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ARTICLE

Chemical Synthesis of Outer Core Oligosaccharide of *Escherichia coli* R3 and Immunological Evaluation

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Lipopolysaccharides (LPS), major virulence determinants in Gram–negative bacteria, are responsible for many pathophysiological responses and can elicit strong immune responses. In order to better understand the role of LPS in host–pathogen interactions and elucidate the immunogenic properties of LPS outer core oligosaccharide, an all α–linked *Escherichia coli* R3 outer core pentasaccharide was first synthesized with a propyl amino linker at the reducing end. This oligosaccharide was also covalently conjugated to a carrier protein (CRM_{197}) via the reducing end propyl amino linker. An immunological analysis demonstrated that this glycoconjugate can elicit specific anti-pentasaccharide antibodies with *in vitro* bactericidal activity. These findings will contribute to further exploring this pentasaccharide antigen as a vaccine candidate.

Introduction

Lipopolysaccharides (LPS) are important cell envelope components of Gram–negative bacteria, embedded in the outer leaflet of the asymmetric outer membrane and exposed on the cell surface.[1] LPS contribute to the integrity of outer membrane and protect the cells against various environmental stresses including lipophilic antibiotics and host immune system.[2] Moreover, LPS act as strong stimulators of innate and adaptive immunity in diverse eukaryotic species ranging from insects to humans.[2-3] Therefore, the study of LPS has attracted considerable interest with respect to the development of vaccines, therapeutics and diagnostics.[4] LPS typically consist of three parts: an endotoxic lipid A comprising a bisphosphorylated and acylated β–(1→6)–linked glucosamine disaccharide backbone, a core oligosaccharide, and a distal polysaccharide called O–antigen whose composition widely varies in different bacterial species.[3b, 5] Structurally, the core oligosaccharide region can be further subdivided into an inner core region typically consisting of 3–deoxy–D-manno–oct–2–ulosonic acid (Kdo) and L–glycero–D–manno–heptopyranonse residues, and an outer core region that contains various hexoses and hexosamines.[3b, 6]

In *Escherichia coli* (*E. coli*), the core oligosaccharides have limited variation with only five core structures named R1, R2, R3, R4 and K12.[7] The R3 core type is of biomedical interest because it is found in most verotoxigenic isolates such as O157:H7,[8] which causes approximately 210 million cases of diarrhea and 380,000 deaths in the developing world each year.[9] The structure of *E. coli* O157:H7 LPS has been determined and reported in previous literatures (Figure 1A).[8, 10] *E. coli* O157:H7 has a typical LPS consisting of a lipid moiety, a core oligosaccharide (inner core and outer core) and an O–antigen. The development of LPS–based vaccines, therapeutics, and diagnostics requires well-defined oligosaccharides conjugated to carrier proteins for immunological studies to identify the structural motifs that can elicit protective antibody responses.[4c, 11] Although oligosaccharides can be isolated from bacteria, the homogeneity cannot be achieved due to the

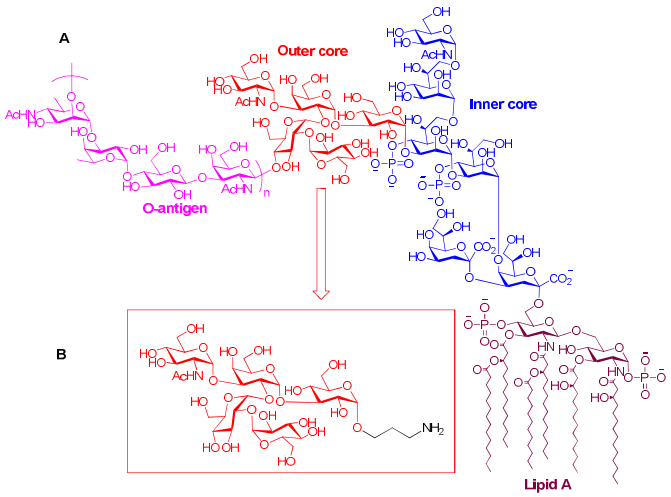


Figure 1. (A) The structure of *E. coli* O157:H7 LPS; (B) The structure of *E. coli* R3 outer core oligosaccharide with propyl amino linker

variations of natural oligosaccharides during the processes of isolation and conjugation to carriers.[12] Chemical synthesis of well-defined oligosaccharides can overcome this problem by utilizing an artificial linker at the reducing end that allows conjugation to carrier proteins.[11c, 13] Moreover, it can provide various sub–structures for structure–activity relationships study to determine the minimal epitope that can elicit protective immune response.[11b, 14] Herein, an all α–linked pentasaccharide of *E. coli* R3 outer core was chemically synthesized with a propyl amino linker at the reducing end (Figure 1B), and was conjugated to the nontoxic mutant of diphtheria toxin CRM_{197} to afford a glycoprotein. Furthermore, levels of IgG and IgM antibodies against the pentasaccharide and *in vitro* bactericidal activity of these antibodies against *E. coli* O157:H7 were evaluated.

Results and discussion

Initial attempt to synthesize outer core pentasaccharide

The chemical synthesis of the outer core pentasaccharide of *E. coli* R3 is challenging, because it is highly branched and consists of all α–linked glycosidic bonds. Furthermore, the pentasaccharide contains a number of glycosides that are difficult to install in a stereo–selective fashion. Especially, the introduction of α–glucosides and α–galactosides often leads to the formation of a mixture of anomers, which are difficult to separate and result in lower yields of the desired products. Installing the highly crowded *cis*–1,2,3–α–linked galactose (B) with glucose (C) and *N*-acetylglucosamine (D) needs to establish an optimal order of glycosylation. As illustrated in Figure 2, the fully protected pentasaccharide was initially envisioned to be synthesized by a convergent and stereo–controlled [2 + 3] approach. Unfortunately, the desired product was not detected by ESI mass spectrometry analysis, and substantial decomposition of the glycosyl donor (trichloroacetimidate) and acceptor were observed by TLC. It was considered that the steric hindrance resulted in inaccessibility of C–2 hydroxy group for the third glycosylation after C–1 and C–3 hydroxy groups of galactose (B) were glycosylated. Therefore, a [4 + 1] coupling approach was carried out by initially installing C–2 hydroxy group of galactose (B), followed by glycosylation of C–3 and C–1 hydroxy groups. Stereo–controlled glycosylations were assisted by solvent effects and temperature control. Moreover, C–3 and C–1 of the galactose building block (B) were modified by orthogonal protecting groups benzoyl ester (Bz) and 4–methoxyphenyl (MP), respectively. The orthogonal protecting groups made it possible to establish the proper order of glycosylation to assemble the highly branched pentasaccharide.

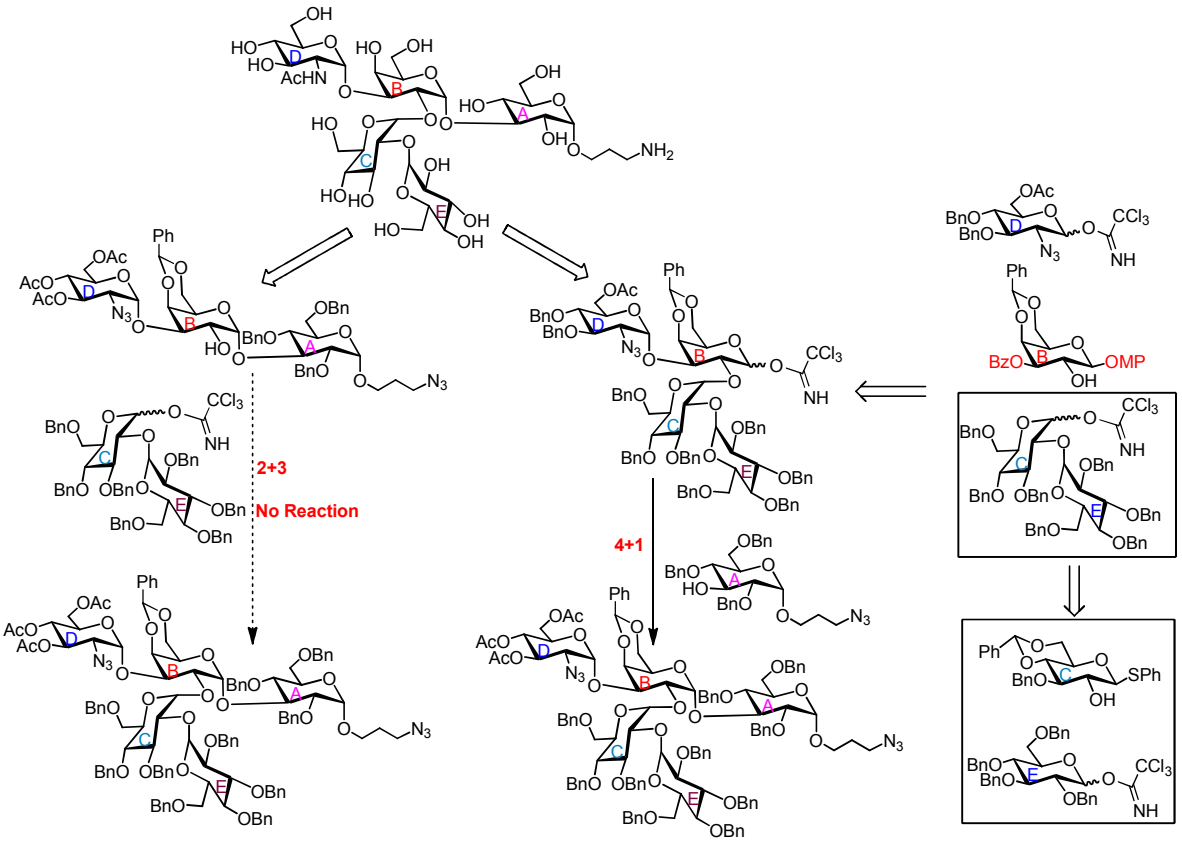
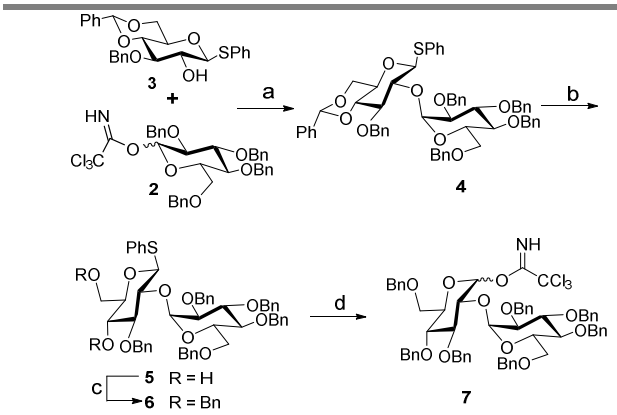


Figure 2 Retrosynthetic analysis of outer core oligosaccharide of *E. coli* R3

Synthesis of disaccharide donor 7

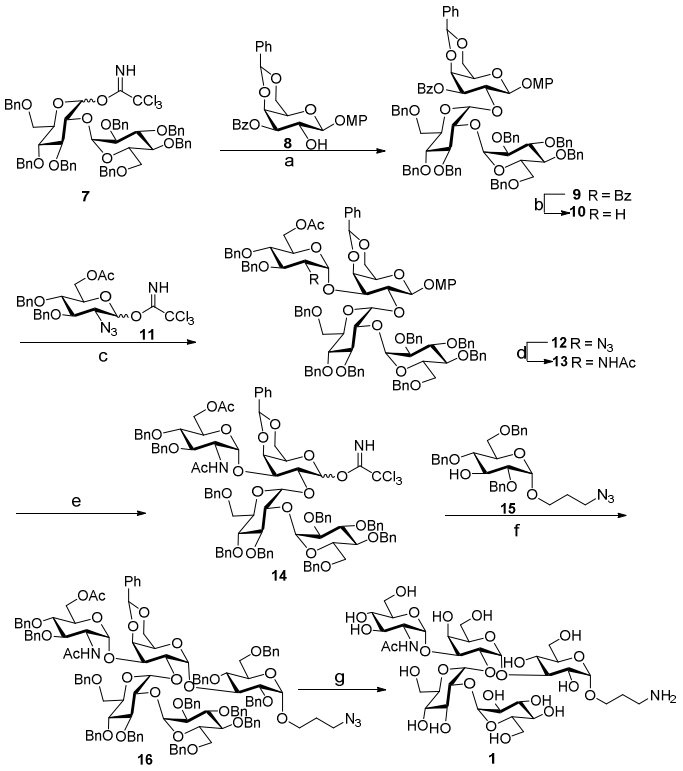
Having established the optimal order of glycosylation, *cis*–1,2–α–linked disaccharide donor **7** needed to be synthesized (Scheme 1). Assisted by solvent effects (diethyl ether as co-solvent with dichloromethane), glycosylation of the donor **2** with a nonparticipation benzyl ether at C–2 and the acceptor **3** synthesized in our previous report [15] predominantly afforded α–anomer ($^1J_{H\text{-}H}$ = 3.6 Hz, α/β=10:1) at –40 °C.[16] The isolated disaccharide **4** was contaminated by the rearrangement product of trichloroacetimidate donor **2**. The contaminated compound **4** was directly subjected to removal of the benzylidene acetal using trifluoroacetic acid (TFA) in a mixture of CH_2Cl_2 and water,[17] which made it easy to separate the byproduct to give desired diol **5**. Benzylation of compound **5** with benzyl bromide and NaH in DMF gave the purified benzyl ether **6** in 53% yield over three steps. The compound **6** was treated with *N*–bromosuccinimide (NBS) to afford the resulting lactol,[18] which was converted into the corresponding trichloroacetimidate **7** using trichloroacetonitrile and 1,8–diazabicycloundec–7–ene (DBU) in 68% yield over two steps.



Scheme 1. Reagents and conditions: a) TMSOTf, 4 Å MS, Et_2O/CH_2Cl_2 1:1, –40 °C to room temperature; b) DCM/TFA/H_2O 10:1:0.1; c) NaH, BnBr, DMF, 53% (three steps); (d) i: NBS, acetone/H_2O 9:1; ii: CCl_3CN, DBU, DCM, 68% (two steps)

Assembly of pentasaccharide 1

A successful synthetic strategy of compound **1** was illustrated in Scheme 2. With the disaccharide donor **7** in hand, coupling of the trichloroacetimidate **7** with the acceptor **8** in the presence of catalytic amount of TMSOTf as the promoter in diethyl ether/dichloromethane (1:1) solvent system provided a trisaccharide at –20 °C, which was purified to afford **9** as main α–anomer ($^1J_{H\text{-}H}$= 3.6 Hz, α/β ⩾20:1) in 55% yield.[16] Removal of benzoyl group (Bz) was easily accomplished by treatment with base (NaOMe) to afford trisaccharide acceptor **10** in 96% yield. The partially benzylated donor **11** was much more stable and easier to use compared to the corresponding extremely reactive fully benzylated donor.[15] The trisaccharide **10** was coupled with glycosyl donor **11** using a catalytic amount of TMSOTf in diethyl ether/dichloromethane (1:1) solvent system to afford the desired α-linked tetrasaccharide **12** ($^1J_{H\text{-}H}$ = 3.2 Hz) in 76% yield, no β–anomer byproduct was detected. The azide moiety of **12** was reduced with $NaBH_4$ and $NiCl_2{\cdot}H_2O$ to give amine, which was immediately acetylated to afford the resulting **13** in excellent yield (94%).[19] Oxidative cleavage of the anomeric MP (4–methoxyphenyl) moiety of **13** using ceric ammonium nitrate gave a lactol, which was converted into the corresponding trichloroacetimidate **14** using trichloroacetonitrile and DBU in 53% yield over two steps.[20] A TMSOTf–mediated coupling of trichloroacetimidate **14** with the acceptor **15** in a diethyl ether/dichloromethane (1:1) solvent system led to desired α-linked pentasaccharide **16** ($^1J_{H\text{-}H}$= 3.2 Hz, α/β ⩾20:1) in 42% yield. Finally, the deprotection of **16** started with removal of the acetyl groups using base (NaOMe), followed by using Pearlman's catalyst ($Pd(OH)_2/C$) and H_2 to afford the desired target compound **1** in 72% yield.

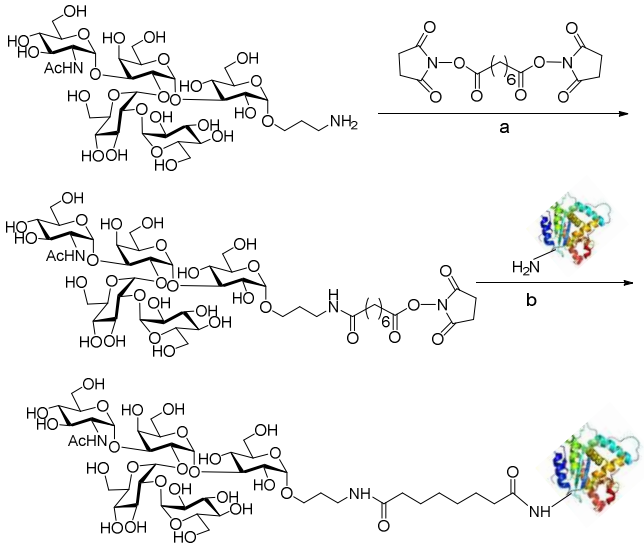


Scheme 2. Reagents and conditions: a) TMSOTf, 4 Å MS, Et_2O/CH_2Cl_2 1:1, –20 °C to room temperature, 55%; b) $NaOCH_3$, CH_3OH, 45 °C, 96%; c) TMSOTf, 4 Å MS, Et_2O/CH_2Cl_2 1:1, –20 °C to room temperature, 76%; d) i: $NaBH_4$, $NiCl_2{\cdot}H_2O$, CH_2Cl_2/MeOH 1:1.5; ii: Ac_2O, 94% (two steps); e) i: $(NH_4)_2Ce(NO_3)_6$, toluene/CH_3CN/H_2O 1:1.5:1; ii: CCl_3CN, DBU, DCM, 53% (two steps); f) TMSOTf, 4 Å MS, Et_2O/CH_2Cl_2 1:1, –25 °C to room temperature, 42%; g) i: $NaOCH_3$, CH_3OH; ii: H_2, $Pd(OH)_2/C$, 72% (two steps).

Conjugation of the pentasaccharide to carrier protein

To perform immunological experiments, the outer core pentasaccharide of *E. coli* R3 was conjugated to the nontoxic mutant of diphtheria toxin CRM_{197}.[21] CRM_{197} is an immunogenic carrier protein, which can improve the immunogenicity of oligosaccharide antigens and induce a T-cell dependent immune response.[22] This particular carrier

protein has been used as a constituent of licensed conjugate vaccines against *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* type B.[23] The outer core pentasaccharide–CRM_{197} glycoconjugate was prepared as illustrated in Scheme 3. The primary amine in the propyl amino linker of pentasaccharide **1** was treated with an excess of di–*N*–hydroxysuccinimidyl suberate in the presence of triethylamine and DMF to afford activated monoester.[24] The modified pentasaccharide with an activated ester was then conjugated to amino groups of the protein (the molar ratio: carbohydrate/protein 50:1) in PBS buffer (pH 7.4) to give the glycoprotein.[25] This glycoprotein was confirmed by SDS–PAGE analysis, displaying a shift toward a higher mass of glycoprotein compared with unconjugated CRM_{197} (Supplementary Information Figure S1). The corresponding saccharide loading ratio was 17.5 by MALDI–TOF mass spectrometry analysis (Supporting Information Figure S1), and the conjugation efficiency was 35%.[13b] Based on the same procedure, another glycoprotein pentasaccharide–BSA (Supplementary Information Figure S2) was also prepared as a coating antigen to install the plates for reflecting specific binding between penatsaccharide moiety and the induced anti–penatascchride antibody in following ELISA assay.[21]



Scheme 3. Reagents and conditions: (a) Et_3N, DMF; (b) CRM_{197} or BSA, 3 x PBS buffer, pH 7.4

Immunological evaluation of the pentasaccharide–CRM_{197} glycoconjugate

The resulting pentasaccharide–CRM197 glycoconjugate was evaluated for its ability to elicit antibody responses to pentasaccharide **1** in a mouse model. Freund's adjuvant was selected, because it is an effective adjuvant in mice that has been used to improve antibody responses to a synthetic oligosaccharide antigen.[21d] The initial immunization was performed with Freund's complete adjuvant (FCA), and the second and third immunizations were performed with Freund's incomplete adjuvant (FIA). Female BALB/c mice were subcutaneously immunized three times at biweekly intervals with 2.5 μg carbohydrate based doses of pentasaccharide–CRM_{197} conjugate formulated with adjuvant (FCA/FIA). The same dose of CRM_{197} formulated with adjuvant (FCA/FIA), adjuvant (FCA/FIA), and only PBS were used as negative controls. The serum antibodies of immunized mice against pentasaccharide were measured by ELISA assay (Figure 3). The total IgG titers were significantly increased in the serum of mice immunized with pentasaccharide–CRM_{197} compared with the other immunized groups ($P<0.001$). Moreover, higher IgM titers were also detected in the serum of mice immunized with pentasaccharide–CRM_{197} compared with the other immunized groups ($P<0.01$). These results indicated that the pentasaccharide–CRM_{197} glycoconjugate effectively elicited humoral immune responses in this mouse model.

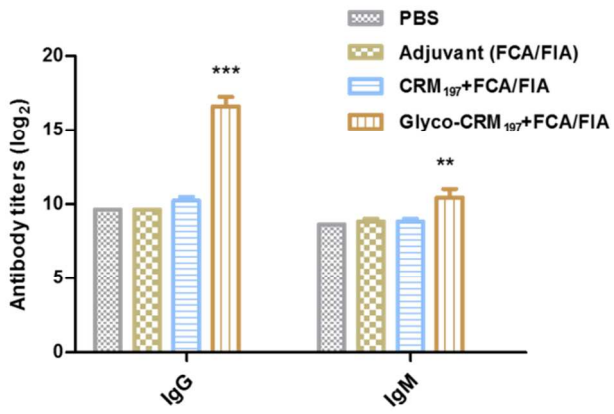


Figure 3. Evaluation of anti–pentasaccharide antibody titers (IgG and IgM) after the third immunization. The cutoff value was OD $_{\text{negative control}}$ ×2.1. Results were expressed as the arithmetic mean ± SD indicated by error bars. Differences of two groups were generated by t-test and indicated with symbols (**: $P < 0.01$ and ***: $P < 0.001$).

In order to illuminate the nature of immune response produced in mice immunized with pentasaccharide–CRM_{197} and its potential relevance to overall T cell phenotype, IgG subclass profiles were evaluated by ELISA assay (Figure 4). In pentasaccharide–CRM_{197} immunized mice, the antibody titers of IgG1 and IgG2b were dramatically increased (Figure 4A and C), indicating a Th2–type response.[26] Moreover, predominantly higher titers of IgG2a were produced in serum of the immunized mice with pentasaccharide–CRM_{197} compared with other groups (Figure 4B), which indicated that a Th1–type response was evoked in mice immunized with pentasaccharide–CRM_{197}.[26] In addition, an increase in IgG3 titers in group of pentasaccharide–CRM_{197} was also observed (Figure 4D), which is correlated with a Th1–type response.[26] The IgG subclass profiles indicated that not only a Th2–type response but also a Th1–type response was induced in mice immunized with pentasaccharide–CRM_{197}.

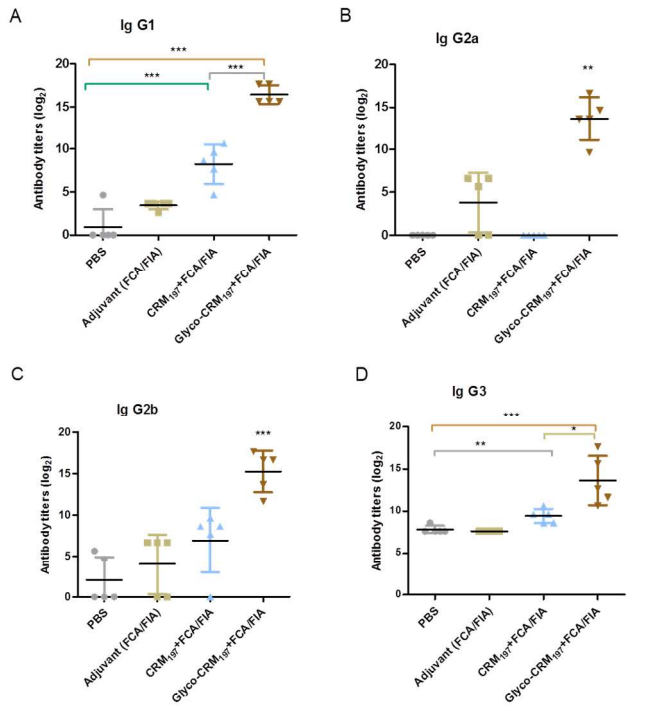


Figure 4. Antibody IgG subclass profiles after the third immunization, (A) IgG1 titers, (B) IgG2a titers, (C) IgG2b titers, (D) IgG3 titers. The cutoff value was $OD_{\text{negative controls}}$ ×2.1. Each point showed the titers for an individual mouse, and the horizontal lines indicated the means for the group of five mice. Results were represented as the arithmetic mean ± SD indicated by error bars. Differences of two groups were indicated with symbols (*: P < 0.05, **: P < 0.01 and ***: P < 0.001).

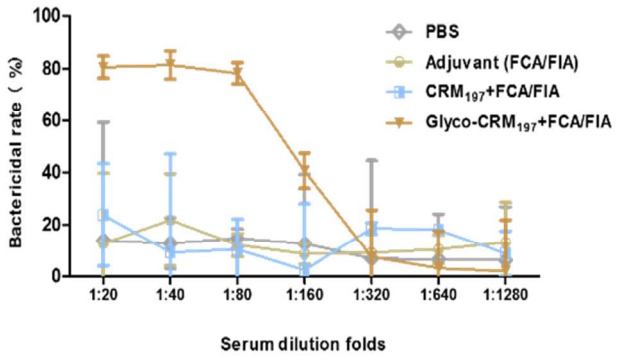


Figure 5. Bactericidal activity of serum from the immunized mice against *E. coli* O157:H7. The Bactericidal rate was described as the artithmetic mean ± SD indicated by error bars.

Bactericidal activity

To evaluate the bactericidal activity induced by pentasaccharide–CRM_{197} conjugate against an *E. coli* strain containing R3 outer core structure, diluted mouse serum samples were incubated with *E. coli* O157:H7 in rabbit sera and then developed by Cell Counting Kit–8 (CCK–8), which allows sensitive colorimetric assays for the determination of cell viability.[27] An approximate 50% killing of *E. coli* O157:H7 was observed with a 160–fold dilution of the serum from mice immunized with pentasaccharide–CRM_{197}, while 50% bactericidal activity was not achieved even with only a 20–fold dilution of the serum from the other groups. When the serum was diluted 80–fold from mice immunized with pentasaccharide–CRM_{197}, about 80% of bacteria were killed (Figure 5). These results indicated that the serum from mice immunized with pentasaccharide–CRM_{197} showed remarkable bactericidal activity against *E. coli* O157:H7.

Conclusions

In summary, we described the first total synthesis of the outer core pentasaccharide of *E. coli* R3 using a [4 + 1] coupling strategy. The orthogonal protecting groups modified galactose building block (B) made it possible to establish the optimal order of glycosylation for synthesis of the highly crowded 1,2,3–*cis* configured oligosaccharide. Furthermore, solvent effects and temperature were exploited to control the anomeric selectivity of glycosylation. These strategies will be significant for preparation of other highly branched oligosaccharides. The immunological evaluation of the pentasaccharide–CRM_{197} glycoconjugate indicated that this glycoconjugate was able to elicit specific anti-pentasaccharide antibodies with *in vitro* bactericidal activity against *E. coli* O157:H7. Overall, this work represented a new perspective in the design and synthesis of carbohydrate antigens to be explored as vaccine candidates.

Experimental

General procedures

All reagents were purchased from commercially sources and were used without further purification. All solvents were available with commercially dried or freshly dried and distilled prior to use. Reactions were monitored by thin layer chromatography (TLC) using silica gel GF_{254} plates with detection by short wave UV light (λ = 254 nm) and staining with 10% phosphomolybdic acid in EtOH or *p*–anisaldehyde solution (ethanol/*p*–anisaldehyde/acetic acid/sulfuric acid 135:5:4:1.5), followed by heating on a hot plate. Column chromatography was conducted by silica gel (200–300 mesh) with ethyl acetate and hexane as eluent. Optical rotation values were measured using a JASCO DIP-360 polarimeter at the ambient temperature in specified solvents. ^{1}H NMR and ^{13}C NMR were recorded with Bruker AV 400 spectrometer at 400 MHz (^{1}H NMR), 100 MHz (^{13}C NMR) using $CDCl_3$ and CD_3OD as solvents. Chemical shifts were reported in δ (ppm) from $CDCl_3$ (7.26 ppm for ^{1}H NMR, 77.00 ppm for ^{13}C NMR), CD_3OD (3.31 ppm for ^{1}H NMR, 49.00 ppm for ^{13}C NMR). Coupling constants were reported in hertz. High–resolution mass spectra (HRMS) were obtained on a Varian QFT–ESI mass spectrometer, and glycoproteins were analyzed by Bruker ultrafleXtreme MALDI TOF/TOF mass spectrometer.

Phenyl 2,3,4,6–tetra–*O*–benzyl–α–D–glucopyranosyl–(1→2)–*O*–3,4,6–tri–*O*–benzyl–1–thio–β–D–glucopyranoside (6)

A mixture of donor **2** (1.39 g, 2.03 mmol) and acceptor **3** (610 mg, 1.36 mmol) and 4 Å molecular sieves (2.0 g) in 10 mL dry

Et_2O/CH_2Cl_2 (1:1) was stirred at room temperature under Ar for 30 min. Then this mixture was cooled –40 °C, and TMSOTf (35 µL, 0.20 mmol) was added. The reaction was slowly warmed to room temperature in 1 h, TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 5:1, R_f = 0.36). The reaction was quenched by the addition of 0.1 mL triethylamine and filtered. The filtrate was concentrated in vacuum and purified by silica gel chromatography (hexane/ethyl acetate 10:1) to afford a syrup **4** (965 mg) contaminated by the rearrangement product of trichloroacetimidate donor **2**. ESI HRMS: m/z calcd for $C_{60}H_{64}NO_{10}S$ $[M+NH_4]^+$ 990.4251, found 990.4254.

This disaccharide syrup **4** (965 mg, 0.99 mmol) was dissolved in 10 mL CH_2Cl_2, 1 mL trifluoroacetic acid and 0.1 mL water was added. The mixture was stirred for 30 min at room temperature. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 1:1, R_f = 0.42). The reaction was quenched by the addition of 1.0 mL triethylamine. The mixture was washed with water, the organic layer was dried (Na_2SO_4) and filtered. The filtrate was concentrated in vacuum and purified by silica gel chromatography (hexane/ethyl acetate 2:1) to afford corresponding diol **5** as syrup (698 mg). ESI HRMS: m/z calcd for $C_{53}H_{56}O_{10}SNa$ $[M+Na]^+$ 907.3492, found 907.3478.

The above isolated diol (698 mg, 0.79 mmol) was dissolved in 10 mL DMF, and benzyl bromide (0.28 mL, 2.36 mmol) was added. Sodium hydride (60% dispersion in mineral oil, 94 mg, 2.36 mmol) was then added slowly at 0 °C. The reaction mixture was stirred at room temperature overnight. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 8:1, R_f = 0.17). The reaction was quenched with MeOH and concentrated in vacuum. The residue was dissolved with CH_2Cl_2, and the organic layer was washed with water, followed by drying and filtration. The filtrate was concentrated in vacuum and purified by silica gel chromatography (hexane/ethyl acetate 8:1) to afford a purified syrup **6** (768 mg, 53% from **3**, three steps). $[\alpha]_D^{20}$ = +61.5 (c = 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.14 (dd, J = 2.0 Hz, J = 10.4 Hz, 1 H), 3.25 (d, J = 10.0 Hz, 1 H), 3.53–3.55 (m, 1 H), 3.64–3.78 (m, 6 H), 3.92–4.01 (m, 2 H), 4.09 (d, J = 10.4 Hz, 1 H), 4.24 (d, J = 12.0 Hz, 1 H), 4.36 (d, J = 11.2 Hz, 1 H), 4.48–4.60 (m, 4 H), 4.73 (d, J = 11.6 Hz, 1 H), 4.76–4.83 (m, 4 H), 4.87 (d, J = 9.6 Hz, 1 H, H–1^C), 4.92 (d, J = 11.6 Hz, 1 H), 4.76-4.83 (m, 2 H), 5.94 (d, J = 3.6 Hz, 1 H, H–1^E), 7.00–7.05 (m, 4 H), 7.10–7.14 (m, 3 H), 7.17–7.32 (m, 31 H), 7.50–7.52 (m, 2 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 67.75, 68.73, 70.08, 73.05, 73.21, 73.30, 74.51, 74.65, 74.75, 75.43, 75.52 77.45, 78.52, 78.77, 79.61, 81.67, 84.62, 86.86 (C–1^C), 95.61(C–1^E), 127.12, 127.18, 127.22, 127.33, 127.39, 127.42, 127.46, 127.57, 127.67, 127.76, 127.87, 127.94, 127.99, 128.09, 128.17, 128.26, 128.81, 131.11, 133.34, 137.66 , 137.70, 137.74 , 137.87, 138.02, 138.61, 138.72 ESI HRMS: m/z calcd for $C_{67}H_{72}NO_{10}S$ $[M+NH_4]^+$ 1082.4877, found 1082.4865.

2,3,4,6–Tetra–*O*–benzyl–α–D–glucopyranosyl–(1→2)–*O*–3,4,6–tri–*O*–benzyl–β–D–glucopyranosyl trichloroacetimidate (7)

To a solution of **6** (750 mg, 0.7 mmol) in acetone (10 mL) and water (1.0 mL) was added NBS (250 mg, 1.4 mmol) at 0 °C. The mixture was stirred for 3 h at room temperature. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 3:1, R_f = 0.25). The reaction was quenched by the addition of 10 mL $Na_2S_2O_{3(sat.)}$ solution. The acetone was removed in vacuum, the crude product was diluted with CH_2Cl_2 (30 mL), washed with H_2O (10 mL). And the aqueous phase was extracted by CH_2Cl_2 (20 mL), the organic fractions were combined, dried over Na_2SO_4 and filtered. The filtrate was concentrated in vacuum and purified by silica gel chromatography (hexane/ethyl acetate 3:1) to afford corresponding hemiacetal as syrup (540 mg). ESI HRMS: m/z calcd for $C_{61}H_{68}NO_{11}$ $[M+NH_4]^+$ 990.4792, found 990.4805.

A mixture of the above isolated hemiacetal (540 mg, 0.55 mmol), CCl_3CN (1 mL) and DBU (0.1 mL) in anhydrous CH_2Cl_2 (8 mL) was stirred for 2 h at room temperature. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 3:1, R_f = 0.70). The mixture was concentrated in vacuum and purified by silica gel chromatography (hexane/ethyl acetate 5:1) to afford a syrup **7** (532 mg, 68%, two steps) for next coupling step quickly.

4–Methoxyphenyl 2,3,4,6–tetra–*O*–benzyl–α–D–glucopyranosyl–(1→2)–*O*–3,4,6–tri–*O*–benzyl–α–D–glucopyranosyl–(1→2)–3–*O*–benzoyl–4,6–*O*–benzylidene–β–D–galactopyranoside (9)

A mixture of donor **7** (503 mg, 0.45 mmol) and acceptor **8** (258 mg, 0.54 mmol) and 4 Å molecular sieves (1.0 g) in 8 mL anhydrous Et_2O/CH_2Cl_2 (1:1) was stirred at room temperature under Ar for 30 min. Then this mixture was cooled –20 °C, TMSOTf (12 µL, 0.068 mmol) was added. The reaction was warmed slowly room temperature in 1 h, TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 3:1, R_f = 0.25). The reaction was quenched by the addition of 0.1 mL triethylamine and filtered. The filtrate was concentrated in vacuum and purified by silica gel chromatography (hexane/ethyl acetate 5:1) to afford a syrup trisaccharide **9** (354 mg, 55%). $[\alpha]_D^{20}$ = +83.5 (c = 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.26 (dd, J = 3.6 Hz, J = 9.6 Hz, 1 H), 3.46 (dd, J = 3.2 Hz, J = 10.0 Hz, 1 H), 3.54–3.64 (m, 3 H), 3.67–3.70 (m, 2 H), 3.74 (s, 3 H, Ph*OCH*$_3$), 3.86–3.87 (m, 2 H), 3.95–4.08 (m, 4 H), 4.18 (d, J = 12.4 Hz, 1 H), 4.23 (d, J = 11.6 Hz, 1 H), 4.31 (d, J = 11.6 Hz, 1 H), 4.40–4.43 (m, 3 H), 4.46–4.52 (m, 2 H), 4.57–4.66 (m, 5 H), 4.67–4.72 (m, 2 H, H–1^B, Ph*CH*$_2$), 4.74–4.86 (m, 4 H), 5.06 (d, J = 3.6 Hz, 1 H, H–1^C), 5.28 (dd, J = 3.6 Hz, J = 10.0 Hz, 1 H), 5.48 (s, 1 H, Ph*CH*), 5.92 (d, J = 2.0 Hz, 1 H, H–1^E), 6.75–6.85 (m, 6 H), 6.94–6.98 (m, 2 H), 7.08–7.13 (m, 3 H), 7.17 (m, 5 H), 7.19–7.25 (m, 10 H), 7.27–7.36 (m, 14 H), 7.40–7.48 (m, 7 H), 8.01-8.03 (m, 2 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 55.55 (Ph*OCH*$_3$), 65.76, 68.14, 68.34, 68.64, 69.10, 70.21, 70.33, 71.43, 73.07, 73.27, 73.31, 73.44, 73.73, 74.26, 74.78, 75.33, 76.13, 77.64, 79.45, 80.32, 81.69, 92.88 (C–1^C), 93.58 (C–1^E), 100.69 (Ph*CH*), 101.22 (C–1^B), 114.49, 117.00, 126.17, 127.02, 127.08, 127.29, 127.38, 127.49, 127.51, 127.67, 127.75, 127.86, 127.95, 128.00, 128.11, 128.18, 128.28, 128.31, 128.63, 128.80, 129.55, 129.78, 133.18, 137.63, 137.99, 138.15, 138.21, 138.53, 138.68, 138.72, 139.32, 150.37, 154.99, 166.22 (Ph*CO*); ESI

HRMS: m/z calcd for $C_{88}H_{88}O_{18}Na$ $[M+Na]^+$ 1455.5868, found 1455.5809.

4–Methoxyphenyl 2,3,4,6–tetra–*O*–benzyl–α–D–glucopyranosyl–(1→2)–*O*–3,4,6–tri–*O*–benzyl–α–D–glucopyranosyl–(1→2)–*O*–4,6–benzylidene–β–D–galactopyranoside (10)

A solution of trisaccharide (215 mg, 0.15 mmol) in dry methanol (4 mL) was added sodium methoxide (10 mg, 0.18 mmol). The solution was stirred at 45 °C overnight. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 2:1, R_f = 0.23). Then the acid resin (Dowex® 50WX2 H^+–form) was added and stirred to adjust pH 7, followed by filtration. The filtrate was concentrated in vacuum and purified by silica gel chromatography (hexane/ethyl acetate 2:1) to afford a syrup **10** (191 mg, 96%). $[\alpha]_D^{20}$ = +75.0 (c = 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.13 (s, br, 1 H, OH), 3.23–3.26 (m, 2 H), 3.42–3.49 (m, 2 H), 3.63–3.78 (m, 9 H), 3.95 (dd, J = 4.0 Hz, J = 9.6 Hz, 1 H), 4.01–4.08 (m, 3 H), 4.11 (dd, J = 3.2 Hz, J = 9.2 Hz, 1 H), 4.16 (dd, J = 7.6 Hz, J = 9.6 Hz, 1 H), 4.23–4.28 (m, 3 H), 4.36 (d, J = 11.6 Hz, 1 H), 4.43–4.49 (m, 3 H), 4.54–4.61 (m, 2 H), 4.62–4.65 (m, 2 H, H–1^B, PhCH_2), 4.70 (d, J = 11.6 Hz), 4.75–4.79 (m, 2 H), 4.81–4.86 (m, 2 H), 4.98 (d, J = 10.4 Hz, 1 H), 5.22 (d, J = 3.2 Hz, 1 H, H–1^C), 5.55 (s, 1 H, Ph*CH*), 5.79 (d, J = 4.0 Hz, 1 H, H–1^E), 6.75–6.77 (m, 2 H), 6.85–6.87 (m, 2 H), 7.01–7.05 (m, 2 H), 7.13–7.17 (m, 10 H) 7.23–7.34 (m, 26 H), 7.48–7.56 (m, 2 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 55.57 (PhOCH_3), 66.21, 68.60, 68.79, 70.02, 70.51, 71.38, 73.15, 73.23, 73.95, 74.00, 74.77, 75.34, 75.73, 76.24, 77.71, 78.15, 79.79, 81.22, 81.58, 92.57 (C–1^C), 94.41 (C–1^E), 100.61 (Ph*CH*), 101.37 (C–1^B), 114.51, 116.92, 126.32, 127.09, 127.30, 127.38, 127.49, 127.61, 127.67, 127.92, 128.08, 128.13, 128.15, 128.24, 128.30, 128.61, 129.04, 137.49, 137.87, 138.25, 138.35, 138.37, 138.55, 138.75, 139.15, 150.62, 154.94; ESI HRMS: m/z calcd for $C_{81}H_{84}O_{17}Na$ $[M+Na]^+$ 1351.5606, found 1351.5599.

4–Methoxyphenyl 2,3,4,6–tetra–*O*–benzyl–α–D–glucopyranosyl–(1→2)–*O*–3,4,6–tri–*O*–benzyl–α–D–glucopyranosyl–(1→2)–[6–*O*–acetyl–2–azido–3,4–di–*O*–benzyl–2–deoxy–α–D–glucopyranosyl–(1→3)]–4,6–*O*–benzylidene–β–D–galactopyranoside (12)

A mixture of donor **11** (103 mg, 0.18 mmol) and acceptor **10** (160 mg, 0.12 mmol) and 4 Å molecular sieves (200 mg) in 8 mL anhydrous Et_2O/CH_2Cl_2 (1:1) was stirred at room temperature under Ar for 30 min. Then this mixture was cooled –20 °C, TMSOTf (3.3 µL, 0.018 mmol) was added. The reaction was slowly warmed room temperature in 2 h, TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 2:1, R_f = 0.18). The reaction was quenched by the addition of 0.1 mL triethylamine and filtered. The filtrate was concentrated in vacuum and purified by silica gel chromatography (hexane/ethyl acetate 2:1) to afford as a syrup tetrasaccharide **12** (158 mg, 76%). $[\alpha]_D^{20}$ = +86.0 (c = 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.95 (s, 3 H, CH_3CO), 3.12–3.35 (m, 2 H), 3.49 (dd, J = 3.2 Hz, J = 10.4 Hz, 1 H), 3.57 (dd, J = 2.4 Hz, J = 9.2 Hz, 1 H), 3.63–3.69 (m, 2 H), 3.74–3.76 (m, 4 H), 3.81–3.87 (m, 2 H), 3.88–3.93 (m, 2 H), 3.98–3.99 (m, 2 H), 4.02–4.08 (m, 3 H), 4.12 (dd, J = 4.4 Hz, J = 10.4 Hz, 1 H), 4.25–4.28 (m, 2 H), 4.31–4.35 (m, 2 H), 4.38–4.44 (m, 2 H), 4.45–4.50 (m, 3 H), 4.53–4.57 (m, 5 H), 4.59–4.64(m, 4 H, H–1^B, PhCH_2), 4.75–4.81 (m, 6 H), 4.84–4.89 (m, 2 H), 5.02 (d, J = 3.2 Hz, 1 H, H–1^D), 5.25 (d, J = 3.6 Hz, 1 H, H–1^C), 5.56 (s, 1 H, Ph*CH*), 6.06 (s, 1 H, H–1^E), 6.76–6.78 (m, 2 H), 6.83–6.85 (m, 2 H), 6.97–7.00 (m, 6 H), 7.08–7.13 (m, 10 H), 7.15–7.25 (m, 15 H), 7.27–7.33 (m, 17 H), 7.54–7.56 (m, 2 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 20.79 (CH_3CO), 55.56 (PhOCH_3), 62.19, 63.20, 65.85, 68.31, 68.47, 68.96, 69.66, 70.07, 70.27, 70.43, 71.68, 73.27, 73.49, 74.43, 74.80, 75.05, 75.11, 75.35, 75.94, 77.22, 77.74, 78.10, 79.33, 80.17, 81.19, 81.83, 93.01 (C–1^E), 93.15 (C–1^C), 93.26 (C–1^D), 100.58 (Ph*CH*), 101.71 (C–1^B), 114.55, 117.16, 126.01, 127.09, 127.18, 127.27, 127.31, 127.37, 127.43, 127.47, 127.62, 127.73, 127.82, 127.90, 127.96, 128.09, 128.17, 128.24, 128.36, 128.49, 128.75, 137.43, 137.49, 137.84, 138.16, 138.24, 138.44, 138.75, 138.77, 139.05, 150.46, 155.08, 170.20 (CH_3CO); ESI HRMS: m/z calcd for $C_{103}H_{107}N_3O_{22}Na$ $[M+Na]^+$ 1760.7244, found 1760.7251.

4–Methoxyphenyl 2,3,4,6–tetra–*O*–benzyl–α–D–glucopyranosyl–(1→2)–*O*–3,4,6–tri–*O*–benzyl–α–D–glucopyranosyl–(1→2)–[6–*O*–acetyl–2–acetamido–3,4–di–*O*–benzyl–2–deoxy–α–D–glucopyranosyl–(1→3)]–4,6–*O*–benzylidene–β–D–galactopyranoside (13)

To a stirred solution of tetrasaccharide **12** (140 mg, 0.08 mmol) in 5 mL DCM/MeOH (1:1.5) was cooled at 0 °C, $NiCl_2{\cdot}6H_2O$ (114 mg, 0.48 mmol) and $NaBH_4$ (30 mg, 0.8 mmol) was added. The color of the solution changed from green to black. The reaction mixture was maintained at 10 °C for 2 h, TLC analysis showed the starting material disappeared, followed by adding Ac_2O (0.1 mL) at 10 °C. The reaction mixture was stirred at 10 °C for 1 h, TLC analysis showed a major product (hexane/ethyl acetate 1:2, R_f = 0.42), then the reaction was quenched with 0.1 mL triethylamine. The mixture was concentrated in vacuo to give a residue, which was purified by silica gel chromatography (hexane/ethyl acetate 2:3) to afford a syrup **13** (132 mg, 94%). $[\alpha]_D^{20}$ = +83.8 (c = 0.16, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.33 (s, 3 H, NHCOCH_3), 2.01 (s, 3 H, CH_3CO), 3.23 (dd, J = 3.6 Hz, J = 9.6 Hz, 1 H), 3.29 (dd, J = 3.6 Hz, J = 10.8 Hz, 1 H), 3.56–3.66 (m, 3 H), 3.73 (s, 3 H, PhOCH_3), 3.76–3.82 (m, 2 H), 3.89–4.03 (m, 7 H), 4.09–4.15 (m, 2 H), 4.22 (d, J = 12.4 Hz, 1 H), 4.32–4.33 (m, 1 H), 4.38–4.48 (m, 6 H), 4.51–4.58 (m, 6 H), 4.60–4.66 (m, 5 H, H–1^B, PhCH_2), 4.71–4.88 (m, 8 H), 5.07 (d, J = 3.6 Hz, 1 H, H–1^D), 5.09 (d, J = 4.0 Hz, 1 H, H–1^C), 5.42 (s, 1 H, Ph*CH*), 5.99 (d, J = 2.8 Hz, 1 H, H–1^E), 6.54 (d, J = 10.0 Hz, 1 H, Ac*NH*), 6.75–6.83 (m, 4 H), 6.95–7.07 (m, 9 H), 7.09–7.21 (m, 20 H), 7.25–7.30 (m, 17 H), 7.37–7.43 (m, 4 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 20.76 (NHCOCH_3), 22.20 (CH_3CO), 52.12, 55.42, 62.32, 65.71, 68.21, 68.41, 68.86, 69.79, 69.92, 71.67, 72.22, 72.36, 72.90, 73.02, 73.58, 74.75, 74.84, 74.87, 75.21, 76.07, 76.88, 77.00, 77.55, 77.81, 79.54, 81.29, 81.44, 81.50, 92.31 (C–1^C), 92.65 (C–1^D), 93.01 (C–1^E), 101.19 (C–1^B), 101.25 (Ph*CH*), 114.44, 116.72, 126.26, 126.75, 127.19, 127.28, 127.37, 127.44, 127.52, 127.64, 127.89, 127.93, 128.00, 128.06, 128.11, 128.16, 128.24, 128.29, 129.02, 137.39, 137.61, 137.68, 137.76, 138.02, 138.11,

138.31, 138.34, 138.47, 139.01, 150.20, 154.96, 170.36 (NH*CO*CH_3),170.46 (CH_3*CO*); ESI HRMS: m/z calcd for $C_{105}H_{111}NO_{23}Na$ $[M+Na]^+$ 1776.7445, found 1776.7452.

2,3,4,6–tetra–*O*–benzyl–α–D–glucopyranosyl–(1→2)–*O*–3,4,6–tri–*O*–benzyl–α–D–glucopyranosyl–(1→2)–[6–*O*–acetyl–2–acetamido–3,4–di–*O*–benzyl–2–deoxy–α–D–glucopyranosyl–(1→3)]–4,6–*O*–benzylidene–β–D–galactopyranosyl trichloroacetimidate (14)

To a solution of **13** (123 mg, 0.07 mmol) in 1:1.5:1 toluene–MeCN–water (7 ml) was added ceric ammonium nitrate (192 mg, 0.35 mmol) at 0 °C. The mixture was stirred vigorous and was warmed room temperature in 30 min. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 1:2, R_f = 0.40), then the mixture was diluted with ethyl acetate, washed with water, saturated $NaHCO_{3\,(aq)}$, and brine, dried (Na_2SO_4) and filtered. The filtrate was concentrated in vacuum and purified by silica gel chromatography (hexane/ethyl acetate 2:3) to afford the corresponding hemiacetal (83 mg). ESI HRMS: m/z calcd for $C_{98}H_{105}NO_{22}Na$ $[M+Na]^+$ 1670.7026, found 1670.7032.

To a solution of the above isolated hemiacetal (83 mg, 0.05 mmol) in dry DCM was added 0.5 mL CCl_3CN and 0.05 mL DBU. The mixture solution was stirred at room temperature for 3 h under the Ar atmosphere. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 1:2, R_f = 0.74). The mixture was concentrated in vacuum to give a residue, which was purified by silica gel chromatography (hexane/ethyl acetate 1:1) to afford a yellow syrup **14** (66 mg, 53% from **13**) for next coupling step quickly.

3–Azidopropyl 2,3,4,6–tetra–*O*–benzyl–α–D–glucopyranosyl–(1→2)–*O*–3,4,6–tri–*O*–benzyl–α–D–glucopyranosyl–(1→2)–[6–*O*–acetyl–2–acetamido–3,4–di–*O*–benzyl–2–deoxy–α–D–glucopyranosyl–(1→3)]–*O*–4,6–benzylidene–β–D–galactopyranosyl–(1→3)–2,4,6–tri–*O*–benzyl–α–D–glucopyranoside (16)

A mixture of donor **14** (65 mg, 0.036 mmol) and acceptor **15** (28.8 mg, 0.054 mmol) and 4 Å molecular sieves (100 mg) in dry 6 mL Et_2O/CH_2Cl_2 (1:1) was stirred at room temperature under Ar for 30 min. Then this mixture was cooled –25 °C, TMSOTf (1 μL, 0.0054 mmol) was added. The reaction was slowly warmed room temperature in 2 h, TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 1:1, R_f = 0.37). The reaction was quenched by the addition of 0.1 mL triethylamine and filtered. The filtrate was concentrated in vacuum and purified by silica gel chromatography (hexane/ethyl acetate 3:2) to afford a syrup **16** (33 mg, 42%). $[\alpha]_D^{20}$ = +87.4 (c = 0.8, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.33 (s, 3 H, NHCO*CH*$_3$), 1.72 (s, 3 H, *CH*$_3$CO), 1.92 (m, 2 H, $OCH_2CH_2CH_2N_3$), 3.32 (m, 2 H), 3.43 (m, 3 H), 3.62–3.71 (m, 8 H), 3.76–3.80 (m, 6 H), 3.91–4.08 (m, 7 H), 4.10–4.14 (m, 2 H), 4.23 (m, 3 H), 4.33–4.38 (m, 5 H), 4.43–4.90 (m, 3 H), 4.54–4.60 (m, 5 H), 4.66–4.76 (m, 6 H), 4.83–4.87 (m, 5 H), 4.89–4.98 (m, 4 H), 5.16 (d, J = 3.6 Hz, 1 H, H–1^D), 5.22 (s, 1 H, PhC*H*), 5.31 (d, J = 3.2 Hz, 1 H, H–1^B), 5.51-5.58 (m, 2 H, H–1^C and AcN*H*), 5.91 (s, 1 H, H–1^E), 6.97–7.00 (m, 2 H), 7.11–7.22 (m, 24 H), 7.29–7.42 (m, 37 H), 7.57 (m, 2 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 20.69 (NHCO*CH*$_3$), 22.58 (*CH*$_3$CO), 28.79 ($OCH_2CH_2CH_2N_3$), 48.20 ($OCH_2CH_2CH_2N_3$), 52.30, 61.43, 62.02, 64.57, 68.48, 68.88, 69.27, 69.57, 70.00, 70.12, 70.47, 71.73, 72.62, 73.00, 73.44, 74.93, 75.00, 75.20, 75.47, 76.15, 77.26, 77.73, 78.89, 79.09, 80.60, 81.37, 81.82, 93.58 (C–1^C), 93.71 (C–1^B), 93.78 (C–1^D), 95.87 (C–1^E), 95.88 (C–1^A), 100.98 (PhC*H*), 126.23, 127.19, 127.31, 127.35, 127.44, 127.48, 127.53, 127.64, 127.75, 127.86, 128.08, 128.12, 128.17, 128.23, 128.26, 128.31, 128.37, 128.44, 128.57, 128.73, 129.07, 137.72, 137.77, 137.88, 138.13, 138.23, 138.29, 138.40, 138.84, 139.22, 170.04 (NH*CO*CH_3), 170.08 (CH_3*CO*); ESI HRMS: m/z calcd for $C_{128}H_{139}N_4O_{27}$ $[M+H]^+$ 2163.9627, found 2163.9648.

3–aminopropyl α–D–glucopyranosyl–(1→2)–α–D–glucopyranosyl–(1→2)–[2–acetamido–2–deoxy–α–D–glucopyranosyl–(1→3)]–α–D–galactopyranosyl–(1→3)–α–D–glucopyranoside (1)

A solution of 16 (22 mg, 0.01 mmol) in dry methanol (2 mL) was added catalytic amount of sodium methoxide (pH 9–10). The solution was stirred at room temperature for 2 h. TLC analysis showed complete conversion of starting material to a major product (hexane/ethyl acetate 1:2 R_f = 0.33). Then the acid resin (Dowex® 50WX2 H^+–form) was added and stirred to adjust pH 7, followed by filtration. The filtrate was concentrated in vacuum to afford a syrup (16 mg). ESI HRMS: m/z calcd for $C_{126}H_{137}N_4O_{26}$ $[M+H]^+$ 2121.9521, found 2121.9507.

To a stirred solution of the above syrup (16 mg, 0.0075 mmol) in 2 mL methanol was added $Pd(OH)_2/C$ (20 mg) at 50 psi H_2 atmosphere for 3 days. The catalyst was filtered off, the filtrate was concentrated to afford a white solid **1** (6.7 mg, 72% from **16**). $[\alpha]_D^{20}$ = +178.0 (c = 0.2, H_2O); ^{1}H NMR (CD_3OD, 400 MHz): δ 1.97–2.00 (m, 2 H, $OCH_2CH_2CH_2N_3$), 2.02 (s, 3 H, NHCO*CH*$_3$), 3.15–3.20 (m, 2 H), 3.35–3.44 (m, 6 H), 3.58–3.61 (m, 3 H), 3.68–3.77 (m, 10 H), 3.85–3.87 (m, 2 H), 3.91–3.99 (m, 6 H), 4.09 (m, 2 H), 4.25–4.30 (m, 3 H), 5.04–5.05 (m, 2 H, H–1^A and H–1^D), 5.17 (d, J = 3.6 Hz, 1 H, H–1^B), 5.47 (d, J = 3.2 Hz, 1 H, H–1^C), 5.79 (s, H–1^E), ^{13}C NMR (CD_3OD, 100 MHz): δ 20.43 (*CH*$_3$CO), 28.34 ($OCH_2CH_2CH_2N_3$), 37.91 ($OCH_2CH_2CH_2N_3$), 52.91, 59.42, 60.10, 60.56, 60.73, 64.29, 66.30, 66.83, 68.93, 69.01, 69.18, 69.60, 70.23, 70.40, 70.55, 71.03, 71.21, 71.28, 71.49, 71.66, 72.18, 72.57, 75.15, 76.84, 90.77 (C–1^C), 91.96 (C–1^B), 93.67 (C–1^D), 95.98 (C–1^E), 98.76 (C–1^A), 167.49 (CH_3*CO*); ESI HRMS: m/z calcd for $C_{35}H_{62}N_2O_{26}Na$ $[M+Na]^+$ 949.3489, found 949.3484.

Preparation of NHS activated pentasaccharide

A solution of the propyl amino–linked pentasaccharide **1** (2.0 mg, 0.002 mmol) in DMF (0.5 mL) containing triethylamine (50 μL), was added dropwise to a stirred solution of di–*N*–hydroxysuccinimidyl suberate (7.4 mg, 0.02 mmol) in DMF (0.5 mL). The reaction was kept under gentle stirring at room temperature for 3 h. TLC analysis showed complete conversion of starting material to a major product (ethyl acetate/methanol/water/acetic acid 5:1.5:0.75:0.15, R_f = 0.50). The reaction was then concentrated, the residue was added 0.5 mL water,

extracted with EtOAc (1 mL x 4). The aqueous layer was lyophilized to give NHS activated pentasaccharide as a white power (2.0 mg). ESI HRMS: m/z calcd for $C_{47}H_{77}N_3O_{31}Na$ $[M+Na]^+$ 1202.4439, found 1202.4453.

Conjugate NHS activated pentasaccharide to CRM_{197} and BSA

The NHS activated pentasaccharide was conjugated to CMR_{197} (or BSA) at a molar ratio 50:1 in 3 x PBS buffer (pH 7.4). The solution was incubated overnight at room temperature. Then the resultant solution was ultrafiltrated and washed with 1 x PBS buffer using Amicon Centrifugal Filter Devices (Ultracel 10, 000). The glycoproteins solution was lyophilized to give a white solid. Glycoproteins were analyzed by Bruker ultrafleXtreme MALDI TOF/TOF mass spectrometer.

SDS–PAGE

The glycoproteins (5 μg) and CRM_{197} (or BSA) (5 μg) were suspended in 10 μL of sample buffer (5% (w/v) SDS, 10% (v/v) glycerol, 25 mM Tris–HCl, pH 6.8, 10 mM DTT, 0.01% (w/v) bromophenol blue), loaded into different lanes of a 1.5–mm–thick, 12% (w/v) SDS–PAGE gel, and visualized by Coomassie Brilliant Blue R–250 staining.

Immunization of mice

The mice were purchased from Vital River Laboratories. Groups of 5 female BALB/c mice (6–8 week old) were subcutaneously (several different sites with a total of 150 μL) immunized on days 0, 14, and 28 with 2.5 μg carbohydrate antigen based doses of pentasaccharide–CRM_{197} conjugate formulated with Freund's adjuvant (mixing equal volume of conjugate and Freund's adjuvant, v/v,1:1). PBS, Freund's adjuvant and CRM_{197}/Freund's adjuvant (v/v,1:1） were used as controlled groups. For immunizations with Freund's adjuvant, the first immunization was performed with Freund's complete adjuvant (FCA) (Sigma–Aldrich, F5881), and the second and third immunizations with Freund's incomplete adjuvant (FIA) (Sigma–Aldrich, F5506). Seven days after the third immunization, the blood was taken via lateral saphenous, and then centrifuged for 20 min at 4,000 RPM to collect the sera (without anticoagulants). These collected sera were ready to the following ELISA assay and bactericidal activity assay.

ELISA assay

The ELISA 96–well plates (Corning®, #3590) were coated with 1 μg/mL pentasaccharide–BSA in 1 x PBS buffer (pH 7.4) overnight at 4 °C. The coated plates were washed three times with PBS buffer containing 0.05% (v/v) Tween 20 (PBST) (pH 7.4), and then blocked for 2 hours at room temperature with 2% BSA (w/v) in PBST. The coated plates were incubated with 100 μL of sera diluted with dilution buffer (1% BSA, PBST) 2–fold from 1:200 to 204,800 for 2 hours at room temperature after being washed three times with PBST. Then quintuple PBST washing was performed, and 100 μL/well of 1:3000-1:20000 HRP–conjugated goat anti-mouse IgG (Invitrogen, USA), IgG1, IgG2a, IgG2b, IgG3 and IgM (Abcam) were added respectively, and incubated 1 hour at room temperature. After the plates were washed with PBST, 100 μL/well of enzyme substrate tetramethylbenzidine (TMB) solution was added and incubated for 15 min (a blue color developed for the sera with antibodies) before the enzymatic reaction was terminated by adding 1 M HCl, and the OD of each sample was measured at 450 nm with a microplate spectrophotometer (Biorad). Antibody titers were expressed by evaluating the logarithm of the reciprocal of sera dilution based two. The statistical and graphical analysis was performed using GraphPad Prism 5 software.

Bactericidal activity

Complement-dependent bactericidal activity was measured in serum of day 35 (one week after the third immunization). *E. coli* O157:H7 containing R3 outer core was used as the test strain. 104 bacteria per mL suspended in 1% peptone supplemented with 5% 3–week–old rabbit sera as the complement source were added to 96–well plates (Costar®, # 3590), and mixed with serum diluted with 2–folds PBS (pH 7.4) from 1:20 to 1:12,800. The mixture was incubated at 37°C for 1 h. 10 μL/well of the CCK8 was added, and plates were incubated for 6 h. The absorbance of plates at 450 nm was read by a microplate spectrophotometer (Biorad). Percent bactericidal activity was calculated as the proportion of the dead bacteria exposed to immunized–serum compared with the number of bacteria in control culture condition.

Statistical analysis

All the statistical analyses and figures were generated by GRAPHPAD PRISM software version 5.0. Data were shown as means ± standard deviation (SD). The difference between two groups was compared by *t* test. For multiple comparisons, one–way ANOVA was used. A probability (P) value ≤ 0.05 was considered statistically significant.

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