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Structural studies of the capsular polysaccharide from *Actinobacillus pleuropneumoniae* serotype 12*

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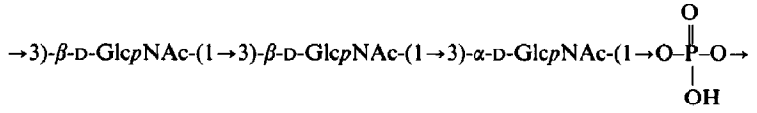
ABSTRACT

The capsular polysaccharide of *A. pleuropneumoniae* is composed of 2-acetamido-2-deoxy-D-glucose (3 parts) and phosphate (1 part). The arrangement of these components in the repeating unit was determined by dephosphorylation, methylation, g.l.c.–m.s., and 1D and 2D n.m.r. spectroscopic methods. The polysaccharide was found to be a high molecular weight polymer of repeating trisaccharide units, joined through phosphate diester linkages, having the structure,



INTRODUCTION

Actinobacillus pleuropneumoniae is the causal agent of swine pleuropneumonia, a disease characterized by hemorrhagic, fibrinous lesions of the respiratory tract. The capsules of *A. pleuropneumoniae* have been implicated in lesion production and bacterial virulence. Inoculation of heavily encapsulated *A. pleuropneumoniae* serotype 5 into swine resulted in the development of necrohemorrhagic lesions and respiratory disease, whereas minimally encapsulated strains did not produce these effects[1,2]. Variations in capsular thickness and structure among the *A. pleuropneumoniae* serotypes may explain in part why all strains do not show equal virulence[3].

Chemical characterization of the capsular polysaccharides should afford a better understanding of their serology and biological activities and, to this end, we have embarked on the structural analyses of the capsules of the known twelve serotypes of *A. pleuropneumoniae*[4–9]. This paper records the results of the structural analysis of the capsular polysaccharide of *A. pleuropneumoniae* serotype 12.

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RESULTS AND DISCUSSION

Isolation and characterization. — *A. pleuropneumoniae* serotype 12 capsular polysaccharide was obtained *via* its insoluble cetyltrimethylammonium salt[10] from the 2.5% saline wash of fermentor-grown cells. On Sephadex G-50 gel filtration chromatography, the polysaccharide eluted as a sharp peak at the void volume of the system (K_{av} 0.03), indicating it has a high molecular weight. On DEAE-Sephacel ion-exchange chromatography, it eluted as a discrete homogeneous fraction in a sodium chloride gradient. The purified polysaccharide had $[\alpha]_D$ +28.2 (*c* 1.1, water) and was homogeneous with respect to aminodeoxyaldose and phosphate. *Anal.* Found: C, 38.99; H, 5.97; N, 5.94; and ash, 5.49%. The phosphate content of the polymer was determined colorimetrically to be 9.4% (as PO_4).

Complete acid hydrolysis of the polysaccharide and g.l.c.–m.s. of the derived alditol acetates showed only 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxyglucitol, while analysis of the nitrous acid-deaminated, reduced ($NaBH_4$), and acetylated products of hydrolysis showed 1,3,4,6-tetra-*O*-acetyl-2,5-anhydromannitol. The absolute configurations of the aminodeoxyglycose residues were established as D by conversion to the corresponding (*R*)-2-butyl glycosides[11] and g.l.c.–m.s. of the peracetylated derivatives.

The ^{1}H-n.m.r. spectrum (Fig. 1) of the type 12 polysaccharide contained three signals for anomeric protons at 5.44 (dd, 1 H, $J_{1,2}$ 3.2 Hz; $^3J_{H,P}$ 8.6 Hz), 4.66 (d, 1 H, $J_{1,2}$ 8.4 Hz), and 4.64 p.p.m. (d, 1 H, $J_{1,2}$ 7.8 Hz), and, in addition, three signals at 2.02, 2.04, and 2.13 p.p.m. for the CH_3 of the *N*-acetyl groups. Consistent with these findings, the ^{13}C-n.m.r. spectrum contained resonances for anomeric carbons at 95.0, 101.6 and 101.7 p.p.m., the $^1J_{C,H}$ values of which were 176, 165, and 165 Hz, respectively. Also present were signals for the carbonyl (175.4, 174.8, and 174.5 p.p.m.) and methyl groups (23.6

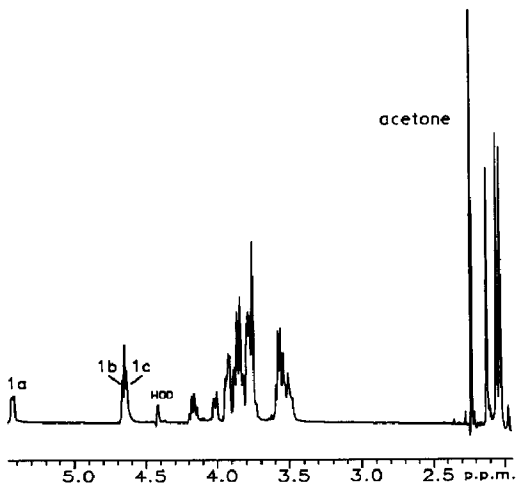


Fig. 1. ^{1}H-N.m.r. spectrum of the capsular polysaccharide of *A. pleuropneumoniae* serotype 12, recorded at 57°.

and 23.2 p.p.m.) of the acetamido functions of the three 2-acetamido-2-deoxy-D-glucose residues of the repeating unit. A ^{13}C-DEPT experiment (Fig. 2) showed resonances for three primary hydroxyl-group carbons (60–62 p.p.m.), suggesting that none of the sugars in the repeating unit were O-6 linked.

The combined analytical results were consistent with the type 12 polysaccharide being composed of a trisaccharide repeating unit joined by phosphodiester groups containing one α-linked and two β-linked 2-acetamido-2-deoxy-D-glucopyranose residues.

Dephosphorylation of the polysaccharide. — Depolymerization of the polysaccharide with 48% aqueous hydrofluoric acid, followed by fractionation of the product on Bio-Gel P-2, afforded the phosphate-free trisaccharide **1** (K_{av} 0.39) which was pure by t.l.c. (R_F 0.32) and had $[\alpha]_D$ −19.8° (*c* 0.96, water). The ^{1}H-n.m.r. spectrum of reduced **1** showed signals for anomeric protons from the two β-linked 2-acetamido-2-deoxy-D-glucopyranoside residues at 4.63 (d, 1 H, $J_{1,2}$ 7.7 Hz) and 4.62 p.p.m. (d, 1 H, $J_{1,2}$ 8.3 Hz), together with resonances for the three methyl groups of the acetamido functions at 2.02, 2.04, and 2.08 p.p.m. Signals for the two anomeric carbons were observed in the ^{13}C-n.m.r. spectrum at 102.2 and 101.8 p.p.m., with resonances at 23.4, 23.2, and 23.0 p.p.m. for the methyl carbons, and 175.2, 174.9, and 174.8 p.p.m. for the carbonyl carbons, thus confirming the presence of the acetamido functions. Further assignments of the ^{1}H and ^{13}C-n.m.r. spectra were achieved by homonuclear (COSY) and heteronuclear (CHORTLE) chemical shift correlation (Tables I and II). The disappearance of the anomeric ^{1}H and ^{13}C resonances from the α-linked 2-acetamido-2-deoxy-D-glucose residue, which was present in the original polysaccharide, suggested that this sugar had become the reducing end of **1**.

Reduced, methylated **1** gave a single g.l.c. peak (T_{MS} 3.01) and an e.i.–mass spectrum with diagnostic fragment ions at *m/z* 130, 228, 260, 276, 473, and 505 (Fig. 3). Hydrolysis of reduced, methylated **1** yielded 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-methylamino-D-glucitol, 2-deoxy-3,4,6-tri-*O*-methyl-2-methylamino-D-glucose, and 2-deoxy-4,6-di-*O*-methyl-2-methylamino-D-glucose, in the molar ratios of 0.3:1.0:0.8, as determined by g.l.c.–m.s. analysis of their reduced and acetylated derivatives.

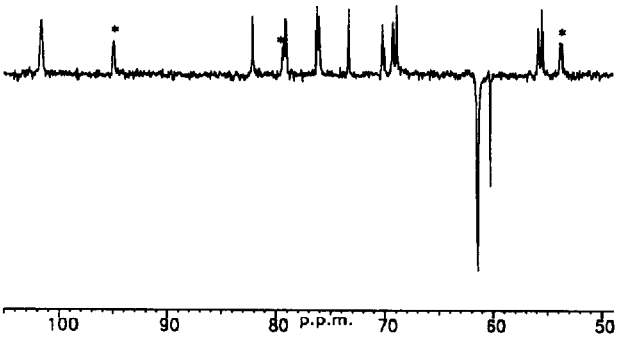


Fig. 2. Partial ^{13}C-DEPT spectrum of the capsular polysaccharide of *A. pleuropneumoniae* serotype 12, recorded at 37°. Carbon–phosphorus couplings are marked with asterisks.

TABLE I

^{1}H-N.m.r. data[a] for *A. pleuropneumoniae* serotype 12 capsular polysaccharide and its dephosphorylated product

Compound	*Unit* **a** α-D-*GlcpNAc*							*Unit* **b** β-D-*GlcpNAc*							*Unit* **c** β-D-*GlcpNAc*						
	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* ($J_{1,2}$)	*H-2* ($J_{2,3}$)	*H-3* ($J_{3,4}$)	*H-4* ($J_{4,5}$)	*H-5*	*H-6* ($J_{5,6}$)	*H-6'* ($J_{5,6'}$) ($J_{6,6'}$)
Capsular Polysaccharide	5.435[b]	4.030	3.869	3.577	3.878	3.856	3.781	4.659	3.838	4.177[c]	3.582	3.530	3.940	3.771	4.640	3.780	3.843	3.565	3.498	3.929	3.785
	(3.2)	(10.5)	(8.6)	(—)[d]		(2.6)	(6.8)	(8.4)	(10.5)	(10.2)	(9.5)		(2.1)	(5.3)	(7.8)	(10.5)	(8.5)	(10.2)		(3.2)	(6.3)
							(12.8)							(12.8)							(12.7)
Dephosphorylated product 1 (reduced)	3.642[e]	4.112	4.123	3.542	3.383	3.651	3.651	4.618	3.680	3.614	3.466	3.472	3.937	3.750	4.628	3.829	3.807	3.542	3.498	3.929	3.759
								(8.3)							(7.7)						

[a] Chemical shifts measured at 57° in p.p.m. with internal acetone as reference (2.225 p.p.m.). [b] $^{3}J_{H,P}$ 8.6 Hz. [c] $^{3}J_{H,P}$ 7.4 Hz. [d] Unresolved. [e] Approximate value for both H-1 and H-1'.

TABLE II

^{13}C-N.m.r. chemical shifts[a] for *A. pleuropneumoniae* serotype 12 capsular polysaccharide and its dephosphorylated product

Compound	*Unit* **a**						*Unit* **b**						*Unit* **c**					
	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*	*C-4*	*C-5*	*C-6*
Native polysaccharide	95.0	53.6	79.1[b]	69.1	73.4	60.4	101.6	55.8	79.3[b]	70.4	76.3	61.5	101.7	55.5	82.3	69.4	76.1	61.5
	(—)[c]	(9.2)[d]						(—)[c]	(6.1)[e]	(—)[c]								
Dephosphorylated product 1 (reduced)	63.7	54.3	76.7	69.7	71.6	61.0	101.8	56.7	74.2	70.8	76.7	61.8	102.2	55.6	82.4	71.2	75.8	61.6

[a] Chemical shifts measured at 57° in p.p.m. downfield from tetramethylsilane, with internal acetone as reference (31.07 p.p.m.). [b] Values taken from the spectrum measured at 37°. [c] Signal broadened, $J_{C,P}$ unresolved. [d] $^{3}J_{C,P}$ (Hz). [e] $^{2}J_{C,P}$ (Hz).

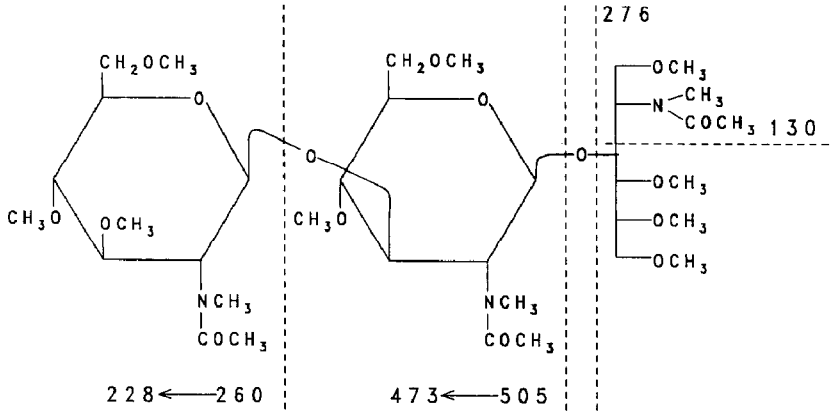


Fig. 3. E.i.–m.s. fragment ions of reduced methylated oligosaccharide **1**.

The above results establish **1** as a trisaccharide with the structure, β-D-Glc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc

Since on dephosphorylation of the native polysaccharide the 2-acetamido-2-deoxy-α-D-glucosyl residue became the reducing end of **1**, it follows that it was this sugar that was glycosidically linked to the phosphate group. The positions of the phosphate group linkages were unequivocally determined by assignment of all ^{1}H and ^{13}C resonances in the n.m.r. spectra of the polysaccharide.

N.m.r. studies of the capsular polysaccharide. — The complete assignment of the ^{1}H-n.m.r. spectrum of the polysaccharide *via* COSY and relay COSY was followed by assignment of the ^{13}C resonances from a CHORTLE experiment. Although the polysaccharide contains only 2-acetamido-2-deoxy-D-glucose residues, delineation of the connectivities defined by cross-peaks observed in the COSY (Fig. 4) and relay COSY (data not shown) spectra led to the identification of three independent subspectra corresponding to the α-linked (residue **a**) and the two β-linked (residues **b** and **c**) D-glucopyranosyl ring systems. The resonances for the anomeric protons, labelled H-1a (5.44 p.p.m.), H-1b (4.66 p.p.m.), and H-1c (4.64 p.p.m.) (Fig. 1), served as starting points from which the seven ^{1}H resonances (H-1–H-6′) associated with each residue were assigned (Table I) from the appropriate cross-peaks (Fig. 4). The resonance for H-1a showed phosphorus coupling ($^{3}J_{H,P}$ 8.6 Hz) confirming that the α-linked 2-acetamido-2-deoxy-D-glucose residue was glycosidically linked to the phosphate group. Similarly, H-3b (4.18 p.p.m.) appeared as a broad quartet ($J_{obs} \sim 10$ Hz) in which additional heteronuclear coupling, apparent from the COSY cross-peaks relating this resonance to the H-2b ($J_{2,3}$ 10.5) and the H-4b ($J_{3,4}$ 10.2) resonances (Fig. 4), indicated C-3 of residue **b** as the site of substitution by the phosphate group. This substitution pattern was further evident from the observed upfield shift (~0.56 p.p.m.) of the H-3b resonance in the phosphate-free oligosaccharide **1** (Table I).

The ^{13}C-n.m.r. spectral data (Table II) support the above evidence. The presence of scalar ^{31}P coupling to the C-2a ($^{3}J_{C,P}$ 9.2 Hz) resonance, together with a broading of the resonance for C-1a is consistent with a phosphate linkage at C-1 of residue **a**. The

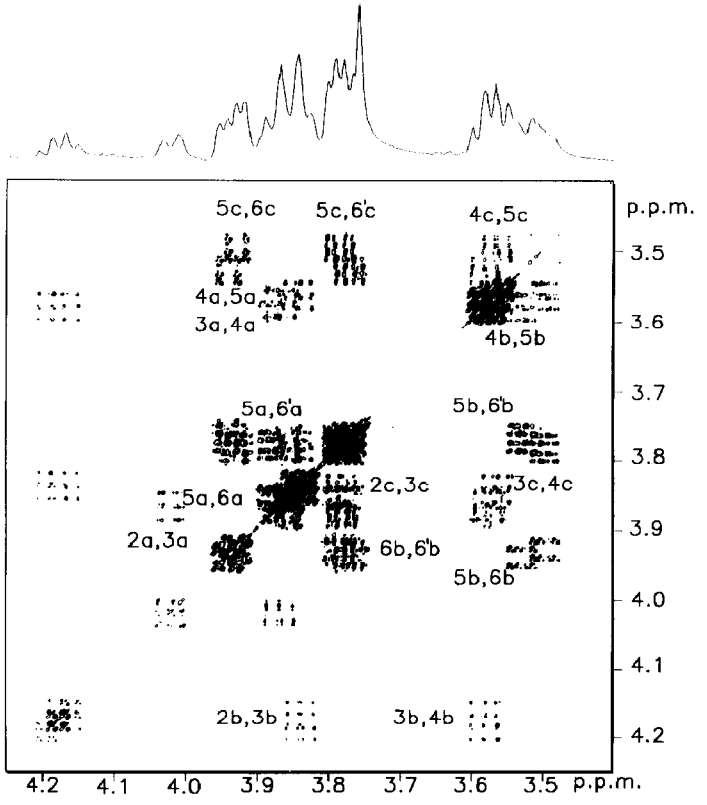


Fig. 4. Phase-sensitive COSY contour plot of the ^{1}H spectral region 4.25–3.40 p.p.m. for the capsular polysaccharide of *A. pleuropneumoniae* serotype 12, recorded at 57°. The 1D n.m.r. spectrum is displayed along the F_2 axis.

$^3J_{C,P}$ value has been found to be greater than that of $^2J_{C,P}$ in 2-acetamido-2-deoxy-α-D-glucose-1-phosphate[12]. The downfield chemical shifts of the C-3 resonances of residues **a**, **b**, and **c** (79.1, 79.3, and 82.3 p.p.m., respectively) compared to those of unsubstituted 2-acetamido-2-deoxy-D-glucopyranosides[12] confirm the O-3 substitution of each residue. The assignment of the signal at 79.3 p.p.m. to C-3b is validated by the presence of scalar ^{31}P coupling ($^2J_{C,P}$ 6.1 Hz).

To verify the sequence of sugars and their positions of substitution in the polysaccharide repeating unit, ^{1}H–^{1}H n.O.e. measurements were made. Since overlapping of the resonances for the anomeric protons of the two β-linked sugars precluded the use of 1D n.O.e. measurements, a 2D NOESY experiment was employed to obtain this information (Fig. 5). Only one n.O.e. was observed for residue **a**, an intraresidue effect between H-1 and H-2, confirming its anomeric configuration as α. The lack of any interresidue n.O.e. is in agreement with residue **a** being anomerically linked to the phosphate moiety. Intraresidue effects between H-1 and H-3, and between H-1 and H-5 of residues **b** and **c** were in accord with these residues being β-linked. In addition, an interresidue n.O.e. was observed between H-1b and H-3c establishing the sequence **b** → **c**. However, no interresidue effects relating H-1c to residue **a** protons were detected,

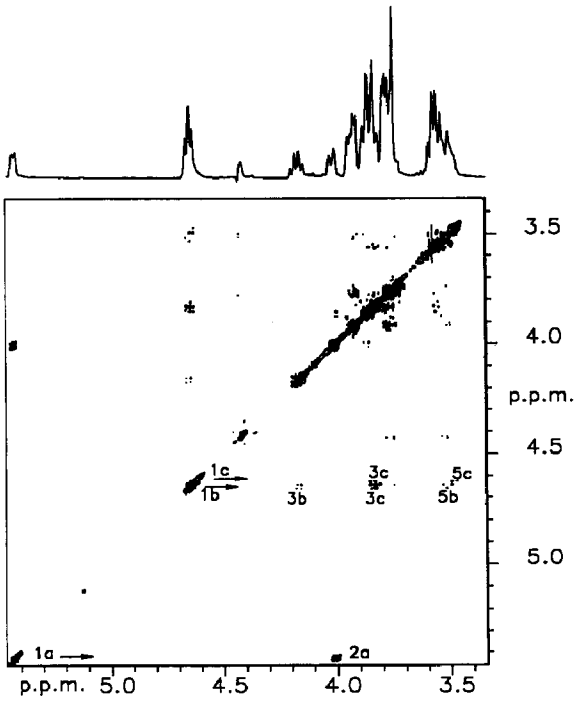
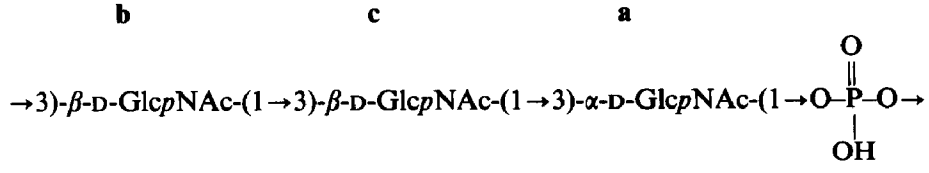


Fig. 5. Contour plot of the ^{1}H spectral region 5.46–3.34 p.p.m. of the phase-sensitive NOESY spectrum for the capsular polysaccharide of *A. pleuropneumoniae* serotype 12, recorded at 57°. Cross-peaks arising from n.O.e. involving the anomeric protons are indicated.

although HSEA calculations[13] on the trisaccharide **1** had predicted the occurrence of an n.O.e. to H-3a.

Thus the combined n.m.r. evidence allows the following linkages to be assigned: **a** → P, P → **b**, and **b** → **c** confirming the sequence of sugars in a linear repeating unit as **b** → **c** → **a**. The structure of the trisaccharide repeating unit of the capsular polysaccharide of *A. pleuropneumoniae* serotype 12 can therefore be identified as,



Like serotypes 1 and 4 (refs. 10 and 14), serotype 12 belongs structurally to the group of *A. pleuropneumoniae* capsular polysaccharides which are composed solely of oligosaccharide repeating units joined by phosphate diester linkages.

EXPERIMENTAL

Production of capsular polysaccharide. — *A. pleuropneumoniae* 12 (NRCC 4267) was grown (yield 3 g wet weight/L), and its capsular polysaccharide was isolated as previously described[15]. Pure polysaccharide was obtained by gel-filtration on a column of Sephadex G-50, followed by ion-exchange chromatography on DEAE-Sephacel, from which it eluted as a single peak in a 0→1M NaCl gradient.

Analytical methods. — Gel-permeation and DEAE-Sephacel chromatography were carried out as previously described[15]. Quantitative colorimetric methods used were the modified Elson–Morgan for aminoglycoses[16] and the Chen method for phosphate[17].

Analytical g.l.c.–m.s. was done with a Hewlett–Packard 5885B system fitted with a flame ionization detector and an OV-17 fused silica capillary column (Quadrex Corp.). The following programs were employed: *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 (*R*)-2-butyl glycosyl acetates) 180° for 2 min, then 6°/min to 240°; and *D* (for methylated trisaccharide alditols) 220° for 1 min, then 7°/min to 280°. The methylated trisaccharide alditol retention time is quoted relative to that of methylated sucrose (T_{MS}).

For analysis of constituent sugars, samples (1 mg) of polysaccharide or oligosaccharide were hydrolysed with 4M trifluoroacetic acid (0.5 mL) for 1 h at 125°. Glycoses were determined by g.l.c.–m.s. of their derived alditol acetates[18].

T.l.c. was performed on silica gel (E. Merck) with 6:3:1 (v/v) propanol–conc. NH_4OH–water.

Dephosphorylation of the capsular polysaccharide. — Polysaccharide (25 mg) was dissolved in 48% (w/v) aqueous hydrofluoric acid (1 mL) and kept for 48 h at 4°C. The reaction was quenched by pouring the mixture into a slurry of solid CO_2–calcium carbonate–dichloromethane[19], and, after removal of the precipitate and dichloromethane by centrifugation, the phosphate-free product **1** was obtained by gel-permeation chromagraphy on Bio-Gel P-2 (yield 7.2 mg). A sample was reduced ($NaBH_4$, 3 h, room temperature), methylated[20], and analysed by g.l.c.–e.i.-m.s., then hydrolysed, and the partially methylated glycoses were converted to their alditol acetates.

N.m.r. spectroscopy. — All spectra were obtained, on solutions in D_2O, at 37° or 57° using a Bruker AM 500 spectrometer equipped with an Aspect 300 computer, operating in the pulsed F.t. mode.

Proton spectra, recorded at 500 MHz, were obtained using a spectral width of 2.5 KHz, a 16-K data block, and a 90° pulse. Chemical shifts are expressed relative to internal acetone (2.225 p.p.m.).

Broad-band proton-decoupled ^{13}C spectra (125 MHz) were obtained using a spectral width of 32 KHz and a 90° pulse width employing WALTZ decoupling[21]. Heteronuclear $^1J_{C,H}$ values were measured using gated decoupling[22], and DEPT spectra[23] were obtained with broad-band decoupling, a 135° proton pulse width, and a 3.3 ms delay between pulses.

Two-dimensional homonuclear COSY, relay COSY, and NOESY experiments

were carried out as previously described[24], and the data were processed to give either magnitude[25] or phase-sensitive[26,27] spectra.

General methods. — Concentrations were made under reduced pressure at $<40°$. Optical rotations were determined at 22° in 10-cm microtubes using a Perkin–Elmer model 243 polarimeter.

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