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THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K60; A NOVEL, STRUCTURAL PATTERN*

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ABSTRACT

Non-linear capsular polysaccharides of *Klebsiella* bacteria usually have a single side-chain per repeating unit, or, less commonly, two side-chains attached to the same unit. The capsular polysaccharide from *Klebsiella* serotype K60 is unique in having three side-chains in the heptasaccharide repeating-unit shown. The structure, including the configuration of the glycosidic linkages, was established mainly by characterization of the oligosaccharides obtained by partial hydrolysis of both the original, capsular polysaccharide and the polymer resulting from the removal, by Smith degradation, of the side chains.

$$\begin{array}{l}\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow \\ \qquad\qquad\qquad\qquad\quad 4 \qquad\qquad\quad 2 \qquad\qquad\quad 2 \\ \qquad\qquad\qquad\qquad\quad \uparrow \qquad\qquad\quad \uparrow \qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\quad 1 \qquad\qquad\quad 1 \qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad \alpha\text{-D-Glc}p \qquad\quad \beta\text{-D-Glc}p \qquad\quad \beta\text{-D-Glc}p\end{array}$$

INTRODUCTION

The *Klebsiella* capsular polysaccharides that are composed[1] of D-glucuronic acid, D-glucose, D-galactose, and D-mannose constitute the largest chemogroup[2] in this genus, with 20 strains represented; half of these incorporate pyruvic acid as an acetal into the polysaccharides[2]. The capsular polysaccharide from *Klebsiella* serotype K60, discussed here, has a structural pattern that is novel in this series.

RESULTS AND DISCUSSION

N.m.r. spectra and composition. — The polysaccharide purified by precipitation with Cetavlon moved as a single band in electrophoresis on cellulose acetate, and had $[\alpha]_D$ +58°, an equivalent weight of 1100, and a molecular weight of 8.1×10^5. N.m.r. spectroscopy (^1H and ^{13}C) indicated seven anomeric protons and carbon

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TABLE I

METHYLATION ANALYSES OF K60 CAPSULAR POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugars[a] *(as alditol acetates)*	*Relative retention times*			*Mole %*[b]			
	Column B (ECNSS-M, 170°)	*Column C (OV-225, 170°)*	*Column D (SP-1000, 220°)*	*I*[c]	*II*	*III*	*IV*
2,3,4,6-Glc	**1.00**	**1.00**	**1.00**	42	48	—	41
2,4,6-Glc	1.85	1.83	1.68	16	16	28	25
2,4,6-Man	1.91	—	1.82	—	—	30	—
2,4,6-Gal	2.12	—	1.91	—	—	24	—
4,6-Man	3.13	2.96	2.60	13	13	—	18
4,6-Gal	3.50	3 26	2.82	14	12	—	—
2,6-Glc	3.50	3 26	2 72	—	11	—	—
2,4-Glc	4.75	—	—	—	—	18	—
2-Glc	7.92	7.61	—	15	—	—	—
2,3,4,6-Gal[d]	—	—	0.92	—	—	—	16

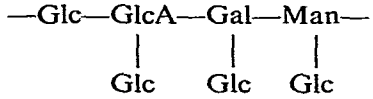
[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, etc. [b]Values were corrected by use of effective carbon-response factors given by Albersheim *et al.*[18] [c]Key: I, original, capsular polysaccharide; II, remethylated after reduction of uronic ester, III, polymer **P1** obtained by Smith degradation; IV, product from β-elimination reaction. [d]3-*O*-Ethyl-2,4,6-tri-*O*-methylgalactose.

atoms, corresponding to three α- and four β-glycosidic linkages; no deoxy sugars, acetyl groups, or acetal-linked pyruvic acid could be detected.

Acid hydrolysis gave mannose, galactose, glucose, glucuronic acid, glucuronolactone, and an aldobiouronic acid. In contrast to the analytical data of Nimmich[1], galactose was detected, but no fucose. Acid hydrolysis of the carboxyl-reduced polysaccharide, and conversion into alditol acetates, gave mannose, galactose, and glucose in the ratios of 1 : 1 : 5. The configuration of the mannose and the glucose was determined to be D by measurement of the circular dichroism[3] (c.d.) of the alditol acetates; galactose was also assigned a D configuration, by c.d. measurements of a methylated derivative subsequently isolated. The sugar ratios obtained, confirmed by the methylation analysis, indicated that the K60 polysaccharide contains D-mannose, D-galactose, D-glucose, and D-glucuronic acid in the ratios of 1 : 1 : 4 : 1.

Methylation analysis. — Methylation[4,5] followed by carboxyl reduction, hydrolysis, and conversion into alditol acetates[6,7] gave the results shown in Table I, column I, and further methylation after carboxyl reduction gave the data presented in Table I, column II. These results show that the polysaccharide consists of a heptasaccharide repeating-unit having three (terminal) glucosyl groups and one residue each of mannose, galactose, and glucuronic acid as the branching points.

A partial structure for the K60 polysaccharide may, therefore, be written in the following form,



it being recognized that the sequence of the main chain was unknown at this stage, and that the 3-linked glucose might equally well be in a side chain. In either case, it is clear that only the three (terminal) glucosyl groups are susceptible to periodate oxidation.

TABLE II

N.M.R. DATA FOR *Klebsiella* K60 CAPSULAR POLYSACCHARIDE, AND DERIVED POLY- AND OLIGO-SACCHARIDES

Compound[a]	Δ[b]	$J_{1,2}$ (Hz)	^{1}H-N.m.r. data		^{13}C-N.m.r. data	
			Integral proton	*Assignment*[c]	*p.p.m.*[b]	*Assignment*[d]
GlcAβ(1→3)Gal-OH	5.30	2	0.4	3-Galα—	104.50	GlcAβ—
A1	4.74	8	1	GlcAβ—	97.04	3-Galβ—
	4.60	7	0.6	3-Galβ—	93.08	3-Galα—
GlcAβ(1→3)Gal-OH β|2 1 Glc	5.38	2	<1	3-Galα— | 2	104.21	GlcAβ—
	4.70	8	1	GlcAβ—	102.46	Glcβ—
A2	4.66	8	>1	Glcβ— 3-Galβ— | 2	92.87	3-Galα— | 2
Glcβ(1→3)GlcAβ(1→3)Gal-OH	5.28	2	0.4	3-Galα—	104.35	3-GlcAβ—
A3	4.80	7	1	Glcβ—	103.52	Glcβ—
	4.77	7	1	3-GlcAβ—	97.04	3-Galβ—
	4.64	7	0.6	3-Galβ—	93.00	3-Galα—
Glcβ(1→2)Manα(1→3)Glc-OH	5.35	2	1	2-Manα—	102.54	Glcβ—
N1	5.23	2	0.6	3-Glcα—	99.85	2-Manα—
	4.65	8	1	Glcβ—	96.80	3-Glcβ—
	4.48	8	0.4	3-Glcβ—	93.07	3-Glcα—

$\xrightarrow{3}$Glc$\xrightarrow[\beta]{1\,3}$GlcA$\xrightarrow[\beta]{1\,3}Gal\xrightarrow[\alpha]{1\,3}Man\xrightarrow[\alpha]{1}$	5.33	2	1	3-Man—$_{\alpha}$	104.19	3-GlcA—$_{\beta}$
P1	5.27	2	1	3-Gal—$_{\alpha}$	103 34	3-Glc—$_{\beta}$
	4.83	7	1	3-Glc—$_{\beta}$	101.42	3-Gal—$_{\alpha}$ / 3-Man—$_{\alpha}$
	4.77	8	1	3-GlcA—$_{\beta}$		
$\xrightarrow{3}$Glc$\xrightarrow[\beta]{1\,3}$GlcA$\xrightarrow[\beta]{1\,3}Gal\xrightarrow[\alpha]{1\,3}Man\xrightarrow[\alpha]{1}$ with α|4, β|2α, α|2α, Glc|1, Glc|1, Glc|1	5.47	2	2	Glc—$_{\alpha}$ / 3-Man— with |2	104.23	3-GlcA—$_{\beta}$ with |4
K60 polysaccharide	5 37	?	1	3-Gal—$_{\alpha}$ with |2	104 02	Glc—$_{\beta}$
					103.07	3-Glc—$_{\beta}$
	5 04	7	1	Glc—$_{\beta}$	102 44	Glc—$_{\beta}$
	4.85	7	1	3-Glc—$_{\beta}$	100 60	3-Man—$_{\alpha}$ with |2
	4.70	7	1	3-GlcA—$_{\beta}$ with |4		
	4 54	7	1	Glc—$_{\beta}$	99.56	Glc—$_{\alpha}$ / 3-Gal—$_{\alpha}$ with |2

[a]For the source of **A1**, **A2**, **A3**, and **N1**, see text. [b]Chemical shift relative to internal acetone; δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate. [c]The numerical prefix indicates the position in which the sugar is substituted; the α or β, the configuration of the glycosidic bond, or the anomer in the case of a (terminal) reducing sugar residue. Thus, 3-Gal—$_{\alpha}$ refers to the anomeric proton of a 3-linked galactosyl residue in the α-anomeric configuration. The absence of a numerical prefix indicates a (terminal) nonreducing group. [d]As in *c*, but for ^{13}C nuclei.

Periodate oxidation. — Periodate oxidation was performed on the sodium salt of the starting material, and the periodate consumption reached a plateau (4.8 molecules of periodate per repeating unit) in 140 h. Reduction of the polyaldehyde, and oxidation of the resulting polyalcohol, caused the consumption of a further 0.7 molecule of oxidant per repeating unit; the theoretical value is 6 moles of periodate per mole of K60 polysaccharide. Reduction followed by mild hydrolysis (Smith degradation) yielded a polysaccharide (**P1**) composed of D-glucose, D-galactose, D-mannose, and D-glucuronic acid in equimolar proportions. The n.m.r. spectrum of **P1** (see Table II)* showed the presence of two α- and two β-linkages, and the

*Previous publications in this series have included tabular n.m.r. data that have been used for assigning anomeric configurations. In the present article, a modification has been made in the method of designating the assignment of each signal in the anomeric regions of the spectra. The symbolism used indicates the position of substitution (if any) of the sugar residue, in addition to the anomeric configuration; it is hoped that this method of presentation will focus attention on the variation of chemical shifts with substitution patterns. See also, Table II, footnote *c*.

methylation data (see Table I, column III) demonstrate that **P1** is a linear polymer: the 3-linked glucose must, therefore, be part of the main chain. It further follows from these data that, of the three lateral, glucose units, one is α- and two are β-linked, and that the side chains are joined to O-4 of the D-glucuronic acid residue and to O-2 of both the D-galactose and D-mannose residues.

The partial structure may now be elaborated to the following,

$$\begin{array}{l}\rightarrow 3)\text{-Glc-}(1\rightarrow 3)\text{-GlcA-}(1\rightarrow 3)\text{-Gal-}(1\rightarrow 3)\text{-Man-}(1\rightarrow \\ \qquad\qquad\quad 4 \qquad\quad 2 \qquad\quad 2 \\ \qquad\qquad\quad \uparrow \qquad\quad \uparrow \qquad\quad \uparrow \\ \qquad\qquad\quad 1 \qquad\quad 1 \qquad\quad 1 \\ \qquad\qquad \text{Glc} \qquad \text{Glc} \qquad \text{Glc}\end{array}$$

again with the proviso that the sequence of the main chain was still unknown, as were the anomeric configurations of all of the linkages. These problems were resolved by isolation, and characterization, of suitable oligosaccharides.

Partial hydrolysis. — In effect, there were two polysaccharides to be studied: the original, K60 capsular polysaccharide, which is highly branched, and the linear polymer **P1** obtained by periodate degradation. It was anticipated that the information obtained from the products of partial hydrolysis of the two polysaccharides would be complementary, and this proved to be the case.

(*a*) *Original capsular polysaccharide.* Partial hydrolysis of the starting material gave two acidic oligosaccharides (**A1** and **A2**), together with a neutral one (**N1**). On the basis of their n.m.r.-spectral data (see Table II) and their methylation analyses (see the experimental section), the structures of these compounds were shown to be as follows.

$$\begin{array}{lll}\beta\text{-GlcA-}(1\rightarrow 3)\text{-Gal} & \beta\text{-GlcA-}(1\rightarrow 3)\text{-Gal} & \beta\text{-Glc-}(1\rightarrow 2)\text{-}\alpha\text{-Man-}(1\rightarrow 3)\text{-Glc} \\ \textbf{A1} & \begin{array}{c}2\\ \uparrow\\ 1\\ \beta\text{-Glc}\end{array} & \textbf{N1} \\ & \textbf{A2} &\end{array}$$

Comparison of the spectral data for the original polysaccharide and **P1** had already demonstrated that, of the three lateral glucose units, one is α- and two are β-linked. The last two were shown directly, by the structures of **A2** and **N1**, to be those linked to galactose and to mannose; by elimination, the third glucose unit must be α-linked to O-4 of the glucuronic acid residue, whence it follows that the formula may now be expanded to the following.

$$\begin{array}{l}\rightarrow 3)\text{-Glc-}(1\rightarrow 3)\text{-}\beta\text{-GlcA-}(1\rightarrow 3)\text{-Gal-}(1\rightarrow 3)\text{-}\alpha\text{-Man-}(1\rightarrow \\ \qquad\qquad\quad 4 \qquad\quad 2 \qquad\quad 2 \\ \qquad\qquad\quad \uparrow \qquad\quad \uparrow \qquad\quad \uparrow \\ \qquad\qquad\quad 1 \qquad\quad 1 \qquad\quad 1 \\ \qquad\quad \alpha\text{-Glc} \qquad \beta\text{-Glc} \qquad \beta\text{-Glc}\end{array}$$

There were still two anomeric linkages unassigned, those of the in-chain glucosyl

residue and the galactosyl residue; according to the spectra of the original polysaccharide, one is α and the other β.

(*b*) *Polysaccharide* **P1**. Partial hydrolysis of polysaccharide **P1** yielded several acidic oligosaccharides (see Experimental section), from which a trisaccharide (**A3**) was purified, and characterized. The n.m.r. spectrum of **A3** (see Table II), taken in conjunction with analytical and methylation data, showed this compound to be as follows.

β-Glc-(1→3)-β-GlcA-(1→3)-Gal

A3

Hence, the galactosyl residue must be α-linked. In view of the known[8] lability of 3-linked galactose residues to acid, it is not surprising that this sugar occupies the (terminal) reducing position in all of the oligosaccharides isolated, and thus the configuration of the galactosyl linkage may be established only by a process of elimination.

CONCLUSIONS

The experiments described lead to the conclusion that the structure of the capsular polysaccharide of *Klebsiella* serotype K60 is based on the following repeating-unit.

$$\begin{array}{l} \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow \\ \qquad\qquad\qquad\qquad\quad 4 \qquad\qquad\quad 2 \qquad\qquad\quad 2 \\ \qquad\qquad\qquad\qquad\quad \uparrow \qquad\qquad\quad \uparrow \qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\quad 1 \qquad\qquad\quad 1 \qquad\qquad\quad 1 \\ \qquad\qquad\qquad\quad \alpha\text{-D-Glc}p \qquad\quad \beta\text{-D-Glc}p \qquad\quad \beta\text{-D-Glc}p \end{array}$$

The structure is of a unique pattern in this series of bacterial polysaccharides, having three separate side-chains per repeating unit. Some aspects of this structure were confirmed by a β-elimination experiment.

Based on the spectral data for the oligosaccharides isolated, it was then possible to assign the signals in the spectra of the original polysaccharide (see Table II).

EXPERIMENTAL

General methods. — The instrumentation used for infrared and n.m.r. spectroscopy, g.l.c.–m.s., and the measurement of optical rotation has been described[9]. 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, and (*3*) 2:1:1 1-butanol–acetic acid–water. Chromatograms were developed with silver nitrate[10]. Preparative paper-chromatography was performed by the descending method, using Whatman No. 3MM paper and solvent *3*. Analytical-g.l.c. separations were performed in stainless-steel columns (1.8 m × 3 mm), with a carrier-gas flow-rate of 20 mL/min. Columns used were (*A*) 3% of SP-2340 on Supelcoport (100–120 mesh), (*B*) 5% of

ECNSS-M, (*C*) 3% of OV-225, and (*D*) 5% of SP-1000, each on Gas Chrom Q (100–120 mesh). Preparative g.l.c. was performed in a column (1 8 m × 6.3 mm) of (*E*) 5% of Silar 10C on Gas Chrom Q (100–120 mesh).

Gel-permeation chromatography was performed in a column (2.5 × 120 cm) of Bio-Gel P-2 (<400 mesh). The column was eluted with 500:5:2 water–pyridine–acetic acid at a flow rate of ~8 mL/h. Fractions (2–3 mL) were collected, freeze-dried, weighed, and (after an elution profile had been obtained) chromatographed on paper. Ion-exchange chromatography for separation of neutral from acidic oligosaccharides was performed in a column (2 × 28 cm) of Bio-Rad AG1-X2 (formate) resin (200–400 mesh). The neutral fraction was eluted with water; the acidic, with 10% formic acid.

Preparation and properties of K60 capsular polysaccharide. — A culture of *Klebsiella* K60 (4463/52) was obtained from Dr. I. Ørskov, Copenhagen, and was grown on a medium of agar (60 g), NaCl (8 g), K_2HPO_4 (4 g), $MgSO_4 \cdot 7\,H_2O$ (1 g), $CaCO_3$ (2 g), sucrose (120 g), and bacto yeast-extract (8 g) in water (4 L) for 3 d. Cells were harvested, and diluted with water containing 1% of phenol, and the suspension was centrifuged for 6 h at 30,000 r.p.m. in a Beckman T4 zonal rotor. The clear, supernatant liquor was poured into ethanol, and the crude polysaccharide precipitated was dissolved in water (800 mL): the polysaccharide was precipitated with 10% Cetavlon, dissolved in 4M NaCl (700 mL), precipitated with ethanol, dissolved in water, and the solution dialyzed against running tap-water for 3 d; yield: 8 g of polysaccharide from 12.5 L of medium; the product had $[\alpha]_D$ +58° (*c* 0.33, water). The purity of the polysaccharide was checked by electrophoresis, using a 1% solution on cellulose acetate strips (Sepraphore III; 15 × 2.5 cm) in veronal buffer, pH 8.6 (LKB-Produkter AB, Stockholm 12, Sweden) at 300 V for 90 min, and then development in either Alcian Blue in citrate-buffered ethanol (pH 4), or with periodate–Schiff reagent.

The ^{1}H-n.m.r. spectrum of the K60 polysaccharide in D_2O at 90° contained signals corresponding to 7 anomeric protons, at δ 5.47 (2 H, $J_{1,2}$ 2 Hz), 5.37 (1 H, $J_{1,2}$ ~2 Hz), 5.04 (1 H, $J_{1,2}$ ~7–8 Hz), 4 85 (1 H, $J_{1,2}$ ~7–8 Hz), 4.70 (1 H, $J_{1,2}$ ~7–8 Hz), and 4.54 (1 H, $J_{1,2}$ ~7–8 Hz) (see Table II for assignments). The ^{13}C-n.m.r. spectrum showed six signals in the anomeric region, at 104.23, 104.02, 103.07, 102.44, 100.60, and 99.56 p.p.m., the signal at 99.56 p p.m. being twice the height of the other five. Several signals between 61.1 and 62.2 p.p.m., due to C-6 of the hexosyl units were also present (see Table II for assignments).

Hydrolysis of the polysaccharide. — Hydrolysis of a sample (20 mg) of the K60 polysaccharide with 2M trifluoroacetic acid overnight at 95°, and removal of the acid by successive evaporations with water, followed by paper chromatography (solvents *1* and *2*), showed D-mannose, D-galactose, D-glucose, D-glucuronolactone, D-glucuronic acid, and an aldobiouronic acid

Sugar analysis was performed as previously described[11]. The alditol acetates of mannose, galactose, and glucose were identified by g l.c. (column *A*; programmed at 195° for 4 min, and then at 2°/min to 260°), and found to be present in the ratios

of 1 : 1 : 5. Preparative g.l.c. (column *E*, programmed from 210° at 4°/min to 250°), followed by measurement of the c.d. spectra, showed both the mannitol hexaacetate and the glucitol hexaacetate to be of the D configuration.

Methylation analysis. — The capsular polysaccharide (300 mg), in the free acid form, obtained by passing the sodium salt through a column of Amberlite IR-120 (H^+) resin, was dissolved in anhydrous dimethyl sulfoxide (30 mL) and methylated by the Hakomori procedure[4,5]. Methylation was incomplete, as shown by hydroxyl absorption in the i.r. spectrum. A subsequent Purdie[12] treatment effected complete methylation; yield, 272 mg. Carboxyl-reduction of the fully methylated polysaccharide with $LiAlH_4$ in anhydrous oxolane, hydrolysis (of a portion) with 2M trifluoroacetic acid (TFA), reduction with sodium borohydride, and acetylation with 1 : 1 acetic anhydride–pyridine gave a mixture of partially methylated alditol acetates which was analyzed by g.l.c. in columns *B*, *C*, and *D*, and by g.l.c.–m.s. (see Table I, column I). The methylated, carboxyl-reduced polysaccharide (30 mg) was further methylated by the Hakomori method, hydrolyzed with 2M TFA, reduced with $NaBH_4$, and acetylated. Analysis of the partially methylated alditol acetates by g.l.c.–m.s. in column *D* showed the replacement of 2-*O*-methylglucose by 2,6-di-*O*-methylglucose (see Table I, column II). Preparative g.l.c. in column *E* (215°, isothermal) afforded a sample of 4,6-di-*O*-methylgalactitol acetate, whose positive c.d. curve indicated that the galactose had the D configuration[3].

Periodate oxidation of the K60 capsular polysaccharide. — A solution of K60 polysaccharide (1.0 g) in water (150 mL) was mixed with 0.1M $NaIO_4$ and 0.4M $NaClO_4$ (150 mL), and the reaction was allowed to proceed at 4° in the dark. The consumption of periodate and release of formic acid were respectively monitored (1-mL aliquots) by the Fleury–Lange method[13] and titration against mM NaOH. Periodate consumption and formic acid production reached a plateau after 140 h (4.8 mol of periodate and 1.7 mol of formic acid per mol of polysaccharide). Ethylene glycol (10 mL) was added, the solution of polyaldehyde was dialyzed overnight, the material reduced with $NaBH_4$ (1.5 g), the base neutralized with 50% acetic acid, and the solution dialyzed and freeze-dried, to yield the polyalcohol (680 mg). This material was further oxidized with periodate (200 mL of 0.05M $NaIO_4$ and 0.2M $NaClO_4$): the periodate consumption was constant after 70 h (0.7 mol of periodate/mol of polysaccharide). Re-isolation gave the polyalcohol (660 mg).

This product was hydrolyzed with 0.5M TFA for 24 h at room temperature[14,15]. Paper chromatography in solvent *1* showed the presence of one mobile compound, identified, by comparison with a standard, as glycerol, and a polymeric product; dialysis afforded 550 mg of polymeric material (**P1**). Total hydrolysis of **P1**, and examination on paper (solvents *1* and *2*) showed D-mannose, D-galactose, D-glucose, D-glucuronic acid, and an aldobiouronic acid. Sugar analysis, as previously described, gave (g.l.c., column *A*) mannitol, galactitol, and glucitol hexaacetates in the ratios of 1 : 1 : 2, one of the glucitol units being derived from the glucuronic acid. Methylation of **P1** (25 mg) by the Hakomori method and one Purdie treatment afforded a fully methylated polysaccharide showing no hydroxyl absorption in the infrared spectrum.

The fully methylated material (10 mg) was reduced with $LiAlH_4$, then hydrolyzed with 2M TFA for 10 h, and the product reduced with $NaBH_4$, and acetylated. The partially methylated alditol acetates were analyzed by g.l.c. and g.l.c.–m.s. in columns *B* and *D*, with the results given in Table I, column III. A portion (10 mg) of the methylated polysaccharide was hydrolyzed with 2M TFA for 5 h, and separated into the acidic and neutral components on Bio-Rad AGl-X2 ion-exchange resin. The acidic fraction was refluxed in 3% HCl/MeOH, the product reduced with $NaBH_4$ in anhydrous methanol, the product hydrolyzed with 2M TFA for 3 h, and the product reduced with $NaBH_4$, and acetylated. The results obtained by g.l.c. in column *B* showed the presence of 2,4,6-tri-*O*-methylgalactose and 2,4-di-*O*-methylglucose in the ratio of 1.0:1.3.

The p.m.r. spectrum of **P1** showed signals at δ 5.33 (1 H, $J_{1,2}$ ~2 Hz), 5.27 (1 H, $J_{1,2}$ ~2 Hz), 4.83 (1 H, $J_{1,2}$ 7 Hz), and 4.77 (1 H, $J_{1,2}$ 7 Hz). The ^{13}C-n.m r. spectrum showed signals at 104.19 (1 C), 103.34 (1 C), and 101.42 p.p m. (2 C) in the anomeric region, and four signals, at 84.11, 83.02, 80.13, and 76.60 p.p.m , due to C-3 of the four hexosyl residues.

Partial hydrolysis of the K60 capsular polysaccharide — A solution of K60 polysaccharide (500 mg) in 0.5M trifluoroacetic acid (100 mL) was refluxed for 3 h. The acid was removed by evaporation, and the acidic were separated from the neutral components in a column of Bio-Rad AG1-X2 resin. The acidic fraction (130 mg) was separated on Bio-Gel P-2, to give 25 mg of pure aldobiouronic acid (**A1**) and 10 mg of pure aldotriouronic acid (**A2**).

The aldobiouronic acid **A1** had R_{Glc} 0.28 (solvent *1*) and $[\alpha]_D$ +12° (*c* 1.05, water). Sugar analysis showed glucitol (from uronic acid) and galactitol hexaacetates to be present in equimolar proportions. The aldotriouronic acid **A2** had R_{Glc} 0.14 (solvent *1*), and, after hydrolysis, examination on paper (solvents *1* and *2*) indicated glucose, galactose, and an aldobiouronic acid. Acid **A2** was reduced with aqueous sodium borohydride, the products methylated, and the ester fraction reduced with lithium aluminum hydride in anhydrous oxolane. After hydrolysis, g.l.c. analysis (column *B*) showed a component of R_T 0.8, together with peaks corresponding to 2,3,4,6-tetra-*O*-methylglucose (R_T 1.0) and 2,3,4-tri-*O*-methylglucose (R_T 1.9). Mass spectrometry showed that the fastest component was 1,4,5,6-tetra-*O*-methylgalactitol

The neutral fraction was separated on Bio-Gel P-2, to give, in pure state, a trisaccharide (**N1**; 30 mg) having R_{Glc} 0.32 (solvent *1*) and $[\alpha]_D$ +31° (*c* 0.32, water). G.l.c. analysis of **N1** showed mannitol and glucitol hexaacetates in the ratio of 1.2 Methylation analysis (columns *C* and *D*) of reduced **N1** gave 1,2,4,5,6-penta-*O*-methylglucitol (R_T 0.42; column *D*), 2,3,4,6-tetra-*O*-methylglucose (R_T 1.00), and 3,4,6-tri-*O*-methylmannose (R_T 1.66).

The n.m.r. data for **A1**, **A2**, and **N1** are presented in Table II.

Partial hydrolysis of polysaccharide **P1** *(from periodate oxidation)*. — A sample (350 mg) of **P1** was hydrolyzed with 1M trifluoroacetic acid (75 mL) for 1 h, and the oligosaccharides were separated into acidic and neutral fractions in a column of Bio-Rad AG1-X2 resin. The acidic fraction (220 mg) was separated by chromato-

graphy in a column of Bio-Gel P-2 gel. Three fractions were collected, corresponding to (*1*) the aldobiouronic acid (43 mg), (*2*) a mixture of two trisaccharides (30 mg), and (*3*) a mixture of two tetrasaccharides (32 mg).

Fraction 2 was purified by paper chromatography (solvent *C*), and the pure aldotriouronic acid was isolated (**A3**; 14 mg), having $[\alpha]_D$ +10° (*c* 1.1, water). Sugar analysis of this compound, as previously described, showed (g.l.c., column *A*) galactitol and glucitol hexaacetates in the ratio of 1:2. Methylation analysis (column *B*) showed the presence of 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylgalactose, and 2,4-di-*O*-methylglucose in approximately equimolar proportions.

Uronic acid degradation[16,17]. — Methylated K60 polysaccharide (30 mg) was carefully dried and then, together with a trace of *p*-toluenesulfonic acid, dissolved in 19:1 dimethyl sulfoxide–2,2-dimethoxypropane (20 mL) under nitrogen in a sealed flask; dimethylsulfinyl anion (10 mL) was added, and allowed to react at room temperature. After 18 h, the product was directly alkylated with ethyl iodide. Following neutralization of the base with 50% acetic acid, and addition of water, the ethylated, degraded product was isolated by partition between chloroform and the aqueous solution. Hydrolysis with 2M TFA, and g.l.c.–m.s. analysis of the alditol acetate derivatives, yielded the results given in Table I, column IV.

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