Synthesis and study of anti-tumor vaccines

S Sarkar

The University of Toledo

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation

http://utdr.utoledo.edu/theses-dissertations/420

This Dissertation is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.
A Dissertation

entitled

Synthesis and Study of Anti-tumor Vaccines

by

Sourav Sarkar

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Chemistry

________________________________________
Dr. Steven J. Sucheck, Committee Chair

________________________________________
Dr. Katherine A. Wall, Committee Member

________________________________________
Dr. L. M. Viranga Tillekeratne, Committee Member

________________________________________
Dr. Kana Yamamoto, Committee Member

________________________________________
Dr. Particia R. Komuniecki, Dean
    College of Graduate Studies

The University of Toledo

December 2012
An Abstract of

Synthesis and Study of Anti-tumor Vaccines

by

Sourav Sarkar

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Doctor of Philosophy Degree in Chemistry

The University of Toledo

December 2012

The inherent weak immune response against carbohydrate antigens has directed several novel approaches towards increasing their immunogenicity for their use as vaccine components. We hypothesized that conjugation of an L-rhamnose (Rha) moiety to a carbohydrate antigen would increase the immune response against the antigen in mice possessing anti-Rha antibodies via an antibody-dependent antigen uptake mechanism. To explore this hypothesis we synthesized a single-molecule three-component vaccine containing the GalNAc-O-Thr (Tn) tumor specific antigen, a 20 amino acid helper T-cell epitope (YAF) derived from an outer membrane protein of Neisseria meningitides and a Rha moiety. Synthesis of the vaccine was achieved by automated Fmoc-based solid phase peptide synthesis and deacetylated by brief treatment with NaOMe. Groups of female BALB/c mice were immunized and boosted with Rha-ovalbumin (Rha-OVA) formulated with either TiterMax® Gold or Sigma Adjuvant System® for a period of 35 days to generate optimum anti-Rha antibodies necessary for evaluating the vaccine. Anti-Rha antibody titers were >100 fold higher in groups of mice immunized with Rha-OVA than the control groups. Mice producing anti-Rha were
challenged with Rha-YAF-Tn or YAF-Tn. Sera collected from the groups initially immunized with Rha-OVA and later challenged with Rha-YAF-Tn showed a two fold increase in anti-Tn titer at 1/100 serum dilution compared to mice not immunized with Rha-OVA. An in vitro T-cell proliferation study using cells primed with either Rha-YAF-Tn or YAF-Tn was performed to examine differences in antigen uptake and presentation on the MHC II in the presence of anti-Rha antibodies. In the presence of anti-Rha antibodies proliferation of T-cells showed a 10-fold decrease in the amount of antigen required. The result strongly suggests that T-cells present in the spleen were presented with higher concentrations of Rha-YAF-Tn as a result of the presence of the anti-Rha antibodies.

MUC1 variable number tandem repeats (VNTRs) conjugated to tumor-associated carbohydrate antigens (TACAs) have been shown to break self-tolerance in humanized MUC1 transgenic mice. Therefore, we hypothesize that a MUC1 VNTR TACA-conjugate can be successfully formulated into a liposome-based anti-cancer vaccine. The immunogenicity of the vaccine should be further augmented by incorporating surface displayed L-rhamnose (Rha) epitopes onto the liposomes to take advantage of a natural antibody-dependent antigen uptake mechanism. To validate our hypothesis we synthesized a 20-amino acid MUC1 glycopeptide containing a GalNAc-O-Thr (Tn) TACA by SPPS and conjugated it to a functionalized Toll-like receptor ligand (TLRL). An L-Rha-cholesterol conjugate was prepared using tetraethylene glycol (TEG) as a linker. The liposome-based anti-cancer vaccine was formulated by the extrusion method using TLRL-MUC1-Tn conjugate, Rha-TEG-cholesterol and 1,2-dipalmitoyl-sn-glycero-
3-phosphocholine (DPPC) in a total lipid concentration of 30 mM. The stability, homogeneity and size characterization of the liposomes was evaluated by SEM and DLS measurements. The formulated liposomes demonstrated positive binding with both anti-Rha and mouse anti-human MUC1 antibodies. Groups of female BALB/c mice were immunized and boosted with a rhamnose-Ficoll (Rha-Ficoll) conjugate formulated with alum as adjuvant to generate the appropriate concentration of anti-Rha antibodies in the mice. Anti-Rha antibody titers were >25-fold higher in the groups of mice immunized with the Rha-Ficoll conjugate than the non-immunized control groups. The mice were then immunized with the TLRL-MUC1-Tn liposomal vaccine formulated either with or without the surface displaying Rha epitopes. Sera collected from the groups of mice initially immunized with Rha-Ficoll and later vaccinated with the Rha-displaying TLRL-MUC1-Tn liposomes showed a >8-fold increase in both anti-MUC1-Tn and anti-Tn antibody titers in comparison to the groups of mice that did not receive Rha-Ficoll. T-cells from BALB/c mice primed with a MUC1-Tn peptide demonstrated increased proliferation to the Rha-liposomal vaccine in the presence of antibodies isolated from Rha-Ficoll immunized mice compared to nonimmune mice, supporting the proposed effect on antigen presentation. The anti-MUC1-Tn antibodies in the vaccinated mice serum recognized MUC1 on human leukemia U266 cells.
Acknowledgements

My entire research has been successful because of the inspiration, generosity and support of many individuals. First of all I would like to pay gratitude to my research advisor Dr. Steven J. Sucheck for giving me an opportunity to work in his esteemed group; motivating, mentoring and inspiring me through every challenge that came in my way throughout my graduate study. I deeply thank my co-advisor Dr. Katherine A. Wall for educating me in the fields of immunology that helped me immensely in the design and execution of the animal studies. I profusely appreciate the helpful suggestions and criticisms of my committee members Dr. L. M. Viranga Tillekeratne and Dr. Kana Yamamoto.

Due acknowledgement is given to my past and present laboratory members for the informative discussions; chemistry related or otherwise, maintenance of the laboratory and for keeping an extremely cordial environment in my work place. I deeply appreciate the training and help provided by Dr. Yong Wah Kim in NMR and MS experiments.

I am extremely grateful to my family especially to my parents and my wife and my friends for their constant encouragement and support throughout my study which made the tedious research look simple and easy.

With deep gratitude I thank everyone who helped me bring this graduate study to a successful completion and made my stay a meaningful one.
# Table of Contents

Abstract ................................................................. iii
Acknowledgements ................................................................... vi
Table of Contents ................................................................ vii
List of Schemes ...................................................................... viii
List of Figures ........................................................................ ix
List of Abbreviations .............................................................. xii

1 Strategies In Carbohydrate Based Anti-Cancer Vaccine Development ............... 1
   1.1 Introduction ................................................................... 1
   1.2 Tumor Associated Carbohydrate Antigens ............................. 3
   1.3 Generating Immune Response against TACAs ....................... 7
   1.4 Strategies for Improving Immunogenicity of TACAs ............ 11
   1.5 Summary ......................................................................... 21
   1.6 References ....................................................................... 23

2 Synthesis of a Single Molecule L-Rhamnose-Containing Three Component Vaccine and Evaluation of Antigenicity in the Presence of Anti-L-Rhamnose Antibodies ................................................................. 31
   2.1 Introduction ....................................................................... 31
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>Results</td>
<td>34</td>
</tr>
<tr>
<td>2.3</td>
<td>Immunological Results and Discussion</td>
<td>43</td>
</tr>
<tr>
<td>2.4</td>
<td>Conclusion</td>
<td>55</td>
</tr>
<tr>
<td>2.5</td>
<td>Experimental Section</td>
<td>57</td>
</tr>
<tr>
<td>2.6</td>
<td>References</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>Synthesis and Immunological Evaluation of a MUC1 Glycopeptide</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Incorporated into L-Rhamnose Displaying Liposomes</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>74</td>
</tr>
<tr>
<td>3.2</td>
<td>Results and Discussion</td>
<td>76</td>
</tr>
<tr>
<td>3.3</td>
<td>Immunological Results and Discussion</td>
<td>87</td>
</tr>
<tr>
<td>3.4</td>
<td>Conclusion</td>
<td>100</td>
</tr>
<tr>
<td>3.5</td>
<td>Experimental Section</td>
<td>102</td>
</tr>
<tr>
<td>3.6</td>
<td>References</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Appendix</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>Supporting Information for Chapter 2</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>Supporting Information for Chapter 3</td>
<td>157</td>
</tr>
</tbody>
</table>
List of Schemes

2.1 Synthesis of Conjugable L-Rhamnopyranosides .................................................37
2.2 Synthesis of L-Rhamnopyranoside conjugates with BSA, OVA and KLH .........38
2.3 Synthesis of Tn-BSA Conjugate ..............................................................................39
2.4 Synthesis of Acetylated Rha-YAF-Tn and YAF-Tn ...............................................40
2.5 Deacetylation of Rha-YAF-Tn and YAF-Tn .............................................................42
3.1 Synthesis of Rha-TEG-Cholesterol and alkyne functionalized Pam₃Cys..............78
3.2 Synthesis of Pam₃Cys-MUC-1-Tn Conjugate .........................................................81
3.3 Synthesis of Rha-Ficoll Conjugate ..........................................................................87
List of Figures

1-1 Schematic representation of TACA subtypes ..........................................................5
1-2 Representative structures of TACAs ............................................................................6
1-3 Schematic representation of interaction between B cells and helper T cells ..........10
1-4 TACA conjugated carrier protein conjugates ..........................................................13
1-5 Representative structure of Tn-CPMV conjugate ....................................................16
1-6 Structure of repeating subunits of ZPSs .................................................................17
1-7 Fully Synthetic carbohydrate based anti-tumor vaccines ....................................20

2-1 Diagramatic representation of cancer antigen uptake mediated by the presence of anti-Rha antibodies ........................................................................................................34
2-2 Anti-rhamnose antibody titre in non-immunized C57BL/6 mouse serum in the absence and presence of 0.1 M galactose or rhamnose .................................................44
2-3 Anti-rhamnose antibody titre in non-immunized Swiss Webster mouse serum in the absence and presence of 0.1 M galactose or rhamnose .................................................45
2-4 Stepwise immunization plan ......................................................................................45
2-5 Group average anti-Rha-BSA antibody titres after second Rha-OVA boost ..........46
2-6 Individual mouse anti-Rha-BSA antibody titres for Group A-F after second Rha-OVA boost at 1/100 serum dilution ..................................................................................47
Group average anti-Tn-BSA antibody titers after second YAF-Tn and Rha-YAF-Tn Boost..........................................................................................................................50

Individual anti-Tn-BSA antibody titres for Groups A, C and E after second Rha-YAF-Tn boost at 1/100 serum dilution.................................................................51

Individual anti-Tn-BSA antibody titres for Groups B, D and F after second YAF-Tn boost at 1/100 serum dilution .................................................................51

Anti Rha-BSA antibody titres after second boost with Rha-YAF-Tn and YAF-Tn at 1/100 serum dilution ................................................................................52

Competitive binding of anti-Tn antibodies with Tn-BSA in the presence of free Tn..............................................................................................................52

T-Cell proliferation measured by $[^3]$Hthymidine incorporation in cells from mice primed with YAF-Tn or Rha-YAF-Tn with no added serum antibodies..............54

T-Cell proliferation measured by $[^3]$Hthymidine incorporation in cells from mice primed with Rha-YAF-Tn with in vitro added Rha and non Rha serum antibodies ..............................................................................................................55

Schematic representation of Fc-Fcγ interaction in the in vivo generated immune complex leading to enhanced antigen uptake by APCs, e.g. dendritic cells........76

Size characterization of liposomes: SEM images at 5 kV acceleration voltage ....83

DLS Measurements at 1/1000 Dilution .................................................................................84

Fluorescence microscope images with Batch 1 liposomes under 60 X magnification ............................................................................................................85

Anti-Rha Antibody Isotype Titers after 4th Boost with Rha-Ficoll (group A) or Rha-OVA (group B) at 1/5 or 1/500 Serum Dilutions respectively .................89
3-6 T-Cell Proliferation in BALB/c mice with MUC1-Tn Peptide in Presence and Absence of Dendritic Cells .................................................................91

3-7 A) T-cell proliferation measured; B) Stepwise immunization plan; C) Group average of anti-Rha antibody titers after 4th boost; D) Group average of anti-MUC1-Tn antibody titers after 1st boost.................................................................92

3-8 Anti-MUC1-Tn Antibody Isotype Titers after 1st boost with Pam3Cys-MUC1-Tn liposomes or Pam3Cys-MUC1-Tn + Rha liposomes at 1/50 Serum Dilutions.....97

3-9 A) Competitive binding of anti-MUC1-Tn antibodies; B) Group average of anti-Tn antibody titer after 1st boost.................................................................99

3-10 Binding of anti-MUC1-Tn antibodies to human leukemia U266 cells............100
List of Abbreviations

AcOH .................... acetic acid
APC ...................... antigen presenting cell
BSA ...................... bovine serum albumin
CFA ...................... complete Freund’s Adjuvant
DC ...................... dendritic cell
DCM ..................... dichloro methane
DIC ..................... 1,3-diisopropylcarbodiimide
DIPEA .................. N,N-diisopropylethylamine
DLS ..................... dynamic light scattering
DMF ..................... dimethyl formamide
DPPC ................... 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
ELISA ................... enzyme linked immunosorbent assay
EtOH .................. ethanol
HOBt .................. 1-hydroxybenzotriazole
HRP .................. horseradish peroxidase
ICF ...................... incomplete Freund’s Adjuvant
KLH ..................... keyhole limpet hemocyanin
MeOH .................. methanol
MHC .................. major histocompatibility complex
MUC1 .................. mucin1
OVA ..................... ovalbumin
PBS ..................... phosphate buffered saline
Rha .................. rhamnose
SAS ............................Sigma Adjuvant System®
SEM ............................scanning electron microscope

TACA ...........................tumor-associated carbohydrate antigen
TEG ............................tetraethylene glycol
TFA ............................2,2,2-Trifluoroacetic acid
TLRL ............................Toll-like receptor ligand
TMG ............................TiterMax® Gold

VNTR ............................variable number tandem repeats

ZPS ............................zwitterionic polysaccharide
Chapter 1

Strategies In Carbohydrate Based Anti-Cancer Vaccine Development

1.1 Introduction

Cancer, medically known as malignant neoplasm broadly, refers to the unregulated and uncontrollable growth of abnormal cells in the body leading to the death of the individual. In humans over two hundred different forms of cancers have been identified affecting more than sixty organs and they have been collectively referred to as one of the most fatal diseases prevailing in the modern era, claiming millions of lives worldwide.\textsuperscript{1a} According to the American Cancer Society, cancer is the second most common cause of death in the United States, superseded only by cardiovascular diseases, cancer accounts for nearly one out of every four deaths.\textsuperscript{1b} In the year 2012 alone, about 1,638,910 new cases are expected to be diagnosed and about 577,190 are expected to lose their fight against cancer in the USA.\textsuperscript{1b} By 2050 experts predict that there will be as many as 27 million new cases accounting for over 17.5 million deaths by this fatal disease. The alarming increase in patients affected by cancer has propelled medical science to hunt for novel and path breaking therapies to treat cancer. As a result several new advances have been made towards the treatment of this life threatening disease over the last few decades.\textsuperscript{2,3}
Traditional methods for cancer treatment include surgery, radiation therapy and chemotherapy, which are often unselective therapies leading to unwanted side effects and often fail to prevent cancer recurrence. Thus new strategies to harness the power of the body’s own immune system to eradicate malignant cells is of extreme importance. The first example of cancer immunotherapy dates back to 1893 by Coley, where he observed that an injection of bacterial toxins into a tumor generated a strong immune response thereby eliminating the existing sarcoma. However, little was understood about our immune system at that time, but this study created a benchmark towards modern day cancer immunotherapy. Our present day understanding directs us to believe that the endotoxins in the bacterial mixture elicit cytokines and interleukins which stimulate an immune response by activation of macrophages, natural killer cells and cytotoxic T-cells. Since then numerous approaches with modification of Coley’s method have been carried out including the use of a patient’s own tumor cells after inactivation as a possible method of vaccination. The benefit of such methods is that the patient’s own tumor cells are used, resulting in a tumor-specific immune response. This concept has been used in the past with genetic modification of both autologous and heterologous malignant cells which express high density of co-stimulatory proteins. However the major drawback of such an approach is the high cost and labor requirement as well as the inability to measure specific immune response.

Another avenue relies on the generation of passive immunity against existing tumors where monoclonal antibodies against the cancer antigen are first generated in a separate animal followed by their introduction into cancer affected patients. The first monoclonal antibody (mAb) to be approved by the US FDA was rituximab, which
targets the CD20 on the B-cell surface of non-Hodgkin’s lymphoma. Since then a number of mAbs targeting malignant cells in humans have been recently approved by the US FDA for cancer treatment that primarily target epithelial cancers such as advanced prostate cancer. However this passive immune therapy approach only provides temporary protection against human malignancies which can only extend the patient’s life up to several months. Therefore the hunt for a vaccine for the permanent eradication of this life threatening disease is of prime importance.

A major difference between classical vaccines which are used to prevent the occurrence of a disease and anti-tumor vaccines is that they are aimed at generating an immune response against an already existing malignancy. In addition anti-tumor vaccines are also able to prevent recurrence of a tumor after its surgical removal. In concept the identification of tumor associated carbohydrate antigens (TACAs) on tumor cell surfaces has evoked a flurry of activity in the field of cancer immunotherapy. The majority of tumor cells are characterized by unusual cell surface glycosylation which over express aberrant short oligosaccharide sequences and show enhanced sialylation of cell-surface glycolipids and O- and N-linked glycoproteins. A shortened glycopeptide exposes parts of the peptide backbone that are typically shielded in the native form thus making them more accessible to the immune system. Moreover several TACA bound glycopeptides are secreted into the serum by the tumor cells thus making them excellent targets for the development of anti-tumor vaccines.
1.2 Tumor-Associated Carbohydrate Antigens

Cell surface protein and lipid bound oligosaccharides in the living system are associated with a number of essential biological processes. Glycoconjugates on tumor cell surface show abnormalities in both structure and distribution and thus play a key role in tumor metastasis and progression. These tumor associated carbohydrate antigens (TACAs) have been extensively studied in the hunt for novel approaches towards cancer therapeutics. Several proposed theories illustrated that changes in the tumor environment and metabolism in tumor cells leads to the changes in the expression of normal genes which are the primary causes for TACA formation. TACAs can be broadly divided into two categories (i) glycolipid antigens where the TACAs are ligated to lipid bilayers by hydrophobic interaction and (ii) glycoprotein antigens where the TACAs are attached to the cell surface proteins via the hydroxyl group to a serine or threonine residue on the peptide backbone (Figure 1). The glycolipids can be further classified into (a) gangliosides such as GD2, GD3, fucosyl GM1, GM2, GM3; (b) globo-series including Globo-H, Gb3, Gb4 and Gb5; (c) lacto-series or the Lewis antigens such as sialyl Lewis\textsuperscript{a} (SLe\textsuperscript{a}), SLe\textsuperscript{x}, SLe\textsuperscript{x}-Le\textsuperscript{x} and Le\textsuperscript{y}. A few well know examples of the protein bound TACAs include Tn, STn and the TF antigens which results from incomplete O-glycan synthesis (Figure 2).
Figure 1. Schematic representation of TACA subtypes.
Figure 2. Representative structures of TACAs.
Some tumor cells are distinguished from normal cells by the overexpression of TACAs on their surface.\textsuperscript{25-27} Moreover similar TACAs are expressed on the surface of numerous cancer cell lines. As for example Tn and Globo H tumor markers are expressed on the surface of numerous epithelial cancer cell lines such as breast, prostrate and ovarian cancers.\textsuperscript{25,28-31} In addition TACAs are highly over expressed in mucins which are a class of membrane bound intensely glycosylated high molecular weight proteins found on malignant cells of epithelial origin.\textsuperscript{32} As for example mucin-1 (MUC-1) found in over 90\% of breast carcinomas expresses high density of Tn, STn, TF, Globo-H as well as Lewis antigens.\textsuperscript{33} MUC-1 is shed into the patient’s sera and has been targeted as a marker for cancer.\textsuperscript{32,33} Another important avenue in cancer immunotherapy is the prevention of cancer recurrence by targeting cancer stem-cells. Extensive studies have shown that TACAs such as Globo H and Gb5 are significantly expressed in cancer stem cells.\textsuperscript{34-36} Therefore TACA incorporated anti-tumor vaccines provides a promising rationale where a single construct can target tumors of various origins.

\section*{1.3 Generating Immune Response Against TACAs}

In spite of the fact that TACAs appear as promising vaccine candidates against cancer, numerous causes restrict their use. The isolation of TACAs from natural sources in pure state and sufficient quantity is practically almost impossible. The generation of these antigens through organic synthesis has provided a path breaking alternative. In the laboratory TACAs can be precisely synthesized in moderate quantities having the same structural integrity as their natural occurring counterparts. Further more the synthesis of
these complex structures has produced interesting chemical approaches such as one-pot synthesis and automated oligosaccharide synthesis.\textsuperscript{37-39}

Another major drawback of using TACAs as vaccine candidates is the fact that TACAs are inherently very weakly immunogenic due to their T-cell independent character. The immune response to TACAs are governed primarily by very short lived low affinity IgM antibodies without the generation of memory cells unlike protein and peptide antigens where the major immune response is effected by the high affinity IgG antibodies. Additionally normal cells also expresses TACAs in low densities on their surfaces and hence TACAs are referred as “self-antigens”. Further tumor cells often shed TACAs into the blood stream thereby building immuno-tolerance against the TACAs in the vaccine constructs.\textsuperscript{7}

Antibodies against TACAs have been shown to destroy cancer cells by complement dependant cytotoxicity (CDC) and/or by antibody-dependant cellular cytotoxicity (ADCC). Antibodies against the tumor cells can be generated either by active immunization (vaccine administration) or passive immunizations (immunization with the antibody itself) both of which have been effective. As for example patients with high titers of naturally occurring antibodies against GM2 have over 90% five year survival rate as compared to the general 40% rate.\textsuperscript{40,41} Also administration of the mAb against TACAs such as anti-GD3 can elicit tumor regression in humans.\textsuperscript{42}

Antibodies are produced by the B-cells of our immune system after they have been activated by their respective antigens. The membrane-bound Ig proteins on the surface of the B-cells recognizes numerous antigens including carbohydrates. These antigens bind to the B-cells thereby inducing cross linking between the Ig proteins which
in turn activates the B-cells to produce low affinity IgM antibodies.\textsuperscript{43} For a class-switch to the high affinity IgG antibodies helper T-cells must be activated and then interact with the B-cells. The activation of the helper T-cells requires the involvement of the antigen presenting cells (APCs).\textsuperscript{44,45} The most common APCs are dendritic cells which capture protein antigens and after internalization and processing, the proteins are broken down into small peptidic fragments that are surface displayed on the APC as a complex with the major histocompatibility complex (MHC) class II molecules. The antigen complexed on the MHC II on the APC now migrates towards the lymphoid organs where it interacts with the naïve T-cells leading to their activation.\textsuperscript{46,47} This activation induces the B-cells and the helper T-cells to migrate towards each other. The MHC II-peptide complex also presented by the B-cells now generates an interaction with the helper T-cells leading to the expression of co-stimulatory proteins. The activated helper T-cells expresses CD40L which binds with the CD40 on the B-cells resulting in the release of cytokines by the helper T-cells. These cytokines now stimulate the B-cells to differentiate into antibody secreting cells. Also there is a considerable amount of generation of memory B cells which differentiate into the high affinity IgG antibody secreting cells on subsequent exposure to the antigens (Figure 3).\textsuperscript{48}
Figure 3. Schematic representation of interaction between B cells and helper T cells; (A) low affinity IgM secretion in absence of helper T cell activation and (B) high affinity IgG secretion in presence of helper T cell activation.

The effectiveness of any vaccine depends on its potential to successfully trigger both the innate and the adaptive immune system against the antigen of interest. In addition to the activation of the B and the T lymphocytes the adaptive immune response requires danger signals that are offered by the innate immune system. Vaccines are often administered along with suitable adjuvants which stimulate cytokine release and provide the optimum condition for APC maturation. In the recent past the Toll-like receptors (TLRLs) have emerged as a promising vaccine adjuvant candidate which triggers the initial innate immune response followed by the initiation and control of the adaptive immune response.49-51 They have also been shown to stimulate the release of several
cytokines resulting in the optimum interaction between the activated APCs with the B and the T lymphocytes.

1.4 Strategies for Improving Immunogenicity of TACAs

The weak immunogenicity of TACAs poses a severe problem in their use as vaccine candidates. Therefore there is always a hunt for new approaches to break this immunotolerance of TACAs. Out of the various strategies employed to render TACAs more potent towards the immune system, their conjugation to large carrier proteins, virus-like particles (VLPs) and zwitterionic polysaccharides have been widely studied. Another important approach towards cancer vaccine development has been the synthesis and study of fully synthetic carbohydrate-based cancer vaccines.

1.4.1 TACA Conjugated Carrier Proteins

The classical approach towards the development of anti-tumor vaccines was the conjugation of the TACAs of interest to large carrier proteins such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid (TT). The carrier proteins along with the conjugated TACAs are picked up by the APCs (including B-cells) and after internalization and processing are displayed on the surface as a complex with MHC II molecules. These are then recognized by the helper T-cells leading to the production of necessary cytokines for the activation of the B-cells. Thus the carrier proteins induce the interaction of the helper T-cells with the B-cells leading to an enhanced immune response towards the conjugated TACAs.
Earlier studies in this field demonstrated that the choice of carrier proteins, nature of conjugation and the adjuvant used greatly influences the immune response against the carbohydrate antigen. Helling and co-workers observed that the best immune response against the ganglioside GD3 was achieved when it was conjugated with KLH and QS-21 was used as an external adjuvant. The first generation of TACA conjugate protein vaccines were monomeric and involved a single carbohydrate antigen such as Globo-H, GM2, Leβ, KH-1, Fucosyl-GM1 and others conjugated to KLH. The vaccines were evaluated in mice models using external adjuvants and were able to elicit both IgM and IgG titers and these antibodies also showed moderate binding with human breast cancer cell lines such as MCF-7. The Globo-H KLH conjugate vaccine entered Phase I clinical trial against prostrate cancer where it was observed that generated IgM antibodies were specific to the Globo-H on the tumor cells. The second generation vaccines consisted of several units of the TACAs clustered and conjugated to the carrier protein. The rationale behind this approach relied on the fact that the TACAs on the tumor cell surfaces are often expressed as clusters and therefore incorporating the clustered carbohydrate antigens onto the carrier protein will mimic the natural circumstances. Several monomeric clustered carbohydrate antigens have been synthesized including Tn, Tf, STn, 2,6-STF and Leβ and implanted onto protein carriers such as KLH. These clustered vaccines demonstrated a more robust immune response than their non-clustered congeners (Figure 4).
A long term goal has been to develop a vaccine candidate that will simultaneously generate an immune response against multiple cancer antigens. One of the approaches towards this strategy was the co-administration of several monomeric TACA-KLH conjugates such as GD3-KLH, Le$^\text{y}$-KLH, MUC1-KLH and MUC2-KLH along with QS-21 adjuvant. Immunological studies in mice revealed that there were considerable titers of both IgM and IgG antibodies whether the conjugate was administered separately or in
a polyvalent fashion.\textsuperscript{66} This study was followed by several hexavalent and heptavalent mixtures of monomeric carbohydrate antigen KLH conjugates which demonstrated similar results.\textsuperscript{67} With these successful preclinical results, a hexavalent monomeric KLH conjugate including GM2, Globo-H, Le\textsuperscript{\gamma}, glycosylated MUC1-32 mer, Tn, and TF and another heptavalent KLH conjugate consisting of GM2, Globo-H, Le\textsuperscript{\gamma}, Tn, STn, TF and Tn-MUC1 co-administered with QS-21 as adjuvant were evaluated in Phase II clinical trials.\textsuperscript{68} These formulations were well tolerated and proved to be effective in the generation of antibodies against multiple cognate carbohydrate antigens. While this strategy seems to be effective there are several drawbacks. One of the major concerns is that there is a substantial use of the carrier protein which can suppress the immune response specific for the cancer antigens leading to the loss of immunogenicity of the vaccine. The synthesis of the multiple KLH constructs are low yielding and often hard to reproduce. Also each component of the vaccine requires non-trivial regulatory validations.\textsuperscript{69} To overcome these issues it is required to develop a vaccine construct which will encode numerous carbohydrate antigens on a single protein back bone. The power of synthetic organic chemistry has paved the way for the generation of such vaccine constructs. In one of the studies by Livingston and co-workers a unimolecular pentavalent construct was synthesized consisting of Globo-H, GM2, STn, TF and Tn and conjugated to KLH.\textsuperscript{70} Vaccination studies in mice with this unique pentavalent vaccine generated high titers of both IgM and IgG antibodies against all the five carbohydrate antigens. Further the generated antibodies recognized all the five different antigens on breast cancer cell lines such as MCF-7. Such vaccines are awaiting clinical trials in the near future.\textsuperscript{69}
1.4.2 TACA Conjugated Virus-Like Particles

Virus-like particles (VLPs) refer to caspids from viruses composed of highly organized structural proteins.\(^7\) The rational behind the conjugation of TACAs to the VLPs is that the immune response is more pronounced when the TACAs are presented in a highly ordered fashion.\(^7\) One of the most frequently used VLP for efficient TACA delivery in the Cowpea Mosaic Virus (CPMV) that consists of an icosahedral arrangement of 60 copies of identical subunits.\(^7\) Previous studies reported that a Tn-KLH conjugate failed to generate a robust immune response towards the cancer antigen. However when a Tn-CPMV construct was synthesized from a maleimide derivative of Tn and a cysteine mutant of CPMV and the immune response evaluated in mice it showed high titers of both IgM and IgG antibodies against Tn (Figure 5).\(^7\) The generated antibodies demonstrated strong binding to the multi-drug resistant ovarian cancer cell line NCI-ADR RES. In a separate study, the synthesis and immunological evaluation of a number of TACA-VLP conjugates including Globo-H, sialyl Lewis\(^x\) and blood group A tetrasaccharides demonstrated high antibody generation against the cancer antigens.\(^7\) These studies indicate that VLPs have high potential as TACA delivery agents.
1.4.3 TACA Conjugated Zwitterionic Polysaccharides

Carbohydrate antigens are inherently very weakly immunogenic due to their T-independent nature. However, zwitterionic polysaccharides (ZPSs) isolated from bacterial capsules have been found to activate the helper T-cells. The high density of alternative positive and negative charges are primarily responsible for binding with MHC class II molecules and presentation to helper T-cells. Several ZPSs have been isolated from different bacterial species such as PS A1 and PS B from Bacteroides fragilis strain 9342, PS A2 from B. fragilis 638 and Sp1 from type 1 Streptococcus pneumonia (Figure 6). Since ZPSs have the ability to induce helper T-cell activation conjugation with TACAs to induce a robust immune response against the cancer antigen is expected. Andreana and co-workers conjugated GalNAc to selectively oxidized PS A1 through oxime bond formation. This construct successfully generated IgG3 antibodies against the carbohydrate when administered alone or with the help of Titermax Gold adjuvant. Studies with numerous other TACA-ZPS conjugates are necessary to amplify this concept.
Figure 6. Structure of repeating subunits of ZPSs.
1.4.4 Fully Synthetic Carbohydrate Based Cancer Vaccines

One of the major drawbacks of using carrier protein conjugated TACAs as vaccine candidates is the fact that the carrier proteins are themselves immunogenic leading to the suppression of the immune response against the carbohydrate antigen.\textsuperscript{79} As an example the Tn-KLH conjugate failed to generate sufficient high affinity antibodies against Tn where as high titers of anti-KLH antibodies were reported.\textsuperscript{80} To eliminate the necessity of a carrier protein, the TACAs must be conjugated to potent antigens or receptors that can activate the necessary immune cells. In this context Toll-like receptors (TLRs) have been conjugated to TACAs to trigger the release of cytokines which activates the APCs, macrophages and the B-cells.\textsuperscript{81} As an example the TLR2 agonist Pam\textsubscript{3}CysSK\textsubscript{4} has been covalently attached to several TACAs including a dimeric and trimeric Tn cluster and a monomeric and trimeric cluster of Le\textsuperscript{y}.\textsuperscript{82} Immunization studies in mice revealed that the major antibodies generated against the respective antigens were IgM with almost negligible class switch to the high affinity IgG even with the co-administration of QS-21 adjuvant. These results indicate that a fully synthetic vaccine requires the presence of a helper T-epitope to induce a class switch to the high affinity IgG antibodies.

Two component vaccines were designed by conjugating the carbohydrate B-cell epitopes with a CD4\textsuperscript{+} T-cell epitope. Initial studies involved the conjugation of a STn antigen glycosylated MUC1 glycopeptide derivative to a CD4+ helper T-cell epitope derived from ovalbumin.\textsuperscript{83} This conjugate was co-administered along with complete Freuend’s adjuvant (CFA) to transgenic mice expressing T-cell receptors specific for the ovalbumin T-epitope and successfully generated highly specific antibodies against the
glycosylated MUC1. Another inspiring example of a two component cancer vaccine was the synthesis of a multi-antigenic glycopeptide (MAG) consisting of a non-immunogenic tetravalent lysine core where each arm is conjugated with a helper T-epitope derived from the polio virus and a trimeric Tn antigen. Immunological evaluation in the mice model after co-administration of this conjugate with a mild alum adjuvant demonstrated high titers of IgG against Tn and increased survival rates in a prophylactic setting.

A tri-component cancer vaccine is designed to incorporate the carbohydrate B-cell epitope, the helper T-cell epitope as well as a potent immune activator such as a TLR ligand that satisfies the minimum requirements to elicit an immune response against the carbohydrate antigen. The inclusion of the TLR ligand in the vaccine construct renders self-adjuvanating character and eliminates the requirement of an external adjuvant. The first example of a three component vaccine was reported by Boons and co-workers where the Tn antigen was conjugated with the 20-amino acid peptide helper T-cell epitope YAF, and the TLR ligand Pam3Cys (Figure 7). The vaccine was tested as a liposomal formulation in mice with or without the external adjuvant QS-21. Though moderate titers of IgG was observed against Tn, this strategy showed a promising avenue for future development.
Figure 7. Fully Synthetic carbohydrate based anti-tumor vaccines; (A) a multi-antigenic glycopeptides based on an oligolysine scaffold and (B) a three component vaccine.

MUC1 is an over glycosylated transmembrane glycoprotein that is expressed at the apical surfaces of human epithelial cells and contains the 20 amino acid HGVTSAPDTRPAGSTAPPAPA tandem repeats. MUC1 is overexpressed on the surface of a number of carcinomas of epithelial origin like breast, ovary, colon, rectum and pancreatic cancers. Several conjugates with the TACA glycosylated MUC1 glycopeptide have been synthesized and studied.\textsuperscript{87,88} Semi synthetic vaccines consisting of the TACA glycosylated MUC1 peptide conjugated to carrier proteins such as KLH and TT were studied by the Kunz and Sorensen groups respectively.\textsuperscript{89,90} Strong and specific antibody responses (mainly of the IgG1 subtype) were observed in both cases against the glycosylated MUC1 peptide thus proving that the MUC1 glycopeptide can be successfully formulated into an anti-tumor vaccine. With the success of the semi synthetic MUC1 based anti-tumor vaccines, several approaches towards the synthesis of their fully synthetic counterparts were evaluated. Kunz and coworkers synthesized a two component vaccine consisting the 20-amino acid tandem repeat of the MUC1 peptide glycosylated with the TF or Tn antigen at several sites conjugated to TLR2 agonist Pam$_3$CysK$_4$.\textsuperscript{91} The vaccine was administered along with Freund’s adjuvant to mice.
Although there was sufficient proof of the generation of anti-MUC1 specific antibodies yet the overall titers were less when compared with those generated against the MUC1-TT conjugate. A reason for the low antibody titers against the MUC1 glycopeptide can be attributed to the fact that the vaccine lacked a helper T-cell epitope. Payne and coworkers demonstrated that a helper T-cell epitope is necessary in the vaccine design by synthesizing a tri component vaccine consisting of the intrinsic adjuvant Pam₃CysSer, the tandem repeats of the MUC1 peptide glycosylated with the Tn or the TF antigen and a helper T-cell epitope and studied the immunological effects in mice. It was observed that heavy glycosylation of the MUC1 peptide with the TACAs actually reduces the immune response presumably due to the masking of the helper T-cell epitope. Also the anti-glycosylated MUC1 antibody titers were much higher in the tri-component vaccine than that observed in case of the di-component construct which lacks the helper T-cell epitope. Another novel tricomponent vaccine construct consisting of TLR2 ligand Pam₃CysSK₄, the tumor associated glycopeptides derieved from MUC1 and the helper T-cell epitope derieved from the polio virus was synthesized by the Boons group. This novel vaccine generated a robust immune response against the glycopeptides antigen and triggered both the humoral and cellular arms of the immune system along with sufficient cytokine induction.

1.5 Summary

Over the last few decades there has been substantial progress in the development of novel strategies in fields of anti-cancer vaccine development. Many of the vaccine constructs have shown great promise as potential candidates for clinical use for the prevention and treatment of cancer but till date the hunt for a universally accepted TACA
based anti-tumor vaccine is still on. The vaccine candidates should be able to trigger both the humoral and the cellular arms of the immune system specifically towards the carbohydrate antigens with minimal side reactions to be able to be considered as a successful vaccine construct. The fully synthetic carbohydrate based anti-tumor vaccine has the advantage over other approaches as it incorporates only those elements required for the desired immune response minimizing unwanted side reactions. However, many different avenues in the development of fully synthetic vaccines need to be explored for the successful generation of an effective anti-tumor vaccine which can find clinical use in the near future.
1.6 References


Chapter 2

Synthesis of a Single Molecule L-Rhamnose-Containing Three Component Vaccine and Evaluation of Antigenicity in the Presence of Anti-L-Rhamnose Antibodies

2.1 Introduction

The glycoconjugates on the surface of cancer cells are often expressed in abnormal quantities and show unique structural modifications in their carbohydrate moieties in comparison to the glycoconjugates found on normal cells. These aberrant carbohydrate epitopes, also known as tumor-associated cancer antigens (TACAs), have been widely used as important markers for cancer detection and disease progression. Studies have shown prolonged survival of cancer patients by the virtue of antibodies generated against these cancer antigens. This observation suggests that the immune system can be enhanced by vaccination with TACAs possibly leading to improved treatment outcomes for cancer patients.

An obstacle to the use of TACAs as cancer vaccines is their inherently week immunogenicity. The generation of high affinity IgG antibodies against cancer depends upon the combined interaction of B-cells and helper T-cells, which requires the antigen to be preferentially displayed on the MHC-II class molecules which occur on the surface

\[^{1}J. \text{Am. Chem. Soc. 2010, 132, 17236-17246.}\]
of antigen presenting cells (APC) such as dendritic cells.\textsuperscript{10} In general, carbohydrate epitopes alone do not activate the helper T-cells, resulting in the production of low affinity IgG and IgM antibodies against cancer antigens. Earlier efforts to break this immunological tolerance have focused on the conjugation of cancer antigens to large immunogenic carrier proteins such as KLH,\textsuperscript{11-18} BSA,\textsuperscript{17,19,20} Toll-like receptor (TLR) agonists\textsuperscript{21} and zwitterionic polysaccharides.\textsuperscript{22} A number of drawbacks have been noted for the carrier protein conjugation strategy.\textsuperscript{21} For example, the conjugation reaction can often be uncontrollable leading to the formation of conjugates with unspecified epitope ratios leading to issues of reproducibility in the immunological experiments.\textsuperscript{23} Further, the carrier protein itself can induce a strong immune response, leading to the suppression of antibody production against the cancer antigen.\textsuperscript{21} In addition, the linkers used for conjugating the carrier proteins to the carbohydrate epitopes can themselves be immunogenic.\textsuperscript{24} More recently smaller antigenic peptides have been conjugated to TACAs and studied as vaccines which may mitigate these issues.\textsuperscript{12,20,25-28} As an example, Boons \textit{et. al} conjugated a 20 amino acid peptide (YAF), derived form the outer membrane of \textit{Neisseria meningitides} and an MHC class II restricted site for human and mouse T-cells, to the cancer antigen $\alpha$-N-acetylgalactoseamine-$\alpha$-threonine (Tn) and a lipopeptide $\text{S-}[\text{R}(R)-2,3\text{-dipalmitoyloxy-propyl}]\text{-N-palmitoyl}\cdot(R)\text{-cysteine}$ (Pam$\text{}_{3}\text{Cys}$), which has strong immuoadjuvant properties and interacts with TLR-2 resulting in production of pro-inflammatory cytokines and chemokines thereby stimulating the antigen-presenting cells (APCs).\textsuperscript{21} The production of high IgG antibody titers against Tn provided strong evidence that small peptides can be successfully used to stimulate helper T-cells.
Another avenue for boosting the immune response to tumor antigens has been the targeting the antigens of interest to APCs.\textsuperscript{29,30} Recent studies by Galili \textit{et al.} had explored the \textit{in vivo} interaction of Gal$\alpha$1-3Gal$\beta$1-4GlcNAc-R ($\alpha$-Gal) epitopes in model vaccines against HIV gp120 and flu virus with naturally occurring anti-Gal antibodies in $\alpha$-1,3-galactosyltransferase knockout mice by an antibody dependant antigen uptake mechanism which showed a higher than 100-fold increase in immunogenicity.\textsuperscript{31,32} Human serum has been reported to contain high amounts of naturally occurring anti-rhamnose antibodies. These antibodies are highly antigen specific and reproducibly high titer results have been reported for a large collection of serum samples from various individuals of different age groups.\textsuperscript{33,34} Herein we hypothesized that the effectiveness of a cancer vaccine can be increased by conjugation of a helper T-cell peptide and B-cell antigen with an L-rhamnose (Rha) carbohydrate epitope by the same antibody-mediated antigen uptake mechanism described by Galili \textit{et al.} (Figure 1). Our study focuses on the idea that the Fc portion of the \textit{in vivo} generated immune complex between an anti-Rha IgG or IgM antibody and the Rha-containing conjugate vaccine can be recognized by the Fc$\gamma$ receptors or other receptors on APCs such as dendritic cells.\textsuperscript{16} This will result in an overall internalization of the vaccine and better presentation on the human MHC. The Rha epitopes on the N-terminus of the synthetic peptide are not expected to interfere with MHC class II binding of the YAF peptide. This idea is supported by promising results by Galili \textit{et al.} where naturally occurring anti-Gal antibodies had been targeted using $\alpha$-Gal epitopes on a model vaccine.\textsuperscript{30} In this study we describe the synthesis of a single molecule three-component vaccine containing a Rha-epitope, the 20 amino acid peptide
(YAF) and the cancer antigen Tn and explored the immunological effects of this vaccine by in vivo and in vitro experiments in mice.

Figure 1. Diagramatic representation of cancer antigen uptake mediated by the presence of anti-Rha antibodies. [APC = antigen presenting cells, e.g. dendritic cells].

2.2 Results

2.2.1 Synthesis of Conjugatable L-Rhamnopyranosides.

To test our hypothesis we synthesized conjugatable L-rhamnopyranosides 3 and 5 (Scheme 1). Tetraacetyl rhamnopyranoside 1 was glycosylated in presence of boron trifluoride etherate with 4-pentene-1-ol to generate the rhamnose pentenyl glycoside 2 (69%). Sodium periodate catalysed oxidation of 2 in presence of ruthenium trichloride afforded the acid 3 (98%). Deacetylation using zemplen conditions followed by ozonolysis of 2 afforded the L-rhamnose aldehyde 5 (87.5%).
2.2.2 Synthesis of Rha-BSA, Rha-KLH, Rha-OVA and Tn-BSA conjugates.

Compound 5 was conjugated with BSA, KLH and OVA by incubation in the presence of sodium cyanoborohydride in 0.1 M phosphate buffered saline (PBS) pH 7.2, to produce conjugates Rha-BSA 6, Rha-KLH 7 and Rha-OVA 8 respectively (Scheme 2). The conjugates were purified by filtration through a dialysis tubing with molecular weight cut-off value of 10000 Da, with 6-7 changes of PBS. The protein concentration of the conjugates were determined by the Bradford method using standard curves of BSA, KLH and OVA as reference. The carbohydrate concentration of 6, 7 and 8 were determined by the phenol-sulfuric acid method using a standard curve of 5. The epitope ratios of 6, 7 and 8 were calculated to be 11.6, 259 and 4.5 Rha/protein molecule respectively. A Tn-BSA conjugate was prepared by deprotection of the Fmoc group in glycosyl amino acid 9 using catalytic hydrogenolysis conditions in the presence of AcOH/MeOH followed by deacetylation with sodium methoxide to generate compound 11 (Scheme 3). Compound 11 was conjugated to BSA by incubation in the presence of 0.2% glutaraldehyde and sodium cyanoborohydride in 0.1 M PBS, pH 7.2, to furnish 12. The protein concentration of 12 was determined by Bradford method using a standard curve of BSA as reference.

2.2.3 Synthesis of Rha-YAF-Tn and YAF-Tn Vaccines.

The TACA-containing vaccines were synthesized by Fmoc strategy using solid phase chemistry. Preloaded Fmoc L-Arg Wang resin was used for the peptide synthesis (Scheme 4). An Arg residue was used at the C-terminus since a preloaded Tn-Wang resin was not available and we were concerned about the potential for poor loading on Wang
resin using the bulky Fmoc-Thr-(GalNAc(Ac)3-α-D)-OH (9) building block. The Fmoc groups were deprotected by 25% piperidine in dimethyl formamide (DMF). The amino acid esters of 1-hydroxybenzotriazole (HOBt) were coupled sequentially using 1,3-diisopropylcarbodiimide (DIC) as the coupling agent. Finally the peptides were cleaved from the resin by a reagent cocktail of 88% TFA, 3% thioanisole, 5% ethanedithiol, 2% water and 2% phenol, filtered, precipitated and centrifuged. The identities of the peptides 16 and 17 were confirmed by MALDI-TOF analysis (Compound 16: [M+H] m/z calcd for C_{158}H_{231}N_{39}O_{49} = 3459.6783, found 3459.6902; Compound 17: [M+H] m/z calcd for C_{142}H_{209}N_{39}O_{40} = 3101.5519, found 3101.5452).

The acetyl groups in compounds 16 and 17 were deprotected by treatment with 6 mmol NaOMe for 2 h²⁷ (Scheme 5). Purification was accomplished by size exclusion chromatography on Bio-Gel (P-2, 45-90 μM). The vaccines were lyophilized to obtain compounds 18 and 19 as white powders (85%). The identities of the vaccines were determined by HR-MALDI-TOF analysis (Compound 18: [M+H] m/z calcd for C_{146}H_{220}N_{39}O_{43} = 3207.614, found 3207.165; Compound 19: [M+H] m/z calcd for C_{136}H_{204}N_{39}O_{37} = 2975.520, found 2975.562).
Scheme 1. Synthesis of Conjugable L-Rhamnopyranosides.\textsuperscript{a}

\textsuperscript{a} Reagents and conditions: (a) 4-Pentene-1-ol, BF\textsubscript{3}.Et\textsubscript{2}O, CH\textsubscript{2}Cl\textsubscript{2}, 0 °C→r.t., 12 h, 69%;
(b) NaIO\textsubscript{4}, RuCl\textsubscript{3}, CH\textsubscript{2}Cl\textsubscript{2}-CH\textsubscript{3}CN-H\textsubscript{2}O (2:2:3), r.t., 18 h, 98%; (c) Na, MeOH, r.t., 1h, quantitative; (d) O\textsubscript{3}, MeOH-CH\textsubscript{2}Cl\textsubscript{2} (1:4), Me\textsubscript{2}S, -70 °C→r.t., 87.5%.
**Scheme 2.** Synthesis of L-Rhamnopyranoside conjugates with BSA, OVA and KLH.\(^a\)

\[\text{Reagents and conditions:} \ (a) \ 	ext{BSA, PBS (pH 7.2), NaBH}_3\text{CN, 37 °C, 24 h}; \ (b) \ KLH, PBS (pH 7.2), NaBH}_3\text{CN, 37 °C, 24 h}; \ (c) \ OVA, PBS (pH 7.2), NaBH}_3\text{CN, 37 °C, 24 h.} \]

\([n = \text{Rha/protein molecule}].\)
Scheme 3. Synthesis of Tn-BSA Conjugate.$^a$

$^a$ Reagents and conditions: (a) MeOH, AcOH, 10% Pd/C, H₂, 3h; (b) NaOMe/MeOH, 2 h; (c) BSA, 0.2% glutaraldehyde, NaBH₃CN, 20 min.
Scheme 4. Synthesis of Acetylated Rha-YAF-Tn and YAF-Tn.\textsuperscript{3}

\[ \text{Fmoc-} \text{N} \equiv \text{NH} \quad \text{Wang Resin} \quad \text{a} \quad \text{Fmoc-} \text{N} \equiv \text{NH} \quad \text{NHPbf} \]

13

\[ \text{Fmoc-} \text{N} \equiv \text{NH} \quad \text{NHPbf} \]

14

b

\[ \text{Fmoc-} \text{N} \equiv \text{NH} \quad \text{NHPbf} \quad \text{FmocHN-YAFKARHANVGRNAFELGLGO} \]

15

c

\[ \text{FmocHN-YAFKARHANVGRNAFELGLGO} \quad \text{c} \quad \text{H}_2\text{C}-\text{O}-\text{YAFKARHANVGRNAFELGLGO} \]

16

d

\[ \text{H}_2\text{N-}\text{YAFKARHANVGRNAFELGLGO} \quad \text{d} \quad \text{H}_2\text{N-}\text{YAFKARHANVGRNAFELGLGO} \]

17
Reagents and conditions: (a) (i) 25% piperidine/DMF (ii) HOBr ester of 9, DIC/NMP (b) Repeat step a with G, L, F, L, E, F, A, N, R, G, V, N, A, H, R, A, Y, K, F, A, Y in sequence (c) (i) 25% piperidine/DMF (ii) HOBr ester of 3, DIC/NMP (iii) 88% TFA, 3% thioanisole, 5% ethanedithiol, 2% water and 2% phenol (d) (i) 25% piperidine/DMF (ii) 88% TFA, 3% thioanisole, 5% ethanedithiol, 2% water and 2% phenol. [Pbf = 2,2,4,6,7-pentamethyl-dihydrobenzofurane-6-sulfonyl].
Scheme 5. Deacetylation of Rha-YAF-Tn and YAF-Tn.$^a$

$^a$ Reagents and Conditions: (a) NaOMe/MeOH, r.t., 2 h, 85%.
2.3 Immunological Results and Discussion.

2.3.1 Anti-Rhamnose Antibody Generation.

The first goal of the immunological study was to determine whether mice contain naturally occurring anti-rhamnose antibodies in their serum. Serum samples from two different non-immunized mice (C57BL/6 and Swiss Webster) were screened against Rha-BSA conjugate 6 using an ELISA (Figures 2 and 3). Competition with free rhamnose and galactose added to the serum was also performed to test the competitive binding of the naturally occurring anti-rhamnose antibodies in the serum (if any) with the bound rhamnose on the plates. Absorbances at 620 nm showed that anti-rhamnose antibody concentrations in non-vaccinated mice were similar to the background at all serum dilutions. All the absorbances decreased comparably with serial dilutions of the serum samples from both mouse types. From this study we concluded that laboratory mice do not contain significant titers of naturally occurring anti-rhamnose antibodies.

Six groups of mice (groups A-F) were then immunized on day 0 with either phosphate buffered saline (PBS control, groups A and B) or Rha-OVA/TiterMax® Gold (TMG) adjuvant (groups C and D) or Rha-OVA/Sigma Adjuvant System® (SAS) (groups E and F) and boosted on day 25 (Figure 4). An ELISA on the serum collected from the mice showed that the antibodies against Rha-BSA in groups C-F were 100-fold higher than the control groups A and B (Figures 5 and 6). This immunization step confirmed that anti-rhamnose antibodies were artificially generated in the experimental groups of mice (C-F).

We also determined the anti-rhamnose antibody titers from groups A-F against a Rha-KLH conjugate 7 (data not shown). These titers were compared with those
determined using Rha-BSA conjugate 6 to evaluate if there were any measurable differences when Rha-conjugates with different epitope ratios were used to detect anti-rhamnose antibody titer. It was observed that the anti-rhamnose antibody titers were comparable at every serum dilution when either conjugates 6 or 7 were used. As an example the absorbances at 620 nm for group E at 1/100 serum dilution were 1.629 and 1.663 for conjugates 6 and 7 respectively. The results show that the anti-rhamnose antibody titer determined using the Rha-BSA conjugate did not differ markedly from the titers determined using the Rha-KLH conjugate. Therefore we used Rha-BSA conjugate for our future assays.

![Graph](image)

**Figure 2.** Anti-rhamnose antibody titre in non-immunized C57BL/6 mouse serum in the absence and presence of 0.1 M galactose or rhamnose.
**Figure 3.** Anti-rhamnose antibody titre in non-immunized Swiss Webster mouse serum in the absence and presence of 0.1 M galactose or rhamnose.

**Figure 4.** Step wise immunization plan. A-F represents six groups of female BALB/c mice. Stage I: Groups A and B were immunized with PBS, groups C and D with Rha-
OVA/TMG and groups E and F with Rha-OVA/SAS. Stage II: Vaccine challenge on groups A, C and E was done with Rha-YAF-Tn/CFA (boosted with Rha-YAF-Tn/ICF) and groups B, D and F with YAF-Tn/CFA (boosted with YAF-Tn/ICF). [Rha-YAF-Tn = rhamnose vaccine, YAF-Tn = non rhamnose vaccine, TMG = TiterMax® Gold adjuvant, SAS = Sigma Adjuvant System®, CFA = Complete Freund’s Adjuvant, ICF = Incomplete Freund’s Adjuvant].

![Graph](image)

**Figure 5.** Group average anti-Rha-BSA antibody titres after second Rha-OVA boost. [Groups A and F represents an average of a group of 3 mice each where as groups B-E represents an average of a group of 4 mice each].
Figure 6. Individual mouse anti-Rha-BSA antibody titres for Group A-F after second Rha-OVA boost at 1/100 serum dilution.

2.3.2 Vaccination with YAF-Tn and Rha-YAF-Tn Conjugates.

After successful generation of anti-rhamnose antibodies in our experimental groups of mice, the next step was to challenge the mice with either Rha-YAF-Tn 18 or YAF-Tn 19 and compare the anti-Tn antibodies in titers. Therefore, mice groups A, C and E were challenged in vivo on day 52 with 80 µg of Rha-YAF-Tn 18 per mouse in 50 µL emulsions containing equal volumes of 0.01 M PBS (pH 7.2) and Complete Freund’s Adjuvant (CFA), whereas mice groups B, D and F were challenged in vivo with 80 µg of YAF-Tn 19 per mouse in 50 µL emulsions of equal volume 0.01 M PBS (pH 7.2) and CFA.21,26,39,47 The mice were then boosted in vivo on day 81 with 50 µL equal volume emulsions of 0.01 M PBS (pH 7.2) and Incomplete Freund’s Adjuvant (ICF) containing either 80 µg per mouse of Rha-YAF-Tn (groups A, C and E) or YAF-Tn (groups B, D and F). The serum was pooled on day 91 and the anti-Tn antibody titers determined using
an ELISA. A plot of absorbance (at 620 nm) vs log_{10} [1/serum dilution] showed that anti-Tn antibody titers fall close to the background for all the groups beyond 1/100 serum dilutions (Figure 7). A careful investigation at 1/100 serum dilution revealed that the anti-Tn antibody ELISA absorbance recorded at 620 nm for groups C (0.900) and E (0.678) were 2 and 1.5 fold greater respectively than the anti-Tn antibody titer of group A (0.400) (Figure 8). The absorbances at 620 nm corresponding to the anti-Tn antibody titers for groups B (0.690), D (0.704) and F (0.601), which received the vaccine challenge without the rhamnose epitope were comparable. The data shows that groups C and E which had previously been immunized with Rha-OVA were generating more anti-Tn antibodies after vaccination with the Rha conjugate vaccine Rha-YAF-Tn in comparison to mice vaccinated with the vaccine lacking the Rha moiety. The increased anti-Tn antibody titer is presumably due to anti-rhamnose antibodies mediating antigen uptake.\textsuperscript{30,31} This was evident from the low anti-Tn antibody generation for the group A where the mice did not generate anti-rhamnose antibodies due to the prior lack of immunization with Rha-OVA conjugate 8. Comparable anti-Tn antibody production for the groups B, D and F was also justified as the challenge vaccine did not contain the rhamnose epitope and therefore would not be expected to be influenced by anti-rhamnose antibodies (Figure 9). We also measured the anti-rhamnose antibody titers in the serum after the boost with Rha-YAF-Tn or YAF-Tn (Figure 10) to confirm the presence of anti-rhamnose antibodies and therefore prove that the increase in the production of anti-Tn antibodies was a result of an anti-rhamnose antibody mediated antigen uptake mechanism. The cumulative absorbances recorded at 620 nm for the control groups A and B were 1.385 and 0.681. These results indicated that the anti-rhamnose antibodies in titers were 2 times greater in
group A which was challenged with Rha-YAF-Tn than in Group B where the mice were never vaccinated with a rhamnose epitope. The cumulative absorbances for groups C-F which were all immunized with Rha-OVA conjugate 8 were 1.536, 1.029, 1.730, 1.602 and were considerably higher than group B confirming that the mice in these groups had considerable amounts of anti-rhamnose antibodies in their blood serum to form an in vivo immune complex with the rhamnose epitope in the vaccine. Also, mice that received Rha conjugates twice (Groups C and E) had similar anti-Rha titers to mice that had received them only once (Groups A, D, and F), indicating that the presence of Rha is not directing the immune response to expand the anti-Rha response at the expense of the anti-Tn response.

The specificity of the antibodies reactive against Tn-BSA was evaluated by a competitive binding assay by screening serum samples after the boost with Rha-YAF-Tn or YAF-Tn with or without prior incubation with free Tn 11 against BSA-Tn conjugate 12. Serum dilutions of 1/100 from two mice in groups B and C were incubated with free Tn 11 at concentrations of 0, 10^{-5}, 10^{-4}, 10^{-3} M in 0.1 M PBS (pH 7.2) prior to addition of the serum dilutions in the 96 well ELISA plates. Also here we used both horseradish peroxidase (HPR) goat anti-mouse IgG + IgM as well as only IgG recognizing secondary antibodies to confirm the presence of both IgG and IgM against Tn in the mice sera. The absorbances decreased uniformly with increase in the free Tn concentration in the serum dilutions. As an example the absorbances at 620 nm for the serum dilution of a group C mouse at free Tn concentrations of 0, 10^{-5}, 10^{-4}, 10^{-3} M using HPR goat anti-mouse IgG + IgM as secondary antibody were 0.432, 0.386, 0.301, 0.307. This trend was followed for all other serum dilutions (Figure 11). These results confirmed that the antibodies
produced after vaccinations were specific towards Tn. The results also showed that there was considerable amount of IgG antibody generation against Tn.

**Figure 7.** Group average anti-Tn-BSA antibody titers after second YAF-Tn and Rha-YAF-Tn Boost. [Groups A, B-E and F represents an average of a group of 3, 4 and 2 mice each].
**Figure 8.** Individual anti-Tn-BSA antibody titres for Groups A, C and E after second Rha-YAF-Tn boost at 1/100 serum dilution. A t-test confirms that groups A and E differ with a P value of <0.05.

**Figure 9.** Individual anti-Tn-BSA antibody titres for Groups B, D and F after second YAF-Tn boost at 1/100 serum dilution.
Figure 10. Anti Rha-BSA antibody titres after second boost with Rha-YAF-Tn and YAF-Tn at 1/100 serum dilution.

Figure 11. Competitive binding of anti-Tn antibodies with Tn-BSA in the presence of free Tn. [Each point represents an average of a duplicate].
2.3.3 T-Cell Proliferation Study.

T-Cell proliferation assays were performed to further assess whether or not Rha conjugate vaccines are more efficiently presented by APC in the presence of anti rhamnose antibodies. Two female BALB/c mice were used for this experiment. Mouse 1 was immunized (day 0) by a subcutaneous injection of 100 µL emulsion of YAF-Tn/CFA (12.8 µg) and mouse 2 was injected subcutaneously with 100 µL emulsion of Rha-YAF-Tn/CFA (12.8 µg). The mice were sacrificed on day 7 and the spleens were removed and separate single cell suspensions were prepared and combined with normal serum antibodies or anti-Rha serum antibodies. Vaccine solutions (Rha-YAF-Tn or YAF-Tn) were then added to the cell preparations. In the absence of added antibodies the proliferative response of spleen T cells from cells primed with YAF-Tn and challenged with either YAF-Tn (6008, 2932, 1844, 1476 cpm at of $10^{-5} - 10^{-8}$ M antigen concentrations) or Rha-YAF-Tn (7220, 3412, 2475, 2295 cpm at $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$ M antigen concentrations, respectively) were comparable indicating that Rha conjugation did not interfere with the presentation of the YAF epitope (Figure 12). Spleen T cells primed with Rha-YAF-Tn in absence of added antibodies responded slightly better to Rha-YAF-Tn (4959, 2314, 1031, 522 cpm at $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$ M antigen concentrations respectively) \textit{in vitro} than to YAF-Tn (3363, 1314, 454, 425 cpm at $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$ M antigen concentrations respectively). When anti-rhamnose antibodies were added at initiation of the culture, Rha-YAF-Tn primed T cells showed similar stimulation at a 10 fold lower concentration when the antigen contained rhamnose. For example, cells reached around 9000 cpm with $10^{-7}$ M Rha-YAF-Tn with anti-Rha antibodies and with $10^{-6}$ M Rha-YAF-Tn with non-Rha antibodies (Figure 13).
When the YAF-Tn peptide was used to challenge the cells, no difference between addition of anti-rhamnose and non-rhamnose antibodies was observed in the proliferation of the T-cells at all antigen concentrations. The effect of anti-rhamnose antibodies was more pronounced in the mice primed with Rha-YAF-Tn than in YAF-Tn for reasons that are under further investigation. This gives us insight into the role the anti-rhamnose antibodies in allowing the better uptake of the YAF peptide by the antigen presenting cells which in turn provides overall internalization of the vaccine and better presentation on the MHC.

![Graph](image)

**Figure 12.** T-Cell proliferation measured by $[^3]$H]thymidine incorporation in cells from mice primed with YAF-Tn or Rha-YAF-Tn with no added serum antibodies.
Figure 13. T-Cell proliferation measured by $[^3H]$thymidine incorporation in cells from mice primed with Rha-YAF-Tn with in vitro added Rha and non Rha serum antibodies. [The boxed region highlights the incorporation at $10^{-6}$ and $10^{-7}$ M antigen concentrations].

2.4 Conclusion.

A fully synthetic L-rhamnose containing three component vaccine has been prepared. Laboratory mice do not produce naturally occurring anti-rhamnose antibodies and therefore anti-rhamnose antibodies were generated in the experimental groups of mice via immunization with Rha-OVA conjugate 8. Vaccine challenge with Rha-YAF-Tn in groups A, C and E (Figure 8) showed that anti-Tn antibody generation was 2 and 1.5 times greater in groups C and E respectively than the control group A. However the anti-Tn antibody titers were comparable for groups B, D and F where YAF-Tn was used for the vaccine challenge. This observation was attributed to the fact that the YAF peptide was better displayed on the MHC II as a result of the in vivo formation of an immune complex between the rhamnose epitope in the vaccine and the anti-rhamnose
antibodies in the serum. This hypothesis was further justified by a T-cell proliferation assay which showed a 10-fold decrease in the amount of antigen needed to stimulate the T-cells in the presence of anti-Rha antibodies. The vaccine did not induce an unwanted dominant response to rhamnose itself, which agrees with the predicted properties of natural antibodies to antigens such as rhamnose. Overall, the results suggest that rhamnose conjugates can be used to harness endogenous anti-rhamnose antibodies for enhancing the immune response.
2.5 Experimental Section.

*General Methods.* All fine chemicals such as L-rhamnose, 4-pentene-ol, sodium periodate, ruthenium(III) chloride and dimethyl sulfide and anhydrous solvents such as anhydrous methanol were purchased from Acros Organics, Boron trifluoride etherate was from Aldrich. The chemicals were used without further purification. All solvents were obtained from Fisher and used as received except dichloromethane, which was dried and distilled following the standard procedures. Silica (230-400 mesh) for flash column chromatography was obtained from Sorbent Technologies; thin-layer chromatography (TLC) precoated plates were from EMD. TLCs (silica gel 60, f$_{254}$) were visualized under UV light or by charring (5% H$_2$SO$_4$-MeOH). Flash column chromatography was performed on silica gel (230-400 mesh) using solvents as received. $^1$H NMR were recorded either on a Varian VXRS 400 MHz or an INOVA 600 MHz spectrometer in CDCl$_3$ or CD$_3$OD using residual CHCl$_3$ and CHD$_2$OH as internal references, respectively. $^{13}$C NMR was recorded on a Varian VXRS 100 MHz in CDCl$_3$ using the triplet centered at δ 77.3 or CD$_3$OD using the septet centered at δ 49.0 as internal reference. High resolution mass spectrometry (HRMS) was performed on a TOF mass spectrometer. The peptides were synthesized on a Omega 396 synthesizer (Advanced ChemTech, Louisville, Ky). Preloaded Fmoc-L-Arg (pbf)-Wang resin all other Fmoc-L-amino acids were procured from Anaspec (San Jose, CA). Secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). All mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

**Pentenyl 2,3,4-tri-O-acetyl-α-L-rhamnopyranoside (2).** To a solution of 1,2,3,4-tetra-O-acetyl-D-rhamnopyranose, (1) (1.41 g, 4.07 mmol) in CH$_2$Cl$_2$ (2.78 mL) was
added 4-pentene-1-ol (0.61 mL, 6.11 mmol) and BF₃·Et₂O (1.54 mL, 12.22 mmol) at 0 °C and the resulting solution was stirred at ambient temperature under N₂ atmosphere. The reaction was monitored by TLC and appeared complete after 12 h. The reaction mixture was diluted with CH₂Cl₂ (20 mL) and washed with saturated NaHCO₃ (20 mL), 10% NaCl (10 mL) and water (25 mL). The organic layer was dried (anhydrous Na₂SO₄). Excess solvent was removed under reduced pressure and the crude material was purified by silica gel flash column chromatography (10.3 x 5.1 cm). Elution with 1:4 EtOAc-hexanes produced 2 as colorless glassy solid: yield 0.995 g (69%); Rf = 0.41 (1:4 EtOAc-hexanes); ¹H NMR (600 MHz, CDCl₃): δ 1.22 (d, 3 H, J = 6 Hz, C-5 CH₃), 1.71 (m, 2 H, O-CH₂-CH₂), 1.99 (s, 3 H, COCH₃), 2.05 (s, 3 H, COCH₃), 2.14 (m, 2 H, O-CH₂-CH₂-CH₂), 2.16 (s, 3 H, COCH₃), 2.44 (m, 1 H, O-CH), 3.70 (m, 1 H, O-CH), 3.88 (m, 1 H, H-5), 4.71 (d, 1 H, J = 1.2 Hz, H-1), 4.99 (dq, 1 H, J = 1.8 Hz, 10.2 Hz, terminal olefinic CH), 5.06 (dq, 1 H, J = 1.8 Hz, J = 16.5, terminal olefinic CH), 5.06 (t, 1 H, J = 10.2 Hz, H-4), 5.24 (dd, 1 H, J = 1.8 Hz, J = 3.6 Hz, H-2), 5.31 (dd, 1 H, J = 3.6 Hz, 6.6 Hz, H-3), 5.81 (m, 1 H, olefinic CH); ¹³C NMR (100 MHz, CDCl₃): δ 17.56 (CH₃), 20.92, 20.99, 21.12, 28.61, 30.35, 66.38, 67.55, 69.31, 70.11, 71.31, 97.57 (C-1), 115.25, 137.98, 170.14 (C=O), 170.17 (C=O), 170.33 (C=O); mass spectrum (HRMS), [M+Na] m/z calc. for C₁₇H₂₆NaO₈ is 381.1525, found 381.1495.

4-O-(2,3,4-tri-O-acetyl-α-L-rhamnopyranosyl)-butanoicacid (3). To a solution of 2 (0.1 g, 0.279 mmol) in a mixture of CH₂Cl₂-CH₃CN-H₂O (2:2:3) was added NaIO₄ (0.24 g, 1.12 mmol) in a single portion and anhydrous RuCl₃ (0.001 g, 0.005 mmol) and the resulting solution was stirred at ambient temperature under N₂ atmosphere for 2 h. NaIO₄ (0.24 g, 1.12 mmol) was further added and the resulting solution was stirred at
ambient temperature under N\textsubscript{2} atmosphere.\textsuperscript{2} The reaction was monitored by TLC and appeared complete after 18 h.\textsuperscript{36} The reaction mixture was diluted with CH\textsubscript{2}Cl\textsubscript{2} (2.5 mL) and H\textsubscript{2}O (3.8 mL). The product was extracted from the aqueous layer by washing it with CH\textsubscript{2}Cl\textsubscript{2} (0.5 mL x 4). The combined organic layers was dried (anhydrous. Na\textsubscript{2}SO\textsubscript{4}). Excess solvent was removed under reduced pressure and the crude material was purified by silica gel flash column chromatography (13.5 x 3.2 cm). Elution with 1:9:60:130 AcOH-MeOH-EtOAc-hexanes produced 3 as colorless glassy solid: yield 0.103 g (98%); \(R_f\) = 0.57 (1:1:9 AcOH-MeOH-EtOAc-hexanes); \(^1\)H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 1.23 (d, 3 H, \(J\) = 6.4 Hz, C-5 CH\textsubscript{3}), 1.98 (t, 2 H, \(J\) = 6 Hz, O-CH\textsubscript{2}-CH\textsubscript{2}), 2.00 (s, 3 H, COCH\textsubscript{3}), 2.07 (s, 3 H, COCH\textsubscript{3}), 2.16 (s, 3 H, COCH\textsubscript{3}), 2.46 (t, 2 H, \(J\) = 7.2 Hz, O-CH\textsubscript{2}-CH\textsubscript{2}-CH\textsubscript{2}), 3.49 (m, 1 H, O-CH), 3.79 (m, 1 H, O-CH), 3.87 (m, 1 H, H-5), 4.73 (d, 1 H, \(J\) = 1.6 Hz, H-1), 5.08 (t, 1 H, \(J\) = 9.6 Hz, H-4), 5.26 (dd, 1 H, \(J\) = 3.6 Hz, \(J\) = 6.4 Hz, H-3); \(^{13}\)C NMR (100 MHz, CDCl\textsubscript{3}): \(\delta\) 17.52 (CH\textsubscript{3}), 20.86, 20.93, 21.03, 24.59, 24.71, 66.58, 67.09, 69.38, 69.93, 71.14, 97.52 (C-1), 170.22 (C=O), 170.32 (C=O), 178.45 (COOH); mass spectrum (HRMS), [M+Na] \textit{m/z} calc. for C\textsubscript{16}H\textsubscript{24}NaO\textsubscript{10} is 399.1267, found 399.1248.

\textbf{Pentenyl-\(\alpha\)-L-rhamnopyranoside. (4).} To a solution of 2 (0.188g, 0.33 mmol) in MeOH (5 mL), metallic Na (0.015g) was added and the resulting solution was stirred at ambient temperature under N\textsubscript{2} atmosphere.\textsuperscript{37} The reaction was monitored by TLC and appeared complete after 1 hour. Excess solvent was removed under reduced pressure and the crude material was purified by silica gel flash column chromatography (9 x 3 cm). Elution with 2:23 MeOH-CH\textsubscript{2}Cl\textsubscript{2} produced 4 as a colorless solid: yield 0.118 g (quantitative); \(R_f\) = 0.14 (1:19 MeOH-CH\textsubscript{2}Cl\textsubscript{2}); \(^1\)H NMR (600 MHz, CDCl\textsubscript{3}): \(\delta\) 1.30 (d, 3 H, \(J\) = 6.6 Hz, C-5 CH\textsubscript{3}), 1.66 (m, 2 H, O-CH\textsubscript{2}-CH\textsubscript{2}), 2.10 (m, 2 H, O-CH\textsubscript{2}-CH\textsubscript{2}-CH\textsubscript{2}),
3.40 (m, 1 H, O-CH), 3.46 (t, 1 H, J = 9.6 Hz, H-4), 3.62 (m, 1 H, O-CH), 3.66 (m, 1 H, H-5), 3.75 (d, 1 H, J = 7.8 Hz, H-3), 3.92 (s, 1 H, H-2), 4.46 (br. s, 1 H, O-H), 4.54 (br. s, 1 H, O-H), 4.74 (s, 1 H, H-1), 4.86 (br. s, 1 H, O-H), 4.97 (dd, 1 H, J = 1.2 Hz, J = 10.2 Hz, terminal olefinic C-H), 5.02 (dd, 1 H, J = 1.2 Hz, J = 17.1 Hz, terminal olefinic C-H), 5.79 (m, 1 H, olefinic C-H); 13C NMR (100.57 MHz, CDCl3): δ 17.72 (CH3), 28.73, 30.40, 67.19, 71.28, 71.98, 72.95, 99.92 (C-1), 115.25, 138.07; mass spectrum (HRMS), [M+Na] m/z calc. for C11H20NaO5 is 255.1208, found 255.1205.

4-(O-α-L-rhamnopyranosyl)-butanal (5). A solution of 4 (0.04 g, 0.17 mmol) in 1:4 MeOH-CH2Cl2 (10 mL) was cooled to -70 ºC. Oxygen gas was passed through solution for 10 mins. Oxygen gas was then bubbled into the solution at -70 ºC for 15 mins. to ensure complete conversion of the starting compound followed by the passage of O2 gas for 2 mins. The reaction was monitored by TLC. Me2S (0.2 mL) was added and the reaction mixture was allowed to warm to ambient temperature. Excess solvent was removed under reduced pressure and the crude material was purified by silica gel flash column chromatography (11.5 x 1.3 cm). Elution with 2:23 MeOH-CH2Cl2 produced 5 as a colorless glassy solid: yield 0.035 g (87.5%); Rf = 0.16 (1:9 MeOH-CH2Cl2); 1H NMR (600 MHz, CD3OD): δ 1.26 (d, 3 H, J = 6 Hz, C-5 CH3), 1.64 (m, 2 H, O-CH2-CH2), 2.16 (m, 2 H, O-CH2-CH2-CH2), 3.31 (m, 1 H, O-CH), 3.36 (t, 1 H, J = 9.6 Hz, H-4), 3.41 (m, 1 H, O-CH), 3.57 (m, 1 H, H-5), 3.63 (dd, 1 H, J = 9.6 Hz, J = 3 Hz, H-3), 3.78 (d, 1 H, J = 1.2 Hz, H-2), 4.65 (d, 1 H, J = 1.2 Hz, H-1), 9.71 (s, 1 H, CHO); 13C NMR (100 MHz, CD3OD): 18.16 (CH3), 26.12, 34.82, 68.42, 69.91, 72.47, 72.57, 74.12, 101.75 (C-1); mass spectrum (HRMS), [M+Na] m/z calc. for C10H18NaO6 is 257.1001, found 257.1000.
4-(O-α-L-rhamnopyranosyl)-butanal-BSA conjugate (6). Compound 5 (0.005 g, 0.02 mmol) was dissolved in 0.1 M phosphate buffered saline (PBS), pH 7.2 (1 mL) and 15 mg of BSA was added followed by the addition of sodium cyanoborohydride (0.005 g, 0.080 mmol) and the mixture incubated under gentle agitation at 37 °C for 24 h. After 16 h, sodium cyanoborohydride (0.002 g, 0.040 mmol) was further added and the incubation continued. The mixture was filtered using a dialysis tubing with molecular weight cut-off value 10000 Da, with 6-7 changes of PBS at 4 °C. The protein concentration in the conjugate was estimated by the Bradford method using BioRad dye reagent. Absorbances were recorded at 595 nm. A standard curve was prepared with concentrations of BSA in the range of 0.2 mg/mL to 1 mg/mL prepared in 0.1 M PBS, pH 7.2. The absorbance of 6 was recorded following a 10 fold dilution and the concentration of the BSA in 6 was calculated to be 7.27 mg/mL from the standard curve. The concentration of Rha epitope in 6 was determined by phenol-sulfuric acid method. Absorbances were recorded at 480 nm. A standard curve was prepared with concentration of 5 in the range 0.05 mg/mL to 0.8 mg/mL in double distilled water. The absorbance of 6 was recorded after 2 times dilution and the concentration of the Rha epitope in 6 was calculated to be 0.313 mg/mL from the standard curve. The epitope ratio in 6 was calculated to be 11.61.

4-(O-α-L-rhamnopyranosyl)-butanal-KLH conjugate (7). Compound 5 (0.005 g, 0.02 mmol) was dissolved in 0.1 M phosphate buffered saline (PBS), pH 7.2 (1 mL) and 15 mg of KLH was added followed by the addition of sodium cyanoborohydride (0.005 g, 0.08 mmol) and the mixture incubated under gentle agitation at 37 °C for 24 h. After 16 h, sodium cyanoborohydride (0.0025 g, 0.04 mmol) was further added and the
incubation continued. The mixture was filtered using a dialysis tubing with molecular weight cut-off value 10000 Da, with 6-7 changes of PBS at 4 °C.39 The protein concentration in the conjugate was estimated by the Bradford method using BioRad dye reagent.40 Absorbances were recorded at 595 nm. A standard curve was prepared with concentrations of KLH in the range of 0.2 mg/mL to 1 mg/mL prepared in 0.1 M PBS, pH 7.2. The absorbance of 7 was recorded following a 10 fold dilution and the concentration of the KLH in 7 was calculated to be 5.51 mg/mL from the standard curve. The concentration of Rha epitope in 7 was determined by phenol-sulfuric acid method.41 Absorbances were recorded at 480 nm. A standard curve was prepared with concentration of 5 in the range 0.05 mg/mL to 0.4 mg/mL in double distilled water. The absorbance of 7 was recorded after 2 times dilution and the concentration of the Rha epitope in 7 was calculated to be 0.728 mg/mL from the standard curve. The epitope ratio in 7 was calculated to be 259.

4-(O-α-L-Rhamnopyranosyl)-butanal-OVA conjugate (8). Compound 5 (0.005 g, 0.02 mmol) was dissolved in 0.1 M phosphate buffered saline (PBS), pH 7.2 (1 mL) and 15 mg of OVA was added followed by the addition of sodium cyanoborohydride (0.005 g, 0.08 mmol) and the mixture incubated under gentle agitation at 37 °C for 24 h. After 16 h, sodium cyanoborohydride (0.0025 g, 0.04 mmol) was further added and the incubation continued. The mixture was filtered using a dialysis tubing with molecular weight cut-off value 10000 Da, with 6-7 changes of PBS at 4 °C.39 The protein concentration in the conjugate was estimated by the Bradford method using BioRad dye reagent.40 Absorbances were recorded at 595 nm. A standard curve was prepared with concentrations of OVA in the range of 0.2 mg/mL to 1 mg/mL prepared in 0.1 M PBS,
pH 7.2. The absorbance of 8 was recorded following a 10 fold dilution and the concentration of the OVA in 7 was calculated to be 17.45 mg/mL from the standard curve. The concentration of Rha epitope in 8 was determined by phenol-sulfuric acid method.\textsuperscript{41} Absorbances were recorded at 480 nm. A standard curve was prepared with concentration of 5 in the range 0.05 mg/mL to 0.40 mg/mL in double distilled water. The absorbance of 8 was recorded after 2 fold dilution and the concentration of the Rha epitope in 8 was calculated to be 1.12 mg/mL from the standard curve. The epitope ratio in 8 was calculated to be 4.5.

3-\textit{O}-(2-Acetamido-2-deoxy-\textit{\alpha}-D-galactopyranosyl)-L-threonine (11). To a solution of 9 (200 mg, 0.298 mmol) in dry MeOH (10 mL) was added 10\% activated Pd/C (133 mg) followed by the addition of glacial AcOH (1.0 mL). The mixture was stirred under H\textsubscript{2} atmosphere for 3 hours. The reaction mixture was filtered through celite, concentrated and co distilled with toluene to remove excess AcOH.\textsuperscript{43} The byproduct was extracted with 1:1 diethyl ether-toluene (3 x 3 mL) and the residue was dried under vacuum overnight. The residue was dissolved in dry MeOH (2 mL) and NaOMe (1 M) was added until the solution became alkaline (pH 8-9) and stirred at ambient temperature for 2 hours. The reaction mixture was neutralized with Amberlite H\textsuperscript{+} exchange resin, filtered and concentrated.\textsuperscript{44} The residue was then dissolved in deionized water and lyophilized to afford 11 as a white solid: yield = 0.05 g (52\%); \(R_f = 0.08\) (4:1:1 \textit{n}-butanol-AcOH-H\textsubscript{2}O); \textit{\textsuperscript{1}}H NMR (600 MHz, D\textsubscript{2}O) and \textit{\textsuperscript{13}}C NMR (100 MHz, D\textsubscript{2}O) chemical shifts for 11 correlated with the reported literature values;\textsuperscript{45} mass spectrum (ESIMS), [M+Na] \textit{m/z} calc. for C\textsubscript{12}H\textsubscript{22}N\textsubscript{2}NaO\textsubscript{8} is 345.3, found 345.2.
**GalNAc-α-O-Thr-BSA Conjugate (12).** Compound 11 (6 mg, 18.62 μmol) was dissolved in PBS (0.5 mL, pH 7.2). To the solution was added BSA (6 mg) in PBS (1 mL, pH 7.2). The solution was mixed thoroughly and 0.2% aqueous glutaraldehyde solution (0.41 mL) was added and incubated under gentle agitation at ambient temperature for 1 hour. NaBH₃CN in PBS (0.4 mL, pH 7.2) was added to the protein solution and incubated for another 20 minutes at ambient temperatures. The mixture was filtered using a dialysis tubing with molecular weight cut-off value 10000 Da, with 6-7 changes of PBS at 4 °C. The protein concentration in the conjugate was estimated by the Bradford method using BioRad dye reagent. Absorbances were recorded at 595 nm. A standard curve was prepared with concentrations of BSA in the range of 0.2 mg/mL to 1 mg/mL prepared in 0.1 M PBS, pH 7.2. The absorbance of 12 was recorded following a 10 fold dilution and the concentration of the BSA in 12 was calculated to be 2.15 mg/mL from the standard curve.

**Glycopeptides (16) and (17).** The glycopeptides were synthesized by Fmoc strategy on an Omega 396 synthesizer (Advanced ChemTech, Louisville, Ky) using solid phase chemistry. The peptide synthesis was performed by coupling amino acid esters of HOBt using DIC as the coupling agent. A six-fold excess of Nα-Fmoc amino acid esters of HOBt in NMP were used in the synthesis. A 1:1 ratio of amino acid to DIC was used in all the coupling reactions. Deprotection of Nα-Fmoc group was accomplished by treatment with 25% piperidine in dimethylformamide twice; first for 5 minutes and then a second time for 25 minutes. After the synthesis was completed, the peptides were cleaved from the solid support and deprotected using a modified reagent K cocktail consisting of 88% TFA, 3% thioanisole, 5% ethanedithiol, 2% water and 2% phenol. 4 mL of cleavage

---

1synthesized at The Cleveland Clinic.
cocktail was added to the dried peptide-resins in a 15 mL glass vial blanketed with nitrogen. Cleavage was carried out for 2.5 hrs with gentle magnetic stirring. At the end cleavage time, the cocktail mixture was filtered on a Quick-Snap column. The filtrate was collected in 20 mL ice-cold butane ether. The peptides were allowed to precipitate for an hour at -200 C, centrifuged, and washed twice with ice-cold methyl-t-butyl ether. The precipitate was dissolved in 25% acetonitrile and lyophilized to complete dry powder. Quality of peptides was analyzed by analytical reverse phase HPLC and MALDI-TOF-TOF (matrix assisted laser desorption ionization time-of-flight) mass spectrometer, model 4800 from Applied Biosystems (Compound 16 : [M+H] m/z calcd for C\textsubscript{158}H\textsubscript{232}N\textsubscript{39}O\textsubscript{49} = 3459.6783, found 3459.6902; Compound 17 : [M+H] m/z calcd for C\textsubscript{142}H\textsubscript{210}N\textsubscript{39}O\textsubscript{40} = 3101.5519, found 3101.5452).

**Glycopeptide (18).** Compound 16 (.007g, 2.02 \( \mu \)mol) was dissolved in 2 mL of dry methanol and 12 \( \mu \)L of freshly prepared 1 M sodium methoxide was added and the reaction mixture was stirred at ambient temperature under nitrogen atmosphere for 2 h. The reaction mixture was neutralized with solid carbon dioxide. The reaction mixture was concentrated and purified by Bio-Gel (P-2, fine 45-90 \( \mu \)m) size exclusion chromatography using deionized water as solvent. Lyophilization of the elutants afforded 18 as a white powder: yield 0.0055 g, 85%; HR-MALDI-MS calcd for C\textsubscript{146}H\textsubscript{220}N\textsubscript{39}O\textsubscript{43} [M+H] m/z = 3207.614, found 3207.165.

**Glycopeptide (19).** Compound 17 (.0055g, 1.77 \( \mu \)mol) was dissolved in 2 mL of dry methanol and 12 \( \mu \)L of freshly prepared 1 M sodium methoxide was added and the reaction mixture was stirred at ambient temperature under nitrogen atmosphere for 2 h. The reaction mixture was neutralized with solid carbon dioxide. The reaction mixture
was concentrated and purified by Bio-Gel (P-2, fine 45-90 µm) size exclusion chromatography using deionized water as solvent. Lyophilization of the elutants afforded 19 as a white powder: yield 0.0045 g, 85.38%; HR-MALDI-MS calcd for C\textsubscript{136}H\textsubscript{204}N\textsubscript{39}O\textsubscript{37} [M+H] m/z = 2975.520, found 2975.562.

**Immunizations.** Twenty four 6-8 week old female BALB/c mice (The Jackson Laboratory) were used for the immunological study and were grouped into 6 groups A-F containing 4 mice each. Groups A and B were injected (day 0) subcutaneously with 100 µL of phosphate buffer saline (PBS, pH 7.2) and served as the control groups. Groups C and D were subcutaneously injected with a 100 µL equivolume emulsion of Rha-OVA conjugate 8 (prepared in PBS) and TMG, whereas groups E and F were injected subcutaneously with a 100 µL equivolume emulsion of Rha-OVA conjugate 8 (prepared in PBS) and SAS. Groups C and D received 100 µg and groups E and F received 75 µg of Rha-OVA respectively. Mice were boosted (day 25) by subcutaneous injections of 100 µL PBS (groups A and B), 100 µL emulsion of Rha-OVA/TMG (groups C and D; 50 µg of Rha-OVA per mouse) and 100 µL of Rha-OVA/ICF (groups E and F; 100 µg of Rha-OVA per mouse). The mice were bled on day 36 and the collected sera were tested for anti-Rha antibody.

**ELISA for measuring anti-Rha antibody titers.** 96 Well plates (Immulon 4 HBX) were coated with Rha-BSA conjugate 6 (2 µg/mL) in 0.01 M PBS (pH 7.2) and incubated overnight at 4 °C. The plates were washed 5 times with PBS containing 0.1% Tween-20. Blocking was achieved by incubating the plates for 1 h at room temperature with BSA in 0.01 M PBS (1mg/mL). The plates were then washed 5 times and incubated for 1 h with serum dilutions in PBS. Unconjugated antibody in the serum was removed
by washing and the plates were incubated for 1 h at room temperature with Horseradish Peroxidase (HRP) goat anti-mouse IgG + IgM (Jackson Immunoresearch Laboratories) diluted 2000 times in PBS/BSA. The plates were washed and TMB (3,3',5,5'-tetramethylbenzidine) One component HRP microwell substrate (Bio FX, Owings Mills, MD) was added and allowed to react for 10 mins. Absorbances were recorded at 620 nm and were plotted against $\log_{10} 1/$serum dilution.

**ELISA for comparing anti-Rha antibody attachment capacity of Rha-BSA and Rha-KLH.** Half of a 96 well plate (Immulon 4 HBX) was coated with Rha-BSA conjugate 6 (2 µg/mL) in 0.01 M PBS (pH 7.2) and the other half was coated with Rha-KLH conjugate 7 (2 µg/mL) in 0.01 M PBS (pH 7.2) and incubated overnight at 4 °C. The ELISA was then performed as described above.

**Vaccinations.** Vaccine challenge was done on day 52. Two separate emulsions were prepared by mixing 1.1 mg of glycopeptide 18 (Rha-YAF-Tn) in 400 µL of 0.01 M PBS (pH 7.2) with 400 µL of Complete Freuend’s Adjuvant (Rha-Vacc) and 1.1 mg of glycopeptide 19 (YAF-Tn) in 400 µL of 0.01 M PBS (pH 7.2) with 400 µL of CFA (Vacc). Groups A, C and E were challenged with Rha-Vacc (50 µL emulsion subcutaneous injections containing 80 µg of Rha-YAF-Tn per mouse). Groups B, D and F were challenged with Vacc (50 µL emulsion injections containing 80 µg of YAF-Tn per mouse). Mice were boosted with the challenge on day 81. Groups A, C and E were subcutaneously injected with 50 µL emulsion of Rha-YAF-Tn/ICF (80 µg of Rha-YAF-Tn per mouse) where as groups B, D and F were subcutaneously injected with 50 µL emulsion of YAF-Tn/ICF (80 µg YAF-Tn per mouse). Mice were bled on day 91 and the collected sera were tested for anti-Tn antibodies.
**ELISA for measuring anti-Tn antibody titers.** 96 well plates (Immulon 4 HBX) were coated with Tn-BSA conjugate 12 (2 μg/mL) in 0.01 M PBS (pH 7.2) and incubated overnight at 4 °C. The ELISA was continued as described above.

**ELISA for competitive binding with free Tn.** A 96 well plate (Immulon 4 HBX) was coated with Tn-BSA conjugate 12 (2 μg/mL) in 0.01 M PBS (pH 7.2) and incubated overnight at 4 °C. The plate was washed 5 times with PBS containing 0.1% Tween-20. Blocking was achieved by incubating the plate for 1 h at room temperature with BSA in 0.01 M PBS (1mg/mL). The plate was then washed 5 times and incubated for 1 h with serum dilutions of 1/100 in PBS with or without prior mixing with varying concentrations of free Tn (compound 11) from 0, 10^{-5}, 10^{-4}, 10^{-3} M in 0.01 M PBS (pH 7.2). Unconjugated antibody in the serum was removed by washing and the plate was incubated for 1 h at room temperature with Horseradish Peroxidase (HRP) goat anti-mouse IgG + IgM (secondary antibody) diluted 2000 times in PBS/BSA or HPR goat anti-mouse IgG diluted to 1000 times in PBS/BSA. The plate was washed and TMB 1 component HRP microwell substrate was added and allowed to react for 10 mins. Absorbances were recorded at 620 nm and was plotted against log_{10} [1/free Tn concentration].

**T-Cell Proliferation Study.**

**Immunization.** Two female BALB/c mice (The Jackson Laboratory) were used for this experiment. Mouse 1 was immunized (day 0) by a subcutaneous injection of 100 μL emulsion of YAF-Tn/CFA (12.8 μg) and mouse 2 was injected subcutaneously with 100 μL emulsion of Rha-YAF-Tn/CFA (12.8 μg).
**Preparation of anti-Rha Antibodies.** Sera was pooled from the PBS and Rha-OVA immunized mice bleed on day 36 of the previous experiment. Antibody fractions from each pool were prepared by precipitation at 40% saturation of ammonium sulfate. The mixtures were incubated overnight and centrifuged at 10000 g for 10 minutes and then resuspended in 0.5 mL water. The antibody solutions were concentrated and buffer was changed twice with PBS using an Ultrafree 0.5 centrifugal filter device (Millipore, Billerica, MA) having a molecular cut off of 50000 D. Aborbances of the antibody solutions were recorded at 280 nm to calculate the concentrations and the normal and anti-Rha antibody solutions were diluted to 0.5 mg/mL.

**Preparation of Spleen Cell Suspensions and Assay Setup.** On day 7, the mice were sacrificed and the spleens were removed and placed separately in freshly prepared spleen cell culture medium (5 mL). Single cell suspensions were prepared using two separate sterile glass homogenizers. The cells were washed three times with culture medium and brought to 2.5 x 10^6 cells/mL. 100 μL of the spleen cell suspensions were added to 96 well plates (2.5x10^5 cells per well) followed by the addition of normal serum antibodies or anti-Rha serum antibodies (5 μg per well). Control wells were deprived of the antibody treatment. Next vaccine solutions (Rha-YAF-Tn or YAF-Tn) prepared in 0.01 M PBS (pH 7.2) were added into the wells in the concentration range of 0.01 μM – 10 μM. The plates were incubated at 37 °C for 5 days. On day 5 the cells were pulsed with [3H]-Thymidine (40 μCi/mL, 25 μL per well) and incubated overnight at 37 °C. The cells were harvested on glass-fiber filters and incorporation was determined by measurements on a Top Count scintillation counter (Packard, Downers Grove, IL).
2.6 References.


(33) Oyelaran, O.; McShane, L. M.; Dodd, L.; Gildersleeve, J. C. *J. Proteome Res.* **2009**, 8, 4301-4310.


(40) Bio-Rad Manufactures manual.


Chapter 3

Synthesis and Immunological Evaluation of a MUC1 Glycopeptide Incorporated into L-Rhamnose Displaying Liposomes

3.1 Introduction

Tumor-associated carbohydrate antigens (TACAs) have been extensively used as markers for cancer detection and for monitoring the advancement of the disease. TACAs on tumor cells are over expressed demonstrating modification in structure and distribution relative to normal cells which renders them as excellent targets for anti-cancer vaccines. Numerous TACAs have been identified from the glycoprotein MUC1 obtained from cancer cells of epithelial origin. Some of these antigens include the Thomsen-Friedenreich (TF), Tn, STn as well as α-2,6-sialyl-TF and α-2,3-sialyl-TF antigens. A recent finding made by Finn and coworkers reported that MUC1 variable number tandem repeats (VNTRs) containing TACAs were more potent at breaking self tolerance in MUC1 transgenic mice than the unglycosylated VNTR. According to the authors the glycopeptide is a more ‘foreign’ like epitope in comparison to the unglycosylated MUC1 which is more ‘self’ like. Vaccination with TACA-containing MUC1 was successful in generating specific antibodies for the glycopeptides and boosting previously suppressed MUC1 specific T-cell responses. Further, they identified under review: Bioconjugate Chem.
a population of dendritic cells (DCs) that display the VNTRs bearing the GS(GalNAc-O-Thr) epitope on MHC class-II molecules.

Several approaches in which a MUC1 glycopeptide could be formulated into an anti-cancer vaccine with improved immunogenicity were considered. We believe that an interesting approach would be to incorporate xenoantigens onto the target antigen. The xenoantigens would complex naturally occurring cognate antibodies which would facilitate uptake of the target antigen by antigen presenting cells (APCs). Past studies have focused on the installation of α-Gal epitopes on vaccine constructs to boost the immune response with promising results,\(^6\) since human serum is abundant in anti-α-Gal antibodies.\(^7\) However, recent studies by both Bovin and Gildersleeve on human serum have revealed that even more abundant human natural anti-carbohydrate antibodies are present against the xenoantigen L-rhamnose (Rha).\(^8\) Further, anti-Rha antibodies can be generated in non-transgenic mice in contrast to anti-α-Gal antibodies,\(^9,10\) In this study we have explored the Rha epitope as a noncovalently-linked ligand displayed on the surface of a liposomal vaccine for enhancing the immune response against a tumor-associated glycopeptide fragment of MUC1 in mice producing anti-Rha antibodies.\(^10\)

A successful anti-cancer vaccine will modulate the efficient delivery of the antigens to the APCs.\(^11\) Liposomes have been effective in the delivery of viral, bacterial and tumor antigens to APCs\(^12\) and have the advantage of protecting peptide-based antigens against proteolysis \emph{in vivo}. Liposomes also generate multivalency in the vaccine, promoting numerous antigen-antibody interactions facilitating opsonization of the vaccine. Additional mechanisms may also involve Rha epitopes interacting with endogenous B-cell receptors (BCRs) to uptake and present antigens on B-cells.\(^7a\)
Vaccine immunogenicity can also be improved by the introduction of an adjuvant. Toll-like receptor ligands (TLRL), for example, have potent adjuvant activity.\textsuperscript{13} Therefore we incorporated the TLR-2 ligand, Pam\textsubscript{3}Cys, into our vaccine synthesis to serve the dual role of adjuvant and lipid anchor to a liposome. Further, we envisioned a liposome capable of displaying Rha epitopes, the later designed to bind endogenous anti-Rha antibodies in human serum. The resulting Ig-vaccine complex would then be taken up by APCs (Figure 1).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic representation of Fc-Fc\textgamma interaction in the \textit{in vivo} generated immune complex leading to enhanced antigen uptake by APCs, e.g. dendritic cells.}
\end{figure}

3.2 Results and Discussion

3.2.1 Synthesis of Rha-TEG-Cholesterol.

To validate our hypothesis we first synthesized an L-Rha-cholesterol conjugate using tetraethylene glycol (TEG) as a linker which is reported to facilitate the formation
of small-sized homogenous liposomes and allows good binding interaction of the head
group$^{14,15}$ (Scheme 1A). Cholesterol tetraethylene glycol 1$^{15}$ was glycosylated with
peracetyl rhamnose in presence of boron trifluoride etherate to afford peracetyl
rhamnose-TEG-cholesterol 2 (32%) which was deacetylated under Zemplén conditions to
generate Rha-TEG-cholesterol 3 (85%).$^{10}$ In a liposomal formulation the cholesterol
fragment in 3 will anchor the Rha epitopes on the surface of the liposomes thereby
facilitating anti-Rha antibody binding.

### 3.2.2 Synthesis of Alkyne Functionalized Pam$_3$Cys.

Our next target was to synthesize a functionalized Toll-like receptor ligand
(TLRL) which will serve the purpose of an immunoadjuvant for our vaccine candidate
and also anchor the MUC1-Tn conjugate on the surface of the liposome. We focused our
attention towards the synthesis of a conjugable form of the lipopeptide $S-[(R)-2,3$
-dipalmitoyloxy-propyl]-N-palmitoyl-(R)-cysteine (Pam$_3$Cys) which has been identified as
a TLR-2 agonist and has been successfully used in the past as an immunoadjuvant in the
design of three component vaccines.$^{16}$

We planned to incorporate an alkyne functionality through an amide linkage at the
C-terminal of Pam$_3$Cys which can be conjugated to an azide moiety on a MUC1-Tn
construct by a simple copper-catalyzed ‘click reaction’. To synthesize a conjugatable
Pam$_3$Cys alkyne (Scheme 1B), first the tert-butyl protection of O-palmitoylated Fmoc L-
cystine tert-butyl ester 4$^{17}$ was cleaved by a brief treatment with trifluoroacetic acid
(TFA). The free acid was coupled with propargyl amine in the presence of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxy-
benzotriazole (HOBt) and N,N-Diisopropylethylamine (DIPEA) to yield 5 (66% over 2 steps).\textsuperscript{17,18} Finally the Fmoc group in compound 5 was removed by treatment with a mixture of acetonitrile–dichloromethane–diethyl amine (2:1:2) followed by subsequent palmitoylation by coupling with palmitic acid, PyBOP, HOBt and DIPEA to afford our target alkyne functionalized Pam\textsubscript{3}Cys amide derivative 6 (80% over 2 steps).\textsuperscript{18,19}

**Scheme 1.** Synthesis of Rha-TEG-Cholesterol and alkyne functionalized Pam\textsubscript{3}Cys.\textsuperscript{a}

\textsuperscript{a}Reagents and conditions: (A) (a) peracetyl L-rhamnose, BF\textsubscript{3}.OEt\textsubscript{2}, CH\textsubscript{2}Cl\textsubscript{2}, 0°C r.t., 18 h, 32%; (b) NaOMe, MeOH, r.t., 1 h, 85% [TEG= CH\textsubscript{2}CH\textsubscript{2}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{3}]; (B) (a) (i) TFA, r.t., 1 h; (ii) propargyl amine, PyBOP, HOBt, DIPEA, 4 Å mol. sieves, CH\textsubscript{2}Cl\textsubscript{2}, r.t.,
4 h, 66% (2 steps); (b) (i) CH$_3$CN-CH$_2$Cl$_2$-Et$_2$NH (2:1:2), r.t., 2 h; (ii) PamOH, PyBOP, HOBt, DIPEA, CH$_2$Cl$_2$, 4 Å mol. sieves, r.t., 4 h, 80% (2 steps) [Pam = CH$_3$(CH$_2$)$_{14}$CO].

3.2.3 Synthesis of Pam$_3$Cys-MUC1-Tn Conjugate.

For synthesis of the MUC1-Tn construct, we targeted the 20-amino acid tandem repeat of MUC1 which included the GS(GalNAc-O-T)A epitope identified by Finn et al.$^5$ We planned to install a terminal azido group into the glycopeptide which would make the ‘click’ conjugation to the Pam$_3$Cys alkyne feasible. The glycopeptide azide was synthesized by Fmoc strategy on an Omega 396 synthesizer (Advanced ChemTech, Louisville, KY) starting from preloaded Fmoc-L-Ala Wang resin using solid-phase chemistry (Scheme 2). The peptide synthesis was performed by coupling amino acid esters of HOBt usingDIC as the coupling agent. A 6-fold excess of N$^{\alpha}$-Fmoc amino acid esters of HOBt in NMP were used in the synthesis. A 1:1 ratio of amino acid to DIC was used in all the coupling reactions. Deprotection of N$^{\alpha}$-Fmoc group was accomplished by treatment with piperidine in DMF. After the synthesis was complete, the peptide was cleaved from the solid support and deprotected using a modified reagent K cocktail consisting of TFA-thioanisole-ethanedithiol-water-phenol (88:3:5:2:2). The cocktail mixture was filtered through a Quick Snap column, purified by C18 reverse phase HPLC and lyophilized to afford 9. The acetyl groups in compound 9 were deprotected by treatment with 6 mM sodium methoxide in methanol.$^{20}$ The product was purified by Bio-Gel (P-2, fine 45-90 μm) size exclusion chromatography using deionized water as solvent. Lyophilization of the elutants afforded 10 (100%) as a white powder.
Our next challenge was the conjugation of alkyne functionalized Pam\textsubscript{3}Cys derivative 6 with the glycopeptide azide 10. Our initial efforts to conjugate the alkyne and the azide fragments via a copper catalyzed ‘click’ reaction using copper sulfate pentahydrate and sodium ascorbate failed. To counteract this problem we used a Cu (I) stabilizing agent tris [(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA) which is known to increase the yield of copper catalyzed click reactions significantly by stabilizing the in situ generated Cu(I) intermediate.\textsuperscript{21}

Conjugation of 10 (1 equiv.) with 6 (3 equiv.) in presence of copper sulfate pentahydrate (12 equiv.), TBTA (12 equiv.) and sodium ascorbate (12 equiv.) in H\textsubscript{2}O-MeOH-THF (1:1:2) as solvent at ambient temperatures afforded our target Pam\textsubscript{3}Cys-MUC1-Tn conjugate 11 after 40 h. Compound 11 was purified by LH20 using MeOH-dichloromethane (1:1) as solvent. The elutants were lyophilized to afford 11 as a white solid.
Scheme 2. Synthesis of Pam$_3$Cys-MUC-1-Tn Conjugate.$^a$

$^a$Reagents and conditions: (a) (i) 25% piperidine, DMF, r.t. 30 min; (ii) HOBr, DIC, NMP, FmocNH-Ser(Ot-Bu)-OH, repeat steps with T, V, G, H, A, P, P, A, T(Ac$_3$GalNAc), S, G, P, A, P, R, T, D, P, 5-azido pentanoic acid; (b) 88% TFA, 3% thioanisole, 5% ethanedithiol, 2% water and 2% phenol; (c) NaOMe, MeOH, r.t., 2 h, 100%; (d) 6, CuSO$_4$.5H$_2$O, Na-ascorbate, TBTA, water-methanol-THF (1:1:2), r.t., 40 h, (100%).

3.2.4 Liposome Formulation and Characterization.

For the preparation of the liposomes we used 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). The liposomes were formulated by the extrusion method in a total lipid concentration of 30 mM.$^{22}$ To test specific antibody binding to the surface-displayed Rha and glycosylated MUC1 epitopes we prepared three batches of the liposomes. Batch 1 was our positive batch of the liposomes and was formulated with Rha-TEG-cholesterol (10%), Pam$_3$Cys-MUC1-Tn 11 (0.69 μM), DPPC (80%) and cholesterol (10%). Batch 2 lacked the surface displayed Rha epitopes and was formulated with Pam$_3$Cys-MUC1-Tn 11 (0.69 μM), DPPC (80%) and cholesterol (20%). Batch 3
was our control and was formulated with only DPPC (80%) and cholesterol (20%). Particle size can be an important modulator of the immune response for neutral liposomes. Therefore, the homogeneity, stability as well as size characterization of the liposomes were evaluated by scanning electron microscope (SEM) imaging (Figure 2) and dynamic light scatter scattering (DLS) measurements (Figure 3). All batches of liposomes were found to be stable at 4 °C for 2 days and were around 100 nm in diameter. An antibody binding study showed positive binding of the Batch 1 liposomes with both our previously generated anti-Rha antibodies as well as mouse anti-human-MUC1(CD 227, BD Biosciences, San Jose, CA) antibodies using FITC goat anti-mouse IgG/IgM secondary antibodies and fluorescence imaging of the coated liposomes (Figure 4). The binding assay proved that the Rha and the MUC1-Tn epitopes of each conjugate were displayed on the surface of the liposomes. No such antibody binding (both anti-Rha and anti-human MUC1) was observed for the Batch 1 liposomes. Batch 2 liposomes only demonstrated mouse anti-human-MUC1 antibody binding (not shown).
Figure 2. Size characterization of liposomes: SEM images at 5 kV acceleration voltage (A) Batch 1 liposomes under 50000 X magnification, (B) Batch 1 liposomes under 250000 X magnification.
Figure 3: DLS Measurements at 1/1000 Dilution (A) Batch 1 liposomes, (B) Batch 2 liposomes, (C) Buffer (HEPES pH = 7.4).
Figure 4. Fluorescence microscope images with Batch 1 liposomes under 60 X magnification (A) Images with control antibodies (antibodies isolated from pre-immunization serum) 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} images: brightfield, FITC and overlay; (B) Images with anti-Rha antibodies, 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} images: brightfield, FITC and overlay; (C) Images with anti-MUC 1 antibodies, 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} images: brightfield, FITC and overlay.

3.2.5 Synthesis of Rha-Ficoll Conjugate.

To evaluate the efficacy of our vaccine in a mouse model we generated mice with anti-Rha antibodies using a Rha-Ficoll immunization. The naive animals do not contain significant amounts of anti-Rha antibodies.\textsuperscript{10} We were interested in immunizing the mice with a Rha conjugate which would show a minimal T-dependant immune response. We focused our attention on the synthesis of a Rha-Ficoll conjugate since the carrier, Ficoll, has been reported to be excellent for generating a T-independent immune response.\textsuperscript{24} For
the synthesis of the Rha-Ficoll conjugate 16 (Scheme 3), tetraacetyl rhamnopyranoside 12 was glycosylated with 2-azido ethanol in presence of boron trifluoride etherate to generate the peracetylated rhamnose 2-azidoethyl glycoside 13 (83 %).\textsuperscript{25,26} Deacetylation of 13 under Zemplén conditions afforded rhamnose 2-azidoethyl glycoside 14 (86 %) which was reduced by treatment with Pd-charcoal under H\textsubscript{2} atmosphere to furnish the rhamnose 2-aminoethyl glycoside 15 (quantitative).\textsuperscript{27,28} Commercially available Ficoll 400 was oxidized by sodium periodate in acetate buffer (pH 4.7) followed by conjugation with 15 via reductive amination using sodium cyanoborohydride in borate buffer (pH 8.0) to produce Rha-Ficoll conjugate 16.\textsuperscript{29} The conjugate was purified by filtration through a dialysis tubing with a molecular weight cut off value of 10000 Da. The epitope ratio of 16 was calculated to be 9.44 Rha/Ficoll molecule by hydrolysis of 16 followed by derivatization with 4-amino-N-[2-(diethylamino)ethyl] benzamide (DEAEAB) and comparison of the UV-HPLC peak area with standard curve obtained from DEAEAB derivative of 14 by the methods described by Dalpathado and coworkers.\textsuperscript{30}
**Scheme 3.** Synthesis of Rha-Ficoll Conjugate.\(^a\)

\[
\begin{align*}
12 & \xrightarrow{a} 13 & 14 \\
Ficoll 400 & \xrightarrow{d} \text{oxidized Ficoll 400} & \xrightarrow{e} \text{Rha-Ficoll}
\end{align*}
\]

\(^a\)Reagents and conditions: (a) 2-azidoethanol, BF\(_3\)-OEt\(_2\), CH\(_2\)Cl\(_2\), 0 °C - r.t., 12 h, 83%; 
(b) NaOMe / MeOH, r.t., 2 h, 86%; (c) H\(_2\) / Pd-C / MeOH, 12 h, quantitative; (d) NaIO\(_4\), acetate buffer, 2 h; (e) 15, borate buffer, Na(CN)BH\(_3\), 12 h, epitope ratio = 9.44 (Rha per Ficoll molecule).

3.3 Immunological Results and Discussion.

3.3.1 Comparison of Anti-Rha Antibody Titers generated against Rha-Ficoll and Rha-OVA.

The first goal of the immunological study was to immunize mice with the Rha-Ficoll conjugate and elicit anti-Rha antibody titers in order to have a model animal that could simulate the naturally occurring anti-Rha antibodies found in human serum. Two groups of five female BALB/c mice each were immunized on day 0 with Rha-Ficoll/Alum adjuvant (group A) or Rha-OVA/complete Freund’s adjuvant (CFA) (group B). The mice were boosted three more times on days 14, 28 and 42 with either Rha-
Ficoll/Alum (group A) or Rha- OVA/incomplete Freund’s adjuvant (ICF) (group B). Sera was collected separately from groups A and B after the 3rd boost and the anti-Rha antibodies in the sera from the two groups of mice were isotyped by screening against Rha-BSA (Figure 5). The results demonstrated the anti-Rha antibody titers in the Rha-OVA immunized mice groups were 100-fold higher than those from the Rha-Ficoll immunized mice. However, the isotype distribution confirmed that Rha-Ficoll and Rha-OVA produced the anti-Rha antibody subclasses in different proportions. Anti-Rha antibodies from Rha-OVA immunization were dominated by IgG1 (65 %) while Rha-Ficoll immunization produced antibodies which comprised mainly of IgG3 (48 %) and IgM (25%). IgG1 and IgG3 act similarly in that they both stimulate high affinity FcγRI receptors which trigger responses from macrophages. However, IgG1 also stimulates low affinity FcγRIIB receptors which inhibit the signals from the FcγRI and B cell receptors thereby diminishing B-cell activity and immunogenicity of macrophages. On the other hand the anti-Rha antibody isotypes from Rha-Ficoll immunized mice serum resemble those naturally occurring in the human serum which is presumed to be generated through a T-independent response. Thus, we anticipated that initial immunization with Rha-Ficoll prior to the vaccine challenge will be a more realistic animal model for the human.
3.3.2 T-Cell Proliferation Study.

T-cell proliferation assays were performed to determine if the combination of anti-Rha antibodies and Rha-modified liposomal vaccine would potentiate a T-cell proliferative response. In the first part of the study we optimized the proliferation assay conditions. BALB/c mice were immunized (day 0) and boosted (days 14, 28 and 42) with 100 μL emulsions of MUC1-Tn 10/Sigma adjuvant system (SAS) (50 μg peptide per mouse, each injection). The mice were sacrificed (day 49), the spleens were removed and single cell suspensions were prepared and incubated with MUC1-Tn (8.8 x 10^{-3} – 1.1 μg/mL) alone or with syngeneic bone marrow dendritic cells (DCs) previously pulsed with the same doses of antigen. We observed that DCs enhanced proliferation (Figure 6), as had been previously observed for C57BL/6 mice.\textsuperscript{5a} To test the ability of anti-Rha antibodies to enhance antigen presentation, spleen cells from BALB/c mice immunized as above were prepared. DCs from BALB/c bone marrow were pulsed liposomes at antigen
concentrations of $8.8 \times 10^{-3} - 0.22 \, \mu g/mL$ together with antibodies isolated from either Rha-Ficoll or Rha-OVA immunized mice or nonimmune mice. The pulsed DCs were added to the spleen cells and proliferation assessed after 3 days. The spleen T-cells proliferated better in presence of anti-Rha antibodies (from both Rha-Ficoll and Rha-OVA immunized mice serum) than in the presence of control serum antibodies over the antigen concentration range of $8.8 \times 10^{-3} - 0.22 \, \mu g/mL$ (Figure 7A). Also the T-cell proliferation was higher in presence of anti-Rha antibodies generated against Rha-Ficoll (6328, 6045 and 6521 counts per minute (cpm) at antigen concentrations of $8.8 \times 10^{-3}$, 0.044 and 0.22 μg/mL) than those against Rha-OVA (5018, 4926 and 4880 cpm at antigen concentrations of $8.8 \times 10^{-3}$, 0.044 and 0.22 μg/mL), even though the titer of anti-Rha antibodies was higher in the serum of Rha-OVA immunized mice. The results strongly suggest that the Rha-modified antigen was more effectively internalized and presented by the APCs in the presence of anti-Rha antibodies, particularly those less-inhibitory isotypes characteristic of natural antibodies and generated by Rha-Ficoll immunization. Therefore, we concluded that BALB/c mice in which anti-Rha antibodies are generated with Rha-Ficoll 16 immunization will be an appropriate model for the immunogenicity of the Rha-conjugated MUC1-Tn liposomes.
Figure 6. T-Cell Proliferation in BALB/c mice with MUC1-Tn 10 Peptide in Presence and Absence of Dendritic Cells.
Figure 7A. T-cell proliferation measured by $[^3]H$thymidine incorporation in T-cells from mice spleen cells primed with MUC1-Tn 10 and challenged with Pam$_3$Cys-MUC1-Tn 11 + Rha liposomes in the presence of anti-Rha antibodies (abs) or control abs [anti Rha(OVA) and anti Rha(Ficoll) abs are the antibodies isolated from the serum of Rha-OVA and Rha-Ficoll immunized mice respectively].
Figure 7B. Stepwise immunization plan. Groups A1, A2, B1 and B2 each represents four groups of female BALB/c mice. Stage I: groups A2 and B2 were immunized with Rha-Ficoll/Alum whereas groups A1 and B1 were non-immunized. Stage II: Vaccination; groups A1 and A2 vaccinated and boosted with Pam₃Cys-MUC1-Tn liposomes whereas groups B1 and B2 were vaccinated with Pam₃Cys-MUC1-Tn + Rha liposomes.
Figure 7C. Group average of anti-Rha antibody titers after 4th boost with Rha-Ficoll/Alum.
Figure 7D. Group average of anti-MUC1-Tn antibody titers after 1st boost with Pam₃Cys-MUC1-Tn liposomes or Pam₃Cys-MUC1-Tn + Rha liposomes.

3.3.3 Anti-Rha Antibody Generation.

Four groups of five female BALB/c mice each (groups A1, A2, B1 and B2) (6-8 weeks old) were used for this vaccination study. Groups A2 and B2 were immunized (day 0) and boosted (days 14, 28, 42 and 56) with 100 μL equivolume emulsion of Rha-Ficoll (prepared in PBS) and alum adjuvant. Groups A1 and B1 served as the control groups and were deprived of the Rha-Ficoll/Alum immunization (Figure 7B). The mice were bled on day 66 and the ELISA performed by screening the sera from the different groups against Rha-BSA showed that the anti-Rha antibody titers in groups A2 and B2 were 25-fold higher than the control groups (Figure 7C). Thus immunization with Rha-Ficoll confirmed the generation of anti-Rha antibodies in the experimental groups of mice.
3.3.4 Vaccination with Rha and non-Rha-displaying MUC1-Tn Liposomes.

Two separate liposomal formulations were prepared. The first contained DPPC, cholesterol and Pam\textsubscript{3}Cys-MUC1-Tn 11 (2 nmol) (Pam\textsubscript{3}Cys-MUC1-Tn liposomes) and the second contained DPPC, cholesterol, Rha-TEG-cholesterol 3 and Pam\textsubscript{3}Cys-MUC1-Tn 11 (2 nmol) (Pam\textsubscript{3}Cys-MUC1-Tn + Rha liposomes). In both formulations the total lipid concentration was 30 mmol. The vaccination was performed on day 77. Groups A1 and A2 were given 100 μL subcutaneous injections of the Pam\textsubscript{3}Cys-MUC1-Tn liposomes (2 nmol of peptide per mouse) and groups B1 and B2 were given 100 μL subcutaneous injections of the Pam\textsubscript{3}Cys-MUC1-Tn + Rha liposome (2 nmol peptide per mouse). The mice were boosted on day 91 with either the Pam\textsubscript{3}Cys-MUC1-Tn liposome (groups A1 and A2, 2 nmol peptide per mouse) or the Pam\textsubscript{3}Cys-MUC1-Tn + Rha liposome (groups B1 and B2). The mice were bled on day 101 and the sera evaluated for anti-MUC1-Tn and anti-Tn antibodies (Figure 7D).

Anti-MUC1-Tn antibody titers were determined by screening the sera against the MUC1-Tn conjugate 10 (Figure 7D). The data showed that groups A1, A2 and B1 had similar titers at 1/25, 1/50, 1/100 and 1/200 serum dilutions. This proved that prior immunization with Rha-Ficoll does not affect the response to a non-Rha conjugated vaccine (groups A1 and A2). In addition the Rha epitopes on the vaccine do not alter the inherent immunogenicity of the MUC1-Tn epitopes on the vaccine (groups A1 and B1). The anti-MUC1-Tn titers for group B2 showed an 8-fold increase compared to groups A1, A2 and B1 which was mediated by the anti-Rha antibody dependant antigen uptake. The anti-MUC1-Tn antibodies from each group were isotyped (Figure 8) which showed
that group B2 showed an increase in IgG1, IgG2a, IgG2b, and IgM isotypes relative to
the other 3 groups. The specificity of the antibodies towards MUC1-Tn antigen was
determined by a competitive binding experiment (Figure 9A). Serum from every group at
1/100 dilution was incubated with the MUC1-Tn conjugate 10 at concentrations of 0, 10^{-5}, 10^{-4}, and 10^{-3} M in 0.01 M PBS prior to addition in the ELISA plates coated with the
conjugate 10. The absorbances decreased uniformly with increasing concentrations of
free MUC1-Tn in the serum dilutions for each group. As an example, the absorbances at
620 nm for the serum dilution of group B2 at free MUC1-Tn concentrations of 0, 10^{-5}, 10^{-4}
and 10^{-3} M were 0.790, 0.601, 0.577 and 0.512 respectively. These results confirmed
the specificity of the anti-MUC1-Tn antibodies towards the respective antigen.

![Figure 8](image_url)

**Figure 8.** Anti-MUC1-Tn Antibody Isotype Titers after 1st boost with Pam$_3$Cys-MUC1-
Tn liposomes or Pam$_3$Cys-MUC1-Tn + Rha liposomes at 1/50 Serum Dilutions.
The antibody titer generated solely against the TACA was determined by screening serum dilutions from every group against a Tn-BSA conjugate (Figure 9B). Here also we observed a >8-fold increase in the anti-Tn antibody titers for group B2 in comparison to groups A1, A2 and B1 which was again attributed to the better uptake of the antigen in presence of the anti-Rha antibodies by an antibody-dependant antigen-uptake mechanism. An interesting finding of this study was that the anti-MUC1-Tn antibody titers were higher than the corresponding anti-Tn antibody titers for the same serum dilutions for every group, assuming similar levels of antigen on the plate. As an example, for the group B2 the absorbances at 620 nm for the anti-MUC1-Tn and the anti-Tn antibody titers at 1/100 serum dilutions were 0.922 and 0.509 respectively. This observation demonstrated that the Rha-displaying MUC1-Tn vaccine successfully generated antibodies against both the MUC1 peptide and the TACA.

The ability of the anti-MUC1-Tn antibodies in the vaccinated mice serum to bind to MUC1-Tn on human tumor cells was demonstrated with U266 human leukemia cells. These cells express MUC1 on their surface as shown by binding with mouse anti-human MUC1 antibodies (CD 227, BD Biosciences, San Jose, CA) (Figure 10A). Serum from group B2 mice also recognized the MUC1 on the tumor cells with similar efficiency relative to background (Figure 10B). This demonstrates that the antibodies generated against the glycopeptide recognize the MUC1 protein in its native environment.
9A.

![Graph A](image1)

**Figure 9.** A) Competitive binding of anti-MUC1-Tn antibodies with bound MUC1-Tn in presence of free MUC1-Tn. B) Group average of anti-Tn antibody titer after 1st boost with Pam3Cys-MUC1-Tn liposomes or Pam3Cys-MUC1-Tn + Rha liposomes.
Figure 10. Binding of anti-MUC1-Tn antibodies to human leukemia U266 cells. (A) 2nd antibody alone, ...; with mouse anti-human MUC1 antibodies, ... (B) with 1/5 dilution of non-immunized mouse serum, ...; with 1/5 dilution of group B2 mice serum, ...

3.4 Conclusion.

In conclusion a fully synthetic two component vaccine containing the lipopeptide adjuvant Pam₃Cys appended to a 20-amino acid MUC1 peptide containing the TACA GalNAc-O-Thr (Tn) was synthesized and was successfully formulated into liposomes along with an Rha cholesterol conjugate. The resulting liposomes were homogenous in size and were stable at 4 °C for two days. Binding studies with both anti-Rha and mouse anti-human MUC1 antibodies revealed that the Rha and the MUC1 glycopeptide epitopes were surface displayed on the liposomes. A Rha-Ficoll conjugate was synthesized for the generation of anti-Rha antibodies in mice. The in vitro proliferation of MUC1-Tn primed mice spleen T-cells showed increased proliferation to Rha-liposomes in the presence of antibodies from Rha-Ficoll immunized mice relative to nonimmune mice. Vaccination studies with Rha- and non-Rha- displaying MUC1-Tn liposomes in mice either non-
immunized or immunized with Rha-Ficoll illustrated that anti-MUC1-Tn and anti-Tn antibodies were >8-fold higher in the groups of mice previously immunized with Rha-Ficoll and later vaccinated with the Pam3Cys-MUC1-Tn + Rha liposomes. The anti-MUC1-Tn antibodies in the serum of the vaccinated mice recognized the aberrant MUC1 on human leukemia U266 cells. Overall this vaccine successfully triggered both T cell and humoral immunity enhanced by anti-Rha antibody dependant antigen uptake.
3.5 Experimental Section.

**General Methods.** All fine chemicals such as L-rhamnose, cholesterol, \( p \)-toluene sulfonyl chloride, copper sulfate etc. and anhydrous solvents such as anhydrous methanol were purchased from Acros Organics. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Boron trifluoride etherate was from Aldrich. The chemicals were used without further purification. All solvents were obtained from Fisher and used as received except dichloromethane, which was dried and distilled following the standard procedures\(^3\). Silica (230-400 mesh) for flash column chromatography was obtained from Sorbent Technologies; thin-layer chromatography (TLC) precoated plates were from EMD. TLCs (silica gel 60, \( f_{254} \)) were visualized under UV light or by charring (5% \( \text{H}_2\text{SO}_4-\text{MeOH} \)). Flash column chromatography was performed on silica gel (230-400 mesh) using solvents as received. \(^1\)H NMR was recorded either on a Varian VXRS 400 MHz or an INOVA 600 MHz spectrometer in CDCl\(_3\) or CD\(_3\)OD using residual CHCl\(_3\) and CHD\(_2\)OH as internal references, respectively. \(^{13}\)C NMR was recorded on a Varian VXRS 100.56 MHz or an INOVA 150.84 MHz in CDCl\(_3\) using the triplet centered at \( \delta \, 77.273 \) or CD\(_3\)OD using the septet centered at \( \delta \, 49.0 \) as internal reference. High resolution mass spectrometry (HRMS) was performed on a TOF mass spectrometer. The peptide was synthesized on an Omega 396 synthesizer (Advanced ChemTech, Louisville, KY). Tris [(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA), preloaded Fmoc-L-Ala-Wang resin and all other Fmoc-L-amino acids were procured from Anaspec (San Jose, CA). Ficoll\(_®\) 400 and Imject\(_®\) Alum were purchased from Sigma and Thermo Scientific respectively. FITC goat anti-mouse IgG/IgM and purified mouse anti-human CD227 (anti-human MUC1)
were obtained from BD-biosciences (San Jose, CA). Scanning electron microscope imaging was done on a JEOL JSM-7500F field scanning electron microscope. Dynamic light scattering measurements was done DynaPro Titan temperature controlled microsampler (Wyatt Technology Corporation). Fluorescence microscopy was done on a Nikon TiU microscope. All other secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Female BALB/c mice (6-8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME). U266 human leukemia cells were purchased from American Type Culture Collection (Manassas, VA).

(5-Cholesten-3α-yloxy)-3n3-y-trixaundecanyl 2,3,4-Tri-O-acetyl-α-L-rhamnopyranoside (2). To a solution of 1,2,3,4-tetra-O-acetyl rhamnopyranose (0.64 g, 1.92 mmol) in CH2Cl2 (3 mL) was added (5-cholesten-3α-yloxy)-n3-y-trixaundecan-1-ol (1.30g, 2.30 mmol) in CH2Cl2 and the mixture was cooled to 0 °C. BF3.OEt2 (486 mL, 3.84 mmol) was added dropwise to the reaction mixture and the resulting solution was stirred at ambient temperature under N2 atmosphere. The reaction was monitored by TLC (EtOAc:hexanes = 1:1) and appeared complete after 18 h. The reaction mixture was diluted with CH2Cl2 (25 mL) and washed with saturated NaHCO3 (25 mL), water (25 mL) and brine (25 mL) after which the organic layer was dried over anhydrous Na2SO4. Excess solvent was evaporated under reduced pressure and the residue was purified by silica gel flash column chromatography using 30% EtOAc in hexanes as solvent to afford 2 as a light yellow solid (0.51 g, 32%). 1H NMR (600 MHz, CDCl3): δ 0.67 (s, 3H, cholesterol), 0.85-1.15 (23H, cholesterol), 1.21 (d, 3H, J = 6 Hz, C-5 CH3), 1.24-1.52 (12H, cholesterol), 1.80-1.95 (5H, cholesterol), 1.98 (s, 3H, COCH3), 2.05 (s, 3H, COCH3), 2.15 (s, 3H, COCH3), 3.17 (m, 1H, -OCH-cholesterol), 3.63-3.66 (16H, -CH2-
CH$_2$O-TEG), 3.92 (m, 1H, H-5), 4.77 (d, 1H, $J = 1.8$ Hz, H-1), 5.06 (t, 1H, $J = 10.2$ Hz, H-4), 5.26 (dd, 1H, $J = 1.8$, 3.6 Hz, H-2), 5.30 (dd, 1H, $J = 4.2$, 9.9 Hz, H-3), 5.33 (m, 1H, -C=CH-cholesterol). $^{13}$C NMR (100.56 MHz, CDCl$_3$): $\delta$ 12.04, 17.61, 18.90, 19.58, 20.95, 21.03, 21.14, 21.25, 22.76, 23.02, 24.01, 24.48, 28.21, 28.43, 28.53, 29.90, 32.07, 32.13, 35.97, 36.37, 37.07, 37.42, 39.23, 39.70, 39.96, 42.50, 50.36, 53.63, 56.32, 56.96, 66.46, 67.30, 67.46, 69.30, 70.03, 70.24, 71.35, 79.68, 97.74 (C-1), 121.74 (C=C), 141.15 (C=C), 170.18 (COCH$_3$), 170.25 (COCH$_3$), 170.32 (COCH$_3$). HRMS [M + Na] $m/z$: caled for C$_{47}$H$_{78}$NaO$_{12}$, 857.5391; found, 857.5396.

(5-Cholest en-3α-yloxy)-3n$_3$-triax aundecanyl Rhamnopyranoside (3). To a solution of 2 (0.45 g, 0.54 mmol) in MeOH (10 mL) was added metallic sodium (0.03 g) and the resulting solution was stirred at ambient temperature under N$_2$ atmosphere. The reaction was monitored by TLC (5% MeOH in CH$_2$Cl$_2$) and appeared complete after 1 h. The solution was neutralized by Amberlite H$^+$ exchange resin. Excess solvent was evaporated under reduced pressure and the residue was purified by silica gel flash column chromatography using 5% MeOH in CH$_2$Cl$_2$ as solvent to afford 3 as a yellowish white solid (0.32 g, 85%). $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 0.68 (s, 3H, cholesterol), 0.86 – 1.25 (24H, cholesterol), 1.32 (d, 3H, $J = 6$ Hz, C-5 CH$_3$), 1.44 – 1.53 (16H, cholesterol), 2.83 (s, 1H, C-4 OH), 3.08 (d, 1H, $J = 3$ Hz, H-1), 3.20 (m, 1H, -O-CH-cholesterol), 3.43 (t, 1H, $J = 9.6$ Hz, H-4), 3.62 – 3.71 (16H, -CH$_2$-CH$_2$O-TEG), 3.73 (m, 1H, H-5), 3.83 (dd, 1H, $J = 3$, 6.9 Hz, H-3), 3.98 (s, 1H, H-2), 4.87 (s, 1H, C-2 OH), 5.31 (s, 1H, C-3 OH), 5.35 (m, 1H, -C=CH-cholesterol). $^{13}$C NMR (100.56 MHz, CDCl$_3$): $\delta$ 12.07, 17.83, 18.92, 19.60, 21.27, 22.78, 23.04, 24.03, 24.50, 28.23, 28.44 (2), 32.08, 32.15, 35.99, 36.39, 37.06, 37.39, 39.09, 39.73, 39.97, 42.53, 50.36, 56.34, 56.97, 66.74, 67.32, 68.07,
70.48, 70.63, 70.75, 70.81, 70.93, 71.05, 71.80, 73.81, 79.87, 99.98 (C-1), 121.96 (C=C-cholesterol), 140.98 (C=C-cholesterol). HRMS [M + Na] m/z: calcd for C$_{41}$H$_{72}$NaO$_{9}$, 731.5074; found, 731.5090.

**N-propargyl Pam$_{2}$FmocCys Amide Derivative 5.** Pam$_{2}$FmocCys tertiary butyl ester (0.30 g, 0.32 mmol) was dissolved in minimum volume of neat TFA (1 mL) and stirred at ambient temperature under N$_2$ atmosphere. TLC (EtOAc:hexanes = 1:4) indicated the completion of the reaction after 1 h. The reaction mixture was evaporated to dryness under vacuum and the residue was dissolved in CH$_2$Cl$_2$ (3 mL). PyBOP (198 mg, 0.38 mmol), HOBt (58 mg, 0.38 mmol), DIPEA (78 µL, 0.47 mmol) and 4 Å mol. sieves (2-3 beads) were added sequentially and the mixture was stirred for 5 minutes at room temperature followed by the addition of propargyl amine (25 µL, 0.38 mmol) and stirred at ambient temperatures under N$_2$ atmosphere. The reaction was monitored by TLC (EtOAc:hexanes = 1:4) and appeared complete after 4 h. The reaction mixture was filtered, washed with phosphate buffer (10 mL) and extracted with CH$_2$Cl$_2$ (3 x 10 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated. The residue was purified by silica gel flash column chromatography using EtOAc-hexanes (1:4) as solvent to afford 5 as a white solid (192 mg, 66%). $^1$H NMR (600 MHz, CDCl$_3$): δ 0.87 (t, 6H, J = 7.2 Hz, Pam-CH$_3$), 1.14 - 1.65 (m, 52H, Pam-CH$_2$), 1.68 (s, 1H, alkyne-CH), 2.18-2.35 (m, 4H, COCH$_2$), 2.83 (m, 1H, Cys-CHH), 2.89 (dd, 1H, J = 7.2, 14.4 Hz, S-glyceryl-O-CHH), 3.01 (dd, 1H, J = 6, 14.4 Hz, cys-CHH), 4.06 (dd, 1H, J = 3, 4.8 Hz, S-glyceryl-O-CHH), 4.08 (s, 2H, CO-NH-CH$_2$), 4.18 (dd, 1H, J = 6, 11.4 Hz, S-glyceryl-O-CHH), 4.23 (t, 1H, J = 7.2 Hz, Fmoc-CH), 4.39 (m, 1H, NH-CH-CO), 4.42 (m, 2H, Fmoc-CH$_2$), 5.12 (m, 1H, S-glyceryl-O-CH), 5.73 (d, 1H, J = 7.8 Hz, Pam-NH), 6.89 (s, 1H, CO-NH-
CH$_2$), 7.31 – 7.81 (m, 8H, Fmoc-ArH). $^{13}$C NMR (150.84 MHz, CDCl$_3$): $\delta$ 14.35 – 36.70 (30C, Pam-C), 47.29 (2), 53.32, 63.58, 67.48, 70.61, 71.78, 72.07, 79.09, 79.85, 120.22, 125.30, 127.30 (2), 127.97 (2) 141.49, 141.50, 143.85, 143.89 (Aromatic-C), 170.07, 173.66 (2), 174.04 (Cys-CO). HRMS [M + Na] $m/z$: calcd for C$_{56}$H$_{86}$N$_2$NaO$_7$S, 953.6053; found, 953.6073.

*N*-propargyl Pam$_3$Cys Amide Derivative 6. Compound 5 (192 mg, 0.21 mmol) was dissolved in a mixture of CH$_3$CN-CH$_2$Cl$_2$-Et$_2$NH (2:1:2, 2.50 mL) and stirred at ambient temperature under N$_2$ atmosphere. TLC (EtOAc:hexanes = 1:4) indicated the complete deprotection of the Fmoc group after 2 h. The reaction mixture was evaporated to dryness under vacuum. Palmitic acid (64 mg, 0.25 mmol), PyBOP (128 mg, 0.25 mmol), HOBt (38 mg, 0.25 mmol) were dissolved in CH$_2$Cl$_2$ (3 mL) followed by the addition of DIPEA (51 μL, 0.31 mmol). The mixture was stirred for 5 minutes and added to the residue of the Fmoc deprotected product from compound 5 containing 4 Å mol. sieves (2-3 beads). The reaction mixture was stirred at ambient temperature under N$_2$ atmosphere. The reaction was monitored by TLC (EtOAc:hexanes = 1:4) and appeared complete after 4 h. The reaction mixture was diluted with CH$_2$Cl$_2$ (15 mL), filtered and evaporated to dryness. The residue was purified by silica gel flash column chromatography using EtOAC – hexanes (1 : 4) as solvent to afford 6 as a pale yellow solid (156 mg, 80%). $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 0.88 (t, 12H, $J = 6.6$ Hz, Pam-CH$_3$), 1.10 – 1.63 (m, 78H, Pam-CH$_2$), 2.23 (s, 1H, Alkyne-CH), 2.24 – 2.36 (m, 6H, COCH$_2$), 2.71 (dd, 1H, $J = 7.8$, 14.4 Hz, Cys-CH$_2$H), 2.86 (m, 6H, COCH$_2$), 2.95 (dd, 1H, $J = 6$, 14 Hz, Cys-CH$_2$H), 4.06 (m, 2H, CO-NH-CH$_2$), 4.18 (dd, 1H, $J = 6.6$, 12 Hz, S-glyceryl-O-CH$_3$H), 4.40 (dd, 1H, $J = 3$, 12 Hz, S-glyceryl-O-CH$_2$H), 4.64 (q, 1H, $J = 6$ Hz, NH-CH-
CO), 5.12 (m, 1H, S-glyceryl-OCH), 6.64 (d, 1H, J = 8.4 Hz, Pam-NH), 7.02 (t, 1H, J = 5.4 Hz, CO-NH-CH₂). ¹³C NMR (150.84 MHz, CDCl₃): δ 14.35 – 42.19 (48C, Pam-C, Cys-Cβ, S-glyceryl-C, NH-C), 51.30 (Cys-Cα), 63.65, 70.61, 71.98, 79.08, 170.40 (Cys-CO), 173.70, 173.86, 174.06 (Pam-CO). HRMS [M + Na] m/z: calcd for C₅₇H₁₀₆N₂NaO₆S, 969.7669; found, 969.7682.

**Glycopeptide Azide 9.¹** The glycopeptide azide was synthesized by Fmoc strategy on an Omega 396 synthesizer (Advanced ChemTech, Louisville, KY) using solid phase chemistry. The peptide synthesis was performed by coupling amino acid esters of HOBt using DIC as the coupling agent. A 6-fold excess of Nα-Fmoc amino acid esters of HOBt in NMP were used in the synthesis. A 1:1 ratio of amino acid to DIC was used in all the coupling reactions. Deprotection of Nα-Fmoc group was accomplished by treatment with 25% piperidine in dimethylformamide twice; first for 5 minutes and then a second time for 25 minutes. After the synthesis was complete, the peptide was cleaved from the solid support and deprotected using a modified reagent K cocktail consisting of 88% TFA, 3% thioanisole, 5% ethanedithiol, 2% water and 2% phenol. 4 mL of cleavage cocktail was added to the dried peptide-resin in a 15 mL glass vial blanketed with nitrogen. Cleavage was carried out for 2.5 hrs with gentle magnetic stirring. At the end of the cleavage time, the cocktail mixture was filtered on a Quick-Snap column. The filtrate was collected in 20 mL ice-cold butane ether. The peptide was allowed to precipitate for an hour at -200 °C, centrifuged, and washed twice with ice-cold methyl-tert-butyl ether. The precipitate was dissolved in 25% acetonitrile and lyophilized to complete dry powder. Quality of peptides was analyzed by analytical reverse phase HPLC and MALDI-TOF (matrix assisted laser desorption ionization time-of-flight) mass spectrometer, model

¹synthesized at The Cleveland Clinic.
Glycopeptide Azide 10. Compound 9 (5 mg, 2.24 μmol) was dissolved in 2 mL of dry methanol and 12 μL of freshly prepared 1 M sodium methoxide was added and the reaction mixture was stirred at ambient temperature under N₂ atmosphere for 2 h. The reaction mixture was neutralized with solid carbon dioxide. The reaction mixture was concentrated and purified by Bio-Gel (P-2, fine 45-90 μm) size exclusion chromatography using deionized water as solvent. Lyophilization of the elutants afforded 10 as a white powder (4.7 mg, 100%). HR-MALDI-MS: [M+H] m/z calcd for C₁₀₀H₁₅₅N₂₉O₃₇, 2355.1172; found, 2355.1753.

Lipopeptide 11. CuSO₄.5H₂O (134 μg, 0.54 μmol) and TBTA (2.14 mg, 4.04 μmol) were dissolved in H₂O – THF (1:1, 0.40 mL) and to it Na-ascorbate (0.80 mg, 4.04 μmol) was added and stirred for 5 minutes. Compound 6 (1.27 mg, 1.35 μmol) in THF (0.40 mL) was added to the reaction mixture and stirred for 15 minutes followed by the addition of a solution of compound 10 (1 mg, 0.45 μmol) in H₂O-MeOH (1:3, 0.4 mL). The reaction mixture was stirred at ambient temperature under N₂ atmosphere for 40 h. The reaction mixture was concentrated, dissolved in CH₂Cl₂-MeOH (1:1) and purified by a short LH 20 size exclusion column using CH₂Cl₂-MeOH (1:1) as solvent. Lyophilization of the elutants afforded 11 as a white solid (1.9 mg, 100%). HR-MALDI-MS: [M+H] m/z calcd for C₁₅₁H₂₅₅N₃₁O₄₀S, 3175.593; found 3175.425. A mass peak corresponding to a protonated methyl ester of the product was also observed.

Liposome Formulation: Lipid stock solutions were prepared by dissolving each lipid into chloroform inside glass vials. Aliquots of the stock solutions were mixed in
proportions in another small glass vial to give a solution with a total lipid concentration of 30 mM in a total volume of 2 mL (Batch 1: DPPC 80%, Cholesterol 10%, Rha-cholesterol 10% and Pam3cys-MUC1-Tn 0.69 µM; Batch 2: DPPC 80%, cholesterol 20%, Pam3cys-MUC1-Tn 0.69 µM; Batch 3: DPPC 80%, cholesterol 20%). Chloroform was removed by subjecting the lipid solutions to a constant stream of nitrogen. The resulting lipid films were dried under vacuum overnight. The dried lipid films were hydrated with 2 mL of HEPES buffer (pH = 7.4). The suspensions of the lipids in the buffer were agitated at 43 °C for 40 mins. The suspensions were subjected to 10 freeze-thaw cycles (dry ice/acetone and water at 40 °C). Final liposomes were prepared by extrusion (21 times) using a LipoFast Basic fitted with a 100 nm polycarbonate membrane to control the liposome size.

**Liposome characterization:**

**Liposome Size Characterization:** Size determination of the liposomes was done by scanning electron microscope (SEM) imaging and dynamic light scattering (DLS) measurements. For SEM characterization the liposome samples were diluted 1000 times with HEPES buffer (pH = 7.4) and freeze dried over copper studs fitted with a carbon conducting tape and the images recorded at an acceleration voltage of 5 kV. DLS measurements were done after dilution of the liposome samples 10000 times with HEPES buffer (pH = 7.4).

**Anti-Rha and Anti-MUC1 Antibody Binding to Surface Exposed Rha and MUC1 Epitopes on Liposomes:** One million liposomes from each batch in 50 µL phosphate buffered saline (PBS) were added separately into a 1.5 mL Eppendrof tube followed by 50 µL of primary antibody solution in deionized water containing 5-50
µg/mL of antibodies [either control IgG (isolated from the serum of non-immunized mice) or anti-Rha IgG isolated from the serum of Rha-ovalbumin immunized mice\textsuperscript{10} or mouse anti-human CD227 monoclonal antibodies (anti-human MUC1)] and incubated on ice for 30 mins. 1 mL PBS-0.1% Tween was added to each tube and vortexed. Liposomes were centrifuged at 14000 rpm in an Eppendorf centrifuge at 4 °C for 5 mins. The supernatants were carefully discarded and the washing and centrifugation steps were repeated 2 more times for a total of 3 washes. Liposomes were then resuspended in 50 µL of PBS-0.1% Tween. 50 µL of diluted FITC goat anti-mouse IgG/IgM secondary antibody were added (2-30 µg/mL) to the tubes, mixed and covered with aluminum foil to protect from light and incubated on ice for 30 mins. After washing 3 times with PBS-0.1% Tween and centrifugation, the supernatants were removed and pellets were resuspended in 1 mL PBS-0.1% Tween. 10 µL of the resuspended solutions were put on glass slides and imaged under a fluorescence microscope.

2-Azidoethyl-2,3,4-Tri-O-acetyl-α-L-rhamnopyranoside (13). To a solution of 1,2,3,4-tetra-O-acetylrhamnopyranoside (12) (2.00 g, 6.02 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (5.00 mL) were added 2-azidoethanol (0.79 g, 9.03 mmol) and BF\textsubscript{3}·OEt\textsubscript{2} (1.53 mL, 12.04 mmol) at 0 °C and the resulting solution was stirred at ambient temperature under N\textsubscript{2} atmosphere. The reaction was monitored by TLC and appeared to be complete after 12 h. The reaction mixture was diluted with CH\textsubscript{2}Cl\textsubscript{2} (15 mL) and washed with water (2 x 20 mL), saturated NaHCO\textsubscript{3} (2 x 20 mL) and brine (20 mL), after which the organic layer was dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}. Excess solvent was removed under reduced pressure and the crude material was purified silica gel flash column chromatography (3.3 x 8.5 cm). Elution with 1:5 EtOAc/hexanes afforded 13 as a colorless solid (1.78 g, 83%). $^1$H NMR (600 MHz,
CDCl₃): δ 1.24 (d, 3H, J= 6.6 Hz, C-5 CH₃), 1.99 (s, 3H, COCH₃), 2.06 (s, 3H, COCH₃), 2.16 (s, 3H, COCH₃), 3.42 (m, 1H, -CH₃-N₃), 3.48 (m, 1H, -CH₂-N₃), 3.64 (m, 1H, -O-CH₃), 3.87 (m, 1H, -O-CH₂), 3.93 (m, 1H, H-5), 4.79 (d, 1H, J = 1.8 Hz, H-1), 5.09 (t, 1H, J = 10.2 Hz, H-4), 5.27 (dd, 1H, J = 1.2, 3.3 Hz, H-2), 5.31 (dd, 1H, J = 3.3, 9.9 Hz, H-3). ¹³C NMR (100 MHz, CDCl₃): δ 17.66 (CH₃), 20.93, 21.03, 21.13, 50.58, 66.91, 66.99, 69.08, 69.87, 71.09, 97.79 (C-1), 170.09 (C=O), 170.24 (C=O), 170.30 (C=O). HRMS [M + Na] m/z: calcd for C₁₄H₂₁N₃O₈, 382.1226; found, 382.1215.

2-Azidoethyl α-L-Rhamnopyranoside (14). To a solution of 13 (1.53 g, 4.26 mmol) in MeOH (5 mL) was added metallic Na (0.01 g) and the resulting solution was stirred at ambient temperature under N₂ atmosphere. The reaction was monitored by TLC and appeared complete after 2 h. Excess solvent was removed under reduced pressure and the crude material was purified by silica gel flash column chromatography (3.3 x 8.5 cm). Elution with 2:23 MeOH/CH₂Cl₂ yielded 14 as a colorless solid (0.85 g, 86%). ¹H NMR (600 MHz, CDCl₃): δ 1.34 (d, 3H, J= 6.6 Hz, C-5 CH₃), 3.41 (m, 2H, -CH₂-N₃), 3.49 (t, 1H, J = 9.3 Hz, H-4), 3.63 (m, 1H, -O-CH₃), 3.69 (m, 1H, H-5), 3.81 (dd, 1H, J = 3.3, 9.3 Hz, H-3), 3.89 (m, 1H, -O-CH₂), 3.99 (q, 1H, J = 1.6 Hz, H-2), 4.83 (d, 1H, J = 1.2 Hz, H-1). ¹³C NMR (100 MHz, CDCl₃): δ 17.75 (CH₃), 50.71, 66.72, 68.49, 70.98, 71.76, 73.27, 100.02 (C-1). HRMS [M + Na] m/z: calcd for C₈H₁₅N₃O₅, 256.0909; found, 256.0906.

2-Aminoethyl α-L-Rhamnopyranoside (15). To a solution of 14 (0.42 g, 1.82 mmol) in MeOH (3 mL) was added activated Pd/charcoal (0.025 g) and the resulting solution was stirred at ambient temperature under H₂ atmosphere. The reaction was monitored by TLC and appeared to be complete after 12 h. The reaction mixture was
diluted with MeOH (2 mL), filtered through celite and concentrated under reduced pressure to yield 15 as a colorless gel (0.46 g, quantitative) which was used without further purification for subsequent reactions. ESIMS [M + H] m/z: cacld for C₈H₁₇NO₅, 208.2243; found, 208.30.

2-Aminoeethyl α-L-Rhamnopyranoside-Ficoll Conjugate (16). Ficoll 400 (1.00 g, 0.0025 mmol) was dissolved in acetate buffer (10 mL, pH 4.7) and NaIO₄ (0.01 g, 0.047 mmol) was added and the reaction mixture was stirred at ambient temperature for 2 h in the dark. Excess NaIO₄ was removed by dialysis against the acetate buffer (pH 4.7) through dialysis tubing with a molecular weight cutoff value of 10000 Da with six to seven changes of the buffer at 4 °C. The oxidized Ficoll 400 was transferred to a round bottom flask and excess solvent was evaporated to dryness under reduced pressure. The residue was dissolved in borate buffer (20 mL, pH 8.0) followed by the addition of 15 (0.05 g, 0.25 mmol) and stirred at ambient temperature for 2 h. To the reaction mixture was added NaBH₃CN (0.094 g, 1.50 mmol) and the resulting solution was incubated overnight at 4 °C. The mixture was dialyzed through dialysis tubing with a molecular weight cutoff value of 10000 Da with six to seven changes in buffer at 4 °C to afford 16. The epitope ratio of 16 was calculated to be 9.44 (Rha:Ficoll) by hydrolysis of 16 followed by derivatization with 4-amino-N-[2-(diethylamino)ethyl] benzamide (DEAEAB) and comparison of the UV-HPLC peak area with standard curve obtained from DEAEAB derivative of 14 by the methods described by Dalpathado and coworkers. Briefly, the standard curve was generated by refluxing Compound 14 (0.007 g, 0.031 mmol) with 1 N HCl at 100 °C for 4 h and the reaction mixture was evaporated to dryness. The residue was dissolved in tetrahydrofuran (2 mL) and DEAEAB (0.011 g,
0.037 mmol) and Et₃N (0.007 mL, 0.046 mmol) were added and the resulting solution was refluxed for 2 h. The reaction mixture was evaporated to dryness and the residue was dissolved in MeOH (2 mL) followed by the addition of NaB(OAc)₃H and the resulting solution refluxed for 8 h. The solution was evaporated to dryness and the residue was dissolved in MeOH (2 mL) and filtered through a syringe filter. Serial dilutions from this stock solution were prepared and the components were separated on a reverse phase HPLC using a C18 column. Water containing 0.1% TFA (A) and 95% ACN/H₂O (B) were used as the mobile phases using a linear gradient (5-20% B in 20 min) at the flow rate of 1 mL/min. Absorbances were recorded at 289 nm. The standard curve was generated by plotting the UV-HPLC peak area against the concentration in mmol of DEAEAB derivative of 14.

**Comparison of Anti-L-Rha Antibodies generated against Rha-Ficoll versus Rha-OVA**

**Immunizations.** Ten BALB/c mice were divided into two groups A and B containing five mice each. Group A was subcutaneously injected (day 0) with a 100 μL equivolume emulsion of Rha-Ficoll conjugate 16 and Alum (50 μg of Rha-Ficoll per mouse) whereas group B was subcutaneously injected with a 100 μL equivolume emulsion of Rha-OVA conjugate and complete Freund’s adjuvant (CFA) (50 μg of Rha-OVA per mouse). Groups A and B were boosted four times on days 14, 28, 42 and 56 with Rha-Ficoll/Alum (50 μg of Rha-Ficoll per mouse, each boost) and Rha-OVA/incomplete Freund’s adjuvant (ICF) (50 μg of Rha-OVA per mouse, each boost) respectively. The mice were bled on day 66 and the collected sera from each group were tested for anti-Rha antibody subclass identification.
**Anti-Rha Antibody Subclass Identification.** 96 Well plates (Immuron 4 HBX) were coated with Rha-bovine serum albumin (BSA) conjugate (2 μg/mL) in 0.01 M phosphate buffered saline (PBS, pH 7.2) and incubated overnight at 4 °C. The plates were washed 4 times with PBS containing 0.1% Tween-20. Blocking was achieved by incubating the plates for 1 h at room temperature with BSA in 0.01 M PBS (1mg/mL). The plates were then washed 4 times and incubated for 1 h with 1/100 serum dilution in PBS. Unconjugated antibody in the serum was removed by washing and the plates were incubated overnight at 4 °C with subclass specific (IgG1, IgG2a, IgG2b, IgG3, IgA and IgM) rabbit anti-mouse antibody (Zymed Laboratories mouse monoAb-ID kit). The plates were washed and incubated with horseradish peroxidase (HRP)-goat anti-rabbit IgG (H+L) for 1 h at room temperature. The plates were washed and ABTS substrate buffer (diluted 50 times) was added and allowed to react for 30 min. Absorbances were recorded at 405 nm and compared for each antibody subclass in each group.

**T-Cell Proliferation Study to Test the Cell Mediated Immune Response in BALB/c Mice against the MUC1-Tn 10 Glycopeptide.**

**Immunization.** Female BALB/c mice were primed (day 0) and boosted three times (days 14, 28 and 42) with 100 μL subcutaneous injections of an equivolume emulsion of the MUC1-Tn 10 (prepared in phosphate buffer saline-PBS) and Sigma adjuvant system (SAS) (50 μg of peptide per mouse, each injection).
Bone Marrow Dendritic Cell (BMDC) Culture from BALB/c mice. Bone marrow from the femur and tibia of nonimmune BALB/c mice was forced out by flushing with cell culture medium in a syringe. Single cell suspensions were washed twice and cleared of red blood cells. The cells were resuspended in cell culture medium to a concentration of $10^6$ cells/mL. Granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/mL) and interleukin 4 (IL-4) (10ng/mL) were separately added to each cell suspension for the two mice strains and incubated at 37 °C for 3 days. The cell suspensions were centrifuged and 75% of the supernatant was discarded and replenished with fresh cell culture medium followed by the addition of GM-CSF (10 ng/mL) and IL-4 (10 ng/mL). The cell suspensions were incubated at 37 °C for 3 days.

Preparation of Spleen Cell Suspensions and Assay Setup. On day 49, the mice were sacrificed and spleen cell suspensions were prepared, washed and brought to $5 \times 10^6$ cells/mL. 100 μL of the spleen cell suspensions were added to 96 well plates ($5 \times 10^5$ cells per well). The dendritic cell (DC) suspensions from each mouse were pulsed separately by incubating with MUC1-Tn 10 at antigen concentrations of $8.8 \times 10^{-3} – 1.1 \mu g/mL$ at 37 °C for 4 h. 100 μL of the pulsed DCs were added to wells containing the respective spleen cells ($5 \times 10^4$ DCs per well). To the spleen cell suspensions in the other wells was added 100 μL of MUC1 VNTR-Tn solution ($8.8 \times 10^{-3} – 1.1 \mu g/mL$) in PBS. Control wells were deprived of any antigen. The plates were incubated at 37 °C for 4 days. On day 4 the cells were pulsed with $[^3]$H-thymidine (40 μCi/mL, 25 μL per well) and incubated overnight at 37 °C. The cells were harvested on glass-fiber filters and incorporation was measured.

determined by measurements on a Top Count scintillation counter (Packard, Downers Grove, IL).

**T-Cell Proliferation Study. Immunization.** One female BALB/c mouse (6-8 weeks old, The Jackson Laboratory) was primed (day 0) and boosted three times (days 14, 28 and 42) with 100 μL subcutaneous injections of an equivolume emulsion of the MUC1-Tn conjugate 10 (prepared in phosphate buffer saline-PBS) and sigma adjuvant system (SAS) (50 μg of peptide per mouse, each injection).

**Preparation of Anti-Rha Antibodies.** The Rha-Ficoll and the Rha-OVA immunized mice were bled on day 66 and the sera was pooled. IgG fractions from each pool were prepared by precipitation at 40% saturation of ammonium sulfate. The mixtures were incubated overnight and centrifuged at 10000 x g for 10 minutes and then resuspended in 0.5 mL water. The antibody solutions were concentrated and buffer was changed twice with PBS using an Ultrafree 0.5 centrifugal filter device (Millipore, Billerica, MA) having a molecular cut off of 50000 D. Aorbances of the antibody solutions were recorded at 280 nm to calculate the concentrations and the anti-Rha antibody solutions generated and isolated from the Rha-Ficoll and the Rha-OVA immunized mice were each diluted to 1.0 mg/mL.

**Preparation of Spleen Cell Suspensions and Assay Setup.** On day 49, the mouse was sacrificed and the spleen was removed and placed in 5 mL of freshly prepared spleen cell culture medium (DMEM with 10% fetal calf serum). Single cell suspension was prepared using sterile glass homogenizers. The cells were washed three times with culture medium and brought to 5 x 10⁶ cells/mL. 100 μL of the spleen cell suspensions were added to 96 well plates (5x10⁵ cells per well). The dendritic cell (DC) suspension
cultured from the bone marrow of a BALB/c mouse was pulsed with the antigen by incubating with the Rha-displaying MUC1-Tn liposomes at antigen concentrations of $8.8 \times 10^{-3} – 1.1 \mu g/mL$ at 37 °C for 4 h together with anti-Rha antibodies generated from either Rha-Ficoll or Rha-OVA immunized mice sera (5 μg per well) or with control antibodies isolated from non-immunized mice serum. 100 μL of the pulsed DCs was added to the wells containing the spleen cells (5 x 10^4 DCs per well). The plates were incubated at 37 °C for 4 days. On day 4 the cells were pulsed with [\textsuperscript{3}H]-thymidine (40 μCi/mL, 25 μL per well) and incubated overnight at 37 °C. The cells were harvested on glass-fiber filters and incorporation was determined by measurements on a Top Count scintillation counter (Packard, Downers Grove, IL).

**Immunizations.** The 20 female BALB/c mice used for this study were divided into four groups A1, A2, B1 and B2 containing 5 mice each. Groups A1 and B1 served as the control groups and were not immunized. Groups A2 and B2 were injected subcutaneously (day 0) with a 100 μL equivolume emulsion of Rha-Ficoll conjugate and Alum (100 μg of Rha-Ficoll per mouse). The mice were boosted with 100 μL subcutaneous injections of Rha-Ficoll/Alum on days 14, 28, 42 and 56 (100 μg of Rha-Ficoll per mouse, each boost). The mice in each groups A1, A2, B1 and B2 were bled on day 66 and the collected sera was tested for anti-Rha antibodies.

**ELISA for Measuring Anti-Rha Antibody Titers.** 96 Well plates (Immulon 4 HBX) were coated with Rha-BSA conjugate (2 μg/mL) in 0.01 M PBS and incubated overnight at 4 °C. The plates were washed 5 times with PBS containing 0.1% Tween-20. Blocking was achieved by incubating the plates for 1 h at room temperature with BSA in 0.01 M PBS (1mg/mL). The plates were then washed 5 times and incubated for 1 h with
serum dilutions in PBS. Unbound antibody in the serum was removed by washing and the plates were incubated for 1 h at room temperature with Horseradish Peroxidase (HRP) goat anti-mouse IgG + IgM (Jackson Immunoresearch Laboratories) diluted 5000 times in PBS/BSA. The plates were washed and TMB (3,3’,5,5’-tetramethylbenzidine) One component HRP microwell substrate (Bio FX, Owings Mills, MD) was added and allowed to react for 10 mins. Absorbances were recorded at 620 nm and were plotted against log10 [1/serum dilution].

**Vaccinations.** Vaccination was performed on day 77. Two separate liposomal formulations were prepared with DPPC (80%), cholesterol (20%) and Pam3Cys-MUC1-Tn 11 (2 nmol) (Pam3Cys-MUC1-Tn liposomes) and DPPC (80%), cholesterol (10%), Rha-TEG-cholesterol 3 (10%) and Pam3Cys-MUC1-Tn 11 (2 nmol) (Pam3Cys-MUC1-Tn +Rha liposomes) in total lipid concentrations of 30 mmol. Groups A1 and A2 were vaccinated with 100 μL subcutaneous injections of the Pam3Cys-MUC1-Tn liposomes (2 nmol of peptide per mouse) and groups B1 and B2 were vaccinated with 100 μL subcutaneous injections of the Pam3Cys-MUC1-Tn +Rha liposomes (2 nmol peptide per mouse). The mice were boosted on day 91 with either the Pam3Cys-MUC1-Tn liposomes (groups A1 and A2, 2 nmol peptide per mouse) or the Pam3Cys-MUC1-Tn +Rha liposomes (groups B1 and B2). The mice were bled on day 101 and the sera collected were tested for anti-MUC1-Tn and anti-Tn antibodies.

**ELISA for Measuring Anti-MUC1-Tn Antibody Titers.** 96 Well plates (Immulon 4 HBX) were coated with MUC1-Tn conjugate 10 (15 μg/mL) in 0.01 M PBS and incubated overnight at 4 °C. The ELISA was continued as described above.
**ELISA for Measuring Anti-Tn Antibody Titers.** 96 Well plates (Immulon 4 HBX) were coated with Tn-BSA conjugate (15 μg/mL) in 0.01 M PBS and incubated overnight at 4 °C. The ELISA was continued as described above.

**Anti-MUC1-Tn Antibody Subclass Identification.** 96 Well plates (Immulon 4 HBX) were coated with MUC1-Tn conjugate 10 (15 μg/mL) in 0.01 M PBS and incubated overnight at 4 °C. The plates were washed 4 times with PBS containing 0.1% Tween-20. Blocking was achieved by incubating the plates for 1 h at room temperature with BSA in 0.01 M PBS (1mg/mL). The plates were then washed 4 times and incubated for 1 h with 1/100 serum dilution in PBS. Unbound antibody in the serum was removed by washing and the plates were incubated overnight at 4 °C with subclass specific (IgG1, IgG2a, IgG2b, IgG3, IgA and IgM) rabbit anti-mouse antibody (Zymed Laboratories mouse monoAb-ID kit). The plates were washed and incubated with HRP-goat anti-rabbit IgG (H+L) for 1 h at room temperature. The plates were washed and ABTS substrate buffer (diluted 50 times) was added and allowed to react for 30 min. Absorbances were recorded at 405 nm and compared for each antibody subclass in each group.

**ELISA for Competitive Binding with Free MUC1-Tn.** A 96 well plate (Immulon 4 HBX) was coated with MUC1-Tn conjugate 10 (15 μg/mL) in PBS and incubated overnight at 4 °C. The plate was washed 5 times with PBS containing 0.1% Tween-20. Blocking was achieved by incubating the plate for 1 h at room temperature with BSA in M PBS (1mg/mL). The plate was then washed 5 times and incubated for 1 h with serum dilutions of 1/100 in PBS with or without prior mixing with varying concentrations of free MUC1-Tn (compound 10) from 0, 10^-5, 10^-4, 10^-3 M in PBS. Unbound antibody in the serum was removed by washing and the plate was incubated for
1 h at room temperature with Horseradish Peroxidase (HRP) goat anti-mouse IgG + IgM (secondary antibody) diluted 5000 times in PBS/BSA. The plate was washed and TMB 1 component HRP microwell substrate was added and allowed to react for 10 mins. Absorbances were recorded at 620 nm and were plotted against $\log_{10} [1/\text{free Tn concentration}]$.

**Tumor Cell Staining.** U266 cells (American Type Culture Collection, Manassas, VA), were cultured in RPMI 1640 with 15% fetal calf serum (FCS). Cells were stained with purified mouse anti-human MUC1 antibodies (CD227, 0.5 μg), non-immune BALB/c mice serum (1/5 dilution) and group B2 mice serum (1/5 dilution). The cells were then stained with FITC-conjugated goat anti-mouse IgG + IgM (0.5 μg) and fluorescence was quantified with a BD FACS Calibur.
3.6 References.


References

Chapter 1


117, 5701-5711.

(57) Raghupathi, G.; Deshpande, P. P.; Coltart, D. M.; Kim, H. M.; Williams, L. J.;

(58) Cappello, S.; Liu, N. X.; Musselli, C.; Brezicka, F. T.; Livingsto, P. O.;

O.; Danishefsky, S. J.; Livingston, P. O. Angew. Chem. Int. Ed. 1997, 36, 125-
128.

(60) Raghupathi, G.; Slovin, S. F.; Adluri, S.; Sames, D.; Kim, I. J.; Kim, H. M.;
Spassova, M.; Bornmann, W. G.; Lloyd, K. O.; Scher, H. I.; Livingston, P. O.;

(61) Slovin, S. F.; Raghupathi, G.; Adluri, S.; Ungers, G.; Terry, K.; Kim, S.; Spassova,
M.; Bornmann, W. G.; Fazzari, M.; Dantis, L.; Olkiewicz, K.; Lloyd, K. O.;
Livingston, P. O.; Danishefsky, S. J.; Scher, H. I. Proc. Natl. Acad. Sci. USA
1999, 96, 5710-5715.

(62) Kuduk, S. D.; Schwarz, J. B.; Chen, X. -T.; Glunz, P. W.; Sames, D.; Raghupathi,
G.; Livingston, P. O.; Danishefsky, S. J. J. Am. Chem. Soc. 1998, 120, 12474-
12485.


Chapter 2


(33) Oyelaran, O.; McShane, L. M.; Dodd, L.; Gildersleeve, J. C. J. Proteome Res. 2009, 8, 4301-4310.


(40) Bio-Rad Manufactures manual.


Chapter 3


(9) Chen, W.; Gu, L.; Zhang, W.; Motari, E.; Cai, L.; Styslinger, T. J.; Wang, P. G. 

(10) Sarkar, S.; Lombardo, S. A.; Herner, D.N.; Talan, R. S.; Wall, K. A.; Sucheck, S. 

Pope, M.; Stamatatos, L. Blood 2000, 96, 3505-3513. (b) Foged, C.; Arigita, C.; 

(12) (a) Mallick, A. I.; Sinha, H.; Chaudhuri, P.; Nadeem, A.; Khan, S. A.; Dar, K. A.; 
Owasis, M. Vaccine 2007, 25, 3692-3704. (b) Jérôme, V.; Graser, A.; Müller, R.; 


(14) Bardonnet, P.-L.; Faivre, V.; Pirot, F.; Boullanger, P.; Falson. F. Biochem. 


Ed. 2005, 44, 5985-5988.

(17) Metzger, J. W.; Wiesmüller, K.-H.; Jung, G. Int. J. Peptide Protein Res. 1991, 38, 
545-554.


Prod. 2001, 64, 883-891.


Appendix A

Supporting Information for Chapter 2

Synthesis of a Single Molecule L-Rhamnose-Containing Three Component Vaccine and Evaluation of Antigenicity in the Presence of Anti-L-Rhamnose Antibodies

Page 143: $^1$H NMR of Pentenyl 2,3,4-tri-$O$-acetyl-$\alpha$-L-rhamnopyranoside 2.

Page 144: $^{13}$C NMR of Pentenyl 2,3,4-tri-$O$-acetyl-$\alpha$-L-rhamnopyranoside 2.

Page 145: $^1$H NMR of 4-$O$-(2,3,4-tri-$O$-acetyl-$\alpha$-L-rhamnosyl)-butanoic acid 3.

Page 146: $^{13}$C NMR of 4-$O$-(2,3,4-tri-$O$-acetyl-$\alpha$-L-rhamnosyl)-butanoic acid 3.

Page 147: $^1$H NMR of Pentenyl-$\alpha$-L-rhamnopyranoside 4.

Page 148: $^{13}$C NMR of Pentenyl-$\alpha$-L-rhamnopyranoside 4.

Page 149: $^1$H NMR of compound 4-$O$-(O-$\alpha$-L-rhamnopyranosyl)-butanal 5.

Page 150: $^{13}$C NMR of compound 4-$O$-(O-$\alpha$-L-rhamnopyranosyl)-butanal 5.

Page 151: $^1$H NMR of compound 3-$O$-(2-Acetamido-2-deoxy-$\alpha$-D-galactopyranosyl)-L-threonone 11.

Page 152: $^{13}$C NMR of compound 3-$O$-(2-Acetamido-2-deoxy-$\alpha$-D-galactopyranosyl)-L-threonone 11.

Page 153: HR-MALDI-TOF of Glycopeptide 16

Page 154: HR-MALDI-TOF of Glycopeptide 17

Page 156: HR-MALDI-TOF of Glycopeptide 19.
$^1$H NMR of Pentenyl 2,3,4-tri-$O$-acetyl-$\alpha$-L-rhamnopyranoside 2.
$^{13}$C NMR of Pentenyl 2,3,4-tri-O-acetyl-α-L-rhamnopyranoside 2.
$^1$H NMR of 4-\(O\)-(2,3,4-tri-\(O\)-acetyl-\(\alpha\)-L-rhamnosyl)-butanoic acid 3.
$^{13}$C NMR of 4-O-(2,3,4-tri-O-acetyl-α-L-rhamnosyl)-butanoic acid 3
$^1$H NMR of Pentenyl-α-L-rhamnopyranoside 4.
$^{13}$C NMR of Pentenyl-α-L-rhamnopyranoside 4.
$^1$H NMR of compound 4-($O$-$\alpha$-$L$-rhamnopyranosyl)-butanal 5.
$^{13}$C NMR of compound 4-$(O$-$\alpha$-$L$-rhamnopyranosyl)$-butanal 5.
$^1$H NMR of compound 3-O-(2-Aacetamido-2-deoxy-α-D-galactopyranosyl)-L-threonone 11.
$^{13}$C NMR of compound $3-O$-(2-Acetamido-2-deoxy-$\alpha$-$\text{D}$-galactopyranosyl)-$\text{L}$-threonone 11.
HR-MALDI-TOF of Glycopeptide 16
HR-MALDI-TOF of Glycopeptide 17
HR-MALDI-TOF of Glycopeptide 18.
HR-MALDI-TOF of Glycopeptide 19.
Supporting Information for Chapter 3

Synthesis and Immunological Evaluation of a MUC1 Glycopeptide Incorporated into L-Rhamnose Displaying Liposomes

Page 159: $^1$H NMR of (5-cholesten-3α-yloxy)-3$n_3^3$-trixaundecanyl-2,3,4-tri-O-acetyl-α-L-rhamnopyranoside (2).

Page 160: $^{13}$C NMR of (5-cholesten-3α-yloxy)-3$n_3^3$-trixaundecanyl-2,3,4-tri-O-acetyl-α-L-rhamnopyranoside (2).

Page 161: $^1$H-gCosy of (5-cholesten-3α-yloxy)-3$n_3^3$-trixaundecanyl-2,3,4-tri-O-acetyl-α-L-rhamnopyranoside (2).

Page 162: HRMS of (5-cholesten-3α-yloxy)-3$n_3^3$-trixaundecanyl-2,3,4-tri-O-acetyl-α-L-rhamnopyranoside (2).

Page 163: $^1$H NMR of (5-cholesten-3α-yloxy)-3$n_3^3$-trixaundecanyl rhamnopyranoside (3).

Page 164: $^{13}$C NMR of (5-cholesten-3α-yloxy)-3$n_3^3$-trixaundecanyl rhamnopyranoside (3).

Page 165: $^1$H-gCosy of (5-cholesten-3α-yloxy)-3$n_3^3$-trixaundecanyl rhamnopyranoside (3).

Page 166: HRMS of (5-cholesten-3α-yloxy)-3$n_3^3$-trixaundecanyl rhamnopyranoside (3).

Page 167: $^1$H NMR of N-propargyl Pam$_2$FmocCys amide derivative 5.

Page 168: $^{13}$C NMR of N-propargyl Pam$_2$FmocCys amide derivative 5.

Page 169: $^1$H-gCosy of N-propargyl Pam$_2$FmocCys amide derivative 5.

Page 170: HRMS of N-propargyl Pam$_2$FmocCys amide derivative 5.
Page 171: $^1$H NMR of $N$-propargyl Pam$_3$Cys amide derivative 6.

Page 172: $^{13}$C NMR of $N$-propargyl Pam$_3$Cys amide derivative 6.

Page 173: $^1$H-gCosy of $N$-propargyl Pam$_3$Cys amide derivative 6.

Page 174: HRMS of $N$-propargyl Pam$_3$Cys amide derivative 6.


Page 177: HR-MALDI-TOF of lipopeptide 11.

Page 178: $^1$H NMR of 2-Azidoethyl-2,3,4-Tri-O-acetyl-$\alpha$-L-rhamnopyranoside (13).

Page 179: $^{13}$C NMR of 2-Azidoethyl-2,3,4-Tri-O-acetyl-$\alpha$-L-rhamnopyranoside (13).

Page 180: $^1$H-gCosy of 2-Azidoethyl-2,3,4-Tri-O-acetyl-$\alpha$-L-rhamnopyranoside (13).

Page 181: HRMS of 2-Azidoethyl-2,3,4-Tri-O-acetyl-$\alpha$-L-rhamnopyranoside (13).

Page 182: $^1$H NMR of 2-Azidoethyl $\alpha$-L-rhamnopyranoside (14).

Page 183: $^{13}$C NMR of 2-Azidoethyl $\alpha$-L-rhamnopyranoside (14).

Page 184: $^1$H-gCosy of 2-Azidoethyl $\alpha$-L-rhamnopyranoside (14).

Page 185: HRMS of 2-Azidoethyl $\alpha$-L-rhamnopyranoside (14).

Page 186: ESI MS of 2-Aminoethyl $\alpha$-L-rhamnopyranoside (15).
\(^1\)H NMR of (5-Cholesten-3α-yl oxy)-3\textsubscript{a} tria undecanyl-2,3,4-tri-\(O\)-acetyl-\(\alpha\)-L-rhamnopyranoside (2).
$^{13}$C NMR of (5-Cholen-3α-yloxy)-3α-triaundecanyl-2,3,4-tri-O-acetyl-α-L-rhamnopyranoside (2).
$^1$H-gCosy of (5-Cholesten-3α-yloxy)-3m$_3$-trixaundecanyl 2,3,4-Tri-O-acetyl-α-L-rhamnopyranoside (2).
HRMS of (5-Cholesten-3α-yloxy)-3α-triaundecanyl 2,3,4-Tri-O-acetyl-α-L-rhamnopyranoside (2).
$^1$H NMR of (5-Cholesten-3α-yloxy)-3u$_3$-triaundecanyl Rhamnopyranoside (3).
$^{13}$C NMR of (5-Cholesten-3α-yloxy)-3α-trixaundecanyl Rhamnopyranoside (3).
$^1$H-gCosy of (5-Cholesten-3α-yloxy)-3n$_3$-triaundecanyl Rhamnopyranoside (3).
HRMS of (5-Cholesten-3α-yloxy)-3α-trixaundecanyl Rhamnopyranoside (3).
$^1$H NMR of $N$-Propargyl Pam$_2$FmocCys Amide Derivative (5).
$^{13}$C NMR of N-Propargyl Pam$_2$FmocCys Amide Derivative (5).
$^1\text{H-gCosy of \textit{N-Propargyl Pam}\textsubscript{2}FmocCys Amide Derivative (5).}$
HRMS of N-Propargyl Pam₂FmocCys Amide Derivative (5).

Calculated M+Na 953.6053
Measured M+Na 953.6073
2.1ppm

Internal Standard

TOF MS ES+
3.30e4
$^1$H NMR of $N$-Propargyl Pam$_3$Cys Amide Derivative (6).
$^{13}$C NMR of N-Propargyl Pam$_3$Cys Amide Derivative (6).
$^1$H-gCosy of N-Propargyl Pam$_3$Cys Amide Derivative (6).
HRMS of $N$-Propargyl Pam$_3$Cys Amide Derivative (6).
HR-MALDI-TOF of Glycopeptide Azide (9).
HR-MALDI-TOF of Glycopeptide Azide (10).

Calculated M+H 2229.0895
Observed M+H 2229.0959
2.5 ppm
HR-MALDI-TOF of Lipopeptide (11).
$^1$H NMR of 2-Azidoethyl-2,3,4-Tri-O-acetyl-$\alpha$-L-rhamnopyranoside (13).

\[ \text{Diagram of molecule with chemical shifts on the graph.} \]
$^{13}$C NMR of 2-Azidoethyl-2,3,4-Tri-$O$-acetyl-$\alpha$-L-rhamnopyranoside (13).
1H-gCosy of 2-Azidoethyl-2,3,4-Tri-O-acetyl-α-L-rhamnopyranoside (13).
HRMS of 2-Azidoethyl-2,3,4-Tri-\(O\)-acetyl-\(\alpha\)-L-rhamnopyranoside (13).
$^1$H NMR of 2-Azidoethyl α-L-rhamnopyranoside (14).
$^{13}$C NMR of 2-Azidoethyl α-L-rhamnopyranoside (14).
$^1$H-gCosy of 2-Azidoethyl $\alpha$-L-rhamnopyranoside (14).
HRMS of 2-Azidoethyl α-L-rhamnopyranoside (14).
ESI MS of 2-Aminoethyl α-L-rhamnopyranoside (15).