receptors is necessary to deliver the biochemical signals to the B-cell in order to initiate the process of activation.

In those cases, the oligosaccharide antigens were equipped with an allyl linker, which is subjected to ozonolysis to provide an aldehyde group and enables conjugation to a protein carrier by reductive amination.[187, 188, 192] An alternative method involves the use of maleimide derived proteins that can be reacted with thiolated carbohydrate antigens. The conjugates have been evaluated in mice and typically both IgM and IgG antibodies were elicited which were able to recognize natural epitopes expressed by tumor cells and induce complement-mediated lysis of tumor cells.

Boons and coworkers have developed both a solid support and solution phase synthesis of the Le^y,[170, 186] Le^x,[186, 195] and the KH-1(Le^y-Le^x)[173] antigens. In the synthesis of the KH-1 antigen (**18**), which was equipped with an artificial aminopropyl spacer, two different orthogonally protected lactosamine building blocks (**1** and **9**) were employed (Scheme 1.2). In addition to the orthogonal Fmoc, Lev, Troc,[186, 196] and silyl protecting groups, a *p*-(benzoyl)-benzyl goup was used as a novel anomeric protecting group, which could be selectively removed at a late stage in the synthesis, thus offering the benefit of enhanced flexibility. The approach provided easy access to a Le^y glycosyl donor (**8**) and a Le^x acceptor (**16**) that could

be coupled in one key glycosylation to provide the hetero dimeric Lewis antigen (Scheme 1.2). The KH-1 antigen derivatized with a thio acetyl was conjugated to KLH that had been activated with electrophilic 3-(bromoacetamido)propionyl groups. Immunizations with the conjugate in combination with the adjuvant QS-21 evoked a strong immune response against the heptasaccharide in mice. Studies of the cross-reactivity revealed that the antibodies also recognized the terminal Le^y antigen, albeit with much lower titers. However, the antibody recognition of the reducing end Le^x trisaccharide was low, clearly demonstrating that the raised antibodies recognized an epitope spanning the two Lewis antigen monomers. These findings support the notion that it may be possible to develop a tumor specific anti-cancer vaccine targeting carbohydrate antigens.

A number of carbohydrate-protein conjugates have been examined in Phase I, II, and III clinical trials.[197-209] The results reported to date indicate that the carbohydrate-conjugated vaccines are well-tolerated, do not induce autoimmune reactions, and appear most promising when used in combination with a potent adjuvant, such as the saponin QS-21, the immunomodulator cyclophosphamide, and stem cell rescue.[210, 211] A clear correlation between vaccine-induced antibody responses and clinical course after immunizations has been found. However, even when optimized immunization protocols were used, it was difficult to induce high titers of high-affinity IgG antibodies in most patients. The results of the pre-clinical and clinical studies indicate that many factors influence the antigenicity of tumor-associated antigens conjugated to carrier proteins. The choice of carrier protein, the conjugation method, the nature of the linker, carbohydrate-loading onto the protein, and the immunoadjuvant can greatly influence the magnitude and specificity of the elicited immune response.[170, 212-215]



Scheme 1.2. Synthesis of the Le^y-Le^x dimer. (a) DCM-Et_3N (5/1, v/v) 95%; (b) NH_2NH_2-HOAc, MeOH, DCM, 87%; (c) NIS, TESOTf, DCM, 0 ^oC; (d) H_2O_2, Et_3N, THF, 82%; (e) DDQ, DCM-H_2O 95/5, 78%; (f) CCl_3CN, DBU, DCM, 91%; (g) DCM-Et_3N (5/1, v/v), 95%; (h) NIS, TESOTf, DCM, 0 ^oC, 74%; (i) H_2O_2, Et_3N, THF, 80%; (j) DDQ, DCM-H_2O (95/5, v/v), 81%; (k) CCl_3CN, DBU, DCM, 90%; (l) BF_3-Et_2O, DCM, 86%; (m) TBAF, HOAc, THF, 82%; (n) NIS, TBSOTf, DCM, -30 ^oC, 62%; (o) 1: Zn, HOAc; 2: Ac_2O, pyridine; 3: $Pd(OAc)_2$, H_2, HOAc-EtOH (1/5 v/v); 4: NaOMe, MeOH, pH 10, 52% over four steps; (p) 1. SAMA-OPfp, Et_3N, DMF; 2. 7% NH_3 in DMF.

1.5.3 Problems with carbohydrate-protein conjugate vaccines

The attachment of a carbohydrate to a carrier protein represents a problematic aspect of conjugate vaccine development. In general, the conjugation chemistry is difficult to control and may result in conjugates with ambiguities in the composition and structure and batch-wise variations of prepared glycoconjugates. As a general rule, a higher loading of tumor-associated oligosaccharide antigen onto the protein induces a stronger immune response and thus batch variations in loading may be detrimental to the vaccine efficacy.[216] In addition, the linkers that are employed for the conjugation of carbohydrate to a carrier protein can be immunogenic, leading to epitope suppression.[170, 217] For example, Boons and co-workers have found that the rigid cyclohexyl maleimide linker, which is often employed in conjugation chemistry because of its rapid and selective reaction with thiol derivatives at near neutral pH, dramatically reduced the immune response of mice towards the Le^y antigen. It was found that mainly IgM and IgG anti-linker antibodies have been elicited.[170] In this study, the carrier protein KLH was activated with a maleimide and then reacted with the Le^y antigen derivatized with a thiol linker (Figure 1.9). Higher titers of anti-Le^y antibodies were obtained when the smaller and more flexible 3-(bromoacetamido)-propionate linker was used for protein activation and attachment of the Le^y antigen. In this case, the immune response toward the linker was reduced, which led to the improved immune response of the Le^y antigen.

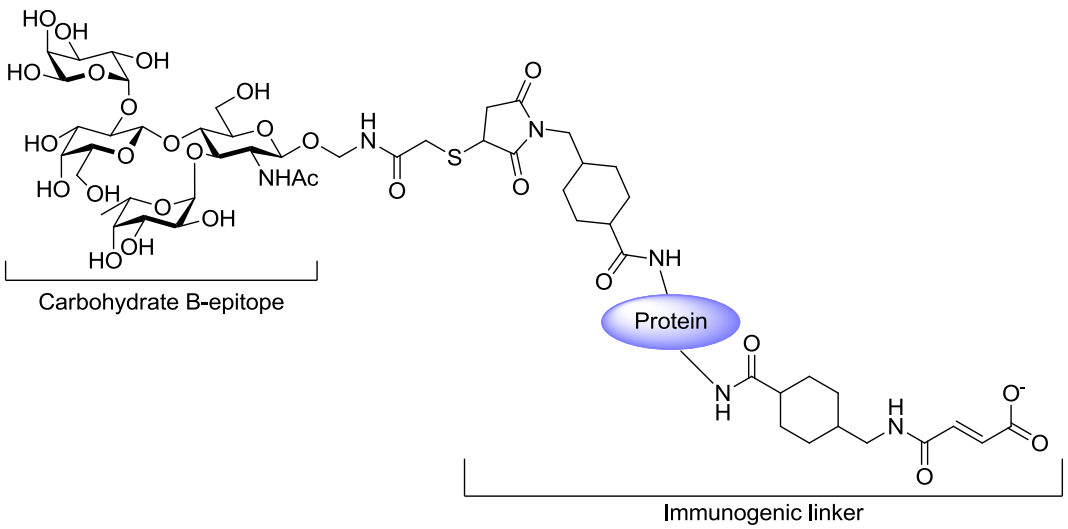


Figure 1.9. The maleimide linker connected with a thiol *vs*. unreacted hydrolyzed linker.

Another major drawback of using carrier proteins is that they are highly immunogenic themselves and will inevitably elicit strong B-cell responses. This feature can lead to carrier-induced epitope suppression, which is in particular a problem when “self-antigens” such as tumor-associated carbohydrates are employed. As a result, novel strategies have been pursued to more efficiently present a tumor-associated carbohydrate epitope to the immune system, resulting in a class switch to IgG antibodies. In particular, attention has been focused on subunit vaccines, which are devoid of any unnecessary immunogenic components, comprising only of those elements necessary for evoking an innate and humoral immune response, which results in a more focused and antigen-specific immune response.

1.5.4 Fully-synthetic carbohydrate-based cancer vaccines

1.5.4.1 Two-component vaccines

One approach to improve the presentation of a TACA to relevant immune cells is to attach the antigen to a receptor ligand which can target or activate appropriate immune cells. Mannosylation of antigens, for example, may result in selective targeting to antigen-presenting cells that carry mannose receptors.

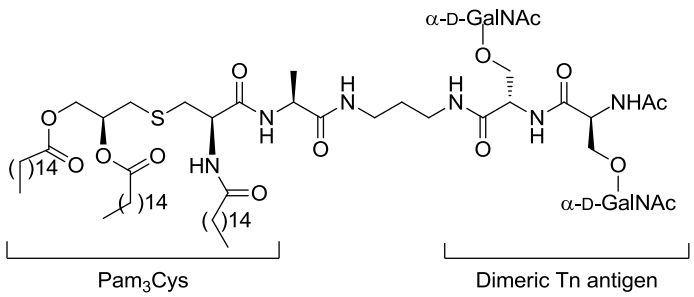


Figure 1.10. A two-component cancer vaccine consisting of a TLR ligand and dimeric Tn-antigen.

Toll-like receptor (TLR) ligands, such as the lipopeptide Pam_3Cys, a TLR2 ligand, has been attached to TACAs. TLR activation by Pam_3Cys leads to cytokine production, which in turn activates dendritic cells, macrophages, and B-cells.[218-221] An example utilizing Pam_3Cys in this fashion was reported by Toyokuni and co-workers, who covalently linked a dimeric Tn-antigen to Pam_3Cys (Figure 1.10).[222, 223] Although low titers of IgG antibodies were elicited, the study showed that a small synthetic carbohydrate antigen could generate an immune response against the carbohydrate without a macromolecular carrier.

Danishefsky and co-workers have utilized a similar strategy and several TACAs including monomeric Le^y, a trimeric cluster of Le^y, and a trimeric Tn-antigen cluster were attached to Pam_3Cys .[191, 213, 214, 224] Mice immunized with the vaccine constructs elicited antibodies that recognized the natural epitope expressed by relevant cancer cell lines. However, mainly IgM antibodies were detected and it was found that the co-administration with the external immunoadjuvant QS-21 did not induce a class switch to IgG antibodies. For the Tn-antigen trimeric cluster, it was found that the trimeric presentation of this antigen gave higher titers of antibodies, which displayed enhanced recognition of Tn-expressing cancer cells. These results highlight that a lack of a helper T-epitope, which is required to induce a class switch to IgG antibodies and affinity maturation, results mainly in the production of IgM antibodies.

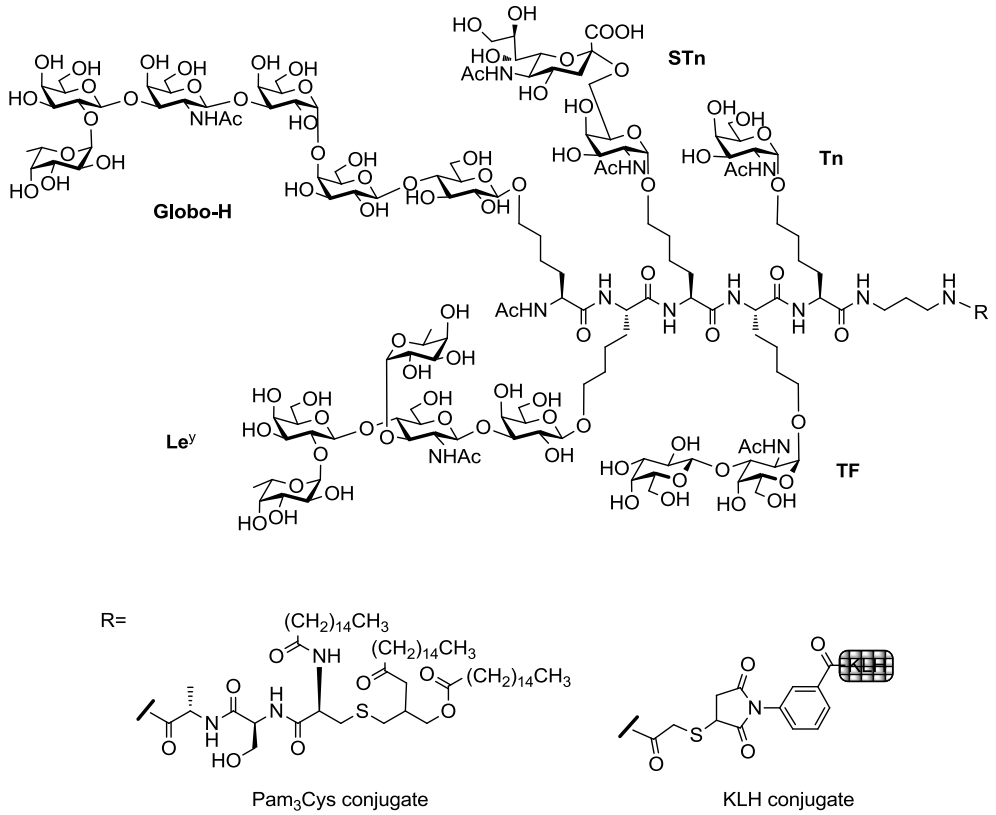


Figure 1.11. Polyantigenic vaccine construct developed by Danishefsky and coworkers.

A commendable chemical synthesis was undertaken to obtain a unimolecular multi-antigenic construct comprising of the Globo-H, Le^y, STn-, TF-, and Tn-antigens all attached to the same peptide backbone (Figure 1.11).[225, 226] The rationale of a polyantigenic construct is that it combines TACAs that are closely related to a particular type of cancer, in this case prostate cancer.[227-229] The oligosaccharides were synthesized using the glycal assembly method and equipped with pentenyl or allyl spacers, which subsequently were used to produce norleucine amino acid building blocks carrying the glycans on the side chain. These building blocks were then used to synthesize the Pam_3Cys-containing construct using conventional peptide chemistry. Mice were inoculated with the candidate vaccine in the presence of the adjuvant QS-21, and IgM antibodies against all antigens were detected. When the multi-antigenic construct was linked to the carrier protein KLH and co-administered with QS-21 in a murine host, both IgM

and IgG antibodies were elicited; the antibodies recognized three different tumor cell lines, all expressing two or more of the five antigens on their respective cell surfaces.

Two-component cancer vaccines composed of a TACA and a CD4+ T-cell epitope have been designed and synthesized to enhance the interaction between the helper T-cell and B-cell, thereby inducing higher titers of antibodies and achieving a class switch to IgG antibodies. In one attempt, a MUC1 derived glycopeptide carrying a single STn moiety was linked to a CD4+ T-cell epitope derived from ovalbumin using a polar non-immunogenic linker (Figure 1.12).[230] The vaccine candidate was administered, together with complete Freund's adjuvant, to transgenic mice expressing T-cell receptors specific for the ovalbumin T-epitope. It was found that an IgG antibody response was mounted and the concentration of serum antibodies increased after each boost. It was also found that antibodies were highly specific for the glycosylated MUC1 peptide when compared to the unglycosylated MUC1 peptide.

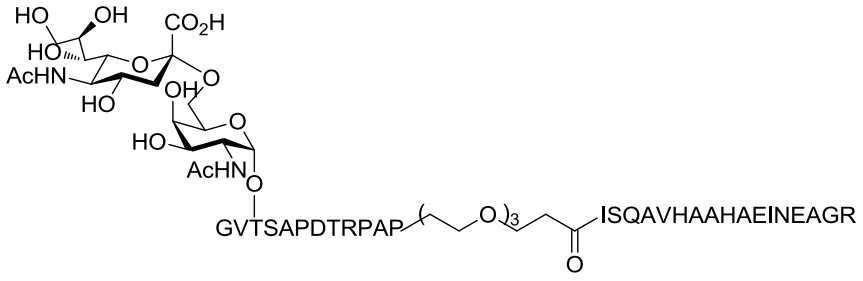


Figure 1.12. Two-component cancer vaccine that incorporates a glycopeptide B-cell epitope and a peptide helper T-cell epitope.

To target the heterogeneity in glycosylation of MUC1 derived peptides, a construct containing three different B-cell epitopes, namely unglycosylated, Tn-, and TF-modified MUC1 and one copy of the universal PADRE helper peptide T-epitope, was evaluated in mice (Figure 1.13).[231] IgG antibodies were raised towards all three B-cell epitopes and the antisera recognized native tumor epitopes expressed by human mammary adenocarcinoma cells.

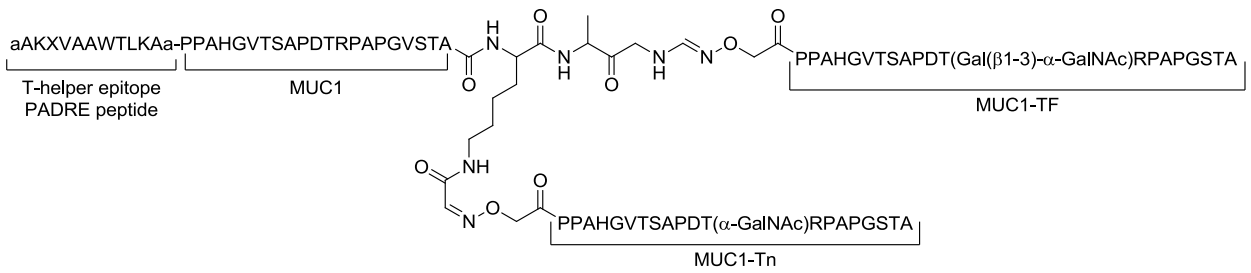


Figure 1.13. A fully synthetic trimeric anti-cancer vaccine consisting of three B-cell epitopes and a helper T-cell peptide, PADRE.

A multi-antigenic glycopeptide (MAG) has been based on a non-immunogenic polylysine scaffold and has successfully been pursued for eliciting antibodies against the Tn-antigen (Figure 1.14). A four-arm lysine core, with each arm extended by a CD4+ peptide T-helper epitope derived from polio virus or the PADRE peptide and a trimeric Tn-antigen has been examined in mice and non-human primates.[232-235] The induced immune response promoted an increase in survival in murine tumor studies, using both a prophylactic and therapeutic setting. In the therapeutic setting, administration of CY, which is reported to increase anti-tumor response, increased the survival rate from 40% to 80%.[234] The clustered MAG construct induced superior titers of anti-Tn IgG antibodies when compared to a KLH conjugate carrying trimeric Tn clusters.[235] The MAG construct elicited good titers of IgG antibodies in the presence of the mild adjuvant Alum, whereas the clustered KLH conjugate required co-administration with the more potent adjuvant QS-21.

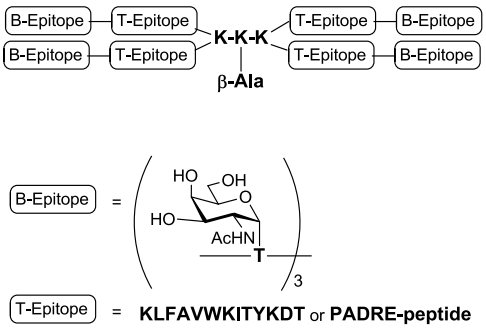


Figure 1.14. Schematic representation of a multiple antigen glycopeptide cancer vaccine candidate containing a tumor associated Tn-antigen cluster and a universal T-cell epitope.

1.5.4.2 Multi-component vaccines

A tri-component vaccine which contains a carbohydrate B-cell epitope, a helper T-cell epitope, and a potent immune activator/modulator, such as a TLR ligand or a cytokine, would incorporate the minimal subunits necessary to evoke an immune response against a carbohydrate.[236-238] In a first report, a fully synthetic three-component anti-cancer vaccine composed of the Tn-antigen, a helper T-epitope derived from *Neisseria meningitis*, and the TLR ligand Pam_3Cys was designed and synthesized using a block synthetic approach.[236] The vaccine candidate was incorporated into phospholipid-based liposomes and then evaluated for its immunogenicity in mice, in the presence or absence of the external adjuvant QS-21. Although only low-to-moderate titers of IgG antibodies were raised against the Tn-antigen, the results indicated promising possibilities for further strategy development.

In a subsequent study, two additional tri-component vaccine candidates composed of the tumor-related MUC1 glycopeptide, a well-documented helper T-cell epitope from the polio virus, and either Pam_2CysSK_4 or Pam_3CysSK_4 as built in immunoadjuvants, were designed (Figure 1.15).[239] Pam_2CysSK_4 is a potent activator of TLR2 and TLR6, while Pam_3CysSK_4 induces cellular activation through TLR1 and TLR2. Compound **19** was prepared by solid-phase peptide synthesis using Rink Amide AM resin and conventional Fmoc-protected amino acid building blocks. After assembly of the glycopeptide, the acetyl esters of the saccharide moiety were cleaved by treatment with 80% hydrazine in methanol. The lipid anchor, *N*-Fmoc-Pam_2Cys-OH, was coupled manually and after cleavage of the *N*-Fmoc group, the glycopeptide was cleaved off the resin and purified. Unfortunately, a similar linear synthesis of vaccine candidate **20** gave a product that was difficult to purify to homogeneity. Therefore, cancer vaccine **20** was prepared by *liposome-mediated* native chemical ligation of building blocks **24**, **25**, and **28** (Scheme 1.3).[240] Boons and coworkers found that the rate and yield of the NCL

reaction was improved if the reactants were embedded in liposomes, especially with the reaction of hydrophobic peptide **28**, which has limited solubility in commonly used ligation

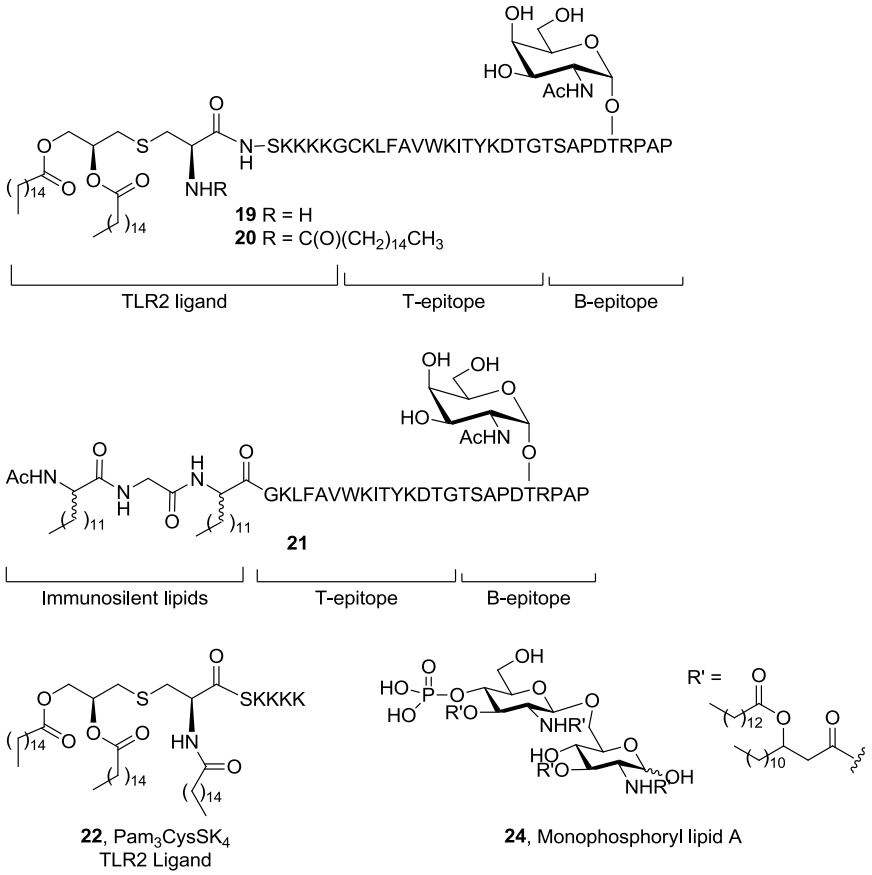


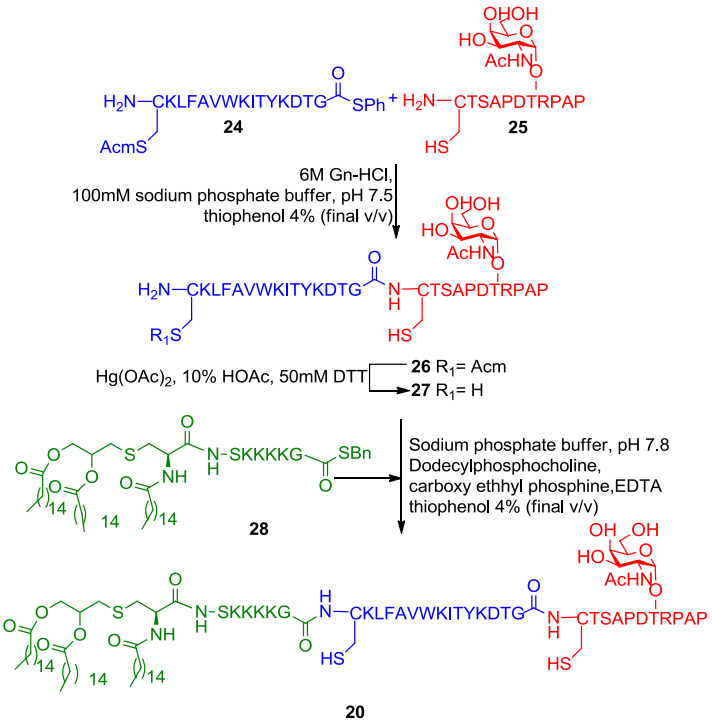
Figure 1.15. Chemical structures of synthetic antigens.

buffers and solvents. In a typical protocol, a film of dodecylphosphocholine, thioester **24**, and thiol **25** was hydrated in a phosphate buffer (pH 7.5) in the presence of tris(2-carboxyethyl)phosphine and EDTA. The liposomes were sized by extrusion and the ligation was initiated by 2-mercaptoethane sulfonate (MESNa). The acetamidomethyl (Acm) thiol protecting group was removed using mercuric acetate and then a subsequent *liposome-mediated* NCL of deprotected glycopeptide **27** and lipopeptide thioester **28** gave Pam_3CysSK_4-containing vaccine **20**. The vaccine candidates were incorporated into phospholipid-based liposomes and their antigenicity was evaluated in BALB/c mice. Compound **20** induced exceptionally high IgG

antibody titers. Further subtyping of antibodies revealed high titers of IgG3 antibodies, which are typical for an anti-carbohydrate response, and a bias towards a Th2 response, as the levels of IgG1 antibodies were high. Co-administration with an external immunoadjuvant QS-21 did not alter the titers of IgG antibodies. However a shift toward a mixed Th1-Th2 response was induced. Interestingly, it was found that vaccine candidate **19**, which incorporates the TLR2 and TLR6 ligand Pam_2CysSK_4, raised lower titers of anti-MUC1 IgG antibodies. The elicited antibodies were shown to bind to MCF7 tumor cells, which express the MUC1 antigen.

The influence of covalent attachment of the various components of the vaccine candidate on antigenic responses and the importance of liposomal preparation of the vaccine were further investigated in mice. Uptake and proteolytic processing of antigen for subsequent presentation of a peptide-MHC class II complex on the surface is critical for eliciting IgG antibodies. It could be argued that by incorporating the three components into a liposome, proteolytic processing would be rendered unnecessary and thus a more robust immune response would be seen. However it was shown that both the covalent attachment of the three components and the liposomal preparation were critical for achieving good antibody titers. The lipid adjuvant moiety of the vaccine aids in presenting the tumor-related antigen in a multivalent fashion to B-cell Ig receptors, which is required to be clustered to induce activation of B-cells. It was also shown that the TLR2 ligand Pam_3CysSK_4 induces cytokines, such as tumor necrosis factor-α (TNF-α), in a TLR-2 dependent manner and facilitated uptake and internalization of the vaccine candidate by cells expressing TLR2. The covalent attachment of the lipid adjuvant also ensures that cytokines are produced locally at the site where the vaccine interacts with relevant immune cells and facilitates uptake by APCs that express TLR2. The importance of the TLR-engagement was further investigated using **21**, containing an immunosilent lipopeptide anchor based on lipidated amino acids rather than on the TLR agonist (Figure 1.15).[241] Lipidated glycopeptide **21** was synthesized in a straightforward manner using solid-phase peptide

synthesis. The compound elicited significantly lower titers of IgG antibodies, demonstrating that TLR engagement is critical for optimum antigenic responses. When compound **21** was co-administered with Pam_3CysSK_4 (**22**) or monophosphoryl lipid A (**23**), similar titers of IgG antibodies were raised in mice. However, the resulting antisera had an impaired ability to recognize cancer cells.



Scheme 1.3. Synthesis of a three-component anti-cancer vaccine.

Recently a multi-epitope vaccine consisting of a cluster of the Tn-antigen as the B-epitope, a $CD4^+$ T-cell epitope, a $CD8^+$ T-cell epitope, and palmitic acid, serving as a built-in adjuvant, was reported.[242, 243] The vaccine was based on the regioselectively addressable functionalized template (RAFT), which is a cyclic decapeptide consisting of proline, glycine, and lysine residues. The side chains of the lysine residues provided opportunities for selective incorporation of different antigens on opposite faces of the RAFT via classical ligation chemistry.

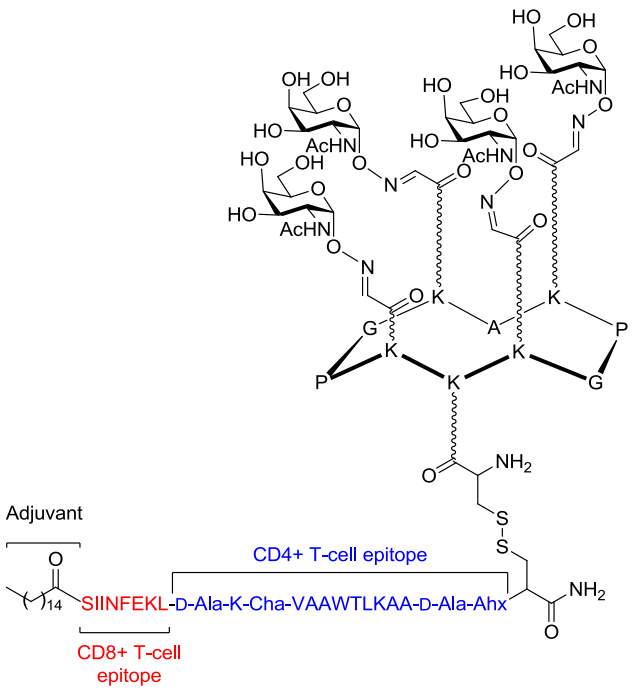


Figure 1.16. Multi-epitope vaccine based on the RAFT scaffold consisting of a cluster of the Tn-antigen as the B-epitope, a $CD4^+$ T-epitope, a $CD8^+$ T-epitope, and a palmitic acid adjuvant.

The candidate vaccine was delivered in an adjuvant-free setting and showed no adverse effects in a murine host. The elicited antibodies were shown to recognize human breast tumor cells MCF7, which express the Tn-antigen. The vaccine also induced strong specific $CD4^+$ T-cell and $CD8^+$ T-cell responses. In prophylactic tumor studies with MO5 tumor cells, none of the twenty mice developed a tumor in the monitoring period of 90 days. In contrast, the survival rate for mice immunized with a vaccine candidate lacking the palmitic acid adjuvant and CpG as an external adjuvant was determined to be 80%.

Fully synthetic anti-cancer vaccines targeting tumor-associated carbohydrates provide an attractive option for the treatment of cancer. Recent developments in the synthesis of complex carbohydrates and glycopeptides have made it possible for the evaluation in pre-clinical and clinical settings. The research has provided important insight into which components influence and are necessary to evoke an immune response capable of eradicating tumor cells. Recent reports have pointed out the importance of including TLR agonists in synthetic subunit

vaccines which are capable of activating the innate immune system. Thus far, there are only two examples of fully synthetic multi-component vaccines that incorporate a tumor-associated glycopeptide antigen, a helper T-cell epitope, and a built-in adjuvant that serves as a potent immune activator/modulator. Contrary to previous understanding, it is now accepted that glycopeptides can mediate classical MHC immune responses. Thus, cytotoxic T-lymphocytes, as opposed to helper T-cells, are expected to react with tumor cells, thus presenting an additional opportunity for glycopeptide-based cancer vaccines.[109] Native MUC1 glycopeptides have been shown to bind to MHC class I both *in vitro* and *in vivo* and high-affinity glycopeptides carrying the Tn- or TF-antigen have been used to induce a carbohydrate-specific cytotoxic T-cell response in mice.[110, 111] Two-component vaccines, consisting of a $CD8^+$ glycopeptide and a helper T-cell epitope have shown promising results in tumor models.[113] Although these results are promising, further pre-clinical and clinical research is necessary to access the full potential of these vaccine candidates and their usefulness in cancer therapy.

The research described in this thesis is aimed at the design of a cancer vaccine candidate that will generate a specific immune response by using chemically well-defined synthetic glycopeptide antigens as immunogens. In this thesis, we describe improved methods for the synthesis of glycolipopeptide cancer vaccine candidates. Chapter II describes the *microwave-assisted* liposome-mediated native chemical ligation for the rapid synthesis of glycolipopeptides. In chapter III, the immunotherapeutic efficacy of fully synthetic three-component vaccine candidates is evaluated in MUC1.tg mice. In chapter III, a prophylactic tumor challenge study is reported in which the vaccine candidate is composed of a Tn-antigen containing MUC1 glycopeptide as the B-epitope, a helper T-cell peptide derived from the polio virus, and the TLR2 ligand Pam_3CysSK_4 as the built-in immunoadjuvant. The tumor-associated carbohydrate antigen, MUC1, is expected to interact with Ig receptors of B-cells, thus directing an antibody response against this component of the vaccine. In addition, the glycopeptide

MUC1 serves as an MHC class I ligand and is expected to induce a carbohydrate-specific cytotoxic T lymphocyte response. After internalization of the glycolipopeptide, the T-helper peptide will be complexed with MHC class II and presented on the surface of B-cells to facilitate the necessary interaction with a T-helper cell. The T-helper epitope is also presented as a complex with MHC-II on the surface of antigen-presenting cells, which results in activation of naïve T-cells. The activated T-cells then migrate to the T-cell zone, where they interact with B-cells. Finally, Pam_3CysSK_4 is a ligand for TLR2 and thus initiates the production of necessary cytokines. The lipopeptide immunoadjuvant also facilitates the incorporation of the vaccine candidate into liposomes, which is necessary for multivalent presentation of the antigen to the immune system. The ability of the cancer vaccine candidate to eradicate tumor cells in addition to the antibody and cytotoxic T lymphocyte responses will be discussed. Chapter IV describes an improved synthetic protocol for the linear synthesis of glycosylated lipopeptide vaccine candidates utilizing microwave-assisted solid-phase peptide synthesis. In chapter IV, the synthesis of vaccine candidates which contain both B-epitopes and T-helper epitopes derived MUC-1 and varying glycosylation patterns are synthesized and evaluated in MUC1.Tg mice. The abilities of the vaccine candidates to induce humoral immune responses are examined. Chapter V discusses the total synthesis of a sialylated MUC1 glycolipopeptide using microwave-assisted solid-phase peptide synthesis.

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CHAPTER II

MICROWAVE-ASSISTED LIPOSOME-MEDIATED NATIVE CHEMICAL LIGATION FOR THE RAPID SYNTHESIS OF GLYCOLIPOPEPTIDES*

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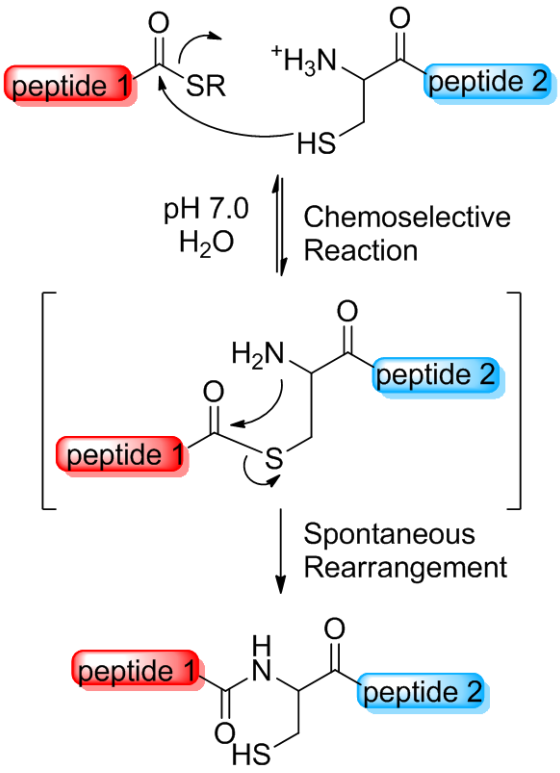
*__Pamela S. Thompson__, Therese Buskas, Geert-Jan Boons. To be submitted to *Org. Lett.*

2.1 Abstract

The over-expression of oligosaccharides, such as Globo-H, Lewis^Y, and Tn antigens, is a common feature on tumor cells. Traditional cancer vaccine candidates composed of a tumor associated carbohydrate conjugated to a carrier protein (e.g. KLH or BSA) have failed to elicit sufficient titers of IgG antibodies. We have developed fully synthetic three-component vaccine candidates composed of a tumor-associated antigen, a promiscuous peptide T-helper epitope, and a lipopeptide adjuvant. In our first approach the compounds were synthesized by solid-phase peptide synthesis (SPPS) combined with native chemical ligation (NCL). Here, we compare our initial approach with microwave-assisted NCL. The results of our study demonstrate that the incorporation of a lipopeptide thioester and an *N*-terminal cysteine glycopeptide into DPC-liposomes and the use of microwave irradiation greatly facilitates NCL to afford a range of glycolipopeptides. The method described here provides the glycolipopeptide product after a reaction time of 20 minutes using only 2 equivalents of the expensive peptide thioester reactant.

2.2 Introduction

Native chemical ligation (NCL) is a chemoselective reaction that occurs at physiological pH between an *N*-terminal cysteine residue and a *C*-terminal peptide thioester (Scheme 2.1).[1-3] In the first step of the ligation, a reversible *trans*-thioesterification occurs between the *C*-terminal thioester and the sulfhydryl of the *N*-terminal cysteine. The ligated peptide thioester undergoes a spontaneous intramolecular *S* → *N* shift, generating the thermodynamically favored native amide bond at the ligation junction.

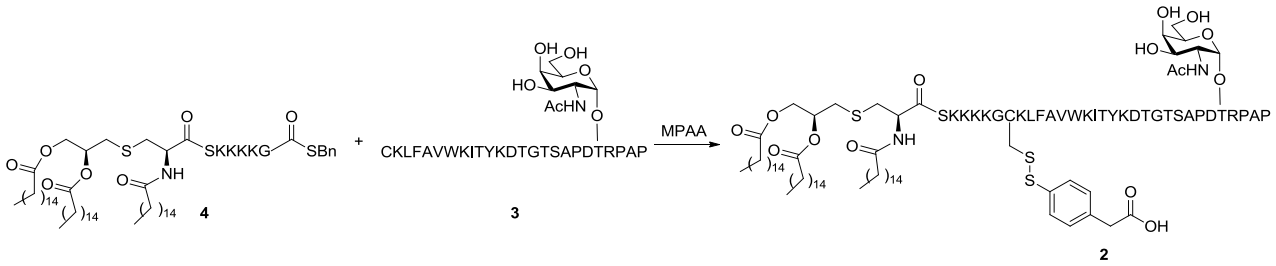


Scheme 2.1. Generalized mechanism of native chemical ligation (NCL).

NCL is a powerful method for the synthesis of proteins and peptides; the reaction is limited, however, when peptide segments are poorly soluble in the aqueous buffer. We have previously shown that glycolipopeptides could be synthesized using liposome-mediated native chemical ligation.[4] The use of liposomes greatly increases the reaction rates of ligations of sparingly soluble peptide reactants. This methodology is attractive for the NCL of lipophilic peptides, which usually gives low yields of products under classical reaction conditions. In our attempts to further optimize the liposome-mediated native chemical ligation, we concluded that 5 equivalents of thioester were required for the reaction to progress with consistent yields.

It has been reported that using 4-mercaptophenylacetic acid (MPAA) as the initiator in native chemical ligations facilitates a faster reaction rate, with excellent yields.[5] We expanded our method to include the use of MPAA and interestingly found that in addition to longer reaction times and 5 equivalents of **4**, the major ligation product was the disulfide of the desired

glycolipopeptide and MPAA (**2**) (Scheme 2.2). It was envisaged that increasing the reaction rate and decreasing the reaction time would decrease the chance of disulfide formation, which could be achieved by using microwave-assisted liposome-mediated native chemical ligation.



Scheme 2.2. Native chemical ligation using MPAA resulted in the formation of a disulfide beween initiator and product.

The use of microwave irradiation in organic synthesis has become increasingly popular in both pharmaceutical industry and academia. Thermally-driven organic transformations can take place in either conventional heating or microwave-accelerated heating.[6] In microwave-accelerated heating, microwaves couple directly with molecules of the entire reaction mixture, resulting in a rapid rise in temperature. Since this process is not limited by the thermal conductivity of the vessel, the result is an instantaneous localized superheating of any substance that will respond to either dipole rotation or ionic conduction.[7, 8] Enhanced microwave synthesis is a method that has been developed to ensure a constant level of microwave energy is applied to a reaction.[6] By externally cooling the reaction vessel with compressed air, while simultaneously administering microwave irradiation, energy can be introduced into the reaction, while keeping the reaction temperature low.

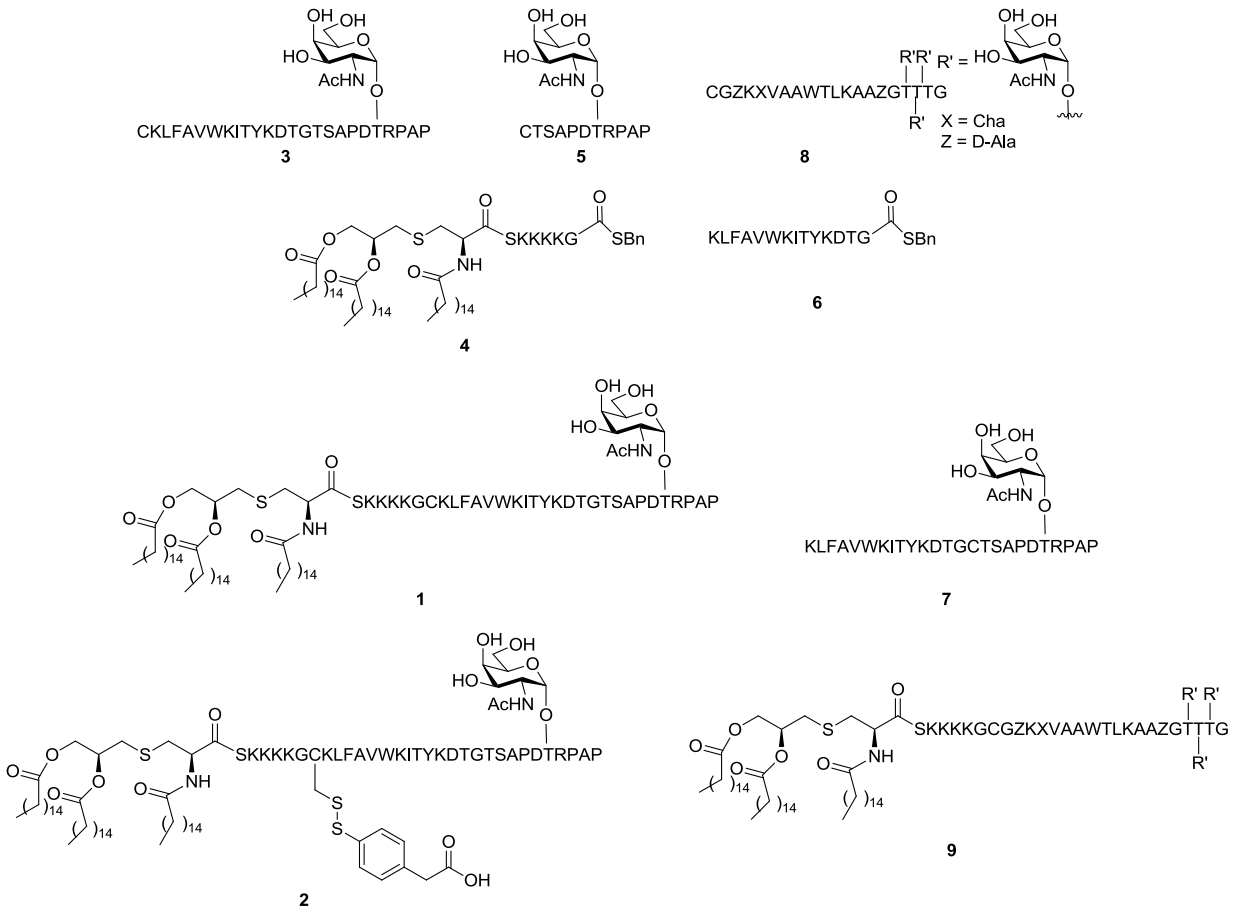


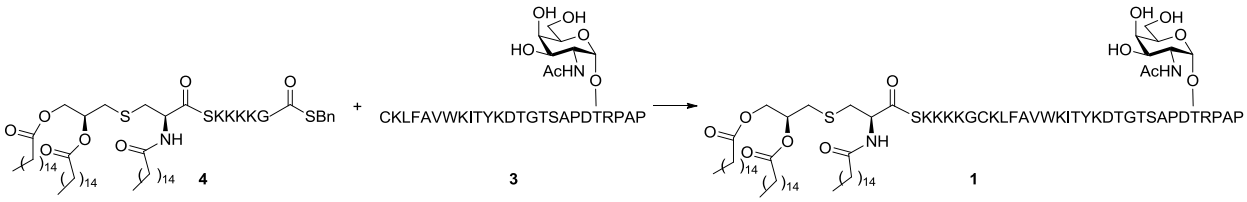
Figure 2.1. Synthetic compounds for *Microwave-assisted* liposome-mediated NCL

We have previously shown that compound **1**, which is composed of a tumor-associated glycopeptide B-cell epitope derived from MUC-1, a polio virus-derived T-cell epitope,[9] and the lipopeptide adjuvant Pam_3CysSK_4,[10, 11] elicits a robust immune response in BALB/c mice.[12, 13] We believed that this compound could be prepared from building blocks **3** and **4** using microwave-assisted native chemical ligation (MW NCL). The NCL between the cysteine moiety of **3** and the thioester of **4** should provide the desired glycolipopeptide **1**.[13]

2.3 Results and discussion

Dodecylphosphocholine (DPC) liposome-mediated chemical ligation was performed using a sodium phosphate buffer (pH 7.8) containing 2 mM TCEP and 0.3% EDTA.[4] The ligation was catalyzed by the addition of 1 mM MPAA and the progress was monitored by RP-

HPLC and MALDI-TOF. After a reaction time of 6 hours, conversion of **3** and **4** into **2** and hydrolysis of the thioester was observed (Scheme 2.1). Following the reduction of disulfide **2** using 20 mM TCEP and 20 mM EDTA in 6M Guanidine hydrochloride and 200 mM sodium phosphate buffer,[14] glycolipopeptide **1** was obtained with 50% yield. In an attempt to improve ligation yields, microwave-assisted native chemical ligation was examined using a variety of reaction conditions.



Scheme 2.3. Synthesis of glycolipopeptide **1** by native chemical ligation.

We first examined microwave-assisted traditional NCL under standard conditions using a phosphate buffer (pH 7.5) containing 6M guanidinium hydrochloride.[15] The ligation was catalyzed by the addition of 4% thiophenol and the progress was monitored by RP-HPLC and MALDI-TOF. After a reaction time of 20 minutes at 37 ^{o}C in an open vessel, 10 % conversion of **3** and **4** into **1** and hydrolysis of the thioester was observed.

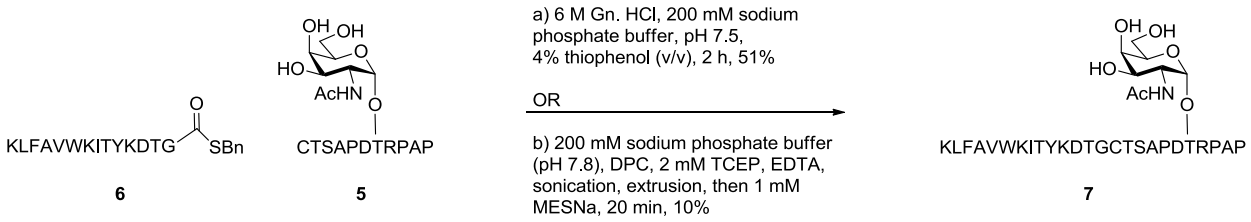
Microwave-assisted liposome-mediated proved to be a higher yielding method. This method was examined using both MPAA and MESNa as initiators, at a variety of temperatures in both sealed and open vessel systems. Each microwave-assisted liposome-mediated NCL ligation was performed in sodium phosphate buffer (pH 7.8) containing 2 mM TCEP and 0.3% EDTA and was catalyzed by the addition of 1 mM of initiator (either MPAA or MESNa) and the progress was monitored by RP-HPLC and MALDI-TOF.

Table 2.1. Reaction conditions explored for the synthesis of glycolipopeptide **1**.

Initiator	***Buffer***	***Reaction Protocols***	***% yield***
4% Thiophenol	1:1.5 GnHCl: 200 mM Sodium phosphate (pH 7.5)	18 hours at 37 ^{o}C	23%
1mM MESNa	200 mM Sodium phosphate (pH 7.8), 2.0 mM TCEP, 0.3% EDTA, degassed 1.5 hr (final pH 7.4) *Liposomal preparation*	2 hours at 37 ^{o}C	35 – 45%
1 mM MPAA		5 hours at 37 ^{o}C	40- 50%
4% Thiophenol	1:1.5 GnHCl: 200 mM Sodium phosphate (pH 7.5)	25 W, 37 ^{o}C, 1 atm, 20 min	10%
1 mM MESNa	200 mM Sodium phosphate (pH 7.8), 2 mM TCEP, 0.3% EDTA, degassed 1.5 hr (final pH 7.4) *Liposomal preparation*	25 W, 37 ^{o}C, 1 atm, 20 min	80%
		25 W, 75 ^{o}C, 1 atm, 20 min	50%
		25 W, 90 ^{o}C, 1 atm, 20 min	75%
		25 W, 90 ^{o}C, 200 psi, 20 min	60%
1 mM MPAA		25 W, 37 ^{o}C, 1 atm, 20 min	>1%
		25 W, 75 ^{o}C, 1 atm, 20 min	0%
		25 W, 90 ^{o}C, 1 atm, 20 min	34%
		25 W, 90 ^{o}C, 200 psi, 20 min	17%

The use of MPAA as initiator proved to be inefficient. After reacting for 20 minutes in an open vessel at 37 ^{o}C w 25W microwave irradiation, less than 1% of **1** was isolated. It was envisaged that increasing the reaction temperature or pressure would increase the reaction yields. At 75 ^{o}C, however, no product was isolated after HPLC purification. At 90 ^{o}C, glycolipopeptide **1** was isolated in a low 34% yield, which was due to disulfide formation between **3** and MPAA.

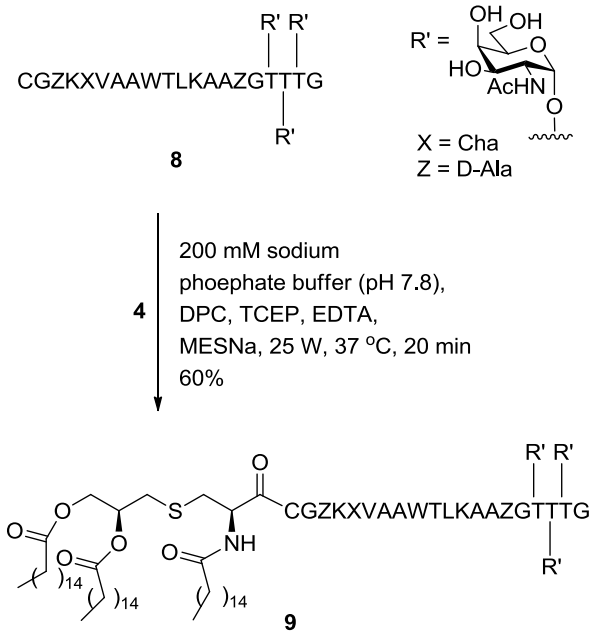
Using MESNa as the initiator, it was found that after a reaction time of 20 minutes at 37 ^{o}C in an open vessel, conversion of **3** and **4** into **1** was observed. Purification by RP-HPLC gave **1** with 80% yield. At increased temperatures of 75 ^{o}C and 90 ^{o}C, the reaction proceeded with slightly lower yields, due to the hydrolysis of thioester **4** at higher temperatures. It was found that performing the reaction under sealed conditions at 200 psi did not have an effect on the efficiency of the reaction at higher temperatures.



Scheme 2.2. Synthesis of glycopeptide **7** under microwave irradiation at 25 W and 37 ^{o}C.

The scope of this method was expanded to include more soluble peptide fragments. Peptides **5** and **6** were used to synthesize **7**, which contains a polio-derived T-helper peptide and a tumor associated glycopeptide. We found that microwave irradiation of peptides **5** and **6** at 25 W at 37 ^{o}C using thiophenol as the initiator provided glycopeptide **7** in 51% yield within 2 hours.

In our initial attempts we were unable to synthesize glycolipopeptide **9**, which consists of a clustered Tn antigen, a promiscuous T-helper peptide (PADRE)[16-18], and lipopeptide adjuvant Pam_3CysSK_4 , utilizing conventional Fmoc-based solid-phase peptide synthesis. Encouraged by the preparation of **1**, we focused our attention to glycopeptide **9**, this time using microwave-assisted liposome-mediated native chemical ligation. A liposomal preparation of peptide **8** and **4** was prepared and subjected to the microwave-assisted liposome-mediated reaction conditions employed for the synthesis of **1**. The ligation was catalyzed by the addition of MESNa, and after a reaction time of 20 minutes at 37 ^{o}C in an open vessel, the product was purified by RP-HPLC to give **9** with a yield of 60%.



Scheme 2.3. Synthesis of Pam_3CysSK_4-PADRE-Tn_3 cancer vaccine candidate.

2.4 Conclusion

In summary, we have successfully exploited microwave-assisted liposome-mediated native chemical ligation to obtain glycolipopeptides for use as cancer vaccine candidates using a highly efficient protocol. The results of our study demonstrate that the incorporation of a lipopeptide thioester and an *N*-terminal cysteine glycopeptide into DPC-liposomes and the use of microwave irradiation greatly facilitates NCL to afford a range of glycolipopeptides. The method described here provides the glycolipopeptide product after a reaction time of 20 minutes using only 2 equivalents of the expensive peptide thioester reactant.

2.4 Experimental Procedure

Reagents and general experimental procedure: Amino acid derivatives and resins were purchased from NovaBioChem; DMF was purchased from EM Science and NMP from Applied Biosystems. Dodecylphosphocholine was obtained from Avanti Polar Lipids. All other chemical reagents were purchased from Aldrich, Acros, Alfa Aesar, and Fischer and used without further

purification. All solvents employed were reagent grade. All microwave reactions were performed using CEM Discover Labmate (open vessel) and Discover Benchmate (sealed vessel) units utilizing external cooling with compressed air. Reverse Phase HPLC was performed on an Agilent 1100 series system equipped with an autosampler, UV detector, and fraction collector. RP-HPLC was carried out using a Zorbax Eclipse C18 analytical column (5 µm, 9.4 x 250 mm) at a flow rate of 1.5 mL/min, a Jupiter C4 semi-preparative column (5 µm, 10 x 250 mm) at a flow rate of 3 mL/min, and a Vydac C4 analytical column (5 µm, 4.6 x 250 mm) at a flow rate of 1 mL/min. All runs used linear gradients of 0 – 100% solvent B in A over a 40 minute period unless otherwise specified. (A: 95% Water, 5% Acetonitrile, 0.1% TFA; B: 95% Acetonitrile, 5% Water, 0.1% TFA) High resolution mass spectra were obtained by using MALDI-ToF (Applied Biosystems 4700 Proteomics Analyzer) with α-cyano-4-hydroxycinnamic acid as an internal standard matrix.

General methods for Solid-Phase Peptide Synthesis (SPPS): Peptides were synthesized by established protocols on an Applied Biosystems, ABI 433A peptide synthesizer equipped with a UV detector using N^{α}-Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) as the activating reagents. Single coupling steps were performed with conditional capping. The coupling of the glycosylated amino acid N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc), *N*-fluorenylmethoxycarbonyl-*R*-(2,3-bis(palmitoyloxy)-(2*R*-propyl)-(R)-cysteine, and palmitic acid were carried out manually. The manual couplings were monitored by standard Kaiser test.

General methods for liposome preparation: A pH 7.8 200 mM sodium phosphate buffer containing 2 mM tris(2-carboxyethyl)phosphine (TCEP) and 0.3% EDTA was prepared. The buffer was degassed for 1 hour. The cysteine-containing peptide (1 eq.), thioester (2 eq.), and dodecylposphocholine (13 eq.) were dissolved in 1:1 $CHCl_3$:Trifluoroethanol and the solvents were removed. The lipid/peptide film was then hydrated in an incubator at 41 ^{o}C for 4 hours.

The mixture was sonicated and the peptide/lipid suspension was extruded through 1.0 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^{o}C to obtain uniform vesicles.

Synthesis of glycolipopeptide 2:

Peptide thioester **4** (1.1 mg, 0.674 μmol), peptide **3** (1.0 mg, 0.337 μmol), and dodecylphosphocholine (1.5 mg, 4.38 μmol) were dissolved in a mixture of 1:1 $CHCl_3$:Trifluoroethanol (5 mL). The solvents were removed under reduced pressure to give a lipid/peptide film. The lipid/peptide film was hydrated for 4 hours at 41 ^{o}C using a 200 mM sodium phosphate buffer containing 2 mM TCEP and 0.3% EDTA. The mixture was sonicated and the peptide/lipid suspension was extruded through 1.0 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^{o}C to obtain uniform vesicles. To the vesicle suspension was added 4-mercaptophenylacetic acid (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was carried out in an incubator at 37 ^{o}C. The reaction was monitored by RP-HPLC and after 6 hours the reaction mixture was purified by RP-HPLC on an analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 minutes. Lyophilization of the appropriate fractions afforded **2** as the major product (75%). $C_{225}H_{373}N_{45}O_{55}S_2$ HR MALDI-ToF MS: observed 4681.4409, calculated 4681.6936 (M+).

Synthesis of glycolipopeptide 1:

Condition A: Peptide thioester **4** (0.56 mg, 0.337 μmol), peptide **3** (0.5 mg, 0.168 μmol), and dodecylphosphocholine (0.77 mg, 2.184 μmol) were dissolved in a mixture of 1:1 $CHCl_3$:Trifluoroethanol (5 mL). The solvents were removed under reduced pressure to give a lipid/peptide film. The lipid/peptide film was hydrated for 4 hours at 41 ^{o}C using a 200 mM sodium phosphate buffer containing 2 mM TCEP and 0.3% EDTA. The mixture was sonicated and the peptide/lipid suspension was extruded through 1.0 μm polycarbonate membranes

(Whatman, Nucleopore, Track-Etch Membrane) at 50 ^{o}C to obtain uniform vesicles. To the vesicle suspension was added 4-mercaptophenylacetic acid (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was carried out in a CEM Discover Labmate unit at 25 W and 37 ^{o}C under open vessel conditions. After 20 minutes, the reaction mixture was purified by RP-HPLC on an analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 minutes. Lyophilization of the appropriate fractions afforded **1** (>1%). $C_{217}H_{367}N_{45}O_{53}S$ HR MALDI-ToF MS: observed 4516.5283, calculated 4516.6926 (M+H).

Condition B: Peptide thioester **4** (0.56 mg, 0.337 μmol), peptide **3** (0.5 mg, 0.168 μmol), and dodecylphosphocholine (0.77 mg, 2.184 μmol) were dissolved in a mixture of 1:1 $CHCl_3$:Trifluoroethanol (5 mL). The solvents were removed under reduced pressure to give a lipid/peptide film. The lipid/peptide film was hydrated for 4 hours at 41 ^{o}C using a 200 mM sodium phosphate buffer containing 2 mM TCEP and 0.3% EDTA. The mixture was sonicated and the peptide/lipid suspension was extruded through 1.0 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^{o}C to obtain uniform vesicles. To the vesicle suspension was added 4-mercaptophenylacetic acid (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was carried out in a CEM Discover Labmate unit at 25 W, 75 ^{o}C, and atmospheric pressure under open vessel conditions. After 20 minutes, the reaction mixture was purified by RP-HPLC on an analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 minutes. Analysis of the fractions showed no product was formed.

Condition C: Peptide thioester **4** (0.56 mg, 0.337 μmol), peptide **3** (0.5 mg, 0.168 μmol), and dodecylphosphocholine (0.77 mg, 2.184 μmol) were dissolved in a mixture of 1:1 $CHCl_3$:Trifluoroethanol (5 mL). The solvents were removed under reduced pressure to give a lipid/peptide film. The lipid/peptide film was hydrated for 4 hours at 41 ^{o}C using a 200 mM sodium phosphate buffer containing 2 mM TCEP and 0.3% EDTA. The mixture was sonicated

and the peptide/lipid suspension was extruded through 1.0 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^{o}C to obtain uniform vesicles. To the vesicle suspension was added 4-mercaptophenylacetic acid (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was carried out in a CEM Discover Labmate unit at 25 W and 90 ^{o}C under open vessel conditions. After 20 minutes, the reaction mixture was purified by RP-HPLC on an analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 minutes. Lyophilization of the appropriate fractions afforded **1** (34%). $C_{217}H_{367}N_{45}O_{53}S$ HR MALDI-ToF MS: observed 4516.6650, calculated 4516.9626 (M+H).

Condition D: Peptide thioester **4** (0.56 mg, 0.337 μmol), peptide **3** (0.5 mg, 0.168 μmol), and dodecylphosphocholine (0.77 mg, 2.184 μmol) were dissolved in a mixture of 1:1 $CHCl_3$:Trifluoroethanol (5 mL). The solvents were removed under reduced pressure to give a lipid/peptide film. The lipid/peptide film was hydrated for 4 hours at 41 ^{o}C using a 200 mM sodium phosphate buffer containing 2 mM TCEP and 0.3% EDTA. The mixture was sonicated and the peptide/lipid suspension was extruded through 1.0 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^{o}C to obtain uniform vesicles. To the vesicle suspension was added 4-mercaptophenylacetic acid (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was carried out in a CEM Discover Benchmate unit at 25 W, 90 ^{o}C, and 200 psi under sealed vessel conditions. After 20 minutes, the reaction mixture was purified by RP-HPLC on an analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 minutes. Lyophilization of the appropriate fractions afforded **1** (17%). $C_{217}H_{367}N_{45}O_{53}S$ HR MALDI-ToF MS: observed 4516.6650, calculated 4516.6926 (M+H).

Condition E: Peptide thioester **4** (1.1 mg, 0.674 μmol), peptide **3** (1.0 mg, 0.337 μmol), and dodecylphosphocholine (1.5 mg, 4.38 μmol) were dissolved in a mixture of 1:1 $CHCl_3$:Trifluoroethanol (5 mL). The solvents were removed under reduced pressure to give a

lipid/peptide film. The lipid/peptide film was hydrated for 4 hours at 41 ^{o}C using a 200 mM sodium phosphate buffer containing 2 mM TCEP and 0.3% EDTA. The mixture was sonicated and the peptide/lipid suspension was extruded through 1.0 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^{o}C to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was carried out in a CEM Discover Labmate unit at 25 W and 37 ^{o}C under open vessel conditions. After 20 minutes, the reaction mixture was purified by RP-HPLC on an analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 minutes. Lyophilization of the appropriate fractions afforded **1** (80%). $C_{217}H_{367}N_{45}O_{53}S$ HR MALDI-ToF MS: observed 4517.7515, calculated 4517.6926 (M+2H).

Condition F: Peptide thioester **4** (1.1 mg, 0.674 μmol), peptide **3** (1.0 mg, 0.337 μmol), and dodecylphosphocholine (1.5 mg, 4.38 μmol) were dissolved in a mixture of 1:1 $CHCl_3$:Trifluoroethanol (5 mL). The solvents were removed under reduced pressure to give a lipid/peptide film. The lipid/peptide film was hydrated for 4 hours at 41 ^{o}C using a 200 mM sodium phosphate buffer containing 2 mM TCEP and 0.3% EDTA. The mixture was sonicated and the peptide/lipid suspension was extruded through 1.0 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^{o}C to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was carried out in a CEM Discover Labmate unit at 25 W and 75 ^{o}C under open vessel conditions. After 20 minutes, the reaction mixture was purified by RP-HPLC on an analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 minutes. Lyophilization of the appropriate fractions afforded **1** (50%). $C_{217}H_{367}N_{45}O_{53}S$ HR MALDI-ToF MS: observed 4517.6479, calculated 4517.6926 (M+2H).

Condition G: Peptide thioester **4** (1.1 mg, 0.674 µmol), peptide **3** (1.0 mg, 0.337 µmol), and dodecylphosphocholine (1.5 mg, 4.38 µmol) were dissolved in a mixture of 1:1 $CHCl_3$:Trifluoroethanol (5 mL). The solvents were removed under reduced pressure to give a lipid/peptide film. The lipid/peptide film was hydrated for 4 hours at 41 ^oC using a 200 mM sodium phosphate buffer containing 2 mM TCEP and 0.3% EDTA. The mixture was sonicated and the peptide/lipid suspension was extruded through 1.0 µm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^oC to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was carried out in a CEM Discover Labmate unit at 25 W and 90 ^oC under open vessel conditions. After 20 minutes, the reaction mixture was purified by RP-HPLC on an analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 minutes. Lyophilization of the appropriate fractions afforded **1** (75%). $C_{217}H_{367}N_{45}O_{53}S$ HR MALDI-ToF MS: observed 4521.7842, calculated 4521.6926 (M+6H).

Condition H: Peptide thioester **4** (1.1 mg, 0.674 µmol), peptide **3** (1.0 mg, 0.337 µmol), and dodecylphosphocholine (1.5 mg, 4.38 µmol) were dissolved in a mixture of 1:1 $CHCl_3$:Trifluoroethanol (5 mL). The solvents were removed under reduced pressure to give a lipid/peptide film. The lipid/peptide film was hydrated for 4 hours at 41 ^oC using a 200 mM sodium phosphate buffer containing 2 mM TCEP and 0.3% EDTA. The mixture was sonicated and the peptide/lipid suspension was extruded through 1.0 µm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^oC to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was carried out in a CEM Discover Benchmate unit at 25 W, 90 ^oC, and 200 psi under sealed vessel conditions. After 20 minutes, the reaction mixture was purified by RP-HPLC on a an analytical C-4 reversed phase column

using a gradient of 0 – 100% B in A over a period of 40 minutes. Lyophilization of the appropriate fractions afforded **1** (60%). $C_{217}H_{367}N_{45}O_{53}S$ HR MALDI-ToF MS: observed 4518.1719, calculated 4518.6926 (M+3H).

Condition I: Peptide thioester **4** (0.56 mg, 0.337 µmol) and peptide **3** (0.5 mg, 0.168 µmol) were dissolved in 6 M Guanidine HCl and 200 mM sodium phosphate (pH 7.5) as 1:1.5 ratios to obtain a final concentration of 1 mM. The ligation was initiated by the addition of 4% thiophenol (v/v). The reaction was carried out in a CEM Discover Labmate unit at 25 W and 37 ^{o}C under open vessel conditions. After 20 minutes, the reaction mixture was purified by RP-HPLC on an analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 minutes. Lyophilization of the appropriate fractions afforded **1** (10%). $C_{217}H_{367}N_{45}O_{53}S$ HR MALDI-ToF MS 4521.7842: observed, calculated 4521.6926 (M+5H).

Synthesis of KLFAVWKITYKDTG-COSBn 5: Synthesis of the protected peptide thioester was carried out on preloaded H-Gly-sulfamylbutyryl Novasyn TG resin (0.1 mmol) as described in the general methods section for peptide synthesis.

Activation and Cleavage of peptide thioester 5: The resin-bound peptide was washed thoroughly with DCM (5 mL x 2) and *N*-methyl-2-pyrrolidone (NMP) (5 mL x 3). After initial washings, the resin was swollen in DCM (5 mL) for 1 h. The resin was treated with DIPEA (0.5 mL, 3 mmol), iodoacetonitrile (0.36 mL, 5 mmol) in NMP (6 mL). It is important to note that the iodoacetonitrile was filtered through a plug of basic alumina before addition to the resin. The resin was agitated under the exclusion of light for 24 h, filtered, and washed with NMP (5 mL x 4), DCM (5 mL x 4), and THF (5 mL x 4). The activated *N*-acyl sulfonamide resin was swollen in DCM for 1 h, drained, and transferred to a round bottom flask. To the resin-containing flask was added THF (4 mL), benzyl mercaptan (0.64 mL, 5 mmol), and sodium thiophenolate (27 mg, 0.2 mmol). After agitation for 24 h, the resin was filtered and washed with THF (10 mL). The

combined filtrate and washings were collected and contrated in *vacuo*. The thiol impurities were removed by passing the crude peptide through a LH-20 column (1:1 DCM:MeOH). The appropriate fractions were collected and the solvent was removed to recover the fully protected peptide thioester.

Side chain deprotection of thioester 5: The protected peptide was treated with reagent B (5 mL, 88% TFA, 5% phenol, 5% H_2O, 2% TIPS) for 6 h at room temperature. The TFA solution was added dropwise to a screw cap centrifuge tube containing ice-cold diethyl ether (25 mL) and the resulting suspension was left overnight at 4 ^oC, after which the precipitate was collected by centrifugation at 3000 rpm (5 ^oC, 20 min). After decanting the ether, the peptide precipitate was re-suspended in ice-cold diethyl ether (25 mL) and the process of washing was repeated twice. The peptide was lyophilized and purified by RP-HPLC using a semi-preparative C-18 reversed phase column using a linear gradient of 0-100% B in A over a period of 50 min. Lyophilization of the appropriate fractions afforded **5** (40%, based on resin loading capacity). $C_{88}H_{130}N_{18}O_{19}S$ HR MALDI-ToF MS: observed 1814.6802, calculated 1813.9117 (M+K).

Synthesis of glycopeptide 7: Peptide thioester **5** (µmol) and glycopeptide **6** (µmol) were dissolved in 6 M Guanidine HCl and 200 mM sodium phosphate (pH 7.5) as 1:1.5 ratios to obtain a final concentration of 1 mM. The ligation was initiated by the addition of 4% thiophenol (v/v). The reaction was carried out in a CEM Discover Labmate unit at 25 W and 37 ^oC under open vessel conditions. After 2 hours, the reaction mixture was purified by RP-HPLC on a semi-prep C-18 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 minutes. Lyophilization of the appropriate fractions afforded **7** (51%). $C_{134}H_{210}N_{34}O_{30}S$ HR MALDI-ToF MS: observed 3007.5903, calculated 3006.4801 (M+K).

Synthesis of Cys-PADRE-Tn_3 glycopeptide 8: SPPS was performed on Rink Amide AM LL resin (0.25 mmol) as described in the general methods section for peptide synthesis. Side

chain protection was as follows: Fmoc-Lys(Boc) and Fmoc-Trp(Boc)-Thr($\psi^{Me,Me}$pro). Manual coupling was carried out for the first amino acids using Fmoc-Gly (0.5 mmol) and Fmoc-Thr-(AcO_3-α-D-GalNAc) (0.5 mmol), with HATU (0.5 mmol) and DIPEA (1 mmol) in DMF for 12 hours. The coupling reactions were monitored by Kaiser test. The glycopeptide was then elongated on the peptide synthesizer. The resin was thoroughly washed with DMF (5 mL x 2), DCM (5 mL x 5), and MeOH (5 mL x 5) and dried in *vacuo* to a constant weight. The resin was then swelled in DCM (5 mL) for 1 h, after which it was treated with 94% TFA, 2.5% water, 2.5% EDT, and 1% TIPS (10 mL) for 2 h at room temperature. The resin was filtered and washed with neat TFA (2 mL x 2). The filtrate was concentrated in *vacuo* to approximately 1/3 of its original volume. The peptide was then precipitated using diethyl ether (0 ^oC) and recovered by centrifugation at 3000 rpm at 5 ^oC for 15 min. The crude glycopeptide was purified by RP-HPLC on a semi-preparative C18 reversed phase column using a gradient of 0-100% B in A over a period of 50 min. Treatment of the acetylated peptide with 1 M NaOMe in MeOH afforded crude glycopeptide **8**. The crude glycopeptide was purified by RP-HPLC on a semi-preparative C-18 reversed phase column using a gradient of 0-100% B in A over a period of 50 min. Lyophilization of the appropriate fractions afforded glycopeptide **8** (90%). $C_{110}H_{183}N_{27}O_{39}S$ HR MALDI-ToF MS: observed 2537.261, calculated 2538.2887 (M+).

Synthesis of glycolipopeptide 9: Peptide thioester **4** (1.5 mg, 0.759 μmol), peptide **8** (0.5 mg, 0.380 μmol), and dodecylphosphocholine (1.7 mg, 4.93 μmol) were dissolved in a mixture of 1:1 $CHCl_3$:Trifluoroethanol (5 mL). The solvents were removed under reduced pressure to give a lipid/peptide film. The lipid/peptide film was hydrated for 4 hours at 41 ^oC using a 200 mM sodium phosphate buffer containing 2 mM TCEP and 0.3% EDTA. The mixture was sonicated and the peptide/lipid suspension was extruded through 1.0 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^oC to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (1 mM) to initiate the

reaction (1.5 mM final peptide concentration). The reaction was carried out in a CEM Discover Labmate unit at 25 W and 37 ^{o}C under open vessel conditions. After 20 minutes, the reaction mixture was purified by RP-HPLC on a semi-preparative C-18 reversed phase column using a gradient of 0 – 100% B in A over a period of 50 minutes. Lyophilization of the appropriate fractions afforded **1** (1.1 mg, 75%). $C_{193}H_{340}N_{38}O_{52}S_2$ HR MALDI-ToF MS: observed 4094.8025, calculated 4094.5196 (M+8H).

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Chapter III

IMMUNE RECOGNITION OF TUMOR-ASSOCIATED MUC1 IS ACHIEVED BY A FULLY SYNTHETIC ABERRANTLY GLYCOSYLATED MUC1 TRIPARTITE VACCINE*

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3.1 Abstract

The mucin MUC1 is typically aberrantly glycosylated by epithelial cancer cells manifested by truncated *O*-linked saccharides. The resultant glycopeptide epitopes can bind cell surface major histocompatibility complex (MHC) molecules and are susceptible to recognition by cytotoxic T-lymphocytes (CTLs), while aberrantly glycosylated MUC1 protein on the tumor cell surface can be bound by antibodies to mediate antibody-dependent cell-mediated cytotoxicity (ADCC). Efforts to elicit CTLs and IgG antibodies against cancer-expressed MUC1 have not been successful when nonglycosylated MUC1 sequences were used for vaccination, probably due to conformational dissimilarities. Immunizations with densely glycosylated MUC1 peptides have also been ineffective due to impaired susceptibility to antigen processing. Given the challenges to immuno-target tumor-associated MUC1, we have identified the minimum requirements to consistently induce CTLs and ADCC-mediating antibodies specific for the tumor form of MUC1 resulting in a therapeutic response in a mouse model of mammary cancer. The vaccine is composed of the immunoadjuvant Pam_3CysSK_4, a peptide T_{helper} epitope and an aberrantly glycosylated MUC1 peptide. Covalent linkage of the three components was essential for maximum efficacy. The vaccine produced CTLs, which recognized both glycosylated and nonglycosylated peptides, whereas a similar nonglycosylated vaccine gave CTLs which recognized only nonglycosylated peptide. Antibodies elicited by the glycosylated tripartite vaccine were significantly more lytic compared to the unglycosylated control. As a result, immunization with the glycosylated tripartite vaccine was superior in tumor prevention. Besides its own aptness as a clinical target, these studies of MUC1 are likely predictive of a covalent linking strategy applicable to many additional tumor-associated antigens.

3.2 Introduction

A large number of carcinomas of breast, ovary, colon, rectum, pancreas and prostate exhibit a striking overexpression of MUC1 resulting in a loss of polarization and altered glycosylation [1, 2]. MUC1 is a heavily glycosylated type 1 transmembrane mucin that is expressed

on the apical surface of glandular epithelial cells at low levels and at very high levels following transformation. Human MUC1 is composed of a cytoplasmic signaling peptide, a transmembrane domain and an ectodomain composed of a variable number tandem repeats of twenty amino acids. Each repeat contains 5 potential *O*-glycosylation sites. The glycosylation pattern depends on the tissue of origin and the physiological state of the tissue [1, 3]. Tumor-associated MUC1 is aberrantly glycosylated due to a lack of core 1,3-galactosyltransferase (T-synthase) [4], producing truncated carbohydrate structures such as Tn (αGalNAc-Thr), STn (αNeu5Ac-(2,6)-αGalNAc-Thr) and Thomsen-Friedenreich (TF) antigen (βGal-(1,3)-αGalNAc-Thr). Recently, the NCI Translational Research Working Group prioritized cancer vaccine targets based on therapeutic function, immunogenicity, role of Ag in oncogenicity, specificity, expression level, stem cell expression, percentage of patients with antigen positive cancer and cellular location [5]. MUC1 was ranked second of 75 tumor-associated antigens. In this respect, MUC1 displays nearly ubiquitous expression in a wide variety of tumor types, it is found on cancer stem cells and has a functional role in tumorigenesis.

Humoral responses to MUC1 have been observed in benign diseases and carcinoma patients and the presence of circulating antibodies against MUC1 at the time of cancer diagnosis has been correlated with a favorable disease outcome in breast cancer patients [6, 7]. The MUC1-derived peptide sequences RPAPGS, PPAHGVT, and PDTRP have been identified as the most frequent minimal epitopes [8, 9]. Furthermore, modification of the peptides with αGalNAc (Tn-antigen) led to stronger antibody binding. It has been proposed that the improved binding is due to saccharide induced conformational change of the peptide backbone [10-12].

Cytotoxic T-lymphocytes (CTLs) isolated from patients with breast carcinoma can recognize epitopes present on MUC1 tandem repeat peptide [13]. It has been proposed that T-cell epitopes from the MUC1 core domain are packaged within tumor cells in their truncated glycosylation state into major histocompatibility complex (MHC) class I molecules, leading to natural MHC-restricted recognition of “hypoglycosylated’ epitopes [14-17]. Several MUC1-derived HLA-A2–

binding peptides have been identified including STAPPAHGV and SAPDTRPAPG [13, 18, 19].

Early efforts to develop MUC1-based cancer vaccines focused on the use of unglycosylated MUC1 tandem repeat peptides of different lengths, conjugated to different carriers and/or administered with an adjuvant [8, 20-27]. In general, these strategies have failed to elicit effective immune responses to MUC1-expressing cancer cells, probably due to the conformational disparities between nonglycosylated vaccine sequences and tumor-expressed, aberrantly glycosylated MUC1 [10-12]. The immunogenicity of carbohydrate epitopes (Tn-, or sialyl-Tn) conjugated to an antigenically irrelevant carrier protein has been examined in mice, however, these constructs elicited only modest IgM and IgG antibody responses [28-31]. Such vaccine candidates suffer from immune suppression by the carrier protein and, in addition, cannot activate CTL responses. A synthetic 60-mer MUC1 tandem repeat peptide, which was glycosylated by polypeptide GalNAc transferases to give saturating *O*-glycan occupancy (five sites per repeat), elicited only modest antibody responses [32]. Recent clarifying studies have shown that a densely glycosylated MUC1 glycopeptide cannot be processed by antigen presenting cells (APCs) [17] thereby compromising the presentation of class I and class II glycopeptides, and consequently, T_{helper} cells and CTLs will not be activated. Interestingly, glycopeptides carrying the Tn- or TF-antigens have been used to induce a carbohydrate-specific cytotoxic T-cell response in mice [33]. Two-component vaccines, consisting of an MHC I glycopeptide and a T_{helper} epitope, have shown promise in tumor models [34]. These vaccine candidates do, however, not induce antibody responses. Thus, a MUC1-based cancer vaccine that consistently elicits relevant humoral and cellular immunity has not yet been developed.

We show here that a glycosylated MUC1-derived glycopeptide covalently linked to a Toll-like receptor (TLR) agonist can elicit potent humoral and cellular immune responses and is efficacious in reversing tolerance and generating a therapeutic response. The examination of a number of control compounds demonstrate that the therapeutic effect of the three-component vaccine is due to nonspecific antitumor responses elicited by the adjuvant, and specific humoral

and cellular immune responses elicited by the MUC1-derived glycopeptide. It has been found that glycosylation of the MUC1 peptide and covalent attachment of the TLR agonist is critical for inducing optimal responses immune responses.

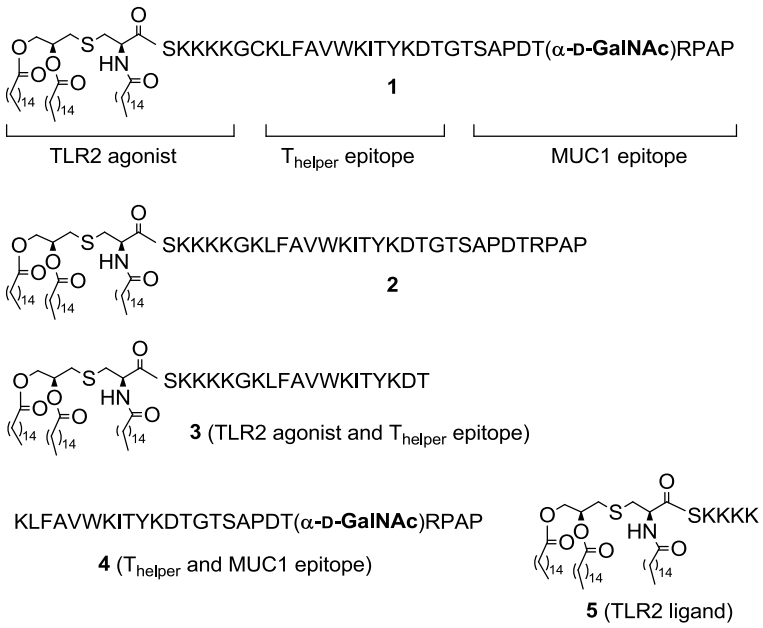
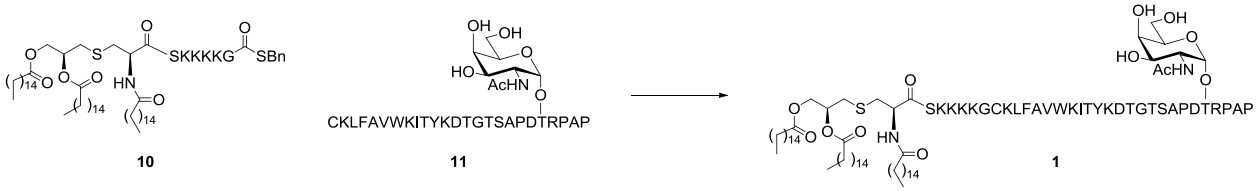


Figure 3.1 . Chemical structures of synthetic antigens.

3.3 Results

Antigen design and tumor challenge studies. The efficacy of liposomal preparations of compounds **1**, **2**, **3**, a mixture of **4** and **5**, and **5** alone (Figure 3.1) were examined in a well-established mouse model for mammary cancer [35]. The multi-component vaccine candidate **1** contains a tumor-associated glycopeptide derived from MUC1 [1, 3], the well-documented murine MHC class II restricted T_{helper} epitope KLFAVWKITYKDT derived from polio virus [36], and the lipopeptide Pam_3CysSK_4, which is a potent agonist of Toll-like receptor 2 (TLR2) [37]. Previously, the MUC1-derived glycopeptide SAPDT(αGalNAc)RPAP, was identified as the antigenic-dominant domain of the tandem repeat of MUC1 [8, 9]. Furthermore, this epitope can also be presented in complex with MHC class I (K^b) resulting in the activation of CTLs [38]. The MHC class II restricted T_{helper} epitope of **1** was expected to induce a class switch from IgM to IgG antibody production and facilitate the presentation of exogenous glycopeptides on MHC class 1.

Finally, the Pam_3CysSK_4 moiety of **1** will function as an inbuilt adjuvant by eliciting relevant cytokines and chemokines [37]. To determine the importance of the carbohydrate moiety of **1**, construct **2** was examined, which has a similar structure as **1** except that the threonine of the MUC1 peptide is not glycosylated. Compound **3** lacks the MUC1 (glyco)peptide epitope of **1** and **2** and was examined to account for possible therapeutic effects due to immune activation by the adjuvant. Finally, a mixture of the glycopeptide **4** and adjuvant Pam_3CysSK_4 **5** was examined to establish the importance of covalent attachment of the adjuvant to the MUC1 glycopeptide and T_{helper} epitope.



10 **11** **1**

Scheme 3.1. Synthesis of **1** by *microwave-assisted* liposome-mediated native chemical ligation of compounds **10** and **11**.

The multi-component vaccine **1** was prepared by liposome-mediated native chemical ligation of the thiobenzyl ester of Pam_3CysSK_4 [39] and the glycopeptide CKLFAVWKITYKDTGTSAPDT(αGalNAc)RPAP (**11**, Scheme 3.1). Compounds **2**, **3**, **4** were synthesized by a linear SPPS protocol using a Rink amide AM resin, Fmoc protected amino acids and Fmoc-Thr-(3,4,6-tri-*O*-acetyl-α-D-GalNAc). The resulting compounds were incorporated into phospholipid-based small unilamellar vesicles (SUVs) by hydration of a thin film of the synthetic compounds, egg phosphatidylcholine, phosphatidylglycerol and cholesterol in a HEPES buffer (10 mM, pH 6.5) containing NaCl (145 mM) followed by extrusion through a 100 nm Nuclepore® polycarbonate membrane. Groups of MUC1.Tg mice (C57BL/6; H-2^b) that express human MUC1 were immunized three-times at biweekly intervals with liposomal preparations of compounds **1**, **2**, **3**, a mixture of **4** and **5**, and **5** alone. After 35 days, the mice were challenged with MMT mammary tumor cells (positive for MUC1 and Tn) followed by one

more boost after one week. One week after the last immunization, the mice were sacrificed and the efficacy of the vaccines determined by tumor weight. Furthermore, the robustness of humoral immune responses was assessed by titers of MUC1-specific antibodies and the ability of the antisera to lyse MUC1-bearing tumor cells. In addition, cellular immune responses were evaluated by determining the number of IFN-γ producing $CD8^+$ T-cells and the ability of these cells to lyse cells.

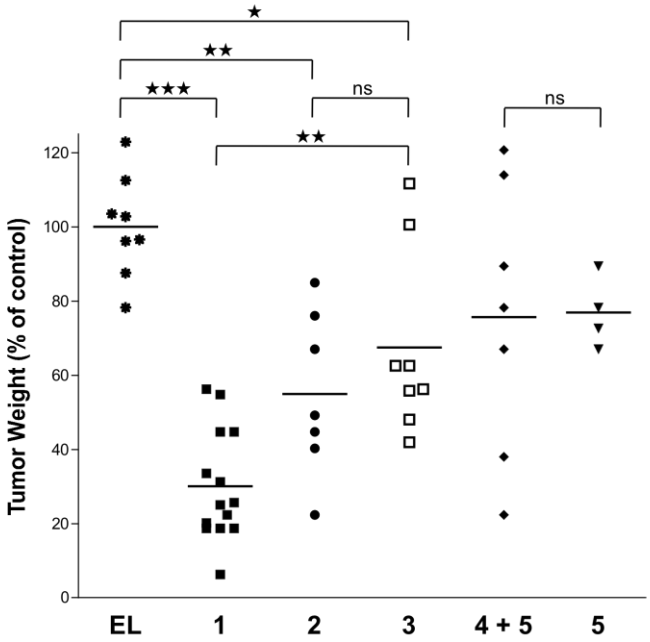


Figure 3.2. Glycosylated multi-component vaccine reduces MMT tumor burden in MUC1.Tg mice. MUC1.Tg mice were immunized with empty liposomes (EL) as control or with liposomes containing **1**, **2**, **3**, **4 + 5** or **5** (25 μg containing 3 μg of carbohydrate). Three bi-weekly immunizations were given prior to a tumor challenge with MUC1-expressing MMT tumor cells (1×10^6 cells) followed by one boost one week after. The animals were sacrificed 7 days after the last injection and tumor wet weight was determined. Data are presented as percentage of control (mice vaccinated with empty liposomes). Each data point represents an individual mouse and the horizontal lines indicate the mean for the group of mice. Asterisks indicate statistically significant difference (★P<0.05, ★★P<0.01, and ★★★P<0.001) and ns indicates no significant difference.

Immunization with multi-component vaccine candidate **1** led to a significant reduction in tumor burden compared to empty liposomes or treatment with compound **3**, which does not contain a MUC1 glycopeptide epitope (Figure 3.2). Interestingly, immunizations with compound

3 led to somewhat smaller tumors compared to the application of empty liposomes, indicating antitumor properties due to nonspecific adjuvant effects. Unglycosylated multi-component vaccine candidate **2** and a mixture of compounds **4** and **5** did not exhibit a significant improvement of anti-cancer properties compared to control immunizations. In these cases, large dispersion in tumor weights was observed whereas immunization with compound **1** led to substantial reduction in tumor weight in all mice.

Table 3.1. ELISA anti-MUC1 and anti-T_{helper} antibody titers[a] after 4 immunizations with various preparations.

Immunization[b]	IgG total MUC1	IgG1 MUC1	IgG2a MUC1	IgG2b MUC1	IgG3 MUC1	IgM MUC1	IgG total T_{helper}
EL[c]	1,500	200	0	300	300	100	100
1 (NT)[d]	31,900	10,600	10,000	15,500	3,900	100	2,100
1	30,200	16,000	6,600	10,700	3,900	50	3,000
2	12,900	10,400	4,100	4,500	700	100	1000
3	1,300	0	100	900	0	0	50
4 + **5**	300	0	0	200	0	0	1,000
5	0	0	200	0	0	50	50

[a] Anti-MUC1 and anti-T_{helper} antibody titers are presented as median values for groups of four to thirteen mice. ELISA plates were coated with BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate for anti-MUC1 antibody titers or NeutrAvidin-biotin-T_{helper} for anti-T_{helper} antibody titers. Titers were determined by linear regression analysis, with plotting of dilution versus absorbance. Titers are defined as the highest dilution yielding an optical density of 0.1 or greater relative to normal control mouse sera. [b] Liposomal preparations were employed. MMT tumors were induced between the 3rd and 4th immunization. [c] EL = empty liposomes. [d] No tumor induced.

Humoral Immunity. Anti-MUC1 antibody titers were determined by coating microtiter plates with the MUC1-derived glycopeptide CTSAPDT(αGalNAc)RPAP conjugated to maleimide-modified BSA. Compound **1** had elicited robust IgG antibody responses, and subtyping of the antibodies indicated a mixed Th1/Th2 response (Table 3.1 and Figure 3.3). Mice immunized with **1** but not challenged with MMT tumor cells elicited similar titers of antibodies, indicating that immune suppression by cancer cells was probably reversed. Inhibition ELISA using the MUC1-derived (glyco)peptides TSAPDT(αGalNAc)RPAP and

TSAPDTRPAP as inhibitors showed that the polyclonal sera had slightly higher affinities for the glycosylated MUC1 epitope (Table 3.2 and Figure 3.4). Furthermore, low titers of antibodies

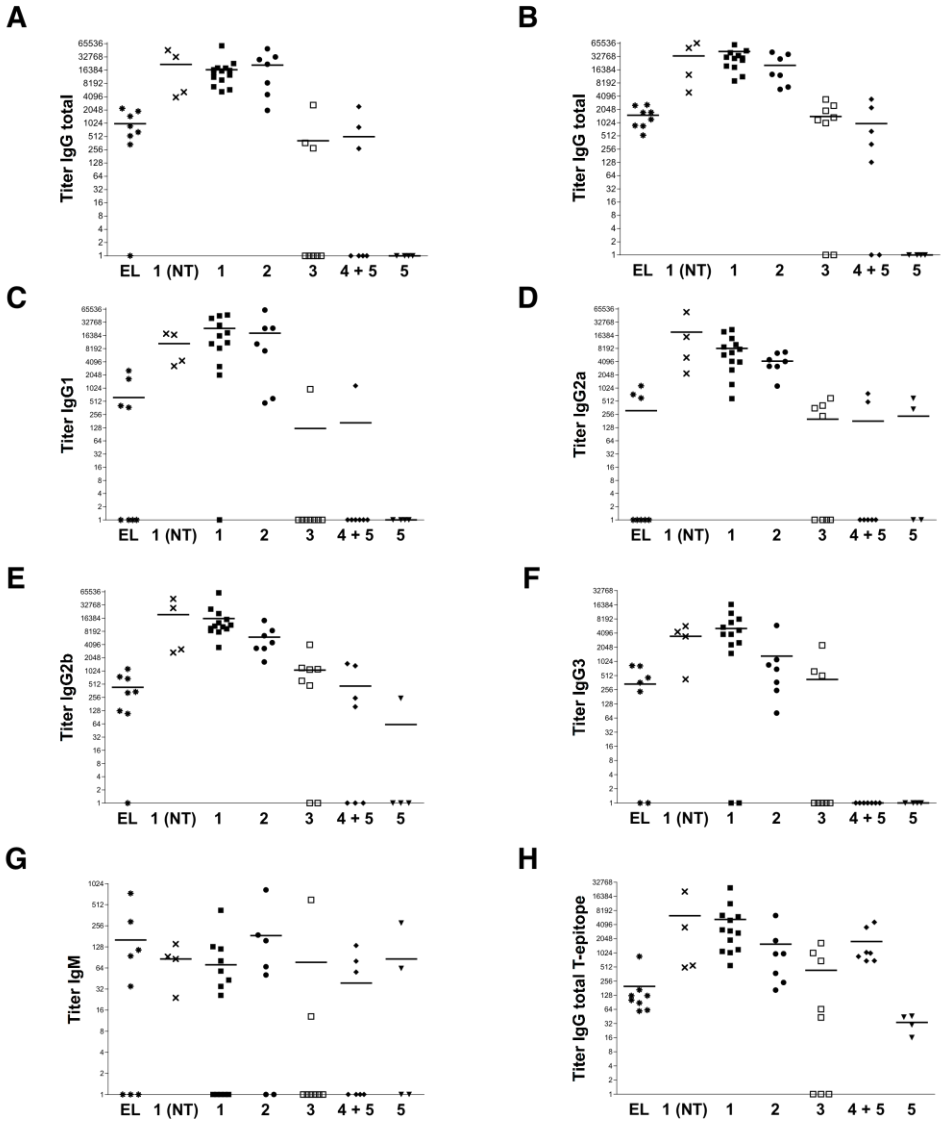


Figure 3.3. ELISA anti-MUC1 and anti-T_{helper} antibody titers after 3 (*A*) or 4 (*B-H*) immunizations with **1**, **2**, **3**, **4 + 5** or **5** with or without (NT) tumor induction as indicated. ELISA plates were coated with BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate (*A-G*) or NeutrAvidin-biotin-T_{helper} (*H*) and titers were determined by linear regression analysis, plotting dilution *vs.* absorbance. Titers were defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera. Each data point represents the titer for an individual mouse after 4 immunizations and the horizontal lines indicate the mean for the group of mice.

against the T_{helper} epitope were measured indicating that the candidate vaccine does not suffer from immune suppression. Although compound **2** does not contain a carbohydrate moiety, the resulting antisera could recognize the CTSAPDT(αGalNAc)RPAP epitope. However, in this case, no IgG3 antibodies were detected, consistent with an absence of carbohydrate in the vaccine. Interestingly, the mixture of compounds **4** and **5** had elicited low titers of antibodies, highlighting the importance of covalent attachment of the Pam_3CysSK_4 to the glycopeptide epitope for robust antigenic responses. As expected, the controls that did not contain a MUC1-derived epitope (**3** and **5**) did not elicit anti-MUC1 antibody responses.

Table 3.2. Competitive inhibition ELISA[a].

Immunization	IC_{50} inhibitors (μM)	
	SAPDT(αGalNAc)RPAP (**6**)	SAPDTRPAP (**7**)
1	3.01	7.19
	(2.54 to 3.59)	(6.23 to 8.29)
2	3.63	6.30
	(2.88 to 4.56)	(5.36 to 7.41)

[a] ELISA plates were coated with BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate. Serum samples of groups of 7 mice after immunizations with **1** or **2**, diluted to obtain in the absence of an inhibitor an OD of approximately 1 in the ELISA, were first mixed with glycopeptide **6** (SAPDT(αGalNAc)RPAP) or peptide **7** (SAPDTRPAP) (0-500 μM final concentration) and then applied to the coated microtiter plate. Optical density values were normalized for the optical density values obtained with serum alone (0 μM inhibitor, 100%). Inhibition data were fit with the following logistic equation: $Y = Bottom + (Top - Bottom) / (1 + 10^{(X - Log\ IC50)})$, where Y is the normalized optical density, X is the logarithm of the concentration of the inhibitor and IC_{50} is the concentration of the inhibitor that reduces the response by half. The IC_{50} values are reported as best-fit values and as 95% confidence intervals.

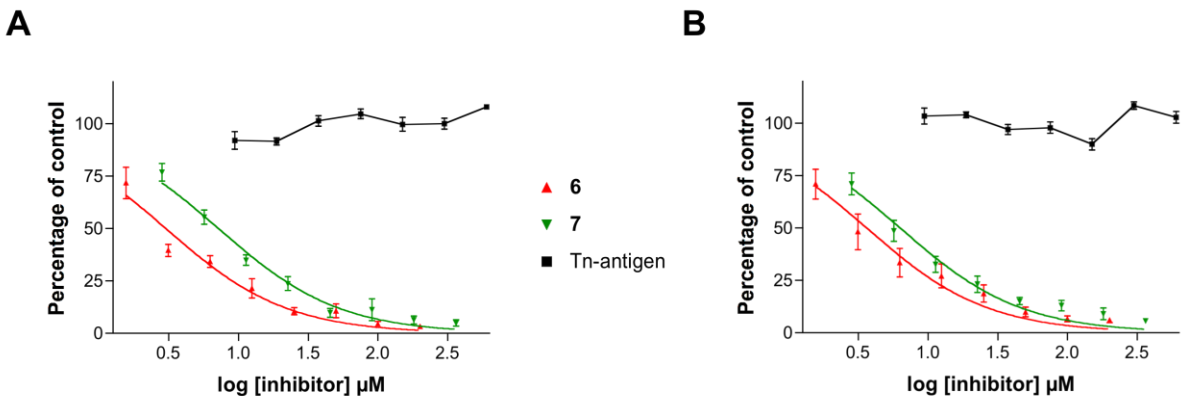


Figure 3.4. Competitive inhibition of antibody binding to BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate by glycopeptide **6** (SAPDT(αGalNAc)RPAP), peptide **7** (SAPDTRPAP) and the Tn-antigen (α-O-GalNAc-Thr). ELISA plates were coated with BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate. Serum samples after immunizations with (*A*) **1** and (*B*) **2**, diluted to obtain in the absence of an inhibitor an OD of approximately 1 in the ELISA, were first mixed with **6**, **7** or Tn-antigen (0-500 µM final concentration) and then applied to the coated microtiter plate. Optical density values were normalized for the optical density values obtained with serum alone (0 µM inhibitor, 100%). The data are reported as the means ± SEM of groups of mice (*n*=7).

Antibody-dependent cell-mediated cytotoxity (ADCC) was examined by labeling two MUC1- expressing cancer cell types with ^{51}Cr, followed by the addition of antisera and cytotoxic effector cells (NK cells) and measurement of released ^{51}Cr. The antisera obtained by immunization with **1** was able to significantly increase cancer cell lysis compared to the control compound **3** (Figure 3.5*A* and *B*). Importantly, antibodies elicited by compound **2** were significantly less efficacious in cell lysis compared to compound **1**, highlighting the importance of glycosylation for relevant antigenic responses. As expected, the antisera derived from a mixture of **4** and **5** and the control derivatives lacking the MUC1 glycopeptide did not induce significant cell lysis.

Cellular Immunity. To assess the ability of the vaccine candidates to activate CTLs, $CD8^+$ T-cells from lymph nodes of the mice were isolated by magnetic cell sorting and incubated with irradiated dendritic cells (DCs) pulsed with the immunizing peptides on ELISPOT plates. As expected, vaccine candidates **1** and **2** exhibited robust $CD8^+$ responses compared to control (Figure 3.6*A*, **1** and **2** *vs.* **3**). Interestingly, a mixture of glycopeptides **4** and adjuvant **5**

(Pam_3CysSK_4) induced the activation of a smaller number of $CD8^+$, indicating that covalent attachment of the MUC1 and T_{helper} epitope to the adjuvant is important for optimal activation of CTLs.

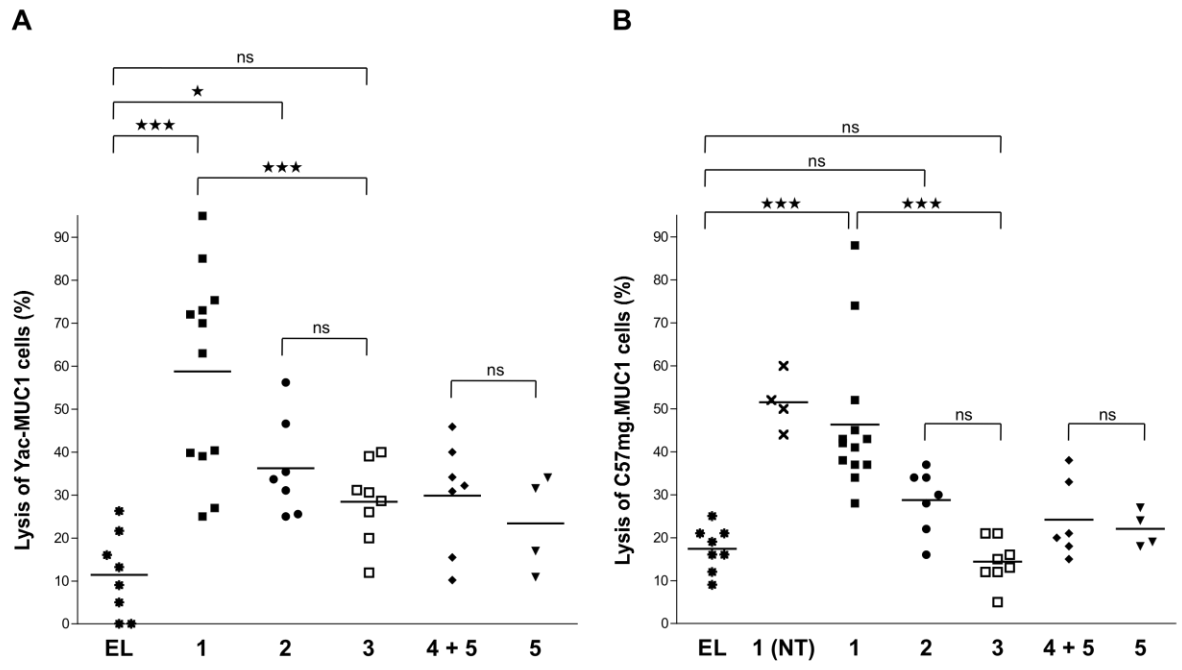


Figure 3.5. Induction of antibody-dependent cell-mediated cytotoxicity (ADCC). Tumor cells, (*A*) Yac-MUC1 and (*B*) C57mg.MUC1, were labeled with chromium for 2 h and then incubated with serum (1:25 diluted) obtained from mice immunized with empty liposomes (EL) or liposomes containing **1**, **2**, **3**, **4 + 5** or **5** with or without (NT) tumor induction as indicated for 30 min at 37 °C. The tumor cells were then incubated with effector cells (NK cells KY-1 clone) for 4 h. Effector to target ratio is 50:1. Spontaneous release was below 20% of complete release. Each data point represents an individual mouse and the horizontal lines indicate the mean for the group of mice.

The lytic activity of the isolated $CD8^+$ cells without *in vitro* stimulation was examined by a ^{51}Cr-release assay in which DCs were pulsed with the MUC1-derived glycopeptide SAPDT(αGalNAc)RPAP (**6**) or the peptide SAPDTRPAP (**7**) in case of immunization **2**. CTLs activated by compounds **1** and **2** exhibited significantly greater cytotoxicity compared to controls (Figure 3.6*B*). Furthermore, mice immunized with a mixture of **4** and **5** exhibited a reduced lytic activity, further demonstrating the importance of covalent attachment of the various epitopes.

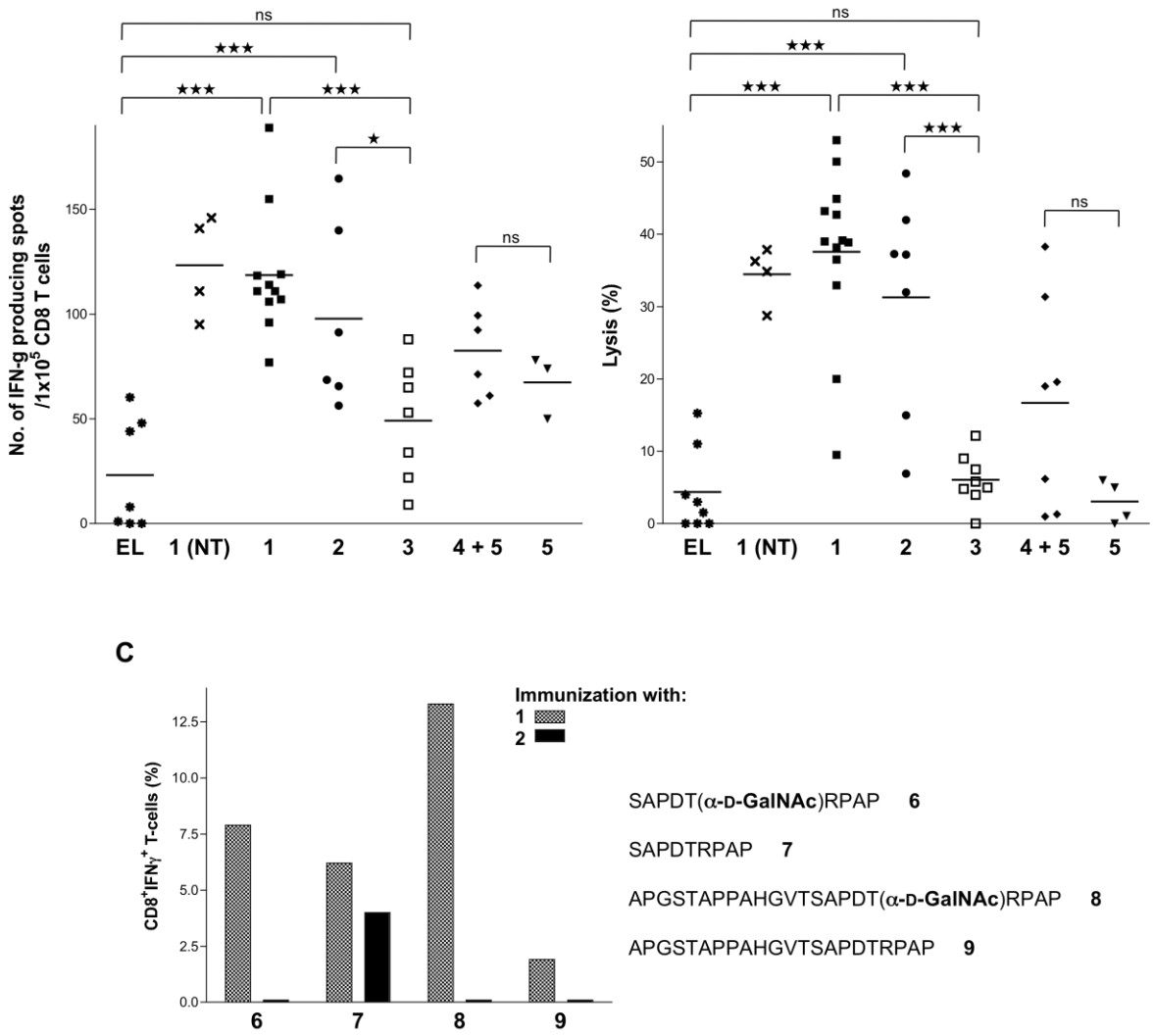


Figure 3.6. Induction of cytotoxic T-cell responses. (*A*) IFN-γ producing $CD8^+$ T-cells in MUC1.Tg mice. $CD8^+$ T-cells isolated from lymph nodes of mice immunized with empty liposomes (EL) or liposomes containing **1**, **2**, **3**, **4 + 5** or **5** with or without (NT) tumor induction as indicated were analyzed for MUC1-specific IFN-γ spot formation without *in vitro* stimulation. Each data point represents an individual mouse and the horizontal lines indicate the mean for the group of mice. (*B*) Induction of $CD8^+$ cytolytic T-cells in MUC1.Tg mice. $CD8^+$ T-cells were isolated from lymph nodes of mice immunized with empty liposomes (EL) or liposomes containing **1**, **2**, **3**, **4 + 5** or **5** with or without (NT) tumor induction as indicated and subjected to a ^{51}Cr-release assay without any *in vitro* stimulation. DCs pulsed with glycopeptide SAPDT(αGalNAc)RPAP (**6**) for **1** (NT), **1**, **3**, **4 + 5** and **5**, peptide SAPDTRPAP (**7**) for **2** or unpulsed for EL were used as targets. Spontaneous release was below 15% of complete release. Each data point represents an individual mouse and the horizontal lines indicate the mean for the group of mice. (*C*) Epitope requirements of $CD8^+$ T-cells. Mice were immunized with liposomes containing **1** or **2**. Lymph note derived T-cells expressing low levels of CD62L were obtained by cell sorting and cultured for 14 days in the presence of DCs pulsed with glycopeptide **6** for **1** or peptide **7** for **2**. The resulting cells were analyzed by ICC for the presence of $CD8^+IFN\gamma^+$ T-cells after exposure to DCs pulsed with (glyco)peptides **6**-**9**.

To investigate in detail the epitope requirements of the $CD8^+$ cells, groups of five MUC1.Tg were immunized with liposomal preparations of compounds **1** and **2**, followed by sorting $CD62^{Low}$ T cells from lymph nodes, which were stimulated *in vitro* for 2 days by DCs pulsed with glycopeptide SAPDT(αGalNAc)RPAP (**6**) and peptide SAPDTRPAP (**7**), respectively and then allowed to expand for 14 days by culturing with IL-2, IL-7 and IL-15. The percentage of IFN-γ producing $CD8^+$ cells was established after pulsing DCs with MUC1-derived (glyco)peptides **6**-**9**. Compound **1** had activated a diverse range of CTL that could be activated by glycosylated and nonglycosylated structures, whereas those obtained by immunization with **2** only showed responsiveness with unglycosylated peptide **7**. Furthermore, $CD8^+$ cells obtained from immunizing with **1** could lyse DCs pulsed with glycosylated and unglycosylated structures (Figure 3.6*C*).

These results indicate that CTLs activated by immunizations with **1** recognize a wider range of structures including glycosylated and unglycosylated MUC1-derived peptides whereas CTLs obtained from compound **2** exhibit a strong preference for unglycosylated peptides.

Cytokine induction. The lipopeptide moiety of the three-component vaccine is required for initiating the production of necessary cytokines and chemokines by interacting with TLR2 on the surface of mononuclear phagocytes [37, 40, 41]. To examine the activity of TLR2 moiety of the vaccine candidates, primary DCs obtained by an established method [42] were exposed over a wide range of concentrations to the compounds **1**-**3** and *E. coli* 055:B5 LPS and the supernatants examined for mouse TNF-α, IFN-β, RANTES, IL-6, IL-1β, IL-10, IP-10, IL-12p70, and IL-12/23p40 using commercial or in-house developed capture ELISAs (Tables 3.3 and 3.4 and Figure 3.7). The compounds induced the secretion of TNF-α, RANTES, IL-6, IL-1β and IL-12/23p40 with similar efficacies and potencies indicating that attachment of glycopeptide did not affect activity. The compounds did not induce the secretion of immunosuppressant IL-10, and furthermore, IFN-β and IP-10 were not detected, which is in agreement with TRIF-dependent cellular activation of these cytokines [43].

Table 3.3. Cytokine plateau values[a] (pg/mL) of dose-response curves of liposome preparations loaded with compound **1**, **2** or **3** and *E. coli* LPS obtained after incubation of primary DCs for 24 h.

Cytokine (pg/mL)	**1**	**2**	**3**	LPS
TNF-alpha	836 ± 103	695 ± 50	854 ± 67	3,265 ± 96
IFN-beta	nd[b]	nd	nd	505 ± 34
RANTES	584 ± 59	553 ± 54	536 ± 28	8,869 ± 416
IL-6	298 ± 28	316 ± 40	401 ± 43	668 ± 34
IL-1beta	60 ± 10	84 ± 13	77 ± 4	209 ± 15
IL-1beta/ATP	187 ± 50	181 ± 26	194 ± 14	596 ± 24
IL-10	nd	nd	nd	91 ± 6
IP-10	nd	nd	nd	2,196 ± 44
IL-12 p70	nd	nd	nd	623 ± 19
IL-12/23 p40	13,668 ± 496	10,692 ± 853	11,192 ± 382	27,679 ± 460

[a] Plateau values as reported by Prism as best-fit values ± SEM using non-linear least squares curve fitting as picogram of cytokine per µg of total protein.
[b] nd indicates not detected.

Table 3.4. Cytokine log EC_{50} values[a] (nM) of liposome preparations loaded with compound **1**, **2** or **3** and *E. coli* LPS in primary DCs.

Cytokine (pg/mL)	**1**	**2**	**3**	LPS
TNF-alpha	3.08 ± 0.25	2.99 ± 0.14	4.17 ± 0.10	-2.38 ± 0.12
IFN-beta	nd[b]	nd	nd	-3.04 ± 0.24
RANTES	3.12 ± 0.17	2.88 ± 0.19	3.66 ± 0.09	-2.25 ± 0.16
IL-6	3.58 ± 0.16	2.88 ± 0.23	4.05 ± 0.14	-3.15 ± 0.18
IL-1beta	3.52 ± 0.28	3.99 ± 0.21	4.01 ± 0.08	-0.80 ± 0.22
IL-1beta/ATP	2.48 ± 0.48	2.44 ± 0.31	3.06 ± 0.13	-0.37 ± 0.12
IL-10	nd	nd	nd	nd
IP-10	nd	nd	nd	-2.59 ± 0.09
IL-12 p70	nd	nd	nd	-1.67 ± 0.14
IL-12/23 p40	3.15 ± 0.07	3.10 ± 0.16	3.51 ± 0.06	-1.89 ± 0.06

[a] Log EC_{50} values as reported by Prism as best-fit values ± SEM using non-linear least squares curve fitting.
[b] nd indicates not detected at levels for accurate EC_{50} determination.

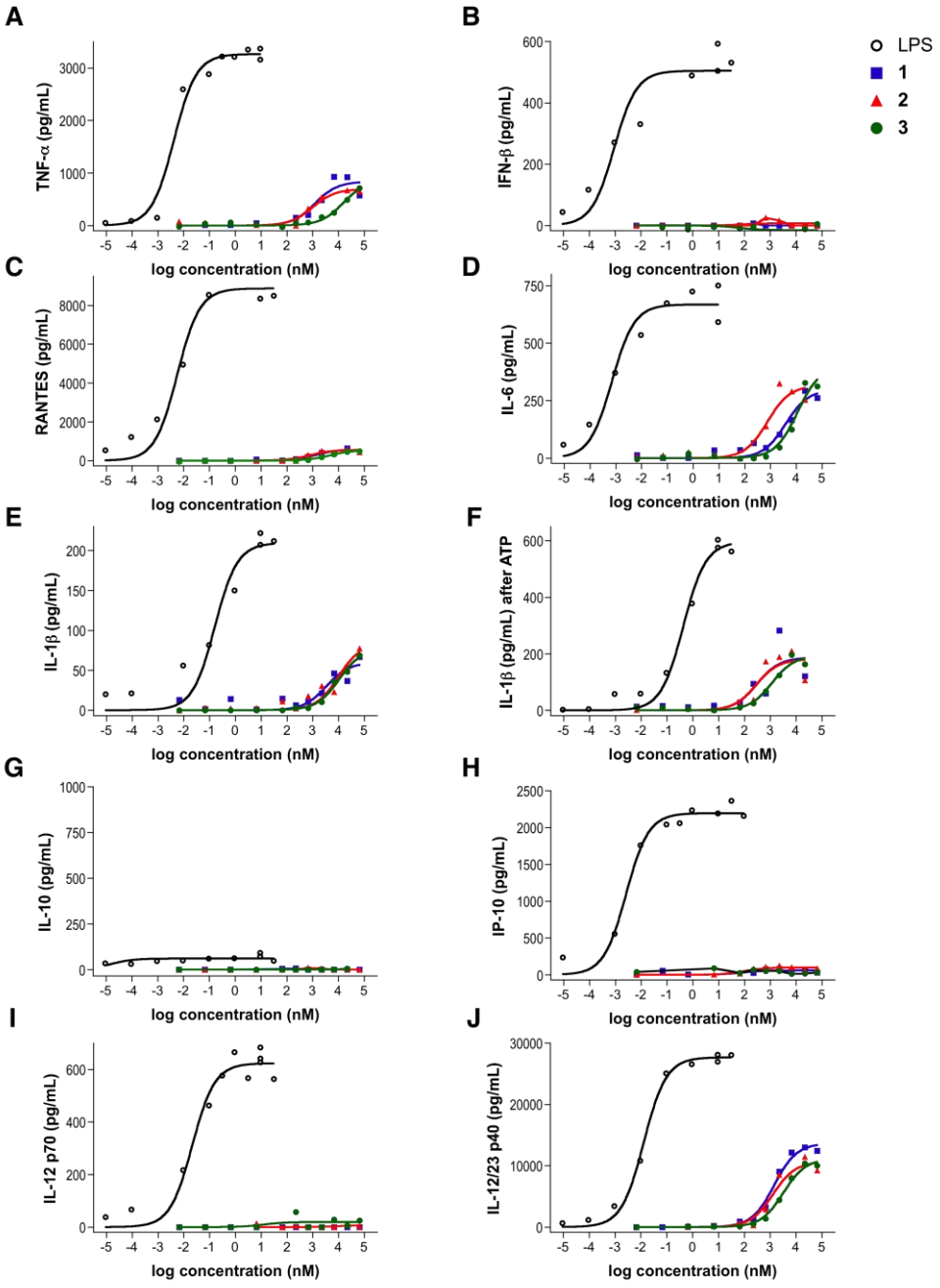


Figure 3.7. Cytokine production by DCs after stimulation with liposome preparations loaded with compound **1**, **2** or **3**, or *E. coli* LPS for 24 h. Primary mouse DCs were incubated for 24 h with increasing concentrations of liposome preparations loaded with compound **1**, **2** or **3**, or *E. coli* LPS as indicated. (*A*) TNF-α, (*B*) IFN-β, (*C*) RANTES, (*D*) IL-6, (*E* and *F*) extracellular IL-1β, (*G*) IL-10, (*H*) IP-10, (*I*) IL-12 p70 and (*J*) IL-12/23 p40 in cell supernatants were measured using ELISAs. For estimation of IL-1β secretion after ATP treatment, cells were incubated with ATP (5 mM) for 30 min subsequent to the 24 h incubation with inducers. The data are reported as the means ± SD of triplicate treatments.

3.4 Discussion

Evidence is emerging that successful cancer vaccine should be multimodal and activate several aspects of the immune system at once [44]. Although cellular and humoral immune responses against MUC1 have been observed in some cancer patients, it has been difficult to design cancer vaccine candidates that can elicit both of these responses [2]. Previously, we found that a tripartite vaccine composed of a glycopeptide derived from MUC1, a promiscuous T_{helper} peptide and a TLR2 agonist can elicit in wild-type mice exceptionally high titers of IgG antibodies [45]. Here, we report a detailed mechanistic study using a humanized mouse model of mammary cancer that demonstrates that the tripartite vaccine can elicit IgG antibodies that can lyse MUC1 expressing cancer cells, stimulate cytotoxicity of T-lymphocytes, and activate innate immune responses thereby reversing tolerance and generating a therapeutic response. The tumor model was selected because it is convenient for screening a relatively large number of compounds and resembles a model for treatment of a minimal residual disease in which cancer patients, in particular, breast cancer patients, are apparently cancer free after surgery, radiation, and/or chemotherapy but are in danger of relapse due to the presence of micro-metastatic tumors. It is the expectation that a cancer vaccine can destroy remaining cancer cells, thereby improving long-term survival.

Analysis of control compounds revealed that reduction in tumor burden mediated by the tripartite vaccine was caused by specific immunity against MUC1 and by nonspecific adjuvant effects mediated by the TLR2 agonist. Evidence is emerging that TLRs are widely expressed by tumor cells and their activation can result in inhibition or promotion of tumorigenicity[46]. Furthermore, cytokines and chemokines, which are produced following the activation of the TLRs, can stimulate the expression of a number of co-stimulatory proteins for optimum interactions between helper T-, B- and antigen presenting cells. A recent study indicates that TLR1/2 agonists have a unique ability to reduce the suppressive function of $Foxp3^+$ regulatory T

cells (Tregs) and enhance the cytotoxicity of tumor-specific CTL *in vitro* and *in vivo* and potentially have more favorable antitumor effects than other TLR agonists [47].

The studies presented here also demonstrated that covalent attachment of the TLR2 agonist to the glycolipoptide epitope is critical for eliciting antibodies and optimal CTL function. Lipidation with the TLR2 agonist makes it possible to formulate the candidate vaccine in a liposomal preparation, which probably will enhance its circulation time. Furthermore, a liposomal preparation presents the glycopeptide epitopes in a multivalent manner, thereby providing an opportunity for efficient clustering of Ig receptors of B-cells, which is required to initiate B-cell signaling and antibody production. Furthermore our previous studies have shown that covalent attachment of the TLR2 agonist Pam_3CysSK_4 facilitates selective internalization by TLR2-expressing immune cells such B-cells and APCs [45]. Uptake and processing of antigen and subsequent presentation of the T_{helper} epitope as a complex with MHC class I or II on the cell surface of APCs, is critical for eliciting IgG antibodies. Over the past decade, numerous studies have shown that selective targeting of antigens to APCs will result in improved immune responses [48, 49]. For example, oxidized mannan, heat shock proteins, bacterial toxins, and antibodies targeting cell surface receptors of DCs have been attached to antigens to increase uptake by DCs. Although these uptake strategies are attractive, they have as a disadvantage that the targeting device is antigenic, which may result in immune suppression of tumor-associated carbohydrates. The attractiveness of Pam_3CysSK_4 for facilitating uptake by APCs lies in its low intrinsic immunity. Thus, the three-component vaccine will facilitate uptake without suffering immune suppression.

Finally, we have found that glycosylation of the MUC1 epitope was critical for optimal reduction in tumor burden. The mechanistic studies provided a rationale for these observations and it was found that immunization with compound **1** led to somewhat higher titers of antibodies that were significantly more lytic compared to the use of compound **2** which lacks the Tn-antigen. Conformational studies by NMR complemented by light scattering measurements have

indicated that deglycosylation of MUC1 results in a less extended and more globular structure [50]. Similar studies using MUC1 related *O*-glycopeptides have shown that the carbohydrate moieties exert conformational effects [10-12], which may provide a rationale for differences in immune responses. Also, the use of glycosylated **1** led to the efficient activation of CTLs, which were able to recognize glycosylated and unglycosylated structures, with the former ones being preferred. On the other hand, immunizations with unglycosylated compound **2** led to CTLs that mainly recognize unglycosylated structures. It is known that short *O*-linked glycans such as the Tn and STn on MUC1 tandem repeats remain intact during DC processing in the MHC class II pathway [14-17] and thus it is possible to elicit glycopeptide selective CTL responses. Moreover, there is evidence that MUC1 glycopeptides can bind more strongly to the MHC class I mouse allele H-2K^b compared with the corresponding unglycosylated peptide [38]. The progression of carcinomas is not only associated with the modification of MUC1 with truncated saccharides such as the Tn-antigen but these structures are present at much higher densities and thus effective immunotherapy needs to elicit responses that are directed to such structures.

In conclusion, a tripartite vaccine engineered to emulate glycosylated MUC1 was unique in its capacity to generate CTL and ADCC-mediating antibodies, which recognized tumor-associated MUC1. This was associated with a significantly superior therapeutic anti-tumor effect. We hypothesize that a tumor-specific anti-MUC1 response is attainable, but only when the MUC1 component of the vaccine contains the conformational elements of aberrant glycosylation.

3.5 Experimental section

General Methods for Automated Synthesis of Solid-Phase (Glyco)(lipo)peptides 1-11. (Glyco)(lipo)peptides and were synthesized on RinkAmide AM resin (0.1 mmol, unless otherwise stated) by established protocols on an Applied Biosystems, ABI 433A peptide synthesizer equipped with a UV detector using N$^\alpha$-Fmoc-protected amino acids and the following side chain protection was employed: *N*-α-Fmoc-Asp-Thr(ΨMe,Me pro)-OH, *N*-α-Fmoc-

Ile-Thr(ΨMe,Me pro)-OH, *N*-α-Fmoc-*N*-ε-*tert*-Boc-*L*-lysine, *N*-α-Fmoc-*O*-*tert*-butyl-*L*-serine, *N*-α-Fmoc-*O*-*tert*-butyl-*L*-threonine, *N*-α-Fmoc-*O*-*tert*-butyl-*L*-tyrosine. The lipid moiety was installed using *N*-α-Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine. The activating reagent was 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt). Single coupling steps were performed with conditional capping. The Tn moiety was installed manually using N^{α}-Fmoc-Thr-(AcO_3-α-D-GalNAc) (134 mg, 0.2 mmol) in DMF (2 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) as the activating reagent. The manual coupling was monitored by standard Kaiser test. The resulting glycopeptides were purified by reversed-phase high performance liquid chromatography (RP HPLC) on an Agilent 1100 series system equipped with an autosampler, UV detector and fraction collector using a Zorbax Eclipse semi-preparative C-18 column using a linear gradient of 0→100% B (acetonitrile 95%, water 5%, TFA 0.1%) in A (water 95%, acetonitrile 5%, TFA 0.1%) over 40 min. Lipopeptides **1**-**3** and **10** were purified by RP-HPLC on either an Phenomenex Jupiter analytical or semi-preparative C-4 reversed phase column using a gradient of 0 – 100% B in A over 40 min.

Synthesis of Compound 1: Pam_3CysSK_4 thioester **10** (S1) (1.1 mg, 0.674 µmol), glycopeptide **11** (1.0 mg, 0.337 µmol) and dodecylphosphocholine (1.5 mg, 4.38 µmol) were dissolved in a mixture of $CHCl_3$/trifluoroethanol (1/1 v/v, 5 mL). The solvents were removed under reduced pressure to give a thin film, which was hydrated for 4 h at 41 ^{o}C using a sodium phosphate (200 mM) buffer containing TCEP (2 mM) and EDTA (0.3%). The mixture was sonicated and the suspension was extruded through polycarbonate membranes (1.0 µm Whatman, Nucleopore, Track-Etch Membrane) at 50 ^{o}C to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was carried out in a CEM Discover Labmate unit at 25 W and 37 ^{o}C under open vessel conditions. After 20 min, the reaction mixture was purified by RP-HPLC on an analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of

40 min. Lyophilization of the appropriate fractions afforded **1** (0.76 mg, 50%). $C_{217}H_{367}N_{45}O_{53}S_2$ HR MALDI-ToF MS: observed 4516.9668 [M+H]; calculated 4516.685 [M+H].

Synthesis of Compound 2: Lipopeptide **2** was synthesized by SPPS following the general protocol on Rink Amide AM resin (0.1 mmol). After the assembly of the peptide, the remaining steps were performed manually. *N*-α-Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (180 mg, 0.2 mmol) was dissolved in DMF (5 mL), and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and added to the resin. The coupling reaction was monitored by the Kaiser test and was complete. Upon completion of the coupling, the *N*-Fmoc group was cleaved using 20% 4-methyl piperidine in DMF (6 mL). Palmitic acid (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%; 10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^{o}C; 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude lipopeptide was purified a Phenomenex Jupiter C-4 semi preparative column using a linear gradient of 0-100% solvent B in A over a period of 40 min, and the appropriate fractions were lyophilized to afford **2**. $C_{206}H_{349}N_{43}O_{47}S$ HR MALDI-ToF MS: observed, [M+2H] 4211.0464; calculated [M+2H] 4211.5962.

Synthesis of Compound 3: Lipopeptide **3** was synthesized on Rink Amide AM resin (0.1 mmol) following the general protocol for peptide synthesis and the procedure for lipidation described for compound **2**. The resulting crude lipopeptide was purified by HPLC on a Phenomenex Jupiter C4 semi preparative column using a linear gradient of 0-100% solvent B in A over a period of 40 min, and the appropriate fractions were lyophilized to afford **3.** $C_{162}H_{279}N_{29}O_{31}S$, MALDI-ToF MS: observed, [M+H] 3160.237; calculated, [M+H] 3156.087.

Synthesis of glycopeptide 4: Glycopeptide **4** was synthesized using MW-SPPS on a Discover SPS system utilizing HBTU/HOBt activation protocol on Rink Amide AM LL Resin (0.1 mmol). The glycosylated amino acid N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc) (134 mg, 0.2 mmol) was dissolved in DMF (2 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and were added to the resin. The microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The resin was then returned to the automated synthesizer for further elongation. The *N*-terminal Fmoc residue was then removed using 20% 4-methyl piperidine in DMF and the resulting amine was capped using 10% Ac_2O, 5% DIPEA in DMF. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 hours. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. After which it was treated with 95% TFA, 2.5% TIPS, and 2.5% H_2O (10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^{o}C; 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycopeptide was purified by HPLC on a semi preparative C-18 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min, and the appropriate fractions were lyophilized to afford **4**. $C_{133}H_{207}N_{33}O_{40}$, MALDI-ToF MS: observed, [M+] 2906.9919; calculated, [M+] 2906.518.

Synthesis of lipopeptide 5: Lipopeptide **4** was synthesized on Rink Amide AM resin (0.1 mmol) following the general protocol for peptide synthesis and the procedure for lipidation described for compound **2**. The resulting crude lipopeptide was purified by HPLC on a Phenomenex Jupiter C4 semi preparative column using a linear gradient of 0-100% solvent B in A over a period of 40 min, and the appropriate fractions were lyophilized to afford **5.** $C_{81}H_{157}N_{11}O_{12}SNa$, MALDI-ToF MS: observed, [M+Na] 1531.117; calculated, [M+Na] 1531.163.

Synthesis of glycopeptide 6: Glycopeptide **6** was synthesized on Rink Amide Am resin (0.1 mmol) following the general protocol for peptide synthesis. The glycosylated amino acid N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc) (134 mg, 0.2 mmol) was dissolved in DMF (2 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and were added to the resin. The manual coupling reaction was monitored by Kaiser test and was complete after 18 hours. The resin was then returned to the automated synthesizer for further elongation. The *N*-terminal Fmoc residue was then removed using 20% 4-methyl piperidine in DMF and the resulting amine was capped using 10% Ac_2O, 5% DIPEA in DMF. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 hours. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 hours. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. After which it was treated with 95% TFA, 2.5% TIPS, and 2.5% H_2O (10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^{o}C; 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycopeptide was purified by HPLC on a semi preparative C-18 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min, and the appropriate fractions were lyophilized to afford **6**. $C_{48}H_{79}N_{14}O_{19}$, MALDI-ToF MS: observed, [M+H] 1155.645; calculated, [M+H] 1155.564.

Synthesis of peptide 7: Peptide **7** was synthesized on Rink Amide Am resin (0.1 mmol) following the general protocol for peptide synthesis. The *N*-terminal Fmoc residue was removed using 20% 4-methyl piperidine in DMF and the resulting amine was capped using 10% Ac_2O, 5% DIPEA in DMF. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 hours. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was

swelled in DCM (5 mL) for 1 h. After which it was treated with 95% TFA, 2.5% TIPS, and 2.5% H_2O (10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^oC; 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycopeptide was purified by HPLC on a semi preparative C-18 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min, and the appropriate fractions were lyophilized to afford **7**. $C_{40}H_{66}N_{13}O_{14}$, MALDI-ToF MS: observed, [M+H] 952.485; calculated, [M+H] 952.485.

Synthesis of glycopeptide 8: Glycopeptide **8** was synthesized on Rink Amide AM resin (0.1 mmol) following the general protocol for peptide synthesis. The glycosylated amino acid N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc) (134 mg, 0.2 mmol) was dissolved in DMF (2 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and were added to the resin. The manual coupling reaction was monitored by Kaiser test and was complete after 18 hours. The resin was then returned to the automated synthesizer for further elongation. The *N*-terminal Fmoc residue was then removed using 20% 4-methyl piperidine in DMF and the resulting amine was capped using 10% Ac_2O, 5% DIPEA in DMF. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 hours. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. After which it was treated with 95% TFA, 2.5% TIPS, and 2.5% H_2O (10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^oC; 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycopeptide was purified by HPLC on a semi preparative C-18 reversed phase column using a linear gradient of 0-100%

solvent B in A over a 40 min, and the appropriate fractions were lyophilized to afford **8**. $C_{98}H_{156}N_{29}O_{35}$, MALDI-ToF MS: observed, [M+H] 2299.354; calculated, [M+H] 2299.132.

Synthesis of peptide 9: Peptide **9** was synthesized on Rink Amide Am resin (0.1 mmol) following the general protocol for peptide synthesis. The *N*-terminal Fmoc residue was removed using 20% 4-methyl piperidine in DMF and the resulting amine was capped using 10% Ac_2O, 5% DIPEA in DMF. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 hours. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. After which it was treated with 95% TFA, 2.5% TIPS, and 2.5% H_2O (10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^oC; 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycopeptide was purified by HPLC on a semi preparative C-18 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min, and the appropriate fractions were lyophilized to afford **9**. $C_{90}H_{142}N_{28}O_{30}$, MALDI-ToF MS: observed, [M+H] 2096.906; calculated, [M+H] 2096.052.

Synthesis of lipopeptide thioester 10: Lipopeptide thioester **10** was synthesized on a preloaded H-Gly-sulfamylbutyryl Novasyn TG resin (0.1 mmol) as described in the general methods section for peptide synthesis and the lipidation protocol for lipopeptide **2**.

*Activation and Cleavage of peptide thioester **10**:* The resin-bound peptide was washed thoroughly with DCM (5 mL x 2) and *N*-methyl-2-pyrrolidone (NMP) (5 mL x 3). After initial washings, the resin was swollen in DCM (5 mL) for 1 h. The resin was treated with DIPEA (0.5 mL, 3 mmol), iodoacetonitrile (0.36 mL, 5 mmol) in NMP (6 mL). It is important to note that the iodoacetonitrile was filtered through a plug of basic alumina before addition to the resin. The resin was agitated under the exclusion of light for 24 h, filtered, and washed with NMP (5 mL x

4), DCM (5 mL x 4), and THF (5 mL x 4). The activated *N*-acyl sulfonamide resin was swollen in DCM for 1 h, drained, and transferred to a round bottom flask. To the resin-containing flask was added THF (4 mL), benzyl mercaptan (0.64 mL, 5 mmol), and sodium thiophenolate (27 mg, 0.2 mmol). After agitation for 24 h, the resin was filtered and washed with THF (10 mL). The combined filtrate and washings were collected and contrated in *vacuo*. The thiol impurities were removed by passing the crude peptide through a LH-20 column (1:1 DCM:MeOH). The appropriate fractions were collected and the solvent was removed to recover the fully protected peptide thioester.

*Side chain deprotection of thioester **10**:* The protected peptide was treated with reagent B (5 mL, 88% TFA, 5% phenol, 5% H_2O, 2% TIS) for 6 h at room temperature. The TFA solution was added dropwise to a screw cap centrifuge tube containing ice-cold diethyl ether (25 mL) and the resulting suspension was left overnight at 4 ^oC, after which the precipitate was collected by centrifugation at 3000 rpm (5 ^oC, 20 min). After decanting the ether, the peptide precipitate was re-suspended in ice-cold diethyl ether (25 mL) and the process of washing was repeated twice. The peptide was lyophilized and purified by RP-HPLC using a semi-preparative C-4 reversed phase column using a linear gradient of 0-100% B in A over a period of 50 min. Lyophilization of the appropriate fractions afforded **10**. $C_{90}H_{165}N_{11}O_{13}S_2Na$, MALDI-ToF MS: observed, [M+Na] 1965.137, calculated, [M+Na] 1965.192.

Synthesis of glycopeptide 11: Glycopeptide **11** was synthesized on Rink Amide AM resin (0.1 mmol) following the general protocol for peptide synthesis. The glycosylated amino acid N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc) (134 mg, 0.2 mmol) was dissolved in DMF (2 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and were added to the resin. The manual coupling reaction was monitored by Kaiser test and was complete after 18 hours. The resin was then returned to the automated synthesizer for further elongation. The *N*-terminal Fmoc residue was then removed using 20% 4-methyl piperidine in DMF and the resulting amine was capped using 10% Ac_2O, 5% DIPEA in DMF. The resin was washed

thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 hours. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. After which it was treated with 94% TFA, 2.5% EDT, 2.5% H_2O, and 1% TIPS (10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^oC; 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycopeptide was purified by HPLC on a semi preparative C-18 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min, and the appropriate fractions were lyophilized to afford **6**. $C_{134}H_{211}N_{34}O_{40}S$, MALDI-ToF MS: observed, [M+H] 2968.399; calculated, [M+H] 2968.524.

Liposome Preparation for Immunizations. Each glycolipopeptide was incorporated into phospholipid-based small unilamellar vesicles (SUVs) by hydration of a thin film of the synthetic compounds, egg phosphatidylcholine, phosphatidylglycerol, and cholesterol in a HEPES buffer (10 mM, pH 7.4) containing NaCl (145 mM) followed by extrusion through a 0.1 µm Nucleopore® polycarbonate membrane.

Immunizations and Tumor Palpation. Eight to 12-week-old MUC1.Tg mice (C57BL/6; H-2b) that express human MUC1 at physiological level were immunized three-times at biweekly intervals at the base of the tail intradermally with liposomal preparations of three-component vaccine constructs (25 µg containing 3 µg of carbohydrate) and the respective controls which lack the tumor-associated MUC1 epitope. After 35 days, the mice were challenged with MMT mammary tumor cells (1×10^6 cells), which express MUC1 and Tn. On day 42, one more immunization was given. Palpable tumors were measured by calipers, and tumor weight was calculated according to the formula: grams = [(length) X (width) 2]/ 2, where length and width are measured in centimeters. On day 49, the mice were sacrificed, the tumors were surgically removed and tumor weight was determined.

^{51}Chromium (Cr) Release Assay. Cytolytic activity was determined by a standard ^{51}Cr release method using $CD8^+$ T-cells from tumor-draining lymph nodes without any *in vitro* stimulation as effector cells and ^{51}Cr labeled DCs pulsed with respective peptide as target cells at a 100:1 ratio for 6 h. Target cells were loaded with 100 µCi ^{51}Cr (Amersham Biosciences) per 10^6 target cells for 2 h before incubation with effectors. Radioactive ^{51}Cr release was determined using the Topcount Microscintillation Counter (Packard Biosciences) and specific lysis was calculated: (experimental cpms – spontaneous cpms/complete cpms – spontaneous cpms) x 100. Spontaneous lysis was <15% of total lysis.

Determination of ADCC. Tumor cells (Yac-MUC1 or C57mg.MUC1) were labeled with 100 µCi ^{51}Cr for 2 h at 37 ˚C, washed and incubated with serum (1 in 25 dilutions) obtained from the vaccinated mice for 30 min at 37 ˚C. NK cells, which have high expression of CD16 receptor, were used as effectors. These cells were stimulated with IL-2 (200 units/mL) for 24 h prior to assay. Effector cells were seeded with the antibody-labeled tumor cells in 96-well culture plates (Costar high binding plates) at an effector:target cell ratio of 50:1 for 4 h. The release of ^{51}Cr was determined by the Top Count. Spontaneous and maximum release of ^{51}Cr was determined and was below 20%. The percentage of specific release was determined: (release-spontaneous release/maximal release-spontaneous release) x 100.

IFN-γ ELISPOT Assay. At time of sacrifice, MAC sorted $CD8^+$ T-cells from tumor-draining lymph notes were isolated from treated MUC1.Tg mice and used as responders in an IFN-γ ELISPOT assay as described previously [34]. Spot numbers were determined using computer-assisted video image analysis by ZellNet Consulting, Inc. (Fort Lee, NJ). Splenocytes from C57BL/6 mice stimulated with Concavalin A were used as a positive control.

Serologic Assays. Anti-MUC1 IgG, IgG1, IgG2a, IgG2b, IgG3, and IgM antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [51]. Briefly, ELISA plates (Thermo Electron Corp.) were coated with a MUC1 glycopeptide conjugated to BSA through a maleimide linker (BSA-MI-CTSAPDT(αGalNAc)RPAP). Serial dilutions of the

sera were allowed to bind to immobilized MUC1. Detection was accomplished by the addition of phosphate-conjugated anti-mouse antibodies and *p*-nitrophenyl phosphate (Sigma). To determine antibody titers against the T_{helper} (polio) epitope, Reacti-bind NeutrAvidin coated and pre-blocked plates (Pierce) were incubated with biotin-labeled T_{helper} (10 μg/mL; 100 μL/well) for 2 h. Next, serial dilutions of the sera were allowed to bind to immobilized T_{helper} epitope. Detection was accomplished as described above. The antibody titer was defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera.

Inhibition ELISAs. Serum samples were diluted in diluent buffer to give without inhibitor expected final optical density values of approximately 1. The diluted serum samples (60 μL) were mixed in an uncoated microtiter plate with diluent buffer, glycopeptide SAPDT(αGalNAc)RPAP (**6**), peptide SAPDTRPAP (**7**) or (α-O-GalNAc-Thr (Tn-antigen) in diluent buffer (60 μL) with a final concentration of 0-500 μM. After incubation at room temperature for 30 min, the mixtures (100 μL) were transferred to a plate coated with BSA-MI-CTSAPDT(αGalNAc)RPAP. The microtiter plates were incubated and developed as described above using an alkaline phosphatase-conjugated detection antibody for IgG total. Optical density values were normalized for the optical density values obtained with monoclonal antibody alone (no inhibitor, 100%).

Cytokine Assays. DCs were prepared from mouse bone marrow cultures as previously described [52, 53]. On the day of the exposure assay mature DCs were plated as 4×10^6 cells/well in 1.8 mL in 24-well tissue culture plates. Cells were then incubated with different stimuli (200 μL, 10X) for 24 h in a final volume of 2 mL/well. Stimuli were given at a wide concentration range (corresponding to final concentrations of 0.1 ng/mL to 100 μg/mL Pam_3CysSK_4 for **1**, **5**, or **6** in liposomes and 0.001 ng/mL to 10 μg/mL for *E. coli* LPS). Supernatants were collected. For estimation of the effect of ATP on IL-1β secretion, DCs were re-incubated for 30 min in the same volume of medium containing ATP (5 mM; Sigma), after which supernatants were

harvested. Cytokine quantification of mouse TNF-α, RANTES, IL-6, IL-1β, IL-10, IP-10, IL-12 p70, IL-12/23 p40 and IFN-β was performed by ELISA as described before[54].

Statistical Analysis. Multiple comparisons were performed using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. Differences were considered significant when $P < 0.05$.

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CHAPTER IV

HUMORAL IMMUNE RESPONSES BY IMMUNIZATIONS WITH FULLY SYNTHETIC THREE-COMPONENT CANCER VACCINE CANDIDATES*

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4.1 Abstract

The mucin MUC1 is typically aberrantly glycosylated by epithelial cancer cells manifested by truncated *O*-linked saccharides. The resultant glycopeptide epitopes can bind cell surface major histocompatibility complex (MHC) molecules and are susceptible to recognition by cytotoxic T-lymphocytes (CTLs), while aberrantly glycosylated MUC1 protein on the tumor cell surface can be bound by antibodies to induce antibody-dependent cell-mediated cytotoxicity (ADCC). Efforts to elicit CTLs and IgG antibodies against cancer-expressed MUC1 have not been successful when unglycosylated MUC1 sequences were used for vaccination, likely due to conformational differences between the unglycosylated peptide sequences and the aberrantly glycosylated MUC1 which is present on tumor cells. Immunizations with densely glycosylated MUC1 peptides have also been ineffective due to impaired susceptibility to antigen processing. While many vaccine candidates have shown promise in murine tumor models, they failed to elicit humoral immune responses. We have found that vaccination with constructs containing aberrantly glycosylated long MUC1 peptide sequences in addition to the toll-like receptor 2 ligand Pam_3CysSK_4 could be utilized to provide glycopeptide-specific antibody responses. Furthermore, we were able to streamline the synthesis of glycolipopeptide cancer vaccine candidates by exploiting microwave-assisted solid-phase peptide synthesis for their linear assembly.

4.2 Introduction

A large number of breast, ovarian, colorectal, pancreatic, and prostate cancers exhibit a striking overexpression of MUC1, resulting in a loss of polarization.[1] Furthermore, tumor-associated MUC1 is aberrantly glycosylated due to a lack of core 1,3-galactosyltransferase, producing truncated carbohydrate structures such as the Tn (α-GalNAc-Thr), STn (α-Neu5Ac-(2,6)-αGalNAc-Thr), and Thomsen-Friedenreich (β-Gal-(1,3)-αGalNAc-Thr) antigens. Recently,

the NCI Translational Research Working group prioritized cancer antigens based on various criteria, including therapeutic function, immunogenicity, the role of the antigen in oncogenicity, specificity, expression level, and the percentage of patients with antigen positive cancer.[2] MUC1 was ranked second out of the 75 tumor-associated antigens screened. In this respect, MUC1 displays nearly ubiquitous expression in a wide variety of tumor types, is found on cancer stem cells, and has a functional role in tumorigenesis.

Humoral responses to MUC1 have been observed in benign diseases and carcinoma patients and the presence of circulating antibodies against MUC1 at the time of cancer diagnosis has been correlated with a favorable disease outcome in breast cancer patients.[3, 4] The MUC1-derived peptide sequences RPAPGS, PPAHGVT, and PDTRP have been identified as the most frequent epitopes.[5, 6] It has also been observed that peptides which have been modified with αGalNAc exhibited strong antibody binding. It has been proposed that the improved binding is due to saccharide-induced conformational change of the peptide backbone.[7-9]

It has been proposed that T-cell epitopes from the MUC1 core domain are packaged within tumor cells in their truncated glycosylation state into MHC class I molecules, leading to natural MHC-restricted recognition of “hypoglycosylated’ epitopes.[10-12] There is evidence that MUC1 glycopeptides can bind more strongly to the MHC class I mouse allele *H2k*^{b} compared to the corresponding unglycosylated peptide and induce activation of cytotoxic T-lymphocytes (CTL) in human/MUC1 transgenic mice.[13] Several MUC1-derived HLA-A2–binding peptides have been identified including STAPPAHGV and SAPDTRAPG.[14-16]

Early efforts towards the development of MUC1-based cancer vaccines focused on the use of unglycosylated MUC1 tandem repeat peptides of varying lengths conjugated to different carrier proteins and were administered with or without an external adjuvant.[6, 17-24] These

strategies have generally failed to elicit effective immune responses to MUC1 expressing cancer cells. This is likely due to conformational differences between unglycosylated peptide sequences and the aberrantly glycosylated MUC1 which is present on tumor cells.[7-9] The immunogenicity of carbohydrate epitopes (Tn, or sialyl-Tn) conjugated to a carrier protein has been examined in mice. These constructs, however, elicited only modest IgM and IgG antibody responses.[25-28] A synthetic 60-mer MUC1 tandem repeat peptide with saturated *O*-glycan occupancy (five sites per repeat) was found to induce modest humoral responses.[29] Recent studies have shown that a densely glycosylated MUC1 glycopeptide cannot be processed by antigen-presenting cells (APCs), which compromises the presentation of class I and class II glycopeptides and, hence, T_{helper} cells and CTLs will not be activated.[10] Interestingly, glycopeptides carrying the Tn- or TF-antigens have been used to induce carbohydrate-specific cellular immune responses in mice.[30] Two-component vaccines, consisting of an MHC I glycopeptide and a T_{helper} epitope, have shown promise in tumor models, but failed to induce antibody responses.[31]

We have previously shown that compound **1**, which is composed of a tumor-associated glycopeptide B-cell epitope derived from MUC-1, a polio virus-derived T-cell epitope, and the lipopeptide adjuvant Pam_3CysSK_4, elicits specific humoral and cellular immune responses in MUC1.Tg mice. It has been reported that short *O*-linked glycans, like αGalNAc, on MUC1 tandem repeats remain intact during dendritic cell processing and in the major histocompatibility complex (MHC) class II pathway.[12] As glycosylation of the MUC1 peptides is known to contribute to the extent and site specificity of cathepsin-mediated proteolysis,[32] we proposed that vaccination with constructs **2 – 4** (Figure 4.1), which contain aberrantly glycosylated long MUC1 peptide sequences, could be utilized to determine the optimal MHC-II binding epitopes to provide peptide- or glycopeptide-specific antibody responses.

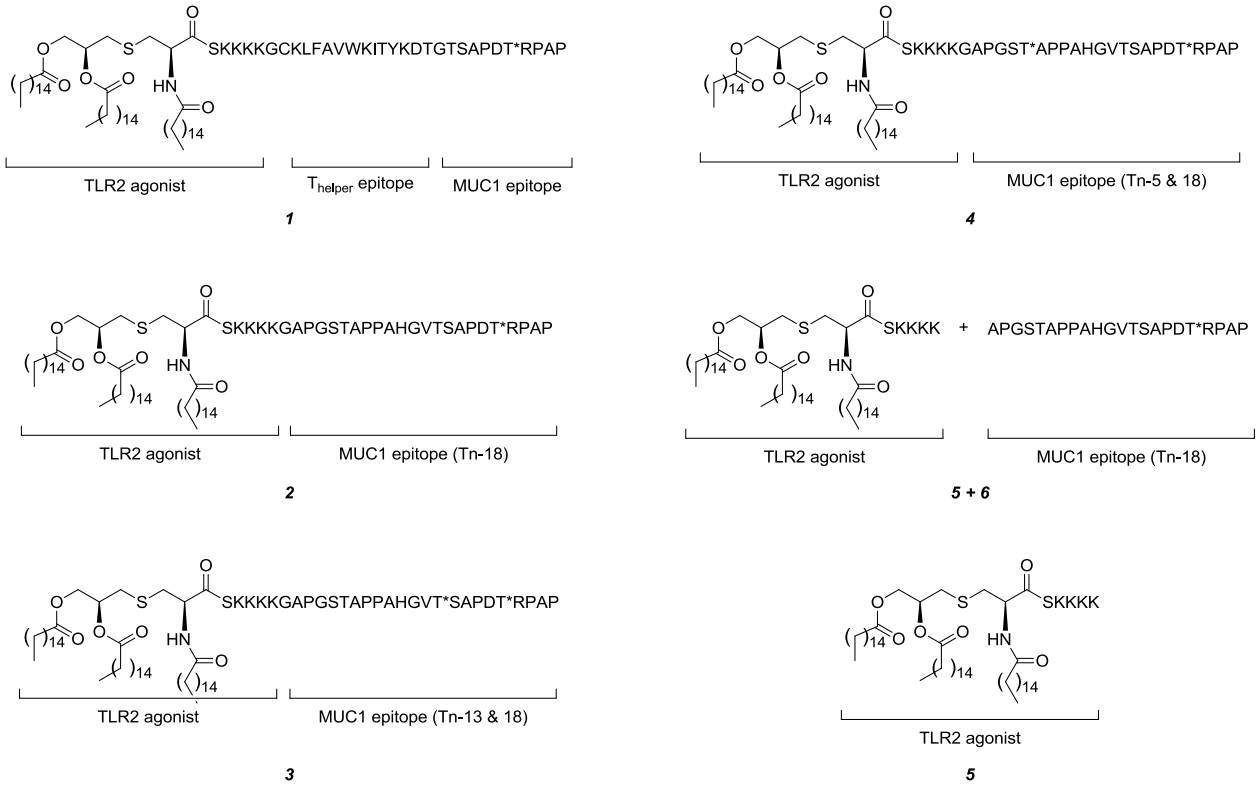


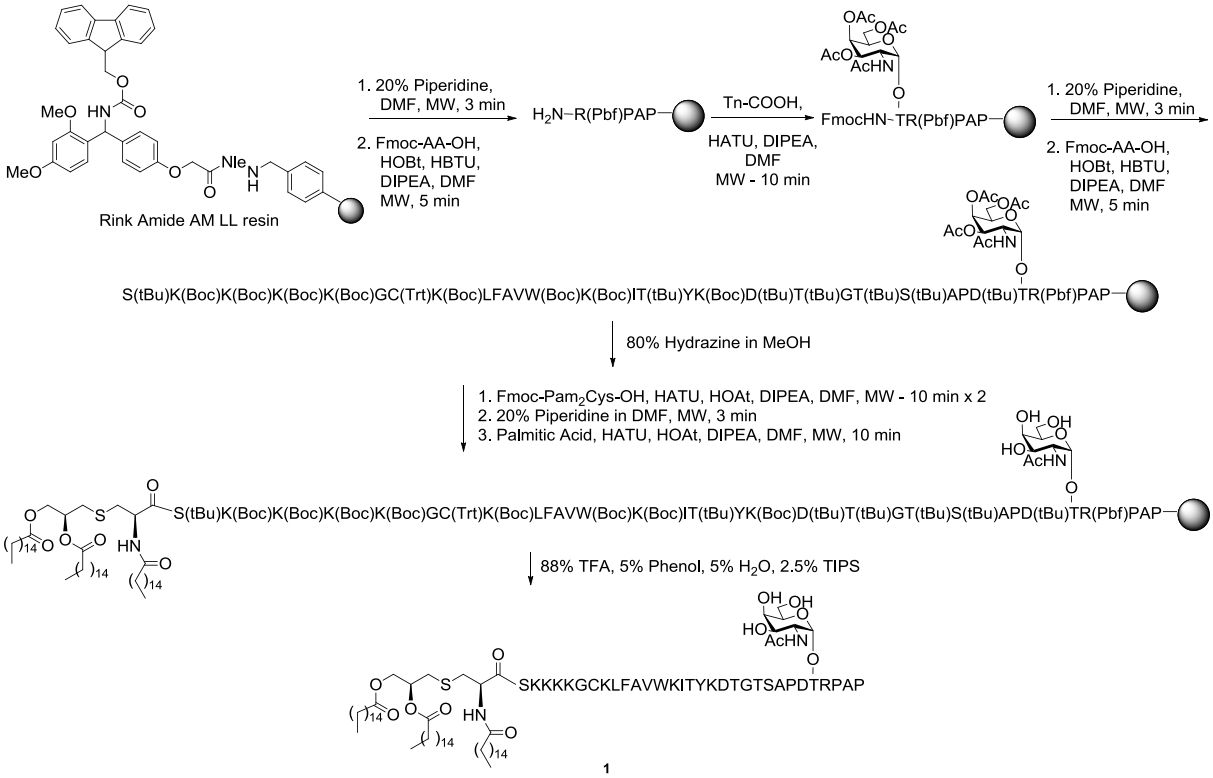
Figure 4.1. Chemical structures of synthetic vaccine constructs.

4.3 Results and discussion

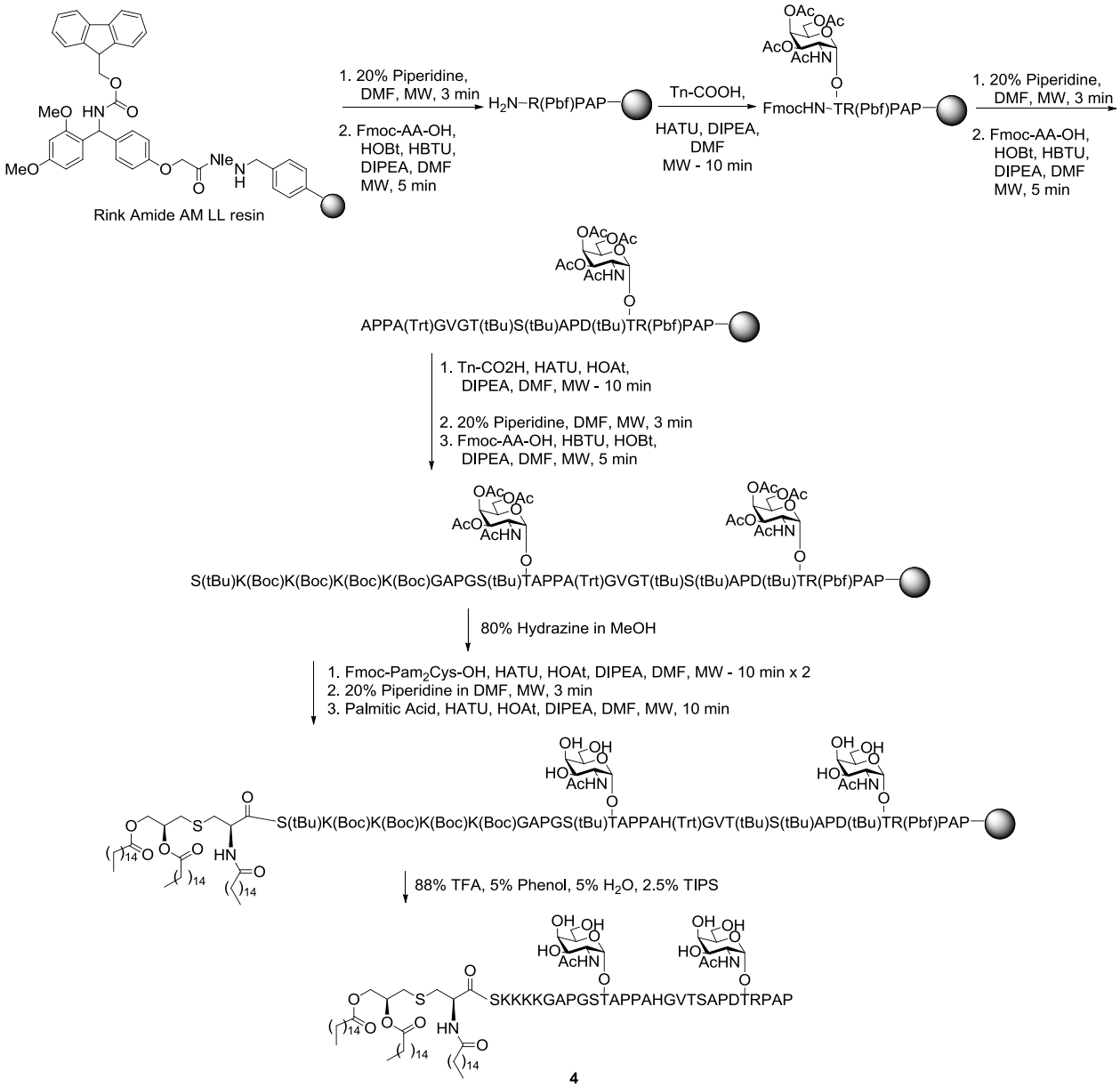
We previously prepared vaccine construct **1** utilizing microwave-assisted liposome-mediated native chemical ligation. Using this method, it was found that the combined use of microwaves and liposomes greatly increases the reaction rates of ligations of sparingly soluble peptide reactants. There have been many reports suggesting that microwave-assisted synthesis of peptides reduces reaction times while providing peptides of high purity.[33] We therefore hypothesized that microwave-assisted solid phase peptide synthesis (MW-SPPS) could be utilized for the linear synthesis of the vaccine candidates.

Glycolipopeptide **1** was assembled using MW-SPPS (Scheme 4.1). Using Rink Amide AM LL resin, the first four amino acids were introduced using a CEM Liberty 12-channel automated microwave peptide synthesize, which utilizes an HBTU-mediated HOBt ester activation protocol. A Fmoc-protected Tn antigen was introduced manually using an

HATU/HOAt activation protocol under microwave irradiation. The resin was then returned to the automated peptide synthesizer to further elongate the peptide. Following the installation of the final serine residue, the resin was removed from the synthesizer and was treated with 70% hydrazine in methanol to remove the acetyl esters of the saccharide. The Fmoc-Pam_2Cys and palmitic acid residues were coupled manually using HATU/HOAt in the presence of DIPEA in DMF under microwave irradiation. The concomitant amino acid side chain deprotection and cleavage from the resin was accomplished using 88% TFA, 5% phenol, 5% H_2O, and 2% TIPS. The glycolipopeptide was then obtained following purification by RP-HPLC using a C4 column.



Scheme 4.1. Synthesis of glycolipopeptide **1** by MW-SPPS.



Scheme 4.2. Synthesis of glycolipopeptide **4** by MW-SPPS.

Encouraged by the successful preparation of **1**, we focused our attention on the synthesis of cancer vaccine candidates **2**-**4**, which contain a MUC1-derived B-epitope and T-helper peptide sequence, in addition to the TLR2 ligand Pam_3CysSK_4. Using the combination of automated and manual MW-SPPS described above, glycolipopeptides **2**-**4** were successfully synthesized in a linear fashion (Scheme 4.2). Likewise, lipopeptide **5** and glycopeptide **6** were synthesized under microwave irradiation.

The resulting compounds were incorporated into phospholipid-based small unilamellar vesicles by hydration of a thin film of the synthetic compounds, egg phosphatidylcholine, phosphatidylglycerol, and cholesterol in a HEPES buffer (10 mM, pH 6.5) containing NaCl (145 mM) followed by extrusion through a 100 nm Nucleopore polycarbonate membrane. Groups of MUC1.Tg mice (C57BL/6; H-2^b) which express human MUC1 were immunized three times at biweekly intervals with liposomal preparations of compounds of **1**, **2**, **3**, **4**, a mixture of **5** and **6**, and **5** alone. After 35 days, the mice were challenged with MC-38 colon tumor cells, followed by one final boost after one week. One week after the last immunization, the mice were sacrificed and the robustness of humoral immune responses was assessed by determining anti-MUC1 antibody titers.

Anti-MUC1 antibody titers were determined by coating microtiter plates with the MUC1-derived glycopeptides CTSAPDT(αGalNAc)RPAP, CAPGSTAPPAHGVTSAPDT(αGalNAc)RPAP, CAPGSTAPPAHGVT(αGalNAc)SAPDT(αGalNAc)RPAP, and CAPGST(αGalNAc)APPAHGVTSAPDT(αGalNAc)RPAP conjugated to maleimide-modified BSA (final concentration of MUC1 0.21 μg/mL). Immunizations with compounds **1**, **2**, **3**, and **4** resulted in a robust production of IgG antibodies (Table 4.1). This demonstrates that immunization with vaccine constructs consisting of the TLR2 ligand Pam_3CysSK_4 and a 22-mer glycopeptide derived from MUC1 are able to elicit humoral immune responses in MUC1.Tg mice. Immunization with compound **3** resulted in slightly lower IgG titers, indicating that the dendritic cell processing and MHC-II presentation of a glycopeptide which is glycosylated at the Tn-13 position is not as efficient. As expected, the mixture of compounds **5** and **6** had elicited lower titers of antibodies than compound **2**, highlighting the importance of covalent attachment of the Pam_3CysSK_4 to the glycopeptide epitope for robust antigenic responses. Supporting our previous observations,[34] there was a broad range in the antibody titers of the group immunized

with compounds **5** and **6** (Figure 4.2), further highlighting the significance of the covalent attachment of the TLR2 ligand to the glycopeptide epitope.

Table 4.1. ELISA anti-MUC1 antibody titers[a] after 3rd & 4th immunizations with various preparations.

Coating	IgG total MUC1		IgG total LMUC1 Tn-18		IgG total LMUC1 Tn-13 & 18		IgG total LMUC1 Tn-5 & 18	
Immunization[b]	After 3rd	After 4th	After 3rd	After 4th	After 3rd	After 4th	After 3rd	After 4th
1 (n=7)	14,600	41,000						
2 (n=7)			11,100	44,500				
3 (n=7)					9,300	32,800		
4 (n=7)							10,200	40,000
5 + 6 (n=7)			1,500	35,700				

[a] Anti-MUC1 antibody titers are presented as mean values for groups of four to seven mice. ELISA plates were coated with BSA-MI-CTSAPDT(αGalNAc)RPAP (MUC1), BSA-MI-CAPGSTAPPAHGVTSAPDT(αGalNAc)RPAP (LMUC1 Tn-18), BSA-MI-CAPGSTAPPAHGVT(αGalNAc)SAPDT(αGalNAc)RPAP (LMUC1 Tn-13 & 18), or BSA-MI-CAPGST(αGalNAc)APPAHGVTSAPDT(αGalNAc)RPAP (LMUC1 Tn-5 & 18) conjugate for anti-MUC1 antibody titers. Titers were determined by linear regression analysis, with plotting of dilution versus absorbance. Titers are defined as the highest dilution yielding an optical density of 0.1 or greater relative to normal control mouse sera. [b] Liposomal preparations were employed. MC-38 colon tumors were induced between the 3rd and 4th immunization.

Lipidation with the TLR2 agonist Pam_3CysSK_4 makes it possible to formulate the vaccine candidates in liposomal preparations, which likely enhances the circulation time. Moreover, a liposomal preparation presents the glycopeptide epitope multivalently to B-cell receptors, thereby providing an opportunity for efficient clustering of Ig receptors of B-cells, which is required to initiate B-cell signaling and antibody production. We have previously shown that covalent attachment of the TLR2 agonist Pam_3CysSK_4 facilitates selective internalization by TLR2-expressing immune cells such B-cells and APCs.[35] Uptake and processing of the antigen

and subsequent presentation of the MUC1-derived T_{helper} sequence as a complex with MHC class II on the cell surface of APCs are critical for eliciting IgG antibodies.

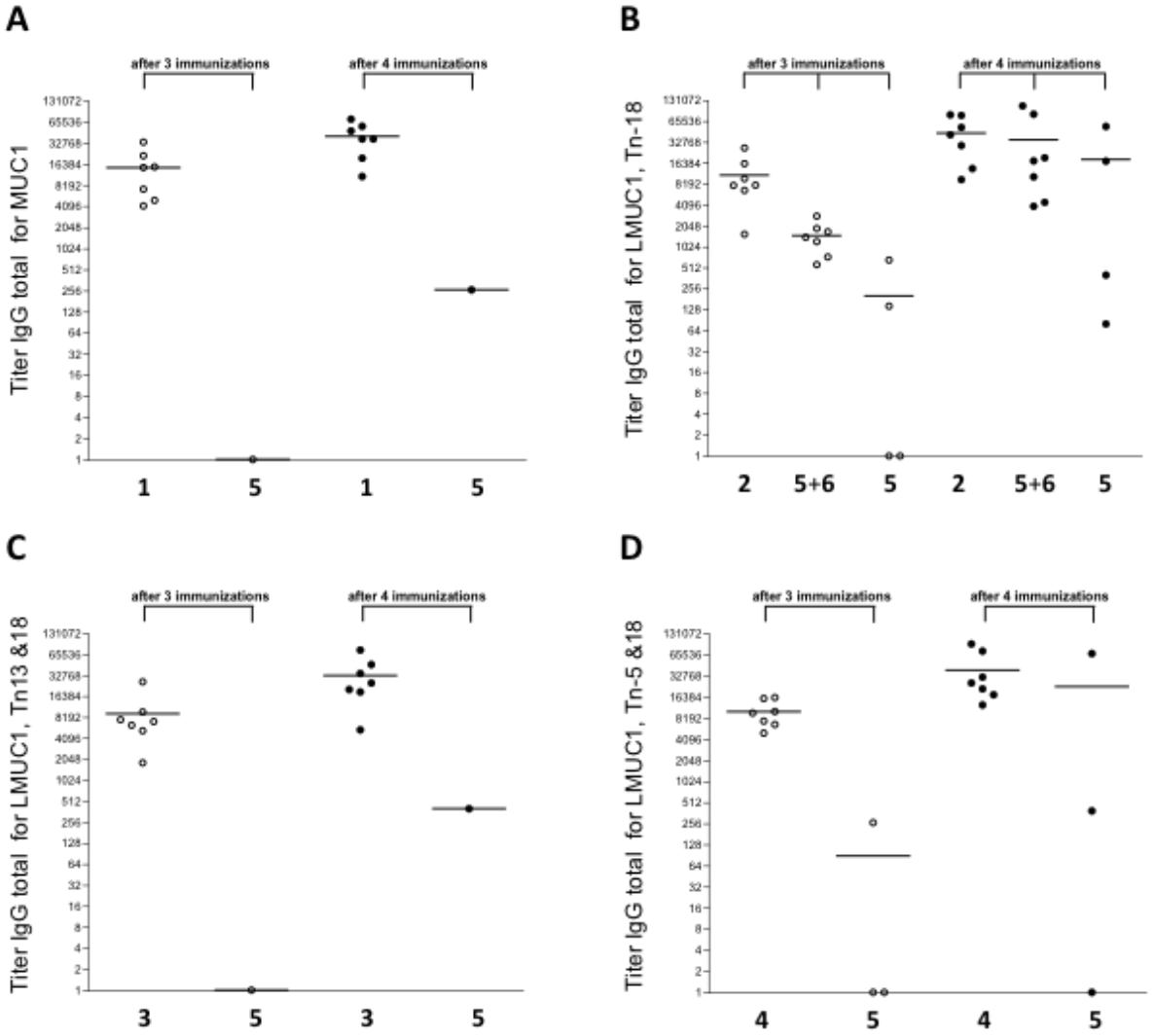


Figure 4.2. ELISA anti-MUC1 antibody titers after immunizations with **1**, **2**, **3**, **4**, **5 + 6**, or **5** alone. ELISA plates were coated with BSA-MI-CTSAPDT(αGalNAc)RPAP (MUC1) (*A*), BSA-MI-CAPGSTAPPAHGVTSAPDT (αGalNAc)RPAP (LMUC1, Tn-18) (*B*) BSA-MI-CAPGSTAPPAHGVT(αGalNAc)SAPDT (αGalNAc)RPAP (LMUC1, Tn-13 & 18) (*C*), or BSA-MI-CAPGST(αGalNAc)APPAHGVTSAPDT(αGalNAc)RPAP (LMUC1, Tn-5 & 18) (*D*) and titers were determined by linear regression analysis, plotting dilution *vs.* absorbance. Titers were defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera. Each data point represents the titer for an individual mouse after 3 or 4 immunizations as indicated and the horizontal lines indicate the mean for the group of mice.

4.4 Conclusion

In summary, we have successfully exploited microwave-assisted solid-phase peptide synthesis for the linear synthesis of glycolipopeptides for use as cancer vaccine candidates. We believe this method could streamline the synthesis of carbohydrate-based cancer vaccine candidates. In addition, we have found that vaccination with constructs **2** – **4**, which contain aberrantly glycosylated long MUC1 peptide sequences, could induce glycopeptide-specific antibody responses.

4.5 Experimental Procedure

Reagents and general experimental procedure: Amino acid derivatives and Rink Amide AM LL resin were purchased from NovaBioChem; DMF was purchased from Aldrich. All other chemical reagents were purchased from AnaSpec and Aldrich and were used without further purification. All solvents employed were reagent grade. Reverse Phase HPLC was performed on an Agilent 1100 series system equipped with an autosampler, UV detector, and fraction collector. RP-HPLC was carried out using a Zorbax Eclipse C18 analytical column (5 μm, 9.4 x 250 mm) at a flow rate of 1.5 mL/min and a Vydac C4 analytical column (5 μm, 4.6 x 250 mm) at a flow rate of 1 mL/min. All runs used linear gradients of 0 – 100% solvent B in A over a 40 min period unless otherwise specified. (A: 95% Water, 5% Acetonitrile, 0.1% TFA; B: 95% Acetonitrile, 5% Water, 0.1% TFA) High resolution mass spectra were obtained by using MALDI-ToF (Applied Biosystems 5800 Proteomics Analyzer) with α-cyano-4-hydroxycinnamic acid as an internal standard matrix.

General methods for solid-phase peptide synthesis (SPPS): Peptides were synthesized on a 0.1 mmol scale with established protocols on a CEM Liberty peptide synthesizer equipped with a UV detector using N^{α}-Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) as the

activating reagents. Side chain protection was as follows: *N*-α-Fmoc-*O*-*tert*-butyl-Asp-OH, *N*-α-Fmoc-*N*-ε-*tert*-Boc-*L*-lysine, *N*-α-Fmoc-*O*-*tert*-butyl-*L*-serine, *N*-α-Fmoc-*O*-*tert*-butyl-*L*-threonine, *N*-α-Fmoc-*O*-*tert*-butyl-*L*-tyrosine. The coupling of the glycosylated amino acid N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc)-OH, *N*-α-fluorenylmethoxycarbonyl-*R*-(2,3-bis(palmitoyloxy)-(2*R*-propyl)-(R)-cysteine, and palmitic acid were carried out manually using (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HATU) and 1-Hydroxy-7-azabenzotriazole (HOAt) as activators and a CEM Discover SPS instrument. The manual couplings were monitored by standard Kaiser test.

Synthesis of tricomponent vaccine glycolipopeptide (1): Glycolipopeptide **1** was synthesized using MW-SPPS following the above protocol on Rink Amide AM LL Resin (0.1 mmol). The first four amino acids were introduced using the fully automated CEM Liberty system. The resin was then removed from the synthesizer. N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc)-OH (100 mg, 0.15 mmol) was dissolved in DMF (2 mL) and HATU (57 mg, 0.15 mmol), HOAt (20 mg, 0.15 mg), and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and were added to the resin. The manual microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The peptide was then elongated under MW-SPPS conditions described above until the final serine residue; the remaining steps were performed manually. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 h. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. *N*-α-Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (180 mg, 0.2 mmol) was dissolved in DMF (5 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and then added to the resin. The microwave-irradiated coupling reaction was monitored by the Kaiser test and was complete after 10 min. Upon completion of the coupling, the *N*-α-Fmoc group was cleaved using 20% 4-methyl piperidine in DMF (5 mL) under microwave irradiation.

Palmitic acid (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%; 10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^{o}C, 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycolipopeptide was purified by HPLC on a Jupiter analytical C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over 40 min, and the appropriate fractions were lyophilized to afford **1**. $C_{217}H_{367}N_{45}O_{53}S_2$ HR MALDI-ToF MS: observed 4516.9668 [M+H]; calculated 4516.685 [M+H].

Synthesis of tricomponent vaccine glycolipopeptide (2): Glycolipopeptide **2** was synthesized using MW-SPPS following the above protocol on Rink Amide AM LL Resin (0.1 mmol). The first four amino acids were introduced using the fully automated CEM Liberty system. The resin was then removed from the synthesizer. N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc)-OH (100 mg, 0.15 mmol) was dissolved in DMF (2 mL) and HATU (57 mg, 0.15 mmol), HOAt (20 mg, 0.15 mg), and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and then added to the resin. The manual microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The peptide was then elongated under MW-SPPS conditions described above until the final serine residue; the remaining steps were performed manually. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 h. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. *N*-α-Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (180 mg, 0.2 mmol) was dissolved in DMF (5 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2

min and then added to the resin. The microwave-irradiated coupling reaction was monitored by the Kaiser test and was complete after 10 min. Upon completion of the coupling, the *N*-α-Fmoc group was cleaved using 20% 4-methyl piperidine in DMF (5 mL) under microwave irradiation. Palmitic acid (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%; 10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^{o}C, 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycolipopeptide was purified by HPLC on a Jupiter analytical C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over 40 min, and the appropriate fractions were lyophilized to afford **2**. $C_{179}H_{310}N_{40}O_{47}S$, MALDI-ToF MS: observed, [M+H] 3805.575; calculated, [M+H] 3805.289

Synthesis of tricomponent vaccine glycolipopeptide (3): Glycolipopeptide **3** was synthesized using MW-SPPS following the above protocol on Rink Amide AM LL Resin (0.1 mmol). The first four amino acids were introduced using the fully automated CEM Liberty system. The resin was then removed from the synthesizer. N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc)-OH (100 mg, 0.15 mmol) was dissolved in DMF (2 mL) and HATU (57 mg, 0.15 mmol), HOAt (20 mg, 0.15 mg), and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and were added to the resin. The manual microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The next four amino acid residues were then introduced using the automated peptide synthesizer. N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc)-OH (100 mg, 0.15 mmol) was dissolved in DMF (2 mL) and HATU (57 mg, 0.15 mmol), HOAt (20 mg, 0.15 mg), and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and were added to the resin. The manual

microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The peptide was then elongated under MW-SPPS conditions described above until the final serine residue; the remaining steps were performed manually. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 hours. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. *N*-α-Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (180 mg, 0.2 mmol) was dissolved in DMF (5 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min, and was added to the resin. The microwave-irradiated coupling reaction was monitored by the Kaiser test and was complete after 10 min. Upon completion of the coupling, the *N*-α-Fmoc group was cleaved using 20% 4-methyl piperidine in DMF (5 mL) under microwave irradiation. Palmitic acid (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%; 10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^{o}C, 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycolipopeptide was purified by HPLC on a Jupiter analytical C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over 40 min, and the appropriate fractions were lyophilized to afford **3**. $C_{187}H_{323}N_{41}O_{52}S$, MALDI-ToF MS: observed, [M+H] 4008.284; calculated, [M+H] 4008.369.

Synthesis of tricomponent vaccine glycolipopeptide (4): Glycolipopeptide **4** was synthesized using MW-SPPS following the above protocol on Rink Amide AM LL Resin (0.1 mmol). The first four amino acids were introduced using the fully automated CEM Liberty

system. The resin was then removed from the synthesizer. N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc)-OH (100 mg, 0.15 mmol) was dissolved in DMF (2 mL) and HATU (57 mg, 0.15 mmol), HOAt (20 mg, 0.15 mg), and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and then added to the resin. The manual microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The peptide was then elongated under MW-SPPS conditions described above until for the next twelve amino acid residues. The resin was removed from the synthesizer and a mixture of N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc)-OH (100 mg, 0.15 mmol), HATU (57 mg, 0.15 mmol), HOAt (20 mg, 0.15 mg), and DIPEA (67 µL, 0.4 mmol) in 2 mL DMF was added to the resin. The manual microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The peptide was then elongated under MW-SPPS conditions described above until the final serine residue; the remaining steps were performed manually. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 70% hydrazine in methanol for 2 h. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. *N*-α-Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (180 mg, 0.2 mmol) was dissolved in DMF (5 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and then added to the resin. The microwave-irradiated coupling reaction was monitored by the Kaiser test and was complete after 10 min. Upon completion of the coupling, the *N*-α-Fmoc group was cleaved using 20% 4-methyl piperidine in DMF (5 mL) under microwave irradiation. Palmitic acid (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%; 10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^{o}C, 30 mL) and

recovered by centrifugation at 3,000 rpm for 15 min. The crude glycolipopeptide was purified by HPLC on a Jupiter analytical C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over 40 min, and the appropriate fractions were lyophilized to afford **4**. $C_{187}H_{323}N_{41}O_{52}S$, MALDI-ToF MS: observed, [M+H] 4008.512; calculated, [M+H] 4008.369.

Synthesis of Lipopeptide 5: Lipopeptide **5** was synthesized on Rink Amide AM LL resin (0.1 mmol) following the general protocol for peptide synthesis and the procedure for lipidation described for compound **1**. The resulting crude lipopeptide was purified by HPLC on a Phenomenex Jupiter C4 semi preparative column using a linear gradient of 0-100% solvent B in A over a period of 40 min, and the appropriate fractions were lyophilized to afford **5.** $C_{162}H_{279}N_{29}O_{31}S$, MALDI-ToF MS: observed, [M+H] 3160.237; calculated, [M+H] 3156.087.

Synthesis of Glycopeptide 6: Glycopeptide **6** was synthesized on Rink Amide AM LL Resin (0.1 mmol) following the general protocol for peptide synthesis described above. The glycosylated amino acid N^{α}-Fmoc-Thr-(Ac_3-α-D-GalNAc) (134 mg, 0.2 mmol) was dissolved in DMF (2 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and then added to the resin. The microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The resin was then returned to the automated synthesizer for further elongation. Next, the *N*-terminal Fmoc residue was removed using 20% 4-methyl piperidine in DMF and the resulting amine was capped using 10% Ac_2O, 5% DIPEA in DMF. The resin was washed thoroughly with DCM (10 mL x 5) and then treated with 70% hydrazine in methanol for 2 h. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. After which it was treated with 95% TFA, 2.5% TIPS, and 2.5% H_2O (10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^{o}C; 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycopeptide was

purified by HPLC on a semi preparative C-18 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min, and the appropriate fractions were lyophilized to afford **6**. $C_{98}H_{155}N_{29}O_{35}$, MALDI-ToF MS: observed, [M+H] 2299.351; calculated, [M+H] 2299.132.

Liposome Preparation for Immunizations. Each glycolipopeptide was incorporated into phospholipid-based small unilamellar vesicles (SUVs) by hydration of a thin film of the synthetic compounds, egg phosphatidylcholine, phosphatidylglycerol, and cholesterol in a HEPES buffer (10 mM, pH 7.4) containing NaCl (145 mM) followed by extrusion through a 0.1 μm Nucleopore® polycarbonate membrane.

Immunization Protocol. Eight to 12-week-old MUC1.Tg mice (C57BL/6; H-2b) that express human MUC1 at physiological level were immunized three-times at biweekly intervals at the base of the tail intradermally with liposomal preparations of three-component vaccine constructs (25 μg containing 3 μg of carbohydrate) and the respective controls which lack the vaccine constructs. After 35 days, the mice were challenged with MC-38 colon tumor cells (1×10^6 cells). On day 42, one more immunization was given. On day 49, the mice were sacrificed and serum was collected.

Serologic Assays. Anti-MUC1 IgG antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) as described previously.[36] Briefly, ELISA plates (Thermo Electron Corp.) were coated with a MUC1 (glyco)peptide conjugated to BSA through a maleimide linker (BSA-MI-CTSAPDT(αGalNAc)RPAP, BSA-MI-CAPGSTAPPAHGVTSAPDT(αGalNAc)RPAP, BSA-MI-CAPGSTAPPAHGVT(αGalNAc)SAPDT(αGalNAc)RPAP, and BSA-MI-CAPGST(αGalNAc)APPAHGVTSAPDT(αGalNAc)RPAP). Serial dilutions of the sera were allowed to bind to immobilized MUC1. Detection was accomplished by the addition of phosphate-conjugated anti-mouse antibodies and *p*-nitrophenyl phosphate (Sigma). The

antibody titer was defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera.

4.6 References

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CHAPTER V

MICROWAVE-ASSISTED SYNTHESIS OF A THREE-COMPONENT CANCER VACCINE CONSISTING OF A SIALYLATED MUC1 GLYCOPEPTIDE AND TOLL-LIKE RECEPTOR 2 LIGAND PAM_3CYSSK_4*

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*__Pamela S. Thompson__, Geert-Jan Boons. To be submitted to *ChemComm*.

5.1 Abstract

A large number of epithelial cancers, such as breast, ovarian, and pancreatic cancers, exhibit striking alterations in the level of expression and glycosylation profile of mucins. The membrane-bound glycoprotein MUC1 is highly overexpressed on the majority of carcinomas and is therefore considered an important target in the development of efficient cancer vaccines. As the complexity of the glycans on the vaccine candidates increases, difficulty arises in their assembly. A synthetic protocol needs to be developed for the synthesis of complex glycolipopeptide vaccine candidates. We report here the efficient synthesis of a glycolipopeptide which is composed of a MUC1 glycopeptide containing the sialyl-Tn antigen, a T-cell epitope derived from the polio virus, and the TLR2 ligand Pam_3CysSK_4 utilizing microwave-assisted solid phase peptide synthesis. A strategically designed sialyl-Tn building block was synthesized in a steroselective manner by exploiting participating solvents in the glycosylation steps. Employing this appropriately protected sialyl-Tn building block during the amino acid coupling steps allowed for the rapid linear construction of the glycolipopeptide cancer vaccine candidate using microwave-assisted solid-phase peptide synthesis.

5.2 Introduction

A large number of epithelial cancers, such as breast, ovarian, and pancreatic cancers, exhibit striking alterations in the level of expression and glycosylation profile of mucins.[1, 2] Mucins are high molecular weight glycoproteins containing numerous *O*-linked carbohydrate side chains such as Tn and STn, where they function as protective barriers and provide lubrication due to their hydration capacity. There are about twenty different mucin glycoproteins that have been identified. MUC1 is a high molecular weight trans-membrane protein with a large and highly glycosylated extracellular domain consisting of a variable number of tandem repeats of twenty amino acids (TAPPHAGVTSAPDTRPAPGS); each repeat has five potential sites for *O*-glycosylation.[3] The membrane-bound glycoprotein MUC1 is highly overexpressed by

the majority of carcinomas and is therefore considered an important target in the development of efficient cancer vaccines.

We have previously shown that a three-component cancer vaccine composed of a tumor-associated carbohydrate B epitope, a promiscuous peptide T-helper epitope, and a Toll-like receptor (TLR) circumvented immune suppression caused by a carrier protein.[4, 5] The exceptional antigenic properties of this three-component vaccine was attributed to the absence of unnecessary features which were antigenic and contained all the mediators required for eliciting relevant IgG immune responses. Attachment of the TLR2 agonist Pam_3CysSK_4[6, 7] to the B- and T-epitopes ensured cytokines were produced at the site where the vaccine interacted with the immune cells. We have also applied this technology toward the synthesis of vaccine candidates to generate monoclonal antibodies specific for *O*-GlcNAc, which led to the identification of more than 200 mammalian *O*-GlcNAc modified proteins.[8]

In our initial attempts, we found that the linear synthesis of these molecules gave products that were difficult to purify to homogeneity. Therefore, the glycolipopeptides were synthesized by solid-phase peptide synthesis (SPPS) combined with *liposome-mediated* native chemical ligation (NCL).[9] Recently, other groups have tried to emulate this vaccine technology, using a glycopeptide covalently attached to Pam_3CysSK_4 as vaccine candidates. However, in their approaches, an unnatural PEG linker was incorporated into the peptide backbone, between the TLR and the glycopeptide epitope.[10-12] There are disadvantages to this: firstly, the PEG linker may be antigenic, and secondly, the presence of an unnatural linker may interfere with antigen/peptide processing.

As the complexity of the glycans on the vaccine candidates increases, difficulty arises in their assembly. A synthetic protocol needs to be developed for the synthesis of complex glycolipopeptide vaccine candidates. There have been many reports suggesting that microwave-assisted synthesis of peptides would reduce reaction times while providing peptides of high purity.[13] We report here the efficient synthesis of a glycolipopeptide that is composed of

a MUC1 glycopeptide containing the sialyl Tn antigen, a T-cell epitope derived from the polio virus,[14] and the TLR2 ligand Pam_3CysSK_4 utilizing microwave-assisted solid phase peptide synthesis (Figure 5.1).

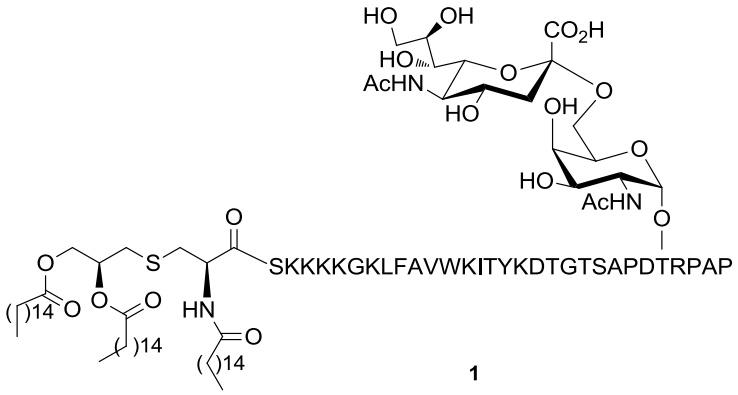

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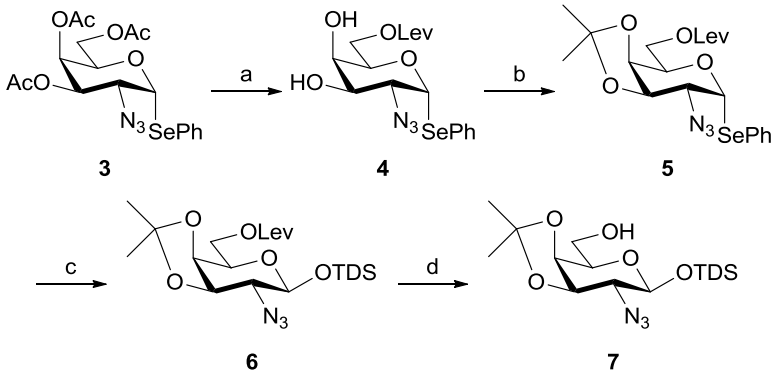
Figure 5.1. Target molecule

5.3 Results and discussion

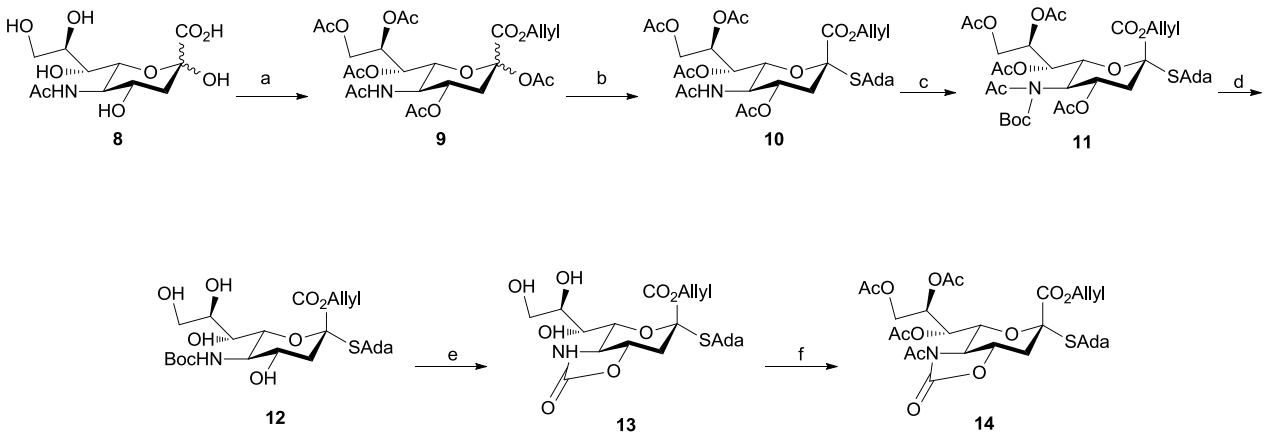
The first challenge we faced was the incorporation of STn into the peptide. The synthesis of sialylated glycopeptides usually involves using sialic acid residues that are protected with a methyl ester. In our initial approach, we were unable to deprotect the methyl ester of glycopeptides without β-elimination of the glycan from the peptide. We therefore turned our focus to preparing sialic acid derivatives which contain an allyl ester, which could easily be removed under mild conditions.

A properly protected sialyl-Tn antigen **2** was designed which would be compatible with Fmoc-based MW-SPPS (Scheme 5.4). The carboxylic acid of sialic acid was protected as an allyl ester, which could be removed in the presence of the palmitoyl esters of Pam_3Cys, while the acetyl moieties protecting the hydroxyls and the acetamido functionality of sialic acid could be removed on resin using hydrazine in methanol prior to installing the Pam_3Cys moiety during MW-SPPS.



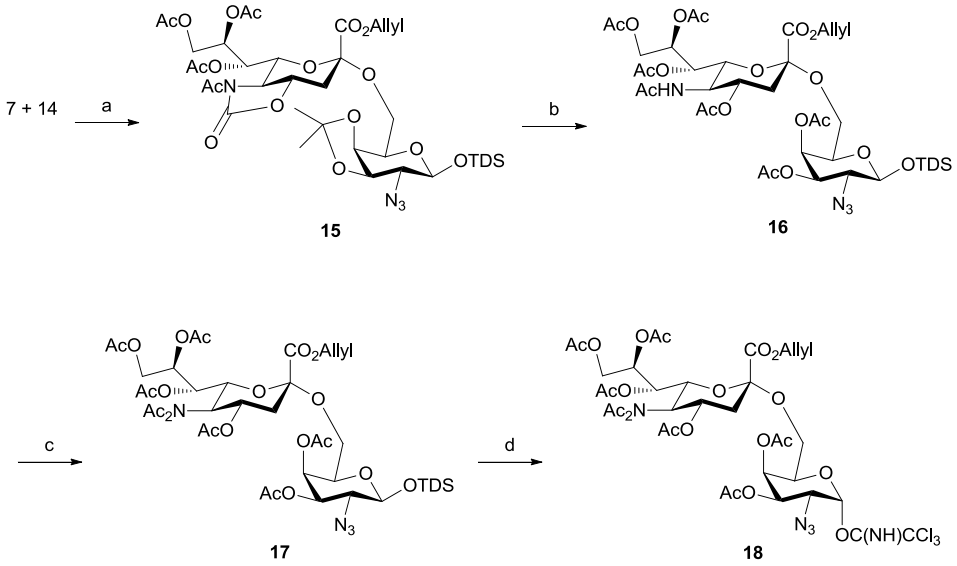
Scheme 5.1. Synthesis of Galactosyl Acceptor 7. (a) i) NaOMe, MeOH, ii) Levulinic acid, 2-chloro-1-methylpyridinium iodide, Et_3N, dioxane, 65% over 2 steps; (b) 2,2-dimethoxypropane, PTSA, 75%; (c) i) $HgCl_2$, $CaCO_3$, ii) TDS-Cl, Imidazole, 71% over 2 steps; (d) Hydrazine acetate, EtOH/Toluene, 99%.

Galactosyl acceptor **7** was prepared to ensure a regioselective glycosylation at the C-6 position. A levulinoyl ester was regioselectively installed on the C-6 hydroxyl,[15] followed by isopropylidene protection of the cis diols. Hydrolysis of the selenophenyl moiety was achieved using mercuric chloride and calcium carbonate, and subsequent installation of an anomeric TDS provided **6**. Finally, removal of the levulinoyl group using hydrazine acetate gave galactosyl acceptor **7** (Scheme 5.1).



Scheme 5.2. Synthesis of Sialyl Donor 14. (a) i) Ac_2O, Pyridine, ii) Cs_2CO_3, AllylBr, DMF, 91% over 2 steps; (b) 1-adamantanethiol, $BF_3{\cdot}Et_2O$, DCM, 84%; (c) Boc_2O, DMAP, THF, 91%; (d) NaOMe, AllylOH, 67%; (e) i) TFA, ii) 4-$NO_2C_6H_4OCOCl$, $NaHCO_3$, H_2O, MeCN, 61% over 2 steps; (f) i) Ac_2O, Pyridine, ii) AcCl, DIPEA, DCM, 80% over 2 steps.

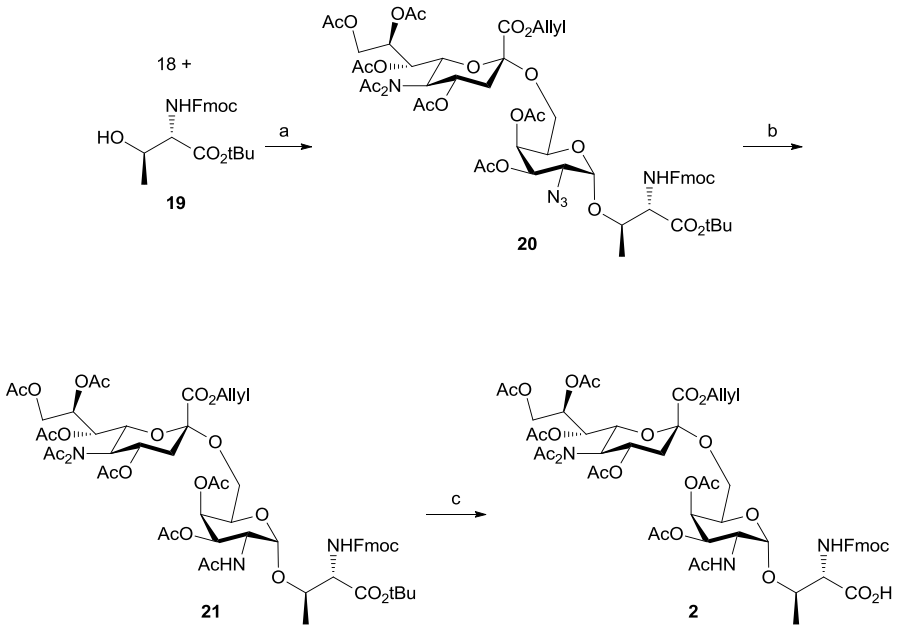
It is well-known that *N*-acetyl-5-*N*-4-*O*-oxazolidinone protected 1-adamantylthio sialosides give excellent yields and α-selectivities in linking various carbohydrates under NIS-TfOH in situ activation conditions.[16, 17] We selected sialyl thioglycoside **14**, with allyl ester protection of the carboxylate moiety (Scheme 5.2). We found that glycosylation of the *N*-acetyl-5-*N*-4-*O*-oxazolidinone donor with galactosyl acceptor **7** under NIS/TfOH activation conditions at -78 ^{o}C in 1:1 DCM/MeCN provided disaccharide **15** in excellent yields with complete α-selectivity.



Scheme 5.3. Synthesis of Disaccharide Donor 18. (a) NIS, TfOH, DCM/MeCN, -78 ^{o}C, 86%; (b) i) NaOMe, AllylOH, ii) 70% AcOH (aq), 70 ^{o}C, iii) Ac_2O, Py, 65% over 3 steps; (c) Isopropenyl Acetate, CSA, 65 ^{o}C, 99%; (d) i) HF/pyridine, THF, ii) CCl_3CN, DBU, 76% over 2 steps.

Cleavage of the oxazolidinone and isopropylidene moieties provided sialyl disaccharide **16**. We found that glycosylation of threonine **19**[18] with a sialyl disaccharide which contains a sialyl N-acetyl moiety resulted in an inseparable α/β mixture. Incorporation of the sialyl-Tn antigen into a glycopeptide for the purposes of vaccine development requires anomerically pure material. Therefore, we explored other functionalities on the sialyl acetamido group. We found that by installing an *N*,*N*-diacetate on sialic acid[19] allowed for glycosylation at 0 ^{o}C in diethyl ether, providing glycosylated amino acid **20** with complete α selectivity. Reductive acetylation of

the galactosyl azide, followed by removal of the tBu protection of the threonine provided the desired properly protected sialyl Tn derivative **2** (Scheme 5.4).



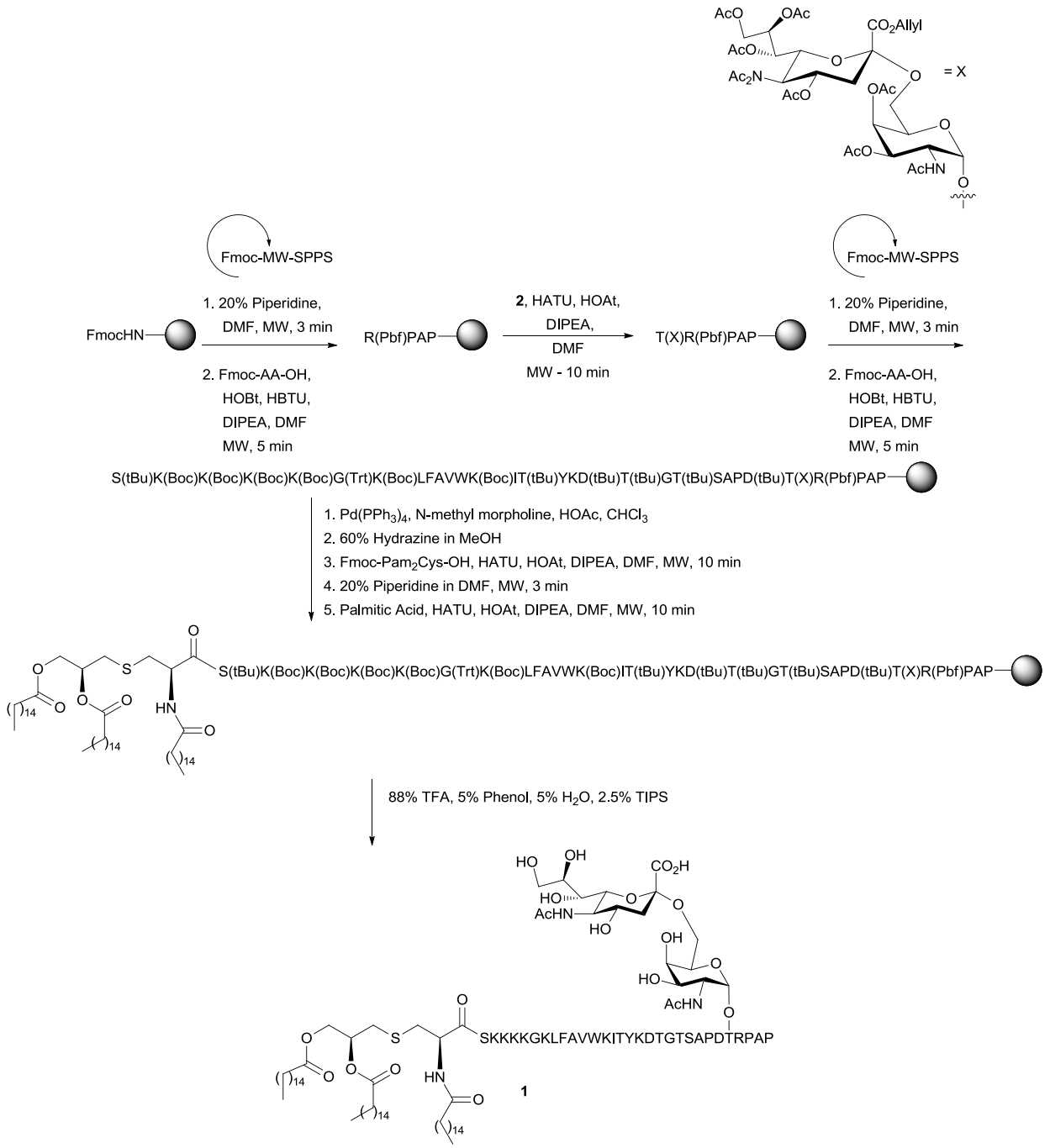
Scheme 5.4. Synthesis of STn antigen 2. (a) TMSOTf, Et_2O, 0 ^oC, 85%; (b) Zn, $CuSO_4$, THF, Ac_2O, AcOH, 65%; (c) TFA/DCM (1/1), 99%.

Glycopeptide **1** was synthesized using MW-SPPS (Scheme 5.5). Using Rink Amide AM LL resin, the first four amino acids were introduced using a CEM Liberty 12-channel automated microwave peptide synthesize, which utilizes an HBTU/HOBt activation protocol. Glycosylated amino acid **2** was introduced manually using an HATU/HOAt activation protocol under microwave irradiation. The resin was then returned to the automated peptide synthesizer to further elongate the peptide. Following the installation of the final serine residue, the resin was removed from the synthesizer and was treated with The resin was then treated with $Pd(PPh_3)_4$, in $CHCl_3$, acetic acid, and *N*-methyl morpholine to remove the allyl ester of sialic acid.[20] Subsequently, 60% hydrazine in methanol was added to the resin to remove the acetyl esters of the disaccharide.[5] The Fmoc-Pam_2Cys and palmitic acid residues were coupled manually using HATU/HOAt in the presence of DIPEA in DMF under microwave irradiation.. The amino acid

side chain deprotection and cleavage from the resin was accomplished using 88% TFA, 5% phenol, 5% H_2O, and 2% TIPS. The glycolipopeptide was then obtained following purification by RP-HPLC using a C4 column.

5.4 Conclusion

In summary, we have successfully exploited microwave-assisted solid-phase peptide synthesis for the linear synthesis of glycolipopeptides for use as cancer vaccine candidates. A sialyl-Tn building block was synthesized in a steroselective manner by exploiting participating solvents in the glycosylations. Employing a properly protected sialyl-Tn building block during the amino acid coupling steps allowed for the linear construction of the vaccine candidate containing the TLR2 ligand Pam_3CysSK_4. Microwave-assisted solid-phase peptide synthesis enabled rapid construction of the glycolipopeptide with high purity. We believe this method could streamline the synthesis of carbohydrate-based cancer vaccine candidates.



Scheme 5.5. Microwave-Assisted SPPS of glycolipopeptide **1**.

5.5 Experimental Procedure

General remarks:

All reactions were carried out under nitrogen with anhydrous solvents, unless otherwise stated. CH_2Cl_2 was distilled from CaH_2 prior to use in each reaction. Chemicals used were reagent

grade as supplied except where noted. *N*-iodosuccinimide was used after recrystallization in 1,4-dioxane/CCl_4. Column chromatography was performed on silica gel G60 (60 – 200 µm 60 Å); reactions were monitored by TLC on Silicagel 60 F_{254}. The compounds were detected by examination under UV light and visualized by charring with cerium ammonium molybdate in 20% sulfuric acid. Solvents were removed under reduced pressure at ≤ 35 ^{o}C. 1H-NMR, gCOSY, and gHSQC spectra were recorded in $CDCl_3$ at 300 MHz or 500 MHz on a Varian Inova spectrometer with tetramethylsilane as an internal standard, unless otherwise stated. ^{13}C-NMR data reported from gHSQC spectra, unless otherwise stated. Reverse Phase HPLC was performed on an Agilent 1100 series system equipped with an autosampler, UV detector, and fraction collector. RP-HPLC was carried out using a Jupiter C4 analytical column (5 µm, 4.6 x 250 mm) at a flow rate of 1 mL/min. (A: 95% Water, 5% Acetonitrile, 0.1% TFA; B: 95% Acetonitrile, 5% Water, 0.1% TFA) High resolution mass spectra were obtained by using MALDI-ToF (Applied Biosystems 5800 Proteomics Analyzer) with 2,5-dihydroxybenzoic acid or α-cyano-4-hydroxycinnamic acid as an internal standard matrix.

General methods for automated microwave-assisted solid-phase peptide synthesis (MW-SPPS): Peptides were synthesized by established protocols on a CEM Liberty Automated Microwave Peptide Synthesizer equipped with a UV detector using *N*-α-Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) as the activating reagents. Deprotection of the N-α-Fmoc was achieved using 20% 4-methyl piperidine in DMF.

General methods for manual MW-SPPS: Peptides were synthesized by established protocols on a CEM Discover SPS Microwave Peptide Synthesizer using *N*-α-Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) as the activating reagents. The couplings of the glycosylated amino acid *N*-α-Fmoc-Thr-(Ac_3-α-D-GalNAc), *N*-α-fluorenylmethoxycarbonyl-*R*-(2,3-

bis(palmitoyloxy)-(2*R*-propyl)-(R)-cysteine, and palmitic acid were carried out using (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HATU). Each manual coupling was monitored by standard Kaiser test. Deprotection of the N-α-Fmoc was achieved using 20% 4-methyl piperidine in DMF.

Phenyl (2-azido-6-*O*-levulinoyl)-1-seleno-α-D-galactopyranoside (4):

Peracetylated galactose azide **3** (13.6 g, 29.1 mmol) was dissolved in methanol (200 mL) and treated with 1M NaOMe/MeOH until pH = 9. The reaction stirred at room temperature for 3 hours. The reaction mixture was neutralized by the addition of acetic acid (1.5 mL). The mixture was concentrated *in vacuo* and dried on a high vacuum pump. The crude compound was dissolved in dioxane (370 mL) with CMPI (13.1 g, 58.1 mmol). Levulinic acid (4.04 g, 34.9 mmol) and triethylamine (24 mL, 174.3 mmol) were added. The reaction stirred at room temperature overnight. The reaction mixture was diluted with EtOAc and washed with sat. aq. $NaHCO_3$ (200 mL x 3) and brine (200 mL). The organic layer was dried ($MgSO_4$), filtered, and evaporated to dryness. The residue was purified by silica gel chromatography (0% → 55% EtOAc in hexanes) to afford compound **4** in 65% yield. Analytical data for **4**: R_f = 0.43 (1:3 hexanes-EtOAc); ^{1}H NMR: δ 7.67 – 7.54 (3 H, m, aromatic), 7.39 – 7.22 (2 H, m, aromatic), 5.97 (1 H, d, *J* 5.3, H-1), 4.46 (2 H, q, *J* 6.7, H-5, H-6$_a$), 4.10 (2 H, td, *J* 8.7, 3.6, H-6$_b$, H-2), 4.02 (1 H, t, *J* 3.3, H-4), 3.83 (1 H, d, *J* 3.1, H-3), 3.02 (1 H, d, *J* 3.6, C4-O*H*), 2.83 – 2.67 (3 H, m, CO_2CH_2, C3-O*H*), 2.58 – 2.50 (2 H, m, CH_2(C=O)), 2.23 – 2.14 (3 H, m, CH_3) ppm. ^{13}C NMR: δ 134.87, 134.83, 134.36, 129.02, 128.85, 84.94 (C-1), 71.04, 80.86, 68.11, 62.47, 61.59, 37.96, 29.72, 27.68 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{17}H_{21}N_3O_6Se$ $[M+Na]^+$ calc 466.0493, found 466.0672.

Phenyl (2-azido-3,4-*O*-isopropylidene-6-*O*-levulinoyl)-1-seleno-α-D-galactopyranoside (5):

Compound **4** (1.7 g, 3.9 mmol) was dissolved in 2,2-dimethoxypropane (20 mL) and *p*-Toluenesulfonic acid (0.078 mmol) was added. The reaction mixture stirred for 6 hours and was then neutralized with triethylamine (0.5 mL). The mixture was concentrated *in vacuo* and purified by silica gel chromatography (0 → 35% EtOAc in hexanes) to afford compound **5** in 65% yield. Analytical data for **5**: R_f = 0.48 (2:1 hexanes-EtOAc); 1H NMR: δ (300 MHz, $CDCl_3$) 7.60 (2 H, dd, *J* 6.5, 3.0, aromatic), 7.37 – 7.18 (3 H, m, aromatic), 5.84 (1 H, d, *J* 5.2, H-1), 4.60 (1 H, d, *J* 2.5, H-5), 4.40 – 4.15 (3 H, m, H-6, H-4, H-3), 3.99 (1 H, dd, *J* 6.7, 5.3, H-2), 2.70 (2 H, t, *J* 6.6, CO_2CH_2), 2.52 (2 H, t, *J* 6.6, CH_2(C=O)), 2.17 (3 H, s, CH_3), 1.52 (3 H, s, C(CH_3)), 1.35 (3 H, s, C(CH_3)) ppm. ^{13}C NMR: δ 134.48, 134.42, 134.36, 128.86, 128.70, 83.10 (C-1), 75.27, 72.69, 69.59, 64.09, 62.55, 38.17, 30.07, 28.21, 28.21, 26.28 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{20}H_{25}N_3O_6Se$ $[M+Na]^+$ calc 506.0806, found 506.1049.

Thexyldimethylsilyl (2-azido-3,4-*O*-isopropylidene-6-*O*-levulinoyl)-α-D-galactopyranoside (6):

To a solution of compound **5** in 10:1 acetonitrile/water was added mercuric chloride and calcium carbonate. The reaction mixture stirred at room temperature overnight and was then concentrated *in vacuo*. The residue was suspended in $CH_2Cl_2$and then filtered. The filtrate was then evaporated to dryness and purified by silica gel column chromatography. The purified hemiacetal was then dissolved in $CH_2Cl_2$and TDS-chloride and imidazole was added to the solution at 0 ^{o}C. The reaction was stirred at room temperature for 3 hours. The reaction mixture was diluted with $CH_2Cl_2$and washed with sat. aq. $NaHCO_3$. The organic layer was dried ($MgSO_4$), evaporated to dryness, and purified by silica gel chromatography (0 → 25% EtOAc in hexanes) to afford **6** in 71% yield. Analytical data for **6**: R_f = 0.60 (2:1 hexanes/EtOAc); ^{1}H NMR: δ (300 MHz, $CDCl_3$) 4.22 (1 H, d, *J* 8.2, H-1), 4.15 (2 H, dd, *J* 6.0, 4.9, H-6), 3.87 (1 H, dd, *J* 5.3, 2.2, H-4), 3.76 – 3.63 (2 H, m, H-5, H-3), 3.10 (1 H, t, *J* 8.1, H-2), 2.62 – 2.52 (2 H, m, C*H*H-

C*H*H), 2.44 – 2.35 (2 H, m, C*H*H-C*H*H), 2.00 (3 H, s, CH_3), 1.55 – 1.40 (1 H, m, SiC$(CH_3)_2$C$(CH_3)_2$C*H*$(CH_3)_2$, 1.35 (6 H, s, C(CH_3)), 1.14 (3 H, s, C(CH_3)), 0.70 (12 H, dd, *J* 4.3, 2.4, SiC$(CH_3)_2$C(C$H_3)_2$CH(C$H_3)_2$), 0.00 (6 H, d, *J* 2.4, SiC(C$H_3)_2$C$(CH_3)_2$CH$(CH_3)_2$) ppm. ^{13}C NMR: δ 96.77 (C-1),77.87, 73.15, 71.38, 68.13, 64.29, 38.13, 30.34, 28.57, 28.57, 26.80, 20.30, -1.31 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{22}H_{39}N_3O_7Si$ $[M+Na]^+$ calc 508.2455, found 508.3337.

Thexyldimethylsilyl (2-azido-3,4-*O*-isopropylidene)-α-D-galactopyranoside (7):

To a solution of **6** (0.6 g, 1.27 mmol) in 2:1 ethanol/toluene (21 mL) was added hydrazine acetate (0.57 g, 6.35 mmol). The reaction stirred at room temperature for 7 hours. The reaction mixture was diluted with ethyl acetate (100 mL) and was washed with sat. aq. $NaHCO_3$ and brine (100 mL). The organic layer was dried ($MgSO_4$), evaporated to dryness, and purified by silica gel chromatography (0 → 25% EtOAc in hexanes) to afford **6** in 99% yield. Analytical data: R_f = 0.57; ^{1}H NMR: δ (300 MHz, $CDCl_3$) 4.24 (1 H, d, *J* 8.1, H-1), 3.88 (1 H, dd, *J* 5.4, 2.1, H-4), 3.73 (2 H, ddd, *J* 13.9, 10.3, 6.9, H-6_a, H-5), 3.66 – 3.54 (2 H, m, H-3, H-6_b), 3.10 (1 H, t, *J* 8.2, H-2), 1.72 (1 H, dd, *J* 9.3, 3.6, C6-O*H*), 1.48 (1 H, dt, *J* 13.7, 6.9, SiC$(CH_3)_2$C$(CH_3)_2$C*H*$(CH_3)_2$), 1.35 (3 H, s, C(CH_3)), 1.14 (3 H, s, C(CH_3)), 0.69 (12 H, dd, *J* 3.8, 3.1, SiC$(CH_3)_2$C(C$H_3)_2$CH(C$H_3)_2$), -0.00 (6 H, s, SiC(C$H_3)_2$C$(CH_3)_2$CH$(CH_3)_2$) ppm. ^{13}C NMR: δ 96.73 (C-1), 77.69, 74.01, 73.48, 67.97, 62.58, 28.43, 26.62, 20.05, -1.43 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{17}H_{33}N_3O_5Si$ $[M+Na]^+$ calc 410.2087, found 410.2278.

Allyl (1-adamantanyl-5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-glycero-α-D-galacto-non-2-ulopyranoside)oate (10):

1-Adamantanethiol (1.05 g, 6.2 mmol) and compound **9** (2.92 g, 5.2 mmol) were dissolved in CH_2Cl_2 (52 mL). $BF_3{\cdot}OEt_2$ (1.6 mL, 13 mmol) was added at room temperature and the reaction mixture stirred for 19 hours. The mixture was diluted with CH_2Cl_2 (50 mL) and was washed with sat. aq. $NaHCO_3$ (100 mL). The organic layer was dried with $MgSO_4$, filtered, and evaporated to

dryness. The residue was purified by silica gel chromatography (0 → 30% acetone in toluene) to afford **10** in 84% yield. Analytical data: R_f = 0.38 (3:1 toluene/acetone); ^{1}H NMR: δ (300 MHz, $CDCl_3$) 6.05 – 5.87 (1 H, m, C*H*=CH_2), 5.49 – 5.12 (5 H, m, CH=CH_2, H-7, H-8, H-4), 4.90 (1 H, dd, *J* 12.3, 1.7, H-9_a), 4.82 – 4.59 (2 H, m, CH_2- CH=CH_2), 4.55 (1 H, dd, *J* 10.5, 2.7, H-6), 4.39 – 4.24 (1 H, m, H-9_b), 4.16 – 4.02 (1 H, m, H-5), 2.54 (1 H, dd, *J* 13.5, 4.7, H-3_a), 2.18 – 1.57 (122 H, m, OAc x 4, NAc, H-3_b, Ada) ppm. ^{13}C NMR δ: 119.81, 74.01, 72.82, 69.48, 69.04, 66.72, 63.45, 49.83, 43.78, 39.92, 36.08, 29.80, 23.14, 20.96, 20.68, 20.67, 20.65 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{32}H_{45}NO_{12}S$ $[M+Na]^+$ calc 690.2560, found 690.3072.

Allyl (1-adamantanyl-5-acetamido-5-*N*-*tert*-butoxycarbonyl-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-glycero-α-D-galacto-non-2-ulopyranoside)oate (11):

To a solution of compound **10** (1.9 g, 2.8 mmol) in THF (70 mL) was added Boc_2O (3.85 mL, 16.8 mmol) and DMAP (68 mg, 0.56 mmol). The reaction mixture was stirred at 60 ^{o}C for 24 hours. The reaction mixture was quenched with methanol and evaporated to dryness. The residue was purified by silica gel chromatography (0 → 20% EtOAc in hexanes) to afford **11** in 91% yield. Analytical data: R_f = 0.28 (3:1 hexanes/EtOAc); $_1$H NMR: δ (300 MHz, $CDCl_3$) 5.96 (1 H, ddd, *J* 11.8, 10.5, 5.3, C*H*=CH_2), 5.64 (1 H, td, *J* 11.1, 4.7, H-4), 5.45 – 5.22 (4 H, m, CH=CH_2, H-6, H-7), 5.15 (1 H, dd, *J* 9.4, 5.1, H-8), 4.87 (1 H, dd, *J* 11.4, 6.8, H-9_a), 4.81 – 4.58 (3 H, m, H-5, CH_2CH=CH_2), 4.31 (1 H, dd, *J* 12.3, 8.9, H-9_b), 2.63 (1 H, dd, *J* 13.5, 4.8, H-3_a), 2.34 (3 H, s, N-COCH_3), 2.20 – 1.79 (23 H, m, H-3_b, OAc x 4, Ada), 1.74 – 1.57 (9 H, m, C(CH_3)$_3$), 1.48 (3 H, dd, *J* 18.2, 12.7, Ada) ppm. ^{13}C NMR δ: 131.31, 119.73, 74.10, 73.72, 73.10, 72.00, 69.33, 66.83, 66.36, 62.8, 53.23, 43.60, 41.72, 35.92, 29.68, 28.01, 26.94, 20.95, 20.81, 20.78, 20.58 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{37}H_{53}NO_{14}S$ $[M+Na]^+$ calc 790.3084, found 790.3265.

Allyl (1-adamantanyl- 5-*N*-*tert*-butoxycarbonyl- 3,5-dideoxy-2-thio-glycero-α-D-galacto-non-2-ulopyranoside)oate (12):

Compound **11** (2.1 g, 2.69 mmol) was dissolved in allyl alcohol (30 mL) and 1M NaOMe was added until pH = 9. The reaction was stirred under vacuum for 4 hours and was quenched neutralized with the addition of acetic acid (0.5 mL). The mixture was concentrated *in vacuo* and the residue was purified by silica gel chromatography (0 → 30% acetone in $CHCl_3$) to afford **12** in 80% yield. Analytical data: R_f = 0.38 (1:1 toluene/acetone); HR-MALDI-ToF/MS: m/z for $C_{27}H_{43}NO_9S$ $[M+Na]^+$ calc 580.2556, found 580.3585.

Allyl (1-adamantanyl-5-*N*-4-*O*-carbonyl-3,5-dideoxy-2-thio-glycero-β-D-galacto-non-2-ulopyranoside)oate (13):

Compound **12** (1.0 g, 1.79 mmol) was stirred in TFA (4 mL) for 1 hour. The mixture was coevaporated with toluene and dried under high vacuum overnight. The residue was dissolved in acetonitrile (8 mL) and water (16 mL) and $NaHCO_3$ (0.75 g, 8.9 mmol) was added. The reaction mixture was cooled to 0 ^oC and 4-nitrophenylchloroformate (0.89 g, 4.45 mmol) was added. The reaction stirred at 0 ^oC for 4 hours. The mixture was washed with EtOAc (100 mL x 3) and the organic layer was washed with brine (50 mL x 2), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (0 → 50% acetone in $CHCl_3$) to afford **13** in 61% yield. Analytical data: R_f = 0.31 (1:1 $CHCl_3$/acetone), ^1H NMR: δ (300 MHz, $CDCl_3$) 6.04 (1 H, s), 6.01 – 5.87 (1 H, m, C*H*=CH_2), 5.37 (2 H, ddd, *J* 13.8, 11.5, 1.2, CH=CH_2), 4.78 (1 H, dd, *J* 13.0, 5.9, *CH*HC H=CH_2), 4.72 – 4.60 (2 H, m *CH*H*C*H=CH_2,H-4), 4.47 (1 H, dd, *J* 9.8, 4.9, H-6), 3.97 – 3.74 (4 H, m, H-9, H-7, H-8), 3.52 (1 H, d, *J* 3.9, C8-O*H*), 3.43 (1 H, t, *J* 10.4, H-5), 3.32 (1 H, d, *J* 3.9, C7-O*H*), 2.74 (1 H, dd, *J* 12.8, 3.6, H-3_a), 2.62 (1 H, d, *J* 6.6, C9-O*H*), 2.17 (1 H, t, *J* 12.7, H-3_b), 1.94 (5 H, m, Ada), 1.64 (10 H, d, *J* 9.5, Ada). ^{13}C NMR: δ (75 MHz) 170.46 (*C*O_2Allyl), 159.70 (N(*C*=O)O, 130.98, 120.21,

86.18, 77.65, 77.43, 77.22, 76.80, 75.68, 72.41, 70.79, 67.26, 63.98, 59.25, 51.32, 43.77, 39.32, 36.16, 30.05. HR-MALDI-ToF/MS: *m*/*z* for $C_{23}H_{33}NO_8S$ $[M+Na]^+$ calc 506.1825, found 506.2570.

Allyl (1-adamantanyl-5-acetamido-7,8,9-tri-*O*-acetyl-5-*N*-4-*O*-carbonyl-3,5-dideoxy-2-thio-glycero-β-D-galacto-non-2-ulopyranoside)oate (14):

Compound **13** (0.53 g, 1.1 mmol) was treated with 2:1 Py/Ac_2O (30 mL) overnight. The mixture was quenched with allyl alcohol. The mixture was co-evaporated with toluene. The crude material was dissolved in CH_2Cl_2 (10 mL) and DIPEA (1.9 mL, 10.9 mmol) was added. The reaction mixture was cooled to 0 ^oC and acetyl chloride (0.62 mL, 8.7 mmol) was added. The reaction stirred at room temperature for 1 hour. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and was washed with sat. aq. $NaHCO_3$ (50 mL x 3). The organic layer was dried ($MgSO_4$), filtered, evaporated to dryness, and purified by silica gel column chromatography (0 →25% EtOAc in hexanes) to afford **14** in 80% yield. Analytical data: R_f = 0.28 (2:1 hexanes/EtOAc); ^1H NMR: δ (500 MHz, $CDCl_3$) 6.05 – 5.90 (1 H, m, C*H*=CH_2), 5.73 (1 H, t, *J* 2.9, H-7), 5.42 (1 H, dd, *J* 17.2, 1.3, CH=C*H*H), 5.33 (2 H, ddd, *J* 10.4, 4.5, 1.8, CH=CH*H*, H-8), 4.74 (5 H, dddd, *J* 22.3, 14.3, 8.9, 2.5, CH_2CH=CH_2, H-9_a, H-4, H-6), 4.23 (1 H, dd, *J* 12.2, 7.9, H-9_b), 3.69 (1 H, dd, *J* 11.3, 9.3, H-5), 2.83 (1 H, dd, *J* 12.8, 3.6, H-3_a), 2.50 (3 H, s, NCOCH_3), 2.20 (1 H, t, *J* 12.8, H-3_b), 2.13 (6 H, d, OAc x 2), 2.08 – 1.97 (6 H, m, OAc, Ada), 1.92 (3 H, d, *J* 11.5, Ada), 1.73 – 1.62 (6 H, m, Ada), 1.56 (4 H, s, Ada) ppm. ^{13}C NMR: δ 131.33, 119.97, 75.69, 74.64, 73.44, 72.37, 66.91, 63.28, 60.48, 43.81, 38.84, 37.56, 29.85, 21.68, 21.11, 20.76 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{31}H_{41}NO_{12}S$ $[M+Na]^+$ calc 674.2247, found 674.580.

Thexyldimethylsilyl [Allyl (5-acetamido-7,8,9-tri-*O*-acetyl-5-*N*-4-*O*-carbonyl-3,5-dideoxy-glycero-α-D-galacto-non-2-ulopyranoside)oate]-(2→6)-*O*- (2-azido-3,4-*O*-isopropylidene)-α-D-galactopyranoside (15):

Sialyl donor **14** (87 mg, 0.13 mmol) and galactosyl acceptor **7** (78 mg, 0.20 mmol) were co-evaporated with toluene and dried overnight under high vacuum. The residue was dissolved in 1:1 acetonitrile/ CH_2Cl_2 (4 mL) and molecular sieves were added. After 2 hours, the mixture was cooled to -78 ^oC and NIS (45 mg, 0.20 mmol) and TfOH (4 µL, 0.04 mmol) were added. The reaction mixture stirred at -78 ^oC in the dark for 30 minutes and was then diluted with CH_2Cl_2 and washed with sat. aq. $NaHCO_3$. The organic layer was dried ($MgSO_4$), filtered, concentrated *in vacuo*, and and purified by silica gel column chromatography (0 → 40% EtOAc in hexanes) to provide **15** in 86% yield. Analytical data: R_f = 0.56 (1:1 hexanes/EtOAc); ^1H NMR: δ (500 MHz, $CDCl_3$) 5.74 (1 H, ddd, *J* 17.1, 6.0, 4.4, C*H*=CH_2), 5.39 (1 H, dd, *J* 7.0, 1.6, H-7'), 5.24 – 5.16 (2 H, m, H-8', CH=C*H*H), 5.14 (1 H, dd, *J* 10.4, 1.0, CH=CH*H*), 4.51 (2 H, qd, *J* 12.8, 6.0, C*H*$_2$CH=CH_2), 4.36 (1 H, dd, *J* 9.4, 1.6, H-6'), 4.29 – 4.13 (1 H, m, H-9_a'), 3.92 (1 H, dd, *J* 12.2, 6.9, H-9_b'), 3.89 – 3.80 (2 H, m, H-4, H-4'), 3.77 (1 H, dd, *J* 10.3, 7.5, H-6_a), 3.74 – 3.61 (2 H, m, H-5, H-3), 3.59 – 3.49 (2 H, m, H-5', H-6_b), 3.08 (1 H, t, *J* 8.1, H-2), 2.70 (1 H, dd, *J* 12.2, 3.6, H-3_a), 2.30 (3 H, d, *J* 6.4, NCOC*H*$_3$), 1.99 – 1.77 (9 H, m, OAc x 3), 1.47 (1 H, dt, *J* 13.7, 6.8, SiC(CH_3)$_2$C(CH_3)$_2$C*H*(CH_3)$_2$), 1.36 (3 H, s, C(C*H*$_3$)), 1.14 (3 H, d, *J* 8.4, C(C*H*$_3$)), 0.75 – 0.66 (12 H, m SiC(CH_3)$_2$C(C*H*$_3$)$_2$CH(C*H*$_3$)$_2$), -0.01 (5 H, dd, *J* 11.2, 5.0, SiC(C*H*$_3$)$_2$C(CH_3)$_2$CH(CH_3)$_2$) ppm. ^{13}C NMR: δ 131.27, 120.71, 97.06 (C-1), 77.55, 95.16, 72.90, 72.41, 72.08, 70.24, 68.00, 67.49, 64.73, 63.43, 36.26, 34.11, 28.66, 26.52, 25.16, 21.69, 21.07, 20.97, 19.53, -2.52 ppm. HR-MALDI-ToF/MS: m/z for $C_{38}H_{58}N_4O_{17}Si$ $[M+Na]^+$ calc 893.3464, found 893.830.

Thexyldimethylsilyl [Allyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-glycero-α-D-galacto-non-2-ulopyranoside)oate]-(2→6)-*O*- (3,4-di-*O*-acetyl-2-azido)-α-D-galactopyranoside (16):

Sialyl disaccharide **15** (330 mg, 0.379 mmol) was dissolved in allyl alcohol (10 mL) and 1M NaOMe was added until pH = 9. The reaction was stirred under vacuum for 3 hours and was neutralized by the addition of acetic acid. The mixture was concentrated *in vacuo* and the residue was dissolved in 70% aq. acetic acid (10 mL) and stirred at 70 ^{o}C for 2 hours. The mixture was co-evaporated with toluene and dried overnight under high vacuum. The residue was then dissolved in 2:1 Py/Ac_2O (6 mL) and stirred for 18 hours. The mixture was quenched with allyl alcohol (3 mL) and was co-evaporated with toluene. The residue was purified by silica gel column chromatography (0 → 100% EtOAc in hexanes) to afford **16** in 65% yield. Analytical data: R_f = 0.52 (100% EtOAc); ^{1}H NMR: δ $_{H}$ (500 MHz, $CDCl_3$) 5.74 – 5.60 (1 H, m, *C*H=CH_2), 5.20 – 4.99 (4 H, m, CH=C*H*$_2$, H-8', H-4), 4.85 (1 H, d, *J* 9.6, N*H*), 4.62 (1 H, dd, *J* 15.0, 7.0, H-4'), 4.55 (1 H, d, *J* 10.9, H-3), 4.49 (1 H, dd, *J* 12.7, 5.8, C*H*HCH=CH_2), 4.40 (2 H, dd, *J* 15.1, 6.7, H-1, CH*H*CH=CH_2), 4.05 (1 H, d, *J* 12.5, H-6_a), 3.83 – 3.70 (3 H, m, H-6_b, H-5, H-5'), 3.56 (2 H, d, *J* 10.8, H-9_a', H-7'), 3.33 (1 H, t, *J* 9.2, H-2), 3.08 (1 H, t, *J* 10.3, H-9_b'), 2.33 (1 H, d, *J* 12.7, H-3_a'), 1.93 (6 H, d, *J* 4.0, Ac x 2), 1.83 (12 H, d, *J* 37.8, Ac x 4), 1.66 (4 H, d, *J* 6.4, Ac, H-3_b'), 1.55 – 1.33 (1 H, m $SiC(CH_3)_2C(CH_3)_2CH(CH_3)_2$), 0.68 (12 H, s, $SiC(CH_3)_2C(CH_3)_2CH(CH_3)_2$), 0.01 (6 H, d, *J* 6.4, $SiC(CH_3)_2C(CH_3)_2CH(CH_3)_2$) ppm. ^{13}C NMR: δ 131.57, 120.53, 97.52 (C-1), 72.45, 72.91, 71.53, 69.00, 67.85, 67.16, 63.94, 63.94, 63.02, 49.90, 38.37, 34.36, 23.52, 21.42, 21.10, 21.03, 20.97, 20.54, 19.64 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{40}H_{62}N_4O_{19}Si$ $[M+Na]^+$ calc 953.3675, found 953.3832.

Thexyldimethylsilyl [Allyl (5-*N*-acetylacetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-glycero-α-D-galacto-non-2-ulopyranoside)oate]-(2→6)-*O*-3,4-di-*O*-acetyl-2-azido-α-D-galactopyranoside (17):

Disaccharide **16** (230 mg, 0.25 mmol) was dissolved in isopropenyl acetate (2.5 mL) and CSA (6 mg, 0.025 mmol) was added. The reaction mixture was heated at 65 ^{o}C overnight. The mixture was neutralized by the addition of triethylamine and was concentrated *in vacuo*. The residue was purified by column chromatography (0 → 65% EtOAc in hexanes) to afford **17** in 99% yield. Analytical data: R_f = 0.55 (1:2 hexanes/EtOAc); ^{1}H NMR: δ (300 MHz, $CDCl_3$) 6.04 – 5.85 (1 H, m, C*H*=CH_2), 5.47 (2 H, ddd, *J* 18.5, 9.2, 3.2, H-4', CH=C*H*H), 5.40 – 5.23 (3 H, m, CH=CH*H*, H-4, H-8'), 5.12 (1 H, dd, *J* 8.5, 1.7, H-7'), 4.92 (1 H, dd, *J* 10.1, 1.7, H-6'), 4.84 – 4.58 (4 H, m, H-3, C*H*$_2$CH=CH_2, H-1), 4.29 (1 H, dd, *J* 12.4, 2.7, H-9$_a$'), 4.12 (2 H, dt, *J* 12.5, 6.8, H-5', H-9$_b$'), 3.87 – 3.73 (2 H, m, H-5, H-6$_a$), 3.60 – 3.38 (2 H, m, H-2, H-6$_b$), 2.74 (1 H, dd, *J* 12.9, 5.1, H-3$_a$'), 2.40 – 2.23 (6 H, m, NAc x 2), 2.22 – 1.93 (18 H, m, OAc x 6), 1.73 (2 H, ddd, *J* 20.5, 13.2, 9.1, H-3$_b$', SiC(CH_3)$_2$C(CH_3)$_2$C*H*(CH_3)$_2$), 0.92 (12 H, d, *J* 5.3, SiC(CH_3)$_2$C(C*H*$_3$)$_2$CH(C*H*$_3$)$_2$), 0.22 (6 H, dd, *J* 7.6, 4.1, SiC(C*H*$_3$)$_2$C(CH_3)$_2$CH(CH_3)$_2$) ppm. ^{13}C NMR: δ 120.07, 97.24 (C-1), 71.88, 71.44, 69.99, 68.42, 67.33, 66.93, 66.91, 66.87, 63.69, 62.94, 62.16, 57.16, 38.94, 34.02, 28.09, 26.07, 21.22, 20.99, 20.92, 20.84, 20.18, 18.72, -1.68 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{42}H_{64}N_4O_{20}Si$ $[M+Na]^+$ calc 995.3781, found 995.2717.

Allyl (5-*N*-acetylacetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-glycero-α-D-galacto-non-2-ulopyranoside)oate-(2→6)-*O*-(3,4-di-*O*-acetyl-2-azido-α-D-galactopyranosyl) trichloroacetimidate (18):

Compound **17** (80 mg, 0.0822 mmol) was dissolved in 3 mL THF and 70% HF/Py was added (0.86 mL, 32.886 mmol). The reaction mixture was stirred at room temperature for 5 hours and was diluted with CH2Cl2 and washed with sat. aq. $NaHCO_3$. The organic layer was dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 (1 mL) and

treated with trichloroacetonitrile (82 µL, 0.822 mmol) and DBU (0.3 µL, 0.00246 mmol) for 2 hours. The reaction mixture was evaporated to dryness and purified by silica gel column chromatography (0 → 70% EtOAc in hexanes) to afford **18** in 76% yield. Analytical data: Rf = 0.54 (1:2 hexanes/EtOAc).

_N_-(9-Fluorenylmethyloxycarbonyl)-_O_-[Allyl (5-_N_-acetylacetamido-4,7,8,9-tetra-_O_-acetyl-3,5-dideoxy-glycero-α-D-galacto-non-2-ulopyranoside)oate-(2→6)-_O_- (3,4-di-_O_-acetyl-2-azido-α-D-galactopyranosyl)]-L-threonine _tert_-butyl ester (20):

Acid-washed molecular sieves were added to a solution of sialyl disaccharide donor **18** (61 mg, 0.0623 mmol) and threonine acceptor **19** (75 mg, 0.188 mmol) in 0.5 mL Et_2O. The mixture was stirred for 1 hour and then cooled to 0 ^oC. TMSOTf (2.3 µL, 0.01251 mmol) was added and the reaction was complete in 15 minutes. The mixture was diluted with $CH_2Cl_2$and was filtered into sat. aq. $NaHCO_3$ (20 mL). The organic layer was dried ($MgSO_4$), filtered, concentrated *in vacuo*, and purified by silica gel column chromatography (0 → 50% EtOAc in hexanes) to afford **20** in 78% yield. Analytical data: R_f = 0.32 (1:1 hexanes/EtOAc); 1H NMR: δ (500 MHz, acetone) 7.95 – 7.82 (8 H, m, aromatic), 7.73 (4 H, dd, *J* 14.6, 8.0, aromatic), 7.49 – 7.25 (4 H, m, aromatic), 6.16 – 5.89 (1 H, m, C*H*=CH_2), 5.58 – 5.23 (6 H, m, H-4', CH=C*H*$_2$, H-4, H-8', H-3), 5.23 – 5.08 (1 H, m, H-7'), 4.91 (2 H, m, H-6'), 4.89 – 4.77 (1 H, m, C*H*HCH=CH_2), 4.77 – 4.57 (1 H, m, CH*H*CH=CH_2), 4.52 – 4.21 (8 H, m, OC*H*CH_3 threonine, H-5, CHC*H*$_2$ Fmoc, H-9$_a$', C*H*CO_2Allyl, C*H*CH_2 Fmoc, H-5'), 4.09 (1 H, ddt, *J* 34.3, 13.4, 6.6, H-9$_b$'), 3.99 – 3.87 (1 H, m, H-6$_a$), 3.82 (3 H, dd, *J* 11.1, 3.3, H-2), 3.48 (1 H, dd, *J* 9.9, 5.4, H-6$_b$), 2.92 – 2.69 (1 H, m, acetone-d^6, H-3$_a$'), 2.35 (6 H, d, *J* 15.7, NAc x 2), 2.20 – 1.89 (18 H, m, OAc x 3), 1.80 (1 H, t, *J* 12.0, H-3$_b$'), 1.62 – 1.44 (9 H, m, C(CH_3)$_3$), 1.44 – 1.22 (3 H, m, CH_3 threonine) ppm. ^{13}C NMR: δ 127.94, 127.25, 125.57, 120.23, 118.80, 99.64 (C-1), 77.20, 70.27, 68.77, 68.69, 68.37, 67.75, 67.48, 66.95, 66.59, 66.57, 63.31, 62.13, 58.25, 56.90, 47.61, 38.77, 27.2, 26.87, 25.27, 20.70, 20.64, 20.32,

20.23, 18.40 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{57}H_{71}N_5O_{24}$ $[M+Na]^+$ calc 1232.4387, found 1232.5597.

_N_-(9-Fluorenylmethyloxycarbonyl)-_O_-[Allyl (5-_N_-acetylacetamido-4,7,8,9-tetra-_O_-acetyl-3,5-dideoxy-glycero-α-D-galacto-non-2-ulopyranoside)oate-(2→6)-_O_- (2-acetamido-3,4-di-_O_-acetyl-α-D-galactopyranosyl)]-L-threonine _tert_-butyl ester (21):

Glycosylated amino acid **20** (180 mg, 0.149 mmol) was dissolved in 3:2:1 THF/Ac_2O/HOAc (3 mL) and treated with Zn (126 mg, 1.93 mmol) and sat. aq. $CuSO_4$ (0.25 mL) for 45 minutes. The mixture was filtered over celite and co-evaporated with toluene. The residue was purified by silica gel column chromatography (0 → 85% EtOAc in hexanes) to afford **21** in 78% yield. Analytical data: R_f = 0.33 (1:3 hexanes/EtOAc) ^{1}H NMR: δ (500 MHz, acetone) 7.80 – 7.64 (2 H, m, aromatic), 7.64 – 7.47 (4 H, m, aromatic), 7.28 (2 H, dd, *J* 26.1, 20.5, aromatic), 5.99 – 5.71 (1 H, m, C*H*=CH_2), 5.47 – 5.28 (1 H, m, H-4'), 5.25 – 5.08 (3 H, m, CH=C*H*$_2$, H-4, H-8'), 5.08 – 4.96 (1 H, m, H-7'), 4.85 – 4.79 (3 H, m, H-6', H-3, H-1), 4.75 – 4.65 (1 H, m, C*H*HCH=CH_2), 4.63 – 4.52 (1 H, m, C*H*HCH=CH_2), 4.46 – 4.18 (6 H, m, CHC*H*$_2$ Fmoc, H-2, OC*H*CH_3 threonine, H-9$_a$'), 4.15 – 3.87 (4 H, m, C*H*CH_2 Fmoc, C*H*CO_2Allyl, H-5, H-9$_b$'), 3.86 – 3.76 (1 H, m, H-6$_a$), 3.49 – 3.31 (1 H, m, H-6$_b$), 2.82 – 2.54 (1 H, m, H-3$_a$'), 2.21 (6 H, d, *J* 16.6, NAc x 2), 2.09 – 1.74 (21 H, m, NAc, OAc x 6), 1.68 (9 H, dd, *J* 20.3, 8.4, H-3$_b$'), 1.48 – 1.29 (9 H, m, C(C*H*$_3$)$_3$), 1.29 – 1.19 (3 H, m, C*H*$_3$ threonine) ppm. ^{13}C NMR: δ 128.03, 127.34, 125.46, 120.39, 119.14, 99.95 (C-1), 76.25, 70.47, 69.96, 68.82, 68.55, 67.98, 67.47, 66.57, 66.53, 66.39, 63.82, 61.75, 59.85, 56.87, 47.46, 47.04, 39.04, 29.51, 27.58, 27.30, 25.43, 22.77, 20.44, 20.42, 20.31, 20.16, 20.01, 19.33 ppm. HR-MALDI-ToF/MS: m/z for $C_{57}H_{73}N_3O_{24}$ $[M+Na]^+$ calc 1248.4587, found 1248.5901.

***N*-(9-Fluorenylmethyloxycarbonyl)-*O*-[Allyl (5-*N*-acetylacetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-glycero-α-D-galacto-non-2-ulopyranoside)oate-(2→6)-*O*- (2-acetamido-3,4-di-*O*-acetyl-α-D-galactopyranosyl)]-L-threonine (2):**

Compound **21** (140 mg, 0.114 mmol) was treated with 1:1 TFA/ CH_2Cl_2 (9 mL) for 3 hours. The mixture was concentrated *in vacuo* and purified by silica gel column chromatography (0 → 20% methanol in CH_2Cl_2) to afford **2** in 99% yield. Analytical data: R_f = 0.8 (20:1 CH_2Cl_2/MeOH); ^{1}H NMR: δ (500 MHz, acetone) 7.88 (2 H, d, *J* 7.5, aromatic), 7.72 (2 H, t, *J* 7.2, aromatic), 7.43 (2 H, t, *J* 7.4, aromatic), 7.35 (2 H, td, *J* 7.4, 2.7, aromatic), 6.72 (1 H, d, *J* 8.6, N*H*), 6.08 – 5.94 (1 H, m, C*H*=CH_2), 5.54 (1 H, td, *J* 10.7, 5.2, H-4'), 5.45 (1 H, d, *J* 17.3, CH=C*H*H), 5.40 – 5.25 (3 H, m, H-4, H-8', CH=CH*H*), 5.17 (1 H, d, *J* 7.8, H-7'), 5.05 (2 H, dd, *J* 8.2, 3.2, H-1, H-3), 4.99 (1 H, d, *J* 10.1, H-6'), 4.85 – 4.76 (1 H, m, CH*H*CH=CH_2), 4.72 (1 H, dd, *J* 13.2, 5.7, CH*H*CH=CH_2), 4.41 (4 H, p, *J* 10.4, H-2, CHCH_2 Fmoc, OC*H*CH_3 threonine), 4.35 – 4.20 (5 H, m, H-9_a', C*H*CH_2 Fmoc, C*H*CO_2Allyl, H-5', H-5), 4.13 (1 H, dd, *J* 12.4, 5.7, H-9_b'), 3.94 (1 H, dd, *J* 10.0, 7.0, H-6_a), 3.46 (1 H, dd, *J* 10.0, 5.6, H-6_b), 3.16 – 2.69 (1 H, m, H-3_a'), 2.34 (6 H, d, *J* 18.6, NAc x 2), 2.09 (6 H, dd, *J* 26.5, 11.1, OAc x 2), 2.00 – 1.85 (15 H, m, NAc, OAc x 4), 1.83 (1 H, dd, *J* 19.2, 7.6, H-3_b'), 1.34 (3 H, d, *J* 6.4, CH_3 threonine) ppm. ^{13}C NMR: δ 131.74, 127.93, 127.80, 127.33, 125.42, 118.87, 99.44 (C-1), 76.59, 70.26, 69.07, 68.96, 68.39, 67.92, 67.67, 66.81, 66.47, 66.28, 63.44, 61.90, 59.07, 57.15, 47.62, 47.47, 38.87, 29.49, 25.22, 25.33, 22.50, 20.55, 20.32, 20.10, 18.23 ppm. HR-MALDI-ToF/MS: *m*/*z* for $C_{53}H_{65}N_5O_{24}$ $[M+Na]^+$ calc 1192.3961, found 1192.6213.

Sialyl Tn-glycolipopeptide (1):

Glycolipopeptide **1** was synthesized using MW-SPPS on Rink Amide AM LL Resin (0.1 mmol) using an automated CEM-Liberty instrument equipped with a UV-detector and a CEM-Discover SPS instrument. Side chain protection was as follows: *N*-α-Fmoc-*O*-*tert*-butyl-Asp-OH, *N*-α-Fmoc-*N*-ε-*tert*-Boc-*L*-lysine, *N*-α-Fmoc-*O*-*tert*-butyl-*L*-serine, *N*-α-Fmoc-*O*-*tert*-butyl-*L*-threonine,

N-α-Fmoc-*O*-*tert*-butyl-*L*-tyrosine. Glycosylated amino acid **2** (157 mg, 0.13 mmol) was dissolved in DMF (2 mL) and HATU (51 mg, 0.13 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min and were added to the resin. The manual microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The peptide was then elongated under MW-SPPS conditions described above until the final serine residue; the remaining steps performed manually. The resin was then treated with $Pd(PPh_3)_4$ (171 mg, 0.15 mmol) in $CHCl_3$/HOAc/NMM (37:2:1, 5 mL) for 3 hours. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 60% hydrazine in methanol for 2 hours. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h. *N*-α-Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (180 mg, 0.2 mmol) was dissolved in DMF (5 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min, and was added to the resin. The microwave-irradiated coupling reaction was monitored by the Kaiser test and was complete after 10 min. Upon completion of the coupling, the *N*-α-Fmoc group was cleaved using 20% 4-methyl piperidine in DMF (5 mL) under microwave irradiation. Palmitic acid (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%; 10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^{o}C, 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycolipopeptide was purified by HPLC on a Jupiter analytical C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over 40 min, and the appropriate fractions were lyophilized to afford **1** (34% based on resin loading capacity). HR-MALDI-ToF/MS: m/z for $C_{225}H_{379}N_{45}O_{60}S$ $[M+2H]^+$ calc 4705.7710, found 4705.939.

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Chapter VI

CONCLUSIONS

The over-expression of oligosaccharides, such as Globo-H, Lewis^Y, and Tn antigens, is a common feature on tumor cells. Traditional cancer vaccine candidates composed of a tumor associated carbohydrate conjugated to a carrier protein (e.g. KLH or BSA) have failed to elicit sufficient titers of IgG antibodies. We have developed fully synthetic three-component vaccine candidates composed of a tumor-associated antigen from MUC1, a promiscuous peptide T-helper epitope derived from the polio virus, and the TLR2 ligand Pam_3CysSK_4. In our initial approach, glycopeptides and lipid thioesters were synthesized by solid-phase peptide synthesis (SPPS), followed by native chemical ligation (NCL) to construct the final vaccine construct. In an effort to improve the synthesis of our cancer vaccine constructs, we have successfully exploited *microwave-assisted* liposome-mediated native chemical ligation to obtain glycolipopeptides for use as cancer vaccine candidates using a highly efficient protocol. The results of our study demonstrate that the incorporation of a lipopeptide thioester and an *N*-terminal cysteine glycopeptide into DPC-liposomes and the use of microwave irradiation greatly facilitates NCL to afford a range of glycolipopeptides. The method described here provides the glycolipopeptide product in high yields after a reaction time of 20 minutes using only 2 equivalents of the expensive peptide thioester reactant.

The mucin MUC1 is typically aberrantly glycosylated by epithelial cancer cells manifested by truncated *O*-linked saccharides. The resultant glycopeptide epitopes can bind cell

surface major histocompatibility complex (MHC) molecules and are susceptible to recognition by cytotoxic T-lymphocytes (CTLs), while aberrantly glycosylated MUC1 protein on the tumor cell surface can be bound by antibodies to mediate antibody-dependent cell-mediated cytotoxicity (ADCC). Given the challenges to immuno-target tumor-associated MUC1, we have identified the minimum requirements to consistently induce CTLs and ADCC-mediating antibodies specific for the tumor form of MUC1 resulting in a therapeutic response in a mouse model of mammary cancer. The vaccine is composed of the immunoadjuvant Pam_3CysSK_4, a peptide T_{helper} epitope derived from the polio virus and an aberrantly glycosylated MUC1 peptide. Covalent linkage of the three components was found to be essential for maximum efficacy. The vaccine produced CTLs, which recognized both glycosylated and nonglycosylated peptides, whereas a similar unglycosylated vaccine gave CTLs which recognized only unglycosylated peptide. Antibodies elicited by the glycosylated tripartite vaccine were significantly more lytic compared to the unglycosylated control. As a result, immunization with the glycosylated tripartite vaccine was superior in tumor prevention. Besides its own aptness as a clinical target, these studies of MUC1 are likely predictive of a covalent linking strategy applicable to many additional tumor-associated antigens.

It has been reported that short *O*-linked glycans, like αGalNAc, on MUC1 tandem repeats remain intact during dendritic cell processing and in the major histocompatibility complex (MHC) class II pathway. As glycosylation of the MUC1 peptides is known to contribute to the extent and site specificity of cathepsin-mediated proteolysis, we proposed that vaccination with constructs which contain aberrantly glycosylated long MUC1 peptide sequences could be utilized to determine the optimal MHC-II binding epitopes to provide peptide or glycopeptide-specific humoral responses. Utilizing *microwave-assisted* liposome-mediated native chemical ligation, it was found that the combined use of microwaves and liposomes greatly increases the reaction rates of ligations of sparingly soluble peptide reactants. There

have been many reports suggesting that microwave-assisted synthesis of peptides reduces reaction times while providing peptides of high purity. We therefore hypothesized that microwave-assisted solid phase peptide synthesis (MW-SPPS) could be utilized for the linear synthesis of the vaccine candidates. We found that vaccine constructs that contain the lipopeptide adjuvant Pam_3CysSK_4 could be efficiently synthesized in a linear fashion utilizing microwave irradiation for the amino acid coupling and *N*-Fmoc deprotection steps in combination with on-resin deacetylation of the acetyl protected αGalNAc residue(s) prior to the installation of the Pam_3Cys residue. We have successfully exploited microwave-assisted solid-phase peptide synthesis for the linear synthesis of a library of glycolipopeptides for use as cancer vaccine candidates. In addition, we have found that that immunization with multicomponent vaccine candidates that contain aberrantly glycosylated long MUC1 peptide sequences and the TLR2 ligand Pam_3CysSK_4 could induce the production of high affinity IgG antibodies in MUC1.Tg mice. Switching from a MMT breast tumor model to a MC-38 colon tumor model proved to be problematic; there was a significant increase in antibody production after the introduction of MC-38 colon tumor cells in all groups, including those immunized with empty liposomes, suggesting that the tumors themselves may be inherently immunogenic.

We have successfully extended MW-SPPS for the linear synthesis of glycolipopeptides containing complex glycans. A sialyl-Tn building block was synthesized in a steroselective manner by exploiting participating solvents in the glycosylations. Employing a properly protected sialyl-Tn building block during the amino acid coupling steps enabled the linear construction of the vaccine candidate containing the TLR2 ligand Pam_3CysSK_4. We found that the vaccine construct could be efficiently synthesized in a linear fashion utilizing microwave irradiation for the amino acid coupling and *N*-Fmoc deprotection steps in combination with on-resin deallylation and subsequent deacetylation of the acetyl protecting groups of the αNeu5Ac-(2,6)-αGalNAc- residue prior to the installation of the Pam_3Cys residue. Microwave-assisted solid-

phase peptide synthesis enabled rapid construction of the glycolipopeptides with high purity. We believe this method could streamline the synthesis of carbohydrate-based cancer vaccine candidates.

With the technology developed in our lab for the efficient synthesis of glycosylated lipopeptides, a vast future lies ahead for the synthesis of carbohydrate-based cancer vaccine candidates. Vaccine constructs can be synthesized in which both the T-helper peptide sequence and tumor-associated carbohydrate antigen are varied. In this manner, a variety of cancer vaccine candidates can be screened for their immunotherapeutic efficacy.