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STRUCTURE OF THE LIPOPOLYSACCHARIDE ANTIGENIC O-CHAIN PRODUCED BY *Actinobacillus pleuropneumoniae* SEROTYPE 4 (ATCC 33 378)*

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ABSTRACT

The structure of the antigenic O-polysaccharide part of the S-type lipopolysaccharide produced by *Actinobacillus pleuropneumoniae* serotype 4 has been determined by periodate oxidation, methylation, partial hydrolysis, and ^{1}H- and ^{13}C-n.m.r. spectroscopy. The O-polysaccharide structure has a branched-tetrasaccharide repeating unit, {→3)-β-D-Gal*p*-(1→4)-[β-D-Glc*p*-(1→3)]-β-D-Gal*p*NAc-(1→4)-α-L-Rha*p*-(1-}$_n$. The structure resembles that of the lipopolysaccharide O-chain of *A. pleuropneumoniae* serotype 7, and their common epitopes may account for the apparent serological cross-reactivity observed between the two serotypes when incompletely adsorbed, anticapsular-typing sera are used.

INTRODUCTION

The lipopolysaccharide components of *Actinobacillus pleuropneumoniae* are implicated in the pathogenesis of severe respiratory disease in pigs[1]. So far, ten serotypes of *A. pleuropneumoniae,* based on their different capsular polysaccharides, have been described[2–6]. Our structural studies of these capsular materials[7] indicated that their structures are sufficiently diverse that monospecific-typing antisera for the differentiation of the serotypes would be easy to obtain. However, serological cross-reactivities between the serotypes have been reported[8].

To date, each *A. pleuropneumoniae* serotype examined has revealed a unique lipopolysaccharide (LPS) differing in the structure of their O-polysaccharide component. Since antibodies raised against killed whole cells of *A. pleuropneumoniae* contain antibody directed to both the capsule and lipopolysaccharide antigens, it is possible that the use of unabsorbed or partially absorbed typing-antisera may give apparent serotype cross-reactions due to antibody directed towards lipopolysaccharide O-chain antigens rather than the capsules. A structural identification of

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the O-polysaccharide associated with each *A. pleuropneumoniae* serotype should provide a basis for the understanding of observed serological cross-reactions and subsequently assist in the production of specific serogroup-typing antisera.

We describe herein the structural determination of the lipopolysaccharide O-chain produced by *A. pleuropneumoniae* serotype 4 and relate the structure to the lipopolysaccharide O-chain of *A. pleuropneumoniae* serotype 7, thus offering an explanation of the reported serological cross-reaction between these two serotypes.

EXPERIMENTAL

Production of lipopolysaccharide and O-polysaccharide. — *Actinobacillus pleuropneumoniae* (ATCC 33378) serotype 4 was grown in Bacto PPLO broth (yield, 3 g wet weight/L) and LPS was isolated by the aqueous phenol-extraction procedure as previously described[9]. Solutions of LPS (0.2 g) in aqueous 1% acetic acid (100 mL) were kept at 100° for 1 h, the precipitated Lipid A was removed by low-speed centrifugation, and the O-polysaccharide was recovered by Sephadex G-50 gel filtration as previously described[9].

Analytical methods. — Quantitative methods for glycose analysis and g.l.c. and g.l.c.–m.s. methods used were the same as previously described[9]. A sample (0.5 mg) of oligo- or poly-saccharides was hydrolyzed in a sealed glass-tube with 10M HCl (1 mL) for 15 min at 90°, or with 2M trifluoroacetic acid (1 mL) for 17 h at 100°, followed by concentration to dryness. Glycoses were determined by g.l.c. of their alditol acetate derivatives[10] using *myo*-inositol as an internal standard. The configuration of glycoses was established by capillary g.l.c. of their per(trimethylsilyl)ated[11] or peracetylated (−)-2-butyl glycosides[12]. Lipids were identified by g.l.c.–m.s. (program C) of their methyl esters derived by sealed-tube methanolysis of samples (1 mg) with 3% methanolic HCl for 4 h at 100°. T.l.c. was performed on silica gel (Merck) with 6:3:1 propanol–conc. NH_3–water. Gel filtration was performed on columns of Sephadex G-50 (2 × 100 cm) or Bio-Gel P-2 (200–400 mesh) (Bio-Rad Labs.). Sodium dodecyl sulfate(SDS)–PAGE and methylation analyses were made under the same previously recorded conditions, as were periodate oxidation studies[9], except that the Smith-type hydrolysis of the periodate-oxidized and reduced ($NaBH_4$) product was effected with 0.5M trifluoroacetic acid for 48 h at 20°, followed by gel filtration of the products on a column of Bio-Gel P-2.

Partial hydrolysis. — O-Polysaccharide (10 mg) in 0.5M trifluoroacetic acid (5 mL) was heated for 2 h at 100° and, after successive additions of water and evaporations, the products in water (5 mL) containing methanol (0.3 mL) were *N*-acetylated by treatment with acetic anhydride (0.1 mL) for 2 h at 20°. After evaporation of the solution, the residue in water (1 mL) was treated with NH_3 (0.1 mL), and the products were fractionated by gel filtration on a column of Bio-Gel P-2.

N.m.r. spectroscopy. — ^{1}H-Decoupled ^{13}C-n.m.r. spectra (125 MHz) were

recorded at 37° for a 25-kHz spectral width, by use of a $\pi/2$ pulse and a 32 K data set on a Bruker AM500 spectrometer. Chemical shifts are expressed relative to the signal of internal acetone (1%, δ 31.07). ^{1}H-N.m.r. spectra (500 MHz) were recorded at 37° with a spectral width of 2.5-kHz, a $\pi/2$ pulse, and a 16 K data set for digital resolution of 0.3 Hz/point. Chemical shifts are expressed relative to the signal of internal acetone (0.1%, δ 2.225) and coupling constants are reported in Hz. Polysaccharide samples were twice exchanged with D_2O, and then examined as solutions in 99.8% D_2O (5-mm diam. tubes) at a concentration of 15 mg/mL.

^{1}H-Homonuclear-correlated, 2D-n.m.r. experiments, phase-sensitive COSY[13,14], and relay COSY[15] were performed at 37° using the standard software provide by Bruker DISNMR. Quadrature detection in both dimensions was employed in the COSY experiments. In the phase-sensitive COSY, the initial (t_1,t_2), matrices of 512 × 2048 data points were zero filled to 2048 × 2048 data points to provide 1 Hz/point digital resolution in both domains. Resolution enhancement in both domains was done by nonshifted sine-bell function prior to Fourier transformation. The number of transients f.i.d. was 8 for the COSY. The n.O.e. experiments were performed in the difference mode with sequential irradiation of each line in a multiplet[16,17]. The total irradiation time was 200 ms.

A heteronuclear ^{13}C–^{1}H shift-correlation experiment was done on a Bruker AM500 spectrometer using a CHORTLE (carbon–hydrogen correlation from one-dimensional polarization transfer spectra by the least-squares analysis) technique[18] at 37° with a solution (15 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 4000 transients/f.i.d.

RESULTS AND DISCUSSION

Extraction of *Actinobacillus pleuropneumoniae* serotype 4 cells (275 g, wet weight) by a modified lysozyme–phenol–water procedure[19], followed by purification of the LPS by repeated ultracentrifugation, afforded an aqueous-phase LPS (540 mg) and a phenol–phase soluble LPS (373 mg). Both LPS preparations gave identical patterns on SDS–PAGE analysis[20] and showed a typical banding indicative of S-type LPS[21]. The aqueous phase LPS was found to be contaminated by a capsular polysaccharide and was not examined further.

Fission of the phenol-phase soluble LPS with hot 2% acetic acid (2 h) resulted in a degradation of the O-chain. Partial hydrolysis of the phenol-phase LPS (200 mg) under milder conditions (1% acetic acid, 1 h, 100°) gave an insoluble Lipid A (64 mg). Sephadex G-50 gel filtration of the water-soluble products afforded an O-chain which was separated into three fractions according to the molecular weight (K_{av} 0.03, 7.4 mg; K_{av} 0.07, 6.2 mg; and K_{av} 0.17, 8.1 mg), a core oligosaccharide (K_{av} 0.67, 13.0 mg), and a monosaccharide fraction (K_{av} 0.83, 17.7 mg) containing 3-deoxy-2-octulosonate.

The O-chain polysaccharide, fraction K_{av} 0.03, had $[\alpha]_D^{22}$ −21.8° (*c* 0.74, water). *Anal.* Found: C, 40.52; H, 6.07; N, 1.84; ash, 0.0. On quantitative analysis,

TABLE I

G.L.C.-M.S. OF THE PRODUCTS OF METHYLATION ANALYSIS OF THE O-CHAIN OF *A. pleuropneumoniae* SEROTYPE 4

Derivative	T_{GM}	*Molar ratio*
1,4,5-Tri-*O*-acetyl-2,3-di-*O*-methyl-L-(1-^{2}H)rhamnitol	0.89	0.65
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-(1-^{2}H)glucitol	1.00	1.00
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-(1-^{2}H)galactitol	1.45	1.21
1,3,4,5-Tetra-*O*-acetyl-2-deoxy-6-*O*-methyl-2-(*N*-methyl-acetamido)-D-(1-^{2}H)galactitol	4.20	0.33

the O-chain was found to be composed of D-glucose, D-galactose, 2-acetamido-2-deoxy-D-galactose, and L-rhamnose in the molar ratios of 1.0:1.0:0.4:0.6. The configuration and identification of the glycoses were established by their isolation by paper chromatography, followed by g.l.c. of their acetylated (−)-2-butyl glycoside derivatives[12]. The L configuration of rhamnose was demonstrated by the method of Gerwig *et al.*[11]. Subsequent analysis of the lower-molecular-weight fractions of the O-chain (K_{av} 0.07 and K_{av} 0.17) revealed the presence of D-rhamnose (~5–6%), which was probably present in the region close to the core of the LPS.

The ^{1}H-n.m.r. spectrum (500 MHz, 37°) of the O-chain polysaccharide contained four signals for anomeric protons at δ 5.044 (unresolved, 1 H), 4.822 (d, 1 H, $J_{1,2}$ 6.8 Hz), 4.807 (d, 1 H, $J_{1,2}$ 8.2 Hz), and 4.483 (d, 1 H, $J_{1,2}$ 7.8 Hz); one signal for CH_3 of NAc at δ 2.043 (s, 3 H); and one signal for CH_3 of 6-deoxyglycose at δ 1.320 (d, 3 H, $J_{5,6}$ 6.1 Hz). Consistent with these results, the ^{13}C-n.m.r. spectrum (125 MHz, 37°) contained signals for four anomeric carbon atoms at δ 105.21, 103.74, 102.90, and 102.82; one signal for C-2 of the 2-acetamido-2-deoxyglycopyranosyl residue at δ 52.88; signals for one *N*-acetyl group at δ 23.15 and 175.87; and one signal for C-6 of a 6-deoxyglycose at δ 17.92.

The methylated and hydrolyzed 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 that the O-chain of *A. pleuropneumoniae* serotype 4 is composed of a tetrasaccharide repeating-unit having at the 2-acetamido-2-deoxy-D-galactopyranosyl residue, which is linked through O-3 and O-4, a branch with a D-glucopyranosyl terminal group.

In order to determine the sequence of the glycose units in the proposed branched tetrasaccharide repeating-unit, the anomeric configuration of the component monosaccharides, and their ring sizes, a complete assignment of the ^{1}H and ^{13}C resonances was achieved, and was followed by one-dimensional n.O.e. difference spectroscopy.

Assignments of the proton resonances were made from a phase-sensitive COSY[13,14] and relay COSY[15] experiments. The residues in the O-chain polysaccharide were arbitrarily labelled *a* to *d* according to the order of their H-1 resonances in the ^{1}H-n.m.r. spectrum, the connectivities were traced *via* cross-

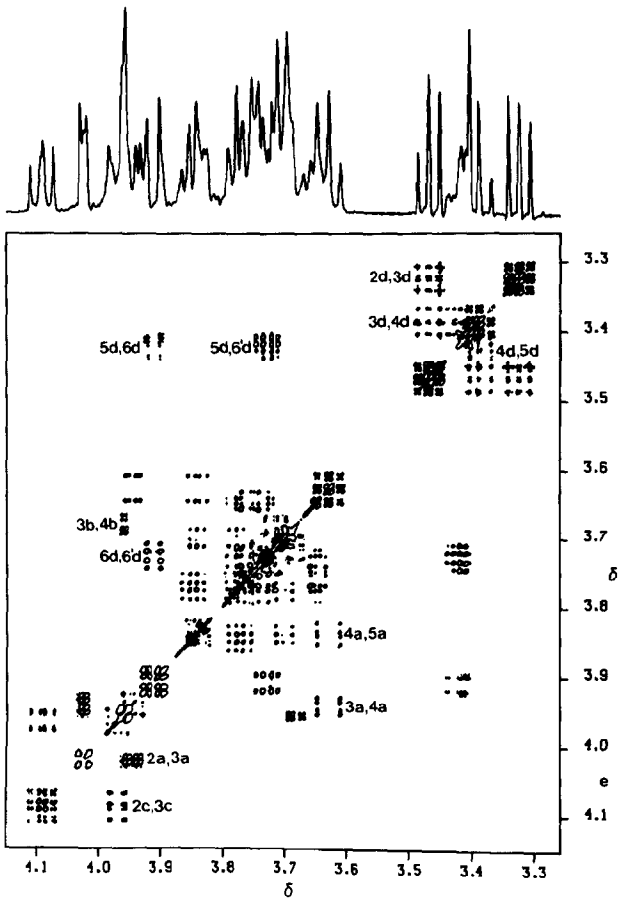


Fig. 1. Phase-sensitive COSY contour plot of the ring proton region of the O-polysaccharide of *A. pleuropneumoniae* serotype 4.

TABLE II

^{1}H-CHEMICAL SHIFTS[a] FOR THE O-CHAIN POLYSACCHARIDE OF *Actinobacillus pleuropneumoniae* SEROTYPE 4

Proton	*Unit* a [→4)-α-L-*Rha*p-(1→]	*Unit* b [→3)-β-D-*Gal*p-(1→]	*Unit* c [→4)-β-D-*GalpNAc*-(1→] 3 ↑	*Unit* d [β-D-*Glc*p-(1→]
H-1	5.044[b]	4.822 (6.8)[c]	4.807 (8.2)[c]	4.483 (7.8)[c]
H-2	4.024	3.71	4.090	3.324
H-3	3.94	3.68	3.97	3.47
H-4	3.62	3.96	4.407	3.39
H-5	3.84	3.64	3.70	3.42
H-6a	1.320	3.73	3.84	3.91
H-6b		3.77	3.77	3.725
NAc			2.043	

[a]In δ values measured at 37° for a solution in D_2O with 0.1% acetone as the internal reference (δ 2.225).
[b]Not resolved. [c]$J_{1,2}$ in Hz.

peaks in the COSY spectrum (Fig. 1), and the majority of the proton resonances were assigned in this manner (Table II). These were confirmed by correlation with the corresponding ^{13}C resonances by a ^{13}C–^{1}H shift-correlated experiment CHORTLE[18], which also served to establish the ^{13}C assignments (Table III). Since all proton resonances were assigned, it was possible to determine the monosaccharide sequence by one-dimensional n.O.e. difference experiments[16,17]. Irradiation of each anomeric resonance *a–d* gave an interresidue n.O.e. which provided the linkage information (Fig. 2). Thus, the sequence **1** was established. In the case of residue *b*, saturation of the H-1*b* resonance (Fig. 2b) produced intraresidue n.O.e's to both H-3*c* and H-4*c*. The linkage substitution of residue *c* was obtained from the Smith degradation and partial hydrolysis studies (see below).

N.O.e. experiments were also used to confirm the anomeric configuration of the glycose units. A β-D-glycopyranosyl unit exhibits significant intraresidue n.O.e.'s between H-1, -3, and -5, whereas none are seen for the corresponding protons in an α-D-glycopyranosyl unit. Thus, the residues *b*, *c*, and *d* have β-D configurations and residue *a* has an α-L configuration (Fig. 2). Based on the ^{1}H-chemical-shift data, $J_{1,2}$ values, and results of the n.O.e. difference experiments, residue *a* was assigned to the α-L-rhamnopyranosyl unit, residue *b* ($J_{1,2}$ 6.8 Hz) to the β-D-galactopyranosyl unit, residue *c* ($J_{1,2}$ 8.2 Hz) to the 2-acetamido-2-deoxy-β-D-galactopyranosyl unit, and residue *d* ($J_{1,2}$ 7.8 Hz) to the β-D-glucopyranosyl unit.

Further proof for the proposed structure of the tetrasaccharide repeating-unit was obtained from the partial hydrolysis of the O-chain with 0.5M trifluoroacetic acid. *N*-Acetylation of the product, followed by gel filtration on Bio-Gel P-2 afforded a disaccharide (K_{av} 0.65) which gave a single spot on t.l.c. (R_F 0.37) and, on hydrolysis, afforded 2-amino-2-deoxy-D-galactose and D-glucose (1:1). The ^{1}H-n.m.r. spectrum (200 MHz, 27°) of reduced ($NaBH_4$) disaccharide showed a single anomeric proton signal at δ 4.54 (d, 1 H, $J_{1,2}$ 7.7 Hz) and a signal at δ 2.05 (s, 3 H, CH_3 of NAc). The ^{13}C-n.m.r. spectrum (50 MHz, 27°) contained a signal for one

TABLE III

^{13}C-CHEMICAL SHIFTS[a] FOR THE O-CHAIN POLYSACCHARIDE[b] OF *Actinobacillus pleuropneumoniae* SEROTYPE 4

Carbon atom	*Unit* a [→4)-α-L-*Rha*p-(1→]	*Unit* b [→3)-β-D-*Gal*p-(1→]	*Unit* c [→4)-β-D-*Gal*p*NAc*-(1→] 3 ↑	*Unit* d [β-D-*Glc*p-(1→]
C-1	102.90	103.74	102.82	105.21
C-2	69.29	71.48	52.88	73.84
C-3	71.11	81.13	80.36	76.26
C-4	81.36	71.11	75.40	70.55
C-5	68.35	75.78	74.88	76.68
C-6	17.92	61.77	61.17	61.69
$NHCOCH_3$			23.15	
$NHCOCH_3$			175.87	

[a]In δ values measured at 37° with 1% acetone as an internal reference (δ 31.07). [b]Assignments confirmed by a ^{13}C–^{1}H-shift correlation experiment.

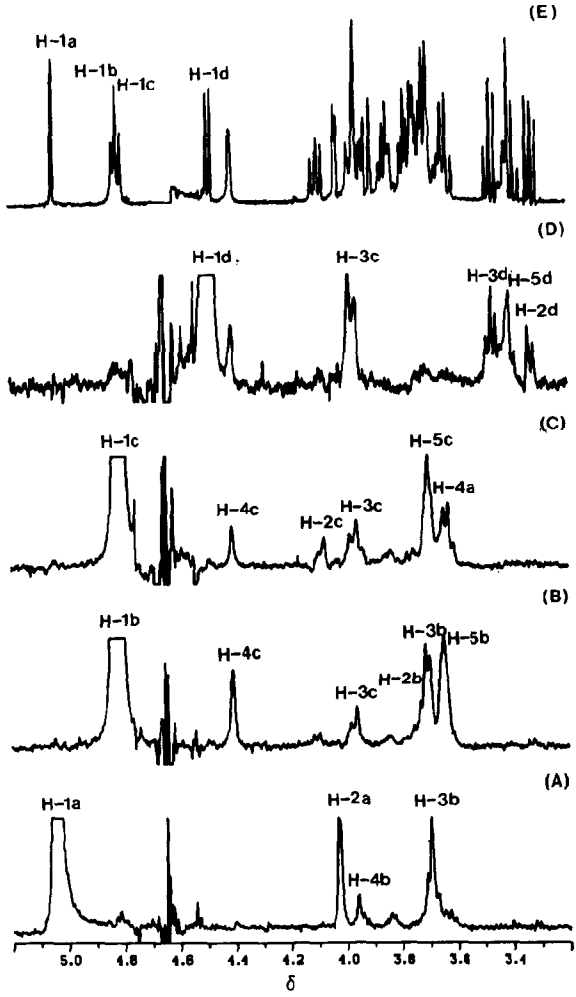
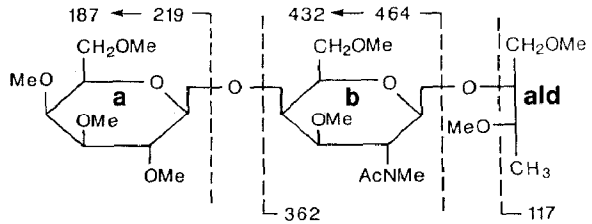


Fig. 2. N.O.e. difference spectra for the O-polysaccharide on saturation of the resonances: (A) H-1*a*, (B) H-1*b*, (C) H-1*c*, (D) H-1*d*, and (E) an off-resonance control spectrum, measured at 37°. See text for the notation.

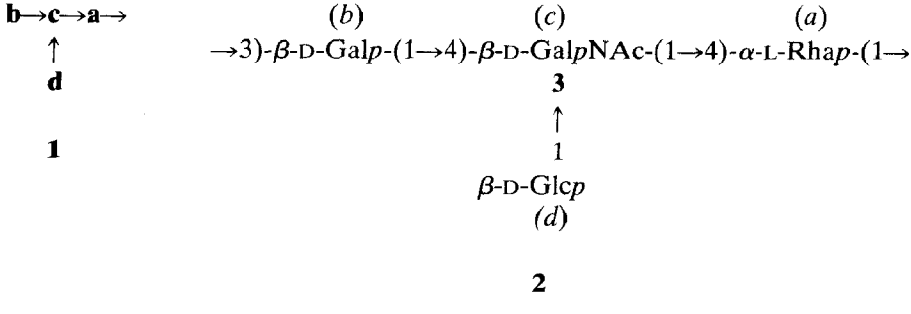
anomeric carbon atom at δ 105.8. Hydrolysis of the permethylated, reduced disaccharide yielded products which, after reduction ($NaBD_4$) and acetylation, on g.l.c.–m.s. analysis (program B) gave 2,3,4,6-tetra-*O*-methyl-D-(1-^{2}H)glucitol and 2-deoxy-1,4,5,6-tetra-*O*-methyl-1-(*N*-methylamino)D-galactitol (1.0:0.2). Consideration of the composition, g.l.c.–m.s., and n.m.r. evidence allowed a disaccharide to be identified as D-Glc*p*-(1→3)-D-GalNAc. These results indicated that the terminal D-glucopyranosyl group was attached to O-3 of the 2-acetamido-2-deoxy-D-galactopyranosyl residue. Periodate oxidation was used to confirm the sequence of glycoses in the main chain. Smith-type hydrolysis of the reduced ($NaBH_4$),

periodate-oxidized product with 0.5M trifluoroacetic acid and subsequent gel filtration chromatography of the products on Bio-Gel P-2 afforded a trisaccharide (K_{av} 0.61), which gave a single spot on t.l.c. (R_F 0.47) and, on hydrolysis, afforded 1-deoxyerythritol, D-galactose, and 2-amino-2-deoxy-D-galactose (0.5:1.0:1.3). The ^{1}H-n.m.r. spectrum (500 MHz, 27°) of the trisaccharide showed two anomeric signals at δ 4.66 (d, 1 H, $J_{1,2}$ 8.4 Hz) and 4.59 (d, 1 H, $J_{1,2}$ 6.6 Hz); one signal for CH_3 of NAc at δ 2.05; and one signal for CH_3 of 1-deoxyerythritol at δ 1.17 p.p.m. The ^{13}C-n.m.r. spectrum showed signals at 105.2 (anomeric C), 102.4 (anomeric C), 23.0 (CH_3 of NAc), and 17.7 (CH_3 of 1-deoxyerythritol). G.l.c.–m.s. analysis of the permethylated compound gave a mass-spectrum fragmentation pattern consistent with the presence of 1-deoxytetritol at the potential reducing end of the oligosaccharide. The following fragments were obtained (Scheme 1): primary ions



Scheme 1. Fragmentation pattern of the methylated oligosaccharide obtained after Smith degradation of the O-polysaccharide with some primary and secondary fragments.

of the A series[22], characteristic of the terminal, nonreducing hexosyl residue, were at *m/z* 219 (**aA**$_1$) and 187 (**aA**$_2$). Those of the 1-deoxytetritol residue were at *m/z* 117 (**ald**), 177 (**ald J**$_1$), 362 (**bald**), and 422 (**bald J**$_1$). Expected secondary ions appeared at *m/z* 101, 88, 71, and 45. The g.l.c.–m.s. analysis in the c.i. mode confirmed the presence of one hexosyl, one 2-acetamido-2-deoxyhexosyl, and a 1-deoxytetritol residue, *i.e.*, peaks at *m/z* 117 (**ald**), 219 (**aA**$_1$), 362 (**bald**), and 464 (**abA**$_1$); and parent peak $M^+ + 1$ at *m/z* 598. Hydrolysis of the permethylated trisaccharide yielded products, which after reduction ($NaBD_4$) and acetylation, on g.l.c.–m.s. analysis (program B) were identified as 2,3,4,6-tetra-*O*-methyl-D-galactitol and 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylamino)-D-galactitol (1.0:0.4). The aforementioned combined results allowed the structure of the trisaccharide to



be given as D-Gal*p*-(1→4)-D-Gal*p*NAc-(1→3)-1-deoxyerythritol. The 1-deoxyerythritol was derived from an oxidation of the L-rhamnopyranosyl residue.

The combined chemical and n.m.r. evidence permits the structure of the O-chain polysaccharide to be established as the branched repeating tetrasaccharide unit **2**. Structural similarity between O-antigenic chains of *A. pleuropneumoniae* serotype 4 and serotype 7, the latter differing in the presence of a terminal β-D-galactopyranosyl group[23], could account for a serological cross-reactivity between serotypes 4 and 7[24]). The "lipid A" isolated was not structurally examined, but it was found to be composed of the following fatty acids: 3-hydroxydodecanoic acid (1.3%), n-tetradecanoic acid (26.3%), 3-hydroxytetradecanoic acid (56.3%), n-hexadecanoic acid (8.2%), and *cis*-9-hexadecenoic acid (4.6%). It also contained 2-amino-2-deoxy-D-glucose (7.0%) and a phosphoric ester (2.2%).

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