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Structural studies of the saccharide part of the cell envelope lipopolysaccharide from *Haemophilus influenzae* strain AH1-3 (*lic3* +) *

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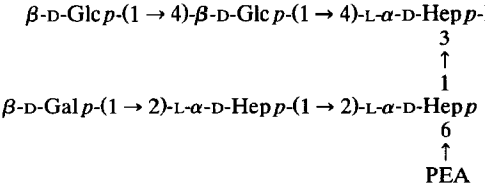
ABSTRACT

The structure of the saccharide part of the lipopolysaccharide from *Haemophilus influenzae* strain AH1-3 (*lic3* +) has been investigated. The saccharide was obtained from the lipopolysaccharide by mild acid hydrolysis followed by high-performance anion-exchange chromatography, and isolated fractions were studied by methylation analysis, NMR spectroscopy, and FAB mass spectrometry. The major saccharide is a heptasaccharide with the following structure,



in which Kdo is 3-deoxy-D-*manno*-oct-2-ulosonic acid and PEA is 2-aminoethyl phosphate. Hep is identified as L-*glycero*-D-*manno*-heptose. The absolute configuration of the phosphorylated heptose is tentative only.

INTRODUCTION

Haemophilus influenzae lacks structures equivalent to the polysaccharide chain built by repeating oligosaccharide units in enterobacterial O-antigenic lipopolysaccharide (LPS) and can be described as rough. In the *H. influenzae* lipopolysaccharide, lipid A is joined via 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) to a heterogeneous polymer of neutral sugars, including heptose, glucose, and galactose[1]. At

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least three chromosomal loci (*lic1, lic2*, and *lic3*) are involved in the biosynthesis of the saccharide part of the LPS[2]. Phase variation is observed for *H. influenzae*, which means that the expression of LPS antigenic structures can vary reversibly from generation to generation, making elucidation of fine structural details difficult[3].

The mutant AH1-3 of *H. influenzae* type b has deletions in *lic1* and *lic2*, and expresses only *lic3*. The structure of the saccharide part of its lipopolysaccharide has been investigated.

RESULTS AND DISCUSSION

LPS was obtained by extraction of the bacteria as described by Galanos et al.[4], followed by precipitation from the phenol phase with diethyl ether–acetone[5], and was transformed into saccharides by hydrolysis with acid under mild conditions (1% acetic acid, 100°C, 2 h) followed by reduction with sodium borohydride. The saccharides were applied to a high-performance anion-exchange liquid-chromatography system (DIONEX). The heterogeneity of the saccharide fraction was evident (Fig. 1A). Two fractions, I and II, representing 42 and 36% of the total amount of saccharides, were isolated as major products and rechromatographed. The rechromatography profile of I is shown in Fig. 1B. Oligosaccharides OS-1 and OS-3 were

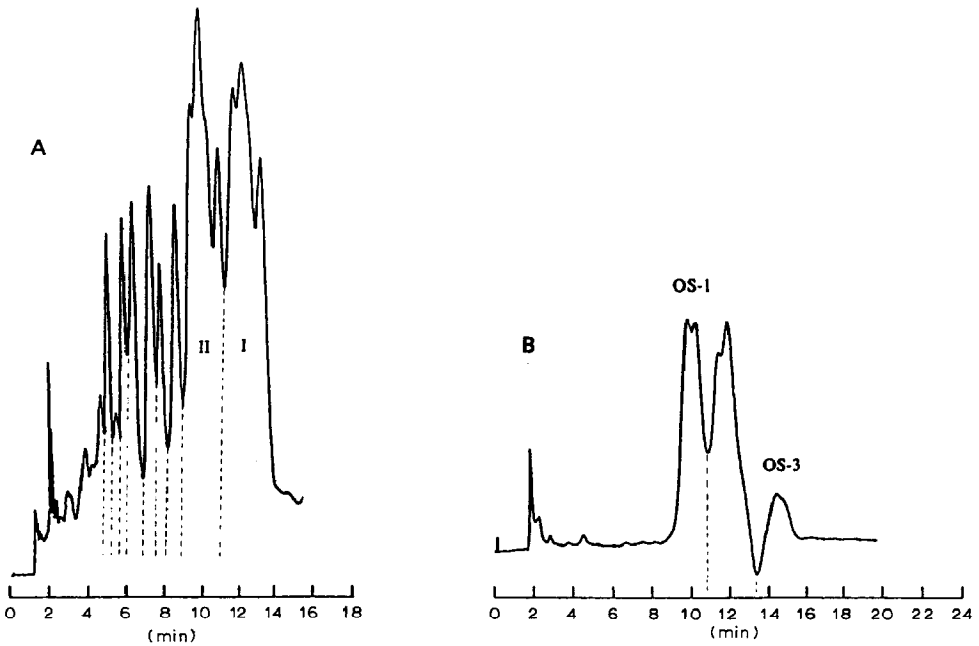


Fig. 1. (A) Elution profile (DIONEX) of the saccharides obtained from LPS after mild acid hydrolysis. (B) Rechromatography profile of fraction I obtained in A.

TABLE I

Methylation analyses of oligosaccharide samples

Sugar [a]	t_R [b]	Mol%		
		OS-1	OS-2	OS-3
2,3,4,6-Glc	1.00	31	31	49
2,3,4,6-Gal	1.05	27	24	15
2,3,6-Glc	1.29	15	4	11
3,4,6,7-Hep	1.83	16	24	5
2,6,7-Hep	1.98	11	17	3
2,3,4,6,7-Hep	1.54			16

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. [b] Retention time of the corresponding alditol acetate at 160°C (2 min) → 220°C at 2°C/min, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a DB-1 column.

isolated from I, and OS-2 resulted from II. The yields of OS-1, OS-2, and OS-3 were 15, 6, and 3%, respectively, of the total saccharides.

All fractions contained Kdo (evident by NMR data) and phosphate. Sugar analysis of OS-1 showed D-glucose, D-galactose, and L-*glycero*-D-*manno*-heptose in the relative proportions 2:1:2. The absolute configurations of the sugars were determined by the method devised by Gerwig et al.[6]. Methylation analysis of this sample gave terminal D-glucose, terminal D-galactose, 4-linked D-glucose, 3,4-linked L,D-heptose, and 2-linked L,D-heptose (Table I).

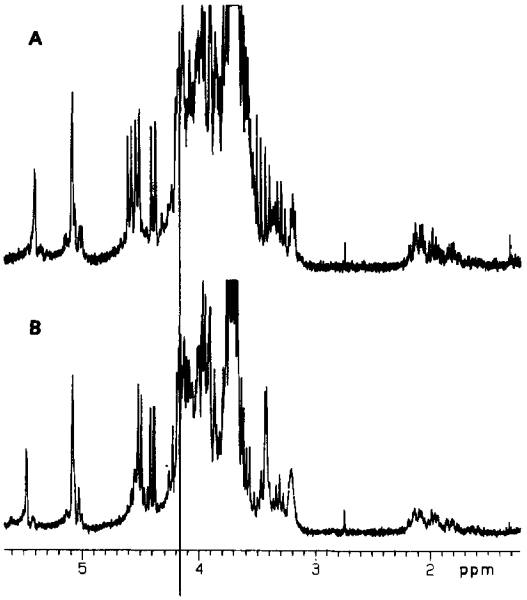


Fig. 2. 270-MHz ^{1}H NMR spectra of OS-1 (A) and OS-2 (B) run at 85°C.

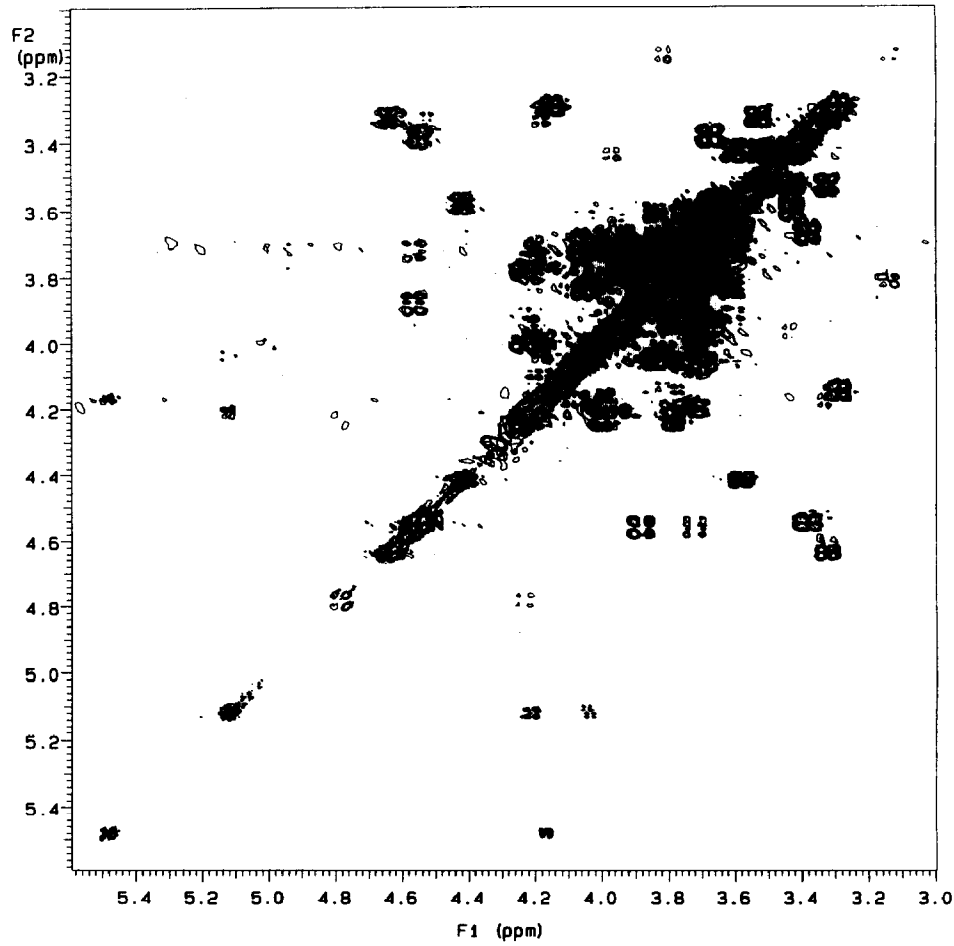


Fig. 3. Part of the DQF-COSY spectrum of OS-1 at 70°C.

The ^{1}H NMR spectra of OS-1 (Figs. 2A and 3) reflected the sample microheterogeneity observed by chromatographic methods (Fig. 1B). ^{1}H NMR chemical shifts are given in Table II. In the low-field region, signals were observed at δ 5.49 (1 H, J 2.0 Hz), 5.13 (1 H, J 2.0 Hz), 5.13 (0.5 H, J 1.60 Hz), 5.11 (0.5 H, J 1.60 Hz), 4.64 (1 H, J 7.9 Hz), 4.57 (1 H), 4.56 (1 H, J 7.9), and 4.43 (1 H, J 7.60 Hz). Major signals for deoxy protons derived from Kdo were found in the high-field region at δ 2.12, 2.00, 2.17, and 1.85, which implied that the Kdo moiety existed in different forms. This was in agreement with results from FABMS on native material (discussed below), which indicated that the Kdo residue had been transformed into a mixture of anhydro-Kdo units in the course of the mild acid hydrolysis. The anomeric resonances at δ 5.11 and 5.13, each corresponding to 0.5 H and coupling to δ 4.07 and 4.05, respectively, accounted for one anomeric proton which was noticeably affected by the microheterogeneity. Cross-peaks

TABLE II

^{1}H NMR chemical shifts [a] of OS-1 and OS-2 at 70°C

Sugar residue		Chemical shift (δ)								
		H-1	H-2	H-3	H-4	H-5	H-6	H-6′	H-7	H-7′
β-D-Glc*p*-(1 →	I	4.64	3.34	3.54	3.44	3.61	3.74	3.92		
		4.52	3.31	3.47	3.47	3.70	3.83	3.83		
→ 4)-β-D-Glc*p*-(1 →	II	4.56	3.40	3.68	3.68	3.63	3.83	4.04		
β-D-Gal*p*-(1 →	III	4.43	3.59	3.68	3.88	3.82				
		4.42	3.57	3.63	3.92					
→ 2)-L-α-D-Hep*p*-(1 →	IV	5.49	4.18	4.01	4.01	3.73	4.57		3.90	3.90
		5.52	4.16	3.95	3.95	3.67				
→ 2)-L-α-D-Hep*p*-(1 →	V	5.13	4.22	3.93	3.97	3.91				
		5.12	4.18	3.92						
→ 3/4)-L-α-D-Hep*p*-(1 →	VI [b]			4.18	4.21	3.77				
		5.10	4.00							
PEA		3.30	4.16							
		3.26	4.16							
Kdo [c]				2.12	4.16					
				2.00						
				2.17						
				1.85						

[a] Chemical shifts are given in ppm relative to internal sodium 3-trimethylsilylpropanoate-d_4 (0.00 ppm); PEA, 2-aminoethyl phosphate residue. The top row shows the chemical shifts of OS-1 and the second row shows chemical shifts of OS-2. [b] H-1 and H-2 of VI are split into two signals, respectively, evident from DQF-COSY run in a phase-sensitive mode. H-1 resonates at δ 5.11/5.13 and H-2 at δ 4.07/4.05, respectively. [c] Chemical shifts for Kdo are only given for OS-1.

obtained in a COSY spectrum from the signal at δ 4.56 to 3.90 and 3.73 showed complex coupling patterns due to an additional coupling (J not resolved) which indicated this proton to be a proton with a vicinal phosphate group and not an anomeric proton. A broad signal at δ 3.30 with a cross-peak at δ 4.16 indicated that the phosphate was substituted with ethanolamine. 2-Aminoethyl phosphate is found, *inter alia*, in glycosphingolipids[7] and the chemical shifts recorded for the signals from this substituent are in agreement with published values. The ^{13}C NMR spectrum of OS-1 contained, *inter alia*, signals for anomeric carbons at δ 103.6 ($J_{C,H}$ 159 Hz), 103.4 ($J_{C,H}$ 161 Hz), 103.3 ($J_{C,H}$ 161 Hz), 100.8 ($J_{C,H}$ 177 Hz), 100.4 ($J_{C,H}$ 173 Hz), and 99.2 ($J_{C,H}$ 173 Hz). The three signals at $\delta \sim 103$ correspond to β-linked hexoses. The remaining signals are thus from the heptoses which are α-linked. The FAB mass spectrum (run in the negative mode) of underivatised OS-1 showed main signals at m/z 1428 and 1406. The molecular weight of a heptasaccharide containing 3 hexoses, 3 heptoses, reduced Kdo, and 2-aminoethyl phosphate is 1425. It is suggested that the signals at m/z 1428 and 1406 correspond to $(M - 18 - 2 + Na)^-$ and $(M - 18 - 1)^-$, respectively. Kdo-con-

taining compounds dehydrate only to a minor degree due to lactonisation[8]. Auzanneau et al.[9] have reported on the production of anhydro-Kdo moieties, as artifacts of the mild hydrolysis procedure, by β-elimination of a phosphate substituent from C-4 of Kdo. The existence of anhydro-Kdo in OS-1 as a result of delipidation is a reasonable explanation for the difference of 18 Da in mass between the expected pseudomolecular ions and the observed ions. It is concluded that the microheterogeneity of the sample is mostly due to the heterogeneity of the Kdo residue obtained after acid hydrolysis.

It is evident that a heptose is substituted with the 2-aminoethyl phosphate group since the ratio in the sugar analysis gave only 2 heptoses versus 3 hexoses. The amount of 1,2,5-tri-*O*-acetyl-3,4,6,7-tetra-*O*-methylheptitol compared with the amount of 1,3,4,5-tetra-*O*-acetyl-2,6,7-tri-*O*-methylheptitol in the methylation analysis is 1.45:1.00, indicating that the phosphorylated heptose residue is 2-linked. The possibility of a 3-linkage was ruled out since no significant *m/z* 117 was detected in the mass spectrum of the methylated derivative. Results from methylation analysis of dephosphorylated material confirmed the existence of two 2-linked heptoses.

Sugar analysis of OS-2 gave D-glucose, D-galactose, and L-*glycero*-D-*manno*-heptose in the relative proportions 1:1:2. The ^{1}H NMR spectrum of OS-2 is shown in Fig. 2B. ^{1}H NMR chemical shift data are given in Table II. In the low-field region, signals are observed at δ 5.52 (1 H, *J* 2.0 Hz), 5.10 (2 H, *J* not resolved), 4.54 (1 H), 4.52 (1 H, *J* 7.6 Hz), and 4.42 (1 H, *J* 7.2 Hz). The signal at δ 4.54 showed complex coupling patterns (*J* not resolved) in cross-peaks to δ 3.80 and 3.67, and was thus considered to be for a non-anomeric proton as observed for OS-1. Signals for Kdo and 2-aminoethyl phosphate were also present. These data indicated that the material was composed of 2 hexoses and 3 heptoses. Methylation analysis of OS-2 gave terminal D-glucose, terminal D-galactose, 2-linked L,D-heptose, and 3,4-linked L,D-heptose (Table I). With the assumption that OS-2 is an incomplete form of OS-1, the existence of **1** as a part of OS-1 was indicated.

β-D-Glc*p*-(1 → 4)-β-D-Glc*p*

1

Methylation analysis of OS-3 showed relatively less terminal galactose but the appearance of terminal heptose, giving evidence for **2**.

Two ROESY experiments with mixing times of 150 and 250 ms (Fig. 4) resulted in information about most interresidue ROEs, and this is summarised in Table III. From the data on ROEs and the information of the linkage positions, the presence of the disaccharide elements **1**–**3** was evident.

β-D-Gal*p*-(1 → 2)-L-α-D-Hep*p* β-D-Glc*p*-(1 → 4)-L-α-D-Hep*p*

2 **3**

Overlapping signals of V and VI in the ROE spectrum and the fact that all

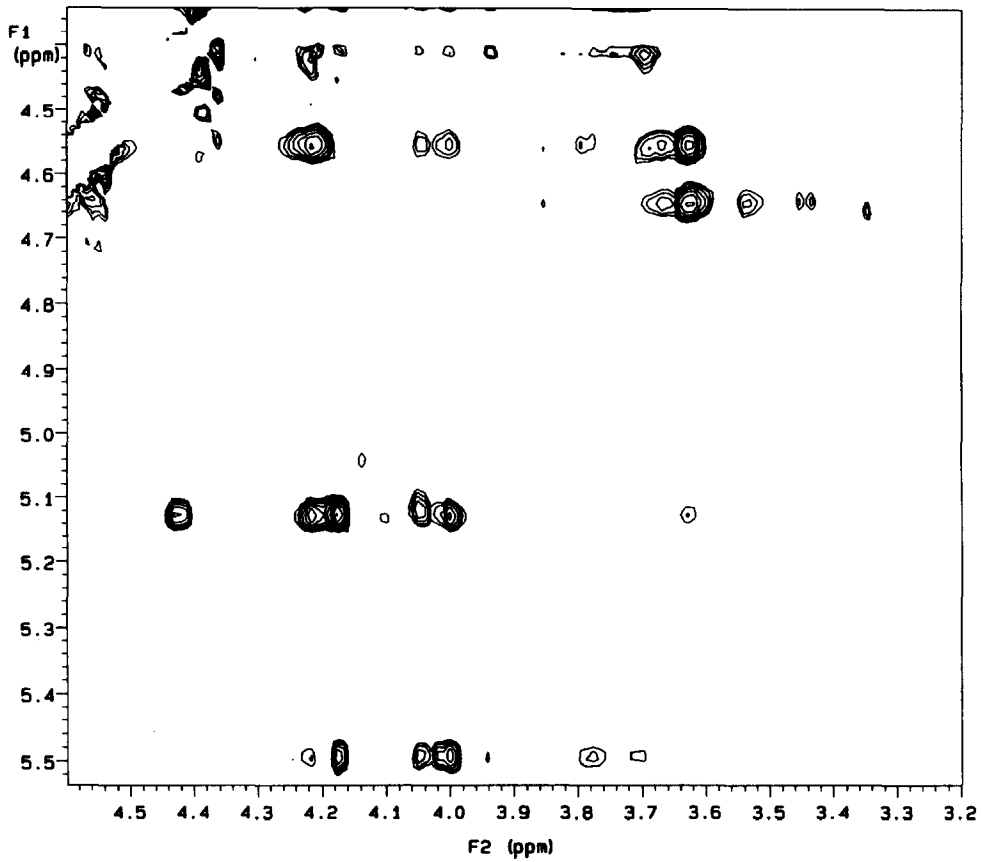


Fig. 4. Part of the 2D-ROESY spectrum of OS-1 (mixing time, 250 ms) run at 70°C.

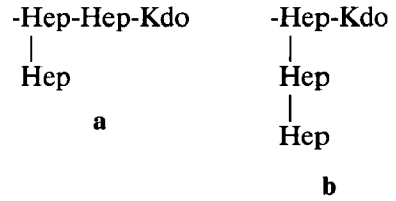
TABLE III

Observed ROE contacts from anomeric protons of OS-1

Anomeric proton	ROE contacts to [a]
4.64 (t-Glc, I)	3.54 (Glc, I, H-3), 3.61 (Glc, I, H-5), 3.68 (→ 4-Glc, II, H-4)
4.56 (→ 4-Glc, II)	3.63 (→ 4-Glc, II, H-5), 3.68 (Glc, II, H-3), 4.21 (→ 3/4-Hep, H-4, VI), 4.01 (n.r.), 4.05 (n.r.)
4.43 (t-Gal, III)	3.68 (Gal, III, H-3), 3.82 (Gal, III, H-5), 4.22 (→ 2-Hep, V, H-2), 5.13 (→ 2-Hep), V, H-1)
5.49 (→ 2-Hep, IV)	4.18 (→ 2-Hep, IV, H-2), 4.01 (n.r.) 4.05 (n.r.), 5.13 (→ 2-Hep, V, H-1)
5.13 (→ 2-Hep, V)	4.22 (→ 2-Hep, V, H-2), 4.43 (Gal, III, H-1), 4.18 (n.r.), 4.01 (n.r.)
5.11/5.13 (→ 3/4-Hep, VI)	4.05/4.07 (→ 3/4-Hep, VI, H-2), 4.18 (n.r.)

[a] n.r., Not rationalised.

resonances for the Kdo residue could not be determined allowed two possibilities, **a** and **b**, for arrangement of the heptose residues.



An ROE signal due to spin diffusion (data not shown) was observed for the anomeric proton of residue IV to the anomeric proton of residue V. Cross-peaks in 2D-NOE experiments, due to spin diffusion from one anomeric proton to the anomeric proton of an adjacent residue, have been described by Jansson et al.[10] and indicate in the OS-1 saccharide the existence of structure element **4**, which favours arrangement **b** for the heptoses. That all heptose residues are α-linked was confirmed by the existence of ROEs from their anomeric protons to the respective H-2 resonances. All hexoses showed ROEs to their H-3 and H-5 resonances, which is expected for β-linked residues.

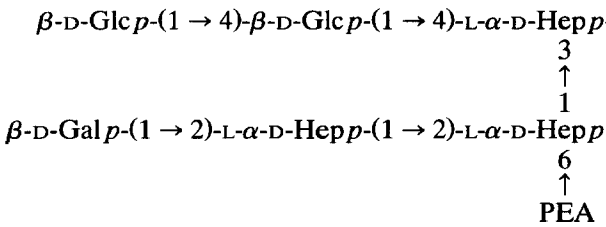
→ 2)-L-α-D-Hep*p*-(1 → 2)-L-α-D-Hep*p*

4

The 2-aminoethyl phosphate group is located at O-6 of residue IV.

The FAB mass spectra (run in a positive mode) of methylated OS-1 and OS-2 showed main signals in the corresponding molecular ion region which could not be rationalised, probably due to the complicated nature of the Kdo residue. However, a major fragment ion was found at *m/z* 867. Upon deuteriomethylation, this ion was shifted to *m/z* 909, which is in agreement with the addition of 14 deuteriomethyl groups. The stability of *i.a.* 2-aminoethyl phosphate to permethylation protocols has been reported[8], and we suggest that *m/z* 867 is consistent with Gal–Hep–(PEA)Hep$^+$.

From the combined data presented above, **5** is proposed to be part of the structure for the lipopolysaccharide oligosaccharide from mutant AH1-3 of *Haemophilus influenzae* type b.



5

To our knowledge, this is the third reported structure of a *H. influenzae* lipopolysaccharide[11,12].

The mutant strain expresses only the *lic3* locus, which has facilitated and made the structural analysis possible. However, the saccharide part of the AH1-3 lipopolysaccharide still showed considerable heterogeneity (Fig. 1). The major fraction OS-1 has the structure given above, whereas fraction OS-2 lacks the β-(1 → 4)-linked D-glucose residue and fraction OS-3 the β-(1 → 2)-linked D-galactose residue.

Fractions OS-1, OS-2, and OS-3 accounted for ca. 25% of the saccharides, and were the only ones which gave enough material for structural studies. The heterogeneity pattern is reproducible and has been found in all four batch cultures of *H. influenzae* AH1-3 which we have studied.

The *H. influenzae* AH1-3 lipopolysaccharide differs from those of *Escherichia coli*[13], *Salmonella*[13], *Shigella*[13], *Neisseria meningitidis*[14,15], and *N. gonorrheae*[16] in having a L-α-D-Hep*p*-(1 → 2)-L-α-D-Hep*p* disaccharide. This heptose disaccharide has been found in the lipopolysaccharide of *Aeromonas hydrophila*[17–19]. A monoclonal antibody, MAHI 3, elicited by immunisation with *H. influenzae* RM 7004 (the parent strain of AH1-3), bound to the *H. influenzae* AH1-3 as well as *A. hydrophila* chemotype I, II, and III lipopolysaccharide, but to none of a series of *E. coli, Salmonella*, and *Shigella* lipopolysaccharides extracted from rough mutants of various chemotypes (unpublished results). Available evidence suggests that the L-α-D-Hep*p*-(1 → 2)-L-α-D-Hep*p* disaccharide is an essential part of the epitope defining the specificity of this monoclonal antibody. The structure of the LPS of *H. influenzae* nontypable strain 2019 was recently published[12], while our study was near completion. The heptose region α-Hep-(1 → 2)-α-Hep-(1 → 3)-α-Hep-(1 → 5)-Kdo appears to be identical in *H. influenzae* strains AH1-3 and 2019. However, the hexose regions differ. Whereas strain AH1-3 has a cellobiose unit, that of strain 2019 has a lactose disaccharide. In addition, strain AH1-3 has a β-(1 → 2)-linked D-galactose. This suggests that the frequent phase variation observed in *H. influenzae* may be a result of variation in the hexose part.

EXPERIMENTAL

General methods.—Concentrations were performed under diminished pressure at 35°C (bath) or at room temperature flushing with N_2. For GLC, a Hewlett–Packard 5890 instrument fitted with a flame-ionisation detector was used. Separations of alditol acetates and partially methylated alditol acetates were performed on DB-5 and DB-1 fused-silica capillary columns, respectively, using a temperature gradient of 160°C (2 min) → 220°C at 2°C/min. GLC–MS was performed with a Hewlett–Packard 5890 gas chromatograph equipped with an electron-impact Hewlett–Packard 5970 mass spectrometer, using the above conditions. FAB mass spectra were recorded on a NERMAG R10-10L quadrupole instrument and on a JEOL SX 102 instrument. Ions were produced from a matrix of triethanolamine for native oligosaccharides and from a matrix of thioglycerol for methylated material.

Methylation analysis was performed essentially as described[20]. Phosphorus was determined according to Chen et al.[21].

Bacteria and cultivation.—The double mutant RM7004-XP1-AH1-3 has deletions in *lic1* and *lic2* loci containing genes required for the expression of several variable saccharide structures defined by monoclonal antibodies 6A2, 12D9 (*lic1*), and 5G8 4C4 (*lic2*)[22,23]. Bacteria were cultivated to late logarithmic phase in brain–heart infusion broth (Difco) supplemented with yeast extract (10g/L, Difco), hemin (10 mg/L, Sigma), and nicotinamide adenine dinucleotide (10 mg/L, Sigma). Cultivation was done at constant pH 7.0 with aeration at 37°C in a 35-L fermentor (Belach Bioteknik AB, Stockholm, Sweden). The bacteria were harvested with a Sorvall RC-5B centrifuge at 4°C and 9000*g* for 30 min, washed with deionised water, and lyophilised.

Preparation of LPS.—LPS was extracted from lyophilised bacteria as described by Galanos et al.[4], with the modification of using 6 vol of 1:5 diethyl ether–acetone instead of water to 1 vol of the phenol solution[5].

Separation of oligosaccharides.—Oligosaccharides were obtained after mild hydrolysis (1% acetic acid, 100°C, 2 h) of the LPS. They were separated on a high-performance anion-exchange chromatography system (DIONEX, Sunnyvale, CA) consisting of a quaternary gradient pump and a pulsed amperometric detector with a gold electrode. A CarboPac PA 1 column (9 × 250 mm) was used for the carbohydrate separation. Samples were eluted with 2 eluents: *A* (100 mM NaOH) and *B* (100 mM NaOH–500 mM NaOAc). The gradient used was 35 → 50% *B* in 30 min, and the flow rate was 5 mL/min. Subfractionation was performed using eluent *B* and water in the ratio 18/82.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 70 and 85°C. Chemical shifts are reported in ppm, using internal sodium 3-trimethylsilylpropanoate-d_4 (δ 0.00, ^1H) and external 1,4-dioxane (δ 67.4, ^{13}C) as references. COSY and 1D-HOHAHA experiments were performed on a JEOL GX-270 instrument with standard JEOL pulse sequences. The mixing times in the 1D-HOHAHA varied from 20 to 300 ms. The DQF-COSY, 2D-HOHAHA, and 2D-ROESY experiments were performed on a Varian Unity-500 spectrometer operating at 500 MHz with mixing times of 80 ms (HOHAHA) and 150/250 ms (ROESY). C–H Coupling constants were established using the INEPT experiment.

NMR assignments.—Chemical shifts observed for the signals of H-1 to H-7′ of the sugar residues in OS-1 and OS-2 are given in Table II. The H-1 signals of the heptoses were readily recognised by their small H-1–H-2 coupling constants, and the remaining H-1 signals thus belong to the hexoses which were β-linked due to their large coupling constants.

Assignments for OS-1 were made as follows: the COSY spectrum gave the chemical shifts of the H-2 signals. All proton signals of residue I could be assigned by 1D-HOHAHA and were in agreement with resonances found for terminal glucose. H-4 of the galactose was recognised both in 1D- and 2D-HOHAHA by its typical coupling pattern; H-1 to H-3 resonances for the galactose residue were

assigned by these experiments. The H-5 resonance was identified in the ROESY spectrum. The remaining H-1 signal for a hexose thus belonged to the 4-linked glucose. The assignments of its H-1 to H-6 resonances were obtained by combining results from 1D- and 2D-HOHAHA experiments. The signals of H-1 to H-5 for residue IV were identified in the 1D- and 2D-HOHAHA spectra and their assignments were obtained by combining results from the different experiments. A cross-peak in the COSY spectrum from the H-5 signal to δ 4.57 gave the chemical shift of H-6 which had a cross-peak to δ 3.90, the chemical shift of H-7 of this residue. Signals for H-1 to H-5 for residue V are found in the 2D-HOHAHA spectrum and their assignment was done by combining the results from all NMR experiments. No separate signals of residue VI, except for H-2, are found in the 2D-HOHAHA spectrum, indicating overlapping signals with residue V. H-3 of VI was identified by a cross-peak, observed in both DQF-COSY and 2D-HOHAHA, from the H-2 signal to δ 4.18. Since we observed a signal at δ 3.78 of this residue in the 1D-HOHAHA spectrum, which is H-5, we could identify H-4 of VI by a cross peak from the H-5 signal to δ 4.21. Assignments for OS-2 were done in the same way as described for OS-1.

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