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PRIMARY STRUCTURE OF *Klebsiella* SEROTYPE K22 CAPSULAR POLYSACCHARIDE: ANOTHER GLYCAN CONTAINING 4-*O*-[(*S*)-1-CARBOXYETHYL]-D-GLUCURONIC ACID*

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ABSTRACT

The primary structure of the acidic capsular polysaccharide isolated from *Klebsiella* serotype K22 has been investigated using methylation analysis, hydrolysis, bacteriophage-borne enzyme degradation, and n.m.r. spectroscopy. The repeating unit comprises the chain disaccharide →3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ substituted by 4-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p*A-(1→6)-α-D-Glc*p*-(1→ at O-4 of the galactose. The galactose carries an *O*-acetyl group on position 6.

INTRODUCTION

Seventy seven *Klebsiella* K-antigens have been distinguished serologically[1]; of these, only K22, K29, K42, K43, K65, and K71 remain to be characterised. The K22 antigen has been found[2,3] to contain an unusual acidic sugar which was not fully characterised. We have now completed this study and report the primary structure of the *Klebsiella* serotype K22 repeating-unit.

RESULTS AND DISCUSSION

Preparation of K22 polysaccharide. — Two different batches of polysaccharide were prepared. In the first batch, *Klebsiella* 1996/49 (O1:K22) bacteria were grown on $D_{1.5}$ agar for 3 days and the acidic polysaccharide was isolated by the phenol–water–cetyltrimethylammonium bromide procedure[4,5]. After treatment with RNAse, the product was treated with alkali to afford polysaccharide PS-A,

*Dedicated to Professor Bengt Lindberg.

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TABLE I

METHYLATION DATA FOR K22 PS-A AND DERIVED PRODUCTS

Methylated sugars[a]	*Molar ratios*[c]		
	I[b]	*II*	*III*
1,2,5,6-Gal[d]			0.7
2,3,4,6-Glc	0.1	0.5	1.0
2,4,6-Gal		0.3	
2,3,6-Glc	1.0	1.0	
2,3,4-Glc	0.9	0.1	0.9
2,6-Gal	1.0	0.5	

[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.* [b]I, K22 polysaccharide PS-A; II, partially hydrolysed polysaccharide; III, oligomer P1-A. [c]Determined on a glass column (1.52 m by 0.2 cm) of ECNSS-M on Gas-Chrom Q. [d]Deuterated at C-1.

which had $[\alpha]_D$ +8.5°. In the second batch, bacteria were grown on sucrose-rich nutrient agar and the polysaccharide was isolated and purified as described previously[6], to afford polysaccharide PS-C which had $[\alpha]_D$ +9.0°.

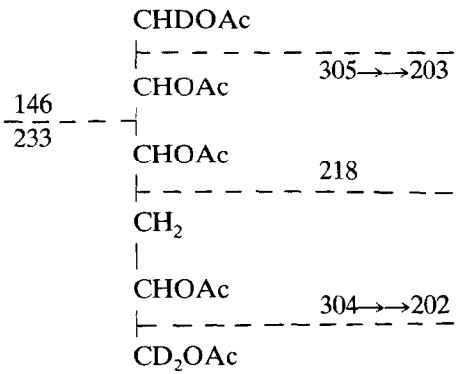
Composition of PS-A. — Paper chromatography and electrophoresis of an acid hydrolysate of PS-A indicated the presence of glucose, galactose, and an unusual acidic sugar (XA). Quantitative determination of the hexoses by g.l.c. as well as enzymically, and of the unknown acidic component with carbazole–sulphuric acid[7], using D-glucuronic acid as standard, indicated that the polymer contained D-glucose, D-galactose, and XA in the ratios 2:1:1. PS-A exhibited an u.v. absorption at λ_{max} 235 nm, which was eliminated by hydrogenation, indicating unsaturation in the molecule.

Methylation analysis and partial hydrolysis of PS-A. — The polysaccharide was methylated and hydrolysed, and the derived alditol acetates were analysed by g.l.c.–m.s. (column I, Table I). Although the acidic sugar was not observed, the substitution patterns of the three other sugars indicate that it must be in a terminal position. The repeating unit of PS-A therefore consists of a tetrasaccharide with a galactose branch. When PS-A was treated briefly with acid, the resulting neutral polymer was composed of glucose and galactose in the ratio 1.5:1.0, indicating that the acidic sugar must have been located in the side chain. Methylation analysis of the neutral polymer (column II, Table I) revealed (*a*) that all of XA and some 6-linked glucose were removed during acid treatment of PS-A, (*b*) that the branch in PS-A consists of the unit XA-(1→6)-Glc-(1→, and (*c*) that this unit is linked to O-4 of the galactosyl residue in the chain disaccharide →4)-Glc-(1→3)-Gal-(1→.

Bacteriophage degradation of PS-A. — *Klebsiella* phage ϕ22 (ref. 8) forms plaques with acapsular haloes[9] on *Klebsiella* 1996/49 and has been shown to depolymerise the K22 glycan[8]. The virus has an isometric head of 55 nm in diameter and a flexible tail of 182 nm, carrying four spikes of ~10 nm (ref. 2). The viral depolymerase is assumed to be associated with these latter organelles[9]. After

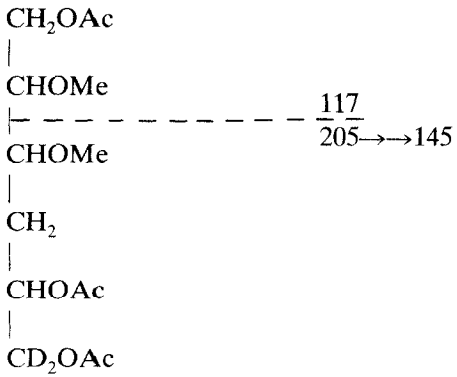
exhaustive incubation of PS-A with purified particles of ϕ22, three major oligosaccharides, a tetra-, octa-, and dodeca-saccharide, were isolated after ion-exchange chromatography and designated P1-A, $[\alpha]_D$ +60°; P2-A, $[\alpha]_D$ +44°; and P3-A, $[\alpha]_D$ +41°. Each had the same composition as PS-A when analysed by g.l.c. and with carbazole–sulphuric acid. Reduction of P1-A and P2-A with NaB^3H_4 and analysis of the amount of tritium present in the g.l.c. fractions after hydrolysis indicated that galactose was the reducing terminus in each case. However, a small amount of tritiated glucose was detected; this is attributed to the presence of a small amount of oligomer terminated by glucose and points to the fact that the viral enzyme is not 100% bond-specific. This phenomenon of a partial lack of specificity has been observed also with other bacteriophage-associated glycanases[10].

P3-A was hydrogenated and partially hydrolysed with dilute acid, and the major acidic component was isolated by paper electrophoresis. The product was esterified with diazomethane, reduced with $Ca(B^2H_4)_2$, acetylated, and analysed by g.l.c.–m.s. The fragmentation obtained (shown below) indicates that the product is a 3-deoxyhexitol penta-acetate which must have been derived from a hex-4-enuronic acid (XA) in P3-A.



P1-A was treated with diazomethane, reduced with $Ca(B^2H_4)_2$, methylated, and hydrolysed, and the derived alditol acetates were examined by g.l.c.–m.s. (column III, Table I). The results indicate that the cleavage of the polysaccharide by the viral enzyme occurred between the branched galactose and the in-chain 4-linked glucose.

P3-A was hydrogenated, methylated, reduced with $Ca(B^2H_4)_2$, and hydrolysed, and the products were converted into alditol acetates. Examination of the partially methylated alditol acetate derived from XA gave the result shown below.



This result confirms that XA possesses hydroxyl groups in positions 2 and 3, that the double bond is located between C-4 and C-5, and that the hex-4-enuronic acid occupies a terminal position in the polysaccharide.

N.m.r. spectroscopy and anomeric configurations of the components of PS-A. — It was not possible to obtain an n.m.r. spectrum of K22 PS-A because of the high viscosity of its solution; however, good ^{1}H-n.m.r. spectra of P1-A and P3-A were obtained and the results are summarised in Table II. The partially hydrolysed polysaccharide, which contained glucose and galactose in a 1.5:1.0 ratio, also gave a reasonable ^{1}H-n.m.r. spectrum and, by comparing the spectra, it was possible to assign the anomeric configurations of the component sugars. The ^{1}H-n.m.r. spectrum of P1-A (Na^+) showed one α- and one β-anomeric signal integrating for one proton each, as well as the partial α- and β-signals for reducing-end galactose. Two other 1-proton resonances were observed at δ 5.15 (J 5 Hz) and 6.06 (J 4 Hz). These are absent from the spectrum of the partially hydrolysed polymer and must therefore belong to the acidic sugar. These values are in agreement with those of H-1 and H-4 of a hex-4-enuronic acid[11]. In the latter spectrum, two 1-proton β-anomeric signals were observed as well as an α-anomeric signal at δ 4.92 (J 3 Hz) integrating for only ~0.4 H. As mentioned before, the methylation results of this polymer indicate that only ~0.5 mol of terminal glucose is present, and so the signal at δ 4.92 can be assigned to H-1 of the terminal α-glucose and the in-chain sugars are thus β-linked. The repeating unit of K22 PS-A can thus be written as follows, where XA is a hex-4-enuronic acid.

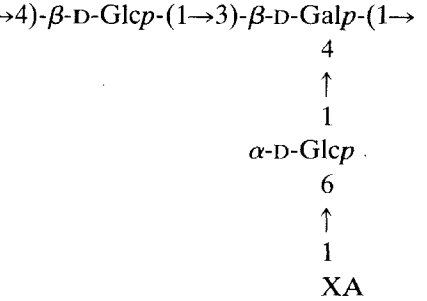


TABLE II

^{1}H-N.M.R. DATA FOR K22 PS-A PRODUCTS

Compound	*δ (p.p.m.)*[a]	*No. of H*	$J_{1,2}$ *(Hz)*	*Assignment*[b]
Partially hydrolysed PS-A	4.92	~0.4	3	α-Glc-
	4.62	~1	7–8	β-Gal-,
→4)-β-Glc-(1→3)-β-Gal-	4.46	~1	7–8	β-Glc-
4				
⋮				
1				
(40%) α-Glc				
P1-A	6.06	1.0	4	H-4 XA
β-Glc-(1→3)-Gal	5.31	0.5	2	α-Gal
4	5.15	1.0	5	XA
↑	4.96	1.0	3.5	α-Glc-
1	4.66	0.5	8	β-Gal
α-Glc	4.64	1.0	8	β-Glc-
6				
↑				
1				
XA				
P3-A	6.13	0.8	3	H-4 XA
	5.16	0.8	5	XA
	4.93	1.0	4	α-Glc-
	4.55	2.0		β-Gal-, β-Glc-

[a]Chemical shift in p.p.m. relative to internal acetone at δ 2.17 downfield from TSP, measured on a Bruker HFX-90 spectrometer at 70°. [b]α-Glc- connotes the anomeric proton of a glucose residue in the α-anomeric configuration; the absence of a hyphen indicates a reducing residue. [c]Overlapped signals.

Composition and n.m.r. spectra of PS-C. — The ^{1}H-n.m.r. spectrum of an ultrasonicated sample of PS-C (Na^+) showed (Table III) two 3-proton signals in the high-field region at δ 1.33 (doublet, *J* 6.1 Hz) and 2.17 (singlet). The latter resonance is ascribed to the methyl protons of an OAc group in the repeating unit while the former is indicative of a methyl substituent adjacent to a methine group[12]. In the low-field region of the spectrum, two very small signals were observed at δ 5.81 and 5.08. These signals probably indicate the presence of a small amount of hex-4-enuronic acid[11] in the polymer. Five signals were observed in the δ 4.3–5.0 region, one of which (δ 4.46) integrated for two protons. PS-C was treated with potassium methylsulphinylmethanide, and the ^{1}H-n.m.r. spectrum of the product (PS-CB) revealed that the OAc groups had been removed, that the signal at δ 4.46 now integrated for one proton only, that a signal at δ 4.36 was absent, and that only four signals were now present in the anomeric region of the spectrum. These results demonstrate that the acetate groups in the native polysaccharide cause two protons to be deshielded into the anomeric region of the spectrum and suggest that the OAc groups are located[13] at position 6 of a hexose residue. The results further indicate that the repeating unit of native K22 polysaccharide (PS-C) is a tetrasaccharide possessing one α- and three β-linked sugars.

TABLE III

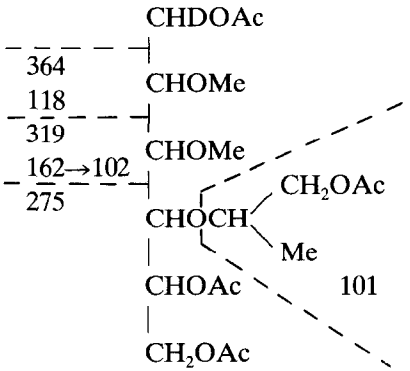
^{1}H- AND ^{13}C-N.M.R. DATA FOR POLYSACCHARIDE PS-C AND DERIVED PRODUCTS

Compound[a]	*^{1}H-N.m.r. data (500 MHz)*				*^{13}C-N.m.r. data (125 MHz)*
	δ (p.p.m.)[b]	*J (Hz)*	*No. of H*	*Assignment*[c]	*δ (p.p.m.)*[d]
Polysaccharide	1.33(d)	6.1	3	CH_3 Lactyl	
PS-C[f]	2.17(s)		3	CH_3 Acetyl	
	5.81		~0.1		
	5.08		~0.1		
	4.94		1	α-Glc-	
	4.73		1	β-Glc-	
	4.61	7.5	~0.9	β-Gal-	
	4.46		2	Lac-β-GlcA-, H-6 β-Gal-OAc	
	4.36		1	H-6′ β-Gal-OAc	
Polysaccharide	1.34(d)	6.0	3	CH_3 Lactyl	
base-treated	4.97	n.o.	1	α-Glc-	
PS-CB[f]	4.72	~7.5	1	β-Glc-	
	4.59	~7.5	1	β-Gal-	
	4.47	~7.5	1	Lac-β-GlcA-	
P1-C[g]	1.374(d)	6.9	3.0	CH_3 Lactyl	19.083
OAc	2.126(s)		1.6	CH_3 Acetyl	21.039
|	2.124(s)		1.0		
6	3.201(dd)	9.3	0.6	H-2 β-Glc-	
β-Glc-(1→3)-Gal	3.202(dd)	9.3	0.4		
4	3.480(dd)	9.3	1.0	H-2 α-Glc-	
↑	3.680(dd)	9.4	0.6	H-2 β-Gal	
1	4.053(dd)	9.0	0.4	H-2 α-Gal	
α-Glc	4.281(q)	6.9	1.0	CH Lactyl	77.777
6	4.548	8.1	1.0	Lac-β-GlcA-	103.211
↑	4.614	7.9	0.4	β-Glc-	105.456
1	4.622	7.6	0.6		105.508
4-*O*-Lac-β-GlcA	4.698	7.8	0.6	β-Gal	97.071
	4.932	3.7	1.0	α-Glc-	99.985. 100.034
	5.324	3.3	0.4	α-Gal	93.092
	3.402(dd)	7.9	1.0	C/H-2 Lac-β-GlcA-	73.618 (74.79)[e]
	3.686(t)	9.3	1.0	C/H-3 Lac-β-GlcA-	75.839 (76.53)
	3.619(t)	9.3	1.0	C/H-4 Lac-β-GlcA-	81.007 (72.79)
	4.054(d)	9.3	1.0	C/H-5 Lac-β-GlcA-	74.764 (76.90)
					174.549
				COOH Lac-β-GlcA-,	174.614 (176.82)
				C=O Acetyl	173.261
				COOH Lactyl	178.454

[a]For the sources of compounds, see text. [b]Chemical shift relative to internal acetone, δ 2.230 downfield from DSS. [c]β-Glc- connotes the anomeric proton/carbon of a glucose residue in the β-anomeric configuration, the absence of a hyphen indicates a reducing residue; s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet. [d]Chemical shift in p.p.m. relative to internal acetone, 31.070 p.p.m. downfield from DSS. [e]Values in parentheses refer to chemical shifts of β-D-GlcA. [f]Recorded at 95°. [g]Recorded at 30°.

PS-C was methanolysed, carboxyl-reduced, and hydrolysed, and g.l.c. of the derived alditol acetates showed derivatives of glucose, galactose, and a slow-eluting compound, T_{Gal} 2.58, in the ratios 1.9:1.0:0.6. The last compound had the same retention time (DB-225) as the alditol acetate derived from carboxyl-reduced 4-*O*-[(*S*)-1-carboxyethyl]-D-glucuronic acid (from *Klebsiella* K37 polysaccharide)[14] and differed from that (T_{Gal} 2.66) of the alditol acetate derived by carboxyl-reduction of 4-*O*-[(*R*)-1-carboxyethyl]-D-glucose (from *Klebsiella* K66 polysaccharide)[12]. Lindberg *et al.* have reported[15] the g.l.c. separation of derivatives of the *R* and *S* isomers of 4-*O*-hydroxypropyl-D-glucose.

Bacteriophage depolymerisation of PS-C. — Bacteriophage ϕ22 depolymerisation of PS-C followed by gel-permeation chromatography of the digest afforded oligosaccharides P1-C, P2-C, and P3-C. Oligosaccharides P1-C and P2-C were reduced with NaB^2H_4, methylated, methanolysed, reduced, hydrolysed, reduced with NaB^2H_4, and acetylated, and the products were examined by g.l.c.–m.s. The results (columns I and II, Table IV) indicated that P1-C was a tetrasaccharide and P2-C was an octasaccharide, each having galactose at the reducing terminus. The e.i.-mass spectral fragmentation pattern of the alditol acetate with the retention time of 4.29 relative to internal 2,3,4,6-tetra-*O*-methylglucitol diacetate was compatible with the following structure.



Characterisation of the acidic sugar of PS-C. — K22 PS-C was methanolysed, carboxyl-reduced, and hydrolysed, and the fastest eluting component was isolated by paper chromatography and purified by gel-permeation chromatography. ^{13}C-N.m.r. spectroscopy (Table V) of the product showed sixteen resonances whose chemical shifts were in keeping with the α and β forms of 4-*O*-hydroxypropyl-glucose. The signals for C-4 are displaced by ~9 p.p.m. from those of α- and β-glucose, confirming the position of substitution by the hydroxypropyl unit. The β-effect can be observed for C-5 but not for C-3. This phenomenon is consistent with previous observations[16].

The sugar had $[\alpha]_D$ +60° in keeping with that of authentic 4-*O*-[(*S*)-hydroxypropyl]-D-glucose[15], and different from that of the *R* isomer, $[\alpha]_D$ +49°. After de-etherification with 48% aqueous hydrogen bromide[17], g.l.c. analysis of the derived

TABLE IV

METHYLATION DATA FOR OLIGOMERS P1-C AND P2-C

Methylated sugar[a]	R_{TMG}[b]	*Molar ratios*		
		I[c]	*II*	*III*
1,2,5,6-Gal[d]	0.89	0.74	0.58	
2,3,4,6-Glc	1.00	0.99	0.95	1.07
2,3,4-Glc	1.51	1.00	1.95	3.00
2,3,6-Glc	1.97		0.89	
2,6-Gal	2.47		1.00	0.33
2-Gal	2.78			1.53
2,3,4[R]-Glc	4.29	0.51	0.89	0.76

[a]2,3,4,6-Glc refers to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol *etc.* [b]Retention time relative to that of 2,3,4,6-Glc, determined on a DB-225 column (30 m × 0.25 mm) at 205°. [c]I, P1-C alditol; II, P2-C alditol; III, P2-C methylated while preserving the OAc groups, 235°. [d]Deuterated at C-1. [R]Refers to hydroxypropyl.

TABLE V

^{13}C-N.M.R. DATA FOR 4-*O*-[(*S*)-2-(1-HYDROXY)PROPYL]-D-GLUCOSE FROM K22

	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1'*	*C-2'*	*C-3'*
4-*O*-hp-α-Glc[a]	92.74	72.29	73.78	79.25	71.45	61.08	66.08	77.86	17.25
α-Glc[b]	92.9	72.5	73.8	70.6	72.3	61.6			
4-*O*-hp-β-Glc[a]	96.68	74.93	76.72[c]	79.25	76.08[c]	61.22	66.00	77.73	17.25
β-Glc[b]	96.7	75.1	76.7	70.6	76.8	61.7			

[a]Chemical shift in p.p.m. as for Table III; hp connotes hydroxypropyl. [b]Data taken from ref. 30. [c]Values may be interchanged.

acetylated aldononitriles[18] revealed 40% of glucose and 60% of unchanged 4-*O*-hydroxypropylhexose. The glucose so produced was converted into the acetylated (−)-2-octyl glycosides[19], and g.l.c. indicated that it was D. These results, when considered together with the n.m.r. data for P1-C (Table III), conclusively prove that the K22 acidic sugar is 4-*O*-[(*S*)-1-carboxyethyl]-D-glucuronic acid and is identical to that found in *Klebsiella* K37 polysaccharide.

N.m.r. spectroscopy of P1-C and location of the acetyl group. — ^{1}H-N.m.r. spectroscopy (Table III) indicated P1-C (H^+) to be a tetrasaccharide containing close to 1 mol of OAc groups. Apart from the partial α- and β-anomeric signals of the reducing galactose, one α- and two β-anomeric signals were observed. Comparison of the chemical shifts of the H-1 resonances of P1-A (Table II) with those of P1-C (Table III) permits the assignment of the resonance at δ 4.932 to H-1 of the α-glucose residue and the twinned resonances at δ 4.622 and 4.614 to H-1 of the β-glucose residue. The signal at δ 4.548 in the spectrum of P1-C, which was not observed in the spectrum of P1-A, may therefore be attributed to H-1 of the acidic

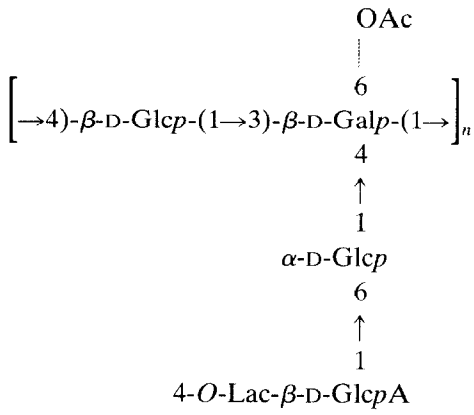
sugar. The ^{13}C-n.m.r. spectrum of P1-C (Table III) contains four signals in the carbonyl region. Two of the signals are attributed to acetate carbonyls (see later) and the remaining two must therefore arise from the carboxyl groups of the acidic sugar. The isolation of 4-*O*-hydroxypropylglucose from methanolysed, reduced PS-C and the presence in the ^{1}H-n.m.r. spectrum of P1-C of resonances at δ 1.37 (3 H, J 6.9 Hz) and 4.281 (1 H, J 6.9 Hz) confirm that the acidic sugar is a 4-*O*-lactyl-β-glucuronic acid (see above). The protons of the OAc group were also twinned due to the mutarotation and resonated at δ 2.124 and 2.126. This suggests that the OAc groups reside on the reducing galactose residues in P1-C.

The ^{13}C-n.m.r. spectrum of P1-C showed the two β-anomeric signals, an α-anomeric signal which was twinned, and the partial signals for the reducing galactose. A DEPT[20] spectrum showed seven signals due to C-6 resonances. The signals at δ 68.537 and 68.625 can be assigned to C-6 of the α-glucose which is 6-linked; the twinning may be due to the mutarotation of the reducing galactose. The signal at δ 62.209 is assigned to the unlinked C-6 of the β-glucose residue. The shifts of the two signals at δ 63.435 and 63.587 are in keeping with C-6 carbons that are *O*-acetylated[13], and since they are twinned can be assigned to *O*-acetylated C-6 carbons of the reducing galactose. The low-intensity twinned signals at δ 60.759 and 60.853 are assigned to the C-6 carbons of the small number of galactose residues that are unacetylated.

In the COSY-45 spectrum of P1-C, the connectivities between all the H-1 and H-2 signals were observed; however, many of the other connectivities were overlapped due to complication of the spectrum by the mutarotation of the reducing residue and by the incomplete acetylation. The terminal 4-*O*-lactylglucuronic acid was the only unit not affected and the chemical shifts of all the protons could be readily extracted from the COSY spectrum. The ^{13}C chemical shifts of this unit were obtained from the ^{13}C–^{1}H shift-correlated spectrum of P1-C.

Confirmation that the OAc groups are located on C-6 of the reducing galactose was achieved by methylating P2-C under acidic conditions that preserve OAc groups. P2-C was methylated with methyl triflate in trimethyl phosphate[21], then methanolysed, reduced, and hydrolysed, and the products were analysed by g.l.c. as alditol acetates (column III, Table III). The decrease in the value of 2,6-di-*O*-methylgalactose (from ~2 mol to 0.33 mol) and the appearance of 1.53 mol of 2-*O*-methylgalactose indicates clearly that the OAc substituents are carried on position six of the branched reducing galactose. The small amount of 2,6-di-*O*-methylgalactose shows that not quite 1 mol of OAc group is present in P2-C; this inference is borne out by the ^{1}H-n.m.r. spectrum. The structure of K22 polysaccharide PS-C is thus as shown below.

The acidic residue in PS-A thus appears to be an artefact produced by elimination of the lactate group. PS-A was obtained by treatment of the native polysaccharide with alkali and thus may suggest that the elimination was alkali-mediated. However, the presence of a small amount of hexenuronic acid in PS-C,



which was not treated with alkali, suggests otherwise. Preliminary experiments strongly indicate that removal of lactate occurs during the production and not the isolation and purification of the polysaccharide. It appears that *Klebsiella* K22 bacteria are able to produce an enzyme to eliminate lactate for use as a source of carbon when nutrients become depleted during growth. Further experiments are in progress.

Although the acidic polysaccharides of *Klebsiella* K22 and K37 have the same sequence of sugars, they differ in their serology. This difference appears to be due to the presence of OAc substituents in the former. The only other K-antigen in the *Klebsiella* series to possess a lactyl group is K66, which has 4-*O*-[(*R*)-1-carboxyethyl]-D-glucose as a constituent[12].

EXPERIMENTAL

Materials and methods. — Optical rotations were measured for aqueous solutions in a 1-cm cell at 21–23°, using a Perkin–Elmer model 141 polarimeter. Solutions were evaporated under diminished pressure at bath temperatures ≤40°. Descending paper chromatography was conducted with Schleicher and Schüll paper No. 2043a and b, or Whatman No. 1 paper, and the following solvent systems: *A*, ethyl acetate–pyridine–water (8:2:1 or 4:1:1); *B*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); and *C*, ethyl acetate–acetic acid–water (3:1:1). High-voltage electrophoresis was conducted on the No. 2043b paper, using pyridine acetate buffer at pH 5.3 and application of 80–130 mA for 45–90 min. Sugars were detected with alkaline silver nitrate[22]. G.l.c. was performed on a Finnigan 9500 chromatograph, with flame-ionisation detection, a 1.52 m × 0.2 cm glass column of ECNSS-M on Gas Chrom Q, and nitrogen as carrier gas. For g.l.c.–m.s., the same chromatograph was coupled to a Finnigan model 3200E-003 mass spectrometer

with helium as the carrier gas. G.l.c. and g.l.c.–m.s. were also carried out using a Hewlett-Packard 5890A chromatograph, with flame-ionisation detection, a DB-225 bonded-phase capillary column (30 m × 0.25 mm) having a film thickness of 0.25 μm, and helium as carrier gas; and a Hewlett-Packard 5988A g.l.c.–mass spectrometer using the same column. N.m.r. spectra were recorded with a Bruker HFX-90 or Bruker WM-500 Ft spectrometer at 30° (in some cases at 70° and 95° for ^{1}H-n.m.r.). Samples were deuterium-exchanged by freeze-drying from D_2O. Acetone was used as the internal standard. COSY-45, ^{13}C–^{1}H correlation, and DEPT spectra were acquired using standard Bruker software.

Hexoses were determined enzymically using fungal D-glucose oxidase (EC 1.1.3.4)[23] and D-galactose dehydrogenase from *P. fluorescens* (EC 1.1.1.48)[24]. Hexuronic acid was determined with carbazole–sulphuric acid[7] in the intact samples, using D-glucuronic acid as standard. For carboxyl-reduction, dried samples were treated with boiling methanolic 3% hydrogen chloride for 16 h at 80° and, after neutralisation, the products were reduced with $NaBH_4$ in anhydrous methanol. Hydrolyses were carried out with 2M trifluoroacetic acid for 12–16 h at 100° or 0.25M H_2SO_4 for 8–10 h at 100°. Alditol acetates were prepared by reduction of sugars with $NaBH_4$ or NaB^2H_4 and acetylation with pyridine–acetic anhydride (1:1) for 1 h at 100°. Methylation was effected by the Hakomori method following experimental details given by Hellerquist *et al.*[25], and also using the method of Phillips and Fraser[26].

Preparation and composition of polysaccharides. — (*a*) Polysaccharide PS-A was prepared from *Klebsiella* 1996/49 (O1:K22) which was grown on $D_{1,5}$ agar (Merck). The bacteria were harvested and dried, and the acidic polysaccharide was extracted by the phenol–water–cetyltrimethylammonium bromide (CTAB) procedure[4] (26 g of dry bacteria were obtained from 100 15-cm plates and yielded 1 g of polysaccharide). The product was treated with RNAse from bovine pancreas (EC 3.1.4.22) to remove residual RNA[27] and further treated with 0.25M NaOH for 4 h[5] at 56° to yield polysaccharide PS-A which had a λ_{max} of 235 nm.

A portion of PS-A was hydrolysed and the constituent sugars were analysed enzymically[23,24], by carbazole–sulphuric acid[7], and by g.l.c. of the derived alditol acetates. PS-A (40 mL of a 0.1% aqueous solution) was hydrogenated over 50 mg of $Pd/BaSO_4$ (10% Pd, Merck), the air above the solution was replaced by nitrogen and then by hydrogen (1 atm.), and the mixture was stirred at room temperature until the product no longer absorbed at 235 nm. The catalyst was removed by filtration and the filtrate was lyophilised to yield the hydrogenated product (47 mg). PS-A (100 mg) was partially hydrolysed with 0.1M trifluoroacetic acid (20 mL) for 90 min at 100°. The residual polymer was precipitated with ethanol (96%, 70 mL), and a solution in distilled water was dialysed and lyophilised to yield partially hydrolysed PS-A (43 mg).

(*b*) Polysaccharide PS-C was isolated from *Klebsiella* 1996/49 which was grown on sucrose-rich nutrient agar as previously described[6]. The acidic polysaccharide was separated from the cells by ultracentrifugation and purified with

CTAB (1.39 g of PS-C was obtained, 92 mg per litre of nutrient medium). The acid form of PS-C (100 mg) in Me_2SO (10 mL) was stirred with potassium methylsulphinylmethanide (10 mL) for 16 h at room temperature. After neutralisation of the base with acetic acid (10%) and dialysis, the polysaccharide PS-CB was recovered by lyophilisation. PS-C (15 mg), *Klebsiella* K37 polysaccharide (15 mg, prepared as for K22), and *Klebsiella* K66 polysaccharide (12 mg, kindly supplied by Professor B. Lindberg) were methanolysed, reduced, and hydrolysed, and the products were analysed by g.l.c. as alditol acetates.

Bacteriophage degradations. — *Klebsiella* bacteriophage $\phi 22$ was used throughout. For the degradation of PS-A, the production of high-titre phage stocks and the purification of the virus particles were carried out essentially as described[10] for *Klebsiella* $\phi 11$. The final yield of purified $\phi 22$, after isopycnic centrifugation through CsCl, was $\sim 2 \times 10^{13}$ plaque-forming units (PFU) from 1 L of culture. Solutions containing ~3 mg of PS-A and 10^{11} PFU of $\phi 22$ particles per mL of PBS (phosphate-buffered saline, pH 7) were incubated at 37°, and the decrease in viscosity (Ostwald viscometer) and increase in reducing power were monitored. The viscosity became constant after 2 h and maximum reducing power was reached after 3 h (6.5 μg of degradation products equivalent to 1 μg of glucose). After 48 h, the digest was desalted (Sephadex G-10) and lyophilised. The oligomers so obtained were taken up in 0.05M Tris/HCl buffer (pH 7.2), adsorbed on to a column of DEAE-Sephadex A-25, and eluted with a linear NaCl gradient 0–0.5M in the same buffer. Most of the material appeared in three fractions, P1-A 13%, P2-A 21%, and P3-A 27%, eluting at 0.16, 0.24, and 0.28M NaCl, respectively, which were desalted and lyophilised. Paper electrophoresis showed them to be homogeneous having M_{GlcA} 0.48, 0.54, and 0.78, respectively. For determination of the composition and d.p., the oligomers were reduced with $NaBH_4/NaB^3H_4$ and hydrolysed, and the hexoses were determined enzymically, by g.l.c. of alditol acetates, and by monitoring of single g.l.c. fractions for radioactivity.

P1-A was treated with diazomethane, reduced with $Ca(B^2H_4)_2$[28], and methylated. The product was hydrolysed sequentially with formic and suphuric acids[25] and analysed by g.l.c. of the alditol acetates (column III, Table I). P3-A (16 mg) was hydrogenated and partially hydrolysed with 0.1M trifluoroacetic acid, as for PS-A. After evaporation of the acid, the major acidic component (M_{GlcA} 1.05) was isolated by preparative paper electrophoresis and reduced with $Ca(B^2H_4)_2$ after esterification with diazomethane[28]. The product (2.2 mg) was acetylated, purified on Sephadex LH-20, and analysed by g.l.c.–m.s. Another portion of P3-A was hydrogenated, methylated, and reduced with $Ca(B^2H_4)_2$. After hydrolysis with formic and sulphuric acids, the sample was analysed by g.l.c.–m.s. of the derived alditol acetates.

For phage degradation of PS-C, high titres of $\phi 22$ were prepared according to the methods described by Dutton *et al.*[29], but omitting any concentration steps. PS-C (500 mg) was dissolved in a solution of 6.9×10^{12} PFU of $\phi 22$ in dialysed nutrient broth (Difco, 275 mL) and stirred gently for 72 h at 37° in the presence of

some $CHCl_3$. After filtration and lyophilisation, the digest was taken up in water and dialysed against distilled water. The diffusates were collected 12-hourly (× 5), concentrated (lyophilised), and passed down an ice-jacketed column of Amberlite IR-120(H^+) resin (3 ×) and then freeze-dried (yield, 871 mg). Chromatography on Bio-Gel P-4, in aqueous pyridinium acetate (pH 5.3) gave three fractions P1-C (49 mg), P2-C (154 mg), and P3-C (205 mg). P1-C and P2-C were reduced to alditols with NaB^2H_4 and methylated (potassium dimsyl), methanolysed, reduced, hydrolysed, reduced (NaB^2H_4), acetylated, and analysed by g.l.c.–m.s. (columns I and II, Table IV). P2-C (6 mg) was methylated with methyl triflate (100 μL) in trimethyl phosphate (1 mL), in the presence of 2,6-di-*tert*-butyl-4-methylpyridine (150 mg), for 2 h[21] at 50°. The methylated oligomer was extracted into $CHCl_3$, washed sequentially with aqueous 10% H_2SO_4, water, saturated aqueous $NaHCO_3$, and water, and dried (Na_2SO_4). After further purification on Sephadex LH-20, the material was methanolysed, reduced, hydrolysed, and analysed by g.l.c.–m.s. as alditol acetates (column III, Table IV).

Isolation and characterisation of 4-O-[(S)-*1-carboxyethyl*]-D-*glucuronic acid.* — PS-C (150 mg) was methanolysed, reduced, and hydrolysed, and the component having R_{Glc} 2.0 was isolated by paper chromatography (solvent *C*, 16 h). The material (17.2 mg) was further purified on Bio-Gel P-2 to afford a compound (10 mg) which had $[\alpha]_D$ +60° (*c* 1). A portion (2.4 mg) was treated with aqueous 48% hydrogen bromide (0.8 mL) for 6 min[17] at 100°, the acid was neutralised (Ag_2CO_3), and the salts were removed by centrifugation. The residue, after evaporation of the solvent, was converted into the acetylated aldononitriles[18] and analysed by g.l.c. on DB-225.

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