Published in final edited form as:
Chembiochem. 2009 December 14; 10(18): 2893–2899. doi:10.1002/cbic.200900587.

Synthesis, Conjugation, and Immunological Evaluation of the Serogroup 6 Pneumococcal Oligosaccharides

Dr. Archana R. Parameswar[a], **Dr. In Ho Park**[b], **Dr. Rina Saksena**[c], **Dr. Pavol Kováč**[c], **Prof. Moon H. Nahm**[b], and **Prof. Alexei V. Demchenko**[*,a]

[a]Department of Chemistry and Biochemistry, University of Missouri, One University Boulevard, St. Louis, MO 63121 (USA)

[b]Departments of Pathology and Microbiology, University of Alabama at Birmingham, 845 19th Street South, BBRB 614, Birmingham, AL 35294 (USA)

[c]NIDDK, LBC, National Institutes of Health, Bethesda, MD 20892-0815 (USA)

Abstract

The first synthesis of the newly discovered oligosaccharide of pneumococcal serotype 6C and its spacer-containing analogue is reported. Conjugation of the spacer-containing oligosaccharides of pneumococcal saccharides 6A, 6B, 6C and derivatives thereof with bovine serum albumin (BSA) protein carrier was carried out by using squaric-acid approach to obtain the oligosaccharide–protein conjugates in excellent yields. The conjugates have been tested with a rabbit antiserum pool (Pool B) used for pneumococcal serotyping. The results showed that synthetic carbohydrate conjugates express epitopes found in native capsular polysaccharides of serotypes 6A, 6B, and 6C.

Keywords

carbohydrates; glycoconjugates; oligosaccharides; *Streptococcus pneumoniae*; synthetic vaccines

Introduction

Streptococcus pneumoniae (SPn) is one of the most common Gram-positive pathogens that cause many life-threatening diseases. These pathogenic pneumococci display one of many structurally diverse polysaccharide (PS) capsules, which in turn serve as a cover against external influences and render the bacterium more or less resistant to nonspecific host defense.[1] Amongst 91 elucidated SPn serotypes,[2, 3] SPn6A and SPn6B are nearly equally important causes of bacterial infections[4] that account for 4.7 and 7%, respectively, of all cases of invasive pneumococcal disease in the US. Also, on the world-wide scale, the SPn6-serogroup has been consistently ranked within the top three causes of invasive pneumococcal disease.[5] This high medical importance stimulated extensive structural studies to establish the structures of capsular PSs of both SPn6A and SPn6B.[6–8]

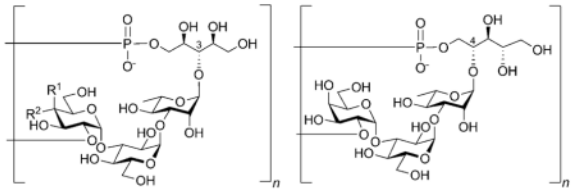
Recently, two individual subtypes of pneumococci with notable genetic and structural differences[9] have been identified within the serotype that was previously known as SPn6A.[3] Structural studies of these “two SPn6A isolates” showed that the major subtype produces a capsular polysaccharide with galactose–glucose–rhamnose–ribitol phosphate as

Fax: (+1) 1-314-516-5342 demchenkoa@umsl.edu.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900587.

the repeating unit, the sequence that was previously referred to as SPn6A.[3] The minor subtype was found to be composed of the previously unknown sequence of glucose–glucose–rhamnose–ribitol phosphate as the repeating unit. Considering serological and structural similarities to other SPn6-serogroup PSs, this minor subtype was designated as the new serotype SPn6C, the third member of SPn6-serogroup, and the 91st pneumococcal serotype.[3]

Since the pneumococci bacterial cell is surrounded by a PS capsule, which makes the organism resistant to phagocytosis, preventive vaccination is a viable tool against the bacterial invasion.[10, 11] A common approach to vaccine development is based on the natural PS isolates used either neat[12, 13] or as conjugates with a protein carrier.[14–17] Usually, serotype-specific antibodies are formed in response to vaccination with a PS or its conjugate. However, due to similarity in the carbohydrate-core structures of SPn6A and SPn6B (**1a** and **1b**, positions 3 and 4, respectively), which differ in the way the ribitol unit is connected, only hydrolytically more stable and, hence, the more easily accessible natural isolate of SPn6B was selected and included in all currently licensed multicomponent vaccines.[7, 13, 15,18] Although it was suggested that the SPn6B-based vaccines produce the 6B-specific antibodies that cross-react with SPn6A at a much lower rate than that of SPn6B,[4, 19–21] this cross-reactivity-based approach has been applied to preventive vaccination for many years.



Evidently, there are two structural variants between SPn6B (**1b**), and SPn6C (**1c**)—namely galactose vs. glucose in addition to the ribitol attachment—currently used in vaccines. Due to this more significant structural difference, the cross-protection against SPn6C may be inadequate; expectedly much weaker than that of SPn6A. Arguably, this may result in an outbreak of SPn6C-derived pneumococcal infections,[3] just as it previously occurred for serotype SPn19A. Indeed, the first results were very worrisome: following the introduction of the polysaccharide conjugate vaccine containing SPn6B, the occurrence of SPn6A has decreased, but the prevalence of SPn6C has increased.[22, 23] Therefore, the importance of a new, versatile vaccine that would provide efficient cross-protection against the entire serogroup 6 has come to the forefront.

In an effort to investigate a new experimental pneumococcal conjugate vaccine based on chemically synthesized carbohydrates, we have already reported the efficient chemical synthesis of oligosaccharides of SPn6A, SPn6B, and structural variants thereof.[24, 25] In continuation of our recent studies towards the synthesis of pneumococcal oligosaccharides, herein we report the first synthesis of the repeating unit of the newly discovered serotype SPn6C. The BSA-conjugates of various SPn6-serogroup oligosaccharides were also obtained and their antigenicities were tested in rabbit antisera specific for SPn6-serogroup capsular polysaccharides. We expect that the studies reported herein will complement previous extensive studies of synthetic oligosaccharides SPn6B as well as mimetics and conjugates thereof reported by Kamerling, Vliegenthart and their co-workers.[20, 26–30]

Results and Discussion

Oligosaccharide synthesis

SPn6C repeating unit represents a complex pseudotetrasaccharide[31] **2** (Scheme 1), which in the natural polysaccharide is connected through a phosphate (5→2‴) linkage (see **1a**–**c**). In our previous work, we demonstrated that the pseudo-di-, -tri-and -tetrasaccharides of SPn6A and SPn6B series could be synthesized by using a selective activation strategy in overall excellent yields and complete stereoselectivity.[24, 25] Conceptually similar approaches were planned for application to the synthesis of the repeating unit of SPn6C (**2**) and its spacer-equipped analogue **3**, suitable for the conjugation to a protein carrier. Known building blocks **4**–**10** have been selected for the syntheses of **2** and **3** as highlighted in Scheme 1.

Two approaches have been envisaged for the synthesis of pseudotetrasaccharide **2**. As depicted in Scheme 2, the first method involved glycosidation of the SBox glucosyl donor **4**[32, 33] with the SEt disaccharide acceptor **11**, obtained from building blocks **5**[34] and **8**,[35] as previously reported.[24] The trisaccharide **12**, obtained in 60 % yield with complete α-selectivity, was then coupled with ribitol acceptor **9**[24] in the presence of *N*-iodosuccinimide (NIS) and trimethylsilyl trifluoromethane-sulfonate (TMSOTf). The resulting pseudotetrasaccharide derivative **13 a**, obtained in 89 % yield with complete α-stereoselectivity, was then subjected to a two-step deprotection. Thus, sequential deacylation (NaOMe in MeOH) and hydrogenation using Pd/C in EtOH afforded the pseudotetrasaccharide **2** in 85 % yield.

The pseudotetrasaccharide **2** was also obtained through a more efficient convergent manner as depicted in Scheme 3. Glucosyl donor **4**, which bears the SBox leaving group was selectively activated in the presence of the thioglycoside acceptor **6**.[36] This reaction was carried out in dichloromethane by using AgOTf as promoter, and the disaccharide **14** was obtained in 75 % yield with complete α-stereoselectivity. For the synthesis of the disaccharide intermediate **15**, rhamnose building block **7**[37] was glycosidated with ribitol acceptor **9** to afford the requisite disaccharide in 65 % yield. To complete the assembly, the disaccharide donor **14** was coupled with the disaccharide acceptor **15** in the presence of NIS and a catalytic amount of TfOH. The resulting derivative **13 b**, obtained in 66 % yield, was deprotected as described above to afford pseudotetrasaccharide **2** in 75 % yield.

Synthesis of the spacer-containing pseudotetrasaccharide **3** was accomplished from building blocks **4**, **6**, **7**, and **10**,[25] as described for the second synthesis of compound **2** (Scheme 3). In the key glycosylation step, the disaccharide donor **14** was coupled with the disaccharide acceptor **16**, obtained from building blocks **7** and **10**, as previously reported.[25] This glycosylation step was accomplished in the presence of NIS/TfOH promoter system, and the requisite pseudotetrasaccharide **17** was obtained with complete 1,2-*cis* stereo-selectivity in 74 % yield. The deprotection of **17** was carried out by deacylation using a 1M solution of NaOMe in methanol followed by hydrogenation under acidic conditions[25] to afford the target tetrasaccharide **3** in 72 % yield.

Conjugation

It is well-known that capsular PS-based vaccines are T-cell independent antigens and hence the immune response to such “plain” PS-based vaccines is very poor in children younger than eighteen months of age, the elderly, and those individuals whose immune system is compromised.[14] Low-molecular-weight oligosaccharides are even less immunogenic, but such haptens can be transferred into T-cell-dependent antigens by conjugation to proteins. Hence, in order to enhance the immunogenicity of the synthetic oligosaccharides of SPn6A–C and, in turn, their efficiency in subsequent immunization studies, we decided to convert

the synthesized oligosaccharides into polyvalent neoglycoconjugates. There are many existing methods for conjugation of carbohydrates to proteins.[2, 38] The method we chose for the synthesis of neoglycoconjugates of SPn6 oligosaccharides is based on the selective reaction of amines with squaric acid diesters, which was originally described by Tietze.[39] This approach involves a two-step, pH-dependent coupling of two different amines. First, the free amino group of the spacer moiety in oligosaccharide **3** and in previously synthesized oligosaccharides **18**–**23**[25] (Scheme 4), and second is a free amino group of a protein. The amine of the spacer was first reacted with one of the ester groups of 3,4-di-ethoxy-3-cyclobutene-1,2-dione (**24**, squaric acid diethyl ester) at pH 7 to form the corresponding monoamides **25**–**31**, which were purified by reverse phase chromatography.

The remaining ester group of monoamides **25**–**31** was subsequently coupled with amino groups of the protein in a much more basic medium (pH 9). Bovine serum albumin (BSA, molecular mass 66 430 Da) has 59 lysine residues[40, 41] each bearing a terminal amino group of which about 30–35 are accessible for chemical coupling.[42] Hence, BSA was chosen as a model protein carrier for the development of SPn6 neoglyco-conjugates, which were prepared through controlled formation (borate buffer, pH 9) of 1,2-bisamides of the squaric acid **32**–**38** from the corresponding monoamides **25**–**31** (Scheme 4, Table 1). For preliminary studies, our aim was to obtain the conjugates with oligosaccharide/BSA ratio of 4–8:1. The progress of the reaction was monitored by surface-enhanced laser desorption ionization time of flight (SELDI-TOF) mass spectrometry.[43] When the desired loading was achieved, the conjugation reactions were terminated by neutralization followed by ultrafiltration. The remaining material was lyophilized to obtain the pure SPn6–BSA conjugates, as follows: tetrasaccharide SPn6C–BSA (**32**), disaccharide of SPn6A/6C–BSA (**33**), trisaccharide of SPn6A/6C (**34**), tetrasaccharide SPn6A–BSA (**35**), disaccharide of SPn6B–BSA (**36**), trisaccharide of SPn6B (**37**), and tetrasaccharide SPn6B–BSA (**38**).

Biological tests

In order to determine whether chemically synthesized neoglycoconjugates express the epitope(s) recognized by anti-sero-group 6 antibodies, a rabbit antiserum (Pool B, Statens Serum Institut, Copenhagen, Denmark) was tested for its binding to the synthetic oligosaccharide–BSA conjugates in ELISA. Pool B contains antibodies to serotypes 6A, 6B, and 6C and is used to serotype pneumococci.[44] For this experiment, microplates were coated with SPn saccharide–BSA conjugates (**32**–**38**) or various natural pneumococcal capsular PSs (2 μg mL^{-1}). Interestingly, it was determined that Pool B bound to three SPn tetrasaccharide–BSA conjugates: tetrasaccharide SPn6C–BSA (**32**), tetrasaccharide SPn6A–BSA (**35**), and tetrasaccharide SPn6B–BSA (**38**, Figure 1), whereas it neither bound SPn di- and trisaccharide–BSA conjugates (**33**, **34**, **36**, and **37**) nor plain BSA. This suggested that the immobilized tetrasaccharides are the minimal oligosaccharide sequences required to express sero-group 6 epitopes.

In order to demonstrate that the SPn6A and SPn6C tetrasaccharide–BSA conjugates **35** and **32**, respectively, express serogroup SPn6 epitopes found in free PSs, we investigated whether free natural SPn6A, SPn6B, or SPn6C PSs could inhibit the binding of rabbit antibodies to the tetrasaccharide–BSA conjugates. We found that free SPn6A and SPn6C PSs can inhibit the binding, whereas free SPn6B PS could not inhibit the binding as depicted in Figure 2. This result suggested that 6A–and 6C–tetrasaccharide conjugates have epitopes found in free 6A and 6C PS, and some of the epitopes are different from those of free 6B PS.

To test immunogenicity of synthetic carbohydrate conjugates, mice (five per group) were intraperitoneally immunized with 10 μg of synthetic SPn oligosaccharide–BSA conjugates at week 0, week 7 and week 10, and were bled at week 0 (preimmune) and week 11 (postimmune). Preimmune sera (open symbols) and postimmune sera (closed symbols) were

tested at 1:100 dilution for binding to ELISA plates coated with different pneumococcal PS, and the OD value was determined at 405 nm after incubation for 3 h at RT (Figure 3). Post-immune sera from mice immunized with BSA did not display binding to any pneumococcal PSs. In contrast, sera from mice immunized with SPn6A-tetra BSA (**35**) or SPn6B-tetra BSA (**38**) showed antibodies binding to SPn6A, SPn6B, or SPn6C PSs, as well as to two unrelated pneumococcal PSs (serotype SPn14 or pneumococcal cell wall PS). In order to see the binding specificity in more detail, one postimmune serum in each test group was allowed to bind to various pneumococcal PSs in the presence of free capsular PSs that acted as inhibitors. We observed that postimmune sera did not bind to serotype SPn6A PS and SPn6C PS not only in the presence of serogroup SPn6 capsules but also in the presence of serotype SPn2 PS, sero-type SPn14 PS, and cell wall PS (data not shown). This suggested that the mouse antibodies developed in response to the synthetic oligosaccharide–BSA conjugate immunization are poly-reactive and are not specific for pneumococcal capsule of serotypes SPn6A, SPn6B, or SPn6C.

Conclusions

In conclusion, the first-reported chemical synthesis of the 91st pneumococcal serotype SPn6C (discovered in 2007)[3] was accomplished by using an efficient and convergent approach. A range of neoglycoconjugates of SPn6A, SPn6B, and SPn6C series with the carrier protein (BSA) was also obtained and the glycoconjugation process was monitored by SELDI-TOF mass spectrometry. The conjugates have been tested with a rabbit antiserum pool (Pool B), which is used for pneumococcal sero-typing. The results showed that synthetic carbohydrate conjugates express epitopes found in native capsular PSs of sero-types SPn6A, SPn6B, and SPn6C. As compared to a BSA control, serogroup SPn6 tetrasaccharide–BSA conjugates elicited antibody reacting with serogroup SPn6 PS, but the immune sera bound to the unrelated pneumococcal PSs. Currently, it is unknown whether these broadly cross-reactive antibodies would be protective against pneumococcal infection but it is interesting to note that some antibodies (called natural antibodies) are known to cross-react with many different unrelated antigens.[45] Further studies are needed to understand whether the synthetic antigens display many different conformational configurations that are inducing production of natural antibodies of broad specificity. Additional studies and experiments involving antibody binding affinity, epitope search, antibody functionality, and potential autoimmunity are currently under way.

Experimental Section

Typical glycosylation procedures. Preparation of oligosaccharides

Method A. AgOTf-Promoted activation of the SBox glycosyl donors—A mixture of the glycosyl donor (0.11 mmol), glycosyl acceptor (0.10 mmol), and freshly activated molecular sieves (3 &, 200 mg) in $ClCH_2CH_2Cl$ or CH_2Cl_2 (2 mL) was stirred under argon for 1.5 h. Freshly conditioned AgOTf (0.22 mmol) was added and the reaction mixture was stirred for 15 min at RT, and diluted with CH_2Cl_2. The solid was filtered off and the residue was washed with CH_2Cl_2. The combined filtrate (30 mL) was washed with $NaHCO_3$ (20% aq., 15 mL) and water (3 × 10 mL), the organic phase was separated, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (ethyl acetate/hexane, 0:100 to 20:80, 5 % gradient elution) to afford an oligosaccharide derivative.

Method B. NIS/TfOH-promoted activation of S-ethyl glycosyl donors—A mixture of the glycosyl donor (0.13 mmol), glycosyl acceptor (0.10 mmol), and freshly activated molecular sieves (4 &, 200 mg) in $ClCH_2CH_2Cl$ (2 mL) or diethyl ether/$ClCH_2CH_2Cl$ (5:1, 2 mL) was stirred for 1 h under argon. NIS (0.25 mmol) and TfOH

(0.025 mmol) were added at 0°C and the reaction mixture was stirred for 10 min. Upon completion, the solid was filtered off and the residue was washed with CH_2Cl_2. The combined filtrate (30 mL) was washed with $Na_2S_2O_3$ (20 % aq., 15 mL), and water (3 × 10 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (ethyl acetate/hexane, 0:100 to 20:80 5% gradient elution) or Sephadex LH-20 (methanol/dichloromethane, 1:1, *v*/*v* elution) to afford an oligosaccharide derivative.

Deprotection. Synthesis of oligosaccharides 2 and 3

O-(α-D-Glucopyranosyl)-(1→3)-O-(α-D-glucopyranosyl)-(1→3)-O-(α-L-rhamnopyranosyl)-(1→3)-D/L-ribitol (2)—1 M NaOMe (~0.1 mL) was added to a solution of **13** (**a** or **b**, 100 mg, 0.103 mmol) in dry methanol (1.0 mL) until pH of 10 was reached. The reaction mixture was stirred for 15 h at RT, then neutralized with Dowex (H^+) resin, filtered, and the filtrate was concentrated in vacuo. The crude residue was dissolved in ethyl acetate/ethanol (1:1, *v*/*v*, 2.0 mL) and 10 % Pd/C (20 mg) was added. The reaction mixture was stirred under an atmosphere of H_2 for 15 h. The catalyst was then filtered off, washed with methanol, and the combined filtrate was concentrated under reduced pressure. The residue was purified by column chromatography on Sephadex G-15 (water elution) to afford compound **2** as a film (26.8 mg, 85 %). Analytical data for **2**: R_f = 0.35 (methanol/dichloromethane/water, 9:9:2, *v*/*v*/*v*); $[\alpha]_D^{27}$ +69.2° (*c* =1.0, H_2O); ^{1}H NMR (500 MHz, D_2O): δ = 1.35 (d, 3H; $_D$ +69.2 H-6′), 3.50 (dd, $J_{4''',5'''}$ = 9.7 Hz, 1 H; H-4‴), 3.58–3.62 (m, 3 H; H-4′, 5″, 2‴), 3.66–3.95 (m, 14 H; H-1, 2, 5, 3′, 5′, 2″, 5″, 3‴, 6a″, 6b″, 6a‴, 6b‴), 3.97–4.01 (m, 2 H; H-4, 4″), 4.04–4.09 (m, 3 H; H-3, 3″, 5‴), 4.25 (dd, $J_{2',3'}$ = 2.4 Hz, 1 H; H-2′), 5.06 (d, $J_{1',2'}$ = 1.6 Hz, 1 H; H-1′), 5.14 (d, $J_{1'',2''}$ = 3.8 Hz, 1 H; H-1″), 5.41 ppm (d, $J_{1''',2'''}$ = 3.8 Hz, 1 H; H-1‴); ^{13}C NMR (125 MHz, D_2O): δ = 17.0, 60.4, 60.6, 62.7, 62.8, 63.0, 67.3, 69.7, 69.8, 69.9, 70.2, 70.4, 70.5, 71.1, 71.8, 72.0, 72.1 (× 2), 72.4, 73.3, 75.7, 80.1, 80.3, 95.9, 99.7, 100.5 ppm; HR-FAB MS calcd for $C_{23}H_{42}O_{19}Na$: 645.2218 [*M*+Na] $^+$; found 645.2210.

O-(α-D-Glucopyranosyl)-(1→3)-O-(α-D-glucopyranosyl)-(1→3)-O-(α-L-rhamnopyranosyl)-(1→3)-1-O-(4-aminobutyl)-D-ribitol (3)—NaOMe (1 M, ~0.1 mL) was added to a solution of **17** (60 mg, 0.06 mmol) in dry methanol (1.0 mL) until a pH of 9 was reached. The reaction mixture was stirred for 15 h at RT, then neutralized with Dowex (H^+) resin, filtered, and concentrated in vacuo. The crude residue was dissolved in a mixture of ethanol/HCl (10 mL, 12:0.03, *v*/*v*) and 10 % Pd/C (70 mg) was added. The reaction mixture was stirred under an atmosphere of H_2 for 8 h. After that, the solid was filtered off, the filtrate was neutralized with Dowex (OH^-) resin, and concentrated under reduced pressure. The residue was coevaporated with water (2 × 2 mL) and then purified by column chromatography on Sephadex G-15 (water elution) to afford the title compound **3** as a clear film in 72 % yield. Analytical data for **3**: $[\alpha]_D^{27}$ +69.4° (*c* = 1.0, H_2O); ^{1}H NMR (500 MHz, D_2O): δ = 1.35 (d, 3H; H-6′), 1.73–1.83 (m, 4 H; 2 CH_2sp), 3.08–3.10 (m, 2H; CH_2sp), 3.51 (dd, $J_{4''',5'''}$ = 9.9 Hz, 1 H; H-4‴), 3.60–3.68 (m, 5 H; H-4′, 5″, 2‴, CH_2sp), 3.71–3.93 (m, 14 H; H-1, 2, 5, 3′, 5′, 2″, 5″, 3‴, 6a″, 6b″, 6a‴, 6b‴), 3.95–4.02 (m, 2 H; H-4, 4″), 4.06–4.12 (m, 3H; H-3, 3″, 5‴), 4.24–4.27 (m, 1H; H-2′), 5.05 (d, $J_{1',2'}$ = 1.7 Hz, 1 H; H-1′), 5.15 (d, $J_{1'',2''}$ = 3.6 Hz, 1 H; H-1″), 5.41 ppm (d, $J_{1''',2'''}$ = 3.7 Hz, 1H; H-1‴); ^{13}C NMR (125 MHz, D_2O): δ=17.1, 24.1, 26.1, 39.7, 60.5, 60.6, 62.7, 67.2, 69.7 (× 2), 69.8, 70.2 (× 2), 70.4 (× 2), 70.7, 71.1, 71.8, 72.1 (× 2), 73.2, 75.6, 80.1, 80.4, 95.8, 99.6, 100.5 ppm; HR-FAB MS calcd for $C_{27}H_{52}NO_{19}$: 694.3134 [*M*+H]$^+$; found 694.3141.

General procedure for preparation of squarate monoamide intermediates (25–31)—Diethyl squarate (24, 0.08 mmol) was added to a solution of the amine spacer

containing oligosaccharides **3** and **18**–**23** (0.04 mmol) in a potassium phosphate buffer (pH 7.0, 2.0 mL). The mixture was stirred for 15 h at RT, after which TLC on silica gel ($CHCl_3$/MeOH/AcOH/H_2O, 4:3:3:2, *v*/*v*/*v*/*v*) showed complete conversion of the starting material to a faster moving product. The reaction mixture was concentrated and the residue was purified by using Strata-X reverse phase cartridges conditioned by washing with MeOH (70 mL), followed by water (90 mL). The compound was eluted with water (3 × 10 mL), followed by stepwise gradient of MeOH in water (→5%, 10 mL). An aqueous solution of the purified material was filtered through a Sterile Millipore syringe filter unit (0.22 μm) and the filtrate was lyophilized to give white or pale yellow solids **25**–**31**.

General procedure for the preparation of neoglycoconjugates (32–38)

Squaric acid adduct (**25**–**31**, 1.0 mg, 0.0018 mmol) was transferred into the glass reaction vessel containing BSA (20 mg, 0.0003 mmol) by using borate buffer (pH 9, 45 μL, 40 mmol). The reaction was gently stirred at RT and the progression of the conjugation was periodically monitored by SELDI-TOF mass spectrometry. When the desired loading was achieved (see Table 1), the reaction mixture was neutralized with phosphate buffer (pH 7). The resulting mixture was subjected to centrifugal ultrafiltration (4000 r.p.m., 12 min) and washed with $(NH_4)_2CO_3$ (10 mM aq., 7 × 15 mL, 12 min each) to remove low-molecular-mass materials. A solution of the retained material was filtered through a Sterile Millipore syringe filter (0.22 μm) and lyophilized to afford the conjugates **32**–**38** as white solids in good yields (see Table 1).

General procedure for SELDI-TOF MS analysis

Blank preparation—BSA (1 mg) was diluted with water (6 μL) in a plastic Eppendorf centrifuge tube. A portion of the stock solution (2 μL) was applied on the Protein Chip®. The chip was force-air dried, washed twice with water (5 μL) with drying in between the washes. Finally, saturated solution of sinapinic acid (2 μL, prepared by dissolving 15 mg of sinapinic acid in acetonitrile/1 % TFA, 1:1, *v*/*v*, and centrifugating twice at 4000 r.p.m. for 3 min) was applied and dried.

Sample preparation—A sample of the reaction mixture (1 μL) was diluted with water (9 μL) in a centrifuge tube. A portion of this solution (2 μL) was applied on the chip. The chip was air dried, washed with water (2 × 5 μL) with drying in between the washes. Finally saturated solution of sinapinic acid (2 μL) was applied and dried.

ELISA—Conventional direct ELISA was performed to determine whether rabbit antibodies specific for serogroup SPn6 can recognize the synthetic oligosaccharides conjugated with BSA. ELISA plates (Corning Costar 9017, Corning, Acton, MA, USA) were coated with oligosaccharide–BSA antigens by placing the conjugate (2 $\mu g mL^{-1}$ in 0.1M sodium bicarbonate buffer pH 9.6) in the wells for overnight at 4 °C. The plates were then washed with phosphate buffered saline containing Tween 20 (0.05 %, PBS-T), and blocked with PBS-T containing skim milk (2 %) for 1 h at RT. After washing the plates with PBST, the plates were loaded with various dilutions of Pool B (Statens Serum Institut, Copenhagen, Denmark) and incubated for 2 h at RT. Plates were then washed with PBS-T and loaded with alkaline phosphatase (AP)-conjugated goat anti-rabbit Igs (Biosource, Camarillo, CA, USA). After second incubation for 2 h at RT, plates were loaded with a solution of *p*-nitrophenyl phosphate in diethanolamine buffer (100 μL of a 1 mg mL^{-1}, pH 9.8) and incubated for 1–2 h at RT. The substrate reaction was stopped by addition of 3 N NaOH (50 μL). Optical density was measured at 405 nm using an ELISA plate reader.

To confirm the specificity of antibody binding to oligosaccharide–BSA conjugates, an inhibition ELISA was performed. ELISA plates were coated with the oligosaccharide–BSA

antigens as described above, and the plates were loaded with Pool B (50 μL, diluted 1:400) and variously diluted pneumococcal capsular PSs (50 μL). Pneumococcal capsular PSs used are serotypes SPn2, 6A, 6B, 6C and 14. The remaining steps were identical to the ELISA described above. Percent inhibition was calculated according to the formula: [(1−A_{405} of serum with inhibitor)/A_{405} of serum without inhibitor] × 100 %.

To show antibodies in mouse serum, the conventional direct ELISA was performed as described above with few modifications. The ELISA plates were coated with a different natural pneumococcal PS (10 mg L^{-1}) and were loaded with diluted of mouse serum (1:100). Enzyme-conjugated antibodies to mouse immunoglobulin was used in place of antibodies to rabbit immunoglobulin.

Mouse immunization—C57BL/6 mice (female, 6 weeks old) were immunized with 10 μg of oligosaccharide-BSA conjugates in Freund's adjuvant on days 0, 49 and 70 and bled on days 0 and 77. The experiment was performed under the protocol approved by the IACUC of University of Alabama at Brirmingham (Protocol APN090406646).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was supported with NSF (CHE-0547566) and NIH awards (AI-067494) to A.V.D. and NIH funds (AI-031473, AI-30021) to M.H.N.

References

1. Avery OT, Dubos R. J Exp Med. 1931; 54:73–89. [PubMed: 19869903]
2. Tomasz, A., editor. Molecular Biology & Mechanisms of Disease. Vol. 3. Mary Ann Liebert; Larchmont: 2000. "Pneumococcal Polysaccharides: A Chemical View", J. P. Kamerling in Streptococcus pneumoniae; p. 81-114.
3. Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MCC, Nahm MH. J Clin Microbiol. 2007; 45:1225–1233. [PubMed: 17267625]
4. Väkeväinen M, Eklund C, Eskola J, Käyhty H. J Infect Dis. 2001; 184:789–793. [PubMed: 11517443]
5. Robinson DA, Briles DE, Crain MJ, Hollingshead SK. J Bacteriol. 2002; 184:6367–6375. [PubMed: 12399507]
6. Rebers PA, Heidelberger M. J Am Chem Soc. 1961; 83:3056–3059.
7. Robbins JB, Lee CJ, Rastogi SC, Schiffman G, Henrichsen J. Infect Immun. 1979; 26:1116–1122. [PubMed: 43290]
8. Robbins JB, Austrian R, Lee CJ, Rastogi SC, Schiffman G, Henrichsen J, Makela PH, Broome CV, Facklam„ RR. J Infect Dis. 1983; 148:1136–1159. [PubMed: 6361173]
9. Park IH, Park S, Hollingshead SK, Nahm MH. Infect Immun. 2007; 75:4482–4489. [PubMed: 17576753]
10. Austrian R. Drugs Aging. 1999; 15(Suppl 1):1–10. [PubMed: 10690790]
11. Wuorimaa T, Kayhty H. Scand J Immunol. 2002; 56:111–129. [PubMed: 12121432]
12. Lucas AH, Reason DC. Immunol Rev. 1999; 171:89–104. [PubMed: 10582166]
13. Moreau M, Schulz D. J Carbohydr Chem. 2000; 19:419–434.
14. Pozsgay V. Adv Carbohydr Chem Biochem. 2000; 56:153–199. [PubMed: 11039111]
15. Ledwith M. Curr Opin Paediatr. 2001; 13:70–74.
16. Adam D, Scholz H. Klin Paediatr. 2001; 213:109–113.
17. Pozsgay V. Curr Topics Med Chem. 2008; 8:126–140.

18. Alonso De Velasco E, Verheul AFM, Verhoef J, Snippe H. Microbiol Rev. 1995; 59:591–603. [PubMed: 8531887]
19. Saeland E, Jakobsen H, Ingolfsdottir G, Sigurdardottir ST, Jonsdottir I. J Infect Dis. 2001; 183:253–260. [PubMed: 11110649]
20. Jansen WTM, Hogenboom S, Thijssen MJL, Kamerling JP, Vliegenthart JFG, Verhoef J, Snippe H, Verheul AFM. Infect Immun. 2001; 69:787–793. [PubMed: 11159969]
21. Quataert S, Martin D, Anderson P, Giebink GS, Henrichsen J, Leinonen M, Granoff DM, Russell H, Siber G, Faden H, Barnes D, Madore DV. Immunol Invest. 2001; 30:191–207. [PubMed: 11570640]
22. Park IH, Moore MR, Treanor JJ, Pelton SI, Pilishvili T, Beall B, Shelly MA, Mahon BE, Nahm MH. J Infect Dis. 2008; 198:1818–1822. [PubMed: 18983249]
23. Nahm MH, Lin J, Finkelstein JA, Pelton SI. J Infect Dis. 2009; 199:320–325. [PubMed: 19099489]
24. Parameswar AR, Pornsuriyasak P, Lubanowski NA, Demchenko AV. Tetrahedron. 2007; 63:10083–10091.
25. Parameswar AR, Hasty SJ, Demchenko AV. Carbohydr Res. 2008; 343:1707–1717. [PubMed: 18439990]
26. Slaghek TM, van Vliet MJ, Maas AAM, Kamerling JP, Vliegenthart JFG. Carbohydr Res. 1989; 195:75–86. [PubMed: 2636047]
27. Slaghek TM, van Oijen AH, Maas AAM, Kamerling JP, Vliegenthart JFG. Carbohydr Res. 1990; 207:237–248. [PubMed: 2076519]
28. Slaghek TM, Maas AAM, Kamerling JP, Vliegenthart JFG. Carbohydr Res. 1991; 211:25–39. [PubMed: 1773430]
29. Thijssen MJL, van Rijswijk MN, Kamerling JP, Vliegenthart JFG. Carbohydr Res. 1998; 306:93–109. [PubMed: 9691442]
30. Thijssen MJL, Bijkerk MHG, Kamerling JP, Vliegenthart JFG. Carbohydr Res. 1998; 306:111–125. [PubMed: 9691443]
31. Note: IUPAC describes alditols as acyclic polyols with the general formula $HOCH_2[CH(OH)]_nCH_2OH$ (formally derivable from an aldose by reduction of the carbonyl group). We believe that because the aldehyde group, which is the major attribute of the sugar, is lost during this derivatization alditols should be referred to as pseudosugars. Hence, herein all di-, tri-, and tetrasaccharides containing the ribitol unit are referred to as pseudo-di-, -tri- and -tetrasaccharides. This agrees with the IUPAC nomenclature that describes monosaccharide as a term which includes aldoses, ketoses, and a wide variety of compounds derived by oxidation, deoxygenation, introduction of other substituents, alkylation, and acylation of hydroxy groups, and chain branching.
32. Demchenko AV, Kamat MN, De Meo C. Synlett. 2003:1287–1290.
33. Kamat MN, Rath NP, Demchenko AV. J Org Chem. 2007; 72:6938–6946. [PubMed: 17676918]
34. Pornsuriyasak P, Demchenko AV. Chem Eur J. 2006; 12:6630–6646.
35. Pozsgay V. Carbohydr Res. 1992; 235:295–302. [PubMed: 1473109]
36. Garegg PJ, Kvarnstrom I, Niklasson A, Niklasson G, Svensson SCT. J Carbohydr Chem. 1993; 12:933–953.
37. Auzanneau FI, Bundle DR. Carbohydr Res. 1991; 212:13–24.
38. Kuberan B, Linhardt RJ. Curr Org Chem. 2000; 4:653–677.
39. Tietze LF, Schroeter C, Gabius S, Brinck U, Goerlach-Graw A, Gabius HJ. Bioconjugate Chem. 1991; 2:148–153.
40. Hirayama K, Akashi S, Furuya M, Fukuhara KI. Biochem Biophys Res Commun. 1990; 173:639–646. [PubMed: 2260975]
41. Hou SJ, Saksena R, KovQc P. Carbohydr Res. 2008; 343:196–210. [PubMed: 18048016]
42. Van Regenmortel, MHV.; Briand, JP.; Muller, S.; Plaue, S. Synthetic Polypeptides as Antigens. Vol. 19. Elsevier; Amsterdam: 1988. Laboratory Techniques in Biochemistry and Molecular Biology.
43. Saksena R, Chernyak A, Karavanov A, KovQc P. Methods Enzymol. 2003; 362:125–139. [PubMed: 12968361]

44. Sørensen UB. J Clin Microbiol. 1993; 31:2097–2100. [PubMed: 8370735]
45. Bouvet JP, Dighiero G. Infect Immun. 1998; 66:1–4. [PubMed: 9423831]

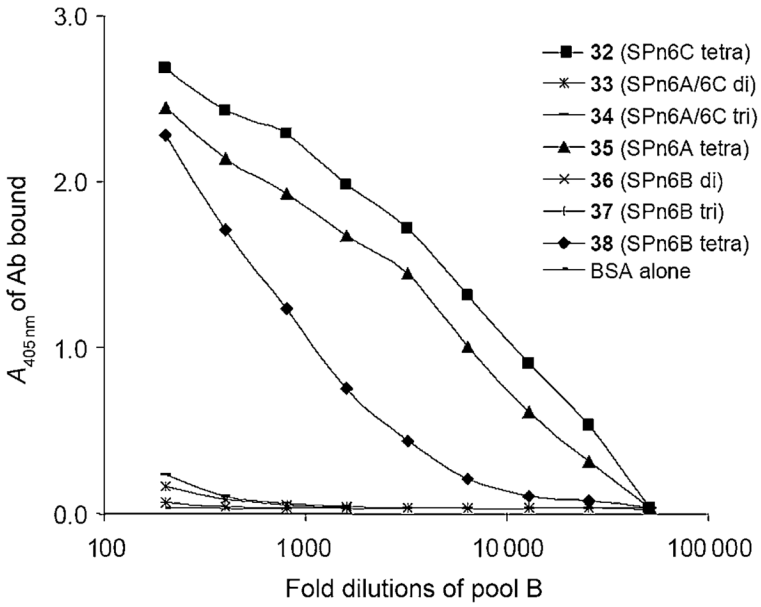


Figure 1.

Antibodies bound (*y*-axis) to ELISA plates coated with synthetic SPn oligosaccharide–BSA conjugates at various dilutions of Pool B (*x*-axis). ELISA plates were first coated with various synthetic conjugates (2 μg mL^{-1}) and then incubated with Pool B (Statens Serum Institut, Copenhagen, Denmark) at various dilutions. The amount of rabbit antibody bound to ELISA plates was then determined.

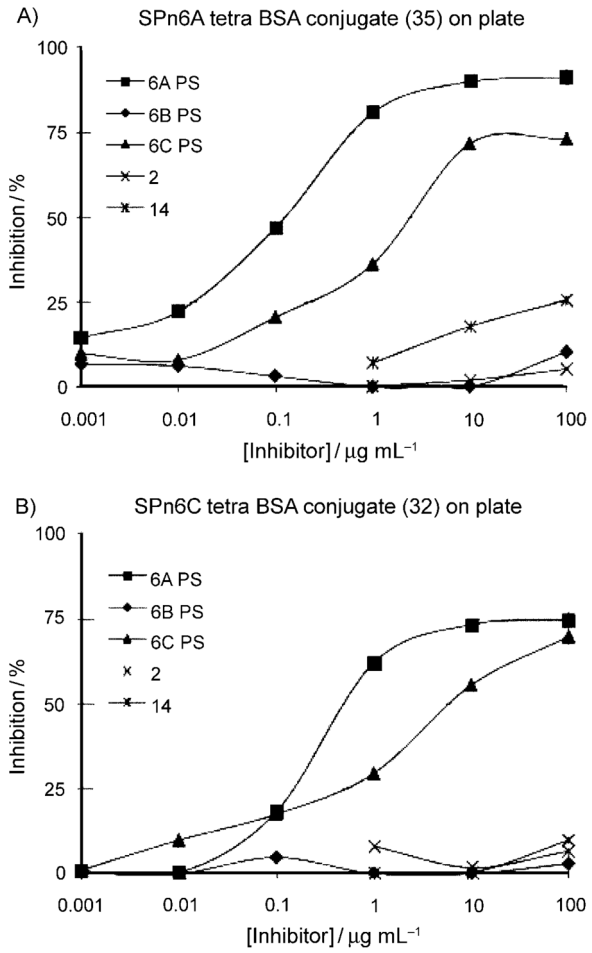


Figure 2.

Inhibition (*y*-axis) of Pool B binding to A) SPn6A or B) SPn6C tetrasaccharide–BSA conjugates by various concentrations (x-axis) of different pneumococcal polysaccharides. Free SPn6A and SPn6C PS inhibited the binding but free serotypes SPn2, SPn6B, and SPn14 PSs did not inhibit the binding even at high PS concentrations.

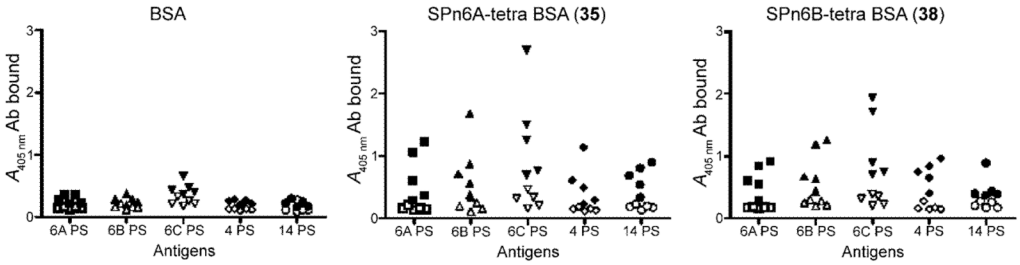
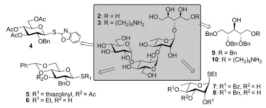
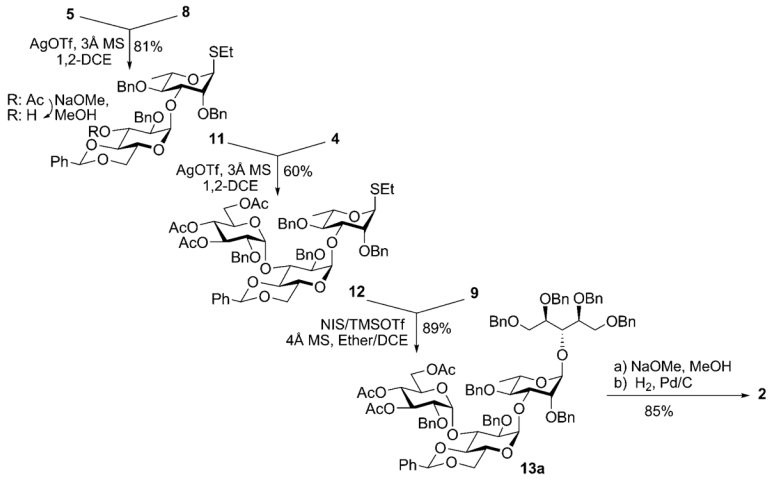


Figure 3.

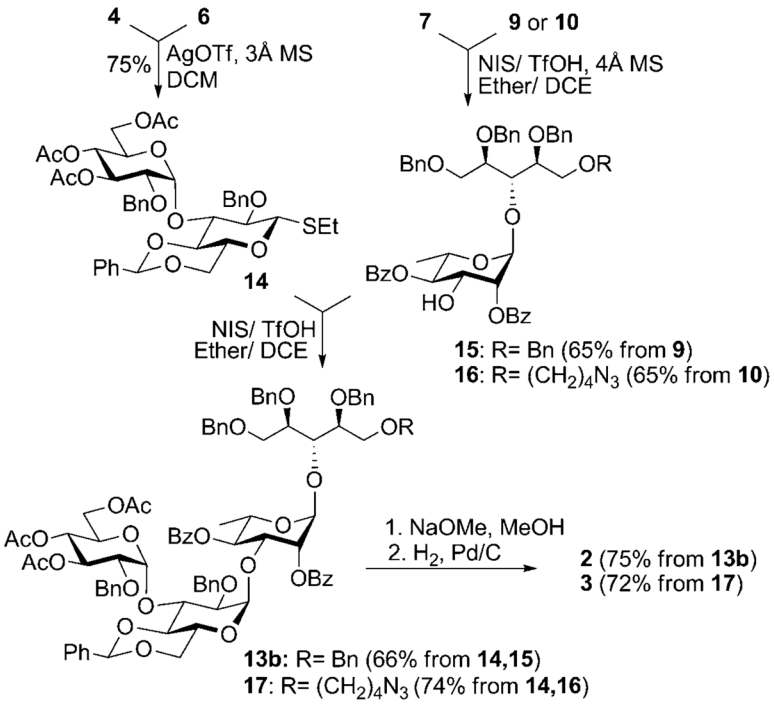
Amount of mouse antibody (*y*-axis) bound to ELISA wells coated with different capsular PSs (indicated in the *x*-axis). Legend. Mice were immunized with BSA (left), SPn6A tetrasaccharide–BSA (**35**, middle panel) or SPn6B tetrasaccharide–BSA (**38**, right). Each group consisted of five C57BL/6 mice (six weeks old), and each symbol represents a mouse. Open symbols represent preimmune sera and solid symbols represent postimmune sera.



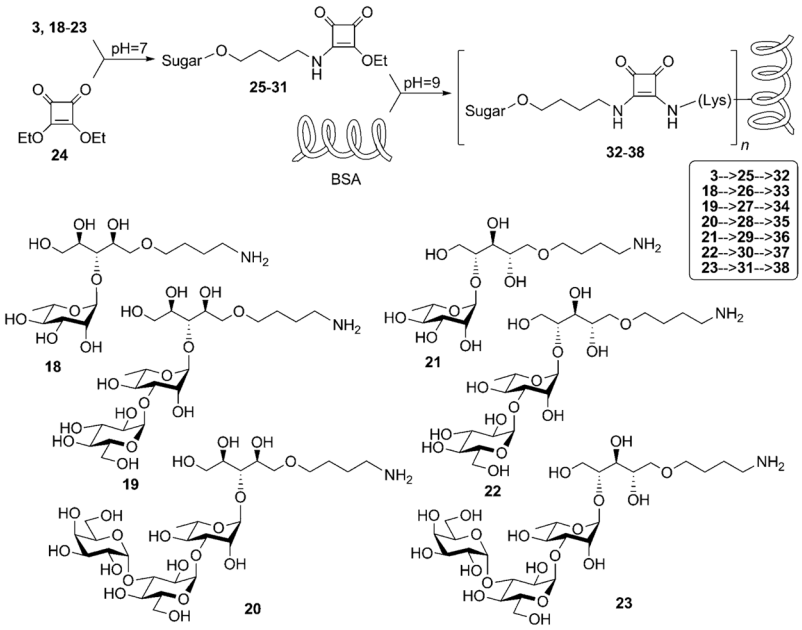
Scheme 1.
Retrosynthetic analysis of the repeating unit of SPn6C.



Scheme 2.
Synthesis of pseudotetrasaccharide **2**.



Scheme 3.
Convergent synthesis of pseudotetrasaccharides **2** and **3**.



Scheme 4.

Conjugation of spacer-containing SPn oligosaccharides **3**, **18**–**23** with BSA by using squaric acid. Synthesis of neoglycoconjugates **32**–**38**.

Table 1

Data sheet for the synthesis of SPn6 conjugates **32**–**38**.

Oligosaccharide→ amide→conjugate	Starting ratio (amide/BSA)	Loading[a]	Conjugate M_W	Yield [%]	Conjugation efficiency [%]
3→25→32	12:1	5.2	70502.19	98	43
18→26→33	10:1	7.3	69713.19	95	73
19→27→34	6:1	5.0	69493.86	98	83
20→28→35	8:1	4.0	69527.50	55	50
21→29→36	12:1	5.1	68734.70	84	42
22→30→37	12:1	8.3	71534.90	96	69
23→31→38	6:1	5.0	70344.94	93	83

[a] In the oligosaccharide–BSA conjugate (*n* in Scheme 4).