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STRUCTURE OF THE CAPSULAR POLYMER OF *Actinobacillus pleuropneumoniae* SEROTYPE 6*

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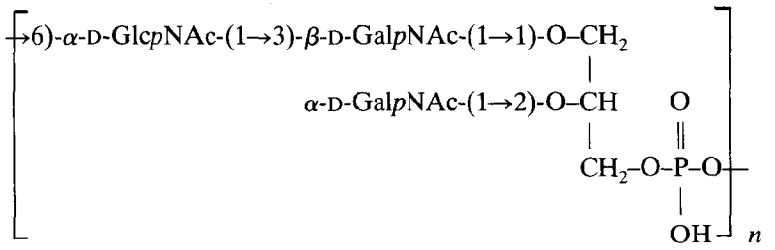
ABSTRACT

The capsular polymer of *Actinobacillus pleuropneumoniae* serotype 6 (ATCC 33590) was investigated by methylation, dephosphorylation, partial hydrolysis, and ^{1}H- and ^{13}C-n.m.r. methods. It was found to be a high-molecular-weight polymer composed of triglycosylglycerol phosphate repeating-units joined through monophosphate diester linkages, and has the structure:



INTRODUCTION

Actinobacillus pleuropneumoniae is one of the most common causal agents of pleuropneumonia in pigs and is responsible for severe economic losses in the swine industry. The serotyping of *A. pleuropneumoniae* is related to capsular antigens and, at present, ten serotypes of *A. pleuropneumoniae* are recognized[1–5]. Serological cross-reactions have been reported between *A. pleuropneumoniae* serotypes 1 and 9, serotypes 3, 6, and 8, and serotypes 4 and 7[6,7], but the immunochemical basis for these observations is not known. It is of interest to determine, from the point of view of epidemiological studies and vaccine development, whether the capsules or somatic lipopolysaccharide antigenic components are involved in the polyagglutinability of *A. pleuropneumoniae* serotypes. As part of a systematic study of the structures of the capsules and lipopolysaccharides of *A. pleuropneumoniae*, we now report on the structure of the serotype 6 specific capsular polymer.

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EXPERIMENTAL

Cell production. — *Actinobacillus pleuropneumoniae* serotype 6 (ATCC 33590) was grown in brain heart infusion (BHI, Difco) supplemented with NAD and 1% of D-glucose, using a 75L IF-75 fermentor (New Brunswick Scientific) in an 18-h fermentation at 37°. Cells were killed with phenol (0.75%, final concentration) prior to harvesting using a Sharples continuous centrifuge (yield ~3 g wet weight/L).

Isolation and purification of capsular polymer. — Cells were washed with 2.5% saline and, following low speed centrifugation, the dialyzed saline extract was digested with ribonuclease, deoxyribonuclease, and trypsin, and the crude capsular polymer was recovered by precipitation with 5 volumes of aqueous 95% ethanol. The polymer was further purified by precipitation of, and recovery from, its insoluble cetyltrimethylammonium complex as previously described[8]. Pure capsular polymer was obtained from the latter material by gel filtration on Sephadex G-50 followed by ion-exchange chromatography on DEAE-Sephacel from which it was eluted with ~0.1M sodium chloride.

DEAE-Sephacel chromatography. — A solution of the crude capsular polymer (50 mg) in water (1 mL) was applied to a column (1.2 × 35 cm) of DEAE-Sephacel (Pharmacia Fine Chemicals) equilibrated with 0.05M Tris-HCl buffer (pH 7.2), and the column was irrigated with the buffer (50 mL) followed by a gradient of 0→0.5M NaCl in the same buffer, and the collection of eluted fractions (1 mL).

Gel filtration. — Gel filtration was done on a column (2 × 100 cm) of Sephadex G-50 (Pharmacia Fine Chemicals), using 0.05M pyridinium acetate (pH 4.5) as the eluant, or on a column (1.6 × 87 cm) of Bio-Gel P2 (200–400 mesh, Bio-Rad Labs.), using distilled water as the eluant.

Column eluants were continuously monitored using a Waters 403 refractometer, and samples (100 μL) of collected fractions were analyzed colorimetrically for aldose, aminodeoxyaldose, and phosphate.

Gas–liquid chromatography. — G.l.c. was done with a Hewlett–Packard 5830A chromatograph fitted with a flame-ionization detector, using the following conditions: capillary column (0.32 mm × 25 m) 007 series bonded phase, fused silica OV-17 (Quadrex Corp.); temperature programs, *A* 180° (2 min) → 240° at 4°/min; *B* 200° (2 min) → 240° at 1°/min. Nitrogen was the carrier gas at 30–40 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 performed with a Hewlett–Packard 5985B system, using the conditions *A* and *B* and an ionization potential of 70 eV.

The configurations of glycoses were established by capillary g.l.c. of their acetylated (−)-2-butyl glycosides[9], and the identity of each glycose was established by comparison of its g.l.c. retention time and mass spectrum with those of a reference sample.

Analytical methods. — Quantitative methods used were (*a*) the phenol–

sulfuric acid methods for neutral glycose[10], (*b*) the modified Elson–Morgan method for aminodeoxyglycoses[11], and (*c*) the method of Chen *et al.*[12] for phosphate. Glycoses were determined by g.l.c. of their derived alditol acetates[13], using inositol as the internal standard. For neutral glycoses, samples (1 mg) were hydrolyzed with 2M HCl (1 mL) for 16 h at 100° and the hydrolyzates were concentrated to dryness. T.l.c. was done on Silica Gel 60 (Merck), using 1-propanol–conc. ammonia–water (6:3:1).

Methylation analysis. — Samples (1–2 mg) were methylated according to the Hakomori method[14], the products were recovered by extraction with chloroform (5 mL), and the extracts were washed with water (5 × 5 mL). The products were hydrolyzed by the method of Stellner *et al.*[15] and, following concentration to dryness, the residues were dissolved in water (4 mL) and reduced with sodium borodeuteride (15 mg) for 17 h at 20°. Each reaction mixture was acidified with dilute acetic acid and concentrated to dryness, and methanol (5 × 10 mL) was distilled from the residue to remove borate. The residual alditols were acetylated by heating with acetic anhydride (1 mL) for 2 h at 115° and the products were analyzed directly by g.l.c.–m.s. (program *B*).

Dephosphorylation of capsular polymer. — A solution of the polymer (23 mg) in aqueous 48% hydrofluoric acid (0.5 mL) was kept for 72 h at 4° and then concentrated to dryness in high vacuum over NaOH. A solution of the residue in water (0.5 mL) was neutralized with aqueous 5% ammonium hydroxide and then fractionated by gel filtration on Bio-Gel P2.

N.m.r. spectroscopy. — Proton-coupled and -decoupled ^{13}C-n.m.r. spectra (125 MHz) were recorded at 27° for a 25-kHz spectral width, using a $\pi/2$ pulse and a 32k data set on a Bruker AM500 spectrometer. DEPT experiments[16] were performed for a 12.5-kHz spectra 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.).

^{1}H-N.m.r. spectra (500 MHz) were recorded at 27°, 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 solution of capsular polymer (50 mg) in water (3 mL) was passed through Chelex-100 resin (3 mL, Bio-Rad Labs.), and the lyophilized product was exchanged twice with D_2O before being examined as a solution in 99.8% D_2O (25 mg/mL, pD 9.0) in 5-mm diameter tubes.

Proton homonuclear-correlated 2D-n.m.r. experiments, phase sensitive COSY[17,18] and relay COSY[19], were performed at 27° using the standard software provided 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 × 4096 data points were zero filled to 2048 × 4096 data points, to provide 1 Hz/point digital resolution in both domains. Resolution enhancement in

both domains was done by non-shifted sine bell function prior to Fourier transformation. The number of transients per FID was 8 for the COSY.

A heteronuclear ^{13}C–^{1}H shift-correlated spectrum[20,21] was recorded on a polysaccharide sample (100 mg/mL, 5-mm diameter tube) with proton decoupling in the F_1 domain. The initial (t_1, t_2) matrix of 256 × 4096 points was Fourier-transformed to 512 × 4096 points, corresponding to a digital resolution of 5.2 Hz/point in the F_2 domain and 2.0 Hz/point in the F_1 domain.

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

RESULTS AND DISCUSSION

Actinobacillus pleuropneumoniae serotype 6 capsular polymer was obtained by regeneration from its insoluble cetyltrimethylammonium complex formed from the dialyzed saline wash of fermentor-grown cells (yield, 80 mg of polysaccharide/100 g wet weight of cells). On ion-exchange chromatography on DEAE-Sephacel, the pure polymer was eluted in a salt gradient at ~0.1M NaCl. On gel filtration on Sephadex G-50, the polymer was eluted at the void volume of the system (K_{av} 0.03), indicating it to have a high molecular weight and by analysis to be homogeneous with respect to aminodeoxyaldose and phosphate composition. The polymer had $[\alpha]_D$ +114° (*c* 1.5, water) and was essentially free from nucleic acid and protein, (*Anal.* Found: C, 40.39; H, 5.88; N, 5.38; and ash, 7.10%).

Quantitative g.l.c. analyses (program *A*) of the reduced ($NaBH_4$) and acetylated products of hydrolysis of the polymer showed 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxyglucitol (T_{GA} 1.27), 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxygalactitol (T_{GA} 1.32), and glycerol triacetate (T_{GA} 0.17) (0.7:2.0:0.1), while analysis of the nitrous acid-deaminated, reduced ($NaBH_4$) and acetylated products of hydrolysis by g.l.c. (program *A*) showed 1,3,4,6-tetra-*O*-acetyl-2,5-anhydromannitol (T_{GA} 0.68), 1,3,4,6-tetra-*O*-acetyl-2,5-anhydrotalitol (T_{GA} 0.73), and glycerol triacetate (T_{GA} 0.17) (1.0:1.2:0.8). Capillary g.l.c. of the acetylated (−)-2-butyl glycoside derivatives of the polymer hydrolysate confirmed the D configurations of the aminodeoxyglycoses, and colorimetric analysis showed the polymer to contain 7.8% of phosphate. The above analyses indicated the polymer to be composed of 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, glycerol, and phosphate in the molar ratios 1:2:1:1.

The ^{1}H-n.m.r. spectrum of the polymer contained signals for anomeric protons at δ 5.111 (d, 1 H, $J_{1,2}$ 3.8 Hz), 5.037 (d, 1 H, $J_{1,2}$ 3.7 Hz), and 4.565 (d, 1 H, $J_{1,2}$ 8.4 Hz), and two signals for CH_3 of NAc at δ 2.086 (s, 6 H) and 2.038 (s, 3 H). Consistent with these results, the ^{13}C-n.m.r. spectrum (125 MHz, 27°) of the polymer showed signals for three *N*-acetyl groups at 22.8, 23.1, 23.3, 175.1, 175.2, and 175.4 p.p.m., three signals for C-2 of 2-acetamido-2-deoxyglycopyranosyl residues at 50.6, 51.5, and 54.0 p.p.m., and three signals for anomeric carbon atoms

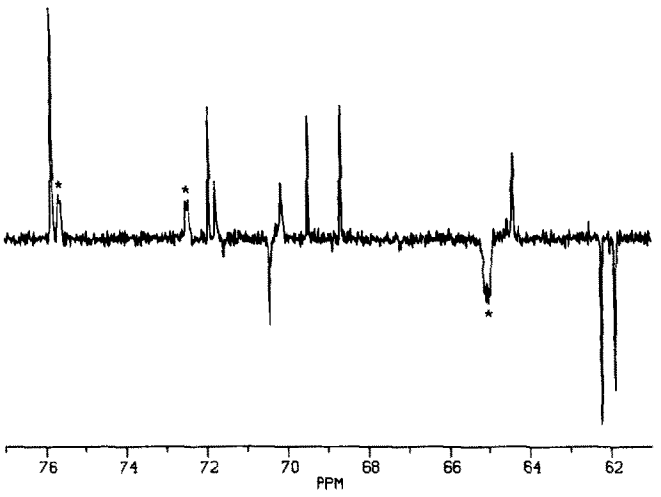
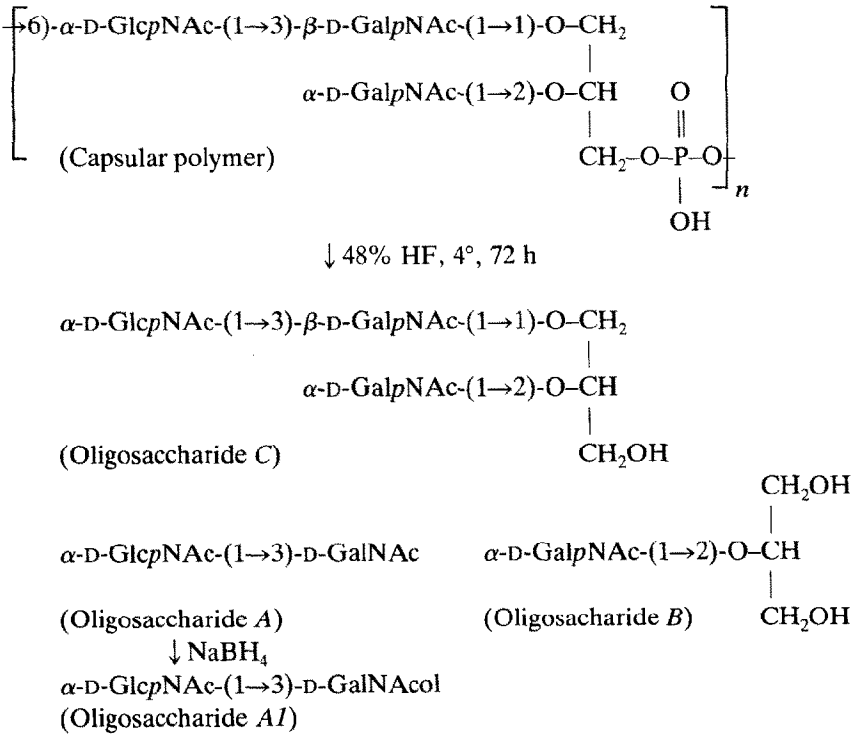


Fig. 1. Partial ^{13}C-DEPT spectrum of the capsular polymer of *A. pleuropneumoniae* serotype 6. Carbon–phosphorus couplings are marked with asterisks.



Scheme 1. Hydrolysis product of *A. pleuropneumoniae* serotype 6 capsular polymer.

at 94.1, 97.5, and 102.1 p.p.m. The ^{13}C-DEPT[16] spectrum of the polymer showed four primary-hydroxyl-group carbon atoms, suggesting the presence of an alditol moiety in addition to the three 2-acetamido-2-deoxyglycopyranosyl residues (Fig. 1). The presence of at least three phosphorus-coupled carbon atoms indicated a teichoic acid-type structure for the polymer (Scheme 1).

Treatment of the polymer with cold aqueous 48% hydrofluoric acid followed by fractionation of the products on Bio-Gel P2 afforded three oligosaccharides (*A*, *B*, *C*) (see Scheme 1).

Oligosaccharide *A* (K_{av} 0.59) had $[\alpha]_D$ +90° (*c* 0.3, water), gave a single spot in t.l.c. (R_F 0.48), and hydrolysis of the reduced ($NaBH_4$) oligosaccharide (*A1*) followed by g.l.c. analysis (program *A*) of the reduced ($NaBD_4$) and acetylated products gave 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-glucitol-*1-d* and 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-galactitol (0.8:1). The ^{1}H-n.m.r. spectrum of *A1* contained signals at δ 5.090 (d, 1 H, $J_{1,2}$ 3.7 Hz), 2.059 (s, 3 H, CH_3 of NAc), and 2.050 p.p.m. (s, 3 H, CH_3 of NAc), while the ^{13}C-n.m.r. spectrum showed signals at 175.2 ($NHCOCH_3$), 98.8 (anomeric C), 22.9 ($NHCOCH_3$), and 22.7 p.p.m. ($NHCOCH_3$). A consideration of the g.l.c.–m.s. data of methylated *A1* (Table I), in conjunction with the methylation analysis and ^{1}H- and ^{13}C-n.m.r. data, identified *A* as α-D-Glc*p*NAc-(1→3)-D-GalNAc.

Oligosaccharide *B* (K_{av} 0.65) had $[\alpha]_D$ +147° (*c* 0.3, water), gave a single spot in t.l.c. (R_F 0.54), and on hydrolysis afforded 2-amino-2-deoxy-D-galactose and glycerol (1.0:0.1). The ^{1}H-n.m.r. spectrum of *B* contained a single anomeric proton signal at δ 5.080 (d, 1 H, $J_{1,2}$ 3.7 Hz) and a signal at δ 2.041 (s, 3 H, CH_3 of NAc). The ^{13}C-n.m.r. spectrum of *B*, as expected from its composition, contained 11 signals, including the characteristic signals at 175.5 ($NHCOCH_3$), 98.0 (anomeric C), and 22.8 p.p.m. ($NHCOCH_3$) as well as three hydroxymethyl signals at 62.2,

TABLE I

G.L.C.–M.S. OF THE PRODUCTS OF THE METHYLATION ANALYSES OF *A. pleuropneumoniae* SEROTYPE 6 CAPSULAR POLYMER AND ITS OLIGOSACCHARIDE DEGRADATION PRODUCTS

Derivative	T_{GM}	*Molar ratios*		
		Capsular polymer	*Oligosaccharide C*	*Oligosaccharide A1*
3-*O*-Acetyl-2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol	1.90			0.5
1,5-Di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol-*1-d*	2.70		1.0	1.0
1,5-Di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol-*1-d*	3.05	1.0	0.4	
1,5,6-Tri-*O*-acetyl-2-deoxy-3,4-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol-*1-d*	3.89	0.5		
1,3,5-Tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol-*1-d*	3.90	0.6	0.3	

62.0, and 61.2 p.p.m., indicating that both hydroxymethyl groups of the glycerol moiety were not substituted[22] and implying that the glycerol is substituted at O-2.

G.l.c.–m.s. analysis of methylated *B* confirmed the presence of a non-reducing 2-acetamido-2-deoxy-D-galactopyranosyl substituent and a glycerol moiety: *m/z* 260 ($a'A_1$), 228 ($a'A_2$), and a base peak at *m/z* 163 (a'ald J_1)[23]. G.l.c.–m.s. analysis of methylated *B* in the c.i. mode also gave fragments consistent with the proposed structure: a base peak at *m/z* 260 ($a'A_1$), a peak at *m/z* 228 ($a'A_2$), and a parent peak (M + 1) 380. Consideration of the composition, m.s. data, and n.m.r. evidence allow *B* to be identified as α-D-Gal*p*NAc-(1→2)-glycerol.

Oligosaccharide *C* (K_{av} 0.28) had $[\alpha]_D$ +98° (*c* 0.6, water), gave a single spot in t.l.c. (R_F 0.42), and g.l.c. analysis of its hydrolysis products indicated it to be composed of 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, and glycerol (1:2:1). The ^{1}H-n.m.r. spectrum of *C* contained three anomeric proton signals at δ 5.077 (d, 1 H, $J_{1,2}$ 3.5 Hz), 5.036 (d, 1 H, $J_{1,2}$ 3.5 Hz), and 4.521 (d, 1 H, $J_{1,2}$ 8.4 Hz), and three signals from CH_3 of NAc at 2.058 (s, 3 H), 2.042 (s, 3 H) and 2.037 p.p.m. (s, 3 H). The ^{13}C-n.m.r. spectrum of *C* contained three signals for C=O of NAc at 175.5, 175.3, and 175.1 p.p.m., three signals of equal intensity for anomeric carbons at 102.3, 97.9, and 94.3 p.p.m., and three signals for CH_3 of NAc at 22.7, 22.6, and 22.1 p.p.m. Consistent with the n.m.r. and compositional data, g.l.c.–m.s. of methylated *C* confirmed the presence of three 2-acetamido-2-deoxyhexosyl residues and a glycerol moiety: a base peak at *m/z* 260 (aA_1 and $a'A_1$), and peaks at *m/z* 505 (abA_1), 593 (abaldA_1 and a'aldbA_1), and 348 (a'aldA_1)[23]. G.l.c.–m.s. analysis in the c.i. mode gave fragments which were also consistent with the tetrasaccharide structure: base peak at *m/z* 260 (aA_1), a peak at *m/z* 505 (abA_1), and parent peaks (M − 1) at *m/z* 868 and (M + 1) at *m/z* 870 (Fig. 2).

G.l.c.–m.s. (program *B*) analysis of the acetylated, reduced ($NABD_4$) products of hydrolysis of methylated *C* identified 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol-*1-d*, 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol-*1-d*, and 1,3,5-tri-*O*-acetyl-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol-*1-d* (1.0:0.4:0.3) (Table I). These

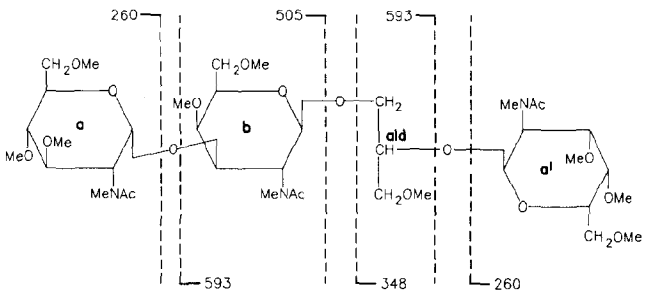


Fig. 2. G.l.c.–m.s. of methylated oligosaccharide *C* (see Scheme 1), with some primary fragments.

results indicate that *C* possesses an unusual structure having two terminal non-reducing residues and a glycerol moiety forming a di-*O*-substituted unit. The prior characterization of oligosaccharides *A* and *B* permits the sequence of *C* to be established as:

D-Glc*p*NAc-(1→3)-D-Gal*p*NAc-(1→1)-glycerol
2
↑
1
D-Gal*p*NAc

The results of the methylation analysis of the original polymer (Table I) suggest that the phosphate group is located at the O-6 position of the 2-acetamido-2-deoxy-D-glucopyranosyl residues, and the combined g.l.c.–m.s. and n.m.r. evidence so far described indicates that the capsular polymer is of the teichoic type and composed of triglycosylglycerol phosphate repeating-units joined through monophosphate diester bridges.

The position of the phosphate linkage was confirmed by 2D-n.m.r. studies. A complete assignment of the proton resonances was made from phase-sensitive COSY[17,18] and relay COSY[19] experiments (Table II). The units in the polymer were arbitrarily labelled *a–d* according to the decreasing order of the chemical shifts of their H-1 resonances in the ^{1}H-n.m.r. spectrum. Most of the proton resonances could be assigned through their cross-peaks (Fig. 3). The assignments of the H-6 and H-6′ signals were confirmed by correlation with the corresponding ^{13}C resonances *via* ^{13}C–^{1}H shift-correlation experiments[20,21], which also enabled complete assignment of the resonances in the ^{13}C-n.m.r. spectrum of the polymer, which contained several pairs of coincident resonances, such as C-3*d*, C-6*b* and C-3*c*, C-5*c*.

TABLE II

^{1}H-N.M.R. CHEMICAL SHIFTS[a] FOR THE CAPSULAR POLYMER OF *A. pleuropneumoniae* SEROTYPE 6

Proton	*Unit* a α-D-*Gal*p*NAc-(1→*	*Unit* b *→6)*-α-D-*Glc*p*NAc-(1→*	*Unit* c *→3)*-β-D-*Gal*p*NAc-(1→*	*Unit* d *→1)-Glycerol-(3→* *2* ↑
H-1	5.111 (3.8)[b]	5.037 (3.7)[b]	4.565 (8.4)[b]	4.13
H-1′				3.81
H-2	4.22	3.98	4.08	4.06
H-3	3.91	3.62	3.79	4.02
H-3′				3.93
H-4	3.98	3.55	4.11	
H-5	4.13	3.67	3.66	
H-6	3.76	4.09	3.76	
H-6′	3.80	4.05	3.81	

[a]In p.p.m. measured at 27° in D_2O with acetone (0.1%) as the internal reference (2.225 p.p.m.). [b]$J_{1,2}$ in Hz.

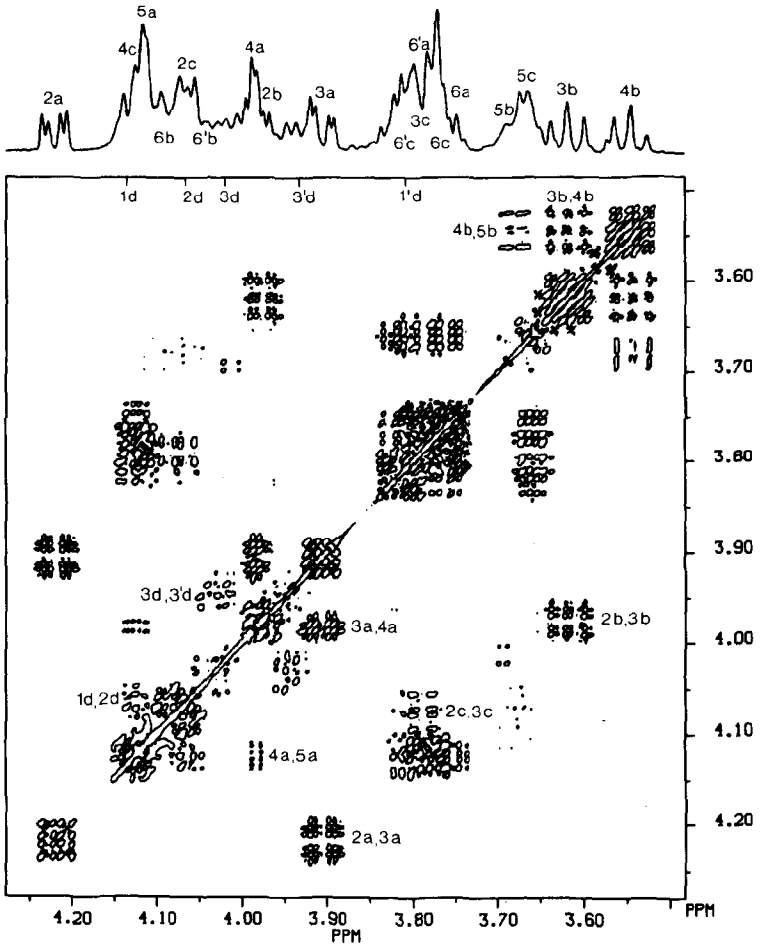


Fig. 3. Phase-sensitive COSY contour plot of the ring proton region of *A. pleuropneumoniae* serotype 6 capsular polymer.

Comparison of the ^{13}C chemical shifts (Table III) with those of literature values[22] indicated that three hexopyranosyl residues (*a*, *b*, and *c*) were involved in glycosidic linkages. As expected from the results of methylation analysis of the polysaccharide, C-1*a*, C-6*b*, C-3*c*, and the three glycerol carbon atoms C-1*d*, C-2*d*, and C-3*d* experienced considerable deshielding. Based on the polymer composition, proton chemical shift data, and $J_{1,2}$ values (3.8 Hz), unit *a* was assigned to the 2-acetamido-2-deoxy-α-D-galactopyranosyl unit, *b* to the 2-acetamido-2-deoxy-α-D-glucopyranosyl residue ($J_{1,2}$ 3.7 Hz), and *c* to the 2-acetamido-2-deoxy-β-D-galactopyranosyl residue ($J_{1,2}$ 8.4 Hz). These conclusions are in agreement with the results obtained using anomeric one-bond $^{13}C,^{1}H$-coupling constants ($^{1}J_{C,H}$)[24]. The values

TABLE III

^{13}C-N.M.R. CHEMICAL SHIFTS[a] FOR THE CAPSULAR POLYMER[b] OF *A. pleuropneumoniae* SEROTYPE 6

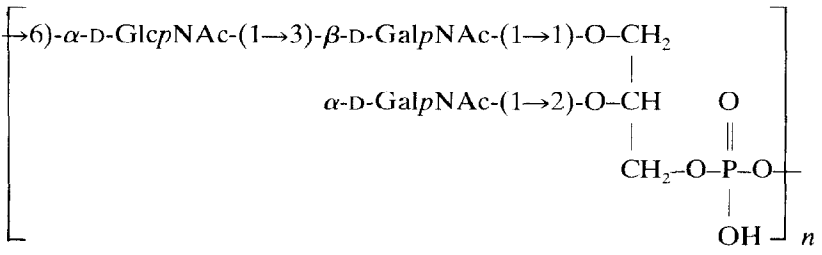
Carbon atom	*Unit* a α-D-*GalpNAc-(1→*	*Unit* b →6)-α-D-*GlcpNAc-(1→*	*Unit* c →3)-β-D-*GalpNAc-(1→*	*Unit* d →1)-*Glycerol*-(3→ 2 ↑
C-1	97.47 (172)[c]	94.12 (171)[c]	102.10 (163)[c]	70.44
C-2	50.60	54.03	51.48	75.66 (7.1)[d]
C-3	68.69	71.78	75.85	65.02 (~4)[e]
C-4	69.51	70.16	64.42	
C-5	71.96	72.46 (5.2)[d]	75.85	
C-6	62.23	65.02 (~4)[e]	61.90	

[a]In p.p.m. measured at 27° with acetone (1%) as internal reference (31.07 p.p.m.). [b]Assignments confirmed by a ^{13}C–^{1}H shift-correlation experiment. [c]$^{1}J_{C,H}$ in Hz. [d]$^{3}J_{^{31}P,^{13}C}$ in Hz. [e]$^{2}J_{^{31}P,^{13}C}$ in Hz.

of $^{1}J_{C,H}$ for the residue *a* and *b* were, respectively, 172 and 171 Hz, which are consistent with the assigned α-D configurations, while the value of $^{1}J_{C,H}$ for residue *c* (163.5 Hz) confirms its assigned β-D configuration.

The ^{13}C–^{31}P couplings for C-6*b* and C-3*d* ($^{2}J_{^{31}P,^{13}C}$ 4 Hz) and the large three-bond coupling for C-5*b* ($^{3}J_{^{31}P^{13}C}$ 5.2 Hz) and C-2*d* ($^{3}J_{^{31}P^{13}C}$ 7.1 Hz) located the monophosphate diester group at the O-6 position of the 2-acetamido-2-deoxy-α-D-glucopyranosyl residues and at O-3 of the glycerol moieties.

The results of the analysis of the capsular polymer of *A. pleuropneumoniae* serotype 6 lead to the conclusion that it is a polymer composed of repeating triglycosylglycerol phosphate units linked through monophosphate diester linkages and has the structure shown:



Capsular polymers of *A. pleuropneumoniae* serotypes 2[25] and 3[26] as well as those of other Gram-negative bacteria[27–29] have glycosylglycerol phosphate structures of the teichoic acid type. However, the glycosylation of O-2 of the glycerol moiety as found in the present study has previously been reported only for bacterial cell-wall glycerol teichoic acids[30].

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