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Synthesis of a tetrasaccharide and its glycoconjugate corresponding to the capsular polysaccharide of *Neisseria meningitidis* serogroup X and its immunochemical studies†

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A tetrameric unit of the capsular polysaccharide of *Neisseria meningitidis* X (MenX) consisting of α-linked 2-acetamido-2-deoxy-D-glucosyl phosphate moieties has been prepared with excellent yield using a sequential stereoselective coupling strategy and conjugated with the tetanus toxoid protein to furnish the glycoconjugate derivative. Good yields were achieved in all intermediate steps. The immunochemical properties of the tetrasaccharide and semi-synthetic glycoconjugate derivative have been evaluated for the development of a potential MenX vaccine candidate.

Introduction

Bacterial meningitis[1,2] is a major cause of mortality and morbidity among the viral and microbial infections.[3] Sub-Saharan Africa is known as the meningitis belt due to the prevalence of *Neisseria meningitidis* (Men).[4] Among the 13 serogroups of *N. meningitidis* the most common pathogenic strains belong to MenA, B, C, W-135 and MenY serogroups. However, after the introduction of MenA vaccine (MenAfriVac)[5] and other conjugate vaccines consisting of MenA, C, Y and W (Menactra, Menveo and Nimenrix)[6,7] the increase in prevalence of MenX infection may be possible which has been found to be responsible for epidemics in the meningitis belt.[8] To date there is no vaccine available to prevent against infection caused by serogroup MenX, therefore there is an urgency to develop a MenX vaccine. Currently available meningococcal vaccines have been developed using the capsular polysaccharides (CPS) of various strains of *N. meningitidis.* Hence, the CPS of MenX has been targeted for the development of a MenX vaccine candidate. The MenX CPS is a homopolymer of α-linked 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate moiety (Fig. 1), which was initially reported by Bundle *et al.*[9] and later confirmed by Xie *et al.*[10]

Polysaccharide vaccines were initially introduced for the eradication of infections due to capsulated bacteria. However, these vaccines were effective in adults and older children only due to the T-cell independent immune response and were ineffective in children below two years of age.[11] In order to overcome this issue, glycoconjugate vaccines which are highly effective in adults as well as children[12] were developed. Many of the glycoconjugate vaccines consist of CPS antigens prepared by bacterial fermentation which have several disadvantages such as handling of live bacterial strains, presence of biological impurities, batch to batch variations, epitopic modification during conjugation *etc.* In order to overcome the above mentioned shortcomings, use of the synthetic oligosaccharides with precise structures for the preparation of glycoconjugate derivatives would be a better option.[13] In the recent past, synthetic oligosaccharide derived conjugate vaccines have proved to be equally or more immunogenic than native polysaccharide vaccines.[14,15] The advent of elegant synthetic methodologies[16] made it possible to prepare these glycans in a cost effective manner.[17,18] The synthetic oligosaccharides can be engineered to get specific functional groups attached to it for their use during conjugation with proteins. Moreover, due to the homogeneous nature of the synthetic glycan, it is expected that the final glycoconjugates would be comparatively more homogenous as compared to conventional conjugates.

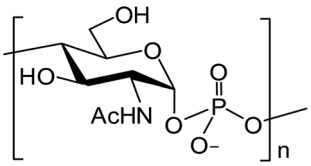

Fig. 1 Structure of the repeating unit of the CPS of *Neisseria meningitidis* serogroup X.

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Recently, Morelli *et al.* reported the chemical synthesis of monomer, dimer and trimer fragments of the MenX CPS[19] as well as evaluation of their antigenic potential.[20] However, the reported synthetic strategy suffers from a number of shortcomings, such as low yielding reaction conditions, use of moisture sensitive reagents in the phosphorylation steps and longer reaction time *etc.* These oligosaccharide fragments were attached to a 3-aminopropyl spacer through a phosphate linker, which may have a concern about the stability in various reaction conditions. Furthermore, the oligosaccharide fragments are short and not effective enough to produce the significant immunogenicity during the immunological studies.[20] So, it is desirable to identify the optimum (minimum) length of the oligosaccharide fragment as the effective epitope for the development of a glycoconjugate vaccine. Therefore, it was envisaged that synthesis of a higher oligomeric repeating unit of the MenX CPS fragment, with a spacer linker of optimum length attached to the reducing end through a stable oxygen linked glycoside bond could reduce the risk of instability of the molecule and improve the conjugation with the protein as well as improve the antigenic potential. The length of the spacer linker has also significant influence on the conjugation of the glycan moiety with the protein as well as immunogenicity of the glycoconjugate.[21] In this endeavour, efficient synthesis of a phosphate linked tetrasaccharide repeating unit of the MenX CPS as its 6-aminohexyl glycoside is reported. The 6-aminohexyl group linked to the reducing end of the MenX tetrasaccharide (**1**) has been used to connect the synthetic glycan moiety with the protein (tetanus toxoid; TT). A preliminary immunochemical study of the synthetic glycoconjugate derivative is also reported herein (Fig. 2).

Results and discussion

The synthesis of the target compound **1** is quite challenging because of the following issues: (a) maintenance of the 1,2-*cis* stereochemistry around the glycosyl phosphodiester linkages; (b) handling of the relatively unstable glycosyl 1-phosphonate derivatives during synthesis, purifications and oligomer synthesis; (c) yield optimization in the phosphate linkage formation steps *etc.* The reducing terminal monosaccharide unit connected with 6-(*N*-benzyloxycarbonyl)aminohexyl linker (**4**) and the glycosyl α-*H*-phosphonate intermediate (**2**) were prepared from the common starting material D-glucosamine hydrochloride using reported reaction conditions.[19] Reported reaction conditions for the preparation of compound **2**[19] (Scheme 1) from the suitably protected hemiacetal derivative (**5**)[19] did not result in a satisfactory yield, which led us to develop two-step, high yielding stereoselective reaction conditions along with a significant decrease in reaction time. Compound **5** was treated with diphenyl phosphite in the presence of pyridine to produce anomeric mixture of glycosyl-*H*-phosphonate derivatives,[22] which was further treated with phosphorous acid in a prolonged anhydrous reaction condition[23] to furnish 3-*O*-acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphonate (**2**)[19] with overall 65% yield. The formation of α-*H*-phosphonate derivative **2** was confirmed by ^{1}H NMR spectral analysis [signals at δ 7.70–6.40 (d, $J_{H,P}$ = 640 Hz, 1H) for H-P and δ 5.75 (dd, J = 8.8, 3.3 Hz) for H-1 in ^{1}H NMR spectrum]. Stereoselective glycosylation[24] of *p*-methylphenyl-4-*O*-acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-1-thio-α-D-glucopyranoside (**3**)[25] with 6-(*N*-benzyloxycarbonyl)amino hexanol in the presence of a combination of *N*-iodosuccinimide (NIS) and triflic acid (TfOH) followed by removal of the *O*-acetyl group using sodium methoxide[26] furnished 6-(*N*-benzyloxycarbonyl)aminohexyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (**4**) in 50% over all yield together with the undesired beta product (26%), which was separated by column chromatography (Scheme 1). Compound **4** has been used as the starting point for the elongation of the phosphate linked glycan chain by the stereoselective sequential phosphorylations using compound **2** as glycosyl donor in the presence of pivaloyl chloride as a coupling agent (Scheme 2).

Having two reaction intermediates **2** and **4** at hand, attempts were made to couple them together in a stereoselective manner through phosphate linkage. A few reaction conditions are available in the literature for the coupling of phosphonate derivatives with alcohols with a number of shortcomings in terms of low yield and requirement of complex reagents.[27–30] A

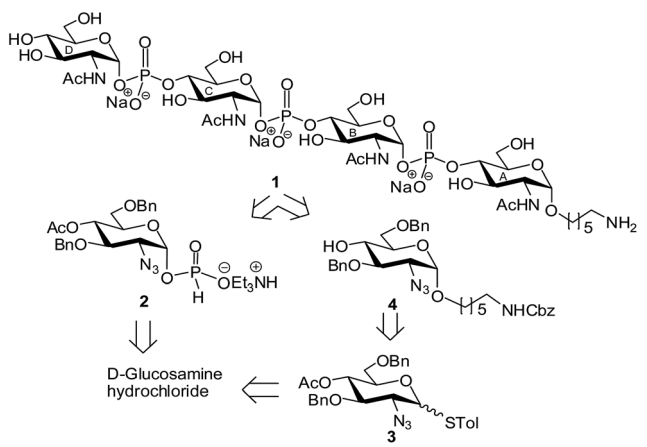
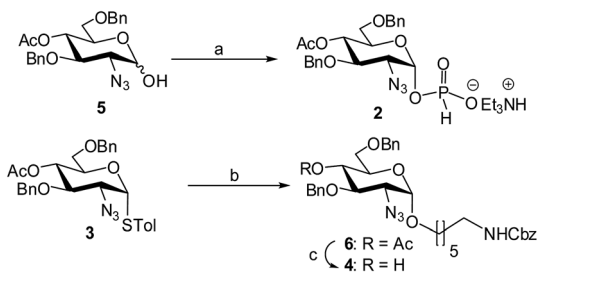
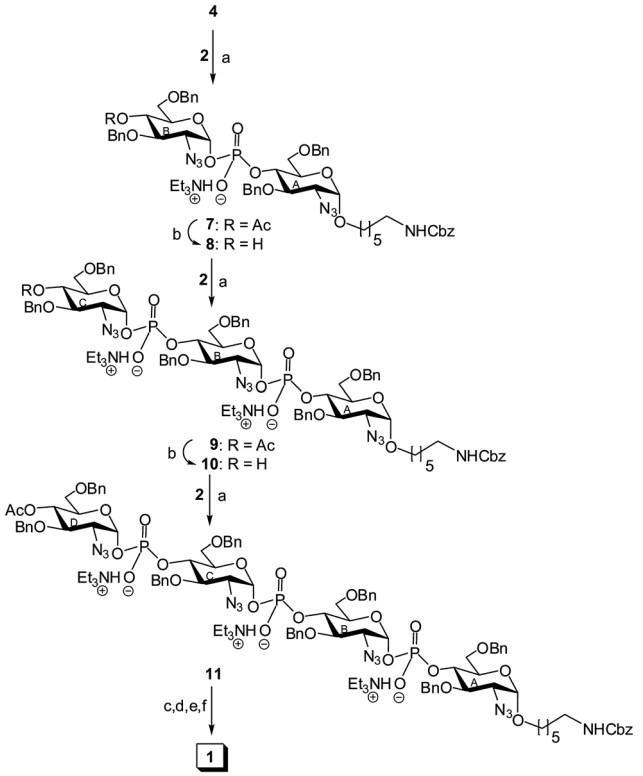


Fig. 2 Structure of the synthesized tetrasaccharide (**1**) as its 6-aminohexyl glycoside corresponding to the capsular polysaccharide of *N. meningitidis* serogroup X and its synthetic intermediates.



Scheme 1 Reagent and conditions: (a) (i) diphenyl phosphite, pyridine, room temperature, 2 h, TEA : H_2O (1 : 1), 1 h; (ii) H_3PO_3, CH_3CN, room temperature, 4 days, overall 65%; (b) 6-(*N*-benzyloxycarbonyl)amino hexanol, NIS, TfOH, THF, −5 °C, 1 h; (c) NaOMe, CH_3OH, room temperature, 50 °C, 1 h, 50% over two steps.



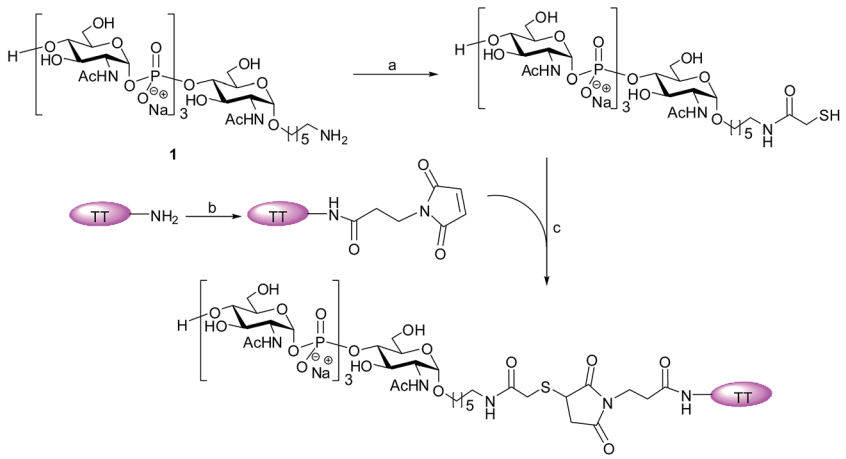
Scheme 2 Reagent and conditions: (a) pivaloyl chloride, pyridine, room temperature, 30 min, then −40 °C, iodine, pyridine–H_2O (9.75 : 0.25), 1.5 h, 86% for compound **7**; 60% for compound **9**; 53% for compound **11**; (b) CH_3ONa, CH_3OH, room temperature, 55 °C, 1.5 h, quantitative for compound **8**; 89% for compound **10**; (c) thioacetic acid, pyridine, room temperature, 18 h; (d) CH_3ONa, CH_3OH, 50 °C, 2 h; (e) H_2, $Pd(OH)_2$–C (20%), CH_3OH–H_2O (1 : 1), 104 psi, room temperature, 16 h, 45% over all.

two-step reaction condition involving pivaloyl chloride mediated coupling[31] of compound **4** with compound **2** followed by oxidation in the presence of molecular iodine in moist pyridine furnished phosphodiester linked disaccharide derivative **7** in 86% yield, which was de-*O*-acetylated using sodium methoxide at an elevated temperature to give disaccharide acceptor **8** in quantitative yield. Stereoselective formation of compound **7** was confirmed from its NMR spectral analysis [signals at δ 5.64 (dd, J = 7.0, 3.5 Hz, 1H, H-1_B), 4.81 (d, J = 3.5 Hz, 1H, H-1_A) in ^{1}H NMR and at δ 97.5 (C-1_A), 94.6 (C-1_B) in ^{13}C NMR spectra]. Following similar reaction condition, compound **8** was allowed to couple with compound **2** in a stereoselective manner in the presence of pivaloyl chloride followed by the treatment with molecular iodine to furnish phosphodiester linked trisaccharide derivative **9** in 60% yield, which was confirmed from its NMR spectral analysis [signals at δ 5.70–5.80 (m, 2H, H-1_B, H-1_C), 4.88 (d, J = 3.5 Hz, 1H, H-1_A) in ^{1}H NMR and at δ 97.3 (C-1_A), 93.8 (2C, C-1_B, C-1_C) in ^{13}C NMR spectra]. Removal of the *O*-acetyl group using sodium methoxide at an elevated temperature gave trisaccharide acceptor **10** in 89% yield. Repeating the similar reaction conditions, stereoselective coupling of compound **10** with compound **2** in the presence of pivaloyl chloride followed by iodine resulted in the formation of the tetrasaccharide derivative **11** in 53% yield. NMR spectral analysis of compound **11** unambiguously supported its stereoselective formation [signals at δ 5.70–5.73 (m, 3H, H-1_B, H-1_C, H-1_D), 4.90 (br s, 1H, H-1_A) in ^{1}H NMR and at δ 97.4 (C-1_A), 94.2 (2C, C-1_B, C-1_C), 94.0 (C-1_D) in ^{13}C NMR spectra]. Finally, compound **11** was subjected to a series of reactions involving (a) conversion of azido group to acetamido group on treatment with thioacetic acid;[32] (b) removal of acetyl group using sodium methoxide at an elevated temperature; (c) removal of benzyl ethers and Cbz group using hydrogenolysis over Pearlman's catalyst[33] to furnish compound **1** in 45% over all yield. Spectral analysis of compound **1** unambiguously confirmed its formation [δ 5.52–5.56 (m, 3H, H-1_B, H-1_C, H-1_D), 4.89 (d, J = 2.5 Hz, 1H, H-1_A) in ^{1}H NMR and 96.4 (C-1_A), 94.4 (C-1_D), 94.1 (2C, C-1_B, C-1_C) in ^{13}C NMR spectra] (Scheme 2).

Preparation of synthetic MenX tetrasaccharide-tetanus toxoid (compound 1–TT) conjugate and measurement of antigenicity

The conjugates of the synthesized MenX tetrasaccharide (compound **1**) with tetanus toxoid (TT) were prepared through the amino linker present at the reducing end of compound **1** following recently developed reaction conditions.[21] The free amino group of compound **1** was reacted with *S*-acetylthioglycolic acid-*N*-hydroxysuccinimide ester (SATA) followed by treatment with hydroxylamine hydrochloride to furnish glycan derivative attached to a linker with free thiol (SH) group.[21] The oligosaccharide and SH content were analyzed by the methods of Chen[34] and Ellman,[35] respectively. In another experiment, the TT protein was modified by reacting with 3-(maleimido) propionic acid-*N*-hydroxysuccinimide ester (BMPS) to generate TT–maleimide conjugate derivative.[21] Protein content was determined by Lowry[36] method and maleimide labelling was estimated by Ellman method.[35] Thiolated compound **1** and maleimide linked TT were coupled together to furnish compound **1**–TT conjugate (Scheme 3). The saccharide content in the compound **1**–TT conjugate was calculated by Chen's assay[34] and protein content was calculated by Lowry assay.[36] The glycan–protein w/w ratio was theoretically calculated by dividing saccharide content by protein content and was found to be in the range of 0.3 to 0.35. As the carbohydrate portion of the conjugate is responsible for specific MenX antibodies, we have considered only the yield specific to the compound **1** input in the beginning (thiolation) to the end of the conjugation scheme and we got a recovery of ~30% (5.5 mg conjugated compound **1** from 18 mg compound **1** used initially in the thiolation reaction analyzed using Chen's method[34]).

The antigenicity of MenX tetramer (compound **1**) and compound **1**–TT conjugate was estimated in relation to no-antigen control in a competitive enzyme-linked immunosorbent assay (Inhibition-ELISA) experiment along with MenX bacterial CPS (MenX CPS) as control. The bacterial CPS (used at concentrations ranging from 10–1000 μg mL^{-1}) gave rise to significant inhibition of the anti-MenX antibodies as evident by reduction in optical density of the wells as compared to the no antigen control. The inhibition increased with increase in saccharide concentration. The compound **1** and compound



Scheme 3 Reagents: (a) *S*-acetylthioglycolic acid-*N*-hydroxysuccinimide ester (SATA), 0.1 M HEPES buffer, room temperature, 1 h, then $NH_2OH \cdot HCl$; (b) BMPS, 0.1 M HEPES buffer, room temperature, 2 h; (c) 0.1 M HEPES, 8 °C, 16 h, 30%.

1–TT conjugate were also able to neutralize the specific antiserum against *N. meningitidis* serogroup X significantly and inhibit the binding of antibodies to the bacterial MenX polysaccharide coated on the plate (Fig. 3). Compound **1** (unconjugated synthetic MenX tetramer) showed lower inhibition (up to 68% inhibition) compared to its conjugate formulation (up to 89% inhibition) at all different concentrations tested (10–1000 μg mL^{-1}). Also, bacterial MenX CPS showed higher inhibition than the test candidates at all respective antigen concentrations used. Data obtained from the inhibition ELISA experiment demonstrated that both compound **1** and compound **1**–TT conjugate were antigenic and are able to neutralize the rabbit antiserum against *N. meningitidis* serogroup X. The inhibition increased with concentration of antigen used in the assay. Interestingly, there was no inhibition of compound **1** alone till the concentration of 100 μg mL^{-1}, whereas, the compound **1**–TT conjugate showed observable inhibitions from the starting concentration of 10 μg mL^{-1} itself. This difference might indicate towards the need for a larger molecule to be able to neutralize the antibodies. Such observations of lack of inhibition with smaller synthetic oligomers and lesser inhibition as compared to the full length bacterial polysaccharide have been reported in earlier literature.[37] These findings potentially target towards the development of an immunogenic semi-synthetic glycoconjugate derivative prepared using compound **1** and tetanus toxoid (TT).

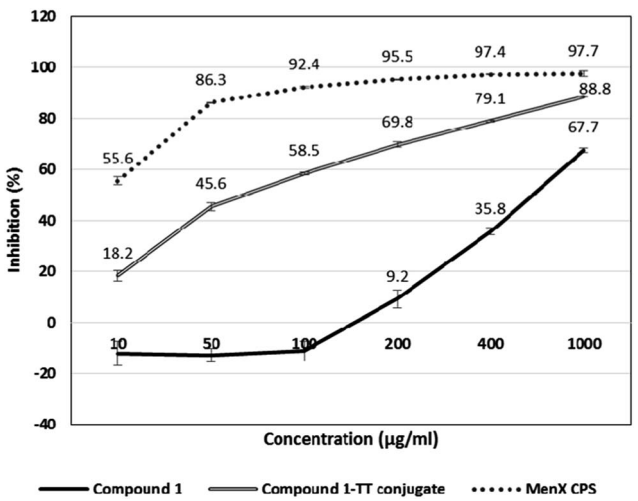


Fig. 3 Inhibition of anti-MenX antibodies with different concentrations of compound **1** and compound **1**–TT conjugate and MenX CPS.

Conclusion

In conclusion, an efficient synthetic strategy for the tetrameric repeating unit of the capsular polysaccharide of *N. meningitidis* serogroup X has been developed using common reaction intermediates and a generalized reaction condition. The stereoselective outcome of each phosphodiester linkage formation was highly satisfactory. To the best of our knowledge, this is the first report on the synthesis of tetrameric repeating unit of MenX CPS and its conjugation with tetanus toxoid protein to give the glycoconjugate derivative. The inhibition ELISA shows homologous reaction of tetramer and tetramer conjugates with MenX hyperimmune serum indicating the immunogenic potential of the candidate.

Experimental section

General methods

All reactions were monitored by thin layer chromatography over silica gel coated TLC plates. The spots on TLC were visualized by warming ceric sulphate or 2% 2,4-DNP in 0.1 M H_2SO_4 in methanol sprayed plates in hot plate. Silica gel 230–400 mesh or 100–200 mesh was used for column chromatography. NMR spectra were recorded on Bruker Avance 500 MHz using $CDCl_3$ as solvent and TMS as internal reference unless stated otherwise. Chemical shift value is expressed in δ ppm. The complete assignment of proton and carbon spectra was carried out by using a standard set of NMR experiments, *e.g.* ^{1}H NMR, ^{13}C NMR, ^{13}C DEPT 135, 2D COSY and 2D HSQC *etc.* MS were

recorded on a MALDI-TOF/TOF (AB Sciex 5800). Optical rotations were recorded in a Jasco P-2000 polarimeter. Microanalysis was carried out on Carlo Erba analyzer. Commercially available grades of organic solvents of adequate purity were used in all reactions.

4-*O*-Acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl hydrogenphosphonate triethylammonium salt (2)

To a solution of the hemiacetal derivative **5** (4.5 g, 10.5 mmol) in pyridine (45 mL) was added diphenyl phosphite (14.1 mL, 73.7 mmol) at room temperature and it was allowed to stir at room temperature for 2 h. The reaction mixture was cooled to 0 °C, diluted with TEA : H_2O (40 mL; 1 : 1 v/v) and it was stirred further for 30 min. The solvents were removed under reduced pressure and co-evaporated with toluene (2 × 50 mL). The residue was diluted with CH_2Cl_2 (100 mL), washed with satd. $NaHCO_3$ (100 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified over SiO_2 using CH_2Cl_2–CH_3OH–TEA (95 : 5 : 1) as an eluent to furnish anomeric mixture of phosphonate derivative as pale yellow syrup (6.4 g). To a solution of the obtained phosphonate derivative (6.4 g) in anhydrous CH_3CN (50 mL) was added phosphorous acid (1 g) and the solution was allowed to stir at room temperature for 4 days. The reaction was quenched by adding triethylamine (2.5 mL) at 0 °C, concentrated under reduced pressure. The residue was diluted with CH_2Cl_2 (100 mL), washed with satd. $NaHCO_3$ (100 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified over SiO_2 using CH_2Cl_2–CH_3OH–Et_3N (97 : 3 : 1) as an eluent to give pure compound **2** (4.1 g, 65%). Brown syrup; $[\alpha]_D^{25}$ +38 (*c* 0.5, $CHCl_3$); IR (neat): 3015, 2110, 1741, 1370, 1214, 912, 752 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 12.3 (br s, 1H, Et_3NH), 7.20–7.37 (m, 10H, Ar-H), 7.70–6.40 (d, $J_{H,P}$ = 640 Hz, 1H, H-P), 5.75 (dd, J = 8.8 and 3.3 Hz, 1H, H-1), 5.17 (t, J = 9.7 Hz, 1H, H-4), 4.82 (d, J = 11.3 Hz, 1H, PhC*H*), 4.63 (d, J = 11.3 Hz, 1H, PhC*H*), 4.53 (d, J = 11.6, 1H, PhC*H*), 4.48 (d, J = 12.0 Hz, 1H, PhC*H*), 4.23–4.21 (m, 1H, H-5), 4.09 (t, J = 9.8 Hz, 1H, H-3), 3.46–3.55 (m, 3H, H-6_{ab}, H-2), 2.95–3.10 (m, 6H, $(CH_3CH_2)_3N$), 1.86 (s, 3H, CH_3CO), 1.23–1.33 (m, 12H, $(CH_3CH_2)_3N$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.5 (CH_3CO), 137.5 (Ar-*C*), 127.5–137.4 (Ar-*C*), 92.8 (C-1), 77.7 (C-3), 74.5 (PhCH_2), 73.2 (PhCH_2), 70.5 (C-4), 69.8 (C-5), 68.5 (C-6), 63.2 (C-2), 45.3 ($(CH_3CH_2)_3N$), 20.6 (CH_3CO), 8.3 ($(CH_3CH_2)_3N$); MS (ESI) *m*/*z*: 490.08 $[M - NHEt_3]^-$; anal. calcd for $C_{28}H_{41}N_4O_8P$ (592.62): C, 56.75; H, 6.97%; found: C, 56.60; H, 7.15%.

6-(*N*-Benzyloxycarbonyl)aminohexyl 2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (4)

To the stirred solution of thioglycoside **3** (6.8 g, 12.7 mmol) and benzyl (6-hydroxyhexyl) carbamate (3.8 g, 15.2 mmol) in THF (80 mL) were added NIS (3.5 g, 15.5 mmol) and triflic acid (50 μL) over 5 min at −5 °C under argon. The reaction mixture was stirred at the same temperature for 1 h. The reaction mixture was diluted with EtOAc (150 mL) and washed with 5% aq. $Na_2S_2O_3$ and satd. $NaHCO_3$ (200 mL each). The organic layer was dried over (Na_2SO_4), filtered and concentrated under reduced pressure. To a solution of the crude mass in methanol (70 mL) was added CH_3ONa (2.2 g) portion wise over 10 min. The reaction mixture was stirred at 50 °C for 1 h and cooled to room temperature. The reaction mixture was neutralized using Amberlite IR-120 (H^+) resin, filtered, and concentrated under reduced pressure. The crude product was purified over SiO_2 using Petroleum ether–EtOAc (4 : 1) as an eluent to afford pure compound **4** (3.9 g, 50%). Yellow syrup; $[\alpha]_D^{25}$ +55 (*c* 0.5, $CHCl_3$); IR (neat): 3356, 3064, 3032, 2934, 2862, 2110, 1703, 1524, 1497, 1454, 1361, 1075, 910, 734 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.37–7.52 (m, 15H, Ar), 5.18–5.21 (m, 2H, Cbz), 5.02 (d, J = 11.0 Hz, 1H, PhC*H*), 4.99 (d, J = 3.5 Hz, 1H, H-1), 4.94 (d, J = 11.0 Hz, 1H, PhC*H*), 4.82–4.88 (m, 1H, N*H*), 4.64–4.73 (ABq, J = 12.0 Hz, 2H, PhCH_2), 3.96 (t, J = 9.0 Hz each, 1H, H-4), 3.86–3.91 (m, 1H, H-5), 3.76–3.85 (m, 4H, H-3, H-6_{ab}, –OC*H*–), 3.55–3.61 (m, 1H, –OC*H*–), 3.39 (dd, J = 10.0, 3.5 Hz, 1H, H-2), 3.25–3.35 (m, 2H, NCH_2–), 1.45–1.80 (m, 8H, –CH_2–); ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.3 (NH*C*O), 127.5–138.0 (Ar-*C*), 97.7 (C-1), 79.6 (C-3), 74.8 (PhCH_2), 73.5 (C-4), 71.9 (PhCH_2), 70.2 (C-5), 69.5 (C-6), 68.2 (Cbz), 66.4 (OCH_2), 62.6 (C-2), 40.7 (NCH_2), 29.6 (–CH_2–), 29.2 (–CH_2–), 26.2 (–CH_2–), 25.6 (–CH_2–); MS (ESI) *m*/*z*: 617.35 $[M - H]^-$; anal. calcd for $C_{34}H_{42}N_4O_7$ (618.72): C, 66.00; H, 6.84%; found: C, 65.83; H, 7.03%.

6-(*N*-Benzyloxycarbonyl)aminohexyl-*O*-(4-*O*-acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside, triethylammonium salt (7)

A mixture of α-*H*-phosphonate derivative **2** (2.87 g, 4.84 mmol) and acceptor **4** (1.5 g, 2.42 mmol) was co-evaporated with anhydrous pyridine under vacuum for three times. The mixture was dissolved in anhydrous pyridine (50 mL) and pivaloyl chloride (0.9 mL, 10 mmol) was added to it drop-wise at room temperature under nitrogen atmosphere and the stirring was continued for 30 min. The reaction mixture was cooled to −40 °C and to the cooled reaction mixture was added a solution of I_2 (1.2 g, 4.85 mmol) in pyridine : H_2O (8 mL; 9.75 : 0.25 v/v) over 15 min and stirred for 1 h. The reaction mixture was quenched by addition of aq. $Na_2S_2O_3 \cdot 5H_2O$ solution (200 mL, 1 M). The reaction mixture was diluted with H_2O (200 mL) and extracted using CH_2Cl_2 (2 × 300 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude product was purified over SiO_2 using CH_3OH–CH_2Cl_2 (4 : 96) + 1% Et_3N as eluent to give pure compound **7** (2.5 g, 86%). Yellow liquid; $[\alpha]_D^{25}$ +43.4 (*c* 0.5, $CHCl_3$); IR (neat): 3355, 2928, 2108, 1746, 1715, 1233, 1051 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 11.95 (br s, 1H, Et_3NH), 7.13–7.46 (m, 25H, Ar), 5.64 (dd, J = 7.0, 3.5 Hz, 1H, H-1_B), 5.12 (d, J = 10.5 Hz, 1H, PhC*H*), 5.06 (t, J = 10.0 Hz each, 1H, H-4_B), 5.01 (br s, 2H, PhCH_2), 4.81 (d, J = 3.5 Hz, 1H, H-1_A), 4.73 (d, J = 10.5 Hz, 1H, PhC*H*), 4.64–4.70 (m, 1H, N*H*), 4.51–4.61 (3d, J = 11.0 Hz each, 3H, 3PhC*H*), 4.43–4.49 (m, 1H, H-4_A), 4.30–4.40 (3d, J = 11.0 Hz each, 3H, 3PhC*H*), 4.04–4.07 (m, 1H, H-5_B), 3.92–3.98 (m, 2H, H-3_A, H-6_{aA}), 3.86–3.91 (m, 1H, H-5_A), 3.76–3.84 (m, 2H, H-3_B, H-6_{bA}), 3.61–3.67 (m, 1H, OC*H*–), 3.36–3.40 (m, 3H, H-2_B, H-6_{aB}, OC*H*–), 3.32 (dd, J = 11.0, 4.5 Hz, 1H, H-6_{bB}), 3.23 (dd, J = 10.5, 3.0 Hz, 1H,

H-2_A), 3.05–3.12 (m, 2H, NCH_2), 2.80–2.90 (m, 6H, N(CH_2CH$_3$)$_3$), 1.69 (s, 3H, COCH_3), 1.25–1.60 (m, 8H, –CH_2–), 1.12–1.17 (m, 9H, N(CH$_2$CH_3)$_3$); ^{13}C NMR (125 MHz, CDCl$_3$): δ 169.5 (CH$_3$*C*O), 156.3 (NH*C*O), 121.2–139.1 (Ar-*C*), 97.5 (C-1_A), 94.6 (C-1_B), 78.9 (C-3_A), 78.0 (C-3_B), 75.3 (C-4_A), 74.8 (Ph*C*H$_2$), 74.5 (Ph*C*H$_2$), 73.5 (Ph*C*H$_2$), 73.4 (Ph*C*H$_2$), 70.7 (C-5_A), 70.6 (C-4_B), 70.1 (C-5_B), 69.3 (C-6_A), 68.6 (C-6_B), 68.1 (O*C*H$_2$), 66.6 (Ph*C*H$_2$), 63.5 (C-2_B), 62.9 (C-2_A), 45.5 [N(*C*H$_2$CH$_3$)$_3$], 41.0 (N*C*H$_2$), 29.8 (*C*H$_2$), 29.2 (*C*H$_2$), 26.4 (*C*H$_2$), 25.8 (*C*H$_2$), 20.7 (*C*H$_3$CO), 8.5 [N(*C*H$_3$CH$_2$)$_3$]; MS (MALDI) *m*/*z*: 1106.43 [M – NHEt$_3$]$^-$; anal. calcd for $C_{62}H_{81}N_8O_{15}P$ (1209.32): C, 61.58; H, 6.75%; found: C, 61.40; H, 6.97%.

6-(*N*-Benzyloxycarbonyl)aminohexyl-*O*-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside, triethylammonium salt (8)

A solution of compound **7** (2.5 g, 2.04 mmol) in 0.25 M CH$_3$ONa in methanol (25 mL) was allowed to stir at 55 °C for 1.5 h. The reaction mixture was cooled to room temperature and neutralized with Amberlite IR-120 (H$^+$) resin, filtered and concentrated. The crude product purified over SiO$_2$ using CH$_3$OH–CH$_2$Cl$_2$ (4 : 96) + 1% Et$_3$N as an eluent to give pure compound **8** (2.4 g, quantitative). Brown syrup; $[\alpha]_D^{25}$ +56 (*c* 1.5, CHCl$_3$); IR (neat): 3347, 3010, 2933, 2108, 1710, 1102, 1052, 755 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$): δ 12.2 (br s, 1H, N$^+$*H*(CH$_2$CH$_3$)$_3$), 7.25–7.57 (m, 25H, Ar-H), 5.73 (br s, 1H, H-1_B), 5.32 (d, J = 11.0 Hz, 1H, PhC*H*), 5.10–5.15 (m, 2H, Cbz), 4.94 (br s, 1H, H-1_A), 4.63–4.84 (m, 6H, PhC*H*), 4.44–4.55 (m, 3H, H-4_A, N*H*, PhC*H*), 4.01–4.25 (m, 3H, H-3_A, H-5_A, H-5_B), 3.98 (m, 2H, H-6_{abA}), 3.56–3.82 (m, 5H, H-3_B, O*C*H$_2$, H-6_{abB}), 3.40–3.52 (m, 2H, H-2_B, H-4_B), 3.35 (dd, J = 10.0 Hz, 3.0 Hz, 1H, H-2_A), 3.15–3.20 (m, 2H, NCH_2), 2.67–2.78 [m, 6H, N(CH_2CH$_3$)$_3$], 1.35–1.65 (m, 8H, –CH_2–), 1.10–1.13 [m, 9H, N(CH$_2$CH_3)$_3$]; ^{13}C NMR (125 MHz, CDCl$_3$): δ 156.3 (Cbz), 127.1–139.0 (Ar-*C*), 97.3 (C-1_A), 94.0 (C-1_B), 79.9 (C-3_A), 79.0 (C-3_B), 74.9 (C-4_A), 74.6 (Ph*C*H$_2$), 74.2 (Ph*C*H$_2$), 73.5 (Ph*C*H$_2$), 73.1 (Ph*C*H$_2$), 72.3 (C-4_B), 71.1 (C-5_A), 70.8 (C-5_B), 69.8 (C-6_A), 69.5 (C-6_B), 67.8 (O*C*H$_2$), 66.4 (Cbz), 63.4 (C-2_B), 62.8 (C-2_A), 44.9 (N(*C*H$_2$CH$_3$)$_3$), 40.9 (N*C*H$_2$), 29.7 (–*C*H$_2$–), 29.2 (–*C*H$_2$–), 26.4 (–*C*H$_2$–), 25.7 (–*C*H$_2$–), 8.21 ((*C*H$_3$CH$_2$)$_3$N); MS (MALDI) *m*/*z*: 1064.41 [M – NHEt$_3$]$^-$; anal. calcd for $C_{60}H_{79}N_8O_{14}P$ (1167.29): C, 61.74; H, 6.82%; found: C, 61.57; H, 7.00%.

6-(*N*-Benzyloxycarbonyl)aminohexyl-*O*-(4-*O*-acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside, bis-triethylammonium salt (9)

A mixture of α-*H*-phosphonate derivative **2** (2.55 g, 4.29 mmol) and disaccharide acceptor **8** (1.7 g, 1.43 mmol) were co-evaporated with anhydrous pyridine under vacuum for three times. The residue was dissolved in anhydrous pyridine (40 mL) and pivaloyl chloride (1.07 mL, 8.58 mmol) was added to it drop wise at room temperature over 10 min and stirred for 30 min. The reaction mixture was cooled to −40 °C and to the cooled reaction mixture was added solution of I_2 (2.1 g, 8.58 mmol) in pyridine : water (10 mL, 9.75 : 0.25, v/v) over 15 min. The cooling was stopped and the reaction mixture left to stir for additional 1 h. The reaction was quenched by addition of saturated solution of $Na_2S_2O_3 \cdot 5H_2O$ (50 mL). The reaction mixture was cooled to room temperature, diluted with water (100 mL) and extracted with CH_2Cl_2 (400 mL). The separated organic layer was dried (Na_2SO_4), filtered and concentrated under reduced pressure. The residue was purified over SiO_2 using CH$_3$OH–CH$_2$Cl$_2$ (5 : 96) + 1% Et$_3$N as eluent, to furnish pure compound **9** (1.5 g, 60%). Yellow syrup; $[\alpha]_D^{25}$ +30.5 (*c* 1.2, CHCl$_3$); IR (neat): 3019, 2253, 2110, 1739, 1716, 1216, 908, 734 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$): δ 12.0 (s, 2H, N$^+$*H*(CH$_2$CH$_3$)$_3$), 7.23–7.53 (m, 35H, Ar-H), 5.70–5.80 (m, 2H, H-1_B, H-1_C), 5.27–5.32 (m, 2H, 2PhC*H*), 5.15–5.08 (m, 3H, H-4_C, Cbz), 4.88 (d, J = 3.5 Hz, 1H, H-1_A), 4.63–4.93 (m, 8H, N*H*, 7PhC*H*), 4.30–4.51 (m, 6H, H-3_A, H-3_B, H-5_B, 3PhC*H*), 3.85–4.22 (m, 7H, H-2_C, H-3_C, H-4_B, H-5_A, H-5_C, H-6_{abA}), 3.60–3.73 (m, 1H, OC*H*–), 3.22–3.58 (m, 8H, H-2_A, H-2_B, H-4_A, H-6_{abB}, H-6_{abC}, OC*H*–), 3.10–3.20 (m, 2H, NCH_2), 2.70–2.86 (m, 12H, N(CH_2CH$_3$)$_3$), 1.77 (s, 3H, COCH_3), 1.30–1.66 (m, 8H, CH_2), 1.07–1.10 (m, 18H, N(CH$_2$CH_3)$_3$); ^{13}C NMR (125 MHz, CDCl$_3$): δ 169.5 (CH$_3$*C*O), 156.3 (NH*C*O), 127.1–138.8 (Ar-*C*), 97.3 (C-1_A), 93.8 (2C, C-1_B, C-1_C), 79.1 (C-3_C), 77.9 (C-3_A), 74.6 (3C, C-3_B, C-4_A, Ph*C*H$_2$), 73.3 (3C, 3Ph*C*H$_2$), 73.2 (Ph*C*H$_2$), 71.0 (Ph*C*H$_2$), 70.6 (2C, C-4_C, C-5_A), 69.9 (C-4_B), 69.7 (C-5_C), 69.2 (C-5_B), 68.6 (2C, C-6_A, C-6_B), 67.9 (2C, C-6_C, O*C*H$_2$), 66.4 (Cbz), 63.7 (C-2_C), 63.3 (C-2_B), 62.9 (C-2_A), 45.1 (N(*C*H$_2$CH$_3$)$_3$), 40.9 (N*C*H$_2$), 29.8 (*C*H$_2$), 29.2 (*C*H$_2$), 26.8 (*C*H$_2$), 25.5 (*C*H$_2$), 20.7 (CO*C*H$_3$), 8.3 ((*C*H$_3$CH$_2$)$_3$N); MS (MALDI) *m*/*z*: 1553.57 [M – 2NHEt$_3$]$^-$; anal. calcd for $C_{88}H_{118}N_{12}O_{22}P_2$ (1757.89): C, 60.13; H, 6.77%; found: C, 60.00; H, 6.95%.

6-(*N*-Benzyloxycarbonyl)aminohexyl-*O*-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside, bis-triethylammonium salt (10)

A solution of compound **9** (1.5 g, 0.85 mmol) in 0.25 M CH$_3$ONa in CH$_3$OH (20 mL) was allowed to stir at 60 °C for 2 h. The reaction mixture was cooled to room temperature, diluted with methanol (20 mL) and neutralized with Amberlite IR-120 (H$^+$) resin, filtered and concentrated. The crude product purified over SiO$_2$ using CH$_3$OH–CH$_2$Cl$_2$ (5 : 96) + 1% Et$_3$N as eluent to give pure compound **10** (1.3 g, 89%). Yellow syrup; $[\alpha]_D^{25}$ +58.5 (*c* 1.5, CHCl$_3$); IR (neat): 3347, 2934, 2866, 2245, 2108, 1710, 1454, 1238, 1052 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$): δ 12.15 (br s, 2H, N$^+$*H*(CH$_2$CH$_3$)$_3$), 7.21–7.56 (m, 35H, Ar-H), 5.77 (br s, 1H, H-1_B), 5.72 (br s, 1H, H-1_C), 5.25–5.35 (m, 2H, 2PhC*H*), 5.11 (br s, 2H, Cbz), 4.91 (br s, 1H, H-1_A), 4.90 (br s, 1H, N*H*), 4.62–4.80 (m, 9H, 9PhC*H*), 4.43–4.51 (m, 4H, H-3_A, H-3_B, H-5_B, PhC*H*), 4.30–4.40 (m, 1H, H-3_C), 4.02–4.09 (m, 4H, H-2_C, H-4_B, H-5_A, OC*H*–), 3.82–3.95 (m, 4H, H-4_C, H-5_C, H-6_{abA}), 3.62–3.69 (m, 4H, H-2_A, H-2_B, H-6_{abB}), 3.30–3.45 (m, 4H, H-4_A, H-6_{abC}, OC*H*–), 3.10–3.20 (m, 2H, NCH_2), 2.60–2.72 (m, 12H, N(CH_2CH$_3$)$_3$), 1.26–1.65 (m, 8H, CH_2), 0.97–1.08 (m, 18H, N(CH$_2$CH_3)$_3$); ^{13}C NMR (125 MHz, CDCl$_3$):

δ 156.3 (Cbz), 127.0–139.1 (Ar-*C*), 97.3 (C-1_A), 94.0 (C-1_B), 93.8 (C-1_C), 80.0 (C-3_C), 79.0 (2C, C-3_A, C-3_B), 77.2 (2C, C-4_A, Ph*C*H_2), 74.9 (3C, 3Ph*C*H_2), 74.4 (Ph*C*H_2), 73.5 (Ph*C*H_2), 73.2 (C-4_B, C-4_C), 73.1 (C-5_A), 72.4 (C-5_B), 71.8 (C-5_C), 70.8 (C-6_A), 69.9 (C-6_C), 69.3 (C-6_B), 67.8 (O*C*H_2), 66.4 (Cbz), 63.7 (C-2_B), 63.3 (C-2_C), 63.0 (C-2_A), 44.9 (N(*C*H_2CH$_3$)$_3$), 40.9 (N*C*H_2), 29.7 (–*C*H_2–), 29.1 (–*C*H_2–), 26.4 (–*C*H_2–), 25.8 (–*C*H_2–), 8.2 ((*C*H_3CH$_2$)$_3$N); MS (MALDI) *m*/*z*: 1715.54 [M + 1]$^-$; anal. calcd for $C_{86}H_{116}N_{12}O_{21}P_2$ (1715.86): C, 60.20; H, 6.81%; found: C, 60.02; H, 7.02%.

6-(*N*-Benzyloxycarbonyl)aminohexyl-*O*-(4-*O*-acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside, tris-triethylammonium salt (11)

A mixture of compound **10** (1.3 g, 0.75 mmol) and α-*H*-phosphonate derivative **2** (1.3 g, 2.25 mmol) were co-evaporated with anhydrous pyridine under vacuum for three times. The mixture was dissolved in anhydrous pyridine (30 mL) and pivaloyl chloride (0.65 mL, 5.25 mmol) was added to it drop wise at room temperature over 10 min under argon. The reaction mixture was left to stir for 30 min. The reaction mixture was cooled to −40 °C and to the cooled reaction mixture was added a solution of I_2 (1.3 g, 5.25 mmol) in pyridine : water (3 mL, 9.75 : 0.25) over 15 min. The cooling was stopped and the reaction mixture left to stir for additional 1 h. The reaction was quenched by addition of saturated solution of $Na_2S_2O_3 \cdot 5H_2O$ (50 mL). The reaction mixture was warmed to room temperature, diluted with water (100 mL) and extracted by CH_2Cl_2 (2 × 200 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated under reduced pressure. The residue was purified over SiO_2 using CH_3OH–CH_2Cl_2 (5 : 95) + 1% Et_3N as an eluent to furnish compound **11** (0.9 g, 53%). Yellow syrup; $[\alpha]_D^{25}$ +41 (*c* 1.0, $CHCl_3$); IR (neat): 2937, 2253, 2110, 1741, 1718, 1454, 1375, 908, 733 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 12.0 (br s, 3H, N$^+$*H*(CH$_2$CH$_3$)$_3$), 7.21–7.53 (m, 45H, Ar-H), 5.70–5.73 (m, 3H, H-1_B, H-1_C, H-1_D), 5.22–5.36 (m, 3H, 3PhC*H*), 5.10 (br s, 3H, H-4_D, Cbz), 4.90 (br s, 1H, H-1_A), 4.89 (br s, 1H, N*H*), 4.60–4.80 (m, 10H, H-4_A, 9PhC*H*), 4.30–4.54 (m, 9H, H-3_A, H-3_B, H-4_C, H-5_A, H-5_B, 4PhC*H*), 3.80–4.20 (m, 8H, H-3_C, H-3_D, H-4_B, H-5_D, H-6_{abA}, H-6_{abC}), 3.65–3.74 (m, 1H, OC*H*–), 3.25–3.55 (m, 7H, H-2_B, H-2_C, H-6_{abB}, H-6_{abD}, OC*H*–), 3.00–3.20 (m, 5H, H-2_A, H-2_D, H-5_C, NC*H*$_2$), 2.70–2.72 (m, 18H, N(C*H*$_2$CH$_3$)$_3$), 1.76 (s, 3H, C*H*$_3$CO), 1.35–1.60 (m, 8H, C*H*$_2$), 1.07 (br s, 27H, ((C*H*$_3$CH$_2$)$_3$N); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.5 (CH$_3$*C*O), 156.3 (Cbz), 125.2–138.9 (Ar-*C*), 97.4 (C-1_A), 94.2 (2C, C-1_B, C-1_C), 94.0 (C-1_D), 78.9 (3C, C-3_B, C-3_C, C-3_D), 78.1 (C-3_A), 75.1 (2C, 2Ph*C*H$_2$), 74.7 (3C, 3Ph*C*H$_2$), 74.5 (2C, 2Ph*C*H$_2$), 73.3 (3C, C-4_A, C-5_A, Ph*C*H$_2$), 73.2 (3C, C-4_B, C-4_C, C-4_D), 71.9 (C-5_C), 71.7 (C-5_B), 70.9 (C-5_D), 70.6 (C-6_C), 69.9 (C-6_D), 69.3 (C-6_B), 68.6 (C-6_A), 67.9 (O*C*H$_2$), 66.5 (Cbz), 63.5 (3C, C-2_B, C-2_C, C-2_D), 63.0 (C-2_A), 45.0 (N(*C*H$_2$CH$_3$)$_3$), 40.9 (–N*C*H$_2$–), 29.8 (–*C*H$_2$–), 29.2 (–*C*H$_2$–), 26.4 (–*C*H$_2$–), 25.8 (–*C*H$_2$–), 20.7 (CO*C*H$_3$), 8.22 ((*C*H$_3$CH$_2$)$_3$N); MS (MALDI) *m*/*z*: 2000.63 [M − 3(NHEt$_3$)]$^-$; anal. calcd for $C_{114}H_{155}N_{16}O_{29}P_3$ (2306.46): C, 59.36; H, 6.77%; found: C, 59.17; H, 7.00%.

6-Aminohexyl-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-(2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-(2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate)-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranoside, trisodium salt (1)

To a solution of compound **11** (0.9 g, 0.4 mmol) in anhydrous pyridine (5 mL) was added thioacetic acid (3.2 mL) under inert atmosphere and the reaction mixture was stirred for 15 h. The reaction mixture was diluted with cold water (30 mL) followed by saturated solution of sodium bicarbonate (20 mL) and extracted with CH_2Cl_2 (2 × 70 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated under reduced pressure. The solid residue was purified by washing with 8% CH_2Cl_2 in petroleum ether to give *N*-acetylated product (700 mg). A solution of the *N*-acetylated compound (700 mg) in 1 M CH_3ONa (15 mL) in CH_3OH was stirred at 60 °C for 1.5 h. The reaction mixture was cooled to room temperature, neutralized using Amberlite IR-120 (H^+) resin. The reaction mixture was filtered and concentrated under reduced pressure to give the deacetylated product. To the solution of the de-*O*-acetylated product (500 mg) in CH_3OH : water, 1 : 1 (30 mL) was added 20% $Pd(OH)_2$–C (150 mg) under inert atmosphere at room temperature. The reaction mixture was allowed to stir under H_2 (104 psi) at room temperature for 16 h. The reaction mixture was filtered through a Celite bed and the filtering bed was washed with H_2O (3 × 30 mL). The filtrate was concentrated under reduced pressure and the crude mass was recrystallized in methanol to give pure compound **1** (220 mg, 45%). White powder; HPSEC purity: 98%; $[\alpha]_D^{25}$ −42 (*c* 0.5, H_2O); IR (KBr): 3436, 2517, 1632, 1383, 1443, 1204 cm^{-1}; ^{1}H NMR (500 MHz, D_2O): δ 5.52–5.56 (m, 3H, H-1_B, H-1_C, H-1_D), 4.89 (d, J = 2.5 Hz, 1H, H-1_A), 4.01–4.12 (m, 4H, H-2_A, H-2_B, H-3_A, H-3_B), 3.90–4.00 (m, 8H, H-2_C, H-2_D, H-3_C, H-4_C, H-5_A, H-5_B, H-5_C, H-5_D), 3.80–3.89 (m, 9H, H-3_D, H-6_{abA}, H-6_{abB}, H-6_{abC}, H-6_{abD}), 3.75–3.79 (m, 2H, H-4_A, H-4_B), 3.70–3.74 (m, 1H, OC*H*–), 3.56 (t, J = 9.5 Hz each, 1H, H-4_D), 3.47–3.53 (m, 1H, OC*H*–), 2.97–3.03 (m, 2H, NC*H*$_2$), 2.0–2.20 (m, 12H, NHCOC*H*$_3$), 1.35–1.70 (m, 8H, C*H*$_2$); ^{13}C NMR (125 MHz, D_2O): δ 174.5 (*C*OCH$_3$), 174.4 (*C*OCH$_3$), 174.2 (*C*OCH$_3$), 174.0 (*C*OCH$_3$), 96.4 (C-1_A), 94.4 (C-1_D), 94.1 (2C, C-1_B, C-1_C), 74.5 (C-3_C), 73.9 (2C, C-3_A, C-3_B), 72.9 (C-3_D), 72.0 (2C, C-4_C, C-5_A), 70.8 (C-4_A), 70.7 (C-4_B), 70.1 (C-5_B), 70.0 (2C, C-5_C, C-5_D), 69.4 (C-4_D), 68.0 (O*C*H$_2$–), 60.7 (C-6_A), 60.4 (C-6_B), 60.2 (C-6_C), 60.0 (C-6_D), 53.6 (2C, C-2_B, C-2_C), 53.5 (2C, C-2_A, C-2_D), 39.4 (N*C*H$_2$), 28.2 (*C*H$_2$), 26.7 (*C*H$_2$), 25.3 (*C*H$_2$), 24.9 (*C*H$_2$), 22.0 (2C, 2CO*C*H$_3$), 21.8 (2C, 2CO*C*H$_3$); MS (MALDI) *m*/*z*: 1212.34 [M − Na]$^-$; anal. calcd for $C_{38}H_{67}N_5Na_3O_{30}P_3$ (1235.84): C, 36.93; H, 5.46%; found: C, 36.76; H, 5.68%.

Preparation of the compound 1 (MenX tetramer) and tetanus toxoid (TT) conjugate

To a solution of compound **1** (18 mg, 14.56 mmol) in 0.1 M HEPES buffer containing 0.15 M NaCl, 10 mM EDTA, pH 7.5,

was added a solution of *S*-acetylthioglycolic acid *N*-hydroxysuccinimide ester (SATA) (10 mg, 43.7 mmol) in dimethyl sulfoxide (218 μL) and the reaction mixture was stirred for 1 hour at room temperature. The resulting solution was purified by Sephadex G-10 chromatography to remove excess SATA. The resulting solution was reacted with hydroxylamine hydrochloride (35 mg, 35 equiv.) and solution was stirred at room temperature for 2 hours and stored at −20 °C (13 mg, 70%) and used within 48 hours.

A solution of tetanus toxoid (20 mg mL^{-1}) in 0.1 M HEPES, pH 7.6 was added to a solution of 3-(maleimido) propionic acid *N*-hydroxysuccinimide ester (7.2 mg) in 1-methyl-2-pyrrolidinone (135 μL) and the reaction mixture was stirred for 2 hour at room temperature. The resulting solution was diafiltered against 0.1 M PBS containing 0.15 M NaCl, 5 mM EDTA, pH 6.8 through 50 kDa cutoff membrane leading to a recovery of 16 mg (80%) modified TT.

A solution of thiolated oligosaccharide (13 mg) in buffer containing 0.1 M HEPES, 0.15 M NaCl, 10 mM EDTA, pH 7.5, was mixed with a solution of maleimide linked tetanus toxoid (10 mg) in 0.1 M PBS, 0.15 M NaCl, 5 mM EDTA, pH 6.8. The resulting solution was gently stirred overnight at 4 °C. At the end of the reaction, solution was diafiltered against buffer containing 0.1 M MES, 0.2 M NaCl, pH 6.5 through 50 kDa cutoff membrane to achieve purified compound **1**–TT conjugate (5.5 mg conjugated compound **1** in the compound **1**–TT conjugate).

Determination of antigenic properties of compound 1 (MenX tetramer) and compound 1–TT conjugate

Eight thousand fold diluted rabbit antiserum against *N. meningitidis* serogroup X (228801; BD) was incubated in triplicate for 1 hour at 37 °C with both the test antigens (compound **1** and compound **1**–TT conjugate) and MenX CPS (as positive control) at various concentrations that is 10, 50, 100, 200, 400 and 1000 μg mL^{-1} diluted in phosphate-buffered saline (PBS) containing 0.1% v/v Brij 35 and 5% fetal bovine serum (FBS) in 96 well micro titer plate (plate A). A separate plate (plate B) was coated with a mixture of MenX CPS and methylated Human Serum Albumin (m-HSA) and subsequently blocked with 5% FBS after overnight incubation at 4 °C. To this plate B, antitoxin–antigen mix from plate A was added and incubated at 37 °C for 1 hour and then kept at room temperature for 1 hour. The plate was washed with phosphate-buffered saline, pH 7.4 containing 0.1% Brij 35. The plate was incubated for 60 minutes at room temperature with peroxidase labelled anti-rabbit IgG antibodies in PBS, 0.1% Brij 35 and 5% FBS. Plate was washed again and incubated for 10 min at room temperature with the 100 μL peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine-H_2O_2 in sodium acetate buffer. The reaction was stopped by adding 50 μL of 2 M H_2SO_4. The absorbance (A_{450}) was recorded on an ELISA reader (Tecan micro plate reader). The control consisted of MenX CPS coated wells incubated without the inhibitors (serum + diluent buffer) and had the maximum optical density, referred as optical density for no antigen control (OD_{NAC}). The percentage inhibition for the test antigens and positive control was calculated by using the equation:

$$\text{Inhibition}\% = (OD_{NAC} - OD_A)/(OD_{NAC} - OD_B) \times 100$$

OD_B is the optical density of blank wells. Optical density for test antigens (compound **1** and compound **1**–TT conjugate) and positive control are referred as OD_A. The data were reported as the mean ± SD of the measurements performed in triplicate.

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