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A STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* K69

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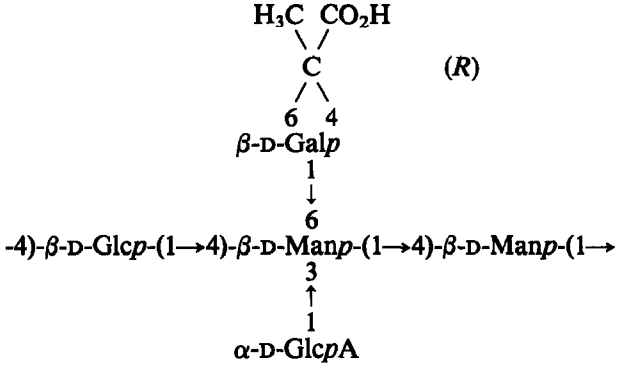
ABSTRACT

The structure of the capsular polysaccharide isolated from *Klebsiella* serotype K69 has been investigated by a combination of chemical and spectroscopic methods. The repeating structure of the deacetylated polysaccharide is shown to be of the "3 + 1 + 1" type, and it carries a 1-carboxyethylidene acetal at positions 4 and 6 of a terminal galactosyl group. The location of acetyl groups in the polysaccharide has not been established. The repeating unit of the deacetylated polysaccharide has the following structure.



INTRODUCTION

Klebsiella serotypes K7, K13, K26, K30, K31, K33, K35, K46, and K69 are strains[1] whose capsular polysaccharides are composed of D-galactose, D-glucose, D-glucuronic acid, D-mannose, and pyruvic acid. Three of these strains [K30 (ref. 2), K33 (ref. 3), and K69] have, in addition, *O*-acetyl substituents, and, in this subgroup, only the structure of K69 has not been established.

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RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — *Klebsiella* K69 bacteria were grown on an agar medium, and the acidic capsular polysaccharide was purified by precipitation with cetyltrimethylammonium bromide[4]. The purified product had $[\alpha]_D$ +55° and was shown to be monodisperse (M_r 1.5 × 10^7) by gel-permeation chromatography. Hydrolysis of the polysaccharide and g.l.c. analysis of the peracetylated aldononitriles[5] derived from the products showed the presence of mannose, glucose, and galactose in the molar ratios 1.7:1.0:1.4. After reduction of the uronic acid present, following methanolysis, these sugars appeared in the ratios 2.0:2.0:1.4. The increase in the molar proportion of glucose identifies the uronic acid as glucuronic acid, while the increase in the molar proportion of mannose suggests that the glucuronic acid is linked to a mannosyl residue. This was later confirmed by a β-elimination reaction[6,7] and the partial hydrolysis study. All the constituent sugars were shown to have the D configuration by g.l.c. analysis of their (−)-2-octyl glycoside acetates[8].

The high viscosity of the native polysaccharide resulted in a poorly resolved ^{1}H-n.m.r. spectrum. A much improved spectrum was obtained after the acid form of the polysaccharide had been heated in water for 30 min at 100°. The spectrum (Table I) contained five signals in the anomeric region, integrating for six protons. The 2-proton singlet (unresolved doublets) at δ 4.77 may be assigned to the anomeric protons of two β-mannopyranosyl residues[9,10]. Of the remaining four signals, only three are due to anomeric protons (*cf.* sugar ratios). Signals which frequently resonate in, or close to, the anomeric region in ^{1}H-n.m.r. spectra are those for H-4 and H-5 of α-galactopyranosyluronic acid residues[11–13] and H-5 of α-glucopyranosyluronic acid residues[9,14]. The latter resonance displays the largest coupling constant ($J_{4,5}$ ~10 Hz) and, thus, it is possible to assign the signal at δ 4.47 ($J_{4,5}$ 10 Hz) to H-5 of an α-glucopyranosyluronic acid residue. The solitary α-signal at δ 5.19 must therefore be due to H-1 of the uronic acid. The two remaining signals in the β-region of the spectrum are due to the anomeric protons of a galactose and a glucose residue. The assignment of the "anomeric" signals in the ^{1}H-n.m.r. spectrum of the partially hydrolysed polysaccharide was confirmed by comparison with the data obtained from the spectra of the oligosaccharides isolated during the partial hydrolysis study. The ^{1}H-n.m.r. spectrum also showed signals at δ 1.48 and 2.16 which were assigned to the methyl protons of 1-carboxyethylidene and acetate, respectively. The ratios of anomeric, 1-carboxyethylidene, and acetate protons were 5:3:1. This finding indicates that each pentasaccharide repeating-unit in the partially hydrolysed polysaccharide carries a 1-carboxyethylidene group while approximately every third repeating unit is *O*-acetylated. Despite the ^{13}C-n.m.r. spectrum of K69 being poorly resolved, the methyl resonance of the 1-carboxyethylidene group was seen at 26.0 p.p.m.

Methylation analysis. — Methylation of the K69 polysaccharide followed by g.l.c.–m.s. of the derived alditol acetates gave the results shown in Table II, column I; g.l.c. analysis of the reduced, methylated polysaccharide gave the data shown in column II. The results, when considered in conjunction with the partial hydrolysis study (see below), indicate that the polysaccharide has a pentasaccharide repeating-

TABLE I

N.M.R. DATA FOR *Klebsiella* K69 POLYSACCHARIDE AND OLIGOSACCHARIDES DERIVED THEREFROM

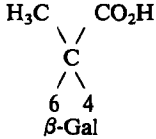
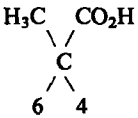
Compound[a]	*^{1}H-N.m.r. data*			
	δ[b] (*p.p.m.*)	$J_{1,2}$ (*Hz*)	*No. of H*	*Assignment*[c]
	5.19	n.o.[d]	1	α-GlcA
→4)-β-Glc-(1→4)-β-Man-(1→4)-β-Man-(1→	4.77	n.o.	2	→4)-β-Man
				
K69 Polysaccharide	4.62	7	1	β-Gal
	4.50	7	1	→4)-β-Glc
	4.47	10	1	H-5 of α-GlcA
	2.16	s[e]	1	CH_3 of OAc
	1.48	s	3	CH_3 of pyruvate acetal
α-GlcA-(1→3)-β-Man-(1→4)-Man	5.29	3	1	α-GlcA
A1	5.22	n.o.	0.6	→4)-α-Man
	4.94	n.o.	0.4	→4)-β-Man
	4.77	n.o.	0.6	→3)-β-Man-(1→4)-α-Man
	4.76	n.o.	0.4	→3)-β-Man-(1→4)-β-Man
α-GlcA-(1→3)-β-Man-(1→4)-Man-ol	5.29	3	1	α-GlcA
A1-alditol	4.81	n.o.	1	→3)-β-Man
	4.30	10	1	H-5 of α-GlcA
β-Glc-(1→4)-β-Man-(1→4)-Man	5.19	3.5	1	α-GlcA
3	5.19	n.o.	0.6	→4)-α-Man
↑	4.90	n.o.	0.4	→4)-β-Man
1	4.77	n.o.	0.6	→4)-β-Man-(1→4)-α-Man
α-GlcA				3
				↑
A2	4.76	n.o.	0.4	→4)-β-Man-(1→4)-β-Man
				3
				↑
	4.52	8	1	β-Glc
β-Glc-(1→4)-β-Man-(1→4)-Man-ol	5.23	3	1	α-GlcA
3				
↑	4.83	n.o.	1	→4)-β-Man
1				3
α-GlcA				↑
	4.51	8	1	β-Glc
A2-alditol	4.42	10	1	H-5 of α-GlcA

TABLE I *(continued)*

Compound[a]	*^{1}H-N.m.r. data*			
	δ[b] *(p.p.m.)*	*$J_{1,2}$ (Hz)*	*No. of H*	*Assignment*[c]
β-Gal 1 ↓ 6 β-Glc-(1→4)-β-Man-(1→4)-Man 3 ↑ 1 α-GlcA	5.18	n.o.	1	α-Glc
	5.18	n.o.	0.6	→4)-α-Man
	4.90	n.o.	0.4	→4)-β-Man
A3	4.77	n.o.	0.6	→-4)-β-Man-(1→4)-α-Man
	4.76	n.o.	0.4	→4)-β-Man-(1→4)-β-Man
	4.58	8	1	β-Gal
	4.47	8	1	β-Glc
	4.31	11	1	H-5 of α-GlcA

[a] For sources of *A1–3*, see text. [b]Chemical shift relative to that of acetone, δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS). [c] H-1 unless stated otherwise. [d] Not observed. [e] Singlet.

TABLE II

METHYLATION ANALYSIS OF *Klebsiella* K69 POLYSACCHARIDE AND DERIVED POLY- AND OLIGO-SACCHARIDES

Methylated sugars[a] *(as alditol acetates)*	T[b] *(OV-225)*	*Molar ratios*[c]					
		I	*II*	*III*	*IV*	*V*	*VI*
1,2,3,5,6-Man	0.54				0.41	0.36	0.70
2,3,4,6-Glc	1.00					0.55	0.84
2,3,4,6-Gal	1.11						0.70
2,4,6-Man	1.60				1.00		
2,3,6-Man	1.62	0.92	0.98	1.00			
2,3,6-Glc	1.81	0.98	0.97	0.64			
2,3,4-Glc	1.81		0.55		0.51	0.37	0.49
2,6-Man	2.28					1.00	
2,3-Man	2.73			0.95			
2,3-Gal	3.24	1.00	1.00	0.83			
2-Man	3.90	0.79	0.98				1.00

[a] 2,3,6-Man = 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol, *etc*, [b] Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on column DB-225 (J + W fused-silica capillary column, 0.25-μm film thickness, 30 m × 0.25 mm), isothermal at 205°. [c] Molar ratios: I, methylated native polysaccharide; II, methylated, reduced native polysaccharide; III, methylated product of uronic acid degradation; IV, reduced, methylated, reduced *A1*; V, reduced, methylated, reduced *A2*; VI, reduced, methylated, reduced *A3*.

unit containing two terminal groups and a double branch-point. The results identify a 4,6-pyruvalated galactose and the glucuronic acid as the non-reducing end-groups. The ~20% increase in the concentration of the 2-*O*-methylmannose residues after reduction of the uronic acid residues indicates that the glucuronic acid is directly linked to the mannose branch-point.

Base-catalysed degradation. — In a single operation, the permethylated polysaccharide was treated with methylsulphinyl carbanion and directly alkylated with methyl iodide[6,7]. A polymeric product was obtained, the analysis of which is given in Table II, column III. The results confirm that the glucuronic acid is terminal and show that it is linked to O-3 of the doubly branched mannose residue.

Partial hydrolysis. — Partial hydrolysis of the polysaccharide with acid, followed by gel-permeation chromatography, gave three acidic oligosaccharides (*A1*–*A3*).

α-GlcA-(1→3)-β-Man-(1→4)-Man

A1

β-Glc-(1→4)-β-Man-(1→4)-Man
3
↑
1
α-GlcA

A2

β-Gal
1
↓
6
β-Glc-(1→4)-β-Man-(1→4)-Man
3
↑
1
α-GlcA

A3

A1 had $[\alpha]_D$ +27°, a d.p.[15] of 3, and mannose as the reducing terminus. G.l.c. analysis (peracetylated aldononitriles) of *A1*-alditol gave mannose, glucuronic acid, and mannitol in the ratios 1:1:1. In the ^{1}H-n.m.r. spectrum of *A1* (Table I), the fractional resonances at δ 5.22 and 4.94 represent the anomeric protons of the α and β forms of the terminal reducing mannopyranose residue. The twin signals at δ 4.77 and 4.76 are in the same ratio as that of the anomeric protons of the reducing mannose, and are thus attributed to a β-linked mannosyl residue adjacent to the reducing end. This conclusion is confirmed by the spectrum of *A1*-alditol (Table I). Here, the reducing mannose residue has been reduced, causing the replacement of the β-mannose signals by a 1-proton singlet (unresolved doublet) at δ 4.81. The remaining anomeric signal at δ 5.29 in the spectrum of *A1* is therefore assigned to H-1 of an α-glucuronic acid residue. G.l.c. and g.l.c.–m.s. analysis of the alditol acetates derived from carboxyl-reduced, methylated *A1*-alditol are given in Table II, column IV. The methylation results are consistent with the structure for *A1*.

A2 had $[\alpha]_D$ +32°, ad.p.[15] of 4, mannose as the reducing terminus, and mannose, glucose, and glucuronic acid in the ratios 2:1:1. Comparison of the ^{1}H-

n.m.r. data for *A2* and its alditol with those for *A1* and its alditol reveals an additional resonance at δ 4.52 and 4.51 in the spectra of *A2* and *A2*-alditol, respectively. These signals must be due to H-1 of glucose residues in *A2* and *A2*-alditol, which are therefore β-linked. Methylation analysis of *A2*-alditol shows (Table II, column V) that the glucose residue occurs as a non-reducing terminus attached to O-4 of the interior mannosyl residue.

A3 had $[\alpha]_D$ +35°, ad.p.[15] of 5, mannose as the reducing terminus, and the same sugar composition as the native polysaccharide. Comparison of the ^{1}H-n.m.r. data for *A3* with those for *A2* (Table I) and of the methylation data for the respective alditols (Table II, columns VI and V) shows that *A3* has a terminal β-galactosyl group linked to O-6 of the central mannosyl residue.

The low proportions of 1,2,3,5,6-penta-*O*-methylmannitol acetate and 2,3,4-tri-*O*-methylglucitol triacetate in the methylation analysis of the alditols of *A1*, *A2*, and *A3* may be ascribed to losses sustained during the work-up procedures in the case of the former (because of its high volatility) and to incomplete reduction of the uronic acid group in the case of the latter. Attempts to isolate an oligosaccharide fragment carrying acetate from the partial hydrolysate were unsuccessful. Bacteriophage-mediated degradation of capsular polysaccharides has proved to be a successful method for locating such labile substituents as pyruvate and acetate. This approach is currently being pursued for K69 polysaccharide.

Comparison of the experimental data for the polysaccharide with those for *A3* clearly demonstrate that *A3* represents the chemical repeating-unit of depyruvalated, deacetylated *Klebsiella* K69 polysaccharide. The remaining information required to specify fully the structure of deacetylated K69 polysaccharide is the position of attachment of the β-mannopyranosyl residue in the repeating unit and the absolute configuration of the 1-carboxyethylidene group. A comparison of the methylation results of *A3* and the polysaccharide shows that the β-mannopyranosyl residue is linked to O-4 of the β-glucopyranosyl residue. The appearance of the methyl resonance of the 1-carboxyethylidene group at 26.0 p.p.m. in the ^{13}C-n.m.r. spectrum of the polysaccharide indicates that this group has the *R* configuration[16].

Thus, the structure of the deacetylated capsular polysaccharide of *Klebsiella* K69 is as shown in the Abstract. It is interesting to note that the structure for K69 polysaccharide differs from the structures of deacetylated K30 and K33 polysaccharides only in the location of the 1-carboxyethylidene group. In the latter two polysaccharides, the group is attached to O-3 and O-4 of the galactopyranosyl residue.

EXPERIMENTAL

General methods. — Optical rotations were measured at 21–23° with a Perkin–Elmer model 141 polarimeter for aqueous solutions in a 1-cm cell. Solutions were concentrated under reduced pressure at ⩽40° (bath). Descending p.c. was performed on Whatman No. 1 paper with *A*, 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; and *B*, 5:1:5:3 ethyl acetate–acetic acid–pyridine–water; and de-

tection with alkaline silver nitrate[17] or periodate-benzidine[18].

^{1}H-n.m.r. spectra (internal acetone, δ 2.23) were recorded with a Bruker WM 500 Ft spectrometer at 30° and at 95°. Samples were deuterium-exchanged by freeze-drying solutions in D_2O (99.7 and 99.96%). Gel-permeation chromatography was performed on columns of Sepharose 4B CL, Sephacryl S500, and Bio-Gel P-2, calibrated with dextrans, and elution with M NaCl or 0.1M pyridine acetate buffer (pH 5.2). Fractions (1 mL) were analysed by the phenol–sulfuric acid method[19]. Analytical g.l.c. was performed at 205° and 225°, using a Hewlett–Packard 5890 A gas chromatograph fitted with flame-ionisation detectors, a 3392A recording integrator, a DB-225 bonded-phase capillary column (30 m × 0.25 mm) having a film thickness of 0.25μm, and with helium as the carrier gas. G.l.c.–m.s. was conducted with a Micromass 16F spectrometer, with an ionisation energy of 40 eV and an ion-source temperature of 170°.

Preparation and properties of K69 polysaccharide. — A culture of *Klebsiella* K69, obtained from Dr. I. Ørskov (Copenhagen), was propagated on Mueller–Hinton agar. The acidic capsular polysaccharide, isolated and purified using cetyltrimethylammonium bromide[4], had $[\alpha]_D$ +55° and, on elution from a column (60 × 1.6 cm) of Sephacryl S500 with pyridine acetate buffer, was shown to be monodisperse (M_r = 1.5 × 10^7, calibration with dextrans).

Sugar composition. — The polysaccharide (5 mg) was hydrolysed with 2M trifluoroacetic acid (16 h) at 100° and the acid was then evaporated. The products were converted into the acetylated aldononitriles[5] and analysed by g.l.c. Dried polysaccharide (10 mg) was treated with refluxing methanolic 3% hydrogen chloride for 16 h at 80°, the mixture was then neutralised, and the products were reduced with $NaBH_4$ in anhydrous methanol and then hydrolysed with 2M trifluoroacetic acid (16 h, 100°). The products were converted into the acetylated aldononitriles and examined by g.l.c.

Methylation analysis. — The polysaccharide (40 mg), in the acid form, was methylated by a modified Hakomori method[20], using equal volumes of methyl sulphoxide and tetramethylurea as solvent. The methylated polysaccharide (15 mg) was treated with methanolic 3% hydrogen chloride for 16 h at 80°, and the mixture was neutralised and then concentrated. One portion of the residue was hydrolysed with 2M trifluoroacetic acid overnight at 100°, and the products were reduced with $NaBH_4$ and then acetylated. The other portion was reduced with $NaBH_4$ in dry methanol and then hydrolysed with 2M trifluoroacetic acid, and the products were treated as for the first portion. The partially methylated alditol acetates of both portions were analysed by g.l.c. and g.l.c.–m.s. (Table II, columns I and II).

Base-catalysed degradation. — Methylated K69 polysaccharide (20 mg) was degraded by base[6,7] and then re-alkylated with methyl iodide. The polymeric product was hydrolysed with 2M trifluoroacetic acid, and the products were reduced with $NaBH_4$, acetylated with 1:1 acetic anhydride–pyridine, and analysed by g.l.c. and g.l.c.–m.s. (Table II, column III).

Partial hydrolysis. — Samples (3 × 250 mg) of polysaccharide were separately

heated for 1.5, 3, and 5.5 h at 100°. The acid was removed from each sample by co-evaporation with water and each product was eluted from a column (65 × 2.6 cm) of Bio-Gel P-2 with water. Fractions corresponding to tri-, tetra-, and pentasaccharides were combined and re-chromatographed to give *A1* (63 mg), R_{GAL} 0.56 (solvent *B*), $[\alpha]_D$ +27°, d.p.[15] 3, and a glucuronic acid–mannose ratio of 1:21; *A2* (59 mg), R_{GAL} 0.39, $[\alpha]_D$ +32°, d.p.[15] 4, and glucuronic acid–mannose–glucose ratios of 1:2:1; *A3* (40 mg) R_{GAL} 0.27, $[\alpha]_D$ +35°, d.p.[15] 5, and glucuronic acid–mannose–glucose–galactose ratios of 1:2:1:1.

A1 (26 mg), *A2* (19 mg), and *A3* (9 mg) were reduced with $NaBD_4$, a portion of each alditol was methylated, purified (Sephadex LH-20), and methanolysed, and the products were reduced ($NaBH_4$), hydrolysed with 2M trifluoroacetic acid, converted into partially methylated alditol acetates, and examined by g.l.c. and g.l.c.–m.s. (Table II, columns IV–VI).

Determination of absolute configuration. — Polysaccharide (10 mg) was methanolysed, and the products were reduced ($NaBH_4$), then hydrolysed with 2M trifluoroacetic acid, and converted into the (−)-2-octyl glycoside acetates[8] which were examined by g.l.c.

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