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STRUCTURAL STUDIES ON THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* SEROTYPE K64

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ABSTRACT

Partial hydrolysis with acid, methylation analysis (including uronic acid degradation), Smith degradation, and p.m.r. spectroscopy have been used to determine the primary structure of the capsular polysaccharide of *Klebsiella* serotype K64. The hexasaccharide repeating-unit, which also contains one *O*-acetyl substituent, comprises a 4)-α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→4)-α-D-Man*p*-(1→ chain with a 4,6-*O*-(1-carboxyethylidene)-β-D-glucopyranosyl and an L-rhamnosyl group attached to the 4-linked D-mannosyl residue at O-2 and O-3, respectively.

INTRODUCTION

The bacterial genus *Klebsiella* produces numerous, type-specific, capsular polysaccharides (K-antigens), of which ~80 different types have been identified on the basis of their serological reactions. A qualitative study of these polysaccharides by Nimmich[1,2] led to their being grouped in chemotypes according to their component monosaccharides. Certain of these chemotypes are unique, whereas others may include up to seventeen different strains.

Klebsiella K64 is one of two strains producing capsular polysaccharides that are known to contain residues of D-glucuronic acid, D-glucose, D-mannose, and L-rhamnose. The structure of an acidic polysaccharide from a strain of *Aerobacter aerogenes* N.C.T.C. 8172 (*Klebsiella* serotype K64) was investigated by Stacey and co-workers[3] in 1958; periodate oxidation studies showed that large numbers of glucosyl, mannosyl, and glucosyluronic acid residues were either linked (1→3) or were involved in branching, and that the rhamnose was present either as end groups or linked through O-2 or O-4. An aldobiouronic acid obtained by partial hydrolysis was identified as a glucosyluronic-mannose. A more-recent publication[4] dealt with the molecular-weight distributions of the products of graded, acid hydrolysis of the K64 polysaccharide. A detailed, structural analysis of this polysaccharide is now presented.

TABLE I

PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE OLIGOSACCHARIDES FROM *Klebsiella* K64 CAPSULAR POLYSACCHARIDE

Fraction	*Oligomer*	$[\alpha]_D$ *(degrees)*	R_{Glc} *(Solvent a)*	*Mol. wt.*	*Equivalent weight*	*Neutral sugars*[b] *(molar ratios)*	*Methylated sugars*[c] *(alditol acetates)*[d]
1	Neutral disaccharide mixture	−6	0.74	340	—	Glc (1.0) Man (1.0)	2,3,4,6-Glc 2,3,6-Man, 3,4,6-Man
2	Aldobiouronic acid	+40	0.74	360	n.d.[a]	Man	2,4,6-Man
3	Aldotriouronic acid	+68	0.42	520	n.d.[a]	Glc (1.0) Man (0.7)	2,4,6-Glc 2,4,6-Man
4	Acidic oligosaccharide	+64	0.15	930	440	Glc (2.0) Man (2.2)	2,3,4,6-Glc (tr), 2,4,6-Glc 2,4,6-Man, 2,3,6-Man, 3,6-Man (tr)
5	Acidic oligosaccharide	+59	0.04	1100	530	Glc (2.0) Man (2.7)	2,3,4,6-Glc, 2,4,6-Glc 2,4,6-Man, 2,3,6-Man (tr), 3,6-Man

[a]n.d. = not determined. [b]By g.l.c. of their derived alditol acetates on column *b*. [c]Positions of *O*-methyl groups given by locants. [d]G.l.c. on column *a*.

RESULTS AND DISCUSSION

The capsular polysaccharide used in the present study was obtained from a culture (N.C.T.C. 9184), and had the same characteristics as those previously reported[4]. Homogeneity tests, by electrophoresis on cellulose acetate strips and by ultracentrifugation, indicated that the polysaccharide was monodisperse. The proton magnetic resonance spectrum of the polysaccharide showed the presence of rhamnosyl, pyruvic acetal, and *O*-acetyl groups, and six anomeric protons. The molar proportions of the neutral sugars in a hydrolyzate were found by g.l.c. analysis to be rhamnose (1.0), mannose (1.4), and glucose (2.0), in agreement with those reported previously[4]. On the basis of the circular dichroism spectra of their alditol acetates, the D configuration was assigned to the mannose and glucose, and the L configuration to the rhamnose.

Partial hydrolysis with acid; isolation and identification of oligosaccharides. — Partial hydrolysis with acid was used to generate a number of structurally significant oligosaccharides on a preparative scale. Fractions separated by gel-permeation chromatography were purified by preparative paper-chromatography, to yield five components, the properties of which are shown in Table I. The fraction having a molecular weight of 350 contained the aldobiouronic acid 3-*O*-(α-D-glucopyranosyluronic acid)-D-mannose (**2**), and a mixture (**1**) of 4-*O*-β-D-glucopyranosyl-D-mannose (**1a**) and 2-*O*-β-D-glucopyranosyl-D-mannose (**1b**) in the ratio of 3 : 1. Mixture **1** was separated into its components by g.l.c. of the per(trimethylsilyl) derivatives, and the positions of linkage were determined by mass spectrometry according to the method of Haverkamp *et al.*[5], the results being confirmed by methylation analysis of the mixture. The aldotriouronic acid **3**, identified as *O*-(α-D-glucopyranosyluronic acid)-(1→3)-*O*-α-D-mannopyranosyl-(1→3)-D-glucose, yielded the aldobiouronic acid **2** plus D-glucose on mild hydrolysis with acid, and the higher oligosaccharides, **4** and **5**, were found to contain the same three types of sugar residues.

β-D-Glc*p*-(1→4)-D-Man*p* + β-D-Glc*p*-(1→2)-D-Man*p*
1a **1b**

α-D-Glc*p*A-(1→3)-D-Man*p*
2

α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-D-Glc*p*
3

The assignment of structures **4** and **5** to these higher oligosaccharides was based primarily on the evidence obtained from determinations of equivalent weight and from methylation analyses. That a D-glucosyluronic acid group is located at the non-reducing end of each oligosaccharide was corroborated by methanolysis and by g.l.c. of their respective, methylated derivatives, which gave methyl 2,3,4-tri-*O*-methyl-D-glucuronate, in addition to methyl glycosides of the methylated sugars in Table I.

α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→4)-α-D-Man*p*-(1→4)-α-D-Glc*p*A-(1→3)-D-Man*p*

4

α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→4)-α-D-Man*p*-(1→4)-α-D-Glc*p*A-(1→3)-D-Man*p*

2
↑
1
β-D-Glc*p*

5

A trace of 2,3,6-tri-*O*-methyl-D-mannose found on methylation analysis of **5** is attributed to the presence of a small proportion of **4** in this fraction, due to incomplete separation of these two oligosaccharides by the gel-permeation and paper-chromatographic methods used in their isolation. Identification of 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,6-di-*O*-methyl-D-mannose in the hydrolyzate of the methylated derivative of **5** indicates the branched nature of this oligosaccharide; the presence of traces of these two methylated sugars in the hydrolyzate of methylated **4** is probably the result of some contamination of this oligosaccharide with **5**. Loss of the substituent D-glucosyl group from **5** gives oligosaccharide **4**. The structures of the lower oligosaccharides (**1**–**3**) isolated from the polysaccharide hydrolyzate support the assignment to components **4** and **5** of the structures depicted.

As no L-rhamnose was detected in the hydrolyzates of oligosaccharides **4** and **5**, it may be assumed that the sugar constitutes substituent groups in the polysaccharide structure. Comparison of the methylation analysis of oligosaccharide **5** with that of the original polysaccharide (see Table II) indicates attachment of the L-rhamnosyl groups at O-3 of D-mannosyl residues.

Autohydrolysis of the capsular polysaccharide A. — Autohydrolysis of the K64 capsular polysaccharide (*A*) yielded pyruvic acid, a trace of rhamnose, and a residual

TABLE II

METHYLATION ANALYSES OF *Klebsiella* K64 CAPSULAR POLYSACCHARIDE (*A*) AND AUTOHYDROLYZED POLYSACCHARIDE (*B*)

Sugar	T[a]	*Molar proportions (%)*		
		A[b]	A[c]	B[c]
2,3,4-Rha	0.59	21.4	19.5	17.8
2,3,4-6-Glc	1.01	—	—	18.7
2,4,6-Man	1.19	11.5	18.2	16.6
2,4,6-Glc	1.34	20.8	20.4	20.8
3,6-Man	1.63	—	—	6.8
2,3-Glc	2.12	24.0	21.0	—
6-Man	2.33	20.8	20.8	19.3

[a]Retention time of corresponding, per(trimethylsilyl)ated alditol, relative to that of 2,3,4,6-tetra-*O*-methyl-D-galactose on a column of SE-52 at 140°. [b]Hydrolyzate examined after hydrolysis for 6 h. [c]Hydrolyzate examined after hydrolysis for 18 h.

polysaccharide (*B*). Proton magnetic resonance spectroscopy of polysaccharide *B* showed the absence of pyruvic acetal, and gel-permeation chromatography indicated a high degree of polymerization. A sample of polysaccharide *B* was methylated, and the product was used for the methylation analysis described later.

Methylation analysis of polysaccharide A. — Samples of the fully methylated K64 polysaccharide were hydrolyzed for periods of 6 and 18 h, respectively, and a portion of each hydrolyzate was analyzed by paper chromatography and by g.l.c. of the per(trimethylsilyl)ated, derived alditols, giving the results shown in Table II. It is evident that the number of branch points in the structure is higher than can be accounted for in terms of the substituent L-rhamnosyl groups found. The low proportion of 2,4,6-tri-*O*-methyl-D-mannose in the hydrolyzate after 6 h may be ascribed to incomplete hydrolysis of the aldobiouronic acid linkages involving residues of mannose. The residues of 2,3-di-*O*-methyl-D-glucose in methylated *A* cannot represent branch points in the polysaccharide chain, as no terminal units in equivalent number are found. However, from the structure of oligosaccharide **5**, it is evident that substituent D-glucosyl groups are present in the K64 polysaccharide. The pyruvic acid must, therefore, be acetal-linked to O-4 and O-6 of these D-glucosyl groups; this would account for the proportion of 2,3-di-*O*-methyl-D-glucose found in the hydrolyzate of methylated *A*. Identification of 6-*O*-methyl-D-mannose in this hydrolyzate indicates that mannose constitutes a double-branch point in the polysaccharide chain. As the L-rhamnosyl groups had already been shown to form one branch point at O-3, the 4,6-*O*-(1-carboxyethylidene)-D-glucosyl group must be attached to either O-2 or O-4 of this same D-mannose residue. However, the presence of 4-linked, chain residues of D-mannose in oligosaccharides **4** and **5** shows that the second branch point is at O-2.

The identities of the methylated sugars produced on hydrolysis of methylated polysaccharide *A* were confirmed by isolation, and characterization, of the individual sugars. A large-scale hydrolyzate of the methylated polysaccharide was fractionated into neutral and acidic components by means of ion-exchange resins. The mixture of methylated, neutral sugars was separated into its components by column chromatography on cellulose, and, in some cases, these were further purified by preparative paper-chromatography. 2,3,4-Tri-*O*-methyl-L-rhamnose, a mixture of 2,4,6-tri-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-mannose [separated by means of preparative g.l.c. of their per(trimethylsilyl)ated, derived alditols], 2,3-di-*O*-methyl-D-glucose, and 6-*O*-methyl-D-mannose were isolated in this way, and were characterized by the methods outlined in the Experimental section.

The methylated, acidic sugars were converted into the methyl ester methyl glycosides, which were ester-reduced with lithium aluminum hydride[6], and the products hydrolyzed. Analysis of the hydrolyzate by paper chromatography and by g.l.c. (as their derived, alditol acetates) showed only 2,4,6-tri-*O*-methylmannose and 2,3-di-*O*-methylglucose to be present in the mixture. Recovery of the glucose derivative followed by *O*-demethylation gave glucitol, which was shown to be of the D configuration by measurement of the c.d. spectrum of its hexaacetate. As no residues of 2,3-di-*O*-methyl-D-glucose were found in the methylated, acidic sugars before reduc-

tion, their appearance here must be due to reduction of the glucosyluronic acid residues present in the original polysaccharide; these must, therefore, also be of the D configuration. These results confirmed that the aldobiouronic acid obtained on hydrolysis of polysaccharide *A* is 3-*O*-(D-glucopyranosyluronic acid)-D-mannose (**2**), and that the D-glucuronic acid is not in a terminal position, or involved in branching.

Lindberg degradation of methylated polysaccharide A. — The fully methylated *Klebsiella* K64 polysaccharide *A* was degraded with alkali according to the method of Lindberg *et al.*[7], and the product was directly re-alkylated. The modified polysaccharide was hydrolyzed, and the resulting methylated sugars were characterized, by paper chromatography and by g.l.c. of the derived alditol acetates, as 2,3,4-tri-*O*-methyl-L-rhamnose, 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-glucose, and 2,3-di-*O*-methyl-D-glucose. These results showed the complete loss of the D-glucosyluronic acid residues, and confirmed that these are linked to O-3 of D-mannose. The absence of 6-*O*-methyl-D-mannose from the hydrolyzate of the base-degraded polysaccharide indicates that the D-glucosyluronic acid residues are linked through O-4 in the polysaccharide chain.

Methylation analysis of degraded polysaccharide B. — Methylation analysis of the degraded polysaccharide *B*, obtained by autohydrolysis of *A*, showed (see Table II) complete disappearance of 2,3-di-*O*-methyl-D-glucose, which was replaced by 2,3,4,6-tetra-*O*-methyl-D-glucose in significant molar proportion; this confirmed that the pyruvic acid group must be linked to O-4 and O-6 of a substituent D-glucosyl group in the original polysaccharide *A*. The small proportion of 3,6-di-*O*-methyl-D-mannose found in the hydrolyzate of methylated *B* arises from the loss of substituent L-rhamnosyl groups on autohydrolysis of *A*, and again shows that these are linked to O-3 of D-mannose. As only O-6 of this D-mannosyl residue is unsubstituted, the D-glucosyl residue and group in the polysaccharide must be linked to O-4 and O-2 of this D-mannosyl residue to produce, on hydrolysis, the neutral disaccharides mentioned.

Reduction of the methylated derivatives of polysaccharides A *and* B. — Reduction of fully methylated polysaccharide *A* with lithium aluminum hydride gave a product (*C*) containing no methyl ester groups (no i.r. absorption at 1740 cm^{-1}). Paper chromatography of a hydrolyzate failed to reveal any new neutral sugars resulting from the reduction of the D-glucosyluronic acid residues in the chain. However, g.l.c. analysis indicated a substantial rise in the proportion of 2,3-di-*O*-methyl-D-glucose in the hydrolyzate (see Table III); this increase must be due to the reduction of 4-linked D-glucosyluronic acid residues in the polysaccharide chain.

In order to confirm this conclusion, the fully methylated derivative of the autohydrolyzed polysaccharide *B* was similarly reduced, and the product (*D*) examined as already described. Analysis of a hydrolyzate of product *D*, shown in Table III, again indicated the presence of a substantial proportion of 2,3-di-*O*-methyl-D-glucose. As methylated polysaccharide *B* contained no such residues, their appearance here could only have resulted from the reduction of 4-linked D-glucosyluronic acid residues in the polysaccharide chain.

TABLE III

METHYLATION ANALYSES OF THE REDUCED, METHYLATED DERIVATIVES (*C* AND *D*) OF POLYSACCHARIDES *A* AND *B*

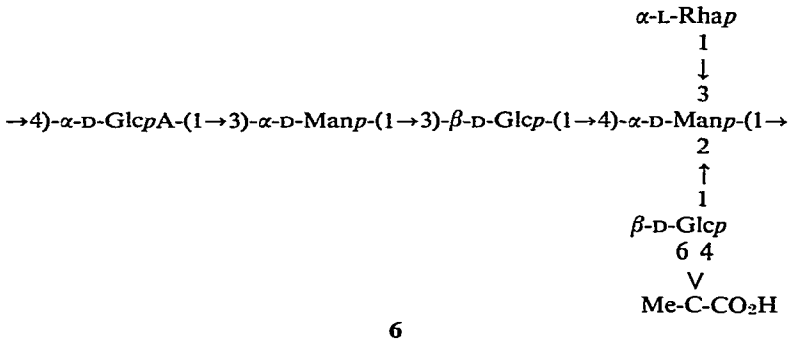
Sugar	T[a]	*Molar proportions (%)*	
		C[b]	D[b]
2,3,4-Rha	0.59	17.0	13.6
2,3,4,6-Glc	1.01	—	18.5
2,4,6-Man	1.19	17.8	16.9
2,4,6-Glc	1.34	16.8	17.3
3,6-Man	1.63	—	6.8
2,3-Glc	2.12	33.8	11.5
6-Man	2.33	14.6	15.4

[a]As for Table II. [b]Hydrolyzates examined after hydrolysis for 6 h. *C* is reduced, methylated *A*; *D* is reduced, methylated *B*.

Smith-degradation studies of polysaccharide A. — Polysaccharide *A* was oxidized with periodate, 0.49 mol per hexosyl residue being consumed, and the product was reduced and then hydrolyzed. The acidic oligosaccharide obtained had a molecular weight of 560 according to gel-permeation chromatography. From the p.m.r. spectrum of this oligosaccharide in D_2O, it could be seen that one β-D-glucosyl and two α-D-mannosyl linkages were present. G.l.c. analysis of a hydrolyzate of a sample showed that D-mannosyl and D-glucosyl units were present in the ratio of 1.9:1. The i.r. spectrum of the fully methylated oligosaccharide indicated a methyl ester grouping, which disappeared on reduction of the methylated product with lithium aluminum hydride. The reduced, methylated oligosaccharide was hydrolyzed, and the hydrolyzate was analyzed by paper chromatography and by g.l.c.; 2,3,4,6-tetra-*O*-methyl-D-mannose (1.0), 2,4,6-tri-*O*-methyl-D-glucose (0.8), and 2,3,6-tri-*O*-methyl-D-mannose (1.0) were found to be present in the molar ratios shown.

The identification of a 4-linked residue of D-mannose in the product obtained by Smith degradation of the K64 polysaccharide further proves that 4-*O*-β-D-glucopyranosyl-D-mannose (**1a**) forms part of the chain within the repeating unit, and that 2-*O*-β-D-glucopyranosyl-D-mannose (**1b**) is obtained as a result of the attachment of a D-glucosyl group to O-2 of the same D-mannosyl residue. The higher proportion of the (1→4)-linked isomer obtained on mild hydrolysis of the polysaccharide with acid is also consistent with this assignment.

Anomeric configurations of constituent sugars. — The modes of linkage of the D-glucosyluronic acid, D-glucosyl, and D-mannosyl residues in the polysaccharide were determined by p.m.r. spectroscopy and by measurement of the optical rotations of the oligosaccharides mentioned previously. The L-rhamnosyl group in the repeating unit does not appear in any of these structures. However, the values calculated for the specific rotation of polysaccharide *A* on the basis of Hudson's rules of isorotation[8] are +33° if this L-rhamnosyl group is assumed to be α-L-linked, and +57° if it is β-L-linked; the actual value, +28°, is much closer to the former, which indicates that these groups are probably α-L-linked. The increase in optical rotation accompanying



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the release of L-rhamnose during the initial stages of mild hydrolysis[4] with acid is also indicative of this mode of linkage.

Postulated structure of the repeating unit in Klebsiella *K64 capsular polysaccharide.* — The results of Smith degradation, methylation analyses, and partial hydrolysis with acid, together with the identification of a number of structurally significant oligosaccharides, are all consistent with the presence of a doubly-branched hexasaccharide repeating-unit (**6**) in the capsular polysaccharide from *Klebsiella* serotype K64. It has not yet been possible to locate the position of the *O*-acetyl group in this structure. The results of the present study on *Klebsiella* K64 are in agreement with the earlier work of Stacey *et al.*[3], except for the fact that D-glucuronic acid is neither 3-linked, nor involved in branching.

Comparison with other Klebsiella *capsular polysaccharides.* — A number of the structural features found in the capsular polysaccharide from K64 have been encountered in other *Klebsiella* polysaccharides. Recent studies[9] have shown that the K33 polysaccharide has a doubly-branched pentasaccharide repeating-unit in which a residue of D-mannose is linked through O-4 in the polysaccharide chain and carries substituent groups at O-3 and O-6. Branched hexasaccharide units have also been found in the K18 (ref. 10), K27 (ref. 11), K28 (ref. 12), K36 (ref. 13), and K52 (ref. 14) polysaccharides; of these, only K36 has 4,6-*O*-(1-carboxyethylidene)-D-glucose in a side chain. Pyruvic acid has also been found attached to 3-linked residues of D-glucose in the polysaccharide chains of K7 (ref. 15), K27 (ref. 11), and K56 (ref. 16), and the aldobiouronic acid 3-*O*-(α-D-glucopyranosyluronic acid)-D-mannose (**2**) forms part of the polysaccharides of K2 (ref. 17), K4 (ref. 18), K13 (ref. 19), K21 (ref. 20), K24 (ref. 21), and K33 (ref. 9); this is the aldobiouronic acid unit most often found in the *Klebsiella* polysaccharides examined thus far. The neutral fragment 3-*O*-α-D-mannopyranosyl-D-glucose occurs in the polysaccharides of K7 (ref. 15), K24 (ref. 21), K28 (ref. 12), and K59 (ref. 22), but not in combination with D-glucuronic acid to form the aldotriouronic acid unit found in K64. However, results of studies[18] on the polysaccharide from *Klebsiella* K4 indicated that the same aldotriouronic acid structure occurs here in a linear repeating-unit. *Klebsiella* K2 contains[17] both the aldobiouronic acid and the neutral disaccharide 4-*O*-β-D-glucopyranosyl-D-mannose (**1a**) found in K64, in a tetrasaccharide repeating-unit in which the D-glucosyluronic acid residue occupies a terminal position. Residues of

D-glucuronic acid linked through O-4 have been reported to occur in a number of polysaccharides, but not preceded in the chain by an α-D-linked residue of D-mannose as found in that from K64.

EXPERIMENTAL

General methods. — Paper chromatography was performed with Whatman No. 1 paper and the following solvent systems (all v/v): (*a*) 1-butanol–acetic acid–water, 2:1:1; (*b*) 1-butanol–ethanol–water (4:1:5, upper phase); (*c*) 1-butanol–ethanol–water, 2:1:1; (*d*) 8:2:1 ethyl acetate–pyridine–water; (*e*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; and (*f*) butanone–water azeotrope. Spots were made visible by heating the papers for 5–10 min at 110° after spraying with one of the following reagents: (*i*) *p*-anisidine hydrochloride in aqueous 1-butanol; (*ii*) aqueous ammoniacal silver nitrate; and (*iii*) *o*-phenylenediamine in aqueous trichloroacetic acid (for pyruvic acid[23]). Chromatograms of methylated sugars were washed with hot water after being heated for 10 min. Preparative paper-chromatography was conducted with Whatman No. 3MM paper; the relevant strips were excised, and eluted with water for 6 h, and the aqueous extracts were filtered, concentrated *in vacuo*, and finally freeze-dried. R_{Glc} values reported for oligosaccharides (see Table I) are their mobilities relative to that of D-glucose on Whatman No. 1 paper in the solvent system specified.

The g.l.c. analysis of per(trimethylsilyl)ated sugars[24] and derived alditols[25] was conducted with a Beckman GC-4 gas chromatograph with a flame-ionization detector, and glass columns (1.80 m × 6.25 mm o.d.) packed with 3% (w/w) of SE-52 on Chromosorb W (80–100 mesh; acid washed), at a flow rate of helium carrier-gas of 60 mL.min^{-1}, isothermally at 140°. Retention times (T values) were measured relative to that of di-*O*-(trimethylsilyl)ated 2,3,4,6-tetra-*O*-methylgalactitol (actual retention-time ~5 min). Methyl glycosides[26] were analyzed with the same instrument, using glass columns (1.80 m × 6.25 mm o.d.) packed with 14% (w/w) of ethylene glycol succinate polyester on Chromosorb W (80–100 mesh; acid washed), at a flow rate of helium carrier-gas of 50 mL.min^{-1}, isothermally at 150°. Analyses of partially methylated alditol acetate mixtures[27] were performed with a Pye 104 gas chromatograph, using glass columns (2 m × 4 mm o.d.) of (*a*) 3% of ECNSS-M on Gas-Chrom Q (100–120 mesh), at a flow rate of nitrogen carrier-gas of 40 mL.min^{-1}, isothermally at 175°; and (*b*) 3% of OV-225 on Chromosorb W (80–100 mesh; acid washed), isothermally at 170°. Alditol acetate mixtures were analyzed quantitatively[28] by using column (*b*), isothermally at 190°. Preparative g.l.c. was conducted with the Pye instrument, using a glass column (2 m × 6 mm o.d.) fitted with a 10:1 splitter, and packed with 3% of ECNSS-M on Gas-Chrom Q (100–120 mesh), at a flow rate of nitrogen carrier-gas of 60 mL.min^{-1}, isothermally at 190°. Each fraction collected was examined for purity by re-injection of a sample into column (*b*).

Analytical-scale, gel chromatography was conducted by the method reported earlier[4]. Preparative, gel-permeation chromatography was performed on a column

(112 × 4 cm) of Bio-Gel P-2, using water as the eluent. The hydrolyzates (~1 g in 10 mL of water) were applied to the column, and fractions (25–50 mL) of the effluent were collected, and screened for carbohydrate by the phenol–sulfuric acid method[29].

Electrophoresis was performed for 45 min at 220 V, using cellulose acetate strips (17.4 × 2.4 cm) in pyridine–formate buffer (0.1M) at pH 3. Aqueous solutions (2 mg/mL) of the polysaccharides were applied to the strips (presoaked in buffer, and partially dried), by means of a Beckman sample-applicator. The polysaccharide bands were detected by immersion of the strips in a 10% aqueous solution of Alcian Blue.

Ultracentrifugation was performed at 60 000 r.p.m. with a Beckman Model L2-65B ultracentrifuge fitted with a Schlieren analyzer. Concentrations of polysaccharides in water varied between 2 mg/mL and 10 mg/mL, with 10 mg of sodium chloride added to each solution.

Circular dichroism spectra were recorded with a Jasco JA 40A spectropolarimeter, using a quartz cell (capacity 0.3 mL, path-length 0.1 cm). Compounds were dissolved in high-purity acetonitrile, and their spectra were recorded[30] in the range of 250–205 nm. Optical rotations were measured for aqueous solutions (unless otherwise stated) at 20 ±3° with a Perkin–Elmer Model 141 polarimeter. The infrared spectra of methylated derivatives (in chloroform) were recorded with a Perkin–Elmer Model 237 spectrophotometer. Proton magnetic resonance spectra were recorded with a Varian XL 100 or a Bruker WH-90 instrument. Samples were prepared by dissolving in D_2O after freeze-drying (2–3 times) of D_2O solutions. Spectra were recorded at temperatures ranging from 28 to 90°, with tetramethylsilane as the external standard. All τ values are given relative to that for internal sodium 4,4-dimethyl-4-silapentanesulfonate taken as τ 10. Mass spectra were recorded with a Varian 311A mass spectrometer at 70 eV. The position of methoxyl substitution was determined by using the data of Lindberg and co-workers[31], and, where possible, by comparison of mass spectra with those of authentic compounds.

All solutions were evaporated *in vacuo* at 40°. Hydrolyses were performed in trifluoroacetic acid under nitrogen in sealed tubes at 100°; the acid was subsequently removed by co-distillation with methanol. Alditols produced by reduction of the compounds in hydrolyzates with sodium borohydride were acetylated with 1:1 (v/v) acetic anhydride–pyridine under nitrogen in sealed tubes for 2 h at 100°.

Methylation analyses. — Methylation of polysaccharides and oligosaccharides was conducted by the methods of Hakomori[32] and Purdie and Irvine[33]. Before analysis, the methylation products were passed through a column of Sephadex LH-20, with 2:1 (v/v) ethanol–chloroform as the eluent (methylated polysaccharides), or Merckogel OR-PVA 500, with methanol as the eluent (methylated oligosaccharides). Samples (10 mg) of the fully methylated derivatives were hydrolyzed in 2M trifluoroacetic acid (2 mL) for 18 h at 100°, and the sugars in the hydrolyzates were reduced with sodium borohydride. After acidification, and removal of borate (by distillation with methanol), the residue was dissolved in anhydrous pyridine (1 mL) and treated with hexamethyldisilazane (0.2 mL) and chlorotrimethylsilane (0.1 mL) for 5 min. The solution was evaporated to dryness, and the mixture of per(trimethylsilyl)ated

alditols, extracted from the residue with hexane, was analyzed quantitatively by g.l.c.

In some cases, methylated derivatives were methanolyzed with 10% methanolic hydrogen chloride for 18 h at 100°. After neutralization of the acid with silver carbonate, filtration, and evaporation of the filtrate to dryness *in vacuo*, a drop of methanol was added to each residue, and the methyl glycosides were qualitatively analyzed by g.l.c.

Properties of Klebsiella *K64 capsular polysaccharide.* — Capsular polysaccharide *A*, grown and purified as before[4], had $[\alpha]_D^{20}$ +28° (*c* 0.1), contained N 0.4%, and moved as a single component in ultracentrifugation and electrophoresis. The weight-average molecular weight was ~1.7 × 10^6 (gel-permeation chromatography), and the equivalent weight was ~570 (as acid by titration), in agreement with the values previously reported[4]. The p.m.r. spectrum of a 2% solution of the polysaccharide in D_2O showed sharp singlets at τ 7.72 (acetate CH_3) and 8.40 (pyruvate CH_3), a doublet at τ 8.62 (L-rhamnose CH_3), and three signals in the anomeric region. Integration of the spectrum indicated ratios of 1:1:1 for the CH_3 groups, and the presence of six anomeric protons.

Hydrolysis of polysaccharide A; *sugar analysis.* — After hydrolysis of a sample of polysaccharide *A* in 2M trifluoroacetic acid for 18 h at 100°, paper chromatography (solvents *a* and *d*), showed rhamnose, mannose, glucose, glucuronic acid, and pyruvic acid. Analysis of the neutral sugars by g.l.c. of the alditol acetates gave rhamnose, mannose, and glucose in the ratios of 1.0:1.4:2.0. Preparative g.l.c. of these acetates allowed the recovery of L-rhamnitol pentaacetate as a syrup, and the hexaacetates of D-mannitol and D-glucitol as crystalline compounds. Comparison of the circular dichroism spectra of these derivatives with those of standard samples confirmed the configurational assignments[30].

Partial hydrolysis of polysaccharide A *with acid; separation of oligosaccharides.* — Polysaccharide *A* (1 g) was hydrolyzed with 0.01M trifluoroacetic acid (120 mL) for 48 h at 100°, and the hydrolysis products were separated by preparative, gel-permeation chromatography into four components which gave single peaks at molecular weights 1100, 930, 520, and 350 when examined by gel-permeation chromatography on Bio-Gel P-10. The structures of these oligomers were proved by standard techniques, including partial-hydrolysis studies and methylation analysis, as well as the use of p.m.r. spectroscopy and measurement of optical rotation to determine the configurations of anomeric linkages. The results obtained are summarized in Table I. Proportions of neutral sugars were determined by g.l.c. (alditol acetate method) after hydrolysis of the oligosaccharides with 2M trifluoroacetic acid for 8 h at 100°.

The component having molecular weight 350 was shown by paper chromatography (solvent *c*) to be a mixture of an aldobiouronic acid and a neutral fragment having the mobility of a disaccharide. Separation of the mixture by preparative paper-chromatography (solvent *c*) gave compounds **1** and **2**. Oligosaccharides **1–5** were further characterized as follows.

Compound **1**. A sample of the disaccharide (20 mg) was dissolved in water

(2 mL) and reduced with sodium borohydride (20 mg) for 24 h. After acidification with Amberlite IR-120 (H^+) resin and removal of borate, the product was dissolved in D_2O (2 mL) and freeze-dried. The p.m.r. spectrum of this compound (in D_2O) showed a doublet at τ 5.36 (6 Hz), indicating a β-D linkage. Hydrolysis of the reduction product followed by paper chromatography (solvent *d*) gave only glucose. Analysis of the neutral compound (by courtesy of Dr. J. P. Kamerling, Utrecht) by g.l.c.–mass spectrometry of the per(trimethylsilyl) derivative indicated that neutral disaccharides having (1→2)- and (1→4)-type linkages were present in the ratio of 1:3. Methylation of the mixture followed by hydrolysis, and g.l.c. analysis, as the derived alditol acetates (column *a*), of the methylated sugars present in the hydrolyzate, gave 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-mannose, and 3,4,6-tri-*O*-methyl-D-mannose.

Compound **2**. On methanolysis and g.l.c., the methylated aldobiouronic acid gave methyl glycosides of methyl 2,3,4-tri-*O*-methyl-D-glucuronate (removed by saponification) and 2,4,6-tri-*O*-methyl-D-mannose. The identity of the 2,4,6-tri-*O*-methyl-D-mannose was confirmed by hydrolysis of the methylated aldobiouronic acid, and analysis of the hydrolyzate by paper chromatography (solvents *b* and *f*) and by the g.l.c. retention-time of the per(trimethylsilyl)ated, derived alditol. The p.m.r. spectrum of the aldobiouronic acid in D_2O had anomeric signals at τ 4.73 (3.5 Hz), 4.82 (2 Hz) and 5.08 (2 Hz), which are in good agreement with those published for 3-*O*-(α-D-glucopyranosyluronic acid)-D-mannose[17].

Compound **3**. Reduction of the aldotriouronic acid with sodium borohydride, followed by hydrolysis with 2M trifluoroacetic acid for 6 h at 100° and treatment for 4 h with hot, M sodium hydroxide under oxygen, yielded D-glucitol (identified by g.l.c. of its hexaacetate). Hydrolysis of the aldotriouronic acid, followed by paper chromatography of the hydrolyzate (solvent *a*), gave D-glucose, D-mannose, and the aldobiouronic acid **2**. On methanolysis and g.l.c., methylated **3** gave the methyl glycosides of methyl 2,3,4-tri-*O*-methyl-D-glucuronate, 2,4,6-tri-*O*-methyl-D-mannose, and 2,4,6-tri-*O*-methyl-D-glucose. The identity of the 2,4,6-tri-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-mannose was confirmed by hydrolysis of the methylated aldotriouronic acid with 2M trifluoroacetic acid for 8 h at 100°, and g.l.c. of the trimethylsilyl derivatives of the anomers of the tri-*O*-methyl sugars. The p.m.r. spectrum in D_2O of the borohydride-reduced aldotriouronic acid showed anomeric signals at τ 4.69 (3 Hz) and 4.80 (1.5 Hz), consistent with those expected for D-glucuronic acid (α) and D-mannose (α) residues.

Compound **4**. Hydrolysis of the components having molecular weight 930, followed by paper chromatography of the hydrolyzate (solvent *a*), gave D-mannose, D-glucose, and the aldobiouronic acid **2**. The fully methylated derivative of **4** was hydrolyzed with 2M trifluoroacetic acid for 18 h at 100°, and the sugars present in the hydrolyzate were analyzed by paper chromatography (solvents *b* and *f*) and by g.l.c. of the derived alditol acetates, which showed the presence of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-mannose, and 3,6-di-*O*-methyl-D-mannose.

Compound **5**. Paper chromatography (solvent *a*) of a hydrolyzate of the oligomer having molecular weight 1100 revealed the same components as were found on hydrolysis of oligosaccharide **4**. Methylation, followed by hydrolysis with 2M trifluoroacetic acid for 18 h at 100°, and analysis of the sugars present in the hydrolyzate by paper chromatography (solvents *b* and *f*) and by g.l.c. of the derived alditol acetates, gave 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-glucose, 3,6-di-*O*-methyl-D-mannose, and a small amount of 2,3,6-tri-*O*-methyl-D-mannose.

Methanolysis and g.l.c. of the fully methylated derivatives of **4** and **5** gave methyl 2,3,4-tri-*O*-methyl-D-glucuronate, in addition to the sugars identified as methylated alditol acetates.

Autohydrolysis of polysaccharide A. — Polysaccharide *A* (0.1 g) was dissolved in water (initial pH ~3) and heated for 3 h at 100°. Paper chromatography (solvent *e*) of the concentrated autohydrolyzate showed a fast-moving spot, chromatographically identical with pyruvic acid and giving the same characteristic, white fluorescence as the authentic material when sprayed with *o*-phenylenediamine and examined[23] under u.v. light. A similar chromatogram, sprayed with *p*-anisidine, showed the presence of L-rhamnose and a large amount of material at the origin. The autohydrolyzate was diluted with water and dialyzed against distilled water (1 L) for 24 h. The non-dialyzable material (17 mg) was recovered by freeze-drying of the aqueous solution. The autohydrolyzed polysaccharide (*B*), which had $[\alpha]_D^{22}$ +25° (*c* 0.1), $\overline{M}_w$ 1.5 × 10^6 (by gel-permeation chromatography on agarose gel) and was found to contain no pyruvic acid (according to its p.m.r. spectrum), was used for the methylation studies described later.

Methylation analysis of polysaccharide A. — A sample (1.26 g) of capsular polysaccharide *A* in the acid form was methylated once by Hakomori's method and twice by the method of Purdie and Irvine, to give an amorphous product (1.17 g), $[\alpha]_D^{20}$ +20° (*c* 1.1, chloroform), that showed no absorption at 3700–3200 cm^{-1} (OH) and strong absorption at 1740 cm^{-1} (methyl ester CO); n.m.r. data (chloroform-*d*): τ 8.60 (s, pyruvic acetal).

A sample (20 mg) of the fully methylated polysaccharide was hydrolyzed with 2M trifluoroacetic acid for 18 h at 100°. Samples were removed after hydrolysis for 6 and 18 h, reduced, the products per(trimethylsilyl)ated, and the ethers analyzed by g.l.c., to give the results shown in Table II. The methylated polysaccharide (0.72 g) was hydrolyzed with 2M trifluoroacetic acid for 18 h at 100°, and the neutral sugars (0.36 g) produced were separated from the acidic sugars (0.12 g) by chromatography on a column of Amberlite IR-45 (OH^-) ion-exchange resin. The neutral-sugar mixture was applied to a column (65 × 4 cm) of cellulose, water-jacketed at 28°, and separated into its components by elution with butanone–water azeotrope. In some cases, mixtures were resolved by preparative paper-chromatography (solvent *b*). The fractions obtained were characterized as follows.

Fraction I (19 mg) was chromatographically identical with 2,3,4-tri-*O*-methyl-L-rhamnose. The per(trimethylsilyl)ated alditol and alditol acetate derivatives were

identical in their retention characteristics in g.l.c. with an authentic sample of this methylated sugar.

Fraction II (90 mg). On reduction and per(trimethylsilyl)ation, this gave derivatives of 2,4,6-tri-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-glucose in the ratio of 7:10. The mixture was separated into its pure components by g.l.c., using a preparative column (2 m × 6 mm o.d.) packed with 15% of SE-52 on Chromosorb W (80–100 mesh), isothermally at 140°.

Fraction III (33 mg), $[\alpha]_D^{20}$ +39° (*c* 0.3), was indistinguishable from 2,3-di-*O*-methyl-D-glucose by paper chromatography and by p.m.r. spectroscopy. The g.l.c. retention-times of both the per(trimethylsilyl)ated and alditol acetate derivatives were identical with those of the authentic material.

Fraction IV (35 mg), $[\alpha]_D^{20}$ +8° (*c* 0.1), was characterized as 6-*O*-methyl-D-mannose by (*a*) its paper-chromatographic color and mobility, (*b*) its p.m.r. spectrum in D_2O, which showed signals at τ 4.78 (1.5 Hz), 5.07 (2 Hz), and 6.54, in good agreement with those given in the literature[34] for this compound, and (*c*) the g.l.c. retention-time of its derived alditol acetate[35].

The identity of each of these methylated sugars was confirmed by mass spectrometry of the derived alditol acetate.

The methylated, acidic sugars were converted into their methyl ester methyl glycosides by heating with 1% methanolic hydrogen chloride under nitrogen in a sealed tube for 6 h at 100°. After neutralization of the acid with silver carbonate, filtration, and evaporation, the residue was dissolved in tetrahydrofuran (10 mL) and reduced with lithium aluminum hydride (700 mg). A sample (30 mg) of the reduction product was hydrolyzed with 2M trifluoroacetic acid for 5 h at 100°, and the sugars present in the hydrolyzate were analyzed by paper chromatography (solvents *b* and *f*), which showed the presence of 2,4,6-tri-*O*-methyl-D-mannose and 2,3-di-*O*-methyl-D-glucose. Quantitative analysis of these sugars by g.l.c. of the per(trimethylsilyl)ated, derived alditols gave 2,4,6-tri-*O*-methyl-D-mannose and 2,3-di-*O*-methylglucose in the ratio of 5:8. Reduction, acetylation, and preparative g.l.c. gave the individual, methylated, alditol acetates, which were further characterized by mass spectrometry. A sample of the 2,3-di-*O*-methylglucitol tetraacetate was demethylated[36] with BCl_3, and the product acetylated, to give glucitol hexaacetate, the circular dichroism spectrum of which was recorded.

Lindberg degradation. — A solution of dried, methylated polysaccharide (60 mg) and *p*-toluenesulfonic acid (2 mg) in 19:1 dimethyl sulfoxide–2,2-dimethoxypropane (10 mL) was prepared in a flask fitted with a rubber serum-cap. The flask was flushed with nitrogen before the addition of 1.5M methylsulfinyl anion in dimethyl sulfoxide (10 mL). The solution was kept under nitrogen, and stirred for 48 h. After being cooled in an ice bath, methyl iodide (15 mL) was added, and the solution was stirred for a further 30 min at room temperature. The remethylated product (53 mg) was isolated by partition between chloroform and water, and a sample (20 mg) was hydrolyzed with 2M trifluoroacetic acid for 8 h at 100°. After removal of the acid, the hydrolyzate was analyzed by paper chromatography (solvents *b* and *f*) and by g.l.c.

of the derived alditol acetates (column *a*). The individual acetates were recovered by preparative g.l.c., and further characterized by mass spectrometry.

Methylation analysis of degraded polysaccharide B. — Polysaccharide *B* (15 mg), produced on autohydrolysis of *A*, was methylated by Hakomori's method, to give a derivative (18 mg) that showed no hydroxyl absorption in the i.r. spectrum. Hydrolysis of the methylated product with 2M trifluoroacetic acid for 18 h at 100°, followed by paper chromatography of the hydrolyzate (solvents *b* and *f*) showed the absence of 2,3-di-*O*-methyl-D-glucose and the appearance of a new spot having the same mobility as 2,3,4,6-tetra-*O*-methyl-D-glucose and giving the same pink color when sprayed with *p*-anisidine. The hydrolyzate was reduced, the products per(trimethylsilyl)ated, and the ethers analyzed by g.l.c., to give the results shown in Table II.

Reduction of methylated polysaccharides A *and* B. — Fully methylated polysaccharide *A* (122 mg) was reduced with lithium aluminum hydride (700 mg) in tetrahydrofuran (10 mL), to give a product having $[\alpha]_D^{20}$ +14° (*c* 0.1, chloroform). Hydrolysis of a sample with 2M trifluoroacetic acid for 8 h at 100°, followed by paper chromatography (solvent *b*) and g.l.c. analysis of the sugars present in the hydrolyzate (as the trimethylsilylated, derived alditols), gave the proportions shown in Table III. The methylated derivative (6 mg) of polysaccharide *B* was reduced, the product hydrolyzed, and the hydrolyzate analyzed in the same way.

Smith degradation of A. — Capsular polysaccharide *A* (0.25 g) was dissolved in 113mM aqueous sodium metaperiodate (50 mL), and the solution was kept in the dark for 4 days, the consumption of periodate being monitored by the arsenite method[37]. After this time, the reaction was terminated by the addition of ethylene glycol (1.5 mL), and the solution was stirred for 2 h and then dialyzed against running tap-water for 3 days. Reduction with sodium borohydride (2 g), de-ionization with Amberlite IR-120 (H^+) resin, freeze-drying, and removal of borate, yielded the polyalcohol (106 mg). This product was dissolved in M trifluoroacetic acid (20 mL) and stirred for 110 h at room temperature. Gel-permeation chromatography on Bio-Gel P-2 was used to monitor the change in molecular weight during the hydrolysis; this showed that, after 24 h, a single product, molecular weight 560, was generated, and no change in this molecular weight was apparent when samples removed after 48 and 100 h were examined. The trifluoroacetic acid was removed by freeze-drying, and the residue was dissolved in methanol and examined by paper chromatography (solvent *b*). When sprayed with silver nitrate, the paper chromatogram showed the presence of glycolaldehyde and a large proportion of slow-moving material near the origin. The oligosaccharide was recovered by evaporation of the methanol and extraction of the residue with absolute ethanol; the ethanol-insoluble material was dissolved in water and freeze-dried, to yield the Smith-degraded product (67 mg), which had $[\alpha]_D^{20}$ +24° (*c* 0.1).

This oligosaccharide (5 mg) was hydrolyzed with 2M trifluoroacetic acid (1 mL) for 5 h at 100°, and the hydrolyzate was analyzed by g.l.c. of the derived alditol acetates. The p.m.r. spectrum of the oligosaccharide in D_2O showed signals in the anomeric region at τ 4.78 (1 Hz) and 5.14 (6 Hz).

The Smith-degraded oligosaccharide (18 mg) was methylated, to give a product (15 mg) whose i.r. spectrum showed no absorption in the hydroxyl region and strong absorption at 1720 cm^{-1} (methyl ester). After reduction of the methylated product with lithium aluminum hydride (100 mg) in tetrahydrofuran (3 mL), the i.r. spectrum showed no absorption at 1720 cm^{-1}. The reduced, methylated oligosaccharide was heated with 2M trifluoroacetic acid for 8 h at 100°, and the sugars present in the hydrolyzate were analyzed by paper chromatography (solvents *b* and *f*) and by g.l.c. of the derived alditol acetates. The acetates, recovered by preparative g.l.c., were further characterized by mass spectrometry.

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