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The lipooligosaccharide (LOS) of *Neisseria meningitidis* Serogroup B Strain NMB contains L2, L3, and novel oligosaccharides, and lacks the lipid-A 4′-phosphate substituent

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Abstract

The complete structure of the lipooligosaccharide (LOS) from *Neisseria meningitidis* strain NMB (serotype 2b:P1.2,5), a serogroup B cerebrospinal fluid isolate, was determined. Two oligosaccharide (OS) fractions and lipid-A were obtained following mild acid hydrolysis of the LOS. The structures in these fractions were determined using glycosyl composition and linkage analyses, N spectroscopy and mass spectrometry. One oligosaccharide fraction (OS1) consists of a molecule having a glycosyl sequence identical to that previously reported for the LOS from immunotype L2 *N. meningitidis* [A. Gamain, M. Beurret, F. Michon, J.-R. Brisson, and H.J. Jennings, *J. Biol. Chem.*, 267, (112) 922–925] i.e., a lacto-*N*-neotetraose is attached to heptose I (Hep I), with terminally linked *N*-acetylglucosaminosyl and glucosyl residues attached to Hep II of the inner core. Approximately 70% of this structure is acetylated at O-6 of the terminally linked α-*N*-acetylglucosaminosyl residue. As with the L2 structure, the NMB LOS contained phosphoethanolamine (PEA) at *O*-6 or *O*-7 of the Hep II residue. The second oligosaccharide fraction (OS2) contains a mixture of three different molecules, all of which vary from one another in their glycosyl substitution patterns of the Hep II residue. The most abundant molecule in OS2 has a structure identical to that of OS1, i.e., it has the L2 glycosyl sequence. A second molecule (OS2a) lacks the terminal glucosyl residue at O–3 of Hep II; i.e., it has a glycosyl sequence identical to that of the mild acid released oligosaccharide of *N. meningitidis* immunotype L3, L4, or L7 LOSs. The third molecule (OS2b) is a novel structure that lacks the terminal *N*-acetylglucosaminosyl residue linked to O-2 of Hep II. Overall, 76% of OS released from NMB LOS has the L2 structure, 15% is OS2a (L3), and 9% is OS2b. A portion (20%) of the molecules in the NMB LOS preparation also contained terminally linked sialic acid attached to O-3 of the lacto-*N*-neotetraose galactosyl residue, which is also consistent with the L3, or L4 LOS structures. In contrast to the previously reported structure of *N. meningitidis* lipid-A [V. A. Kulshin, U. Zähringer, B. Linder, C.E. Frasch, C.-M. Tsai, B.A. Dmitriev, and E.T. Rietschel, *J. Bacteriol.*, 174, (1992) 1793–1800], only 30% of the lipid-A from NMB LOS possesses 4′-phosphate. Comparison with the lipid-A of LOS purified

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from an isogenic acapsulate mutant, M7, revealed that the 4′-position was almost completely occupied with phosphate. These data emphasize the structural heterogeneity of the OS and phosphate substituents of Hep II, and 4′-phosphorylation of lipid-A of meningococcal LOS.

Keywords: *Neisseria meningitidis*; Lipooligosaccharides; Serogroup B

1. Introduction

Neisseria meningitidis is an important obligate human pathogen [1–3]. A variety of clinical syndromes are produced by systemic meningococcal infection, including limited benign bacteraemia, meningitis and fulminant meningococcal septicemia [4]. The severity of the disease has been correlated to high levels of circulating endotoxin and the resultant proinflammatory response [5]. It has been shown in various biological systems that the lipid-A moiety of the *N. meningitidis* lipooligosaccharide (LOS) is the effector of endotoxic activities [6,7]. Meningococcal LOS is also involved in the colonization and invasion of epithelial cells [8,9] and is required for resistance to serum bactericidal activity [10,11]. Phase variation of the oligosaccharide chain (α-chain) attached to the first heptose (Hep I) of the LOS inner core generates structural heterogeneity and antigenic diversity of meningococcal LOSs [12]. The expression of the lacto-*N*-neotetraose α-chain, which mimics human glycosphingolipids [13,14], has been correlated with invasive meningococcal disease [15,16].

Intense research has focused upon elucidating the chemical structures and biosynthetic pathway for meningococcal LOSs. Meningococcal LOSs have been serologically divided into at least 12 immunotypes [17]. Structural studies on the major oligosaccharides obtained from the Ll and L6 [18], L2 [19], L3 [20], L4 [21], L5 [22], and L7 [21] immunotypes have identified regions of structural differences and similarities which account for both the immunotype specificity and cross-reactivity exhibited by various meningococcal LOS antibodies [23,24]. Structural heterogeneity also exists among the oligosaccharides obtained from the LOS of a single meningococcal strain. This heterogeneity is generated by the addition or deletion of sugar residues [22], as well as by the presence and location of phosphoethanolamine (PEA) [18] [19] and O-acetyl groups [22]. Sialylation of the terminal galactopyranosyl residue of the lacto-*N*-neotetraose α-chain is also variable and strain dependent [25,26]. In this paper, the structural determination of the LOS of the serogroup B strain NMB (2B: P1.2,5:L3,7,9), which was originally isolated from the cerebrospinal fluid of a patient with meningococcal meningitis) is described. This LOS differs in both the Hep II oligosaccharides and lipid-A from previously published [19,20,27,28] structures and extends our understanding of the heterogeneity of meningococcal LOS.

2. Results

The isolation of NMB LOS and the OSs released by mild acid hydrolysis.—A portion of purified LOS (60 mg) was mild acid hydrolyzed, and the oligosaccharides (OSs) were separated by gel-filtration chromatography using a Bio-Gel P-4 column. Two oligosaccharide peaks, OS1 and OS2, eluted just after the void volume (figure not shown). The yields of OS1 and OS2 were 7 mg and 15 mg, respectively.

Composition analysis.—Glycosyl and fatty acid composition analysis of purified LOS, LOS treated with aqueous hydrofluoric acid (LOS-HF), OS1, OS1-HF, OS2-HF and lipid-A are shown in Table 1. The intact LOS has a 2:2:1:2 glycosyl molar ratio of glucose:galactose:heptose:glucosamine, with significant levels of both Kdo and sialic acid. The glycosyl molar ratio after HF treatment is 1:1:1:1 due to an increase in the amount of heptose. Since the procedure and columns used for GLC analysis do not allow measurement of phosphorylated glycosyl residues, the increase in heptose after HF treatment is due to its detectability after the removal of phosphate groups. The sialic acid content, as determined by analysis of its per-*O*-trimethylsilylated methyl glycoside, is approximately 20% of the glucose or galactose amount, indicating that approximately 20% of the of the molecules in the LOS preparation are sialylated. The level of glucosamine is most likely low due to the resistance of its glycoside bond to acid hydrolysis. Fatty acid analysis of the lipid-A showed that

Table 1
Composition analysis of *N. meningitidis* strain NMB LOS, LOS-HF, OS1, OS1-HF, OS2-HF and lipid-A

	Composition (relative mol % for glycosyl residue)[a,b]					
Residue	LOS	LOS-HF	OS1	OS1-HF	OS2-HF	Lipid-A
Glucose	29	26	34	28	27	–
Galactose	27	24	30	25	26	–
l,D-Heptose	15	23	14	26	28	–
Glucosamine	29	27	22	21	19	+
Kdo	+	+	+	+	+	–
Sialic acids	+	+	–	–	–	–
C12:0	ND	ND	–	–	–	0.18
3-OH-C12:0	ND	ND	–	–	–	0.18
3-OH-C14:0	ND	ND	–	–	–	0.18

[a] Fatty acyl residues were calculated in mol/mg lipid-A from *N. meningitidis* lipid-A.
[b] +, present; –, absent; ND, not determined.

Table 2
Glycosyl linkage analysis of *N. meningitidis* strain NMB LOS, OS1, OS1-HF, OS2-HF, and lipid-A

	Composition (relative mol %)				
Glycosyl linkage	LOS	OS1	OS1-HF	OS2-HF	Lipid-A
Terminal Glc	14	17	15	12	—
Terminal Gal	11	16	13	15	—
4-linked Glc	16	18	15	15	—
3-linked Gal	18	14	11	11	—
2,3,6-linked Hep[a]	—	—	6	—	—
2,3,7-linked Hep[a]	—	—	4	—	—
2-linked Hep	—	—	—	3	—
3-linked Hep	—	—	—	2	—
3,4-linked Hep	13	14	14	14	—
2,3-linked Hep	Trace	Trace	2	10	—
Terminal GlcNAc	8	10	9	8	42
4-linked GlcNAc	9	11	11	10	—
6-linked GlcNAc	11	—	—	—	58

[a] Ethylation of permethylated HF treated OS results in ethyl substitution at the 6- or 7-position.

it contained dodecanoic, 3-hydroxydodecanoic, and 3-hydroxytetradecanoic acids in a ratio consistent with that previously reported for *N. meningitidis* strain M986-NCV1 [27].

The composition of OS1 before and after HF treatment gives essentially the same results as those described above for the LOS, with the exception that sialic acid is not present due to its removal by the mild acid hydrolysis procedure. The OS2-HF sample has essentially the same composition as the OS1-HF sample. Determination of the absolute configurations of the hexoses present in OS1 revealed that all hexoses and hexosamines have the D-configuration, and heptoses have the L-glycero-D-manno-configuration.

Glycosyl linkage and mass spectrometric analyses of the NMB LOS and OS fractions.—The glycosyl linkage results, shown in Table 2, for the intact LOS, OS1, and OS1-HF, are consistent with the previously reported the L2 immunotype LOS from *N. meningitidis* strain 2241 [19]. The molar ratio of the various glycosyl residues for OS1 is 1.1:1.0:1.2:0.9:0.8:0.7:0.7 of terminal glucose:terminal galactose:4-linked glucose:3-linked galactose:3,4-linked heptose (Hep I):terminal-*N*-acetylglucosamine:4-linked *N*-acetylglucosamine. In addition, trace amounts of 2,3-linked heptose (Hep II) were detected. The procedure and columns used did not allow the detection of phosphorylated glycosyl residues. However, glycosyl-linkage analysis after aqueous 48% HF treatment of OS1 (OSl-HF), which removes phosphate and phosphoethanolamine (PEA) substituents, resulted in an increased amount of 2,3-linked heptose (data not shown). This result indicated that the Hep II residue contained a phosphate or a PEA substituent. The locations of PEA or phosphate substituents were determined by methylation of OS1, followed by HF treatment and ethylation. Preparation and analysis of the partially methylated/ethylated alditol acetates showed the presence two heptosyl derivatives, 1,2,3,5-tetra-*O*-acetyl-6-*O*-ethyl-4,7-di-*O*-methyl-heptitol and 1,2,3,5-tetra-*O*-acetyl-7-*O*-ethyl-4,6-di-*O*-methylheptitol, due to 2,3-linked heptosyl residues substituted with a PEA group at O-6 and O-7 (see Table 2), respectively. The location of a PEA group at O-6 or O-7 of the Hep II residue is consistent with the previously reported structure for the immunotype L2 LOS from an *N. meningitidis* strain 2241 [19]. The trace amounts of 2,3-linked heptose in OS1 (prior to HF treatment) is consistent with the presence of a small amount of OS1 which lacks the PEA substitution on Hep II, a result also consistent with the previously reported L2 LOS structure [19].

Methylation analysis of the intact LOS showed the same glycosyl linkages as those observed for OS1 with the exception that the LOS sample contained less terminally linked galactose with a corresponding increase in 3-linked galactose. Methylation analysis using the modified procedure designed to determine Kdo and sialic acid linkages showed that the intact LOS contained terminally linked and 4,5-linked Kdo, as well as terminally linked sialic acid. Thus, the increase in terminally linked galactose and decrease in 3-linked galactose after mild acid

hydrolysis (compare the LOS and OS1 results shown in Table 2) are due to the removal of sialic acid terminally linked to O-3 of the lacto-*N*-neotetraose galactosyl residue. Therefore, some (i.e., 20%, see above composition studies) of the intact LOS molecules from NMB are sialylated.

Glycosyl linkage analysis of OS2-HF gave results very similar to those obtained for OS1-HF with the exception that low but significant levels of 2-linked and 3-linked heptosyl residues were observed. Table 2 shows that the ratio of 3,4-linked heptose (Hep I) to the lacto-*N*-neotetraose glycosyl residues (i.e., to terminally linked galactose, 4-linked glucose, 3-linked galactose, and 4-linked *N*-acetylglucosamine) in OS2-HF is that same as the ratio found for OS1-HF. This indicates that all of the molecules in the OS2-HF preparation have the lacto-*N*-neotetraose portion of the LOS. Additionally, the overall similarity in the methylation results of OS2-HF with those for OS1-HF indicate that a large proportion of the OS2-HF fraction consists of the same structure as found for OS1-HF; namely, the L2 structure. The locations of PEA substituents in OS2 was not determined; however, it is likely that the majority are present at the O-6 or O-7 positions of Hep II since a large portion of this fraction has the same structure as that for OS1. The presence of 2-linked and 3-linked heptosyl residues indicates that the OS2 fraction, in addition to having the same structure found in OS1, contains other molecules having heterogeneity in the substituents linked to O-2 and O-3 of the Hep II residue; one of these molecules lacks the O-3 substituent (OS2a), and a second lacks the O-2 substituent (OS2b). Based on the likely possibility that the major oligosaccharide from NMB LOS is of the L2 structure, and based on the previously reported structure for the L2 LOS [19], it is likely that OS2a is missing the glucosyl residue terminally linked to Hep II, and OS2b is missing the *N*-acetylglucosaminosyl residue terminally linked to Hep II. Thus, OS2a would likely have a glycosyl sequence the same as reported for the LOS oligosaccharides from immunotypes L3 [20], L4 [21], or L7 [21]. Since OS2a is a minor structure (see next paragraph) of the OS2 fraction, it was not possible to accurately determine the location of the PEA group; the PEA group is reported to be located at O-3 for both L3 and L7 molecules and at O-6 for the L4 molecule [20,21]. Also, the L4 structure is reported to have an acetyl group at O-3 of the terminal *N*-acetylglucosminosyl residue. The presence of the O-acetyl substituent in the NMB LOS is described below.

That the OS2-HF fraction contained the above three molecules was further supported by FABMS analysis of this sample. The spectrum (not shown) gave three molecular ions ($[M + Na - H_2O]^+$) of *m*/*z* 1681, 1519, and 1478. The loss of water is due to the presence of Kdo as a lactone or anhydro residue. Based on the glycosyl compositions and linkages shown in Tables 2 and 3, these ions are consistent with molecules of the following compositions: *m*/*z* 1681 = $Gal_2GlcNAc_2Glc_2Hep_2Kdo$, *m*/*z* 1519 = $Gal_2GlcNAc_2Glc_1Hep_2Kdo$, and *m*/*z* 1478 = $Gal_2GlcNAc_1Glc_2Hep_2Kdo$. Analysis of OS1-HF showed only the ion at *m*/*z* 1681.

The methylation data, together with the relative amounts of OS1 (7 mg) and OS2 (15 mg), permits the estimation of the relative proportion of the various HF-treated oligosaccharides derived from the NMB LOS preparation. The methylation and FABMS data for OS2-HF indicates that all of the molecules in that preparation contain the lacto-*N*-neotetraose, and, therefore, the molar ratios of 2-linked: 3,4-linked heptose, and of 3-linked:3,4-linked heptose represent the fractions of OS2-HF which are OS2a and OS2b, respectively. Using these ratios the amount of OS2a is 3.2 mg ([3/14][15 mg]), and the amount of OS2b is 2.1 mg ([2/14][15 mg]). The remainder of the OS2-HF, 9.7 mg, has the same structure as OS1-HF. Thus, of the total 22 mg of oligosaccharide obtained from the NMB LOS preparation, 16.7 mg (76%) consists of the OS 1 structure, 3.2 mg (15%) of OS2a, and 2.1 mg (9%) OS2b.

Table 3
Observed molecular mass and composition derived from electrospray mass spectrometry of the *N. meningitidis* NMB O-deacylated LOS

Observed ion	Proposed composition[a]	Calculated Mr[b]	Experimental Mr[b]
970.0	$(M_A + 2H + NH_3NH_2)^{3+}$	2875.75	2875.0
980.0	$(M_A + H + 2NH_3NH_2)^{3+}$	2875.75	2873.0
997.0	$(M_B + 2H + NH_3NH_2)^{3+}$	2955.73	2956.0
911.5	$(M_C + 2H + NH_4)^{3+}$	2713.6	2714.5
937.0	$(M_D + 2H + NH_4)^{3+}$	2793.6	2791.0

[a] M_A = $GlcNAc_2$,Gal_2,Glc_2,Hep_2,Kdo_2,PEA,P,β-OH-Cl4:O_2, $GlcN_2$ M_B = $GlcNAc_2$,Gal_2,Glc_2,Hep_2,Kdo_2.PEA.P_2,β-OH-Cl4:O_2,$GlcN_2$ M_C = $GlcNAc_2$,G1c_2,Glc,Hep_2,Kdo_2,PEA,P,β-OH-CI4:O_2.GICN_2 M_D = $GlcNAc_2$,Gal_2,Glc,Hep_2,Kdo_2,-PEA,P_2, β-OH-Cl4:O_2.$GlcN_2$

[b] Since the triply charged ions have a possible error of about 0.5 mass units, the error in the molecular weights of these molecules can be as much as 2 mass units.

Analysis of the NMB lipid-A.—Glycosyl-linkage analysis of the lipid-A showed that it contained terminally linked and 6-linked glucosamine (Table 3). If the 4′-position of the lipid-A were completely phosphorylated, only the 6-linked glucosaminosyl residue would have been detected since the analytical methods used do not permit the detection of phosphorylated sugars. Therefore, the presence of terminally linked glucosamine indicates that a significant portion of the lipid-A in NMB is not phosphorylated at the 4′-position. Table 2 shows that the ratio of terminally to 6-linked glucosamine is 0.7:1.0 suggesting that 70% of the NMB lipid-A lacks the 4′-phosphate. However, any quantitative interpretation should be regarded with caution, since the 6-linked glucosamine at the reducing end of the lipid-A is subject to partial degradation during the methylation procedure. The lack of a 4′-phosphate on the lipid-A differs from the previously published report by Kulshin et al. [27]. Their analysis of the lipid-A from a nonencapsulated serogroup B isolate, *N. meningitidis* strain M986-NCV1, found that 85% of the lipid-A was phosphorylated and esterified with phosphoethanolamine. Since NMB is a capsule-producing strain, it was decided to repeat the methylation analysis of its lipid-A and include the lipid-A from a non-encapsulated genetically defined mutant of NMB, termed M7. Fig. 1 clearly shows that the lipid-A from mutant M7 does not contain any detectable terminally linked glucosamine, while the NMB lipid-A contains relatively large amounts of this residue. Both lipid-A samples contain 6-linked glucosamine as expected. Thus, this result shows that the lipid-A from *N. meningitidis* mutant M7 still contains 4′-phosphate, and probably has a structure similar or identical to that previously reported for the lipid-A from the nonencapsulated serogroup B isolate, *N. meningitidis* strain M986-NCV1 [27].

The lack of the 4′-phosphate on the lipid-A from NMB was further indicated by ^{31}P NMR spectroscopy, and by ESIMS analysis. The presence of the various lipid-A phosphates can be determined by ^{31}P NMR [29]. The resonance for the 4′-monophosphate occurs at about δ 4.6 [29]. Analysis of both NMB and M7 O-deacylated lipid-A samples (spectra not shown) showed that the NMB sample contained a resonance at δ 4.62 of very low intensity, while the M7 lipid-A showed an intense resonance at this chemical shift. ESIMS analysis of the NMB O-deacylated LOS gave three triply charged

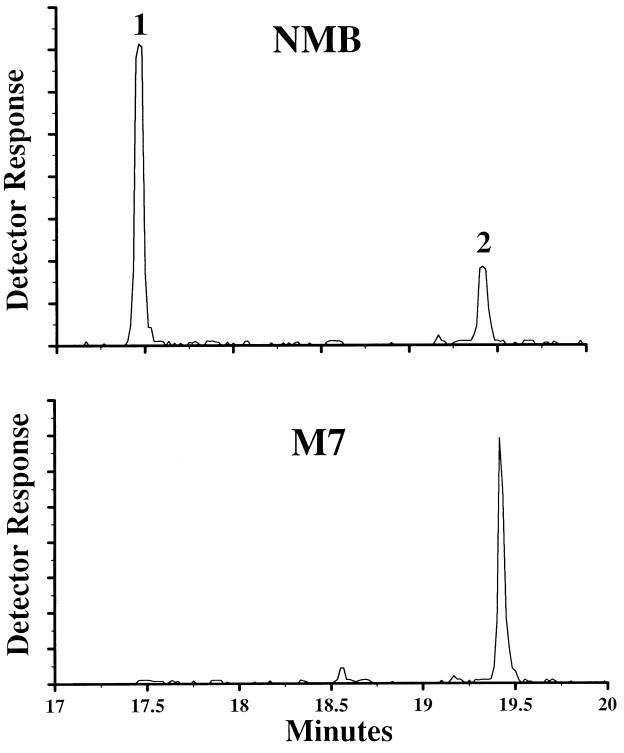


Fig. 1. The GLC profile of the partially methylated alditol acetates (PMAAs) of the lipid-A from NMB (top) and strain M7 (bottom). The figure shows the selective ion profile (*m*/*z* 159) for the lipid-A from each strain. This ion is characteristic of the PMAA derivatives of the lipid-A glucosaminosyl residues. Peak 1 is the PMAA derived from terminally linked glucosamine (primary fragments are *m*/*z* 290, 203, 161, 159), and peak 2 is the PMAA of 6-linked glucosamine (*m*/*z* 318, 203, 189, 203, 159).

ions at *m*/*z* 970, 980, and 997 (see Fig. 2 and Table 3). These three ions are due to two molecular species, MA and MB: $[M_A + NH_2NH_3 + 2H]^{3+} = 970$, $[M_A + 2NH_2NH_3 + H]^{3+} = 980$, and $[M_B + NH_2NH_3 + 2H]^{3+} = 997$. The masses are consistent with MA having a composition of $GlcNAc_2Gal_2Glc_2Hep_2Kdo_2PEA_1P_1\beta$-OH-C14:$O_2GlcN_2$ and MB having a composition the same as MA but with one additional phosphate. Presumably, the major structure, MA, lacks the 4′-phosphate while the minor structure contains this phosphate substituent. The compositions of both the MA and MB structures are consistent with the immunotype L2 structure. The ESIMS analysis also showed ions at *m*/*z* 911.5 and 937.0. These ions are consistent with triply charged ions of two additional molecular species, MC and MD, where $[M_C + 2H + NH_4]^{3+} = 911.5$, and $[M_D + 2H + NH_4]^{3+} = 937.0$. in which the composition of Mc is $GlcNAc_2Gal_2Glc_1Hep_2Kdo_2PEA_1P_1\beta$-OH-C14:$O_2GlcN_2$, and MD has the same composition but

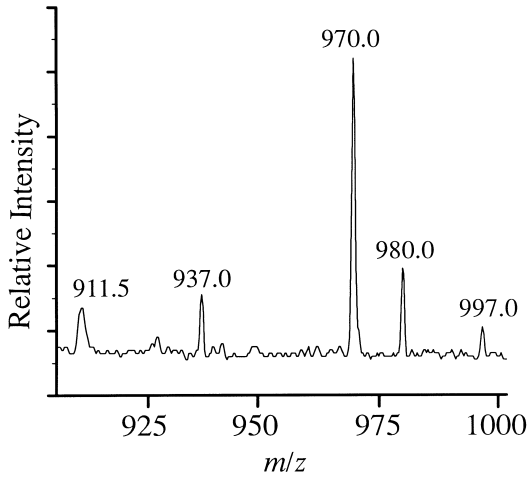


Fig. 2. The ESIMS spectrum of O-deacylated NMB LOS. The ions shown are triply charged ions as defined in the text and in Table 3.

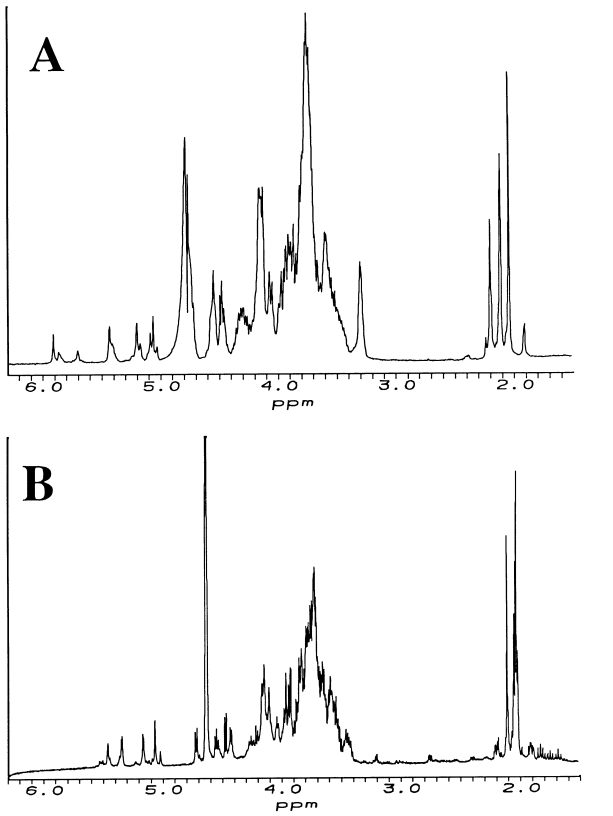


Fig. 3. The proton spectra of OS1 before (top) and after (bottom) treatment with aqueous HF.

with one additional phosphate. Again, Mc is probably a molecular species which lacks the 4′-phosphate. Thus, methylation, ESIMS and ^{31}P NMR analyses all support the conclusion that the major form of NMB lipid-A lacks the 4′-phosphate moiety. Lastly, the ESIMS results show that both Mc and Md have glycosyl and PEA compositions consistent with immunotype L7 [21], L3 [21] or L4 [21] structures, but without the sialic acid residue. Since the composition and methylation data clearly show that at least 20% of this LOS is sialylated, it is likely that the acidic conditions used for positive-mode ESIMS analysis resulted in the loss of the terminally linked sialic acid residue.

NMR spectroscopic analysis.—The above results strongly suggested that NMB LOS consisted of a mixture of several oligosaccharide structures very similar or identical to those reported for immunotypes L2 (the major structure), L3 (or L4 or L7), and a novel structure that contains the lacto-*N*-neotetraose but lacks the terminally linked *N*-acetylglucosaminosyl residue linked to O-2 of Hep II. The glycosyl sequence of the major oligosaccharide was further characterized by NMR spectroscopy.

Except for possible heterogeneity in the location of the PEA group on either O-6 or O-7 of Hep II, the stoichiometric ratio of the glycosyl residues in OS1 (see Table 2) indicated that this fraction contained one structure. Therefore, the OS1 fraction was used for the NMR studies. Comparison of the ^{1}H NMR spectrum of OS1 with that of OS1-HF (Fig. 3) shows that HF treatment removed both PEA and O-acetyl groups as indicated by the loss of their respective characteristic resonances at δ 3.33 and 2.19. Integration of the O- and N-acetyl methyl protons indicated that 70% of the OS1 sample is O-acetylated.

The glycosyl sequence of OS1-HF was determined from a series of two-dimensional NMR experiments. With the aid of 2D COSY (spectrum not shown), TOCSY (Fig. 4B), and broad-band decoupled HSQC (spectrum not shown) spectra, most of the protons and many carbon resonances of OS1-HF could be assigned and are listed in Table 4. Assigning some proton and carbon resonances was facilitated by the similarity of the OS1-HF NMR data with those previously reported for the LOS oligosaccharides from *N. meningitidis* immunotypes L2 [19], L3 [20], L4 [21].

The 1D proton spectrum in Fig. 3 shows that OS1-HF gives signals for eight anomeric protons. These were labelled **A** to **I**. Resonances **A** to **D** were assigned to β-linked residues ($J_{1,2}$ 8 Hz), and residues **E** to **H** were assigned to the α-linked residues ($J_{1,2} < 2$–3 Hz). Anomeric protons **A** and **B** had TOCSY traces with characteristic chemical shifts and *J*-coupling values ($J_{4,5} \sim 5$ Hz) of β-galactosyl residues. The downfield chemical shift of **A**-C-3 (δ 83.15) showed that residue **A** is 3-linked.

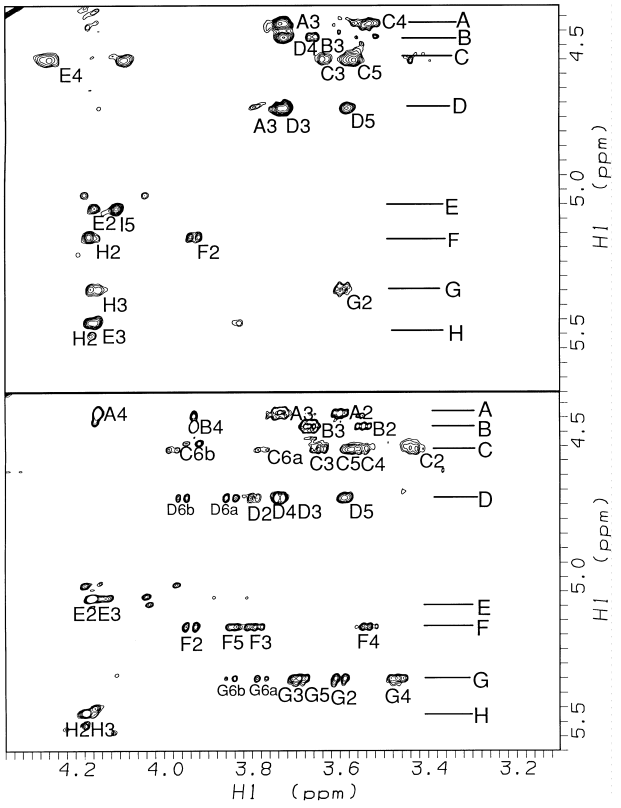


Fig. 4. The TOCSY (bottom) and NOESY (top) spectra of OS1-HF. The various assignments are shown and are defined in both the text and in Tables 4 and 5.

Residue **B** had shifts compatible with a terminally linked galactose [30]. The *β*-*N*-acetylglucosaminosyl residues, **D** and **F**, were identified based on the TOCSY crosspeak patterns and chemical shifts of C-2 at *δ* 56.5 and *δ* 55.3, respectively. The *N*-acetyl methyl signals were observed at *δ* 2.05 and *δ* 2.10. The downfield chemical shift of **D**-C-4 (*δ* 79.44) showed that it is 4-linked. Residue **F** would then be the terminally linked *N*-acetylglucosamine, as expected from the glycosyl linkage analysis (Table 2). The residue **C** carbon and proton chemical shifts were typical for a *β*-glucosyl residue [30]. The downfield chemical shift of **C**-C-4 (*δ* 79.96) showed that it is 4-linked. Residue **G** showed proton and carbon chemical shifts consistent with a terminally linked *α*-glucosyl residue [30]. The remaining **E** and **H** residues were assigned to the two L-*glycero*-D-*manno*-heptosyl residues. The chemical shift of **E**-C-4 was significantly downfield relative to **H**-C-4, suggesting that **E** was the 3,4-linked heptosyl residue (Hep I). Similarly, **H**-C-2 was also downfield at *δ* 80.95, indicating that it must be the 2,3-linked heptosyl residue (Hep II). The overlap in proton chemical shifts did not allow unambiguous assignment of H-3 of the two Hep residues, a result similar to that reported for immunotype L2 LOS [19]. Additionally, signals between *δ* 1.76 and *δ* 2.21 were characteristic for Kdo-H-3 protons. There are multiple Kdo signals due to the fact that mild acid hydrolysis can lead to oligosaccharides having a reducing end Kdo pyranosyl, Kdo furanosyl, or anhydro-Kdo residues. The Kdo residue was designated as **I**.

The complete sequence of glycosyl residues in OS1-HF was deduced from the NOESY (Fig. 4A) data which are summarized in Table 5. Please refer to the glycosyl sequence given in the structure below during the following explanation of the NOE data. Starting from the anomeric proton of **B**, there is a strong NOE to **D**-H-4 showing that **B** is linked to O-4 of residue **D**. Residue **D**, in turn, is linked to O-3 of **A** as indicated by a strong NOE between **D**-H-1 and **A**-H-4. It should be noted that since the chemical shifts for **D**-H-3 and **A** H-3 overlap, the NOE crosspeak from **D**-H-1 to this position is a summation of both inter- and intra-residue NOEs. Residue **A** is linked to O-4 of **C** as indicated by the strong NOE between **A**-H-1 and **C**-H-4. The H-1 of residue **C** has an NOE contact with **E**-H-4, showing that **C** is linked to O-4 of E. Because of the multiple reducing end forms of Kdo, i.e., possible pyranose, furanose and anhydro structures, direct NMR evidence for a 1 → 5 linkage between residues **E** and **I** was not obtained. However a crosspeak was observed between **E**-H-1 and a Kdo proton. This Kdo proton has a chemical

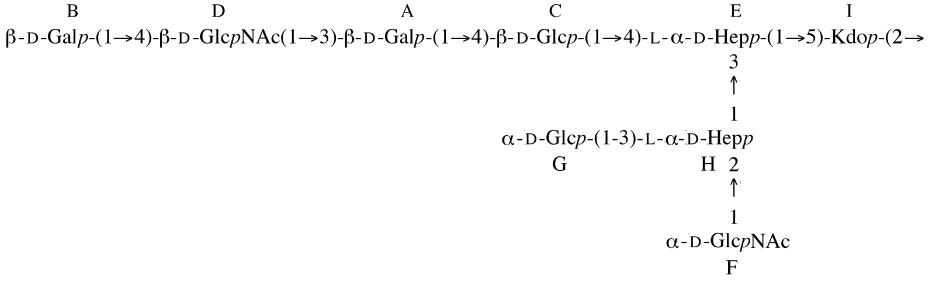


Table 4
^{1}H and ^{13}C NMR chemical shift data for OS1-HF derived from *N. meningitidis* serotype B strain NMB LOS. Chemical shifts are expressed relative to DSS signal 0.00 ppm at 35 °C

	Chemical shifts $^{1}H/^{13}C$							
Glycosyl residue	1	2	3	4	5	6a	6b	
→3)-β-D-Gal*p*-(1→	4.44	3.60	3.74	4.15				
A	104.11	71.18	83.15	69.50				
β-D-Gal*p*-(1→	4.48	3.55	3.68	3.94				
B	104.00	72.12	73.68	69.71				
→4)-β-D-Glc*p*-(1→	4.56	3.44	3.65	3.56	3.57	3.78	3.98	
C	103.12	74.10	75.42	79.96	76.30	61.63	61.63	
→4)-β-D-Glc*p*NAc-(1→	4.73	3.80	3.73	3.74	3.59	3.85	3.96	
D	103.74	56.29	73.08	79.44	75.74	61.07	61.07	
→3,4)-L-α-D-Hep*p*-(1→	5.08	4.16	4.15	4.27	4.14			
E	102.11	71.37	78.53	74.11	72.50			
α-D-Glc*p*NAc-(1→	5.18	3.94	3.79	3.54	3.84			
F	100.06	55.04	71.90	70.86	73.15			
α-D-Glc*p*-(1→	5.36	3.60	3.70	3.47	3.68	3.78	3.85	
G	101.65	72.87	73.20	70.40	73.68			
→2,3)-L-α-D-Hep*p*-(1→	5.47	4.18	4.16	4.23				
H	100.54	80.95	78.83	67.56				
	H-3ax	H-3eq	H-4	H-5	H-6	H-7	H-8a	H-8b
→5)-α-D-Kdo*p*-(2→	2.20/2.21	1.84/1.76	4.02/3.99	3.91/3.88				
Kdo (derivative)	2.08	1.91	4.12					

Table 5
NOE data for the OS1-HF derived from *N. meningitidis* LOS strain NMB

Anomeric proton		NOE contact to proton			
Glycosyl	δ	δ	Intensity[a]	Residue,	atom
→3)-α-D-Gal*p*-(1→	4.44	3.56	s	C	H-4
A		3.74	w	A	H-3
β-D-Gal*p*-(1→	4.48	3.68	w	B	H-3
B		3.74	s	D	H-4
→4)-β-D-Glc*p*-(1→	4.56	3.57	w	C	H-5
C		3.65	w	C	H-3
		4.27	s	E	H-4
→4)-β-D-Glc*p*NAc-(1→	4.73	3.59	w	D	H-5
D		3.74	s	A	H-3
		3.73	w	D	H-3
→3,4)-L-α-D-Hep*p*-(1→	5.08	3.91	w	Kdo(p)	H-5
E		4.12	s	Kdo(d)	
		4.16	w	E	H-2
α-D-Glc*p*NAc-(1→	5.18	3.94	w	F	H-2
F		4.18	s	H	H-2
α-D-Glc*p*-(1→	5.36	3.60	w	G	H-2
G		4.16	s	H	H-3
→2,3)-L-α-D-Hep*p*-(1→	5.47	4.15	s	E	H-3
H		4.18	s	H	H-2

[a] The intensities are estimated and are given as, s = strong, m = medium, and w = weak.

shift consistent with either H-4 or H-5. The glycosyl linkage analysis of the NMB LOS and the previously reported *N. meningitidis* LOS structures [19,20] clearly support a 5-linked residue. Therefore, it is concluded that residue **E** is linked to residue **I** at O-5. The terminal α-N-acetylglucosaminosyl residue **F** has a clear NOE contact to H-2 of heptosyl residue **H**, and, therefore, **F** is linked to O-2 of **H**. Similarly, the terminal α-glucosyl residue **G** has an NOE contact compatible with either H-3 or H-2 of heptosyl residue **H**. Since residue **F** has already been assigned to O-2 of residue **H**, **G** must be linked to O-3 of **H**. Finally, heptosyl residue **H** has an NOE contact to **E**-H-3

showing that **H** is linked to **E** at O-3. From the combined glycosyl linkage analysis and NMR analysis results, OS1-HF is proposed to have structure **1** which is the same as the previously reported [19] L2 structure.

The location of the O-acetyl group was determined from a TOCSY experiment on OS1. A sub-spectrum through H-1 of the terminally linked α-*N*-acetylglucosaminosyl residue (**F**) (Fig. 5) showed that the **F**-H-6 proton resonances were shifted downfield compared to those for OS1-HF which lacks the O-acetyl group (see Table 4). These results support the conclusion that the O-acetyl group of OS1 is present at O-6 of the α-*N*-acetylglucosaminosyl residue linked to O-2 of Hep II.

3. Discussion

The heterogeneity of NMB LOSs are summarized by the structures shown in Fig. 6. The major oligosaccharide, OS1, and the major portion of OS2, accounting for approximately 76% of the LOS oligosaccharides, has the immunotype L2 structure. A small portion of these L2 structures do not have a PEA substituent, while the vast majority

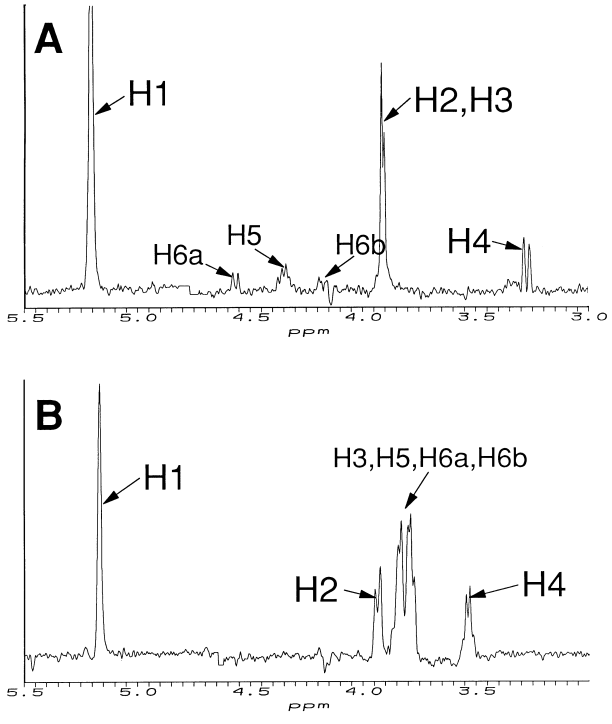
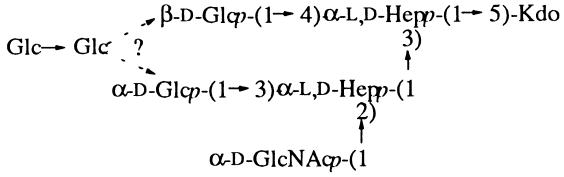


Fig. 5. The sub-spectra through H-1 of the terminal α-*N*-acetylglucosaminosyl residue from the TOCSY analysis of OS1 (top) and OS1-HF (bottom). The resonances of the various protons are as indicated

have a PEA group at the Hep Il O-6 or O-7. The terminally linked *N*-acetylglucosaminosyl residue of this L2 structure is acetylated at O-6. The location of the O-acetyl group on the previously reported L2 structure was not determined [19]. The predominance of an L2 structure in NMB is consistent with mass spectrometry data of an earlier report [28] suggesting that the major LOS species has a composition consistent with such a structure.

A second oligosaccharide (OS2a-HF), which accounts of 15% of the structures, has the same glycosyl sequence as the oligosaccharides reported for the L3, L4, and L7 immunotypes [19,20]. Since the percentage of OS2a (15%) and the level of terminal sialic acid residues (20%) in the LOS preparation are very similar, this may indicate that the OS2a structure may be sialylated. However, further analysis must be done to determine whether the terminal sialic acide is attached to the L3 structure or is distributed on both the L2 and L3 structures. Since OS2a is a minor component of the OS2 fraction, it was not possible to determine whether or not OS2a contains a PEA substituent at the Hep II O-3 (an L3 structure [20]), or at O-6 (an L4 structure [21]) position.

The third oligosaccharide (OS2b) has a novel structure not previously reported for *N. meningitidis*. The mass spectrometry data show that OS2b lacks one of the two *N*-acetylglucosaminosyl residues and has a composition of $Hex_4GlcNAc_1Hep_2Kdo_2$. An oligosaccharide of this composition was reported by Lee et al. [28] for NMB and its SS3 mutant. In that report the suggested structure was:

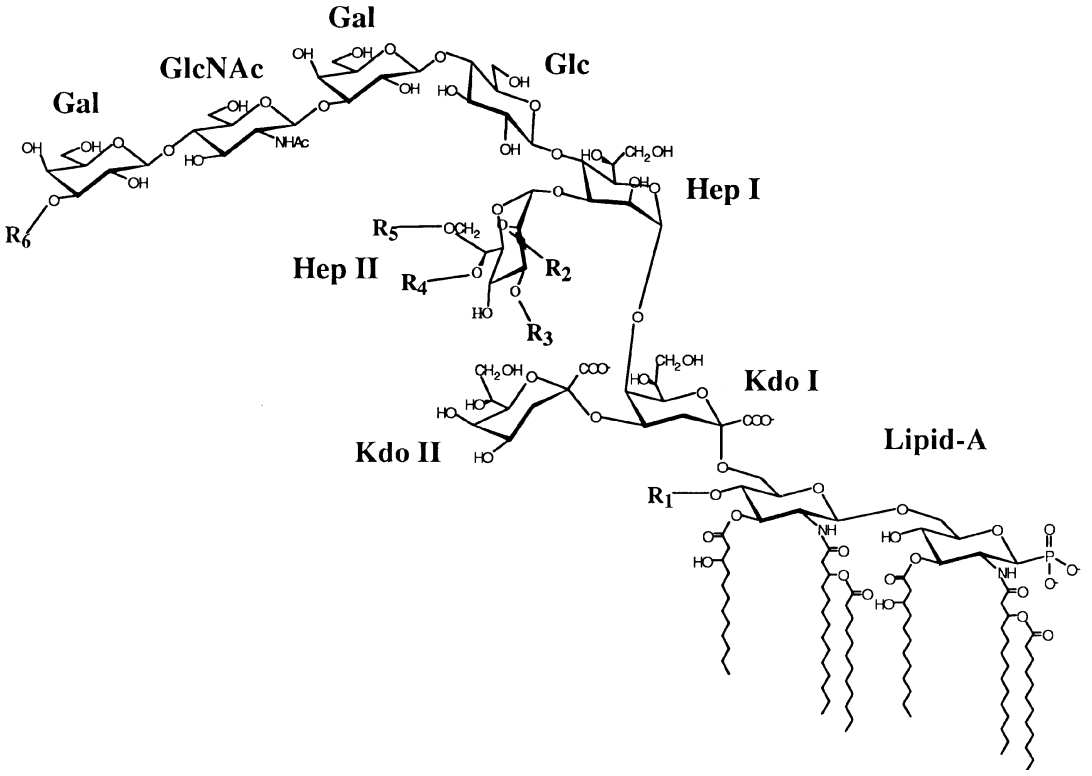


However, the methylation data, together with the mass spectrometric data, presented in this report clearly indicate that the $Hex_4GlcNAc_1Hep_2Kdo_2$ oligosaccharide from NMB LOS contains both the lacto-*N*-neotetraose portion of the molecule and an Hep II residue that is glycosylated only at O-3, supporting the following structure for OS3b: The existence of structure OS2b in NMB LOS was unexpected since previous work showed that the presence of this terminally linked α-*N*-acetylglucosaminosyl residue in the LOS inner core is

β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-α-L,D-Hep*p*-(1→5)-Kdo
3
↑
1
α-D-Glc*p*-(1→3)-α-L,D-Hep*p*

necessary for the addition of the lacto-*N*-neotetraose portion of the molecule [31]. A possible explanation may be that the terminal *N*-acetylglucosaminosyl residue was removed after LOS synthesis.

Another unusual feature of the NMB LOS is that a great portion of the molecules, perhaps 70%, lack the lipid-A 4′phosphate moiety. In *E. coli*, the presence of the 4′-phosphate is apparently required for the addition of the Kdo residues to the lipid-A precursor, Kdo_2lipid-IVA [32,33]. In *R. iegumino-sarum*, the mature lipid-A does not have the 4′-phosphate [34], but does have all the enzymes required to synthesize Kdo_2lipid-IVA [35] and a unique phosphatase that removes this moiety [36]. It may be that NMB contains a similar enzyme which removes the 4′-phosphate at some point during LOS synthesis. Kulshin et al [27] found that



LOS-derived Oligosaccharides	LOS Type	[1]R1	R2	R3	R4	R5	R6
OS1 (76%)	L2	H/P	α-GlcNAc	α-Glc	[3]H/PEA	[3]H/PEA	H
OS2a (15%)	L3	H/P	α-GlcNAc	PEA([2]?)	H	H	α-NeuNAc
OS2b (9%)	?	H/P	H	α-Glc	[4]?	[4]?	H

Fig. 6. A summary of the various LOS structures found in NMB. [1]Approximately 70% of the lipid-A in this NMB LOS preparation is without the 4′-phosphate. It is not known if this lack of the 4′-phosphate is distributed among all of the LOS types or is specifically present in one type: e.g., only present on the L2-type LOS. [2]The location of the PEA at this position for the LOS carrying OS2a has not been determined. However, since NMB LOS does carry an L3 immunotype, it is presumed that this structure will carry this PEA group at the same position. As reported for the L3 structure [24]. [3]The vast majority of the L2-type LOS molecules carry the PEA group at O-6 or O-7 of Hep II. Only trace levels of molecules have no PEA groups at either of these two positions. [4]It is not known if this novel LOS structure carries PEA group on Hep II.

85% of the lipid-A structure expressed by the non-encapsulated serogroup B isolate, *N. meningitidis* strain M986-NCV1, possessed phosphate head groups esterified with PEA. An interesting observation was that the non-encapsulated genetically defined mutant of NMB, termed M7, does have the lipid-A 4′-phosphate. Since NMB is a capsule forming strain, this result may indicate that the removal of the 4′-phosphate is related in some, as yet unknown, manner to capsule formation. It should also be noted that the lack of a 4′-phosphate is not consistent with the mass spectrometric data of O-deacylated NMB LOS presented in an earlier report [28]. A possible explanation for these conflicting results may be the significant difference in the procedures used for LOS purification: hot phenol–water [28] versus chloroform–petroleum ether extraction (this report).

The results presented in this report show that NMB LOS contains immunotype L2 and most probably L3 structures, as well as a structure not previously observed. In addition, the presence or absence of PEA substituents, as well as the lack of the lipid-A 4′-phosphate contributes to the LOS heterogeneity. Furthermore, the heterogeneity of NMB LOS may be determined, not only by enzymes involved in addition of glycosyl residues, phosphate, and PEA to the LOS, but also by enzymes that remove substituents from (i.e., process) certain LOS structures.

The position of the phosphates on the hydrophilic backbone of lipid-A, as well as the degree of substitution of these positions with phosphoethanolamine, have been shown to profoundly affect the endotoxic activity of *E. coli* lipid-A. Monophosphoryl lipid-A is less bioactive than diphosphoryl lipid-A by the rabbit pyrogenicit test, chicken embyro lethal dose test, and Schwartzmann reaction [37], as well as by a reduced ability to stimulate monokine induction in murine macrophage cell lines [24]. The phosphorylation patterns of meningococcal lipid-A may be more diverse than is currently known. A brief study of the ability of meningococcal and gonococcal LOS to clot Limulus amebocyte lysate revealed that this activity was strain dependent and possibly related to the amount of phosphorylated lipid-A expressed by each isolate [6]. A much more detailed survey, including detailed structural analyses of the lipid-A from a variety of meningococcal isolates, is required to determine the importance of these observations in relation to meningococcal disease.

4. Experimental

Bacterial strains and condition of growth.—*Neisseria meningitidis* serogroup B (serotype 2b:P1.2,5) strain NMB (CDC8201085) was isolated from the cerebrospinal fluid of a patient with meningococcal meningitis in Pennsylvania (USA) 1982. Strain M7 is a genetically defined unencapsulated variant of strain NMB due to a Class II Tn*916* insertion in *synA(X)*, the first gene of the serogroup B capsule biosynthesis operon [38]. These strains were grown under aerobic conditions with 3.5% CO_2 at 37 °C on GC agar (Difco) supplemented with 0.4% glucose and 0.68 mM $Fe(NO_3)_3$. Liquid cultures were vigorously aerated at 37 °C in GC broth with the same supplements and 0.5 M sodium bicarbonate. [39].

Isolation of LOS and preparation of LOS oligosaccharides (OSs).—The LOSs were prepared from 5 g (dry weight) of bacteria as previously described [30]. An LOS sample (20 mg) was hydrolyzed in aqueous 1% acetic acid (10 mL) for 2 h at 100 °C. The hydrolysate was centrifuged at 10,000×*g* for 20 min, and the supernatant was collected. The pellet was washed once with 5 mL of water and centrifuged again. The water wash was added to the supernatant, and any remaining lipid-A was extracted with diethyl ether (three times, 5-mL volumes each time). The aqueous phase, containing the OSs, was lyophilized. The lyophilized OSs were dissolved in 0.5 mL of water, filtered with Microfilterfuge tubes containing 0.45-μm pore size Nylon-66 membrane filters, and applied to a Bio-Gel P-4 column (70×1.6 cm), and eluted with water containing 1% 1-butanol. Fractions were assayed for carbohydrate by phenol–sulfuric acid assay. Fractions representing the OS peaks were pooled and lyophilized.

O-Deacylation.—The LOS or lipid-A samples were deacylated according to the procedure of Helander et al. [40]. Approximately 8 mg of LOS was incubated with 1 mL of anhydrous hydrazine for 20 min at 37 °C. The solution was cooled to −20 °C, and 5 mL of chilled acetone was added dropwise to precipitate O-deacylated LOS. The sample was then centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatant was removed, and the pellet was washed again with cold acetone and centrifuged. The precipitated O-deacylated LOS was then resuspended in 1 mL of water and lyophilized.

Preparation of LOS-HF.—The LOS and OS samples (8 mg) were placed in 1.5 mL polypropylene

tubes. The samples were treated with cold aqueous 48% hydrogen fluoride (HF) (100 μL) and kept for 24 h at 4 °C [41]. The HF was removed by flushing under a stream of air, followed by addition of diethyl ether (600 μL) and drying with a stream of air. This step was repeated three times. The dry pellet was dissolved in water and lyophilized. The lyophilized, dephosphorylated OSs were dissolved in 0.5 mL of water, filtered with Microfilterfuge tubes containing 0.45-μm-pore-size Nylon-66 membrane filters, and applied to a Bio-Gel P-4 column (70×1.6 cm), and eluted with water containing 1% 1-butanol. Fractions were assayed for carbohydrate by phenol–sulfuric acid assay. Fractions representing the OS peaks were pooled and lyophilized.

Nuclear magnetic resonance (NMR) spectroscopy.—Samples were prepared for NMR analysis by a two-fold lyophilization from D_2O, dissolution in D_2O and analysis. Spectra were recorded at 35 °C. Proton chemical shifts are reported in ppm, using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, δ_H 0.00). Carbon chemical shifts were calculated using indirect referencing to internal DSS, and the values were adjusted to correspond with 1,4-dioxane at δ_c 67.4 [30,42]. All NMR spectra were recorded on Bruker AMX 500 or DRX 600 MHz spectrometers. Two-dimensional DQF-COSY [43], TOCSY [44,45], and NOESY [46] datasets were collected in the phase-sensitive mode using the States-TPPI [47] method. In these experiments, low-power presaturation was applied to the residual HDO signal. Typically, datasets of 2048 (t_2)×512 (t_1) complex points were collected with 16 scans per FID, and a sweep width in both dimensions of 6 ppm. The TOCSY experiments contained MLEV17 [48] mixing sequences ranging from 60 ms to 320/ms, and the NOESY mixing delay was 200 ms.

A gradient HSQC [49] dataset was collected using the echo-antiecho method for pure absorption data. Datasets of 2048(t_2)×512(t_1) complex points were acquired, with 32 scans per FID for the HSQC. The sweep widths were 7 ppm for proton (F2) and 60 ppm for carbon (F1). The GARP [50] sequence was used for ^{13}C decoupling during acquisition. Data were processed typically with a Lorentzian-to-Gaussian weighting function applied to t_2 and a shifted squared sinebell function and zero-filling applied to t_1. Data shown was processed with Felix software (Molecular Simulations, Inc.).

^{31}P NMR spectroscopy.—O-deacylated lipid-A was dissolved in D_2O containing 5 mM EDTA and 2% (w/v) sodium deoxycholate, and the pH was adjusted with triethylamine [29]. ^{31}P NMR spectra were recorded with a Bruker AMX 500 MHz spectrometer, using ^1H broadband decoupling during acquisition. The pulse flip angle was 90°, and the relaxation delay between scans was 20 s. The data were referenced to external 85% phosphoric acid (0.00 ppm).

Glycosyl composition analyses.—The glycosyl compositions of LOS and OS (0.5 mg each) were determined by hydrolysis in 2 M trifluoroacetic acid (0.5 mL) in a closed vial at 120 °C for 3 h. The resulting glycoses in the hydrolysate were reduced with $NaBH_4$, acetylated, and were analyzed by GLC and combined GLC–MS [51]. For the determination of *N*-acetylneuraminic acid present in LOS, the LOS sample (0.5 mg) was dried in vacuum, methanolyzed in 1 mL of MeOH, 2 N HCl at 80 °C for 4 h. The released methyl glycoside residues were trimethylsilylated, and the mixture was analyzed by GLC–MS [46]. The absolute configurations of the glycoses present in the OSs were determined from the preparation and analysis of their trimethylsilylated-(+)-(*S*)-2-butyl and (−)-(*S*)-2-butyl glycosides [52,53].

Glycosyl-linkage analyses.—Glycosyl linkage analyses were carried out using a modified NaOH method [54]. Samples (1 mg each) were dissolved in dimethyl sulfoxide (100 μL), powdered NaOH (100 mg) was added, and the reaction mixture were stirred rapidly for 30 min. Methylation was performed by the sequential additions of iodomethane (10, 10, and 20 μL) at 10 min intervals. After an additional 20 min stirring, the methylated glycans were recovered in the organic phase after addition of chloroform (0.5 mL×3) and M sodium thiosulfate (1 mL). The permethylated product was further purified by reversed-phase chromatography on a Sep-Pak C_{18} cartridge [55]. The permethylated glycans were hydrolyzed with 2 M trifluoroacetic acid (12 °C, 3 h), reduced with $NaBH_4$ or $NaB(^2H)_4$, acetylated, and analyzed by GLC and GLC–MS [51]. For the determination of the Kdo and sialic acid linkages, the permethylated LOS was methanolyzed in 1 mL of methanoic 2 N HCl at 80 °C for 4 h. The released methyl glycoside residues were acetylated, and the mixture was analyzed by GLC–MS [56].

Lipid-A purification.—Lipid-A was released from the LPS (10 mg) by hydrolysis in aqueous 1%

acetic acid (5 mL) at 100 °C for 2 h. The precipitated lipid-A was collected by centrifugation (10,000×*g*). The precipitate was resuspended with water (2 mL) and partitioned with chloroform (2 mL). The chloroform layer containing purified lipid-A was concentrated to dryness.

Fatty acid analysis.—Total fatty acids were released by methanolysis of lipid-A with methanoic M HCl at 80 °C for 16 h, and were trimethylsilylated. The resulting fatty acid methyl esters were analyzed by GLC–MS [51]. Ester- and amide-linked fatty acids were distinguished by preferential release of the ester-linked fatty acids using anhydrous sodium methoxide [57], and the products were analyzed by GLC–MS.

Chromatographic and spectrometric techniques.—GLC and GLC–MS analyses were performed by fused silica capillary columns (length, 30 m; inner diameter, 0.32 mm) with helium as the carrier gas. A DB-5 column (J & W Scientific) was used for aminoglycosyl derivatives, and an SP2330 column (Supelco, Bellefonte, PA) was used for the neutral glycosyl derivatives. GLC equipment consisted of an HP5890 gas chromatograph equipped with a flame-ionization detector (Hewlett–Packard). GLC–MS (EI) was performed on a Hewlett–Packard 5970 MSD.

Mass spectrometry.—O-Deacylated LOS was analyzed by electrospray-ionization mass spectrometry (ESIMS) with a SCIEX API-III mass analyzer operated in the positive-ion mode with an orifice potential of 50 V. Spectra are the accumulation of 10–15 scans collected over the mass range of 400 to 2000. The O-deacetylated LOS sample was dissolved in distilled water at a final concentration of 2 μg/μL, and the sample solution mixed with equal volume of ESIMS solution (aqueous 30% methnol containing 1% HCl) and pumped into the mass spectrometer at a rate of 3 μL min^{-1}.

Fast-atom-bombardment mass spectrometry (FABMS) was performed using a JEOL (Tokyo, Japan) SX/SX 102A tandem four-sector mass spectrometer, which was operated at 10 kV accelerating potential. Ions were produced by fast-atom bombardment (FAB) with xenon using a JEOL FAB gun operated at 6 kV in a conventional FAB ion source. Spectra acquired are averaged profile data of three scans as recorded by a JEOL complement data system. These spectra were acquired from 200 to 2000 *m*/*z* at a Mte that would scan from *m*/*z* 0 to *m*/*z* 2500 in 1 min. A filtering rate of 100 Hz, and an approximate resolution of 1000 was used in acquiring these spectra. The samples were dissolved in dimethyl sulfoxide and 1 μL aliquots were mixed with an equal volume of the FAB matrix, thioglycerol (TG), on the probe tip.

Acknowledgements

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