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STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K35

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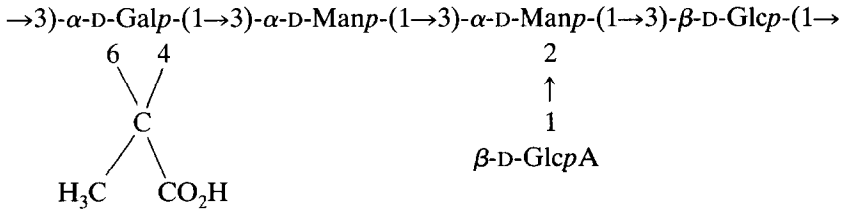
ABSTRACT

The structure of the capsular polysaccharide (K antigen) of *Klebsiella* K35 has been established as having the pentasaccharide repeating unit shown ("four plus one" type). The structural investigation utilized the techniques of methylation, β-elimination, Smith degradation, and partial hydrolysis. N.m.r. spectroscopy (^1H and ^{13}C) was used extensively to establish the configurations of the anomeric linkages and to delineate the sequence of the sugars in the structure of the polysaccharide.



INTRODUCTION

Klebsiella serotype K35 is one of 17 strains whose capsular polysaccharides are composed of D-glucuronic acid, D-galactose, D-glucose, and D-mannose[1,2]. Eight of these polysaccharides have 1-carboxyethylidene substituents, and in this subgroup, the structures of the polysaccharides from *Klebsiella* K7, K13, K26, K30, K31, K46, and K69 have been established[3].

As part of our continuing study to explain the serology and immunochemistry of the capsular polysaccharides of this genus on a structural basis, we now report our results on K35, the eighth member of the subgroup.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — *Klebsiella* K35 bacteria were grown on an agar medium and the acidic polysaccharide was purified by two precipitations with

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cetyltrimethylammonium bromide[4,5]. The product was monodisperse by gel-permeation chromatography ($M_r = 9 \times 10^6$) and had $[\alpha]_D$ +66°, which compares reasonably well with the value of +59° calculated by using Hudson's rules of isorotation[6]. The presence of galactose, glucose, mannose, and an aldobiouronic acid in the acid hydrolyzate of the polysaccharide was observed by paper chromatography. G.l.c. analysis of this same hydrolyzate gave the molar ratio of galactose:glucose:mannose as 1.00:1.08:1.03 (see Table I, column I). When the uronic acid was reduced, following methanolysis[7], the molar proportions of glucose and mannose increased (see Table I, column II). This indicated that the glucuronic acid is linked to a mannosyl unit, which was later confirmed by a β-elimination reaction[8] and the isolation of an aldobiouronic acid. The increase in glucose and mannose was less than expected, presumably due to incomplete reduction of the uronic acid. A composition of Gal:Man:Glc:GlcA of 1:2:1:1 was, however, corroborated both by n.m.r. spectroscopy and by methylation analysis. The ^{1}H-n.m.r. spectrum of the polysaccharide indicated (Fig. 1a) the presence of five anomeric protons, corresponding to three α- and two β-linkages[9]; also, one 1-carboxyethylidene acetal group[10] per repeating unit was detected. These results were further substantiated by the ^{13}C-n.m.r. spectrum[11] (see Fig. 2a and Table II). Circular dichroism measurements[12] on the alditol acetates showed glucuronic acid, glucose, and mannose to have the D-configuration. Galactose and the branch-point mannose were shown to possess the D-configuration by the circular dichroism curves of their 2,4,6-tri-*O*-methyl and 4,6-di-*O*-methyl derivatives[12] respectively.

Methylation analyses[13,14]. — Analyses were conducted (a) on the original polysaccharide, (b) after reduction of the uronic ester following methylation, (c) after remethylation of product *b*, and (d) on the depyruvylated polysaccharide with reduction of the uronic ester following methylation. The results, presented in Table III, columns I to IV, confirm the concept of a pentasaccharide repeating unit and show that (a) the branch point is a mannosyl residue linked at O-2 and O-3, (b) the glucuronic acid is a terminal, nonreducing group, and (c) the 1-carboxyethylidene acetal group is linked at O-4 and O-6 of the galactosyl residue. A β-elimination

TABLE I

SUGAR ANALYSIS OF *Klebsiella* K35 POLYSACCHARIDE AND DERIVED PRODUCTS

Sugar (as alditol acetate)	*Molar ratios*[a,b]				
	I	*II*	*III*	*IV*	*V*
Galactose	1.00	1.00	1.14	0.93	1.00
Glucose	1.08	1.21	1.00	1.00	—
Mannose	1.03	1.40	2.07	0.93	0.98

[a]Determined on an SP-2340 column programmed at 195° for 4 min, and then at 2°/min to 260°. [b]I, original acidic K35 polysaccharide; II, carboxyl-reduced polysaccharide, III, Smith-degraded polysaccharide, IV, carboxyl-reduced trisaccharide, V, neutral disaccharide

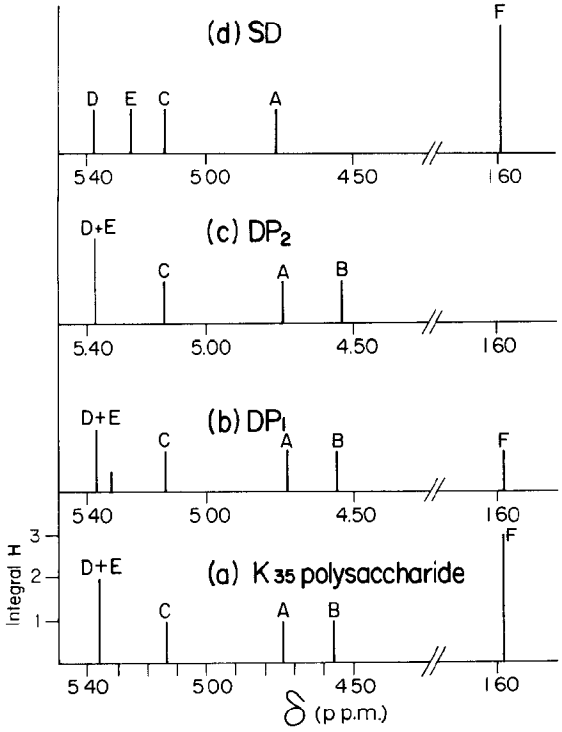


Fig. 1. ^{1}H-n.m.r. spectra (400 MHz) of (a) K35 polysaccharide, (b) partially depyruvylated (~66%) K35 polysaccharide, (c) completely depyruvylated K35 polysaccharide, and (d) Smith-degraded polysaccharide. The height of each vertical line is proportional to the proton integral.

experiment[8] demonstrated that the uronic acid is directly linked to the mannose at O-2 (see Table III, column V).

Smith degradation[15,16]. — Periodate oxidation of the native polysaccharide was followed by reduction and selective hydrolysis with dilute acid. The recovered material **SD** was polymeric, confirming that the uronic acid is laterally attached, and the n.m.r. spectra (see Table II) showed the disappearance of a β-signal at δ 4.56 ($J_{1,2}$ 7 Hz) (^{1}H) and at 103.45 p.p.m. (^{13}C). The full retention of the pyruvic acid methyl signal at δ 1.59 in the ^{1}H-n.m.r., indicates the stability to acid of the pyruvic acetal group linked to positions 4 and 6 of the galactose unit. This is in agreement with the difficulty encountered during depyruvylation (see Experimental). In contrast, the acyclic acetal product obtained by reduction following periodate oxidation of the polysaccharide was highly susceptible to hydrolysis with dilute acid. Sugar analysis of **SD** showed only neutral sugars (see Table I, column III), whereas the methylation analysis gave results identical to those obtained by β-elimination (see Table III, column VI).

Partial hydrolysis. — Partial hydrolysis of the native polysaccharide with acid was followed by separation of the acidic and neutral fractions *via* ion-exchange

TABLE II

N M R DATA (400 MHz) FOR *Klebsiella* K35 CAPSULAR POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES

Compound[a]	*^1H*				*^{13}C*	
	δ[b] *(p.p.m.)*	$J_{1,2}$[c] *(Hz)*	*Integral (no. of H)*	*Assignment*[d] *and symbol*[e]	*δ*[f] *(p.p.m.)*	*Assignment*[g] *and symbol*[h]
GlcA$\underset{\beta}{\overset{1\ 2}{}}$Man	5.30	s	0.73	$\overset{2}{}$Man$_{\alpha}$	102.51	GlcA$_{\beta}$
(A2)	4.95	s	0 27	$\overset{2}{}$Man$_{\beta}$	94.55	$\overset{2}{}$Man$_{\beta}$
	4.54	8	1.00	GlcA$_{\beta}$	92.90	$\overset{2}{}$Man$_{\alpha}$
GlcA$\underset{\beta}{\overset{1\ 2}{}}Man\underset{\alpha}{\overset{1\ 3}{}}$Glc	5.35	s	1.00	$\overset{2}{}$Man$_{\alpha}$	102.44	GlcA$_{\beta}$
(A3)	5.24	3	0.40	$\overset{3}{}$Glc$_{\alpha}$	99.75	$\overset{2}{}$Man$_{\alpha}$
	4 66	8	0.60	$\overset{3}{}$Glc$_{\beta}$	96.80	$\overset{3}{}$Glc$_{\beta}$
	4.54	8	1.00	GlcA$_{\beta}$	93.14	$\overset{3}{}$Glc$_{\alpha}$
Gal$\underset{\alpha}{\overset{1\ 3}{}}$Man	5.29	s	1.00	Gal$_{\alpha}$		
(N2)	5.17	s	0.66	$\overset{3}{}$Man$_{\alpha}$		
	4.91	s	0.34	$\overset{3}{}$Man$_{\beta}$		

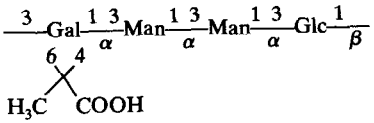
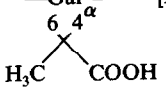
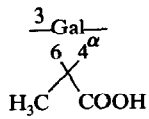
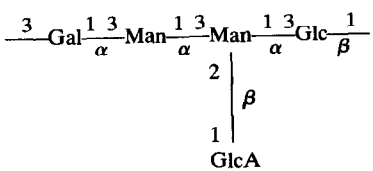
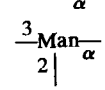
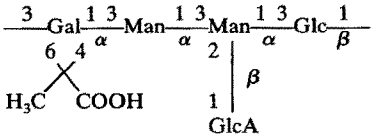
 **(SD)**	5.38	s	1.00		[D]	104.85		[A]
	5.25	s	1.00		[E]	102.89		[C]
	5.15	s	1.00		[C]	101.84		[D]
	4.76	8	1.00		[A]			
	1.59	s	3.00	CH_3 of pyr	[F]	101.54		[E]
						25.84	CH_3 of pyr	[F]
 **(DP2)**	5.37	b	2.00		[D]	104.40		[A[1]]
					[E]	103.32	GlcA$_{\beta}$	[B]
	5.15	s	1.00		[C]	102.89		[C]
	4.74	7	1.00		[A]	101.35		[D[1]]
	4.54	7	1.00	GlcA$_{\beta}$	[B]	99.38		[E]

TABLE II (continued)

Compound[a]	*^1H*					*^{13}C*			
	δ[b] *(p.p.m.)*	$J_{1,2}$[c] *(Hz)*	*Integral (no. of H)*	*Assignment*[d] *and symbol*[e]		*δ*[f] *(p.p.m.)*		*Assignment*[g] *and symbol*[h]	
						62.19 61.97 61.71 61.52		C-6 of hexoses	
3-Gal(1,3)-Man(1,3)-Man(1,3)-Glc(1) α α α β; Gal 6,4 → H_3C COOH (33%); Man 2 → GlcA 1 β	5.37	b	2.00	3-Gal α	[D,D[1]]	104.89	β	3-Glc(1,3)-Gal; Gal 6,4 → H_3C COOH	[A]
(DP1)				3-Man α; 2 →	[E]	104.54		3-Glc(1,3)-Gal β	[A[1]]
	5.15	b	1.00	3-Man α	[C]	103.50		GlcA β	[B]
						103.12		3-Man α	[C]
	4.73	7	1.00	3-Glc β	[A,A[1]]	101.78		3-Gal α; 6,4 → H_3C COOH	[D]
	4.56	7	1.00	GlcA β	[B]	101.38		3-Gal α	[D[1]]
	1.58	s	1.00	CH_3 of pyr	[F]	99.51		3-Man α; 2 →	[E]
						25.83		CH_3 of pyr	[F]



K35 polysaccharide

5.36	b	2.00	^{3}Gal$_{\alpha}$ (6,4 → H_3C COOH)	[D]	173.73	C=O of GlcA	
			^{3}Man$_{\alpha}$ (2 →)	[E]	172.85	C=O of pyr	
5.14	2	1.00	^{3}Man$_{\alpha}$	[C]	104.87	^{3}Glc$_{\beta}$	[A]
4.74	7	1.00	^{3}Glc$_{\beta}$	[A]	103.45	GlcA$_{\beta}$	[B]
4.57	7	1.00	GlcA$_{\beta}$	[B]	103.05	^{3}Man$_{\alpha}$	[C]
1.58	s	3.00	CH_3 of pyr	[F]	101.84	^{3}Gal$_{\alpha}$ (6,4 → H_3C COOH)	[D]
					99.43	^{3}Man$_{\alpha}$ (2 →)	[E]
					25.81	CH_3 of pyr (*R* config)	[F]

[a]For the origin of compounds **A2, A3, N2, SD, DP2,** and **DP1,** see text. [b]Chemical shift downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS); measured from internal acetone, $\delta = 2.23$ p.p.m. [c]Key: b = broad, unable to assign accurate coupling constant; s = singlet. [d]For example, ^{3}Man$_{\alpha}$ refers to the anomeric proton of a 3-linked mannosyl residue in the α-anomeric configuration. The absence of a numerical prefix indicates a (terminal) nonreducing group. [e]See Figs. 1 and 3. [f]Chemical shift in p.p.m. downfield from DSS. [g]As indicated in *d* but for anomeric ^{13}C nuclei. [h]See Figs. 2 and 3.

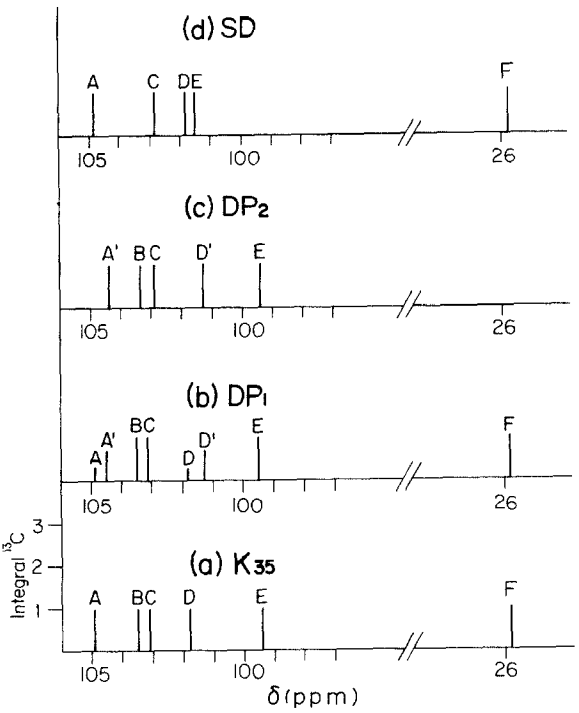


Fig. 2. ^{13}C-N.m.r. spectra (100.6 MHz) of (a) K35 polysaccharide, (b) partially depyruvylated (~66%) K35 polysaccharide, (c) completely depyruvylated K35 polysaccharide, and (d) Smith-degraded polysaccharide. Due to the n O.e effect, peak quantitation is inaccurate. Peak heights for anomeric signals are derived from the corresponding ^{1}H-spectrum.

TABLE III

METHYLATION ANALYSES OF *Klebsiella* K35 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar[a] *(as alditol acetate)*	*Mole %*[b,c]								
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*
2,3,4,6-Gal	—	—	—	—	—	—	—	—	52
2,4,6-Gal	—	—	—	20	—	—	—	—	—
2-Gal	24	18	19	—	23	22	—	—	
2,3,4,6-Glc	—	—	21	—	—	—	—	—	
2,4,6-Glc	26	21	21	20	25	27	—	33	—
2,3,4-Glc	—	21	—	20	—	—	44	34	—
4,6-Man	24	20	19	20	—	—	—	—	—
3,4,6-Man	—	—	—	—	—	—	56	33	—
2,4,6-Man	26	20	20	20	54	51	—	—	48

[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, etc. [b]Determined on an SP-1000 column at 220° isothermal. Values are corrected by use of the effective carbon-response factors given by Albersheim *et al.*[20]. [c]I, original acidic polysaccharide; II, uronic ester reduced after methylation; III, remethylation after reduction of uronic ester; IV, depyruvylated polysaccharide with uronic ester reduced after methylation; V, product from β-elimination and remethylation; VI, Smith-degraded polysaccharide; VII, methylated and reduced disaccharide **A2**; VIII, methylated and reduced trisaccharide **A3**, IX, neutral disaccharide **N2**.

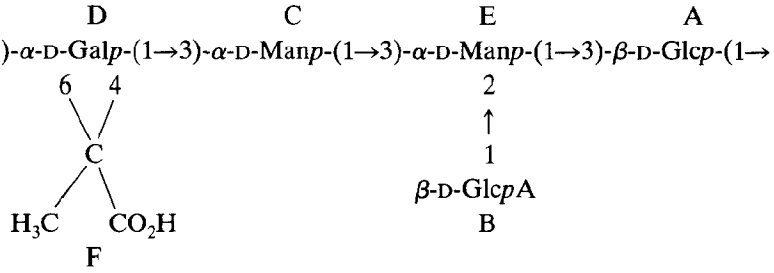
chromatography. The neutral fraction contained predominantly monosaccharides and a disaccharide **N2**, which was separated by paper chromatography. Separation of the acidic fraction, by paper chromatography, yielded three oligosaccharides **A2, A3,** and **A4** (which was indicated to be a mixture of oligosaccharides). On the basis of their n.m.r.-spectral data (see Table II) and their methylation analyses (see Table III, columns VII to IX), the structures of these compounds were shown to be as follows:

β-GlcA-(1→2)-Man **A2**

β-GlcA-(1→2)-α-Man-(1→3)-Glc **A3**

α-Gal-(1→3)-Man **N2**

The sum of these experiments establishes the structure of the *Klebsiella* K35 polysaccharide as



which is consistent with the n.m.r. spectra obtained. The individual sugar residues in the structure above have been designated by the letters A–E to facilitate discussion of these spectra in the following section.

N.m.r. study on original, depyruvylated, and Smith-degraded polysaccharides. — The numerical n.m.r. data for these polysaccharides are assembled in Table II, and the different spectra are compared schematically in Figs. 1 (^{1}H) and 2 (^{13}C), where the height of each vertical line is proportional to the number of protons or carbon atoms. From the chemical shift of the methyl group of the acetal (F, Figs. 1a and 2a), it was possible to assign the *R* configuration to the acetal carbon atom. It has been shown[10] that the chemical shifts for the methyl groups of the acetal differ according to whether these groups are axial or equatorial. The difference is especially pronounced in ^{13}C spectra, where the values are ~18 p.p.m. for the axial methyl groups and ~26 p.p.m. for the equatorial[10].

Partial depyruvylation of a poly- or oligo-saccharide may lead to the twinning of certain signals[17]. Mild hydrolysis of K35 polysaccharide gave a product **DP1** whose ^{13}C-n.m.r. spectrum showed pairs of signals A,A^1 and D,D^1 for residues A and D, respectively. The ratios of the heights of A:A^1 and D:D^1 were similar, and

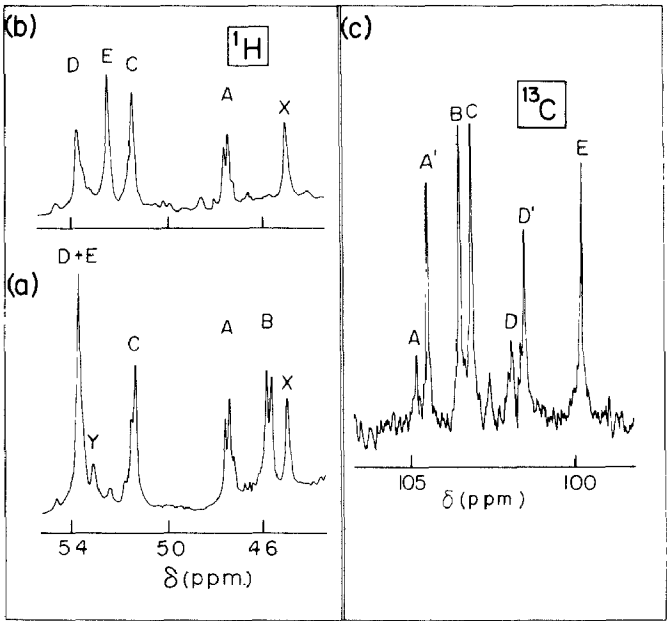


Fig. 3. (a) ^{1}H-N.m.r spectrum (400 MHz) of partially depyruvylated K35 polysaccharide, (b) Smith degradation product **SD**; (c) ^{13}C-spectrum (100.6 MHz) of partially depyruvylated K35 polysaccharide

as hydrolysis of the acetal progressed to completion (product **DP2**) only signals A^1 and D^1 were observed. This is illustrated by comparison of Figs. 2b and 2c, while the actual anomeric region of **DP1** is depicted in Fig. 3c. Twinning was less noticeable in the proton spectrum but the small unassigned peak Y in Fig. 3a, which was larger in the spectrum of **DP1**, may be due to this phenomenon, although this signal was absent from the spectrum of **DP2**. Signal X is unassigned, but it integrated for less than one proton. It may be noted that removal of the acetal group caused an upfield shift of the signals A and D. Twinning of the signal due to the α-D-galactopyranosyl residue, which is substituted by the pyruvic acid, is to be expected. The effect on the anomeric signal of the β-D-glucopyranosyl residue may be rationalized by inspection of a Framework molecular model, which shows that the carboxyl function of the acetal group on the galactose should have deshielding effect on the anomeric carbon of its posterior neighbor (3-Glc$_{\overline{\beta}}$) but minimal influence on its anterior neighbor (3-Man$_{\overline{\alpha}}$). The assignment of signal A to the β-D-glucopyranosyl unit, and of signal B to the β-D-glucopyranosyluronic acid, follows from the disappearance of the latter signal, in both the ^{1}H and ^{13}C-n.m.r. spectra, on Smith degradation of the K35 polysaccharide (compare Figs. 1a and 1d, 2a and 2d, and 3a and 3b). The spectra also demonstrate that the anomeric signals of the branch point D-mannose residue (E) shifted on removal of the uronic acid side chain, upfield in the ^{1}H case and downfield in ^{13}C case.

A referee has suggested that we should consider a repeating unit of the form

-D–E–A–C–, but this would not account for the formation of oligosaccharide **N2** (D–C). Furthermore, a molecular model shows that the carboxyl group of the acetal would now deshield α-D-mannopyranosyl residue C, yet these signals (^1H and ^{13}C) are clearly unperturbed by progressive hydrolysis of the acetal substituent.

CONCLUSION

The polysaccharide from *Klebsiella* K35 has a "4 + 1" type pattern, similar to the polysaccharides isolated from *Klebsiella* K9 and K59 (ref. 3), wherein the immunodominant group, a terminal, nonreducing D-glucuronic acid, is in the side-chain. However, in K35 there is an additional immunodominant feature, namely a 1-carboxyethylidene substituent[18] linked to O-4 and O-6 of a D-galactose residue.

EXPERIMENTAL

General methods. — The instrumentation used for infrared and n.m.r. spectroscopy, g.l.c., g.l.c.–m.s., and circular dichroism and optical rotation measurements has been described previously[19]. Analytical paper chromatography was performed by the descending method, using Whatman No. 1 paper and the following solvent systems: (1) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, (2) freshly prepared 2:1:1 1-butanol–acetic acid–water, (3) 8:2:1 ethyl acetate–pyridine–water, and (4) the upper phase of 4:1:5 1-butanol–ethanol–water. Chromatograms were developed with silver nitrate, or by spraying with *p*-anisidine hydrochloride in aqueous 1-butanol and heating the papers for 5–10 min at 110°. Preparative paper chromatography was performed by the descending method, using Whatman No. 1 paper and solvent 2. Analytical g.l.c. separations were achieved in stainless-steel columns (1.8 m × 3 mm) with a nitrogen carrier-gas flow-rate of 20 mL/min. The columns used were (A) 3% of SP-2340 on Supelcoport (100–120 mesh), programmed at 195° for 4 min, and then at 2°/min to 260°; and (B) 5% of SP-1000 on Gas Chrom Q (100–120 mesh), isothermal at 220°. Preparative g.l.c. was conducted in column C (1.8 m × 6.3 mm): 3% of SP-2340 on Supelcoport (100–120 mesh), programmed from 190–260° at 4°/min.

Preparation and properties of K35 capsular polysaccharide. — A culture of *Klebsiella* K35, obtained from Dr. Ida Ørskov, Copenhagen, was grown as previously described[4,5], and the polysaccharide was purified by two precipitations with Cetavlon. The purified polysaccharide (1.5 g from 7.5 L of medium) had $[\alpha]_D$ +66° (*c* 0.15, water). Analysis by gel chromatography (100 × 9 cm column of Sepharose 4B with 5 mL/h of M NaCl as eluant) showed it to be homogeneous with a molecular weight of 9×10^6 (column calibrated with dextrans). N.m.r. spectroscopy (^1H and ^{13}C) was performed on the original K35 polysaccharide; the principal signals and their assignments are recorded in Table II.

Hydrolysis of the polysaccharide. — A sample (11 mg) of K35 polysaccharide was hydrolyzed with 2M trifluoroacetic acid (TFA) for 15 h at 95°, then the acid was removed by successive evaporations with water. Paper chromatography (solvents 1

and 3) showed the presence of galactose, glucose, mannose, and an aldobiuronic acid (R_{Glc} 0.47). A quantitative analysis of the neutral sugars as their alditol acetates is presented in Table I, column I. A portion of the K35 polysaccharide (10 mg) was methanolyzed[7] overnight in 3% HCl–MeOH, and the products were reduced with sodium borohydride, hydrolyzed, and analyzed, with the results shown in Table I, column II. Alditol acetates for c.d. measurements[12] were obtained by g.l.c., using column C.

Methylation analysis. — A sample of K35 polysaccharide in the free acid form (30 mg) was methylated by the Hakomori procedure[13,14]. The product recovered after dialysis and extraction with methylene chloride showed complete methylation (no hydroxyl absorption in the i.r. spectrum). One part (11 mg) was hydrolyzed with 2M TFA, after which paper chromatography in solvent 4 revealed five components, namely 2,4,6-tri-*O*-methylglucose (R_{TMG} 0.80), 2,4,6-tri-*O*-methylmannose (0.75), 4,6-di-*O*-methylmannose (0.50), 2-*O*-methylgalactose (0.18), and a relatively faint pink spot (0.09) which is attributed to an acid compound. Analytical figures for the neutral sugars are presented in Table III, column I. A second portion (22 mg) of the methylated polysaccharide was reduced with lithium aluminum hydride in refluxing oxolane. The product (19 mg) was divided into halves, one of which was hydrolyzed and analyzed with the results shown in Table III, column II. The other half was remethylated[13,14], hydrolyzed, and converted to partially methylated alditol acetate derivatives. The g.l.c. result is given in Table III, column III. All methylation analyses were confirmed by g.l.c.–m.s.[19].

Depyruvylated K35 polysaccharide. — An aqueous solution of K35 polysaccharide (70 mg, pH 2.75) was autohydrolyzed for 4 h on a steam bath. Dialysis (mol. wt. cut-off 3500) against distilled water followed by lyophilization of the retentate yielded 55 mg of product. ^{1}H-N.m.r. analysis of this product showed that only ~30% of the acetal-linked pyruvic acid had been removed. After further hydrolysis (0.02M TFA, 1.5 h, 95°) of the polysaccharide and workup by a similar procedure, n.m.r. analyses (see **DP1** in Table II) indicated that ~33% of the pyruvate still remained intact. A third autohydrolysis (4.5 h, 95°) conducted on the partially depyruvylated polysaccharide accomplished complete depyruvylation (see **DP2** in Table II).

A sample (10 mg) of the depyruvylated K35 polysaccharide was successively methylated by the Hakomori procedure[13,14], reduced with lithium aluminum hydride in refluxing oxolane, hydrolyzed, and converted to partially methylated alditol acetates. The g.l.c. result is given in Table III, column IV. The disappearance of 2-*O*-methylgalactose (see columns I–III) and the appearance of 2,4,6-tri-*O*-methylgalactose shows that pyruvate is linked as an acetal to O-4 and O-6 of the galactose residue. The partially methylated alditol acetates for c.d. measurements[12] were obtained by g.l.c., using column C.

Uronic acid degradation[8]. — Methylated K35 polysaccharide (11 mg) dried *in vacuo* was dissolved in 19:1 Me_2SO-2,2-dimethoxypropane (8 mL) containing a trace of *p*-toluenesulfonic acid, and the solution was stirred under nitrogen. Sodium

methylsulfinylmethylide (2M, 3 mL) was added, and the mixture was stirred for 18 h at room temperature. The mixture was frozen, methyl iodide was added, the mixture was melted and stirred for 2 h at room temperature, then the excess methyl iodide was evaporated. The methylated, degraded product was isolated by partition between chloroform and water (3 × 15 mL), the product was dried and hydrolyzed with 2M TFA, and the sugars released were analyzed as described previously for the methylation analysis (see Table III, column V).

Smith degradation[15,16]. — A solution of K35 polysaccharide (53 mg) in water (40 mL) was mixed with $NaIO_4$ (0.5 g) and $NaClO_4$ (0.2 g), and kept stirring in the dark at room temperature. After 60 h, ethylene glycol (0.5 mL) was added, and then the mixture was reduced with $NaBH_4$. The excess of $NaBH_4$ was decomposed with formic acid (50%) and the product was dialyzed (mol. wt. cut-off 3500) against 0.25M TFA for 48 h. Further dialysis against distilled water, and passage through a column of Amberlite IR 120 (H^+) resin, followed by concentration and lyophilization gave the polyol **SD** (30 mg). N.m.r. analyses (see Table II) of **SD** showed that the terminal glucuronic acid had been degraded, and that the pyruvic acid acetal structure was unaffected by the mild hydrolytic conditions used. Results for the sugar and methylation analyses are given in Table I, column III and Table III, column VI, respectively.

Partial hydrolysis. — The K35 polysaccharide (450 mg) was hydrolyzed with 0.5M TFA for 2 h at 95°. After removal of the TFA by successive evaporations with water, the hydrolyzate was dialyzed (mol. wt. cut-off 3500) against distilled water. The lyophilized retentate (170 mg) was subjected to further hydrolysis (0.5M TFA, 1 h, 95°) and the same workup procedure followed. A solution of the combined, lyophilized dialyzate (380 mg) was separated on a column of Bio-Rad AG1-X2 (formate) ion-exchange resin, to give a neutral (150 mg) and an acidic fraction (200 mg). The neutral fraction was eluted with water and the acidic fraction with formic acid (10%). Preparative paper chromatographic separation of the acidic fraction, using solvent 2, gave an aldobiuronic acid **A2** (18 mg), an aldotriuronic acid **A3** (26 mg), a mixture **A4** (12 mg) of aldotetrauronic acids (as analyzed by p.m.r. and methylation analysis), and higher molecular weight oligosaccharides (65 mg) which remained at the origin. Paper chromatography of the neutral fraction showed that it comprised predominantly monosaccharides (galactose, glucose, and mannose), plus a neutral disaccharide **N2** (11 mg) which was separated by preparative paper chromatography using solvent 2.

Each oligosaccharide was subjected to sugar analysis and methylation analysis. For sugar analysis the acidic oligosaccharides were treated with 3% HCl in anhydrous methanol for 8 h on a steam bath, then the methyl ester groups in the products were reduced with sodium borohydride in anhydrous methanol. This was followed by hydrolysis with 2M TFA, reduction to the alditols, and acetylation with 1:1 acetic anhydride–pyridine. The results of the g.l.c. analyses (column A) are given in Table I. The neutral disaccharide **N2** was initially hydrolyzed and analyzed similarly. Methylations were conducted by the method of Hakomori[13,14] (the acidic

oligosaccharides being reduced with lithium aluminium hydride in anhydrous oxolane after methylation), after which hydrolysis with 2M TFA, sodium borohydride reduction, and acetylation gave the partially methylated alditol acetates, which were analyzed by g.l.c. and g.l.c.–m.s. (see Table III). The n.m.r. data for each oligosaccharide are given in Table II.

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