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- (71) **Applicant (for all designated States except US):**
ANCORA PHARMACEUTICALS, INC. [US/US]; 200
Boston Avenue, Suite 4975, Medford, MA 02155 (US).
- (72) **Inventors; and**
- (75) **Inventors/Applicants (for US only):** CHRIST, William,
J. [US/US]; 10 Sutherland Street, Andover, MA 01810-
1419 (US). CAMPBELL, A., Stewart [US/US]; 4 Shee-
han Circle, Framingham, MA 01701 (US). ORGUEIRA,
Hernan [AR/AR]; Yatay 1163 3a, Caba, CP1184 (AR).
- (74) **Agents:** ELLIOTT, Joshua T. et al.; Brinks Hofer Gilson
& Lione, P.O. Box 110285, Research Triangle Park, NC
27703 (US).

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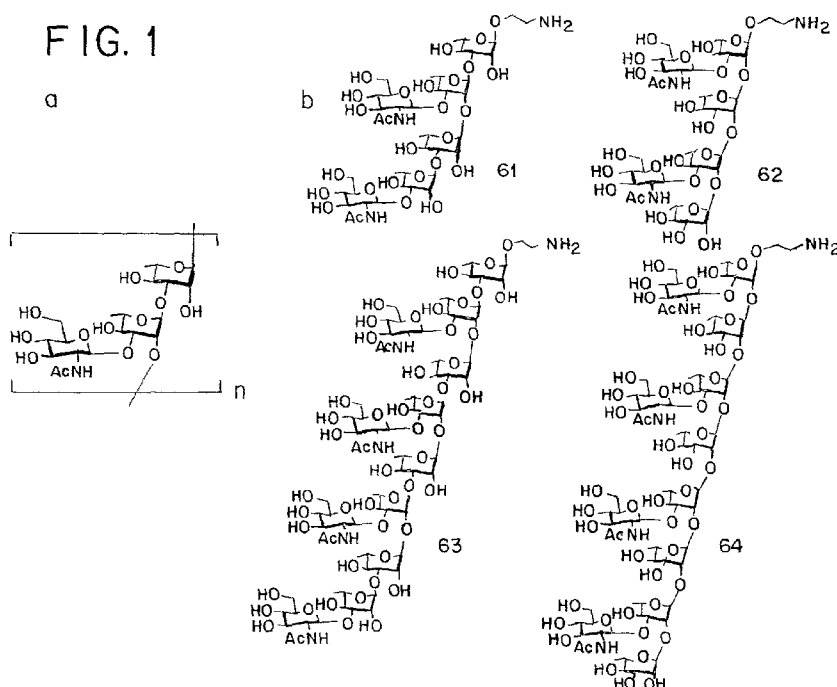
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(54) **Title:** SYNTHETIC OLIGOSACCHARIDE GROUP A STREPTOCOCCUS

FIG. 1



(57) **Abstract:** The present invention provides novel synthetic poly-L-rhamnose oligosaccharides, compositions containing the same, and methods of preventing Group A Streptococcus infections.

SYNTHETIC OLIGOSACCHARIDE GROUP A STREPTOCOCCUS

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Patent Application No. 61/422,533, filed December 13, 2010, the entire content of which is hereby incorporated by reference.

BACKGROUND

10 **1. Technical Field text**

 The present invention provides synthetic oligosaccharides which confer immuno-protection against Group A *Streptococcus* (GAS) and synthetic intermediates for their production. A series of oligosaccharides based on the GAS-PS structure were prepared by chemical synthesis and
15 conjugated to a carrier. When tested in mice, the conjugates containing the synthetic oligosaccharides conferred levels of immuno-protection comparable to those elicited by the native conjugate. Antisera from immunized rabbits promoted phagocytosis of encapsulated GAS strains.

2. Background Information

20 Group A *Streptococcus* (GAS) infections represent a significant healthcare concern throughout the world. GAS is responsible for a broad spectrum of diseases ranging from asymptomatic colonization, uncomplicated pharyngeal and skin infections, to life-threatening invasive illnesses including sepsis, necrotizing fasciitis and toxic shock syndrome.
25 Pharyngitis may lead to delayed sequelae as rheumatic fever. In developing countries rheumatic fever remains endemic and can be followed by rheumatic heart disease, causing hundreds of thousands of deaths every year. Currently, no vaccine to prevent GAS infections exists although GAS has been on the WHO priority prevention list for decades.

30 The diversity of GAS strains is the major challenge for the development of an anti-GAS vaccine, and therefore the identification of a common protective antigen is highly desirable. GAS bacteria contain a surface polysaccharide consisting of repeating $[\rightarrow 3)\alpha\text{-L-Rhap}(1\rightarrow 2)[\beta\text{-D-}$

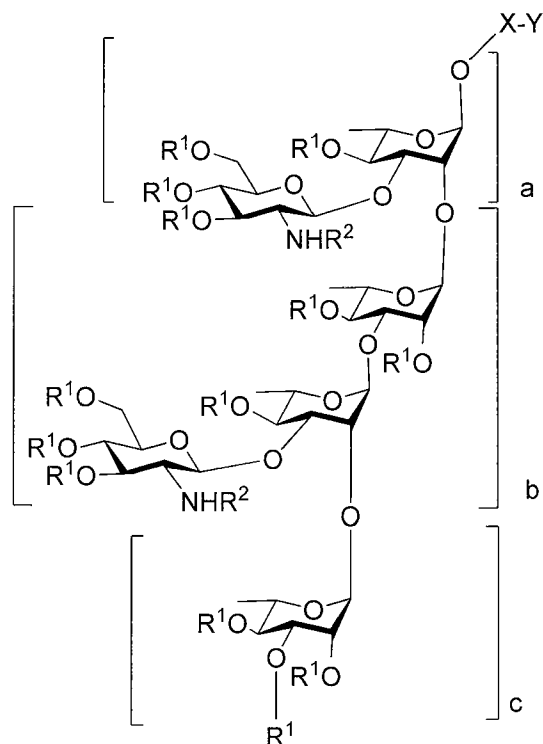
Glc₆NAc(1→3)] α -L-Rhap(1-)]_n units (Fig. 1a). These long polysaccharide chains adopt a helical conformation, where the rhamnoses form the helix core and the immunodominant *N*-acetylglucosamine residues are exposed on the periphery. The GAS polysaccharide (GAS-PS) is conserved and abundantly expressed in the vast majority of M+ GAS strain serotypes. This feature has been used to develop the “Rapid Strep Test,” which detects the presence of the Group A *Streptococcus* carbohydrate antigen in the throat of infected patients.

Human anti-GAS-PS antibodies have been associated with protection against GAS throat colonization and promote bacterial phagocytosis. Accordingly, GAS-PS has been considered attractive for the development of an anti-GAS conjugate vaccine. Purified GAS-polysaccharide has been conjugated to tetanus toxoid carrier and elicited a protective immune response in a mouse challenge model [9]. Efforts to determine the antibody-binding epitope of GAS-PS revealed a hexamer structure of two repeating units as core antigenic determinant which is believed to be recognized by human anti-GAS humoral immune response [11]. To date it has not been established whether a minimal GAS-PS core antigen determinant can elicit an immunoprotective response.

In contrast to conventional polysaccharide isolation, chemical synthesis provides access to pure, homogeneous oligosaccharides of well-defined structure that contain single reactive groups for covalent conjugation. Access to pure synthetic oligosaccharides is of particular value as the isolated GAS-PS may potentially contain minor contaminants like M protein or hyaluronic acid capsular polysaccharide, the former associated with pathologic cross-reactive autoimmune response and the latter representing a self-antigen. Recent progress in the chemical synthesis of oligosaccharides associated with introduction of automated systems facilitates the implementation of a synthetic vaccine approach at industrial scale, avoiding fermentation of pathogenic bacteria [15].

BRIEF SUMMARY

The present invention provides a synthetic oligosaccharide I:



where a and c are each 0 or 1; b is an integer from 1 to 8; each R^1 is independently H or a hydroxyl-protecting group; each R^2 is independently H or an amino-protecting group; X is a bond or a linker; Y is H, R or a carrier, where R is a hydroxyl-protecting group (which can be the same or different than an R^1). When $a+b+c=2$ or 3, the compound is also referred to herein as a building block or intermediate.

The present invention provides intermediates, including mono-, di-, and tri-saccharides useful for synthesis of oligosaccharides I.

The present invention provides compositions and methods for synthesizing the oligosaccharides and conjugates that have a specific number of monosaccharide units.

The present invention further provides immunogenic and immunoprotective compositions containing synthetic oligosaccharides I and antibodies derived therefrom for diagnosing, treating, and preventing infections caused by bacteria such as *Streptococcus aureus* and others.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Structures of the repeating unit of the cell-wall polysaccharide of GAS (1a) and synthetic oligosaccharide structures 1-4 (1b).

5 Figure 2. Representative end-stage synthesis of GAS oligosaccharides. All synthetic oligosaccharides were prepared from a common set of mono-, di-, and trisaccharide building blocks, which were assembled in a highly convergent approach.

10 Figure 3. Expansion of the region of the ¹H NMR spectrum of purified GAS cell wall polysaccharide.

 Figure 4. Characterization of synthetic oligosaccharide conjugates. a) CRM₁₉₇ glycoconjugates were analyzed by SDS-PAGE in 7% Tris-Acetate gel; the glycoconjugates migrate with diffuse bands which cover a region consistent with the expected increase of Mw as compared to CRM₁₉₇. Lane numbers indicate CRM₁₉₇ (1), CRM₁₉₇-61/1 (2), CRM₁₉₇-61/2 (3), CRM₁₉₇-62/1 (4), CRM₁₉₇-62/2 (5), CRM₁₉₇-63/1 (6), CRM₁₉₇-63/2 (7), CRM₁₉₇-63/3 (8), CRM₁₉₇-64/1 (9), CRM₁₉₇-64/2 (10), CRM₁₉₇-GAS-PS (11). The numbers on the right indicate the numbers of GAS-PS chains attached to CRM₁₉₇. b) Size-exclusion HPLC profile of the glycoconjugates.

 Figure 5. MS spectra of the glycoconjugate CRM₁₉₇-63/2 and CRM₁₉₇-GAS-PS. A.u. – arbitrary units.

 Figure 6. Immunogenicity of synthetic oligosaccharide conjugates. a) Total IgG antibody titres against GAS-PS. Mice were immunized with unconjugated GAS-PS, CRM₁₉₇-GAS-PS and CRM₁₉₇-glycoconjugates of synthetic oligosaccharides. The graph shows data of groups of 8-16 mice immunized independently; each dot represents single mouse sera; horizontal bars indicate GMT of group with 95% confidence interval statistical bars. GMT values are shown in Table 1, supra. b) Example of distribution of the anti-GAS-PS IgG subclasses and IgM for one immunization group.

 Figure 7. Flow cytometry analysis of M1 3348 and M23 DSM2071 strains. Bacteria were stained with preimmune mouse sera (negative

control), type-specific M proteins (positive control) and CRM₁₉₇-GAS-PS followed by R-Phycoerythrin-conjugated goat anti-mouse secondary antibodies. Grey shaded histograms indicate staining of bacteria with preimmune sera, while black histograms highlight the signal obtained using specific immune antisera.

Figure 8. Protection against i.p. challenge with M1 and M23 GAS serotypes. M1 3348 challenge: protection levels obtained immunizing mice with Alum alone (n=48), GAS-PS (n=40, p>0.05), M1 protein (n=24, p<0.001), CRM₁₉₇-GAS-PS (n=64, p<0.001), CRM₁₉₇-61 (n=40, p<0.001), CRM₁₉₇-62 (n=48, p<0.001), CRM₁₉₇-63 (n=48, p<0.05), CRM₁₉₇-64 (n=24, p<0.001). M23 2071 challenge: protection levels obtained immunizing mice with Alum alone (n=64), M23 protein (n=32, p<0.001), CRM₁₉₇-GAS-PS (n=64, p<0.05), CRM₁₉₇-61 (n=16, p<0.001).

Figure 9. Opsonophagocytic properties of antisera from rabbits immunized with synthetic and native GAS-PS glycoantigens. Bacteria were incubated with antisera from rabbits immunized with the glycoconjugates or with Alum alone (control). Results are expressed as mean percent killing $\pm$ standard deviation of at least 4 replicate experiments.

Figure 10. Depiction of a series of reaction schemes for assembling building blocks used in FIG. 11.

Figure 11. Depiction of a reaction scheme for synthesizing an oligosaccharide of the present invention.

DETAILED DESCRIPTION OF THE DRAWINGS AND THE PRESENTLY PREFERRED EMBODIMENTS

Definitions

In order to provide a clear and consistent understanding of the specification and claims, the following definitions are provided.

Units, prefixes, and symbols may be denoted in their SI accepted form. Numeric ranges recited herein are inclusive of the numbers defining the range and include and are supportive of each integer within the defined range. Unless otherwise noted, the terms “a” or “an” are to be construed as

meaning “at least one of.” The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including but not limited to patents, patent applications,
5 articles, books, and treatises, are hereby expressly incorporated by reference in their entirety for any purpose.

As used herein, “oligosaccharide” refers to a compound containing two or more monosaccharides. Oligosaccharides are considered to have a reducing end and a non-reducing end, whether or not the monosaccharide at
10 the reducing end is in fact a reducing sugar. In accordance with accepted nomenclature, oligosaccharides are depicted herein with the non-reducing end on the left and the reducing end on the right. All oligosaccharides described herein are described with the name or abbreviation for the non-reducing monosaccharide (e.g., Gal), preceded by the configuration of the
15 glycosidic bond (α or β), the ring bond, the ring position of the reducing monosaccharide involved in the bond, and then the name or abbreviation of the reducing monosaccharide (e.g., GlcNAc). The linkage between two sugars may be expressed, for example, as 2,3, 2 $\rightarrow$ 3, or 2-3. Each monosaccharide is a pyranose or furanose.

20 As used herein, “monosaccharide” refers to a single sugar residue in an oligosaccharide, including derivatives therefrom. Within the context of an oligosaccharide, an individual monomer unit is a monosaccharide which is (or can be) bound through a hydroxyl group to another monosaccharide.

25 As used herein, “endotoxin-free” refers to an oligosaccharide that does not contain endotoxins or endotoxin components normally present in bacterial isolates.

30 As used herein, “synthetic” refers to material which is substantially or essentially free from components, such as endotoxins, glycolipids, oligosaccharides, etc., which normally accompany a compound when it is isolated. Typically, synthetic compounds are at least about 90% pure, usually at least about 95%, and preferably at least about 99% pure. Purity can be indicated by a number of means well known in the art. Preferably,

purity is measured by HPLC. The identity of the synthetic material can be determined by mass spectroscopy and/or NMR spectroscopy.

As used herein, the term “carrier” refers to a protein, peptide, lipid, polymer, dendrimer, virosome, virus-like particle (VLP), or combination thereof, which is coupled to the oligosaccharide to enhance the immunogenicity of the resulting oligosaccharide-carrier conjugate to a greater degree than the oligosaccharide alone.

As used herein, “protein carrier” refers to a protein, peptide or fragment thereof, which is coupled or conjugated to an oligosaccharide to enhance the immunogenicity of the resulting oligosaccharide-protein carrier conjugate to a greater degree than the oligosaccharide alone. For example, when used as a carrier, the protein carrier may serve as a T-dependent antigen which can activate and recruit T-cells and thereby augment T-cell dependent antibody production.

As used herein, “conjugated” refers to a chemical linkage, either covalent or non-covalent, that proximally associates an oligosaccharide with a carrier so that the oligosaccharide conjugate has increased immunogenicity relative to an unconjugated oligosaccharide.

As used herein, “conjugate” refers to an oligosaccharide chemically coupled to a carrier through a linker and/or a cross-linking agent.

As used herein, “passive immunity” refers to the administration of antibodies to a subject, whereby the antibodies are produced in a different subject (including subjects of the same and different species) such that the antibodies attach to the surface of the bacteria and cause the bacteria to be phagocytosed or killed.

As used herein, “protective immunity” means that a vaccine or immunization schedule that is administered to a animal induces an immune response that prevents, retards the development of, or reduces the severity of a disease that is caused by a pathogen or diminishes or altogether eliminates the symptoms of the disease. Protective immunity may be predicted based on the ability of serum antibody to activate complement-mediated bactericidal activity or confer passive protection against a bacterial infection in a suitable animal challenge model.

As used herein, “immunoprotective composition” refers to a composition formulated to provide protective immunity in a host.

As used herein, “in a sufficient amount to elicit an immune response” (e.g., to epitopes present in a preparation) means that there is a detectable difference between an immune response indicator measured before and after administration of a particular antigen preparation. Immune response indicators include but are not limited to: antibody titer or specificity, as detected by an assay such as enzyme-linked immunoassay (ELISA), bactericidal assay (e.g., to detect serum bactericidal antibodies), flow cytometry, immunoprecipitation, Ouchter-Lowry immunodiffusion; binding detection assays of, for example, spot, Western blot or antigen arrays; cytotoxicity assays, and the like.

As used herein, “antibody” encompasses polyclonal and monoclonal antibody preparations, as well as preparations including hybrid antibodies, altered antibodies, $F(ab')^2$ fragments, $F(ab)$ molecules, F_v fragments, single chain fragment variable displayed on phage (scFv), single domain antibodies, chimeric antibodies, humanized antibodies, and functional fragments thereof which exhibit immunological binding properties of the parent antibody molecule.

As used herein, “monoclonal antibody” refers to an antibody composition having a homogeneous antibody population. The term is not limited by the manner in which it is made. The term encompasses whole immunoglobulin molecules, as well as Fab molecules, $F(ab')_2$ fragments, F_v fragments, single chain fragment variable displayed on phage (scFv), and other molecules that exhibit immunological binding properties of the parent monoclonal antibody molecule.

As used herein, “specifically binds to an antibody” or “specifically immunoreactive with”, when referring to an oligosaccharide, protein or peptide, refers to a binding reaction which is based on and/or is probative of the presence of the antigen in a sample which may also include a heterogeneous population of other molecules. Thus, under designated immunoassay conditions, the specified antibody or antibodies bind(s) to a particular antigen or antigens in a sample and does not bind in a significant

amount to other molecules present in the sample. Specific binding to an antibody under such conditions may require an antibody or antiserum that is selected for its specificity for a particular antigen or antigens.

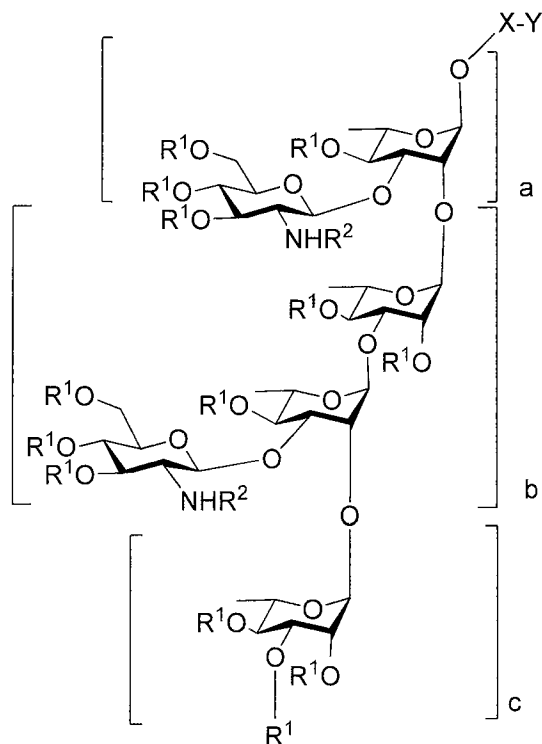
5 As used herein, “antigen” refers to include any substance that may be specifically bound by an antibody molecule.

As used herein, “immunogen” and “immunogenic composition” refer to an antigenic composition capable of initiating lymphocyte activation resulting in an antigen-specific immune response.

10 As used herein, “epitope” refers to a site on an antigen to which specific B cells and/or T cells respond. The term is also used interchangeably with “antigenic determinant” or “antigenic determinant site.” B cell epitope sites on proteins, oligosaccharides, or other biopolymers may be composed of moieties from different parts of the macromolecule that have been brought together by folding. Epitopes of this
15 kind are referred to as conformational or discontinuous epitopes, since the site is composed of segments the polymer that are discontinuous in the linear sequence but are continuous in the folded conformation(s). Epitopes that are composed of single segments of biopolymers or other molecules are termed continuous or linear epitopes. T cell epitopes are generally restricted
20 to linear peptides. Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen.

Synthetic oligosaccharides

25 The present invention provides a synthetic oligosaccharide I:



where a and c are each 0 or 1;

b is an integer from 1 to 8;

5 each R^1 is independently H or a hydroxyl-protecting group;

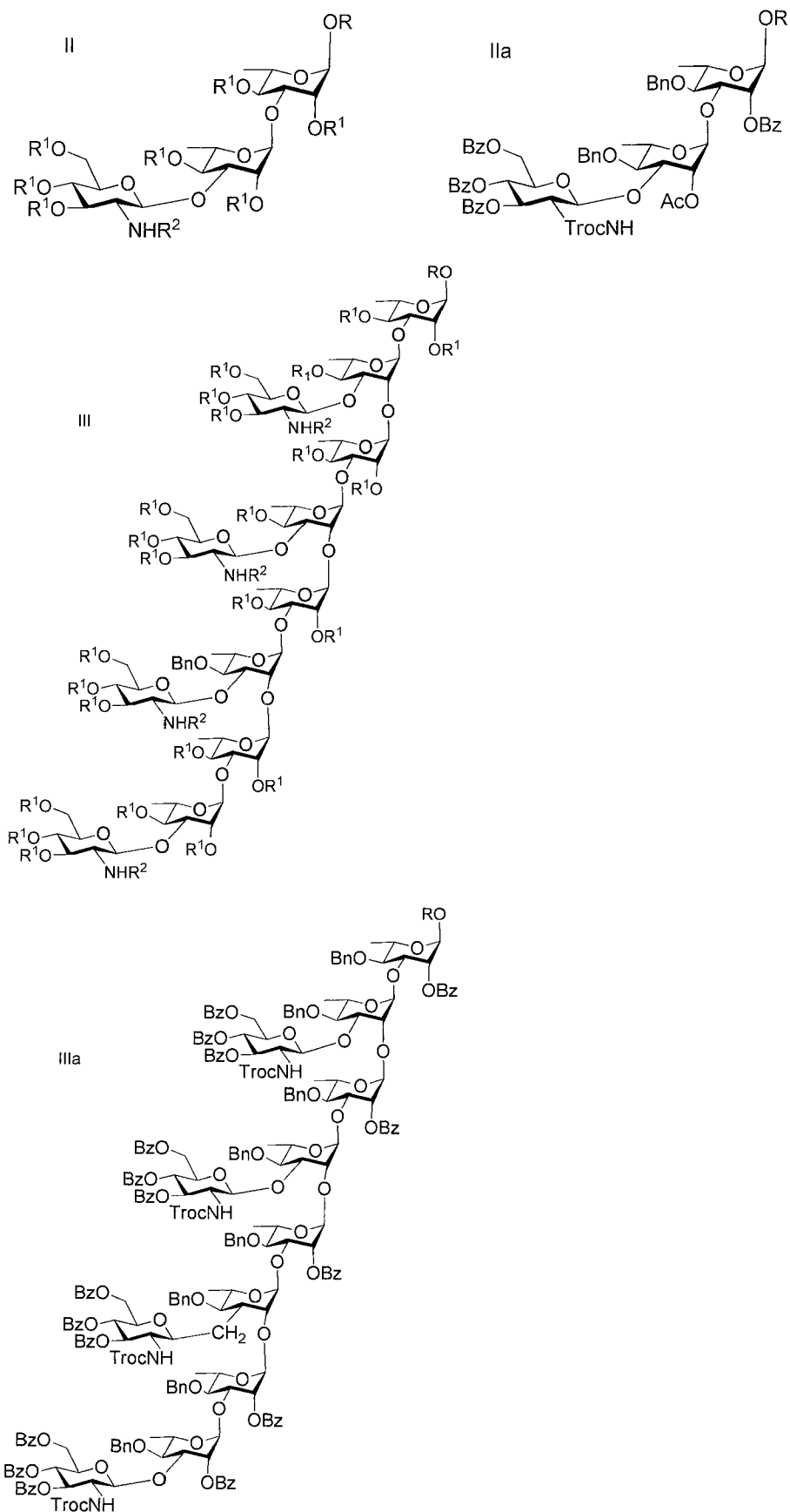
each R^2 is independently H or an amino-protecting group;

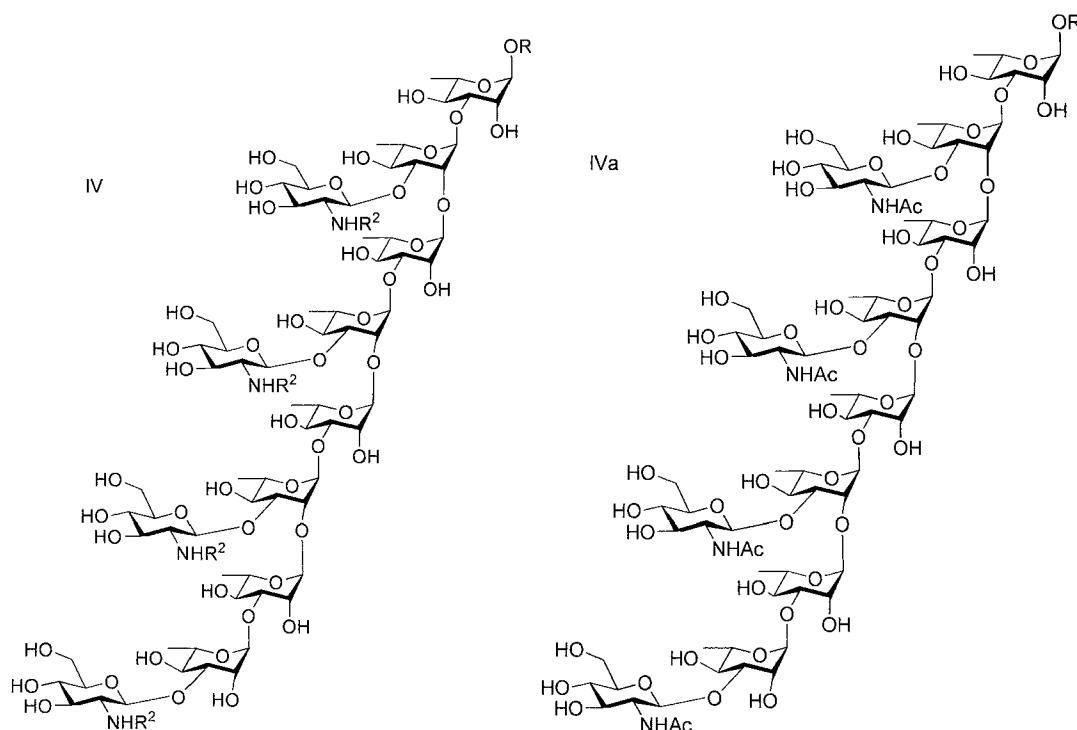
X is a bond or a linker;

Y is H, R or a carrier, where R is a hydroxyl-protecting group
(which can be the same or different than an R^1).

10 In one embodiment, one a is 1 and c is 0. In another embodiment, a is 0 and c is 1. In another embodiment, b is an integer from 2 to 8. In another embodiment, X is a bond and Y is R.

Some embodiments of the present invention are shown below in II, IIa, III, IIIa, IV and IVa. In each of these formula, R^1 , R^2 , R, X and Y are
15 as defined for I.





Oligosaccharides in accordance with the present invention can be formed from the building blocks A, B, C, D, E and E1 shown in Figure 10. An exemplary synthesis is shown in Figure 11.

5 The present invention includes oligosaccharides 61, 62, 63, 64 as well as novel intermediates described below in the examples.

Suitable hydroxyl and amino protecting groups are those customarily considered in carbohydrate chemistry, including those mentioned in "Protective Groups in Organic Synthesis", 3rd edition, T. W. Greene and P. G. M. Wuts (Ed.), John Wiley and Sons, New York, 1999.

10 Exemplary amino-protecting groups (such as R²) are silyl groups such as trimethylsilyl, triisopropylsilyl, tributylsilyl, t-butyldimethylsilyl and t-butyldiarylsilyl; carbamates such as (trichloroethyl) carbamate (Troc), t-butoxycarbonyl, benzyloxycarbonyl, 4-methoxybenzyloxycarbonyl and 4-nitrobenzyloxycarbonyl; formyl, acetyl (Ac), benzoyl and pivaloyl, methoxymethyl, t-butyl, benzyl and tetrahydropyranyl. Troc and Ac are more preferred.

15 Exemplary hydroxy-protecting groups (such as R¹ and Y) are formyl, acetyl, substituted acetyl, propionyl, butynyl, pivalamido, benzoyl, biphenylcarbonyl, substituted biphenylcarbonyl, ethoxycarbonyl, t-

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butoxycarbonyl, benzyloxycarbonyl, phenoxycarbonyl, benzyl, diphenylmethyl, triphenylmethyl, t-butyl, tetrahydropyranyl, allyl (All), N-phenylcarbamate, N-imidazolyl carbamate, trialkylsilyl, isopropylidialkylsilyl, alkylidiisopropylsilyl, triisopropylsilyl and t-butylidialkylsilyl. Among these, benzoyl, biphenylcarbonyl and substituted biphenylcarbonyl are more preferred for R¹. When Y is a hydroxyl protecting group, it can be any group described herein, preferably All or - (CH₂)₂N₃.

Suitable linkers comprise at one end a grouping able to enter into a covalent bonding with a reactive functional group of the carrier, e.g. an amino, thiol, or carboxyl group, and at the other end a grouping likewise able to enter into a covalent bonding with a hydroxyl group of an oligosaccharide according to the present invention. Between the two functional groups of the linker molecule there is a biocompatible bridging molecule of suitable length, e.g. substituted or unsubstituted heteroalkylene, arylalkylene, alkylene, alkenylene, or (oligo)alkylene glycol groups. Linkers preferably include substituted or unsubstituted alkylene or alkenylene groups containing 1-10 carbon atoms.

Linkers able to react with thiol groups on the carrier are, for example, maleimide and carboxyl groups; preferred groupings able to react with aldehyde or carboxyl groups are, for example, amino or thiol groups. Preferred covalent attachments between linkers and carriers include thioethers from reaction of a thiol with an α -halo carbonyl or α -halo nitrile, including reactions of thiols with maleimide; hydrazides from reaction of a hydrazide or hydrazine with an activated carbonyl group (e.g. activated NHS-ester or acid halide); triazoles from reaction of an azide with an alkyne (e.g. via "click chemistry"); and oximes from reaction of a hydroxylamine and an aldehyde or ketone as disclosed, for example, in Lees et al., *Vaccine*, 24:716, 2006. Although amine-based conjugation chemistries could be used in principle for coupling linkers and/or spacers to the oligosaccharides described herein, these approaches would typically sacrifice uniformity inasmuch as the oligosaccharides of the present

invention typically contain a plurality of amines bonded to second carbon of the respective monosaccharide units.

Further suitable linker molecules are known to skilled workers and commercially available or can be designed as required and depending on the functional groups present and can be prepared by known methods.

Suitable carriers are known in the art (See e.g., Remington's Pharmaceutical Sciences (18th ed., Mack Easton, PA (1990)) and may include, for example, proteins, peptides, lipids, polymers, dendrimers, virosomes, virus-like particles (VLPs), or combinations thereof, which by themselves may not display particular antigenic properties, but can support immunogenic reaction of a host to the oligosaccharides of the present invention (antigens) displayed at the surface of the carrier(s).

Preferably, the carrier is a protein carrier, including but are not limited to, bacterial toxoids, toxins, exotoxins, and nontoxic derivatives thereof, such as tetanus toxoid, tetanus toxin Fragment C, diphtheria toxoid, CRM (a nontoxic diphtheria toxin mutant) such as CRM 197, cholera toxoid, *Staphylococcus aureus* exotoxins or toxoids, *Escherichia coli* heat labile enterotoxin, *Pseudomonas aeruginosa* exotoxin A, including recombinantly produced, genetically detoxified variants thereof; bacterial outer membrane proteins, such as *Neisseria meningitidis* serotype B outer membrane protein complex (OMPC), outer membrane class 3 porin (rPorB) and other porins; keyhole limpet hemocyanine (KLH), hepatitis B virus core protein, thyroglobulin, albumins, such as bovine serum albumin (BSA), human serum albumin (HSA), and ovalbumin; pneumococcal surface protein A (PspA), pneumococcal adhesin protein (PsaA); purified protein derivative of tuberculin (PPD); transferrin binding proteins, polyamino acids, such as poly(lysine:glutamic acid); peptidyl agonists of TLR-5 (e.g. flagellin of motile bacteria like *Listeria*); and derivatives and/or combinations of the above carriers. Preferred carriers for use in humans include tetanus toxoid, CRM 197, and OMPC.

Depending on the type of bonding between the linker and the carrier, and the structural nature of the carrier and oligosaccharide, a carrier

may display on average, for example, 1 to 500, 1 to 100, 1 to 20, or 3 to 9 oligosaccharide units on its surface.

Methods for attaching an oligosaccharide to a carrier, such as a carrier protein are conventional, and a skilled practitioner can create conjugates in accordance with the present invention using conventional methods. Guidance is also available in various disclosures, including, for example, U.S. Pat. Nos. 4,356,170; 4,619,828; 5,153,312; 5,422,427; and 5,445,817; and in various print and online Pierce protein cross-linking guides and catalogs (Thermo Fisher, Rockford, IL).

In one embodiment, the carbohydrate antigens of the present invention are conjugated to CRM 197, a commercially available protein carrier used in a number of FDA approved vaccines. CRM-conjugates have the advantage of being easier to synthesize, purify and characterize than other FDA approved carriers such as OMPC. Carbohydrate antigens may be conjugated to CRM via thiol-bromoacetyl conjugation chemistry. CRM activation may be achieved by reacting the lysine side chains with the NHS ester of bromoacetic acid using standard conditions as previously described in U.S. Pat. Appl. Publ. 2007-0134762, the disclosures of which are incorporated by reference herein. CRM may be functionalized with 10-20 bromoacetyl groups per protein (n=10-20) prior to conjugation. Conjugation may be performed at pH=9 to avoid aggregation of CRM. Careful monitoring of pH must be employed to ensure complete CRM reaction with NHS-bromoacetate while minimizing background hydrolysis of CRM. Activated CRM may be purified by size exclusion chromatography prior to conjugation. Antigen-CRM conjugates may be synthesized by reacting thiol-terminated carbohydrate antigens with bromoacetamide-activated CRM.

CRM conjugates may be purified via size exclusion chromatography to remove and recover any unreacted carbohydrate. MBTH (specific for GlcNAc residues) and Bradford assays may be used to determine carbohydrate:protein ratio and protein content, respectively, as previously described (Manzi et al., Curr. Prot. Mol. Biol., section 17.9.1 (Suppl. 32), 1995. In preferred embodiments, a minimum carbohydrate content of about

15% by weight for each conjugate may be generated. Typically, a conjugate may include about 3-20 antigens per protein carrier.

5 In another embodiment, carbohydrate antigens may be conjugated to one or more carriers suitable for development of diagnostic assays, including ELISAs and microarrays. Exemplary carriers for use in such assays include bovine serum albumin (BSA), keyhole limpet hemocyanine (KLH), biotin, a label, a glass slide or a gold surface. By way of example, synthetic carbohydrate antigens may be conjugated to BSA by a thiol-maleimide coupling procedure. Maleimide-BSA contains 15-20 maleimide groups per protein (n=15-20). Accordingly, oligosaccharide antigens may be conjugated to maleimide functionalized BSA, whereby a 20-fold molar excess of the antigen is reacted with commercially available Inject maleimide BSA (Pierce) in maleimide conjugation buffer (Pierce). Conjugation may be performed at pH=7.2 to avoid hydrolysis of the maleimide group during conjugation.

15 In another aspect, the present invention provides compositions containing GAS-PS oligosaccharides I and a pharmaceutically acceptable vehicle. The compositions are preferably immunogenic and immunoprotective.

20 The present invention contemplates the use of single- and multi-valent vaccines comprising any of the synthetic oligosaccharides described herein. The identification of a single oligosaccharide antigen eliciting a protective immune response can facilitate development of a single-antigen vaccine candidate against one or more bacterial target(s) expressing GAS-PS. Thus, in one embodiment, the compositions may contain a single oligosaccharide I.

25 The present invention further contemplates multi-antigen vaccine candidates and vaccines thereof. In one embodiment, the invention provides a composition containing two, three, four or more different oligosaccharides I.

30 Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences (18th ed., Mack Easton Pa. (1990)).

Pharmaceutically acceptable vehicles may include any vehicle that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable vehicles may include large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers; inactive virus particles, insoluble aluminum compounds, calcium phosphate, liposomes, virosomes, ISCOMS, microparticles, emulsions, and VLPs.

The compositions of the present invention may further include one or more adjuvants. An oligosaccharide-protein conjugate composition may further include one or more immunogenic adjuvant(s). An immunogenic adjuvant is a compound that, when combined with an antigen, increases the immune response to the antigen as compared to the response induced by the antigen alone so that less antigen can be used to achieve a similar response. For example, an adjuvant may augment humoral immune responses, cell-mediated immune responses, or both.

Those of skill in the art will appreciate that the terms “adjuvant,” and “carrier,” can overlap to a significant extent. For example, a substance which acts as an “adjuvant” may also be a “carrier,” and certain other substances normally thought of as “carriers,” for example, may also function as an “adjuvant.” Accordingly, a substance which may increase the immunogenicity of the synthetic oligosaccharide or carrier associated therewith is a potential adjuvant. As used herein, a carrier is generally used in the context of a more directed site-specific conjugation to an oligosaccharide of the present invention, whereby an adjuvant is generally used in a less specific or more generalized structural association therewith.

Exemplary adjuvants and/or adjuvant combinations may be selected from the group consisting of mineral salts, including aluminum salts, such as aluminum phosphate and aluminum hydroxide (alum) (e.g., Alhydrogel™, Superfos, Denmark) and calcium phosphate; RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate, and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion, whereby any of the 3 components MPL,

TDM or CWS may also be used alone or combined 2 by 2; toll-like receptor (TLR) agonists, including, for example, agonists of TLR-1 (e.g. tri-acyl lipopeptides); agonists of TLR-2 (e.g. peptidoglycan of gram-positive bacteria like streptococci and staphylococci; lipoteichoic acid); agonists of TLR-3 (e.g. double-stranded RNA and their analogs such as poly 1:C); agonists of TLR-4 (e.g. lipopolysaccharide (endotoxin) of gram-negative bacteria like Salmonella and E. coli); agonists of TLR-5 (e.g. flagellin of motile bacteria like Listeria); agonists of TLR-6 (e.g. with TLR-2 peptidoglycan and certain lipids (diacyl lipopeptides)); agonists of TLR-7 (e.g. single-stranded RNA (ssRNA) genomes of such viruses as influenza, measles, and mumps; and small synthetic guanosine-base antiviral molecules like loxoribine and ssRNA and their analogs); agonists of TLR-8 (e.g. binds ssRNA); agonists of TLR-9 (e.g. unmethylated CpG of the DNA of the pathogen and their analogs; agonists of TLR-10 (function not defined) and TLR-11-(e.g. binds proteins expressed by several infectious protozoans (Apicomplexa), specific toll-like receptor agonists include monophosphoryl lipid A (MPL[®]), 3 De-O-acylated monophosphoryl lipid A (3 D-MPL), OM-174 (E. coli lipid A derivative); OM triacyl lipid A derivative, and other MPL- or lipid A-based formulations and combinations thereof, including MPL[®]-SE, RC-529 (Dynavax Technologies), AS01 (liposomes+MPL+QS21), AS02 (oil-in-water PL + QS-21), and AS04 (Alum + MPL)(GlaxoSmith Kline, Pa.), CpG-oligodeoxynucleotides (ODNs) containing immunostimulatory CpG motifs, double-stranded RNA, polyinosinic:polycytidylic acid (poly I:C), and other oligonucleotides or polynucleotides optionally encapsulated in liposomes; oil-in-water emulsions, including AS03 (GlaxoSmith Kline, Pa.), MF-59 (microfluidized detergent stabilized squalene oil-in-water emulsion; Novartis), and Montanide ISA-51 VG (stabilized water-in-oil emulsion) and Montanide ISA-720 (stabilized water/squalene; Seppic Pharmaceuticals, Fairfield, NJ); cholera toxin B subunit; saponins, such as Quil A or QS21, an HPLC purified non-toxic fraction derived from the bark of Quillaja Saponaria Molina (STIMULON[™] (Antigenics, Inc., Lexington, Mass.) and saponin-based adjuvants, including immunostimulating complexes

(ISCOMs; structured complex of saponins and lipids) and other ISCOM-based adjuvants, such as ISCOMATRIX™ and AbISCO®-100 and -300 series adjuvants (Isconova AB, Uppsala, Sweden); QS21 and 3 D-MPL together with an oil in water emulsion as disclosed in U.S. Pat. Appl. No. 2006/0073171; stearyl tyrosine (ST) and amide analogs thereof; virus-like particles (VLPs) and reconstituted influenza virosomes (IRIVs); complete Freund's adjuvant (CFA); incomplete Freund's adjuvant (IFA); *E. coli* heat-labile enterotoxin (LT); immune-adjuvants, including cytokines, such as IL-2, IL-12, GM-CSF, Flt3, accessory molecules, such as B7.1, and mast cell (MC) activators, such as mast cell activator compound 48/80 (C48/80); water-insoluble inorganic salts; liposomes, including those made from DNPC/Chol and DC Chol; micelles; squalene; squalane; muramyl dipeptides, such as N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP) as found in U.S. Pat. No. 4,606,918, N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), and N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'2'-dipalmitoyl-n-glycero-3-hydroxyphosphoryl; SAF-1 (Syntex); AS05 (GlaxoSmith Kline, Pa.); and combinations thereof.

In preferred embodiments, adjuvant potency may be enhanced by combining multiple adjuvants as described above, including combining various delivery systems with immunopotentiating substances to form multi-component adjuvants with the potential to act synergistically to enhance antigen-specific immune responses in vivo. Exemplary immunopotentiating substances include the above-described adjuvants, including, for example, MPL and synthetic derivatives, MDP and derivatives, oligonucleotides (CpG etc), ds RNAs, alternative pathogen-associated molecular patterns (PAMPs)(*E. coli* heat labile enterotoxin; flagellin, saponins (QS-21 etc), small molecule immune potentiators (SMIPs, e.g., resiquimod (R848)), cytokines, and chemokines.

Methods of Treating or Preventing GAS infections

Oligosaccharide compositions

In one embodiment, the present invention provides pharmaceutically acceptable immunogenic or immunoprotective oligosaccharide

compositions and their use in methods for preventing GAS infection in a patient in need thereof. In one embodiment, comprising administering an effective amount of an oligosaccharide of the present invention. An immunogenic or immunoprotective composition will include a “sufficient amount” or “an immunologically effective amount” of a GAS-PS -protein conjugate according to the present invention, as well as any of the above mentioned components, for purposes of generating an immune response or providing protective immunity, as further defined herein.

Administration of the oligosaccharide- or oligosaccharide conjugate compositions or antibodies, as described herein may be carried out by any suitable means, including by parenteral administration (e.g., intravenously, subcutaneously, intradermally, or intramuscularly); by topical administration, of for example, antibodies to an airway surface; by oral administration; by in ovo injection in birds, for example, and the like. Preferably, they are administered intramuscularly.

Typically, the compositions may be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection. An aqueous composition for parenteral administration, for example, may include a solution of the immunogenic component(s) dissolved or suspended in a pharmaceutically acceptable vehicle or diluent, preferably a primarily aqueous vehicle. An aqueous composition may be formulated as a sterile, pyrogen-free buffered saline or phosphate-containing solution, which may include a preservative or may be preservative free. Suitable preservatives include benzyl alcohol, parabens, thimerosal, chlorobutanol, and benzalkonium chloride, for example. Aqueous solutions are preferably approximately isotonic, and its tonicity may be adjusted with agents such as sodium tartrate, sodium chloride, propylene glycol, and sodium phosphate. Additionally, auxiliary substances required to approximate physiological conditions, including pH adjusting and buffering agents, tonicity adjusting agents, wetting or emulsifying agents, pH buffering substances, and the like, including sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium

chloride, sorbitan monolaurate, triethanolamine oleate, etc. may be included with the vehicles described herein.

5 Compositions may be formulated in a solid or liquid form for oral delivery. For solid compositions, nontoxic and/or pharmaceutically acceptable solid vehicles may include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition may be formed by incorporating any of the normally employed excipients, 10 including those vehicles previously listed, and a unit dosage of an active ingredient, that is, one or more compounds of the invention, whether conjugated to a carrier or not. Topical application of antibodies to an airway surface can be carried out by intranasal administration (e.g., by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the antibodies to an airway surface can 15 also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the antibodies as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatuses for administering respirable particles of 20 pharmaceutical formulations are well known, and any conventional technique can be employed. Oral administration may be in the form of an ingestible liquid or solid formulation.

25 The preparation of such pharmaceutical compositions is within the ordinary skill in the art, and may be guided by standard reference books such as Remington the Science and Practice of Pharmacy, Lippincott Williams & Wilkins; 21 ed., May 1, 2005, which is incorporated herein by reference.

30 The concentration of the oligosaccharides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 0.1% to as much as 20% to 50% or more by weight, and may be selected on the basis of fluid volumes, viscosities, stability, etc., and/or in accordance with the particular mode of

administration selected. A human unit dose form of the compounds and composition is typically included in a pharmaceutical composition that comprises a human unit dose of an acceptable vehicle, preferably an aqueous vehicle, and is administered in a volume of fluid that is known by those of skill in the art to be used for administration of such compositions to humans, and is adjusted according to commonly understood principles for a particular subject to be treated. Thus in one embodiment, the invention provides a unit dosage of the vaccine components of the invention in a suitable amount of an aqueous solution, such as 0.1-3 ml, preferably 0.2-2 mL.

The compositions of the present invention may be administered to any animal species at risk for developing an infection by a microbial species expressing a GAS-PS antigen.

The present invention can also be used to treat or prevent other bacteria infections where the bacterium is known or suspected to express GAS-PS. Suitable bacteria that can be treated with the present invention include *Streptococcus* species, such as *S. pyogenes*. Accordingly, specific GAS-PS oligosaccharides may be modified, depending on the specific compositional makeup, including acetylation profiles of these antigens in their respective bacterial species.

The treatment may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of treatment may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable treatment schedules include: (i) 0, 1 month and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired responses expected to reduce disease symptoms, or reduce severity of disease.

The amounts effective for inducing an immune response or providing protective immunity will depend on a variety of factors, including the oligosaccharide composition, conjugation to a carrier, inclusion and nature of adjuvant(s), the manner of administration, the weight and general

state of health of the patient, and the judgment of the prescribing physician.

By way of example, the amounts may generally range for the initial immunization (that is for a prophylactic administration) from about 1.0 μg to about 5,000 μg of oligosaccharide for a 70 kg patient, (e.g., 1.0 μg , 2.0 μg , 2.5 μg , 3.0 μg , 3.5 μg , 4.0 μg , 4.5 μg , 5.0 μg , 7.5 μg , 10 μg , 12.5 μg , 15 μg , 17.5 μg , 20 μg , 25 μg , 30 μg , 35 μg , 40 μg , 45 μg , 50 μg , 75 μg , 100 μg , 250 μg , 500 μg , 750 μg , 1,000 μg , 1,500 μg , 2,000 μg , 2,500 μg , 3,000 μg , 3,500 μg , 4,000 μg , 4,500 μg or 5,000 μg). The actual dose administered to a subject is often, but not necessarily, determined according to an appropriate amount per kg of the subject's body weight. For example, an effective amount may be about 0.1 μg to 5 $\mu\text{g}/\text{kg}$ body weight.

A primary dose may optionally be followed by boosting dosages of from about 1.0 to about 1,000 of peptide (e.g., 1.0 μg , 2.0 μg , 2.5 μg , 3.0 μg , 3.5 μg , 4.0 μg , 4.5 μg , 5.0 μg , 7.5 μg , 10 μg , 12.5 μg , 15 μg , 17.5 μg , 20 μg , 25 μg , 30 μg , 35 μg , 40 μg , 45 μg , 50 μg , 75 μg , 100 μg , 250 μg , 500 μg , 750 μg , 1,000 μg , 1,500 μg , 2,000 μg , 2,500 μg , 3,000 μg , 3,500 μg , 4,000 μg , 4,500 μg or 5,000 μg) pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific T cell activity in the patient's blood.

The immunogenic compositions comprising a compound of the invention may be suitable for use in adult humans or in children, including young children or others at risk for contracting an infection caused by a GAS-PS-expressing bacterial species. Optionally such a composition may be administered in combination with other pharmaceutically active substances, and frequently it will be administered in combination with other vaccines as part of a childhood vaccination program.

Antibody Compositions

In another embodiment, the invention provides an antibody preparation against one or more oligosaccharides I in accordance with the present invention. The antibody preparation may include any member from the group consisting of polyclonal antibody, monoclonal antibody, mouse monoclonal IgG antibody, humanized antibody, chimeric antibody, fragment thereof, or combination thereof.

Pharmaceutical antibody compositions may be used in a method for providing passive immunity against a bacterial target species of interest, including *S. pyogenes* and other GAS-PS-expressing bacteria. A pharmaceutical antibody composition may be administered to an animal subject, preferably a human, in an amount sufficient to prevent or attenuate the severity, extent of duration of the infection by the bacterial target species of interest.

The administration of the antibody may be either prophylactic (prior to anticipated exposure to a bacterial infection) or therapeutic (after the initiation of the infection, at or shortly after the onset of the symptoms). The dosage of the antibodies will vary depending upon factors as the subject's age, weight and species. In general, the dosage of the antibody may be in a range from about 1-10 mg/kg body weight. In a preferred embodiment, the antibody is a humanized antibody of the IgG or the IgA class. The route of administration of the antibody may be oral or systemic, for example, subcutaneous, intramuscular or intravenous.

Antibodies in diagnostic assays

In a further aspect, the present invention provides compositions and methods for inducing production of antibodies for diagnosing, treating, and/or preventing one or more infections caused by GAS-PS expressing bacteria.

Antisera to GAS-PS conjugates may be generated in New Zealand white rabbits by 3-4 subcutaneous injections over 13 weeks. A pre-immune bleed may generate about 5 mL of baseline serum from each rabbit. A prime injection (10 μ g antigen equivalent) may be administered as an emulsion in complete Freund's adjuvant (CFA). Subsequent injections (5 μ g antigen equivalent) may be given at three week intervals in incomplete Freund's adjuvant (IFA). Rabbits may be bled every two weeks commencing one week after the third immunization. Approximately 25 – 30 mL of serum per rabbit may be generated from each bleeding event and frozen at -80°C. Serum may be analyzed by ELISA against the corresponding GAS-PS conjugate as described below. In addition, antisera from later bleeds may be affinity purified as further described below.

The oligosaccharides and antibodies generated therefrom can be used as diagnostic reagents for detecting GAS-PS structures or antibodies thereagainst, which are present in biological samples. The detection reagents may be used in a variety of immunodiagnostic techniques, known to those of skill in the art, including ELISA- and microarray-related technologies. In addition, these reagents may be used to evaluate antibody responses, including serum antibody levels, to immunogenic oligosaccharide conjugates. The assay methodologies of the invention typically involve the use of labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, and/or secondary immunologic reagents for direct or indirect detection of a complex between an antigen or antibody in a biological sample and a corresponding antibody or antigen bound to a solid support.

Such assays typically involve separation of unbound antibody in a liquid phase from a solid phase support to which antibody-antigen complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

Typically, a solid support is first reacted with a first binding component (e.g., an anti- GAS-PS antibody or GAS-PS oligosaccharide) under suitable binding conditions such that the first binding component is sufficiently immobilized to the support. In some cases, mobilization to the support can be enhanced by first coupling the antibody or oligosaccharide to a protein with better binding properties, or that provides for immobilization of the antibody or antigen on the support without significant loss of antibody binding activity or specificity. Suitable coupling proteins include, but are not limited to, macromolecules such as serum albumins including bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art. Other molecules that can be

used to bind antibodies the support include polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and the like. Such molecules and methods of coupling these molecules are well known to those of ordinary skill in the art and are described in, for example, U.S. Pat. No. 7,595,307, U.S. Pat. Appl. No. US 2009/0155299, the disclosures and cited references therein of which are incorporated by reference herein.

The following examples are included for purposes of illustration and are not intended to limit the scope of the invention.

CRM₁₉₇ conjugates with the native GAS-PS and with four different synthetic GAS oligosaccharides varying in length and composition of the reducing end (Fig. 1b) were prepared and characterized by physico-chemical methods. conjugation of the synthetic antigens yielded glycoconjugates of more homogeneous nature compared to conjugates with isolated GAS-PS was observed (Fig. 4a). The more homogeneous nature of the synthetic oligosaccharides glycoconjugates is an important feature that may facilitate control over the conjugation process and establishment of the immunogenicity correlates. Importantly, native GAS-PS isolated from bacterial fermentation has contamination with its polyrhamnose variant species, the presence of which might potentially interfere with standardization of the antigen preparation, again pointing in favor of synthetic approach applied to anti-GAS vaccines.

CRM₁₉₇ conjugates of both synthetic oligosaccharides and native GAS-PS elicited high levels of serum GAS-PS-specific IgG with a bias towards IgG1 production (Fig. 6). Carbohydrate antigen formulation with Alum was suitable, as it favored a Th2 response that is preferred for vaccination against extracellular pathogens. The synthetic oligosaccharides and GAS-PS were antigenically similar and induced antibodies with hexasaccharide paratope.

Trying to establish a few variables related to the immunogenicity of the synthetic GAS oligosaccharides conjugates, the saccharide chain length, the conjugate glycosylation degree and the terminal non-reducing sugar were varied. A saccharide chain length of six, corresponding to the

minimal size of the epitope, is sufficient to elicit a protective response characterized by antibodies which recognize the native polysaccharide. Concerning the glycosylation degree of the conjugates, a loading between 6 and 8 carbohydrate chains per protein molecule, has been found optimal for the glycoconjugates with dodecasaccharide 63, while higher loading of 12 chains led to a decrease in the immunogenicity (Fig. 6 and Table 1).

Table 1. Glycoconjugate composition and geometric mean titres (GMT) of serum anti-GAS-PS antibodies induced after mice immunization and MALDI-TOF.

Conjugate code	Average CHO/Protein Mol/mol		Total IgG GMT	AI ^a , M NaSCN
	Chemical assays ^b	MALDI-TOF		
CRM ₁₉₇ -61/1	3.4	Nd	2912	1.29
CRM ₁₉₇ -61/2	11.6	14.2	5980	0.87
CRM ₁₉₇ -62/1	5.1	Nd	1895	1.08
CRM ₁₉₇ -62/2	7.6	7.5	2465	0.64
CRM ₁₉₇ -63/1	5.8	Nd	10262	0.81
CRM ₁₉₇ -63/2	7.5	8.8	17780	1.24
CRM ₁₉₇ -63/3	12.2	13.6	639	0.68
CRM ₁₉₇ -64/1	7.6	Nd	4063	0.92
CRM ₁₉₇ -64/2	9.2	11.5	2108	0.85
GAS-PS	2-3	3-4	12086	0.86

^a The avidity index (AI) is expressed as the concentration of NaSCN needed to reduce the OD₄₅₀ by 50%. Pooled sera were analyzed.

^b See example section below.

Synthetic conjugates induced protective immunity in mice, against two GAS isolates of M1 and M23 serotypes. Interestingly, despite the fact that both strains appeared highly encapsulated, GAS-PS on their surface was accessible for antibody binding as revealed by flow cytometry analyses (Fig. 7). The conjugates obtained with all four types of synthetic GAS oligosaccharides were able to induce significant levels of protection against M1 challenge in mice (Fig. 8). Furthermore, immunization with CRM₁₉₇-61, one of the minimal protective units of GAS-PS, conferred 56% protection against M23 strain. Using an M1 strain it was shown that anti-GAS-PS antibodies induced by the four synthetic glycoconjugates in

rabbits, promote opsonophagocytic killing of GAS in a whole blood assay (Fig. 9).

Thus, synthetic GAS-PS structures display similar immunogenicity to that of natural GAS-PS. The glycoconjugates CRM₁₉₇-63/1 and CRM₁₉₇-63/2, exposing optimal number of dodecasaccharides containing GAS-PS antigenic core and immunodominant GlcNAc sugar on the non-reducing terminus, elicited specific IgG titres in mice comparable to those induced by CRM₁₉₇-GAS-PS. Immunoprotection studies in a mouse challenge model and opsonophagocytosis *in vitro* assays with specific rabbit antisera demonstrated that synthetic conjugate vaccine candidates have similar efficacy to conjugates of isolated GAS-polysaccharide.

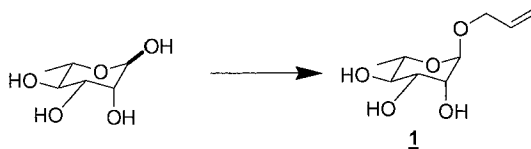
Synthetic oligosaccharides are valuable for carbohydrate conjugate vaccine development against Group A *Streptococcus* as has been reported for other pathogens. Until recently, the major obstacle to the development of synthetic carbohydrate vaccines were the challenges associated with the chemical synthesis of oligosaccharides. Automated oligosaccharide synthesis provides now ready access to synthetic antigens and tools such as glycan microarrays to assess immune response and to map antibody epitopes. Synthetic conjugate vaccines have been shown to generate protective immune responses against a growing number of infectious agents including *Streptococcus pneumoniae* type 3, 6A, 6B and 14, *Shigella dysenteriae* type 1, *Haemophilus influenzae* type b. Synthetic glyco-antigens formed also the basis for tumor vaccines candidates as well as *Bacillus anthracis*, *Plasmodium falciparum* and *Leishmania* conjugate vaccines.

Synthetic oligosaccharides representing the conserved GAS glycan can be used in a optimized multivalent formulation based on glycoconjugates and recombinant protein antigens.

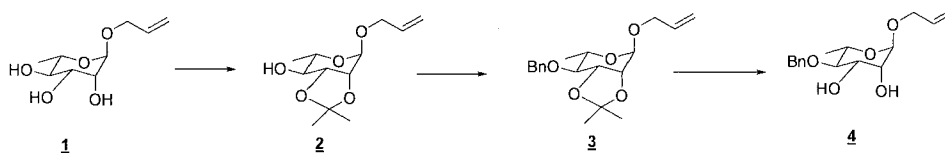
EXAMPLES

Synthesis of building blocks

Various building blocks can be synthesized as shown in Figures 10 and 11 and as exemplified below.



Synthesis of compound 1: To a 2L three neck flask, fitted with a reflux condenser, a mechanical paddle stirrer, and a nitrogen bubbler, was added 100 g (0.55 mol) L-rhamnose monohydrate, 300 mL allyl alcohol, and 1.0 g (2.0 mmol) of scandium (III) triflate. The resulting mixture was heated to reflux under nitrogen with stirring for 10 hours. TLC [dichloromethane : methyl alcohol, (5:1 v/v)] indicated the consumption of starting material with the formation of a faster running product 1 spot at 0.5 R_f . The mixture was allowed to cool to room temperature, and was followed by the addition of 30 mL triethylamine. The reaction mixture was concentrated under reduced pressure to a thick syrup. The resulting syrup was taken up in 1.0 L of a 9:1 (v/v) solution of dichloromethane and methyl alcohol. The crude product solution was applied via suction to a 16 cm (d) x 10 cm (h) pad of 800 g 40-63 μ m silica, which had been pre-eluted via suction with 2.0 L of 9:1 (v/v) dichloromethane: methyl alcohol. The loaded pad was then eluted via suction by the application and collection of 1.0 L fractions of: dichloromethane (2.0 L total), followed by 9:1 (v/v) dichloromethane: methyl alcohol (5.0 L total). The product containing fractions were concentrated under reduced pressure to give 100 g (0.49 mol) desired product 1 as a yellowish syrup in 89% yield.



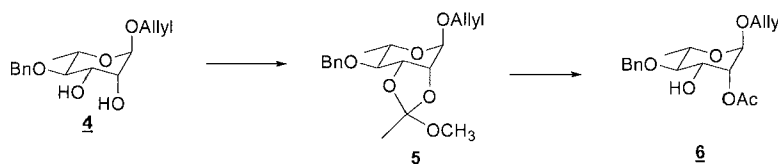
Synthesis of compound 4: A mixture of 84 g (0.41 mol) 1, 200 mL anhydrous acetone, 200 mL 2,2-dimethoxypropane, and 500 mg ($\pm$)-camphor sulfonic acid was stirred for 1h at 23°C. TLC [dichloromethane : methyl alcohol, 10:1 (v/v)] indicated the consumption of starting material 1 with the formation of a faster running product spot 2 at 0.8 R_f . The reaction mixture was neutralized by careful addition of 100 mL saturated sodium bicarbonate solution and extracted with 1.0 L ethyl acetate. The product

containing extract was dried by stirring over 200 g anhydrous sodium sulfate for 15 minutes and filtered. The filtrate was concentrated under reduced pressure giving 2 as a crude syrup, which, after further drying by co-evaporation with 300 mL portions of anhydrous toluene 3 times, was used directly in the next step.

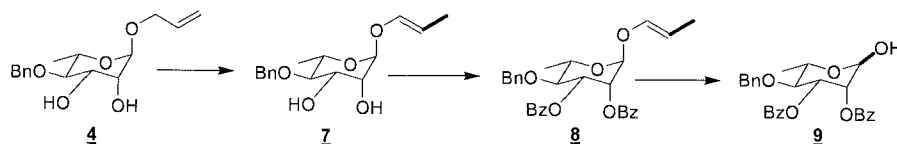
Crude acetonide 2 was dissolved in 100 mL of anhydrous tetrahydrofuran. The resulting solution was transferred via Teflon[®] cannula over 15 minutes to a stirred suspension of 20 g of 60% sodium hydride (oil dispersion) in 200 mL of a 4:1 (v/v) mixture of anhydrous tetrahydrofuran: anhydrous 1-methyl-2-pyrrolidinone, which had been pre-cooled 0°C, under a slow nitrogen stream. The resulting solution was stirred for 5 minutes, then 40 g of benzyl bromide was added dropwise over 30 minutes via dropping funnel. The reaction mixture was then allowed to warm to room temperature and stirred 12 hours longer. TLC [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 2 with the formation of a faster running product 3 spot at 0.7 R_f. The reaction mixture was cooled to 0°C and excess sodium hydride quenched by the dropwise addition of 20 mL of methyl alcohol over 15 minutes. The quenched reaction mixture was warmed to room temperature, stirred for 1 hour, then diluted by the addition of 500 mL tetrahydrofuran and 200 mL brine. The upper product containing organic layer was again washed with 200 mL brine. The organic layer was dried over anhydrous magnesium sulfate with stirring for 5 minutes, filtered, and concentrated under reduced pressure to a syrup. The crude syrup 3 was used directly in the next step.

Crude 3 was dissolved with stirring at room temperature in 500 mL of a 4:1:1 (v/v/v) solution of tetrahydrofuran: water: trifluoroacetic acid. After 12 hours, TLC [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of starting material with the formation of a slower running product 4 spot at 0.4 R_f. The reaction mixture was then diluted with 200 mL brine. The upper product-containing organic layer was again washed with 200 mL brine. The organic layer was dried over anhydrous sodium sulfate with stirring for 5 minutes, filtered, and concentrated under reduced pressure to a thick syrup. The crude product 4 was dissolved in 200 mL

dichloromethane and applied via suction to a 16 cm (d) x 10 cm (h) pad of 800 g 40-63 μ m silica, which had been pre-eluted via suction with 2.0 L of 8:1 (v/v) heptane : ethyl acetate. The loaded pad was then eluted via suction by the application and collection of 1.0 L fractions of: 8:1 (v/v) heptane : ethyl acetate (4.0 L total), followed by 4:1 (v/v) heptane : ethyl acetate (4.0 L total). The product containing fractions were concentrated under reduced pressure to give 76 g (0.26 mol) of desired product 4 as a yellowish syrup in a 3 step overall yield of 63%.



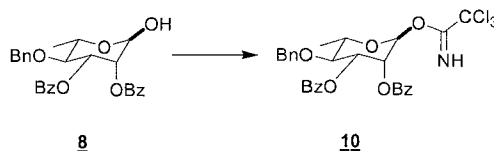
Synthesis of compound 6: A mixture of 129 g (0.44 mol) compound 4, 300 mL anhydrous acetonitrile, 112 mL trimethylorthoacetate, and 10 g ($\pm$)-camphor-10-sulfonic acid was stirred at room temperature for 30 minutes. TLC [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of starting material 4 with the formation of a faster running product 5 spot at 0.6 R_f . To the reaction mixture was added 10 mL of a 8:1 (v/v) solution of acetic acid: water and stirring was continued for 30 minutes longer. TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 5 with the formation of a slower running product spot 6 at 0.4 R_f . The reaction mixture was concentrated under reduced pressure to dryness giving crude 6 as a yellow syrup. The crude 6 was dissolved in 200 mL dichloromethane and applied via suction to a 16 cm (d) x 10 cm (h) pad of 800 g 40-63 μ m silica, which had been pre-eluted via suction with 2.0 L of 8:1 (v/v) heptane : ethyl acetate. The loaded pad was then eluted via suction by the application and collection of 1.0 L fractions of: 8:1 (v/v) heptane : ethyl acetate (4.0 L total), followed by 4:1 (v/v) heptane : ethyl acetate (4.0 L total). The product 6 containing portions were concentrated under reduced pressure to give 135 g (0.40 mol) desired product as a yellowish syrup in a 2 step overall yield of 91%.



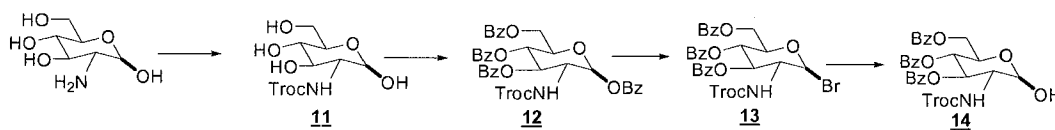
Synthesis of compound 9: A 50 g (0.17 mol) portion of 4 was dried by dissolving in 200 mL anhydrous toluene and evaporating to a syrup 3 times. To the dried 4 was added 48 g (0.43 mol) potassium tert-butoxide and 300 mL anhydrous dimethylsulfoxide. The resulting mixture was heated to 85°C, under nitrogen, for 12 hours. TLC analysis [heptane : tert-butyl methyl ether, 1:1 (v/v), 2 elutions] indicated the consumption of 4 with the formation of a faster running product spot 7, at 0.3 R_f. The reaction was cooled to room temperature, diluted with 500 mL tert-butyl methyl ether and washed with 400 mL brine. The upper layer containing product 7 was stirred with 100 g anhydrous sodium sulfate, 50 g silica, and 10 g activated charcoal for 10 minutes, then filtered through 50 g Celite[®] 545. The filtrate was concentrated under reduced pressure giving product 7 as a yellow orange syrup, which was used directly in the next step.

To the crude 7 syrup was added 200 mL anhydrous pyridine followed by the dropwise addition of 30 mL benzoyl chloride over 15 minutes. The resulting mixture was stirred for 12 hours, at room temperature. TLC analysis [heptane : tert-butyl methyl ether, 1:1 (v/v)] indicated the consumption of 7 with the formation of a faster running product spot 8, at 0.8 R_f. The reaction mixture was concentrated under reduced pressure to a thick syrup, taken up in 500 mL ethyl acetate and washed with 300 mL brine. The product 8 containing upper layer was concentrated under reduced pressure to a yellowish syrup. The syrup was taken up in 300 mL tetrahydrofuran, 20 mL of 2N hydrochloric acid was added and the resulting mixture heated to 50°C, with stirring, for 12 hours. TLC analysis [heptane : tert-butyl methyl ether, 1:1 (v/v)] indicated the consumption of 8 with the formation of a slower running product spot 9 at 0.4 R_f. The reaction mixture was concentrated under reduced pressure to a syrup and dissolved in 20 mL dichloromethane. The crude product 9 solution was applied via suction to a 16 cm (d) x 10 cm (h) pad of 800 g 40-

63 μ m silica, which had been pre-eluted via suction with 2.0 L 9:1 (v/v) heptane : ethyl acetate. The loaded pad was then eluted via suction by the application and collection of 1.0 L fractions of: 9:1 (v/v) heptane : ethyl acetate (3.0 L total), 6:1 (v/v) heptane : ethyl acetate (3.0 L total), and 4:1 (v/v) heptane : ethyl acetate (4.0 L total). The product containing fractions were concentrated under reduced pressure to give 55 g (0.12 mol) of desired product 9 as a yellowish solid in a 3 step overall yield of 70%.



Synthesis of compound 10: A 101g (0.22 mol) portion of 8 was taken up in 150 mL trichloroacetonitrile and 20 mL of anhydrous dichloromethane at room temperature. To the resulting solution was added 100g anhydrous potassium carbonate powder (-325 mesh). After 40 minutes, TLC analysis [heptane : ethyl acetate, 4:1 (v/v)] indicated the consumption of 8 with the formation of a faster running product spot 10, at 0.75 R_f . The reaction mixture was filtered through a pad of 50 g Celite[®] 545, followed by a 100 mL dichloromethane wash of the pad. The filtrate and wash were combined and applied via suction to a 5 cm (d) x 5 cm (h) pad of 100 g 40-63 μ m silica, which had been pre-eluted via suction with 200 mL of 4:1 (v/v) heptane: ethyl acetate. The loaded pad was eluted via suction by the application and collection of 1 L fractions of 4:1 (v/v) heptane : ethyl acetate (5.0 L total). The product containing fractions were concentrated under reduced pressure to give 115 g (0.19 mol) of desired product 10 as a yellowish syrup in yield of 87%.



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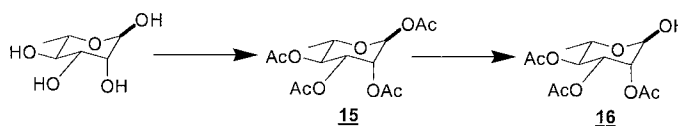
Synthesis of compound 14: To a mechanically stirred solution of 305 g (1.4 mol) D-(+)-glucosamine hydrochloride in 600 mL of water was added 119 g sodium bicarbonate portionwise, so that foaming of the

resulting mixture was controlled. The resulting mixture was then cooled to 0°C and 310 g 2,2,2-trichloroethyl chloroformate were added dropwise at such a rate to keep the reaction temperature below 10°C and to keep foaming under control. The reaction was allowed to warm to room temperature and stirred another 6 hours. The mixture was filtered and the off-white solid obtained was dried for 12 hours under 1 torr vacuum to give 187 g of 11, which was used without further treatment in the next step.

Intermediate 11 was taken up in 1.4 L anhydrous pyridine, cooled to 0°C with mechanical stirring, and 455 mL benzoyl chloride were added dropwise. The addition rate was adjusted to keep the reaction temperature below 20°C. The resulting mixture was stirred at room temperature 12 hours longer. TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 11 with the formation of a faster running pair of close running product spots 12 at 0.8 R_f. The reaction mixture was cooled to 5°C, and 500 mL methyl alcohol was slowly added so that the resulting exothermic quenching of the excess benzoyl chloride could be kept below 20°C. After stirring 1 hour longer, the reaction mixture was concentrated under reduced pressure to a thick syrup, dissolved in 3 L ethyl acetate and the resulting solution washed with 2 L of water. The upper organic layer was washed with 1 L of brine and stirred over 500 g anhydrous sodium sulfate for 1 hour. The dried solution was filtered and concentrated under reduced pressure giving intermediate 12 as a yellowish syrup, which was used without further treatment in the next step.

The crude syrup 12 was dissolved in 1.0 L of anhydrous dichloromethane, cooled to 10°C, and 693 g of 26% hydrogen bromide in acetic acid (wt/wt) solution was added, keeping the temperature below 10°C. After 3 hours TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 12 with the formation of a faster running pair of close running product spots 13 at 0.9 R_f. The reaction mixture was diluted with 1.0 L dichloromethane, and the resulting solution washed twice with 1.0 L portions of water followed by 3 washes with 500 mL portions of saturated sodium bicarbonate solution. The washed organic layer was concentrated under reduced pressure to a thick syrup, taken up in 1.0 L of a

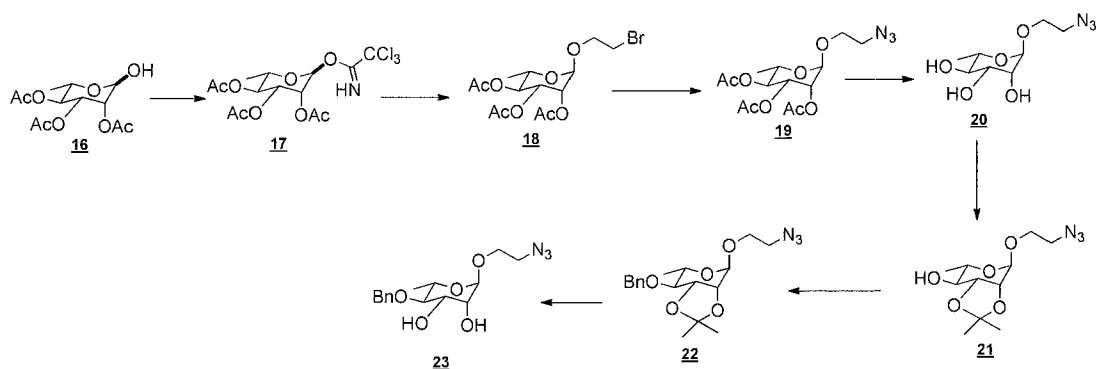
4:1 (v/v) solution of acetonitrile: water, and stirred at room temperature for 24 hours. TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 13 with the formation of a slower running pair of close running product spots 14, at 0.4 R_F. The reaction solution was concentrated under reduced pressure to a thick syrup and dissolved in 2.0 L ethyl acetate. The reaction solution was washed 3 times with 500 mL portions of saturated sodium bicarbonate solution, then twice with 500 mL portions of brine. The washed organic layer was concentrated under reduced pressure to a thick syrup, dissolved in 400 mL dichloromethane and loaded via suction onto a 16 cm (d) x 20 cm (h) pad of 2.0 kg 40-63 μm silica, which had been pre-eluted via suction with 2.0 L of 6:1 (v/v) heptane: ethyl acetate. The loaded pad was then eluted via suction by the application and collection of 1.0 L fractions with: 6:1 (v/v) (2.0 L total), 4:1 (v/v) (6.0 L total), 3:1 (v/v) (4.0 L total), 2:1 (v/v) (4.0 L total), and 1:1 (v/v) (6.0 L total). The product-containing fractions were concentrated under reduced pressure to give 224 g (0.34 mol) of desired product 14 as a solid in a 4 step overall yield of 24%.



Synthesis of compound 16: To 100 g (0.55 mol) L-rhamnose monohydrate was added 0.4 L acetic anhydride at 20°C, with mechanical stirring. To the resulting mixture was added 0.75 g iodine, which was then stirred at room temperature 12 hours longer. TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of the L-rhamnose monohydrate with the formation of a faster running product spot 15, at 0.7 R_F. The reaction mixture was concentrated under reduced pressure to a thick syrup, dissolved in 1.0 L ethyl acetate and the resulting solution washed with 1.0 L of saturated thiosulfate solution. The upper organic layer was washed with 1.0 L of brine and the upper organic layer stirred over 500 g anhydrous sodium sulfate for 1 hour. The solution was filtered

and concentrated under reduced pressure giving intermediate 15 as a yellowish syrup, which was used without further treatment in the next step.

Intermediate 15 was taken up in 350 mL tetrahydrofuran and cooled to 0°C. Ammonia gas was slowly bubbled through the cooled solution for 2 hours, the saturated solution was allowed to warm to room temperature, sealed, and allowed to stir for an additional 12 hours. TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 15 with the formation of a slower running pair of close running product spots 16 at 0.6 R_f. The reaction mixture was diluted with 500 mL tetrahydrofuran and washed with 500 mL brine. The upper organic layer was concentrated under reduced pressure to give crude solid 16. The crude solid was taken up in 500 mL ethyl acetate, washed with 250 mL portions of water 3 times, and concentrated under reduced pressure to a solid, which after drying gave 71.9 g (0.25 mol) of an off white solid 16 in 45% 2 step overall yield.



Synthesis of compound 23: A 283 g (0.98 mol) portion of 16 was taken up in 150 mL trichloroacetonitrile and 20 mL of anhydrous dichloromethane, at room temperature, with stirring. To the resulting mixture was added 300 g anhydrous potassium carbonate powder (-325 mesh). After 40 minutes, TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 16 with the formation of a faster running product spot 17, at 0.75 R_f. The reaction mixture was then filtered through a 50 g pad of Celite® 545, followed by a 50 mL dichloromethane wash of the pad. The dichloromethane filtrate and wash were then applied via

suction to a 5 cm (d) x 5 cm (h) pad of 100 g 40-63 μ m silica, which had been pre-eluted via suction with 200 mL of 1:1 (v/v) heptane: ethyl acetate. The loaded pad was then eluted via suction by the application and collection of in 1 L fractions with 1:1 (v/v) heptane : ethyl acetate (5 L total). The product-containing fractions were concentrated under reduced pressure to give 403 g (0.93 mol) of desired product 17 as a clear syrup a in yield of 95%, which was used without further treatment in the next step.

Intermediate 17 was dried by mixing with 200 mL portions of anhydrous toluene and evaporating to a syrup 3 times. The dried 17 was dissolved in 100 mL anhydrous dichloromethane under nitrogen and 230 g 2-bromoethanol was added. At room temperature 1.0 mL of trimethylsilyl trifluoromethanesulfonate was added dropwise over 10 minutes. After 30 minutes TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 17 with the formation of a faster running product spot 18 at 0.8 R_f . The reaction was quenched by the addition of 100 mL saturated sodium bicarbonate solution and extracted with 1.0 L ethyl acetate. The ethyl acetate extract was washed with 100 mL brine, and the organic layer dried over anhydrous magnesium sulfate with stirring for 10 minutes. The dried solution was filtered and concentrated under reduced pressure to give crude 18, which was used without further treatment in the next step.

Crude intermediate 18 was mixed with 300 mL dimethylformamide and 282 g sodium azide, heated to 65°C under nitrogen with stirring for 4 hours. TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 16 with the formation of a slower running product spot 19 at 0.7 R_f . The reaction mixture was cooled to room temperature, diluted with 1.0 L ethyl acetate, and the resulting solution washed with two 300 mL portions of brine. The upper organic layer was dried over 300 g anhydrous sodium sulfate with stirring for 30 minutes. Filtration and stripping of the dried solution gave a yellowish syrup of crude 19. Intermediate 19 was further dried by addition of 300 mL portions of toluene and stripping to a thick syrup 3 times. The dried 19 was used without further treatment in the next step.

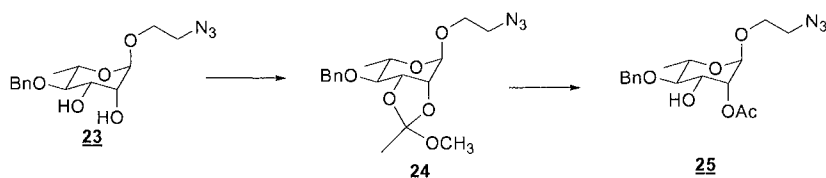
The dried intermediate 19 was then dissolved with stirring in 200 mL anhydrous methyl alcohol and cooled to 0°C. A solution made by the dropwise addition of 20 mL acetyl chloride to 200 mL of anhydrous methyl alcohol, which had been pre-cooled to 0°C, was then added to the stirred cold solution of intermediate 19. The resulting solution was allowed to warm to room temperature, capped, and stirred for 12 hours longer. TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 19 with the formation of a slower running product spot 20 at 0.1 R_f. The reaction mixture was then concentrated under reduced pressure to a thick syrup and used without further treatment in the next step.

Crude intermediate 20 was dissolved in 400 mL anhydrous acetone at room temperature with stirring. To the resulting solution was added 100 mL 2,2-dimethoxypropane and 1.0 g (±)-camphor-10-sulfonic acid. The resulting solution was allowed to stir 12 hours longer. TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 19 with the formation of a faster running product spot 21 at 0.9 R_f. The reaction mixture was slowly poured into a stirred biphasic mixture of 1.0 L saturated sodium bicarbonate solution and 2.0 L of ethyl acetate. The upper organic layer was washed twice with 200 mL portions of brine and dried by stirring over 300 g of anhydrous magnesium sulfate for 15 minutes. The mixture was filtered and concentrated under reduced pressure to give crude intermediate 21 as a yellowish syrup. Crude 21 was further dried by the addition of 300 mL anhydrous toluene and stripping to a thick syrup three times. The dried 21 was used without further treatment in the next step.

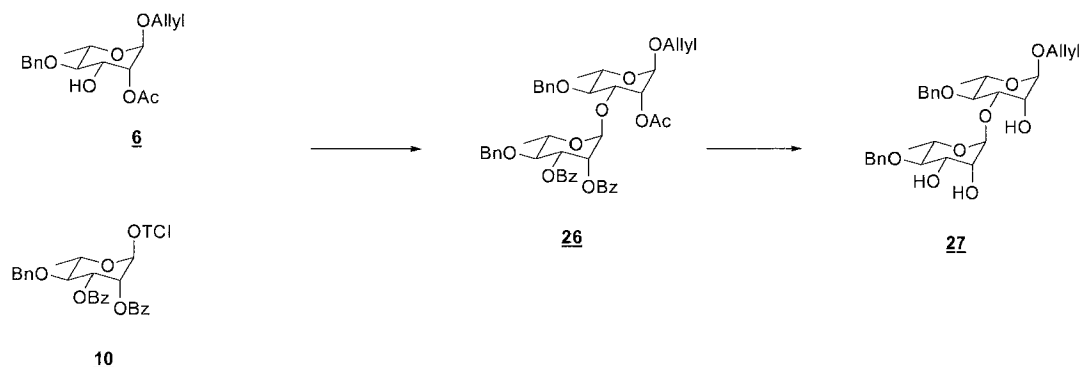
Crude acetonide 21 was dissolved in 100 mL of anhydrous tetrahydrofuran and added via Teflon[®] cannula over 15 minutes using nitrogen pressure, to a stirred suspension of 40 g of 60% sodium hydride (oil dispersion) and 300 mL of a 4:1 (v/v) mixture of anhydrous tetrahydrofuran : anhydrous 1-methyl-2-pyrrolidinone, which had been pre-cooled 0°C, under a slow nitrogen stream. The resulting mixture was stirred for 5 minutes, then 64 g of benzyl bromide was added dropwise via dropping funnel over 30 minutes. The resulting reaction mixture was warmed to room temperature and stirred 12 hours longer. TLC [heptane :

ethyl acetate, 3:1 (v/v)] indicated the consumption of starting material with the formation of a faster running product 22 spot at 0.5 R_f . The reaction mixture was cooled to 0°C and excess sodium hydride quenched by the dropwise addition of 100 mL of methyl alcohol over 15 minutes. The resulting mixture was warmed to room temperature and stirred 1 hour longer. The quenched reaction mixture was then diluted by the addition of 500 mL tetrahydrofuran and 200 mL brine. The upper product- containing organic layer was again washed with 200 mL brine and dried over anhydrous magnesium sulfate with stirring for 5 minutes. The dried organic layer was filtered and concentrated under reduced pressure to give crude product 22 as a syrup, which was used directly in the next step.

Crude product 22 was dissolved at room temperature with stirring in 500 mL of a 4:1:1 (v/v/v) solution of tetrahydrofuran :water : trifluoroacetic acid. After 12 hours TLC [heptane : ethyl acetate, 2:1 (v/v)] indicated the consumption of starting material 22 with the formation of a slower running product 23 spot at 0.2 R_f . The reaction mixture was diluted with 200 mL brine the upper organic layer was washed again with 200 mL brine. The organic layer was dried over anhydrous sodium sulfate with stirring for 5 minutes, filtered, and concentrated under reduced pressure to a syrup. The crude product was dissolved in 200 mL dichloromethane and applied via suction to a 16 cm (d) x 10 cm (h) pad of 800 g 40-63 μ m silica, which had been pre-eluted via suction with 2.0 L of 6:1 (v/v) heptane: ethyl acetate. The loaded pad was then eluted via suction by the application and collection of 1 L fractions with: 8:1 (v/v) heptane : ethyl acetate (4 L total), followed by 4:1 (v/v) heptane : ethyl acetate (4 L total). The product-containing fractions were concentrated under reduced pressure to give 100 g (0.31 mol) of desired product 23 as a crystalline solid in a 7 step overall yield of 32%.

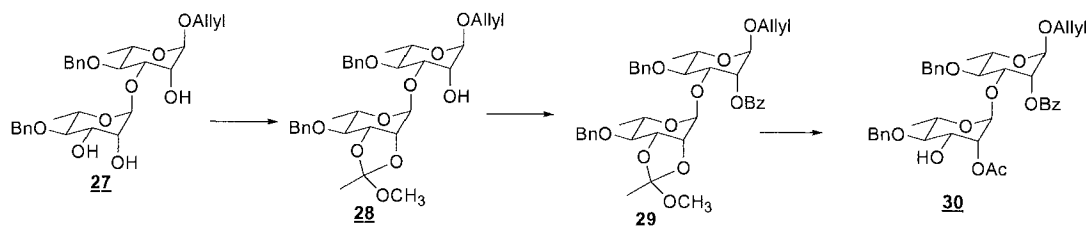


Synthesis of compound 25: A mixture of 100 g (0.31 mol) compound 23, 200 mL anhydrous acetonitrile, 78 mL trimethylorthoacetate, and 14 g ($\pm$)- camphor-10-sulfonic acid was stirred at room temperature for 20 minutes. TLC [heptane : ethyl acetate, 2:1 (v/v)] indicated the consumption of starting material 23 with the formation of a faster running product 24 spot at 0.7 R_f and a second spot corresponding to 25 at 0.5 R_f . To the reaction mixture was added 5.0 mL water and stirring was continued for 20 minutes longer. TLC analysis [heptane : ethyl acetate, 2:1 (v/v)] indicated the consumption of 24 with the increase in intensity of the slower running product spot 25 , at 0.5 R_f . The reaction mixture was concentrated under reduced pressure to dryness giving crude 25 as a yellow syrup. The crude 25 was dissolved in 200 mL dichloromethane and applied via suction to a 16 cm (d) x 10 cm (h) pad of 800 g 40-63 μ m silica, which had been pre-eluted via suction with 2.0 L of 6:1 (v/v) heptane: ethyl acetate. The loaded pad was then eluted via suction by the application and collection of 1.0 L fractions of: 8:1 (v/v) heptane : ethyl acetate (4 L total), followed by 4:1 (v/v) heptane : ethyl acetate (4 L total). The fractions containing 25 were concentrated under reduced pressure to give 95 g (0.26 mol) desired product as a yellowish syrup in a 2 step overall yield of 84%.



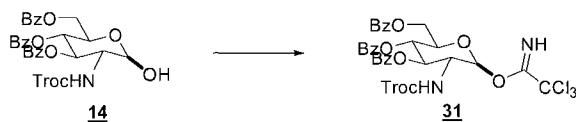
Synthesis of compound 27: A mixture of 115g (0.19 mol) intermediate 10 and 57.5g (0.17 mol) intermediate 6 were dried by mixing with 200 mL portions of anhydrous toluene and evaporating to a syrup 3 times. The dried mixture was dissolved in 300 mL anhydrous dichloromethane under nitrogen with stirring at room temperature. To the solution 0.4 mL of trimethylsilyl trifluoromethanesulfonate was added dropwise over 10 minutes. After 60 minutes TLC analysis [heptane : ethyl acetate, 3:1 (v/v)] indicated the consumption of 10 and 6 with the formation of product spot 26 at 0.5 R_f . The reaction was quenched by the addition of 100 mL saturated sodium bicarbonate solution and extracted with 1.0 L ethyl acetate. The ethyl acetate extract was washed with 100 mL brine and dried over anhydrous magnesium sulfate with stirring for 10 minutes. The dried solution was filtered and concentrated under reduced pressure to give crude 26, to which 200 mL heptane was added, followed by a minimum amount of dichloromethane to make the resulting solution homogeneous. The solution was cooled to 5°C and stirred for 1 hour. The crystallized trichloroacetamide by-product was filtered and the filtrate concentrated under reduced pressure to give crude 26 coupling product.

Crude intermediate 26 was dissolved in 300 mL anhydrous tetrahydrofuran. To the solution of 26 was added 300 mL anhydrous methyl alcohol and 20 mL of a 25% (wt/wt) sodium methoxide in methyl alcohol solution. The resulting solution was heated to 60°C with stirring for 30 minutes. TLC analysis [heptane : ethyl acetate, 2:1 (v/v)] indicated the consumption of 26 with the formation of product spot 27 at 0.2 R_f . The reaction mixture was cooled to room temperature, diluted with 300 mL tetrahydrofuran, and washed twice with 100 mL brine. The upper organic layer was concentrated under reduced pressure to a crude solid which was recrystallized from heptane/ethyl acetate giving 68 g (0.13 mol) intermediate 27 in 76% 2 step overall yield.

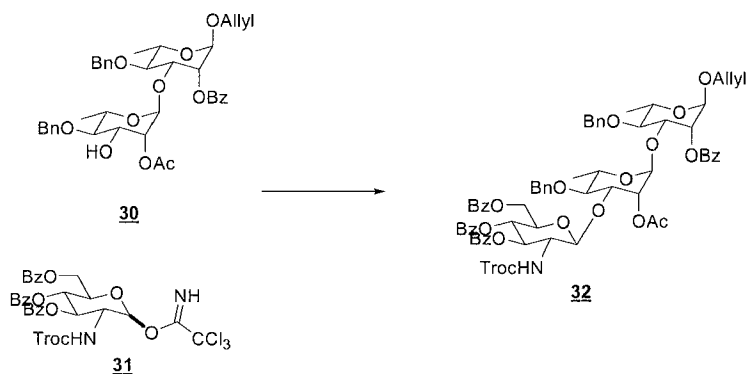


Synthesis of compound 30: A mixture of 68 g (0.13 mol) compound 27, 200 mL anhydrous acetonitrile, 33 mL trimethylorthoacetate, and 3.0 g ($\pm$)-camphor-10-sulfonic acid was stirred at room temperature for 60 minutes. TLC [heptane : ethyl acetate, 2:1 (v/v)] indicated the consumption of starting material 27 with the formation of a faster running product 28 spot at 0.5 R_f . To the reaction mixture was added 20 mL triethylamine and mixture was concentrated under reduced pressure to a syrup. To the crude 24 syrup was added 200 mL anhydrous pyridine followed by the dropwise addition of 17 mL benzoyl chloride over 15 minutes. After stirring for 30 minutes at room temperature, TLC analysis [heptane : ethyl acetate, 2:1 (v/v)] indicated the consumption of 28 with the formation of a faster running product spot 29 at 0.8 R_f . The reaction mixture was concentrated under reduced pressure to dryness and dissolved in 200 mL ethyl acetate. The ethyl acetate solution was washed with 100 mL water, 100 mL brine, and concentrated under reduced pressure to a crude syrup. The crude 29 was taken up in 200 mL of a mixture of 8:1 (v/v) glacial acetic acid: water and stirred at room temperature for 30 minutes longer. TLC analysis [heptane : ethyl acetate, 2:1 (v/v)] indicated the consumption of 29 with the formation of a slower running product spot 30 at 0.7 R_f . The reaction mixture was concentrated under reduced pressure to a crude syrup, dissolved in 100 mL dichloromethane and applied via suction to a 16 cm (d) x 10 cm (h) pad of 800 g 40-63 μ m silica, which had been pre-eluted via suction with 2.0 L of 8:1 (v/v) heptane: ethyl acetate. The loaded pad was then eluted via suction by the application and collection of 1.0 L fractions of: 8:1 (v/v) heptane : ethyl acetate (4 L total), followed by 4:1 (v/v) heptane : ethyl acetate (4 L total), and 2:1 (v/v) heptane : ethyl acetate (4 L total). The product-containing fractions were

concentrated under reduced pressure to give 28 g (0.04 mol) desired product as a yellowish syrup in a 3 step overall yield of 32%.

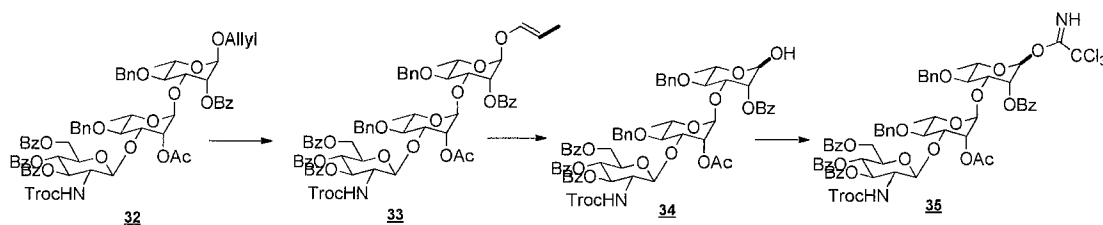


Synthesis of compound 31: A 20g (0.03 mol) portion of 14 was taken up with stirring in 40 mL trichloroacetonitrile and 30 mL of anhydrous dichloromethane at room temperature. To the resulting mixture was added 40g anhydrous potassium carbonate powder (-325 mesh). After 10 minutes, TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 14 with the formation of a faster running product spot 31 at 0.8 R_f. The reaction mixture was filtered through a pad of 50 g Celite[®] 545 followed by a 20 mL dichloromethane wash of the pad. The dichloromethane filtrate and wash were applied via suction to a 5 cm (d) x 5 cm (h) pad of 20 g 40-63 μ m silica, which had been pre-eluted via suction with 200 mL of 1:1 (v/v) heptane: ethyl acetate. The loaded pad was eluted via suction by application and collection of 100 mL fractions of 1:1 (v/v) heptane : ethyl acetate (500 mL total). The product- containing portions were concentrated under reduced pressure to give 23 g (0.028 mol) of desired product 31 as a yellowish solid in yield of 95%.



Synthesis of compound 32: A mixture of 23g (28 mmol) intermediate 31 and 14.5g (21.5 mmol) intermediate 30 were dried by mixing with 200 mL portions of anhydrous toluene and evaporating to a syrup 3 times. The dried mixture was dissolved in 10 mL anhydrous dichloromethane under nitrogen with stirring at room temperature. To the

solution was then added dropwise 30 μ L of trimethylsilyl trifluoromethanesulfonate over 10 minutes. After 15 minutes TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 31 and 30 with the formation of product spot 32 at 0.7 R_f . The reaction was quenched by the addition of 100 mL saturated sodium bicarbonate solution and extracted with 500 mL ethyl acetate. The ethyl acetate extract was washed with 100 mL brine and dried over anhydrous magnesium sulfate with stirring for 10 minutes. The dried solution was filtered and concentrated under reduced pressure to give crude 32, to which 200 mL heptane was added, followed by a minimum amount of dichloromethane to make the resulting solution homogeneous. The solution was cooled to 5°C and stirred for 1 hour. The crystallized trichloroacetamide by-product was filtered and the filtrate concentrated under reduced pressure to give crude coupling product 32. The crude syrup was dissolved in 10 mL dichloromethane and applied via suction to a 7 cm (d) x 20 cm (h) pad of 400 g 40-63 μ m silica, which was pre-eluted via suction with 1 L of 9:1 (v/v) heptane: ethyl acetate. The loaded pad was then eluted via suction by the application and collection of 125 mL fractions of: 9:1 (v/v) heptane : ethyl acetate (1.0 L total), followed by 6:1 (v/v) heptane : ethyl acetate (1.0 L total), and 4:1 (v/v) heptane : ethyl acetate (2.0 L total). The product 32 containing fractions were concentrated under reduced pressure to give 23 g (17.2 mmol) of desired product as a syrup in a yield of 80%.

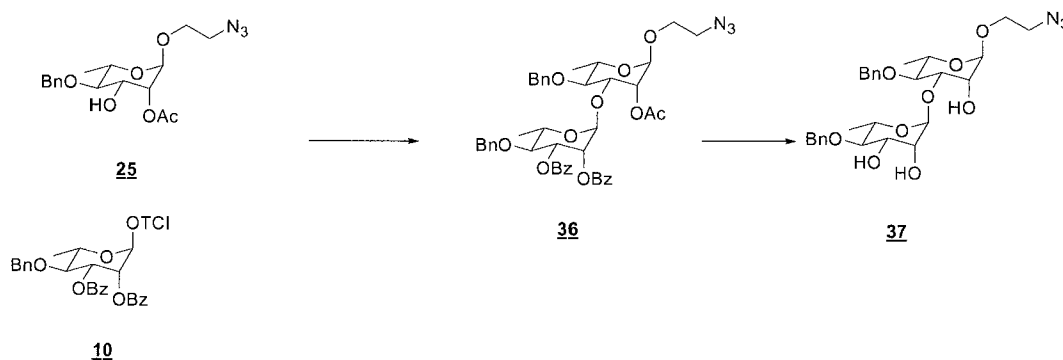


Synthesis of compound 35: In a glove box under anhydrous oxygen- free conditions 1.0 g of (1,5-cyclooctadiene)bis(methyldiphenylphosphine)iridium (I) hexafluorophosphate (Felkin's catalyst) and 100 mL anhydrous tetrahydrofuran were combined. The septum- sealed flask was removed from the glove box and hydrogen gas was bubbled through until all the

suspended solids had dissolved and the color of the solution became a pale yellow. The resulting solution was purged of hydrogen by bubbling oxygen- free argon through the solution for 20 minutes. The activated catalyst solution was transferred to a flask containing 60 g of (45 mmol) intermediate 33 via a Teflon[®] cannula using oxygen- free argon gas pressure. The resulting solution was stirred under oxygen- free argon, for 30 minutes. TLC analysis [heptane : ethyl acetate, 1:1(v/v)] indicated the consumption of 32 with the formation of a faster running product spot 33 at 0.8 R_f. To the isomerized product 33 solution were added 100 mL of a 50 wt % in water solution of 4-methylmorpholine N-oxide and 100 mg of osmium tetroxide. The resulting solution was shielded from light and allowed to stir open to the atmosphere at room temperature for 12 hours. TLC analysis [heptane : ethyl acetate, 1:1(v/v)] indicated the consumption of 33 with the formation of a lower running product spot 34 , at 0.3 R_f. Saturated thiosulfate solution (25 mL) was added and the resulting mixture stirred for 1 hour longer. The dark reaction mixture was diluted with 200 mL tetrahydrofuran and the upper organic layer was washed with 100 mL brine and dried by stirring with 20 g anhydrous magnesium sulfate for 10 minutes. Filtration and stripping of the dried solution gave a dark crude syrup. The crude syrup was dissolved in 10 mL dichloromethane and applied via suction to a 7 cm (d) x 20 cm (h) pad of 600 g 40-63 μ m silica, which was pre-eluted via suction with 1 L of 9:1 (v/v) heptane: ethyl acetate . The loaded pad was eluted via suction by application and collection of 125 mL fractions of: 9:1 (v/v) heptane : ethyl acetate (1.0 L total), followed by 4:1 (v/v) heptane : ethyl acetate (1.0 L total), and 2:1 (v/v) heptane : ethyl acetate (2.0 L total). The product 34 containing fractions were concentrated under reduced pressure to give 55 g (42 mmol) desired product as a syrup in a yield of 95%.

A 55 g (42 mmol) portion of 34 was taken up in 100 mL trichloroacetonitrile and 30 mL of anhydrous dichloromethane with stirring at room temperature. To the resulting mixture was added 100g anhydrous potassium carbonate powder (-325 mesh). After 1 hour TLC analysis [heptane : ethyl acetate, 2:1 (v/v)] indicated the consumption of 34 with the

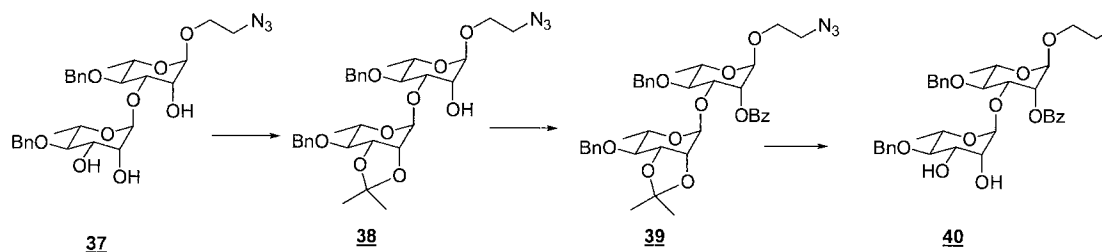
formation of a faster running product spot 35 at 0.3 R_f. The reaction mixture was filtered through a pad of 50 g Celite[®] 545, followed by a 100 mL dichloromethane wash of the pad. The dichloromethane filtrate and wash were applied via suction to a 5 cm (d) x 5 cm (h) pad of 20 g 40-63 μ m silica, which had been pre-eluted via suction with 200 mL of 2:1 (v/v) heptane: ethyl acetate. The loaded pad was then eluted via suction by the application and collection of 100 mL fractions with 2:1 (v/v) heptane : ethyl acetate (500 mL total). The product containing fractions were concentrated under reduced pressure to give 60 g (41 mmol) of desired product 35 as a syrup in yield of 98%.



Synthesis of compound 37: A mixture of 17g (28 mmol) intermediate 10 and 7.8g (21 mmol) intermediate 25 were dried by mixing with 200 mL portions of anhydrous toluene and evaporating to a syrup 3 times. The dried mixture was dissolved under nitrogen in 50 mL anhydrous dichloromethane with stirring at room temperature. To the solution was added dropwise 0.1 mL of trimethylsilyl trifluoromethanesulfonate over 10 minutes. After 60 minutes, TLC analysis [heptane : ethyl acetate, 3:1 (v/v)] indicated the consumption of 10 and 25 with the formation of product spot 36 at 0.5 R_f. The reaction was quenched by the addition of 100 mL saturated sodium bicarbonate solution and extracted with 500 mL ethyl acetate. The ethyl acetate extract was washed with 100 mL brine and dried over anhydrous magnesium sulfate with stirring for 10 minutes. The dried solution was filtered and concentrated under reduced pressure to give crude 36, to which 200 mL heptane was added, followed by a minimum amount of dichloromethane to make the

resulting solution homogeneous. The solution was cooled to 5°C and stirred for 1 hour. The crystallized trichloroacetamide by-product was filtered and the filtrate concentrated under reduced pressure to give crude coupling product 36.

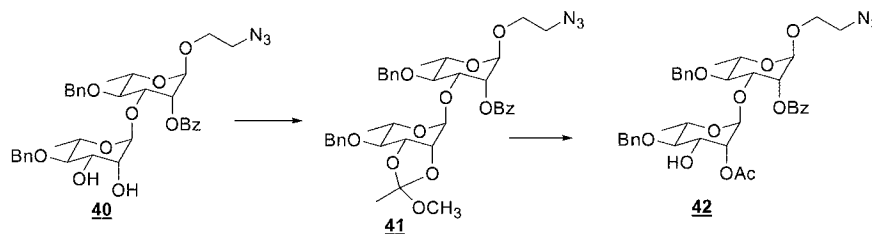
Crude intermediate 36 was dissolved in 40 mL anhydrous tetrahydrofuran, followed by the addition of 40 mL anhydrous methyl alcohol and 2.0 mL of a 25% (wt/wt) sodium methoxide in methyl alcohol solution. The resulting solution was heated to 40°C with stirring for 2 hours. TLC analysis [heptane : ethyl acetate, 1:2 (v/v)] indicated the consumption of 36 with the formation of product spot 37 at 0.5 R_f. The reaction mixture was then cooled to room temperature, diluted with 200 mL tetrahydrofuran, and washed twice with 100 mL brine. The upper organic layer was concentrated under reduced pressure to a crude crystalline mass which was used without further purification in the next step.



Synthesis of compound 40: Crude intermediate 37 was dissolved in 40 mL anhydrous acetone and 30 mL 2,2-dimethoxypropane. To the resulting mixture was added 14 g Amberlyst® 15 hydrogen form resin and the resulting mixture stirred at room temperature for 30 minutes. TLC [heptane : ethyl acetate, 1:2 (v/v)] indicated the consumption of starting material 37 with the formation of a faster running product 38 spot at 0.8 R_f. The reaction mixture was filtered and the solvents concentrated under reduced pressure to a syrup. To the crude 38 syrup was added 50 mL anhydrous pyridine followed by the dropwise addition of 2.7 mL benzoyl chloride over 10 minutes. After stirring for 1 hour at room temperature TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 38 with the formation of a faster running product spot 39 at 0.8 R_f. The reaction mixture was concentrated under reduced pressure to dryness and

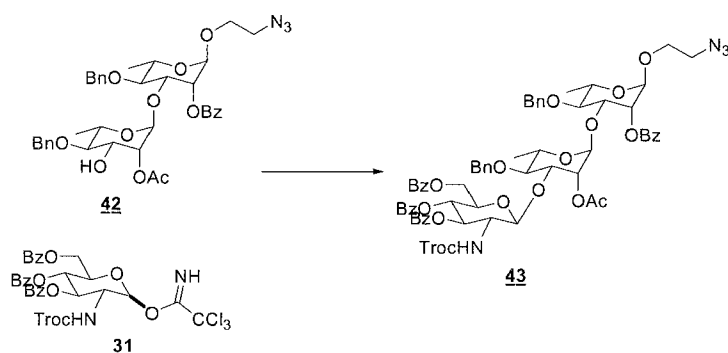
dissolved in 200 mL ethyl acetate. The ethyl acetate solution was washed with 100 mL water, 100 mL brine, and concentrated under reduced pressure to a crude syrup. Crude 39 was then taken up in a mixture of 8:1 (v/v) glacial acetic acid: water and stirred at room temperature for 12 hours.

5 TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 39 with the formation of a slower running product spot 40 at 0.4 R_f . The reaction mixture was concentrated under reduced pressure to a crude syrup, dissolved in 10 mL dichloromethane and applied via suction to a 7 cm (d) x 20 cm (h) pad of 300 g 40-63 μ m silica, which was pre-eluted via suction with 1.0 L of 9:1 (v/v) heptane: ethyl acetate. The loaded pad was then
10 eluted via suction by the application and collection of 125 mL fractions of: 9:1 (v/v) heptane : ethyl acetate (1.0 L total), followed by 4:1 (v/v) heptane : ethyl acetate (1.0 L total), and 2:1 (v/v) heptane : ethyl acetate (2.0 L total). The product 40 containing fractions were concentrated under
15 reduced pressure to give 9.5 g (14.3 mmol) desired product as a syrup in a 4 step overall yield of 51%.



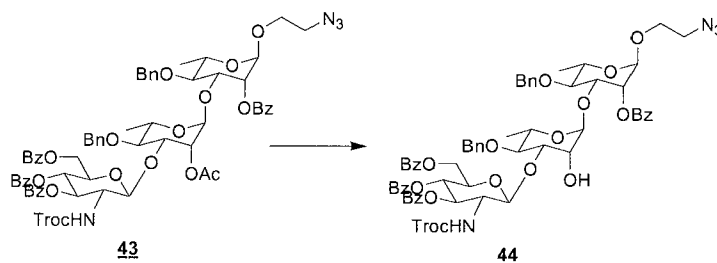
Synthesis of compound 42: A mixture of 9.5 g (14.3 mmol)
20 compound 40, 50 mL anhydrous acetonitrile, 3.8 mL trimethylorthoacetate, and 0.5 g ($\pm$)-camphor-10-sulfonic acid was stirred at room temperature for 20 minutes. TLC [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of starting material 27 with the formation of a faster running product 41 spot at 0.8 R_f . To the reaction mixture were added 1.0 mL
25 water and 20 mL dichloromethane. The resulting mixture was stirred at room temperature for 10 minutes longer. TLC analysis [heptane : ethyl acetate, 1:1 (v/v)] indicated the consumption of 41 with the formation of a slower running product spot 42 at 0.6 R_f . The reaction mixture was

concentrated under reduced pressure to a crude syrup, dissolved in 10 mL dichloromethane and applied via suction to a 7 cm (d) x 20 cm (h) pad of 300 g 40-63 μ m silica, which was pre-eluted via suction with 1 L of 9:1 (v/v) heptane: ethyl acetate. The loaded pad was then eluted via suction by the application and collection of 125 mL fractions of: 9:1 (v/v) heptane : ethyl acetate (1.0 L total), followed by 4:1 (v/v) heptane : ethyl acetate (1.0 L total), and 2:1 (v/v) heptane : ethyl acetate (2.0 L total). The product 42 containing fractions were concentrated under reduced pressure to give 8.0 g (11.3 mmol) of a yellowish syrup in a 2 step overall yield of 79%.



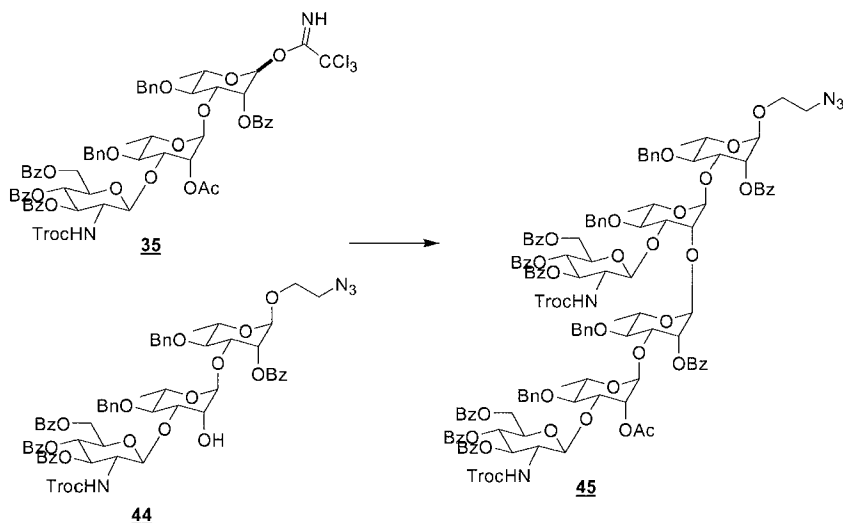
Synthesis of compound 43: A mixture of 12.0 g (15 mmol) intermediate 31 and 8.0 g (11 mmol) intermediate 42 were dried by mixing with 200 mL portions of anhydrous toluene and evaporating to a syrup 3 times. The dried mixture was dissolved in 10 mL anhydrous dichloromethane under nitrogen with stirring at room temperature. To the solution was added dropwise 30 μ L of trimethylsilyl trifluoromethanesulfonate over 10 minutes. After 15 minutes, TLC analysis [heptane : ethyl acetate, 2:1 (v/v)] indicated the consumption of 31 and 42 with the formation of product spot 43 at 0.6 R_f . The reaction was quenched by the addition of 100 mL saturated sodium bicarbonate solution and extracted with 500 mL ethyl acetate. The ethyl acetate extract was washed with 100 mL brine and dried over anhydrous magnesium sulfate with stirring for 10 minutes. The dried solution was filtered and concentrated under reduced pressure to give crude 43, to which 200 mL heptane was added, followed by a minimum amount of dichloromethane to make the resulting solution homogeneous. The solution was cooled to 5°C and

stirred for 1 hour. The crystallized trichloroacetamide by-product was filtered and the filtrate concentrated under reduced pressure to give crude coupling product 43. The crude syrup was dissolved in 10 mL dichloromethane and applied via suction to a 7 cm (d) x 20 cm (h) pad of 400 g 40-63 μ m silica, which was pre-eluted via suction with 1.0 L of 9:1 (v/v) heptane: ethyl acetate. The loaded pad was then eluted via suction by application and collection of 125 mL fractions of: 9:1 (v/v) heptane : ethyl acetate (1.0 L total), followed by 6:1 (v/v) heptane : ethyl acetate (1.0 L total), and 4:1 (v/v) heptane : ethyl acetate (2.0 L total). The product 43 containing fractions were concentrated under reduced pressure to give 13.5 g (9.9 mmol) desired product as a syrup in a yield of 90%.



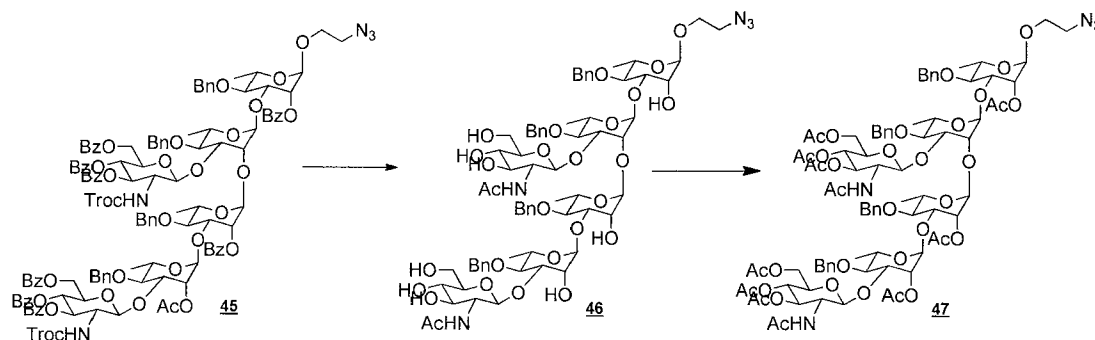
The intermediate 43 [13.5 g (9.9 mmol)] was dissolved with stirring in 20 mL anhydrous methyl alcohol and 5.0 mL anhydrous dichloromethane, then cooled to 0°C. A solution made by the dropwise addition of 5.0 mL acetyl chloride to 20 mL of anhydrous methyl alcohol at 0°C, was then added to the stirred cold solution of intermediate 43. The resulting solution was allowed to warm to room temperature, capped, and stirred for 12 hours longer. TLC analysis [heptane : ethyl acetate, 2:1 (v/v)] indicated the consumption of 43 with the formation of a slower running product spot 44, at 0.5 R_f . The reaction mixture was poured into a saturated sodium bicarbonate solution and extracted with 200 mL ethyl acetate. The ethyl acetate extract was washed with 50 mL brine and dried over 20 g anhydrous magnesium sulfate with stirring for 10 minutes. The dried extract was filtered and concentrated under reduced pressure to a thick syrup. The crude 44 syrup was dissolved in 10 mL dichloromethane. The resulting solution was applied via suction to a 7 cm (d) x 20 cm (h) pad of 400 g 40-63 μ m silica, which was pre-eluted via suction with 1.0 L of 9:1

(v/v) heptane: ethyl acetate . The loaded pad was then eluted via suction by application and collection of 125 mL fractions of: 9:1 (v/v) heptane : ethyl acetate (1.0 L total), followed by 6:1 (v/v) heptane : ethyl acetate (1.0 L total), and 4:1 (v/v) heptane : ethyl acetate (2.0 L total). The product 44 containing fractions were concentrated under reduced pressure to give 10.0 g (7.6 mmol) desired product as a syrup in a yield of 77%.



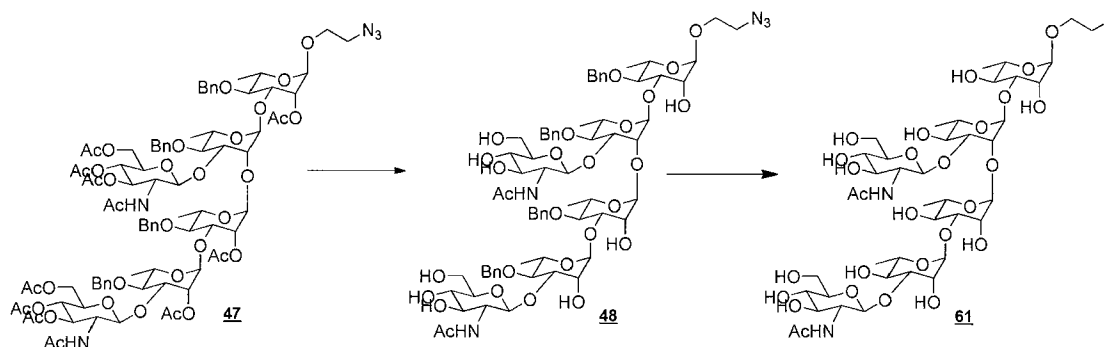
Synthesis of compound 45: A mixture of 10.0 g (7.6 mmol) intermediate 44 and 14.0 g (9.9 mmol) intermediate 35 were dried by mixing with 200 mL portions of anhydrous toluene and evaporating to a syrup 3 times. The dried mixture was dissolved in 10 mL anhydrous dichloromethane under nitrogen with stirring at room temperature. To the solution was added dropwise 30 μ L of trimethylsilyl trifluoromethanesulfonate over 10 minutes. After 15 minutes TLC analysis [heptane : ethyl acetate 1:1 (v/v)] indicated the consumption of 44 and 35 with the formation of product spot 45, at 0.4 R_f. The reaction was quenched by the addition of 100 mL saturated sodium bicarbonate solution and extracted with 500 mL ethyl acetate. The ethyl acetate extract was washed with 100 mL brine and dried over anhydrous magnesium sulfate with stirring for 10 minutes. The dried solution was filtered and concentrated under reduced pressure to give crude 45, to which 200 mL heptane was added, followed by a minimum amount of dichloromethane to

make the resulting solution homogeneous. The solution was cooled to 5°C and stirred for 1 hour. The crystallized trichloroacetamide by-product was filtered and the filtrate concentrated under reduced pressure to give crude 45 coupling product. The crude syrup was dissolved in 10 mL dichloromethane and applied via suction to a 7 cm (d) x 20 cm (h) pad of 400 g 40-63 μ m silica, which was pre-eluted via suction with 1.0 L of 9:1 (v/v) heptane: ethyl acetate. The loaded pad was eluted via suction by application and collection of 125 mL fractions of: 9:1 (v/v) heptane : ethyl acetate (1.0 L total), followed by 6:1 (v/v) heptane : ethyl acetate (1.0 L total), and 4:1 (v/v) heptane : ethyl acetate (2.0 L total). The product 45 containing fractions were concentrated under reduced pressure to give 12.0 g (4.6 mmol) desired product as a syrup in a yield of 61%.



Synthesis of compound 47: A 6.0g (2.3 mmol) portion of intermediate 45 was dissolved in 120 mL of tetrahydrofuran. To this solution was added 40 mL water, followed by 6.8 g sodium hydroxide pellets. The resulting mixture was heated to 60°C under nitrogen and stirred for 48 hours. TLC analysis [dichloromethane : methyl alcohol, 5:1 (v/v)] indicated the consumption of 45 with the formation of a single product spot 46 at 0.4 R_f . The reaction mixture was cooled to room temperature and the upper organic layer separated. To the organic layer was added 50 mL of acetic anhydride and 50 mL of anhydrous pyridine. The resulting solution was concentrated under reduced pressure to a white solid. This procedure was repeated twice. Finally, 50 mL of acetic anhydride and 50 mL of anhydrous pyridine were added to the solid and the resulting mixture was stirred at 60°C under nitrogen 12 hours longer. TLC analysis

[dichloromethane : methyl alcohol, 5:1 (v/v)] indicated the consumption of 46 with the formation of a faster moving product spot 47 at 0.9 R_f . The cooled solution was concentrated under reduced pressure to a white solid, dissolved in 200 mL dichloromethane and washed with 100 mL water. The lower organic layer was separated and dried over 20 g anhydrous sodium sulfate with stirring for 30 minutes. The solution was filtered and applied via suction to a 4 cm (d) x 20 cm (h) pad of 200 g 40-63 μ m silica, which was pre-eluted via suction with 500 mL of 1:1 (v/v) heptane: ethyl acetate. The loaded pad was eluted via suction by application and collection of 100 mL fractions of: 1:1 (v/v) heptane : ethyl (500 mL total), followed by 1:2 (v/v) heptane : ethyl acetate (500 mL total), and ethyl acetate (500 mL total). The product 47 containing fractions were concentrated under reduced pressure to give 3.56 g (2.0 mmol) desired product as a white solid in a 2 step overall yield of 84%.



Synthesis of compound 61: A 1.0g (0.55 mmol) portion of intermediate 47 was dissolved in 20 mL of anhydrous tetrahydrofuran. To this solution was added 20 mL anhydrous methyl alcohol followed by 100 μ L of a 25% (wt/wt) sodium methoxide in methyl alcohol solution. The resulting solution was heated to 60°C with stirring for 2 hours. TLC analysis [dichloromethane : methyl alcohol, 5:1 (v/v)] indicated the consumption of 47 with the formation of a slower moving product spot 48 at 0.6 R_f . The reaction mixture was then cooled to room temperature and 100 μ L concentrated hydrochloric acid was added. The reaction mixture was diluted with 100 mL tetrahydrofuran, washed with 30 mL brine and the

upper organic layer separated. The organic layer was concentrated under reduced pressure to a white solid giving 1.23 g of crude 48 which was used without further processing in the next step.

5 Crude intermediate 48 (1.23 g) was dissolved in 50 mL of tetrahydrofuran and transferred to a 500 mL glass pressure bottle. Water was added to the solution dropwise until the resulting solution became slightly cloudy. To this solution was then added 100 μ L of concentrated hydrochloric acid. The pressure bottle was purged with nitrogen and 1.0 g 10% palladium on carbon was added. The pressure bottle containing the
10 reaction mixture was then evacuated until the reaction mixture began to bubble, then backfilled with nitrogen to 30 psi. The evacuation/backfilling procedure was repeated 3 times. The evacuation/backfilling procedure was then repeated 3 more times using hydrogen instead of nitrogen as the backfilling gas. The purged reaction solution was then stirred, at room
15 temperature, under a 30 psi hydrogen atmosphere for 72 hours. TLC analysis [n-butyl alcohol: ethyl alcohol : water : ammonium hydroxide, 2:2:2:1(v/v/v/v)] indicated the consumption of 48 with the clean formation of a slower moving product spot 61 , at 0.6 R_f. The reaction mixture was purged of hydrogen via a evacuation/backfilling operation previously
20 described using nitrogen for backfilling. The pressure was then brought to ambient and the catalyst was filtered through a plug of 5 g Celite[®] 545. The catalyst/Celite[®] 545 plug was then washed with 50 mL water. The filtrates were combined, and concentrated under reduced pressure to a crude white solid 61. The crude 61 was dissolved in 3 mL water and loaded onto a 26
25 mm x 900 mm column fine P-2 Bio-Gel[®] that had been pre-equilibrated with water (pH 3.8-4) . The column was then eluted with water at a rate of 1 mL/minute, collecting 5.0 mL fractions. The product containing fractions, as determined by HPLC method X, were combined and filtered through a 0.45 μ m Teflon[®] filter. The filtrate was then lyophilized to give
30 304 mg (0.29 mmol) 61 as a hydrochloride salt in 52% 2 step overall yield.

Synthesis of other building blocks: The above building blocks can be combined in various ways to synthesize an assortment of other bi-, tri- and oligo-saccharides, as shown in Figures 10 and 11.

Synthesis of oligosaccharide conjugates

Synthetic GAS oligosaccharides 61 – 64, equipped with an amino-terminated two-carbon linker, were synthesized from mono-, di- and tri-saccharide building blocks as exemplified above. Final compounds were purified by size exclusion chromatography (P-4 Bio-Gel, Pharmacia XK26 x 100 cm bed, pH 3.9 H₂O). Fractions analyzing for greater than 90% purity by HPLC were combined and lyophilized to give the final material as the hydrochloride salt. Each oligosaccharide was characterized by ¹H NMR, MALDI-MS, elemental analysis, HPLC-ELSD and the free amine content was determined by the Habeeb assay (REF). CRM₁₉₇ was produced internally in Novartis V&D, Siena, Italy.

Streptococcal strain GAS SF370 M1 was obtained from the University of Siena, Italy. GAS M1 3348 was obtained from Istituto Superiore di Sanità, Rome, Italy. GAS M23 DSM2071 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures). Bacterial strains were stored at –80°C and routinely grown at 37°C in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) (Difco).

General analytical methods

Total saccharide concentration was determined by HPAEC-PAD analyses (ICS-3000 Dionex system). Briefly, the GAS-PS preparation was hydrolyzed in 4 M trifluoroacetic acid for 2 h at 100°C, dried and dissolved in water. Samples of 20 µL were injected into CarboPac PA1 analytical column (250 mm x 4 mm i.d., Dionex) with CarboPac PA1 guard column (50 mm x 4 mm i.d., Dionex). Isocratic separations were performed using a 15-min 50 mM NaOH followed by a 5-min 500 mM NaOH regeneration step and 10-min re-equilibration, set to a flow rate of 1.0 mL min⁻¹. Monosaccharide peaks were detected directly by using quadruple-potential waveform pulsed amperometry on a gold electrode and an Ag/AgCl reference electrode. Raw data were elaborated on a Chromeleon 6.8 chromatography software (Dionex) with 0.5-10 µg/mL GlcNAc calibration curve. Rapid hexose quantification was achieved by Phenol-H₂SO₄ method [19]. Unconjugated saccharide concentration was analysed by passing 1

mL of purified neoglycoconjugate preparation at 10 µg/mL saccharide concentration through C4 hydrophobic interaction column (0.5 mL resin, Bioselect, Grace Vydac). Unconjugated carbohydrate was eluted with 1 mL of 20% acetonitrile in water and subsequently estimated by HPAEC-PAD analysis. Protein concentration was determined by MicroBCA and Bradford protein assay kit (Thermo Fisher Scientific). Hyaluronic acid concentration was determined using a commercial test kit (Corgenics) and DNA concentration was determined spectrophotometrically [20]. Size exclusion HPLC for glycoconjugate profiling was performed on Ultimate-3000 HPLC system (Waters) with a TSK Gel G4000SW column. All the samples were eluted at 0.5 mL/min flow with a mobile phase buffer containing 0.1 M sodium phosphate, 0.1 M sodium sulphate, 5% acetonitrile, pH 7.2. The amount of bacterial capsule was revealed by capsule quantification colorimetric assay kit (Corgenix HA Test kit, Corgenix Medical Corporation) [21]. GAS strains were grown in 10 mL of THY to OD₆₀₀=0.4. Cells were collected by centrifugation, washed twice and resuspended in PBS. Twenty microliters of bacterial suspension (approximately 2×10^6) were used. Hyaluronic acid concentration was calculated by comparing the absorbance at 450 nm of the sample against a reference curve prepared from the reagent blank and five HA reference solutions (50, 100, 200, 500, and 800 ng/mL) included in the kit.

NMR analyses

Lyophilized CHO samples were dissolved in deuterium oxide (D₂O, 99.9% atom D, Aldrich) to produce a uniform solution. ¹H NMR experiments were recorded at 25°C on a Bruker Avance™ 600 MHz spectrometer, using a 5 mm broadband probe (Bruker). The XWINNMR™ software package (Bruker) was used for data acquisition and processing; 32k data points were collected over a 10 ppm spectral width for the proton spectra. The transmitter was set at the HDO frequency, which was also used as reference signal (4.79 ppm). ¹D proton NMR spectra were collected using a standard one-pulse experiment.

Size exclusion coupled to Multi-Angle Laser Light Scattering photometer (SEC-MALLS)

[00193] The SEC-MALLS system consisted of an Alliance 2695 solvent delivery module (Waters, Millipore) and a TSK-Gel[®] G2000SWXL column (Tosoh Bioscience) connected in series and coupled to a Dawn EOS multi-angle light scattering photometer (Wyatt Technology Corp.). The polysaccharide sample was injected at the concentration of 11.6 mg/mL in 10 mM NaPi pH 7.0 and eluted at the flow rate of 0.5 mL/min. Data were collected and processed using the software ASTRA[™] (Wyatt technology Corp.). The absolute molecular mass was determined according to the equation $R(\Theta) = K^*McP(\Theta)[1 - 2A_2McP(\Theta)]$, where $R(\Theta)$ is the excess Rayleigh ratio, K^* the polymer constant for a particular scattering system, M the molecular mass, c the solute concentration (g/mL), $P(\Theta)$ the form factor related to the mean square radius r_g of the particle and A_2 is the second virial coefficient, a measure of solute-solvent interaction, which to a first approximation can be taken as zero [22]. The dn/dc value, which is squared in the expression for the optical constant K^* , was measured with an interferometric refractometer Optilab DSP (Wyatt Technology Corp.) pre-calibrated with NaCl samples. Six different polysaccharide samples with concentration of 0.039-1.158 mg/mL in 10 mM NaPi pH 7.0 was injected into the refractometer, starting with the lowest concentration. Data were processed using the software DNDC[™] (Wyatt Technology Corp.).

Mass Spectrometry

MALDI-TOF mass spectra of CRM₁₉₇ and glycoconjugates were recorded by an UltraFlex III MALDI-TOF/TOF instrument (Bruker Daltonics) in linear mode and with positive ion detection. All the samples for analysis were prepared by mixing 1 μ L product and 1 μ L of sinnapinic acid matrix in 0.1% TFA and 30% acetonitrile. 1 μ L of each mixture has been deposited on samples plate, dried at room temperature for 10 min and subjected to the spectrometer.

Isolation of native GAS-PS

GAS-PS was released from bacterial culture by reductive acidic treatment and purified by several steps of ultrafiltration and chromatography [9]. Briefly, acid-treated cell pellet suspension was

clarified by orthogonal filtration using 0.65 μm pore glass fiber filter (Sartopure GF2 capsule, Sartorius). Then permeate was diafiltrated with 30K cut-off membrane (Hydrosart, Sartorius) against 1M NaCl and water. The obtained material was further purified by anion exchange chromatography (Q-Sepharose FF resin, AKTA systems, GE Healthcare). PS-containing fractions as evidenced by phenol sulphuric acid test, were concentrated by TFF using 5K membrane (Sartorius).

Preparation and characterization of glycoconjugates

In a typical experiment 1 μmol of synthetic oligosaccharide was treated with disuccinimidyl adipate (10 μmol) in 1.5 mL DMSO containing 0.01 mL triethylamine. After 2 h of vigorous stirring the activated oligosaccharide was purified by chromatography on silica gel (0.035-0.70 mm, 60 $\text{\AA}$, Sigma-Aldrich) using a gradient of 0-70% methanol in ethylacetate. Eluted fractions were analyzed by TLC (ethylacetate-methanol 1:1) with detection by charring with 10% ethanolic H_2SO_4 and ninhydrin (1.5 mg/mL in 38:1.75:0.25 1-BuOH/ H_2O /HOAc). Fractions containing pure activated oligosaccharide were merged with subsequent determination of active ester groups (yield of ester introduction 13-39%) [23]. An alternative method pursued to purify the activated oligosaccharide consisted in precipitation of the reaction mixture in 9 volumes of ethylacetate; the pellet obtained by centrifugation was washed two times with 1 mL of ethylacetate and vacuum dried (yield of ester group introduction 42-51%).

The activated oligosaccharide was then reacted, with a conjugation stoichiometry of 30:1 active ester group /protein (mol/mol) with CRM₁₉₇ or human serum albumin (HSA) in 100 mM NaPi buffer pH 7.0 (10-20 mg/mL). The mixture was incubated overnight at RT, mixing very gently with a magnetic stirrer. The conjugation of the native GAS-PS to CRM₁₉₇ or HSA was instead achieved by direct reductive amination in the presence of NaBH_3CN [24]. The reaction was carried out in 200 mM NaPi pH 8.0 with stoichiometry 4:2:1 (wt:wt:wt) of GAS-PS: NaBH_3CN :protein. The solution was 0.22 μm filtered and kept at 37°C for 2 days. In all cases the

conjugates were purified from excess of unconjugated carbohydrate using ultrafiltration spin columns with 30K or 100K cut-off (Vivaspin, Sartorius).

Active immunizations

5 Animal experimental guidelines set forth by the Novartis Animal Care Department were followed in the conduct of all animal studies.

Groups of 8-16 female CD-1 mice (5-6 week old) were immunized on days 1, 21 and 35 with 10 µg of conjugated carbohydrate antigen formulated with 2 mg/mL aluminium hydroxide adjuvant. Adsorption was confirmed to be > 80% for all carbohydrate conjugates tested, as assessed by SDS-PAGE analysis of formulation supernatants and desorbed material. All immunizations were performed by administering a 200 µL dose volume via intraperitoneal route. Adjuvant alone or non conjugated GAS-PS (10 µg, formulated with alum,) were used for negative control groups, while serotype-specific M protein (10 µg) was used for positive control groups. Serum samples were collected before the first immunization and 14 days after the third immunization (day 49).

New Zealand white rabbits (2 kg) were immunized on days 1, 21 and 35 with 100 µg of conjugated carbohydrate antigen formulated with 2 mg/mL aluminium hydroxide, or with adjuvant alone. Immunizations were performed by administering 1 mL dose volume via subcutaneous route. Serum samples were collected 14 days after the third immunization.

ELISA assays

Titration of carbohydrate-specific antibodies was performed on individual sera 2 weeks after the last immunization. For that purpose 96-well Maxisorp plates (Nunc, Thermo Fisher Scientific) were coated with 100 µL/well of 1 µg/mL HSA-GAS-PS conjugate in PBS. Plates were incubated for 3 hours at room temperature, then washed three times with TPBS (0.05% Tween 20 in PBS, pH 7.4) and blocked with 250 µL/well of 2% BSA (Sigma-Aldrich) for 1h at 37°C. Each incubation step was followed by triple TPBS wash. Serum samples were initially diluted 1:500-1:1000 in 2 % BSA in TPBS, transferred into coated-blocked plates (200 µL) and serially two-fold diluted followed by 2 h incubation at 37°C. Then 100 µL/well of 1:2000-1:5000 diluted alkaline phosphatase-conjugated goat

anti-mouse IgM (μ -chain specific, Sigma Aldrich), anti-mouse IgG, anti-rabbit IgG or anti-mouse IgG subclass (whole molecule, Sigma Aldrich) were added and left for 2 h at 30°C. Bound alkaline phosphatase was visualized by adding 100 μ L/well of 3 mg/mL para-nitrophenyl-phosphate (pNPP) disodium hexahydrate (Sigma Aldrich) in 1M diethanolamine buffer (pH 9.8). After 10 minutes of development at RT, plates were analysed at 405 nm in a microplate spectrophotometer. Antibody titres were calculated by interpolating ODs into a reference calibration curve and expressed in ELISA units (EU) per mL. The thiocyanate elution ELISA for affinity studies is described elsewhere [25]. Absorbance values of sera without NH₄SCN were at least 1.0.

Flow cytometry of group A Streptococci incubated with GAS-PS-specific antisera

Bacteria were grown in THY to OD₆₀₀ = 0.4, washed twice with PBS, suspended in newborn calf serum (Sigma), incubated for 20 min at room temperature, and dispensed in a 96-well plate (20 μ L per well). Eighty microliters of pre-immune or immune sera from mouse immunized with CRM₁₉₇-GAS-PS were diluted in PBS/0.1% BSA and then added to the bacterial suspension to a final dilution of 1:200. Incubation was performed on ice for 30 min. Bacteria were washed and then incubated on ice for 30 min in 10 μ L of R-Phycoerythrin conjugated F(ab)₂ goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) in PBS/0.1% BSA/20% newborn calf serum to a final dilution of 1:100.

Stained bacteria were then fixed in 2% paraformaldehyde and, after washing, cells were resuspended in PBS. Samples were analyzed with a FACS Calibur cytometer (Becton Dickinson), using the FlowJo Software.

Immunoabsorption of anti-hexasaccharide antibodies from mouse sera

HSA conjugate (100 μ g) of oligosaccharide 1 (Fig. 1b) was coupled to 200 μ L N-hydroxysuccinimide-activated sepharose resin (GE Healthcare) following the manufacturer instructions. Excess of active groups on the resin was blocked with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3. Operations with small amount of resin were performed in clarification

spin column (Vivapure, Sartorius) with 0.22 μm membrane filter. Resin was equilibrated in PBS, incubated with 1:20 PBS-diluted mice sera for 10 min and then spun. The flow through was cycled five times through the column. The resin was regenerated by 100 mM glycine, pH 2.7 and re-equilibrated with PBS. Flow-through serum fractions (1:100 diluted) were tested for reactivity against HSA conjugates of 1, 3 (Fig. 1b) and GAS-PS in ELISA assay. Reference anti-CRM197 titration was performed to allow the recalculation of antibody titres due to unspecific absorption. Similarly HSA conjugate of oligosaccharide 2 (Fig. 1b) was coupled to the activated sepharose resin and used for depletion of mice antiserum to be consequently tested for its reactivity against HSA conjugates of 2, 4 (Fig. 1b) and GAS-PS in ELISA.

In vivo protection assays

Animals immunized as described above were intraperitoneally (i.p.) challenged on day 56, three weeks after the last immunization, with 50 (for M23 DSM2071 strain) or 2.5×10^6 (for M1 3348 strain) colony forming units (cfu) per mouse (lethal dose 90, LD90). Animals were monitored on a daily basis and euthanized when they exhibit defined humane endpoints that were pre-established for the study in agreement with Novartis Animal Welfare Policies. Bacterial cultures for infection experiments were grown in Todd-Hewitt broth until mid-log phase, appropriately diluted in fresh medium, and 200 μL were administered to each mouse by i.p. injection. Samples of the inoculum were plated on THY plates (Difco) supplemented with 5% sheep blood to verify the infectious dose. The plates were incubated at 37°C overnight, and the number of colonies was counted on the next day.

Whole-blood bactericidal assay

The capacity of rabbit antisera to mediate bacterial killing in a whole-blood assay was assessed as described by Lancefield [26] with some modifications. Samples of GAS strain M1 3348 were grown in THY for 16h at 37°C. 125 μL of bacteria diluted in PBS (corresponding to 15-50 CFU) were mixed with 125 μL of tested antisera, 1000 μL of whole blood from a naïve animal and 10% baby rabbit complement. Each reaction

mixture tube was rotated end over end at 37°C for 5 hours. Following incubation, cell counting was performed by plating each mixture onto triptic soy agar plates (BD) supplemented with 5% sheep blood. Percentage of the bacterial killing was calculated using the following equation: [(mean CFU_{negative control}) – (mean CFU_{test sample}) / mean CFU_{negative control}] x 100, where the negative control corresponds to the serum from rabbit immunized with adjuvant alone.

Statistics

Unpaired t-test was applied to natural logarithm values of serum ELISA titres to determine the differences between GMT of the immunization groups. Mann-Whitney t-test was used to determine the differences in protection level and opsonophagocytosis-mediated killing. Comparison was performed between negative control group (Alum alone) and the immunization group of interest.

Design and Synthesis of GAS Oligosaccharides

Oligosaccharides 61 –64 (Fig. 1b) were designed to evaluate the influence of antigen length and sequence on immunogenicity and immunoprotection *in vivo*. Based on the previously described immunodominant hexamer epitope [11, 27], two hexamers and two dodecamers were designed and synthesized with each pair differing at their non-reducing termini. An amine-terminated two-carbon linker was incorporated at the reducing end of each oligosaccharide to accommodate a variety of spacers and conjugation chemistries.

The four oligosaccharides were assembled using a convergent block coupling approach starting from a set of mono-, di- and trisaccharide building blocks leading to a fully-protected form of each target (Fig. 2). As an example a four-step deprotection sequence provided oligosaccharide 63 in crude form (Fig. 2), with 57% overall yield from compound 65 (Fig. 2). Final oligosaccharide products were purified by size exclusion chromatography, lyophilized and obtained as the hydrochloride salts. All oligosaccharides were fully characterized by ¹H NMR, MALDI-MS, elemental analysis, and HPLC.

GAS-PS Isolation and Purification

Native GAS-PS was extracted from GAS SF370 M1 strain, a clinical isolate that expresses limited amount of hyaluronic acid capsule. Final GAS-PS preparation contained <0.3% of DNA and <0.005% hyaluronic acid. MicroBCA assay detected 2-3% protein contamination. However, neither silver stained SDS-PAGE, nor ESI-qTOF and MALDI-TOF investigations were able to reveal the presence of protein or peptide contamination, suggesting that the MicroBCA detection was likely influenced by reducing groups of GAS-PS [28, 29]. The average molecular weight of GAS-PS was 8.9 ± 1.0 kDa, corresponding to 18 repeating units as determined by MALLS using the experimental dn/dc value of 0.168 mL/g.

The structure of purified GAS-PS was determined by 1H NMR analysis (Fig. 3). The 1H NMR spectrum was consistent with the published structure [4]. The so-called variant GAS polysaccharide, – the poly-rhamnose $[\rightarrow 3)\alpha\text{-L-Rhap}(1\rightarrow 2)\alpha\text{-L-Rhap}(1-)]_n$ backbone devoid of branching GlcNAc residues, was detected typically at < 10%. In certain cases this variant carbohydrate can substitute typical GAS-PS in the bacterial cell wall after passage in mice [8, 11]. The 1H -NMR peak of poly-rhamnose H_1^{RhaB} was shifted in comparison to conventional GAS CHO H_1^{RhaB} , while both variant and conventional H_1^{RhaA} signals were merged (Fig. 3). A molar ratio between poly-rhamnose and GlcNAc-containing species of GAS-PS was calculated by integration of the variant H_1^{RhaB} and the merged H_1^{RhaA} peaks: % polyrhamnose = $[H_1^{RhaB}_{VAR}/(H_1^{RhaA} + H_1^{RhaA}_{VAR})] \times 100$.

Generation and Characterization of Glycoconjugates

A well-established method to improve the typically poor immunogenicity of carbohydrate antigens relies on conjugation to a protein carrier that provides T cell epitopes. The glycans were coupled to lysine residues of CRM₁₉₇, a non-toxic mutant diphtheria toxin that has been extensively used as a carrier for glycoconjugate vaccines in humans. Carbohydrate haptens were covalently linked to the lysine residues present in the carrier protein. The synthetic oligosaccharide antigens carry an amino group at the end of a two carbon spacer (Fig. 1b) which was

derivatized with disuccinimidyl adipate and subsequently coupled to the CRM₁₉₇. Native GAS-PS was instead conjugated via its reducing end group by direct reductive amination. This procedure yielded conjugates that, after purification by ultrafiltration, were characterized by MS analyses, SDS-PAGE, size exclusion HPLC, protein and carbohydrate content. As determined by SDS- PAGE and size-exclusion HPLC, all synthetic oligosaccharides produced glycoconjugates with more homogeneous pattern than CRM₁₉₇ -GAS-PS. The conjugated GAS-PS showed a significant polydispersion in molecular size, likely due to the larger size of the carbohydrate hapten (Fig. 4a and 4b). A representative MALDI-TOF spectrum of the glycoconjugate CRM₁₉₇ -3/2 and CRM₁₉₇ -GAS-PS is shown in Fig. 5. The profile of CRM₁₉₇ -3/2 is composed of a polydispersion centered at 77922.9 m/z corresponding to approximately nine oligosaccharide chains per CRM₁₉₇ molecule; profile of CRM₁₉₇ -GAS-PS is composed of a polydispersion centered at 87356.4 and 96778.3 m/z corresponding to three and four GAS-PS chains per CRM₁₉₇ molecule, respectively.

In both cases the spectrum contains the polydispersion corresponding to the doubly charged adducts.

The carbohydrate/protein ratio of the conjugates made with native GAS-PS ranged from 40 to 50% (wt/wt) corresponding to 2-3 on molar basis; the molar carbohydrate/protein ratio of conjugates made with synthetic GAS oligosaccharides ranged from 3 to 12, as determined by chemical methods (Table 1, supra). In all cases the amount of unconjugated sugar in the glycoconjugates was <10%.

Anti-GAS-PS Immunogenicity of Glycoconjugates

CD-1 mice received one prime and two boosting doses of the carbohydrate-protein conjugates (10 µg each, formulated with alum). Control groups were immunized either with alum only or unconjugated GAS-PS formulated with alum. Sera were collected on day 49, two weeks after the third immunization to assess antibody responses by ELISA, using GAS-PS coupled to HSA as coating reagent.

All synthetic conjugates regardless their saccharide chain length, carbohydrate loading and the nature of their terminal sugar, were able to induce GAS-PS specific IgG in mice while, as expected, the unconjugated GAS-PS was not immunogenic (Fig. 6a). The antibody responses to most conjugates with synthetic oligosaccharides were lower than those elicited by CRM₁₉₇-GAS-PS ($P < 0.05$); however a notable exception was represented by conjugates CRM₁₉₇-63/1 (notation is oligosaccharide 63, conjugated to 1 antigen and linked to CRM₁₉₇) and CRM₁₉₇-63/2, with a carbohydrate loading of 5.8 and 7.5 respectively (Table 1, supra), which induced a level of anti-GAS-PS IgG comparable to that obtained with the native GAS-PS glycoconjugate ($P = 0.452$ and 0.088 respectively). On the other hand, CRM₁₉₇-63/3 with a carbohydrate loading of 12 induced significantly lower titres ($P < 0.05$). This observation seems to indicate that, for a given chain length, differences in the conjugate glycosylation degree are crucial for the induction of an optimal antibody response.

Concerning the influence of the terminal non-reducing sugar on the ability to induce specific antibody responses against the native GAS-PS, a direct comparison between CRM₁₉₇-1/1 and CRM₁₉₇-2/1 groups (same length, similar loading, but different terminal non-reducing sugar) did not reveal a significant difference ($P = 0.608$), although the GMT induced by the GlcNAc-terminating oligoconjugate was higher (2911 EU/mL vs 1895 EU/mL, Table 1, supra). Similar observations could be made for the pair of the dodecasaccharide glycoconjugates CRM₁₉₇-63/2 and CRM₁₉₇-64/1 (GMT 17780 EU/mL vs 4063 EU/mL, respectively, $P = 0.102$).

The analysis of IgG subclasses revealed that the major IgG isotype induced by both synthetic oligosaccharides and GAS-PS glycoconjugates is IgG1, showing a bias toward a Th2 response, consistent with the use of Alum as adjuvant (Fig. 6b), all conjugates elicited also specific IgM. Avidity of specific IgG detected with thiocyanate elution ELISA was not significantly different between the immunization groups (Table 1, supra).

Specificity of Anti-GAS-Polysaccharide Polyclonal Antibodies

In order to determine whether carbohydrate chains longer than the hexasaccharides represent any additional epitopes for antibody recognition,

sera from a group of mice immunized with CRM₁₉₇-63 were pooled and depleted of anti-61 antibodies by affinity chromatography. Loss of serum reactivity against 61 in ELISA assay verified the effectiveness of the affinity resin. The depleted serum pool was tested further against an HSA conjugate of 63. IgG levels against 63 were less than 1% or undetectable, suggesting that although 63 represents an elongated structure and contains more repeating units than the hexasaccharide 61, it did not elicit any antibodies specific for larger epitopes. The same procedure was repeated for the serum pool from a group of mice immunized with GAS-PS glycoconjugate. No anti-GAS-PS reactivity was observed after depletion of antibodies specific for the hexasaccharide 61 indicating equal IgG specificity. Similarly, pooled antisera from mice immunized with CRM₁₉₇-64 were depleted for anti-62 antibodies, and no reactivity against 64 or GAS-PS was detected after depletion.

Exposure of GAS-PS on surface of bacterial cells

GAS virulence in humans and animal models closely correlates with the expression of the hyaluronic acid capsule. Encapsulation interferes with antibody-mediated opsonization of bacteria and may potentially shield protective antigens [35, 36]. GAS-PS was described to be highly abundant on the surface of multiple GAS strains and furthermore is commonly used for diagnostic purposes. However, its accessibility on the encapsulated strains has not been deeply studied. Two clinical GAS strains were chosen possessing robust capsule to test the protective efficacy of GAS-PS glycoconjugates, GAS M1 3348 and M23 DSM2071. Surface exposure of the GAS-PS on M1 3348 and M23 DSM2071 was revealed by flow cytometry after the incubation of live bacteria with antigen-specific mice antisera (Fig. 7). The analysis revealed that both strains were positive for the anti-polysaccharide antibody binding.

Protection against i.p. challenge with GAS

Mice immunized with three 10 µg doses of Alum-formulated glycoconjugates or serotype-specific M proteins were challenged with an appropriate LD₉₀ dose of GAS strain. Survival was monitored for 14 days (Fig. 8 A) and the differences in the protection extent induced in each group

compared to the negative control were analysed by Mann-Whitney t-test. Immunization with strain-relevant M protein conferred over 75% protection (Fig. 8). Mice immunized with all conjugated carbohydrate antigens and challenged with an M1 strain had significantly lower mortality than control mice immunized with alum alone ($P<0.05$), with survival rates ranging from 29% to 50% 14 days after the challenge. Cross-protection properties were verified using the conjugate obtained from hexasaccharide 61, the smallest antigenic determinant recognized by human anti GAS immune response, which conferred 56% protection against the M23 challenge strain ($P<0.05$) (Fig. 8).

To address the question whether anti-GAS-PS antibodies would promote opsonophagocytic killing in an *in vitro* assay system, a preliminary experiment was carried out where rabbits were immunized with three 100 µg doses of each of the glycoconjugates formulated in Alum (1 animal/vaccine, as described herein). After having determined the presence of specific anti-GAS-PS IgG, the opsonophagocytic properties of rabbit antisera were tested in a whole blood assay using the M1 3348 strain. GAS cells were incubated with antisera prior to addition of whole blood from naïve animals. Antisera raised against CRM₁₉₇-61, CRM₁₉₇-62, CRM₁₉₇-63, CRM₁₉₇-64 and CRM₁₉₇-GAS-PS promoted similar levels of killing compared to the control sample ($p<0.05$) (Fig. 9).

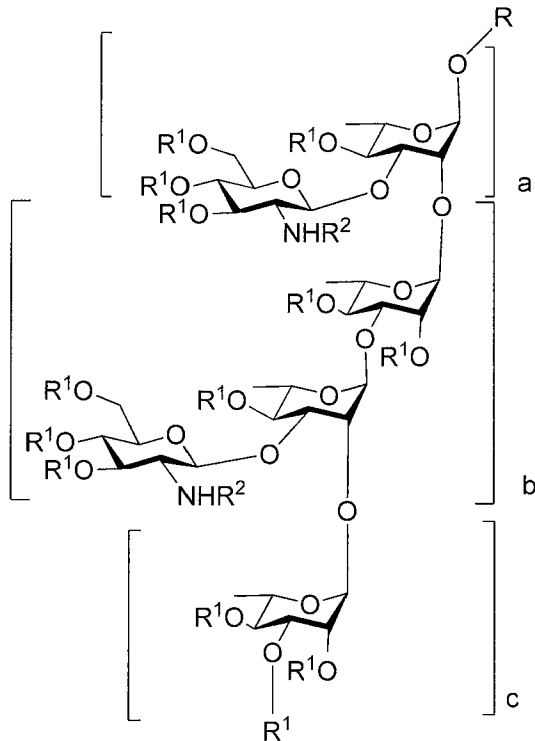
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CLAIMS

1. A synthetic oligosaccharide I:



where a and c are each 0 or 1;

- 5 b is an integer from 1 to 8;

each R^1 is independently H or a hydroxyl-protecting group;

each R^2 is independently H or an amino-protecting group; and

R is a hydroxyl-protecting group.

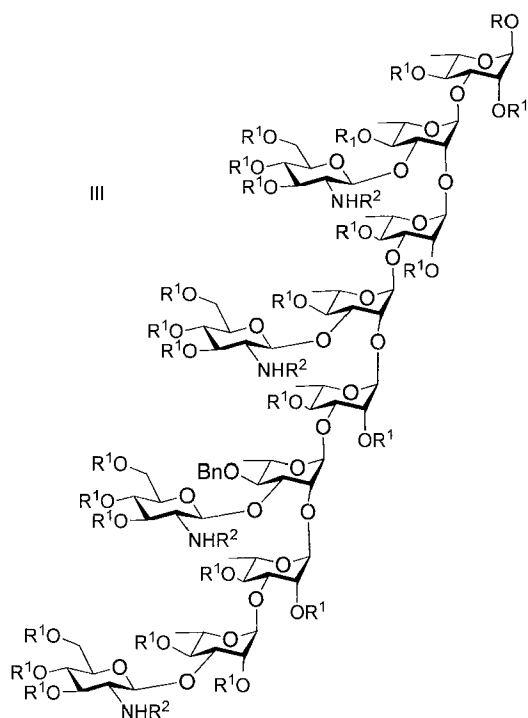
- 10 2. The synthetic oligosaccharide of claim 1, wherein a is 0.

3. The synthetic oligosaccharide of claim 1, wherein c is 0.

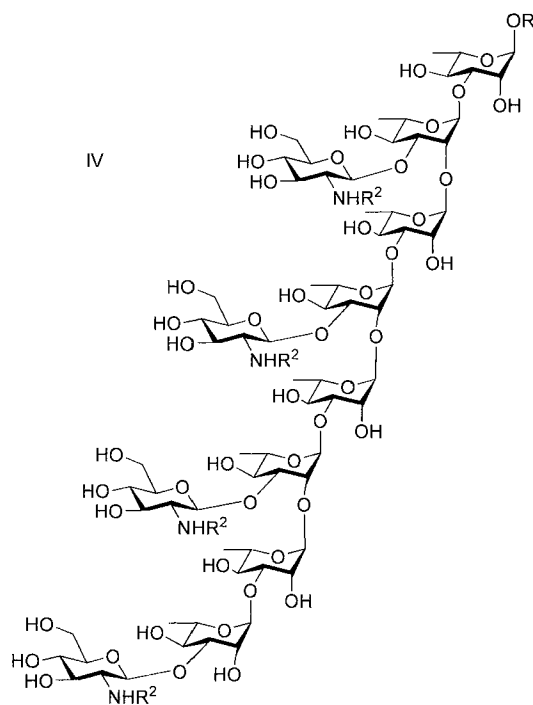
4. The synthetic oligosaccharide of claim 1, wherein b is an integer from 2 to 8.

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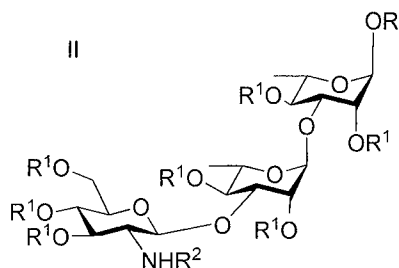
5. The synthetic oligosaccharide of claim 1, which is



6. The synthetic oligosaccharide of claim 1, which is



7. The synthetic oligosaccharide of claim 1, which is

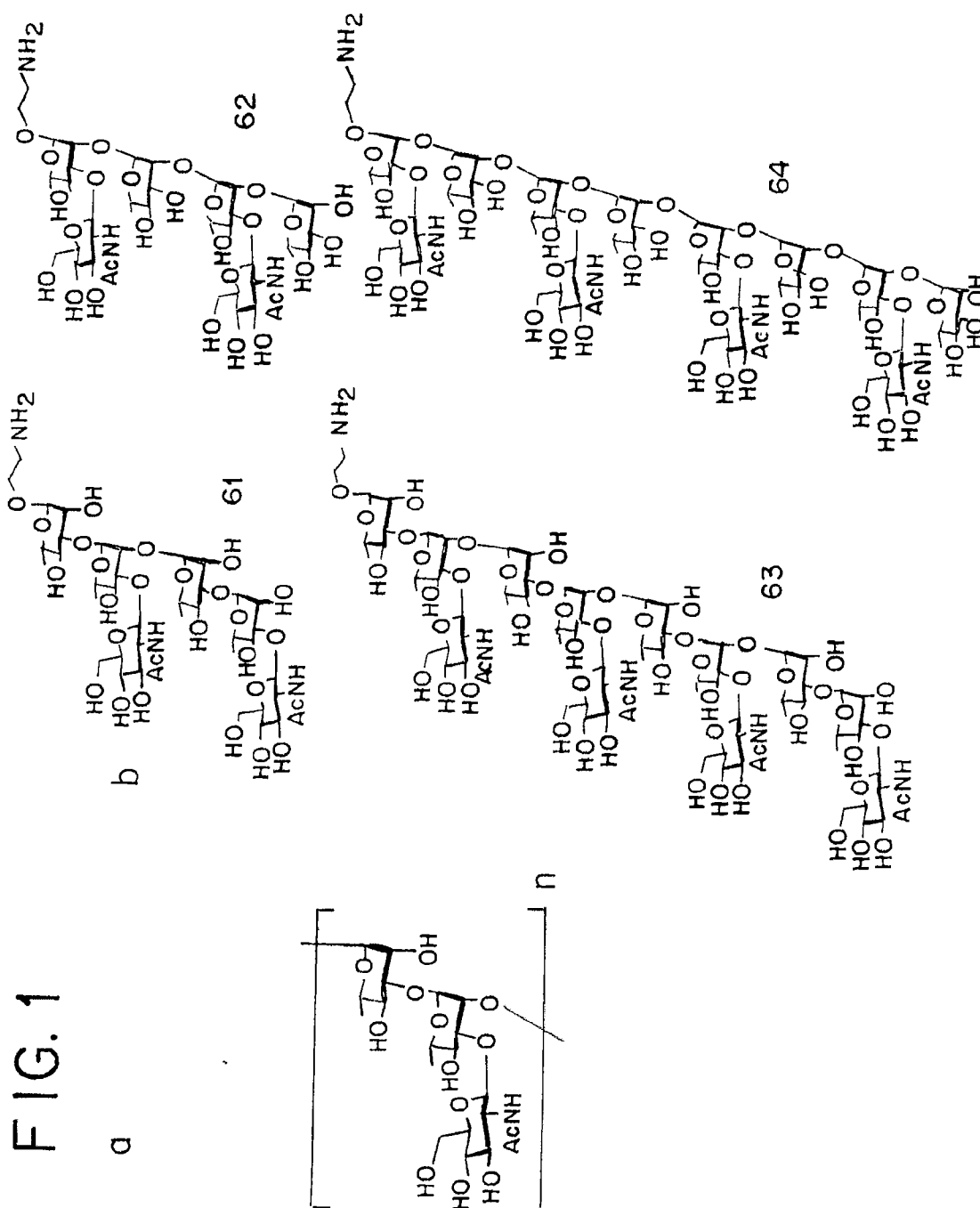


8. The synthetic oligosaccharide of claim 1, where each R^2 is independently selected from the group consisting of a silyl group, a carbamate, formyl, acetyl, benzoyl, pivaloyl, methoxymethyl, t-butyl, benzyl and tetrahydropyranyl.

9. The synthetic oligosaccharide of claim 1, where each R^1 and R is independently selected from the group consisting of formyl, acetyl, substituted acetyl, propionyl, butynyl, pivalamido, benzoyl, biphenylcarbonyl, substituted biphenylcarbonyl, ethoxycarbonyl, t-butoxycarbonyl, benzyloxycarbonyl, phenoxycarbonyl, benzyl, diphenylmethyl, triphenylmethyl, t-butyl, tetrahydropyranyl, allyl, N-phenylcarbamate, N-imidazolyl carbamate, trialkylsilyl, isopropylalkylsilyl, alkyl-diisopropylsilyl, triisopropylsilyl and t-butylalkylsilyl.

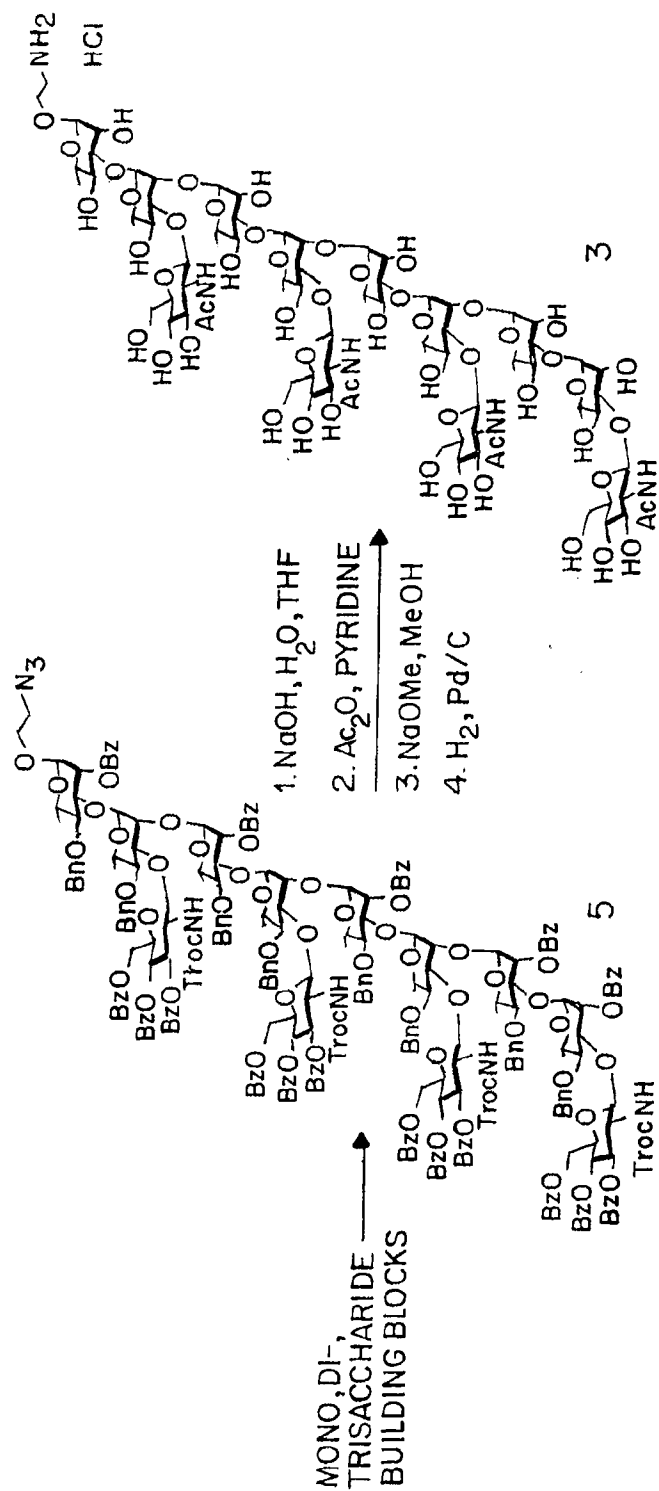
10. The synthetic oligosaccharide of claim 1, where each R^1 is hydrogen, each R^2 is acetyl and R is hydroxyl or $-\text{CH}_2\text{CH}_2\text{NH}_2$.

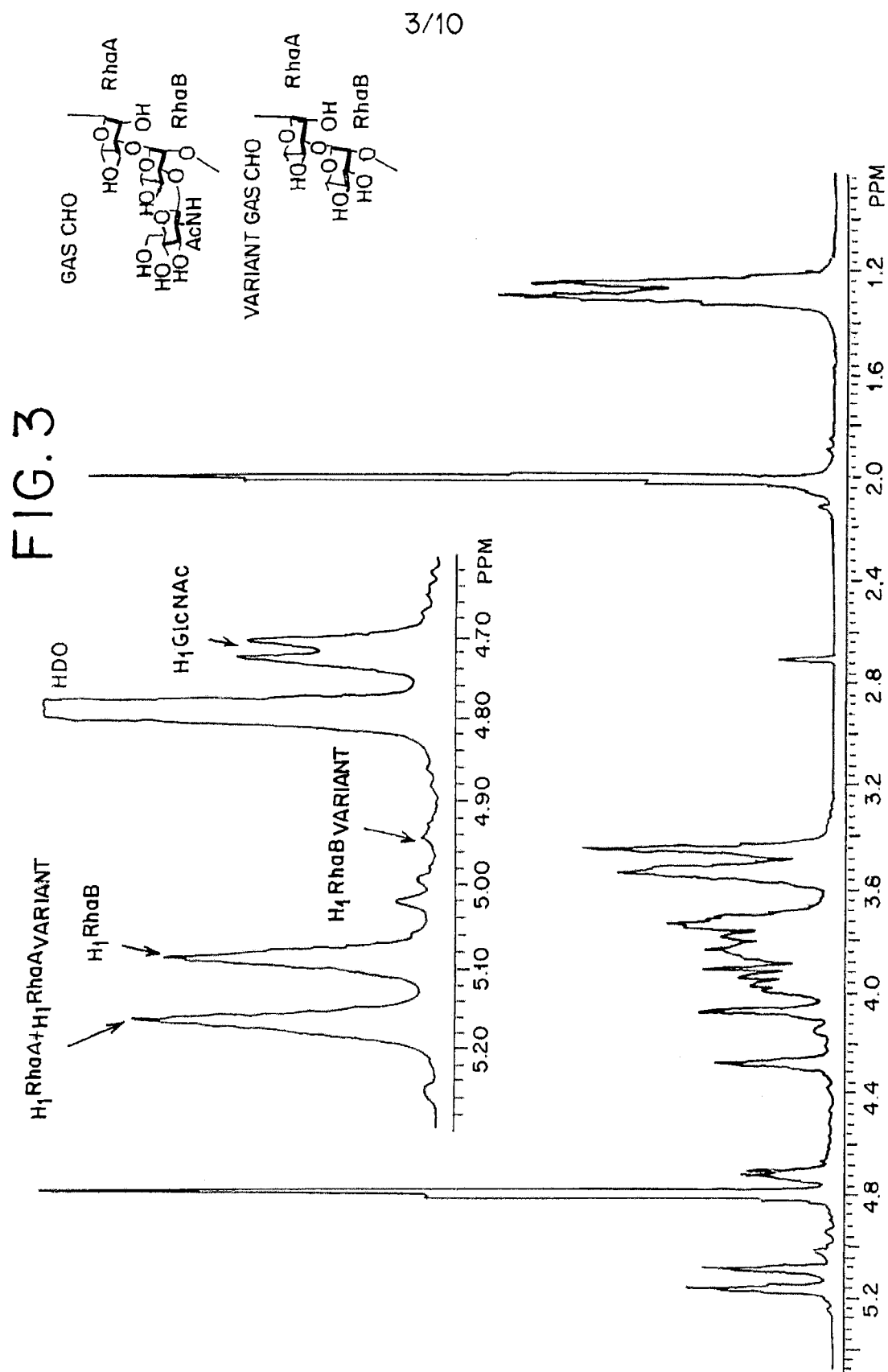
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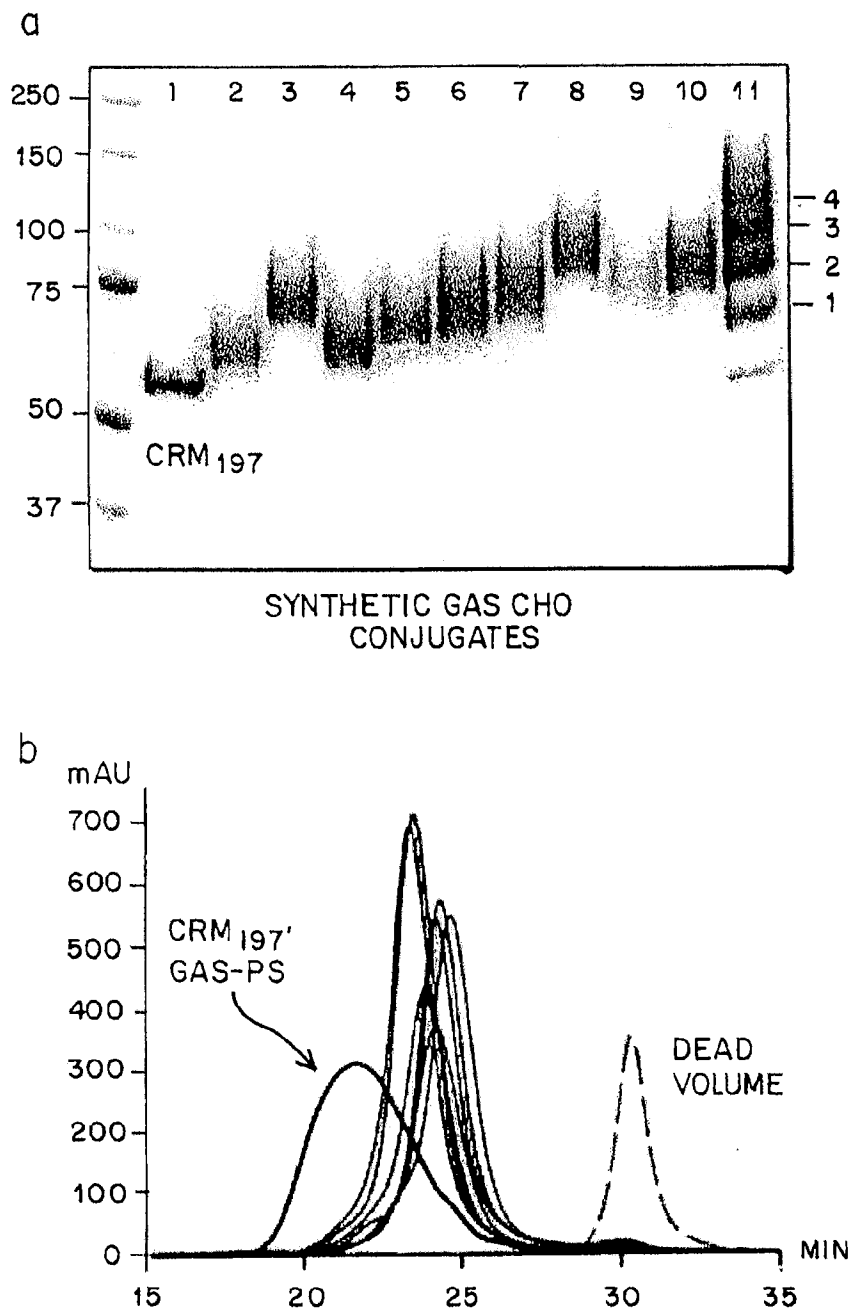
FIG. 2





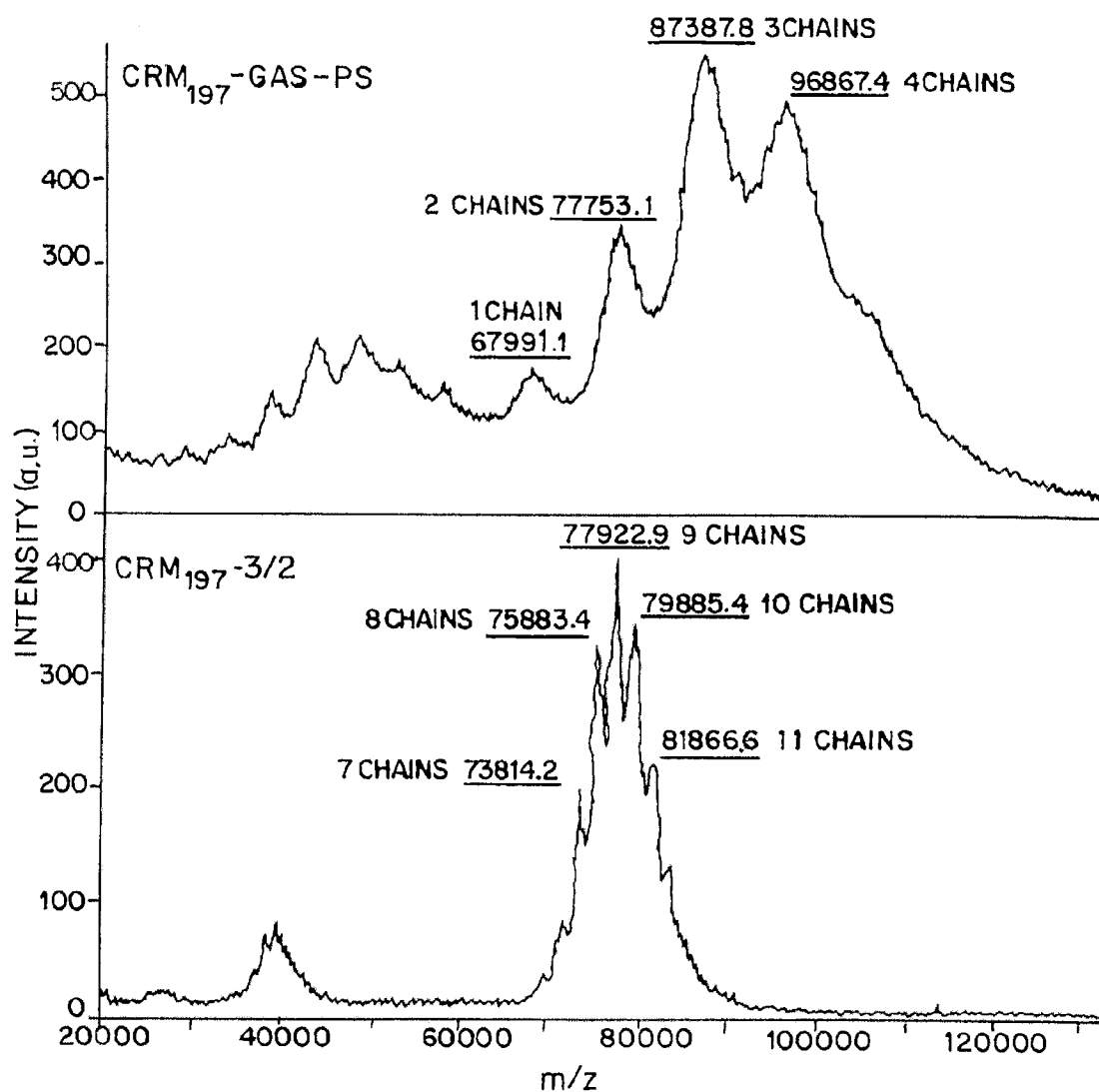
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FIG. 4



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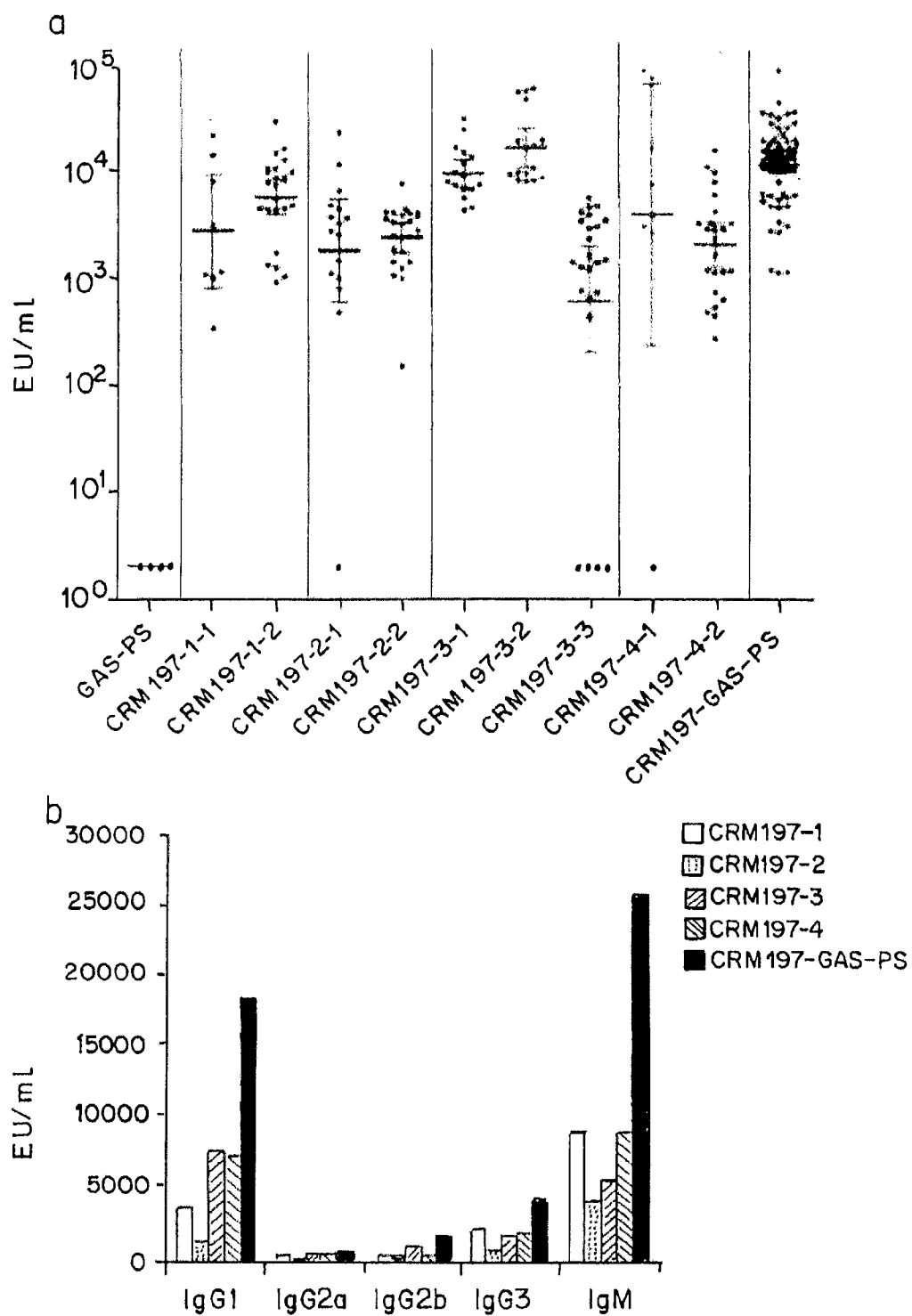
FIG. 5



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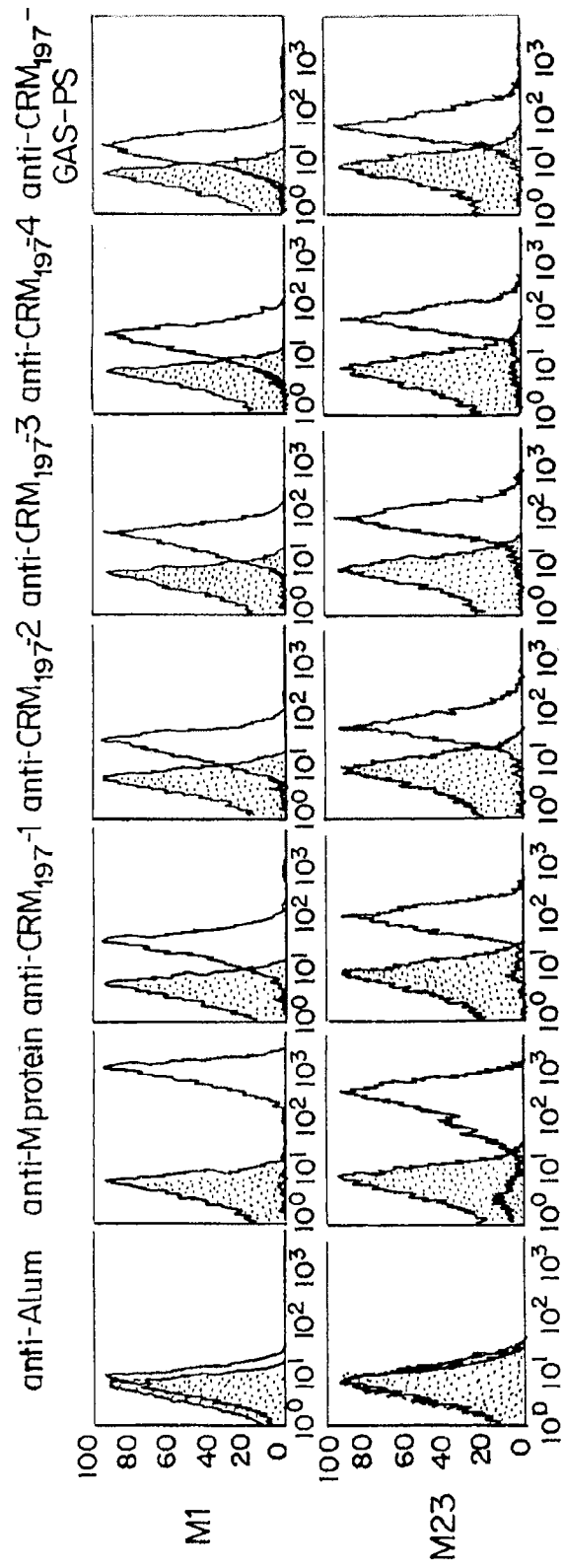
FIG. 6

VACCINE, KABANOVA et al., FIG. 6



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FIG. 7



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FIG. 8

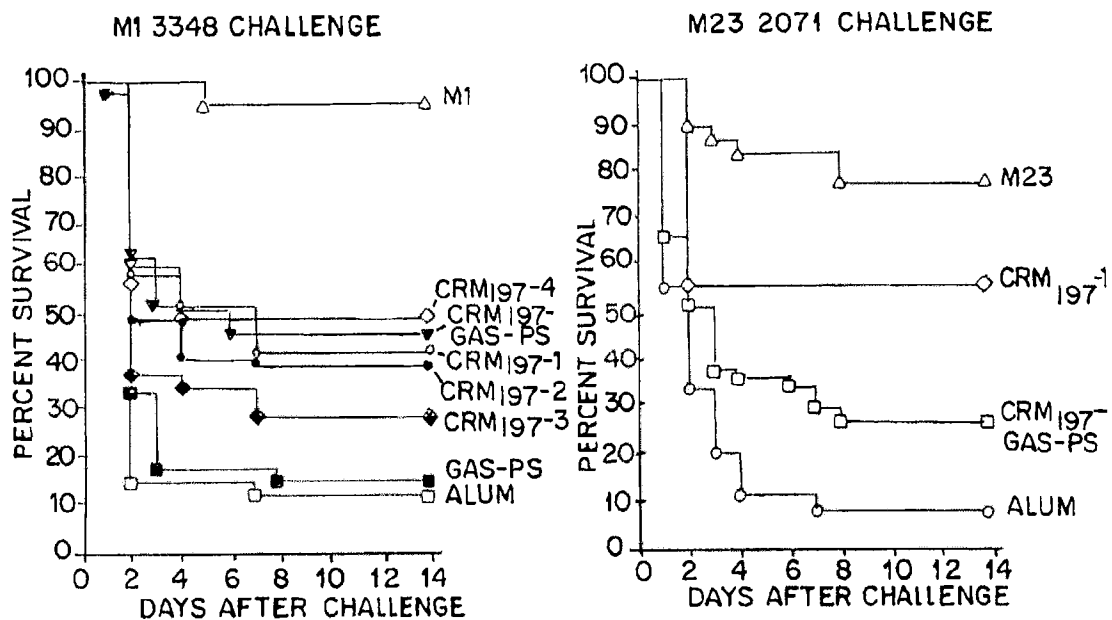


FIG. 9

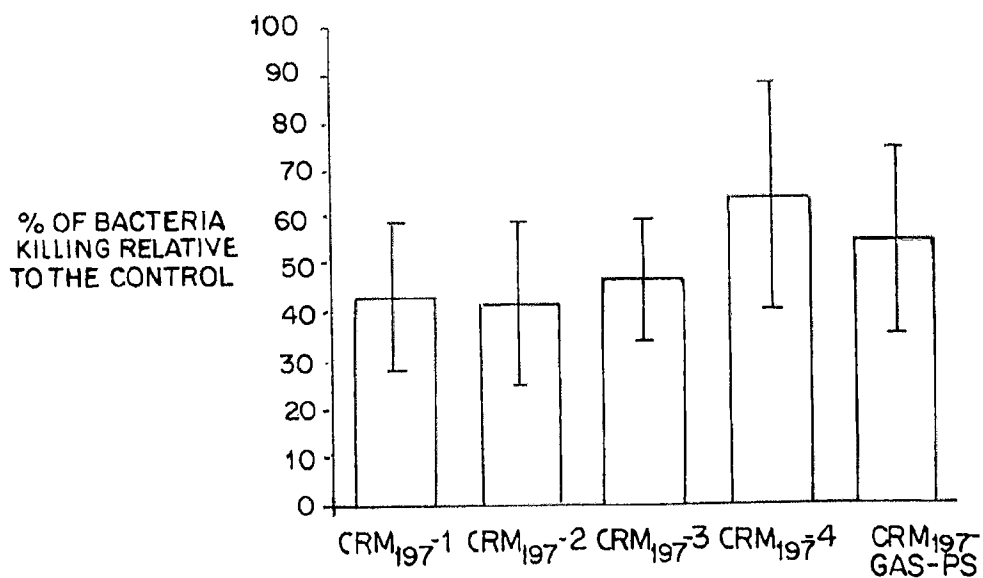
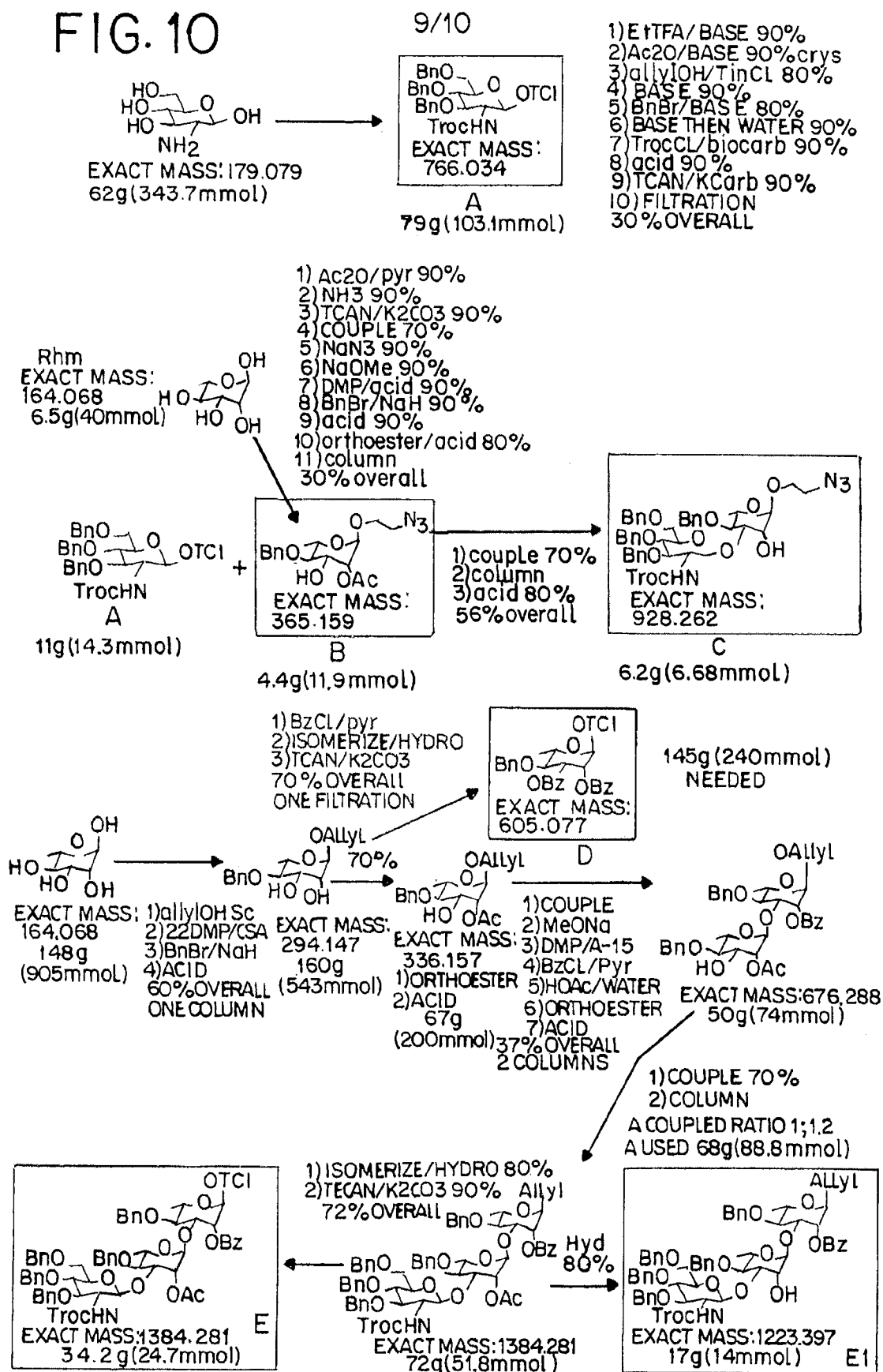


FIG. 10



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/64446

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01N 43/04; A61K 31/70 (2011.01)

USPC - 514/38-39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 514/38-39

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC: 514/23, 27, 32, 40, 54 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Electronic Database Searched: PUBWEST (PGPB,USPT,USOC,EPAB,JPAB), Google. Search Terms Used Oligosaccharide, synthetic oligosaccharide, group a streptococcus, protecting group, formyl, acetyl, propionyl, butenyl, pivalamido, benzoyl, biphenyl carbonyl

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0047225 A1 (Zhu et al.) 25 February 2010 (25.02.2010) entire document, especially para [0079], [0088]	1-10
Y	US 4,418,192 A (Tanaka et al.) 29 November 1983 (29.11.1983) especially col 4, ln 4-25, formula	1-10

☐ Further documents are listed in the continuation of Box C.


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Date of the actual completion of the international search

28 March 2012 (28.03.2012)

Date of mailing of the international search report

04 APR 2012

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