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# Structural analysis of the lipopolysaccharide from *Neisseria meningitidis* strain BZ157 *galE*: localisation of two phosphoethanolamine residues in the inner core oligosaccharide☆

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**Abstract**

The structure of the phase-variable lipopolysaccharide (LPS) from the group B *Neisseria meningitidis* strain BZ157 *galE* was elucidated. The structural basis for the LPS's variation in reactivity with a monoclonal antibody (MAb) B5 that has specificity for the presence of phosphoethanolamine (PEtn) at the 3-position of the distal heptose residue (HepII) was established. The structure of the *O*-deacylated LPS was deduced by a combination of monosaccharide analyses, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. These analyses revealed the presence of a novel inner core oligosaccharide (OS) structure in the MAb B5 reactive (B5+) LPS that contained two PEtn residues simultaneously substituting the 3- and 6-positions of the HepII residue. The determination of this structure has identified a further degree of variability within the inner core OS of meningococcal LPS that could contribute to the interaction of meningococcal strains with their host. 

*Keywords:* *Neisseria meningitidis*; Phosphoethanolamine; LPS; NMR; Mass spectrometry; Biosynthesis

## 1. Introduction

Meningococcal lipopolysaccharide (LPS) lacks the repeating O-antigens that are typically present in the LPS of enteric pathogens. Instead, the LPS of *Neisseria meningitidis* comprises a core oligosaccharide unit with an inner core di-heptose-*N*-acetyl-glucosamine backbone, wherein the two heptose residues can provide a point of attachment for the outer core oligosaccharide residues.[1] Meningococcal LPS has been classified into 12 distinct LPS immunotypes (L1–L12), originally defined by serological cross-reactivities,[2] but further defined by structural analyses. The structures of LPS from immunotypes L1/6,[3,4] L2,[5] L3,[6] L4/7,[7] L5[8] and L9[9] have been elucidated. The structural basis of the immunotyping scheme is governed by the location of a phosphoethanolamine (PEtn) moiety on the distal heptose residue (HepII) at either the 3- or 6-position or by its absence. The length and nature of oligosaccharide extension from the proximal heptose residue (HepI) and the presence or absence of a glucose sugar at HepII also dictates the immunotype. Oligosaccharides extending from HepI often mimic human glycolipid structures and include sialylated lacto-*N*-neotetraose[5–8] (L2, L3, L4, L5), lactose (L8) and the Pk antigen[3] (L1). Genetic control of meningococcal LPS biosynthesis is well understood, and all of the genes encoding the glycosyltransferases are known including *lgtA*, *lgtB* and *lgtE*,[10,11] *lgtC*,[12] *rfaC*,[13] *lgtF*, *rfaK*,[14,15] *rfaF*[16] and *lgtG*.[17] A characteristic of meningococcal LPS is reversible, high-frequency phase variation of terminal structures mediated by mutation in homopolymeric DNA tracts present in LPS biosynthetic genes.[18] These homopolymeric tracts are absent in inner core LPS biosynthetic genes such as *rfaC*, *rfaK*, *rfaF* and *lgtF*, making this structure relatively stable and an attractive

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candidate for incorporation into a vaccine. A clear understanding of the biosynthesis of *N. meningitidis* LPS has provided us with the genetic tools to construct antigens comprising truncated LPS for production and epitope mapping of antibodies to defined inner core LPS structures. The absence of galactose residues in the conserved inner core structure of meningococcal LPS has led to the utilisation of mutants defective in the enzyme UDP glucose-4-epimerase (GalE) in the construction of truncated LPS structures. This enzyme is essential for *N. meningitidis* to synthesise UDP-Gal for incorporation of galactose into its LPS and is encoded by the gene *galE*.[19] Mutation of this gene results in truncation of the LPS's oligosaccharide chain at the glucose residue at HepI. In an attempt to investigate LPS epitopes for potential inclusion into a vaccine against serogroup B meningococcal disease, we have previously identified an inner core LPS epitope defined by a monoclonal antibody designated B5 (MAb B5) that was developed against the LPS from a *galE* mutant of immunotype L3. The cognate epitope of MAb B5 was found to be present on 70% of all *N. meningitidis* strains tested.[20] We have found that MAb B5 recognises LPS inner core structures that contain PEtn attached specifically at the 3-position of HepII. L1, L3, L7, L8 and L9 LPS immunotypes of *N. meningitidis* containing PEtn at the 3-position of HepII were MAb B5-reactive (B5+), whereas those containing PEtn in an exocyclic position (L2, L4 and L6 immunotypes), or glycoforms that completely lack PEtn (L5 immunotype) were MAb B5 nonreactive (B5−). Recently we successfully utilised MAb B5 to identify a gene (*lpt-3*) that encodes a PEtn transferase, which specifically transfers a PEtn molecule to the 3-position of the HepII residue.[21] Previous studies utilising MAb B5 had investigated several clinical isolates of *N. meningitidis* that did not react with this MAb.[20] One strain of particular interest was the group B strain BZ157 that was found to vary in terms of its reactivity with MAb B5.[20,21] Genetic analyses subsequently revealed that this variation was due to expression of the phase-variable gene *lgtG* that encodes an α-(1→3)-glucosyl transferase that attaches a glucose residue to the 3-position of HepII as was first described in *N. gonorrhoeae*.[17] The *lgtG* gene contains a homopolymeric 5′-$(C)_n$-3′ tract. Molecular biological techniques on the BZ157 strain[21] revealed that if the number of cytosine residues (11 Cs) left the remainder of the gene in frame with the start codon then a functional LgtG was expressed and a glucose residue was transferred to the 3-position of HepII. Absence of a PEtn moiety at this 3-position precludes MAb B5 reactivity. However, if the number of 5′-$(C)_n$-3′ repeats (9 Cs) left the remainder of the gene out of frame with the start codon, LgtG was not expressed, and no glucose residue was therefore attached to the 3-position of HepII, and the PEtn transferase (Lpt-3) could now attach the PEtn moiety to this location creating a MAb B5 reactive epitope.[21] In this way the group B strain BZ157 can phase vary its inner core LPS epitope with respect to MAb B5 reactivity. Structural analyses were therefore undertaken in order to confirm these molecular biological and immunological observations. The *galE* mutant of strain BZ157 was chosen for these studies, as the truncated nature of the LPS derived from this mutant would simplify the structural analyses. During the structural analyses it became apparent that a novel structural feature for meningococcal LPS had been identified, namely the presence of two PEtn residues in the core OS. The localisation of these two PEtn residues and elucidation of this rare structure are described.

## 2. Results

A colony of *N. meningitidis* strain BZ157 *galE* that was found to be reactive with MAb B5 (B5+) by colony hybridisation was selected and elaborated. After growth of this BZ157 *galE* B5+ strain in the 28-L fermenter, an aliquot was taken and a further colony hybridisation was performed with MAb B5 that revealed that greater than 80% of the cells were still B5+. Similarly, a MAb B5 nonreactive (B5−) colony of strain BZ157 *galE* was selected and elaborated. ELISA of the LPS and *O*-deacylated LPS (data not shown) were consistent with the expected phenotypes. LPS was isolated from both the B5+ and B5− phase variants by standard methods. Sugar analysis of the LPS-derived alditol acetates revealed for both strains approximately equimolar amounts of glucitol and glucosaminitol. L-*glycero*-D-*manno*-heptitol was also identified in LPS from both strains, and the small amount of ribitol identified suggested that both LPSs were relatively RNA free. *O*-deacylated LPS from *N. meningitidis* strains BZ157 *galE* B5+ and B5− were prepared by hydrazinolysis, and initial analyses were carried out by electrospray-ionisation (ESIMS) and capillary electrophoresis–mass spectrometry (CE–MS) (Table 1). Determination of the size of the lipid A moiety by MS–MS revealed a variation in the degree and nature of phosphorylation of lipid A for both the B5+ and B5− *O*-deacylated LPS as has been reported previously.[21,22] The size of the core oligosaccharides (OS) was thus deduced. This MS data was consistent with the immunological data for these strains as all B5− strains contained at least two glucose (Glc) residues in the inner core LPS. The presence of additional Glc containing glycoforms for the B5− *O*-deacylated LPS has been observed previously for *galE* mutants of meningococcal strains.[20,23,24] The 3-Glc-containing glycoform was assumed to correspond to substitution of the Glc residue at HepI with an additional

Glc residue, along with the LgtG transferred α-Glc residue at the 3-position of HepII. The presence of the Glc disaccharide at HepI is thought to be due to the fact that in the *galE* background no galactose residues are available for attachment to the Glc at HepI, and hence this acceptor remains available for a previously outcompeted glucosyl transferase. Only approximately 20% of B5+ glycoforms contained two Glc residues, and no 3-Glc-containing glycoforms were observed. The presence of some glycoforms containing two Glc residues in the B5+ *O*-deacylated LPS is again consistent with the previously observed behaviour for *galE* mutants of meningococcal strains.[20,23,24] The most interesting observation from initial MS analysis of the B5+ *O*-deacylated LPS was the identification of two PEtn residues in the majority of the core OS. In order to determine the location of the two PEtn residues in the core OS, CE–MS studies were performed on the *O*-deacylated LPS from the B5+ strain (Fig. 1). The mixture of glycoforms was separated by capillary electrophoresis (CE) (Fig. 1(a)), and initially isomeric glycoforms with $m/z$ $795^{3-}$ corresponding to a molecular mass of 2389 amu were analysed by MS–MS (Fig. 1(b)). This revealed that one glycoform contained an additional PEtn residue on the lipid A molecule as deduced from the asterisked doubly and singly charged ions at $m/z$ $536.5^{2-}$ and $1074.5^{-}$, respectively, corresponding to 1075 amu, which is 123 amu higher than

Table 1
Negative-ion ESIMS data and proposed compositions of *O*-deacylated LPS from *galE* mutant of *N. meningitidis* BZ157 $B5^{+}$ and $B5^{-}$ strains[a]

| Strain | Observed ions ($m/z$) | | Molecular mass (Da) | | Relative intensity | Lipid A[b] | Core OS | Proposed composition |
|---|---|---|---|---|---|---|---|---|
| | $(M-2H)^{2-}$ | $(M-3H)^{3-}$ | Observed | Calculated | | | | |
| BZ157 | | | | | | | | |
| $B5^{+}$ | 1131.5 | 754.0 | 2265.0 | 2266.1 | 2.8 | 952 | 1313 | Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A-OH |
| | 1192.8 | 795.1 | 2388.3 | 2389.1 | 16.7 | 952 | 1436 | Glc, GlcNAc, 2Hep, 2PEtn, 2Kdo, Lipid A-OH |
| | | | | | | 1075 | 1313 | Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A-OH |
| | 1254.6 | 836.0 | 2511.1 | 2512.2 | 27.3 | 1075 | 1436 | Glc, GlcNAc, 2Hep, 2PEtn, 2Kdo, Lipid A-OH |
| | 1274.0 | 849.0 | 2550.0 | 2551.3 | 10.6 | 952 | 1598 | 2Glc, GlcNAc, 2Hep, 2PEtn, 2Kdo, Lipid A-OH |
| | | | | | | 1075 | 1475 | 2Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A-OH |
| | 1294.5 | 862.7 | 2591.1 | 2590.4 | 21.7 | 1155 | 1436 | Glc, GlcNAc, 2Hep, 2PEtn, 2Kdo, Lipid A-OH |
| | | | | | | 1278 | 1313 | Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A-OH |
| | 1335.5 | 890.0 | 2673.0 | 2674.3 | 7.8 | 1075 | 1598 | 2Glc, GlcNAc, 2Hep, 2PEtn, 2Kdo, Lipid A-OH |
| | 1356.0 | 903.7 | 2714.1 | 2713.4 | 10.6 | 1278 | 1436 | Glc, GlcNAc, 2Hep, 2PEtn, 2Kdo, Lipid A-OH |
| | 1437.0 | 957.7 | 2876.1 | 2875.4 | 2.5 | 1278 | 1598 | 2Glc, GlcNAc, 2Hep, 2PEtn, 2Kdo, Lipid A-OH |
| $B5^{-}$ | 1212.7 | 808.0 | 2427.2 | 2428.2 | 27.0 | 952 | 1475 | 2Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A-OH |
| | | | | | | 1075 | 1352 | 2Glc, GlcNAc, 2Hep, 2Kdo, Lipid A-OH |
| | 1274.1 | 848.9 | 2550.0 | 2551.3 | 46.0 | 1075 | 1475 | 2Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A-OH |
| | 1355.4 | 903.3 | 2712.8 | 2713.4 | 27.0 | 1075 | 1637 | 3Glc, GlcNAc, 2Hep, PEtn, 2Kdo, Lipid A-OH |

[a] Average mass units were used for calculation of molecular weight based on proposed composition as follows: Glc, 162.15; Hep, 192.17; GlcNAc, 203.19; Kdo, 220.18; PEtn, 123.05. The average molecular weight of the *O*-deacylated lipid A (Lipid A-OH) is as indicated.

[b] As determined by MS–MS analyses.

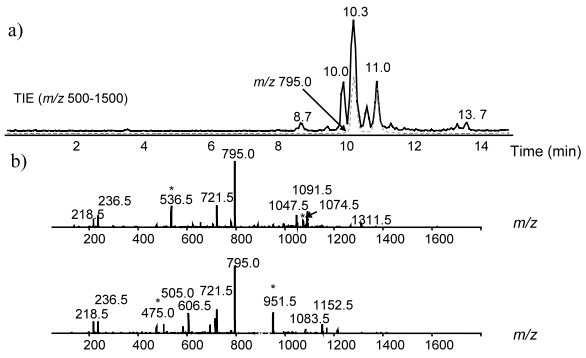



Figure 1. Negative-ion capillary electrophoresis–electrospray ionisation mass spectrum (CE–ESIMS) of *O*-deacylated LPS from *N. meningitidis* strain BZ157 *galE* B5+. (a) Total ion electrophoresogram and single ion monitoring for $m/z\ 795^{3-}$; (b) MS–MS of $m/z\ 795^{3-}$. Ions indicative of lipid A molecule are marked with an asterisk.

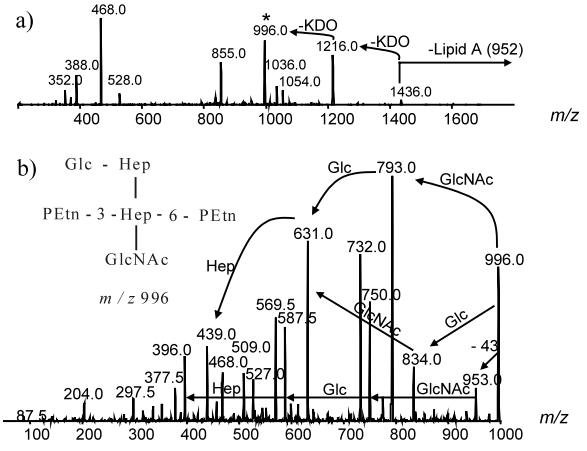
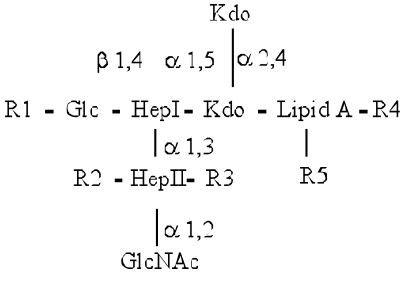



Figure 2. Positive-ion capillary electrophoresis–electrospray ionisation mass spectrum (CE–ESIMS) of *O*-deacylated LPS from *N. meningitidis* strain BZ157 *galE* B5+. (a) MS–MS of $m/z\ 1195^{2+}$; (b) MS–MS of $m/z\ 996^{2+}$. Ion at $m/z\ 996^{2+}$ is highlighted with an asterisk in Fig. 2(a)).

the commonly encountered diphosphorylated lipid A species with molecular mass 952 amu. Subtraction of the molecular mass of the lipid A molecule from the molecular mass of this glycoform revealed the core OS to have a mass of 1313 amu consistent with a composition of Glc, GlcNAc, 2Hep, PEtn, 2Kdo. The other isomeric glycoform contained two PEtn moieties in the core OS as deduced from the asterisked doubly and singly charged fragment ions at $m/z\ 475^{2-}$ and $951.5^{-}$, respectively, corresponding to 952 amu, the minimal lipid A structure, thus revealing a core OS of mass 1436 amu consistent with the composition Glc, GlcNAc, 2Hep, 2PEtn, 2Kdo. Whilst analysis in negative-ion mode identified two PEtn residues in the core OS, accurate localisation of these residues in the core OS was not possible. This was achieved by CE–ESIMS analysis on this glycoform in the positive-ion mode, whereby MS–MS analysis was performed on the doubly charged ion at $m/z\ 1195^{2+}$ (which corresponds to the triply charged ion at $m/z\ 795^{3-}$). In this spectrum (Fig. 2(a)) an abundant, asterisked singly charged ion at $m/z\ 996^{+}$, derived from the complete *O*-deacylated LPS molecule following the loss of the lipid A moiety and two Kdo molecules was identified. Utilising a high orifice voltage, $m/z\ 996^{+}$ was fragmented in a MS–MS experiment in which the product ions localised both PEtn moieties to the HepII residue (Fig. 2(b)). The singly charged ion at $m/z\ 996^{+}$ showed abundant fragment ions arising from consecutive losses of the terminal glucose and *N*-acetylglucosamine residues, resulting in singly charged ions of $m/z\ 834^{+}$, $793^{+}$ and $631^{+}$. Subsequent loss of the HepI residue resulted in the formation of an ion of $m/z\ 439^{+}$ corresponding to PEtn-Hep-PEtn. A second series of ions originating from a loss of 43 amu from the ion at $m/z\ 996^{+}$ were presumed to arise from loss of an ethanolamine moiety on fragmentation. Similar analyses revealed that HepII was consistently diphosphorylated in all glycoforms that contained two PEtn groups in the core OS (data not shown). The conclusion from the MS data that HepII and not HepI is the heptose residue where the two PEtn molecules are attached, was based upon the universally accepted inner core meningococcal LPS structure, wherein PEtn has only ever been identified at HepII, and the fact that these glycoforms arose from a MAb B5 reactive strain which therefore infers the presence of PEtn at the 3-position of the HepII residue.

The range of glycoforms produced by the B5+ *galE* strain are summarised in Table 2. Clearly isomeric

Table 2
Summary of CE–ESIMS data for *O*-deacylated LPS from *N. meningitidis* strain BZ157 *galE* B5+




| Time (min) | Observed Ions (*m/z*) $(M-3H)^{3-}$ | Proposed Composition | | | | |
|---|---|---|---|---|---|---|
| | | Core | | | LipidA | |
| | | R1 | R2 | R3 | R4 | R5 |
| 11.00 | 754 | H | PEtn | H | H | H |
| | 795 | H | PEtn | PEtn | H | H |
| | 863 | H | PEtn | PEtn | H | P PEtn |
| 10.72 | 863 | H | PEtn | H | PEtn | P PEtn |
| 10.30 | 795 | H | PEtn | H | PEtn | H |
| | 836 | H | PEtn | PEtn | PEtn | H |
| | 849 | Glc | PEtn | PEtn | H | H |
| | 904 | H | PEtn | PEtn | PEtn | P PEtn |
| 10.00 | 849 | Glc | PEtn | H | PEtn | H |
| | 890 | Glc | PEtn | PEtn | PEtn | H |
| | 958 | Glc | PEtn | PEtn | PEtn | P PEtn |

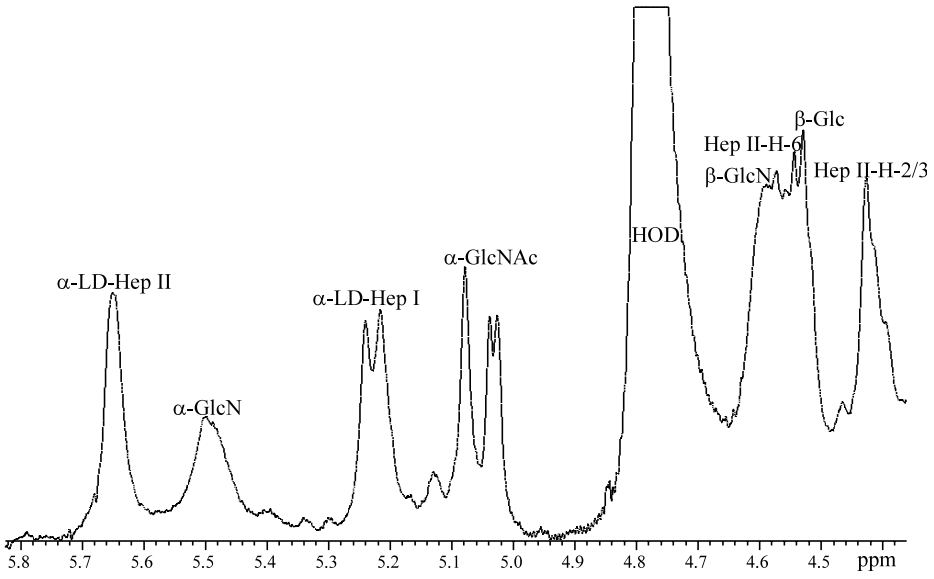



Figure 3. Anomeric region of the 1D $^1$H NMR spectrum of the *O*-deacylated LPS from *N. meningitidis* strain BZ157 *galE* B5+. The spectrum was recorded in $D_2O$ at pH 7.0 and 295 K.

Table 3
$^1$H NMR chemical shifts for the *O*-deacylated LPS from strain *N. meningitidis* BZ157 *galE* B5+[a]

| | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 | H-7 | NOEs |
|---|---|---|---|---|---|---|---|---|
| HepI | 5.24 | 4.12 | 4.12 | 4.28 | nd | 4.07 | nd | 4.32 Kdo H-5 |
| | 5.22 | 4.12 | 4.12 | nd | nd | nd | nd | 4.32 Kdo H-5 |
| HepII | 5.66 | 4.43 | 4.41 | 4.15 | 3.80 | 4.58 | 3.81<br>3.71 | 4.12 HepI H-3 |
| | 5.65 | 4.19 | 4.02 | nd | nd | nd | nd | 4.12 HepI H-3 |
| α-GlcNAc | 5.08 | 3.89 | 3.51 | 4.09 | nd | nd | – | 4.43 HepII H-2<br>5.66 HepII H-1 |
| | 5.03 | 3.87 | 3.51 | 4.09 | nd | nd | – | 4.19 HepII H-2<br>5.65 HepII H-1 |
| α-GlcN | 5.49 | 3.85 | nd | nd | nd | nd | – | – |
| β-Glc | 4.53 | 3.37 | 3.48 | 3.58 | 3.39 | nd | – | 4.28 HepI H-4<br>4.07 HepI H-6 |
| β-GlcN | 4.61 | 3.89 | 3.83 | nd | nd | nd | – | nd |

[a] Recorded at 25 °C, in $D_2O$. Chemical shifts referenced to internal acetone at 2.225 ppm.

glycoforms exist that separate by CE, presumably due to the different locations of the PEtn moiety. For example, the triply charged ion at $m/z$ 849 is observed at two retention times: 10:00 and 10:30 min. The glycoform at 10:00 min was found to have a lipid A of 1075 amu by MS–MS analysis; however, the isomeric glycoform at 10:30 min had a lipid A of 952 amu, and consequently the core OS of this glycoform contained two PEtn species, which by the techniques described above were both localised at the HepII residue.

In order to elucidate the exact locations on the HepII residue where the PEtn moieties are attached, NMR studies were performed. Initially BZ157 *galE* B5+ *O*-deacylated LPS gave a poor $^1$H NMR spectrum; however, on addition of deuterated SDS (5 mg) and EDTA (0.5 mg) a resolved spectrum was obtained (Fig. 3).

The assignment of $^1$H resonances of the sugars of the B5+ *galE O*-deacylated LPS was achieved by employing standard homonuclear 2D NMR COSY and TOCSY techniques and by comparison with reported data for the meningococcal oligosaccharides.[7] These assignments are summarised in Table 3, and the anomeric region of the TOCSY spectrum is shown in Fig. 4.

In the spectrum from the B5+ *O*-deacylated LPS, spin systems arising from heptose residues (HepI and HepII) were readily identified from their anomeric $^1$H resonances at 5.24, 5.22, 5.66 and 5.65 ppm from their small $J_{1,2}$ ($\sim$1 Hz) and $J_{2,3}$ ($\sim$3 Hz) coupling constant

values, which pointed to *manno*-pyranosyl ring systems. The α-configurations were evident for these residues from the occurrence of intraresidue NOEs between the H-1 and H-2 resonances only. The remaining resolved residues in the α-anomeric region at 5.49, 5.08 and 5.03 ppm were determined to be *gluco*-pyranose amino sugars, based upon the coupling constants of $J_{1,2}$ (3 Hz) and $J_{2,3}$, $J_{3,4}$, $J_{4,5}$ (8–10 Hz) and that the H-2 resonances of 3.85, 3.89 and 3.87 ppm correlated in $^{13}C-^{1}H$ HSQC experiments to $^{13}C$ resonances of $\sim$56.0 ppm, this $^{13}C$ chemical shift being diagnostic of amino-substituted carbons. The occurrence of two sets of signals for each inner core residue (HepI, HepII and GlcNAc) was apparent from this initial analysis. The explanation for this behaviour was due to the heterogeneity of the sample, a fact that became clear on further studies (see below). The remainder of the anomeric resonances in the low-field region (4.45–6.00 ppm) of the spectrum were all attributable to β-linked residues by virtue of their high $J_{1,2}$ ($\sim$8 Hz) coupling constants. Two of these resonances at 4.53 and 4.61 ppm were assigned to the *gluco*-configuration by virtue of their $J_{2,3}$, $J_{3,4}$, $J_{4,5}$ (8–10 Hz) vicinal ring-proton coupling constants. The residue at 4.61 ppm was deemed to be an amino sugar as its H-2 resonance correlated in a $^{13}C-^{1}H$ HSQC experiment to a $^{13}C$ resonances of $\sim$55 ppm. An additional signal in this region of the spectrum at 4.58 ppm was assigned to the H-6 proton of the HepII residue due to cross-peaks in the TOCSY spectrum to resonances at 3.81 and 3.71 ppm characteristic of the H-7a and H-7b protons of the HepII residue, respectively.

The chemical shifts of the H-2 (4.43 ppm) and H-3 (4.41 ppm) resonances of the HepII residue in the B5+ *O*-deacylated LPS were characteristic of PEtn substitution at the 3-position.[7] The absence of PEtn at the 3-position from some HepII residues in the B5+ *O*-deacylated LPS was evident from the upfield-shifted value of the H-2 (4.19 ppm) and H-3 (4.02 ppm) protons of this heptose residue. The signal at 4.58 ppm was characteristic of PEtn substitution at the 6-position of HepII.[7]

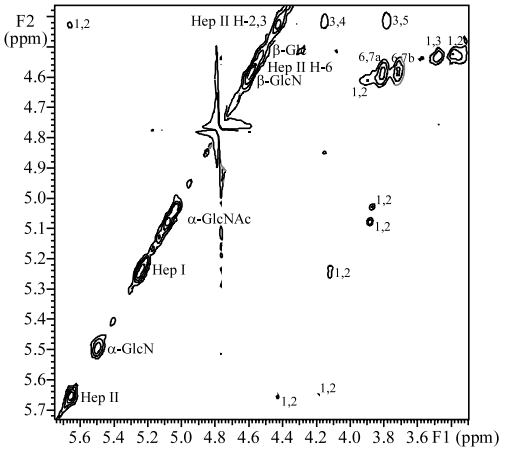



Figure 4. TOCSY spectrum of the α-anomeric region of the *O*-deacylated LPS from *N. meningitidis* strain BZ157 *galE* B5+. The spectrum was recorded in $D_2O$ at pH 7.0 and 295 K.

A $^{31}P-^{1}H$ HSQC experiment showed cross-peaks from $^{31}P$ signals to $^{1}H$ resonances at 4.58 and 4.41 ppm (Fig. 5), in addition to the expected cross peaks from the lipid A phosphate moieties (5.49 and 3.85 ppm) and within the PEtn residues (4.08 ppm), which confirms the identification of resonances at 4.58 and 4.41 ppm as sites of phosphorylation.

The sequences of glycosyl residues within the oligosaccharides were determined from interresidue $^{1}H-^{1}H$ NOE measurements between anomeric and aglyconic protons on adjacent glycosyl residues. The observed NOE connectivities and $^{1}H$ chemical shifts were indicative of the typical meningococcal inner core structure and are shown in Fig. 6 and summarised in Table 3, and included an NOE connectivity from the anomeric $^{1}H$ resonance of GlcI to the H-4 and H-6 $^{1}H$ resonances of HepI at 4.28 and 4.07 ppm, respectively. Similarly, a typical NOE connectivity was observed from the anomeric $^{1}H$ resonance of HepI to the H-5 and H-7 $^{1}H$ resonances of the Kdo residue at 4.32 and 3.84 ppm, respectively. The NOE data also explained the observation of two sets of resonances for the α-GlcNAc, HepI and HepII residues. The anomeric $^{1}H$ resonance of the α-GlcNAc residue at 5.08 ppm had interresidue NOE connectivities to the H-1 and H-2 $^{1}H$ resonances at 5.66 and 4.43 ppm of the HepII residue in which PEtn is attached at the 3-position. Whereas the anomeric $^{1}H$ resonance of the α-GlcNAc residue at 5.03 ppm had interresidue NOE connectivities to the H-1 and H-2 $^{1}H$ resonances at 5.65 and 4.19 ppm of the HepII residue in which the 3-position is not substituted (Fig. 6). The relative intensity of these resonances is therefore indicative of the amount of PEtn present at the 3-position of the HepII residue and the approximate ratio of 4:1 is in good agreement with the initial colony hybridisation data for the B5+ strain.

Whilst MS data provided evidence for the presence of LPS populations containing two PEtn substituents at the HepII residue, and NMR evidence was consistent with substitution of HepII residues at both the 3- and 6-positions, unequivocal proof for substitution of the same HepII residue by PEtn moieties at both the 3- and 6-positions simultaneously had not been established. To this end, selective excitation experiments were performed, selectively irradiating the H-3 proton of the 3-substituted HepII residue (i.e., 4.41 ppm) in a 1D TOCSY experiment with increasing mixing times (30–150 ms) to optimise magnetisation transfer through the HepII ring system. However, in *manno*-configured heptose sugars the coupling constant between the H-5 and

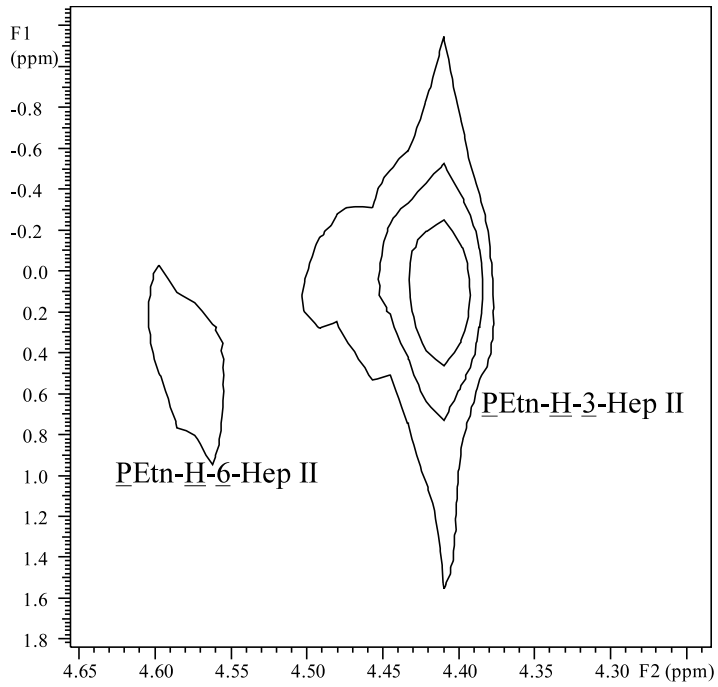



Figure 5. Region of the 2D $^{31}P-^{1}H$ HSQC spectrum of the *O*-deacylated LPS from *N. meningitidis* strain BZ157 *galE* B5+ illustrating the di-PEtn-Hep II connectivities. The spectrum was recorded in $D_2O$ at pH 7.0 and 295 K.

H-6 protons is so small that it is not possible in a TOCSY experiment to access the H-6 $^{1}H$ resonance from the H-3 $^{1}H$ resonance. However, once the H-3 $^{1}H$ resonance had been selectively irradiated, the H-4 and H-5 $^{1}H$ resonances were revealed (optimised with a TOCSY mixing time of 60 ms) (Fig. 7(a)). In a subsequent TOCSY–NOESY selective experiment,[25] it would therefore be possible to selectively irradiate this H-5 proton in a NOESY step following the selective excitation of the H-3 $^{1}H$ resonance in a TOCSY step, in order to reveal the H-6 $^{1}H$ resonance. This was successfully achieved and is shown in Fig. 7(b). Therefore, identification of the H-3 (4.41 ppm) and H-6 (4.58 ppm) $^{1}H$ resonances in the same spin system by selective irradiation has unequivocally demonstrated that the HepII residue is simultaneously substituted by two PEtn molecules at the 3- and 6-positions in those glycoforms that contain two PEtn residues in their core OS. The major inner core LPS glycoforms produced by strain BZ157 *galE* B5+ are illustrated in Fig. 8.

## 3. Discussion

Structural analysis of the *O*-deacylated LPS from the group B meningococcal strain BZ157 *galE* B5+ has identified the novel structural feature of an inner core OS containing two PEtn residues, and, furthermore, these two residues have both been localised to the distal heptose residue of the inner core and found to substitute this residue at the 3- and 6-positions. Previous studies on meningococcal LPS had suggested the presence of a second PEtn molecule in the core OS, but without localisation.[26,27] Earlier studies on gonococcal LPS have also alluded to the presence of additional phosphorylation sites in the core OS, but none have localised specifically the attachment points of these

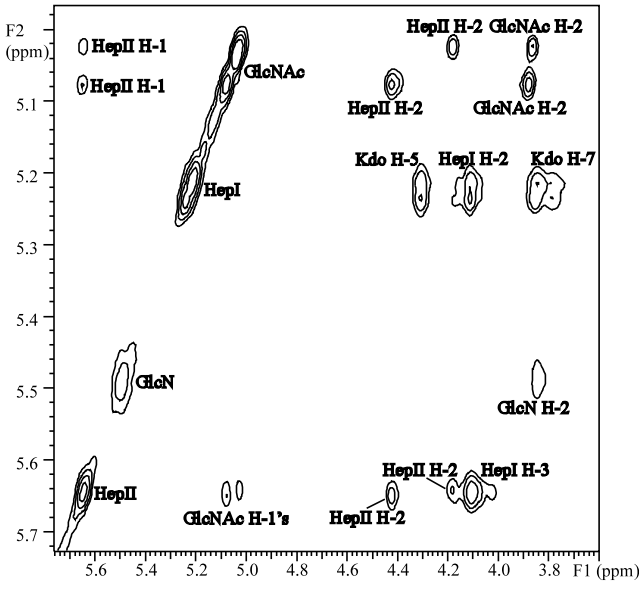



Figure 6. NOESY spectrum of the α-anomeric region of the *O*-deacylated LPS from *N. meningitidis* strain BZ157 *galE* B5+. The spectrum was recorded in $D_2O$ at pH 7.0 and 295 K.

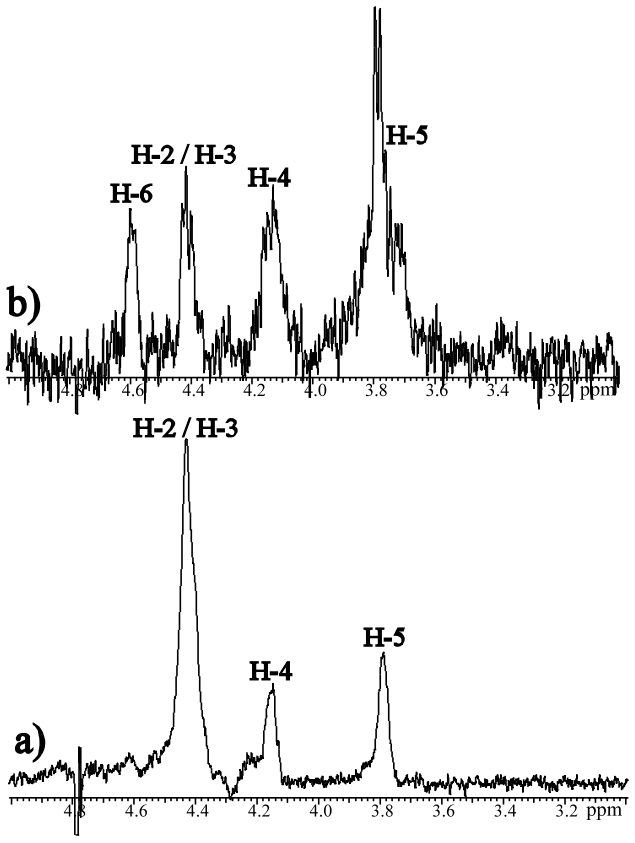



Figure 7. (a) 1D TOCSY and (b) 1D TOCSY–NOESY spectrum using selective excitation of H-2, H-3 of HepII in the TOCSY step and H-5 of HepII in the NOESY step. The spectrum was recorded in $D_2O$ at pH 7.0 and 295 K. The assignments of the $^1H$ resonances of the HepII residue are indicated.

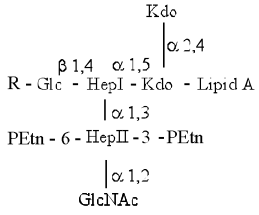



Figure 8. Chemical structure of the major glycoforms from the LPS of *N. meningitidis* strain BZ157 *galE* B5 + . R is H or Glc.

phosphate-containing groups.[28] One study described an analogous situation to that described here, where a mutation of the *lgtG* gene in gonococcal strain 15253 led to the simultaneous gain of a PEtn moiety, although the exact location of the additional PEtn moiety was not determined.[17] Other studies on gonococcal LPS described the presence of a PEtn moiety at the HepI residue of the wild type F62 strain, but without any strong evidence for this localisation.[26] Recently, Rahman et al,[29] identified a similar pattern for di-PEtn substitution in the *rfaK* mutant of the genome strain NMB. The *rfaK* gene encodes an α-(1 → 2)-*N*-acetylglucosamine transferase, whereby a GlcNAc residue is attached to the 2-position of the HepII residue. Surprisingly, there were no di-PEtn molecules in the wild-type NMB strain when the GlcNAc residue was still present.[30] It is conceivable that in the *rfaK* mutant the arrangement of residues in the inner core LPS molecule would differ significantly from that found in the fully assembled inner core structure, which in all likelihood could affect subsequent steps in the biosynthesis of the inner core oligosaccharide. In the present study it is very unlikely that the *galE* mutation would influence this phenomenon, as the presence or absence of galactose in the outer core of the LPS would have negligible affect on the inner core presentation.

The genetic control of the phosphorylation pattern observed at HepII in BZ157 LPS is intriguing. Many of the genes involved in the substitution of this HepII residue are known, with *rfaK* encoding the *N*-acetylglucosamine-α-(1 → 2) transferase, *lgtG* encoding the glucose-α-(1 → 3) transferase and *lpt-3* encoding the PEtn transferase to the 3-position. The gene responsible for the transfer of PEtn to the 6-position of HepII is not currently known. Clearly the interplay between *lgtG* and *lpt-3* dictates the arrangement of residues at HepII, and this interplay is affected by the phase-variable nature of the *lgtG* gene. It is evident that when *lgtG* is in frame and the glucosyl transferase is expressed, this enzyme seems to have a higher affinity for the 3-position of HepII and outcompetes the *lpt-3* gene product for this acceptor. However, when *lgtG* is out of frame and the glucosyl transferase is not expressed, the *lpt-3* gene product is then able to utilise the HepII residue as an acceptor. The gene responsible for transfer of the PEtn residue to the 6-position of HepII seems to function whether glucose, PEtn or no residue is present at the 3-position of HepII. Studies are underway in our laboratories to ascertain the structural consequences, specifically in terms of phosphorylation of HepII, of a mutation in the *lpt-3* gene and phase locking on or off the *lgtG* gene. Additionally, studies are underway to attempt to identify the PEtn transferase specific for the 6-position of HepII in meningococcal LPS. MAbs specific for inner core LPS that contain PEtn at this 6-position have been developed in our laboratories and are being utilised to attempt to identify this gene.

The biological relevance of this di-PEtn phosphorylation pattern is of interest. Recently studies performed in our laboratory on a group Y meningococcal clinical isolate have identified an identical structural arrangement in terms of the PEtn substitution pattern at HepII to that found for the group B clinical isolate BZ157 described here. The LPS of this group Y strain stoichiometrically presented two PEtn moieties at the HepII residue and has been shown to have an unusually high ability to activate complement.[31] Therefore, this struc-

tural motif would actually appear to be detrimental for the infecting strain. It would be interesting to ascertain the immunocompetence of the hosts of these strains that contain two PEtn residues in the core OS of their LPS.

These analyses have therefore revealed further potential for variation in the inner core LPS of meningococcal strains. The potential to vary the degree and nature of phosphorylation at the HepII residue provides *N. meningitidis* with additional mechanisms to alter the LPS epitopes and affect its interaction with the host.

## 4. Experimental

*Growth of organism and isolation of lipooligosaccharide (LPS).*—*Neisseria meningitidis* strains BZ157 *galE* B5+ and B5− (NRCC #6094 and 6095) were initially grown overnight at 37 °C in 10% $CO_2$ on 50% Todd–Hewitt 50% Columbia (THC) agar plates. A B5+ and B5− colony were selected by colony hybridisation (see below) and elaborated. Starter plates were used to heavily inoculate starter cultures (1 L) and grown in (THC) broth at 37 °C for 18 h. Starter cultures were used to inoculate the 28-L fermenter (New Brunswick Scientific Microferm) with the same media, and grown as for starter cultures. The bacterial growth was harvested by continuous centrifugation, and the percentage of B5+ reactivity was determined by colony hybridisation of a 5-mL aliquot after dilution plating on THC plates. A plate with 25–40 distinct colonies was blotted using nitrocellulose membranes (Millipore). The primary antibody was MAb B5 and the secondary antibody was alkaline phosphatase goat anti-mouse IgG. Colour development was achieved using nitroblue–tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT–BCIP alkaline phosphatase substrate). LPS was extracted by the hot phenol–water method as described previously[32] and purified from the aqueous phase by ultracentrifugation (45 K, 4 °C, 5 h). Solid-phase ELISA with MAb B5 confirmed the LPS phenotype as described.[20] *O*-deacylated LPS was prepared as described previously.[20]

*Analytical methods.*—Sugars were determined as their alditol acetate derivatives by GLC–MS as described previously.[32]

*Mass spectrometry.*—All electrospray-ionisation (ESIMS) analyses were carried out as described previously.[20] Capillary electrophoresis–ESIMS (CE–ESIMS) analysis was performed on a crystal Model 310 CE instrument (AYI Unicam, Boston, MA, USA) that was coupled to an API 3000 mass spectrometer (Perkin–Elmer/Sciex, Concord, Canada) via a microionspray interface. A sheath solution (2:1 2-propanol–methanol) was delivered at a flow rate of 1 μL/min to a low dead volume tee (250 μm i.d., Chromatographic Specialities, Brockville, Canada). All aqueous solutions were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) before use. An electrospray stainless steel needle (27 gauge) was butted against the low dead volume tee and enabled the delivery of the sheath solution to the end of the capillary column. Separation was obtained on ~90 cm length of bare fused-silica capillary (192 μm o.d. × 50 μm i.d. [Polymicro Technologies, Phoenix, AZ, USA]) using 30 mM morpholine in deionised water (negative-ion mode), pH 9.0, containing 5% methanol and 15 mM ammonium acetate–ammonium hydroxide in deionised water (positive-ion mode), pH 9.0, containing 5% methanol. A voltage of 25 kV was typically applied at the injection. The outlet of the capillary was tapered to ca. 15 μm i.d. using a laser puller (Sutter Instruments, Novato, CA, USA). Mass spectra were acquired with dwell times of 3.0 ms per step of 1 $m/z$ unit in full-mass scan mode. In the CE–ESIMS, 10 nL of sample was typically injected by using 100 mbar for duration of 0.1 min. For CE–ESIMS–MS experiments, about 30 nL of sample was introduced using 300 mbar for 0.1 min. The MS–MS data were acquired with dwell times of 2.0 ms per step of 1 $m/z$ unit. Fragment ions formed by collision activation of selected precursor ions with nitrogen in the RF-only quadrupole collision cell were mass analysed by scanning the third quadrupole.

*NMR spectroscopy.*—Nuclear magnetic resonance (NMR) experiments were performed on Varian INOVA 600, 500, 400 and 200 NMR spectrometers using a 5 mm triple resonance probe with Z gradient. Measurements were made at 25 °C at concentrations of ~2 mg/mL in $D_2O$, subsequent to several lyophilisations with $D_2O$. For the proton chemical shift reference, the methyl resonance of internal acetone at 2.225 ppm was used. All the NMR data were acquired using Varian sequences provided with the VNMR 6.1B software. The same program was used for processing. All NMR spectra were acquired as described previously.[32] The mixing times for the NOESY and TOCSY experiments were 400 and 80 ms, respectively.

The 1D $^{31}P$ experiment was carried out on a Varian Inova 200 spectrometer with a sweep width of 40 ppm, 20000 transients and acquisition time of 1.6 s. The 2D $^{1}H-^{31}P$ HSQC experiment was acquired on a Varian Inova 400 spectrometer for 6 h. The coupling constant was optimised at 12 Hz by performing an array of 1D–HSQC experiments. The sweep width in the $F2$ ($^{1}H$) dimension was 6.0 ppm and in the $F1$ ($^{31}P$) dimension was 16.2 ppm. Water presaturation during the relaxation delay was 1.5 s, acquisition time in $t_2$ was 0.21 s, and 32 increments with 180 (HMQC) scans per increment were obtained.

The selective 1D analogue of the 3D TOCSY–NOESY experiment[25] was performed using a 270° Gaussian pulse (1024 points) truncated at 2.5% for

selective excitation. The pulse width of the selective pulses was 50–80 ms. For the TOCSY experiment the number of transients was 3072 with a 10 ppm sweep width, and the mixing time was an array from 30–150 ms. For the TOCSY–NOESY experiment, the number of transients was 32,768 with a sweep width of 10 ppm. The spin lock mixing time for the TOCSY step was optimised at 60 ms. The mixing time for the NOESY step was 500 ms.

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