

SYNTHESIS AND IMMUNOLOGICAL EVALUATION OF A THREE-COMPONENT
CANCER VACCINE CANDIDATE

by

SAMPAT INGALE

(Under the Direction of Geert-Jan Boons)

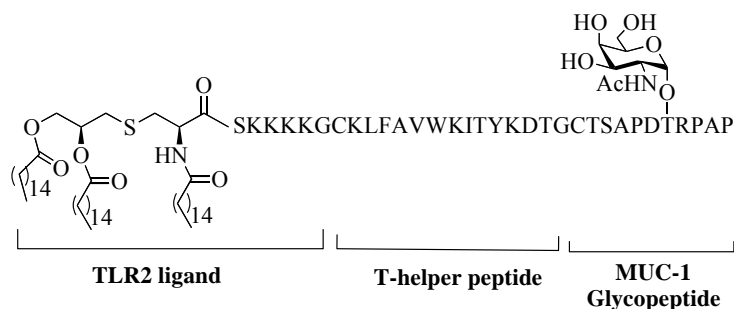
ABSTRACT

Cancer is an invincible menace that contributes to about 7 million deaths annually, or 12.5% of total annual deaths worldwide. However, advances in surgery, chemotherapy and radiation therapy have reduced mortality rates. In this thesis, a novel approach towards the development of a fully synthetic carbohydrate-based cancer vaccine for various forms of human cancers is presented which contains all the necessary components required for cancer immunotherapy.

Although the idea of vaccination on a mass scale is more than 200 years old, carbohydrate-based cancer vaccines are still in a developmental and experimental stage. Carbohydrate-based cancer vaccine development has been complicated by the difficulty of eliciting high titers of IgG antibodies in patients. It appears that the induction of IgG antibodies against a tumor-associated carbohydrate is much more difficult than the induction of similar antibodies against viral and bacterial carbohydrates because carbohydrates are auto-antigens and well tolerated by the immune system. Also, carbohydrates are poor immunogens. Some of the obstacles that inhibit the development of a carbohydrate-based cancer vaccine are the availability of pure oligosaccharides, and poor immunogenicity of oligosaccharides. In this research, we have developed a three-component vaccine candidate composed of a tumor-associated antigen, a

promiscuous peptide T-helper epitope and a lipopeptide adjuvant. A three-component vaccine has a number of distinctive advantages over a traditional conjugate vaccine. For example, the minimal subunit vaccine does not suffer from epitope suppression, which is a characteristic of carbohydrate-protein conjugates. Apart from providing danger signals, lipopeptide Pam₃CysSK₄ also facilitates the incorporation of the antigen into liposomes.

Finally, a highly convergent method based on sequential native chemical ligation is developed, which allows the construction of three-component vaccine candidates in an efficient manner. This method also allows studying structure-activity relationship (SAR) studies for various components of the vaccine. Besides synthetic chemistry, the research focuses vaccine formulation and immunology.



INDEX WORDS: cancer, vaccine, carbohydrate, glycopeptide, glycolipopeptide.

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DEDICATION

To my wonderful parents

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
LIST OF TABLES	viii
LIST OF FIGURES	ix
CHAPTER	
1 Introduction.....	1
1.1 Immune System.....	1
1.2 Vaccine Development Background.....	11
1.3 Immune System and Cancer.....	15
1.4 Cell Surface Carbohydrates.....	19
1.5 Research Objective.....	37
1.6 References	39
2 Solid Phase Peptide Synthesis	47
2.1 Chemical Protein Synthesis.....	47
2.2 Solid-Phase Peptide Synthesis.....	47
2.3 Chemoselective Ligation.....	54
2.4 Native Chemical Ligation	57
2.5 Synthesis of C-Terminal Peptide Thioesters	60
2.6 Chemical Synthesis of Glycopeptides.....	66
2.7 Chemo-enzymatic synthesis of Glycopeptides.....	67

2.8	References	69
3	Synthesis of Glyco(lipo)peptides by Liposome Mediated Native	
	Chemical Ligation	74
3.1	Experimental Procedures.....	86
3.2	References	98
4	Overcoming Immune Suppression of Tumor-Associated Carbohydrates by	
	Employing a Fully Synthetic Three-Component Vaccine	101
4.1	Experimental Procedures.....	118
4.2	References	130
5	Immunological Evaluation of Toll Like Receptor Ligands as a Adjuvants in a Three-	
	Component Cancer Vaccine Candidate.....	132
5.1	Experimental Procedures.....	142
5.2	References	146
6	Structure Activity Relationship of a Three-Component Cancer Vaccine	
	Candidates.....	149
6.1	Experimental Procedures.....	157
6.2	References	162
7	Conclusions.....	164

LIST OF TABLES

	Page
Table 1.1: Main types of vaccines	13
Table 1.2: Potential targets for cancer vaccines.....	17
Table 1.3: Representative cancer vaccine strategies.....	18
Table 2.1: Chemoselective ligation methods used for the synthesis of Native proteins by	
Ligation of unprotected peptide fragments	56
Table 4.1: ELISA anti-MUC1 antibody titers after 4 immunizations with various preparations.	109
Table 4.2: ELISA anti-MUC1 antibody titers one week after the third, fourth and fifth	
immunization.....	109
Table 5.1: Liposomal preparation for immunization	140

LIST OF FIGURES

	Page
Figure 1.1: Innate and adaptive immunity	1
Figure 1.2: Innate immunity and recognition	2
Figure 1.3: Types of adaptive immunity	3
Figure 1.4: Presentation of extracellular and cytosolic antigens	4
Figure 1.5: Two signal requirement for lymphocyte activation	5
Figure 1.6: Mechanisms of helper T-cell mediated B cell activation	5
Figure 1.7: Effector functions of Th1 and Th2 cells	6
Figure 1.8: Stimulation of immune response by adjuvant effect	9
Figure 1.9: Components of vaccine	14
Figure 1.10: Antigen processing by virus and cancer	16
Figure 1.11: Composition of typical mammalian cell	20
Figure 1.12: Cell surface oligosaccharides	20
Figure 1.13: Monosaccharides present in mammals	21
Figure 1.14: Biosynthesis of cell surface carbohydrates	22
Figure 1.15: Cell surface oligosaccharides involved in various functions	23
Figure 1.16: Representative examples of TACAs	25
Figure 1.17: Cartoon of MUC1 glycoprotein	26
Figure 1.18: Design of carbohydrate-based cancer vaccine	29
Figure 1.19: T _N 3-PAM cluster	32
Figure 1.20: Le ^y Serine-PAM cluster	33

Figure 1.21: MUC1-OVA ₍₃₂₃₋₃₃₉₎ vaccine conjugate.....	34
Figure 1.22: Fully synthetic vaccine candidate.....	34
Figure 1.23: Multiple antigenic glycopeptide.....	35
Figure 1.24: Regioselectively addressable functional template.....	36
Figure 1.25: Gold nanoparticles as new scaffolds for antigen presentation	36
Figure 2.1: Structures of protecting groups	53
Figure 3.1: Fully synthetic vaccine candidate 1	74
Figure 4.1: Synthetic compounds for the immunological evaluation	102
Figure 4.2: ELISA anti-MUC1 antibody titers after 4 immunization	110
Figure 4.3: Flow cytometry analysis of specific anti-MUC1 antibodies	111
Figure 4.4: Involvement of TLR2 in NF- κ B activation	112
Figure 4.5: TNF- α production by murine macrophages	112
Figure 4.6: Induction of dendritic cell maturation	113
Figure 4.7: Cellular uptake of compound 9	116
Figure 5.1: Immune response by TLRs and non-TLRs.....	133
Figure 5.2: Immune response by non-TLR alone	133
Figure 5.3: Synthetic targets to study the adjuvant effect.....	134
Figure 5.4: Immune response and role of B and T cells	135
Figure 5.5: Synthetic compounds to study the role TLR in immune response.....	136
Figure 6.1: Glyco(lipo)peptide constructs with <i>Neisseria meningitidis</i> T-helper peptide.....	150
Figure 6.2: ELISA anti-MUC1 antibody titers after 4 immunizations	151
Figure 6.3: Fully synthetic vaccine candidates with different architecture	152
Figure 6.4: Cancer vaccine candidates with MUC-1 glycopeptide containing sialic acid antigen.....	154

LIST OF ABBREVIATION

AA	any of the 20 natural amino acid
Ac	acetyl
Acm	acetamidomethyl
Ac ₂ O	acetic anhydride
APC	antigen presenting cells
BAL	backbone amide linker
Bn	benzyl
Boc	<i>N-tert.</i> -butoxycarbonyl
BSA	bovine Serum Albumin
CBD	chitin binding domain
CTL	cytotoxic T-lymphocytes
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DC	dendritic cell
DCC	dicyclohexylcarbodiimide
DCM	methylene chloride
DIPEA	diisopropylethylamine
DMAP	<i>N, N</i> -Dimethylaminopyridine
DMF	<i>N, N</i> - dimethylformamide
DMSO	dimethylsulfoxide

DPC	dodecylphosphocholine
DTT	dithiothreitol
EDT	1,2-diethane thiol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ESI-MS	electronspray ionization mass spectrometry
Et	ethyl
EtOH	ethanol
Fmoc	fluorenylmethoxycarbonyl
Fuc	fucoside
Gal	galactose
GAG	glycosylamino glycan
GalNAc	<i>N</i> -acetyl galactosamine
Glc	glucose
GlcNAc	<i>N</i> -Acetyl glucosamine
Gn.HCl	guanidinium hydrochloride
h	hours
HATU	<i>O</i> -(7-Azabenzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyl- uronium hexafluorophosphate
HBTU	2-(1H-Benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyl-uronium hexafluorophosphate
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV	human immunodeficiency virus
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
IgM	immunoglobulin M
KLH	keyhole limpet hemocyanine
LC-MS	liquid chromatography mass spectrometry
LPS	lipopolysaccharide
MAG	multiple antigenic glycopeptide
MALDI-ToF	matrix assisted laser desorption ionization time of flight mass spectrometry
MeOH	methanol
m/z	mass to charge ratio
Me	methyl
Min	minute
mM	millimolar
mmol	millimole
MCF7	human breast adenocarcinoma cell line
MHC	major histocompatibility complex
MS	molecular sieves
MUC1	mucin 1 glycoprotein
NCL	native chemical ligation

NF- κ B	nuclear factor-kappa B
NMP	<i>N</i> -methylpyrrolidone
Pal	palmitoyl
Pam ₃ Cys	<i>N</i> -palmitoyl- <i>S</i> -[2,3-bis(palmitoyloxy)-propyl]-(<i>R</i>)- cysteine
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
Ph	phenyl
PRR	pattern recognition receptors
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
PV	poliovirus
RAFT	regioselectively addressable functionalized template
RP-HPLC	reversed phase high performance liquid chromatography
SARS	severe acute respiratory syndrome
SDS	sodium dodecyl sulfate
SK-MEL-28	human melanoma cells
SPPS	solid phase peptide synthesis
SUV	small unilamellar vesicles
TAA	tumor associated antigen
TACA	tumor-associated carbohydrate antigen
TCEP	tris-(2-carboxyethyl)phosphine
TCR	T-cell receptor

TEM	transmission electron microscopy
TFA	trifluoroacetic acid
Th1/Th2	T-helper cell 1/ T-helper cell 2
THF	tetrahydrofuran
TFE	trifluoroethanol
TIS	triisopropylsilane
TLC	thin layer chromatograph
TLR	toll like receptors
Tn	<i>N</i> - α -Fmoc-Thr-(AcO ₃ - α -D-GalNAc)-OH
TNF- α	tumor necrosis factor- α
TRD	tandem repeat domain
TT	tetanus toxoid
QS-21	<i>Quillaja saponaria</i>

CHAPTER 1

Introduction

1.1 Immune system

Historically, immunity meant protection from disease and, more specifically, infectious disease. The cells and molecules responsible for immunity constitute the immune system, and their collective and coordinated response to the introduction of a foreign substance is called the immune system. The physiologic function of the immune system is defense against infectious microbes. The mammalian immune system is classified into two broad categories: innate (natural) immunity and adaptive (acquired) immunity.¹

1.1.1 Innate Immunity

Innate immunity consists of natural anatomical barriers such as skin and mucous membranes (Figure 1.1), and physiological barriers such as the elevation of temperatures and acid in digestive organs whose purpose is to kill harmful bacteria.

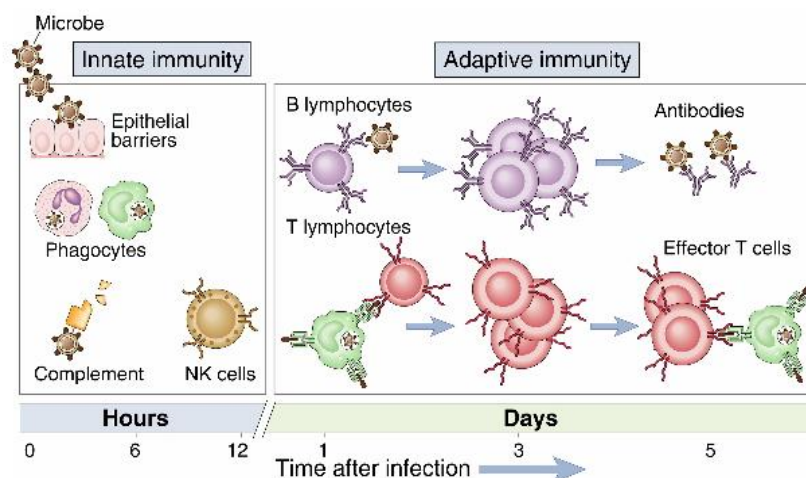


Figure 1.1 | Innate and Adaptive immunity¹

The faster-acting innate immune system is the first line of defense and reacts quickly within minutes to molecular patterns found in microbes. Later, the immune response remains triggered over days to weeks, targeted precisely to develop a specific adaptive immune response.

To distinguish from self-components the innate immune system uses a variety of relatively invariable receptors and pattern recognition receptors (PRRs) that recognize a wide range of chemical structures (Figure 1.2) conserved in pathogens (pathogen associated molecular patterns, PAMPs) such as, lipopolysaccharide (LPS), unmethylated CpG-DNA, and flagellin.²⁻⁴ PRRs are differentially expressed on a variety of immune cells including neutrophils, macrophages, dendritic cells, natural killer cells, B cells and non-immune epithelial and endothelial cells. These PRRs can be: (i) secreted molecules found in blood and lymph that are associated with complement and opsonization, (ii) surface receptors on phagocytic cells that are associated with endocytosis, or (iii) Toll like receptors (TLRs), a family of receptors found on macrophages, dendritic cells (DCs) and epithelial cells that have been conserved from insects to mammals.

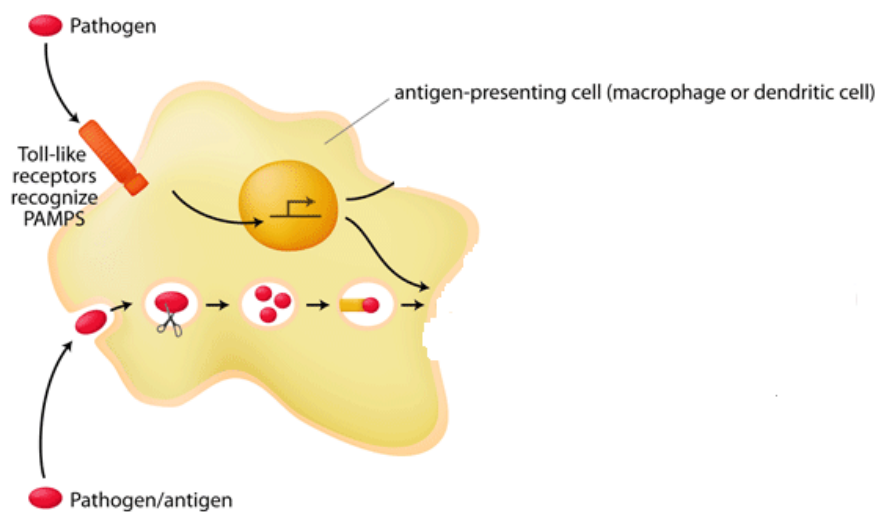


Figure 1.2 | Innate immunity and recognition⁴

Once a PAMP binds to PRR, a “danger signal” is received which leads to a cascade of events leading to the expression of cytokines, chemokines, and co-stimulatory molecules that are essential to trigger adaptive immunity.

1.1.2 Adaptive Immunity and Antigen Processing

The adaptive (or acquired) immune responses of the immune system are very specific and slower than the innate immune response.¹ Adaptive immune responses follow innate immune response and are dependent on recognition of specific antigens by antigen receptors present on B and T cells. There are two types of adaptive immunity: cell mediated immunity, and humoral immunity (Figure 1.3)^{4,5}

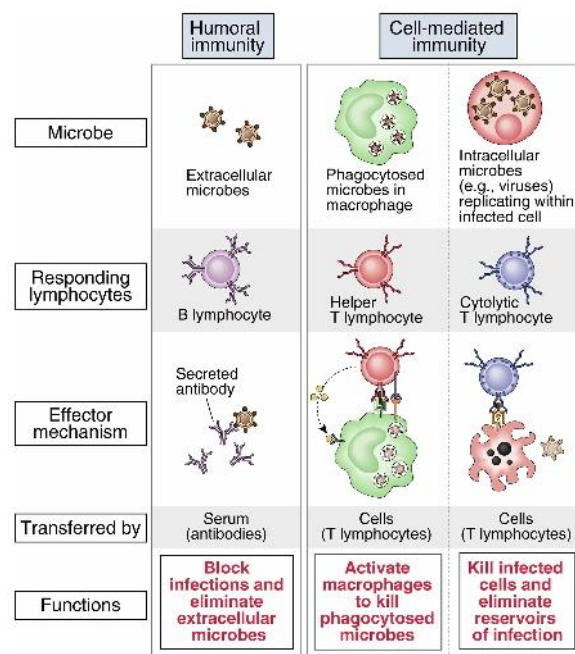


Figure 1.3 | Types of adaptive immunity¹

T-lymphocytes are responsible for cell-mediated immunity and B-lymphocytes take part in the development of humoral immunity. Adaptive immunity uses selection and clonal expansion of immune cells harboring made-to-order somatically rearranged receptor genes (T- and B-cell receptors), which recognize antigens from the pathogen, thereby

providing specificity and long-lasting immunological memory. Innate immune response leads to a rapid burst of inflammatory cytokines and activation of APCs such as macrophages and DCs. The processing and presentation of protein antigens by APCs to T is dependent on whether the antigen is intracellular (endogenous) or extracellular (exogenous) (Figure 1.4).

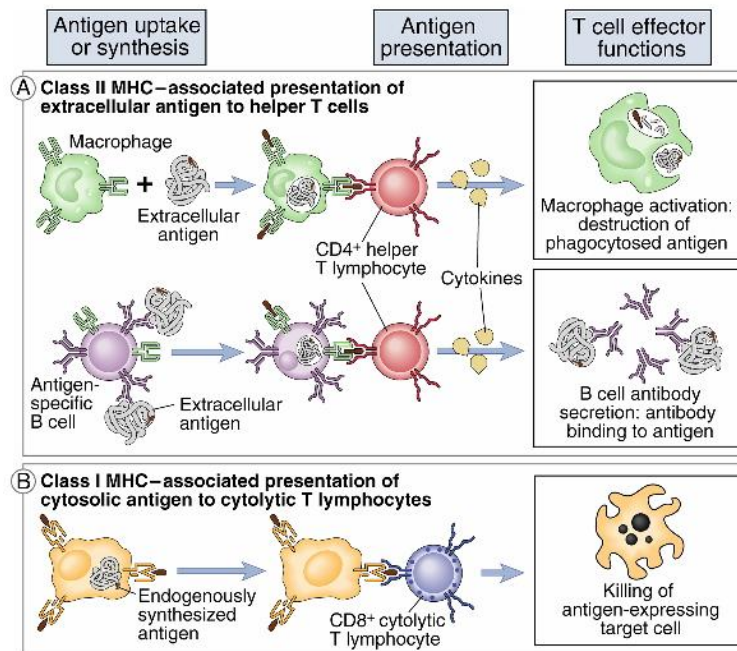


Figure 1.4 | Presentation of extracellular and cytosolic antigens¹

Endogenous antigens (e.g. viral antigens produced or delivered into the cell cytosol) are degraded into 8-13 amino acid long peptides that are transported into the endoplasmic reticulum (ER) and loaded onto Major-Histocompatibility Complex (MHC) class I to form MHC-I-peptide complexes.⁶ These complexes are exported via the Golgi apparatus to the APC surface for presentation and activation of CD8⁺ T cells.¹ Exogenous antigens (e.g. bacteria) are taken up by APCs via endocytosis and degraded in the lysosomes into peptides of 13-18 amino acids. These peptides, bound to MHC class II molecules that are embedded in the lysosome membrane to form MHC-II-peptide

complexes, are delivered by exocytosis to the cell membrane for presentation to CD4+ T cells.

1.1.3 Th1-Th2 Polarization

CD4+ and CD8+ cells recognize antigens present on APCs as complexes with MHC class II or I respectively.

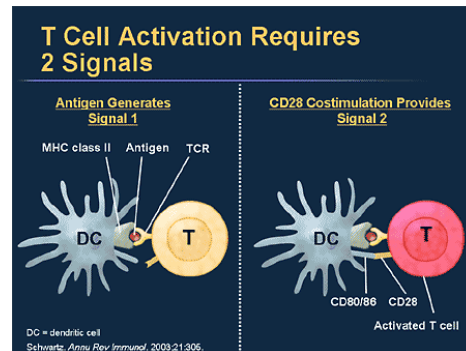


Figure 1.5 | Two signal requirement for lymphocyte activation⁷

The process mediated by the TCR leads to the activation of T cells and production of effector cells that are capable of secreting cytokines.⁵ Two signals are required for T cell activation (Figure 1.5): one derived from the interaction of the TCR with the antigen-MHC complex,⁷⁻⁹ and the other a co-stimulatory signal delivered by the interaction of ligands such as B7-1 or B7-2 present on the APCs, with the CD28 receptors on T cells (Figure 1.6).

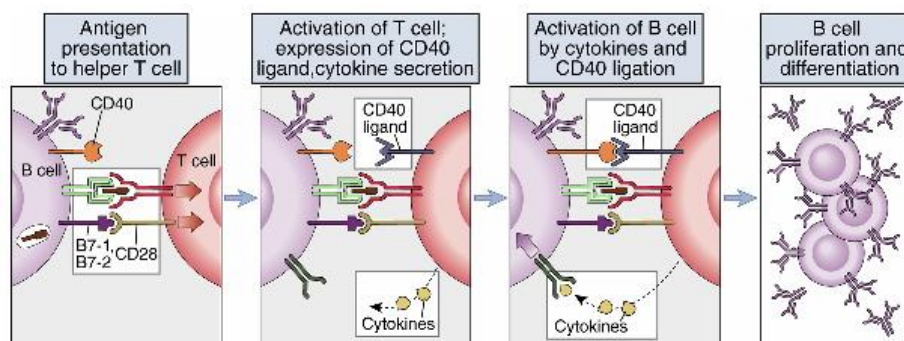


Figure 1.6 | Mechanisms of helper T cell-mediated B cell activation¹

The activated T cells produce two major types of effector cells: helper (Th), and cytotoxic (Tc) cells derived from CD4 and CD8 cells, respectively. CD4 cells interact with APCs carrying MHC-II-antigen complexes to yield Th1 or Th2 cells. This selection appears to depend on the origin of the activated DC that interacts with the CD4 T-cell. Several studies indicate that the B7-1 signal preferentially promotes the development of Th1 cells leading to production of pro-inflammatory cytokines such as interferon gamma (IFN- γ), IL-2, and tumor necrosis factor β (TNF- β), and stimulate the production of cytotoxic T lymphocytes (CTL). The B7-2 signal leads to the development of Th2 cells that produce IL-4 and IL-10 cytokines that favor antibody production and class switching (Figure 1.7), and also inhibit Th cells from entering the Th1 path¹⁰⁻¹⁷

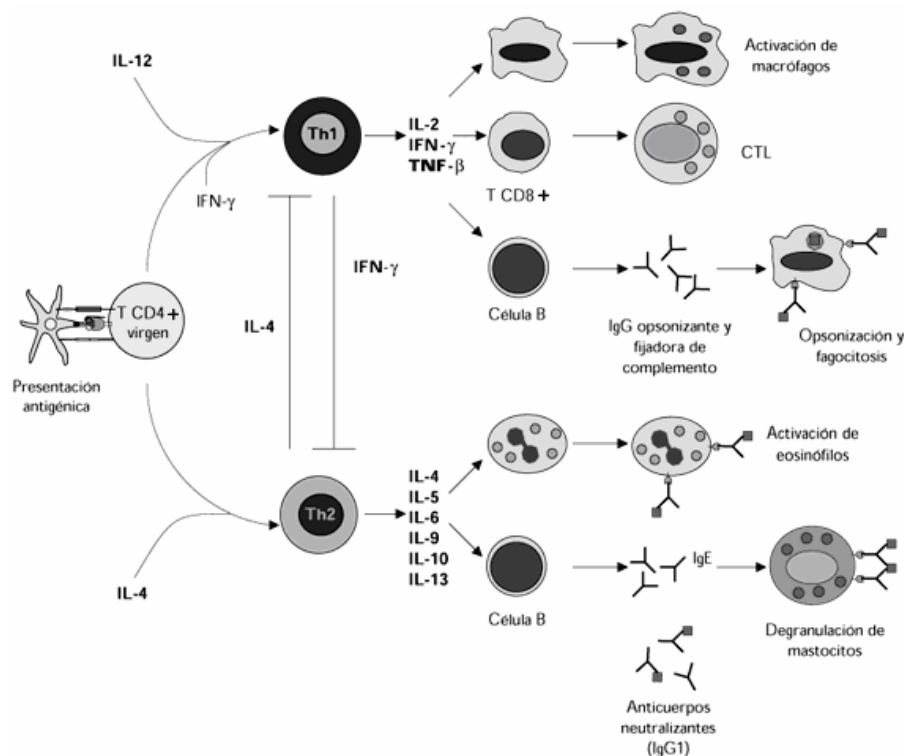


Figure 1.7 | Effector functions of Th1 and Th2 cells²⁰

Following activation, T cells express the new surface antigen CTLA-4, which binds tightly to B7 ligands and arrests T cell activation. Failure to promote a CD28- based co-

stimulatory signal leads to T cell anergy (unreactivity), which is also known as ‘immune tolerance’. As a result, modulation of this co-stimulatory signal provides an avenue for immune stimulation by adjuvants that can substitute for B7-1 or B7-2 ligands.¹⁸⁻²⁰ Thus, there is a growing interest in developing novel well-defined immunostimulatory adjuvants that *bias* the immune system toward Th1 or Th2 response.

1.1.4 Immunological Adjuvants

The term ‘adjuvant’ is derived from the Latin term ‘adjuvare’, which means to ‘help’, and is defined as a product that increases or modulates the immune response against an antigen.²¹⁻²⁴ Adjuvants are typically immune potentiators consisting of characteristic molecular patterns derived from pathogens. Adjuvants serve as powerful companions to antigens, to which the immune system is expected to mount an immune response. The influence of adjuvants on the enhancement of overall immune response to a given antigen is both quantitative and qualitative. As a result, characteristics of adjuvants designed for infectious diseases *vs* cancer vaccines may vary or differ. Despite two centuries of vaccine use, only a few adjuvants and delivery systems are licensed for human use, in part because traditional vaccines are based on live or attenuated live organisms. The invasive properties of these organisms provide efficient delivery to antigen-presenting cells and various naturally occurring components of the pathogens stimulate the innate immune system.⁶

Although dozens of different adjuvants have been shown to be effective in preclinical and clinical studies, only aluminium-based salts (Alum) and squalene-oil-water emulsion (MF59) have been approved for human use, because adjuvants used in human vaccines must fulfill stringent requirements which are rarely met. Thus, the availability of suitable

adjuvants is limited. Some of the necessary properties for an effective adjuvant include^{2,4}

1) non-toxicity or negligible toxicity at the dose range; 2) ability to stimulate a strong humoral and/or T-cell immune response; 3) good immunological memory or long-term immunity; 4) inability to induce autoimmunity; 5) lack of mutagenic, carcinogenic, teratogenic and pyrogenic properties; 6) stability at a wide range of storage conditions including duration, temperature, and pH.⁴

1.1.5 Adjuvant Classification

Adjuvants are classified according to their chemical nature, origin, and physical and chemical properties of related compounds based on immunomodulating properties or they can be classified according to their capacity to stimulate either innate or adaptive immunity.²⁵

Innate Immunity adjuvants:

- Microbial products (PAMPs) (e.g. CpG-DNA, LPS)
- Bind to broadly specific TLRs and PRRs on APCs
- Activate Nuclear Factor- κ B to:
 - Produce inflammatory cytokines
 - Increase B7 ligand production
- Adaptive immunity is activated

Adaptive Immunity adjuvants:

- Natural and synthetic products, such as cytokines, quillaja saponins, tucaresol
- Bind to highly specific receptors on T cells
- Activate T cells by
 - Co-stimulatory signals
 - Induction of cytokine-regulated genes

Furthermore, various saponins differ in their capacity to stimulate Th1 or Th2 immunity, allowing classification according to their capacity to stimulate Th1 or Th2 immunity (associated with cell mediated and humoral immunity, respectively). Adjuvants are also sub-classified according to their chemical structures to establish structure-activity

relationships that would allow the identification of pharmacophores responsible for immune modulation.

1.1.6 Mechanism of Immune Stimulation

Adjuvants exert their effects by mechanisms that may act in a concerted manner. Structural characterization of several adjuvants and the identification of their cellular receptors, such as toll like receptors (TLRs) and co-stimulatory ligand receptors, elicit better understanding of the mechanism of action of adjuvants at the molecular level.^{2, 26,27} One mechanism of action is depot formation (Figure 1.8), which is observed with alum, emulsion based, and insoluble adjuvants whereby antigens and adjuvants are sequestered at the injection site and released over time to stimulate antigen-presenting cells (APCs) such as macrophages and, particularly, DCs, which are referred to as ‘professional APCs’.²⁴

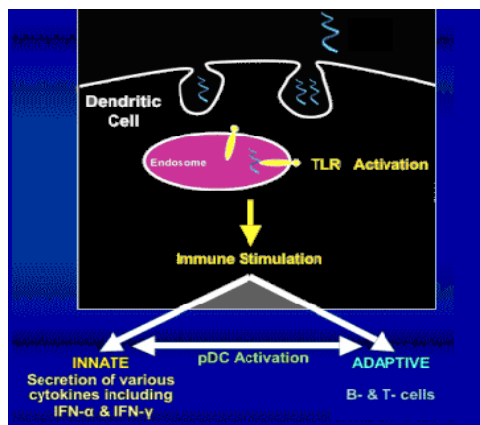


Figure 1.8 | Stimulation of immune response by adjuvant effect²⁴

Adjuvants also promote an increase in the number of antigen-specific lymphocytes in the lymph nodes. Furthermore, particulate adjuvants bind to antigens to form aggregates that are engulfed by APCs via endocytosis to form endosomes. Targeting the adjuvant to specific receptors is another mechanism by which an adjuvant-antigen complex is

delivered to APCs for processing. More effective targeting is achieved by using adjuvants with residues recognized by receptors on APCs. For example, the mannose receptor that belongs to the endocytic-Pattern Recognition Receptors (PRRs) binds compounds containing mannose, *N*-acetylglucosamine or fucose residues and sulfated oligosaccharides. Binding of these adjuvant-antigen complexes to PRRs initiates efficient receptor-mediated endocytosis and antigen processing.

1.1.7 Challenges of Adjuvant Discovery

Due to the growing need for qualitatively specific immune responses, no single adjuvant is sufficient for broad applications.^{4, 25, 28} Thus; the need for a tailor-made vaccine-specific adjuvant is likely. Available methods of delivery may be inadequate for small molecular adjuvants and therefore may require specialized formulations for the delivery of small molecular compounds into cells and retention at the injection site. On the other hand potent activation of innate immune responses may lead to toxic side effects, which may necessitate delivery systems to limit diffusion from injection sites. Intracellular targets of immune potentiators may require development of membrane permeable and metabolically stable compounds. To fully exploit our recent insight into the innate immune system, expertise in various disciplines such as chemistry, biology, high-throughput instrumentation and formulation and testing of new adjuvants are required. Regulatory guidelines on the licensing of new adjuvants are still being formulated. Uncertainties remain regarding the scope of preclinical characterization and toxicity testing that will be required for approval.

1.2 Vaccine Development Background

It has been more than two centuries since Edward Jenner first coined the term vaccine, which refers to antigenic presentation used to establish immunity. In 1798, Edward Jenner administered the cowpox virus, a crude inoculation into humans to protect against small pox infection, which revolutionized the science of disease prevention and control.²⁹

More than a century later, it was firmly established that inoculation with live attenuated or inactivated organisms could introduce a protective immunological response. It is now believed that the early vaccines devised to protect the host from bacterial and viral infections have saved more lives than all therapeutic drugs discovered in history.³⁰⁻³⁵

The majority of available vaccines are protective in nature. The main function of protective vaccines is to prime the memory of the immune system.² Vaccination, has eradicated infectious diseases such as smallpox and is expected to eradicate others such as polio. Furthermore, about twenty-six infectious diseases are preventable through vaccination, making prevention of disease by vaccination one of the most significant medical achievements of humankind.³⁶ Current approaches to vaccine development are vastly different from earlier approaches and many new vaccines have been replaced with better versions at a higher frequency than ever before, with new vaccines developed at a rapid rate of almost one per year over the past 25 years as compared to one every 5 years.³⁷

There are four major types of vaccines licensed for use in humans (Table 1.1)³⁰: (i) live attenuated vaccines, (ii) inactivated (killed) vaccines, (iii) toxoid vaccines, and (iv) subunit or component vaccines. Live attenuated vaccines usually are created from the naturally occurring organism itself, making them cost effective and easy to produce.

However, there are intrinsic risks associated with this type of vaccine as the organisms used in the vaccines can cause infection, although rarely causing serious disease. The concept of using live organisms to elicit protective immunity has been adopted for vaccines against several bacterial and viral pathogens.

A second general method of vaccine preparation is to inactivate (kill) an otherwise pathogenic organism and induce a lower level and shorter duration of immunity than that of live organisms. All antigens of the pathogen are available to the immune system, yet the organism itself is rendered harmless. Toxoid vaccines are created by treating *toxins* (or poisons) produced by organisms with heat or chemicals such as formaldehyde to destroy their ability to cause illness. Although toxoids do not cause disease, they stimulate the body to produce protective immunity in much the same way as the organism's natural toxins.

Subunit or component vaccines contain an antigenic subunit or component of the disease-causing organism. The rationale behind their design is that an antigenic fragment can create an immune response similar to that of a completely inactivated or attenuated microorganism. Although the new well-characterized antigens offer advantages in the selection of antigenic epitopes and safety, their poor immunogenicity is a general drawback. Unfortunately, the immune system does not respond strongly to these antigens, which results in an immune response insufficient to exert full protection. The poor immunogenicity of these pure antigens has created an urgent need for the identification of pharmaceutically acceptable methods capable of initiating a strong immune response.

Table 1.1 | Main Types of vaccines³⁰

Vaccine type	Selected disease targets	Vaccine preparation
Live attenuated	Smallpox Tuberculosis Yellow fever Polio Chickenpox Rotavirus Influenza	Crude preparation of cowpox infected calf skin <i>M. bovis</i> BCG grown in media Purified, attenuated virus grown in eggs Purified, attenuated virus grown in tissue culture with cells Purified, attenuated virus grown in tissue culture with cells Purified, attenuated virus grown in tissue culture with cells Purified, attenuated virus grown in eggs
Killed	Typhoid fever Plague Whooping cough Influenza Polio Hepatitis A	Inactivated <i>S. typhi</i> grown in media Inactivated <i>Y. pestis</i> grown in media Inactivated whole-cell <i>B. pertusis</i> grown in media Inactivated virus grown on eggs Inactivated virus grown in tissue culture cells Inactivated virus grown in tissue culture cells
Purified subunit	Diphtheria Tetanus Pneumococcus Meningococcus Haemophilus influenzae B Pertussis Anthrax	Inactivated toxin from <i>C. diphtheriae</i> grown in media Inactivated toxin from Clostridium tetani grown in media Polysaccharides from 23 <i>Streptococcus pneumoniae</i> strains grown in media Polysaccharides from <i>N. Meningitidis</i> strains grown in media Polysaccharides from <i>H. influenzae</i> chemically conjugated to carrier protein Acellular extract <i>B. pertusis</i> grown in media Culture supernatant of <i>B. anthracis</i> grown on media
Recombinant subunit	Hepatitis B <i>Borrelia burgdorferi</i>	Purified, recombinant HBsAg VLP produced in tissue culture cells Purified, recombinant OspA protein produced in tissue culture cells (no longer available)

Although vaccines are considered as one of the most successful medical interventions against infectious diseases, many significant obstacles remain, which include the improvement of suboptimal vaccines, development of new vaccines against several parasitic, bacterial and viral diseases such as chagas, malaria, tuberculosis and hepatitis C for which no vaccines yet exist, and the rapid response to newly emerging infections. Modern times have also brought new diseases such as HIV and SARS leading to an extensive research effort aimed at identifying components for use as a vaccine to

stimulate the immune system for encounters with a pathogen or cancer and the generation of a long lasting immunity.^{4, 21}

In designing effective vaccines, several key elements are required (Figure 1.9)^{30, 36}. First, an antigen against which adaptive immune responses are to be elicited is needed. It is important to consider antigens on the basis of their selection (in cases where there may be many to choose from, such as for bacterial pathogens) and presentation (where tertiary structure must be preserved, such as for the induction of conformation-specific antibodies). Second, immune potentiators are required to stimulate the innate immune system. Stimulation of the innate immune system is known to play an important role in the evolution of the adaptive immune system. The inclusion of immune potentiators triggers early innate immune responses to aid in the generation of robust and long-lasting adaptive immune responses, which are crucial to vaccine effectiveness. Finally, delivery systems that target the vaccine (both antigen and immune potentiators) to appropriate cells of the immune system will ensure optimal stimulation as they deliver the vaccine to the right cells at right time.

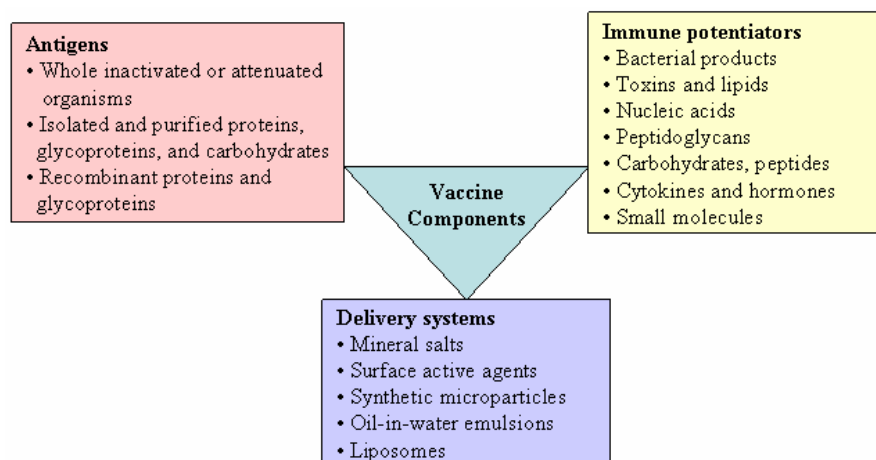


Figure 1.9 | Components of Vaccine³

1.3 Immune System and Cancer

The theory of developing immunotherapy to control cancer growth has now been shown to be partially correct, as strong immune responses against cancer cells are difficult to generate. This is because cancer cells have developed numerous ways to evade the immune system. For example, cancerous cells shed certain types of molecules that inhibit the ability of the body to attack cancer cells. As a result, cancers become less “visible” to the immune system.³⁶ Given today’s knowledge of most cell types of the immune system and their functions, it is clear that cooperation between innate and adaptive arms of the immune system is vital to the initiation of signaling processes and immunological memory against invading cancerous cells.^{36, 38-40} Therefore, the goal of a cancer vaccine is not to prevent cancer, but rather stimulate the immune system by various means. Vaccines available for the prevention of infectious diseases are prophylactic (preventive) in nature, meaning they are administered to healthy individuals to stimulate the immune system to prevent infection.^{22, 41, 42} This aim can be exemplified by progress made in the development of successful vaccines against cervical cancer, which is caused by the human papilloma virus. In contrast, most cancer vaccines are therapeutic (designed to destroy a pre existing tumor) in nature as they are administered to patients to treat cancer by stimulating the immune response to recognize and attack cancer cells without harming healthy cells. However, to date immunological responses to other types of cancer vaccines have been rarely robust enough to achieve measurable clinical responses or disease regression, possibly because not all cancers are caused by infectious agent but are rather caused by genetic defects that encode for proteins involved in cell growth. Since these proteins are very similar to those found in healthy cells, it is difficult to develop

vaccines targeting the cancer cells without harming healthy cells. Moreover, to remain healthy the immune system must be able to “tolerate” healthy cells and recognize and attack abnormal cells. The various components of the immune system do not recognize or respond to defective genes but do recognize and respond to abnormal proteins, a phenomenon which can be exploited to stimulate and boost the body’s immune system to respond through a network of specialized cells and tissues that fight infection and disease.

1.3.1 Cancer Vaccine Targets

Cancer vaccines are based on the principle that the host immune system is capable of generating immune responses and stimulating defense mechanisms against tumor cells. Although vaccine therapies for the prevention of infectious diseases have been used for centuries, potential cancer therapies are faced with particular challenges such as tumor cells already exposed to the immune system, resulting in the induction of a certain degree of tolerance within the host (Figure 1.10). Moreover, tumor cells are generally considered to be "poor immunogens."

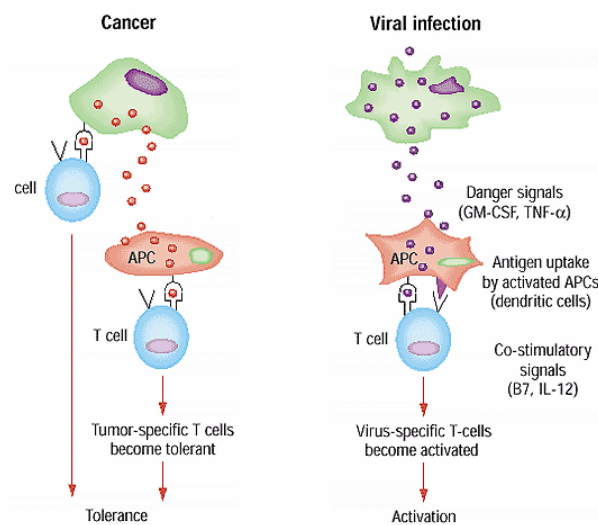


Figure 1.10 | Antigen processing by Virus and Cancer⁴⁰

As a result, approaches to cancer immunotherapy have been either to heighten the immune recognition of tumor cells by making tumor cells appears more "foreign" to the host immune system, or enhancement of the host response against tumor cells by increased lymphocytic activation. It is clear that many changes in tumor cell antigen expression potentially recognized by the host T-cells.⁴³⁻⁴⁵ Thus, identification of tumor-associated antigens (TAA) has generated renewed enthusiasm for the application of immune-based therapies to the treatment of malignancies. Tumor antigens recognized by human T-cells fall into several general categories and examples, of well-defined tumor antigens from each category are listed in Table 1.2.⁴⁴

Table 1.2 | Potential targets for Cancer Vaccines⁴⁴

Antigen Type	Tumor Antigen	Neoplasia
Tissue-specific Ag	Prostate-specific Ag	Prostate cancer
	Prostate-specific membrane Ag	Prostate cancer
	Tyrosinase	Melanoma
	Gp100	Melanoma
	α -fetoprotein	Liver cancer
Tumor-specific Ag	Immunoglobulin idiotype	B-cell NHL, Myeloma
	TCR	T-cell NHL
	Bcr-abl fusion product	CML
	Mutant p53	Lung, colorectal, head cancer,
Cancer-testis Ag	MAGE-1, MAGE-3	Melanoma, lung, colorectal
	NY-ESO-1	Melanoma, breast cancer
Over-expressed Ag	Her-2/neu	Breast, lung, ovarian cancer
	MUC-1	Pancreatic, lung, breast, colorectal cancer
Viral Ag	HPV E6/E7	Cervical, penile cancer
	EBV LMP2a	EBV + Hodgkin's disease
	HCV	Liver cancer
	HHV-8	Kaposi sarcoma

Ag = antigen, NHL = non-Hodgkin's lymphoma, CML = chronic myeloid leukemia.

1.3.2 Cancer Vaccine Strategies

In general, the goal of cancer immunotherapy is to introduce the immunizing "foreign" antigen to antigen-presenting cells (APCs) and elicit long-lasting immunologic memory from T cells. Antigens may be introduced to APCs by various methods such as

exogenous delivery of antigens (whole cell and protein vaccines), direct transduction (recombinant viral and bacterial vaccines), and direct loading of peptide fragments onto APCs. Active immunization elicits specific or nonspecific reactivity against a tumor antigen by stimulating the patient's own immune system, while *passive* immunization administers antitumor antibodies or cells against a tumor antigen.

The exquisite specificity of the immune system can be exploited to precisely target cancer cells without harming healthy cells.⁴⁹ This hope has motivated the development of novel immunologic approaches to design vaccine candidates that can target and eliminate cancer, although with limited success to date. The identification of human TAAs has made possible innovative approaches to antigen-specific vaccination.^{46-49, 50} Several approaches have been designed in defining the immunogenic epitopes of TAAs and in augmenting their immunogenicity, along with new information on the mechanisms of TAA presentation. Furthermore, appropriate routes for delivering these antigens to the immune system are being discovered in an attempt to optimize vaccination in humans. These strategies are summarized in Table 1.3.⁴³

Table 1.3 | Representative Cancer Vaccine Strategies

Vaccine	Adjuvant/Carrier
Antigenic Epitopes <ul style="list-style-type: none"> • Peptides • Gangliosides 	KLH, Detox, QS-21
Dendritic cells <ul style="list-style-type: none"> • Peptides, immunoglobulin idiotype • Tumor lysates • DNA or RNA 	KLH, Detox, QS-21, dendritic cells, Vaccinia virus, BCG, plasmids (conjugated T-cell epitopes)
Tumor cells unmodified or modified <ul style="list-style-type: none"> • Autologous • Allogenic • Mixed autologus-allogenic 	BCG, Complete Freund's adjuvant, dendritic cells, vaccinia virus, Newcastle disease virus
Tumor-APCs hybrid	Detox, Antigen presenting cells
DNA alone (naked DNA), recombinant viruses (adenovirus, vaccinia, others)	KLH, Detox, QS-21, dendritic cells, Vaccinia virus, BCG, plasmids (conjugated T-cell epitopes)

1.3.4 Requirements of Cancer Vaccine

An ideal cancer vaccine would induce a strong anti-tumor response in the host while sparing healthy tissue. Therefore, the major components required for any cancer vaccine are: 1) antigenic targets which can be either intracellular or surface antigens, and over-expressed by tumors cells but not expressed in healthy cells; 2) a platform for antigen presentation for determining the type of immune response generated; depending on the platform used both CD8⁺ and CD4⁺ T helper responses can be generated, although CD4⁺ T helper responses tend to predominant; and 3) immune stimulants/immunological adjuvants which influence the quality of the immune response and T-cell activation through the use of different adjuvants or immune-stimulants to promote antigen presentation.

Certain carbohydrate epitopes are often over-expressed on the surface of transformed cells than that of healthy cells. The developmental reasons for the over-expression of these carbohydrate epitopes are not completely understood. The isolation and identification of differential tumor associated carbohydrate antigens (TACAs) has spurred intense research into the exploitation of these TACA for the development of a carbohydrate-protein conjugate or peptide conjugate as chemically homogeneous or partially homogenous synthetic anticancer vaccines.

1.4 Cell Surface Carbohydrates

Glycans are comprised of oligosaccharides; polysaccharides and carbohydrates are one of the four basic components of the cell (Figure 1.11), constituting a significant class of natural biopolymers.⁵¹⁻⁵³

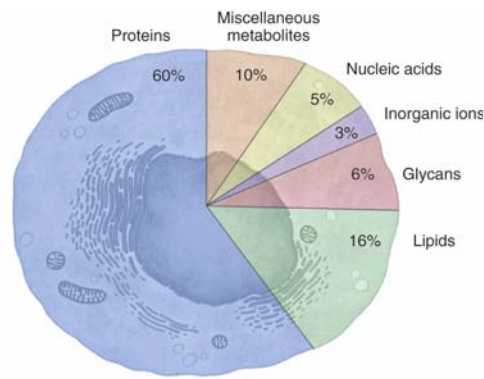


Figure 1.11 | Composition of Typical Mammalian Cell⁵¹

The biological importance of carbohydrates has become increasingly significant in the last two decades, during which they have been identified in various physiological and pathological processes. It is now an indisputable fact that carbohydrates are involved in sophisticated, and complex roles in mammals. The surface of all cells is decorated with carbohydrates (Figure 1.12). However, carbohydrates differ among the cell types and are regulated in development and differentiation.⁵⁴⁻⁵⁷

For example, cells display different patterns of carbohydrates depending on their stage of development and environment. There are ten different monosaccharides present in mammalian systems (Figure 1.13).

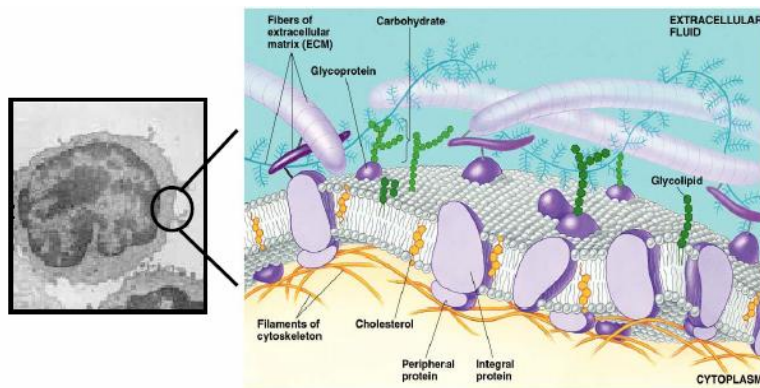


Figure 1.12 | Cell Surface Oligosaccharides

However, the structural diversity of the cell surface carbohydrates is much more complex than that of the protein molecules. Using these monosaccharides, each cell employs its metabolic pathways to manufacture a unique yet complimentary mixture of oligosaccharides. The assembly of the monosaccharides into complex carbohydrates is a straightforward process in which the monosaccharides obtained by the cell from dietary sources and recycling salvage processes are converted into nucleotide-sugar donors.^{58, 59}

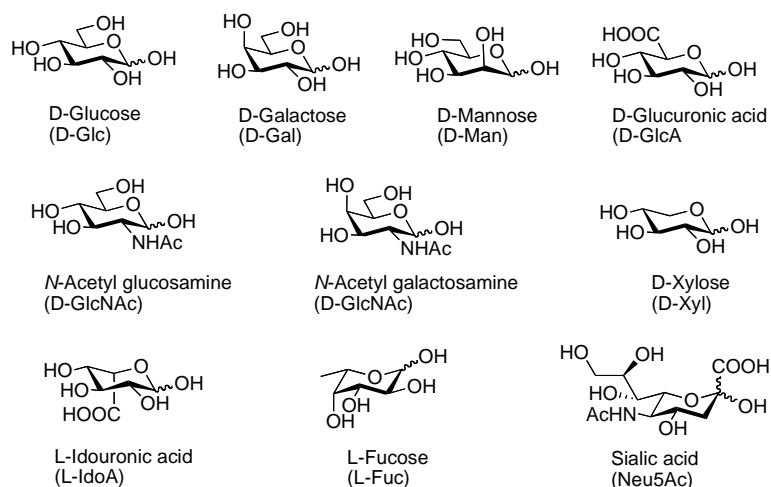


Figure 1.13 | Monosaccharides present in Mammals⁵⁸

These conversions typically entail the phosphorylation of hydroxyl groups on the monosaccharide under a sequential action of the glycosyltransferase and glycosidase enzymes. Additionally, the stereo-centers of the monosaccharides can be inverted to create related epimers, and one monosaccharide can be transformed into a different sugar. Each step in the process involves one specific glycosyltransferase, so that the exact composition of the final product is determined by allowing (or avoiding) contact with a particular glycosyltransferase. After the assembly, the obtained oligosaccharides are delivered to the surface of mammalian cells, where the diverse oligosaccharide structures exist in natural form (Figure 1.14).⁶⁰⁻⁶¹

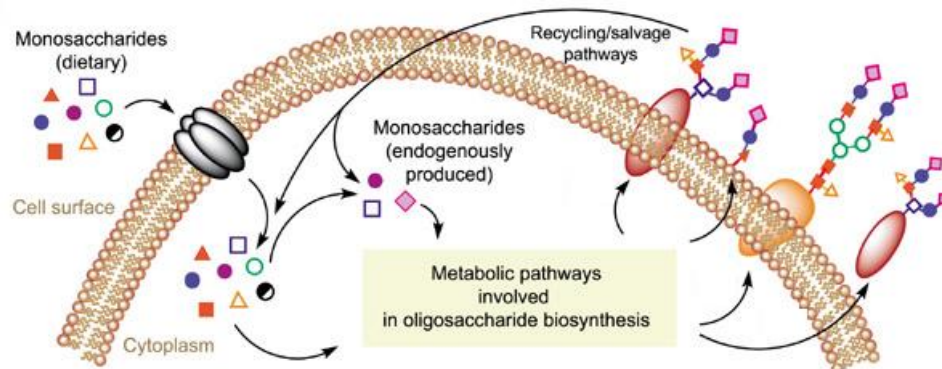


Figure 1.14 | Biosynthesis of Cell Surface Carbohydrates⁵⁸

On the cell surface, carbohydrates are presented in the form of glycoconjugates such as glycolipids, glycoproteins and Proteoglycans. Glycolipids are oligosaccharides that are covalently bound to a lipid moiety. The lipid portion of the molecule is non-polar and fits well into the hydrophobic cell membrane as an anchor, which express the oligosaccharides to the extracellular environment.⁵¹⁻⁵³

Glycoproteins are oligosaccharides that are covalently attached to the polypeptide backbone. There are two main types of protein glycosylation: *N*-glycosylation, whereby in which the glycan is attached to an asparagine residue present in a tripeptide consensus sequence (Asn-X-Ser/Thr, where X can be any amino acid except proline), and *O*-glycosylation, whereby the glycan is attached to a hydroxylated amino acid, most commonly serine or threonine residues. For *O*-linked glycoproteins, there is no common core structure, while all cell surfaces containing *N*-linked glycoproteins have an identical pentasaccharide core. Proteoglycans comprise the third group of glycoconjugates, and are composed of a protein and polysaccharide with a molecular weight of up to 30,000. The polysaccharide is a glycosaminoglycan (GAG) in which the repeating unit is a disaccharide moiety. These disaccharides typically carry a negative charge.

Cell surface carbohydrates play a central role in a wide range of biological phenomenon such as cell-cell recognition and adhesion, cell growth and differentiation, cell development, lymphocyte migration, inflammatory processes, fertilization, embryogenesis, neuronal development, hormone activities, oncogenesis and viral infection (Figure 1.15). Thus, it is necessary to understand and probe the role of these carbohydrates in specific diseases to facilitate the development of novel therapies and vaccines.⁵⁴⁻⁵⁷

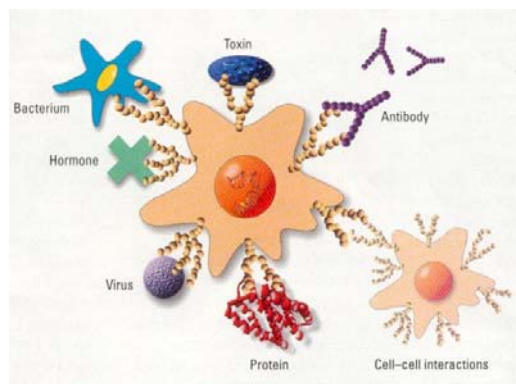


Figure 1.15 / Cell Surface Oligosaccharides involved in Various Functions⁵⁷

The cell surface carbohydrates play an important role in immune system. For example, glycoconjugates serve as a point of attachment for pathogens (bacteria, viruses, and toxins), other cells, and molecules (hormones, antibodies, and other carbohydrates). Furthermore, cell surface carbohydrates are exploited by bacterial and viral pathogens for attachment before invasion. The presentation of glycoconjugates on the cell surface is not static but rather dynamic and ever changing, a system that evolves with the development and differentiation of the cell.

Glycosylation is a process involving the most complex and diverse set of post-translationally modified structures on intracellular and secreted proteins. Changes in glycosylation are often a hallmark of disease states. For example, major changes in

carbohydrate expression occur during the onset and progression of various diseases such as cancer and rheumatoid arthritis. These disease states frequently display carbohydrates at different levels or with fundamentally different structures than those observed on normal cells, a phenomenon first described in the early 1970s. Over the past decade, advances made in genomics, proteomics and mass spectrometry now enable the association of specific glycan structures with disease states. In some cases, the functional significance of disease-associated changes in glycosylation has been revealed. In addition, the site of protein glycosylation is critical to their expression and folding and increases their thermal and proteolytic stability. Although there have been substantial advances towards understanding the effect of glycosylation in a variety of biological processes, progress towards an understanding of the specific roles of glycan structures has been limited due to their complexity and heterogeneity.⁶¹

1.4.1 Carbohydrate-Based Cancer Vaccine

The identification of distinct glycoprotein and glycolipid constructs that are over-expressed on the cell surface of malignant cells has spurred intense research into the exploitation of these tumor associated carbohydrate antigens (TACA) for the development of a carbohydrate-protein or peptide conjugate as chemically homogenous or partially synthetic anticancer vaccines. TACAs such as Globo-H, GM1 and lacto series are anchored to the cell surface by a lipid tail, while carbohydrates such as Tn, STn, TF, sialyl-Le^a (sLe^a), sLe^x, Le^y, and other antigens are linked to the protein backbone via serine or threonine residues (Figure 1.16).⁶²⁻⁶⁵

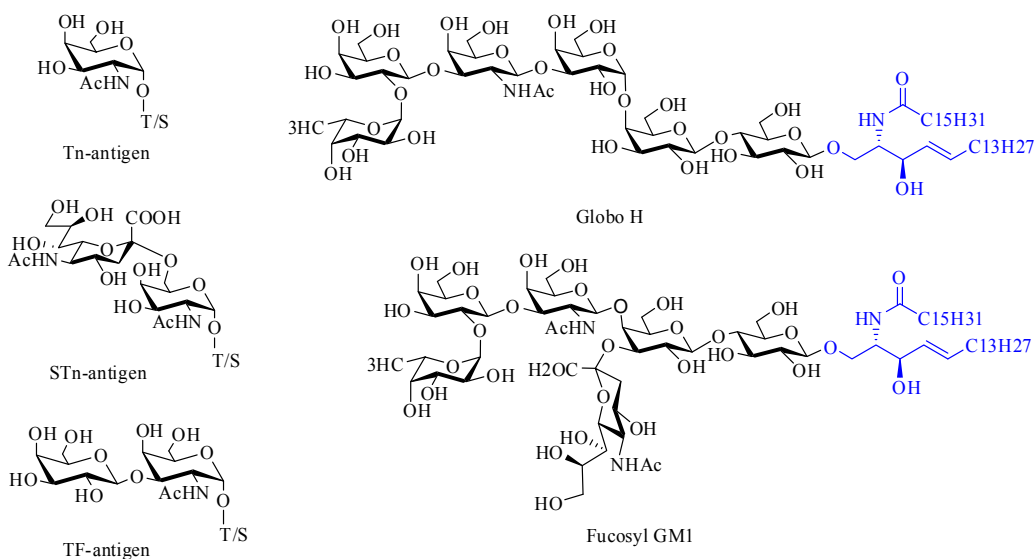


Figure 1.16/ Representative examples of TACAs⁶²⁻⁶⁵

A common feature of oncogenically transformed cells is the aberrant expression of TACAs that differ from those found on their healthy counterparts. Numerous studies show that some of these TACA can promote metastasis and are associated with a poor prognosis hence its expression is strongly correlated with the poor survival rates of cancer patients. Furthermore, abnormal glycosylation is an important criterion for the stage, direction, and fate of tumor progression. Therefore, TACAs are considered as an important target for active specific and passive immunotherapy after tumor resection and chemotherapy treatments to avoid cancer recurrence.⁶⁶⁻⁶⁸

A large number of epithelial cancers such as breast, ovarian, colorectal, pancreatic, and prostate exhibit striking alterations in the level of expression and glycosylation profile of mucins.⁶⁹⁻⁷² Mucins (from the Latin word *mucus*, meaning slime) are high molecular weight glycoproteins containing numerous *O*-linked carbohydrate side chains such as Tn, T, sialyl-Tn, and sialyl-T antigens (Figure 1.17), where they function as protective barriers and provide lubrication due to their hydration capacity. Beyond bulk properties, mucins can modulate protein functions such as intracellular trafficking and the regulation

of half-lives of chemokines and hormones as well as determine the aggregation states of membrane-bound glycoproteins.⁷³ The carbohydrate moiety of mucins can also serve as a ligand for cell adhesion. There are about twenty different mucin glycoproteins having similar overall architecture with an *N*-terminal region followed by a region containing a variable number of tandem repeat units. The variable number of tandem repeats of the different mucins can contain 8 to 23 amino acids, which are rich in serine (10%), threonine (15%) and proline (25%) residues.

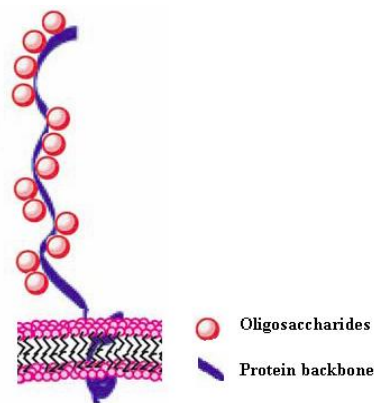


Figure 1.17 | Cartoon of MUC glycoprotein

Currently known mucins can be divided into two groups depending on their structural properties and biosynthetic routes namely membrane-bound proteins or secreted proteins.

The membrane bound glycoprotein MUC1, also referred to as episialin, polymorphic epithelial mucin (PEM), is highly over expressed by the majority of carcinomas and considered an important target in the development of efficient cancer vaccines.^{74, 75}

MUC-1 is a high molecular weight transmembrane protein with a large and highly glycosylated extracellular domain consisting of a variable number of tandem repeats of twenty amino acids, specifically TAPPHAGVTSAPDTRPAPGS, of which each repeat has five potential sites for *O*-glycosylation, namely two serine and three threonine residues. The structural diversity created by the branched *O*-oligosaccharide chain is

immense, as about 50-80% of the high molecular weight of MUC-1 results from the *O*-linked oligosaccharide side chains whose main components are *N*-acetylgalactosamine, galactose, fucose, *N*-acetylglucosamine, and neuraminic acid.^{76, 77}

Many TACAs are embryonic in origin or expressed at low levels in normal tissues and elevated levels on tumors. Thus, eventual development of a cancer vaccine is based on the theory that it may be possible to stimulate the immune system to target cancer cells on the basis of their differential glycosylation patterns on the cell surface.

Despite the significance of TACAs in cancer immunotherapy, there are mechanisms that allow these antigens to be seen as “self” by the immune system, in which case B cells expressing high-affinity antibodies for these structures would have been eliminated during development.⁷⁸ The aim of cancer immunotherapy is to override the suppressive mechanisms that enable cancer cells to avoid the immune system and to potentiate existing immune responses against cancer cells expressing TACAs. Furthermore, Carbohydrates are typically poor immunogen, difficult to purify in large quantities, difficult to synthesize, and usually result in short lived IgM-type antibodies. Thus, early studies on the development of cancer vaccines have relied on upon biological approaches, including the use of immunogens expressing TACAs based on cancer cell lines, recombinant proteins, plasmid DNA, and viral vectors. Although some evidence of efficacy is reported, most of these attempts are not successful owing to microheterogeneity of natural glycoproteins, leading to non-specific immune responses.

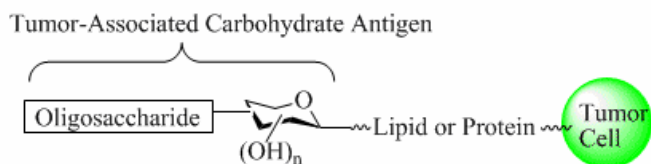
Fortunately, great strides have been made during the last decade to synthesize these complex carbohydrates through chemical and chemo-enzymatic methods. As a result, complete carbohydrate epitopes can be obtained in high purity and relatively large

amounts by organic synthesis. Furthermore, organic synthesis allows the incorporation of various functionalities with unique reactivity that allows selective conjugation to a carrier protein.⁷⁹⁻⁸⁶ Although, the use of peptides, carbohydrates, and glycopeptides in cancer immunotherapy is an attractive idea, TACAs exhibit low immunogenicity because of their inability to activate helper T-lymphocytes has complicated their development as vaccines. For most immunogens, including carbohydrates, antibody production depends on the cooperative interaction of two type's lymphocytes, the B-cells and helper T-cells. Saccharides alone cannot activate helper T-cells and therefore have a limited immunogenicity as manifested by low affinity IgM antibodies and the absence of IgG antibodies. In order to overcome the T-cell independent properties of carbohydrates, past research has focused on the conjugation of saccharides to a foreign carrier protein such as KLH (Figure 1.18),⁸⁷⁻⁹⁰ tetanus toxoid, and BSA.^{91, 92}

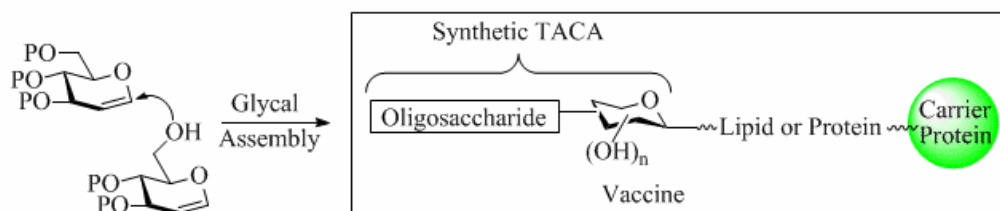
In this approach, the carrier protein enhances the presentation of the carbohydrate to the immune system and provides T-epitopes (peptide fragments of 12-15 amino acids) that can activate T-helper cells. As a result, a class switch from low affinity IgM to high affinity IgG antibodies can be accomplished. The first approved human vaccine utilizing this approach to prevent disease from *Haemophilus influenzae* type b (HiB) infection has been commercialized in Cuba.⁹³

Danishefsky *et al* have extensively exploited the differential expression of TACAs targets for cancer immunotherapy. They have chemically synthesized monomeric oligosaccharides comprising antigens Fucosyl GM1, Le^y and Globo-H, as well as multivalent oligosaccharides bearing copies of the same antigen, such as the T_N, T_F, ST_N, and Le^y antigens.^{64, 87-89}

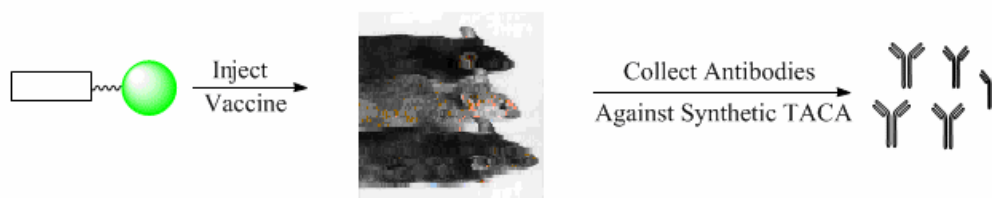
a) Cell surface glycoproteins and glycolipids displaying TACA



b) Design of a carbohydrate based antitumor vaccine



c) Induction of antibodies to synthetic TACA



d) Evaluation of reactivity of antibodies against cell surface TACAs



Figure 1.18 | Design of Carbohydrate-based Cancer Vaccine⁶⁴

These compounds are equipped with an anomeric allyl moiety that allowed selective conjugation to the carrier protein KLH, are being investigated in clinical trials against several cancer types, including breast, prostate, ovarian and small-cell lung carcinomas. Overall, these conjugates have raised high titers of IgM and low titers of IgG antibodies, which could induce a complement-mediated lysis of cancer cells and prolonged disease-free periods for patients. A number of other research groups have also reported elegant syntheses and immunological evaluations of TACAs.⁹⁴⁻¹⁰³

Mucins also constitute potential targets for cancer immunotherapy, because of their expression in many human carcinomas.¹⁰⁴⁻¹⁰⁶ For example, the tumor-associated forms of the MUC1 exhibit altered levels of glycosylation within the 20 amino acid tandem repeat domain (TRD). The immunogenicity of the MUC1 glycoprotein has been extensively studied for non-glycosylated as well as glycosylated backbones of protein or peptide. A comparative immunogenicity studies directed at exploring the optimal vaccine design for T_N-targeted immunotherapy revealed that glycosylation at three or five sites of the TRD of MUC1 conjugated to KLH induced strong antibody response against T_N and tumor cells expressing T_N antigens. Furthermore, a 32 mer MUC1 peptide glycosylated at three sites per TRD produced significantly higher anti-Tn antibody titers than a Tn-KLH conjugate having cluster of T_N (T_N(c)-KLH). This observation is attributed to the multivalent effect. Kunz *et al*^{92, 107-109} has developed anti-cancer vaccines based on defined synthetic T_N, T, and sialyl-T_N glycopeptides from MUC1. These constructs are derived from glycopeptide sequence from the MUC1 tandem repeat carrying a sialyl-Tn side chain conjugated through a polar spacer amino acid with a partial T-cell epitope from tetanus toxoid. To get the strong T-helper-cell-dependent antibody production that is crucial for the development of an efficient anticancer vaccine they have conjugated glycopeptide antigens to carrier proteins such as BSA or KLH. MUC1-KLH conjugate constructs induce antibodies that are highly reactive with glycopeptides, but only weakly reactive with carbohydrate haptens and the non-glycosylated peptide.

1.4.2 Limitations Carbohydrate-Conjugate Semi-Synthetic Vaccines:

A number of carbohydrate-protein conjugate have been examined in preclinical and phase I, II, and II clinical trials. The results indicate that carbohydrate-conjugate vaccines

are most promising when used in combination with a potent immunostimulator such as QS-21. However, in an optimized immunization protocols it was difficult to induce high levels of high affinity IgG antibodies in most patients. It appears that the induction of IgG antibodies against TACAs is much more difficult than the induction of similar antibodies against viral and bacterial carbohydrate antigens. This could be because TACAs are auto-antigens and consequently well tolerated by the immune system. The shedding of carbohydrates by the growing tumor reinforces this tolerance. Furthermore, the conjugation of a carbohydrate to a carrier protein also introduces several new problems. In general, carbohydrate-protein conjugation chemistry is difficult to control as it results in conjugates with ambiguities in composition and structure, which may affect reproducibility of an immune response. In addition, a foreign carrier protein such as KLH can elicit a strong B-cell response, which may lead to suppression of an antibody response against the carbohydrate epitope. Also, linkers used for the conjugation of carbohydrates to proteins can be immunogenic, leading to epitope suppression.¹¹⁰⁻¹¹²

With the above in mind, it is clear that the successful development of carbohydrate-based cancer vaccines requires novel strategies for the more efficient presentation of tumor associated carbohydrate epitopes to the immune system and results in a more efficient class switch from IgM to IgG antibodies. There are relatively few examples of vaccine constructs that incorporate such epitopes in fully synthetic vaccines without the inclusion of carrier proteins.

1.4.3 Totally Synthetic Vaccines

Fully synthetic vaccines are of great interest to cancer immunotherapy as they are comprised of pure chemically defined homogenous structures. Some of the synthetic

vaccines tested in mice are based on glycopeptide structures, whereby researchers have developed linear and dendrimeric glycopeptides in which the TACA is associated with a peptide containing a CD4⁺ T-cell epitope to allow the T and B cell cooperation required for increasing the level and affinity of the antibody response. Such glycopeptides containing a T_N-antigen and murine T cell epitope (poliovirus peptide, PV) have shown production of IgG antibodies in mice that can recognize T_N antigens on the surface of human tumor cells. Different linear glycopeptides based on clusters of three (T_N3, Figure 1.19) and six (T_N6) Tn have also been synthesized and shown to induce high levels of anti-Tn antibodies.¹¹³⁻¹¹⁵

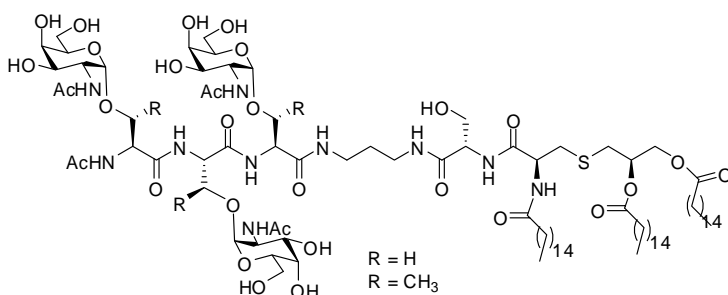


Figure 1.19 | T_N3-PAM Cluster¹¹³⁻¹¹⁵

Other fully synthetic vaccines are based on tripalmitoyl-S-glyceryl-cysteinyserine (PAM), which is potent immune activator acting through TLR2. PAM is derived from the immunologically active N-terminal sequence of an *E.coli* lipoprotein that has been widely used to augment the immunogenicity of peptides and carbohydrates. When glycopeptides containing the Le^y-serine (Figure 1.20)¹¹⁶ or the T_N-serine epitopes conjugated to the PAM moiety were administered to mice together with QS-21, they induced both IgG and IgM antibodies. Interestingly, such a compound containing a cluster of three contiguous Le^y serine epitopes was found to be superior to the mono Le^y serine construct in eliciting anti-tumor antibodies. Mice immunized with T_N(c)-PAM also

developed anti-T_N IgM antibodies, antibodies found to be less effective than those of T_N(c)-KLH. However, these glycoconjugates have never yet been tested for their capacity to promote further tumor rejection.

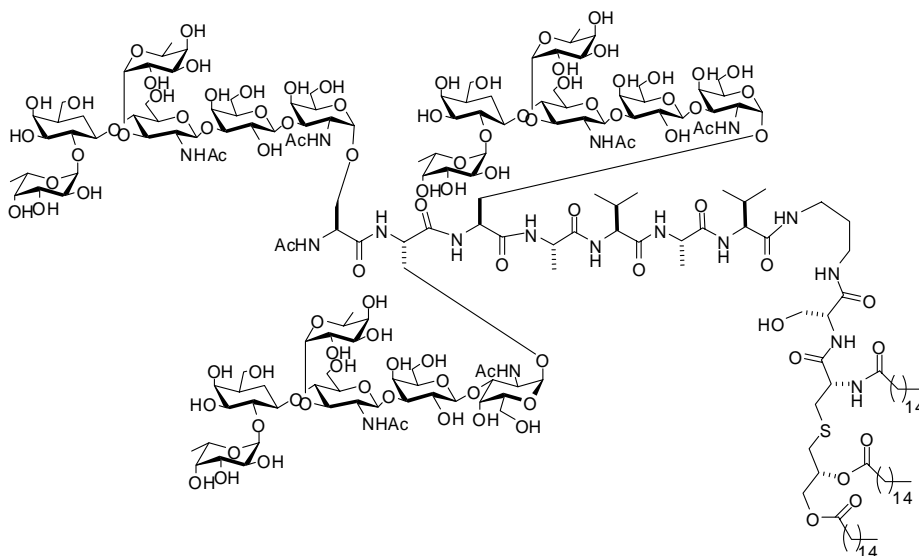


Figure 1.20 | Le^Y Serine-PAM Cluster¹¹⁶

Recently, Kunz *et al*⁷⁵ reported the construction of anticancer vaccine in which a tumor-associated sialyl-T_N (ST_N) glycopeptide antigen from the tandem repeats region of MUC1 is connected to a T_H-cell peptide from ovalbumin (OVA₃₂₃₋₃₂₉) (Figure 1.21). Immunization of mice with this construct in combination with Freund's adjuvant gave high titers of IgG antibodies response in one third of the mice. These antibodies had high specificity for the exact structure of the MUC1 glycopeptide antigen, but neither to the non-glycosylated peptide of identical peptide sequence, nor the ST_N saccharide linked to the TRD.

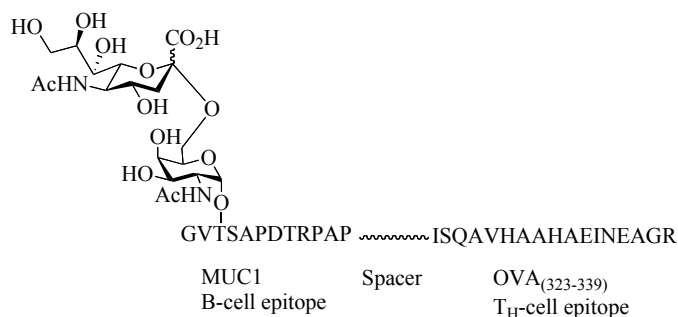


Figure 1.21 | MUC1-OVA₍₃₂₃₋₃₃₉₎ vaccine conjugate⁷⁵

The first fully synthetic three-component glycopeptide cancer vaccine is reported by our group (Figure 1.22)¹¹⁷ has a tumor associated carbohydrate antigen, as a B-epitope, a universal T-cell epitope that has been identified as being MHC class II restricted for human, and the lipopeptide adjuvant PAM.

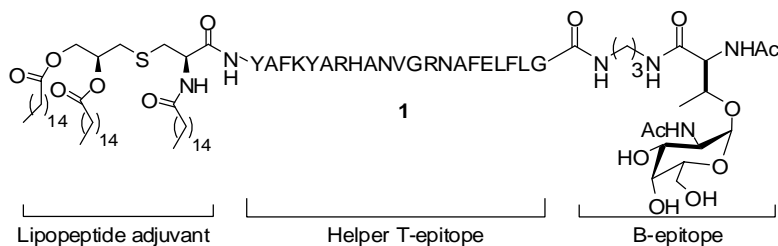


Figure 1.22 | Fully synthetic vaccine candidate¹¹⁷

Mice immunized with the liposomal preparation of this vaccine construct elicited moderate titers of IgG and IgM antibodies against T_N antigen. The presence of IgG antibodies indicates that the activation of helper T-lymphocytes by helper T-epitope peptide. Furthermore, the IgG antibodies were raised by mice which were immunized with liposomal preparation of vaccine construct indicate that the built-in adjuvant PAM had triggered appropriate signals for the maturation of DC and their subsequent activation of helper T-cells. Co-administration of liposomal preparation of vaccine construct with QS-21 elicited high titers of anti T_N-antibodies.

1.4.5 New Delivery Vehicles for Cancer Vaccines

Most commonly used vehicles for the delivery of potential cancer vaccines candidates have relied upon liposomal formulations using the lipopeptide PAM or carrier proteins such as KLH and BSA. Cytokines are also considered valuable in cancer immunotherapy; however, their potential as carrier molecules in the context of cancer vaccine is still under evaluation. The multiple antigen glycopeptide (MAG) is based on non-immunogenic lysine core capable accommodating four copies of the antigen (MAG: TN) have been synthesized (Figure 1.23).¹¹⁸

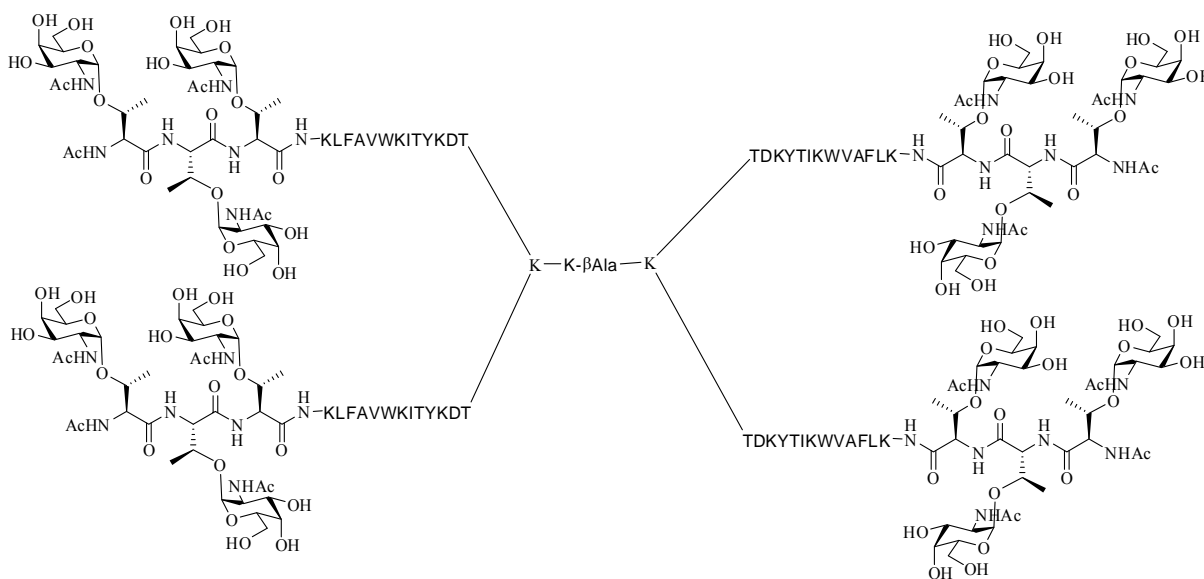


Figure 1.23 | Multiple antigenic glycopeptide (MAG)¹¹⁸

The MAG constructs, in addition to being multivalent, are multi-component vaccines displaying the TN antigen as a cluster on a peptide motif (B-cell epitope) and peptide fragments such as those derived from the poliovirus (PV) and tetanus toxoid (TT), both CD4⁺ T-cell epitopes. Immunization studies with mice, a MAG: T_N3-PV construct found to be a superior vaccine compared with a KLH conjugate bearing the T_N-cluster.

An extension of the MAG technology uses regioselectively addressable functionalized templates (RAFTs) as new scaffolds for the development of cancer vaccines. (Figure 1.24). Immunization of mice with these constructs resulted in the formation of IgG antibodies that are able to recognize native form of the T_N-antigen in the human tumor cell line.¹¹⁹

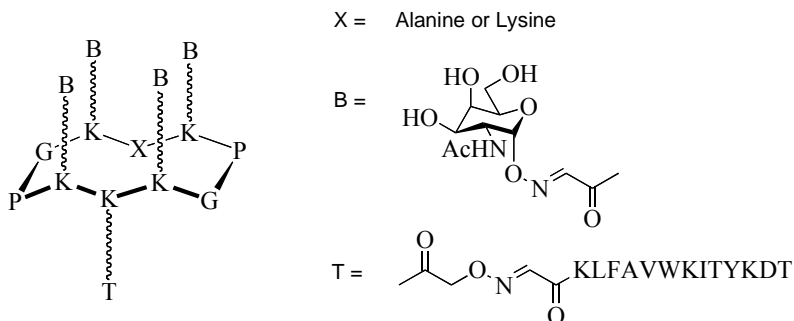


Figure 1.24 | Regioselectively addressable functional template (RAFT) vaccines¹¹⁹

Perhaps the most exciting new development in the multivalent presentation of TACAs in cancer vaccine therapies relies on the use of nanotechnology (Figure 1.25). Although exciting attempts to characterize this construct have failed because of problems associated with insolubility.¹²⁰

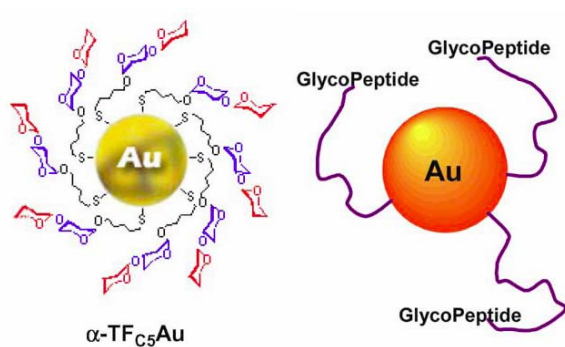


Figure 1.25 | Gold nanoparticles as new scaffolds for antigen presentation¹²⁰

1.5 Research Objective

The goal of the project described in this thesis is the design of a cancer vaccine candidate that will generate a specific immune response by using chemically well-characterized synthetic antigens as immunogens found on the surface of tumor cells. In this thesis we describe the synthesis and immunological evaluation of a fully synthetic three-component construct that is based on our previously reported vaccine candidate. We anticipate that several improvements could be made. For example, it has found that antibodies against T_N-antigen poorly recognize cancer cells. Clustering or presentating the T_N antigen as part of MUC1 elicits antibodies with improved binding characteristics. The T-epitope employed in this construct is known to be a MHC class II restricted epitope for humans. Thus, a more efficient class switch to IgG antibodies is expected when murine T-epitope is used. Furthermore, it has found that the lipopeptide Pam₃CSK₄, is more potent immunoadjuvant than Pam₃Cys.

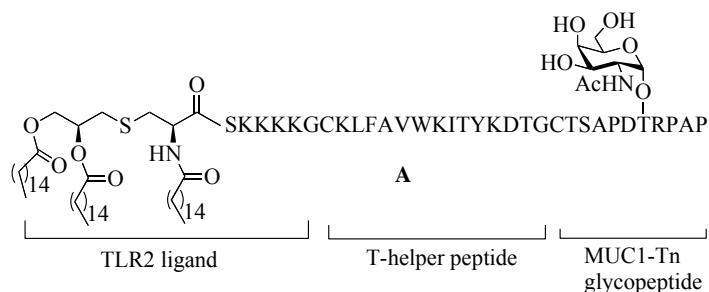


Figure 1.26 | Fully synthetic three-component vaccine candidate

Thus, based on these considerations, vaccine constructs **A** is designed (Figure 1.26), which contains as a B-epitope the MUC1 glycopeptide, the well-documented murine helper T-cell peptide KLF_{AVWKITYKDT} (KLF) derived from polio virus, and the lipopeptide Pam₃CysSK₄.

The TACA of the fully synthetic vaccine is expected to interact with Ig receptors of a B-cell and thus direct the antibody response against this part of the vaccine. After internalization, the peptide T-helper peptide will be complexed with MHC and then presented on the cell surface of B-cells to facilitate the necessary interaction with a T-helper cell. The T-helper epitope will also be presented as a complex with MHC on the surface of antigen presenting cells, which will result in the activation of the naïve T-cell. Subsequently, the activated T-cells will migrate to the T-cell zone where they will interact with B-cells. Finally, Pam₃CSK₄ is ligand for TLR2 and therefore will initiate the production of necessary cytokines. Apart from “danger signals”, the lipopeptide Pam₃CSK₄ also facilitates the incorporation of the antigen into liposomes. A liposomal formulation is attractive because it presents the antigen in a very efficient (multivalent) way to the immune system.

This project is divided into two broad components. The primary objective focuses on the development of a novel synthetic methodology for the synthesis of glyco(lipo)peptides. Large quantities of a number of glyco(lipo)peptides were synthesized. The second objective of the project is aimed at the immunological evaluation of the glyco(lipo)peptide to identify the minimum structural component necessary to increase immunogenicity and also develop structure-activity relationships within various glyco(lipo)peptides.

Since the synthetic methodology used in this thesis is based on solid phase peptide synthesis (SPPS) and glycopeptide synthesis, brief introduction to these methods is presented in chapter 2.

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CHAPTER 2

Solid Phase Peptide Synthesis

2.1 Chemical Protein Synthesis

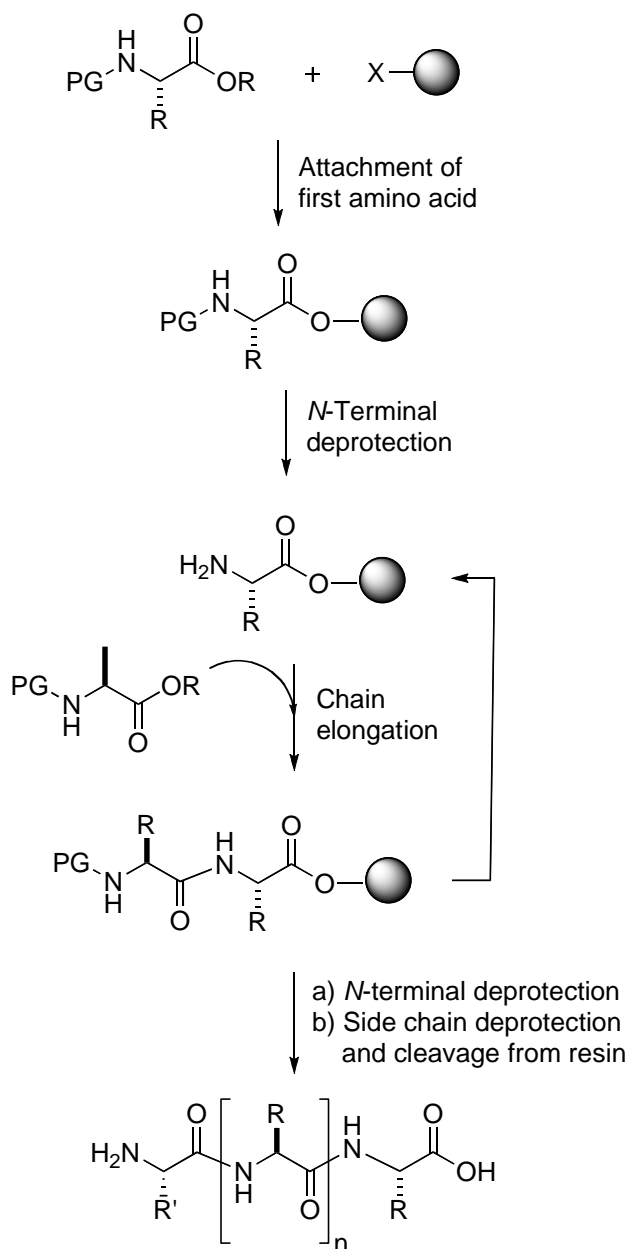
Merrifield's revolutionary idea of synthesizing peptides on a solid support as opposed to the traditional method of peptide assembly in solution provides the basis for modern chemical peptide and protein synthesis.¹ Either the entire protein is produced by solid-phase peptide synthesis (SPPS) or, more often, smaller peptide fragments are assembled by SPPS and then joined together. There are number of methods available to ligate two peptide fragments either in solution or on solid phase. Although a short introduction to modern SPPS and the predominant methods for chemically synthesizing proteins is given here, several recent reviews on this quickly evolving field of modern research are referred to.²⁻⁵

2.2 Solid-Phase Peptide Synthesis

Traditional solution-phase peptide synthesis is performed by coupling an *N*-terminally protected and *C*-terminally activated amino acid to another amino acid or peptide with an unprotected *N*-terminal in an organic solvent. If necessary, side chains of both fragments are blocked by various compatible protecting groups. After standard workup and purification, the *N*-terminal protecting group of the newly formed peptide is removed and another amino acid can be coupled following the same procedure.

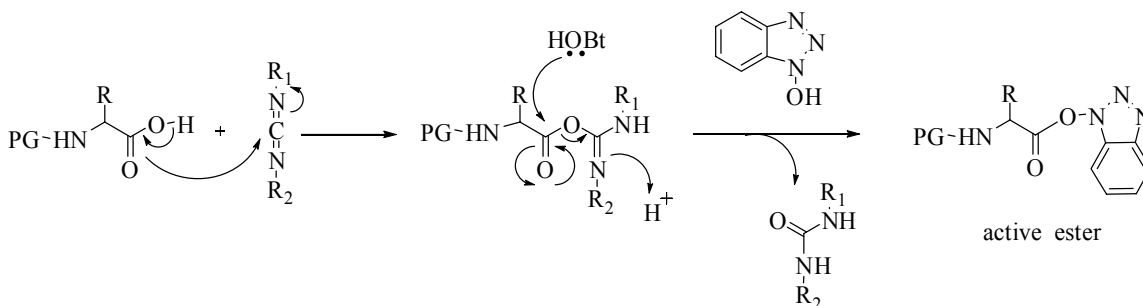
Depending on the sequence and length of the peptide, solubility problems and purification difficulties can thereafter affect the success of an attempted synthesis. These obstacles were largely overcome with the introduction of SPPS. First, byproducts can be

removed by simple filtration. Second, solubility problems are overcome by conducting the amide bond formation under heterogeneous conditions. Furthermore, the repetitive character of solid-phase peptide assembly renders it ideal for automation. Scheme 2.1 shows the basic principles of SPPS.



Scheme 2.1 | General Procedure for Solid Phase Peptide Synthesis (SPPS)

The solid support is often a copolymer of styrene and 1% divinylbenzene, functionalized with alcohol or amine and is covalently derivatized with the first amino acid or various linkers. The side chain and the amine of the incoming amino acid are protected and its C-terminus is activated, for example as an ester with a 1-hydroxybenzotriazole (HOBt, Scheme 2.2).



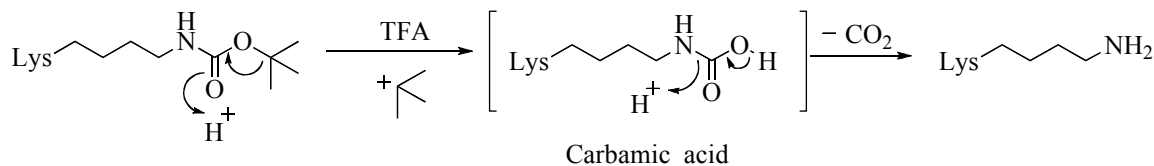
Scheme 2.2 | Activation of an amino acid to form an active ester

Numerous reagents and methods are available to activate the C-terminus⁵⁻⁶ of amino acids. After coupling is complete, the *N*-terminal protecting group is removed and the next amino acid added. After successful coupling, the cycle of deprotection and coupling is repeated until the desired peptide has been assembled. Upon the removal of the last *N*-terminal protecting group, the peptide is released from the resin and the side chain protecting groups are often cleaved simultaneously.

The crude SPPS product is then purified usually by HPLC and characterized. Modern mass spectrometric methods such as electrospray mass spectrometry (ESI-MS) and matrix-assisted laser desorption ionization (MALDI) have become increasingly important for determining the mass and sequence of the peptide.²

The Boc and Fmoc methods are two different chemical SPPS methodologies available for the synthesis of the polypeptides and their names are derived from the *N*-terminal protecting groups for amino acids, either as an *N*-*tert*-butoxycarbonyl (Boc) or 9-

fluorenylmethoxycarbonyl (Fmoc) group.⁷ Boc chemistry was developed first⁸⁻⁹ and is considered as the more traditional method. The Boc method relies on the graduated acid lability of the *N*-terminal and side chain protecting groups.⁷ The *N*^α-Boc group is removed by acidolytic cleavage (*Scheme 2.3*) by trifluoroacetic acid (TFA) during chain assembly.



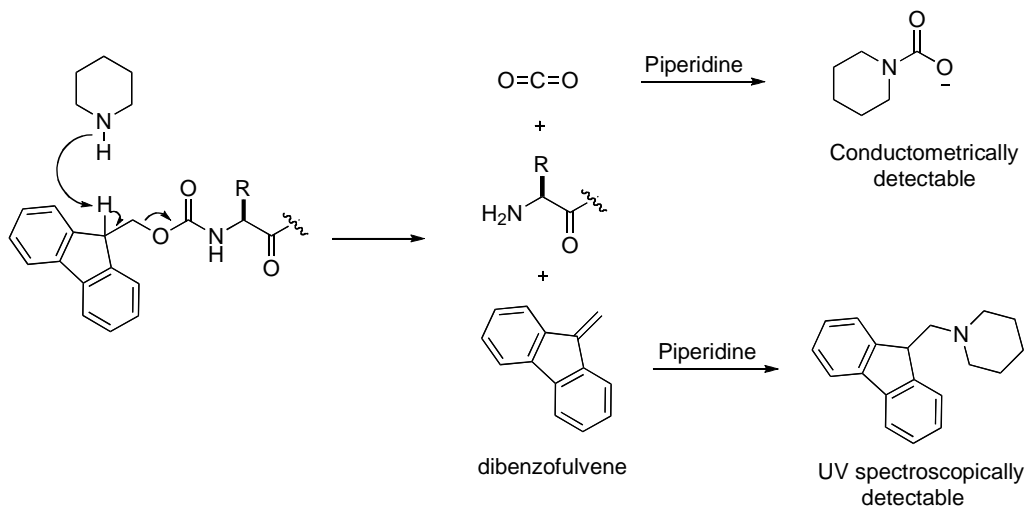
Scheme 2.3 | Deprotection of the Boc protecting group by TFA.

After each deprotection step, a neutralization step is required in order to convert the deprotected *N*-terminus from an ammonium ion into a nucleophilic amine, for which the diisopropyl ethyl amine (DIPEA) is often utilized. The incoming amino acid is often activated as a mixed anhydride or an OBt ester (*Scheme 2.2*). After the synthesis has been completed, the peptide is removed from the resin and the side chain protecting groups simultaneously cleaved by reaction with anhydrous hydrogen fluoride (HF). The use of highly toxic HF requires special precautions and represents the major drawback of Boc chemistry. A highly optimized protocol for chain assembly following Boc chemistry has been described¹⁰ and used to assemble the HIV-1 protease, a protein consisting of 99 amino acids in one stretch.¹¹

The second methodology, or Fmoc chemistry was introduced in 1978¹²⁻¹⁴ and uses repeated exposure to a secondary amine, often piperidine, to remove the *N*^α-protecting group via β -elimination.

An advantageous aspect of Fmoc-SPPS is that the progress of an ongoing synthesis can be monitored (*Scheme 2.4*). After nucleophilic attack by piperidine on the proton at C-9

of the aromatic system, a β -elimination takes place and the carbamate decomposes to dibenzofulvene, carbon dioxide and the free amine of the growing chain. Piperidine can react with CO_2 and form an ionic species.



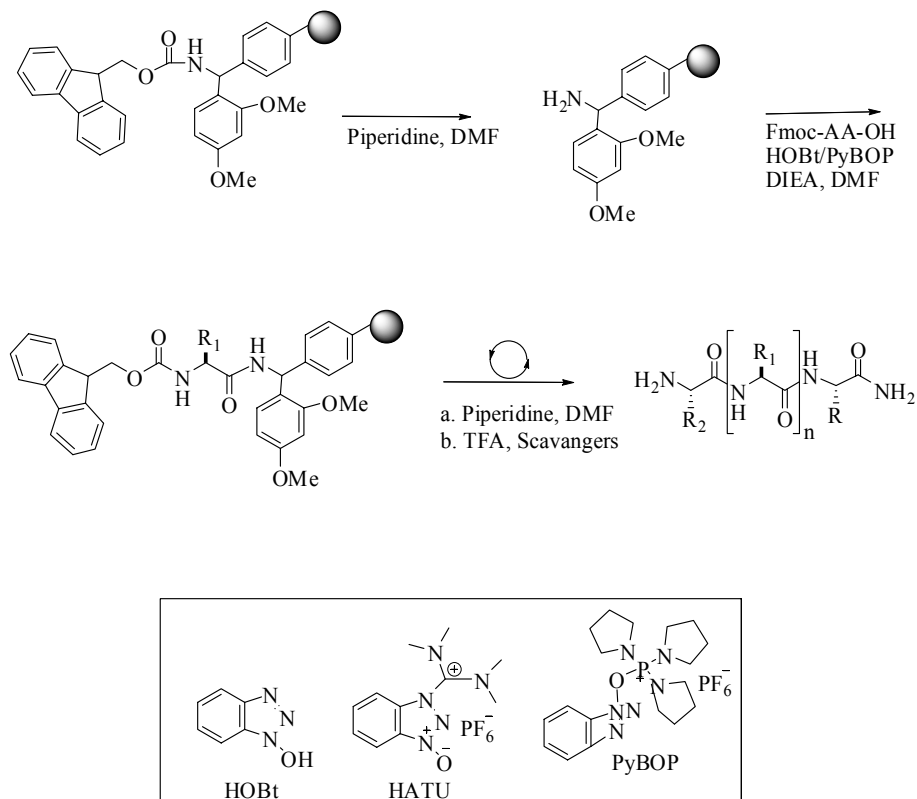
Scheme 2.4 | Deprotection of Fmoc-group.

The overall conductivity of the resulting solution is indicative of the amount of the compound present. Furthermore, piperidine nucleophilically attacks the benzofulvene to form a tertiary amine, a compound that absorbs between 300 and 320 nm, of which change in absorption can be used to monitor the release of the Fmoc group. In Boc chemistry, monitoring of the ongoing synthesis can only be achieved by the quantitative ninhydrin reaction.¹⁵

Since the peptides described in the thesis were synthesized applying SPPS according to Fmoc methodology, the conditions chosen are discussed in some detail. Scheme 2.5 provides an overview.

Removal of the Fmoc group is achieved by adding a mixture of piperidine and *N,N*-dimethylformamide (DMF). The incoming amino acid is activated prior to coupling. A 1:1 mixture of HOBt, Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium

hexafluorophosphate (PyBOP), or 2-(1-*H*-benzotriazole-1-yl)-oxy-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU) in DMF is used and the amino acid is converted to the corresponding OBt ester¹⁶ (structures of HOBt, HBTU, and PyBOP in scheme 2.5). DIPEA is added to the activation reaction, so a neutralization step after deprotection of the *N*-terminal amine is not necessary.



Scheme 2.5 | General method for Fmoc-SPPS

Side chain protecting groups are labile to TFA and released simultaneously with cleavage from the solid support and the linker shown in scheme 2.5 is the often used Rink amide, which yields peptides with a C-terminal amide upon acidolytic cleavage of the peptide-resin.

The abbreviations of the commonly used protected derivatives utilized in Fmoc chemistry are Arg(pbf), Asn(Trt), Asp(OtBu), Cys(Trt), Cys(Acm), Gln(Trt), Glu(OtBu), His(Trt),

Lys(Boc), Ser(*t*Bu), Thr(*t*Bu), Tyr(*t*Bu), and Trp(Boc) and the structures of the protecting groups are shown in figure 2.1.

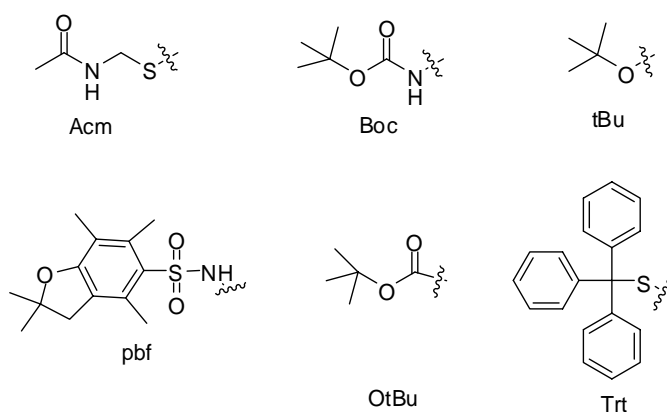


Figure 2.1 | Structures of protecting group

In order to trap reactive co-products generated upon cleavage of the side chain protecting groups, scavengers are added to the cleavage mixture. Typically, phenol, water, triisopropyl silane or thiols are used to react with these compounds to prevent addition of the reactive cations such as trityl or butyl to the peptide chain.

Despite the availability of highly optimized coupling protocols and powerful coupling agents, both Boc and Fmoc SPPS have limitations. Lack of quantitative reaction eventually leads to the formation significant levels of resin-bound byproducts. While synthesis of short peptides (<20 amino acids, AA) can be considered relatively routine, production of polypeptides of ≥ 50 amino acid residues only works in favorable cases under highly optimized conditions. Synthesis of even a small protein often presents a major challenge.

2.3 Chemoselective Ligation

Considering the limitations of linear polypeptide assembly on a solid phase, methods to ligate peptide fragments obtained by SPPS in solution would be useful for the creation of larger peptides and shorter proteins. Classically, fully protected peptide segments are condensed in organic solvents, but there are problems associated with this approach. First, general purification methods for such protected segments are not available. Second, fully protected peptides are difficult to characterize analytically. Finally, depending on the sequence, protected peptides tend to be poorly soluble in solvents useful for peptide synthesis.² Furthermore peptide segments in organic solvents undergoes epimerization at the C-terminal resulting in an unnatural product formation.

These problems are generally less severe with unprotected peptide segments, which often have good solubility in aqueous media, and chaotropes such as guanidinium hydrochloride (GnHCl) can be added to increase solubility. The peptide segments can be purified easily by RP-HPLC, and characterization by ESI-MS is straightforward. For these reasons, chemoselective ligation of unprotected peptide segments is frequently the method of choice to obtain small proteins. Chemoselective ligation refers to the aqueous covalent coupling of unprotected, highly functionalized biomolecules that contain mutually and uniquely reactive functional groups. Underlying principle of chemoselective ligation is bioorthogonality. In other words, pair of the functional groups that undergoes ligation must react together exclusively within a biological environment. Chemoselective ligations can be divided into two classes; non-native ligations and acyl transfer ligations. The non-native ligations as the name suggest, do not form amide bond at the site of ligation. In these types of ligations unique reactive functionalities that are

absent from protein side chains forms hydrazone, oxime, thioester and thioether bonds at the ligation site. Several strategies have been reported and are described in Table 1.1. All these methods have produced fully active proteins, but the presence of an unnatural structure at the ligation site is generally a potential drawback since it could potentially perturb structure and function.

The first strategy involves a novel bond between two peptides formed by nucleophilic attack of a thioacid or an α -bromo ketone resulting in a thioester. Kent employed this method to produce an engineered HIV-1 protease.¹⁷ The second strategy is closely related to the first that it relies on the attack of a nucleophilic sulfur moiety (a thiol) on an α -bromo ketone. This method is advantageous because the resulting thioether is more stable than a thioester to hydrolysis and is demonstrated by Alewood synthesis of an HIV-1 protease analogue with ligation strategy.¹⁸ The third strategy joins two fragments by forming a disulfide linkage (entry 3). The *N*-terminal peptide contains a mixed disulfide at the *C*-terminus, obtained from reaction of the free thiol with 2, 2'-dipyridyl sulfide. A thiolytic displacement reaction takes place with the *N*-terminal thiol of the second peptide serving as a nucleophile. Since a thioacid has a pKa of about 3 whereas an aliphatic thiol has a pKa of about 9, ligation reactions in entries 1 and 3 can be combined by performing the thioester forming reaction at low pH. A tethered dimer of HIV-1 has been chemically synthesized exactly according to this strategy.¹⁹

Entry 4 illustrates a completely different approach for the ligation of peptides in aqueous solution. In this ligation reaction, the first peptide contains several aldehyde moieties, and the second peptide has an *N*-terminal aminooxyacetal group. When these two compounds react, homogenous artificial proteins are obtained through the formation of oxime

bonds.²⁰ The last entry shown in Table 1.1 establishes a new (poly)peptide from two fragments through an interesting cascade of reaction steps resulting in a thiazolidine moiety. The C-terminal peptide has a cysteine at its N-terminus which has an electrophilic C-terminal aldehyde. The two fragments first condense to form an imine, which is attacked by the neighboring thiol to give a heterocycle. Subsequent acyl transfer from oxygen to nitrogen yields the peptide bond to a novel proline type analog. This method has been successfully used to obtain an epidermal growth factor-like polypeptide²¹ and an HIV-1 protease analog.²²

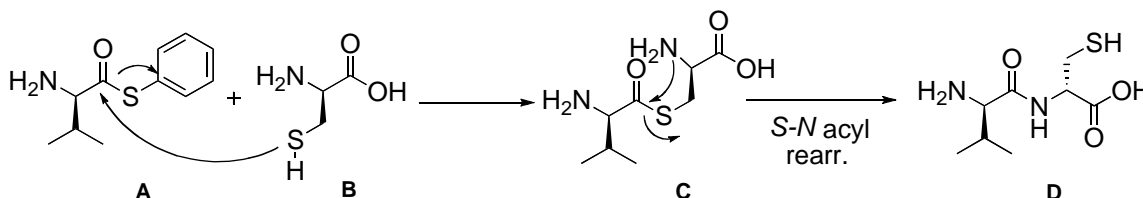
Table 1| Chemoselective Methods Used for the Synthesis of Native Proteins by Ligation of Unprotected Peptide Segments.²

Entry	Chemistry	Ligation reaction	Reference
1	Thioester-forming ligation	$\text{Pep}_1\text{-C(=O)SH} + \text{Br-CH}_2\text{-C(=O)Pep}_2 \xrightarrow[\text{pH 4.3}]{6\text{M Gn.HCl}} \text{Pep}_1\text{-C(=O)S-CH}_2\text{-C(=O)Pep}_2$	17
2	Thioether-forming ligation	$\text{Pep}_1\text{-C(=O)NH-CH}_2\text{-SH} + \text{Br-CH}_2\text{-C(=O)Pep}_2 \xrightarrow[\text{pH 8.5}]{6\text{M Gn.HCl}} \text{Pep}_1\text{-C(=O)NH-CH}_2\text{-S-CH}_2\text{-C(=O)Pep}_2$	18
3	Directed disulfide formation	$\text{Pep}_1\text{-C(=O)NH-CH}_2\text{-S-SR} + \text{HS-CH}_2\text{-C(=O)Pep}_2 \xrightarrow[\text{pH 5.0}]{6\text{M Gn.HCl}} \text{Pep}_1\text{-C(=O)NH-CH}_2\text{-S-S-CH}_2\text{-C(=O)Pep}_2$	19
4	Oxime-forming ligation	$\text{Pep}_1\text{-C(=O)CHO} + \text{H}_2\text{N-O-CH}_2\text{-C(=O)Pep}_2 \xrightarrow[\text{pH 4.8}]{} \text{Pep}_1\text{-C(=O)CH=N-O-CH}_2\text{-C(=O)Pep}_2$	20
5	Thiazolidine-forming ligation	$\text{Pep}_1\text{-C(=O)O-CH}_2\text{-CHO} + \text{H}_2\text{N-CH(CH}_2\text{SH)-C(=O)Pep}_2 \xrightarrow[\text{pH 3-4}]{} \left[\text{Pep}_1\text{-C(=O)O-CH}_2\text{-CH=N-CH(CH}_2\text{SH)-C(=O)Pep}_2 \right] \rightarrow \text{Pep}_1\text{-C(=O)N-CH}_2\text{-CH}_2\text{-C(=O)Pep}_2$	21-23

In case of acyl transfer ligations, intramolecular acyl transfer at the ligation site forms native amide bond. They are further divided as Native chemical ligation and Staudinger ligation.

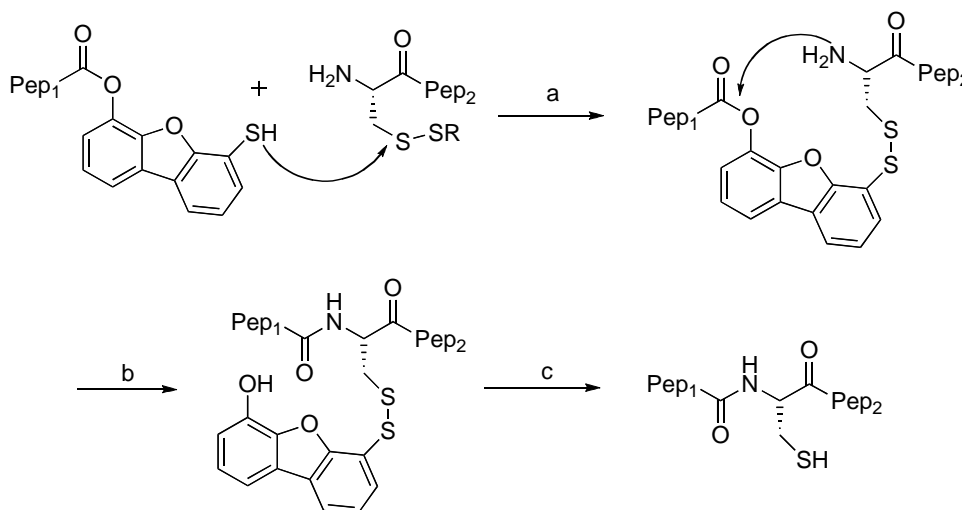
2.4 Native Chemical Ligation

Wieland in 1953 first reported^{23a}, intramolecular thioester exchange reaction between valine thiophenyl ester **A** and cysteine **B** to give *N*-valylcysteine **D** via *S*-valylcysteine **C** (Scheme 2.6 a)



Scheme 2.6a | Wieland's *S-N* acyl rearrangement reaction^{23a}

Later on in 1980 Kemp and coworkers employed *O-N* acyl transfer successfully in prior thiol capture method. Model studies showed that *O-N* acyl transfer could be initiated through a 12-membered ring intermediate using dibenzofuran template. Kemp's thiol capture ligation strategy was an important subsequent step since it resulted in novel peptides with a normal amide bond at the ligation site.²⁴⁻²⁶ Scheme 2.6 depicts the reaction sequence which yields this condensation.

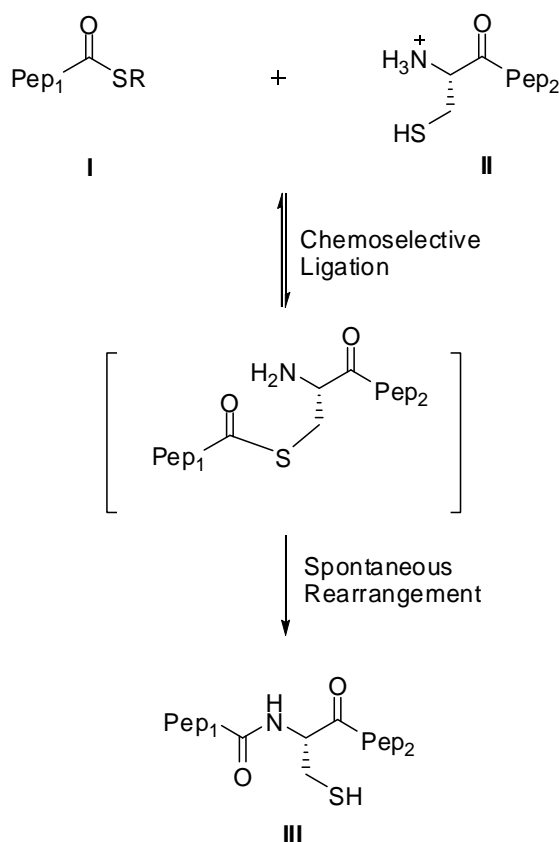


Scheme 2.6 | Kemp's thiol captures method. Conditions: a) Hexafluoro-2-propanol (HFIP)/water 4:1; b) DMSO; c) Tributylphosphine.²⁶

First, an unsymmetrical disulfide bond between two peptides is formed through attack of the C-terminal 4-mercaptobenzofuran of the first fragment on the N-terminal cysteine side chain, which is present as mixed disulfide. The intramolecular *O*-*N* acyl transfer takes place through a 12-membered ring intermediate using dibenzofuran template resulting in an amide bond. Reductive cleavage of the side chain disulfide produces a fully unprotected peptide. The basic idea was to convert the otherwise intermolecular amide bond formation into a more efficient intramolecular reaction. Ligation of the two fragments through chemoselective thiol-disulfide exchange rendered the *O*-*N*-acyl transfer step an intramolecular process.

Forty years after Wieland's original discovery, Kent and co-workers in 1994²⁷⁻²⁹ established Native Chemical Ligation for the direct synthesis of native protein. This procedure also extends Kemp's thiol capture strategy an important step further and has broad applicability due to its simplicity and efficiency. (Scheme 2.7). The essence of native chemical ligation lies in the trans-thioesterification step between thioester **I** in one peptide and the sulfhydryl group from the terminal cysteine residue **II** of the other peptide to generate a ligated thioester intermediate, which undergoes spontaneous *S* to *N* acyl rearrangement to yield the final ligated product **III**, containing a native peptide bond at the ligation junction. The initial transthioesterification reaction is catalyzed by a suitable thiol additive to maintain reduction of the cysteine thiols and enhance the reactivity of the C-terminal thioester through *in situ* thiol exchange, resulting in the corresponding phenyl thioester^{30, 31} and reversible under physiological conditions. The subsequent intramolecular nucleophilic attack by the α - amino group of the *N*- terminal cysteine to form the final amide bond is irreversible and highly favorable due to the intramolecular

five- membered ring formation, which leads to subsequent formation of the thermodynamically stable amide bond. A key feature of this reaction is that it is highly chemoselective, occurring exclusively at the *N*-terminal cysteine of the peptide, even in the presence of other unprotected side chain residues including internal cysteine residues.



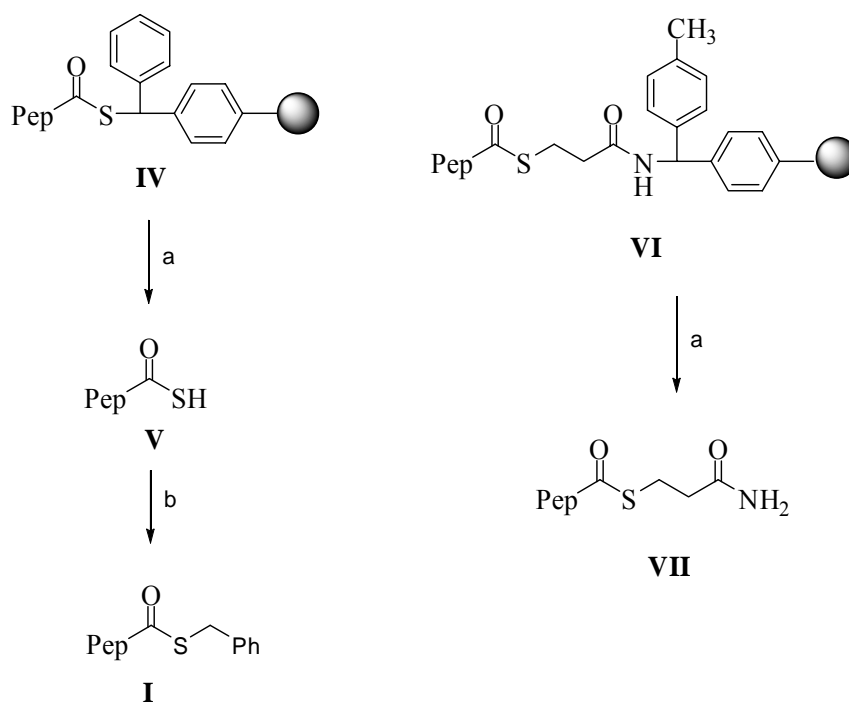
Scheme 2.7 | Native Chemical Ligation of peptide thioester **I** with a second peptide containing an *N*-terminal cysteine **II**. The nucleophilic sulfur attacks thioester **I** to form an intermediate thioester that rearranges spontaneously to the more stable amide **III**.

The rate of ligation with model peptides LYRAX-thioester and CRANK, where X represents all 20 natural amino acids, has been investigated. Ligations with peptide thioesters containing a *C*-terminal leucine, threonine, valine, isoleucine or proline were shown to proceed very slowly for steric reasons. For all other amino acids, ligations were complete within 24 hours.³² Furthermore, racemization is not detected in the coupling reaction.³³ Native chemical ligation is by far the most popular method to obtain proteins

by chemical means and has been the focus of numerous publications.³⁴ Other ligation strategies follow a similar procedure: histidine-mediated ligation³⁵, methionine-mediated ligation³⁶, the use of a removable auxiliary, and the so-called Staudinger ligation.^{3,5, 37-39} Enzymatic ligation of peptide segments has been used to form amide bonds and is exemplified by the enzyme “subtiligase”, an engineered serine protease, which has been used for several ligations including the synthesis of unnatural residues of ribonuclease A variants.⁴⁰

2.5 Synthesis of C-Terminal Peptide Thioesters

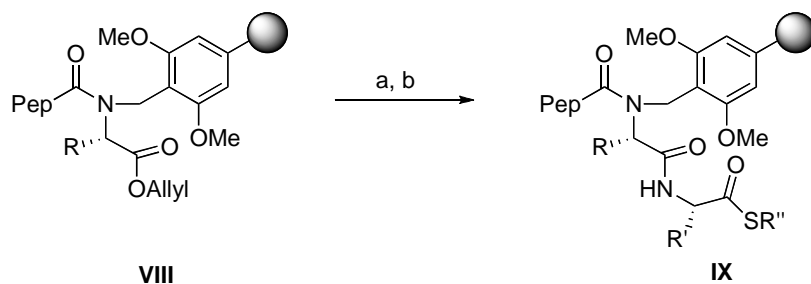
Native Chemical ligation requires access to C-terminal peptide thioesters (Scheme 2.7). A variety of methods to obtain these compounds have been developed, of which two Boc compatible approaches to thioesters are frequently used. The first approach (Scheme 2.8, left) exploits a 4-(α -mercaptobenzyl)phenyl resin.⁴¹⁻⁴³ In this approach, the peptide chain is assembled by standard ways on resin **IV** and then cleaved by anhydrous hydrogen fluoride to give a fully deprotected peptide as C-terminal thio acid **V**. The thioacid is then converted into thioester **I** by reaction with electrophiles such as benzyl bromide or bromoacetic acid. In the second method (Scheme 2.8, right), 3-mercaptopropionate is coupled to a p-methyl benzhydrylamine resin, followed by standard Boc-SPPS. The resulting thioester-linked peptide resin **VI** is treated with anhydrous hydrogen fluoride and the fully deprotected C-terminal peptide thioester **VII** is directly obtained.^{44,45} This method is advantageous because no additional transformations are required following cleavage from the resin, therefore permitting broad application.



Scheme 2.8 | Boc-SPPS compatible methods to obtain C-terminal peptide thioesters. Conditions: a) Cleavage of peptide from resin and side chain deprotection with HF; b) Benzyl bromide.

In contrast, thioester-linked peptide resins cannot be used in Fmoc chemistry as they are unstable to repeated exposure to piperidine and subject to epimerization under basic conditions. Several strategies to address these problems have been reported⁴⁶ of which thioester-compatible Fmoc cleavage cocktails have been successfully applied. Aimoto reported a mixture of 1-methylpyrrolidone (25%), hexamethylamine and HOBt (2%) in NMP/dimethylsulfoxide (DMSO, 1:1) to effectively replace piperidine and was able to obtain a 25-residue peptide thioester.⁴⁷ Wade and coworkers utilized non-nucleophilic base 1, 8-diaza-bicyclo [5.4.0] undec-7-ene (DBU) to remove the Fmoc group without harming the thioester.⁴⁸

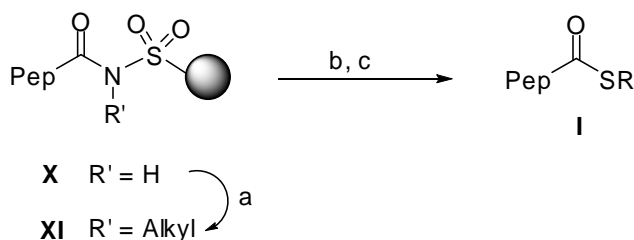
An alternate strategy introduces the labile thioester at the end of solid-phase synthesis. Barany described the use of the backbone amide linker (BAL) for this purpose (Scheme 2.9)⁴⁹⁻⁵¹



Scheme 2.9 | Backbone amide linker (BAL) strategy for C-terminal peptide thioester synthesis. Conditions: a) $\text{Pd}(\text{PPh}_3)_4$ (5 equiv.); b) H-AA-SR'' /base/activation agent. AA=Amino acid.

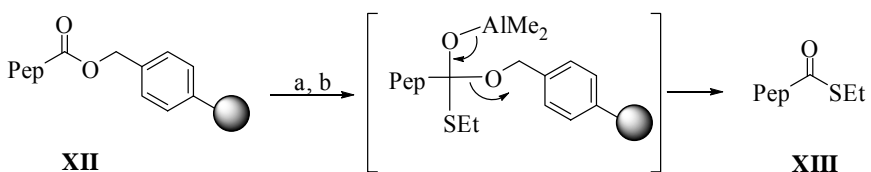
In this strategy, the *penultimate* amino acid, which has its C-terminal orthogonally blocked is attached to the benzaldehyde group of the BAL handle via reductive amination. Then, the next amino acid is introduced by coupling to the newly formed secondary amine using a non-standard procedure to prevent on-resin diketopiperazine formation.⁵⁰ The remainder of the peptide is assembled by standard Fmoc-SPPS, resulting in peptide-resin **VIII**. The C-terminus is then deprotected and a preformed C-terminal amino acid thioester introduced **IX**. Finally, the peptide-thioester is then released from the resin with a standard TFA cleavage cocktail. Due to its rather complicated and labor-intensive nature, the method has not found broad use.

A more general approach to introduce the thioester moiety at the end of the solid-phase synthesis relies on the sulfonamide or “safety-catch” linker (Scheme 2.10),^{52,53} on which the peptide is assembled by standard protocols.



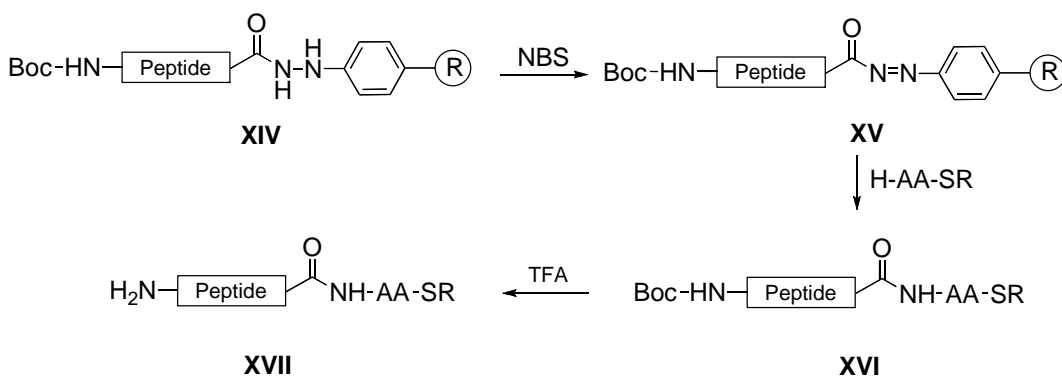
Scheme 2.10 | “Safety-catch linker” for C-terminal peptide thioester synthesis. Conditions: a) TMS-CHN_2 or Iodoacetonitrile/ DIPEA; b) Thiol; c) TFA and scavengers.

Another method to obtain a C-terminal peptide thioester that does not rely on any special linkers or complex mixtures to replace piperidine has been reported by Hilvert *et al*⁵⁴ and is based on a report by Corey *et al* that alkylaluminium thiolate, when prepared from trimethyl aluminum (AlMe_3) and the corresponding mercaptan, reacts with simple esters in methylene chloride (CH_2Cl_2) to produce thioesters in high yield.^{55, 56} The feasibility of cleaving thioesters from resins has also been reported.⁵⁴ However, Hilvert *et al* found that an excess of AlMe_3 or dimethyl aluminum chloride (AlMe_2Cl) and ethane thiol (EtSH) added to a suspension of peptide-resin **XII** in CH_2Cl_2 resulted in the desired ethyl thioester **XIII** in good yield (Scheme 2.11). Epimerization of the C-terminal residue was lowest with the use of AlMe_2Cl , but the use of this aluminum reagent resulted in significant thioesterification of protected Asp and Glu side chains. This side reaction is suppressed with AlMe_3 , but significant epimerization at the C-terminus limits the use of the method for the synthesis peptide thioester.



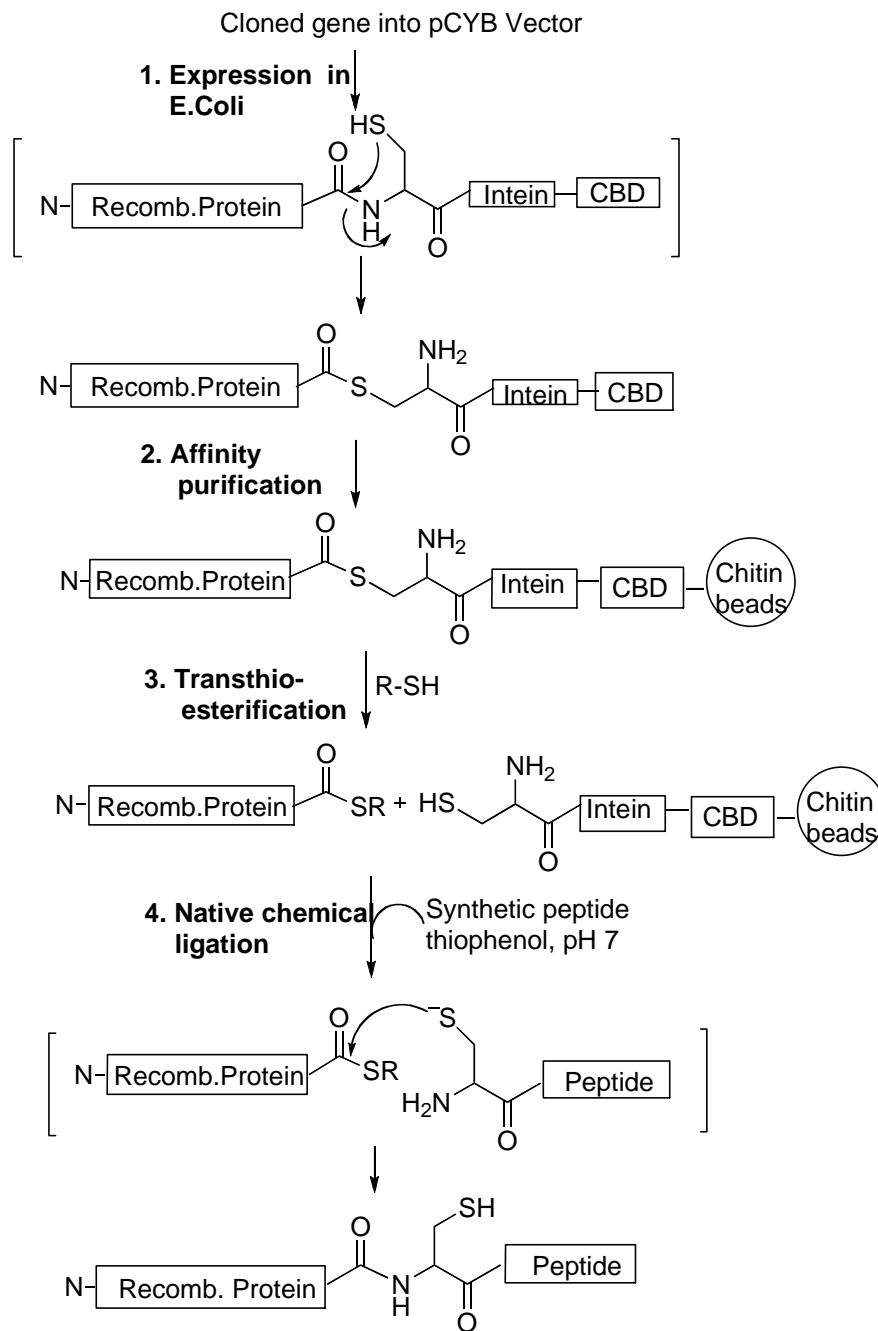
Scheme 2.11 / Lewis acid mediated C-terminal peptide thioester synthesis. Conditions: a) EtSH , AlMe_3 or AlMe_2Cl , CH_2Cl_2 ; b) TFA, water, scavengers.

Camarero *et al*⁵⁷ described a method based on the use of an aryl hydrazine linker, which is totally stable to conditions required for SPPS. After the completion of the peptide synthesis the linker is activated by mild oxidation. This step converts the aryl hydrazine group into a highly reactive diazene intermediate, which reacts with an α -amino acid alkyl thioester (H-AA-SR) to yield the corresponding peptide α -thioester.



Scheme 2.12 | Aryl hydrazine mediated C-terminal peptide thioester synthesis.

Recombinant methodologies based on intein-mediated protein splicing have made C-terminal thioesters available commercially.⁵⁸ Protein splicing involves thioester intermediates, which can be trapped with a suitable thiol and is depicted in Scheme 2.13. The protein interest is appended to the N-terminus of an engineered intein or protein splicing domain and the fusion protein produced in *E.coli* and purified by affinity chromatography. The intein segment of the fusion protein has an N-terminal cysteine, which attacks the neighboring carbonyl group to give a transient thioester intermediate, a step, which is essentially the reverse of the final step in native chemical ligation (Scheme 2.7). The intermediate is captured by an exogenous thiol and ligated with a second peptide and the resulting semi synthetic protein isolated simply by washing the resin beads. This method, referred to as ‘expressed protein ligation’, has significantly widened the scope of native chemical ligation since it creates much larger polypeptides with C-terminal thioesters than the previously used SPPS-methodology.⁵⁹ Expressed protein ligation has been used to obtain several recombinant proteins, including segmentally isotopically labeled proteins for NMR studies⁶⁰ and proteins containing spectroscopic probes.⁶¹

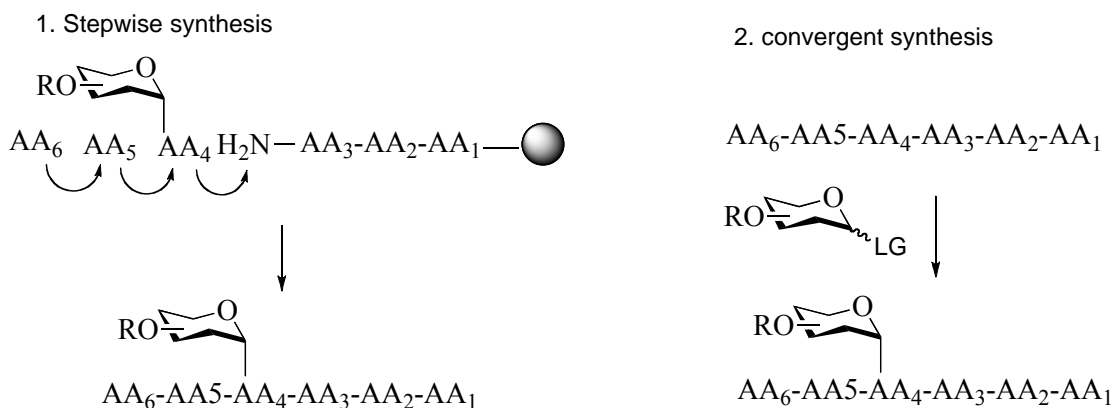


Scheme 2.13 | Mechanism of Expressed Protein Ligation. CBD = chitin binding domain.⁵⁹

2.6 Chemical Synthesis of Glycopeptides

Glycoproteins occur naturally in a number of forms (glycoforms) that possess the same peptide backbone but differ in the nature and site of glycosylation. The different properties exhibited by each component within these micro heterogeneous mixtures present regulatory difficulties and problems in determining exact function through structure activity relationships, therefore creating an urgent need for alternative sources of homogenous glycoproteins. The only methods available currently to access these well-defined structures are chemical and enzymatic syntheses.⁶²⁻⁶⁸

A crucial step in the chemical synthesis of glycopeptides is the incorporation of the saccharide into the peptide. To accomplish this, two approaches can be considered: the direct glycosylation of a properly protected full length peptide, or the use of a preformed glycosylated amino acid building block for the stepwise synthesis of the peptide backbone (Scheme 2.14).



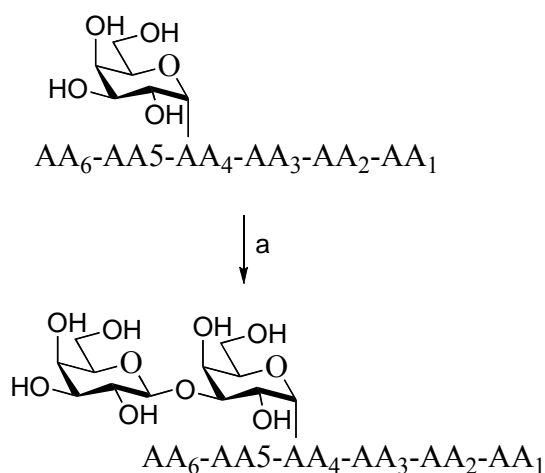
Scheme 2.14 | Different strategies of glycopeptide synthesis. AA = Amino acid.

An advantage of the direct glycosylation method is that the route is more convergent, permitting fast access to glycopeptides differing in glycan structure. However, direct *O*-glycosylation is often plagued by low yields due to the low reactivity of the side-chain

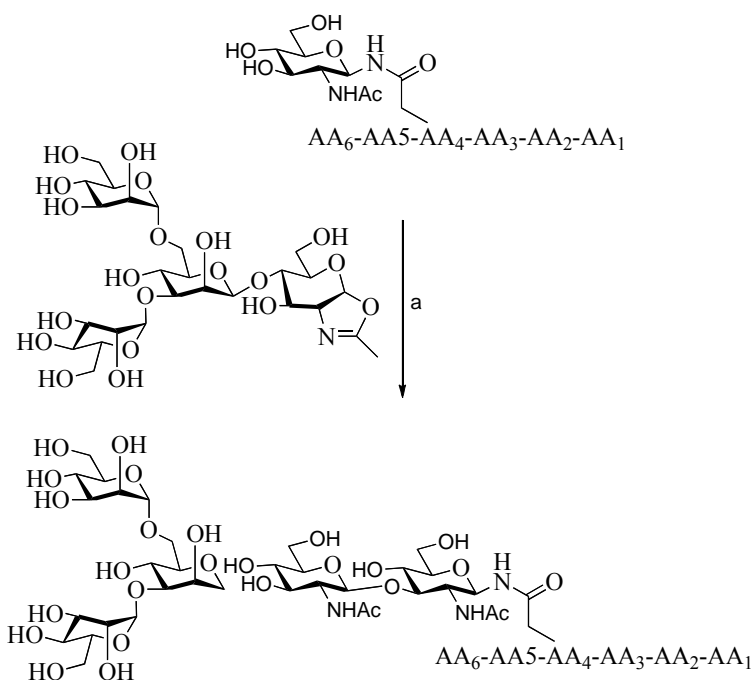
hydroxyls and the low solubility of the peptides under conditions commonly employed for chemical glycosylation. There are number of methods available for the chemical synthesis of *O*-linked as well as *N*-linked glycopeptides.⁶⁹

2.7 Chemo-Enzymatic Synthesis of Glycopeptides

The incorporation of amino acids carrying large and complex glycans into long peptides remains a significant challenge. The use of enzymatic transfer of individual monosaccharides or large oligosaccharides to glycopeptides containing simple glycans offers an attractive alternative to the tedious chemical synthesis of complex oligosaccharides. In this approach, a simple monosaccharide can be introduced into the peptide in a stepwise or convergent manner. The purified glycopeptides then function as acceptors for the specific glycosyltransferases for oligosaccharide elongation, and the oligosaccharide can be built through the stepwise addition of monosaccharide residues using various glycosyltransferases (*Scheme 2.15*), or single transfer of a fully assembled oligosaccharide using endo- β -Nacetylglucosaminidases (ENGases) (*Scheme 2.16*).^{70,71}



Scheme 2.15 | Chemo-enzymatic synthesis of a glycopeptide. Conditions a) glycosyltransferases (UDP-Gal, β -1,3-GalT).



Scheme 2.16 | Chemo-enzymatic synthesis of a glycopeptide. Conditions a) endo-β-N-acetylglucosaminidases (Endo-A).

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CHAPTER 3

Synthesis of Glyco(lipo)peptides by Liposome Mediated Native Chemical Ligation

NCL is a chemo-selective reaction that occurs at physiological pH between an *N*-terminal cysteine residue and a *C*-terminal peptide thioester.¹⁻³ In the first step of ligation, a reversible *trans*-thioesterification takes place between the *C*-terminal thioester and the sulfhydryl group from the *N*-terminal cysteine residue. The ligated peptide thioester then undergoes a rapid, irreversible and spontaneous intramolecular S→N shift, generating the thermodynamically favored native amide bond at the ligation junction. NCL occurs uniquely at an *N*-terminal cysteine residue regardless of the presence of any additional internal cysteine residues and, as this ligation method is compatible with both carbohydrates and peptides, provides access to glycopeptides. NCL is emerging as a powerful tool to assemble highly complex (glyco)peptides and small proteins.

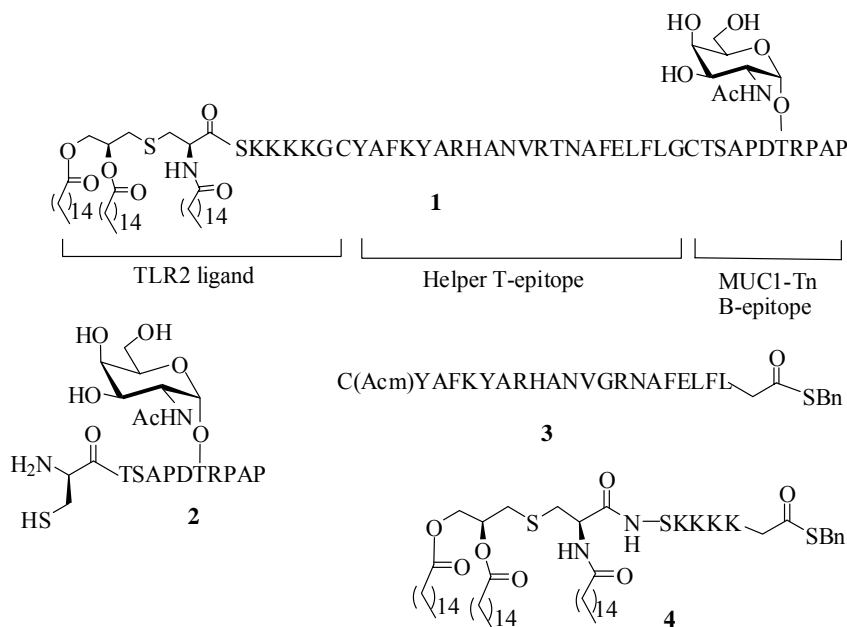
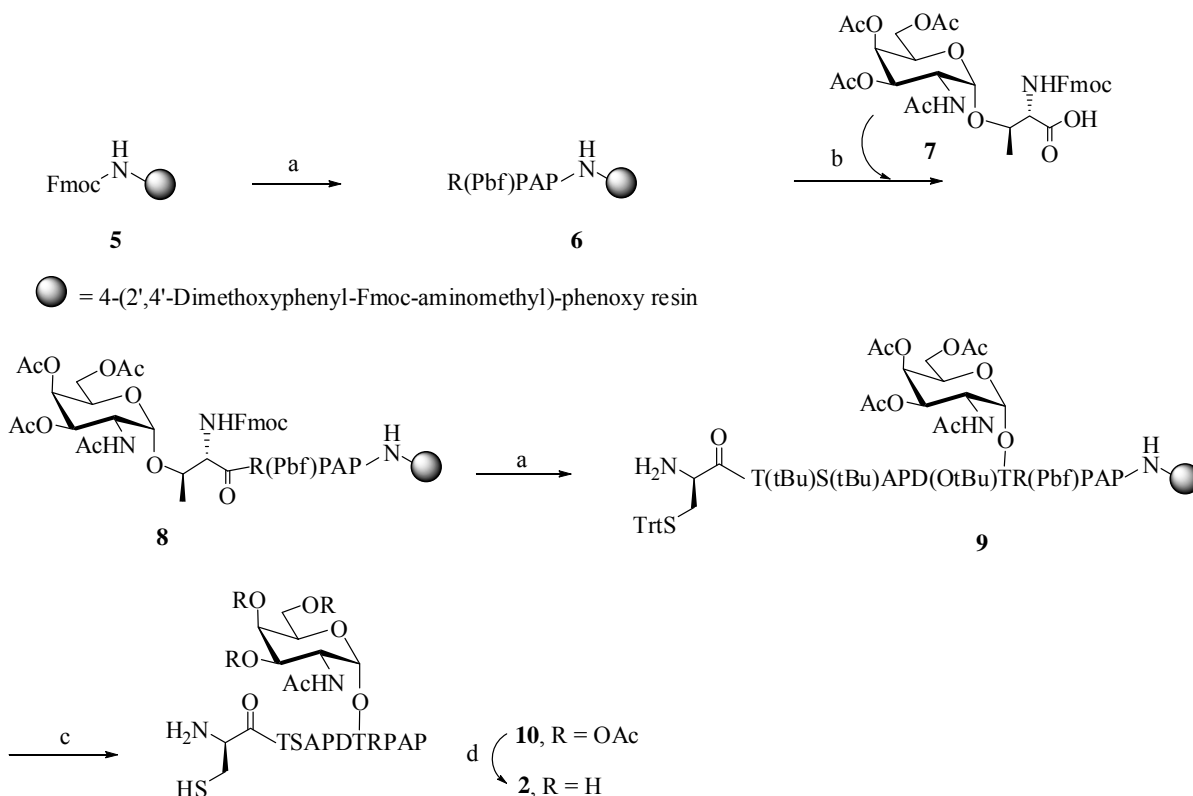


Figure 3.1 | Fully synthetic vaccine candidate 1

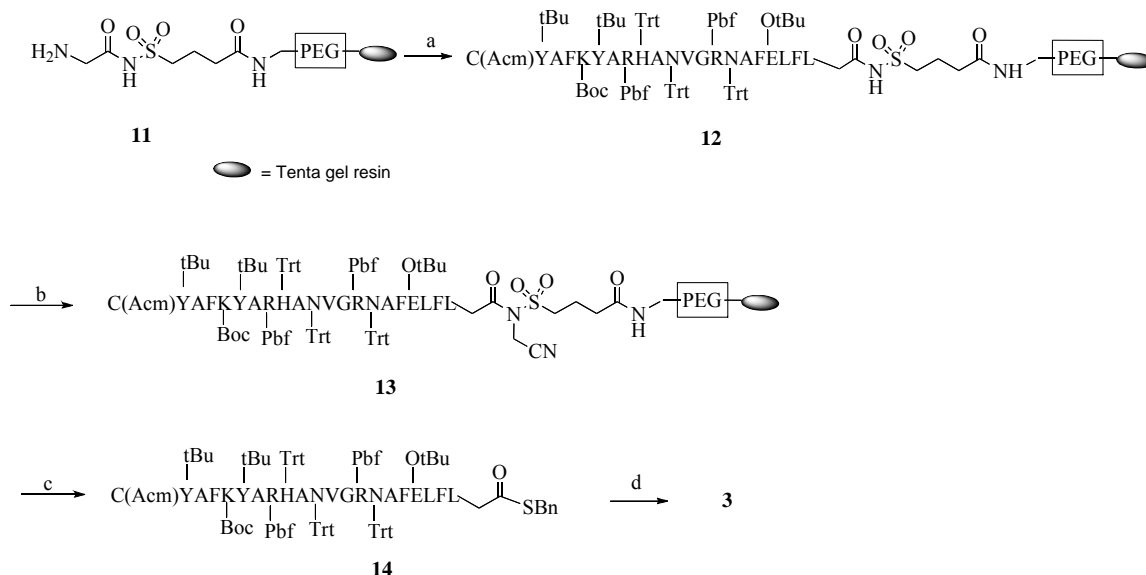
Vaccine candidate **1** (Figure 3.1), which is composed of the tumor-associated glycopeptide derived from MUC-1,⁴ the well-documented T-cell epitope YAFKYARHANVGRNAFELFL (YAF),⁵ and the lipopeptide Pam₃CysSK₄,^{6,7} was selected as a synthetic target.

It was envisaged that compound **1** could be prepared from building blocks **2**, **3**, and **4** by sequential NCL. The MUC-1 epitope **2** was assembled by solid-phase peptide synthesis (SPPS) using Fmoc protected amino acids and *N*- α -Fmoc-Thr(α -AcO₃-D-GalNAc)-OH (Tn antigen)⁸ on Rink amide resin **5** (Scheme 3.1).



Scheme 3.1 | Synthesis of the Cys-glycopeptide 3. Reagents and Conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) **7**, HATU/HOAt, DIPEA, NMP, overnight; c) TFA (94.0%), water (2.5%), EDT (2.5%), TIS (1%); d) 5% aqueous hydrazine, excess of DTT.

Thus, first four amino acids were coupled by stepwise solid phase peptide synthesis using an automated HBTU-mediated HOBt ester activation protocol⁹ to give compound **6**. Next, the glycosylated amino acid building block **7** was manually coupled using HATU/HOAt in the presence of DIPEA in NMP to give compound **8**. The peptide sequence was then elongated by using a standard protocol to give compound **9**. After the completion of the synthesis, the glycopeptide was cleaved from the resin by treatment with TFA (94.0%), water (2.5%), ethanedithiol (2.5%) and TIS (1%) to give compound **10**. Finally, the acetyl esters of the saccharide moiety were cleaved by treatment of 5% aqueous hydrazine in the presence of DTT to give glycopeptide **2** in good overall yield.



Scheme 3.2 | Synthesis of the Cys(Acm)- α -thioester **2 using the alkanesulfonamide “safety-catch” linker.** Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) ICH₂CN, DIEA, NMP, 24 hr; c) BnSH, Na-Thiophenate, THF, 24 hr; d) Reagent B (TFA (88%), Phenol (5%), H₂O (5%), TIS (2 %)), 4 h.

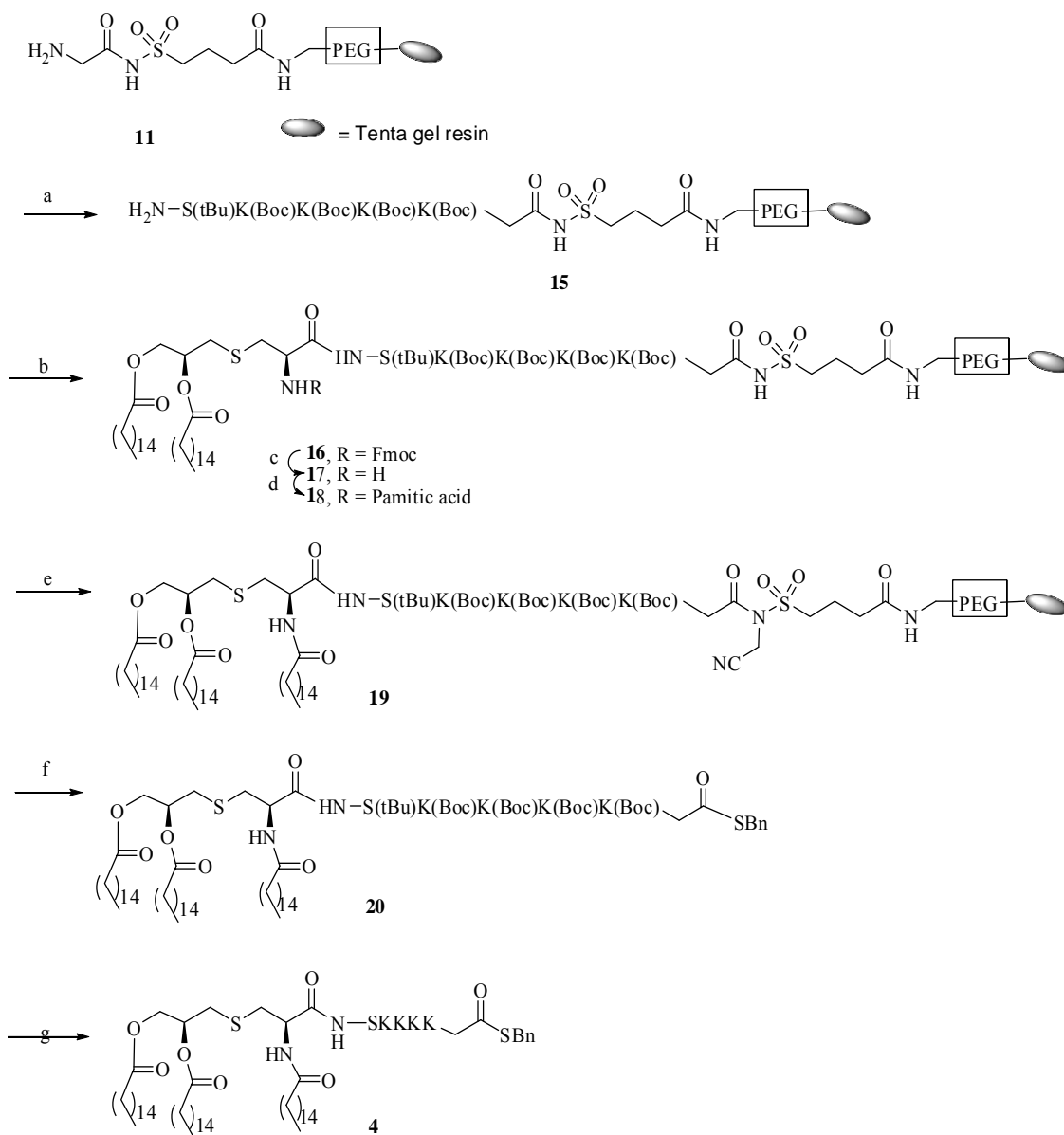
The synthesis of peptide thioester **3** was carried out on a sulfonamide "safety-catch" linker¹⁰⁻¹² **11** (Scheme 3.2) using automated Fmoc based stepwise solid phase peptide synthesis methods to give support bound fully protected compound **12**.

After the completion of the synthesis, cleavage of the fully assembled peptide from the support was accomplished by a two-step procedure entailing alkylation of the sulfonamide with iodoacetonitrile to afford the support-bound *N,N*-cyanomethylacylalkane sulfonamide active ester **13** followed by treatment with benzyl mercaptan in the presence of sodium thiophenate to give a protected peptide having a C-terminal thioester **14**.

The amino acid protecting groups of the peptide were removed by treatment with reagent B (TFA, phenol, water and TIS; 88/5/5/2) to give **3** in excellent yield (79%) after purification by RP-HPLC on a C18 column. This compound is equipped with an *N*-terminal cysteine residue carrying the orthogonal actaimomethyl (Acm) thiol protecting group, which is stable under conventional side-chain deprotection with TFA but can be cleaved using Hg(II) or Ag(I), or oxidatively by using I₂.¹³

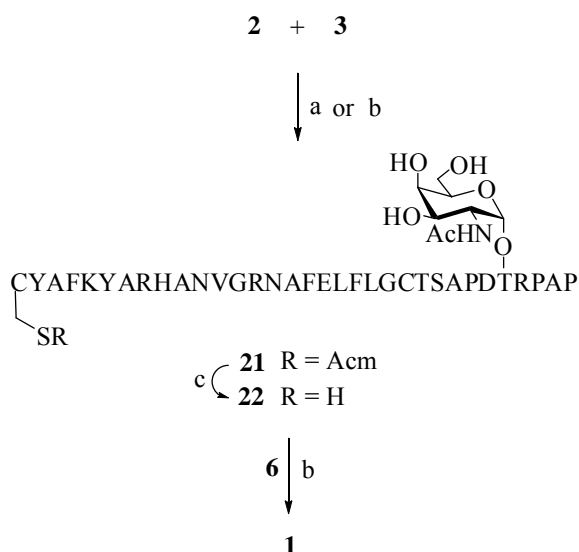
The synthesis of Pam₃CysSK₄- α -thioester **4** (Scheme 3.3) was accomplished using the solid support used for the preparation of compound **3**. The initial amino acids couplings were carried out by standard protocols for Fmoc chemistry to afford compound **15**, while the remaining steps were performed manually. Thus, the coupling of Pam₂Cys-OH^{14,14a} in the presence of PyBOP/HOBt containing DIPEA in DMF gave compound **16**. After the completion of this reaction, the *N*-Fmoc group was removed using 20% piperidine in DMF and the resulting free amine of the resulting compound **17** was coupled with palmitic acid using PyBOP/HOBt in the presence of DIPEA in DMF to give fully protected support bound lipopeptide **18**. The lipopeptide thioester was then released from the solid support by two step protocol described for compound **3** to get fully protected lipopeptide thioester **20**. The acid sensitive protecting groups were then removed by

treatment of reagent B (TFA 88%, Water 5%, Phenol 5%, TIS 2%) to give lipopeptide thioester **6** in good yield.



Scheme 3.3 | Synthesis of the Lipopeptide- α -thioester **6 using the alkanesulfonamide “safety-catch” linker.** Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) Manual coupling of Pam₂Cys-OH, PyBOP, HOBt in the presence of DIPEA in DMF; c) 20 % Piperidine in DMF; d) Coupling of Palmitic acid, PyBOP, HOBt in the presence of DIPEA in DMF; e) ICH₂CN, DIEA, NMP, 24 h; f) BnSH, Na-Thiophenolate, THF, 24 h; g) Reagent B (TFA (88%), Phenol (5%), H₂O (5%), TIS (2%)), 4 h.

Having building blocks **2**, **3**, and **4** at hand, attention was focused on the preparation of glycolipopeptide **1** by sequential NCL. The ligation of **2** with **3** was performed under standard conditions (Scheme 3.4) using a phosphate buffer (pH 7.5) containing 6 M of guanidinium-hydrochloride.¹⁰ The ligation was promoted by the addition of 4% thiophenol (v/v)¹⁵ and the progress of the reaction monitored by LC/MS (conditions a, Scheme 3.4). The reaction was rather sluggish and after a reaction time of 18 hours partial conversion of **2** and **3** into **21** and some hydrolysis of the thioester was observed. Purification by semi-preparative RP-HPLC gave **21** in a yield of 48%.



Scheme 3.4 | Synthesis of three-component vaccine by NCL. Reagents and conditions: a) 6 M Gn.HCl, 200 mM sodium phosphate buffer (pH 7.5), thiophenol 4% (final v/v); b) 200 mM phosphate buffer (pH 7.5), DPC, TCEP, EDTA, sodium 2-mercaptoethane sulfonate 2% (final w/v). c) Hg(OAc)₂, 10% aq HOAc, 50 mM DTT.

Next, the AcM group of **21** was removed using mercury(II) acetate to give glycopeptide **22**, containing a free sulfhydryl moiety. Unfortunately, a second NCL of compound **22** with the thioester **4** in a phosphate buffer containing 6 M guanidinium-hydrochloride and thiophenol did not provide target compound **1**. The failure of this reaction is probably due to the poor solubility of **4**. Addition of detergents such as SDS¹⁶ and DPC,¹⁷ at ambient

and elevated reaction temperatures (40-50°C) did not improve the ligation. Furthermore, the use of alternative promoters such as a mixture of sodium thiophenate and thiophenol or sodium 2-mercaptoethane sulfonate did not lead to product formation. Attempts to perform the ligation in a phosphate buffer containing 8 M urea and use of trifluoroethanol as a reaction solvent also led to failure.

We envisaged that the incorporation of compounds **22** and **4** into liposomes would facilitate solubilization^{18,19} and hence increase the rate of ligation. Thus, a film of dodecylphosphocholine, thiol **22**, and thioester **4** was hydrated by incubation at 37°C for 4 hours in a phosphate buffer (pH 7.5) in the presence of carboxyethyl phosphine and EDTA. The latter two reagents were added to suppress disulfide formation. The mixture was ultra-sonicated for 1 minute and the resulting vesicles were sized to 1 µm by passing through a polycarbonate membrane filter. The ligation was promoted by the addition of sodium 2-mercaptoethane sulfonate²⁰ and, surprisingly after a reaction time of 2 hours, LC-MS showed completion of the reaction. After purification by RP-HPLC over a C-4 column, compound **1** was obtained in a high yield of 83%. The use of thiophenol as a promoter resulted in a significantly slower reaction rate and after 4 hours the reaction had proceeded to only ~ 60% completion. After a reaction time of 16 hours, LC-MS revealed significant hydrolysis of palmitoyl esters.

Encouraged by the successful preparation of **1**, attention was again on the synthesis of glycopeptide **21** employing new methodology (conditions b, Scheme 3.4). The preparation of this compound by traditional NCL was relatively low yielding due to the poor solubility of **3** in a phosphate buffer containing 6 M guanidinium-hydrochloride. It was envisaged that incorporation of **2** and **3** into liposomes would increase the solubility

and hence a higher yield of product may be expected. Thus, a liposomal preparation of glycopeptide **2** and peptide **3** was prepared using the conditions employed for the preparation of **1**. The ligation was promoted by the addition of sodium 2-mercaptoethane sulfonate and, after a reaction time of 2 hours, the product was purified by RP-HPLC to give **21** in an excellent yield of 78% (conditions b, Scheme 3.4).

Interestingly, no product formation was observed when a solution of **2** was added to a liposomal preparation of **3** using sodium 2-mercaptoethane sulfonate as the promoter (compound **2** has reasonable solubility in phosphate buffer). The results of these experiments indicate that NCL takes place within the lipid environment of the liposome and not at the water-liposome interface.

To examine the generality of the novel approach, vaccine candidates **23**, **24**, and **25** (Figure 3.2), which differ in (glyco)peptide and lipid composition, were prepared by sequential *liposome*-mediated NCL starting from building blocks **2**, **3**, **4**, **26**, and **27**.

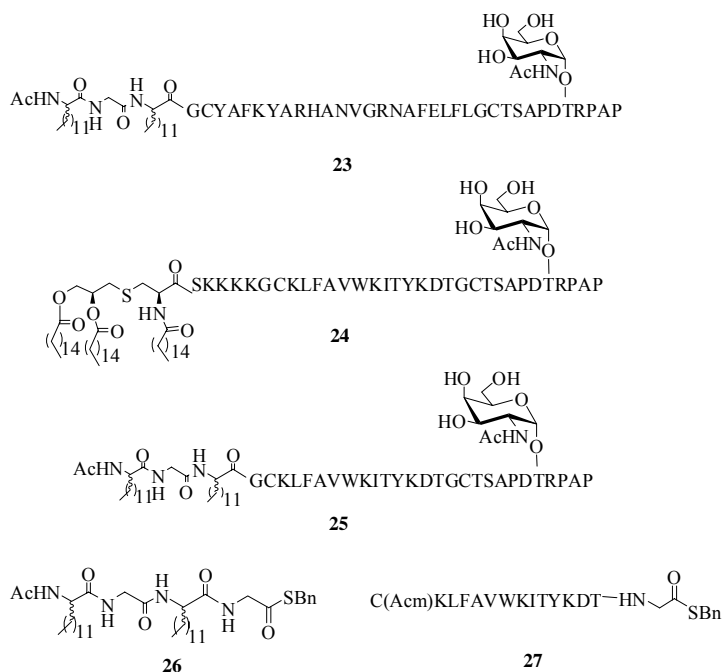
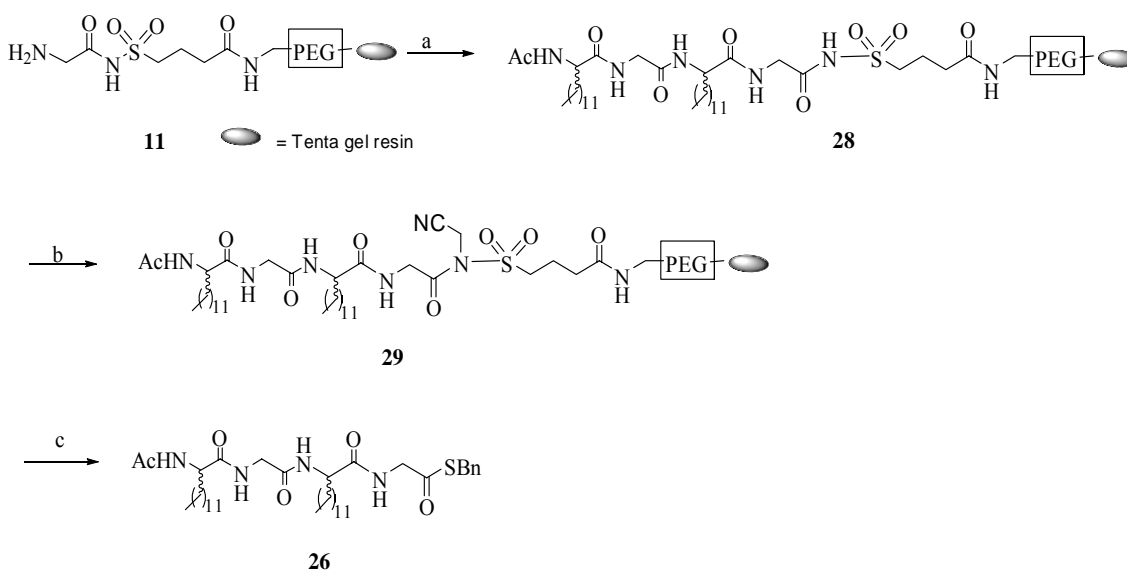


Figure 3.2 | Fully synthetic vaccine candidates **23**, **24**, and **25**

Synthesis of lipopeptide thioester **26** (Scheme 3.5) was accomplished by using sulfanoamide “safety-catch linker” **11**. The synthesis of this compound was carried out manually by using PyBOP/HOBt ester activation protocol. Thus, the coupling of an *N*- α -Fmoc-lipidated amino acid^{21,22} was carried out by using PyBOP/HOBt in the presence of DIPEA in NMP. Upon completion, the *N*-Fmoc group was deprotected using 20% piperidine in DMF and the free amine of the resulting compound was coupled with *N*- α -Fmoc-Gly-OH in the presence of PyBOP /HOBt containing DIPEA in NMP. Upon completion of this reaction one more cycle of coupling with *N*- α -Fmoc-lipidated amino acid^{21,22} was carried out to complete the synthesis.

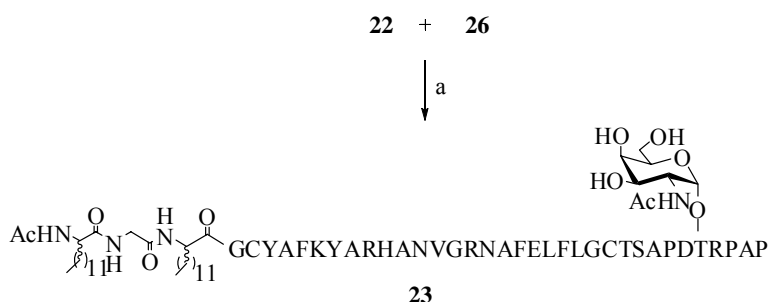


Scheme 3.5 | Synthesis of the Lipopeptide- α -thioester **8 using the alkane sulfonamide “safety-catch” linker.** Reagents and conditions: a) i. Manual coupling of Fmoc-lipidated amino acid with PyBOP/HOBt in the presence of DIPEA in DMF; ii. 20% Piperidine in DMF; iii. Manual Coupling of Fmoc-Gly-OH with PyBOP/HOBt in the presence of DIPEA in DMF; iv. 20% Piperidine in DMF; v. Manual coupling of Fmoc-lipidated amino acid with PyBOP/HOBt in the presence of DIPEA in DMF; vi. 20% Piperidine in DMF; vii. 10% Ac₂O, 5% DIPEA in NMP for 10 min; b) ICH₂CN, DIEA, NMP, 24 h; c) BnSH, Na-thiophenate, THF, 24 h.

Finally the *N*-Fmoc was deprotected to give free amine at the *N*-terminus, which was then acylated on the support to give compound **28**. The lipopeptide thioester **26** was then

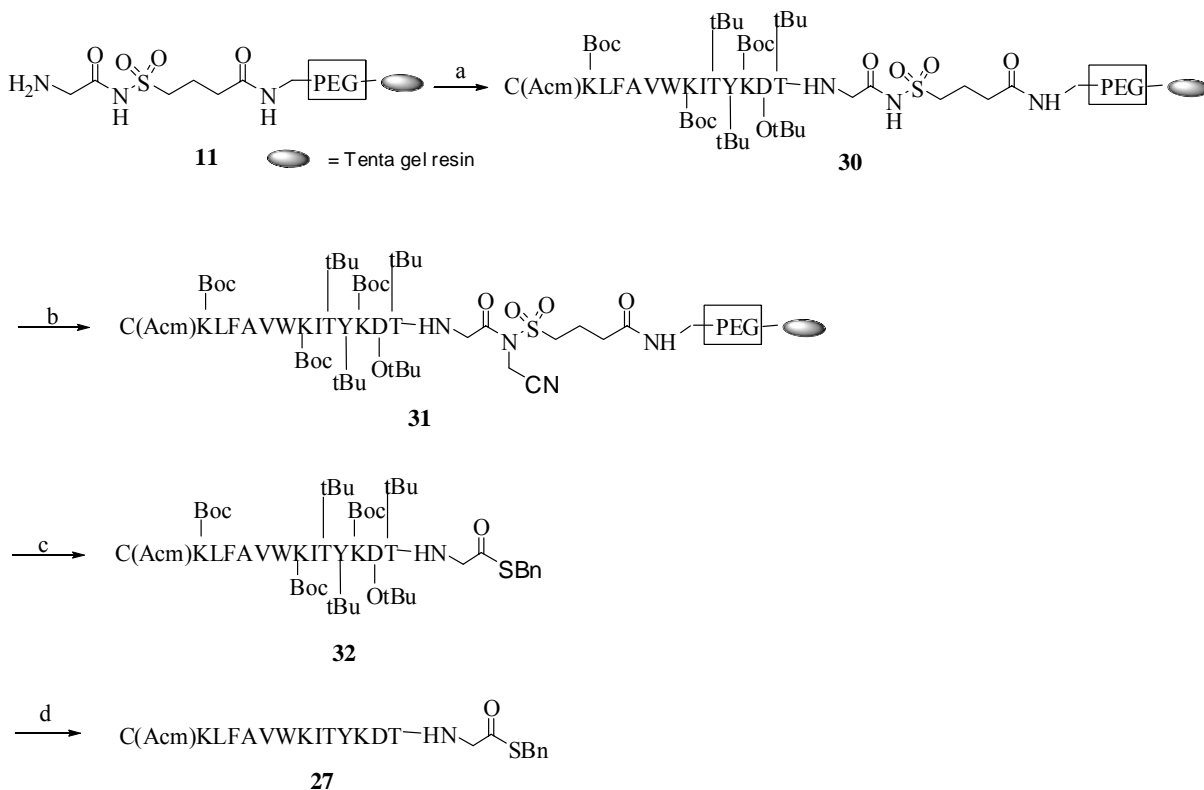
released from the solid support by two step procedure employed for the synthesis of compound **3**.

Glycolipopeptide **23** (Scheme 3.6) was easily obtained in 78% yield by the ligation of **22**, which was prepared from compounds **2** and **3** with thioester **26** by using liposome mediated native chemical ligation.

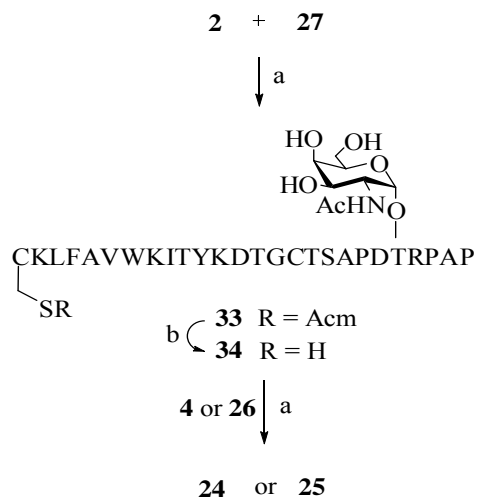


Scheme 3.6 | Preparation of **23** from **22** and **26**. Reagents and conditions: a) 200 mM phosphate buffer (pH 7.5), DPC, TCEP, EDTA, sodium 2-mercaptoethane sulfonate 2% (final w/v).

For the synthesis of derivatives **24** and **25**, the compound **27** (Scheme 3.7) was synthesized. This compound was obtained by using the protocol described for compound **3** (Scheme 3.2). The peptide thioester **27** was obtained in a yield of 74%, has an an *N*-terminal cysteine residue carrying the orthogonal Acn protecting group. Next, the ligation of **2** with **27** using *liposome*-mediated native chemical ligation conditions gave glycopeptide **33** in excellent yield (79%), which after removal of the Acn group¹³ (\rightarrow **34**) was ligated with thioesters **4** or **26** to give glyco(lipo)peptides **26** and **27** respectively (Scheme 3.8). Each *liposome*-mediated NCL was completed within 2 hours as determined by LC-MS, and after purification by semi-preparative RP-HPLC provided the glycopeptides or glycolipopeptides in high yield.



Scheme 3.7 | Synthesis of the Cys(Acm)- α -thioester **9 using the alkanesulfonamide “safety-catch” linker.** Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) ICH₂CN, DIEA, NMP, 24 h; c) BnSH, Na-Thiophenate, THF, 24 h; d) Reagent K (TFA (88%), Phenol (5%), H₂O (5%), TIS (2%)), 4 h.



Scheme 3.8 | Preparation of **24 and **25** from **2**, **4**, **26**, and **27**.** Reagents and conditions: a) 200 mM phosphate buffer (pH 7.5), DPC, TCEP, EDTA, sodium 2-mercaptoethane sulfonate 2% (final w/v). b) Hg(OAc)₂, 10% aq HOAc, 50 mM DTT.

Previously, Kochendoerfer and co-workers¹⁵ performed a NCL between a synthetic hydrophobic polypeptide incorporated into a cubic lipidic phase and a tetrapeptide, which was added to the membrane preparation. This mode of ligation is different from the approach described here because only one of the two reactants is incorporated into the membrane. Furthermore, Otaka and coworkers¹⁶ reported that lipid bilayer assisted NCL between a thioester and an *N*-terminal cysteine peptide can successfully be used for the synthesis of membrane protein segments possessing two transmembrane regions and one extracellular domain. In this approach, peptides were embedded in a palmitoyloleoyl phosphatidylcholine membrane and the reaction was promoted by the addition of thiophenol.

The results of our study demonstrate that incorporation of a lipophilic (lipo)peptide thioester and an *N*-terminal cysteine glycopeptide into DPC-liposomes facilitates NCL to afford a range of glycopeptides and glycolipopeptides. Surprisingly, the new approach is not limited to peptides that have a trans- and an extra cellular domain. Furthermore, it was found that 2-mercaptoethane_sulfonate is a more effective promoter compared to thiophenol. In this respect, it was observed that the *liposome*-mediated NCLs were completed within 2 hours, which is remarkably fast for the type of substrates employed. The high reaction rate can probably be attributed to a concentration effect in the liposomes.

In conclusion, we have developed a novel approach for native chemical ligation by the entrapment of reactants in liposomes. The new methodology is particularly suited for the synthesis of lipophilic (glyco)peptides of biological importance.²³⁻²⁸ For example, it allows the synthesis of a range of three-component vaccine candidates by a modular

approach using an array of B- and T-epitopes and lipopeptide adjuvants. A modular approach is attractive because it provides greater synthetic flexibility than linear synthesis. In this respect, each building block can be used for the preparation of several different target compounds. Furthermore, compared to conventional linear SPPS, a block synthetic approach will minimize by-product build-up in the growing peptide chain. In this respect, the DT sequence of the MUC-1 glycopeptide is prone to aspartimide formation,²⁹ which can occur at each coupling step. In a convergent block synthesis, the individual building blocks can be purified by RP-HPLC and characterized by NMR and MS prior to assembly, providing a sound basis for highly pure final products.

3.1 Experimental procedures:

Reagents and general procedures: Amino acid derivatives and resins were purchased from NovaBioChem and Applied Biosystems; *N, N*-dimethylformamide (DMF) from EM Science; and *N*-methylpyrrolidone (NMP) from Applied Biosystems. Dodecyl phosphocholine (DPC) was obtained from Avanti Polar Lipids. All other chemical reagents were purchased from Aldrich, Acros, Alfa Aesar and Fischer and used without further purification. All solvents employed were reagent grade. Reverse Phase high performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 series system equipped with an autosampler, UV-detector and fraction-collector by using a Zorbax Eclipse C8 analytical column (5 μ m, 4.6 x 150 mm) at a flow rate of 1 ml/min, a semi preparative C8 column (5 μ m, 10 x 250 mm) at a flow rate of 4 ml/min, a Synchropak C4 analytical column (5 μ m, 4.6 x 100 mm) at a flow rate of 1 ml/min and a Vydac C4 semi preparative column (5 μ m, 4.6 x 250 mm) at a flow rate of 2 ml/min. All runs used linear gradients of 0-100% solvent B in A over a 40 min. period was used

unless otherwise specified. (A = water 95%, acetonitrile 5% and 0.1% TFA, B= water 5%, acetonitrile 95% and 0.1% TFA). MALDI-ToF mass spectra were recorded on a ABI 4700 proteomic analyzer.

General methods for Solid-Phase Peptide Synthesis (SPPS): Peptides were synthesized by established protocols on a Applied Biosystems, ABI 433A peptide synthesizer equipped with UV-detector using *N*- α -Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyl hexafluorophosphate (HBTU)/1-Hydroxybenzotriazole (HOBt) as the activating reagents. Single coupling steps were performed with conditional capping. The coupling of the glycosylated amino acid *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc) and *N*- α -Fmoc-*R*-(2,3-bis(palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine was carried out manually. The manual couplings were monitored by standard Kaiser test.³⁰

Synthesis of glycopeptide 10: SPPS was performed on Rink amide resin (0.1 mmol) as described above. Side chain protection was as follows: *N*- α -Fmoc-*N*^G-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*L*-arginine, *N*- α -Fmoc-*L*-aspartic acid α -*tert*-butyl ester, *N*- α -Fmoc-*S*-trityl-*L*-cysteine, *N*- α -Fmoc-*O*-*tert*-butyl-*L*-serine, *N*- α -Fmoc-*O*-*tert*-butyl-*L*-threonine. The first four amino acids, Arg-Pro-Ala-Pro were coupled on the peptide synthesizer using a standard protocol. After the completion of the synthesis, a manual coupling was carried out using *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc) (134 mg, 0.2 mmol), with HATU (76 mg, 0.2 mmol), HOAt (0.2 mmol, 27 mg) and DIPEA (70 μ l, 0.4 mmol) in NMP for 12 h. The coupling reaction was monitored by standard Kaiser test. The resin was washed with NMP (6 ml x 1) and DCM (6 ml x 1), and resubjected to the same coupling conditions to ensure complete coupling. The

glycopeptide was elongated on the peptide synthesizer. The resin was washed thoroughly with NMP (6 ml x 1), DCM (6 ml x 1) and MeOH (6 ml x 1) and dried *in vacuo*. The resin was swelled in DCM (5 ml) for 1 h, after which it was treated with TFA 94%, water 2.5%, EDT 2.5%, and 1 TIS % (10 ml) for 2 h at room temperature. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated *in vacuo* to approximately 1/3 of its original volume. The peptide was precipitated by the addition of diethyl ether (30 ml) (0°C) and recovered by centrifugation at 3000 rpm for 15 min. The crude glycopeptide was purified by RP-HPLC on a semi-preparative C-18 reversed phase column using a linear gradient of 0-100% solvent B in A over a period of 40 min., and lyophilization of the appropriate fractions afforded **10** (90% based on resin loading capacity). C₅₉H₉₄N₁₆O₂₄S, MALDI-ToF MS: observed, 1443.8918Da; calculated, 1443.5371Da.

Deacetylation of glycopeptide 10: The glycopeptide **10** (5 mg, 3.4 µmol) was treated with 5% aqueous hydrazine (2 ml) containing DTT (12 mg, 68 µmol). The reaction was monitored by MALDI-ToF MS. After standing for 1 hr at room temperature, the crude product was neutralized with acetic acid (1.5 ml) and purified by RP-HPLC on a semi-preparative C-18 reversed phase column using a linear gradient of 0-100% solvent B in A over a period of 40 min., to afford after lyophilization compound **2** (4.0 mg, 88%). C₅₃H₈₈N₁₆O₂₁S, MALDI-ToF MS: observed, 1317.9580Da; calculated, 1317.4271Da.

Synthesis of Peptide thioester 3: The synthesis of Ac^m protected peptide thioester **3** was carried out on preloaded H-Gly-sulfamylbutyryl Novasyn TG resin (0.1 mmol) as described in the general method section for peptide synthesis. The following side chain protection was employed: *N*-α-Fmoc-N^G- (2,2,4,6,7-pentamethyldihydrobenzofuran-5-

sulfonyl)-L-arginine, *N*- α -Fmoc-*N*- β -trityl-L-asparagine, *N*- α -Fmoc-S-acetamidomethyl-L-cysteine, *N*- α -Fmoc-L-glutamic acid γ -*tert.*-butyl ester, *N*- α -Fmoc-*N*-im-trityl-L-histidine, *N*- α -Fmoc-*N*- ϵ -*tert.*-Boc-L-lysine, *N*- α -Fmoc-*O*-*tert.*-butyl-L-threonine, *N*- α -Fmoc-*O*-*tert.*-butyl-L-tyrosine.

Activation and Cleavage of peptide thioester 3. The resin bound peptide was washed thoroughly with DCM (5 ml x 2) and *N*-methyl-2-pyrrolidone (NMP) (5 ml x 2). After initial washings it was swelled in DCM (5 ml) for 1 h. The resin was treated with DIPEA (0.5 ml, 3 mmol), iodoacetonitrile (0.36 ml, 5 mmol) in NMP (6 ml). It is important to note that the iodoacetonitrile was filtered through a plug of basic alumina before addition to the resin. The resin was agitated under the exclusion of light for 24 h, filtered and then washed with NMP (5 ml x 4), DCM (5 ml x 4) and THF (5 ml x 4). The activated *N*-acyl sulfonamide resin was swollen in DCM (5 ml) for 1 h, drained and transferred to a 50 ml round bottom flask. To the resin-containing flask was added THF (4 ml) and benzyl mercaptan (0.64 ml, 5 mmol), and sodium thiophenate (27 mg, 0.2 mmol). After agitation for 24 h, the resin was filtered and washed with hexane (10 ml). The combined filtrate and washings were collected and concentrated in *vacuo* to approximately 1/3 of its original volume. The crude product was precipitated by the addition of *tert*-butyl methyl ether (0°C) (60 ml) and recovered by centrifugation at 3000 rpm for 15 min., and after the decanting of the ether the peptide precipitate was dissolved in mixture DCM and Methanol (1.5 ml/1.5 ml). The thiol impurities present in the peptide precipitate was then removed by passing it through a LH-20 column. The fractions containing product were mixed together and solvents were removed to recover the fully protected peptide thioester.

Side chain deprotection of peptide thioester 3: The protected peptide was treated with a reagent B (5 ml, (TFA 88%, phenol 5%, H₂O 5%, TIS 2%)) for 6 h at room temperature. The TFA solution was added drop wise to a screw cap centrifuge tube containing ice cold *tert*-butyl methyl ether (40 ml) and the resulting suspension was left overnight at 4°C, after which the precipitate was collected by centrifugation at 3000 rpm (20 min), and after the decanting of the ether the peptide precipitate was re-suspended in ice cold *tert*-butyl methyl ether (40 ml) and the process of washing was repeated twice. The crude peptide was purified by semi preparative C-8 reversed phase column using a linear gradient of 0-100% solvent B in A over a period of 40 min., and lyophilization of the appropriate fractions afforded **3** (79% based on resin loading capacity). C₁₂₈H₁₈₂N₃₄O₂₉S₂, MALDI-ToF MS: observed, [M+Na] 2748.2439Da; calculated, [M+Na] 2748.1584Da.

Synthesis of lipopeptide thioester 4. The synthesis of **4** was carried out on a H-Gly-sulfamylbutyryl Novasyn TG resin (0.1 mmol) as described in the general method section for peptide synthesis. After coupling of the first five amino acids, the remaining steps were performed manually. *N*- α -Fmoc -*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (Pam₂Cys-OH) (267 mg, 0.3 mmol) was dissolved in DMF (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) were premixed for 2 min, and was added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 12 h. Upon completion of the coupling, the *N*-Fmoc group was cleaved using 20% piperidine in DMF (6 ml). Palmitic acid (77 mg, 0.3 mmol) was coupled to the free amine as described above using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) in DMF.

The resin was washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2) and MeOH (5 ml x 2) and then dried in *vacuo*. The activation, cleavage and side chain deprotection of the resin bound peptide was carried out by using same method described for peptide **3**. The crude peptide was purified by HPLC on a semi preparative C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min., and the appropriate fractions were lyophilized to afford **4** (65% based on resin loading capacity). $C_{90}H_{165}N_{11}O_{13}S_2$, MALDI-ToF MS: observed, $[M+Na]$ 1695.2335Da; calculated, $[M+Na]$ 1695.4714Da.

Ligation between 2 and 3 to give 21: Conditions a (Scheme 3.4): The peptide thioester **3** (10 mg, 3.6 μ mol) and peptide **2** (7.24 mg, 5.5 μ mol) were dissolved in 6 M Gn.HCl, 200 mM sodium phosphate (pH 7.5) as 1:1.5 ratios to obtain final concentration of 1 mM. The ligation was initiated by the addition of 4% thiophenol (300 μ l, 2.0 μ mol) based on the volume of the reaction mixture. The ligation reaction was carried out in an incubator at 37°C and the progress of the reaction was periodically monitored by RP-HPLC and LC-MS. After a reaction time of 18 h, the reaction was diluted with 20% 2-mercaptoethanol in ligation buffer (3 ml). The resulting mixture was then purified by C-8 semi-preparative reversed phase column using linear gradients of 0-100% solvent B in A over a 40 min., and the appropriate fractions were collected and lyophilized to give **21** (6.7 mg, 48%). The Acm protecting group of the ligated product was removed by dissolving the glycopeptide in 10% aq. AcOH (2 ml, pH 4.0) followed by the treatment of Hg (II) acetate (8.18 mg, 25 μ mol) for 30 min., the reaction was quenched by addition of DTT (5.27 mg, 30 μ mol). The Acm deprotected product was purified by semi-

preparative RP-HPLC using a water/acetonitrile gradient to yield **22** (5.7 mg, 87%).

C₁₇₁H₂₅₇N₄₉O₄₉S₂, MALDI-ToF MS: observed, 3847.6615 Da, calculated, 3847.3031 Da.

Condition b (Scheme 3.4): The peptide thioester **3** (2 mg, 0.73 μ mol) and peptide **2** (1.44 mg, 1.1 μ mol), and dodecyl phosphocholine (1.5 mg, 4.4 μ mol) were dissolved in a mixture of trifluoroethanol and CHCl₃ (2.5 ml/ 2.5 ml). The solvents were removed under reduced pressure to give a lipid/peptide film on the surface of the round bottom flask. The lipid/peptide film was hydrated for 4 h at 37°C using 200 mM phosphate buffer (pH 7.5, 2 ml) in the presence of tris(carboxyethyl)phosphine (2% w/v, 40.0 μ g) and EDTA (0.1% w/v, 2.0 μ g). The mixture was ultrasonicated for 1 min. The peptide/lipid suspension was extruded through 1.0 μ m polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50°C to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (2% w/v, 40.0 μ g) to initiate the ligation reaction. The reaction was carried out in an incubator at 37°C and was complete within 2 hours. The reaction was then diluted with 20% 2-mercaptoethanol in ligation buffer (2 ml). The resulting mixture was purified by RP-HPLC on a semi-preparative C-8 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min., and the fraction possessing the expected product as determined by MALDI-ToF were collected and lyophilized to give **21** (2.2 mg, 78%). The Acm protecting group of the ligated product was removed by dissolving the glycopeptide in 10% aq. AcOH (2 ml) (pH 4.0) followed by the treatment of Hg(II)acetate (2.7 mg, 7 μ mol) for 30 min., the reaction was quenched by addition of DTT (1.7 mg, 9 μ mol). The Acm deprotected product was purified by semi-preparative RP-HPLC using a water/acetonitrile gradient to yield **22** (1.9

mg, 89%). $C_{171}H_{257}N_{49}O_{49}S_2$, MALDI-ToF MS: observed, 3847.6015 Da, calculated, 3847.3031Da.

Ligation between 22 and 4 to give 1: The peptide **22** (3.0 mg, 0.77 μ mol) and peptide thioester **4** (1.96 mg, 1.1 μ mol), and dodecyl phosphocholine (1.5 mg, 4.6 μ mol) were dissolved in a mixture of trifluoroethanol and $CHCl_3$ (2.5 ml/ 2.5 ml). The solvents were removed under reduced pressure to give a lipid/peptide film on the surface of the round bottom flask. The lipid/peptide film was hydrated for 4 h at 37°C using 200 mM phosphate buffer (pH 7.5, 2 ml) in the presence of tris(carboxyethyl)phosphine (2% w/v, 40.0 μ g) and EDTA (0.1% w/v, 2.0 μ g). The mixture was ultrasonicated for 1 min. The peptide/lipid suspension was extruded through 1.0 μ m polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50°C to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (2% w/v, 40.0 μ g) to initiate the ligation reaction. The reaction was carried out in an incubator at 37°C and the progress of the reaction was periodically monitored by MALDI-ToF, which showed that the reaction was complete within 2 h. and was complete within 2 hours. The reaction was then diluted with 20% 2-mercaptoethanol in ligation buffer (2 ml). The crude peptide was purified by semi preparative C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min., and lyophilization of the appropriate fractions afforded **1** (3.5 mg, 83%). $C_{254}H_{414}N_{60}O_{62}S_3$, MALDI-ToF MS: observed, 5392.9712Da, calculated, 5392.0171Da.

Synthesis of Lipopeptide thioester 26. The synthesis of **26** was carried out on a H-Gly-sulfamylbutyryl Novasyn TG resin (0.1 mmol) by a manual procedure. *N*- α -Fmoc-Lipidated amino acid (139 mg, 0.3 mmol) was coupled to the free amine of the resin

using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) in DMF. The coupling reaction was monitored by Kaiser test and was complete after standing for 12 h. Upon completion of the coupling, the *N*-Fmoc group was cleaved using 20% piperidine in DMF (6 ml). *N*- α -Fmoc-Gly-OH (90 mg, 0.3 mmol) was dissolved in DMF (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) were premixed for 2 min, and the mixture was added to the resin. The coupling reaction was monitored by Kaiser test and was complete after standing for 12 h. Upon completion of the coupling, the *N*-Fmoc group was cleaved using 20% piperidine in DMF (6 ml). One more cycle of coupling with *N*- α -Fmoc-Lipidated amino acid was carried out as described above to complete the synthesis. Finally, the *N*-Fmoc group was cleaved using 20% piperidine in DMF (6 ml) and acetylated using 5 ml of 10% Ac₂O, 5% DIPEA in NMP for 10 min. The resin was washed thoroughly with DMF (10 ml), DCM (10 ml) and MeOH (10 ml) and dried in *vacuo*. The activation and cleavage of the resin bound peptide was carried out by using same method described for peptide **3**. The crude peptide was purified by HPLC on a semi preparative C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over a period of 40 min., and the appropriate fractions were lyophilized to afford **26** (69% based on resin loading capacity). C₄₁H₇₀N₄O₅S, MALDI-ToF MS: observed, [M+Na] 753.4871Da; calculated, [M+Na] 753.5067Da.

Synthesis of peptide thioester 27: The synthesis of Ac₂S protected peptide thioester was carried out on preloaded H-Gly-sulfamylbutyryl Novasyn TG resin (0.1 mmol) as described in the general methods section for peptide synthesis. Side chain protection was as follows: *N*- α -Fmoc-*L*-aspartic acid β -*tert*.-butyl ester, *N*- α -Fmoc-*S*-acetamidomethyl-

L-cysteine, *N*- α -Fmoc-*N*- ϵ -*tert*.-Boc-*L*-lysine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-threonine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-tyrosine. Activation, cleavage and side chain deprotection was performed by the using same method described for compound **3**. The crude peptide was purified by semi preparative C-8 reversed phase column using a linear gradient of 0-100% of solvent B over A over period of 40 min., and lyophilization of the appropriate fractions afforded **27** in good yield (74% based on resin loading capacity). C₉₄H₁₄₀N₂₀O₂₁S₂, MALDI-ToF MS: observed, [M+Na] 1972.1240Da; calculated, [M+Na] 1973.3716Da

Ligation between 22 and 26 to give 23: The peptide **22** (2 mg, 0.51 μ mol) and peptide thioester **26** (0.53 mg, 0.72 μ mol) and, dodecyl phosphocholine (0.9 mg, 3.0 μ mol) were dissolved in a mixture of trifluoroethanol and CHCl₃ (2.5 ml/ 2.5 ml). The solvents were removed under reduced pressure to give a lipid/peptide film on the surface of the round bottom flask. The lipid/peptide film was hydrated for 4 h at 37°C using 200 mM phosphate buffer (pH 7.5, 2 ml) in the presence of tris(carboxyethyl)phosphine (2% w/v, 40.0 μ g) and EDTA (0.1% w/v, 2.0 μ g). The mixture was ultrasonicated for 1 min. The peptide/lipid suspension was extruded through 1.0 μ m polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50°C to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (2% w/v, 40.0 μ g) to initiate the ligation reaction. The reaction was carried out in an incubator at 37°C and the progress of the reaction was periodically monitored by MALDI-ToF, which showed that the reaction was complete within 2 h. and was complete within 2 hours. The reaction was then diluted with 20% 2-mercaptoethanol in ligation buffer (2 ml).The crude peptide was purified by semi preparative C-4 reversed phase column using a linear gradient of 0-

100% solvent B in A over a 40 min., and lyophilization of the appropriate fractions afforded **23** (1.7 mg, 78%). $C_{205}H_{319}N_{53}O_{54}S_2$, MALDI-ToF MS: observed, 4454.0313Da, calculated, 4454.1791Da.

Ligation between 2 and 27 to give 34: The peptide **2** (5.6 mg, 4.3 μ mol) and peptide thioester **27** (6.0 mg, 3.0 μ mol) and dodecyl phosphocholine (8.0 mg, 25.0 μ mol) were dissolved in a mixture of trifluoroethanol and $CHCl_3$ (2.5 ml/ 2.5 ml). The solvents were removed under reduced pressure to give a lipid/peptide film on the surface of the round bottom flask. The lipid/peptide film was hydrated for 4 h at 37°C using 200 mM phosphate buffer (pH 7.5, 2 ml) in the presence of tris(carboxyethyl)phosphine (2% w/v, 40.0 μ g) and EDTA (0.1% w/v, 2.0 μ g). The mixture was ultrasonicated for 1 min. The peptide/lipid suspension was extruded through 1.0 μ m polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50°C to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (2% w/v, 40.0 μ g) to initiate the ligation reaction. The reaction was carried out in an incubator at 37°C and was complete within 2 hours. The reaction was then diluted with 20% 2-mercaptoethanol in ligation buffer (2 ml). The resulting reaction mixture was purified by using RP-HPLC on a semi-preparative C-8 reversed phase column using linear gradients of 0-100% solvent B in A over a 40 min., the fraction possessing the expected mass were collected and lyophilized to give **33** (7.4 mg, 79%). The Acm protecting group of the ligated product was removed by dissolving the glycopeptide in 10% aq. AcOH (2 ml, pH 4.0) followed by the treatment of Hg(II)acetate (11.5 mg, 30 μ mol) for 30 min. After which the reaction was quenched by addition of DTT (7.4 mg, 40 μ mol). The Acm deprotected product was purified by semi preparative RP-HPLC using a water/acetonitrile gradient to

yield **34** (5.6 mg, 77%). $C_{137}H_{215}N_{35}O_{41}S_2$, MALDI-ToF MS: observed, 3073.7275Da, calculated, 3072.5129Da.

Ligation between 34 and 4 to give 24: The peptide **34** (1.5 mg, 0.48 μ mol) and peptide thioester **4** (0.98 mg, 0.58 μ mol) and, dodecyl phosphocholine (0.9 mg, 2.88 μ mol) were dissolved in a mixture of trifluoroethanol and $CHCl_3$ (2.5 ml/ 2.5 ml). The solvents were removed under reduced pressure to give a lipid/peptide film on the surface of the round bottom flask. The lipid/peptide film was hydrated for 4 h at 37°C using 200 mM phosphate buffer (pH 7.5, 2 ml) in the presence of tris(carboxyethyl)phosphine (2% w/v, 40.0 μ g) and EDTA (0.1% w/v, 2.0 μ g). The mixture was ultrasonicated for 1 min. The peptide/lipid suspension was extruded through 1.0 μ m polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50°C to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (2% w/v, 40.0 μ g) to initiate the ligation reaction. The reaction was carried out in an incubator at 37°C and the progress of the reaction was periodically monitored by MALDI-ToF, which showed that the reaction was complete within 2 h. and was complete within 2 hours. The reaction was then diluted with 20% 2-mercaptoethanol in ligation buffer (2 ml). The crude peptide was purified by semi preparative C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min., and lyophilization of the appropriate fractions afforded **24** (1.8 mg, 85%). $C_{220}H_{372}N_{46}O_{54}S_3$, MALDI-ToF MS: observed, 4622.3549Da, calculated, 4621.7785Da.

Ligation between 34 and 28 to give 25: The peptide **34** (3.081 mg, 1.0 μ mol) and peptide thioester **26** (1.1 mg, 1.5 μ mol) and, dodecyl phosphocholine (1.9 mg, 6.0 μ mol) were dissolved in a mixture of trifluoroethanol and $CHCl_3$ (2.5 ml/ 2.5 ml). The solvents

were removed under reduced pressure to give a lipid/peptide film on the surface of the round bottom flask. The lipid/peptide film was hydrated for 4 h at 37°C using 200 mM phosphate buffer (pH 7.5, 2 ml) in the presence of tris(carboxyethyl)phosphine (2% w/v, 40.0 µg) and EDTA (0.1% w/v, 2.0 µg). The mixture was ultrasonicated for 1 min. The peptide/lipid suspension was extruded through 1.0 µm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50°C to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (2% w/v, 40.0 µg) to initiate the ligation reaction. The reaction was carried out in an incubator at 37°C and the progress of the reaction was periodically monitored by MALDI-ToF, which showed that the reaction was complete within 2 h. and was complete within 2 hours. The reaction was then diluted with 20% 2-mercaptoethanol in ligation buffer (2 ml). The crude peptide was purified by semi preparative C-8 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min., and lyophilization of the appropriate fractions afforded **25** (2.6 mg, 73%). C₁₇₁H₂₇₇N₃₉O₄₆S₂, MALDI-ToF MS: observed, 3679.6072Da, calculated, 3679.3928Da.

3.2 References

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CHAPTER 4

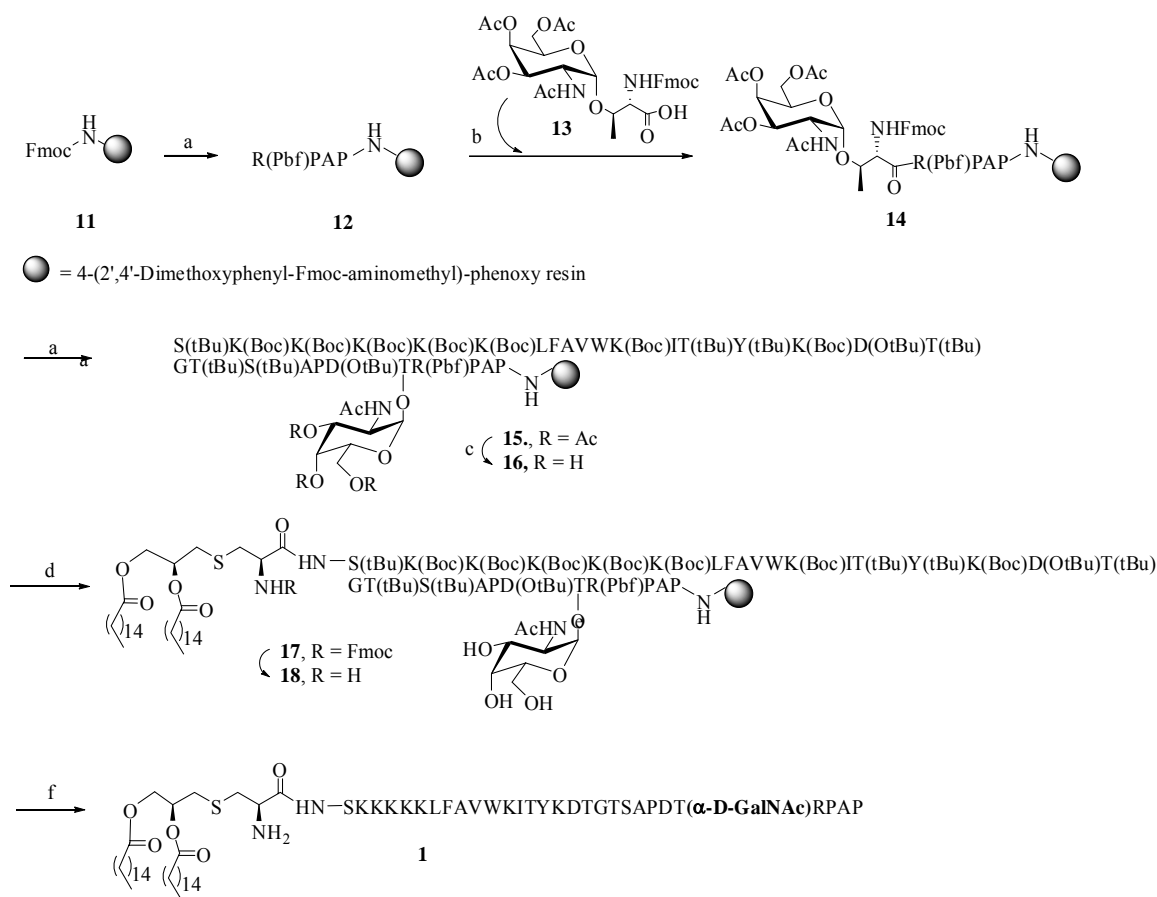
Overcoming Immune Suppression of Tumor-Associated Carbohydrates by

Employing a Fully Synthetic Three-Component Vaccine

A broad and expanding body of preclinical and clinical studies demonstrates¹⁻⁴ that naturally acquired, passively administered or actively induced antibodies against carbohydrate-associated tumor antigens are able to eliminate circulating tumor cells and micro-metastases in cancer patients. Tumor-associated saccharides are, however, of low antigenicity, because they are self-antigens and consequently tolerated by the immune system. The shedding of antigens by the growing tumor reinforces this tolerance. In addition, foreign carrier proteins such as KLH and BSA and the linker that attach the saccharides to the carrier protein can elicit strong B-cell responses, which may lead to the suppression of antibody responses against the carbohydrate epitope.^{5,6} It is clear that the successful development of carbohydrate-based cancer vaccines requires novel strategies for the more efficient presentation of tumor-associated carbohydrate epitopes to the immune system, resulting in a more efficient class switch to IgG antibodies.⁷⁻¹³

We reasoned that a three-component vaccine composed of a tumor-associated carbohydrate B-epitope, a promiscuous peptide T-helper epitope, and a TLR ligand will circumvent immune suppression caused by a carrier protein or the linker region of a classical conjugate vaccine. Such a vaccine candidate contains, however, all mediators required for eliciting a strong and relevant IgG immune response. Furthermore, the fully synthetic nature of a three-component vaccine ensures that it can be prepared in a

coupled by stepwise solid phase peptide synthesis using an automated HBTU-mediated HOBt ester activation¹⁷ protocol to give compound **12**. Next, the glycosylated amino acid building block **13** was manually coupled using HATU/HOAt in the presence of DIPEA in DMF to give compound **14**. The peptide sequence was then elongated by using standard protocol to give compound **15**. After assembly of the glycopeptide, the acetyl esters of the saccharide moiety were cleaved by treatment with 80% hydrazine in MeOH^{18, 19} on the resin to afford compound **16**.

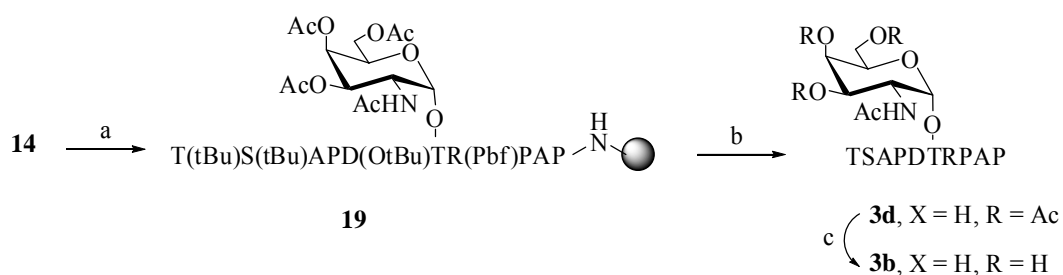


Scheme 4.1 | Synthesis of compound 1. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) **13**, HATU/HOAt, DIPEA, NMP, overnight; c) hydrazine (80%) in MeOH, 2 h; d) manual coupling of Pam₂Cys-OH, PyBOP, HOBt in the presence of DIPEA in DMF; e) piperidine (20%) in DMF; f) reagent B (TFA 88%, phenol 5%, H₂O 5%, TIS 2%), 2 h.

The acetyl esters of the saccharide moiety were cleaved first to avoid the hydrolysis of palmitic esters by the basic conditions used at the end of the synthesis. Next, *N*- α -Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (Pam₂Cys-OH)²⁰ was manually coupled to the *N*-terminal amine of the glycopeptide **16** using PyBOP/HOBt in the presence of DIPEA in DMF to give the resin bound glyco(lipo)peptide **17**. The *N*-Fmoc group of **17** was removed using 20% piperidine in DMF to afford compound **18**. The amino acid side chain deprotection and cleavage of the resin was then accomplished by treating resin bound glyco(lipo)peptide **18** with reagent B (TFA 88%, Phenol 5%, Water 5%, TIS 2%) for 2h. The glyco(lipo)peptide **1** was then recovered by RP-HPLC purification using C4 column in moderate yield (30%).

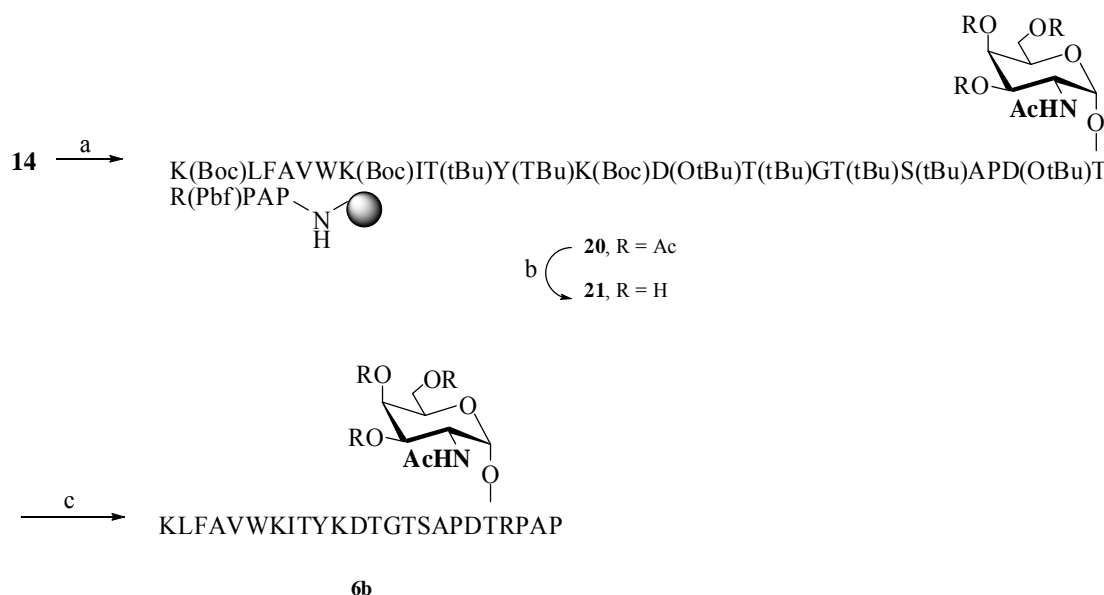
Unfortunately, a similar linear synthesis of compound **2** gave a product that was difficult to purify to homogeneity. Therefore, **2** was prepared by *liposome*-mediated native chemical ligation (NCL) of building blocks **3a**, **4**, and **5**.²¹ The chemical synthesis of these compounds have been described in *chapter 3*, compounds **3**, **9**, and **6** respectively.

The synthesis of glycopeptide **3b** (Scheme 4.2) was carried out by SPPS starting from compound **14** (Scheme 4.1). The peptide sequence was then assembled by using standard protocol to afford compound **19**. After the completion of the synthesis, the glycopeptide was cleaved from the resin by treatment with reagent B (TFA 88%, Phenol 5%, Water 5%, TIS 2%). Finally, the acetyl esters of the saccharide moiety were cleaved by treatment of 5% aqueous hydrazine to give glycopeptide **3b** in excellent overall yield (86%).



Scheme 4.2 | Synthesis of compounds 3b. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) reagent B (TFA 88%, Phenol 5%, Water 5%, TIS 2%); c) aqueous hydrazine (5%).

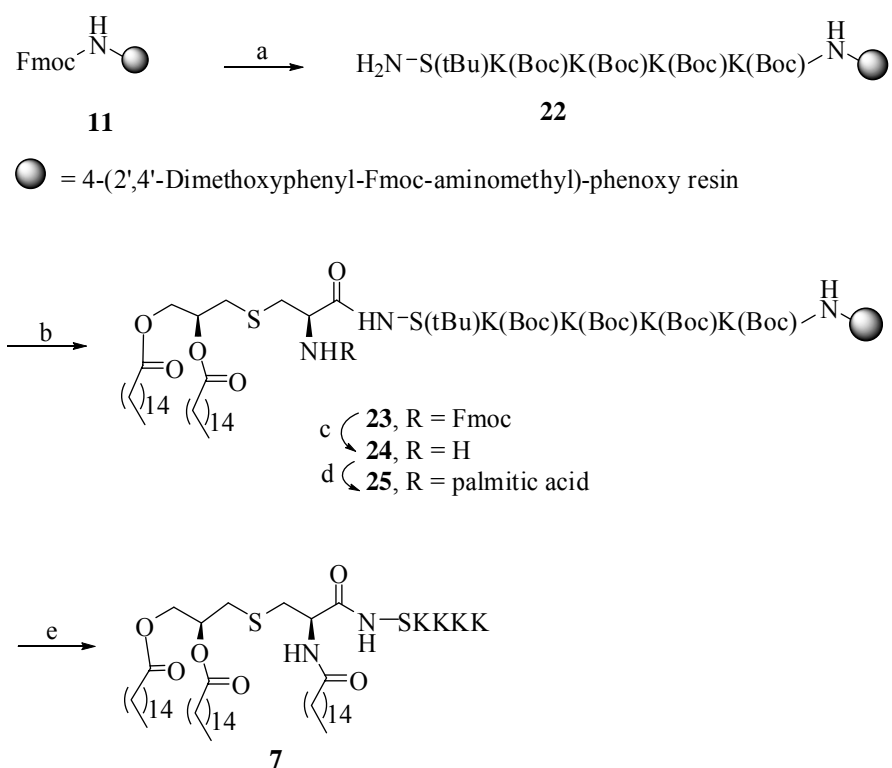
Glycopeptide **6b** (Scheme 4.3) was also synthesized from starting compound **14** (scheme 4.1). The glycopeptide was assembled using standard protocol for Fmoc method. Thus, the peptide sequence was assembled to afford resin bound compound **20**. The acetyl esters of the saccharide moiety were cleaved by treatment with 80% hydrazine in MeOH^{18,19} on the resin to afford compound **21**.



Scheme 4.3 | Synthesis of compound 6b. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) hydrazine (80%) in MeOH, 2 h; c) reagent B (TFA 88%, phenol 5%, H₂O 5%, TIS 2%), 2 h.

Finally side chain deprotection and cleavage of the glycopeptide from the resin was accomplished by treatment of reagent B for 2 h to afford compound **6b** in 65% overall yield.

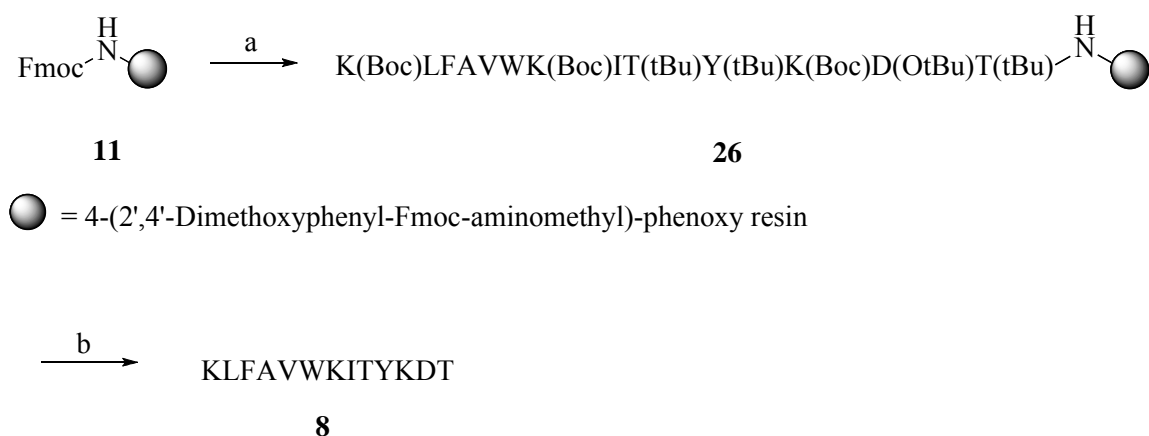
The synthesis of compound **7** (Scheme 4.4) was carried out by SPPS protocol using Rink amide resin. After the assembly of the initial peptide sequence by using an automated HBTU-mediated HOBt ester activation protocol to give compound **22**, the rest of the synthetic steps were performed manually. Thus, Pam₂Cys-OH²⁰ was coupled to the *N*-terminal amine of the peptide **22** using PyBOP/HOBt in the presence of DIPEA in DMF to give the resin bound lipopeptide **23**.



Scheme 4.4 | Synthesis of compound 7. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) manual coupling of Pam₂Cys-OH, PyBOP, HOBt in the presence of DIPEA in DMF; c) piperidine (20%) in DMF; d) coupling of palmitic acid, PyBOP, HOBt in the presence of DIPEA in DMF; e) reagent B (TFA 88%, Phenol 5%, Water 5%, TIS 2%).

The *N*-Fmoc group of **23** was removed using 20% piperidine in DMF and the free amine of the resulting compound **24** was coupled with palmitic ester in the presence of PyBOP/HOBt containing DIPEA in DMF to give fully protected resin bound lipopeptide **25**. The amino acid side chain deprotection and cleavage of the lipopeptide from the resin was accomplished by treating resin bound lipopeptide **25** with reagent B (TFA 88%, Phenol 5%, Water 5%, TIS 2%) for 2h. The lipopeptide **7** was then recovered by RP-HPLC purification using C4 column in good overall yield (65%).

Finally, the synthesis of compound **8** is outlined in scheme 4.5. The peptide **8** was obtained in 60% yield after purification by RP-HPLC over a C8 column.



Scheme 4.5 | Synthesis of compound 8. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) reagent B (TFA 88%, phenol 5%, H₂O 5%, TIS 2%), 2 h.

Next, compounds **1** and **2** were incorporated into phospholipid-based small uni-lamellar vesicles (SUVs) by hydration of a thin film of the synthetic compounds, egg phosphatidylcholine, phosphatidylglycerol and cholesterol in a HEPES buffer (10 mM, pH 6.5) containing NaCl (145 mM) followed by extrusion through a 100 nm Nuclepore® polycarbonate membrane. Groups of five female BALB/c mice were immunized intraperitoneal four times at weekly intervals with liposomes containing 3 µg of saccharide.

To explore the adjuvant properties of the vaccine candidates, liposomes were administered with or without the potent saponin immuno-adjuvant QS-21.

Anti-MUC1 antibody titers were determined by coating microtiter plates with CTSAPDT(α -DGalNAc)RPAP **3a**, conjugated to bromoacetyl modified BSA and detection was accomplished with anti-mouse IgG antibodies labeled with alkaline phosphatase. Mice were immunized with **2** elicited exceptionally high titers of anti-MUC1 IgG antibodies (Table 4.1, 4.2, and Fig. 4.2). Sub-typing of the IgG antibodies (IgG1, IgG2a, IgG2b, and IgG3) indicated a bias towards a Th2 response.²² Furthermore; the observed high IgG3 titer is typical of an anti-carbohydrate response.

Co-administering of adjuvant QS-21 did not lead to a significant increase of IgG antibodies; however, in this case a mixed Th1/Th2 response was observed. Surprisingly, the use of glycolipopeptide **1**, which contains Pam₂CysSK₄ instead of Pam₃CysSK₄, gave lower titers of IgG antibodies. Importantly, compounds **1** and **2** elicited low titers of antibodies against the T-epitope indicating that the vaccine does not suffer from immune suppression.

The influence of covalent attachment of the various components of the vaccine candidate on antigenic responses was investigated by immunizing mice with a liposomal preparation of compounds **6b**, which is composed of the T-epitope linked to the B-epitope, and the adjuvant Pam₃CysSK₄ (**7**). Surprisingly, significantly lower titers of IgG antibodies were determined compared to the use of **2** (Table 4.1), which demonstrates that covalent attachment of Pam₃CysSK₄ to the T-B-epitope is important for optimal immune responses. No or very low IgG antibody responses were observed when the two compounds were administered as a saline solution indicating that a liposomal preparation

contributes to antigenicity. The importance of covalent attachment was further highlighted in an experiment in which the B-epitope (**3b**), T-epitope (**8**), and adjuvant Pam₃CysSK₄ (**7**) were administered as a liposomal preparation resulting in low titers of IgG antibodies.

Table 4.1 | ELISA anti-MUC1 antibody titers* after 4 immunizations with various preparations.

Immunization**	IgG total***	IgG1	IgG2a	IgG2b	IgG3
1	20,906	66,947	700	930	7,292
1 / QS-21	30,158	113,060	22,972	6,586	17,837
2	169,607	389,270	56,482	42,693	116,831
2 / QS-21	322,819	371,290	378,903	56,842	263,471
6b / 7	16,561	26,767	3,330	3,118	7,758
6b / 7 (saline)	628	360	380	782	431
3b / 7 / 8	2,270	8,374	0	0	0

Table 4.2 | ELISA anti-MUC1 antibody titers* one week after the third, fourth, and fifth immunization.

Immunization**	IgG total	IgG1	IgG2a	IgG2b	IgG3
2 (3 rd imm.)	18,922	31,682	3,190	5,654	22,863
2 (4 th imm.)	169,607	389,270	56,482	42,693	116,831
2 (5 th imm.)	252,929	726,668	64,887	92,413	163,052
2 / QS-21 (3 rd imm.)	50,799	71,139	37,717	16,619	83,377
2 / QS-21 (4 th imm.)	322,819	371,290	378,903	56,842	263,471
2 / QS-21 (5 th imm.)	140,353	292,546	274,048	32,296	259,926

* Anti-MUC1 antibody titers are presented as the median for groups of five mice. ELISA plates were coated with BSA-BrAc-MUC1 conjugate and titers were determined by linear regression analysis, plotting dilution vs. absorbance. Titers are defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera.

** Liposomal preparations were employed, except for **6b** / **7** (saline).

*** A statistical significant difference ($P < 0.05$) was observed between **1** vs. **2**, **2** vs. **6b** / **7**, **2** vs. **6b** / **7** (saline), **2** vs. **3b** / **7** / **8**, and **6b** / **7** vs. **6b** / **7** (saline).

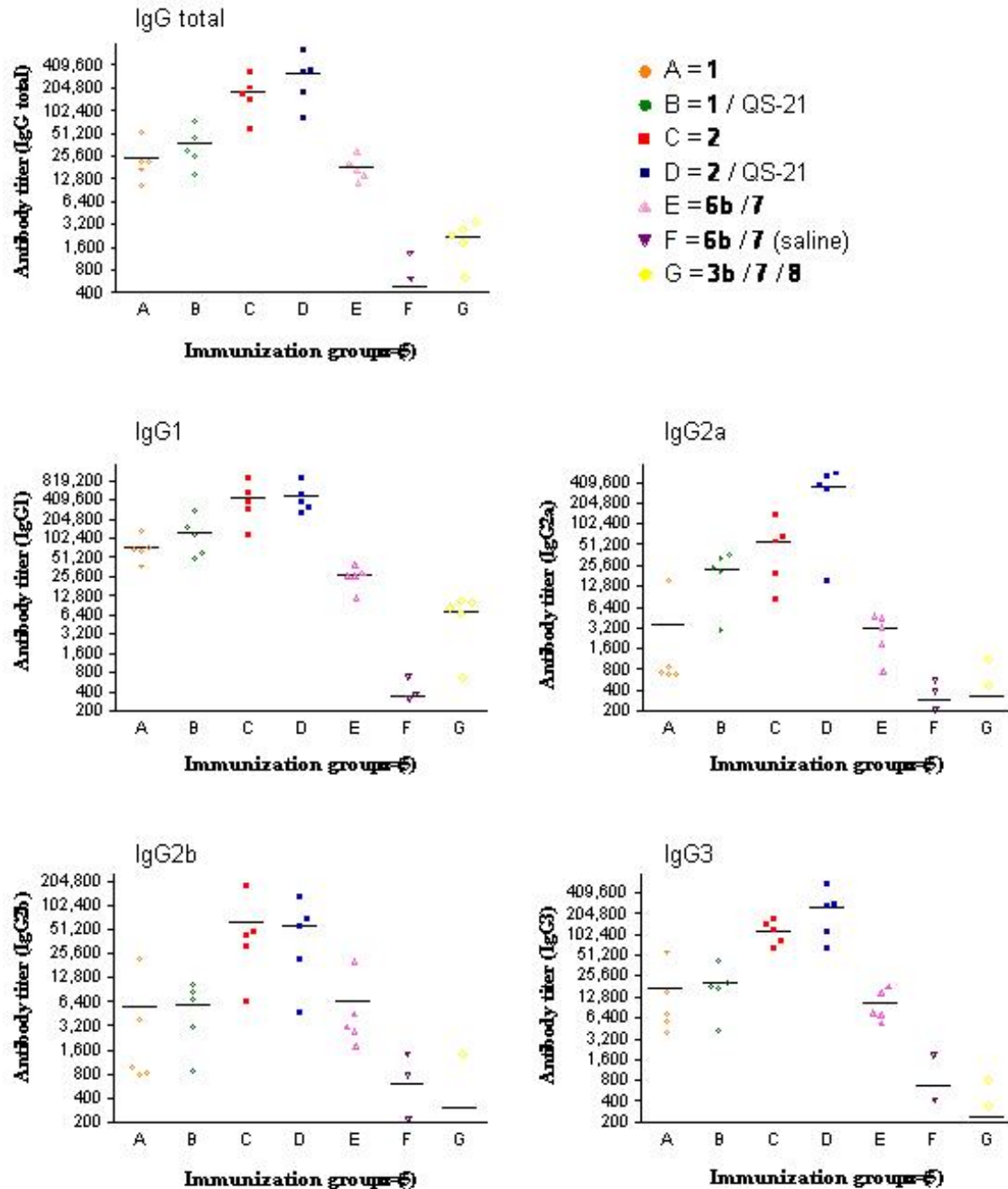


Figure 4.2 | ELISA anti-MUC1 antibody titers after 4 immunizations with 1, 1 / QS-21, 2, 2 / QS-21, 6b / 7, 6b / 7 (saline), and 3b / 7 / 8. ELISA plates were coated with BSA-BrAc-MUC1 conjugate and titers were determined by linear regression analysis, plotting dilution vs. absorbance. Titers were defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera. Each data point represents the titer for an individual mouse after 4 immunizations and the horizontal lines indicate the mean for the group of five mice.

To ensure that the mouse sera were able to recognize the native MUC1 antigen present on cancer cells, binding of the sera to the MUC1 expressing MCF7 human breast cancer cell line was examined by flow cytometry. The anti-sera elicited against **1** and **2** reacted strongly with the MUC1 positive tumor cells whereas no binding was observed when SK-MEL 28 cells, which do not express the MUC1 antigen, were employed (Fig. 4.3). Further studies showed that both compounds induced the secretion of cytokines such as TNF- α in a TLR2-dependent manner leading to the up-regulation of co-stimulatory proteins such as CD80, CD83, and CD86 (Figs. 4.4, 4.5, and 4.6).

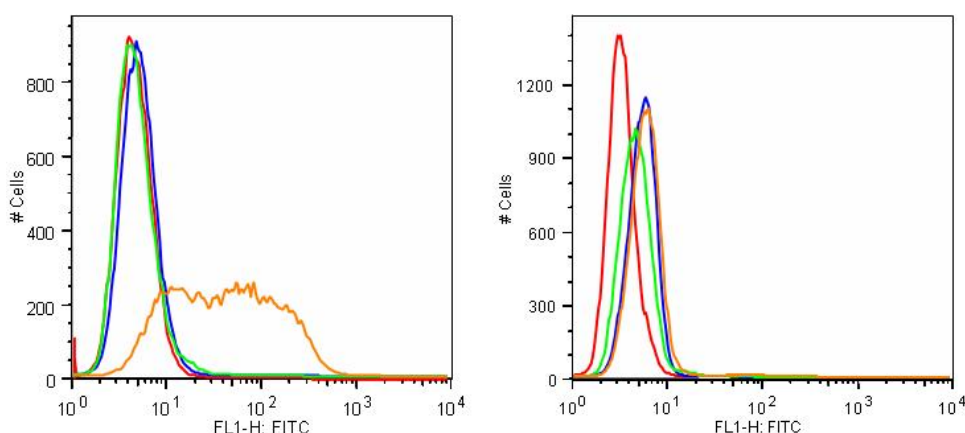


Figure 4.3 | Flow Cytometry analysis of specific anti-MUC-1 antibodies. Reactivity was tested in MCF7 (A) and SK-MEL-28 (B) cells. Fluorescence intensity of serum (1:50 diluted) was assessed before (serum control; green) and after 4 immunizations with **2** (orange). Also shown are medium (red) and conjugate (blue) controls. This is a representative example of the group of mice immunized with **2** (n=5). Similar results were obtained for mice immunized with **2** in the presence of QS-21 and **1** in the absence and presence of QS-21.

Uptake and proteolytic processing of antigen and subsequent presentation of the peptide T-epitope as a complex with MHC class II on the cell surface of APCs, is critical for eliciting IgG antibodies. Thus, immunizations with a liposomal preparation of the individual components of **2** will not require such processing and hence may lead to even more robust antigenic responses.

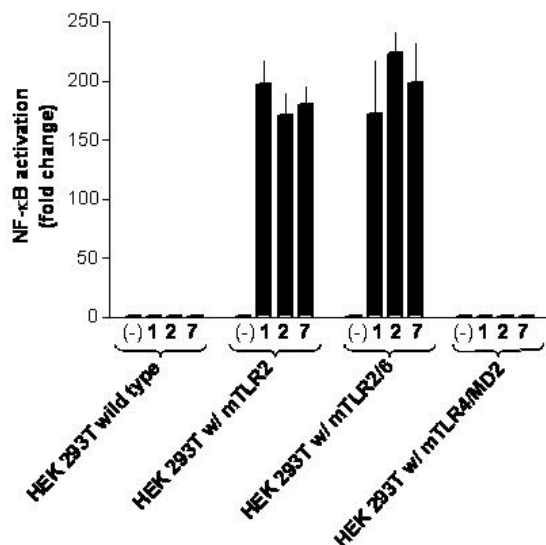


Figure 4.4 | Involvement of TLR2 in NF- κ B activation by compounds 1, 2, and 7. Induction of NF- κ B activation was determined in triplicate cultures of HEK293T cells (wild type and stable transfected with murine TLR2, TLR2/TLR6, or TLR4/MD2) transiently transfected with the expression vectors pELAM-Luc and pRL-TK. Forty-four h post-transfection, cells were treated with the synthetic compounds 1, 2, and 7 (6.1 μ M each) or were left untreated (control; (-)). Forty-eight h post-transfection, NF- κ B activation was determined by firefly luciferase activity relative to *Renilla* luciferase activity. In the transfection experiment shown, human TNF- α (10 ng/mL) induced 145 ± 11 , 82 ± 25 , 141 ± 10 , and 42 ± 2 fold activation of NF- κ B in wild type and stable transfected murine TLR2, TLR2/TLR6, or TLR4/MD2 cells, respectively.

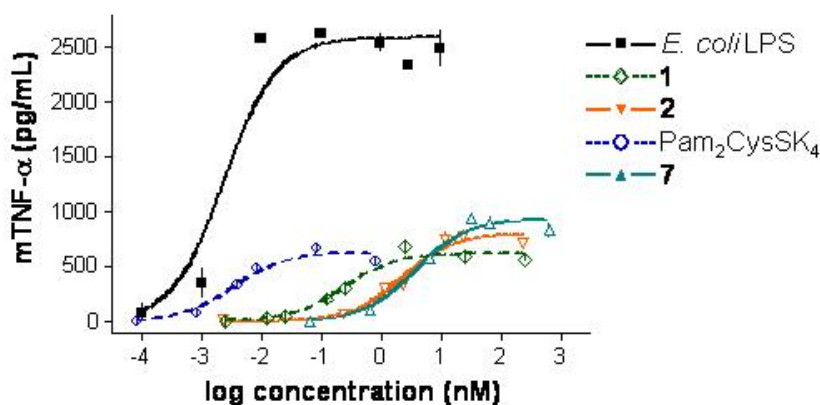


Figure 4.5 | TNF- α production by murine macrophages after stimulation with LPS and synthetic compounds 1, 2, Pam₂CysSK₄, and 7. Murine 264.7 RAW γ NO(-) cells were incubated for 5.5 h with increasing concentrations of *E. coli* LPS or synthetic compounds 1, 2, Pam₂CysSK₄, and 7 as indicated.

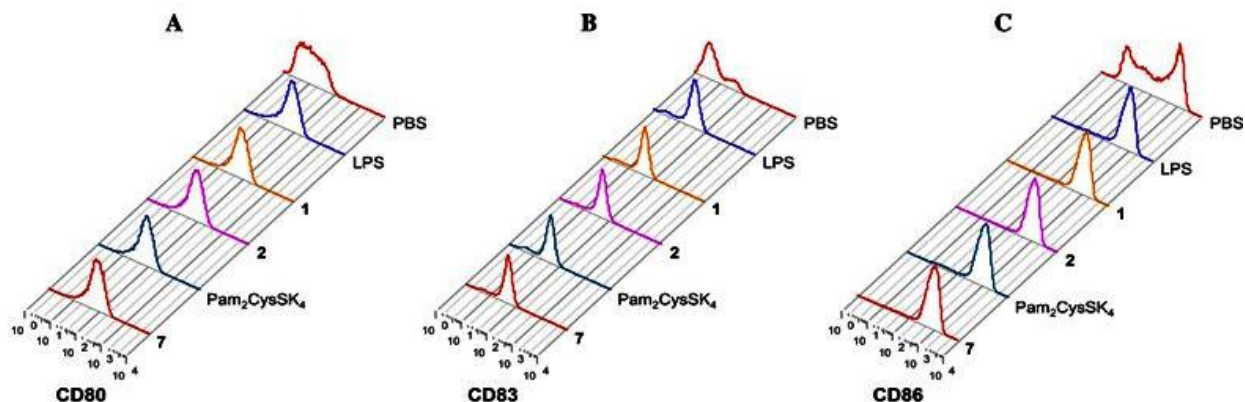
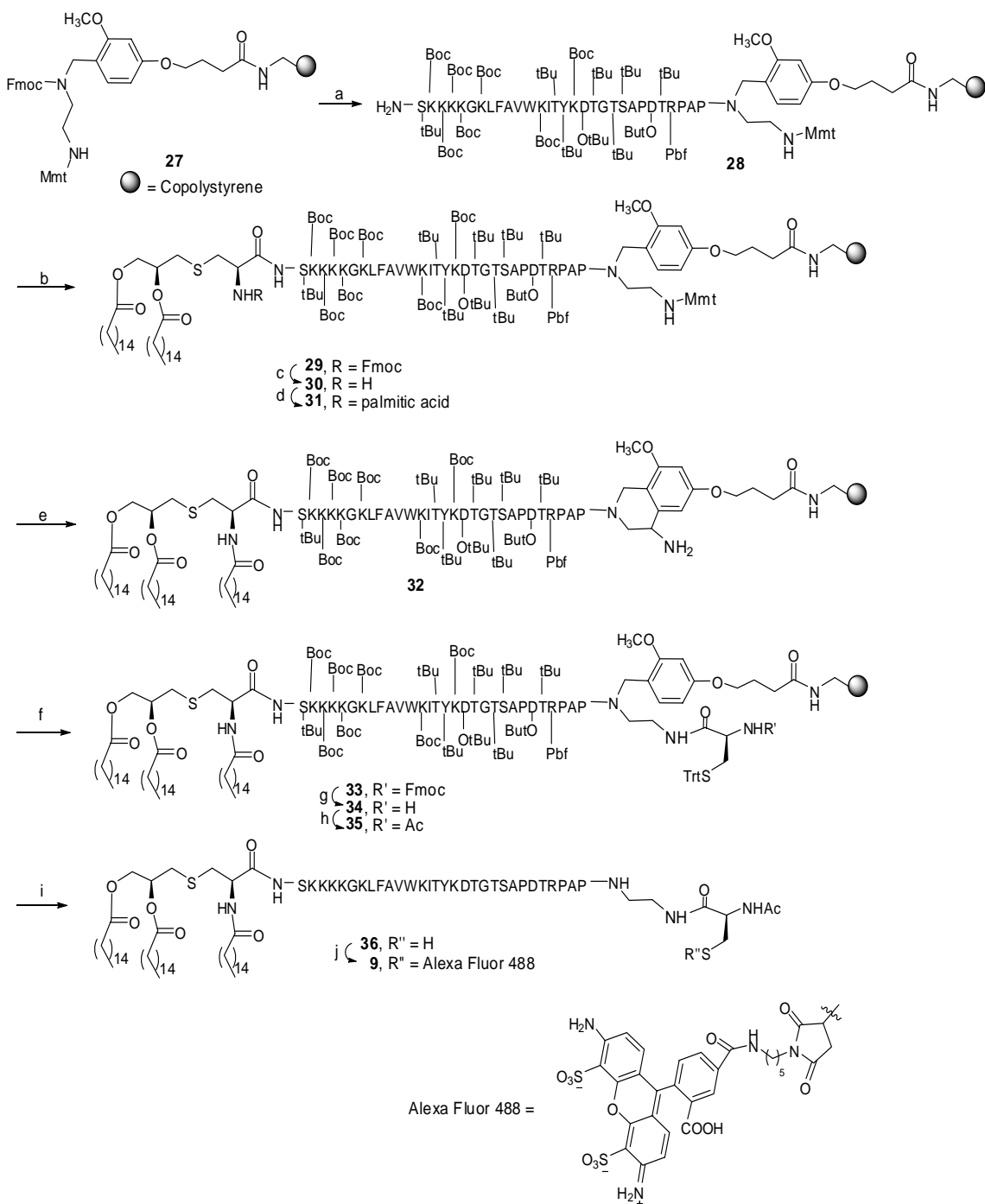


Figure 4.6 | Induction of dendritic cell maturation. After exposure of MDDCs to PBS (negative control), LPS (1 $\mu\text{g/mL}$; positive control), and compounds **1**, **2**, Pam₂CysSK₄, and **7** (1 $\mu\text{g/mL}$ each) for 16-24 h, expression of CD80 (A), CD83 (B), and CD86 (C) was determined by flow cytometry analysis.

It was anticipated that in addition to initiating the production of cytokines and stimulating the up regulation of co-stimulatory proteins, the lipopeptide Pam₃CysSK₄ (**7**) may facilitate selective targeting and uptake by antigen presenting cells in a TLR2-dependent manner. To test this hypothesis, compound **9**, which contains an Alexa Fluor® 488 fluorescence label, was prepared (Scheme 4.6). The synthesis of compound **9** was carried out on a universal NovaTag ® resin **27** containing bifunctional linker for selective attachment of a cysteine moiety, which could be conjugated to Alexa Fluor® 488 C5-maleimide. Thus, peptide was assembled using an automated HBTU-mediated HOBt ester activation protocol to give compound **28**. The Pam₂Cys-OH²⁰ was then coupled with *N*-terminal amine of the peptide **28** using PyBOP/HOBt in the presence of DIPEA in DMF to give the resin bound lipopeptide **29**. The *N*-Fmoc group of **29** was cleaved by using 20% piperidine in DMF and the free amine of the resulting compound **30** was coupled with palmitic ester in the presence of PyBOP/HOBt containing DIPEA in DMF to give fully protected resin bound lipopeptide **31**. The pendant Mmt group of **31** was

then removed by treatment of HOBt (1M) in TFE/DCM for 2 h to obtain **32**. *N*- α -Fmoc-Cys(Trt)-OH was coupled to the free amine of **32** using PyBOP/HOBt in the presence of DIPEA in DMF to give **33**. Upon completion of the coupling, the *N*-Fmoc group was cleaved using 20% piperidine in DMF to give **34** and the free amino group was acylated to obtain **35**. The lipopeptide was then cleaved from the resin by treatment of TFA (94%), EDT (2.5%), H₂O (2.5%), TIS (1%) for 4 h at room temperature. After purification by RP-HPLC lipopeptide **36** was obtained in 25% overall yield. The lipopeptide **36** was then treated with Alexa Fluor® 488 C5 maleimide in the presence of catalytic amount of TCEP and DIPEA in DMSO at room temperature for 12 h. The crude reaction mixture was then purified by RP-HPLC to afford compound **9** in 68% overall yield.

The compound **9** was then administered to mouse macrophages. After 30 min, the cells were harvested, lysed, and the fluorescence measured. To account for possible cell surface binding without internalization, the cells were also treated with trypsin before lyses and then examined for fluorescence. A significant quantity of **9** was internalized, whereas a small amount was attached to the cell surface. To determine whether the uptake was mediated by TLR2, uptake studies were also performed using wild type HEK 293T cells and HEK 293T cells stable transfected with murine TLR2, TLR2/TLR6, or TLR4/MD2 (Fig. 4.7.). Importantly, significant uptake was only observed when the cells were transfected with TLR2, indicating that uptake is mediated by this receptor. It is important to note that macrophage galactose type C-lectin (MGL) may also facilitate uptake of the vaccine candidates by binding to the Tn-antigen, which is not probed by compound **9**.²³



Scheme 4.6 | Synthesis of compound 9. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) manual coupling of Pam₂Cys-OH, PyBOP, HOBt in the presence of DIPEA in DMF; c) piperidine (20%) in DMF; d) coupling of palmitic acid, PyBOP, HOBt in the presence of DIPEA in DMF; e) HOBt (1M) in TFE/DCM, 2 h; f) Fmoc-Cys(Trt)-OH, PyBOP, HOBt in the presence of DIPEA in DMF, 4 h; g) piperidine (20%) in DMF; h) Ac₂O (10%), DIPEA (5%) in NMP, 30 min; i) TFA (94%), EDT (2.5%), H₂O (2.5%), TIS (1%), 4 h; j) Alexa Fluor® 488 C5 maleimide ester, TCEP, DIPEA in DMSO, RT, 12 h.

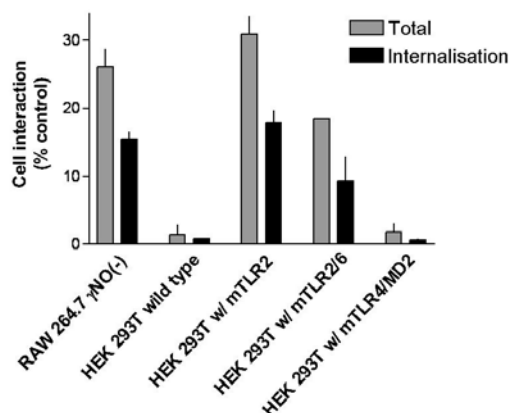


Figure 4.7 | Cellular uptake of compound 9. Cells (RAW 264.7 γ NO(-), HEK 293T wild type, and HEK 293T stable transfected with murine TLR2, TLR2/TLR6, or TLR4/MD2) were exposed to Alexa fluor 488-labeled compound **9** (1 μ g/mL) for 30 min. After cells were washed and lysed (total cell interaction; grey) or washes, treated with trypsin, and then lysed (internalization only; black) fluorescence (absorbance 485 nm, emission 538 nm) was measured. Fluorescence values were normalized for maximum possible fluorescence (100%).

Most efforts aimed at developing carbohydrate-based cancer vaccines have been focused on the use of chemically synthesized tumor-associated carbohydrates linked through an artificial linker to a carrier protein.¹⁻⁴ It has been established that the use of KLH as a carrier protein in combination with the powerful adjuvant QS-21 gives the best results. However, a drawback of this approach is that KLH is a very large and cumbersome protein that can elicit high titers of anti-KLH-antibodies, leading to immune suppression of the tumor-associated carbohydrate epitope.²⁴ Furthermore, the conjugation chemistry is often difficult to control as it results in conjugates with ambiguities in composition and structure, which may affect the reproducibility of immune responses.

Also, the linker moiety can elicit strong B-cell responses.^{5,6} Not surprisingly, preclinical and clinical studies with carbohydrate-protein conjugates have led to results of mixed merit. For example, mice immunizations with a trimeric cluster of Tn-antigens conjugated to KLH (Tn(c)-KLH) in the presence of the adjuvant QS-21 elicited modest

titers of IgG antibodies.²⁵ Examination of the candidate vaccine in a clinical trial of relapsed prostate cancer patients gave low median IgG and IgM antibody titers.²⁶ In another study, a number of MUC1-derived glycopeptides conjugated to KLH were investigated as immunogens.²⁷ It was found that only glycopeptides composed of multiple repeat units that are highly glycosylated with Tn antigens elicited IgG antibodies that could recognize cancer cells.

The excellent antigenicity of the here reported three-component vaccine is attributed to a number of unique features. Firstly, it does not have any unnecessary features that are antigenic and may induce immune suppression. The chemical attachment of the TLR2 agonist Pam₃CysSK₄ to the B- and T-epitopes ensures that cytokines are produced at the site where the vaccine interacts with immune cells. This should lead to a high local concentration of cytokines facilitating maturation of relevant immune cells. It also facilitates uptake by TLR2-expressing cells such as APCs, which will assist antigen processing and full activation. Finally, a fully synthetic approach makes it possible to optimize the various components of the candidate vaccine by structure-activity relationship studies. In this respect, proper design of the three-component vaccine is essential, because previously we showed that a compound composed of the inferior Pam₃Cys adjuvant and a human helper T-epitope elicits low titers of IgG antibodies.¹² Furthermore, compound **1** which contains the TLR1/2 agonist Pam₂CysSK₄ gave lower titers than compound **2**, which is modified by the TLR2/6 agonist Pam₃CysSK₄. It has also been found that covalent attachment of the three components is important for optimal antigenic responses. Probably, the lipid moiety of the vaccine candidate facilitates proper presentation and retention into liposomes. In this respect, liposomes are

an attractive carrier due to their low intrinsic immunogenicity. Furthermore, liposomes can present the B-epitopes multivalently facilitating B-cell receptor clustering resulting in B-cell activation.

4.1 Experimental procedures

Reagents and general procedures: Amino acid derivatives and resins were purchased from NovaBioChem and Applied Biosystems; DMF from EM Science; and NMP from Applied Biosystems. Egg phosphatidylcholine (PC), Phosphatidylglycerol (PG), Cholesterol, and Dodecyl phosphocholine (DPC) was obtained from Avanti Polar Lipids. All other chemical reagents were purchased from Aldrich, Acros, Alfa Aesar and Fischer and used without further purification. All solvents employed were reagent grade. Reverse Phase HPLC was performed on an Agilent 1100 series system equipped with an autosampler, UV-detector and fraction-collector using a Zorbax Eclipse C8 analytical column (5 μ m, 4.6 x 150 mm) at a flow rate of 1 ml/min, a semi preparative C8 column (5 μ m, 10 x 250 mm) at a flow rate of 2 ml/min, a Synchropak C4 analytical column (5 μ m, 4.6 x 100 mm) at a flow rate of 1 ml/min and a Vydac C4 semi preparative column (5 μ m, 4.6 x 250 mm) at a flow rate of 2 ml/min. All runs used linear gradients of 0-100% solvent B in A over a 40 min. period was used unless otherwise specified. (A = water 95%, acetonitrile 5% and 0.1% TFA 0, B= water 5%, acetonitrile 95% and 0.1% TFA). MALDI-ToF mass spectra were recorded on a ABI 4700 proteomic analyzer.

General methods for Solid-Phase Peptide Synthesis (SPPS): Peptides were synthesized by established protocols on a Applied Biosystems, ABI 433A peptide synthesizer equipped with UV-detector using *N*- α -Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyl hexafluorophosphate (HBTU)/1-

Hydroxybenzotriazole (HOBt) as the activating reagents. Single coupling steps were performed with conditional capping. The coupling of the glycosylated amino acid *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc) and *N*- α -Fmoc-*R*-(2,3-bis(palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine was carried out manually. The manual couplings were monitored by standard Kaiser test.²⁸

Chemical synthesis of compounds 3a, 4, 5, 6a, and 2 has been described in chapter 3 compounds 3, 9, 6, 14, and 11 respectively.

Synthesis of compound 1: The synthesis of **1** was carried out on a Rink amide resin (0.1 mmol) as described in the general method. Side chain protection was as follows: *N*- α -Fmoc-*N*^G-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*L*-arginine, *N*- α -Fmoc-*L*-aspartic acid α -*tert*.-butyl ester, *N*- α -Fmoc-*N*- ϵ -*tert*.-Boc-*L*-lysine, *N*- α -Fmoc-*O*-*tert*-butyl-*L*-serine, *N*- α -Fmoc-*O*-*tert*-butyl-*L*-threonine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-threonine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-tyrosine. The first four amino acids, Arg-Pro-Ala-Pro, were coupled on the peptide synthesizer using a standard protocol. After the completion of the synthesis, a manual coupling was carried out using *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc) (0.4 mmol, 268 mg), with HATU (0.4 mmol, 152 mg), HOAt (0.4 mmol, 55 mg), and DIPEA (0.4 mmol, 70 μ L) in NMP for 12 h. The coupling reaction was monitored by standard Kaiser test. The resin was washed with NMP (6 ml x 1) and DCM (6 ml x 1) and resubjected to the same coupling conditions to ensure complete coupling. The glycopeptide was then elongated on the peptide synthesizer. The resin was washed thoroughly with NMP (6 ml x 1), DCM (6 ml x 1), and MeOH (6 ml x 1) and dried *in vacuo*. The resin was then swelled in DCM (5 ml) for 1 h, after which it was treated with hydrazine (80%) in MeOH (10 ml) for 1 h. This procedure was repeated twice for 30 min

to ensure complete deprotection of acetyl esters of the saccharide moiety. The resin was then washed thoroughly with DMF (5 ml x 4), DCM (5 ml x 4), and MeOH (5 ml x 4) and then dried *in vacuo*. The resin was swelled in DCM (5 ml) for 1 h. *N*- α -Fmoc--*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (267 mg, 0.3 mmol) was dissolved in DMF (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol), and DIPEA (67 μ L, 0.4 mmol) were premixed for 2 min, and was added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 12 h. Upon completion of the coupling, the *N*-Fmoc group was cleaved using piperidine (20%) in DMF (6 ml). Palmitic acid (77 mg, 0.3 mmol) was coupled to the free amine as described above using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol), and DIPEA (67 μ L, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2), and MeOH (5 ml x 2) and then dried *in vacuo* to constant weight. The resin was swelled in DCM (5 ml) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2.5%) (10 ml) for 2 h at room temperature. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated *in vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 °C) (30 ml) and recovered by centrifugation at 3000 rpm for 15 min. The crude glycolipopeptide was purified by RP-HPLC on a semi preparative C-4 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a 40 min period and the appropriate fractions were lyophilized to afford **1** (30% based on resin loading capacity). C₁₉₆H₃₂₈N₄₃O₅₀S, MALDI-ToF MS: observed [M⁺], 4120.3638Da; calculated [M⁺], 4119.0246Da.

Synthesis of compound 3b: The synthesis of compound **3b** was carried out starting from resin bound glycopeptide **14**. The glycopeptide was elongated on the peptide synthesizer. The resin was washed thoroughly with DMF (6 ml x 1), DCM (6 ml x 1), and MeOH (6 ml x 1) and dried *in vacuo*. The resin was swelled in DCM (5 ml) for 1 h after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%) (10 ml) for 2 h at room temperature. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated *in vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0°C) (30 ml) and recovered by centrifugation at 3000 rpm for 15 min. The crude glycopeptide was purified by RP-HPLC on a semi-preparative C-18 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a period of 40 min and lyophilization of the appropriate fractions afforded **3b** (5.0 mg, 86%). C₅₀H₈₃N₁₅O₂₀, MALDI-ToF MS: observed [M+], 1214.7357Da; calculated, [M+] 1214.2825Da.

Synthesis of compound 6b: The synthesis of compound **3b** was carried out starting from resin bound glycopeptide **14**. The glycopeptide was elongated on the peptide synthesizer. The resin was washed thoroughly with DMF (6 ml x 1), DCM (6 ml x 1), and MeOH (6 ml x 1) and dried *in vacuo*. After the completion of the synthesis resin was treated with hydrazine (80%) in MeOH^{26, 27} (10 ml) for 1 h. This procedure was repeated twice for 30 min to ensure complete deprotection of acetyl esters of saccharide moiety. The resin was washed thoroughly with DMF (5 ml x 4), DCM (5 ml x 4), and MeOH (5 ml x 4) and then dried *in vacuo* to constant weight. The resin was swelled in DCM (5 ml) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%) (10 ml) for 2 h at room temperature. The resin was filtered and washed with neat TFA (2

ml). The filtrate was concentrated *in vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 °C) (30 ml) and recovered by centrifugation at 3000 rpm for 15 min. The crude glycopeptide was purified by RP-HPLC on a semi preparative C-18 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a 40 min period and the appropriate fractions were lyophilized to afford **6b** (65% based on resin loading capacity). C₁₃₁H₂₀₅N₃₃O₃₉, MALDI-ToF MS: observed [M+], 2866.6770Da; calculated [M+Na], 2866.2271Da.

Synthesis of compound 7: The synthesis of **7** was carried out on a Rink amide resin (0.1 mmol) as described in the general method. After coupling of the first five amino acids, the remaining steps were performed manually. *N*- α -Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (Pam₂Cys-OH) (267 mg, 0.3 mmol) was dissolved in DMF (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBT (40 mg, 0.3 mmol), and DIPEA (67 μ l, 0.4 mmol) were premixed for 2 min, and was added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 12 h. Upon completion of the coupling, the *N*-Fmoc group was cleaved using piperidine (20%) in DMF (6 ml). Palmitic acid (77 mg, 0.3 mmol) was coupled to the free amine as described above using PyBOP (156 mg, 0.3 mmol), HOBT (40 mg, 0.3 mmol), and DIPEA (67 μ l, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2), and MeOH (5 ml x 2) and then dried *in vacuo*. The resin was swelled in DCM (5 ml) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5% and TIS 2%) (10 ml) for 2 h at room temperature. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated *in vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 °C) (30 ml) and recovered by

centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by RP-HPLC on a semi preparative C-4 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a 40 min period and the appropriate fractions were lyophilized to afford **7** (65% based on resin loading capacity). $C_{81}H_{156}N_{11}O_{12}S$, MALDI-ToF MS: observed $[M+Na]$, 1531.2430Da; calculated $[M+Na]$, 1531.1734Da.

Synthesis of compound 8: The synthesis of **8** was carried out on a Rink amide resin (0.1 mmol) as described in the general method. After the completion of synthesis the resin was washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2), and MeOH (5 ml x 2) and then dried *in vacuo*. The resin was swelled in DCM (5 ml) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%) (30 ml) for 2 h at room temperature. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated *in vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 °C) (30 ml) and recovered by centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by RP-HPLC on a semi preparative C-8 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a 40 min period and the appropriate fractions were lyophilized to afford **8** (60% based on resin loading capacity). $C_{79}H_{122}N_{18}O_8$, MALDI-ToF MS: observed $[M+]$, 1611.9182Da; calculated $[M+]$, 1611.9238Da.

Synthesis of compound 9: The synthesis of **9** was carried out on a universal NovaTag resin (**27**; 0.1 mmol) as described in the general method. After coupling of the amino acids on a peptide synthesizer to obtain **28**, the remaining steps were performed manually. *N*- α -Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (267 mg, 0.3 mmol) was dissolved in DMF (5 ml), and PyBOP (156 mg, 0.3 mmol), HOBT (40 mg, 0.3

mmol), and DIPEA (67 μ l, 0.4 mmol) were premixed for 2 min, and was added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 12 h. Upon completion of the coupling, the *N*-Fmoc group of **29** was cleaved using piperidine (20%) in DMF (6 ml) to give **30**. Palmitic acid (77 mg, 0.3 mmol) was coupled to the free amine of **30** as described above using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol), and DIPEA (67 μ l, 0.4 mmol) in DMF to afford **31**. The resin was washed thoroughly with DMF (5 ml x 2) and DCM (5 ml x 2). The pendant Mmt group of **31** was then removed by treatment of HOBt (1M) in TFE/DCM (10 ml) for 2 h to obtain **32**. Next, *N*- α -Fmoc-Cys(Trt)-OH (175.8 mg, 0.3 mmol) was coupled to the free amine of **32** using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol), and DIPEA (67 μ L, 0.4 mmol) in DMF to give **33**. Upon completion of the coupling, the *N*-Fmoc group was cleaved using piperidine (20%) in DMF (6 ml) to give **34** and the free amine was acetylated using Ac₂O (10%), DIPEA (5%) in NMP (10 ml) for 30 min to obtain **35**. The lipopeptide was then cleaved from the resin by treatment of TFA (94%), EDT (2.5%), H₂O (2.5%), TIS (1%) (10 ml) for 4 h at room temperature. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated *in vacuo* to approximately one third of its original volume. The peptide was precipitated using diethyl ether (0°C) (40 ml) and recovered by centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by HPLC on a semi preparative C-4 reversed phase column using a linear gradient of 0 to 95% solvent B (acetonitrile (95%), water (5%), TFA (0.01%)) in solvent A (water (95%), acetonitrile (5%), TFA (0.01%)) over a 40 min period and the appropriate fractions were lyophilized to give **36** (25% based on resin loading capacity). MALDI-ToF MS: observed [M⁺], 4341.4347Da; calculated [M⁺],

4342.6288Da. The lipopeptide **36** (0.5 mg, 0.11 μ mol) was then treated with Alexa Fluor® 488 C5 maleimide (0.4 mg, 0.55 μ mol) in the presence of catalytic amount of TCEP and DIPEA in DMSO (1 ml) at room temperature for 12 h. The crude reaction mixture was purified by RP-HPLC on a semi preparative C-4 reversed phase column using a linear gradient of 0 to 95% solvent B (acetonitrile (95%), water (5%), TFA (0.01%)) in solvent A (water (95%), acetonitrile (5%), TFA (0.01%)) over a 40 min period and the appropriate fractions were lyophilized to afford **9** (0.4 mg, 68%). MALDI-ToF MS: observed [M+], 5040.12Da; calculated [M+] 5039.76Da.

Cell culture: RAW 264.7 γ NO(-) cells, derived from the RAW 264.7 mouse monocyte/macrophage cell line, were obtained from ATCC. The cells were maintained in RPMI 1640 medium with L-glutamine (2 mM), adjusted to contain sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), HEPES (10 mM), and sodium pyruvate (1.0 mM) and supplemented with penicillin (100 u/mL) / streptomycin (100 μ g/mL; Mediatech), and FBS (10%; Hyclone). The human embryonic kidney cell line (HEK 293T), obtained from ATCC, was grown in Dulbecco's modified Eagle's medium (ATCC) with L-glutamine (4 mM), glucose (4.5 g/L), and sodium bicarbonate (1.5 g/L) supplemented with penicillin (100 u/mL) / streptomycin (100 μ g/mL; Mediatech), Normocin (100 μ g/mL; InvivoGen), and FBS (10%). HEK293T cells, stable transfected with mouse TLR4/MD2/CD14, mouse TLR2, or mouse TLR2 and TLR6, were obtained from InvivoGen and grown in the same growth medium as for HEK 293T cells supplemented with blasticidin (10 μ g/mL) and for the HEK w/ hTLR4/MD2/CD14 also with HygroGold (50 μ g/mL). Human breast adenocarcinoma cells (MCF7),²⁹ obtained from ATCC, were cultured in Eagle's minimum essential medium with L-glutamine (2 mM) and Earle's BSS, modified

to contain sodium bicarbonate (1.5 g/L), non-essential amino acids (0.1 mM), and sodium pyruvate (1 mM), and supplemented with bovine insulin (0.01 mg/mL; Sigma) and FBS (10%). Human skin malignant melanoma cells (SK-MEL-28) were obtained from ATCC and grown in Eagle's minimum essential medium with L-glutamine (2 mM) and Earle's BSS, adjusted to contain sodium bicarbonate (1.5 g/L), non-essential amino acids (0.1 mM), and sodium pyruvate (1 mM), and supplemented with FBS (10%). Freshly isolated human monocytes were cultured in R-5-RPMI 1640 (Invitrogen) supplemented with heat-inactivated FBS (5%; Invitrogen), sodium pyruvate (1%; Sigma), 2-mercaptoethanol (50 μ M; Invitrogen), HEPES buffer (1 M; Sigma), and gentamicin (50 μ g/ml; Invitrogen). All cells were maintained in a humid 5% CO₂ atmosphere at 37 °C.

Dose and immunization schedule: Groups of five mice (female BALB/c, age 8-10 weeks) were immunized four or five times at 1-week intervals. Each boost included 3 μ g of saccharide in the liposome formulation. In some immunizations, the external immuno-adjuvant QS-21 (10 μ g; Antigenics Inc.) was included. Serum samples were obtained before immunization (pre-bleed) and one week after the final immunization. The final bleeding was done by cardiac bleed.

Serologic assays: Anti-MUC1 IgG, IgG1, IgG2a, IgG2b, and IgG3 antibody titers were determined by enzyme-linked immunosorbent assay (ELISA), as described previously.³⁰ Briefly, ELISA plates (Thermo Electron Corp.) were coated with a conjugate of the MUC1 glycopeptide conjugated to BSA through a bromoacetyl linker (BSA-BrAc-MUC1). Serial dilutions of the sera were allowed to bind to immobilized MUC1. Detection was accomplished by the addition of phosphate-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.), IgG1 (Zymed), IgG2a (Zymed), IgG2b

(Zymed), or IgG3 (BD Biosciences Pharmingen) antibodies. After addition of *p*-nitrophenyl phosphate (Sigma), the absorbance was measured at 405 nm with wavelength correction set at 490 nm using a microplate reader (BMG Labtech). The antibody titer was defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera.

Flow cytometry analysis: Pre- and post-immunization sera were diluted 50-fold and incubated with MCF7 and SK-MEL-28 single-cell suspensions for 30 min on ice. Next, the cells were washed and incubated with goat anti-mouse IgG g-chain specific antibody conjugated to FITC (Sigma) for 20 min. Cells were analyzed by flow cytometry using the FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems) and data analysis was performed with FlowJo software (Tree Star, Inc.).

Cytokine assays: RAW 264.7 γ NO(-) cells were plated on the day of the exposure assay as 2×10^5 cells/well in 96-well plates (Nunc) and incubated with different stimuli for 5.5 h in the presence or absence of polymyxin B. Culture supernatants were collected and stored frozen (-80 °C) until assayed for cytokine production. Concentrations of TNF- α were determined using the TNF- α DuoSet ELISA Development kit from R&D Systems. Concentration-response data were analyzed using nonlinear least-squares curve fitting in Prism (GraphPad Software, Inc.). These data were fit with the following four parameter logistic equation: $Y = E_{\max} / (1 + (EC_{50}/X)^{\text{Hill slope}})$, where Y is the TNF- α response, X is the concentration of the stimulus, E_{\max} is the maximum response, and EC_{50} is the concentration of the stimulus producing 50% stimulation. The Hill slope was set at 1 to be able to compare the EC_{50} values of the different inducers. All TNF- α values are

presented as the means \pm SD of triplicate cultures, with each experiment being repeated three times.

Evaluation of materials for contamination by LPS: To ensure that any increase in cytokine production was not caused by LPS contamination of the solutions containing the various stimuli, the experiments were performed in the absence and presence of polymyxin B, an antibiotic that avidly binds to the lipid A region of LPS, thereby preventing LPS-induced cytokine production.³¹ Cytokine concentrations in supernatants of cells preincubated with polymyxin B (30 μ g/mL; Bedford Laboratories) for 30 minutes before incubation with *E. coli* O55:B5 LPS for 5.5 h showed complete inhibition of cytokine production, whereas preincubation with polymyxin B had no effect on cytokine synthesis by cells incubated with the synthetic compounds **1**, **2**, Pam₂CysSK₄, and **7**. Therefore, LPS contamination of the latter preparations was inconsequential.

Dendritic cell maturation: Monocytes were set-up on day 1 and were allowed to enrich for 3 days. On day 4 the monocyte-derived dendritic cells (MDDCs) were exposed to LPS (1 μ g/mL), PBS, and compounds **2** or **7** (1 μ g/mL each) for 16-24 h. Next, cells were washed and incubated with the following monoclonal antibodies: anti-CD80 conjugated to PE (BD BioSciences), anti-CD83 conjugated to FITC (BD BioSciences), and anti-CD86 conjugated to PE (BD BioSciences) for 30 min. Cells were washed, fixed, and analyzed by flow cytometry.

Transfection and NF- κ B activation assay: The day before transfection, HEK293T cells and stable transfected HEK293T cells with murine TLR2, TLR2/TLR6, or TLR4/MD2 were plated in 96-well cell culture plates to reach the next day approximately 60% confluency. Cells were transiently transfected with expression plasmids using PolyFect

Transfection Reagent (Qiagen). Briefly, HEK293T cells and stable transfected HEK293T cells with TLR2, TLR2/TLR6, or TLR4/MD2 were transfected with pELAM-Luc (NF- κ B-dependent firefly luciferase reporter plasmid, 50 ng/well),³² and pRL-TK (Renilla luciferase control reporter vector, 1 ng/well; Promega) as an internal control to normalize results. The empty vector pcDNA3 (Invitrogen) was used as a control and to normalize the DNA concentration for all of the transfection reactions (total DNA 70 ng/well). Forty-four h post-transfection, cells were exposed to the stimuli at the indicated concentrations for 4 h, after which cell extracts were prepared. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and the Fluoroskan Accent FL combination luminometer/fluorometer (Thermo Electron Corporation). Expression of the firefly luciferase reporter gene was normalized for transfection efficiency with expression of Renilla luciferase. The data are reported as the means \pm SD of triplicate treatments. The transfection experiments were repeated at least twice.

Binding and uptake assay: RAW 264.7 γ NO (-) cells, HEK293T cells, and HEK293T cells stable transfected with murine TLR2, TLR2/TLR6, or TLR4/MD2 (2.6×10^6 cells/mL) were exposed to Alexa Fluor 488-labeled compound **9** (1 μ g/mL) for 30 min at 37 °C. Cells were harvested and washed in HNE buffer (HEPES, 20 mM; NaCl, 150 mM; EDTA, 1 mM). Samples that were assessed for internalization only were treated with trypsin (500 μ g/mL) for 1 min and washed in HNE buffer. Next, cells were lysed in Passive Lysis Buffer (Promega) and fluorescence (absorbance 485 nm, emission 538 nm) of the cell lysates was measured using the POLARstar OPTIMA combination luminometer/fluorometer (BMG Labtech). Fluorescence values were normalized for maximum possible fluorescence (100%), using untreated cell lysates spiked with the

fluorescent compound. The data are presented as the means \pm SD of triplicate treatments, with each experiment being repeated three times.

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CHAPTER 5

Immunological Evaluation of Toll like Receptor Ligands as a Adjuvants in a Three-Component Cancer Vaccine Candidate

Worldwide, eradication of smallpox by vaccination illustrates that vaccination is arguably the most cost-effective public health intervention against infection. The successful vaccination against infectious or neoplastic diseases is to prime the hosts immune system to generate an efficient defense and memory immune response.^{1, 2} However, the generation of strong immune responses to poorly immunogenic antigens, requires the help of an immunostimulatory adjuvant, which can amplify the immune response. Although dozens of different adjuvants have been shown to be effective in preclinical and clinical studies, only aluminum based salts (alum) and squalene oil-water emulsion (MF59) have been approved for human use.^{3, 4} Adjuvants are extremely important for the development of therapeutic vaccines, such as cancer vaccines in generating a robust immune response, and yet their mechanism of action is unknown. Current understanding of the role of adjuvant is that they serve as a danger signal, which is detected by a group of pattern recognition receptors (PRRs) like Toll like receptors (TLRs) expressed on macrophages and dendritic cells.⁵⁻⁸ There are 10 different functional TLRs in humans (12 in mice) and collectively they sense a wide array of microbial stimuli. Intracellular TLR signaling within dendritic cells is mediated by different proteins followed by activation of naïve antigen-specific T-cells leading to subsequent development of a strong and specific acquired immune response.⁹⁻¹² As a result, there is a growing interest for the development

of synthetic ligands that stimulate specific TLRs in developing novel vaccine adjuvants, particularly in the context of vaccines capable of eliciting Th1 immune response.¹³⁻¹⁶

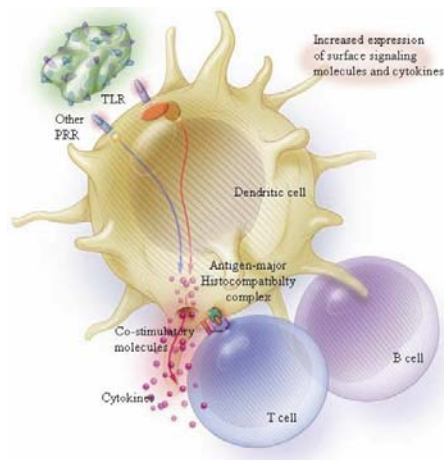


Figure 5.1 | Immune response by TLRs and non-TLRs¹⁷

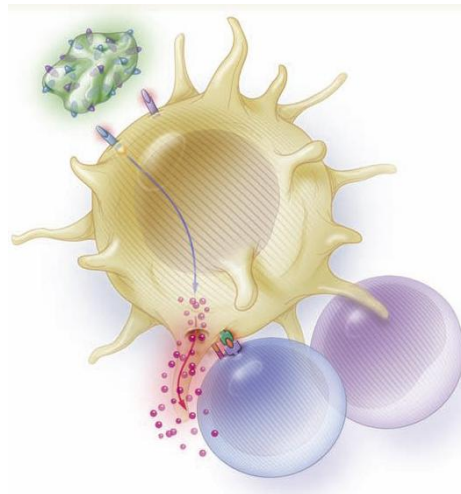


Figure 5.2 | Immune response by non-TLRs alone

However, recent studies by Gavin *et al* concludes that TLRs signaling is not essential for the induction of an antibody response to protein antigen administered in alum, complete Freund's adjuvant, incomplete Freund's adjuvant, and Ribi.^{18, 19} Furthermore, data suggests that the robust antibody responses to moderate doses of antigen can be achieved in the total absence of TLR ligands. Hence, TLR ligands can be excluded from the vaccine adjuvants as they might raise the possibility of unwanted side effects. Their data

is inconsistent with the model that suggest the important role of TLRs in early microbial suppression, reputation of antibody class, and sustaining antibody secretion at delayed times after immunization, rather than as an essential component of the self-non-self discrimination of the adaptive immune response.

To understand the importance of TLR ligand in the context of three-component cancer vaccine, we have designed a fully synthetic compound without TLR ligand. The glyco(lipo)peptide **1** (Figure 5.3) contains, as a B-epitope tumor associated carbohydrate antigen derived from MUC-1,²⁰ the well documented MHC-Class II restricted T-helper peptide derived from the Polio virus,²¹ and the immunosilent lipid moiety.

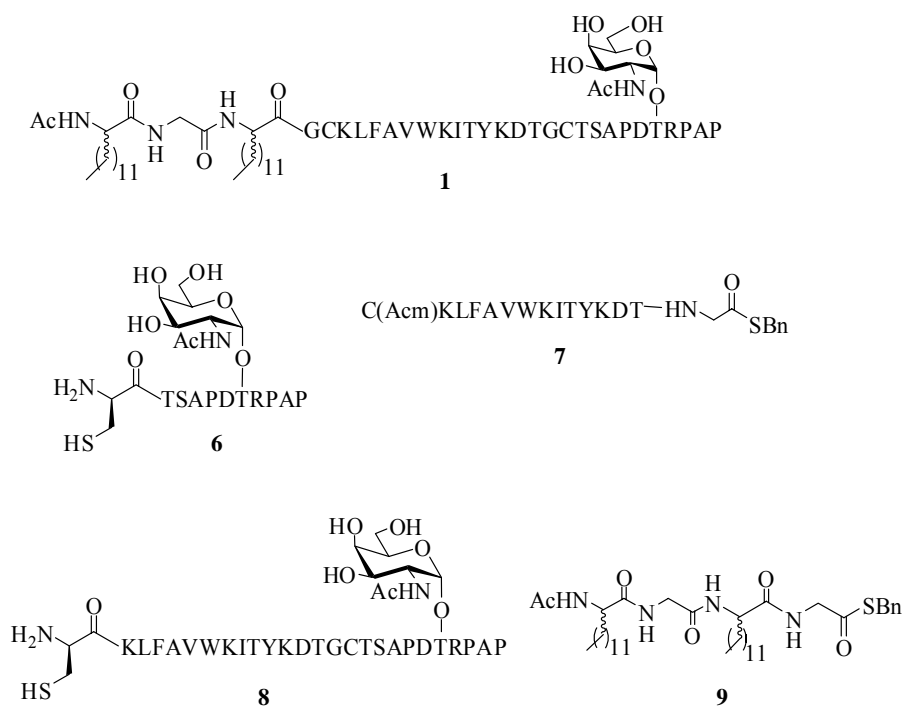


Figure 5.3 | Synthetic targets to study the adjuvant effect.

It was believed that the B-epitope will interact with Ig receptors of B-cells,²²⁻²⁴ and direct antibody response. After internalization and proteolytic processing in endosomes, the T-helper peptide will be presented as a complex with a MHC II on the surface of APC.

Subsequently, the APCs will migrate to lymphoid organs where the peptide complexed with MHC class II will interact with T-cell receptors of naïve CD4⁺ T-lymphocytes. This interaction will result in the activation and migration of T-cells to the T-cell zone where they will interact with B-cells resulting in B-cell maturation and the production of IgG antibodies.²⁵⁻²⁸ This interaction is also called as “signal 1” for the activation of naïve T-lymphocytes.

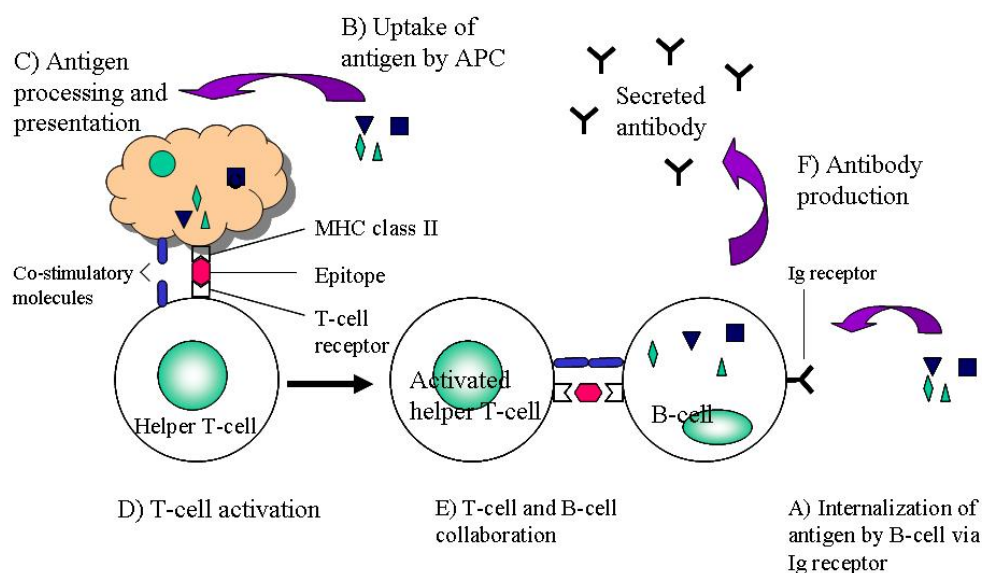


Figure 5.4 | Immune response and role of B and T cells

The immunosilent moiety attached to the T-and B-epitopes, is non-immunogenic in nature; as a result, this will not provide “signal 2” through the co-stimulatory molecules for the activation of T-lymphocytes, and might lead to a weak immune response to the non-immunogenic antigen. It has been known that insufficient presentation or co-stimulation may result in a weak immune response and even the induction of T-cell anergy.²⁹

It was envisaged that the vaccine candidate **1** can be prepared by *liposome*-mediated native chemical ligation (NCL) of building blocks **6**, **7**, and **8**.³⁰ Thus, a film of

dodecylphosphocholine, thiol **6**, and peptide thioester **7** was hydrated in a phosphate buffer (pH 7.5) in the presence of carboxyethyl phosphine and EDTA and then ultrasonicated. The ligation was initiated by the addition of sodium 2-mercaptoethane sulfonate and the reaction was monitored by LC-MS. Next, the actamidomethyl (Acm)³¹ protecting group of the ligation product was removed using mercury(II) acetate and the product was purified by RP-HPLC over a C-8 column. A second liposome mediated NCL of the free sulfhydryl moiety of **8** with thioester **9** gave glycolipopeptide **1** in good overall yield.

Next, to investigate the role of different TLR ligands liposomal preparation of vaccine candidate **1** was co-administered with TLR2 (Pam₃CSK₄, **10**)^{32,33} and TLR4 (MPL-A, **11**)^{34,35} ligands (figure 5.5).

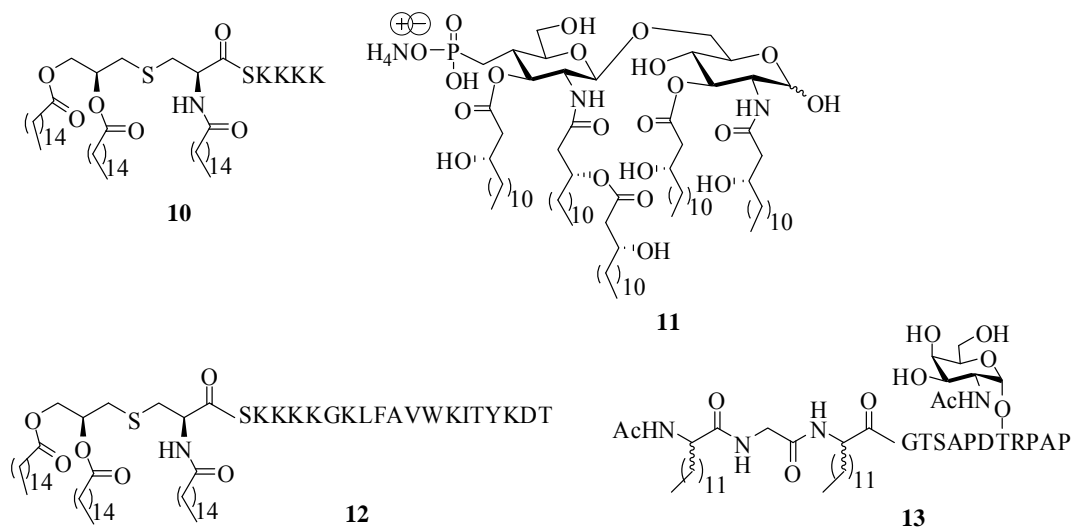
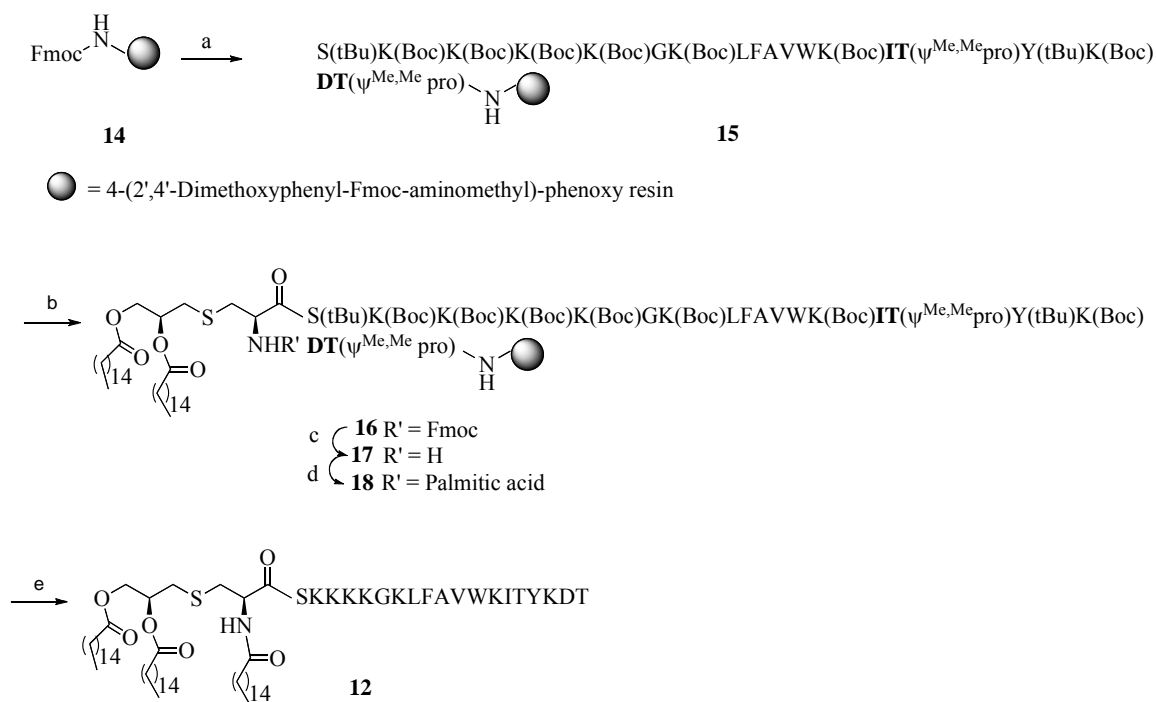


Figure 5.5 | Synthetic compounds to study the role TLR in immune response.

Furthermore, to investigate the importance of covalent linkage or topology between T-epitope, B-epitope, and immunosilent moiety, compound **12** containing TLR2 ligand attached to T-epitope and compound **13** containing an immunosilent moiety coupled to B-epitope was synthesized.

The chemical synthesis of compounds **1**, **2**, **3**, **4**, and **5** have been described in chapter 3 (compounds **1**, **2**, **3**, **4**, and **5**) respectively.

Compound **12** was prepared by a SPPS using a Rink amide resin **14**, Fmoc protected amino acids, and di-peptide amino acid building blocks for difficult sequences (Scheme 5.1). The *N*- α -Fmoc-Ile-Thr($\Psi^{\text{Me,Me}}$ pro)-OH, di-peptide building block was used to avoid the difficulties encountered during the coupling of IT sequence, while *N*- α -Fmoc-Asp-Thr($\Psi^{\text{Me,Me}}$ pro)-OH was used to avoid the aspartimide formation.³⁶



Scheme 5.1 | Chemical synthesis of Pam₃CSK₄-T-epitope 4. Reagents and Conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) Manual coupling of Pam₂Cys-OH, PyBOP, HOBt in the presence of DIPEA in DMF; c) 20% Piperidine in DMF; d) Coupling of Palmitic acid, PyBOP, HOBt in the presence of DIPEA in DMF; e) Reagent B, TFA (88%), Phenol (5%), Water (5%), TIS (2%), 2 h.

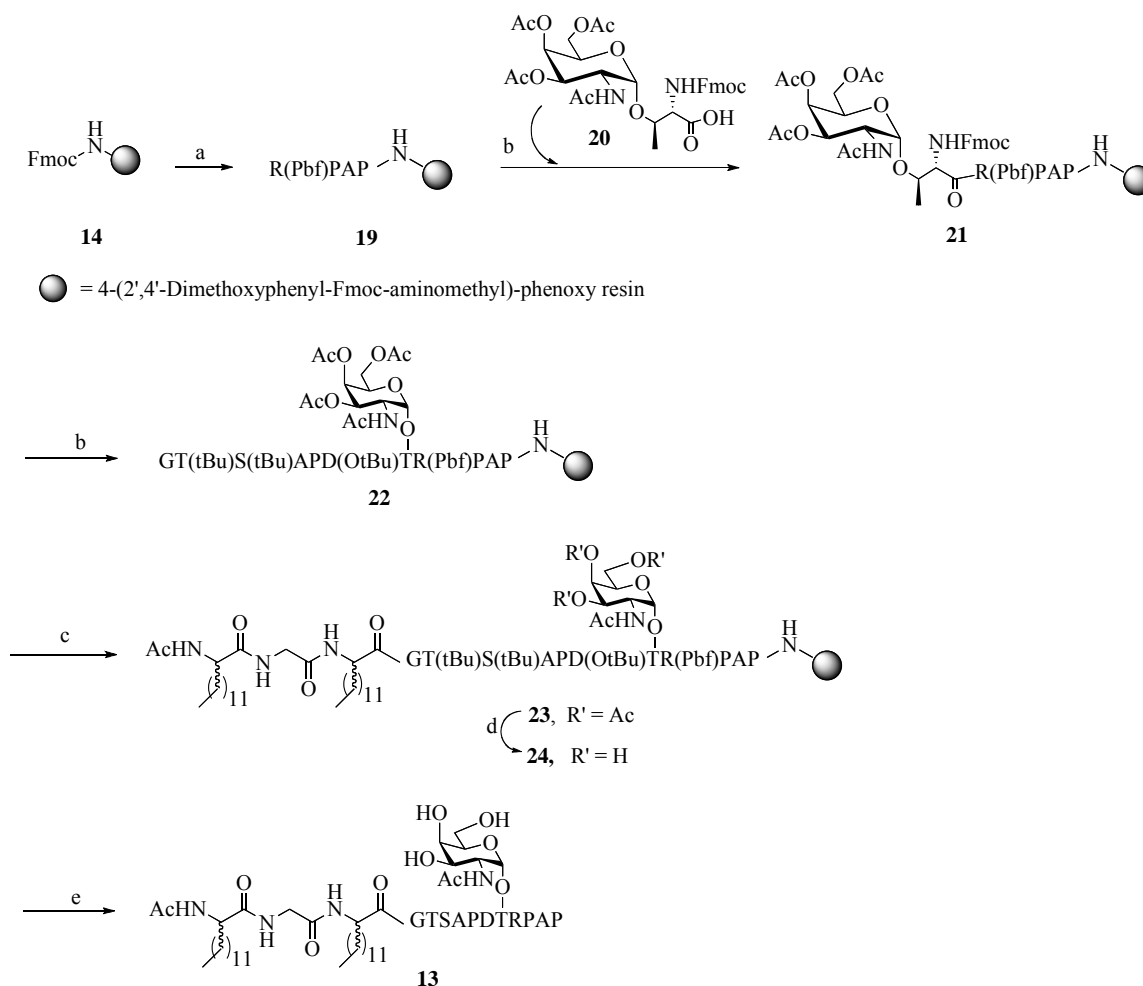
The initial amino acids couplings were carried out by standard protocol for Fmoc chemistry to afford compound **15** on a peptide synthesizer, while the remaining steps were performed manually. Thus, the coupling of Pam₂Cys-OH³⁷ in the presence of PyBOP/HOBt containing DIPEA in DMF gave compound **16**. After the completion of

this reaction, the *N*-Fmoc group was removed using 20% piperidine in DMF and the resulting free amine of the compound **17** was coupled with palmitic acid in the presence of PyBOP/HOBt containing DIPEA in DMF to give fully protected support bound lipopeptide **18**. The simultaneous side chain deprotection and cleavage from the resin was carried out by treating lipopeptide **18** with reagent B (TFA 88%, Water 5%, Phenol 5%, TIS 2%) for 2 h to give lipopeptide **12** in moderate overall yield (37%).

The synthesis of **13** was performed by a SPPS protocol using a Rink amide resin **14**, Fmoc protected amino acids and *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc) (Scheme 5.2). After the coupling of first four amino acids using an automated HBTU-mediated HOBt ester activation protocol to give compound **19**, the glycosylated amino acid **20** was coupled manually by using HATU/HOAt in the presence of DIPEA in DMF as the activation cocktail to obtain compound **21**. The remaining peptide sequence was then assembled by SPPS to afford compound **22**. Next, the *N*- α -Fmoc-Lipidated amino acid^{38,39} was coupled manually by using PyBOP/HOBt in the presence of DIPEA in NMP. Upon completion of the coupling, the *N*-Fmoc group was cleaved using standard protocol followed by the coupling *N*- α -Fmoc-Gly-OH by using PyBOP/HOBt in the presence of DIPEA in NMP. Upon completion of this reaction the *N*-Fmoc group was cleaved by using standard protocol. Next, the *N*- α -Fmoc-Lipidated amino acid was then coupled using PyBOP/HOBt in the presence of DIPEA in NMP.

Finally, the *N*-Fmoc group was cleaved using standard protocol followed by the acylation of the free amine group to afford compound **23**. After assembly of the glycolipopeptide, acetyl esters of the saccharide moiety were cleaved by treatment with 60% hydrazine in MeOH to obtain compound **24**. The side chain deprotection and cleavage of the

glycolipopeptide **24** was carried out by the treatment of reagent B (TFA 88%, Phenol 5%, water 5%, TIS 2%) to give compound **13** in moderate overall yield.



Scheme 5.2 | Chemical synthesis of lipidated B-epitope 5. Reagents and Conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) i. **13**, HATU/HOAt, DIPEA, NMP, overnight, ii. SPPS using Fmoc-chemistry, coupling with HBTU/HOAt in the presence of DIPEA in NMP; c) i. Manual coupling of Fmoc-lipidated amino acid with PyBOP/HOBt in the presence of DIPEA in NMP; ii. 20% Piperidine in DMF; iii. Manual Coupling of Fmoc-Gly-OH with PyBOP/HOBt in the presence of DIPEA in NMP; iv. 20% Piperidine in DMF; v. Manual coupling of Fmoc-lipidated amino acid with PyBOP/HOBt in the presence of DIPEA in NMP; vi. 20% Piperidine in DMF; vii. 10% Ac₂O, 5% DIPEA in NMP for 10 min; (d) 60% hydrazine in MeOH, 2 hr; e) Reagent B, TFA (88%), Phenol (5%), Water (5%), TIS (2%), 2 hr.

Next, the attention was focused on immunizing mice and characterizing the antibody response. Thus compound **1** was incorporated into phospholipid-based small uni-lamellar

vesicles (SUVs) by hydration of a thin film of the synthetic compound, ⁴⁰ egg phosphatidylcholine, phosphatidylglycerol and cholesterol in a HEPES buffer (10 mM, pH 7.4) containing NaCl (145 mM) followed by extrusion through a 100 nm Nuclepore® polycarbonate membrane. Groups of five female BALB/c mice were immunized four times at weekly intervals with liposomes containing 3 µg of saccharide. To explore the adjuvant properties of the vaccine candidate, liposomes were administered with or without the potent TLR ligands as summarized in Table 5.1. The influence of covalent attachment of the various components of the vaccine candidate on antigenic responses was investigated by immunizing mice with a liposomal preparation of compounds **12**, which is composed of the T-epitope linked to adjuvant Pam₃CysSK₄, and compound 13, containing immunosilent moiety and B-epitope.

Table 5.1 | Liposomal preparation for Immunization

Entry	Liposomal preparation	Additional comments
1	Compound 1	Intraperitoneal Injection
2	Compound 1 + Pam ₃ CSK ₄ (10)	TLR2 ligand
3	Compound 1 + MPL-A (11)	TLR4 ligand
4	Compound 12 + Compound 13	Injection at same site
5	Compound 12 Compound 13	Subcutaneous injection at different site

Next, anti-MUC1 antibody titers were determined by coating microtiter plates with CTSAPDT (α-D-GalNAc)RPAP conjugated to bromoacetyl modified BSA and detection was accomplished with anti-mouse IgG antibodies labeled with alkaline phosphatase. Mice immunized with **1** elicited significantly low titers of anti-MUC1 antibodies (Figure 5.6). Furthermore, the antigen specific IgG1 antibodies are significantly lower, whereas IgG2 antibodies are completely abolished in this group of mice. Co-administrating TLR2 ligand as an adjuvant (Table 5.1, entry 2), however, gave exceptionally high titers of anti-

MUC1 IgG antibodies. Sub-typing of the IgG antibodies indicated the presence of IgG1 and IgG2b indicating that TLR signaling is important for the induction of these antibodies. Also, this group of mice gave high titers of IgG3 antibodies, which requires the cytokine production by accessory cells. Thus, TLR plays an important role for the production of cytokines and the stimulation of accessory cells. Importantly, antibodies obtained in this group did not produce antibodies against the T-epitope.

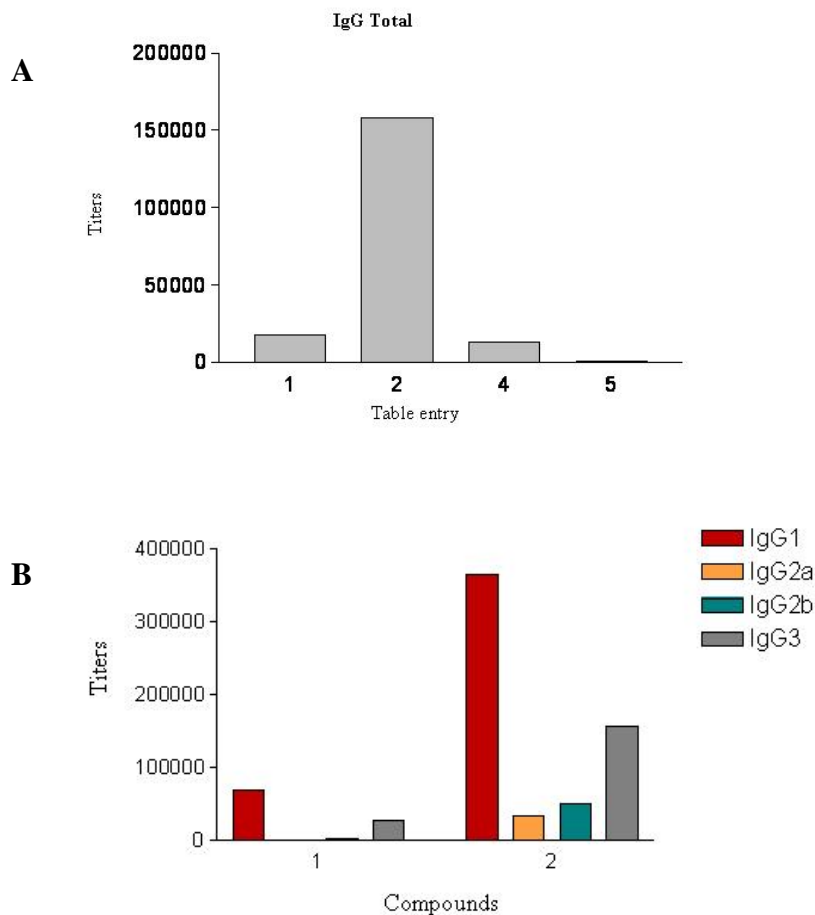


Figure 5.6 | ELISA anti-MUC1 antibody titers after 4 immunizations. ELISA plates were coated with BSA-Br-Ac-MUC1 conjugate and titers were determined by linear regression analysis, plotting dilution vs. absorbance. Titers were defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera. All titers are medians for a group of five mice. **A)** Total IgG antibodies, and **B)** Subtyping of IgG antibodies for entry 1 and 2 (table 5.1).

Immunological data for group 3 (Table 5.1, entry 3) with co-administration of TLR4 ligand is awaited, while group 4 and group 5 resulted very low production of anti-MUC1 antibodies. We believe, this liposomal preparation is taken up and processed by different APC cells. As a result, they do not induce a strong antibody response.

From our study we can conclude that the TLRs are essential for the immunogens that are not highly immunogenic in nature. Furthermore, TLR ligands induce strong activation of innate immune cells and subsequent stimulation of a robust adaptive immune response.

5.1 Experimental procedures

Reagents and general procedures: Amino acid derivatives and resins were purchased from NovaBioChem and Applied Biosystems; DMF from EM Science; and NMP from Applied Biosystems. Egg phosphatidylcholine, Phosphatidylglycerol, Cholesterol, and Dodecyl phosphocholine was obtained from Avanti Polar Lipids. All other chemical reagents were purchased from Aldrich, Acros, Alfa Aesar and Fischer and used without further purification. All solvents employed were reagent grade. Reverse Phase HPLC was performed on an Agilent 1100 series system equipped with an autosampler, UV-detector and fraction-collector using a Zorbax Eclipse C8 analytical column (5 μ m, 4.6 x 150 mm) at a flow rate of 1 ml/min, a semi preparative C8 column (5 μ m, 10 x 250 mm) at a flow rate of 4 ml/min, a Synchropak C4 analytical column (5 μ m, 4.6 x 100 mm) at a flow rate of 1 ml/min and a Jupiter C4 semi preparative column (5 μ m, 4.6 x 250 mm) at a flow rate of 2 ml/min. All runs used linear gradients of 0-100% solvent B in A over a 40 min. period was used unless otherwise specified. (A = water 95%, acetonitrile 5% and 0.1% TFA 0, B= water 5%, acetonitrile 95% and 0.1% TFA). MALDI-ToF mass spectra were recorded on a ABI 4700 proteomic analyzer.

General methods for Solid-Phase Peptide Synthesis (SPPS): Peptides were synthesized by established protocols on a Applied Biosystems, ABI 433A peptide synthesizer equipped with UV-detector using *N*- α -Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyl hexafluorophosphate (HBTU)/1-Hydroxybenzotriazole (HOBt) as the activating reagents. Single coupling steps were performed with conditional capping. The coupling of the glycosylated amino acid *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc), *N*- α -Fmoc-*R*-(2,3-bis(palmitoyloxy)-(2*R*-propyl)-(i>R)-cysteine, and *N*- α -Fmoc-Lipidated amino acid was carried out manually. The manual couplings were monitored by standard Kaiser test.

Synthesis of lipopeptide 12: SPPS was performed on Rink amide resin (0.1 mmol). Side chain protection was as follows: *N*- α -Fmoc-Asp-Thr($\Psi^{\text{Me,Me}}$ pro)-OH, *N*- α -Fmoc-Ile-Thr($\Psi^{\text{Me,Me}}$ pro)-OH, *N*- α -Fmoc-*N*- ϵ -tert.-Boc-*L*-lysine, *N*- α -Fmoc-*O*-tert-butyl-*L*-serine, *N*- α -Fmoc-*O*-tert-butyl-*L*-threonine, *N*- α -Fmoc-*O*-tert-butyl-*L*-tyrosine. After the assembly of the peptide by using standard SPPS, the remaining steps performed manually. *N*- α -Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(i>R)-cysteine (267 mg, 0.3 mmol) was dissolved in DMF (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) were premixed for 2 min, and was added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 12 h. Upon completion of the coupling, the *N*-Fmoc group was cleaved using 20% piperidine in DMF (6 ml). Palmitic acid (77 mg, 0.3 mmol) was coupled to the free amine as described above using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2) and MeOH (5 ml x 2) and then dried in *vacuo*. The

resin was swelled in DCM (5 ml) for 1 h. After which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%) (10 ml) for 2 h. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0°C) (30 ml) and recovered by centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by HPLC on a semi preparative C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min., and the appropriate fractions were lyophilized to afford **12** (37% based on resin loading capacity). C₁₆₂H₂₇₈N₂₉O₃₁S, MALDI-ToF MS: observed, [M+] 3160.9423Da; calculated, [M+] 3160.1814Da.

Synthesis of glycolipopeptide 13: SPPS was performed on Rink amide resin (0.1 mmol) as described above. Side chain protection was as follows: *N*- α -Fmoc-*N*^G-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*L*-arginine, *N*- α -Fmoc-*L*-aspartic acid α -*tert*.-butyl ester, *N*- α -Fmoc-*S*-trityl-*L*-cysteine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-serine, *N*- α -Fmoc-*O*-*tert*-butyl-*L*-threonine. The first four amino acids, Arg-Pro-Ala-Pro were coupled on the peptide synthesizer using a standard protocol. After the completion of the synthesis, a manual coupling was carried out using *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc) (0.2 mmol, 134 mg), with HATU (0.2 mmol, 76 mg), HOAt (0.2 mmol, 27 mg) and DIPEA (0.4 mmol, 70 μ l) in NMP for 12 h. The coupling reaction was monitored by standard Kaiser test. The resin was washed with NMP (6 ml x 1) and DCM (6 ml x 1), and resubjected to the same coupling conditions to ensure complete coupling. The glycopeptide was then elongated on peptide synthesizer. After the completion of the synthesis, the resin was washed thoroughly with NMP (6 ml x 1), DCM (6 ml x 1) and MeOH (6 ml x 1) and dried in *vacuo*. The rest of the peptide sequence was completed

manually. Next, *N*- α -Fmoc-Lipidated amino acid (139 mg, 0.3 mmol) was dissolved in NMP (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) were premixed for 2 min, and was added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 8 h. Upon completion of the coupling; the *N*-Fmoc group was cleaved using 20% piperidine in NMP (6 ml). *N*- α -Fmoc-*L*-glycine (90 mg, 0.3 mmol) was dissolved in NMP (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) were premixed for 2 min, and were added to the resin. The coupling reaction was monitored by Kaiser test and was complete after standing for 4 h. Upon completion of the coupling; the *N*-Fmoc group was cleaved using 20% piperidine in NMP (6 ml). One more cycle of coupling of *N*- α -Fmoc-Lipidated amino acid (139 mg, 0.3 mmol) was carried out as described above using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) in NMP (5 ml). Finally, the *N*-Fmoc group was cleaved using 20% piperidine in NMP (6 ml) and acetylated using 5 ml of 10% Ac₂O, 5% DIPEA in NMP for 10 min. The resin was washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2) and MeOH (5 ml x 2) and dried in *vacuo*. The resin was swelled in DCM (5 ml) for 1 h. The resin was treated with 60% hydrazine in MeOH (10 ml) for 2 h and washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2) and MeOH (5 ml x 2) and dried in *vacuo*. The resin was then swelled in DCM (5 ml) for 1 hr. After which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%) (10 ml) for 2 h. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The glycolipopeptide was precipitated using diethyl ether (0°C) (40 ml) and recovered by centrifugation at 3000 rpm for 15 min. The

crude glycolipopeptide was purified by RP-HPLC on a semi preparative C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min., and the appropriate fractions were lyophilized to afford **13** (35% based on resin loading capacity). C₈₄H₁₄₅N₁₉O₂₅, MALDI-ToF MS: observed, [M⁺] 1821.1991Da; calculated, [M⁺] 1821.1624Da.

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CHAPTER 6

Structure Activity Relationship of Three-Component Cancer Vaccine Candidates

The three-component vaccine construct consisting of a tumor-associated carbohydrate antigen, a promiscuous T-helper peptide epitope and a Toll like receptor ligand induced an efficient class switch from the low affinity IgM to high affinity IgG antibodies.¹ Therefore, we believe that to get a desired immune response, vaccine constructs should contain components, such as the carbohydrate B-epitope as an antigen, the promiscuous T-helper peptide and the TLR ligand. Thus, this chapter focuses on developing structure activity relationship between various three-component vaccine constructs for eliciting a strong and relevant immune response. The rationale for synthesis of these vaccine constructs was to investigate the role of T-helper peptide epitope, the effect of tumor associated carbohydrate B-epitope, and finally the optimum architecture of the three-component cancer vaccine construct. Furthermore, vaccine constructs will be synthesized with or without TLR ligand, which would help in understanding the role of TLR ligand as an adjuvant for inducing a strong antibody response. Thus, three-component vaccine constructs that differ in T-epitope peptide, B-epitope, topology, and TLR ligands were synthesized.

It is well known that the MHC class II requires peptides approximately 12-16 amino acids for optimum binding. Moreover, as MHC genes are polymorphic in nature, different individual recognizes different peptides as a T-helper peptide. As a result, a number of peptide motifs have been identified that can be recognized by many people. The peptide YAFKYARHANVGRNAFELFL, originated from *Neisseria Meningitidis*,²

is known to bind MHC class II in humans. Thus, three-component vaccine construct containing a carbohydrate antigen, T-helper peptide and a TLR ligand **1** was synthesized by using liposome mediated native chemical ligation. The influence of covalent attachment of the various components of the vaccine candidate on antigenic responses was investigated by immunizing mice with a liposomal preparation of compounds **3**, which is composed of the T-epitope linked to the B-epitope, and the adjuvant Pam₃CysSK₄ (**4**). Furthermore, vaccine candidate **2** without TLR ligand was also synthesized. Immunological evaluation of this compound can be carried out in the absence or presence of TLR2 ligand **4** as an adjuvant.

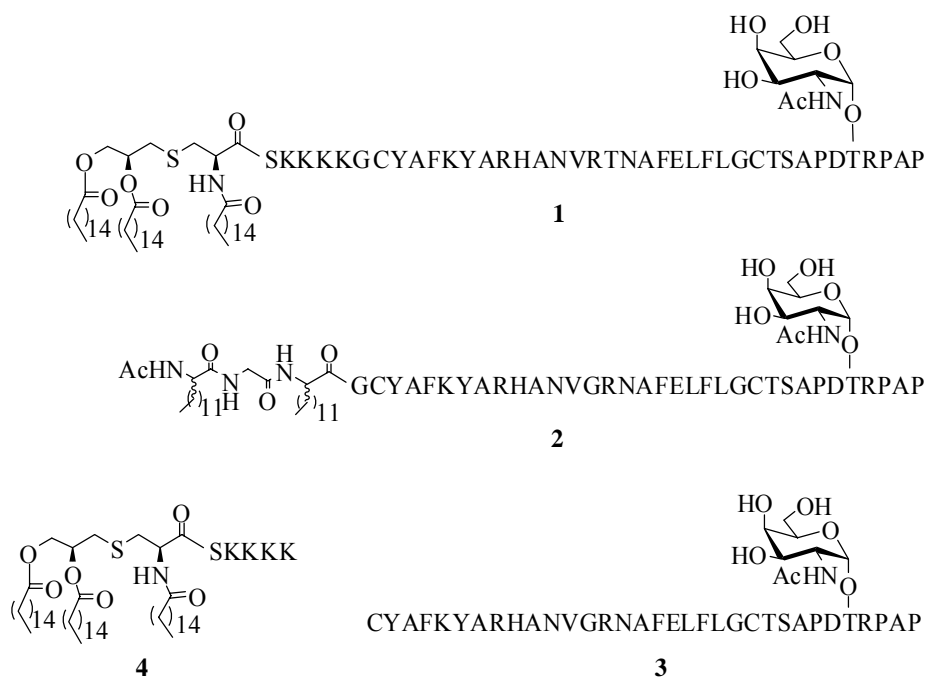


Figure 6.1| Glyco(lipo)peptide constructs with *Nisseria Meningitidis* T-helper epitope

It was envisaged that the vaccine candidates **1**, **2**, and **3** was can be synthesized by *liposome*-mediated native chemical ligation³ and has been described in chapter 3 (compounds **1**, **23**, and **22** respectively), while chemical synthesis of compound **4** has been described in chapter 4 (compound **7**).

Next, attention was focused on immunizing mice and characterizing the antibody responses. Thus compound **1** was incorporated into phospholipid-based small unilamellar vesicles (SUVs) by hydration of a thin film of the synthetic compound, egg phosphatidylcholine, phosphatidylglycerol and cholesterol in a HEPES buffer (10 mM, pH 7.4) containing NaCl (145 mM) followed by extrusion through a 100 nm Nuclepore® polycarbonate membrane. Groups of five female BALB/c mice were immunized intraperitoneal four times at weekly intervals with liposomes containing 3 µg of saccharide.¹ To investigate the adjuvant properties of TLR2 ligand, compound **2** was co-administered with compound **4**. Furthermore, a mixture of glycopeptide **3** and lipopeptide **4** in HEPES buffer was administered four times at weekly intervals prior to sera harvesting.

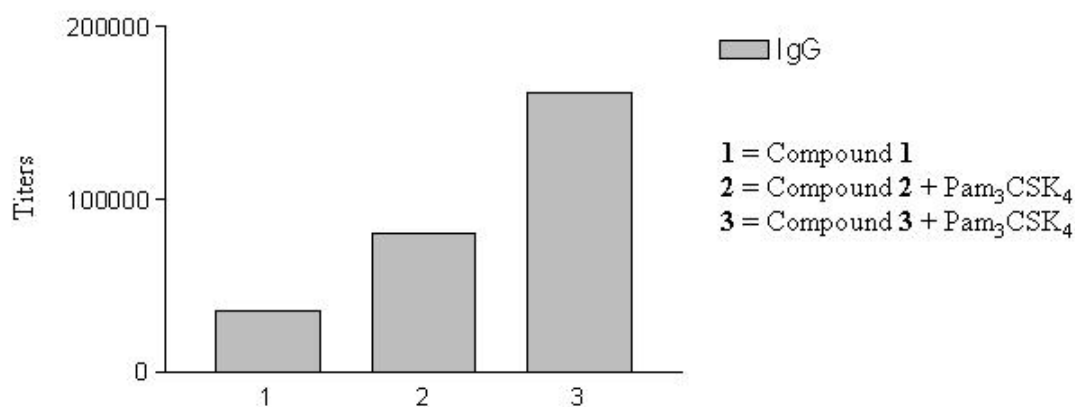


Figure 6.2 | ELISA anti-MUC1 antibody titers after 4 immunizations. Anti-MUC1 antibody titers are presented as means of groups of five mice. Titers are defined as the highest dilution yielding an optical density of 0.1 or greater over background of blank sera.

The total IgG anti-MUC1 antibody titers were measured by coating microtiter plates with CTSAPDT(α -D-GalNAc)RPAP conjugated with alkaline phosphatase. Mice immunized with **1** gave modest antibody titers as against for compound **2** and **3** (Figure 6.2). Further, immunological analyses of these compounds are under evaluation.

It is believed that after cellular uptake, the T-epitope of the three-component vaccine must be released by the proteases before it can be presented to MHC. Therefore, the three-component vaccine constructs that differ in the topology may be important for the stimulation of an immune response. Thus, the compounds **5** and **6** were synthesized in different order than the compound **1** and **2** (chapter 4) respectively. Compound **5** has a TLR4 ligand (Pam₂CSK₄), and compound **6** contain a TLR2 ligand (Pam₃CSK₄)^{4, 5} covalently attached to MUC-1 glycopeptide as a B-epitope and a murine T-helper peptide derived from poliovirus.

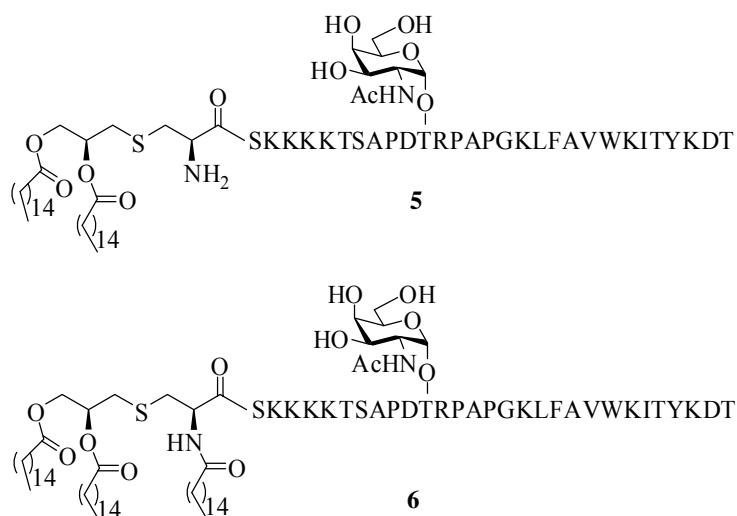
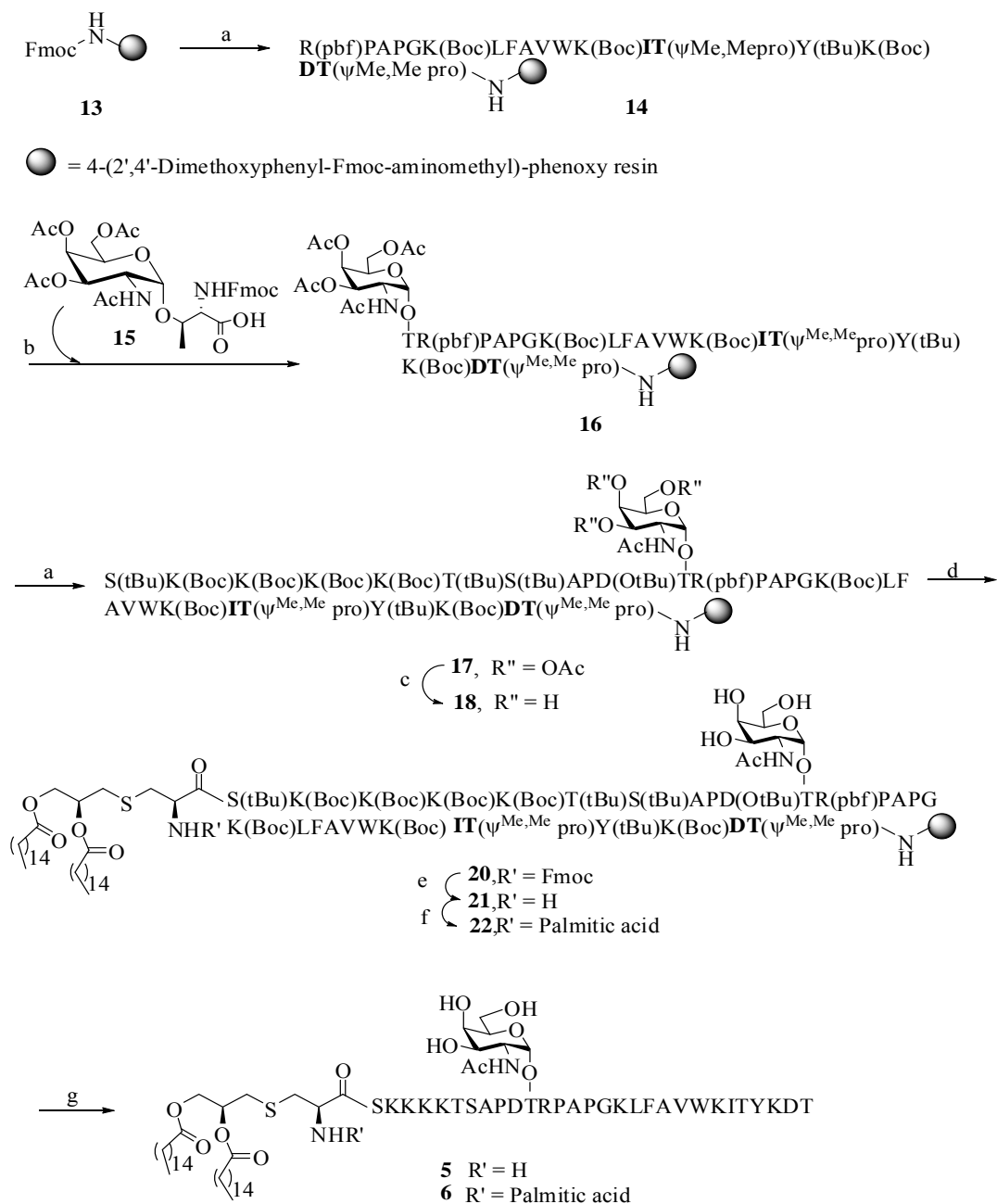


Figure 6.3 | Fully synthetic vaccine candidates with different architecture

The chemical synthesis of compounds **5** and **6** was carried out by a SPPS protocol using a Rink amide resin, Fmoc protected amino acids and *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc).⁶ After assembly of the peptide by using an automated HBTU-mediated HOBt ester activation protocol to give compound **14**, the coupling of the glycosylated amino acid **15** was carried out manually using HATU/HOAt in the presence of DIPEA in NMP to give compound **16**. The acetyl esters of the saccharide moiety were cleaved by the

treatment of 60% hydrazine in MeOH,^{7, 8} to afford compound **18**.



Scheme 6.1 | Chemical synthesis of glyco(lipo)peptide. Conditions, (a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) **15**, HATU/HOAt, DIPEA, NMP, overnight; c) 60% hydrazine in MeOH, 2 hr; d) Manual coupling of Pam₂Cys-OH, PyBOP, HOBt in the presence of DIPEA in DMF; (e) 20 % Piperidine in DMF; (f) Coupling of Palmitic acid, PyBOP, HOBt in the presence of DIPEA in DMF; (g) Reagent B, (TFA 88%, Phenol 5%, H₂O 5%, TIS 2%), 2 hr.

The peptide was elongated by manual coupling with Pam₂Cys-OH⁹ to give resin bound lipopeptide **20**. The *N*-Fmoc was removed by using 20% piperidine in DMF to give compound **21**, the glyco(lipo)peptide **5** was obtained by treatment of reagent B (TFA 88%, Phenol 5%, Water 5%, and TIS 2%) in 62% overall yield after purification by RP-HPLC over a C4 column. On the other hand, compound **21** on manual coupling with the palmitic acid; followed by of reagent B (TFA 88%, Phenol 5%, Water 5%, and TIS 2%) to gave compound **6** in overall yield of 39% (Scheme 6.1).

Immunological analysis of these compounds is under evaluation.

Having synthesized vaccine candidates that differ T-helper peptide, topology the attention was focused on the synthesis of three-component glyco(lipo)peptide construct that contain different tumor-associated carbohydrate B-epitope.

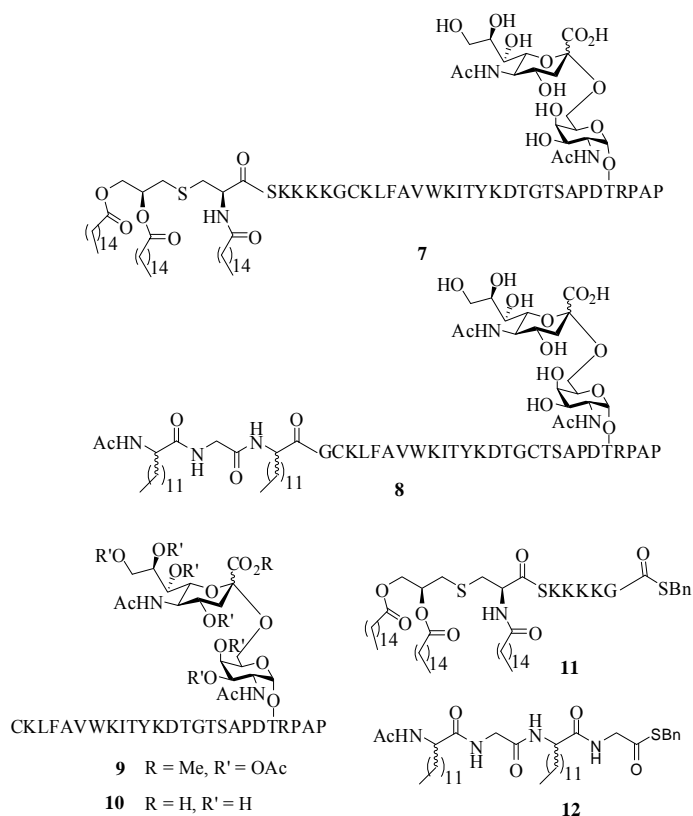
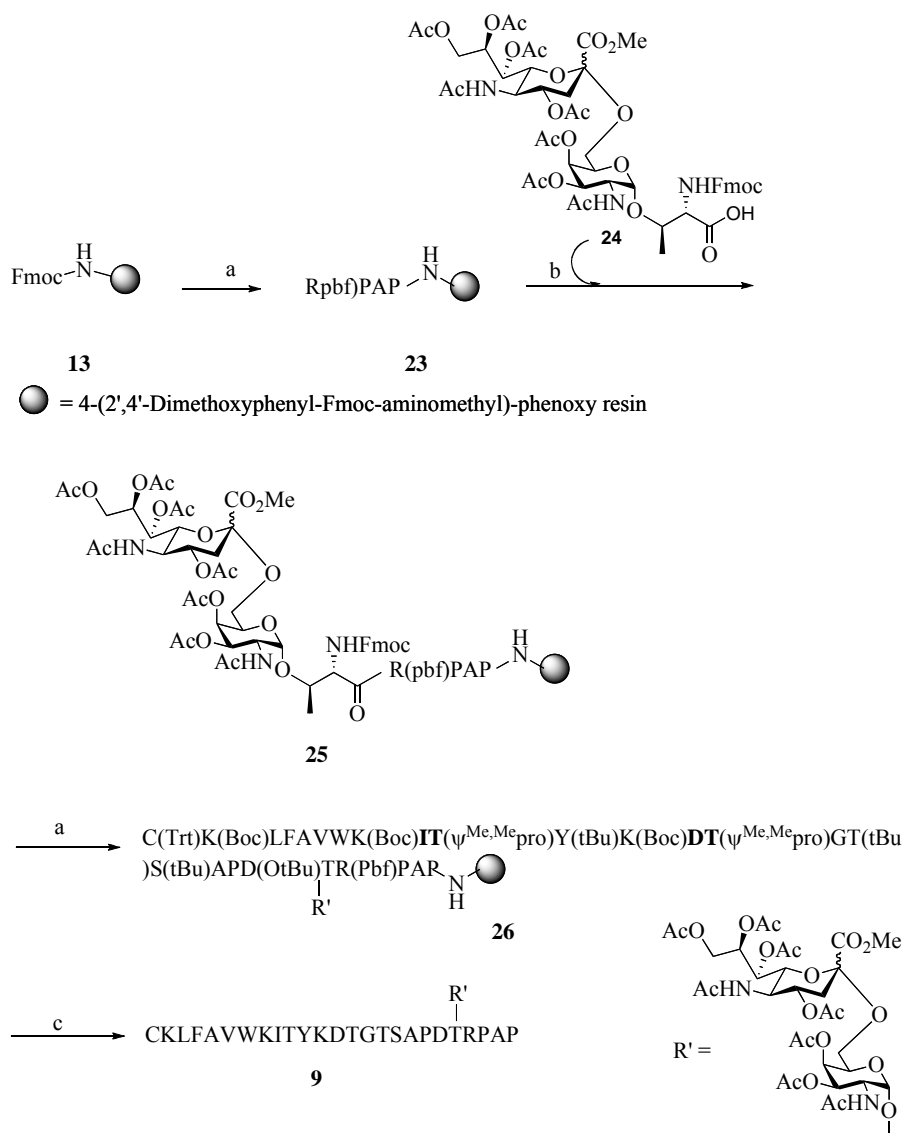


Figure 6.4 | Cancer vaccine candidates with MUC-1 glycopeptide containing sialic acid antigen

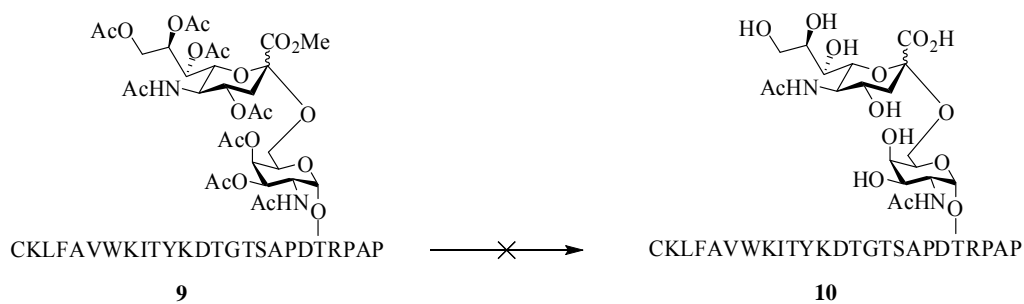
To this end, compound-containing MUC1 peptide modified by a STn (sialyl Tn, NeuAc- α -2 \rightarrow 6- α -D-GalNAc \rightarrow O-Thr)¹⁰⁻¹⁵ **7** moiety was prepared by using *liposome*-mediated native chemical ligation. For the synthesis of this series of compound, it was decided first to synthesize the compound **10** which on ligation with compound **11** would give desired glyco(lipo)peptide **7** containing TLR2 ligand. At the same time it would also allow the synthesis of three-component construct without TLR2 ligand **8** on reaction with compound **12**.

The synthesis of glycopeptide **10** was carried out as shown in scheme 6.2 using solid phase peptide synthesis, and manual coupling, First four amino acids, were coupled by using a standard protocol on peptide synthesizer followed by manual coupling of STn 24 in the presence of HATU/HOAt in the presence of DIPEA in NMP to give **25**, the resin bound glycopeptide was then further elongated by using standard SPPS protocol to afford compound **26**, simultaneous side chain deprotection of protecting groups of amino acids along with cleavage from the resin glycopeptide **9** was obtained in good yield (41%).

Next, step in the synthesis is the deprotection of acetyl esters, and methyl ester on the saccharide moiety. To carry out these reactions there are several procedures available in the literature,¹⁶⁻¹⁸ however, multiple attempts made to deprotect methyl ester moiety failed to give any product formation (Scheme 6.3).



Scheme 6.2 | Synthesis of glycopeptide containing STn antigen. Reagents and conditions: a) SPPS; b) **24**, HATU/HOAt, in the presence of DIPEA in NMP, Overnight; c) Reagent K (TFA 94%, water 2.5%, EDT 2.5%, TIS 1%), 4 h.



Scheme 6.3 | Deprotection of saccharide protecting groups

It is our expectation that from this series of compound, we will be able to develop structure-activity relationship between various components of three-component vaccine. Furthermore, it will also provide access to wide array of compounds, which would allow the better understanding of the immune system and immune response towards the three-component cancer vaccine.

6.1 Experimental procedures:

Reagents and general procedures: Amino acid derivatives and resins were purchased from NovaBioChem and Applied Biosystems; DMF from EM Science; and NMP from Applied Biosystems. PC, PG, Cholesterol, and DPC was obtained from Avanti Polar Lipids. All other chemical reagents were purchased from Aldrich, Acros, Alfa Aesar and Fischer and used without further purification. All solvents employed were reagent grade. Reverse Phase HPLC was performed on an Agilent 1100 series system equipped with an autosampler, UV-detector and fraction-collector using a Zorbax Eclipse C8 analytical column (5 μ m, 4.6 x 150 mm) at a flow rate of 1 ml/min, a semi preparative C8 column (5 μ m, 10 x 250 mm) at a flow rate of 4 ml/min, a Synchropak C4 analytical column (5 μ m, 4.6 x 100 mm) at a flow rate of 1 ml/min and a Jupiter C4 semi preparative column (5 μ m, 4.6 x 250 mm) at a flow rate of 2 ml/min. All runs used linear gradients of 0-100% solvent B in A over a 40 min. period was used unless otherwise specified. (A = water 95%, acetonitrile 5% and 0.1% TFA, B= water 5%, acetonitrile 95% and 0.1% TFA). MALDI-ToF mass spectra were recorded on a ABI 4700 proteomic analyzer.

General methods for Solid-Phase Peptide Synthesis (SPPS): Peptides were synthesized by established protocols on a Applied Biosystems, ABI 433A peptide synthesizer equipped with UV-detector using *N*- α -Fmoc-protected amino acids and 2-

(1H-bezotriazole-1-yl)-oxy-1,1,3,3-tetramethyl hexafluorophosphate (HBTU)/1-Hydroxybenzotriazole (HOBt) as the activating reagents. Single coupling steps were performed with conditional capping. The coupling of the glycosylated amino acid *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc) and *N*- α -Fmoc-*R*-(2,3-bis(palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine was carried out manually. The manual couplings were monitored by standard Kaiser test.

Synthesis of glycolipopeptide 5: SPPS was performed on Rink amide resin (0.1 mmol). Side chain protection is as follows: *N*- α -Fmoc-Asp-Thr($\Psi^{\text{Me,Me}}$ pro)-OH, *N*- α -Fmoc-*N*^G-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*L*-arginine, *N*- α -Fmoc-Ile-Thr($\Psi^{\text{Me,Me}}$ pro)-OH, *N*- α -Fmoc-*N*- ϵ -*tert*.-Boc-*L*-lysine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-serine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-threonine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-tyrosine. After the assembly of the peptide by using standard SPPS, a manual coupling was carried out using *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc) (0.2 mmol, 134 mg), with HATU (0.2 mmol, 77 mg), HOAt (0.2 mmol, 27 mg) and DIPEA (0.4 mmol, 70 μ l) in NMP for 12 h. The coupling reaction was monitored by standard Kaiser test. The resin was washed with NMP (6 ml) and DCM (6 ml), and resubjected to the same coupling conditions to ensure complete coupling. The glycopeptide was elongated on peptide synthesizer. The resin was washed thoroughly with NMP (6 ml x 1), DCM (6 ml x 1) and MeOH (6 ml x 1) and dried in *vacuo*. The resin was swelled with DCM (5 ml) for 1 h. The resin was treated with 60% hydrazine in MeOH (10 ml) for 2 h and washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2) and MeOH (5 ml x 2) and dried in *vacuo*. Then resin was swelled in DCM (5 ml) for 1 h. *N*- α -Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (267 mg, 0.3 mmol) was dissolved in DMF (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBt (40

mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) were premixed for 2 min, and was added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 12 h. Upon completion of the coupling, the *N*-Fmoc group was cleaved using 20% piperidine in DMF (6 ml). The resin was washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2) and MeOH (5 ml x 2) and then dried in *vacuo*. The resin was then swelled in DCM (5 ml) for 1 h. After which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%) (10 ml) for 2 h. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0°C) (30 ml) and recovered by centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by HPLC on a semi preparative C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min., and the appropriate fractions were lyophilized to afford **5** (62% based on resin loading capacity). C₁₉₆H₃₂₈N₄₃O₅₀S, MALDI-ToF MS: observed, [M⁺] 4120.3638Da; calculated, [M⁺] 4119.0246Da.

Synthesis of glyco(lipo)peptide 6: SPPS was performed on Rink amide resin (0.1 mmol). Side chain protection is as follows: *N*- α -Fmoc-Asp-Thr($\Psi^{\text{Me,Me}}$ pro)-OH, *N*- α -Fmoc-*N*^G-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*L*-arginine, *N*- α -Fmoc-Ile-Thr($\Psi^{\text{Me,Me}}$ pro)-OH, *N*- α -Fmoc-*N*- ϵ -*tert.*-Boc-*L*-lysine, *N*- α -Fmoc-*O*-*tert.*-butyl-*L*-serine, *N*- α -Fmoc-*O*-*tert.*-butyl-*L*-threonine, *N*- α -Fmoc-*O*-*tert.*-butyl-*L*-tyrosine. After the assembly of the peptide by using standard SPPS, a manual coupling was carried out using *N*- α -Fmoc-Thr-(AcO₃- α -D-GalNAc) (0.2 mmol, 134 mg), with HATU (0.2 mmol, 77 mg), HOAt (0.2 mmol, 27 mg) and DIPEA (0.4 mmol, 70 μ l) in NMP for 12 h. The coupling reaction was monitored by standard Kaiser test. The resin was washed with

NMP (6 ml x 1) and DCM (6 ml x 1), and resubjected to the same coupling conditions to ensure complete coupling. The glycopeptide was elongated on peptide synthesizer. The resin was washed thoroughly with NMP (6 ml x 1), DCM (6 ml x 1) and MeOH (6 ml x 1) and dried in *vacuo*. The resin was swelled with DCM (5 ml) for 1 h. The resin was treated with 60% hydrazine in MeOH (10 ml) for 2 h and washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2) and MeOH (5 ml x 2) and dried in *vacuo*. Then resin was swelled in DCM (5 ml) for 1 h. *N*- α -Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (267 mg, 0.3 mmol) was dissolved in DMF (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) were premixed for 2 min, and was added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 12 h. Upon completion of the coupling; the *N*-Fmoc group was cleaved using 20% piperidine in DMF (6 ml). Next, palmitic acid (77 mg, 0.3 mmol) was coupled to the free amine as described above using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μ l, 0.4 mmol) in DMF (5 ml). The resin was washed thoroughly with DMF (5 ml x 2), DCM (5 ml x 2) and MeOH (5 ml x 2) and dried in *vacuo*. The resin was swelled in DCM (5 ml) for 1 hr. After which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%) (10 ml) for 2 h. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0°C) (30 ml) and recovered by centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by HPLC on a semi preparative C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over a 40 min., and the appropriate fractions were lyophilized to afford **6** (39% based on resin loading capacity).

C₂₁₂H₃₅₈N₄₃O₅₁S, MALDI-ToF MS: observed, [M⁺] 4358.0142Da; calculated, [M⁺] 4357.4334Da.

Synthesis of glycopeptide 9: SPPS was performed on Rink amide resin (0.1 mmol) as described above. Side chain protection was as follows: *N*- α -Fmoc-Asp-Thr($\Psi^{\text{Me,Me}}$ pro)-OH, *N*- α -Fmoc-*N*^G-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*L*-arginine, *N*- α -Fmoc-*S*-trityl-*L*-cysteine, *N*- α -Fmoc-Ile-Thr($\Psi^{\text{Me,Me}}$ pro)-OH, *N*- α -Fmoc-*N*- ϵ -*tert*.-Boc-*L*-lysine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-serine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-threonine, *N*- α -Fmoc-*O*-*tert*.-butyl-*L*-tyrosine. Thus first four amino acids, Arg-Pro-Ala-Pro were coupled on the peptide synthesizer using a standard protocol. After the completion of the synthesis, a manual coupling was carried out using silyl Tn (NeuAc- α -2 \rightarrow 6- α -D-GalNAc \rightarrow *O*-Thr) (0.3 mmol, 331 mg), with HATU (0.4 mmol, 152 mg), HOAt (0.4 mmol, 55 mg) and DIPEA (0.4 mmol, 70 μ l) in NMP for 12 h. The coupling reaction was monitored by standard Kaiser test. The resin was washed with NMP (6 ml x 1) and DCM (6 ml x 1), and resubjected to the same coupling conditions to ensure complete coupling. The glycopeptide was elongated on peptide synthesizer. The resin was washed thoroughly with NMP (6 ml x 1), DCM (6 ml x 1) and MeOH (6 ml x 1) and dried in *vacuo*. The resin was swelled in DCM (5 ml) for 1 hr. After which it was treated with reagent K (TFA 94%, water 2.5%, EDT 2.5%, and TIS 1%) (10 ml) for 2 h at room temperature. The resin was filtered and washed with neat TFA (2 ml). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0°C) (40 ml) and recovered by centrifugation at 3000 rpm for 15 min. The crude glycopeptide was purified by RP-HPLC on a semi-preparative C-18 reversed phase column using a linear gradient of 0-100% solvent B in A over a

period of 40 min., and lyophilization of the appropriate fractions afforded **9** (41% based on resin loading capacity). C₁₅₈H₂₄₁N₃₅O₅₄S, MALDI-ToF MS: observed, 3526.3965Da; calculated, 3526.8712Da.

Deprotection of glycopeptide (10): 1) The glycopeptide **9** (5 mg, 1.4 µmol) was treated with 0.1 M NaOH solution (2 ml), pH 10.0, containing DTT (12 mg, 14 µmol), the reaction was monitored by MALDI-ToF MS. After standing for 12 h at room temperature, the LC-MS showed the beta-elimination product. This reaction was also repeated at different pH conditions with similar result. 2) The glycopeptide **9** (5 mg, 1.4 µmol) was treated with 0.3 M NaOH solution (2 ml), pH 10.0, containing DTT (12 mg, 14 µmol), the reaction was monitored by MALDI-ToF. After standing for 12 h at room temperature, the LC-MS the showed beta-elimination product. This reaction was also repeated at different pH conditions and at elevated temperature with similar result. 3) The glycopeptide **9** (5 mg, 1.4 µmol) dissolved in ethanol (1 ml) and H₂O (1 ml) was added LiOH.H₂O (1.76 mg, 42 µmol) containing DTT (12 mg, 14 µmol), at room temperature. After being stirred at 80 °C for 17 h, the reaction mixture was evaporated in *vacuo*. The residue obtained was beta-elimination product.

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CHAPTER 7

Conclusions

The novel synthetic methodology described in **chapter 3** allows the synthesis of a range of three-component vaccine candidates by a modular approach using an array of B- and T-epitopes and lipopeptide adjuvants. This *liposome*-mediated native chemical ligation approach is attractive because it provides greater synthetic flexibility than linear synthesis. In this respect, each building block can be used for the preparation of several different target compounds. Furthermore, compared to conventional linear SPPS, a block synthetic approach will minimize by-product build up in the growing peptide chain.

As with every biological system, tumors are opportunistic and redundant mechanisms, which guarantee their survival and development. The robustness of these processes makes cancer immunotherapy a challenge. Thus, a successful cancer immunotherapy should depend on optimal tumor-specific immune activation, which is facilitated by the use of appropriate structural motifs. Moreover, the treatment of cancer with vaccines should rely on adjuvants designed not just to activate the immune system but also to induce system recovery from tumor-induced immunosuppression. The research described in **chapter 4** demonstrates the versatility of a three-component construct in cancer vaccine design, which is attributed to a number of unique features. First, it does not have any unnecessary features that are antigenic and may induce immune suppression. The chemical attachment of the TLR2 agonist Pam₃CysSK₄ to the B- and T-epitopes ensures that cytokines are produced at the site where the vaccine interacts with immune cells, which should lead to a high local concentration of cytokines facilitating maturation of

relevant immune cells. The treatment also facilitates uptake by TLR2-expressing cells such as APCs, which will assist antigen processing and full activation.

The fully synthetic approach developed in the thesis makes it possible to optimize the various components of the candidate vaccine by developing a structure-activity relationship. Thus, compounds synthesized in **chapter 5** focuses on the requirement of TLR the ligand to induce a necessary signal to the immune system. The result of the study clearly shows that the presence of a TLR ligand in either bound or unbound form helps to stimulate the accessory cells leading to priming of the immune system.

In **Chapter 6**, the synthesis of three-component vaccine constructs that are related to the different topology of B-and T-epitopes is discussed. The immunological evaluation of these compounds indicates that by designing well-defined molecules it is possible to stimulate immune response.