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(54) Titre : GLYCOLIPOPEPTIDES A TROIS COMPOSANTS
(54) Title: THREE COMPONENT GLYCOLIPOPEPTIDES

(57) Abrégé/Abstract:
Carbohydrate antigens are a common feature of cancer cells and pathogens, such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV). Carbohydrates cannot activate helper T-cells and have poor immunogenicity. This invention provides a novel glycolipopeptide to generate antibodies to carbohydrate antigens. The glycolipopetide includes at least one B-epitope, at least one T-epitope, and a lipid component. The invention also includes a method for making the glycolipopeptide of the invention. The invention also provides a polyclonal or monoclonal antibody against the glyeolipopetitde of the invention. The invention also includes a pharmaceutical composition including the glycolipopeptide.

76433-138

Abstract

Carbohydrate antigens are a common feature of cancer cells and pathogens, such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV). Carbohydrates cannot activate helper T-cells and have poor immunogenicity. This invention provides a novel glycolipopeptide to generate antibodies to carbohydrate antigens. The glycolipopetide includes at least one B-epitope, at least one T-epitope, and a lipid component. The invention also includes a method for making the glycolipopeptide of the invention. The invention also provides a polyclonal or monoclonal antibody against the glycolipopetitde of the invention. The invention also includes a pharmaceutical composition including the glycolipopeptide.

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THREE COMPONENT GLYCOLIPOPEPTIDES

This application claims the benefit of U.S. Applications Serial Nos. 60/755,881, filed January 3, 2006; 60/796,769, filed May 2, 2006; and 60/809,272, filed May 30, 2006.

STATEMENT OF GOVERNMENT RIGHTS

This invention was made with government support under a grant from the National Cancer Institute of the National Institute of Health (Grant No RO1 CA88986). The U.S. Government has certain rights in this invention.

BACKGROUND

A large number of tumor-associated carbohydrate antigens (TACA) are expressed on human cancer cells in the form of glycolipids and glycoproteins. A common feature of oncogenic transformed cells is the over-expression of oligosaccharides, such as Globo-H, Lewis^{Y}, and Tn antigens. Numerous studies have shown that this abnormal glycosylation can promote metastasis and hence it is strongly correlated with poor survival rates of cancer patients.

The differential expression that is characteristic of these tumor-associated carbohydrate antigens renders them attractive targets for immunotherapy and the development of cancer vaccines. Recently, several elegant studies have attempted to capitalize on the differential expression of tumor-associated carbohydrates for the development of cancer vaccines (e.g., Raghupathi, Cancer Immunol. 1996, 43, 152-157; Musselli et al., J. Cancer Res. Clin. Oncol. 2001, 127, R20-R26; Sabbatini et al., Int. J. Cancer, 2000, 87, 79-85; Lo-Man et al., Cancer Res. 2004, 64, 4987-4994; Kagan et al., Immunol. Immunother. 2005, 54, 424-430).

Carbohydrate antigens are also abundant on the surface the human immunodeficiency virus (HIV), the causative agent of acquired immune

deficiency syndrome (AIDS). Hepatitis C virus (HCV) is also known to contain carbohydrate antigens.

For most immunogens, including carbohydrates, antibody production depends on the cooperative interaction of two types of lymphocytes, B-cells and helper T-cells. Carbohydrates alone, however, cannot activate helper T-cells and therefore are characterized by poor immunogenicity. The formation of low affinity IgM antibodies and the absence of IgG antibodies manifest this limited immunogenicity. It has proven difficult to overcome the immunotolerance that characterizes these antigens.

In an effort to activate helper T cells, researchers have conjugated carbohydrate antigens to a foreign carrier protein, *e.g.* keyhole limpet hemocyanin (KLH) or detoxified tetanus toxoid (TT). The carrier protein enhances the presentation of the carbohydrate to the immune system and supplies T-epitopes (typically peptide fragments of 12-15 amino acids) that can activate T-helper cells.

However, conjugation of carbohydrates to a carrier protein poses several new problems. The conjugation chemistry is difficult to control, resulting in conjugates with ambiguities in composition and structure that may affect the reproducibility of an immune response. In addition, the foreign carrier protein may elicit a strong B-cell response, which in turn may lead to the suppression of an antibody response against the carbohydrate epitope. The latter is particularly a problem when self-antigens are employed such as tumor-associated carbohydrates. Also, linkers employed for conjugating carbohydrates to proteins can themselves be immunogenic, leading to epitope suppression. See also McGeary et al., for a review of lipid and carbohydrate based adjuvant/carriers in vaccines (J. Peptide Sci. 9 (7): 405-418, 2003).

Not surprisingly, several clinical trials with carbohydrate-protein conjugate cancer vaccines failed to induce sufficiently strong helper T-cell responses in all patients. Therefore, alternative strategies need to be developed for the presentation of tumor associated carbohydrate epitopes that will result in a more efficient class switch to IgG antibodies. These strategies may potentially prove useful as well for the development of vaccines based on other carbohydrate epitopes, particularly those form pathogenic viruses such as HIV and HCV.

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SUMMARY OF THE INVENTION

The present invention provides a glycolipopeptide, also referred to herein as a lipidated glycopeptide, as well as pharmaceutical compositions containing such glycolipopeptide and methods of making and using such glycolipopeptide. In a preferred embodiment, the glycolipopeptide of the invention is fully synthetic.

The glycolipopotide preferably contains at least 2 epitopes: a B-epitope and a T-epitope, as well as a lipid component. The glycolipopeptide is thus able to elicit both a humoral response to the B-epitope and a cellular immune response to T-epitope. In a preferred embodiment, the glycolipopetide of the invention advantageously combines features from a B-epitope glycan or glycopeptide and a T-epitope peptide derived from glycoproteins of mammalian (preferably human or murine) or microbial origin.

Lipidation may potentially confer several additional advantages to the glycolipopeptide. It may potentially help the glycolipopeptides self assemble into vesicles, and may also facilitate the incorporation of the immunogen into a liposome which in turn may potentially improve the presentation of the immunogen to the immune system. Additionally, the lipid component may potentially serve as a built-in adjuvant. Cellular uptake of the glycopeptide may potentially also be facilitated by the lipidation. Cytokine production may potentially also be enhanced by inclusion of the lipid component.

Accordingly, in one aspect, the invention provides a glycolipopeptide containing at least one carbohydrate component having a B-epitope; at least one peptide component having a T-epitope; and at least one lipid component. The carbohydrate component and the peptide component may be heterologous with respect to each other; or they may be homologous with respect to each other.

The carbohydrate component of the glycolipopeptide can include a glycoconjugate, for example, a glycosylated protein, a glycosylated peptide, a glycosylated lipid, a glycosylated amino acid, a DNA or an RNA. The B-epitope of the carbohydrate component may be from a microorganism, such as a virus, a bacterium, a fungus, and a protozoan. Exemplary viruses as sources for the B-epitope include human immunodeficiency virus and hepatitis C virus, without limitation. The B-epitope can therefore constitute all or part of a viral antigen, such as a viral antigent from human immunodificienty virus or hepatitis

C virus. Alternatively or additionally, the B-epitope can constitute all or part of a self-antigen. For example, the B-epitope can be one that is overexpressed on a cancer cell. An exemplary self-antigen is MUC-1 glycopeptide.

The peptide component of the glycolipopeptide, which includes a T-epitope, preferably includes a helper T-epitope.

The lipid component of the glycolipopeotide is preferably an immunostimulatory lipid. For example, the lipid component can include a Toll-like receptor (TLR) ligand, such as a PamCys-type lipid. Examples of a PamCys-type lipid include Pam_3Cys or Pam_3CysSK_n, where n = 0, 1, 2, 3, 4 or 5. A particularly preferred lipid component includes Pam_3CysSK_4. In another preferred embodiment, the lipid component binds to a Toll-like receptor ligand and facilitates internalization of the glycolipopeptide by a target cell. Exemplary lipid components can be found, for example, in Scheme 8 hereinbelow. The lipid may serve as an internal (covalently linked) adjuvant.

Optionally, the glycolipopeptide of the invention includes at least one linker component. The linker component may link one or more of the carbohydrate component, peptide component and/or lipid component to each other or to a different component or structure.

A particularly preferred embodiment of the glycolipopeptide is one that contains at least one carbohydrate component that includes a self-antigen having a B-epitope, for example a MUC-1 glycopeptide; at least one peptide component comprising a helper T-epitope; and at least one lipid component, for example a Toll-like receptor ligand. In another particularly preferred embodiment, the glycolipopeptide of the invention includes at least one carbohydrate component that has a B-epitope; at least one peptide component that has a helper T-epitope; and at least one lipid component that binds to a Toll-like receptor and facilitates uptake of the glycolipopeptide by a target cell that includes the Toll-like receptor; wherein the carbohydrate component and the peptide component are heterologous with respect to each other. In another particularly preferred embodiment, the glycolipopeptide includes at least one carbohydrate component that includes a self-antigen that has a B-epitope; at least one peptide component that has a helper T-epitope; and at least one lipid component that binds to a Toll-

like receptor and facilitates uptake of the glycolipopeptide by a target cell that includes the Toll-like receptor.

In another aspect, the invention provides a pharmaceutical composition. In one embodiment, the pharmaceutical composition includes a glycolipopeptide of the invention, without limitation. Optionally, the pharmaceutical composition contains plurality of glycopeptides, which may include glycolipopeptides having different or the same B-epitopes, having different or the same T-epitopes and/or having different or the same lipid components. In another embodiment, the pharmaceutical composition includes an antibody against a glycolipopeptide of the invention, without limitation. The antibody can be a monoclonal or polyclonal antibody, and may be a humanized antibody. Techniques for humanizing antibodies are well known in the art.

Optionally, the pharmaceutical composition contains a liposome. Formulations with liposomes, micelles, or other lipid vesicles may facilitate delivery of the glycopeptide to a subject in need thereof. The glycolipopeptide may be covalently or noncovalently incorporated into the liposome, micelle or other lipid vesicle.

The pharmaceutical composition preferably includes a pharmaceutically acceptable carrier. In one embodiment, the pharmaceutical composition does not contain an external adjuvant. In another embodiment, the pharmaceutical composition contains an external adjuvant. An example of an external adjuvant is QS-21.

The pharmaceutical composition of the invention may potentially be used as a vaccine, for example to potentially treat or prevent an infection, disease or disorder. Additionally, the glycolipopeptide of the invention may be used for the manufacture of a medicament which may potentially be used to treat or prevent an infection, disease or disorder.

Accordingly, in another aspect, the invention may potentially provide a method for treating or preventing an infection, disease or disorder in a subject that involves administering a pharmaceutical composition of the invention to a subject in need thereof. Inclusion of QS-21 as an external adjuvant may skew the immune response of the subject toward a Th1 response, compared to a comparable pharmaceutical composition that does not include QS-21. The infection, disease

or disorder that is treated or prevented may be one that is caused by a microorganism, such as a virus, a bacterium, a fungus, and a protozoan. Viral infections that can be treated or prevented include, without limitation, those caused by a human immunodeficiency virus or a hepatitis C virus. Alternatively, the infection, disease or disorder that is treated or prevented can include cancer, a precancerous condition, or an autoimmune disease.

In another aspect, the invention includes a method for making the glycolipopeptide of the invention. The carbohydrate component, the peptide component and the lipid component are synthetically linked, for example by using chemical or in vitro enzymatic methods.

In another aspect, the invention provides polyclonal or monoclonal antibody against the glycolipopeptide of the invention. Humanized antibodies are encompassed by the invention.

In yet another aspect, the invention provides a method for identifying a Toll-like receptor (TLR) ligand. A Toll-like receptor ligand is useful for inclusion in a glycolipopeptide vaccine of the invention. The method includes contacting a candidate compound with a target cell containing a TLR, and determining whether the candidate compound binds to the TLR. Optionally, the method also includes determining whether the candidate compound is internalized by the target cell. In a preferred embodiment, the candidate compound includes a lipid, and the TLR ligands thus identified are useful as the lipid component for the glycolipopeptide of the invention. Accordingly, another embodiment of the glycolipopeptide of the invention includes at least one carbohydrate component having a B-epitope; at least one peptide component having a helper T-epitope; and at least one lipid component identified using the method of identifying a TLR ligand as described herein.

In another aspect, the invention provides a glycolipopeptide comprising at least one carbohydrate component comprising a B-epitope, wherein the B-epitope is from a microorganism; at least one peptide component comprising a helper T-epitope; and at least one lipid component comprising a Toll-like receptor (TLR) ligand comprising Pam_3CysSK_n,

wherein n = 1, 2, 3, 4 or 5; wherein the carbohydrate component and the peptide component are heterologous with respect to each other.

In another aspect, the invention provides a glycolipopeptide comprising: at least one carbohydrate component comprising a self-antigen comprising a B-epitope, wherein the B-epitope is overexpressed on a cancer cell; at least one peptide component comprising a helper T-epitope; and at least one lipid component comprising a Toll-like receptor (TLR) ligand comprising Pam_3CysSK_n, wherein n = 1, 2, 3, 4 or 5.

In another aspect, the invention provides a pharmaceutical composition comprising: a glycolipopeptide comprising: at least one carbohydrate component comprising a B-epitope, wherein the B-epitope is overexpressed on a cancer cell; at least one peptide component comprising a helper T-epitope; and at least one lipid component comprising a Toll-like receptor (TLR) ligand comprising Pam_3CysSK_n, wherein n = 1, 2, 3, 4 or 5; and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a pharmaceutical composition comprising: a glycolipopeptide comprising: at least one carbohydrate component comprising a B-epitope, wherein the B-epitope is from a microorganism; at least one peptide component comprising a helper T-epitope; and at least one lipid component comprising a Toll-like receptor (TLR) ligand comprising Pam_3CysSK_n, wherein n = 1, 2, 3, 4 or 5; and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a pharmaceutical composition comprising: an antibody which specifically binds the glycolipopeptide as described herein; and a pharmaceutically acceptable carrier.

In another aspect, the invention provides the use of the glycolipopeptide as described herein for the manufacture of a medicament to cause antibody production.

In another aspect, the invention provides the use of the pharmaceutical composition as described herein for causing antibody production in a subject.

In another aspect, the invention provides a method for making the glycolipopeptide as described herein comprising synthetically linking the carbohydrate component, the peptide component and the lipid component.

In another aspect, the invention provides a polyclonal or monoclonal antibody which specifically binds the glycolipopeptide as described herein.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an exemplary glycolipopeptide of the invention.

Figure 2 shows flow cytometry analysis for specific anti-MUC-1 antibodies. Reactivity was tested on MCF-7 (A) and SK-MEL-28 (B) cells.

Fluorescence intensity of serum (1:50 diluted) was assessed before (serum control; open peak) and after immunization with 3 (filled peak).

Figure 3 shows TNF-α production by murine macrophages after stimulation with LPS and synthetic compounds. Murine RAW γNO(-) cells were incubated for 5.5 hours with increasing concentrations of *E. coli* LPS (■), **1** (●), Pam_2CysSK_4 (▼), **2** (♦), Pam_3CysSK_4 (▲), or **3** (□) as indicated.

Figure 4 shows the effect of TLR ligand on cellular uptake.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The glycolipopeptide of the invention includes at least one B-epitope, at least one T-epitope, and a lipid component. In a preferred embodiment, the glycolipopeptide consists essentially of three main components: at least one carbohydrate component that contains a B-epitope; at least one peptide component that contains a helper T-epitope; and at least one lipid component. Exemplary carbohydrate, peptide and lipid components are described herein and also, for example, in references cited herein, including Koganty et al., US Patent Publication 20060069238, published March 30, 2006; see also Koganty et al., Drug Disc. Today, 1 (5): 190-198, 1996. The three components are covalently linked, either directly or indirectly, to form a single glycolipopeptide molecule. Indirect linkage involves the use of an optional linker component "L" to link two or more of the main components together. The three main components can be linked together (directly or indirectly) in any order. For example, the lipid and carbohydrate component can each be covalently linked to the peptide component to form the glycolipopeptide. Alternatively, the lipid component and the peptide component can each be covalently linked to the carbohydrate component. Likewise, the carbohydrate component and the peptide component can each be covalently linked to the lipid component. Or, all three components can be linked such that each of the three components is covalently linked to each of the other two components. Intermolecular crosslinking is also possible, as described in more detail below.

In a preferred embodiment, of the glycolipopeptide of the invention, the peptide component is flanked by the lipid and carbohydrate components. In this

embodiment, the lipid component is attached, directly or indirectly, to one terminus, preferably the N-terminus, of the peptide component, and the carbohydrate component is attached, directly or indirectly, to the other terminus, preferably the C-terminus, of the peptide component. Alternatively, one or the other of the lipid or carbohydrate components, or both, can be attached to an internal site on the peptide component. For example, a carbohydrate component could be attached via the hydroxyl group of a threonine or serine residue, or a cysteine sulfhydryl group, regardless of where it is located in the peptide sequence. Likewise, for example, a lipid component could be attached via a side chain nitrogen in an arginine or lysine, regardless of where it is located in the peptide sequence. The glycopeptide of the invention is not limited by any particular conjugation chemistry or by the location of the conjugation sites.

In a preferred embodiment, the glycolipopeptide of the invention contains one carbohydrate component, one peptide component, and one lipid component. In another embodiment, the glycoplipopeptide contains a plurality of carbohydrate components, which may be the same, or may be different. Likewise, in another embodiment, the glycolipopeptide contains a plurality of peptide components, which may be the same, or may be different. Further, in another embodiment, the glycolipopeptide contains a plurality of lipid components, which may be the same, or may be different. Thus, various embodiments of the glycolipopeptide of the invention may contain one or more carbohydrate components, one or more peptide components, and/or one or more lipid components. For example, the concept of "multiple antigenic glycopeptides" (Bay et al., U.S. Pat. No. 6,676,946, Jan. 13, 2004, Bay et al.; WO 98/43677, published October 8, 1998, Bay et al.) can be adapted for use in the present invention. High antigen density can be achieved using a core, for example a poly-lysine core, to which extended peptidic "arms" (the peptide component of the glycolipopeptide of the invention) are attached, which peptidic arms display the carbohydrate antigen components of the glycolipopeptide in clustered presentation. The lipid component of the glycolipopeptide can likewise extend from the lysine core, particularly in embodiments wherein the peptide component is attached to the lysine core via a nonterminal amino acid. High antigen density can also be achieved by using a liposome as a delivery

vehicle, as exemplified in Examples II and III. Additionally or alternatively, the glycolipopeptides can be optionally cross-linked to form a multi-molecular complex, thereby increasing the antigen density.

The various carbohydrate, peptide and lipid components of the glycolipopeptide can be structurally derived from or based on, and/or can mimic, those found in naturally occurring biological molecules. The glycolipopeptide components preferably contain molecular structures or parts of structures (including epitopes) that are identical to or similar to those found in a living organism. Typically, while the components of the glycolipopeptide are derived from, are structurally based on, and/or mimic naturally occurring structures, they are prepared synthetically, using chemical or in vitro enzymatic methods, for example. In some embodiments, epitopes that are formed in the naturally occurring antigen from molecular elements that are close in space but distant from each other in terms of chemical bonding can be formed in the glycolipopeptide of the invention by a different chemical structure (with different bonding order or pattern) that forms the same or a similar epitope.

The three component glycolipopeptide of the invention can be viewed as cassette, wherein the carbohydrate component, the peptide component, and the lipid component are each independently selected for inclusion in the glycolipopeptide. Any combination (i.e., mixing and matching) of carbohydrate component, peptide component and lipid component as described herein to form a glycolipopeptide is encompassed by the invention.

Carbohydrate component

The carbohydrate component of the glycolipopeptide can be any component that contains a carbohydrate. Examples of suitable carbohydrate components include oligosaccharides, polysaccharides and monosaccharides, and glycosylated biomolecules (glycoconjugates) such as glycoproteins, glycopeptides, glycolipids, glycosylated amino acids, DNA, or RNA. Glycosylated peptides (glycopeptides) and glycosylated amino acids, which contain one or more carbohydrate moieties as well as a peptide or amino acid, are particularly preferred as the carbohydrate component of the glycolipopeptide of the invention. An example of a glycopeptide is CD52, which is expressed on

virtually all human lymphocytes and believed to play an important role in the human immune system. An example of a glycosylated amino acid is the Tn antigen. It should be understood that when the carbohydrate component is a glycopeptide, the peptide part of the glycopeptide optionally includes a T-epitope and thus may serve as a peptide component of the glycolipopeptide.

The carbohydrate component of the glycolipopeptide of the invention includes a carbohydrate that contains one or more saccharide monomers. For example, the carbohydrate can include a monosaccharide, a disaccharide or a trisaccharide; it can include an oligosaccharide or a polysaccharide. An oligosaccharide is an oligomeric saccharide that contains two or more saccharides and is characterized by a well-defined structure. A well-defined structure is characterized by the particular identity, order, linkage positions (including branch points), and linkage stereochemistry (α, β) of the monomers, and as a result has a defined molecular weight and composition. An oligosaccharide typically contains about 2 to about 20 or more saccharide monomers. A polysaccharride, on the other hand, is a polymeric saccharide that does not have a well defined structure; the identity, order, linkage positions (including brand points) and/or linkage stereochemistry can vary from molecule to molecule. Polysaccharides typically contain a larger number of monomeric components than oligosaccharides and thus have higher molecular weights. The term "glycan" as used herein is inclusive of both oligosaccharides and polysaccharides, and includes both branched and unbranched polymers. When the carbohydrate component contains a carbohydrate that has three or more saccharide monomers, the carbohydrate can be a linear chain or it can be a branched chain. In a preferred embodiment, the carbohydrate component contains less than about 15 saccharide monomers; more preferably in contains less than about 10 saccharide monomers.

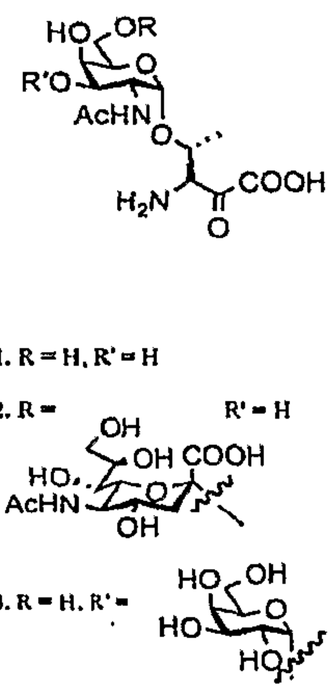
The carbohydrate component of the glycolipopeptide includes a carbohydrate that contains a B-epitope. The B-epitope can be a naturally occurring epitope or a non-naturally occurring epitope. Preferably, two or more saccharide monomers of the carbohydrate interact to form a conformational epitope that serves as the B-epitope. A B-epitope is an epitope recognized by a

B cell. Any antigenic carbohydrate that contains a B-epitope can be used as the carbohydrate component, without limitation.

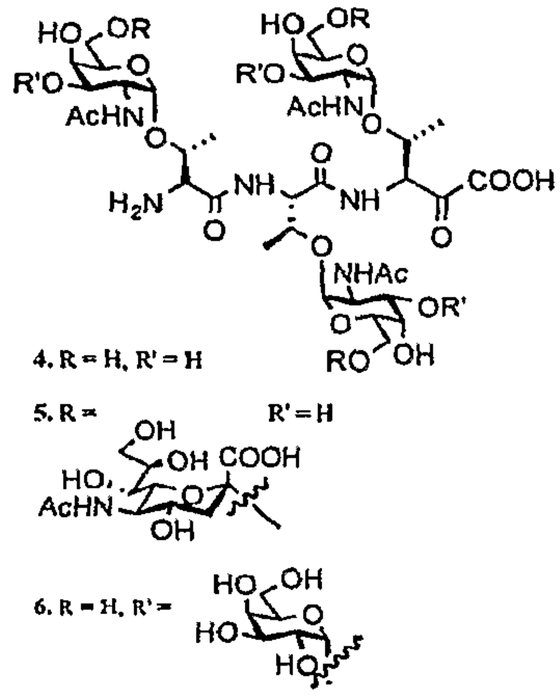
In one embodiment, the carbohydrate component contains all or part of a self-antigen. Self-antigens are antigens that are normally present in an animal's body. They can be regarded as "self-molecules," e.g., the molecules present in or on the animal's cells, or proteins like insulin that circulate in the animal's blood. An example of a self-antigen is a carbohydrate-containing component derived from a cancer cell of the animal, such as a tumor-associated carbohydrate antigen (TACA). Typically, such self-antigens exhibit low immunogenicity. Examples include tumor-related carbohydrate B-epitope such as Le^y antigen (a cancer related tetrasaccharide; e.g., Fucα(1,2)-Galβ(1,4)-[Fucα(1,3)]-GalNAc); Globo-H antigen (e.g., L-Fucα(1,2)-Galβ(1,3)-GalNAcβ(1,3)-Galα(1,4)-Galβ(1,4)-Glu); T antigen (e.g., Galβ(1,3)-GalNAcα-O-Ser/Thr); STn antigen (sialyl Tn, e.g., NeuAcα(2,6)-GalNAcα-O-Ser/Thr); and Tn antigen (e.g., α-GalNAc-O-Ser/Thr). Another example of a self-antigen is a glycopeptide derived from the tandem repeat of the breast tumor-associated MUC-1 of human polymorphic epithelial mucin (PEM), an epithelial mucin (Baldus et al., Crit. Rev. Clin. Lab. Sci., 41(2):189-231 (2004)). A MUC-1 glycopeptide comprises at least one Tn and/or sialyl Tn (sialyl α-6 GalNAc, or "STn") epitope; preferably linked to a threonine (T-Tn or T-STn).

Structures of exemplary tumor-associated carbohydrate antigens (TACA) that can be used as a component of the glycolipopeptide include, without limitation, the structures shown in Schemes 1 and 2.

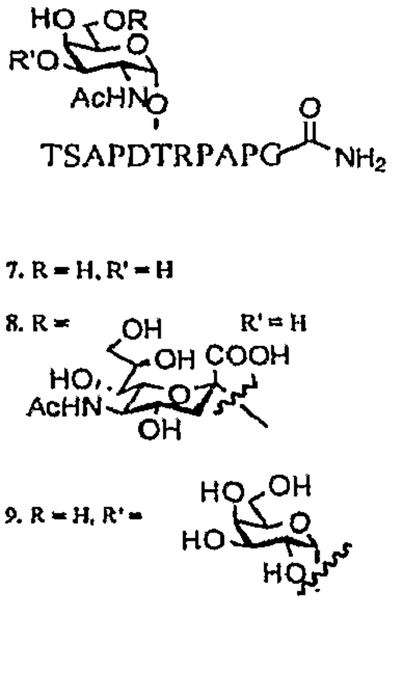
Tn, STn, and TF antigens



Tn3, STn3, and TF3



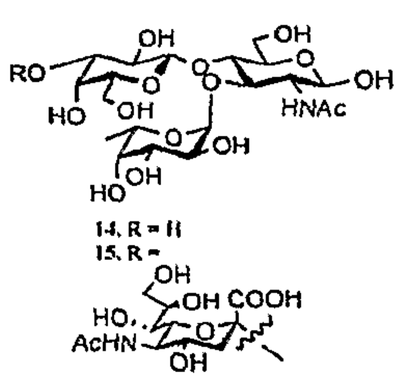
MUC-1 with Tn, STn, and TF



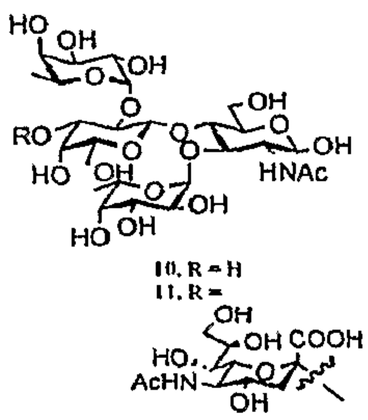
Scheme 1

It should be noted that the Tn, STn, and TF structures shown in Scheme 1 (monomeric, trimeric, clustered) are all shown with a threonine residue. The corresponding serine analogues are also suitable structures. In the case of Tn3, STn3, TF3 and their respective clusters, all possible homo- and hetero-analogues with differences in the threonine/serine composition of the backbone are included.

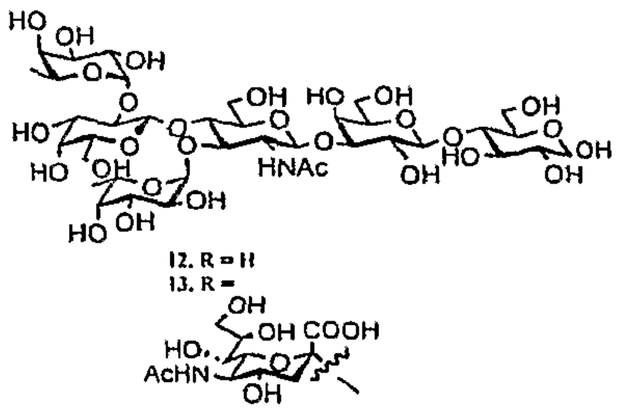
Lewis^a and SLewis^a



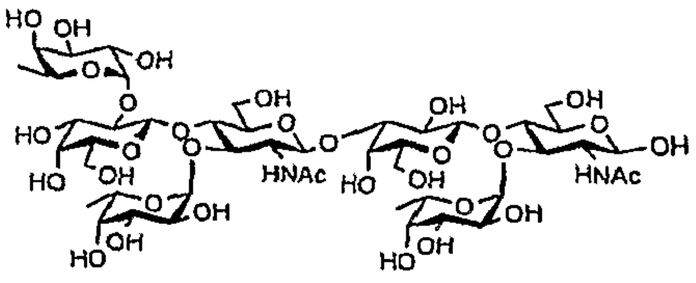
Lewis^y and SLewis^y



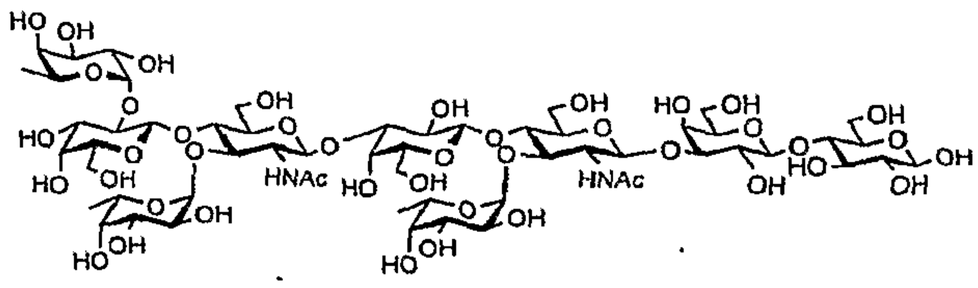
Lewis^y-lactose and SLewis^y-lactose



Lewis^y-Lewis^a dimer



The KH-1 antigen

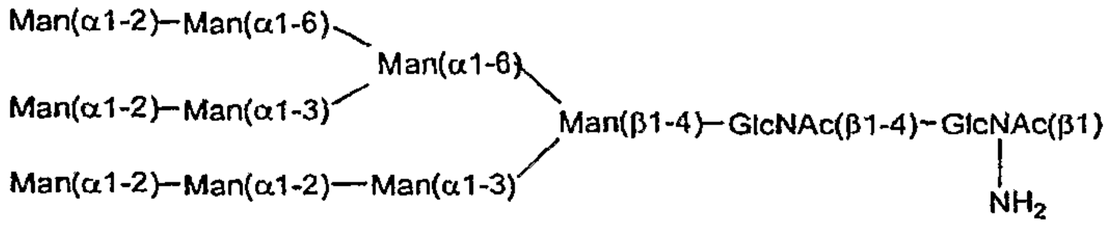


Scheme 2

In another embodiment, the carbohydrate component contains all or part of a carbohydrate antigen (typically a glycan) from a microorganism, preferably a pathogenic microorganism, such as a virus (e.g., a carbohydrate present on gp120, a glycoprotein derived from the HIV virus), a Gram-negative or Gram-positive bacterium (e.g., a carbohydrate derived from *Haemophilus influenzae*, *Streptococcus pneumoniae*, or *Neisseria meningitides*), a fungus (e.g., a 1,3-β-linked glucan) a parasitic protozoan (e.g., a GPI-anchor found in protozoan parasites such as *Leishmania* and *Trypanosoma brucei*), or a helminth. Preferably, the microorganism is a pathogenic microorganism.

An exemplary glycan from viral pathogens, Man9 from HIV-1 gp120, is shown in Scheme 3.

Man9 from HIV-1 gp120



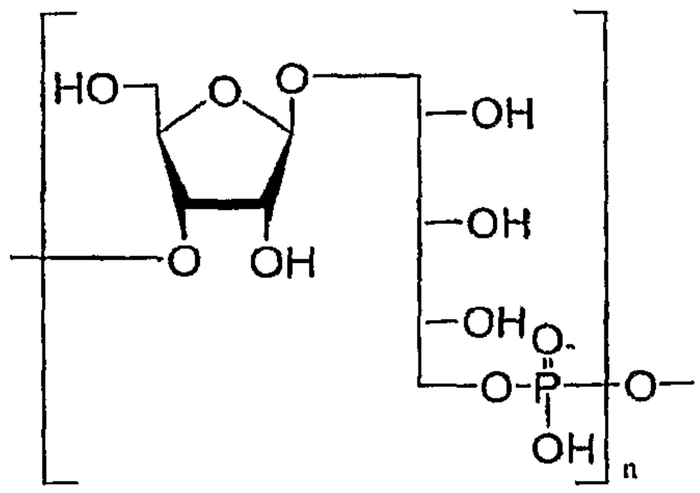
Scheme 3

Exemplary HIV carbohydrate and glycopeptide antigens are described in Wang et al., Current Opinion in Drug Disc. & Develop., 9(2): 194-206 (2006), and include both naturally occurring HIV carbohydrates and glycopeptides, as well as synthetic carbohydrates and glycopeptides based on naturally occurring HIV carbohydrates and glycopeptides.

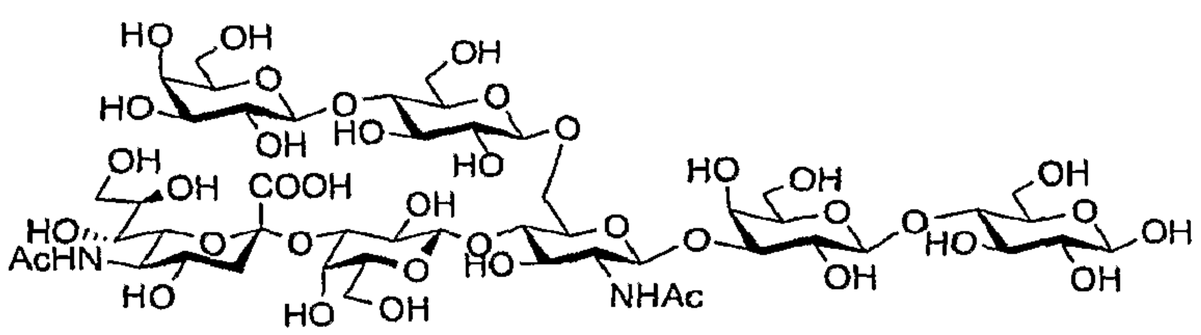
Exemplary HCV carbohydrate and glycopeptide antigens are described in Koppel et al. *Cellular Microbiology* 2005;*7*(2):157-165 and Goffard et al. *J. of Virology* 2005;*79*(13):8400-8409, and include both naturally occurring HCV carbohydrates and glycopeptides, as well as synthetic carbohydrates and glycopeptides based on naturally occurring HCV carbohydrates and glycopeptides.

Exemplary glycans from bacterial pathogens are shown in Scheme 4.

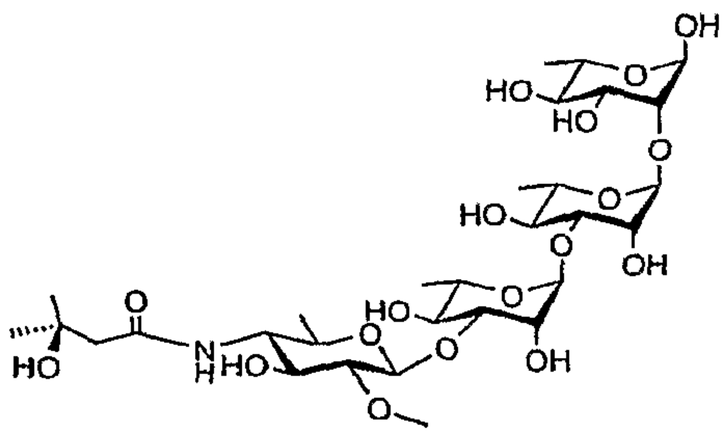
Haemophilus influenzae Type b, CPS repeating unit

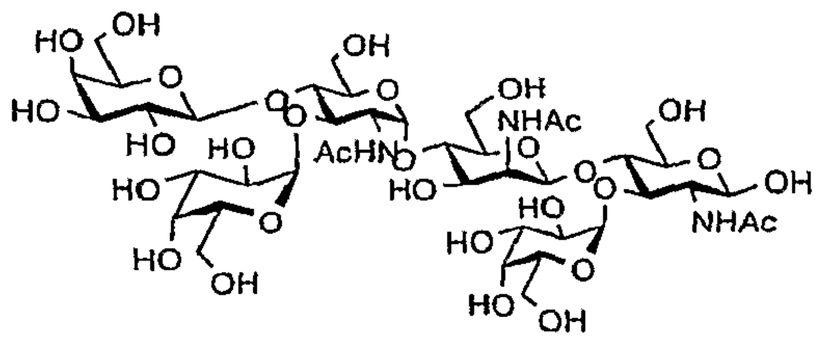


Group B type III *Streptococcus*

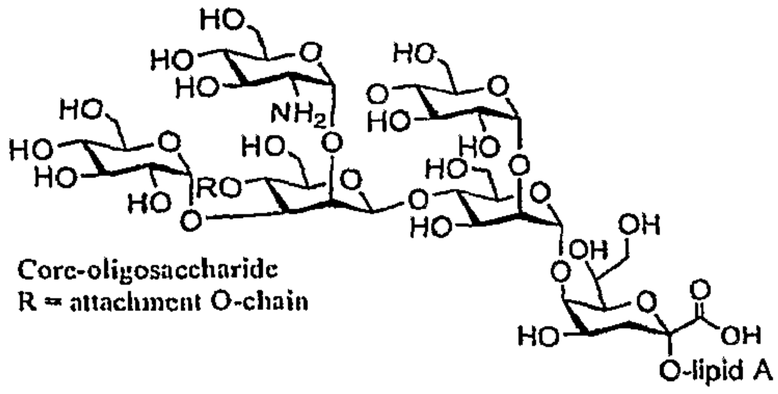


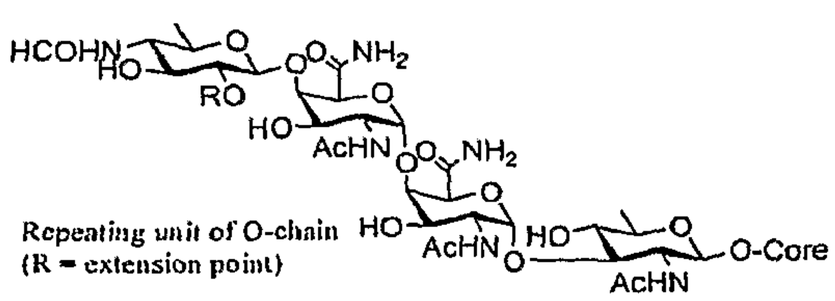
Cell-wall Carbohydrates from *Bacillus anthracis, Ames, Sterne, Pasteur*



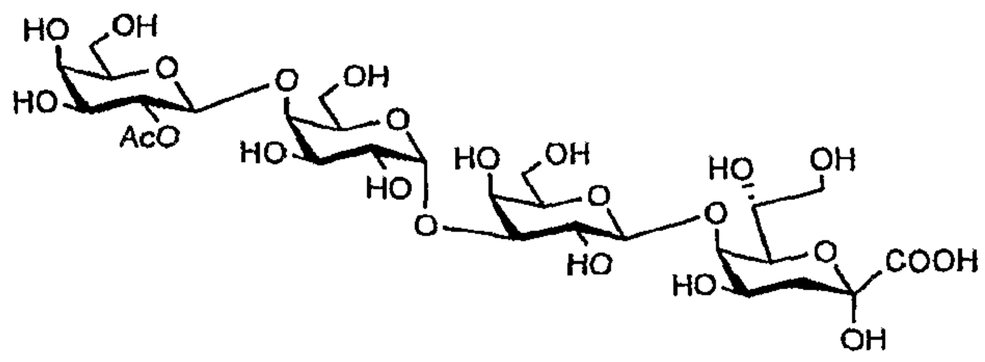


Francisella tularencis, Core region and O-side chain repeating unit





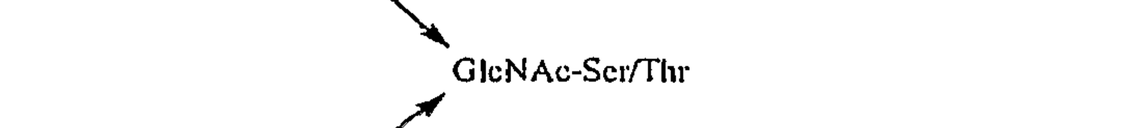
Burkholdaria pseudomallei, exo-polysaccharide



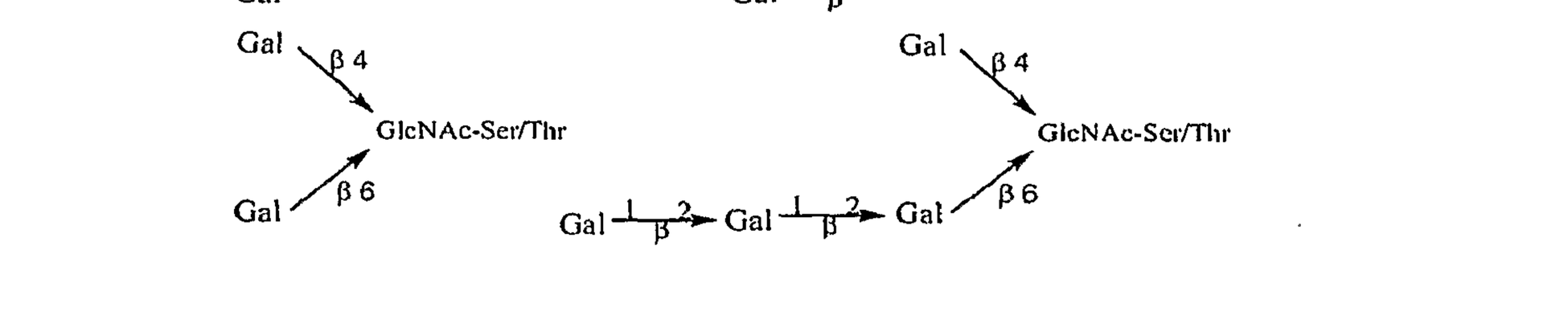
Scheme 4

Exemplary glycans from protozoan pathogens are shown in Scheme 5.

Plasmodium falsiparum, Malaria parasite



Leishmania species antigenic glycan

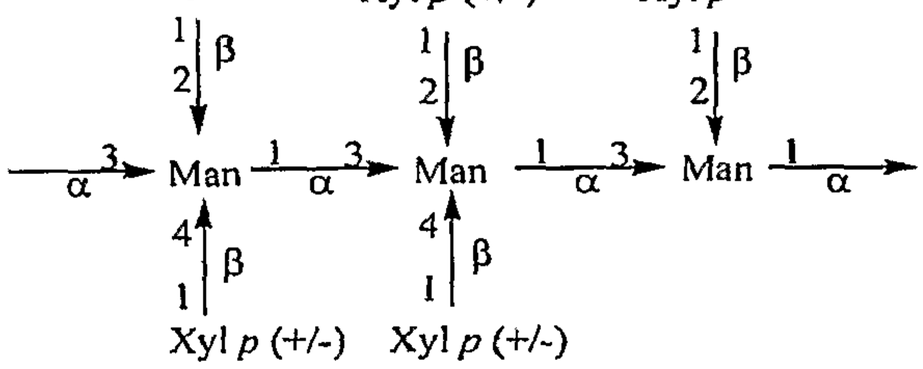


Trypanozoma cruzi

Scheme 5

An exemplary glycan from a fungal pathogen is shown in Scheme 6.

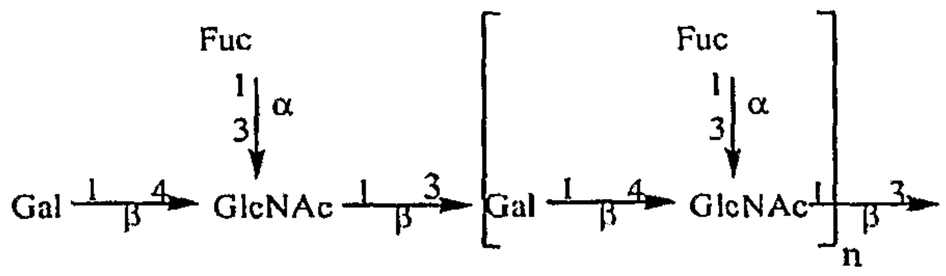
Cryptococcus neoformans Capsular polysaccharide



Scheme 6

An exemplary glycan from helminth pathogen is shown in Scheme 7.

Schistosoma



Scheme 7

It will be appreciated by one of skill in the art that while numerous antigenic carbohydrate structures are known, many more exist, since only a small fraction of the antigenic or immunogenic carbohydrates have been identified thus far. Examples of the many carbohydrate antigens discovered thus far can be found in Kuberan et al., Curr. Org. Chem, 4, 653-677 (2000); Ouerfelli et al., Expert Rev. Vaccines 4(5):677-685 (2005); Hakomori et al., Chem. Biol. 4, 97-104 (1997); Hakomori, Acta Anat. 161, 79-90 (1998); "The use of carbohydrate antigens for the preparation of vaccines for therapy in breast cancer," Drugs of Today 38(11):759-768 (2002); Mendonca-Previato et al., Curr Opin. Struct. Biol. 15(5):499-505 (2005); Jones, Anais da Academia Brasileira de Ciencias 77(2):293-324 (2005); Goldblatt, J. Med. Microbiol. 47(7):563-567 (1998); Diekman et al., Immunol. Rev., 171: 203-211, 1999; Nyame et al., Arch.

Biochem. Biophys., 426 (2): 182-200, 2004; Pier, Expert Rev. Vaccines, 4 (5): 645-656, 2005; Vliegenthart, FEBS Lett., 580 (12): 2945-2950, Sp. Iss., 2006; Ada et al., Clin. Microbiol. Inf., 9 (2): 79-85, 2003; Fox et al., J. Microbiol. Meth., 54 (2): 143-152, 2003; Barber et al., J. Reprod. Immunol., 46 (2): 103-124, 2000; and Sorensen, Persp. Drug Disc. Design, 5: 154-160, 1996. Any antigenic carbohydrate derived from a mammal or from an infectious organism can be used as the carbohydrate component of the glycolipopeptide of the invention, without limitation.

Peptide component

The peptide component of the glycolipopeptide includes a T-epitope, preferably a helper T epitope. The peptide component can be any peptide-containing structure, and can contain naturally occurring and/or non-naturally occurring amino acids and/or amino acid analogs (e.g., D-amino acids). Preferably peptide component contains less than about 20 amino acids and/or amino acid analogs. Examples of peptide components include the universal helper T peptide, QYIKANSKFIGITEL ("QYI") (SEQ ID NO:1), the universal helper T peptide YAFKYARHANVGRNAFELFL ("YAF") (SEQ ID NO:2), the murine helper T peptide KLFAVWKITYKDT ("KLF") (SEQ ID NO:3) derived from polio virus, and pan-DR binding (PADRE) peptides (PCT WO 95/07707; Alexander et al., Immunity 1:751-761 (1994); Alexander et al., J. Immunol. 2000 Feb 1;164(3):1625-33; U.S Pat. No. 6,413,935 (Sette et al., July 2, 2002)).

Preferred immunogenic peptide components for use in the glycolipopeptide of the invention include universal (degenerate or "promiscuous") helper T-cell peptides, which are peptides that are immunogenic in individuals of many major histocompatibility complex (MHC) haplotypes. Numerous universal helper T-cell peptide structures are known; however, it should be understood that additional universal T-epitopes, including some with similar or even higher potency, will be identified in the future, and such peptides are well-suited for use as the peptide component the glycolipopeptide of the invention.

Exemplary T-cell peptides for use in the glycolipopeotide include, without limitation:

Synthetic, nonnatural PADRE peptide, DAla-Lys-Cha-Val-Ala-Ala-Trp-Thr-Leu-Lys-Ala-Ala-DAla, including all the analogues described by J Alexander et al. in Immunity, Vol. 1, 751-761, 1994;

Peptides derived from tetanus toxin, e.g., (TT830-843) QYIKANSKFIGITEL (SEQ ID NO:1), (TT1084-1099) VSIDKFRIFCKANPK (SEQ ID NO:4), (TT1174-1189) LKFIIKRYTPNNEIDS (SEQ ID NO:5), (TT1064-1079) IREDNNITLKLDRCNN (SEQ ID NO:6), and (TT947-967) FNNFTVSFWLRVPKVSASHLE (SEQ ID NO:7);

Peptides derived from polio virus, e.g., KLFAVWKITYKDT (SEQ ID NO:3);

Peptides derived from *Neisseria meningitidis*, e.g., YAFKYARHANVGRNAFELFL (SEQ ID NO:2); and

Peptides derived from *P. falsiparum* CSP, e.g., EKKIAKMEKASSVFNVNN (SEQ ID NO:8)

The peptide component of the glycolipopeptide contains a T-epitope. A T-epitope is an epitope recognized by a T cell. The T-epitope can elicit a CD4+ response, thereby stimulating the production of helper T cells; and/or it can elicit a CD8+ response, thereby stimulating the production of cytotoxic lymphocytes. Preferably, the T-epitope is an epitope that stimulates the production of helper T cells (i.e., a helper T-cell epitope or Th-epitope), which in turn make possible a humoral response to the B-epitope supplied by the carbohydrate component of the glycolipopeptide of the invention.

It should be understood that the glycolipopeptide of the invention can contain multiple T-epitopes, which may be the same or different. Further, T-epitopes may be present on the carbohydrate component and/or the lipid component (e.g., in embodiments that include glycopeptides and/or glycolipids as the carbohydrate and/or lipid components) in addition to, or in place of, the peptide component.

In one embodiment, the B-epitopes and the T-epitopes are homologous; that is, they are derived from the same organism, sometimes even the same biomolecule. For example, in a glycolipopeptide potentially suitable for use as a vaccine

against a microbial pathogen, the T-epitope in addition to the B-epitope may be epitopes that are present in the microbial pathogen. In another embodiment, the B-epitopes and the T-epitopes are heterologous; that is, they are not derived from the same organism. For example, a glycolipopeptide potentially suitable for use as an anti-cancer vaccine may have a B-cell epitope from a cancer cell, but a T-cell epitope from a bacterium or virus.

Lipid component

It was originally postulated that a glycopeptide having just two main components, i.e., a carbohydrate component and a peptide component, would be effective to elicit an immune response in an animal. The helper T-cell epitope was expected to induce a T-cell dependent immune response, resulting in the production of IgG antibodies against a tumor-related carbohydrate B-epitope such as Le^y and Tn. However, in some applications, the two component vaccine was not found to be very effective. It was postulated that the B-cell and helper T-cell epitopes lack the ability to provide appropriate "danger signals" for dendritic cell (DC) maturation. In the present disclosure, a lipid component was included in the compound, resulting in the glycolipopeptide of the invention.

The lipid component can be any lipid-containing component, such as a lipopeptide, fatty acid, phospholipid, steroid, or a lipidated amino acids and glycolipids such as Lipid A derivatives. Preferably, the lipid component is non-antigenic; that is, it does not elicit antibodies directed against specific regions of the lipid component. However, the lipid component may potentially and preferably does serve as an immunoadjuvant. The lipid component may potentially serve as a carrier or delivery system for the multi-epitopic glycolipopeotide. It may potentially assist with incorporation of the glycolipopeptide into a vesicle or liposome to facilitate delivery of the glycolipopeptide to a target cell, and it may potentially enhance uptake by target cells, such as dendritic cells. Further, the lipid component may potentially stimulate the production of cytokines.

One class of preferred lipid components for use in the glycolipopeptide of the invention comprises naturally occurring and synthetic molecular ligands of the various Toll-like receptors (TLRs). There are many known subclasses of Toll-like receptors (e.g., TLR1, TLR2, TRL3, TLR4, TLR5, TLR6, TLR7,

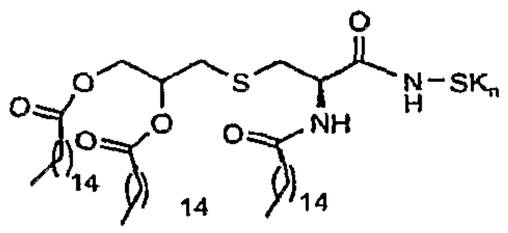
TLR8, TLR9, TLR10, TLR11, TLR12, TLR13, TLR14, TLR15 and TLR16). See Roach et al., PNAS 2005,102:9577-9582, for a review of the relationships between and evolution of Toll-like receptors; and Duin et al., TRENDS Immunol., 2006, 27:49-55, for a discussion of TLR signaling in vaccination. Particularly preferred are lipid components that interact with TLR2 and TLR4. TLR2 is involved in the recognition of a wide array of microbial molecules from Gram–positive and Gram–negative bacteria, as well as mycoplasma and yeast. TLR2 ligands include lipoglycans, lipopolysaccahrides, lipoteichoic acids and peptidoglycans. TLR4 recognizes Gram-negative lipopolysaccharide (LPS) and lipid A, its toxic moiety. TLR ligands are widely available commercially, for example from Apotech and InvivoGen. Preferably, the lipid component is a TLR ligand that facilitates uptake of the glycolipopeptide by antigen presenting cells (see Example III).

Suitable lipids for use as the lipid component of the glycolipopeptide of the invention include PamCys-type lipid structures, such as those derived from Pam_3Cys (*S*-[(*R*) -2, 3-dipalmitoyloxy-propyl]-*N*-palmitoyl-(*R*) - cysteine) and Pam_2Cys (*S*-[(*R*) -2, 3-dipalmitoyloxy-propyl]-(*R*) - cysteine), which lacks the N-palmitoyl group of Pam_3Cys. Pam_3Cys and Pam_2Cys are derived from the immunologically active N-terminal sequence of the principal lipoprotein of *Escherichia coli*. This class of lipids also includes Pam_3CysSK_4 (*N*-palmitoyl-*S*-[(*R*)-2,3-bis(palmitoyloxy)-propyl]-(*R*)–cysteinyl-(*S*)-seryl-(*S*)-lysine-(*S*)-lysine-(*S*)-lysine-(*S*)-lysine-(*S*)-lysyne) and Pam_2CysSK_4 (*S*-[(*R*)-2,3-bis(palmitoyloxy)-propyl]-(*R*)–cysteinyl-(*S*)-seryl-(*S*)-lysine-(*S*)-lysine-(*S*)-lysine-(*S*)-lysyne), which lacks the N-palmitoyl group of $Pam_3CysSK4$; it should be understood that the number of lysines in these structures can be 0, 1, 2, 3, 4, 5 or more (i.e., K_n where n = 0, 1, 2, 3, 4, 5 or more).

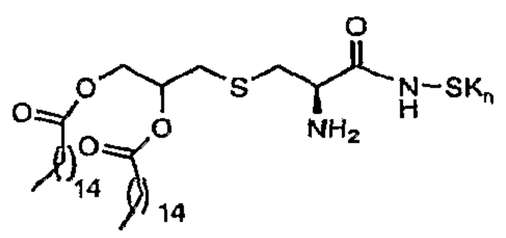
Another preferred class of lipids includes Lipid A (LpA) type lipids, including Lipid A analogs and derivatives. Exemplary Lipid As are derived from *E. coli, S. typhimurium* and *Neisseria meningitidis*. The Lipid As can be attached to the carbohydrate component (containing a B-epitope) of the glycolipopeptide and/or to the peptide component (containing a T-epitope) through a linker that is connected, for example, to the anomeric center or anomeric phosphate, the C-4' phosphate or the C-6' position. The phosphates

can be modified, for example, to include one or more phosphate ethanolamine diesters. Exemplary Lipid A derivatives are described in, for example, Caroff et al., Microbes Infect. 4, 915-926 (2002); Raetz et al., Annu. Rev. Biochem. 71, 635-700 (2002); and Dixon et al., J. Dent. Res. 84, 584-595 (2005).

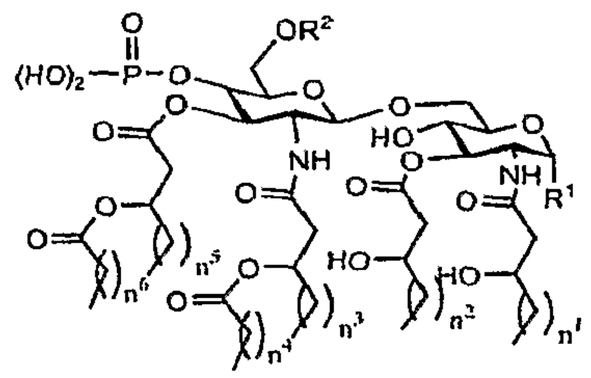
Below, in Scheme 8, are exemplary immunostimulatory lipids for the incorporation into the glycolipopeptide of the invention. The first structure in the first row is Pam_3CysSK_n; the second structure in the first row is Pam_2CysSK_n; and the last 4 structures are Lipid A derivatives.



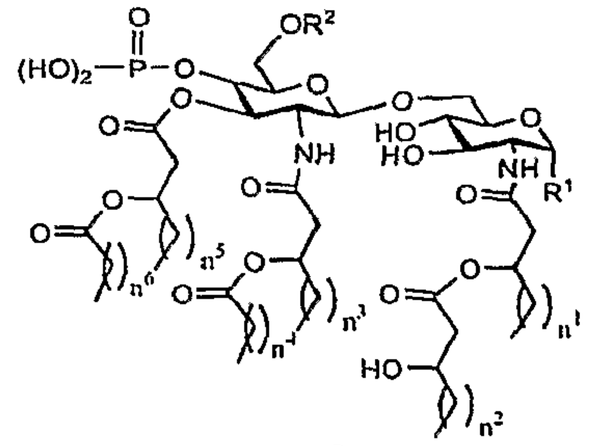
n = 0, 1, 2, 3, 4, 5



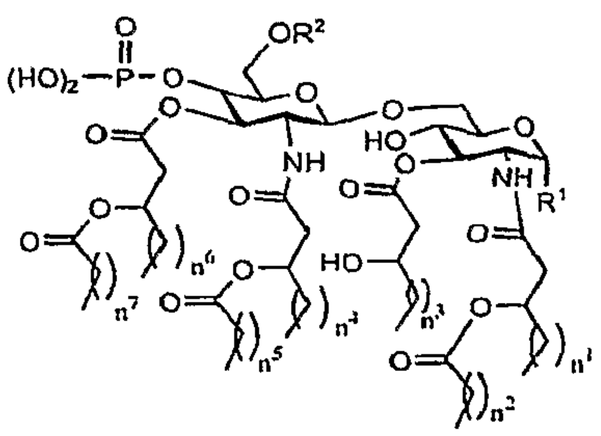
n = 0, 1, 2, 3, 4, 5



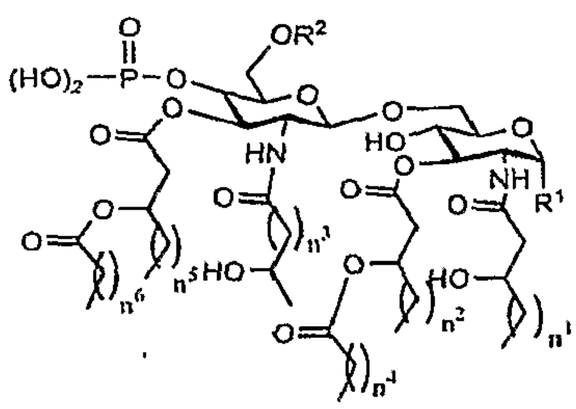
$R^1 = OP(O)(OH)_2$, or $R^1 = H$; $R^2 = H$ or KDO
$n^1, n^2, n^3, n^4, n^5, n^6 = 8, 9, 10, 11, 12, 13, 14, 15, 16, 17$ or 18



$R = OP(O)(OH)_2$, or $R = H$; $R^2 = H$ or KDO
$n^1, n^2, n^3, n^4, n^5, n^6 = 8, 9, 10, 11, 12, 13, 14, 15, 16, 17$ or 18



$R = OP(O)(OH)_2$, or $R = H$; $R^2 = H$ or KDO
$n^1, n^2, n^3, n^4, n^5, n^6, n^7 = 8, 9, 10, 11, 12, 13, 14, 15, 16, 17$ or 18



$R = OP(O)(OH)_2$, or $R = H$; $R^2 = H$ or KDO
$n^1, n^2, n^3, n^4, n^5, n^6 = 8, 9, 10, 11, 12, 13, 14, 15, 16, 17$ or 18

Scheme 8

Lipids that are structurally based on Pam_3Cys are particularly preferred for use as the lipid component. Pam_3Cys is derived from the immunologically active *N*-terminal sequence of the principal lipoprotein of *Escherichia coli*.

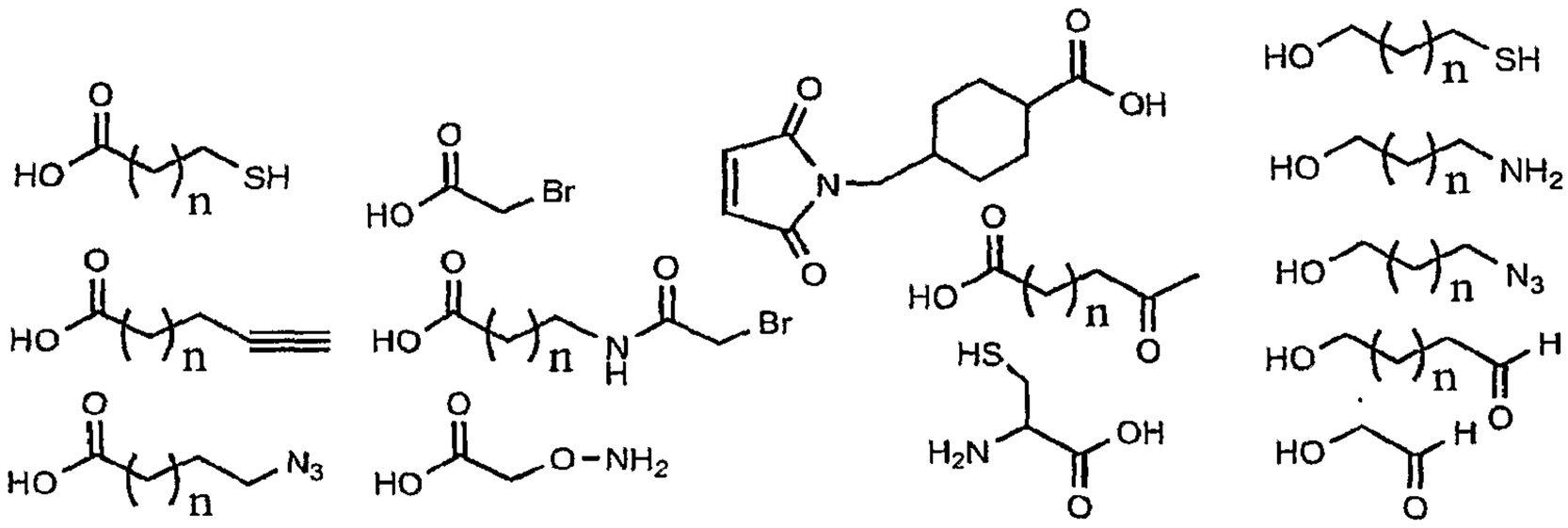
These lipopeptides are powerful immunoadjuvants. Recent studies have shown that Pam_3Cys exerts its activity through the interaction with Toll-like receptor-2 (TLR2).

Interaction between the lipid component and a TLR results in the production of pro-inflammatory cytokines and chemokines, which, in turn, stimulates antigen-presenting cells (APCs), and thus, initiating helper T cell development and activation. The lipopeptide also facilitates the incorporation of the glycolipopeptide into liposomes. Liposomes have attracted interest as vectors in vaccine design due to their low intrinsic immunogenicity, thus, avoiding undesirable carrier-induced immune responses.

Optional linker

One or more linkers ("L") are optionally used for assembly of the three components of the glycolipopeptide. In one embodiment, the linker is a bifunctional linker that has functional groups in two different places, preferably at a first and second end, in order to covalently link two of the three components together. A bifunctional linker can be either homofunctional (i.e., containing two identical functional groups) or heterofunctional (i.e., containing two different functional groups). In another embodiment, the linker is trifunctional (hetero- or homo-) and can link all three components of the glycolipopeptide together. A suitable functional group has reactivity toward or comprises any of the following: amino, alcohol, carboxylic acid, sulfhydryl, alkene, alkyne, azide, thioester, ketone, aldehyde, or hydrazine. An amino acid, e.g., cysteine, can constitute a linker.

Bifunctional linkers are exemplified in Scheme 9.



n = 0, 1, 2, 3...

Scheme 9

Fig. 1 shows an exemplary fully synthetic glycolipopeptide of the invention containing a carbohydrate-based B-epitope, a peptide T-epitope and a lipopeptide. The compound shown in Fig. 1 contains a L-glycero-D-manno-heptose sugar that acts as a B-epitope, the peptide sequence YAFKYARHANVGRNAFELFL (SEQ ID NO:2) that has been identified as a MHC class II restricted recognition site for human T-cells and is derived from an outer-membrane protein of *Neisseria meningitidis*, and the lipopeptide S-2-3[dipalmitoyloxy]-(R/S)-propyl-N-palmitoyl-R-Cysteine (Pam_3Cys). As noted elsewhere herein, lipopeptide Pam_3Cys and the related compound Pam_3CysSK_4 are highly potent B-cell and macrophage activators.

Methods of making the glycolipopeptide, as exemplified in the Examples, are also encompassed by the invention. Preferably, the method for making the glycolipopeptide utilizes chemical synthesis, resulting in a fully synthetic glycolipopeptide. In embodiments that make use of one or more linkers, the optional linker component is functionalized so as to facilitate covalent linkage of one of the main components to another of the main components. For example, the linker can be functionalized with at each end with a thiol-reactive group, such as maleimide or bromoacetyl, and the components to be joined are modified to include reactive thiols. Other options for ligation chemistry include Native Chemical Ligation, the Staudinger Ligation

and Huisgen ligation (also known as "Click Chemistry"). Example II illustrates how the carbohydrate component, in that case an oligosaccharide, and the peptide component can be functionalized with a thiol-containing linker. Preferably, the linker component, if used, is nonantigenic.

The glycolipopeptide of the invention is capable of generating an immune response in a mammal. The glycolipopeptide is antigenic, in that it can generate a humoral response, resulting in the activation of B cells and production of antibodies (immunoglobulins) such as IgM. Additionally, the glycolipopeptide is immunogenic, in that it can generate a cellular response; for example, it facilitates the activation of T cells, particularly helper T cells which are also instrumental in the generation of a more complex antibody response that includes the production of IgG. Ultimately, the immune response elicited in the animal includes the production of anti-carbohydrate antibodies.

Because it is antigenic and immunogenic, the glycolipopeptide of the invention is well-suited for use in a pharmaceutical composition. The invention thus includes pharmaceutical compositions that include a glycolipopeptide of the invention as well as a pharmaceutically acceptable carrier. In a preferred embodiment, the pharmaceutical composition contains liposomes, for example phospholipid-based liposomes, and the glycolipopeptide is incorporated into liposomes as a result of noncovalent interactions such as hydrophobic interactions. Alternatively, the glycolipopeptide can be covalently linked to a component of the liposome. The liposome formulation can include glyocolipopeptides that have the same or different B-epitopes; the same or different T-cell epitopes; and/or the same or different lipid components.

The glycolipopeptide of the invention may potentially be formulated as a pharmaceutical composition for veterinary or human use. The pharmaceutical composition optionally includes excipients or diluents that are pharmaceutically acceptable as carriers and compatible with the glycolipopeptide. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other ingredients of a composition and not deleterious to the recipient thereof or to the live attenuated virus. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol, or the

like and combinations thereof. In addition, if desired, the pharmaceutical composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, salts, and/or adjuvants which enhance the effectiveness of the immune-stimulating composition. For oral administration, the glycolipopeptide can be mixed with proteins or oils of vegetable or animal origin. Methods of making and using such pharmaceutical compositions are also included in the invention.

The pharmaceutical composition of the invention may potentially be administered to any subject including humans and domesticated animals (e.g., cats and dogs). In a preferred embodiment, the pharmaceutical composition may potentially be useful as a vaccine and contains an amount of glycolipopeotide effective to induce an immune response in a subject. Dosage amounts, schedules for vaccination and the like for the glycolipopeptide vaccine of the invention are readily determinable by those of skill in the art. The vaccine can be administered to the subject using any convenient method, preferably parenterally (e.g., via intramuscular, intradermal, or subcutaneous injection) or via oral or nasal administration. The potentially useful dosage to be administered will vary, depending on the type of animal to be vaccinated, its age and weight, the immunogenicity of the attenuated virus, and mode of administration.

Inclusion of an adjuvant, such as alum or QS-21 , in the pharmaceutical composition is optional. However, it has been found that as long as the three main components of the glycolipopeptide are covalently linked, an adjuvant is not needed in order to effectively generate an immune response in an animal. Moreover, the inclusion of QS-21 may skew the immune response, resulting in a change in the relative amounts Th1 and Th2 T cells produced (see Example III). QS-21 can be included as an adjuvant in the pharmaceutical composition when, for example, a shift toward a Th1 response is desired, as opposed to a bias toward a Th2 response that is observed in the absence of QS-21.

As noted, the pharmaceutical composition may potentially be useful as a vaccine. The vaccine may potentially be a prophylactic or protective vaccine, administered before or after contact with a pathogen but prior to the development of infection or disease. Likewise, the vaccine may potentially be a therapeutic vaccine, administered after infection

with a pathogen, or the development of a disease or disorder such as cancer, precancerous conditions, or autoimmune disease. Thus, in an embodiment, vaccines that include a glycolipopeptide as described herein, including antimicrobial (e.g., anti-viral or anti-bacterial) and anti-cancer vaccines, may potentially be encompassed by the present invention. Cancers that may potentially be effectively treated or prevented include, but are not limited to, prostate cancer, bladder cancer, colon cancer and breast cancer.

The glycolipopeptide of the invention may potentially also be used in passive immunization methods. For example, the glycolipopeptide can be administered to a host animal such as a rabbit, mouse, rat, chicken or goat to generate antibody production in the host animal. Protocols for raising polyclonal antibodies in host animals are well known. The T-epitope or T-epitopes included in the glycolipopeptide optionally are selected to be the same as or similar to the corresponding T-epitope of the host animal in which the antibody is raised. The antibodies are isolated from the animal, then may potentially be administered to a mammalian subject, preferably a human subject, prophylactically or therapeutically to potentially treat or prevent disease or infection. Monoclonal antibodies against the glycolipopeptide of the invention can be isolated from hybridomas prepared in accordance with standard laboratory protocols; they can also be produced using recombinant techniques such as phage display. Such antibodies may potentially also be useful for passive immunization. Optionally, the anti-glycolipopeptide monoclonal antibodies are human antibodies or humanized antibodies. The B-epitope or B-epitopes included in the glycolipopeptide used to create the polyclonal or monoclonal antibodies is selected with reference to the potential purpose of treatment. The invention encompasses polyclonal and monoclonal anti-glycolipopeptide antibodies, as well as methods for making and using them.

Finally, as noted elsewhere herein, it has been surprisingly found that covalent attachment of a Toll-like receptor (TLR) ligand to a glycopeptide comprising a carbohydrate component (containing a B epitope) and a peptide component (containing a T-epitope) enhances uptake and internalization of the glycopeptide by a target cell (see Example III). TLR ligands thus identified that are characterized as lipids are preferred lipid components for use in the glycolipopeptide of the invention. The invention thus further provides a method for identifying TLR ligands, preferably lipid ligands, that includes contacting a candidate compound with a target cell containing a Toll-like receptor (TLR), and

determining whether the candidate compound binds to the TLR (i.e., is a TLR ligand). Preferably, the candidate compound is internalized by the target cell through the TLR. Lipid-containing TLR ligands identified by binding to a TLR and, optionally, by internalization into the target cell are expected to be immunostimulatory and are well-suited for use as the lipid component of the glycolipopeptide of the invention. The invention therefore also encompasses glycolipopeptides which include, as the lipid component(s), one or more lipid-containing TLR ligands identified using the method of the invention.

EXAMPLES

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope of the invention as set forth herein.

Example I. Towards a Fully Synthetic Carbohydrate-based Anti-cancer Vaccine: Synthesis and Immunological Evaluation of a Lipidated Glycopeptide Containing the Tumor-associated Tn-antigen.

In this Example, a fully synthetic candidate cancer vaccine, composed of a tumor associated Tn-antigen, a peptide T-epitope and the lipopeptide Pam_3Cys was prepared by a combination of polymer-supported and solution phase chemistry. Incorporation of the glycolipopeptide into liposomes gave a formulation that was able to elicit a T-cell dependent antibody response in mice.

A common feature of oncogenic transformed cells is the over-expression of oligosaccharides, such as Globo-H, $Lewis^Y$, and Tn antigens (Lloyd, Am. J Clin. Pathol. 1987, 87, 129; Feizi et al., Trends in Biochem. Sci. 1985, 10, 24-29; Springer, J. Mol. Med. 1997, 75, 594-602; Hakomori, Acta Anat. 1998, 161, 79-90). Numerous studies have shown that this abnormal glycosylation can promote metastasis (Sanders et al., Mol. Pathol. 1999, 52, 174-178) and hence its expression is strongly correlated with poor survival rates of cancer patients.

Several elegant studies have exploited the differential expression of tumor-associated carbohydrates for the development of cancer vaccines (Ragupathi, Cancer Immunol. 1996, 43, 152-157; Musselli et al., J Cancer Res. Clin. Oncol. 2001, 127, R20-R26). The inability of carbohydrates to activate helper T-lymphocytes has complicated, however, their use as vaccines Kuberan et al., Current Organic Chemistry 2000, 4, 653-677). For most immunogens, including carbohydrates, antibody production depends on the cooperative interaction of two types of lymphocytes, B-cells and helper T-cells (Jennings et al., Neoglycoconjugates, preparation and application, Academic, San Diego, 1994). Saccharides alone cannot activate helper T-cells and therefore have a limited immunogenicity. The formation of low affinity IgM antibodies and the absence of IgG antibodies manifest this limited immunogenicity.

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 (*e.g.* Keyhole Limpet Hemocyanin (KLH) detoxified tetanus toxoid). 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.

However, the conjugation of carbohydrates to a carrier protein poses several problems. In general, the conjugation chemistry is difficult to control, resulting in conjugates with ambiguities in composition and structure, which may affect the reproducibility of an immune response (Anderson et al., J. Immunol. 1989, 142, 2464-2468). In addition, the foreign carrier protein can elicit a strong B-cell response, which may lead to the suppression of an antibody response against the carbohydrate epitope. The latter is a greater problem when self-antigens are employed such as tumor-associated carbohydrates. Also linkers for the conjugation of carbohydrates to proteins can be immunogenic, leading to epitope suppression (Buskas et al., Chem. Eur. J. 2004, 10, 3517-3523). Not surprisingly, several clinical trials with carbohydrate-protein conjugate cancer vaccines failed to induce sufficiently strong helper T-cell responses in all patients (Sabbatini et al., Int. J. Cancer 2000, 87, 79-85). Therefore, alternative strategies need to be developed for the presentation of tumor associated carbohydrate epitopes that will result in a more efficient class switch to IgG

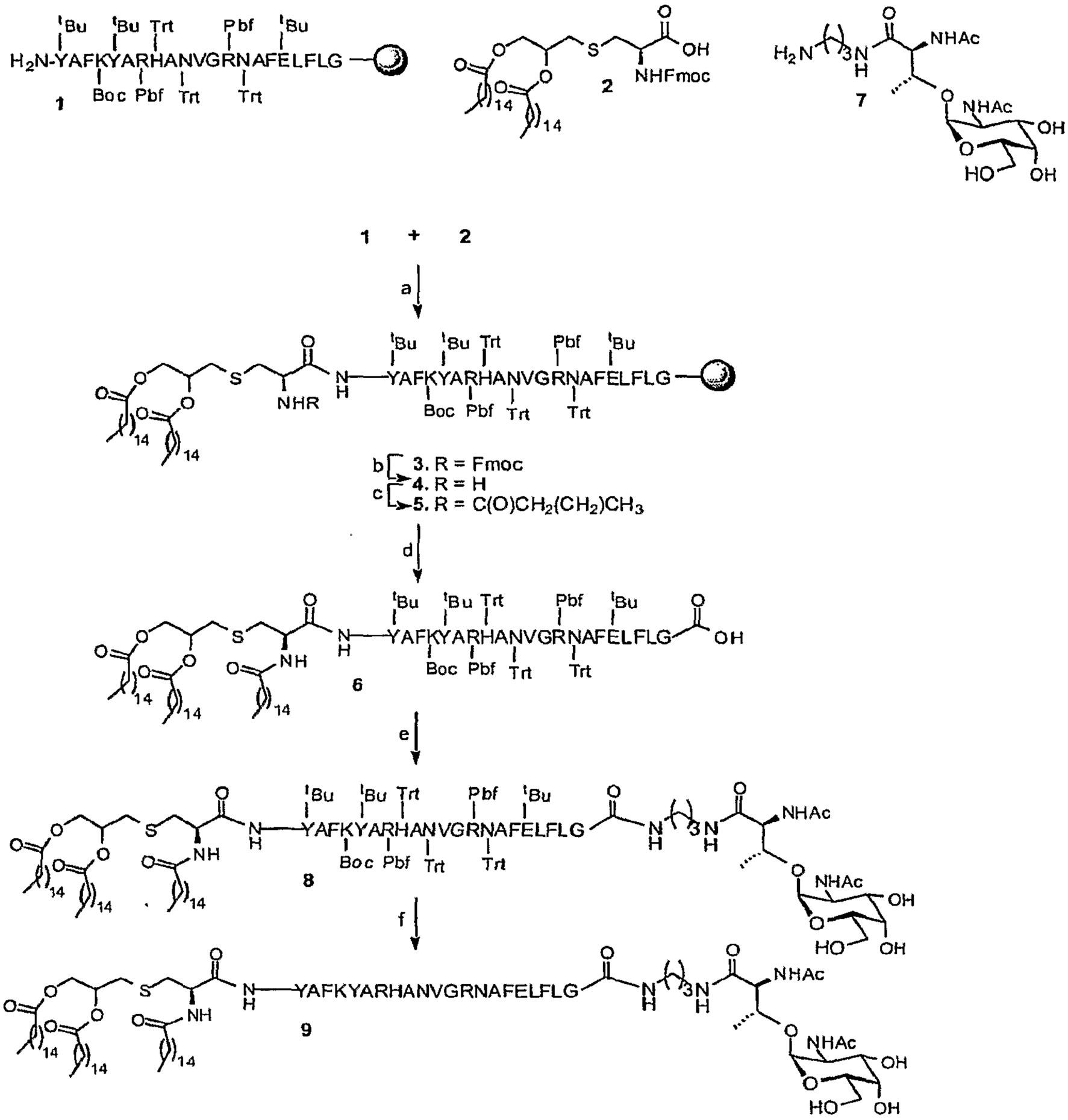
antibodies (Keil et al., Angew. Chem. Int. Ed. 2001, 40, 366-369; Angew. Chem. 2001, 113, 379-382; Toyokuni et al., Bioorg. & Med. Chem. 1994, 2, 1119-1132; Lo-Man et al., Cancer Res. 2004, 64, 4987-4994; Kagan et al., Cancer Immunol. Immunother. 2005, 54, 424-430; Reichel et al., Chem. Commun. 1997, 21, 2087-2088).

Here we report the synthesis and immunological evaluation of a structurally well-defined fully synthetic anti-cancer vaccine candidate (compound **9**) that constitutes the minimal structural features required for a focused and effective T-cell dependent immune response. The vaccine candidate is composed of the tumor-associated Tn-antigen, the peptide T-epitope YAFKYARHANVGRNAFELFL (YAF) (SEQ ID NO:2), and the lipopeptide S-[(R)-2,3-dipalmitoyloxy-propyl]-*N*-palmitoyl-(R)-cysteine (Pam_3Cys). The Tn-antigen, which will serve as a B-epitope, is over-expressed on the surface of human epithelial tumor-cells of breast, colon, and prostate. This antigen is not present on normal cells, and thus rendering it an excellent target for immunotherapy. To overcome the T-cell independent properties of the carbohydrate antigen, the YAF peptide was incorporated. This 20 amino acid peptide sequence is derived from an outer-membrane protein of *Neisseria meningitides* and has been identified as a MHC class II restricted site for human T-cells (Wiertz et al., J. Exp. Med. 1992, 176, 79-88). It was envisaged that this helper T-cell epitope would induce a T-cell dependent immune response resulting in the production of IgG antibodies against the Tn-antigen. The combined B-cell and helper T-cell epitope lacks the ability to provide appropriate "danger signals" (Medzhitov et al., Science 2002, 296, 298-300) for dendritic cell (DC) maturation. Therefore, the lipopeptide Pam_3Cys, which is derived from the immunologically active *N*-terminal sequence of the principal lipoprotein of *Escherichia coli* (Braun, Biochim. Biophys. Acta 1975, 415, 335-377), was incorporated. This lipopeptide has been recognized as a powerful immunoadjuvant (Weismuller et al., Physiol. Chem. 1983, 364, 593) and recent studies have shown that it exerts its activity through the interaction with Toll-like receptor-2 (TLR-2) (Aliprantis et al., Science 1999, 285, 736-73). This interaction results in the production of pro-inflammatory cytokines and chemokines, which, in turn, stimulates antigen-presenting cells (APCs), and

thus, initiating helper T cell development and activation (Werling et al., Vet. Immunol. Immunopathol. 2003, 91, 1-12). The lipopeptide also facilitates the incorporation of the antigen into liposomes. Liposomes have attracted interest as vectors in vaccine design (Kersten et al., Biochim. Biophys. Acta 1995, 1241, 117-138) due to their low intrinsic immunogenicity, thus, avoiding undesirable carrier-induced immune responses.

The synthesis of target compound **9** requires a highly convergent synthetic strategy employing chemical manipulations that are compatible with the presence of a carbohydrate, peptide and lipid moiety. It was envisaged that **9** could be prepared from spacer containing Tn-antigen **7**, polymer-bound peptide **1**, and *S*-[2,3-bis(palmitoyloxy)propyl]-*N*-Fmoc-Cys (Pam_2FmocCys, **2**, (Metzger et al., Int. J. Peptide Protein Res. 1991, 38, 545-554)). The resin-bound peptide **1** was assembled by automated solid-phase peptide synthesis using Fmoc protected amino acids in combination with the hyper acid-sensitive HMPB-MBHA resin and 2-(1H-benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole (HBTU/HOBt) (Knorr et al., Tetrahedron Lett. 1989, 30, 1927-1930) as the activation cocktail (Scheme 10). The HMPB-MBHA resin was selected because it allows the cleavage of a compound from the resin without concomitant removal of side-chain protecting groups. This feature was important because side-chain functional groups of aspartic acid, glutamic acid and lysine would otherwise interfere with the incorporation of the Tn-antigen derivative **7**. Next, the Pam_2FmocCys derivative **2** was manually coupled to the N-terminal amine of peptide **1** using PyBOP (Martinez et al., J. Med. Chem. 1988, 28, 1874-1879) and HOBt in the presence of DIPEA in a mixture of DMF and dichloromethane to give the resin-bound lipopeptide **3**. The Fmoc group of **3** was removed under standard conditions and the free amine of the resulting compound **4** was coupled with palmitic acid in the presence of PyBOP and HOBt to give the fully protected and resin-bound lipopeptide **5**. The amine of the Pam_2Cys moiety was palmitoylated after coupling with **1** to avoid racemization of the cysteine moiety. Cleavage of compound **5** from the resin was achieved with 2% TFA in dichloromethane followed by the immediate neutralization with 5% pyridine in methanol. After purification by LH-20 size exclusion chromatography, the C-

terminal carboxylic acid of lipopeptide **6** was coupled with the amine of Tn-derivative **7**, employing DIC/HOAt/DIPEA (Carpino, J. Am. Chem. Soc 1993, 115, 4397-4398) as coupling reagents to give, after purification by Sephadex LH-20 size-exclusion chromatography, fully protected lipidated glycopeptide **8** in a yield of 79%. Mass spectrometric analysis by MALDI-TOF showed signals at *m/z* 5239.6 and 5263.0, corresponding to $[M+H]^+$ and $[M+Na]^+$, respectively. Finally, the side-chain protecting groups of **8** were removed by treatment with 95% TFA in water using 1,2-ethanedithiol (EDT) as a scavenger. It was found that the alternative use of triisopropyl silane (TIS) resulted in the formation of unidentified by-products. The target compound **9** was purified by size-exclusion chromatography followed by RP-HPLC using a Synchropak C4 column. MALDI mass analysis of **9** showed a signal at m/z 3760.3 corresponding to $[M+Na]^+$.



Scheme 10. a) PyBOP, HOBt, DIPEA, DMF/DCM (5/1, v/v); b) piperidine/DMF (1/5, v/v); c) $CH_3(CH_2)_{14}COOH$, PyBOP, HOBt, DMF/DCM (1/5, v/v); d) 2% TFA in DCM; e) 7, DIC, HOAt, DIPEA, DMF/DCM (2/1, v/v), 79%; f) TFA/H_2O/EDT (95/2.5/2.5, v/v/v), 79%.

Next, the compound **9** was incorporated into phospholipid-based liposomes. Thus, after hydration of a lipid-film containing **9**, cholesterol, phosphatidylcholine and phosphatidylethanolamine, small uni-lamellar vesicles (SUVs) were prepared by extrusion through 100 nm Nuclepore® polycarbonate membranes. Transmission electron microscopy (TEM) by negative stain

confirmed that the liposomes were uniformly sized with an expected diameter of approximately 100 nm (see Figure 1 of Buskas et al., Angew. Chem. Int. Ed. 2005, 44, 5985-5988). The liposome preparations were analyzed for *N*-acetyl galactosamine content by hydrolysis with TFA followed by quantification by high pH anion exchange chromatography. Concentrations of approximately 30 μg/mL of GalNAc were determined, which corresponded to an incorporation of approximately 10% of the starting compound **9**.

Groups of five female BALB/c mice were immunized subcutaneously at weekly intervals with freshly prepared liposomes containing 0.6 μg carbohydrate. To explore the adjuvant properties of the built-in lipopeptide Pam_3Cys, the antigen-containing liposomes were administered with or without the potent saponin immuno-adjuvant QS-21 (Antigenics Inc., Lexington, MA). Anti-Tn antibody titers were determined by coating microtiter plates with a BSA-Tn conjugate and detection was accomplished with anti-mouse IgM or IgG antibodies labeled with alkaline phosphatase. As can be seen in Table 1, the mice immunized with the liposome preparations elicited IgM and IgG antibodies against the Tn-antigen (Table 1, entries 1 and 2). The presence of IgG antibodies indicated that the helper T-epitope peptide of **9** had activated helper T-lymphocytes. Furthermore, the observation that IgG antibodies were raised by mice which were only immunized with liposomes (group 1) indicated that the built-in adjuvant Pam_3Cys had triggered appropriate signals for the maturation of DCs and their subsequent activation of helper T-cells. However, the mice which received the liposomes in combination with QS-21 (group 2), elicited higher titers of anti Tn-antibodies. This stronger immune response may be due to a shift from a mixed Th1/Th2 to a Th1 response (Moore et al., Vaccine 1999, 17, 2517-2527).

Table 1. ELISA anti-Tn antibody titers[a] after 4 immunizations with the glycolipopeptide/liposome formulation.

Entry	Group	IgM Titers	IgG Titers
1.	1. Pam_3Cys-YAF-Tn	250	1410
2.	2. Pam_3Cys-YAF-Tn + QS-21	170	2675

[a] ELISA plates were coated with a BSA-BrAc-Tn conjugate. All titers are means for a group of five mice. Titers were determined by regression analysis, plotting log_{10} dilution vs. absorbance. The titers were calculated to be the highest dilution that gave 0.1 or higher than the absorbance of normal saline mouse sera diluted 1:100.

The results presented herein provide, for the first time, a proof-of-principle for the potential use of lapidated glycopeptides as a minimal subunit vaccine. It is to be expected that several improvements can be made. For example, it has been found that a clustered presentation of the Tn-antigen is a more appropriate mimetic of mucins, and hence antibodies raised against this structure recognize better Tn-antigens expressed on cancer cells (Nakada et al., J. Biol. Chem. 1991, 266, 12402-12405; Nakada et al., Proc. Natl. Acad. Sci. USA 1993, 90, 2495-2499; Reddish et al., Glycoconj. J. 1997, 14, 549-560; Reis et al., Glycoconj. J. 1998, 15, 51-62). The Th-epitope employed in this study is known to be a MHC class II restricted epitope for humans. Thus, a more efficient class-switch to IgG antibodies may be expected when a murine Th-epitope is employed. On the other hand, compound 9 is a more appropriate vaccine candidate for use in humans. A recent report indicated that Pam_2Cys is a more potent immunoadjuvant than Pam_3Cys (Jackson et al., Proc. Nat. Acad. Sci. USA 2004, 101, 15440-15445). It has also been suggested that the Pam_2Cys adjuvant has improved solubility properties (Zeng et al., J. Immunol. 2002, 169, 4905-4912), which is a problematic feature of compound 9. Studies addressing these issues are ongoing.

This work is reported in Buskas et al., Angew. Chem. Int. Ed. 2005, 44, 5985-5988.

Supporting Information

Reagents and general experimental procedures. Amino acids and resins were obtained from Applied Biosystems and NovaBiochem; DMF from EM science; and NMP from Applied Biosystems. Phosphatidylethanolamine (PE), cholesterol, phosphatidylcholine (PC; eggyolk), and phosphatidylglycerol (PG; egg yolk) were from purchased from Sigma-Aldrich and Fluka. All other chemicals were purchased from Aldrich, Acros, and Fluka and used without further purification. All solvents employed were of reagent grade and dried by refluxing over appropriate drying agents. TLC was performed using Kieselgel 60 F_{254} (Merck) plates, with detection by UV light (254 nm) and/or by charring with 8% sulfuric acid in ethanol or by ninhydrine. Column chromatography was performed on silica gel (Merck, mesh 70-230). Size exclusion column chromatography was performed on Sephadex LH-20. Extracts were concentrated under reduced pressure at ≤ 40 °C (water bath). An Agilent 1100 series HPLC system equipped with an autosampler, UV-detector and fraction-collector and a Synchropak C4 column 100x4.6 mm RP with a flow rate of 1 mL/min was used for analysis and purifications. Positive ion matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra were recorded using an HP-MALDI instrument using gentisic acid as a matrix. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Inova300 spectrometer, a Varian Inova500 spectrometer, and a Varian Inova600 spectrometer all equipped with Sun workstations. ^{1}H spectra recorded in $CDCl_3$ were referenced to residue $CHCl_3$ at 7.26 ppm or TMS, and ^{13}C spectra to the central peak of $CDCl_3$ at 77.0 ppm. Assignments were made using standard 1D experiments and gCOSY/DQCOSY, gHSQC and TOCSY 2D experiments.

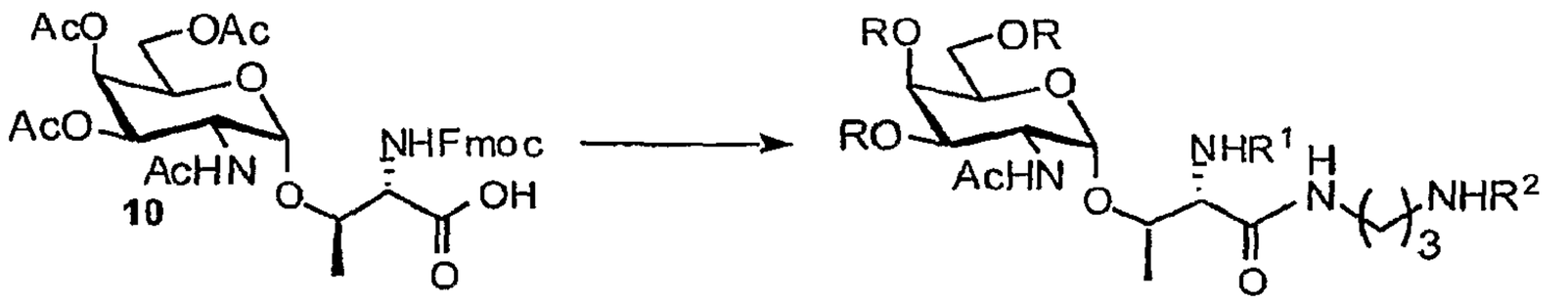
Lipopeptide **6**. Compound **1** was synthesized on HMPB-MBHA resin (maximum loading, 0.1 mmol). The synthesis of peptide **1** was carried out on an ABI 433A peptide synthesizer equipped with a UV-detector using Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) as the coupling reagents. Single coupling steps were performed with conditional capping as needed. After completion of the synthesis of peptide **1**,

the remainding steps were performed manually. *N*-Fluorenylmethoxycarbonyl-S-(2,3-bis(palmitoyloxy)-(2R-propyl)-(R)-cysteine **2** (120 mg, 0.13 mmol) was dissolved in DMF (5 mL) and PyBOP (0.13 mmol), HOBt (0.13 mmol), and DIPEA (0.27 mmol) were added. After premixing for 2 min., DCM (1 mL) was added and the mixture was added to the resin. The coupling step was performed twice. Upon completion of the coupling, as determined by the Kaiser test, the N-Fmoc group was cleaved using 20% piperidine in DMF (5 mL). Palmitic acid (77 mg, 0.3 mmol) was coupled to the free amine as described above using PyBop (0.3 mmol), HOBt (0.3 mmol) and DIPEA (0.6 mmol) in DMF. The resin was thoroughly washed with DMF and DCM and dried under vacuum for 4h. The fully protected lipopeptide **6** was released from the resin by treatment with 2% trifluoroacetic acid in DCM (2.5 mL) for 2 min. The mixture was filtered into 5% pyridine in methanol solution (5 mL). The procedure was repeated and fractions containing the lipopeptide were pooled and concentrated to dryness. The crude product was purified by size-exclusion chromatography (LH-20, DCM/MeOH, 1:1) to give lipo-peptide **6** (275 mg, 0.057 mmol) as a white solid: R_f = 0.57 (DCM/MeOH 9:1); selected NMR data ($CDCl_3/CD_3OD$ 1/1 v/v 600MHz): ^1H, δ 0.48-0.90 (m, 27 H, Pam CH_3, Leu CH_3, Val CH_3), 0.96-1.61 (m, Leu CH_2, Leu CH, Lys CH_2, ^{t}Bu CH_3, Boc CH_3, Ala CH_3, Arg CH_2), 1.18 (br s, 72H, Pam CH_2), 1.95, 1.99 (s, 4x3H, Pbf CH_3C), 2.36, 2.41, 2.44 (s, 6x3H, Pbf CH_3), 2.48 (s, 2x2H, Pbf CH_2) 2.65-2.73 (m, 6H, S-C*H*$_2$-glyceryl, His CH_2, Cys$^{\beta}$), 3.47 (m, 2H, Gly$^{\alpha}$), 3.57 (m, 2H, Gly$^{\alpha}$), 4.06 (m, 1H, S-glyceryl-C*H*$_2^{b}$O), 4.32 (m, 1H, S-glyceryl-C*H*$_2^{a}$O), 3.65-4.39 (m, 17H, Phe$^{\alpha}$, Ala$^{\alpha}$, His$^{\alpha}$, Lys$^{\alpha}$, Val$^{\alpha}$, Asn$^{\alpha}$, Glu$^{\alpha}$, Tyr$^{\alpha}$, Arg$^{\alpha}$), 4.45 (m, 1H, Cys$^{\alpha}$), 5.06 (m, 1H, S-glyceryl-C*H*), 6.72-7.39 (m, 70H, His CH, Tyr aromat, Phe aromat, Trt aromat), 7.48-8.29 (m, NH). MALDI-MS calcd for $C_{269}H_{373}N_{33}O_{42}S_3$ [M+Na] *m/z* = 4860.22: found 4860.31.

Protected glycolipopeptide **8**. A solution of lipopeptide **6** (22 mg, 4.6 μmol), HOAt (6.3 mg, 46 μmol), and DIC (7 μL, 46 μmol) in DCM/DMF (2/1 v/v, 1.5 mL) was stirred under argon atm. at ambient temperature for 15 min. Compound **7** (8 mg, 19 μmol) and DIPEA (14 μL, 92 μmol) in DMF (1.5 mL) was added to the stirred mixture of lipopeptide and the reaction was kept at room

temperature for 18h. The mixture was diluted with toluene and concentrated to dryness under reduced pressure. Purification of the residue by size-exclusion chromatography (LH-20, DCM/MeOH 1:1) gave compound **8** (19 mg, 79%) as a white solid: selected NMR data ($CDCl_3/CD_3OD$ 1/1 v/v 600MHz): ^1H, δ 0.60-0.90 (m, 27 H, Pam CH_3, Leu CH_3, Val CH_3), 0.96-1.61 (m, Leu CH_2, Leu CH, Lys CH_2, ^{t}Bu CH_3, Boc CH_3, Ala CH_3, Arg CH_2), 1.18 (br s, 72H, Pam CH_2), 1.94, 1.98, 1.99, 2.00 (s, 6x3H, Pbf CH_3C, HNAc CH_3), 2.36, 2.41, 2.45 (s, 6x3H, Pbf CH_3), 2.48 (s, 2x2H, Pbf CH_2), 3.42-4.31 (m, Phe$^\alpha$, Ala, Lys, Val, Asp, Glu, Tyr, Arg, Gly, Leu, His, Asn CH_2, Tyr CH_2, Phe CH_2, Arg CH_2), 3.71 (H-3), 3.88 (H-4) 4.06 (S-glyceryl-CH_2^βO), 4.20 (t, 1H, H-2), 4.32 (m, 1H, S-glyceryl-CH_2^αO), 4.42 (m, 1H, Cys$^\alpha$), 4.82 (d, 1H, H-1, *J*=3.68Hz), 5.06 (m, 1H, S-glyceryl-C*H*), 6.72-7.39 (m, 70H, His CH, Tyr aromat, Phe aromat, Trt aromat), 7.48-8.29 (m, NH). MALDI-MS calcd for $C_{286}H_{403}N_{37}O_{49}S_3$ [M+Na] *m/z* = 5262.67: found 5262.99.

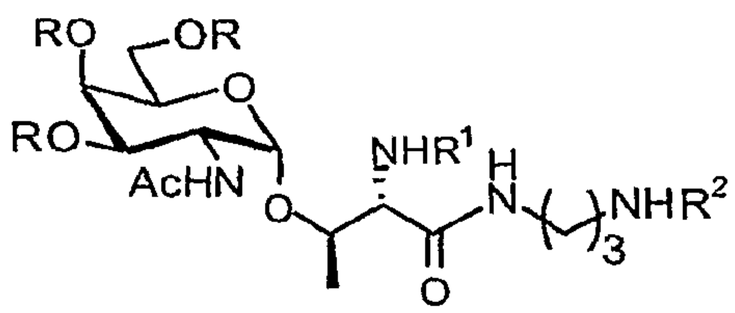
Glycolipopeptide **9**. Compound **8** (12 mg, 2.3 μmol) in a deprotection cocktail of TFA/H_2O/ethane-1,2-dithiol (95:2.5:2.5, 3 mL) was stirred at room temperature for 1h. The solvents were removed under reduced pressure and the crude compound was first purified by a short size-exclusion LH-20 column (DCM/MeOH 1:1) and the then by HPLC using a gradient of 0-100% acetonitrile in H_2O (0.1% TFA) to give, after lyophilization, compound **9** (6.8 mg, 79%)as a white solid: selected NMR data ($CDCl_3/CD_3OD$ 600MHz): ^1H, δ 0.74-0.96 (m, 27H, Pam CH_3, Leu CH_3, Val CH_3), 1.11-2.35 (Leu CH_2, Leu CH, sp CH_2, Lys CH_2, Glu CH_2, Ala CH_3, Val CH, Asp CH_2), 1.29 (br S, 72H, Pam CH_2), 2.43-3.87 (Ala$^\alpha$, Gly$^\alpha$, S-glyceryl-OCH_2, Cys$^\beta$, H-2, H-3, H-4, H-5, H-6), 4.05-4.73 (m, Cys$^\alpha$, Phe$^\alpha$, Tyr$^\alpha$, His$^\alpha$, Leu$^\alpha$, Lys$^\alpha$, Asp$^\alpha$, Val$^\alpha$, Arg$^\alpha$, Glu$^\alpha$, H-1), 5.12 (m, 1H, S-glyceryl-C*H*), 6.64-6.71 (dd+dd, 2H, His CH, NH), 6.86-7.12 (dd+dd 2H, His CH, NH) 7.16-8.23 (m, Tyr aromat, Phe aromat, NH). HR-MALDI-MS calcd for $C_{186}H_{297}N_{37}O_{41}S$ [M+Na] *m/z* = 3760.1911: found 3760.3384.



11. R = Ac, R^1 = Fmoc, R^2 = Boc

Tn derivative **11**. Compound **10** was dissolved in DMF (10 mL) and di-isopropylcarbodiimide (DIC) (82 μL, 0.53 mmol) and HOAt (216 mg, 1.58 mmol) were added. After stirring for 15 min., 3-(N-(*tert.* butyloxycarbonyl)-amino)propanol (111 mg, 0.63 mmol) was added and the reaction was kept at ambient temperature for 15 h. The mixture was concentrated to dryness under reduced pressure and the residue was purified by silica gel column chromatography (0-5% MeOH in DCM) and LH-20 size-exclusion chromatography (DCM/MeOH 1:1) to give compound **11** (363 mg, 83%). R_f = 0.63 (DCM/MeOH 9:1); $[\alpha]_D$ +4.4 (c 1.0 mg/mL, CH_2Cl_2); NMR data ($CDCl_3$, 500MHz): ^{1}H, δ 1.27 (d, 3H, CH_3 Thr), 1.43 (s, 9H, ^{t}Bu CH_3), 1.46-1.61 (m, 2H, CH_2), 1.99 (s, 3H, CH_3 Ac), 2.05 (s, 6H, CH_3 Ac), 2.06 (s, 3H, CH_3 Ac), 2.17 (s, 3H, CH_3 Ac), 3.17-3.27 (m, 3H, CH_2, CH_{2a}), 3.48-3.50 (m, 1H, CH_{2b}), 4.07-4.28 (m, 6H, H-6, H-5, Thr^{α}, Thr^{β}, CH Fmoc), 4.43-4.51 (m, 2H, CH_2 Fmoc), 4.62 (dd, 1H, H-2), 4.89 (br t, 1H, NH), 5.04-5.11 (m, 2H, H-1, H-3), 5.41 (d, 1H, H-4), 5.75 (br d, 1H, NH T), 6.81 (br d, 1H, NH GalNAc), 7.17-7.79 (m, 8H, aromatic H); ^{13}C ($CDCl_3$, 75MHz) δ17.19, 20.92, 20.99, 21.09, 23.30, 28.55, 30.69, 35.87, 36.92, 47.43, 47.77, 58.57, 62.36, 67.47, 68.68, 77.46, 80.08, 99.88, 120.25, 125.34, 127.35, 128.00, 128.76, 129.13, 141.55, 143.94, 144.01, 156.51, 157.52, 169.68, 170.66, 170.94, 170.99.

HR-MALDI-MS calcd for $C_{41}H_{54}N_4O_{14}$ [M+Na] *m/z* = 849.3535: found 849.3391.



11. R = Ac, R^1 = Fmoc, R^2 = Boc
12. R = Ac, R^1 = Ac, R^2 = Boc
13. R = H, R^1 = Ac, R^2 = Boc
7. R = H, R^1 = Ac, R^2 = H

Tn derivative 7. A solution of compound **11** (194 mg, 0.24 mmol) in 20% piperidine in DMF (5 mL) was stirred at ambient temperature for 1h. The mixture was concentrated to dryness and the residue was treated with pyridine/acetic anhydride (3:1, 5 mL) for 2 h. The reaction mixture was diluted with toluene and concentrated to dryness. The residue was dissolved in dichloromethane and washed with 1M HCl and sat. aq. $NaHCO_3$, dried with $MgSO_4$, filtered and concentrated. Purification of the residue by size-exclusion chromatography (LH-20, DCM/MeOH 1:1) furnished compound **12** (167 mg, 91%): NMR data ($CDCl_3$, 300MHz): ^{1}H, δ 1.24 (d, 1H, Thr CH_3), 1.42 (s, 9H, ^{t}Bu CH_3), 1.55-1.59 (m, 2H, $NHCH_2CH_2CH_2NH$), 1.95, 2.02, 2.03, 2.12, 2.14 (s, 15H, CH_3 Ac), 3.13-3.23 (m, 3H, CH_2 + CH_{2a}), 3.36-3.41 (m, 1H, CH_{2b}), 4.03-4.12 (m, 2H), 4.19-4.23 (m, 2H, Thr^{β}), 4.54-4.61 (m, H-2, Thr^{α}), 4.88 (m, 1H, NH), 4.96 (s, 1H, *J*=3.57 Hz, H-1), 5.07 (dd, 1H, H-3), 5.35 (d, 1H, H-4), 6.43 (br S, 1H, NH), 6.72 (br S, 1H, NH). MALDI-MS calcd for $C_{28}H_{46}N_4O_{13}$ [M+Na] *m/z* = 669.296: found 669.323. Compound **12** was deprotected by stirring with 5% hydrazine-hydrate in methanol (5 mL) at room temperature for 35 min. The reaction mixture was diluted with toluene and concentrated. The residue was co-evaporated twice with toluene. Purification by silica gel column chromatography (DCM/MeOH 5:1) yielded **13** (119 mg, 89%): NMR data (CD_3OD, 300MHz): ^{1}H, δ 1.26 (d, 3H, Thr CH_3), 1.43 (s, 9H, ^{t}Bu CH_3), 1.57-1.63 (m, 2H, $NHCH_2CH_2CH_2NH$), 2.06, 2.10 (s, 2x3H NHAc), 2.12-3.09 (m, 2H, CH_2), 3.15 (m, 2H, CH_2), 3.31 (br s, 2H, H-6), 3.68-3.76 (m, 2H, H-3, H-5), 3.88 (d, 1H, H-4), 4.22-4.26 (m, 2H, H-2, Thr^{β}), 4.46 (m, 1H, Thr^{α}), 4.84 (d, 1H, H-1), 6.60 (br m, 1H, NH), 7.50 (br d, 1H, NH). MALDI-MS calcd. for $C_{22}H_{40}N_4O_{10}$ [M+Na] *m/z* = 543.264: found 543.301. A solution of **13** in

trifluoro acetic acid (4 mL) was stirred under an argon atmosphere at ambient temperature for 45 min. The reaction mixture was then diluted with DCM and concentrated to dryness. The crude product was purified by column chromatography (Iatro beads, EtOAc/MeOH/H_2O 2:2:1 → MeOH/H_2O 1:1). After concentration of the pooled fractions, the solid was lyophilized from H_2O to give compound **7** (91 mg, 0.21 mmol, 95%) as a white powder. $R_f = 0.17$ (EtOAc/MeOH/H_2O 6:3:1); $[\alpha]_D$ -37 (c 1.0 mg/mL, H_2O); NMR data (D_2O, 300MHz): ^1H, δ 1.15 (d, 3H, $J = 6.3$ Hz, Thr CH_3), 1.73-1.77 (m, 2H, CH_2), 1.95 (s, 3H, NHAc), 2.04 (s, 3H, NHAc), 2.82-2.87 (m, 2H, CH_2), 3.11-3.15 (m, 1H, CH_{2a}), 3.22-3.26 (m, 1H, CH_{2b}), 3.65 (m, 2H, H-6), 3.76 (dd, 1H, $J = 2.9$, 11.2 Hz, H-3), 3.87 (d, 1H, $J = 2.9$ Hz, H-4), 3.92 (t, 1H, H-5), 3.99 (dd, 1H, $J = 3.41$, 11.2 Hz, H-2), 4.28-4.30 (m, 1H, Thr^{β}), 4.32 (d, 1H, $J = 2.4$ Hz, Thr^{α}) 4.78 (d, 1H, $J = 3.56$ Hz, $J = 3.9$ Hz, H-1), 7.97 (br d, 1H, NH), 8.17 (br t, 1H, NH), 8.27 (br d, 1H, NH); ^{13}C (D_2O, 75MHz), δ 18.17 Thr CH_3), 21.93, 22.33 (2xNAc) 26.98 (CH_2), 36.55 (CH_2), 37.22 (CH_2), 49.98 (C-6), 58.30 (C-3), 61.46 (C-4), 67.76 (C-5), 68.65 (C-2), 71.54 (C-Thr^{β}), 74.60 (C-Thr^{α}), 98.60 (C-1), 172.09, 174.37, 175.18 (3x C=O, NHAc). HR-MALDI-MS calcd for $C_{17}H_{32}N_4O_8$ [M+Na] $m/z = 443.2118$: found 443.2489.

Liposome preparation. Liposomes were prepared from PC, PG, cholesterol, and the glycolipopeptide **9** (15 μmol, molar ratio 65:25:50:10). The lipids were dissolved in DCM/MeOH (3/1, v/v) under an atmosphere of argon. The solvent was then removed by passing a stream of dry nitrogen gas, followed by further drying under high vacuum for one hour. The resulting lipid film was suspended in 1 mL 10 mM Hepes buffer, pH 6.5, containing 145 mM NaCl. The solution was vortexed on a shaker (250 rpm), under Ar atmosphere at 41 °C for 3 hours. The liposome suspension was extruded ten-times through 0.6 μm, 0.2 μm and 0.1 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 °C to obtain SUV.

Immunizations. Groups of five mice (female BALB/c, 6 weeks) were immunized subcutaneously on days 0, 7, 14 and 21 with 0.6 μg of carbohydrate-containing liposomes and 10 μg of the adjuvant QS-21 in each boost. The mice

were bled on day 28 (leg-vein) and the sera were tested for the presence of antibodies.

ELISA. 96-well plates were coated over night at 4 °C with Tn–BSA, (2.5 μg mL^{-1}) in 0.2 M borate buffer (pH 8.5) containing 75 mM sodium chloride (100 μL) per well). The plates were washed three times with 0.01 M Tris buffer containing 0.5% Tween 20% and 0.02% sodium azide. Blocking was achieved by incubating the plates 1 h at room temperature with 1% BSA in 0.01 M phosphate buffer containing 0.14 M sodium chloride. Next, the plates were washed and then incubated for 2 h at room temperature with serum dilutions in phosphate buffered saline. Excess antibody was removed and the plates were washed three times. The plates were incubated with rabbit anti-mouse IgM and IgG Fcγ fragment specific alkaline phosphatase conjugated antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 2 h at room temperature. Then, after the plates were washed, enzyme substrate (*p*-nitrophenyl phosphate) was added and allowed to react for 30 min before the enzymatic reaction was quenched by addition of 3 M aqueous sodium hydroxide and the absorbance read at dual wavelengths of 405 and 490 nm. Antibody titers were determined by regression analysis, with $\log_{10}$ dilution plotted against absorbance. The titers were calculated to be the highest dilution that gave two times the absorbance of normal mouse sera diluted 1:120.

Example II. Non-Covalently Linked Diepitope Liposome Preparations

In a first set of experiments, the tumor-related carbohydrate B-epitope and the universal T-epitope peptide were incorporated separately into preformed liposomes to form a diepitopic construct. Additionally, the lipopeptide Pam_3Cys was incorporated into the liposome with the expectation that it would function as a built-in adjuvant, and thus circumvent the necessity of using an additional external adjuvant, such as QS-21.

The liposomes were prepared from lipid anchors carrying two different thiol-reactive functionalities, maleimide and bromoacetyl, at their surface. The Pam_3Cys adjuvant was also incorporated into the preformed liposome and included a maleimide functionality. Conveniently, the maleimide and the

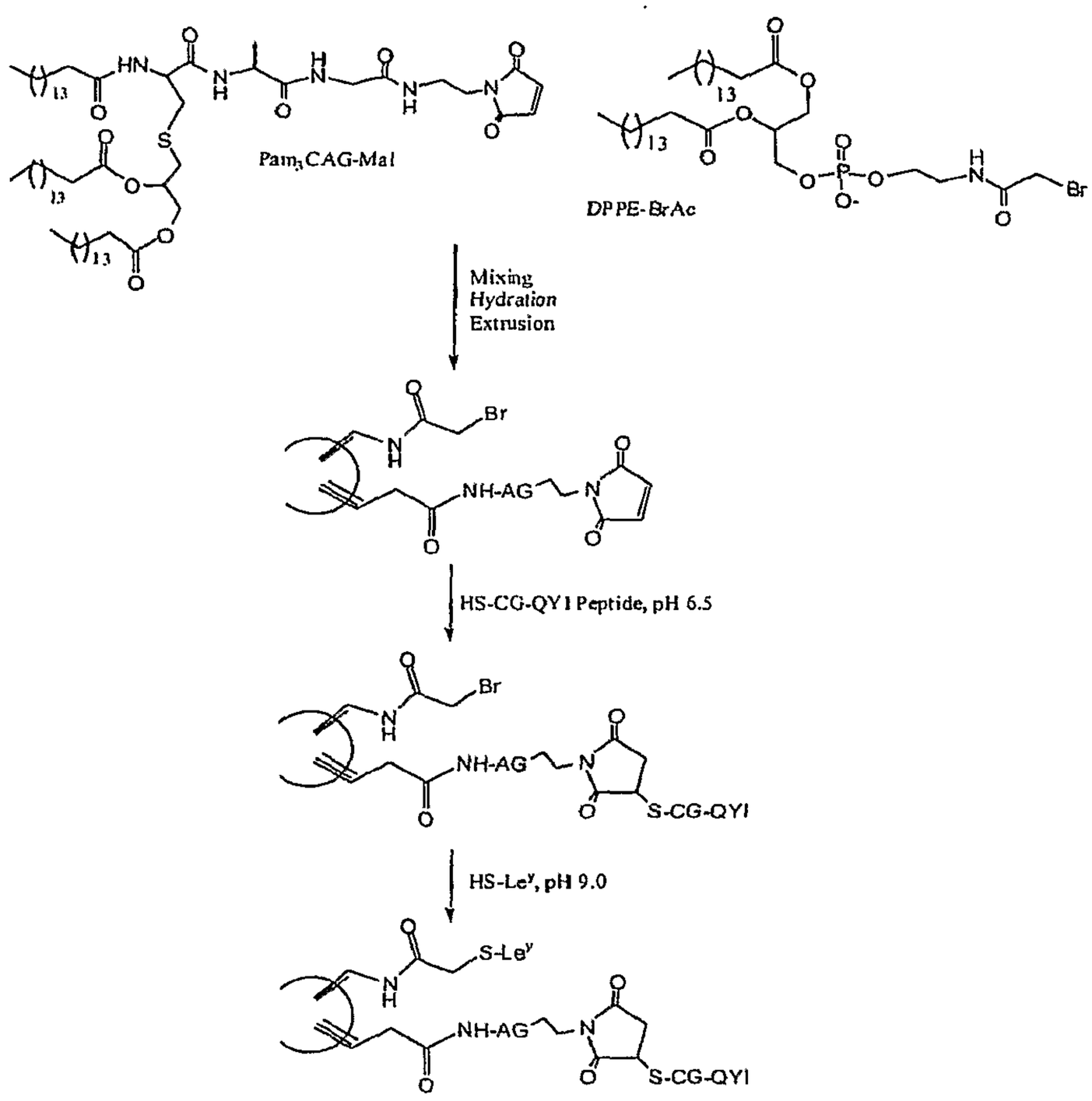
bromoacetyl group show a marked difference in their reactivity towards sulfhydryl groups. The maleimide reacts rapidly with a sulfhydryl compound at pH 6.5, whereas the bromoacetyl requires slightly higher pH 8-9 to react efficiently with a thiol compound.

By exploiting this difference in reactivity, a diepitope liposome construct carrying the cancer related Le^y tetrasaccharide and the universal T helper peptide QYIKANSKFIGITEL (QYI) (SEQ ID NO:1) was prepared (Scheme 11). For the conjugation to the thiol-reactive anchors, both the oligosaccharide and the peptide were functionalized with a thiol-containing linker. The two-step consecutive conjugation to preformed liposomes has a great advantage: it is a very flexible approach that makes it easy to prepare liposomes carrying an array of different carbohydrate B-epitopes. The yield of conjugation, as based on quantitating the carbohydrate and peptide covalently coupled to the vesicles, was high, 70-80% for the oligosaccharide and 65-70% for the peptide, and the results were highly reproducible.

It is important to note that in these first diepitope liposome constructs, the carbohydrate B-epitope and peptide T-epitope are not themselves joined together by covalent linkages, but rather are held in proximity by their respective lipid anchors to which they are conjugated, and by hydrophobic interactions. It has been shown in several reports in the literature regarding vaccine candidates with pathogen-related peptide B-epitopes that this approach is successful leading to good titers of both IgM and specific IgG antibodies. These studies also indicate that the built-in adjuvant Pam_3Cys is sufficient to induce a proper immune response.

However, in our study with the tumor-related carbohydrate B-epitope Le^y, immunizations of mice using the non-covalently linked diepitope liposome preparation described in this Example resulted in only very low titers of IgM antibodies. No IgG anti-Le^y antibodies were detected. Even more surprising, co-administering the liposomal vaccine candidate with the powerful external adjuvant, QS-21, did not improve the outcome. Additionally, it was found that mice that had been immunized with an un-coated liposome control, *i.e.* a liposome that carried nothing but the maleimide and bromoacetyl functional groups on the surface, elicited high titers of IgG antibodies as detected by

ELISA. More detailed ELISA studies of the anti-sera from this group of mice using a variety of protein conjugates revealed that the mice had responded to and elicited antibodies towards the maleimide linker. Also the anti-sera from the mice immunized with the liposomes coated with the Le^y antigen and the QYI peptide were screened for anti-linker antibodies and it was found that also these mice had elicited IgG antibodies towards the maleimide linker.



Scheme 11

Due to its high reactivity at near neutral pH, the maleimide linker is widely used in conjugation chemistry to reach glyco- and peptide-protein conjugates that are further used in immunization studies. There are commercially available protein conjugation kits (Pierce Endogen Inc.) that utilize the maleimide linker both for the antigenic conjugate and the detection

conjugate. Our data show that using these kits can lead to false positive results, especially when working with antigens of low immunogenicity (See T. Buskas, Y. Li and G-J. Boons, Chem. Eur. J.,10:3517-3523, 2004).

To test whether the highly immunogenic maleimide linker suppressed the immune response towards the Le^y tetrasaccharide, we prepared the non-covalent diepitope liposome using only the bromoacetyl linker. In this experiment, the thiol-containing Le^y tetrasaccharide and the universal T helper peptide were conjugated, in separate reactions, to lipids containing the bromoacetyl linker. The conjugated lipids were then mixed together to form lipid vesicles. Administering this new liposome formulation to mice, with or without the external adjuvant QS-21, raised only low titers of anti-Le^y antibodies. Thus, the lack of an effective immune response toward the Le^y tetrasaccharide was not due solely to the immunogenic maleimide linker.

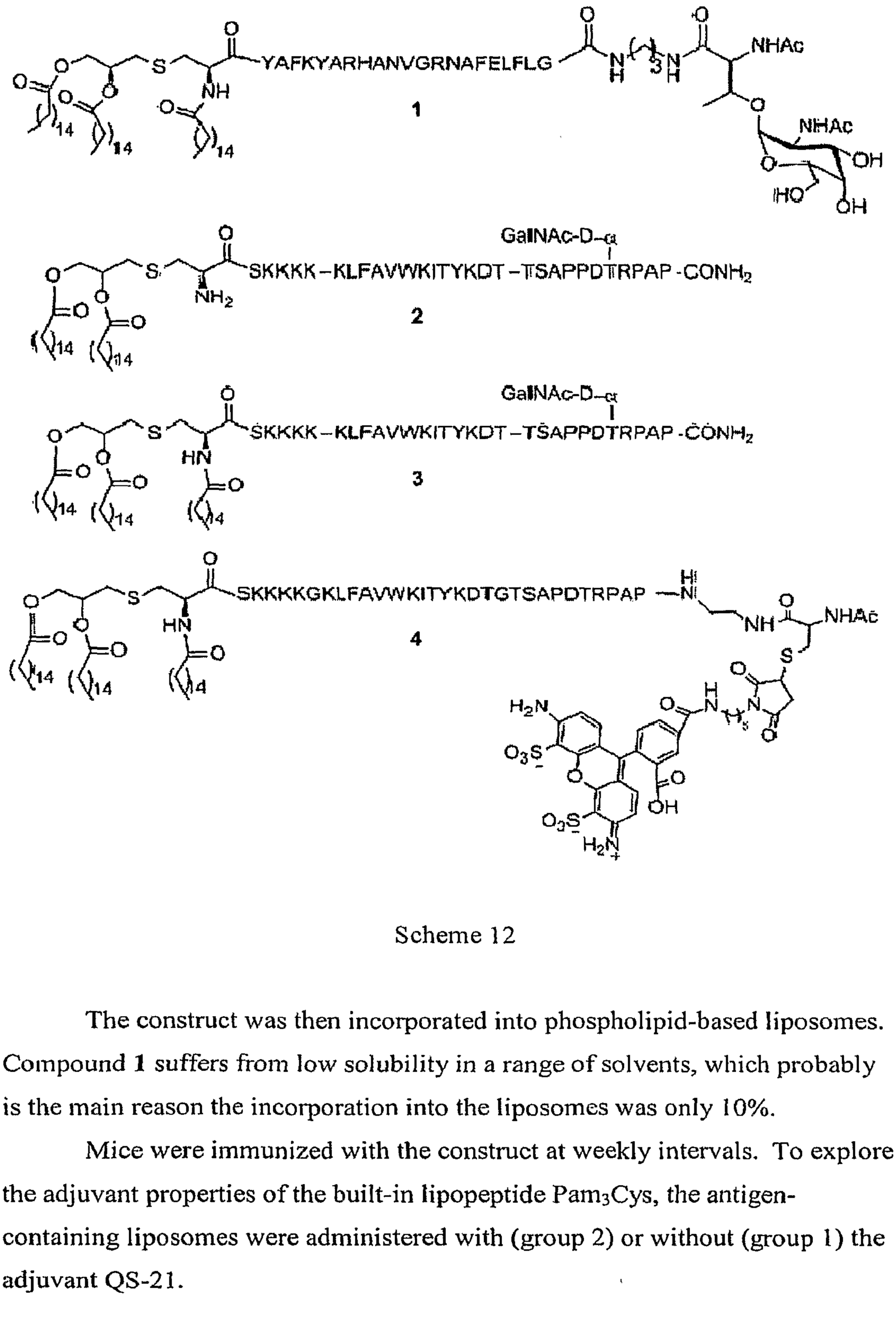
Since the tumor-associated Le^y tetrasaccharide is known to be only weakly immunogenic, we prepared another diepitope liposomal construct where the more immunogenic Tn(cluster) antigen was used as a target B-epitope. However, the same negative results were obtained with this antigen. Again, immunizations of mice resulted in only very low titers of anti-Tn(c) IgM antibodies. Co-administering with QS-21 as an external adjuvant did nothing to enhance the immune response.

From these results we concluded that the non-covalently linked diepitope liposome approach that has proven successful for a range of peptide antigens failed when a tumor-associated carbohydrate antigen of low immunogenicity was used as a B-epitope. Thus, we reasoned that the tumor-associated carbohydrate B-epitope and the helper T-epitope needed to be presented differently to the immune system to evoke a T-cell dependent immune response.

Example III. Covalently Linked Diepitope Liposome Preparations

We speculated that in order to achieve a better presentation of the carbohydrate B-epitope and peptide T-epitope, perhaps they needed to be covalently linked together. To test this idea we synthesized construct **1** (Scheme 12), a structurally well-defined anti-cancer vaccine candidate containing the structural features needed for a focused and effective T-cell dependent immune response. The vaccine candidate is composed of the tumor-associated Tn-antigen, the peptide T-epitope YAFKYARHANVGRNAFELFL (YAF) (SEQ ID NO:2) (*Neisseria meningitides*) and the lipopeptide Pam_3Cys. Due to difficulties in the synthesis using the original helper T-epitope peptide QYI, a different universal T-epitope (YAF) that displayed better solubility properties was used in this study.

Compound **1** was synthesized in a highly convergent manner by a combination of solid-phase and solution phase synthesis.



Scheme 12

The construct was then incorporated into phospholipid-based liposomes. Compound **1** suffers from low solubility in a range of solvents, which probably is the main reason the incorporation into the liposomes was only 10%.

Mice were immunized with the construct at weekly intervals. To explore the adjuvant properties of the built-in lipopeptide Pam_3Cys, the antigen-containing liposomes were administered with (group 2) or without (group 1) the adjuvant QS-21.

As can be seen in Table 1 (Example I), the mice immunized with the liposome preparations elicited both IgM and IgG antibodies against the Tn-antigen (Table 1, entries 1 and 2). The presence of IgG antibodies indicated that the helper T-epitope peptide of 1 had activated helper T-lymphocytes. Furthermore, the observation that IgG antibodies were raised by mice which were immunized with liposomes in the absence of the external adjuvant QS-21 (group 1) indicated that the built-in adjuvant Pam_3Cys had triggered appropriate signals for the maturation of DCs and their subsequent activation of helper T-cells. However, the mice which received the liposomes in combination with QS-21 (group 2) elicited higher titers of anti Tn-antibodies. This stronger immune response may be due to a shift from a mixed Th1/Th2 to a Th1 skewed response.

The results provide, for the first time, a proof-of-principle for the potential use of a lipidated glycopeptide that contains a carbohydrate B-epitope, a helper T-cell epitope and a lipopeptide adjuvant as a minimal, self-contained subunit vaccine. It was also concluded that to evoke a T-cell dependent immune response toward the tumor-associated carbohydrate antigen, it is not enough that the carbohydrate B-epitope and the peptide T-epitope are presented together in a non-covalent manner on the surface of a adjuvant-containing liposome; rather, the entities are preferably covalently joined together. Finally, it was observed that an external adjvuant (QS-21) was not needed when the three components (carbohydrate B-epitope, helper T-cell epitope and lipopeptide) are covalently linked to form the lipidated glycopeptides.

Alternative glycolipopeptide components

Several improvements can be made to compound **1**. For example, it has been found that antibodies elicited against the Tn-antigen poorly recognize cancer cells. However, clustering (Nakada et al., Proc. Natl. Acad. Sci. USA 1993, 90, 2495-2499; Reddish et al., 1997, 14, 549-560; Zhang et al., Cancer Res. 1995, 55, 3364-3368; Adluri et al., Cancer Immunol. Immunother 1995, 41, 185-192) or presenting the Tn antigen as part of the MUC-1 glycopeptide elicits antibodies with improved binding characteristics (Snijdewint et al., Int. J. Cancer 2001, 93,97-106).The T-epitope employed in compound **1** is a MHC class II restricted epitope for humans. Thus, a more efficient class-switch to IgG

antibodies may be expected when a murine T-epitope is used. Furthermore, it has been found that the lipopeptide Pam_2Cys or Pam_3CysSK_4 are more potent immunoadjuvants than Pam_3Cys (Spohn et al., Vaccine 2004, 22, 2494-2499). However, it was not known whether attachment of Pam_2Cys or Pam_3CysSK_4 to the T- and B-epitope would affect their efficacies and potencies. Thus, based on these considerations, compounds **2** and **3** (Scheme 12) were designed, which contain the MUC-1 glycopeptide as a B-epitope, the well-documented murine helper T-cell epitope KLFAVWKITYKDT (KLF) (SEQ ID NO:3) derived from Polio virus (Leclerc et al., J. Virol. 1991, 65, 711-718) as the T-epitope, and the lipopeptide Pam_2Cys or Pam_3CysSK_4, respectively.

Glycolipopeptides **2** and **3** were incorporated into phospholipid-based liposomes as described for compound **1**. Surprisingly, the solubility problems that plagued compound **1** were not an issue for compounds **2** and **3**. Female BALB/c mice were immunized four times at weekly intervals with the liposome formulations with or without the external adjuvant QS-21 (Kensil et al., J. Immunol. 1991, 146, 431-437). Anti-Muc1 antibody titers were determined by coating microtiter plates with CTSAPDT(αGalNAc)RPAP conjugated to BSA and detection was accomplished with anti-mouse IgG antibodies labeled with alkaline phosphatase. The results are summarized in Tables 2 and 3.

Table 2. ELISA anti-MUC-1 antibody titers* after 4 immunizations with the glycolipopeptide/liposome formulations.

Entry	Group	IgG total
1.	**2**	24,039
2.	**2** + QS-21	36,906
3.	**3**	183,085
4.	**3** + QS-21	450,494

* ELISA plates were coated with a BSA-BrAc-MUC-1 conjugate. Anti-MUC-1 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 mouse sera.

Table 3. ELISA anti-MUC-1 antibody titers* after 4 immunizations with the glycolipopeptide/liposome formulations.

Entry	Group	IgG1	IgG2a	IgG2b	IgG3
1.	**2**	74,104	3,599	5,515	17,437
2.	**2** + QS-21	126,754	22,709	5,817	20,017
3.	**3**	448,023	57,139	61,094	115,131
4.	**3** + QS-21	653,615	450,756	70,574	305,661

* ELISA plates were coated with a BSA-BrAc-MUC-1 conjugate. 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 mouse sera.

As can be seen in Table 2, mice immunized with liposomal preparations of compounds **2** and **3** elicited high titers of anti-MUC-1 IgG antibodies. Surprisingly, mice that were immunized with the Pam_3CysSK_4-based vaccine elicited higher titers of antibodies than mice immunized with Pam_2Cys derivative. These results are contradictory to reports that have compared adjuvancy of Pam_2Cys and Pam_3CysSK_4. Sub-typing of the IgG antibodies (IgG1, IgG2a, IgG2b and IgG3) indicated a bias towards a Th2 immune response (entries 1 and 3, Table 3). Co-administering of the adjuvant QS-21 did not lead to a significant increase of IgG antibody, however, in these cases a mixed Th1/Th2 response was observed (entries 2 and 4, Table 3).

To ensure that the mouse sera were able to recognize native MUC-1 glycopeptide present on cancer cells, the binding of the sera to the MUC-1 expressing MCF-7 human breast cancer cell line was examined. Thus, the cells were treated with a 1:50 diluted sera for 30 minutes after which goat anti-mouse IgG antibodies labeled with FITC was added. The percentage of positive cells and mean fluorescence was determined by flow cytometry analysis. As can be seen in (Figure 2), the anti-sera reacted strongly with the MUC-1 positive tumor cells whereas no binding was observed for sera obtained from naïve mice. Furthermore, no binding was observed when SK-MEL 28 cell were employed,

which do not express the MUC-1 glycopeptide. These results demonstrate that anti-MUC-1 antibodies induced by **3** recognize the native antigen on human cancer cells. Further ELISA studies showed that titers against the T-epitope were very low, showing that no significant epitope suppression had occurred.

The lipopeptide moiety of the three-component glycolipopeptide is required for initiating the production of necessary cytokines and chemokines (danger signals) (Bevan, Nat. Rev. Immunol. 2004, 4, 595-602; Eisen et al., Curr. Drug Targets 2004, 5, 89-105; Akira et al., Nat. Immunol. 2001, 2, 675-680; Pasare et al., Immunity 2004, 21, 733-741; Dabbagh et al., Curr. Opin. Infect. Dis. 2003, 16, 199-204; Beutler, Mol. Immunol. 2004, 40, 845-859). The results of recent studies indicate that the lipopeptide initiates innate immune responses by interacting with the Toll-like receptor 2 on the surface of mononuclear phagocytes. After activation, the intracellular domain of TLR-2 recruits the adaptor protein MyD88, resulting in the activation of a cascade of kinases leading to the production of a number of cytokines and chemokines. On the other hand, lipopolysaccharides induce cellular responses by interacting with the Toll-like receptor 4 (TLR4)/MD2, which results in the recruitment of the adaptor proteins MyD88 and TRIF leading to a more complex pattern of cytokine. TNF-α secretion is the prototypical measure for activation of the MyD88-dependent pathway, whereas secretion of IFN-β is commonly used as an indicator of TRIF-dependent cellular activation (Akira et al., Nat. Immunol. 2001, 2, 675-680; Beutler, Mol. Immunol. 2004, 40, 845-859).

To examine whether attachment of a glycopeptide containing a T epitope and a B epitope to the TLR ligand affects cytokine production, the efficacy (EC_{50}) and potency (maximum responsiveness) of TNF-α and IFN-β secretion induced by compounds **1**, **2** and **3** was determined and the results compared with those of Pam_2CysSK_4, Pam_3CysSK_4 and LPS. Thus, RAW NO^- mouse macrophages were exposed over a wide range of concentrations to compounds **1**, **2** and **3**, Pam_2CysSK_4, Pam_3CysSK_4 and *E. coli* 055:B5 LPS. After 5.5 hours, the supernatants were harvested and examined for mouse TNF-α and IFN-β using commercial or in-house developed capture ELISA assays, respectively.

Table 4. EC_{50} and E_{max} values of concentration-response curves of *E. coli* LPS and synthetic compounds for TNF-α production by mouse macrophages (RAW γNO(-) cells).

	EC_{50} (nM)*	E_{max} (pg/mL)*
E. coli LPS	0.002	2585
1	10.230	363
Pam_2CysSK_4	0.003	631
2	0.223	622
Pam_3CysSK_4	3.543	932
3	2.151	802

* Values of EC50 and Emax are reported as best-fit values according to Prism (GraphPad Software, Inc). Concentration-response data were analysed using nonlinear least-squares curve fitting in Prism.

As can be seen in Figure 3 and Table 4, glycolipopeptide **3** and Pam_3CysSK_4 induced the secretion of TNF-α with similar efficacies and potencies indicating that attachment of the B-epitope and T-epitope had no effect on cytokine and chemokine responses. Surprisingly, attachment of the B-epitope and the T-epitope to Pam_2CysSK_4 led to a significant reduction in potency and thus in this case the attachment of the B-epitope and the T-epitope led to a reduction in activity. Compound **1** which contains the Pam_3Cys moiety is significantly less active than the compounds **2** and **3**, which may explain the poor antigenicity of compound **1**. Compounds **1**, **2** and **3** did not induce the production of INF-β. Surprisingly, *E. coli* 055:B5 displayed much larger potencies and efficacies for TNF-α induction compared to compounds **1**, **2**, **3**, and Pam_3CysSK_4. In addition, it was able to stimulate the cells to produce INF-β. *E. coli* LPS is too active resulting in over-activation of the innate immune system, leading to symptoms of septic shock.

It was speculated that in addition to initiating the production of cytokines and chemokines, the lipopeptide may facilitate selective targeting and uptake by antigen presenting cells in a TLR2 dependent manner. To test this hypothesis, compounds **4**, which contains a fluorescence label, was administered to RAW

NO^- mouse macrophages and after 30 minutes the cells were harvested, lysed and the fluorescence measured. To account for possible cell surface binding without internalization, the cells were also trypsinized before lyses and then examination for fluorescence. As can be seen in Figure 4, a significant quantity of the **4** was internalized whereas a small amount was attached to the cell surface. To determine whether the uptake was mediated by TLR2, the uptake studies were repeated using native HEK297 cell and HEK297 cell transfected with either TLR2 or TLR4/MD2. Importantly, significant uptake was only observed when the cells were transfected with TLR2 indicating that uptake is mediated by this receptor. These studies show that TLR2 facilitates the uptake of antigen, which is an important step in antigen processing and immune responses.

Experimental procedures

Cell culture

RAW 264.7 γNO(-) 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 μg/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 μg/mL; Mediatech), Normocin (100 μg/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 μg/mL) and for the HEK w/ hTLR4/MD2/CD14 also with HygroGold (50 μg/mL). Human breast adenocarcinoma cells (MCF7), 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%). All cells were maintained in a humid 5% CO_2 atmosphere at 37°C.

Serologic assays

Anti-MUC1 IgG, IgG1, IgG2a, IgG2b, and IgG3 antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). 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 (Molecular Devices). The antibody titer was defined as the highest dilution yielding an optical density of 0.1 or greater over background of blank 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 γ-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 γNO(-) 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 hours 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 and concentrations of IFN-β were determined as follows. ELISA plates were coated with rabbit polyclonal antibody against mouse IFN-β (PBL Biomedical Laboratories). IFN-β in standards and samples was allowed to bind to the immobilized antibody. Rat anti-mouse IFN-β antibody (USBiological) was then added. Next horseradish peroxidase-conjugated goat anti-rat IgG (H+L) antibody (Pierce) and a chromogenic substrate 3,3',5,5'-tetramentylbenzidine (Pierce) were added. After the reaction was stopped, the absorbance was measured at 450 nm with wavelength correction set to 540 nm using a microplate reader (Molecular Devices). 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: $Y = E_{max} / (1 + (EC_{50}/X)^{Hill\ slope})$, where Y is the cytokine 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 cytokine values are presented as the means ± SD of triplicate cultures.

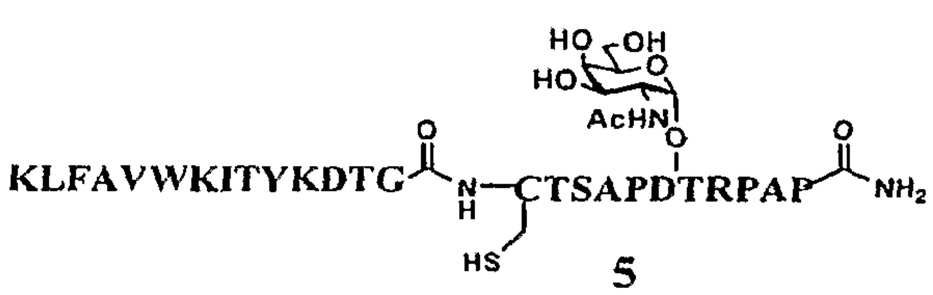
Binding and uptake assay

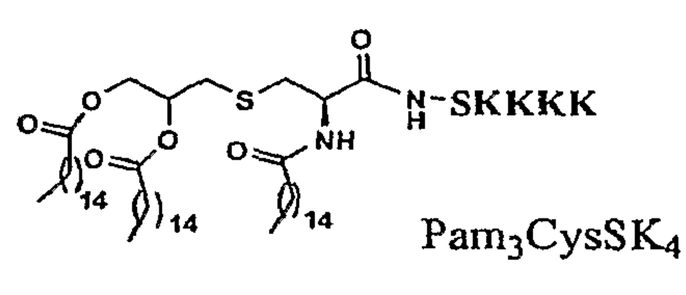
RAW NOγ(-) cells, HEK293T cells, and HEK293T cells stable transfected with murine TLR2, TLR2/TLR6, or TLR4/MD2 (2.6×10^6 cells/mL) were exposed to Alexa Fluor 488-labeled compound 5 (1 μg/mL) for 30 min at 37 °C. Cells were harvested and washed in HNE buffer (HEPES, 20 mM; NaCl, 150 mM; EDTA, 1 mM). Samples that were assessed for internalization only were treated with trypsin (500 μg/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 of triplicate treatments.

Example IV. Covalent Attachment of the Lipid Component

To establish the importance of covalent attachment of the TLR ligand to the vaccine candidate, compound **5** (Scheme 13) which only contains the B-epitope and the T-epitope was designed and synthesized. Mice were immunized four times at weekly intervals with this compound in the presence of PAM_3CysSK_4. Interestingly, the mixture of glycopeptide **5** and the adjuvant Pam_3CysSK_4 elicited no- or very low titers of IgG antibodies, demonstrating that covalent attachment of Pam_3CysSK_4 to the B-epitope and T-epitope is critical for strong immune responses.



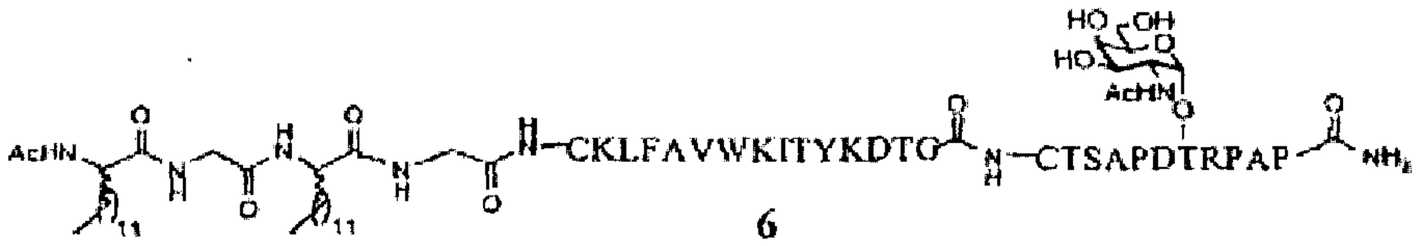


Scheme 13

Example V. Lipid Component

To determine the importance of lipidation with a ligand of a Toll like receptor, compound **6** (Scheme 14) was designed and synthesized. This compound is composed of the B- epitope and T-epitope linked to non-immunogenic lipidated amino acids. Mice were immunized with a liposomal preparation of compound **6**, similar to the procedure employed for compound **1** and **2**. Liposomes containing compound **6** induced titers that were significantly lower than those elicited by compound **3**, demonstrating that a TLR ligand of the three-component vaccine is important for optimal immune responses.

76433-138



6

Scheme 14

Conclusions

The three-component carbohydrate-based glycolipopeptide potentially has a number of distinctive advantages over a traditional conjugate vaccine. For example, the glycolipopetide potentially does not suffer from epitope suppression, a characteristic of carbohydrate-protein conjugates. Apart from providing danger signals, the lipopeptide Pam_3CysSK_4 may potentially also facilitate the incorporation of the antigen into liposomes. A liposomal formulation may be attractive because it potentially presents efficiently the antigen to the immune system. A unique feature of the glycolipopeptide is that Pam_3CysSK_4 potentially promotes selective targeting and uptake by antigen presenting cells, T-helper cells and B-lymphocytes, which express Toll-like receptors (Example III). Finally, a fully synthetic compound has as an advantage that it can be fully characterized, which facilitates its production in a reproducible manner.

The foregoing description and examples have been given for clarity and understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined in the claims.

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 76433-138 Seq 29-JUN-09 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

SEQUENCE TABLE

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<150> US 60/775,881
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<150> US 60/796,769
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<220>
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<220>
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<212> PRT
<213> artificial

<220>
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<220>
<223> T-cell peptide

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20

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<220>
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1 5 10 15
Asn Asn

CLAIMS:

1. A glycolipopeptide comprising

at least one carbohydrate component comprising a B-epitope, wherein the B-epitope is from a microorganism;

at least one peptide component comprising a helper T-epitope; and

at least one lipid component comprising a Toll-like receptor (TLR) ligand comprising Pam_3CysSK_n, wherein n = 1, 2, 3, 4 or 5;

wherein the carbohydrate component and the peptide component are heterologous with respect to each other.

2. The glycolipopeptide of claim 1 wherein the microorganism is selected from the group consisting of a virus, a bacterium, a fungus, and a protozoan.

3. The glycolipopeptide of claim 1 wherein the microorganism is a human immunodeficiency virus.

4. The glycolipopeptide of claim 1 wherein the microorganism is a hepatitis C virus.

5. The glycolipopeptide of any one of claims 1 to 4 wherein the carbohydrate component comprises a glycosylated peptide.

6. The glycolipopeptide of any one of claims 1 to 5 further comprising at least one linker component that covalently links two or more of the glycolipopeptide components.

7. The glycolipopeptide of claim 6 wherein the linker component comprises a thiol-reactive linker component.

8. A glycolipopeptide comprising:

at least one carbohydrate component comprising a self-antigen comprising a B-epitope, wherein the B-epitope is from a cancer cell;

at least one peptide component comprising a helper T-epitope; and

at least one lipid component comprising a Toll-like receptor (TLR) ligand comprising Pam_3CysSK_n, wherein n = 1, 2, 3, 4 or 5.

9. The glycolipopeptide of claim 8 wherein the self-antigen comprises a MUC-1 glycopeptide.

10. The glycolipopeptide of any one of claims 1 to 9, wherein at least one peptide component comprises a helper T-epitope derived from polio virus.

11. The glycolipopeptide of claim 8 or 9, said glycolipopeptide comprising a glycopeptide that comprises both the carbohydrate component and the peptide component.

12. The glycolipopeptide of claim 11, wherein the glycopeptide is derived from a glycoprotein of mammalian origin.

13. The glycolipopeptide of claim 11 or 12 wherein the glycopeptide comprises both a B-epitope and a T-epitope.

14. The glycolipopeptide of claim 13 wherein the T-epitope comprises a $CD4^+$ epitope.

15. The glycolipopeptide of claim 13 wherein the T-epitope comprises a $CD8^+$ epitope.

16. The glycolipopeptide of any one of claims 11 to 15 wherein the glycopeptide comprises at least one GalNAc moiety, GlcNAc moiety, or combination thereof.

17. The glycolipopeptide of claim 9 wherein the MUC-1 glycopeptide comprises the amino acid sequence TSAPDT(αGalNAc)RPAP.

18. The glycolipopeptide of claim 8 or 9 wherein the B-epitope comprises the amino acid sequence PDT(αGalNΛc)R.

19. The glycolipopeptide of any one of claims 1 to 18 wherein the lipid component comprises Pam_3CysSK_4.

20. The glycolipopeptide of any one of claims 1 to 9 and 11 to 19 wherein the helper T-epitope comprises SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

21. A pharmaceutical composition comprising:

a glycolipopeptide comprising:

at least one carbohydrate component comprising a B-epitope, wherein the B-epitope is from a cancer cell;

at least one peptide component comprising a helper T-epitope; and

at least one lipid component comprising a Toll-like receptor (TLR) ligand comprising Pam_3CysSK_n, wherein n = 1, 2, 3, 4 or 5; and

a pharmaceutically acceptable carrier.

22. A pharmaceutical composition comprising:

a glycolipopeptide comprising:

at least one carbohydrate component comprising a B-epitope, wherein the B-epitope is from a microorganism;

at least one peptide component comprising a helper T-epitope; and

at least one lipid component comprising a Toll-like receptor (TLR) ligand comprising Pam_3CysSK_n, wherein n = 1, 2, 3, 4 or 5; and

a pharmaceutically acceptable carrier.

23. The pharmaceutical composition of claim 21 or 22 further comprising a liposome.

24. The pharmaceutical composition of claim 23 wherein the glycolipopeptide is covalently or noncovalently incorporated into the liposome.

25. The pharmaceutical composition of claim 24 wherein a plurality of glycolipopeptides are covalently or noncovalently incorporated in the liposome.

26. The pharmaceutical composition of claim 25 wherein at least some of the glycolipopeptides contain different B-epitopes.

27. The pharmaceutical composition of claim 25 wherein at least some of the glycolipopeptides contain the same B-epitopes.

28. The pharmaceutical composition of claim 21 or 22 which does not contain an external adjuvant.

29. The pharmaceutical composition of claim 21 or 22 further comprising an external adjuvant.

30. The pharmaceutical composition of claim 29 wherein the external adjuvant comprises QS-21.

31. The pharmaceutical composition of any one of claims 21 to 30, wherein the lipid component comprises Pam_3CysSK_4.

32. The pharmaceutical composition of any one of claims 21 to 31, wherein the helper T epitope comprises SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

33. A pharmaceutical composition comprising:

an antibody which specifically binds the glycolipopeptide of any one of claims 1 to 20; and

a pharmaceutically acceptable carrier.

34. Use of the glycolipopeptide of any one of claims 1 to 20 for the manufacture of a medicament to cause antibody production.

35. Use of the pharmaceutical composition of any one of claims 21 to 32 for causing antibody production in a subject.

36. A method for making the glycolipopeptide of any one of claims 1 to 20 comprising synthetically linking the carbohydrate component, the peptide component and the lipid component.

37. A polyclonal or monoclonal antibody which specifically binds the glycolipopeptide of any one of claims 1-20.

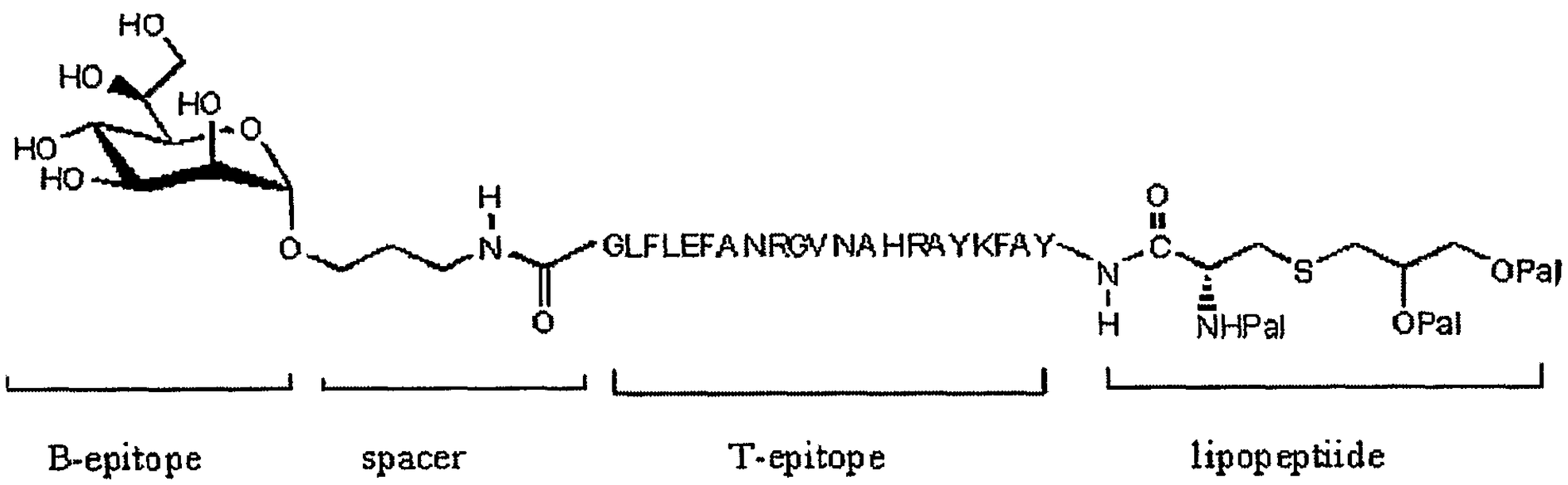

HO
HO
HO
HO
HO
O
O
N
O
GLFLEFANRGVNAHRAYKFAY
N
H
C
NHPal
S
OPal
OPal
B-epitope
spacer
T-epitope
lipopeptiide

Figure 1

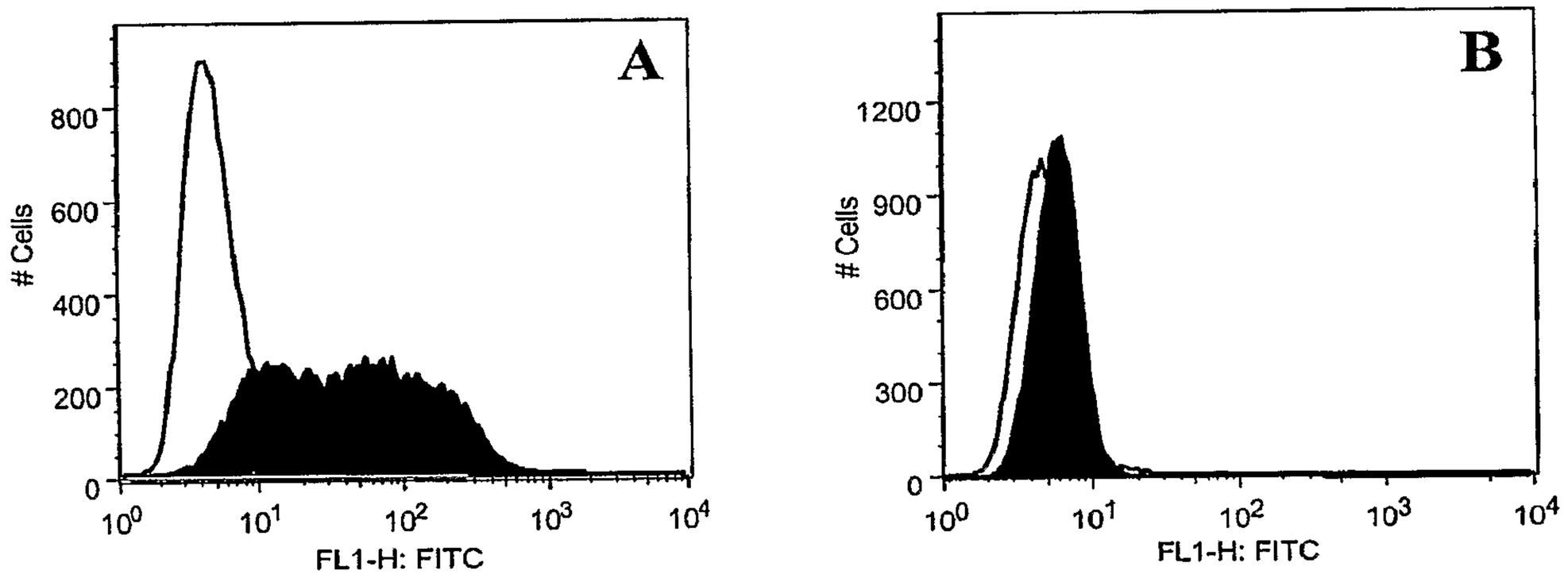

A
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600
400
200
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Cells
10^0
10^1
10^2
10^3
10^4
FL1-H: FITC
B
1200
900
600
300
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Cells
10^0
10^1
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10^3
10^4
FL1-H: FITC

Figure 2

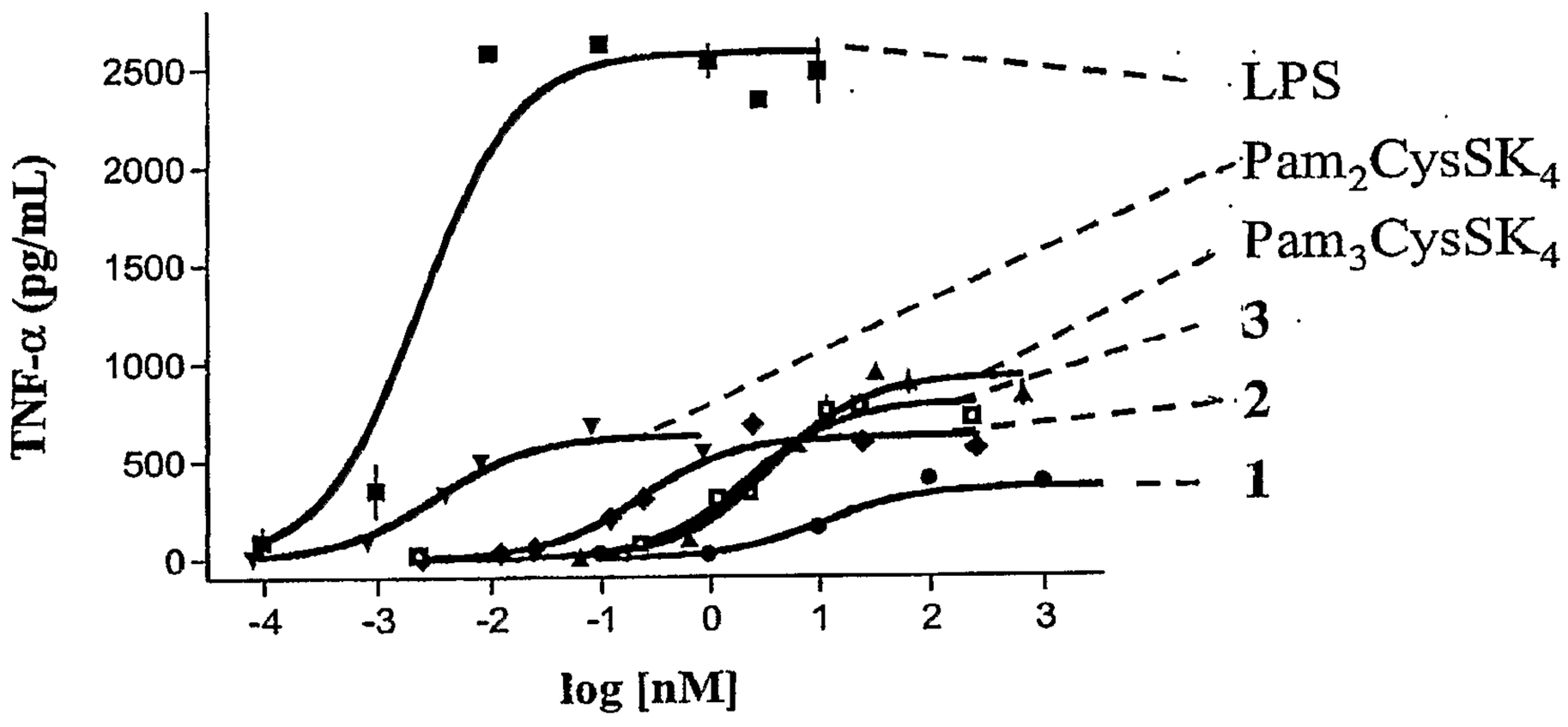

TNF-α (pg/mL)
2500
2000
1500
1000
500
0
-4
-3
-2
-1
0
1
2
3
log [nM]
LPS
Pam_2CysSK_4
Pam_3CysSK_4
3
2
1

Figure 3

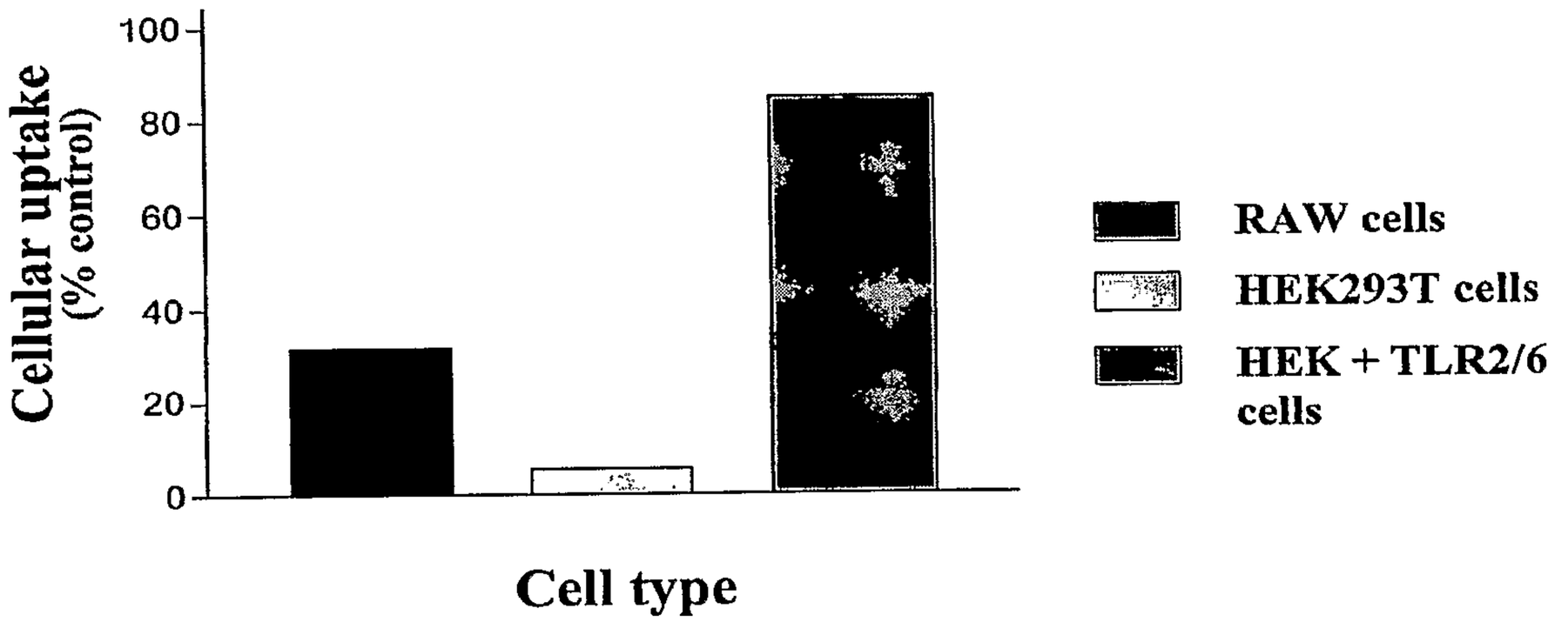

Cellular uptake
(% control)
100
80
60
40
20
0
Cell type
RAW cells
HEK293T cells
HEK + TLR2/6 cells

Figure 4