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STRUCTURAL INVESTIGATION OF *Klebsiella* SEROTYPE K18 POLYSACCHARIDE

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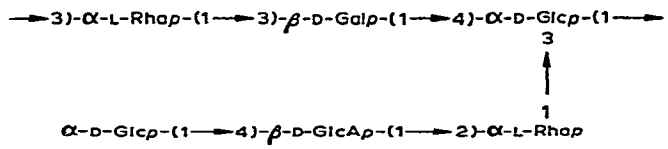
ABSTRACT

Klebsiella K-type 18 is one of five strains belonging to the chemotype containing D-glucuronic acid, D-glucose, D-galactose, and L-rhamnose, but lacking a 1-carboxyethylidene acetal group. The main features of the capsular polysaccharide were apparent from the results of methylation and periodate oxidation studies, but the precise structure was only determined by application of a β-elimination reaction-sequence. The capsular polysaccharide of *Klebsiella* serotype K18 has the structure shown, and represents a new pattern (among these antigens) of a main chain of three sugar residues carrying a side chain also composed of three monosaccharide units (three plus three).



INTRODUCTION

The 81 different serotypes of *Klebsiella* capsular polysaccharides may be divided into ~20 chemotypes[1,2], one of which comprises residues of glucuronic acid, glucose, galactose, and rhamnose. There are 10 strains in this one chemotype[3], in five of which the antigenic capsular polysaccharides carry (1-carboxyethylidene) groups (K12, 36, 45, 55, and 70). The structures of the capsular polysaccharides of K36, K55, and K70 have already been established[4–6], and we now report the results of our investigation on the capsular polysaccharide of *Klebsiella* K18, a member of the sub-group (K18, 19, 23, 41, and 79) that lacks (1-carboxyethylidene) substituents. A preliminary account of the nature of the polysaccharide from *Klebsiella* K41 has been published[7].

*Commonwealth Scholar from New Zealand, 1973–1977.

RESULTS AND DISCUSSION

Composition and properties. — Isolation and purification of the polysaccharide were conducted as previously described[4,6]. One precipitation with Cetavlon was performed, and the purified material had $[\alpha]_D$ +77.3°. Proton nuclear magnetic resonance (^{1}H-n.m.r.) spectroscopy of a 2% solution of the polysaccharide in deuterium oxide showed two doublets of equal intensity, at τ 8.74 and 8.67, characteristic of the methyl groups of ω-deoxy sugars[8]. The anomeric region of the spectrum indicated that the repeating unit is composed of six sugar residues, of which two are β-linked and four are α-linked[9]. Integration of the signals from the methyl groups and from the anomeric protons showed two of the six sugar residues to be ω-deoxy sugars (rhamnose residues).

Acid hydrolysis of the polysaccharide rapidly liberated glucose, and complete hydrolysis gave rhamnose, glucose, galactose, and glucuronic acid. The three neutral sugars were obtained in the ratios of 2:2:1, or 2:3:1 after reduction of the glucuronic acid. The configuration of the rhamnose was shown to be L, and that of the glucose to be D, by measurement of the circular dichroism (c.d.) spectra of their alditol acetates[10]. In a similar manner, the glucuronic acid (as the 6,3-lactone) was shown to have the D configuration, as had the galactose, based on the c.d. spectrum of a sample of the 2,4,6-tri-*O*-methylgalactose isolated during the methylation analysis.

Methylation. — The polysaccharide was methylated[11], and the product hydrolyzed, and the sugars thus liberated were separated into neutral and acidic fractions. The acidic fraction was converted into the methyl ester methyl glycosides, and these were reduced, and the products hydrolyzed. Alternatively, the methylated polysaccharide was reduced before hydrolysis. The results of these analyses are presented in Table I, columns I, II, and III; they show that one glucose residue is terminal and one constitutes a branch point, and they also demonstrate the nature of the aldobiouronic acid. It was impossible to resolve, by g.l.c. on column 1 (Apiezon L),

TABLE I

METHYLATION ANALYSIS OF *Klebsiella* K18 CAPSULAR POLYSACCHARIDE

Methylated sugars[a] (*as alditol acetates*)	*T* (*min*)		*Mole %*		
	Column 1[b] (*Apiezon* L)	*Column 2*[c] (*ECNSS-M*)	*I*[d]	*II*	*III*
3,4-Rha	18.6		0.7	+	1.0
2,4-Rha	20.7		1.0		1.0
2,3,4,6-Glc	23.6		1.0		1.0
2,4,6-Gal	35.3	7.5	1.0		1.0
2,6-Glc		11.5	1.0		1.0
2,3-Glc	42.9			++	1.0

[a]3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol, etc. [b]Column 1 at 212°, He at 90 mL/min. [c]Column 2 at 220°, He at 50 mL/min. [d]I, neutral fraction; II, acidic fraction; III, reduced (see text for details).

the alditol acetates derived from 2,4,6-tri-*O*-methylgalactose and 2,6-di-*O*-methylglucose, but they were separable on column 2 (ECNSS-M).

Partial hydrolysis. — Partial hydrolysis of the polysaccharide was conducted under two sets of conditions, namely, by heating with trifluoroacetic acid (0.5M) for (*a*) 4 h, to obtain an aldobiouronic acid (A_2) as the preponderant component, together with a small proportion of a neutral disaccharide (N_1), and (*b*) 30 min, to produce a trisaccharide (A_2) and higher oligomers. The hydrolyzates were examined, as described in the Experimental section, by use of ion-exchange resins, a charcoal–

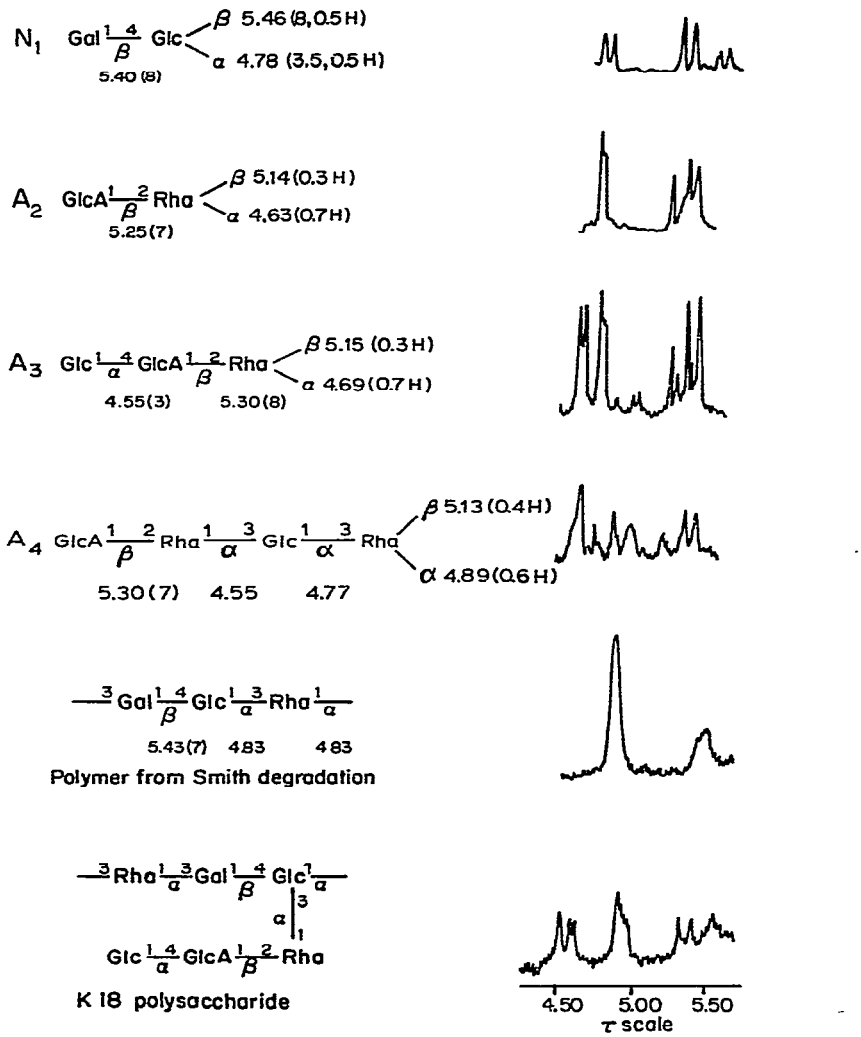


Fig. 1. ^{1}H-N.m.r. spectra at 100 MHz of K18 polysaccharide, isolated oligosaccharides, and periodate-degraded polymer in D_2O at ~90°. (Only the region of the anomeric protons is shown; see text for details.)

Celite column, and, in particular, paper chromatography. Despite many tedious separations, the higher oligomers A_4, A_5, and A_6 could not be obtained pure, although ^{1}H-n.m.r. spectral data suggested that A_4 was a tetrasaccharide, A_{5a} a mixture of pentasaccharides, and A_{6a} a mixture of hexasaccharides.

The structures of the three components obtained pure (N_1, A_2, and A_3), together with that of the main component of A_4, are given (with selected ^{1}H-n.m.r. data) in Fig. 1. Some physical properties of these oligosaccharides are given in Table II, along with the results of methylation analysis of these and higher oligomers. At the time the experiments described here were performed, the analysis of fractions A_4, A_{5a}, and A_{6a} served only to show that they were mixtures. When the sequence of the monosaccharide units in *Klebsiella* K18 capsular polysaccharide was finally established by degradation involving a β-elimination reaction[12] (see later), it became clear that fraction A_4 is primarily composed of residues FEDB, with a minor proportion of EDBC (see Scheme 1); the methylation analyses of fractions 5a and 6a could be similarly rationalized.

TABLE II

OLIGOSACCHARIDES ISOLATED BY PARTIAL HYDROLYSIS OF *Klebsiella* K18 POLYSACCHARIDE: PHYSICAL PROPERTIES AND METHYLATION DATA

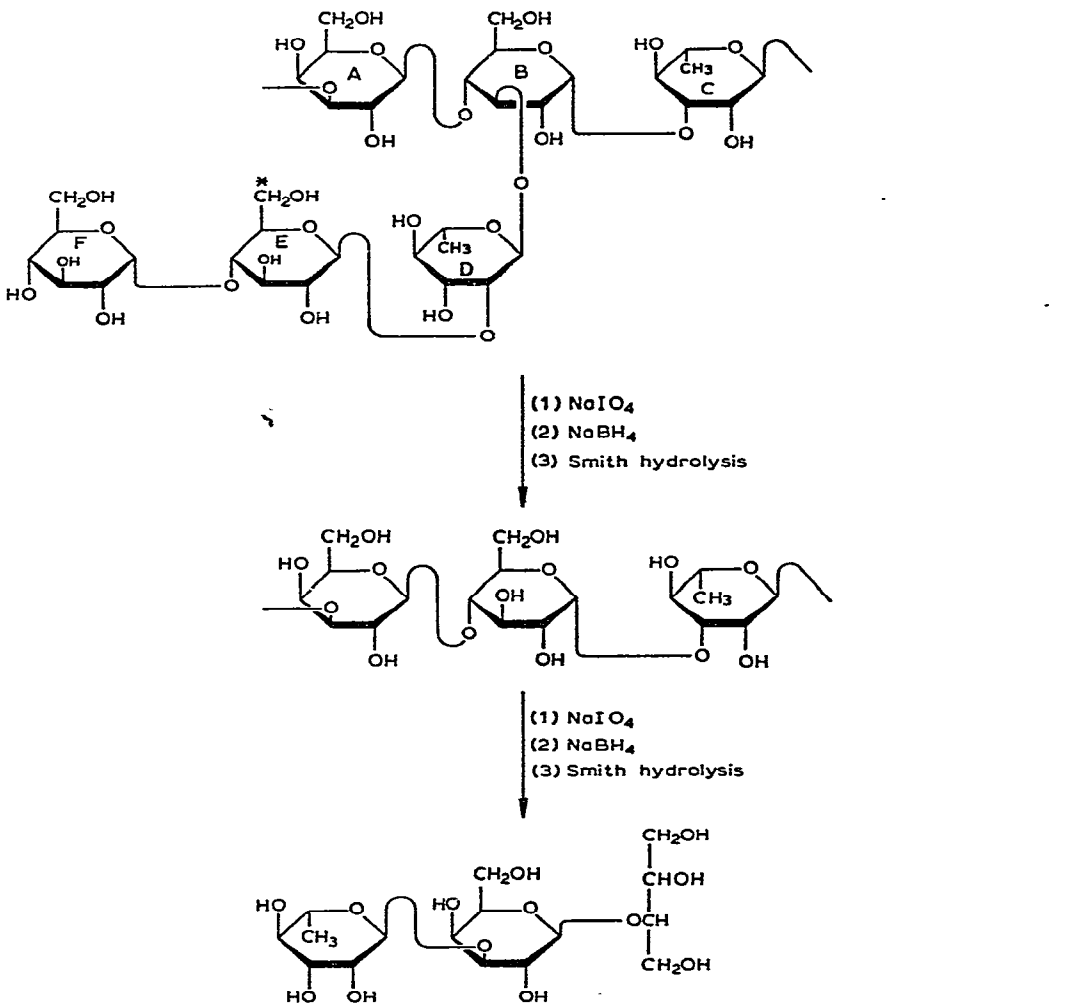
Oligosaccharide[a]	$[\alpha]_D$ (H_2O) (*degrees*)	R_{GlcA} (*solvent C*)	*Methylation data*[b]
N_1			2,3,4,6-Gal 2,3,6-Glc
A_2	−12.8	1.8	2,3,4-Glc[c] 3,4-Rha
A_3	+50.0	0.53	2,3,4,6-Glc 3,4-Rha 2,3-Glc[c]
A_4	+30.0	0.44	2,3,4,6-Glc 2,4,6-Glc 2,4-Rha 3,4-Rha
A_{5a}	+38.0	~0.23	
A_{6a}	+62.0	~0.07	

[a]For structures, see Fig. 1. [b]As in footnore *a* to Table I. [c]After reduction of the uronic acid.

Periodate oxidation. — Both the original, acidic polysaccharide, and the neutral polymer obtained following reduction of the uronic acid by use of the carbodiimide procedure[13], were oxidized. Similar results were obtained in each case. Oxidation of the neutral polysaccharide is discussed here, and the Experimental section contains, in addition, a summary of the experiment on the acidic starting-material.

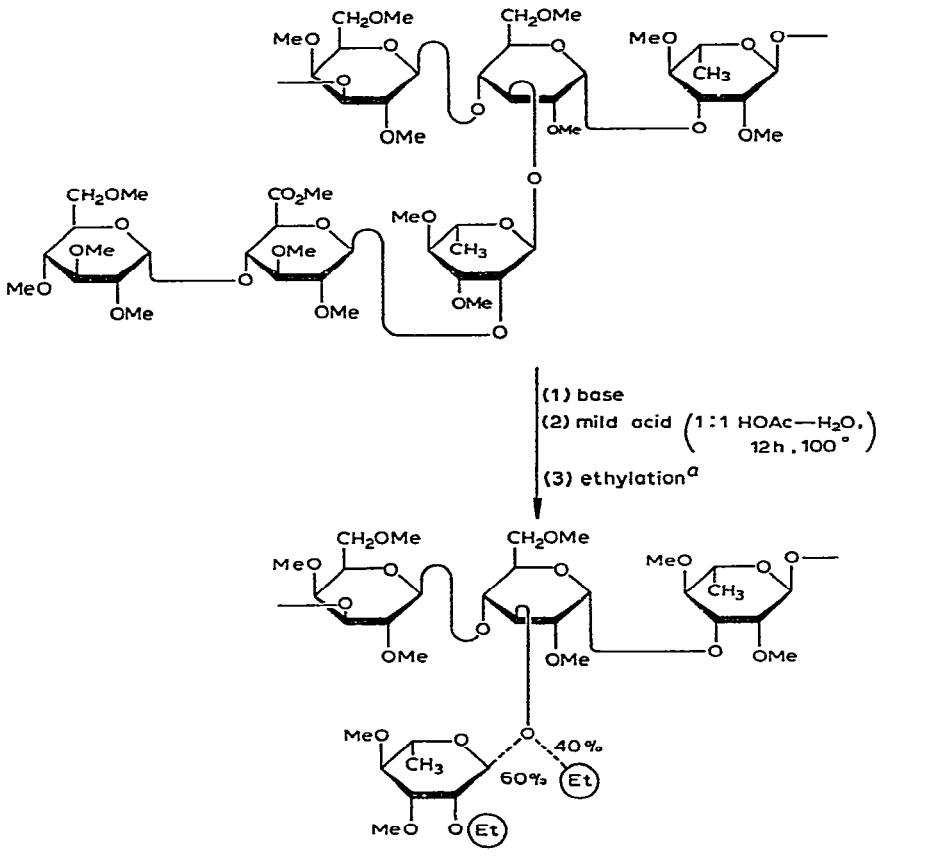
The carboxyl-reduced polysaccharide consumed the theoretical amount of periodate (four molecules per repeating unit of six sugars) in 60 h. Hydrolysis of a

sample of the derived polyol, obtained by reduction with borohydride, dialysis, and lyophilization, gave 1,2-propanediol, glycerol, erythritol, rhamnitol, galactitol, and glucitol in approximately equimolar amounts; this indicated that one rhamnose and two glucose residues were oxidized. The major part of the polyol was subjected to a Smith degradation[14], using trifluoroacetic acid, and, after removal of the acid, the product was dialyzed against a constant volume of water. The dialyzate contained 1,2-propanediol, glycerol, and erythritol. The retentate was examined by ^{1}H-n.m.r. spectroscopy; the spectrum showed signals for the methyl group of an ω-deoxy sugar, and anomeric protons corresponding to one β- and two α-linked sugars (see Fig. 1).



Scheme 1. The sequential, periodate oxidation of reduced *Klebsiella* K18 capsular polysaccharide; the group marked with an asterisk in unit E was a carboxyl group in the original polysaccharide (see text for details).

A portion of the non-dialyzable material was methylated, and hydrolysis of the product yielded 2,4-di-*O*-methylrhamnose, 2,3,6-tri-*O*-methylglucose, and 2,4,6-tri-*O*-methylgalactose. This result established the presence of three sugar residues in the main chain, but left their sequence indeterminate; a second periodate oxidation resolved this point (see Scheme 1). When the non-dialyzable, polymeric material was



	Relative proportions[b]
2,4,6-tri-*O*-methylgalactose	1.0
2,4-di-*O*-methylrhamnose	1.0
2,6-di-*O*-methylglucose	0.6
3-*O*-ethyl-2,6-di-*O*-methylglucose	0.4
2-*O*-ethyl-3,4-di-*O*-methylrhamnose	0.3[c]

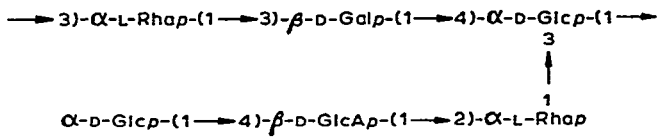
Scheme 2. The degradation, by β-elimination, of methylated capsular polysaccharide from *Klebsiella* K18; see text for details. ([a]The ethyl groups are circled for clarity. [b]Column 2. [c]Volatile component, recovery not quantitative.)

subjected to a second, sequential Smith-degradation, there was obtained in almost quantitative yield a disaccharide glycoside. The nature of the products of hydrolysis of this glycoside, and of its fully methylated derivative, coupled with the ^{1}H-n.m.r. spectrum of the glycoside, permitted the structure of this compound to be written as α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→2)-D-erythritol. Although it was now possible to write a unique structure for the K18 polysaccharide, further proof was sought by application of the sequence of reactions based on uronic acid degradation[12].

Uronic acid degradation. — A carefully dried sample of fully methylated K18 polysaccharide was treated overnight with methylsulfinyl anion, and then with 50% aqueous acetic acid[12]. Following dialysis overnight, a polymeric product was recovered by lyophilization. This material was ethylated, the ether was hydrolyzed, the products were reduced, the alditols were acetylated, and the acetates analyzed by g.l.c., with the results shown in Scheme 2. Study of Scheme 2 reveals that, under the conditions of a β-elimination sequence, the 2-linked rhamnose residue attached to the branch point should not, theoretically, be degraded. The glycosidic linkage of a 6-deoxyhexose is, however, noticeably acid-labile, and the results presented in Scheme 2 are readily rationalized if it is assumed that 60% of these rhamnose residues were hydrolyzed by the treatment with acetic acid.

These experiments confirm the existence of three sugar residues in the main chain, and demonstrate that it is a rhamnose residue that is attached to the branch point.

It is thus apparent that the data from methylation, partial hydrolysis, and sequencing experiments are self-consistent, and permit the structure of the hexasaccharide repeating-unit of K18 capsular polysaccharide to be formulated as follows.



In the systematic study of these capsular antigens now being undertaken in several laboratories, the polysaccharide from *Klebsiella* K41 was the first shown[7] to contain a side chain comprising three sugar residues, but these are part of a repeating unit based on seven monosaccharide units. The structure of the K18 polysaccharide is novel, in that it represents a new pattern of "three plus three".

EXPERIMENTAL

General methods. — The instrumentation used and the general methods employed have been described[4–6]. For descending, paper-chromatography, the following solvent systems (v/v) were used: (*A*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, (*B*) 4:1:1 ethyl acetate–pyridine–water, (*C*) 2:1:1 1-butanol–acetic

acid–water, (*D*) butanone–water azeotrope, and (*E*) 4:1:5 1-butanol–ethanol–water. Analytical and preparative g.l.c. were initially performed by use of an F & M model 720 instrument fitted with dual thermal-conductivity detectors, and, later, analytical separations were conducted with a Hewlett–Packard 5700 instrument fitted with dual flame-ionization detectors. Columns used were: (1) 20% of Apiezon L on 60–80 mesh Diatoport S (2.5 m × 6.3 mm); (2) 3% of ECNSS-M on 100–120 mesh Gas Chrom Q (1.25 m × 6.3 mm); and (3) 0.2% of polyethylene glycol succinate, 0.2% of polyethylene glycol adipate, and 0.4% of XF-1150 on 100–120 mesh Gas Chrom Q (1.8 m × 6.3 mm). Peak areas were measured with an Infotronics CRS-100 electronic integrator.

Isolation and properties of K18 capsular polysaccharide. — A culture of *Klebsiella* K18 (1754-49) was grown as described[4] for K36; 4 L of medium yielded a mixture of slime and cells (450 mL) which was diluted with an equal volume of 2% phenol, and centrifuged at 68,000 *g* for 1 h. The clear, supernatant liquor was treated with 5% Cetavlon (40 mL), the precipitate was dissolved in 2M sodium chloride, and the acidic polysaccharide was recovered by precipitation into ethanol. This product was decationized by use of Amberlite IR-120 (H^+) ion-exchange resin, dialyzed against distilled water, and lyophilized, to give 4.2 g of pure capsular polysaccharide having $[\alpha]_D$ +77.3° (*c* 0.96, water). Elemental analysis showed the absence of ash and nitrogen and there was no absorption at 260 or 280 nm.

After dialysis and lyophilization, the supernatant liquor from the Cetavlon precipitation yielded 1.8 g of neutral polysaccharide(s) that was shown, following hydrolysis, to contain mainly galactose, with lesser proportions of glucose and mannose; this material was not further examined.

The p.m.r. spectrum of a 2% solution of the purified, acidic polysaccharide in D_2O at 95° showed (see Fig. 1) signals of anomeric protons at τ 5.55 (1 H), 5.33 (1 H, $J_{1,2}$ 7 Hz), 4.91 (2 H), 4.58 (1 H), and 4.50 (1 H), and methyl protons at τ 8.74 (3 H, $J_{5,6}$ 3 Hz) and 8.68 (3 H, $J_{5,6}$ 3 Hz).

A sample of polysaccharide (10 mg) was heated in 2M hydrochloric acid for 8 h at 97°. The solution was cooled, passed through a column of Duolite A-4 (OH^-) ion-exchange resin, to remove all acidic compounds, and evaporated; the neutral fraction was analyzed by g.l.c. (column 2 programmed from 150° at 3°/min, helium flow 60 mL/min) of the derived alditol acetates, and found to contain rhamnose (11.1 min), galactose (30.1 min; m.p. and mixed m.p. with galactitol hexaacetate 160–162°), and glucose (35.5 min; m.p. and mixed m.p. with D-glucitol hexaacetate 98–99°) in the approximate ratios of 2:1:2.

A second sample of polysaccharide (15 mg) was depolymerized by refluxing with 3% methanolic hydrogen chloride, the acid neutralized (Ag_2CO_3), the sugars reduced with sodium borohydride in methanol, and the mixture further processed as described[15] for *Klebsiella* K62 polysaccharide, to yield the same three alditol acetates in the ratios 2:1:3.

Individual components were collected, and dissolved in acetonitrile, and their c.d. spectra were measured at 213 nm; rhamnitol pentaacetate $\Delta\varepsilon_{213}^{MeCN}$ −1.50, glucitol

hexaacetate +0.33. For the c.d. of galactose and glucuronic acid, see, respectively, the methylation and partial hydrolysis experiments.

Methylation of the capsular polysaccharide. — An excess of methylsulfinyl anion (2M, 25 mL) was added to a solution of vacuum-dried polysaccharide (1 g) in anhydrous dimethyl sulfoxide (40 mL), and the mixture was stirred for 12 h. Methyl iodide (7 mL) was added slowly, while stirring and maintaining the temperature below 20°. After dialysis for 30 h against running tap-water, and lyophilization, the recovered material (1.05 g) had a small absorption at 3600 cm^{-1} and so it was successively remethylated according to the methods of Kuhn and Purdie, to give a product devoid of hydroxyl-group absorption in the i.r. spectrum.

A sample (0.1 g) of the fully methylated polysaccharide was digested in 90% formic acid for 1 h at 97°, the solution concentrated, and the concentrate heated in 2M hydrochloric acid for 6 h at 100°. Examination of the hydrolyzate by paper chromatography (solvents *D*, and *E*) showed the presence of 2,3,4,6-tetra-*O*-methylglucose (R_F 0.82, *D*; 0.84, *E*), 2,4-di-*O*-methylrhamnose (R_F 0.65, 0.74; green color with *p*-anisidine spray) slightly ahead of, but incompletely resolved from, 3,4-di-*O*-methylrhamnose (brown), 2,4,6-tri-*O*-methylgalactose (R_F 0.40, 0.62), 2,6-di-*O*-methylglucose (R_F 0.19, 0.54), and an acidic component (R_F 0.00, ~0.25).

The hydrolyzate was separated into neutral and acidic fractions by use of a column of Duolite A-4 (OH^-) ion-exchange resin. After conversion of the neutral components into alditol acetates, analysis by g.l.c. gave the results shown in Table I, column I. The individual fractions were collected, and the fourth fraction was resolved into its two components on column 2. The positions of the methoxyl groups in each sugar were determined by mass spectrometry. Samples of 2,6-di- and 2,3,4,6-tetra-*O*-methylglucitol acetates were separately *O*-demethylated with boron trichloride, the alditol acetylated, and the acetate purified by g.l.c., to give D-glucitol hexaacetate, m.p. 92–94°, undepressed by admixture with an authentic sample. The c.d. curve of the 2,4,6-tri-*O*-methylgalactitol triacetate isolated was identical ($\Delta\varepsilon_{213}^{MeCN}$ +1.40) to that of an authentic standard having the D configuration.

The acidic fraction, eluted from the ion-exchange column with 10% formic acid, was refluxed with 3% methanolic hydrogen chloride, the acid neutralized (Ag_2CO_3), the esters reduced with sodium borohydride in methanol, and the products hydrolyzed. Examination by paper chromatography (solvent *D*) revealed a minor spot corresponding to 3,4-di-*O*-methylrhamnose (R_F 0.65) and a major one equivalent to 2,3-di-*O*-methylglucose (R_F 0.30). The derived alditol acetates were analyzed by g.l.c., with the results shown in Table I, column II.

A sample (100 mg) of the fully methylated polysaccharide in tetrahydrofuran was reduced with lithium aluminum hydride, and the product hydrolyzed, initially with 95% formic acid, and then with 2M trifluoroacetic acid. Analysis of the liberated monosaccharides as their alditol acetates gave, in g.l.c., the results shown in Table I, column III. Fraction 4 was collected, and separated on column 2 into equimolar amounts of the two components, as previously described. In addition, fraction 5 was

O-demethylated, and the product reacetylated, to give D-glucitol hexaacetate, m.p. and mixed m.p. 94–96°.

Partial hydrolysis of capsular polysaccharide with acid. — Autohydrolysis of the polysaccharide (pH 2.5) for 13 h at 97° liberated glucose as the only neutral sugar, and traces of a component with R_{Glc} 0.39 (solvent *A*). When the polysaccharide was treated with 0.5M trifluoroacetic acid at 97°, heating for 10 min gave mainly glucose; oligomeric material was obtained after 30 min; and when the time of reaction was extended to 4 h, an aldobiouronic acid was the major product.

In one experiment, 1 g of polysaccharide was heated with 0.5M trifluoroacetic acid for 4 h at 97°, and the hydrolyzate was separated into neutral and acidic fractions by use of Duolite A-4 (OH^-) ion-exchange resin. Chromatographic separation of the neutral fraction on Whatman No. 1 paper (solvent *B*) gave a disaccharide (N_1, 10 mg; R_{Glc} 0.20), in addition to monosaccharides. The acidic fraction (190 mg) was separated on Whatman 3MM paper by use of solvent *C*, to give glucuronic acid (A_1, 21 mg), a component A_2 (75 mg; R_{Glc}= 1.10), and small amounts of slower-moving components. The glucuronic acid was evaporated, with the addition of hydrochloric acid, and the c.d. spectrum of the derived glucuronolactone was measured. The sample had $\Delta\varepsilon_{219}^{H_2O}$ +2.90, and standard D-glucurono-6,3-lactone, +3.32.

In a second experiment, the polysaccharide (2 g) was heated with 0.5M trifluoroacetic acid for 30 min at 97°. The hydrolyzate was evaporated to dryness, and water was twice added to, and evaporated from, the residue. The resulting syrup was applied to a charcoal–Celite column (10 × 1.4 cm, containing 60 g of a 1:1 mixture) that was eluted with water (2 L), 20% aqueous ethanol (1 L), and warm, 25% aqueous isopropyl alcohol (1.5 L). The aqueous eluate mainly contained monosaccharides (0.58 g). The two alcoholic eluates were combined (1.03 g), and the components were separated on Whatman 3MM paper (solvent *C*). The properties of the oligosaccharides isolated are shown in Table II, and their structures are presented in Fig. 1.

Periodate oxidation of neutral polysaccharide. — Acidic, capsular polysaccharide was carboxyl-reduced by two treatments according to the carbodiimide procedure of Taylor and Conrad[13]. A solution of this reduced polysaccharide (300 mg) in 0.05M sodium periodate (300 mL) was kept in the dark for 60 h at 0°, ethylene glycol (10 mL) was added, and the solution was kept for 1 h and then dialyzed against tap-water overnight. Reduction with sodium borohydride (500 mg) was followed by dialysis and lyophilization, to yield 240 mg of the polyol, a 5-mg portion of which was hydrolyzed with 2M trifluoroacetic acid for 12 h at 95°, the sugars reduced, and the alditols acetylated. G.l.c.–m.s. (column 3) revealed the presence of the peracetates corresponding to glycerol, erythritol, rhamnitol, galactitol, and glucitol in the proportions 21:18:19:22:20.

The periodate-oxidized and reduced polysaccharide (235 mg) was subjected to a Smith hydrolysis with 0.5M trifluoroacetic acid for 16 h at room temperature. Removal of the trifluoroacetic acid by repeated evaporations with water gave a syrup; this was dialyzed against a fixed volume of water. The dialyzable and non-dialyzable materials were individually lyophilized. Paper chromatography (solvents *B* and *C*) of

the former fraction indicated the presence of 1,2-propanediol, glycerol, and erythritol. Acetylation, and preparative g.l.c., gave erythritol tetraacetate (m.p. 80–82°) as the only crystalline product.

The non-dialyzable material (144 mg) was examined by n.m.r. spectroscopy. The ^{1}H-n.m.r. spectrum showed the presence of signals in the anomeric region at τ 4.83 (2 H, broad singlet) and 5.43 (1 H, broad doublet, $J_{1,2}$ ~7 Hz). A sharp signal at τ 8.65 (3 H, $J_{1,2}$ 6 Hz), attributable to the CH_3 of rhamnose, was also apparent. Methylation of the non-dialyzable, polymeric material obtained from the periodate oxidation and Smith hydrolysis gave a permethylated product that was subsequently hydrolyzed, the sugars reduced, and the alditols acetylated. G.l.c.–m.s. (column 2) confirmed the presence of the alditol acetates corresponding to 2,4-di-*O*-methylrhamnose, 2,3,6-tri-*O*-methylglucose, and 2,4,6-tri-*O*-methylgalactose.

Part of the foregoing, polymeric material (70 mg) was re-treated in the dark with 0.05M periodate for 48 h at 0°. After the excess of periodate had been decomposed by the addition of ethylene glycol, the mixture was dialyzed, reduced with borohydride, and lyophilized to yield 50 mg of product. Treatment of this material with 0.5M trifluoroacetic acid for 16 h at room temperature gave a syrup which was applied to the top of a column (100 × 2.6 cm) of Sephadex G-25. Irrigation of the column at a flow rate of 7 mL/h with a 5:2:500 pyridine–acetic acid–water buffer gave an oligomer P (30 mg, R_{Glc} 0.81, solvent *A*) that showed $[\alpha]_D$ −14.8° (*c* 1.03, water). ^{1}H-N.m.r. spectroscopy of P gave resonances attributable to protons at nonreducing, anomeric positions: τ 4.93 (1 H, $J_{1,2}$ 2 Hz) and 5.45 (1 H, $J_{1,2}$ 7.5 Hz). A doublet at τ 8.70 (3 H, $J_{5,6}$ 6 Hz) was also present. After hydrolysis of a portion of oligosaccharide P, paper chromatography (solvent *A*) indicated the presence of rhamnose, galactose, and erythritol. Methylation of P was performed by the Hakomori procedure, and the product was purified on silica gel (R_F 0.43; ethyl acetate). The electron-impact mass spectrum of permethylated P showed peaks at (*inter alia*) *m/e* 45 (19), 71 (45), 88 (38), 89 (23), 101 (51), 115 (36), 159 (36), 189 (100), 207 (22), 235 (8), and 411 (8). Hydrolysis of permethylated P (2M trifluoroacetic acid for 6 h at 95°), reduction of the sugars, acetylation of the alditols, and g.l.c. analysis gave peaks corresponding to 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; the volatile 2-*O*-acetyl-1,3,4-tri-*O*-methyl-D-erythritol was lost under diminished pressure during processing.

Periodate oxidation of acidic polysaccharide. — Capsular polysaccharide (977 mg) was dissolved in 0.05M sodium periodate (200 mL), and the solution was kept in the dark at 1°. The consumption of periodate, determined spectroscopically at 222.5 nm, became constant (at 0.64 molecule of oxidant per repeating unit) by 360 h, when the reaction was stopped by the addition of ethylene glycol. Dialysis, reduction with sodium borohydride, de-ionization, and lyophilization yielded the desired polyol (0.77 g).

A 10-mg portion of this polyol was hydrolyzed with 2M hydrochloric acid; paper chromatography (solvent *B*) revealed the presence of galactose, glucose, rhamnose (R_{Glc} 3.13), glycerol (R_{Glc} 3.35), erythrono-1,4-lactone (R_{Glc} 5.26, R_F 0.60),

and 1,2-propanediol (R_{Glc} 8.95, R_F 0.80). The lactone was distinguished by spraying with hydroxylamine–ferric chloride[16].

Analysis of the reduced, acetylated hydrolyzate by g.l.c. on column 1, programmed from 160° at 1°/min (He flow, 43 mL/min), confirmed the presence of glycerol (5.2 min), erythritol (16.4 min; m.p., and mixed m.p. with erythritol tetraacetate, 80–82°), rhamnose (29.2 min), galactose (60.8 min; m.p., and mixed m.p. with galactitol hexaacetate, 160–162°), and glucose (64.1 min; m.p., and mixed m.p. with D-glucitol hexaacetate, 98–99°) in ratios of 0.4:1:1:1:1.

Degradation of uronic acid of methylated polysaccharide. — A carefully dried sample (116 mg) of methylated *Klebsiella* K18 polysaccharide was dissolved in a solution of *p*-toluenesulfonic acid (3 mg) in dimethyl sulfoxide (19 mL) and 2,2-dimethoxypropane (1 mL). The solution was stirred for 4 h at room temperature, and then 2M methylsulfinyl anion (15 mL) was added; stirring was continued under nitrogen overnight. Sufficient 50% aqueous acetic acid was added to lower the pH to 6.0, and the solution was diluted to 100 mL with water, and extracted with chloroform (3 × 25 mL). The extracts were combined, washed with water (3 × 25 mL), and evaporated to a syrup; this was treated with 50% aqueous acetic acid for 12 h at 100°. The acetic acid was removed under diminished pressure, and a solution of the residue in methanol was treated with decolorizing charcoal, and evaporated, to give a honey-colored syrup. This material was dialyzed against tap-water overnight, and lyophilization then gave 53 mg of the degraded material.

The degraded polymer was ethylated by use of ethyl iodide and silver oxide. Subsequent hydrolysis, reduction, and acetylation gave a mixture of partially etherified alditol acetates that was examined by g.l.c.–m.s. (column 3). The alditol acetates corresponding to 2-*O*-ethyl-3,4-di-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose, 3-*O*-ethyl-4,6-di-*O*-di-*O*-methylglucose, 2,4,6-tri-*O*-methylgalactose, and 2,6-di-*O*-methylglucose were identified.

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