Carbohydrate Vaccine

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Synthesis of *Neisseria meningitidis* Serogroup W135 Capsular Oligosaccharides for Immunogenicity Comparison and Vaccine Development**

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Neisseria meningitidis (meningococcus) is a Gram-negative human pathogen that causes meningococcal diseases, such as meningococcal meningitis and meningococcal septicemia.[1] Based on the surface capsular oligosaccharides of the organism, 13 serogroups of *N. meningitidis* have been identified, among which A, B, C, Y, and W135 are the major pathogenic strains.[2] Serogroup A is the pathogen most often implicated in the seasonal epidemic disease in the developing countries of Asia and sub-Saharan Africa.[3] Serogroups B and C cause the majority of cases in industrialized countries.[4] Serogroups W135 and Y are responsible for the remaining cases in developing countries.

The capsular polysaccharide plays an important role in bacterial pathogenesis; its antiphagocytic properties help the bacteria to escape from antibodies and complement deposition.[5] On the other hand, the unique structure of the capsular polysaccharide also makes a good target for vaccine design. The first polysaccharide vaccine was developed in 1974; however, owing to the poor immunogenicity of polysaccharides, these vaccines typically elicit an IgM response and fail to induce T-cell-dependent immunity.[6] Moreover, polysaccharide vaccines are poor in immunological memory, especially for children under two-years old, who belong to the major risk group of meningococcal diseases. Consequently, polysaccharide–protein conjugate vaccines were developed to improve immunogenicity.[7] Currently, the major source of polysaccharides for vaccine preparation is from acidic lysis of bacteria and column chromatography purification.[8] Owing to limits of purification, the obtained polysaccharide is heterogeneous and therefore, vaccine quality is inconsistent. Hence, we were interested in developing a synthesis of capsular polysaccharides with defined lengths for a homogeneous vaccine. Recently, we developed a chemical method to prepare pure *N. meningitidis* serogroup C capsular polysaccharide, an α-(2→9) oligosialic acid glycans of defined length.[9] Herein, we focus on the synthesis of *N. meningitidis* serogroup W135 capsular oligosaccharide, which consists of a glycan repeating unit of →6)-α-D-Gal*p*-(1→4)-α-D-Neu*p*5Ac(9OAc)-(2→ (Figure 1). To the best of our knowledge, the total synthesis of this molecule has not been reported.

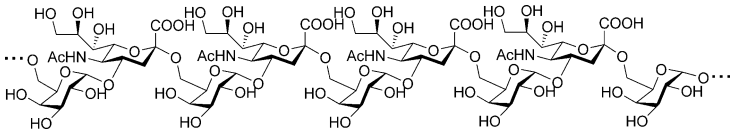

Figure 1. Formula of *N. meningitidis* serogroup W135 capsular oligosaccharide composed of a repeating unit of →6)-α-D-Gal*p*-(1→4)-α-D-Neu*p*5Ac(9OAc)-(2→.

From a structural point of view, there are two major challenges for such a synthesis: α-selectivity of the sialic acid linkage and 1,2-*cis* galactoside formation. α-Sialylation is challenging because of 1) the lack hydroxy group on the C-3 position for neighboring participation, 2) the strong electron-withdrawing carboxylic group on the C-1 position, which reduces the formation of the oxocarbenium, and 3) the occurrence of a 2,3-elimination reaction during the glycosylation reaction.[10] Many sialic acid donors designed to address these problems have focused on three main characteristics: leaving-group optimization, structure modification, or a combination of both.[11] Moreover, the use of a nitrile solvent can increase the α-selectivity.[12] Another synthetic challenge comes from the 1,2-*cis* linkage for glyco-synthesis. Although many approaches have been used to construct such a glycan linkage, there is no general method to achieve this purpose. The methods used to tackle this challenge include: 1) remote electron-donating group participation;[13] 2) addition of a bulky group at C-6 position for long-range participation;[14] 3) C-2 neighboring group participation.[15]

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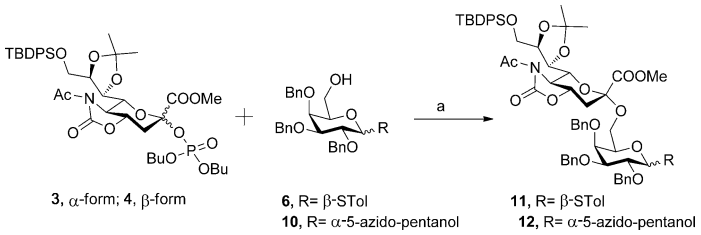
Considering all these challenges, a strategy to synthesize the *N. meningitidis* serogroup W135 capsular oligosaccharide with various lengths is highly interesting. *N. meningitidis* serogroup W135 capsular oligosaccharides of defined lengths can be used to study the detailed relationship between oligosaccharide length and immunogenicity. After careful analysis of the structure of the *N. meningitidis* serogroup W135 capsular oligosaccharide, we decided to begin the synthesis from suitably protected disaccharides **11** and **12** (Figure 2). These disaccharides can be selectively opened at the *N*-acetyl-5-*N*,4-*O*-carbonyl oxazolidinone ring under weakly basic



Scheme 1. Synthesis of disaccharide building blocks **11** and **12**. Reagents and conditions: a) TMSOTf, CH_2Cl_2, −70 °C, about 90%. TMSOTf = trimethylsilyl trifluoromethanesulfonate.

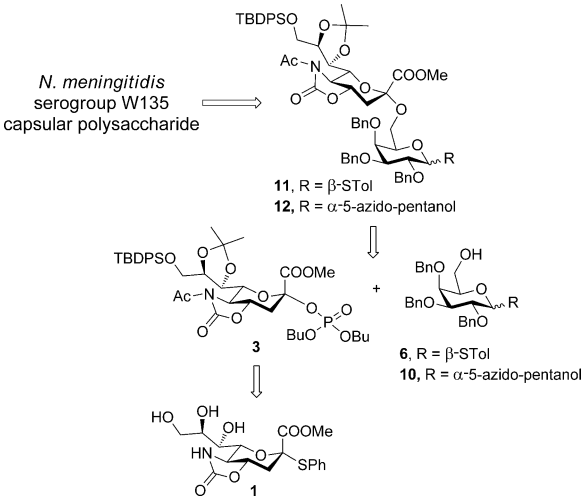


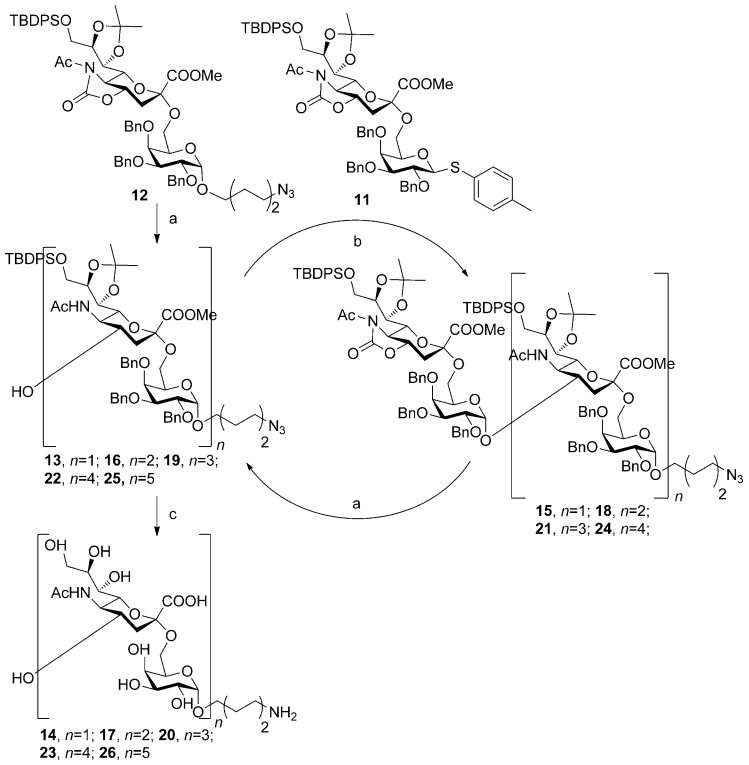
Figure 2. Retrosynthesis of *N. meningitidis* capsular oligosaccharide. TBDPS = *tert*-butyldiphenylsilyl; STol = *p*-methylphenyl thio.

conditions[16] to give the 4-OH compound without affecting the other protecting groups. The disaccharide STol leaving group at the reducing end of the galactose residue was designed for the next coupling reaction, and the azidopentane linker at the reducing end was designed for immobilization onto *N*-hydroxysuccinimide (NHS)-coated slides or conjugation to a carrier protein as vaccine candidates. Disaccharides **11** and **12** can be further disconnected into a suitably protected sialyl phosphate donor **3** and thiol galactosides **6** and **10** owing to the concern of regio-deprotection or stereoselective glycosylation. Compound **3** combined oxazolidinone and the phosphate leaving group to increase the α-selectivity[16,17] and was easily prepared from compound **1**.[17a] Detailed synthetic procedures for the monosaccharide building blocks **3**, **6**, **10** are shown in the Supporting Information.

We examined the conditions for reacting α- and β-phosphate sialoside with galactoside. The α-phosphate sialoside **3** reacted with **6** under the activation of TMSOTf in CH_2Cl_2 at −78 °C for ten minutes to give Neu5Ac-α-(2→6)-Gal disaccharide **11** as a single isomer in 91% yield (Scheme 1). The configuration of the disaccharide was examined by NMR spectroscopy, and the newly formed α-glycosidic bond was confirmed by a $^3J(C_1\text{-}H_{3ax}) = 5.7$ Hz coupling constant. A previous report indicated that β-phosphate sialoside was less reactive than α-phosphate sialoside and required a higher temperature for activation.[17b] In this case, however, the β-phosphate sialoside **4** was fully activated even at −70 °C with the galactoside acceptor **6**. We also obtained the α-isomer only product (**12**) in 91% yield using **3** as a donor and **10** as an acceptor.

Oligosaccharide elongation was accomplished by way of an iterative glycosylation and deprotection strategy, using disaccharide **11** as a common donor for the [2+*n*] glycosylation reaction to construct up to decasaccharides. We used **12** as a reducing-end building block and selectively removed its oxazolidinone ring using Zemplén conditions to obtain **13** in 78% yield. Glycosylation of the disaccharide donor **11** and alcohol acceptor **13** in the presence of NIS/TfOH activation in CH_2Cl_2 at −40 °C for one hour gave the fully protected tetrasaccharide **15** in 64% yield. Compound **15** was further treated with Zemplén conditions to open the oxazolidinone ring and gave tetrasaccharide acceptor **16** in 75% yield. The fully protected hexasaccharide **18** was synthesized by [2+4] glycosylation using **11** and **16** with 52% yield as a single stereoisomer. Repeating the oxazolidinone ring opening and the same [2+*n*] glycosylation strategy, octasaccharide **21** and decasaccharide **24** were synthesized. But with increasing length of the oligosaccharide, the yields decreased to 46% and 35%, respectively. Fortunately, these products were also obtained as a single α isomer. The alcohol products of the hexasaccharide **19**, octasaccharide **22**, and decasaccharide **25** were obtained from the fully protected oligosaccharide using Zemplén conditions in 70–80% yield (Scheme 2).

Finally, the completely deprotected compounds were obtained by global deprotection of the alcohol compounds **13**, **16**, **19**, **22**, and **25**. First, the TBDPS and isopropylidene groups of the alcohol compounds were removed in the presence of excess $BF_3{\cdot}OEt_2$ at 0 °C for three hours owing to the fluoride and acidic property of BF_3. Second, the methyl ester group was removed using a strong base, NaOH in MeOH. Finally, all benzyl groups were removed by a hydrogenation reaction with $Pd(OH)_2$ catalyst and H_2 in MeOH/H_2O. The final deprotected products **14**, **17**, **20**, **23**, and **26** were obtained in 45–60% yields over three steps (Scheme 2). Notably, the estimated coupling constant for the anomeric proton of galactose was about 3–4 Hz, and we confirmed that only α-linked Gal-(1→4)-Neu5Ac oligosaccharides were produced by the small value of this coupling constant (for detailed synthetic procedures and compound characterization, see the Supporting Information).

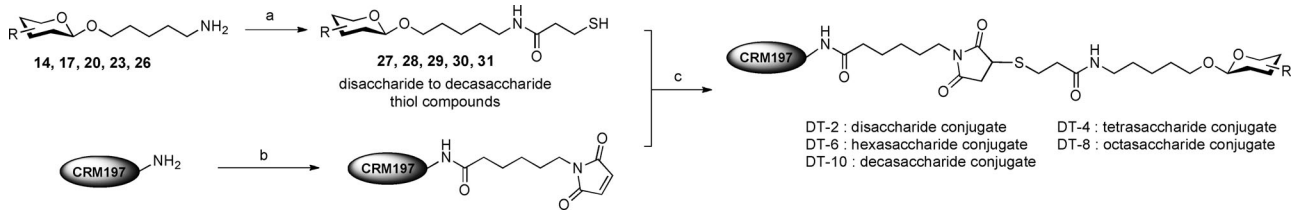


Scheme 2. Synthesis of oligosaccharides. Reagents and conditions: a) NaOMe, MeOH, RT, 65–75%; b) NIS, TfOH, CH_2Cl_2, −40°C, 64% for **15**, 52% for **18**, 46% for **21**, 35% for **24**; c) $BF_3{\cdot}OEt_2$, acetonitrile, 0°C; NaOH, $MeOH/H_2O$; $Pd(OH)_2/H_2$, $MeOH/H_2O$, 35–50%, over three steps. NIS = *N*-iodosuccinimide.

Many chemical approaches have been developed to crosslink carbohydrates and proteins: 1) the Staudinger ligation employs a substituted phosphite to react with the azide-modified protein to form the carbohydrate–protein conjugate through the formation of an amide bond;[18] 2) oxime conjugation introduces an aminooxy group on the protein to react with an oligosaccharide containing an aldehyde or ketone group;[19] 3) Michael addition often uses thiol group addition to a maleimide to form a stable thioester linkage;[20] and 4) copper (I)-catalyzed cycloaddition of azides to alkynes (click chemistry) provides efficient glycoconjugation.[21] However, the triazole ring from the click chemistry reaction produces an undesired immune response. Therefore, we adopted the thiol–maleimide coupling method for carbohydrate–protein conjugation because of its high efficiency in sialic acid-rich compounds.[22] We began by using the commercially available amine-reactive reagent 3,3-dithiobis(sulfosuccinimidylpropionate) (DTSSP) to react with the deprotected amine compounds **14**, **17**, **20**, **23**, and **26** in PBS buffer (pH 7.4) overnight. The disulfide bond was then cleaved with dithiothreitol (DTT) at 40°C for one hour to give the free thiol products **27**, **28**, **29**, **30**, and **31** as Michael donors in 70–75% yield. To generate a reactive maleimide group on the protein, diphtheria toxin (DT) mutant CRM197 was reacted with *N*-(ε-maleimidocaproyloxy)sulfosuccinimide ester (sulfo-EMCS) in PBS buffer (pH 8.0) for one hour. The number of maleimide linkers on the protein was determined by MALDI-TOF mass spectrometry. On average, 20 maleimide linkers were coupled to one CRM197 molecule. Oligosaccharides were conjugated to the carrier protein CRM197 by mixing thiol-modified oligosaccharides **27**, **28**, **29**, **30**, and **31** and maleimide-modified CRM197 in PBS buffer (pH 7.4) for one hour (Scheme 3) to obtain the glycoconjugates DT-2, DT-4, DT-6, DT-8, and DT-10 with various carbohydrate epitopes on the DT. Again, the number of oligosaccharides conjugated to the CRM197 was determined by MALDI-TOF mass spectrometry (Supporting Information, Table S1).

We examined the immunogenicity of these synthetic glycoconjugates using a mouse-serum assay. In the experimental group, 6- to 8-week-old female BALB/c mice ($n = 5$) were injected intramuscularly with conjugates containing 2 μg of oligosaccharide in 100 μL PBS buffer at two-week intervals, and the antigens were formulated with 2 μg of an α-galactosylceramide derivative C34 or alum adjuvant. PBS buffer alone was injected into the control group of mice. Seven days after the third boost, blood samples were collected for each mouse for serological immune analysis by glycan microarray.

N. meningitidis W135 capsular di- to decasaccharides **14**, **17**, **20**, **23**, and **26**, plus 70 other synthetic amine-containing oligosaccharides (the formulas of these glycans are shown in Table S2) were printed on NHS-coated glass slides. Details of the microarray fabrication and detection procedure are described in the Supporting Information. To investigate the immunogenicity of different-length oligosaccharides, sera collected from the mice were diluted 200-fold in PBST buffer containing 3% BSA. The diluents were incubated with the microarray at 4°C for one hour to allow the induced antibodies to bind to the oligosaccharides. Excess serum antibodies were then washed out and the microarrays were



Scheme 3. Oligosaccharide conjugation to the carrier protein. Reagents and conditions: a) DTSSP, PBS buffer (pH 7.4), RT; DTT, 40°C, 70–75%; b) sulfo-EMCS, PBS buffer (pH 8.0), RT; c) PBS buffer (pH 7.4), RT.

incubated with fluorescently labeled goat anti-mouse IgG antibodies as the secondary antibody at 4 °C for one hour. Finally, the slides were washed thoroughly and scanned at 635 nm wavelength with a microarray fluorescence chip reader.

In the group using C34 as an adjuvant, mice immunized with DT-2 elicited antibody against the *N. meningitidis* serogroup W135 capsular disaccharide **14**, but did not cross react with tetra- or longer oligosaccharides. This antibody also recognized other similar disaccharides on the slide, including Neu5Gc-α-(1→6)-Gal-α-(2→ and Neu5Ac-α-(1→6)-Gal-β-(2→. In contrast, antibodies induced by DT-4 bound tetra- to decasaccharides **17**, **20**, **23**, and **26**, but did not recognize **14** or other oligosaccharides on the slide (Figure S1). Antibodies induced by DT-6, DT-8, and DT-10 also showed the same pattern on the microarray as the DT-4 induced antibodies. Therefore, we concluded that the antibodies induced by DT-4, DT-6, DT-8, and DT-10 were very similar but different from the antibodies induced by DT-2. Moreover, based on the fluorescence intensity, DT-4, DT-6, DT-8, and DT-10 induced antibodies bound to longer oligosaccharides with higher affinity and DT-8 induced the most abundant antibody titers (Figure 3).

In the group that used alum as an adjuvant, the antibodies induced by DT-4, DT-6, DT-8, and DT-10 also differed from DT-2 (Figure S2), and the patterns were overall very similar

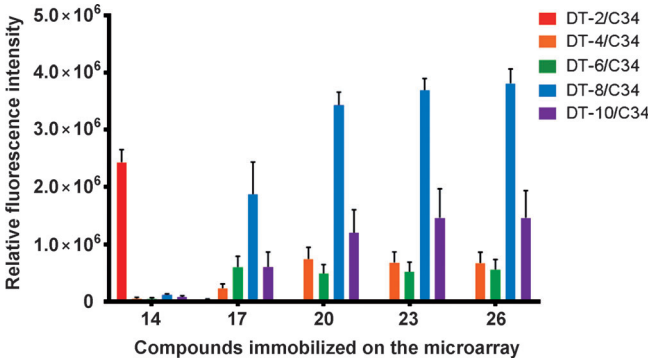


Figure 3. Comparison of the immunogenicity by microarray. Antibodies elicited by DT-4–DT-10 conjugates had higher binding affinity with longer oligosaccharides, and DT-8 recruited the highest quantity of antibodies.

to the vaccines that used C34 as an adjuvant. However, the titer of the antibodies induced using the alum adjuvant was lower than using C34.

When the carbohydrate itself was used as an antigen, the immune system produced a thymus-independent (TI) response, leading to predominantly IgM antibody. In contrast, the carbohydrate–protein conjugates elicited a thymus-dependent (TD) response,[23] and the ratio of IgG and IgM changed. We used DT-8/C34-induced serum antibodies for analysis of the antibody isotypes and subclasses. We first determined DT-8 induced IgG and IgM antibody titers by microarray by we defining the antibody titer as an s/N ratio of fluorescence intensity lower than three. The results showed that the anti-DT-8 IgG antibody titer was greater than 5×10^5, but the titer of IgM antibody was only 200 (Table S3). Therefore, the use of a TD-antigen resulted in antibody isotype switching from IgM to IgG.

We further looked into the distribution of the IgG subclasses by incubating the oligosaccharide-coated microarray with secondary anti-mouse IgG1, IgG2a, IgG2b, IgG2c, and IgG3 antibodies after the serum antibody binding. The anti-IgG antibody in serum contained IgG1, IgG2b, IgG2c, and IgG3 but no significant amount of IgG2a (Figure S4). We observed that the IgG1 subclass was highest in the serum and IgG3, a typical anti-carbohydrate antibody,[24] displayed a high level in the serum.

A serum bactericidal assay (SBA) was used to demonstrate the bactericidal abilities of the antibodies. Details of the SBA are described in the Supporting Information.[25] Our results showed that the bactericidal ability was roughly correlated with the antibody level on the microarray. Accordingly, serum from mice immunized with DT-2 showed no bactericidal ability. The SBA titers in mice immunized with DT-4 and DT-8 were 1/8 and 1/16 (Table 1). To summarize, DT-2 does not induce antibodies with bactericidal abilities, DT-4 was the minimum length required to induce bactericidal antibodies, and DT-8 elicited the most abundant antibodies with bactericidal effects.

Table 1: Serum bactericidal titers.[a]

Sera	Titer
DT-2/C34	n.d.[b]
DT-4/C34	1/8
DT-6/C34	1/8
DT-8/C34	1/16
DT-10/C34	1/4

[a] Bactericidal titer from sera of mice immunized with different length oligosaccharide–protein conjugate. [b] No bactericidal activity.

In conclusion, we have synthesized *Neisseria meningitidis* serogroup W135 capsular disaccharides to decasaccharides with excellent stereoselectivity for each glycosylation step in good yields. The highly α-stereoselective sialylation is due to the combination of an *N*-acetyl-5-*N*,4-*O*-carbonyl protecting group and a dibutyl phosphate as leaving group; while the high α-stereoselectivity for galactosylation may be caused by the long range participation of a bulky 6-*O*-group and a benzyl group at the C-4 position. To overcome the intrinsically poor immunogenicity of oligosaccharides, these oligosaccharides were conjugated to a carrier protein (CRM197) in the average number of 3–6, depending on the length of the oligosaccharide. The carrier protein CRM197 provides peptides to interact with MHC class II molecules on the antigen-presenting cell (APC), followed by stimulation of the Th cell for antibody maturation. Our results showed that induced IgG antibody titers were much higher than IgM antibody titers. Furthermore, analysis of the distribution of IgG subclasses showed that the antigen predominantly elicited IgG1 antibodies. Also, IgG3, a typical anti-carbohydrate antibody, was found at high levels in the serum. Consequently, the oligosaccharide–protein conjugate is a TD antigen, which

processes antibody isotype switching from IgM to IgG1. Antibodies induced by DT-2 recognized only disaccharides, but could not cross react with tetrasaccharides or longer. In contrast, antibodies induced by DT-4, DT-6, DT-8, and DT-10 all recognized tetra- to decasaccharides but not disaccharides. Patterns on the glycan microarray were the same for both adjuvants, alum and C34. Therefore, we concluded that antibodies induced by DT-4–DT-10 were similar, but different from DT-2. Antibodies elicited by DT-2 showed no bactericidal ability in the SBA experiment, while antibodies elicited DT-4–DT-10 had good bactericidal ability. These results imply that the minimum length of oligosaccharide required to make effective antibodies is a tetrasaccharide, and further isotope-labeling experiments are ongoing. Furthermore, with glycans in a single and homogeneous form, we can control the number of glycans on the protein to modulate the immunogenicity. Thus, we have demonstrated that synthetic tetrasaccharide–protein conjugates are a promising candidate for producing anti-*Neisseria meningitidis* serogroup W135 vaccines.

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