Pathogen Detection

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Plague Detection by Anti-carbohydrate Antibodies**

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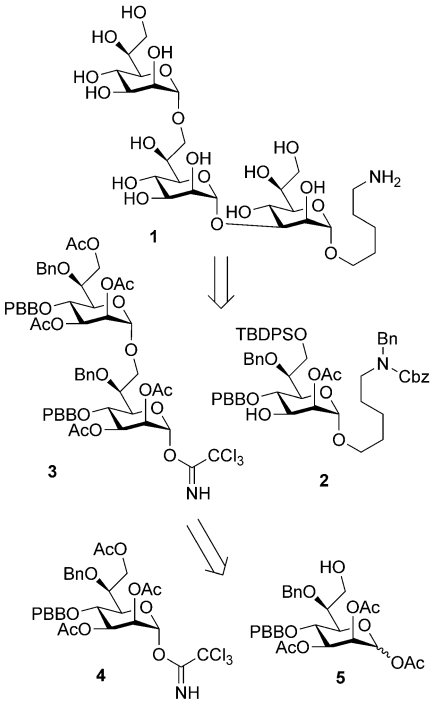
Dedicated to the Bayer company on the occasion of its 150th anniversary

"Black Death" decimated the world population during the middle ages. In total, three plague pandemics killed more than 200 million people over the past 1500 years. The Bubonic plague is caused by the non-sporulating bacterium *Yersinia pestis*.[1] Recent plague cases have been reported in Africa and Asia.[2] *Y. pestis* is a category-A biothreat agent owing to its ready person-to-person dissemination and high lethality.[3] The intentional dissemination of plague by aerosolization would cause fulminant pneumonia in exposed individuals.[4] Since pneumonic plague is typically fatal when untreated, early detection is essential to implement effective preventive measures.[5] Currently, *Y. pestis* samples are tested by polymerase chain reaction (PCR)-based assays or traditional phenotyping[6] that are accurate means of detection, but are complex, expensive, and slow.[7] Antibody binding to surface protein antigens is a promising and less complicated option for plague detection.[8] But higher false-positive and negative readouts as well as differentiation between related bacteria have made it difficult to create selective, reliable, antibody-based detection systems.[9] The inner core of *Yersinia* bacterial surface lipopolysaccharide (LPS) has a unique structure that, unlike most Gram-negative LPS, does not contain O-side chains.[10] The LPS from *Y. pestis* is immunodominant and LPS specific antibodies in patient sera are of diagnostic value.[10a]

Herein, we describe the synthesis of a plague-specific oligosaccharide antigen that facilitates fabricating glycan arrays to screen patient sera and other biological samples. LPS-specific monoclonal antibodies (mAbs) serve to detect *Y. pestis* bacteria. Incorporation of anti-*Y. pestis* cell surface LPS mAbs into point-of-care diagnostic systems would provide the basis for specific detection.

Since the isolation of LPS from *Y. pestis* is difficult because of variations in LPS expression,[11] these antigens are produced synthetically. The triheptose motif from the inner core structure (-L-α-D-Hep*p*-(1→7)-L-α-D-Hep*p*-(1→3)-L-α-D-Hep*p*) served as the antigen target for antibody generation (Scheme 1). Trisaccharide hapten **1** was designed to carry a primary amine at the reducing terminus through a linker for conjugation to a protein carrier. Retrosynthetic analysis revealed heptoside disaccharide trichloroacetimidate **3** and heptoside **2**[12] as key intermediates (Scheme 1). Disaccharide **3** in turn can be derived from heptose building blocks **4** and **5**.



Scheme 1. Retrosynthetic analysis of the *Y. pestis* LPS inner core heptose trisaccharide.

The synthesis of heptose building blocks **2** and **4** commenced with reduction of lactone **6**[12] with lithium tri-*tert*-butoxyaluminum hydride to give hemiacetals **7** and **8** (Scheme 2). Migration of the *tert*-butyldimethylsilyl (TBS) ether from C-2 to C-3 occurred under the basic conditions. Desilylation of both **7** and **8** using TBAF and subsequent acetylation provided intermediate **9**. Trichloroacetimidate **4** was obtained in good to excellent yields from a selective deacetylation of the anomeric position of **9** followed by reaction with trichloroacetonitrile and 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU). The installation of a linker on the mono-heptose **4**, and deacetylation, provided intermediate **11**. The primary hydroxy group was then masked as

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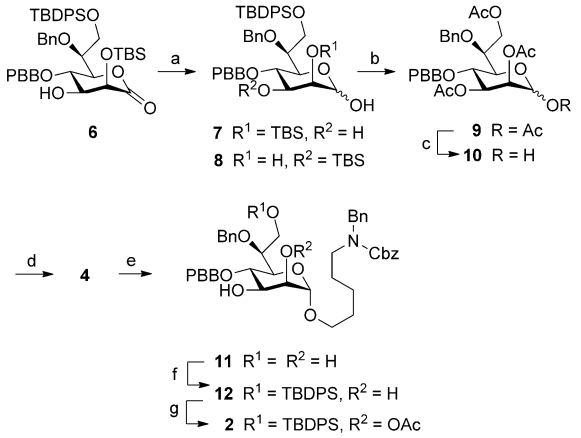
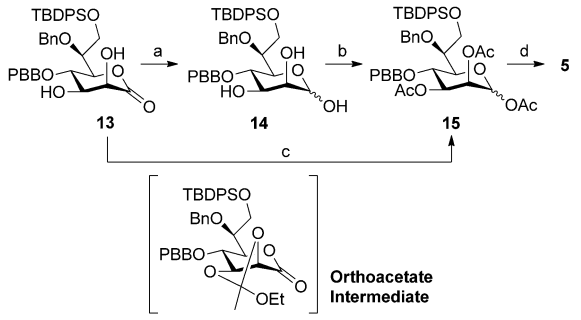


Scheme 2. Synthesis of building blocks **2** and **4**. Reagents and conditions: a) $LiAlH(OtBu)_3$, THF, −20 to −15 °C, **7** 30%, **8** 38%; b) 1. TBAF, THF; 2. Ac_2O, pyridine, quant.; c) NH_3, MeOH, quant.; d) Cl_3CCN, DBU, CH_2Cl_2, 70% to quant; e) 1. *N*-benzyl-*N*-benzyloxycarbonyl-5-aminopentan-1-ol, TMSOTf, CH_2Cl_2, −30 °C; 2. NaOMe, MeOH, 35%; f) TBDPSCl, imidazole, DMAP, CH_2Cl_2, 76%; g) 1. triethyl orthoacetate, PTSA, toluene; 2. 80% aq. AcOH, THF, 70 to 78%. DBU: 1,8-Diazabicyclo[5.4.0]undec-7-ene; DMAP: 4-Dimethylaminopyridine; PTSA: *p*-Toluenesulfonic acid; TBAF: Tetra-*n*-butylammoniumfluoride; TMSOTf: Trimethylsilyl trifluoromethanesulfonate; TBDPSCl: *tert*-Butyl(chloro)diphenylsilane, PBB: *p*-Bromobenzyl.

a TBDPS ether to give **12**. A cyclic orthoacetate was then formed on the 2,3-diol by treatment of triethyl orthoacetate and subsequently opened with aqueous acetic acid to exclusively provide building block **2** in good yield. The reduction of lactone **13**[12] to hemiacetal **14** was carried out under the same conditions as the reduction of **6**. The reaction, however, was much slower and was not completed even after two days (Scheme 3, Condition a). Subsequent acetylation of **14** and TBDPS cleavage provided building block **5**.

Alternatively, when the diol on lactone **13** was initially masked as the orthoacetate (Scheme 3, Condition c), the reduction was completed much faster (under 30 min). The resulting lactol was then treated with aqueous acetic acid and acetylated to furnish the intermediate **15** in 70% yield over four steps.



Scheme 3. Synthesis of building block **5**. Reagents and conditions: a) $LiAlH(OtBu)_3$, THF, room temperature, 40%; b) Ac_2O, pyridine, quant.; c) 1. triethyl orthoacetate, PTSA, toluene; 2. $LiAlH(OtBu)_3$, THF, −20 to −15 °C; 3. 80% aq. AcOH, THF; 4. Ac_2O, pyridine, 70% (4 steps); d) HF·pyridine, THF, quant.

Union of building blocks **4** and **5** yielded disaccharide **16**. Subsequent anomeric deacetylation and conversion into the glycosyl trichloroacetimidate provided disaccharide glycosylating agent **3**. Coupling of **2** and **3** followed by deacetylation, desilylation, and hydrogenolysis yielded trisaccharide **1**.

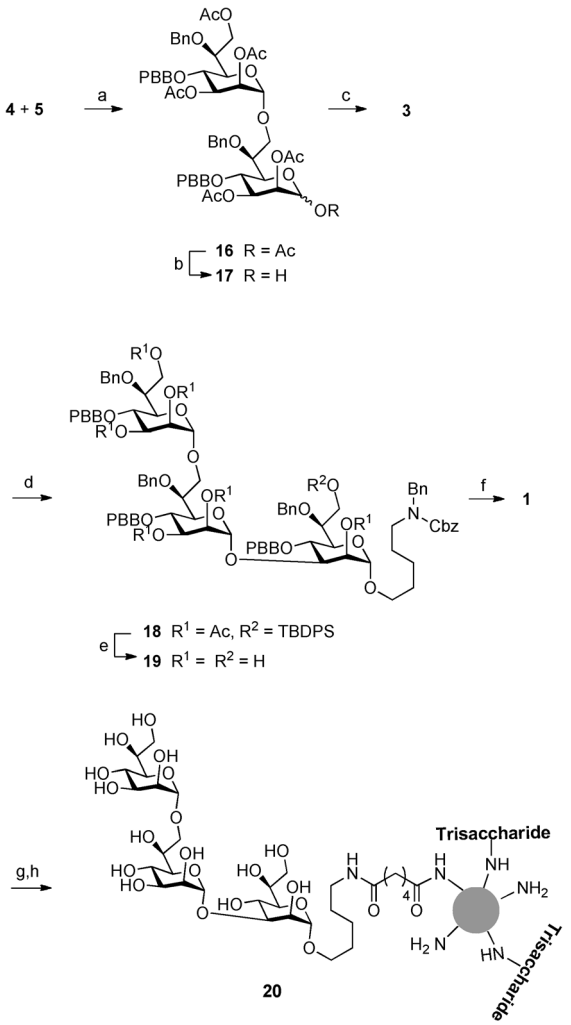
Carbohydrate antigens elicit a T cell-independent immune response, but do not induce an immunoglobulin class switch, thus, carbohydrate antigens are conjugated to immunogenic carrier proteins that induce a T cell dependent immune response.[13] Synthetic hapten **1** was conjugated to diphtheria toxoid CRM197 as carrier protein.[14] CRM197 is a constituent of licensed vaccines.[15] The amine group of the linker moiety in triheptose **1** was treated with one of the *N*-hydroxy succinimide (NHS) activated esters of disuccinimido adipate (DSAP) in DMSO with catalytic amounts of triethylamine to form the corresponding spacer-activated oligosaccharide (Scheme 4).[16]

Residual DSAP was subsequently removed by extraction with chloroform. The spacer-activated glycan was coupled with the CRM197 lysine amino groups in 50 mM phosphate buffer at pH 7.4 to afford neoglycoconjugate **20**. Conjugation was confirmed by SDS-PAGE and the oligosaccharide/CRM197 ratio was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The mass spectrum of the neoglycoconjugate revealed mass peaks between 59.9 and 68.8 kDa indicating that, on average, seven oligosaccharides were loaded onto CRM197.

To test the immunogenicity of the heptose trisaccharide hapten, C57BL/6 mice were immunized with glycoconjugate **20**. One prime and two boosting doses of the carbohydrate–protein conjugates that were formulated with Complete Freund's adjuvant were delivered. The anti-hapten **1** antibody titers were monitored by glycan microarray analysis and showed that all immunized mice formed IgG antibodies that specifically bound hapten **1** (Figure 1).

Immunized mice showed robust IgG responses against the trisaccharide antigen with significantly higher antibody titers in comparison to pre-immunized serum levels. Serum antibodies showed higher binding to triheptose **1** even in the presence of 150 mM thiocyanate indicating higher affinities for the antigen. Sera from immunized mice also contained antibodies against both the carrier proteins as well the spacer construct employed in the glycoconjugates (Figure 1). The response against **1** was comparatively higher and the carrier protein did not suppress the anti-trisaccharide response. Glycan array analysis showed the presence of higher amounts of IgG1 and IgG2 antibodies than IgG3 indicating isotype switching has occurred. Immunized mice showed a robust secondary response after boosting as a two-fold increase in endpoint titers was observed (Figure 1). Both class switch and robust boosting response can be attributed to transformation of a T-independent oligosaccharide hapten to a T-dependent glycoconjugate antigen.[17] T-cell help for triheptose specific B-cells was recruited and promoted isotype switching and memory B-cell differentiation.

Mouse splenocytes were isolated for hybridoma development. After subcloning and iterative selection, five hybridomas were established. Glycan array analyses at each



Scheme 4. Synthesis of trisaccharide hapten **1** and conjugation to carrier protein CRM197 to yield neoglycoconjugate **20**. Reagents and conditions: a) TMSOTf, CH_2Cl_2, −35 to −10°C, 65 to 86%; b) hydrazine acetate, DMF, 74% or NH_3, MeOH, 87%; c) Cl_3CCN, DBU, CH_2Cl_2, 80%; d) **2**, TMSOTf, CH_2Cl_2, −40 to −30°C, 68%; e) 1. NaOMe, MeOH; 2. TBAF, THF, quant.; f) 10% Pd/C, H_2, MeOH/H_2O/AcOH 10:10:1, 60 to 95%. g) Et_3N, disuccinimido adipate, DMSO, 37°C; h) CRM197 carrier protein, PBS buffer solution (pH 7.4), 37°C. CBz: carboxybenzyl, PBS: Phosphate-buffered saline.

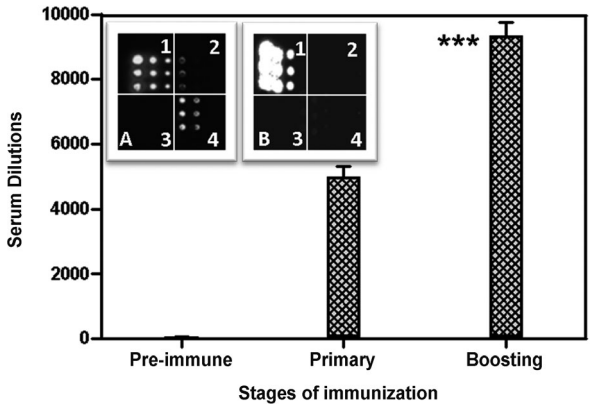


Figure 1. Anti-triheptose IgG responses from sera of immunized mice determined by glycan array analysis. Endpoint titers are plotted on the *y*-axis. Inset: A representative well from glycan array depicting fluorescent dots indicating antigen–antibody responses. A) represent antibody binding from serum and B) represent binding of purified mAbs. Triheptose hapten **1** is printed on quadrant 1, CRM 197 in quadrant 2, an unrelated glycan as a negative control in quadrant 3 and control spacer construct in quadrant 4. Bars represent mean (±) standard deviation of six microarray spots and *** represents $P \leq 0.001$ (2-sided Student's t-test).

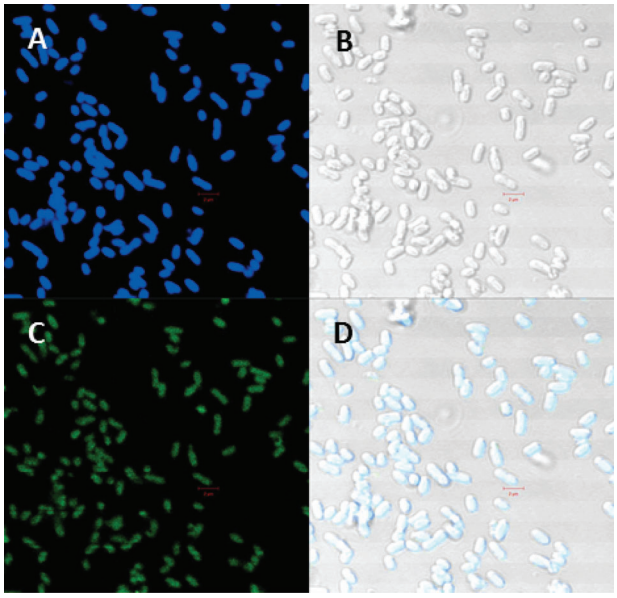


Figure 2. Indirect immunofluorescent staining of *Y. pestis* by anti-hapten **1** mAbs. CLSM images of immunostained *Y. pestis*; A) counter staining of bacterial DNA with DAPI, B) DIC images showing unstained bacteria, C) FITC specific fluorescence indicating binding of secondary antibody, and D) overlay of all three layers.

selection step helped to identify the clones that secreted antibodies with high specificity and affinity for **1**. Selected hybridomas were expanded in bioreactors and secreted antibodies from the supernatant were purified to homogeneity using protein G affinity chromatography. To investigate whether monoclonal anti-**1** antibodies recognize the LPS, fixed *Y. pestis* bacteria were analyzed by immunofluorescence and imaged using confocal laser microscopy (CLSM). Specific bacterial labeling was observed by localized green fluorescence of a secondary antibody (anti-mouse IgG FITC). The green fluorescence co-localized with the blue fluorescence of DAPI used to stain bacterial DNA. Moreover, the fluorescence was associated only with bacteria as evident from the DIC (Differential interference contrast) images. Purified mAbs as well as Abs from sera bound specifically to the bacterial surface, thus providing the basis for the detection of *Y. pestis* in biological samples (Figure 2).

Using synthetic oligosaccharide glycan arrays containing structures from related bacteria, we determined the binding specificity and the capability of mAbs to distinguish between different cell-surface glycans. Monoclonal antibody binding against LPS oligosaccharides found on Gram-negative bacteria was tested using a glycan array. Monoclonal antibodies

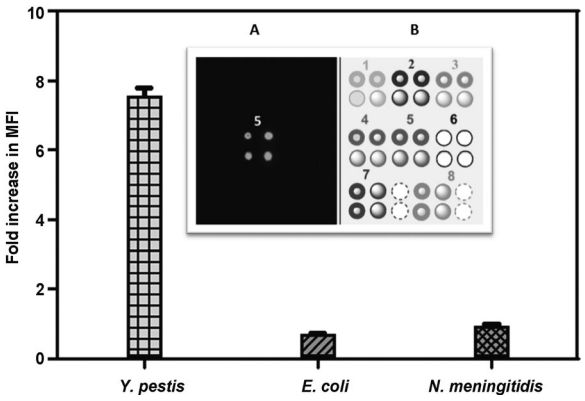


Figure 3. Anti-hapten **1** mAbs binding to *Y. pestis*, *E. coli*, and *N. meningitidis*. Inset: Representative glycan array image of mAbs binding to LPS based oligosaccharides from various bacteria A) image and B) printing pattern. Spot 1: is heptose monosaccharide, 2: Kdo-monosaccharide, 3: Heptose-Kdo disaccharide from all Gram-negative bacteria, 4: Kdo-Kdo-Kdo trisaccharide from *C. trachomatis*, 5: triheptose **1** from *Y. pestis*, 6: buffercontrol, 7: inner core tetrasaccharide from *N. meningitidis* LPS and 8: conserved trisaccharide from LPS structures of all Gram negative bacteria). MFI = Mean fluorescence intensity; bars represent mean (±) standard deviation of three labeling experiments.

to **1** exclusively bound to antigen **1** (Figure 3, spot 5) and none of the other synthetic glycans on the array. One of the antibodies weakly bound to the heptose monosaccharide. The binding specificity was also tested on native isolated LPS from related bacteria by a surface plasmon resonance (SPR) based binding assay. Isolated LPS from *E. coli*, *Salmonella typhi*, and *Neisseria meningitidis* were used in the study. The mAbs were captured using anti-IgG surfaces and used for binding isolated LPS. Capturing mAbs and binding LPS in solution allowed the variations arising from differential immobilization of structurally different LPSs to be limited. Compared to the synthetic *Y. pestis* LPS derivative, binding to the mAbs by *E. coli* O55 and *N. meningitidis* LPS was negligible. Even a delipidated derivative of *E. coli* O55:B5 LPS did not bind to the mAb. Since *E. coli* show strain specific LPS expression, LPS isolated from another strain, *E. coli* O127:B8, was used in the binding study. Both LPS from *E. coli* O127:B8 and *S. typhi* showed significant cross reactivity to the anti-*Y. pestis*-mAbs but the binding was weaker than to the *Y. pestis* LPS derivative (Supporting Information). The specificity of binding of mAbs to *Y. pestis* LPS can be explained by unique structural features of *Y. pestis* LPS. The inner core region of *E. coli* and *N. meningitidis* LPS is often decorated with nonstoichiometric additions of other glycans and with phosphate, pyrophosphorylethanolamine, or phosphorylcholine residues.[18] Preliminary saturation-transfer difference (STD) NMR spectroscopy studies of mAb binding to *Y. pestis* LPS derivative **1** indicated a role for the side-chain hydroxy groups in antibody recognition (data not shown). These groups are often derivatized in *N. meningitidis* and *E. coli* LPS which serves to explain how mAbs discriminate the different LPSs.

To further determine the binding specificity, the interaction of the mAbs to *E. coli* as well as *N. meningitidis*, was analyzed by flow cytometry using an anti-mouse IgG-FITC secondary antibody. Increased mean fluorescence intensity of bacteria-associated fluorescence indicated mAb binding to the bacteria. Monoclonal antibodies to *Y. pestis* bound specifically to this pathogen but not to *N. meningitidis* and *E. coli* bacteria. The mAbs are highly specific, can selectively detect *Y. pestis* and differentiate related Gram negative bacteria.

In conclusion, we demonstrate that synthetic oligosaccharides based on unique cell surface glycans can be exploited to create immunological agents for bacterial detection. Heptose trisaccharide-specific mAbs are the basis for highly sensitive and specific detection systems for *Y. pestis*. Efforts to incorporate these antibodies to point-of-care diagnostic platforms are currently underway.

Experimental Section

1: Disaccharide trichloroacetimidate **3** (64 mg, 0.050 mmol) and alcohol **2** (38 mg, 0.036 mmol) were dried and dissolved in anhydrous CH_2Cl_2 (0.5 mL) and 4 Å MS (100 mg) was added. The mixture was cooled to −40 °C and TMSOTf (4 μL, 0.022 mmol) was added. The reaction mixture was stirred at −40 to −30 °C for 1 h, and then quenched with saturated $NaHCO_3$ aqueous solution. The organic phase was dried over $MgSO_4$, solids removed by filtration, and concentrated. The crude product was purified by flash column chromatography (hexane/EtOAc 2:1) to give **18** (52 mg, 67 %) as a colorless oil. NaOMe was added (0.2 mL, 0.5 M in MeOH) to a solution of **18** (50 mg, 0.023 mmol) in MeOH (1.5 mL) and the reaction mixture was stirred at room temperature for 15 h. After neutralization by Amberlite IR-120, the mixture was filtered and concentrated. The residue was then dissolved in THF (1 mL) and TBAF (0.2 mL, 1 M in THF) was added. The reaction mixture was stirred at room temperature for 48 h and then concentrated in vacuo. The resulting residue was purified by a Sephadex LH-20 column chromatography (MeOH) to give **19** as colorless oil. Pd/C (10 %, 100 mg) was added to a solution of **19** (37.5 mg, 0.022 mmol) in MeOH/H_2O/AcOH (2 mL, 50:50:1). The mixture was stirred under H_2 atmosphere for 48 h, filtered and concentrated in vacuo. The crude product was purified by Sephadex LH-20 column chromatography (H_2O) to give **1** (9 mg, 60 %) as a white powder after lyophilisation.

*Conjugation of trisaccharide **1** to CRM197 carrier protein*: A solution of trisaccharide **1** (4.27 mmol) in DMSO was added to a solution of disuccinimido adipate (0.43 mmol) and triethylamine (10 μL) in DMSO (100 μL), at room temperature. After 1.5 h, 0.5 mL of phosphate buffer (0.1 M pH 7.4) was added to the reaction mixture and the residual linker extracted with chloroform. The extraction procedure was repeated three times and the resultant aqueous layer was centrifuged (300 g, 5 min) to separate traces of chloroform. The aqueous layer was separated and added to 1 mL of protein solution (CRM197, 1 mg mL^{-1} in 0.1 M phosphate buffer pH 7.4). The reaction was allowed to continue for 5–6 h with gentle stirring at pH 7.4. The glycoconjugate was purified either by using size exclusion chromatography or by ultrafiltration.

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