Development of a Liposaccharide-Based Delivery System and Its Application to the Design of Group A Streptococcal Vaccines

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Group A streptococcus (GAS) is associated with many human diseases, ranging in severity from benign to life-threatening. A promising strategy for developing vaccines against GAS involves the use of carbohydrates as carriers for peptide antigens. This study describes the optimized synthesis of D-glucose and D-galactose derived carriers, bearing an adipate linker and four *tert*-butoxycarbonyl protected aminopropyl groups. Prophylactic GAS vaccine candidates were synthesized by conjugating multiple copies of a single GAS M protein derived peptide antigen (either J8 or J14) onto the carbohydrate carriers. These antigens contain peptide sequences, which are highly conserved and offer the potential to prevent infections caused by up to 70% of GAS strains. Lipophilic amino acids were also conjugated to the D-glucose anomeric carbon to produce a self-adjuvanting liposaccharide vaccine. High serum IgG antibody titers against each of the incorporated peptide epitopes were detected following subcutaneous immunization of B10.BR (H-2^k) mice with the liposaccharide vaccine candidates.

Introduction

Vaccines are considered to be one of the safest and most cost-effective public health interventions, preventing up to three million deaths each year.[1] Problems with conventional vaccines including instability (necessitating their transport and storage under cold chain conditions), the paucity of carriers and adjuvants suitable for human use, and the potential dangers of using live microorganisms (risk of infection/reversion to virulence, allergies, and autoimmune responses) have led to the development of synthetic vaccines.[2] Peptide vaccines have many advantages over conventional vaccines. For example, peptide vaccines may incorporate only the minimal microbial components required to elicit an effective immune response, thereby reducing the risk of side effects and autoimmune responses associated with the administration of unnecessary microbial components. The development of synthetic peptide vaccines still faces many hurdles, however (e.g., toxicities associated with the administration of adjuvants). Although many experimental adjuvants are available, very few (e.g., squalene oil–water emulsions, calcium- and aluminum-based salts, cholera toxin B subunit, and monophosphoryl lipid A) have been used in vaccines approved for human administration.[3] This lack of suitable adjuvants for human use has spawned a need to develop new adjuvants. One such approach involves the conjugation of lipids to peptides to impart adjuvant activity upon the conjugated peptide antigens. These lipopeptide vaccines may constitute an ideal strategy for producing vaccines against pathogens that infect mucosal surfaces and may hold potential for the delivery of nonimmunogenic peptides and drugs orally.[4,5] Lipopeptide vaccine approaches have been studied for the development of vaccines against human immunodeficiency virus type-1 (HIV-1),[6] hepatitis B[7] and C viruses,[8] and malaria.[9]

The coupling of lipids to peptide antigens may be performed via an amplifying dendrimer carrier system such as the multiple antigen peptide (MAP) system,[10] which polymerizes peptide antigens through a branched polylysine core. In animal studies, the immune responses elicited against a peptide antigen were increased when the antigen was conjugated to a MAP system compared to when the peptide antigen was administered on its own.[11] Recent studies[12–15] have investigated vaccine development using the lipid core peptide (LCP[a]) system, a vaccine delivery system that conjugates lipoamino acids (α-amino acids with long alkyl side chains) through a MAP system to multiple copies of one or more peptide antigens. The LCP system provides similar immune responses to antigens coadministered with conventional adjuvants and represents a promising system for mucosal vaccine development.[16]

The advent of new, efficient, and sensitive analytical and synthetic methods in carbohydrate chemistry, together with an increased understanding of glycobiology and glycoimmunology, makes the development of carbohydrate-based vaccines possible. Carbohydrates, as carriers, similarly to the MAP system,[13] provide numerous attachment points for the conjugation of one or more copies of same or different peptide antigens. Furthermore, by using different carbohydrates, the stereochemistry of the vaccine may be altered to provide an optimal orientation for the recognition of peptide epitopes by cells of the immune system. Carbohydrate carriers also help to reduce degradation of the attached peptide antigens. Therefore, the conjugation of peptide antigens to lipids (as an adjuvant) and carbohydrates (as a carrier) represents a highly promising strategy for the development of peptide vaccines.

Group A streptococcal infection is caused by the bacterium *Streptococcus pyogenes* and may cause a variety of health problems ranging from a sore throat (e.g., *Streptococcal*

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[a] Abbreviations: Boc, *t*-butoxycarbonyl; CFA, complete Freund's adjuvant; GAS, group A streptococcus; LCP, lipid core peptide; PBS, phosphate-buffered saline; *p*MBHA, *p*-methylbenzhydrylamine; RF, rheumatic fever; RHD, rheumatic heart disease.

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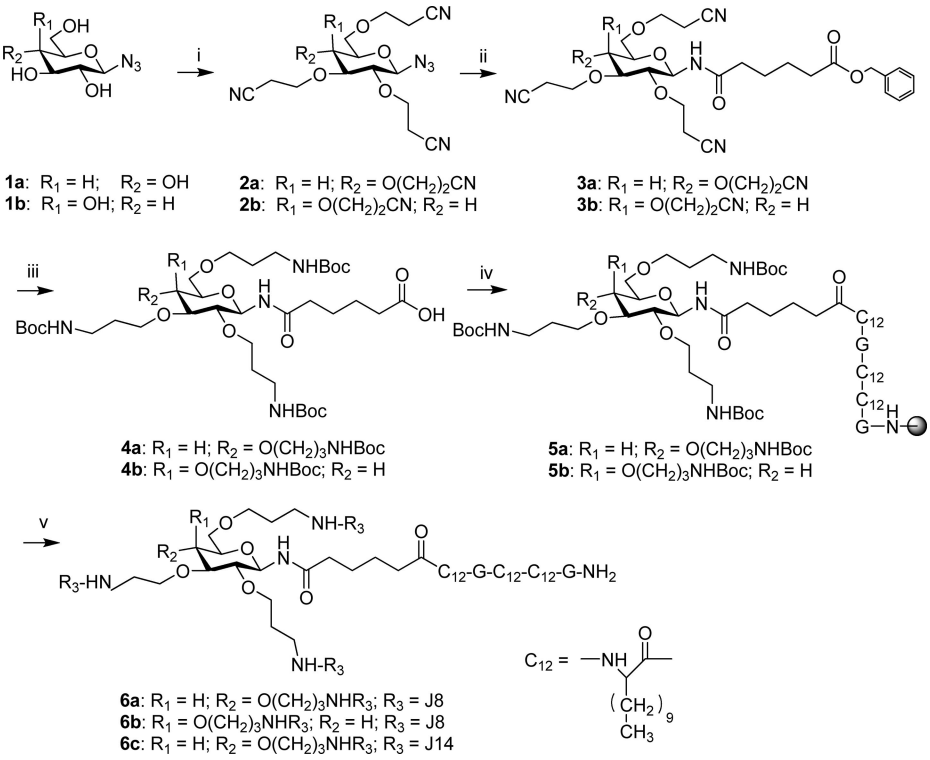
Scheme 1. Synthesis of the Glucose/Galactose-Lipid Core Templates (6a, 6b, and 6c) Bearing R = J8 or J14 Peptide Antigens, Respectively[a]



[a] Reagents and conditions: (i) acrylonitrile, DBU, ACN; (ii) H_2/Pd-C, THF; HBTU, DIPEA, THF, monobenzyl adipate; (iii) $NaBH_4$, $CoCl_2 \cdot 6H_2O$, Boc_2O, MeOH; H_2/Pd-C; (iv) C_{12}-G-C_{12}-C_{12}-G-NH-MBHA resin; (v) stepwise solid-phase peptide synthesis of J8 (QAEDK VKQ**SR EAKKQ VEKAL** KQLED KVQ) or J14 (KQAEDK VK**ASR EAKKQ VEKAL E**QLED KVK); HF cleavage.

pharyngitis) to the potentially fatal poststreptococcal *sequelae* rheumatic fever (RF) and rheumatic heart disease (RHD). Together, RF and RHD are responsible for more than 517000 deaths per annum (World Health Organization report).[17,18] The GAS cell-surface M protein, of which over 150 different types have been identified,[19] is considered to be an important target for the development of prophylactic GAS vaccines. Current vaccination approaches include the use of multiepitope vaccines incorporating epitopes derived from the highly variable M protein amino-terminus and/or the M protein carboxy-terminal region,[18,20,21] which is more highly conserved between GAS strains.

The current study describes the design of a synthetic subunit vaccine delivery system incorporating a carbohydrate template, which acts as a carrier for multiple copies of the J8 or J14 antigens; chimeric peptides derived from the carboxy-terminal *C*-repeat region of the GAS coiled-coil α-helical surface M protein (peptide 145).[22] This glycopeptide in turn was conjugated to a lipid moiety, synthesized using lipidic amino acids, to impart adjuvant activity upon the vaccine construct. To optimize the delivery system, different carbohydrate entities (D-glucose and D-galactose) were synthesized and characterized prior to their incorporation into lipid−carbohydrate−peptide (lipoglycopeptide) vaccines. Subcutaneous immunization of B10.BR (H-2^k) mice was performed and the vaccines' capacity to elicit high-titer antigen specific systemic IgG antibodies against J8 or J14 was assessed using an enzyme-linked immunosorbent assay (ELISA).

Results and Discussion

The described vaccine delivery system consists of three components: (1) a carbohydrate core (a carrier); (2) an immunostimulant lipid moiety (an adjuvant); and (3) multiple copies of a B-cell epitope containing antigen. The carbohydrate cores (**4a**, **4b**; Scheme 1) were designed to be conjugated to the immunostimulant lipid moiety at the anomeric position, with the other carbohydrate hydroxyl groups conjugated to Boc-protected amine containing spacers to enable the coupling of multiple peptide epitopes to the carbohydrate carrier.

Carbohydrate core synthesis was initiated with the cyanoethylation of β-D-glucopyranosyl azide[23] (**1a**) using acrylonitrile and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile (Scheme 1). Acrylonitrile was freshly distilled before use to prevent the formation of inseparable byproduct resulting from its dimerization. Pure tetra-*O*-(cyanoethyl) D-glucopyranosyl azide (**2a**; 66% yield), after selective reduction (catalytic hydrogenation) of the azido group, was immediately conjugated with freshly prepared monobenzyl adipate,[24] using the peptide coupling reagents, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), and *N*,*N*-diisopropylethylamine (DIPEA), to give benzyl ester **3a** in 52% yield.

The β-D-galactopyranosyl derivatives (**1b**−**4b**; Scheme 1) were prepared according to the procedure described by McGeary et al.[25] Problems were encountered, however, with the synthesis of the β-D-glucopyranosyl core **4a**. Reduction of the four cyanoethyl groups to amines (using sodium borohydrate and cobalt(II) chloride in methanol), *t*-butoxycarbonyl (Boc)-protection (with di-*tert*-butyl dicarbonate), and benzyl cleavage (by saponification with lithium hydroxide) were performed in situ. Due to the partial Boc-deprotection, caused by lowering the pH of the aqueous phase during the separation of the product, the formation of byproduct difficult to separate was observed. To optimize the synthesis, catalytic hydrogenation over 10% Pd/C instead of saponification with lithium hydroxide was done to

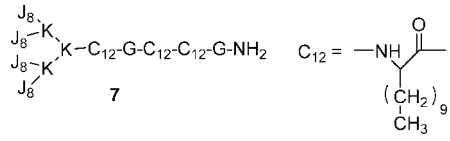


Figure 1. Structure of polylysine core with J8 antigen (**7**).

reduce these problems. This resulted in higher yield of the β-D-glucosyl derivative **4a**, featuring a carboxylic acid at the anomeric position (88% instead of 46%).

The lipid moiety used in the current study has been previously demonstrated to enhance the elicitation of antigen-specific serum IgG antibody responses against incorporated peptide antigens[2,13–15,26,27] The synthesis of the lipidic adjuvant was performed on *p*-methylbenzhydrylamine (*p*MBHA) resin as previously described.[28] The lipid adjuvant consists of three lipoamino acids (C_{12} = 2-amino-D,L-dodecanoic acid) with glycine spacers (C_{12}-G-C_{12}-C_{12}-G). The Boc-protected glucosyl (**4a**) and galactosyl (**4b**) carbohydrate cores were coupled to the resin bound lipid adjuvant using HBTU/DIPEA in situ neutralization.[29] Couplings were repeated where necessary to ensure a coupling efficiency greater than 99.6%, as confirmed by quantitative ninhydrin analysis.[30]

Synthetic peptide sequences, J8 or J14,[22,31] were synthesized by stepwise solid-phase peptide synthesis, and in situ neutralization chemistry,[29] following deprotection of the Boc-protected carbohydrate amine groups. Finally, the products **6a**, **6b**, and **6c** (Scheme 1) were cleaved from the resin using anhydrous hydrofluoric acid, and purified by preparative reverse phase high performance liquid chromatography (RP-HPLC) on a C4 column using acetonitrile-water gradients containing 0.1% trifluoroacetic acid over 60 min and characterized by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS), electrospray ionization mass spectrometry (ESI-MS), and analytical RP-HPLC.

As a positive control for the immunological studies a convential polylysine lipid-core peptide system (**7**; Figure 1) containing four copies of the J8 peptide antigen was produced.

Immunological assessment was performed in female B10.BR (H-2^k) mice. The mice (n = 5/group) were injected subcutaneously on days 0, 21, 31, and 41 with 30 μg of compounds **6a**, **6b**, **6c**, and **7** in phosphate-buffered saline (PBS). As positive controls, two groups of mice were administered the peptide antigens J8 or J14 emulsified in complete Freund's adjuvant (CFA). It has been previously reported that peptide antigens J8 and J14 did not elicit antibody response when administered subcutaneously without CFA.[15,31] For negative controls, mice were administered CFA or PBS. Sera were collected from the tail artery one day before each injection and nine days following the last injection and then analyzed by ELISA for total antigen-specific (J8 or J14) serum IgG antibodies. While all vaccine candidates (**6a**, **6b**, **6c**, and **7**) were unable to elicit significant titers of J8 or J14-specific IgG antibodies following primary immunization, a rapid increase in antigen-specific systemic IgG antibody titers was observed following each boost (see Supporting Information). At the final bleed (day 50), all mice immunized with **6a**−**6c** elicited high levels of specific serum IgG antibodies (Figure 2). The vaccine structure–activity response relationship was examined by comparing candidates with different carbohydrate core (D-glucose or D-galactose) and peptide epitope (J8 or J14) in reference to polylysine core (**7**), see Figure 2. No significant difference was observed between the levels of J8 or J14-specific serum IgG antibodies elicited by carbohydrate core-based vaccines **6a**−**6c** (administered without adjuvant) and the positive control group (administered J8 or J14 mixed with CFA; **6a** and **6b** vs J8+CFA, $P > 0.05$; **6c** vs J14+CFA, $P > 0.05$), which demonstrates the potential for using carbohydrate cores in the design of future subunit vaccines. Moreover, the levels of specific serum IgG antibodies elicited by vaccine candidates **6a** or **6b** were comparable to the polylysine core-based vaccine **7** (**6a** and **6b** vs **7**, $P > 0.05$). Changing the carbohydrate core did not have an effect on IgG antibody levels (**6a** vs **6b**, $P > 0.05$) as well as changing the peptide antigen (**6a** vs **6c**, $P > 0.05$; Figure 2).

It has been previously demonstrated[31,32] that both J8 and J14 peptides formulated with diphtheria toxoid and proteosomes, respectively, are capable of inducing opsonic antibodies and can protect outbred mice from virulent challenge. Therefore, induction of opsonic antibodies, protective activity, and mucosal immunization by liposaccharide vaccine candidates **6a**−**6c** are now under investigation. Success of this research could result in a vaccine that not only prevents pharyngitis, but could save many lives that are lost through RF and RHD, as it is believed that they are associated with throat infection with GAS.

Conclusion

The danger of rheumatic fever and the increasing number of life-threatening invasive GAS infections in many countries of the world lends support to attempts to develop safe and effective vaccines against GAS infections. The development of self-adjuvanting vaccines is emerging as a promising approach for human vaccination in the future. Our vaccine delivery system employs a glycolipid core acting as an adjuvant and a carrier coupled to multiple copies of immunogenic peptides to induce antibody responses without the coadministration of additional potentially toxic adjuvants. Mice immunized with the described liposaccharide vaccine candidates elicited comparable levels of systemic J8-specific IgG antibodies to mice immunized with a polylysine analogue. Although the fact that changing the carbohydrate core did not have an effect on IgG antibody levels, using different carbohydrate units as vaccine carriers provides a unique possibility to engineer the vaccine system due to the possibility to direct and change positions of peptide epitopes in three-dimensional space and thus providing better peptide recognition by cells and higher immune response. Moreover, the results of this study suggest that this system could potentially be used to develop vaccines against other infectious organisms, and it could be potentially used to produce therapeutic vaccines, which require cellular type immune responses.[33]

Experimental Section

Materials and Methods. All chemicals used in this study were of analytical grade or equivalent unless otherwise stated. *N*-α-Boc-L-amino acids and *p*MBHA resin were purchased from Novabiochem (Läufelfingen, Switzerland), Renanal (Budapest, Hungary) and Peptides International (Louisville, Kentucky). Peptide synthesis grade *N*,*N*′-dimethylformamide (DMF), dichloromethane (DCM), trifluoroacetic acid (TFA), HBTU, and di-*t*-butyldicarbonate were purchased from Auspep (Melbourne, VIC, Australia). HPLC grade acetonitrile (ACN), isopropyl alcohol (IPA), and methanol (MeOH) were supplied by Labscan (Dublin, Ireland) or Honeywell Burdick and Jackson (Morristown, NJ). Hydrogen bromide in acetic acid and ninhydrin were obtained from Merck (Kilsyth, VIC, Australia). Palladium (10%) on carbon was purchased from Lancaster Synthesis (Lancashire, England). Anhydrous hydrofluoric acid (HF) was supplied by BOC gases (Sydney, NSW, Australia). All other reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

A Kel-F HF apparatus (Peptide Institute, Osaka, Japan) was used for HF cleavage. Flash chromatography was performed on silica

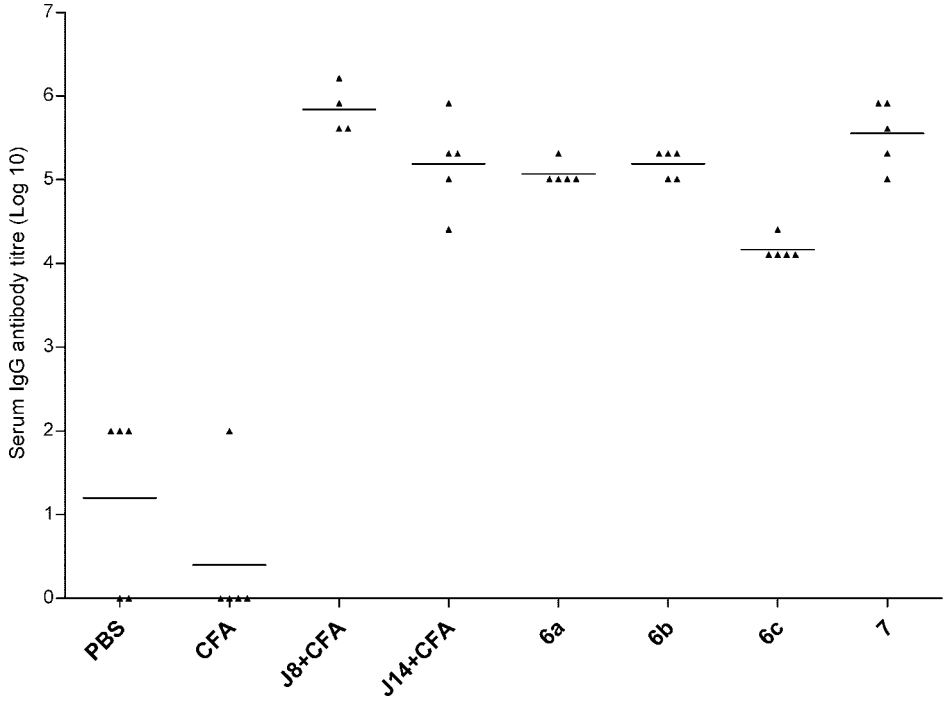


Figure 2. J8 or J14 specific serum IgG antibody titers (log10) at the final bleed (day 50) elicited in response to immunization of B10.BR (H-2^k) mice at the tail base with the carbohydrate core-based vaccine candidates (**6a**−**6c**) and the polylysine core-based vaccine (**7**), as determined by ELISA. Statistical analysis was performed using a one-way ANOVA followed by the *t*-student posthoc test (no significant difference $p > 0.05$ between all vaccine candidates and their positive controls).

gel 60 (230–400 mesh; Lomb scientific, Taren Point, NSW, Australia). Thin layer chromatography (TLC) was performed on Merck (Darmstadt, Germany) aluminum-backed silica gel 60 F254 plates. Detection was achieved using 20% (v/v) H_2SO_4 in EtOH, and compounds were revealed after heating. LC-ESI-MS and ESI-MS were performed on a Perkin-Elmer-Sciex API3000 using the Analyst 1.4 (Applied Biosystems/MDS Sciex, Toronto, Canada) software. Samples (1–10 μL) were injected into ACN−H_2O mobile phases containing 0.1% (v/v) acetic acid and run in positive ion mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on either a Bruker Avance 500 MHz spectrometer (Bruker Biospin, Germany) or a Bruker Avance 300 MHz spectrometer (Bruker Biospin, Germany) at 298 K in deuterated chloroform ($CDCl_3$; Cambridge Isotope Laboratories, Andover, MA). Chemical shifts are reported in parts per million (ppm), with chemical shifts referenced to the residual $CHCl_3$ peak (δ 7.24 ppm). NMR spectra were processed using Topspin 1.3 (Bruker Biospin, Germany). Analytical RP-HPLC was performed using Shimadzu (Kyoto, Japan) instrumentation (Class Vp 6.12 software, SCL-10AVp controller, SIL-10A auto-injector, LC-10AT pump, LC-10AD pump, Waters 486 tunable absorbance detector) using a 0–100% linear gradient of solvent B over 30 min with a 1 mL/min flow rate and detection at 214 nm. Solvent A consisted of 0.1% (v/v) aqueous TFA and solvent B consisted of 90% ACN/H_2O + 0.1% TFA (method 1) or 90% IPA/H_2O + 0.1% TFA (method 2). Separation was achieved on a Vydac (Hesperia, CA) analytical C4 column (214TP54; 5 μm; 4.6 × 250 mm). Preparative RP-HPLC was performed on a Waters Delta 600 system in linear gradient mode using a 10 mL/minute flow rate, with detection at 230 nm. Separations were performed on a Vydac C4 preparative column (214TP1022; 10 μm; 22 × 250 mm).

Synthesis of Carbohydrate Core 2,3,4,6-Tetra-*O*-(cyanoethyl)-β-D-glucopyranosyl Azide (2a). DBU (0.27 mL; 1.76 mmol) and freshly distilled acrylonitrile (2.88 mL; 43.7 mmol) were added to a stirred solution of glucopyranosyl azide[23] **1a** (0.9 g; 4.4 mmol) in acetonitrile (22.5 mL) at RT. After four hours, additional portions of DBU (0.27 mL; 1.76 mmol) and acrylonitrile (2.88 mL; 43.7 mmol) were added, and the reaction was left to stir overnight. The reaction mixture was then condensed (rotary evaporation), and the crude product was purified by flash chromatography on silica (3:1 ethyl acetate–hexane) to give compound **2a** as a white powder (1.2 g; 2.9 mmol; 66% yield). R_f = 0.53 (3:1 EtOAc−hexane). ESI-MS: $C_{18}H_{23}N_7O_5$ (417.1839) *m/z* $[M + H^+]^+$ 418 (calcd, 418.2), $[M + NH_4^+]^+$ 435.6 (calcd, 435.2), $[M + Na^+]^+$ 440.5 (calcd, 440.2). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.59–2.70 (m, 8H, 4 × CH_2CN), 3.11 (t, 1H, H-3, J = 8.8 Hz), 3.36 (t, 1H, H-2, J = 9.1 Hz), 3.42–3.48 (m, 1H, H-5), 3.55 (t, 1H, H-4, J = 9.3 Hz), 3.70–4.18 (m, 10H, 5 × CH_2O), 4.58 (d, 1H, H-1, J = 8.6 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.0, 19.2, 19.3, 19.4, 66.3, 67.2, 67.5, 68.2, 76.4, 76.5, 76.6, 81.8, 83.9, 89.9, 118.0, 118.1, 118.5, 118.6.

6-(2,3,4,6-Tetra-*O*-(cyanoethyl)-β-D-glucopyranosylamino)-6-oxohexanoic Acid Benzyl Ester (3a). Cyanoethylated β-D-glucopyranosyl azide **2a** (4.2 g; 10 mmol) was dissolved in THF (260 mL) and hydrogenated at atmospheric pressure over 10% Pd/C (0.42 g) for 2 h. Upon completion (determined by TLC; eluent EtOAc), adipic acid monobenzyl ester[24] (2.6 g; 11 mmol), HBTU (3.99 g; 10.6 mmol), and DIPEA (2.1 mL; 12 mmol) were added to the reaction mixture and stirred at room temperature under an argon atmosphere for 16 h. The reaction mixture was then filtered through Celite, which was thoroughly washed with methanol afterward. The filtrate was condensed (rotary evaporation), dissolved in EtOAc (200 mL), and washed with 5% aqueous HCl (3 × 100 mL), saturated aqueous $NaHCO_3$ (2 × 100 mL), and saturated aqueous NaCl (50 mL), dried ($MgSO_4$), and condensed (rotary evaporation). Pure product **3a** (3.17 g; 5.19 mmol; 52% yield) was obtained as colorless oil after flash chromatography on silica (EtOAc). R_f = 0.4 (EtOAc). ESI-MS: $C_{31}H_{39}N_5O_8$ (609.2875) *m/z* $[M + H^+]^+$ 610.7 (calcd, 610.3), $[M + NH_4^+]^+$ 627 (calcd, 627.3), $[M + Na^+]^+$ 632 (calcd, 632.3). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.62–1.7 (m, 4H, CH_2CH_2), 2.25 (t, 2H, CH_2CON, J = 6.8 Hz), 2.36 (t, 2H, CH_2COO, J = 6.9 Hz), 2.50–2.66 (m, 8H, 4 × CH_2CN), 3.23 (t, 1H, $H6_a$, J = 9.0 Hz), 3.36–3.42 (m, 2H, CH_2O), 3.51 (t, 1H, $H6_b$, J = 9.5 Hz), 3.59–4.15 (m, 10H, 3 × CH_2O, 4 × CHO), 5.03–5.06 (m, 1H, H-1), 5.08 (s, 2H, CH_2OPh), 6.47 (d, 1H, NH, J = 9.5 Hz), 7.33 (br s, 5H, 5 × Ar-H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.0, 19.2, 19.3, 19.4, 24.2, 24.5, 33.6, 36.1, 66.3, 67.4 (2 ×), 67.6, 68.0,

68.9, 74.0, 75.9, 76.9, 78.8, 81.5, 85.0, 118.4, 118.5, 118.6 (2×), 118.7, 128.1, 128.2, 128.6, 135.9, 173.4, 173.6.

6-(2,3,4,6-Tetra-*O*-(butoxycarbonylaminopropyl)-β-D-glucopyranosylamino)-6-oxohexanoic Acid (5a). Compound **3a** (1.0 g; 1.6 mmol), cobalt chloride hexahydrate (3.1 g; 13 mmol), and di-*tert*-butyl dicarbonate (1.8 g; 8.3 mmol) were dissolved in methanol (45 mL) and cooled to 0 °C (iced water). Aliquots of sodium borohydride (2.48 g; 65.5 mmol) were then added to the mixture over a 30 min period, and subsequently, the reaction was stirred for 4 h at RT. Upon completion of the Boc-protection (determined by TLC; eluent EtOAc), the mixture was filtered and evaporated under reduced pressure to give crude compound **4a** (1.5 g), which was then dissolved in dry THF (80 mL), degassed, and hydrogenated over 10% Pd/C (120 mg) with stirring at room temperature under H_2(g) overnight. After evaporation, the crude product was dissolved in 10% methanol in $CHCl_3$, filtered through Celite, concentrated, and purified by flash chromatography on silica using the same eluent to give **5a** (0.92 g; 0.99 mmol; 88% yield) as colorless foam. R_f = 0.3 (10% methanol in $CHCl_3$). ESI-MS: $C_{44}H_{81}N_5O_{16}$ (935.5757) *m/z* $[M + H^+]^+$ 936.9 (calcd, 936.6), $[M + Na^+]^+$ 959 (calcd, 958.6), $[MH\text{-}BOC]^+$ 837 (calcd, 836.6).^{1}H NMR (300 MHz, $CDCl_3$): δ 1.42 (br s, 36H, 4 × CMe_3), 1.55–1.83 (m, 12H, 6 × CH_2), 2.25–2.38 (m, 4H, 2 × CH_2CO), 2.90–3.95 (m, 22H, 4 × CH_2N, 5 × CH_2O, 4 × CHO), 4.67 (br s, 1H, H-1), 4.77–5.22 (m, 5H, 5 × NH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 24.3, 24.6, 24.9, 28.2, 28.4, 29.7, 29.9, 30.4, 30.6, 30.7, 33.4, 36.1, 38.2, 38.7, 41.0, 65.3, 66.0, 69.2, 70.2, 70.5, 70.8, 71.0, 71.4, 76.3, 77.2, 78.0, 79.0, 79.6, 79.8, 81.6, 85.2, 127.0, 127.6, 128.5, 156.0, 156.4, 156.6, 173.8.

Synthesis of Peptides and Lipopeptides. Lipopeptide vaccines (**6a**−**6c**) were synthesized by stepwise solid-phase peptide synthesis on *p*MBHA resin (0.4 mmol NH_2/g; 0.2 mmol scale), using HBTU/DIPEA in situ neutralization and Boc-chemistry.[29] The *p*MBHA resin was swollen in anhydrous DMF (10 mL/g of resin) for over an hour followed by neutralization of the hydrochloride salt of the resin-bound amine by 3 × 15 min treatments with 10% (v/v) DIPEA in anhydrous DMF (10 mL/g of resin). Coupling cycles consisted of Boc-deprotection (2 × 1 min treatments with neat TFA), a DMF flow-wash (1 min), and couplings with 4.1 equiv of preactivated amino acid (20–60 min). Amino acid activation (1 min) was achieved by dissolving amino acids (0.82 mmol; 4.1 equiv) in a 0.5 M HBTU-DMF solution (1.6 mL; 0.8 mmol; 4 equiv), followed by the addition of DIPEA (212 μL; 1.22 mmol). Coupling efficiency was determined using the quantitative ninhydrin test,[30] with couplings repeated, if necessary, to give coupling yields greater than 99.7%. Where coupling yields of greater than 99.7% could not be achieved after three couplings, the remaining amines were blocked by acetylation. This was achieved by treating the resin with acetic anhydride (0.5 mL; 5.29 mmol), DIPEA (0.47 mL; 2.70 mmol), and DMF (14 mL) for 5 min, and then repeating the treatment for 15 min. When coupling to a proline residue, the chloranil test[34] was used to qualitatively assess coupling yields to the praline secondary amine. Boc-amino acids with the following side-chain protection were utilized for the synthesis: Arg(Tos), Asp(OcHx), Gln(Xan), Glu(OcHx), Lys(2-Cl-Z), Lys(Boc), Ser(Bzl). After the coupling of glutamine, the resin was washed with DCM before and after Boc-deprotection to prevent high-temperature-catalyzed pyrrolidone carboxylic acid (Pca) formation.[29] Synthetic racemic 2-(*t*-butoxycarbonylamino)-D,L-dodecanoic acid (Boc-C_{12}-OH)[35] was utilized for lipid core synthesis. The lipid core was synthesized by coupling Boc-Gly OH to *p*MBHA resin, followed by two cycles of Boc-C_{12}-OH, then Boc-Gly OH and Boc-C_{12}-OH. Carbohydrate cores (**4a**, **4b**) were attached to the lipid core on *p*MBHA resin (0.25 mmol NH_2/g; 0.1 mmol scale) using the same procedure as used for the coupling of amino acids. First, activation of the carbohydrate cores **4a** and **4b** (1 min) was achieved by dissolving **4a** and **4b** (0.2 mmol; 2 equiv) in a 0.5 M HBTU-DMF (0.4 mL; 0.2 mmol; 2 equiv) followed by the addition of DIPEA (70 μL; 0.4 mmol). Then, the activated carbohydrate cores were added to the lipid core conjugated *p*MBHA resin and coupled for 10 h. Four peptide attachment points were provided by the carbohydrate (**4a** or **4b**) or poly lysine cores which were coupled to the lipid core. Following Boc-deprotection, four copies of the peptide antigen, J8 (QAEDKVKQ**SREAKKQVEKAL**KQLEDKVQ) or J14 (KQAEDKVK**ASREAKKQVEKALE**QLEDKVK), were synthesized by stepwise solid phase peptide synthesis. After the synthesis of each peptide was complete, the amino-terminal Boc protecting groups were removed with TFA. The peptidyl-resins were then thoroughly washed with DMF, DCM, and MeOH and dried under vacuum prior to cleavage using anhydrous HF. HF cleavage (10 mL HF/g resin) was performed at 0 °C for two hours in the presence of 5% (v/v) *p*-cresol. Following the cleavage, the HF was removed under reduced pressure, and the peptides were precipitated in ice-cold diethyl ether, filtered, dissolved in 40% ACN/H_2O + 0.1% TFA, and lyophilized. The lyophilized products were purified by preparative RP-HPLC on a C4 column using a 10–100% solvent B gradient over 60 min. The collected fractions were analyzed by RP-HPLC and LC-ESI-MS and combined to give **6a** (34 mg), **6b** (24 mg), and **6c** (38 mg) following lyophilization. Compound **7** has been prepared and characterized as described previously.[36]

Compound **6a**: Purification yield, 57%; HPLC, t_R = 18.6 min (method 1, C4 column), t_R = 17.2 min (method 2, C4 column); LC-ESI-MS, $[M + 13H^+]^{13+}$ *m/z* 1101.2 (calcd, 1101.0), $[M + 14H^+]^{14+}$ *m/z* 1020.3 (calcd, 1022.4), $[M + 15H^+]^{15+}$ *m/z* 952.1 (calcd, 954.3), $[M + 16H^+]^{16+}$ *m/z* 892.7 (calcd, 895.0), $[M + 17H^+]^{17+}$ *m/z* 839.9 (calcd, 842.2); MW 14299.5 g/mol.

Compound **6b**: Purification yield, 42%; HPLC, t_R = 20.0 min (method 1, C4 column), t_R = 16.9 min (method 2, C4 column); LC-ESI-MS, $[M + 18H^+]^{18+}$ *m/z* 795.1 (calcd, 795.4), $[M + 19H^+]^{19+}$ *m/z* 755.4 (calcd, 753.6), $[M + 21H^+]^{21+}$ *m/z* 684.4 (calcd, 682.0), $[M + 23H^+]^{23+}$ *m/z* 624.5 (calcd, 623.0), $[M + 24H^+]^{24+}$ *m/z* 596.5 (calcd, 596.0), $[M + 26H^+]^{26+}$ *m/z* 554.6 (calcd, 551.0); MW 14299.5 g/mol.

Compound **6c**: Purification yield, 63%; HPLC, t_R = 18.7 (method 1, C4 column), t_R = 17.1 min (method 2, C4 column); LC-ESI-MS, $[M + 12H^+]^{12+}$ *m/z* 1216.7 (calcd, 1214.1), $[M + 15H^+]^{15+}$ *m/z* 973.5 (calcd, 975.3), $[M + 17H^+]^{17+}$ *m/z* 862.2 (calcd, 859.0), $[M + 18H^+]^{18+}$ *m/z* 809.9 (calcd, 811.0); MW 14587.92 g/mol.

Immunization Protocol. All protocols were approved by the Queensland Institute of Medical Research Animal Ethics Committee and were carried out according to Australian National Health and Medical Research Council guidelines. Female B10.BR (H-2^k) mice (4–6 week-old, Animal Resource Centre, Perth, Western Australia, Australia) were used for immunization. Mice (n = 5/group) were primed subcutaneously at the tail base with 30 μg of immunogens (**6a**−**6c**) in a total volume of 50 μL of sterile-filtered PBS. Mice received a further three boosts (30 μg of immunogens in a total volume of 50 μL of PBS) at 10 day intervals (days 21, 31, and 41) with. Two positive control groups received 30 μg of either J8 or J14 emulsified in a total volume of 50 μL of CFA/PBS (1:1). Two negative controls were administered a 50 μL of either CFA/PBS (1:1) or PBS alone. All controls were boosted with 50 μL of PBS. Blood was collected from the tail artery of each mouse one day prior to each injection and nine days after the last immunization. The blood was left to clot at 37 °C for 1 h and then centrifuged for 10 min at 3000 RPM to remove clots. Sera were then stored at −20 °C.

Enzyme-Linked Immunosorbent Assay (ELISA). Determination of serum IgG antibodies against the J8 or J14 epitopes was performed using a previously described ELISA.[37] Optical density was read at 450 nm in a microplate reader following the addition of peroxidase-conjugated goat antimouse IgG, and *O*-phenylenediamine. The antibody titer was defined as the lowest dilution with an optical density more than three standard deviations greater than the mean absorbance of control wells containing normal mouse serum.

Statistics. Statistical analysis of antibody titers was performed using a one-way ANOVA followed by the *t*-student posthoc test. GraphPad Prism 4 software was used for statistical analysis, with $p < 0.05$ taken as statistically significant.

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Supporting Information Available: Immunological data for liposaccharide vaccine candidates **6a**−**6c** and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) O'Hagan, D. T.; Rappuoli, R. Novel approaches to pediatric vaccine delivery. *Adv. Drug Delivery Rev.* **2006**, *58* (1), 29–51.
(2) Olive, C.; Toth, I.; Jackson, D. Technological advances in antigen delivery and synthetic peptide vaccine developmental strategies. *Mini-Rev. Med. Chem.* **2001**, *1*, 429–438.
(3) Mesa, C.; Fernandez, L. E. Challenges facing adjuvants for cancer immunotherapy. *Immunol. Cell Biol.* **2004**, *82* (6), 644–650.
(4) Bergeon, J. A.; Toth, I. Enhancement of oral drug absorption—Effect of lipid conjugation on the enzymatic stability and intestinal permeability of l-Glu-l-Trp-NH_2. *Bioorg. Med. Chem.* **2007**, *15* (22), 7048–7057.
(5) Falconer, R. A.; Toth, I. Design, synthesis and biological evaluation of novel lipoamino acid-based glycolipids for oral drug delivery. *Bioorg. Med. Chem.* **2007**, *15* (22), 7012–7020.
(6) Pialoux, G.; Gahery-Segard, H.; Sermet, S.; Poncelet, H.; Fournier, S.; Gerard, L.; Tartar, A.; Gras-Masse, H.; Levy, J. P.; Guillet, J. G. Lipopeptides induce cell-mediated anti-HIV immune responses in seronegative volunteers. *AIDS* **2001**, *15* (10), 1239–1249.
(7) Livingston, B. D.; Crimi, C.; Grey, H.; Ishioka, G.; Chisari, F. V.; Fikes, J.; Grey, H.; Chesnut, R. W.; Sette, A. The hepatitis B virus-specific CTL responses induced in humans by lipopeptide vaccination are comparable to those elicited by acute viral infection. *J. Immunol.* **1997**, *159* (3), 1383–1392.
(8) Lechmann, M. P. D.; Jake Liang, T. M. D. Vaccine development for hepatitis C. *Semin. Liver Dis.* **2000**, (02), 211–226.
(9) Benmohamed, L.; Thomas, A.; Bossus, M.; Brahimi, K.; Wubben, J.; Gras-Masse, H.; Druilhe, P. High immunogenicity in chimpanzees of peptides and lipopeptides derived from four new Plasmodium falciparum pre-erythrocytic molecules. *Vaccine* **2000**, *18* (25), 2843–2855.
(10) Tam, J. P. Synthetic peptide vaccine design: Synthesis and properties of a high-density multiple antigenic peptide system. *Proc. Natl. Acad. Sci. U.S.A* **1988**, *85* (15), 5409–5413.
(11) Herrera, S.; de Plata, C.; Gonzalez, J. M.; Perlaza, B. L.; Bettens, F.; Corradin, G.; Arevalo-Herrera, M. Antigenicity and immunogenicity of multiple antigen peptides (MAP) containing P. vivax CS epitopes in Aotus monkeys. *Parasite Immunol.* **1997**, *19* (4), 161–170.
(12) Hayman, W. A.; Toth, I.; Flinn, N.; Scanlon, M.; Good, M. F. Enhancing the immunogenicity and modulating the fine epitope recognition of antisera to a helical group A streptococcal peptide vaccine candidate from the M protein using lipid-core peptide technology. *Immunol. Cell Biol.* **2002**, *80* (2), 178–187.
(13) Moyle, P. M.; Olive, C.; Ho, M. F.; Good, M. F.; Toth, I. Synthesis of a highly pure lipid core peptide based self-adjuvanting triepitopic group A streptococcal vaccine, and subsequent immunological evaluation. *J. Med. Chem.* **2006**, *49* (21), 6364–6370.
(14) Horvath, A.; Olive, C.; Karpati, L.; Sun, H. K.; Good, M.; Toth, I. Toward the development of a synthetic group A streptococcal vaccine of high purity and broad protective coverage. *J. Med. Chem.* **2004**, *47* (16), 4100–4104.
(15) Olive, C.; Hsien, K.; Horvath, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. Protection against group A streptococcal infection by vaccination with self-adjuvanting lipid core M protein peptides. *Vaccine* **2005**, *23* (17–18), 2298–2303.
(16) Olive, C.; Hsien Kuo, S.; Mei-Fong, H.; Dyer, J.; Horvath, A.; Toth, I.; Good, M. F. Intranasal administration is an effective mucosal vaccine delivery route for self-adjuvanting lipid core peptides targeting the group A streptococcal M protein. *J. Infect. Dis.* **2006**, *194* (3), 316–324.
(17) Carapetis, J. R.; Steer, A. C.; Mulholland, E. K.; Weber, M. The global burden of group A streptococcal diseases. *Lancet Infect. Dis.* **2005**, *5* (11), 685–694.
(18) Bisno, A. L.; Brito, M. O.; Collins, C. M. Molecular basis of group A streptococcal virulence. *Lancet Infect. Dis.* **2003**, *3* (4), 191–200.
(19) McArthur, J. D.; Walker, M. J. Domains of group A streptococcal M protein that confer resistance to phagocytosis, opsonization and protection: Implications for vaccine development. *Mol. Microbiol.* **2006**, *59* (1), 1–4.
(20) Olive, C.; Batzloff, M. R.; Toth, I. Lipid core peptide technology and group A streptococcal vaccine delivery. *Exp. Rev. Vac.* **2004**, *3* (1), 43–58.
(21) Cunningham, M. W. Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev.* **2000**, *13* (3), 470–511.
(22) Hayman, W. A.; Brandt, E. R.; Relf, W. A.; Cooper, J.; Saul, A.; Good, M. F. Mapping the minimal murine T cell and B cell epitopes within a peptide vaccine candidate from the conserved region of the M protein of group A streptococcus. *Int. Immunol.* **1997**, *9* (11), 1723–1733.
(23) Gyorgydeak, Z.; Szilagyi, L.; Paulsen, H. Synthesis, Structure and Reactions of Glycosyl Azides. *J. Carbohydr. Chem.* **1993**, *12* (2), 139–163.
(24) English, A. R.; Girard, D.; Jasys, V. J.; Martingano, R. J.; Kellogg, M. S. Orally effective acid prodrugs of the beta-lactamase inhibitor sulbactam. *J. Med. Chem.* **1990**, *33* (1), 344–347.
(25) McGeary, R. P.; Jablonkai, I.; Toth, I. Carbohydrate-based templates for synthetic vaccines and drug delivery. *Tetrahedron* **2001**, *57* (41), 8733–8742.
(26) Peter, M.; Moyle, C. O. M. F. G. I. T. Method for the synthesis of highly pure vaccines using the lipid core peptide system. *J. Pept. Sci.* **2006**, *12* (12), 800–807.
(27) Moyle, P. M.; Olive, C.; Karpati, L.; Barozzi, N.; Ho, M. F.; Dyer, J.; Sun, H. K.; Good, M.; Toth, I. Synthesis and immunological evaluation of M protein targeted tetra-valent and tri-valent group A streptococcal vaccine candidates based on the lipid-core peptide system. *Int. J. Pept. Protein Res.* **2006**, *12* (3), 317–326.
(28) Horváth, A.; Olive, C.; Wong, A.; Clair, T.; Yarwood, P.; Good, M.; Toth, I. A lipophilic adjuvant carrier system for antigenic peptides. *Lett. Pept. Sci.* **2001**, *8* (3), 285–288.
(29) Schnolzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. In situ neutralization in Boc-chemistry solid-phase peptide-synthesis—Rapid, high-yield assembly of difficult sequences. *Int. J. Pept. Protein Res.* **1992**, *40* (3–4), 180–193.
(30) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem.* **1981**, *117* (1), 147–157.
(31) Batzloff, M. R.; Yan, H. R.; Davies, M. R.; Hartas, J.; Lowell, G. H.; White, G.; Burt, D. S.; Leanderson, T.; Good, M. F. Toward the development of an antidisease, transmission-blocking intranasal vaccine for group A streptococcus. *J. Infect. Dis.* **2005**, *192* (8), 1450–1455.
(32) Batzloff, M. R.; Hayman, W. A.; Davies, M. R.; Zeng, M.; Pruksakorn, S.; Brandt, E. R.; Good, M. F. Protection against group A streptococcus by immunization with J8-diphtheria toxoid: Contribution of J8 and diphtheria toxoid-specific antibodies to protection. *J. Infect. Dis.* **2003**, *187* (10), 1598–1608.
(33) Moyle, P. M.; Olive, C.; Ho, M. F.; Pandey, M.; Dyer, J.; Suhrbier, A.; Fujita, Y.; Toth, I. Toward the development of prophylactic and therapeutic human papillomavirus type-16 lipopeptide vaccines. *J. Med. Chem.* **2007**, *50* (19), 4721–4727.
(34) Christensen, T. Qualitative test for monitoring coupling completeness in solid-phase peptide-synthesis using chloranil. *Acta Chem. Scand.* **1979**, *33* (10), 763–766.
(35) Gibbons, W. A.; Hughes, R. A.; Charalambous, M.; Christodoulou, M.; Szeto, A.; Aulabaugh, A. E.; Mascagni, P.; Toth, I. Lipidic peptides 0.1. Synthesis, resolution and structural elucidation of lipidic amino-acids and their homo-oligomers and heterooligomers. *Liebigs Ann. Chem.* **1990**, (12), 1175–1183.
(36) Olive, C.; Batzloff, M. R.; Horvath, A.; Wong, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. A lipid core peptide construct containing a conserved region determinant of the group A streptococcal M protein elicits heterologous opsonic antibodies. *Infect. Immun.* **2002**, *70* (5), 2734–2738.
(37) Pruksakorn, S.; Galbraith, A.; Houghten, R. A.; Good, M. F. Conserved T-cell and B-cell epitopes on the M-protein of group-a streptococci—Induction of bactericidal antibodies. *J. Immunol.* **1992**, *149* (8), 2729–2735.

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