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**Pneumococcal polysaccharides and their use in immunogenic polysaccharide-carrier protein conjugates**

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(71) Applicant(s)  
**Merck Sharp & Dohme LLC**

(72) Inventor(s)  
**PORAMBO, Richard J.;ABEYGUNAWARDANA, Chitrananda;MUSEY, Luwy Kavuka;KOSINSKI, Michael J.;CUI, Yadong Adam;MCHUGH, Patrick;KONIETZKO, Janelle**

(74) Agent / Attorney  
**Spruson & Ferguson, GPO Box 3898, Sydney, NSW, 2001, AU**

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## (71) Applicant: MERCK SHARP &amp; DOHME CORP.

[US/US]; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US).

## (72) Inventors; and

## (71) Applicants (for US only): PORAMBO, Richard, J.

[US/US]; 770 Sumneytown Pike, West Point, Pennsylvania 19486 (US). ABEYGUNAWARDANA, Chitrananda

[US/US]; 351 N. Sumneytown Pike, North Wales, Pennsylvania 19454 (US). MUSEY, Luwy Kavuka [US/US]; 351 N. Sumneytown Pike, North Wales, Pennsylvania 19454 (US). KOSINSKI, Michael, J. [US/US]; 770 Sumneytown Pike, West Point, Pennsylvania 19486 (US). CUI, Yadong Adam [US/US]; 351 N. Sumneytown Pike, North Wales, Pennsylvania 19454 (US). MCHUGH, Patrick [US/US]; 770 Sumneytown Pike, West Point, Pennsylvania 19486 (US). KONIETZKO, Janelle [US/US]; 770 Sumneytown Pike, West Point, Pennsylvania 19486 (US).

## (74) Common Representative: MERCK SHARP &amp; DOHME

CORP.; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US).

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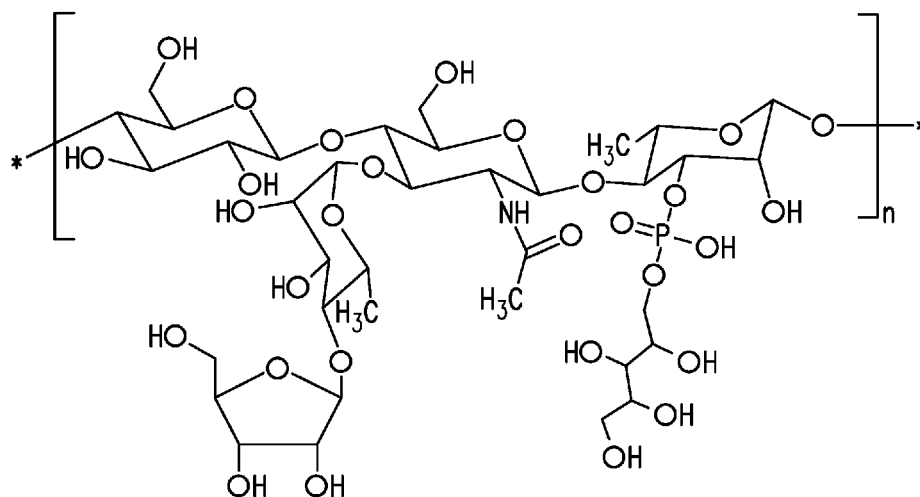


FIG.1A

(57) **Abstract:** The present invention provides capsular polysaccharides from *Streptococcus pneumoniae* serotypes identified using NMR. The present invention further provides polysaccharide-protein conjugates in which capsular polysaccharides from one or more of these serotypes are conjugated to a carrier protein such as CRM197. Polysaccharide-protein conjugates from one or more of these serotypes may be included in multivalent pneumococcal conjugate vaccines having polysaccharides from multiple additional *Streptococcus pneumoniae* serotypes.



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## TITLE OF THE INVENTION

PNEUMOCOCCAL POLYSACCHARIDES AND THEIR USE IN IMMUNOGENIC  
POLYSACCHARIDE-CARRIER PROTEIN CONJUGATES

## 5 FIELD OF INVENTION

The present invention provides purified capsular polysaccharides from *Streptococcus pneumoniae* serotype 24F, and polysaccharide-protein conjugates having polysaccharides from this serotype. Polysaccharide-protein conjugates from this serotype may be included in multivalent pneumococcal conjugate vaccines.

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## BACKGROUND OF THE INVENTION

*Streptococcus pneumoniae*, one example of an encapsulated bacterium, is a significant cause of serious disease world-wide. In 1997, the Centers for Disease Control and Prevention (CDC) estimated there were 3,000 cases of pneumococcal meningitis, 50,000 cases of pneumococcal bacteremia, 7,000,000 cases of pneumococcal otitis media and 500,000 cases of pneumococcal pneumonia annually in the United States. See Centers for Disease Control and Prevention, MMWR Morb Mortal Wkly Rep 1997, 46(RR-8):1-13. Furthermore, the complications of these diseases can be significant with some studies reporting up to 8% mortality and 25% neurologic sequelae with pneumococcal meningitis. See Arditi *et al.*, 1998, Pediatrics 102:1087-97.

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The multivalent pneumococcal polysaccharide vaccines that have been licensed for many years have proved invaluable in preventing pneumococcal disease in adults, particularly, the elderly and those at high-risk. However, infants and young children respond poorly to unconjugated pneumococcal polysaccharides. Bacterial polysaccharides are T-cell-independent immunogens, eliciting weak or no response in infants. Chemical conjugation of a bacterial polysaccharide immunogen to a carrier protein converts the immune response to a T-cell-dependent one in infants. Diphtheria toxoid (DTx, a chemically detoxified version of DT) and CRM197 have been described as carrier proteins for bacterial polysaccharide immunogens due to the presence of T-cell-stimulating epitopes in their amino acid sequences.

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The pneumococcal conjugate vaccine, Prevnar<sup>®</sup>, containing the 7 most frequently isolated serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) causing invasive pneumococcal disease in young children and infants at the time, was first licensed in the United States in February 2000. Following universal use of Prevnar<sup>®</sup> in the United States, there has been a significant reduction

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in invasive pneumococcal disease in children due to the serotypes present in Prevnar<sup>®</sup>. See Centers for Disease Control and Prevention, MMWR Morb Mortal Wkly Rep 2005, 54(36):893-7. However, there are limitations in serotype coverage with Prevnar<sup>®</sup> in certain regions of the world and some evidence of certain emerging serotypes in the United States (for example, 19A and others). See O'Brien *et al.*, 2004, Am J Epidemiol 159:634-44; Whitney *et al.*, 2003, N Engl J Med 348:1737-46; Kyaw *et al.*, 2006, N Engl J Med 354:1455-63; Hicks *et al.*, 2007, J Infect Dis 196:1346-54; Traore *et al.*, 2009, Clin Infect Dis 48:S181-S189.

Prevnar 13<sup>®</sup> is a 13-valent pneumococcal polysaccharide-protein conjugate vaccine including serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F. See, e.g., U.S. Patent Application Publication No. US 2006/0228380 A1, Prymula *et al.*, 2006, Lancet 367:740-48 and Kieninger *et al.*, Safety and Immunologic Non-inferiority of 13-valent Pneumococcal Conjugate Vaccine Compared to 7-valent Pneumococcal Conjugate Vaccine Given as a 4-Dose Series in Healthy Infants and Toddlers, presented at the 48<sup>th</sup> Annual ICAAC/ISDA 46<sup>th</sup> Annual Meeting, Washington DC, October 25-28, 2008. See, also, Dagan *et al.*, 1998, Infect Immun. 66: 2093-2098 and Fattom, 1999, Vaccine 17:126.

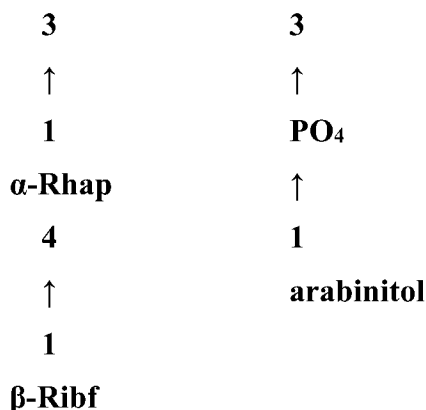
*S. pneumoniae* has been categorized into more than ninety serotypes based on the structure of the capsular polysaccharide. A list of known pneumococcal capsular polysaccharide structures is provided in Geno, 2015, Clinical Microbiology Reviews 28:871-899.

The current multivalent pneumococcal conjugate vaccines have been effective in reducing the incidence of pneumococcal disease associated with those serotypes present in the vaccines. However, the prevalence of the pneumococci expressing serotypes not present in the vaccine has been increasing. Accordingly, there is a need to identify and characterize emerging pneumococcal serotypes for inclusion in future vaccines.

## SUMMARY OF THE INVENTION

The present invention provides purified capsular polysaccharides from *Streptococcus pneumoniae* serotypes 24F, and polysaccharide protein conjugates having this serotype. The present invention is based, in part, on the structural identification of capsular polysaccharides from this serotype.

Accordingly, in a first aspect, the present invention provides a polysaccharide-carrier protein conjugate, wherein the polysaccharide comprises a repeating unit of the following structure:



wherein the carrier protein is CRM197; and further wherein the polysaccharide-carrier protein conjugate has a molecular weight from 1000 kDa to 10,000 kDa.

In a second aspect, the present invention provides an immunogenic composition comprising the polysaccharide carrier-protein conjugate of the first aspect, and a pharmaceutically acceptable carrier.

In a third aspect, the present invention provides a method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide, comprising administering to a human an immunologically effective amount of the immunogenic composition of the second aspect.

In a fourth aspect, the present invention provides use of the immunogenic composition of the second aspect in the manufacture of a medicament for inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide in a human.

In a fifth aspect, the present invention provides an immunogenic composition comprising the polysaccharide carrier-protein conjugate of the first aspect, and a pharmaceutically acceptable carrier.

In a sixth aspect, the present invention provides a method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide, comprising administering to a human an immunologically effective amount of the immunogenic composition of the fifth aspect.

In a seventh aspect, the present invention provides use of the immunogenic composition of the fifth aspect in the manufacture of a medicament for inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide in a human.

In one embodiment, the present invention provides a polysaccharide with the following repeating unit:



3

3

 $\uparrow$  $\uparrow$ 

1

PO<sub>4</sub> $\alpha\text{-Rhap}$ 

4

 $\uparrow$ 

1

arabinitol

 $\uparrow$ 

1

 $\beta\text{-Rib}f$ .

A polysaccharide from *Streptococcus pneumoniae* serotype 24F can be represented by



3

3

 $\uparrow$  $\uparrow$ 

1

PO<sub>4</sub> $\alpha\text{-Rhap}$ 

4

 $\uparrow$ 

1

arabinitol

 $\uparrow$ 

1

 $\beta\text{-Rib}f$ 

where n represents the number of repeating units.

In certain embodiments, the polysaccharide has between 5 and 5000 repeating units. In certain aspects, the polysaccharide has between 10 and 3,000, 20 and 2,000, 50 to 1,500, or 100 to 1,000 repeating units.

In certain embodiments, the polysaccharide has a molecular weight from 5 kDa to 5,000 kDa. In certain aspects, the polysaccharide has a molecular weight from 10 kDa to 2,000 kDa, 50 kDa to 1,500 kDa, or 100 kDa to 1,000 kDa.

The present invention further provides activated polysaccharides produced from any of the above embodiments wherein the polysaccharide is activated with a chemical reagent to produce reactive groups for conjugation to a linker or carrier protein. In certain embodiments, the activation occurs at the arabinitol-1-PO<sub>4</sub>. In certain embodiments, the polysaccharide is activated with periodate. In certain aspects of this embodiment, the activation occurs on the carbon 2 position of arabinitol-1-PO<sub>4</sub>.

The present invention further provides polysaccharide-protein conjugates in which polysaccharides or activated polysaccharides as provided for above are conjugated to a carrier protein. In certain aspects, the carrier protein is selected from CRM197, diphtheria toxin

fragment B (DTFB), DTFB C8, Diphtheria toxoid (DT), tetanus toxoid (TT), fragment C of TT, pertussis toxoid, cholera toxoid, *E. coli* LT, *E. coli* ST, and exotoxin A from *Pseudomonas aeruginosa*. In one specific aspect, the carrier protein is CRM197.

In certain aspects, the polysaccharide-protein conjugates are prepared using reductive amination chemistry under aqueous conditions or in an aprotic solvent such as dimethyl sulfoxide (DMSO). In a specific aspect, the polysaccharide-protein conjugates are prepared using reductive amination chemistry in DMSO.

In one embodiment, the present invention provides a multivalent immunogenic composition comprising unconjugated polysaccharides or polysaccharide-protein conjugates from *Streptococcus pneumoniae* serotype 24F, and unconjugated polysaccharides or polysaccharide-protein conjugates from one or more of *Streptococcus pneumoniae* serotypes 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 7B, 7C, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15A, 15B, 15C, 16F, 17F, 18B, 18C, 19A, 19F, 20, 21, 22A, 22F, 23A, 23B, 23F, 24B, 27, 28A, 31, 33F, 34, 35A, 35B, 35F, and 38. In one subembodiment, a multivalent immunogenic composition comprises unconjugated polysaccharides or polysaccharide-carrier protein conjugates but not both. In one subembodiment, a multivalent immunogenic composition comprises a mixture of unconjugated polysaccharides or polysaccharide-carrier protein conjugates. In certain subembodiments, a multivalent immunogenic composition of the invention has up to 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, or 90 serotypes.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**Figures 1A-B** depict graphical representation of the repeating unit structure of A) *S. pneumoniae* serotype 24F polysaccharide; and B) periodate activated *S. pneumoniae* serotype 24F repeating structure. Note that the structure shown in Figure 1B is to show the predominant activation site (as an activated saccharide). Within a polysaccharide chain, it is not necessary that all of the repeating units will be activated as drawn in Figure 1B.

**Figure 2** depicts the 600 MHz one-dimensional <sup>1</sup>H NMR spectrum of the capsular polysaccharide from *S. pneumoniae* serotype 24F in D<sub>2</sub>O at 50°C. Signals arising from internal standards (DMSO and DSS-d<sub>6</sub>), residual water (HOD) and other residual components from the purification process; ethanol (EtOH), isopropanol (IPA) and acetate, are marked. Minor signals marked by \* are due to *S. pneumoniae* cell wall residuals such as C-polysaccharide and/or peptidoglycans.



**Figure 3** depicts the one-dimensional (1D)  $^1\text{H}$  NMR identity region to be used for serotype identifications of *S. pneumoniae* serotype 24F. Signal positions of each anomeric proton of the repeating unit from each monosaccharide residue is marked.

**Figure 4** depicts partial two-dimensional (2D)  $^1\text{H} - ^{13}\text{C}$  multiple bond correlation NMR

5 spectrum of *S. pneumoniae* serotype 24F establishing covalent linkages between sugar residues in the repeating structure. Correlation establishing glycosidic linkages are labeled in the figure.

**Figure 5** depicts establishment of phosphodiester linkages in the capsular polysaccharide repeating unit of *S. pneumoniae* serotype 24F.

**Figure 6** Top spectrum; 1D TOCSY of activated serotype 24F polysaccharide with excitation

10 centered at the peak at 5.19 ppm showing a single correlation to signal at 3.86 ppm. Bottom spectrum is a standard 1D proton spectrum of serotype 24F identifying the triplet at 5.19 ppm.

**Figure 7** Top spectrum shows the 1D TOCSY of activated serotype 24F polysaccharide with excitation centered at the signal at 5.19 ppm. Bottom figure is the multiplicity edited gHSQCAD. Filled ovals are methyl and methine groups, open ovals are methylenes.

15 **Figure 8** shows ELISA IgG antibody titers (post-dose 2) for rabbits immunized with *S. pneumoniae* monovalent serotypes conjugated to CRM197 and formulated with aluminum phosphate adjuvant (APA). Error bars represent the geometric mean + 95% confidence interval.

**Figure 9** shows serotype specific OPA titers (post-dose 2) for rabbits immunized with *S. pneumoniae* monovalent serotypes conjugated to CRM197 and formulated with aluminum

20 phosphate adjuvant (APA). Error bars represent the geometric mean + 95% confidence interval.

**Figure 10** shows protection from *S. pneumoniae* serotype 24F intratracheal challenge of C57BL/6 mice immunized with 24F-CRM197 monovalent conjugate vaccine formulated with aluminum phosphate adjuvant (APA). Mantel Cox log-rank test indicates that C57BL/6 mice immunized with 24F-CRM197/APA vaccine were significantly protected from challenge

25 compared to both APA immunized mice ( $P=0.0004$ ) and naïve mice ( $P<0.0001$ ).

**Figure 11** shows protection from *S. pneumoniae* serotype 24F intratracheal challenge of CD-1 mice immunized with 24F-CRM197 monovalent conjugate vaccine formulated with aluminum phosphate adjuvant (APA). Mantel Cox log-rank test indicates that CD-1 mice immunized with 24F-CRM197/APA vaccine were significantly protected from challenge compared to both APA

30 immunized mice ( $P=0.0099$ ) and naïve mice ( $P=0.0076$ ).  
**Figure 12** shows protection from *S. pneumoniae* serotype 24F intratracheal challenge of Swiss Webster mice immunized with 24F-CRM197 monovalent conjugate vaccine formulated with aluminum phosphate adjuvant (APA). Mantel Cox log-rank test indicates that Swiss Webster

mice immunized with 24F-CRM197/APA vaccine were significantly protected from challenge compared to both APA immunized mice ( $P < 0.0001$ ) and naïve mice ( $P = 0.0002$ ).

**Figure 13** shows serotype specific (*S. pneumoniae* serotypes 16F, 23A, 23B, 24F, 31) pre-immune, PD1 and PD2 geometric mean antibody titers for rabbits immunized with a multivalent pneumococcal conjugate vaccine (2 µg/PnPs). Error bars represent 2 standard errors of the geometric mean titer of each serotype (X-axis).

**Figure 14** shows serotype specific (*S. pneumoniae* serotypes 16F, 23A, 23B, 24F, 31) pre-immune, PD1 and PD2 OPA dilution titers for rabbits immunized with a multivalent pneumococcal conjugate vaccine (2 µg/PnPs). Symbols indicate the individual titers and error bars represent the 95% confidence intervals (CIs) of the geometric mean titers (GMTs). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns=not significant.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is based, in part, on the identification of novel pneumococcal polysaccharide structure(s) by NMR technology. It is believed that the structure provided herein is the first identification or the first correct identification of *S. pneumoniae* serotype 24F.

The *S. pneumoniae* serotype 24F polysaccharide was produced from its respective strain and purified. The produced (and purified) polysaccharides were used to generate individual Ps-CRM197 conjugates. *S. pneumoniae* serotype 24F has a unique polysaccharide, which results in a unique conjugate production process. The resulting conjugate(s) were demonstrated to be immunogenic in animal studies.

As used herein, the term "polysaccharide" (Ps) is meant to include any antigenic saccharide element (or antigenic unit) commonly used in the immunologic and bacterial vaccine arts, including, but not limited to, a "saccharide", an "oligosaccharide", a "polysaccharide", a "liposaccharide", a "lipo-oligosaccharide (LOS)", a "lipopolysaccharide (LPS)", a "glycosylate", a "glycoconjugate", a "derivatized or activated polysaccharide or oligosaccharide", and the like. Unless otherwise specified, the polysaccharide nomenclature used herein follows the IUB-IUPAC Joint Commission on Biochemical Nomenclature (JCBM) Recommendations 1980. See JCBN, 1982, J. Biol. Chem. 257:3352-3354.

As used herein, "immunogenic composition" refers to a composition containing an antigen, such as a bacterial capsular polysaccharide or a polysaccharide-protein conjugate, that has the ability to elicit an immune response in a host such as a mammal, either humorally or

cellularly mediated, or both. The immunogenic composition may serve to sensitize the host by the presentation of the antigen in association with MHC molecules at a cell surface. In addition, antigen-specific T-cells or antibodies can be generated to allow for the future protection of an immunized host. Immunogenic compositions thus can protect the host from infection by the bacteria, reduced severity, or may protect the host from death due to the bacterial infection. . Immunogenic compositions may also be used to generate polyclonal or monoclonal antibodies, which may be used to confer passive immunity to a subject. Immunogenic compositions may also be used to generate antibodies that are functional as measured by the killing of bacteria in either an animal efficacy model or via an opsonophagocytic killing assay.

As used herein, the term “isolated” in connection with a polysaccharide refers to isolation of *S. pneumoniae* serotype specific capsular polysaccharide from purified polysaccharide using purification techniques known in the art, including the use of centrifugation, depth filtration, precipitation, ultrafiltration, treatment with activate carbon, diafiltration and/or column chromatography. Generally an isolated polysaccharide refers to partial removal of proteins, nucleic acids and non-specific endogenous polysaccharide (C-polysaccharide). The isolated polysaccharide contains less than 10%, 8%, 6%, 4%, or 2% protein impurities and/or nucleic acids. The isolated polysaccharide contains less than 20% of C-polysaccharide with respect to type specific polysaccharides.

As used herein, the term “purified” in connection with a bacterial capsular polysaccharide refers to the purification of the polysaccharide from cell lysate through means such as centrifugation, precipitation, and ultra-filtration. Generally, a purified polysaccharide refers to removal of cell debris and DNA.

As used herein, the term “Mw” refers to the weight averaged molecular weight and is typically expressed in Da or kDa. Mw takes into account that a bigger molecule contains more of the total mass of a polymer sample than the smaller molecules do. Mw can be determined by techniques such as static light scattering, small angle neutron scattering, X-ray scattering, and sedimentation velocity.

As used herein, the term “Mn” refers to a number average molecular weight and is typically expressed in Da or kDa. Mn is calculated by taking the total weight of a sample divided by the number of molecules in the sample and can be determined by techniques such as gel permeation chromatography, viscometry via the (Mark–Houwink equation), colligative methods such as vapor pressure osmometry, end-group determination or proton NMR. Mw/Mn reflects polydispersity.

As used herein, the term “molar ratio” is a fraction typically expressed as a decimal to the tenths or hundredths place. For example, a molar ratio of from 0 or 0.1 to 1.0 expressed in tenths will include any of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1.0.

As used herein, the abbreviation “PnPs” refers to pneumococcal polysaccharide.

5 As used herein, the term "comprises" when used with the immunogenic composition of the invention refers to the inclusion of any other components (subject to limitations of "consisting of" language for the antigen mixture), such as adjuvants and excipients. The term "consisting of" when used with the multivalent polysaccharide-protein conjugate mixture of the invention refers to a mixture having those particular *S. pneumoniae*  
10 polysaccharide protein conjugates and no other *S. pneumoniae* polysaccharide protein conjugates from a different serotype.

As used herein, the phrase “activation site” on a sugar means that the site can be chemically modified to form a reactive group. Activation site takes into account the preferred tendency of an activation agent to react at a specific site.

15 As used herein, the phrase “activated polysaccharide” refers to a polysaccharide that has been chemically modified to form reactive groups in a polysaccharide chain. An activated polysaccharide does not necessarily mean that all the available activation sites have been chemically modified.

As used herein, the phrase “extent of activation” on a polysaccharide chain refers  
20 to the overall ratio between the number of activated chemical group to the number of repeat units on the polysaccharide chain.

Unless otherwise specified, all ranges provided herein are inclusive of the recited lower and upper limits.

Monosaccharide analysis of *S. pneumoniae* serotype 24F showed Glc, Rha, GlcN,  
25 Rib (ribose), and Rib-ol-*P* (ribose phosphate) as constituents (but no structure information has been available). See Kamerling, 2000, Pneumococcal polysaccharides: a chemical view, p. 81-114. In Tomasz (ed), *Streptococcus pneumoniae* molecular biology & mechanisms of disease. Mary Ann Liebert, Inc., Larchmont, NY. The structure identified in the Examples is consistent with the saccharide composition previously identified. No structure information has been  
30 available to date for serotype 24A or 24B polysaccharides for comparison.

The identification of the structure for these polysaccharide serotype(s) may allow their incorporation into pneumococcal vaccines, either unconjugated or as a polysaccharide-

protein conjugate. Conjugate vaccines comprising streptococcal and pneumococcal Ps are well-known in the art. See e.g., U.S. Pat. Nos. 6,248,570; 5,866,135; and 5,773,007.

#### Capsular polysaccharides

5                   Capsular polysaccharides from *Streptococcus pneumoniae* from the serotype(s) of the invention can be prepared by standard techniques known to those skilled in the art. For example, polysaccharides can be isolated from bacteria and may be sized to some degree by known methods (see, e.g., European Patent Nos. EP497524 and EP497525); and preferably by microfluidisation accomplished using a homogenizer or by chemical hydrolysis. In one  
10   embodiment, *S. pneumoniae* strains corresponding to each polysaccharide serotype are grown in a soy-based medium. The individual polysaccharides are then purified through standard steps including centrifugation, precipitation, and ultra-filtration. See, e.g., U.S. Patent Application Publication No. 2008/0286838 and U.S. Pat. No. 5,847,112. Polysaccharides can be sized in order to reduce viscosity and/or to improve filterability of subsequent conjugated products.  
15   Chemical hydrolysis may be conducted using acetic acid. Mechanical sizing may be conducted using High Pressure Homogenization Shearing.

                  In some embodiments, the purified polysaccharides before conjugation have a molecular weight of between 5 kDa and 4,000 kDa. Molecular weight can be calculated by size exclusion chromatography (SEC) combined with multiangle light scattering detector (MALS)  
20   and refractive index detector (RI). In other such embodiments, the polysaccharide has an average molecular weight of between 10 kDa and 4,000 kDa; between 50 kDa and 4,000 kDa; between 50 kDa and 3,000 kDa; between 50 kDa and 2,000 kDa; between 50 kDa and 1,500 kDa; between 50 kDa and 1,000 kDa; between 50 kDa and 750 kDa; between 50 kDa and 500 kDa; between 100 kDa and 4,000 kDa; between 100 kDa and 3,000 kDa; between 5 kDa and  
25   2,000 kDa; between 10 kDa and 2,000 kDa; between 50 kDa and 2,000 kDa; between 100 kDa and 2,000 kDa; between 100 kDa and 1,500 kDa; between 100 kDa and 1,000 kDa; between 100 kDa and 750 kDa; between 100 kDa and 500 kDa; between 100 and 400 kDa; between 200 kDa and 4,000 kDa; between 200 kDa and 3,000 kDa; between 200 kDa and 2,000 kDa; between 200 kDa and 1,500 kDa; between 200 kDa and 1,000 kDa; or between 200 kDa and 500 kDa. In  
30   certain embodiments, the polysaccharide has a molecular weight from 50 kDa to 5,000 kDa. In certain aspects, the polysaccharide has a molecular weight from 10 kDa to 2,000 kDa, 50 kDa to 1,500 kDa, or 75 kDa to 200 kDa.

In certain embodiments, the *S. pneumoniae* serotype 24F polysaccharide has between 5 and 2,000 repeating units. In certain aspects, the polysaccharide has between 15 and 1,500, 20 and 400, 30 to 300, 40 to 250, or 50 to 200 repeating units.

## 5 Carrier Protein

Polysaccharides from one or more of the serotypes can be conjugated to a carrier protein ("Pr") to improve immunogenicity in children, the elderly and/or immunocompromised subjects. Where more than one serotype is used in a multivalent composition, the serotypes may be prepared with the same carrier protein or different carrier proteins. Each capsular polysaccharide of the same serotype is typically conjugated to the same carrier protein.

In a particular embodiment of the present invention, CRM197 is used as a carrier protein. CRM197 is a non-toxic variant of diphtheria toxin (DT). The CRM197 carrier protein is a mutant form of DT that is rendered non-toxic by a single amino acid substitution in Fragment A at residue 52. In one embodiment, the CRM197 carrier protein is isolated from cultures of *Corynebacterium diphtheria* strain C7 ( $\beta$ 197) grown in casamino acids and yeast extract-based medium. In another embodiment, CRM197 is prepared recombinantly in accordance with the methods described in U.S. Pat. No. 5,614,382. Typically, CRM197 is purified through a combination of ultra-filtration, ammonium sulfate precipitation, and ion-exchange chromatography. In some embodiments, CRM197 is prepared in *Pseudomonas fluorescens* using Pfenex Expression Technology™ (Pfenex Inc., San Diego, CA).

Other suitable carrier proteins include additional inactivated bacterial toxins such as DT, Diphtheria toxoid fragment B (DTFB), TT (tetanus toxoid) or fragment C of TT, pertussis toxoid, cholera toxoid (e.g., as described in International Patent Application Publication No. WO 2004/083251), *E. coli* LT (heat-labile enterotoxin), *E. coli* ST (heat-stable enterotoxin), and exotoxin A from *Pseudomonas aeruginosa*. Bacterial outer membrane proteins such as outer membrane complex c (OMPC), porins, transferrin binding proteins, pneumococcal surface protein A (PspA; See International Application Patent Publication No. WO 02/091998), pneumococcal adhesin protein (PsaA), C5a peptidase from Group A or Group B streptococcus, or *Haemophilus influenzae* protein D, pneumococcal pneumolysin (Kuo *et al.*, 1995, Infect Immun 63; 2706-13) including ply detoxified in some fashion for example dPLY-GMBS (See International Patent Application Publication No. WO 04/081515) or dPLY-formol, PhtX, including PhtA, PhtB, PhtD, PhtE and fusions of Pht proteins for example PhtDE fusions, PhtBE fusions (See International Patent Application Publication Nos. WO 01/98334 and WO

03/54007), can also be used. Other proteins, such as ovalbumin, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or purified protein derivative of tuberculin (PPD), PorB (from *N. meningitidis*), PD (*Haemophilus influenzae* protein D; see, e.g., European Patent No. EP 0 594 610 B), or immunologically functional equivalents thereof, synthetic peptides (See  
 5 European Patent Nos. EP0378881 and EP0427347), heat shock proteins (See International Patent Application Publication Nos. WO 93/17712 and WO 94/03208), pertussis proteins (See International Patent Application Publication No. WO 98/58668 and European Patent No. EP0471177), cytokines, lymphokines, growth factors or hormones (See International Patent Application Publication No. WO 91/01146), artificial proteins comprising multiple human CD4+  
 10 T cell epitopes from various pathogen derived antigens (See Falugi *et al.*, 2001, Eur J Immunol 31:3816-3824) such as N19 protein (See Baraldoi *et al.*, 2004, Infect Immun 72:4884-7), iron uptake proteins (See International Patent Application Publication No. WO 01/72337), toxin A or B of *C. difficile* (See International Patent Publication No. WO 00/61761), and flagellin (See Ben-Yedidia *et al.*, 1998, Immunol Lett 64:9) can also be used as carrier proteins.

15 Other DT mutants can also be used as the carrier protein, such as CRM176, CRM228, CRM45 (Uchida *et al.*, 1973, J Biol Chem 218:3838-3844); CRM9, CRM45, CRM102, CRM103 and CRM107 and other mutations described by Nicholls and Youle in Genetically Engineered Toxins, Ed: Frankel, Maecel Dekker Inc, 1992; deletion or mutation of Glu-148 to Asp, Gln or Ser and/or Ala 158 to Gly and other mutations disclosed in U.S. Pat. No.  
 20 4,709,017 or U.S. Pat. No. 4,950,740; mutation of at least one or more residues Lys 516, Lys 526, Phe 530 and/or Lys 534 and other mutations disclosed in U.S. Pat. No. 5,917,017 or U.S. Pat. No. 6,455,673; or fragment disclosed in U.S. Pat. No. 5,843,711.

Where multivalent vaccines are used, a second carrier protein can be used for one or more of the antigens. The second carrier protein is preferably a protein that is non-toxic and  
 25 non-reactogenic and obtainable in sufficient amount and purity. The second carrier protein is also conjugated or joined with an antigen, e.g., a *S. pneumoniae* polysaccharide to enhance immunogenicity of the antigen. Carrier proteins should be amenable to standard conjugation procedures. In one embodiment, each capsular polysaccharide not conjugated to the first carrier protein is conjugated to the same second carrier protein (e.g., each capsular polysaccharide  
 30 molecule being conjugated to a single carrier protein). In another embodiment, the capsular polysaccharides not conjugated to the first carrier protein are conjugated to two or more carrier proteins (each capsular polysaccharide molecule being conjugated to a single carrier protein). In

such embodiments, each capsular polysaccharide of the same serotype is typically conjugated to the same carrier protein.

### Conjugation

5 Prior to conjugation, the purified polysaccharides can be chemically activated to make the saccharides capable of reacting with the carrier protein to form an activated polysaccharide. As used herein, the term “activated polysaccharide” refers to a polysaccharide that has been chemically modified as described below to enable conjugation to a linker or a carrier protein. The purified polysaccharides can optionally be connected to a linker. Once  
10 activated or connected to a linker, each capsular polysaccharide is separately conjugated to a carrier protein to form a glycoconjugate. The polysaccharide conjugates may be prepared by known coupling techniques.

The present invention further provides activated polysaccharides produced from any of the above embodiments wherein the polysaccharide is activated with a chemical reagent  
15 to produce reactive groups for conjugation to a linker or carrier protein. In certain embodiments, the activation occurs at the arabinitol-1-PO<sub>4</sub>. In certain embodiments, the polysaccharide is activated with periodate. In certain aspects of this embodiment, the activation occurs on the carbon 2 position of arabinitol-1-PO<sub>4</sub>. See Figure 1B for the structure of the periodate activated serotype 24F polysaccharide. The formation of an aldehyde predominantly  
20 at the second carbon was surprising in that it requires selective cleavage of 2,3-carbons with vicinal diols or preferred continued series of oxidation at higher numbered carbons with vicinal diols (or aldehyde) until finally oxidation at 2<sup>nd</sup> carbon of the arabinitol sugar. In certain embodiments, the present invention provides activated polysaccharides and mixtures of activated polysaccharides wherein greater than 50%, 60%, 70% or 80% of the aldehyde groups (through  
25 periodate activation) are at the arabinitol. In one aspect of this embodiment, the activated polysaccharide has a structure as shown in Figure 1B.

In certain embodiments, the polysaccharide can be coupled to a linker to form a polysaccharide-linker intermediate in which the free terminus of the linker is an ester group. The linker is therefore one in which at least one terminus is an ester group. The other terminus is  
30 selected so that it can react with the polysaccharide to form the polysaccharide-linker intermediate.

In certain embodiments, the polysaccharide can be coupled to a linker using a primary amine group in the polysaccharide. In this case, the linker typically has an ester group



at both termini. This allows the coupling to take place by reacting one of the ester groups with the primary amine group in the polysaccharide by nucleophilic acyl substitution. The reaction results in a polysaccharide-linker intermediate in which the polysaccharide is coupled to the linker via an amide linkage. The linker is therefore a bifunctional linker that provides a first  
5 ester group for reacting with the primary amine group in the polysaccharide and a second ester group for reacting with the primary amine group in the carrier molecule. A typical linker is adipic acid N-hydroxysuccinimide diester (SIDEA).

In certain embodiments, the coupling can also take place indirectly, i.e. with an additional linker that is used to derivatise the polysaccharide prior to coupling to the linker. The  
10 polysaccharide is coupled to the additional linker using a carbonyl group at the reducing terminus of the polysaccharide. This coupling comprises two steps: (a1) reacting the carbonyl group with the additional linker; and (a2) reacting the free terminus of the additional linker with the linker. In these embodiments, the additional linker typically has a primary amine group at both termini, thereby allowing step (a1) to take place by reacting one of the primary amine  
15 groups with the carbonyl group in the polysaccharide by reductive amination. A primary amine group is used that is reactive with the carbonyl group in the polysaccharide. Hydrazide or hydroxylamino groups are suitable. The same primary amine group is typically present at both termini of the additional linker. The reaction results in a polysaccharide-additional linker intermediate in which the polysaccharide is coupled to the additional linker via a C—N linkage.

In certain embodiments, the polysaccharide can be coupled to the additional  
20 linker using a different group in the polysaccharide, particularly a carboxyl group. This coupling comprises two steps: (a1) reacting the group with the additional linker; and (a2) reacting the free terminus of the additional linker with the linker. In this case, the additional linker typically has a primary amine group at both termini, thereby allowing step (a1) to take place by reacting one of  
25 the primary amine groups with the carboxyl group in the polysaccharide by EDAC activation. A primary amine group is used that is reactive with the EDAC-activated carboxyl group in the polysaccharide. A hydrazide group is suitable. The same primary amine group is typically present at both termini of the additional linker. The reaction results in a polysaccharide-additional linker intermediate in which the polysaccharide is coupled to the additional linker via  
30 an amide linkage.

In one embodiment, the chemical activation of the polysaccharides and subsequent conjugation to the carrier protein by reductive amination can be achieved by means described in U.S. Pat. Nos. 4,365,170, 4,673,574 and 4,902,506, U.S. Patent Application

Publication Nos. 2006/0228380, 2007/184072, 2007/0231340 and 2007/0184071, and International Patent Application Publication Nos. WO2006/110381, WO2008/079653, and WO2008/143709. The chemistry may entail the activation of pneumococcal polysaccharide by reaction with any oxidizing agent which a primary hydroxyl group to an aldehyde, such as  
5 TEMPO in the presence of oxidant (WO2104/097099), or reacting two vicinal hydroxyl groups to aldehydes, such as periodate (including sodium periodate, potassium periodate, or periodic acid). The reactions lead to a random oxidation of primary hydroxyl groups or random oxidative cleavage of vicinal hydroxyl groups of the carbohydrates with the formation of reactive aldehyde groups.

10 In this embodiment, coupling to the carrier protein is by reductive amination via direct amination to the lysyl groups of the protein. For example, conjugation is carried out by reacting a mixture of the activated polysaccharide and carrier protein with a reducing agent such as sodium cyanoborohydride in the presence of nickel. The conjugation reaction may take place under aqueous solution or in the presence of dimethyl sulfoxide (DMSO). See, e.g., U.S. Patent  
15 Application Publication Nos. US2015/0231270 and US2011/0195086 and European Patent No. EP 0471 177 B1. Unreacted aldehydes are then capped with the addition of a strong reducing agent, such as sodium borohydride.

Reductive amination involves two steps, (1 ) oxidation of the polysaccharide to form reactive aldehydes, (2) reduction of the imine (Schiff base) formed between activated  
20 polysaccharide and a carrier protein to form a stable amine conjugate bond. Before oxidation, the polysaccharide is optionally size reduced. Mechanical methods (e.g. homogenization) or chemical hydrolysis may be employed. Chemical hydrolysis may be conducted using acetic acid. The oxidation step may involve reaction with periodate. For the purpose of the present invention, the term "periodate" includes both periodate and periodic acid; the term also includes  
25 both metaperiodate ( $\text{IO}_4^-$ ) and orthoperiodate ( $\text{IO}_6^{5-}$ ) and includes the various salts of periodate (e.g. , sodium periodate and potassium periodate). In an embodiment the capsular polysaccharide is oxidized in the presence of metaperiodate, preferably in the presence of sodium periodate ( $\text{NaIO}_4$ ). In another embodiment the capsular polysaccharide is oxydized in the presence of orthoperiodate, preferably in the presence of periodic acid.

30 In an embodiment, the oxidizing agent is a stable nitroxyl or nitroxide radical compound, such as piperidine-N-oxy or pyrrolidine-N-oxy compounds, in the presence of an oxidant to selectively oxidize primary hydroxyls (as described in, for example, International Patent Application Publication No. WO 2014/097099). In said reaction, the actual oxidant is the

N-oxoammonium salt, in a catalytic cycle. In an aspect, said stable nitroxyl or nitroxide radical compound are piperidine-N-oxy or pyrrolidine-N-oxy compounds. In an aspect, said stable nitroxyl or nitroxide radical compound bears a TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) or a PROXYL (2,2,5,5-tetramethyl-1-pyrrolidinyloxy) moiety. In an aspect, said stable nitroxyl radical compound is TEMPO or a derivative thereof. In an aspect, said oxidant is a molecule bearing a N-halo moiety. In an aspect, said oxidant is selected from the group consisting of N-Chlorosuccinimide, N-Bromosuccinimide, N-Iodosuccinimide, Dichloroisocyanuric acid, 1,3,5-trichloro-1,3,5-triazine-2,4,6-trione, Dibromoisocyanuric acid, 1,3,5-tribromo-1,3,5-triazine-2,4,6-trione, Diiodoisocyanuric acid and 1,3,5-triiodo-1,3,5-triazine-2,4,6-trione. Preferably said oxidant is N-Chlorosuccinimide.

In certain aspects, the oxidizing agent is 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO) free radical and N-Chlorosuccinimide (NCS) as the cooxidant (as described in International Patent Application Publication No. WO2014/097099). Therefore in one aspect, the glycoconjugates from *S. pneumoniae* are obtainable by a method comprising the steps of: a) reacting a saccharide with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and N-chlorosuccinimide (NCS) in an aqueous solvent to produce an activated saccharide; and b) reacting the activated saccharide with a carrier protein comprising one or more amine groups (said method is designated "TEMPO/NCS-reductive amination" thereafter).

Optionally the oxidation reaction is quenched by addition of a quenching agent.

The quenching agent maybe selected from vicinal diols, 1,2-aminoalcohols, amino acids, glutathione, sulfite, bisulfate, dithionite, metabisulfite, thiosulfate, phosphites, hypophosphites or phosphorous acid (such as glycerol, ethylene glycol, propan-1,2-diol, butan-1,2-diol or butan-2,3-diol, ascorbic acid).

The second step of the conjugation process for reductive amination is the reduction of the imine (Schiff base) bond between activated polysaccharide and a carrier protein to form a stable conjugate bond (so-called reductive amination), using a reducing agent. Reducing agents which are suitable include the cyanoborohydrides (such as sodium cyanoborohydride) or sodium borohydride. In one embodiment the reducing agent is sodium cyanoborohydride.

In certain embodiments of the methods of the invention, the reductive amination reaction is carried out in aprotic solvent (or a mixture of aprotic solvents). In an embodiment, the reduction reaction is carried out in DMSO (dimethyl sulfoxide) or in DMF (dimethylformamide) solvent. The DMSO or DMF solvent may be used to reconstitute the

activated polysaccharide and carrier protein, if lyophilized. In one embodiment, the aprotic solvent is DMSO.

At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, which may be capped or quenched using a suitable capping or  
 5 quenching agent. In one embodiment this capping or quenching agent is sodium borohydride ( $\text{NaBH}_4$ ). Suitable alternatives include sodium triacetoxymethylborohydride or sodium or zinc borohydride in the presence of Bronsted or Lewis acids), amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, *t*-BuMe'PrN- $\text{BH}_3$ , benzylamine- $\text{BH}_3$  or 5-ethyl-2-methylpyridine borane (PEMB) or borohydride exchange resin.

10 Glycoconjugates prepared using reductive amination in an aprotic solvent are generally used in multivalent pneumococcal conjugate vaccines. Thus, in certain embodiments for multivalent compositions where not all the serotypes are prepared in an aprotic solvent, the reduction reaction for the remaining serotypes is carried out in aqueous solvent (e.g., selected from PBS (phosphate buffered saline), MES (2-(*N*-morpholino)ethanesulfonic acid), HEPES, (4-  
 15 (2-hydroxyethyl)-1-piperazineethanesulfonic acid), Bis-tris, ADA (N-(2-Acetamido)iminodiacetic acid), PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid)), MOPSO (3-Morpholino-2-hydroxypropanesulfonic acid), BES (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid), MOPS (3-(N-morpholino)propanesulfonic acid), DIPSO (3-Bis(2-hydroxyethyl) amino-2-hydroxypropane-1-sulfonic acid), MOBS (4-(N-  
 20 morpholino)butanesulfonic acid), HEPPSO (N-(2-Hydroxyethyl)piperazine-N-(2-hydroxypropanesulfonic acid)), POPSO (Piperazine-1,4-bis(2-hydroxy-3-propanesulfonic acid)), TEA (triethanolamine), EPPS (4-(2-Hydroxyethyl)piperazine-1-propanesulfonic acid), Bicine or HEPB, at a pH between 6.0 and 8.5, 7.0 and 8.0, or 7.0 and 7.5).

In some embodiments, the glycoconjugates of the present invention comprise a  
 25 polysaccharide having a molecular weight of between 10 kDa and 10,000 kDa. In other such embodiments, the polysaccharide has a molecular weight of between 25 kDa and 5,000 kDa. In other such embodiments, the polysaccharide has a molecular weight of between 50 kDa and 1,000 kDa. In other such embodiments, the polysaccharide has a molecular weight of between 70 kDa and 900 kDa. In other such embodiments, the polysaccharide has a molecular weight of  
 30 between 100 kDa and 800 kDa. In other such embodiments, the polysaccharide has a molecular weight of between 200 kDa and 600 kDa. In further such embodiments, the polysaccharide has a molecular weight of 100 kDa to 1,000 kDa; 100 kDa to 900 kDa; 100 kDa to 800 kDa; 100 kDa to 700 kDa; 100 kDa to 600 kDa; 100 kDa to 500 kDa; 100 kDa to 400 kDa; 100 kDa to 300

kDa; 150 kDa to 1,000 kDa; 150 kDa to 900 kDa; 150 kDa to 800 kDa; 150 kDa to 700 kDa; 150 kDa to 600 kDa; 150 kDa to 500 kDa; 150 kDa to 400 kDa; 150 kDa to 300 kDa; 200 kDa to 1,000 kDa; 200 kDa to 900 kDa; 200 kDa to 800 kDa; 200 kDa to 700 kDa; 200 kDa to 600 kDa; 200 kDa to 500 kDa; 200 kDa to 400 kDa; 200 kDa to 300; 250 kDa to 1,000 kDa; 250 kDa to 900 kDa; 250 kDa to 800 kDa; 250 kDa to 700 kDa; 250 kDa to 600 kDa; 250 kDa to 500 kDa; 250 kDa to 400 kDa; 250 kDa to 350 kDa; 300 kDa to 1,000 kDa; 300 kDa to 900 kDa; 300 kDa to 800 kDa; 300 kDa to 700 kDa; 300 kDa to 600 kDa; 300 kDa to 500 kDa; 300 kDa to 400 kDa; 400 kDa to 1,000 kDa; 400 kDa to 900 kDa; 400 kDa to 800 kDa; 400 kDa to 700 kDa; 400 kDa to 600 kDa; or 500 kDa to 600 kDa.

In certain embodiments, the conjugation reaction is performed by reductive amination wherein nickel is used for greater conjugation reaction efficiency and to aid in free cyanide removal. Transition metals are known to form stable complexes with cyanide and are known to improve reductive methylation of protein amino groups and formaldehyde with sodium cyanoborohydride (S Gidley *et al.*, *Biochem J.* 1982, 203: 331-334; Jentoft *et al.* *Anal Biochem.* 1980, 106: 186-190). By complexing residual, inhibitory cyanide, the addition of nickel increases the consumption of protein during the conjugation of and leads to formation of larger, potentially more immunogenic conjugates.

Suitable alternative chemistries include the activation of the saccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated saccharide may thus be coupled directly or via a spacer (linker) group to an amino group on the carrier protein. For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using GMBS) or a haloacetylated carrier protein (for example using iodoacetimide [e.g. ethyl iodoacetimide HCl] or N-succinimidyl bromoacetate or SIAB, or SIA, or SBAP). Preferably, the cyanate ester (optionally made by CDAP chemistry) is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein using carbodiimide (e.g., EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described in International Patent Application Publication Nos. WO 93/15760, WO 95/08348 and WO 96/29094; and Chu *et al.*, 1983, *Infect. Immunity* 40:245-256.

Other suitable techniques use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EDC, TSTU. Many are described in International Patent Application Publication No. WO 98/42721. Conjugation may involve a

carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with CDI (See Bethell *et al.*, 1979, J. Biol. Chem. 254:2572-4; Hearn *et al.*, 1981, J. Chromatogr. 218:509-18) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional

- 5 protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

Following the conjugation (the reduction reaction and optionally the capping or quenching reaction), the glycoconjugates may be purified (enriched with respect to the amount  
10 of polysaccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration, ultrafiltration, precipitation/elution, column chromatography (ion exchange chromatography, multimodal ion exchange chromatography, DEAE, or hydrophobic interaction chromatography), and depth filtration. See, e.g., U.S. Pat. No. 6,146,902. In an embodiment, the  
15 glycoconjugates are purified by diafiltration or ion exchange chromatography or size exclusion chromatography.

One way to characterize the glycoconjugates of the invention is by the number of lysine residues in the carrier protein (e.g., CRM197) that become conjugated to the saccharide, which can be characterized as a range of conjugated lysines (degree of conjugation). The  
20 evidence for lysine modification of the carrier protein, due to covalent linkages to the polysaccharides, can be obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation results in a reduction in the number of lysine residues recovered, compared to the carrier protein starting material used to generate the conjugate materials. In a preferred embodiment, the degree of conjugation of the glycoconjugate of the invention is  
25 between 2 and 15, between 2 and 13, between 2 and 10, between 2 and 8, between 2 and 6, between 2 and 5, between 2 and 4, between 3 and 15, between 3 and 13, between 3 and 10, between 3 and 8, between 3 and 6, between 3 and 5, between 3 and 4, between 5 and 15, between 5 and 10, between 8 and 15, between 8 and 12, between 10 and 15 or between 10 and 12. In an embodiment, the degree of conjugation of the glycoconjugate of the invention is about 2, about  
30 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14 or about 15. In a preferred embodiment, the degree of conjugation of the glycoconjugate of the invention is between 4 and 7. In some such embodiments, the carrier protein is CRM197.

The glycoconjugates of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein. In some embodiments, the ratio of polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.5 and 3.0 (e.g., about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 2.6, about 2.7, about 2.8, about 2.9, or about 3.0). In other embodiments, the saccharide to carrier protein ratio (w/w) is between 0.5 and 2.0, between 0.5 and 1.5, between 0.8 and 1.2, between 0.5 and 1.0, between 1.0 and 1.5 or between 1.0 and 2.0. In further embodiments, the saccharide to carrier protein ratio (w/w) is between 0.8 and 1.2. In a preferred embodiment, the ratio of capsular polysaccharide to carrier protein in the conjugate is between 0.9 and 1.1. In some such embodiments, the carrier protein is CRM197. The glycoconjugates and immunogenic compositions of the invention may contain free saccharide that is not covalently conjugated to the carrier protein, but is nevertheless present in the glycoconjugate composition. The free saccharide may be non-covalently associated with (i.e., non-covalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate.

In a preferred embodiment, the glycoconjugate comprises less than about 50%, 45%, 40%, 35%, 30%, 25%, 20% or 15% of free polysaccharide compared to the total amount of polysaccharide. In a preferred embodiment the glycoconjugate comprises less than about 25% of free polysaccharide compared to the total amount of polysaccharide. In a preferred embodiment the glycoconjugate comprises less than about 20% of free polysaccharide compared to the total amount of polysaccharide. In a preferred embodiment the glycoconjugate comprises less than about 15% of free polysaccharide compared to the total amount of polysaccharide.

#### Multivalent polysaccharide-protein conjugate vaccines

In certain embodiments of the invention, multivalent polysaccharide vaccines comprising unconjugated polysaccharides or polysaccharide-protein conjugates from *Streptococcus pneumoniae* serotype 24F and capsular polysaccharides from one or more of *S. pneumoniae* serotypes 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 7B, 7C, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15A, 15B, 15C, 16F, 17F, 18B, 18C, 19A, 19F, 20, 21, 22A, 22F, 23A, 23B, 23F, 24B, 27, 28A, 31, 33F, 34, 35A, 35B, 35F, and 38 either as free polysaccharides, a component of a polysaccharide-protein conjugate or a combination thereof, to provide a multivalent pneumococcal vaccine. In certain embodiments of the invention, the immunogenic composition comprises, consists essentially of, or consists of capsular polysaccharides from 2, 3, 4, 5, 6, 7, 8,

9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, or 44 *S. pneumoniae* serotypes individually conjugated to one or more carrier proteins. Preferably, saccharides from a particular serotype are not conjugated to more than one carrier protein.

5                   After the individual glycoconjugates are purified, they are compounded to formulate the immunogenic composition of the present invention. These pneumococcal conjugates are prepared by separate processes and bulk formulated into a single dosage formulation.

#### 10   Pharmaceutical/Vaccine Compositions

                  The present invention further provides compositions, including pharmaceutical, immunogenic and vaccine compositions, comprising, consisting essentially of, or alternatively, consisting of any of the polysaccharide *S. pneumoniae* serotype combinations described above together with a pharmaceutically acceptable carrier and an adjuvant.

15                  Formulation of the polysaccharide-protein conjugates of the present invention can be accomplished using art-recognized methods. For instance, individual pneumococcal conjugates can be formulated with a physiologically acceptable vehicle to prepare the composition. Examples of such vehicles include, but are not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions.

20                  In a preferred embodiment, the vaccine composition is formulated in L-histidine buffer with sodium chloride.

                  As defined herein, an "adjuvant" is a substance that serves to enhance the immunogenicity of an immunogenic composition of the invention. An immune adjuvant may enhance an immune response to an antigen that is weakly immunogenic when administered alone, e.g., inducing no or weak antibody titers or cell-mediated immune response, increase  
25                  antibody titers to the antigen, and/or lowers the dose of the antigen effective to achieve an immune response in the individual. Thus, adjuvants are often given to boost the immune response and are well known to the skilled artisan. Suitable adjuvants to enhance effectiveness of the composition include, but are not limited to:

30                  (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.;

                  (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (defined below) or bacterial cell wall



components), such as, for example, (a) MF59 (International Patent Application Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, (c) Ribi™ adjuvant system (RAS), (Corixa, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of 3-O-deacylated monophosphorylipid A (MPL™) described in U.S. Pat. No. 4,912,094, trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL+CWS (Detox™); and (d) a Montanide ISA;

(3) saponin adjuvants, such as Quil A or STIMULON™ QS-21 (Antigenics, Framingham, MA) (see, e.g., U.S. Pat. No. 5,057,540) may be used or particles generated therefrom such as ISCOM (immunostimulating complexes formed by the combination of cholesterol, saponin, phospholipid, and amphipathic proteins) and Iscomatrix® (having essentially the same structure as an ISCOM but without the protein);

(4) bacterial lipopolysaccharides, synthetic lipid A analogs such as aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa, and which are described in U.S. Pat. No. 6,113,918; one such AGP is 2-[(R)-3-tetradecanoyloxytetradecanoylamino]ethyl 2-Deoxy-4-O-phosphono-3-O-[(R)-3-tetradecanoyloxytetradecanoyl]-2-[(R)-3-- tetradecanoyloxytetradecanoylamino]-b-D-glucopyranoside, which is also known as 529 (formerly known as RC529), which is formulated as an aqueous form or as a stable emulsion;

(5) synthetic polynucleotides such as oligonucleotides containing CpG motif(s) (U.S. Pat. No. 6,207,646);

(6) cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, IL-15, IL-18, etc.), interferons (e.g., gamma interferon), granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), costimulatory molecules B7-1 and B7-2, etc; and

(7) complement, such as a trimer of complement component C3d.

In another embodiment, the adjuvant is a mixture of 2, 3, or more of the above adjuvants, e.g., SBAS2 (an oil-in-water emulsion also containing 3-deacylated monophosphoryl lipid A and QS21).

Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanine-2-(1',2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

In certain embodiments, the adjuvant is an aluminum salt. The aluminum salt  
5 adjuvant may be an alum-precipitated vaccine or an alum-adsorbed vaccine. Aluminum-salt adjuvants are well known in the art and are described, for example, in Harlow, E. and D. Lane (1988; *Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory) and Nicklas, W. (1992; *Aluminum salts. Research in Immunology* 143:489-493). The aluminum salt includes, but is not limited to, hydrated alumina, alumina hydrate, alumina trihydrate (ATH), aluminum  
10 hydrate, aluminum trihydrate, Alhydrogel<sup>®</sup>, Superfos, Amphogel<sup>®</sup>, aluminum (III) hydroxide, aluminum hydroxyphosphate (Aluminum Phosphate Adjuvant (APA)), amorphous alumina, trihydrated alumina, or trihydroxyaluminum.

APA is an aqueous suspension of aluminum hydroxyphosphate. APA is manufactured by blending aluminum chloride and sodium phosphate in a 1:1 volumetric ratio to  
15 precipitate aluminum hydroxyphosphate. After the blending process, the material is size-reduced with a high-shear mixer to achieve a monodisperse particle size distribution. The product is then diafiltered against physiological saline and steam sterilized.

In certain embodiments, a commercially available Al(OH)<sub>3</sub> (e.g. Alhydrogel<sup>®</sup> or Superfos of Denmark/Accurate Chemical and Scientific Co., Westbury, NY) is used to adsorb  
20 proteins. Adsorption of protein is dependent, in another embodiment, on the pI (Isoelectric pH) of the protein and the pH of the medium. A protein with a lower pI adsorbs to the positively charged aluminum ion more strongly than a protein with a higher pI. Aluminum salts may establish a depot of Ag that is released slowly over a period of 2-3 weeks, be involved in nonspecific activation of macrophages and complement activation, and/or stimulate innate  
25 immune mechanism (possibly through stimulation of uric acid). See, e.g., Lambrecht *et al.*, 2009, *Curr Opin Immunol* 21:23.

Monovalent bulk aqueous conjugates are typically blended together and diluted. Once diluted, the batch is sterile filtered. Aluminum phosphate adjuvant is added aseptically to target a final concentration of 4 µg/mL for all *S. pneumoniae* serotypes except serotype 6B,  
30 which is diluted to a target of 8 µg/mL, and a final aluminum concentration of 250 µg/mL. The adjuvanted, formulated batch will be filled into vials or syringes.

In certain embodiments, the adjuvant is a CpG-containing nucleotide sequence, for example, a CpG-containing oligonucleotide, in particular, a CpG-containing

oligodeoxynucleotide (CpG ODN). In another embodiment, the adjuvant is ODN 1826, which may be acquired from Coley Pharmaceutical Group.

"CpG-containing nucleotide," "CpG-containing oligonucleotide," "CpG oligonucleotide," and similar terms refer to a nucleotide molecule of 6-50 nucleotides in length that contains an unmethylated CpG moiety. See, e.g., Wang *et al.*, 2003, Vaccine 21:4297. In another embodiment, any other art-accepted definition of the terms is intended. CpG-containing oligonucleotides include modified oligonucleotides using any synthetic internucleoside linkages, modified base and/or modified sugar.

Methods for use of CpG oligonucleotides are well known in the art and are described, for example, in Sur *et al.*, 1999, J Immunol. 162:6284-93; Verthelyi, 2006, Methods Mol Med. 127:139-58; and Yasuda *et al.*, 2006, Crit Rev Ther Drug Carrier Syst. 23:89-110.

#### Administration/Dosage

The compositions and formulations of the present invention can be used to protect or treat a human susceptible to infection, e.g., a pneumococcal infection, by means of administering the vaccine via a systemic or mucosal route. In one embodiment, the present invention provides a method of inducing an immune response to a *S. pneumoniae* capsular polysaccharide conjugate, comprising administering to a human an immunologically effective amount of an immunogenic composition of the present invention. In another embodiment, the present invention provides a method of vaccinating a human against a pneumococcal infection, comprising the step of administering to the human an immunologically effective amount of an immunogenic composition of the present invention.

Optimal amounts of components for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. For example, in another embodiment, the dosage for human vaccination is determined by extrapolation from animal studies to human data. In another embodiment, the dosage is determined empirically.

"Effective amount" of a composition of the invention refers to a dose required to elicit antibodies that significantly reduce the likelihood or severity of infectivity of a microbe, e.g., *S. pneumoniae*, during a subsequent challenge.

The methods of the invention can be used for the prevention and/or reduction of primary clinical syndromes caused by microbes, e.g., *S. pneumoniae*, including both invasive

infections (meningitis, pneumonia, and bacteremia), and noninvasive infections (acute otitis media, and sinusitis).

Administration of the compositions of the invention can include one or more of: injection via the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or via  
5 mucosal administration to the oral/alimentary, respiratory or genitourinary tracts. In one embodiment, intranasal administration is used for the treatment of pneumonia or otitis media (as nasopharyngeal carriage of pneumococci can be more effectively prevented, thus attenuating infection at its earliest stage).

The amount of conjugate in each vaccine dose is selected as an amount that  
10 induces an immunoprotective response without significant, adverse effects. Such amount can vary depending upon the pneumococcal serotype. Generally, for polysaccharide-based conjugates, each dose will comprise 0.1 to 100  $\mu$ g of each polysaccharide, particularly 0.1 to 10  $\mu$ g, and more particularly 1 to 5  $\mu$ g. For example, each dose can comprise 100, 150, 200, 250, 300, 400, 500, or 750 ng or 1, 1.5, 2, 3, 4, 5, 6, 7, 7.5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 22,  
15 25, 30, 40, 50, 60, 70, 80, 90, or 100  $\mu$ g of each polysaccharide.

Optimal amounts of components for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. For example, in another embodiment, the dosage for human vaccination is determined by extrapolation from animal studies to human data. In another embodiment, the dosage is  
20 determined empirically.

In one embodiment, the dose of the aluminum salt is 10, 15, 20, 25, 30, 50, 70, 100, 125, 150, 200, 300, 500, or 700  $\mu$ g, or 1, 1.2, 1.5, 2, 3, 5 mg or more. In yet another embodiment, the dose of aluminum salt described above is per  $\mu$ g of recombinant protein.

Generally, each 0.5 mL dose is formulated to contain: 2  $\mu$ g of each *S. pneumoniae*  
25 polysaccharide, except for serotype 6B polysaccharide at 4  $\mu$ g; about 32  $\mu$ g CRM197 carrier protein (e.g., 32  $\mu$ g  $\pm$  5  $\mu$ g,  $\pm$  3  $\mu$ g,  $\pm$  2  $\mu$ g, or  $\pm$  1  $\mu$ g); 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; and sodium chloride and L-histidine buffer. The sodium chloride concentration is about 150 mM (e.g., 150 mM  $\pm$  25 mM,  $\pm$  20 mM,  $\pm$  15 mM,  $\pm$  10 mM, or  $\pm$  5 mM) and about 20 mM (e.g., 20 mM  $\pm$  5 mM,  $\pm$  2.5 mM,  $\pm$  2 mM,  $\pm$  1 mM, or  $\pm$  0.5 mM)  
30 L-histidine buffer.

According to any of the methods of the present invention and in one embodiment, the subject is human. In certain embodiments, the human patient is an infant (less than 1 year of

age), toddler (approximately 12 to 24 months), or young child (approximately 2 to 5 years). In other embodiments, the human patient is an elderly patient (> 65 years). The compositions of this invention are also suitable for use with older children, adolescents and adults (e.g., aged 18 to 45 years or 18 to 65 years).

5                    In one embodiment of the methods of the present invention, a composition of the present invention is administered as a single inoculation. In another embodiment, the composition is administered twice, three times or four times or more, adequately spaced apart. For example, the composition may be administered at 1, 2, 3, 4, 5, or 6 month intervals or any combination thereof. The immunization schedule can follow that designated for pneumococcal  
10                    vaccines. For example, the routine schedule for infants and toddlers against invasive disease caused by *S. pneumoniae* is 2, 4, 6 and 12-15 months of age. Thus, in a preferred embodiment, the composition is administered as a 4-dose series at 2, 4, 6, and 12-15 months of age.

                    The compositions of this invention may also include one or more proteins from *S. pneumoniae*. Examples of *S. pneumoniae* proteins suitable for inclusion include those identified  
15                    in International Patent Application Publication Nos. WO 02/083855 and WO 02/053761.

#### Formulations

                    The compositions of the invention can be administered to a subject by one or more method known to a person skilled in the art, such as parenterally, transmucosally,  
20                    transdermally, intramuscularly, intravenously, intra-dermally, intra-nasally, subcutaneously, intra-peritoneally, and formulated accordingly.

                    In one embodiment, compositions of the present invention are administered via epidermal injection, intramuscular injection, intravenous, intra-arterial, subcutaneous injection, or intra-respiratory mucosal injection of a liquid preparation. Liquid formulations for injection  
25                    include solutions and the like.

                    The composition of the invention can be formulated as single dose vials, multi-dose vials or as pre-filled syringes.

                    In another embodiment, compositions of the present invention are administered orally, and are thus formulated in a form suitable for oral administration, i.e., as a solid or a  
30                    liquid preparation. Solid oral formulations include tablets, capsules, pills, granules, pellets and the like. Liquid oral formulations include solutions, suspensions, dispersions, emulsions, oils and the like.

Pharmaceutically acceptable carriers for liquid formulations are aqueous or non-aqueous solutions, suspensions, emulsions or oils. Examples of nonaqueous solvents are propylene glycol, polyethylene glycol, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Examples of oils are those of animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, olive oil, sunflower oil, fish-liver oil, another marine oil, or a lipid from milk or eggs.

The pharmaceutical composition may be isotonic, hypotonic or hypertonic. However, it is often preferred that a pharmaceutical composition for infusion or injection is essentially isotonic when it is administered. Hence, for storage the pharmaceutical composition may preferably be isotonic or hypertonic. If the pharmaceutical composition is hypertonic for storage, it may be diluted to become an isotonic solution prior to administration.

The isotonic agent may be an ionic isotonic agent such as a salt or a non-ionic isotonic agent such as a carbohydrate. Examples of ionic isotonic agents include but are not limited to NaCl, CaCl<sub>2</sub>, KCl and MgCl<sub>2</sub>. Examples of non-ionic isotonic agents include but are not limited to sucrose, trehalose, mannitol, sorbitol and glycerol.

It is also preferred that at least one pharmaceutically acceptable additive is a buffer. For some purposes, for example, when the pharmaceutical composition is meant for infusion or injection, it is often desirable that the composition comprises a buffer, which is capable of buffering a solution to a pH in the range of 4 to 10, such as 5 to 9, for example 6 to 8.

The buffer may for example be selected from the group consisting of Tris, acetate, glutamate, lactate, maleate, tartrate, phosphate, citrate, carbonate, glycinate, L-histidine, glycine, succinate and triethanolamine buffer.

The buffer may furthermore for example be selected from USP compatible buffers for parenteral use, in particular, when the pharmaceutical formulation is for parenteral use. For example the buffer may be selected from the group consisting of monobasic acids such as acetic, benzoic, gluconic, glyceric and lactic; dibasic acids such as aconitic, adipic, ascorbic, carbonic, glutamic, malic, succinic and tartaric, polybasic acids such as citric and phosphoric; and bases such as ammonia, diethanolamine, glycine, triethanolamine, and Tris.

Parenteral vehicles (for subcutaneous, intravenous, intraarterial, or intramuscular injection) include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Examples are

sterile liquids such as water and oils, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. In general, water, saline, aqueous dextrose and related sugar solutions, glycols such as propylene glycols or polyethylene glycol, Polysorbate 80 (PS-80), Polysorbate 20 (PS-20), and Poloxamer 188 (P188) are preferred liquid carriers, particularly  
5 for injectable solutions. Examples of oils are those of animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, olive oil, sunflower oil, fish-liver oil, another marine oil, or a lipid from milk or eggs.

The formulations may also contain a surfactant. Preferred surfactants include, but are not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the  
10 Tweens), especially PS-20 and PS-80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol) being of particular interest; (octylphenoxy)polyethoxyethanol (IGEPAL CA-630/NP-40);  
15 phospholipids such as phosphatidylcholine (lecithin); nonylphenol ethoxylates, such as the Tergitol™ NP series; polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); and sorbitan esters (commonly known as the SPANs), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. A preferred surfactant for including in the emulsion is PS-20 or PS-80.

20 Mixtures of surfactants can be used, e.g. PS-80/Span 85 mixtures. A combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan monooleate (PS-80) and an octoxynol such as t-octylphenoxypolyethoxyethanol (Triton X-100) is also suitable. Another useful combination comprises laureth 9 plus a polyoxyethylene sorbitan ester and/or an octoxynol.

25 Preferred amounts of surfactants are: polyoxyethylene sorbitan esters (such as PS-80) 0.01 to 1% w/v, in particular about 0.1% w/v; octyl- or nonylphenoxy polyoxyethanols (such as Triton X-100, or other detergents in the Triton series) 0.001 to 0.1% w/v, in particular 0.005 to 0.02% w/v; polyoxyethylene ethers (such as laureth 9) 0.1 to 20% w/v, preferably 0.1 to 10% w/v and in particular 0.1 to 1% w/v or about 0.5% w/v.

30 In certain embodiments, the composition consists essentially of L-histidine (20 mM), saline (150 mM) and 0.2% w/v PS-20 at a pH of 5.8 with 250 µg/mL of APA (Aluminum Phosphate Adjuvant). PS-20 can range from 0.005 to 0.1% w/v with the presence of PS-20 or PS-80 in formulation controlling aggregation during simulated manufacture and in shipping

using primary packaging. Process consists of combining blend of up to 44 *S. pneumoniae* polysaccharide serotypes in L-histidine, sodium chloride, and PS-20 then combining this blended material with APA and sodium chloride with or without antimicrobial preservatives.

The choice of surfactant may need to be optimized for different drug products and drug substances. For multivalent vaccines containing 15 or more *S. pneumoniae* polysaccharide serotypes, PS-20 and P188 are preferred. The choice of chemistry used to prepare the conjugate can also influence the stabilization of the formulation. In particular, as exemplified below, pneumococcal polysaccharide-protein conjugates prepared in aqueous or DMSO solvent and combined in a multivalent composition show significant differences in stability depending on the particular surfactant systems used for formulation.

For the formulations described herein, a poloxamer generally has a molecular weight in the range from 1,100 Da to 17,400 Da, from 7,500 Da to 15,000 Da, or from 7,500 Da to 10,000 Da. The poloxamer can be selected from poloxamer 188 or poloxamer 407. The final concentration of the poloxamer in the formulations of the invention is from 0.001 to 5% w/v, or 0.025 to 1% w/v. A surfactant system comprising a poloxamer must further comprise a polyol. In certain aspects, the polyol is propylene glycol and is at final concentration from 1 to 20% w/v. In certain aspects, the polyol is polyethylene glycol 400 and is at final concentration from 1 to 20% w/v.

Suitable polyols for the formulations are polymeric polyols, particularly polyether diols including, but are not limited to, propylene glycol and polyethylene glycol, Polyethylene glycol monomethyl ethers. Propylene glycol is available in a range of molecular weights of the monomer from ~425 Da to ~2,700 Da. Polyethylene glycol and Polyethylene glycol monomethyl ether is also available in a range of molecular weights ranging from ~200 Da to ~35,000 Da including but not limited to PEG200, PEG300, PEG400, PEG1000, PEG MME 550, PEG MME 600, PEG MME 2000, PEG MME 3350 and PEG MME 4000. A preferred polyethylene glycol is polyethylene glycol 400. The final concentration of the polyol in the formulations may be 1 to 20% w/v or 6 to 20% w/v.

The formulation also contains a pH-buffered saline solution. The buffer may, for example, be selected from the group consisting of Tris, acetate, glutamate, lactate, maleate, tartrate, phosphate, citrate, carbonate, glycinate, L-histidine, glycine, succinate, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MOPS (3-(N-morpholino)propanesulfonic acid), MES (2-(N-morpholino)ethanesulfonic acid) and triethanolamine buffer. The buffer is capable of buffering a solution to a pH in the range of 4 to 10, 5.2 to 7.5, or 5.8 to 7.0. In certain



aspects, the buffer selected from the group consisting of phosphate, succinate, L-histidine, MES, MOPS, HEPES, acetate or citrate. The buffer may furthermore, for example, be selected from USP compatible buffers for parenteral use, in particular, when the pharmaceutical formulation is for parenteral use. The concentrations of buffer will range from 1 mM to 50 mM or 5 mM to 50 mM. In certain aspects, the buffer is L-histidine at a final concentration of 5 mM to 50 mM, or succinate at a final concentration of 1 mM to 10 mM. In certain aspects, the L-histidine is at a final concentration of 20 mM  $\pm$  2 mM.

While the saline solution (i.e., a solution containing NaCl) is preferred, other salts suitable for formulation include but are not limited to, CaCl<sub>2</sub>, KCl and MgCl<sub>2</sub> and combinations thereof. Non-ionic isotonic agents including but not limited to sucrose, trehalose, mannitol, sorbitol and glycerol may be used in lieu of a salt. Suitable salt ranges include, but not are limited to 25 mM to 500 mM or 40 mM to 170 mM. In one aspect, the saline is NaCl, optionally present at a concentration from 20 mM to 170 mM.

In a preferred embodiment, the formulations comprise a L-histidine buffer with sodium chloride.

In another embodiment, the pharmaceutical composition is delivered in a controlled release system. For example, the agent can be administered using intravenous infusion, a transdermal patch, liposomes, or other modes of administration. In another embodiment, polymeric materials are used; e.g. in microspheres in or an implant.

The compositions of this invention may also include one or more proteins from *S. pneumoniae*. Examples of *S. pneumoniae* proteins suitable for inclusion include those identified in International Patent Application Publication Nos. WO 02/083855 and WO 02/053761.

#### Analytical Methods

##### *Molecular weight and concentration analysis of conjugates using HPSEC/UV/MALS/RI assay*

Conjugate samples are injected and separated by high performance size-exclusion chromatography (HPSEC). Detection is accomplished with ultraviolet (UV), multi-angle light scattering (MALS) and refractive index (RI) detectors in series. Protein concentration is calculated from UV280 using an extinction coefficient. Polysaccharide concentration is deconvoluted from the RI signal (contributed by both protein and polysaccharide) using the dn/dc factors which are the change in a solution's refractive index with a change in the solute concentration reported in mL/g. Average molecular weight of the samples are calculated by Astra software (Wyatt Technology Corporation, Santa Barbara, CA) using the measured

concentration and light scattering information across the entire sample peak. There are multiple forms of average values of molecular weight for polydispersed molecules. For example, number-average molecular weight  $M_n$ , weight-average molecular weight  $M_w$ , and z-average molecular weight  $M_z$  (Molecules, 2015, 20:10313-10341). Unless specified, the term  
5 “molecular weight”, as used throughout the specification, is the weight-average molecular weight.

*Determination of lysine consumption in conjugated protein as a measure of the number of covalent attachments between polysaccharide and carrier protein*

10 The Waters AccQ-Tag amino acid analysis (AAA) is used to measure the extent of conjugation in conjugate samples. Samples are hydrolyzed using vapor phase acid hydrolysis in the Eldex workstation, to break the carrier proteins down into their component amino acids. The free amino acids are derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). The derivatized samples are then analyzed using UPLC with UV detection on a C18  
15 column. The average protein concentration is obtained using representative amino acids other than lysine. Lysine consumption during conjugation (i.e., lysine loss) is determined by the difference between the average measured amount of lysine in the conjugate and the expected amount of lysine in the starting protein.

20 *Free polysaccharide testing*

Free polysaccharide (i.e., polysaccharide that is not conjugated with CRM197) in the conjugate sample is measured by first precipitating free protein and conjugates with deoxycholate (DOC) and hydrochloric acid. Precipitates are then filtered out and the filtrates are analyzed for free polysaccharide concentration by HPSEC/UV/MALS/RI. Free polysaccharide is  
25 calculated as a percentage of total polysaccharide measured by HPSEC/UV/MALS/RI.

*Free protein testing*

Free polysaccharide, polysaccharide-CRM197 conjugate, and free CRM197 in the conjugate samples are separated by capillary electrophoresis in micellar electrokinetic  
30 chromatography (MEKC) mode. Briefly, samples are mixed with MEKC running buffer containing 25 mM borate, 100 mM SDS, pH 9.3, and are separated in a preconditioned bare-fused silica capillary. Separation is monitored at 200 nm and free CRM197 is quantified with a

CRM197 standard curve. Free protein results are reported as a percentage of total protein content determined by the HPSEC/UV/MALS/RI procedure.

Having described various embodiments of the invention with reference to the accompanying description and drawings, it is to be understood that the invention is not limited to those precise embodiments, and that various changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims.

The following examples illustrate but do not limit the invention.

## EXAMPLES

### EXAMPLE 1: Preparation of *S. pneumoniae* Capsular Polysaccharides

Methods of culturing pneumococci are well known in the art. See, e.g., Chase, 1967, Methods of Immunology and Immunochemistry 1:52. Methods of preparing pneumococcal capsular polysaccharides are also well known in the art. See, e.g., European Patent No. EP 0 497 524 B1. The process described below generally follows the method described in European Patent No. EP 0 497 524 B1 and is generally applicable to all pneumococcal serotypes, except where specifically modified.

Isolates of pneumococcal subtype 24F were obtained from the Merck Culture Collection. Where needed, subtypes can be differentiated on the basis of Quelling reaction using specific antisera. See, e.g., U.S. Pat. No. 5,847,112. The obtained isolates were further clonally isolated by plating serially in two stages on agar plates consisting of an animal-component free medium containing soy peptone, yeast extract, and glucose without hemin. Clonal isolates for each serotype were further expanded in liquid culture using animal-component free media containing soy peptone, yeast extract, HEPES, sodium chloride, sodium bicarbonate, potassium phosphate, glucose, and glycerol to prepare the pre-master cell banks.

The production of each serotype of pneumococcal polysaccharide consisted of a cell expansion and batch production fermentation followed by chemical inactivation prior to downstream purification. A thawed cell bank vial from each serotype was expanded using a shake flask or culture bottle containing a pre-sterilized animal-component free growth media containing soy peptone or soy peptone ultrafiltrate, yeast extract or yeast extract ultrafiltrate, HEPES, sodium chloride, sodium bicarbonate, potassium phosphate, and glucose. The cell expansion culture was grown in a sealed shake flask or bottle to minimize gas exchange with

temperature and agitation control. After achieving a specified culture density, as measured by optical density at 600 nm, a portion of the cell expansion culture was transferred to a production fermentor containing pre-sterilized animal-component free growth media containing soy peptone or soy peptone ultrafiltrate, yeast extract or yeast extract ultrafiltrate, sodium chloride, potassium phosphate, and glucose. Temperature, pH, pressure, and agitation were controlled. Airflow overlay was also controlled as sparging was not used.

The batch fermentation was terminated via the addition of a chemical inactivating agent, phenol, when glucose was nearly exhausted. Pure phenol was added to a final concentration of 0.8 - 1.2% to inactivate the cells and liberate the capsular polysaccharide from the cell wall. Primary inactivation occurs for a specified time within the fermentor where temperature and agitation continue are to be controlled. After primary inactivation, the batch was transferred to another vessel where it was held for an additional specified time at controlled temperature and agitation for complete inactivation. This was confirmed by either microbial plating techniques or by verification of the phenol concentration and specified time. The inactivated broth was then purified.

#### *Purification of Ps*

The purification of the pneumococcal polysaccharide consisted of several centrifugation, depth filtration, concentration/diafiltration operations, and precipitation steps. All procedures were performed at room temperature unless otherwise specified.

Inactivated broth from the fermentor cultures of *S. pneumoniae* were flocculated with a cationic polymer (such as BPA-1000, Petrolite "Tretolite" and "Spectrum 8160" and poly(ethyleneimine), "Millipore pDADMAC"). The cationic polymers bind to the impurity protein, nucleic acids and cell debris. Following the flocculation step and an aging period, flocculated solids were removed via centrifugation and multiple depth filtration steps. Clarified broth was concentrated and diafiltered using a 100 kDa to 500 kDa MWCO (molecular weight cutoff) filter. Diafiltration was accomplished using Tris, MgCl<sub>2</sub> buffer and sodium phosphate buffer. Diafiltration removed residual nucleic acid and protein.

Further impurities removal was accomplished by reprecipitation of the polysaccharide in sodium acetate and phenol with denatured alcohol and/or isopropanol. During the phenol precipitation step, sodium acetate in sodium phosphate saline buffer and phenol (liquefied phenols or solid phenols) was charged to the diafiltered retentate. Alcohol fractionation of the polysaccharide was then conducted in two stages. In the first stage a low

percent alcohol was added to the preparation to precipitate cellular debris and other unwanted impurities, while the crude polysaccharide remained in solution. The impurities were removed via centrifugation followed by a depth filtration step. The polysaccharide was then recovered from the solution by adding additional isopropanol or denatured alcohol to the batch. The precipitated polysaccharide pellet was recovered by centrifugation, triturated and dried as a powder and stored frozen at -70°C.

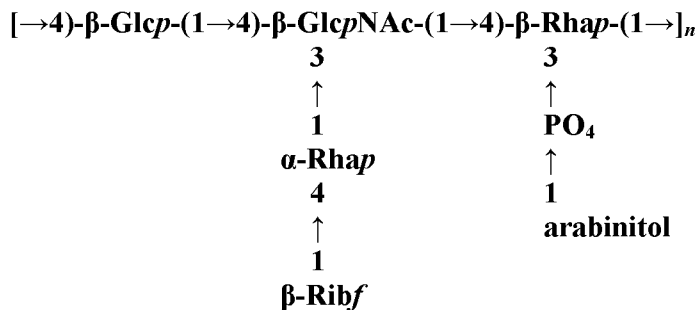
#### EXAMPLE 2: NMR Structure Analyses of Polysaccharides

The strategy for determining polysaccharide structure involved a multiple step process performed substantially as described in Abeygunawardana *et al.*, Determination of the Chemical Structure of Complex Polysaccharides by Heteronuclear NMR Spectroscopy in Advances in Biophysical Chemistry 1993, Vol 3, pages 199-249, JAI Press Inc. The purified polysaccharides were examined using standard 1D and 2D NMR techniques. Finally, the polysaccharides were examined for the presence of phosphate using  $^{31}\text{P}$  NMR.

Assignments of the monosaccharide residues were carried out through  $^1\text{H}$ - $^1\text{H}$  COSY, double quantum filtered homonuclear COSY and total correlation spectroscopy (TOCSY).  $^{13}\text{C}$  chemical shifts were assigned by heteronuclear single quantum coherence spectroscopy (HSQC) and combination HSQC-TOCSY. Multiplicity-edited HSQC was used to distinguish methylene from methine groups. Inter-residue linkages were determined through a combination of HMBC and NOESY spectroscopy. The anomeric configuration of the residues was determined from the anomeric proton and carbon chemical shifts,  $^3J_{\text{H1,H2}}$  and  $^1J_{\text{H1,C1}}$  values. The anomeric configuration of furanose rings is difficult to assess from  $^3J_{\text{H1,H2}}$  and  $^1J_{\text{H1,C1}}$  values, therefore long range couplings of ring protons from the anomeric carbon was utilized for this determination.

1D Phosphorus NMR spectroscopy indicated serotype 24F polysaccharides contained phosphorus in the structure. Assignment of the phosphorus linkage site was done through  $^1\text{H}$ - $^{31}\text{P}$  HMBC spectroscopy on purified (Fig. 5A) and periodate oxidized (Fig. 5B) polysaccharide.

Based on the NMR data in Figs. 2-5, the structure for *S. pneumoniae* serotype 24F polysaccharide was determined to be as follows:



wherein  $n$  represents the number of repeating units constituting the polysaccharide. See also Fig. 1A.

The sugar residues include rhamnose (Rha), ribose (Rib), glucose (Glc) and arabinitol. One of the glucose residues is N-acetylated. Ribose is in the form of furanose.

The italicized letters ( $p$  and  $f$ ) refer to pyranose (a closed ring consisting of six atoms) and furanose (a closed ring consisting of five atoms).

The  $\alpha$  and  $\beta$  refer to the configuration of the proton attached to anomeric carbon of the sugar unit. The anomeric carbon is always number 1 when labeling the carbon atom in a sugar unit (usually 1 through 6).  $\alpha$  means the anomeric proton is in the equatorial position in the 3D structure.  $\beta$  means the anomeric proton is in the axial position.

The numbers associated with arrows refer to how the individual sugar units are connected to each other. For example, the nomenclature  $\alpha\text{-Rhap-(1}\rightarrow 3)\text{-}\alpha\text{-Glc}p\text{-}$  means the number 1 carbon of Rhamnose is linked to the number 3 carbon of Glucose ( $p$  means they are both pyranose rings).

#### *Identification of the main activation site on S. pneumoniae serotype 24F polysaccharide*

The main activation site on *S. pneumoniae* serotype 24F was found to be at the second carbon of the arabinitol sidechain.

Oxidized serotype 24F polysaccharide was lyophilized then redissolved with deuterium oxide for NMR analysis. NMR experiments were carried out at 600 MHz at probe temperature of 37°C using a cryogenically cooled probe. A one dimensional (1D) proton spectrum was acquired using a 90 degree pulse with 16 transients and a 10 second delay between pulses (including 3 seconds of acquisition time). Gradient COSY and TOCSY data were acquired with 4 and 16 transients (respectively) in the first dimension and 512 increments in the second dimension. 1D TOCSY, centered on the triplet at 5.19 ppm, was acquired with 1024 transients with a recycle time of 10 seconds (including 3 seconds of acquisition time) and a

mixing time of 90 ms. A multiplicity edited gHSQCAD was acquired with 64 transients and 512 increments.

Compared to un-activated polysaccharide, the 1D proton spectrum showed an additional signal (triplet) in the anomeric region at 5.19 ppm. gCOSY and TOCSY data showed a cross peak from the signal 5.19 ppm to a signal at 3.86 ppm. 1D TOCSY with selective band centered at 5.19 ppm only showed a single correlation to a triplet at 3.86 ppm (Figure 6). The gHSQCAD data gave carbon chemical shifts of 91.13 ppm (signal at 5.19 ppm) and 70.79 ppm (signal at 3.86 ppm). The carbon chemical shift at 91.13 ppm is slightly upfield from where anomeric signals are typically observed indicating this to be a hydrated aldehyde. The other signal at 70.69 ppm was identified as a methylene (methylene carbons have opposite phase of methine and methyl carbons in the multiplicity edited gHSQCAD experiment; Figure 7). This data indicates the proton at 5.19 ppm (from the hydrated aldehyde) is adjacent to methylene group (3.86 ppm proton and 70.69 ppm carbon). The only place this could occur, based on the structure of 24F polysaccharide, is on the arabinitol sidechain at carbon 2.

The formation of an aldehyde predominantly at the second carbon was surprising in that it requires selective cleavage of 2,3-carbons with vicinal diols or preferred continued series of oxidation at higher numbered carbons with vicinal diols (or aldehyde) until the final oxidation at 2<sup>nd</sup> carbon of the arabinitol sugar.

### EXAMPLE 3: Conjugation of *S. pneumoniae* Serotype 24F Polysaccharide to CRM197 using Reductive Amination in Dimethylsulfoxide

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in dimethyl sulfoxide (DMSO).

Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

#### *Polysaccharide size reduction and oxidation*

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was size-reduced by acid hydrolysis by adding acetic

acid to 200 mM, incubating at 92°C for 50 minutes, then neutralizing by adding cold potassium phosphate pH 7 buffer to 400 mM.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 5 NMWCO tangential flow ultrafiltration membrane.

5           The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The sodium metaperiodate added was 0.18-0.21 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles  
10   aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

15

#### *Polysaccharide conjugation to CRM197*

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.0 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

20           Activated polysaccharides were formulated for lyophilization at 2 mg Ps/mL with sucrose concentration of 10% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The  
25   polysaccharide solution was spiked with sodium chloride to a final concentration of 20-50 mM. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 1.4-1.5 g Ps/L (grams polysaccharide/liter) and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating  
30   unit) was added, and conjugation proceeded for 2 hours at 22°C.

#### *Reduction with sodium borohydride*



Sodium borohydride (2 mole per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. For some batches, the batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

#### *Final filtration and product storage*

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at  $\leq -60^{\circ}\text{C}$ .

Table 1 shows attributes for *S. pneumoniae* serotype 24F polysaccharide conjugate prepared in DMSO.

Table 1. Attributes of *S. pneumoniae* serotype 24F polysaccharide conjugate from DMSO conjugation

Oxidized Ps Mw	Conjugate Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
100 kD	4875 kD	0.94	7.3	4.4%	1.4%
101 kD	3970 kD	0.81	7.9	5.8%	4.6%

#### EXAMPLE 4: Formulation of Monovalent Conjugates

Pneumococcal polysaccharide-CRM197 conjugates were prepared as described in Example 3. The required volume of bulk conjugates needed to obtain the target concentration of individual serotypes were calculated based on batch volume and concentration of individual bulk polysaccharide concentrations. Bulk conjugate of *S. pneumoniae* serotype 24F was combined with excipients, sterile filtered and added to APA under mixing conditions. The final

concentration of *S. pneumoniae* serotype 24F monovalent conjugate vaccine was 4 µg/mL (w/v PnPs) with 20 mM Histidine, 150 mM NaCl, 0.2% (w/v) PS-20 and 0.250 mg/mL (w/v Al) in the form of APA.

5    **EXAMPLE 5: Monovalent Conjugate New Zealand White Rabbit Immunogenicity Study**

          The immunogenicity of the monovalent conjugates was evaluated in a New Zealand White Rabbit (NZWR) model. Adult New Zealand White rabbits (NZWR, n=3/group) were intramuscularly (IM) immunized with 0.25 ml of respective monovalent conjugate vaccine on day 0 and day 14 (alternating sides). Monovalent pneumococcal vaccine was dosed at 1 µg  
10    PnPs (*S. pneumoniae* serotype 24F polysaccharide conjugated to CRM197) with 62.5 µg aluminum phosphate adjuvant (APA) per immunization. Sera were collected prior to study start (pre-immune) and on days 14 (post-dose 1, PD1) and 28 (post-dose 2, PD2). NZWRs were observed at least daily by trained animal care staff for any signs of illness or distress. The vaccine formulations in NZWRs were deemed to be safe and well tolerated. All animal  
15    experiments were performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The NZWR experimental protocol was approved by the Institutional Animal Care and Use Committees at both Merck & Co., Inc (Kenilworth, NJ) and Covance (Denver, PA).

          NZWR sera were tested in ELISA assays to evaluate IgG immunogenicity using a  
20    1-2 mg/ml respective PnPs coating concentration. Functional antibody was determined through opsonophagocytosis assays (OPA) based on previously described protocols. See, e.g., Caro-Aguilar *et al.*, 2017, Vaccine 35:865-72 and Burton *et al.*, 2006, Clin Vaccine Immunol 13(9):1004-9.

          Monovalent pneumococcal conjugate vaccines from *S. pneumoniae* serotype 24F  
25    were found to be immunogenic in rabbits (Figure 8) and generate functional antibody which killed the respective bacterial strain (Figure 9).

**EXAMPLE 6: Monovalent Conjugate Mouse Challenge Study**

          To verify the immunogenicity of *S. pneumoniae* serotype 24 glycoconjugates,  
30    they were tested in a mouse challenge model. Young mice (6-8 weeks old) were immunized with 0.1 ml of 24F-CRM197 monovalent conjugate vaccine on day 0, day 14, and day 28 (n=10/group). C57BL/6 mice and Swiss Webster mice were intraperitoneally (IP) immunized with 0.1 ml injected at one site. CD-1 mice were intramuscularly (IM) immunized with 0.05 ml

injected at two sites. The monovalent pneumococcal vaccine was dosed at 0.4 µg PnPs (24F polysaccharide conjugated to CRM197) with 25 µg aluminum phosphate adjuvant (APA) per immunization. Mice were observed at least daily by trained animal care staff for any signs of illness or distress. The vaccine formulations in mice were deemed to be safe and well tolerated.

5                   On day 52 the mice were intratracheally challenged with *S. pneumoniae* serotype 24F. Exponential phase cultures of *S. pneumoniae* were centrifuged, washed, and suspended in sterile PBS. Mice were anesthetized with isoflurane prior to challenge. 10<sup>6</sup> cfu of *S. pneumoniae* in 0.1ml of PBS was placed in the throat of mice hung upright by their incisors. Aspiration of the bacteria was induced by gently pulling the tongue outward and covering the  
10                   nostrils. Mice were weighed daily and euthanized if weight loss exceeded 20% of starting weight. Blood was collected at 24 hours, 48 hours, and 72 hours to assess for bacteremia. Mice were observed at least twice daily by trained animal care staff for any signs of illness or distress. All animal experiments were performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The mouse  
15                   experimental protocol was approved by the Institutional Animal Care and Use Committee at Merck & Co., Inc.

                  The 24F-CRM197 monovalent pneumococcal conjugate vaccine was found to be protective in all three strains of mice following challenge with *S. pneumoniae* 24F. See Figures 10-12.

#### 20                   EXAMPLE 7: Formulation of Pneumococcal Conjugate Vaccines for Rabbit Polyvalent Study

                  A multivalent pneumococcal conjugate vaccine consisting of different conjugate bulk blend preparations (including from *S. pneumoniae* serotypes 16F, 23A, 23B, 24F and 31) was prepared using pneumococcal polysaccharide-CRM197 conjugates and was formulated in  
25                   20 mM histidine pH 5.8 and 150 mM sodium chloride and 0.1% w/v Polysorbate-20 (PS-20) at 4 µg/mL each serotype for a total polysaccharide concentration of 84 µg/mL. The conjugates were prepared by individually conjugating the CRM197 protein to pneumococcal polysaccharide (PnPs) types (including from *S. pneumoniae* serotypes 16F, 23A, 23B, 24F and 31). The required volume of bulk conjugates needed to obtain the target concentration of individual  
30                   serotypes was calculated based on batch volume and concentration of individual bulk polysaccharide concentrations. The individual conjugates were added to a solution of histidine, sodium chloride and Polysorbate-20 (PS-20) to create the conjugate blend. The formulation vessel containing the conjugate blend was mixed using a magnetic stir bar, and sterile filtered

into another vessel. The formulations were then filled into plastic syringes, glass syringes, or vials and stored at 2-8°C.

EXAMPLE 8: Immunogenicity of a Multivalent Pneumococcal Conjugate Vaccine in New Zealand White Rabbits

Adult New Zealand White rabbits (NZWR, n=5/group) were intramuscularly (IM) immunized with 0.5 ml of the 21-valent pneumococcal conjugate vaccine described in Example 7 on day 0 and day 14 (alternating sides). PCV21 pneumococcal vaccine was dosed at 2 µg of each conjugated PnPs per immunization. Sera were collected prior to study start (pre-immune) and on days 14 (post-dose 1, PD1) and 28 (post-dose 2, PD2). NZWRs were observed at least daily by trained animal care staff for any signs of illness or distress. The vaccine formulations in NZWRs were deemed to be safe and well tolerated. All animal experiments were performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The NZWR experimental protocol was approved by the Institutional Animal Care and Use Committees at both Merck & Co., Inc and Covance (Denver, PA).

NZWR sera were evaluated for IgG immunogenicity using a multiplexed electrochemiluminescence (ECL) assay. This assay was developed for use with rabbit serum based on the human assay described by Marchese *et al.* (Optimization and validation of a multiplex, electrochemiluminescence-based detection assay for the quantitation of immunoglobulin G serotype-specific antipneumococcal antibodies in human serum. *Clin Vaccine Immunol.* 16(3): 387-96 (2009)) using technology developed by MesoScale Discovery (a division of MesoScale Diagnostics, LLC, Gaithersburg, MD) which utilizes a SULFO-TAG™ label that emits light upon electrochemical stimulation. SULFO-TAG™-labeled anti-rabbit IgG was used as the secondary antibody for testing NZWR serum samples. Functional antibody was determined through multiplexed opsonophagocytic assays (MOPA) based on previously described protocols available online at the Bacterial Respiratory Pathogen Reference Laboratory at the University of Alabama at Birmingham using Opsotiter® 3 software (UAB Research Foundation, Caro-Aguilar *et al.*, 2017, *supra*, Burton *et al.*, 2006, *supra*).

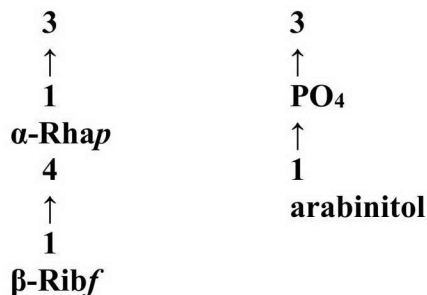
Polysaccharide-protein conjugates prepared from *S. pneumoniae* serotypes 16F, 23A, 23B, 24F, and 31 in a multivalent pneumococcal conjugate vaccines were found to be immunogenic for both post dose 1 (PD1) and post dose 2 (PD2) in rabbits (Figure 13). They also generated functional antibody which killed vaccine-type bacterial strains (Figure 14).

Rabbits immunized with the multivalent pneumococcal conjugate vaccine at the 2 µg dose had significantly higher PD1 MOPA titers for four serotypes compared to pre-immune rabbit sera (Figure 14). Rabbits immunized with PCV21 at the 2 µg dose had significantly higher PD2 MOPA titers for all five serotypes compared to pre-immune rabbit sera (Figure 14). Log

5 Transformed data were analyzed by One-way ANOVA with Dunnett's test to determine significance.

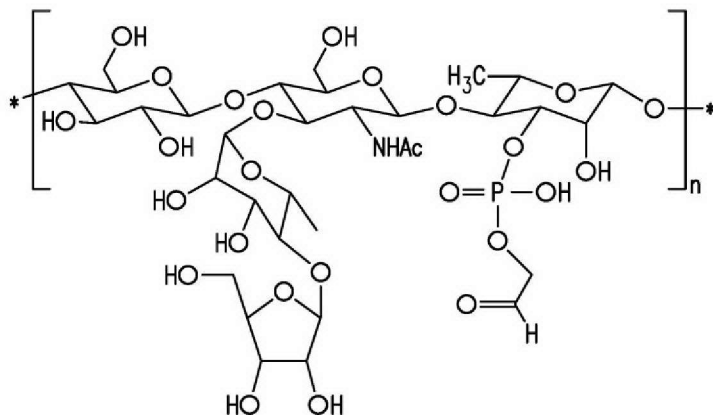
**CLAIMS:**

1. A polysaccharide-carrier protein conjugate, wherein the polysaccharide comprises a repeating unit of the following structure:



wherein the carrier protein is CRM197; and further wherein the polysaccharide-carrier protein conjugate has a molecular weight from 1000 kDa to 10,000 kDa.

2. The polysaccharide-carrier protein conjugate of claim 1, wherein the carrier protein is conjugated to the polysaccharide at the aldehyde of an activated repeating unit of the following structure:



3. The polysaccharide-carrier protein conjugate of claim 1 or 2, wherein the polysaccharide has a molecular weight of between 50 kDa and 1,000 kDa, between 100 kDa and 800 kDa, or between 100 kDa and 300 kDa.

4. The polysaccharide-carrier protein conjugate of any one of claims 1 to 3, wherein the degree of conjugation of the conjugate is between 2 and 15, or between 3 and 10, or between 5 and 10.

5. The polysaccharide-carrier protein conjugate of any one of claims 1 to 4, wherein the polysaccharide-carrier protein conjugate has a polysaccharide to carrier protein mass ratio from 0.5 to 3.0.

6. The polysaccharide-carrier protein conjugate of claim 5, wherein the polysaccharide-carrier protein conjugate has a polysaccharide to carrier protein mass ratio from 0.8 to 1.2.

7. The polysaccharide-carrier protein conjugate of any one of claims 1 to 6, wherein the polysaccharide-carrier protein conjugate has a polysaccharide to carrier protein mass ratio of about 0.94.

8. The polysaccharide-carrier protein conjugate of any one of claims 1 to 7, wherein the polysaccharide-carrier protein conjugate has a polysaccharide to carrier protein mass ratio of about 0.81.

9. The polysaccharide-carrier protein conjugate of any one of claims 1 to 8, wherein the polysaccharide-carrier protein conjugate comprises:

- less than about 30% of non-covalently associated polysaccharide compared to the total amount of polysaccharide;

- less than about 25% of non-covalently associated polysaccharide compared to the total amount of polysaccharide;

- less than about 20% of non-covalently associated polysaccharide compared to the total amount of polysaccharide; or

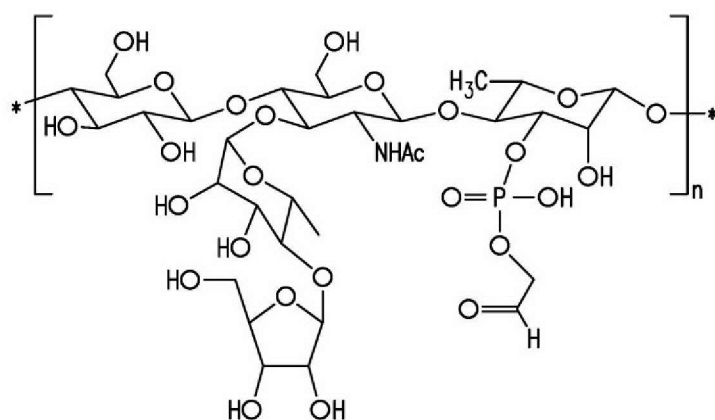
- less than about 15% of non-covalently associated polysaccharide compared to the total amount of polysaccharide.

10. The polysaccharide-carrier protein conjugate of claim 1, wherein the degree of conjugation of the conjugate is between 5 and 10; wherein the mass ratio of polysaccharide to carrier protein in the conjugate is between 0.8 and 1.2; and wherein the

conjugate comprises less than about 15% of free polysaccharide compared to the total amount of polysaccharide.

11. The polysaccharide-carrier protein conjugate of claim 2, wherein the carrier protein is conjugated to the polysaccharide at the aldehyde in at least 50% of the repeating units.

12. The polysaccharide-carrier protein conjugate of claim 1, wherein the carrier protein is conjugated to the polysaccharide at the aldehyde of an activated repeating unit of the following structure:



wherein the carrier protein is conjugated to the polysaccharide at the aldehyde in at least 50% of the repeating units; wherein the degree of conjugation of the conjugate is between 5 and 10; wherein the mass ratio of polysaccharide to carrier protein in the conjugate is between 0.8 and 1.2; and wherein the conjugate comprises less than about 15% of free polysaccharide compared to the total amount of polysaccharide.

13. An immunogenic composition comprising the polysaccharide carrier-protein conjugate of any one of claims 1 to 12, and a pharmaceutically acceptable carrier.

14. The immunogenic composition of claim 13, further comprising one or more additional polysaccharide-carrier protein conjugates, wherein each of the conjugates comprises a polysaccharide of a particular *S. pneumoniae* serotype conjugated to CRM197, and wherein the *S. pneumoniae* serotypes are selected from the group consisting of: 1, 2, 3, 4, 5, 6A,



6B, 6C, 6D, 7B, 7C, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15A, 15B, 15C, 17F, 18C, 19A, 19F, 20, 21, 22A, 22F, 23A, 23B, 23F, 24F, 27, 28A, 31, 33F, 34, 35A, 35B, 35F, and 38.

15. The immunogenic composition of claim 14, wherein the immunogenic composition is formulated to comprise: 0.4 to 4  $\mu\text{g/mL}$  of each polysaccharide present, except for serotype 6B polysaccharide which, if present, comprises 0.8 to 8  $\mu\text{g/mL}$  of polysaccharide; and CRM197 carrier protein in an amount from about 0.5x to 3x the total amount of polysaccharide.

16. The immunogenic composition of claim 15, wherein the immunogenic composition is formulated to further comprise 150 mM sodium chloride, 20 mM L-histidine buffer, and 0.05 to 2% w/v surfactant.

17. The immunogenic composition of claim 16, further comprising an adjuvant selected from the group consisting of aluminum phosphate, aluminum sulfate, and aluminum hydroxide, wherein the adjuvant is present at a concentration from 0.05 to 0.5 mg/mL.

18. A method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide, comprising administering to a human an immunologically effective amount of the immunogenic composition of any one of claims 13 to 17.

19. Use of the immunogenic composition of any one of claims 13 to 17 in the manufacture of a medicament for inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide in a human.

20. The method of claim 18 or the use of claim 19, wherein the immunogenic composition is a single 0.5 mL dose formulated to comprise: 2  $\mu\text{g}$  of each polysaccharide present, except for serotype 6B polysaccharide which, if present, is 4  $\mu\text{g}$ ; about 32  $\mu\text{g}$  CRM197 carrier protein; 0.125 mg aluminum phosphate adjuvant; 150 mM sodium chloride, 20 mM L-histidine buffer, and 0.2% w/v PS-20.

21. An immunogenic composition comprising the polysaccharide carrier-protein conjugate of claim 12, and a pharmaceutically acceptable carrier.

22. The immunogenic composition of claim 21, further comprising one or more additional polysaccharide-carrier protein conjugates, wherein each of the conjugates

comprises a polysaccharide of a particular *S. pneumoniae* serotype conjugated to CRM197, and wherein the *S. pneumoniae* serotypes are selected from the group consisting of: 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 7B, 7C, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15A, 15B, 15C, 17F, 18C, 19A, 19F, 20, 21, 22A, 22F, 23A, 23B, 23F, 24F, 27, 28A, 31, 33F, 34, 35A, 35B, 35F, and 38.

23. A method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide, comprising administering to a human an immunologically effective amount of the immunogenic composition of claim 21 or 22.

24. Use of the immunogenic composition of claim 21 or 22 in the manufacture of a medicament for inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide in a human.

25. The method of claim 23 or the use of claim 24, wherein the immunogenic composition is a single 0.5 mL dose formulated to comprise: 2 µg of each polysaccharide present, except for serotype 6B polysaccharide which, if present, is 4 µg; about 32 µg CRM197 carrier protein; 0.125 mg aluminum phosphate adjuvant; 150 mM sodium chloride, 20 mM L-histidine buffer, and 0.2% w/v PS-20.

**Merck Sharp & Dohme Corp.**

**Patent Attorneys for the Applicant/Nominated Person**

**SPRUSON & FERGUSON**

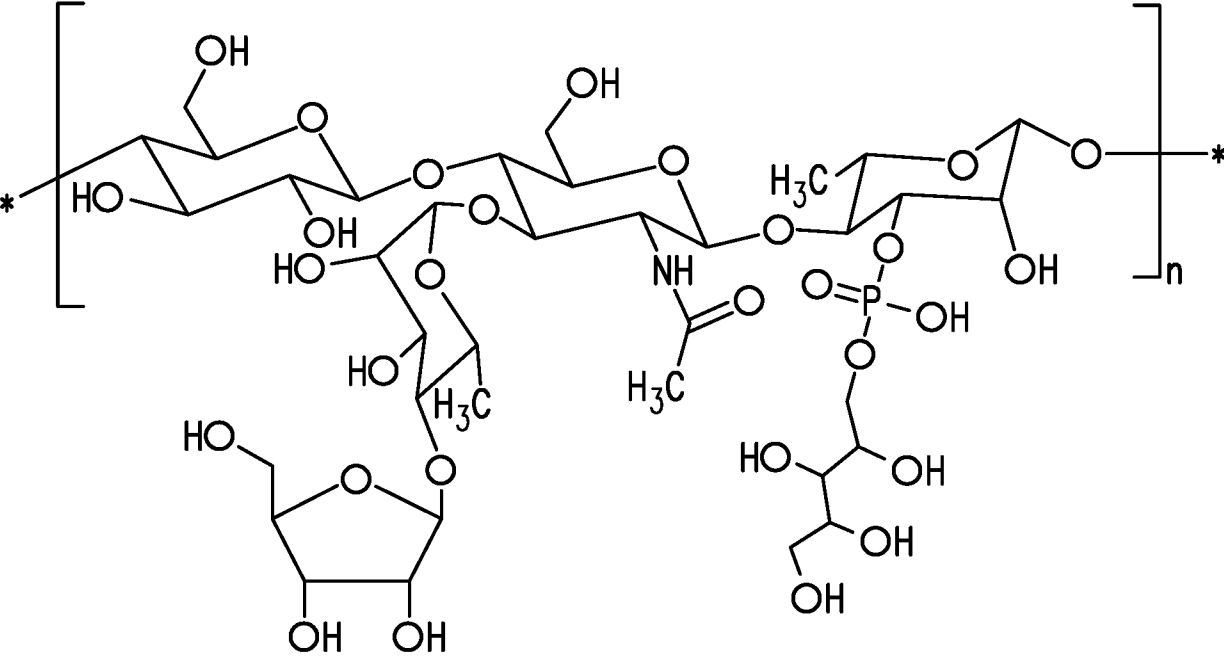


FIG.1A

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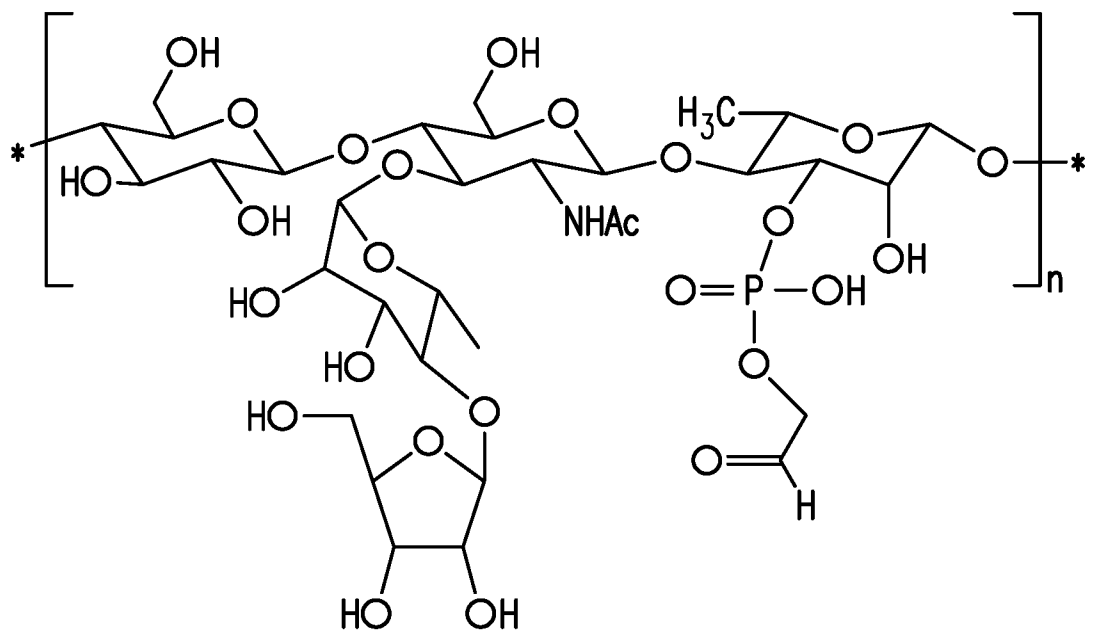


FIG.1B

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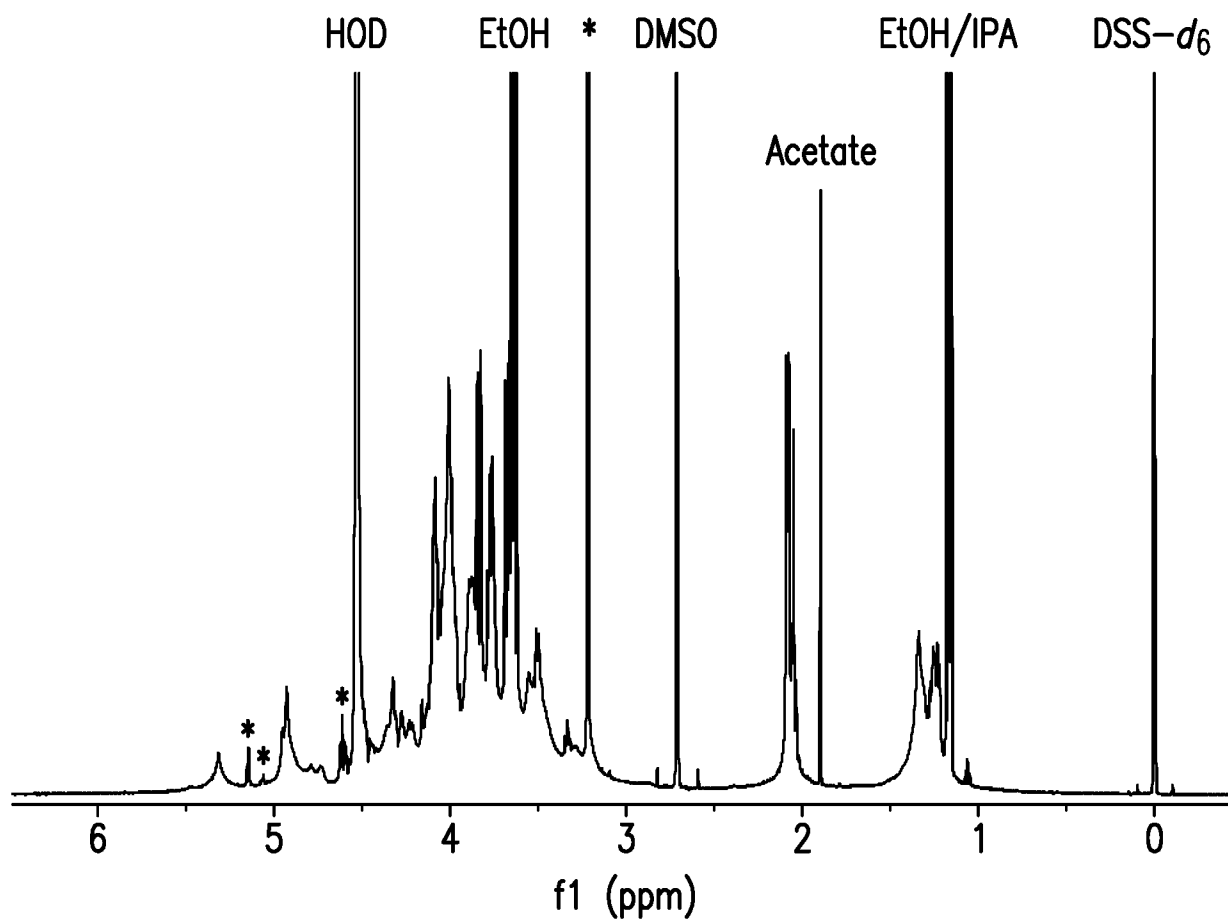


FIG.2

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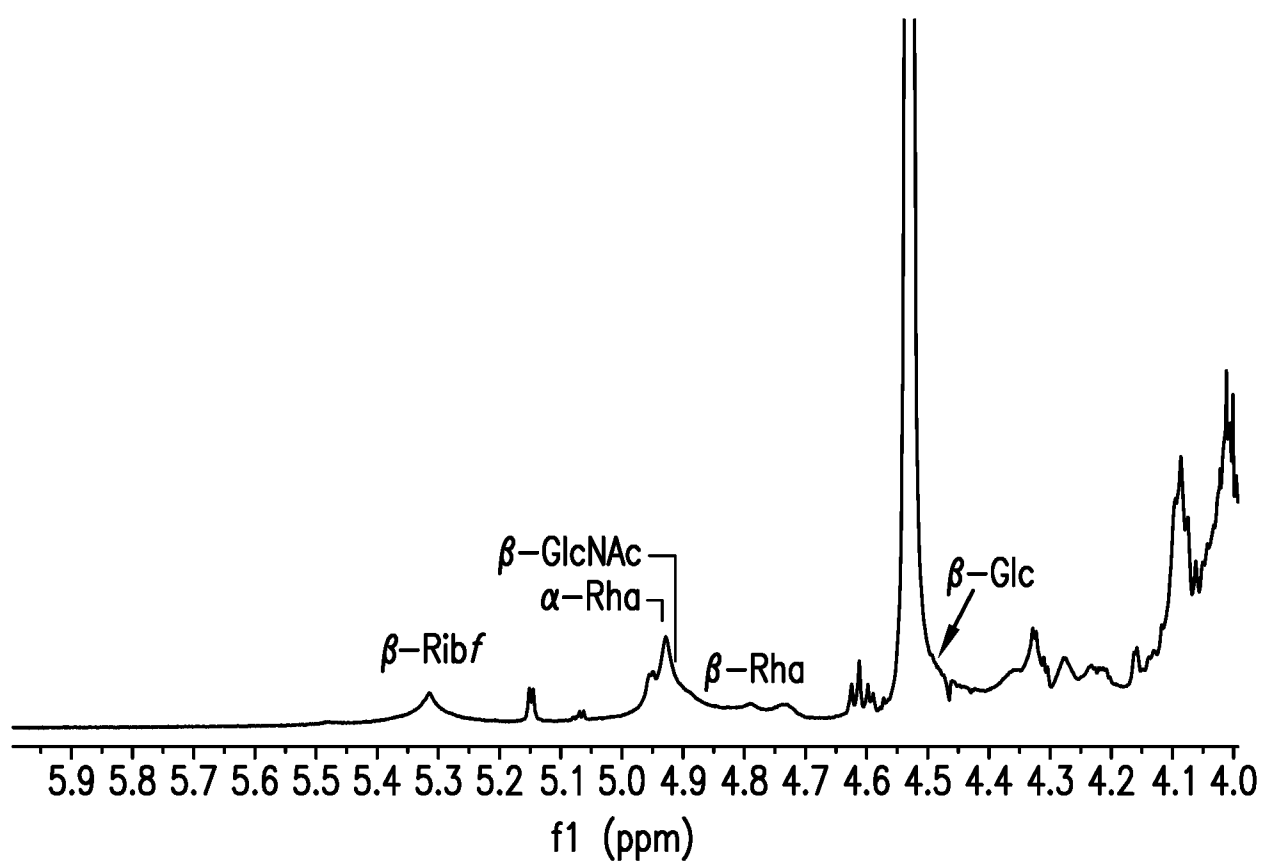


FIG.3

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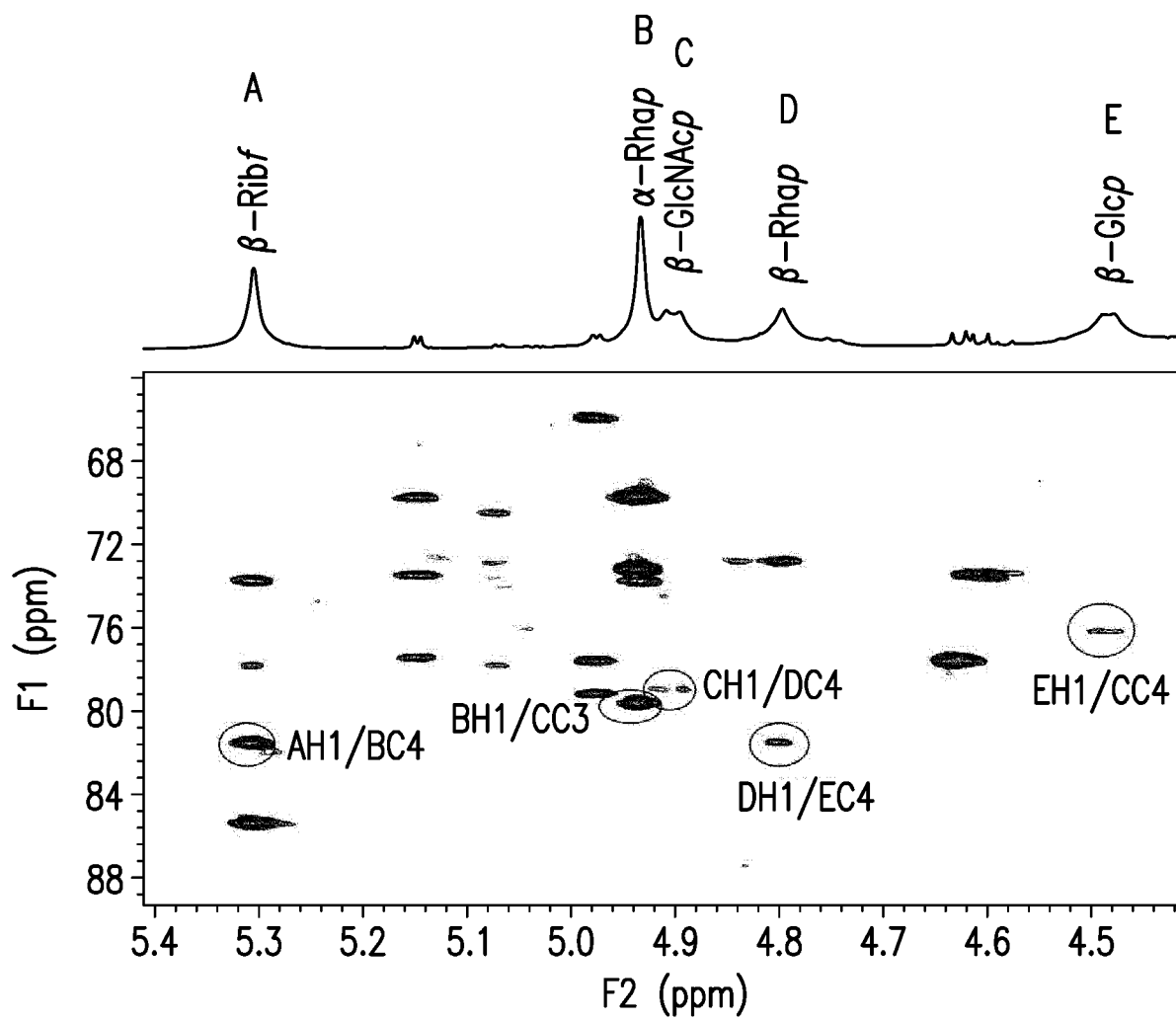


FIG.4

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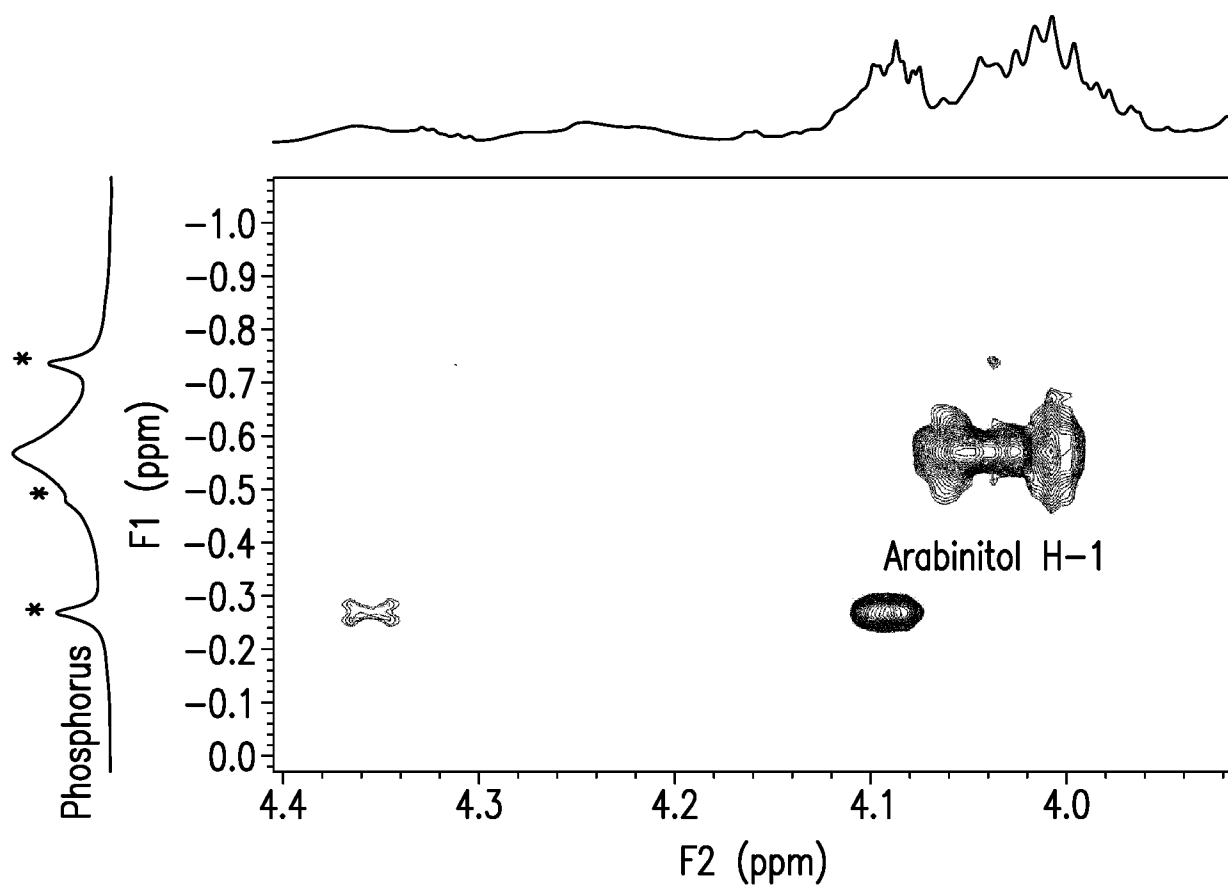


FIG. 5A

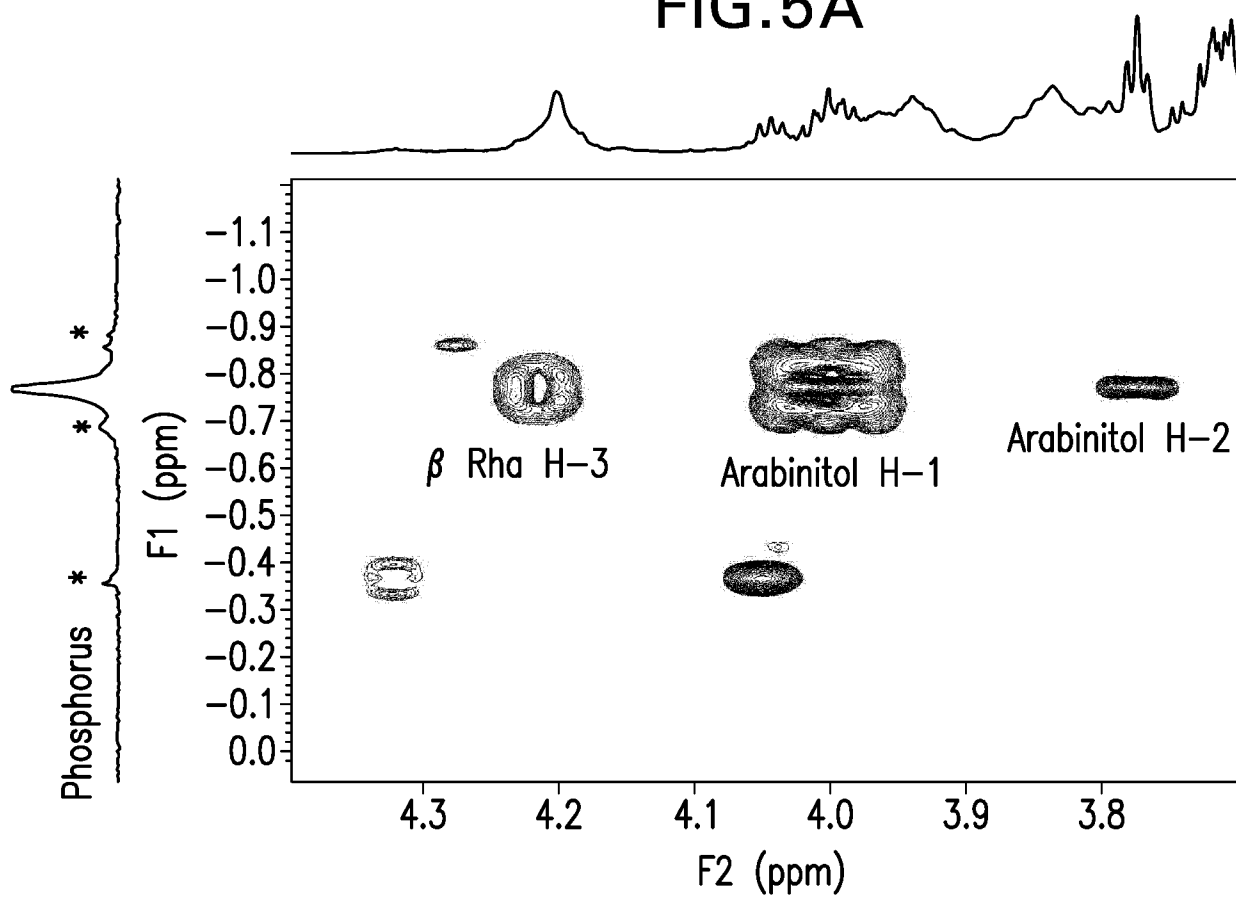


FIG. 5B



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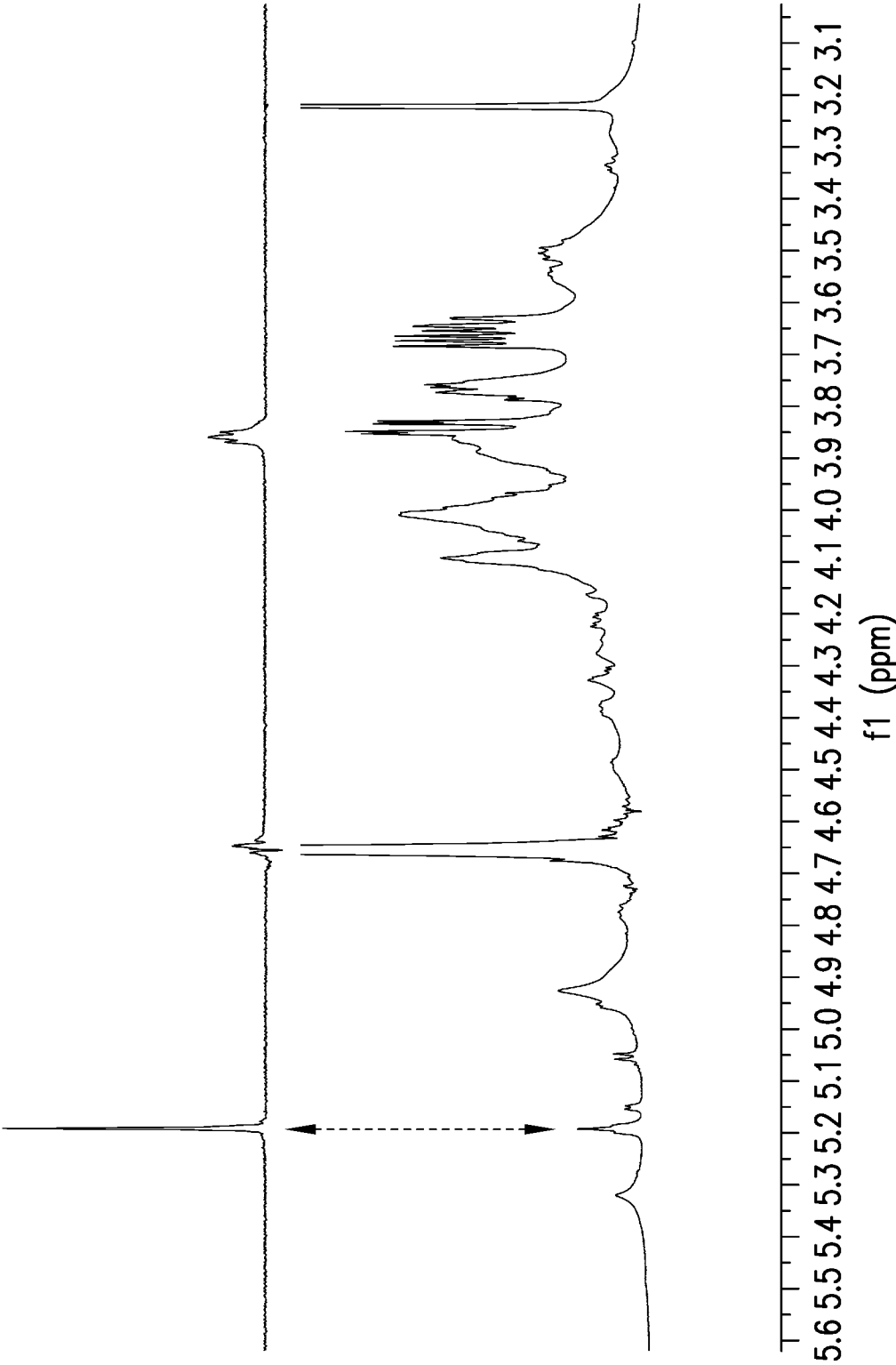


FIG. 6

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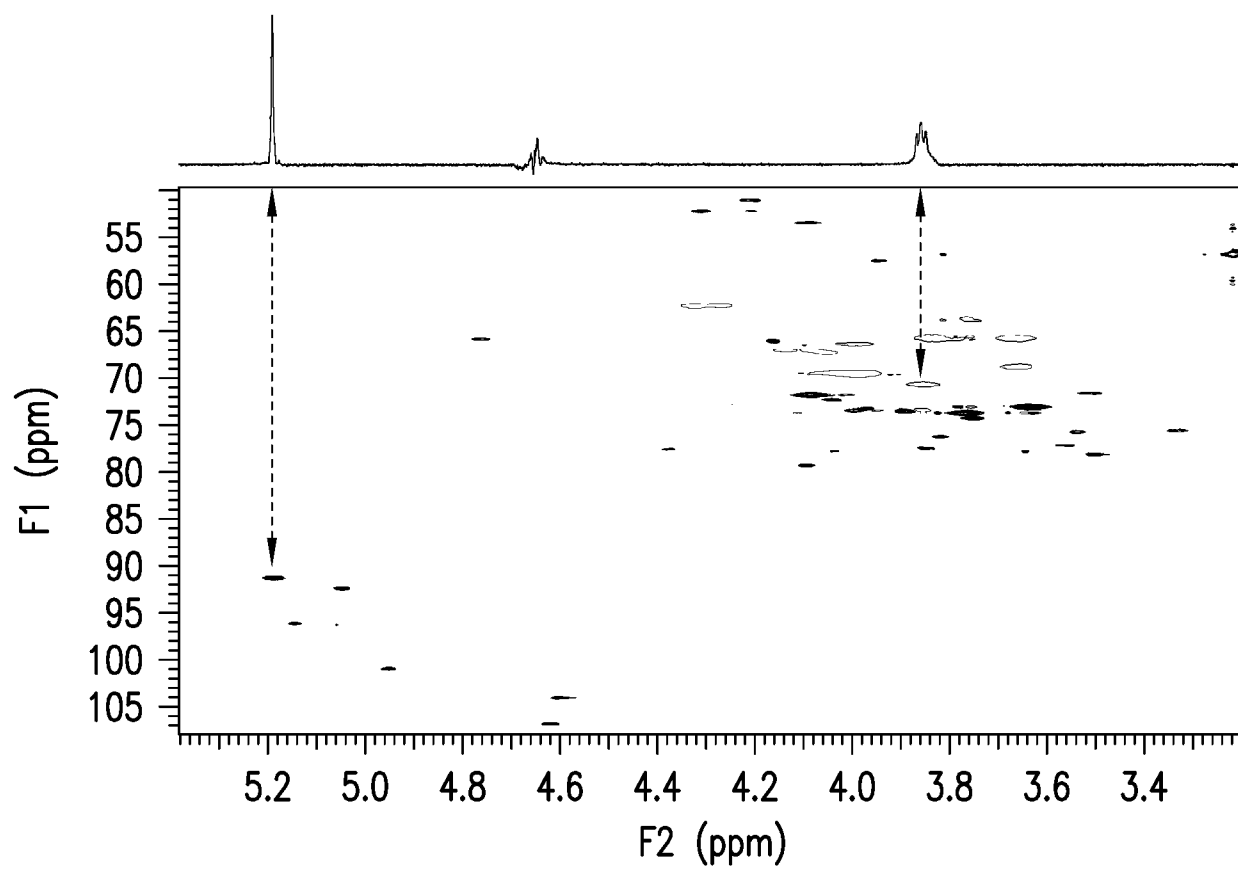


FIG. 7

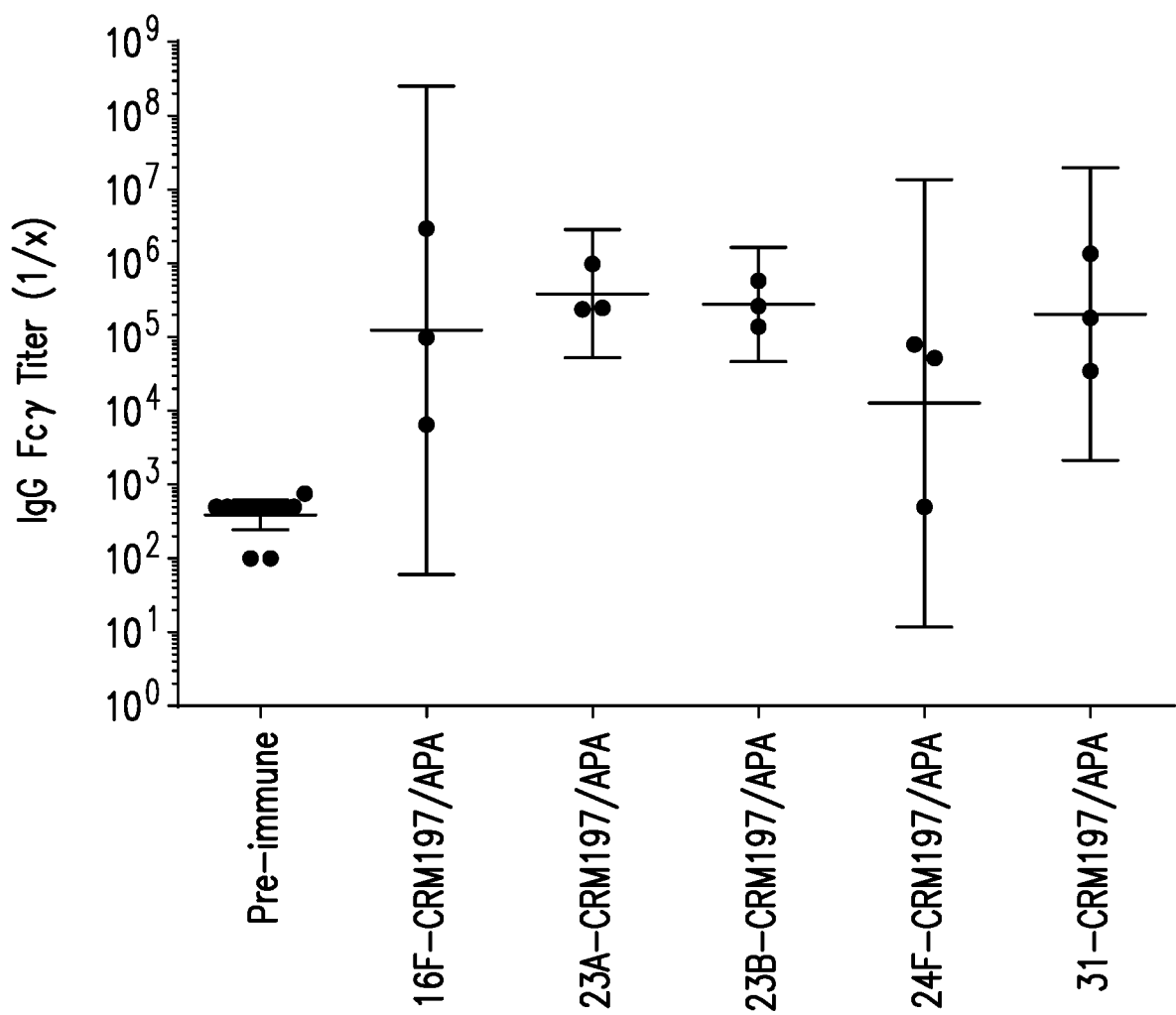


FIG.8

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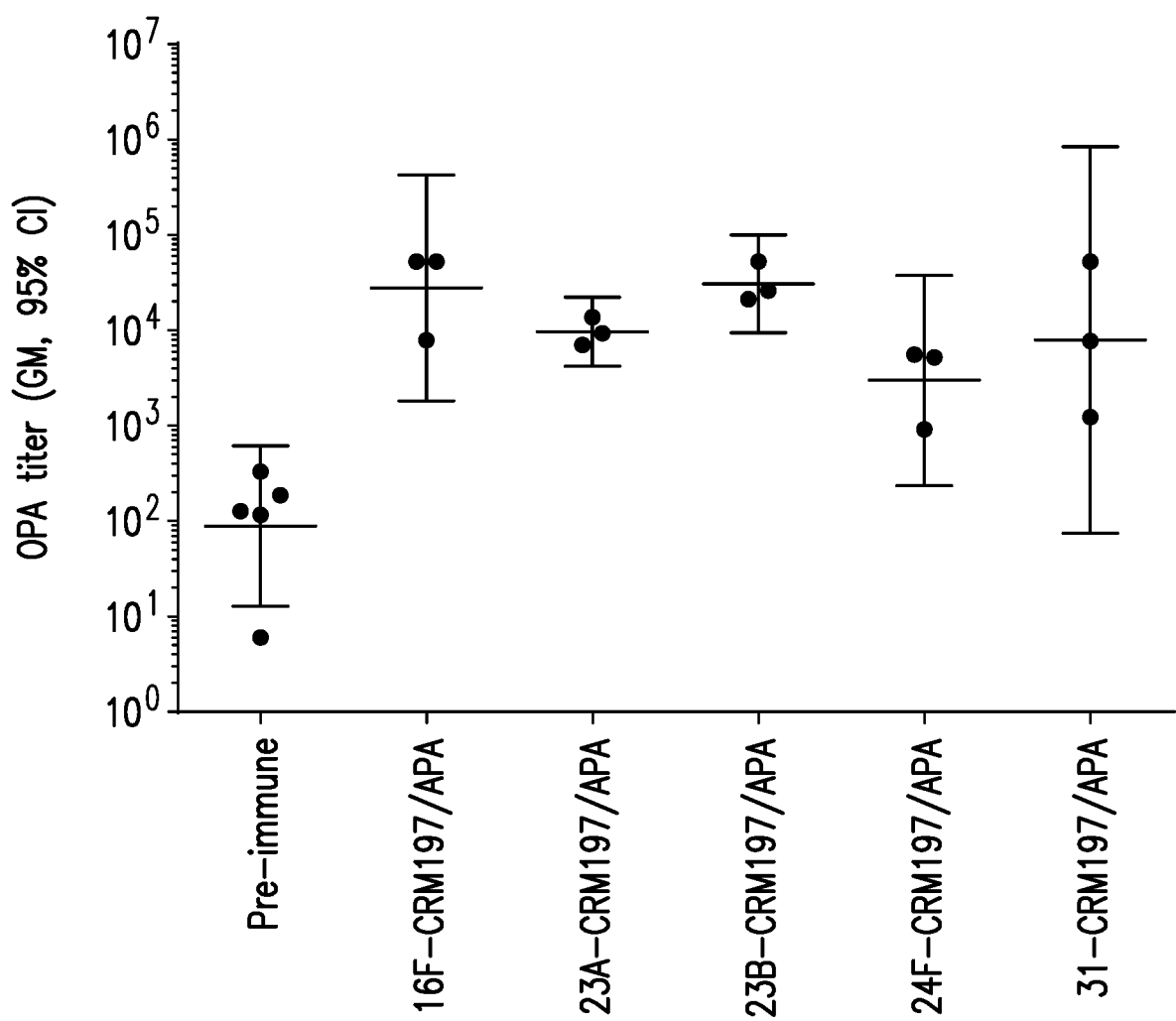


FIG.9

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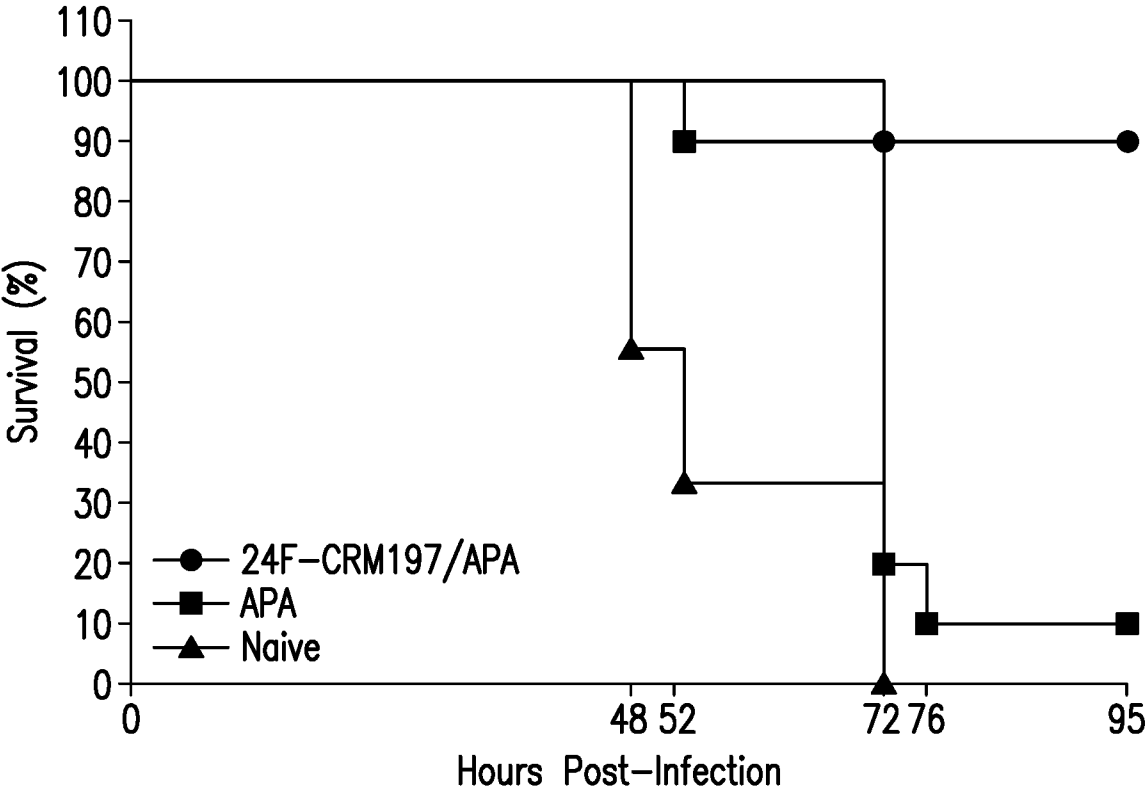


FIG.10

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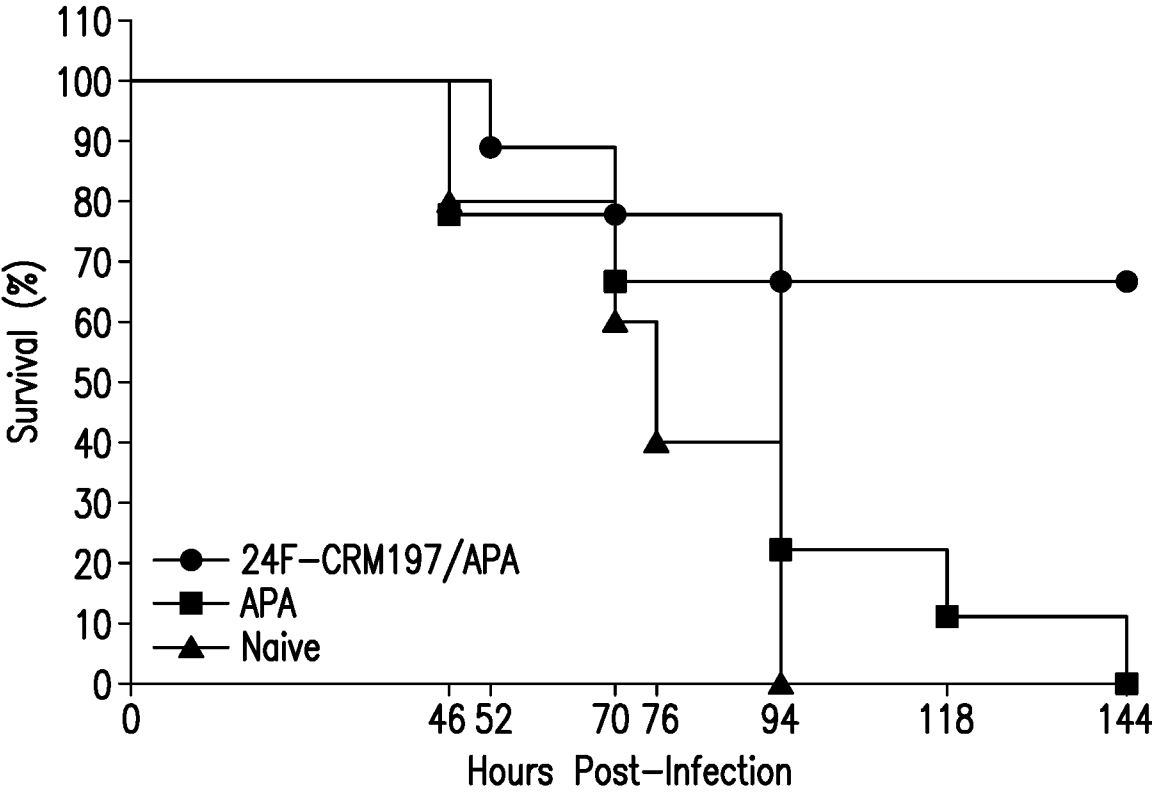


FIG.11

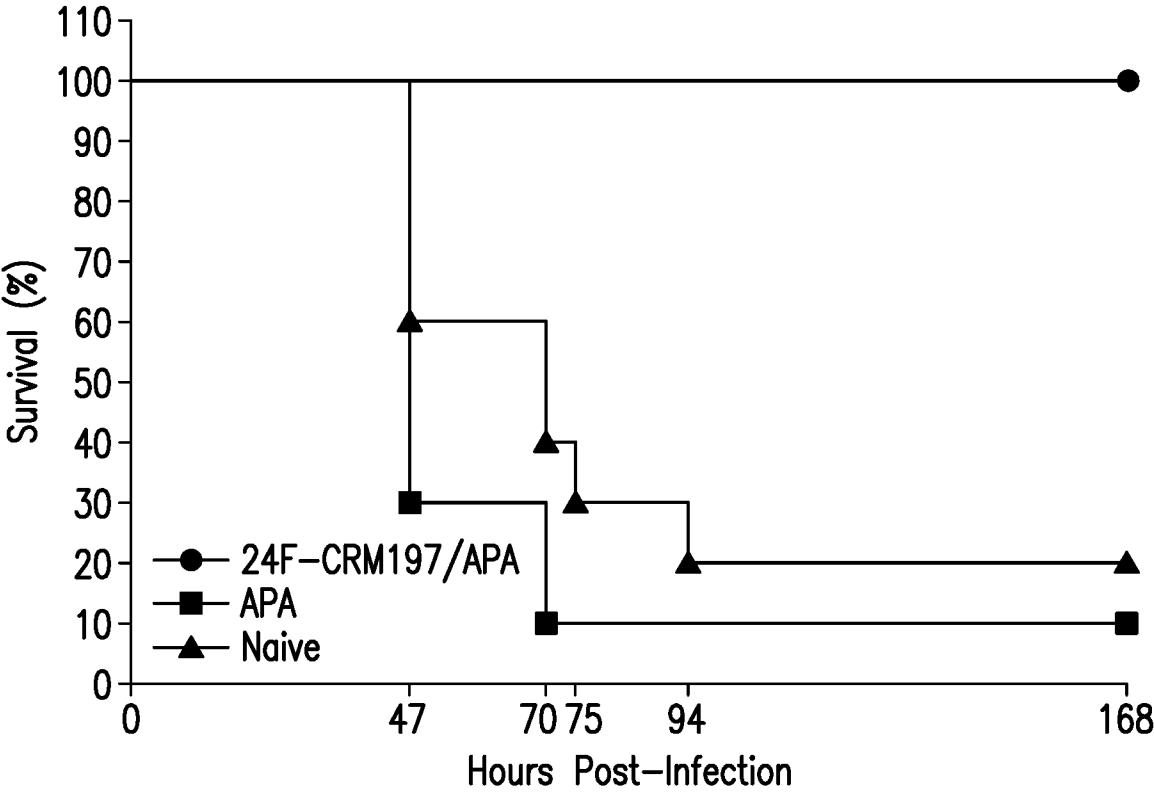


FIG.12

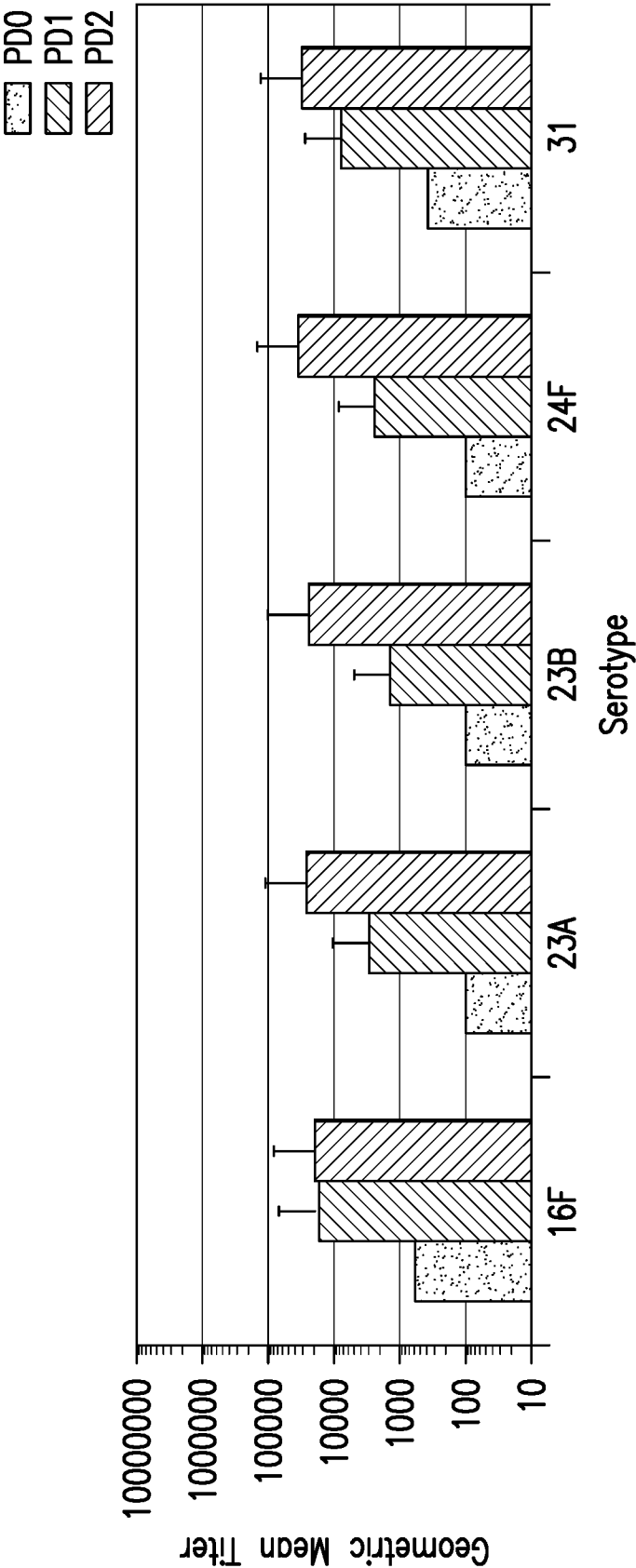


FIG.13



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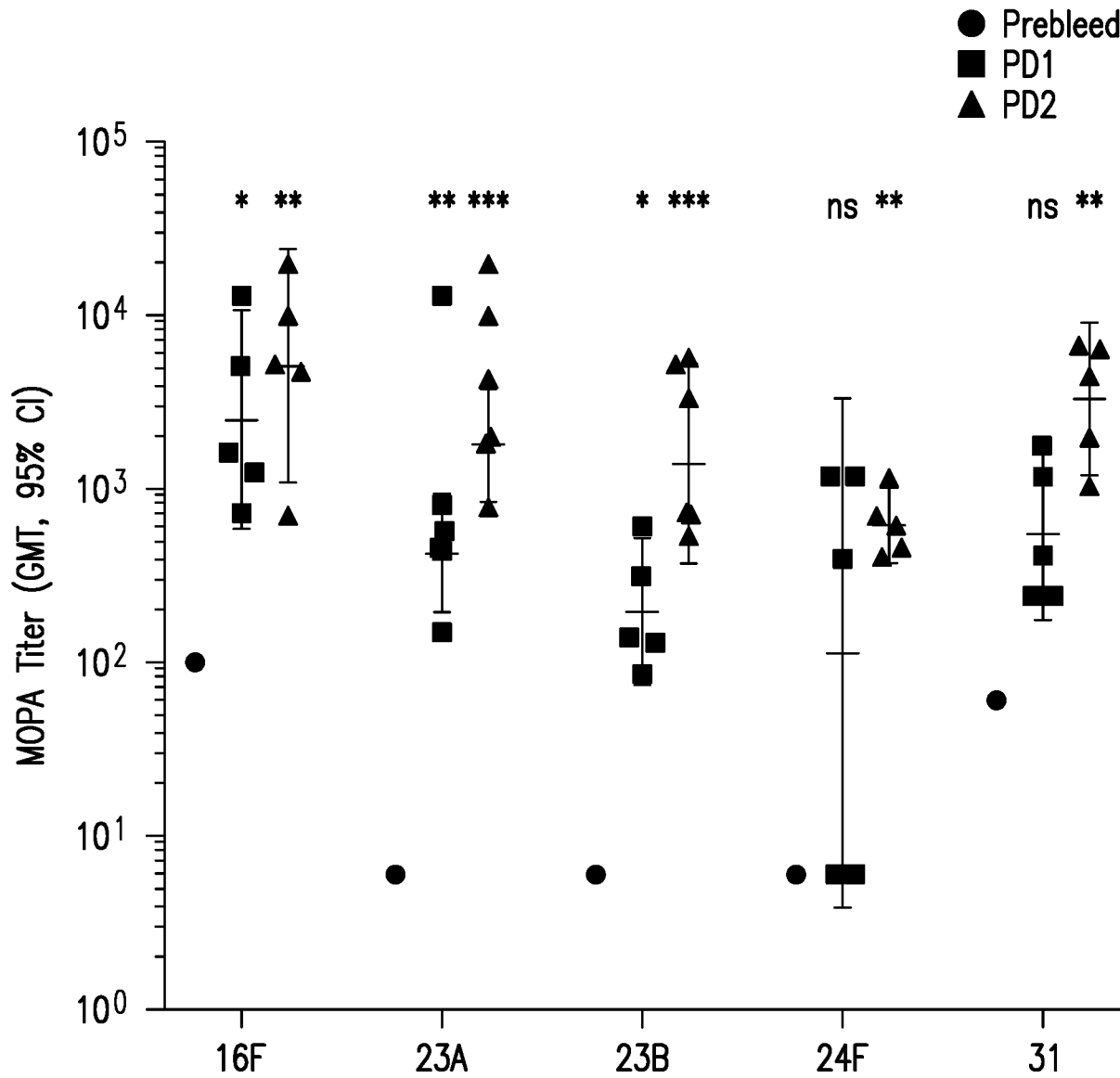


FIG.14