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STRUCTURAL INVESTIGATION OF *Klebsiella* SEROTYPE K70 POLYSACCHARIDE

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ABSTRACT

By using the techniques of methylation analysis, uronic acid degradation, partial hydrolysis, and periodate oxidation, the structure of the capsular polysaccharide from *Klebsiella* serotype K70 has been investigated. Nuclear magnetic resonance was used extensively to characterize fragments obtained as a result of the various degradation procedures. The existence of a linear, hexasaccharide repeating unit having a 1-carboxyethylidene group attached to a 2-linked α-L-rhamnosyl residue in every second repeating unit has been demonstrated.

INTRODUCTION

Of the 81 different *Klebsiella* serotypes, the structural analyses of ~30 of the capsular polysaccharides produced by these bacteria have been reported. *Klebsiella* K70 polysaccharide has been shown to contain residues of glucuronic acid, galactose, glucose, and rhamnose, and it is one of 11 serologically different, K-types having this same qualitative composition[1,2]. We now report the results of our structural investigation of this polysaccharide.

RESULTS AND DISCUSSION

The polysaccharide, isolated as previously described[3], had $[\alpha]_D$ +43° (*c* 2.8, water). Proton magnetic resonance (^{1}H-n.m.r.) spectroscopy indicated the presence of one carboxyethylidene group per 12 sugar residues[4,5]. In the anomeric region (τ 4.5–5.5), six proton signals were observed, and a nine-proton doublet at τ 8.7, due to the methyl groups of 6-deoxy sugars, was also apparent (see Table I). Carbon-13 magnetic resonance (^{13}C-n.m.r.) spectroscopic information[6] was in agreement with the ^{1}H-n.m.r. data, and, in addition, indicated the presence of two hexoses (two signals were observed between 60 and 62 p.p.m., indicative of the signals from C-6 of two hexoses). The ^{1}H- and ^{13}C-n.m.r. spectra therefore indicate the presence

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TABLE I

N.M.R. DATA FOR *Klebsiella* K70 POLYSACCHARIDE AND OLIGOSACCHARIDES ISOLATED[a]

Compound	τ[b]	$J_{1,2}$[c]	^1H-*n.m.r. data*		^{13}C-*n.m.r. data*	
		(Hz)	*Integral*	*Assignment*[d]	*p.p.m.*[e]	*Assignment*[f]
GlcA$\frac{1\,4}{\beta}$Rha-OH	4.89	1.8	0.6 H	α-Rha-OH	103.70	β-GlcA
	5.14	s	0.4 H	β-Rha-OH	94.55	α-Rha-OH
	5.28	8	1 H	β-GlcA	93.23	β-Rha-OH
1	8.70	6 ($J_{5,6}$)	3 H	CH_3 of Rha	17.82	CH_3 of Rha
Glc$\frac{1\,3}{\alpha}$Gal$\frac{1\,2}{\beta}$Rha-OH	4.59	1.8	0.6 H	α-Rha-OH	105.51 }	β-Gal[g]
	4.85	3.5	1 H	α-Glc	104.77 }	
	5.13	s	0.4 H	β-Rha-OH	96.33	α-Glc
2	5.34	7*	1 H	β-Gal	93.92	α-Rha-OH
	8.70	6 ($J_{5,6}$)	3 H	CH_3 of Rha	93.59	β-Rha-OH
					17.56	CH_3 of Rha
Gal$\frac{1\,2}{\beta}$Rha$\frac{1\,3}{\alpha}$erythritol	4.78	1.8	1 H	α-Rha	(16 signals overall)	
	5.43	7.5	1 H	β-Gal	105.75	β-Gal
	8.70	6 ($J_{5,6}$)	3 H	CH_3 of Rha	100.19	α-Rha
5					63.19	C-6 of Gal
					61.77	C-1 of erythritol
					61.74	C-4 of erythritol
					17.35	CH_3 of Rha
Gal$\frac{1\,2}{\beta}$glycerol	5.45	7.5		β-Gal	(9 signals overall)	
					103.33	β-Gal
					62.44 }	C-6 of Gal
					61.90 }	C-1, C-3 of glycerol
6					61.78 }	

TABLE I *(continued)*

Compound	τ^b	$J_{1,2}$[c]	*^{1}H-n.m.r. data*		*^{13}C-n.m.r. data*	
		(Hz)	*Integral*	*Assignment*[d]	*p.p.m.*[e]	*Assignment*[f]
Native K70 polysaccharide	4.78	s*	1 H	α-Rha	105.7	six unassigned, anomeric, ^{13}C signals
	4.90	s*	2 H	α-Rha	103.8	
				α-Rha		
	5.03	s*	1 H	α-Glc	102.9	
	5.23	7	1 H	β-GlcA	101.7	
	5.45	7*	1 H	β-Gal	100.9	
	8.41	s	1.5 H	CH_3 of acetal	95.7	
	8.70	6 ($J_{5,6}$)	9 H	CH_3 of Rha	62.20	C-6 of Gal
					61.30	C-6 of Glc
					17.50	CH_3's of Rha's

[a]For origin of oligosaccharides **1**, **2**, **5**, and **6** see text. [b]Chemical shift relative to internal acetone; τ 7.77 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D. S. S.). [c] s = singlet. Those values marked with an asterisk were broad signals. [d]For example, α-Rha = proton on C-1 of α-linked L-Rha residue (Gal = D-Gal). [e]Chemical shift in p.p.m. downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. from D.S.S. [f]As for [d], but for anomeric ^{13}C nuclei. [g]Two values are given for ^{13}C-1 of β-Gal, as the chemical shift of this carbon atom is affected by α,β equilibrium of the reducing Rha residue, 105.51 = β-Gal(α-Rha), 104.77 = β-Gal(β-Rha).

of three rhamnose residues and two hexose residues. Knowing the qualitative composition of K70 capsular polysaccharide, it may be deduced that the remaining sugar residue in the repeating unit must be that of glucuronic acid.

Hydrolysis of K70 polysaccharide, and paper chromatography of the hydrolyzate, indicated the presence of glucose, glucuronic acid, galactose, and rhamnose. Progressive hydrolysis of the polysaccharide, monitored by paper chromatography, did not show the favored release of any one sugar, and this indicated that the K70 polysaccharide is probably a linear polysaccharide. Methanolysis of *Klebsiella* K70 polysaccharide, reduction of the product with sodium borohydride in dry methanol, and then hydrolysis of the product, yielded a mixture of sugars that was shown by paper chromatography to contain only glucose, galactose, and rhamnose. Reduction and acetylation, to convert this mixture into alditol acetates, and analysis by gas–liquid chromatography (g.l.c.), confirmed the presence of these three sugars in the proportions 31:17:52. This result indicated that the K70 polysaccharide contains glucose, glucuronic acid, galactose, and rhamnose residues in the respective ratios of 1:1:1:3, and that the polysaccharide consists of a hexasaccharide repeating unit. The glucose and rhamnose were shown to be respectively of the D and L configuration by the circular dichroism[7] curves of the corresponding alditol acetates. The configuration of the galactose was shown to be D by the circular dichroism curve of the 2,4,6-tri-*O*-methyl derivative obtained from methylation analysis.

Methylation analysis[8,9] of the native polysaccharide and of a sample of K70 polysaccharide that had been autohydrolyzed at pH 2.2 for 16 h confirmed the existence of a hexasaccharide repeating unit (see Table II). The concomitant increase of 3,4-di-*O*-methyl-L-rhamnose and loss of L-rhamnose after the 1-carboxyethylidene group had been removed, located the acetal on a 2-linked L-rhamnosyl residue, and confirmed the existence of this substituent on every second repeating unit of *Klebsiella* K70 polysaccharide.

TABLE II

METHYLATION ANALYSES OF NATIVE AND DEACETALATED[a] *Klebsiella* K70 CAPSULAR POLYSACCHARIDE

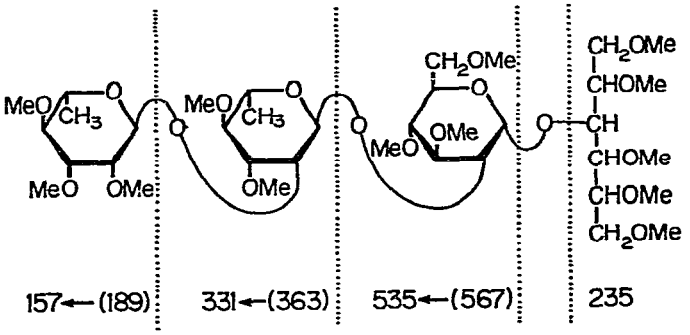
Methylated sugars[b] *(as alditol acetates)*	*T*[c]	*Mole %*[d]	
		I	II
3,4-Rha	0.89	22.6	33.1
2,3-Rha	1.00	17.0	16.9
3,4,6-Glc	1.50	17.2	17.3
Rha	1.58	10.2	
2,4,6-Gal	1.67	16.6	17.3
2,3-Glc (from D-GlcA)	2.40	16.3	15.3

[a]Deacetalated = K70 polysaccharide autohydrolyzed at pH 2.2 for 16 h at 95°. [b]3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol, etc. [c]Retention time of partially methylated alditol acetates, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on a column of 3% of HIEFF 1B on Gas-Chrom Q (100–120 mesh) programmed at 160° for 8 min, and then 2° per min to 190°. [d]I, native polysaccharide; II, deacetalated polysaccharide.

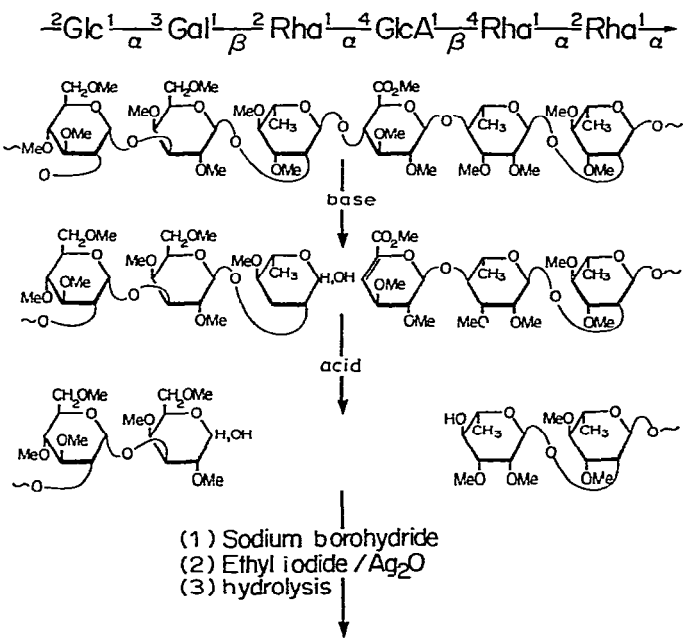
Partial, acid hydrolysis of K70 polysaccharide was performed with 0.5M trifluoroacetic acid for 45 min at 95°. Following separation of acidic and neutral material by ion-exchange chromatography, each fraction was analyzed by using a gel-permeation column of Bio-Gel P-4. From the acidic fraction, a pure oligomer (**1**), $[\alpha]_D$ −30°, was isolated[10]. The ^{1}H- and ^{13}C-n.m.r. spectra were in agreement with formulation of **1** as an aldobiouronic acid (see Table I). Compound **1** was reduced with lithium borodeuteride, and the product methylated. Subsequent reduction with lithium aluminum hydride, hydrolysis, and g.l.c.–m.s.[9,11] analysis, as alditol acetates, of the partially methylated monosaccharides liberated gave components confirming the structure of **1** as being β-D-GlcA*p*-(1 → 4)-L-Rha*p*.

The neutral material obtained from the partial hydrolysis was also separated on a column of Bio-Gel P-4. Oligomer **2**, $[\alpha]_D$ +10°, was isolated, and from the ^{1}H- and ^{13}C-n.m.r. spectra (see Table I) seemed to be a trisaccharide. The ^{13}C-n.m.r. spectrum of **2** was of particular interest, in that the (terminal) reducing sugar residue (easily recognizable in ^{1}H- and ^{13}C-n.m.r. spectra as a rhamnose residue) affected the chemical shift of the nonterminal (middle) anomeric carbon atom. Hence, the C-1 signals from the (reducing) rhamnose residue at 93.92 (α) and 93.59 p.p.m. (β) influenced the anomeric carbon atom of the (middle) glycosyl residue, so that it gave two signals, at 105.51 and 104.77 p.p.m. The chemical shift (~105 p.p.m.) of this double signal allowed it to be assigned to a β-hexose. Reduction of **2** with lithium borodeuteride, and subsequent methylation, yielded a product which was hydrolyzed; the product mixture was reduced, acetylated, and analyzed by g.l.c.–m.s.[9,11]. Identification of the three components observed (see Experimental section) established **2** as being α-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 2)-L-Rha*p*.

A sample of *Klebsiella* K70 polysaccharide from which the 1-carboxyethylidene group had been selectively removed by autohydrolysis was permethylated, and the product subjected to a uronic acid degradation[12]. This technique can provide valuable information in sequencing a linear polysaccharide, even though the β-elimination reactions may not proceed to completion. After treatment with methylsulfinyl anion in dimethyl sulfoxide, and subsequent, mild treatment with acid to cleave the enol ethers formed during the elimination reactions, the mixture of products was reduced with sodium borohydride in dry methanol. Methylation of this reduced mixture with Purdie's reagents, and chromatography of the product on silica gel afforded a component, **3a**, whose ^{1}H-n.m.r. spectrum had signals at τ 4.88 (2 H, $J_{1,2}$ broad) and 5.08 (1 H, $J_{1,2}$ 3 Hz) in the anomeric region. The electron-impact (e.i.) mass spectrum was in agreement with that expected for a trisaccharide alditol[11]. The origin of some pertinent fragments is outlined. It is, perhaps, noteworthy that, besides the fragments aA_1, baA_1, and $cbaA_1$, by which the sequence of **3a** can be determined, the main fragments arise from the cJ_1, bcJ_1, and $abcJ_1$ ions. The latter fragments are in agreement with the interglycoside linkages shown in Scheme A. Hydrolysis of **3a**, and analysis of the products by g.l.c.–m.s.[9,11] of the alditol acetates gave peaks corresponding to 1,2,4,5,6-penta-*O*-methylgalactitol, 2,3,4-tri-*O*-methylrhamnose, 3,4-di-*O*-methylrhamnose, and 3,4,6-tri-*O*-methylglucose.



Scheme A



4-*O*-ethyl-2,3-di-*O*-methyl-L-rhamnose +
3,4-di-*O*-methyl-L-rhamnose +
3,4,6-tri-*O*-methyl-D-glucose +
1,5-di-*O*-ethyl-2,4,6-tri-*O*-methyl-D-galactitol

Scheme B

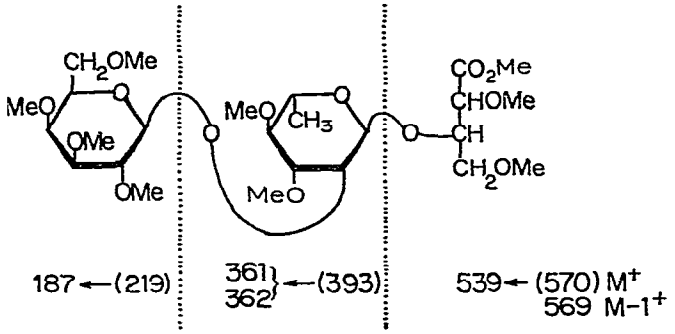
Ethylation of the material obtained by reduction of the uronic acid degradation product with sodium borohydride gave a component, **3b**, and analysis of this material as described for the permethylated product **3a** gave 1,5-di-*O*-ethyl-2,3,6-tri-*O*-methyl-galactitol, 4-*O*-ethyl-2,3-di-*O*-methylrhamnose, 3,4-di-*O*-methylrhamnose, and 3,4,6-

tri-*O*-methylglucose. Bearing in mind that the sequence in compound **2** (obtained by partial hydrolysis) was already known, it was possible to write the structure of **3a** as the permethylated derivative of α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → 2)-α-D-Glc*p*-(1 → 3)-D-galactitol. The 4-*O*-ethyl-2,3-di-*O*-methyl-L-rhamnose obtained from **3b** indicates that the terminal rhamnosyl group is linked through O-4 in the polysaccharide. The sequence of reactions in the β-elimination of K70 polysaccharide is outlined in Scheme B.

The results obtained from partial hydrolysis and uronic acid degradation studies are sufficient to establish the repeating unit of K70 polysaccharide as being → 4)-β-D-GlcA*p*-(1 → 4)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → 2)-α-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 2)-α-L-Rha*p*(1 → . The 1-carboxyethylidene group, shown by methylation analysis and ^{1}H-n.m.r. spectroscopy of K70 polysaccharide to occur on only 50% of the repeating units, is present as an acetal spanning O-3 and O-4 of a 2-linked rhamnosyl residue. The presence of two 2-linked rhamnosyl residues in the repeating unit made it necessary to locate the acetal more precisely; this was done by using periodate oxidation[13].

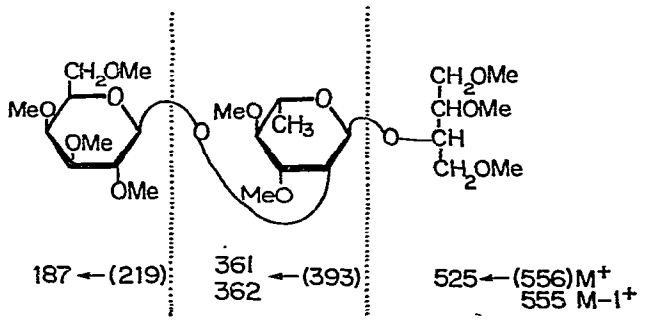
When *Klebsiella* K70 polysaccharide was treated with sodium periodate[14], a plateau value was obtained corresponding to the consumption of six molecules of oxidant per true repeating unit (taken as twelve sugar residues), a value three molecules less than the theoretical value. However, after reduction of the polyaldehyde product, dialysis, and re-treatment with the periodate solution, a further 4.2 molecules of periodate were consumed, and this value rose to 5 molecules after 156 h. These results indicate that hemiacetal formation[15] occurs during the initial treatment with periodate. By ^{1}H-n.m.r. spectroscopy, it was shown that the twice periodate-oxidized polyalcohol contained only a trace of the 1-carboxyethylidene acetal, and it was assumed that this had been cleaved by hydrolysis at the reaction pH of 4.2. Another sample of *Klebsiella* K70 polysaccharide was subjected to oxidation in a buffered solution (pH 5.0) of periodate, and, after 24 h, the product was reduced with sodium borohydride, and the resulting polyalcohol was re-oxidized under the same conditions. The polyalcohol derived from this sequence of reactions was then methylated, the product reduced with lithium aluminum hydride, and hydrolyzed, and the partially methylated sugars were analyzed, as their alditol acetates, by g.l.c.–m.s.[9,11]. Only 1,2,3,4,5-penta-*O*-acetylrhamnitol and 1,3,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol were found to be present (in the ratio of 1:2). This result indicated that the acetal was still present on the doubly oxidized polymer, and that complete periodate oxidation had been achieved. The polyalcohol from the fully oxidized polymer was then subjected to a Smith hydrolysis[16], and the products reduced with sodium borohydride. The mixture of products was separated by gel-permeation chromatography, giving three pure oligomers; **4**, **5**, and **6**. Compound **4** had $[\alpha]_D$ −16°, and ^{1}H-n.m.r. spectroscopy (see Table I) revealed signals attributable to anomeric protons at τ 4.72 (1 H, $J_{1,2}$ 1.8 Hz) and 5.43 (1 H, $J_{1,2}$ 7.5 Hz). After hydrolysis of **4**, paper chromatography showed the presence of galactose, rhamnose, and two other components (indistinguishable from erythronic acid and erythronolactone). Reduction

of **4** with sodium borohydride gave an oligomer (which was chromatographically indistinguishable from oligomer **5**) and some unchanged starting-material. Methylation of **4** gave a product that had in its i.r. spectrum a strong absorption at 1750 cm^{-1}. The mass spectrum of permethylated **4**, using the chemical ionization (c.i.) mode[17], was consistent with the structure given. The source of some pertinent fragments is illustrated in Scheme C. Oligomer **4** is, therefore, β-D-Gal*p*-(1 → 2)-α-L-Rha*p*-(1 → 3)-L-erythronic acid.



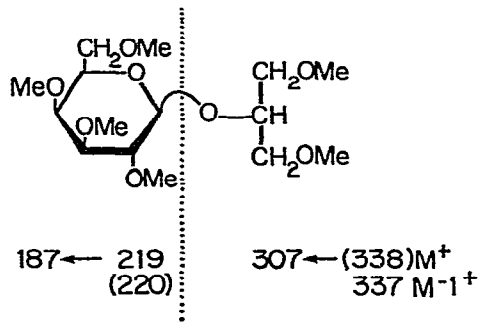
Scheme C

Compound **5** had $[\alpha]_D$ +4.5°, and its ^{1}H-n.m.r. spectrum showed signals at τ 4.78 (1 H, $J_{1,2}$ 2 Hz) and 5.43 (1 H, $J_{1,2}$ 7.5 Hz) in the anomeric region. In the ^{13}C-n.m.r. spectrum, 16 signals overall were observed; two signals occur in the anomeric region at 105.75 and 100.19 p.p.m., and three in the region (60–63 p.p.m.) associated with –CH_2OH groups (see Table I). After hydrolysis of **5**, paper chromatography showed the presence of rhamnose, galactose, and erythritol. Methylation of **5** yielded a product that, on hydrolysis, and conversion of the products into alditol acetates, gave components corresponding to 2,3,4,6-tetra-*O*-methylgalactose and 3,4-di-*O*-methylrhamnose. The c.i. mass spectrum of permethylated **5** was consistent with the structure shown, for which some pertinent fragments are indicated in Scheme D. Compound **5** is, therefore, established as being β-D-Gal*p*-(1 → 2)-α-L-Rha*p*-(1 → 2)-D-erythritol.



Scheme D

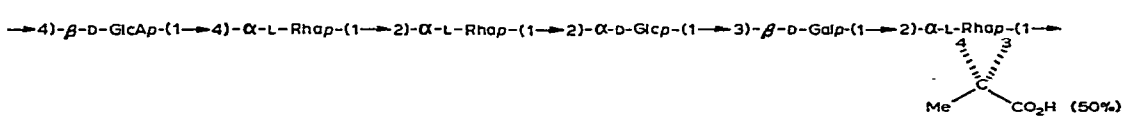
Compound **6**, $[\alpha]_D$ +3.7°, was shown by ^{13}C-n.m.r. spectroscopy to contain nine carbon atoms overall. In the anomeric region, one signal at 103.33 p.p.m. was apparent, and three signals were observed between 60 and 63 p.p.m. In the ^{1}H-n.m.r. spectrum, only one signal, at τ 5.45 (1 H, $J_{1,2}$ 7.5 Hz) was apparent in the anomeric region (see Table I). After hydrolysis of **6**, paper chromatography showed the presence of galactose and glycerol. Methylation of **6**, and subsequent hydrolysis, gave a component corresponding to 2,3,4,6-tetra-*O*-methyl-D-galactose (as its alditol acetate in g.l.c.–m.s.). The c.i.-mass spectrum of permethylated **6** was consistent with the structure shown (Scheme E), in which some pertinent fragments are indicated. Oligomer **6** is thus established as being β-D-Gal*p*-(1 → 2)-glycerol.



Scheme E

The isolation of oligomers **4**, **5**, and **6** is compatible with the linear sequence of K70 polysaccharide, shown earlier. Compound **5** results from the spontaneous lactonization of periodate-oxidized D-glucuronic acid residues during the double oxidation-reduction procedure used, and the subsequent, partial reduction of this lactone with sodium borohydride results in the reduction of the carboxyl group to a primary alcohol group. Some of the D-glucuronic acid residues give rise to erythronic acid, and some, to erythritol, each of which is found as a terminating glycon, in **4** and **5**, respectively. The survival of the 2-linked rhamnose throughout the periodate oxidation indicates that the 1-carboxyethylidene group remains attached to this sugar residue.

The evidence presented for *Klebsiella* K70 polysaccharide is consistent with the structure shown. Because of the good resolution obtained in ^{1}H- and ^{13}C-n.m.r. spectroscopic studies of K70 polysaccharide, it is probable that the 1-carboxyethylidene acetal is distributed evenly throughout the polysaccharide, and is therefore present on every second repeating unit.



EXPERIMENTAL

General methods. — The instrumentation used has been described[3]. For de-

scending, paper chromatography, the following solvent systems (v/v) were used: (*A*) freshly prepared 2:1:1 1-butanol–acetic acid–water, and (*B*) 4:1:1 ethyl acetate–pyridine–water. Analytical, g.l.c. separations were performed in stainless-steel columns (1.83 m × 3.17 mm) with a carrier-gas flow-rate of 20 mL/min. Columns used were (1) 0.2% of poly(ethylene glycol succinate), 0.2% of poly(ethylene glycol adipate), and 0.4% of XF 1150 on Gas Chrom Q (100–120 mesh); and (2) 3% of HIEFF 1B on the same support. Analogous columns (1.8 m × 6.3 mm) were used for preparative, g.l.c. separation.

Preparation and properties of Klebsiella K70 *capsular polysaccharide.* — A culture of *Klebsiella* K70, obtained by courtesy of Dr. I. Ørskov, was grown and isolated as described[3] for *Klebsiella* K36. The polysaccharide had $[\alpha]_D$ −43° (*c* 2.8, water). The ^{1}H-n.m.r. spectrum of K70 polysaccharide was recorded for a solution, 20 mg/mL of D_2O at ~90°, and the ^{13}C-n.m.r. spectrum for a solution, 150 mg/mL of 1:1 H_2O–D_2O, at 40°.

Sugar analysis. — Analysis was performed as described[3], *viz.*, by methanolysis, reduction with sodium borohydride in anhydrous methanol, hydrolysis, reduction, and acetylation. The alditol acetates derived from rhamnose, galactose, and glucose were separated by g.l.c. (column 1, programmed from 120 to 190° at 1°/min), and found to be present in the ratios of 31:17:52. Preparative g.l.c. gave galactitol hexacetate (m.p. 166–168°) and glucitol hexaacetate (m.p. 99°). Circular dichroism (c.d.) of the rhamnitol pentaacetate showed ε_{213}^{MeCN} −1.52, and of the glucitol hexaacetate, ε_{213}^{MeCN} +1.83; by comparison with authentic standards, this confirmed the L and D configurations of the respective parent sugars. The configuration of the galactosyl residue was determined by examining the c.d. curve of the 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol obtained during methylation analysis. This component showed ε_{213}^{MeCN} +2.26, and, by comparison with an authentic standard, this established the galactose as being in the D configuration.

Methylation analysis[8,9]. — A sample of *Klebsiella* K70 polysaccharide that had previously been passed through a column of Amberlite IR-120 (H^+) ion-exchange resin was methylated by the Hakomori[8] procedure. Methylation was found to be incomplete, and Purdie treatment with silver oxide in methyl iodide was needed in order to give a product showing no absorbance at 3600 cm^{-1} in the i.r. spectrum. The ^{1}H-n.m.r. spectrum of this material showed a broad doublet at τ 8.76 (due to the CH_3 of rhamnose) which integrated as 9 protons, and a sharp singlet at τ 8.43 (due to the CH_3 of the 1-carboxyethylidene acetal); these two signals were in the ratio of 6:1. In the anomeric region, signals were observed at τ 4.81 (2 H, $J_{1,2}$ broad), 5.05 (2 H, $J_{1,2}$ 2 Hz), 5.27 (1 H, $J_{1,2}$ 7 Hz), and 5.52 (1 H, $J_{1,2}$ 7 Hz).

Reduction of a sample of methylated K70 polysaccharide with lithium aluminum hydride in refluxing tetrahydrofuran overnight yielded a product having no carbonyl absorption (1750 cm^{-1}) in its i.r. spectrum. Hydrolysis with 2M trifluoroacetic acid overnight at 95°, and subsequent reduction with sodium borohydride, followed by acetylation with acetic anhydride–pyridine, yielded a mixture of partially methylated alditol acetates. This mixture was analyzed[9,11] by g.l.c.–m.s. The alditol acetates of

3,4-di-*O*-methylrhamnose, 2,3-di-*O*-methylrhamnose, rhamnose, 3,4,6-tri-*O*-methylglucose, 2,4,6-tri-*O*-methylgalactose, and 2,3-di-*O*-methylglucose were identified in the ratios of 3:2:1:2:2:2 (see Table II for exact ratios, and for the g.l.c. column used.)

A sample of *Klebsiella* K70 polysaccharide that had been autohydrolyzed at pH 2.5 on a steambath for 16 h was methylated as described for the native K70 polysaccharide. A singlet at τ 8.43 was absent from the ^{1}H-n.m.r. spectrum. Reduction with lithium aluminum hydride, hydrolysis, reduction with sodium borohydride, and acetylation (as for the non-degraded, methylated material) yielded a mixture of partially methylated alditol acetates that was analyzed[9,11] by g.l.c.–m.s. The same components as those obtained from methylated, native K70 polysaccharide were identified, with the exception that no rhamnose was present. The ratios of the five components were 2:1:1:1:1 (see Table II for exact ratios, and for the g.l.c. column used.) The concomitant loss of the 1,2,3,4,5-tetra-*O*-acetylrhamnitol and gain in the proportion of 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol indicates that the 1-carboxyethylidene group was present in the native polysaccharide as an acetal spanning O-3 and O-4 of a 2-linked rhamnose residue.

Partial, acid hydrolysis. — Klebsiella K70 polysaccharide (500 mg) was hydrolyzed with 0.5M trifluoroacetic acid for 45 min at 95°. After removal of the acid by several successive evaporations with water, the material was dialyzed against tap water overnight. The nondialyzable material (250 mg) was lyophilized, and then resubjected to the same hydrolysis and dialysis. The dialyzable material (400 mg) from the hydrolyzates was then separated into neutral and acidic components by using Dowex-1 X2 (formate) ion-exchange resin.

The acid components were neutralized with sodium hydroxide, and applied to the top of a column (1.8 × 100 cm) of Bio-Gel P-4 which was then irrigated with water at a flow rate of 4 mL/h. Fractions (1–2 mL) were collected, lyophilized, and analyzed by paper chromatography. A major component (**1**, 50 mg), having R_{Glc} 1.0 (solvent *A*) and $[\alpha]_D$ −30° (*c* 1.3, water)[10], was obtained. The ^{1}H-n.m.r. spectrum of **1** showed signals, attributable to anomeric protons, at τ 5.28 (1 H, $J_{1,2}$ 8 Hz), 4.89 (0.6 H, $J_{1,2}$ 1.8 Hz), and 5.14 (0.4 H, s). A 6-Hz doublet (3 protons) at τ 8.70 was also apparent. This spectrum is consistent with formulation of **1** as an aldobiouronic acid having a (reducing) rhamnose residue. In the ^{13}C-n.m.r. spectrum, signals at 103.70 p.p.m. (nonreducing C-1) and at 94.55 and 94.23 p.p.m. (reducing C-1) were observed in the anomeric region. No signal attributable to C-6 of a hexose was observed, whereas a signal at 17.82 p.p.m. (CH_3 of an ω-deoxy sugar) was apparent.

Reduction of **1** with lithium borodeuteride, and subsequent methylation, hydrolysis (2M trifluoroacetic acid, overnight at 95°), reduction with sodium borohydride, and acetylation, yielded two components identified by g.l.c.–m.s. (Column 2) as 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol and 4-*O*-acetyl-1,2,3,5-tetra-*O*-methylrhamnitol. The latter component was monodeuterated at C-1. (Some of the tetramethylrhamnitol derivative was lost under diminished pressure during processing.)

The neutral oligomers from the partial hydrolysis of K70 polysaccharide were separated by gel chromatography on Bio-Gel P-4. Besides large proportions of monosaccharides, an oligomer (**2**, 12 mg), having R_{Glc} 0.50 (solvent *A*) and $[\alpha]_D$ +10° (*c* 1.2, water), was isolated. The ^{1}H-n.m.r. spectrum of **2** (D_2O, 90°) gave signals in the anomeric region as follows: τ 5.34 [1 H, $J_{1,2}$ 7 Hz (broad)], 4.85 (1 H, $J_{1,2}$ 3.5 Hz), 4.59 (0.6 H, $J_{1,2}$ 1.8 Hz), and 5.13 (s, 0.4 H). A doublet at τ 8.70 (6 Hz, integrating as three protons) was also observed. In the ^{13}C-n.m.r. spectrum, besides a signal at 17.56 p.p.m., attributable to the CH_3 of an ω-deoxy sugar, five signals assigned to anomeric carbon atoms were observed. Two signals from C-6 of hexoses were also apparent (see Discussion, and Table I, for the assignment of the signals in the anomeric region).

Compound **2** was reduced with lithium borodeuteride, and the product methylated. Hydrolysis with 2M trifluoroacetic acid, reduction with sodium borohydride, and acetylation, yielded alditol acetates corresponding to 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose, together with 2-*O*-acetyl-1,3,4,5-tetra-*O*-methylrhamnitol. G.l.c. and g.l.c.–m.s. were performed with column 2. Some 50% of the tetra-*O*-methylrhamnitol derivative, monodeuterated at C-1, was lost under diminished pressure during processing.

Uronic acid degradation[12]. — A sample of *Klebsiella* K70 polysaccharide that had been autohydrolyzed at pH 2.2 for 16 h at 95° (to remove the 1-carboxyethylidene group) was methylated, and the product carefully dried. To a solution of this material (140 mg) in 19:1 dimethyl sulfoxide–2,2-dimethoxypropane (20 mL) was added *p*-toluenesulfonic acid (5 mg), and the mixture was stirred under nitrogen for 2 h. Methylsulfinyl anion (2M, 15 mL) was then added, and the mixture was stirred overnight. Acetic acid–water (1:1) was added to adjust the pH to 6.0, and the solution was extracted with chloroform (3 × 25 mL). The extracts were combined, washed with water (25 mL), and evaporated, and the residue was treated with 1:9 acetic acid–water for 1 h at 95°. Removal of the acetic acid was achieved by lyophilization. The degraded material was then reduced overnight with sodium borohydride in dry methanol, to give component **3**, and this reduction product was divided into two equal portions. (*a*) One portion was methylated with methyl iodide and silver oxide. Purification on silica gel (ethyl acetate) yielded component **3a** (7 mg); R_F 0.15 (ethyl acetate); ^{1}H-n.m.r. ($CDCl_3$): signals in the anomeric region at τ 4.88 (2 H, $J_{1,2}$ broad) and 5.08 (1 H, $J_{1,2}$ 3 Hz). Two doublets (6 Hz each) at τ 8.72 and 8.76, each integrating as three protons, were also apparent. Electron-impact m.s. of **3a** showed, *inter alia*, the following peaks: *m/e* 88(45), 101(28), 157(29), 189(100), 235(26), 295(19), 331(9), 363(9), 499(4), 535(3), 567(2), and 673(4). Hydrolysis of **3a** (2M trifluoroacetic acid for 6 h at 90°) and g.l.c.–m.s. analysis of the alditol acetate derivatives of the partially methylated sugars gave peaks corresponding to 1,2,4,5,6-penta-*O*-methylgalactitol, 2,3,4-tri-*O*-methylrhamnose, 3,4-di-*O*-methylrhamnose, and 3,4,6-tri-*O*-methylglucose. (*b*) The second portion of **3** was ethylated with ethyl iodide and silver oxide, and, following purification on silica gel (ethyl acetate), yielded component **3b** (6.2 mg), R_F 0.19 (ethyl acetate). Hydrolysis of **3b**, and analysis

(as alditol acetates) by g.l.c.–m.s., gave peaks corresponding to 4-*O*-ethyl-2,3-di-*O*-methylrhamnose, 1,5-di-*O*-ethyl-2,4,6-tri-*O*-methylgalactitol, 3,4-di-*O*-methylrhamnose, and 3,4,6-tri-*O*-methylglucose.

Periodate oxidation of K70 polysaccharide. — A sample (400 mg) of the native polysaccharide was dissolved in a solution (100 mL) of $NaIO_4$ (0.05M) and $NaClO_4$ (0.2M), pH 4.2. The reaction was allowed to proceed at 4° in the dark, and the periodate consumption was monitored by removing 5-mL aliquots, which were analyzed by the Müller–Friedberger method[18]. After 26 h, periodate consumption reached a plateau value of 6 molecules per repeating unit of *Klebsiella* K70 polysaccharide (theoretical; 9 molecules). Following the addition of ethylene glycol, dialysis, reduction with sodium borohydride, and lyophilization, the modified polysaccharide was subjected to a second oxidation as just described. After 20 h, a further 4.2 molecules of periodate per repeating unit of K70 polysaccharide had been consumed, and this value gradually increased to 5.0 molecules after 156 h. Conversion of this material into the polyalcohol (as just described) yielded a non-dialyzable polymer (250 mg) that, by ^{1}H-n.m.r. spectroscopy, contained no 1-carboxyethylidene acetal.

Periodate oxidation of K70 polysaccharide (1 g) was also performed with a solution of $NaIO_4$ (0.05M) plus $NaClO_4$ (0.2M), buffered at pH 5.0 with a sodium acetate buffer. After 24 h, the reaction was stopped by the addition of ethylene glycol, the mixture was dialyzed overnight against running tap-water, and the material was reduced with sodium borohydride. The excess of hydride was decomposed with acetic acid, and the pH of the solution was adjusted to 6.5. Dialysis and lyophilization yielded 950 mg of a polymeric material. Re-treatment with $NaIO_4$–$NaClO_4$ at pH 5.0 as just described, and subsequent processing, yielded 700 mg of a polymer considered to have undergone complete periodate oxidation.

A sample of the periodate-oxidized material was methylated by the Hakomori procedure, and the product was reduced overnight with lithium aluminum hydride in refluxing tetrahydrofuran. Subsequent hydrolysis (2M trifluoroacetic acid, overnight at 95°), reduction, and acetylation, yielded a mixture of alditol acetates that was analyzed by g.l.c.–m.s. (Column 1). The alditol acetates of rhamnose and 2,4,6-tri-*O*-methylgalactose were found to be present in the ratio of 1:2

The rest of the periodate-oxidized material was then subjected to Smith hydrolysis with 0.5M trifluoroacetic acid overnight at room temperature, and the product reduced with sodium borohydride. The mixture of products was applied to the top of a column (160 × 2.5 cm) of Bio-Gel P-2 which was irrigated with distilled water. Three pure oligomers (**4**, **5**, and **6**) were isolated. Compound **4** (60 mg), R_{Glc} 0.92 (solvent *A*), had $[\alpha]_D$ −16° (*c* 2.1, water). In the ^{1}H-n.m.r. spectrum, signals attributable to anomeric protons were observed at τ 4.78 (1 H, $J_{1,2}$ 1.8 Hz) and 5.43 (1 H, $J_{1,2}$ 7.5 Hz); see Table I. The ^{13}C-n.m.r. spectrum of **4** could not be obtained, owing to the very slow relaxation of the terminal, erythronic acid residue. After hydrolysis of **4** with 2M trifluoroacetic acid for 6 h at 95°, paper chromatography (solvent *A*) revealed the presence of rhamnose, galactose, and two further components

having the respective mobilities of erythronolactone and erythronic acid. Methylation of **4** gave a component that had a strong absorbance at 1750 cm^{-1} in its i.r. spectrum. The c.i. mass spectrum of permethylated **4** showed, *inter alia*, the following peaks: *m/e* 111(64), 155(17), 186(15), 187(99), 219(55), 303(17), 361(100), 362(96), 539(13), 568(2), 569(3.0), and 570(1.5). Reduction of permethylated **4** with sodium borohydride in dry methanol, and subsequent hydrolysis, reduction, and acetylation gave a mixture of alditol acetates that was analyzed by g.l.c.–m.s. The alditol acetates of 2,3,4,6-tetra-*O*-methylgalactose and 3,4-di-*O*-methylrhamnose, in equal amounts, were identified. (The very volatile di-*O*-methylerythritol derivative was lost under diminished pressure during processing.)

Oligomer **5** (60 mg), R_{Glc} 0.96 (solvent *A*), had $[\alpha]_D$ +4.5° (*c* 1.5, water). In its ^{1}H-n.m.r. spectrum (D_2O, 90°), signals were observed in the anomeric region at τ 4.78 (1 H, $J_{1,2}$ 2 Hz) and 5.43 (1 H, $J_{1,2}$ 7 Hz). A doublet (6 Hz) at τ 8.70, integrating as three protons, was also present. In the ^{13}C-n.m.r. spectrum, 16 signals overall were observed. Two signals (105.75 and 100.19 p.p.m.) were assigned as arising from anomeric carbon atoms, and three signals appeared in the region associated with $-CH_2OH$ groups (60–62 p.p.m.). The ^{13}C-n.m.r. spectrum is in agreement with that for an oligomer comprising a hexose, an ω-deoxy sugar, and a four-carbon aglycon. Hydrolysis of **5** with 2M trifluoroacetic acid for 4 h at 95° gave a solution containing galactose, rhamnose, and erythritol (paper chromatography, solvent *A*). Methylation of **5** yielded a product having R_F 0.23 (ethyl acetate). The c.i. mass spectrum of permethylated **5** showed, *inter alia*, the following peaks: *m/e* 71(65), 88(55), 101(49), 103(40), 112(100), 133(75), 145(39), 155(68), 163(75), 186(60), 220(99), 307(64), 337(30), and 338(6). Hydrolysis of permethylated **5** and subsequent derivatization yielded alditol acetates corresponding to 2,3,4,6-tetra-*O*-methylgalactose and 3,4-di-*O*-methylrhamnose. The very volatile tri-*O*-methylerythritol derivative was lost under vacuum during processing.

Compound **6** (20 mg), R_{Glc} 1.14 (solvent *A*), had $[\alpha]_D$ +3.7° (*c* 0.8, water). In its ^{1}H-n.m.r. spectrum (D_2O, 90°), only one signal, at τ 5.45 (1 H, $J_{1,2}$ 7.5 Hz), was observable in the anomeric region. In the ^{13}C-n.m.r. spectrum, nine signals were observed overall. The presence of one signal (103.33 p.p.m.) in the anomeric region, and three signals between 60 and 62 p.p.m., is in agreement with formulation of **6** as a hexose linked through C-1 to a three-carbon fragment. After hydrolysis of **6** with 2M trifluoroacetic acid for 4 h at 95°, paper chromatography (solvent *A*) showed the presence of galactose and glycerol. Methylation of **6** gave a compound having R_F 0.34 (ethyl acetate), and the c.i. mass spectrum of this component showed, *inter alia*, the following peaks: *m/e* 71(65), 75(24), 88(55), 101(49), 103(40), 112(100), 127(33), 133(75), 145(37), 155(68), 163(75), 186(60), 220(99), 307(64), and 337(30). Hydrolysis of this permethylated derivative yielded only 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactose (after derivatization as alditol acetates). The very volatile di-*O*-methylglycerol was lost during processing.

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