THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 268, No. 19, Issue of July 5, pp. 14146-14152, 1993
Printed in U.S.A.

Structure of the Sialylated L3 Lipopolysaccharide of *Neisseria meningitidis**

(Received for publication, December 9, 1992, and in revised form, March 25, 1993)

Viliam Pavliak‡, Jean-Robert Brisson, Francis Michon, Dušan Uhrín§, and Harold J. Jennings¶

From the Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada

The L3 immunotype lipopolysaccharide (LPS) of *Neisseria meningitidis* was subjected to degradation procedures, which produced a number of different oligosaccharide fragments. The high resolution ^1H and ^{13}C NMR spectroscopic analyses of these oligosaccharides yielded structural information on a number of different regions of the LPS. For example, from one oligosaccharide, it was found that the endogenous sialylation of the meningococcal LPS occurs at O-3 of the terminal β-D-galactopyranosyl residue of its lacto-*N*-neotetraose antenna in the α-D-configuration. From another, it was also established that the dominant structural feature responsible for L3 epitope specificity is the presence of a phosphorylethanolamine substituent at O-3 of the penultimate heptopyranosyl residue of its other antenna. In addition from information obtained with another oligosaccharide the structure of the 3-deoxy-D-*manno*-octulosonic acid disaccharide region of the L3 LPS was also elucidated. From all the above cumulative data plus some published data, it was then possible to reconstruct the complete structure of the entire native L3 LPS.

Neisseria meningitidis can be serologically divided into at least 12 immunotypes based on its LPS[1] (1-3). The LPS epitopes are located in the glycose moieties of the LPS (4), the latter having been identified as small oligosaccharides of the type normally associated with LPS of the R-type (5). Although heterogeneous, structural studies on the largest of the oligosaccharides obtained from the L1 and L6 (6), L3 (5), L5 (7), and L2 (8) 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). Heterogeneity even exists among the oligosaccharides obtained from the LPS of individual meningococci, and the structural basis of this heterogeneity has been elucidated. Heterogeneity can be generated by the addition or deletion of glycose units (7), as well as by the presence and location of phosphorylethanolamine substituents (6, 8) and possibly *O*-acetyl groups (7). Heterogeneity can also be generated by the endogenous sialylation of the meningococcal LPS (9, 10), in which sialylation occurs at the terminal galactopyranosyl residue of the lacto-*N*-neotetraose antenna of the oligosaccharide (10). This paper describes work that resulted in the structural determination of the complete sialylated L3 LPS of *N. meningitidis*.

EXPERIMENTAL PROCEDURES

Materials—Group Y *N. meningitidis* 406Y (NRCC 4030, serotype L3) was grown in Frantz medium in a New Brunswick Scientific 28L Microferm fermentor. The cells were grown at 37 °C for 18 h with stirring (200 rpm) and 20 liters/min of aeration. Isolation and purification of LPS was done as follows: 412 g (wet weight) bacterial cells were suspended in 600 ml of 40 mM sodium phosphate buffer, pH 7.0, containing 5 mM sodium EDTA and 0.05% sodium azide. Cells were treated with lysozyme (Sigma, 51100 units/mg), and LPS was extracted with 45% phenol (final concentration) at 68-70 °C and finally purified by gel filtration according to procedures described by Gu and Tsai (11).

Analytical Methods—Solutions were evaporated under diminished pressure below 40 °C in a rotary evaporator. Gel filtration was done on columns (1.6 × 90 cm) of Bio-Gel P4 (400-mesh) and P2 (Bio-Rad) at 20 °C using pyridine acetate buffer (0.02 M, pH 5.4) at a flow rate of approximately 12 ml/h. Individual fractions were monitored using a Waters R403 differential refractometer. Combined gas-liquid chromatography-mass spectrometry (GLC-MS) was carried out on a Jeol EX505H instrument using a capillary column (0.25 mm × 20 m) of fused silica DB17 (J and W Scientific) employing an ionization potential of 70 eV.

Nuclear Magnetic Resonance—All NMR experiments were performed on a Bruker AMX 500 spectrometer using a broad band probe with ^1H coil nearest to the sample. ^{13}C, ^1H, and ^{31}P NMR spectra were recorded at 295 and 340 K in 5-mm tubes at concentrations of 1-8 mg of oligosaccharide in 0.5 ml of D_2O at neutral pH. Phosphoric acid (25%) was used as an external chemical shift reference for ^{31}P NMR (σ 0.00 ppm). Dioxane was used as an external standard (σ 67.4 ppm) for ^{13}C. The proton chemical shifts in deuterium oxide (D_2O) are expressed relative to the HOD signal (4.81 ppm at 295 K, 4.348 ppm at 340 K). All experiments were carried out without sample spinning.

The two-dimensional experiments (COSY, TOCSY, NOESY, HMQC) were carried out as described previously (12). The ^1H detected, ^1H-^{13}C correlated two-dimensional HMQC-TOCSY experiment was performed according to Lerner and Bax (13). Selective one-dimensional TOCSY experiments were employed using a half-gaussian pulse with spin-lock mixing times of 30-270 ms (14). If the transfer of magnetization in TOCSY was not possible due to small coupling constants, the spin-lock period was followed by a nonselective COSY step in a relayed TOCSY (15).

Chemical Methods—De-*O*-acylation of LPS was performed as follows. To remove the fatty acids the LPS (50 mg) was dried overnight over P_2O_5, dissolved in anhydrous hydrazine (5 ml), and stirred for 30 min. at 37 °C. After the addition of chilled (−20 °C) acetone, the precipitate was collected by centrifugation, washed with acetone,

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‡ Present address: Univax Biologics Inc., 12280 Wilkins Ave., Rockville, MD 20892.

§ Permanent address: Inst. of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia 84238.

¶ To whom correspondence should be addressed.

[1] The abbreviations used are: LPS, lipopolysaccharide; KDO, 3-deoxy-*D-manno*-octulosonic acid; NOE, nuclear Overhauser effect; LPS-OH, de-*O*-acylated LPS; GLC-MS, gas-liquid chromatography-mass spectrometry; COSY, correlated spectroscopy; NOESY, NOE spectroscopy.

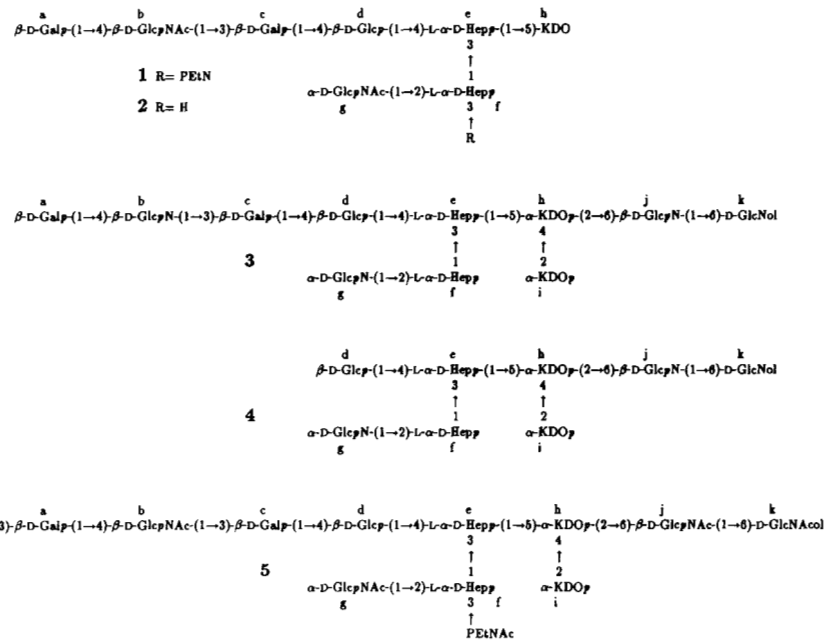

FIG. 1. **Structures of the different oligosaccharides (1–5) obtained when the L3 LPS was subjected to different degradation procedures.**

TABLE I

Methylation analysis of oligosaccharide cores derived from the L3 lipopolysaccharide

Methylated sugar[a,b]	Molar ratios[c]	
	1	2
2,3,4,6-Me_4Gal	1.00	1.00
2,4,6-Me_3Gal	0.35	1.05
2,3,6-Me_3Glc	1.00	0.85
3,4,6,7-Me_4Hep		0.80
3,4,6-Me_3GlcNMeAc	0.60	0.65
2,6,7-Me_3Hep	0.80	0.85
4,6,7-Me_3Hep	0.5	
3,6-Me_2GlcNMeAc	0.55	0.60

[a] Upon hydrolysis, removal of phosphatidylethanolamine located at O-3 of the heptosyl residue occurred.

[b] KDO was detected in **1** as its 1,2,4,6,7,8-hexamethyl-3-deoxy-5-OAc derivative (5).

[c] **1**, acetic acid released core; **2**, dephosphorylated acetic acid released core.

dissolved in water (5 mL), reprecipitated with acetone, dried, dissolved in water, and lyophilized yielding 36.4 mg of de-*O*-acylated LPS (LPS-OH) (73%).

The core oligosaccharides were obtained by heating the LPS (10 mg/ml) in 1% acetic for 2 h at 100 °C. The insoluble lipid A was removed from the hydrolysis mixture by centrifugation at 10,000 rpm. The acid hydrolyzed oligosaccharides (yield ≈ 50%) were then purified by gel filtration on Bio-Gel P4 using 0.02 M pyridinium acetate buffer, pH 5.4, as eluent yielding one main oligosaccharide **1** with $K_{av} = 0.37$.

Dephosphorylation of the de-*O*-acylated LPS (LPS-OH) and acetic acid released core oligosaccharides was accomplished by treating them with aqueous HF at 4 °C for 72 h (6). The excess of HF in the core oligosaccharide sample was removed in a vacuum, over NaOH pellets at 4 °C. In the case of LPS-OH, HF was removed by dialysis against water (5 × 100 ml).

De-*N*-acylation of reduced LPS-OH was performed in anhydrous hydrazine at 85 °C for 7 days as described by Holst *et al.* (16). Re-*N*-acetylation of the product was achieved by treating the sample with acetic anhydride in 5% (w/v) $NaHCO_3$(aqueous) for 1 h at room temperature.

Determination of Sialic Acid by Treatment of the LPS-OH with Neuraminidase—2 mg of LPS-OH dissolved suspended in 1 ml of 25 mM sodium acetate pH 6.8 was treated with 10 millunits of neuraminidase (Genzyme, Cambridge, MA) at 37 °C for 2 h as previously described (10). An additional 10 milliunits of enzyme was added, the mixtures were incubated again at 37 °C overnight and filtered with a 0.22-μm pore size membrane, the filtrate was loaded onto a Sep-Pak C18 cartridge (Waters, Milford, CA), and the cartridge was washed with water. The eluent and washes were pooled and lyophilized. The same steps were used for the control, using a heat-inactivated enzyme. Analysis of sialic acid was done by high performance anion-exchange chromatography. The residue dissolved in 500 μl of H_2O was analyzed for sialic acid with a Carbo Pac PA1 column (4 × 250 mm) and pulsed amperometric detection (Dionex Corp., Sunnyvale, CA). Eluant A consisted of 150 mM NaOH; eluent B consisted of 150 mM NaOH + 1 M NaOAc. Eluting conditions were as follows: 5 min of isocratic 95% A + 5% B, then gradient increasing concentration of B by 1.58% per minute, flow rate 1 ml/min. Retention time for sialic acid was 11.4 min. Xylose was used as internal standard. Retention time was 3.64 min.

Determination of Sialic Acid by GLC-MS—1 mg of de-*O*-acylated LPS was methanolyzed in 2 ml of MeOH, 2 N HCl at 80 °C for 2 h. Released glycose residues were trimethylsilylated, and the mixture was analyzed by GLC-MS (17).

RESULTS AND DISCUSSION

The application of different degradation procedures on the native L3 meningococcal LPS produced a number of its constituent oligosaccharide fragments, of which the structure of five (**1**, **2**, **3**, **4**, and **5**) are shown in Fig. 1. Using this and other structural information, it was then possible to reconstruct a composite structure of the entire L3 LPS. The presence of sialic acid in the LPS was first detected by analysis of a water-soluble preparation in which only the ester-linked fatty acid were removed from the LPS by mild hydrazinolysis (LPS-OH). LPS-OH was treated with either intact or heat-inactivated neuraminidase and analyzed by strong anion-exchange chromatography with pulsed amperometric detection using xylose and sialic acid as standards. The neuraminidase treated LPS-OH gave a single peak with the same retention time as that of sialic acid (11.16 min), whereas an identical sample of LPS-OH treated with heat-inactivated neuraminidase gave no detectable peaks. The presence of sialic acid was also confirmed by GLC-MS of its pertrimethylsilyl methyl glycoside following methanolysis of the native LPS (17).

In order to obtain structural information on the basic core oligosaccharide of L3 serotype, the LPS was hydrolyzed with 1% acetic acid at 100 °C. Subsequent elution of the hydrolysate from a Bio-Gel P4 column afforded one major oligosac-

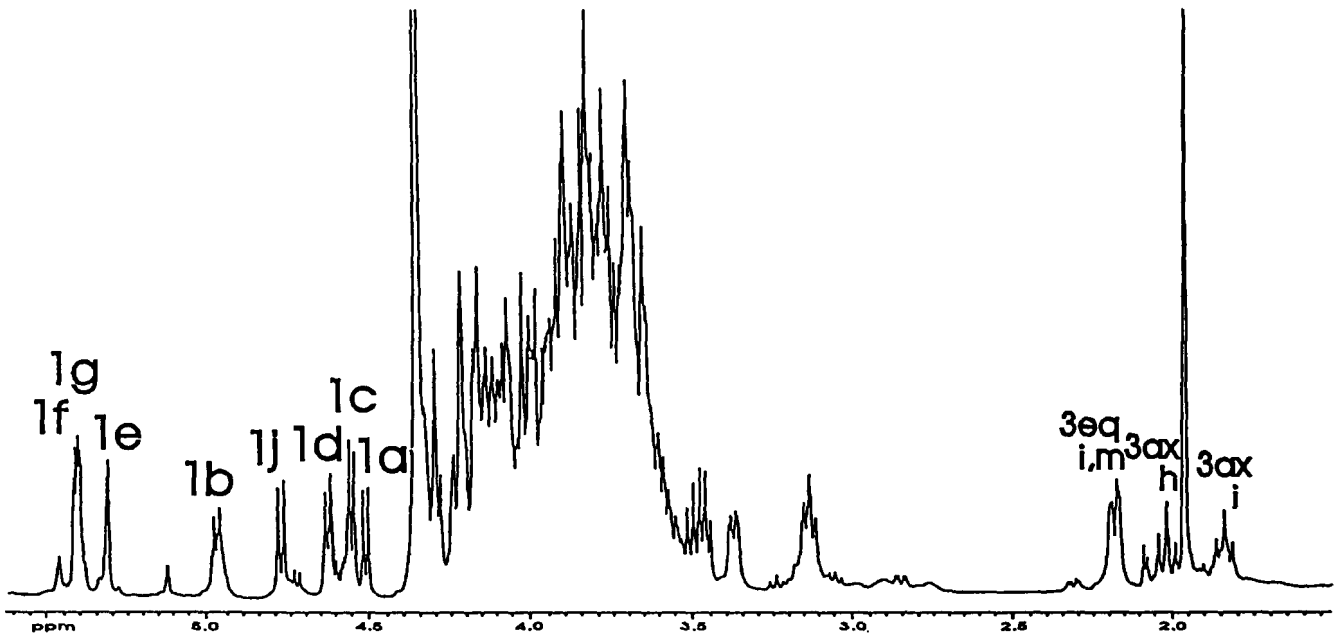

FIG. 2. **^{1}H NMR spectrum (500 MHz, 340 K) of oligosaccharide 3.**

charide (**1**) with K_{av} = 0.37. A further constituent oligosaccharide was produced by the removal of the phosphorylethanolamine substituent from **1** to yield oligosaccharide **2** (Fig. 1). The chemical shifts of the protons of the individual residues of **1** and **2** were assigned by two-dimensional NMR methods (12) as described below for oligosaccharides **3** and **4**, and are listed in Table II.

The ^{31}P NMR spectrum of **1** exhibited one sharp signal at +0.19 ppm, indicating that **1** was phosphorylated. The presence of a doublet at 40.8 ppm ($^3J_{C,P}$ = 8.0 Hz) in the ^{13}C NMR spectrum of **1** and a signal at 3.32 ppm in the ^{1}H NMR of **1** characteristic of a CH_2NH_2 group (6) indicated the presence of a phosphorylethanolamine substituent (data not shown). From these signals, the chemical shifts of the CH_2O group of phosphorylethanolamine in the ^{13}C and ^{1}H NMR spectra (σ 62.5 and 4.18 ppm, respectively) were assigned using ^{1}H-^{13}C two-dimensional HMQC-TOCSY (data not shown). The ^{1}H-^{31}P correlated spectrum (HMQC) of **1** showed only one cross-peak at σ 4.18 ppm in the ^{1}H dimension assigned to H-3 of heptose **f** and CH_2O of phosphorylethanolamine. Dephosphorylation of **1** caused an upfield shift in the H-3 signal to σ 4.06 ppm (Table II), confirming substitution of O-3 of **f** with phosphorylethanolamine. Removal of this substituent afforded oligosaccharide **2** previously identified, and structurally determined using mainly chemical procedures (5), as the L3 core determinant common to a number of different strains of *N. meningitidis*. The location of phosphorylethanolamine was also confirmed by methylation analysis of oligosaccharides **1** and **2**. The results are shown in Table I and indicate that the 4,6,7-trimethyl-heptopyranosyl residue present in phosphorylated oligosaccharide **1** was replaced by a 3,4,6,7-tetramethyl-heptopyranosyl residue in **2**. This confirms the localization of the phosphorylethanolamine group in **1** at O-3 of residue **f**. Sialic acid was not detected in oligosaccharide **1** demonstrating that it is quantitatively removed when subjected to these hydrolysis conditions.

To obtain information about the structure of the KDO region of the L3 LPS, the aforementioned LPS-OH was dephosphorylated, reduced, and de-*N*-acylated. Gel filtration on a Bio-Gel P2 column afforded two distinct oligosaccharides **3** and **4**. The ^{1}H NMR spectrum (Fig. 2) of the faster moving oligosaccharide **3** showed eight signals in the anomeric region (5.6–4.5 ppm), each integrating for one anomeric proton (Table II). These signals were assigned to residues **a**–**g** and similar signals in the spectra of **1** and **2** indicated that they also contained the same residues. In addition, the spectrum contained signals for the geminal protons of two 3-deoxy-D-*manno*-octulosonic acids (KDO): 2.01 ppm (1H, t, H-3ax of residue **h**), 1.84 ppm (1H, t, H-3ax of residue **i**), 2.18 ppm (2H, dd, H-3eq of **h** and **i**). A doublet in the anomeric region at 4.77 ppm ($^3J_{1,2}$ = 8.38 Hz) of β-linked 2-amino-2-deoxyglucose (residue **j**) and signals for the borohydride-reduced (open chain) form of 2-amino-2-deoxy-β-D-glucopyranosyl residue (**k**) are indicative of the remnants of the lipid A structure of the native LPS (18).

Assignments of the proton resonances for **3** and **4** are listed in Table II and the carbon resonances for **3** in Table III. The nuclear Overhauser effects were used to obtain sequence information of residues in **3** and for determination of their anomeric configuration, since the absolute configuration of the component sugars was known (5). Cross-sections for the anomeric protons from two-dimensional NOE spectrum are shown in Fig. 3. Cross-peaks of H-1–H-3 (**a**), H-1–H-5 (**a**), H-1–H-3 (**c**), and H-1–H-5 (**c**) indicated that the D-galactopyranosyl residues **a** and **c** are in the β-D-galacto configuration. Residue **a** is linked to O-4 of **b** and residue **c** is linked to O-4 of **d**. H-1 (**b**) and H-1 (**j**) showed NOEs with H-3 (**b**), H-5 (**b**), H-3 (**c**) and H-3 (**j**), H-5 (**j**), H-6 (**k**), respectively, confirming the β-configuration of 2 amino-2-deoxy-D-glucopyranosyl residues **b** and **j** and indicating that residue **b** is linked to O-3 of **c** and residue **j** to O-6 of residue **k**. NOEs of H-1 (**d**) to H-3 (**d**), H-5 (**d**), and H-4 (**e**) indicated that the D-glucopyranosyl residue **d** has the β-configuration and furthermore is linked to O-4 of residue **e**. H-1 of residues **e**, **f**, and **g** showed NOEs to corresponding H-2 but not to H-5 or H-3, indicating the α-configuration of D-glycero-D-*manno*-heptopyranosyl residues **e** and **f** and 2-amino-2-deoxy-D-glucopyranosyl residue **g** (Fig. 1). The NOEs of H-1 (**e**) to H-5 (**h**) and to H-7 (**h**) indicated that **e** is linked to O-5 of one of the KDO residue (**h**). These NOEs are typical of an α-D-

TABLE II

^1H NMR chemical shift data for oligosaccharides derived from N. meningitidis serotype L3 lipopolysaccharide

Chemical shifts are expressed relative to HOD signal (4.348 at 340 K).

Sugar unit	Proton atom	Chemical shifts 1	2	3	4
Residue **a**	1	4.52	4.52	4.50	
β-D-Gal*p*-(1→	2	3.59	3.59	3.61	
	3	3.70	3.69	3.70	
	4	3.98	3.98	3.98	
	5	3.78	3.78	3.78	
	6	3.76	3.75	3.85	
	6′	3.76	3.75	3.85	
Residue **b**	1	4.81	4.80	4.97[a]	
→4)-β-D-Glc*p*NAc-(1→	2	3.82	3.81	3.14[a]	
	3	3.78	3.77	3.85[a]	
	4	3.75	3.73	3.78[a]	
	5	3.62	3.63	3.68[a]	
	6	3.89	3.88	3.88[a]	
	6′	4.00	3.99	3.99[a]	
	NAc	2.08	2.09		
Residue **c**	1	4.51	4.51	4.54	
→3)-β-D-Gal*p*-(1→	2	3.64	3.65	3.80	
	3	3.79	3.79	3.90	
	4	4.18	4.18	4.21	
	5	3.82	3.82	3.82	
	6	3.76	3.75	3.85	
	6′	3.76	3.75	3.85	
Residue **d**	1	4.60	5.60	4.62	4.58
→4)-β-D-Glc*p*-(1→	2	3.45	3.47	3.45	3.39
	3	3.67	3.69	3.73	3.52
	4	3.53	3.52	3.64	3.31
	5	3.60	3.63	3.64	3.48
	6	3.75	3.71	4.07	3.78
	6′	4.02	4.06	3.85	3.98
Residue **e**	1	5.13	5.10	5.29	5.24
→4)-L-α-D-Hep*p*-(1→	2	4.14	4.15	4.16	4.12
3	3	4.05	4.04	4.12	4.07
↑	4	4.30	4.28	4.27	4.27
	5	4.18	4.16	4.18	4.18
	6	4.03	4.00	4.02	3.98
	7	3.75	3.75	3.77	3.72
	7′	3.65	3.68	3.68	3.67
Residue **f**	1	5.54	5.54	5.40	5.45
→2)-L-α-D-Hep*p*-(1→	2	4.42	4.16	4.32	4.27
	3	4.18	4.06	4.12	4.06
	4	4.12	3.96	3.98	3.98
	5	3.68	3.68	3.68	3.68
	6	4.05	4.03	4.10	4.08
	7	3.79	3.72	3.76	3.72
	7′	3.75	3.67	3.70	3.67
	CH_2NH_2	3.32			
	CH_2O	4.18			
Residue **g**	1	5.17	5.08	5.38[a]	5.30[a]
α-D-Glc*p*NAc-(1→	2	3.96	3.93	3.38[a]	3.31[a]
	3	3.82	3.82	3.94[a]	3.89[a]
	4	3.57	3.56	3.56[a]	3.58[a]
	5	3.89	3.89	3.90[a]	3.78[a]
	6	3.78	3.84	3.86[a]	3.85[a]
	6′	3.86	3.89	3.90[a]	3.92[a]
	NAc	2.16	2.17		
Residue **h**	3ax			2.01	1.97
→5)-α-KDO*p*-(2-	3eq			2.18	2.15
4	4			4.23	4.19
↑	5			4.29	4.25
	6			3.68	3.65
	7			3.87	3.84
	8			3.66	3.93
	8′			4.01	3.63
Residue **i**	3ax			1.84	1.80
α-KDO*p*-(2→	3eq			2.18	2.13
	4			4.07	4.07
	5			4.07	4.07
	6			3.68	3.65
	7			3.96	3.98
	8			3.95	3.98
	8′			3.83	3.82
Residue **j**	1			4.77	4.68
→6)-β-D-Glc*p*N-(1→	2			3.13	3.02
	3			3.69	3.58
	4			3.48	3.43
	5			3.65	3.65
	6			3.57	3.55
	6′			3.66	3.63
Residue **k**	1			3.82	3.77
→6)-GlcNol	1			3.95	3.90
	2			3.58	3.58
	3			4.16	4.13
	4			3.75	3.76
	5			3.99	4.00
	6			3.73	3.73
	6′			4.02	4.02

[a] De-*N*-acetylated residues.

TABLE III

^{13}C NMR chemical shift data of ***3***

Data were measured at 340 K in D_2O with dioxane as external standard (σ 67.4 ppm).

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
(**a**) β-D-Gal*p*(1→	103.92	72.10	73.30	69.80	76.25	62.12		
	(97.37)[a]	(72.96)	(73.78)	(69.69)	(75.93)	(61.84)		
(**b**) →4)-β-D-Glc*p*N(1→	101.80	56.87	72.30	79.60	76.10	61.27		
(**c**) →3)-β-D-Gal*p*(1→	103.35	71.20	83.00	69.60	76.20	62.12		
	(97.37)	(72.96)	(73.78)	(69.69)	(75.93)	(61.84)		
(**d**) →4)-β-D-Glc*p*(1→	102.78	74.52	75.25	80.53	76.10	62.12		
	(96.84)	(75.20)	(76.76)	(70.71)	(76.76)	(61.84)		
(**e**) →4)-L-α-D-Hep*p*[3→](1→	100.47	71.7	76.05	80.66	72.90	70.51	64.22	
	(95.5)	(72.0)	(71.9)	(67.7)	(72.5)	(70.3)	(64.4)	
(**f**) →2)-L-α-D-Hep*p*(1→	101.24	80.66	71.60	68.02	72.83	70.4	64.10	
	(95.5)	(72.0)	(71.9)	(67.7)	(72.5)	(70.3)	(64.4)	
(**g**) α-D-Glc*p*N(1→	98.94	55.82	71.23	70.52	73.81	61.22		
(**h**) →5)-α-KDO*p*-[4→](2→	174.87		35.20	71.16	74.2	73.91	70.82	65.20
	(176.5)	(102.5)	(35.2)	(67.4)	(67.1)	(72.5)	(70.5)	(64.2)
(**i**) α-KDO*p*(2→	175.33		35.75	67.44	67.40	73.91	71.22	65.30
	(176.5)	(102.5)	(35.2)	(67.4)	(67.1)	(72.5)	(70.5)	(64.2)
(**j**) →6)-β-D-Glc*p*N	100.65	56.87	76.12	71.53	73.91	63.15		
(**k**) →6)GlcNol	60.40	56.53	67.55	71.52	70.52	64.22		

[a] Data in parentheses were obtained from the corresponding monosaccharides (23, 32, 33).

pyranoside linked to O-5 of KDO (19). The cross-peak of H-1 (**f**) to H-3 (**e**) indicated the linkage of residue **f** to O-3 of D-glycero-D-*manno*-heptopyranosyl residue **e**. The cross-peaks from H-1 (**f**) to H-5 (**g**) and H-1 (**g**) to H-2 (**f**) indicated that residue **g** is linked to O-2 of residue **f** (20). The observed ^{13}C NMR chemical shift values of the C-1 signals 175.33 ppm (**i**), 174.870 ppm (**h**), and ^{1}H NMR data were consistent with the fact that both KDO residues have the α-configuration (21, 22). The HMQC-TOCSY spectrum of **3** indicated that C-4 and C-5 of one of the KDO residues (**h**) experienced significant deshielding (71.16 and 74.2 ppm, respectively) (Table III), showing that this residue is linked at both O-4 and O-5. These and the ^{1}H NMR results are in accordance with those published for a similarly linked KDO residue (23). That the other KDO residue (**i**) was terminal was ascertained by the similarity of its ^{13}C and ^{1}H NMR chemical shifts (Tables II and III) with those previously reported for the pyranose form of unsubstituted 3-deoxy-D-*manno*-octulosonate (22, 24). Thus, it can be concluded that the KDO region of *N. meningitidis* L3 LPS consists of two KDO residues. One residue (**i**) is terminal, and the second residue (**h**), which is substituted by heptose (**e**) to O-5 and by KDO (**i**) to O-4, forms the link between the core oligosaccharide to the lipid A backbone. The ^{13}C NMR chemical shifts of residues **j** and **k** were in accordance with those previously published for 6-*O*-substituted β-pyranosidic glucosamine and 6-*O*-substituted glucosaminitol (25). This data also indicates that the core is bound to the primary hydroxyl group (O-6) of the β-D-(1→6)-linked glucosamine disaccharide of the lipid A backbone (18).

Residues **a**, **b**, and **c** were not present in oligosaccharides **4** (Tables II and III). Deletion of these residues was not accomplished either following acetic acid treatment (preparation of oligosaccharide **1**) or after strong hydrazinolysis of the de-*O*-acylated LPS used in the preparation of oligosaccharide **5** (see below); therefore, these residues must have been lost during the dephosphorylation of LPS-OH using aqueous HF. Selective one-dimensional TOCSY experiments were used in order to assign chemical shifts for the protons of residues **e**, **f**, **g** and KDO residues **h** and **i** (Fig. 4). To assign H6**h** and H6**i** the one-dimensional relayed TOCSY experiment was employed (Fig. 4, *f* and *g*).

For determination of the sialic acid location, the de-*O*-acylated LPS (LPS-OH) was reduced and de-*N*-acylated. Gel filtration on Bio-Gel P2 yielded one major oligosaccharide, which, after re-*N*-acetylation, gave a signal of chemical shift σ 2.819 ppm in its ^{1}H NMR spectrum. This signal is characteristic for H-3eq of sialic acid indicating its presence in this oligosaccharide. Assignment of chemical shifts in **5** was done by two-dimensional TOCSY and COSY experiments. The results are shown in Table IV and indicate deshielding of H-3 of α-D-galactose (residue **a**) from σ 3.68 ppm in **1** to δ 4.14 ppm in **5**, indicating 2,3-sialylation of terminal galactose (**a**).

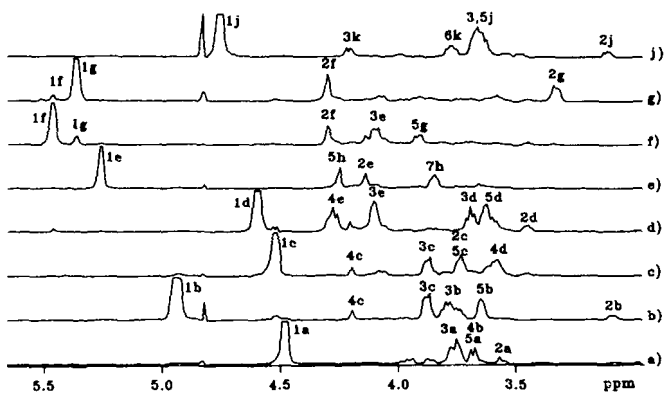

FIG. 3. **Cross-sections from a two-dimensional NOE spectrum of oligosaccharide 3 showing the NOEs for the anomeric protons of residues a–g and j.** The mixing time was set to 600 ms, and the spectrum was measured at 295K in D_2O (δ 4.81 ppm, HOD).

TABLE IV

Comparison of ^{1}H NMR chemical shift data obtained from the sialylated N. meningitidis serotype L3 LPS **5**, *acetic acid released core oligosaccharide* **1**, *and synthetic oligosaccharide* α-D-*NeuAc(2→3)*β-D-*Galp(1→4)*β-D-*GlcpNAc-OCH*$_3$ **6**

Sugar unit	Proton	Chemical shifts[a]		
		5	**1**	**6**
α-D-NeuAc(2→	3ax	1.83		1.79
	3eq	2.82		2.75
→3)β-D-Galp(1→	1	4.57	4.52	4.54
(**a**)	2	3.63	3.59	3.56
	3	4.14	3.70	4.11
	4	4.02	3.98	4.00
→4)β-D-Glc*p*NAc(1→	1	4.79	4.81	4.45
(**b**)	2	3.83	3.82	3.73
	3	3.78	3.78	
	4	3.75	3.75	

[a] Obtained from Ref. 27.

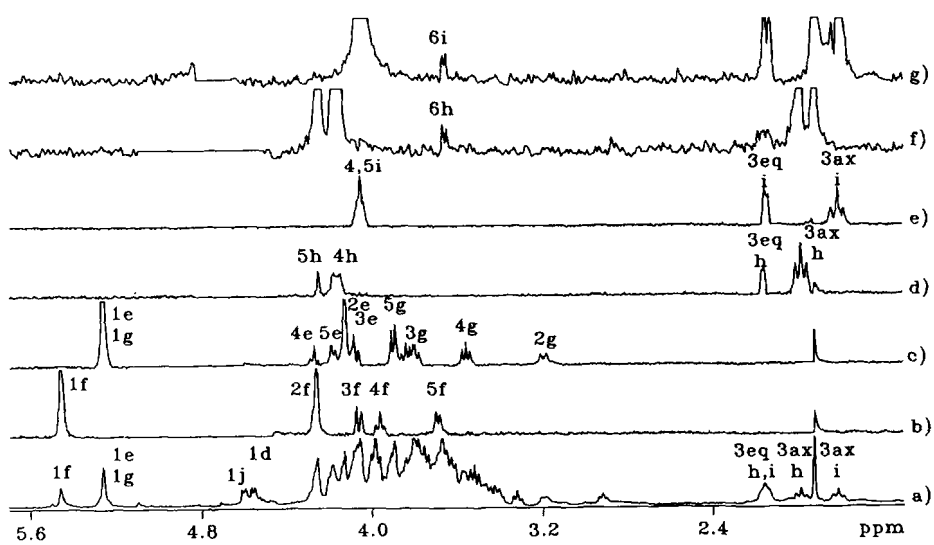

FIG. 4. **Proton spectrum (*trace a*), one-dimensional TOCSY (*traces b–e*), and one-dimensional relayed TOCSY (*traces f* and *g*) spectra of 4 after selective irradiation of H-1 (f) (*trace b*) H-1(e,g) (*trace c*), H-3ax (h) (*trace d*), H-3ax(i) (*trace e*), H-3ax(h) (*trace f*), and H-3ax(i) (*trace g*), respectively.** A half-Gaussian pulse was used in all experiments; spin-lock time was 270 ms (*traces b* and *c*) and 110 ms (*traces d–g*), respectively. Final spin-echo periods of one-dimensional relayed TOCSY experiments (*traces f* and *g*) were set to 120 ms.

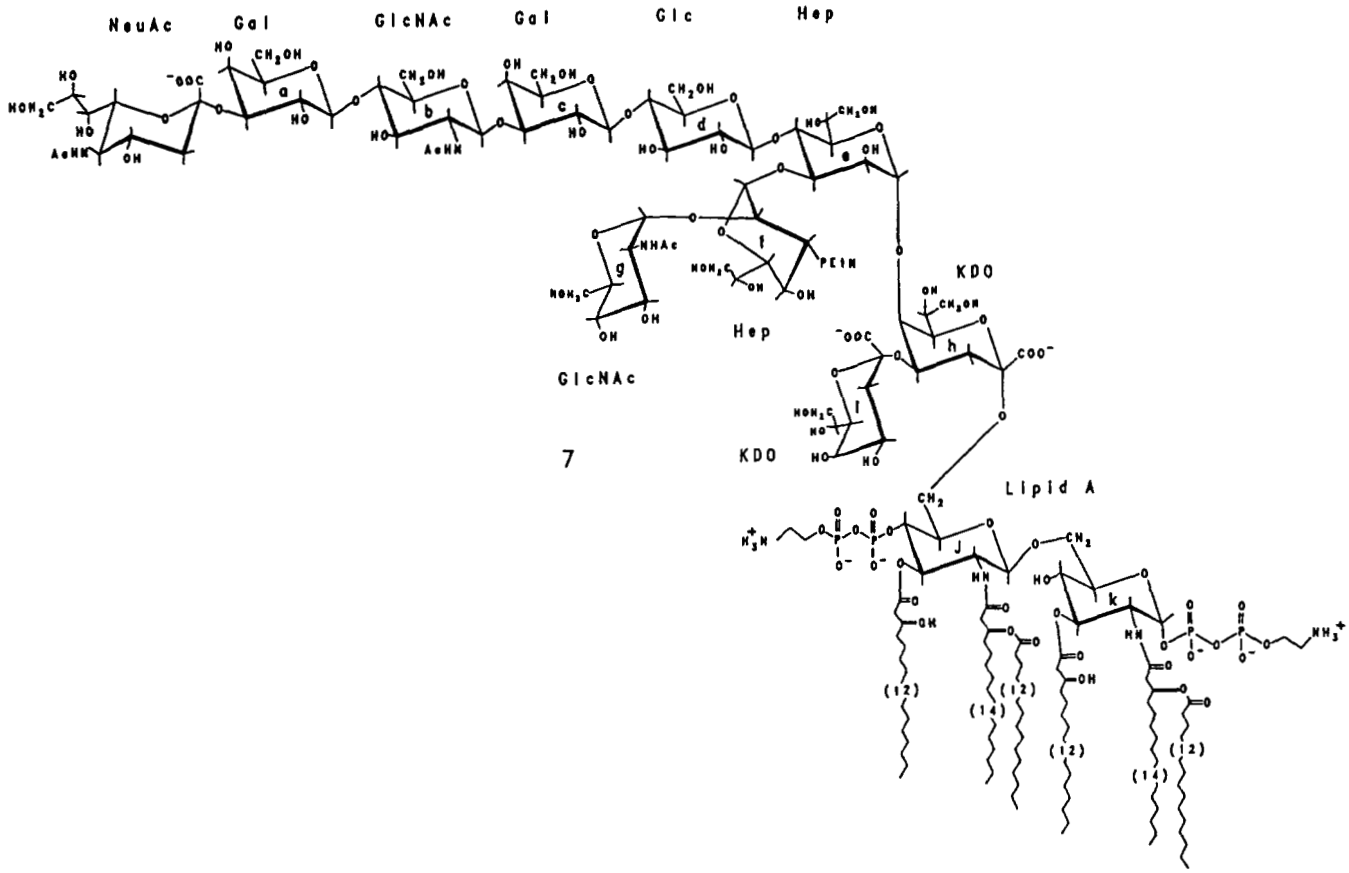

FIG. 5. **Structure of the native sialylated L3 LPS of *N. meningitidis*.**

Large deshielding of aglyconic hydrogen (H-3 of terminal galactose **a**) is characteristic for 2,3-sialosides (26). Chemical shifts for H-3ax (σ 1.784 ppm, $J_{3ax,3eq}$ = 12.2 Hz) and H3eq (σ 2.78 ppm, $J_{3eq,4}$ = 4.3 Hz) were similar to the data found in synthetic 2,3-sialosides (27). From this result it can be deduced that sialic acid is 2,3-linked to terminal galactose in meningococcal L3 LPS.

Using the above structural data, a complete structure (**7**) of the sialylated meningococcal L3 LPS could be proposed (shown in Fig. 5). It was also possible to include the lipid A portion in **7** because the meningococcal lipid A structure was recently elucidated by Kulshin *et al.* (18). We have demonstrated that sialylation of the L3 LPS occurs at O-3 of the terminal galactopyranosyl residue of its lacto-*N*-neotetraose antenna. Lacto-*N*-neotetraose has also been identified in the L2 (9), L4[2], L5 (7), L7[2], and L8[2] immunotypes, and evidence that all these LPS can be sialylated has been reported (9) on the basis of SDS-gel electrophoresis studies. Lacto-*N*-neotetraose is also found in a variety of glycoproteins and glycolipids associated with human tissue (28, 29); because of this, it is possible to hypothesize that structural mimicry could be a virulence factor for meningococci. Interestingly this mimicry with human tissue is also perpetuated even after the addition of the α(2–3)-linked-sialic acid residue to the lacto-*N*-neotetraose antenna, because it then constitutes a structure manifested by the S1 and S3 glycolipids of human granulocytes (30). Because many of the meningococcal LPS immunotypes contain the above structures, the ability to immunotype then must be due to structural differences in their inner cores (31). One important structural feature that has been identified in immunotype designation is the presence or absence of phosphorylethanolamine substituents and on differing locations of this substituent when it is present (8, 31). We have now determined that the L3 immunotype is based on the location of phosphorylethanolamine at O-3 of the heptose penultimate to the terminal 2-acetamido-2-deoxy-α-D-glucopyranosyl residue (Fig. 5).

Acknowledgment—We thank Fred Cooper for the GLC-MS analyses.

[2] H. J. Jennings and F. Michon, unpublished observations.

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