Supporting Information

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SI Materials and Methods

Chemicals. The following chemicals were used: sodium chloride (NaCl), Tris-(hydroxymethyl)-aminomethane (Tris), hydrochloric acid (HCl) (37%), cetyl trimethylammonium bromide (CTAB) (Merck); absolute ethanol (EtOH) and sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$) (Carlo Erba); adipic acid dihydrazide (ADH), EDTA disodium salt, triethylamine (TEA), sodium cyanoborohydride ($NaBH_3CN$), sodium acetate (AcONa), dimethyl sulfoxide (DMSO), dioxane, 6-aminohexanoic acid, sodium phosphate monobasic (NaH_2PO_4), 2,4,6-trinitrobenzenesulfonic acid solution (1 M in water) (TNBS); 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO); trichloroisocyanuric acid (TCC); dimethylformamide (DMF) (Sigma-Aldrich); adipic acid bis(*N*-hydroxysuccinimmide) (SIDEA) (Pfanstiehl Laboratories); sodium hydroxide (NaOH pellets) (Riedel-de Haën); acetonitrile (CH_3CN) (LC-MS Chromasolv); sodium hydroxide (NaOH) (50% solution) (J. T. Baker), sodium nitrate ($NaNO_3$), and tetrabutyl ammonium bromide (TBABr) (Fluka), and sodium acetate salt (pre-weighed reagent) (Dionex) for high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis. CRM_{197} was manufactured by Novartis Vaccines and Diagnostics (NV&D).

Purification Materials. The following materials were used for purification of MenX PS: Sartocon Slice Cassette, 0.1-m^2 filter area, M_r cutoff 100-kDa Hydrosart membrane (3051446801 E-SG) (Sartorius), ZetaPlus Biocap 25 SP10 filter 25 cm^2 [Cuno, Zeta Plus BioCap, BC0025L10SP (3M)], ZetaCarbon 25 filter 25 cm^2 [Cuno, Zeta Plus BioCap, BC0025LR53SP (3M)], Sartobran H9 0.45 + 0.22-μm filter (Sartorius).

Sartocon Slice 200, 200-cm^2 filter area, M_r cutoff 30-kDa PES membrane (3081465902E-SG), Sartocon Slice 200, 200-cm^2 filter area, M_r cutoff 2-kDa Hydrosart membrane (3081441902E-SG) (Sartorius), and Sepharose Q Fast Flow resin (GE Healthcare) were used for selecting the desired range of OS sizes. Sepharose SP resin was used for MenX counterion exchange and Sephadex G-15 resin for desalting of the samples (GE Healthcare). Sephacryl S-300 HR was used for packing the column for conjugates purification (GE Healthcare).

Syringe filters, 0.2-μm PES (Nalgene, 194-2520), were used for 0.2-μm filtration steps of unconjugated and conjugated MenX samples.

Purification of MenX PS. MenX 5967 strain growth and PS production were tested in three different culture media:

(*i*) Modified Catlin v.6: casamino acids (10 g/L), NaCl (5.8 g/L), glucose (10 g/L), K_2HPO_4 (4 g/L), NH_4Cl (1 g/L), K_2SO_4 (1 g/L), $MgCl_2{\cdot}6\ H_2O$ (0.4 g/L), $CaCl_2{\cdot}2\ H_2O$ (0.03 g/L), Fe(III) citrate (0.5 mg/L), pH 7.2;
(*ii*) MCDM1: glucose (10 g/L), soy peptone (15 g/L), NaCl (5.80 g/L), K_2SO_4 (1 g/L), K_2HPO_4 (4 g/L), L-glutamic acid (5 g/L), L-arginine (0.3 g/L), L-serine (0.5 g/L), L-cysteine (0.23 g/L), $MgCl_2$ (0.19 g/L), $CaCl_2$ (0.021 g/L), $FeSO_4$ (0.002 g/L);
(*iii*) Modified Frantz: L-glutamic acid (1.6 g/L), $Na_2HPO_4{\cdot}2H_2O$ (15.5 g/L), KCl (0.09 g/L), NH_4Cl (1.25 g/L), pH 7.6, supplemented with glucose (50 g/L), $MgSO_4{\cdot}7H_2O$ (30 g/L), 25 g/L ultrafiltered yeast extract, L-cysteine (1.5 g/L).

Bacteria were grown on GC agar plates and inoculated in 7 mL of media at 37 °C for 3–4 h, from OD_{600} 0.1 to about OD_{600} 0.8. The preinoculum was transferred to 50-mL shaking flasks and grown overnight. The following day, bacteria cultures were inactivated with 1% formaldehyde at 37 °C for 1 h. A fermentation batch was performed at 18-L scale using culture medium 3, in a B-Braun Sartorius bioreactor.

Culture supernatants were analyzed by NMR sequence with a diffusion filter to cut off all the signals deriving from lower molecular weight (MW) species and highlight the signals of higher MW MenX PS. Fig. S1 shows the spectrum of the culture supernatant post desalting by Vivaspin 10 kDa (Fig. S1*A*) in comparison to the spectrum in filter diffusion (Fig. S1*B*) and the spectrum of the purified MenX PS (Fig. S1*C*). Further analysis of the corresponding pellets by ^1H High-Resolution Magic Angle Spinning NMR (HR-MAS NMR) in solid state did not show MenX PS signals (Fig. S1*D*), indicating that the majority of the PS was released in the supernatant (considering the limit of detection of this assay, the maximum amount of PS remained attached on the bacteria should be 1/8 of the starting amount). Similar results were obtained for each of the three media.

In a standard purification, 5 L of clarified liquid culture were processed. The first step of MenX PS purification process was a tangential flow filtration (TFF) through a 100-kDa M_r cutoff, 0.1-m^2 membrane. The fermentation broth containing MenX PS was concentrated 32 times (Pin, 1.0 bar; Pout, 0.5 bar) followed by 15 vol of diafiltration against 1 M NaCl, 0.1 M Tris, 0.02 M EDTA buffer, pH 7.3 (Pin, 1.6 bar; Pout, 0.4 bar), and then 10 cycles of diafiltration against water (Pin, 1.8 bar; Pout, 0 bar). A solution of 10% CTAB (wt/vol) was added to the MenX PS retentate under continuous stirring to a final concentration of 2% CTAB. After mixing at room temperature for at least 30 min, the MenX–CTA paste was recovered by centrifugation (18,000 × *g* at 25 °C for 10 min) and resuspended in 96% (vol/vol) ethanol, adding two times the volume obtained after the 10% CTAB addition. The mixture was stirred for at least 2 h, centrifuged (18,000 × *g* at 25 °C for 10 min), and the ethanol solution was subsequently filtered at constant flow through an SP10 depth filter and then through a carbon filter (Cuno). Before proceeding with filtration, the devices were washed with water and equilibrated with 96% (vol/vol) ethanol. Finally, MenX–CTA was precipitated by adding 1 M NaCl to the MenX ethanol solution under agitation, to a final concentration of 0.2 M NaCl. After 1 h, the precipitate was recovered by centrifugation (18,000 × *g* at 25 °C for 5 min) and dried under vacuum overnight. The purified MenX was dissolved in water, at a concentration of 3–5 mg/mL, sterile filtered, and stored at −20 °C.

Characterization of MenX PS. Total sugar content was quantified by HPAEC-PAD in all of the purification process intermediates, starting from the fermentation broth, for calculation of the yield step by step.

Protein concentration was estimated by micro-BCA, using BSA as a reference following the manufacturer's instructions (Thermo Scientific). Analysis of nucleic acids was performed by UV spectroscopy at a wavelength of 260 nm assuming that a nucleic acid concentration of 50 μg/mL gives an $OD_{260} = 1$. Protein and nucleic acid content were expressed as ratio percentage relative to sugar content (wt/wt). Chromogenic kinetic Limulus amoebocyte lysate was used to measure endotoxin level (Charles River Endosafe-PTS instrument).

To reduce matrix interference in the analysis, a pretreatment was applied to some samples representing intermediates of MenX purification process:

(*i*) to calculate MenX concentration and percentage impurities in clarified culture broth, the sample was dialyzed against water using Vivaspin 10 kDa;

(*ii*) ethanolic extracts were dried and resolubilized in 1 M NaCl before quantification of sugar and impurities. Dilutions were performed with 1 M NaCl to avoid MenX–CTA precipitation.

Purified MenX samples were characterized by high-performance liquid chromatography–size exclusion chromatography (HPLC-SEC) for estimation of the molecular size distribution (Fig. S2), and by ^{1}H and ^{31}P NMR (1) for confirming the structure and identity of the PS.

MenX OS from Mild Hydrolysis. The PS depolymerization was performed by mild acid hydrolysis and monitored in process by ultra performance liquid chromatography–size exclusion chromatography (UPLC-SEC) and phosphorus (^{31}P) NMR spectroscopy, and it was quenched by neutralization when the desired average degree of polymerization (avDP) was reached.

For MenX OS of avDP 80–100, the hydrolysis was performed in 50 mM NaOAc with saccharide concentration of 2.5 mg/mL, at pH 4.7 and 100 °C for ∼1 h, and quenched by neutralization with NaOH when ^{31}P NMR indicated an avDP of 55–60. TFF (30-kDa) was then performed to eliminate lower M_r chains, diafiltering against 20 vol of distilled water (Pin, 0.8 bar; Pout, 0.0 bar).

For MenX OS of avDP 15–20, the hydrolysis was performed at 80 °C, pH 4.0, for 4–5 h, and quenched when an avDP of 10–13 was reached. Fig. S3 shows the UPLC-SEC profiles of samples collected during one of these hydrolyses and the corresponding avDP calculated by ^{31}P NMR. For selecting OS of avDP 15–20, two purification steps were required. TFF through a 30-kDa M_r cutoff membrane was performed to eliminate the higher avDP chains. The sample was diafiltered against 20 vol of 5 mM NaOAc, 200 mM NaCl buffer, pH 7.2 (Pin, 1.6 bar; Pout, 0.7 bar). The permeate was further purified by anion exchange chromatography using a Sepharose Q resin to remove OS at lower M_r. Approximately 5 mg of MenX OS were loaded per 1 mL of resin at 150 cm/h, in 5 mM NaOAc, 200 mM NaCl buffer, pH 7.2, and the fractions containing MenX OS avDP 15–20 were collected by elution with 5 mM NaOAc, 1 M NaCl buffer, pH 7.2.

HPAEC-PAD profiles of native MenX PS with avDP >100 in comparison with hydrolyzed material of avDP 10 and the final purified MenX OS of avDP 20 (Fig. S4) provide more detailed information of the saccharide polydispersion.

Characterization of MenX OS. Total sugar content was quantified by HPAEC-PAD. UPLC-SEC and HPAEC-PAD were used for estimating the average M_r distribution. The avDP was calculated both by ^{31}P (1) and ^{1}H NMR analysis. ^{31}P NMR signals of phosphodiester in chain groups (P_{Int}) and phosphomonoester end groups (P_{Ter}) were integrated and used for avDP calculation ($avDP = [(P_{Int}/P_{Ter}) + 1]$). ^{1}H NMR signals of proton at C-1 in chain ($H_{1\ Int}$) and at reducing end terminus ($H_{1\ Ter}$ as a sum of $H_{1\alpha\ Ter}$ and $H_{1\beta\ Ter}$) were also integrated and used for avDP calculation ($avDP = [(H_{1\ Int}/H_{1\ Ter}) + 1]$).

Derivatization of MenX OS Reducing End with NH_4OAc by Reductive Amination, Followed by Reaction of MenX-NH_2 with SIDEA and Conjugation with CRM_{197}. MenX OS (avDP 15–20), in 5 mM NaOAc, 1 M NaCl buffer, pH 7.2, at a concentration of 3–5 mg/mL, were added of NH_4OAc and $NaBH_3CN$ as solids, at a final concentration of 300 and 49 mg/mL, respectively. The solution was mixed at 37 °C for 5 d. The reaction mixture was purified by TFF (2-kDa), diafiltering against 20 vol of 1 M NaCl and then 10 vol of distilled water (Pin, 1.5 bar; Pout, 0.5 bar). The derivatized product, recovered in the retentate, was designed as MenX–NH_2.

For introduction of the second linker, SIDEA, dried MenX–NH_2 was dissolved in water/DMSO 1:9 (vol/vol) at NH_2 group concentration of 4 μmol/mL. When the saccharide was completely solubilized, TEA (molar ratio TEA/total NH_2 groups = 5) and then SIDEA (molar ratio SIDEA/total NH_2 groups = 12) were added. The solution was mixed at RT for 3 h. The purification was performed precipitating MenX–NH_2–SIDEA by addition of dioxane (90% volume in the resulting solution) and then washing the pellet with the same organic solvent (10 times with one-third of the volume added for the precipitation) to remove free SIDEA. The pellet was finally dried under vacuum. The product was designated as MenX–NH_2–SIDEA.

For protein conjugation, CRM_{197} and NaH_2PO_4 buffer, pH 7.2, were added to MenX–NH_2–SIDEA at a final protein concentration of 25 mg/mL, final buffer capacity of 25 mM, and a molar ratio of active ester groups to CRM_{197} of 13:1. The reaction was mixed at RT for 15 h. The conjugate was designated as MenX–NH_2–SIDEA–CRM_{197}.

Derivatization of MenX OS Reducing End with ADH by Reductive Amination, Followed by Reaction of MenX-ADH with SIDEA and Conjugation with CRM_{197}. MenX OS (avDP 15–20), after being desalted against water on a SEC Sephadex G-15 column (∼0.3 mg of OS loaded per 1 mL of resin at 30 cm/h) and dried, were solubilized in 100 mM NaOAc, pH 4.5, at a concentration of 40 mg/mL; ADH and then $NaBH_3CN$ were added as solid powders, both at a ratio 1.2:1 by weight with respect to the MenX OS. The solution was mixed at 30 °C for 1 h. The reaction mixture was desalted against water on a SEC Sephadex G-15 column, and the derivatized product was designed as MenX–ADH.

For the introduction of the second linker, SIDEA, and for conjugation to CRM_{197}, the same procedures previously described for MenX–NH_2–SIDEA–CRM_{197} were followed.

Characterization of MenX–CRM_{197} Conjugates. Total saccharide was quantified by HPAEC-PAD (2), protein content by micro-BCA, and the ratio of saccharide to protein was calculated. MenX–CRM_{197} conjugates profiles were compared with free CRM_{197} and free MenX OS by HPLC-SEC and SDS–polyacrylamide gel electrophoresis (SDS/PAGE). Free protein was estimated by HPLC-SEC, running a calibration curve of the unconjugated protein in the range 5–50 μg/mL under the same conditions as for the conjugate. The percentage of unconjugated CRM_{197} was calculated by dividing the amount of free protein detected by HPLC-SEC by the total amount of protein quantified in the sample by micro-BCA. A method for free saccharide determination is under development.

Derivatized MenX OS intermediates were characterized by HPAEC-PAD for sugar content. Introduction of NH_2 groups was verified by TNBS colorimetric method (3) using 6-aminohexanoic acid and ADH as standard for NH_2 quantification on MenX–NH_2 and MenX–ADH, respectively.

Aldehyde groups introduced by oxidation with TEMPO were quantified by NMR (Fig. S5).

Total active ester groups introduced with SIDEA were quantified by A260 (4). Free ADH and free SIDEA were detected by RP-HPLC (5).

HPLC-SEC. MenX PS samples were eluted on a TSK gel 6000PW (30 cm × 7.5 mm) column (particle size, 17 μm; Sigma 8-05765) connected in series with a TSK gel 5000PW (30 cm × 7.5 mm) column (particle size, 17 μm; Sigma 8-05764) with TSK gel PWH guard column (7.5 mm ID × 7.5 cm L; particle size, 13 μm; Sigma 8-06732) (Tosoh Bioscience). The mobile phase was 0.1 M NaCl, 0.1 M NaH_2PO_4, 30% CH_3CN, pH 7.2, at the flow rate of 0.5 mL/min (isocratic method for 60 min).

MenX–CRM_{197} conjugates were analyzed, in comparison with free CRM_{197} and free MenX OS used for conjugation, on a TSK gel 3000PWXL (30 cm × 7.8 mm; particle size, 7 μm; cod. 808021) with TSK gel PWXL guard column (4.0 cm × 6.0 mm; particle size, 12 μm; cod. 808033) (Tosoh Bioscience). The mobile phase was 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% CH_3CN, pH 7.2, at the flow rate of 0.5 mL/min (isocratic method for 30 min).

Void and bed volume calibration was performed with λ-DNA (λ-DNA Molecular Weight Marker III, 0.12–21.2 kbp; Roche) and sodium azide (NaN_3) (Merck), respectively. Saccharide peaks were detected by differential refractive index (dRI) and at 214 nm. Protein and conjugate peaks were detected at 214 and 280 nm and by fluorescence (emission spectrum at 336 nm, with excitation wavelength at 280 nm).

For the K_d (measure of distribution coefficient) determination, the following equation was used: $K_d = [(Te - T_0)/(Tt - T_0)]$, where Te is elution time of the analyte, T_0 is elution time of the bigger fragment of λ-DNA, and Tt is elution time of NaN_3.

HPAEC-PAD for MenX Quantification (2). MenX samples were treated with TFA at a final concentration of 2 M diluted to a total volume of 600 μL in the range 0.5–8 μg/mL. Samples were heated at 100 °C for 2.5 h in a closed screw-cap test tube, and then chilled at 2–8 °C for about 30 min, added to 700 μL of 2 M NaOH, and filtered with 0.45-μm Acrodisc (PALL) filters before analysis. A pure preparation of MenX PS or the synthetic monomer *N*-acetylglucosamine-4-phosphate (GlcNAc-4P), prepared in our laboratory, titered through the colorimetric method for total phosphorus content (6), was suitable for building the calibration curve, set up with standards in the range of 0.5–8 μg/mL. HPAEC-PAD was performed with a Dionex ICS3000 equipped with a CarboPac PA1 column (4 × 250 mm; Dionex) coupled with PA1 guard column (4 × 50 mm; Dionex). Samples were run with a flow rate of 1 mL/min, using a gradient in 10 min from 100 to 500 mM NaOAc in 100 mM NaOH. The effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. A quadruple-potential waveform for carbohydrates was applied. The resulting chromatographic data were processed using Chromeleon software 6.8. The analysis by NMR indicated that the species detected by HPAEC-PAD analysis was the monomer glucosamine GlcN-4P. This was further confirmed by comparison with the synthetic monomer GlcNAc-4P prepared in our laboratory, which also generates GlcN-4P when hydrolyzed in the same conditions. The structural identity of synthetic GlcNAc-4P was assessed by ^{1}H NMR analysis (Fig. S6).

UPLC-SEC. MenX OS from acid hydrolysis of the native PS were eluted with 0.1 M NaH_2PO_4, 0.1 M NaCl, 30% CH_3CN, pH 7.2, and with a flow rate of 0.5 mL/min (isocratic method for 7 min) on an Acquity UPLC BEH200 1.7-mm column (4.6 × 150 mm). Five microliters of samples were injected with MenX concentration of ∼1 mg/mL, and detection was set at 214 nm.

HPAEC-PAD for MenX OS Profiling. MenX OS were run on a CarboPac PA200 column (4 mm × 250 mm; Dionex) with guard column (4 mm × 50 mm; Dionex) connected to an ICS 3000 Dionex system equipped with a pulsed amperometric detector. The 100 mM NaOH, 10 mM $NaNO_3$ was used for column equilibration and a three-steps gradient with increasing amount of $NaNO_3$ (100 mM NaOH + 10, 250, 500 mM $NaNO_3$ for 80, 15, and 3 min, respectively) was used for elution. A flow rate of 0.4 mL/min was used for the entire run of 120 min. The 20-μL samples were injected at a MenX concentration of ∼1 mg/mL. The effluent was monitored, and the resulting chromatographic data were processed as described above.

NMR Spectroscopy (NMR). All of the NMR experiments were recorded by a Bruker Avance III 400 MHz spectrometer. The TOPSPIN software package (Bruker) was used for data acquisition and processing.

Liquid-state experiments. Structural identity of MenX PS was verified by NMR analysis. Approximately 0.5–1.0 mg of MenX was dissolved in 0.75 mL of deuterium oxide (D_2O) (99.9% atom D; Aldrich). ^{1}H NMR and ^{31}P spectra were recorded at 25 ± 0.1 °C using a 5-mm broadband probe (Bruker).

^{1}H NMR spectra were collected with 32,000 data points over a 10-ppm spectral width. The spectra were weighted with 0.2-Hz line broadening and Fourier transformed. The transmitter was set at the water frequency, which was used as the reference signal (4.79 ppm).

^{31}P NMR spectra were recorded at 161.9 MHz, with 32,000 data points over a 20-ppm spectral width. The spectra were weighted with 3.0-Hz line broadening and Fourier transformed. The 85% phosphoric acid in deuterium oxide was used as an external standard (0 ppm).

All of the ^{1}H and ^{31}P NMR spectra were obtained in quantitative manner using a total recycle time to ensure a full recovery of each signal (5 × longitudinal relaxation time T_1).

For verifying MenX PS presence in the culture medium, at this stage ^{1}H spectra were acquired on the solution post desalting by Vivaspin (10 kDa) with a diffusion filter pulse sequence with gradient pulses (diffusion filter, 95%), to highlight the PS signals and remove the low–molecular-mass species free in solution.

Solid-state experiments. MenX cells recovered from bacterial growth were inactivated by 1% (vol/vol) formaldehyde treatment and then washed three times with PBS in D_2O. Approximately 50 μL of compact pellet was inserted into a Kel-F disposable insert and then in a 4 mm MAS ZrO_2 rotor (Bruker). Proton high-resolution magic angle spinning NMR (HR-MAS NMR) experiments were recorded using a Bruker 4-mm HR-MAS probe. The transmitter was set at the hydrogen-deuterium-oxygen frequency, which was also used as reference signal (4.79 ppm). The spectra were recorded at a 4,500-Hz spin rate and 25 °C. The ^{1}H spectra were acquired with a diffusion filter pulse sequence with gradient pulses (diffusion filter, 95%) to remove the low–molecular-mass species free in solution.

SDS/PAGE. Conjugates were characterized in comparison with free CRM_{197} by SDS/PAGE using 7% Tris·acetate gels (NuPAGE, from Invitrogen). The samples (5–20 μL with a protein content of 5 μg) were mixed with 0.5 M DTT (1/5, vol/vol) and NuPAGE LDS sample buffer (1/5, vol/vol). The mixtures were heated at 100 °C for 1 min. The gel, containing loaded samples, was electrophoresed at 45 mA in NuPAGE Tris-Acetate SDS running buffer (20×; Invitrogen) and stained with Simply Blue Safe Stain (Invitrogen).

ELISA Analysis. Ninety six-well Maxisorp plates (Nunc, Thermo Fisher Scientific) were coated with MenX PS by adding 100 μL per well of a 5 μg/mL PS solution in PBS buffer at pH 8.2. Plates were incubated over night at 4 °C, and then washed three times with TPBS (0.05% Tween 20 in PBS, pH 7.4) and blocked with 100 μL per well of BSA (Sigma-Aldrich) solution at 3% in TPBS for 1 h at 37 °C. Each incubation step was always followed by triple TPBS wash. Serum samples were initially diluted 1:25 for preimmune (serum used as negative control), 1:200 for a reference serum (serum obtained from previous immunization and used as internal reference for the following studies), from 1:100 to 1:200 for test sera in TPBS, transferred into coated–blocked plates (200 μL), and serially twofold diluted followed by 2-h incubation at 37 °C. For high-avidity ELISA protocol, the same sera dilutions were applied using TPBS added of 0.1 M ammonium thiocyanate (chaotropic agent) as buffer. Then 100 μL per well of 1:10,000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Sigma-Aldrich) were added and left for 1 h at 37 °C. Visualization of bound alkaline phosphatase was performed by adding 100 μL per well of 1 mg/mL paranitrophenylphosphate disodium hexahydrate (Sigma-Aldrich) in 0.5 M diethanolamine buffer, pH 9.6. After 30 min of development at RT, the OD was measured at 405 nm with a microplate spectrophotometer (Bio-Rad). Antibody titers were expressed as the reciprocal of sera dilution corresponding to OD 1.0 (around the inflection point of OD vs. log dilution sigmoidal curve) (7).

Statistical significance was calculated using the nonparametric Mann–Whitney *t* test; graphical and statistical analyses were done using GraphPad 5.0 software.

Complement-Mediated Bactericidal Activity (Rabbit Serum Bactericidal Activity Assay). Serum bactericidal activity (SBA) against *Neisseria meningitidis* strains was evaluated as previously described by Borrow et al., with minor modifications (8) using pooled baby rabbit serum (Pel-Freez) as complement source.

Briefly, MenX strains were grown overnight on chocolate agar plates (Biomerieaux) at 37 °C in 5% CO_2. Colonies were inoculated in Mueller–Hinton broth containing 0.25% glucose at OD_{600} 0.05–0.08 and incubated at 37 °C with shaking until early-log growth ($OD_{600} \sim 0.25$). The cultured bacteria were diluted in Gey's balanced salt solution containing 1% BSA (Sigma) at the working dilution of 10^4 to 10^5 colony-forming units per mL. All mouse sera to be tested were heat inactivated for 30 min at 56 °C. The total volume in each well was 50 μL, with 25 μL of serial twofold dilutions of test serum, 12.5 μL of bacteria at the working dilution, and 12.5 μL of baby rabbit complement (25%).

Negative controls included bacteria incubated, separately, with the complement serum without the test serum and with test immune sera and heat-inactivated complement. Immediately after the addition of the baby rabbit complement, the controls were plated on Mueller–Hinton agar plates using the tilt method (time 0). The plate was incubated for 1 h at 37 °C; then each sample was spotted on Mueller–Hinton agar plates, and the controls were plated on Mueller–Hinton agar plates using the tilt method (time 1). The agar plates were incubated for 18 h at 37 °C, and the colonies corresponding to time 0 and time 1 were counted.

Serum bactericidal titers were defined as the serum dilution resulting in 50% decrease in colony-forming units per milliliter after 60 min (time 1) incubation of bacteria with reaction mixture, compared with control colony-forming units per milliliter at time 0.

Typically, bacteria incubated with the negative control antibody in the presence of complement showed a 150–200% increase in colony-forming units per milliliter during the 60-min incubation.

SBA analyses were performed in duplicates on pooled sera, and differences were in the range of the variability of the test.

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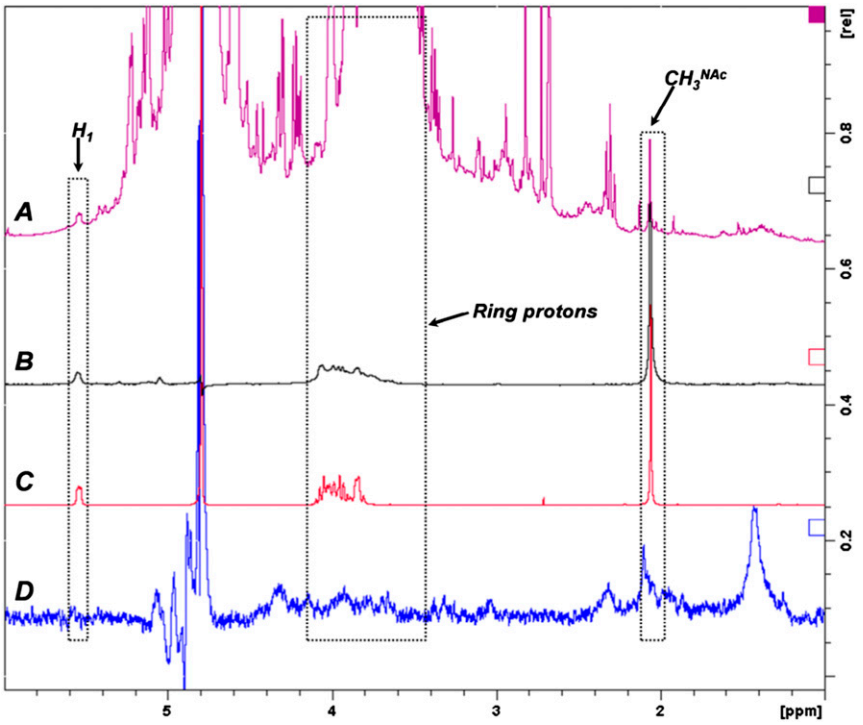

Fig. S1. NMR spectra in the liquid state of the liquid culture post desalting by Vivaspin 10 kDa (*A*), and in filter diffusion (*B*); of purified MenX PS (*C*) and ^{1}H HRMAS in solid-state NMR of corresponding bacterial pellet (*D*). Culture supernatants were analyzed by NMR sequence with a diffusion filter to cut off all of the signals deriving from lower M_r species and highlight the signals of higher M_r MenX PS (*B*). Analysis of the corresponding pellets by ^{1}H high-resolution magic angle spinning NMR (HR-MAS NMR) in solid state (*D*) did not show MenX PS signals, indicating that the majority of the PS was released in the supernatant (considering the limit of detection of this assay, the maximum amount of PS remained attached on the bacteria should be one-eighth of the starting amount).

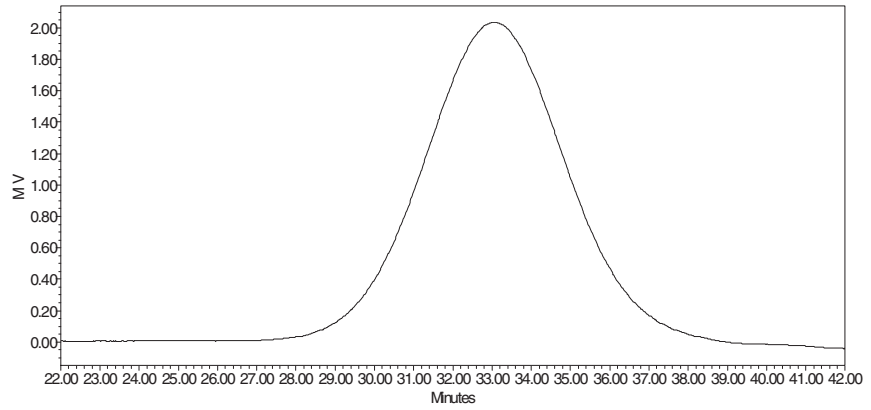

Fig. S2. HPLC-SEC elution profile of purified MenX PS as monitored by dRI. The sample was eluted in 0.1 M NaCl, 0.1 M NaH_2PO_4, 30% CH_3CN, pH 7.2, at the flow rate of 0.5 mL/min on a TSK gel 6000 PW + G5000 PW column series.

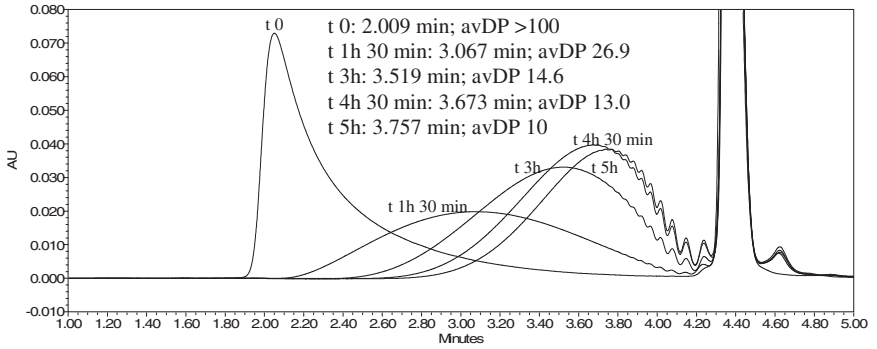

Fig. S3. UPLC-SEC profiles of samples collected at different times of hydrolysis and analyzed in process to obtain purified MenX OS at avDP 15–20. Elution with 0.1 M NaH_2PO_4, 0.1 M NaCl, 30% CH_3CN, pH 7.2, at 0.5 mL/min (isocratic method for 7 min) on a Acquity UPLC BEH200 1.7-mm column (4.6 × 150 mm); V0: 1.944 min; Vtot: 4.365 min. Five microliters of samples injected with MenX concentration of ∼1 mg/mL and detection set at 214 nm. avDP was calculated by ^{31}P NMR.

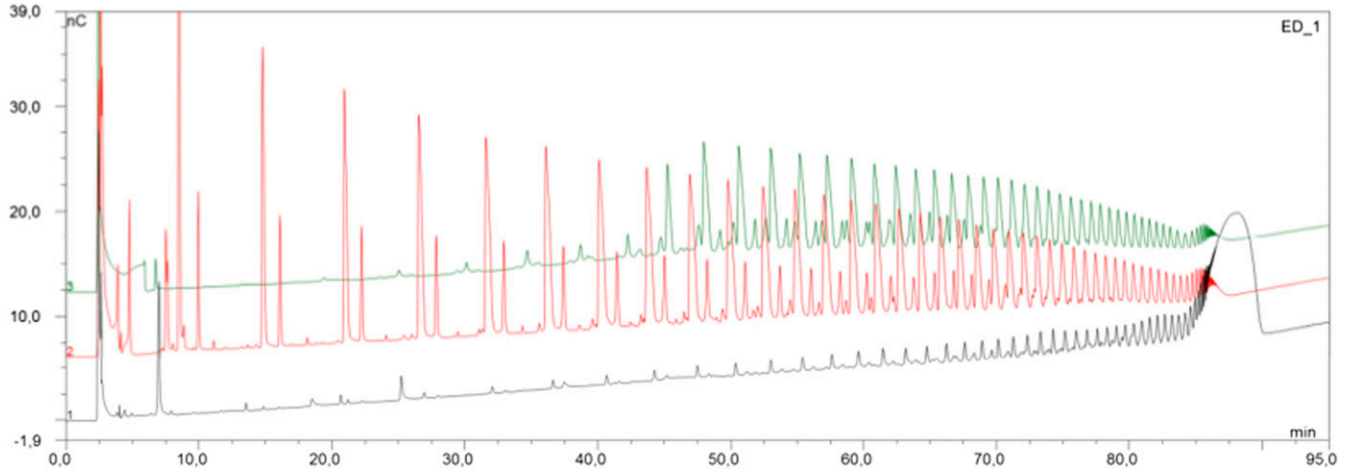

Fig. S4. HPLC profiling on PA200 column (PAD) of native MenX PS avDP >100 (black), hydrolyzed material avDP 10 (red), and final purified MenX OS avDP 20 (green).

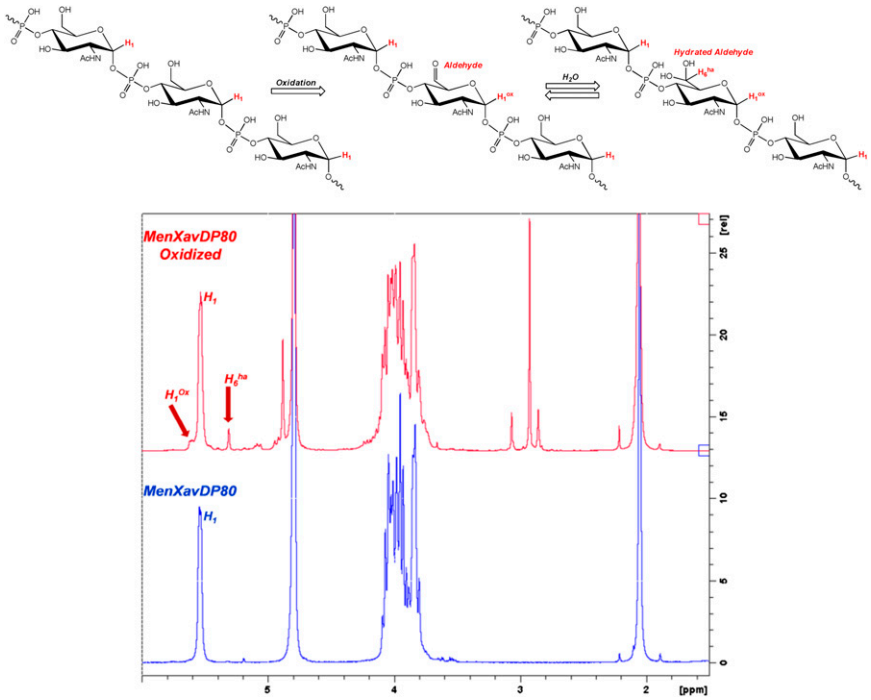

Fig. S5. NMR spectra of MenX OS avDP 80 (blue) and oxidized MenX OS avDP 80 (red): proton NMR signals were revealed for the hydrate aldehyde form. Integration of hydrate aldehyde peak at position C_6 (H_{6ha}) in comparison with H_1 peaks (protons at position C_1) provides the oxidation level.

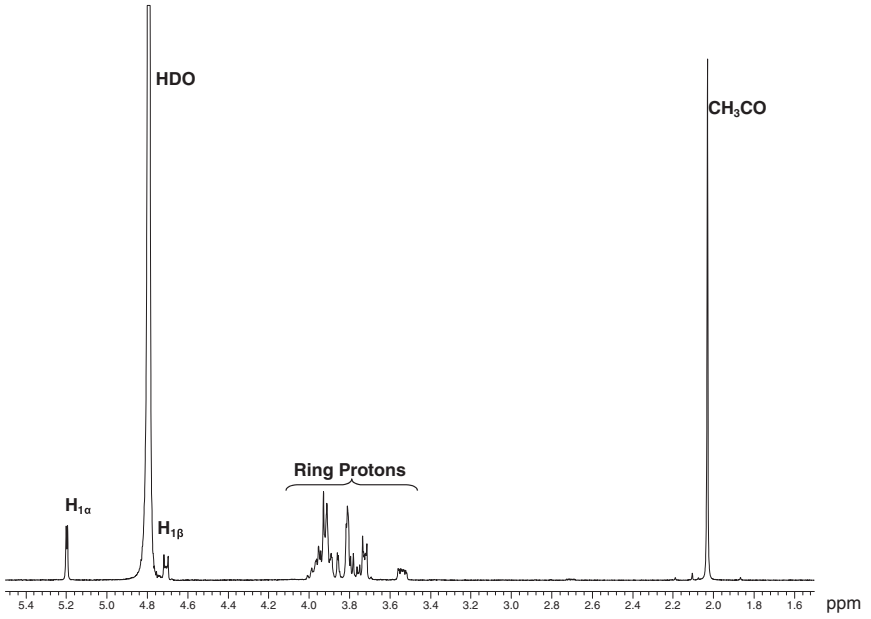

Fig. S6. ^{1}H NMR spectrum registered at 400 MHz and 25 °C of synthetic GlcNAc-4P. Some labels are reported to facilitate the peaks assignment.