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STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K67*

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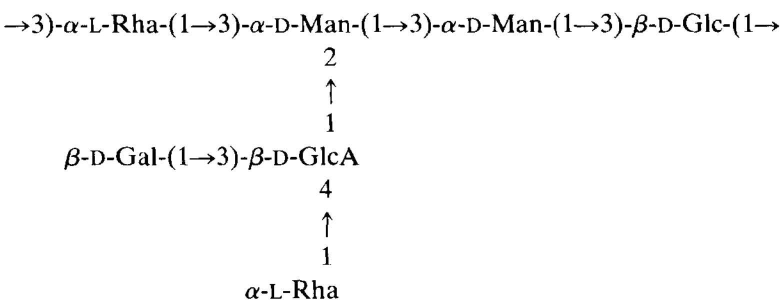
ABSTRACT

The determination of the structure of the capsular polysaccharide from *Klebsiella* K67 involved the use of methylation, periodate oxidation, partial hydrolysis, and β-elimination. The nature of the anomeric linkages was established by using ^{1}H- and ^{13}C-n.m.r. spectroscopy, and was further confirmed by chromic acid oxidation of the fully acetylated polysaccharide. The polysaccharide was found to have the heptasaccharide repeating unit shown. A structure having a branched side-chain is unique in this series of capsular polysaccharides.



$$\begin{array}{rcl}
\rightarrow 3)\text{-}\alpha\text{-L-Rha-}(1\rightarrow 3)\text{-}\alpha\text{-D-} & \text{Man} & \text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc-}(1\rightarrow \\
 & 2 & \\
 & \uparrow & \\
 & 1 & \\
\beta\text{-D-Gal-}(1\rightarrow 3)\text{-}\beta\text{-D-} & \text{GlcA} & \\
 & 4 & \\
 & \uparrow & \\
 & 1 & \\
\alpha\text{-L-} & \text{Rha} &
\end{array}$$

INTRODUCTION

Klebsiella serotype K67 is one of the two strains whose capsular polysaccharides are composed of D-glucuronic acid, D-galactose, D-glucose, D-mannose, and L-rhamnose. The other strain, *Klebsiella* K14, has a 1-carboxyethylidene disubstituent[1].

The heptasaccharide repeating-unit of K67 is unique in the series of *Klebsiella* capsular polysaccharide in the sense that the side-chain D-glucuronic acid is branched. Thus, the pattern may be considered to be a "four-plus-two-plus-one", instead of "four-plus-three" (which would imply a linear branch of three sugar residues).

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RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — Previously described methods were used in order to isolate and purify the polysaccharide[2–4]. The purified product obtained after Cetavlon precipitation was shown to be homogeneous by gel-permeation chromatography ($\bar{M}_w = 1.8 \times 10^6$). The ^{1}H-n.m.r. spectrum of the polysaccharide indicated the presence of seven anomeric protons, corresponding to 3 α, 3 β, and one borderline signal which was proved by chromic acid oxidation[5] to be an α linkage, resulting in 4 α and 3 β linkages. The ^{13}C-n.m.r. data also helped to confirm some of these results.

The presence of galactose, glucose, mannose, rhamnose, and glucuronic acid in the acid hydrolyzate of the polysaccharide was observed by paper chromatography. Determination of the neutral sugars as the alditol acetates, and as the peracetylated aldononitrile (PAAN) derivatives[6], gave rhamnose, mannose, glucose, and galactose in the ratios of 2.4:1.6:1.2:1.0. The carboxyl-reduced polysaccharide gave rhamnose, mannose, glucose, and galactose in the ratios of 2.1:2.0:1.6:1.0, indicating that the uronic acid is glucuronic acid. The glucose and mannose were proved to be of the D, and rhamnose to be of the L, configuration by circular dichroism measurements[7] made on the alditol acetates. Galactose was assigned the D configuration from circular dichroism measurements made on a partially methylated derivative.

Methylation analysis[8]. — Methylation of the K67 polysaccharide, followed by hydrolysis, conversion of the neutral sugars into alditol acetates, and g.l.c.–m.s. analysis thereof, gave the values shown in Table I, column I. Reduction of the methylated polysaccharide with lithium aluminum hydride, and analysis of the

TABLE I

METHYLATION ANALYSIS OF NATIVE, AND DEGRADED K67 POLYSACCHARIDE

Methylated sugars[a] *(as alditol acetates)*	T[b]			*Mole %*[c]		
	Column B[d] *(ECNSS-M)*	*Column* C[e] *(OV-225)*	*Column* D[e] *(SP1000)*	*I*[f]	*II*	*III*
2,3,4-Rha	0.44	0.51	0.52	23.4	12.8	16.1
2,4-Rha	0.99	0.93	1.00	17.7	14.8	11.6
2,3,4,6-Gal	1.20	1.13	1.14	21.9	15.6	18.8
2,4,6-Gal	1.92	1.63	1.71	15.0	15.0	12.2
2,4,6-Man	1.97	1.70	1.82	15.0	15.0	12.2
4,6-Man	3.20	2.53	2.58	7.0	14.4	14.4
2,6-Glc	3.52	2.94	2.69	—	—	14.4
2-Glc	8.58	5.04	4.92	—	13.1	

[a]2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, *etc*. [b]Relative retention-time referred to 2,3,4,6-Glc as 1.00. [c]Values are corrected by use of the effective, carbon-response factors given by Albersheim *et al.*[11] [d]Isothermal, 170°. [e]Isothermal, 220°. [f]I, Original capsular polysaccharide; II, compounds from $LiAlH_4$ reduction of methylated K67; III, compounds from remethylation of the reduced, methylated polymer.

product, gave a new peak due to the reduced uronic acid (see Table I, column II). Remethylation of the lithium aluminum hydride-reduced polymer, and analysis of the product, gave the values shown in Table I, column III. These results indicate that there are two branch points, one on the uronic acid and the other on one of the D-mannosyl residues.

β-Elimination (uronic acid degradation). — A sample of the methylated K67 polysaccharide was treated with dimethylsulfinyl anion overnight, the product treated with methyl iodide, and the ether dialyzed against running water. Analysis of the nondialyzable product showed the presence of three principal sugars: 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylglucose, and 2,4-di-*O*-methylrhamnose in the ratios of 1.9:1.0:1.0. Small amounts of a tri-*O*-methylrhamnose and a tetra-*O*-methylgalactose were also observed. This result indicates that the uronic acid is in the side chain, and that both a (terminal) rhamnosyl and a (terminal) galactosyl group are attached to the uronic acid. It also proves that the uronic acid is linked to O-2 of the D-mannosyl branch point.

Partial hydrolysis. — Partial hydrolysis of the native polysaccharide with acid was followed by separation of the acidic and neutral fractions by using ion-exchange chromatography. The neutral fraction contained monosaccharides only. The acidic fraction yielded four oligosaccharides (**A**$_2$, **A**$_3$, **A**$_4$, and **A**$_5$), which were separated by paper chromatography. The n.m.r.-spectral data for these oligosaccharides (see Table II), and methylation analysis (g.l.c.–m.s.) (see Table III) proved the structures to be as follows.

β-GlcA-(1→2)-Man
A$_2$
β-GlcA-(1→2)-α-Man-(1→3)-Man
A$_3$
β-GlcA-(1→2)-α-Man-(1→3)-α-Man-(1→3)-Glc
A$_4$
β-GlcA-(1→2)-α-Man-(1→3)-α-Man-(1→3)-β-Glc-(1→3)-Rha
A$_5$

Periodate oxidation. — The native polymer consumed four mol of periodate per repeating unit, which is consistent with the concept that only the two (terminal) rhamnosyl and galactosyl groups are oxidized. Hydrolysis under mild conditions (Smith degradation) yielded a polymeric material (**P1**) whose n.m.r. spectra and analysis demonstrated the loss of one rhamnosyl and one galactosyl unit. Polymer **P1** was methylated, and the product was divided into three portions. (*i*) Hydrolysis, and estimation of the resulting neutral sugars, showed the absence of terminal units of rhamnose and galactose, in comparison to the native polysaccharide, whereas the remaining sugars were unchanged, and present in approximately equimolar proportions. (*ii*) Reduction of methylated **P1** before hydrolysis gave an additional peak, identified as that of 2,3,4-tri-*O*-methylglucose, and (*iii*) reduction and re-

TABLE II

N.M.R. DATA FOR *Klebsiella* K67 POLYSACCHARIDE AND THE DERIVED OLIGOSACCHARIDES[a]

Carbohydrate	*¹H-N.m.r. data*				*¹³C-N.m.r. data*	
	Δ^b	$J_{1,2}$ *(Hz)*	*Integral proton*	*Assignment*[c]	*p.p.m.*[d]	*Assignment*[e]
$\text{GlcA}\xrightarrow[\beta]{1\ 2}\text{Man-OH}$ **A_2**	5.30	s	0.8	$\text{2-Man}\underset{\alpha}{\longrightarrow}\text{OH}$		
	4.97	2	0.2	$\text{2-Man}\underset{\beta}{\longrightarrow}\text{OH}$		—
	4.55	8	1.0	$\text{GlcA}\underset{\beta}{\longrightarrow}$		
$\text{GlcA}\xrightarrow[\beta]{1\ 2}\text{Man}\xrightarrow[\alpha]{1\ 3}\text{Man-OH}$ **A_3**	5.29	s	1.0	$\text{2-Man}\underset{\alpha}{\longrightarrow}$	102.6	$\text{GlcA}\longrightarrow$
	5.17	s	0.6	$\text{3-Man}\underset{\alpha}{\longrightarrow}\text{OH}$	100.6	$\text{2-Man}\underset{\alpha}{\longrightarrow}$
	4.91	2	0.4	$\text{3-Man}\underset{\beta}{\longrightarrow}\text{OH}$	97.8	$\text{3-Man}\underset{\beta}{\longrightarrow}\text{OH}$
	4.55	8	1.0	$\text{GlcA}\underset{\beta}{\longrightarrow}$	94.5	$\text{3-Man}\underset{\alpha}{\longrightarrow}\text{OH}$
$\text{GlcA}\xrightarrow[\beta]{1\ 2}\text{Man}\xrightarrow[\alpha]{1\ 3}\text{Man}\xrightarrow[\alpha]{1\ 3}\text{Glc-OH}$ **A_4**	5.27	2	1.4	$\text{2-Man}\underset{\alpha}{\longrightarrow}$ $\text{3-Glc}\underset{\alpha}{\longrightarrow}\text{OH}$	102.5	$\text{GlcA}\underset{\beta}{\longrightarrow}$

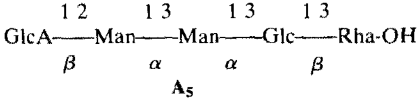
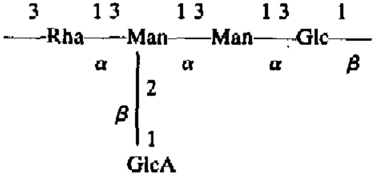
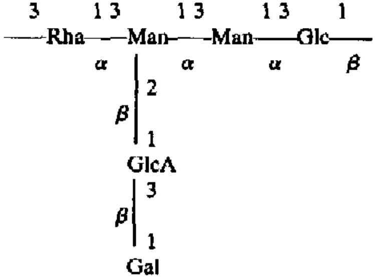
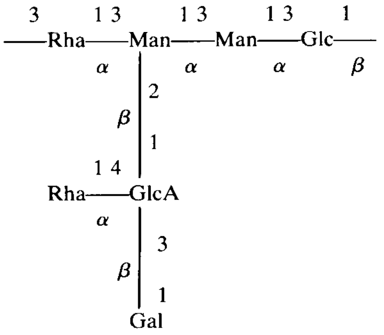
	5.23	s	1.0	3-Man$\underset{\alpha}{\text{——}}$	101.6 101.5	3-Man$\underset{\alpha}{\text{——}}$
	4.66	8	0.6	3-Glc$\underset{\beta}{\text{——}}$OH	100.8	2-Man$\underset{\alpha}{\text{——}}$
	4.57	8	1.0	GlcA$\underset{\beta}{\text{——}}$	96.7	3-Glc$\underset{\beta}{\text{——}}$OH
					93.0	3-Glc$\underset{\alpha}{\text{——}}$OH
 $\mathrm{GlcA}\underset{\beta}{\overset{1\ 2}{\text{——}}}\mathrm{Man}\underset{\alpha}{\overset{1\ 3}{\text{——}}}\underset{\mathbf{A_5}}{\mathrm{Man}}\underset{\alpha}{\overset{1\ 3}{\text{——}}}\mathrm{Glc}\underset{\beta}{\overset{1\ 3}{\text{——}}}\mathrm{Rha\text{-}OH}$	5.26	s	1.0	2-Man$\underset{\alpha}{\text{——}}$	102.3	GlcA$\underset{\beta}{\text{——}}$
	5.22	s	1.0	3-Man$\underset{\alpha}{\text{——}}$	101.5	3-Glc$\underset{\beta}{\text{——}}$
	5.03	2	0.4	3-Rha$\underset{\alpha}{\text{——}}$OH	101.45	3-Man$\underset{\alpha}{\phantom{\text{——}}}$
					101.42	2-Man$\underset{\alpha}{\text{——}}$
	4.90	2	0.4	3-Rha$\underset{\beta}{\text{——}}$OH		
					100.9	3-Rha$\underset{\beta}{\text{——}}$OH
	4.68	8	1.0	3-Glc$\underset{\beta}{\text{——}}$	95.9	3-Rha$\underset{\alpha}{\text{——}}$OH
	4.55	8	1.0	GlcA$\underset{\beta}{\text{——}}$	19.9	C-6 of Rha
	1.28	s	3.0	C-6 of Rha		

Table II (cont.)

Carbohydrate	^{1}H-N.m.r. data				^{13}C-N.m.r. data	
	Δ^b	$J_{1,2}$ (Hz)	Integral proton	Assignment^c	p.p.m.^{d}	Assignment^c
 **P$_1$**	5.33	1	1.0	2-Man$\underset{\alpha}{\text{——}}$		
	5.27	1	1.0	3-Man$\underset{\alpha}{\text{——}}$		
	5.10	1	1.0	3-Rha$\underset{\alpha}{\text{——}}$		
	4.73	8	1.0	3-Glc$\underset{\beta}{\text{——}}$		
	4.53	8	1.0	GlcA$\underset{\beta}{\text{——}}$		
	1.30	4	3.0	C-6 of Rha		
 **SH**	5.29	1	1.0	2-Man$\underset{\alpha}{\text{——}}$	104.7	3-GlcA$\underset{\beta}{\text{——}}$
	5.26	1	1.0	3-Man$\underset{\alpha}{\text{——}}$	103.7	3-Glc$\underset{\beta}{\text{——}}$
	5.04	2	1.0	3-Rha$\underset{\alpha}{\text{——}}$	103.1	3-Gal$\underset{\beta}{\text{——}}$
	4.86	8	1.0	Gal$\underset{\beta}{\text{——}}$	101.5	3-Man$\underset{\alpha}{\text{——}}$
	4.72	6	1.0	3-Glc$\underset{\beta}{\text{——}}$	101.1	2-Man$\underset{\alpha}{\text{——}}$
	4.54	8	1.0	3-GlcA$\underset{\beta}{\text{——}}$	99.6	3-Rha$\underset{\alpha}{\text{——}}$
	1.30	7	2.0	C-6 of Rha	17.5	C-6 of Rha

 **K67**	5.29	1	1.0	2-Man—— α	105.1	3-GlcA—— β
	5.26	1	1.0	3-Man—— α	105.0	3-Glc—— β
	5.03	1	1.0	3-Rha—— α	103.3	3-Gal—— β
	4.90	1	1.0	Rha—— α	101.6	3-Man—— α
	4.84	8	1.0	Gal—— β	101.2	2-Man—— α
	4.72	8	1.0	3-Glc—— β	99.5	3-Rha—— α
	4.50	8	1.0	3-GlcA—— β	96.2	Rha—— α
					17.4	C-6 of Rha

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[a]For the source of A_2, A_3, A_4, A_5, **P1**, and **SH**, see text. [b]Chemical shift relative to internal acetone; Δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). [c]The numerical prefix indicates the position at which the sugar is substituted; α or β, the configuration of the glycosidic bond, or the anomer in the case of a (terminal) reducing sugar residue. Thus, $\underset{\beta}{\text{3-Glc——}}$ refers to the anomeric proton of a 3-linked glucosyl residue in the β-anomeric configuration. The absence of a numerical prefix indicates a (terminal) nonreducing group. [d]Chemical shift in p.p.m. downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from DSS. [e]As in *c*, but for ^{13}C nuclei.

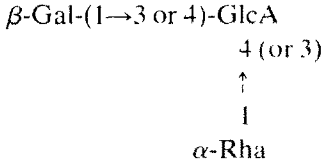
TABLE III

ANALYSIS OF THE OLIGOSACCHARIDES FROM PARTIAL HYDROLYSIS OF *Klebsiella* K67 POLYSACCHARIDE

Oligosaccharide	*Sugar analysis*	*As alditol acetates (molar proportions)*	*Methylation analysis*	*As alditol acetates (molar proportions)*
A$_2$	Man	1^a	2,3,4-Glc	1
	Glc(GlcA)	1	2,4,6-Man	1
A$_3$	Man	2^a	—	
	Glc(GlcA)	1		
A$_4$	Man	1.5^b	2,4,6-Man	1
	Glc	1.0	3,4,6-Man	1
			2,4,6-Glc	1
			2,3,4-Glc	1
A$_5$	Man	1.5^b	2,4,6-Man	1
	Glc	1.0	3,4,6-Man	1
	Rha	0.6	2,4,6-Glc	1
			2,4-Rha	0.8
			2,3,4-Glc	1

^{a}Including GlcA as Glc. ^{b}Neutral sugars only.

methylation gave 2,3,4,6-tetra-*O*-methylglucose (on columns *C* and *D*). These results indicated that both of the terminal sugars are linked to glucuronic acid, and they enabled a partial structure to be drawn, as follows.



$$\begin{array}{l}\beta\text{-Gal-}(1\rightarrow 3 \text{ or } 4)\text{-GlcA} \\ \qquad\qquad\qquad\quad 4\ (\text{or } 3) \\ \qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\quad 1 \\ \qquad\qquad\quad \alpha\text{-Rha}\end{array}$$

Two attempts to oxidize either the galactosyl or the rhamnosyl terminal group selectively[9] were unsuccessful. In the first experiment, no oxidation was observed at the end of 20 min, and, in the second, both sugars had been decomposed at the end of 30 min (with no further change up to 120 min).

Selective hydrolysis with acid. — Treatment of the polysaccharide with 0.1M trifluoroacetic acid (TFA) for 25 min at 95°, and dialysis against distilled water, afforded a nondialyzable, polymeric material and a dialyzate. The dialyzable material contained only rhamnose. Analysis of the polymeric material showed that 60% of the terminal rhamnosyl group had been removed, and methylation analysis yielded 2,4-di-*O*-methylglucose (from GlcA) or, after remethylation, 2,4,6-tri-*O*-methylglucose, establishing O-4 as the position of linkage of the rhamnose. N.m.r. data for the polymer (**SH**) are shown in Table II.

CONCLUSIONS

The structure of the capsular polysaccharide from *Klebsiella* serotype K67 is thus based on the heptasaccharide repeating-unit shown. The structure is unique in this series, but bears a superficial resemblance to the pattern of K46, where a 1-carboxyethylidene group is attached to a lateral, but nonterminal, mannosyl residue[3].

EXPERIMENTAL

General methods. — Paper chromatography was conducted by the descending method, using Whatman No. 1 paper and the following solvent systems (v/v): (*1*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, (*2*) 8:2:1 ethyl acetate–pyridine–water, (*3*) 2:1:1 1-butanol–acetic acid–water, and (*4*) 4:1:5 1-butanol–ethanol–water (upper phase). Chromatograms were developed with silver nitrate, or by spraying with *p*-anisidine hydrochloride in aqueous 1-butanol and heating the papers for 5–10 min at 110°. Preparative paper-chromatography was performed by the descending method, using Whatman No. 1 paper and solvents (*1*) and (*3*). Ion-exchange chromatography, for separation of neutral from acidic oligosaccharides, was performed in a column (3 × 28 cm) of Bio-Rad AG1-X2 (formate) resin (200–400 mesh). The neutral fraction was eluted with water, and the acidic, with 10% formic acid.

Analytical g.l.c. separations were performed with a Hewlett–Packard 5700 instrument fitted with dual flame-ionization detectors. Stainless-steel columns (1.8 m × 3 mm) were used, with a carrier-gas flow rate of 20 mL/min. The packing materials and conditions used were (*A*) 3% of SP-2340 on Supelcoport (100–120 mesh), programmed from 195° for 4 min, and then at 2°/min to 260°; (*B*) 5% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 170°, isothermal; (*C*) 3% of OV225 on Gas Chrom Q (100–120 mesh) at 220°, isothermal; and (*D*) 5% of SP1000 on Gas Chrom Q (100–120 mesh) at 220°, isothermal, unless stated otherwise.

G.l.c.–m.s. was performed with a V.G. Micromass 12 instrument fitted with a Watson–Biemann separator. Spectra were recorded at 70 eV, with an ionization current of 100 μA and an ion-source temperature of 200°.

Preparative g.l.c. was conducted with an F & M model 720 dual-column instrument fitted with thermal conductivity detectors. Stainless-steel columns (1.8 m × 6.3 mm) were used with a carrier-gas (helium) flow rate of 60 mL/min. The packings and conditions used were (*a*) 5% of SP2340 on Supelcoport (100–120 mesh) at 190°, and 4°/min to 260°; and (*b*) 5% of OV225 on Supelcoport (100–120 mesh) at 200°, and 4°/min to 260°.

I.r. spectra were recorded with a Perkin–Elmer model 457 spectrophotometer. Circular dichroism was measured with a Jasco J-20 automatic, recording spectropolarimeter.

^{1}H-N.m.r. spectra were recorded with a Bruker WH-400 instrument at 90 ±5°, or at ambient temperature. Acetone was used as the internal standard (δ

2.23), and all values are given relative to that of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Samples were prepared by dissolving in D_2O and freeze-drying the solutions 2–3 times. ^{13}C-N.m.r. spectra were recorded with the same instrument at ambient temperature. Samples were dissolved in D_2O, and acetone was used as the internal standard (31.07 p.p.m.).

Preparation and properties of K67 capsular polysaccharide. — A culture of *Klebsiella* K67, obtained from Dr. Ida Ørskov, Copenhagen, was grown as previously described[2–4], and the polysaccharide was purified by one precipitation with Cetavlon. The isolated polysaccharide (4.7 g) had $[\alpha]_D$ −17.3° (*c* 0.196, water). The average molecular weight was determined by gel chromatography (courtesy of Dr. S. C. Churms, University of Cape Town, South Africa) to be 1.8×10^6 N.m.r. spectroscopy (^1H and ^{13}C) was performed on the original K67 polysaccharide, but an improved p.m.r. spectrum was obtained at high, rather than at ambient, temperature. The principal signals and their assignments, for both the ^1H- and ^{13}C-n.m.r. spectra, are recorded in Table II.

Hydrolysis of the polysaccharide. — Hydrolysis of a sample (10 mg) of K67 polysaccharide with 2M trifluoroacetic acid (TFA) for 18 h at 95°, removal of the acid by successive evaporations with water, followed by paper chromatography (solvents *1* and *2*), showed mannose, galactose, glucose, glucuronic acid, and rhamnose. Neutral sugars were quantitatively determined by g.l.c. as their alditol acetates or PAAN derivatives. The uronic acid was reduced by refluxing a sample (20 mg) of K67 polysaccharide with 3% HCl in methanol (10 mL) overnight, neutralizing the HCl with $PbCO_3$, removing $PbCl_2$, treating the dried product with $NaBH_4$ (50 mg) in anhydrous methanol (10 mL), and stirring overnight. The excess of $NaBH_4$ was neutralized with Amberlite IR-120 (H^+) resin, and the boric acid, as methyl borate, was evaporated with methanol. The sample was then hydrolyzed, the alditol acetates were prepared, and those from rhamnose, mannose, galactose, and glucose were identified (in both cases) by g.l.c. (column *A*). Preparative g.l.c. (column *a*), followed by measurements of the circular dichroism spectra, showed[7] the mannitol hexaacetate and glucitol hexaacetate to be of the D configuration, and the rhamnitol pentaacetate to be of the L configuration.

Methylation analysis. — The polysaccharide (235 mg), converted into the free acid form by passing the sodium salt through a column of Amberlite IR-120 (H^+) resin, was dissolved in dry dimethyl sulfoxide (25 mL) and methylated by the Hakomori procedure[8]. The product (180 mg), recovered after dialysis against tap water, showed complete methylation (no hydroxyl absorption in the i.r. spectrum). A portion of this product (15 mg) was hydrolyzed with 2M trifluoroacetic acid, the sugars were reduced with sodium borohydride, and the alditols acetylated with 1:1 acetic anhydride–pyridine, and analyzed by g.l.c. in columns *B*, *C*, and *D* (see Table I, column I). G.l.c.–m.s. was conducted with column *D* 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol was isolated by preparative g.l.c. (column *b*), and found to give a positive c.d. curve, indicating that the galactose had the D configuration[7].

Another portion of the fully methylated polysaccharide (30 mg) was subjected to carboxyl reduction with lithium aluminum hydride in anhydrous oxolane. Half of the product was hydrolyzed with 2M TFA, and converted into the alditol acetates, and these were analyzed by g.l.c. in columns *B*, *C*, and *D* (see Table I, column II); column *D* was used for g.l.c.–m.s. The other half was remethylated by the Hakomori method[8], and the product converted into the alditol acetates as before. G.l.c. analysis was conducted in columns *B*, *C*, and *D* (see Table I, column III), and g.l.c.–m.s., in column *D*.

Partial hydrolysis. — A solution of the K67 polysaccharide (997 mg) in 0.5M trifluoroacetic acid (50 mL) was heated for 3 h on a steam bath. After removal of the acid by successive evaporations with water, the acidic and neutral fractions were separated on a column of Bio-Rad AG1-X2 ion-exchange resin. The acidic fraction (434 mg) was separated by preparative chromatography, using solvent *1*. The fractions A_3, A_4, and A_5 were repurified by the same procedure, but solvent *3* was used, in order to give better separation and to remove contaminants present.

The subsequent yields of pure fractions were 26 mg of aldobiouronic acid (A_2), 45 mg of aldotriouronic acid (A_3), 63 mg of aldotetraouronic acid (A_4), and 80 mg of an aldopentaouronic acid. Paper chromatography of the neutral fraction showed only galactose, glucose, mannose, and rhamnose, and there was no indication of the presence of oligosaccharides. This material was not examined further.

The analyses performed on the oligosaccharides were as follows. *(a) Sugar analysis.* (*i*) Acidic oligosaccharides (A_2 and A_3) were treated with 3% HCl in anhydrous methanol for 8 h on a steam bath. The methyl ester obtained was reduced with sodium borohydride in anhydrous methanol, followed by hydrolysis of the product with 2M TFA, reduction to the alditols, and acetylation with 1:1 acetic anhydride–pyridine. The alditol acetates obtained were analyzed by g.l.c. in column *A*. (*ii*) Acidic oligosaccharides (A_4 and A_5) were hydrolyzed with 2M TFA, the products reduced, the alditols acetylated, and the alditol acetates analyzed by g.l.c. in column *A*.

(b) Methylation analysis. All of the methylations were conducted by the method of Hakomori[8]. Portions of the methylated oligosaccharides were hydrolyzed with 2M TFA, the products converted into partially methylated alditol acetates, and these analyzed by g.l.c. and g.l.c.–m.s. in column *D*. The remainder was reduced with $LiAlH_4$ in anhydrous oxolane, hydrolyzed with 2M TFA, the products converted into alditol acetates, and these analyzed by g.l.c. and g.l.c.–m.s. in column *D*.

The results obtained for each oligosaccharide are given in Table III, and the n.m.r. data in Table II.

Periodate oxidation. — A solution of K67 polysaccharide (21.9 mg) in water (5.0 mL) was mixed with 0.03M $NaIO_4$ (5.0 mL), and stirred in the dark at room temperature (23°). After 95 h (consumption of periodate, 3.7 mol per mol of K67), ethylene glycol (0.2 mL) was added. The polyaldehyde was dialyzed overnight, reduced to the polyalcohol with $NaBH_4$ (200 mg), the base neutralized with 50% acetic acid, and the solution dialyzed, and freeze-dried. A fraction of the product (2 mg) was analyzed for the sugars present by treatment with 2M TFA overnight on a steam bath, and conversion into alditol acetates. Analysis by g.l.c. in column *A* showed rhamnitol, mannitol, and glucitol in the ratios of 1:2:1, and there was no galactose present. The rest of the material was treated with 0.5M TFA for 24 h at room temperature, and the acid was removed by repeated addition and evaporation of water. The n.m.r. data for this product (**P1**) are given in Table II. Permethylated **P1** was (*i*) analyzed for neutral sugars; (*ii*) reduced with $LiAlH_4$, and analyzed; and (*iii*) reduced, remethylated, and analyzed (columns *C* and *D*).

Selective, periodate oxidation[9]. — Two series of experiments were performed. In the first, K67 polysaccharide (216 mg) was oxidized with 0.02M $NaIO_4$ (20 mL), and aliquots (5 mL) were withdrawn at 5, 10, 15, and 20 min. In the second, polysaccharide (263 mg) was oxidized with 0.02M $NaIO_4$ (20 mL), and aliquots were withdrawn at 30, 60, 90, and 120 min. Each aliquot was added to ethylene glycol (0.4 mL), and analyzed as the alditol acetates, following dialysis, borohydride reduction, and hydrolysis.

Uronic acid degradation[10] — A sample (40 mg) of methylated K67 polysaccharide was dried, and dissolved in 19:1 dimethyl sulfoxide–2,2-dimethoxypropane (15 mL), together with a trace of *p*-toluenesulfonic acid. The flask was sealed under N_2, and to its contents was added dimethylsulfinyl anion (8 mL); the solution was kept overnight at room temperature, cooled, and methyl iodide (6 mL) was added. The excess of base was neutralized with 50% acetic acid, and the methylated, degraded product was isolated as the nondialyzable fraction after dialyzing for 2 d. G.l.c. analysis as the alditol acetates, after hydrolysis, was conducted in column *D*.

Chromic acid oxidation of the fully acetylated polysaccharide[5]. — To a solution of the original K67 polysaccharide (10 mg) in formamide (5.0 mL) were added acetic anhydride (1.5 mL) and pyridine (1.5 mL), and the mixture was stirred for 20 h. Following dialysis for 2 d, the nondialyzable material was freeze-dried, and the product dissolved in acetic anhydride (2.0 mL); CrO_3 (150 mg) was added, the solution was stirred for 2 h at 50°, and the product was isolated by partition between $CHCl_3$ and water. Hydrolysis and analysis (as alditol acetates) by g.l.c. in column *A* gave rhamnose and mannose in the ratio of 1:1. Glucose and galactose were both absent.

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