Carbohydrate Research, 209 (1991) 211–223
Elsevier Science Publishers B.V., Amsterdam

Structure of the capsular polysaccharide from *Actinobacillus pleuropneumoniae* serotype 7*

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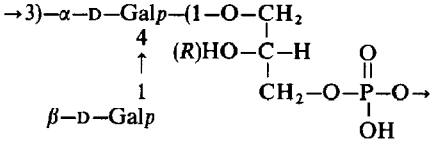
(Received April 20th, 1990; accepted for publication, June 4th, 1990)

ABSTRACT

The structure of the capsular polysaccharide antigen of *A. pleuropneumoniae* serotype 7 was determined using 1D- and 2D-n.m.r. methods. Dephosphorylation, methylation, and g.l.c.–mass spectrometry methods were used to confirm the analysis. The type-7 specific polysaccharide was a high-molecular-weight teichoic acid-type polymer of D-galactose, glycerol, and phosphate (2:1:1), composed of glycosylglycerol repeating units joined through monophosphate diester linkages and with the structure:



INTRODUCTION

The Gram-negative bacterium *Actinobacillus pleuropneumoniae* is the most frequently isolated causal agent of contagious, and often fatal, respiratory swine infections. Twelve serotypes of *A. pleuropneumoniae* have been described[1–6] based on their serotype-specific capsular polysaccharides, each of which has a unique structure[7].

We now report the structure of the serotype-7 specific capsular polysaccharide as part of a systematic study of the immunochemistry of the capsules and lipopolysaccharides of the 12 serotypes of *A. pleuropneumoniae*.

RESULTS AND DISCUSSION

Isolation and characterisation of the polysaccharide. — *A. pleuropneumoniae* serotype-7 capsular polysaccharide was obtained *via* its insoluble cetyltrimethylammonium salt[8] from the saline wash of fermentor-grown cells. Gel-filtration chromatography of the polysaccharide preparation on Sephadex G-50 afforded the homogeneous capsular polymer (K_{av} 0.03), which had $[\alpha]_D$ +125° (*c* 0.3, water) and showed a positive reaction in gel-diffusion against type-7 specific rabbit antisera. *Anal.* Found: C, 34.49;

* NRCC No. 31905

0008-6215/91/$ 03.50

H, 6.06; and N, 0.22%. The phosphate content of the polymer was determined colorimetrically[9] to be 13.6% (PO_4).

Complete acid hydrolysis of the polysaccharide and g.l.c. (program A) of the alditol acetates derived from the products showed galactose as the only detectable glycose, together with glycerol. The absolute configurations of the galactose residues were established as D by g.l.c.–m.s. of their derived acetylated (+)-2-butyl glycosides[10].

The ^{1}H-n.m.r. spectrum of the type-7 polysaccharide contained two signals for anomeric protons at 5.02 (d, 1 H, $J_{1,2}$ 3.7 Hz) and 4.71 p.p.m. (d, 1 H, $J_{1,2}$ 7.9 Hz). The corresponding ^{13}C-n.m.r. resonances were at 93.3 and 104.1 p.p.m., and the $^1J_{C,H}$ values were 171 and 163 Hz, respectively. The ^{13}C-DEPT spectrum (Fig. 1) showed resonances for four primary hydroxyl-group carbons, suggesting the presence of an alditol residue in addition to two glycosyl residues.

The combined analytical results suggested that the polysaccharide is composed of

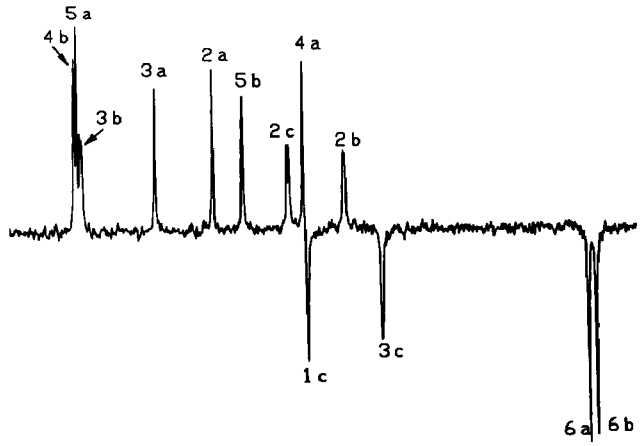


Fig. 1. Partial ^{13}C-DEPT spectrum of the capsular polysaccharide of *A. pleuropneumoniae* serotype 7 recorded at 27°.

a disaccharide repeating-unit containing both α- and β-D-galactopyranosyl residues, glycerol, and phosphate.

N.m.r. studies of the capsular polysaccharide. — The structure of the repeating unit was determined by high-resolution 1D and 2D ^{1}H- and ^{13}C-n.m.r. techniques. The determination of the anomeric configurations of the component monosaccharides, their ring sizes, and the linkage positions of the phosphate groups was achieved by complete assignment of the ^{1}H-n.m.r. spectrum *via* COSY and relay COSY experiments, followed by subsequent assignment of ^{13}C resonances from a ^{13}C–^{1}H chemical shift correlation experiment (CHORTLE). The sequence of the monomeric groups within the repeating unit was then determined from proton n.O.e. measurements.

Examination of the connectivities defined by cross-peaks observed in the COSY (Fig. 2) and relay COSY spectra (data not shown) led to the identification of three

independent sub-spectra corresponding to the two galactopyranosyl ring systems and the glycerol residue in the repeating unit. The resonances for anomeric protons, labelled H-1**a** (4.71 p.p.m.) and H-1**b** (5.02 p.p.m.), served as the starting points from which the seven ^{1}H resonances (H-1→H-6′) associated with each of the galactosyl residues were assigned from the appropriate cross-peaks (Table I). The methylene proton double-doublet (^3J 3.7, ^2J 10.6 Hz) observed at relatively high field (3.65 p.p.m.) provided easy access into the spin-system associated with the glycerol residue **c** (Fig. 2). The ^{13}C resonance (69.5 p.p.m.) corresponding to this methylene proton was identified from the DEPT spectrum (Fig. 1) and the ^{13}C–^{1}H chemical shift correlation experiments. The connectivity between the two methylene protons in the COSY spectrum at 3.65 (H-1_S) and 3.79 p.p.m. (H-1_R), and between each of these resonances and the methine (H-2) resonance at 4.12 p.p.m., was established from the appropriate cross-peaks. Cross-peaks relating all five ^{1}H resonances of the glycerol residue were apparent along the line parallel to the F_2 axis at the chemical shift value of the central methine proton (4.12

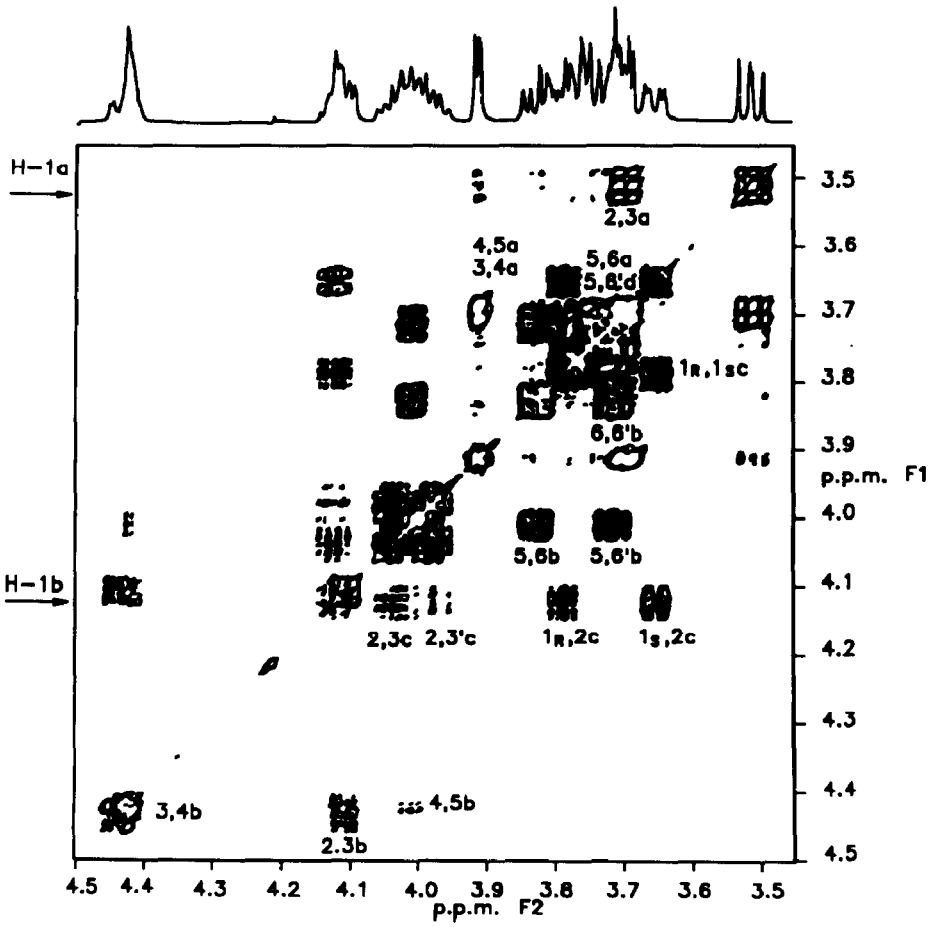


Fig. 2. COSY contour plot of the ring-proton region (4.50–3.45 p.p.m.) of the *A. pleuropneumoniae* serotype-7 capsular polysaccharide recorded at 27°.

TABLE I

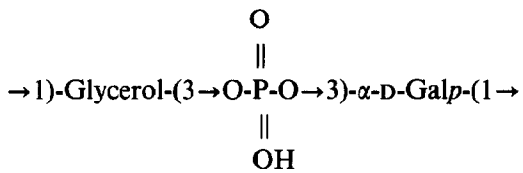
Proton chemical shifts and coupling constants (in parentheses) for the capsular polysaccharide of *A. pleuropneumoniae* serotype 7 and the dephosphorylated oligosaccharide **3**[a]

Compound	*Unit* **a** *β*-D-*Galp*-(*1*→							*Unit* **b** →3)-α-D-*Galp*-(*1*→ 4 ↑							*Unit* **c** →*1*)-*Glycerol*-(*3*→				
	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'* ($J_{5,6'}$) ($J_{6,6'}$)	*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'* ($J_{5,6'}$) ($J_{6,6'}$)	*H-1*$_R$[c] ($J_{1R,2}$)	*H-1*$_S$[c] ($J_{1S,2}$) ($J_{1R,1S}$)	*H-2*	*H-3* ($J_{2,3}$)	*H-3'* ($J_{2,3'}$) ($J_{3,3'}$)
Native CPS[b]	4.711 (7.9)	3.511 (10.0)	3.696 (3.2)	3.911 (0.8)	3.709	3.750 (6.7)	3.750 (4.1) (−11.9)	5.019 (3.7)	4.105 (10.5) (8.7)[d]	4.427 (3.0)	4.419 (1.1)	4.010	3.825 (5.2)	3.718 (4.7) (−12.0)	3.786 (5.8)	3.652 (3.7) (−10.6)	4.120	4.032 (4.4) (7.4)[d]	3.979 (6.2) (−10.7) (6.6)[d]
β-D-Gal*p*-(1→4)-	4.580	3.586	3.659	3.674	3.676	3.771	3.771	4.973	3.910	3.981	4.230	3.970	3.835	3.745	3.75	3.65	3.947	3.650	3.650
α-D-Gal*p*-(1→1)-	(7.6)	(10.0)	(3.0)	(−)[f]		(4.8)	(4.2)	(3.3)	(9.6)	(3.6)	(−1)		(5.4)	(6.6)	(−)[g]	(−)[g]		(4.2)	(6.0)
Glycerol[e] (**3**)							(−10.8)							(−11.4)					(−10.8)

[a] Measured at 27° in p.p.m. from internal acetone (δ 2.225). [b] Data refined by spectral simulation. [c] Stereochemical assignments follow from the occurrence of a specific n.O.e. between H-1b and H-1$_S$[15]. [d] $^3J_{H,P}$ value (Hz). [e] Observed first-order coupling constants. [f] Unresolved. [g] Not determined.

p.p.m.), leading to the assignment of the resonances corresponding to H-3 (4.03 p.p.m.) and H-3′ (3.98 p.p.m.).

The ring proton coupling constants (Table I) established that the D-galactosyl residues **a** and **b** were present as β- and α-pyranosyl units, respectively, both having the $^4C_1$ conformation[11]. The H-3**b** resonance (4.43 p.p.m.) showed, in addition to the homonuclear coupling to H-4**b** ($^3J_{3,4}$ 3.0 Hz) and H-2**b** ($^3J_{2,3}$ 10.5 Hz), heteronuclear coupling to ^{31}P ($^3J_{H,P}$ 8.7 Hz), which indicated C-3**b** to be the site of phosphate substitution. In addition, heteronuclear coupling to ^{31}P was observed for H-3**c** ($J_{H,P}$ 7.4 Hz) and H-3′**c** ($J_{H,P}$ 6.6 Hz), establishing the location of phosphate at C-3 of the glycerol residues in the polysaccharide. The $^3J_{H,P}$ values were estimated by simulation of the ^{1}H-n.m.r. spectrum, using the experimental δ and ^{1}H–^{1}H J values measured from the COSY contour plot. The proton chemical shifts and coupling constants were refined (Table I) until good agreement between the observed and calculated spectra was obtained (Fig. 3). The positioning of the phosphate groups between the α-D-galactopyranosyl and the glycerol residues was confirmed from a 2D ^{31}P–^{1}H chemical shift correlation experiment. Correlations between the ^{31}P resonance and H-3**b** (4.42 p.p.m.), and H-3**c** and H-3′**c** (4.03 and 3.98 p.p.m., respectively), were observed, establishing the connectivity of the phosphate group to the respective carbon atoms, C-3**b** and C-3**c**. Since only a single phosphate group was indicated by ^{31}P-n.m.r. spectroscopy (0.70 p.p.m.), it follows from the evidence obtained so far that the repeating unit contains the phosphate diester fragment **1**.



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The location of the β-D-galactopyranosyl residue **a** was indicated from the considerable deshielding of the H-4**b** resonance (Table I), suggesting **a** to be present as a terminal unit linked to position C-4 of the α-D-galactopyranosyl residue **b**. In order to verify this conclusion, ^{1}H–^{1}H n.O.e. measurements were made (Fig. 4) to establish connectivities between the anomeric and aglyconic protons of the adjacent residues. The through-space connectivities are shown in Fig. 5. Saturation of H-1**a** (4.71 p.p.m.) showed a strong n.O.e. at H-4**b** and, in addition, n.O.e.'s at H-3**a** and H-5**a** (Fig. 4b), firmly establishing that residue **a** must be β-linked to O-4 of residue **b**. Upon saturation of H-1**b** (5.02 p.p.m.), an intraresidue n.O.e. was observed at H-2**b**, together with an n.O.e. at one of the terminal methylene protons (Fig. 4a), H-1_S**c** (3.65 p.p.m.), showing **b** to be α-linked to O-1 of the glycerol residue. It follows that the polysaccharide is composed of a branched repeating-unit **2** linked through phosphate diester groups.

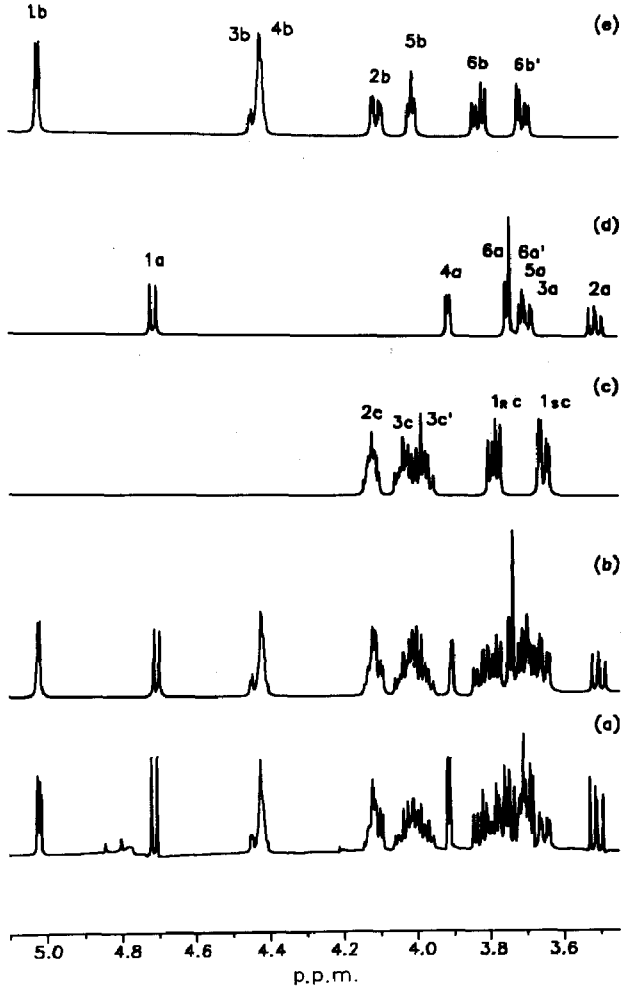
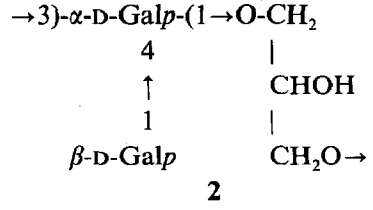


Fig. 3. ^{1}H-N.m.r. spectra of the capsular polysaccharide of *A. pleuropneumoniae* serotype 7 at 27°: (*a*) observed spectrum; (*b*) simulated spectrum. The contributions to the simulated spectrum are from (*c*) the glycerol moiety **c**, (*d*) the terminal non-reducing β-D-galactopyranosyl unit **a**, and (*e*) the branched α-D-galactopyranosyl residue **b**.



The ^{13}C–^{1}H heteronuclear shift-correlated experiment (CHORTLE) enabled the unambiguous assignment of all the carbon resonances in the repeating unit (Table II). As expected, the resonances corresponding to C-2**b** and C-3**b**, and to C-2**c** and C-3**c**, were split due to phosphorus coupling. Moreover, the DEPT experiment confirmed the

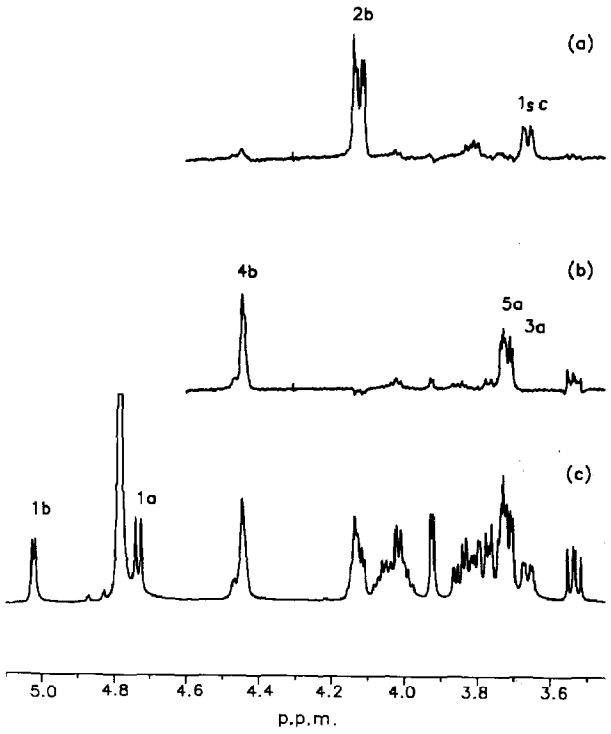


Fig. 4. N.O.e. difference spectra obtained for the capsular polysaccharide of *A. pleuropneumoniae* serotype 7 by saturation of the H-1 resonance of (*a*) the α-D-galactopyranosyl residue **b**, and (*b*) the β-D-galactopyranosyl residue **a**. The off-resonance control spectrum is shown in (*c*).

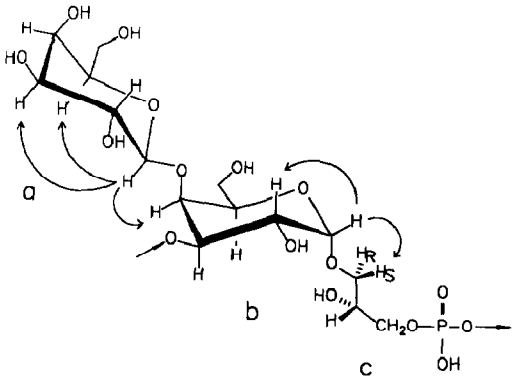


Fig. 5. Structure of the *A. pleuropneumoniae* serotype-7 capsular polysaccharide repeating-unit, showing the short-range through-space connectivities identified by n.O.e. experiments.

TABLE II

^{13}C Chemical shifts and ^{31}P–^{13}C coupling constants (in parentheses) for the capsular polysaccharide of *A. pleuropneumoniae* serotype 7 and the dephosphorylated oligosaccharide *3*[a]

Compound	*Unit* **a** *β*-D-*Galp*-(1→						*Unit* **b** →*3*)-*α*-D-*Galp*-(*1*→ 4 ↑						*Unit* **c** →*1*)-*Glycerol*-(*3*→		
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*
Native CPS[b]	104.1	72.0	73.6	69.6	75.7	61.8	99.3	68.5 (3.9)[c]	75.6 (3.9)[d]	75.8 (−)[c,e]	71.2	61.6	69.5	70.0 (7.9)[c]	67.5 (4.7)[d]
β-D-Gal*p*-(1→4)-*α*-D-Gal*p*-(1→1)-Glycerol (**3**)	105.2	72.3	73.7	69.6	76.0	61.8	99.3	69.7	70.8	79.1	71.1	61.6	69.5	71.3	63.3

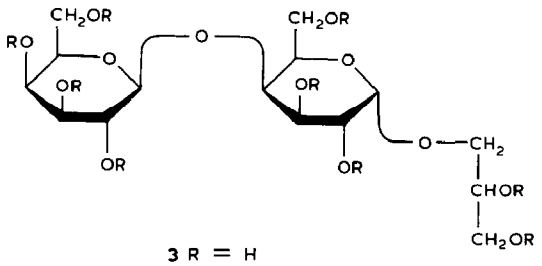
[a] In p.p.m. from internal acetone (δ 31.07). [b] Assigned *via* ^{13}C–^{1}H chemical shift correlation. [c] $^{3}J_{C,P}$ value (Hz). [d] $^{2}J_{C,P}$ value (Hz). [e] Unresolved.

identity of the ^{13}C resonances arising from the four methylene carbon atoms (Fig. 1). The downfield-shifted methylene resonances at 69.5 (s) and 67.5 p.p.m. (d, $J_{C,P}$ 4.7 Hz) were attributed to C-1**c** and C-3**c**, respectively, where the relatively deshielded value of the methylene singlet at 69.5 p.p.m. is consistent with substitution at that position.

Depolymerisation of the polysaccharide. — Treatment of the polysaccharide with aqueous 48% hydrogen fluoride, followed by fractionation of the product on Bio-Gel P-2, afforded the phosphate-free trisaccharide **3**, which had $[\alpha]_D$ +100° (*c* 0.8, water). Identification of **3** as β-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→1)-glycerol was confirmed by its ^1H- and ^{13}C-n.m.r. spectra. Assignment of the ^1H-n.m.r. spectrum was made *via* a COSY experiment (Table I), and the ^{13}C-n.m.r. spectrum, which showed fifteen signals, from a CHORTLE experiment (Table II). In comparison with the ^{13}C resonances for the polysaccharide, the resonances assigned to C-3**b** and C-3**c** of **3** (Table II) were shifted significantly upfield (4.8 and 4.2 p.p.m., respectively), an observation which is in agreement with these carbon atoms being phosphate substituted in the polysaccharide. Concomitantly, the resonances for C-2**b**, C-4**b**, and C-2**c** were shifted downfield (1.2, 3.3, and 1.3 p.p.m., respectively). The large downfield shift for C-4**b** points to substitution at that position.

Saturation of H-1**a** of **3** produced an inter-residue n.O.e. at H-4**b** and saturation of H-1**b** produced a n.O.e. at H-1$_S$**c**, providing confirmation of the sequence of the residues.

In agreement with the proposed structure for **3**, its methylated derivative **4**, on analysis by g.l.c.–m.s. (program *C*), gave an $[M + NH_4]^+$ ion at *m/z* 560 (c.i.) along with characteristic fragment ions[12] at *m/z* 101 (alditol), 219 (aA_1), and 423 (baA_1) (e.i.). Methylation analysis of **3** gave 2,3,4,6-tetra-*O*-methylgalactose and 2,3,6-tri-*O*-methylgalactose in the molar ratio 1.0:1.0.

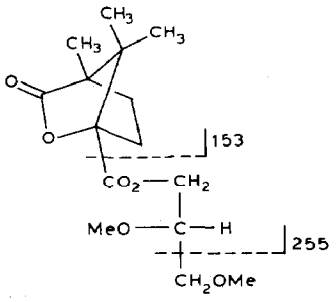


3 R = H

4 R = CH_3

Absolute configuration of the glycerol 3-phosphate residues. — In other teichoic acid-type polysaccharides, both (*R*)- and (*S*) (L- and D)-glycerol 3-phosphate residues have been identified[13–15]. The absolute configuration of the glycerol 3-phosphate residues in the *A. pleuropneumoniae* serotype 7 polysaccharide was indicated from the n.O.e. observed between H-1**b** and H-1$_S$**c** (Fig. 5) and the relative magnitude of the ^3J values ($J_{1S,2}$ and $J_{1R,2}$)[15]. This was evident from the magnitude of $J_{1R,2}$ (5.8 Hz), which is greater than that of $J_{1S,2}$ (3.7 Hz) (Table I), indicating the glycerol 3-phosphate residues to have *R*-chirality (*i.e.*, L configuration) at C-2**c**.

The foregoing conclusion was confirmed by hydrolysis of **4** which afforded, without loss of chirality at C-2, 2,3-di-*O*-methylglycerol, which was converted into the corresponding (−)-camphanate ester **5**. The chirality of **5** was identified as *S* by g.l.c. (program *B*), since it gave a single peak (T_{GA} 0.74) having the same retention time and mass spectrum as an authentic sample derived from L-glyceric acid[16].



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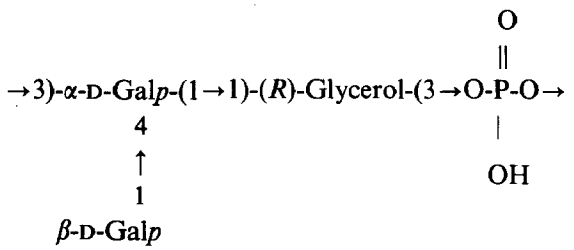
The capsular polysaccharides of *A. pleuropneumoniae* can be divided into three types: (*a*) those which have a teichoic acid-like structure (serotypes 2, 3, 6 and 8), (*b*) those which are composed solely of glycose residues (serotypes 5a and 5b), and (*c*) those which have oligosaccharide units joined by phosphate diester linkages (serotypes 1 and 4)[7,17]. The evidence presented here establishes the repeating unit of *A. pleuropneumoniae* serotype 7 capsular polysaccharide as belonging to type (*a*) (**6**).



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EXPERIMENTAL

Cell production. — *A. pleuropneumoniae* serotype 7 (WF 83, NRCC 4221), provided by Dr. S. Rosendal, University of Guelph, 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, ~3.3 g wet weight/L).

Isolation and purification of capsular polymer. — Cells were washed with 2.5% saline and, following low-speed centrifugation, the dialysed saline extract was digested with ribonuclease, deoxyribonuclease, and trypsin. The polymer was recovered by precipitation with 5 volumes of aqueous 95% ethanol and further purified by precipitation and recovery from its insoluble cetyltrimethylammonium complex[8]. Pure polysaccharide was obtained by gel-filtration chromatography on Sephadex G-50.

Analytical methods. — Gel-permeation chromatography was performed on a column (2 x 100 cm) of Sephadex G-50 (Pharmacia), using 0.05M pyridinium acetate (pH 4.5) as the eluant, or a column (1.5 x 80 cm) of Bio-Gel P-2 (−400 mesh, Bio-Rad), using distilled water as the eluant.

Column eluates were continuously monitored using a Waters 403 refractometer, and samples (100 μL) of collected fractions were analysed colorimetrically for aldoses[18].

Analytical g.l.c. was done with a Hewlett–Packard 5830A chromatograph fitted with a flame-ionisation detector and an OV-17 fused-silica column (Quadrex Corp.). The following programs were used: *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 oligosaccharides), 220° for 1 min then 7°/min to 280°. G.l.c.–m.s. was performed with a Hewlett–Packard 5985B system, using conditions *A* to *C* and an ionising potential of 70 eV (e.i.) or with ammonia as reagent gas (c.i.).

For analysis of constituent sugars, samples of polysaccharide or oligosaccharide (1 mg) were hydrolysed with 4M trifluoroacetic acid (0.5 mL) for 1 h at 125°. The glycoses released were determined by g.l.c. of their derived alditol acetates[19].

The configuration of D-galactose was established by capillary g.l.c. of the acetylated (+)-2-butyl glycoside and comparison of its g.l.c. retention time and mass spectrum with those of a standard[10] (program *A*).

N.m.r. spectroscopy. — All spectra were obtained, at 27° on solutions in D_2O, using a Bruker AM-500 spectrometer equipped with an Aspect 3000 computer, operating in the pulsed-F.t. mode, with quadrature detection using standard Bruker software.

Proton spectra, recorded at 500 MHz, were obtained using a spectral width of 1.1 kHz, a 16k data block, and a 90° pulse. Chemical shifts are expressed relative to internal acetone (2.225 p.p.m.). N.O.e. difference spectra were obtained by sequential irradiation at each line in a multiplet[20], for a total irradiation time of either 200 ms for polysaccharide samples or 800 ms for oligosaccharide samples.

Broad-band proton-decoupled ^{13}C-n.m.r. spectra were obtained at 125 MHz, using a spectral width of 31 kHz, a 32k data block, and a 90° pulse employing WALTZ decoupling[21]. Heteronuclear $^1J_{C,H}$ coupling constants were measured using gated decoupling[22]. DEPT spectra[23] were obtained with broad-band proton decoupling, a 135° proton pulse, and a 3.3-ms delay between pulses. Heteronuclear ^{13}C–^1H chemical shift correlations were carried out by using the CHORTLE pulse sequence[24] as described[25]. Chemical shifts are expressed relative to internal acetone (31.07 p.p.m.).

Homonuclear 2D chemical-shift-correlated (COSY and Relay COSY) experiments were carried out using the conventional pulse sequences[26–28]. Spectra were obtained using matrices (t_1 x t_2) of 512 x 2048 or 256 x 2048 data points for a spectral width of 1082 Hz, a recycle delay of 1.2 s, and 16 transients for each t_1 value. The data were zero-filled to give 1024 x 2048 points, and resolution enhancement in both dimensions was done by unshifted sine-bell-window functions prior to Fourier transformation. Magnitude spectra, symmetrised about the diagonal, were used to represent the data.

Spectral simulations were performed using the Bruker PANIC program. The spin systems corresponding to the two D-galactopyranosyl and the glycerol residues were simulated separately and were then added, employing line widths of 2.0 Hz, to give a composite simulated spectrum.

Phosphorus-31 spectra were recorded at 202 MHz on solutions (~2 mL) in 10-mm sample tubes by employing a spectral width of 10 kHz, a 16k data set, and a 90° pulse. Chemical shifts are referenced to that of an external sample of aqueous 85% phosphoric acid. The heteronuclear 2D ^{31}P–^{1}H chemical-shift-correlated spectrum[29] was recorded using spectral widths of 8065 and 1300 Hz for the respective ^{31}P (F_2) and ^{1}H (F_1) chemical shift domains, a recycle delay of 1.2 s, and fixed delays τ_1 and τ_2 of 62.5 and 31.3 ms, respectively, to select for all multiplicities. The initial data matrix of 128 x 2048 points was zero-filled to give a final matrix of 512 x 2048 points. Resolution enhancement was applied in both dimensions by means of a Lorentz-to-Gauss transformation and the data were processed to give a power spectrum.

Depolymerisation of the capsular polysaccharide. — A solution of the polysaccharide (50 mg) in aqueous 48% (w/v) hydrofluoric acid (2 mL) was kept for 42 h at 4°. The reaction was quenched by pouring the mixture into a slurry of solid CO_2–calcium carbonate–dichloromethane[30], and, after removal of the dichloromethane in a stream of N_2, the precipitate was removed by centrifugation. The phosphate-free product **3** was obtained by gel-permeation chromatography on Bio-Gel P-2 (yield, 10.8 mg). A sample (2 mg) was methylated[31] (**4**), analysed by g.l.c.–m.s., and hydrolysed (4M CF_3CO_2H, 1 h, 125°), and the partially methylated glycoses were converted into their alditol acetates.

Configuration of the glycerol 3-phosphate residue. — A sample of **4** (2 mg) was hydrolysed with 4M trifluoroacetic acid (125°, 1 h) and excess of acid removed in a stream of N_2. The residue in dry pyridine (0.5 mL) was treated with an excess of (1*S*,4*R*)-(−)-camphanoyl chloride (Aldrich) (~10 mg), and the solution was stirred at room temperature for 3 h. The reaction was quenched by addition of aqueous sodium carbonate (5%, 1 mL), and the product, 2,3-di-*O*-methyl-(*S*)-glycerol camphanate (**5**), was extracted into dichloromethane (1 mL). The extract was dried (Na_2SO_4) and analysed by g.l.c.–m.s. using program *B*.

General methods. — Commercial reagents and solvents were analytical grade. Concentrations were made under reduced pressure at 37°. Optical rotations were determined at 22° in 10-cm microtubes, using a Perkin–Elmer model 243 polarimeter.

ACKNOWLEDGMENTS

We thank Mr. F. P. Cooper for g.l.c.–m.s. analysis, Mr. D. W. Griffith for the large-scale production of cells, and Dr. S. Lariviere, University of Montreal, Saint Hyacinthe, for *A. pleuropneumoniae* type-7 specific rabbit antisera.

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