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Structural basis of the *Neisseria meningitidis* immunotypes including the L4 and L7 immunotypes

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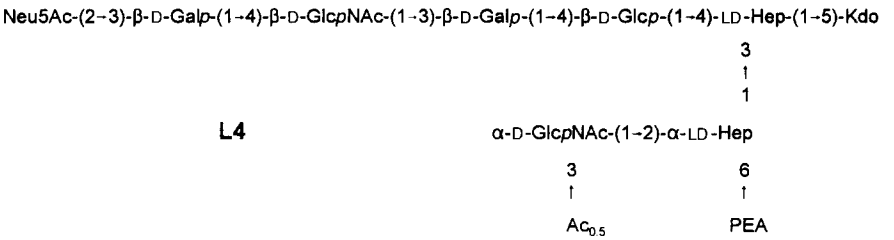
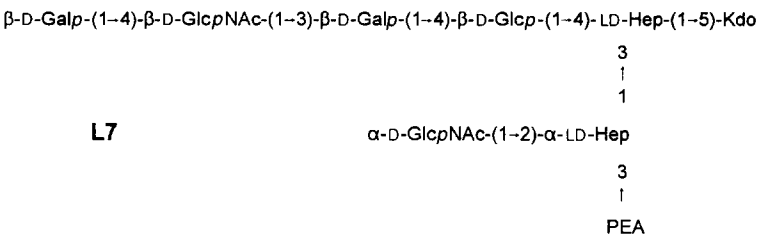
Abstract

The application of high-resolution ^{1}H, ^{13}C and ^{31}P NMR and MS analyses to the oligosaccharide moieties of the L4 and L7 immunotypes of *Neisseria meningitidis* revealed that they had the following structures:





The fact that the L7 LPS is not sialylated at O-3 of its terminal β-D-galactopyranosyl residue implies that it is a mutant strain unable to endogenously sialylate its lacto-*N*-neotetraose

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antenna. With the structural elucidation of the L4 and L7 LPS immunotypes, a more comprehensive structural profile of the LPS involved in disease isolates can now be assembled. This provides valuable insights into the structural basis of the *N. meningitidis* immunotyping system which could be of use in formulating an LPS-based vaccine against meningococcal meningitis.

Keywords: *Neisseria meningitidis*; Lipopolysaccharide; NMR; MS

1. Introduction

Neisseria meningitidis can be serologically divided into at least 12 immunotypes based on its lipopolysaccharide (LPS) [1–3]. The epitopic source of this immunotyping system is the glycose moieties of its LPS [4], the latter having been identified as small oligosaccharides associated with LPS of the R-type [5]. Structural studies on the oligosaccharides obtained from the L1 and L6 [6], L3 [7], L5 [8], and L2 [9] immunotypes have identified regions of structural difference and structural similarity, which account for both the immunotype specificity and cross-reactivity exhibited by the meningococcal LPS [2,4,10]. The immunological profile of the latter is further complicated by the fact that heterogeneity even exists among the oligosaccharides obtained from the LPS of individual meningococci [8,11,12]. Heterogeneity can be generated by the addition or deletion of glycose units [8], as well as by the presence and location of phosphoethanolamine substituents [6,9], and possibly *O*-acetyl groups [8,13]. Heterogeneity can also be generated by the endogenous sialylation of the meningococcal LPS [7,14,15] in which sialylation occurs at O-3 of the terminal galactopyranosyl residue of the lacto-*N*-neotetraose antenna of the oligosaccharide [7,16].

This paper describes the structural elucidation of the L4 and L7 immunotypes and these structures together with those obtained previously provide insights into the structural basis of the meningococcal LPS immunotyping system.

2. Results

The negative-ion mode FABMS spectra of the L4 and L7 core oligosaccharides showed four peaks corresponding to the pseudomolecular ions $[M - H]^-$ with *m/z* 1637 (Hex_3, $HexNAc_2$, Hep_2, Kdo, PEA), 1618 (lactonized form of the free acid form with *m/z* 1636), and 1660 and 1678 for the same components bearing a single acetyl substituent (difference 42 mass units = acetyl). The signals of the molecular ions bearing acetyl substituents were of much less intensity in the case of L7 oligosaccharide indicating that the latter was *O*-acetylated to a much lesser degree than the L4 core oligosaccharide. In the positive-ion mode, four molecular ions $[M + H]^+$ were observed analogous to those in the negative spectra, namely with *m/z* 1638 (free acid form), 1620 (lactone of the free acid form), 1680 (free acid form plus one acetyl group), and 1662 (lactone plus one acetyl group). Two additional ions $[M + H]^+$ with *m/z* 1728 and 1770 were observed which correspond to thioester adducts (+108 mu) of the two lactones. As in the negative-ion spectra, the intensities of the peaks corresponding to ions bearing *O*-acetyl substituents were much lower for the L7 oligosaccharide.

Sugar analysis [17] of the *O*-deacetylated and dephosphorylated L4 and L7 core oligosaccharides showed that they were both composed of D-galactose, D-glucose, *N*-acetyl-D-glucosamine, L-*glycero*-D-*manno*-heptose, and Kdo in ratios 2.0:1.0:1.8:1.9:0.9. The ^{31}P NMR spectra of both the L4 and L7 core oligosaccharides showed one sharp signal (at +0.35 ppm for L4 and −0.32 ppm for L7) indicating that they were both phosphorylated and that they had different patterns of phosphorylation. The presence of a doublet at ca. 41.1 ppm ($^3J_{C,P}$ 7.5 Hz) in both ^{13}C NMR spectra together with a singlet at ca. 3.33 ppm in both ^1H NMR spectra is characteristic of a CH_2NH_2 group [7] indicating the presence of a phosphoethanolamine substituent (PEA). This is corroborated by the above molecular-ion mass data obtained by FABMS, and thus both oligosaccharides appear to be similar in composition to the L3 lipopolysaccharide [7].

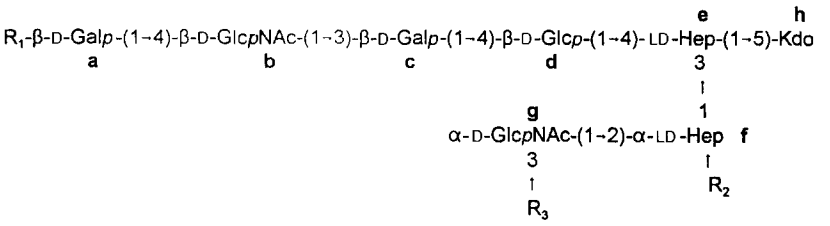
In order to obtain structural information on the basic core oligosaccharides the L4 and L7 LPS were hydrolyzed with 1% acetic acid at 100 °C. Subsequent elution of the hydrolyzates from a Bio-Gel P-4 column afforded essentially one major oligosaccharide from each LPS. The structures of the oligosaccharides are shown in Fig. 1 in which the individual sugar components of the oligosaccharides are

marked **a–h**. Identification of the sugar units in the *N. meningitidis* L4 core oligosaccharide and complete assignment of their proton signals were performed using two-dimensional (2D) homocorrelated COSY, TOCSY, and NOESY NMR techniques [18]. The sequence of sugars was obtained from the interglycosidic connectivity of the sugar residues which were established on the basis of the observed NOEs between the respective anomeric protons and the protons at the site of substitution. The position of substitution was confirmed independently using a heterocorrelated HMBC technique [19] that reveals long-range coupling between the anomeric carbons and the protons at the substitution sites of adjacent sugar units. The anomeric configuration of the constituent sugar units was established in the case of the sugars having the gluco or galacto configuration on the basis of their proton $^{3}J_{1,2}$ coupling constants, and in the case of the two L-*glycero*-D-*manno*-heptoses on the basis of a NOESY experiment that showed intraresidual cross-peaks between their anomeric protons and in the corresponding H-2, but not with H-3 and H-5. The structure of the core oligosaccharide of *N. meningitidis* L4 is shown in Fig. 1. Complete assignments of its ^{1}H and ^{13}C NMR signals are recorded in Tables 1 and 2, respectively.

The position of *O*-acetyl substitution in the L4 oligosaccharide was established from its ^{13}C NMR spectrum which contained inter alia three signals in the region of N-linked carbons of *N*-acetylamino sugars. The signal at 55.97 ppm corresponds to a β-linked *N*-acetylglucosamine (**b**) [20], while two signals of half the intensity at 55.08 and 54.93 ppm correspond to an α-linked *N*-acetylglucosamine (**g**) [21]. Similar intensities were observed in the signals of methyl carbons at 22.96, 22.76, and 22.71 ppm, the intensity of each of the two latter signals being approximately half that at 22.96 ppm. Since there are only two amino sugars in the L4 oligosaccharide, the splitting of the C-2 and the methyl carbon of its α-linked *N*-acetylglucosamine (**g**) results from its partial acetylation at O-3. This was confirmed when, after the *O*-deacetylation of the oligosaccharide by mild alkaline hydrolysis, the splitting of the signals of C-2 and methyl carbon of **g** disappeared. Quantification of the intensity of the signals corresponding to *O*-acetylated and non-acetylated carbons, as well as of the intensities of the $[M + H]^{+}$ peaks at *m/z* 1680 (acetylated) and 1638 (non-acetylated) in the FABMS spectrum of the oligosaccharide implied that the degree of *O*-acetylation was approximately 50%.

The position of substitution of PEA in the L4 oligosaccharide was established using a proton–phosphorus correlated HMQC experiment and the subsequent $^{31}P(\omega_1)$-half filtered (^{1}H, ^{1}H) COSY experiment on the L4 core oligosaccharide. The HMQC spectrum showed two cross-peaks at 4.18 and 4.58 ppm. The former proton signal was assigned to the CH_2O group of phosphoethanolamine since it gave a cross-peak in a COSY experiment with the signal at 3.33 ppm that belonged to the CH_2N group of PEA. A subsequent $^{31}P(\omega_1)$-half filtered (^{1}H, ^{1}H) COSY experiment that only showed cross-peaks of the phosphorus coupled to protons, revealed that the proton resonating at 4.58 ppm had a three-bond coupling with the protons resonating at 3.72, 3.80, and 3.90 ppm. These signals were previously assigned [7] to H-5, H-7, and H-7′ of heptose **f** (Table 1), and therefore the PEA substituent is located at O-6 of **f**.

The ^{1}H and ^{13}C NMR spectra of the L7 core oligosaccharide were similar to the corresponding spectra of the L4 oligosaccharide except for the



L4 R_1 = α-Neu5Ac-(2→3)
R_2 = 6PEA
R_3 = Ac (50%)

L7 R_1 = H
R_2 = 3PEA
R_3 = H

Fig. 1. Structures of the L4 and L7 core oligosaccharides.

Table 1
^{1}H chemical shift data for the *O*-deacetylated L4 and L7 [a] core oligosaccharides

Unit	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	H-7	H-7′
a β-D-Gal	4.490 (4.53)	3.57 (3.56)	3.75 (3.69)	3.95 (3.98)	3.77 (3.83)	3.80			
b β-D-GlcNAc	4.76 (4.81)	3.82 (3.83)	3.75 (3.76)	3.72 (3.75)	3.59 (3.64)	3.95	4.00		
c β-D-Gal	4.485 (4.53)	3.62 (3.61)	3.79 (3.81)	4.15 (4.18)	3.72 (3.78)	3.80			
d β-D-Glc	4.56 (4.59)	3.48 (3.47)	3.73 (3.68)	3.49 (3.58)	3.60 (3.60)	4.02	4.05		
e α-LD-Hep	5.08 (5.13)	4.12 (4.03)	4.13 (4.07)	4.28 (4.17)	4.18	4.03		3.75	3.65
g α-D-GlcNAc	5.06 (5.18)	3.90 (3.92)	3.83 (3.82)	3.50 (3.55)	4.00	3.85	3.79		
f α-LD-Hep	5.59 (5.54)	4.13 (4.38)	4.03 (4.46)	4.03 (4.13)	3.72 (3.80)	4.58		3.90	3.80
PEA	3.33 (3.35)	4.18 (4.20)							
→ 5)-Kdo			ax 1.89	4.11	4.18 (4.12)				

[a] Data in parentheses.

Table 2
^{13}C chemical shift data for the *O*-deacetylated L4 and L7 [a] core oligosaccharides

Unit	C-1	C-2	C-3	C-4	C-5	C-6	C-7	CH_3
a β-D-Gal	104.03 (103.82)	72.06 (71.03)	73.30	70.09 (69.53)	76.07 (75.67)	61.74 (61.83)		
b β-D-GlcNAc	103.27 (103.27)	56.29 (56.26)	74.27	79.69 (79.67)	75.50 (75.55)	61.14 (61.13)		23.16 (23.26)
c β-D-Gal	103.84 (103.82)	71.95 (72.21)	82.95 (82.90)	69.55 (69.53)	76.22 (76.20)	61.74 (61.83)		
d β-D-Glc	103.11 (103.27)	74.67 (74.21)	74.70	80.79 (80.3)	75.20 (75.90)	61.38 (61.43)		
e α-LD-Hep	101.90 (99.97)	69.23	73.02	74.00	71.90	71.00	64.28	
g α-D-GlcNAc	100.10 (100.00)	55.26 (54.89)	72.65	71.30	73.20	61.84		22.96 (23.15)
f α-LD-Hep	100.40 (100.90)	80.54 (78.84)	71.40 (76.20)	67.28 (69.29)	74.00 (73.19)	75.58	64.04	
PEA	41.11 (41.17)	62.80 (62.70)						
→ 5)-Kdo			34.56		73.10			

[a] Data in parentheses.

absence of *O*-acetyl groups as indicated by the absence of splitting in the ^{13}C NMR signals of *N*-acetylglucosamine residue **g** in the spectrum of L7 oligosaccharide. Using the aforementioned NMR spectroscopic procedures, the structure of the L7 core oligosaccharide was established in the same way as the structure of that of L4. The structure is shown in Fig. 1 and the assignments of the ^{1}H and ^{13}C signals are presented in Tables 1 and 2, respectively. The observed NOEs were identical with those in the L4 oligosaccharide. Thus, both oligosaccharides have identical sequences of constituent monosaccharide units, which is also the same sequence as that found in the L3 core [7]. However, the ^{31}P–^{1}H HMQC spectrum of the L7 oligosaccharide indicated that it had a different phosphorylation pattern to that of L4. The spectrum had two cross-peaks at 4.18 and 4.46 ppm of which the former signal, also present in the L4 spectrum, corresponds to the CH_2O group of PEA, while the latter signal is different from that observed at 4.58 ppm in the spectrum of the L4 oligosaccharide, which implied that the L7 oligosaccharide had a different position of phosphorylation. The $^{31}P(\omega_1)$-half filtered (^{1}H, ^{1}H) COSY experiment showed that the signal at 4.46 ppm gave cross-peaks with the protons resonating at 4.38 and 4.13 ppm. These two signals were assigned to H-2 and H-4 of L-*glycero*-α-D-*manno*-heptose **f** and the signal at 4.46 ppm to H-3 of **f**. Therefore, in the L7 core oligosaccharide PEA substitution occurs at position O-3 of heptose **f** as previously established for the oligosaccharide.

For the determination of the presence of sialic acid and its location, the native L4 and L7 lipopolysaccharides were *O*-deacylated by mild hydrazinolysis [7], and the chromatographically purified product (LPS-OH) was investigated using ESMS. The ESMS spectrum of LPS-OH of L4 contains three sets of peaks designated A, B, and C, corresponding to species with masses of 3084, 3267, and 2793. These species gave rise to the triply charged ions $[M - 3H]^{3-}$ with *m/z* 1026.8 (A_3), 1087.8 (B_3), and 930.0 (C_3), as well as quadruply charged $[M - 4H]^{4-}$ and quintuply charged $[M - 5H]^{5-}$ counterparts. Species A has the composition Neu5Ac, Hex_3, $HexNAc_2$, Hep_2, Kdo_2, PEA with attached *O*-deacylated diphosphoryl lipid A (calculated molecular mass 3084.8). Species B differs from species A by 183 mass units and represents species A with an additional molecule of lauric acid that was not removed by hydrazinolysis. Lauric acid is known to be an *O*-acyl substituent of β-hydroxymyristic acid in the lipid A of the *Neisseria sp* [22]. Species C with the molecular mass 2793 represents species A lacking sialic acid.

In contrast the ESMS spectrum of the LPS-OH *N. meningitidis* L7 revealed the presence of only one species that produced triply, quadruply and quintuply charged ions $[M - nH]^{n-}$ that corresponded to a molecular ion with molecular mass of 2793. As mentioned above, this species is derived from an LPS devoid of sialic acid. Not even minor peaks associated with any sialylated species were observed. Since the growth conditions used in the cultivation of the L4 and L7 immunotypes of *N. meningitidis* were identical, it is likely that the L7 immunotype produces a non-sialylated LPS.

In order to establish the position of sialylation in the L4 lipopolysaccharide, a ^{1}H NMR investigation of its *N*,*O*-deacylated derivative was carried out [7]. The integral intensity of the characteristic signals of $H\text{-}3_{ax}$ and $H\text{-}3_{eq}$ of sialic acid with the anomeric signal of unit a implied that the extent of sialylation of the L4 LPS was about 75%. Comparison of the ^{1}H NMR chemical shifts of unit **a** of the L4 oligosaccharide (Table 1) with those of the corresponding β-D-galactopyranosyl unit in its *N*,*O*-deacylated LPS (assignment of its ^{1}H NMR signals was performed using a 1D TOCSY technique) showed that sialylation caused a significant downfield displacement in its H-3 signal from 3.75 to 4.35 ppm. In addition, a selective ROESY experiment indicated a correlation between $H\text{-}3_{ax}$, of sialic acid and this proton. This implies the L4 LPS is sialylated (to the extent of 75%) at O-3 of the terminal β-D-galactopyranosyl unit of its lacto-*N*-neotetraose antenna.

To determine the phosphorylation pattern of the intact L4 and L7 LPS immunotypes, a ^{31}P NMR analysis was carried out on their *O*-deacylated LPS preparations (LPS-OH) which were obtained using hydrazinolysis [7], sodium acetate, or triethylamine treatment [22]. ESMS fragmentation spectra of these *O*-deacylated preparations showed fragments with the *m/z* values consistent with simple monophosphate substitution at the O-1 and O-4′ positions of the L4 and L7 lipid A which are in agreement with the proposed conserved structure of *O*-deacylated lipid A [23], and not with the presence of pyrophosphate substituents as proposed by Kulshin et al. [22].

However, in the ^{31}P NMR spectrum of the intact LPS in a solution containing both EDTA and sodium deoxycholate [23] we did observe signals in the region of −10 to −11 ppm that could be assigned to pyrophosphate substituents [23,24], but the intensity of these signals was 10 times less than those of the

monophosphate substituents (−0.8 ppm). Kulshin et al. [22], reported this signal at +11 ppm probably using an alternative convention. Our inability to obtain pyrophosphorylated LPS can be best explained by the fact that unlike us, Kulshin probably used growth conditions more favourable to its production. This is supported by the observation [23] that the pattern of phosphorylation in the meningococcal LPS varies with different cultivation conditions.

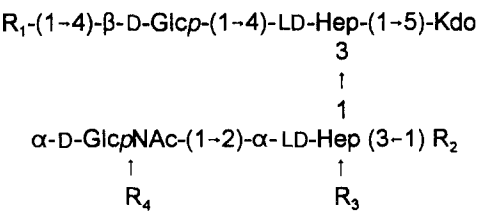
3. Discussion

Interest in the potential use of the LPS of *N. meningitidis* as a vaccine candidate has been sustained by the poor immunogenicity of the group B meningococcal capsular polysaccharide both in its native [25] and protein conjugated form [26]. The meningococcal LPS have been implicated in the immune response to natural infection [27,28] and have been serologically divided into at least 12 immunotypes [1–3]. The source of the immunotype epitopes is contained in the oligosaccharide moieties of the LPS [4] and structural studies on some of these oligosaccharides have identified regions of structural difference and structural similarities, which account for both the immunotype specificity and cross-reactivity exhibited by the meningococcal LPS. Structural elucidation of the L4 and L7 immunotypes now provides an even more comprehensive insight into the structural basis of the immunotyping system and all the known structures are shown in Fig. 2.

The L4 and L7 LPS are closely related to that of L3 [7], because on dephosphorylation and *O*-deacetylation they all give identical diantennary oligosaccharide structures having lacto-*N*-neotetraose as the longest antenna. Lacto-*N*-neotetraose, first identified in the L3 immunotype LPS [5], is also a structural feature of the L2 [12] and L5 [8] immunotypes. Like the L3 immunotype LPS [7] it has now been established that the L4 immunotype can be sialylated at O-3 of the terminal β-D-galactopyranosyl residue of its lacto-*N*-neotetraose antenna, and on the basis of SDS-gel electrophoresis studies it has been reported [14] that all immunotype LPS including L7 having this structural feature can be exogenously sialylated. However, despite the presence of lacto-*N*-neotetraose in the L7 immunotype we were unable to obtain it in its sialylated form. The best explanation for this is that the meningococcal strain (M982B) from which we obtained the L7 LPS for structural studies, and which was typed as L7-specific in the original immunotyping system [2], is a phenotypic variant strain unable to sialylate its lacto-*N*-neotetraose antenna. Because the L3 and L7 immunotype LPS are otherwise structurally identical (Fig. 2) the presence of terminal sialic acid only in the former probably accounts for the ability to differentiate the L3 and L7 immunotypes using the original typing system [1,2].

The predominant immunotype-specific responses [1–3] exhibited by many of the meningococcal LPS containing lacto-*N*-neotetraose and its sialylated anologue (Fig. 2) confirms that the latter structural features are not immunodominant [29]. This lack of immunodominance is due to extensive structural homology with mammalian tissue antigens [29,30] which probably also indicates that these structures are virulence factors for the meningococci. Therefore, the epitopes responsible for LPS immunotyping reside in the inner core of the oligosaccharide moieties [29]. PEA substitution is one such structural variation which, although confined to the heptose residue of the shorter antennae, does vary in location on this residue. Despite extensive structural similarity in the L3 and L4 oligosaccharides (Fig. 2) their respective LPS can be readily immunotyped [2], and ignoring the possible participation of *O*-acetyl groups, this can be attributed to the differing locations of their phosphoethanolamine substituents (at O-3 for L3 and O-6 for L4). Interestingly, like that of the L3 LPS, the oligosaccharide of L1 also has a phosphoethanolamine substituent at O-3 of the same heptose residue [6], and cross-reactions between the two LPS have been reported [2,10]. The ability to immunotype these two LPS therefore must reside in differences in the structures of their long antennae (Fig. 2). In the L1 immunotype an α-D-Gal residue is substituted for the terminal *N*-acetyllactosamine of the L3 lacto-*N*-neotetraose antenna. This results in the formation of yet another epitope [α-D-Gal-(1 → 4)-β-D-Glc] expressed on mammalian cells (human epithelial cells), which interestingly enough is also expressed by other human pathogenic bacteria, e.g. *Haemophilus influenzae* and *N. gonnorhoeae* [31]. If it is true that structures which mimic mammalian tissue are not immunodominant, then the inner core epitope of the L1 immunotype would either have to include the antenna structure or be conformationally controlled by it.

Meningococci of the L2 immunotype express two LPS which differ only in the location of PEA substituents in their oligosaccharide moieties. One oligosaccharide has this substituent at O-6 of its branch heptose residue and the other at O-7 of the same



L1 R_1 = α-D-Gal*p*-(1→4)-β-D-Gal*p*, R_2 = H, R_3 = 3PEA, R_4 = H

L2 R_1[a] = β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*,
R_2 = α-D-Glc*p*,
R_3 = 6 PEA and 7 PEA,
R_4 = Ac (40%)

L3 R_1 = α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*
R_2 = H,
R_3 = 3PEA
R_4 = H

L4 R_1 = α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*
R_2 = H
R_3 = 6PEA
R_4 = 3Ac (50%)

L5 R_1[a] = β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*
R_2 = α-D-Glc*p*
R_3 = H
R_4 = Ac (60%)

L6 R_1 = β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*
R_2 = H
R_3 = 7PEA
R_4 = H

L7 R_1[a] = β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*
R_2 = H
R_3 = 3PEA
R_4 = H

[a] Terminally sialylated as shown by gel-electrophoresis

Fig. 2. Structures of the L1–L7 core oligosaccharides.

residue (Fig. 2). Although the L4 LPS is also similarly substituted at O-6 of its branch heptose residue, its immunotype specificity probably resides in the fact that it lacks the additional terminal α-D-Glc residue of the L2 immunotype situated on the same heptose residue (**f**) as the PEA substituent. The same structural feature probably generates unique L2 immunotype epitopes. This structural feature is also found in the L5 LPS, but in this case the immunotype specificity probably resides in the insertion of an additional β-D-Glc between its lacto-*N*-neotetraose antenna and its inner core (Fig. 2). The same reasoning could be applied to the immunotyping of the L6 LPS, which like the second L2 LPS has a PEA substituent at O-7 of its branch heptose residue. However, in this case the L6 LPS also has another unique structural feature which could account for its specificity, in that it is a phenotypic variant unable to attach the terminal β-D-Gal of its lacto-*N*-neotetraose antenna.

Where it occurs *O*-acetylation of the meningococcal LPS is restricted to the terminal α-D-GlcNAc of its oligosaccharide moieties (Fig. 2). Partial but substantial *O*-acetylation has been demonstrated in the L2, L4, and L5 immunotypes but only in one (L4) has the position of *O*-acetylation (at O-3 of its termi-

nal α-D-GlcNAc) been established. The role of *O*-acetylation in the immunotyping system is difficult to define because the pattern of *O*-acetylation on the isolated oligosaccharides (Fig. 2) may not be representative of that exhibited on the native LPS. This is because in all probability *O*-acetyl groups are partially removed during the mild acid hydrolysis required to obtain the oligosaccharides from the LPS.

An examination of Fig. 2 would indicate that a universal LPS-based vaccine against group B meningococcal meningitis would need to contain LPS of a number of different immunotypes, although this number could be considerably reduced, if it were based on the inclusion of the immunotypes most frequently encountered in disease isolates. The assertion that this is a useful strategy is supported by the fact that the identification of the most frequent isolates by two different groups, one in North America [2] (L2, L3, L4, and L7), and the other in Europe [28] (L1, L2, and L3,7,9) indicate that there is considerable geographical conformity in the immunotypes of both these isolates. Another method of simplifying an LPS-based vaccine would be to use minimal inner core oligosaccharides, common to all immunotypes (Fig. 2), as haptens to construct a broadly cross-reactive oligosaccharide–protein conjugate vaccine [10]. Unfortunately this approach has met with little success to date particularly in the ability of these vaccines to induce bactericidal antibodies [10].

4. Experimental

Materials.—Group C *N. meningitidis* strain 89I (serotype L4) and strain M982B (serotype L7) were grown in Frantz medium in a New Brunswick 28 L Microferm fermentor. Growth conditions as well as isolation of the LPS have been previously described [7].

Analytical methods.—All evaporations were performed at reduced pressure below 40 °C in a rotary evaporator. Gel-permeation chromatography was carried out on columns (1.6 × 90 cm) of Bio-Gel P-4 (400 mesh) and Bio-Gel P-2 (200–400 mesh) at room temperature using pyridine acetate buffer (0.02 M, pH 5.4) at a flow rate of ca. 15 mL/h. Column eluates were monitored using a Waters R401 differential refractometer. Combined GLC-MS was carried out on a Varian Saturn II instrument equipped with a DB-17 capillary column (0.25 mm × 30 m) using an ionization potential of 70 eV. FABMS was carried out in both positive and negative modes on a thioglycerol or a 3:1 dithiothreitol–dithioerythritol matrix at room temperature using a Jeol JMSAX5O5H instrument. ESMS of the *O*-deacylated lipopolysaccharides was performed using a VG Quattro mass spectrometer with an electrospray ion source. Scans were taken in the negative-ion mode over the *m/z* range of 50–2500 and spectra were averaged over 10 scans. For calculations of the predicted masses, the following average mass units were used: H, 1.00794; H_2O, 18.05; hexose (Hex), 162.142; heptose (Hep), 192.169; *N*-acetylhexosamine (HexNAc), 203.195; keto-3-deoxyoctonic acid (Kdo), 220.179; sialic acid (Neu5Ac), 291.258; phosphoethanolamine (PEA), 123.048; *O*-deacylated diphosphoryl lipid A moiety containing two N-linked β-hydroxymyristic acids, 953.009 [32].

Nuclear magnetic resonance.—All NMR experiments were performed on a Bruker AMX 500 spectrometer using a 5 mm broad band probe with the ^1H coil nearest to the sample. ^1H, ^{13}C, and ^{31}P NMR spectra were recorded at 300 and 340 K in 5 mm tubes at concentrations of 1–10 mg oligosaccharide in 0.5 mL D_2O at neutral pH. Acetone was used as an internal standard with the CH_3 resonance at 31.07 ppm for ^{13}C spectra and 2.225 ppm for ^1H spectra. Phosphoric acid (25%) was used as an external standard for ^{31}P spectra (0.00 ppm). All experiments were carried out without sample spinning. 2D homo- and hetero-correlated experiments (COSY, TOCSY, NOESY, HMQC) were performed as described previously [18]. ^1H-detected multiple-bond correlation (HMBC) experiments were carried out according to Bax and Summers [19]. Two-dimensional $^{31}P(\omega_1)$-half filtered (^1H, ^1H) COSY experiments were carried out as previously described [20].

Chemical methods.—The core oligosaccharides were obtained by heating the L4 and L7 LPS in 1% HOAc for 2 h at 100 °C. Gel-filtration of the soluble hydrolyzate on a Bio-Gel P-4 column yielded essentially one oligosaccharide from each of the L4 and L7 immunotypes. *O*-Deacetylation and dephosphorylation of the core oligosaccharides was performed as previously described [6]. Constituent glycoses were determined as their alditol acetates [21] and their absolute configuration was established by capillary GLC of their silylated (−)-2-butyl glycosides [33]. Preparation of the *N,O*-deacylated LPS was performed by heating it in 4 M KOH at 120 °C for 16 h. The resultant mixture was adjusted to pH 6.0 with 2 M HCl and the precipitated fatty acids were separated by centrifugation at 10,000 rpm. The supernatant was subjected to extensive dialysis, freeze-dried, and the *N,O*-deacylated LPS was purified by gel-filtration.

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