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Structure of the O-antigen of *Actinobacillus pleuropneumoniae* serotype 7 lipopolysaccharide*

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ABSTRACT

The structure of the O-antigen polysaccharide of *A. pleuropneumoniae* serotype 7 was investigated by methylation analysis, partial acid hydrolysis, periodate oxidation, and ^{1}H- and ^{13}C-n.m.r. spectroscopy. The polysaccharide repeating-unit consists of a branched tetrasaccharide having the following structure.

$$[\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow]_n$$

$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \beta\text{-D-Gal}p \end{array}$$

INTRODUCTION

The lipopolysaccharides (endotoxins) associated with *A. pleuropneumoniae* strains are implicated in the pathogenesis of a hemorrhagic, necrotising pneumonia in pigs[1]. Outbreaks of *A. pleuropneumoniae* were first described[2] in England in 1961 and, in recent years, there has been an increase in the world-wide incidence of the disease correlated with intensified production and confinement. The pathogenesis of porcine pleuropneumonia is not well understood and endotoxin or encapsulation, or both, may play a role in protecting the bacteria from humoral and cellular defence mechanisms. Apparent serological cross-reactions have been observed[3] between serotypes 1 and 9, serotypes 3, 6, and 8, and serotypes 4 and 7, and structural studies of capsular and O-antigens undertaken by this laboratory suggest that these may be due to antigenic lipopolysaccharide components[4].

We now report the structure of the O-specific polysaccharide of *A. pleuropneumoniae* type 7, which closely resembles that of the type 4 O-antigen[4].

RESULTS AND DISCUSSION

Extraction of *A. pleuropneumoniae* serotype 7 cells (500 g, wet weight) by a modified lysozyme phenol–water procedure[5], followed by ultracentrifugation, afforded

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an aqueous-phase lipopolysaccharide (LPS) (1.5 g) and a phenol-phase LPS (0.4 g). SDS-PAGE analysis[6] of both LPS showed a typical banding pattern indicative[7] of S-type LPS. However, the phenol-phase LPS was heavily contaminated by capsular material and all subsequent work was carried out on the aqueous-phase LPS.

Partial hydrolysis of this LPS (200 mg) with aqueous 1% acetic acid (100 mL, 100°) for 2 h gave an insoluble lipid A (73.6 mg). Gel filtration, on Sephadex G50, of the water-soluble products afforded an O-chain polysaccharide (K_{av} 0.13, 42.9 mg), a core oligosaccharide (K_{av} 0.67, 27 mg), and a low-molecular-weight fraction (K_{av} 0.83) containing 3-deoxy-D-*manno*-2-octulosonate (Kdo).

The O-chain polysaccharide had $[\alpha]_D$ +3.8° (*c* 0.5, water) (*Anal.* Found: C, 40.32; H, 6.00; N, 1.28; Ash, 1.80%). Total acid hydrolysis (4M trifluoroacetic acid, 1 h, 125°) afforded D-galactose, 2-amino-2-deoxy-D-galactose, and L-rhamnose in the molar ratios 2.0:0.8:0.6. The configurations of the glycoses were established by g.l.c. of their (−)-2-octyl glycoside derivatives[8].

The ^{1}H- and ^{13}C-n.m.r. data for the O-polysaccharide were consistent with the analytical data and indicated the presence of aldohexose, 6-deoxyaldohexose, and 2-acetamido-2-deoxyhexose residues. The ^{1}H-n.m.r. spectrum of the O-polysaccharide contained four resonances of equal signal area (1 H) for anomeric protons at 5.04 (d, $J_{1,2}$ 1.5 Hz), 4.86 (d, $J_{1,2}$ 7.5 Hz), 4.81 (d, $J_{1,2}$ 8.2 Hz), and 4.42 p.p.m. (d, $J_{1,2}$ 7.5 Hz), together with characteristic high-field resonances at 1.32 (d, 3 H, $J_{5,6}$ 6.2 Hz) and 2.04 p.p.m. (s, 3 H) from the methyl protons of the L-rhamnosyl residues and the *N*-acetyl groups of the 2-acetamido-2-deoxy-D-galactosyl residues, respectively. Consistent with these results, the ^{13}C-n.m.r. spectrum (Fig. 1) showed four resonances between 100 and 110 p.p.m., for the anomeric carbons of the constituent monosaccharides, clearly indicating the polysaccharide to be composed of tetrasaccharide repeating-units. Diagnostic ^{13}C resonances were observed at 18.1 p.p.m. from the methyl carbon of the L-rhamnosyl residues and at 53.1, 176.0, and 23.5 p.p.m. from C-2 and the acetamido carbons of the aminodeoxyglycosyl residues[9]. In addition, resonances for three primary hydroxyl groups were identified, by a ^{13}C-DEPT experiment, at 61.2, 61.4, and 61.9 p.p.m., which could be attributed to the C-6 of the three hexopyranosyl residues.

The methylated and hydrolysed O-chain afforded a product that, after reduction ($NaBD_4$) and acetylation, gave g.l.c.–m.s. (program *B*) results (Table I) which indicated the polysaccharide to be composed of branched tetrasaccharide repeating-units containing 3-linked galactopyranosyl, 4-linked rhamnopyranosyl, terminal galactopyranosyl, and 2-acetamido-2-deoxygalactopyranosyl residues as di-*O*-substituted branch-points linked through O-3 and O-4.

Periodate oxidation and Smith hydrolysis[10]. — In order to establish the sequence of the glycoses in the tetrasaccharide repeating-unit, the polysaccharide was subjected to periodate oxidation, followed by reduction with sodium borohydride. Smith-type hydrolysis (aqueous 1% acetic acid, 3 h, 100°) of the modified polysaccharide, and subsequent gel-filtration chromatography on Bio-Gel P-2, gave trisaccharide **1** (K_{av} 0.27), which showed a single spot in t.l.c. (R_{GAL} 1.25). The ^{1}H-n.m.r. spectrum of **1** (Table II) contained doublets ($J_{1,2}$ 8.0 Hz) for the anomeric protons at 4.66 and 4.61 p.p.m.,

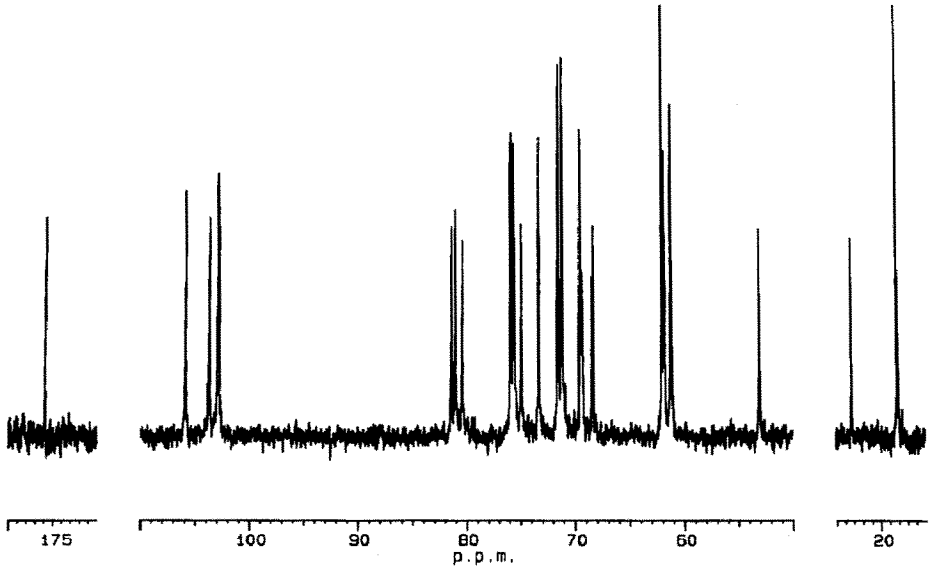


Fig. 1. Proton-decoupled ^{13}C-n.m.r. spectrum of the O-polysaccharide of *A. pleuropneumoniae* serotype 7 recorded at 37°.

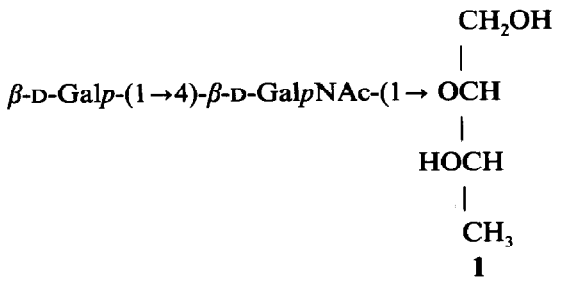
TABLE I

Methylation analysis data for *A. pleuropneumoniae* O-antigen and degradation products

Methylated sugar[a] *(as alditol acetate)*	T_{MG}[c]	*Molar ratio*[b]		
		I	*II*	*III*
2,3-Rha	0.81	0.8		
2,3,4,6-Gal	1.00	1.0	1.0	1.0
2,4,6-Gal	1.42	1.6		
1,2,5,6-GalNAc	2.00			0.5
2,3,6-GalNAc	2.63		0.4	
2,6-GalNAc	3.89	1.2		

[a] 2,3,4,6-Gal represents 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, *etc.* [b] I, native polysaccharide; II, periodate-oxidation and Smith-degradation product **1**; III, partial hydrolysis product **2**. [c] Retention times relative to that of 2,3,4,6-Gal.

indicating the two glycopyranosyl units to be β-linked, an *N*-acetyl methyl singlet at 2.05 p.p.m., and a methyl doublet ($J_{3,4}$ 6.6 Hz) at 1.17 p.p.m. G.l.c.–c.i.-m.s. of methylated **1** gave a single peak (T_{MS} 3.60) with an $[M + H]^+$ ion at *m/z* 598, along with fragment ions at *m/z* 464 (baA_1), 432 (baA_2), 219 (aA_1), 187 (aA_2), and 177 (bald J_1); the J_1 fragment at *m/z* 177 being indicative of a terminal 1-deoxytetritol residue (Fig. 2). Methylation analysis of **1** gave 2,3,4,6-tetra-*O*-methylgalactose and 2-deoxy-3,6-di-*O*-methyl-2-methylaminogalactose (Table I). These results allow **1** to be assigned the following structure.



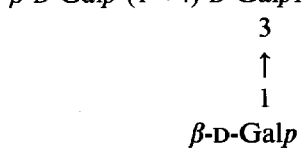
1

Since the 1-deoxyerythritol residue can arise only from the O-4-substituted L-rhamnopyranosyl residues, the trisaccharide backbone of the repeating unit can be identified as

→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→4)-L-Rha*p*-(1→.

Partial acid hydrolysis. — Further structural evidence for the O-chain was obtained from its partial hydrolysis using 0.05M trifluoroacetic acid. Gel-filtration chromatography on Bio-Gel P-2 afforded three main products **2**, **3**, and **4** (K_{av} 0.67, 0.60, and 0.73, respectively), each giving a single spot in t.l.c. (R_{Suc} 0.38, 0.50, and 0.98, respectively).

Total acid hydrolysis of **2** and g.l.c.–m.s. of the derived alditol acetates showed galactose and 2-amino-2-deoxygalactose in the ratio 2:1. G.l.c.–e.i.-m.s. of reduced, methylated **2** showed a single peak (T_{MS} 3.32) with fragment ions at *m/z* 670, 626, 378, 219, and 130 (Fig. 3). Methylation analysis of **2** gave 2,3,4,6-tetra-*O*-methylgalactose and 2-deoxy-1,5,6-tri-*O*-methyl-2-methylaminogalactose (Table I). The ^{1}H-n.m.r. spectrum of reduced **2** showed two signals in the anomeric region at 4.53 and 4.44 p.p.m., both having a $J_{1,2}$ value of 8.0 Hz, and a signal for the methyl protons of 2-acetamido-2-deoxygalactitol at 2.07 p.p.m. The foregoing results allow the following identification of **2**.



2

G.l.c.–e.i.-m.s. of reduced methylated **3** showed two peaks (in the ratio 2.0:1.0), having similar retention times (T_{MS} 4.32 and 3.79 respectively), which indicated the oligosaccharide fraction to consist of two components (**3A** and **3B**). The methylated derivative of the major component **3A** gave fragment ions at *m/z* 654, 480, 393, 219, and

TABLE II

N.m.r. data for oligosaccharides derived from type 7 O-antigen[a]

Oligosaccharide	^{1}*H-N.m.r. data*				^{13}*C-N.m.r. data*	
	δ (p.p.m.)	J *(Hz)*	*Integral (no. of H)*	*Assignment*	*δ (p.p.m.)*	*Assignment*
β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-	4.66	8	1	H-1 of β-GalNAc	105.17	C-1 of β-Gal
-(1→2)-1-deoxyerythritol	4.61	8	1	H-1 of β-Gal	102.36	C-1 of β-GalNAc
(Smith-product, **1**)	4.20	1–2	1	H-4 of β-GalNAc	61.86	C-6 of β-Gal
	2.05	s	3	CH_3 of *N*-acetyl	61.46	C-6 of β-GalNAc
						C-1 of deoxyerythritol
	1.17	6.6	3	CH_3 of deoxyerythritol	54.15	C-2 of β-GalNAc
					23.00	CH_3 of *N*-acetyl
					17.66	CH_3 of deoxyerythritol
β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-ol	4.53	8	1	H-1 of β-Gal	175.09	C=O of *N*-acetyl
3						
↑						
1						
β-D-Gal*p*	4.44	8	1	H-1 of β-Gal	105.11	C-1 of β-Gal
					103.74	C-1 of β-Gal
(**2**)	4.21	m	-	Unassigned	62.77	C-1 of β-GalNAc
	2.07	s	3	CH_3 of *N*-acetyl	61.67	C-6 of β-Gal
					61.39	C-6 of β-GalNAc
					51.89	C-2 of β-GalNAc
					22.87	CH_3 of *N*-acetyl

[a] Measured at 300K in D_2O.

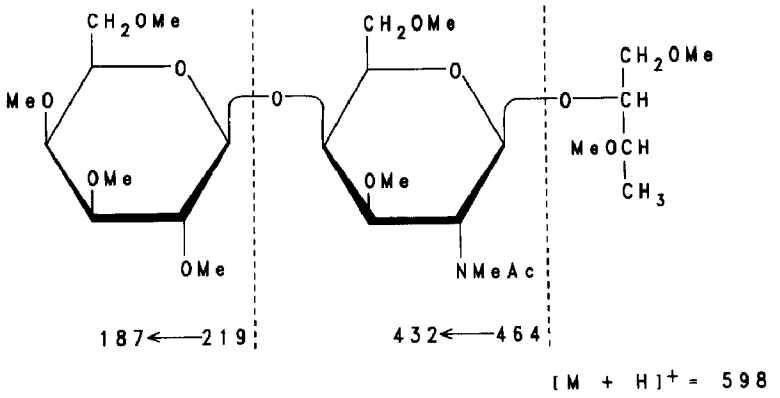


Fig. 2. G.l.c.–c.i.-m.s. fragmentation ions from the methylated Smith-product **1**.

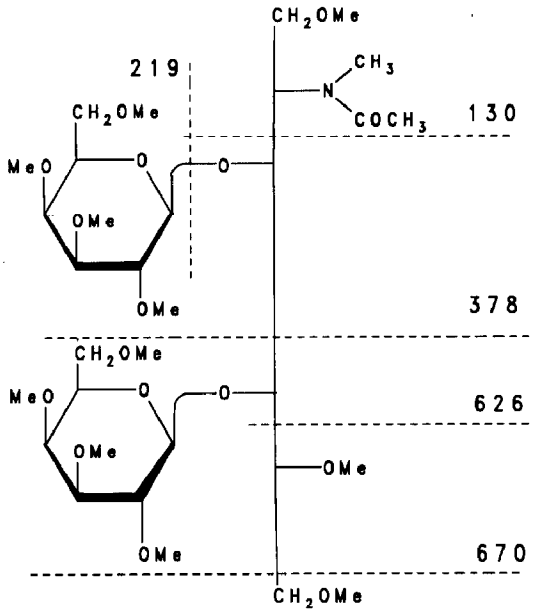


Fig. 3. G.l.c.–e.i.-m.s. fragmentation ions from the reduced and methylated partial hydrolysis product **2**.

189 (Fig. 4), which suggested **3A** to be a tetrasaccharide arising from cleavage of the *β*-D-Gal*p*NAc glycosidic linkage in the repeating unit.

L-Rha*p*-(1→3)-*β*-D-Gal*p*(1→4)-D-Gal*p*NAc
3
↑
1
β-D-Gal*p*

3A

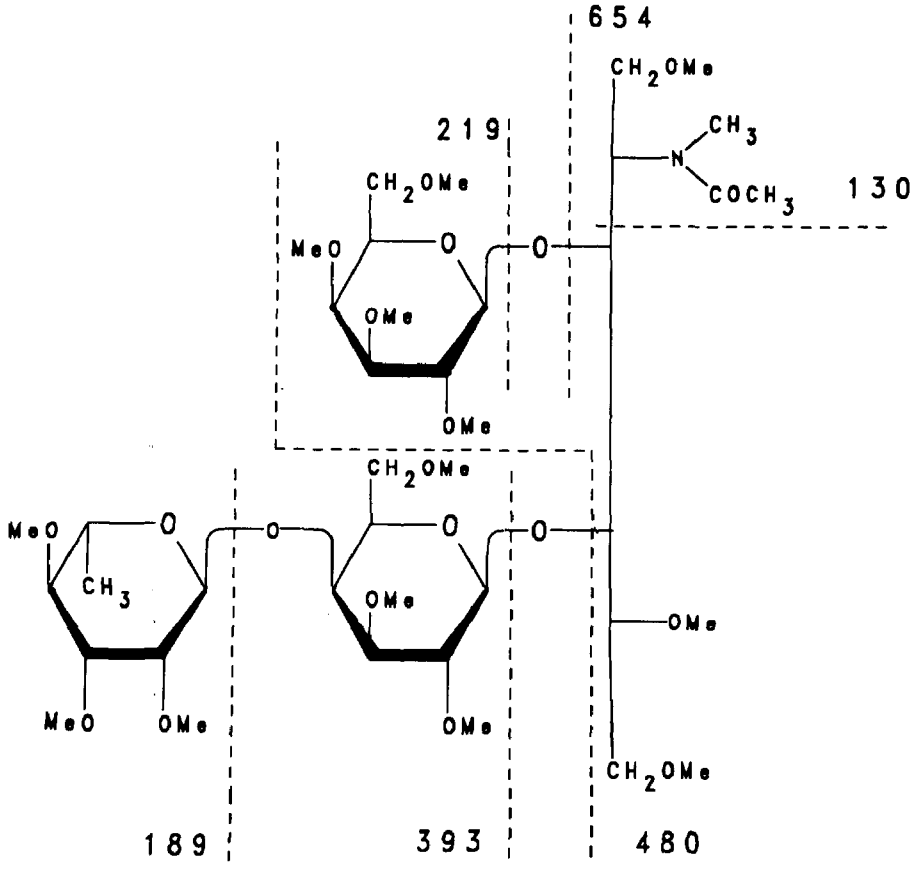


Fig. 4. G.l.c.–e.i.-m.s. fragmentation ions from the reduced and methylated partial hydrolysis product **3A**.

The fragment ions exhibited by the methylated derivative of the minor component **3B** were less diagnostic. However, the presence of fragment ions at *m/z* 219 and 205 was consistent with the tetrasaccharide product having arisen by hydrolysis of the L-Rha*p* glycosidic linkage in the repeating unit.

β-D-Gal*p*-(1→4)-*β*-D-Gal*p*NAc-(1→4)-L-Rha*p*
3
↑
1
β-D-Gal*p*

3B

The tetrasaccharide **3B** was obtained as the major product when the type 7 O-antigen was hydrolysed with dilute sulphuric acid (0.01M, 100°, 3 h).

G.l.c.–e.i.-m.s. of reduced methylated **4** also gave two peaks, in the ratio 1.0:0.6

(T_{MS} 1.88 and 1.92, respectively), indicating **4** to be a mixture of disaccharides (**4A** and **4B**). Each methylated component gave fragment ions characteristic of a hexosyl-2-acetamido-2-deoxyhexitol, *i.e.*, at *m/z* 219 (aA_1), 187 (aA_2), 276 (ald), and 130 (Figs. 5a and 5b). Furthermore, reduced methylated **4B** gave fragment ions at *m/z* 174 and 142, which identified it as having a (1→4) linkage (Fig. 5b). These data are consistent with **4** being a mixture of β-D-Gal*p*-(1→3)-D-Gal*p*NAc (**4A**) and β-D-Gal*p*-(1→4)-D-Gal*p*NAc (**4B**). Thus, the combined chemical evidence permits the branched tetrasaccharide repeating-unit of the O-chain polysaccharide to be identified as

[→4)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→],
3
↑
1
β-D-Gal*p*

5

where the anomeric configurations of the L-rhamnopyranosyl residues were determined by n.m.r. methods (see below) which also served to confirm the structure of the O-chain.

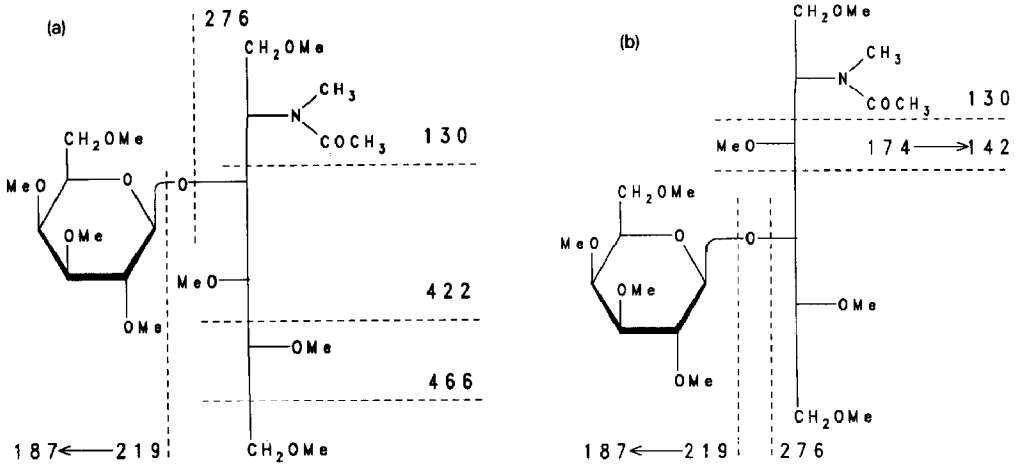


Fig. 5. G.l.c.–e.i.-m.s. fragmentation ions from the reduced and methylated partial hydrolysis products (*a*) **4A**, and (*b*) **4B**.

N.m.r. analysis of the O-polysaccharide. — The ^1H- and ^{13}C-n.m.r. spectra of the O-polysaccharide were assigned completely from homo- and hetero-nuclear correlation experiments. Assignment of the ^1H chemical shifts was achieved by 2D homonuclear chemical shift correlation and n.O.e. measurements. The connectivities observed between coupled protons in the COSY and Relay COSY spectra led to the identification of the spin-systems corresponding to the four glycosyl residues within the repeating unit, which were designated **a, b, c,** and **d** according to the order of the anomeric ^1H chemical

shifts. The chemical shift data and the ^3J values, determined from the appropriate cross-peaks in the COSY spectrum, are recorded in Table III. Assignment of the corresponding ^{13}C resonances was then possible by heteronuclear ^1H–^{13}C correlation, using the CHORTLE pulse sequence[11] (Table IV).

TABLE III

Proton chemical shifts and coupling constants for the LPS O-polysaccharide of *A. pleuropneumoniae* serotype 7[a]

Glycosyl residue	*H-1* ($J_{1,2}$)	*H-2* ($J_{2,3}$)	*H-3* ($J_{3,4}$)	*H-4* ($J_{4,5}$)	*H-5*	*H-6* ($J_{5,6}$)	*H-6'* ($J_{5,6'}$, $J_{6,6'}$)
→4)-α-L-Rha*p*-(1→	5.04	4.02	3.96	3.63	3.84	1.32	
(a)	(1.5)	(3.1)	(9.3)	(9.1)		(6.2)	
→3)-β-D-Gal*p*-(1→	4.86	3.71	3.74	3.97	3.68	~3.76	~3.76
(b)	(7.5)	(9.7)	(~3)	(~1)[b]		(-)[c]	(-,-)[c]
→3,4)-β-D-Gal*p*NAc-(1→	4.81	4.09	3.98	4.40	3.70	3.85	3.77
(c)	(8.2)	(10.3)	(3.2)	(~1)[b]		(6.8)	(6.8, 11.6)
β-D-Gal*p*-(1→	4.42	3.55	3.63	3.92	3.66	3.80	3.76
(d)	(7.5)	(9.6)	(3.3)	(~1)[b]		(6.0)	(5.8, 11.6)

[a] Observed first-order chemical shifts and coupling constants (Hz) determined at 310 K in D_2O. [b] $J_{4,5} \leq 1.5$ Hz. [c] Not determined due to strong coupling.

TABLE IV

Carbon-13 chemical shifts for the LPS O-polysaccharide of *A. pleuropneumoniae* serotype 7[a]

Glycosyl residue	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
→4)-α-L-Rha*p*-(1→	103.0	71.2	71.2[b]	81.5	68.5	18.1
(a)						
→3)-β-D-Gal*p*-(1→	103.9	71.7	81.2	69.5[b]	75.8	61.9
(b)						
→3,4)-β-D-Gal*p*NAc-(1→	102.8	53.1	80.6	75.7	75.0	61.4
(c)[c]						
β-D-Gal*p*-(1→	106.0	71.6	73.5	69.6	76.0	62.2
(d)						

[a] Measured at 310 K in D_2O; assignments were made by ^1H–^{13}C chemical shift correlation. [b] Assignments may be reversed. [c] NAc chemical shifts: C=O, 176.0; CH_3, 23.5 p.p.m.

Delineation of the respective connectivity pathways defined by the cross-peaks in the COSY spectrum (Fig. 6) was straightforward. The L-rhamnopyranosyl residue (**a**) was readily identified from (*a*) the observed small $J_{1,2}$ and $J_{2,3}$ values which were indicative[12] of the axial disposition of the C-2 hydroxyl group, and (*b*) a connectivity relating H-5 (3.84 p.p.m.) and the high-field methyl doublet (1.32 p.p.m.). The anomeric configuration of the residue **a** was indicated to be α from the $^1J_{C,H}$ value (171 Hz) observed for the corresponding anomeric carbon resonance (103.0 p.p.m.) in the ^1H-coupled ^{13}C-n.m.r. spectrum[13] and this was confirmed from ^1H n.O.e. measurements.

The three D-hexose residues (**b, c, d**) each gave spin-systems composed of seven ^{1}H resonances, from which the *β-galacto*-pyranosyl configuration was established for each from the ring-proton ^{3}J values (Table III). Apart from residue **b**, the connectivity pathways from H-1 to H-5 were easily identified in the COSY spectrum (Fig. 6). For the residue **b**, the cross-peaks relating H-2 and H-3 were close to the diagonal, but a definitive assignment of the latter resonance was evident from a connectivity between H-1**b** and H-3**b** in the Relay COSY spectrum. The cross-peaks relating H-4 and H-5 from the residues having the *galacto* configuration were very weak and difficult to identify, particularly the 4,5**b** and 4,5**c** connectivities (Fig. 6). However, the respective H-5 assignments were verified from intraresidue n.O.e., which established H-1/H-3 and H-1/H-5 connectivities within each of the *β*-pyranosyl ring systems (Fig. 7). A direct ^{1}H–^{13}C correlation between the resonances of H-2**c** (4.09 p.p.m.) and C-2**c** (53.1 p.p.m.)

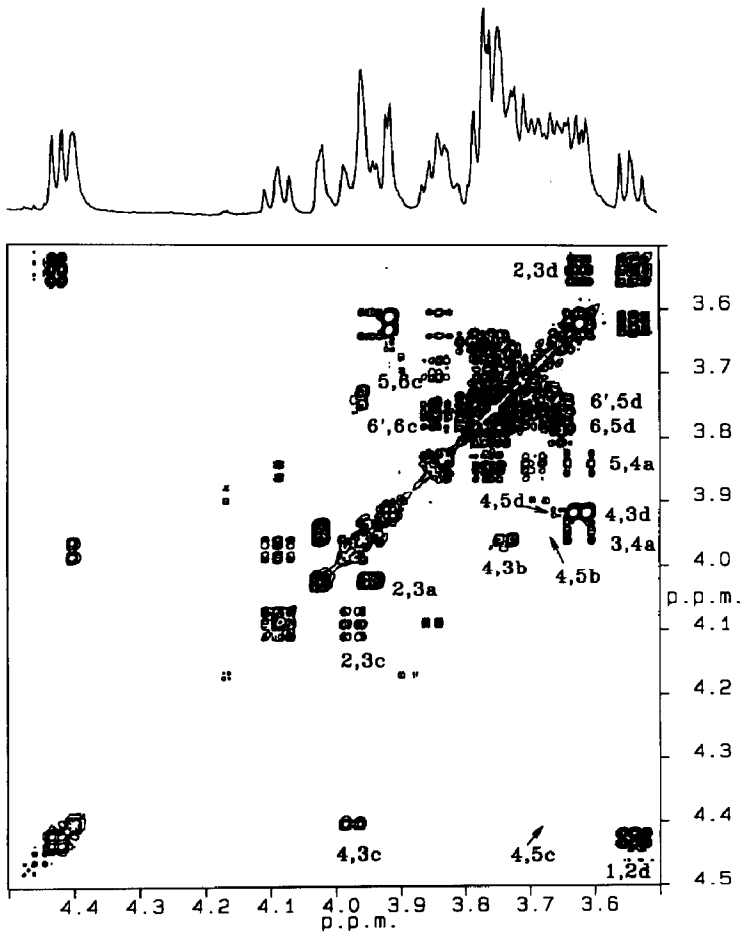


Fig. 6. Contour plot of part of the COSY spectrum (3.5–4.5 p.p.m.) of the *A. pleuropneumoniae* type 7 O-polysaccharide recorded at 37°. Cross-peak assignments are indicated; those for 4,5**c** and 4,5**d** are not visible at the indicated contour level.

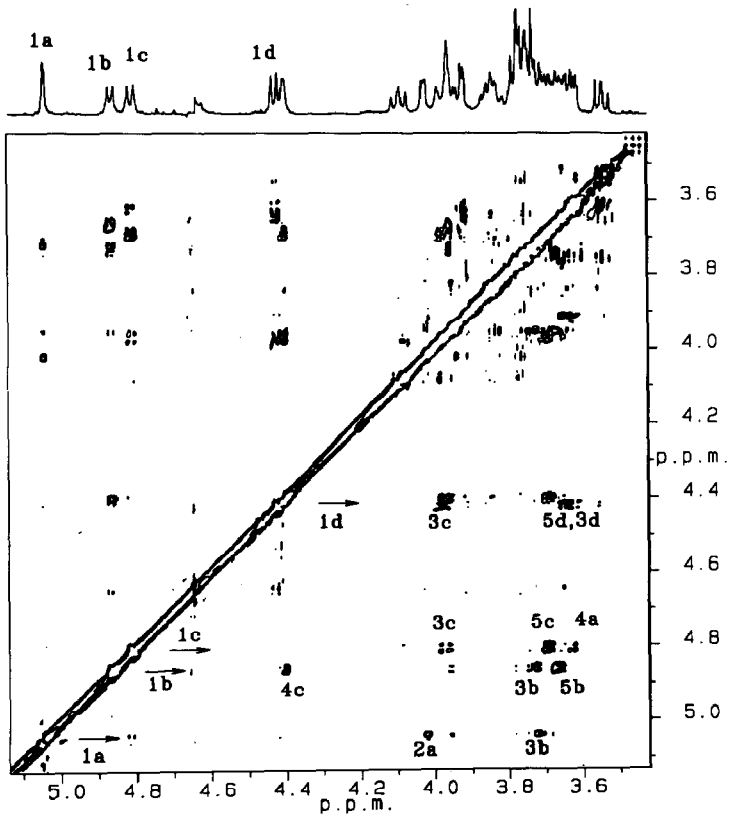


Fig. 7. Contour plot of the ring-proton region (5.13–3.42 p.p.m.) of the *A. pleuropneumoniae* type 7 O-polysaccharide recorded at 37°. Cross-peaks arising from n.O.e. involving the anomeric protons are indicated.

permitted **c** to be identified as the 2-acetamido-2-deoxy-D-galactose residue while **b** and **d** correspond to the two D-galactose residues revealed by chemical analysis.

In agreement with the methylation analysis data, significant deshielding of the ^{13}C resonances assigned to C-4**a**, C-3**b**, C-3**c**, and C-4**c** (Table IV) indicated those positions as the sites of the glycosyl linkages[9]. The ^{13}C chemical shifts associated with residue **d** were similar to values reported for methyl β-D-galactopyranoside[9], suggesting **d** to be the non-reducing end group present in the repeating unit.

Proton n.O.e. measurements confirmed the glycose sequences and the linkage positions[14]. A 2D phase-sensitive NOESY experiment readily provided the n.O.e. information (Fig. 7) while avoiding possible complications arising from the proximity of the H-1**d** (4.42 p.p.m.) and H-4**c** (4.40 p.p.m.) resonances[15]. Thus, the occurrence of inter-residue n.O.e. relating the H-1**a**/H-3**b**, H-1**b**/H-4**c**, and H-1**c**/H-4**a** pairs (Fig. 7) establishes the linear sequence of glycoses within the main chain of the repeating unit as →**a**→**b**→**c**→. Moreover, the inter-residue n.O.e. observed between H-1**d** and H-3**c** confirms the linkage of the non-reducing β-D-galactopyranosyl end-groups (**d**) to O-3 of

the 3,4-di-*O*-substituted 2-acetamido-2-deoxy-β-D-galactopyranosyl residues (**c**).

Thus, the combined evidence permits the repeating unit of the *A. pleuropneumoniae* type 7 O-polysaccharide to be assigned the structure **5**.

The LPS O-polysaccharide of *A. pleuropneumoniae* serotype 4 is also composed of a branched tetrasaccharide repeating-unit with the same main chain structure as the serotype 7 O-chain, but differs in the nature of the non-reducing end-groups[4]. In the O-chain of serotype 4, the di-*O*-substituted 2-acetamido-2-deoxy-β-D-galactopyranosyl residues are substituted by (1→3)-linked β-D-glucopyranosyl residues instead of the (1→3)-linked β-D-galactopyranosyl residues present in the O-chain of serotype 7.

EXPERIMENTAL

Production of lipopolysaccharide and O-polysaccharide. — *A. pleuropneumoniae* (WF 83) serotype 7 was grown in Bacto PPLO broth (yield, 500 g wet weight/L), and the LPS was isolated by the aqueous phenol-extraction procedure[5]. Solutions of LPS (0.2 g) in aqueous 1% acetic acid (100 mL) were kept at 100° for 2 h, and the O-polysaccharide was recovered by gel filtration on Sephadex G-50 and DEAE-Sephacel as previously described[16].

Analytical methods. — Analytical methods were essentially as described[17] except that the configurations of glycoses were determined by g.l.c. of the acetylated (−)-2-octyl derivatives[8]. The following g.l.c.–m.s. conditions were employed using an OV-17 fused-silica capillary column (Quadrex Corp.): *A.* (for alditol acetates), 180° for 2 min then 4°/min to 240°; *B* (for partially methylated alditol acetates), 180° for 2 min then 2°/min to 240°; *C* (for methylated disaccharides), 220° for 1 min then 7°/min to 280°; *D* (for methylated trisaccharides), 240° for 1 min then 10°/min to 280°. For analysis of higher molecular weight oligosaccharide, a DB-1 capillary column (J & W Scientific) was used with program *E*, 180° to 350° at 10°/min.

T.l.c. was performed on silica gel (Merck) with 6:3:1 1-propanol–conc. NH_3–water, and mobilities are recorded relative to galactose (R_{Gal}) or sucrose (R_{Suc}). Sodium dodecyl sulphate (SDS)–PAGE was performed under the conditions previously recorded[6].

Methylation analyses. — Samples (2 mg) were methylated according to the Hakomori procedure[18] and the products were isolated by partition between dichloromethane and water. Methylated oligosaccharides were analysed directly by g.l.c.–m.s. (programs *C, D, E,* or *F*), and retention times are recorded relative to that of methylated sucrose (T_{MS}). Methylated products were hydrolysed with 4M trifluoroacetic for 1 h at 125° and the released partially methylated glycoses were reduced with aqueous sodium borodeuteride for 1 h at 24°. The solutions were acidified with 10% acetic acid in methanol and concentrated to dryness, and methanol (3 × 1 mL) was evaporated from the residues which were then acetylated with acetic anhydride–pyridine (1:1, 0.5 mL) for 30 min at 100°. The products were analysed by g.l.c.–m.s. (program *B*).

Periodate oxidation[10]. — A solution of polysaccharide (50 mg) in 0.1M sodium metaperiodate (10 mL) was kept in the dark for 4 days at 24°. Excess of periodate was

reduced with ethylene glycol (0.5 mL), and the oxidised polymer was reduced with an excess of sodium borohydride. After storage for 16 h at 24°, the cooled solution was neutralised with 50% acetic acid, dialysed, and lyophilised. Smith-type hydrolysis of the periodate-oxidised and reduced polymer was effected with aqueous 1% acetic acid for 3 h at 100°, and the degradation products were fractionated on a column of Bio-Gel P-2.

Partial hydrolysis. — A solution of polysaccharide (50 mg) in 0.05M trifluoroacetic acid (100 mL) was boiled under reflux for 3 h. The acid was neutralised with aqueous 1% ammonium hydroxide and, after lyophilisation, the degradation products were fractionated by gel-permeation chromatography on Bio-Gel P-2.

N.m.r. spectroscopy. — Measurements were made at 27 or 37° on solutions in D_2O with a Bruker AM-500 spectrometer, equipped with an Aspect 3000 computer, using standard Bruker software. One-dimensional ^1H- and ^{13}C-n.m.r. experiments were obtained for spectral widths of 2.2 and 25 kHz, respectively, as described[17]. Chemical shifts are referenced relative to those of the methyl resonances from internal acetone (δ_H 2.225 p.p.m., δ_C 31.07 p.p.m.).

Two-dimensional COSY[19] and Relay COSY[20] spectra were measured over the full spectral width (2400 Hz), using data sets ($t_1 \times t_2$) of 256 × 2048 points, and processed to give magnitude spectra[17]. The COSY was also carried out using a narrow sweep-width (990 Hz) and a data set of 512 × 2048 points (Fig. 6).

The NOESY experiment was carried out in the phase-sensitive mode[21,22], using a 256 × 2048 point data set, a spectral width of 2400 Hz, a 200-ms mixing time, and a 1.2-s recycle delay; 64 transients were acquired for each t_1 value. Resolution enhancement was achieved by means of a sine-bell window function phase-shifted by $\pi/32$ and $\pi/16$ in the t_1 and t_2 dimensions, respectively. The data were zero-filled to 2048 × 2048 points and processed to give an absorption spectrum with symmetrisation.

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

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