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An antibacterial vaccination strategy based on a glycoconjugate containing the core lipopolysaccharide tetrasaccharide Hep_2Kdo_2

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Certain non-mammalian cell wall sugars are conserved across a variety of pathogenic bacteria. This conservation of structure, combined with their structural differences when compared with mammalian sugars, make them potentially powerful epitopes for immunization. Here, we report the synthesis of a glycoconjugate that displays the so-called 'inner core' sugars of Gram-negative bacterial cell walls. We also describe an antibacterial vaccination strategy based on immunization with the glycoconjugate and the subsequent administration of an inhibitor that uncovers the corresponding epitope in pathogenic bacteria. The core tetrasaccharide, Hep_2Kdo_2, a common motif in bacterial lipopolysaccharides, was synthesized and attached via a chain linker to a diphtheria toxin mutant carrier protein. This glycoconjugate generated titres of antibodies towards the inner core tetrasaccharide of the lipopolysaccharide, which were capable of binding the cell-surface sugars of bacterial pathogenic strains including *Neisseria meningitidis*, *Pseudomonas aeruginosa* and *Escherichia coli*. Exposure of bacterial lipopolysaccharide in *in vitro* experiments, using an inhibitor of capsular polysaccharide transport, enabled potent bacterial killing with antiserum.

Despite the twentieth century's triumph in terms of the creation of antibiotics against most bacterial infections, the continuous emergence of resistant strains[1] in more and more bacterial species has led to a focus on the development of vaccines as vital alternatives, especially in the form of carbohydrate-based glycoconjugates[2–4]. Tremendous achievements have been made in the development of capsular polysaccharide (CPS)-based vaccines against infections caused by most of the serogroups of key pathogens. However, some remain untargeted. For example, *Neisseria meningitidis* serogroup B bears a CPS that resembles the surface carbohydrates in the human nervous system and is therefore not a suitable target for the development of a CPS-based vaccine[5].

Other sugar targets exist in bacterial cell walls, such as lipopolysaccharides (LPS). However, lack of access to key LPS fragments has prevented the effective development of corresponding vaccines. Thus, although the extracted and *O*-deacetylated LPS derived from four meningococcal mutant strains has been used for heterogeneous glycoconjugate preparation and so has given some hope that reactivity might be generated towards wild-type and mutant strains of *Neisseria meningitidis*[6–8], these extracts are mixed and lack some of the most potent potential targets.

Hep_2Kdo_2 linked to lipid A has been identified as a conserved molecular target in bacteria because it is essential for the biosynthesis of LPS[9]. This Hep_2Kdo_2 core tetrasaccharide in LPS is also found across various *N. meningitidis* strains[10–15] and other pathogenic bacterial species[16–21]. However, a major limitation in accessing this inner core of LPS has been its minimal availability from bacterial isolates and the heterogeneity of the attached (so-called outer core) glycan structures, which shield access to it, in combination with the CPS, during immune responses[7,22].

Here, we show that chemical synthesis can offer an alternative solution in the quest for an effective LPS-based vaccine candidates. Although related structures have been synthesized previously[23–26], we report what we believe to be the first chemical synthesis of this important tetrasaccharide, the creation of an LPS mimic, and its conjugation to a carrier protein for the production of specific antibodies. The synthesis uses a single chiral pool starting material (D-mannose) to access the complete structure through a strategy of carbon-chain extension homologizations (both pre- and post-glycosylation) plus challenging associated glycosylations achieved with unusual reagents. We also reveal an acid sensitivity of this structure, which may account for its previously poor extraction from natural sources. Immunological evaluation of serum antibodies generated by the conjugate show target specificity and potency, allowing its combined use in potent bacterial killing with an inhibitor[27] of the transporter of CPS.

Results

Despite continuing advances in glycan synthesis[28–31], including some portions of the inner-core LPS[32–34], the target core tetrasaccharide Hep_2Kdo_2 has remained an unsolved and particularly challenging synthetic target. This tetrasaccharide (Fig. 1) is near unique in its complexity: none of its component sugar units is readily available as a starting material (necessitating *de novo* preparation of key building blocks), and all four units are higher sugars (with longer carbon chains than the typical C_5 or C_6 normally found). Unlike many other areas of oligosaccharide synthesis, this has greatly reduced the availability of established strategies for their assembly. Prime among the issues to be resolved is the strikingly poor efficiency of the Kdo sugar unit in chemical glycosylations[35],

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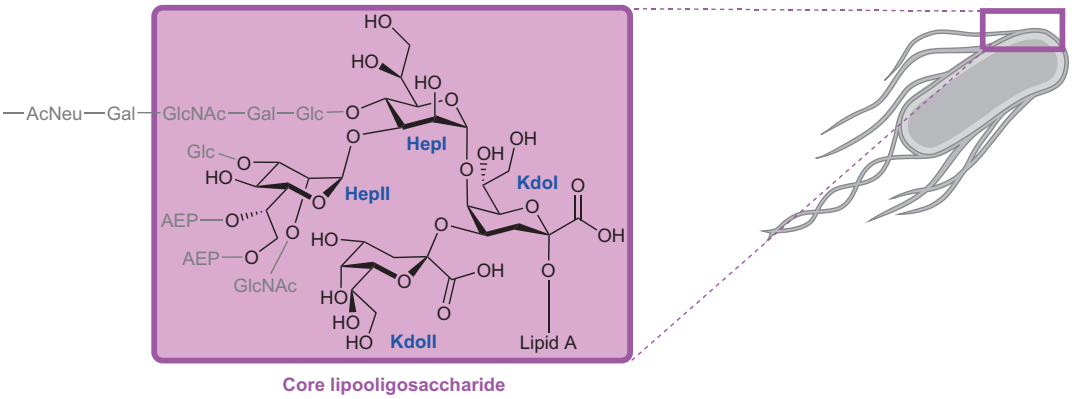

Figure 1 | The target core tetrasaccharide of the lipooligosaccharide (LOS) of Gram-negative bacteria. LPS core structure of wild-type serogroup B *N. meningitidis* strain NMB[9,53]. The so-called 'inner core' tetrasaccharide structure is shown as the full structural formula in black, highlighted by the pink box. Example elaborating sugars that extend to the full LPS are shown in grey. This inner core is common to other Gram-negative bacteria.

compounded by its double occurrence in such a sterically congested motif (Figs 1, 2).

To probe and overcome this inefficiency, a convergent '2+2' (disaccharide donor **3** and disaccharide acceptor **4**) glycosylation approach was chosen (Fig. 2). We chose to survey a number of possibilities for the critical disaccharide α-Hep-(1→3)-α-Hep donor **3**. We also decided to exploit a stereoselective approach to the '2+2' glycosylation assisted by neighbouring group participation[36] for α-Hep-Kdo linkage selectivity (Fig. 2), which could be enabled by the presence of an ester at C2. Moreover, we envisaged the incorporation of a hydrophobic spacer into the reducing terminus of **4** that would, in part, allow the placement of a hydrophobic motif in a similar region as the lipid A moiety that is found naturally at the same position.

For the creation of key donors **3** we chose a novel and unusual strategy of post-glycosylation homologization (an extension of the carbon chain of the sugar, known as an 'ascension'). The vast majority of glycan assembly methods use a logical approach that is programmed around disconnections to complete and intact glycosyl units. Instead, we reasoned that donor disaccharide **3** could be synthesized from mannose trichloroacetimidate **7** (ref. 37) through glycosylation with a suitable mannose acceptor to give α-Man-(1→3)-α-Man (**5**). This would exploit the known[38] excellently stereoselective formation of the Man-α-Man disaccharides in a less congested motif and would allow us to then potentially perform the chain-extension ascension at C6 of both Man hexosyl units of compound **5** to yield donor **3**. In parallel, the α-Kdo-(2→4)-α-Kdo disaccharide acceptor **4** could be achieved through the glycosylation of a derivatized Kdo acceptor bearing an α-linked hydrophobic spacer with Kdo donor **6** (refs 32, 39; Fig. 2).

Peracetylated mannosyl trichloroacetamidate **7** (ref. 37) was used as the starting material for the synthesis of both halves of the important pre-homologization intermediate α-Man-(1→3)-α-Man **5**. We chose to create a partially protected triol glycosyl acceptor **8** to reduce protecting group manipulation. This necessitated a simultaneously stereoselective and regioselective (one (OH3) from three hydroxyls (OH2, 3, 4)) glycosylation to unite **7** and **8**. We reasoned that the target equatorial OH3 hydroxyl in **8** is in the least hindered position and that neighbouring group participation of the C2 acetyl in **7** could facilitate the stereoselective α linkage. Thus, glycosylation of benzyl alcohol with **7**, deacetylation and subsequent selective protection of the C6 hydroxyl with tert-butyl diphenylsilylchloride afforded compound **8** in excellent yields (Supplementary Fig. 8). As desired, regio- and stereoselective glycosylation of **8** with **7** afforded the desired α-(1→3)-linked disaccharide **9** in reasonable yield. Disaccharide **9** was readily converted to **14** (Supplementary Fig. 8) by deacetylation, with selective protection of the C6-OH by TBDPSCl. Conversion of **14** to **5** to gain selective access to only the OH6 and OH6′ hydroxyl groups was achieved via benzylation of the remaining hydroxyls and then selective removal of the two TBDPS protecting groups (Supplementary Fig. 8).

Key intermediate α-Man-(1→3)-α-Man **5** was homologized to α-Hep-(1→3)-α-Hep **10** through simultaneous non-reducing-terminus, carbon chain ascension[40,41] of both mannosyl moieties (six carbon) to heptosyl (Hep) moieties (seven carbon). A six-transformation sequence of dual Swern oxidation of the two primary (OH6, OH6′) hydroxyls, treatment with vinyl magnesium bromide followed by further benzylation of the resulting hydroxyl group furnished **16** (Supplementary Fig. 8) in a good (34% over six transformations) yield and with useful stereoselectivity (d.r. = 77:23). A second six-transformation sequence of dual dihydroxylation, periodate oxidative cleavage and reduction then converted **16** to HepHep **10** in excellent (51% over six transformations) yields. Global debenzylative hydrogenolysis (93% yield) and peracetylation (76% yield) readily gave peracetylated Hep-(1→3)-α-Hep disaccharide **3a**. A direct comparison and agreement of the full characterization data (see Supplementary Methods and spectra) with previous reports[42,43] vitally confirmed the excellent stereoselectivity and regioselectivity of our synthesis. Importantly, this novel dual ascension strategy allowed improved access to the Hep-α-(1→3)-Hep disaccharide (**3a**) (eight instead of sixteen[33] steps) when compared with previously reported syntheses. Disaccharide **3a** then acted as the key precursor for donor disaccharides **3b–e** (Supplementary Fig. 8).

Following the success of this stereo- and regioselective assembly of the HepHep disaccharide unit from D-mannose, we adopted a similar approach to the assembly of the acceptor disaccharide unit α-Kdo-(2→4)-α-Kdo unit **4**. This was achieved through a two-carbon reducing-terminus carbon chain extension[39,40] of the mannosyl moieties (six carbon) to Kdo moieties (eight carbon), performed before glycosylation.

Glycosylation to yield the Kdo–Kdo disaccharide again exploited a simultaneously stereoselective and regioselective (one (OH4) from two hydroxyls (OH4,5)) glycosylation to minimize protecting group manipulation. We reasoned that the target equatorial OH4 hydroxyl in diol acceptor **11** is in the least hindered position and that inherent stereoelectronic bias towards axial (O–C) bond formation by the oxonium ion (or ion-like intermediate) formed from donor **6** (refs 39, 40) would facilitate the selective formation of a (2→4)-α-linkage[35]. Donor **6** was converted to **11** by first introducing the hydrophobic linker moiety: glycosylation of Cbz-protected **17** with **6** furnished the Kdo-lipid monosaccharide **18** in good yield and with good stereoselectivity (>5:1 α, Supplementary Fig. 9a). Chemoselective and regioselective

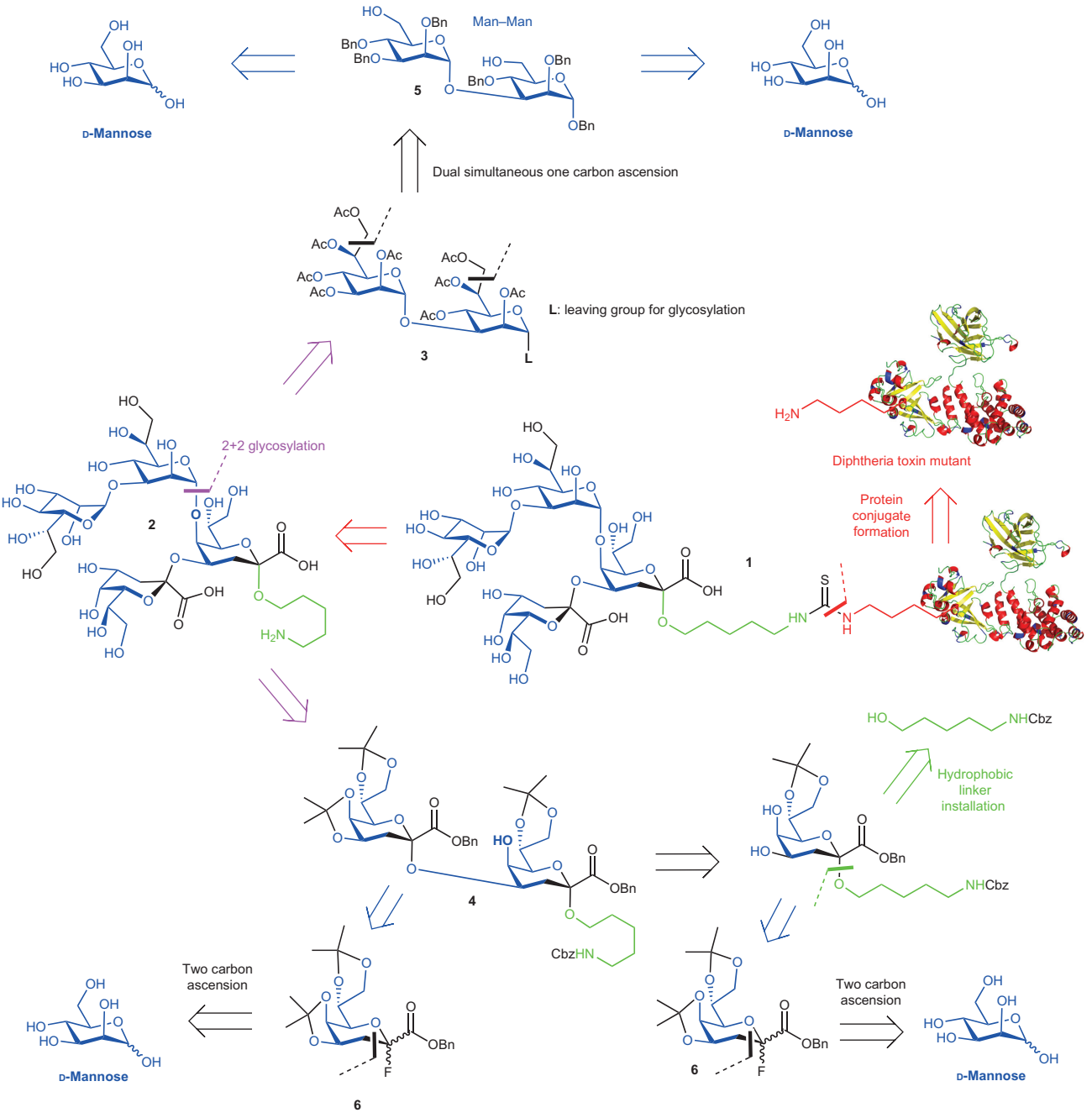

Figure 2 | Design of glycoconjugate with the core tetrasaccharide of the LOS of Gram-negative bacteria. Retrosynthetic analysis of the synthesis of putative vaccine glycoconjugate **1** containing the LPS core tetrasaccharide of the serogroup B *N. meningitidis*. The entire glycan may be derived from the single parent sugar D-mannose using either pre- or post-glycosylation carbon chain ascensions to create key disaccharide Hep-Hep (**3**) and Kdo-Kdo (**4**) building blocks. These were assembled to the tetrasaccharide in a convergent 2+2 glycosylation.

protecting group manipulation (ketal hydrolysis without cleavage of the Cbz then terminal isopropylidene formation) gave diol **11** in good yield. Pleasingly, the key glycosylation between glycosyl fluoride donor **6** and Kdo acceptor **11** was efficiently activated by $BF_3{\cdot}Et_2O$ as a Lewis acid (Supplementary Fig. 9b) and regio- and stereoselectively furnished the Kdo-Kdo disaccharide acceptor **4**. Despite the sluggish nature of the reaction and the formation of this testing linkage, it proceeded with apparently exclusive regioselectivity (only (2→4) linked disaccharides were detected) and with good stereoselectivity (d.r. of α:β = 9:1) in 25% yield (40% based on recovered starting material, Supplementary Fig. 9). Full characterization of **2** (*vide infra*) confirmed the excellent regio- (2→4) and stereo- (α, $^3J_{C1\text{-}H3ax}$ and $^3J_{C1\text{-}H3eq} < 1$ Hz) selectivity of this glycosylation (see Supplementary Information and Supplementary Fig. 5). To the best of our knowledge this represents the first successful synthesis of the Kdoα-(2→4)-Kdo motif.

With the key intermediates in hand, heptose donors **3b–e** (L = Br, SEt, $OC(NH)CCl_3$, $OC(NPh)CF_3$) were prepared in 76–80% yield from **3a** (Supplementary Fig. 8) and then activated using appropriate Lewis acids. Notably, only the Koenigs–Knorr-type bromide donor **3b** (L = Br) proved effective (on activation by AgOTf in the presence of DTBMP); it is rare for this traditional donor sugar type to prove more effective than modern donor variants[38]. Subsequent purification (Supplementary Fig. 1) and global deprotection (ketal hydrolysis, debenzylation, deacetylation, decarbamoylation removing 18 protected groups) successfully yielded HepHepKdoKdo-lipid **2** in 34%

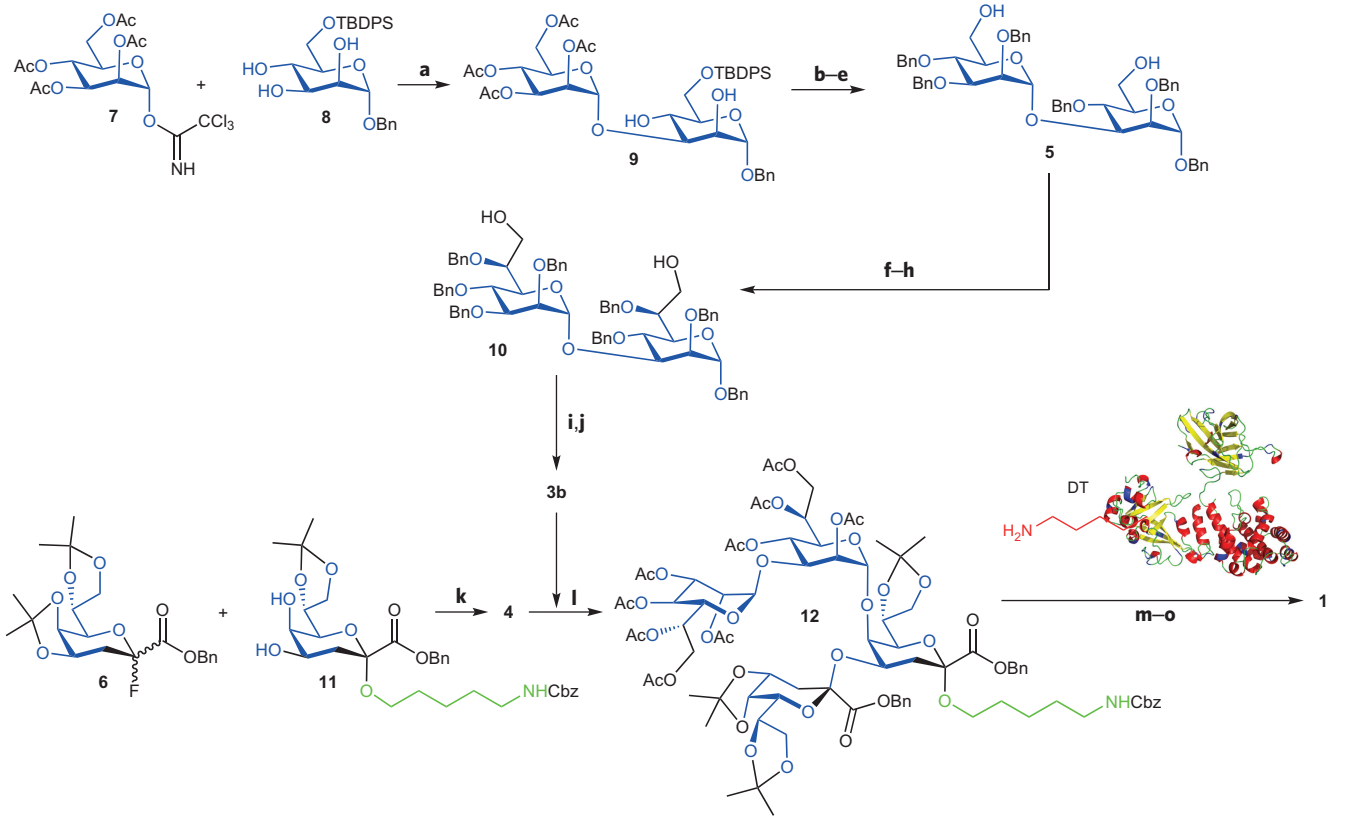

Figure 3 | Synthesis of glycoconjugate vaccine 1. Reagents and conditions for synthetic steps **a**–**o**: **a**, TMSOTf, DCM, 45%; **b**, NaOMe, MeOH; **c**, TBDPSCl, pyridine, 79%; **d**, NaH, BnBr, DMF, 57%; **e**, TBAF, THF, 88%; **f**, i, $COCl_2$, DMSO, Et_3N, DCM; ii, vinyl MgBr, THF, 48% in two steps; **g**, NaH, BnBr, DMF, 70%; **h**, i, OsO_4, NMO, acetone/water, 71%; ii, $NaIO_4$, MeOH/water, 79%; iii, $NaBH_4$, MeOH/water, 91%; **i**, i, Pd/C, H_2, EtOH, 93%; ii, Ac_2O, pyr., 76%; **j**, HBr/AcOH, 80%; **k**, $BF_3{\cdot}Et_2O$, DCM, 0 °C, 25%; **l**, AgOTf, DTBMP, DCM; **m**, TFA/H_2O (3:2), DCM; **n**, i, Pd/C, H_2, EtOH:water = 1:1; ii, 1 N NaOH, 34% over four steps; **o**, $NaHCO_3$, Na_2CO_3, $CSCl_2$, THF; DT-mutant, $NaHCO_3$ pH 9 buffer.

yield (over four steps, 19 transformations) despite competing donor hydrolysis and poor reactivity of the disaccharide Kdo acceptor[35] (Supplementary Fig. 10). Again, full characterization confirmed the excellent regio- and stereo- (α, $^3J_{C1\text{-}H3ax}$ and $^3J_{C1\text{-}H3eq} < 1$ Hz for both KdoI and KdoII) selectivity of the glycosylations used to assemble **2** (Supplementary Fig. 5). Importantly, attempts to remove the isopropylidene protecting groups under typically effective conditions (80% acetic acid, 60 °C, 10 h) led to cleavage of the ketosidic bond that forms the Kdo-Kdo linkage in **12** and **20** (Supplementary Fig. 10), and more mild, careful conditions proved essential. This observed sensitivity to exposure to acid leading to the destruction of the Kdo-Kdo moiety is consistent with observed difficulties in generating isolated samples of Kdo-Kdo in naturally derived samples[44]. Indeed, recent crystal structures of mAbs raised against and crystallized with such natural samples show an absence of the Kdo moiety that displayed lability in our synthesis (KdoII)[8]. This highlights a need for the synthetic methods adopted here (as an alternative to accessing samples through extraction) in the creation of possible Kdo-Kdo-containing vaccines.

The hydrophobic linker moiety in **17** was intentionally equipped with a terminal primary amine group to allow direct protein coupling. This was achieved in a controlled Lys-selective manner[45] through the generation of an intermediate isothiocyanate, using thiophosgene. Subsequent reaction with heterologously expressed diphtheria toxin (DT) mutant (DT-Lys51Glu/Glu148Lys, hereafter referred to as DT)[46] allowed the ready creation of thiourea-linked glycoconjugate vaccine candidate **1** (Fig. 3). Mutants of diphtheria toxin (for example, DT-Asp52Glu, 'cross-reacting material (CRM197)') have been used previously as effective carrier proteins in licensed vaccines effective against multiple pathogens[47]. Quantitative matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) analysis[45] revealed an average loading of four copies of Hep_2Kdo_2-lipid (Fig. 4) to form $(Hep_2Kdo_2)_4$-DT **1**.

The potential of the vaccine candidate glycoconjugate **1** ($(Hep_2Kdo_2)_4$-DT) was evaluated by immunization of New Zealand White rabbits (Supplementary Methods). Enzyme-linked immunosorbent assay (ELISA) analysis of terminal bleed sera showed increased titres in comparison to the pre-bleed sera with a dilution factor to achieve half binding of anti-LPS antibodies to glycoconjugate **1** of ~1:400 (Fig. 5a, blue). Next, to dissect the potential recognition epitopes responsible for this binding affinity, a high-avidity binding ELISA was performed in the presence of NaSCN (150 mM) as a chaotropic agent that induces unfolding of the vaccine antigen protein scaffold but leaves the glycan core LPS tetrasaccharide–lipid moiety intact in the presented antigen[34,48]. Essentially identical serum binding was observed (Fig. 5a, red), consistent with an IgG specificity directed towards the tetrasaccharide–lipid motif. The same sera generated by the glycan core LPS tetrasaccharide–lipid glycoconjugate **1** also showed a general and strong immune response towards not only the LPS from four key target *N. meningitidis* strains MC58, M01, H44 and N298 (Fig. 5b), but also those from other *N. meningitidis* strains (M01 240101, M00 242922, M01 240355, NZ98/254, M06 240052 and M06 240097), *E. coli* (W3110) and even *Pseudomonas aeruginosa* (PAK serotype O6 reference strain) that all share the common Hep_2Kdo_2 tetrasaccharide in their inner core LPS (Fig. 5c). Notably, the sera (pre-bleed and terminal bleed) obtained from immunization (identical procedure, Supplementary Methods) with a corresponding conjugate bearing only Kdo monosaccharide ($(Kdo)_{11}$-DT (**21**)[21]) displayed bound immunogen **21** but showed no binding of the lipid A core of the

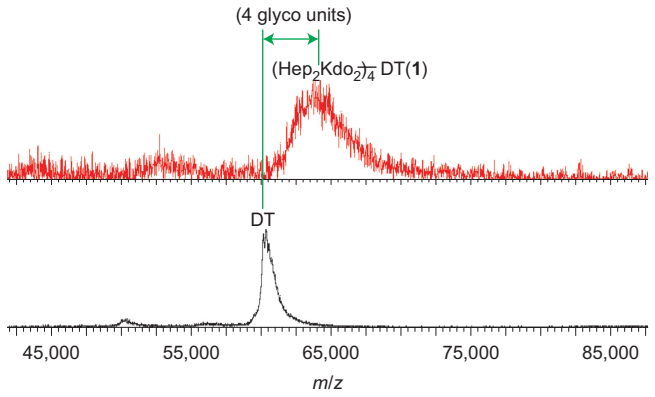

Figure 4 | Mass spectrometric (MALDI-TOF) analysis of glycoconjugate 1. Analysis showed successful attachment of an average of four copies of the LPS core tetrasaccharide to the DT-Lys51Glu/Glu148Lys protein scaffold in glycoconjugate **1**.

LPS in the carbohydrate extracts from different *N. meningitidis* strains (Supplementary Fig. 3). When tested against a conjugate ($(Hep_2Kdo_2)_4$-BSA) built on the alternative protein scaffold bovine serum albumin (BSA), essentially identical titres were observed (Fig. 5a, green) further confirming that the sera contained sugar-specific antibodies.

These promising *in vitro* antigenic data suggested that we had been successful for the first time in generating not only an immune response against this common core LPS motif, as our designed target, but also in generating a 'broad vaccination' response capable of creating a polyclonal response against the sugars of many pathogenic bacteria. In intact bacterial cells, CPS creates a highly effective barrier that blocks critical access of serum antibodies to this core LPS structure. We reasoned that if our designed core LPS immunization were correctly targeted, accessibility to the target should be improved in the absence of shielding CPS. We first confirmed that the antibodies in the Hep_2Kdo_2 antiserum were indeed capable of binding to the bacterium after the CPS layer was partially inhibited[27] by $am_8\gamma CD$ in a low micromolar range (Supplementary Fig. 7). Next, to probe the target and mechanism of our immunization strategy we tested the killing ability of the sera against pathogenic bacteria both with and without CPS.

Consistent with the need for access to the LPS core that is provided by blocking the production of CPS, no significant killing of live *E. coli* (E69 strain) and *N. meningitidis* (MC58, 44/76-SL, NZ98/254) was observed in direct bactericidal assays when serum (containing antibody or simply normal serum) was used alone at 25% (Fig. 5f, lanes 1, 3, 5) on bacteria with intact CPS. Following inhibition of CPS[27] ($am_8\gamma CD$ at 100 μM), complement in normal serum was capable of partial killing (lane 6, only ~40%). However, the combined use of antisera following vaccination and the removal of CPS (through inhibition or in CPS(−) genetic strains) caused a dramatic increase in killing, especially with the use of Hep_2Kdo_2-specific antiserum. At a concentration of 35% (35% serum in RPMI1640 medium, vol/vol), complete killing in the presence of 100 μM $am_8\gamma CD$ was observed. The concentration of $am_8\gamma CD$ required to kill half of the bacteria in the presence of 35% Hep_2Kdo_2-specific antiserum (EC_{50}) was 2.0 ± 0.4 μM ($P < 0.04$, $n = 3$; Fig. 5g). Consistent with a mode of action that targets LPS following vaccination towards the LPS core, the CPS-free mutant strain CWG281 was readily killed by antiserum alone (lanes 7 and 8).

The attachment of pathogenic bacteria to host cells is the very first stage of bacterial invasion/infection of host cells/tissues. Polysaccharides, in particular the outermost CPS, play an essential role, not only in avoiding immune attack but also in enabling effective attachment to the host cells[49–51]. We therefore tested one plausible mechanistic scenario, that the administration of vaccine and then CPS inhibitor as a drug following the occurrence of a bacterial infection could 'rescue' host cells from the attachment of pathogen. The Hep_2Kdo_2 antiserum alone caused a 25% drop in the rabbit peripheral blood mononuclear cells (PBMCs, as the host cell model) attached to fluorescent *E. coli* E69 cells when compared with normal rabbit serum (NRS, blue and black traces in Fig. 5h; the second and first sets of bars in Fig. 5i, respectively). Pleasingly, the combined use of Hep_2Kdo_2 antiserum and $am_8\gamma CD$ (10 μM) rescued 80% of the PBMCs from attachment by the E69 cells within 45 min of incubation (red trace in Fig. 5h; third set of bars in Fig. 5i). Notably, the Hep_2Kdo_2 antiserum is more effective as this 'detachment' in CPS-free CWG281 cells than the Kdo antiserum (cyan and purple traces in Fig. 5h; the fifth and sixth sets of bars in Fig. 5i, respectively). These results together imply that it is feasible to halt a bacterial infection by using a novel two-stage approach of administration: (1) induction of an initial but mostly dormant immune defence through vaccination with the Hep_2Kdo_2 glycoconjugate **1** and (2) ready activation of this 'dormant' immunity with the CPS inhibitor $am_8\gamma CD$ when the bacterial infection is diagnosed and clearance of the invading pathogens is required.

Discussion

We have developed the first chemically synthesized core tetrasaccharide (Hep_2Kdo_2)-based glycoconjugate as a potential vaccine candidate against the infection caused by *N. meningitidis* serogroup B and other bacteria. Because our successful antigenic target, the core tetrasaccharide of LPS Hep_2Kdo_2, is universal in most Gram-negative bacteria, antibodies from this tetrasaccharide-glycoconjugate immunized serum could bind the same core in other pathogenic bacterial species (Fig. 5d,e), as we have shown here. To enable synergistic killing, inhibitors of the CPS transporter in other species (for example, *N. meningitidis* strains) is required. However, such an inhibitor has not yet been discovered. Our earlier research led to the discovery of the first general Wza inhibitor in *E. coli* strain E69 and potentially many strains of other Gram-negative bacterial species, that is, $am_8\gamma CD$ (ref. 27). Protein sequence alignment of Wza_{K30} in *E. coli* E69 and Wza_{MC58} in *N. meningitidis* MC58 (ref. 52) reveals only a low similarity (<30%). Although the primary binding site residues of Wza_{K30} that are targeted by inhibitor $am_8\gamma CD$ (E369, Y373[27]) are not present in Wza_{MC58}, the availability of not only other variants of the inhibitor[27] but also ready routes to create further variants suggests the logic and feasibility of a future screening programme directed at Wza_{MC58} (and others). Notably, support for this ability to extend this unique synergistic strategy to other pathogens comes from the observation that in bacterial laboratory mutants with truncated serogroup B LPS, the key core tetrasaccharide (Hep_2Kdo_2)–lipid motif is indeed exposed[53].

This novel synergistic concept of the combined use of a glycoconjugate vaccine candidate targeting the general inner-core oligosaccharide and a CPS inhibitor is advantageous when compared with the current scenario in antibacterial therapies in that (1) there is a reduced chance of the development of vaccine or inhibitor/antibiotic resistance in pathogenic bacteria, (2) either component alone is non-toxic to either the commensals or the targeted bacterial pathogens, and (3) the maximum killing effects occur only when the specificity of the glycoconjugate matches that of the inhibitor.

It should be noted that the progress of many bacterial infections to potential lethality is so rapid (sometimes within hours, for example, for meningitis) that vaccination upon detection would not be feasible or sensible. Our results suggest that a two-stage process of treatment is feasible: prior induction of anti-inner-core

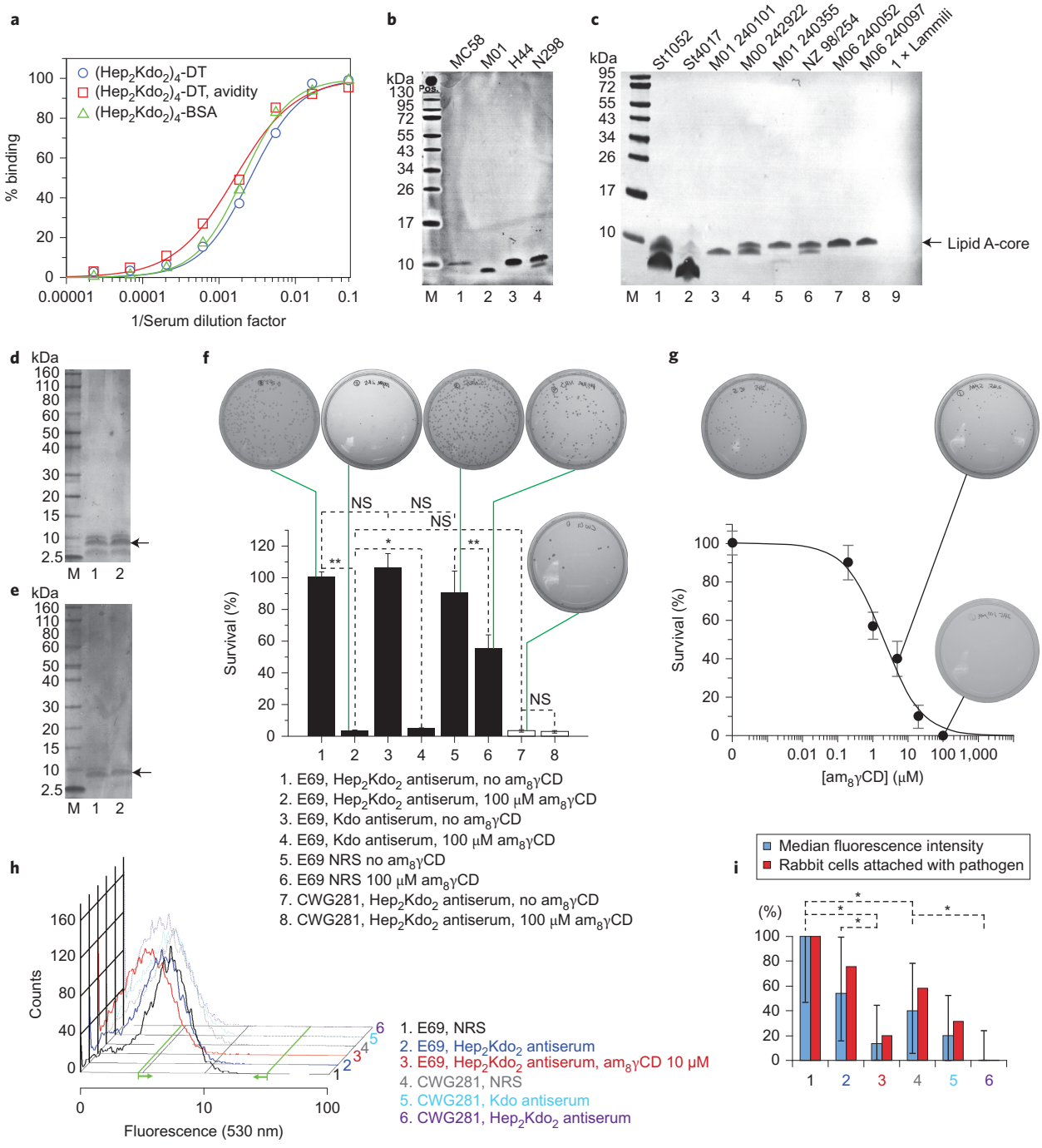

Figure 5 | Immune responses and antibacterial activity from the putative vaccine glycoconjugate 1. a, ELISA analysis of rabbit serum against $(Hep_2Kdo_2)_4$-diphtheria toxin mutant (**1**) under native conditions (blue curve) and under avidity ELISA conditions following chaotropic denaturation using NaSCN (150 mM, red curve). Near identical binding was seen for display of Hep_2Kdo_2 on BSA (green), confirming the sugar-specific nature of the response. **b**, Western blot analysis of carbohydrate extracts from *N. meningitidis* strains MC58, M01, H44 and N298 reacted with anti-**1** serum from rabbit. M: marker. **c**, Western blot analysis of carbohydrate extracts from *N. meningitidis* strains M01 240101, M00 242922, M01 240355, NZ98/254, M06 240052, M06 240097, *E. coli* strain St1052 (W3110) and *P. aeruginosa* PAK serotype O6 reference strain (St4017) reacted with anti-**1** serum from rabbit. M: marker. **d**, Western blot analysis of carbohydrate extracts from strains E69 and CWG281 reacted with anti-**1** serum from rabbit. **e**, Western blot analysis of carbohydrate extracts from strains E69 and CWG281 reacted with anti-**21** serum from rabbit. **f**, Survival (means ± s.d.) of *E. coli* strains E69 (bars 1–6) and CWG281 (bars 7 and 8) in the presence of 25% vol/vol normal rabbit serum (NRS), anti-**1** and anti-**21** sera. Standard deviations (s.d.) were calculated from the data of at least three independent experiments. **g**, Survival (mean ± s.d.) of *E. coli* strain E69 in the presence of 35% vol/vol anti-**1** serum. Standard deviations (s.d.) were calculated from data from at least three independent experiments. *P* values were calculated from the one-tailed Student's *t*-test. Samples in **f** and **g** were incubated at 37 °C for 4 h, RPMI 1640. $*P = 0.01$; $**P = 0.001$; NS, not significant. The inhibitor of CPS transporter Wza, $am_8\gamma CD$ (100 μM), was added to samples 2, 4, 6 and 8 in **f**. The concentrations of $am_8\gamma CD$ ranged from 200 nm to 100 μM in **g**. **h**,**i**, Rescue of rabbit PBMCs from pathogen attachment. Green arrows in **h** mark the fluorescence intensity between which PBMCs were considered attached by *E. coli* E69 or CWG281. Median values and interquartile range for the fluorescence intensities of 10,000 PBMCs are shown as error bars. Significance levels ($*P < 0.02$) were calculated from a one-tailed Mann–Whitney *U*-test.

responses through vaccination and then, when infection develops, rapid blocking of the CPS through inhibition to allow access to the inner core. Thus, one implementation of this 'vaccination + block' approach would be the vaccination of an at-risk cohort (for example, adolescent high-school/undergraduate students at risk from meningitis). On detection of symptoms (perhaps even before full diagnosis of bacterial type given the generality of the inner-core protection against Gram-negative bacteria), a CPS blocker could be administered. We note that widescale vaccination in some countries (for example, the BCG anti-tuberculosis or anti-human papillomavirus programmes in the UK) can prove highly feasible when coordinated through universal access to socialized medical provision (for example, through the UK National Health Service).

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Author contributions

L.K., B.V., A.F., M.K. and B.G.D. designed the experiments. L.K., J.P., A.N.Z. and B.V. conducted the carbohydrate syntheses. B.V. generated protein scaffolds and constructed the corresponding glycoconjugates. L.K., B.V. and L.N. performed the immunological experiments. L.K., B.V., M.K. and B.G.D. analysed the results. L.K., B.V. and B.G.D. wrote the paper. All authors discussed the results and commented on the manuscript.

Additional information

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Competing financial interests

M.K., L.N. and A.F. are employed by GlycoVaxyn.