SYNTHESIS AND IMMUNOLOGICAL EVALUATION OF MUC1 GLYCOPEPTIDE-BASED MULTICOMPONENT CANCER VACCINE CANDIDATES

by

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(Under the Direction of Geert-Jan Boons)

ABSTRACT

Cancer is one of the leading causes of deaths worldwide. Cancer results from a number of molecular events that changes the normal functions of the cells. In tumor cells, the natural control system that prevents cell overgrowth and invasion to other tissues are inactivated leading to its spread to other parts of the body. For many cancer types, combination therapy is the best approach, in which surgery or radiation therapy is performed to treat locally confined cancer followed by chemotherapy which kills the tumor cells that have spread to other parts of the body. However, one of the major drawbacks of these treatments is that they affect healthy cells along with cancer cells. Due to the recent advances in the field of cancer biology and immunology, new strategies such as hormone therapy, stem cell transplant and immunotherapy have evolved as alternative treatments. The mucin MUC1 is a large glycoprotein that is expressed on the apical surface of normal epithelial cells and highly over-expressed in various tumors, including colon, ovarian, and breast cancers. Therefore, MUC1 is an important target for the development of antitumor vaccines. A predominant characteristic of MUC1 is the VNTR region in its extracellular domain. The VNTR region is comprised of repeating units of a 20-amino

acid sequence (APGSTAPPAHGVTSAPDTRP). In healthy epithelia, the VNTR is highly glycosylated on serine and threonine with long and branched O-linked carbohydrates while the cancerous cells express short and truncated TACAs such as Tn and STn. In this presented study we have reported multicomponent full-length MUC1 based cancer vaccine candidates that are capable of inducing humoral and cellular immunity against the tumor-associated MUC1, producing CTLs and ADCC-mediating antibodies. These vaccine candidates consist of a full-length MUC1 glycopeptide bearing one or more Tn antigens and contain immunodominant peptide motifs (PDTRP and PPAHGV) of MUC1 and a built-in immunoadjuvant, TLR2 ligand Pam_3CysSK_4. In this research, we have successfully utilized the highly efficient microwave-assisted solid phase synthesis protocols for linear synthesis of mono-and multi-glycosylated vaccine candidates. However, immunological evaluation of some of the cancer vaccine candidates is still underway.

INDEX WORDS: cancer, vaccine, MUC1, VNTR, peptide, glycopeptide, glycolipopeptide, microwave, TLR2, NOD2, PGN, multicomponent, monoglycosylated, pentaglycosylated.

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DEDICATION

I would like to dedicate my thesis to my beloved parents, my lovely wife, and my sweet daughter.

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ABBREVIATIONS

Å	Angstrom
Ac	Acetyl
AcOH	Acetic acid
Ac_2O	Acetic anhydride
ACN	Acetonitrile
ADCC	Antibody-dependent cell-mediated cytotoxicity
$CHCl_3$	Chloroform
APC	Antigen-presenting cell
Ar	Aromatic
BF_3•Et_2O	Borontrifluoride diethyletherate
Bn	Benzyl
Boc	tert-Butyloxycarbony
BSA	Bovine serum albumin
Bz	Benzoyl
CDC	Complement-dependent cytotoxicity
CFA	Complete Freund's adjuvant
CpG-ODN	Phosphate-guanine-containing oligodeoxynucleotide
CSA	Camphorsulfonic acid
CTL	Cytotoxic T-lymphocyte
$CuSO_4$	Copper sulfate

d	Doublet
Da	Dalton
DC	Dendritic cell
DCM	Dichloromethane
DDQ	2,3-Dicyano-5,6-dichloro quinone
DIPEA	N,N-Diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMF	N,N-Dimethylformamide
DNA	Deoxyribonucleic acid
EL	Empty liposomes
ELISA	Enzyme-linked immunosorbent assay
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethyl alcohol
Et_3SiH	Triethylsilane
Fmoc	Fluorenylmethyloxycarbonyl
GlcNAc	N-Acetyl glucosamine
Hr	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
HF	Hydrogen fluoride

HLA	Human leukocyte antigen
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
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IL	Interleukin
KLH	Keyhole limpet hemocyanin
Lex	Lewisx
Ley	Lewisy
LPS	Lipopolysaccharide
Min	Minutes
m/z	Mass to charge ratio
MAb	Monoclonal antibody
MAG	Multi-antigenic glycopeptide
MDP	Muramyl dipeptide
MeOH	Methanol
MESNa	Sodium 2-mercaptoethanesulfonate
MHC	Major histocompatibility complex

MI	Maleimide
mM	Millimolar
µM	Micromolar
mmol	Millimole
MMT	Mouse mammary tumor
MS	Molecular sieves
MUC1	Mucin 1
MurNAc	*N*-acetylmuramic acid
MW	Microwave
MW-SPPS	Microwave-assisted solid-phase peptide synthesis
NaH	Sodium hydride
NAP	Naphthyl
NCI	National Cancer Institute
NCL	Native chemical ligation
NH_2NH_2	Hydrazine
NIS	N-Iodosuccinimide
NK	Natural killer
NLR	NOD like receptors
NMP	N-methyl pyrrolidone
NMR	Nuclear Magnetic Resonance
NOD	Nucleotide-binding oligomerization domain
Pam	Palmitoyl
PAMP	Pathogen-associated molecular pattern

PEG	Polyethylene glycol
PGN	Peptidoglycan
q	Quartet
Rf	Retention factor
RNA	Ribonucleic acid
RP-HPLC	Reversed-phase high performance liquid chromatography
rt	Room temperature
s	Singlet
sLex	Sialyl Lewisx
SPPS	Solid-phase peptide synthesis
STn	Sialyl-Tn
SUV	Small unilamellar vesicles
t	Triplet
TACA	Tumor-associated carbohydrate antigen
TBAF	Tetrabutyl ammoniumfluoride
TBTA	Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
t-BuOH	tert-Butyl alcohol
TDS	Thexyl dimethyl silyl
TEA	Triethylamine
TeNT	Tetanus neurotoxin
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	Triethylsilyl trifluoromethanesulfonate
TNF	Tumor nerosis factor
TT	Tetanus toxoid
Troc	Trichloroethoxy carbonyl
UV	Ultraviolet
VNTR	Variable number of tandem repeat units

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction to Cancer

Cancer is a group of over 100 diseases involving uncontrolled growth of abnormal cells with very high potential to spread to other body parts. Untreated cancers can cause serious illness and may lead to the death of the individual. In humans, more than two hundred various kinds of cancers have been known that affect over sixty organs and claiming millions of lives worldwide.[1] In recent times, cancer is one of the major public health problems worldwide. Based on the GOBOCAN report, about 14.1 million new cancer cases and 8.2 million deaths occurred worldwide in 2012.[2] It is one of the deadliest diseases and continues to be on the list of top five diseases with highest mortality rate. According to the American Cancer Society, in the United States 1,658,370 new cancer cases are expected to be diagnosed, out of which 589,430 patients are expected to die in 2015.[3] Currently, cancer is the second most common cause of death in the US after heart diseases, causing the deaths of about 1,620 people per day and is expected to become number one within next few years.[4] The significant increase in a number of cancer patients every year has propelled the interest of scientists to search for new therapies to treat a variety of cancers. As a result, many advances have been made over the last two decades towards the diagnosis and treatment of this deadly disease.[5-11]

1.2 Cancer and Immune System

Traditional treatment for cancer includes surgery, radiation therapy, and chemotherapy. Recently, a combination of two or more therapies, in which surgical removal of the tumor cells often supplemented with other treatments, such as radiation and chemotherapy, have become more popular. However, these treatments are frequently nonspecific and adversely affect normal cells, resulting in many unwanted side effects. The conceptual and technical advances in the field of biology, especially in immunology, have led to a new understanding of cellular and molecular interactions of the immune system and cancer. Due to these advancements, novel strategies such as checkpoint inhibitor therapy, gene therapy, and immunotherapy have evolved as alternative treatments.[12-17]

Immunotherapy takes advantage of the body's immune system to kill tumor cells. In 1909, Paul Ehrlich first proposed that the body's immune system plays a pivotal role in detecting and killing tumor cells in early stages.[18] About five decades later, two scientists, Lewis Thomas and Frank MacFarlane Burnet hypothesized that a particular type of immune cells called T cells were vital in the immune system's response against cancer.[19] This explanation headed to the concept of "immune surveillance" or "immunosurveillance". The idea of immunosurveillance continued to be a debated issue until very recently. In 2001, Schreiber and his colleagues provided a breakthrough by presenting experimental evidence showing that the immune system has the ability to prevent cancer cells from developing and thus generating substantial protection against cancer.[20] Since then, many research findings have provided a detailed understanding of cellular and molecular interplays between the immune system and a tumor.[21] However,

generating a robust immune response against the tumor is difficult because tumor cells have found a number of ways to trick the body's immune system. More specifically, tumor cells can release special molecules which can compromise the immune system's ability to attack growing tumor cells. Thus, the tumor cells become invisible to the cells of immune system, which leads to spreading to other organs.[22] Scientific findings over the past decade have proven that efficient crosstalk between body's innate and adaptive arm of immune system is critical for activating various signaling pathways and thereby generating strong immunological memory against growing cancer cells.[23-24] As such, immunotherapy has been shown effective in recent years. For example, administration of antibodies from an external source is believed to mediate cancer cell progression as shown in following figure 1.1. In recent past, the US Food and Drug Administration (FDA) have approved many monoclonal antibodies (mAbs) to treat various types of cancers. For example, alemtuzumab (Campath®) for treatment of chronic lymphocytic leukemia (CLL), trastuzumab (Herceptin®) for treatment of breast cancers, and Rituxan® for the treatment of non-Hodgkin's lymphoma.[25]

1.2.1 Cancer Vaccines

Cancer vaccine immunotherapies against existing cancer include active and passive immunomodulatory approaches. Active immunotherapy mainly enhances the ability of the patient's immune system to generate strong immune response against the tumor's specific antigens and eradicate the cancerous cells. Passive immunotherapy involves the administration of components produced outside of patient's body such as lymphocytes or antibodies to initiate an immune response. On the other hand, the immunomodulatory approach is unspecific and involves global stimulation of the innate

immune system.[26] These immunomodulatory agents increase the body's immune responsiveness and are intended to amplify anticancer immunity.[27]

Active immunotherapies have been in clinical use for a long time. The fact that the body's own immune system can be exploited by vaccination to eradicate tumor cells has been demonstrated in animal models and under clinical trials in human.[28-29] Malignant cells continuously undergo mutations and are very unstable genetically, giving rise to multiple changes in the repertoire of epitope they present. This way tumor cells trick the immune system by becoming less "visible" and prevent attack by T lymphocytes.

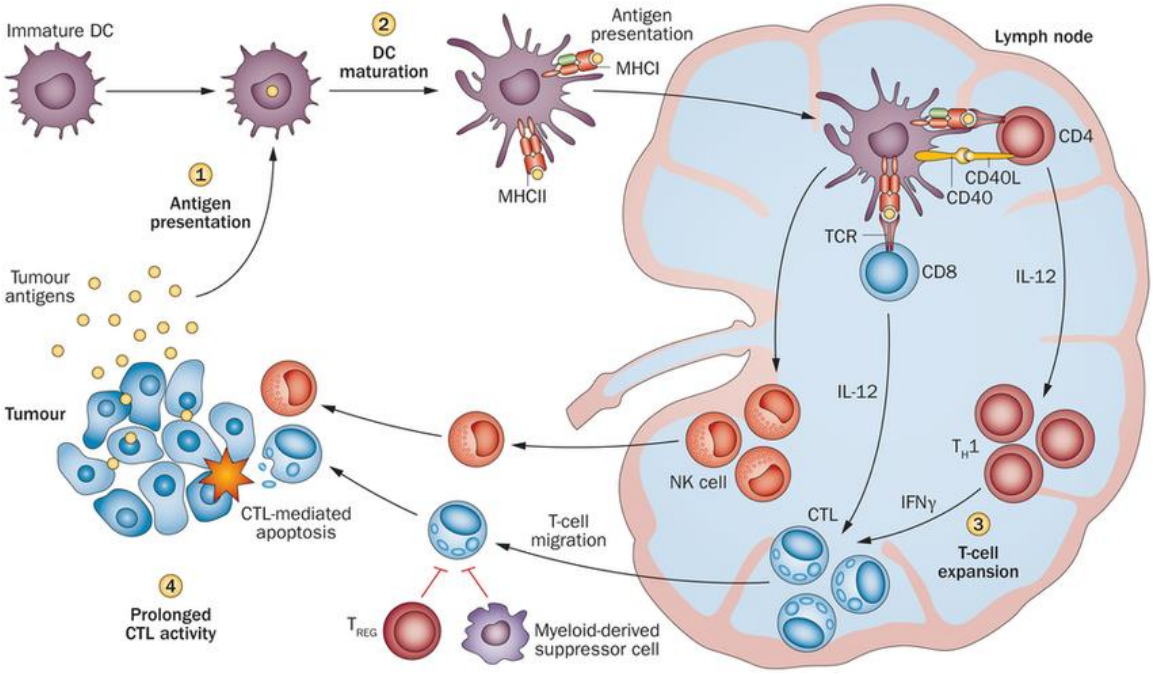


Figure 1.1. Steps in the development of a cellular immune response against TAA

The mechanisms required for a tumor to be visible to the immune system, thus triggering an effective antitumor response, is shown in figure 1.1.[30] There are multiple steps and processes involved in the initiation and augmentation of an immune response

against the tumor cells. The immune system has the ability to recognize tumor-specific antigens with help from a special type of cells known as antigen-presenting cells (APCs), for example, dendritic cells (DCs). Dendritic cells play a very critical role in mounting T-cell specific responses against cancer cells. In the very first step, DCs capture, process, and present tumor-associated antigens (TAAs) that are directly presented by malignant cells to T lymphocytes. In the second step, after the introduction to TAAs, DCs differentiate and migrate to lymph nodes and present TAAs to naïve T cells. This activation of DCs takes place in the presence of suitable activation and/or maturation signals through a number of co-stimulatory protein molecules such as CD40L and various adjuvants. In the third step, expansion of T cells takes place in sufficient numbers so as to identify and eliminate malignant cells. Finally, these activated T cells migrate from lymph node to tumor site and persist long enough to kill cancerous cells.

The innate immune system also complements the adaptive immune system by stimulation and presentation of antigens to B cells and T cells. As opposed to the innate immunity, B-and-T- lymphocytes have the ability to develop an immunological memory that has been exploited in the development of cancer immunotherapy.[19, 31] When T- cells gets activated by tumor antigen presented by antigen-presenting cells such as DCs, they produce certain cytokines and chemokines leading to lysis of tumor cells. Whereas, B-cells differentiate into antibody generating plasma cells that facilitate the death of tumor cells by antibody-dependent cell mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) performed by natural killer (NK) cells.[32] Besides NK cells are important as they also have the ability to kill cancer cells directly.

Over the years, vaccination has considerably reduced the burden of many infectious diseases such as measles and chicken pox. More recently, cancer vaccines are also emerging as an effective anti-cancer treatment.[14, 33-35] Currently US FDA have approved, only two prophylactic anti-cancer vaccines to protect the individual against cancers caused by viruses. In 1981, FDA approved the first cancer preventive vaccine that protects against hepatitis B virus (HBV), which can lead to certain liver cancers.[36] Engerix-B® (GlaxoSmithKline Biologics) and Twinrix® (Rixensart, Belgium) have been approved as preventive vaccines against HBV and are shown to have long-term effects.[37-38] There are certain cancers caused by some strains of the human papillomavirus (HPV) that have been associated with cervical, throat and anal cancers. Gardasil® (Merck and Company) and Cervarix® (GlaxoSmithKline Biologics) have been used to prevent cancers associated with HPV types 16 and 18 that cause approximately 70 % of total cases of cervical cancer worldwide.[39-40] In addition, Gardasil® is effective against HPV types 6 and 11, which are account for about 90 % of total cases of genital warts in males and females, but do not lead to cervical cancer. [41-42]

Alternative to preventative vaccines, therapeutic vaccines are designed to treat cancers that have already developed in the body.[29, 34] The main purposes of such vaccines is to delay or stop tumor cell growth, decrease tumor size, or to eradicate cancerous cells that have escaped other forms of cancer treatment such as surgery and radiation therapy. For developing effective therapeutic cancer vaccine, a detailed understanding of interactions between cancer cells and immune cells is critical. Most of the time, the immune system fails to recognize cancer cells as foreign and thereby does not mount a strong attack against such tumor cells.

There are several factors that make it difficult for the immune system to distinguish cancer cells from healthy cells and attack them. One of the most important factors is that tumor cells express normal self-antigens along with TAAs. Also tumor cells sometimes undergo genetic mutations leading to loss of TACs. Moreover, cancer cells can elicit specific chemical signals, which can suppress immune response by NK cells. Eventually, even if the immune system recognizes invading cancer as a danger, cancer can still evade attack by the immune system.

Due to these facts, developing effective therapeutic anti-cancer vaccines is more difficult and challenging compared to prophylactic cancer vaccines.[43] Effective cancer vaccines must meet two criteria. First, it must stimulate a specific immune response against the correct target (*i.e.* tumor antigen). Second, the immune system must elicit a very strong immune response to overcome barriers produced by cancer cells to protect themselves from attack by B cells and killer T cells. The recent developments in immunology about interplay between immune cells and cancer cells are helping scientists to design a better therapeutic vaccine that achieves both the goals.[44-45]

The identification and isolation of various TAAs have provided major breakthroughs in the field of therapeutic vaccines. In 2010, the US FDA approved the first cancer treatment vaccine, Sipuleucel-T (Provenge®, manufactured by Dendreon) to be used in patients with metastatic prostate cancer.[46-47] This vaccine is designed to mount a strong immune response against prostatic acid phosphatase (PAP), an antigen overexpressed in prostate cancer cells. Interest in therapeutic cancer vaccine is growing since approval of Sipuleucel-T and currently several therapeutic cancer vaccines are in clinical trials.[48-50]

As over or under expression of the TAAs has a direct correlation to cancer progression, identification of the correct antigenic target is critical in vaccine development. Aberrant glycosylation pattern on the cell surface is directly associated with a number of diseases including cancers. [51-54] Cancer cells show abnormal cell surface glycosylation pattern. They have a tendency to overexpress certain unusual glycans, short and truncated versions of carbohydrates, increased level of *N*- and *O*-linked glycoproteins as well as an elevated level of sialylation on the cell surface glyco-lipids. These abnormal and truncated glycosylation patterns also expose parts of the peptide core that are protected in the native forms so that they become more reachable to the cells of the immune system.Also there are several tumor-associated carbohydrate antigens (TACAs) bound glycopeptides which are released into body fluids by cancer cells making them attractive targets for therapeutic cancer vaccines development.[51, 55-61]

1.3 Cell-Surface Carbohydrates

Carbohydrates play a variety of important roles in many cellular events ranging from structural to signaling and recognition.[62] These various biological processes require interactions between cells and the surrounding matrix, which are often mediated by complex carbohydrates.[63] Almost all cells are surrounded by covalently linked monosaccharides and/or oligosaccharides.[64] The carbohydrates commonly found on the cell surface are present in their conjugate forms mostly with proteins or lipids and are known as glycoproteins and glycolipids respectively.[52] In glycoproteins, protein is decorated with one or more sugar molecules covalently linked to the polypeptide backbone. The glycans found on the cell surface are two major types; *N*- and *O*- linked. An *N*-linked oligosaccharide/glycan is a sugar chain covalently linked to an asparagine

amino acid (β-linked) in the polypeptide chain. *N*-glycan commonly contains *N*-acetylglucosamine (GlcNAc) residue and consensus peptide sequence: Asn-X-Ser/Thr, where X can be any amino acid except proline. Similarly, *O*-linked glycans are frequently connected to the polypeptide backbone via a α-linked *N*-acetylgalactosamine (GalNAc) mostly to the hydroxy group of serine and threonine or sometimes hydroxyproline amino acid. On the other hand, in lipid-based glycans, glycosphingolipids (GSLs) are the components of the

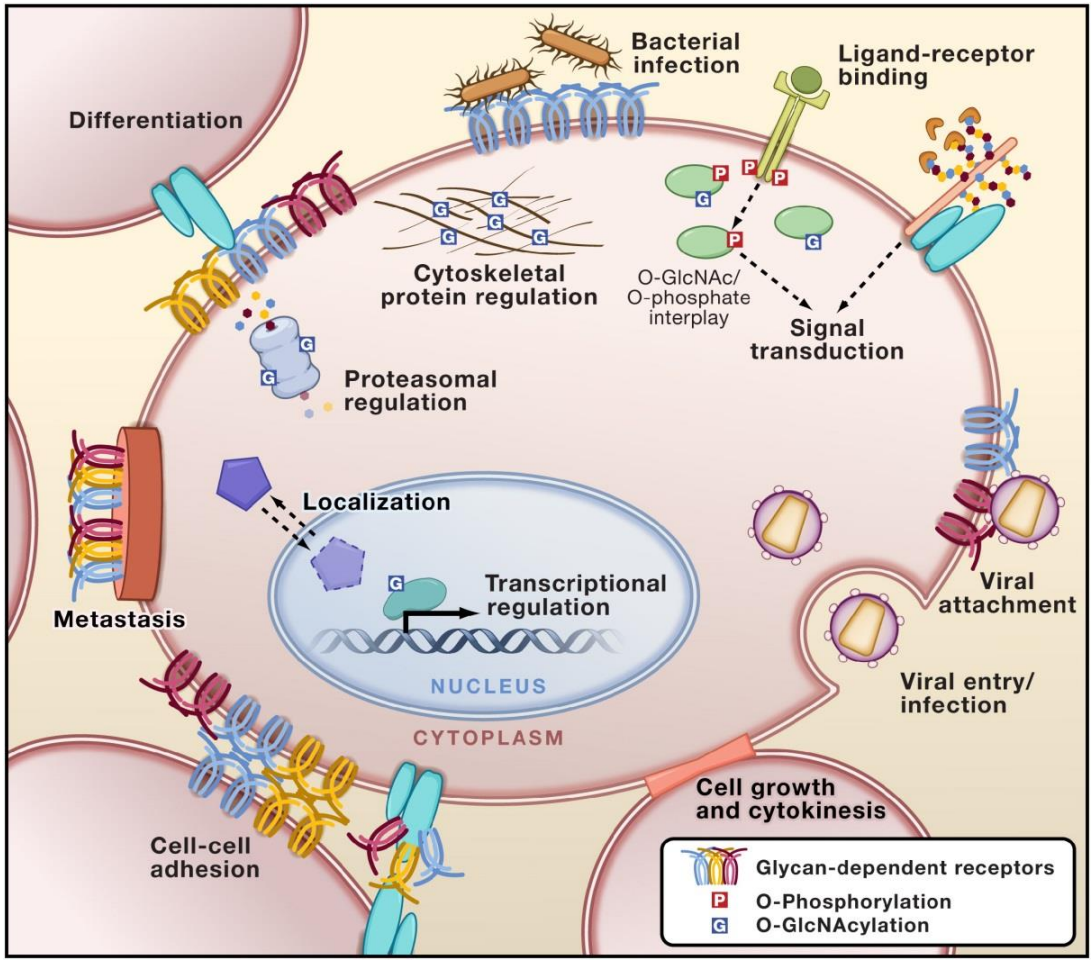


Figure 1.2. Role of Glycans in Cellular Biology[68]

plasma membranes of all eukaryotic cells. So far, approximately 300 different structures of GSL have been identified. In GSL, a glycan core is usually linked via glucose or

galactose to the terminal hydroxyl group of ceramide or sphingoid long chain aliphatic amino alcohols. One characteristic feature of the glycolipids is that the lipid tail is anchored into the hydrophobic cell membrane exposing glycan portion to the extracellular environment.[65-67]

Most of these glycans are on the outer surface of cells and play a crucial role in various biological processes. The biological function of the carbohydrates in cell-cell adhesion and communication, cell differentiation, and fertilization is well documented (Figure 1.2).[68] In addition, the role of glycans in disease is of immense importance due to their role in the intrusion and attachment of various pathogens like bacteria and viruses, blood group immunology, cancer metastasis and other diseases. Hence, to develop new cancer therapies and anti-vaccines, understanding role of these carbohydrates in particular biological processes and disease is critical.[69-70]

1.4 Tumor-associated carbohydrate antigens (TACAs)

Cell surface carbohydrate domains of glycoconjugates are synthesized and dictated by a series of highly organized glycosyltransferases and glycosidases in the endoplasmic reticulum and Golgi apparatus.[59] Any defects in this glycosylation machinery lead to altered glycan patterns, which are used as diagnostic and prognostic indicators for numerous diseases including cancer.[60, 71] Thus, glycosylation changes that occur in cancer lead to the expression of tumor-associated carbohydrate antigens.[55, 62-63, 72] Glycoconjugates such as glycoproteins or glycolipids on such tumor cell surfaces, exhibit abnormalities in both structure and distribution and play a critical role in tumor progression.[73] Glycolipid tumor-associated carbohydrates can be divided into three major subtypes: (i) gangliosides which include GD2, GD3, fucosyl GM1,GM2, GM3; (ii)

Globo subtype include Globo-H, Gb3, Gb4 and Gb5; (iii) lacto or Lewis antigen subtype contain sialyl Lewis^{a} (SLe^{a}), SLe^{x}-Le^{x} and Le^{y}. On the other hand, glycoprotein bound tumor-associated antigens contain the truncated Tn antigen, TF antigen, sialylated Tn antigen (STn) and Globo-H and Le^{y} (Figure 1.3).[52, 74-75]

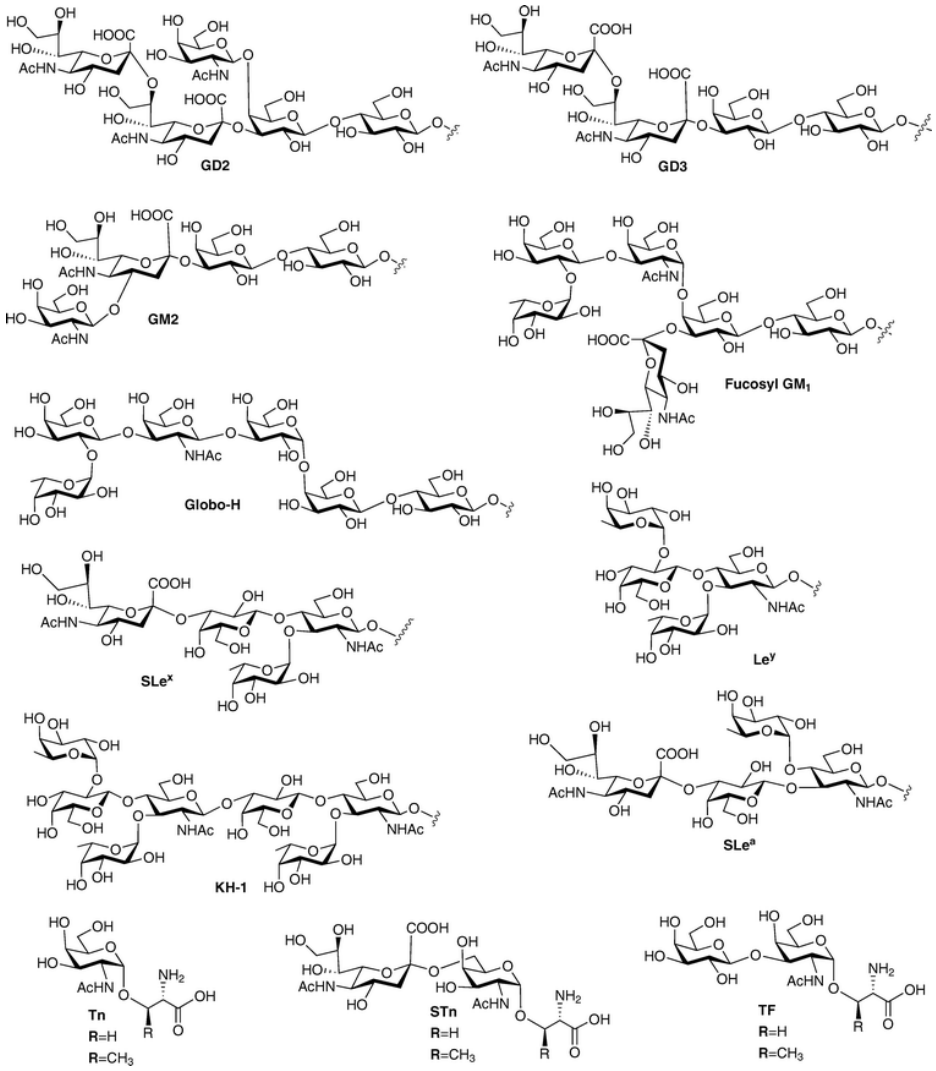


Figure1.3. Structures of Tumor-associated carbohydrate antigens

The tumor-associated gangliosides are overexpressed in various melanomas and in other cancer types and are important targets in cancer research due to their role in tumor growth and spread.[76] GM2 found on melanoma and lung cancer is shown to have a

role in cellular interactions and adhesion during tumor growth. GD2 is associated with neuroblastoma and functions as an attachment motif for a tumor cell to matrices. GD3, which plays a role in the regulation of cell growth angiogenesis during tumor progression, is a diagnostic indicator of melanoma and small cell lung cancer. Similarly, fucosyl GM1 is also associated with small-cell lung cancer. In globo series, Globo H is a hexasaccharide that is highly expressed on various types of cancers, specifically in breast, prostate and lung cancers.[77-78] Numerous tumor-associated glycolipids act as adhesion molecules in normal cell function, but they also aid tumor cell invasion and metastasis. For example, Lewis antigen series contain sialyl Lewis^{a} (SLe^{a}), SLe^{x}, SLe^{x}-Le^{x} and Le^{y} has been found to be the human TAAs (Figure 1.3). The Le^{y} is a tetrasaccharide and found in high levels on carcinomas such as colon, breast, lung and prostate cancers. The heterodimeric form of Le^{x}-Le^{y}, KH-1 is a heptasaccharide which is found on cell surface of colonic adenocarcinoma and has not been found in normal colon tissue, thus it serves as an important biomarker for malignancies.[75, 79-80]

The glycoprotein bound antigens such as Tn, STn, and TF antigens arises due to incomplete *O*-glycan synthesis. Recently, it been found that the loss of expression of active T-synthase/Cosmc can lead to overexpression of the Tn antigen and its sialylated version, STn antigen, is observed in several carcinomas.[54, 71, 81] Such antigens are commonly not found on normal cells surfaces but are shown to be immunoreactive in numerous carcinomas, hence serving as an important target in cancer vaccine research. For example, Mucin1 (MUC1)-a member of the family of epithelial high molecular weight glycoproteins (Mucin), is found overexpressed in breast carcinomas and serves as diagnostic tool for breast cancer.[82-85]

1.5. Mucins

Mucins (MUC), are a family of large, heavily glycosylated proteins with a complex molecular organization. To date, 21 mucin genes have been identified. These mucins are classified into two subtypes based on their structural organization: secreted mucins and membrane-bound mucins.[86] Mucins can be either *O*-glycosylated or *N*-glycosylated and are characterized by a variable number of tandem repeats (VNTR), termed as mucin domain. The tandem repeats of the mucins contain large number of a serine, threonine, and proline amino acid residues with 8 to 23 amino acids per repeat. Both of these mucin subclasses play a role in epithelial cell homeostasis by acting as a protective layer against the entry for toxins and pathogenic microbes.[87]

Mucins play an essential role in cancer pathogenesis, and aberrant expression of the mucins is observed in various malignancies.[88] For example, MUC1 is associated with breast cancer pathogenesis as it affects a number of signaling pathways that influence tumor metastasis. The C-terminal subunit of MUC1 (MUC1-C) acts as an oncoprotein via its interaction with tyrosine kinases receptors such as EGFR and ErbB2, which results in activation of PI3K-Akt and MEK-ERK signaling pathways in breast cancer.[89] In addition, increased expression of MUC1 glycoprotein is also found in pancreatic and ovarian cancers.[90-91] Several studies implicate that another member of transmembrane mucin glycoproteins, MUC4, is involved in several aspects of tumor progression such as metastasis, evasion of apoptosis, and even in drug resistance induction.[92]

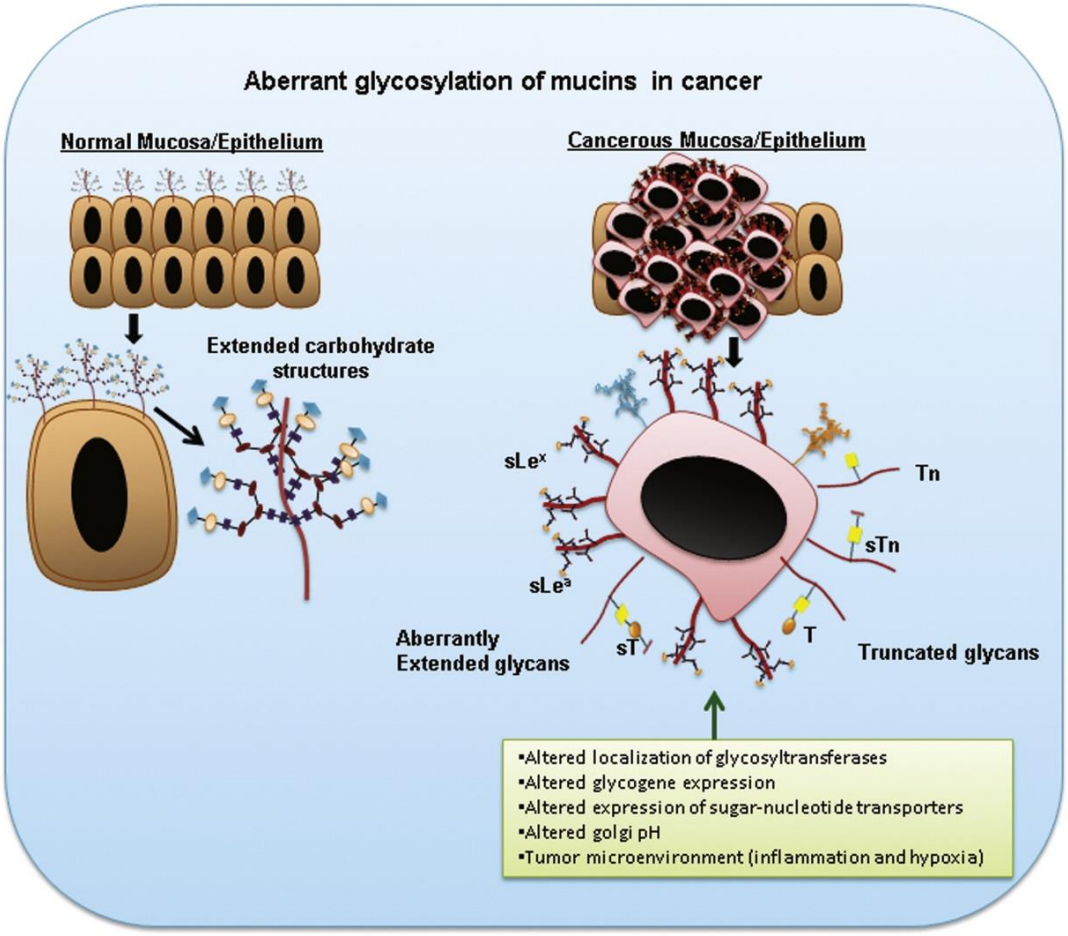


Figure 1.4. Aberrant glycosylation of mucins during cancerous conditions[93]

Typical mucins are composed of ~75% carbohydrate and ~25% amino acids. In various malignancies, alterations in the structure and composition of mucin glycans have been observed. During carcinogenesis, Tumor-associated carbohydrate antigens (TACAs) are found on various glycoproteins including mucins. These unusual glycan forms can be the result of incomplete glycosylation that leads to truncated structures such as Tn, STn, T, and ST or aberrant extension of carbohydrate chains like SLe^a and SLe^x (Figure 1.4).[93-94] For example, mucins containing reduced core 1 and core 3 glycan structures in addition to overexpression of TACAs such as Tn And STn have been observed in pancreatic, colon, and intestinal cancers.[85] Literature reports suggest that these altered glycosylation

patterns are the result of several factors, including but not limited to, altered expression of glycogenes and sugar nucleotide transporters, mislocalization of glycosyltransferases, abnormal Golgi pH, and the tumor microenvironment.[54, 93, 95-96] Since the role of these mucin related TACAs is well documented in cancer biology such as tumor cell adhesion, invasion, proliferation, angiogenesis, and altering the interaction of tumor cells with other cells; they have been extensively used in cancer vaccine development.[86, 97]

1.5.1 Role of mucin glycans in cancer

Literature evidence suggests that deregulated expression of mucin carbohydrates can have a significant impact on the function of the mucins in various pathological conditions and cancer biology. The interactions of mucin carbohydrates with sugar-binding proteins have been shown to have an influence on tumor growth, the escape of immune surveillance, and metastasis.

Sialoglycans overexpressed on mucins affect tumor growth. For example, the MUC1 sialoglycan SLe^X has been shown to mediate its binding with Siglec-9, a sialoglycan-binding protein found on immune cells (figure 1.5a). Mucin glycans also play a critical role in immune surveillance. Tumor tissues have capabilities to evade immune cells, such as macrophages, NK cells, and cytotoxic T cells. This ability of tumor cells to evade immune surveillance is a result of the activation of one or more immunosuppressive pathways. The cancer cells often involve cell surface glycan epitopes to activate these pathways. Elongated mucin carbohydrates beyond Tn antigens increase their susceptibility toward cytotoxic T cell and NK cell mediated cytotoxicity (figure 1.5b). For example, knockout of Cosmc protein inhibited glycan elongation beyond Tn in

the breast (T47D) and pancreatic cancer cells which increased their sensitivity towards immune attack via NK cell-mediated ADCC and cytotoxic T lymphocytes.[98]

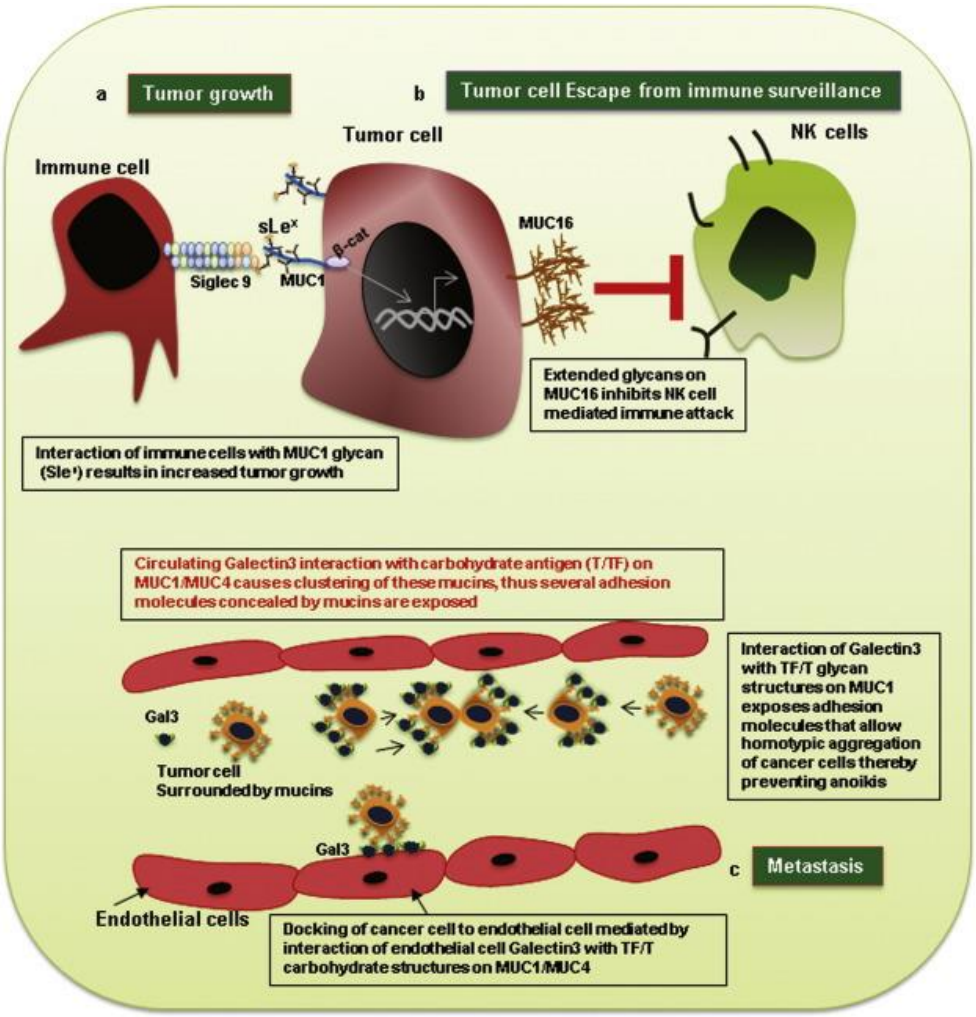


Figure 1.5. Role of Mucins in cancer

Furthermore, the altered glycan pattern of MUC1 on the cell surface has a role in cancer metastasis. For successful metastasis of cancer cells, they must leave their site of origin by degrading extracellular matrix and enter into the blood stream (intravasation) where they become a circulating tumor. When these cells reach the endothelia of target organs, they extravasate, leaving the blood vessel, and start colonizing at secondary

sites.[99] In 2010, Havrylov and co-workers showed that aberrant glycans on MUC1 play a key role in the invasion as well as the migration of tumor cells by affecting the interaction of MUC1 with CIN85, a protein involved in invasion and cytoskeletal alterations.[100]

It has been shown that overexpression of mucin glycans also play a role in cancer metastasis. The interaction of MUC glycans with membrane bound or circulating galectins leads to metastasis. For example, MUC4 interacts with Galectin-3 via T antigen on its surface.[101] This binding leads to MUC4 clustering, which exposes integrin-like adhesion molecules previously shielded by MUC4. Thus, this process facilitates the attachment of cancer cells to endothelial cells (Figure 1.5c). In a similar way, overexpression of circulating Galectin-3 in some cancers has been shown to interact with TF or T antigens on MUC1 by exposing surface ligands which were concealed earlier due to the presence of dense glycan pattern on MUC1 such as CD44 to endothelial cells. Thus, interactions of mucins with galectins play a critical role in metastasis by eliminating the protective layer (shield effect) that prevents the adhesion of tumor cells to endothelial cells (Figure 1.5c).[102] In addition, glycosylation plays a pivotal role in membrane trafficking of mucins. Both *O*- and *N*- linked glycans for some mucins store information for their apical sorting. For example, in Madin-Darby canine kidney (MDCK) cells, *O*- linked tandem repeats on the MUC1 act as an apical sorting signal.[103]

1.6 Carbohydrate-based cancer vaccines

Due to the pivotal role of carbohydrates in cancer biology they have been exploited in the development of novel cancer vaccines. Over the years, several research groups have made a significant contribution in the field of novel glycoconjugate vaccines and fully synthetic carbohydrate-based anticancer vaccines as described in the following sections.

1.6.1 Glycoconjugate vaccines

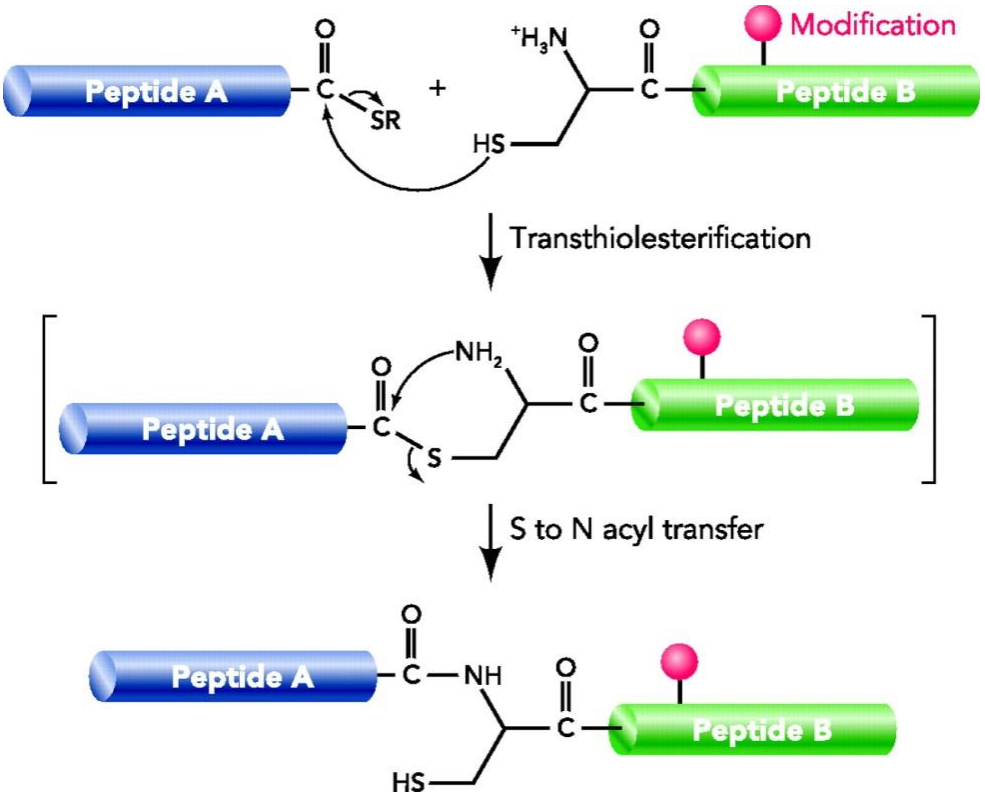
Glycans are functionally important because of their presence on cell surfaces of various biomolecules, including mucins. These glycans modulate cellular functions during various physiological and pathological conditions.[104] As described in section 1.3.2, mucin glycans play a crucial role in tumor cell proliferation, invasion, metastasis, and angiogenesis, and thus they have become very attractive targets for cancer treatment. Unfortunately, TACAs alone are known to have low immunogenicity due to the fact that they are T cell independent and elicit the innate immune response mainly through pathogen-associated molecular pattern (PAMP) receptors on the cell surface and very weak adaptive immune response via cross-linking with B cell receptors.[105-106] However, glycoconjugates have been demonstrated to be an effective strategy for the design of carbohydrate-based cancer vaccines. These approaches involve conjugation of saccharide antigen to various carrier proteins in order to increase the immunogenicity of the vaccine. In this regard, a number of different carrier proteins have been investigated, giving rise to T-cell dependent glycoconjugates. Moreover, most of the cancer vaccines are administered along with an immunoadjuvant to enhance the stimulation of the innate immune response.

Advances in synthetic organic chemistry have made it possible to synthesize complex TACAs. As the development of carbohydrate-based anticancer vaccines is largely based on efficient synthetic methods, researchers have focused on improving synthetic protocols. Several research groups around the world have made a significant contribution to this field.[70, 75, 107-111] However, the synthesis of complex glycans and glycopeptides is still a challenging task because of multi-step synthetic procedures that

are required for the synthesis of glycosyl acceptors and donors, as well as the requirement of stereo- and regioselectivity during glycosidic bond formation. A number of novel anomeric leaving groups and neighboring group participating functionalities have been proposed and utilized, which can be installed orthogonally under milder conditions and are very stable for column purification and storing purposes.[112] Some of the most stable anomeric leaving groups, such as fluorides, thioglycosides, and trichloroacetimidates, give high product yields as well as anomeric ratios.[113-117] The use of anomeric sulfonium ions, glycal assembly methods, and dehydrative glycosylation procedures are also becoming popular for complex oligosaccharide synthesis.[118-119] The glycosylation reactions of these functional groups could be performed under mild conditions and provide high yield with desirable anomeric ratios. More recently, convergent synthesis protocols for oligosaccharides involving complex structures have become available which provides appropriately protected building blocks in a less number of steps. Specifically, one-pot-multi-step synthesis and solid phase oligosaccharide synthesis are being used, which eliminate workup as well as purification steps, thus speeding up the synthesis.[120-123] In addition, researchers also have reported different glycosylation techniques such as orthogonal, chemoselective, and iterative glycosylation that take advantage of differences in the reactivities of leaving groups at the anomeric position, allowing several glycosyl donors to react in a sequential manner, thus resulting in the desired oligosaccharide product. [114, 124]

The conjugation of glycan to specific amino acids in a peptide is an important and difficult step in the synthesis of glycopeptide-based vaccines. A variety of new approaches has been employed for the synthesis of glycopeptides. Glycans can be

attached to amino acids and are introduced into solid phase peptide synthesis. Similarly, other well-established conjugation chemistries can be used to attach functionalized oligosaccharides and peptide fragments, for example, oxime formation followed by reductive amination, hydrazone formation, disulfide, and thioether formation chemistry. Copper-assisted and strain promoted click chemistry is also a powerful technique to connect two peptide or glycopeptide fragments together.[125-126] However, a rigid triazole ring introduced by click reaction can be immunogenic and may suppress the already low immunogenicity of TACAs. Native chemical ligation (NCL) chemistry has become one of the commonly used techniques to form large polypeptides, glycopeptides, and proteins.[127-128] NCL is more advantageous as it is chemoselective and forms a non-immunogenic amide bond as shown in scheme 1.1.[129]



Scheme 1.1 Mechanism of Native Chemical Ligation

During NCL, the thiol group of an N-terminal cysteine of an unprotected peptide attacks the C-terminal thioester of a second unprotected peptide. This reversible trans-thioesterification step is chemo- and regioselective and leads to form a thioester intermediate which then rearranges by an intramolecular *S* to *N*-acyl transfer forming a peptide bond between two fragments (Scheme 1.1).

In the chemical synthesis of vaccines, the type of linker and conjugation chemistry employed play critical roles in the design of anticancer vaccines. Thus, the right choice of carrier protein, adjuvant, and linker as well as conjugation chemistry, play important roles in determining the efficacy of cancer vaccine. Carrier proteins such as tetanus toxoid (TT), bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH), have been used for carbohydrate-based cancer vaccines. These carrier proteins, after internalization and proteolysis, offer helper T-epitope short peptides that can be presented on the surface of an APC in complex with MHC. This antigen presentation is important for the class switch from low-affinity immunoglobulin M (IgM) antibody to high-affinity immunoglobulin G (IgG) antibody. Since IgG antibodies are most abundantly found in the human body and provides long-term protection against bacterial and viral infections as opposed to IgM antibodies, which are a first and short-term response to pathogenic attacks, the class switch from IgM to IgG is essential for a successful vaccine development. Thus, these protein carriers augment the presentation of the TACAs and initiate activation of helper T-cells and can also possess adjuvant-like properties that lead to the production of cytokines by activation of the innate immune system. Danishefsky and co-workers developed a first generation monovalent glycoconjugate vaccine, where a single type of TACA was conjugated to an immunogenic KLH protein molecule. [130]

Globo H-KLH, a mono-epitopic glycoconjugate construct also showed promising results as a cancer vaccine candidate. The combination of QS-21, an immunoadjuvant, and the Globo H-KLH conjugate generated IgM and IgG titers in mice model.[131]

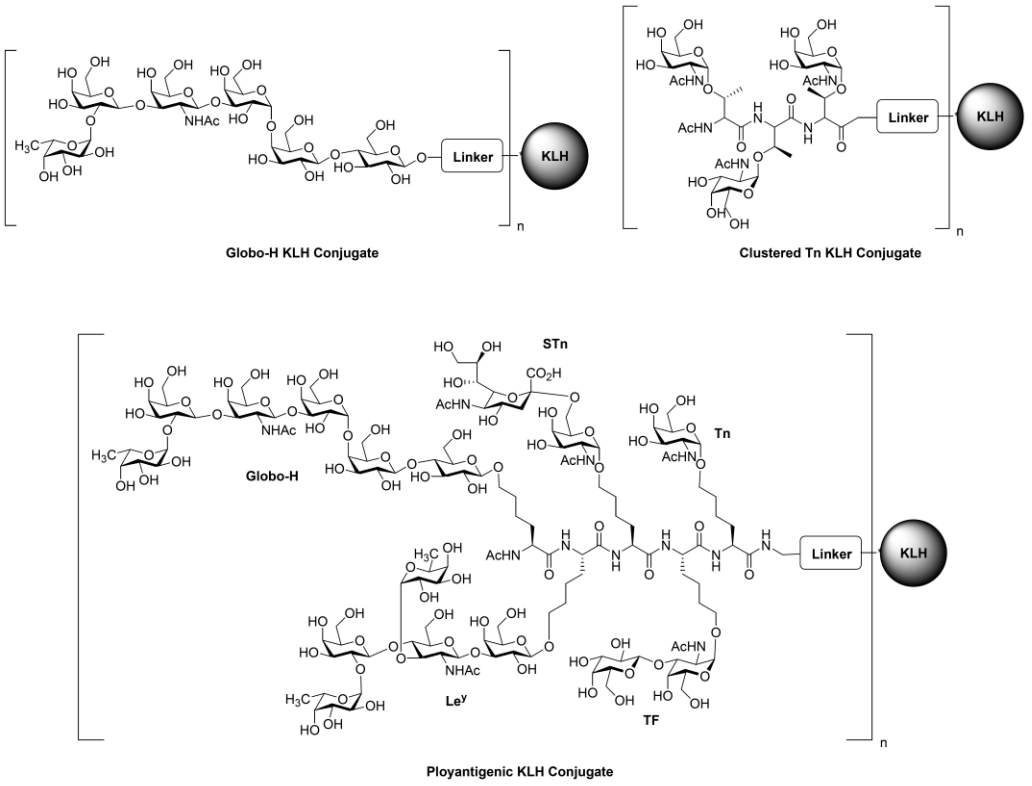


Figure 1.6. TACA-KLH protein conjugates

Other examples of the monovalent vaccine candidates that show promising results include KLH conjugates with Le^y pentasaccharide, KLH-fucosyl GM1 hexasaccharide conjugate and KLH conjugate with synthetic KH-1 antigen (Figure 1.6).[132-133] A recently reported conjugate of KLH-sLe^a hexasaccharide elicited high IgM and IgG antibody titers.[134] Even though mono-epitopic conjugate vaccines showed promising results in mice models and reached clinical trials, they failed to generate sufficient IgG antibodies (T-cell mediated immunity). However, these studies spurred research in developing

tumor-specific cancer vaccines that target carbohydrate antigens. More specifically, these results played critical roles in stimulating interest in the field of glycopeptide-based vaccines development that mimics tumor cell surfaces. In the mucin family, cell surface carbohydrate epitopes are mostly exhibited in the group of two to five (clustered TACAs) and recently these clustered TACAs have become preferred targets for cancer vaccine development. Furthermore, these clustered TACA glycopeptides are often conjugated to a carrier protein to enhance immunogenicity of the vaccine constructs. However, the synthesis of glycopeptides is a challenging task. In particular, maintaining stereochemical control during formation of the α-*O*-glycosidic bond between sugar domains and amino acids (serine and threonine) can be difficult. Danishefsky and co-workers have developed a methodology termed the "cassette approach" where the construct can serve as a general insert that can be later attached to the target sugar containing a glycosyl donor at its reducing end.[135] This approach is advantageous over convergent approach as there is no need for direct coupling of an amino acid (serine/threonine) side chain hydroxyl group of a complex oligosaccharide donor. In this approach, a primary building block carbohydrate is first stereospecifically linked to a serine/threonine. This product is then employed in subsequent glycosylations with appropriate glycosyl donors to prepare a complex oligosaccharide conjugated to serine/threonine, which is then utilized in glycopeptide synthesis as shown in figure 1.7.

N-acetylgalactosamine α-*O*-linked to a serine/threonine is one of the highly conserved moieties in various glycoprotein families including the glycophorin family. Consequently, researchers have synthesized α-GalNAc-O-Ser/Thr building blocks containing different acceptor sites for synthesis of various of glycophorin antigens.[136] The

cassette approach has successfully been employed in vaccine candidate development. KLH conjugates of synthetic trimeric clustered glycopeptides containing Tn, TF, STn 2,6-Sialyl TF and Le^y tumor antigens have been studied, however only combinations of Tn-KLH and TF-KLH clusters along with immunoadjuvant QS-21 have progressed to phase I clinical trials.[135, 137-139]

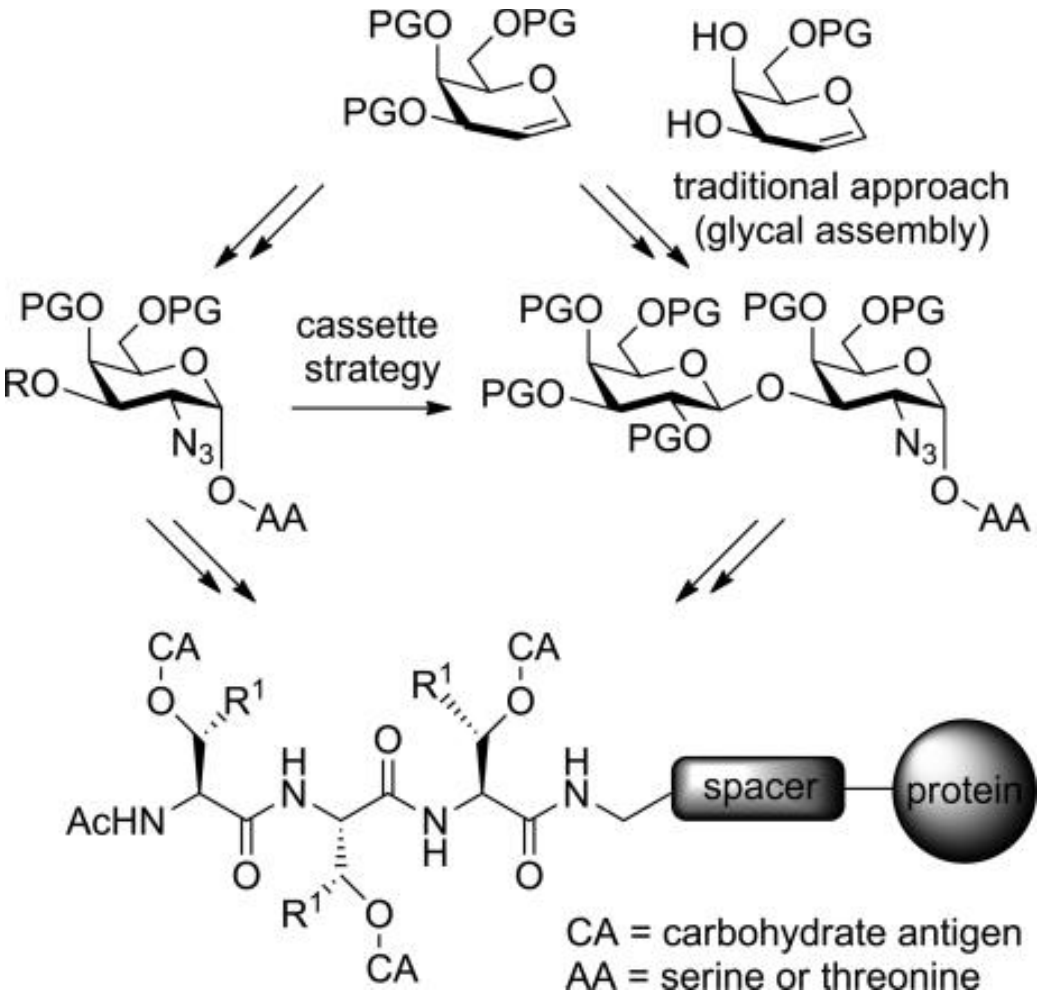


Figure 1.7. Cassette Approach[135]

Even though the carbohydrate-protein conjugate vaccine showed promising results in preclinical and clinical studies, there are some inevitable problems associated with it. First of all, the conjugation of carbohydrates to proteins is hard to control, which

can result in variations in structure and composition of the glycoconjugates. In general, the higher the ratio of TACA to protein the stronger the immune response, hence batch to batch differences in the composition can give ambiguous results and can adversely affect vaccine efficacy.[140-141] Moreover, as immunogenic linkers can lead to epitope suppression, the type of linker used in conjugation chemistry can alter immunological outcomes.[142-143] For example, the cyclohexyl maleimide linker is one of the most commonly used linkers due to its selectivity and reactivity towards thiol groups; however, this linker significantly reduced immunogenicity of the Le^y antigen in mice models. Boons and coworkers described that glycoconjugates containing a maleimide linker elicited mainly IgM and IgG anti-linker antibodies, but when another smaller in length and more flexible 3- (bromoacetamido)-propionate linker (Figure 1.8) was utilized for conjugation, immune response towards linker was significantly reduced and immunogenicity towards Le^y was significantly increased.[143]

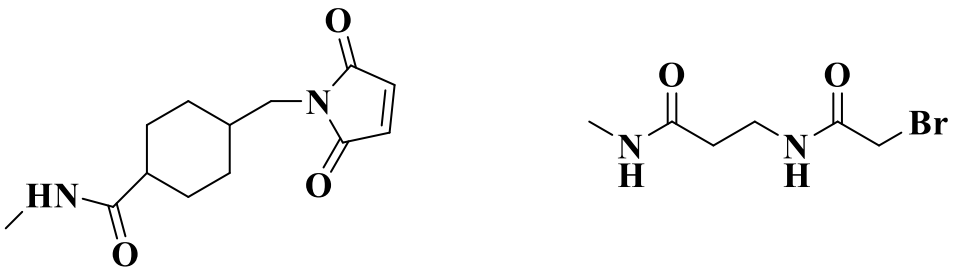


Figure 1.8. 4-(N-Maleimidomethyl)-cyclohexane-1-carboxylate and 3-(bromoacetamido)propionate linkers

Furthermore, another major problem associated with the use of foreign carrier proteins is their self-immunogenicity, which can result in high B cell related responses. This phenomenon can be problematic when self-antigens like TACAs are used as protein carrier, which can induce epitope suppression. Because of these issues, researchers have

focused on developing novel strategies for the presentation of TACAs more effectively to immune cells to generate a robust immune response. In this regard, much research is going towards subunit vaccines that have only the necessary components for evoking innate as well as humoral immunity and no any unnecessary immunogenic elements to allow for a stronger and highly antigen-specific response to be generated.

1.6.2 Fully Synthetic Vaccines

To improve the presentation of TACAs and eliminate the necessity of a carrier protein, TACAs must be attached to a ligand that can activate immune cells. TLRs have been well established to play a central role in activation of innate immunity identifying specific microbe-associated patterns.[144-145] In this regard, TLR ligands have been attached to antigens to initiate production of necessary cytokines, which then activates B cells, macrophages, and APCs.[146] , For example, the Pam_3Cys, one of the TLR2 ligands has been covalently linked to TACAs such as dimeric and trimeric Tn-antigen as well as to a monomeric and trimeric Le^y antigen.[147-149] Mice immunization studies have established that these vaccines when co-administered with the QS-21, an immunoadjuvant, elicited high titers of IgM, but failed to class switch to high-affinity IgG. These, along with several other literature reports, suggested that fully synthetic cancer vaccine candidates may need a T helper epitope for the efficient class switch from low-affinity IgM to high-affinity IgG titers.

Several research groups have pursued the chemical synthesis of two component and multicomponent component vaccine candidates containing carbohydrate B-cell epitopes attached to the T-cell epitope. In one of the early attempts, the Cantacuzene group proposed the concept of multiple antigen glycopeptides (MAG) vaccine

candidates.[150-151] These candidates consist of a Tn-antigen conjugated to a lysine backbone containing helper T-cell epitope derived from poliovirus. This MAG vaccine was able to elicit Tn-specific monoclonal antibodies in a mouse model.

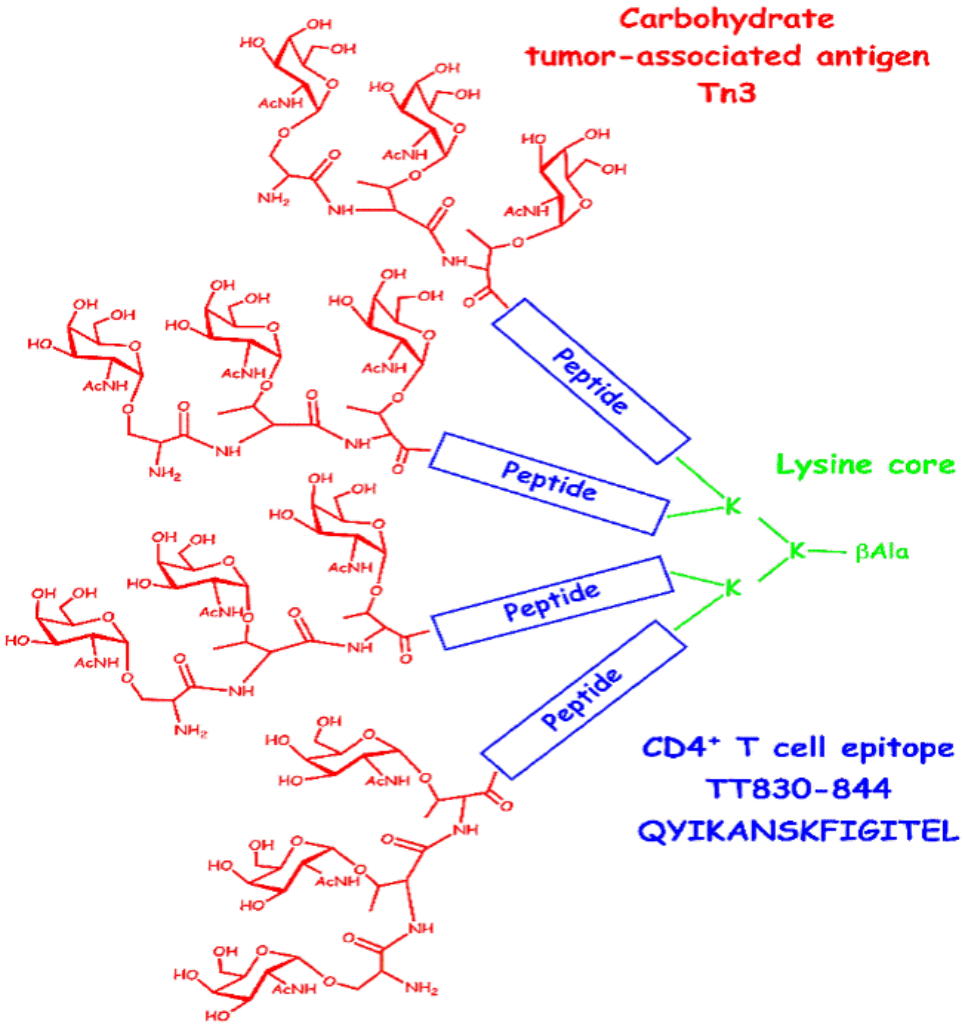


Figure 1.9. Schematic representation of the MAG-Tn3 vaccine.

Early this year in a similar study, Leclerc, and coworkers designed MAG-Tn3, a fully synthetic vaccine based on three consecutive Tn moieties that are *O*-linked to a CD4+ T cell epitope, a tetanus toxoid derived peptide TT830-844 (Figure 1.9).[152] This MAG-Tn3 consists of a lysine amino acid core containing four copies of the TT peptide further extended with a trimer of the Tn antigen. The formulation of this vaccine with

immunostimulant AS15 was able to elicit an anti-Tn antibody response in mice. Furthermore, they showed that this vaccine was also successful in generating anti-Tn antibodies in cynomolgus monkeys, which targeted Tn-antigen expressing cancer cells and mediated tumor cell death *in vitro* as well as *in vivo*. This MAG-Tn3 vaccine is currently in phase I clinical trials.

Danishefsky and co-workers developed poly antigenic vaccine candidates where they attached a specific type of cancer-related TACAs to the peptide backbone. In particular, they successfully synthesized poly antigenic targets where Gobo-H, Tn, STn, TF and Le^y antigens were linked to the peptide core (Figure 1.10).[153-155] The synthesis of the complex carbohydrates was achieved utilizing glycal assembly technique containing allyl or pentenyl spacers, which later can be converted to norleucine amino acids containing the oligosaccharide on side chain for incorporation into the peptide backbone. Upon co-administering these multi-antigenic compounds with an immunoadjuvant (QS-21) in mice, detectable levels of IgM antibodies were found. In addition, when conjugates of these poly antigenic construct with KLH were administered along with QS-21 in murine host, Globo-H–Le^y–Tn glycopeptide (compound 4, Figure 1.10) were found to be more immunogenic compared TF–Le^y–Tn construct (compound 3, Figure 1.10) and elicited IgM as well as IgG antibodies.[155] These results demonstrated that a single vaccine candidate containing several different TACAs can have the ability to stimulate a multifaceted immune response.

In another study, Kunz and coworkers covalently linked the tumor-associated STn MUC1 glycopeptide B cell epitope to an ovalbumin-derived T cell epitope through a nonimmunogenic amino acid linker.[156] The mice immunization study with this vaccine

candidate generated strong and highly specific humoral immune response. The antibodies generated were very specific and recognized only the glycopeptide B cell epitope containing MUC1 peptide linked to STn and did not recognize peptide or STn structures alone.

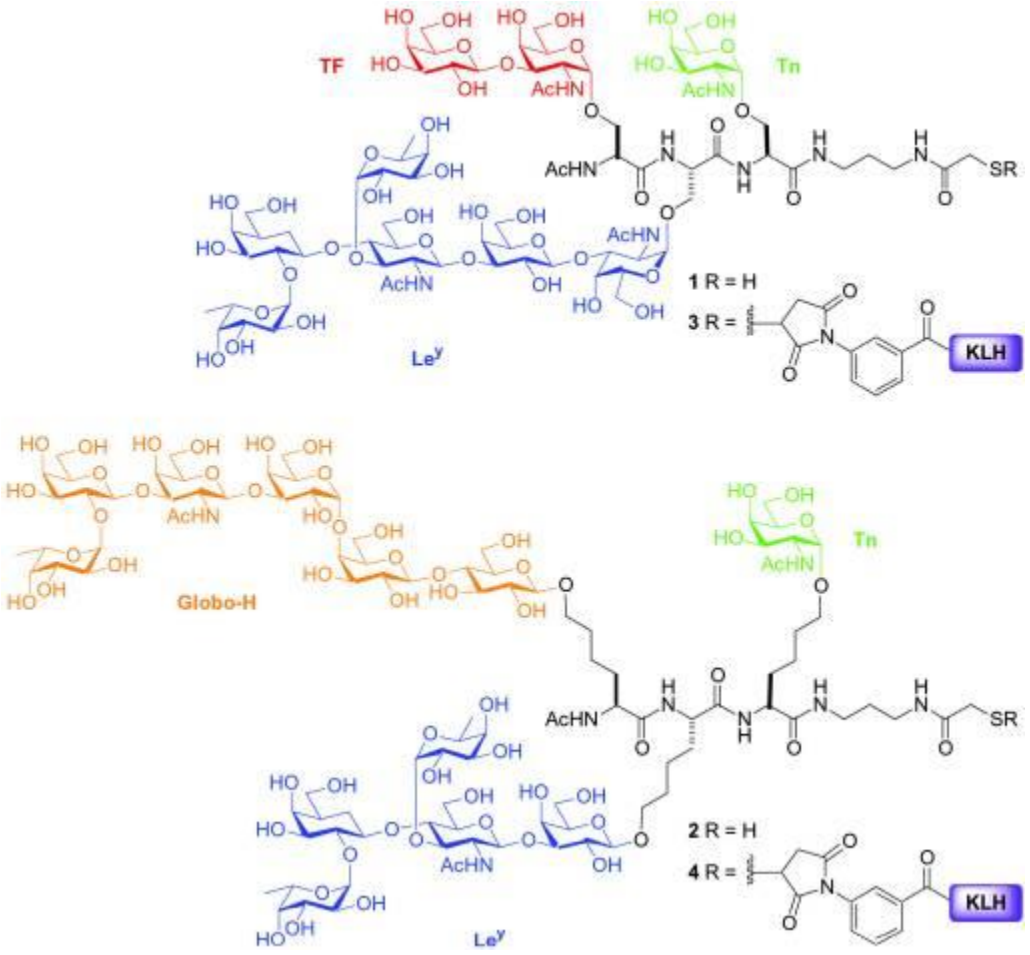


Figure 1.10. Fully synthetic multivalent constructs of TF, Tn, Le^y and Globo-H antigens.

More recently in 2011, Li and coworkers reported fully synthetic MUC1-based anticancer vaccines containing mono-, di- and tetravalent B cell epitopes conjugated to TLR2 ligand (Figure 1.11).[157] The B cell epitope, MUC1 glycopeptide (HGVTSAPDT*RPAPGS*TAPPA) containing Tn and/or T antigen and TLR2 ligand, Pam_3CysSK_4 were prepared by solid-phase synthesis. Conjugation of MUC1

glycopeptide epitope and multivalent alkyne-functionalized lipopeptide (TLR2 ligand) was performed by using Cu^{+} catalyzed click chemistry (Figure 1.11). The tetravalent glycopeptide-lipopeptide candidate bearing the STn-antigen was more immunogenic compared to its monovalent and divalent counterparts, and the tetravalent candidate (Figure 1.11c) was able to initiate CDC-mediated killing of tumor cells.[158]

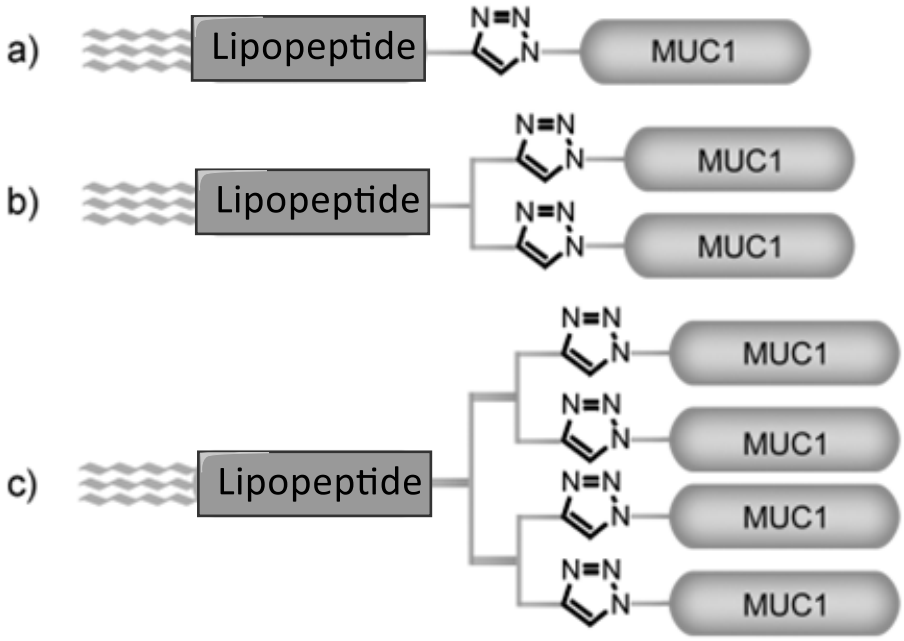


Figure 1.11. Structural representation of multivalent MUC1 glycopeptide conjugates with the TLR 2 ligand.

Another two-component therapeutic glycopeptide vaccine developed by Dr. Li's group contained the MUC1 tandem repeat sequence covalently attached to BSA or various tetanus toxoid derived T-cell peptide epitopes.[159] In this study, the MUC1 tandem repeat glycopeptide sequence HGVTSAPDTRPAPGSTAPPA that was decorated with various combination of T-,Tn- and STn-antigen, was coupled to three different universal T-helper cell epitope peptides, P2 ($TT_{830–843}$- QYIKANSKFIGITE), P4 ($TT_{1273–1284}$-GQIGNDPNRDIL), and P30 ($TT_{947–967}$-FNNFTVSFWLRVPKVSASHLE) and to BSA

(Figure 1.12). It was found that the vaccine conjugate containing the P30 epitope peptide was more effective compared to conjugates with P2 and P4 epitope peptides as well as BSA conjugates. Also the P30 conjugated vaccine with three glycans, more specifically three Tn in the immunodominant motifs PDTRP and GSTAP (Figure 1.12. Peptide-4-P30) elicited a much higher immune response. Interestingly the addition of an external adjuvant, such as Freund's adjuvant, was found to have immunosuppressive effect in this study.

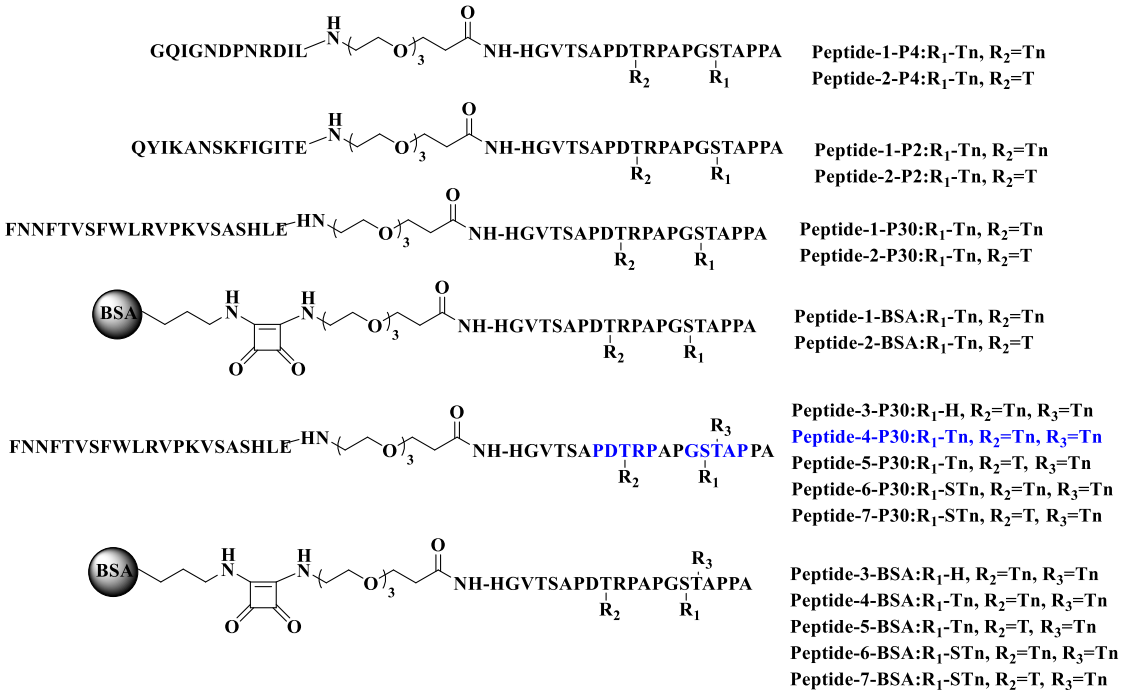


Figure 1.12. Structures of MUC1 glycopeptides conjugated with T-helper epitope peptides and BSA.

In the recent past, Boons and coworkers have made a significant contribution to the development of a three-component cancer vaccine. The three component vaccines are composed of a tumor-associated carbohydrate B epitope, a promiscuous peptide helper T cell (Th) epitope and an immune modulator like a TLR2 ligand. This three-component

vaccine strategy is advantageous over other traditional approaches as it consists of a minimum number of subunits required to elicit carbohydrate antigen-specific immune response and circumvents immune suppression caused by a carrier protein.[160 161] Initially, the fully synthetic three-component vaccine was comprised of the tumor-associated Tn-antigen, a helper T epitope derived from *Neisseria meningitis* and Pam_3Cys as a TLR2 ligand. Mice immunization studies of the vaccine compound were performed by incorporating the vaccine candidate into phospholipid-based liposomes and it was co-administered with and without an external adjuvant QS-21. This first generation vaccine candidate could only elicit low to moderate Tn-antigen specific IgG antibodies, but this result made a significant contribution to further cancer vaccine strategy development.

The second generation Boons group cancer vaccine candidates consisted of a B-epitope, a tumor-associated short glycopeptide derived from MUC1 and the well-established MHC class II restricted Th epitope peptide KLFAVWKITYKDT derived from poliovirus. Additionally, these compounds contained the built-in immune activator lipopeptides Pam_2CysSK_4, which is a very potent TLR2/6 activator, or Pam_3CysSK_4, which is a TLR2 activator. The incorporation of the TLR ligand in addition to the B cell epitope and T-cell epitope in the vaccine rendered self-adjuvant character to the vaccine construct, thus eliminating the necessity of an external adjuvant. In earlier reports, these epitope peptides, glycopeptides, and lipopeptides were synthesized using solid phase synthesis on a traditional automated peptide synthesizer. The three-component vaccine **5** was assembled by reported liposome-mediated native chemical ligation[162] of the thiobenzyl ester of Pam_3CysSK_4 and the glycopeptide CKLFAVWKITYKDTGTSAPDT(αGalNAc)RPAP (Figure 1.13)[163]. The vaccine **5**

(Figure.1.13) produced CTLs that recognized glycosylated as well as a non-glycosylated peptide, however, the non-glycosylated vaccine **6** (Figure 1.13) produced CTLs that recognized only the non-glycosylated peptide. The vaccine **5** elicited high titers of IgG antibodies and was more lytic compared to non-glycosylated counterpart vaccine **6**.

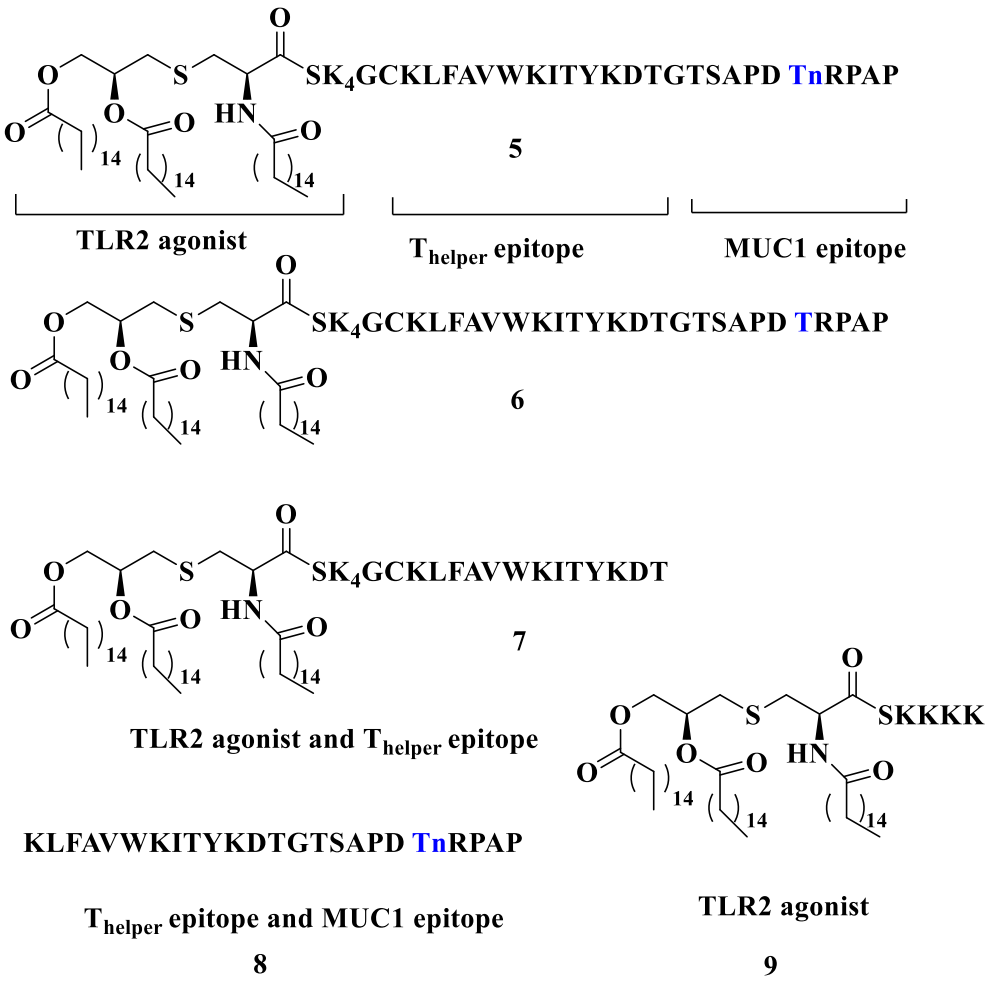


Figure 1.13. Chemical structures of Boons group three-component cancer vaccines

Immunization with a liposomal formulation of multicomponent vaccine **5** could significantly reduce tumor burden compared with empty liposomal control or compound without the MUC1 B-cell epitope (comp.**7**). These studies demonstrated that this tripartite vaccine containing glycosylated MUC1 has the unique ability to generate CTL and ADCC-mediated antibodies that recognized tumor- associated MUC1.

In 2013, the Kunz and Li groups worked together to synthesize a library of two- and three- component vaccines.[164] Their vaccine candidate was composed of previously reported MUC1 glycopeptides B cell epitopes, a TT-derived T cell epitope peptide, and Pam_3CysSK_4 as the TLR2 ligand. These subunits were linked together covalently by an immunologically inert spacer, using a new thioether-ligation method as shown in figure 14. Efficacy of these two- and three-component vaccines containing various Tn glycosylation patterns on MUC1 as well as different T-cell epitopes (P2, P4 or P30) was tested in mice model. These vaccines were administered as liposomes or in combination with complete or incomplete Freund's adjuvant or in aqueous buffer solution. The three-

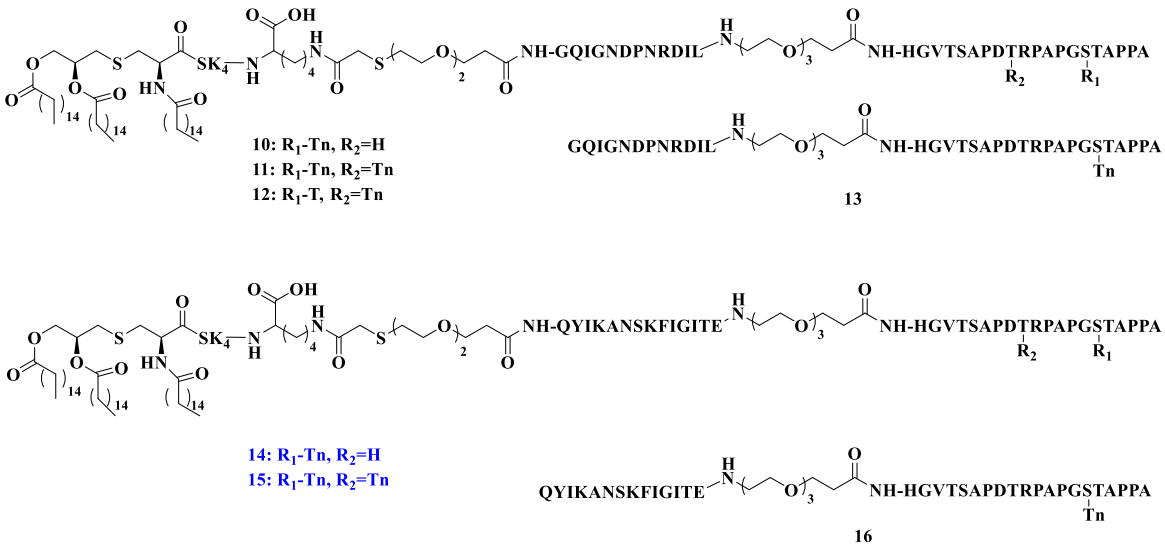


Figure 1.14. Kunz and Li group two-and three-component vaccine candidates.[164]

component vaccines **14** and **15** (Figure 1.14), which consisted of tumor-associated MUC1 (Tn_{15}), the P2 peptide as a T-cell epitope and Pam_3CysSK_4 as TLR2 ligand, showed high titers of IgG especially when not administered with CFA or as liposomal formulations, but instead in aqueous buffer solution.

Summary

Fully synthetic carbohydrate-based cancer vaccines offer an attractive alternative for cancer treatment. The recent progress in the strategy and development of complex carbohydrate and glycopeptide synthesis has made it possible for the preparation of complex vaccines to test their efficacy in pre-clinical and clinical settings. Multiple vaccine candidates have shown very promising results in pre-clinical studies and have the potential to be candidates for cancer treatment and prevention. However, the search for an unanimously accepted carbohydrate based cancer vaccine is still on to qualify as a successful anti-cancer vaccine. For a cancer vaccine candidate to be considered as a successful vaccine, it must be able to induce both humoral as well as cellular immunity against the carbohydrate antigen. Cancer vaccine research over last few years has highlighted the importance, influence, and necessity of the various components of the vaccine required for the activation of immune system. Recent studies have provided insight into the need of inbuilt TLR immunoadjuvant such as Pam_3CSK_4, in addition to the other subunits of the vaccine construct for immune activation. Currently, only a few examples of a fully synthetic two- and three- component vaccine containing TACAs, a T-cell epitope peptide, and inbuilt TLR adjuvant have shown potent immune activation.[161, 163-164] The synthetic carbohydrate-based cancer vaccine is an attractive and advantageous strategy as it includes only the necessary subunits required for an eliciting appropriate immune response while avoiding unnecessary side reactions. In this regard, anti-tumor vaccine candidates consisting a partial and full-length MUC1 glycopeptide as one of the subunits have shown promising immunological results and have the potential to be a successful therapeutic anti-cancer vaccine.[163, 165-168] However, multiple areas can be

further explored, such as the choice of the right peptide and glycopeptide epitopes and their synthesis, conjugation techniques and linker chemistry as well as the quest for new vaccine adjuvants, to prepare a successful anti-cancer vaccine.

The aim of our current research described in this dissertation is the design and synthesis of a full-length MUC1 based multi-component vaccine candidate, consisting of a built-in immunoadjuvant that can elicit a specific immune response. In the second chapter, we report the microwave-assisted solid phase synthesis and immunological outcomes of the monoglycosylated full-length vaccine candidate. Based on immunological outcomes of our first study we felt compelled to synthesize pentaglycosylated full-length MUC1 vaccine candidate and test its efficacy in the mouse model as described in chapter three. The aim of the fourth chapter is to study the immunological outcomes of the spacing effect between TLR ligand and MUC1 subunits in vaccine candidates. For that work, we have synthesized a vaccine candidate containing a covalently attached Pam_3CSK_4 and full MUC1 either via an acid sensitive or an acid stable linker and we are in the process to evaluate the effect of these linkers in the generation of antibodies in a mouse model. Chapter five is aimed at the search for an unmet clinical need for new adjuvants that promote immunity against pathogens on mucosal surfaces. In this regard, we report the synthesis and functional characterization of novel chimeric compounds that can activate pattern recognition receptors stimulated by Gram-positive bacteria. Here, we have covalently linked two PAMPs; TLR2 and NOD2 using Cu (I) assisted click chemistry; we anticipate that these synthetic compounds may activate multiple cellular pathways synergistically and elicit desired protective immune responses.

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CHAPTER 2

SYNTHESIS AND IMMUNOLOGICAL EVALUATION OF MONOGLYCOSYLATED FULL-LENGTH MUC1-BASED MULTICOMPONENT CANCER VACCINE CANDIDATE.

***Nitin T Supekar**, Margreet Wolfert, Sandra Gendler, Geert-Jan Boons. *To be submitted to Angewandte Chemie International Edition.*

2.1 Abstract

Mucins (MUC) are high molecular weight transmembrane glycoproteins expressed on the apical surface of various epithelial cells in the lung, eyes, stomach, gastrointestinal tract as well as several other organs. They are involved in multiple biological processes including cell-to-cell and cell-to-matrix adhesion, signal transduction, and modulation of the immune system. Mucin-1 (MUC1) is highly overexpressed in the majority of carcinomas with significantly short and truncated glycan pattern compared to normal MUC1 and therefore could serve as a potent target for cancer immunotherapy. The extracellular domain of MUC1 consists of multiple 20 amino acids a variable number of tandem repeats (VNTR) with potential five sites for glycosylation. Unfortunately, MUC1 glycopeptide alone is weakly immunogenic and needs an additional stimulator to elicit a strong immune response. We have designed vaccine candidates comprising a full-length MUC1 sequence and built-in Pam_3CSK_4 immunoadjuvant. We anticipate that the full-length sequence of MUC1 offers a tantalizing antigen that contains multiple epitopes that can activate B cells, helper T cells, and cytotoxic T-lymphocytes. The sequence SAPDT(*O*-GalNAc)RPAP has been established as an attractive B-epitope. However, it is also an MHC class I (K^b) epitope that can activate cytotoxic lymphocytes. Glycosylation of this peptide is critical for eliciting antibodies and activate $CD8^+$ cells that can eliminate MUC1-expressing cancer cells. Furthermore, STAPPAHGV and PAHGVTSA are predicted MHC class I (A^b) epitopes. Analysis of the full-length sequence by Rankpep indicated that STAPPAHGVTSA of MUC1 can function as a *promiscuous* helper T-epitope (class II).

The resulting fully synthetic multi-component vaccine candidate was tested in MUC1 transgenic mouse model. As expected, the vaccine-induced humoral immunity by producing robust IgG antibody titers that recognized mono-and pentaglycosylated LMUC1 glycopeptide epitopes as well as being able to induce cellular immunity. Furthermore, ADCC results showed that the antiserum obtained by immunization with LMUC1 vaccine was significantly more cytotoxic compared to the control groups.

2.2 Introduction

The identification of novel tumor-associated antigens (TAAs) has made it possible to develop antigen-specific cancer immunotherapies. Mucin 1 (MUC1), an epithelial mucin glycoprotein, is one of the well-characterized TAA, which is highly overexpressed in most of the adenocarcinomas including breast, lung, pancreas and prostate. In addition, tumor-associated MUC1 is either aberrantly glycosylated or hypoglycosylated compared to normal MUC1 most likely due to lack of core 1-3 galactosyltransferase (T-synthase) activity, an increase in sialylation and/or downregulation of glycosyltransferase genes, giving rise to novel carbohydrate antigens such as STn (α Neu5Ac-(2,6)- α GalNAc-Thr) and Tn(α-GalNAc-O-serine/threonine).[1-3] This striking structural variation in MUC1 glycosylation pattern between cancerous and normal tissues makes it an interesting target for cancer immunotherapy. And because of this, MUC1 has been ranked second out of seventy-five cancer antigens for translational research by National Cancer Institute (NCI).[4] MUC1, an *O*-linked glycoprotein characterized by a variable number of tandemly repeated, proline-rich fragments of 20 amino acids (VNTR) consisting of five sites of glycosylation at serine/threonine amino acid residues. Aberrant glycosylation pattern on tumor cells exposes parts of peptide core

making it more accessible to the immune system. As MUC1 is highly immunogenic and is capable of inducing humoral as well as cellular immune responses, it has become an excellent target for the development of cancer immunotherapy.

Since its discovery as TAA, MUC1 has shown promising results in pre-clinical and clinical settings.[5-9] In vivo studies with non-glycosylated MUC1 based vaccine elicited MUC1 peptide-specific CD4+ and CD8+ T cell responses.[10-12] However, it has been shown that glycosylation on MUC1 is necessary for antigen uptake, processing, and MHC class II presentation thereby triggering robust antibody response as well as for activating CD8+ that can eliminate MUC1-expressing tumor cells.[13-14] As a result, researchers have explored MUC1-based vaccines containing both non-glycosylated as well as glycosylated versions of VNTR units. Most of these vaccines contain MUC1 VNTR conjugated to various carrier proteins such as keyhole limpet hemocyanin (KLH), Tetanus Toxoid (TT) and bovine serum albumin (BSA) to generate robust immune response.[15-17] However, the carrier protein approach has limitations. First of all, protein conjugation techniques are often low-yielding and may have reproducibility issues. In addition, the carrier protein by itself is highly immunogenic and has the ability to suppress the antibody response against weakly immunogenic MUC1 glycopeptide epitopes.[18] Hence, recently fully synthetic multicomponent vaccine constructs with built-in immunoadjuvant and specific T helper epitopes have been evaluated.

Recently, our group designed several vaccine candidates identifying minimum structural requirements for consistent induction of cytotoxic T-lymphocytes (CTLs) and antibody-dependent cell mediated cytotoxicity (ADCC) mediated antibodies for the tumor form of MUC1. The lead three component vaccines contained an aberrantly

glycosylated short MUC1 peptide, a T helper peptide epitope derived from poliovirus and inbuilt immunoadjuvant TLR-2 ligand Pam_3CysSK_4. The vaccine was very effective and elicited both humoral as well as cellular immunity. Furthermore, the importance of TLR-2 immunoadjuvant was highlighted in another study containing a different immunoadjuvant, TLR 9 ligand CpG oligodeoxynucleotide (CpG-ODN).[19] Several studies have shown that CpG-ODN can augment the immunity of MUC1-based experimental cancer vaccines, as well as conjugation of GpG-ODN to carrier protein antigen, can enhance T cell response.[20-25] However, the Pam_3CysSK_4 containing vaccine was more effective that significantly reduced the tumor burden and induced both humoral and cellular immunity compared to a vaccine containing CpG-ODN. Another vaccine candidate consisting of sialyl-Tn antigen in MUC1 glycopeptide vaccine along with the polio T helper cell epitope and Pam_3CysSK_4 elicited both humoral and cellular immune responses against STn MUC-1 and tumor-associated MUC1 in an MUC1 transgenic mouse .[26]

Studies have shown that antibodies induced with MUC1 isolated from tumor tissues have recognized MUC1 peptide motifs PDTRP, PPAHGV as well as RPAPGS as the most frequent immunodominant domains of the MUC1 tandem repeat.[27] The specificity of these anti-MUC1 antibodies have been verified when these peptides are modified with Tn- and T- antigens. It has been hypothesized that the improved binding is due to carbohydrate-induced conformational change of the peptide motif.[27-30] It is well established that T-cell epitopes of the MUC1 domain are bundled within cancer cells in their shortened and truncated glycosylation form into MHC class I molecules, resulting in the natural MHC-restricted recognition of MUC1 epitopes containing truncated

glycosylation pattern.[31-36]. It has also been established that glycosylated MUC1 epitopes have a higher binding affinity towards MHC class I mouse allele *H2k*^{b} compared with their non-glycosylated counterparts and can activate cytotoxic T-lymphocytes (CTL).[37] Recent studies have identified numerous MUC1-based HLA-A2-binding peptide epitopes that include SAPDTRAPG and STAPPAHGV. [38-41]

On the basis of these observations, we felt compelled to investigate the vaccine composed of full-length MUC1 VNTR unit covalently linked Pam_3CysSK_4 (Figure 2.1). We designed these vaccines in such a way that it contain multiple MUC1 immunodominant motifs like PPAHGV and glycosylated version of PDTRP, STAPPAHGV, and PAHGVTSA (predicted MHC class I (A^b) epitopes) and sequence STAPPAHGVTSA which can function as a promiscuous helper T-epitope.

2.3 Result and Discussion

Chemical Synthesis of monoglycosylated vaccine candidate 1.

In earlier reports, native chemical ligation techniques have been employed extensively to synthesize vaccines containing larger peptides, glycopeptides, and lipopeptides.[42-45] Native chemical ligation chemistry involves the thiolate of an *N*-terminal cysteine residue of one peptide fragment attacks the *C*-terminal thioester of a second peptide fragment to effect trans-thioesterification followed by an amide linkage formation after rapid *S*→*N* acyl transfer. However, recent literature reports suggest that microwave assisted solid phase synthesis have significantly improved the efficiency of solid phase synthesis by providing peptide products with higher purity with shorter reaction time.[46-47] Hence we felt compelled to develop and utilize microwave assisted

solid phase peptide synthesis chemistry in preparing cancer vaccine candidate in a linear fashion.

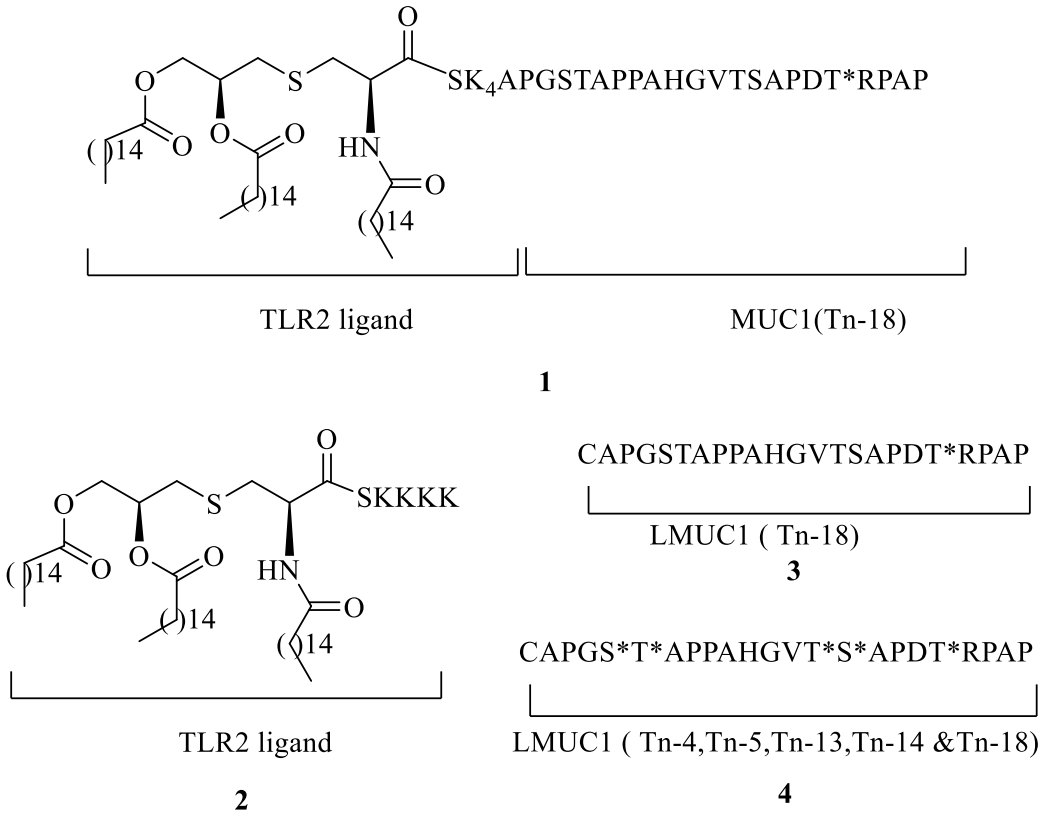


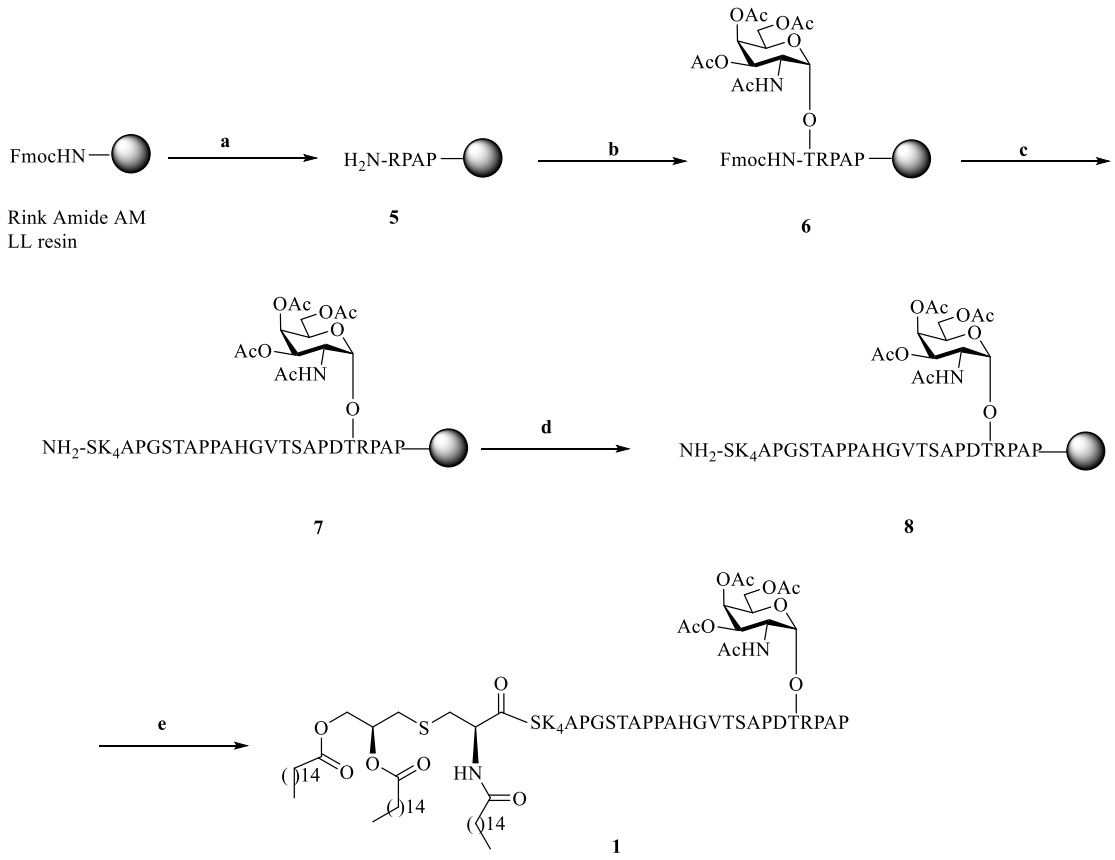
Figure 2.1. Chemical Structure of multicomponent MUC1 vaccine and glycopeptides (* represents glycosylation with α-GalNAc, TLR2 ligand is linked to the *N*-terminus of MUC1 peptide)

The multicomponent vaccine candidate 1 containing a monoglycosylated MUC1-derived B-epitope and T-helper peptide sequences, in addition to the TLR2 ligand, Pam_3CysSK_4 was synthesized using MW-SPPS as shown in scheme 2.1. The first four amino acids, RPAP were assembled on Rink Amide AM LL resin using a CEM Liberty 12-channel automated peptide synthesizer equipped with microwave heating assembly. The amino acid couplings on the synthesizer were achieved using HBTU-mediated HOBt ester activation chemistry and Fmoc-deprotection of the amino acids was accomplished using a solution of 20% 4-methylpiperidine in DMF. The introduction of sugar amino

acid, Fmoc-protected Tn was performed manually under microwave irradiation using HATU/HOAt activation protocol. After coupling of Tn-antigen the Rink Amide AM resin was returned to CEM automated peptide synthesizer to add subsequent amino acid residues. After coupling of serine amino acid, the resin was removed from automated peptide synthesizer and was then treated with a solution of 70% hydrazine in methanol to remove acetyl groups of the Tn-antigen. Following the deacetylation, the Fmoc-Pam_2Cys, as well as final palmitic acid residues were coupled to glycopeptide manually under microwave irradiation using HATU/HOTU in the presence of base DIPEA. The global deprotection of amino acid side chains and glycolipopeptides cleavage from the resin was performed under acidic condition using cocktail containing 88% TFA, 5% phenol, 5% H_2O, and 2% TIPS. The crude product was then purified by RP-HPLC to get final glycolipopeptide vaccine **1**.

Chemical Synthesis of monoglycosylated and pentaglycosylated LMUC1-glycopeptides for BSA conjugation

Encouraged by the successful preparation of vaccine candidate **1**, we focused our attention on the synthesis of monoglycosylated and pentaglycosylated LMUC1 peptides **3** and **4** required for preparation of BSA-MI conjugates**.** These conjugates were crucial to investigate if vaccine **1** elicits antibodies that recognize these synthetic LMUC1 epitopes. Synthesis of the monoglycosylated peptide was straightforward and was prepared using the similar protocol as that for vaccine candidate **1.**



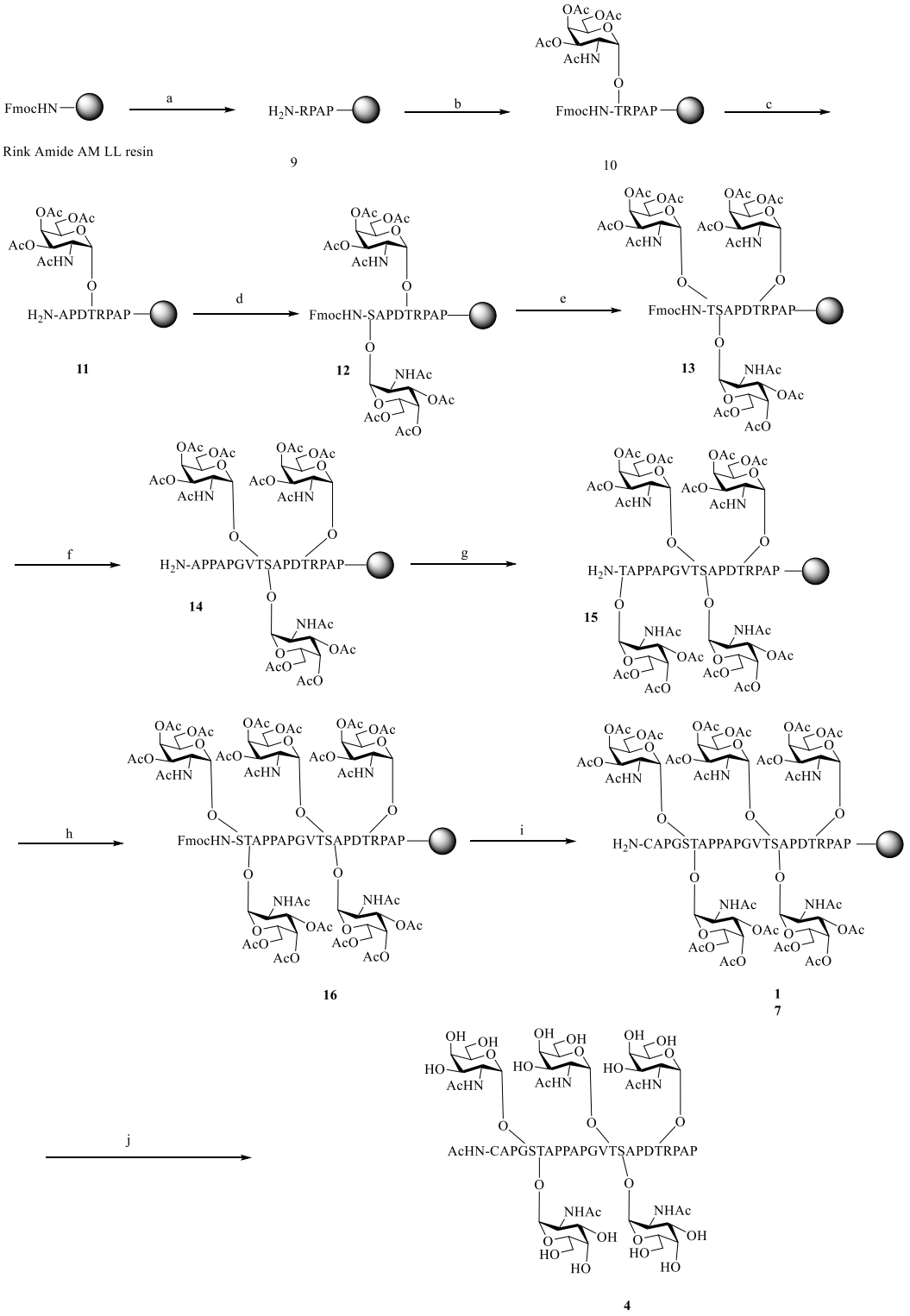
Scheme 2.1. Linear synthesis of monoglycosylated multicomponent LMUC1 vaccine

Reagents and conditions: (a).(i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF MW-SPPS (b) Tn-COOH, HATU, HOAt, DIPEA, DMF (1.2 eq.) MW - 5 min (c).(i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii). Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF MW-SPPS (d) 70% Hydrazine in MeOH, 2 h. (e) (i). Fmoc-Pam_2Cys-OH, HATU, HOAt, DIPEA, DMF, MW - 10 min x 2 (ii). 20% 4-Methylpiperidine in DMF, MW, 3 min (iii) Palmitic Acid, HATU, HOAt, DIPEA, DMF, MW, 10 min (iv). 88% TFA, 5% Phenol, 5% H_2O, 2.5% TIPS

After successful preparation of vaccine candidate **1** and glycopeptide **3**, we attempted a synthesis of penta-glycosylated glycolipopeptide **4**, which also consists of a full length containing terminal cysteine residue for conjugation to BSA, it additionally contains five glycosylated amino acids. During the synthesis of the penta-glycosylated peptide, we encountered numerous synthetic difficulties. After installation of the second glycosylated amino acid followed by deprotection of *N*-terminus Fmoc protecting group,

10-20% of acetyl groups migrated to the free amino group giving rise to chain termination. Similar results were observed during coupling of 3rd, 4^{th,} and 5th glycosylated amino acids. This resulted in a significant decrease in the yield of desired product. It is our belief that higher reaction temperature and longer microwave irradiation may have been an issue for these couplings containing glycosylated amino acids. To overcome this difficulty, microwave assisted methods for coupling and deprotection of glycosylated amino acids were developed and successfully employed. It is worth noting that the amount of glycosylated amino acid, lower coupling temperature, and time were the key factors to overcome chain termination. The following final scheme 2.2 was developed after multiple unsuccessful attempts. As shown in the scheme 2.2, first four amino acids were introduced by using automated peptide synthesizer followed by manual coupling of Fmoc-protected Tn-antigen as usual to get intermediate **10**. Following this, the resin was returned to automated peptide synthesizer for coupling of next three amino acids. The coupling of glycosylated serine was performed manually under microwave irradiation to afford intermediate **12**. Limiting the amount of glycosylated amino acid to 1 equivalent, lowering temperature to 60^{o}C in addition to shorter reaction time (3 minutes) was used for further glycosylated amino acid coupling reaction to avoid unwanted acetyl group migration. The coupling of all glycosylated amino acids was performed manually using this modified coupling protocol. Kaiser test was also performed after coupling of each glycosylated amino acid and any unreacted amino groups were capped using acetic anhydride. After extending glycopeptide till final cysteine residue on automated peptide synthesizer, it was removed from the synthesizer and then deacetylated and cleaved from the solid support using TFA cocktail solution manually. Thus as shown in the scheme

2.2, the synthesis of per-glycosylated LMUC1 glycopeptide **4** was achieved using modified protocol with the help of automated and manual MW-SPPS.



Scheme 2.2 Linear synthesis of pentaglycosylated LMUC1 glycopeptid

Reagents and conditions: (a) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (b)Tn-COOH (1.2 eq.), HATU, HOAt DIPEA, DMF MW - 5 min (c) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (d) (i) Fmoc-Tn-COOH (1 eq.), HATU, HOAt, DIPEA, DMF MW, 5 min (ii) 20% 4-Methylpiperidine, DMF, MW, 3 min (e) Fmoc-Tn-COOH(1 eq.), HATU, HOAt, DIPEA, DMF, MW 5 min. (f) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (g) (i) Fmoc-Tn-COOH (1 eq.), HATU, HOAt DIPEA, DMF MW, 5 min (ii) 20% 4-Methylpiperidine, DMF, MW, 3 min (h) Fmoc-Tn-COOH(1 eq.), HATU, HOAt, DIPEA, DMF, MW 5 min (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (j) (ii)10% Ac_2O, 5%DIPEA in DMF (ii)70% Hydrazine in MeOH, 3 hr (iii) 94% TFA, 2.5% EDT, 2.5% H_2O, 1% TIPS

After successful preparation of vaccine candidates and required glycopeptides for the experiment, attention was focused on exploring the immunological properties of the LMUC1 vaccine. Groups of MUC1.Tg mice (C57BL/6; H-2b) that express human MUC125 were immunized with the liposomal preparation of 1, 2, and empty liposomes five times intradermally at the base of the tail at biweekly intervals. One week after the last immunization, the mice were sacrificed and the humoral immune responses were assessed by titers of MUC1-specific antibodies by ELISA and the ability of the antisera to lyse MUC1-bearing tumor cells. In addition, cellular immune responses were evaluated by ELISPOT assay.

Mice immunized with compound **1,** LMUC1 vaccine candidate elicited robust IgG antibody titers compared to TLR2 and empty liposomes. It was also found that antibodies produced by vaccine candidate **1**, could recognize both mono-and pentaglycosylated versions of LMUC1 epitopes. The IgG subtyping showed a significant number of IgG1, IgG2a, and IgG2b titers indicated a mixed Th1/Th2 response. Furthermore the higher number of IgG3 titers suggesting an anti-carbohydrate response. It is worth to mentioning that very low number of IgM was detected, which is typical for

the efficient class switch from low-affinity IgM titers to high-affinity IgG titers (Table 1.1 and Figure 2.2).

Table 1.1 ELISA anti-MUC1 antibody titers in endpoint serum samples

a) ELISA anti-LMUC1 (Tn-18) antibody titers in endpoint serum samples

#	Compound	IgG tot	IgG1	IgG2a	IgG2b	IgG3	IgM
	EL	2,000	600	300	800	500	0
1	Pam_3CysSK_4-LMUC1(Tn-18)	23,200	29,200	1,200	3,600	5,800	300
2	Pam_3CysSK_4	300	0	0	0	0	0

b) ELISA anti-LMUC1 (5Tn) antibody titers in endpoint serum samples

#	Compound	IgG tot	IgG1	IgG2a	IgG2b	IgG3	IgM
	EL	1,200	500	600	400	300	0
1	Pam_3CysSK_4-LMUC1(Tn-18)	21,700	29,900	2,000	4,800	3,500	500
2	Pam_3CysSK_4	0	0	0	0	0	100

Antibody titers are presented as median values for groups of mice. ELISA plates were coated with BSA-MI-CAPGSTAPPAHGVTSAPDT(αGalNAc)RPAP conjugate for anti-LMUC1(Tn-18) antibody titers and BSA-MI-CAPGS(αGalNAc)T(αGalNAc)APPAHGVT(αGalNAc)S(αGalNAc)APDT(αGalNAc)RPAP conjugate for anti-LMUC1(5Tn) antibody titers. Titers were determined by linear regression analysis, with the 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.

Antibody-dependent cell-mediated cytotoxicity (ADCC) was examined by labeling MUC1-expressing mammary cancer cells 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** significantly increase cancer cell lysis compared to control groups, TLR2 and empty liposomes (Figure 2.3A).

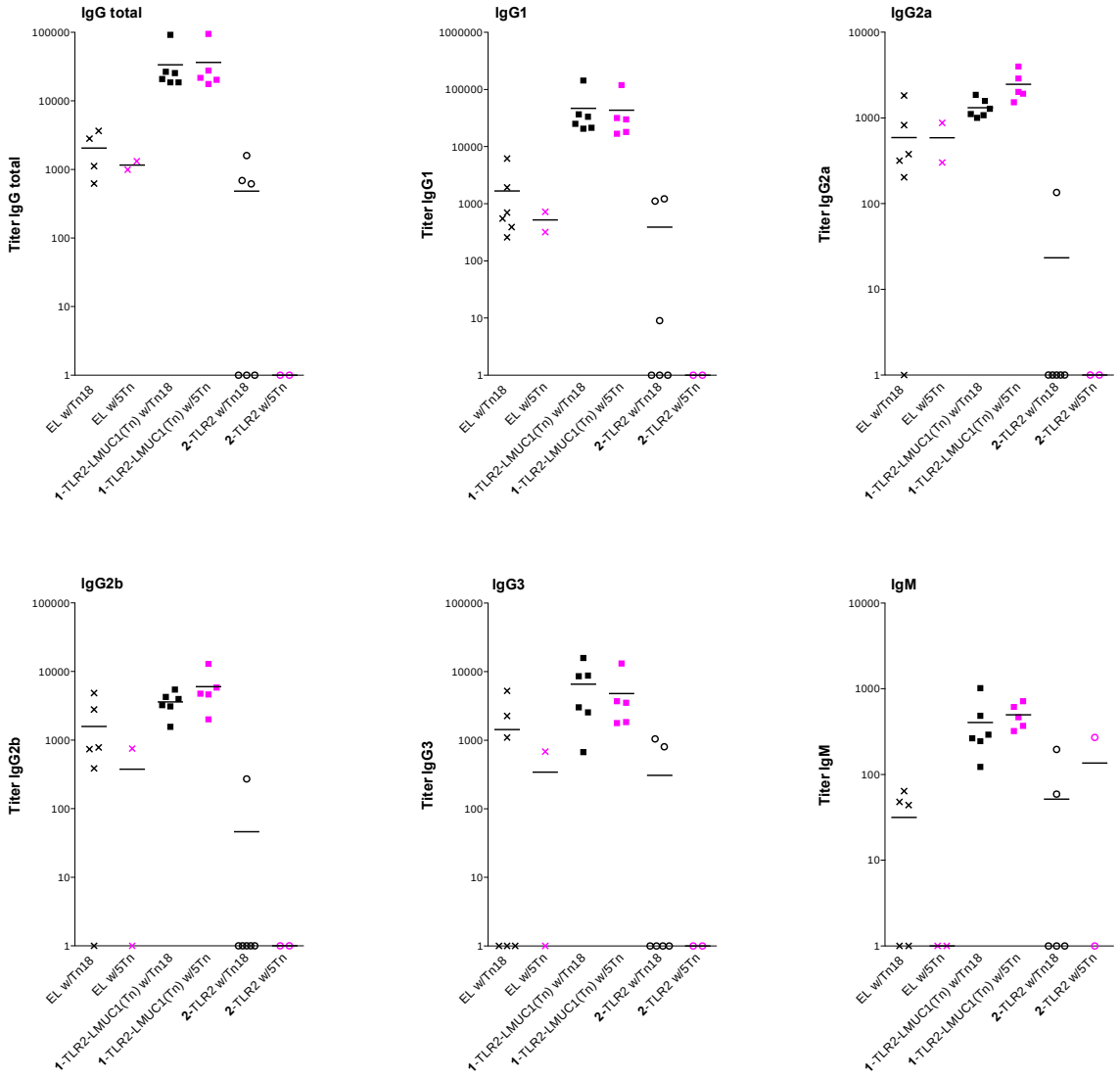


Figure 2.2 Anti-MUC1 IgG and IgM antibody titers determined by enzyme-linked immunosorbent assay (ELISA). Plates were coated with BSA-MICAPGSTAPPAHGVTSAPDT(**α**GalNAc)RPAP conjugate for anti-LMUC1(Tn18) antibody titers and BSA-MICAPGS(**α**GalNAc)T(**α**GalNAc)APPAHGVT(**α**GalNAc)S(**α**GalNAc)APDT(**α**GalNAc)RPAP conjugate for anti-LMUC1(5Tn) antibody titers. Serial dilutions of the sera were allowed to bind to immobilized LMUC1. Detection was accomplished by the addition of phosphate-conjugated anti-mouse antibodies and *p*-nitrophenyl phosphate. Titers were determined by linear regression analysis, plotting dilution *vs.* absorbance. Antibody 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 an individual mouse and the horizontal lines indicate the mean for the group of mice.

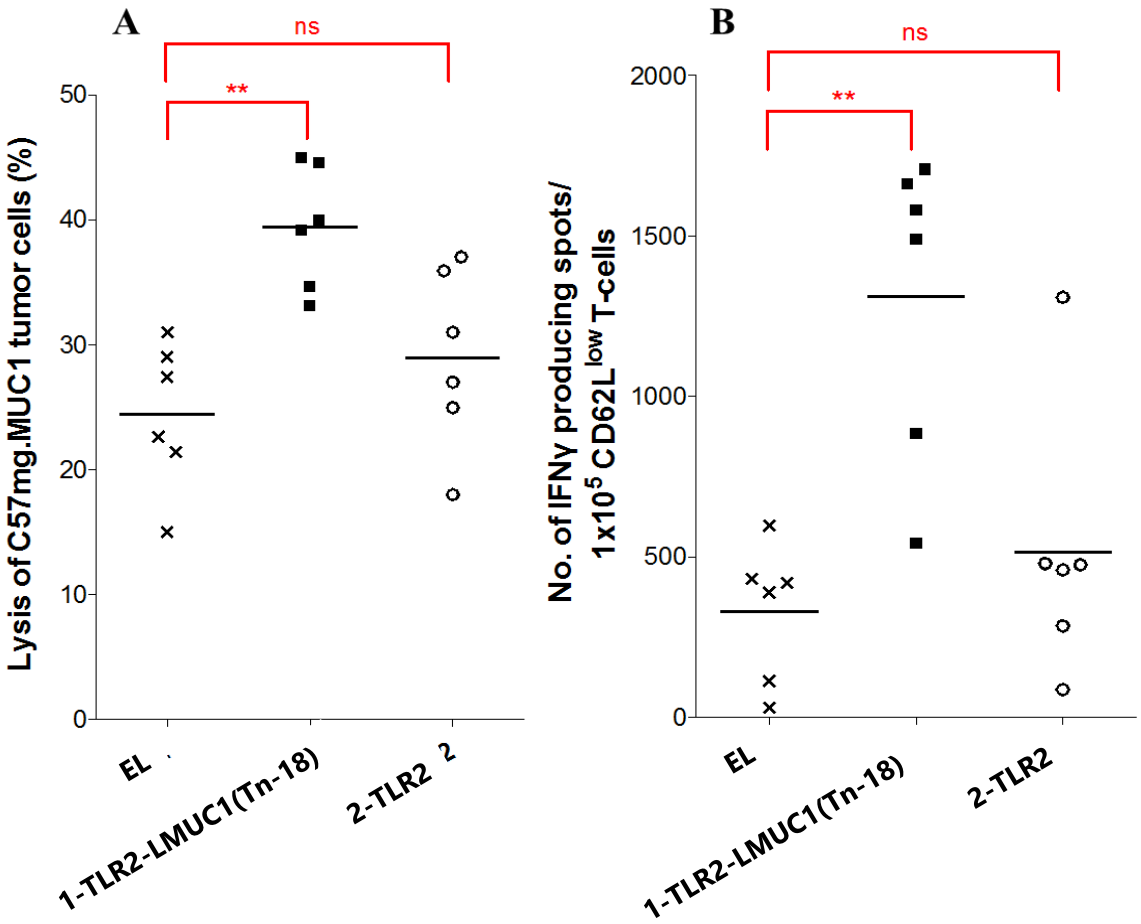


Figure 2.3 (A) Induction of antibody-dependent cell mediated cytotoxicity (ADCC) with C57mg MUC1 tumor cells. C57mg.MUC1 tumor cells were labeled with chromium for 2 h and then incubated with serum (1:25 diluted) obtained from mice immunized with empty liposomes (EL) or liposomes containing **1** or **2** for 30 min at 37 °C. The tumor cells were then incubated with effector cells (NK cells, KY-1 clone) at an effector to target ratio of 50:1 for 4 h after which radioactive ^{51}Cr release was determined. Spontaneous release was 14% of complete release. (B) Induction of cytotoxic $CD62L^{low}$ T-cell response in MUC1.Tg mice. $CD62L^{low}$ T-cells, isolated from tumor-draining lymph nodes, of mice immunized with empty liposomes (EL) or liposomes containing **1** or **2** 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.

To assess the ability of LMUC1 vaccine candidate to activate CTLs, CD62Llow T-cells isolated from tumor-draining lymph nodes by magnetic cell sorting of the

immunized mice were incubated with dendritic cells (DCs) without in vitro stimulation and then were analyzed for MUC1-specific IFN-γ spot formation on ELISPOT plates. It was observed that LMUC1 vaccine candidate **1** exhibited a robust response compared to control groups indicating that the cancer vaccine successfully induced cellular immunity (Fig. 2.3B).

2.4 Conclusions

In summary, we report here the linear preparation of a fully synthetic MUC1-based multi-component cancer vaccine candidate devoid of any artificial linkers that elicit robust antibody titers recognizing monoglycosylated and pentaglycosylated tumor-associated LMUC1 derivatives. Furthermore, our ADCC experiments showed that antiserum of the vaccine candidate was effective in killing cancer cells. Thus, in the presented study, we have found that a fully synthetic multi-component LMUC1 vaccine can generate potent humoral as well cellular immune responses.

2.5 Experimental Procedure

Reagents and general experimental procedure:

Amino acid derivatives and Rink Amide AM LL resin were purchased from Merck Millipore; DMF was purchased from Fisher Scientific. All other chemical reagents were purchased from AnaSpec and Aldrich and were used without further purification. All solvents employed were ACS reagent grade. Reverse Phase HPLC was performed either on an Agilent 1100 or 1200 series system equipped with an autosampler, UV detector, and fraction collector. RP-HPLC was carried out using an Agilent Eclipse XDB-C18 analytical column (5 μm, 4.6 x 250 mm) at a flow rate of 1 mL/min and a Phenomenex Jupiter 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 (95% Acetonitrile, 5% Water, 0.1% TFA) in A (95% Water, 5% Acetonitrile, 0.1% TFA) over a 40 min period unless otherwise specified. High-resolution mass spectra were obtained by using MALDI-ToF (Applied Biosystems 5800 Proteomics Analyzer) with either α-cyano-4-hydroxycinnamic acid or 2,5-Dihydroxybenzoic acid as an internal standard matrix.

General methods for microwave assisted solid phase peptide synthesis (MW-SPPS):

Peptides, glycopeptides and glycolipopeptides were synthesized on a 0.05 or 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 and *N*,*N*-Diisopropylethylamine (DIPEA) as the base for amino acid couplings. Side chain protecting groups for amino acids were 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 Fmoc-Thr[GalNAc(Ac)3-α-D]-OH, Fmoc-Ser[GalNAc(Ac)3-α-D]-OH, *N*-α-fluorenylmethoxycarbonyl-*R*-(2,3-bis(palmitoyloxy)-(2*R*-propyl)-(R)-cysteine, and palmitic acid were carried out manually under microwave irradiation using (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HATU) and 1-Hydroxy-7-azabenzotriazole (HOAt) as activating reagents in presence of *N*,*N*-Diisopropylethylamine (DIPEA) as the base on a CEM Discover SPS instrument. The manual amino acid couplings were monitored by mass spectrometry and standard Kaiser Test.

Synthesis of monoglycosylated vaccine compound 1. The glycolipopeptide vaccine **1** was synthesized by microwave assisted solid phase peptide synthesis (MW-SPPS) on Rink Amide AM LL resin (0.1 mmol). The first four amino acid residues (RPAP) were assembled on resin support using the fully automated CEM LIBERTY 908505 peptide synthesizer equipped with a UV detector. The resin was then removed from the synthesizer and washed with DMF. The Tn moiety was installed manually using N^{α}-Fmoc-Thr-(AcO_3-α-D-GalNAc) (134 mg, 0.2 mmol) in DMF (3 mL), 2-(7-aza-1H-benzotriazole-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (76 mg, 0.2 mmol), 1-Hydroxy-7-azabenzotriazole (HOAt) (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) as the activating reagents. The reaction mixture was microwave-irradiated for 10 minutes and completion of coupling was monitored by Kaiser Test. A small amount of peptide was cleaved from the resin using TFA to confirm completion of coupling reaction by mass spectrometry (MALDI-TOF). The resin was then returned to automated peptide synthesizer to extend the peptide until serine residue. The resin was then removed from a synthesizer, washed with DCM (10 Ml x 3) and further steps were performed manually. The resin containing peptide was then treated with 70% hydrazine in methanol solution for 2.5 hr to remove acetyl groups on the sugar moiety. The resin was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then dried under vacuum. The resin was swelled in DCM for 30 minutes and the lipid moiety was installed manually using N-$^{\alpha}$-Fmoc-R-(2,3-bis(palmitoyloxy)-(2R-propyl)-(R)-cysteine (180 mg, 0.2 mmol) in DMF (5 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) under microwave irradiation. The coupling reactions were monitored by the Kaiser test. Upon completion of the coupling, the resin

was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3). Following that resin was treated with 20% 4-methylpiperidine in DMF (6 mL) under microwave irradiation for 3 minutes to remove *N*-Fmoc group. The final palmitic acid residue (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) in DMF (3 mL). The resin was washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then dried in vacuo. The resin was swelled in DCM (10 mL) for 30 minutes and then treated with the cleavage cocktail (TFA 88%, water 5%, phenol 5%, and TIPS 2%; 10 mL) for 3 h. The resin was filtered and washed with TFA (3 mL). The filtrate was concentrated on a rotary evaporator under vacuum till approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0^{o}C; 25 mL) and recovered by centrifugation at 2,500 rpm for 20 min. The crude glycolipopeptide vaccine construct **1** was 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 with a Phenomenex Jupiter analytical C-4 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 and appropriate fractions were lyophilized to afford pure compound **1**. HR MALDI-TOF MS calculated for $C_{177}H_{307}N_{39}O_{46}S$ [M+H] 3747.2603; observed, 3747.3723.

Synthesis of TLR2 Lipopeptide 2: Lipopeptide 3 was synthesized on Rink Amide AM LL resin (0.1 mmol) following the above described general protocol for MW-SPPS and similar procedure for Lipidation described for compound 1 & 2. After cleavage of lipopeptide from the resin, resulting crude product was 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 with a Phenomenex Jupiter analytical C-4 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 and appropriate fractions were lyophilized to afford pure lipopeptide **2**. $C_{81}H_{158}N_{11}O_{12}S$, MALDI-TOF MS: calculated, [M+H] 1509.1812; observed, [M+H] 1509.2773

Synthesis of monoglycosylated peptide 3 and pentaglycosylated peptide 4: Synthesis of monoglycosylated peptide 1 was straightforward and the similar protocol was used as that for compound **1** except coupling of Pam_3CysSK_4. The pentaglycosylated glycolipopeptide **4** was also synthesized by microwave assisted solid phase peptide synthesis (MW-SPPS) on Rink Amide AM LL resin (0.1 mmol). The first four amino acid residues (RPAP) were assembled on resin support using the fully automated CEM LIBERTY 908505 peptide synthesizer equipped with a UV detector. The resin was then removed from the synthesizer and washed with DMF (5 mL x 2). The first Tn-antigen moiety was installed manually using N^{α}- Fmoc-Thr[GalNAc(Ac)3-α-D]-OH (80.4 mg, 0.12 mmol) in DMF (3 mL), 2-(7-aza-1H-benzotriazole-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (76 mg, 0.2 mmol), 1-Hydroxy-7-azabenzotriazole (HOAt) (27 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) as the activating reagents. The reaction mixture was microwave-irradiated for 10 minutes, and completion of coupling was monitored by Kaiser Test. A small amount of peptide was cleaved from the resin using TFA to confirm completion of coupling reaction by mass spectrometry (MALDI-TOF). The resin was then returned to automated peptide synthesizer to couple next three amino acids until alanine residue. The resin was then removed from a synthesizer, washed with

DCM (10 mL x 3) and coupling of next two glycosylated amino acids were performed manually. The second Tn-antigen moiety was installed using N^{α}- Fmoc-Ser[GalNAc(Ac)3-α-D]-OH (66 mg, 0.1 mmol) in DMF (3 mL) and HATU (38 mg, 0.1 mmol), (HOAt) (14 mg, 0.1 mmol) and DIPEA (67 µL, 0.4 mmol) as the activating reagents. This reaction mixture was subjected to microwave irradiation for 5 minutes at 60^{o}C. After completion of the coupling reaction, Fmoc deprotection of the resulting glycopeptide was achieved using 20% 4-methylpiperidine in DMF (6 mL) under microwave irradiation for 3 minutes. The resin was thoroughly washed with DMF (5 mL x 3) and then with DCM (5 mL x 3). The introduction of third Tn-antigen was also performed manually using by addition of premixed solution of glycosylated amino acid, Fmoc-Thr[GalNAc(Ac)3-α-D]-OH (66 mg, 0.1 mmol), HATU (38 mg, 0.1 mmol), HOAT (14 mg, 0.1 mmol) DIPEA (34 µL, 0.2 mmol) in DMF (3 mL) was added under microwave irradiation for 5 minutes at 60^{o}C. The coupling efficiency was monitored by standard Kaiser Test as well as by MALDI-TOF. The resin was then returned to automated peptide synthesizer to extend next seven amino acid residues on the peptide until alanine residue. The resin was then removed from a synthesizer, washed with DCM (10 Ml x 3) and couplings of fourth and fifth Tn were performed manually using similar conditions as that of conditions for second and third glycosylated amino acids. After successful coupling of fifth Tn-antigen to the peptide backbone, the resin was then returned to automated peptide synthesizer to couple next four amino acids until final cysteine residue. The acetylation of terminal cysteine was also performed on automated peptide synthesizer. The resin containing peptide was then treated with 70% hydrazine in methanol solution for 1.5 hrs. to remove acetyl groups on the sugar moiety. This step was

repeated once more to make sure all acetyl groups are removed. The resin was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then dried under vacuum overnight. The resin was swelled in DCM (10 mL) for 30 minutes and then treated with the cleavage cocktail (TFA 94%, EDT 2.5%, water 2.5%, TIPS 1%; 10 mL) for 3 h. The resin was filtered and washed with TFA (3 mL). The filtrate was concentrated on a rotary evaporator under vacuum till approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0^{o}C; 25 mL) and recovered by centrifugation at 2,500 rpm for 20 min. The crude glycolipopeptide **4** was 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 with a Phenomenex Jupiter analytical 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 and appropriate fractions were lyophilized to afford pure pentaglycosylated glycopeptide compound **4**. This purified glycopeptide was conjugated to maleimide activated BSA. HR MALDI-TOF MS calculated for $C_{133}H_{212}N_{33}O_{57}S$ [M+H] 3215.4420; observed, 3215.4451.

Preparation of Liposomal Formulation for Immunizations: The vaccine candidate **1**, and control TLR2 agonist **2** were incorporated into phospholipid-based small unilamellar vesicles (SUVs) by hydration of a thin film of the synthetic lipid compounds, egg phosphatidylcholine, phosphatidylglycerol and cholesterol in a HEPES buffer (10 mM, pH 6.5) containing NaCl (145 mM) followed by extrusion through the Nuclepore® polycarbonate membranes of pore sizes 1000 nm, 800 nm, 400 nm, 200 nm and 100 nm to achieve uniform sized liposomes.

Immunizations. Eight to 12-week-old MUC1.Tg mice (C57BL/6; H-2b) that express human MUC1 at a physiological level were immunized four times at biweekly intervals at the base of the tail intradermally with liposomal preparations of the vaccine construct (25 µg containing 3 µg of carbohydrate) or with empty liposomes. The endpoint was one week after 4^{th} immunization.

Determination of ADCC. Tumor cells (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 (KY-1 clone), which have high expression of the CD16 receptor, were used as effectors. These cells were stimulated with IL-2 (200 units mL^{-1}) 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. Radioactive ^{51}Cr release was determined using the Topcount Microscintillation Counter (Packard Biosciences). The spontaneous and maximum release of ^{51}Cr was determined. The percentage of specific release was calculated according to the formula: (release-spontaneous release/maximal release-spontaneous release) x 100.

IFN-γ ELISPOT assays. At the time of sacrifice, MAC sorted CD62Low T cells from tumor-draining lymph nodes were isolated from immunized MUC1.Tg mice and used as responders in an IFN-γ ELISPOT assay as described previously.[Mukherjee, 2007, Vaccine 25:1607-1618] 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.

2.6 References

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CHAPTER 3

LINEAR SYNTHESIS AND IMMUNOLOGICAL EVALUATION OF PENTAGLYCOSYLATED LMUC1 MULTICOMPONENT CANCER VACCINE CANDIDATE

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3.1 Abstract

The mucin MUC1, is a large glycoprotein that is expressed on the apical surface of healthy epithelial cells and strongly over-expressed in various tumors, including colon, ovarian, and breast cancers. Therefore, MUC1 is an important target in the development of antitumor vaccines. A predominant characteristic of MUC1 is the variable number of tandem repeats (VNTR) region in its extracellular domain. The VNTR region is comprised of repeating unit of a 20-amino acid sequence (APGSTAPPAHGVTSAPDTRP). In healthy epithelia, the VNTR is highly glycosylated on serine and threonine with long and branched O-linked carbohydrates while the cancerous cells express short and truncated Tumor Associated Carbohydrate Antigens (TACAs) such as Tn. Our previous experiments have established that vaccination with monoglycosylated LMUC1 vaccine elicited robust IgG titers that recognized monoglycosylated and pentaglycosylated LMUC1 glycopeptides so felt compelled to synthesize pentaglycosylated vaccine candidate to test its efficacy in a mouse model compared to the monoglycosylated vaccine. In the presented study, we have synthesized using a linear approach monoglycosylated (Tn-18) as well as pentaglycosylated (Tn-4,Tn-5,Tn-12,Tn-13, and Tn-18) MUC1 vaccine candidates covalently linked to the immunoadjuvant Pam_3CSK_4. The linear synthesis of vaccine candidates was carried out by using microwave assisted solid phase peptide synthesizer with good yields and high HPLC purity. These resulting fully synthetic vaccine candidates were used to immunize MUC1 transgenic mice, however, the additional immunological study is still underway. After four biweekly immunizations, IgG and IgM antibody responses against these vaccine candidates will be measured and compared. Thus, it is to be expected that the

results of this experiment will provide useful insights regarding antigen internalization and processing by APC.

3.2 Introduction

Glycoconjugates are one of the most structurally as well as functionally diverse compounds in nature. The role of protein and lipid-bound glycans in the biological processes such as fertilization, embryogenesis, neuronal development, hormone activities and the proliferation of cells and their organization into specific tissues is well established.[1-3] In addition, the carbohydrates of the host cell are exploited by pathogens for immunological invasion. Carbohydrates also have the ability to induce a protective antibody response during infection by pathogens[4]. More specifically significant changes occur in cell surface carbohydrates during tumor progression, which is an important biomarker for the stage, direction, and fate of tumor growth and progression.[5-10] Numerous tumor-associated carbohydrates have been identified as adhesion molecules, for example, the Lewis antigens sialyl Lewis^x (SLe^x), SLe^x-Le^x and Le^y, which play a critical role in tumor cell invasion as well as metastasis.[11-12]

A large number of epithelial cancer types such as ovarian, breast, colorectal, prostate, and pancreatic cancers exhibit significant alterations in the level of expression and glycosylation pattern of the mucins.[13-16] Mucins are high molecular weight, heterogeneous glycoproteins containing either *O*- or *N*-linked oligosaccharides and are found on the apical surface of epithelial cells. Twenty-one mucins have been identified that possess a similar overall architecture with an *N*-terminal domain followed by a domain containing a variable number of tandem repeats (VNTR), termed as mucin domain. Polymorphic epithelial mucin (PEM, MUC1) is expressed by glandular epithelial

cells and over-expressed by as much as 50-fold and aberrantly glycosylated by the majority of carcinomas.[17-25] Tumor-associated MUC1 can have various glycosylation pattern and can carry the antigens Tn (αGalNAc-Thr), STn (αNeu5Ac-(2,6)-αGalNAc-Thr) and the Thomsen-Friedenreich (TF or T) antigen (αGal-(1,3)-αGalNAc-Thr. These structures can be formed in cancer cells due to a lack of core 1,3-galactosyltransferase (T-synthase). The studies have demonstrated that the expression of T-synthase is regulated by a key molecular chaperone, Cosmc, which resides in the ER and any mutations that lead to loss of function of Cosmc lead to loss of T-synthase activity.[26-27]

Recently, the National Cancer Institute (NCI) identified and prioritized cancer antigens based on various criteria. These criteria were as follows: (a) therapeutic function, (b) immunogenicity, (c) role of the antigen in oncogenicity, (d) specificity, (e) expression level and percent of antigen-positive cells, (f) stem cell expression, (g) number of patients with antigen-positive cancers, (h) number of antigenic epitopes, and (i) cellular location of antigen expression.[28] In this research project, the mucin MUC1 was ranked second out of 75 tumor-associated antigens, which highlights the importance of MUC1 in the development of cancer vaccines. Humoral immune responses against MUC1 have been observed in benign diseases as well as carcinomas. The existence of circulating antibodies against MUC1 during cancer diagnosis directly correlates with promising disease outcome in breast cancer patients.[29-31] The studies have shown that antibodies induced with MUC1 isolated from tumor tissues have recognized MUC1 peptide motifs PDTRP, PPAHGV as well as RPAPGS as the most frequent immunodominant domains of the MUC1 tandem repeat.[32] The specificity of these anti-MUC1 antibodies have been verified when these peptides are modified with Tn- and T-

antigens. It is been hypothesized that the improved binding is due to carbohydrate-induced conformational change of the peptide motif.[22, 32-34]

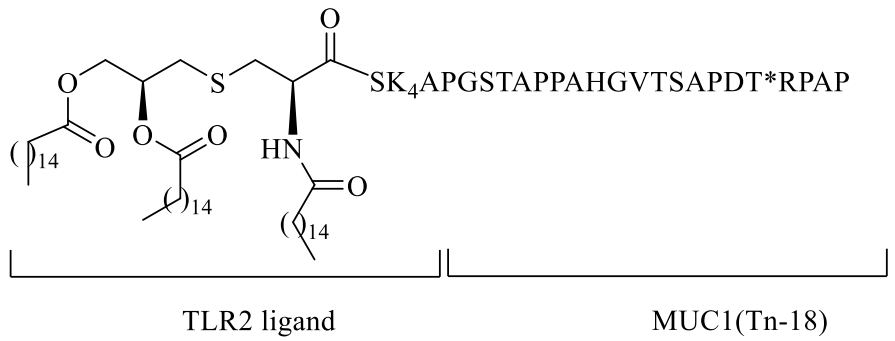
The studies have shown that T-cell epitopes of the MUC1 domain are bundled within cancer cells in their shortened and truncated glycosylation form into MHC class I molecules, resulting in the natural MHC-restricted recognition of MUC1 epitopes containing truncated glycosylation pattern.[35-40] It is also been established that glycosylated MUC1 epitopes have a higher binding affinity towards MHC class I mouse allele $H2k^b$ compared with their non-glycosylated counterparts and can activate cytotoxic T-lymphocytes (CTL).[41] The recent studies have identified numerous MUC1-based HLA-A2-binding peptide epitopes that include SAPDTRAPG and STAPPAHGV. [42-45]

Until very recently, the attempts to develop the MUC1-derived anti-cancer vaccine were mainly concentrated on utilizing different length non-glycosylated MUC1 tandem repeat peptides conjugated to carrier proteins and were co-administered with or without an adjuvant.[46-54] In general such approaches failed mainly due to their inability to generate effective immune responses to MUC1 expressing tumor cells.[47] For example, one such lung cancer vaccine, Stimuvax® containing unglycosyated full length MUC1 showed promising results in phase I and II clinical trials; however, failed to demonstrate a significant improvement in overall survival in phase III study.[55-56] In this regard, conformational studies by NMR and light scattering measurements have shown that non-glycosylated MUC1 peptides form less extended and globular structures compared to their glycosylated counterparts. The carbohydrates on the MUC1 are responsible for conformational changes and hence differences were seen in antibody binding of MUC1 related glycopeptides and peptides.[57] The immunological studies of Tn-or STn conjugate

with carrier proteins have been performed, however, these vaccines could not elicit significant IgG or IgM antibody titers.[58-61] One of the major drawbacks of such vaccines is immune suppression by the carrier protein and hence cannot activate CTL responses. Clauson and coworkers previously reported chemoenzymatically synthesized extended MUC1 TR glycopeptides containing different densities of Tn and STn conjugated to KLH carrier protein. These 60-mer MUC1 derived vaccines with different pattern of Tn-glycosylations could only elicit modest immune responses; however, this study along with other recent studies highlighted the importance of MUC1 glycosylation pattern in the development of anti-cancer vaccine.[62-63]

Boons and coworkers have previously reported the synthesis and immunological evaluation of the vaccine candidate containing MUC1 derived glycopeptide as a B-cell epitope and polio virus-based T-cell peptide epitope covalently linked to immunoadjuvant lipopeptide Pam_3CSK_4. The immunological study of this MUC1 tripartite vaccine showed that this glycolipopeptide was able to elicit specific humoral and cellular immune responses and could reduce tumor size in MUC1.Tg mice.[64] In addition, Finn and coworkers reported that carbohydrates such as *O*-linked GalNAc on the MUC1 glycopeptides are removed during glycoprotein processing by dendritic cells in the major histocompatibility complex (MHC) class II pathway.[65] Since the pattern of *O*-linked glycans on MUC1 control glycoprotein processing by APCs and play a critical role in the extent and site specificity of cathepsin-mediated proteolysis[66] and our previous immunological study have established that monoglycosylated LMUC1 vaccine elicited robust IgG titers that recognized not only monoglycosylated LMUC1 epitope but also pentaglycosylated LMUC1 epitope, we here report the synthesis and immunological

evaluation vaccine candidate consisting pentaglycosylated full-length MUC1 peptide sequences and built-in immunoadjuvant, Pam_3CSK_4 (Figure 3.1) . We expect that the findings of this experiment will be used to identify the MHC-II binding glycopeptide epitopes that can elicit specific antibody responses.



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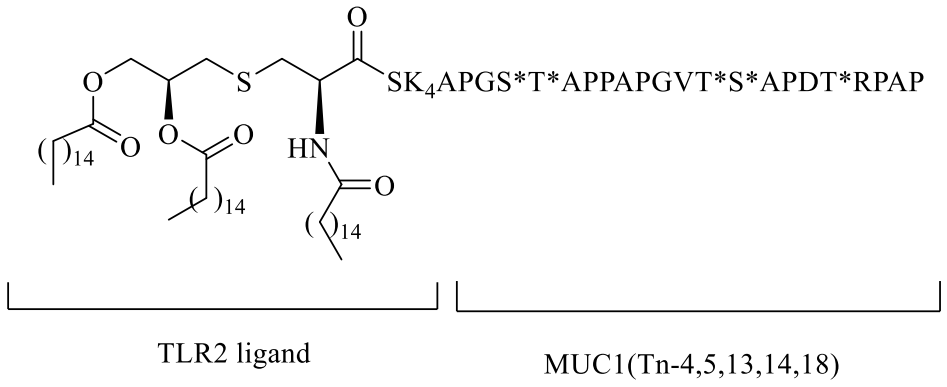

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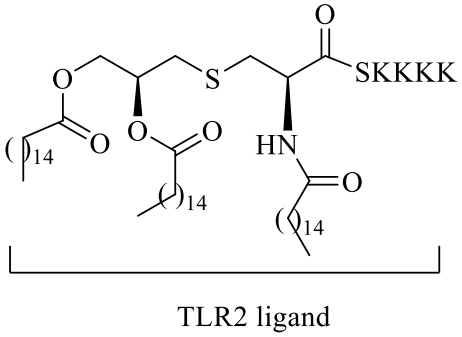

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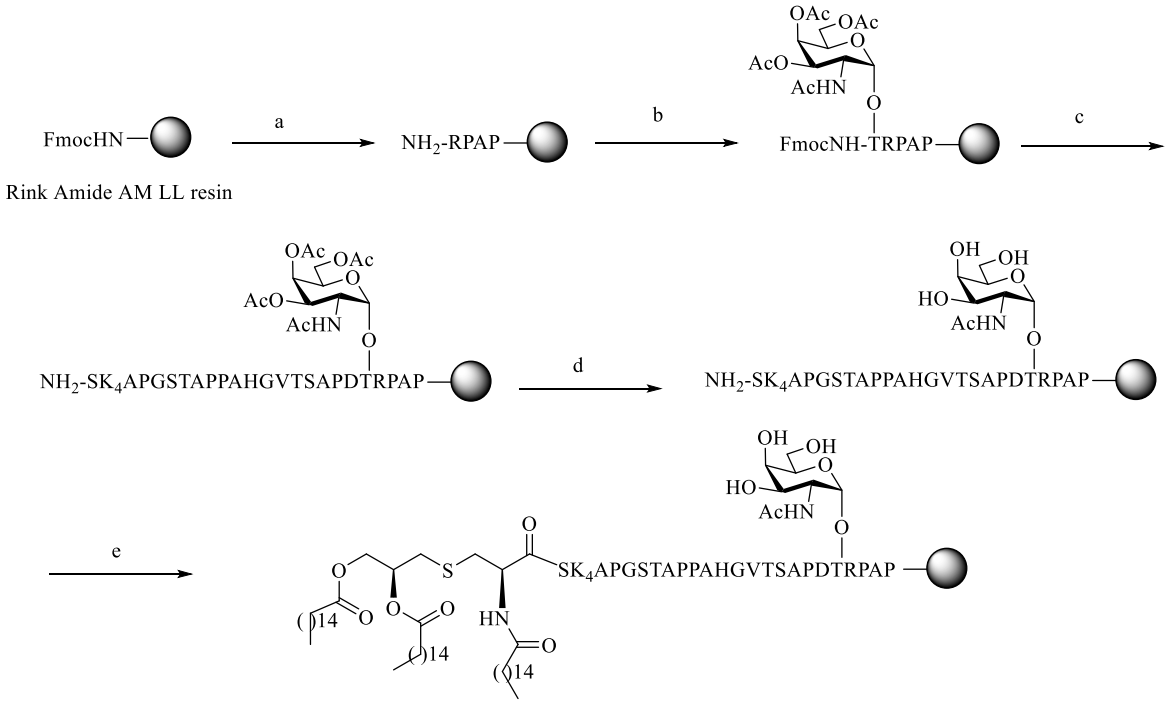
Figure 3.1 Chemical Structures of Synthetic Vaccine Candidates

3.3 Result and Discussion

Researchers have used native chemical ligation to synthesize vaccines containing larger peptides, glycopeptides, and glycolipopeptides. Native chemical ligation chemistry involves the thiolate of an *N*-terminal cysteine residue of one peptide fragment attacking the *C*-terminal thioester of a second peptide fragment to effect trans-thioesterification followed by an amide linkage formation after rapid *S*→*N* acyl transfer. However, recent literature reports suggest that microwave assisted solid phase synthesis have greatly improved the efficiency of solid phase synthesis by providing peptide products with higher purity with shorter reaction time.[67-68] Hence we utilized microwave assisted solid phase peptide synthesis chemistry in preparing cancer vaccine candidate in a linear fashion.

The vaccine candidate **1** containing a MUC1-derived B epitope and T helper peptide sequence, in addition to the TLR2 ligand, Pam_3CysSK_4 was synthesized by following protocols described before with the help of MW-SPPS as shown in the scheme 3.1. The first four amino acids, RPAP were assembled on Rink Amide AM LL resin using a CEM Liberty 12-channel automated peptide synthesizer equipped with microwave heating assembly. The amino acid couplings on the synthesizer were achieved using HBTU-mediated HOBt ester activation chemistry and Fmoc-deprotection of the amino acids was accomplished using a solution of 20% 4-methylpiperidine in DMF. The introduction of sugar amino acid, Fmoc-protected Tn was performed manually under microwave irradiation using HATU/HOAt activation protocol. After coupling of Tn-antigen the Rink Amide AM resin was returned to CEM automated peptide synthesizer to add subsequent amino acid residues. After coupling of serine amino acid, the resin was

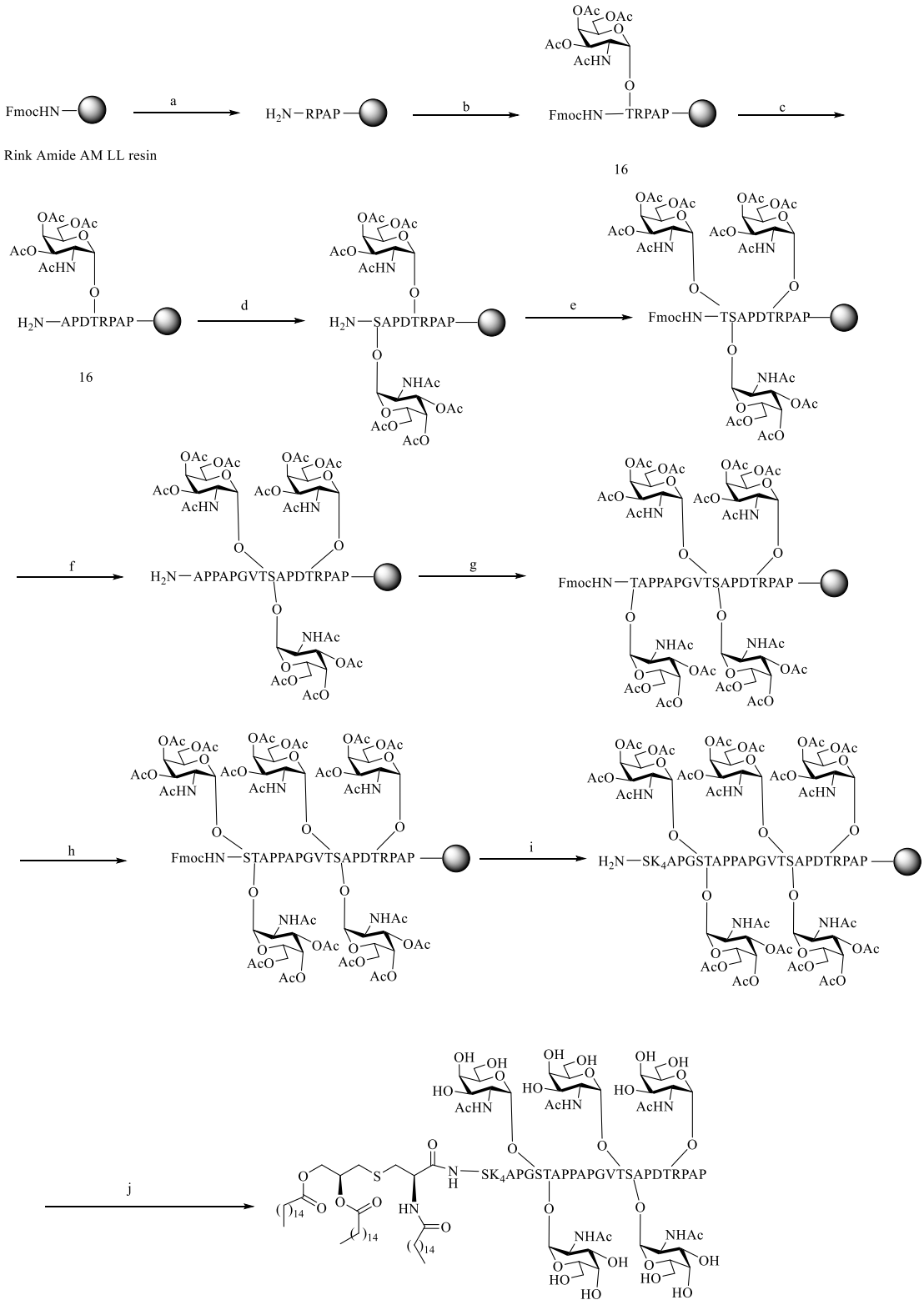
removed from automated peptide synthesizer and was then treated with a solution of 70% hydrazine in methanol to remove acetyl groups of the Tn-antigen. Following the deacetylation, the Fmoc-Pam_2Cys, as well as final palmitic acid residues, were coupled to glycopeptide manually under microwave irradiation using HATU/HOTU in presence of base, DIPEA in DMF. The global deprotection of amino acid side chains and glycolipopeptides cleavage from the resin was performed under acidic condition using cocktail containing 88% TFA, 5% phenol, 5% H_2O, and 2% TIPS. The crude product was then purified by RP-HPLC using C4 column to get final glycolipopeptide **1**.



Scheme 3.1 Synthesis of Vaccine Candidate 1 using MW-SPPS

Reagents and conditions: (a) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (b) Tn-COOH(1.2 eq), HATU, HOAt, DIPEA, DMF, MW 5 min. (c) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (d) 70% Hydrazine in MeOH, 2.5 hr (e) (i) Fmoc-Pam_2Cys-OH, HATU, HOAt, DIPEA, DMF, MW - 10 min x 2 (ii) 20% 4-Methylpiperidine in DMF, MW, 3 min (iii) Palmitic Acid, HATU, HOAt, DIPEA, DMF, MW, 10 min (iv) 87.5% TFA, 5% Phenol, 5% H_2O, 2.5% TIPS

After preparation of vaccine candidate **1**, we focused on a synthesis of penta-glycosylated glycolipopeptide vaccine candidate **2**, which also consists of an MUC1-derived B-epitope and T-helper peptide sequence but additionally it contains five glycosylated amino acids and a covalently linked TLR2 ligand Pam_3CysSK_4. During the synthesis of the pentaglycosylated peptide, multiple synthetic difficulties were encountered as described in our earlier study. After installment of the second glycosylated amino acid followed by deprotection of *N*-terminus Fmoc protecting group, 10-20% of acetyl groups migrated to the free amino group giving rise to chain termination. Similar results were observed during coupling of 3^{rd}, $4^{th,}$ and 5^{th} glycosylated amino acids. This resulted in a significant decrease in the yield of desired product. To overcome this difficulty, microwave assisted methods for coupling and deprotection of glycosylated amino acids were developed and employed. It is worth noting that the amount of glycosylated amino acid, lower coupling temperature and coupling time was the key factors to overcome chain termination. The following final scheme 3.2 was developed after multiple unsuccessful attempts. As shown in the scheme 3.2, first four amino acids were introduced by using automated peptide synthesizer followed by manual coupling of Fmoc-protected Tn-antigen. Following this, the resin was returned to automated peptide synthesizer for coupling of next three amino acids. The coupling of glycosylated serine was performed manually under microwave irradiation. Limiting the amount of glycosylated amino acid to 1 equivalent, lowering temperature to 60^oC in addition to shorter reaction time (3 minutes) was used for further glycosylated amino acid coupling reaction to avoid unwanted acetyl group migration. The coupling of all



Scheme 3.2 Synthesis of pentaglycosylated Vaccine Candidate **2**

Reagents and conditions: (a) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (b)Tn-COOH (1.2 eq.), HATU, HOAt DIPEA, DMF MW - 5 min (c) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (d) (i) Fmoc-Tn-COOH (1 eq.), HATU, HOAt DIPEA, DMF MW, 5 min (ii) 20% 4-Methylpiperidine, DMF, MW, 3 min (e) Fmoc-Tn-COOH(1 eq.), HATU, HOAt, DIPEA, DMF, MW 5 min. (f) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (g) (i) Fmoc-Tn-COOH (1 eq.), HATU, HOAt DIPEA, DMF MW, 5 min (ii) 20% 4-Methylpiperidine, DMF, MW, 3 min (h) Fmoc-Tn-COOH(1 eq.), HATU, HOAt, DIPEA, DMF, MW 5 min (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (j) (i)70% Hydrazine in MeOH, 3 hr (ii) Fmoc-Pam2Cys-OH, HATU, HOAt, DIPEA, DMF, MW - 10 min x 2 (iii) 20% 4-Methylpiperidine, DMF, MW, 3 min (iv) Palmitic Acid, HATU, HOAt, DIPEA, DMF, MW, 10 min (v) 87.5% TFA, 5% Phenol, 5% H2O, 2.5% TIPS

glycosylated amino acids were performed manually using this modified coupling protocol. After extending glycopeptide till final serine residue on automated peptide synthesizer, the glycopeptide was deacetylated and then coupled with lipid residues using a similar procedure as that of vaccine candidate **1**. Thus as shown in the scheme 3.2, the synthesis of vaccine candidate **2** was achieved using modified protocol with help of automated and manual MW-SPPS. The TLR2 ligand compound **3**, Pam_3CysSK_4 was also prepared using similar protocol under microwave irradiation conditions. All these compounds were purified by RP-HPLC on C4 column.

The resulting compounds **1**, **2** and **3** were incorporated into phospholipid-based small unilamellar vesicles (SUVs) by hydration of a thin film of the synthetic lipid compounds, egg phosphatidylcholine, phosphatidylglycerol and cholesterol in a HEPES buffer (10 mM, pH 6.5) containing NaCl (145 mM) followed by extrusion through the Nuclepore® polycarbonate membranes of pore sizes 1000 nm, 800 nm, 400 nm, 200 nm and 100 nm to achieve uniform sized liposomes.

The immunological properties of these vaccines were explored using groups of MUC1.Tg mice (C57BL/6; H-2^b) that express human MUC1 at physiological levels. Three different groups if mice were immunized with the liposomal preparation of vaccines **1** and **2** as well as with TLR2 ligand compound **3** (containing 3 μg of carbohydrate) four times intradermally at the base of the tail at biweekly intervals. One week after the third immunization, the blood were collected. The mice final bleed was collected and mice were sacrificed after two weeks of fourth immunization. The humoral immune responses will be assessed by titers of MUC1-specific antibodies.

3.4 Conclusion

In Summary, we proposed and successfully prepared pentaglycosylated LMUC1 vaccine candidate covalently linked to the immunoadjuvant Pam_3CSK_4 using linear approach. The linear synthesis of vaccine candidates was carried out using microwave assisted solid phase peptide synthesizer and modified coupling conditions for glycosylated amino acids that provided glycolipopeptides in high yields and better HPLC purity. Even though biological experiments are underway, we anticipate that our results will provide useful insights regarding antigen uptake, processing, and presentation by APCs to the cells of immune system. Further, it is our belief that this result will help designing next generation cancer vaccine candidate by providing information regarding glycosylation pattern necessary for induction of robust IgG antibody titers.

3.5 Experimental Section

Reagents and general experimental procedure:

Amino acid derivatives and Rink Amide AM LL resin were purchased from Merck Millipore; DMF was purchased from Fisher Scientific. All other chemical reagents were

purchased from AnaSpec and Aldrich and were used without further purification. All solvents employed were ACS reagent grade. Reverse Phase HPLC was performed either on an Agilent 1100 or 1200 series system equipped with an autosampler, UV detector, and fraction collector. RP-HPLC was carried out using an Agilent Eclipse XDB-C18 analytical column (5 μm, 4.6 x 250 mm) at a flow rate of 1 mL/min and a Phenomenex Jupiter 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 (95% Acetonitrile, 5% Water, 0.1% TFA) in A (95% Water, 5% Acetonitrile, 0.1% TFA) over a 40 min period unless otherwise specified. High-resolution mass spectra were obtained by using MALDI-ToF (Applied Biosystems 5800 Proteomics Analyzer) with either α-cyano-4-hydroxycinnamic acid or 2,5-Dihydroxybenzoic acid as an internal standard matrix.

General methods for microwave assisted solid phase peptide synthesis (MW-SPPS):

Peptides, glycopeptides and glycolipopeptides were synthesized on a 0.05 or 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 and *N*,*N*-Diisopropylethylamine (DIPEA) as the base for amino acid couplings. Side chain protecting groups for amino acids were 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 Fmoc-Thr[GalNAc(Ac)3-α-D]-OH, Fmoc-Ser[GalNAc(Ac)3-α-D]-OH, *N*-α-fluorenylmethoxycarbonyl-*R*-(2,3-bis(palmitoyloxy)-(2*R*-propyl)-(R)-cysteine, and palmitic acid were carried out manually under microwave

irradiation using (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HATU) and 1-Hydroxy-7-azabenzotriazole (HOAt) as activating reagents in presence of *N*,*N*-Diisopropylethylamine (DIPEA) as the base on a CEM Discover SPS instrument. The manual amino acid couplings were monitored by mass spectrometry and standard Kaiser Test.

Synthesis of monoglycosylated vaccine compound 1. The glycolipopeptide vaccine **1** was synthesized by microwave assisted solid phase peptide synthesis (MW-SPPS) on Rink Amide AM LL resin (0.1 mmol). The first four amino acid residues (RPAP) were assembled on resin support using the fully automated CEM LIBERTY 908505 peptide synthesizer equipped with a UV detector. The resin was then removed from the synthesizer and washed with DMF. The Tn moiety was installed manually using N^{α}-Fmoc-Thr-(AcO_3-α-D-GalNAc) (134 mg, 0.2 mmol) in DMF (3 mL), 2-(7-aza-1H-benzotriazole-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (76 mg, 0.2 mmol), 1-Hydroxy-7-azabenzotriazole (HOAt) (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) as the activating reagents. The reaction mixture was microwave-irradiated for 10 minutes and completion of coupling was monitored by Kaiser Test. A small amount of peptide was cleaved from the resin using TFA to confirm completion of coupling reaction by mass spectrometry (MALDI-TOF). The resin was then returned to automated peptide synthesizer to extend the peptide until serine residue. The resin was then removed from a synthesizer, washed with DCM (10 Ml x 3) and further steps were performed manually. The resin containing peptide was then treated with 70% hydrazine in methanol solution for 2.5 hr to remove acetyl groups on the sugar moiety. The resin was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then

dried under vacuum. The resin was swelled in DCM for 30 minutes and the lipid moiety was installed manually using N-$^{\alpha}$-Fmoc-R-(2,3-bis(palmitoyloxy)-(2R-propyl)-(R)-cysteine (180 mg, 0.2 mmol) in DMF (5 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) under microwave irradiation. The coupling reactions were monitored by the Kaiser test. Upon completion of the coupling, the resin was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3). Following that resin was treated with 20% 4-methylpiperidine in DMF (6 mL) under microwave irradiation for 3 minutes to remove *N*-Fmoc group. The final palmitic acid residue (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) in DMF (3 mL). The resin was washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then dried in vacuo. The resin was swelled in DCM (10 mL) for 30 minutes and then treated with the cleavage cocktail (TFA 88%, water 5%, phenol 5%, and TIPS 2%; 10 mL) for 3 h. The resin was filtered and washed with TFA (3 mL). The filtrate was concentrated on a rotary evaporator under vacuum till approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0^{o}C; 25 mL) and recovered by centrifugation at 2,500 rpm for 20 min. The crude glycolipopeptide vaccine construct **1** was 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 with a Phenomenex Jupiter analytical C-4 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 and appropriate fractions were lyophilized to afford pure compound **1**.

HR MALDI-TOF MS calculated for $C_{177}H_{307}N_{39}O_{46}S$ [M+H] 3747.2603; observed, 3747.3723.

Synthesis of pentaglycosylated vaccine compound 2. The pentaglycosylated glycolipopeptide vaccine 2 was also synthesized by microwave assisted solid phase peptide synthesis (MW-SPPS) on Rink Amide AM LL resin (0.1 mmol). The first four amino acid residues (RPAP) were assembled on resin support using the fully automated CEM LIBERTY 908505 peptide synthesizer equipped with a UV detector. The resin was then removed from the synthesizer and washed with DMF (5 mL x 2). The first Tn-antigen moiety was installed manually using N^{α}- Fmoc-Thr[GalNAc(Ac)3-α-D]-OH (134 mg, 0.2 mmol) in DMF (3 mL), 2-(7-aza-1H-benzotriazole-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (76 mg, 0.2 mmol), 1-Hydroxy-7-azabenzotriazole (HOAt) (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) as the activating reagents. The reaction mixture was microwave-irradiated for 10 minutes and completion of coupling was monitored by Kaiser Test. A small amount of peptide was cleaved from the resin using TFA to confirm completion of coupling reaction by mass spectrometry (MALDI-TOF). The resin was then returned to automated peptide synthesizer to couple next three amino acids until alanine residue. The resin was then removed from a synthesizer, washed with DCM (10 mL x 3) and coupling of next two glycosylated amino acids were performed manually. The second Tn-antigen moiety was installed using N^{α}- Fmoc-Ser[GalNAc(Ac)3-α-D]-OH (66 mg, 0.1 mmol) in DMF (3 mL) and HATU (38 mg, 0.1 mmol), (HOAt) (14 mg, 0.1 mmol) and DIPEA (67 µL, 0.4 mmol) as the activating reagents. This reaction mixture was subjected to microwave irradiation for 5 minutes at 60^{o}C. After completion of the coupling reaction, Fmoc

deprotection of the resulting glycopeptide was achieved using 20% 4-methylpiperidine in DMF (6 mL) under microwave irradiation for 3 minutes. The resin was thoroughly washed with DMF (5 mL x 3) and then with DCM (5 mL x 3). The introduction of third Tn-antigen was also performed manually using by addition of premixed solution of glycosylated amino acid, Fmoc-Thr[GalNAc(Ac)3-α-D]-OH (66 mg, 0.1 mmol), HATU (38 mg, 0.1 mmol), HOAT (14 mg, 0.1 mmol) DIPEA (34 µL, 0.2 mmol) in DMF (3 mL) was added under microwave irradiation for 5 minutes at 60^{o}C. The coupling efficiency was monitored by standard Kaiser Test as well as by MALDI-TOF. The resin was then returned to automated peptide synthesizer to extend next seven amino acid residues on the peptide until alanine residue. The resin was then removed from a synthesizer, washed with DCM (10 Ml x 3) and couplings of fourth and fifth Tn were performed manually using similar conditions as that of conditions for second and third glycosylated amino acids. After successful coupling of fifth Tn-antigen to the peptide backbone, the resin was then returned to automated peptide synthesizer to couple next eight amino acids until final serine residue. The resin containing peptide was then treated with 70% hydrazine in methanol solution for 1.5 hr. to remove acetyl groups on the sugar moiety. This step was repeated once more to make sure all acetyl groups are removed. The resin was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then dried under vacuum. The resin was swelled in DCM for 30 minutes and the lipid moiety was installed manually using N-$^{\alpha}$-Fmoc-R-(2,3-bis(palmitoyloxy)-(2R-propyl)-(R)-cysteine (180 mg, 0.2 mmol) in DMF (5 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) under microwave irradiation. The coupling reactions were monitored by the Kaiser test. Upon completion of the coupling, the resin

was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3). Following that resin was treated with 20% 4-methylpiperidine in DMF (6 mL) under microwave irradiation for 3 minutes to remove *N*-Fmoc group. The final palmitic acid residue (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) in DMF (3 mL). The resin was washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then dried in vacuo. The resin was swelled in DCM (10 mL) for 30 minutes and then treated with the cleavage cocktail (TFA 88%, water 5%, phenol 5%, and TIPS 2%; 10 mL) for 3 h. The resin was filtered and washed with TFA (3 mL). The filtrate was concentrated on a rotary evaporator under vacuum till approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0^{o}C; 25 mL) and recovered by centrifugation at 2,500 rpm for 20 min. The crude glycolipopeptide vaccine construct **1** was 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 with a Phenomenex Jupiter analytical C-4 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 and appropriate fractions were lyophilized to afford pure pentaglycosylated glycolipopeptide compound **2**. HR MALDI-TOF MS calculated for $C_{209}H_{360}N_{43}O_{66}S$ [M+H] 4560.5856; observed, 4560.3677.

Synthesis of TLR2 Lipopeptide 3: Lipopeptide 3 was synthesized on Rink Amide AM LL resin (0.1 mmol) following the above described general protocol for MW-SPPS and similar procedure for Lipidation described for compound 1 & 2. After cleavage of lipopeptide from the resin, resulting crude product was 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 with a Phenomenex Jupiter analytical C-4 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 and appropriate fractions were lyophilized to afford pure lipopeptide **3**. $C_{81}H_{158}N_{11}O_{12}S$, MALDI-TOF MS: calculated, [M+H] 1509.1812; observed, [M+H] 1509.2773

Preparation of Liposomal Formulation for Immunizations: The vaccine candidate compounds **1**, **2** and **3** were incorporated into phospholipid-based small unilamellar vesicles (SUVs) by hydration of a thin film of the synthetic lipid compounds, egg phosphatidylcholine, phosphatidylglycerol and cholesterol in a HEPES buffer (10 mM, pH 6.5) containing NaCl (145 mM) followed by extrusion through the Nuclepore® polycarbonate membranes of pore sizes 1000 nm, 800 nm, 400 nm, 200 nm and 100 nm to achieve uniform sized liposomes.

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CHAPTER 4

STUDY ON THE EFFECT OF LINKERS IN MONOGLYCOSYLATED LMUC1-BASED MULTICOMPONENT CANCER VACCINE CANDIDATES.

***Nitin T Supekar**, Sourav Sarkar, Anju Sirohiwal, Margreet Wolfert, Sandra Gendler, Geert-Jan Boons. *To be submitted to Chem. Comm.*

4.1 Abstract

Mucins (MUC) are high molecular weight transmembrane glycoproteins expressed on the apical surface of various epithelial cells in the lung, eyes, stomach, gastrointestinal tract as well as several other organs. Mucins are involved in numerous biological processes, which include cell-to-cell and cell-to-matrix adhesion, signal transduction, and modulation of the immune system. Mucin-1 (MUC1), a member of mucin family, is overexpressed in nearly 70% of carcinomas along with significantly short and truncated glycan pattern on these cells compared to normal MUC1.[1] Recently; it was demonstrated that MUC1 can be immunogenic and could be a potent target for cancer immunotherapy. Unfortunately, MUC1 glycopeptide alone is weakly immunogenic and needs additional stimulator to elicit a strong immune response. We have designed vaccine candidates comprising of a full-length MUC1 sequence and built-in immunoadjuvant. We anticipate that the full-length sequence of MUC1 offers a tantalizing antigen that contains multiple epitopes that can activate B-cells, helper T-cells, and cytotoxic T-lymphocytes. The sequence SAPDT(*O*-GalNAc)RPAP has been established as an attractive B-epitope. However, it is also an MHC class I (K^b) epitope that can activate cytotoxic lymphocytes. Glycosylation of this peptide is critical for eliciting antibodies and activate $CD8^+$ cells that can eliminate MUC1-expressing cancer cells. Furthermore, STAPPAHGV and PAHGVTSA are predicted MHC class I (A^b) epitopes. Analysis of the full-length sequence by Rankpep indicated that STAPPAHGVTSA of MUC1 can function as a *promiscuous* helper T-epitope (class II). The lipopeptide Pam_3CSK_4, a ligand of Toll-like receptor 2 (TLR2), plays a key role in the innate immune system and can serve as a self-adjuvanting immune stimulant in

vaccines. In our previous studies, we designed a number of cancer vaccine candidates that are composed of differently glycosylated full-length MUC1 VNTR unit directly linked to Pam_3CSK_4. However, it is possible that the lysine moieties of Pam_3CSK_4 along with amino acids of MUC1 peptide may create neo-epitopes and, as a result, may affect its immunological properties. Therefore a number of compounds were prepared in which the MUC1 glycopeptide was attached to Pam_3CysSK_4 through an acid sensitive and acid stable spacer. These resulting entirely synthetic vaccine candidates were used to immunize MUC1 transgenic mice. After four biweekly immunizations, IgG and IgM antibody responses against these vaccine candidates were measured, and further immunological experiments are underway.

4.2 Introduction

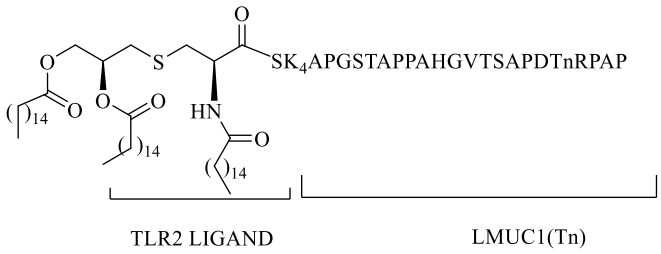
Mucin 1(MUC1) is an important tumor-associated antigen (TAA) as it is overexpressed in most adenocarcinomas. It is a type I transmembrane glycoprotein containing a dense pattern of glycosylation and is overexpressed on the cell surface of many carcinomas.[2-5] Overexpression of MUC1 or glycosylation mostly accompanies the development of cancer and influences various biological processes such as cellular growth, differentiation, adhesion, invasion as well as immune surveillance.[6] In a recent report of National Cancer Institute (NCI), MUC1 was ranked second out of 75 tumor-associated antigens on the basis of their therapeutic function, oncogenicity, immunogenicity, specificity, expression levels and stem cell expression, % positive cells, number of patients with antigen positive cancers, number of epitopes and cellular location of expression.[7] Normal MUC1 and tumor-associated MUC1 display significant structural differences in their glycan density as well as glycan chain lengths. Tumor-

associated MUC1 is much shorter and display less dense carbohydrate distribution pattern compared to normal MUC1.[6] This aberrant glycosylation on tumor cells exposes backbone peptide epitopes as well as TACAs to the immune system.[8] As a result, MUC1 is used as a diagnostic marker in cancer and emerging as an exciting therapeutic target for developing novel approaches for the treatment of epithelial cancer.

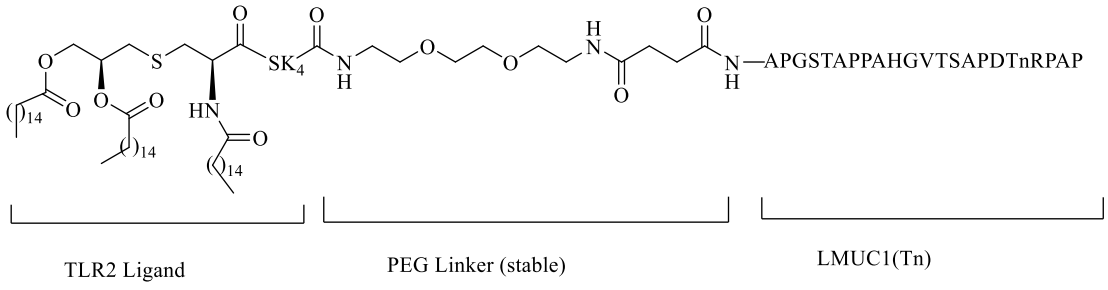
MUC1 in its extracellular part contains a large variable number of tandem repeats (VNTR) consisting of a sequence of 20 amino acids, APGSTAPPAHGVTSAPDTRP with five potential sites for *O*-glycosylation. This glycosylation on MUC1 is critical for its various biological functions.[9] Normally, *O*-glycosylation pattern on the cancer cells is truncated and gives rise to tumor-associated carbohydrate antigens (TACAs) such as Tn antigen, Thomsen–Friedenreich antigen (T antigen) as well as their sialylated versions. However, they are very low immunogenic and need to be conjugated with an external immunoadjuvant.[10] The site and type of glycosylation can have significant impact on the confirmation as well as immunogenic properties of tumor-associated MUC1 glycopeptide epitopes.[11-12] The study of the relationship between the glycosylation type on the peptide and its immunogenicity play a major role in vaccine development. Studies have established that the sequences PDTRP, PPAHGV, and RPAPGS of MUC1 tandem repeat are immunodominant motifs.[13-14] Moreover, studies have shown that T-cell epitopes of the MUC1 domain are bundled within cancer cells in their shortened and truncated glycosylation form into MHC class I molecules, resulting in the natural MHC-restricted recognition of MUC1 epitopes containing truncated glycosylation pattern.[15-20]. It has also been established that glycosylated MUC1 epitopes have a higher binding affinity towards MHC class I mouse allele $H2k^b$ compared with their non-glycosylated counterparts and

can activate cytotoxic T-lymphocytes (CTL).[21] The recent studies have identified numerous MUC1-based HLA-A2-binding peptide epitopes that include SAPDTRAPG and STAPPAHGV. [22-25]

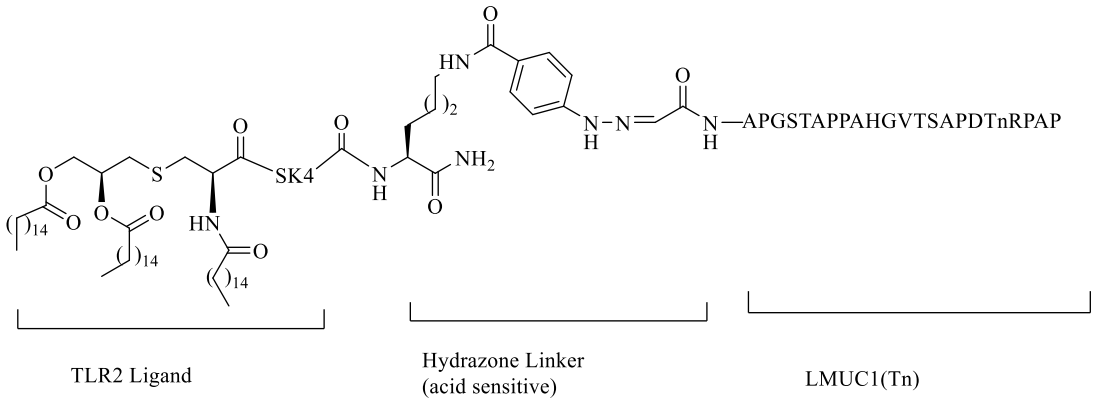
In our earlier experiments, we performed microwave-assisted solid phase synthesis of MUC1 based vaccine candidates containing mono- as well as multiglycosylation with Tn antigen. These vaccine candidates are comprised of full-length VNTR of MUC1 covalently conjugated with immunoadjuvant TLR2 ligand, P_3CSK_4. We designed these vaccines such a way that it contained multiple MUC1 immunodominant motifs like PPAHGV and glycosylated version of PDTRP, STAPPAHGV, and PAHGVTSA (predicted MHC class I (A^b) epitopes) and sequence STAPPAHGVTSA which can function as a promiscuous helper T-epitope (shown by Rankpep analysis). Our earlier findings suggest that monoglycosylated vaccine candidate elicits higher antibody titers compared to pentaglycosylated MUC1 vaccine candidate due to impaired susceptibility to antigen processing. Therefore, in this study, we focused on the synthesis and immunological evaluation of monoglycosylated vaccine candidates containing different linkers between MUC1 peptide sequence and TLR2 ligand. We hypothesize that the lysine moieties of Pam_3CSK_4 along with MUC1 peptide may create a neoepitope that can affect antigen uptake, processing as well as a presentation by APCs. Therefore, a number of vaccine compounds were prepared in which the MUC1 glycopeptide was attached to Pam_3CysSK_4 through an acid sensitive and an acid stable spacer anticipating that they will be proteolysed differently in the acidic environment of lysosome of the APCs. These resulting fully synthetic vaccine candidates were used to immunizing MUC1 transgenic mice antibody titer were measured to test our hypothesis.



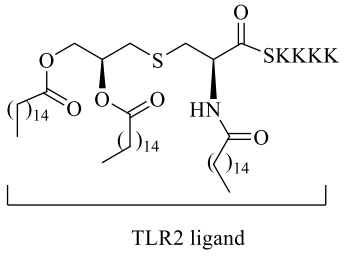
Compound 1



Compound 2



Compound 3



Compound 4

Figure 4.1 Chemical Structures of LMUC1-based Vaccine Candidates with linkers.

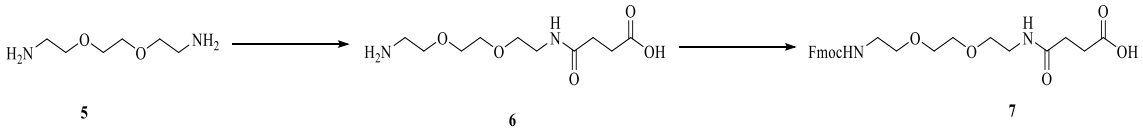
4.3 Result and Discussion

In our previous experiments, we developed a linear approach for the synthesis of vaccine candidate compound **1** using MW-SPPS. We followed the same protocol to the synthesis of compound **1** in this experiment.

4.31 Synthesis of LMUC1 based vaccine Candidates containing PEG linker

4.31a Synthesis of PEG Linker

Diamino-PEG linker **5** was derivatized to mimic amino acid as shown in Scheme 4.1. Building block containing PEG linker **7** required for synthesis of compound **2** was performed as shown in scheme 4.1. 2,2'-(ethane-1,2-diylbis(oxy))diethanamine was reacted with 1 equivalent succinic anhydride in acetonitrile to furnish the acid functionality at one terminus. Amino terminus was then protected by Fmoc using Fmoc-Cl in acetonitrile-water mixture.



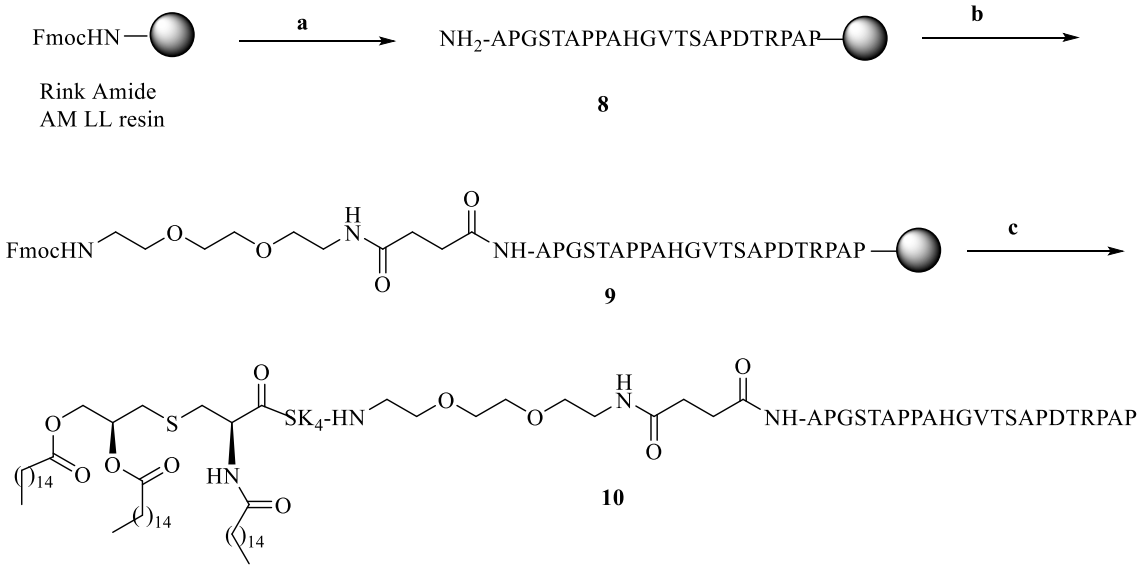
Scheme 4.1 Synthesis of PEG Linker

Reagents and conditions: (a) Succinic anhydride (1.0 eq.), CH_3CN, 4 hr, r.t. (b) Fmoc-Cl, DIPEA (1.0 eq.) CH_3CN:H_2O, overnight, r.t. (62% over 2 steps)

4.31b Synthesis of Model Unglycosylated Vaccine Candidate with PEG linker

Before synthesizing the vaccine candidate with Tn antigen on the backbone of LMUC1 (Vaccine compound **2**), model experiments were carried out to establish the synthetic protocol as shown in scheme 4.2. Full-length MUC1 peptide sequence (intermediate compound **8**) containing free amine at *N*- terminus was assembled on Rink Amide AM LL resin using a CEM Liberty 12-channel automated peptide synthesizer equipped with microwave heating assembly. Attachment of a PEG linker was completed

manually under microwave irradiation using HATU/HOAt activation protocol to get intermediate compound **9**. After coupling of PEG linker, Rink Amide AM resin was returned to CEM automated peptide synthesizer to add subsequent amino acid residues. After coupling of serine amino acid, the resin was removed from automated peptide synthesizer and Fmoc-Pam_2Cys as well as final palmitic acid residues were coupled to glycopeptide manually under microwave irradiation using HATU/HOTU in the presence of base, DIPEA in DMF. The global deprotection of amino acid side chains and lipopeptides cleavage from the resin was performed under acidic condition using a TFA cocktail. The crude product was then purified by RP-HPLC using C4 column to get final model lipopeptide compound **10**.

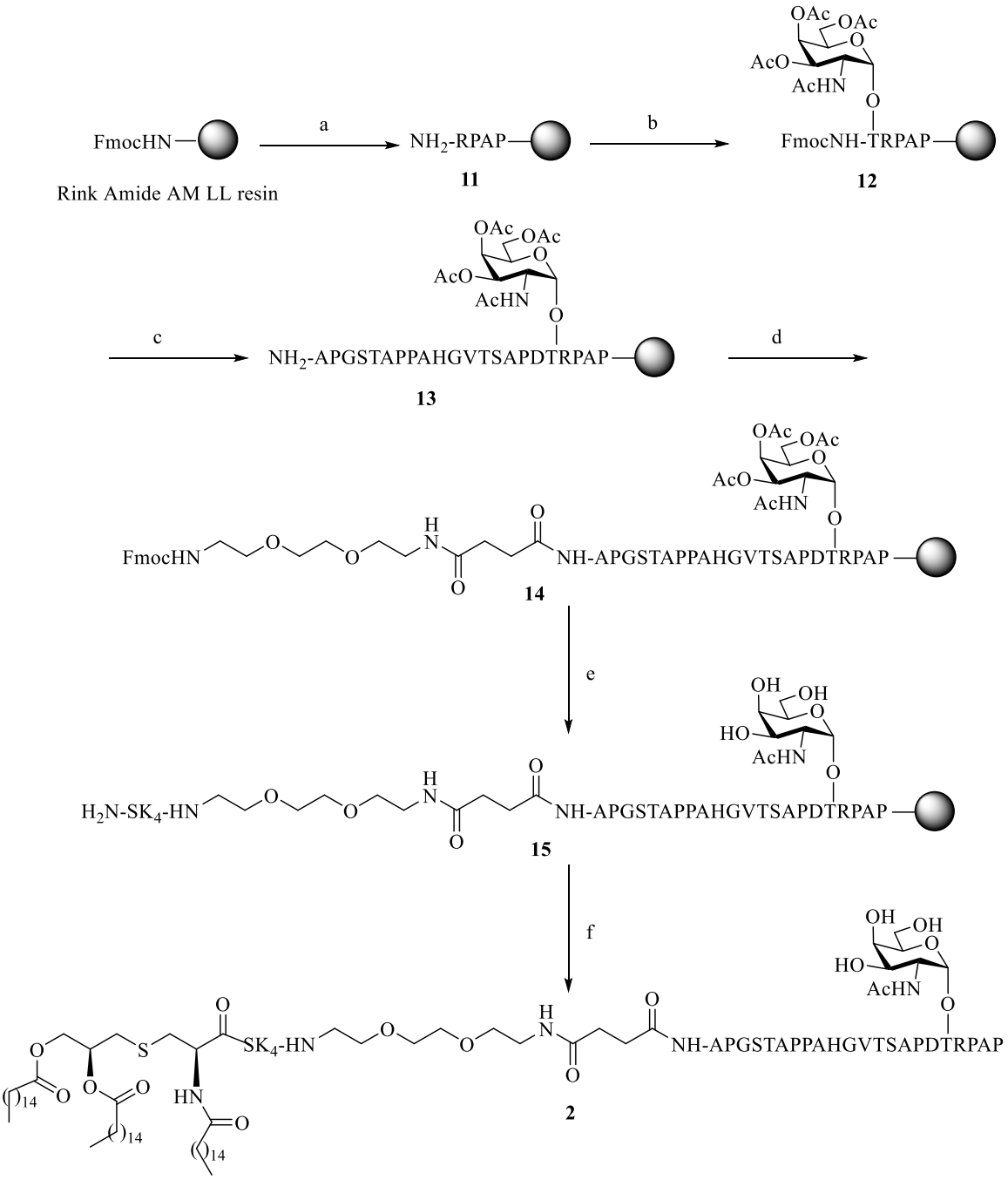


Scheme 4.2 Synthesis of Model Vaccine Candidate without Tn antigen

Reagents and conditions (a) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (b) **7** (2 eq.), HATU, HOAt, DIPEA, DMF, MW 5 min. (c) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (c) (i) Fmoc-Pam_2Cys-OH, HATU, HOAt, DIPEA, DMF, MW - 10 min x 2 (ii) 20% 4-Methylpiperidine in DMF, MW, 3 min (iii) Palmitic Acid, HATU, HOAt, DIPEA, DMF, MW, 10 min (iv) 87.5% TFA, 5% Phenol, 5% H_2O, 2.5% TIPS, 3 hr.

4.32 Synthesis of LMUC1 Vaccine Candidate 2 with Tn antigen

Encouraged by the successful synthesis of model vaccine candidate **10**, we focused our attention on the synthesis of vaccine candidate **2.** The synthesis of lipopeptide vaccine **2** was performed following a standardized protocol used for unglycosylated lipopeptide as described above. The vaccine candidate **2** containing PEG linker between MUC1-derived B-epitope and T-helper peptide sequence and TLR2 ligand, Pam_3CysSK_4 was synthesized using MW-SPPS as shown in scheme 4.3. As described above, the sequence of first four amino acid was assembled on Rink Amide AM LL resin using a CEM Liberty 12-channel automated peptide synthesizer equipped with microwave heating assembly. The introduction of Fmoc-protected Tn was performed manually under microwave irradiation using HATU/HOAt activation protocol to give intermediate **12**. After coupling of Tn-antigen the Rink Amide AM resin was returned to CEM automated peptide synthesizer to extend the peptide sequence till alanine residue. After coupling of alanine amino acid, the resin was removed from automated peptide synthesizer (intermediate compound **13**). The coupling of PEG linker was completed manually under microwave irradiation using HATU/HOAt activation protocol to obtain intermediate compound **14**. Rink Amide AM resin was then returned to CEM automated peptide synthesizer to add subsequent amino acid residues until final serine amino acid. The resin was removed from automated peptide synthesizer and treated with a solution of 70% hydrazine in methanol to remove acetyl groups of the Tn-antigen (intermediate **15**). After deacetylation, the Fmoc-Pam_2Cys, as well as final palmitic acid residues, were coupled to



Scheme 4.3 Synthesis of Vaccine Candidate 2 with Tn antigen

Reagents and conditions (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (b) Tn-COOH(1.2 eq), HATU, HOAt, DIPEA, DMF, MW 5 min. (c) (i) 20% 4-Methylpiperidine, DMF, MW-SPPS (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS. (d) (i) **7** (2 eq.), HATU, HOAt,

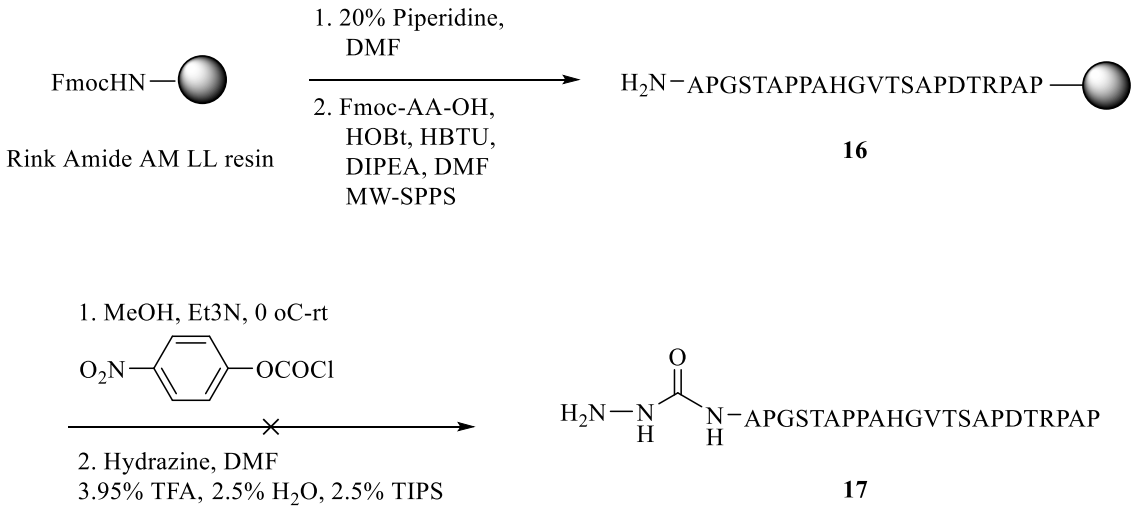
DIPEA, DMF, MW 5 min. (ii) 20% 4-Methylpiperidine, DMF, MW-SPPS (iii) Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (e) 70% Hydrazine in MeOH, 2.5 hr (f) (i) Fmoc-Pam_2Cys-OH, HATU, HOAt, DIPEA, DMF, MW - 10 min x 2 (ii) 20% 4-Methylpiperidine in DMF, MW, 3 min (iii) Palmitic Acid, HATU, HOAt, DIPEA, DMF, MW, 10 min (iv) 87.5% TFA, 5% Phenol, 5% H_2O, 2.5% TIPS, 3 hr.

glycopeptide manually under microwave irradiation using HATU/HOTU in the presence of base, DIPEA in DMF. The global deprotection of amino acid side chains and glycolipopeptides cleavage from the resin was performed under acidic condition using cocktail containing 87.5% TFA, 5% phenol, 5% H_2O, and 2.5% TIPS. The crude product was then purified by RP-HPLC using C4 column to obtain vaccine candidate **2**.

4.40 Synthesis of MUC1 based hydrazone linkage containing vaccine candidate

4.41 Synthesis of hydrazine functionalized Model LMUC1 sequence

At first, a synthetic scheme 4.4 was designed in such a way that *N*-terminus of the LMUC1 contains a free hydrazine group for attachment to aldehyde functionalized Pam_3CSK_4. Microwave assisted linear synthesis of MUC1-based peptide was straightforward and intermediate **16** was prepared without any difficulties. However, the preparation of the hydrazine functionalized MUC1 peptide failed to yield the desired peptide product (compound **17**) even after multiple attempts. MALDI-TOF analysis showed lower molecular weight side-products but did not show product peak. Hence, alternative routes for the synthesis of hydrazone containing vaccine candidates were followed.



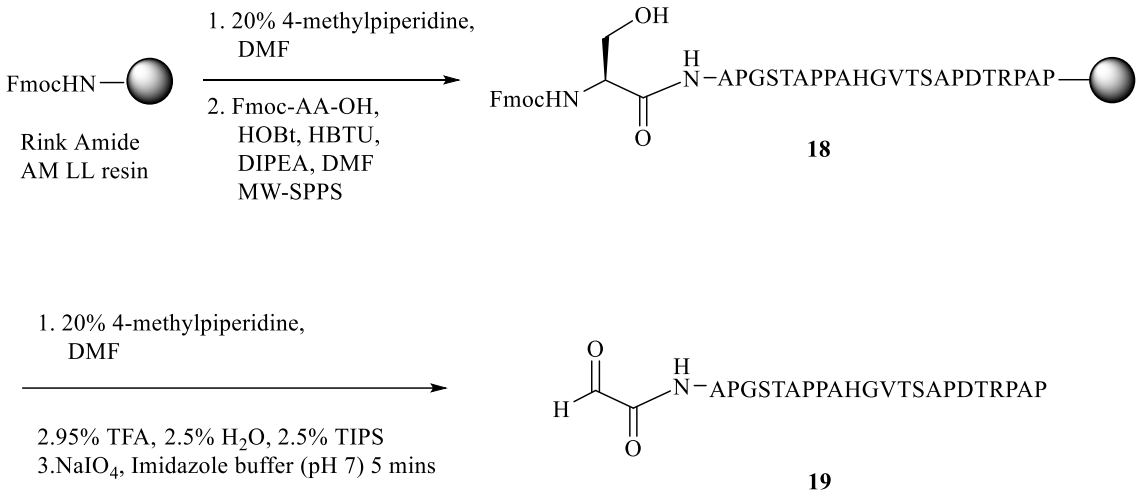
Scheme 4.4 Synthesis of Hydrazine functionalized Model MUC 1 sequence

4.42 Alternative route: Synthesis of Aldehyde-functionalized Model LMUC1 sequence.

Because of difficulties occurred in preparing hydrazine functionalized model compound **3**, an alternative synthetic route was designed. In this synthetic route, *N*-terminal aldehyde functionality was introduced via oxidation of terminal serine. After the screening of a number of oxidation protocols, the following protocol was developed for the synthesis of a model compound. As shown in scheme 4.5, MW-SPPS was utilized to synthesize model MUC1 peptide sequence **18** containing serine residue at the *N*-terminus. The *N*-terminus serine served as masked aldehyde functionality and yielded compound **19** after oxidation in very short reaction time.

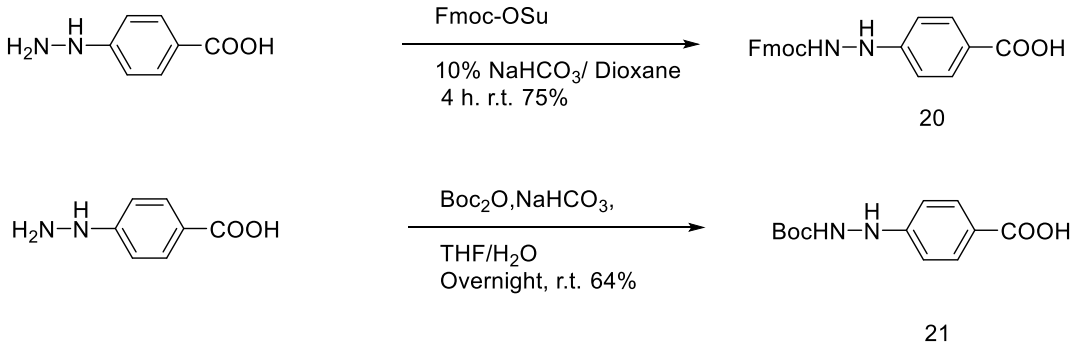
4.43 Synthesis of hydrazine containing linker building blocks

After several unsuccessful attempts for the synthesis of compound **17**, we decided to incorporate hydrazine functionality into the P_3CSK_4 fragment instead of the MUC1 fragment. Following two appropriately protected hydrazino derivatives were prepared for incorporation into lipopeptide fragment (scheme 4.6).



Scheme 4.5 Synthesis of Aldehyde-functionalized Model MUC 1 sequence

Commercially available 4-Hydrazinobenzoic acid was treated with Fmoc *N*-hydroxysuccinimide ester to yield the Fmoc protected hydrazine linker **20**. Similarly, Boc protection of hydrazine functionality was achieved using Boc-anhydride in the presence of sodium bicarbonate to furnish linker compound **21** (Scheme 4.6).

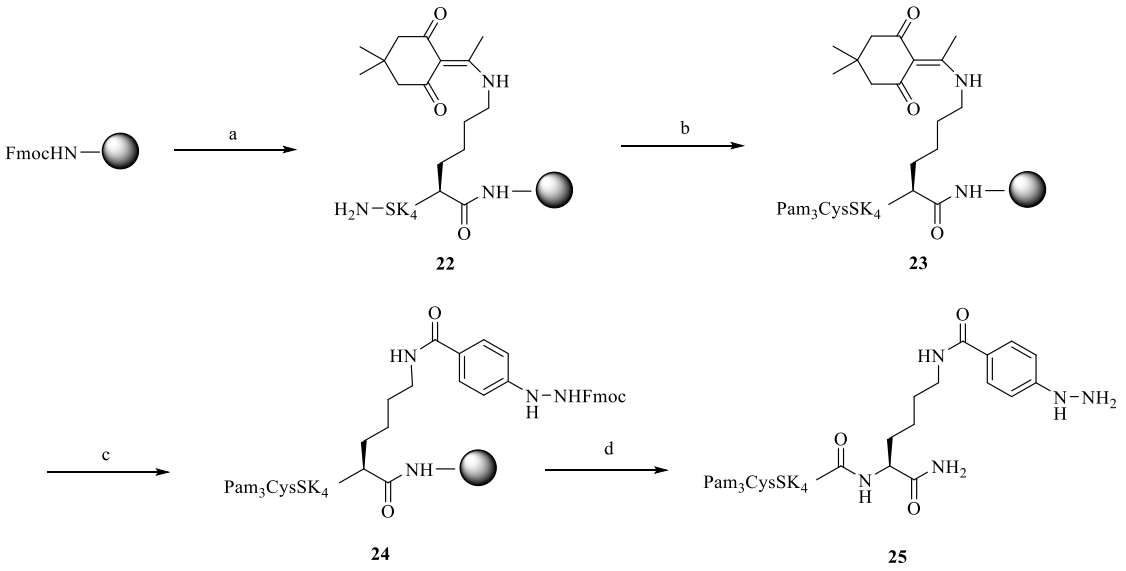


Scheme 4.6 Synthesis of hydrazine containing linker building blocks

4.44 Synthesis of hydrazine functionalized Pam_3CSK_4

In our initial attempts, the base sensitivity of side-chain protecting group (Dde) of lysine amino acid was exploited for attachment of Fmoc protected 4-hydrazinobenzoic

acid (scheme 4.7). To our surprise along with the desired product, inseparable low, as well as high molecular weight (M.W-1735, 1756, 1800 and 1771) impurities, were observed in the final product. Multiple attempts to purify this product using HPLC and SEC failed. We believe that the use of hydrazine hydrate after installment of palmitic acid chains to remove Dde may have been an issue.



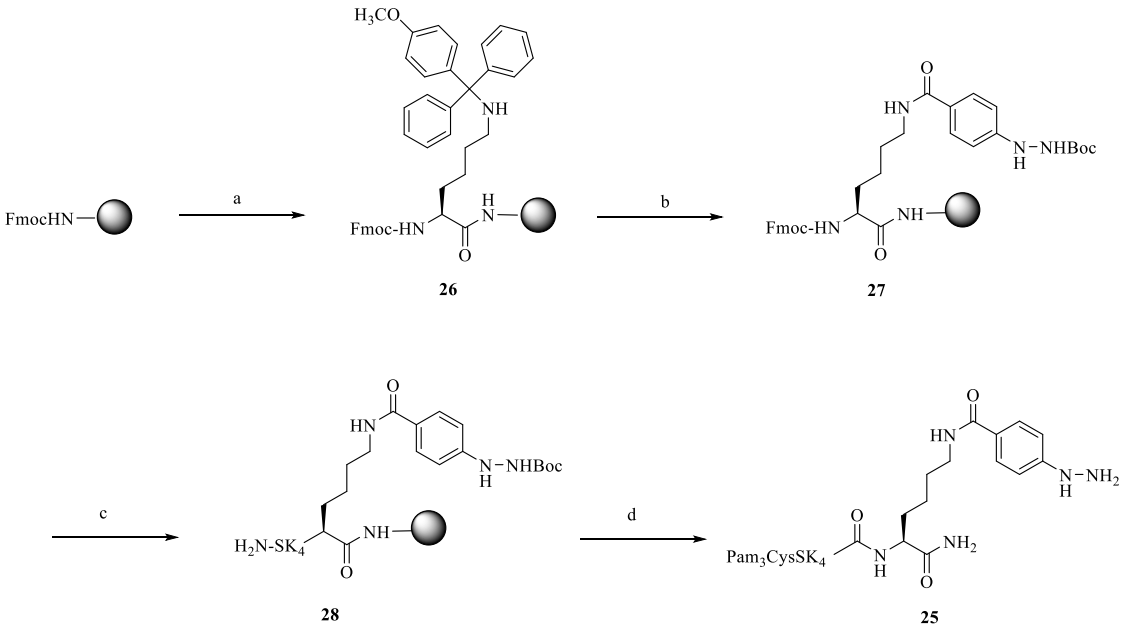
Scheme 4.7 Synthesis of Hydrazine functionalized Pam_3CSK_4

Reagents and conditions: (a) (i) 20% 4-methylpiperidine, DMF (ii) Fmoc-Lys(Dde)-COOH, Fmoc-AA-COOH HOBt, HBTU, DIPEA, DMF MW-SPPS (b) (i) Fmoc-Pam2Cys-OH, HATU, HOAt, DIPEA, DMF, MW - 10 min x 2 (ii) 20% 4-Methylpiperidine in DMF, MW, 3 min (iii) Palmitic Acid, HATU, HOAt, DIPEA, DMF, MW, 10 min (iii) 2% Hydrazine hydrate 10 min (d) (i) **20,** HOBt, HBTU, DIPEA, DMF (ii) 20% 4-methylpiperidine, DMF (iii) 95% TFA, 2.5% H_2O, 2.5% TIPS

4.45 Alternative Route for Synthesis of Hydrazine functionalized Pam_3CSK_4

An alternative method for preparing a hydrazine functionalized compound was designed. This route takes advantage of an acid sensitive Mtt side chain protecting group for amino-containing lysine which can be cleaved under mildly acidic conditions (1% TFA in DCM). By using this route Boc-protected hydrazino benzoic acid was introduced at very early stages of the synthesis as shown in scheme 4.8. The reaction progress was

monitored using MALDI-TOF by cleaving small amount of resin. Acid-sensitive Boc-protected 4-hydrazinobenzoic acid had another advantage as there was no need of one extra deprotection step compared to Fmoc protected 4-hydrazinobenzoic acid. Boc protection could be removed while cleaving the peptide from resin support. This route gave the desired product **25** in good yields with better HPLC purity.



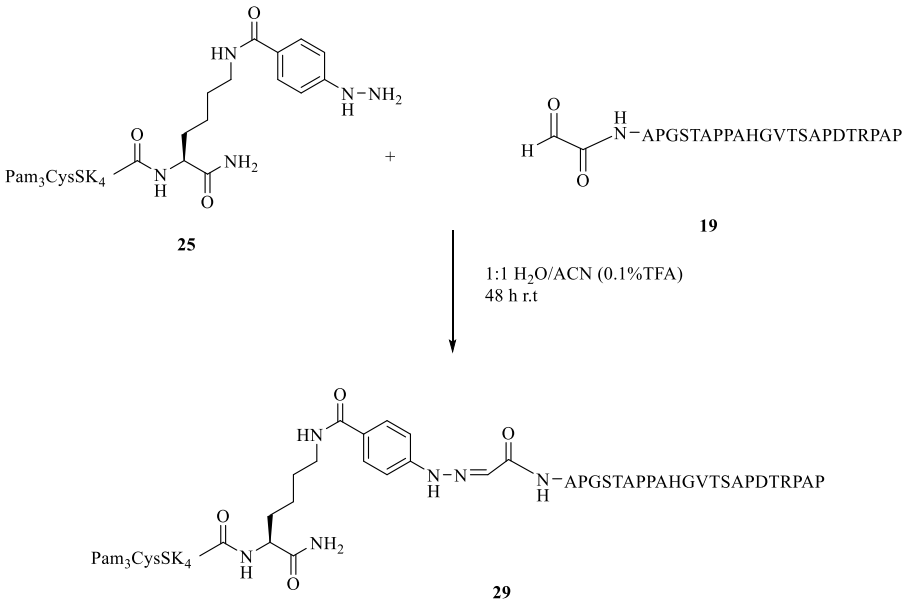
Scheme 4.8 Alternative Route for Synthesis of Hydrazine functionalized Pam_3CSK_4

Reagents and conditions: (a) (i) 20% 4-methylpiperidine, DMF (ii) Fmoc-Lys(Mtt)-COOH, HOBt, HBTU,DIPEA, DMF, MW-SPPS (b) (i) 1%TFA/CH_2Cl_2, 30 min. (ii) **21**, HOBt, HBTU, DIPEA, DMF (c) AA-COOH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (d) (i) 20% 4-Methylpiperidine in DMF, MW, 3 min (ii). Fmoc-Pam_2Cys-OH, HATU, HOAt, DIPEA, DMF, MW- 10 min x 2 (iii) 20% 4-Methylpiperidine in DMF, MW, 3 min (iv). Palmitic Acid, HATU, HOAt, DIPEA, DMF, MW, 10 min (v). 95% TFA, 2.5% H2O, 2.5% TIPS

4.46 Synthesis of Model vaccine compound 4

Once we had building blocks **25** and **19** in hand for the synthesis of the model vaccine candidate, we focused our attention on optimizing the protocol for hydrazine-

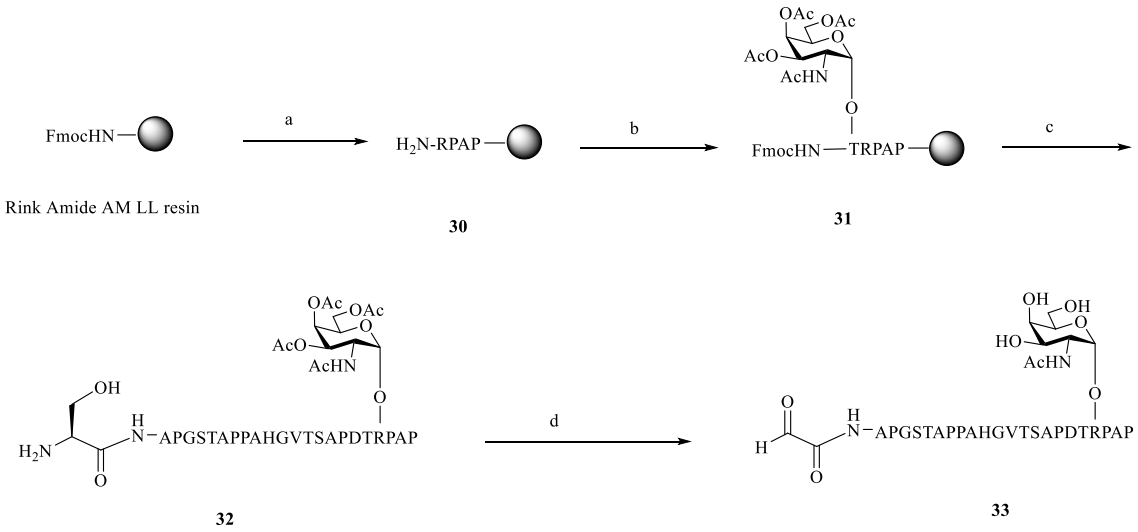
aldehyde coupling. The following protocol for hydrazine-aldehyde coupling was optimized by using a model peptide (Scheme 4.9).



Scheme 4.9 Synthesis of Model vaccine compound containing hydrazone linker

4.47 Synthesis of aldehyde functionalized glycosylated LMUC 1 glycopeptide

After the model synthesis, building blocks for aldehyde functionalized glycosylated LMUC1 peptide were prepared using a similar protocol for preparation of compound **29**. As described before synthesis of glycopeptide **32** containing a terminal serine was straightforward. Oxidation of the terminal serine was performed using sodium periodate followed by deacetylation to yield compound **33** (Scheme 4.10).

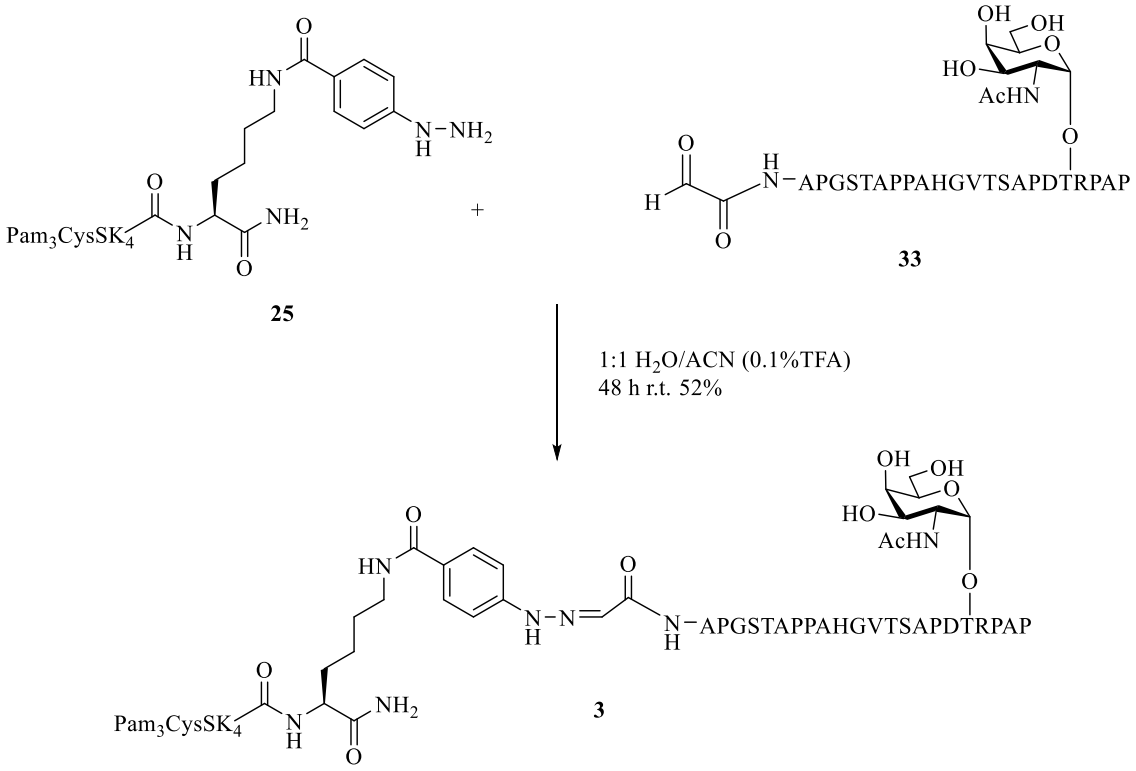


Scheme 4.10 Synthesis of Aldehyde-functionalized MUC 1 glycopeptide

Reagents and conditions (a) (i). 20% 4-methylpiperidine, DMF, MW-SPPS. (ii). Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (b) Tn-COOH, HATU, HOAt, DIPEA, DMF, MW - 5 min (c) (i). 20% 4-methylpiperidine, DMF, MW-SPPS (ii). Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF, MW-SPPS (iii).95% TFA, 2.5% H2O, 2.5% TIPS (d) (i).NaIO4, imidazole buffer (pH 7) 5 mins (ii).6mM NaOMe/MeOH, 2h

4.48 Synthesis of LMUC1 based vaccine candidate 3

An optimized experimental procedure for coupling of hydrazine with aldehyde was used to prepare the vaccine candidate. Coupling of compound **25** and **33** was achieved in acetonitrile-water mixture containing 0.1%TFA to give glycosylated vaccine candidate **3** with the acid sensitive linker.



Scheme 4.11 Synthesis of LMUC1 based vaccine candidate 3

4.4 Immunological Studies:

After successful synthesis of vaccine constructs **1, 2, 3** and **4**, these vaccines compounds were incorporated into lipid-based small unilamellar vesicles by hydration of thin film using egg phosphatidylcholine, phosphatidylglycerol, and cholesterol in an HEPES buffer (10 mM, pH 6.47) containing NaCl (145 mM). Size extrusion was performed by passing liposomes through various sized Nucleopore polycarbonate membranes (1000nm -100 nm). To explore immunological properties of synthetic vaccine candidates, groups of MUC1.Tg mice (C57BL/6; H-2^b) that express human MUC1[25] at physiological levels were immunized with the liposomal preparation of **1, 2, 3** and **4** (containing 3 μg of carbohydrate) four times intradermally at the base of the tail at biweekly intervals. One week after the third immunization, the blood was collected. The

mice final bleed was collected and mice were sacrificed two weeks following the fourth immunization. The humoral immune responses will be assessed by titers of MUC1-specific antibodies.

Further immunological experiments are underway. Briefly, anti-MUC1 IgG, IgG1, IgG2a, IgG2b, and IgG3 antibody titers will be determined by ELISA as described (Boons et.al., 2004, chemistry 10:3517-3524). Briefly, ELISA plates (Thermo Electron) will be coated with a conjugate of the LMUC1 glycopeptide conjugated to BSA through a maleimide linker [BSA-MI-CAPGSTAPPAHGVTSAPDT(αGalNAc)RPAP] conjugate for anti-LMUC1 (Tn18) antibody titers. Serial dilutions of the sera will be allowed to bind to immobilized MUC1. Detection will be accomplished by the addition of phosphate-conjugated anti-mouse antibodies and p-nitrophenyl phosphate (Sigma).

4.5 Conclusion:

In summary, we have successfully developed synthetic protocols for self adjuvanting LMUC1-based vaccine candidates containing acid-stable and acid-labile linkers between TLR2 ligand and LMUC1 glycopeptide. The immunological experiments are underway but we anticipate that our results will provide useful insights regarding generation of neo-epitope between lysine moieties of Pam_3CysSK_4 and LMUC1 glycopeptide residues.

4.6 Experimental Section:

Reagents and general experimental procedure:

Amino acid derivatives and Rink Amide AM LL resin were purchased from Merck Millipore; DMF was purchased from Fisher Scientific. All other chemical reagents were purchased from AnaSpec and Aldrich and were used without further purification. All

solvents employed were ACS reagent grade. Reverse Phase HPLC was performed either on an Agilent 1100 or 1200 series system equipped with an autosampler, UV detector, and fraction collector. RP-HPLC was carried out using an Agilent Eclipse XDB-C18 analytical column (5 μm, 4.6 x 250 mm) at a flow rate of 1 mL/min and a Phenomenex Jupiter 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 (95% Acetonitrile, 5% Water, 0.1% TFA) in A (95% Water, 5% Acetonitrile, 0.1% TFA) over a 40 min period unless otherwise specified. High-resolution mass spectra were obtained by using MALDI-ToF (Applied Biosystems 5800 Proteomics Analyzer) with either α-cyano-4-hydroxycinnamic acid or 2, 5-Dihydroxybenzoic acid as an internal standard matrix.

General method for microwave assisted solid phase peptide synthesis (MW-SPPS):

Peptides, glycopeptides and glycolipopeptides were synthesized on a 0.05 or 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 and *N*,*N*-Diisopropylethylamine (DIPEA) as the base for amino acid couplings. Side chain protecting groups for amino acids were 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 Fmoc-Thr[GalNAc(Ac)3-α-D]-OH, Fmoc-Ser[GalNAc(Ac)3-α-D]-OH, *N*-α-fluorenylmethoxycarbonyl-*R*-(2,3-bis(palmitoyloxy)-(2*R*-propyl)-(R)-cysteine, and palmitic acid were carried out manually under microwave irradiation using (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium

hexafluorophosphate) (HATU) and 1-Hydroxy-7-azabenzotriazole (HOAt) as activating reagents in presence of *N*,*N*-Diisopropylethylamine (DIPEA) as the base on a CEM Discover SPS instrument. The manual amino acid couplings were monitored by mass spectrometry and standard Kaiser Test.

Synthesis of Compound 1. The glycolipopeptide **1** was synthesized by solid phase peptide synthesis (SPPS) on Rink Amide AM resin (0.1 mmol) on a CEM LIBERTY 908505 peptide synthesizer equipped with a UV detector using N^{α}-Fmoc protected amino acids. 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), 2-(7-aza-1H-benzotriazole-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (76 mg, 0.2 mmol), 1-Hydroxy-7-azabenzotriazole (HOAt) (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) as the activating reagents. The lipid moiety was also installed manually using N-$^{\alpha}$-Fmoc-R-(2,3-bis(palmitoyloxy)-(2R-propyl)-(R)-cysteine (180 mg, 0.2 mmol) in DMF (5 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol). The coupling reactions were monitored by the Kaiser test. Upon completion of the coupling, the N-Fmoc group was cleaved using 20% 4-methylpiperidine 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), HOAt (27 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 the cleavage cocktail (TFA 88%, water 5%, phenol 5%, and TIPS 2%; 10 mL) for 3 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 glycol-lipopeptide **1** was 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 with a Phenomenex Jupiter semi-preparative C-4 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 and appropriate fractions were lyophilized to afford **1**. HR MALDI-TOF MS calculated for $C_{177}H_{307}N_{39}O_{46}S$ [M+H] 3747.2603; observed, 3747.3723.

Synthesis of Compound 7. 2,2'-(ethane-1,2-diylbis(oxy))diethanamine (1.46 mL, 10 mmol) was dissolved in acetonitrile (50 mL) and a solution of succinic anhydride (1.0 g, 10 mmol) in acetonitrile (25 mL) was added dropwise for 1h at ambient temperature. The mixture was stirred vigorously for another 3h. After the waxy solid settled, the supernatant was decanted and the residue was washed with acetonitrile (2 x 3 mL) very slowly. The mixture was dissolved in 50% acetonitrile/water (100 mL) and cooled to 0ºC in an ice-bath for 30 min. A solution on Fmoc-OSu (4.4 g, 13 mmol) in acetonitrile (25 mL) was added dropwise for 1 h with vigorous magnetic stirring. The reaction mixture was allowed to warm up to ambient temperature and stirred overnight. The pH of the reaction mixture was maintained between 8 and 9 by the addition of DIPEA throughout the reaction. The solvents were evaporated and the residue dissolved in 5% sodium bicarbonate (100 mL) and washed with ethyl acetate (3 x 100 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified by silica gel flash column chromatography to afford compound 7 (2.86 g, 62% over 2 steps). ^{1}H NMR (300 MHz, DMSO-D_6): δ 2.29 (t, 1H, *J*=6 Hz,

CH*H*-COOH), 2.39 (t, 1H, *J*=6 Hz, C*H*H-COOH), 2.48 (t, 2H, *J*=3 Hz, $C\underline{H_2}$-CH_2-COOH), 3.08-3.40 (2m, 12H, PEG-CH_2), 4.18 (t, 1H, *J*=6 Hz, H-9 Fmoc), 4.27 (d, 2H, $C\underline{H_2}$-O-CO-NH), 7.28-7.88 (m, 8H, Ar-H). ^{13}C NMR (75 MHz, DMSO-D6): δ 25.67, 29.58, 30.30 (2), 47.71 (C-9 Fmoc), 65.76, 69.55 (2), 69.94, 69.98, 120.54-156.59 (12C, Aromatic), 170.48-175.23 (3C, C=O). HR MALDI-TOF MS calculated for $C_{25}H_{30}N_2NaO_7$ [M+Na] 493.1951; observed, 493.1130.

Synthesis of Compound 10. The model lipopeptide compound **10** was synthesized by microwave assisted solid phase peptide synthesis (MW-SPPS) on Rink Amide AM LL resin (0.1 mmol). The peptide sequence containing 22 amino acid residues (APGSTAPPAHGAVTSAPDTRPAP) was assembled on resin support using the fully automated CEM LIBERTY 908505 peptide synthesizer equipped with a UV detector. The resin was then removed from the synthesizer and washed with DMF. The PEG linker was installed manually using compound **7** (94.1mg, 0.2 mmol) in DMF (3 mL), 2-(7-aza-1H-benzotriazole-1yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HATU) (76 mg, 0.2 mmol), 1-Hydroxy-7-azabenzotriazole (HOAt) (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) as the activating reagents. The reaction mixture was microwave-irradiated for 10 minutes and completion of coupling was monitored by Kaiser Test to furnish intermediate compound **9**. A small amount of peptide was cleaved from the resin using TFA to confirm completion of coupling reaction by mass spectrometry (MALDI-TOF). The resin was then returned to automated peptide synthesizer to extend the peptide until serine residue. The resin was then removed from a synthesizer, washed with DCM (10 Ml x 3) and further steps were performed manually. The resin was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then

dried under vacuum. The resin was swelled in DCM for 30 minutes and the lipid moiety was installed manually using N-$^{\alpha}$-Fmoc-R-(2,3-bis(palmitoyloxy)-(2R-propyl)-(R)-cysteine (180 mg, 0.2 mmol) in DMF (5 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) under microwave irradiation. The coupling reactions were monitored by the Kaiser test. Upon completion of the coupling, the resin was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3). Following that resin was treated with 20% 4-methylpiperidine in DMF (6 mL) under microwave irradiation for 3 minutes to remove *N*-Fmoc group. The final palmitic acid residue (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) in DMF (3 mL). The resin was washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then dried in vacuo. The resin was swelled in DCM (10 mL) for 30 minutes and then treated with the cleavage cocktail (TFA 88%, water 5%, phenol 5%, and TIPS 2%; 10 mL) for 3 h. The resin was filtered and washed with TFA (3 mL). The filtrate was concentrated on a rotary evaporator under vacuum till approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0^{o}C; 25 mL) and recovered by centrifugation at 2,500 rpm for 20 min. The crude lipopeptide **10** was 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 with a Phenomenex Jupiter analytical C-4 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 and appropriate fractions were lyophilized to afford pure compound **10**. HR

MALDI-TOF MS calculated for $C_{169}H_{292}N_{38}O_{41}S$ [M+H] 3544.1209; observed, 3544.5624.

Synthesis of Compound 2. The vaccine compound **2** was synthesized by microwave assisted solid phase peptide synthesis (MW-SPPS) on Rink Amide AM LL resin (0.1 mmol). The peptide sequence containing first four amino acid residues (RPAP) was assembled on resin support using the fully automated CEM LIBERTY 908505 peptide synthesizer equipped with a UV detector. The resin was then removed from the synthesizer and washed with DMF. The Tn moiety was installed manually using N^{α}-Fmoc-Thr-(AcO_3-α-D-GalNAc) (134 mg, 0.2 mmol) in DMF (3 mL), 2-(7-aza-1H-benzotriazole-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (76 mg, 0.2 mmol), 1-Hydroxy-7-azabenzotriazole (HOAt) (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) as the activating reagents. The reaction mixture was microwave-irradiated for 10 minutes and completion of coupling was monitored by Kaiser Test. A small amount of peptide was cleaved from the resin using TFA to confirm completion of coupling reaction by mass spectrometry (MALDI-TOF). The resin was then returned to automated peptide synthesizer to extend the peptide until alanine residue (APGSTAPPAHGVTSAPDTnRPAP). The resin was then removed from synthesizer, washed with DCM (10 Ml x 3) and PEG linker was installed manually using N-$^{\alpha}$-Fmoc-3,14-dioxo-2,7,10-trioxa-4,13-diazaheptadecan-17-oic acid (94 mg, 0.2 mmol), in DMF (2 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol). The resin was then returned to automated peptide synthesizer to extend the peptide until serine residue. The resin was then removed from a synthesizer, washed with DCM (10 mL x 3) and further steps were performed manually. The resin containing

peptide was then treated with 70% hydrazine in methanol solution for 2.5h to remove acetyl groups on the sugar moiety. The resin was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then dried under vacuum. The resin was swelled in DCM for 30 minutes and the lipid moiety was installed manually using N-$^{\alpha}$-Fmoc-R-(2,3-bis(palmitoyloxy)-(2R-propyl)-(R)-cysteine (180 mg, 0.2 mmol) in DMF (5 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) under microwave irradiation. The coupling reactions were monitored by the Kaiser test. Upon completion of the coupling, the resin was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3). Following that resin was treated with 20% 4-methylpiperidine in DMF (6 mL) under microwave irradiation for 3 minutes to remove *N*-Fmoc group. The final palmitic acid residue (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) in DMF (3 mL). The resin was washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then dried in vacuo. The resin was swelled in DCM (10 mL) for 30 minutes and then treated with the cleavage cocktail (TFA 88%, water 5%, phenol 5%, and TIPS 2%; 10 mL) for 3 h. The resin was filtered and washed with TFA (3 mL). The filtrate was concentrated on a rotary evaporator under vacuum till approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0^{o}C; 25 mL) and recovered by centrifugation at 2,500 rpm for 20 min. The crude glycolipopeptide vaccine construct **2** was 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 with a Phenomenex Jupiter analytical C-4 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 and appropriate fractions were lyophilized to afford pure compound **2**. HR MALDI-TOF MS calculated for $C_{187}H_{325}N_{41}O_{50}S$ [M+H] 3977.3870; observed, 3977.7151.

Synthesis of Compound 26. The lipopeptide **26** 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 **1**. The N-α-Fmoc-N-ε-4-methyltrityl-L-lysine (Fmoc-Lys(Mtt)-COOH) was installed manually using N^{α}-Fmoc-Lys(Mtt)-COOH (124 mg, 0.2 mmol), in DMF (2 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol). The resin was then treated with 1%TFA in DCM solution for 30 minutes to remove Mtt group on the side chain. Boc-protected hydrazine linker was installed using 4-(2-(tert-butoxycarbonyl)hydrazinyl)benzoic acid (50 mg, 0.2 mmol) in DMF (2 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol). The resin was then returned to automated peptide synthesizer to extend the peptide until serine residue. The resin was then removed from a synthesizer, washed with DCM (10 Ml x 3) and further steps were performed manually. The resin was swelled in DCM for 30 minutes and the lipid moiety was installed manually using N-$^{\alpha}$-Fmoc-R-(2,3-bis(palmitoyloxy)-(2R-propyl)-(R)-cysteine (180 mg, 0.2 mmol) in DMF (5 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) under microwave irradiation. The coupling reactions were monitored by the Kaiser test. Upon completion of the coupling, the resin was then washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3). Following that resin was treated with 20% 4-methylpiperidine in DMF (6 mL) under microwave irradiation for 3 minutes to remove *N*-Fmoc group. The final palmitic acid residue (52 mg, 0.2 mmol) was coupled to the free amine as described

above using HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) in DMF (3 mL). The resin was washed thoroughly with DMF (5 mL x 3), DCM (5 mL x 3), and MeOH (5 mL x 3) and then dried in vacuo. The resin was swelled in DCM (10 mL) for 30 minutes and then treated with the cleavage cocktail (TFA 88%, water 5%, phenol 5%, and TIPS 2%; 10 mL) for 3 h. The resin was filtered and washed with TFA (3 mL). The filtrate was concentrated on a rotary evaporator under vacuum till approximately 1/3 of its original volume. The lipopeptide was precipitated using diethyl ether (0^{o}C; 25 mL) and recovered by centrifugation at 2,500 rpm for 20 min. The crude glycolipopeptide vaccine construct **2** was 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 with a Phenomenex Jupiter analytical C-4 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 and appropriate fractions were lyophilized to yield pure compound **26**. HR MALDI-TOF MS calculated for $C_{94}H_{176}N_{15}O_{14}S$ [M+H] 1771.3242; observed, 1771.1682.

Synthesis of Compound 37. The glycopeptide **37** was synthesized on Rink Amide AM resin (0.1 mmol) following the general protocol for peptide synthesis described for compound **1**. The resulting crude glycopeptide **37** was purified by RP-HPLC on an Agilent Eclipse C18 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 **37**. HR MALDI-TOF MS calculated for $C_{105}H_{165}N_{30}O_{39}$ [M+H] 2469.1772; observed, 2469.4971.

Synthesis of Compound 38. Compound **37** (30 mg, 0.012 mmol; 0.58 µM of terminal serine) was dissolved in water (500 µL) and diluted with Imidazole buffer (2 mL, pH 6.95). Freshly prepared sodium periodate (100 mM, 12 µL i.e. 1.16 µM) was added to the solution and stirred at ambient temperature for 5-10 min. The reaction was quenched by addition of ethylene glycol (24 µL) and the contents were lyophilized. The resulting crude glycopeptide was purified by RP-HPLC on an Agilent Eclipse C18 semi-preparative column using a linear gradient of 0-100% solvent B in A over a period of 40 minutes and the appropriate fractions were lyophilized to afford compound containing aldehyde functionality at *N*-terminus. This compound (25 mg, 0.011 mmol) was dissolved in methanol (2 mL) and freshly prepared 1M sodium methoxide (12 µL, 6 mmol) was added and the reaction mixture was stirred at ambient temperature for 2h. The reaction mixture was neutralized with solid carbon dioxide and the resulting crude glycopeptide **38** was purified by RP-HPLC on an Agilent Eclipse C18 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 **38** (23 mg, 97%). HR MALDI-TOF MS calculated for $C_{98}H_{154}N_{29}O_{36}$ [M+H] 2312.1033; observed, 2312.5662.

Synthesis of Compound 3. Compound **26** (5 mg, 0.002 mmol) and compound **38** (12 mg, 0.006 mmol) were dissolved in a mixture of acetonitrile/water (3:1, 2 mL) and TFA (2 µL, 0.01%) was added and the reaction mixture was stirred at ambient temperature for 48 h. The resulting crude glycolipopeptide **3** was purified by RP-HPLC on a Phenomenex Jupiter C4 semi-preparative column using a linear gradient of 0-100% solvent B in A over a period of 75 min, and the appropriate fractions were lyophilized to afford **3** (5.8

mg, 52%). HR MALDI-TOF MS calculated for $C_{192}H_{327}N_{44}O_{49}S$ [M+H] 4065.4169; observed, 4065.3674.

Synthesis of Compound 20. 4-hydrazino-benzoic acid (2g, 13.14 mmol) was dissolved in 1,4-dioxane (18 mL) and 10 % sodium bicarbonate (36 mL) was added followed by the dropwise addition of Fmoc-Cl (3.74g, 14.46 mmol) in 1,4-dioxane (18 mL) at 0 ºC. The reaction mixture was allowed to warm up to ambient temperature and stirred for 3h. The solvents were evaporated and the product was recrystallized from ethanol/ethyl acetate to afford compound **20** (3.54g, 72%). HR MALDI-TOF MS calculated for $C_{22}H_{18}N_2NaO_4$ [M+Na] 397.1164; observed, 397.0555.

Synthesis of Compound 21. 4-hydrazino-benzoic acid (3g, 19.71 mmol) was dissolved in THF: H_2O mixture (1:1, 100 mL) and sodium bicarbonate (5g, 59.13 mmol) were added followed by the dropwise addition of Boc_2O (5.43mL, 23.66 mmol) in THF (20 mL) at 0 ºC. The reaction mixture was allowed to warm up to ambient temperature and stirred overnight. Completion of the reaction was monitored by TLC. The solvents were evaporated and the product was recrystallized from ethyl acetate/hexane to afford compound **21** (3.15g, 64%). HR MALDI-TOF MS calculated for $C_{12}H_{16}N_2NaO_4$ [M+Na] 275.1008; observed, 274.9915. ^{1}H NMR (300 MHz, DMSO-D_6): δ 1.39 (s, 9H, C-$(CH_3)_3$), 6.64. (d, 2H, *J*=9 Hz, CH-Aromatic), 7.71. (d, 2H, *J*=9 Hz, CH-Aromatic), 8.23 (s,1H, NH-C), 8.89 (s,1H, NH-CAr) 12.17 (s, 1H, COOH).

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CHAPTER 5

SYNTHESIS AND FUNCTIONAL CHARACTERIZATION OF NOVEL CHIMERIC ADJUVANTS THAT ACTIVATE PATTERN RECOGNITION RECEPTORS STIMULATED BY GRAM-POSITIVE BACTERIA.

***Nitin T Supekar**, Anju Sirohiwal, Margreet Wolfert, Geert-Jan Boons. *To be submitted to Chembiochem.*

5.1 Abstract

Adjuvants enhance immunity against the coadministered vaccines or antigens. They act by stimulating innate immune system via various pattern-recognition receptors (PRRs) such as membrane-bound Toll-like receptors (TLRs), cytoplasmic RIG-I-like receptors (RLRs) and intracellular NOD-like receptors (NLRs). Dendritic cells (DCs) are the main target of adjuvants due to their unique role in the stimulation of adaptive immune responses and the induction of tolerance. Adjuvants act directly or indirectly on immature DCs and help their maturation and migration to the draining lymph nodes where the presentation of antigen on major histocompatibility complex (MHC) occurs. This process activates T and B cells to induce humoral immunity against the coadministered vaccine or antigen. Aluminum salt (alum) has been the most widely used adjuvant since its introduction in the 1920s, and it was the only FDA approved adjuvant for several decades. However, for some vaccine formulations, alum does not induce protective and sustained immune responses because alum drives preferentially Th2 responses, and for some pathogens on mucosal surfaces, a Th1 immune response is necessary. Hence, there is an unmet clinical need for new adjuvants that can induce immunity against pathogens on mucosal surfaces. Infected hosts are exposed to multiple pathogen-associated molecular patterns (PAMPs) to generate protective immune responses by activating host PRRs. Therefore, we propose that the compound adjuvants which are derived by linking two PAMPs, may trigger efficient crosstalk between various signal transduction pathways and generate synergistic immune activation. In this particular study, we explored synthesis and biological evaluation compound adjuvants derived from linking TLR2 and NOD ligands.

5.2 Introduction

It is a well-known fact that vaccination is a very efficient strategy to prevent many deadly infectious diseases. For example, Polio was eradicated in the United States of America (USA) by 1979 due to the extensive vaccination program. The purpose of a vaccine is to generate and stimulate protective immunity against the invading pathogen.[1] Protection against the infectious diseases heavily relied on the magnitude, quality, longevity, and location of the produced antibodies within human tissues. Traditional vaccines that induce permanent immunity mostly obtained from live-attenuated viruses or bacterial pathogens; for example, polio[2], vaccinia[3], and anthrax spores[4]. For many years, Aluminium salt was the only adjuvant approved for human use and has been shown effective when added to diphtheria vaccine.[5] Therefore, aluminum salts have been added to other subunit vaccines such as Anthrax Vaccine Adsorbed (AVA), tetanus, and pneumococcal conjugate. However, administration of alum adjuvants to a subunit vaccine against *S. aureus,* V710 (IsdB) did not show any improvement in the generation of antigen-specific antibodies, and that may be one of the causes of its failure in clinical trials.[6] Because of this, there is an unmet need for the development of novel adjuvants which can boost immunity when co-administered with an appropriate antigen against pathogens on mucosal surfaces such as *C. difficile, S. aureus* and *S. pyogenes.*

It is a well-established that adjuvants stimulate innate immunity in the host immune cells by interacting with PRRs such as various TLRs and NLRs.[7] During infection, several PAMPs stimulate these PRRs and hence generate strong innate as well as adaptive immune responses needed for protection against the pathogen.[8] In recent years, bacterial components such as TLR and NLR ligands have attracted the attention of

scientists because their high immunostimulant properties.[9] A number of mammalian PRRs have been identified, however TLRs, and NLRs are well characterized, and known to provide protection against PAMPs in the extracellular environment as well as intracellular compartments. In the case of infection, PRRs activate cellular defense system thereby producing innate protective proteins and proinflammatory cytokines. Besides, PRRs can activate antigen presenting cells (APCs) that can induce pathogen-specific adaptive immune responses.[10]

Activation of innate immune system through stimulation of PRR plays a critical role in various biological processes, eventually resulting in activation of lymphocytes. For example, in vitro study by Blander et al. established that TLR ligands stimulate MHC-II antigen presentation pathway to CD4+ T cells.[11] Also, it has been shown that TLR signals induce phagosomal MHC-I delivery from the endosomal recycling compartment to allow cross-presentation thereby activating CD8+ T cells.[12] Similarly, NLR activation is critical for MHC II presentation in DCs. For example, Cooney et al. showed that NOD2 triggering induces autophagy, which is required for induction of MHC-II antigen-specific CD4 (+) T cell responses in DCs.[13]

Numerous *in vitro* and *in vivo* studies have revealed that use of TLRs as adjuvant in vaccination could enhance adaptive immune responses.[14-15] However, until now TLR4 agonist, monophosphoryl lipid (MPLA) is the only FDA-approved for human use. It is obtained from *Salmonella minnesota* lipopolysaccharide (LPS) and is being used against hepatitis B virus (HBV) and human papillomavirus (HPV) infections. A combination adjuvant of MPLA with alum (AS04) used for HPV vaccination has been shown to be effective compared to administration of only alum along with antigen.[16] The AS04 was

shown to enhance humoral and B-cell mediated immunity as well as produced antibody titers faster than alum.[17] A number of studies that either use TLR adjuvants alone or in combination with other immunoadjuvant are underway to explore the capability of TLR adjuvants for human use. For example, the addition of GPG 7909, a TLR9, to HBV vaccine enhanced its clinical outcomes.[18] In another mice immunization study, it has been shown that administration of nanoparticles containing combination of TLR4 and TLR7 ligands along with antigen synergistically increased antigen-specific, neutralizing antibodies compared to nanoparticles consisting only one TLR immunoadjuvant.[19] TLR2 recognizes bacterial lipoproteins such as thioether linked diacyl-glycerol attached to the *N*-terminal cysteine of secreted proteins that may also carry *N*-linked acyl chains.[20] TLR2 is expressed on multiple cells such as lymphocytes, macrophages, and dendritic cells. The lipopeptide ligands such as Pam_3CysSK_4 that activate these receptors stimulate a wider array of adjuvant functions.[14] Studies have demonstrated that Pam_3CysSK_4 has multiple roles that include DC maturation, activation of macrophages to release cytokines, promoting maturation and stimulation of B cells that produce antigen-specific antibodies and boosting the production of antigen-specific CD8+ T cells.[21-24] Pre-clinical and clinical studies have shown that lipopeptides are safe for use as adjuvants in humans.[25] The mice immunization study by Takeuchi et al has demonstrated that TLR2 and MyD88 knock-out mice were much more susceptible to *S. aureus* infections compared to wild-type mice, which highlights the importance of TLR2 lipopeptide agonists.[26]

NLRs are another widely studied family of PRRs, due to their immunostimulant properties. To date, 22 cytoplasmic NLRs have been identified and well characterized. The member of NLR subfamily, NOD2, consist of *N*-terminal caspase recruitment

domain required for the production of NF-κB and MAPK signaling.[27] A mice immunization study has revealed that NOD2 plays a major role in T cell activation leading to generate strong adaptive immunity.[28] Muramyl dipeptide (MDP), a fragment of bacterial peptidoglycan recognized by NOD2 receptors.[29] Many studies established that MDP displays a broad array of immune-modulatory properties which makes it an attractive target for adjuvant development.[25] However, MDP is known to show some toxicity in humans, more specifically pyrogenicity which lead to a search for the safer derivative of MPD.[25] A number of studies have demonstrated that MDP can tolerate structural modification to improve its activity as an adjuvant and reduce its toxicity. For example, lipidation is one of the attractive approaches. A derivative of MDP, modified by butyl ester retained its adjuvant properties with limiting pyrogenic side effects in human clinical trials.[30-32] Our approach will build on this observation by preparing fusion PAMPs that tether TLR2, a lipopeptide to NOD2, an MDP derivative.

In this study, we hypothesized that covalent attachment of two PAMPs; TLR2-NOD2 agonists will mimic situation as in the case of infection making sure that immune cells are being exposed to both at the same time. This simultaneous presentation will result in efficient crosstalk between multiple signal transduction pathways followed by synergistic immune activation. Thus, these chimeric fusion PAMPs can be co-administered at lower concentrations to avoid unwanted toxic side effects. Therefore, we propose a synthesis of the following compound as shown in figure 5.1. Compound **1** is a MDP (a well-known NOD2 ligand) derivative with alkyne linker at anomeric position. Compound **2** is a disaccharide *N*-acetylmuramic acid (MurNAc) (β1-4)-*N*-acetylglucosamine (MurNAc-GlcNAc), a repeating disaccharide unit of bacterial PGN.

Compound **3** has been designed such a way that Pam_3CysSK_4 (a TLR2 ligand) will have azido handle for its attachment to NOD2 agonists.

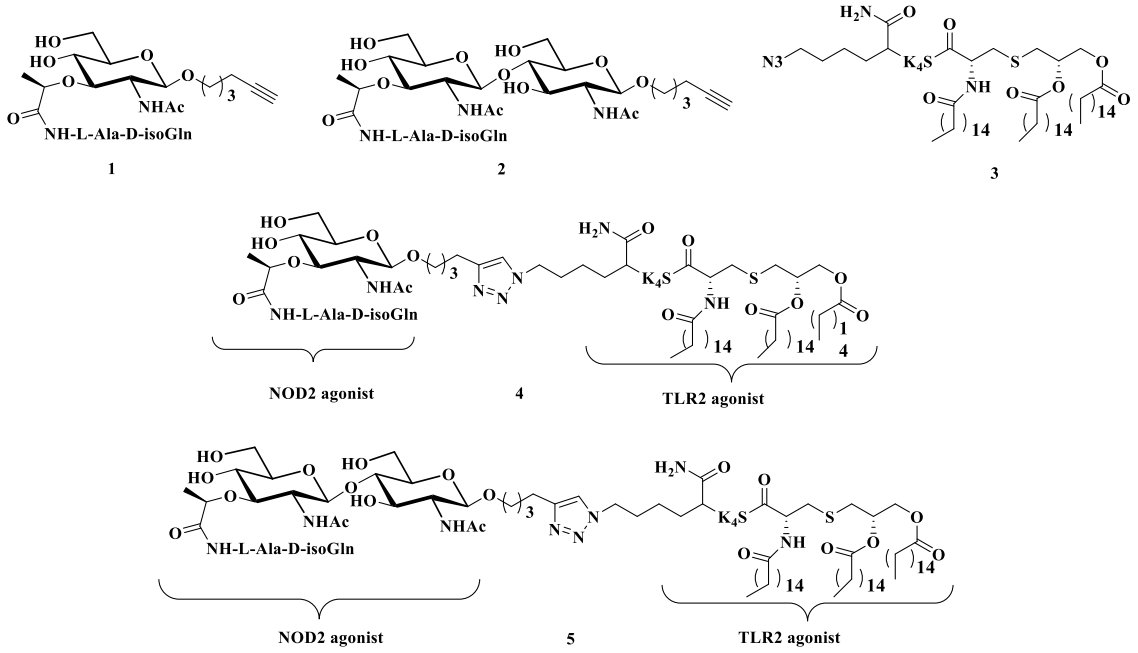
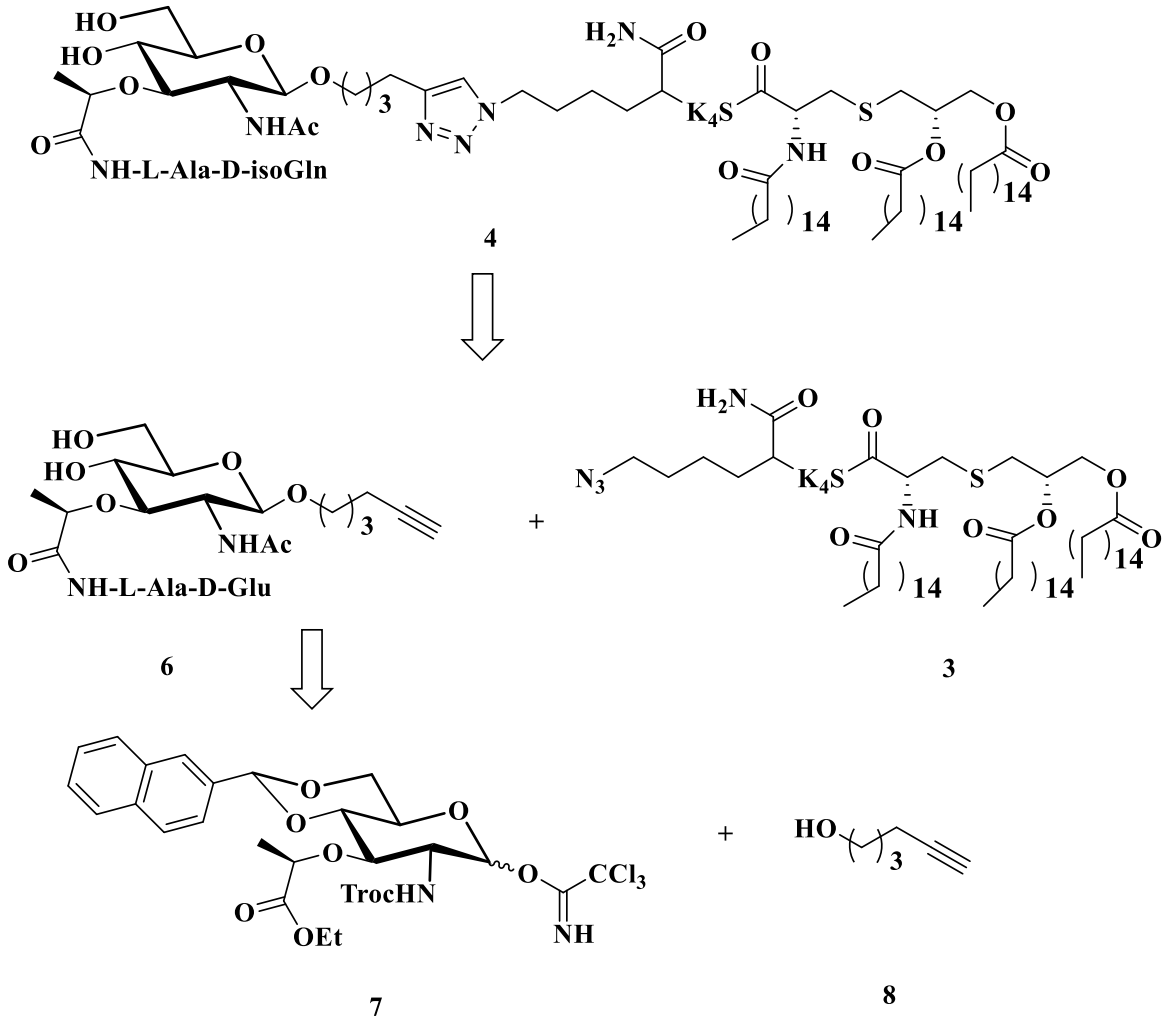
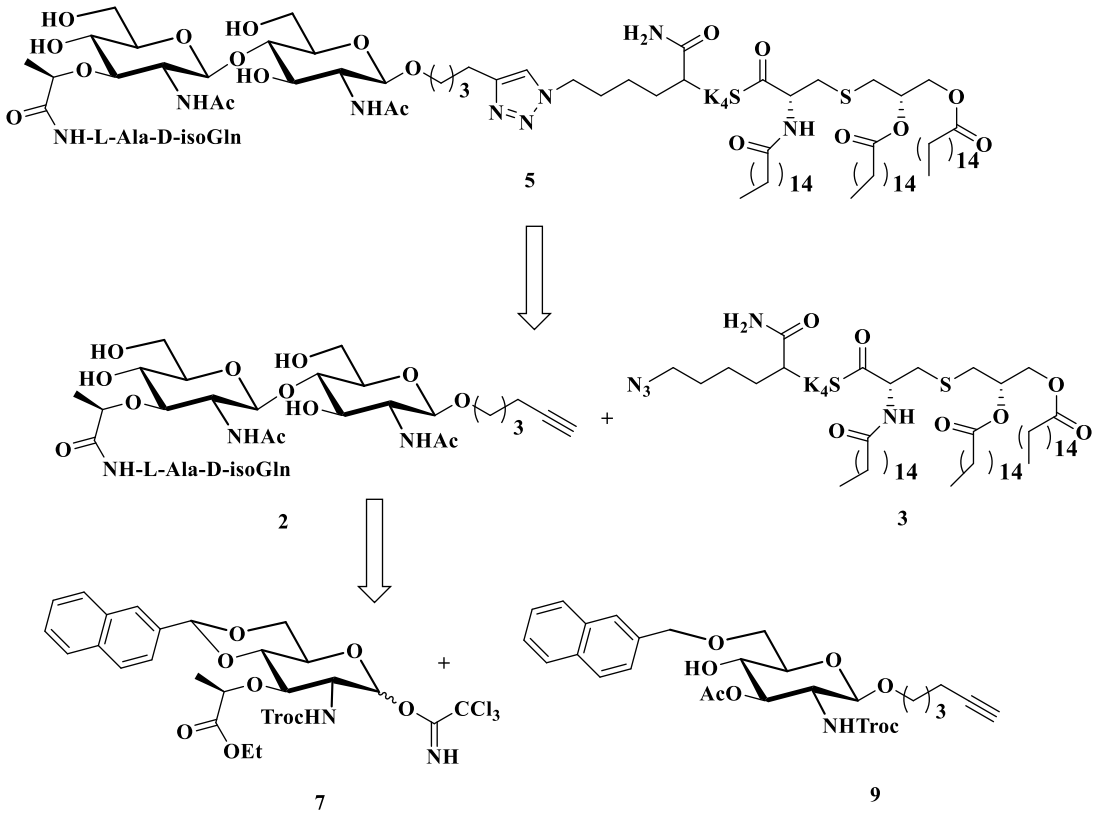


Figure 5.1 Final TLR2 and NOD2 target molecules

Compound **4** consists of MDP covalently linked to Pam_3CysSK_4 via azide-alkyne cycloaddition. Similarly, PGN disaccharide has been covalently attached to TLR2 ligand using Cu (I)-assisted click chemistry (compound **5**). The retrosynthetic analysis of these compounds is shown in Scheme **5.1** and **5.2**. Glycan strand of these compounds will be decorated by a dipeptide [L-Ala-D-*iso*Gln], which is linked to the glycan via an amide bond between the carboxyl group of MurNAc and the amino group of L-Ala at position 1 of the dipeptide. In the NOD2 glycan synthesis, common building blocks have been utilized to achieve both target molecules. Efficacy of these target molecules has been tested for their biological activities.



Scheme 5.1 Retrosynthetic analysis of MDP-TLR2 chimeric compound

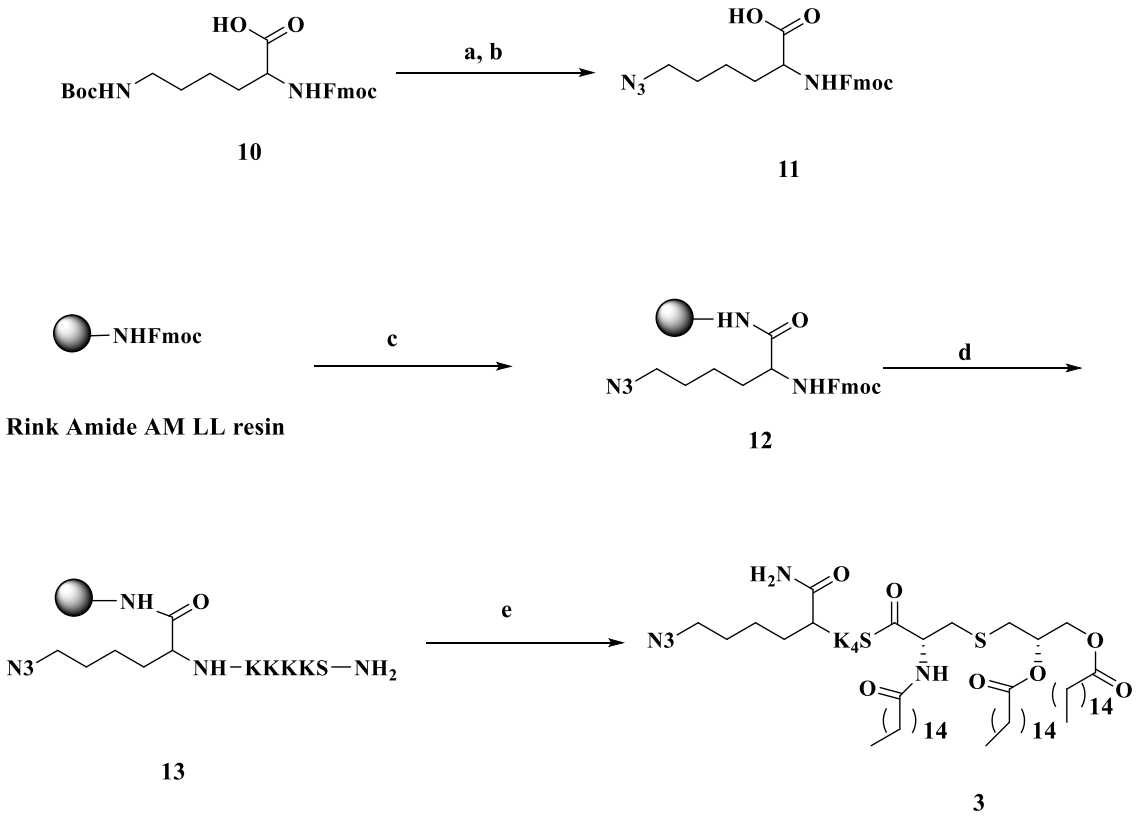


Scheme 5.2 Retrosynthetic analysis of NOD2-TLR2 chimeric compound

5.3 Result and discussion

Synthesis of Fmoc-Azido Lysine derivative 11

Commercially available Fmoc-L-Lys(Boc)-OH **10** was treated with TFA:DCM (1:1) for 30 minutes to remove Boc protection from the amine side chain. The resulting compound Fmoc-L-Lys-OH, K_2CO_3 and copper (II) sulfate pentahydrate was suspended in MeOH/H_2O (8:2 (v/v)), 1H-Imidazole- 1-sulfonyl azide.HCl was added and the pH was adjusted and maintained at 8-9 by the addition of sat. aq. K_2CO_3 during the course of the reaction. Thus Fmoc-Azido Lysine compound **11** was obtained and used in the lipopeptide synthesis (Scheme 5.3).

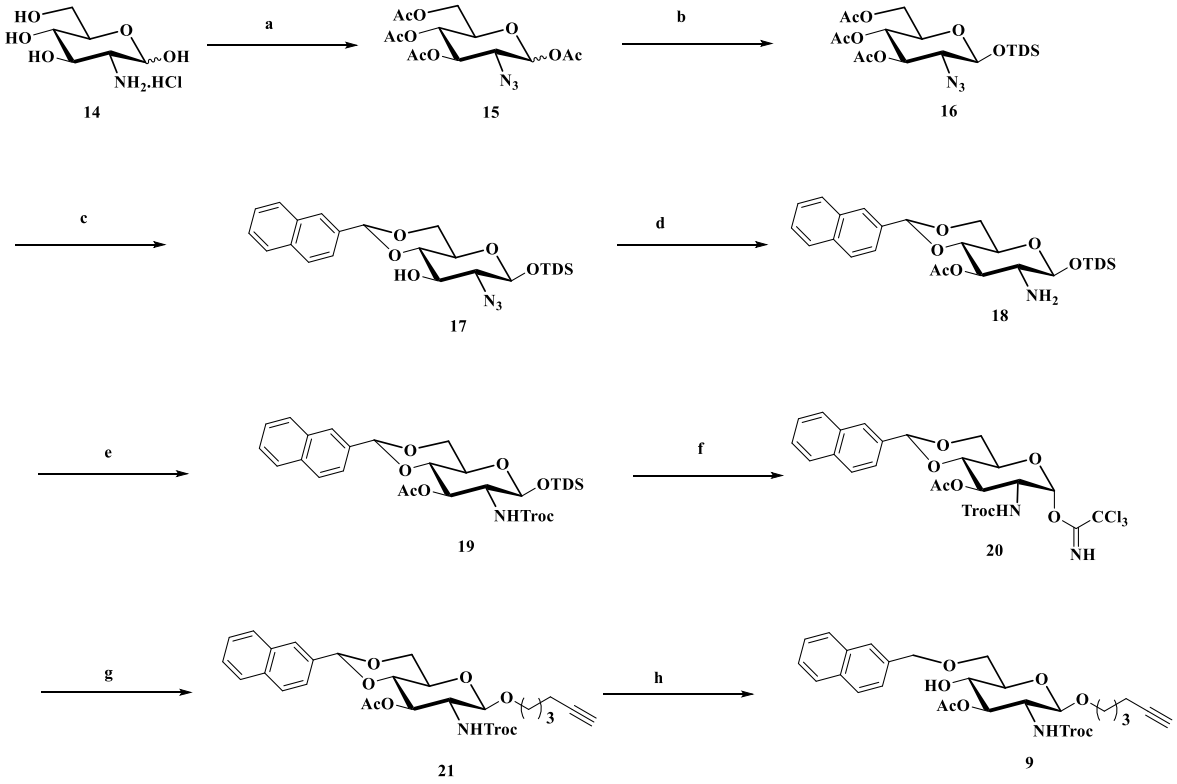


Scheme 5.3 Synthesis of TLR2 ligand with azide handle
Reagents and conditions: (a)50%TFA in DCM (b) 1H-Imidazole-1-sulfonyl azide.HCl, K_2CO_3, MeOH (c) (i) 20% 4-methylpiperidine, DMF, MW (ii) **13**, HOAt, HATU, DIPEA, DMF,MW (d) (i) 20% 4-methylpiperidine, DMF, MW (ii). Fmoc-AA-OH, HOBt, HBTU, DIPEA, DMF MW (e) (i) $Pam_2CysCOOH$, HATU, HOAt, DIPEA, DMF (ii) 20% 4-methylpiperidine in DMF,MW (iii) Palmitic acid, HATU, HOAt, DIPEA, DMF, MW, 10 min (iv) 88% TFA, 5% Phenol, 5% H_2O, 2.5% TIPS

Synthesis of TLR 2 Ligand Lipopeptide 3

Lipopeptide **3** was synthesized using combination manual microwave assisted solid phase peptide synthesizer and automated microwave assisted solid phase peptide synthesizer (MW-SPPS) as shown in Scheme5.3. Fmoc deprotection of amine of Rink Amide AM LL resin was manually performed using 20% 4-methylpiperidine in DMF followed by manual coupling of Fmoc-Azido Lysine derivative **11**, using MW-SPPS to get intermediate **12**. Then next five amino acids were introduced using an HBTU-mediated HOBt ester activation protocol on automated peptide synthesizer to furnish

compound **13**. The Pam_2Cys-OH, lipid derivative, **16** was introduced manually using manual MW-SPPS followed by removal of Fmoc using 20% 4-methylpiperidine in DMF. The resin with the free amino group was then reacted with the palmitic acid in the presence of HATU, and HOAt followed by cleavage of lipopeptide from the solid support to give final lipopeptide TLR2 ligand decorated with azide functionality.

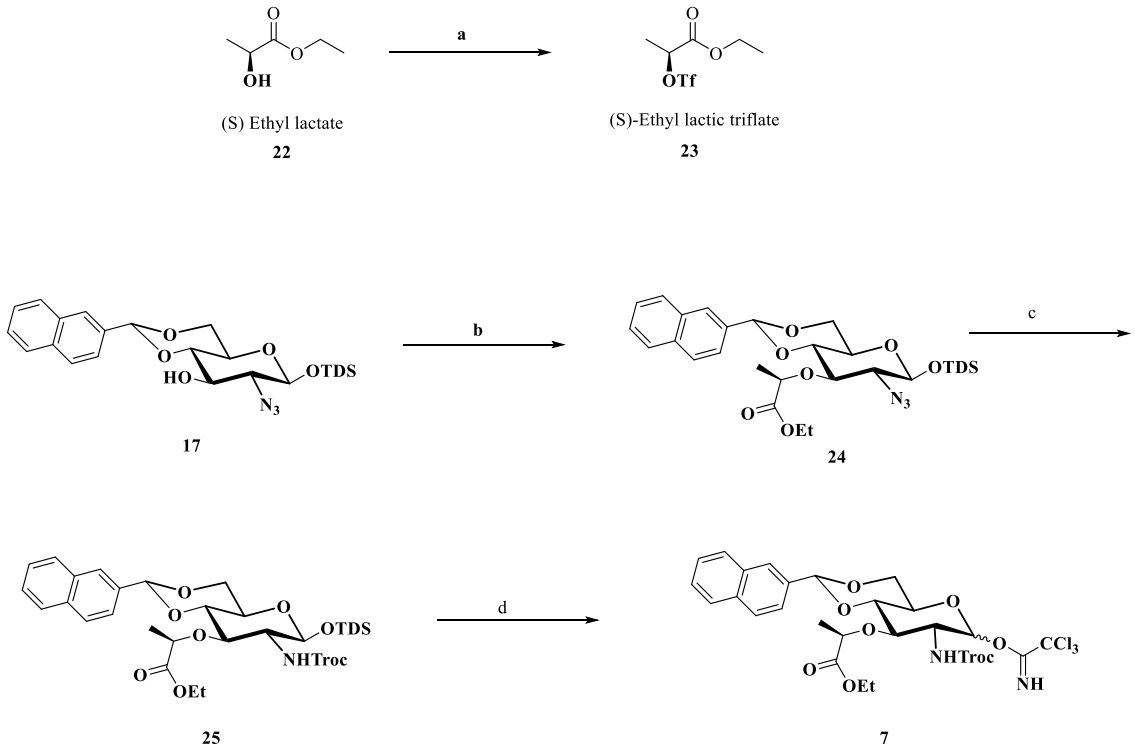


Scheme 5.4 Synthesis of *N-Troc*-Glucosamine Acceptor

Reagents and conditions: (a) (i) Imidazole azide, K_2CO_3, MeOH (ii) 2) Ac_2O, Pyridine 78% over two steps (b) (i) NH_2-NH_2.AcOH DMF 50^oC (ii) TDS-Cl, Imidazole, DMF 87% over two steps (c) (i) NaOMe, MeOH (ii) 2-Napthaldehyde, CSA, ACN, RT 67% over two steps (d) (i) Ac_2O, Pyridine (ii) Zn-Cu, THF:AcOH 75% over two steps (e) Troc-Cl, TMEDA, Dry DCM, 0^oC 75% (f) (i) HF:Py, THF (ii) CCl_3CN, Cs_2CO_3,DCM 80% over two steps (g) (i) 5-Hexyne-1 ol TMSOTf, Dry DCM 4A^o MS, -20^oC, 86% (h) $SiEt_3$ TfOH, Dry DCM, -78^oC, 84%

Synthesis of *N*-Troc-Glucosamine Acceptor, 17

Synthesis of Glucosamine acceptor was started from commercially available D-Glucosamine hydrochloride **14**, which was then treated with *1H*-imidazole- 1-sulfonyl azide.HCl in the presence of K_2CO_3 base in Methanol to convert C2 amine to azide. The free hydroxyls were then acetylated by using Ac_2O in pyridine to furnish compound **15**. Anomeric *O*-acetyl group was converted to a free hydroxyl group using hydrazine acetate then protected with silyl ether to afford compound **16**. Acetyl groups of compound **16** were removed by treatment of sodium methoxide in Methanol followed by 4′,6′-*O*-napthylidene formation to furnish an important intermediate **17** with 67% yield and starting material was recovered and reused**.** This intermediate **17,** containing C3 free hydroxyl group was also used for the synthesis of Muramic acid donor derivative. Acetylation of C3 hydroxyl was achieved by treating it with Ac_2O in pyridine. Reduction of C2 azido group to amine was performed using Zn-Cu couple in acetic acid to obtain compound **18**. The C2 amino group of this intermediate was then protected by neighboring group participating trichloroethoxycarbonyl (Troc) functionality to provide compound **19**. The anomeric silyl ether protection was cleaved in the presence of HF and pyridine in THF and then converted to trichloroacetimidate donor using trichloroacetonitrile in good yields. Next glycosylation of this donor with 5-Hexyne-1 ol linker was achieved in the presence of catalytic amount of TMSOTf in dry DCM at a lower temperature to provide compound **21**. Regioselective 4′,6′-*O*-naphthylidene ring opening was performed by addition of triethylsilane and triflic acid in dry DCM at -78^{o}C to achieve C4 hydroxyl Glucosamine acceptor compound **9** containing alkyne linker at anomeric position (Scheme 5.4).



Scheme 5.5 Synthesis of *N*-Troc Muramic acid donor
Reagents and conditions: (a) Tf_2O, 2,6-Luitidine, DCM, -70^oC (b) NaH, **23**, DCM, 66% (c) (i) Zn-Cu, THF:AcOH (1:1) (ii) Troc-Cl, TMEDA, DCM,0^oC 55% (e) (i) HF:Py, THF, 81% (ii) CCl_3CN, Cs_2CO_3, Dry DCM 0^oC, 93%

Synthesis of Muramic acid donor 30

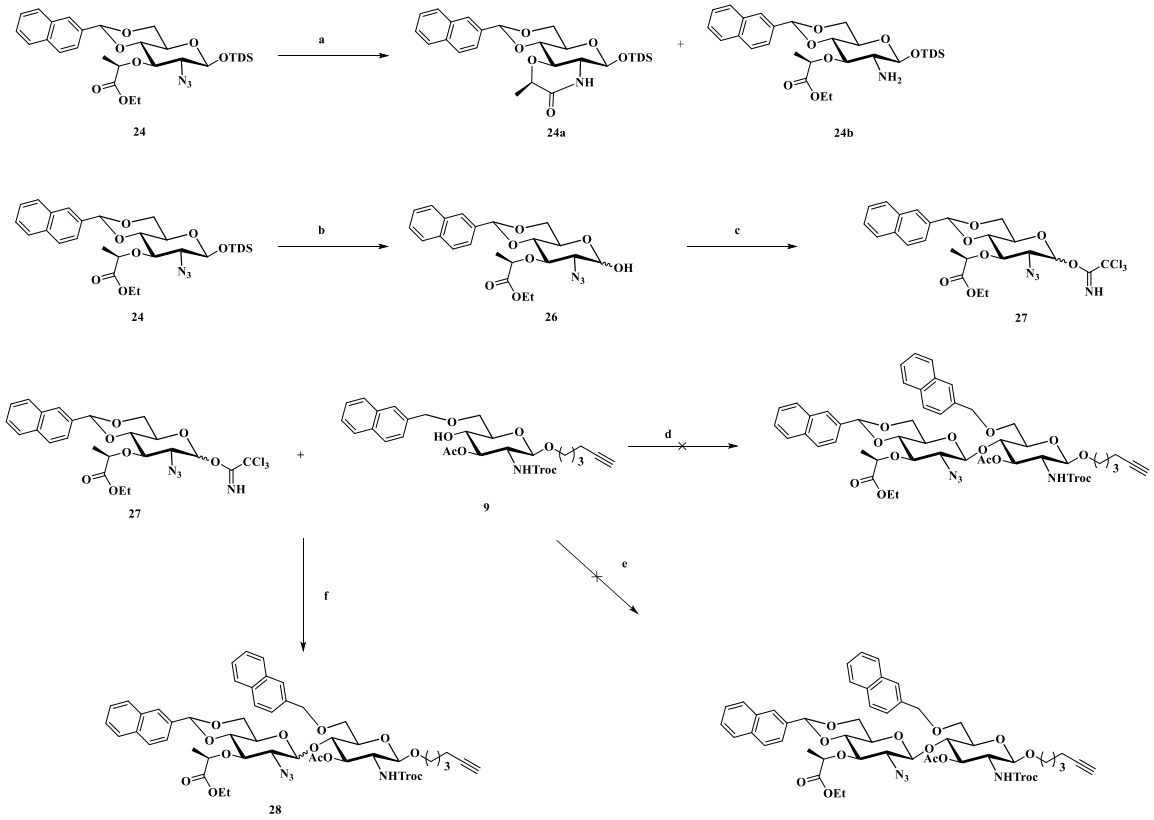
Alkylation of C-2 azido compound **17** was one of the key steps in the synthesis. In the beginning our several attempts to perform alkylation using previously reported[33] protocol which (S)-2-Chloropropionic acid failed may be due to steric bulk at C4-C6 naphthylidene. Based on literature search use of highly reactive trifluoromethanesulfonyl (S)-2-propionic acid provided good alternative.[34] Commercially available (S) Ethyl lactate **22** was utilized for formation of trifluoromethanesulfonyl (S)-2-propionic acid ethyl ester **23**. Previously synthesized intermediate **17** was alkylated at the C3 position by reported protocol that uses treatment of NaH as a base followed by addition of freshly made trifluoromethanesulfonyl (S)-2-propionic acid ethyl ester which afforded Muramic

acid derivative **24** in good yields. The next step in the planned synthetic scheme was the reduction of C2 azido group to amine followed by Troc protection of the Muramic acid derivative. Our initial attempts provided a very low yield of desired amino product and undesired lactam was obtained as a major product. Several other reaction conditions were tried to avoid the formation of this cyclic intermediate but it was formed either in the reduction step or upon addition of the base during next Troc protection step.[35] To address this problem other synthetic routes were sought to make disaccharide using C2 azido Muramic acid donor as shown in scheme 5.6.[34] The reactions without C2 neighboring participating group either failed or yielded a very low percentage of desired beta product (scheme 5.6). After multiple unsuccessful attempts to reduce azide new optimized protocol was used, which yielded desired product as shown in scheme 5.5. The azide group of the derivative **24** was reduced using Zn in THF/AcOH (1:1), and the liberated amino group was in situ protected with Troc group by addition of 10 equivalents of Troc-Cl to achieve compound **25**. It is worth mentioning that a short reaction time and low temperature is crucial to avoid the formation of lactam. Removal of anomeric silyl ether protection of compound 25 was performed in presence of HF and pyridine in THF followed by treatment of trichloroacetonitrile in dry DCM in presence of mild base furnished Muramic acid trichloroacetimidate donor derivative 7 (Scheme 5.5).

Synthesis of MDP with linker and MDP-TLR2 chimeric compound

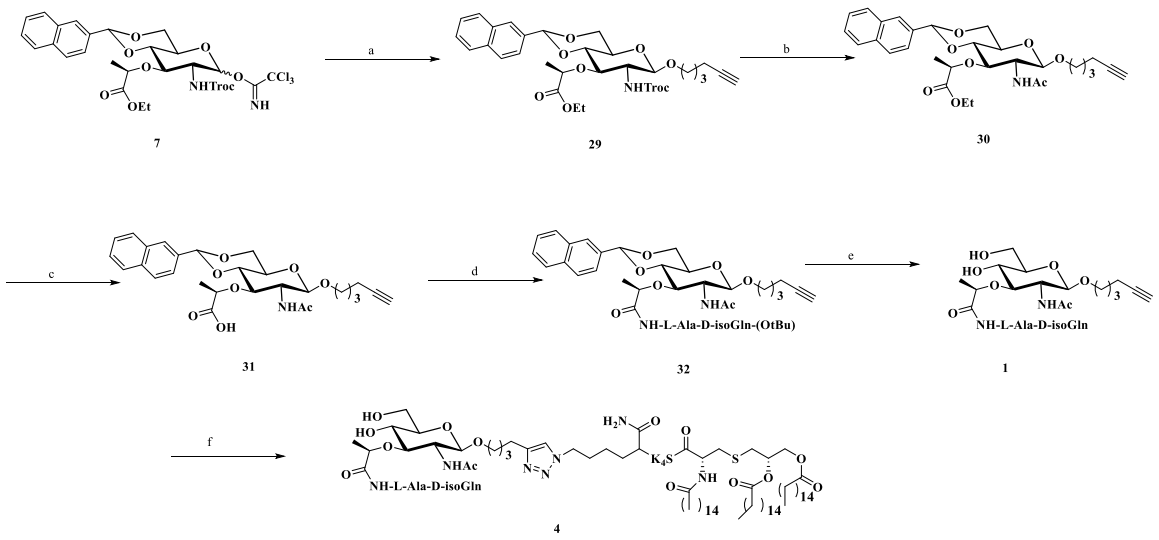
Trichloroacetimidate donor **7** was coupled with 5-Hexyne-1-ol by using trifluoromethanesulfonic anhydride (TMSOTf) as a promoter to give **29** with 96% yield. The Troc group was removed by treating it with Zinc (Zn) dust in acetic acid at r.t., followed by in situ protection with acetyl using acetic anhydride to give **30** with 76%

yield. Hydrolysis of C3 lactyl ester was performed by Lithium hydroxide afforded *N*-acetyl Muramic acid derivative **31**. The L-Ala-D-isoGln-OtBu dipeptide was introduced



Scheme 5.6 Alternative routes for synthesis of PGN fragment
Reagents and conditions: (a) Zn-Cu, THF:AcOH (1:1) (b) HF:Py, THF (c) CCl_3CN, Cs_2CO_3, Dry DCM 0^oC (d) TfOH, Dry ACN, 4A^o MS,-20^oC (e) $Yb(OTf)_3$, Dry ACN, 4A^o MS,-20^oC(f) $BF_3.EtO_2$, Dry DCM:Hexanes, 4A^o MS,-78^oC, 25% (α/β:10/1)

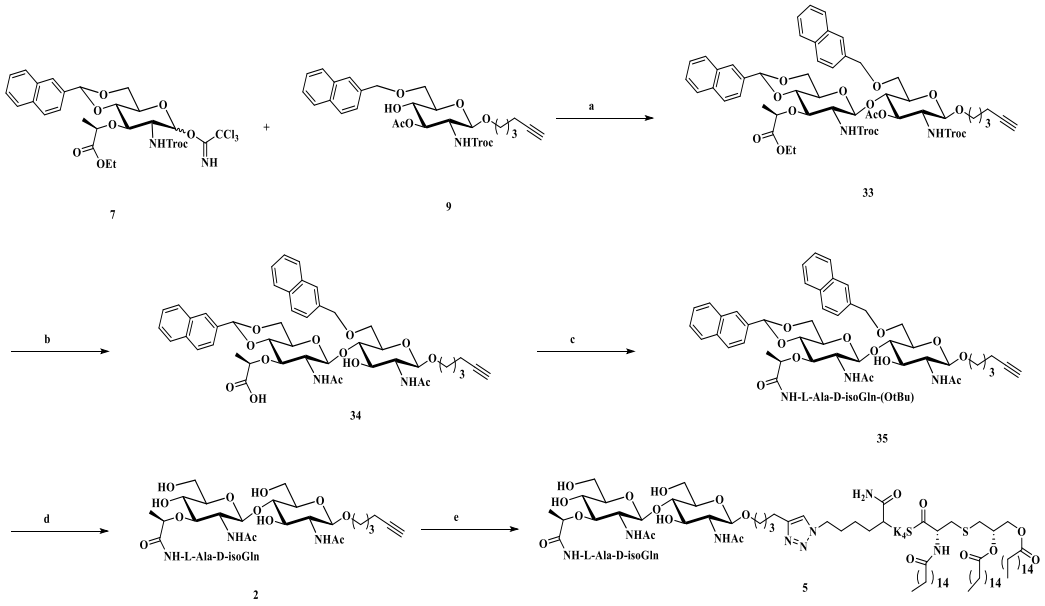
to the liberated carboxylic acid **31** by using HATU, HOAt, and triethylamine (TEA). Removal tBu group of dipeptide and C4-C6 naphthylidene was achieved using CH_2Cl_2/TFA (1:1). The final Cu (I)-assisted cycloaddition between alkynyl MDP derivative and azido TLR2 agonist derivative was performed using combination of previously reported procedures in presence of TBTA and NaAsc to furnish chimeric compound **4** with 75% yield.[36-37]



Scheme 5.7 Synthesis of MDP alkyne derivative and NOD2-TLR2
Reagents and conditions: (a) 5-Hexyne-1-ol TMSOTf, Dry DCM 4A^{o} MS-20^{o}C, 96% (b) Zn-Cu THF:Ac_2O:AcOH (c) LiOH, THF/1-4 Dioxane/H_2O(4:2:1), 76% over two steps (d) HOBt, DIPEA, NH_2-L-Ala-D-isoGln-(OtBu), DMF, 80% (e) (i) DDQ, CH_2Cl_2, MeOH (ii) TFA/CH_2Cl_2 (1:2), 81% (f) $CuSO_4$, TBTA, NaAsc, H_2O:tBuOH, 75%

Synthesis of PGN disaccharide derivative and NOD2-TLR2 chimeric compound

For the synthesis of PGN (MurNAc-GlcNAc) disaccharide previously synthesized, *N*-Troc protected Muramyl acid donor derivative **7** and C4 Hydroxyl of *N*-Troc Glucosamine acceptor **9** containing anomeric alkynyl linker was utilized. The glycosylation was performed under catalytic amount triflic acid conditions to furnish disaccharide **33.** Neighboring group participation by 2,2,2-trichloroethoxy-carbonyl (Troc) group furnished exclusively desired beta (β) product. The obtained compound was then treated with Zn-Cu in THF/AcOH/Ac_2O to remove the *N*-Troc groups and formation of the *N*-acetylated compound. Subsequent deprotection of the ester groups by Lithium hydroxide afforded disaccharide **34**. This disaccharide was then condensed with appropriately protected dipeptide (L-Ala-D-isoGln-OtBu) by using HOAt, HATU, and



Scheme 5.8 Synthesis of PGN disaccharide derivative and NOD2-TLR2 chimeric compound
Reagents and conditions: (a) TfOH, 4A^{o}MS, Dry DCM, -20^{o}C, 95% (b) (i) Zn-Cu, AcOH/Ac_2O/THF, (ii) 2) LiOH, THF/1-4 Dioxane/H_2O, 59% over two steps (c) NH_2-Ala-D-isoGln-OtBu HOAt, HATU, DIPEA, DMF, 87% (d) (i) TFA:DCM (ii) DDQ, CH_2Cl_2: MeOH, 67% over two steps (e) **4**, $CuSO_4$, TBTA, NaAsc, H_2O/tBuOH, 78%

DIPEA in DMF to give PGN disaccharide with dipeptide compound **35**. Final global deprotection of *t*Bu group, naphthylidene acetal and naphthyl ether were achieved by treatment of TFA/DCM and DDQ respectively to furnish PGN disaccharide **2** with alkyne linker at anomeric center. The final Cu (I)-assisted cycloaddition between alkynyl PGN disaccharide derivative and azido TLR2 agonist derivative was performed using combination of previously reported procedures in presence of TBTA and NaAsc to furnish chimeric compound **5** with 78% yield (Scheme 5.8).

5.4 Biological Experiments

To understand the structure-activity relationship, synthetic derivatives **1–5** were tested for their ability to induce a cytokine, TNF- α in a human monocytic cell line (Mono Mac 6 cells). The results were compared with TNF- α production by TLR2 ligand,

Pam_3CysSK_4 alone and synergistic effects of modified and unmodified Pam_3CysSK_4 along with synthetic MDP derivatives as shown in figure **5.2** and table **5.1**.

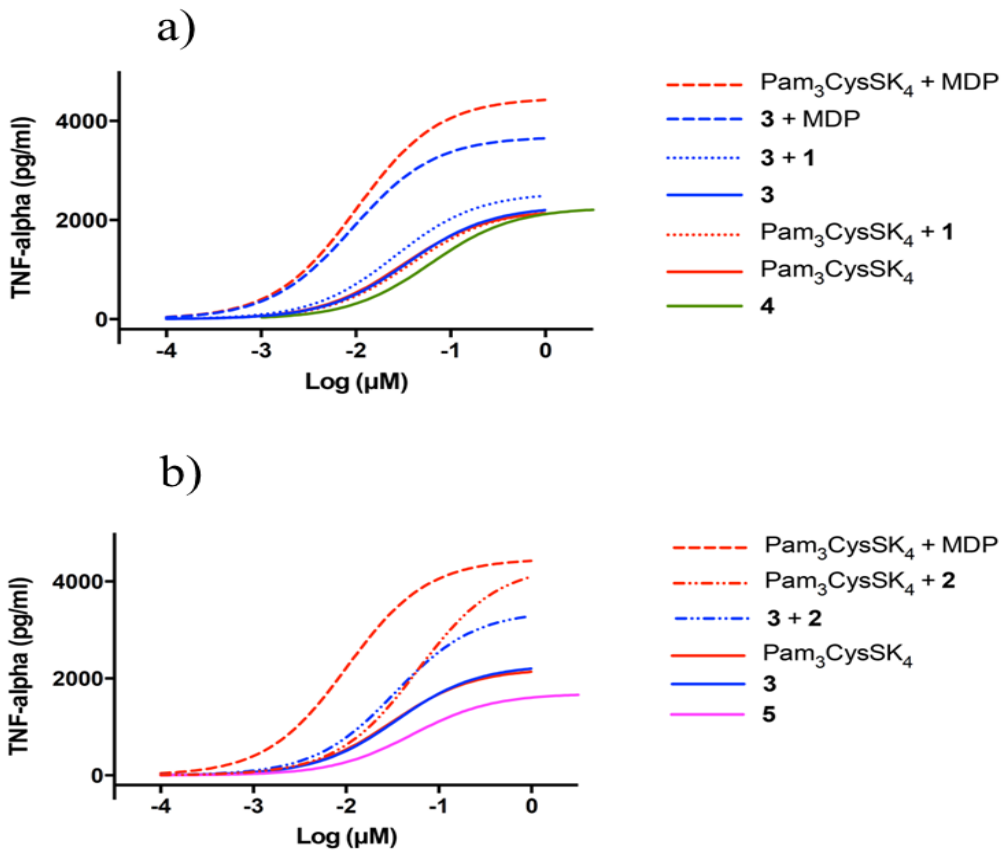


Figure 5.2 TNF-α production by Mono Mac 6 macrophages after stimulation with synthetic compounds 3, 4, 5, and Pam_3CysSK_4 (0-10µM) alone and together with MDP, 1 or 2 (10 µM). MDP, 1 or 2 at10 µM concentration do not produce TNF-α on their own. TNF-α production was measured in supernatants after 5.5 hours incubation.

It was found that the incubation of Mono Mac 6 cells with synthetic compound **3**, which is an azido derivative of Pam_3CysSK_4, did not affect the production of TNF- α compared to unmodified Pam_3CysSK_4 indicating an additional azido lysine moiety did not alter biological properties of Pam_3CysSK_4. However, when synthetic compound **3** was coincubated with unmodified NOD ligand, MDP, a slight drop in the production of TNF-α protein was observed compared with unmodified Pam_3CysSK_4 and MDP. Surprisingly,

a significant drop in the production of TNF-α protein was seen when MM6 cells were incubated with a combination of compound **3** and **1** a modified MDP derivative at the anomeric position. The covalently joined TLR2-NOD2 compound **4** also produced a significant amount of TNF-α protein at the comparable concentration as that of Pam_3CysSK_4 alone (Figure 5.2a). Similarly, incubations with Pam_3CysSK_4 + **2** (modified peptidoglycan disaccharide at the anomeric position) and **3** + **2** did not show a significant difference in the activity. However, covalently linked TLR2-NOD2 compound **5** showed significant drops in the levels of TNF-α protein (figure 5.2b).

Table 5.2 TNF-α Plateau values (pg/ml) and LogEC50 (μM) values for synthetic compounds 3, 4, 5, and Pam_3CysSK_4 alone and together with MDP, 1 or 2.

Compounds	TNF-α Plateau values (pg/ml)	LogEC50 (μM)
Pam_3CysSK_4	2204	-1.503
Pam_3CysSK_4 + MDP	4470	-1.992
Pam_3CysSK_4 + 1	2227	-1.423
Pam_3CysSK_4 + 2	4341	-1.228
3	2278	-1.445
3 + MDP	3683	-2.034
3 + 1	2555	-1.585
3 + 2	3395	-1.476
4	2251	-1.211
5	1688	-1.286

5.5 Conclusion

In summary, we have described the synthesis and biological evaluation of novel TLR2-NOD2, chimeric adjuvants. Furthermore, we report the synthetic strategies to obtain modified MDP and PGN disaccharide fragments from common building blocks. Our biological results suggest that modification of the individual structures of both TLR2 ligand and NOD2 ligand can be tolerated, however, in the case of TLR2-NOD2 fusion

PAMPs, modification at the anomeric position of MDP and PGN may adversely affect their biological properties. It is our belief that further experimentation is needed to explore modification at C6 position of MDP and PGN fragment.

5.6 Experimental Section

Cell Maintenance for cytokine study

Mono Mac 6 cells, provided by Dr. H.W.L. Ziegler-Heitbrock (University of Munich, Germany), were cultured in RPMI 1640 medium with L-glutamine (BioWhittaker) supplemented with 100 u/ml penicillin, 100 µg/ml streptomycin, 1% OPI supplement (Sigma) (containing oxaloacetate, pyruvate and bovine insulin) and 10% heat-inactivated fetal calf serum. The cells were maintained in a humid 5% CO_2 atmosphere at 37°C. New batches of frozen cell stock were grown up every 2 months and growth morphology evaluated. Before the experiment, Mono Mac 6 cells were allowed to differentiate for 2 days in the presence of 10 ng/ml calcitriol (400 µl/flask = 400 µl /40 ml) (Sigma).

Cell Harvesting & Cell Stimulation for Cytokine study

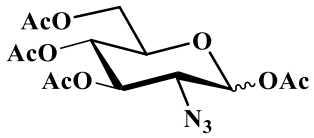
Cells were harvested (four T-75 flasks) by centrifugation (5 min at 1400 rpm). Pellets were combined together into 10 ml media. The number of cells was counted. The cell suspension was further diluted to 1.11 x 106 cells/ml, and aliquoted as 200 µl/well (222000 cells/200 µl) to obtain a final concentration in all samples of 222000 cells/well (= 1.11 x 106 cells/ml). The cell suspension (200 µl/well) was incubated with medium (blank), or synthetic compound (22.2 µl) in triplicate with a range of concentration up to 10 µM. After 5.5 hours the cell suspension was centrifuged for 15 min at 1400 rpm (Sorvall) and the supernatant was harvested and frozen at –80 °C.

All cytokine values are presented as the mean SD of triplicate measurements, with each experiment repeated three times. The supernatants were examined for human TNF-α, using an in-house developed capture ELISA assay repeated three times. Potencies (EC50, concentration producing 50% activity) and efficacies (maximal level of production) were determined by fitting the dose-response curves to a logistic equation using PRISM software.

Synthesis of Compound azido TLR2 ligand compound 3. The lipopeptide **3** was synthesized by solid phase peptide synthesis (SPPS) on Rink Amide AM resin (0.1 mmol) on a CEM LIBERTY 908505 peptide synthesizer equipped with a UV detector using N^{α}-Fmoc protected amino acids. The azidolysine amino acid was installed manually using Fmoc-Azido Lysine (74 mg, 0.19 mmol) in DMF (2 mL), 2-(7-aza-1H-benzotriazole-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (72 mg, 0.19 mmol), 1-Hydroxy-7-azabenzotriazole (HOAt) (26 mg, 0.19 mmol) and DIPEA (70 µL, 0.38 mmol) as the activating reagents. Next, five amino acids were introduced using an automated HBTU-mediated HOBt ester activation protocol on automated peptide synthesizer. Resin was then removed from automated peptide synthesizer and the lipid moiety was also installed manually using N-$^{\alpha}$-Fmoc-R-(2,3-bis(palmitoyl oxy)-(2R-propyl)-(R)-cysteine (178 mg, 0.2 mmol) in DMF (5 mL), HATU (76 mg, 0.2 mmol), HOAt (27 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol). The coupling reactions were monitored by the Kaiser test. Upon completion of the coupling, the N-Fmoc group was cleaved using 20% 4-methylpiperidine 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), HOAt (27 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 overnight. The resin was then swelled in DCM (5 mL) for 1 h, after which it was treated with the cleavage cocktail (TFA 88%, water 5%, phenol 5%, and TIPS 2%; 10 mL) for 3 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 ice-cold diethyl ether (30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude lipopeptide **1** was 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 with a Phenomenex Jupiter semi-preparative C-4 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 and appropriate fractions were lyophilized to afford **1**. HR MALDI-TOF MS calculated for $[M+Na-N_2]^+$ 1657.2486; observed, 1657.2479.

1,3,4,6-tetra-O-acetyl-2-azido-2-deoxy-α/β –D-glucopyranose



15

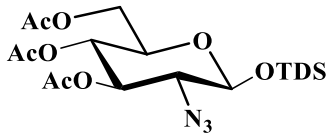
Glucosamine hydrochloride salt (10 g, 46.37 mmol), K_2CO_3 (17.30 g, 125.20 mmol) and $CuSO_4.5H_2O$ (0.115 g, 0.46 mmol) was suspended in 250 mL methanol and stirred overnight at room temperature. The reaction mixture was then concentrated and co-evaporated with toluene (2 x 200 mL). To this, acetic anhydride (34.83 mL, 370.96 mmol) and pyridine (200 mL) was added and stirred for 3 hr. at room temperature. The reaction mixture was then concentrated and the residue was diluted with water (200 mL).

The resulting solution was then extracted with ethyl acetate (3x 200 mL). The combined organic layer was washed with saturated aqueous brine (2x200 mL), dried ($MgSO_4$) and concentrated in *vacuo* to give the crude product, which was purified by silica gel column chromatography (EtOAc/Hexanes, 1/5 v/v) to afford pure product (13.5 g, 78%).

1H NMR (300 MHz,) δ 6.28 (d, J = 3.6 Hz, 1H), 5.54 (d, J = 8.6 Hz, 1H), 5.49 – 5.39 (m, 1H), 5.17 – 4.96 (m, 3H), 4.29 (ddd, J = 12.5, 4.3, 2.4 Hz, 2H), 4.13 – 3.98 (m, 3H), 3.86 – 3.74 (m, 1H), 3.72 – 3.59 (m, 2H), 2.18 (d, J = 0.9 Hz, 6H), 2.13 – 1.98 (m, 18H).

HR MALDI-TOF MS: calculated for $C_{14}H_{19}N_3NaO_9$ [M+Na] 396.1019; observed, $[M+Na]^+$ 396.1092

Dimethylthexylsilyl (3,4,6-tri-O-acetyl)-2-azido-2-deoxy-β –D-glucopyranoside



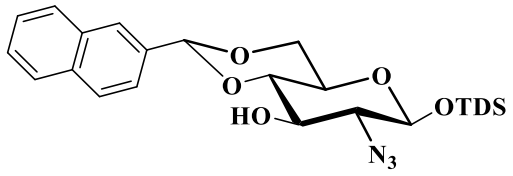
16

Hydrazine acetate (246 mg, 2.6809 mmol) was added to a solution of starting compound (1 g, 2.6809 mmol) in DMF (15 mL) and the reaction mixture was stirred at ambient temperature. After 2 h, TLC analysis showed total consumption of the starting material. DMF was evaporated and the residue was dissolved in EtOAc (50 mL) and then washed with water (2x 50 mL). The organic layer was dried over $MgSO_4$ and evaporated to get the crude compound. A solution of crude compound (850 mg, 2.5757 mmol) in DMF (5 mL) and imidazole (350 mg, 5.1515) was stirred for 5 minutes and TDS-Cl (0.6 mL, 3.0909 mmol) was added dropwise. The reaction mixture was stirred overnight at ambient temperature. The mixture was co-evaporated with toluene *in vacuo* and residue was dissolved in EtOAc (25 mL), washed with water (2x 25 mL), dried over $MgSO_4$ and

concentrated to obtain a crude product. The compound was purified by silica gel column chromatography using a gradient of EtOAc/Hexanes (1/9 v/v) to obtain the product (1.1 g, 87 %).

^{1}H NMR (300 MHz, Chloroform-*d*) δ 5.01 – 4.87 (m, 2H), 4.59 (d, *J* = 7.7 Hz, 1H), 4.22 – 4.03 (m, 2H), 3.70 – 3.57 (m, 1H), 3.48 – 3.31 (m, 1H), 2.09 – 1.95 (m, 9H), 1.73 – 1.54 (m, 1H), 0.92 – 0.82 (m, 12H), 0.18 (d, *J* = 1.8 Hz, 6H). HR MALDI-TOF MS: calculated for $C_{20}H_{35}N_3NaO_8Si$ [M+Na] 496.2091; observed, $[M+Na]^+$ 496.2557

Dimethylthexylsilyl 4,6-*O*-napthylidine-2-azido-2-deoxy-β –D-glucopyranoside

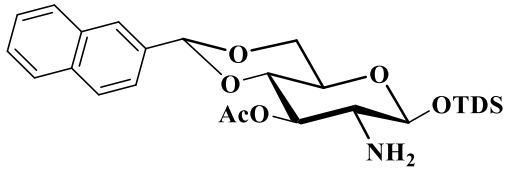


17

A solution of starting material (650 mg, 1.3742mmol) in a NaOMe (70 mg, 1.3742 mmol)in methanol solution (50ml) was stirred at rt for 30min. The mixture was then concentrated to afford the crude product, which was used as it is for next step. Camphor sulfonic acid (127.12 mg, 0.5472 mmol) was added to a solution 2-naphthaldehyde (341.87 mg, 2.1890 mmol) and above crude compound in anhydrous CH_3CN (10ml). After stirring at ambient temperature for 12 h TLC showed approximately half consumption of starting material. The reaction was diluted with ethyl acetate and washed with water (2x10 mL), aqueous saturated $NaHCO_3$ (2x 10 mL), and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude residue was purified by silica gel column chromatography using a gradient of EtOAc/Hexanes (1/8 v/v) to obtain compound (600 mg, 67%) and starting material was recovered.

^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.95 – 7.92 (m, 1H), 7.87 – 7.79 (m, 3H), 7.57 (dd, *J* = 8.6, 1.7 Hz, 1H), 7.50 – 7.45 (m, 2H), 5.65 (s, 1H), 4.59 (d, *J* = 7.6 Hz, 1H), 4.30 (dd, *J* = 10.5, 5.0 Hz, 1H), 3.79 (t, *J* = 10.3 Hz, 1H), 3.64 – 3.53 (m, 2H), 3.44 – 3.25 (m, 2H), 2.74 (s, 1H), 1.72 – 1.55 (m, 1H), 0.90 (d, *J* = 6.9 Hz, 12H), 0.19 (d, *J* = 5.5 Hz, 6H).

Dimethylthexylsilyl 3-*O*-acetyl-4,6-*O*-napthylidine-2-amino-2-deoxy-β –D glucopyranoside



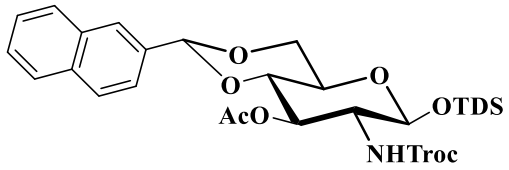
18

Compound 17 (100 mg, 0.1897 mmol) was suspended in mixture of acetic anhydride (0.17 mL, 1.8970 mmol) and pyridine (1 mL) and stirred for 3 hr. at room temperature. The reaction mixture was then concentrated and the residue was diluted with water (5 mL). The resulting solution was then extracted with ethyl acetate (3x 5 mL). The combined organic layer was washed with saturated aqueous brine (2x5 mL), dried ($MgSO_4$) and concentrated in *vacuo* to give the crude product, which was dissolved in 3:1 THF/HOAc (5 mL) and treated with Zn (37 mg, 0.5692 mmol) and sat. aq.$CuSO_4$ (1.25 mL) for 1 hr. The mixture was filtered over celite and co-evaporated with toluene. The residue was purified by silica gel column chromatography using EtOAc/Hexanes (1/8 v/v) gradient to afford free amine compound in 75% yield.

^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.90 – 7.87 (m, 1H), 7.85 – 7.77 (m, 3H), 7.55 – 7.42 (m, 3H), 5.62 (s, 1H), 5.11 (dd, *J* = 10.1, 9.4 Hz, 1H), 4.58 (d, *J* = 7.5 Hz, 1H), 4.31 (dd, *J* = 10.4, 4.9 Hz, 1H), 3.82 (t, *J* = 10.2 Hz, 1H), 3.70 (t, *J* = 9.4 Hz, 1H), 3.54 (td, *J* = 9.7, 4.9 Hz, 1H), 2.83 (dd, *J* = 10.1, 7.6 Hz, 1H), 2.10 (s, 3H), 1.69 – 1.58 (m, 1H), 0.91 –

0.83 (m, 12H), 0.17 (s, 6H). HR MALDI-TOF MS: calculated for $C_{27}H_{39}NNaO_6Si$ $[M+Na]^+$ 524.2444; observed, [M+Na] 524.2440

Dimethylthexylsilyl 3-*O*-acetyl-4,6-*O*-napthylidine-2-deoxy-2-[(2,2,2 trichloroethoxy)carbonyl]amino-β –D glucopyranoside

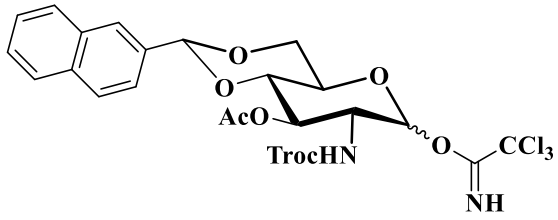


19

Free amine compound (2.25 g, 4.4820 mmol) was dissolved in dry DCM (50 mL) followed by the addition of N, N, N′, N′-Tetramethylethylenediamine (1.34 mL, 8.9640 mmol) and 2,2,2- trichloroethoxycarbonyl chloride (1.854 µL, 13.4462 mmol). The reaction mixture was stirred at RT overnight after which TLC (EtOAc:Hexane, 1:5) showed it was complete. The mixture was diluted with DCM (50 mL) and washed with sat. $NaHCO_3$ (100 mL). The organic layer was dried ($MgSO_4$), filtered and the filtrate was concentrated *in vacuo* and the residue purified by silica gel column chromatography using EtOAc/Hexane (1/9 v/v) as the eluent to afford the title compound as a white foam. 2.2 g (75%)

^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.05 – 7.76 (m, 4H), 7.67 – 7.41 (m, 3H), 5.71 (s, 1H), 5.50 – 5.16 (m, 2H), 4.89 (s, 1H), 4.87 – 4.80 (d, *J* = 7.5 Hz, 2H), 4.62 (d, *J* = 12.0 Hz, 1H), 4.38 (dd, *J* = 10.5, 4.9 Hz, 1H), 4.19 (s, 1H), 3.99 – 3.68 (m, 3H), 3.58 (td, *J* = 9.7, 4.9 Hz, 1H), 2.11 (s, 3H), 1.78 – 1.52 (m, 3H), 1.00 – 0.79 (m, 12H), 0.19 (d, *J* = 5.7 Hz, 6H). HR MALDI-TOF MS: calculated for $C_{30}H_{40}C_{l3}NNaO_8Si$ $[M+Na]^+$ 698.1486; observed, [M+Na] 698.1481

3-*O*-acetyl-4,6-*O*-napthylidine-2-deoxy-2-[(2,2,2 trichloroethoxy)carbonyl]amino-α/β–D glucopyranosyl trichloroacetimidate

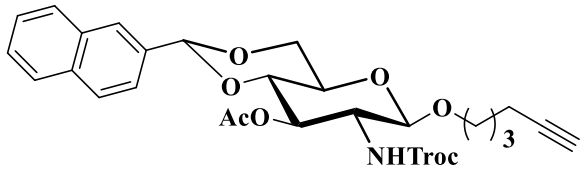


20

To a stirred solution of 18 starting material (1 g, 1.4814 mmol) in THF (20 mL), HF in pyridine (30%, 1.4 mL) was added. After stirring at ambient temperature for 16 h TLC indicated complete consumption of the starting material. The reaction mixture was subsequently diluted with DCM, washed with water, NaHCO3 (Satd.), and brine. The organic phase was dried (MgSO4), filtered, and concentrated under reduced pressure and the resulting white solid was left *in vacuo* for 2 h. Cl_3CCN (766 μL, 7.4074 mmol) and $CsCO_3$ (577 mg, 1.7776 mmol) were added to a solution of the crude material in DCM (25 mL). After stirring for 1 h. at ambient temperature, the mixture was concentrated in vacuo and purified by silica gel column chromatography (EtOAc/Hexane, 1/5 v/v) to give the product (800 mg, 80% after two steps).

^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.77 (s, 1H), 7.97 – 7.78 (m, 4H), 7.60 – 7.43 (m, 3H), 6.43 (d, *J* = 3.8 Hz, 1H), 5.71 (d, *J* = 7.7 Hz, 1H), 5.56 – 5.37 (m, 1H), 5.37 – 5.18 (m, 1H), 4.84 – 4.54 (m, 2H), 4.49 – 4.22 (m, 2H), 3.98 – 3.80 (m, 2H), 2.10 (d, *J* = 6.6 Hz, 3H).

Hex-5-yn-3-*O*-acetyl-4,6-*O*-napthylidine-2-deoxy-2-[(2,2,2 trichloroethoxy)carbonyl]amino-β –D glucopyranoside

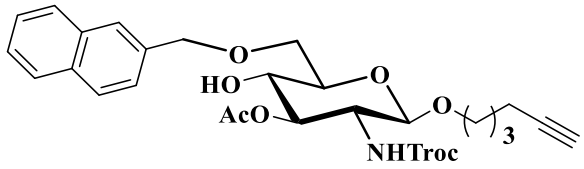


21

A mixture of the donor 19 (90 mg, 0.1325 mmol), acceptor, 5-Hexyn-1-ol (22 μL, 0.1987 mmol), and 4Å flame-dried molecular sieves was stirred in dry DCM (10 mL) at RT for 30 min. The resulting solution was cooled (-60^{o} C), after which TMSOTf (2.4 μL, 0.01987 mmol) was added. The reaction mixture was stirred at -60^{o} C for 45 min. TLC (EtOAc:Hexane, 1:5, v/v) showed the acceptor has been converted into a slightly less polar product. The reaction was then quenched with sat. $NaHCO_3$ (0.5 mL) and the resulting mixture was allowed to warm to RT, after which it was diluted with DCM (5 mL) and washed with sat. $NaHCO_3$ (5 mL). The organic layer was dried ($MgSO_4$), concentrated *in vacuo* and the residue purified by silica gel column chromatography using EtOAc/Hexane (1/9 to 1/6, v/v) as the eluent to afford product (70 mg, 86%).

^{1}H NMR (300 MHz,) δ 7.95 – 7.78 (m, 4H), 7.51 (ddd, *J* = 15.7, 7.3, 2.6 Hz, 3H), 5.67 (s, 1H), 5.35 (t, *J* = 9.9 Hz, 1H), 5.12 (s, 0H), 4.74 (d, *J* = 19.6 Hz, 1H), 4.60 (d, *J* = 8.3 Hz, 1H), 4.41 (dd, *J* = 10.6, 4.9 Hz, 1H), 3.98 – 3.69 (m, 3H), 3.56 (td, *J* = 9.8, 5.4 Hz, 2H), 2.20 (td, *J* = 6.9, 2.6 Hz, 2H), 2.07 (s, 3H), 1.95 (t, *J* = 2.6 Hz, 1H), 1.74 – 1.56 (m, 2H). HR MALDI-TOF MS: calculated for $C_{28}H_{30}C_{l3}NNaO_8$ $[M+Na]^+$ 636.0935; observed, [M+Na] 636.0930

Hex-5-yn-3-*O*-acetyl-6-*O*-(2-methyl-napthyl)-2-deoxy-2-[(2,2,2 trichloroethoxy)carbonyl]amino-β–D glucopyranoside

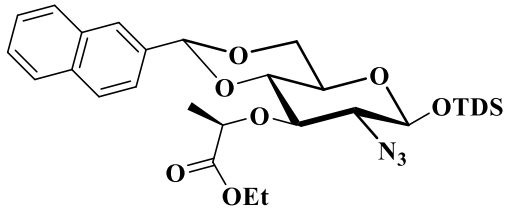


9

A solution of starting material (600 mg, 0.9757 mmol) and activated molecular sieves (4Å, 0.5 g) in dichloromethane (10 mL) was stirred at ambient temperature under an atmosphere of Ar for 1 h. The mixture was cooled to -78^{o}C followed by addition of Et3SiH (311 µl, 1.9515 mmol) and TfOH (130 µL, 1.4635 mmol). After being stirred for 1 h. at -78^{o}C, TLC showed complete consumption of starting material then Et_3N (2 mL) and MeOH (2 mL) were added successively, and the mixture was diluted with DCM and washed with $NaHCO_3$ (satd.), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using a gradient of EtOAc/Hexane (1/9 to 1/2, v/v) to obtain product (500 mg, 84%).

^{1}H NMR (300 MHz,) δ 7.89 – 7.74 (m, 4H), 7.55 – 7.40 (m, 3H), 5.30 (d, *J* = 0.7 Hz, 2H), 5.07 (dd, *J* = 10.8, 9.0 Hz, 2H), 4.84 – 4.62 (m, 4H), 4.48 (d, *J* = 8.3 Hz, 1H), 3.94 – 3.71 (m, 4H), 3.71 – 3.40 (m, 4H), 2.94 (d, *J* = 3.2 Hz, 1H), 2.18 (td, *J* = 6.9, 2.7 Hz, 2H), 2.13 – 2.07 (m, 3H), 1.69 – 1.52 (m, 2H). HR MALDI-TOF MS: calculated for $C_{28}H_{32}C_{l3}NNaO_8$ [M+Na] 638.1091; observed, $[M+Na]^+$ 638.1095

Dimethylthexylsilyl 3-O-{(R)-1-(ethoxycarbonyl)ethyl}-4,6-*O*-napthylidine-2-azido-2-deoxy-β-D-glucopyranoside

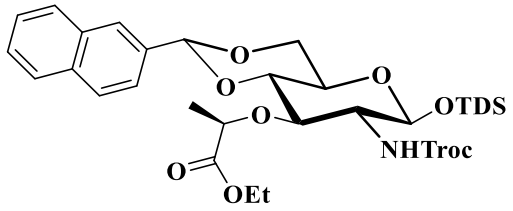


24

To a stirred solution of compound (50 mg, 0.1030 mmol) in dry DCM (5 mL), NaH (3.70 mg, 0.1545 mmol) was added and allowed to stir for 30 min at rt. (S) Ethyl lactic triflate (51.5 mg, 0.2030 mmol) was added and mixture was stirred for 2 h. After 2 h, TLC analysis showed total consumption of the starting material. The reaction mixture was quenched by adding 1 mL ice water. The reaction was then diluted with DCM and washed aqueous saturated $NaHCO_3$ (2x 5 mL), and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude residue was purified by silica gel column chromatography using a gradient of EtOAc/Hexanes (1/20 to 1/15 v/v) to obtain compound (40 mg, 66%).

^{1}H NMR (300 MHz,) δ 7.97 – 7.79 (m, 4H), 7.60 – 7.44 (m, 3H), 5.70 (s, 1H), 4.57 (d, *J* = 7.7 Hz, 1H), 4.45 (q, *J* = 6.9 Hz, 1H), 4.33 (dd, *J* = 10.5, 5.0 Hz, 1H), 4.26 – 4.13 (m, 2H), 3.83 (t, *J* = 10.3 Hz, 1H), 3.72 (t, *J* = 9.2 Hz, 1H), 3.60 (t, *J* = 9.2 Hz, 1H), 3.47 – 3.30 (m, 2H), 1.67 (p, *J* = 6.9 Hz, 1H), 1.54 (d, *J* = 0.8 Hz, 1H), 1.42 (d, *J* = 6.8 Hz, 3H), 1.31 – 1.22 (m, 3H), 0.91 (d, *J* = 7.2 Hz, 12H), 0.19 (d, *J* = 5.0 Hz, 6H). HR MALDI-TOF MS: calculated for $C_{30}H_{43}N_3NaO_7Si$ $[M+Na]^+$ 608.2768; observed, [M+Na] 608.2760

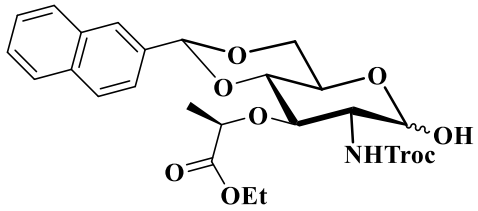
Dimethylthexylsilyl 3-O-{(R)-1-(ethoxycarbonyl)ethyl}-4,6-*O*-napthylidine-2-(2,2,2-trichlo roethoxycarbonylamino)-2-deoxy-β-D-glucopyranoside



25

Compound 23 (300 mg, 0.5125 mmol) was dissolved in 9:1 THF/HOAc (5 mL) and treated with Zn (168 mg, 2.5628 mmol) for 10 minutes. After 10 minutes, TLC analysis showed total consumption of the starting material. To this reaction mixture 2,2,2-trichloroethoxycarbonyl chloride (739 µL, 5.1250 mmol) was added and reaction mixture was stirred at RT for 30 mins. After which TLC (EtOAc:Hexane, 1:5) showed it was complete. The mixture was filtered over celite and co-evaporated with toluene. The residue was dissolved in EtOAc (10 mL) and washed with sat. $NaHCO_3$ (10 mL). The organic layer was dried ($MgSO_4$), filtered and the filtrate was concentrated *in vacuo.* The residue was purified by silica gel column chromatography using EtOAc/Hexanes (1/10 v/v) gradient to afford C2 troc protected compound in 55% yield after two steps. HR MALDI-TOF MS: calculated for $C_{33}H_{46}C_{l3}NNaO_9Si$ $[M+Na]^+$ 756.1905; observed, [M+Na] 756.1900

3-O-{(R)-1-(ethoxycarbonyl)ethyl}-4,6-*O*-napthylidine-2-(2,2,2-trichlo roethoxycarbonylamino)-2-deoxy-α/β-D-glucopyranoside

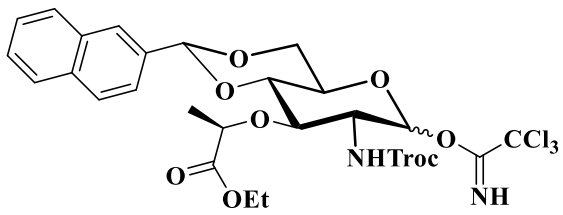


25a

To a stirred solution of 24 starting material (100 mg, 0.1360 mmol) in THF (3 mL), HF in pyridine (30%, 124 µL) was added. After stirring at ambient temperature for 16 h TLC indicated complete consumption of the starting material. The reaction mixture was subsequently diluted with EtOAc, washed with water, $NaHCO_3$ (Sat.), and brine. The organic phase was dried ($MgSO_4$), filtered, and concentrated under reduced pressure and purified by silica gel column chromatography (EtOAc/Hexane, 1/5 v/v) to give the product (65 mg, 81%).

^{1}H NMR (300 MHz,) δ 7.98 – 7.81 (m, 4H), 7.61 – 7.45 (m, 3H), 7.13 (d, *J* = 4.6 Hz, 1H), 5.74 (s, 1H), 5.67 (t, *J* = 3.5 Hz, 1H), 4.82 (d, *J* = 12.0 Hz, 1H), 4.71 (d, *J* = 11.9 Hz, 1H), 4.54 (q, *J* = 7.0 Hz, 1H), 4.39 – 4.05 (m, 4H), 4.00 – 3.67 (m, 4H), 2.86 (d, *J* = 3.7 Hz, 1H), 1.43 (d, *J* = 6.9 Hz, 3H), 1.28 (t, *J* = 7.1 Hz, 3H).

3-O-{(R)-1-(ethoxycarbonyl)ethyl}-4,6-*O*-napthylidine-2-(2,2,2-trichlo roethoxycarbonylamino)-2-deoxy-α/β-D-glucopyranosyl trichloroacetimidate

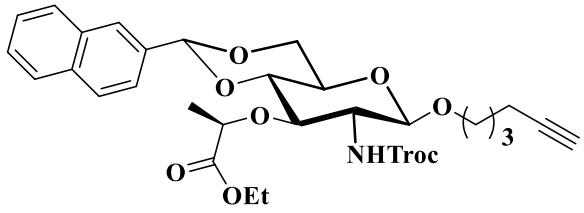


7

To a stirred solution of compound 5 (65 mg, 0.1096 mmol) in dry DCM (7 mL), Cl_3CCN (82 μL, 0.5482 mmol) and $CsCO_3$ (43 mg, 0.1315 mmol) were added. After stirring for 1.5 h. at ambient temperature, TLC showed total consumption of starting material. The mixture was concentrated in vacuo and purified by silica gel column chromatography (EtOAc/Hexane, 1/10 v/v) to give the product as white foam (75 mg, 93%).

^{1}H NMR (300 MHz,) δ 8.65 (s, 1H), 7.99 – 7.81 (m, 4H), 7.52 (ddt, *J* = 10.7, 7.1, 2.8 Hz, 3H), 7.15 (s, 1H), 6.74 (d, *J* = 2.5 Hz, 1H), 5.77 (s, 1H), 4.91 – 4.79 (m, 1H), 4.61 – 4.52 (m, 1H), 4.40 (dd, *J* = 10.3, 4.7 Hz, 1H), 4.31 – 3.95 (m, 5H), 3.94 – 3.79 (m, 2H), 1.47 (d, *J* = 7.0 Hz, 3H), 1.29 (t, *J* = 7.2 Hz, 3H).

Hex-5-yn-3-O-{(R)-1-(ethoxycarbonyl)ethyl}-4,6-*O*-napthylidine-2-(2,2,2-trichlo roethoxycarbonylamino)-2-deoxy-β-D-glucopyranoside



29

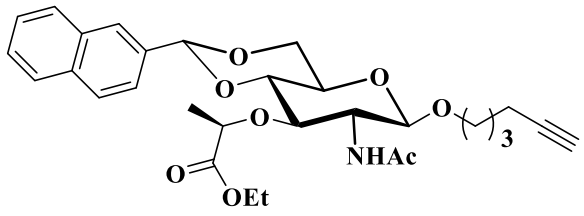
A mixture of the donor 7 (400 mg, 0.5425 mmol), acceptor, 5-Hexyn-1-ol (120 μL, 1.0851 mmol), and 4Å acid washed, flame-dried molecular sieves was stirred in dry DCM (10 mL) at RT for 30 min. The resulting solution was cooled (-60^o C), after which TMSOTf (1 μL, 0.005425 mmol) was added. The reaction mixture was stirred at -60^o C for 45 min. TLC (EtOAc:Hexane, 1:5, v/v) showed the acceptor has been converted into a slightly less polar product. The reaction was then quenched with sat. $NaHCO_3$ (0.3 mL) and the resulting mixture was allowed to warm to RT, after which it was diluted with

DCM (5 mL) and washed with sat. $NaHCO_3$ (5 mL). The organic layer was dried ($MgSO_4$), concentrated *in vacuo* and the residue purified by silica gel column chromatography using EtOAc/Hexane (1/8 to 1/5, v/v) as the eluent to afford the product (350 mg, 96%).

^{1}H NMR (300 MHz,) δ 7.93 – 7.73 (m, 4H), 7.52 – 7.37 (m, 3H), 6.13 (s, 1H), 5.64 (s, 1H), 4.72 (t, *J* = 8.5 Hz, 2H), 4.48 (d, *J* = 8.1 Hz, 1H), 4.43 (d, *J* = 7.0 Hz, 1H), 4.33 (dd, *J* = 10.1, 5.1 Hz, 1H), 4.09 (ddd, *J* = 15.7, 13.1, 7.5 Hz, 2H), 3.82 (dt, *J* = 20.4, 7.6 Hz, 3H), 3.66 (dd, *J* = 10.9, 7.2 Hz, 1H), 3.46 (dtd, *J* = 19.8, 9.8, 9.4, 6.1 Hz, 3H), 2.13 (dq, *J* = 9.7, 3.3, 2.8 Hz, 2H), 1.88 (dt, *J* = 5.4, 2.9 Hz, 1H), 1.71 – 1.48 (m, 4H), 1.33 (d, *J* = 7.1 Hz, 3H), 1.20 (d, *J* = 7.3 Hz, 3H).

HR MALDI-TOF MS Calculated for $C_{31}H_{36}C_{l3}NNaO_9$ $[M+Na]^+$: 694.1353 Observed: 694.1350

Hex-5-yn-3-O-{(R)-1-(ethoxycarbonyl)ethyl}-4,6-*O*-napthylidine-2-acetylamino-2-deoxy-β-D-glucopyranoside

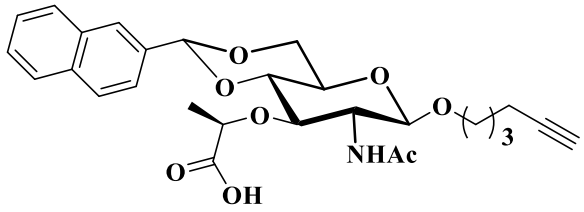


30

The solution of 26 (350 mg, 0.5216 mmol) in 10 mL THF: AcOH: Ac_2O (6:4:1) was cooled to 0^o C. To the resulting mixture was added Zn dust (1.7 g, 26.0804 mmol), 0.5 mL aq. $CuSO_4$ and the reaction mixture was stirred at RT 5 h, after which TLC and MALDI analysis showed the transformation had gone to completion. The solution was filtered through celite, washed with THF (2x5 mL) and the filtrate concentrated, co-

evaporated with toluene and dried *in vacuo*. The residue **30** was used as it is for next step without further purification. HR MALDI-TOF MS Calculated for $C_{30}H_{37}NNaO_8$ $[M+Na]^+$: 562.2417 Observed: 562.2419

Hex-5-yn-3-O-{(R)-1-carboxyethyl}-4,6-*O*-napthylidine-2-acetylamino-2-deoxy-β-D-glucopyranoside

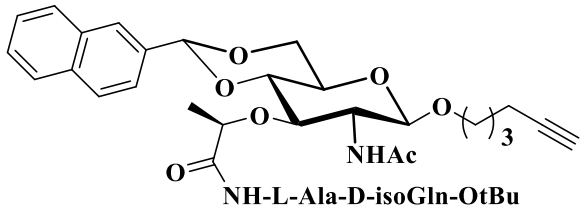


31

The resulting crude compound **30** was dissolved in 5 mL THF: 1-4 Dioxane: H_2O (4:2:1) and added LiOH till pH reaches between 11 to12 and stirred at RT. After 1 hr., TLC and MALDI analysis showed total consumption of starting material. The reaction mixture was then neutralized with Dowex H^+ resin, filtered and freeze dried. Resulting compound was purified by silica gel column chromatography using MeOH: DCM (1:10, v/v) to afford pure product (200 mg, 76 % over two steps).

^{1}H NMR (300 MHz,) δ 7.47 (d, *J* = 1.5 Hz, 1H), 7.42 – 7.30 (m, 3H), 7.13 – 6.94 (m, 3H), 5.28 (s, 1H), 4.12 (d, *J* = 8.3 Hz, 1H), 4.05 – 3.81 (m, 3H), 3.46 – 3.31 (m, 3H), 3.24 (td, *J* = 8.7, 3.1 Hz, 2H), 3.10 – 2.93 (m, 2H), 2.83 (d, *J* = 1.6 Hz, 1H), 1.70 (td, *J* = 6.9, 2.6 Hz, 2H), 1.62 (t, *J* = 2.6 Hz, 1H), 1.52 (s, 3H), 1.28 – 1.01 (m, 4H), 0.89 (d, *J* = 6.9 Hz, 3H).

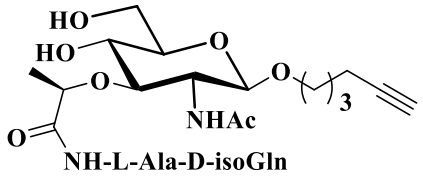
Hex-5-yn-3-O-{(R)-propionyl-(L-alanyl-D-isoglutamine)}-4,6-*O*-napthylidine-2-acetylamino-2-deoxy-β-D-glucopyranoside



32

To a solution of compound **31** (10 mg, 0.0195 mmol) in 3 mL DMF was added HATU (11 mg, 0.0.0293 mmol), HOAt (4 mg, 0.0293 mmol) and DIPEA (10 µL, 0.0586 mmol) and stirred for 5 min. To the resulting yellowish solution added dipeptide (8 mg, 0.0293 mmol) dissolved in 2 mL DMF. The mixture was allowed to stir at RT for 4 h. TLC and MALDI analysis showed complete consumption of Muramic acid derivative. The mixture was diluted with EtOAc (5 mL), washed with water (5 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* and the residue was used for next step without further purification (12 mg, 80%). HR MALDI-TOF MS Calculated for $C_{40}H_{54}N_4NaO_{11}$ $[M+Na]^+$ Exact Mass: 789.3687; Observed 789.3689

Hex-5-yn-3-O-{(R)-propionyl-(L-alanyl-D-isoglutamine)}-2-acetylamino-2-deoxy-β-D-glucopyranoside



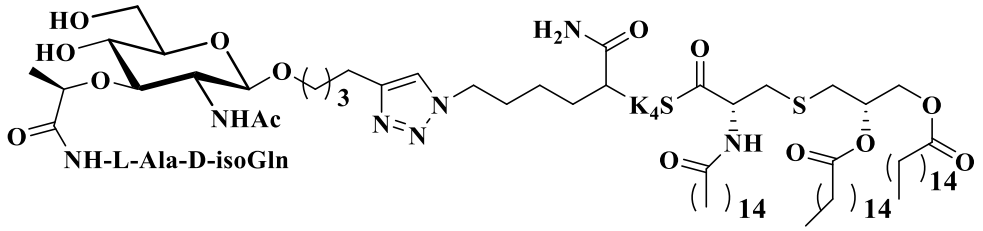
1

The compound (12 mg, 0.0152 mmol) was dissolved in 2 mL TFA: DCM (1:2) and stirred at RT. After 1 h, TLC analysis confirmed complete consumption of starting

material to the desired product. The reaction mixture was concentrated and co-evaporated with toluene (2x2 mL). The residue was purified over itrobeads using MeOH:DCM (1:5, v/v) to give desired product (7 mg, 81%).

^{1}H NMR (300 MHz,) δ 4.58 – 4.33 (m, 1H), 4.03 – 3.59 (m, 4H), 3.59 – 3.14 (m, 4H), 3.14 – 2.75 (m, 6H), 1.92 (t, *J* = 7.5 Hz, 2H), 1.76 – 1.59 (m, 2H), 1.47 (d, *J* = 4.5 Hz, 3H), 1.13 (dq, *J* = 18.7, 7.3, 6.1 Hz, 3H), 0.91 (dd, *J* = 12.4, 6.7 Hz, 6H), 0.79 (s, 1H).

HR MALDI-TOF MS Calculated for $C_{25}H_{40}N_4NaO_{11}$ $[M+Na]^+$ Exact Mass: 595.2591; Observed 595.2596

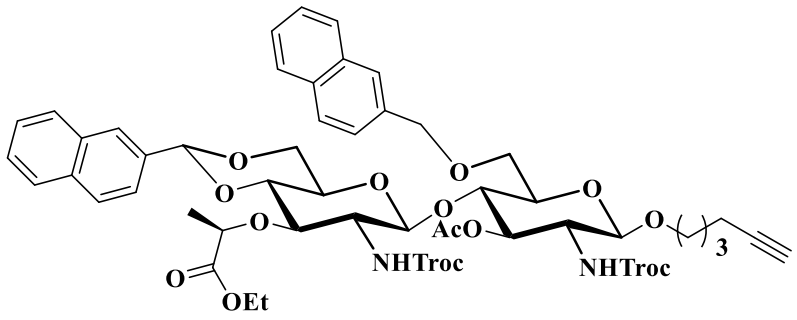


4

To a solution of TLR2 lipopeptide (1.5 mg, 0.9017 µmol) and MDP derivative (0.56 mg, 0.9919 µmol) in H2O:*t*-BuOH (200 µL, 1:1 v/v) added $CuSO_4.5H_2O$ (0.58 mg, 2.7052 µmol), TBTA (0.1433 mg, dissolved in DMSO, 0.2705 µmol) and stirred for 5 min. To this NaAsc (0.53 mg dissolved in H_2O, 2.7052 µmol) was added and vigorously stirred for additional 2 hr. and completion of the reaction was monitored by MALDI analysis. After that reaction mixture was freeze dried, the crude compound was 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 with a Phenomenex Jupiter analytical C-4 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 and appropriate fractions were lyophilized to afford pure compound **1** (1.2

mg, 75%). HR MALDI-TOF MS Calculated for $[M+Na]^+$: 2257.5180 Observed: 2257.5188

Hex-5-yn-3-*O*-acetyl-6--(2-methyl-napthyl)-2-deoxy-4-*O*-[4,6-*O*-napthalidene-2-deoxy-3-*O*-{(R)-1-(ethoxycarbonyl)ethyl}-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranoside



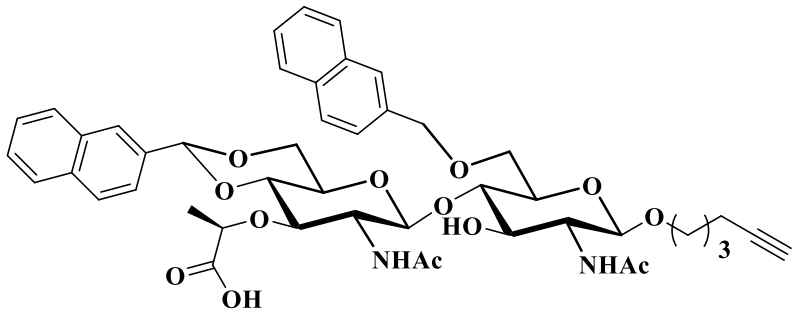
33

A mixture of the donor 5 (30 mg, 0.0486 mmol), acceptor 5 (43 mg, 0.0584 mmol), and 4Å acid washed flame-dried molecular sieves was stirred in dry DCM (5 mL) at RT for 30 min. The resulting solution was cooled (-70^o C) and TfOH (1.2 µL, 0.0097 mmol) was added. The reaction mixture was stirred at -70^o C for 30 min and slowly allowed to warm up to -20^o C and stirred for additional 20 min. TLC analysis (EtOAc:Hexane, 1:2, v/v) showed the acceptor has been converted into a slightly less polar product. The reaction was then quenched with NEt_3 (50 µL) and the resulting mixture was warmed up to RT, after which it was diluted with DCM (5 mL) and washed with sat. $NaHCO_3$ (5 mL). The organic layer was dried ($MgSO_4$), concentrated *in vacuo* and the residue purified by silica gel column chromatography using EtOAc/Hexane (1/10 to 1/6, v/v) as the eluent to afford disaccharide product (55 mg, 95%).

^{1}H NMR (600 MHz,) δ 7.92 – 7.81 (m, 8H), 7.55 – 7.42 (m, 6H), 5.99 (s, 0H), 5.63 (s, 1H), 5.22 (s, 0H), 5.08 (t, *J* = 9.2 Hz, 1H), 4.87 (d, *J* = 12.2 Hz, 1H), 4.77 (d, *J* = 12.0 Hz, 1H), 4.73 (d, *J* = 13.1 Hz, 1H), 4.69 (dd, *J* = 12.1, 6.7 Hz, 2H), 4.39 (d, *J* = 8.1 Hz, 1H), 4.36 (d, *J* = 7.7 Hz, 1H), 4.32 (td, *J* = 9.6, 8.9, 6.0 Hz, 2H), 4.23 (dq, *J* = 10.8, 7.2 Hz, 1H), 4.15 (dq, *J* = 10.8, 7.1 Hz, 1H), 3.97 (t, *J* = 8.6 Hz, 1H), 3.94 – 3.84 (m, 2H), 3.80 – 3.68 (m, 3H), 3.58 (t, *J* = 9.2 Hz, 1H), 3.52 (dt, *J* = 8.9, 3.0 Hz, 1H), 3.47 (dt, *J* = 9.6, 6.4 Hz, 2H), 3.35 (s, 0H), 3.15 (q, *J* = 8.2 Hz, 1H), 2.19 (ddd, *J* = 9.8, 6.3, 2.7 Hz, 2H), 2.00 (s, 3H), 1.94 (t, *J* = 2.6 Hz, 1H), 1.69 (dqt, *J* = 13.8, 9.0, 5.0 Hz, 2H), 1.63 – 1.56 (m, 1H), 1.34 (d, *J* = 7.0 Hz, 3H), 1.26 (t, *J* = 7.1 Hz, 4H).

HR MALDI-TOF MS calculated for $C_{53}H_{58}C_{l6}N_2NaO_{16}$ $[M+Na]^+$ 1211.1815; observed, 1211.1812.

Hex-5-yn-3-*O*-acetyl-6--(2-methyl-napthyl)-2-deoxy-4-*O*-[4,6-*O*-napthalidene-2-deoxy-3-*O*-{(R)-1-carboxyethyl}-2-acetylamino-β-D-glucopyranosyl]-2-acetylamino-β-D-glucopyranoside



34

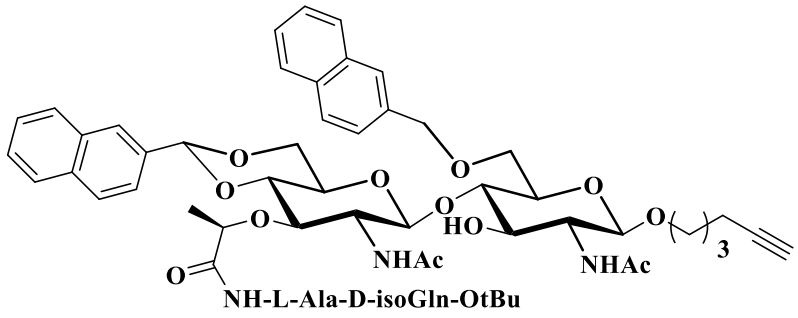
The solution of 29 (25 mg, 0.0210 mmol) in 4 mL THF: AcOH: Ac_2O (3:2:0.5) was cooled to 0^o C in an ice bath. To the resulting mixture was added Zn dust (95 mg, 1.4728 mmol), 0.1 mL aq. $CuSO_4$ and the reaction mixture was stirred at RT overnight, after which TLC showed the transformation had gone to completion. The solution was filtered,

and the filtrate concentrated and co-evaporated in toluene. The residue was purified on silica gel using EtOAc: Hexane (4:1, v/v) affording the title compound. The resulting compound (15 mg, 0.01621 mmol) was dissolved in 2 mL THF:1-4 Dioxane: H_2O (4:2:1) and added LiOH (2 mg, 0.0973 mmol) and stirred at RT. After 1 hr. TLC showed complete consumption of starting material. The reaction mixture was then neutralized with Dowex H^+ resin, filtered and freeze dried. The resulting compound was purified on prep. TLC using MeOH: DCM (1:10, v/v) to afford pure product (10 mg, 59 % over two steps).

Troc reduction: HR MALDI-TOF MS calculated for $C_{51}H_{60}N_2NaO_{14}$ $[M+Na]^+$ 947.3942; observed, 947.3949.

^{1}H NMR (600 MHz,) δ 7.94 – 7.82 (m, 8H), 7.55 – 7.41 (m, 9H), 5.69 (s, 1H), 5.34 (s, 2H), 4.90 (d, *J* = 12.1 Hz, 1H), 4.68 (d, *J* = 12.0 Hz, 1H), 4.42 (d, *J* = 8.2 Hz, 1H), 4.40 – 4.38 (m, 1H), 4.38 – 4.33 (m, 2H), 3.90 (dt, *J* = 9.7, 6.0 Hz, 1H), 3.84 – 3.70 (m, 5H), 3.69 – 3.63 (m, 3H), 3.53 – 3.43 (m, 3H), 3.32 (td, *J* = 9.7, 4.9 Hz, 1H), 2.21 (td, *J* = 7.1, 2.6 Hz, 2H), 2.02 (s, 3H), 2.00 (t, *J* = 2.7 Hz, 1H), 1.97 (s, 3H), 1.70 (ddt, *J* = 12.5, 9.8, 6.3 Hz, 2H), 1.64 – 1.56 (m, 2H), 1.38 (d, *J* = 6.9 Hz, 3H). HR MALDI-TOF MS calculated for $C_{47}H_{54}N_2NaO_{13}$ $[M+Na]^+$ 877.3524; observed, 877.3528.

Hex-5-yn-3-*O*-acetyl-6--(2-methyl-napthyl)-2-deoxy-4-*O*-[4,6-*O*-napthalidene-2-deoxy-3-*O* -[(R)-propionyl-{tert-butyl-(L-alanyl-D-isoglutaminate)}-2-acetylamino-β-D-glucopyranosyl]-2-acetylamino-β-D-glucopyranoside



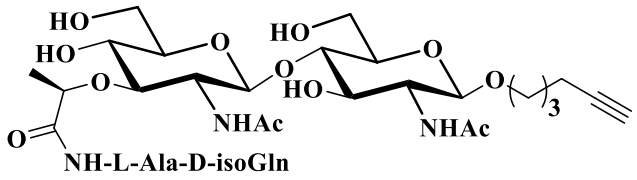
35

To a solution of compound (70 mg, 0.0818 mmol) in 10 mL DMF was added HATU (47 mg, 0.1228 mmol), HOAt (17 mg, 0.1228 mmol) and DIPEA (42 µL, 0.2456 mmol) and stirred for 5 min. To the resulting yellowish solution added dipeptide (34 mg, 0.0818 mmol) dissolved in 5 mL DMF. The mixture was allowed to stir at RT for 4 h. TLC and MALDI analysis showed complete consumption of disaccharide compound. The mixture was diluted with EtOAc (10 mL), washed with water (10 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* and purified on silica gel column chromatography using MeOH/DCM (1/10, v/v) as the eluent to afford desired product (80 mg, 87%).

^{1}H NMR (600 MHz,) δ 8.06 (d, *J* = 8.3 Hz, 1H), 8.00 (d, *J* = 9.1 Hz, 1H), 7.96 – 7.80 (m, 9H), 7.72 (d, *J* = 7.7 Hz, 1H), 7.54 – 7.44 (m, 6H), 7.35 (d, *J* = 7.0 Hz, 1H), 7.28 (s, 1H), 7.05 (s, 1H), 5.83 (s, 1H), 4.70 (s, 2H), 4.67 (d, *J* = 8.1 Hz, 1H), 4.57 (d, *J* = 2.5 Hz, 1H), 4.29 (d, *J* = 5.5 Hz, 1H), 4.27 (dd, *J* = 10.6, 6.5 Hz, 1H), 4.21 (p, *J* = 7.0 Hz, 1H), 4.11 (td, *J* = 8.8, 4.8 Hz, 1H), 4.06 (q, *J* = 6.8 Hz, 1H), 3.80 (td, *J* = 10.4, 9.5, 4.8 Hz, 2H), 3.75 (dt, *J* = 9.1, 4.0 Hz, 2H), 3.72 – 3.58 (m, 4H), 3.52 – 3.33 (m, 6H), 2.72 (t, *J* =

2.5 Hz, 1H), 2.47 (d, *J* = 5.9 Hz, 2H), 2.17 – 2.08 (m, 4H), 1.89 (qt, *J* = 8.1, 5.7, 4.5 Hz, 1H), 1.79 (d, *J* = 3.2 Hz, 5H), 1.70 – 1.60 (m, 1H), 1.52 (p, *J* = 6.6 Hz, 2H), 1.48 – 1.39 (m, 3H), 1.35 (d, *J* = 3.8 Hz, 2H), 1.31 (s, 9H), 1.19 (dd, *J* = 10.8, 6.7 Hz, 6H). HR MALDI-TOF MS calculated for $C_{59}H_{75}N_5NaO_{16}$ $[M+Na]^+$ 1132.5107; observed, 1132.5099.

Hex-5-yn-2-Acetylamino-4-O-[2-acetylamino-2-deoxy-3-O-{(R)-propionyl-(L-alanyl-D-isogl utamine)}-β-D-glucopyranosyl]-2-deoxy-D-glucopyranoside



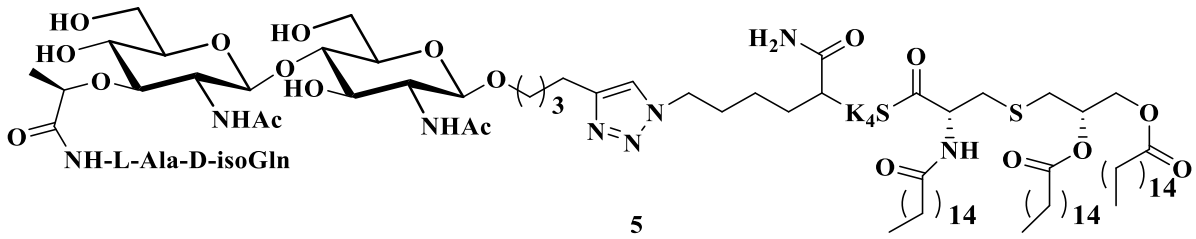
2

The disaccharide compound (22 mg, 0.0198 mmol) was dissolved in 2 mL TFA: DCM (1:2) and stirred at RT. After 1 h, TLC analysis confirmed total consumption of starting material. Reaction mixture was concentrated and co-evaporated with toluene (2x2 mL). Residue was purified over itrobeads using MeOH:DCM (1:4, v/v) to give desired product which was used for next step. To a solution of compound in 10 mL $CHCl_3$:MeOH (1:1) was added DDQ (9 mg, 0.0396 mmol) and stirred for 2 h. Reaction was monitored by MALDI and after completion of reaction, solvent was evaporated and purified over itrobeads using MeOH:DCM (1:1, v/v) to give solid product (10 mg, 67 % over two steps).

^{1}H NMR (500 MHz,) δ 4.44 (d, *J* = 8.5 Hz, 1H), 4.37 (d, *J* = 8.1 Hz, 1H), 4.24 (dd, *J* = 9.8, 4.7 Hz, 1H), 4.13 (dq, *J* = 17.9, 6.9 Hz, 2H), 3.84 – 3.32 (m, 15H), 2.35 – 2.27 (m, 2H), 2.10 (td, *J* = 7.1, 2.6 Hz, 2H), 2.05 (td, *J* = 8.5, 7.9, 5.6 Hz, 1H), 1.92 (s, 3H), 1.87

(s, 3H), 1.85 – 1.79 (m, 1H), 1.58 – 1.48 (m, 2H), 1.46 – 1.35 (m, 2H), 1.31 (d, *J* = 7.2 Hz, 3H), 1.25 (d, *J* = 6.7 Hz, 3H).

HR MALDI-TOF MS calculated for $C_{33}H_{53}N_5NaO_{16}$ $[M+Na]^+$ 798.3385; observed, 798.3380.



5

To a solution of disaccharide compound (0.5 mg, 0.6451µmol) and glycolipopeptide (1.2 mg, 0.7741 µmol) in H2O:*t*-BuOH (200 µL, 1:1 v/v) added $CuSO_4.5H_2O$ (0.11 mg, 0.5160 µmol), TBTA (0.034 mg dissolved in DMSO, 0.0645 µmol) and stirred for 5 min. To this NaAsc (0.13 mg dissolved in H_2O, 0.6451 µmol) was added and vigorously stirred for additional 2 hr. After that reaction mixture was freeze dried the crude compound was 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 with a Phenomenex Jupiter analytical C-4 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 and appropriate fractions were lyophilized to afford pure compound **5** (1.2 mg, 78%). HR MALDI-TOF MS calculated for $C_{120}H_{220}N_{20}NaO_{29}S$ $[M+Na]^+$ 2460.5974; observed, 2460.5972.

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CHAPTER 6

CONCLUSIONS

One of the characteristic features of the cancer cells is over-expression of TACAs on their cell surfaces. However, development of carbohydrate-based cancer vaccine has been problematic due to difficulty in producing high IgG antibody titers. This is due to the fact that TACAs are self-antigens and often tolerated by the immune system or produces weak immune responses. Researchers have addressed this issue by conjugating TACAs to the foreign carrier proteins such as BSA and KLH to induce strong B-cell responses. However, this may result in suppression of carbohydrate-specific antibodies. Hence, for the successful development of carbohydrate-base cancer vaccine, there is need of a novel approach that can efficiently present TACAs to the immune system leading to the production of high-affinity IgG antibody titers.

Mucins (MUC) are high molecular weight transmembrane glycoproteins expressed on the apical surface of various epithelial cells including lungs, eyes, stomach, and gastrointestinal tract as well as several other organs. More specifically, mucin-1 (MUC1) is highly overexpressed in the majority of carcinomas with significantly short and truncated glycan pattern compared to normal MUC1 hence could serve as a potent target for cancer immunotherapy. The extracellular domain of MUC1 consists of multiple 20 amino acids, a variable number of tandem repeats (VNTR) with potential five sites for glycosylation. We have designed vaccine candidates comprised of a full-length MUC1 sequence and built-in immunoadjuvant. We anticipated that the full-length sequence of

MUC1 offers a tantalizing antigen which contains multiple epitopes that can activate B-cells, helper T-cells, and cytotoxic T-lymphocytes. The lipopeptide Pam_3CSK_4, a TLR2 ligand plays a key role in the innate immune system and can serve as a self-adjuvanting immune stimulant in vaccines. Our results showed that our lead vaccine could induce humoral and cellular immunity and produced robust IgG antibody titers that recognized mono-and pentaglycosylated LMUC1 glycopeptide epitopes.

Furthermore, for a better understanding the phenomenon of antigen uptake, processing and presentation of vaccine candidates by APC, we proposed and successfully synthesized a number of vaccine candidates that contained various linkers between monoglycosylated LMUC1 unit and TLR2 ligand as well as a vaccine containing pentaglycosylated LMUC1 unit directly attached to TLR2 ligand. However, immunological studies are still underway.

In an effort to streamline the synthetic methodologies of these long glycolipopeptides, we successfully developed and utilized easily scalable microwave assisted solid phase synthesis protocols that are advantageous over traditional native chemical ligations (NCL) as well as liposome-mediated NCL by providing higher yields with better purity.

Finally, to fulfill the unmet need to develop the vaccine adjuvants that target mucosal surfaces, we proposed and successfully synthesized compound adjuvants. It is our belief that these chimeric adjuvants derived by covalently linking two pathogen-associated molecular patterns (PAMPs) TLR2 and NOD2, could mimic the situation as it occurs during infection and trigger efficient crosstalk between signal transduction pathways and generate synergistic immune activation resulting in enhancement of immune responses

against the co-administered antigens. Further, we tested these synthetic chimeric compounds in MM6 cells for their biological activities. Our results showed that these compounds could produce a significant amount of TNF-α response. However, further structural modifications and more biological experiments are still underway to establish a better understanding of structure-activity relationships of these fusion PAMPs.