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STRUCTURE OF THE O-ANTIGEN POLYSACCHARIDE OF *Haemophilus pleuropneumoniae* SEROTYPE 3 (ATCC 27090) LIPOPOLYSACCHARIDE*†

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ABSTRACT

The structure of the O-antigen polysaccharide of *Haemophilus pleuropneumoniae* serotype 3 (ATCC 27090) S-type lipopolysaccharide was investigated by methylation analysis, partial hydrolysis, periodate oxidation, and ^{1}H- and ^{13}C-n.m.r. spectroscopy, and concluded to be composed of linear pentasaccharide repeating-units having the structure:

$$\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow)_n.$$

INTRODUCTION

The Gram-negative bacterium *Haemophilus pleuropneumoniae* is a major cause of pleuropneumonia in pigs. The organism is encapsulated and ten serotypes, based on the capsular antigens, have been identified[1–3]. The endotoxins are implicated in the pathogenesis of porcine pleuropneumonia[4] and the serologic cross-reactive immunodeterminants are located in the LPS[5].

Recently, the structures of the O-specific polysaccharides of the S-form LPS from *H. pleuropneumoniae* serotypes 1 and 2 have been determined[6,7]. In seeking to understand the immunobiology of *H. pleuropneumoniae* infections in pigs and explain the serology on a structural basis, we now report the structure of the O-specific polysaccharide of *H. pleuropneumoniae* serotype 3 S-type LPS.

EXPERIMENTAL

Bacterial culture. — *Haemophilus pleuropneumoniae* serotype 3 (ATCC 27090), from the collection of the Western College of Veterinary Medicine (University of Saskatchewan, Saskatoon), was grown in Bacto PPLO broth w/o CV (Difco), supplemented with NAD, D-glucose, and horse serum, in a 28-L fermenter

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(New Brunswick Scientific) at 36° ±1° overnight (~18 h). The cells were killed with 0.75% phenol (final concentration) prior to harvesting using a Sharples continuous centrifuge (yield, ~3 g wet-weight/L).

Isolation of the lipopolysaccharide and preparation and purification of the O-polysaccharide. — Saline-washed cells of *H. pleuropneumoniae* serotype 3 were extracted by the lysozyme phenol–water method[8]. LPS was recovered from the dialyzed, separated phenol and aqueous layers by repeated ultracentrifugation at 105,000*g* (18 h at 4°) and were assessed to be pure by the carbocyanine dye assay[9].

In order to obtain the O-polysaccharide, a solution of LPS (477 mg) in aqueous 2% acetic acid (300 mL) was heated for 2 h at 100° and the precipitated "lipid A" was removed by low-speed centrifugation. The supernatant solution was lyophilized and a solution of the residue in 0.05M pyridinium acetate (pH 4.7) was eluted from a column (2 × 100 cm) of Sephadex G-50 (Pharmacia) using the same buffer. Fractions (10 mL) were collected and analyzed colorimetrically for aldose[10], aminodeoxyglycose[11], phosphate[12], and 3-deoxyoctulosonate[13]. The O-polysaccharide was purified by application to a column (1.2 × 35 cm) of DEAE-Sephacel (Pharmacia) equilibrated with 0.05M Tris–HCl buffer (pH 7.2), and elution (1-mL fractions) with the buffer (50 mL) followed by a 0→0.5M gradient of sodium chloride in the same buffer.

Analytical methods. — Quantitative colorimetric methods used were the phenol–sulfuric method for glycoses[10], the modified Elson–Morgan method for 2-amino-2-deoxyglycoses[11], the method of Chen *et al.*[12] for phosphate, and the periodate oxidation–thiobarbituric acid method for 3-deoxyoctulosonate[13].

G.l.c. was done with a Hewlett–Packard model 5830A chromatograph fitted with a flame-ionization detector and a model 18850A electronic integrator, under the following conditions: capillary column (0.32 mm × 25 m), 007 series bonded phase, fused silica OV-17 (Quadrex Corp.); temperature programmes: *A*, 180° for 2 min then → 240° at 4°/min; *B*, 200° for 2 min then → 240° at 1°/min; *C*, a fused-silica capillary column (0.3 mm × 30 m) containing DBWAX (J&W Scientific, Inc.); temperature programme, 180° → 240° at 4°/min.

The carrier gas was dry nitrogen (30 mL/min) and retention times are quoted relative to those of D-glucitol hexa-acetate (T_{GA}) or 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM}). G.l.c.–m.s. was done using a Hewlett–Packard 5985B system, employing the conditions *A*–*C* and an ionization potential of 70 eV.

Glycoses were determined by g.l.c. (programme *A*) of their alditol acetate derivatives[14], using *myo*-inositol as the internal standard. Samples (0.5 mg) of oligo- and poly-saccharide were hydrolyzed in sealed glass tubes with 10M hydrochloric acid (1 mL) for 15 min at 90°, or with 2M acid (1 mL) for 17 h at 100°, followed by concentration to dryness.

The configuration of glycoses was established[15] by capillary g.l.c. of their acetylated (−)-2-butyl glycosides. Lipids were identified by g.l.c.–m.s. (programme *C*) of their methyl esters derived by sealed-tube methanolysis of samples (1 mg) with methanolic 3% hydrogen chloride for 4 h at 100° followed by neutralization

(Ag_2CO_3). T.l.c. was performed on Silica Gel 60 (Merck) with 1-propanol–conc. ammonia–water (6:3:1).

Gel filtration was performed on columns of Sephadex G-50 (2 × 100 cm) (Pharmacia) or Bio-Gel P-2 (200–400 mesh) (Bio-Rad Laboratories). The gel-filtration properties of the eluted materials are expressed in terms of their distribution coefficient K_{av}; $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of the specific material, V_o is the void volume of the system, and V_t is the total volume of the system.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). — Samples (1 μg) of LPS were analyzed in 14% polyacrylamide gels by electrophoresis in the presence of sodium dodecyl sulfate, and bands were detected by silver staining[14].

Methylation analyses. — Samples (1–2 mg) were methylated according to the Hakomori procedure[17] and the products were isolated by partition between chloroform and water. Methylation products were hydrolyzed with 10M hydrochloric acid (1 mL) for 15 min at 90° or with M trifluoroacetic acid (1 mL) for 16 h at 100°. Solutions of the released methylated glycoses in water (2 mL) were treated for 16 h with sodium borodeuteride (12 mg). Each solution was acidified with dilute acetic acid and concentrated to dryness, and methanol (5 × 10 mL) was distilled from the residue which was then acetylated with acetic anhydride (1 mL) for 2 h at 115°. The products were analyzed by g.l.c.–m.s. (programme *B*).

Partial hydrolysis. — A solution of the O-polysaccharide (29 mg) in cold aqueous 48% hydrofluoric acid (1 mL) was kept for 72 h at 4°, then concentrated to dryness in high vacuum over NaOH. The residue was neutralized with aqueous 5% ammonium hydroxide and lyophilized. The products were fractionated by gel filtration on a column of Bio-Gel P-2.

Periodate oxidation. — A solution of the polysaccharide (41 mg) in distilled water (6 mL) was treated with 0.1M sodium metaperiodate (6 mL) in the dark for 6 days at 4°. Excess of periodate was reduced by the addition of ethylene glycol (100 μL), and the oxidized polymer was reduced with sodium borohydride (40 mg). After 16 h at 22°, the cooled solution was neutralized with dilute acetic acid, dialyzed until salt free, and lyophilized.

Smith-type hydrolysis of the periodate-oxidized and reduced polymer was effected with aqueous 2% acetic acid for 2 h at 100°, and the degradation products were fractionated by gel filtration on a column of Bio-Gel P-2 (200–400 mesh).

N.m.r. spectroscopy. — Proton-decoupled ^{13}C-n.m.r. spectra (125 MHz) were recorded at 24° for a 25-kHz spectral width, using a $\pi/2$ pulse and a 32k data set on a Bruker AM 500 spectrometer. DEPT experiments[18] were performed for a 12.5-kHz spectral width, using a 3 $\pi/2$ proton pulse to distinguish between CH and CH_2 resonances. The delay between the pulses $(2J)^{-1}$ was set at 3.4 ms. Chemical shifts are expressed relative to internal acetone (1%, 31.07 p.p.m.).

^1H-N.m.r. spectra (500 MHz) were recorded at 24°, using a spectral width of 2.5 kHz, a $\pi/2$ pulse, and a 16k data set for a digital resolution of 0.3 Hz/point.

Chemical shifts are expressed relative to internal acetone (0.1%, 2.225 p.p.m.) and coupling constants are reported in Hz.

A sample of purified O-polysaccharide was passed through Chelex-100 (Bio-Rad) resin (3 mL) and then exchanged twice with D_2O (99.8%). Spectra were recorded on solutions (25 mg/mL; pD 9.0) in 99.8% D_2O (5-mm-diameter tubes).

Proton homonuclear-correlated 2D-n.m.r. experiments COSY[19], relay COSY[20], and *J*-resolved[21] were performed at 24°, using the standard software provided by Bruker (DISNMR). Quadrature detection in both dimensions was employed in the COSY experiments. The initial (t_1,t_2) matrices of 256 × 2048 data points were zero-filled to 1024 × 2048 data points in order to provide digital resolution of 1 Hz/point in both domains. Resolution enhancement in both dimensions was done by non-shifted sine bell functions prior to Fourier transformation. Magnitude spectra, symmetrized about the diagonal, were used to represent the data. The number of transients per FID was 8 for the COSY experiment.

The 2D *J*-resolved spectrum was obtained using an initial data matrix of 128 × 4096 points that was zero-filled to 256 × 4096 points with a digital resolution of 0.6 Hz per point in the second domain.

A heteronuclear ^{13}C–^1H shift correlation experiment was done on a Bruker AM 500 spectrometer using the CHORTLE (carbon–hydrogen correlations from one-dimensional polarization transfer spectra by least-squares analysis) technique[22]. The experiment was performed at 24° on a solution (70 mg/mL) of O-polysaccharide in D_2O. Four proton-evolution times of 0.24, 1.0, 2.4, and 3.2 ms were used with 4,000 transients per FID. Spin simulations were performed on an Aspect 3000 computer using the Bruker program PANIC. A line width of 2.0 Hz was used in all cases.

General methods. — Concentrations were made under reduced pressure and at <40°. Optical rotations were measured at 20° in 10-cm microtubes, using a Perkin–Elmer 243 polarimeter.

RESULTS AND DISCUSSION

Extraction of *Haemophilus pleuropneumoniae* serotype 3 cells (248 g, wet weight) by a modified lysozyme phenol–water procedure[8], followed by purification of the LPS by repeated ultracentrifugation, afforded an aqueous-phase LPS (2.3 g) and a phenol-phase soluble LPS (96 mg). Both LPS were judged to be pure by the carbocyanine dye assay[9], and SDS–PAGE analysis[16] of both gave a typical separation pattern of an S-type LPS[23] in which the spacing of the bands was indicative of an O-chain having a pentasaccharide repeating-unit.

Partial hydrolysis of the aqueous phase LPS (710 mg) with hot dilute acetic acid gave an insoluble lipid A (122 mg). Gel filtration of the water-soluble products on Sephadex G-50 afforded an O-polysaccharide (K_{av} 0.03, 268 mg), a core oligosaccharide (K_{av} 0.65, 68 mg), and a low-molecular-weight product (K_{av} 0.76, 44 mg) containing 3-deoxy-2-octulosonate. The O-polysaccharide was contaminated

by phosphate-containing material (~10%), presumed to be undegraded LPS[24]. Purification of the O-chain was achieved by ion-exchange chromatography on DEAE-Sephacel; the O-polysaccharide was eluted at the void volume, whereas the phosphate-containing material was eluted at the beginning of the sodium chloride gradient.

The purified O-polysaccharide was readily soluble in water and had $[\alpha]_D$ +32° (*c* 6.7, water) (*Anal.* Found: C, 40.75; H, 5.99; N, 0.86; ash, 2.5%). On the basis of p.c., and g.l.c. of the derived alditol acetates and the acetylated derivatives of their (−)-2-butyl glycosides, the O-polysaccharide was found to be composed of D-glucose and D-galactose in the molar ratio ~2:3. The ^{1}H-n.m.r. spectrum (500 MHz, 24°) of the O-chain polysaccharide contained five signals for anomeric protons at 5.22 (unresolved, 1 H), 5.17 (unresolved, 1 H), 5.06 (d, 1 H, $J_{1,2}$ 3.7 Hz), 4.99 (d, 1 H, $J_{1,2}$ 3.8 Hz), and 4.66 p.p.m. (d, 1 H, $J_{1,2}$ 8.0 Hz), Consistent with these results, the ^{13}C-n.m.r. spectrum (125 MHz, 24°) (Fig. 1) contained signals for anomeric carbons at 110.4, 106.8, 103.3, 99.0, and 98.8 p.p.m. The methylated and hydrolyzed O-chain afforded a product that, after reduction ($NaBD_4$) and acetylation, gave g.l.c.–m.s. (programme *B*) results (Table I) which indicated that the O-chain of *H. pleuropneumoniae* serotype 3 is composed of linear pentasaccharide repeating-units containing →3)-D-Gal*f*-(1-, →3)-D-Glc*p*-(1-, →2)-D-Gal*f*-(1-, →6)-D-Glc*p*-(1-, and →6)-D-Gal*p*-(1-. The methylation data showed that two D-galactose residues were furanoid, a conclusion substantiated by the

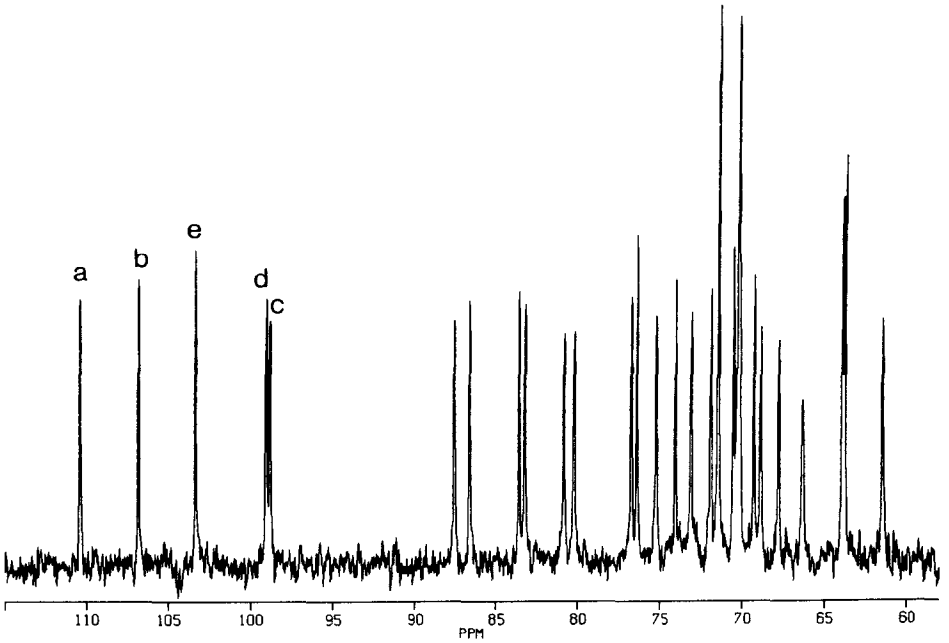


Fig. 1. ^{13}C-N.m.r. spectrum (125 MHz, 24°) of the O-chain from smooth-type LPS of *H. pleuropneumoniae* serotype 3. The recorded assignments are based on a ^{13}C,^{1}H-CHORTLE experiment.

TABLE I

G.L.C.–M.S. OF THE PRODUCTS OF METHYLATION ANALYSIS OF THE O-CHAIN OF *H. pleuropneumoniae* SEROTYPE 3 AND ITS DEGRADATION PRODUCTS

Derivative	T_{GM}[a]	*Molar ratio*			
		I[b]	*II*[c]	*III*[d]	*IV*[e]
1,4-Di-*O*-acetyl-2,3,5-tri-*O*-methyl-L-arabinitol-*1-d*	0.65				0.2
3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-D-galactitol-*1-d*	0.72		0.3	0.4	
1,2,4-Tri-*O*-acetyl-3,5-di-*O*-methyl-L-arabinitol-*1-d*	0.85				0.4
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol-*1-d*	1.00		1.0		
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol-*1-d*	1.09			1.0	
1,3,4-Tri-*O*-acetyl-2,5,6-tri-*O*-methyl-D-galactitol-*1-d* 1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol-*1-d*	1.37	1.8			1.0
1,2,4-Tri-*O*-acetyl-3,5,6-tri-*O*-methyl-D-galactitol-*1-d*	1.42	0.6	1.0		
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol-*1-d*	1.47	1.0	1.4	1.0	
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol-*1-d*	1.62	0.9	1.1		

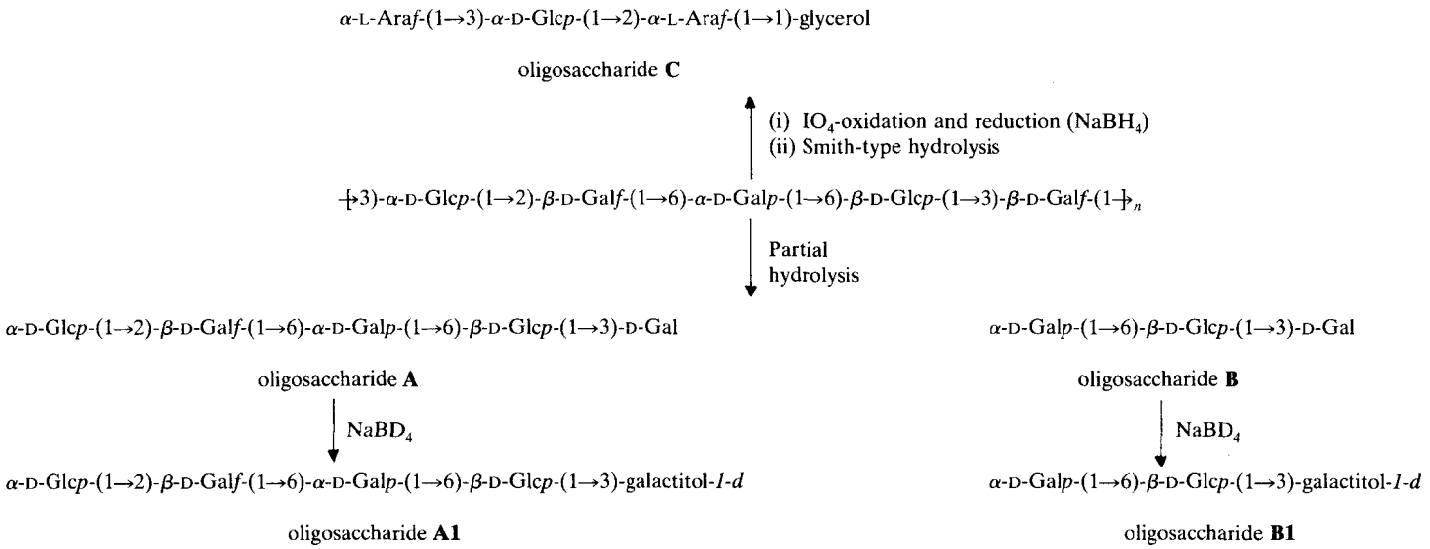
[a]Retention time of the derived alditol acetate relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol-*1-d*. [b]Original O-chain. [c]Oligosaccharide **A1** from partial hydrolysis. [d]Oligosaccharide **B1** from partial hydrolysis. [e]Oligosaccharide **C** obtained on Smith degradation (see text).

observed characteristic signals for anomeric carbons at 110.4 and 106.8 p.p.m. in the ^{13}C-n.m.r. spectrum, and two signals at 5.27 and 5.17 p.p.m. in the region for anomeric protons of the ^{1}H-n.m.r. spectrum of the O-polysaccharide.

In order to sequence the proposed pentasaccharide repeating-unit, advantage was taken of the acid lability of the D-galactofuranosyl residues. Partial hydrolysis of the O-chain with aqueous 48% hydrofluoric acid, followed by gel filtration on Bio-Gel P-2, afforded oligosaccharides **A** and **B** (see Scheme 1).

Oligosaccharide **B** (K_{av} 0.65) had $[\alpha]_D$ +60° (*c* 0.37, water) and gave a single spot in t.l.c. (R_F 0.15). Reduction of **B** with $NaBD_4$ afforded oligosaccharide **B1**, which, on hydrolysis and g.l.c.–m.s. of the derived alditol acetates, was shown to contain D-glucose, D-galactose, and galactitol-*1-d* in the molar ratios 1:1:1. N.m.r. data: ^{1}H (500 MHz, 24°), 5.28 (d, 0.4 H, $J_{1,2}$ 3.1 Hz), 4.99 (d, 1 H, $J_{1,2}$ 2.9 Hz), 4.70 (d, 1 H, $J_{1,2}$ 8.1 Hz), 4.63 p.p.m. (d, 0.6 H, $J_{1,2}$ 7.9 Hz); ^{13}C (50 MHz, 27°); 104.4 (1 C), 98.9 (1 C), 96.9 (0.7 C), 93.0 p.p.m. (0.3 C). G.l.c.–m.s. of methylated **B1** (Table I, column III) in conjunction with the ^{1}H- and ^{13}C-n.m.r. data indicated **B** to be D-Gal*p*-(1→6)-D-Glc*p*-(1→3)-D-Gal.

Oligosaccharide **A** (K_{av} 0.43) had $[\alpha]_D$ +84° (*c* 0.97, water) and gave a single spot in t.l.c. (R_F 0.07). Reduction of **A** with $NaBD_4$ gave oligosaccharide **A1**, which, by g.l.c.–m.s. of the derived alditol acetates, was shown to be a pentasaccharide composed of D-glucose, D-galactose, and galactitol-*1-d* in the molar ratios 2:2:1. N.m.r. data: ^{1}H (500 MHz, 24°), 5.28 (d, 0.3 H, $J_{1,2}$ 3.2 Hz, H-1α), 5.16 (d, 1 H, $J_{1,2}$ <2 Hz), 5.06 (d, 1 H, $J_{1,2}$ 3.6 Hz), 4.99 (d, 1 H, $J_{1,2}$ 3.1 Hz), 4.72 (d, 0.3 H, $J_{1,2}$ 8.3 Hz, H-1′), 4.70 (d, 0.7 H, $J_{1,2}$ 8.1 Hz, H-1′), 4.63 p.p.m. (d, 0.7 H, $J_{1,2}$ 7.9 Hz, H-1β); ^{13}C (50 MHz, 27°), 106.7 (1 C), 104.0 (1 C), 98.8 (2 C), 96.9 (0.6 C), 92.9 p.p.m. (0.4 C).



Scheme 1. Degradation of the O-chain of *H. pleuropneumoniae* serotype 3 by periodate oxidation and by partial hydrolysis.

G.l.c.–m.s. of methylated **A1** (Table I) indicated that the D-glucopyranosyl unit formed the non-reducing end of the pentasaccharide and that the D-galactose residue formed the reducing end. The prior structural identification of **B** now permits the sequence of the pentasaccharide **A** to be established as D-Glc*p*-(1→2)-D-Gal*f*-(1→6)-D-Gal*p*-(1→6)-D-Glc*p*-(1→3)-D-Gal. Further evidence for this structure was obtained from periodate-oxidation studies. Thus, periodate oxidation of the O-polysaccharide resulted in complete cleavage of the exocyclic 5,6-diol moiety of the D-galactofuranosyl residues. Reduction with sodium borohydride, followed by Smith-type hydrolysis with dilute acetic acid and subsequent gel-filtration chromatography of the products on Bio-Gel P-2 (Scheme 1), gave an oligosaccharide **C** (K_{av} 0.52), which had $[\alpha]_D$ −5.6° (*c* 0.36, water), gave a single spot in t.l.c. (R_F 0.45), and, on hydrolysis, reduction, and acetylation, yielded three peaks in g.l.c. identified as the acetates of glycerol (T_{GA} 0.10), arabinitol (T_{GA} 0.64), and glucitol (T_{GA} 1.00) in the molar ratios 0.1:0.6:1.0 (the glycerol arose from the oxidized 6-substituted D-galactopyranosyl residue). N.m.r. data: ^{1}H (500 MHz, 24°), 5.28 (unresolved, 1 H), 5.17 (d, 1 H, $J_{1,2}$ 2.0 Hz), 5.06 p.p.m. (d, 1 H, $J_{1,2}$ 3.5 Hz); ^{13}C (125 MHz, 24°), 109.1 (1 C), 106.9 (1 C), 98.8 p.p.m. (1 C). As expected, the ^{13}C-n.m.r. spectrum of **C** contained 19 signals, with two low-field signals at 109.1 and 106.9 p.p.m. arising from the anomeric carbons of two α-L-arabinofuranosyl residues. G.l.c.–m.s. of methylated **C** gave a fragmentation pattern consistent with the presence of a glycerol moiety at the potential reducing end. The following primary ions of the A series[24] were obtained which are characteristic of the terminal non-reducing pentosyl residue: *m/z* 175 (aA_1), 143 (aA_2), and 111 (aA_3). Those of the glycerol moiety were *m/z* 103 (dA_1), 323 (dcJ_1), and 527 ($dcbJ_1$). Expected secondary ions appeared at *m/z* 101, 89, 75, 71, and 45. G.l.c.–m.s. in the c.i. mode confirmed the presence of two pentosyl residues, one hexosyl residue, and a glycerol moiety: peaks at *m/z* 175 (aA_1), 379 (abA_1), 539 ($abcA_1$), parent peaks $M^+ - 1$ at *m/z* 657 and M^+ at *m/z* 658 (Fig. 2). Methylation analysis of the tetrasaccharide **C** yielded products that, after reduction ($NaBD_4$) and acetylation, gave, in g.l.c.–m.s., the results shown in Table I. The combined ^{1}H- and ^{13}C-n.m.r. evidence together with specific optical rotation and g.l.c.–m.s. data

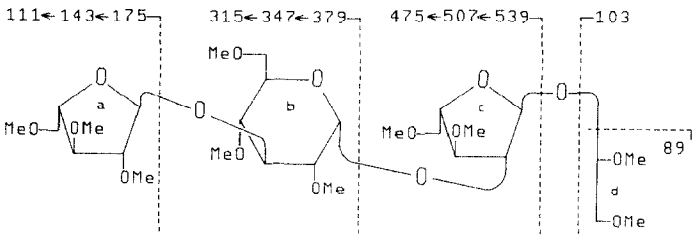


Fig. 2. G.l.c.–m.s. of the methylated oligosaccharide obtained after Smith degradation of the O-polysaccharide, with some primary and secondary fragments.

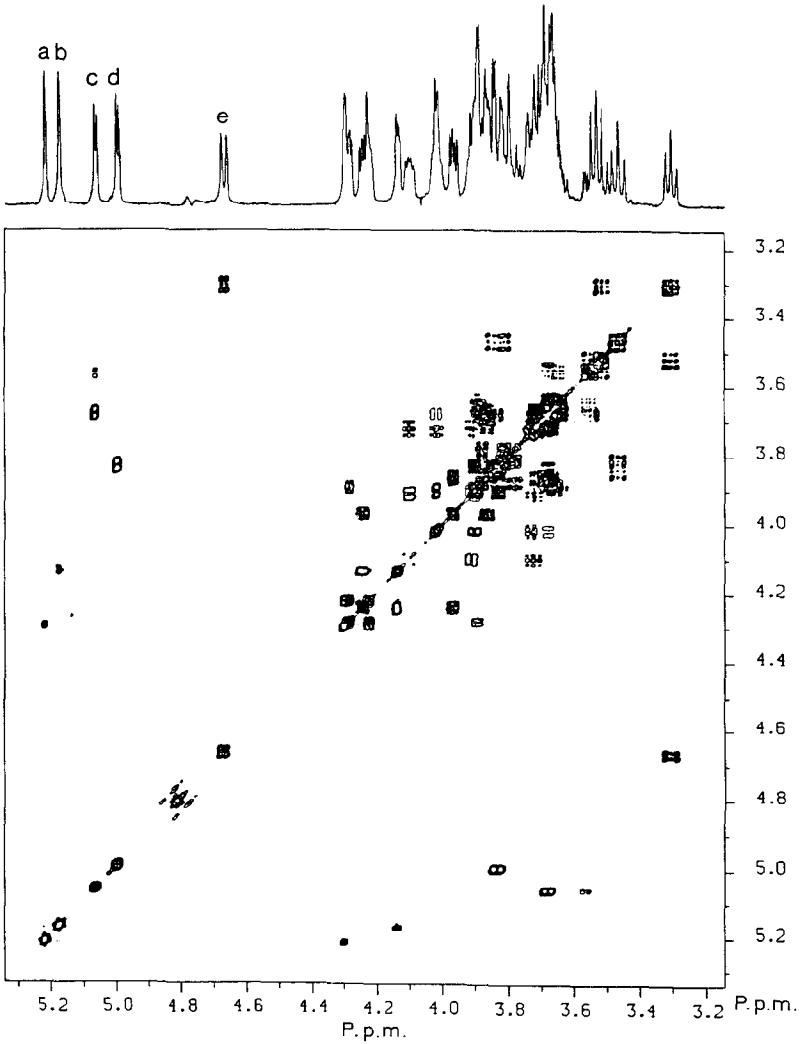


Fig. 3. COSY contour plot of the complete spectrum (5.25–3.15 p.p.m.) of *H. pleuropneumoniae* serotype 3 O-polysaccharide, recorded at 24°. The 1D spectrum is displayed along the F_2 axis.

allows the structure of **C** to be identified as α-L-Ara*f*-(1→3)-α-D-Glc*p*-(1→2)-α-L-Ara*f*-(1→1)-glycerol.

N.m.r. studies. — The determination of the anomeric configurations of the component monosaccharides and their ring sizes was achieved by complete assignment of the ^{1}H-n.m.r. spectra *via* COSY and relay COSY, followed by a ^{13}C–^{1}H shift-correlated experiment (CHORTLE).

Assignments of the proton resonances of the O-chain were made from COSY[19] (Fig. 3) and relayed COSY[20] experiments. The residues in the O-chain

TABLE II

^{1}H CHEMICAL SHIFTS[a] AND COUPLING CONSTANTS[b] FOR THE O-CHAIN POLYSACCHARIDE OF *Haemophilus pleuropneumoniae* SEROTYPE 3

Proton	*Unit* **a**[c] →3)-β-D-*Galf*-(1→	*Unit* **b** →2)-β-D-*Galf*-(1→	*Unit* **c** →3)-α-D-*Glcp*-(1→	*Unit* **d** →6)-α-D-*Galp*-(1→	*Unit* **e** →6)-β-D-*Glcp*-(1→
H-1	5.215 (1.4)	5.171 (2.0)	5.061 (3.7)	4.994 (3.8)	4.667 (8.0)
H-2	4.296 (2.7)	4.135 (4.0)	3.678 (9.8)	3.830 (10.0)	3.304 (9.2)
H-3	4.221 (5.3)	4.240 (7.4)	3.843 (9.3)	3.900 (3.8)	3.518 (9.4)
H-4	4.282 (3.1)	3.966 (3.9)	3.467 (9.3)	4.017 (1.0)	3.547 (9.4)
H-5	3.893 (4.0; 7.5)	3.861 (4.0; 7.5)	3.819 (2.2; 5.4)	4.099 (4.0; 9.0)	3.679 (1.0; 3.5)
H-6	3.669 (−12.0)	3.709 (−12.0)	3.878 (−12.3)	3.906 (−11.5)	3.728 (−11.5)
H-6′	3.646 (−12.0)	3.675 (−12.0)	3.782 (−12.3)	3.718 (−11.5)	4.016 (−11.5)

[a]Simulated parameters obtained from spectra measured at 24° in D_2O (p*D* 9.0) with 0.1% of acetone as internal reference (2.225 p.p.m.). [b]Hz, in parentheses. [c]See formula **1**.

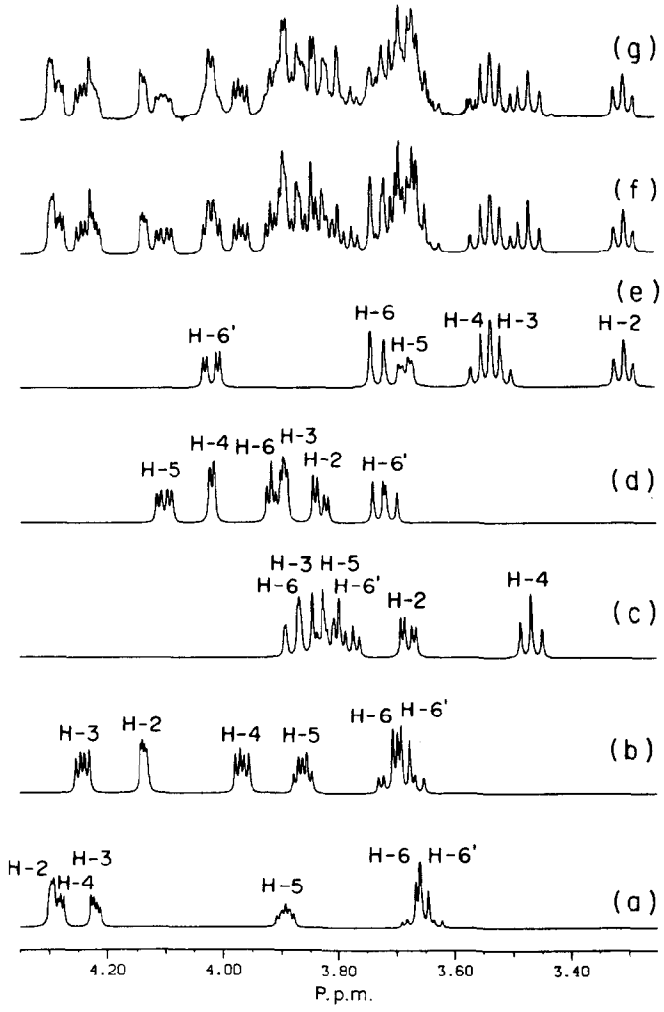


Fig. 4. ^{1}H-N.m.r. spectra of the O-polysaccharide of *H. pleuropneumoniae* serotype 3 at 24°: (g) observed spectrum; (f) simulated spectrum. The contributions to the simulated spectrum are shown in (a) for the →3)-β-D-Gal*f*-(1→ residue, in (b) for the →2)-β-D-Gal*f*-(1→ residue, in (c) for the →3)-α-D-Glc*p*-(1→ residue, in (d) for the →6)-α-D-Gal*p*-(1→ residue, and in (e) for the →6)-β-D-Glc*p*-(1→ residue.

polysaccharide were arbitrarily labelled **a–e** according to the decreasing order of the chemical shifts of the H-1 resonances. Following the cross-peaks, the majority of the proton resonances were readily assigned. Coupling constants were measured from the 2D *J*-resolved[21] spectrum. The proton chemical shifts and coupling constants were refined (Table II) by simulating the ^{1}H-n.m.r. spectrum until a good agreement between the observed and calculated spectra was obtained (Fig. 4). The heteronuclear shift-correlated experiment (CHORTLE[22]) enabled the un-

TABLE III

^{13}C CHEMICAL SHIFTS[a] OF THE O-CHAIN POLYSACCHARIDE[b] FROM *Haemophilus pleuropneumoniae* SEROTYPE 3 AT 24°

Carbon atom	*Unit* **a**[c] *→3)-β-*D*-Galf-(1→*	*Unit* **b** *→2)-β-*D*-Galf-(1→*	*Unit* **c** *→3)-α-*D*-Glcp-(1→*	*Unit* **d** *→6)-α-*D*-Galp-(1→*	*Unit* **e** *→6)-β-*D*-Glcp-(1→*
C-1	110.4	106.8	98.8	99.0	103.3
C-2	80.8	87.6	71.9	69.3	74.1
C-3	86.7	76.4	80.2	70.3	76.8
C-4	83.6	83.3	68.9	70.3	70.0
C-5	71.5	71.5	73.1	70.6	75.3
C-6	63.9	63.8	61.5	67.8	66.3

[a]In p.p.m. from internal acetone (1%, 31.07 p.p.m.). [b]Assignments confirmed by a ^{13}C,^{1}H-CHORTLE experiment. [c]See formula **1**.

ambiguous assignments of all the carbon resonances in the repeating unit of the O-polysaccharide (Table III). Examination of ^{13}C chemical shifts (Table III) and a comparison of these with literature values[25] indicated that two furanose rings (residues **a** and **b**) and three pyranose rings (residues **c**–**e**) were involved in glycosidic linkages. In agreement with the results of the methylation analysis, C-3a, C-2b, C-3c, C-6d, and C-6e experienced significant deshielding. Based on the proton chemical shift data and $J_{1,2}$ values, residues **a** ($J_{1,2}$ 1.4 Hz) and **b** ($J_{1,2}$ 2.0 Hz) were assigned to β-D-Gal*f*, residue **c** to α-D-Glc*p* ($J_{1,2}$ 3.7 Hz), residue **d** to α-D-Gal*p* ($J_{1,2}$ 3.8 Hz), and residue **e** to β-D-Glc*p* ($J_{1,2}$ 8.0 Hz). The combined chemical and n.m.r. evidence permits the structure of the O-antigen chain to be established as the linear repeating pentasaccharide unit **1**.

→3)-α-D-Glc*p*-(1→2)-β-D-Gal*f*-(1→6)-α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*f*-(1→$_n$

(c) (b) (d) (e) (a)

1

Core oligosaccharide. — The core oligosaccharide obtained from the aqueous-phase LPS had $[\alpha]_D$ +51° (*c* 3.4, water) and was composed of D-glucose, D-galactose, D-*glycero*-D-*manno*-heptose, and L-*glycero*-D-*manno*-heptose in the molar ratios 1.4:1.0:0.7:0.9.

Lipid A. — Methanolysis of lipid A with methanolic 2.5% hydrogen chloride for 16 h at 100° followed by g.l.c.–m.s. (programme *C*) showed the lipid A of the aqueous-phase LPS to be composed of the fatty acids: 3-hydroxydodecanoic acid (1.1%), *n*-tetradecanoic acid (27.3%), 3-hydroxytetradecanoic acid (70.4%), and *n*-hexadecanoic acid (1.2%). The lipid A also contained phosphate (3.2%) and 2-amino-2-deoxy-D-glucose (6.9%).

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